CHEMICAL AND CHEMOENZYMATIC SYNTHESSES OF LANTIBIOTICS
AND OTHER BIOACTIVE CYCLIC PEPTIDES

BY

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DISSEPTION

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ABSTRACT

New antibiotics are desperately needed to combat the disturbing rise of pathogenic microorganisms resistant to traditional treatments. Throughout the history of modern medicine, natural products have played a leading role as a source and inspiration of new drugs, particularly antibacterial agents. One emerging family of natural products, the lanthipeptides, is defined by the presence of the thioether-containing crosslinks lanthionine and methyllanthionine and includes many members with promising activities against clinically-relevant bacterial pathogens, including drug-resistant strains. These peptides are biosynthesized by translation of a linear precursor peptide on the ribosome, followed by extensive post-translational modification that imparts substantial structural and functional diversity. In order to improve the pharmacological properties of lanthipeptides and therefore foster their clinical application, a more detailed understanding of their biological activities is essential.

In this thesis, complementary synthetic platforms have been developed to produce lanthipeptides and analogues, and subsequent biological evaluation has revealed important information about the underpinnings of antibacteria activity in several compounds. In a chemoenzymatic approach described in Chapter II, the activity of an engineered biosynthetic enzyme involved in lacticin 481 maturation was reconstituted in vitro and used to produce a panel of lacticin 481 analogues from synthetic precursor peptides, several of which possessed improved antibacterial potency. This enhanced potency was correlated with improved inhibition of transglycosylation during peptidoglycan biosynthesis. Chapter III describes a chemical approach to lanthipeptide production; solid-phase peptide synthesis was utilized to generate the first reported analogues of the potent lanthipeptide epilancin 15X. These efforts provided the first details into structure-activity relationship analysis of this compound and also established it as a pore-forming agent. The total synthesis of lacticin 481 and analogues containing different crosslink stereochemical configurations was also accomplished, which revealed that the natural crosslink configurations are required for biological activity. In Chapter IV, this solid-supported chemical approach was applied to the generation of potent analogues of the immunomodulatory peptide compstatin via substitution of the reduction-labile disulfide moiety for an isosteric thioether that is stable to reduction. The generality of these synthetic platforms allows for similar engineering approaches in other lanthipeptides and other classes of bioactive cyclic peptides, in order to improve the therapeutic potential of these intriguing compounds.
For my wife Amy

“Those who hope in the Lord will renew their strength.
They will soar on wings like eagles.
They will run and not grow weary,
Walk and not grow faint.”
~ Isaiah 40:31

“Come to me, all who labor and are burdened, and I will give you rest.
Take my yoke upon you and learn from me, for I am gentle and humble of heart,
and you will find rest for your souls.
For my yoke is easy, and my burden light.”
~ Matthew 11: 28-30
ACKNOWLEDGMENTS

“Nobody said it was easy. No one ever said it would be this hard.” ~Coldplay, “The Scientist”

The process of scientific research is inherently one of substantial growth and change, of pushing oneself beyond the familiar, and these events do not often come easy. Without any doubt, the past five years have been the most challenging of my life, both professionally and personally. I have been so blessed by God to have the love and support of so many during my time in Champaign. I cannot possibly express my appreciation properly in this space, but know that all of you will be in my thoughts and prayers always.

“This is my dream, my own, just to be at home, and to be all alone... with you.” ~Billy Joel, “Everybody Has a Dream”

No amount of words could properly express my gratitude to my wife and best friend, Amy. She left her own path and leapt into the unknown for me, and will certainly be doing it again as we continue our journey. I often wonder how long I would’ve lasted without her support, and the answer at which I always arrive is “not long.” She has been a constant source of love, peace, and inspiration for me through so much. It’s about time I return the favor, and that’s my plan for the rest of the days God gives us together.

“I’ve seen your face a hundred times, everyday we’ve been apart.” ~Ozzy Osbourne, “Mama, I’m Coming Home”

One of the most difficult parts of my time here has been the distance from my family. To my parents Jim and JoAnn, my sister Allison, and my brother Matt, thanks for all the encouragement and all the love. Many things have changed over the past five years, and that I know I’ve missed a lot of them, but you have always never been far from my mind. Also to my dear friend and fellow Allentown Central Catholic ’04 and Delaware ’08 graduate Elaine Yandrisevits, you’ve been like a twin sister to me, and thanks for everything you’ve done for me and Amy.

“If I leave here tomorrow, would you still remember me? For I must be traveling on now, ‘cause there’s too many places I’ve got to see.” ~Lynyrd Skynyrd, “Free Bird”

My progress towards my doctorate, and my decision to enroll at UIUC in the first place, was made possible by the mentorship of my adviser, Prof. Wilfred van der Donk. Wilfred has
always treated me, my ideas, and my interests with the utmost respect, and this respect has been a critical part of my growth as a scientist. I would like to thank the members of my thesis committee, Prof. Paul Hergenrother, Prof. John Katzenellenbogen, and Prof. Satish Nair, and former committee member Prof. Martin Burke, for their input and suggestions. Many thanks also to my undergraduate research adviser, Prof. Joel Schneider, who greatly helped set the stage for my professional development and has remained an indispensable mentor to this day.

“It’s a long, long road, from which there is no return. While we’re on the way to there, why not share?” ~The Hollies, “He Ain’t Heavy, He’s My Brother”

During my first visit to UIUC, I instantly clicked with a fellow recruit, Noah Bindman, and little did we know that we’d go through this whole process together. Noah has been the yang to my yin, and I’ll always treasure our discussions, whether about religion, politics, or (most commonly) baseball. After all the failed reactions and subgroup meetings and Twins/Phillies playoff losses and softball double-headers, it’s only fitting that we should be hooded together. Another monumental figure is van der Land graduate Dr. Trent Oman, who took me under his wing and became both a great collaborator and friend. Lab never felt quite the same since he moved on to the real world, but our continued career discussions have been an immense help to me.

“How I wish, how I wish you were here. We’re just two lost souls swimming in a fish bowl, year after year.” ~Pink Floyd, “Wish You Were Here”

My dear Martha, how I wish you were still here! Officially, Martha Freeland was the former Program Manager of the Chemistry-Biology Interface Training Grant Program, but to those in van der Land, she was so much more: music connoisseur, social liaison, lunch buddy, advocate, mother hen. I can only hope and pray that our paths will cross again in the future, and thanks so much for always offering a listening ear and a shoulder to cry on. Amy and I will never forget you.

“Thank you for the talk, you know you really eased my mind. I was troubled by the shapes of things to come.” ~Chicago, “Dialogue”

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CHAPTER 1: NATURAL PRODUCTS, PEPTIDES, AND DRUG DISCOVERY

1.1. ANTIBACTERIAL DRUGS AND NATURAL PRODUCTS

Throughout human history, natural products have played a leading role in drug discovery, as the inspiration for or even the direct source of many of the world’s medicines.\textsuperscript{1-3} Furthermore, such molecules have served as a major stimulus for the development of new synthetic strategies and methods, greatly expanding the frontiers of organic chemistry to generate increasingly complex structures.\textsuperscript{4-7} Despite this rich and storied history, recent decades have witnessed natural products making less impact in the pharmaceutical arena due to declining success rates in screening for novel compounds and difficulties in production, among other complications.\textsuperscript{8-11} However, the growing availability of genomic and bioinformatic data has given new life to natural product research by accelerating the discovery of new compounds beyond traditional screening procedures.\textsuperscript{11-14} Indeed, more than one-third of all newly approved small molecule drugs in the United States from 1981-2010 were either natural products or semisynthetic derivatives (Fig. 1.1a), and this number has been trending higher over the past decade.\textsuperscript{15} Even within the subset of “synthetic” compounds, a substantial population contains a pharmacophore based on a bioactive natural product.

Fig. 1.1. Natural products as a source of new drugs in the United States from 1981-2010. (a) Sources of newly approved small molecule drugs. (b) Sources of newly approved antibacterial drugs, including non-small molecule vaccines. Adapted from a survey by Newman and Cragg.\textsuperscript{15}

1.1.1. Mechanisms of antibacterial activity and resistance

Natural product-based drug discovery has been especially fruitful in the pursuit of clinical antibacterials;\textsuperscript{15-18} nearly two-thirds of all newly approved antibacterial drugs from 1981-2010

were small molecule natural products or semisynthetic derivatives (Fig. 1.1b).\textsuperscript{15} Unlike drugs in other medicinal areas, such as oncology or metabolic disorders, antibacterial natural products have evolved specifically to achieve the intended outcome, killing bacteria, as desired for therapeutic application. Harnessing these potent molecules, therefore, taps into nature’s own paradigm by which microorganisms compete against each other. The discovery of new antibiotics is even more pertinent in the context of the development in pathogenic bacteria of resistance to many of the traditional classes of antibacterials, which has been widely recognized as a worldwide crisis for decades.\textsuperscript{19-25} Infections caused by methicillin-resistant \textit{Staphylococcus aureus} (MRSA) have been increasing steadily for over a decade and kill more than 20,000 people in the United States each year.\textsuperscript{26, 27} Other drug-resistant pathogens, such as vancomycin-resistant \textit{Enterococcus} (VRE) and multidrug-resistant \textit{Pseudomonas aeruginosa} and \textit{Acinetobacter baumannii} have also become epidemic in hospital settings.\textsuperscript{19, 28} Furthermore, the emergence of extensively drug-resistant tuberculosis, resistant to multiple first- and second-line treatments, threatens millions in the developing world.\textsuperscript{29, 30} As a striking example of the scarcity in the contemporary antibacterial pipeline, the approval of the diarylquinoline bedaquiline in 2012 represented the first new treatment for tuberculosis in 40 years.\textsuperscript{31} Similarly to antibacterials, alarming rates of resistance development have been recognized in other antimicrobial drugs, including antivirals,\textsuperscript{32} antifungals,\textsuperscript{33} and antimalarials.\textsuperscript{34}

The vast majority of clinical antibacterials exert their biological activity through one of four mechanisms (Fig. 1.2): inhibition of ribosomal polypeptide synthesis (including the aminoglycoside, macrolide, and tetracycline classes), inhibition of nucleic acid synthesis (quinolones, rifamycins), inhibition of cell wall synthesis (\(\beta\)-lactams, glycopeptides), and inhibition of folate synthesis (2,4-diaminopyrimidines, sulfonamides).\textsuperscript{35} Somewhat ironically, the same evolutionary history exploited in the discovery of natural product antibacterials also operates in the other direction; organisms appear to have evolved mechanisms of resistance to such compounds long before their use in human medicine.\textsuperscript{36-38} While each drug or drug class possesses its own resistance profile, several general mechanisms are commonly operative across classes.\textsuperscript{39, 40} The biological target of the drug can be modified in several ways: through genetic mutation, as in the case of penicillin-binding proteins from MRSA; through enzyme-catalyzed alteration, such as ribosome methylation to block macrolide binding; or through molecular substitution, exemplified by alteration of the \(\text{D-Ala-D-Ala}\) motif of nascent peptidoglycan to \(\text{D-}\)
Fig. 1.2. Representative structures of different classes of antibacterial drugs, listing the source of each drug (natural product, semisynthetic derivative of a natural product, synthetic). General mode-of-action is color-coded: inhibition of polypeptide biosynthesis (red), inhibition of nucleic acid biosynthesis (blue), inhibition of cell wall biosynthesis (green), antifolates (purple), cell membrane disruption (gold).
Ala-d-Lac by VRE to evade vancomycin. Drug or multidrug efflux pumps remove the drug from its site of activity inside the cell and have contributed to quinolone and tetracycline resistance in multiple species, including \textit{P. aeruginosa}. In addition, the evolution of enzymatic inactivation of antibacterials has spread across drug classes, including β-lactam hydrolysis, aminoglycoside acetylation or phosphorylation, and macrolide hydrolysis or phosphorylation.

1.1.2. Recent developments in antibacterial natural products

The paucity of biological pathways targeted by clinical antibacterials is suspected to contribute to the rapid development of resistance,\textsuperscript{35} even to next-generation compounds and novel drug classes. The observation of clinical resistance to linezolid, a fully synthetic ribosomal inhibitor that established the oxazolidinone class, followed within a year of its approval in 2000,\textsuperscript{41} although subsequent global appraisals have documented that this resistance has remained very rare.\textsuperscript{42} The glycyclyclines, established by the approval of tigecycline in 2005, are semisynthetic tetracycline derivatives designed to evade common mechanisms of tetracycline resistance, but emerging resistance via efflux pumps was already observed during clinical trials.\textsuperscript{43} Therefore, new antibacterials with entirely novel biological targets and mechanisms-of-action are sorely needed to combat rising rates and prevalence of resistance. However, in the face of this great and growing need, a range of technical and financial aspects have contributed to a general withdrawal of the pharmaceutical industry from antibacterial discovery and development over the past several decades.\textsuperscript{24,44-46}

In spite of these formidable obstacles, several new classes of natural product and natural product-derived antibacterials have reached the market in the past decade (see \textit{Fig. 1.2}). Daptomycin, a macrocyclic lipopeptide isolated from the soil bacterium \textit{Streptomyces roseosporus}, was approved in 2003 for the treatment of Gram-positive skin infections and is active against MRSA and VRE.\textsuperscript{47} This natural product is synthesized via non-ribosomal peptide synthesis (NRPS) machinery\textsuperscript{48} and employs a novel, yet poorly understood, mode of action: calcium-dependent depolarization of the cell membrane.\textsuperscript{47,49} However, like other recently-introduced antibacterial classes, strains of \textit{S. aureus} utilizing diverse resistance mechanisms have been isolated in clinical settings.\textsuperscript{50} Retapamulin is a polycyclic, semisynthetic derivative of the fungal natural product pleuromutilin that was approved in 2007 to treat topical Gram-positive infections.\textsuperscript{51} Similar to many other antibacterials, it inhibits ribosomal peptide synthesis, but
through novel interactions with the 50S ribosomal subunit. Additionally, fidaxomicin established a new class of narrow-spectrum macrocyclic polyene antibacterials with its approval in 2011. This fermentation product of the actinomycete *Dactylosporangium aurantiacum* inhibits RNA polymerase in *Clostridium difficile* while sparing many other species that comprise the intestinal flora, mitigating recurrent infections. While these successes signify a starting point to address the problem of antibacterial resistance, the limited number of compounds currently in development, coupled with the observation of resistance to even newly introduced compounds, beckons for intensified efforts in this area.

1.2. THE IMPACT OF PEPTIDES IN DRUG DEVELOPMENT

Perhaps the converse to small molecule natural products, peptides have garnered greater attention as leads for drug discovery in recent decades after a long period of neglect. When compared to small molecules, peptides hold the potential to improve specificity, decrease off-target effects, and inhibit large biomolecular interactions due to their large size and dense functionality, and screening technologies such as phage display have accelerated the discovery of bioactive peptides as new drug leads. However, limitations in stability and production have curbed enthusiasm for development of natural or synthetic peptides as drugs. In 2012, five of the 39 new drug approvals were peptides: the surfactant lucinactant for respiratory distress syndrome; erythropoietin analogue peginesatide for chronic kidney disease-related anemia; bacterial enterotoxin homologue linaclotide for irritable bowel syndrome; somatostatin analogue pasereotide for Cushing’s disease; and glucagon-like peptide-2 analogue teduglutide for short bowel syndrome. Peptide-based natural products have also been approved for therapeutic use in the past decade, including the cyclic antibacterial lipopeptide daptomycin (see Section 1.1.2) and the disulfide-rich \( \omega \)-conotoxin ziconotide for the treatment of neuropathic pain. In light of this currently modest population of approved peptide drugs, recent advances in peptide stabilization and manufacturing have coalesced in a rich pipeline of bioactive peptides to treat a wide diversity of challenging disease states.

1.2.1. Improving peptides for in vivo applications

In order to address the pharmacological limitations of peptides, the field of peptidomimetics has aimed to replicate the effects of biologically active peptides with more
stable scaffolds that preserve the spatial array of important functional groups.\textsuperscript{66, 67} In the context of peptides themselves, a variety of medicinal chemistry approaches have been used to stabilize lead compounds and reduce clearance rates.\textsuperscript{68-70} N- and C-terminal functionalization, backbone methylation, and introduction of \textalpha{}-amino acids and other non-proteinogenic residues have been widely demonstrated to improve stability to proteolysis.\textsuperscript{71} Conjugation with polyethylene glycol is known to decrease both the typically rapid \textit{in vivo} clearance rate and potential immunogenicity of peptide drugs.\textsuperscript{72} Backbone or side-chain cyclization may have the doubly beneficial effect of improving proteolytic stability and constraining the peptide in a conformation conducive for binding its biological target.\textsuperscript{73, 74} In particular, the disulfide bond, commonly found among therapeutic peptides,\textsuperscript{57} has been the target of considerable engineering efforts to improve stability to cellular reducing agents while preserving the restricted conformation of the peptide.\textsuperscript{66, 75}

1.2.2. Recent developments in peptide synthesis and manufacturing

As interest grows in the potential therapeutic application of peptides, so also grows the need for efficient processes to synthesize longer and more structurally complex molecules.\textsuperscript{76} Improved amino acid activating agents for use in solid-phase peptide synthesis (SPPS) have allowed for improved coupling yields with decreased side-reactions,\textsuperscript{77} but the need for stoichiometric, and often excess, reagents generates considerable waste.\textsuperscript{78} The large-scale production of the 36-residue antiretroviral peptide enfuvirtide via a combination of SPPS and fragment couplings over 106 chemical steps was a landmark achievement in the early 2000s for peptide process chemistry, but the synthesis of 1000 kg of final product necessitated over 45,000 kg of raw materials.\textsuperscript{79, 80} Catalytic amidation reactions, while still in their infancy, have the potential to decrease greatly the waste generated during peptide synthesis.\textsuperscript{81} Novel chemistries for peptide cyclization have also been the subject of considerable scrutiny.\textsuperscript{82}

Chemoselective ligation of unprotected peptide fragments holds promise to streamline the commercial synthesis of therapeutic peptides in a convergent manner and decrease difficulties in purification (Fig. 1.3).\textsuperscript{81} Native chemical ligation between an N-terminal cysteine and a C-terminal thioester, the first such chemistry to be developed, generates a native amide bond under aqueous conditions in the presence of unprotected functionalities.\textsuperscript{83} Recent progress in the use of cysteine surrogates\textsuperscript{84-86} and the solid-phase synthesis of peptide thioesters\textsuperscript{87-89} has further expanded the impact of this powerful strategy, as highlighted by the recent syntheses of the 141
residue parathyroid hormone-related protein\textsuperscript{90} and the 166 residue, tetracyclosylated hormone erythropoietin.\textsuperscript{91} The ketoacid-hydroxylamine (KAHA) ligation first reported in 2006 presents an alternative approach to the amide-forming ligation of fully unprotected peptide fragments;\textsuperscript{92} an N-terminal hydroxylamine and a C-terminal $\alpha$-ketoacid react via a reagentless decarboxylative condensation.\textsuperscript{93} General SPPS-based approaches to the construction of peptides containing both reaction partners have been developed,\textsuperscript{94, 95} and the KAHA ligation has been applied to sequential reactions,\textsuperscript{96} peptide cyclization,\textsuperscript{97} and therapeutic peptide synthesis.\textsuperscript{98} A third approach, the traceless Staudinger ligation between an N-terminal azide and a C-terminal phosphinothioester, has been adapted for use in water,\textsuperscript{99} but lacks the broad functional group and substrate tolerance of native chemical and KAHA ligations.\textsuperscript{100, 101}

1.3. RIBOSOMALLY-SYNTHESIZED PEPTIDE NATURAL PRODUCTS

Considering the difficulties encountered in antibacterial drug discovery as well as the growing potential of peptides as drugs, peptide-based natural products have been suggested as a viable source of new antibacterial drugs.\textsuperscript{17, 18} Peptides produced via NRPS biosynthesis, such as vancomycin and its variants, daptomycin, and bacitracin, have a rich history as clinical antibacterials. However, a separate class of peptide natural products has emerged in the past decade: the ribosomally synthesized and post-translationally modified peptide natural products (RiPPs).\textsuperscript{102} Advances in genome sequencing and bioinformatic analysis have revealed that this class possesses much greater diversity with respect to structure and biological activity than previously anticipated.\textsuperscript{103-106} Over a dozen subfamilies of RiPPs are currently recognized, originating from all three domains of life.\textsuperscript{102} Initially limited to the 20 canonical amino acids
during translation, the diversity of these compounds is greatly enhanced by extensive post-translational modification (PTM), such as heterocyclization, macrocyclization, dehydration, acylation, glycosylation, halogenation, alkylation, and epimerization (Fig. 1.4). Similarly diverse are the biological activities of these compounds, which include antibacterial, antiviral, antimalarial, cytotoxic, hemolytic, and analgesic activity. One RiPP has already reached the clinic, the ω-conotoxin ziconotide, which was approved for the treatment of intractable pain in 2004.

Unlike NRPS systems, the biosynthesis of RiPPs directly connects the final product and the gene encoding its corresponding precursor peptide (Fig. 1.5). This phenomenon renders RiPPs particularly suited for genome-mining strategies, which have demonstrated that these pathways are remarkably widespread throughout nature. The linear, ribosomally translated precursor peptide contains a core peptide, which is post-translationally modified to give the final product, and a leader peptide, which helps to direct the maturation process. A variety of functions of the leader peptide in biosynthesis, export, and immunity have been proposed, which appear to vary from subfamily to subfamily. The precursor peptide may also contain an N-terminal signal peptide to guide subcellular localization in eukaryotic systems and/or a C-
Fig. 1.5. General scheme of RiPP biosynthesis, showing the different portions of the precursor peptide. $X_n^*$ indicates a post-translationally modified residue, which may include cyclization, glycosylation, epimerization, or other alterations. An exception to this general scheme is bottromycin biosynthesis, in which the precursor peptide contains an N-terminal core peptide and a C-terminal follower peptide.\textsuperscript{110,111}

terminal recognition sequence involved in excision and head-to-tail cyclization. Interestingly, in bottromycin biosynthesis, the precursor peptide contains an N-terminal core peptide and a C-terminal follower peptide, the latter of which is removed from the final product and is hypothesized to serve a similar role to the leader peptide of other RiPPs.\textsuperscript{110,111} From a bioengineering standpoint, the relative brevity of RiPP biosynthetic pathways, as well as the substrate promiscuity of the leader-directed biosynthetic machinery, makes RiPPs particularly attractive.\textsuperscript{102,103} Such engineering efforts are needed in order to develop a more full understanding of structure-activity relationships and modes-of-action. This knowledge can then be utilized in the improvement of pharmacological properties, including potency, solubility, and \textit{in vivo} stability, and thus the improvement of the therapeutic potential of these compounds.

1.4. LANTHINEPTIDES AND LANTIBIOTICS

At present, the best-studied subfamily of RiPPs are the lanthipeptides: polycyclic peptides characterized by the presence of the thioether-crosslinked bis-amino acids \textit{meso}-lanthionine (Lan) and (2S,3S,6R)-3-methyllanthionine (MeLan) (Fig. 1.6). Formerly known as lantibiotics for “lanthionine-containing antibiotics,” this family name has been broadened to lanthipeptides to encompass the discovery of compounds with nonantibiotic activities.\textsuperscript{112} Interest in these RiPPs as chemotherapeutics stems from the use of the prototypical lanthipeptide
Fig. 1.6. Sequences, ring topologies, and post-translational modifications of representative lanthipeptides. Modified residues are highlighted, and their chemical structures are shown. The two known lanthipeptide-lipid II binding motifs are also indicated: the nisin motif (green dashed circle) and the mersacidin motif (red dashed circle). Note that the A-ring of haloduracin β has been recently shown to contain (2R,3R,6R)-MeLan.113 (continued on next page)
Fig. 1.6. (continued)
nisin as a food preservative for more than 50 years without substantial incidence of microbial resistance. Lantibiotics possess potent activity against many clinically relevant Gram-positive bacteria, including drug-resistant strains of *Staphylococcus, Streptococcus, Enterococcus,* and *Clostridium,* as well as against select Gram-negative pathogens, such as *Neisseria* and *Helicobacter.* Highlighting the therapeutic potential of this family are several members currently in clinical development, including a natural cinnamycin analogue for the treatment of cystic fibrosis and a semisynthetic derivative of actagardine for the treatment of *Clostridium difficile* infections. Additionally, mutacin 1140 is currently in preclinical development for the treatment of Gram-positive bacterial infections. Other applications, including in food preservation, veterinary medicine, and molecular imaging, are also emerging.

With regard to the molecular topology of these compounds, the arrangement of the Lan/MeLan crosslinks varies widely within the family, from elongated molecules like nisin and lacticin 3147 A2 to more compact, globular structures as seen in cinnamycin (Fig. 1.6). Similarly diverse are the number of crosslinks, which currently ranges between two in lactocin S and seven in geobacillin I. These crosslinks are critical not only for the activity of the mature compounds but also for stability against proteolysis and heat denaturation. Also commonly found are the α,β-unsaturated residues 2,3-didehydroalanine (Dha) and (Z)-2,3-didehydrobutyrine (Dhb), the biosynthetic precursors to Lan and MeLan, respectively (see Section 1.4.1). A wide variety of other tailoring PTMs are also suggested to play roles in stability and/or activity and include cyclic structures such as aminovinylcysteine and lysinoalanine, modified side-chains such as d-alanine, hydroxylated aspartate, and chlorinated tryptophan, and N-terminal acyl moieties (Fig. 1.6).

It was long believed that lanthipeptides were produced only by a small group of Gram-positive Firmicutes and select streptomyceses. However, advances in both genome analysis and isolation studies have allowed for the identification of many new lanthipeptides, which now include approximately 100 isolated compounds, and thousands more have been predicted based on the identification of putative biosynthetic gene clusters. Such genes are widely disseminated in actinomycetes, bacteroidetes, proteobacteria, and cyanobacteria, and have even been detected in mammals. Homologues of lanthipeptide biosynthetic enzymes are also involved in the biosynthesis of other RiPPs, including lanthipeptide dehydratase-like enzymes that install Dha/Dhb in thiopeptides and goadsporin (Fig. 1.4).
Given the rapid pace of genome sequencing and the implementation of freely available, automated \textit{in silico} genome mining software,\textsuperscript{153, 154} the discovery of novel lanthipeptides is expected to continue its acceleration in coming years, potentially yielding further diversity in structure and function.

\textbf{1.4.1. Biosynthesis of lanthipeptides}

Despite the structural diversity across the family, lanthipeptide biosynthesis is remarkably conserved. In all known cases, lanthipeptides are biosynthesized on the ribosome as a linear precursor peptide, generically called a LanA. Like most other RiPP systems, the LanA contains an N-terminal leader peptide and a C-terminal core peptide, the latter of which becomes the mature compound. The distinguishing Lan/MeLan structures are installed post-translationally into the core peptide via a two-reaction sequence: enzymatic dehydration of serine and threonine to the \(\alpha,\beta\)-unsaturated residues Dha and Dhb, respectively, followed by enzymatic Michael-type addition of a cysteinyl thiol to yield the thioether crosslinks Lan and MeLan (Fig. 1.7a). In all cases investigated prior to this year, a \(d\)-configuration is installed at the newly formed \(C_\alpha\)-stereocenter of the crosslink (as depicted in Fig. 1.6),\textsuperscript{125-127, 155-157} which promoted an assumption that the lanthipeptide biosynthetic machinery is only able to produce that particular configuration. Very recently, however, the isomeric \(l\)-configuration was detected in three lanthipeptides, haloduracin \(\beta\) and both peptides of the enterococcal cytolysin, despite the fact that these compounds are biosynthesized by enzymes that have high sequence homology with other lanthipeptide synthetases.\textsuperscript{113} Adding further structural diversity to the lanthipeptides was the discovery of a third type of crosslink, labionin, which is formed by a second Michael addition between the enolate intermediate produced during Lan formation and another Dha to give a carbacycle (Fig. 1.7a).\textsuperscript{158, 159} Following crosslink installation, the maturation process is completed by removal of the leader peptide via proteolysis and export from the producing cell (Fig. 1.7b). If the final product contains other tailoring PTMs, these moieties are installed by additional enzymes encoded in the biosynthetic gene cluster,\textsuperscript{135-137, 160, 161} but the order of these events with respect to other biosynthetic steps remains uncertain.
Biochemical and genetic analyses have revealed four distinct classes of lanthipeptides on the basis of their biosynthetic machinery (Fig. 1.8).\textsuperscript{112, 164} For class I lanthipeptides, such as nisin, microbisporicin, and Pep5, dehydration and cyclization are performed by two separate enzymes: the dehydratase LanB and the cyclase LanC, respectively.\textsuperscript{162, 165, 166} The very recent \textit{in vitro} reconstitution of the nisin dehydratase NisB has finally provided insight into LanB catalysis, which involves glutamylation of substrate serine/threonine residues followed by elimination.\textsuperscript{167} LanC catalysis has been investigated through structural analysis of the nisin cyclase NisC, which revealed a critical active-site zinc ion bound by three conserved residues.
that is believed to activate the cysteinyl thiol for nucleophilic attack.\textsuperscript{168, 169} For class II lanthipeptides, such as mersacidin, lactacin 3147, lactacin 481, and cinnamycin, the dehydration and cyclization reactions are catalyzed by a single bifunctional synthetase, LanM.\textsuperscript{137, 170-172} Mechanistic insights have been provided by the \textit{in vitro} reconstitution of the lactacin 481 synthetase LctM, which uses an N-terminal dehydratase domain to catalyze dehydration via a phosphorylation/elimination sequence with adenosine 5’-triphosphate as cosubstrate\textsuperscript{173} and a C-terminal cyclase domain homologous to LanC.\textsuperscript{174}

The remaining two classes of lanthipeptide biosynthetic machinery have been discovered more recently. Class III lanthipeptides, such as SapB and the labyrinthopeptins, are modified by a trifunctional synthetase, called RamC or LanKC, which contains an N-terminal lyase domain, a central kinase domain, and a C-terminal cyclase domain that lacks many of the conserved active-site residues found in LanC and LanM.\textsuperscript{158, 175, 176} Interestingly, the discovery of additional class III lanthipeptides in the past year has revealed that LanKC is able to catalyze the formation of both Lan and labionin,\textsuperscript{176-178} although the mechanism of cyclization remains unknown. Finally, the class IV synthetase, LanL, has been described in the context of the novel lanthipeptide venezuelin and contains N-terminal lyase and kinase domains as in class III synthetases, but its C-terminal cyclase domain is homologous to LanC.\textsuperscript{112, 179} Taking these studies together, the multiplicity of distinct pathways to generate lanthipeptides demonstrates the convergent evolution of efficient, ribosome-based biosynthetic strategies and highlights the potential evolutionary advantage of accessing high chemical diversity at low genetic cost.\textsuperscript{104, 164, 180}
1.4.2. Modes-of-action of lanthipeptides

As the original class name “lantibiotic” suggests, many lanthipeptides are antibiotics, and more specifically antibacterials that target primarily Gram-positive organisms. The mechanisms by which lantibiotics exert their antibacterial activity have been studied extensively in only a few instances, with nisin as the best understood case at present. Nisin has been demonstrated to employ two related mechanisms involving the essential peptidoglycan precursor lipid II (Fig. 1.9), a combination that results in very potent activity. Nisin binds to the pyrophosphate moiety of lipid II through a hydrogen bond network involving its A- and B-rings; in doing so, it both inhibits the transglycosylation reaction necessary to synthesize peptidoglycan and also sequesters lipid II into nonfunctional locations. Interaction with lipid II is not a novel antibacterial mechanism, as glycopeptides and other antibacterial natural products are known to do the same, but nisin binds at a unique site in lipid II and shows no cross-resistance with vancomycin (Fig. 1.9). Furthermore, once bound to lipid II, nisin is able to

![Fig. 1.9. Schematic of the two major lipid II-based reactions involved in the biosynthesis of peptidoglycan. Transglycosylation adds membrane-bound disaccharide units onto the growing polysaccharide chain, while transpeptidation crosslinks different chains through the lipid II pentapeptide segment. The lipid II binding sites of nisin and vancomycin are indicated.](image)
insert into the membrane and form pore complexes, leading to rapid membrane depolarization and termination of cellular processes. The nisin-lipid II binding motif is found in other class I lanthipeptides (Fig. 1.6), and while these compounds inhibit peptidoglycan biosynthesis, only some of them are able to form pores. Recently, nisin has been shown to bind to intermediates in the wall teichoic acid biosynthetic pathway, which may represent a further diversification of its antibacterial mechanisms. Intriguingly, the class I lanthipeptides Pep5, epilancin K7, and epilancin 15X, which do not possess the nisin-like binding motif, are able to form pores without interacting with lipid II, suggesting that they may bind a different biological target.

A second lipid II binding motif has been described for the class II lanthipeptide mersacidin (Fig. 1.6), which interacts with lipid II though its C-ring and inhibits transglycosylation, but does not form pores. This binding motif contains an essential acidic residue conserved in other class II lanthipeptides; mutation to a non-acidic residue has been shown to reduce severely or abolish activity in a variety of instances. The importance of this conserved residue for interaction with lipid II was recently confirmed in the cases of nukacin ISK-1, a natural variant of lacticin 481, and the α-peptide of haloduracin. For the two-component lanthipeptides lacticin 3147 and haloduracin, the α-peptide shares the mersacidin-like motif and binds lipid II, and this complex then recruits the β-peptide in order to form pores. This mode-of-action explains the observation that the individual peptides of two-component lanthipeptides display modest to negligible activity by themselves, but potent and synergistic activity in combination.

Beyond antibacterial activity, lanthipeptides have demonstrated additional biological activities in recent years. Cinnamycin and its natural variants, the duramycins, bind to the membrane phospholipid phosphatidylethanolamine; this interaction inhibits the release of arachadonic acid by phospholipase A2, which in turn inhibits the eicosanoid biosynthetic pathway. This mechanism has implications in the treatment of cystic fibrosis, and one duramycin is currently in clinical trials for this indication. The class III labyrinthopeptins have demonstrated antiviral and antiallodynic activities, both unique for lanthipeptides, although the underlying mechanisms are not yet known. The class III lanthipeptides SapB and SapT trigger morphogenic changes in streptomycetes, possibly through a surfactant-like mechanism. Furthermore, nisin has recently demonstrated both immunomodulatory and
antitumor activities,\textsuperscript{210} which are very intriguing discoveries for a molecule that is already considered safe for human consumption.

1.5. SUMMARY AND OUTLOOK

In the search for new therapeutic drugs, natural products have been, and continue to be, of utmost importance both as direct medicinal sources and as guides in molecular design. Bioactive peptides, while lacking the storied history of natural products in drug development, have more recently become recognized as viable therapeutic systems, thanks in part to advances in their stabilization and production. Therefore, it seems fitting that peptide-based natural products are emerging as a critical source of new drugs, particularly in the search for new antibacterial agents to combat the rapid rise of drug-resistant bacterial pathogens. One subfamily of peptide natural products, the lanthipeptides, have been investigated for their potent biological activities and their fascinating modes-of-action. However, as with many natural product- and peptide-based drug leads, the clinical application of lanthipeptides and lantibiotics requires general and versatile methods to analyze structure-activity relationships and biological mechanisms, and further to use this information in the design of next-generation compounds with enhanced pharmacological profiles.

This thesis presents the development of synthetic platforms to generate medicinally interesting analogues of lantibiotics and other bioactive cyclic peptides. These approaches were anticipated to yield valuable information about the biological activities of these compounds that in turn would guide engineering efforts to improve therapeutic potential; in this light, several advances have already been made. In Chapter 2 of this thesis, a chemoenzymatic approach to lanthipeptide synthesis is described, in which the biosynthetic machinery of the lantibiotic lacticin 481 was engineered and reconstituted \textit{in vitro} to process synthetic linear substrates into the desired bioactive products in a single step. In this way, a panel of lacticin 481 analogues were produced, and several of these compounds were found to possess improved potency to the natural product. This panel of analogues was further used to establish the mode-of-action of lacticin 481: inhibition of peptidoglycan biosynthesis without pore formation. In Chapter 3, an alternative approach for the generation of lantibiotic analogues via total chemical synthesis is described. A collection of orthogonally-protected Lan/MeLan building blocks were synthesized and utilized to construct analogues of the lantibiotics epilancin 15X and lacticin 481 on the solid-
phase. Epilancin 15X analogues were used to investigate the importance of different PTMs for antibacterial activity and also to determine that epilancin 15X is a pore-forming agent. Lacticin 481 analogues containing stereoisomeric Lan/MeLan crosslinks demonstrated no biological activity, shedding light on the importance of stereochemistry during the evolution of these natural products. Finally, Chapter 4 describes a synthetic approach for the in vivo stabilization of disulfide-containing bioactive peptides by substitution of the disulfide bond with cystathionine, an isosteric monosulfide crosslink. Compstatin, a disulfide-containing immunomodulatory peptide, was used as a model system to demonstrate that this substitution improves the molecule’s reductive stability without sacrificing potency. Taken together, these studies have established versatile synthetic approaches to construct bioactive cyclic peptides, and also have revealed details about the molecular underpinnings of biological activity in two lantibiotics. It is hoped that these platforms will enable future efforts intended to better understand and engineer lantibiotics as potential drugs, as well as to stabilize disulfide-containing therapeutic peptides.

1.6. REFERENCES


CHAPTER 2: BIOSYNTHETIC ENGINEERING AND MODE-OF-ACTION
OF THE LANTIBIOTIC LACTICIN 481*†

2.1. INTRODUCTION

As introduced in Chapters 1.3 and 1.4, large-scale structure-activity relationship (SAR) analyses are necessary in order to develop ribosomally synthesized and post-translationally modified peptide natural products (RiPPs) as clinical drugs. The use of biosynthetic engineering to generate panels of analogues has several key advantages for RiPPs compared to other families of natural products.1, 2 As the precursor peptide is genetically encoded, any mutation to its corresponding gene is directly conveyed to the final product, provided that the mutation is tolerated by the biosynthetic enzymes. The biosynthetic pathways for RiPPs are well suited for bioengineering because they are considerably shorter than those for other natural products, and the enzymes involved in post-translational modification often exhibit substantial substrate promiscuity. This latter observation is likely due to the leader-directed nature of the biosynthesis, which allows for variable core peptides to be recognized and modified by a single biosynthetic enzyme in several natural systems.6-9 Recent successes in the production of analogues of thiopeptides,10, 11 bottromycins,12 and cyanobactins13 have begun to bear out the potential of RiPP biosynthetic engineering.

Of the many subfamilies of RiPPs, the lanthipeptides (see Chapter 1.4 for an introduction) have been particularly fruitful for bioengineering, which has resulted in both an improved understanding of SAR and more potent analogues.14-18 In vivo bioengineering in producing strains and heterologous hosts has resulted in versatile biosynthetic systems for lanthipeptide engineering,15, 19-24 including the introduction of stabilizing lanthionine crosslinks into other therapeutically relevant peptides.25-28 Of particular interest is heterologous expression in Escherichia coli, which greatly expands the genetic toolbox accessible for lanthipeptide bioengineering and sometimes results in improved yields of the desired product compared to natural producing strains.29-34 Alternatively, the in vitro reconstitution of biosynthetic enzymes

allows for a detailed examination of mechanism and substrate scope, and precludes complications inherent to in vivo bioengineering relating to expression, export, and toxicity. The activity of one or more enzymes involved in the biosynthesis of several lanthipeptides, including epidermin,\textsuperscript{35} lacticin 481,\textsuperscript{36, 37} nisin,\textsuperscript{38} haloduracin,\textsuperscript{39} the prochlorosins,\textsuperscript{6} the labyrinthopeptins,\textsuperscript{40} venezuelin,\textsuperscript{41} cinnamycin,\textsuperscript{32} bovicin HJ50,\textsuperscript{33} epilancin 15X,\textsuperscript{42} actagardine,\textsuperscript{30} and catenulipeptin\textsuperscript{43} have been successfully reconstituted in vitro. Of these, the lacticin 481 synthetase LctM has been the most rigorously investigated. As a class II synthetase, LctM catalyzes both dehydration and cyclization of its substrate, the lacticin 481 precursor peptide LctA (Fig. 2.1). The mechanism of dehydration involves phosphorylation of substrate serine and threonine residues using adenosine 5’-triphosphate (ATP) as cosubstrate, followed by elimination of phosphate to generate the α,β-unsaturated structures 2,3-didehydroalanine (Dha) and (Z)-2,3-dedehydrobutyryne (Dhb).\textsuperscript{44} A separate domain in LctM is believed to catalyze cyclization using an active-site zinc ion,\textsuperscript{45} analogous to cyclization by the class I nisin cyclase NisC.\textsuperscript{38, 46}

![Figure 2.1](image_url)

**Figure 2.1.** Biosynthesis of lacticin 481. (a) LctM catalyzes both dehydration and cyclization of the linear precursor peptide LctA. The leader peptide is then removed by the cysteine protease/transporter LctT, resulting in the mature lantibiotic. For the chemical structures of modified residues, see Fig. 1.6. (b) Catalytic mechanism of dehydration by LctM, using ATP as a cosubstrate.
Lacticin 481 is a tricyclic class II lantibiotic from the dairy bacterium *Lactococcus lactis* subsp. *lactis* CNRZ 481, first reported in the early 1990s.\(^{47}\) A panel of natural analogues of lacticin 481 containing an identical ring topology, including nukacin ISK-1 and members of the salivaricins, have been isolated from other Gram-positive bacteria, although little was known about the mechanism(s) of antimicrobial activity of this subfamily at the outset at this work.\(^{48}\) Lacticin 481 appears to contain a motif within its A-ring similar to the C-ring of mersacidin (*Fig. 1.6*), which is known to be involved in binding the essential peptidoglycan precursor lipid II.\(^{49}\) The *in vitro* reconstitution of LctM, and its substantial substrate promiscuity, has permitted the construction of lacticin 481 analogues and other lanthionine-containing peptides, including those bearing non-proteinogenic amino acids.\(^{50-53}\) In a synthetic scheme termed *in vitro* mutasynthesis (IVM), synthetic linear LctA analogues can be generated through chemical synthesis, then treated with LctM to install the desired modifications (*Fig. 2.2*); removal of the leader peptide with a commercial protease yielded a panel of lacticin 481 analogues containing non-proteinogenic amino acids, several of which possessed antibacterial activity.\(^{54}\) However, the number of synthetic manipulations and purifications inherent in this IVM scheme severely restricted its throughput and yield, preventing quantitative analysis of SAR, exploration of mode-of-action, and application to the construction of combinatorial peptide libraries. Additionally, the use of LysC for leader proteolysis resulted in lacticin 481 analogues lacking the N-terminal lysine residue, which reduces potency compared to the full-length natural product.\(^{54}\)

**Figure 2.2.** *In vitro* mutasynthesis of truncated lacticin 481 mutants. Individually mutated residues are shown in the bottom structure. TBTA, tris-(benzyltriazolylmethyl)amine.
In this chapter, an engineered \textit{in vitro} biosynthetic system is described that overcomes the limitations of the IVM approach shown in \textit{Fig. 2.2}. This system has both streamlined the production of lacticin 481 analogues and also challenged previous beliefs about the role of the leader peptide in biosynthesis. In addition, analogues produced using this system were used to determine that, like mersacidin, lacticin 481 inhibits transglycosylation involved in cell wall biosynthesis and contains a conserved glutamic acid residue essential for activity. This work was the result of an extensive collaboration with Dr. Trent Oman, a 2011 doctoral graduate of the University of Illinois at Urbana-Champaign (UIUC), and experimental support from a number of other researchers at UIUC and Harvard University. The individual contributions of each coworker are mentioned throughout the chapter and are listed in detail in the Experimental (Section 2.4).

2.2. RESULTS AND DISCUSSION

2.2.1. \textit{In trans} activity of LctM

The primary source of inefficiency in the IVM approach described above is the need to attach and subsequently remove the leader peptide, which is not incorporated in any fashion into the final product. In the development of LctM-catalyzed IVM, LctM was found to be able to process LctA core peptides, although incompletely, when the LctA leader peptide was provided \textit{in trans}, or even in the complete absence of the leader peptide.\textsuperscript{55} To the best of our knowledge, the only other reported examples of leader-directed post-translational modification showing \textit{in trans} activity are the vitamin K-dependent $\gamma$-carboxylation of glutamate during the maturation of the conopeptide conantokin-G,\textsuperscript{56} \textit{in vitro} dehydration of the labionin-containing lanthipeptide catenulipeptin by the class III synthetase AciKC,\textsuperscript{43} \textit{in vitro} lasso peptide formation in microcin J25 by McjB/McjC,\textsuperscript{57} and partial \textit{in vivo} processing of nisin by NisB/NisC.\textsuperscript{58} These interesting observations contest an existing model in leader-directed biosynthesis, that the leader peptide is absolutely required for substrate recognition by the biosynthetic machinery.\textsuperscript{3-5} Therefore, optimization of the \textit{in trans} activity of LctM to produce fully-modified lacticin 481 and analogues may represent a more efficient and scalable IVM approach.

In order to investigate further the \textit{in trans} activity of LctM, standard solid-phase peptide synthesis (SPPS) was used to construct a panel of LctA core peptides (residues 1-27, \textit{Fig. 2.3}):
wild-type   KGGSGVIHTISHECNMNSTrpQPheVFTCCS  
E13A   KGGSGVIHTISHACNMNSTrpQPheVFTCCS  
N15R/F21H   KGGSGVIHTISHECRMNSTrpQHisVFTCCS  
N15R/F21Pal   KGGSGVIHTISHECRMNStrpQPalVFTCCS  
N15R/W19Nal/F21H   KGGSGVIHTISHECRMNStrpQNalQHisVFTCCS  
N15R/W19Nal/F21Pal   KGGSGVIHTISHECRMNStrpQNalQPalVFTCCS

Figure 2.3. LctA core peptide sequences synthesized and studied in this chapter. Modified Ser and Thr residues are highlighted in green and purple, respectively, and residues mutated from wild-type are shown in orange. Nal, 3-(2-naphthyl)alanine; Pal, 3-(4’-pyridyl)alanine.

The wild-type sequence, a mutant replacing the conserved Glu13 residue (see Section 2.2.6), a double mutant N15R/F21H, and three mutants containing non-proteinogenic amino acids (see Section 2.2.4). For initial studies, the N15R/F21H mutant was used due to its improved solubility to the wild-type sequence; importantly, the final product obtained after processing of this mutant is known to possess comparable antibacterial activity to authentic lacticin 481.\(^{44}\) The LctA leader peptide (residues -24 to -1, Fig. 2.1), synthesized by SPPS, was provided by Dr. Matthew Levengood (UIUC), and LctM was overexpressed in E. coli and purified by Dr. Oman.

In vitro enzymatic assays were conducted similarly to previous reports.\(^{36, 55, 59}\) LctM (2 µM) and the LctA core peptide substrate (20 µM) were incubated at room temperature in the presence of ATP, MgCl\(_2\) and pH 7.5 buffer, and substrate dehydration was monitored by matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF MS). By increasing the concentration of the LctA leader peptide from 2 to 20 µM, the in trans activity of LctM could be improved to the point where the desired four-fold dehydrated substrate was present in substantial amounts, although varying amounts of incompletely processed substrates were also present (Fig. 2.4a). As cyclization cannot be directly monitored by MS, analytical high-performance liquid chromatography (HPLC) and antibacterial assays against the indicator strain Lactococcus lactis subsp. cremoris HP were used to determine if the desired crosslinks were also installed, as all three crosslinks of lacticin 481 are essential for biological activity.\(^{50}\) Indeed, the similar HPLC elution times and antibacterial activities of the purified in trans assay product to those of authentic lacticin 481 confirmed complete cyclization (Fig. 2.4b). This represented the first demonstration that a fully processed, biologically active lacticin 481 analogue could be produced via an in trans enzymatic reaction, as previous in trans reactions
2.4. In trans activity of LctM to process LctA core peptide N15R/F21H. (a) MALDI-TOF mass spectra of N15R/F21H modified by LctM at varying concentrations of LctA leader peptide. Final assay concentrations were 20 μM substrate and 2 μM enzyme, and reactions were run for 3 h. The number above each peak indicates the number of dehydrations; an asterisk indicates a phosphorylated product; SM indicates starting material. (b) Analytical HPLC comparison of purified, four-fold dehydrated product of the in trans LctM reaction (blue) with authentic lacticin 481 (red), showing very similar retention times. Insert: antibacterial activity of the purified product (top, 50 μM, 20 μL) and authentic lacticin 481 (bottom, 50 μM, 20 μL) against L. lactis HP.

only produced incompletely processed intermediates. When compared to the IVM approach in Fig. 2.2, this process reduces the number of chemical transformations needed to convert the synthetic core peptide substrate to the final product from four to one and also preserves the N-terminal lysine of lacticin 481 in the final product.

2.2.2. Development of LctCE-(GS)15, a constitutively active leader-LctM fusion enzyme

The surprising in trans activity of LctM challenges the notion that the leader peptide is absolutely required for post-translational modification of LctA. As the leader and core peptides were not covalently linked, a direct role of the leader peptide in substrate recognition is unlikely. Instead, binding of the leader peptide to LctM may serve to stabilize or “trap” an active conformation of the enzyme, shifting an equilibrium population of conformations from predominantly inactive for catalysis to predominantly active, and thus permitting the processing of the LctA core peptide with greater efficiency (Fig. 2.5a). If this model is accurate, then covalent attachment of the leader peptide to LctM itself may increase the effective molarity
Figure 2.5. Proposed model of LctM activation by the LctA leader peptide. (a) LctM is thought to exist as an equilibrium population of inactive and active conformations, which is shifted upon binding of the LctA leader peptide (red) toward the more active conformation for catalysis. (b) Tethering the leader peptide to LctM via a flexible linker (purple) may result in an enzyme trapped in the active conformation, permitting modification of the leaderless LctA core peptide (green). Figure courtesy of Dr. Trent Oman (UIUC).

between the species and further improve the catalytic activity of the enzyme (Fig. 2.5b). The resulting fusion enzyme would thus possess constitutive activity to modify leaderless core peptide substrates. This design is reminiscent of an approach used successfully to generate a constitutively active protease by fusing the NS3 protease/helicase from hepatitis C virus to its activator protein. Additionally, if the linker region between the leader peptide and LctM could be made of proteinogenic amino acids, the fusion enzyme could be recombinantly expressed. Thus, the overall efficiency of the IVM process could be further improved, as the leader peptide would no longer need to be synthesized separately.

In order to test this hypothesis of a constitutively active fusion LctM, a series of expression constructs were prepared by Dr. Oman, which encoded a hexa-histidine-tagged LctA leader peptide sequence fused to the N-terminus of LctM via a flexible linker composed of varying numbers of repeating glycine and serine residues. These fusion enzymes, termed LctCE-(GS)$_n$ (where $n$ is the number of GlySer repeats), were heterologously expressed in E. coli and purified by immobilized metal affinity chromatography. In vitro reconstitution of enzymatic activity, as for the in trans reactions in Section 2.2.1 except omitting the separate leader peptide, revealed that these fusion enzymes were able to process the leaderless LctA core peptide, and that catalytic efficiency correlated positively with linker length up to (GS)$_{15}$, the longest linker investigated (Fig. 2.6). In the absence of structural information, the exact influence of linker
length on LctCE efficiency is difficult to ascertain, but it can be speculated that the longer linker may allow sufficient conformational freedom for the leader peptide to reach its putative binding site in the synthetase.

2.2.3. Comparison of LctCE-(GS)$_{15}$ and LctM in trans

In order to provide definitive evidence of the importance of tethering the leader peptide to the synthetase, several comparisons between LctCE-(GS)$_{15}$ and LctM with the leader peptide provided in trans were made. To probe ring formation, aliquots of enzymatic assays were treated with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP), a thiol-selective cyanolyzing agent; incomplete ring formation is detected by the presence of cyano adducts of the peptide (Fig. 2.7). At early timepoints in both reaction systems, the dominant species is a three-fold dehydrated intermediate, which possesses between one and three crosslinks. After 60 min incubation, the four-fold dehydrated species in both systems appears and does not react with CDAP. This indicates that all three cysteines in the fully dehydrated assay product were properly cyclized by both LctCE-(GS)$_{15}$ and LctM in trans. The four-fold dehydrated assay products of both reaction systems could be purified by RP-HPLC and demonstrated nearly

Figure 2.6. MALDI-TOF MS analysis of LctA core peptide N15R/F21H processing by LctCE enzymes. Increasing linker length results in progressively improved enzyme efficiency. Final assay concentrations were 20 μM substrate and 2 μM enzyme, and reactions were run for 1 h. The number above each peak indicates the number of dehydrations; as asterisk indicates a phosphorylated product; SM indicates starting material. Figure courtesy of Dr. Trent Oman (UIUC).
identical mass spectra (Fig. 2.8) and antibacterial activity (Fig. 2.9), final indications that the desired lacticin 481 analogues were indeed generated in both cases.

**Figure 2.7.** Analysis of ring formation by LctCE-(GS)\textsubscript{15} and LctM using CDAP. MALDI-TOF mass spectra are shown for assays of LctA core peptide N15R/F21H modified by LctCE-(GS)\textsubscript{15} after (a) 5 min and (b) 60 min, and modified by LctM with the leader provided in trans after (c) 5 min and (d) 60 min. “+\text{x}CN” designates \text{x} number of cyano adducts indicative of incomplete cyclization. Final assay concentrations were 20 μM substrate and 2 μM enzyme, and 20 μM of LctA leader peptide for in trans assays.
Figure 2.8. MALDI-TOF mass spectra of purified, four-fold dehydrated assay products of LctA core peptide N15R/F21H and (a) LctCE-(GS)$_{15}$ or (b) LctM in trans with 20 μM LctA leader peptide. Calc. [M+H]$^+$ 2932.3. Sodium adducts are indicated with an asterisk.

Figure 2.9. Antimicrobial agar diffusion assay against L. lactis HP for the purified assay product of LctA core peptide N15R/F21H incubated with LctCE-(GS)$_{15}$ or LctM with the leader provided in trans (20 μL aliquots).

A comparison of processing activity at different time points revealed the superiority of LctCE-(GS)$_{15}$ to LctM in trans for producing fully-modified lacticin 481 analogues (Fig. 2.10). While both systems generated the partially-processed, three-fold dehydrated intermediate within 5 min, conversion to the four-fold dehydrated product proceeded more quickly with LctCE-(GS)$_{15}$ (Fig. 2.10a) than with LctM in the presence of a 10-fold excess of LctA leader peptide over enzyme (Fig. 2.10b). Considering that the leader peptide is present in an effective stoichiometry of 1:1 with enzyme in the fusion construct, comparison to an in trans system with equimolar leader peptide and LctM was appropriate (Fig. 2.10c). These conditions produced only trace amounts of the four-fold dehydrated product after 3 h, highlighting the importance of covalently fusing the leader and enzyme in LctCE-(GS)$_{15}$ for improved catalytic efficiency.
Figure 2.10. Comparison of the relative processing activity of LctA core peptide N15R/F21H by (a) LctCE-(GS)$_{15}$; (b) LctM in trans with excess LctA leader peptide (20 μM); and (c) LctM in trans with equimolar LctA leader peptide (2 μM). Reactions were quenched as the indicated times and analyzed by MALDI-TOF MS. The number above each peak indicates the number of dehydrations; SM indicates starting material. Final assay concentrations were 20 μM substrate and 2 μM enzyme.

2.2.4. Generation of lacticin 481 analogues

The enzymatic activity of LctCE-(GS)$_{15}$ to produce a bioactive lacticin 481 analogue prompted investigation of its ability to process other LctA core peptide analogues containing non-proteinogenic amino acids, as demonstrated for LctM with leader-containing substrates.$^{51-54}$ Lacticin 481 analogues containing 3-(4'-pyridyl)alanine (Pal) or 3-(2-naphthyl)alanine (Nal) were identified previously to possess near wild-type activity (when normalizing for the truncation of Lys1 in the IVM approach used).$^{54}$ To examine if LctCE-(GS)$_{15}$ preserved the substrate promiscuity of LctM, several double and triple mutants of the LctA core peptide were synthesized containing combinations of the following mutations (Fig. 2.3): Asn15Arg, Phe21His, Phe21Pal, and Trp23Nal. As demonstrated in Fig. 2.11, all four substrates were accepted by LctCE-(GS)$_{15}$, with substantial amounts of the four-fold dehydrated species produced. In most instances, considerable amounts of a three-fold dehydrated intermediate and a three-fold dehydrated, singly phosphorylated intermediate were also present. The incompletely processed residue was identified as Thr24,$^{61}$ which is not involved in ring formation but lies adjacent to two cysteines that are cyclized. The high degree of structural complexity in this region of lacticin 481, coupled with the lack of a leader peptide attached to the substrate, may hinder the enzyme from accessing this residue for phosphorylation or subsequent elimination, resulting in these incompletely-processed species. This observation also agrees with the N-to-
Figure 2.11. MALDI-TOF MS analysis of processing of LctA core peptide mutants by LctCE-(GS)$_{15}$. Starting material (blue) and crude assay products (red) after 3 h incubation are shown, with major products labeled, for (a) N15R/F21H, (b) N15R/F21Pal, (c) N15R/W19Nal/F21H, and (d) N15R/W19Nal/F21Pal. Figure adapted from the doctoral thesis of Dr. Trent Oman (UIUC).

C directionality of processing by LctM proposed in previous work, although those studies were performed with full-length LctA substrate. The four-fold dehydrated assay products could be separated from the other reaction products by RP-HPLC, giving pure lacticin 481 analogues on half-milligram scales for biological evaluation (Fig. 2.12).

Nukacin ISK-1 is a lantibiotic that possesses an identical set of post-translational modifications as lacticin 481, but contains a total of seven mutated residues. To probe further the substrate promiscuity of LctCE-(GS)$_{15}$, the nukacin ISK-1 core peptide NukA(1-27) was synthesized and incubated with the fusion enzyme. While not the dominant species, a substantial amount of the fully-processed, four-fold dehydrated species was obtained (Fig. 2.13). This observation fits well with previous experiments that demonstrated LctM could process a
Figure 2.12. MALDI-TOF mass spectra of LctCE-modified, purified lacticin 481 mutants (a) N15R/F21H, (b) N15R/F21Pal, (c) N15R/W19Nal/F21H, and (d) N15R/W19Nal/F21Pal. Figure adapted from the doctoral thesis of Dr. Trent Oman (UIUC). 61

Figure 2.13. MALDI-TOF MS analysis of processing of the NukA core peptide by LctCE-(GS)₁₅. In the peptide sequence, residues mutated from LctA are highlighted in orange.
chimeric substrate consisting of the LctA leader peptide and NukA core peptide as well as the full-length NukA precursor peptide, despite the divergence between their sequences.\(^{59}\)

**2.2.5. Biological activities of lacticin 481 analogues**

With these purified lacticin 481 analogues in hand, an analysis of biological activity was performed by Dr. Trent Oman (UIUC). Whole-cell growth inhibition assays against the sensitive strain *L. lactis* HP (Fig. 2.14) demonstrated that the lacticin 481 double mutants N15R/F21H (IC\(_{50}\) = 428 ± 21 nM) and N15R/F21Pal (213 ± 9 nM) possessed improved antibacterial potency compared to the authentic compound (785 ± 19 nM). In addition, the triple mutants N15R/W19Nal/F21H (1370 ± 48 nM) and N15R/W19Nal/F21Pal (2420 ± 60 nM) were found to be weaker growth inhibitors, giving a range of potencies over an order of magnitude with authentic lacticin 481 as the median.

As mentioned in Section 2.1, lacticin 481 appears to contain within its A-ring the mersacidin-like lipid II binding motif (TXS/TXD/EC, where X is any residue), which is also present in several other class II lantibiotics known to interact with lipid II and disrupt

![Figure 2.14](image)

**Figure 2.14.** Quantitative growth inhibition analysis for authentic lacticin 481 and analogues against *L. lactis* HP in liquid culture. Calculated IC\(_{50}\) values are shown in the legend. Error bars represent the standard deviation from triplicate experiments. Figure courtesy of Dr. Trent Oman (UIUC).
Evidence that lacticin 481 forms complexes with lipid II was first provided in 2009 using thin-layer chromatography (TLC); more recently, a binding interaction between lipid II and nukacin ISK-1, which has an identical ring topology to lacticin 481, was quantitatively determined using isothermal titration calorimetry. These observations suggested that lacticin 481 likely exerts its antibacterial activity, at least in part, via binding lipid II and inhibiting peptidoglycan formation. Therefore, Dr. Oman, in collaboration with Dr. Tania Lupoli and Prof. Suzanne Walker (Harvard Medical School), investigated whether the changes in antibacterial potency seen in the panel of lacticin 481 analogues could be correlated with inhibition of transglycosylation using an established TLC-based assay. The transglycosylase PBP1b from *Escherichia coli* and a radiolabeled analogue of lipid II were incubated with lacticin 481 and analogues, and the inhibition of transglycosylation was quantitated. The double mutants N15R/F21H (IC$_{50}$ = 7.0 ± 2.9 μM) and N15R/F21Pal (5.4 ± 1.2 μM) exhibited stronger inhibition than lacticin 481 (12 ± 2.3 μM), while the triple mutants N15R/W19Nal/F21H (27 ± 5.6 μM) and N15R/W19Nal/F21Pal (105 ± 34 μM) were weaker inhibitors. These results correlate strongly with the whole-cell antibacterial potencies measured in Fig. 2.14 and are similar to the IC$_{50}$ values determined for known lipid II binders haloduracin.

**Figure 2.15.** Inhibition of PBP1b-catalyzed peptidoglycan (PG) formation by authentic lacticin 481 and analogues, at 4 μM lipid II and 100 nM PBP1b. Calculated IC$_{50}$ values are shown in the legend. Error bars represent the standard deviation from triplicate experiments. Figure courtesy of Dr. Trent Oman (UIUC).
and ramoplanin using the same assay. Furthermore, a whole-cell microscopy study has recently demonstrated that fluorescently-labeled lipid II localizes to the membrane in the same manner as known lipid II binders. Taken together, these results strongly suggest that lacticin 481 exerts its biological activity via binding lipid II and inhibiting peptidoglycan biosynthesis.

2.2.6. Determination of the importance of Glu13 for antibacterial activity

The mersacidin-like lipid II binding motif (see Section 2.2.5) contains a conserved aspartate/glutamate residue that has been established in multiple instances as critical for antibacterial activity; mutation to a non-acidic residue abolishes or severely attenuates the activity of a variety of class II lantibiotics. A previous report stating that the same conserved residue in lacticin 481, Glu13, could be mutated without substantial loss in activity was therefore surprising and merited additional investigation. LctA core peptides representing the wild-type sequence and an E13A mutant (Fig. 2.3) were synthesized by SPPS, and incubation with LctCE-(GS)\textsubscript{15} resulted in production of the four-fold dehydrated product as well as several incompletely processed intermediates (Fig. 2.16). It should be noted that these assays proceeded substantially less to completion than assays with the double/triple mutants (Fig. 2.11). This reduced efficiency is likely due to the decreased solubility of the substrate under these assay conditions, as has been discussed previously. The crude enzymatic assays for these substrates, as well as the more soluble N15R/F21H mutant, were applied to a lawn of L. lactis to test for

![Figure 2.16](attachment:image.png)

**Figure 2.16.** MALDI-TOF MS analysis of LctA core peptide processing by LctCE-(GS)\textsubscript{15}. Crude assay products are shown after 8 h incubation, with major products labeled for (a) wild-type and (b) E13A LctA core peptides.
antibacterial activity; unlike the modified wild-type and N15R/F21H peptides, the modified E13A mutant possessed no discernible activity (Fig. 2.17). With regard to the previous study demonstrating activity for lacticin 481 E13A, the amount of peptide used in the bioassay was not quantified, and a relatively high concentration may have inadvertently been applied that gave the impression of preserved activity. In the case of haloduracin α, the corresponding E22Q mutant was 40-fold less potent than the wild-type, but nonetheless did possess observable activity. In all, these experiments provided further evidence that lacticin 481 behaves similarly to other class II lantibiotics containing the mersacidin-like lipid II binding motif.

![Figure 2.17. Antimicrobial agar diffusion assay against L. lactis HP for crude LctCE-(GS)_{15} assay products (100 μM total peptide concentration, 20 μL) and authentic lacticin 481 (50 μM, 20 μL).](image)

**2.2.7. Lacticin 481 analogue activity against L. lactis CNRZ 481**

One of the inherent complications of *in vivo* mutagenesis of lantibiotics is the potential for exceptionally potent or highly mutated variants to circumvent the producing cell’s immunity mechanisms, killing the cell and preventing discovery in activity screens. However, in the context of developing better clinical antibiotics, such lantibiotics would be of great interest, as they are already capable of overcoming a natural resistance mechanism. In this light, the two lacticin 481 analogues with improved potency (N15R/F21H and N15R/F21Pal, Fig. 2.14) were tested for their ability to kill the natural lacticin 481 producing strain, *L. lactis* subsp. *lactis* CNRZ 481. This strain expresses an ABC transporter complex LctFEG critical for immunity to lacticin 481, likely through expulsion of the compound from the membrane. Crude LctCE-(GS)_{15} assay products were applied to lawns of *L. lactis* 481, and despite their potent activity against the sensitive non-producer *L. lactis* HP, neither of the double mutants showed substantially improved activity compared to wild-type against the producing strain (Fig. 2.18). While the rather modest alterations in the double mutants were not enough to evade the natural
immunity mechanisms, it is feasible that continued diversification of lacticin 481 analogues using this chemoenzymatic platform may produce variants able to kill the producing organism.

2.2.8. Flow cytometry analysis of lacticin 481 membrane disruption

In addition to disrupting cell wall biosynthesis, several lantibiotics have been demonstrated to form pores in the membranes of susceptible bacterial strains, using lipid II as a docking molecule. This phenomenon has been best characterized in the class I lantibiotic nisin\(^79\)\(^-\)\(^81\) and the two-component class II systems lacticin 3147\(^66\) and haloduracin.\(^67\),\(^68\) The combination of pore formation and inhibition of cell wall biosynthesis, mediated by lipid II binding, is critical for the full potency of these molecules. Indeed, several nisin variants unable to form pores still possess antimicrobial activity, although up to 100-fold less potent than the parent molecule.\(^82\) However, shorter lantibiotics such as mersacidin and mutacin 1140 are able to exert potent biological activity without forming pores, perhaps dictated by stronger affinity for lipid II.\(^81\),\(^83\),\(^84\)

To determine if lacticin 481 forms membrane-disrupting pores, two complementary flow cytometry assays were performed (Fig. 2.19). In the first, the membrane potential-sensitive fluorescent dye 3,3’-diethyloxacarbocyanine iodide (DiOC\(_2\)(3)) was used to monitor changes in membrane polarization in the presence of lacticin 481 or nisin.\(^85\) Bacillus subtilis ATCC 6633 was used in these experiments due to the relatively similar potencies of lacticin 481 (IC\(_{50} = 980 \pm 110\) nM, determined by Ms. Chantal Garcia de Gonzalo, UIUC) and nisin (410 ± 170 nM)\(^67\) against this organism, allowing for a direct comparison. Over a concentration range of 0.2 to 20 μM, nisin showed the expected concentration-dependent decrease in cell-associated mean fluorescence intensity (MFI) indicative of membrane depolarization and pore formation, while
Figure 2.19. Flow cytometry analysis of membrane disruption by lacticin 481 and nisin. (a) Membrane depolarization of *Bacillus subtilis* 6633 as measured by DiOC<sub>2</sub>(3) mean fluorescence intensity (MFI). At each lantibiotic concentration, the difference in average MFI of triplicate measurements between lacticin 481 and nisin was statistically significant (*P* < 0.05). (b) Representative histogram of cell count versus DiOC<sub>2</sub>(3) fluorescence intensity. (c) Membrane permeability of *L. lactis* HP as measured by propidium iodide (PI) uptake, demonstrating pore formation for nisin at a concentration 15-fold above its IC<sub>50</sub> value, but no such response for lacticin 481 at concentrations up to 25-fold above its IC<sub>50</sub> value. (d) Representative histogram of cell count versus PI fluorescence intensity.

Lacticin 481 gave no decrease in MFI (*Fig. 2.19a-b*). Interestingly, at the highest lacticin 481 concentration tested, cells exhibited a membrane hyperpolarization; this may represent a cell stress response, as has been reported for rifampicin-resistant *Mycobacterium*. Allocating a membrane integrity assay was also performed using *L. lactis* HP and the fluorescent dye propidium iodide, which can only accumulate in cells in the presence of a pore-forming agent. While nisin gave a large increase in MFI at a concentration 15-fold above its IC<sub>50</sub> value indicative of pore formation, lacticin 481 did not induce increases in MFI above control levels at concentrations up to 20 μM, or 25-fold above its IC<sub>50</sub> value (*Fig. 2.19c-d*). Taken together, these data suggest that...
lacticin 481 does not form membrane-associated pores, and that inhibition of peptidoglycan formation is the most likely mechanism of its antibacterial activity.

2.2.9. Development of a constitutively-active serine/threonine kinase

The production of peptides and proteins with specific sites of phosphorylation has been widely explored in order to investigate the role of phosphorylation in a myriad of signal transduction pathways. The observation that LctM first phosphorylates its substrate, then eliminates the resulting phosphoester to generate α,β-unsaturated residues, was exploited in previous work to develop a leader peptide-dependent serine/threonine kinase; two elimination-deficient mutants of LctM, R399M and T405A, were able to phosphorylate a wide variety of peptides attached to the LctA leader peptide. To determine if the corresponding mutations in LctCE-(GS)₁₅ were able to phosphorylate leaderless substrates, ten diverse serine- or threonine-containing peptides, including known substrates of endogenous kinases, were generated by SPPS (see Section 2.4.8). Incubation of these potential substrates with LctCE-(GS)₁₅ T405A by Dr. Oman resulted in phosphorylation in three of the ten peptides: the AKT/PKB substrate, the PKCμ substrate, and synthetic peptide RWVRSALLI. While it was encouraging that this enzyme was able to accept several leaderless peptides with no relation to its natural substrate, the system lacked the broad promiscuity necessary for development as a general serine/threonine kinase.

2.3. CONCLUSIONS AND OUTLOOK

This chapter has described a unique approach to biosynthetic engineering of RiPPs: construction of a fusion leader-synthetase enzyme, LctCE-(GS)₁₅, which is able to process synthetic substrates lacking a leader peptide to produce analogues of the lantibiotic lacticin 481 in a single step. This in vitro chemoenzymatic platform possesses dramatically improved efficiency and scale compared to previous in vitro systems (as in Fig. 2.2), as the leader peptide no longer needs to be attached to, and subsequently removed from, the core peptide substrate. Importantly, these substrates were amenable to standard SPPS, allowing the freedom to include a variety of non-proteinogenic amino acids in order to explore a wide chemical space. Of the small panel of lacticin 481 analogues produced so far, two have already demonstrated improved antibacterial potency compared to the authentic natural product. Additionally, analogues
produced using this biosynthetic platform have been used to investigate the mode-of-action of lacticin 481, which was found to inhibit transglycosylation involved in cell wall biosynthesis without forming membrane-associated pores.

Larger-scale SAR analysis of lacticin 481, made possible by this technology, may permit the discovery of additional analogues with further improved pharmacological properties. In order to produce interesting analogues in larger quantities for more detailed investigation, *in vivo* expression in *E. coli* can be used, incorporating non-proteinogenic amino acids via stop-codon suppression.\textsuperscript{29, 31, 98} In the case of lacticin 481, *in vivo* coexpression of LctA and LctM, followed by *ex vivo* leader peptide proteolysis to produce lacticin 481 ΔLys1 and mutants, has been carried out successfully by Mr. Noah Bindman (UIUC),\textsuperscript{73, 99} demonstrating the validity of this combined process to discover and scale-up improved lacticin 481 analogues.

Many questions remain answered surrounding the enzymatic activity of the fusion LctCE enzyme. Does LctCE preserve the N-to-C directionality of LctM when processing natural, full-length LctA?\textsuperscript{64} Can the leader-linker region be further optimized, including attachment to the enzyme elsewhere besides the N-terminus, to produce an even more active synthetase or serine/threonine kinase? Biophysical and structural data detailing the leader-LctM interaction, which to date have not been successfully obtained, would greatly facilitate further optimization. Finally, can this fusion approach be applied to other lanthipeptides, or to unrelated leader-directed biosynthetic systems? Efforts to construct a constitutively-active haloduracin synthetase by Drs. Trent Oman and Rebecca Splain (UIUC, unpublished results) have not yet been successful in producing fully-modified haloduracin β, though this compound contains considerably more modifications (seven dehydrations and four cyclizations) than lacticin 481.\textsuperscript{39} Preliminary results suggest that *in trans* activity of the parent synthetase may be able to predict an active fusion enzyme, although this hypothesis requires further investigation.

### 2.4. EXPERIMENTAL

#### 2.4.1. Materials and general methods

Construction of expression plasmids, expression, and purification of LctCE enzymes were performed by Dr. Trent Oman (UIUC).\textsuperscript{61, 99} His\textsubscript{6}-LctM was expressed and purified by Dr. Oman using the known expression plasmid pET28b LctM.\textsuperscript{36} Authentic lacticin 481 was
provided by Dr. Juan Velásquez (UIUC) after isolation from *Lactococcus lactis* subsp. *lactis* CNRZ 481.\textsuperscript{99,100} Lacticin 481 leader peptide (LctA -24—1) was provided by Dr. Matthew Levengood (UIUC).\textsuperscript{55} Nisin was purified from Nisaplin, purchased from Danisco A/S, by Ms. Chantal Garcia de Gonzalo (UIUC) using a known procedure.\textsuperscript{67}

Standard Fmoc-amino acids and resins for solid-phase peptide synthesis (SPPS), peptide coupling reagents $N,N'$-diisopropylcarbodiimide (DIC), $O$-((6-chlorobenzotriazol-1-yl)-$N,N,N',N'$-tetramethyluronium hexafluorophosphate (HCTU), and 1-hydroxybenzotriazole monohydrate (HOBt), and non-proteinogenic amino acids Fmoc-3-(4’-pyridyl)-l-alanine (Fmoc-Pal-OH) and Fmoc-3-(2-naphthyl)-l-alanine (Fmoc-Nal-OH) were purchased from ChemImpex International. Other chemicals and solvents for peptide synthesis, enzymatic reactions, and chromatography were purchased from Sigma Aldrich, ChemImpex International, or Fisher Scientific, and used without further purification. Flow cytometry dyes 3,3’-diethyloxacarbocyanine iodide (DiOC\textsubscript{2}(3)) and propidium iodide (PI) were purchased from Invitrogen/Life Technologies. Cell culture media were purchased from BD Biosciences.

Analytical reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on a Beckman System Gold analytical HPLC system equipped with a Phenomenex Jupiter Proteo C12 (90 Å pore size, 250 mm × 4.6 mm × 10 μm) analytical column at a flow rate of 1 mL/min and a solvent gradient of 2-100% solvent B over 45 min, observing at an absorbance of 220 nm. Preparatory RP-HPLC was performed using a Waters Delta 600 system equipped with a Vydac Delta-Pak C18 (100 Å pore size, 100 mm × 25 mm) column and a flow rate of 8 mL/min or a Phenomenex Jupiter Proteo C12 (90 Å pore size, 250 mm × 15 mm × 10 μm) column and a flow rate of 10 mL/min. Solvent gradients are described for each peptide. All HPLC solvents were filtered with a Millipore filtration system equipped with a 0.22 μm nylon membrane filter prior to use. HPLC solvent compositions: solvent A is 0.1% trifluoroacetic acid (TFA) in H\textsubscript{2}O; solvent B is 80:20 MeCN/H\textsubscript{2}O with 0.087% TFA.

Peptides were analyzed by matrix-assisted laser desorption ionization/time-of-flight (MALDI-TOF) mass spectrometry using an Applied Biosystems Voyager DE-STR spectrometer or a Bruker Daltonics UltraFlexXtreme TOF/TOF spectrometer at the Mass Spectrometry Facility (UIUC) in a matrix solution consisting of saturated α-cyano-4-hydroxycinnamic acid in 1:1:0.1 H\textsubscript{2}O/MeCN/TFA. If desalting was required prior to MS analysis, samples were desalted using Millipore ZipTip\textsubscript{C18} pipette tips and eluted with matrix solution.
2.4.2. Synthesis of LctA core peptide and analogues

Automated Fmoc-based SPPS was performed using either a CEM Liberty microwave peptide synthesizer or an Advanced ChemTech Apex 396 peptide synthesizer at scales of 0.05-0.15 mmol on Fmoc-Ser(tBu)-Wang polystyrene resin. For the Liberty, Fmoc deprotection was performed using 20% piperidine in dimethylformamide (DMF; 7 mL) for two treatments of 1.5 min each, and couplings were performed using 0.2 M Fmoc-amino acid in DMF (2.5 mL), 0.5 M HCTU in DMF (1 mL), and 2 M diisopropylethylamine in N-methylpyrrolidinone (0.5 mL) for 5 min. For the Apex, Fmoc deprotection was performed using 20% piperidine in DMF (4 mL) for two treatments of 6 min each, and couplings were performed using 0.35 M Fmoc-amino acid/0.38 M HOBt in DMF (2 mL), 0.38 M DIC in DMF (2 mL), and DMF (0.5 mL) for one treatment of 1.5 h with an additional treatment if necessary. Solutions containing Fmoc-Pal-OH required at least 15% dimethylsulfoxide in DMF to achieve full solubility. After the synthesis was completed, the resin-bound peptide was washed with CH₂Cl₂ and dried. Concurrent side-chain deprotection and cleavage from resin was performed by stirring the resin-bound peptide in 91.5:5:2.5:1 TFA/water/triisopropylsilane/ethanedithiol for 2 h under N₂. After removing the cleaved resin by filtration, the filtrate was concentrated under a stream of N₂. The peptide was precipitated with cold Et₂O, isolated by centrifugation at 12,000 × g, dissolved in 1:1 H₂O/MeCN, and lyophilized to dryness. The crude peptides were purified by RP-HPLC, employing the solvent gradients listed below, and fractions containing purified material were lyophilized to dryness. See also Notebook I, pages 68, 73-75, 84, 86; Notebook II, pages 96-97; Notebook III, page 7; Notebook IX, pages 29, 31, 43-44.

**LctA core wild type.** Sequence: H-KGGSGVIHTISHECNMNSWQFVFTCCS-OH. RP-HPLC: Phenomenex Jupiter Proteo C12 column and a solvent gradient of 10% solvent B for 1 min, 10-20% over 3 min, 20-48% over 28 min, 48-100% over 1 min. R₂ = 28.3-29.1 min. HRMS (MALDI-TOF) calc. [M+H]⁺ for C₁₂₇H₁₉₁N₃₆O₃₉S₄ 2972.295, found 2972.444.


**LctA core N15R/F21H.** RP-HPLC: Vydac Delta-Pak C18 column and a solvent gradient of 2% solvent B for 2 min, 2-20% over 2 min, 20-27% over 3 min, 27-42% over 30 min, 42-100% over 1 min. R₂ = 29.8-31.0 min. LRMS (MALDI-TOF) calc. [M+H]⁺ 3004.3, found 3004.4.
**LctA core N15R/F21Pal.** RP-HPLC: Vydac Delta-Pak C18 column and a solvent gradient of 2% solvent B for 2 min, 2-18% over 3 min, 18-25% over 3 min, 25-40% over 30 min, 40-100% over 1 min. R_t = 34.0-35.6 min. LRMS (MALDI-TOF) calc. [M+H]^+ 3015.3, found 3015.4.

**LctA core N15R/W19Nal/F21H.** RP-HPLC: same conditions as LctA core N15R/F21H. R_t = 33.5-36.2 min. LRMS (MALDI-TOF) calc. [M+H]^+ 3015.3, found 3016.3.


**NukA core.** Sequence: H-KKKSGViptvshdchmnsfQfVftccs-OH. RP-HPLC: Vydac Delta-Pak C18 column and a solvent gradient of 2% solvent B for 1 min, 2-20% over 2 min, 20-27% over 3 min, 27-43% over 34 min, 43-100% over 1 min. R_t = 37.2-38.6 min. LRMS (MALDI-TOF): calc. [M+H]^+ 3031.6, found 3031.4.

### 2.4.3. *In vitro* reconstitution of LctM and LctCE enzymatic activity

Assays were performed in collaboration with Dr. Oman. For *in trans* LctM assays, LctA core peptide (final concentration 20 µM) and LctA leader peptide (2 or 20 µM) were incubated with purified His<sub>6</sub>-LctM (2 µM) at 25 °C for 3-24 h in a buffer (pH 7.5) consisting of 50 mM Tris, 10 mM MgCl<sub>2</sub>, 2 mM ATP, and 25 µg/mL bovine serum albumin. The extent of reaction was monitored by periodically removing aliquots (9 µL) of the reaction, which were quenched by the addition of 5% aqueous TFA (1 µL; final pH 1-2), desalted by ZipTip<sub>C18</sub>, and analyzed by MALDI-TOF MS. For LctCE assays, the above components and concentrations were used except the leader peptide was omitted and purified LctCE enzyme was used in place of His<sub>6</sub>-LctM. See also Notebook I, page 98; Notebook VIII, page 37-41; Notebook IX, pages 47-48, 61-62.

For examination of ring formation with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP), aliquots (9 µL) of the above reactions were removed after 5 min or 60 min incubation and quenched by the addition of 1% aqueous TFA (0.5 µL). A solution of 1 mM tris(2-carboxyethyl)phosphine/100 mM citrate/pH 3 (10 µL) was added and incubated for 5 min. A solution of 200 mM CDAP/100 mM citrate/pH 3 (10 µL) was then added and incubated for 15 min. The assays were desalted and analyzed as above. See also Notebook VIII, page 45.
For preparative reactions, the total reaction volume was scaled to 5-20 mL and allotted into a series of 1-1.5 mL aliquots due to issues of substrate aggregation at large volumes. The extent of reaction was monitored by MS as above. After 12 h, starting material was generally consumed, at which time the aliquots were pooled, quenched by the addition of TFA (0.5% final concentration), and concentrated to ~1/4 volume by lyophilization. The concentrated reaction mixture was centrifuged at 12,000 × g to remove any insoluble material, and the resulting supernatant was purified by RP-HPLC using a Phenomenex Jupiter Proteo C12 column and a solvent gradient of 10% solvent B for 5 min, then 10-100% over 45 min. Lacticin 481 analogues and partially processed intermediates eluted at 20.8-22.5 min. All fractions were analyzed by MALDI-TOF MS as described above, and purified material was lyophilized to dryness. See also Notebook II, pages 36-37; Notebook VIII, pages 42-44, 48-49.

2.4.4. Solid culture growth inhibition assays

Enzymatic assays (100 μL total volume) were lyophilized to dryness and taken up in sterile deionized water (SDW, 20 μL). Cultures of Lactococcus lactis subsp. cremoris HP (ATCC 11602) or Lactococcus lactis subsp. lactis CNRZ 481 were grown at 30 °C in GM17 medium (40 g/L M17, 0.5% glucose) for 12-18 h, then diluted with fresh GM17 to an optical density at 600 nm (OD\textsubscript{600}) of 1.0. An aliquot of this culture (200 μL) and 20% aqueous glucose (500 μL) were added to melted and cooled GM17 containing 1.5% agar (20 mL), poured into a sterile Nunc dish and allowed to solidify over 20 min. Another aliquot of culture (300 μL) and 20% glucose (750 μL) were added to melted and cooled GM17 agar (30 mL) and poured over the first agar layer. A sterile 96-well PCR tube rack was placed in this second layer, which was allowed to solidify over 30 min. The rack was removed, and the agar was allowed to solidify over an additional 1 h. Redissolved assays were spotted into individual wells, and the plate was incubated at room temperature for 12-24 h, then analyzed. See also Notebook II, pages 5-6; Notebook VIII, pages 35, 51; Notebook IX, pages 33-34, 47-48, 61-62.

2.4.5. Liquid culture growth inhibition assays

Stock concentrations of lacticin 481 and analogues were generated by weighing lyophilized peptide using a Mettler-Toledo MT5 microbalance and dissolving in SDW to give 200 μM stock solutions. Serial two-fold dilutions of peptide stock solutions were prepared in
SDW to give 4x working concentrations. Cultures of *Lactococcus lactis* subsp. *cremoris* HP were grown at 30 °C in GM17 medium for 12-18 h, then diluted with fresh GM17 to an OD$_{600}$ of 0.1. Experiments were performed in triplicate in Corning-Costar 96-well flat-bottom assay plates. Experimental wells contained diluted culture (150 μL) and 4x peptide solution (50 μL). Control wells contained either fresh GM17 (150 μL) and SDW (50 μL, negative control) or diluted culture (150 μL) and SDW (50 μL, positive control). OD$_{600}$ was recorded at hourly intervals using a BioTek Synergy H4 plate reader, and plates were incubated at 30 °C between readings. Plots of blank-corrected OD$_{600}$ vs. peptide concentration were fitted to a dose-response function with the equation: $y = A1 + (A2-A1) / (1 + 10^{(\log x0 - x)p})$, where p = variable Hill slope. Half maximal inhibitory concentration (IC$_{50}$) values were calculated from this fit for each peptide after 5 h incubation, and triplicate calculations were averaged. Assays were performed in collaboration with Dr. Oman. See also Notebook II, pages 43-45.

2.4.6. Inhibition of transglycosylation by lacticin 481 analogues

Thin-layer chromatography-based transglycosylation assays using the transglycosylation enzyme PBP1b from *Escherichia coli* and a [$^{14}$C]GlcNAc-labeled heptaprenyl lipid II analogue were performed by Dr. Oman in collaboration with Dr. Tania Lupoli and Prof. Suzanne Walker (Harvard Medical School) as previously described. See also Notebook II, pages 43-45.

2.4.7. Flow cytometry analysis of membrane disruption

For membrane potential assays using 3,3'-diethyloxacarbocyanine iodide (DiOC$_2$(3)), cultures of *Bacillus subtilis* ATCC 6633 were grown at 37 °C in LB medium for 12-18 h, then diluted with fresh LB to an OD$_{600}$ of 0.1. Cells were combined with DiOC$_2$(3) (final concentration 2 μM), HEPES (1 mM), and glucose (1 mM) and incubated for 20 min at room temperature. Stock solutions of nisin or lacticin 481 were added to final concentrations of 0.2, 2.0, or 20 μM and incubated for an additional 15 min prior to analysis; in the negative control, SDW was added instead of antibiotic. Changes in cell-associated DiOC$_2$(3) fluorescence were measured with a BD Biosciences LSR II flow cytometer at the Roy J. Carver Biotechnology Center (UIUC), using 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 experiments were performed in triplicate. Data analysis to calculate the geometric
mean fluorescence intensity (MFI) of gated cell populations was performed using FCS Express 3.00.0311 V Lite Stand-alone software. Assays involving *B. subtilis* were performed in collaboration with Ms. Garcia de Gonzalo.

For membrane permeability assays using propidium iodide (PI), cultures of *Lactococcus lactis* subsp. *cremoris* HP were grown at 30 °C in GM17 medium for 12-18 h, then diluted with fresh GM17 to an OD$_{600}$ of 0.1. Cells were combined with PI (final concentration 25 μM), HEPES (1 mM), glucose (1 mM), and lacticin 481 (0, 0.2, 2.0, 20 μM) or nisin (0.2 μM), incubated for 15 min at room temperature, and analyzed. Data acquisition and analysis were performed as for membrane potential assays, except emission was measured through a band-pass filter at 695/40 nm. *See also Notebook IX, pages 90-91, 97-98.*

2.4.8. Synthesis of phosphopeptide precursors

Serine/threonine-containing peptides for phosphorylation studies were synthesized via automated Fmoc-SPPS using an Advanced ChemTech Apex 396 synthesizer as described for LctA core peptide analogues (Section 2.4.2) and purified by RP-HPLC using a Vydac Delta-Pak C18 column and solvent gradients as described for each peptide. Phosphorylation assays with LctCE-(GS)$_{15}$ T405A were performed by Dr. Oman.$^{61}$ *See also Notebook III, 42-43, 47-49, 79, 82-91.*

**AKT/PKB substrate.$^{92}$** Sequence: H-ARKRERTYSFGHHA-OH. RP-HPLC gradient: 2% solvent B for 1 min, 2-10% over 2 min, 10-14% over 2 min, 14-26% over 24 min, 26-100% over 1 min. $R_t = 21.2-22.3$ min. LRMS (MALDI-TOF): calc. [M+H]$^+$ 1715.9, found 1716.3.

**Calcineurin/PP2B substrate.$^{93}$** Sequence: H-DLDVPIPGRFDRVSVAAE-OH. RP-HPLC gradient: 2% solvent B for 1 min, 2-16% over 2 min, 16-20% over 2 min, 20-35% over 30 min, 35-100% over 1 min. $R_t = 31.4-32.5$ min. LRMS (MALDI-TOF): calc. [M+H]$^+$ 2112.1, found 2113.0.

**PKCδ substrate.$^{94}$** Sequence: H-RDMRQTAVAVGVIKA-OH. RP-HPLC gradient: 2% solvent B for 1 min, 2-14% over 2 min, 14-18% over 2 min, 18-33% over 30 min, 33-100% over 1 min. $R_t = 26.2-26.9$ min. LRMS (MALDI-TOF): calculated [M+H]$^+$ 1543.9, found 1544.6.

**PKG substrate.$^{95}$** Sequence: H-RKRSRAE-OH. RP-HPLC gradient: 2% solvent B for 1 min, 2-8% over 2 min, 8-18% over 20 min, 18-100% over 1 min. $R_t = 11.0-12.4$ min. LRMS (MALDI-TOF): calculated [M+H]$^+$ 902.5, found 902.7.
**PKC substrate.**\(^96\) Sequence: H-RRGRTGRGRRGIFR-OH. RP-HPLC gradient: same as for AKT/PKB substrate. \(R_t = 21.8-23.6\) min. LRMS (MALDI-TOF): calculated \([M+H]^+ 1701.0\), found 1701.3.

**PKC\(\mu\) substrate.**\(^97\) Sequence: H-AALVRQMSVAFFFK-OH. RP-HPLC gradient: 2\% solvent B for 1 min, 2-24\% over 2 min, 24-28\% over 2 min, 28-43\% over 32 min, 43-100\% over 1 min. \(R_t = 34.3-35.1\) min. LRMS (MALDI-TOF): calculated \([M+H]^+ 1614.9\), found 1615.2.

**Synthetic peptide 1.**\(^91\) Sequence: H-RWVRSALLI-OH. RP-HPLC gradient: 2\% solvent B for 1 min, 2-20\% over 2 min, 20-24\% over 2 min, 24-39\% over 30 min, 39-100\% over 1 min. \(R_t = 32.4-33.5\) min. LRMS (MALDI-TOF): calculated \([M+H]^+ 1113.7\), found 1114.1.

**Synthetic peptide 2.**\(^91\) Sequence: H-RLIKSFAYV-OH. RP-HPLC gradient: 2\% solvent B for 1 min, 2-18\% over 2 min, 18-22\% over 2 min, 22-37\% over 30 min, 37-100\% over 1 min. \(R_t = 25.9-27.3\) min. LRMS (MALDI-TOF): calculated \([M+H]^+ 1096.6\), found 1096.9.

**Synthetic peptide 3.**\(^91\) Sequence: H-RLIKTFAYV-OH. RP-HPLC gradient: same as for synthetic peptide 2. \(R_t = 26.4-28.0\) min. LRMS (MALDI-TOF): calculated \([M+H]^+ 1110.7\), found 1110.9.

**Synthetic peptide 4.**\(^91\) Sequence: H-LRRASVA-OH. RP-HPLC gradient: same as for PKG substrate. \(R_t = 20.3-21.7\) min. LRMS (MALDI-TOF): calculated \([M+H]^+ 772.5\), found 773.7.

### 2.5. REFERENCES


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CHAPTER 3: CHEMICAL SYNTHESIS OF LANTIBIOTICS AND ANALOGUES*†

3.1. INTRODUCTION

As presented in Chapters 1 and 2, the engineering of lanthipeptides and lantibiotics has advanced greatly in recent years due largely to efforts in understanding and manipulating their biosynthetic machinery with powerful in vivo and in vitro platforms.1-5 As many of the enzymes involved in lanthipeptide maturation have demonstrated substantial substrate promiscuity, panels of mutants and analogues have been produced in these ways, including those containing non-proteinogenic amino acids6-10 and additional or alternative crosslinks.11-13 These libraries have been vital to systematic studies of structure-activity relationships, mechanisms-of-action, and stability. However, to date many lantibiotics remain unexplored in this regard as a result of a lack of genetic tools to alter the natural producing organism or an inability to reconstitute the biosynthetic pathway in vitro or in heterologous hosts. Additionally, the generation of mutants using in vivo expression systems can be complicated at multiple stages, including post-translational modification of the precursor peptide, export from the cell, and host immunity,14, 15 which may preclude the discovery of interesting analogues.

As an alternative to in vivo and in vitro biosynthetic platforms, chemical synthesis of lantibiotics presents the ability to remove all dependence on the biosynthetic machinery.16, 17 In this way, complications involving in vivo expression and in vitro reconstitution of enzymatic activity, as well as the necessity of the leader peptide, are eliminated. Furthermore, the chemical space accessible in the generation of analogues is widened greatly. For example, the chemical composition of the crosslinks can be modified beyond lanthionine (Lan) and methyllanthionine (MeLan), fragments of lantibiotics can be generated straightforwardly, and fluorophores and chemical handles can be freely incorporated. Despite the promise of lantibiotic chemical synthesis, the precise control of stereochemistry and regiochemistry involved in crosslink formation is a formidable challenge. Indeed, previous efforts involving the biomimetic syntheses of Lan/MeLan rings in short peptides containing cysteine and α,β-unsaturated residues have

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confirmed these difficulties.\textsuperscript{18-20} Nevertheless, the first total chemical synthesis of a bioactive lantibiotic, nisin, was accomplished by Shiba and coworkers in the 1980s via the coupling of five protected segments synthesized in solution (Fig. 3.1).\textsuperscript{21-25} Lan/MeLan crosslinks were generated via a phosphine-promoted desulfurization reaction from the corresponding disulfides. However, the synthesis was reported over a ten-year span and involved more than a dozen coworkers, certainly far from the ideal timeframe and scope for the generation of panels of analogues.

\begin{center}
\textbf{Figure 3.1.} Solution-phase total synthesis of nisin by Shiba and coworkers.\textsuperscript{25} (a) Construction of Lan/MeLan rings, exemplified by ring A. Desulfurization of cyclic disulfides generated Lan/MeLan, and a Hofmann elimination was used to install Dha/Dhb residues. (b) Iterative coupling and deprotection of the five segments to generate nisin. Prior to treatment with HF, side-chains were protected as follows: Mbh for Asn, Cbz for Lys, and Bn for Ser. Abbreviations: Acm, acetamidomethyl; Bn, benzyl; Boc, \textit{tert}-butoxycarbonyl; Cbz, benzyloxycarbonyl; Mbh, 4,4-dimethoxybenzhydryl; Trt, trityl
\end{center}

In the 2000s, an alternative “building block” approach for lantipeptide synthesis emerged, which has greatly accelerated the development of chemical synthesis as a tool in lantibiotic engineering.\textsuperscript{16, 26} In this approach, orthogonally-protected Lan/MeLan bis-amino acids are used to construct cyclic peptides on the solid-phase (Fig. 3.2). While the solution-phase synthesis of lantipeptides is still being explored in particular situations, as exemplified by
Figure 3.2. SPPS-based approach to synthesize overlapping Lan/MeLan rings in peptides. Lan/MeLan building blocks (boxed) containing orthogonal sets of protecting groups (red and green) can be used to construct complex lanthipeptide topologies with full stereochemical and regiochemical control. Standard coupling conditions allow for cyclization on the solid-phase via amide-bond formation (purple).

The construction of an aminovinylcysteine-containing fragment of mersacidin, the applicability of 9-fluorenymethoxycarbonyl-based solid-phase peptide synthesis (Fmoc-SPPS) is particularly noteworthy; reaction times and the number of purification steps can be dramatically reduced, and yields dramatically increased, compared to a solution-phase synthesis. Using this SPPS-based approach, Vederas and coworkers have recently completed the total syntheses of lactocin S and both peptides of lacticin 3147. Furthermore, this team also produced analogues containing ether, olefin, and methylene/methine crosslinks in place of the natural thioether. The methylene analogue of lactocin S is particularly interesting from an engineering standpoint, as it maintains the antibacterial activity of the parent compound while improving oxidative stability.

In this chapter, the application of the synthetic approach shown in Fig. 3.2 to the construction of two different lantibiotics, epilancin 15X and lacticin 481, and analogues is described. Epilancin 15X (3.1, Fig. 3.3a), a lantibiotic first isolated in 2005 from the clinical strain Staphylococcus epidermidis 15X154, ranks among the most potent lantibiotics against drug-resistant pathogens, including methicillin-resistant S. aureus and vancomycin-resistant Enterococcus. This peptide contains a Lan ring and a set of overlapping MeLan rings toward
Figure 3.3. (a) Sequence and ring topology of epilancin 15X (3.1) and lacticin 481 (3.2). For the chemical structures of modified residues, see Fig. 1.6. (b) Biosynthesis of the N-terminal d-lactyl cap of epilancin 15X. Following modification by ElxB/ElxC and leader proteolysis by ElxP, the N-terminal Dha residue spontaneously hydrolyzes to a pyruvyl group (Pyr), which is reduced to the d-lactyl (DLac) group by ElxO.37

its C-terminus, which resemble the C-terminal portion of nisin (Fig. 3.1) responsible for pore formation.14, 38–40 Toward its N-terminus, epilancin 15X contains an unusual cluster of α,β-unsaturated residues as well as a d-lactyl (DLac) cap, which is installed biosynthetically by the oxidoreductase ElxO following proteolytic cleavage of the leader peptide by serine protease ElxP (Fig. 3.3b).37 No report has yet described biosynthetic manipulation of epilancin 15X in the producing organism, and previous efforts to reconstitute its biosynthesis in heterologous hosts or in vitro have not been successful to date.37 Consequently, no analogues of this natural product have been reported thus far, leaving many questions about its mechanism-of-action unanswered. Intriguingly, neither epilancin 15X41 nor the structurally-similar epilancin K742 bind to lipid II, the most common biological target of lantibiotics; this suggests that these compounds may exhibit their antibacterial activity through binding of a different target.43 The work herein describes the first reported analogues of epilancin 15X, produced through total chemical synthesis, and the first details of its mode-of-action.

As introduced in Chapter 1.4.1, the recent discovery of lantibiotics containing enzymatically-installed crosslinks bearing an LL-configuration,44 instead of the conventional DL-configuration, has prompted questions about the role of Lan/MeLan stereochemistry on biological activity. In order to address this issue systematically, chemical synthesis was again
utilized with lacticin 481 (3.2, Fig. 3.3a, see Chapter 2.1) as a model system. Three analogues of lacticin 481 were generated, in which each of the three \textit{dL-}crosslinks was replaced by its \textit{lL-}stereoisomer, and assayed for antibacterial activity. These efforts not only demonstrated the importance of the natural stereoconfiguration of each crosslink for activity, but also represented only the fifth completed total synthesis of a lanthipeptide.

3.2. RESULTS AND DISCUSSION

3.2.1. Synthesis of Lan and MeLan building blocks

In order to achieve the solid-supported chemical synthesis of lanthipeptides, including those with overlapping ring topologies as outlined in Fig. 3.2, Lan/MeLan building blocks containing orthogonal sets of protecting groups were necessary. These protecting groups would need to fulfill multiple criteria: 1) stability to the basic conditions employed during standard Fmoc-SPPS; 2) mild, solid-phase-amenable, and quantitative deprotection; 3) full orthogonality to other protecting groups used in the synthesis; and ideally 4) straightforward incorporation into building blocks. A review of known protecting groups for amines and carboxylic acids uncovered multiple candidate protecting group strategies: allyl, 4,4-dimethyl-2,6-dioxocyclohexylidene (also called dimedonyl), 4-nitrobenzyl, 2-trimethylsilylethyl, 2,2,2-trichloroethyl, and others.\textsuperscript{45} The peptide synthesis literature offered a variety of examples of the orthogonality among different combinations of these groups as well as conditions to permit mild and quantitative deprotection.\textsuperscript{45-52} Allyl and 4-nitrobenzyl were chosen for initial study, due to their successful incorporation into Lan and Lan-mimicking building blocks in previous reports.\textsuperscript{26, 31, 53} Considering the rings topologies present in epilancin 15X and lacticin 481 (Fig. 3.3a), a total of four building blocks would be necessary for their syntheses (Fig. 3.4): a pair of orthogonal Lan building blocks (\textit{dL-3.3}, \textit{dL-3.4}) and a pair of orthogonal MeLan building (\textit{dL-3.5}, \textit{dL-3.6}).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3_4.png}
\caption{Orthogonal Lan/MeLan building blocks for the chemical synthesis of lanthipeptides. All, allyl; Aloc, allyloxy carbonyl; pNb, \textit{para-}nitrobenzyl; pNz, \textit{para-}nitrobenzyloxycarbonyl.}
\end{figure}
The allyl-protected Lan \textit{dl-3.3} was synthesized with modifications from a previous report (\textit{Scheme 3.1}).\textsuperscript{53} Commercially-available, inexpensive, and enantiomerically-pure amino acid starting materials were straightforwardly elaborated to bromoalanine \textit{d-3.8} and cystine \textit{3.9}. The key transformation involved a one-pot, two-reaction sequence of reduction of \textit{3.9} and subsequent base-promoted, phase-transfer condensation with \textit{d-3.8} to produce the thioether framework of \textit{dl-3.10} in high yields without epimerization at the potentially labile \textit{\alpha}-positions. Acid-promoted removal of the \textit{tert}-butyl ester yielded \textit{dl-3.3}, which was used directly for SPPS. Unfortunately, the direct application of this convergent reaction scheme to nitrobenzyl-protected Lan \textit{3.13} or allyl-protected MeLan \textit{3.16} resulted in epimerization and/or severely reduced yields (\textit{Scheme 3.2}). In the case of \textit{3.13}, the strongly electron-withdrawing nature of the nitrobenzyl ester likely lowered the pK\textsubscript{a} of the \textit{\alpha}-proton of \textit{3.12} sufficiently for abstraction in the basic conditions used for condensation. In the case of \textit{3.16}, a significant amount of allyl-protected dehydrobutyrine, the elimination product of \textit{3.15}, was observed during condensation; Michael addition of the liberated cysteine nucleophile into this dehydrobutyrine would be expected to result in a mixture of diastereomers, i.e. \textit{3.16}. Elimination has been suggested previously as a competing reaction during condensations of haloalanines and thiol nucleophiles,\textsuperscript{26, 54} and the increased steric bulk of the secondary bromide in \textit{3.15} likely permits this elimination to outcompete the desired displacement reaction.

Given these disappointing results, alternative schemes to the nitrobenzyl-protected and MeLan building blocks were necessary to prevent loss of the stereochemical integrity of the

\textbf{Scheme 3.1.} Synthesis of Lan building block \textit{dl-3.3}.\textsuperscript{a}

\begin{center}
\includegraphics[width=\textwidth]{scheme3.1.png}
\end{center}

\textsuperscript{a} Reagents and conditions: a) AlocCl, Na\textsubscript{2}CO\textsubscript{3}, H\textsubscript{2}O, CH\textsubscript{3}CN; b) allyl bromide, NaHCO\textsubscript{3}, DMF, 59\% (two steps); c) PPh\textsubscript{3}, CBr\textsubscript{4}, CH\textsubscript{2}Cl\textsubscript{2}, 78\%; d) HClO\textsubscript{4}, BuOAc; e) FmocOSu, N-methylmorpholine, THF, 79\% (two steps); f) PBu\textsubscript{3}, H\textsubscript{2}O, THF, then \textit{d-3.8}, Bu\textsubscript{4}NBr, NaHCO\textsubscript{3}, H\textsubscript{2}O, EtOAc, 89\%; g) CF\textsubscript{3}CO\textsubscript{2}H, PhSiH\textsubscript{3}, CH\textsubscript{2}Cl\textsubscript{2}, quant.
Scheme 3.2. Unsuccessful syntheses of stereodefined Lan and MeLan building blocks.\(^a\)

\[
\begin{array}{c}
\text{H}_2\text{N}^+\text{CO}_2\text{H} \rightarrow \text{pNzNH}^+\text{CO}_2\text{Nb} \rightarrow \text{pNzNH}^+\text{CO}_2\text{NbBr} \rightarrow \text{FmocHN}^-\text{CO}_2\text{pNb} \\
\text{3.11} \rightarrow \text{3.12} \rightarrow \text{3.13}
\end{array}
\]

\(^a\) Reagents and conditions: a) 4-nitrobenzyl chloroformate, Na\(_2\)CO\(_3\), H\(_2\)O, 1,4-dioxane; b) 4-nitrobenzyl bromide, NaHCO\(_3\), DMF, 83\% (two steps); c) PPh\(_3\), CBr\(_4\), THF, 76\%; d) Fmoc-Cys-O’Bu, Bu\(_4\)NBr, NaHCO\(_3\), H\(_2\)O, EtOAc, 87\% (diastereomers); e) AlocCl, Na\(_2\)CO\(_3\), H\(_2\)O, CH\(_3\)CN; f) allyl bromide, NaHCO\(_3\), DMF, 80\% (two steps); g) PPh\(_3\), CBr\(_4\), CH\(_2\)Cl\(_2\), 80\%; h) Fmoc-Cys-O’Bu, Bu\(_4\)NBr, NaHCO\(_3\), H\(_2\)O, EtOAc, 26\% (diastereomers).

starting materials during synthesis. If the electron-withdrawing nitrobenzyl groups were to blame for the epimerization seen in 3.13, it was hypothesized that switching the protecting groups of the bromoalanine and cysteine might result in more stable intermediates. Gratifyingly, the success of such a strategy was indeed demonstrated for Lan \textit{dl}-3.4 (Scheme 3.3). Fmoc- and \textit{tert}-butyl ester-protected bromoalanine 3.18 condensed cleanly with nitrobenzyl-protected cysteine \textit{dl}-3.20 under the basic phase-transfer conditions described in Scheme 3.1, resulting in the desired thioether \textit{dl}-3.21 in high yield with full preservation of stereochemistry. The diastereomeric purity of \textit{dl}-3.21 was assessed through \textsuperscript{13}C NMR spectroscopy in comparison to the epimerized MeLan 3.13, which exhibited extra “sister signals” indicative of a mixture of diastereomers for both \(\alpha\)- and \(\beta\)-carbons of the molecule (Fig. 3.5).\(^{53}\)

Scheme 3.3. Synthesis of Lan building block \textit{dl}-3.4.\(^a\)

\[
\begin{array}{c}
\text{FmocHN}^-\text{CO}_2\text{Bu} \rightarrow \text{FmocHN}^-\text{CO}_2\text{Bu} \rightarrow \text{FmocHN}^-\text{CO}_2\text{Bu} \\
\text{3.17} \rightarrow \text{3.18}
\end{array}
\]

\(^a\) Reagents and conditions: a) \textit{tert}-butyl 2,2,2-trichloroacetimidate, EtOAc, cyclohexane, 93\%; b) PPh\(_3\), CBr\(_4\), CH\(_2\)Cl\(_2\), 79\%; c) pNzCl, Na\(_2\)CO\(_3\), H\(_2\)O, 1,4-dioxane; d) pNbBr, NaHCO\(_3\), DMF, 94\% (two steps); e) CF\(_3\)CO\(_2\)H, \(^1\text{Pr}_3\)SiH, CH\(_2\)Cl\(_2\), 88\%; f) 3.18, NaHCO\(_3\), Bu\(_4\)NBr, EtOAc, H\(_2\)O, Bu\(_3\)P, 78\%; g) CF\(_3\)CO\(_2\)H, PhSiH\(_3\), CH\(_2\)Cl\(_2\), 95\%;
Figure 3.5. Comparison of the $\alpha$- and $\beta$-carbon $^{13}$C NMR signals of (a) dl-3.21 and (b) 3.13. The extra signals for 3.13 are indicative of the presence of a mixture of diastereomers.

This alternative scheme using bromoalanine 3.18 additionally allowed the stereocontrolled synthesis of MeLan dl-3.5 and dl-3.6 (Scheme 3.4). Protected $\beta$-methylcysteine d-3.25 was synthesized in five steps from $\alpha$-threonine using a published procedure. This compound proved to be a useful intermediate for both building blocks; protecting group manipulation, removal of the 4-methoxybenzyl (Mob) group protecting the thiol, condensation with 3.18, and tert-butyl ester removal resulted in dl-3.5 and dl-3.6 as single diastereomers. Despite the use of hindered secondary thiols, the condensation reactions leading to dl-3.27 and dl-3.29 proceeded in acceptable to high yields.

For the synthesis of lanthipeptides with very complex ring topologies, such as cinnamycin, a third orthogonal set of protecting groups would be necessary. The isovaleryldimedonyl (Ddiv) and 4-[(isovaleryldimedonyl)amino]benzyl ester (ODmab) groups have demonstrated utility in the Fmoc-SPPS of complex peptides, and their deprotection with dilute hydrazine is orthogonal to both allyl and nitrobenzyl protection. A dimedonyl-protected Lan building block was synthesized, but attempted SPPS of peptides containing this residue led invariably to peptides containing cysteine in place of the desired Lan residue (Fig. 3.5).
Scheme 3.4. Synthesis of MeLan building blocks dl-3.5 and dl-3.6.\(^a\)

<table>
<thead>
<tr>
<th>Step</th>
<th>Reagents and conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>a, b</td>
<td>allyl alcohol, TsOH, PhMe, reflux</td>
</tr>
<tr>
<td>c</td>
<td>NsCl, Et(_3)N, CH(_2)Cl(_2), 79% (two steps)</td>
</tr>
<tr>
<td>d</td>
<td>PPh(_3), diisopropylazodicarboxylate, THF, 83%</td>
</tr>
<tr>
<td>e</td>
<td>4-methoxybenzyl mercaptan, BF(_3)OEt(_2), CH(_2)Cl(_2), 86%</td>
</tr>
<tr>
<td>f</td>
<td>4-methoxybenzenethiol, K(_2)CO(_3), MeCN, DMSO, 91%</td>
</tr>
<tr>
<td>g, h</td>
<td>AllocOSu, (\text{Pr}_2)NEt, CH(_2)Cl(_2), 97%</td>
</tr>
<tr>
<td>i</td>
<td>Hg(OAc)(_2), PhOMe, CF(_3)CO(_2)H, then dithiothreitol</td>
</tr>
<tr>
<td>j</td>
<td>NaHCO(_3), Bu(_4)NBr, Bu(_3)P, EtOAc, H(_2)O, 64% (two steps)</td>
</tr>
<tr>
<td>k</td>
<td>CF(_3)CO(_2)H, PhSiH(_3), CH(_2)Cl(_2), 96%</td>
</tr>
<tr>
<td>l</td>
<td>4-nitrobenzyl chloroformate, (\text{Pr}_2)NEt, CH(_2)Cl(_2), 96%</td>
</tr>
<tr>
<td>m</td>
<td>Pd(PPh(_3))(_4), PhNMe, THF</td>
</tr>
<tr>
<td>n</td>
<td>NaHCO(_3), Bu(_4)NBr, Bu(_3)P, EtOAc, H(_2)O, 85% (two steps)</td>
</tr>
<tr>
<td>o</td>
<td>CF(_3)CO(_2)H, PhSiH(_3), CH(_2)Cl(_2), 95%</td>
</tr>
</tbody>
</table>

\(^a\) Reagents and conditions: a) allyl alcohol, TsOH, PhMe, reflux; b) NsCl, Et\(_3\)N, CH\(_2\)Cl\(_2\), 79% (two steps); c) PPh\(_3\), disopropylazodicarboxylate, THF, 83%; d) 4-methoxybenzyl mercaptan, BF\(_3\)OEt\(_2\), CH\(_2\)Cl\(_2\), 86%; e) 4-methoxybenzenethiol, K\(_2\)CO\(_3\), MeCN, DMSO, 91%; f) AllocOSu, \(\text{Pr}_2\)NEt, CH\(_2\)Cl\(_2\), 97%; g) Hg(OAc)\(_2\), PhOMe, CF\(_3\)CO\(_2\)H, then dithiothreitol; h) NaHCO\(_3\), Bu\(_4\)NBr, Bu\(_3\)P, EtOAc, H\(_2\)O, 64% (two steps); i) CF\(_3\)CO\(_2\)H, PhSiH\(_3\), CH\(_2\)Cl\(_2\), 96%; j) 4-nitrobenzyl chloroformate, \(\text{Pr}_2\)NEt, CH\(_2\)Cl\(_2\), 96%; k) Pd(PPh\(_3\))\(_4\), PhNMe, THF; l) 4-nitrobenzyl bromide, NaHCO\(_3\), DMF, 93% (two steps); m) NaHCO\(_3\), Bu\(_4\)NBr, Bu\(_3\)P, EtOAc, H\(_2\)O, 85% (two steps); n) CF\(_3\)CO\(_2\)H, PhSiH\(_3\), CH\(_2\)Cl\(_2\), 95%.

3.6). The instability of this building block likely stems from the use of the Ddiv group; unlike carbamate-based protecting groups, Ddiv may promote a \(\beta\)-elimination reaction, potentially through a oxazolone-like intermediate,\(^56\), \(^57\) resulting in the observed side-product. This complication forced the abandonment of the dimedonyl protecting strategy for Lan/MeLan building blocks. For the synthetic targets described here, only two sets of protecting group (i.e. allyl and nitrobenzyl) were necessary; should a third set be necessary in future studies, the trimethylsilylyethyl strategy as reported previously may serve as a viable addition.\(^50\)

Figure 3.6. Structure of Fmoc-dl-Lan(Ddiv/ODmab)-OH, and observed decomposition during SPPS.
3.2.2. Synthesis of Dha/Dhb-containing peptide fragments

In addition to regio- and stereocontrolled construction of Lan/MeLan rings, another challenge in the synthesis of lanthipeptides is the incorporation of the α,β-unsaturated residues Dha and Dhb, which are not amenable to standard iterative SPPS.\(^5^8\) Following coupling of an N-protected Dha/Dhb residue, deprotection would result in an enamine, which would be present in equilibrium with its corresponding imine. The imine is a weak nucleophile for peptide synthesis and is also prone to hydrolysis in the presence of water to give the corresponding ketone (as in Fig. 3.3b), impeding subsequent couplings. Two alternatives were considered in order to install Dha/Dhb into peptides on the solid phase: use of a “masked” building block that could be straightforwardly coupled, deprotected, and later unmasked to reveal the α,β-unsaturated residue; and coupling of peptide fragments containing Dha/Dhb. In the former approach, alkylcysteines\(^1^8, 1^9\) and alkyl- or arylselenocysteines\(^5^9-\text{61}\) have been described that undergo elimination within a peptide to give the desired α,β-unsaturated residue when treated with an oxidizing agent. Unfortunately, these oxidative conditions are not compatible with thioether crosslinks present in lanthipeptides. Alternatively, a fragment coupling strategy, in which a short peptide containing Dha/Dhb is synthesized separately and coupled as a single unit to the growing peptide, has been used successfully in the synthesis of Dha/Dhb-containing depsipeptides\(^6^2, 6^3\) and lantibiotics.\(^2^9, 3^0\) Furthermore, epimerization of the C-terminal residue, a common complication of fragment-based couplings, can be avoided by placing the α,β-unsaturated residue at the C-terminus of the fragment.

For the synthesis of epilancin 15X (3.1, Fig. 3.3a), three such Dha/Dhb-containing fragments would be necessary: Phe27-Dhb28, Ala2-Dha3, and Lys6-Dhb7-Dhb8. Lacticin 481 (3.2) also contains a Phe-Dhb sequence (residues 23-24), so the same dipeptide fragment used for the synthesis of 3.1 would also be applied to 3.2. The solution-phase synthesis of the three corresponding Fmoc-protected fragments 3.32, 3.35, and 3.39 are shown in Scheme 3.5. Use of the water-soluble activator 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) for amino acid couplings permitted reaction purification without the use of chromatography. The α,β-unsaturated residue in dipeptide fragments 3.32 and 3.35 were constructed via a mesylation-elimination sequence of side chain-unprotected serine or threonine, which in the case of Dhb has been shown previously to be selective for the desired (Z)-olefin.\(^3^2\)
Scheme 3.5. Synthesis of Dha/Dhb-containing fragments for epilancin 15X total synthesis.

\[
\begin{align*}
\text{Boc-Phe-OH} + \text{H-Thr-O\text{Bu}} & \quad \xrightarrow{a} \quad \text{3.30} \\
\text{Boc-Ala-OH} + \text{H-Ser-O\text{Bu}} & \quad \xrightarrow{f} \quad \text{3.33} \\
\text{H-Thr-OH} & \quad \xrightarrow{i,j} \quad \text{3.36} \\
\text{BocHN} & \quad \xrightarrow{m,n} \quad \text{3.39}
\end{align*}
\]

\[\text{Reagents and conditions: a) EDC, HOBT, \text{Pr}_2\text{NEt}, \text{CH}_2\text{Cl}_2, 98%; b) MsCl, \text{Et}_3\text{N}, \text{CH}_2\text{Cl}_2; c) DBU, \text{Cl(CH}_2\text{)}_2\text{Cl, reflux, 90% (two steps); d) CF}_3\text{CO}_2\text{H, CH}_2\text{Cl}_2; e) FmocOSu, Na}_2\text{CO}_3, \text{H}_2\text{O, dioxane, 95% (two steps); f) EDC, HOBT, \text{Pr}_2\text{NEt, CH}_2\text{Cl}_2, 96%; g) DBU, CH}_2\text{Cl}_2, 97% (two steps); h) FmocOSu, Na}_2\text{CO}_3, \text{H}_2\text{O, dioxane, 43% (two steps); i) allyl alcohol, TsOH, PhMe, reflux; j) Boc-Thr-OH, EDC, HOBT, \text{Pr}_2\text{NEt, CH}_2\text{Cl}_2, 92% (two steps); k) Aloc-Lys(Boc)-OH, EDC, HOBT, \text{Pr}_2\text{NEt, CH}_2\text{Cl}_2, 90% (two steps); l) EDC, CuCl, CH}_2\text{Cl}_2, 64%; m) Pd(\text{PPh}_3)_4, \text{PhNHMe, THF; n) FmocOSu, Na}_2\text{CO}_3, \text{H}_2\text{O, dioxane, 89% (two steps).}
\]

For the tripeptide fragment 3.39, an attempted double mesylation-elimination of 3.37 resulted in an inseparable mixture of compounds. Instead, an EDC/CuCl dehydration reaction, also selective for the (Z)-olefin,\(^6^4\) was used to give the desired fragment 3.38, which was elaborated in two steps to 3.39.

A prominent question relating to SAR analysis of epilancin 15X is the importance of the N-terminal \(\alpha\text{Lac} \) cap (Fig. 3.3b), compared to its biosynthetic precursor, a pyruvyl (Pyr) cap.\(^6^5\) Therefore, epilancin 15X analogues containing Pyr instead of \(\alpha\text{Lac} \) were desired. However, while \(\alpha\)-lactic acid can be directly coupled to peptides by SPPS, the coupling of pyruvic acid would be expected to be more challenging, considering the presence of two potential electrophilic sites. Although the direct coupling of pyruvic acid has been reported,\(^6^6\) several attempts with test peptides in relation to this work failed to give the desired Pyr-capped sequences in acceptable yields. Instead, an additional fragment 3.42 was synthesized, bearing N-
terminal Boc protection of a Dha-Ala dipeptide (Scheme 3.6). This fragment could be directly coupled, and upon acidic cleavage/global deprotection, a biomimetic enamine/imine hydrolysis would result in the desired Pyr-capped peptide.\textsuperscript{37}

**Scheme 3.6.** Synthesis of Dha-containing dipeptide fragment for inclusion of N-terminal Pyr.\textsuperscript{a}

\begin{center}
\begin{tikzpicture}[node distance=1.5cm]
  \node (a) {$\text{Boc-Ser-OH} + \text{H-Ala-OMe}$};
  \node (b) [right of=a] {$\text{Bo}_{\text{C}}\text{HN}$};
  \node (c) [right of=b] {$\text{Bo}_{\text{C}}\text{HN}$};
  \node (d) [right of=c] {$\text{Bo}_{\text{C}}\text{HN}$};
  \node (e) [below of=d] {$\text{O}$};
  \node (f) [below of=e] {$\text{O}$};
  \node (g) [below of=f] {$\text{Me}$};
  \node (h) [below of=g] {$\text{Me}$};
  \node (i) [below of=h] {$\text{O}$};
  \node (j) [below of=i] {$\text{O}$};
  \node (k) [below of=j] {$\text{Me}$};
  \node (l) [below of=k] {$\text{Me}$};
  \node (m) [below of=l] {$\text{O}$};
  \node (n) [below of=m] {$\text{O}$};
  \node (o) [below of=n] {$\text{Me}$};
  \node (p) [below of=o] {$\text{Me}$};
  \node (q) [below of=p] {$\text{O}$};
  \node (r) [below of=q] {$\text{O}$};
  \node (s) [below of=r] {$\text{Me}$};
  \node (t) [below of=s] {$\text{Me}$};

  \draw [->] (a) -- (b) node [midway, above] {$a$};
  \draw [->] (b) -- (c) node [midway, above] {$\text{b, c}$};
  \draw [->] (c) -- (d) node [midway, above] {$d$};

  \draw [->] (d) -- (e) node [midway, below] {$\text{O}$};
  \draw [->] (e) -- (f) node [midway, below] {$\text{O}$};
  \draw [->] (f) -- (g) node [midway, below] {$\text{Me}$};
  \draw [->] (g) -- (h) node [midway, below] {$\text{Me}$};
  \draw [->] (h) -- (i) node [midway, below] {$\text{O}$};
  \draw [->] (i) -- (j) node [midway, below] {$\text{O}$};
  \draw [->] (j) -- (k) node [midway, below] {$\text{Me}$};
  \draw [->] (k) -- (l) node [midway, below] {$\text{Me}$};
  \draw [->] (l) -- (m) node [midway, below] {$\text{O}$};
  \draw [->] (m) -- (n) node [midway, below] {$\text{O}$};
  \draw [->] (n) -- (o) node [midway, below] {$\text{Me}$};
  \draw [->] (o) -- (p) node [midway, below] {$\text{Me}$};
  \draw [->] (p) -- (q) node [midway, below] {$\text{O}$};
  \draw [->] (q) -- (r) node [midway, below] {$\text{O}$};
  \draw [->] (r) -- (s) node [midway, below] {$\text{Me}$};
  \draw [->] (s) -- (t) node [midway, below] {$\text{Me}$};

\end{tikzpicture}
\end{center}

\textsuperscript{a} Reagents and conditions: a) EDC, HOBt, \textit{Pr}_2NEt, CH\text{Cl}_2, 85%; b) MsCl, Et\textsubscript{3}N, CH\text{Cl}_2; c) DBU, CH\text{Cl}_2, 80% (two steps); d) LiOH, H\textsubscript{2}O, dioxane, 66%.

### 3.2.3. Total synthesis of epilancin 15X analogues

With these Lan/MeLan and Dha/Dhb-containing building blocks in hand, the solid-supported total chemical synthesis of epilancin 15X (3.1) was explored (Scheme 3.7). Fmoc-SPPS was commenced using preloaded Wang resin, the substitution of which was manually lowered to 0.1-0.15 mmol/g to prevent intermolecular side reactions during cyclization observed at higher resin substitution densities. The use of \textit{N,N}'-diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBt) or 1-hydroxy-7-azabenzotriazole (HOAt) for amino acid couplings effectively minimized side reactions involving the Lan/MeLan building blocks, which were encountered when using conditions that necessitated the addition of base. Dipeptide fragment 3.32 and MeLan building blocks \textit{dL}-3.5 and \textit{dL}-3.6 were successfully coupled to the growing solid-supported peptide backbone using this chemistry, giving linear intermediate 3.43 (Scheme 3.7).

To construct the C-ring, the nitrobenzyl protecting groups of 3.43 were removed via reduction with stannous chloride and catalytic HCl, which left the allyl groups unaltered. Fmoc removal with piperidine revealed the N-terminal amine, which was reacted with the carboxylate of the deprotected MeLan residue using (7-azabenzotriazol-1-yloxy)trippyrrlicinophosphonium hexafluorophosphate (PyAOP),\textsuperscript{67} HOAt, and 2,4,6-collidine to give the monocyclic intermediate 3.44. These optimized cyclization conditions promoted complete conversion in a reasonable reaction time (4 h per cyclization) without epimerization of one or both MeLan residues present in the peptide, as analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC). Uronium-based coupling agents such as HBTU are known to give side reactions during
Scheme 3.7. Solid-supported synthesis of analogues of epilancin 15X.$^{a,b}$

![Diagram of the synthesis process]

Reagents and conditions: a) SPPS; b) SnCl$_2$, HCl, DMF; c) piperidine, DMF; d) PyAOP, HOAt, 2,4,6-collidine, DMF; e) Pd(PPh$_3$)$_4$, PhSiH$_3$, DMF, CH$_2$Cl$_2$; f) CF$_3$CO$_2$H, H$_2$O, PhSiH$_3$ (or PhOMe).

Prior to cleavage from resin (step f), all residues contained appropriate side-chain protecting groups for Fmoc-SPPS: Boc for Lys, Pbf for Arg, and Trt for His. Green residues are those modified from the natural product.

Peptide cyclizations,$^{68}$ and PyAOP was found empirically to outperform DIC as well as the next-generation coupling agent $O$-[(cyano-(ethoxycarbonyl)methyliden)-amino]-yloxytrypyrrolidino-phosphonium hexafluorophosphate (PyOxim)$^{69}$ in conversion and reaction time. In addition, 2,4,6-collidine was used for its known suppression of epimerization in sensitive peptide moieties compared to more common amine bases, such as diisopropylethylamine (iPr$_2$NEt).$^{70}$

Following formation of 3.44, leucine was coupled, setting the stage for a second cyclization to yield the B-ring. The allyl protecting groups were removed using tetrakis(triphenylphosphine)palladium(0) and phenylsilane as a scavenger. Fmoc deprotection and cyclization as detailed above yielded the bicyclic intermediate 3.45. Continued SPPS, including coupling of the allyl-protected Lan$_{DL}$-3.3, gave 3.46, which was deprotected and cyclized as for the B-ring to give intermediate 3.47, containing all three rings of epilancin 15X. To complete the total synthesis, fragments 3.35 and 3.39 were needed to install the remaining Dha and Dhb residues in the peptide. Unfortunately, substantial difficulties were encountered
with tripeptide fragment 3.39. Very poor conversion was observed when coupling 3.39 regardless of activator (DIC, PyAOP, \(O-(6\text{-chlorobenzotriazol-1-yl})-N,N',N'\text{-tetramethyl-uronium hexafluorophosphate (HCTU)}\), and \(\text{fluoro-}N,N,N',N'\text{-bis(tetramethylene)}\text{form-amidinium hexafluorophosphate (TFFH)}\)) and reaction time (up to 48 h), with as much as 95% of peptide remaining uncoupled by RP-HPLC analysis. Furthermore, the product of 3.39 coupling was found to degrade during subsequent treatment with piperidine; mass spectrometric analysis suggested that the amide bond between Dhb residues was hydrolyzed. These difficulties thwarted the original goal of a total synthesis of 3.1.

Epilancin 15X does not contain either of the two described lipid II binding motifs of lantibiotics,\(^{71-73}\) and therefore its unusual N-terminal cluster of uncyclized \(\alpha,\beta\)-unsaturated residues may play a role in target binding, potentially through a covalent mechanism. To explore this possibility and to circumvent the issues involved with fragment 3.39, epilancin 15X analogues were successfully synthesized from intermediate 3.47 (Scheme 3.7) replacing the unsaturated Dha and Dhb residues with their saturated \(\text{L-}\)amino acid counterparts alanine and 2-aminobutyric acid (Abu), respectively. Following SPPS of the linear N-terminal segment and cleavage from resin with trifluoroacetic acid (TFA), epilancin 15X analogue 3.48 (Dha3Ala/Dhb7Abu/Dhb8Abu) was obtained. The N-terminal \(\text{\dagger Lac} \) cap was installed by activating \(\text{\dagger Lactic acid with 3-(diethoxyphosphoryloxy)-1,2,3-benzotrazin-4(3H)}\text{-one (DEPBT), an activator tolerant of residues containing unprotected alcohols.}^{74}\) A second analogue, 3.49, was synthesized that included the three mutations of 3.48 and also an N-terminal Pyr cap produced by coupling and deprotecting fragment 3.42 (Scheme 3.6). Finally, a truncated analogue 3.50 was produced, which replaced the eight N-terminal residues with an acetyl cap. It should be noted that anisole was used in place of triisopropylsilane as the primary scavenging agent during resin cleavage of 3.49, as triisopropylsilane is known to partially reduce peptide ketones.\(^{75}\) Following RP-HPLC, these epilancin 15X analogues were obtained in overall yields of 1.6-1.9%, with an average yield of 92-93% per chemical step. Analytical RP-HPLC and MS confirmed the homogeneity of each peptide after purification (Fig. 3.7), and tandem MS/MS confirmed the desired ring topology of analogue 3.48 compared to the authentic natural product (Fig. 3.8). Importantly, this methodology allowed production of multi-milligram quantities of final product within two weeks, thus permitting follow-up biological studies.
Figure 3.7. Characterization of synthetic epilancin 15X analogues, showing analytical RP-HPLC chromatograms and HR-MALDI-TOF mass spectra for (a) analogue 3.48 (insert: zoom-in of expected product mass); (b) analogue 3.49; and (c) analogue 3.50.
Figure 3.8. MALDI-MS/MS analysis of (a) authentic epilancin 15X, and (b) analogue 3.48. Nearly identical fragmentation patterns are seen in both spectra, indicative of the desired ring topology in both peptides.
3.2.4. GC/MS analysis of epilancin 15X analogues

In order to confirm that the desired $\mathrm{DL}$-configuration of the Lan and MeLan crosslinks remained unchanged over the course of the total synthesis, chiral gas chromatography-mass spectrometry (GC/MS) was used, as has been reported for lanthipeptides previously.\textsuperscript{29, 30, 76} In order to prepare samples for analysis, peptides were first hydrolyzed in refluxing 6 M HCl to their constituent amino acids, which were then derivatized to their corresponding pentafluoropropionamide methyl esters to increase volatility (\textit{Scheme 3.8}). Synthetic standards of derivatized Lan and MeLan amino acids with differing stereochemical configurations, provided by Ms. Weixin Tang (UIUC),\textsuperscript{76} could be resolved using a Varian CP-Chirasil-L-Val column. Additionally, these derivatized Lan and MeLan amino acids fragment during MS analysis to give unique masses compared to proteinogenic amino acids, which permits selected ion monitoring (SIM) at these masses to simplify analysis.

\textbf{Scheme 3.8.} Procedure for peptide sample preparation for chiral GC/MS analysis.

Purified epilancin 15X analogue 3.50 was hydrolyzed and derivatized as outlined in \textit{Scheme 3.8}. GC/MS analysis compared to synthetic standards revealed that both the Lan and MeLan amino acids possessed predominantly the desired $\mathrm{DL}$-configuration (\textit{Fig. 3.9}). The small amounts of non-$\mathrm{DL}$-configurations in the Lan sample is believed to result from epimerization during hydrolysis, as has been reported previously in other lanthionine-containing peptide samples.\textsuperscript{29, 76, 77}

3.2.5. Antibacterial activity of epilancin 15X analogues

In order to assess the effect of each mutation/truncation on antibacterial activity, quantitative growth inhibition assays were performed in liquid culture against the indicator strain \textit{Staphylococcus carnosus} TM300 for each of the synthetic analogues and compared to authentic
Figure 3.9. Chiral GC/MS analysis of hydrolyzed and derivatized 3.50, confirming the desired DL-configuration of Lan and MeLan. (a) SIM at 365 Da of hydrolysate compared to synthetic Lan standards. (b) SIM at 379 Da of hydrolysate compared to synthetic MeLan standards. (c) Representative mass spectrum of derivatized Lan. (d) Representative mass spectrum of derivatized MeLan. Data were recorded at the Metabolomics Center (UIUC).

epilancin 15X (Fig. 3.10), which was isolated from its natural producing strain by Dr. Juan Velásquez (UIUC). Two-fold serial dilutions of each peptide were performed, giving a range of concentrations over three orders of magnitude to test for inhibition of bacterial growth. Replacement of the three N-terminal Dha/Dhb residues of epilancin 15X with their saturated counterparts in analogue 3.48 gave only a modest increase in the half maximal inhibitory concentration (IC₅₀), from 95 ± 9.6 to 270 ± 23 nM. This increase may be due to loss of the planarity of these α,β-unsaturated residues, but a mechanism-of-action involving covalent
attachment of epilancin 15X to its putative biological target can be ruled out. Additional substitution of ωLac for Pyr in 3.49 slightly increased the IC\textsubscript{50} to 354 ± 8 nM. This finding suggests that the N-terminal acyl group only plays a minor role in bioactivity. Though both Pyr and ωLac caps have been demonstrated to protect lantibiotics from aminopeptidases,\textsuperscript{37} the additional enzymatic reaction catalyzed by ElxO to reduce Pyr to ωLac may further stabilize epilancin 15X by removing the ketone, a potential site of nucleophilic attack \textit{in vivo}. Truncation of the eight N-terminal residues in analogue 3.50, however, had a significantly deleterious effect on activity, with a 20-fold increase in IC\textsubscript{50} and a 50-fold increase in minimal inhibitory concentration (MIC) compared to epilancin 15X. This N-terminal tail region, primarily composed of nonpolar residues, may therefore be important for noncovalent binding interactions between epilancin 15X and its putative biological target.

**Figure 3.10.** Growth inhibition activity of authentic epilancin 15X and analogues 3.48, 3.49, and 3.50 against \textit{S. carnosus} TM300. Calculated IC\textsubscript{50} and MIC values are shown in the legend.
3.2.6. Flow cytometry analysis of membrane disruption

The C-terminal portion of epilancin 15X, particularly the arrangement of its crosslinks, strongly resembles the C-terminal portion of nisin, which is responsible for pore formation.\textsuperscript{14, 38-40} It was therefore hypothesized that epilancin 15X may similarly be able to form pores in the membranes of susceptible bacteria. The linear N-terminal tail of epilancin 15X may be involved in target binding, as mentioned above, but it may otherwise be necessary for the molecule to span the length of the membrane. To test these hypotheses, flow cytometry analysis was used to investigate alterations in membrane potential and permeability in \textit{S. carnosus} cells treated with either full-length analogue \textbf{3.48} or truncated analogue \textbf{3.50} (Fig. 3.11). Despite removal of eight residues, \textbf{3.50} retained the ability to disrupt both membrane potential and integrity. Higher concentrations of \textbf{3.50} were required than for \textbf{3.48}, but these correlated well with their relative differences in antibacterial potency (Fig. 3.10). These findings are consistent with the suggested role of the nisin-like C-terminal portion of epilancin 15X for pore-forming activity, and further suggest the N-terminal portion to be involved in binding a putative non-lipid II target to increase potency. Indeed, the pore-forming ability of nisin is enhanced about 1,000-fold through binding lipid II.\textsuperscript{78} The strongly positive charge of epilancin 15X may suggest a negatively-charged binding partner, but to date this putative biological target has not yet been identified.

\textbf{Figure 3.11.} Flow cytometry analysis of pore-forming abilities of \textbf{3.48} and \textbf{3.50} against \textit{S. carnosus} TM300. (a) Membrane depolarization activity as measured by DiOC\textsubscript{2}(3) mean fluorescence intensity (MFI). (b) Membrane permeability activity as measured by propidium iodide (PI) uptake.
3.2.7. Total synthesis of lacticin 481 and ring stereoisomer analogues

Prior to 2013, all characterized Lan/MeLan crosslinks were reported to possess the DL-configuration, as depicted in Fig. 3.4. This enforced an assumption that all lanthipeptide crosslinks contain this configuration, as they are made by homologous biosynthetic enzymes. However, the very recent discovery of LL-crosslinks within biosynthesized lanthipeptides, including both peptides of the enterococcal cytolysin and the β-peptide of haloduracin, has challenged this assumption and also called into question the importance of crosslink stereochemistry to biological activity. This observation, seemingly contradictory to the characteristically high stereochemical fidelity of enzymes, mirrors a growing awareness of natural products produced as stereoisomers, occasionally within the same organism. In order to assess the role of Lan/MeLan stereochemical configuration on antibacterial activity, lacticin 481 (3.2) was chosen as a model system. Although the ring topology of this tricyclic lantibiotic was determined in 1996, the absolute stereochemistry of each ring had not yet been determined. Using the Fmoc-SPPS approach described for the synthesis of epilancin 15X analogues (see Section 3.2.1-3.2.3), Lan building blocks DL-3.3 and DL-3.4, MeLan building block DL-3.6, and dipeptide fragment 3.32 were incorporated into a solid-supported peptide backbone (Scheme 3.9); cyclization of each Lan/MeLan residue using the same conditions described in Section 3.2.3, acidic resin cleavage, and RP-HPLC purification resulted in the total synthesis of lacticin 481 (2).  

Scheme 3.9. Solid-supported synthesis of lacticin 481 (2).  

\(^{a}\) Reagents and conditions: a) SPPS; b) SnCl\(_2\), HCl, DMF; c) piperidine, DMF; d) PyAOP, HOAt, 2,4,6-collidine, DMF; e) Pd(PPh\(_3\))\(_4\), PhSiH\(_3\), DMF, CH\(_2\)Cl\(_2\); f) CF\(_3\)CO\(_2\)H, H\(_2\)O, PhSiH\(_3\).  

\(^{b}\) Prior to cleavage from resin (step f), all residues contained appropriate side-chain protecting groups for Fmoc-SPPS: Boc for Lys and Trp, 'Bu for Ser, O'Bu for Glu, and Trt for Asn, Gln, and His.
synthesis of 3.2. Analytical RP-HPLC (Fig. 3.12a) confirmed co-elution of synthetic 3.2 with the authentic natural product, isolated by Dr. Juan Velasquez (UIUC) from the producing organism *L. lactis* subsp. *lactis* CNRZ 481. Mass spectrometry (Fig. 3.12b) and MS/MS (Fig. 3.13) analyses confirmed the same molecular ion mass and fragmentation pattern. Combined with biological activity (see Section 3.2.9), these data verify the successful synthesis of lacticin 481, only the fifth lanthipeptide to succumb to total chemical synthesis.

**Figure 3.12.** Characterization of synthetic lacticin 481 (3.2). (a) Analytical RP-HPLC chromatogram, showing co-elution with authentic lacticin 481 (red). (b) HR-MALDI-TOF mass spectrum (insert: zoom-in of product mass).

During the course of the synthesis, an appreciable amount of epimerization in one or both of the Lan residues was observed. As epimerization was not seen during the synthesis of epilancin 15X analogues (*Scheme 3.7*), the larger ring sizes of lacticin 481 may be to blame, which necessitate repeated exposure of the ester-protected Lan residues to piperidine during the installation of intervening residues. At least through anecdotal evidence, Lan appears to be less configurationally stable than MeLan, and this repeated basic exposure may slowly erode stereochemical integrity. Fortunately, these epimerized byproducts were successfully separated from the desired full-length peptide during RP-HPLC purification. The use of alternative bases for Fmoc-deprotection, such as 1,8-diazabicycloundec-7-ene or piperazine, may reduce the impact of this phenomenon on yield in future syntheses.
Figure 3.13. MALDI-MS/MS analysis of (a) authentic and (b) synthetic lacticin 418 (3.2). Nearly identical fragmentation patterns are seen in both spectra, indicative of the same ring topology in both peptides.
With the total synthesis of 3.2 established, a systematic evaluation of crosslink stereochemistry was investigated. As the absolute configuration of each Lan/MeLan building block is determined by the amino acid starting material, simply replacing the D-amino acid used to make each DL-building block (Schemes 3.1, 3.3, 3.4) with its corresponding L-enantiomer resulted in the generation of LL-Lan/MeLan building blocks (Fig. 3.14) by an analogous synthetic scheme. In the case of LL-3.6, the use of L-threonine as starting material generated a change of two stereocenters, yielding an overall configuration of (2R,3R,6R) compared to (2S,3S,6R) in DL-3.6. Using these LL- instead of DL-building blocks, each ring of 3.2 was systematically changed using the synthetic scheme outlined in Scheme 3.9. In this way, three additional peptides were constructed, containing the diastereomeric LL-crosslink stereochemistry for the A-ring (LL-A), the B-ring (LL-B) and the C-ring (LL-C) of lacticin 481. Following RP-HPLC purification, these peptides were obtained in average overall yields of 1.3%, or 92% per step. Interestingly, all three stereoisomeric analogues exhibited substantial deviations in analytical RP-HPLC retention time (Fig. 3.15) compared to 3.2 and authentic lacticin 481 (Fig. 3.12a) which may signify a change in the overall three-dimensional structure of the analogues.

![Figure 3.14. Comparison of DL- and LL-Lan/MeLan building blocks used in the synthesis of lacticin 481 and ring diastereomer analogues.](image)

**3.2.8. GC/MS analysis of lacticin 481 and analogues**

To confirm the desired change in absolute Lan/MeLan stereochemistry in each analogue, chiral GC/MS analysis was employed as in Section 3.2.4. The desired all-DL-configuration of hydrolyzed and derivatized 3.2 was observed, as well as LL-MeLan and DL-Lan in LL-A; both LL-B and LL-C exhibited DL-MeLan and an approximately equal mixture of DL- and LL-Lan, as one of the two Lan rings in each peptide was altered (Fig. 3.16). Considering that 3.2, but not any of the stereoisomeric analogues, co-eluted with authentic lacticin 481, an all-DL-configuration of authentic lacticin 481 was therefore verified.
Figure 3.15. Characterization of synthetic lacticin 481 analogues, showing analytical RP-HPLC chromatograms and HR-MALDI-TOF mass spectra for (a) analogue ll-A; (b) analogue ll-B; and (c) analogue ll-C.
Figure 3.16. Chiral GC/MS analysis of the derivatized hydrolysates of (a) 3.2, (b) LL-A, (c) LL-B, and (d) LL-C. Shown is the SIM analysis at both 365 Da and 379 Da for each sample, comparing elution times to synthetic standards (e). Data were recorded at the Mass Spectrometry Laboratory (UIUC).
3.2.9. Antibacterial activity of synthetic lacticin 481 and analogues

The antibacterial activity of synthetic 3.2 and analogues was compared to authentic lacticin 481 against the indicator strain *L. lactis* subsp. *cremoris* HP, analogous to the assays described in Section 3.2.5. As expected, synthetic 3.2 (IC$_{50}$ = 300 ± 70 nM; MIC = 625 nM) possessed comparable potency to the authentic natural product (IC$_{50}$ = 250 ± 50 nM; MIC = 625 nM) (*Fig. 3.17*). However, none of the stereoisomeric analogues possessed detectible activity at concentrations up to the highest tested (10 μM). These results indicate that the all-DL-configuration of crosslinks is essential for biological activity. As homologous LanM enzymes are able to produce both DL- and LL-crosslinks, this observation suggests that the stereochemistry of lacticin 481 evolved specifically to allow tight binding to lipid II, its likely biological target (see Chapter 2), and not because the biosynthetic machinery can only generate the DL-configuration. Changing the stereochemical configuration of any crosslink likely alters the three-dimensional structure of the peptide (*Figs 3.12a and 3.15*), which in turn prevents target binding and abolishes activity.

![Figure 3.17](image.png)

*Figure 3.17.* Growth inhibition activity of authentic and synthetic lacticin 481 and analogues LL-A, LL-B, and LL-C against *L. lactis* HP.
3.3. CONCLUSIONS AND OUTLOOK

In this chapter, the use of total chemical synthesis to construct lantibiotics and analogues was investigated. Due to the independence of this approach from the biosynthetic machinery, the chemical space accessible in the production and engineering of analogues was widened greatly and encompassed modifications of the characteristic Lan/MeLan crosslinks themselves. The development of synthetic schemes to stereochemically-pure, orthogonally-protected Lan/MeLan building blocks allowed for their application to peptide cyclization on the solid-phase, yielding lanthipeptide crosslinks with full control over regiochemistry. Two lantibiotics were pursued as synthetic targets: epilancin 15X, in order to overcome complications in biosynthetic engineering of this compound for SAR analysis; and lacticin 481, in order to probe the importance of crosslink stereochemistry on biological activity. An optimized Fmoc-SPPS approach accessed milligram quantities of analogues of these compounds in average yields of 92-93% per chemical step. Analogues of epilancin 15X with substituted post-translational modifications in its linear N-terminus revealed the importance of this N-terminal portion for potency, potentially through binding of a putative biological target, and of the C-terminal ring system for pore formation. Analogues of lacticin 481 provided for the first time a systematic assessment of the importance of crosslink stereochemistry on biological activity, as replacement of the natural DL-configuration of any crosslink with its LL-isomer abolished antibacterial activity. While wild-type epilancin 15X could not be accessed due to the lability of its Dhb-Dhb motif, the successful synthesis of lacticin 481 represents only the fifth lanthipeptide to succumb to total synthesis, following nisin,\textsuperscript{25} lactocin S,\textsuperscript{29} and both peptides of lacticin 3147.\textsuperscript{30}

With regard to epilancin 15X, a supply of the authentic natural product from the producing organism obviated the need to synthesize the wild-type compound in these studies. However, the total synthesis of epilancin 15X is still desirable. As no information about its putative biological target is currently available, other than that lipid II does not appear to be involved,\textsuperscript{41} mode-of-action studies could be enabled by the chemical synthesis of epilancin 15X analogues containing a variety of chemical handles and probes, such as fluorophores, biotin, bioorthogonal moieties, and photoreactive groups. In addition, other lanthipeptides contain adjacent $\alpha,\beta$-unsaturated residues, such as paenibacillin\textsuperscript{84} and lichenicidin\textsuperscript{p,85, 86} and any synthetic efforts to produce these compounds necessitates a strategy to install such moieties. A large fragment coupling approach reminiscent of the synthesis of lacticin 3147,\textsuperscript{30} in which the N-
A terminal octapeptide is synthesized separately, may permit the total synthesis of epilancin 15X. The Dha/Dhb residues may be installed into the octapeptide through the use of phenylselenocysteine surrogates\textsuperscript{20, 60, 61} or other orthogonal elimination chemistry.\textsuperscript{25, 64} Alternatively, recent advances in peptide ligation chemistry may also be considered for attaching the linear N-terminal portion.\textsuperscript{87-89}

Considering that the stereoisomeric analogues of lacticin 481 lacked biological activity, it appears that the three-dimensional structure of this compound, enforced by the absolute configurations of each of its DL-crosslinks, evolved to bind its biological target. The discovery of other class II lantibiotics that contain both DL- and LL-crosslinks, biosynthesized by homologous LanM synthetases,\textsuperscript{44} begs the question of the role of LL-crosslinks in the bioactivity of these compounds. The SPPS platform described here could be adapted to construct stereoisomeric analogues of enterococcal cytolysin or haloduracin β with the LL-crosslinks replaced by their DL-counterparts in order to address this matter.

3.4. EXPERIMENTAL

3.4.1. Materials and general methods

Authentic epilancin 15X from \textit{Staphylococcus epidermidis} 15X154\textsuperscript{37} and lacticin 481 from \textit{Lactococcus lactis} subsp. \textit{lactis} CNRZ 481\textsuperscript{65} were provided by Dr. Juan Velásquez (University of Illinois at Urbana-Champaign; UIUC) after isolation using known procedures. Amino acids for solution-phase synthesis, standard protected Fmoc-amino acids and resins for solid-phase peptide synthesis (SPPS), \textit{d}-lactic acid, and peptide coupling reagents 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3\textit{H})-one (DEBPT), \textit{N,N'-diisopropylcarbodiimide} (DIC), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), 1-hydroxy-7-azabenzotriazole (HOAt), and 1-hydroxybenzotriazole monohydrate (HOBt) were purchased from ChemImpex International. 7-Azabenzotriazole-1-yl oxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyAOP) was purchased from AAPPTec. Dimethylformamide (DMF), dichloromethane, and tetrahydrofuran (THF) were purchased at reaction grade from Fisher Scientific and dried via a solvent dispensing system prior to use in reactions. Flow cytometry dyes 3,3’-diethyloxycarbocyanine iodide (DiOC\textsubscript{2}(3)) and propidium iodide (PI) were purchased from Invitrogen/Life Technologies. Cell culture media were purchased from BD Biosciences.
Other chemical reagents and solvents were purchased from Sigma Aldrich or Alfa Aesar and used without further purification unless otherwise stated.

All reactions were run under an atmosphere of N₂ unless otherwise stated. Reaction progress and chromatography fractions were monitored by thin layer chromatography (TLC) on silica gel-coated glass plates with a F254 fluorescent indicator. Visualization was achieved by UV absorption by fluorescence quenching or permanganate stain (1.5 g KMnO₄, 10 g K₂CO₃, 1.25 mL of 10% NaOH in 200 mL of H₂O). Flash chromatography was performed using Silicycle SiliaFlash P60, 230-400 mesh silica gel. Analytical reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on an Agilent 1260 Infinity system with a Phenomenex Jupiter Proteo C12 (90 Å pore size, 250 mm × 4.6 mm × 10 μm) analytical column with a flow rate of 1 mL/min and solvent gradients as described for each peptide, observing at an absorbance of 220 nm. Preparatory RP-HPLC was performed using a Waters Delta 600 system equipped with a Phenomenex Jupiter Proteo C12 (90 Å pore size, 250 mm × 15.0 mm × 10 μm) preparative column, a flow rate of 10 mL/min, and solvent gradients as described for each peptide. All HPLC solvents were filtered with a Millipore filtration system equipped with a 0.22 μm nylon membrane filter prior to use. HPLC solvent compositions: solvent A is 0.1% trifluoroacetic acid (TFA) in H₂O; solvent B is 80:20 MeCN/H₂O with 0.087% TFA.

NMR spectra were recorded using a Varian Unity 400 or Unity Inova 500 spectrometer at the NMR Laboratory (UIUC). Small molecules (MW < 800 Da) were analyzed by electrospray ionization/time-of-flight (ESI-TOF) mass spectrometry using a Waters Quattro II quadrupole spectrometer at the Mass Spectrometry Facility (UIUC). Peptides (MW > 800 Da) were analyzed by matrix-assisted laser desorption ionization/time-of-flight (MALDI-TOF) mass spectrometry using a Bruker Daltonics UltrafleXtreme TOF/TOF spectrometer at the Mass Spectrometry Facility (UIUC) in a matrix solution consisting of saturated α-cyano-4-hydroxycinnamic acid in 1:1:0.1 H₂O/MeCN/TFA. If desalting was required prior to MS analysis, samples were desalted using ZipTipC₁₈ pipette tips (Millipore) and eluted with 4 μL of matrix solution, then 2 μL was spotted onto a MALDI target and dried as described above.
3.4.2. Synthesis of allyl-protected Lan building blocks $\text{DL-3}$ and $\text{LL-3}$

Compound $\text{d-3.7}$. $\text{d-Serine (2.10 g, 20.0 mmol)}$ and sodium carbonate (3.18 g, 30.0 mmol) were dissolved in water (30 mL) and MeCN (15 mL) and chilled in an ice bath. A solution of allyl chloroformate (AlocCl, 2.1 mL, 20.0 mmol) in MeCN (15 mL) was added dropwise. The reaction was stirred for 8 h, gradually warming to room temperature. The reaction was concentrated under reduced pressure, then taken up in DMF (50 mL). Sodium bicarbonate (1.68 g, 20.0 mmol) was added, followed by allyl bromide (AllBr, 3.5 mL, 40.0 mmol). The reaction was stirred as a heterogeneous mixture under N$_2$ for 15 h. The reaction was concentrated under reduced pressure, then partitioned between water and EtOAc. The reaction was washed with saturated aqueous NaHCO$_3$, 0.1 M KHSO$_4$ and brine, dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO$_2$, 2:1 hexane/EtOAc) to yield $\text{d-3.7}$ (2.67 g, 11.7 mmol, 59%) as a colorless oil. $R_f$ 0.32 (1:1 hexane/EtOAc). Spectral data match those reported previously.$^{53}$ See also Notebook VII, page 76.

Compound $\text{d-3.8}$. Compound $\text{d-3.7}$ (2.00 g, 8.73 mmol) and carbon tetrabromide (3.47 g, 10.5 mmol) were dissolved in CH$_2$Cl$_2$ (25 mL) and chilled in an ice bath. Triphenylphosphine (2.75 g, 10.5 mmol) was dissolved in CH$_2$Cl$_2$ (10 mL) and added dropwise to the chilled solution. The reaction was warmed to room temperature and stirred for 2.5 h, then washed with water and brine, dried over Na$_2$SO$_4$, filtered and concentrated under reduced pressure. Excess 4:1 hexane/EtOAc was added to precipitate phosphine oxide byproducts, which were removed via filtration through Celite. The filtrate was concentrated under reduced pressure and purified by flash chromatography.
(SiO₂, 5:1 then 4:1 hexane/EtOAc) to yield d-3.8 (1.98 g, 6.78 mmol, 78%) as a colorless oil. Rₚ 0.50 (3:1 hexane/EtOAc). Spectral data match those reported previously.\textsuperscript{53} See also Notebook II, page 78.

**Compound 3.9.** To a solution of l-cystine (6.01 g, 25.0 mmol) in 70% perchloric acid (10 mL) was slowly added tert-butyl acetate (62.5 mL). The solution was stirred for 19 h, gradually becoming homogeneous. Water was added, and the solution was chilled in an ice bath. The pH was adjusted to 10 with 10 M NaOH, and the solution was extracted with EtOAc. The organic layer was dried over Na₂SO₄, filtered, and concentrated under reduced pressure. A portion of the residue (5.95 g, 16.9 mmol) was taken up in THF (85 mL), and N-methylmorpholine (3.85 mL, 34.9 mmol) was added. The solution was chilled in an ice bath, then N-(9-fluorenlymethoxy-carbonyloxy)succinimide (FmocOSu; 11.75 g, 34.9 mmol) was added portionwise as a solid over 10 min. The reaction was allowed to warm to room temperature, then stirred for 19 h. The reaction was concentrated under reduced pressure, taken up in EtOAc, washed with water and brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO₂, 9:1 then 7:3 hexane/EtOAc) to yield 3.9 (10.69 g, 13.4 mmol, 79%) as a white solid. Rₚ 0.55 (2:1 hexane/EtOAc). Spectral data matched those reported previously.\textsuperscript{53} See also Notebook I, pages 38-39.

**Compound dl-3.10.** Tributylphosphine (410 µL, 1.64 mmol) was added to a solution of 3.9 (1.09 g, 1.37 mmol) in THF (15 mL) and stirred for 15 min. Water (1.5 mL) was added, and the reaction was stirred an additional 2.5 h, and then concentrated under reduced pressure. To the resulting oil was added d-3.8 (0.80 g, 2.74 mmol) in N₂-sparged EtOAc (15 mL). Tetrabutylammonium bromide (3.55 g, 10.0 mmol) was dissolved in N₂-sparged 0.5 M aqueous NaHCO₃ (pH adjusted to 8.5, 15 mL), then added to the organic solution. The biphasic mixture was stirred for 18 h, then washed with water and brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO₂, 4:1 hexane/EtOAc) to yield dl-3.10 (1.29 g, 2.11 mmol, 77% over two steps) as a colorless oil. Rₚ 0.28 (3:1 hexane/EtOAc). Spectral data match those reported previously.\textsuperscript{53} See also Notebook VII, page 49.
Compound **DL-3.3.** To a solution of **DL-3.10** (1.80 g, 2.95 mmol) in CH₂Cl₂ (15 mL) was added phenylsilane (400 µL, 3.24 mmol), followed by TFA (15 mL). The reaction was stirred for 2 h, then concentrated under reduced pressure to yield **DL-3.3** (1.64 g, quant.) as a white solid after lyophilization from 1:1 benzene/MeCN. R⁰ 0.20 (25:1:0.1 CH₂Cl₂/MeOH/AcOH). Spectral data matched those reported previously.⁵³ See also Notebook II, page 81.

Compound **L-3.8** was prepared as for its enantiomer **D-3.8** in 34% yield over three steps from L-serine. See also Notebook III, pages 15, 18.

Compound **LL-3.10.** Tributylphosphine (310 µL, 1.23 mmol) was added to a solution of **3.9** (0.82 g, 1.03 mmol) in tetrahydrofuran (10 mL) and stirred under N₂ for 15 min. Water (1.0 mL) was added, and the reaction was stirred an additional 3 h then concentrated under reduced pressure. To the resulting oil was added **L-3.8** (0.60 g, 2.05 mmol) in N₂-sparged EtOAc (10 mL). Tetrabutylammonium bromide (2.64 g, 8.20 mmol) was dissolved in N₂-sparged 0.5 M aqueous NaHCO₃ (pH adjusted to 8.5, 10 mL), then added to the organic solution. The biphasic mixture was stirred under N₂ for 16 h, then washed with water and brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO₂, 4:1 hexane/EtOAc) to yield **LL-3.10** (0.87 g, 1.42 mmol, 70% over two steps) as a colorless oil. R⁰ 0.25 (3:1 hexane/EtOAc).¹³ H NMR (500 MHz, CDCl₃) δ 7.77 (d, J = 7.5 Hz, 2H), 7.62 (d, J = 7.5 Hz, 2H), 7.40 (t, J = 7.5 Hz, 2H), 7.32 (t, J = 7.5 Hz, 2H), 5.93-5.84 (m, 2H), 5.69 (app t, J = 7.0 Hz, 2H), 5.40-5.20 (m, 4H), 4.65-4.55 (m, 5H), 4.50 (m, 1H), 4.41 (m, 2H), 4.24 (t, J = 7.0 Hz, 1H), 3.10-2.95 (m, 4H), 1.49 (s, 9H).¹³ C NMR (125 MHz, CDCl₃) δ 170.3, 169.5, 155.9, 155.7, 143.94, 143.86, 141.4, 132.6, 131.3, 127.8, 127.2, 125.2, 120.1, 119.4, 118.1, 83.2, 67.3, 66.6, 66.1, 54.4, 53.9, 47.2, 35.6, 35.5, 28.1. HRMS (ESI) calc. [M+H]⁺ for C₃₂H₃₉N₂O₈S 611.2427, found 611.2431. See also Notebook VIII, page 16.
Compound **LL-3.3**. To a solution of **LL-3.10** (0.80 g, 1.31 mmol) in CH₂Cl₂ (3 mL) was added phenylsilane (180 µL, 1.44 mmol), followed by trifluoroacetic acid (3 mL). The reaction was stirred under N₂ for 2 h, then concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO₂, 1% MeOH/CH₂Cl₂) to yield **LL-3.3** (0.66 g, 1.19 mmol, 91%) as a white solid after lyophilization from 1:1 benzene/MeCN. Rf 0.03 (1:1 EtOAc/hexane). ¹H NMR (500 MHz, CD₃OD) δ 7.73 (d, J = 7.5 Hz, 2H), 7.63 (m, 2H), 7.34 (t, J = 7.5 Hz, 2H), 7.27 (t, J = 7.5 Hz, 2H), 5.92-5.83 (m, 2H), 5.30-5.24 (m, 2H), 5.20-5.15 (m, 2H), 4.58 (d, J = 5.0 Hz, 2H), 4.51 (m, 2H), 4.44 (m, 2H), 4.30 (m, 2H), 4.18 (t, J = 7.0, 1H), 3.14-3.03 (m, 2H), 2.98-2.88 (m, 2H). ¹³C NMR (125 MHz, CD₃OD) δ 173.7, 171.9, 158.4, 158.1, 145.1, 145.0, 124.4, 134.0, 128.7, 128.1, 126.3, 120.9, 118.8, 117.7, 68.1, 67.0, 66.7, 55.3, 55.1, 48.2, 35.2, 34.9. HRMS (ESI) calc. [M+H]⁺ for C₂₈H₃₁N₂O₈S 555.1801, found 555.1801. See also Notebook VIII, page 17.

### 3.4.3. Synthesis of epimerized Lan building block 3.13

Compound **3.11**. D-Serine (1.00 g, 9.51 mmol) and sodium carbonate (1.01 g, 9.51 mmol) were dissolved in water (10 mL) and acetonitrile (5 mL) and chilled on ice. A solution of 4-nitrobenzyl chloroformate (2.05 g, 9.51 mmol) in acetonitrile (5 mL) was added dropwise to the stirring aqueous solution. The reaction was allowed to warm to room temperature and stirred under N₂ for 7 h. The reaction was concentrated under reduced pressure, and the residue was taken up in DMF (25 mL). Sodium bicarbonate (0.80 g, 9.51 mmol) was added, followed by 4-nitrobenzyl bromide (2.26 g, 10.5 mmol). The reaction was stirred as a heterogeneous mixture for 23 h. Additional 4-nitrobenzyl bromide (1.00 g, 4.6 mmol) was added at 15 h. The reaction was concentrated under reduced pressure, then taken up in EtOAc, washed with saturated aqueous NaHCO₃, water, 0.1 M KHSO₄ and brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude material was precipitated from 1:1 hexane/EtOAc and isolated by filtration, yielding **3.11** (3.33 g, 7.94 mmol, 83%) as a colorless oil. Rf 0.10 (1:1 hexane/EtOAc). ¹H NMR (400 MHz, d₆-
DMSO) \( \delta \) 8.16 (dd, \( J = 8.5 \) Hz, 2.5 Hz, 4H), 7.82 (d, \( J = 7.5 \) Hz, 1H), 7.60-7.53 (m, 4H), 5.26 (d, \( J = 2.5 \) Hz, 2H), 5.17 (d, \( J = 2.5 \) Hz, 2H), 5.06 (t, \( J = 6.0 \) Hz, 1H), 4.23 (m, 1H), 3.71 (m, 2H). LRMS (ESI) calc. [M+H]+ for C\(_{18}\)H\(_{16}\)N\(_3\)O\(_9\) 418.1, found 418.2. See also Notebook III, page 98.

**Compound 3.12.** Compound 3.11 (1.20 g, 2.86 mmol) and carbon tetrabromide (1.14 g, 3.44 mmol) were dissolved in THF (10 mL) and chilled on ice. Triphenylphosphine (0.90 g, 3.44 mmol) was dissolved in THF (5 mL) and added dropwise to the chilled reaction. The reaction was warmed to room temperature and stirred under N\(_2\) for 3 h. The reaction was diluted with EtOAc, forming a precipitate that was removed by filtration through Celite. The filtrate was washed with water and brine, dried over Na\(_2\)SO\(_4\), filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO\(_2\), 3:1 then 3:2 hexane/EtOAc) to yield 3.12 (1.05 g, 2.18 mmol, 76%) as an off-white solid. \( R_f \) 0.50 (1:1 hexane/EtOAc). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta \) 8.22 (t, \( J = 8.0 \) Hz, 4H), 7.51 (t, \( J = 8.0 \) Hz, 4H), 5.72 (d, \( J = 8.0 \) Hz, 1H), 5.38-5.21 (m, 4H), 4.88 (m, 1H), 3.88-3.72 (m, 2H). LRMS (ESI) calc. [M+H]+ for C\(_{18}\)H\(_{15}\)BrN\(_3\)O\(_8\) 480.0, found 480.1. See also Notebook V, page 96.

**Compound 3.13.** Fmoc-Cys-O'Bu\(^{53}\) (0.24 g, 0.59 mmol) and 3.12 (0.30 g, 0.62 mmol) were dissolved in N\(_2\)-sparged EtOAc (3 mL) under N\(_2\). Tetrabutylammonium bromide (0.76 g, 2.36 mmol) was dissolved in N\(_2\)-sparged 0.5 M aqueous NaHCO\(_3\) (pH 8.5, 3 mL), then added to the reaction. The biphasic reaction was stirred for 23 h under N\(_2\). The reaction was diluted with EtOAc, washed with water and brine, dried over Na\(_2\)SO\(_4\), filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO\(_2\), 3:1 then 3:2 hexane/EtOAc) to yield 3.13 (0.39 g, 0.49 mmol, 87%) as a white foam. \( R_f \) 0.19 (2:1 hexane/EtOAc). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta \) 8.16-8.10 (m, 4H), 7.72 (d, \( J = 7.5 \) Hz, 2H), 7.54 (d, \( J = 7.0 \) Hz, 2H), 7.44-7.22 (m, 8H), 5.91 (m, 1H, diastereomers), 5.64 (m, 1H, diastereomers), 5.20-5.11 (m, 4H), 4.62 (m, 1H), 4.44 (m, 1H), 4.32 (m, 2H), 4.17 (t, \( J = 7.0 \) Hz, 1H), 3.12-2.87 (m, 4H), 1.45 (s, 9H). \(^{13}\)C NMR (125 MHz, CDCl\(_3\)) \( \delta \) 170.2, 169.3, 156.0, 155.5, 148.0, 147.7, 143.8, 143.7, 143.5, 142.0, 141.4, 128.6, 128.1, 127.9, 127.2, 125.2, 124.0, 123.8, 120.2, 83.5, 67.4, 67.3, 66.1, 65.7, 54.5-5.43 (diastereomers), 54.1-53.9 (diastereomers), 47.1, 36.1, 35.7
(diastereomers), 35.5-34.9 (diastereomers), 28.1. LRMS (ESI) calc. [M+Na]$^+$ for $\text{C}_{40}\text{H}_{40}\text{N}_4\text{O}_{12}\text{SNa}$ 823.2, found 823.6. See also Notebook V, page 98.

### 3.4.4. Synthesis of epimerized MeLan building block 3.16

Compound 3.14. d-Threonine (4.00 g, 33.6 mmol) and sodium carbonate (3.56 g, 33.6 mmol) were dissolved in water (45 mL) and acetonitrile (20 mL) and chilled in an ice bath. Allyl chloroformate (3.57 mL, 33.6 mmol) was added dropwise. The reaction was allowed to warm to room temperature and stirred under $\text{N}_2$ for 23 h. The reaction was concentrated under reduced pressure, and the residue was taken up in DMF (85 mL). Sodium bicarbonate (2.82 g, 33.6 mmol) was added, followed by allyl bromide (3.20 mL, 37.0 mmol). The reaction was stirred as a heterogeneous mixture for 30 h, with additional allyl bromide (1.50 mL, 17.3 mmol) added after 7 h. The reaction was concentrated under reduced pressure, taken up in EtOAc, washed with saturated aqueous NaHCO$_3$, water, 0.1 M KHSO$_4$, and brine, dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO$_2$, 7:3 then 3:2 hexane/EtOAc) to yield 3.14 (6.55 g, 26.9 mmol, 80%) as a colorless oil. $R_f$ 0.52 (1:1 hexane/EtOAc). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 5.96-5.85 (m, 2H), 5.63-5.48 (m, 1H), 5.38-5.20 (m, 4H), 4.68 (m, 2H), 4.60 (m, 2H), 4.38-4.30 (m, 2H), 2.0-1.8 (bs, 1H), 1.26 (d, $J = 7.0$ Hz, 3H). LRMS (ESI) calc. [M+H]$^+$ for $\text{C}_{11}\text{H}_{18}\text{NO}_5$ 244.1, found 244.2. See also Notebook II, page 69.

Compound 3.15. To a solution of carbon tetrabromide (1.43 g, 4.32 mmol) in benzene (15 mL) was added triphenyl phosphine (1.13 g, 4.32 mmol), and the reaction was stirred for 20 min, forming a precipitate. This mixture was then added to a solution of 3.14 (0.70 g, 2.88 mmol) in benzene (15 mL) and stirred for 4 h under $\text{N}_2$. Solids were removed by filtration through Celite and washed with benzene. The combined filtrates were concentrated under reduced pressure, taken up in EtOAc, washed with brine, dried over Na$_2$SO$_4$, filtered, and concentrated. The crude material was purified by flash chromatography (SiO$_2$, 5:1 hexanes/EtOAc) to yield 3.15 (0.70 g, 2.29 mmol, 80%) as a
colorless oil.  $R_f$ 0.65 (3:1 hexanes/EtOAc).  $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 5.97-5.88 (m, 2H), 5.61 (d, $J$ = 7.5 Hz, 1H), 5.40-5.22 (m, 4H), 4.71 (m, 2H), 4.63-4.58 (m, 3H), 4.38 (m, 1H), 1.81 (d, $J$ = 7.0 Hz, 3H).  LRMS (ESI) calc. [M+H]$^+$ for C$_{11}$H$_{17}$BrNO$_3$ 306.0, found 306.2.  See also Notebook VI, page 91.

Compound 3.16.  Fmoc-Cys-O"Bu$^{53}$ (1.74 g, 4.35 mmol) and 3.15 (1.40 g, 4.57 mmol) were dissolved in N$_2$-sparged EtOAc (20 mL) under N$_2$.  Tetrabutylammonium bromide (5.61 g, 17.4 mmol) was dissolved in 0.5 M aqueous NaHCO$_3$ (pH 8.5, 20 mL), then added to the reaction.  The reaction was stirred vigorously as a heterogeneous mixture for 40 h under N$_2$.  The reaction was diluted with EtOAc, washed with water and brine, dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure.  The crude material was purified by flash chromatography (SiO$_2$, 6:1 hexane/EtOAc) to yield 3.16 (0.39 g, 0.49 mmol, 26%) as a colorless oil and a mixture of diastereomers.  $R_f$ 0.39 (3:1 hexanes/EtOAc).  $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.76 (d, 2H), 7.66 (m, 2H), 7.32 (t, 2H), 5.96-5.86 (m, 2H), 5.77-5.45 (m, 2H, diastereomers), 5.37-5.19 (m, 4H), 4.69-4.40 (m, 6H), 4.38-4.34 (m, 2H), 4.24 (t, 1H), 3.50-3.25 (m, 1H, diastereomers), 3.14-2.93 (m, 2H, diastereomers), 1.49 (s, 9H), 1.35 (d, 1.5H, diastereomers), 1.25 (m, 1.5H, diastereomers).  LRMS (ESI) calc. [M+H]$^+$ for C$_{33}$H$_{41}$N$_2$O$_8$S 625.3, found 625.4.  See also Notebook II, page 77.

3.4.5. Synthesis of nitrobenzyl-protected Lan building blocks DL-3.4 and LL-3.4
Compound **3.17.** To a stirring suspension of *N*-Fmoc-L-serine (8.19 g, 25.0 mmol) in ethyl acetate (125 mL) was added *tert*-butyl 2,2,2-trichloroacetimidate (8.95 mL, 50.0 mmol) in cyclohexane (50 mL) by addition funnel over 15 min. The reaction was stirred under N\(_2\) for 18 h, then washed with saturated aqueous NaHCO\(_3\), water and brine, dried over Na\(_2\)SO\(_4\), filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO\(_2\), 3:1 hexane/EtOAc) to yield **3.17** (8.90 g, 23.2 mmol, 93%) as a white solid. \(R_f\) 0.58 (1:1 hexane/EtOAc). 1H NMR confirms product. Spectral data match those previously reported.\(^{90}\) See also Notebook VII, page 89.

Compound **3.18.** Compound **3.17** (2.00 g, 5.22 mmol) and carbon tetrabromide (2.08 g, 6.26 mmol) were dissolved in CH\(_2\)Cl\(_2\) (10 mL) and chilled in an ice bath. Triphenylphosphine (1.64 g, 6.26 mmol) was dissolved in CH\(_2\)Cl\(_2\) (10 mL) and added dropwise to the chilled solution. The reaction was warmed to room temperature and stirred under N\(_2\) for 2.5 h, then washed with water and brine, dried over Na\(_2\)SO\(_4\), filtered, and concentrated under reduced pressure. Excess 5:1 hexane/EtOAc was added to precipitate phosphine oxide byproducts, which were removed via filtration through Celite. The filtrate was concentrated under reduced pressure and purified by flash chromatography (SiO\(_2\), 15% EtOAc/hexane) to yield **3.18** (1.83 g, 4.10 mmol, 79%) as an amber oil. \(R_f\) 0.56 (3:1 hexane/EtOAc). Spectral data match those reported previously.\(^{91}\) See also Notebook II, page 89.

Compound **3.19.** S-trityl-L-cysteine (1.82 g, 5.00 mmol) and sodium carbonate (0.53 g, 5.00 mmol) were dissolved in water (10 mL) and MeCN (5 mL) and chilled in an ice bath. A solution of 4-nitrobenzyl chloroformate (1.08 g, 5.00 mmol) in MeCN (5 mL) was added dropwise over 10 min. The reaction was stirred for 15 h, gradually warming to room temperature, then concentrated under reduced pressure. The residue was taken up in DMF (10 mL), and sodium bicarbonate (0.50 g, 6.00 mmol) was added, followed by 4-nitrobenzyl bromide (1.30 g, 6.00 mmol). The reaction was stirred as a heterogeneous mixture for 21 h, then concentrated under reduced pressure and the residue taken up in EtOAc. The organic layer was washed with saturated aqueous NaHCO\(_3\), 0.1 M KHSO\(_4\), and brine, dried over Na\(_2\)SO\(_4\), filtered, and concentrated under reduced pressure.
The crude material was purified by flash chromatography (SiO$_2$, 2:1 hexane/EtOAc) to yield d-3.19 (3.20 g, 4.72 mmol, 94%) as a yellow foam. $R_f$ 0.28 (2:1 hexane/EtOAc). $^1$H NMR (500 MHz, CDCl$_3$) δ 8.20 (m, 4H), 7.50 (d, $J = 8.5$ Hz, 2H), 7.46 (d, $J = 8.5$ Hz, 2H), 7.37 (m, 6H), 7.33-7.20 (m, 9H), 5.35 (d, $J = 8.5$ Hz, 1H), 5.28-5.16 (m, 4H), 4.38 (q, $J = 6.5$ Hz, 1H), 2.76 (dd, $J = 12.5$ Hz, 6.5 Hz, 1H), 2.65 (dd, $J = 12.5$ Hz, 4.5 Hz, 1H). $^{13}$C NMR (125 MHz, CDCl$_3$) δ 170.2, 155.3, 148.0, 147.8, 144.2, 143.6, 129.5, 128.6, 128.2, 128.1, 127.2, 67.3, 66.0, 65.7, 53.2, 34.0. HRMS (ESI) calc. [M+Na]$^+$ for C$_{37}$H$_{31}$N$_3$O$_8$SNa 700.1730, found 700.1733. See also Notebook VIII, page 75.

**Compound d-3.20.** To a solution of d-3.19 (2.03 g, 3.00 mmol) in CH$_2$Cl$_2$ (30 mL) under N$_2$ was added triisopropylsilane (0.92 mL, 4.50 mmol), followed by trifluoroacetic acid (3.5 mL, 45.0 mmol). A vivid yellow color appeared immediately, then faded over the reaction time (1.5 h). The reaction was diluted with CH$_2$Cl$_2$ and water, then solid sodium bicarbonate (3.78 g, 45.0 mmol) was slowly added to neutralize the residual acid. The organic layer was separated, washed with brine, dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO$_2$, 2:1 hexane/EtOAc) to yield d-3.20 (1.15 g, 2.64 mmol, 88%) as an amber solid. $R_f$ 0.53 (1:1 hexane/EtOAc). $^1$H NMR (500 MHz, CDCl$_3$) δ 8.20 (app t, $J = 8.5$ Hz, 4H), 5.80 (d, $J = 7.5$ Hz, 1H), 5.35-5.26 (m, 2H), 5.22 (m, 2H), 4.73 (m, 1H), 3.10-2.98 (m, 2H), 1.38 (t, $J = 9.0$ Hz, 1H). $^{13}$C NMR (125 MHz, CDCl$_3$) δ 169.7, 155.3, 148.0, 147.8, 143.5, 142.1, 128.8, 128.3, 124.0, 123.9, 66.2, 65.8, 55.4, 27.1. HRMS (ESI) calc. [M+H]$^+$ for C$_{18}$H$_{18}$N$_3$O$_8$S 436.0815, found 436.0819. See also Notebook VIII, page 76.

**Compound dl-3.21.** Compounds 3.18 (0.58 g, 1.29 mmol) and d-3.20 (0.45 g, 1.03 mmol) were dissolved in N$_2$-sparged EtOAc (5 mL). Tetrabutylammonium bromide (1.33 g, 4.12 mmol) was dissolved in N$_2$-sparged 0.5 M aqueous NaHCO$_3$ (pH adjusted to 8.5, 5 mL), then added to the organic solution. The biphasic mixture was stirred under N$_2$ for 20 h, with tributylphosphine (130 μL, 0.52 mmol) added to the reaction after 5 h. The organic layer was isolated, washed with water and brine, dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO$_2$, 30% EtOAc/hexane) to yield dl-3.21 (0.64 g, 0.80 mmol, 78%) as a colorless foam. $R_f$ 0.39 (2:1 hexane/EtOAc).
hexane/EtOAc). $^1$H NMR (500 MHz, CDCl$_3$) δ 8.15 (m, 4H), 7.74 (d, $J = 7.5$ Hz, 2H), 7.56 (m, 2H), 7.44 (app d, $J = 8.5$ Hz, 4H), 7.39 (t, $J = 7.5$ Hz, 2H), 7.29 (t, $J = 7.5$ Hz, 2H), 5.96 (d, $J = 7.5$ Hz, 1H), 5.74 (d, $J = 7.5$ Hz, 1H), 5.23 (m, 2H), 5.17 (m, 2H), 4.67 (m, 1H), 4.48 (m, 1H), 4.34 (m, 2H), 4.20 (t, $J = 7.0$ Hz, 1H), 3.12 (dd, $J = 14.0$ Hz, 5.0 Hz, 1H), 3.08-3.01 (m, 2H), 2.94 (dd, $J = 14.0$ Hz, 5.0 Hz, 1H), 1.47 (s, 9H). $^{13}$C NMR (125 MHz, CDCl$_3$) δ 170.1, 169.2, 156.0, 155.5, 147.8, 147.6, 143.8, 143.7, 143.5, 142.1, 141.3, 128.5, 128.0, 127.9, 127.2, 125.1, 123.9, 123.8, 120.1, 83.4, 67.4, 66.1, 65.7, 54.5, 54.0, 47.1, 36.1, 35.4, 28.0. HRMS (ESI) calc. [M+H]$^+$ for C$_{40}$H$_{41}$N$_4$O$_{12}$S 801.2442, found 801.2447. See also Notebook IX, page 68.

Compound dl-3.4. To a solution of dl-3.21 (0.50 g, 0.62 mmol) in CH$_2$Cl$_2$ (3 mL) was added phenylsilane (80 µL, 0.66 mmol), followed by trifluoroacetic acid (3 mL). The reaction was stirred under N$_2$ for 2.5 h, then concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO$_2$, 2% MeOH/CH$_2$Cl$_2$) to yield dl-3.4 (0.44 g, 0.59 mmol, 95%) as a colorless foam. $^1$H NMR (500 MHz, CD$_3$OD) δ 8.10 (app t, $J = 8.5$ Hz, 4H), 7.75 (d, $J = 7.5$ Hz, 2H), 7.63 (d, $J = 7.5$ Hz, 2H), 7.51 (app t, $J = 7.5$ Hz, 4H), 7.35 (dt, $J = 7.5$ Hz, 3.5 Hz, 2H), 7.25 (dt, $J = 7.5$ Hz, 3.5 Hz, 2H), 5.25-5.15 (m, 4H), 4.56 (m, 1H), 4.44 (m, 1H), 4.33 (m, 1H), 4.23 (m, 1H), 4.18 (t, $J = 7.0$ Hz, 1H), 3.10 (dd, $J = 14.0$ Hz, 5.0 Hz, 2H), 3.05-2.91 (m, 2H). $^{13}$C NMR (125 MHz, CD$_3$OD) δ 173.7, 171.8, 158.4, 157.9, 148.8, 148.7, 145.7, 145.1, 144.3, 142.4, 129.3, 128.9, 128.8, 128.2, 126.3, 124.51, 124.47, 120.9, 68.2, 66.7, 66.3, 55.8, 55.4, 48.2, 35.7, 35.2. HRMS (ESI) calc. [M+H]$^+$ for C$_{36}$H$_{33}$N$_4$O$_{12}$S 745.1816, found 745.1823. See also Notebook VIII, page 79.

Compound l-3.20 was prepared as for its enantiomer d-3.20 in 63% yield over two steps from S-trityl-l-cysteine. See also Notebook IX, pages 19, 24.
Compound **L\textsubscript{L}-3.21**. Compounds **3.18** (0.56 g, 1.26 mmol) and **t-3.20** (0.50 g, 1.15 mmol) were dissolved in N\textsubscript{2}-sparged EtOAc (8 mL). Tetrabutylammonium bromide (1.48 g, 4.60 mmol) was dissolved in N\textsubscript{2}-sparged 0.5 M aqueous NaHCO\textsubscript{3} (pH adjusted to 8.5, 8 mL), then added to the organic solution. The biphasic mixture was stirred under N\textsubscript{2} for 24 h, with tributylphosphine (140 µL, 0.58 mmol) added to the reaction after 4 h. The organic layer was isolated, washed with water and brine, dried over Na\textsubscript{2}SO\textsubscript{4}, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO\textsubscript{2}, 30\% EtOAc/hexane) to yield **L\textsubscript{L}-3.21** (0.62 g, 0.78 mmol, 68\%) as a colorless foam. \( R_f \) 0.38 (2:1 hexane/EtOAc). \( ^{1}H \) NMR (500 MHz, CDCl\textsubscript{3}) \( \delta \) 8.16 (m, 4H), 7.74 (d, \( J = 7.5 \) Hz, 2H), 7.56 (m, 2H), 7.46-7.36 (m, 6H), 7.29 (t, \( J = 7.5 \) Hz, 2H), 5.91 (d, \( J = 7.5 \) Hz, 1H), 5.65 (d, \( J = 7.0 \) Hz, 1H), 5.23-5.11 (m, 4H), 4.62 (m, 1H), 4.42-4.32 (m, 2H), 4.20 (t, \( J = 7.0 \) Hz, 1H), 3.12-3.03 (m, 3H), 2.91 (dd, \( J = 14.0 \) Hz, 5.5 Hz, 1H), 1.47 (s, 9H). \( ^{13}C \) NMR (125 MHz, CDCl\textsubscript{3}) \( \delta \) 170.2, 169.3, 156.0, 155.5, 147.8, 147.5, 143.7, 143.6, 143.5, 142.1, 141.3, 128.5, 127.9, 127.8, 127.1, 125.1, 123.8, 123.7, 120.1, 83.4, 67.2, 66.0, 65.6, 54.3, 53.9, 47.1, 35.6, 34.8, 28.0. HRMS (ESI) calc. [M+H]\textsuperscript{+} for C\textsubscript{40}H\textsubscript{41}N\textsubscript{4}O\textsubscript{12}S 801.2442, found 801.2443. See also Notebook IX, page 25.

Compound **L\textsubscript{L}-3.4**. To a solution of **L\textsubscript{L}-3.21** (0.55 g, 0.69 mmol) in CH\textsubscript{2}Cl\textsubscript{2} (5 mL) and phenylsilane (80 µL, 0.72 mmol) was added trifluoroacetic acid (5 mL). The reaction was stirred for 2 h, concentrated under reduced pressure and the residue repeatedly redissolved in CH\textsubscript{2}Cl\textsubscript{2} and concentrated to remove residual acid. The crude material was purified by flash chromatography (SiO\textsubscript{2}, 2\%-5\% MeOH/CH\textsubscript{2}Cl\textsubscript{2}) to yield **L\textsubscript{L}-3.4** (0.45 g, 0.61 mmol, 89\%) as a white solid after lyophilization from 1:1 benzene/MeCN. \( R_f \) 0.05 (EtOAc). \( ^{1}H \) NMR (500 MHz, CD\textsubscript{3}OD) \( \delta \) 8.10 (app t, \( J = 8.5 \) Hz, 4H), 7.74 (d, \( J = 7.5 \) Hz, 2H), 7.62 (d, \( J = 7.5 \) Hz, 2H), 7.49 (app t, \( J = 7.5 \) Hz, 4H), 7.34 (t, \( J = 7.5 \) Hz, 2H), 7.26 (t, \( J = 7.5 \) Hz, 2H), 5.26-5.11 (m, 4H), 4.55 (m, 1H), 4.43 (m, 1H), 4.31 (m, 1H), 4.24 (m, 1H), 4.18 (t, \( J = 7.0 \) Hz, 1H), 3.15 (dd, \( J = 14.0 \) Hz, 4.5 Hz, 2H), 2.99 (dd, \( J = 14.0 \) Hz, 3.5 Hz, 1H), 2.90 (dd, \( J = 14.0 \) Hz, 4.0 Hz, 1H). \( ^{13}C \) NMR (125 MHz, CD\textsubscript{3}OD) \( \delta \) 173.7, 171.9, 158.4, 157.9, 148.9, 148.7, 145.7, 145.13, 145.11, 144.3, 142.4, 129.3, 128.9, 128.8, 128.1, 126.3, 124.52, 124.47, 120.9, 68.2, 66.7, 66.3, 55.6,
3.4.6. Synthesis of allyl-protected MeLaN building block dl-3.5

Compound d-3.22. d-Threonine (4.17 g, 35.0 mmol) and para-toluenesulfonic acid monohydrate (7.99 g, 42.0 mmol) were combined in toluene (90 mL). Allyl alcohol (AlOH, 24 mL, 350 mmol) was added, and the reaction was refluxed in an oil bath (110 °C) connected to a Dean-Stark apparatus for 15 h, then concentrated under reduced pressure and dried azeotropically with benzene. The residue was taken up in CH₂Cl₂ (175 mL) and chilled in an ice bath. Triethylamine (14.6 mL, 105 mmol) was added, and the reaction was allowed to stir for 10 min. 4-Nitrobenzenesulfonyl chloride (NsCl, 8.53 g, 38.5 mmol) was added portionwise as a solid, and the reaction was stirred for 4 h at 0 °C. The reaction mixture was washed with 1 M NaH₂PO₄, saturated aqueous NaHCO₃ and brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO₂, 7:3 then 3:2 hexane/EtOAc) to yield d-3.22 (9.48 g, 27.5 mmol, 79% over two steps) as a yellow solid. Rf 0.43 (1:1 hexane/EtOAc). Spectral data match those reported previously. See also Notebook VII, pages 7-8.

Compound d-3.23. A solution of d-3.22 (2.70 g, 7.84 mmol) and triphenylphosphine (2.67 g, 10.2 mmol) in THF (30 mL) was chilled in an ice bath. Diisopropylazodicarboxylate (1.7 mL, 8.63 mmol) was added dropwise, and the reaction was stirred for 2.5 h at 0 °C, then concentrated under reduced pressure. The residue was taken up in EtOAc, washed with saturated aqueous NaHCO₃ and brine, dried over...
Na₂SO₄, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO₂, 5:1 hexane/EtOAc) to yield d-3.23 (2.13 g, 6.53 mmol, 83%) as a yellow solid. Rᵢ 0.54 (2:1 hexane/EtOAc). Spectral data match those reported previously.⁹² See also Notebook VIII, page 20.

Compound d-3.24. A solution of d-3.23 (0.65 g, 2.00 mmol) and 4-methoxybenzyl mercaptan (1.12 mL, 8.00 mmol) in CH₂Cl₂ (20 mL) was chilled in an ice bath. Boron trifluoride diethyl etherate (0.74 mL, 6.00 mmol) was added dropwise to the stirring solution. The reaction was stirred for 21 h at 4 °C, then washed with saturated aqueous NaHCO₃ and brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO₂, 4:1 hexane/EtOAc) to yield d-3.24 (0.82 g, 1.71 mmol, 86%) as a yellow solid. Rᵢ 0.47 (2:1 hexane/EtOAc). Spectral data match those reported previously.⁹² See also Notebook VI, page 39.

Compound d-3.25. To a stirring solution of d-3.24 (2.40 g, 5.00 mmol) in 49:1 MeCN/dimethylsulfoxide (35 mL) were added 4-methoxybenzene thiol (1.84 mL, 15.0 mmol) and potassium carbonate (2.76 g, 20.0 mmol). The reaction was stirred as a heterogeneous mixture for 3 h, then concentrated under reduced pressure. The residue was taken up in EtOAc, washed with water and brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude mixture was purified by flash chromatography (SiO₂, 3:2 then 2:3 hexane/EtOAc) to yield d-3.25 (1.35 g, 4.57 mmol, 91%) as a colorless oil. Rᵢ 0.30 (1:1 hexane/EtOAc). Spectral data match those reported previously.⁹² See also Notebook VII, page 17.

Compound d-3.26. Diisopropylethylamine (0.74 mL, 4.26 mmol) and allyloxy carbonyloxy succinimide (AlocOSu; 0.74 g, 3.73 mmol) were added to a solution of d-3.25 (1.05 g, 3.55 mmol) in CH₂Cl₂ (20 mL) and the reaction was stirred for 12 h. The reaction was washed with water, 10% citric acid and brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO₂, 4:1 hexane/EtOAc) to yield d-3.26 (1.31 g, 3.45 mmol, 97%) as a colorless oil. Rᵢ 0.58 (2:1 hexane/EtOAc). ¹H NMR (500 MHz, CDCl₃) δ 7.20 (dd, J = 9.5 Hz, 2.5 Hz, 2H), 6.84 (dd, J = 9.5 Hz, 2.5 Hz, 2H), 5.95-5.84 (m, 2H), 5.50 (d, J = 9.0 Hz,
1H), 5.37-5.20 (m, 4H), 4.66 (dd, J = 13.0 Hz, 6.0 Hz, 1H), 4.59-4.56 (m, 3H), 4.53 (dd, J = 9.5 Hz, 3.5 Hz, 1H), 3.79 (s, 3H), 3.67 (d, J = 13.0 Hz, 1H), 3.64 (s, J = 13.0 Hz, 1H), 3.31 (m, 1H), 1.30 (d, J = 7.0 Hz, 3H). 

13C NMR (125 MHz, CDCl3) δ 170.6, 158.9, 156.4, 132.7, 131.5, 130.1, 129.7, 119.3, 118.0, 114.1, 66.4, 66.1, 58.6, 55.4, 42.2, 35.2, 19.7.  

HRMS (ESI) calc. [M+H]+ for C19H26NO5S 380.1532, found 380.1532. See also Notebook VII, page 64.

Compound **DL-3.27.** Compound **D-3.26** (0.68 g, 1.80 mmol) was dissolved in TFA (10 mL) and anisole (780 μL, 7.20 mmol). Mercury(II) acetate (1.15 g, 3.60 mmol) was added as a solid, and the purple solution was stirred for 4 h. Dithiothreitol (DTT, 0.56 g, 3.60 mmol) was then added, forming a grey precipitate. This heterogeneous mixture was stirred vigorously for 15 h, then diluted with CH2Cl2 and centrifuged (4600 ×g, 10 min) to remove the solids. The supernatant was concentrated under reduced pressure, taken up in CH2Cl2 and water, and neutralized by slow addition of saturated aqueous NaHCO3 to pH 7. The organic layer was separated, dried over Na2SO4, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO2, 15% EtOAc/hexane) to yield the intermediary thiol, which was used directly for the next reaction without complete concentration or characterization due to its instability. Rf 0.52 (3:1 hexane/EtOAc). To the partially-concentrated thiol was added **3.18** (0.54 g, 1.20 mmol) and N2-sparged EtOAc (6 mL). Tetrabutylammonium bromide (1.55 g, 4.80 mmol) was dissolved in N2-sparged 0.5 M aqueous NaHCO3 (pH adjusted to 8.5, 6 mL), then added to the organic solution. The biphasic reaction was stirred for 5 h, and the pH was adjusted to 8.5 as necessary with 1 M NaOH. Tributylphosphine (150 μL, 0.60 mmol) was added, and the reaction was stirred for an additional 17 h. The organic layer was isolated, washed with water and brine, dried over Na2SO4, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO2, 4:1 hexane/EtOAc) to yield **DL-3.27** (0.48 g, 0.77 mmol, 64% over two steps) as a colorless foam. Rf 0.34 (3:1 hexane/EtOAc). 1H NMR (500 MHz, CDCl3) δ 7.77 (d, J = 7.5 Hz, 2H), 7.62 (d, J = 7.5 Hz, 2H), 7.41 (t, J = 7.5 Hz, 2H), 7.32 (t, J = 7.5 Hz, 2H), 5.95-5.88 (m, 2H), 5.62 (d, J = 7.5 Hz, 1H), 5.57 (d, J = 9.5 Hz, 1H), 5.37-5.20 (m, 4H), 4.70-4.53 (m, 5H), 4.48-4.37 (m, 3H), 4.24 (t, J = 7.0 Hz, 1H), 3.43 (m, 1H), 3.02-2.89 (m, 2H), 1.48 (s, 9H), 1.34 (d, J = 7.0 Hz, 3H). 13C NMR (125 MHz, CDCl3) δ 170.4, 169.4, 156.4, 155.8, 144.0, 143.9, 141.4, 132.6, 131.4, 127.9, 127.2, 125.2, 120.1, 119.6, 118.1, 83.3, 67.3, 66.5, 66.2, 58.5,
Compound DL-3.5. To a solution of DL-3.27 (0.45 g, 0.72 mmol) in CH₂Cl₂ (3 mL) was added phenylsilane (95 µL, 0.76 mmol), followed by TFA (3 mL). The reaction was stirred for 2 h, concentrated under reduced pressure and repeatedly redissolved in CH₂Cl₂ and concentrated to remove residual TFA. The crude material was purified by flash chromatography (SiO₂, 1%-2% MeOH/CH₂Cl₂) to yield DL-3.5 (0.39 g, 0.69 mmol, 96%) as a white solid after lyophilization from 1:1 benzene/MeCN. Rₚ 0.05 (2:1 EtOAc/hexane). ¹H NMR (500 MHz, CD₃OD) δ 7.80 (d, J = 7.5 Hz, 2H), 7.69 (d, J = 7.5 Hz, 2H), 7.39 (t, J = 7.5 Hz, 2H), 7.31 (t, J = 7.5 Hz, 2H), 5.99-5.88 (m, 2H), 5.38-5.29 (m, 2H), 5.24-5.16 (dd, J = 22.5 Hz, 10.5 Hz, 2H), 4.65 (m, 2H), 4.55 (d, J = 5.5 Hz, 2H), 4.47 (d, J = 4.0 Hz, 1H), 4.41-4.30 (m, 3H), 4.25 (t, J = 7.0, 1H), 3.98 (dd, J = 13.5 Hz, 4.5 Hz, 1H), 2.83 (dd, J = 13.5 Hz, 8.5 Hz, 1H), 1.31 (d, J = 7.0 Hz, 3H). ¹³C NMR (125 MHz, CD₃OD) δ 173.7, 171.7, 158.6, 158.4, 145.3, 145.2, 142.6, 134.2, 133.1, 128.8, 128.2, 126.3, 120.9, 119.2, 117.8, 68.2, 67.2, 66.8, 60.2, 55.3, 48.4, 43.8, 34.2, 19.8. HRMS (ESI) calc. [M+H]⁺ for C₂₉H₃₃N₂O₈S 569.1958, found 569.1959. See also Notebook VII, page 38.

3.4.7. Synthesis of nitrobenzyl-protected MeLan building blocks DL-3.6 and LL-3.6

Compound d-3.56. Diisopropylethylamine (1.4 mL, 8.1 mmol) and 4-nitrobenzyl chloroformate (1.46 g, 6.77 mmol) were added to a solution of d-3.25 (2.00 g, 6.77 mmol) in CH₂Cl₂ (35 mL), and the reaction was stirred for 16 h. The reaction was washed with water, 10% citric acid and brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO₂, 4:1 hexane/EtOAc) to yield d-3.56 (3.10 g, 6.53 mmol, 96%) as a
colorless oil. \( R_f \) 0.50 (2:1 hexane/EtOAc). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \( \delta \) 8.21 (d, \( J = 9.0 \) Hz, 2H), 7.51 (d, \( J = 9.0 \) Hz, 2H), 7.19 (d, \( J = 8.5 \) Hz, 2H), 6.83 (dt, \( J = 8.5 \) Hz, 2.0 Hz, 2H), 5.91-5.83 (m, 1H), 5.62 (d, \( J = 9.0 \) Hz, 1H), 5.36-5.32 (dd, \( J = 17.0 \) Hz, 1.0 Hz, 1H), 5.27 (dd, \( J = 10.5 \) Hz, 1.0 Hz, 1H), 5.23 (d, \( J = 13.5 \) Hz, 1H), 5.19 (d, \( J = 13.5 \) Hz, 1H), 4.66 (dd, \( J = 13.0 \) Hz, 6.0 Hz, 1H), 4.58 (dd, \( J = 13.0 \) Hz, 6.0 Hz, 1H), 4.53 (dd, \( J = 9.0 \) Hz, 3.5 Hz, 1H), 3.78 (s, 3H), 3.67 (d, \( J = 13.0 \) Hz, 1H), 3.63 (d, \( J = 13.0 \) Hz, 1H), 3.33 (m, 1H), 1.30 (d, \( J = 7.5 \) Hz, 3H). \(^{13}\)C NMR (125 MHz, CDCl\(_3\)) \( \delta \) 170.3, 158.9, 156.1, 147.7, 143.8, 131.4, 130.0, 129.5, 128.1, 123.9, 119.4, 114.1, 66.5, 65.7, 58.7, 55.4, 42.1, 35.2, 19.8. HRMS (ESI) calc. [M+H]\(^+\) for C\(_{23}\)H\(_{27}\)N\(_2\)O\(_7\)S 475.1539, found 475.1556. See also Notebook VII, page 62.

Compound d-3.28. Tetrakis(triphenylphosphine)palladium(0) (390 mg, 0.34 mmol) was added to a solution of d-3.56 (3.20 g, 6.74 mmol) and N-methylaniline (1.5 mL, 13.5 mmol) in THF (60 mL). The reaction was stirred for 1.5 h, protected from light, then concentrated under reduced pressure. The resulting oil was taken up in DMF (25 mL), and sodium bicarbonate (1.13 g, 13.5 mmol) and 4-nitrobenzyl bromide (3.65 g, 16.9 mmol) were added as solids. The reaction was stirred for 30 h, with additional 4-nitrobenzyl bromide (pNbBr, 1.46 g, 6.74 mmol) added after 11 h. The reaction mixture was concentrated under reduced pressure, taken up in EtOAc, washed with saturated aqueous NaHCO\(_3\), water and brine, dried over Na\(_2\)SO\(_4\), filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO\(_2\), 30% EtOAc/hexane) to yield d-3.28 (3.55 g, 6.23 mmol, 93% over two steps) as an amber foam. \( R_f \) 0.38 (2:1 hexane/EtOAc). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \( \delta \) 8.19 (app. t, \( J = 8.5 \) Hz, 4H), 7.50 (d, \( J = 8.5 \) Hz, 2H), 7.45 (d, \( J = 9.0 \) Hz, 2H), 7.13 (d, \( J = 8.5 \) Hz, 2H), 6.78 (dt, \( J = 8.5 \) Hz, 2.0 Hz, 2H), 5.60 (d, \( J = 9.0 \) Hz, 1H), 5.28-5.14 (m, 4H), 4.59 (dd, \( J = 9.0 \) Hz, 3.5 Hz, 1H), 3.75 (s, 3H), 3.64 (d, \( J = 13.0 \) Hz, 1H), 3.59 (d, \( J = 13.0 \) Hz, 1H), 3.33 (m, 1H), 1.33 (d, \( J = 7.0 \) Hz, 3H). \(^{13}\)C NMR (125 MHz, CDCl\(_3\)) \( \delta \) 170.3, 158.9, 156.0, 147.9, 147.8, 143.6, 142.2, 129.9, 129.5, 128.1, 123.9, 128.2, 123.93, 123.89, 114.1, 66.0, 65.8, 58.8, 55.3, 41.7, 35.0, 19.4. HRMS (ESI) calc. [M+H]\(^+\) for C\(_{27}\)H\(_{28}\)N\(_3\)O\(_9\)S 570.1546, found 570.1566. See also Notebook VII, page 63.

Compound d-3.57. Compound d-3.28 (0.85 g, 1.50 mmol) was dissolved in TFA (6 mL) and anisole (650 µL, 6.00 mmol). Mercury(II) acetate (0.96 g, 3.00 mmol) was added, and the purple solution was stirred for 4 h.
Dithiothreitol (0.46 g, 3.00 mmol) was added, immediately forming a grey precipitate. This heterogeneous mixture was stirred vigorously for 15 h, then diluted with CH$_2$Cl$_2$ and centrifuged (4600 xg, 10 min) to remove the solids. The supernatant was concentrated under reduced pressure, the residue taken up in CH$_2$Cl$_2$ and water, and neutralized by slow addition of saturated aqueous NaHCO$_3$ to pH 7. The organic layer was separated, dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO$_2$, 3:1 hexane/EtOAc) to yield d-3.57, which was used directly for the next reaction. $R_f$ 0.60 (1:1 hexane/EtOAc). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.23 (app. t, $J = 8.5$ Hz, 4H), 7.53 (d, $J = 8.5$ Hz, 4H), 6.66 (d, $J = 9.0$ Hz, 1H), 5.34-5.21 (m, 4H), 4.66 (dd, $J = 9.0$ Hz, 3.0 Hz, 1H), 3.65 (m, 1H), 1.68 (bs, 1H), 1.40 (d, $J = 7.0$ Hz, 3H). $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 170.1, 156.2, 148.3, 143.5, 142.1, 128.8, 128.3, 124.1, 124.0, 66.2, 66.0, 59.8, 37.4, 22.1. HRMS (ESI) calc. [M+H]$^+$ for C$_{19}$H$_{20}$N$_3$O$_8$S 450.0971, found 450.0982. See also Notebook VII, page 28.

Compound dL-3.29. Compounds 3.18 (0.45 g, 1.00 mmol) and d-3.57 (assumed 1.50 mmol) were dissolved in N$_2$-sparged EtOAc (5 mL). Tetrabutylammonium bromide (1.29 g, 4.00 mmol) was dissolved in N$_2$-sparged 0.5 M aqueous NaHCO$_3$ (pH adjusted to 8.5, 5 mL), then added to the organic solution. The biphasic mixture was stirred under N$_2$ for 7 h, and the pH was adjusted to 8.5 as necessary with 1 M NaOH. Tributylphosphine (125 µL, 0.50 mmol) was added, and the reaction was stirred for an additional 17 h. The organic layer was isolated, washed with water and brine, dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO$_2$, 4:1 then 2:1 hexane/EtOAc) to yield dL-3.29 (0.69 g, 0.85 mmol, 85% over two steps) as a colorless foam. $R_f$ 0.24 (2:1 hexane/EtOAc). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.17 (app. t, $J = 8.5$ Hz, 4H), 7.75 (d, $J = 7.5$ Hz, 2H), 7.56 (m, 2H), 7.47 (app. d, $J = 7.0$ Hz, 4H), 7.39 (dt, $J = 7.5$ Hz, 3.0 Hz, 2H), 7.29 (t, $J = 7.5$ Hz, 2H), 6.78 (d, $J = 9.0$ Hz, 1H), 5.66 (d, $J = 7.0$ Hz, 1H), 5.34-5.15 (m, 4H), 4.57 (dd, $J = 9.0$ Hz, 3.0 Hz, 1H), 4.44 (m, 1H), 4.34 (d, $J = 7.0$ Hz, 2H), 4.19 (t, $J = 7.0$ Hz, 1H), 3.48 (m, 1H), 3.01 (dd, $J = 13.5$ Hz, 4.5 Hz, 1H), 2.88 (dd, $J = 13.5$ Hz, 4.5 Hz, 1H), 1.47 (s, 9H), 1.35 (d, $J = 7.0$ Hz, 3H). $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 170.3, 169.2, 156.1, 155.8, 148.0, 147.7, 143.8, 143.7, 143.5, 142.0, 141.4, 128.8, 128.1, 127.9, 127.2, 125.11, 125.08, 123.94, 123.87, 120.2, 83.5, 67.4, 66.1, 65.8, 58.7, 54.5, 47.1, 43.5, 33.8, 28.1, 19.7. HRMS

Compound **dl-3.6.** To a solution of **dl-3.29** (0.65 g, 0.80 mmol) in CH₂Cl₂ (3 mL) and phenylsilane (105 µL, 0.84 mmol) was added TFA (3 mL). The reaction was stirred for 2 h, concentrated under reduced pressure and repeatedly redissolved in CH₂Cl₂ and concentrated to remove residual TFA. The crude material was purified by flash chromatography (SiO₂, 1%-2% MeOH/CH₂Cl₂) to yield **dl-3.6** (0.58 g, 0.76 mmol, 95%) as a white solid. Rf 0.16 (EtOAc).

**¹H NMR** (500 MHz, CD₃OD) δ 8.12 (app. t, J = 8.5 Hz, 4 H), 7.75 (d, J = 7.5 Hz, 2H), 7.63 (d, J = 7.5 Hz, 2H), 7.45 (app. t, J = 7.0 Hz, 4H), 7.35 (t, J = 7.5 Hz, 2H), 7.27 (t, J = 7.5 Hz, 2H), 5.30-5.16 (m, 4H), 4.57 (d, J = 4.5 Hz, 1H), 4.39 (dd, J = 8.5 Hz, 4.5 Hz, 1H), 4.35-4.24 (m, 2H), 4.18 (t, J = 7.0 Hz, 1H), 3.49 (m, 1H), 3.09 (dd, J = 13.5 Hz, 4.5 Hz, 1H), 2.83 (dd, J = 13.5 Hz, 8.5 Hz, 1H), 1.33 (d, J = 7.0 Hz, 3H). **¹³C NMR** (125 MHz, CD₃OD) δ 173.6, 171.5, 158.4, 158.3, 149.0, 148.8, 145.7, 145.2, 145.1, 144.2, 142.5, 129.7, 129.0, 128.8, 128.2, 126.3, 124.6, 124.5, 120.9, 68.2, 66.8, 66.4, 60.4, 55.5, 48.3, 43.7, 34.1, 19.6. HRMS (ESI) calc. [M+H]+ for C₃₇H₃₅N₄O₁₂S 759.1972, found 759.1964. See also Notebook VII, page 34.

**Compound l-3.57** was prepared as for its enantiomer **d-3.57** in 20% yield over nine steps from **l-threonine.** See also Notebook IX, pages 11, 14-18, 22.

**Compound lL-3.29.** Compounds **3.18** (0.66 g, 1.46 mmol) and **l-3.57** (0.53 g, 1.17 mmol) were dissolved in N₂-sparged EtOAc (10 mL). Tetrabutylammonium bromide (1.51 g, 4.68 mmol) was dissolved in N₂-sparged 0.5 M aqueous NaHCO₃ (pH adjusted to 8.5, 10 mL), then added to the organic solution. The biphasic mixture was stirred under N₂ for 24 h, with tributylphosphine (150 µL, 0.59 mmol) added to the reaction after 7 h. The organic layer was isolated, washed with water and brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO₂, 30%
EtOAc/hexane) to yield LL-3.29 (0.74 g, 0.91 mmol, 78%) as a colorless foam. \( R_f \) 0.33 (2:1 hexane/EtOAc). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \( \delta \) 8.17 (d, \( J = 8.5 \) Hz, 2H), 8.12 (d, \( J = 8.5 \) Hz, 2H), 7.74 (d, \( J = 7.5 \) Hz, 2H), 7.56 (m, 2H), 7.49 (d, \( J = 8.5 \) Hz, 2H), 7.40 (m, 4H), 7.29 (t, \( J = 7.5 \) Hz, 2H), 5.89 (d, \( J = 8.5 \) Hz, 1H), 5.62 (d, \( J = 7.5 \) Hz, 1H), 5.29-5.10 (m, 4H), 4.56 (m, 1H), 4.45 (m, 1H), 4.36 (d, \( J = 7.0 \) Hz, 2H), 4.19 (t, \( J = 7.0 \) Hz, 1H), 3.50 (m, 1H), 3.02 (dd, \( J = 14.0 \) Hz, 1H), 2.89 (dd, \( J = 14.0 \) Hz, 1H), 1.48 (s, 9H), 1.35 (d, \( J = 7.0 \) Hz, 3H). \(^{13}\)C NMR (125 MHz, CDCl\(_3\)) \( \delta \) 170.2, 169.3, 156.0, 147.9, 147.6, 143.8, 143.7, 143.5, 142.1, 141.3, 128.7, 128.0, 127.9, 127.2, 125.1, 123.9, 123.8, 120.1, 83.4, 67.4, 66.1, 65.7, 58.9, 54.4, 47.1, 42.9, 34.0, 28.1, 19.4. HRMS (ESI) calc. [M+H]\(^+\) for C\(_{41}\)H\(_{43}\)N\(_4\)O\(_{12}\)S 815.2598, found 815.2598. See also Notebook IX, page 23.

**Compound LL-3.6.** To a solution of LL-3.29 (0.50 g, 0.62 mmol) in CH\(_2\)Cl\(_2\) (5 mL) and phenylsilane (70 \( \mu \)L, 0.66 mmol) was added trifluoroacetic acid (5 mL). The reaction was stirred for 2 h, concentrated under reduced pressure and repeatedly redissolved in CH\(_2\)Cl\(_2\) and concentrated to remove residual acid. The crude material was purified by flash chromatography (SiO\(_2\), 0%-5% MeOH/CH\(_2\)Cl\(_2\)) to yield LL-3.6 (0.42 g, 0.56 mmol, 90%) as a white solid after lyophilization from 1:1 benzene/MeCN. \( R_f \) 0.05 (EtOAc). \(^1\)H NMR (500 MHz, CD\(_3\)OD) \( \delta \) 8.07 (d, \( J = 8.5 \) Hz, 2H), 8.03 (d, \( J = 8.5 \) Hz, 2H), 7.70 (d, \( J = 7.5 \) Hz, 2H), 7.59 (m, 2H), 7.51 (d, \( J = 8.5 \) Hz, 2H), 7.43 (d, \( J = 8.5 \) Hz, 2H), 7.32 (t, \( J = 7.5 \) Hz, 2H), 7.24 (t, \( J = 7.5 \) Hz, 2H), 5.26-5.04 (m, 4H), 4.52 (d, \( J = 4.5 \) Hz, 1H), 4.38 (m, 1H), 4.28-4.22 (m, 2H), 4.15 (t, \( J = 7.0 \) Hz, 1H), 3.46 (m, 1H), 3.08 (dd, \( J = 14.0 \) Hz, 4.5 Hz, 1H), 2.91 (dd, \( J = 13.5 \) Hz, 3.5 Hz, 1H), 1.34 (d, \( J = 6.5 \) Hz, 3H). \(^{13}\)C NMR (125 MHz, CD\(_3\)OD) \( \delta \) 174.0, 171.6, 158.5, 158.2, 149.0, 148.7, 145.6, 145.2, 145.1, 144.3, 142.5, 129.7, 128.9, 128.8, 128.1, 126.3, 124.55, 124.46, 120.9, 68.2, 66.8, 66.4, 60.5, 55.7, 48.3, 43.9, 34.7, 19.6. HRMS (ESI) calc. [M+H]\(^+\) for C\(_{37}\)H\(_{35}\)N\(_4\)O\(_{12}\)S 759.1972, found 759.1962. See also Ting Chen Notebook I, page 21.

### 3.4.8. Synthesis of Dha/Dhb-containing peptide fragments

![Diagram of peptide synthesis](image)
Compound 3.30. l-Threonine tert-butyl ester hydrochloride (0.50 g, 2.36 mmol) was dissolved in CH₂Cl₂ (12 mL) and iPr₂NEt (620 μL, 3.54 mmol). N-Boc-l-phenylalanine (0.63 g, 2.36 mmol), HOBt (0.36 g, 2.36 mmol) and EDC (0.45 g, 2.36 mmol) were added as solids. The reaction was stirred for 14 h, then washed with saturated aqueous NaHCO₃, 10% citric acid and water. Each aqueous wash was back-extracted with CH₂Cl₂. The organic fractions were combined, washed with brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure, yielding 3.30 (0.98 g, 2.32 mmol, 98%) as a white solid. Rf 0.82 (EtOAc).

1H NMR (400 MHz, CDCl₃) δ 7.30-7.19 (m, 5H), 6.67 (d, J = 8.4 Hz, 1H), 5.04 (d, J = 7.6 Hz, 1H), 4.43 (dd, J = 8.4 Hz, 3.2 Hz, 1H), 4.38 (m, 1H), 4.19 (m, 1H), 3.16-3.03 (m, 2H), 2.45 (bs, 1H), 1.46 (s, 9H), 1.39 (s, 9H), 1.15 (d, J = 6.4 Hz, 3H).

13C NMR (125 MHz, CDCl₃) δ 171.9, 169.7, 155.7, 136.6, 129.5, 128.7, 127.0, 82.7, 80.4, 68.7, 58.1, 56.0, 38.1, 28.4, 28.1, 20.0. HRMS (ESI) calc. [M+H]+ for C₂₂H₃₅N₂O₆ 423.2495, found 423.2499. See also Notebook V, page 72.

Compound 3.31. A two-step dehydration selective for the Z-olefin was performed based on the procedure of Pattabiraman et al.²²

Compound 3.30 (1.19 g, 2.82 mmol) was dissolved in CH₂Cl₂ (30 mL) and triethylamine (0.98 mL, 7.05 mmol) and chilled in an ice bath. Methanesulfonyl chloride (MsCl, 0.44 mL, 5.64 mmol) was added dropwise, and the reaction was stirred for 1 h, gradually warming to room temperature. The reaction was concentrated under reduced pressure, then taken up in 1,2-dichloroethane (DCE, 30 mL) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 1.69 mL, 11.3 mmol). The reaction was heated to reflux in an oil bath (90 °C) for 4 h, then concentrated under reduced pressure. The residue was taken up in EtOAc, washed with 10% citric acid, saturated aqueous NaHCO₃ and brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO₂, 7:1 then 4:1 hexane/ EtOAc) to yield 3.31 (1.03 g, 2.55 mmol, 90% over two steps) as a white solid. Rf 0.48 (2:1 hexane/EtOAc).

1H NMR (500 MHz, CDCl₃) δ 7.38 (s, 1H), 7.31-7.27 (m, 2H), 7.24-7.21 (m, 3H), 6.68 (q, J = 7.0 Hz, 1H), 5.00 (m, 1H), 4.49 (m, 1H), 3.19 (dd, J = 13.5 Hz, 6.0 Hz, 1H), 3.07 (m, 1H), 1.67 (d, J = 7.0 Hz, 3H), 1.46 (s, 9H), 1.40 (s, 9H).

13C NMR (125 MHz, CDCl₃) δ 169.5, 163.4, 155.6, 136.6, 132.6, 129.5, 128.8,
Compound 3.32. Compound 3.31 (1.27 g, 3.14 mmol) was dissolved in CH₂Cl₂ (10 mL) and TFA (10 mL) and stirred for 1.5 h. The reaction was concentrated under reduced pressure, repeatedly taken up in CH₂Cl₂ and re-concentrated to remove residual acid. To the resulting residue was added sodium carbonate (0.67 g, 6.28 mmol), water (30 mL) and 1,4-dioxane (30 mL), and the system was chilled in an ice bath. N-(9-fluorenylmethoxycarbonyloxy)succinimide (FmocOSu, 1.06 g, 3.14 mmol) was then added portionwise as a solid. The reaction was stirred for 20 h, gradually warming to room temperature. Volatile components were removed under reduced pressure, then the system was diluted with H₂O and acidified to pH 2 with 2 M HCl. The aqueous suspension was extracted with EtOAc (3x), then the combined organic layers were dried over Na₂SO₄, filtered, and concentrated under reduced pressure to ~20 mL. Hexane (~150 mL) was added to form a precipitate, which was isolated by filtration and dried to yield 3.32 (1.40 g, 2.98 mmol, 95% over two steps) as a white powder. Rᵋ 0.12 (EtOAc). ¹H NMR (400 MHz, CD₃OD) δ 7.75 (d, J = 7.2 Hz, 2H), 7.55 (m, 2H), 7.34 (t, J = 7.2 Hz, 2H), 7.29-7.15 (m, 7H), 6.82 (q, J = 7.2 Hz, 1H), 4.50 (dd, J = 9.6 Hz, 5.2 Hz, 1H), 4.30-4.16 (m, 2H), 4.11 (t, J = 7.0 Hz, 1H), 3.21 (dd, J = 13.8 Hz, 5.2 Hz, 1H), 2.89 (dd, J = 13.8 Hz, 9.6 Hz, 1H), 1.65 (d, J = 7.2 Hz, 3H). ¹³C NMR (125 MHz, CD₃OD) δ 173.1, 167.2, 158.2, 145.2, 142.5, 138.6, 136.8, 130.4, 129.4, 128.7, 128.4, 128.1, 127.7, 126.3, 120.9, 68.0, 57.8, 48.3, 39.1, 14.1. HRMS (ESI) calc. [M+H]+ for C₂₈H₂₇N₂O₅ 471.1920, found 471.1923. See also Notebook VI, page 14.

Compound 3.33. L-Serine tert-butyl ester hydrochloride (1.58 g, 8.00 mmol) was taken up in CH₂Cl₂ (40 mL) and tPr₂NEt (2.1 mL, 12.0 mmol) and stirred for 10 min. N-Boc-L-alanine (1.51 g, 8.00 mmol), HOBt (1.35 g, 8.80 mmol) and EDC (1.53 g, 8.00 mmol) were added as solids. The reaction was stirred for 13 h, then washed with saturated aqueous NaHCO₃, 10% citric acid and water and brine. Each aqueous wash was back-extracted with CH₂Cl₂. The organic fractions
were combined, dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure to yield **3.33** (2.58 g, 7.76 mmol, 97%) as a white solid. $R_f$ 0.64 (EtOAc). $^1$H NMR (500 MHz, CDCl$_3$) δ 7.07 (d, $J = 7.5$ Hz, 1H), 5.36 (d, $J = 6.5$ Hz, 1H), 4.50 (m, 1H), 4.18 (m, 1H), 3.92 (dd, $J = 11.5$ Hz, 3.0 Hz, 1H), 3.86 (dd, $J = 11.5$ Hz, 4.0 Hz, 1H), 1.46 (s, 9H), 1.41 (s, 9H), 1.36 (d, $J = 7.5$ Hz, 3H). $^{13}$C NMR (125 MHz, CDCl$_3$) δ 173.2, 169.4, 155.9, 82.8, 80.4, 63.2, 55.6, 50.6, 28.4, 28.1, 18.4. HRMS (ESI) calc. [M+H]$^+$ for C$_{15}$H$_{29}$N$_2$O$_6$ 333.2026, found 333.2029. See also Notebook VII, page 37.

**Compound 3.34.** Compound **3.33** (1.66 g, 5.00 mmol) was dissolved in CH$_2$Cl$_2$ (25 mL) and triethylamine (1.7 mL, 12.5 mmol) and chilled in an ice bath. Methanesulfonyl chloride (770 µL, 10.0 mmol) was added dropwise, and the reaction was allowed to warm to room temperature and stirred for 1 h. DBU (2.2 mL, 15.0 mmol) was then added, and the reaction was stirred for an additional 2 h, then concentrated under reduced pressure. The residue was taken up in EtOAc, washed with 10% citric acid, saturated NaHCO$_3$ and brine, dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO$_2$, 7:3 hexane/EtOAc) to yield **3.34** (1.51 g, 4.80 mmol, 96% over two steps) as a colorless oil. $R_f$ 0.79 (1:1 hexane/EtOAc). $^1$H NMR (500 MHz, CDCl$_3$) δ 8.42 (bs, 1H), 6.51 (s, 1H), 5.81 (s, 1H), 4.97 (m, 1H), 4.25 (m, 1H), 1.52 (s, 9H), 1.45 (s, 9H), 1.39 (d, $J = 7.5$ Hz, 3H). $^{13}$C NMR (125 MHz, CDCl$_3$) δ 171.5, 162.9, 155.6, 132.1, 108.1, 82.9, 80.5, 51.1, 28.4, 28.0, 18.2. HRMS (ESI) calc. [M+H]$^+$ for C$_{15}$H$_{27}$N$_2$O$_5$ 315.1920, found 315.1919. See also Notebook VII, page 39.

**Compound 3.35.** Compound **3.34** (0.94 g, 3.00 mmol) was dissolved in CH$_2$Cl$_2$ (10 mL) and TFA (10 mL) and stirred 1 h. The reaction was concentrated under reduced pressure, repeatedly taken up in CH$_2$Cl$_2$ and re-concentrated to remove residual acid. To the resulting residue was added sodium carbonate (0.64 g, 6.00 mmol), water (15 mL) and 1,4-dioxane (15 mL), and the system was chilled in an ice bath. FmocOSu (1.01 g, 3.00 mmol) was then added portionwise as a solid. The reaction was stirred for 17 h, gradually warming to room temperature. Volatile components were removed under reduced pressure, then the system was diluted with water and acidified to pH 2 with 2 M HCl. The aqueous suspension was extracted with EtOAc (3x), then the combined
organic layers were dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO$_2$, 1%-5% MeOH/CH$_2$Cl$_2$) to yield 3.35 (0.49 g, 1.23 mmol, 43% over two steps) as a white powder after lyophilization from 1:1 benzene/MeCN. $R_f$ 0.07 (EtOAc). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.66 (bs, 1H), 7.76 (d, $J$ = 7.5 Hz, 2H), 7.57 (m, 2H), 7.40 (t, $J$ = 7.5 Hz, 2H), 7.30 (t, $J$ = 7.5 Hz, 2H), 6.68 (s, 1H), 6.03 (s, 1H), 5.69 (m, 1H), 4.77 (s, 1H), 4.40 (d, $J$ = 7.0 Hz, 2H), 4.20 (t, $J$ = 7.0 Hz, 1H), 1.43 (d, $J$ = 6.5 Hz, 3H). $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 172.1, 166.3, 156.5, 143.7, 143.6, 141.4, 130.7, 127.9, 127.2, 125.2, 120.2, 111.2, 67.7, 50.9, 47.1, 19.2. HRMS (ESI) calc. [M+H]$^+$ for C$_{21}$H$_{21}$N$_2$O$_5$ 381.1450, found 381.1448. See also Notebook VII, page 40.

Compound 3.36. L-Threonine (1.43 g, 12.0 mmol) and para-toluenesulfonic acid monohydrate (2.74 g, 14.4 mmol) were combined in toluene (30 mL). Allyl alcohol (8.2 mL, 120 mmol) was added, and the reaction was refluxed in an oil bath (110 °C) connected to a Dean-Stark apparatus for 8 h, then concentrated under reduced pressure and dried azeotropically with benzene. The resulting material was taken up in CH$_2$Cl$_2$ (60 mL) and $^t$Pr$_2$NEt (6.3 mL, 36.0 mmol) and stirred for 10 min. N-Boc-L-threonine (2.63 g, 12.0 mmol), HOBt (2.02 g, 13.2 mmol) and EDC (2.29 g, 12.0 mmol) were added as solids. The reaction was stirred for 16 h, then washed with saturated aqueous NaHCO$_3$, 10% citric acid, water and brine. Each aqueous wash was back-extracted with CH$_2$Cl$_2$. The organic fractions were combined, dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure to ~30 mL. Upon the addition of 1:1 EtOAc/hexane (150 mL), a precipitate formed, which was isolated by filtration and washed with hexane to yield 3.36 (4.01 g, 11.1 mmol, 92% over two steps) as an off-white solid. $R_f$ 0.57 (EtOAc). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.40 (d, $J$ = 9.0 Hz, 1H), 5.94-5.86 (m, 1H), 5.67 (d, $J$ = 8.0 Hz, 1H), 5.36-5.31 (dd, $J$ = 17.5 Hz, 1.5 Hz, 1H), 5.26 (dd, $J$ = 10.5 Hz, 1.0
Hz, 1H), 4.65 (d, J = 6.0 Hz, 1H), 4.59 (dd, J = 9.0 Hz, 2.5 Hz, 1H), 4.37 (m, 1H), 4.28 (m, 1H), 4.18 (dd, J = 8.0 Hz, 2.0 Hz, 1H), 3.83 (bs, 2H), 1.44 (s, 9H), 1.22 (d, J = 6.5 Hz, 3H), 1.19 (d, J = 6.5 Hz, 3H). \(^{13}\)C NMR (125 MHz, CDCl\(_3\)) δ 172.0, 170.7, 156.5, 131.5, 119.2, 80.6, 68.1, 67.4, 66.5, 58.4, 57.9, 28.4, 20.2, 18.3. HRMS (ESI) calc. [M+H]\(^+\) for C\(_{16}\)H\(_{29}\)N\(_2\)O\(_7\) 361.1975, found 361.1982. See also Notebook VII, page 30.

Compound 3.37. Compound 3.36 (0.94 g, 2.62 mmol) was dissolved in CH\(_2\)Cl\(_2\) (5 mL) and TFA (5 mL) and stirred for 2 h. The reaction was concentrated under reduced pressure, repeatedly taken up in CH\(_2\)Cl\(_2\) and re-concentrated to remove residual acid. The deprotected dipeptide was dissolved in CH\(_2\)Cl\(_2\) (12 mL) and \(^1\)Pr\(_2\)NEt (1.3 mL, 7.50 mmol) and stirred for 10 min. N\(_\alpha\)-Aloc-N\(_\varepsilon\)-Boc-L-lysine\(^95\) (0.83 g, 2.50 mmol), HOBt (0.42 g, 2.75 mmol) and EDC (0.48 g, 2.50 mmol) were added sequentially. The reaction was stirred for 14 h, then washed with saturated aqueous NaHCO\(_3\), 10% citric acid, water and brine. Each aqueous wash was back-extracted with CH\(_2\)Cl\(_2\). The organic fractions were combined, dried over Na\(_2\)SO\(_4\), filtered, and concentrated under reduced pressure to yield 3.37 (1.29 g, 2.25 mmol, 90% over two steps) as a colorless foam. \(R_f\) 0.36 (EtOAc). \(^1\)H NMR (500 MHz, CD\(_3\)OD) δ 6.00-5.89 (m, 2H), 5.38-5.28 (m, 2H), 5.21 (app. dd, J = 22.5 Hz, 10.5 Hz, 2H), 4.65 (d, J = 5.5 Hz, 2H), 4.55 (d, J = 5.0 Hz, 2H), 4.49 (d, J = 3.0 Hz, 1H), 4.44 (d, J = 4.0 Hz, 1H), 4.32 (m, 1H), 4.23-4.14 (m, 2H), 3.03 (m, 2H), 1.81 (m, 1H), 1.68 (m, 1H), 1.51-1.41 (m, 13H), 1.22 (d, J = 6.5 Hz, 3H), 1.19 (d, J = 6.5 Hz, 3H). \(^{13}\)C NMR (125 MHz, CD\(_3\)OD) δ 175.2, 172.7, 171.5, 158.6, 158.5, 134.3, 133.3, 118.7, 117.7, 79.9, 68.4, 68.2, 67.0, 66.7, 59.8, 59.3, 56.5, 41.0, 32.8, 30.6, 28.8, 24.1, 20.4, 19.8. HRMS (ESI) calc. [M+H]\(^+\) for C\(_{26}\)H\(_{45}\)N\(_4\)O\(_{10}\) 573.3136, found 573.3132. See also Notebook VII, page 48.

Compound 3.38. Compound 3.37 (0.42 g, 0.73 mmol) and EDC (0.56 g, 2.94 mmol) were dissolved in 49:1 CH\(_2\)Cl\(_2\)/DMF (15 mL). Copper(I) chloride (0.17 g, 1.76 mmol) was added as a solid, and the reaction was stirred for 24 h, protected from light. Additional portions of EDC (0.56 g, 2.94 mmol) and copper(I) chloride (0.17 g, 1.76 mmol) were added, and the reaction was stirred an additional 24 h. The solution was concentrated under reduced pressure, taken up in 0.2 M aqueous EDTA and
extracted with EtOAc (3x). The organic fractions were combined, washed with 10% citric acid and brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO₂, 3:1 EtOAc/hexane) to yield 3.38 (0.25 g, 0.47 mmol, 64%) as a white solid. \( R_f \) 0.56 (EtOAc). \(^1^H \) NMR (500 MHz, CDCl₃) \( \delta \) 7.87 (s, 1H), 7.68 (bs, 1H), 6.84 (q, \( J = 7.0 \) Hz, 1H), 6.79 (q, \( J = 7.0 \) Hz, 1H), 5.95-5.82 (m, 3H), 5.34-5.26 (m, 2H), 5.21 (app. t, \( J = 11.5 \) Hz, 2H), 4.74 (m, 1H), 4.65 (d, \( J = 5.5 \) Hz, 2H), 4.53 (d, \( J = 5.5 \) Hz, 2H), 4.12 (m, 1H), 3.12 (m, 2H), 1.93 (m, 1H), 1.79 (m, 4H), 1.74 (d, \( J = 7.0 \) Hz, 3H), 1.54-1.42 (m, 13H). \(^{13}^C \) NMR (125 MHz, CDCl₃) \( \delta \) 171.3, 164.3, 162.9, 157.1, 156.9, 135.0, 132.4, 132.1, 129.0, 126.5, 118.4, 79.8, 66.4, 66.0, 56.0, 39.5, 31.0, 29.8, 28.6, 22.5, 14.6, 13.8. HRMS (ESI) calc. [M+H]⁺ for C₂₆H₄₁N₄O₅ 537.2924, found 537.2921. See also Notebook VII, page 33.

**Compound 3.39.** Tetrakis(triphenylphosphine)palladium(0) (70 mg, 0.06 mmol) was added to a solution of 3.38 (0.33 g, 0.62 mmol) and \( N \)-methylaniline (200 µL, 1.86 mmol) in THF (6 mL). The reaction was stirred for 2 h, protected from light, then concentrated under reduced pressure. To the resulting oil was added sodium carbonate (0.13 g, 1.24 mmol), H₂O (6 mL) and 1,4-dioxane (6 mL), and the reaction was chilled in an ice bath. FmocOSu (1.01 g, 3.00 mmol) was then added portionwise as a solid. The reaction was stirred for 22 h, gradually warming to room temperature. Volatile components were removed under reduced pressure, then the system was diluted with water and acidified to pH 2 with 2 M HCl. The aqueous suspension was extracted with EtOAc (3x), then the combined organic layers were dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO₂, 2%-5% MeOH/CH₂Cl₂) to yield 3.39 (0.35 g, 0.55 mmol, 89% over two steps) as a white solid. \( R_f \) 0.02 (EtOAc). \(^1^H \) NMR (500 MHz, CD₃OD) \( \delta \) 7.78 (d, \( J = 7.5 \) Hz, 2H), 7.65 (app. dd, \( J = 15.0 \) Hz, 7.5 Hz, 2H), 7.38 (t, \( J = 7.5 \) Hz, 2H), 7.30 (t, \( J = 7.5 \) Hz, 2H), 6.85 (q, \( J = 7.0 \) Hz, 1H), 6.77 (q, \( J = 7.0 \) Hz, 1H), 4.39-4.31 (m, 2H), 4.18 (t, \( J = 7.0 \) Hz, 1H), 4.09 (t, \( J = 7.0 \) Hz, 1H), 3.05 (t, \( J = 6.5 \) Hz, 2H), 1.84 (m, 1H), 1.78-1.73 (m, 4H), 1.71 (d, \( J = 7.0 \) Hz, 3H), 1.54-1.45 (m, 4H), 1.42 (s, 9H). \(^{13}^C \) NMR (125 MHz, CD₃OD) \( \delta \) 174.6, 167.4, 165.2, 158.9, 158.4, 145.2, 144.9, 142.4, 137.0, 133.8, 130.5, 128.61, 128.60, 128.5, 128.0, 126.06, 125.99, 120.7, 79.7, 67.9, 57.0, 48.2, 40.8, 31.9, 30.4, 28.6, 24.1, 14.1, 13.5. HRMS (ESI) calc. [M+H]⁺ for C₃₄H₄₁N₄O₈ 635.3081, found 635.3082. See also Notebook VII, page 55.
Compound 3.40. L-Alanine methyl ester hydrochloride (0.70 g, 5.00 mmol) was taken up in CH₂Cl₂ (25 mL) and iPr₂NEt (1.3 mL, 7.50 mmol) and stirred for 5 min. N-Boc-L-serine (1.03 g, 5.00 mmol), HOBt (0.84 g, 5.50 mmol) and EDC (0.96 g, 5.00 mmol) were added as solids. The reaction was stirred for 21 h, then washed with saturated aqueous NaHCO₃, 10% citric acid, water and brine. Each aqueous wash was back-extracted with CH₂Cl₂. The organic fractions were combined, dried over Na₂SO₄, filtered, and concentrated under reduced pressure to yield 3.40 (1.24 g, 4.27 mmol, 85%) as a white solid. Rf 0.45 (EtOAc). ¹H NMR (500 MHz, CDCl₃) δ 7.32 (d, J = 7.0 Hz, 1H), 5.69 (d, J = 6.5 Hz, 1H), 4.54 (pent, J = 7.0 Hz, 1H), 4.22 (m, 1H), 3.95 (dd, J = 11.0 Hz, 4.0 Hz, 1H), 3.71 (s, 3H), 3.65 (d, J = 11.0 Hz, 4.0 Hz, 1H), 1.41 (s, 9H), 1.38 (d, J = 7.0 Hz, 3H). ¹³C NMR (125 MHz, CDCl₃) δ 173.4, 171.1, 156.1, 80.4, 63.1, 55.3, 52.7, 48.3, 28.3, 17.8. HRMS (ESI) calc. [M+Na]⁺ for C₁₂H₂₂N₂O₆Na 313.1376, found 313.1377. See also Notebook VIII, page 59.

Compound 3.41. Compound 3.40 (1.08 g, 3.72 mmol) was dissolved in CH₂Cl₂ (40 mL) and triethylamine (1.3 mL, 9.30 mmol) and chilled in an ice bath. Methanesulfonyl chloride (580 µL, 7.44 mmol) was added dropwise, and the reaction was allowed to warm to room temperature and stirred for 1 h. DBU (2.8 mL, 18.6 mmol) was then added, and the reaction was stirred for an additional 2 h, then concentrated under reduced pressure. The residue was taken up in EtOAc, washed with 10% citric acid, saturated aqueous NaHCO₃ and brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO₂, 7:3 hexane/EtOAc) to yield 3.41 (0.81 g, 2.97 mmol, 80% over two steps) as a pale yellow oil. Rf 0.70 (1:1 hexane/EtOAc). ¹H NMR (500 MHz, CDCl₃) δ 7.25 (s, 1H), 6.69 (d, J = 7.0 Hz, 1H), 6.03 (s, 1H), 5.12 (t, J = 1.5 Hz, 1H), 4.61 (pent, J = 7.0 Hz, 1H), 3.77 (s, 3H), 1.46-1.42 (m, 12H). ¹³C NMR (125 MHz, CDCl₃) δ 173.1, 163.7, 152.7, 98.4, 80.6, 52.7, 48.6, 28.2, 18.2. HRMS (ESI) calc. [M+Na]⁺ for C₁₂H₂₂N₂O₆Na 295.1270, found 295.1275. See also Notebook VIII, page 60.
Compound 3.42. To a solution of 3.41 (0.54 g, 2.00 mmol) in 1,4-dioxane (5 mL) was added 1 M aqueous lithium hydroxide (5 mL). The reaction was stirred for 1 h, then volatile components were removed under reduced pressure. The system was diluted with water and acidified to pH 3 with 2 M HCl. The aqueous suspension was extracted with EtOAc (10x), then the combined organic layers were dried over Na₂SO₄, filtered, and concentrated under reduced pressure to yield 3.42 (0.34 g, 1.32 mmol, 66%) as a colorless foam.  

$$R_f = 0.03 \text{ (EtOAc)}.$$  

$^1$H NMR (400 MHz, CDCl₃) δ 7.28 (s, 1H), 6.71 (d, $J = 7.2$ Hz, 1H), 6.02 (s, 1H), 5.14 (s, 1H), 4.63 (pent, $J = 7.2$ Hz, 1H), 1.51 (d, $J = 7.2$ Hz, 3H), 1.45 (s, 9H).  

$^{13}$C NMR (125 MHz, CDCl₃) δ 176.1, 164.1, 159.9, 134.7, 110.0, 81.0, 48.2, 28.4, 18.1. HRMS (ESI) calc. [M+H]$^+$ for C₁₁H₁₉N₂O₅ 259.1294, found 259.1288. See also Notebook VIII, page 61.

3.4.9. General procedure for SPPS

Unless noted otherwise, standard cycles for SPPS were performed as follows, using a fritted glass reaction vessel equipped with a N2 inlet for resin/reagent agitation and a suction outlet for draining. Fmoc deprotection was achieved by agitating resin with 20% piperidine in DMF (5 mL) for 10-20 min. After draining the reaction vessel, the resin was washed with DMF (3 x 5 mL x 30 s) and CH₂Cl₂ (2 x 5 mL x 30 s). The appropriately side-chain protected Fmoc-amino acid (5 equiv.) in DMF (5-10 mL) was pre-activated with DIC and HOBt (5 equiv. each) for 5 min, then added to the resin and agitated for 45-60 min. After draining the reaction vessel, the resin was washed as before. The completion of all couplings was assessed by a Kaiser test; double couplings were performed for the residues following each cyclization and otherwise as needed. Test cleavages were performed after all cyclization steps by removing a small portion of dry resin from the reaction vessel and treating with 90:5:5 TFA/H₂O/triisopropylsilane for 1 h under N₂. After removing the cleaved resin by filtration, the filtrate was concentrated under a stream of N₂. The peptide was precipitated with cold Et₂O, isolated by centrifugation at 12,000 ×g, and dissolved in 1:1 H₂O/MeCN. An aliquot of this solution was spotted onto a MALDI-TOF MS target for analysis.

3.4.10. Total synthesis of epilancin 15X analogues

See also Notebook VII, pages 73-74, 94-96, 100; Notebook VIII, pages 5-7, 55-56, 65-67, 73.
Intermediate 3.43. The substitution of the Fmoc-Lys(Boc)-Wang resin (initial substitution 0.36 mmol/g) was first reduced such that 1 equiv. corresponded to 0.10 mmol/g. To ensure local as well as global reduction in resin substitution, the resin was first swelled in DMF for 20 min, followed by addition of Fmoc-Lys(Boc)-OH (1 equiv.) and Boc-Ala-OH (2 equiv.) that had been pre-activated with DIC and HOBt (3 equiv. each) for 5 min. The reaction was performed for 15 h. Any remaining free resin sites were capped with 1:2:7 Ac₂O/pyridine/DMF (10 mL) for 15 min. Adjusted resin substitution was calculated as follows: Fmoc-protected resin (10 mg) was agitated with 20% piperidine/DMF (1.0 mL) for 15 min. A 20 μL aliquot of this solution was diluted 100:1 with DMF. The absorbance of this solution at 301 nm was recorded after blanking with pure DMF, and the resin substitution was calculated using the equation: substitution = 101(absorbance)/7.8(resin weight). After standard Fmoc deprotection and Fmoc-Gly-OH coupling/deprotection, dipeptide Fmoc-Phe-(Z)-Dhb-OH (3.32, 2 equiv.) was pre-activated with DIC and HOAt (2 equiv. each) and iPr₂NEt (5 equiv.) in DMF for 10 min, reacted with the resin-bound peptide for 12 h, and deprotected by the standard protocol. Fmoc-His(Trt)-OH was coupled/deprotected by the standard protocol. MeLan building block DL-3.6 (1.5 equiv.) was pre-activated with DIC and HOAt (3 equiv. each) in DMF for 5 min, then reacted with the resin-bound peptide for 2 h, and not Fmoc-deprotected, to yield resin-bound intermediate 3.43.

Monocyclic intermediate 3.44. The nitrobenzyl protecting groups of 3.43 were removed with two treatments of 6 M SnCl₂ and 5 mM HCl/dioxane in DMF (5 mL) for 1 h each. Following the second treatment, the reaction vessel was drained, and the resin was washed with DMF (3 x 5 mL x 30 s), 1:1 DMF/H₂O (3 x 10 mL x 1 min), 1:1 THF/H₂O (3 x 10 mL x 1 min), DMF (3 x 5 mL x 30 s), and CH₂Cl₂ (2 x 5 mL x 30 s). The Fmoc group was removed by the standard protocol, followed by washing with DMF (5 x 5 mL x 30 s), CH₂Cl₂ (3 x 5 mL x 30 s), and DMF (2 x 5 mL x 30 s) to remove all traces of piperidine. Cyclization was promoted by adding PyAOP and HOAt (5 equiv. each) in DMF to the resin and
agitating for 5 min, then adding 2,4,6-collidine (10 equiv.) and agitating for 1.5 h. After draining, this treatment was repeated for 1.5 h to yield 3.44. Test cleavage analysis on a small sample was performed as described in Section 3.4.9 to confirm completed cyclization. HRMS (MALDI-TOF) calc. [M+H]+ for C_{56}H_{84}N_{15}O_{15}S_{2} 1270.571, found 1270.572.

Bicyclic intermediate 3.45. Fmoc-Leu-OH was coupled to 3.44 by the standard protocol, but not Fmoc-deprotected. The allyl protecting groups were then removed by agitating resin with tetrakis(triphenylphosphine)palladium(0) (1 equiv.) and phenylsilane (10 equiv.) in 1:1 DMF/CH$_2$Cl$_2$ (10 mL) for 2 h, protected from light. After draining the reaction vessel, the resin was washed with CH$_2$Cl$_2$ (3 x 10 mL x 1 min), 0.5% diethylldithiocarbamate in DMF (3 x 10 mL x 1 min), DMF (3 x 5 mL x 30 s), and CH$_2$Cl$_2$ (2 x 5 mL x 30 s). The Fmoc group was removed by the standard protocol, followed by washing with DMF (5 x 5 mL x 30 s), CH$_2$Cl$_2$ (3 x 5 mL x 30 s), and DMF (2 x 5 mL x 30 s) to remove all traces of piperidine. Cyclization was promoted by adding PyAOP and HOAt (5 equiv. each) in DMF to the resin and agitating for 5 min, then adding 2,4,6-collidine (10 equiv.) and agitating for 2 h. After draining, this treatment was repeated for 2 h to yield 3.45. Test cleavage analysis was performed to confirm completed cyclization. HRMS (MALDI-TOF) calc. [M+H]+ for C_{55}H_{85}N_{16}O_{13}S_{2} 1241.592, found 1241.629.

Tricyclic intermediate 3.47. Fmoc-Phe-OH, Fmoc-Gly-OH and Fmoc-Arg(Pbf)-OH were coupled to 3.45 and deprotected by the standard protocol. Lan building block 2 (1.5 equiv.) was pre-activated with DIC and HOAt (3 equiv. each) in DMF for 5 min and coupled to the resin-bound peptide for 2 h, then deprotected by the standard protocol. Fmoc-Leu-OH and Fmoc-Lys(Boc)-OH were coupled and deprotected by the standard protocol, then a second Fmoc-Lys(Boc)-OH was coupled but not Fmoc-deprotected. Removal of the allyl and Fmoc groups, peptide cyclization and test cleavage analysis were performed as described for 3.45 to yield resin-bound intermediate 3.47. HRMS (MALDI-TOF) calc. [M+H]+ for C_{96}H_{152}N_{29}O_{21}S_{3} 2143.087, found 2143.117.
Analogue 3.48. The next 10 residues were coupled to resin-bound 3.47 and deprotected by the standard protocol. The N-terminus was acylated by treatment with d-lactic acid (5 equiv.), DEPBt (5 equiv.) and iPr2NEt (10 equiv.) for 2 h. The peptide was cleaved from resin and globally deprotected with 90:5:2.5:2.5 TFA/H2O/triisopropylsilane/thioanisole under N2 for 2 h. The cleaved resin was removed by filtration, and the filtrate was concentrated under a stream of N2. The peptide was precipitated with cold Et2O, isolated by centrifugation at 12,000 ×g, dissolved in 1:1 H2O/MeCN, and lyophilized to dryness. Crude 3.48 was dissolved to 10 mg/mL in 5% MeCN/H2O with 0.1% TFA and purified by preparatory RP-HPLC using a solvent gradient of 5% solvent B (see Section 3.4.1) for 1 min, then 5-25% over 4 min, then 25-50% over 25 min, then 50-100% over 1 min. Partially-pure 3.48 eluted in fractions collected over 25.3-29.2 min. These fractions were lyophilized and re-purified under the same conditions, with pure product eluting over 27.1-28.0 min. Lyophilization yielded 3.48 (2.0 mg, 0.63 μmol, 1.6% from a 40 μmol scale synthesis, 93% per step over 59 steps) as a white powder. HRMS (MALDI-TOF) calc. [M+H]+ for C145H240N41O33S3 3179.752, found 3179.794.

Analogue 3.49. The next 9 residues were coupled to resin-bound 3.47 and deprotected by the standard protocol. The N-terminal PyrAla moiety was incorporated by coupling Boc-Dha-Ala-OH (3.38, 4 equiv.) with DIC and HOAt (4 equiv. each) in DMF for 3 h. To prevent reduction of the ketone formed upon Boc deprotection, anisole was used in place of triisopropylsilane in the cleavage cocktail. The peptide was cleaved from resin and globally deprotected with 90:5:2.5:2.5 TFA/H2O/anisole/thioanisole under N2 for 3 h. Crude 3.49 was isolated and purified as described for 3.48. Partially-pure 3.49 eluted in fractions collected over 27.2-30.0 min. These fractions were lyophilized and re-purified under the same conditions, with pure product eluting over 27.5-28.5 min. Lyophilization yielded 3.49 (1.3 mg, 0.41 μmol, 1.6% from a 25 μmol scale synthesis, 93% per step over 57 steps) as a white powder. HRMS (MALDI-TOF) calc. [M+H]+ for C145H240N41O33S3 3177.736, found 3177.765.
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Truncated analogue 3.50. Fmoc-Ala-OH, Fmoc-Lys(Boc)-OH and Fmoc-Ile-OH were coupled to resin-bound 3.47 and deprotected by the standard protocol. The N-terminus was acetylated with 1:2:4 Ac₂O/pyridine/DMF for 30 min. The peptide was cleaved from resin and globally deprotected with 90:5:2.5:2.5 TFA/H₂O/triisopropylsilane/thioanisole under N₂ for 2 h. Crude 3.50 was isolated and purified as for 3.48. Partially-pure 3.50 eluted in fractions collected over 14.4-17.0 min. These fractions were lyophilized and re-purified under the same conditions, with pure product eluting over 16.1-17.0 min. Lyophilization yielded 3.50 (1.2 mg, 0.48 μmol, 1.9% from a 25 μmol scale synthesis, 92% per step over 45 steps) as a white powder. HRMS (MALDI-TOF) calc. [M+H]+ for C₁₁₃H₁₈₂N₃₃O₂₅S₃ 2497.314, found 2497.405.

3.4.11. Total synthesis of lacticin 481 and analogues

See also Notebook IX, pages 5-6, 20-21, 26-28, 36-37, 40-41.

Intermediate 3.51. The substitution of the Fmoc-Ser(‘Bu)-Wang resin (initial substitution 0.55 mmol/g) was first reduced such that 1 equiv. corresponded to 0.10 mmol/g. The resin was swelled in DMF for 20 min, and the reaction vessel was drained. To ensure local as well as global substitution reduction, Lan building block dl-3.4 (for 3.2, lL-A, and LL-B) or lL-3.4 (for LL-C) (1 equiv.) and Boc-Ala-OH (3 equiv.) were pre-activated with DIC/HOAt (4 equiv.) for 5 min, then added to the resin. The reaction was performed for 3 h. Any remaining free resin sites were capped with 1:2:7 Ac₂O/pyridine/DMF for 30 min. Adjusted resin substitution was calculated as follows: Fmoc-protected resin (20 mg) was agitated with 20% piperidine/DMF (1.0 mL) for 15 min. A 20 μL aliquot of this solution was diluted 100:1 with DMF. The absorbance of this solution at 301 nm (A₃₀₁) was recorded after blanking with pure DMF, and the resin substitution was calculated using the equation: Substitution = 101(A₃₀₁)/7.8(mg resin).⁹⁴ After standard Fmoc deprotection, Lan building block dl-3.3 (for 3.2, ll-A, and ll-C) or ll-3.3 (for ll-B) (2 equiv.) was pre-activated with DIC/HOAt (2 equiv) for 5 min, then added to resin and reacted for 2 h. After standard Fmoc deprotection, Fmoc-Phe-(Z)-Dhb-OH (3.32, 3 equiv.) was pre-activated with DIC/HOAt (3 equiv.) in DMF for 10 min and reacted with the resin-bound peptide for 2 h; this coupling was repeated for an
additional 2 h, then the peptide was Fmoc-deprotected by the standard protocol. Val22, Phe21 and Gln20 were coupled/Fmoc-deprotected by the standard protocol, then Trp19 was coupled but not deprotected to yield the resin-bound intermediate 3.51.

Monocyclic intermediate 3.52. The nitrobenzyl protecting groups of 3.51 were removed with two treatments of 6 M SnCl₂ and 5 mM HCl/dioxane in DMF (5 mL) for 1 h each. Following the second treatment, the reaction vessel was drained, and the resin was washed with DMF (3 x 5 mL x 30 s), 1:1 DMF/H₂O (3 x 10 mL x 1 min), 1:1 THF/H₂O (3 x 10 mL x 1 min), DMF (3 x 5 mL x 30 s), and CH₂Cl₂ (2 x 5 mL x 30 s). The Fmoc group was removed by the standard protocol, followed by washing with DMF (5 x 5 mL x 30 s), CH₂Cl₂ (3 x 5 mL x 30 s), and DMF (2 x 5 mL x 30 s) to remove all traces of piperidine. Cyclization was promoted by adding PyAOP and HOAt (5 equiv. each) in DMF to the resin and agitating for 5 min, then adding 2,4,6-collidine (10 equiv.) and agitating for 3 h. After draining, this treatment was repeated for an additional 3 h to yield the resin-bound intermediate 3.52. Test cleavage analysis on a small sample was performed as described in Section 3.4.9 to confirm completed cyclization. HRMS (MALDI-TOF) calc. [M+Na]⁺ for C₆₅H₈₃N₁₃O₁₇S₂Na 1404.537, found 1404.530.

Bicyclic intermediate 3.54. Asn17, Met16 and Asn15 were coupled/Fmoc-deprotected to 3.52 by the standard protocol. MeLan building block DL-3.6 (for 3.2, LL-B, and LL-C) or LL-3.6 (for LL-A) (2 equiv.) was pre-activated with DIC/HOAt (2 equiv) for 5 min, then added to resin and reacted for 2 h. After standard Fmoc-deprotection, Glu13 was coupled and Fmoc-deprotected by the standard protocol, then His12 was coupled but not deprotected. The allyl protecting groups were then removed by agitating resin with tetrakis(triphenylphosphine)palladium(0) (1 equiv.) and phenylsilane (10 equiv.) in 1:1 DMF/CH₂Cl₂ (10 mL) for 2 h, protected from light. After draining the reaction vessel, the resin was washed with CH₂Cl₂ (3 x 5 mL x 1 min), 0.5% diethyldithiocarbamate in DMF (3 x 10 mL x 1 min), DMF (3 x 5 mL x 30 s), and CH₂Cl₂ (2 x 5 mL x 30 s). The Fmoc group was removed by the standard protocol, followed by washing with DMF (5 x 5 mL x 30 s), CH₂Cl₂ (3 x 5 mL x 30 s), and DMF (2 x 5 mL x 30 s) to remove all
traces of piperidine. Cyclization was promoted as for 3.52 to yield the resin-bound intermediate 3.54. Test cleavage analysis was performed to confirm completed cyclization. HRMS (MALDI-TOF) calc. [M+Na]+ for C_{104}H_{130}N_{26}O_{32}S_{4}Na 2405.813, found 2405.835.

Tricyclic intermediate 3.55. Ile10 was coupled to resin-supported 3.54 by the standard protocol but not Fmoc-deprotected. Removal of the nitrobenzyl and Fmoc groups, peptide cyclization and test cleavage analysis were performed as described for 3.52 to yield resin-bound intermediate 3.55. HRMS (MALDI-TOF) calc. [M+Na]+ for C_{95}H_{129}N_{25}O_{26}S_{4}Na 2186.832, found 2186.859.

Lacticin 481 (3.2) and analogues LL-A, LL-B, and LL-C. The remaining eight residues were coupled to resin-supported 3.55 and deprotected by the standard protocol. The peptide was cleaved from resin and globally deprotected with 92.5:5:2.5 TFA/H_{2}O/trisopropylsilane under N_{2} for 2 h. The cleaved resin was removed by filtration, and the filtrate was concentrated under a stream of N_{2}. The peptide was precipitated with cold Et_{2}O, isolated by centrifugation at 12,000 xg, dissolved in 1:1 H_{2}O/MeCN and lyophilized to dryness. Crude peptide was dissolved to 10 mg/mL in 10% MeCN/H_{2}O with 0.1% TFA and purified by preparatory RP-HPLC using a solvent gradient of 10% solvent B (see Section 3.4.1) for 1 min, then 10-25% over 4 min, then 25-50% over 25 min, then 50-100% over 1 min. Partially-pure peptide eluted in fractions collected 23-28 min. These fractions were lyophilized and re-purified under the same conditions, with pure product eluting as described below. Lyophilization yielded the desired peptides 3.2, LL-A, LL-B, and LL-C (average yield 1.9 mg, 0.66 μmol, 1.3% from a 50 μmol scale synthesis, 92% per step over 52 steps) as white powders.

* Synthetic lacticin 481 (3.2). R_{t} = 26.3-27.0 min. HRMS (MALDI-TOF) calc. [M+H]+ for C_{127}H_{183}N_{36}O_{35}S_{4} 2900.252, found 2900.244.

* Lacticin 481 LL-A. R_{t} = 22.6-23.3 min. HRMS (MALDI-TOF) calc. [M+H]+ for C_{127}H_{183}N_{36}O_{35}S_{4} 2900.252, found 2900.278.
**Lacticin 481 LL-B.** \( R_t = 22.7-23.4 \text{ min.} \) HRMS (MALDI-TOF) calc. \([M+H]^+\) for \( \text{C}_{127}\text{H}_{183}\text{N}_{36}\text{O}_{35}\text{S}_4 \) 2900.252, found 2900.279.

**Lacticin 481 LL-C.** \( R_t = 23.0-23.6 \text{ min.} \) HRMS (MALDI-TOF) calc. \([M+H]^+\) for \( \text{C}_{127}\text{H}_{183}\text{N}_{36}\text{O}_{35}\text{S}_4 \) 2900.252, found 2900.256.

### 3.4.12. Chiral GC/MS analysis of synthetic peptides

The enantiomeric purity of Lan/MeLan amino acids produced by hydrolysis of synthetic peptides was confirmed by chiral GC/MS, using a procedure modified from previous reports.\(^{29,95}\)

Lyophilized peptides (0.1-0.2 mg) were dissolved in 6 M HCl (3 mL) and heated at 100 °C in a sealed, high-pressure reaction vessel for 18 h. The reaction was cooled and concentrated with a stream of N\(_2\) over several hours. Methanol (3 mL) was chilled in an ice bath, and acetyl chloride (1 mL) was added dropwise. This solution was added to the dry hydrolysate, and the mixture was sealed and heated at 100 °C for 1 h. The reaction was allowed to cool, then concentrated with a stream of N\(_2\). The dry residue was suspended in CH\(_2\)Cl\(_2\) (3.5 mL) and chilled in an ice bath. Pentafluoropropionic anhydride (0.5 mL) was added, and the mixture was sealed and heated at 100 °C for 20 min. The reaction was allowed to cool, then concentrated with a stream of N\(_2\). The residue was dissolved in methanol and re-concentrated, then dissolved again in methanol (50 μL) for analysis. Lan/MeLan standards of differing stereochemical configurations (DD, DL and LL for Lan; DL and LL for MeLan), synthesized using published procedures and similarly derivatized as their pentafluoropropionamide methyl esters,\(^{30,76}\) were provided by Weixin Tang (University of Illinois at Urbana-Champaign) as solutions in methanol.

The derivatized hydrolysate and standards were analyzed by GC/MS using an Agilent 7890A gas chromatograph equipped with a Varian CP-Chirasil-l-Val fused silica column (25 m x 250 μm x 0.12 μm) and an Agilent 5975C Inert XL EI/CI MS (Metabolomics Center, UIUC) or a Waters Micromass GCT Premier detector (Mass Spectrometry Laboratory, UIUC). Sample solutions in methanol were introduced to the instrument via splitless injection at an inlet temperature of 190-200 °C and flow rate of 1.7-2.0 mL/min helium gas. The temperature gradient used was held at 160 °C for 5 min, then ramped from 160 °C to 180 °C at 3 °C/min, then held at 180 °C for 1-5 min. The MS was operated in scan mode, and data was analyzed by selected ion monitoring (SIM) at known unique fragment masses of 365 Da for Lan and 379 Da for MeLan. All standards eluted as distinct peaks. Some observed epimerization of Lan/MeLan
amino acids is believed to result from hydrolysis, which has been reported previously. See also Notebook VIII, pages 63-64; Notebook IX, pages 7-9.

3.4.13. Liquid culture growth inhibition assays

To generate stock concentrations of each peptide, lyophilized peptide was weighed using a Mettler-Toledo MT5 microbalance and dissolved in sterile deionized water (SDW) to give 200 μM stock solutions. Aliquots of these solutions were analyzed by analytical HPLC at an absorbance of 220 nm, and concentrations were normalized as necessary based on the integration under the expected peptide peak. Serial two-fold dilutions of peptide stock solutions were prepared in SDW to give 4x working concentrations.

For assays with epilancin 15X and analogues, cultures of the indicator strain Staphylococcus carnosus TM300 (5 mL) were grown at 37 °C in bovine heart infusion medium (BHI, 37 g/L) for 12 h, then diluted with fresh BHI to an optical density at 600 nm (OD_{600}) of 0.1. For assays with lacticin 481 and analogues, cultures of Lactococcus lactis subsp. cremoris HP (ATCC 11602) were grown at 30 °C in GM17 medium (40 g/L M17, 0.5% glucose) for 12-18 h, then diluted with fresh GM17 to an OD_{600} of 0.1, corresponding to approximately 1 × 10^8 colony forming units/mL. Corning-Costar 96-well flat-bottom assay plates were used to determine the activity of each peptide against its appropriate indicator strain, and experiments were performed in triplicate. Experimental wells contained diluted culture (150 μL) and 4x peptide solution (50 μL). Control wells contained either fresh media (150 μL) and SDW (50 μL, negative control) or diluted culture (150 μL) and SDW (50 μL, positive control). OD_{600} was recorded at hourly intervals using a BioTek Synergy H4 plate reader, and plates were incubated at 37 °C (for S. carnosus) or 30 °C (for L. lactis) between readings. After subtraction of blanks from experimental measurements, plots of OD_{600} vs. peptide concentration were fitted to a dose-response function with the equation: y = A1 + (A2-A1) / (1 + 10^{(logx0 - x)p}), where p = variable Hill slope. Half maximal inhibitory concentration (IC_{50}) and minimal inhibitory concentration (MIC) values were determined from this fit for each peptide after 5-6 h incubation, and triplicate calculations were averaged. See also Notebook VIII, pages 71-72; Notebook IX, pages 88-89.
3.4.14. Flow cytometry analysis of membrane disruption

Cultures of *S. carnosus* TM300 were grown as described for bioactivity assays and diluted with fresh BHI to an OD$_{600}$ of 0.1. For membrane depolarization assays, cells were combined with DiOC$_2$(3) (final concentration 2 μM), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, 1 mM), and glucose (1 mM) and incubated for 20 min at room temperature. Stock solutions of epilancin 15X analogues 21 or 23 were added to final concentrations of 0.1, 1.0, or 10 μM and incubated for an additional 20 min prior to analysis; SDW was added for the negative control. For membrane permeability assays, cells were combined with PI (final concentration 25 μM), HEPES (1 mM), glucose (1 mM), and epilancin 15X analogues (0.1, 1.0, or 10 μM), incubated for 15 min at room temperature, and analyzed. Changes in cell-associated dye fluorescence were measured with a BD Biosciences LSR II flow cytometer at the Roy J. Carver Biotechnology Center (UIUC), using excitation at 488 nm with an argon laser and measurement of emission through a band-pass filter at 530/30 nm for DiOC$_2$(3) or 695/40 nm for PI. A minimum of 25,000 events were detected for each sample, and each peptide concentration was repeated in triplicate. Data analysis to calculate the geometric mean fluorescence intensity (MFI) of each population was performed using FCS Express 3.00.0311 V Lite Stand-alone software. See also Notebook VIII, pages 90-91, 97-98.

3.5. REFERENCES


CHAPTER 4: SYNTHESIS OF ANALOGUES OF THE IMMUNOMODULATORY PEPTIDE COMPSSTATIN WITH IMPROVED IN VIVO STABILITY*

4.1. INTRODUCTION

4.1.1. Disulfide bond engineering in bioactive peptides

Due to superior proteolytic stability compared to their linear counterparts (see Chapter 1.2), cyclic peptides have received increased attention in recent years as therapeutic agents.\(^1\)-\(^4\) The designation “cyclic” encompasses a variety of structures, but most commonly found is the disulfide bond. Beyond the ubiquitous role of disulfides in protein structure,\(^5\)-\(^7\) several peptide natural product families, including conopeptides,\(^8\)-\(^10\) cyclotides,\(^11\)-\(^13\) and defensins,\(^14\) contain complex topologies enforced by this moiety. Furthermore, a survey of approved peptide drugs reveals a variety of disulfide-containing compounds with numerous indications (Fig. 4.1), including eptifibatide,\(^15\) linaclotide,\(^16\) octreotide,\(^17\) oxytocin,\(^18\) pramlin tide,\(^19\) and ziconotide.\(^20\)

Many of these drugs are produced synthetically.\(^1\) For most bioactive peptides, the structural constraint enforced by one or more disulfide bonds serves both to improve resistance to proteolysis and reduce the entropic cost of binding to their biological targets.\(^2\),\(^13\),\(^21\)-\(^23\) However, the lability of the S-S bond to biological reducing agents, such as glutathione, limits its in vivo stability. Indeed, this phenomenon has been exploited in the development of antibody-drug conjugates and drug delivery vehicles with conditionally-stable disulfide linkages.\(^24\)-\(^27\)

In order to overcome this shortcoming inherent in many peptide drugs, disulfide bond engineering has been investigated extensively using reduction-stable moieties as mimics of the disulfide (Fig. 4.2). Conotoxins containing diselenide bonds have been synthesized and evaluated, and several of these “selenoconotoxins” exhibited improved folding, target binding, and reductive stability compared to the parent disulfide-containing compounds. Analogues of oxytocin containing diselenide, ditelluride, selenylsulfide, methylene, and methine bridges have also demonstrated improved stability and, in some cases, potent activity. Other disulfide mimics, including lactam bridges, triazoles, and noncovalent interactions, have been investigated in different systems with varying levels of success in preserving the biological activity of the parent disulfide. Cystine to lantionine (Lan) substitution, in which one sulfur atom is removed, has been shown to improve the in vivo stability of analogues of octreotide and cyclic enkephalin on the order of two- to five-fold. However, the ring contraction resulting from this substitution decreased potency in the octreotide analogues, as well as in analogues of oxytocin, cell-adhesion molecule, and ligands for the low-density lipoprotein receptor. In comparison, the substitution of cystine with cystathionine (Cth) involves the change of only a single atom: a sulfur to a methylene (Fig. 4.2). This isosteric substitution has been hypothesized to impart minimal structural perturbation in peptides, while removing the reductive lability of the S-S bond. Indeed, Cth-containing analogues of α-conotoxin ImI, oxytocin, anticardiolipid antibody binders, and VCAM/VLA-4 antagonists have been generated through a variety of synthetic approaches that largely maintain the potency of the parent disulfide-containing compounds. Furthermore, two-dimensional NMR analysis of the α-conotoxin analogues revealed no structural perturbation by substituting one or both cystine moieties for Cth, providing additional evidence of the isosteric nature of this substitution.

![Fig 4.2. Examples of cystine disulfide mimics used in peptide disulfide bond engineering.](image-url)
4.1.2. Compstatin, a peptide inhibitor of the complement immune system

As a central part of innate immunity, the complement system serves as a critical line of defense against bacterial infections, a link between innate and adaptive immunity, and a disposal system for immunity- and inflammation-related byproducts. Over 30 proteins are known to constitute this proteolytic cascade, along with multiple mechanisms of initiation and downstream effects. Interestingly, the entire pathway converges at one keystone reaction, the proteolytic activation of complement factor 3 (C3) to produce anaphylatoxin C3a and opsonin C3b, which mediate all subsequent outcomes. However, excessive or inappropriate activation of complement has been associated with host tissue damage in a variety of disease states, including ischemia-reperfusion injury, Alzheimer’s disease, rheumatoid arthritis, systemic lupus erythematosus, sepsis, and transplant rejection. Consequently, complement inhibition has been established as a potential therapeutic approach in these and other disorders.

Compstatin (Fig. 4.3a) is a 13-residue, disulfide-containing peptide inhibitor of the complement pathway discovered by Prof. John Lambris and coworkers at the University of Pennsylvania in 1996, through screening of a phage-display library. This peptide binds directly

![Diagram of Compstatin and complement inhibition.](image)

Fig 4.3. Compstatin and complement inhibition. (a) Structures of compstatin and acetylated analogue 4.1, highlighting altered structures in green. (b) Schematic of the proposed mechanism of compstatin inhibition. Three initiation pathways converge at the formation of the protease C3 convertase, a multiprotein complex, which cannot access its substrate C3 when C3 is bound by compstatin.
to both C3 and C3b, and thus inhibits the central reaction of complement activation via steric blocking of C3 convertase, the endogenous C3 protease, and other necessary binding partners (Fig. 4.3b).\textsuperscript{57, 58} Extensive structure-activity relationship analysis has yielded compstatin derivatives with up to 1000-fold improvement in potency,\textsuperscript{59-64} including an acetylated variant (4.1, Fig. 4.3a) that is currently in clinical trials for the treatment of age-related macular degeneration, a main cause of blindness in the elderly.\textsuperscript{65} The disulfide bond of compstatin, which enforces the constrained conformation necessary to bind C3,\textsuperscript{57} is critical for complement inhibition, as reduction-alkylation of the bond or substitution of cysteine with alanine abolishes biological activity,\textsuperscript{56, 64} and for protecting the peptide from \textit{in vivo} degradation.\textsuperscript{64} Therefore, the identification of strategies to improve the metabolic stability of compstatin through disulfide replacement would be useful in improving its potential and impact in clinical application.

This chapter describes synthetic efforts to improve the pharmacokinetic properties of compstatin analogues. A disulfide engineering approach was used to produce compstatin analogues containing Cth in place of cystine via solid-phase peptide synthesis (SPPS) in order to improve \textit{in vivo} stability. Strategic placement of this structural substitution yielded analogues with no sensitivity to reduction, but largely preserved binding and inhibitory activity. Furthermore, an alternative strategy to increase the \textit{in vivo} half-life of compstatin was explored by appending an N-terminal albumin affinity tag shown in another peptide system to decrease drug clearance rate.\textsuperscript{66} This work was the result of a collaboration with Prof. John Lambris, whose laboratory first discovered compstatin, and his student Dr. Apostolia Tzekou in the Department of Pathology and Laboratory Medicine at the University of Pennsylvania School of Medicine (UPSM). The individual contributions of these coworkers are mentioned throughout the chapter and are listed in detail in the Experimental (Section 4.4).

4.2. RESULTS AND DISCUSSION

4.2.1. Synthesis of Cth building blocks

Several strategies have been reported to generate cyclic Cth-containing peptides, including solid-supported intramolecular thioalkylation.\textsuperscript{46, 47} In this work, considering that Cth is the one-carbon homologue of Lan, an analogous synthetic approach to the SPPS-based construction of lantibiotics discussed in Chapter 3 was sought. In this light, differentially-protected Cth building blocks were necessary. Allyl-based protection was chosen (as in Chapter
3.2) due to its orthogonality to the standard conditions of Fmoc-SPPS and ease of removal on the solid-phase. Additionally, it is important to recognize that there are two possible isomers of Cth as a cystine isostere, depending on which sulfur atom of the original disulfide is replaced; this necessitates the construction of two different allyl-protected Cth building blocks. Indeed, studies on VCAM/VLA-4 antagonists have shown that analogues containing different isomers of Cth exhibit different potencies.\(^{49}\)

The nomenclature used to describe the two Cth isomers was based on the position of the sulfur atom relative to the Fmoc/free acid side of the molecule; the two isomers were consequently labeled γ- and δ-Cth. The synthesis of γ-Cth building block 4.7 (Scheme 4.1) proceeded analogously to the Lan building blocks discussed in Chapter 3.2.1, via a base-promoted phase-transfer condensation of cysteine 4.3 and 4-bromo-2-aminobutyrate 4.5 to generate thioether 4.6, followed by tert-butyl ester removal. The resulting building block 4.7 preserved the enantiomeric purity of the starting materials, as determined by GC/MS analysis (see Section 4.2.3) and \(^{13}\)C NMR spectroscopy. The δ-Cth building block 4.13 (Scheme 4.2) was synthesized similarly to its isomer, again with preservation of stereochanical configuration.

In order to enable the synthesis of overlapping Cth bridges in peptides, a fully orthogonal Cth building block was also desired. An isovaleryldimedonyl (Ddiv)/trimethylsilylethyl (Tmse) ester protection strategy was explored, which has been reported previously for a Lan building block.\(^{67}\) As described in Chapter 3.2.1, these protecting groups are orthogonal to both allyl and Fmoc and can be deprotected on the solid-phase under mild conditions.\(^{68,69}\) The synthesis of this

**Scheme 4.1. Synthesis of γ-Cth building block 4.7.**

\[\text{Reagents and conditions: a) } \text{HClO}_4, \text{BuOAc}; \text{ b) FmocOSu, N-methylmorpholine, THF, 79\% (two steps); c) PBu}_3, \text{H}_2\text{O, THF, 86\%; d) AlocCl, Na}_2\text{CO}_3, \text{H}_2\text{O, CH}_3\text{CN; e) allyl bromide, NaHCO}_3, \text{DMF, 77\% (two steps); f) PPh}_3, \text{CBr}_4, \text{CH}_2\text{Cl}_2, 80\%; g) 4, \text{Bu}_4\text{NBr, NaHCO}_3, \text{H}_2\text{O, EtOAc, 84\%; h) CF}_3\text{CO}_2\text{H, PhSiH}_3, \text{CH}_2\text{Cl}_2, 95\%.}\]
**Scheme 4.2.** Synthesis of δ-Cth building block 4.13.*

δ-Cth building block (4.19, Scheme 4.3) proceeded analogously to Schemes 4.1 and 4.2, except that an allyl ester was used in place of the tert-buty1 ester for temporary carboxylate protection due to the sensitivity of Tmse to the acidic conditions necessary for tert-buty1 ester removal. While this building block could be synthesized in acceptable yields, it was found to decompose via elimination in the presence of piperidine used during Fmoc-SPPS. This instability was attributed to the Ddiv group, as observed for similarly-protected Lan building blocks (see Section 3.2.1).

**Scheme 4.3.** Synthesis of δ-Cth building block 4.19.*

*a Reagents and conditions: a) FmocCl, Na₂CO₃, H₂O, CH₃CN; b) allyl bromide, NaHCO₃, DMF, 64% (two steps); c) PPh₃, CBr₄, CH₂Cl₂, 60%; d) 2-isovaleryldimedone, Pr₂NEt, MeOH, reflux; e) Me₃SiEtOH, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, DMAP, CH₂Cl₂, 55% (two steps); f) CF₃CO₂H, Pr₃SiH, CH₂Cl₂, 79%; g) Bu₄NBr, NaHCO₃, H₂O, EtOAc, 82%; h) Pd(PPh₃)₄, PhNHCH₃, THF, 78%. Ddiv, isovaleryldimedonyl; Tmse, trimethylsilyl ethyl; Trt, trityl.
4.2.2. Synthesis of Cth-containing compstatin analogues

Following the successful syntheses of Cth isomers 4.7 and 4.13, the solid-phase construction of three Cth-containing compstatin analogues commenced (Fig. 4.4). Both possible Cth isomers (4.20, 4.21) of second-generation compstatin derivative 4.161 (Fig. 4.3) were constructed. Based on biological data (see Sections 4.2.4 and 4.2.5), peptide 4.21, utilizing δ-Cth building block 4.13 with the sulfur atom placed closer to the N-terminus, was found to be more active than the γ-Cth-containing analogue. Therefore, the δ-Cth analogue (4.23) of the most potent compstatin derivative known to date (4.22) was also produced, which contains an acetyl cap and four mutations from compstatin, 1-methyltryptophan (Trp(Me)) in position 4, sarcosine (Sar) in position 8, alanine in position 9, and N-methylisoleucine (N-MeIle) in position 13. These modifications yield an overall 1000-fold increase in potency over compstatin due to a free conformation more favorable for binding and improved hydrophobic interactions.59

4.1: Ac-IleCysValTrpGlnAspTrpGlyAlaHisArgCysThr-NH₂  4.22: Ac-IleCysVal[Trp(Me)]GlnAspTrpSarAlaHisArgCys[NMelle]-NH₂
4.20: Ac-IleHcyValTrpGlnAspTrpGlyAlaHisArgAlaThr-NH₂  4.23: Ac-IleAlaVal[Trp(Me)]GlnAspTrpSarAlaHisArgHcy[NMelle]-NH₂
4.21: Ac-IleAlaValTrpGlnAspTrpGlyAlaHisArgHcyThr-NH₂

Figure 4.4. Sequences of compstatin derivatives and Cth-containing analogues studied in this work. Mutations from compstatin are highlighted in green. The two Cth isomers are depicted as Hcy-Ala (red, for γ-Cth) or Ala-Hcy (blue, for δ-Cth). Hcy, homocysteine; Sar, sarcosine; Trp(Me), 1-methyltryptophan.

The Fmoc-SPPS of Cth-containing compstatin analogues (Scheme 4.4) mirrored the chemical synthesis of lantibiotics discussed in Chapter 3. The substitution density of Rink amide AM resin was manually lowered to 0.2 mmol/g to prevent intermolecular reactions from occurring during peptide cyclization. Fmoc-amino acids, including Cth building blocks 4.7 and 4.13, were activated using O-(6-chlorobenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HCTU) and diisopropylethylamine (iPr₂NEt), and Fmoc groups were removed with piperidine. After the construction of solid-supported linear intermediate 4.24, the allyl groups of the Cth residue were removed with tetrakis(triphenylphosphine)palladium(0) and phenylsilane, the N-terminal Fmoc group was removed, and the peptide was cyclized using (benzotriazol-1-ylxylo)trityrroldinophosphonium hexafluorophosphate (PyBOP), 1-hydroxy-7-azabenztiazole (HOAt), and iPr₂NEt. Cyclization was monitored by analytical reversed-phase high-performance liquid chromatography (RP-HPLC) and matrix-assisted laser desorption
**Scheme 4.4.** Solid-supported synthesis of compstatin analogues 4.20, 4.21, 4.23.\(^a\)\(^b\)

\[ \text{AllO}_2\text{C} \rightarrow \text{NHAc} \]

1. \( \text{NHAc} \text{H} \rightarrow \text{Gln} \text{Val} \text{Trp} \)
2. \( \text{Gln} \text{Val} \text{Trp} \rightarrow \text{Asp Ala His Arg} \)
3. \( \text{Asp Ala His Arg} \rightarrow \text{Ctn} \)

**4.20:** \( Y = \text{CH}_2, Z = \text{S} \)
**4.21:** \( Y = \text{S}, Z = \text{CH}_2 \)
**4.23:** \( Y = \text{S}, Z = \text{CH}_2 \)

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\( ^a \) Reagents and conditions: a) SPPS; b) Pd(PPh\(_3\))\(_4\), PhSiH\(_3\), DMF, CH\(_2\)Cl\(_2\); c) piperidine, DMF d) PyBOP, HOAt, 'Pr\(_2\)NEt, DMF; e) CF\(_3\)CO\(_2\)H, H\(_2\)O, 'Pr\(_3\)SiH, ethanedithiol. \(^b\) Prior to cleavage from resin (step e), all residues contained appropriate side-chain protecting groups for Fmoc-SPPS: Boc for Trp, Pbf for Arg, 'Bu for Thr, O'Bu for Asp, and Trt for Gln and His.

**ionization/time-of-flight mass spectrometry (MALDI-TOF MS), which indicated complete conversion to the cyclic intermediate 4.25 within 3 h.** Subsequent isoleucine coupling, N-terminal acetylation, and concurrent resin cleavage and global deprotection afforded Ctn-containing peptides 4.20, 4.21, and 4.23, which were purified by RP-HPLC. The pure peptides were obtained in overall yields of 2.5-5%, or 88-90% per chemical step. Analogue 4.23 gave the lowest yield, which was impacted in part by the difficulty in coupling amino acids to the N-methylated residues at positions 8 and 13. Analytical RP-HPLC and MALDI-TOF MS confirmed the homogeneity of each peptide after purification (Fig. 4.5)

**4.2.3. GC/MS analysis of Ctn building blocks and compstatin analogues**

In order to confirm that the desired absolute stereoconfiguration of the Ctn building blocks as well as Ctn bridges within the compstatin analogues was retained, chiral gas chromatography-mass spectrometry (GC/MS) was used, as described in Chapter 3.2.4 for Lan and reported previously for Ctn.\(^{70}\) δ-Ctn building block 4.13 was globally deprotected, and peptide 4.23 was hydrolyzed in refluxing 6 M HCl. The resulting amino acids were then derivatized to their corresponding pentafluoropropionamide methyl esters and analyzed using a
Figure 4.5. Characterization of Cth-containing compstatin analogues, showing analytical RP-HPLC chromatograms and MALDI-TOF mass spectra for (a) analogue 4.20; (b) analogue 4.21; and (c) analogue 4.23.
Varian CP-Chirasil-L-Val column, compared to derivatized commercial Cth possessing the desired LL-configuration and a synthetic standard with an alternative DL-configuration. The derivatized Cth amino acids fragment during MS analysis to give unique mass ions compared to proteinogenic amino acids, permitting selected ion monitoring (SIM) at these m/z values to unambiguously identify Cth. Both building block 4.13 and hydrolyzed peptide 4.23 contained Cth bearing the desired LL-configuration (Fig. 4.6), indicating that minimal epimerization occurred during building block synthesis and SPPS.

Figure 4.6. Chiral GC/MS analysis of Cth building blocks and peptides, confirming the desired LL-configuration. (a) SIM at 379 Da of deprotected and derivatized 4.13 compared to Cth standards. (b) SIM at 379 Da of hydrolyzed and derivatized 4.23 compared to Cth standards. (c) Representative mass spectrum of derivatized Cth.
4.2.4. Kinetic binding analysis of compstatin analogues

The affinities of Cth-containing compstatin analogues 4.20, 4.21, and 4.23 for C3b were characterized by Dr. Apostolia Tzekou (UPSM) using an established surface plasmon resonance (SPR) assay (Fig. 4.7). Biotinylated C3b was site-specifically captured on a streptavidin-coated sensor chip to prevent surface heterogeneity. An initial kinetic ranking of the Cth-containing analogues indicated that the association and dissociation profiles of δ-Cth-containing 4.21 were comparable to those of the parent disulfide 4.1, while γ-Cth-containing 4.20 displayed much faster dissociation. δ-Cth-containing 4.23 showed a similar association rate, but faster dissociation rate, compared to parent disulfide 4.22.

![Figure 4.7. Kinetic ranking via SPR of compstatin analogues.](image)

Given this initial ranking, a full kinetic analysis was performed by Dr. Tzekou to further characterize and quantitate the relationship between the structure and activity. Due to the very slow dissociation rate constant of analogue 4.22, a single-cycle kinetics approach was used to allow for characterization of interactions with slow dissociation rates in a shorter period of time than does the traditional multi-cycle approach. All peptides showed a binding response that could be fitted to a 1:1 binding model, allowing for the extraction of rate constants $k_a$ and $k_d$ (Fig. 4.8). The γ-Cth analogue 4.20 possessed 5.7-fold reduced affinity for C3b compared to parent 4.1, while δ-Cth analogue 4.21 possessed only a 1.6-fold lower affinity, highlighting the
importance of the exact position of the sulfur atom. δ-Cth analogue 4.23 possessed a 3.8-fold lower affinity than parent 4.22. The chief differences between the Cth-containing analogues and their respective disulfide-containing parent peptides were found in $k_d$, as $k_a$ remained rather similar (Fig. 4.8f). The largely comparable association rate constants within each analogue series suggest that the Cth modification had little effect on complex formation with C3b, maintaining the ability of the peptides to adjust between distinct solution and bound conformations.\(^{57, 59}\) However, it is possible that a slightly changed geometry between cystine and Cth may influence the exact positioning of key residues and thereby affect the complex stability, as measured by $k_d$. This effect appears more pronounced in 4.23, as the mutations in parent peptide 4.22 were made specifically to tailor the peptide to its binding site.

![Figure 4.8. Kinetic titration analysis via SPR of binding between compstatin analogues and C3b.](image)

For (a) 4.1, (b) 4.20, and (c) 4.21, two sets of concentrations are superimposed for a better description of the full binding range and to achieve a well-defined affinity profile. Data were fitted to a 1:1 binding model (orange line), from which kinetic rate constants were extracted. (f) Kinetic rate constants $k_a$ and $k_d$ and binding affinity $K_D$ for each compstatin analogue, showing average and standard deviation of at least six data sets. Figure courtesy of Dr. Apostolia Tzekou (UPSM).
4.2.5. Inhibition of complement activation by compstatin analogues

The biological activities of the Cth-containing analogues were compared to that of their corresponding disulfide-containing parent peptides using an established enzyme-linked immunosorbent assay (ELISA) by Dr. Tzekou (Fig. 4.9).61 The ability of these compounds to inhibit antigen/antibody-induced complement activation was assessed by measuring the deposition of C3b on an ELISA surface, compared to controls with no peptide (Fig. 4.9b). These assays yielded results consistent with the data obtained in the kinetic assays (Section 4.2.4). Analogue 4.20 was again identified as the least active analogue, thereby confirming the unfavorable effect of sulfur-to-methylene substitution on the C-terminal side of the original disulfide. In both series, Cth-containing peptides 4.21 and 4.23 showed decreased inhibitory activity compared to parent peptides 4.1 and 4.22, respectively, but only by a factor of ~2. The combined analyses confirm thioether-restricted compstatin analogues 4.21 and 4.23 as potent complement inhibitors.

Figure 4.9. Inhibitory activity of compstatin analogues. (a) Representative results of at least five assays for inhibition of complement activation by compstatin analogues. The calculated IC_{50} values are shown for each analogue. (b) Schematic representation of the inhibition assay. BSA, bovine serum albumin; OVA, ovalbumin; a-hC3 HRP, anti-human C3 antibody conjugated to horseradish peroxidase. Figure courtesy of Dr. Apostolia Tzekou (UPSM).
4.2.6. Effect of oxidation and reduction on compstatin analogue activity

A disulfide-to-thioether substitution was hypothesized to improve the reductive stability of compstatin analogues; however, thioethers are labile to metabolic oxidation, as has been well documented in the case of methionine-containing polypeptide drugs. In addition, thioether oxidation has been shown to severely decrease the activity of the lantibiotic nisin, although the lantibiotic actagardine contains an enzymatically-oxidized methyllanthionine bridge important for full activity. In this work, Cth building blocks and Cth-containing peptides appeared stable to spontaneous oxidation during synthesis, purification, biological testing, and storage. However, systematic assessment of the effect of oxidative and reductive treatments on analogue activity was desired, as such conditions may be encountered in vivo. Since the binding and inhibitory activities of disulfide-containing 4.22 were largely maintained in δ-Cth-containing 4.23, the effect of oxidation and reduction on these peptides was assessed by Dr. Tzekou. Both peptides were treated with a 500-fold molar excess of tris(2-carboxyethyl)phosphine hydrochloride (TCEP); this treatment resulted in reduction of the disulfide of 4.22 (Fig. 4.10a) but no change in 4.23, confirming its stability to reduction. Similarly, both peptides were also exposed to excess hydrogen peroxide, which resulted in oxidation of the thioether bridge of 4.23 to the corresponding sulfoxide (Fig. 4.10b) but no modification of 4.22.

The oxidant- and reductant-treated 4.22 and 4.23 were tested for complement inhibitory activity by ELISA (see Section 4.2.5). In order to prevent re-formation of the disulfide bond during analysis, reduced 4.22 was treated with iodoacetamide to alkylate the cysteinyl thiols. As expected based on previous studies, reduction and alkylation of 4.22 severely diminished activity compared to 4.22, while treatment with H2O2 had only marginal consequences (Fig. 4.11). This confirmed the importance of both the cyclic structure and reductive stability to inhibitory activity. Conversely, thioether-oxidized 4.23 showed a decrease in potency compared to 4.23, but as the magnitude of this reduction was a mere 4.4-fold, this sulfoxide-containing peptide remained a highly potent, sub-micromolar inhibitor of complement activation. Therefore, while a different metabolic lability was introduced in the thioether-containing analogues, oxidative treatment gave only modest effects on activity compared to disulfide reduction.
Figure 4.10. MALDI-TOF MS analysis of redox treatment on compstatin analogues. (a) Disulfide-containing analogue **4.22** before and after treatment with excess TCEP, showing a +2 Da mass shift indicative of disulfide reduction. (b) Cth-containing analogue **4.23** before and after treatment with excess H$_2$O$_2$, showing a +16 Da mass shift indicative of thioether oxidation to sulfoxide. In the reverse cases, treatment of **4.22** with H$_2$O$_2$ and **4.23** with TCEP, no changes in mass were observed. Figure courtesy of Dr. Apostolia Tzekou (UPSM).
Figure 4.11. Effect of redox modification on complement inhibition by compstatin analogues 4.22 and 4.23. All data sets except reduced/alkylated 4.22 could be evaluated using a dose-response function to determine comparative IC\textsubscript{50} values. Figure courtesy of Dr. Apostolia Tzekou (UPSM).

4.2.7. Synthesis of an albumin affinity tag to improve \textit{in vivo} half-life

Even if rendered stable to proteolysis and redox agents, small peptide therapeutics such as compstatin often suffer from short half-lives \textit{in vivo} due to rapid clearance rates from the human body.\textsuperscript{1, 76-78} One approach to address this limitation is the attachment of serum albumin binding tags to peptide therapeutics, which has been shown to increase the \textit{in vivo} half-lives of bioactive peptides up to 50-fold by slowing clearance rates.\textsuperscript{66, 79} It was therefore hypothesized that a similar approach could be used to broaden the therapeutic application of compstatin, including to systemic disease states. A diphenylcyclohexyl phosphodiester moiety (4.27, Scheme 4.5) was synthesized with modification from a previous report.\textsuperscript{66} A Robinson-type tandem Michael addition/aldol condensation furnished hexenone 4.24, which was reduced by sequential hydrogenation and borohydride treatment to give alcohol 4.25. This alcohol was coupled to phosphoramidite 4.26, and the resulting phosphite was oxidized to the phosphate, followed by cyanoethyl and ethyl ester removal to give 4.27. Conjugates of compstatin derivatives and 4.27 are currently being investigated in the laboratory of Prof. John Lambris (UPSM) for alteration of complement inhibitory activity and \textit{in vivo} clearance rates.
**Scheme 4.5.** Synthesis of albumin affinity tag 4.27.

$a$ Reagents and conditions: a) KOH, EtOH, 0 °C, 70%; b) H₂, Pd/C, THF; then NaBH₄, NaOH, H₂O, THF, 96%; c) DIPEA, CH₂Cl₂, 78%; d) 4.25, 1H-tetrazole, 'BuOOH, MeCN; e) NH₃, MeOH; f) LiOH, H₂O, THF, 54% (three steps).

### 4.3. CONCLUSIONS AND OUTLOOK

In this chapter, the chemical synthesis of thioether-containing analogues of the complement inhibitor compstatin was described, confirming the cystathionine bridge as a viable disulfide mimic in a bioactive peptide system. Replacing the cystine of compstatin with Cth was found largely to preserve the binding and inhibitory activities of the parent peptide while removing lability to reduction. As suggested in other peptides,$^{49}$ the position of the sulfur atom within the Cth bridge played a role in biological activity; placement closer to the N-terminus of the peptide (δ-Cth, as in 4.21) was found to give more potent activity than placement closer to the C-terminus (γ-Cth, as in 4.20). In comparing potential metabolic alterations of disulfide- and thioether-containing analogues, reduction of the disulfide had a severely deleterious effect on potency while oxidation of the Cth thioether gave only a modest decrease, highlighting the benefit of Cth in this context. While N-terminally acetylated compstatin analogues have been shown previously to possess high stability in plasma under normal conditions,$^{64}$ a variety of complement-related pathologies can trigger changes in the reductive environment of blood. For example, compstatin derivatives have shown promise in treating models of bacterial sepsis,$^{80}$ but this disease state is also associated with a large increase in the plasma levels of thioredoxin$^{81}$ that may threaten the disulfide structure. Similarly, complement inhibition by compstatin has therapeutic potential in several hemolytic conditions,$^{54, 82}$ during which the large reservoir of reduced glutathione in erythrocytes (1-3 mM) is released into the plasma.$^{83, 84}$ In these relevant therapeutic applications, protection of compstatin analogues against reduction becomes even more important.
More generally, as the impact of peptide-based drugs has continued to develop, disulfide bond engineering has become a major focus of efforts toward in vivo stabilization (see Section 4.1.1). The versatile nature of this solid-supported synthesis allows for the production of a wide variety of cyclic Cth-containing peptides, in order to probe the effects of disulfide substitution on the activity and stability of other potential drugs. Indeed, Cth-containing analogues replacing one or both of the disulfides of α-conotoxin ImI have been recently reported, which also largely maintain the structure and potency of the parent disulfide-containing peptide. The availability of Cth building blocks bearing orthogonal protecting groups (as described for lanthionine building blocks in Chapter 3) would allow for the controlled synthesis of overlapping Cth bonds. The recent synthesis of novel thioether-containing amino acids has already expanded the known suite of differentially-protected Cth building blocks useful in disulfide bond engineering.

4.4. EXPERIMENTAL

4.4.1. Materials and general methods

Disulfide-containing compstatin derivatives 4.1 and 4.22 were synthesized in the laboratory of Prof. John Lambris (UPSM) as previously described. Unprotected amino acids for solution-phase synthesis, standard protected Fmoc-amino acids for solid-phase peptide synthesis (SPPS), Fmoc-Rink amide AM resin, N-(9-fluorenylmethoxycarbonyloxy)succinimide (FmocOSu), and peptide coupling reagents 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), benzotriazol-1-yl-oxytrityltridotridinophosphonium hexafluorophosphate (PyBOP), O-(6-chlorobenzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HCTU), and 1-hydroxy-7-azabenzotriazole (HOAt) were purchased from ChemImpex International or Advanced ChemTech. N-Fmoc-1-methyl-L-tryptophan (Fmoc-Trp(Me)-OH) was synthesized from 1-methyl-L-tryptophan by an established procedure. Dimethylformamide (DMF), tetrahydrofuran (THF), and dichloromethane were purchased at ACS-grade from Fisher Scientific and dried via a solvent dispensing system prior to use in reactions. Other chemical reagents, including an L-cystathionine standard for GC/MS analysis, and solvents were purchased from Sigma Aldrich, Alfa Aesar, or Fisher Scientific and used without further purification.

All reactions were run under an atmosphere of N₂ unless otherwise stated. Reaction progress and chromatography fractions were monitored by thin layer chromatography (TLC) on silica gel-coated glass plates with a F254 fluorescent indicator. Visualization was achieved by...
UV absorption by fluorescence quenching or permanganate stain (1.5 g KMnO$_4$, 10 g K$_2$CO$_3$, 1.25 mL of 10% NaOH in 200 mL of H$_2$O). Flash chromatography was performed using Silicycle SiliaFlash P60, 230-400 mesh silica gel. Analytical reversed-phase high-performance liquid chromatography (RP-HPLC) was performed using a Beckman System Gold instrument equipped with a Vydc C18 (300 Å pore size, 250 mm × 4.6 mm × 5 μm) analytical column, a flow rate of 1 mL/min, and a solvent gradient of 2% solvent B for 1 min, then 10-100% over 45 min, observing at an absorbance of 220 nm. Preparatory RP-HPLC was performed on a Waters 600 system equipped with a Waters Delta-Pak C18 (100 Å pore size, 100 mm × 25 mm) column or a Phenomenex Luna C18 (100 Å pore size, 250 mm × 10.0 mm × 10 μm) column with a flow rate of 8 mL/min and solvent gradients as described for each peptide. All HPLC solvents were filtered with a Millipore filtration system equipped with a 0.22 μm nylon membrane filter prior to use. HPLC solvent compositions: solvent A is 0.1% trifluoroacetic acid (TFA) in H$_2$O; solvent B is 80:20 MeCN/H$_2$O with 0.087% TFA.

NMR spectra were recorded using a Varian Unity 400 or Unity Inova 500 spectrometer at the NMR Laboratory (UIUC). Small molecules (MW < 800 Da) were analyzed by electrospray ionization/time-of-flight (ESI-TOF) mass spectrometry on a Waters Quattro II quadrupole spectrometer at the Mass Spectrometry Facility (UIUC). Peptides (MW > 800 Da) were analyzed by matrix-assisted laser desorption ionization/time-of-flight (MALDI-TOF) mass spectrometry using an Applied Biosystems Voyager-DE spectrometer at the Mass Spectrometry Facility (UIUC) in a matrix solution consisting of saturated α-cyano-4-hydroxycinnamic acid in 1:1:0.1 H$_2$O/MeCN/TFA.

### 4.4.2. Synthesis of Cth building blocks 4.7, 4.13, and 4.19
Compound 4.2. To a solution of l-cystine (6.01 g, 25.0 mmol) in 70% perchloric acid (10 mL) was slowly added tert-butylacetate (62.5 mL). The solution was stirred for 19 h, gradually becoming homogeneous. Water was added, and the solution was chilled in an ice bath. The pH was adjusted to 10 with 10 M NaOH, and the solution was extracted with EtOAc. The organic layer was dried over Na₂SO₄, filtered, and concentrated under reduced pressure. A portion of the residue (5.95 g, 16.9 mmol) was taken up in THF (85 mL) and chilled in an ice bath, and N-methylmorpholine (3.85 mL, 34.9 mmol) was added. FmocOSu (11.75 g, 34.9 mmol) was then added portionwise over 10 min. The reaction was warmed to room temperature, then stirred for 19 h. The reaction was concentrated under reduced pressure, taken up in EtOAc, washed with water and brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO₂, 9:1 then 7:3 hexane/EtOAc) to yield 4.2 (10.69 g, 13.4 mmol, 79%) as a white solid. R₇ 0.55 (2:1 hexane/EtOAc). Spectral data matched those reported previously. See also Notebook I, pages 38-39.

Compound 4.3. To a solution of 4.2 (3.00 g, 3.66 mmol) in tetrahydrofuran (45 mL) was added tributylphosphine (1.37 mL, 5.50 mmol) under N₂. The reaction was stirred for 30 min, then water (4.5 mL) was added and the reaction stirred an additional 14 h. Solvent was removed under reduced pressure, and the residue was taken up in EtOAc, washed with 10% citric acid and brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO₂, 9:1 then 4:1 hexane/EtOAc) to yield 4.3 (2.53 g, 6.33 mmol, 86%) as a colorless oil. R₇ 0.45 (4:1 hexane/EtOAc). Spectral data matched those reported previously. See also Notebook I, page 42.

Compound 4.4. L-Homoserine (1.01 g, 8.48 mmol) and sodium carbonate (0.90 g, 8.48 mmol) were dissolved in water (10 mL) and acetonitrile (5 mL) and chilled in an ice bath. Allyl chloroformate (900 µL, 8.48 mmol) was added dropwise. The reaction was allowed to warm to room temperature and stirred under N₂ for 18 h. The reaction was concentrated under reduced pressure, and the residue was taken up in DMF (20 mL). Sodium bicarbonate (0.71 g, 8.48 mmol) was added, followed by allyl bromide (810 µL, 9.33 mmol). The reaction was stirred as a heterogeneous
mixture for 48 h, with additional allyl bromide (405 µL, 4.66 mmol) added after 22 h. The reaction was concentrated under reduced pressure, then taken up in EtOAc, washed with saturated aqueous NaHCO₃, water, 0.1 M KHSO₄, and brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO₂, 2:1 then 1:1 hexane/EtOAc) to yield 4.4 (1.58 g, 6.50 mmol, 77%) as a colorless oil. Rₛ 0.39 (SiO₂, 1:1 hexane/EtOAc); ¹H NMR (500 MHz, CDCl₃) δ 5.94-5.86 (m, 2H), 5.67 (d, J = 7.3 Hz, 1H), 5.35-5.21 (m, 4H), 4.65 (d, J = 6.0 Hz, 2H), 4.60-4.53 (m, 3H), 3.76-3.65 (m, 2H), 2.64 (bs, 1H), 2.20-2.14 (m, 1H), 1.77-1.71 (m, 1H). ¹³C NMR (125 MHz, CDCl₃) δ 172.3, 156.7, 132.6, 131.5, 119.1, 118.1, 66.3, 66.2, 58.5, 51.5, 35.6. HRMS (ESI) calc. [M+Na]⁺ for C₁₁H₁₇NO₅Na 266.1004, found 266.1008. See also Notebook I, page 47.

Compound 4.5. To a solution of 4.4 (4.00 g, 16.4 mmol) in CH₂Cl₂ (45 mL) was added carbon tetrabromide (6.53 g, 19.7 mmol), and the reaction was chilled in an ice bath. A solution of triphenylphosphine (5.17 g, 19.7 mmol) in CH₂Cl₂ (20 mL) was added dropwise to the chilled reaction. The reaction was warmed to room temperature and stirred under N₂ for 1 h, then washed with water and brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. Excess 1:1 hexane/EtOAc was added to precipitate the phosphine oxide byproduct, which was removed via filtration through Celite. The filtrate was concentrated under reduced pressure and purified by flash chromatography (SiO₂, 4:1 then 2:1 hexane/EtOAc) to yield 4.5 (4.02 g, 13.1 mmol, 80%) as a colorless oil. Rₛ 0.50 (2:1 hexane/EtOAc). ¹H NMR (500 MHz, CDCl₃) δ 5.95-5.85 (m, 2H), 5.37-5.21 (m, 5H), 4.65 (dt, J = 5.6 Hz, 1.2 Hz, 2H), 4.58 (d, J = 5.6 Hz, 2H), 4.52 (m, 1H), 3.44 (t, J = 7.0 Hz, 2H), 2.48-2.41 (m, 1H), 2.29-2.22 (m, 1H). ¹³C NMR (125 MHz, CDCl₃) δ 171.3, 155.9, 132.5, 131.4, 119.3, 118.1, 66.4, 66.1, 53.0, 35.7, 28.2. HRMS (ESI) calc. [M+H]⁺ for C₁₁H₁₇BrNO₄ 306.0341, found 306.0339. See also Notebook II, page 35.

Compound 4.6. Compounds 4.3 (1.60 g, 4.01 mmol) and 4.5 (1.29 g, 4.21 mmol) were dissolved in N₂-sparged EtOAc (20 mL) under N₂. Tetrabutylammonium bromide (5.17 g, 16.0 mmol) was dissolved in N₂-sparged 0.5 M aqueous NaHCO₃ (pH adjusted to 8.5, 20 mL), then added to the reaction. The biphasic mixture was for 22 h under N₂. The reaction was diluted with EtOAc, washed with water and brine, dried over Na₂SO₄, filtered, and concentrated under
reduced pressure. The crude material was purified by flash chromatography (SiO₂, 4:1 then 2:1 hexane/EtOAc) to yield 4.6 (2.10 g, 3.36 mmol, 84%) as a colorless oil. Rf 0.20 (3:1 hexane/EtOAc). ¹H NMR (500 MHz, CDCl₃) δ 7.76 (d, J = 7.5 Hz, 2H), 7.61 (d, J = 7.5 Hz, 2H), 7.40 (t, J = 7.5 Hz, 2H), 7.32 (t, J = 7.5 Hz, 2H), 5.94-5.85 (m, 2H), 5.69 (d, J = 7.3 Hz, 1H), 5.40 (d, J = 7.1 Hz, 1H), 5.34-5.17 (m, 4H), 4.63 (d, J = 6.0 Hz, 2H), 4.59-4.46 (m, 4H), 4.39 (d, J = 7.0 Hz, 2H), 4.24 (t, J = 7.0 Hz, 1H), 2.97 (dq, J = 13.4 Hz, 4.9 Hz, 2H), 2.65-2.60 (m, 2H), 2.15-1.96 (m, 2H), 1.49 (s, 9H). ¹³C NMR (125 MHz, CDCl₃) δ 171.7, 169.8, 155.9, 144.0, 143.9, 141.4, 132.7, 131.5, 127.8, 127.2, 125.3, 120.1, 119.2, 118.0, 83.1, 67.3, 66.3, 66.1, 54.4, 53.1, 47.3, 34.9, 32.6, 28.8, 28.1. HRMS (ESI) calc. [M+H]+ for C₃₃H₄₁N₂O₈S 625.2584, found 625.2582. See also Notebook II, page 39.

Compound 4.7. To a solution of 4.6 (2.00 g, 3.20 mmol) in CH₂Cl₂ (16 mL) was added phenylsilane (440 µL, 3.52 mmol), followed by TFA (16 mL). The reaction was stirred at room temperature for 2 h. The system was concentrated under reduced pressure, then dissolved in 1:1 benzene/acetonitrile, flash frozen, and lyophilized to yield 4.7 (1.72 g, 3.03 mmol, 95%) as a white powder. Rf 0.34 (25:1 CH₂Cl₂/methanol). ¹H NMR (500 MHz, CD₃OD) δ 7.81 (d, J = 7.5 Hz, 2 H), 7.70 (d, J = 7.5 Hz, 2H), 7.41 (t, J = 7.5 Hz, 2H), 7.35-7.30 (m, 2H), 5.98-5.90 (m, 2H), 5.36-5.17 (m, 4H), 4.63 (m, 2H), 4.55 (m, 2H), 4.43-4.33 (m, 4H), 4.24 (t, J = 7.0 Hz, 1H), 3.12-2.86 (m, 2H), 2.74-2.60 (m, 2H), 2.14-1.97 (m, 2H). ¹³C NMR (125 MHz, CD₃OD) δ 174.0, 173.4, 158.5, 158.4, 145.3, 145.2, 142.5, 134.2, 133.2, 128.8, 128.2, 126.3, 120.9, 118.7, 117.6, 68.2, 66.8, 66.6, 55.2, 54.3, 34.6, 32.4, 29.4. HRMS (ESI) calc. [M+H]+ for C₂₉H₃₃N₂O₈S 569.1958, found 569.1957. See also Notebook II, page 42.
Compound **4.8.** D-Homoserine (2.0 g, 16.8 mmol) and sodium carbonate (1.78 g, 16.8 mmol) were dissolved in water (35 mL) and chilled in an ice bath. A solution of FmocOSu (5.67 g, 16.8 mmol) in 1,4-dioxane (15 mL) was added slowly to the aqueous solution. The reaction was warmed to room temperature and stirred under N\textsubscript{2} for 8 h. The dioxane was removed under reduced pressure, and the remaining mixture was washed with EtOAc, then acidified to pH 2 with 2 M HCl. A precipitate formed, which was extracted with EtOAc, dried over Na\textsubscript{2}SO\textsubscript{4}, filtered, and concentrated under reduced pressure to yield the amine-protected intermediate as a white solid. A portion of this intermediate (1.00 g, 2.93 mmol) was dissolved in CH\textsubscript{2}Cl\textsubscript{2} (20 mL) and tetrahydrofuran (5 mL). Tert-butyl 2,2,2-trichloroacetimidate (1.30 mL, 7.32 mmol) was added, and the reaction was stirred under N\textsubscript{2} for 16 h. The reaction was concentrated under reduced pressure, then taken up in EtOAc, washed with saturated aqueous NaHCO\textsubscript{3} and brine, dried over Na\textsubscript{2}SO\textsubscript{4}, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO\textsubscript{2}, 2:1 hexane/EtOAc) to yield **4.8** (0.61 g, 1.53 mmol, 52%) as a white foam. R\textsubscript{f} 0.43 (1:1 hexane/EtOAc). \textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}) δ 7.77 (d, J = 7.5 Hz, 2H), 7.60 (d, J = 7.5 Hz, 2H), 7.32 (t, J = 7.5 Hz, 2H), 3.75-3.58 (m, 2H), 2.59 (bs, 1H), 2.20-2.12 (m, 1H), 1.67-1.61 (m, 1H), 1.48 (s, 9H). \textsuperscript{13}C NMR (125 MHz, CDCl\textsubscript{3}) δ 171.8, 157.0, 143.9, 143.7, 141.4, 127.8, 127.2, 125.1, 120.1, 82.6, 58.4, 51.6, 51.6, 47.2, 36.1, 28.1. HRMS (ESI) calc. [M+H]\textsuperscript{+} for C\textsubscript{23}H\textsubscript{28}NO\textsubscript{5} 398.1967, found 398.1958. See also Notebook III, page 94; Notebook IV, page 6.

Compound **4.9.** To a solution of **4.8** (0.70 g, 1.76 mmol) in CH\textsubscript{2}Cl\textsubscript{2} (7 mL) was added carbon tetrabromide (0.70 g, 2.11 mmol), and the solution was chilled in an ice bath. A solution of triphenylphosphine (0.55 g, 2.11 mmol) in CH\textsubscript{2}Cl\textsubscript{2} (3 mL) was added dropwise. The reaction was warmed to room temperature and stirred under N\textsubscript{2} for 1 h, then washed with water and brine, dried over Na\textsubscript{2}SO\textsubscript{4}, filtered, and concentrated under reduced pressure. Excess 1:1 hexane/EtOAc was added to precipitate the phosphine oxide byproduct, which was removed via filtration through Celite. The filtrate was concentrated under reduced pressure and purified by flash chromatography (SiO\textsubscript{2}, 5:1 hexane/EtOAc) to yield **4.9** (0.76 g, 1.65 mmol, 94%) as a colorless oil. R\textsubscript{f} 0.45 (3:1 hexane/EtOAc). \textsuperscript{1}H NMR (CDCl\textsubscript{3}, 500 MHz) δ 7.77 (d, J = 7.5 Hz, 2H), 7.60 (d, J = 7.5 Hz,
2H), 7.40 (t, J = 7.5 Hz, 2H), 7.33 (dt, J = 7.5 Hz, 1.5 Hz, 2H), 5.38 (d, J = 7.7 Hz, 1H), 4.46-4.35 (m, 3H), 4.23 (t, J = 7.0 Hz, 1H), 3.42-3.36 (m, 2H), 2.45-2.17 (m, 2H), 1.49 (s, 9H).

13C NMR (CDCl3, 400 MHz) δ 170.6, 156.0, 143.9, 143.8, 141.4, 127.9, 127.2, 125.2, 120.1, 83.0, 67.1, 53.6, 47.3, 36.2, 28.3, 28.1. HRMS (ESI) calc. [M+H]+ for C23H27BrNO4 460.1123, found 460.1126. See also Notebook IV, page 47.

Compound 4.10. S-trityl-γ-cysteine (2.50 g, 6.88 mmol) and sodium carbonate (0.73 g, 6.88 mmol) were suspended in water (14 mL) and acetonitrile (7 mL) and chilled in an ice bath. Allyl chloroformate (0.73 mL, 6.88 mmol) was added dropwise. The reaction was allowed to warm to room temperature and stirred under N2 for 16 h. Acetonitrile was removed under reduced pressure, and additional water was added. The pH was adjusted to 2 with 2 M HCl, forming a precipitate which was extracted with EtOAc. The organic fractions were concentrated under reduced pressure, and the residue was taken up in DMF (18 mL). Sodium bicarbonate (0.58 g, 6.88 mmol) and allyl bromide (0.72 mL, 8.26 mmol) were added, and the reaction was stirred as a heterogeneous mixture for 17 h, with additional allyl bromide (0.30 mL, 3.47 mmol) added after 7 h. The reaction was concentrated under reduced pressure, taken up in EtOAc, washed with saturated aqueous NaHCO3, water, 0.1 M KHSO4, and brine, dried over Na2SO4, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO2, 5:1 hexane/EtOAc) to yield 4.10 (2.34 g, 4.80 mmol, 70%) as pale yellow oil. Rf 0.48 (3:1 hexane/EtOAc). 1H NMR (500 MHz, CDCl3) δ 7.41 (m, 6H), 7.30 (m, 6H), 7.23 (m, 3H), 5.91-5.85 (m, 2H), 5.35-5.21 (m, 5H), 4.62 (dt, J = 5.5 Hz, 1.5 Hz, 2H), 4.56 (d, J = 5.5 Hz, 2H), 4.37 (m, 1H), 2.72-2.58 (m, 2H). 13C NMR (125 MHz, CDCl3) δ 170.2, 155.5, 144.3, 132.6, 131.5, 129.5, 128.1, 127.9, 126.9, 118.8, 117.8, 67.0, 66.3, 65.9, 52.9, 34.2. HRMS (ESI) calc. [M+Na]+ for C29H29NO4SNa 510.1715, found 510.1707. See also Notebook IV, pages 49, 53.

Compound 4.11. To a solution of 4.10 (0.60 g, 1.23 mmol) in CH2Cl2 (12 mL) was added triisopropylsilane (0.50 mL, 2.46 mmol), followed by TFA (1.9 mL, 24.6 mmol) under N2. The reaction was stirred under N2 for 1 h, then partitioned between CH2Cl2 and water. Solid NaHCO3 (2.07 g, 24.6 mmol) was slowly added to neutralize the residual acid. The organic layer was separated, washed with brine, dried over Na2SO4, filtered, and concentrated under reduced pressure. The crude material was purified
by flash chromatography (SiO₂, 6:1 hexane/EtOAc) to yield 4.11 (0.26 g, 1.04 mmol, 85%) as a colorless oil. This compound was used immediately for further reaction, as it is unstable for long-term storage. \( R_f \) 0.51 (3:1 hexane/EtOAc). \(^1\)H NMR (400 MHz, CDCl₃) \( \delta \) 5.96-5.86 (m, 2H), 5.65 (d, \( J = 7.0 \) Hz, 1H), 5.39-5.22 (m, 4H), 4.72-4.64 (m, 3H), 4.59 (d, \( J = 5.6 \) Hz, 2H), 3.08-2.95 (m, 2H), 1.39 (t, \( J = 9.0 \) Hz, 1H). \(^1^3\)C NMR (125 MHz, CDCl₃) \( \delta \) 169.8, 155.6, 132.5, 131.4, 119.4, 118.1, 66.5, 66.1, 55.3, 27.3. HRMS (ESI) calc. [M+H]⁺ for C₁₀H₁₆NO₄S 246.0800, found 246.0797. See also Notebook IV, page 55.

Compound 4.12. Compounds 4.11 (0.24 g, 0.96 mmol) and 4.9 (0.44 g, 0.96 mmol) were dissolved in N₂-sparged EtOAc (5 mL) under N₂. Tetrabutylammonium bromide (1.23 g, 3.82 mmol) was dissolved in N₂-sparged 0.5 M aqueous sodium bicarbonate (pH 8.5, 5 mL), then added to the reaction. The biphasic mixture was stirred for 20 h under N₂. The reaction was diluted with EtOAc, washed with water and brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO₂, 4:1 then 3:1 hexane/EtOAc) to yield 4.12 (0.56 g, 0.89 mmol, 93%) as a colorless oil. \( R_f \) 0.22 (3:1 hexane/EtOAc). \(^1\)H NMR (500 MHz, CDCl₃) \( \delta \) 7.76 (d, \( J = 7.5 \) Hz, 2H), 7.60 (d, \( J = 7.5 \) Hz, 2H), 7.40 (t, \( J = 7.5 \) Hz, 1H), 5.93-5.86 (m, 2H), 5.68 (d, \( J = 7.5 \) Hz, 1H), 5.43 (d, \( J = 7.5 \) Hz, 1H), 5.36-5.18 (m, 4H), 4.65 (d, \( J = 5.5 \) Hz, 2H), 4.62-4.36 (m, 3H), 4.23 (t, \( J = 7.0 \) Hz, 1H), 3.01 (d, \( J = 4.5 \) Hz, 2H), 2.58-2.55 (m, 2H), 2.12-1.88 (m, 2H), 1.48 (s, 9H). \(^1^3\)C NMR (125 MHz, CDCl₃) \( \delta \) 171.0, 170.5, 156.1, 144.0, 141.4, 132.6, 131.4, 127.8, 127.2, 125.2, 120.1, 119.3, 118.1, 82.7, 67.1, 66.1, 53.8, 53.6, 47.2, 34.7, 32.9, 28.7, 28.1. HRMS (ESI) calc. [M+H]⁺ for C₃₃H₄₁N₂O₈S 625.2584, found 625.2592. See also Notebook IV, page 56.

Compound 4.13. To a solution of 4.12 (0.53 g, 0.85 mmol) in CH₂Cl₂ (3 mL) was added phenylsilane (120 µL, 0.93 mmol), followed by TFA (3 mL). The reaction was stirred at room temperature for 2 h. The system was concentrated under reduced pressure, then dissolved in 1:1 benzene/acetonitrile, flash frozen and lyophilized to yield 4.13 (0.48 g, 0.84 mmol, 98%) as a white powder. \( R_f \) 0.33 (25:1 CH₂Cl₂/methanol). \(^1\)H NMR (500 MHz, CD₃OD) \( \delta \) 7.77 (d, \( J = 7.5 \) Hz, 2H), 7.68-7.64 (m, 2H), 7.37 (t, \( J = 7.5 \) Hz, 2H), 7.29 (t, \( J = 7.5 \) Hz, 2H), 5.95-5.88 (m, 2H),
5.34-5.27 (m, 2H), 5.22-5.15 (m, 2H), 4.63 (d, J = 5.5 Hz, 2H), 4.53 (m, 2H), 4.42 (m, 1H), 4.36-4.32 (m, 3H), 4.20 (t, J = 7.0 Hz, 1H), 3.03-2.84 (m, 2H), 2.68-2.56 (m, 2H), 2.15-1.95 (m, 2H).

$^{13}$C NMR (125 MHz, CD$_3$OD) δ 175.4, 172.1, 158.7, 158.2, 145.3, 142.5, 134.2, 133.1, 128.8, 128.1, 126.3, 120.9, 118.8, 117.7, 67.9, 67.0, 66.7, 55.4, 54.0, 34.4, 32.5, 29.5. HRMS (ESI) calc. [M+H]$^+$ for C$_{29}$H$_{33}$N$_2$O$_8$S 569.1958, found 569.1953. See also Notebook IV, page 70.

**Compound 4.14.** L-Homoserine (0.97 g, 8.14 mmol) and sodium carbonate (0.86 g, 8.14 mmol) were dissolved in water (10 mL) and MeCN (5 mL). 9-Fluorenylmethyl chloroformate (FmocCl; 2.11 g, 8.14 mmol) was added slowly as a solid over 5 min, and the reaction was stirred for 18 h. The reaction was concentrated under reduced pressure, and the residue was taken up in DMF (20 mL). Sodium bicarbonate (0.68 g, 8.14 mmol) was added, followed by allyl bromide (780 μL, 8.95 mmol) added at 21 h. The reaction was stirred for 48 h, with additional allyl bromide (350 μL, 4.07 mmol) added at 21 h. The reaction was concentrated under reduced pressure, taken up in EtOAc, washed with saturated aqueous NaHCO$_3$, H$_2$O, 0.1 M KHSO$_4$, and brine, dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO$_2$, 3:2 then 1:1 hexanes/EtOAc) to yield 4.14 (1.98 g, 5.19 mmol, 60%) as a white solid. $R_f$ 0.45 (1:1 hexanes/EtOAc). $^1$H NMR (400 MHz, CDCl$_3$) δ 7.78 (d, 2H), 7.59 (d, 2H), 7.39 (t, 2H), 7.30 (t, 2H), 5.92-5.82 (m, 1H), 5.63 (d, 1H), 5.38-5.22 (m, 4H), 4.62 (d, 2H), 4.57 (m, 1H), 4.48-4.37 (m, 2H), 4.20 (t, 1H), 3.75-3.55 (m, 2H), 2.23-1.65 (m, 2H). LRMS (ESI) calc. [M+H]$^+$ for C$_{22}$H$_{24}$NO$_3$ 382.2, found 382.2. See also Notebook I, page 51.
Compound 4.15. To a solution of 4.14 (4.15 g, 10.9 mmol) in CH$_2$Cl$_2$ (40 mL) was added carbon tetrabromide (5.41 g, 16.3 mmol), and the solution was chilled in an ice bath. A solution of triphenylphosphine (4.28 g, 16.3 mmol) in CH$_2$Cl$_2$ (15 mL) was added via cannula. The reaction was warmed to room temperature and stirred under N$_2$ for 1 h, then washed with H$_2$O and brine, dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure. The triphenylphosphine oxide byproduct was precipitated in 1:1 hexane/EtOAc and removed by filtering through Celite. The filtrate was concentrated under reduced pressure and purified by flash chromatography (SiO$_2$, 4:1 then 2:1 hexanes/EtOAc) to yield 4.15 (2.92 g, 6.57 mmol, 60%) as a colorless oil. $R_f$ 0.52 (3:1 hexanes/EtOAc). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.78 (d, 2H), 7.58 (d, 2H), 7.38 (t, 2H), 7.29 (t, 2H), 5.95-5.83 (m, 1H), 5.41-5.23 (m, 3H), 4.65 (d, 2H), 4.50 (m, 1H), 4.45 (m, 2H), 4.20 (t, 1H), 3.39 (t, 2H), 2.52-2.18 (m, 2H). LRMS (ESI) calc. [M+H]$^+$ for C$_{22}$H$_{23}$BrNO$_4$ 444.1, found 444.2. See also Notebook II, page 31-32.

Compound 4.16. S-trityl-$\alpha$-cysteine (2.00 g, 5.50 mmol) was suspended in MeOH (40 mL), and 2-(3-methylbutyryl)-5,5-dimethyl-1,3-cyclohexandione (DdivOH; 1.09 mL, 5.00 mmol) and iPr$_2$NEt (3.48 mL, 20.0 mmol) were added. A condenser was installed, and the reaction was stirred at reflux for 16 h. The reaction was concentrated under reduced pressure, and the residue was partitioned between EtOAc and dilute aqueous HCl (pH 2). The organic layer was isolated, dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure to ~1/4 volume. Excess cold hexane was added to form a precipitate, which was isolated by filtration and concentrated under reduced pressure to yield the amine-protected intermediate (2.83 g, 4.97 mmol, quant.) as a white powder. A portion of this intermediate (1.30 g, 2.28 mmol) was dissolved in CH$_2$Cl$_2$ (15 mL) and chilled in an ice bath. In succession, 4-dimethylaminopyridine (DMAP; 55 mg, 0.46 mmol) was added as a solid, followed by 2-trimethylsilylethanol (TmseOH; 390 $\mu$L, 2.74 mmol), and lastly EDC (0.61 g, 3.48 mL, 20.0 mmol) were added. A portion of this intermediate (1.30 g, 2.28 mmol) was dissolved in CH$_2$Cl$_2$ (15 mL) and chilled in an ice bath. In succession, 4-dimethylaminopyridine (DMAP; 55 mg, 0.46 mmol) was added as a solid, followed by 2-trimethylsilylethanol (TmseOH; 390 $\mu$L, 2.74 mmol), and lastly EDC (0.61 g, 3.19 mmol). The reaction was warmed to room temperature and stirred under N$_2$ for 5.5 h, then diluted with CH$_2$Cl$_2$, washed with 1 M HCl and brine, dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO$_2$, 9:1 then 7:1 hexane/EtOAc) to yield 4.16 (0.84 g, 1.25 mmol, 55%) as a colorless oil. $R_f$ 0.58 (3:1 hexanes/EtOAc). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.58-7.26 (m, 6H), 7.26-7.19 (m, 9H), 4.25-4.15 (m, 3H), 3.71 (dd, 2H), 2.68 (m, 2H), 2.39 (s, 4H), 1.78 (m,
1H), 1.02 (s, 6H), 0.95-0.80 (m, 8H), 0.02 (s, 9H). LRMS (ESI) calc. [M+H]+ for C₄₀H₅₂NO₄SSi 670.3, found 670.1. See also Notebook I, pages 60-61.

Compound 4.17. To a solution of 4.16 (3.10 g, 4.63 mmol) in CH₂Cl₂ (40 mL) was added triisopropylsilane (1.90 mL, 9.26 mmol), followed by TFA (7.1 mL, 92.6 mmol) under N₂. The reaction was stirred under N₂ for 2 h, then partitioned between CH₂Cl₂ and H₂O. Solid NaHCO₃ (7.78 g, 92.6 mmol) was added slowly to neutralize the acid. The organic layer was isolated, washed with brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO₂, 8:1 then 4:1 hexanes/EtOAc) to yield 4.17 (1.56 g, 3.65 mmol, 79%) as a colorless oil. Rf 0.37 (3:1 hexanes/EtOAc). ¹H NMR (400 MHz, CDCl₃) δ 4.66 (m, 1H), 4.27 (m, 2H), 3.12-2.90 (m, 3H), 2.42 (s, 4H), 1.92 (m, 1H), 1.62 (t, 1H), 1.03-0.92 (m, 14H), 0.03 (s, 9H). LRMS (ESI) calc. [M+H]+ for C₂₁H₃₈NO₄SSi 428.2, found 428.3. See also Notebook II, page 48.

Compound 4.18. Compounds 4.17 (0.34 g, 0.79 mmol) and 4.15 (0.37 g, 0.83 mmol) were dissolved in N₂-sparged EtOAc (8 mL). Tetrabutylammonium bromide (1.02 g, 3.16 mmol) was dissolved in N₂-sparged 0.5 M aqueous NaHCO₃ (pH adjusted to 8.5, 8 mL), then added to the organic solution. The biphasic mixture was stirred under N₂ for 24 h. The organic layer was isolated, washed with water and brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO₂, 4:1-2:1 hexane/EtOAc) to yield 4.18 (0.51 g, 0.65 mmol, 82%) as a colorless foam. Rf 0.33 (3:1 hexanes/EtOAc). ¹H NMR (400 MHz, CDCl₃) δ 7.75 (d, 2H), 7.60 (d, 2H), 7.39 (t, 2H), 7.29 (t, 2H), 5.95-5.88 (m, 1H), 5.85 (d, 1H), 5.38-5.22 (m, 2H), 4.62 (m, 3H), 4.47 (m, 1H), 4.39 (d, 2H), 4.30-4.21 (m, 3H), 3.11-3.02 (m, 3H), 2.63 (m, 2H), 2.40 (s, 4H), 2.18-1.98 (m, 2H), 1.95 (m, 1H), 1.05-0.95 (m, 14H), 0.02 (s, 9H). LRMS (ESI) calc. [M+H]+ for C₂₁H₃₈NO₄SSi 791.4, found 791.0. See also Notebook I, page 59.

Compound 4.19. To a solution of compound 4.18 (1.90 g, 2.40 mmol) in THF (50 mL) was added N-methylaniline (785 µL, 7.21 mmol). The reaction was purged with N₂, then tetrakis(triphenylphosphine)palladium(0) (140 mg, 5 mol%) was added. The reaction was stirred
under N\textsubscript{2} for 1.5 h, protected from light, then diluted with EtOAc, washed with saturated aqueous NH\textsubscript{4}Cl and brine, dried over Na\textsubscript{2}SO\textsubscript{4}, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO\textsubscript{2}, 50:1 then 20:1 CH\textsubscript{2}Cl\textsubscript{2}/MeOH) to yield 4.19 (1.41 g, 1.88 mmol, 78\%) as a pale yellow solid. \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) \(\delta 7.73 (d, 2H), 7.57 (m, 2H), 7.38 (t, 2H), 7.28 (t, 2H), 5.80 (d, 1H), 4.63 (m, 1H), 4.48 (m, 1H), 4.37 (d, 2H), 4.26 (t, 1H), 3.11-2.96 (m, 3H), 2.59 (m, 2H), 2.42 (s, 4H), 2.22-1.95 (m, 2H), 1.87 (m, 1H), 1.07-0.92 (m, 14H), 0.02 (s, 9H). \textsuperscript{LRMS (ESI) calc. [M+H]\textsuperscript{+} for C\textsubscript{40}H\textsubscript{55}N\textsubscript{2}O\textsubscript{8}Si 751.3, found 751.5. See also Notebook II, page 50.

4.4.3. Synthesis of compstatin analogues 4.20, 4.21, and 4.23

Unless noted otherwise, standard cycles for SPPS were performed as follows, using a fritted glass reaction vessel equipped with a N\textsubscript{2} inlet for resin/reagent agitation and a suction outlet for draining. Fmoc deprotection was achieved by agitating resin with 20\% piperidine in DMF (5 mL) for two treatments of 10 min each. After draining the reaction vessel, the resin was washed with DMF (3 x 5 mL x 30 s) and CH\textsubscript{2}Cl\textsubscript{2} (2 x 5 mL x 30 s). The appropriately side-chain protected Fmoc-amino acid (4 equiv.) in DMF (5-10 mL) was pre-activated with HCTU (4 equiv.) and \textsuperscript{3}Pr\textsubscript{2}NEt (8 equiv.) for 5 min, then added to resin and agitated for 45-60 min. After draining the reaction vessel, the resin was washed as before. The completion of all couplings was assessed by a Kaiser test; double couplings were performed as needed. Test cleavages were performed periodically by removing a small portion of dry resin from the reaction vessel and treating with 90:5:5 TFA/H\textsubscript{2}O/triisopropylsilane for 1 h under N\textsubscript{2}. After removing the cleaved resin by filtration, the filtrate was concentrated under a stream of N\textsubscript{2}. The peptide was precipitated with cold Et\textsubscript{2}O, isolated by centrifugation at 12,000 \(\times\)g, and dissolved in 1:1 H\textsubscript{2}O/MeCN. An aliquot of this solution was spotted onto a MALDI-TOF MS target for analysis.

Fmoc-Rink amide AM resin (initial substitution 0.6 mmol/g) was swelled in DMF for 20 min. The Fmoc group was removed, and the first amino acid (Fmoc-Thr(\textsuperscript{t}Bu)-OH for 4.20 and 4.21, Fmoc-N-Me-Ile-OH for 4.23) was pre-activated, coupled, and deprotected by the standard protocol. Resin substitution was then reduced such that 1 equiv. corresponded to 0.2 mmol/g. Cth building blocks 4.7 or 4.13 (1 equiv.) were pre-activated with HCTU (1 equiv.) and \textsuperscript{3}Pr\textsubscript{2}NEt (2 equiv.) in DMF (5 mL) for 5 min, then added to resin and agitated for 3 h. Remaining free resin sites were acetylated with a solution of 1:1:3 Ac\textsubscript{2}O/\textsuperscript{3}Pr\textsubscript{2}NEt/DMF (10 mL) for 30 min. The
resin-bound peptide was Fmoc-deprotected, and the next eight residues were coupled and deprotected by the standard protocol. Fmoc-Val-OH was then coupled by the standard protocol, but not Fmoc-deprotected.

Following the coupling of Fmoc-Val-OH, the allyl protecting groups were removed by agitating resin with tetrakis(triphenylphosphine)palladium(0) (1 equiv.) and phenylsilane (10 equiv.) in 1:1 DMF/CH₂Cl₂ (10 mL) for 2 h, protected from light. After draining the reaction vessel, resin was washed with CH₂Cl₂ (3 x 10 mL x 1 min), 0.5% diethylthiocarbamate in DMF (3 x 10 mL x 1 min), DMF (3 x 5 mL x 30 s), and CH₂Cl₂ (2 x 5 mL x 30 s). The Fmoc group was removed by the standard protocol, followed by washing with DMF (5 x 5 mL x 30 s), CH₂Cl₂ (3 x 5 mL x 30 s), and DMF (2 x 5 mL x 30 s) to remove all traces of piperidine. Cyclization was performed by adding PyBOP (5 equiv.), HOAt (5 equiv.), and iPr₂NEt (10 equiv.) in DMF to the resin and agitating for two treatments of 1.5 h each. Fmoc-Ile-OH was then coupled and deprotected by the standard protocol, and the N-terminus was acetylated with a solution of 1:1:3 Ac₂O/iPr₂NEt/DMF (10 mL) for 30 min. The resin was washed with CH₂Cl₂ and dried. The peptide was cleaved from resin and globally deprotected with 91.5:5:2.5:1 TFA/water/triisopropylsilane/ethanedithiol under N₂ for 2 h. The cleaved resin was removed by filtration, and the filtrate was concentrated under a stream of N₂. The peptide was precipitated with cold Et₂O, isolated by centrifugation at 12,000 xg, dissolved in 1:1 H₂O/MeCN, and lyophilized to dryness. The crude peptide was purified by RP-HPLC employing the conditions described below (see Section 4.1.1). Product-containing fractions were pooled, lyophilized, and analyzed by MALDI-TOF MS. See also Notebook II, pages 51-54, 56; Notebook III, pages 65-66, 70; Notebook V, pages 16-17, 21.

Compstatin analogue 4.20. RP-HPLC: Waters Delta-Pak C18 column and a solvent gradient of 2% solvent B for 1 min, 2-20% over 2 min, 20-26% over 3 min, 26-41% over 30 min, 41-100% over 1 min. Rᵣ = 31.9-32.8 min. Yield: 8 mg pure peptide from a 0.1-mmol scale synthesis (5.0% total yield, 90% per step over 29 steps). LRMS (MALDI-TOF) calc. [M+H]⁺ for C₇₂H₁₀₃N₂₂O₁₈S 1595.7, found 1595.7.

Compstatin analogue 4.21. RP-HPLC: same conditions as 4.20. Rᵣ = 31.8-32.8 min. Yield: 6 mg pure peptide from a 0.1-mmol scale synthesis (3.8% total yield, 89% per step over 29 steps). LRMS (MALDI-TOF) calc. [M+H]⁺ for C₇₂H₁₀₃N₂₂O₁₈S 1595.7, found 1596.0.
Compstatin analogue 4.23. RP-HPLC: Phenomenex Luna C18 column and a solvent gradient of 5% solvent B for 1 min, 5-24% over 2 min, 24-30% over 3 min, 30-45% over 30 min, 45-100% over 1 min. R_t = 26.7-27.8 min. Yield: 12 mg pure peptide from a 0.3-mmol scale synthesis (2.4% total yield, 88% per step over 29 steps). LRMS (MALDI-TOF) calc. [M+H]^+ for C_{77}H_{113}N_{22}O_{17}S 1649.8, found 1649.9.

4.4.4. Chiral GC/MS analysis

The enantiomeric purity of cystathionine building block 4.13 as well as cystathionine derived from hydrolyzed compstatin analogue 4.23 was confirmed by GC/MS after derivatization as the pentafluoropropionamide methyl esters.\textsuperscript{88, 89} Compound 4.13 was prepared for analysis by loading the protected amino acid (23 mg, 0.04 mmol) onto swelled 2-chlorotrityl chloride resin (31 mg, 0.02 mmol) in iPr\textsubscript{2}NEt (28 µL, 0.16 mmol) and 1:1 DMF/CH\textsubscript{2}Cl\textsubscript{2} (2 mL) for 3 h. Allyl protecting groups were removed with a solution of Pd(PPh\textsubscript{3})\textsubscript{4} (23 mg, 0.02 mmol) and PhSiH\textsubscript{3} (21 µL, 0.1 mmol) in 1:1 DMF/CH\textsubscript{2}Cl\textsubscript{2} (3 mL) for 2 h, protected from light. The resin was drained, then washed with CH\textsubscript{2}Cl\textsubscript{2} (3 x 5 mL x 1 min), 0.5% diethyldithiocarbamate in DMF (3 x 5 mL x 1 min), and DMF (3 x 5 mL x 30 s). The Fmoc group was removed with 20% piperidine in DMF (2 mL) for three treatments of 10 min each. The resin-bound amino acid was dried and cleaved from resin with 95:5 TFA/water for 1 h under N\textsubscript{2}. The cleaved resin was removed by filtration, and the filtrate concentrated by a stream of N\textsubscript{2}. The unprotected amino acid was precipitated with cold Et\textsubscript{2}O, isolated by centrifugation, and lyophilized from 1:1 water/acetonitrile. The resulting deprotected cystathionine as well as cystathionine standards were derivatized by dissolving the amino acid (2-5 mg) in 0.2 M HCl (1 mL), sealing in a high-pressure reaction vessel and heating to 100 °C for 10 min. The reaction was cooled and concentrated under reduced pressure. Methanol (3 mL) was chilled in an ice-water bath, and acetyl chloride (1 mL) was added dropwise. This solution was added to the amino acid, sealed in a high-pressure reaction vessel and heated at 100 °C for 45 min. The reaction was allowed to cool and concentrated under reduced pressure, then chilled in an ice-water bath. CH\textsubscript{2}Cl\textsubscript{2} (2.5 mL) and pentafluoropropionic anhydride (1 mL) were added, and the mixture heated at 100 °C for 20 min. The reaction was allowed to cool and concentrated under reduced pressure, then taken up in methanol (200 µL) for analysis.
For compstatin analogue 4.23, lyophilized peptide (0.2-0.4 mg) was dissolved in 6 M HCl (3 mL) and heated at 100 °C in a sealed, high-pressure reaction vessel for 20 hr. The reaction was cooled and concentrated under reduced pressure. Methanol (3 mL) was chilled in an ice-water bath, and acetyl chloride (1 mL) was added dropwise. This solution was added to the hydrolysate, sealed in a high-pressure reaction vessel and heated at 100 °C for 45 min. The reaction was allowed to cool and concentrated under reduced pressure, then chilled in an ice-water bath. CH₂Cl₂ (2.5 mL) and pentafluoropropionic anhydride (1 mL) were added, and the mixture heated at 100 °C for 20 min. The reaction was allowed to cool and concentrated under reduced pressure, then taken up in methanol (50-100 μL) for analysis.

The derivatized samples and standards were analyzed by GC/MS using an Agilent 7890A gas chromatograph equipped with a Varian CP-Chirasil-l-Val fused silica column (25 m x 250 μm x 0.12 μm) fused silica column and an Agilent 5975C Inert XL EI/CI MS (Metabolomics Center, UIUC). Sample solutions in methanol were introduced to the instrument via splitless injection at an inlet temperature of 200 °C and flow rate of 1.5 mL/min helium gas. The temperature gradient used was 160 °C for 5 min, then 160 °C to 200 °C at 3 °C/min, then held at 200 °C for 20 min. The MS was operated in simultaneous scan/single-ion monitoring (SIM) mode, monitoring at a known fragment mass of 379 Da. Cystathionine standards eluted as distinct peaks, with the DL-isomer (19.6-19.8 min) eluting before the LL-isomer (20.0-20.2 min). A small amount of the DL-configuration in the hydrolysate is believed to result from epimerization during hydrolysis, as has been reported previously. See also Notebook IV, pages 97-100; Notebook V, pages 13-14.

4.4.5. Kinetic binding analysis of compstatin analogues

Kinetic analysis via surface plasmon resonance (SPR) of the binding interaction between compstatin analogues and C3b was performed by Dr. Apostolia Tzekou in the laboratory of Prof. John Lambris (UPSM). Briefly, C3b was generated by limited trypsinization of complement factor 3 and labeled with biotin-maleimide using established protocols. Biotinylated C3b was captured site-specifically on a streptavidin chip at approximate densities of 3000, 4000, and 5000 resonance units. Interaction between the immobilized C3b and compstatin analogues were characterized using a GE Healthcare Biacore 3000 instrument in phosphate-buffered saline (10 mM sodium phosphate, 150 mM NaCl, pH 7.4) containing 0.005% Tween-20. An initial kinetic
ranking was performed by injecting a constant peptide concentration of 1 μM over the chip surface for 2 min. Detailed kinetic parameters were obtained via a kinetic titration,\textsuperscript{71} consecutive injections of a set of five increasing peptide concentrations over the chip surface were performed in a single cycle without waiting for full dissociation between injections. A three-fold dilution series (two sets at 0.46-37 nM and 111-9000 nM for \textbf{4.1}, \textbf{4.20}, and \textbf{4.21}, one set at 0.46-37 nM for \textbf{4.22} and \textbf{4.23}) was injected at a flow rate of 30 μL/min; each injection was done for 2 min, and the peptide was allowed to dissociate for 5 min before the next injection was started. After the end of the last injection, a dissociation time of 10 min was used for \textbf{4.1}, \textbf{4.20}, and \textbf{4.21}, and 15 min for \textbf{4.22} and \textbf{4.23}. A series of blank injections was subtracted from the binding signals, and the processed signals were fitted to a single cycle 1:1 binding model using BIAevaluation to extract the kinetic rate constants ($k_a$, $k_d$) and calculate the binding affinity ($K_D = k_d/k_a$).

\textbf{4.4.6. Complement inhibition assays}

Assessment of the complement inhibitory ability of compstatin analogues was performed by Dr. Tzekou, using an established enzyme-linked immunosorbent assay (ELISA) that initiates the classical pathway of complement activation via an ovalbumin-based antigen/antibody complex.\textsuperscript{61} Briefly, ovalbumin was immobilized on the ELISA well surface, which was then treated with normal human plasma containing serial dilutions of compstatin analogues in veronal-buffered saline. Anti-ovalbumin antibody was used to initiate the complement pathway, and after a 15 min incubation, the deposition of C3b on the plate was measured with a polyclonal anti-C3 antibody. The percentage of inhibition was plotted against compstatin analogue concentration and fitted to a logistic dose-response function.

\textbf{4.4.7. Effect of reduction and oxidation on compstatin analogue activity}

The effect of treating compstatin analogues with reducing and oxidizing agents on biological activity was performed by Dr. Tzekou. Briefly, for reductive treatment, peptides \textbf{4.22} and \textbf{4.23} were dissolved in 0.1% aqueous TFA to a concentration of 50 μM. An aqueous solution of tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was added to give a final concentration of 25 mM. Samples were incubated for 1.5 h at room temperature, and reduction was monitored by MALDI-TOF MS. To prevent re-formation of the disulfide bond during further characterization, the TCEP-treated solution of \textbf{4.22} was concentrated by lyophilization,
dissolved in a 1:1 mixture of 100 mM aqueous ammonium bicarbonate (pH 7.8) and 0.1% TFA in acetonitrile in the presence of a 10-fold molar excess of TCEP, and treated with a 20-fold molar excess of iodoacetamide. A mass shift of +114 was observed by MALDI-TOF MS, corresponding to alkylation of both cysteine residues. Peptides were then purified by RP-HPLC.

For oxidative treatment, in order to provide the most general evaluation of the effect of sulfoxide formation on activity, a non-stereoselective sulfur oxidation method was used. Aqueous solutions of 4.22 and 4.23 (20 μM) were brought to slightly basic pH with ammonium hydroxide. Hydrogen peroxide was then added to a final concentration of 0.4% by volume, and oxidation was monitored by MALDI-TOF MS. After several hours of incubation at room temperature, 4.23 displayed a complete mass shift of +16, indicative of sulfoxide formation. Reactions were then concentrated by lyophilization. The reduced/alkylated and oxidized derivatives of 4.22 and 4.23 were tested for their complement inhibitory activity as described in Section 4.4.6.

4.4.8. Synthesis of albumin affinity tag 4.27

Compound 4.24. Diphenylacetaldehyde (3.58 mL, 20.0 mmol) and 3-buten-2-one (2.46 mL, 30.0 mmol) were dissolved in anhydrous ethanol (15 mL) and chilled to 0 °C in an ice bath. Potassium hydroxide pellets (0.56 g, 10.0 mmol) were dissolved in anhydrous ethanol (10 mL) in a separate flask and chilled to 0 °C. The cold hydroxide solution was slowly added to the aldehyde solution over 10 min, and the reaction was stirred at 0 °C for 3 h, over which time a precipitate formed. The reaction was concentrated under reduced pressure, and the resulting residue was partitioned between H₂O and Et₂O. The mixture was acidified to pH 3 with 2 M aqueous HCl, and the layers were separated. The organic fraction was dried over Na₂SO₄,
filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO\(_2\), 9:1 hexanes/EtOAc) to yield 4.24 (3.47 g, 14.0 mmol, 70%) as a white solid. \( R_f \) 0.52 (4:1 hexane/EtOAc). Spectral data matched those reported previously.\(^{91}\) See also Notebook X, page 5.

Compound 4.25. To a solution of 4.24 (3.40 g, 13.7 mmol) in THF (30 mL) was added 10% palladium on carbon (140 mg). The reaction vessel was flushed with hydrogen gas, and the system was sealed under a hydrogen atmosphere (1 atm) and stirred vigorously for 20 h. The catalyst was removed by filtration through Celite and washed with THF (15 mL). The combined filtrate was chilled to 0 °C in an ice bath. Sodium borohydride (0.26 g, 6.85 mmol) was dissolved in 0.1 M aqueous NaOH (7 mL) in a separate flask and chilled to 0 °C, then added dropwise to the organic solution. The system was warmed to room temperature and stirred for 2 h. The reaction was then chilled to 0 °C, quenched with 2 M aqueous HCl (12 mL) and diluted with cold H\(_2\)O (100 mL). The system was stirred at 0 °C for 30 min, over which time a white precipitate formed. The precipitate was isolated by filtration, washed with cold H\(_2\)O, and dried under reduced pressure to yield 4.25 (3.32 g, 13.2 mmol, 96% over two steps) as a white solid. \( R_f \) 0.26 (4:1 hexanes/EtOAc). Spectral data matched those reported previously.\(^{92}\) See also Notebook X, page 6.

Compound 4.26. To a solution of ethyl 6-hydroxyhexanoate (2.44 mL, 15.0 mmol) and \(^{t}\)Pr\(_2\)NEt (10.4 mL, 60.0 mmol) in CH\(_2\)Cl\(_2\) (60 mL) was added dropwise 2-cyanoethyl-\(N,N\)-diisopropylchlorophosphoramidite (3.51 mL, 15.8 mmol). The reaction was stirred for 2 h, then washed with ice-cold saturated aqueous NaHCO\(_3\), dried over Na\(_2\)SO\(_4\), filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO\(_2\), 9:1 hexane/EtOAc with 0.1% Et\(_3\)N) to yield 4.26 (4.20 g, 11.7 mmol, 78%) as a colorless liquid. \( R_f \) 0.80 (2:1 hexane/EtOAc). Spectral data matched those reported previously.\(^{93}\) See also Notebook X, page 10.
Compound 4.27. Compounds 4.26 (2.90 g, 8.05 mmol) and 4.25 (2.13 g, 8.45 mmol) were combined in acetonitrile (30 mL). A 0.45 M solution of 1H-tetrazole in acetonitrile (18.8 mL, 8.45 mmol) was added, and the reaction was stirred under N₂ for 3 h. A 70% w/w aqueous solution of tert-butylhydroperoxide (8 mL) was added, and the reaction stirred for an additional 1 h, then concentrated under reduced pressure. The resulting residue was taken up in EtOAc, washed with 10% Na₂S₂O₃, saturated aqueous NaHCO₃, and brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO₂, 1:1 hexanes/EtOAc with 0.1% Et₃N) to yield a colorless oil (Rₐf 0.13, 1:1 hexanes/EtOAc). This oil was taken up in 2 M ammonia in methanol (80 mL) and stirred under N₂ for 12 h to remove the cyanoethyl substituent. The reaction was concentrated under reduced pressure, and the resulting oil was taken up in THF (10 mL) and chilled to 0 °C in an ice bath. A 2 M aqueous solution of lithium hydroxide (5 mL) was added, and the reaction was warmed to room temperature and stirred for 2 h to hydrolyze the ethyl ester. The reaction was concentrated under reduced pressure and taken up in water (100 mL). The solution was acidified to pH 2 with 2 M aqueous HCl, forming a white precipitate that was extracted with EtOAc (3 x 50 mL). The organic fractions were combined, dried over Na₂SO₄, filtered, and concentrated under reduced pressure to yield 4.27 (1.94 g, 4.35 mmol, 54% over three steps) as an off-white solid after lyophilization from 1:1 benzene/acetonitrile. Rₐf 0.45 (4:1:1 BuOH/H₂O/AcOH). ¹H NMR (500 MHz, CD₃OD) δ 7.32-7.20 (m, 8H), 7.10 (q, J = 7.5 Hz, 2H), 4.33 (m, 1H), 3.89 (q, J = 6.5 Hz, 2H), 2.60 (m, 2H), 2.28 (t, J = 7.5 Hz, 2H), 2.19 (m, 2H), 1.89 (m, 2H), 1.74 (m, 2H), 1.66-1.58 (m, 4H), 1.45-1.39 (m, 2H). ¹³C NMR (125 MHz, CD₃OD) δ 177.5, 149.2, 148.6, 129.3, 129.1, 128.2, 127.9, 126.6, 75.2, 66.8, 46.6, 34.9, 34.0, 31.3, 30.8, 26.4, 25.7. ³¹P NMR (200 MHz, CD₃OD) δ 0.64. HRMS (ESI) calc. [M+H]⁺ for C₂₄H₃₂O₆P 447.1937, found 447.1934. See also Notebook X, pages 11-13.

4.5. REFERENCES


