University of Alberta

Synthesis and Biological Evaluation of the Lantibiotic Peptide Lactocin S and its Analogues

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Chemistry

Spring, 2012
Edmonton, Alberta

Dedication

For my parents

Abstract

Lantibiotics (lanthionine containing antibiotics) are highly post-translationally modified peptides used by bacteria as a form of chemical warfare. In light of the challenges faced today in the field of antibacterial therapy, these potent natural products are useful lead compounds for the development of future antibiotics.

Lactocin S (**98**) is a 37 amino acid lantibiotic peptide isolated from *Lactobacillus sakei* L45. Due to low levels of production by the natural producer organism, complete structural identification has been challenging. The total chemical synthesis of **98** using a combined solution and solid phase chemistry approach is described. The unusual amino acid lanthionine was synthesized in solution with orthogonal protecting groups (**58**) so that it could be cyclized on-resin during the solid phase synthesis of the peptide. An *N*-terminal α-ketoamide precursor was also made in solution. Following coupling to the rest of the peptide on resin, global deprotection of the peptide resulted in the formation of the α-ketoamide containing lactocin S (**98**). A simplified purification of lactocin S (**98**) from *Lactobacillus sakei* L45 was developed. Comparison of the natural and synthetic peptides via activity testing, mass spectrometry and chiral GC/MS analysis confirmed the proposed structure of lactocin S (**98**).

Unfortunately, lactocin S suffers from pH and oxidative instability. This thesis describes the synthesis of analogues developed with a goal of improving stability as well as activity. The lanthionine rings were systematically replaced with diaminopimelate, which contains a methylene carbon in place of the

thioether of lanthionine. Orthogonally protected diaminopimelate (**137**) was synthesized via a key photolysis reaction of diacylperoxide **170** to form the backbone. The solid phase synthesis of the analogues was then accomplished. One of these analogues, A-DAP lactocin S (**151**), exhibited improved activity compared to the natural peptide (**98**). A second site of modification was methionine 12. Leucine (**181**) and norleucine (**182**) analogues were made that exhibit full retention of activity. A final analogue (**183**) was synthesized using an oxazole ring to mimic the dehydrobutyrine-α-ketoamide functionality at the *N*-terminus of lactocin S (**98**). This compound displayed no biological activity. The methodology described, provides access to potentially stable and active analogues of lantibiotics.

Acknowledgements

Thank you to my supervisor, John Vederas, for your insightful advice and the many opportunities you have provided me. I really appreciate the infectious enthusiasm you have for science and I am grateful for the wonderful research group you have assembled. It has been a privilege and a pleasure to work with everyone in the Vederas group, both past and present. I have learned so much from my labmates and had a lot of fun along the way.

Thanks to Dr. Dave Dietrich and Dr. Jenn Chaytor for your help proofreading my thesis and for your many great suggestions for its improvement. I am very glad to have had the chance to work with you both. My thanks to: Larissa Petriw (summer 2009 and 2010), Mike Little (summer 2011), Taylor Seal (Wisest, summer 2009) and Jessica Li (Wisest, 2010) for all their hard work and enthusiasm. Throughout my PhD. many people have given me guidance. I am especially grateful to Dr. Matt Clay, Dr. Darren Derksen, Dr. Patrick Liu, Dr. Leah Martin-Visscher, and Dr. Vijay Pattabiraman for all their suggestions and encouragement.

The Chemistry Department has excellent support from all its service labs, I am especially fortunate to have been assisted by each of the wonderful staff of the Mass Spectrometry Facility. Thanks to Dr. Randy Whittal, Dr. Angie Morales-Izquierdo, Don Morgan, Jing Zheng, Bela Reiz and Lisa Nikolai.

I gratefully acknowledge the Natural Sciences and Engineering Research Council of Canada, the Advanced Food and Materials Network and the University of Alberta for financial support.

My heartfelt thanks to all my family and friends, who have made my life so rich and happy. To my best friend Matt, you are a source of such joy and happiness in my life. Thank you for sharing this adventure with me and I look forward to many more to come.

To Mum and Dad, thank you for everything. Thank you for a lifetime of looking at the world and wondering how it works. Your constant love and encouragement has helped me keep going when I didn't think I could. I am so lucky to have two fantastic friends that also happen to share half their DNA with me!

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List of Abbreviations

$[\alpha]_D^{26}$	specific rotation at 26 °C
ABC	ATP-binding cassette
Abu	aminobutyrine
Acm	acetamidomethyl
Ala	alanine
Alg	allylglycine
Alloc	allyloxycarbonyl
APT	all purpose tween
aq.	aqueous
Ar	aromatic
Asn	asparagine
Asp	aspartic acid
ATP	adenosine triphosphate
BHT	butylated hydroxytoluene
Boc	*tert*-butyloxycarbonyl
Bn	benzyl
br	broad
Bu	butyl
c	concentration
Cbz	carboxybenzyl
CHCA	α-cyano-4-hydroxy cinnamic acid
C-terminal	carboxy terminal

Cy	cyclohexyl
Cys	cysteine
δ	chemical shift
d	day
d	doublet
DAB	2,4-diaminobutyric acid
DAP	diaminopimelic acid
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCC	1,3-dicyclohexylcarbodiimide
DCE	1,2-dichloroethane
deg	degree
Dha	dehydroalanine
Dhb	dehydrobutyrine
DIBAL	diisobutylaluminum hydride
DIC	diisopropylcarbodiimide
DIPEA	diisopropylethylamine
DMAP	4-methylaminopyridine
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNs	2,4-dinitrobenzenesulfonyl
EDC	1-ethyl-3-(3’-dimethylaminopropyl)carbodiimide
equiv	equivalents
ES	electrospray

Et	ethyl
Et_2O	diethylether
Et_3N	triethylamine
EtOAc	ethylacetate
FDA	Food and Drug Administration
Fmoc	9*H*-fluorenylmethyloxycarbonyl
Fmoc-OSu	9*H*-fluorenylmethylsuccinimidyl carbonate
FT	Fourier transform
FTICR	Fourier transform ion cyclotron resonance
GC/MS	gas chromatography mass spectrometry
Gly	glycine
Glu	glutamic acid
GRAS	generally recognized as safe
His	histidine
HOBt	1-hydroxybenzotriazole
HRMS	high resolution mass spectrometry
HPLC	high performance liquid chromatography
hν	light
Ile	isoleucine
IR	infrared
IPA	isopropyl alcohol
J	coupling constant
LAB	lactic acid bacteria

Leu	leucine
Lys	lysine
m	multiplet
MALDI-TOF	matrix assisted laser desorption ionization time of flight
Mbh	4,4’-dimethoxybenzhydryl
Me	methyl
MeCys	methylcysteine
Met	methionine
MeOH	methanol
Mes	mesityl
MHz	megahertz
min	minute
mol	mole
mmol	millimole
MRS	de Man Rogosa and Sharpe
MRSA	methicillin resistant *Staphylococcus aureus*
MS	mass spectrometry
MsCl	methanesulfonyl chloride
MS/MS	tandem mass spectrometry
MW	molecular weight
n-BuLi	*n*-butyllithium
N-terminal	amino terminal
NMM	*N*-methylmorpholine

NMP	*N*-methylpyrrolidone
NMR	nuclear magnetic resonance
NRPS	non-ribosomal peptide synthase
PCR	polymerase chain reaction
PDC	pyridinium dichromate
Ph	phenyl
Phe	phenylalanine
Pmc	2,2,5,7,8-pentamethyl chroman-6-yl sulfonyl
*p*NB	*para*-nitrobenzyl
*p*NZ	*para*-nitrobenzyloxycarbonyl
ppm	part per million
Pro	proline
p-TsOH	*para*-toluene sulfonic acid
PyBOP	benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
q	quartet
res	residue
R_f	retention factor
RP	reversed phase
rpm	revolutions per minute
rt	room temperature
s	singlet
SAR	structure activity relationship

Ser	serine
SIM	selective ion monitoring
S_N2	bimolecular nucleophilic substitution
SPPS	solid phase peptide synthesis
t	triplet
T	tesla
TBA-Br	tetra-*n*-butylammonium bromide
TBAF	tetra-*n*-butylammonium fluoride
TBDMS	*tert*-butyldimethylsilyl
TCEP	tris(2-carboxyethyl)phosphine
TLC	thin layer chromatography
tBu	*tertiary*-butyl
tert-butyl	*tertiary*-butyl
TFA	trifluoroacetic acid
THF	tetrahydrofuran
Thr	threonine
TIPS	triisopropylsilane
Trt or trityl	triphenylmethyl
Ts	*para*-toluenesulfonyl
UV-Vis	ultra violet visible
Val	valine
vol/vol	volume/volume
wt/vol	weight/volume

Tyr	tyrosine

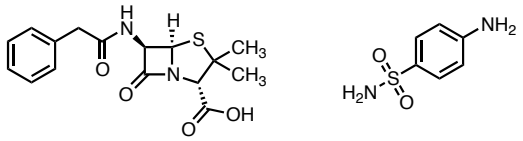
Chapter 1: Introduction

1.1 Importance of Antibiotics

In the last century, antibacterial drug therapy has changed the western world. Average life expectancy in the United States increased from 49 to 77 years between 1900-2005.[1] In 1900, 18.2% of children died before their 5th birthday, but by the year 2005 this number was down to lower than 1%.[1] Antibiotics are not the only reason for these improvements. Multiple factors have contributed to our increased longevity; improved nutrition, better hygiene practices and widespread access to vaccines were all highly influential.[2]

1.1.1 Early antibiotic discovery

The 1920s and 1930s were the dawn of clinically relevant antibiotics, with the discovery of the sulfonamides[3] and the penicillins.[4]



penicillin G (**1**) sulfanilamide (**2**)

Figure 1-1 The early classes of antibiotics, penicillins and sulfonamides.

The famous findings of Alexander Fleming in 1928, that *Penicillium notatum* produced a compound that inhibited the growth of a *Staphylococcus*

strain,[5] were a major driving force for further investigations into antibiotics development. In the decades that followed, natural sources were extensively investigated for their ability to produce useful and varied antibacterial compounds.

1.1.2 The golden age of antibiotic discovery

The middle of the twentieth century saw an explosion of interest and research into the discovery of new antibiotics. Referred to as the "golden age" of human antibacterial discovery (1940s to early 1960s), this era yielded most of the different classes of antibiotics in use today (Table 1-1 and Figure 1-2).[6]

Table 1-1 New classes of antibiotics discovered during the golden age

Class	Source (Natural or Synthetic)	Example
β-lactams[7]	Natural	cephalothin (**3**)[8]
aminoglycosides	Natural	streptomycin (**4**)[9]
rifamycins	Natural	rifampicin (**5**)[10]
glycopeptides	Natural	vancomycin (**6**)[11]
lincosamides	Natural	clindamycin (**7**)[12]
tetracyclines	Natural	tetracycline (**8**)[13]
polypeptides	Natural	polymyxin B1 (**9**)[14]
macrolides	Natural	erythromycin (**10**)[15]
quinolones	Synthetic	ciprofloxacin (**11**)[16]

The new classes of antibiotics discovered throughout this period were almost exclusively from natural sources. The examples given may not themselves be natural products, but they are directly derived from molecules found in nature. The quinolones (e.g. ciprofloxacin (**11**)[16]) are the only major class of unnatural antibiotics that were discovered during this period.

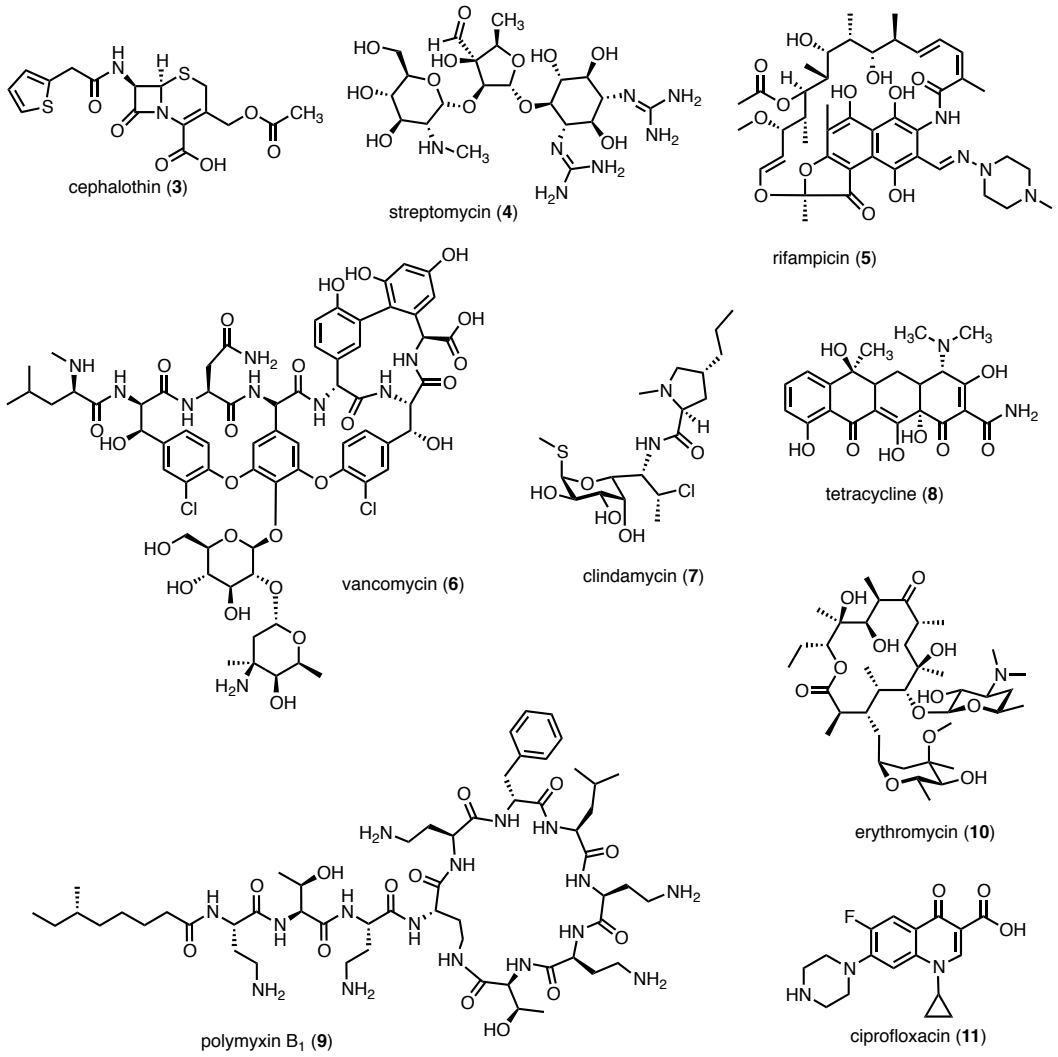


Figure 1-2 Examples of antibiotic classes discovered during the golden age

Inspired by the new scaffolds seen in nature, medicinal chemists began modifying the functionalities of these antibiotic compounds. The result was a huge library of molecules that have very different structures and act in a myriad of ways to kill bacteria.

1.1.3 Mode of action of antibiotics

Figure 1-3 shows many of the cellular targets for antibiotics and some of the compound classes that act on them. For instance, most of the β-lactam-containing antibiotics interfere with the synthesis of the bacterial cell-wall constituent peptidoglycan, by inhibiting important biosynthetic enzymes called D,D-transpeptidases.[17] In contrast the well-known macrolide erythromycin (**10**), disrupts the production of bacterial proteins by interacting with the 50S subunit of the ribosome.[18] The polypeptide called polymyxin B_1 (**9**) acts on the bacterial cell membrane of Gram-negative bacteria, causing increased permeability.[17]

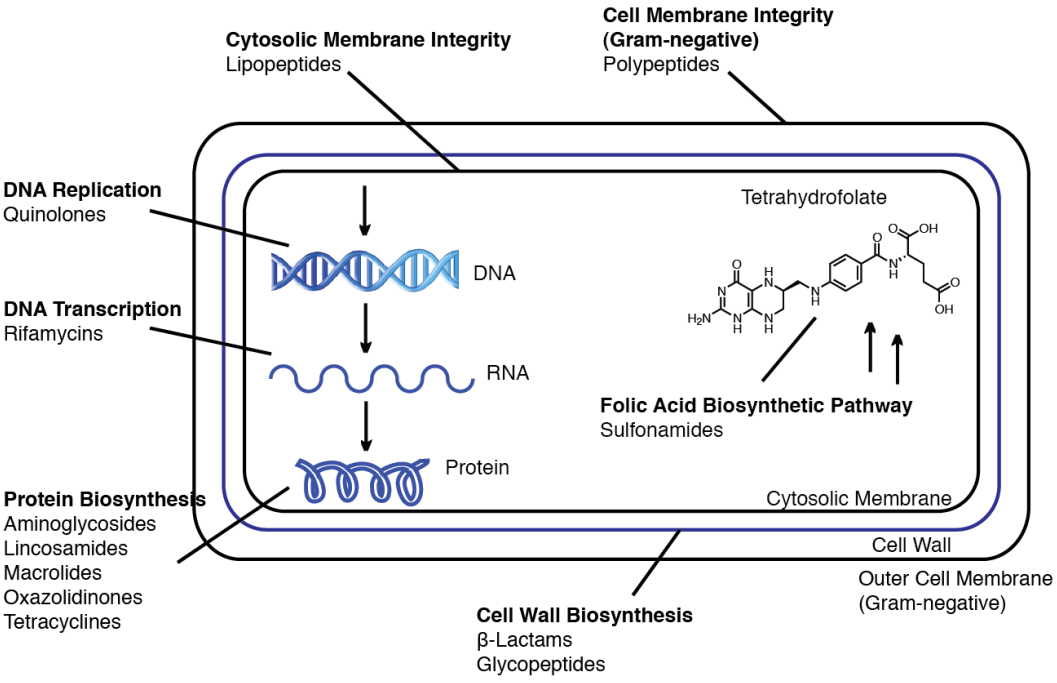


Figure 1-3 Representation of a bacterial cell showing targets for common antibiotics

1.1.4 Bacterial resistance to antibiotics

Following the discovery of many highly effective antibiotics, it was widely touted that bacterially caused infectious diseases would become a problem of the past. In 1967, U.S. Surgeon General William H. Stewart reportedly stated "…. that we had essentially defeated infectious diseases and could close the book on them ……".[19] Sadly, this was not the case.

Within a few years of clinical use, bacterial resistance to antibiotics started to develop and some organisms have actually become resistant to entire classes of drugs.[20,21] Additionally, multi-drug resistant bacteria have developed. Methicillin-resistant *Staphylococcus aureus* (MRSA) is one such organism. By 2003, more than half of the *S. aureus* infections detected in hospitals were methicillin-resistant.[22] MRSA is now treated with the powerful glycopeptide vancomycin (**6**), which is sometimes referred to as a "drug of last resort".[23] However, even vancomycin-resistant MRSA is now being observed, albeit infrequently.[22]

There are as many resistance mechanisms as there are modes of action. Alteration of the cellular target may occur. When the terminal D-Ala-D-Ala motif of peptidoglycan is mutated to D-Ala-D-lactate, this results in decreased binding by vancomycin (**6**).[24] Thickening of the peptidoglycan layer of the bacterial cell wall can also confer vancomycin (**6**) resistance.[22] Enzymatic degradation of the antibacterial compound is another common method. β-lactam containing antibiotics can be hydrolyzed at the central 4-membered ring by β-lactamases.[18] Expulsion of the antibiotic from the cell via efflux pumps is prevalent, for

example tetracycline (**8**) is removed from resistant cells before it can inhibit protein biosynthesis.[6]

Usually there is a small fraction of organisms that are innately resistant to any antibiotic. However, the way we utilize antibiotics makes a huge difference to how quickly these resistant organisms become dominant.[21] When antibiotic treatment of an infection is incomplete (frequently due to patient non-compliance), an optimal environment for the selection of resistant strains is generated.[21] A similar result arises from the unnecessary use of antibiotics in response to viral infections.[25] Furthermore, very large amounts of antibiotics are incorporated into the feed of farmed animals as a preventative measure against bacterial infection.[21]

It is becoming increasingly evident that the treatment of bacterial infections will be an ongoing battle, one in which the target is constantly changing and thus treatments must adapt. Unfortunately, in the last five decades, very few new antibiotic classes have been discovered (Figure 1-4).[6]

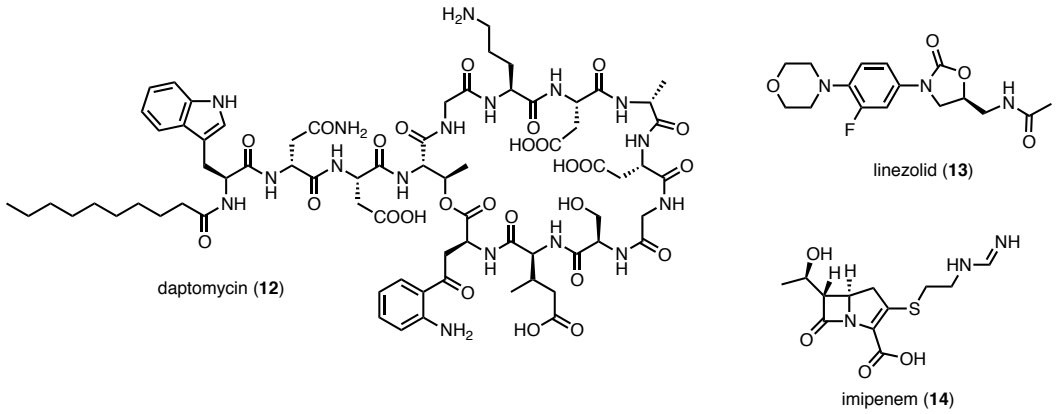


Figure 1-4 Representative examples of recently discovered classes of antibiotics: lipopeptides (daptomycin),[26] carbapenems (imipenem),[27] and oxazolidinones (linezolid).[28]

1.1.5 Challenges in antibiotics development

There are several contributing factors to the large decline in antibiotic discovery in recent decades. Pharmaceutical companies have low incentives to work on antibiotic development. By comparison to anti-depressants or cholesterol lowering drugs for example, antibiotics are used for a short duration and (theoretically) result in a cure. This makes them financially less appealing to companies, despite the considerable need for new compounds.[29,30] The second challenge is that the "easy" natural product antibiotics have already been discovered. Actinobacteria isolated from soil samples are relatively easy to culture and have yielded many of the currently known antibiotic scaffolds. However on average ~1 in 100 of these organisms produce streptomycin (**4**) and so this creates a background bioactivity that must now be accounted for when screening actinobacteria for novel compounds.[29] Daptomycin (**12**), for instance,

occurs in ~1 in 10,000,000 actinobacteria, thus using standard methods one could rediscover streptomycin (**4**) ~100,000 times before finding daptomycin (**12**) again. This type of scaffold rediscovery means that simple small scale screening programs can be very ineffective.

What are the alternatives for antibiotic discovery? Organisms from under-explored environments, such as marine-dwelling bacteria[31] and symbiotic bacteria[32,33] have already yielded promising leads. The use of different techniques such as bioinformatics can be used to identify compounds.[23] Genome mining has also identified promising leads.[34] Previously discovered compounds that haven't been thoroughly investigated provide another source of inspiration.[35]

There are several well-known non-ribosomally produced peptide antibiotics (vancomycin (**6**), polymyxin B1 (**8**) and daptomycin (**12**)). These compounds have found varied uses and are structurally diverse. However, one area that remains underdeveloped for human therapeutics is ribosomally produced antibacterial peptides. Such peptides from bacteria are called bacteriocins and are the focus of this dissertation.

1.2 Bacteriocins From Lactic Acid Bacteria

Bacteriocins are potent ribosomally synthesized antimicrobial peptides, which are made by bacteria and are capable of killing other bacteria.[36] In essence, bacteriocins are a form of chemical warfare.[37] These molecules often act on close relatives of the producing organism but have no toxicity toward the producer.

This phenomenon is called immunity and there are several mechanisms by which bacteria protect themselves.[38,39] As with resistance mechanisms for conventional antibiotics, some bacteria can mitigate the action of their bacteriocins by pumping them out of the cell via an ATP-binding cassette (ABC) transporter.[38] Alternatively, many bacteria produce specific immunity proteins, which physically interact with the bacteriocins and stop them binding to their targets on the producing cell.[38] It is believed that virtually all bacteria are capable of producing at least one bacteriocin.[36] Given the many interesting compounds already found, this suggests that a vast number of potentially valuable peptides remain to be discovered.

The first bacteriocin identified was colicin V from *Escherichia coli* in 1925.[39-41] It is an example of a bacteriocin from a Gram-negative bacterium. Gram-positive bacteria also produce bacteriocins, and the peptides produced by lactic acid bacteria (LAB) are particularly interesting. Many of these organisms are generally non-toxic toward humans and so along with their bacteriocins, some have been classified as GRAS (generally recognized as safe) by the American Food and Drug Administration (FDA).[39] LAB bacteriocins are synthesized by the ribosome and then may undergo differing amounts of post-translational modification.

1.2.1 Classification

The classification system for these bacteriocins is continually being refined.[36,39,42] A currently accepted system proposed by Nes and co-workers is outlined in Table 1-2.[37]

Table 1-2 A recent classification system for LAB bacteriocins[37]

Classes	Sub-Types	Example
Class I Lantibiotics Post-translationally modified peptides containing lanthionine (**17**) and methyllanthionine (**18**)	**Type A** Elongated and cationic	nisin A (**16a**)[43]
	Type B Globular and neutral/basic	mersacidin[44]
Class II Non-Lantibiotics Heat stable peptides that are not post-translationally modified	**Type IIa** pediocin-like	pediocin PA-1 (**15**)[45]
	Type IIb two-component	lactococcin G[46]
	Type IIc miscellaneous	lactococcin A[47]
	Type IId leaderless	enterocin I/L50A and B[48,49]
	Type IIe fragments of larger peptides	propionicin F[50]
Class III Heat Labile Large unmodified heat labile peptides	None	helveticin J[51]
Class IV Circular Peptides post-translationally modified by *N* to *C* terminal cyclization	None	carnocyclin[52]

LAB bacteriocins have seen application in several fields, but thus far their primary use has been in food preservation. These potent antibacterial compounds

can inhibit the growth of food spoilage pathogens. For instance, the type IIa peptide pediocin PA-1 (**15**) is strongly active against *Listeria monocytogenes* and is used to treat meat before packaging.[53] Pediocin PA-1 (**15**) is applied as an extract obtained from fermentation of its producing organism *Pediococcus acidilactici*.[36]

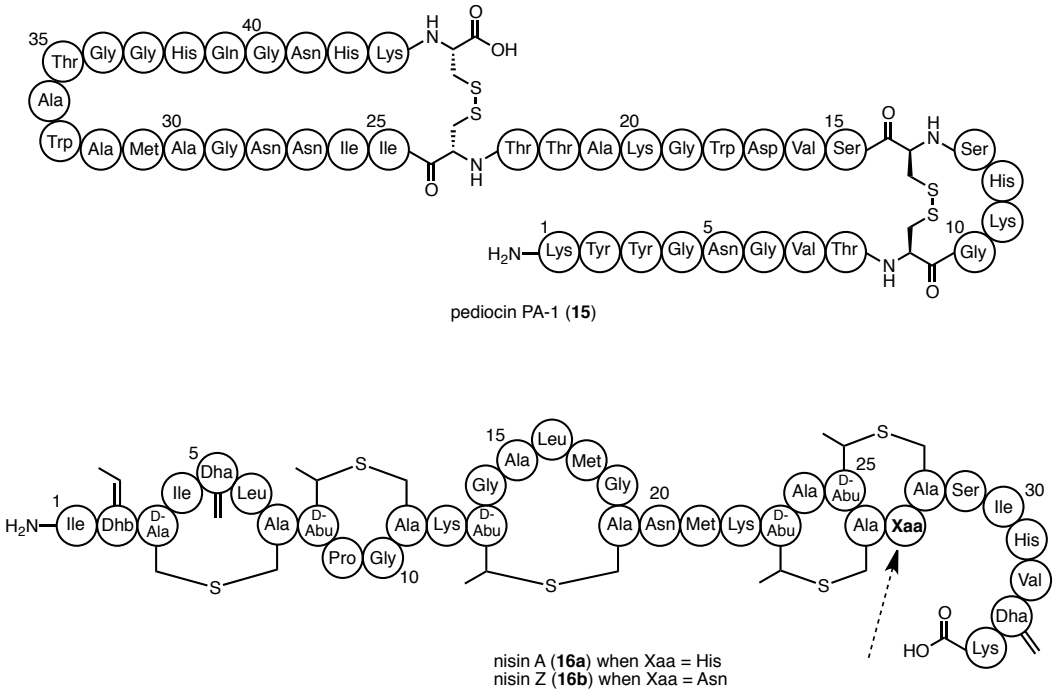


Figure 1-5 Structures of the representative class I and II bacteriocins. Pediocin PA-1 (**15**) and nisin A (**16a**) / nisin Z (**16b**).

Nisin A (**16a**), often called the prototypical lantibiotic (Figure 1-5), has been used extensively in the preservation of food, in particular dairy products. Licensed by the FDA of the United States of America and in over 40 other countries, nisin A (**16a**) has potent activity against many Gram-positive organisms and shows little to no negative interactions with eukaryotic cells.[54] As

mentioned above, nisin A (**16a**) is a lantibiotic peptide (Class I) and these compounds will be discussed in more detail below.

1.3 Lantibiotics

Lantibiotics (lanthionine-containing antibiotics) are highly post-translationally modified bacteriocins that are made by lactic acid bacteria. The most famous example is the aforementioned nisin A (**16a**), which was first identified over 80 years ago.[55,56] Almost all members of the family have potent antimicrobial activity and some target very clinically important organisms such as MRSA.[57] This class of bacteriocins take their name from one of the modified amino acids they typically possess.[58]

1.3.1 Common post-translational modifications in lantibiotics

The lantibiotics contain many unusual amino acids (Figure 1-6), which result from enzymatic processing of a ribosomally produced precursor peptide.[59]

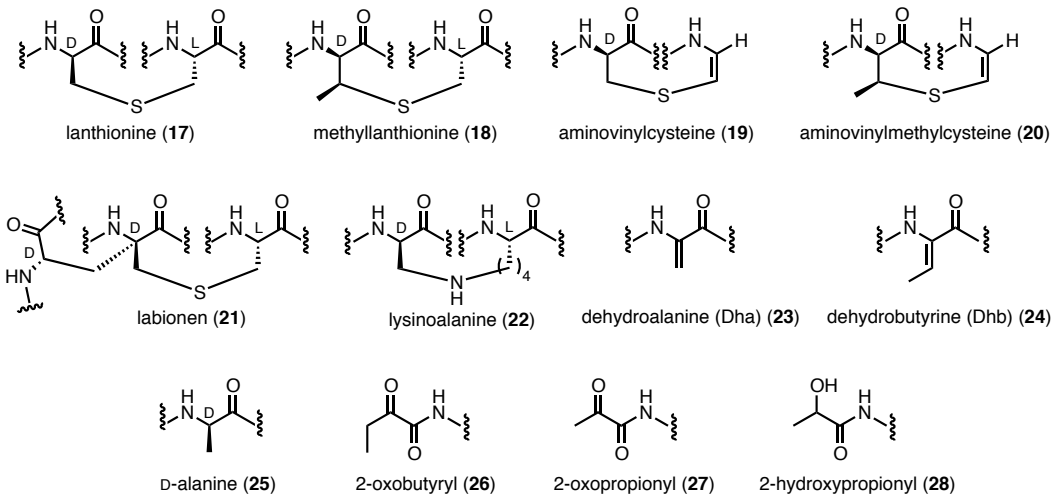


Figure 1-6 Unusual structural features in lantibiotic peptides

These amino acids have the effect of changing not only the primary structure of the peptides, but also the overall three-dimensional shape. Residues **17-22** in Figure 1-6 are all made from the cyclization of two or three amino acid side chains. This restricts the possible conformations of the resulting peptide.

1.3.2 Biosynthesis

Lantibiotics are gene-encoded peptides that undergo considerable post-translational modification by a suite of dedicated enzymes. The genetic information for each peptide and its modification enzymes is clustered together along with genes for immunity, cleavage, transport and regulation (Figure 1-7).[58]

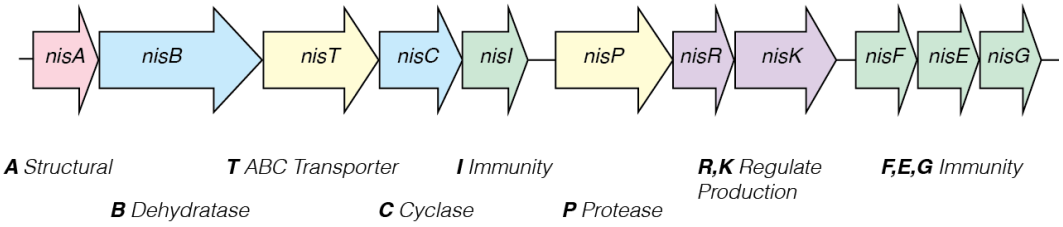
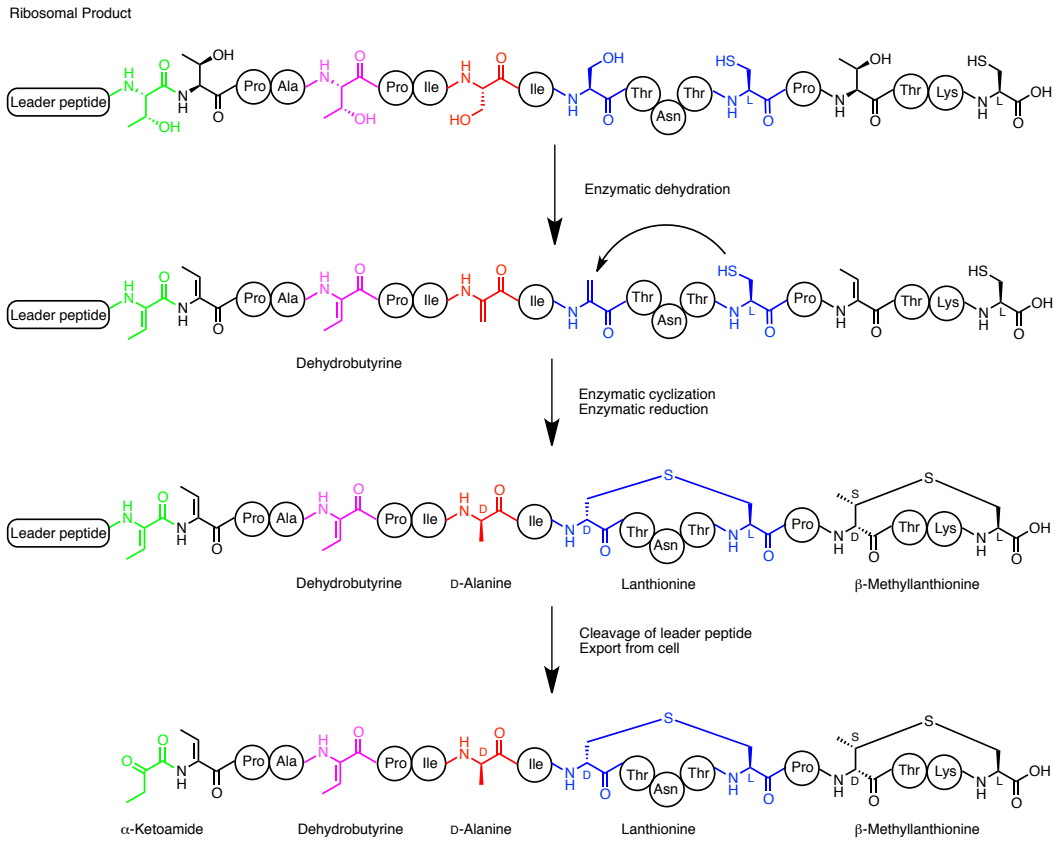


Figure 1-7 Biosynthetic gene cluster for nisin A (**16a**)

Lantibiotic peptides are synthesized by the ribosome as a linear precursor peptide with an *N*-terminal leader sequence. This linear peptide then undergoes multiple transformations to introduce unusual amino acid functionalities. Finally, the leader sequence is cleaved from the now mature peptide, and the peptide is exported from the producing cell into the surrounding environment. The common post-translational modification steps are shown in Scheme 1-1.



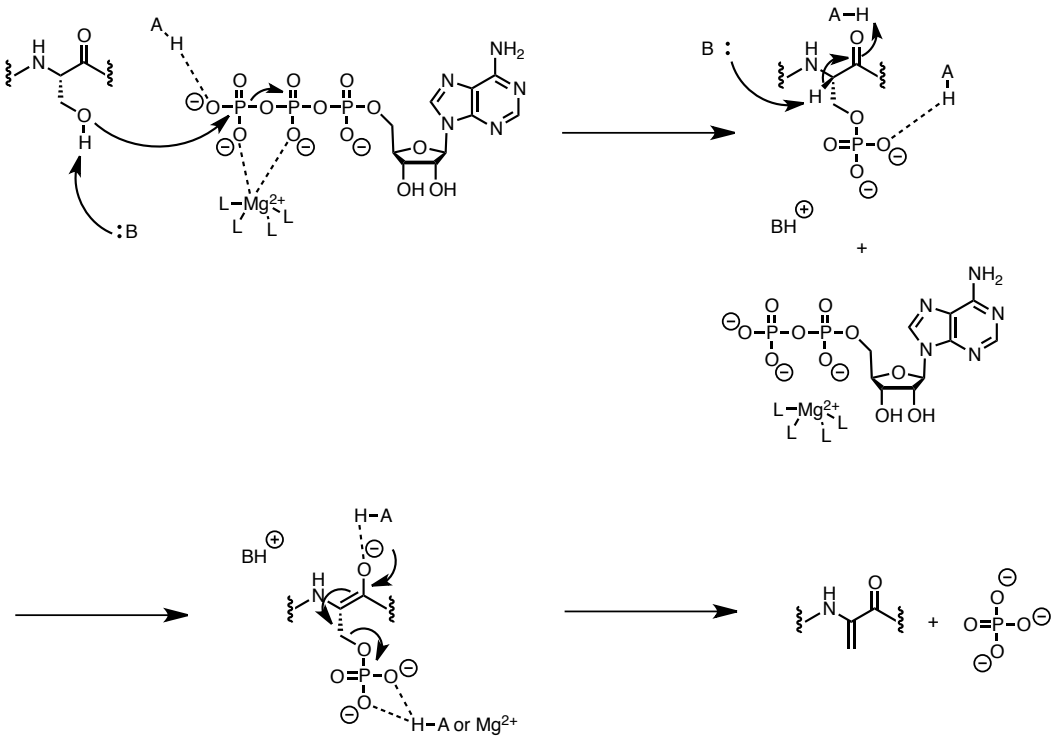
Scheme 1-1 Overview of the biosynthesis of lantibiotics

After ribosomal synthesis, serine and threonine residues within the peptide can be dehydrated to give dehydroalanine (**23**) and dehydrobutyrine (**24**) respectively. Some are then converted to cyclic lanthionine (**17**) and methyllanthionine (**18**) linkages by Michael addition of the thiol of a cysteine residue. Each of these transformations may be done by a dedicated enzyme or by a dual function enzyme that facilitates both the dehydration and cyclization reactions. Whether one or two enzymes are required is peptide dependent. Not all dehydrated moieties are transformed to thioethers; some remain intact in the final

mature peptide, whilst others are reduced selectively.[60,61] One further fate for dehydro amino acids residing at the *N*-terminus of the mature peptide is conversion to an α-ketoamide. Each of these transformations is discussed in more detail below.

1.3.2.a Dehydration of serine and threonine residues

There are several different dehydration enzymes known and although they show limited sequence similarity, it is believed that they all catalyze the reaction in a similar fashion. VenL[62,63] (from venezuelin biosynthesis) and LctM[64-66] (from lacticin 481 biosynthesis) are examples of dual dehydration/cyclization enzymes, and their dehydration function has been reconstituted *in vitro*. Scheme 1-2 shows the proposed mechanism of action for LctM. First, the hydroxyl functionality of a serine reacts with the terminal phosphate of ATP in the presence of a magnesium ion to yield a phosphorylated serine. Deprotonation at the α-carbon gives an enolate, and upon elimination of the phosphate, dehydroalanine is formed.[65] The exact role of the magnesium ion is uncertain, but it is reportedly needed for the phosphorylation reaction. Additionally, its binding pattern to the phosphate oxygens, shown in Scheme 1-2, is a common motif seen in Ser/Thr kinases.[65] The authors also state that while mutagenesis gives some indication of which active site residues might be important for catalyzing this reaction, they have been unsuccessful thus far in obtaining a crystal structure that could shed light on the exact mechanism.

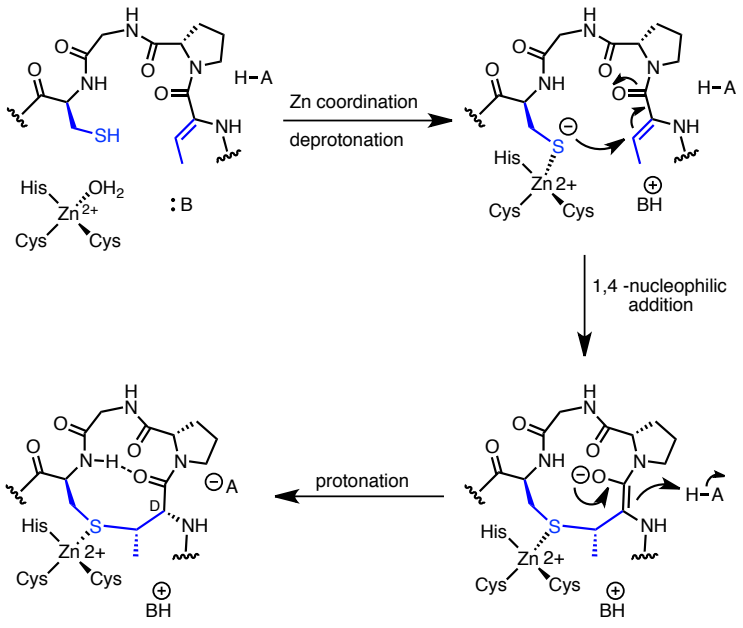


Scheme 1-2 Proposed mechanism of dehydration by LctM, L = Asn 247, Glu 261, Glu 446 (Adapted from You *et al.*)[65]

1.3.2.b Cyclization to form lanthionine and methyllanthionine

Unlike the dehydration enzymes, the cyclization enzymes of lantibiotics show considerable sequence homology. The cyclization portions of both LctM and VenL resemble the dedicated enzyme NisC. NisC is responsible for the formation of lanthionine (**17**) and methyllanthionine (**18**) in nisin A (**16a**), and its activity has been reconstituted *in vitro*. Some investigations of the important active site residues using mutagenesis experiments have been reported.[67,68] Shown in Scheme 1-3 is the mechanism proposed by Li and van der Donk.[67] Zinc is needed for the cyclization reaction to occur, and it was previously observed that a

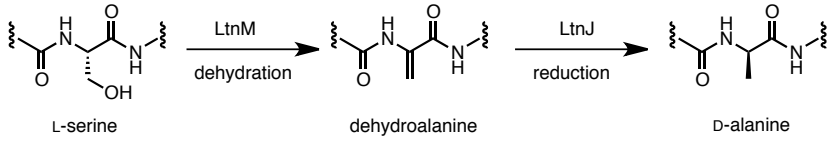
cysteine thiol coordinated to zinc has a lower pKa value than its uncoordinated counterpart.[69] The authors propose that following co-ordination and deprotonation, the thiolate is positioned to attack the dehydrobutyrine in a Michael fashion. The resulting enolate is then protonated to give D-stereochemistry at the newly formed stereocentre. The addition to the double bond of the dehydrobutyrine is anti, and so the authors claim there must be two separate amino acids in the active site responsible for the cysteine deprotonation and the protonation of the enolate.[67] A co-crystal structure would likely enable full characterization of the particular residues involved.



Scheme 1-3 Mechanism of cyclization by NisC (Adapted from Li *et al.*)[67]

1.3.2.c Reduction of dehydroalanine to D-alanine

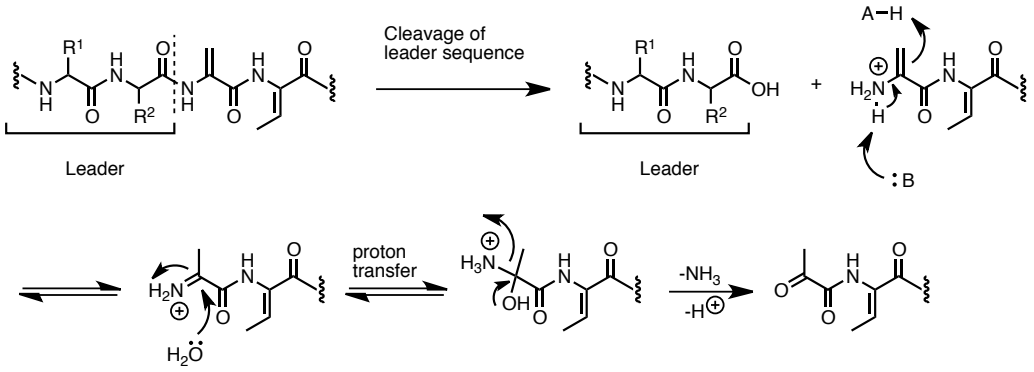
As mentioned previously, some dehydroalanines (**23**) are not retained or converted to lanthionine (**17**). In these cases, stereospecific reduction can also take place, yielding a D-alanine (**25**) residue. This is seen in three lantibiotics, lacticin 3147 A1 (**51**) and A2 (**52**) and lactocin S (**98**).[60,61] The enzyme responsible for the transformation in lactocin S is currently unknown, whereas LtnJ has been identified as the reduction enzyme for lacticin 3147 A1 (**51**) and A2 (**52**).[60] Knockout studies in which LtnJ is made non-functional result in the production of peptides that are two and four mass units smaller than normal A1 (**51**) and A2 (**52**), respectively. This corresponds with peptides containing dehydroalanine (**23**) instead of D-alanine (**25**). This provides strong evidence that LtnJ catalyzes the formation of D-alanine (**25**), however, at this point the enzyme has not been reconstituted *in vitro* and so there is little known about the enzymatic mechanism.



Scheme 1-4 Hypothesized formation of D-alanine residues in lacticin 3147 A1 (**51**) and A2 (**52**)

1.3.2.d Formation of *N*-terminal α-ketoamide

One further fate for dehydroamino acids is conversion into α-ketoamides. When a dehydroalanine (**23**) or dehydrobutyrine (**24**) is the *N*-terminal amino acid of the future peptide, it can be transformed into an α-ketoamide upon cleavage of the leader sequence (Scheme 1-5). This process occurs because of the reactivity of the primary enamine functionality that results from cleavage of the leader. Once the amide bond is broken, the primary enamine readily tautomerizes to an iminium ion and this can then be hydrolyzed by water to give the α-ketoamide.



Scheme 1-5 Formation of *N*-terminal α-ketoamide upon cleavage of leader sequence

1.3.3 Mode of action of lantibiotics

Lantibiotics are active almost exclusively against Gram-positive microorganisms. Some of the peptides have a narrow spectrum of activity, however, they are frequently very potent.[57] These peptides interact with the bacterial cell in several ways to exert their toxic effect. Lantibiotics can bind to

lipid II (see Figure 1-8), and sequester it, thus interfering with the biosynthesis of the bacterial cell wall.[57,70] This is toxic for the bacteria because lipid II is a building block for peptidoglycan, which is a very important structural component of the bacterial cell wall.[71] This is similar to the way that vancomycin (**6**) exerts its antibacterial effect; it recognizes and binds to the D-Ala-D-Ala motif of lipid II. Mersacidin, a globularly shaped lantibiotic, interacts with the sugar-phosphate portion of lipid II. Because lantibiotics interact with lipid II differently than commonly used antibiotics, organisms with structurally altered lipid II that develop resistance to vancomycin (**6**) can still be susceptible to lantibiotics.[58]

There is a second way that many lantibiotics kill bacteria and that is to form pores in the cellular membrane (Figure 1-8). This mechanism is better understood, especially in the case of nisin A/Z (**16a,b**).[72] At high concentrations of peptide and in the absence of lipid II, nisin A/Z (**16a,b**) is able to permeabilize model membranes. It was originally thought that this pore formation and the subsequent loss of cellular contents was responsible for the peptide's bioactivity.[73] However, the concentration of nisin Z (**16b**) required to form pores in the absence of lipid II is far higher (~2.5 μM)[74] than the observed values for activity in nature (~5 nM),[74] suggesting this is not the only cause of the observed activity. It was subsequently discovered that when lipid II is present in the membrane, nisin A (**16a**) interacts with the glycolipid and forms more stable dual-component pores proposed to consist of eight nisin molecules and four lipid II molecules (Figure 1-8).[71,74] The subsequent leakage of cellular contents results in cell death.

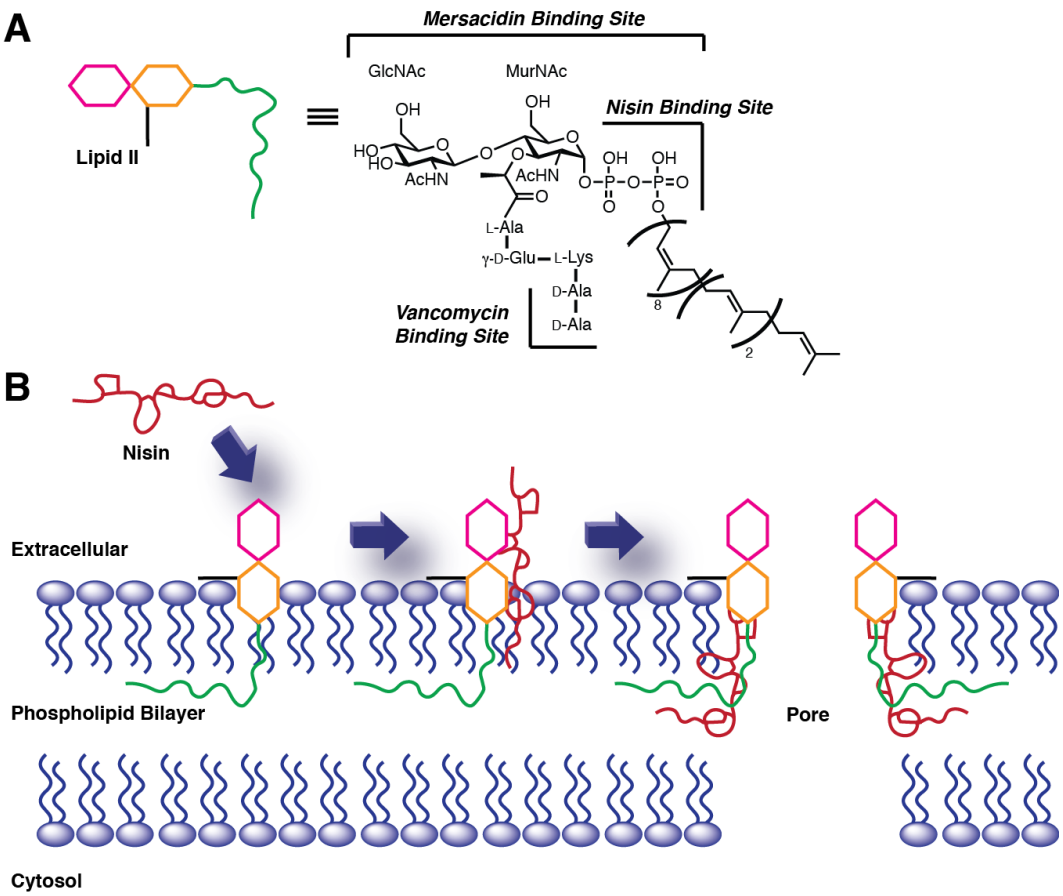


Figure 1-8 **A.** Shows the structure of Lipid II with the binding sites of several antibiotics. **B.** Shows the interaction of nisin A (**16a**) with lipid II[75] and the subsequent formation of transmembrane pores. (Adapted from Breukink *et. al.*)[71]

It is significant that many lantibiotics have more than one mechanism of action.[76] If bacteria develop resistance to one mode, the peptide is still able to exert a toxic effect with the other. This, along with the very potent activity of many lantibiotics, is what makes them such promising candidates for clinical development.

1.3.4 Applications of lantibiotics

Lantibiotics show promise for use in both the preservation of food and as a clinical treatment for bacterial infection. Thus far, the development of food applications has advanced further than the medical direction. This is unsurprising, since lantibiotics are produced by bacteria naturally present in food. For instance, lactic acid bacteria used in cheese production also produce lantibiotics.[36,77] Therefore, humans have in effect been using these antimicrobial peptides for food preservation for a long time without knowing it. As mentioned in Section 1.2, nisin A (**16a**) is widely used in food applications as a purified bacteriocin and is currently the only lantibiotic licensed as a food preservative. However, many other lantibiotics are being investigated for use, as are alternative administration methods such as supplementing with the producing organism to obtain peptide production *in situ*.[78-80]

The use of lantibiotics as human therapeutics is still in the developmental stages, however, these compounds show real promise for the treatment of bacterial infections. Peptides are currently being investigated for the treatment of halitosis,[81] dental caries,[82,83] acne,[84] mastitis,[85] skin infections and systemic infections.[44] Additionally, nisin A (**16a**) and lacticin 3147 A1 (**51**) and A2 (**52**) have spermicidal properties and could be used in prophylactic treatments to not only prevent pregnancy, but also to help protect from sexually transmitted infections.[57,86] Importantly, mersacidin shows promise as an alternative therapy to vancomycin (**6**) for resistant MRSA infections.[87] Both nisin A (**16a**) and lacticin 3147 A1 (**51**) and A2 (**52**) are also active against the debilitating *Clostridium*

difficile.[57,88] While still in development, lantibiotics may in the future provide an additional option for the treatment of bacterial infections.

A current limitation for the use of lantibiotics is the instability of some of the peptides under certain conditions. Many lantibiotics are only stable at low pH.[77,89] Nisin A (**16a**) can only be used in acidic environments and loses activity above pH 7.[78,90] Nisin A (**16a**) is also inactivated by oxidation of the thioethers in methionine, lanthionine (**17**), and methyllanthionine (**18**) residues to sulfoxides.[91] There are a few lantibiotics stable and active at higher pHs. One of these is haloduracin, a two-compotent lantibiotic isolated from *Bacillus halodurans,* which grows in alkaline conditions (pH > 9).[92-94] Although lantibiotics are extremely potent antibacterial compounds, there are limitations to their practical application due to stability. As a result, there is considerable interest in generating peptide analogues with not only improved activity but also increased chemical stability.

1.3.5 Preparing analogues of lantibiotics

Multiple approaches have been used to make analogues of lantibiotics.[57,72,95,96] Genetic manipulation has been utilized to produce variants of lantibiotics that contain simple amino acid replacements. This approach allows for the evaluation of the importance of a single amino acid.[97] Although it is an incredibly useful tool (and the source of the most data thus far), there are several drawbacks to this system. Firstly, analogues are limited to peptides containing

primarily natural amino acids. Secondly, because of the extensive post-translational modifications of the peptides, the organism used for the production of mutants must possess the requisite modification enzymes. Thirdly, if mutant peptides with an altered spectrum of activity are produced, they may not be recognized by the immunity mechanisms of their producing organism. This would result in the death of the producing bacteria and would stop any further synthesis of this active peptide. In this way, peptides with improved activity and specificity could go undetected. Finally, as the production of desired analogues relies upon the modification machinery recognizing/accepting altered substrates, many target analogues may not be produced and so cannot be tested for activity.

Recently, van der Donk and co-workers published an *in vitro* "mutasynthesis" approach.[98] This enables the introduction of noncoded unnatural amino acids in a linear peptide via synthetic chemistry and then subsequent use of enzymes to increase complexity (introduce rings etc.). This approach could prove very useful, so long as the requisite modification enzymes can be used *in vitro* and have sufficiently relaxed substrate specificity.

Total chemical synthesis can provide access to peptide analogues with complete chemical control and few amino acid limitations.[99-101] The analogues produced via chemical synthesis have so far focused on altering the ring structures of lantibiotics. This would be a difficult change to make using current genetic manipulation methods. As mentioned previously, lanthionine (**17**) and methyllanthionine (**18**) are the result of post-translational enzymatic processing, and any change to the enzyme substrate may mean the requisite enzyme no longer

recognizes it. No such problem exists in chemical synthesis and so it has been possible for analogues to be synthesized with different rings. Several studies on the A2 (**52**) peptide of lacticin 3147 have uncovered the importance of lanthionine versus methyllanthionine,[99] as well as the requirements of ring size and heteroatom identity.[100,101] The following section discusses the chemical synthesis of these analogues and the outcome of biological testing.

1.4 Synthesis of Lantibiotics

The synthesis of lantibiotics is important for the production of analogues, and is also a technique for determining if the proposed structures of isolated peptides are correct. Several approaches have been used to synthesize these peptides including solution and solid phase synthesis. There are several features of lantibiotics that make them more synthetically challenging than other peptides. Introducing the side chain cyclized lanthionine (**17**) and methyllanthionine (**18**) residues is one of the greatest challenges. Another is incorporation of the dehydrated residues, dehydroalanine (**23**) and dehydrobutyrine (**24**). Multiple strategies have been used to produce these peptides and several will be described below.

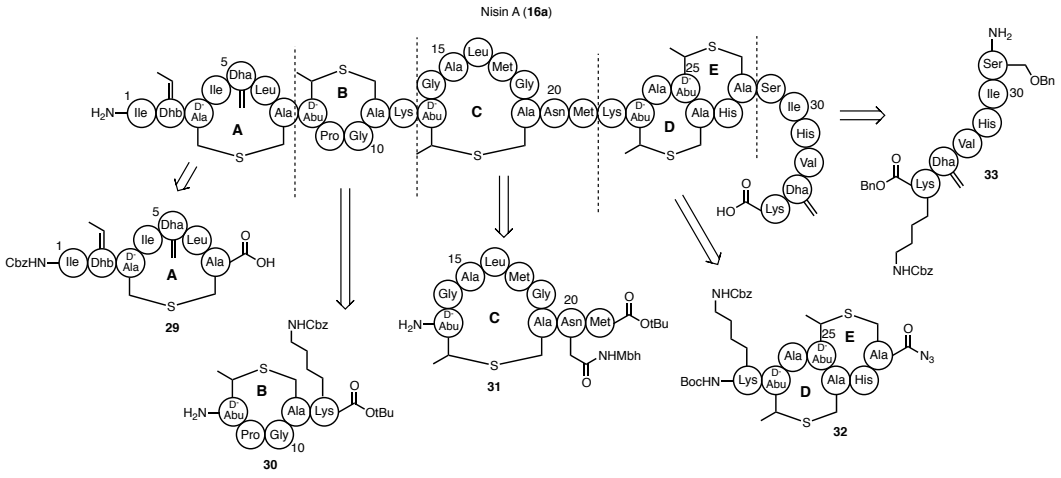
1.4.1 Total synthesis of lantibiotics using solution chemistry

The synthesis of lantibiotic peptides via solution chemistry methods is difficult. As the peptides get longer, solubility and purification become

problematic. Therefore, a convergent synthetic approach is typically employed.[102] Additionally, coupling reactions become more challenging, as bringing the two pieces together in correct orientation is more difficult when the peptide gets longer. Often, the terminal position required to undergo reaction can be buried within the three dimensional structure of the peptide. The extensive use of protecting groups is needed to differentiate between very similar functionalities and developing the right strategy to do this is vital.

1.4.2 The total synthesis of nisin A

The solution synthesis of nisin A (**16a**) was completed twenty years ago by Shiba and co-workers and represents a massive achievement.[103] This was the first chemical synthesis of a lantibiotic peptide and is particularly impressive considering the structure of nisin A (**16a**). This peptide contains dehydrated residues as well as both lanthionine (**17**) and methyllanthionine (**18**). Its complexity is increased further because two of the rings (D and E) overlap each other along the backbone of the peptide generating a knot structure. In Shiba's approach, each ring of the peptide was synthesized individually and then coupled together in a convergent manner (see Scheme 1-6).[103-108] The efforts of the Shiba group to make this peptide covered a ten-year timeframe.



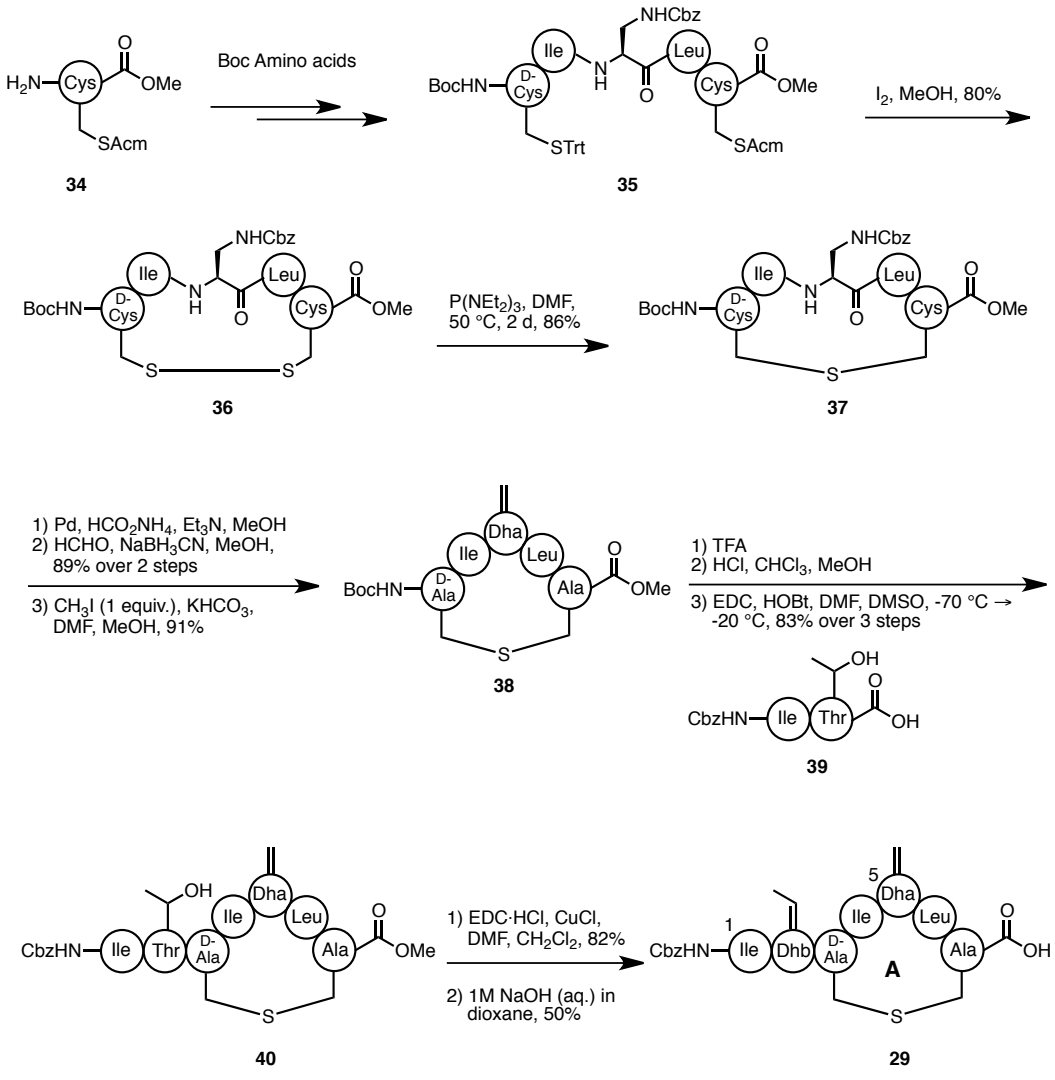
Scheme 1-6 Retrosynthetic analysis of Shiba's nisin A (**16a**) synthesis

The authors used similar methodology to make each ring, so the synthesis of only two of the fragments will be described in detail, along with the final assembly of nisin A (**16a**).

1.4.2.a Synthesis of the A-ring of nisin A

The A-ring fragment **29** contains a lanthionine ring along with a dehydroalanine and a dehydrobutyrine residue.[105] The A-ring was synthesized by first coupling together Boc-protected amino acids to yield a linear pentapeptide (**35**) capped with two cysteine residues (see Scheme 1-7). The thioether linkage was made by treating the cysteines with iodine and methanol to form a disulfide bond (**36**). Subsequent sulfur extrusion with tris(diethylamino)phosphine yielded the thioether lanthionine (**37**).[109] The dehydroalanine residue within the A-ring was formed by an elimination reaction. The 2,3-diaminopropionate residue was

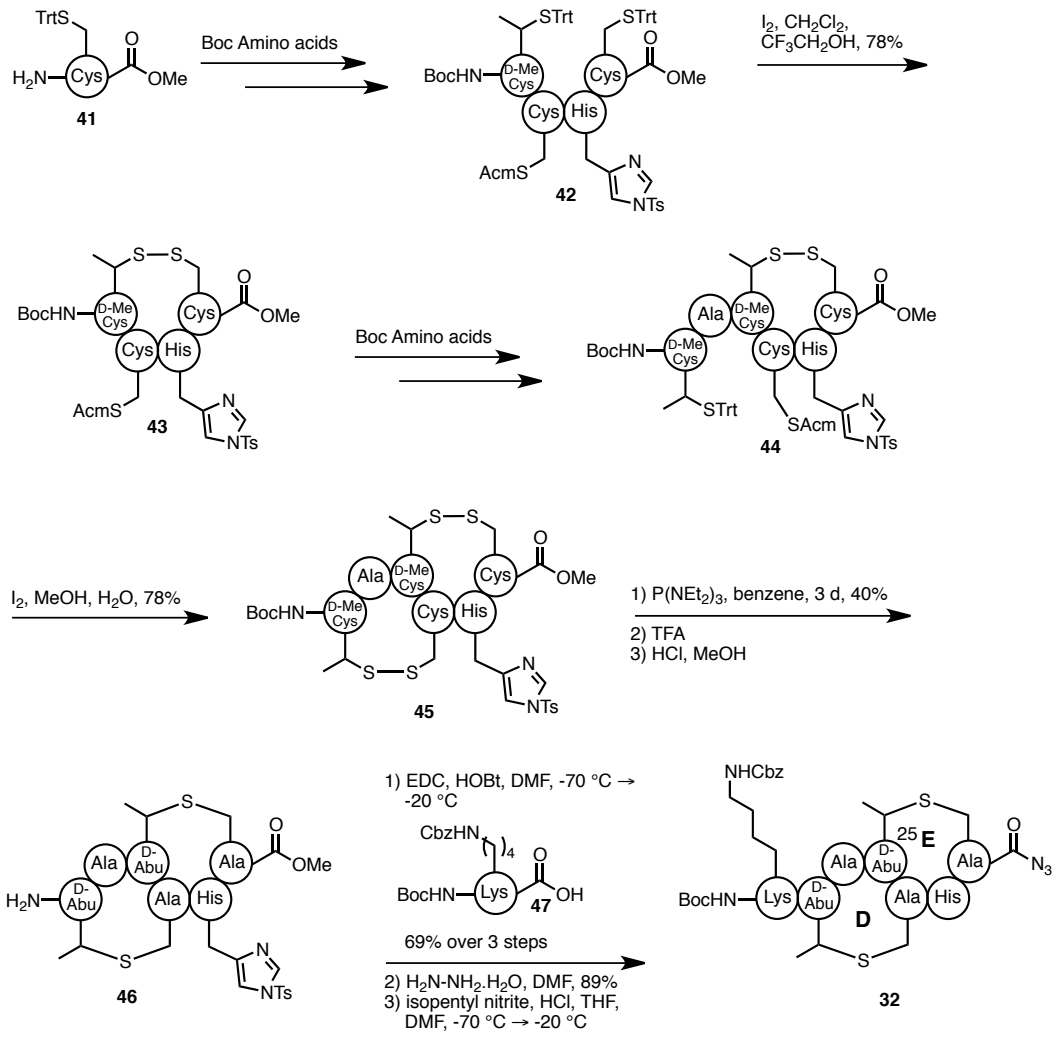
alkylated by reductive amination with formaldehyde and sodium cyanoborohydride, and then the tertiary amine was exposed to Hoffman degradation conditions to generate the desired alkene (**38**).[104] Finally, to introduce the dehydrobutyrine functionality, threonine-containing dipeptide **39** was coupled to the *N*-terminus of the A-ring and then EDC and copper (I) chloride were used to generate the dehydrated residue (**29**). These reaction conditions were chosen after an *O*-mesylation approach had been unsucessful.[104,110]



Scheme 1-7 Synthesis of the A-ring of nisin A (**16a**)[104,105]

1.4.2.b Synthesis of the D/E-rings of nisin A

The synthesis of the D and E-rings of nisin A is more complicated because the two rings are intertwined. This means the cyclization of the rings must be specific so that the correct connectivity is obtained. By using selective protection the authors were able to form the first disulfide bond in the presence of an additional cysteine. Following incorporation of a further 3-methylcysteine, the second disulfide was formed (Scheme 1-8).[108] Linear tetrapeptide **42** contains a cysteine and a 3-methylcysteine protected with trityl groups and one cysteine protected with acetamidomethyl (Acm). When this peptide was treated with iodine in the presence of dichloromethane and trifluoroethanol, the trityl groups were removed whilst the Acm group remained undisturbed.[111] The choice of solvents was important to control the selectivity of this reaction. The deprotected thiols react further under these conditions to yield the disulfide-containing compound **43**.



Scheme 1-8 Synthesis of the D/E-rings of nisin A (**16a**)

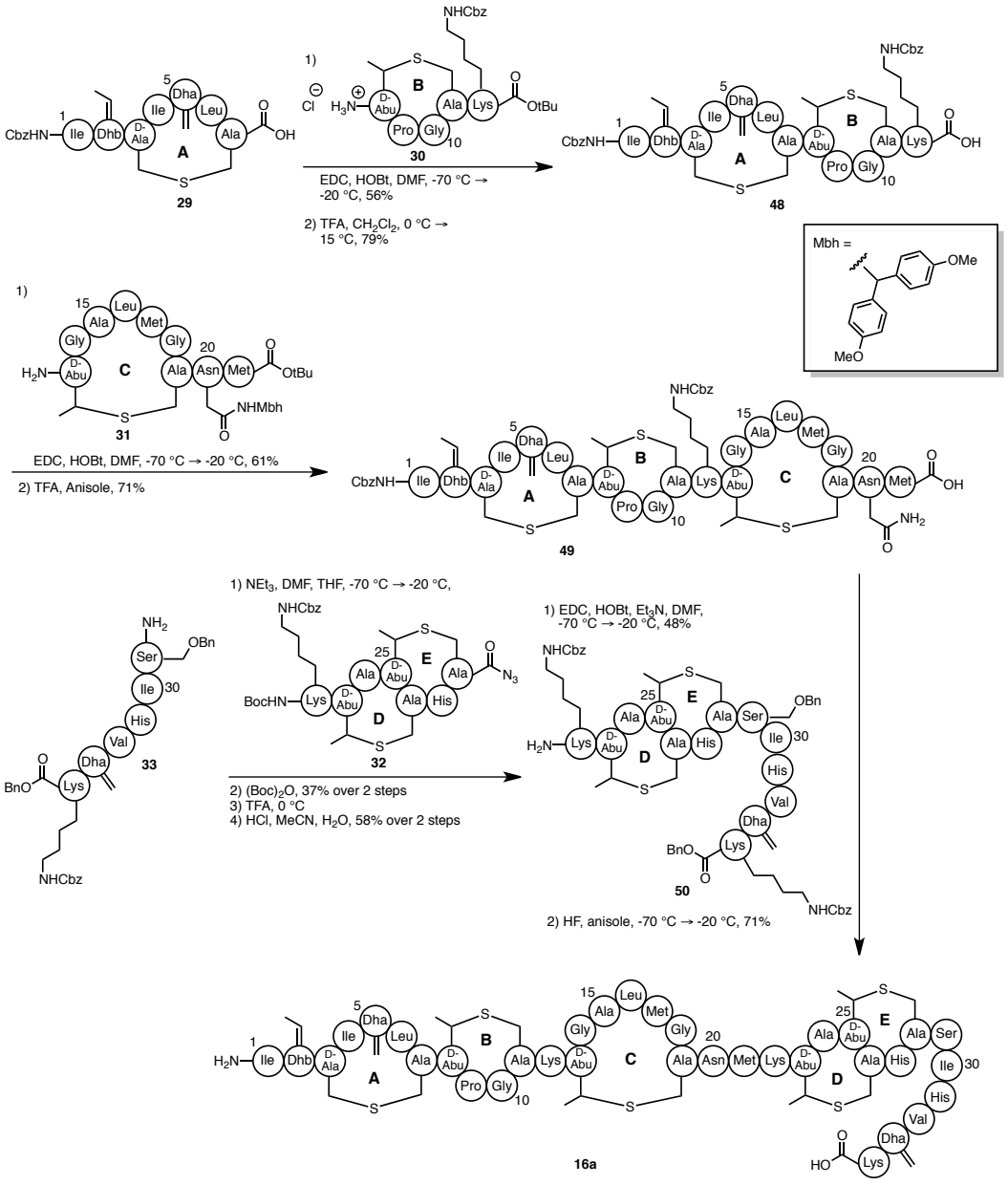
The remaining amino acids of the D-ring were coupled onto the *N*-terminus of **43** and then the second disulfide was formed. This was achieved by removal of the trityl and Acm protecting groups of the remaining cysteine and 3-methylcysteine with iodine in methanol and water. These conditions allow both protecting groups to be removed and facilitate the formation of the second disulfide bond. The resulting knotted disulfide compound **45** was transformed to

the corresponding knotted methyllanthionine core of the D- and E-ring system using tris(diethylamino)phosphine as seen for the A-ring. The authors had also investigated stepwise formation of the D and E methyllanthionine rings. They formed the E-ring disulfide **43** and converted it directly to the methyllanthionine. They then introduced the remaining D-ring residues and converted them to the second disulfide and the corresponding methyllanthionine. The authors found that the stepwise approach had considerably lower yields for the important sulfur extrusion/methyllanthionine formation steps, and so the simultaneous approach was found to be superior.[108] The final step to obtain the desired fragment was to convert the *C*-terminal ester to an acid azide (**32**) using hydrazine followed by isopentyl nitrite under acidic conditions.

1.4.2.c Endgame of the total synthesis of nisin A

Using the different methodologies for synthesis of the thioether rings and dehydroamino acids described above, the fragments for the B- and C-rings along with a *C*-terminal fragment were also obtained (synthesis not shown).[103,104,106,107] The different pieces were then coupled together as shown in Scheme 1-9. The *C*-terminus of the A-ring (**29**) was coupled to the *N*-terminus of the B-ring (**30**) using EDC and HOBt, then the *C*-terminal *tert*-butyl ester was deprotected with TFA and the C-ring fragment (**31**) was coupled via its *N*-terminus using EDC and HOBt. Removal of the *C*-terminal *tert*-butyl ester with TFA and anisole yielded **49** a tricyclic peptide containing the A-, B- and C-rings of nisin A (**16a**). In a

similar fashion, the D/E-ring fragment (**32**) was coupled via its acid azide to the *C*-terminal fragment (**33**). Following removal of the Boc protecting group a bicyclic compound **50** was obtained, this was then coupled to tricyclic peptide **49** and global deprotection with HF gave nisin A (**16a**).[103,104] This synthesis is a monumental achievement and to date is the only total synthesis of a lantibiotic peptide to be completed using solely solution chemistry methods.



Scheme 1-9 Solution synthesis of nisin A (**16a**)

1.4.3 Solid phase peptide synthesis

The synthesis of peptides by chemical means has been revolutionized by the development of solid phase peptide chemistry. In fact, this method is so

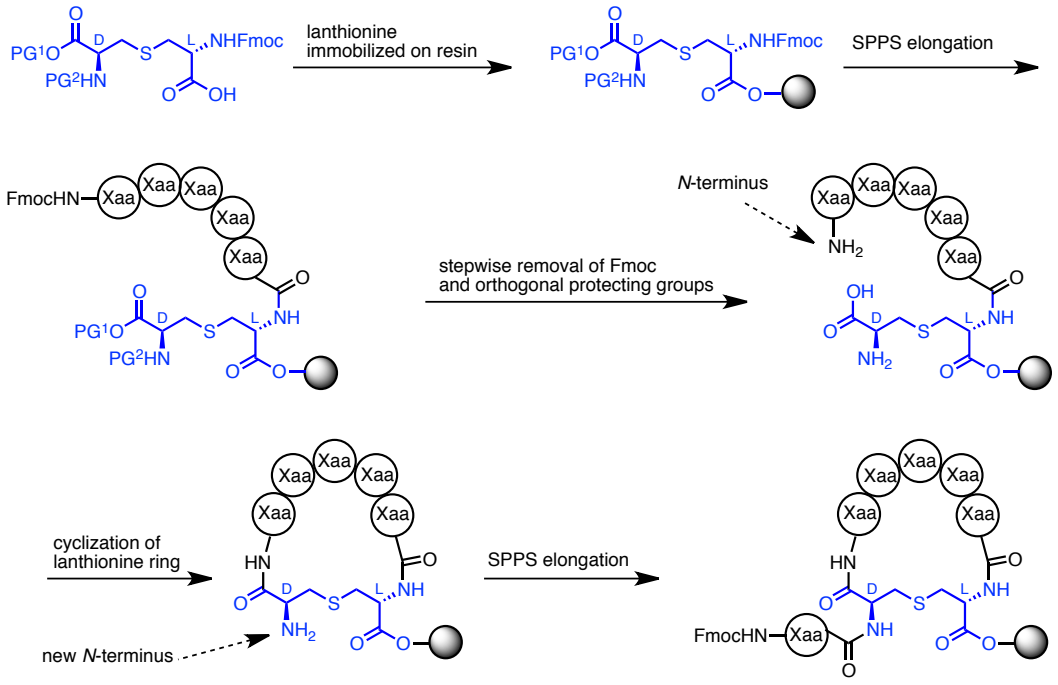
important that Bruce Merrifield, its creator,[112] was awarded the 1984 Nobel Prize in Chemistry for his efforts. The fundamental concept is that a peptide is immobilized at its *C*-terminus on a solid resin bead. Then the *N*-terminus can be reacted with a new amino acid to lengthen the peptide that still remains attached to the resin. In this manner, quite long peptides (~50 amino acids) can be made in a relatively short timeframe.[102] By using compatible protecting groups, one is able to control with considerable selectivity the elongation of the peptide. Once the desired amino acids have been incorporated, a single chemical reaction can be used to break the attachment of the *C*-terminus to the resin and a full length free peptide is obtained. The biggest advantage of SPPS is that it allows for very simple, fast and effective purification of the peptide. Reagents are added to the immobilized peptide in a solution, and then once the reaction is complete, any unreacted reagents can be washed away leaving only the product immobilized on the resin. In this way, reactions can be pushed to near completion by the use of many equivalents of reagents. Unlike solution chemistry that requires column chromatography or crystallization purification at nearly every step, peptides made using SPPS generally only require chromatographic purification at the very end of the synthesis.

There are two main approaches to SPPS and they are based around what protecting group is being used at the *N*-terminus of the growing peptide. The two groups most commonly used are Boc (*tert*-butyloxycarbonyl) and Fmoc (9-fluorenylmethyloxycarbonyl) and they behave quite differently. Fundamentally, the Boc group is cleaved under acidic conditions whilst the Fmoc group is cleaved

under alkaline conditions. Depending on the *N*-terminal protection strategy, the side chains of each amino acid need to be protected such that they are unaffected by the removal of the *N*-terminal protection. Each general strategy has advantages and disadvantages. The cleavage conditions for the Boc approach use the dangerous acid HF, however, not all peptides are compatible with the basic conditions used to deprotect Fmoc.[113,114] These two methods are complementary. The solid phase chemistry discussed in this thesis makes use of Fmoc chemistry.

1.4.4 The synthesis of lantibiotics and their analogues using SPPS

The synthetic approach for lantibiotics on solid support is fundamentally different to the approach used by Shiba to make nisin A. A linear rather than convergent synthesis must be used. In the SPPS syntheses, the unusual amino acids lanthionine or methyllanthionine (or their analogues) can be made in solution first, and then subsequently incorporated into the solid phase synthesis. By protecting the lanthionine residues with orthogonal groups, the cyclic portions of the molecule can in principle be introduced by on-resin cyclization reactions. All of the syntheses described below utilize this approach to make lantibiotics and their analogues with SPPS.



Scheme 1-10 General approach to the synthesis of lantibiotics on resin

Each 'terminus" of the lanthionine is differentially protected such that when the amino acid is attached to the resin, one end can essentially mimic a protected side chain (Scheme 1-10). The peptide can then be elongated via standard SPPS to incorporate the residues of the ring. When the desired linear peptide has been made, the protected terminus of the lanthionine can be unprotected, and an amide bond formed between the *N*-terminal amine of the peptide chain and the carboxyl functionality of the lanthionine. The remaining amine functionality of the lanthionine is now the *N*-terminus of the peptide and can be used to elongate the peptide.

1.4.5 Synthesis of lacticin 3147 A1 and A2 and analogues

This methodology has been used to synthesize lacticin 3147 A1 (**51**) and A2 (**52**). Full length analogues of A2 (**52**) have also been made, replacing the methyllanthionine residues with lanthionine (**53**) residues,[99] the sulfur of **17** and **18** with oxygen (**54**)[101] or with an olefin (**55**).[100] These analogues were designed to improve the stability of lantibiotics and to investigate the effect of the rings on biological activity. Several groups have produced shortened analogues of nisin Z (**16b**) containing one or more rings of the lantibiotic.[115-122]

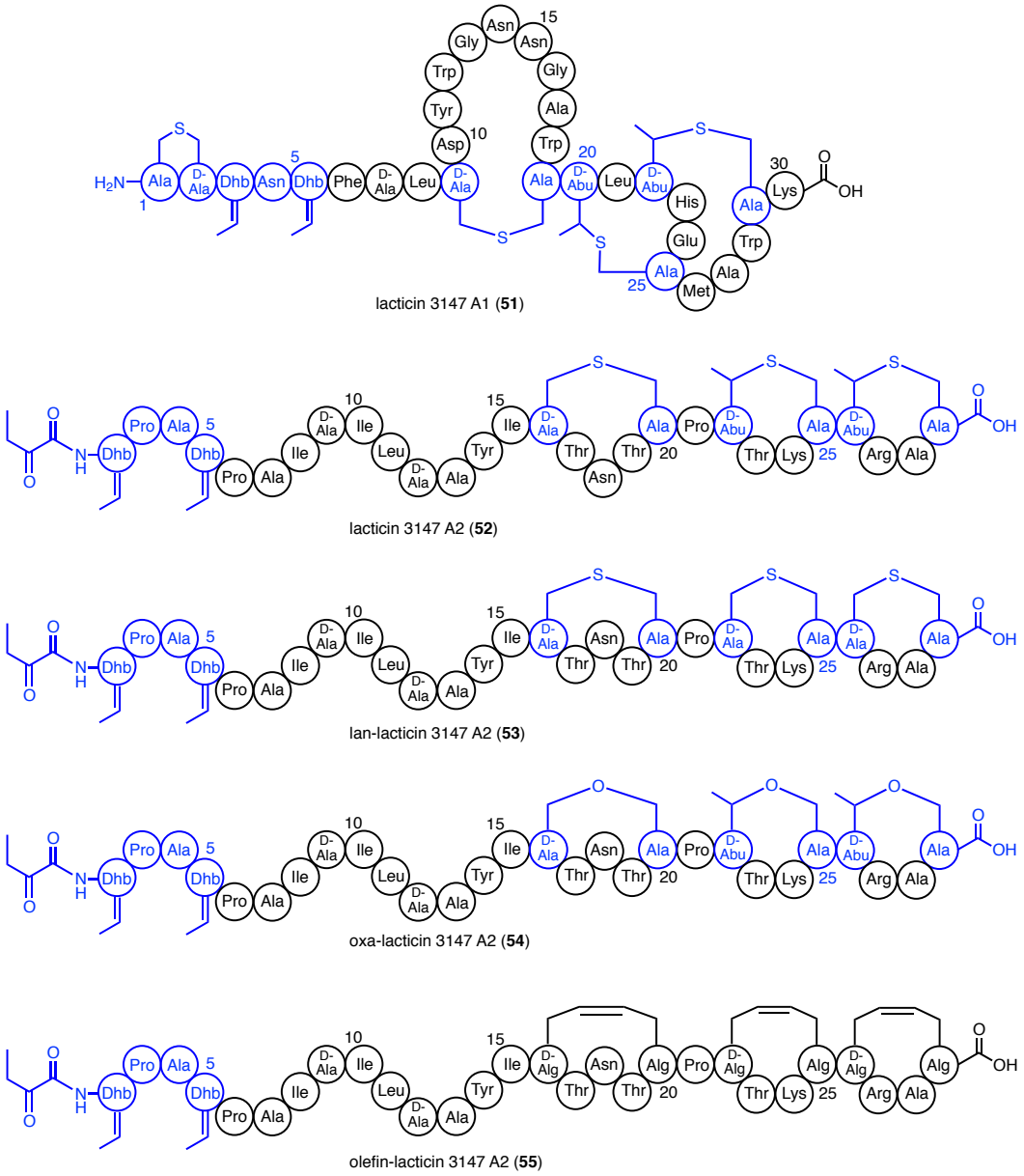


Figure 1-9 The structures of lacticin 3147 A1 (**51**) and A2 (**52**) and their analogues (**53**, **54** and **55**) obtained by SPPS methodology. The portions of the peptides highlighted in blue must first be made in solution before they are incorporated into the SPPS.

The following sections are devoted to the use of SPPS methodology to make the lacticin 3147 derived peptides in Figure 1-9. Section 1.4.6 will focus on

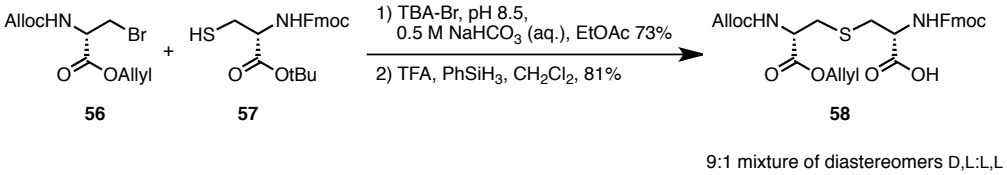
several ways used to synthesize orthogonally protected building blocks in solution. Then Section 1.4.7 will describe the different solution syntheses of the *N*-terminal portions of lacticin 3147 A1 (**51**) and A2 (**52**). Section 1.4.8 will discuss how these are incorporated into SPPS to yield the peptides of interest and Section 1.4.9 will describe the synthesis of an olefin analogue (**55**) of lacticin 3147 A2.

1.4.6 Solution synthesis of orthogonally protected lanthionine, methyllanthionine, and their oxa analogues

Several research groups have achieved the solution synthesis of orthogonally protected lanthionine and methyllanthionine.[120,121,123-129] The examples of orthogonally protected lanthionine and methyllanthionine described below contain either two or three levels of orthogonal protection. If a peptide contains interlocking rings, it is vital to have the third level of protection. If it only contains sequential rings, then two types of protection are sufficient. Two different approaches to the synthesis of these solution building blocks are described. The first is an S_N2 displacement of an alkyl halide by cysteine to give lanthionines. A second approach involves nucleophilic attack on an aziridine to yield either lanthionine or methyllanthionine. This methodology is also used to make oxa-lanthionine analogues.

1.4.6.a Synthesis of orthogonally protected lanthionine by S_N2 displacement of an alkyl bromide

The synthesis of allyl/Alloc protected lanthionine **58,** for use in the SPPS of lanlacticin 3147 A2 (**53**), was done via an S_N2 reaction of protected cysteine **57** and 3-bromo D-alanine **56**.[99] The resulting lanthionine exists as an inseparable mixture of diastereomers due to a side reaction, in which 3-bromo D-alanine **56** eliminates to a dehydroalanine that can be attacked by Michael addition to give both stereoisomers (D,L and L,L).

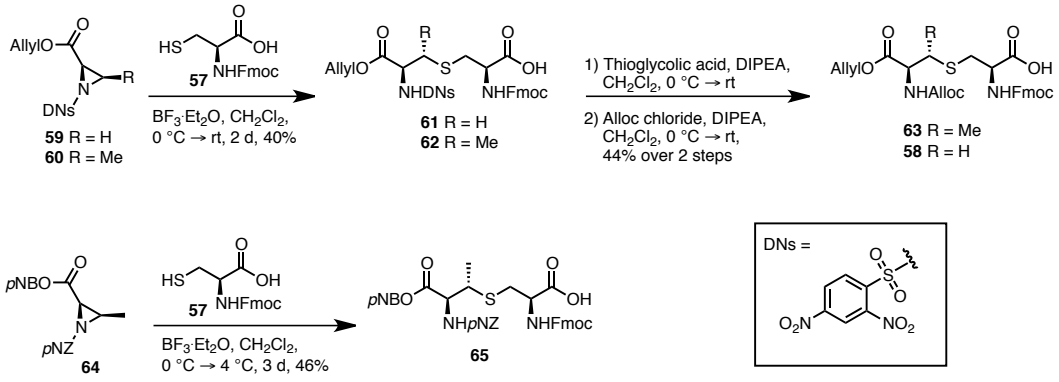


Scheme 1-11 Synthesis of orthogonally protected lanthionine (**58**) by S_N2 displacement of an alkyl bromide

This methodology is based upon an approach published by Tabor and co-workers, in which they produced analogues of the C-ring of nisin A/Z (**16a,b**) by replacing the natural methyllanthionine (**18**) with lanthionine (**17**)[120,126] using allyl/Alloc protected lanthionine **58**. This synthesis of the lanthionine analogue of the C-ring of nisin A/Z (**16a,b**) by Tabor and co-workers was the first time a lanthionine ring had been formed on-resin using an orthogonal protecting approach.[126] Whilst providing useful synthetic methodology, no antibacterial activity tests for this analogue have been reported.

1.4.6.b Synthesis of orthogonally protected lanthionine, methyllanthionine, and their oxalanthionine and methyllanthionine analogues by aziridine ring opening

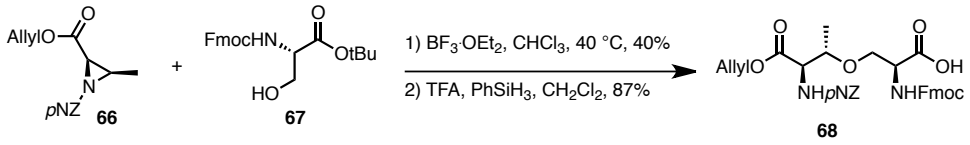
Orthogonally protected lanthionine (**58**) and methyllanthionines (**63** and **65**) for use in the SPPS of the rings in lacticin 3147 A1 (**51**) and A2 (**52**) were made incorporating two different sets of protecting groups (Scheme 1-12). This was because A1 (**51**) contains interlocking rings and greater synthetic control is needed. The authors chose to use an aziridine ring opening reaction to form the thioether linkage, to avoid the formation of diastereomers seen in Section 1.4.6.a.[130]



Scheme 1-12 Synthesis of orthogonally protected lanthionine (**58**) and methyllanthionines (**63, 65**)

Briefly, aziridines **59**, **60,** and **64** each with an electron withdrawing protecting group on the nitrogen can be opened by Fmoc protected cysteine **57**.

Methyllanthionine **65** is ready for SPPS. Following the replacement of DNs protecting groups with Alloc, compounds **58** and **63** are also available by this approach. This methodology had previously been applied to the synthesis of orthogonally protected oxygen analogues of lanthionine (**17**) and methyllanthionine (**18**).[131]



Scheme 1-13 Synthesis of orthogonally protected 3-methyl-oxa-lanthionine (**68**)

The authors produced orthogonally protected oxa-lanthionine and 3-methyl-oxa-methyllanthionine (**68**) using the aziridine ring opening approach, this time using the side chain of Fmoc protected serine **67** as the nucleophile. These building blocks were then used to make oxa-lacticin 3147 A2 (**54**).[101]

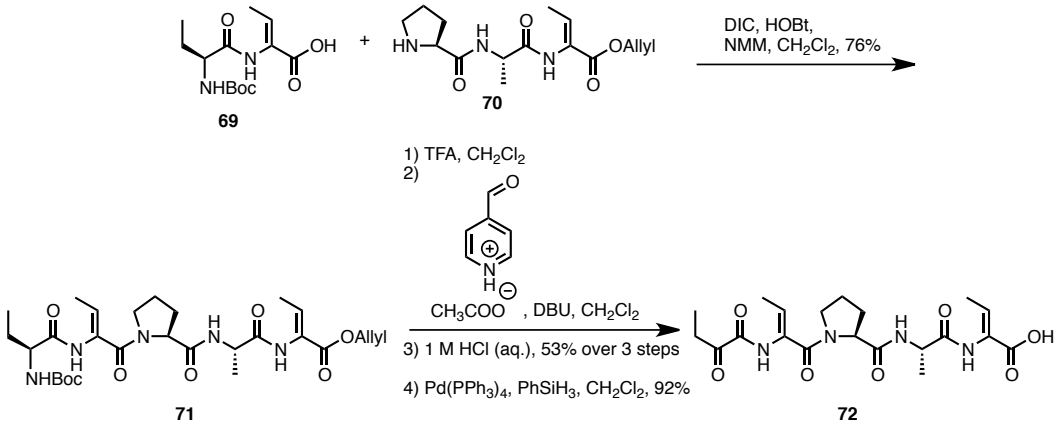
1.4.7 Synthesis of the *N*-terminal five residues in lacticin 3147 A1 and A2

The five residues at the *N*-termini of both lacticin 3147 A1 (**51**) and A2 (**52**) are highly modified. The dehydrobutyrine residues in both are not compatible with the deprotection conditions used during SPPS, as piperidine can attack these residues in a Michael fashion to form undesirable piperidine adducts. The *N*-terminal α-ketoamide of A2 (**52**) is also a synthetic challenge. Each pentapeptide

was made in solution and two different approaches were used to form the α-ketoamide.

1.4.7.a Synthesis of the *N*-terminus of lacticin 3147 A2 by a transamination approach

The first strategy used to make the *N*-terminus of A2 utilizes a transamination reaction to form the α-ketoamide functionality during solution synthesis. A dehydrodipeptide **69** and a dehydrotripeptide **70**, each formed by mesylation-dehydration of a threonine in the precursor, were coupled together using DIC and HOBt to give pentapeptide **71**. Then the terminal α-ketoamide was installed via a transamination using 4-pyridinium carboxaldehyde.[132,133]



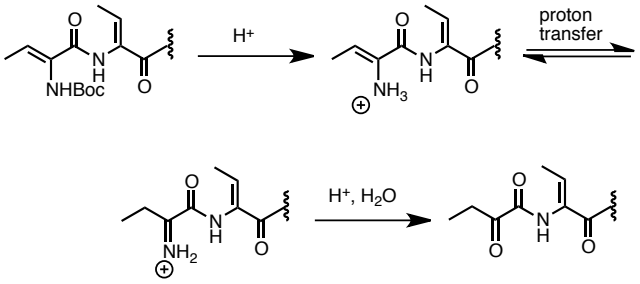
69 **70** **71** **72**

Scheme 1-14 Synthesis of *N*-terminal pentapeptide (**72**)

Following removal of the allyl protecting group, pentapeptide **72** was ready for incorporation into the SPPS of A2 peptides.

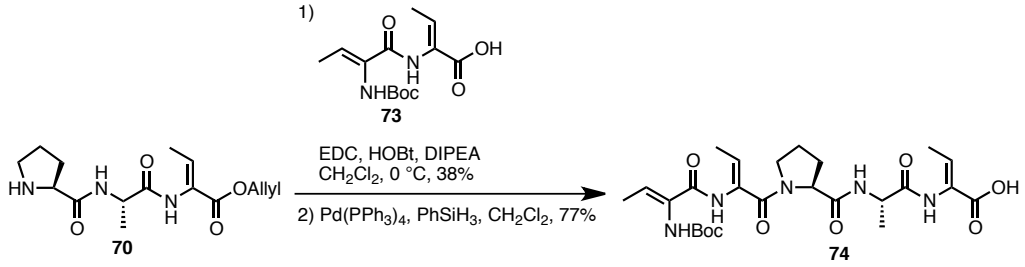
1.4.7.b Synthesis of the *N*-terminus of lacticin 3147 A2 by a biomimetic approach

An alternative synthetic approach for the formation of the α-ketoamide was based upon its biosynthesis (Scheme 1-15). This is achieved by incorporating a Boc protected dehydrobutyrine residue at the *N*-terminus, to mimic the leader sequence that is attached to the dehydrobutyrine at the *N*-terminus in the natural system. A pentapeptide containing an *N*-terminal Boc dehydrobutyrine is synthesized in solution and then coupled to the rest of the peptide on resin.



Scheme 1-15 Formation of an α-ketoamide via hydrolysis of an *N*-terminal iminium ion.

When the peptide is cleaved from the resin using acid, an enamine is formed that tautomerizes to an iminium ion that can be hydrolyzed by water to give the α-ketoamide. Pentapeptide **74** was designed to utilize this approach for the synthesis of A2 (**32**).[134]

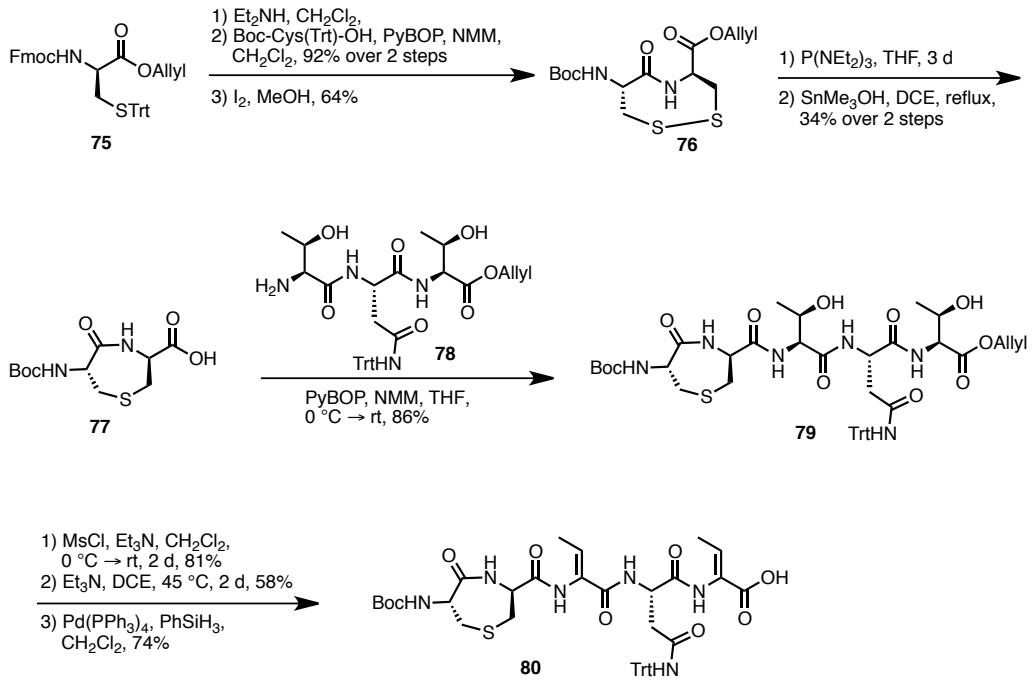


Scheme 1-16 Synthesis of *N*-terminal pentapeptide (**74**)

Dipeptide **73** and tripeptide **70** were synthesized by mesylation of threonine in precursor peptides followed by elimination. They were coupled together using EDC and HOBt, and deprotection of the allyl ester yielded pentapeptide **74** ready for use in the SPPS of lacticin 3147 A2 (**52**).

1.4.7.c Synthesis of the *N*-terminus of lacticin 3147 A1

The *N*-terminus of lacticin 3147 A1 (**51**) was synthesized in solution as the pentapeptide **80** (Scheme 1-17). This fragment contains lanthionine and two dehydrobutyrines. The lanthionine portion (**71**) was made using sulfur extrusion, from the corresponding disulfide **70**, similar to the approach used in Shiba's synthesis of nisin A (**16a**).[103,104]



Scheme 1-17 The synthesis of the *N*-terminal pentapeptide of lacticin 3147 A1 (**51**)

Following coupling of tripeptide **78** to lanthionine **77**, the dehydroamino acids were formed by elimination of the hydroxyl functionalities after activation as mesylates. Deprotection of the allyl ester gave **80** ready for coupling to the resin bound portion of A1 (**51**).

1.4.8 Solid phase synthesis of lacticin 3147 A1 and A2 and their analogues

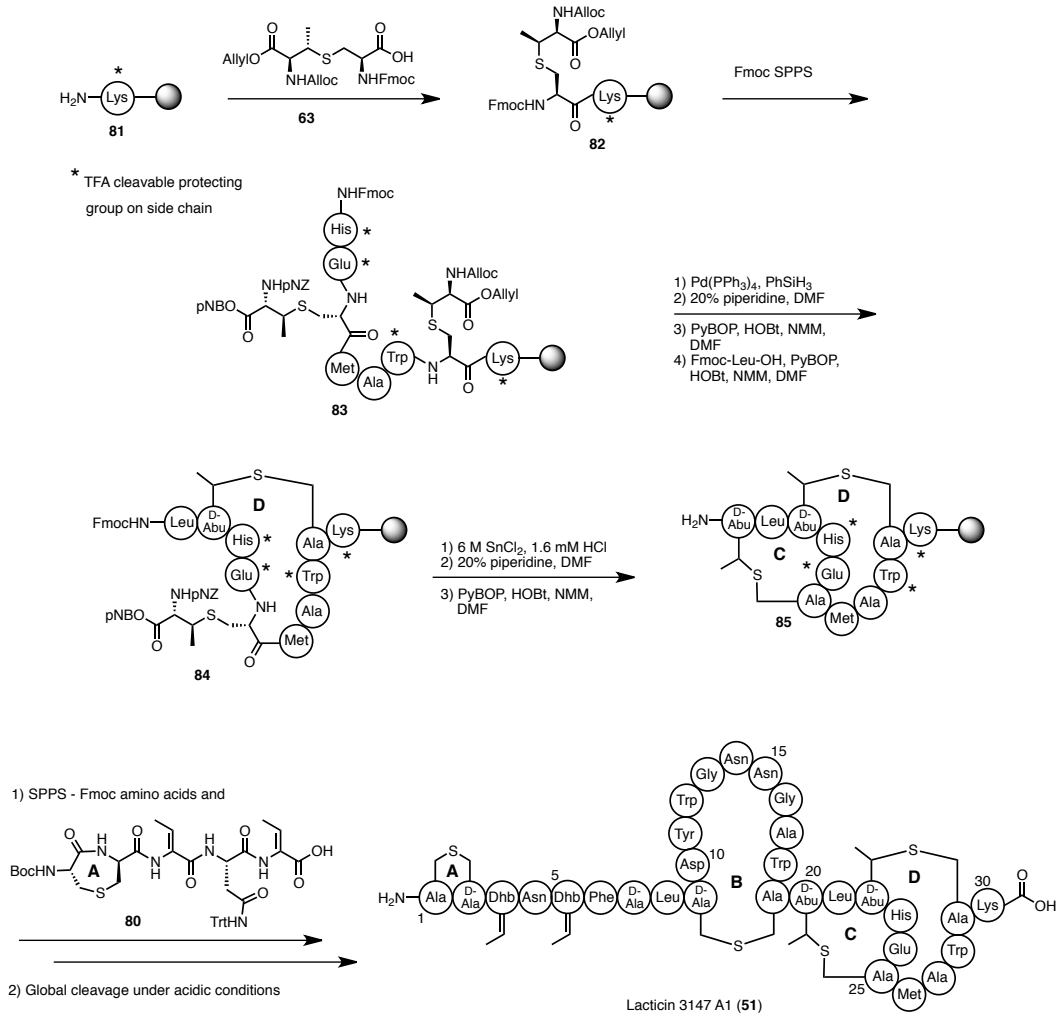
Lacticin 3147 A1 (**51**) and A2 (**52**) is a two-component lantibiotic, consisting of two peptides that function synergistically. The total synthesis of these two peptides was very recently published[134] by our laboratory, and represents only the second synthesis of a natural lantibiotic using SPPS. The

synthesis of lactocin S (**98**),[135] described in this thesis, is the first SPPS of a natural lantibiotic. Indeed some of the work detailed later in this dissertation laid the foundation for the synthesis of lacticin 3147 A1 (**51**) and A2 (**52**).

As mentioned previously, the authors utilize a combination of solution and solid phase chemistry to produce each of the natural A1 (**51**) and A2 (**52**) peptides. In addition, analogues of A2 (**52**) with different rings have also been synthesized.[99-101] Once the necessary building blocks were formed in solution, the solid phase syntheses of the lacticin 3147 A1 (**51**) and A2 (**52**) peptides and their analogues (**53**), (**54**) and (**55**) were completed.

1.4.8.a The total synthesis of lacticin 3147 A1 (51)

To commence the SPPS synthesis of lacticin 3147 A1 (**51**), orthogonally protected methyllanthionine **63** was coupled onto 2-chlorotrityl resin preloaded with a lysine residue (**81**). Using standard Fmoc conditions, the residues of the D-ring (including orthogonally protected methyllanthionine **65** with different protecting groups) were introduced to yield **83**. The *N*-terminal Fmoc was removed along with the allyl/Alloc groups protecting the methyllanthionine of the D-ring, while the groups protecting the methyllanthionine of the C-ring remain intact.

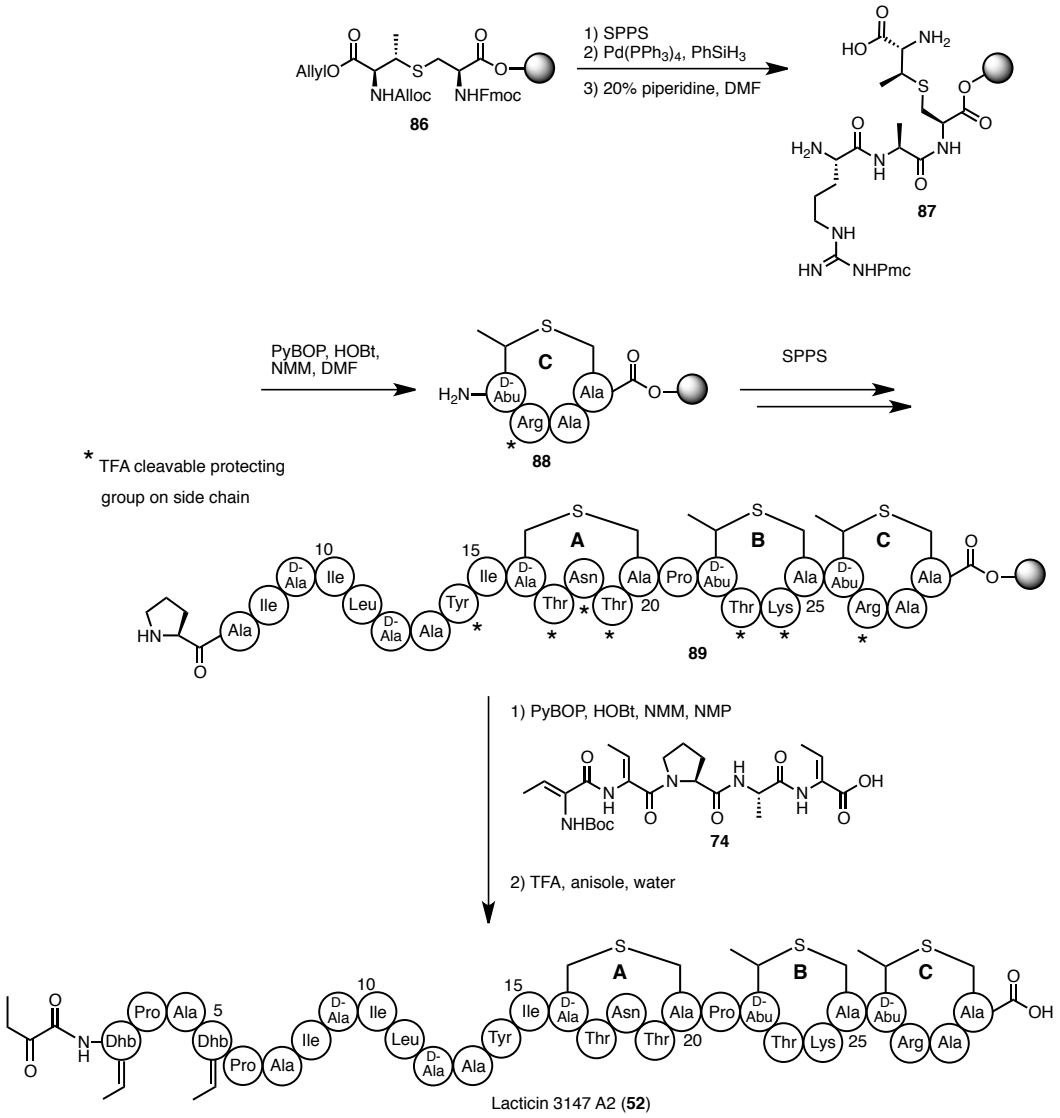


Scheme 1-18 The synthesis of lacticin 3147 A1 (**51**)

Cyclization was affected using PyBOP and HOBt as coupling agents, and then Fmoc-Leucine was introduced to give **84** containing the D-ring of lacticin 3147 A1 (**51**). Removal of the protecting groups on the methyllanthionine of the C-ring and the *N*-terminus, followed by cyclization, gave **85** with the interlocked C/D-ring system in place. In a similar fashion the remaining amino acids were introduced and the final 5 residues were incorporated via pentapeptide **80**. The full-length peptide was cleaved from the resin under acidic conditions to give **51**.

1.4.8.b The total synthesis of lacticin 3147 A2 (52)

The synthesis of lacticin 3147 A2 (**52**) follows a similar approach to that of A1 (**51**), albeit simpler as there is no interlocking ring system. The solid phase synthesis began with the installation of orthogonally protected methyllanthionine (**63**) onto 2-chlorotrityl resin (**86**) (Scheme 1-19).



Scheme 1-19 Synthesis of lacticin 3147 A2 (**52**)

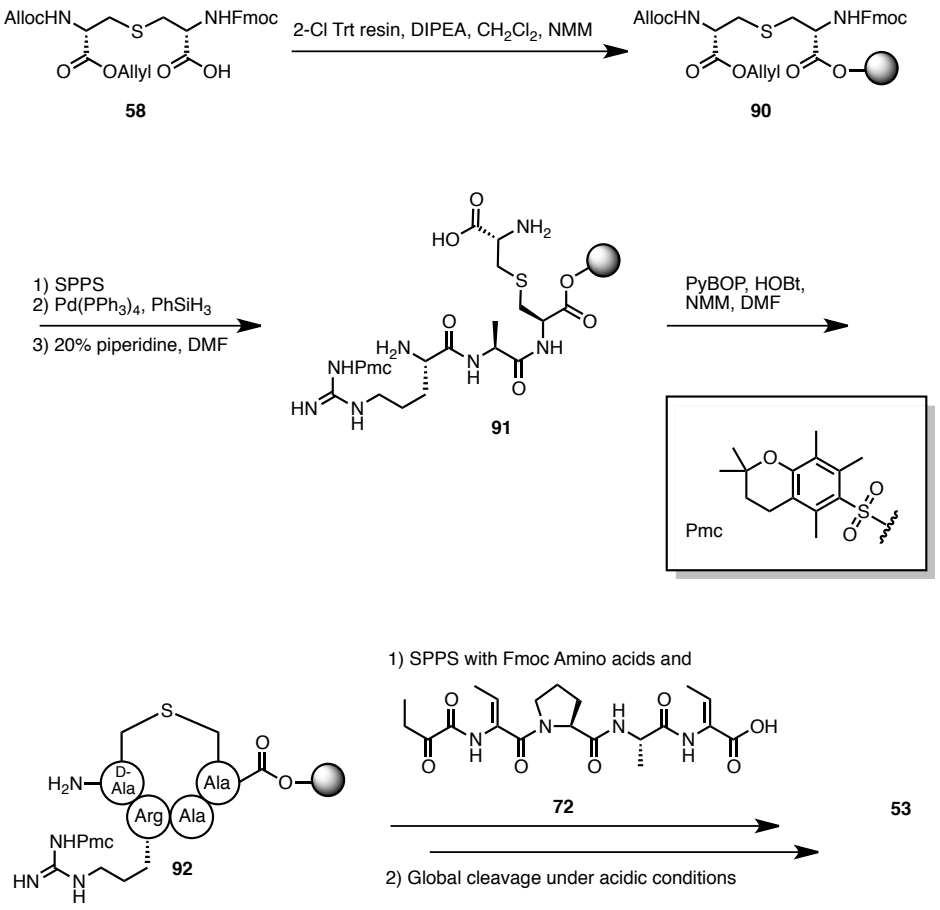
Following introduction of the residues in the C-ring of lacticin 3147 A2 (**52**), the *N*-terminal Fmoc protecting group was removed along with the allyl/Alloc groups on the methyllanthionine to give **87**. Cyclization was affected using PyBOP and HOBt as coupling reagents, thus forming the C-ring (**88**) on resin. The remaining rings and amino acids of **89** were added using the same

approach and then pentapeptide **74** was coupled to the resin-bound peptide. Treatment with TFA in the presence of water and anisole removed the peptide from resin and completed the synthesis of lacticin 3147 A2 (**52**) by forming the α-ketoamide.

The biological activity of synthetically produced lacticin 3147 A1 (**51**) and A2 (**52**) was compared to that of A1 (**51**) and A2 (**52**) isolated from the natural producer organism. The compounds were found to display the same behavior. The authors were also able to confirm the stereochemistry of the lanthionine (**17**) and methyllanthionine (**18**) residues in both peptides by using chiral gas chromatography.[134]

1.4.8.c The total synthesis of lan-lacticin 3147 A2 (53)

A lanthionine analogue (**53**) of lacticin 3147 A2 has been synthesized via SPPS using orthogonally protected lanthionine (**58**) in place of methyllanthionine.[99,120,129] Following attachment of orthogonally protected lanthionine **58** on to 2-chlorotrityl resin to give **90**, the residues of the C-ring were installed via SPPS (Scheme 1-20). Then the orthogonal protecting groups allyl and Alloc were removed along with the *N*-terminal Fmoc protection. Linear peptide **91** was treated with PyBOP and HOBt as coupling agents and the C-ring of lan-lacticin 3147 A2 (**53**) was formed.



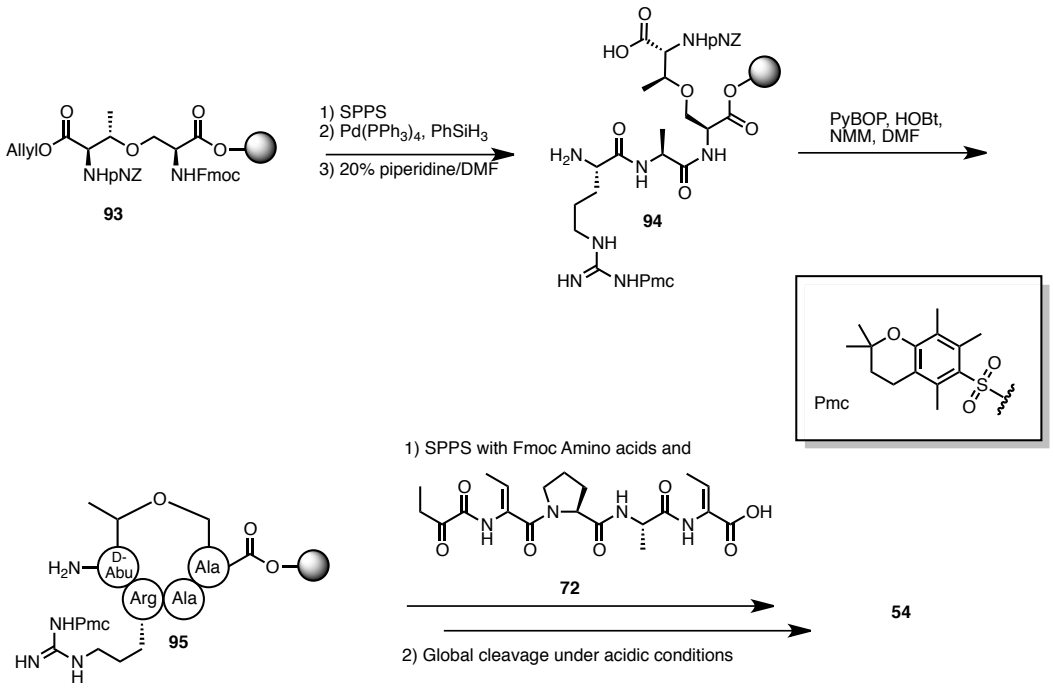
Scheme 1-20 Synthesis of lan-lacticin 3147 A2 (**96**)

In a similar manner, the remaining rings and residues were incorporated until residue 6 and then the final five amino acids were added as the pentapeptide **72**. Following the cleavage of the peptide from resin, lan-lacticin 3147 A2 (**53**) was obtained. When tested for biological activity it was found to be considerably less active than the native peptide **52**. Intriguingly, although the independent activity of lan-lacticin 3147 A2 (**53**) was essentially abolished, the synergistic activity of the peptide when combined with lacticin 3147 A1 (**51**) was still observed, although at a concentration 100 fold higher than the natural peptide.

The authors propose that the analogue **53** may no longer be able to bind and sequester lipid II but in combination with **51** it is still able to form pores in the cell membrane and exert its killing effect via membrane depolarization.[99]

1.4.8.d The total synthesis of oxa-lacticin 3147 A2 (54)

An oxygen-containing analogue (**54**) of lacticin 3147 A2 was also synthesized. The synthesis of oxa-lacticin 3147 A2 (**54**) was completed in the same manner as for lan-lacticin 3147 A2 (**53**) with the exception that orthogonally protected oxa-lanthionine and oxa-methyllanthionine (**68**) were incorporated to replace lanthionine (**17**) and methyllanthionine (**18**) (Scheme 1-21).

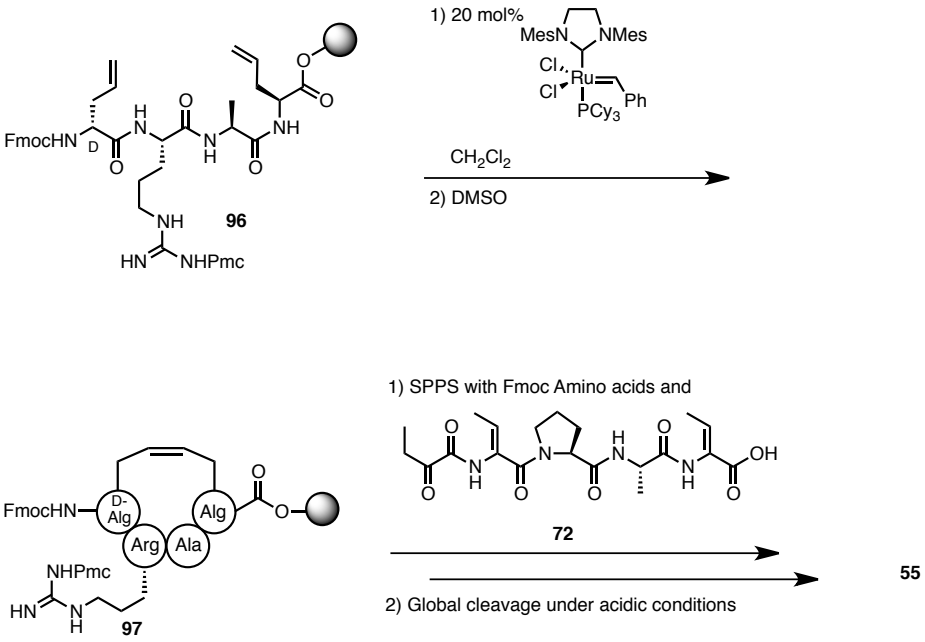


Scheme 1-21 Synthesis of oxa-lacticin 3147 A2 (**54**)

Activity testing of oxa-lacticin 3147 A2 (**54**) found that the analogue was twenty times less active compared to the natural compound. In contrast to lan-lacticin 3147 A2 (**53**), however, the oxa-lacticin 3147 A2 (**54**) no longer displays synergistic activity but is still active alone (albeit at a higher concentration than needed for natural A2 (**52**)). The authors suggest that this may arise because the peptide can still bind to lipid II, possibly via the B and C-rings and so it can sequester lipid II and stop the biosynthesis of peptidoglycan. However, it would appear that the conformational changes brought about by replacing a sulfur with an oxygen may have changed the structure of oxa-lacticin 3147 A2 (**54**) sufficiently that it can no longer recognize or bind to a complex of natural A1 (**51**) and lipid II that would subsequently go on to form pores.[101]

1.4.9 The synthesis olefin-lacticin 3147 A2 using ring closing metathesis

The research group of Liskamp has published the synthesis of several truncated analogues of nisin Z in which the sulfur in lanthionine and methyllanthionine has been replaced with ring expanded alkene[115,118], alkyne[116,119] and alkane[117] functionalities. These peptides were all made utilizing ring closing metathesis.[136] To date no full-length synthetic analogues of nisin A/Z (**16a,b**) have been published.



Scheme 1-22 Synthesis of olefin-lacticin 3147 A2 (**55**)

The third lacticin 3147 A2 (**52**) analogue synthesized was olefin lacticin 3147 A2 (**55**) and its synthesis also utilized ring-closing metathesis.[100] Using SPPS, L- and D-allylglycine were incorporated into a linear peptide along with the other residues in the C-ring of olefin-lacticin 3147 A2 (**55**). Once tetrapeptide **96** was formed, on resin metathesis was done using Grubb's 2nd generation catalyst to give the cyclized C-ring **97** (Scheme 1-22). The same approach was used to introduce the remaining rings and residues of the peptide. The final five amino acids were added as pentapeptide **72**.

Table 1-3 A comparison of lacticin 3147 A2 (**52**) and its analogues

Peptide	Number	Modification	Relative Activity
lacticin 3147 A2	**52**	N/A	++ (alone) +++ (with A1)
lan-lacticin A2	**53**	B, C-rings lanthionine	- (alone) + (with A1)
oxa-lacticin A2	**54**	A-ring oxa-lanthionine and B,C-rings oxa-methyllanthionine	+ (alone) - (with A1)
olefin-lacticin A2	**55**	A,B,C-rings olefin carbocycles	- (alone) - (with A1)

After removal from the resin, full-length olefin-lacticin 3147 A2 (**55**) was obtained and tested for antibacterial activity. The peptide was found to lack any biological activity against the microorganisms that were used for testing (Table 1-3). This may be because the increased ring size of the alkene alters the conformation of the peptide. It could also be that the absence of the methyl group of the methyllanthionine (**18**) residues negatively impacts activity.

1.5 Thesis Overview

This thesis describes research into the structure and synthesis of antimicrobial peptides. It is important to understand not only what structures are responsible for the activity of these peptides but also how they can be made.

Chapter 2 discusses how the primary structure of the lantibiotic peptide lactocin S (**98**) was confirmed (Figure 1-10). By using a combination of chemical synthesis, biological testing, chiral gas chromatography and mass spectral techniques, it was possible to determine that the correct structure was assigned. A highly simplified procedure for the isolation of lactocin S (**98**) from its producing organism *Lactobacillus sakei* L45 was also developed.

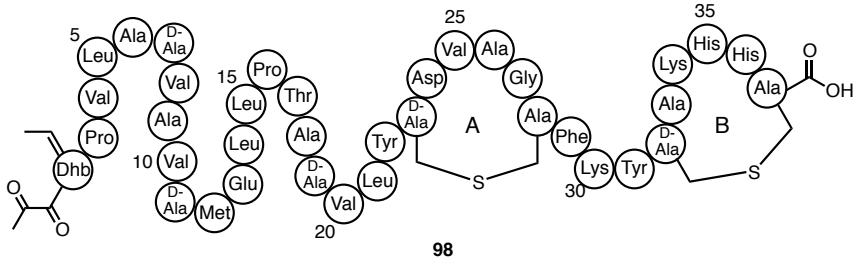


Figure 1-10 Structure of lactocin S (**98**)

Chapter 3 describes the synthesis of analogues of lactocin S (**98**). These compounds were designed to improve understanding of the structure-activity relationship of the molecule and also with a view to increase both the activity and stability of the peptide. Diaminopimelic acid (**136**) (see page 102) was used as an oxidatively stable replacement for lanthionine (**17**), and the importance of

methionine 12 was probed by replacement with carbon analogues. Finally, the *N*-terminus was altered to investigate the significance of both the dehydrobutyrine residue and the α-ketoamide moiety.

Appendix A encompasses work on the characterization of the structure and biosynthetic machinery of novel polymyxins recently isolated from *Paenibacillus polymyxa* PKB1. D-DAB_3-polymyxin B_1 (**A-5**) and D-DAB_3-polymyxin B_2 (**A-6**) are variants of polymyxin B_1 (**A-1**) and B_2 (**A-2**), where the residue at position three has D- rather than L-stereochemistry. This was determined by hydrolysis, derivatization, and chiral gas chromatography-mass spectrometry analysis.

Chapter 2: Lactocin S - Synthesis and Structure Confirmation

2.1 Background

2.1.1 Lactocin S isolation and structure proposal

Nes and co-workers identified a strain of *Lactobacillus sakei*, isolated from fermented sausage, which inhibited the growth of related strains of *Lactobacillus*, *Leuconostoc* and *Pediococcus*.[137] They were able to identify and purify a bacteriocin that was responsible for the antibacterial activity described and they named it lactocin S (**98**).[138-144] Unfortunately, the peptide is only produced in very small amounts (21 μg/L) by the bacteria so structural characterization has been challenging. They proposed a structure for the peptide based upon several different experiments.[61,139]

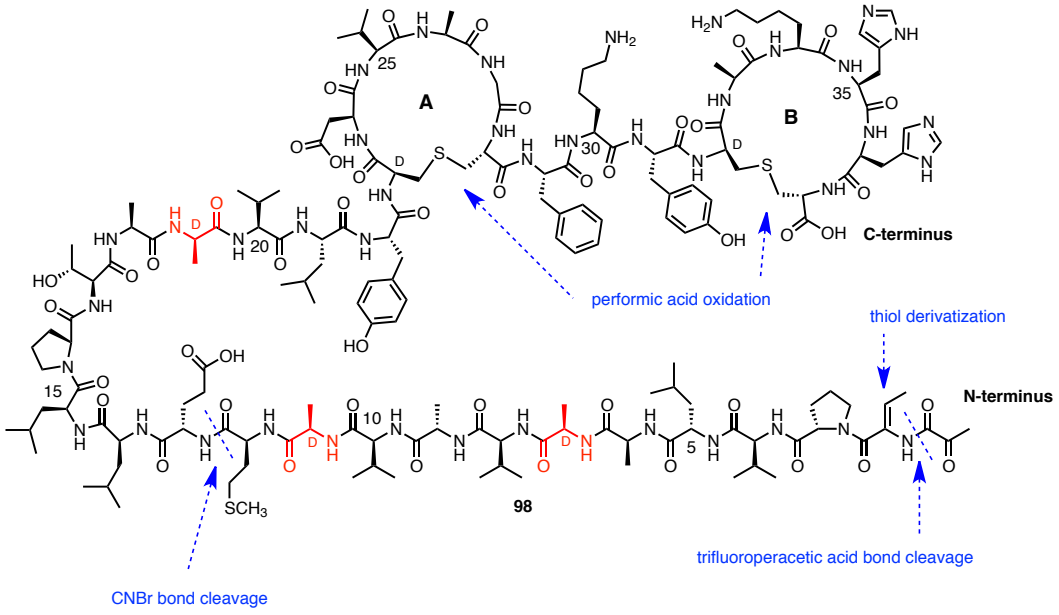


Figure 2-1 Proposed structure of lactocin S (**98**). Blue notation shows points of chemical derivatization to determine structure. Red denotes parts of molecule with inconsistent genetic and Edman sequences.

The *N*-terminus of the peptide is blocked to Edman degradation because of an unusual α-ketoamide and so to obtain sequence information, the peptide was cleaved at methionine 12 using cyanogen bromide (Figure 2-1). Nes and coworkers were then able to determine the first 25 amino acid residues (12-36) in the *C*-terminus of lactocin S (**98**) using Edman sequencing.[139] The lanthionine residues were identified as 'cysteine-like' following a performic acid oxidation that provides information on the number of cysteic acid residues formed.[139] By using trifluoroperacetic acid to cleave off the α-ketoamide, and propanethiol to attack the dehydroamino acids by Michael addition, the *N*-terminally blocked

peptide is rendered susceptible to Edman sequencing and the remaining residues (2-12) were identified.[145]

A portion of the determined amino acid sequence was used to build a DNA template that allowed Nes and coworkers to identify a restriction fragment containing the structural gene (*lasA)* for lactocin S (**98**).[61] Once the DNA was cloned and sequenced[61,146] the authors were able to confirm their previously proposed amino acid assignments and to predict that the *N*-terminus was capped by a dehydrobutyrine-α-ketoamide.[61] The genetic sequence also implied that residues 7, 11 and 19 should be L-serine, however, Edman degradation had identified these residues as alanines. Hydrolysis of lactocin S to its constituent amino acids and then subsequent derivatization as pentafluoropropanamide isopropyl esters, facilitated chiral gas chromatography mass spectrometry analysis of the alanine stereochemistries. Three alanine residues have D-stereochemistry, suggesting that positions 7, 11 and 19 undergo a post-translational modification of L-serine to D-alanine.

2.1.2 Biosynthetic gene cluster of lactocin S

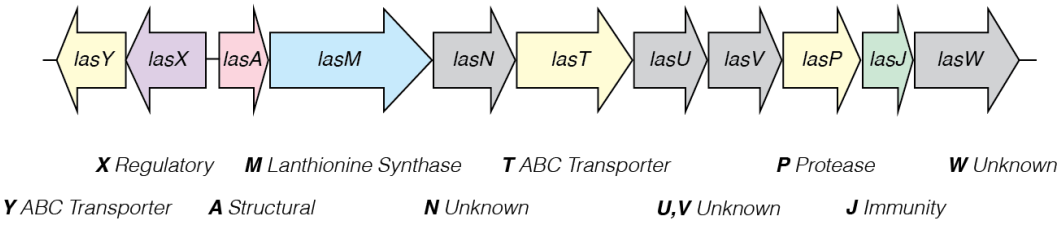


Figure 2-2 Lactocin S (**98**) biosynthetic gene cluster

The biosynthetic gene cluster for lactocin S (**98**) has been identified, and some of the genes classified (Figure 2-2).[146-149] The lanthionine (**17**) residues of the peptide appear to be produced by a single dehydration/cyclization enzyme LasM, and the enzymes responsible for cleavage of the leader sequence and export from the cell are thought to be LasP and LasT, respectively. An enzyme that resembles an immunity protein has been dubbed LasJ[149] (also called LasI in an earlier publication[147]). At this point, no gene product has been identified for the conversion of L-serine to D-alanine.[60] A second ABC transporter gene *lasY* has been identified although its function is not currently known.[149] A regulatory gene *lasX* is involved in the control of transcription of the biosynthetic genes *lasA-W*.[148,149] Classification systems for lantibiotics generally focus on the overall structure of the peptide along with which modification enzymes are used (LanM vs LanB and LanC). Lactocin S (**98**) does not fit well into the standard defined categories and is frequently left "unclassified" or is placed in a group of one. As such, discussion of classification is not useful to understanding this peptide. What

is important is a description of the structural features of the peptide along with identification of portions of the molecule that remain uncertain.

2.1.3 Structural uncertainty for lactocin S

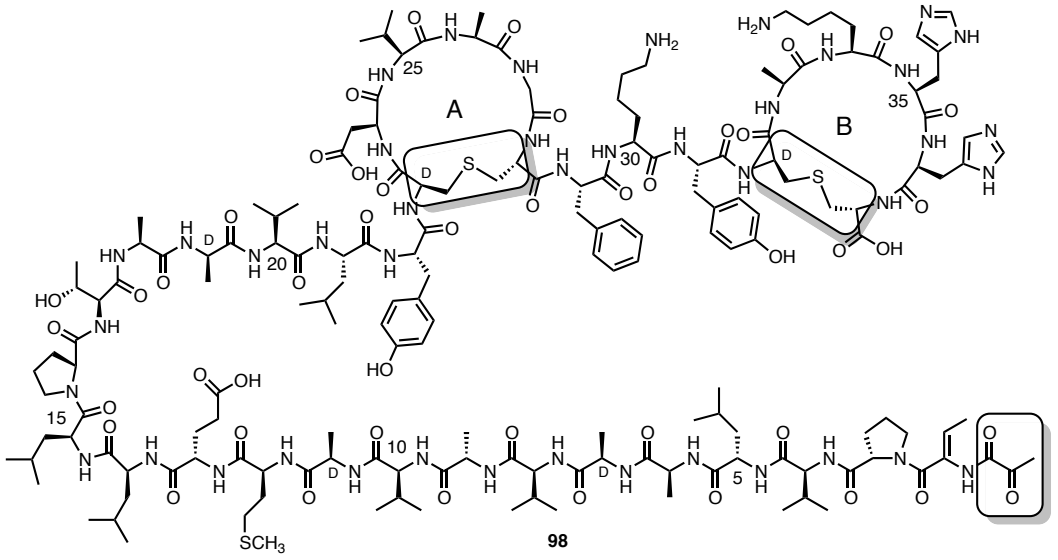


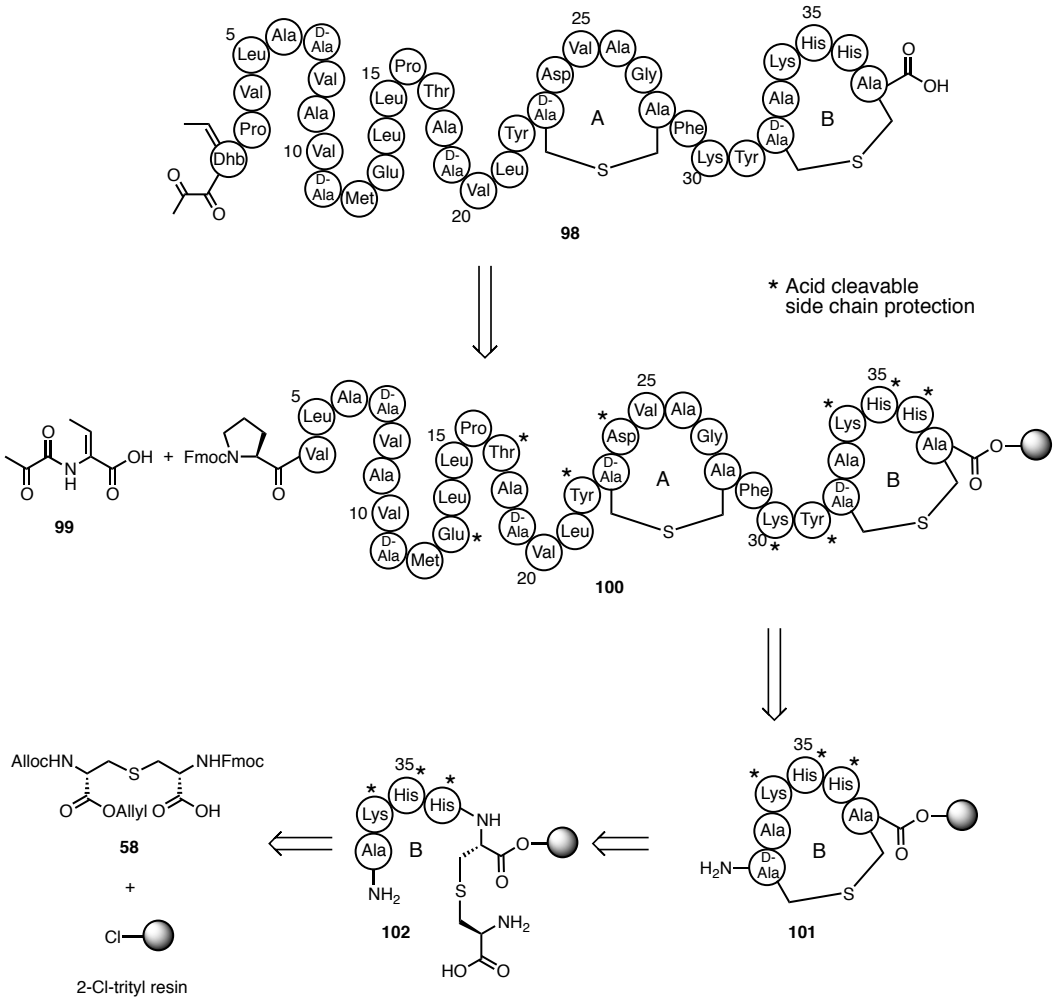
Figure 2-3 Areas of lactocin S (**98**) that are uncertain

Figure 2-3 contains the originally proposed structure for lactocin S (**98**), claimed by Nes and co-workers.[61] This 37 amino acid peptide was proposed to contain two D,L-lanthionine (*meso*-lanthionine) residues in the *C*-terminal half of the peptide, and a flexible and hydrophobic *N*-terminal portion. Within this flexible portion are three D-alanine residues, a dehydrobutyrine, and an *N*-terminal α-ketoamide. The sequencing described above had allowed the authors to determine the identity of almost all of the amino acids present, however, the *N*-

terminal α-ketoamide was merely postulated based upon the genetic sequence and the known instability of dehydroamino acids in this position. A second assumption put forward by the authors was the stereochemistry of the lanthionine residues. Only a limited number of peptides have been investigated for lanthionine (**17**) or methyllanthionine (**18**) stereochemistry and so far they have all had the same D,L-configuration.[150-153] The authors postulated that lactocin S (**98**) would have the same D,L-arrangement as seen previously. Also, because the genetic sequence for the peptide is known, they were able to predict which center in the residue would be L and which would be D. (The center from cysteine should remain L whilst the center derived from serine could be inverted via stereospecific Michael addition to dehydroalanine).

2.1.4 Synthetic attempts to confirm the structure of lactocin S

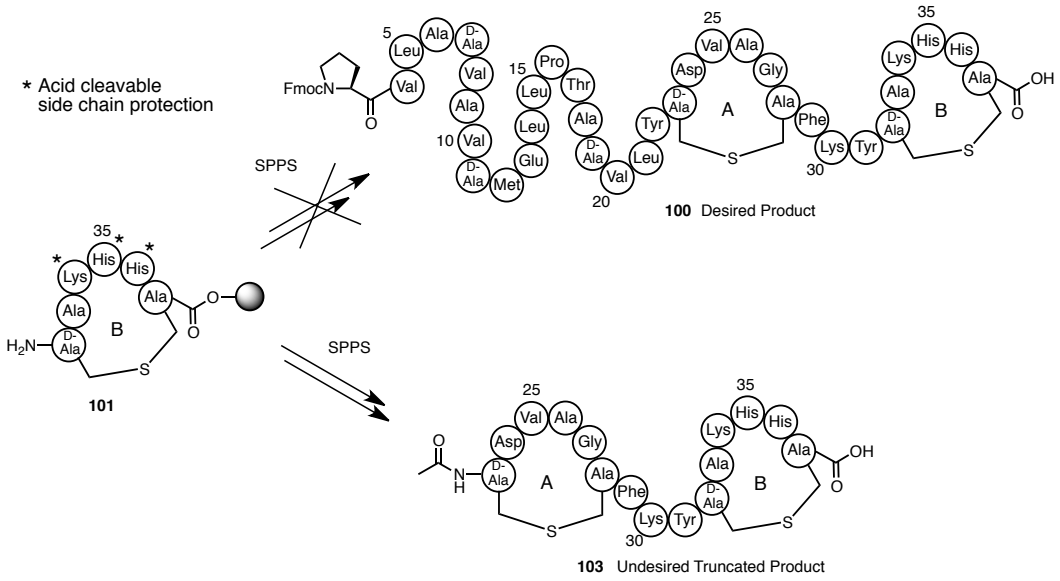
With these uncertainties in mind, former graduate student Vijaya Pattabiraman set out to synthesize lactocin S (**98**) to confirm the proposed structure.[154] Briefly, he proposed to produce the peptide using a combination of solid and solution phase chemistry (Scheme 2-1). The highly modified two residues at the *N*-terminus were to be made in solution, (as was orthogonally protected lanthionine (**58**)) and then each could be incorporated onto the resin at the appropriate point in the synthesis.



Scheme 2-1 Original retrosynthetic approach to lactocin S (**98**)

This approach was very similar to that taken in the synthesis of lan-lacticin 3147 A2 (**53**).[99] Indeed, the same synthesis of orthogonally protected lanthionine **(58)** was employed, and it was incorporated onto the solid support in a similar fashion to produce the B-ring of lactocin S (**101**).[154] After the A-ring was introduced, elongation to include residues 21→3 to give **100** was attempted using

automated and manual procedures (Scheme 2-2). However, both methods were unsuccessful, with a truncated product **103** observed as the major product.



Scheme 2-2 Undesired truncation during attempted synthesis of lactocin S (**98**)

This compound appears to have been end-capped (*N*-terminally acetylated). After each coupling reaction in SPPS, it is standard practice to treat the peptide on resin with a solution of 20% acetic anhydride in DMF. The purpose of this is to acetylate any unreacted terminal amines so that they can no longer be elongated. This minimizes contamination with similarly sized peptides at the end stages of the synthesis. Since the synthesis was only monitored every five or so residues, the exact timing/mechanism of **103** formation is unknown. It may be that the *N*-terminus of the peptide can bend back and react with the carbonyl between tyrosine 22 and lanthionine. This could eliminate a cyclic peptide and leave a free

amine at the N-terminus of the peptide that could be acetylated. The product appears to be formed at some point between addition of residue 15 and residue 9.

At this stage, Dr. Pattabiraman completed his PhD and this project was passed along. As a result of the observations made above, some alterations to the overall project objectives were made.

2.2 Project Objectives

The overall aim of this project is to use total synthesis to confirm the validity of the proposed structure for lactocin S (**98**). It was envisioned that a combination of solution and solid phase chemistry could be utilized. However, preliminary synthetic efforts were hampered by an undesired truncation of the peptide during SPPS as described above. Therefore, before the synthesis can be successfully completed, an investigation into the particulars of this side reaction must take place. The first goal of this project is to identify when the undesired truncation reaction occurs and to develop a synthetic strategy to circumvent this problem. The second major part of this project is to obtain natural lactocin S (**98**), in order to compare it to the synthetic peptide. The isolation procedure published by Nes and coworkers is somewhat tedious, and so the development of a simplified approach would be useful. Finally, once both synthetic and natural lactocin S (**98**) are in hand they will need to be compared, and the important structural features investigated. We proposed the use of both mass spectrometry and biological activity techniques for this analysis.

2.3 Results and Discussion

2.3.1 Synthetic investigation of truncation and a potential solution

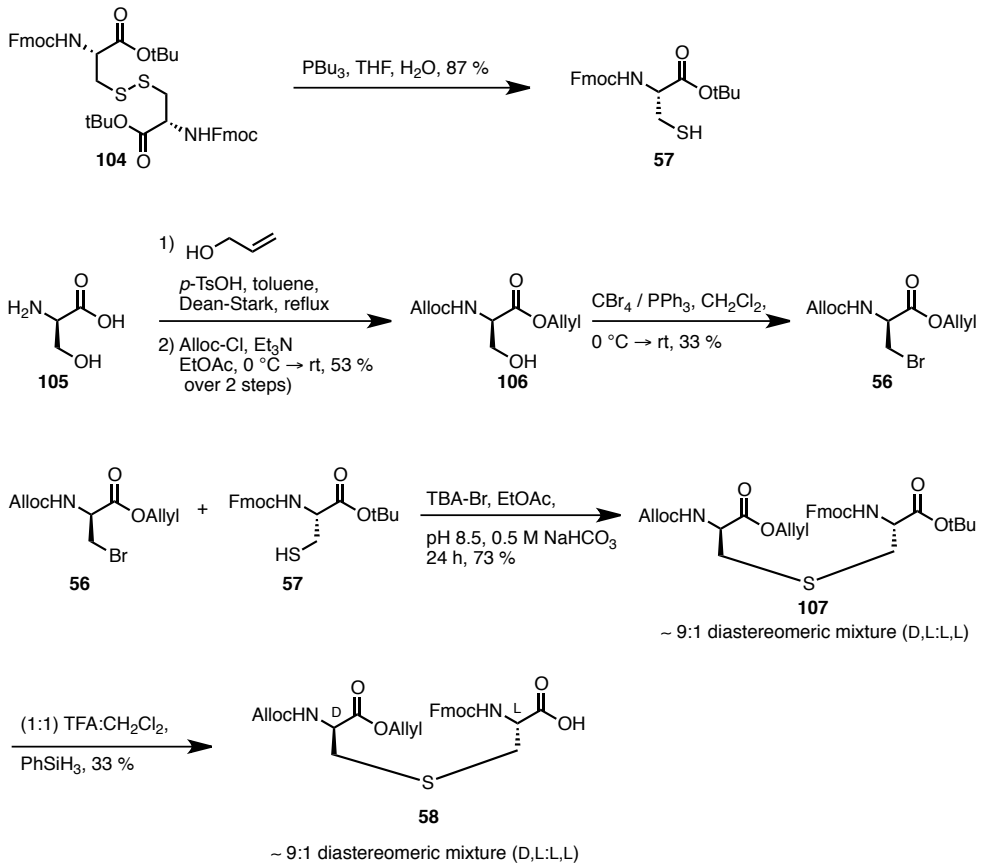
The first goal of this project was to investigate the formation of an undesirable truncated product during the SPPS synthesis of lactocin S (**98**), and to find a way of preventing this. To understand this unwanted reaction, the efforts of Dr. Pattabiraman were duplicated to identify the exact point in the synthesis that the degradation occurs. Based upon information from the previous attempts, it appeared a truncation reaction might occur at some point after the introduction of residue 20, and before the addition of residue 8.

The synthesis of lactocin S (**98**) on resin starts with introduction of orthogonally protected lanthionine (**58**) and the peptide is then elongated through the formation of the bicyclic ring system. At this point, each time a residue is added, a sample of the peptide will be cleaved from the resin and analyzed by MALDI-MS to check for formation of **103**. Before investigating the truncation and a possible alternative synthesis of lactocin S, it was necessary to synthesize orthogonally protected lanthionine (**58**) and incorporate it into SPPS synthesis.

2.3.2 Synthesis of orthogonally protected lanthionine and the A,B-rings of lactocin S

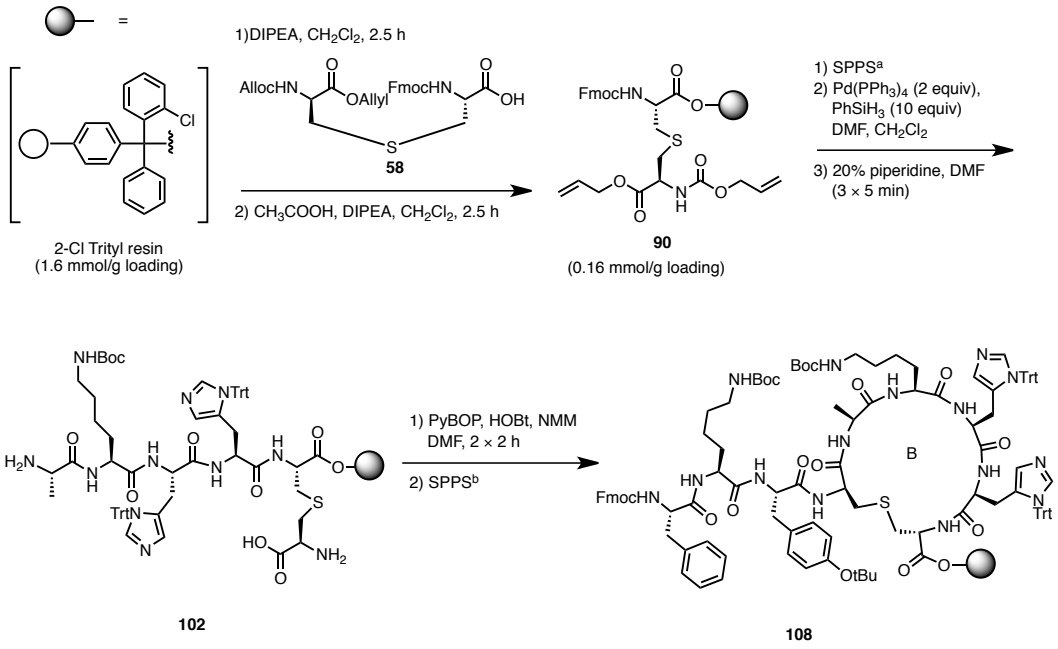
This synthesis follows a literature procedure that was used to make orthogonally protected lanthionine for the synthesis of lan-lacticin 3147 A2

(**53**).[99,120,129] Fmoc/tBu protected cysteine (**57**) was obtained from the corresponding cystine **104** by treatment with PBu_3 in the presence of water in THF (Scheme 2-3). The other half of the lanthionine was formed from D-serine (**105**). The carboxylic acid was converted to an allyl ester by reaction with allyl alcohol and *p*-toluenesulfonic acid. The amine was then protected as an allyloxy carbamate (**106**) by treatment with allyl chloroformate and triethylamine. The Appel reaction was used to convert the alcohol of **106** to the corresponding bromide (**56**), with carbon tetrabromide and triphenylphosphine, providing the second coupling reagent for lanthionine formation.



Scheme 2-3 Synthesis of orthogonally protected lanthionine **(58)**

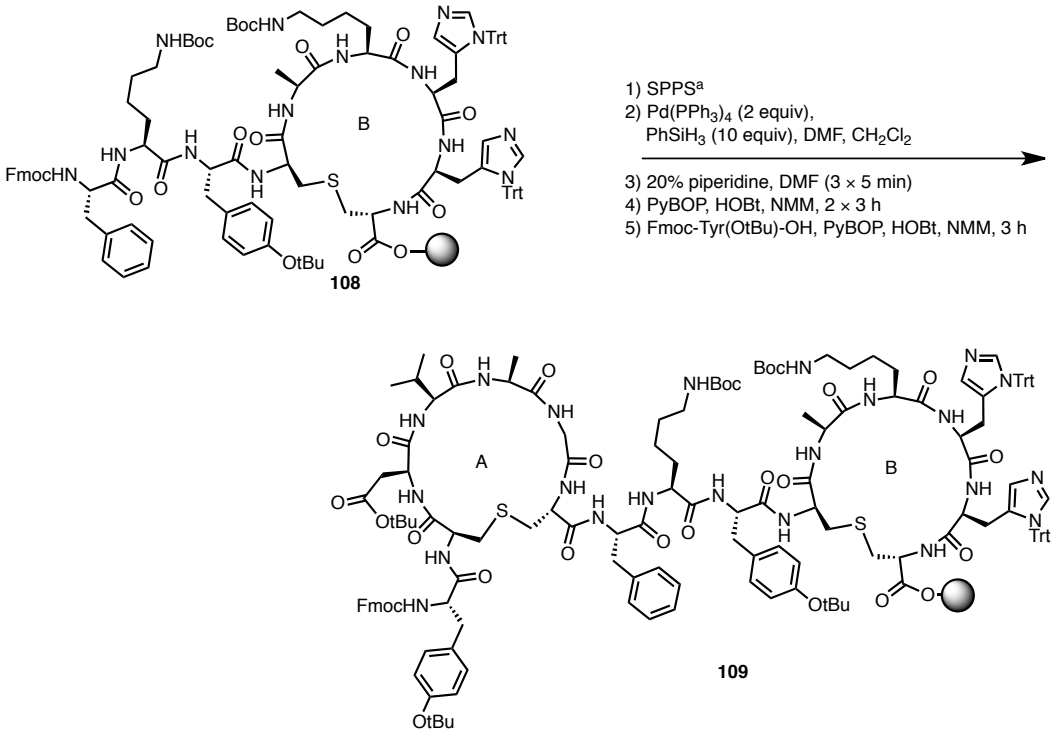
The key step in this synthesis is the reaction of protected cysteine **57** with the 3-bromo D-alanine **56** using phase transfer conditions. While this transformation proceeds in good yield, it does have a drawback: a small amount of an undesired diastereomer is formed. This happens because under the basic conditions of the reaction, some of the 3-bromo D-alanine eliminates to give a dehydroalanine, and non-selective Michael addition by **57** then produces **107** with both D- and L-stereochemistry at the α carbon that was formerly only D.



Scheme 2-4 Loading of orthogonally protected lanthionine **58** onto resin and synthesis of the B-ring of lactocin S (**108**). [a] Conditions for SPPS: (i) 20% piperidine in DMF, (ii) Fmoc-His(Trt)-OH, PyBOP, HOBt, NMM, (iii) Repeat steps (i) and (ii) for Fmoc-His(Trt)-OH, Fmoc-Lys(Boc)-OH and Fmoc-Ala-OH. [b] Conditions for SPPS: (i) Fmoc-Tyr(OtBu)-OH, PyBOP, HOBt, NMM, (ii) 20% piperidine in DMF, (iii) Repeat steps (i) and (ii) for Fmoc-Lys(Boc)-OH and then step (i) for Fmoc-Phe-OH

To initiate the SPPS of lactocin S (**98**), orthogonally protected lanthionine **58** was coupled onto 2-Cl-trityl resin (Scheme 2-4). The initial loading of the resin was 1.6 mmol/g. This proved to be too high for the purposes of this synthesis and so the amount of lanthionine **58** coupled was kept to 0.16 mmol/g. Unreacted sites were capped with acetic acid. The reason a reduced loading is important is that when the A-ring is cyclized, the peptides can either react intramolecularly (desired) or intermolecularly (undesired). If the loading on the resin is lower, it encourages intramolecular over intermolecular bond formation.

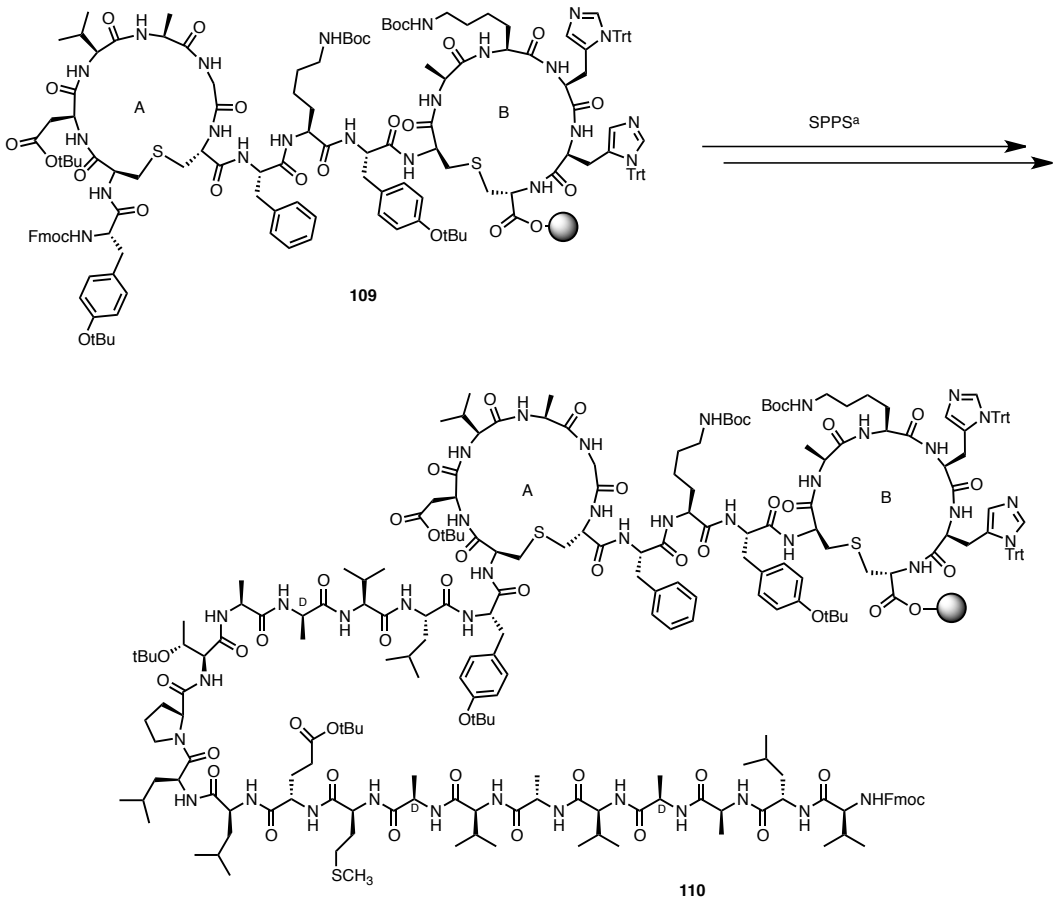
Once orthogonally protected lanthionine was installed on resin, the amino acids found within the B-ring of lactocin S could be added using standard Fmoc SPPS protocols. To prepare the peptide for on-resin cyclization, the orthogonal protecting groups, allyl and Alloc were removed with $Pd(Ph_3)_4$, yielding a free acid and amine. The *N*-terminus of the peptide was deprotected using 20% piperidine in DMF. This yields **102** ready for cyclization to form the B-ring. The resin was treated with the coupling conditions of PyBOP, HOBt and NMM and cyclization occurs between the *N*-terminal amine of alanine 33 and the free acid of the lanthionine residue. The remaining free amine of lanthionine becomes the new *N*-terminus of the peptide, and SPPS was used to incorporate the three residues that reside between the A- and B-rings to give **108**. A second equivalent of orthogonally protected lanthionine **58** was then installed on resin and SPPS used to introduce the residues found within the A-ring of lactocin S (Scheme 2-5). The A-ring was then prepared in the same fashion as the B-ring. Desired bicyclic compound **109** was obtained by introducing Fmoc-Tyr(OtBu)-OH. At this point the peptide on resin was ready for the in-depth investigation of the undesired truncation reaction.



Scheme 2-5 Continued SPPS for the synthesis of **109**. [a] Conditions for SPPS: (i) 20% piperidine in DMF, (ii) **58**, PyBOP, HOBt, NMM, (iii) Repeat steps (i) and (ii) for Fmoc-Gly-OH, Fmoc-Ala-OH, Fmoc-Val-OH, Fmoc-Asp(OtBu)-OH.

2.3.3 Elongation study of lactocin S to isolate point of degradation

The Fmoc protecting the *N*-terminus was removed with 20% piperidine in DMF (Scheme 2-6). Then the next residue, Fmoc-Leu-OH, was coupled using PyBOP and HOBt.



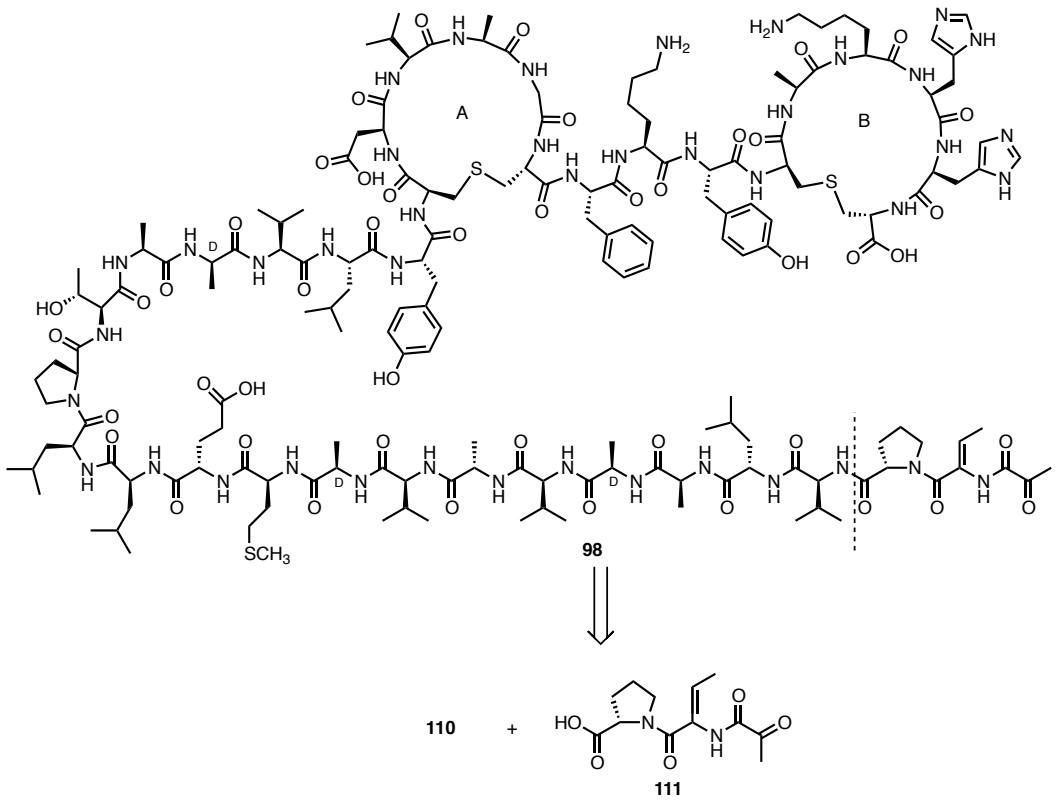
Scheme 2-6 Investigation of the SPPS degradation of lactocin S. [a]Conditions for SPPS: (i) 20% piperidine in DMF, (ii) Fmoc-Leu-OH, PyBOP, HOBt, NMM, (iii) Cleave a small sample with TFA:H_2O:TIPS 95:2.5:2.5 (iv) MALDI-TOF MS analysis (v) Repeat steps (i)-(iv) for Fmoc-Val-OH, Fmoc-D-Ala-OH, Fmoc-Ala-OH, Fmoc-Thr(OtBu)-OH, Fmoc-Pro-OH, Fmoc-Leu-OH, Fmoc-Leu-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Met-OH, Fmoc-D-Ala-OH, Fmoc-Val-OH, Fmoc-Ala-OH, Fmoc-Val-OH, Fmoc-D-Ala-OH, Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Val-OH.

Then a small sample of resin was treated with TFA:H_2O:TIPS 95:2.5:2.5 to cleave the peptide from the resin. MALDI-TOF mass spectrometry was used to analyze the sample, looking for the presence of both the desired peptide and any truncated peptide **103**. Following a positive identification of the desired peptide, the remaining peptide immobilized on resin was treated with a solution of 20%

acetic anhydride in DMF to endcap any unreacted free amines. The cycle began again with the *N*-terminal Fmoc protecting group being removed. All coupling solutions were saved until the MS analysis could confirm the absence of truncation product **103**. In this manner, the amino acids from residue 20 to residue 4 were introduced without the detection of **103** by mass spectrometry. Based upon the observations described in Section 2.1.4, this is an unexpected result, albeit a useful one. It remains unclear how **103** was formed in previous attempts. Because the elongation proceeds smoothly, it is possible to continue directly with the total synthesis of lactocin S (**98**).

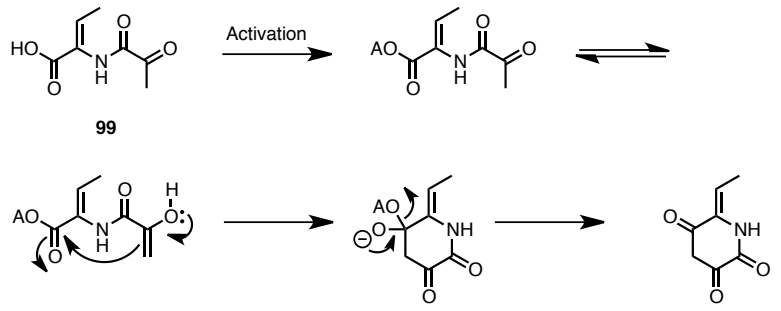
2.3.4 Synthesis of the *N*-terminal portion of lactocin S (98)

The synthesis of lactocin S (**98**) was now nearly complete and all that was required was to form the *N*-terminal portion of the peptide and to couple it onto the rest of the peptide that is immobilized on the resin. Scheme 2-7 shows an altered retrosynthetic approach to lactocin S in which a tripeptide (residues 1-3) is made in solution. This is in contrast to the dipeptide **99** previously proposed.



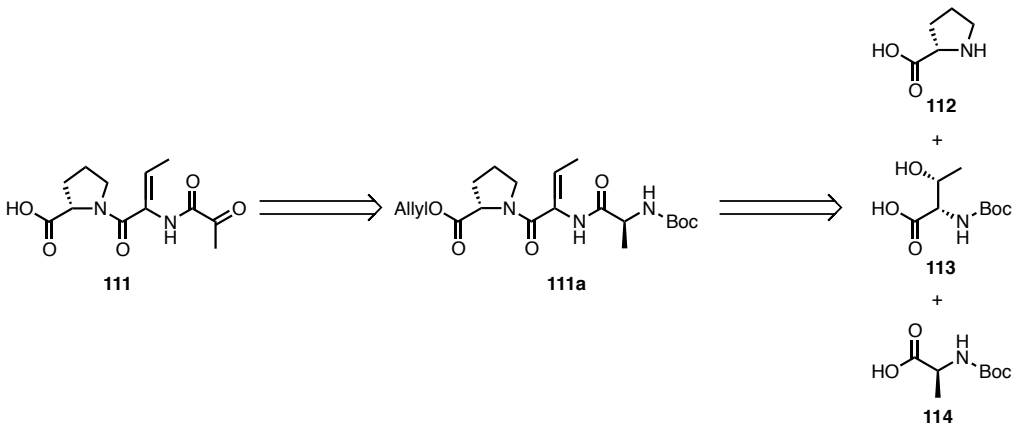
Scheme 2-7 Revised retrosynthetic approach to lactocin S (**98**)

It seemed possible that coupling to the more hindered *N*-terminal proline (residue 3) might be difficult. Additionally, a possible side reaction might take place when the originally proposed dipeptide **99** is activated for coupling. The *N*-terminal ketone could tautomerize to an enol and then attack through its terminal carbon at the carbonyl of the activated acid thus forming a six membered ring (Scheme 2-8).



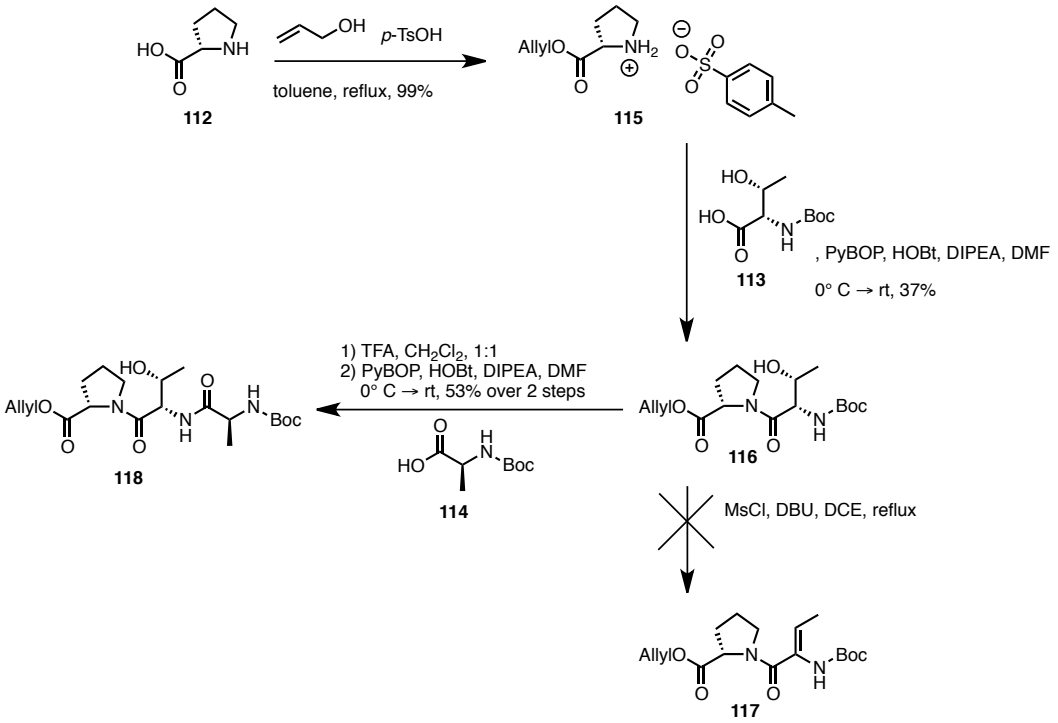
Scheme 2-8 Potential side reaction for dipeptide **99**. "A" represents an activating group.

For these reasons, an *N*-terminal tripeptide was designed instead. The α-ketoamide functionality of **111** could be introduced using transamination in a similar fashion to the preparation of **72** that was utilized in the synthesis of lacticin 3147 A2 analogues **53**, **54** and **55**.[99-101] The transamination precursor **111a** could be made from proline (**112**), Boc-Thr-OH (**113**) and Boc-Ala-OH (**114**) (Scheme 2-9).



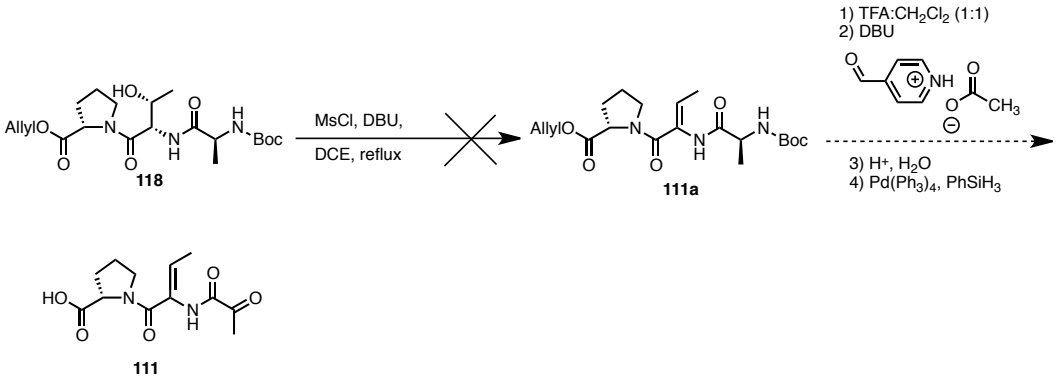
Scheme 2-9 Retrosynthetic approach to *N*-terminal tripeptide **111**

To begin the synthesis of **111**, proline (**112**) was protected as an allyl ester by reaction with allyl alcohol and *p*-toluenesulfonic acid. This protected amino acid (**115**) was then coupled to threonine **113** in the presence of PyBOP, HOBt and DIPEA. At this point of the synthesis a dehydration of threonine was attempted. The hydroxyl group was activated by mesylation with methanesulfonyl chloride, and a dehydration with DBU was undertaken. However, no reaction was observed. In an alternative approach, the *N*-terminal Boc group was removed in acidic conditions using TFA, and then Boc-Ala-OH (**114**) was coupled onto the dipeptide with PyBOP, HOBt and DIPEA.



Scheme 2-10 Synthetic attempts toward *N*-terminal tripeptide **111**

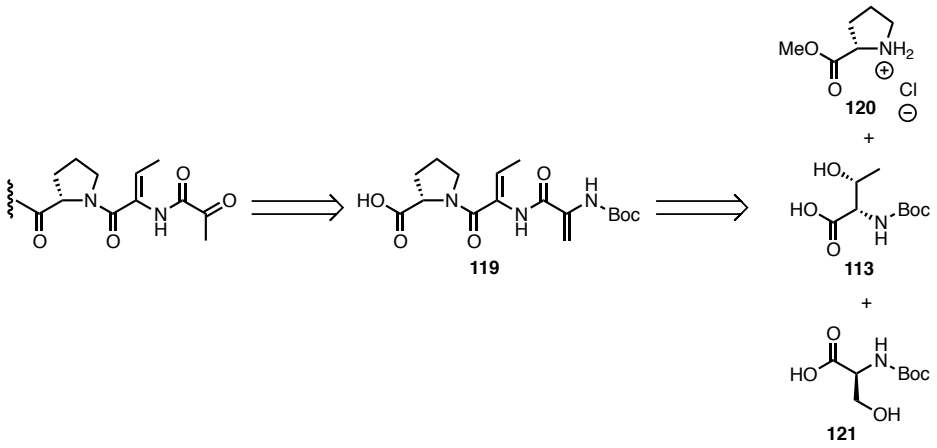
The tripeptide **118** was submitted to mesylation/dehydration conditions with methanesulfonyl chloride and DBU, but the peptide was not converted to the corresponding dehydropeptide **111a**.



Scheme 2-11 Synthetic attempts toward *N*-terminal tripeptide **111**

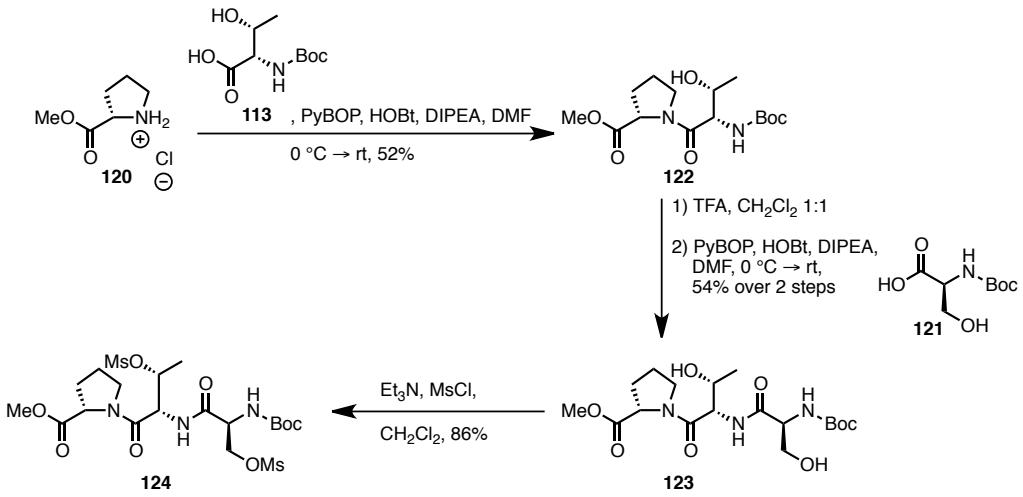
A further revision of the retrosynthetic approach to the synthesis of lactocin S (**98**) was made. Due to synthetic challenges associated with the use of transamination for the formation of the α-ketoamide functionality in **53**, **54** and **55,** namely poor yields, an alternative approach was chosen. Using a biomimetic approach the α-ketoamide can be installed as a masked functionality (Boc-Dha) and then converted to the desired moiety upon exposure to acid and water. This is the same method as was used to make the α-ketoamide in the recent publication[134] of the total synthesis of lacticin 3147 A1 (**51**) and A2 (**52**) (see Scheme 1-15) but it was first developed for this synthesis of lactocin S. Scheme 2-12 shows the retrosynthetic approach to this functionality. Tripeptide **119** could be coupled onto the peptide on resin, and then when the resin is cleaved the *N*-terminal

enamine will be exposed and transformed into the α-ketoamide. Compound **119** could be obtained from the three amino acids **120**, **113** and **121** via coupling and dehydration reactions.



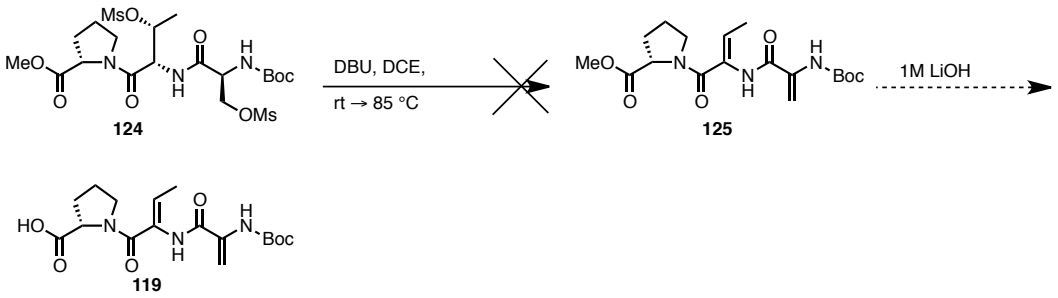
Scheme 2-12 Retrosynthetic approach to an altered *N*-terminal tripeptide **119**

The synthesis of tripeptide **119** was initiated by coupling of proline **120** with threonine **113** using PyBOP, HOBt and DIPEA (Scheme 2-13). Dipeptide **122** was obtained, and the *N*-terminal Boc group was removed by treatment with TFA. Then Boc-Ser-OH (**121**) was introduced by coupling with PyBOP and HOBt to yield tripeptide **123**.



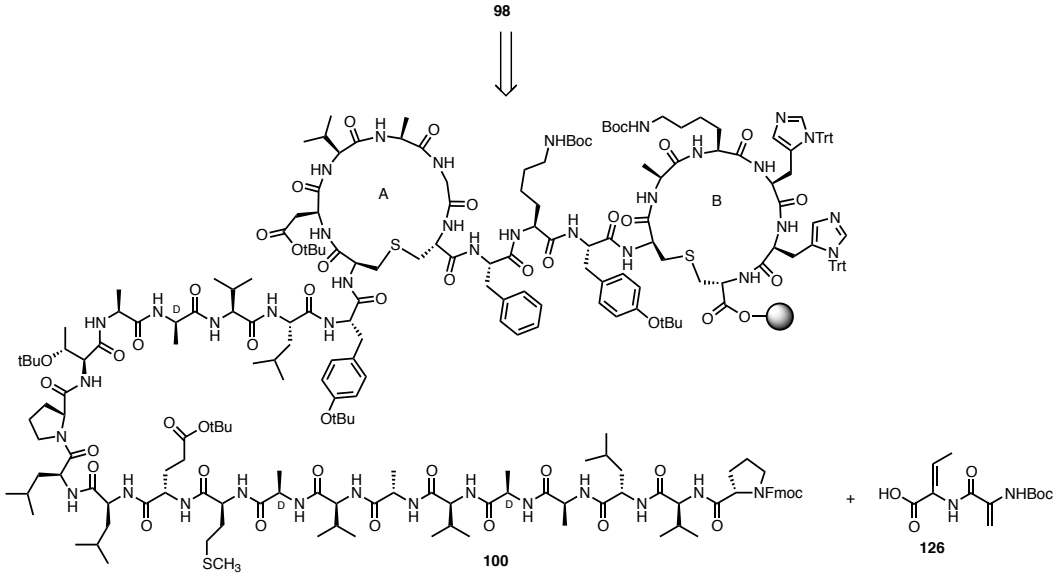
Scheme 2-13 Synthetic attempts toward altered *N*-terminal tripeptide **119**

To form the two dehydro-residues, the alcohols of serine and threonine were converted to the corresponding mesylates by methanesulfonyl chloride in the presence of triethylamine. Elimination of the mesylates in compound **124** was attempted using DBU at elevated temperature, but no desired product was obtained (Scheme 2-14). It may be that the sterically hindering proline residue negatively impacts elimination of the mesylates in this compound. Perhaps DBU is simply too large to attack the proton on the α-carbon of threonine when it is adjacent to a proline residue. However, attempted elimination using unhindered KOH (aq), dibenzo-18-crown-6 in 1,2-dichloroethane (DCE) at elevated temperature also did not give the desired compound **125**.



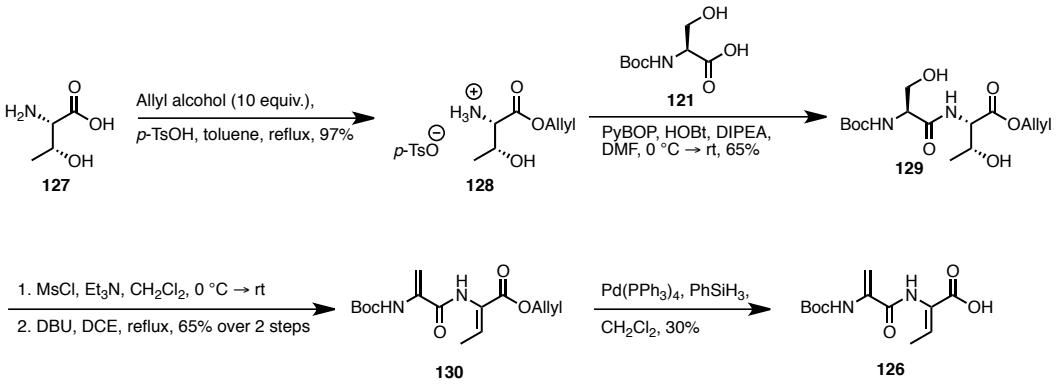
Scheme 2-14 Synthetic attempts toward altered *N*-terminal tripeptide **119**

Due to the challenges faced using proline in the tripeptide, dipeptide **126** (residues 1 and 2) could be used instead, and the proline (residue 3) could be incorporated into the peptide on resin (Scheme 2-15). As the biomimetic methodology uses a masked α-ketoamide in the dipeptide stage there should be no problems with unwanted cyclization as was the concern with **99**.



Scheme 2-15 Retrosynthetic plan for the synthesis of lactocin S (**98**) incorporating an *N*-terminal dipeptide

The synthesis of dipeptide **126** is very similar to the approach taken for tripeptide **119**. Firstly, threonine **127** was protected as the allyl ester **128** by reaction with allyl alcohol and *p*-toluenesulfonic acid. It was then coupled to Boc-Ser-OH **121** using PyBOP, HOBt and DIPEA (Scheme 2-16). The hydroxyl functionalities of this dipeptide (**129**) were converted to mesylates by treatment with methanesulfonyl chloride and triethylamine. Then, under refluxing conditions with DBU, the two amino acids underwent elimination reactions to yield the dehydrated dipeptide **130**. This reaction proceeds smoothly, unlike the previous dehydration attempts with tripeptides **124** and **118** or dipeptide **116**. It appears that proline was indeed the cause of the problem.



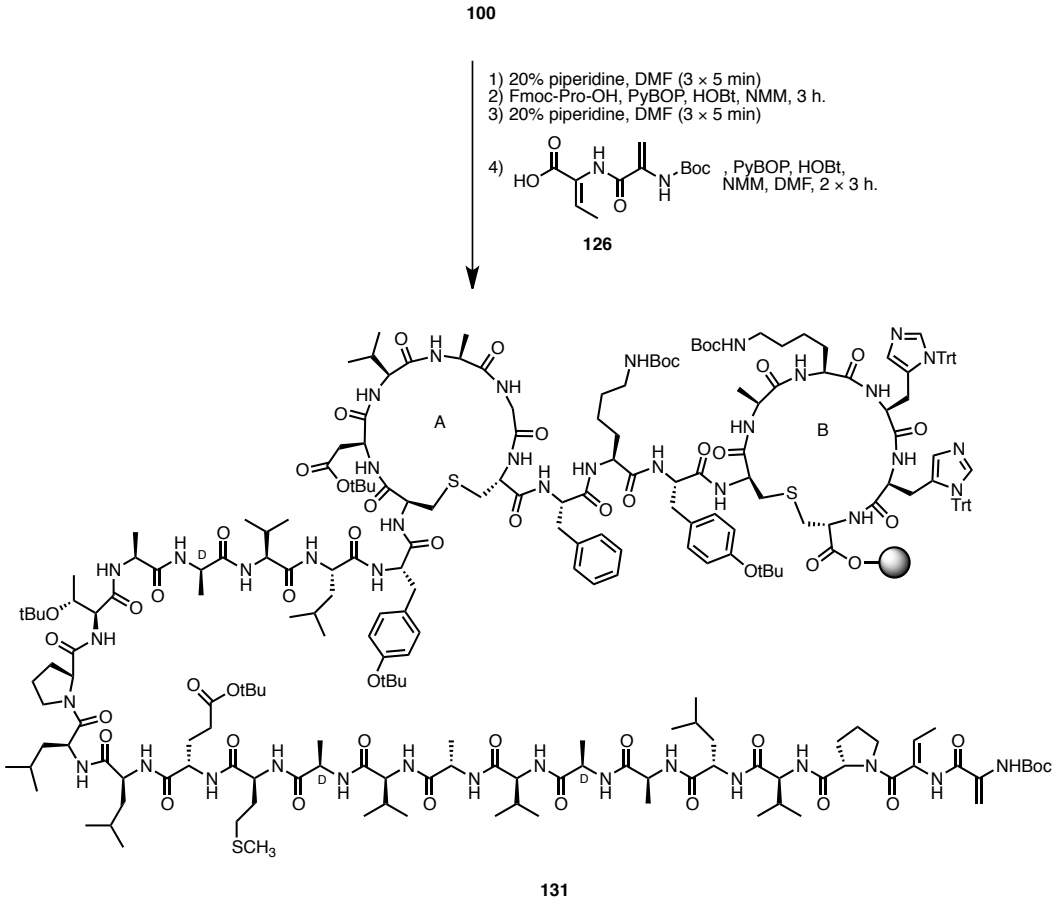
Scheme 2-16 Synthesis of *N*-terminal dipeptide **126**

With **130** in hand, all that remained was to remove the allyl protecting group on the carboxylic acid. This was accomplished using $Pd(PPh_3)_4$ in the

presence of $PhSiH_3$. The resulting free acid **126** is ready for coupling to the peptide immobilized on resin.

2.3.5 Completion of the synthesis of lactocin S

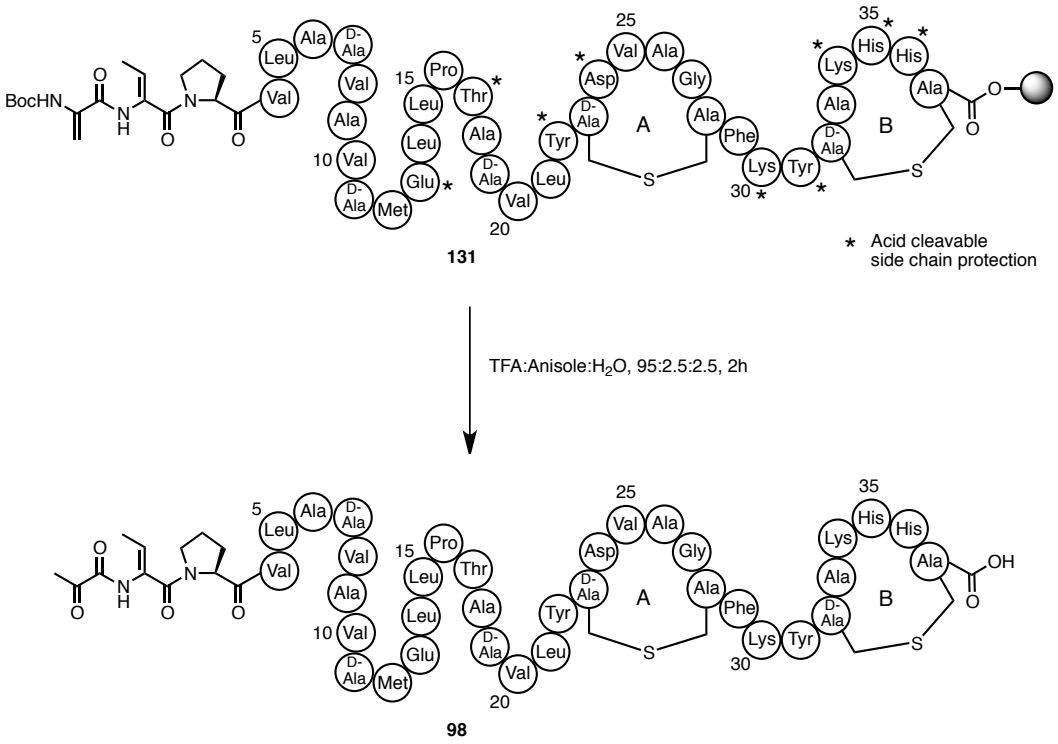
To complete the synthesis of lactocin S (**98**), the peptide immobilized on resin must first be extended to include residue 3 (proline). The *N*-terminal Fmoc protecting group was removed with 20% piperidine in DMF and Fmoc-Pro-OH was coupled onto the peptide with PyBOP, HOBt and NMM (Scheme 2-17). Then, following removal of the Fmoc on proline, the dipeptide representing residues 1 and 2 (**126**) was coupled onto the peptide on resin. The same reagents are used (PyBOP and HOBt). However, as the reaction is more sluggish than a typical coupling reaction, a second round of coupling is necessary to achieve complete conversion to the full-length peptide **131**. Now all that remains is to cleave the peptide from resin to accomplish the synthesis of lactocin S (**98**).



Scheme 2-17 Synthesis of full length lactocin S on resin (**131**)

Total synthesis of lactocin S (**98**) was achieved by cleavage of the full-length peptide from the solid support. Using TFA, with anisole and water as scavengers, the peptide is removed from the resin. Under these conditions, all of the side-chain protecting groups are removed. This includes the acid sensitive Boc group on the *N*-terminus, thus unmasking a primary enamine, that can tautomerize to an iminium ion and be hydrolyzed by the water to give an α-ketoamide. The peptide was purified by reversed phase HPLC using a C2/C18 reversed phase column. A C2/C18 column was used because the hydrophobicity of lactocin S

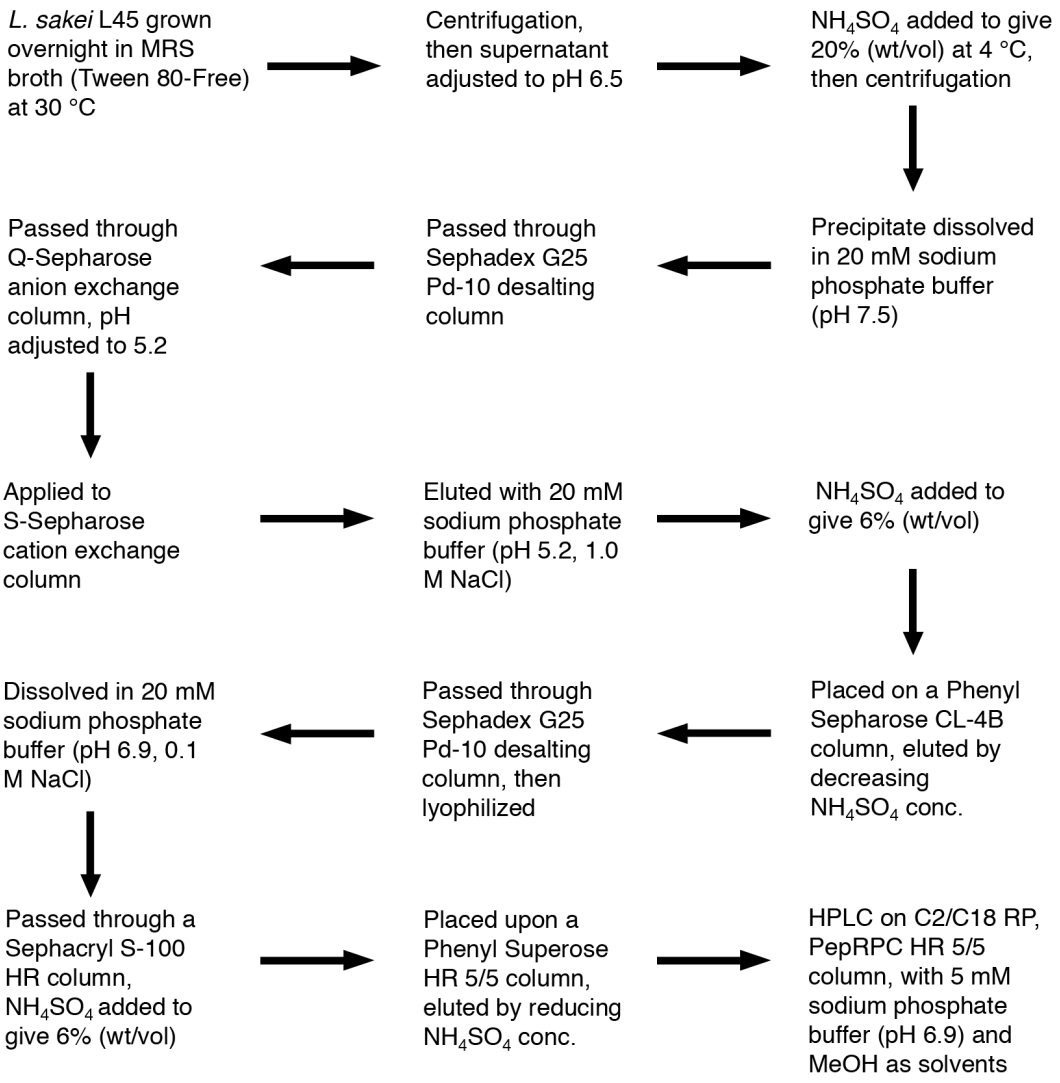
(**98**) prevented elution off a C18 column, even when using 100% organic solvent (acidified acetonitrile, isopropyl alcohol or methanol). Additionally, the oxidative instability of the peptide is such that the HPLC was done with a reducing agent (tris(2-carboxyethyl)phosphine) under an atmosphere of argon. Lactocin S (**98**) was obtained pure with an overall yield of 10% (with an average yield of 97% per step over 33 deprotections and couplings, 2 macrocyclizations, 2 allyl deprotections and 1 cleavage/hydrolysis reaction). MALDI-TOF mass spectrometry confirms that the desired peptide **98** has been formed.



Scheme 2-18 Synthesis of lactocin S (**98**)

2.3.6 Isolation of natural lactocin S from *Lactobacillus sakei* L45

To confirm the proposed structure of lactocin S (**98**), the synthetically produced peptide needs to be compared to the natural compound. This means culturing of *L. sakei* and purification of lactocin S (**98**) from the growth medium must be undertaken. Previous purification procedures published by Nes and co-workers[138,139] were lengthy and required multiple chromatographic steps (Scheme 2-19). Purification of peptides from bacterial sources should be done as quickly as possible to avoid degradation. This is because the longer the peptide is exposed to the enzymes and oxygen, the more likely it is to decompose. Lactocin S (**98**) is prone to inactivation by oxidation of the sulfur atoms in the lanthionine (**17**) and methionine residues by oxygen present in air. This problem means that a simpler/faster purification of lactocin S (**98**) from *L. sakei* would be extremely useful.



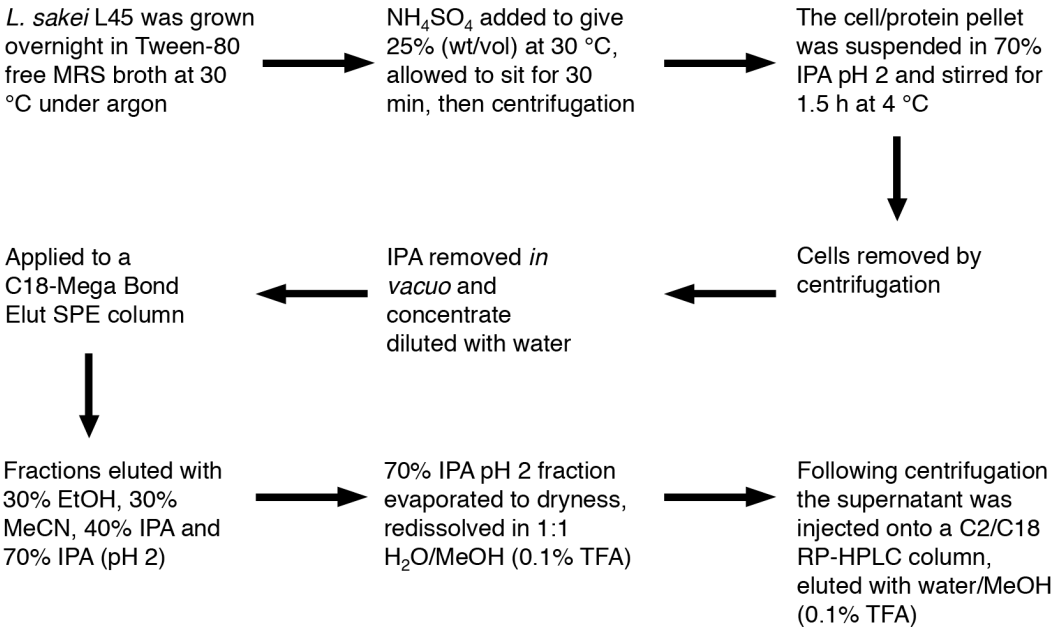
Scheme 2-19 Purification procedure for lactocin S (**98**) published by Nes and co-workers[139]

The purification procedures used by Nes and co-workers were nearly all water based and many of the buffers were close to neutral pH.[139] Work in our lab has found that lanthionine residues oxidize more readily at non-acidic pH, and so the procedure reported by Nes seems challenging.

To simplify the purification, our approach was to try to use fewer chromatographic steps and to incorporate organic solvents in the isolation rather than aqueous buffers. Because lactocin S (**98**) is hydrophobic this should be a reasonable approach. The progress of the purification is monitored by activity against a sensitive indicator organism (*Pediococcus acidilactici* Pac 1.0) and by MALDI-TOF mass spectrometry. The work in this section was completed with the assistance of an undergraduate summer student, Larissa Petriw, and a grade 11 WISEST student, Taylor Seal.

The conditions of the first step in the purification of *L. sakei* L45 are very important. The bacteria grows well in de Man Rogosa and Sharpe (MRS) broth, but one ingredient within this mixture must be removed for the purification to be successful. Tween-80 (polysorbate-80) is a surfactant added to the growth medium to help the bacteria take up nutrients. However, this compound appears to have a high affinity for lactocin S (**98**) and all of our attempts to separate this from the peptide using chromatography (hydrophobic interaction, ion exchange) were unsuccessful. For our purification, MRS broth was made from individual ingredients and Tween-80 was excluded, preventing the need for its subsequent removal. After inoculation, the bacteria was grown overnight, under an atmosphere of argon at 30 °C. It has been reported that *L. sakei* L45 can grow in either aerobic or anaerobic conditions, but more lactocin S is produced in the absence of oxygen.[140] The growth medium was then treated with ammonium sulfate (25% wt/vol) for 30 min at 30 °C to allow proteins to precipitate out of

solution. The cells and solid proteins were then collected using centrifugation, and the pellet suspended in a 70% isopropyl alcohol solution at pH 2. The cationic lantibiotic might adhere to the anionic surface of the bacterial cells making it harder to isolate the peptide directly from the supernatant. This is why the cells were suspended in the 70% isopropyl alcohol pH 2 along with the proteins. The cells were then removed by centrifugation and the resulting supernatant concentrated to remove the alcohol.



Scheme 2-20 Purification of lactocin S (**98**) from *L. sakei* L45

The resulting aqueous protein solution was applied to a C-18 solid phase extraction column (Mega Bond Elut®), and the column washed sequentially with 30% ethanol, 30% acetonitrile, 40% isopropyl alcohol and 70% isopropyl alcohol

pH 2. The final fraction shows activity. After all solvent was removed, the residue was redissolved in 1:1 methanol (0.1% TFA):water (0.1% TFA). A further centrifugation step, to remove insoluble material, was required before the sample was injected on a C2/C18 RP-HPLC column. The product was eluted with methanol and water each containing 0.1% TFA and the reducing agent (tris(2-carboxyethyl)phosphine) (1.5 mg/L). These solvents were also degassed by continuous bubbling with argon gas, in order to eliminate oxygen. The active fraction was collected under a stream of argon and then evaporated to dryness.

2.3.7 Biological evaluation of synthetic lactocin S (98)

To confirm that synthetic lactocin S (**98**) has the same structure as natural lactocin S (**98**), the bioactivity of the two peptides was compared. The antibacterial activity of the two peptides was determined against *P. acidilactici* Pac 1.0 and *L. sakei* L45 using a spot-on-lawn assay (Figure 2-4). *P. acidilactici* Pac 1.0 is a strain that is sensitive to natural lactocin S (**98**). *L. sakei* L45 is the producing strain and, as mentioned earlier, is immune to the antibacterial properties of the peptide.

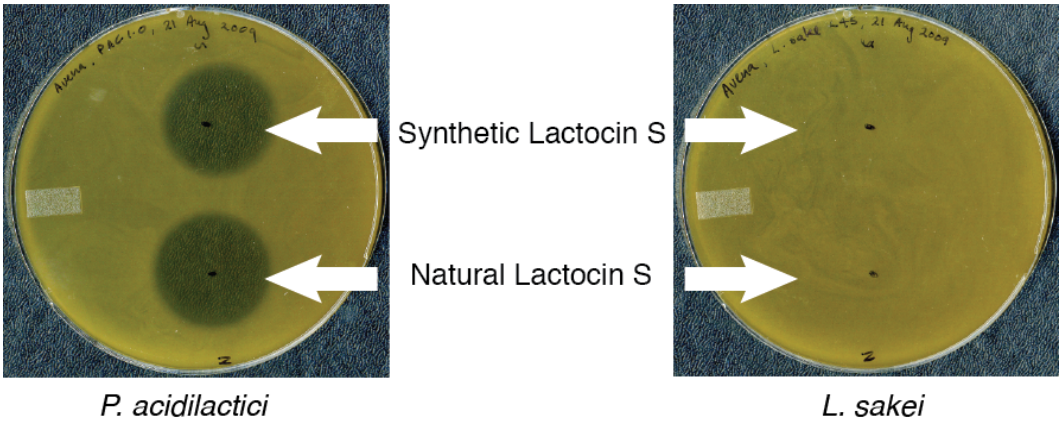


Figure 2-4 Comparison of biological activity of natural and synthetic lactocin S (**98**)

The assay was done by applying a solution of the peptide of interest onto a lawn of hard agar. Once the solvent had evaporated a layer of molten soft agar inoculated with the organism of interest was poured over top. Once the bacteria have grown, any clear zones (no bacterial growth) are indicative of antibacterial activity. Natural and synthetic lactocin S (**98**) show the same biological behavior, inhibiting the growth of the sensitive microorganism but having no effect on the growth of the producing one. This is strong supporting evidence for the proposed structure.

2.3.8 MS/MS sequencing of natural and synthetic lactocin S

To confirm that synthetic lactocin S (**98**) has the same peptide sequence as natural lactocin S (**98**), tandem MS/MS sequencing of both peptides was done.

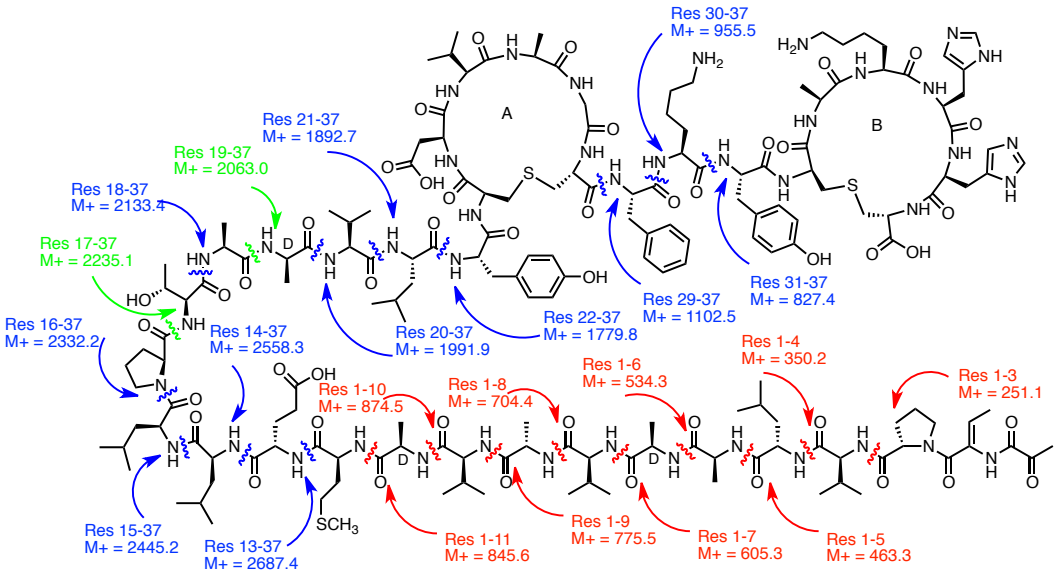
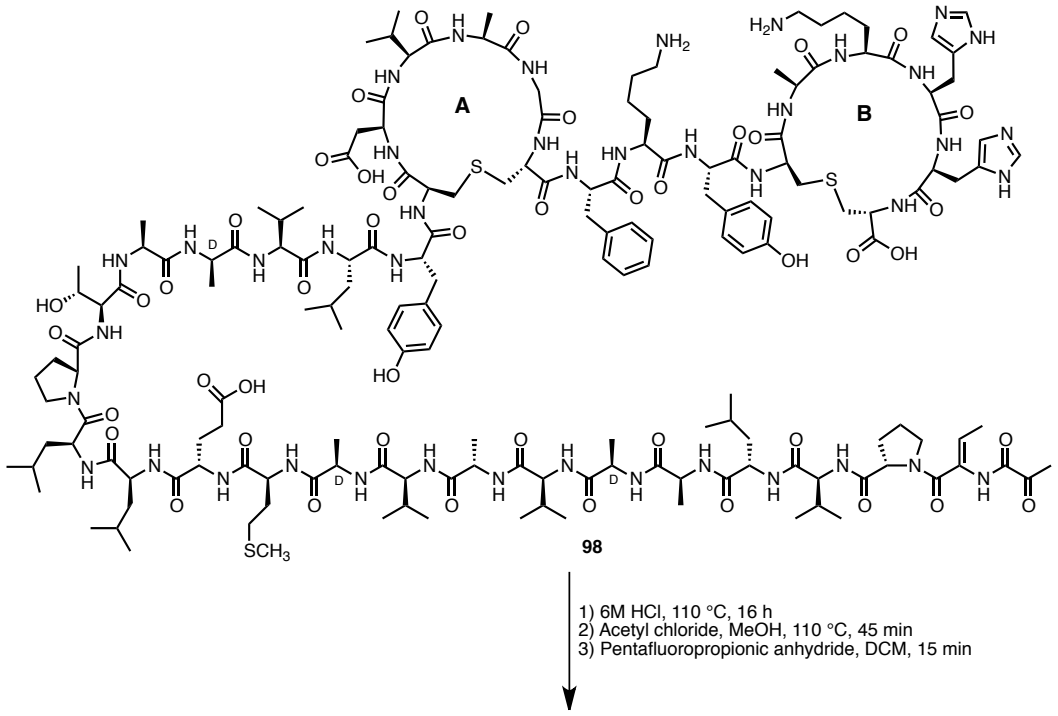


Figure 2-5 MS/MS sequencing data for synthetic lactocin S (**98**)

Figure 2-5 shows the ions found for the synthetic peptide. Analysis of natural lactocin S (**98**) yields the same fragmentation pattern. This is further evidence for the correct assignment of structure for lactocin S. Mass spectral evidence also supports the presence of an α-keto-amide at the *N*-terminus of lactocin S (**98**).

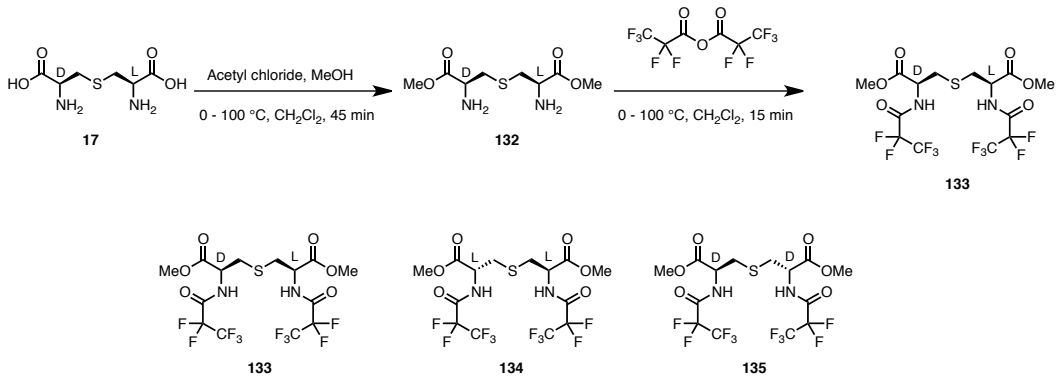
2.3.9 Chiral-GC/MS analysis of lanthionine residues in lactocin S (98)

To investigate the stereochemistry of the lanthionine residues in lactocin S (**98**), chiral gas chromatography mass spectrometry (GC/MS) was used.[150] To do this, each peptide was hydrolyzed under acidic conditions to yield individual amino acids (Scheme 2-21).



Scheme 2-21 Hydrolysis and derivatization of lactocin S (**98**)

To improve the volatility of the amino acids within the GC, they were derivatized to pentafluoropropanamide methyl esters (Scheme 2-22).[150]



Scheme 2-22 Derivatization procedure for lanthionine standards

Briefly, the peptides were each refluxed in 6M HCl for 18 h and then treated with acetyl chloride in methanol to generate methyl esters of the amino acids. Reaction of the amino acid esters with pentafluoropropanoic anhydride gave the fully derivatized amino acids. No purification was undertaken during any of these synthetic steps; the solvent and reagents were simply removed *in vacuo* between each reaction. In addition to derivatizing the hydrolysates of each peptide, amino acid standards for each isomer of lanthionine (L,L or D,D or D,L) were also converted to their pentafluoropropanamide methyl esters **133**, **134** and **135** for use in the GC/MS analysis.

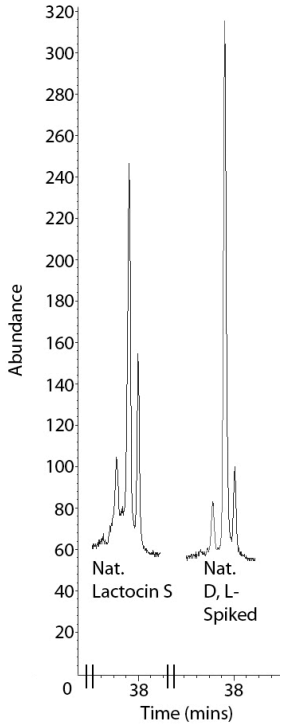


Figure 2-6 GC/MS trace for derivatized lanthionine in natural lactocin S (**98**) hydrolysate and the hydrolysate with derivatized D,L-lanthionine **133** added

The derivatized hydrolysates were injected individually onto a 25 m long Chirasil-L-Val column within a GC coupled to an MS detector. For synthetic lactocin S (**98**), the temperature was held at 160 °C for 10 min and then increased to 180 °C with a temperature ramp of 3 °C/min. For natural lactocin S (**98**) the temperature program started at 100 °C and was increased to 180 °C with a temperature ramp of 2 °C/min. This change is due to the use of different machines to analyze the separate peptides. Both peptides have a large central peak that corresponds to D,L-lanthionine **133** and two small flanking peaks that are the D,D **135** and L,L **134** isomers. It is believed that these peaks represent a small amount

of epimerization, which may result from the prolonged exposure of lanthionine to high temperature and acidity in the hydrolysis of the peptides.[155]

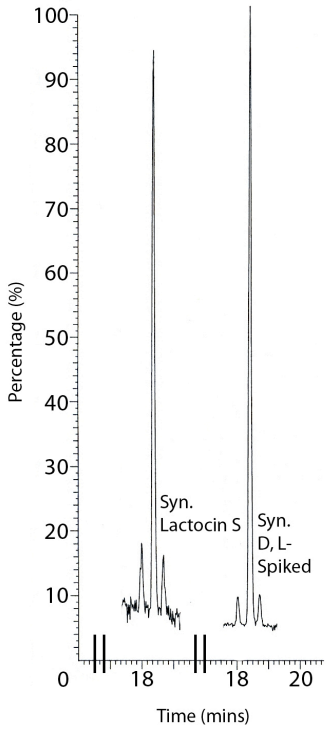


Figure 2-7 GC/MS trace for derivatized lanthionine in synthetic lactocin S (**98**) hydrolysate and the hydrolysate with derivatized D,L-lanthionine (**133**) added

Addition of derivatized standards of lanthionine (D,D **135**, D,L **133**, L,L **134**) results in an increase in the magnitude of one of the three peaks. When D,D **135** is added, the earliest peak is enlarged such that there are two major signals. In the same manner when L,L **134** is added, the last peak increases in size to give two major signals. When D,L **133** is added, the central peak becomes even bigger and there is only one major signal. This is very strong evidence that the lanthionine

residues within lactocin S (**98**) have D,L stereochemistry, as in other lantibiotics examined previously (nisin A (**16a**),[150] epidermin,[151] Pep-5[153] and gallidermin).[152]

2.4 Conclusions

The total synthesis of lactocin S (**98**) has been completed using a combination of solution and solid phase peptide chemistry. By incorporating orthogonally protected lanthionine (**58**) into a solid phase approach, on resin cyclization reactions provide access to the A and B rings of lactocin S (**98**). The final two residues of the peptide are installed on resin, and a biomimetic reaction generates mature lactocin S (**98**) upon cleavage from the resin. This is the first SPPS synthesis of a natural lantibiotic peptide to be reported and only the second total synthesis of a natural lantibiotic. Following isolation of natural lactocin S from *L. sakei* L45, the two peptides were compared. Using biological activity testing, MS/MS sequencing and chiral GC/MS the proposed structure of lactocin S (**98**) was confirmed by comparison of the natural peptide to the synthetic standard.

Chapter 3: Analogues of Lactocin S

3.1 Background

Lantibiotic peptides are potent antibacterial compounds. However, to date only a few have been utilized in food preservation or medical applications.[77] One reason for this is the instability of these compounds under physiological conditions. Many of the peptides are prone to oxidation in air and they often require acidic pH for stability and activity.[89,91] Chemists and biochemists have tried to develop more stable compounds by making analogues.[95,96]

3.1.1 Potential lactocin S analogues

Lactocin S (**98**) is a good lead compound for the design and synthesis of analogues. This peptide requires an acidic environment both for activity and stability.[140] The sulfur atoms within the lanthionine (**17**) and methionine residues all react with atmospheric oxygen rendering the peptide less active. If these functionalities could be replaced with more oxidatively stable groups, it would greatly enhance the utility of this compound. If a suitable mimic for lanthionine can be discovered, the analogue methodology could be expanded to many other important lantibiotics, for example lacticin 3147 A1 (**51**) and A2 (**52**).

Figure 3-1 shows potential sites where lactocin S (**98**) could be altered to produce analogues. Diaminopimelic acid (DAP) is one possible substitute for lanthionine that would likely render the peptide more oxidatively stable.

Analogues of the lantibiotic lacticin 3147 A2 (**52**) have been made where lanthionines (and methyllanthionines) have been replaced with oxa-lanthionine/methyllanthionine (**54**)[101] or olefinic carbocycles (**55**).[100] Both of these analogues were less active than the natural peptide as was discussed in Chapter 1. Using DAP may have advantages over those alterations, as the ring retains the same number of atoms and carbon and sulfur also have very similar electronegativities.

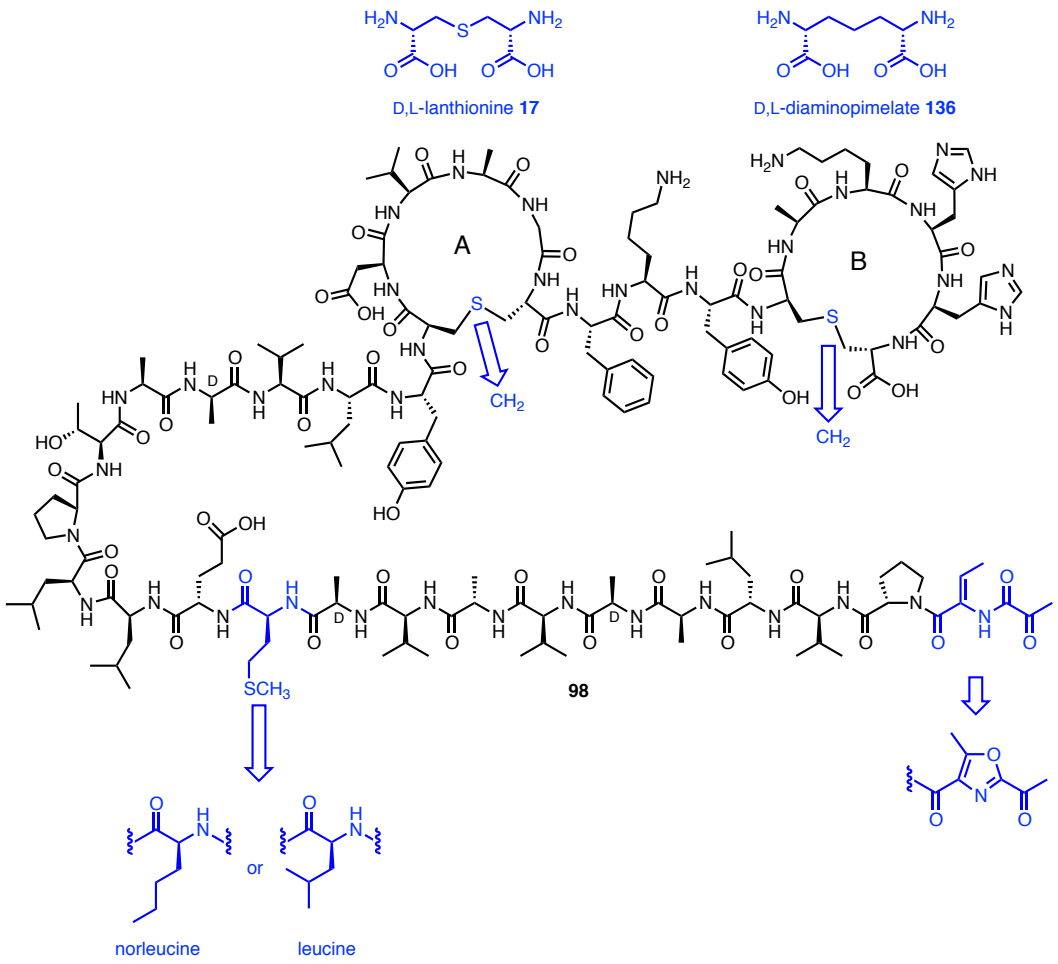


Figure 3-1 The structure of lactocin S (**98**) with potential alterations highlighted in blue.

Methionine could be replaced with amino acids with all-carbon side chains such as leucine and norleucine. Because Met-12 oxidizes very readily in lactocin S (**98**), substitution with one of these amino acids would be a very simple change that could greatly improve stability. This alteration has been successful in another biologically important peptide (neopetrosiamide A) studied by our group.[156]

Although not believed to be involved in oxidative instability, the highly modified *N*-terminal dipeptide of lactocin S (**98**) is also an ideal site for structural investigation. It has been reported that introduction of the relatively planar Fmoc functionality in place of the final five residues of lan-lacticin 3147 A2 (**53**) gave a peptide that unexpectedly retained some activity.[99] Alteration of the *N*-terminus of lactocin S (**98**) to incorporate a planar functionality would give insight to the importance of this unusual dipeptide moiety.

3.1.2 Diaminopimelate

Diaminopimelic acid (DAP) is a naturally occurring metabolite that is utilized by plants and bacteria to synthesize the essential amino acid L-lysine.[157,158] This transformation has been the focus of research trying to find new antibiotic targets.[159] Humans do not biosynthesize L-lysine; instead we must obtain it from our diets. This makes the enzymes of DAP conversion to L-lysine ideal targets for inhibitors.[159] In the same way as lanthionine, DAP can exist as three different isomers, D,D, D,L and L,L. D,L-DAP could be a useful mimic of D,L-

lanthionine because they differ only by the substitution of a methylene unit for a sulfur atom. Having already developed a synthetic methodology for making lactocin S on solid support using orthogonally protected lanthionine as a building block,[135] perhaps this could be expanded to allow the synthesis of DAP peptides using orthogonally protected DAP. Figure 3-2 shows orthogonally protected lanthionine (**58**) and orthogonally protected DAP (**137**) with the structural difference highlighted in red.

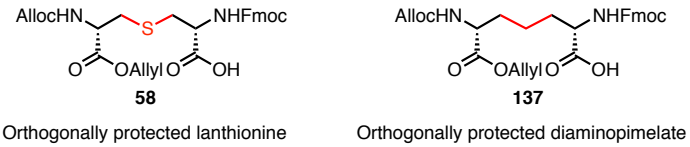


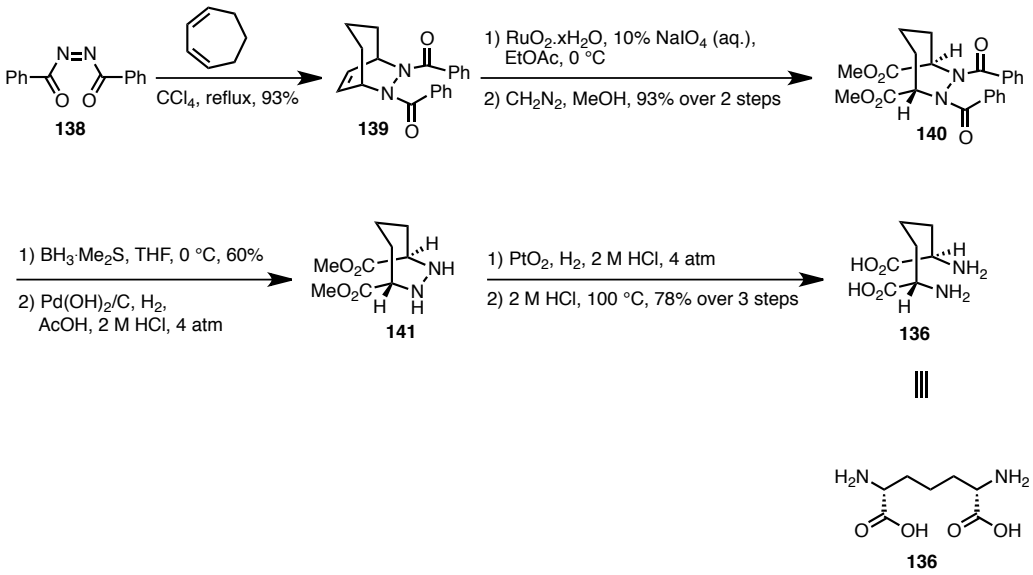
Figure 3-2 Orthogonally protected lanthionine (**58**) and orthogonally protected diaminopimelate (**137**)

Before any solid phase chemistry can be attempted with orthogonally protected DAP (**137**), this building block must be obtained with the correct stereochemistry and the desired protecting groups. Because of its involvement in the biosynthesis of L-lysine, synthetic methods to produce D,L-DAP have been developed.[157]

3.1.3 Synthesis of diaminopimelate

Some methods for D,L-DAP synthesis provide access to the compound in its fully unprotected state. Scheme 3-1 shows an approach to the synthesis of

unprotected DAP utilizing a Diels Alder reaction to form **139**, which contains the backbone of the amino acid.[157,160] The acid functionalities are then formed by oxidative cleavage of the double bond with RuO_4 and then protected as methyl esters using diazomethane to give **140**. To unmask the amine moieties, first the benzoyl protection was reduced with $BH_3{\cdot}SMe_2$ and the resulting benzyl groups were then cleaved off by hydrogenolysis. Subsequent reduction of the N-N bond in **141** with PtO_2 and H_2 gave the desired free amine. A final acid hydrolysis gave fully unprotected D,L-DAP **136**.



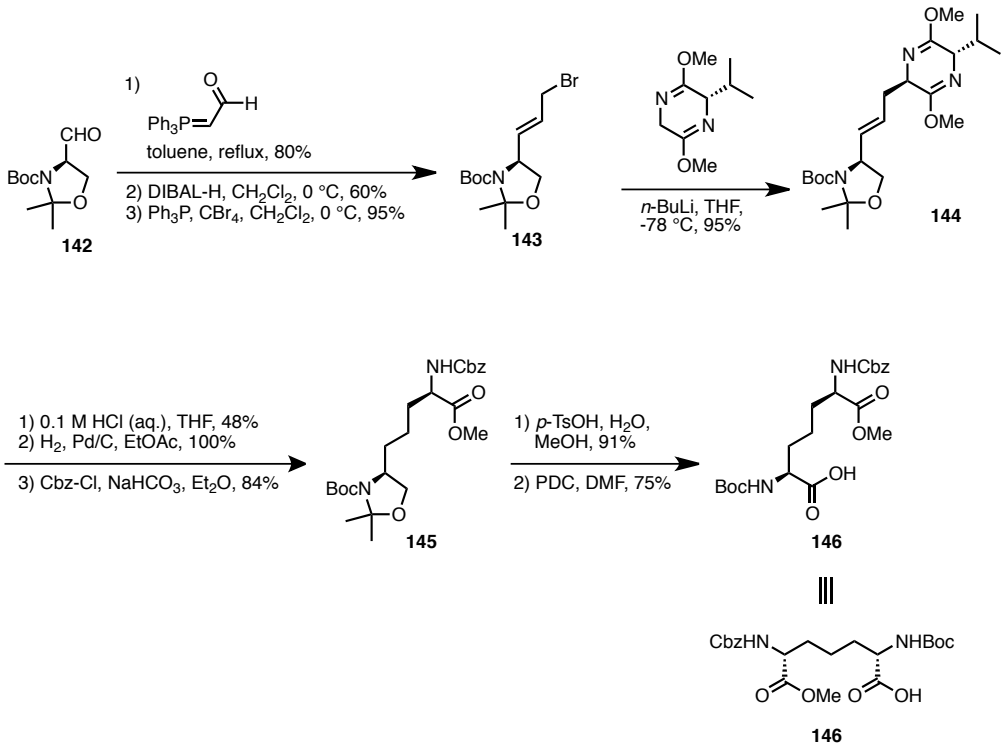
138 **139** **140** **141** **136** **136**

Scheme 3-1 Synthesis of D,L-diaminopimelate **136**

Despite the novel approach used to form D,L-DAP **136**, this synthesis is not useful for the formation of orthogonally protected D,L-DAP (**137**) as required for SPPS. It is very hard to selectively protect the functional groups on D,L-DAP **136** once it has been synthesized because they are nearly impossible to

differentiate. This synthesis is useful in situations requiring the natural product, but is problematic when a protected DAP is needed.

Scheme 3-2 shows the synthesis of D,L-DAP with orthogonal protection,[161,162] starting with Garner's oxazolidine **142**. A Wittig reaction with (triphenylphosphoranylidene)acetaldehyde was used to form the C3 backbone of the amino acid. The resulting aldehyde was then reduced to an alcohol with DIBAL and converted to an alkyl bromide (**143**) by the Appel reaction with CBr_4 and PPh_3. Then, **143** was alkylated with Schöllkopf's bislactim ether derived from L-valine in the presence of the base *n*-BuLi. The authors state that this reaction gave only one isomer and that none of the undesired diastereomer was detected by ^{13}C NMR. Masked D,L-DAP **144** is treated with acid to cleave the bislactim, generating one equivalent of L-valine methyl ester and the methyl ester of one half of DAP. Hydrogenation with Pd/C as a catalyst gave the saturated backbone of DAP, and then the free amine was protected by reaction with Cbz-Cl under basic conditions to yield **145**.

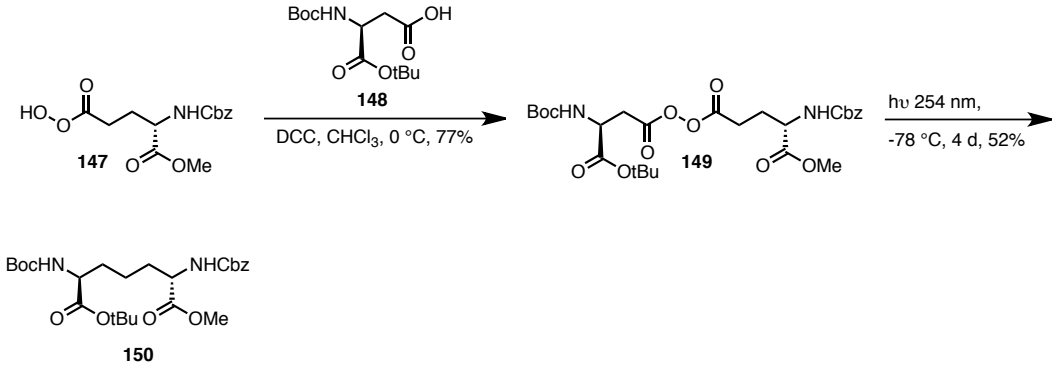


Scheme 3-2 Synthesis of protected D,L-diaminopimelate **146**

Refluxing in wet methanol under acidic conditions hydrolyzed the oxazolidine ring, and the resulting alcohol was oxidized to the acid **146** using pyridinium dichromate. This synthesis provides access to orthogonally protected D,L-DAP with good stereochemical control. However, these protecting groups are not amenable to SPPS, and considerable manipulations would be required to introduce the necessary allyl/Alloc and Fmoc functionalities.

Recently our group published a method for the synthesis of DAP that uses photolysis to form the amino acid backbone.[163] Scheme 3-3 documents the key steps in this reaction, where the side chain peracid of a glutamic acid derivative **147** is coupled to the γ-acid of aspartic acid **148** to form a diacylperoxide (**149**).

Then, diacylperoxide **149** can be irradiated with UV light at low temperature in solid state to form the backbone of L,L-DAP **150**. This reaction proceeds via a radical decarboxylation and recombination mechanism.



Scheme 3-3 Synthesis of orthogonally protected L,L-diaminopimelate **150** via photolysis

The advantages of the photolysis method are that different protecting groups can be introduced on each amino acid prior to the photolysis reaction and formation of DAP. Additionally, because this reaction is done on the sample as a solid, instead of in solution, there is less chance of scrambling of the two halves to form homo-dimers, as is a frequent problem in unsymmetrical Kolbe electrolysis reactions.[164] Finally, this approach ensures the stereochemical integrity of the amino acid remains intact.

3.2 Project Objectives

Lactocin S (**98**) is a lantibiotic peptide with antibacterial activity against other Gram-positive microorganisms. Unfortunately, its application is limited by susceptibility to oxidation of the sulfur-containing amino acids lanthionine (**17**) and methionine. The aim of this project is to synthesize analogues of lactocin S (**98**) in which the lanthionine (**17**) and methionine residues are replaced with carbon-containing amino acids.

There are two lanthionine rings in lactocin S (**98**) and they will each be replaced systematically by D,L-DAP (**136**). In order to use SPPS to make these analogues, a solution synthesis of orthogonally protected DAP (**137**) must first be developed.

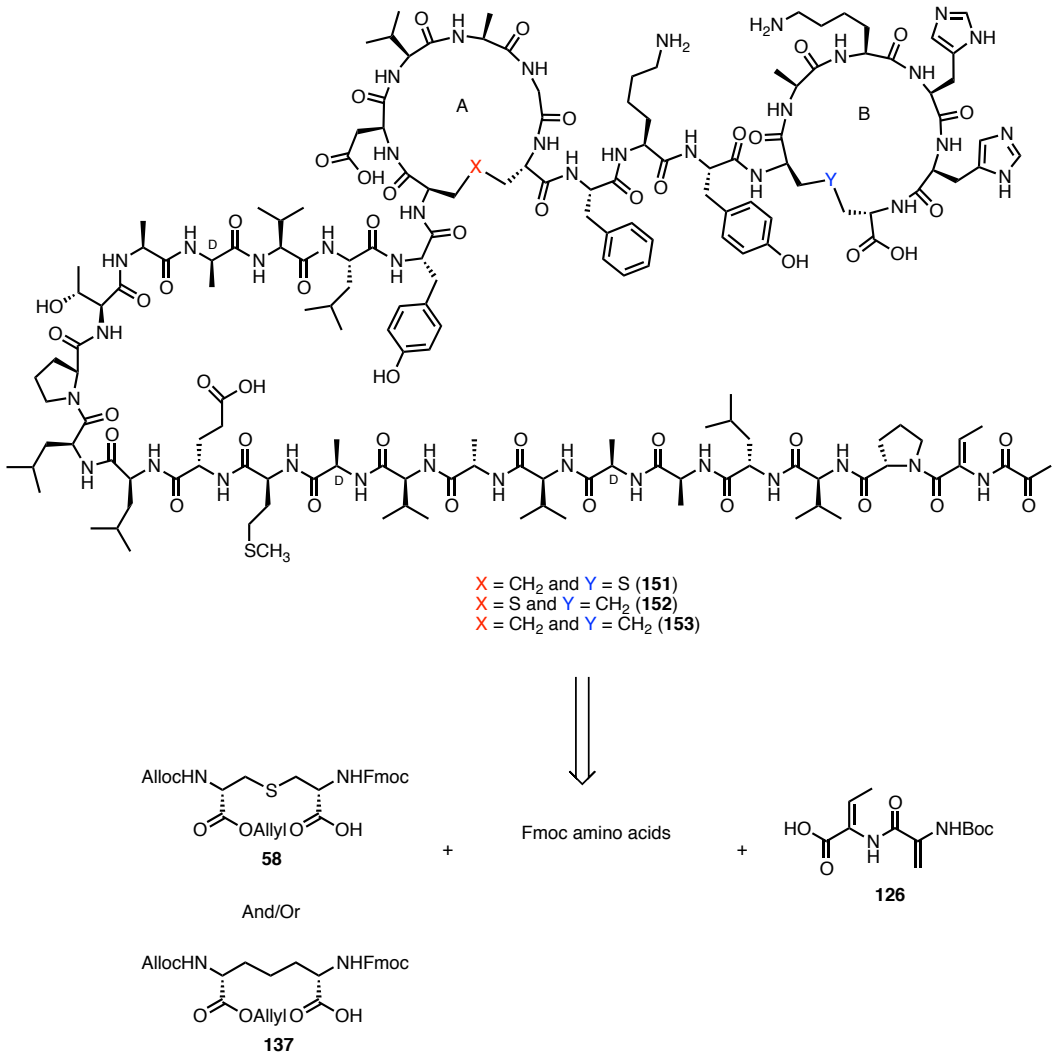
The second aim of this project is to investigate the effect of Met-12 on the activity/stability of lactocin S (**98**). Two analogues will be synthesized, one with the natural amino acid leucine at position 12 (**181**), and one with the unnatural norleucine (**182**).

Finally, the *N*-terminal two "amino acids" of lactocin S (**98**) will be replaced with an oxazole synthesized in solution that is designed to mimic the natural dipeptide but is more planar.

3.3 Results and Discussion

3.3.1 Retrosynthetic approach to DAP analogues of lactocin S

The synthetic approach to the DAP analogues (**151**, **152**, **153**) of lactocin S uses the same methodology as for natural lactocin S (**98**). Orthogonally protected DAP (**137**) and lanthionine (**58**) are made in solution and then incorporated along with standard Fmoc protected amino acids into a solid phase synthesis (Scheme 3-4). By using the same protection strategy for DAP and lanthionine, they can be used interchangeably in the synthesis and this should allow for the production of all three analogues using the same approach.

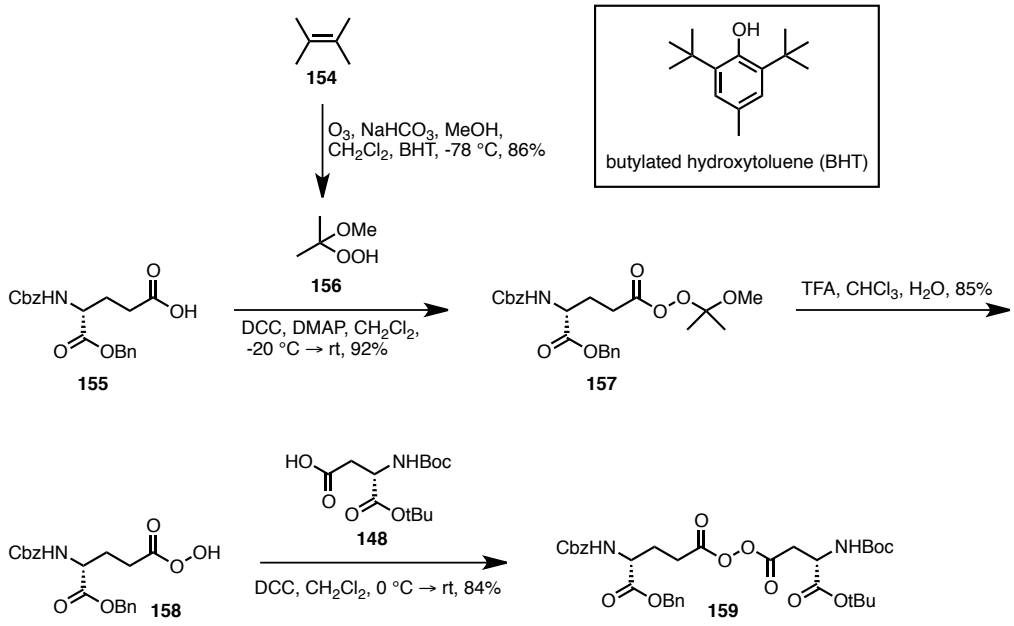


Scheme 3-4 Retrosynthetic approach to DAP-containing lactocin S analogues

Before commencement of the SPPS, it is necessary to synthesize orthogonally protected DAP (**137**). This synthesis is based upon the photolysis strategy described in Section 3.1.3 (Scheme 3-3). The incorporation of allyl/Alloc protecting groups has not been previously described, so our initial approach was to use a proven protection strategy (Bn, Cbz/tBu, Boc),[163] and then introduce allyl and Alloc after the formation of the DAP backbone.

3.3.2 Synthesis of orthogonally protected diaminopimelate using established protecting groups

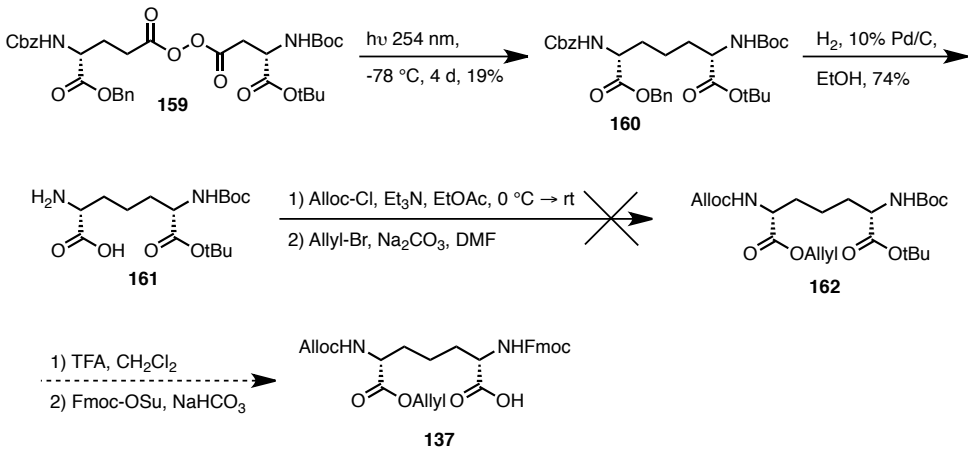
The synthesis of orthogonally protected DAP (**137**) was initiated by the reaction of Cbz/Bn protected D-glutamic acid (**155**) with 2-methoxyprop-2-yl hydroperoxide (**156**) using DCC and DMAP as coupling reagents (Scheme 3-5).



Scheme 3-5 Attempted synthesis of orthogonally protected diaminopimelate

Hydroperoxide **156** was made from 2,3-dimethylbutene (**154**) by ozonolysis in the presence of methanol and sodium bicarbonate. The D-glutamic acid perester **157** was converted to peracid **158** by treatment with TFA and water. It was then immediately coupled to Boc/tBu protected L-aspartic acid **148** in the

presence of DCC. Once formed, **158** must be used directly before it decomposes to carboxylic acid **155**. By contrast, diacylperoxide **159** is relatively stable and can be purified by silica gel chromatography and stored at -20 °C for many months. With **159** in hand, it was treated under the photolysis conditions to yield the DAP backbone **160**. This reaction was done on the solid sample and at a low temperature to ensure the desired product is formed. However, these conditions do result in a slow reaction time (four days) and a low yield (19 %).



Scheme 3-6 Attempted synthesis of orthogonally protected diaminopimelate

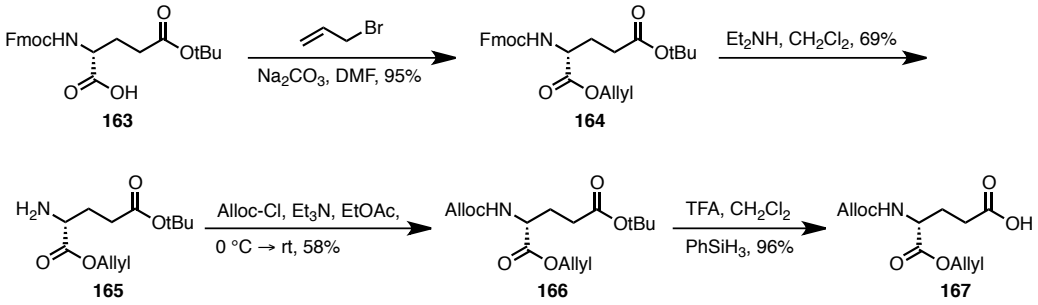
Because the material is in solid state, as the top layer reacts it forms a protective coating of product and effectively shields starting material underneath from the UV irradiation. To alleviate this problem, the solid was dissolved and redeposited partway through the reaction to expose unreacted material. Chromatography allows for the separation of product (**160**) from starting material (**159**). Recovered diacylperoxide **159** can be submitted to the reaction conditions

again. DAP **160** was then hydrogenated with 10% Pd/C to yield **161** deprotected at the L-terminus of DAP. Attempts to introduce the allyl and Alloc protecting groups were unsuccessful. In part, this was due to challenges in solubilizing **161** under the required reaction conditions.

An alternative approach to orthogonally protected DAP (**137**) was being developed simultaneously and ultimately was more successful, hence this route was not investigated further.

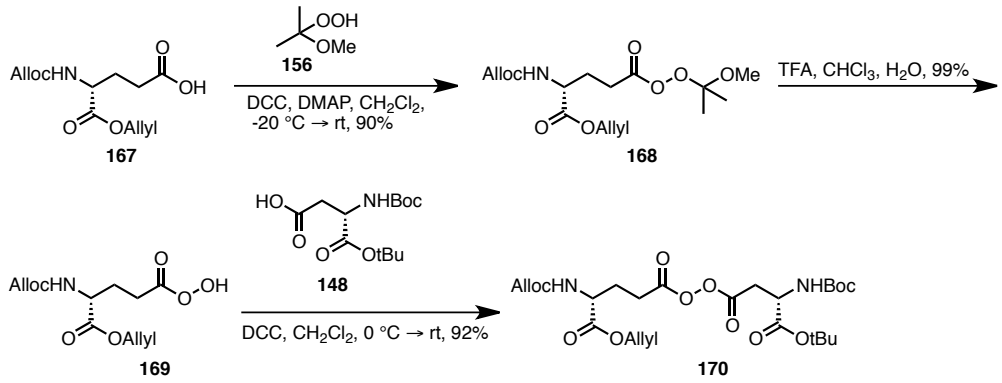
3.3.3 Synthesis of orthogonally protected diaminopimelate with allyl/Alloc protecting groups

An alternative strategy to obtain orthogonally protected DAP (**137**) incorporates the allyl and Alloc protecting groups at a much earlier point in the synthesis, such that fewer protecting group manipulations are required after the formation of the DAP backbone. The synthesis begins with the protection of Fmoc-D-glutamic acid **163** as an allyl ester by treatment with allyl bromide and sodium carbonate (Scheme 3-7). The Fmoc protection on **164** was then removed under basic conditions with diethylamine and the Alloc group installed by reaction of **165** with allyl chloroformate in the presence of triethylamine.



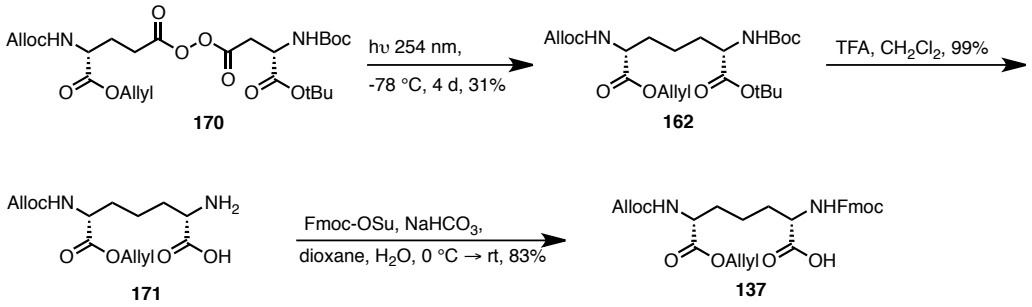
Scheme 3-7 Synthesis of allyl/Alloc protected D-glutamic acid **167**

Finally, the *tert*-butyl ester of **166** was deprotected with TFA to give allyl/Alloc D-glutamic acid **167**. This amino acid was then converted to a perester (**168**) by reaction with hydroperoxide **156** and DCC and DMAP as described earlier (Scheme 3-8). Peracid **169** was generated by addition of TFA and water and then used directly in the next step to couple to Boc/tBu L-aspartic acid with DCC.



Scheme 3-8 Synthesis of orthogonally protected diacylperoxide **170**

The diacylperoxide **170** was then exposed to the photolysis conditions and the desired DAP **171** obtained (Scheme 3-9). To obtain orthogonally protected DAP **137**, Boc and *tert*-butyl protecting groups were removed under acidic conditions to give **171** and Fmoc was introduced onto the amine by the action of Fmoc succinimide and sodium bicarbonate.



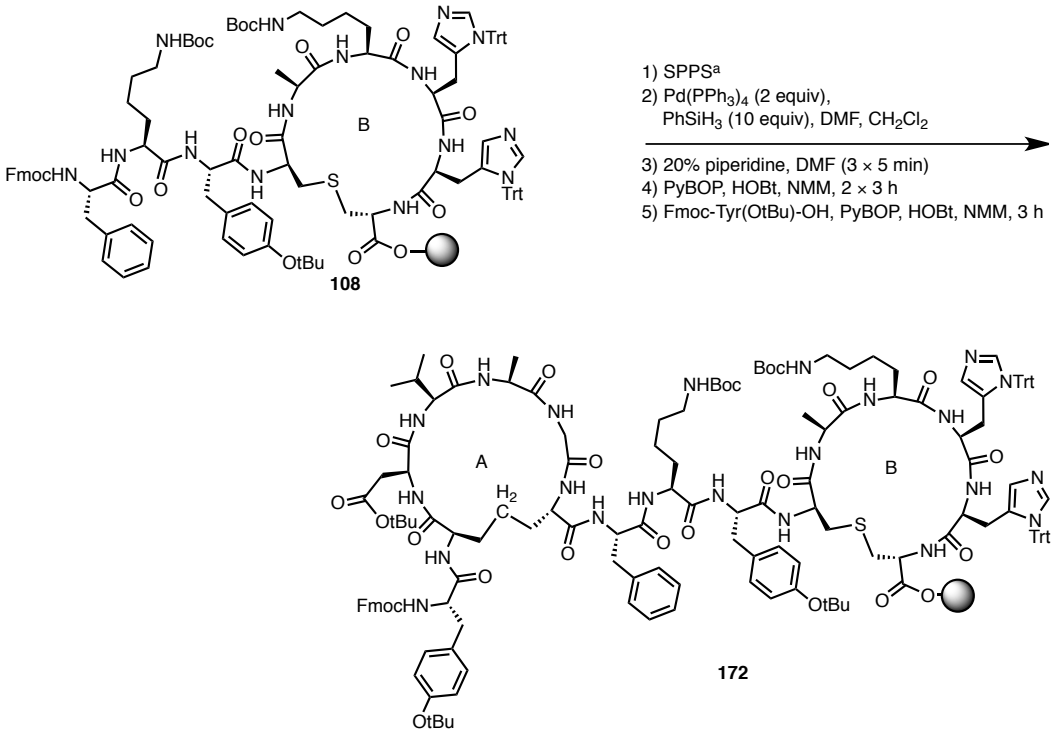
Scheme 3-9 Synthesis of orthogonally protected diaminopimelate **137**

With the completion of this synthesis, orthogonally protected DAP (**137**) is ready to be utilized in the synthesis of analogues of lactocin S (**98**).

3.3.4 Synthesis of A-DAP lactocin S

The first DAP-containing analogue of lactocin S (**98**) to be synthesized contains a DAP residue for the A-ring and a lanthionine residue for the B-ring. This allows the use of an intermediate from the synthesis of lactocin S (**98**) as the starting material. Compound **108**, in which the B-ring of lactocin S has been installed and cyclized on resin was the starting point for the synthesis of A-DAP lactocin S (**151**). Standard solid phase peptide synthesis was then used to

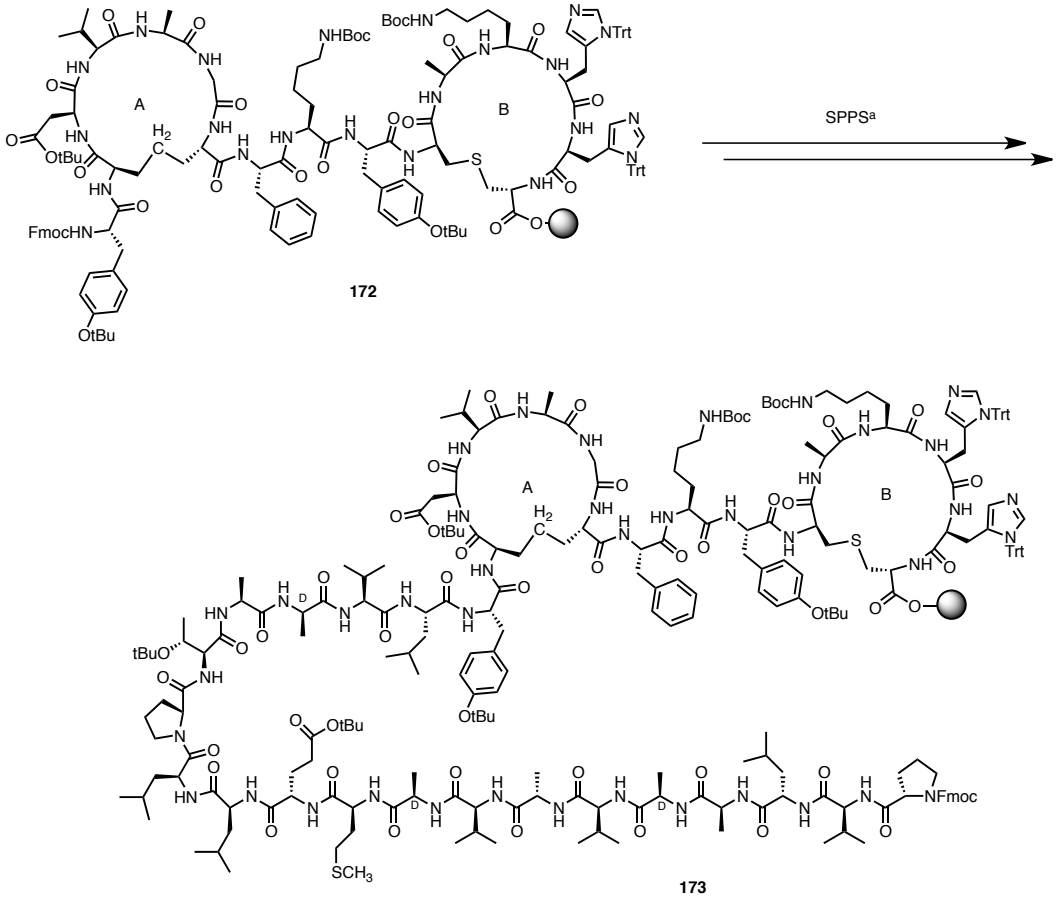
introduce orthogonally protected DAP (**137**) and the amino acids found within the A-ring (Scheme 3-10). The allyl and Alloc groups were removed using $Pd(PPh_3)_4$ and phenylsilane, then the *N*-terminal Fmoc was deprotected with piperidine. The ring can be cyclized by coupling of the resulting amine to the acid of DAP. The free amine of DAP is now the *N*-terminus of the peptide and is coupled to Fmoc-Tyr-OH to give compound **172**.



Scheme 3-10 Synthesis of **172**, consisting of the A/B-rings of A-DAP lactocin S. [a] Conditions for SPPS: (i) 20% piperidine in DMF, (ii) **137**, PyBOP, HOBt, NMM, (iii) Repeat steps (i) and (ii) for Fmoc-Gly-OH, Fmoc-Ala-OH, Fmoc-Val-OH, Fmoc-Asp(OtBu)-OH

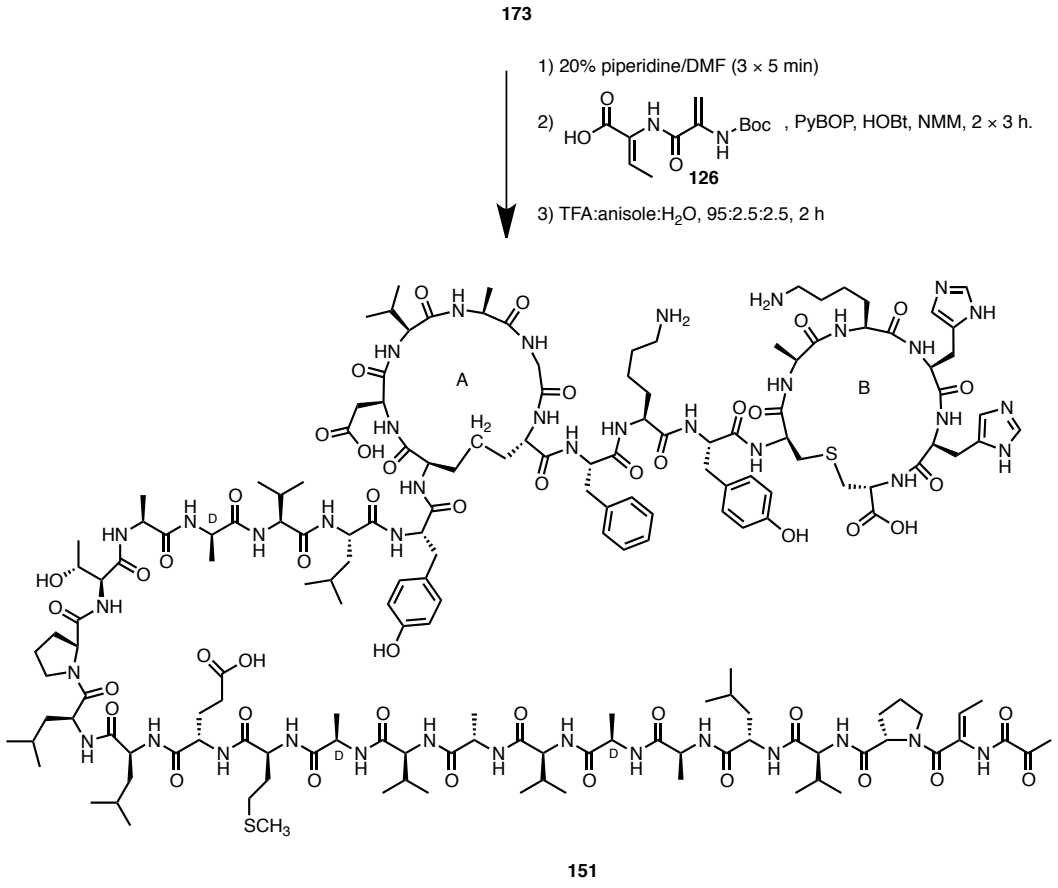
Bicyclic peptide **172** was elongated using SPPS to incorporate the residues 21-3 of A-DAP lactocin S (**151**). This proceeded in a straightforward manner to

yield **173** (Scheme 3-11). To ensure that the synthesis was advancing smoothly, after every second coupling reaction, a small sample of the peptide was cleaved from the resin under acidic conditions and analyzed by MALDI-TOF MS.



Scheme 3-11 The synthesis of **173** consisting of residues 37-3 of A-DAP lactocin S. [a]Conditions for SPPS: (i) 20% piperidine in DMF, (ii) Fmoc-Leu-OH, PyBOP, HOBt, NMM, (iii) Repeat steps (i) and (ii) for Fmoc-Val-OH, Fmoc-D-Ala-OH, Fmoc-Ala-OH, Fmoc-Thr(OtBu)-OH, Fmoc-Pro-OH, Fmoc-Leu-OH, Fmoc-Leu-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Met-OH, Fmoc-D-Ala-OH, Fmoc-Val-OH, Fmoc-Ala-OH, Fmoc-Val-OH, Fmoc-D-Ala-OH, Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Val-OH, Fmoc-Pro-OH.

Upon synthesis of **173**, the *N*-terminal Fmoc group was removed with piperidine and the peptide on resin was coupled with dipeptide **126** to yield full length A-DAP lactocin S on resin. The peptide was cleaved from the resin using a cocktail of TFA:anisole:H_2O 95:2.5:2.5. This also removes all protecting groups and unmasks the *N*-terminus that is subsequently converted to an α-ketoamide in these conditions.

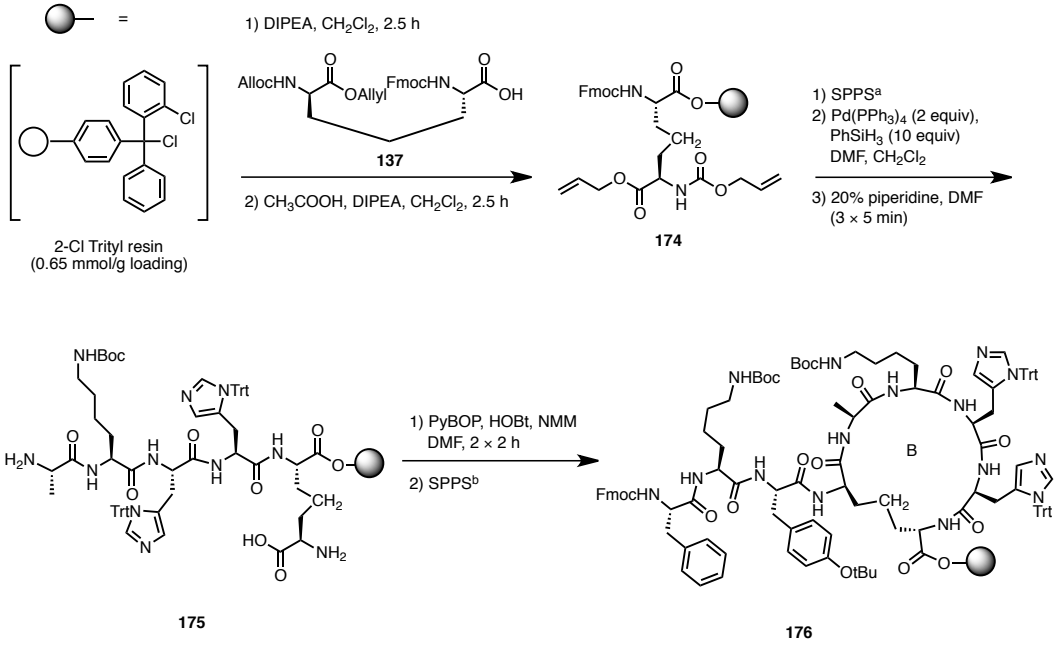


Scheme 3-12 Synthesis of A-DAP lactocin S (**151**)

A-DAP lactocin S (**151**) was purified by reversed phase HPLC using a C2/C18 column, following a procedure similar to that used in the purification of lactocin S (**98**). Pure A-DAP lactocin S (**151**) was obtained with an overall yield of 2.5%, corresponding to an average yield of 95% per step over 33 deprotections and couplings, 2 macrocyclizations, 2 allyl deprotections and 1 cleavage/hydrolysis reaction. MALDI-TOF mass spectrometry confirmed that the desired peptide **151** was formed.

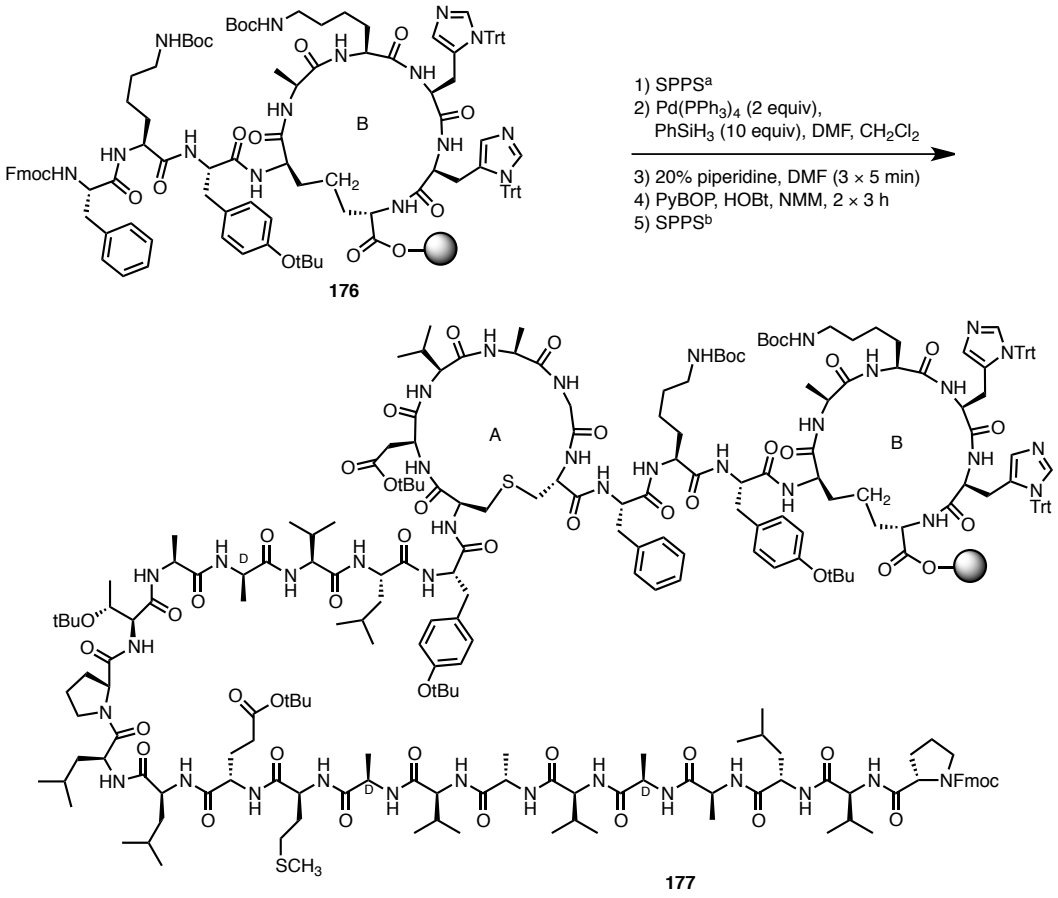
3.3.5 Synthesis of B-DAP lactocin S

The second DAP containing analogue of lactocin S (**98**) has a lanthionine residue for the A-ring and a DAP residue for the B-ring. This synthesis begins with the attachment of orthogonally protected DAP (**137**) onto 2-chlorotrityl resin (Scheme 3-13).



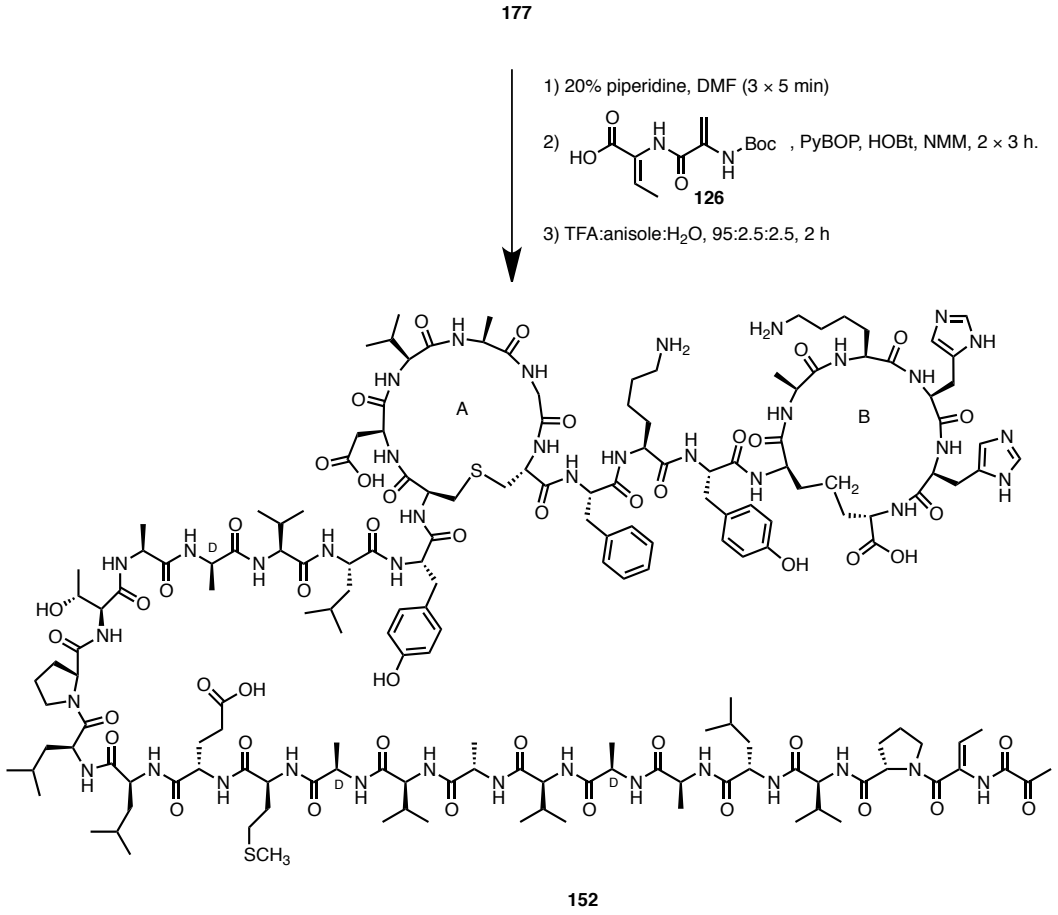
Scheme 3-13 Loading of orthogonally protected DAP (**137**) onto resin and synthesis of the B-ring of B-DAP lactocin S (**152**). [a]Conditions for SPPS: (i) 20% piperidine in DMF, (ii) Fmoc-His(Trt)-OH, PyBOP, HOBt, NMM, (iii) Repeat steps (i) and (ii) for Fmoc-His(Trt)-OH, Fmoc-Lys(Boc)-OH and Fmoc-Ala-OH. [b]Conditions for SPPS: (i) Fmoc-Tyr(OtBu)-OH, PyBOP, HOBt, NMM, (ii) 20% piperidine in DMF, (iii) Repeat steps (i) and (ii) for Fmoc-Lys(Boc)-OH and then step (i) for Fmoc-Phe-OH

The resin loading was reduced from 0.65 mmol/g to 0.16 mmol/g by coupling a smaller amount of **137** to the resin in the presence of DIPEA and then capping the remaining resin sites with acetic acid. Standard Fmoc SPPS was employed to introduce the remaining residues of the B-ring of B-DAP lactocin S. Then deprotection/cyclization and SPPS was used as described previously to generate the B-ring containing compound **176**. The A-ring (Scheme 3-14) was synthesized containing lanthionine and extended by SPPS to yield **177**, consisting of residues 37 to 3 of B-DAP lactocin S.



Scheme 3-14 The synthesis of **177** consisting of residues 37-3 of B-DAP lactocin S. [a]Conditions for SPPS: (i) 20% piperidine in DMF, (ii) **58**, PyBOP, HOBt, NMM, (iii) Repeat steps (i) and (ii) for Fmoc-Gly-OH, Fmoc-Ala-OH, Fmoc-Val-OH, Fmoc-Asp(OtBu)-OH. [b]Conditions for SPPS: (i) 20% piperidine in DMF, (ii) Fmoc-Leu-OH, PyBOP, HOBt, NMM, (iii) Repeat steps (i) and (ii) for Fmoc-Val-OH, Fmoc-D-Ala-OH, Fmoc-Ala-OH, Fmoc-Thr(OtBu)-OH, Fmoc-Pro-OH, Fmoc-Leu-OH, Fmoc-Leu-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Met-OH, Fmoc-D-Ala-OH, Fmoc-Val-OH, Fmoc-Ala-OH, Fmoc-Val-OH, Fmoc-D-Ala-OH, Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Val-OH, Fmoc-Pro-OH.

The synthesis of B-DAP lactocin S (**152**) was completed by introduction of dipeptide **126** and cleavage of the peptide from the resin under acidic conditions.

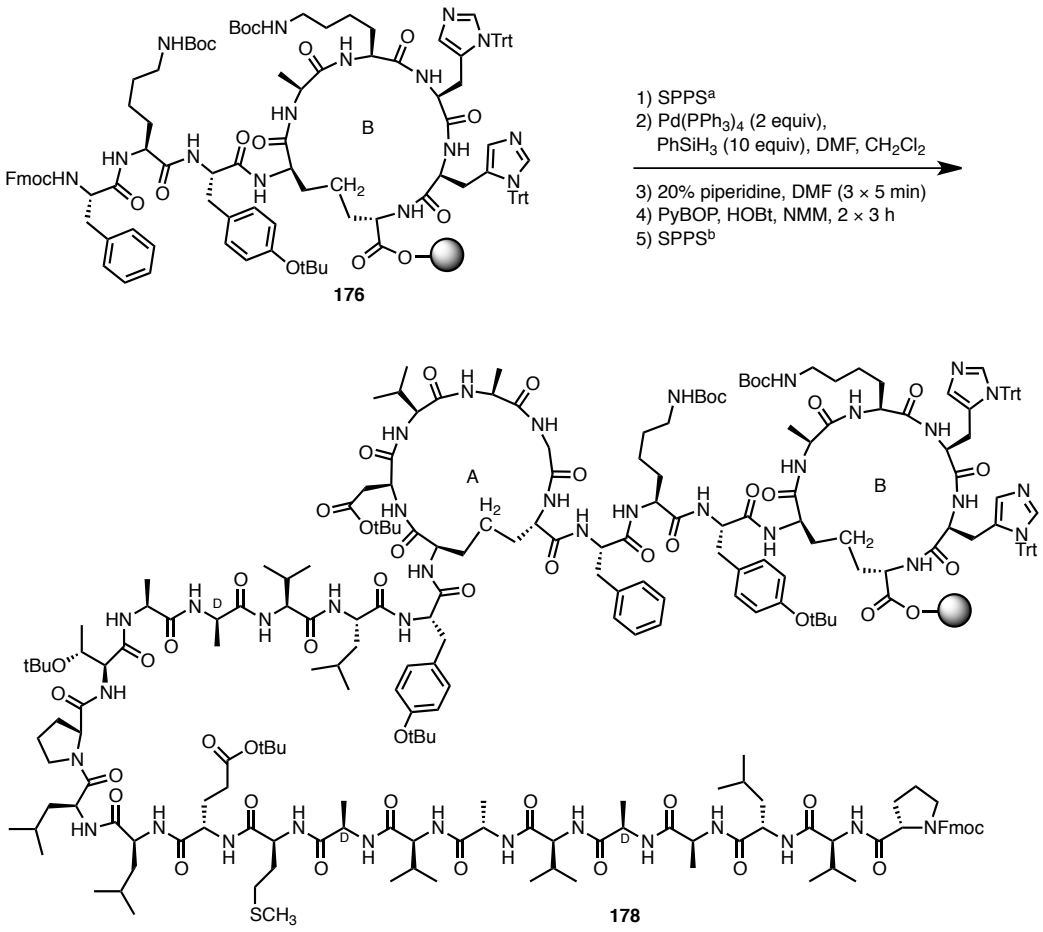


Scheme 3-15 Synthesis of B-DAP lactocin S (**152**)

B-DAP lactocin S (**152**) was purified following a procedure similar to that used in the purification of lactocin S (**98**). Pure B-DAP lactocin S (**152**) was obtained in an overall yield of 1.9%, corresponding to an average yield of 95% per step over 33 deprotections and couplings, 2 macrocyclizations, 2 allyl deprotections and 1 cleavage/hydrolysis reaction. MALDI-TOF mass spectrometry confirmed that the desired peptide **152** is formed.

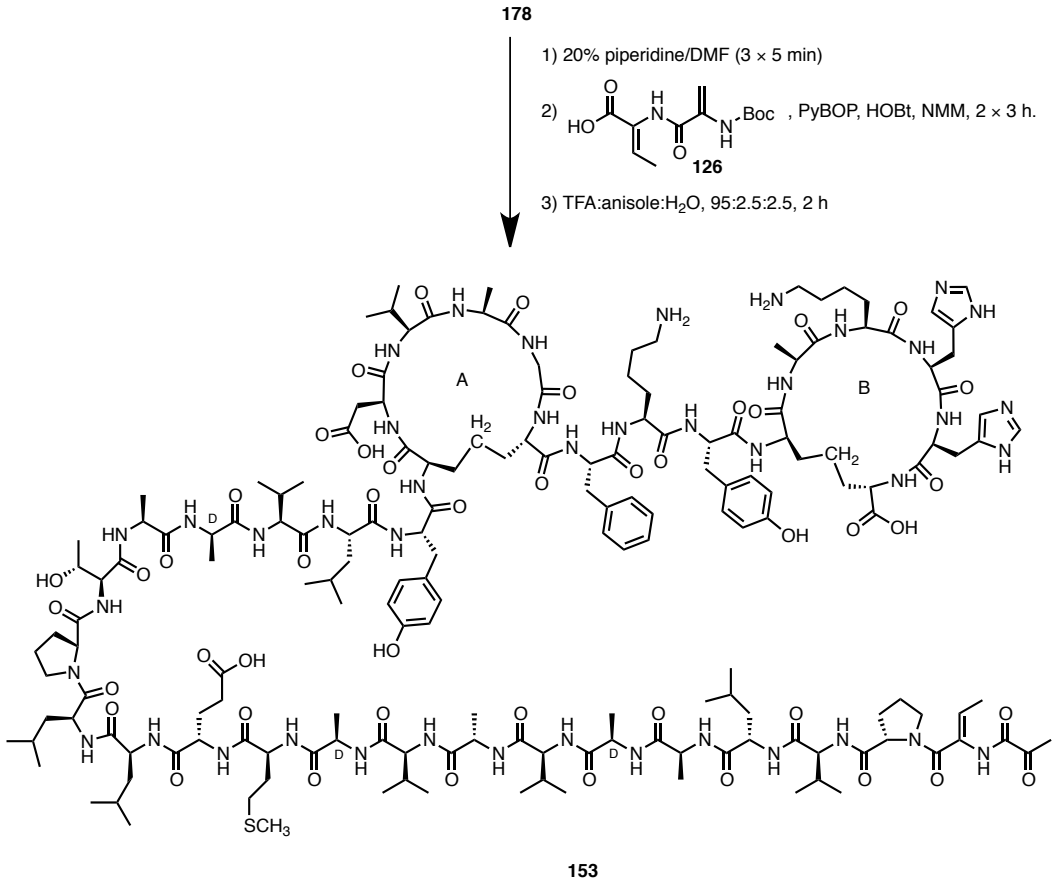
3.3.6 Synthesis of DAP lactocin S

The third and final DAP containing lactocin S analogue has two DAP rings and no lanthionines. The synthesis of this peptide utilizes compound **176**, an intermediate from the synthesis of B-DAP lactocin S (**153**), as starting material. Fmoc SPPS was used to introduce orthogonally protected DAP (**137**) and the rest of the amino acids of the B-ring (Scheme 3-16). After deprotection and cyclization reactions the peptide was extended by SPPS to yield **178**, consisting of residues 3 to 37 of DAP lactocin S.



Scheme 3-16 The synthesis of **178** consisting of residues 3-37 of DAP lactocin S. [a]Conditions for SPPS: (i) 20% piperidine in DMF, (ii) **137**, PyBOP, HOBt, NMM, (iii) Repeat steps (i) and (ii) for Fmoc-Gly-OH, Fmoc-Ala-OH, Fmoc-Val-OH, Fmoc-Asp(OtBu)-OH. [b]Conditions for SPPS: (i) 20% piperidine in DMF, (ii) Fmoc-Leu-OH, PyBOP, HOBt, NMM, (iii) Repeat steps (i) and (ii) for Fmoc-Val-OH, Fmoc-D-Ala-OH, Fmoc-Ala-OH, Fmoc-Thr(OtBu)-OH, Fmoc-Pro-OH, Fmoc-Leu-OH, Fmoc-Leu-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Met-OH, Fmoc-D-Ala-OH, Fmoc-Val-OH, Fmoc-Ala-OH, Fmoc-Val-OH, Fmoc-D-Ala-OH, Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Val-OH, Fmoc-Pro-OH.

The synthesis of DAP lactocin S (**153**) was completed by installation of dipeptide **126** and subsequent removal of the peptide from resin under acidic conditions.



Scheme 3-17 Synthesis of DAP lactocin S (**153**)

DAP lactocin S (**153**) was purified by a similar procedure to that used in the purification of lactocin S (**98**). Pure DAP lactocin S (**153**) was obtained in an overall yield of 1.6%, corresponding to an average yield of 94% per step over 33 deprotections and couplings, 2 macrocyclizations, 2 allyl deprotections and 1 cleavage/hydrolysis reaction. MALDI-TOF mass spectrometry confirmed that the desired peptide **153** was formed.

3.3.7 Activity testing of DAP-lactocin S analogues

The antimicrobial activity of the three DAP analogues was compared to that of lactocin S using spot-on-lawn assays. A panel of organisms was used, including the producing organism (*L. sakei*) and a sensitive strain (*P. acidilactici*). The experimental section lists other organisms used. The three peptides exhibit the same spectrum of activity as lactocin S (**98**). As shown in Figure 3-3, they are all active against *P. acidilactici* and none inhibit the growth of *L. sakei*. This dilution assay demonstrates that B-DAP lactocin S (**153**) and DAP lactocin S (**153**) are half as active as lactocin S. In contrast, A-DAP lactocin S (**152**) appears to retain the activity of lactocin S (**98**) and is somewhat more active against *P. acidilactici*.

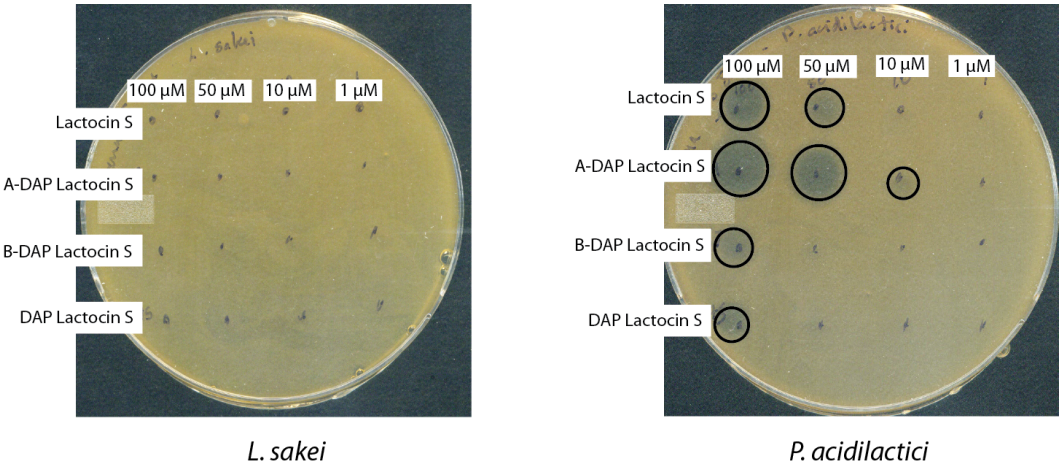


Figure 3-3 Comparative activity testing of DAP containing lactocin S analogues. The black circles indicate zones of clearing, where bacterial growth was inhibited by the peptide.

3.3.8 Implications for SAR of lactocin S

A-DAP lactocin S (**152**) is the first synthetic ring analogue of a lantibiotic to retain full activity compared to the natural peptide. Analogues of another lantibiotic, lacticin 3147 A2 (**52**) where sulfur is replaced with oxygen (**54**)[101] or an olefin (**55**)[100] had twenty times lower activity and no activity, respectively. A further analogue (**53**), with lanthionine rings instead of methyllanthionine rings, had no individual activity but retained synergistic activity (albeit 100 fold less active) with lacticin 3147 A1 (**51**) These results indicate that structural alteration of lanthionine rings can be very deleterious to the bioactivity of lantibiotic peptides and reinforces the significance of our results for the DAP-containing lactocin S compounds.

Little is understood about the mode of action of lactocin S. Preliminary results from a binding assay found that the peptide does not appear to bind with the cell wall precursor lipid II (Personal communication with Wilfred A. van der Donk). It is possible that the peptide forms pores in the cell membrane without complexing with lipid II, as is seen for higher concentrations of nisin A.[71,165] Another possibility is that the peptide interacts with a different membrane lipid, but this has yet to be determined. The activity results obtained for the DAP analogues of lactocin S provide a valuable insight into the SAR of the peptide. The difference in activity between **152** and **153** is marked, and suggests that the two rings of lactocin S may have quite different roles in the antibacterial action of the peptide. Previous reports have suggested that the cationic *C*-terminus of lactocin S (**98**) may interact with the anionic cell membrane of Gram-positive

bacteria.[140,165] There are two histidine residues and a lysine within the B-ring, that would all be positively charged at acidic pH. Perhaps structural changes (e.g. lanthionine to DAP as in **152**) to the B-ring interfere with its conformation, such that interaction between the positively charged residues of the ring and the anionic cell membrane is compromised. A decreased affinity for the bacterial cell membrane could then result in decreased pore formation and lower activity.

In contrast, the A-ring is further from the *C*-terminus and lacks any basic residues. Therefore, it may not be involved in a specific recognition interaction with the cell membrane. The full retention of activity when the A-ring is replaced with DAP (**151**), suggests that sulfur in the A-ring is not crucial for activity, making it more amenable to alteration. Additionally, with the introduction of a methylene carbon in place of the sulfur of lanthionine, this analogue appears by mass spectrometry to be more stable to oxidation by air. This is likely the cause of the small increase in activity observed for this analogue.

The double ring analogue (**153**) shows a similar level of activity to the B-ring analogue **152**. This is consistent with the hypothesis that changing the B-ring reduces activity and the alteration of the A-ring has no substantial effect on the activity. It is important to note that this analogue shows far greater oxidative stability when examined by mass spectrometry.

To gain a stronger understanding of the mode of action of this peptide, it would be useful to test its ability to cause membrane permeabilization, both with and without lipid II. The importance of the basic residues in the B-ring could be

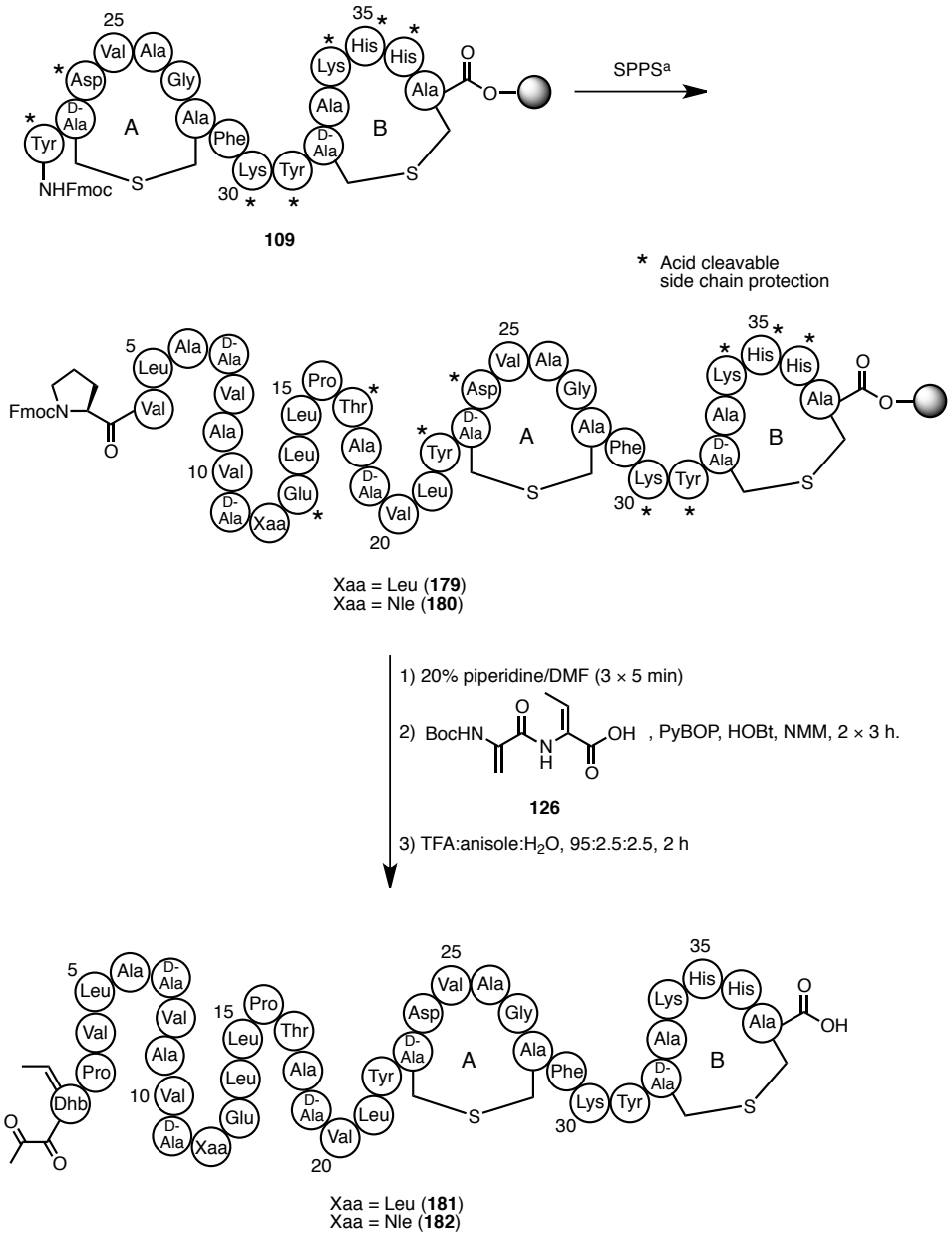
determined using a combination of analogue synthesis, activity testing, and membrane permeability assays.

3.3.9 Lactocin S analogues at methionine

Two analogues of lactocin S that differ at residue 12 were considered. Methionine could be replaced with an isosteric carbon mimic (norleucine, Nle) and a natural amino acid mimic (leucine, Leu). Norleucine has been previously shown to be a good mimic of methionine in the bioactive peptides neopetrosiamide A[156] and pediocin PA-1 (**15**).[166] The work in this section was completed with the assistance of an undergraduate summer student, Michael Little.

3.3.10 Synthesis of methionine 12 analogues of lactocin S

The synthesis of Leu-12 lactocin S (**181**) and Nle-12 lactocin S (**182**) begins with the A and B-rings (**109**) of lactocin S in place. The synthesis is then the same as for lactocin S, except incorporating either Fmoc-Leu-OH or Fmoc-Nle-OH at residue 12 instead of Fmoc-Met-OH (Scheme 3-18).



Scheme 3-18 The synthesis of Leu-12 (**181**) and Nle-12 (**182**) analogues of lactocin S. [a]Conditions for SPPS: (i) 20% piperidine in DMF, (ii) Fmoc-Leu-OH, PyBOP, HOBt, NMM, (iii) Repeat steps (i) and (ii) for Fmoc-Val-OH, Fmoc-D-Ala-OH, Fmoc-Ala-OH, Fmoc-Thr(OtBu)-OH, Fmoc-Pro-OH, Fmoc-Leu-OH, Fmoc-Leu-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Leu-OH or Fmoc-Nle-OH, Fmoc-D-Ala-OH, Fmoc-Val-OH, Fmoc-Ala-OH, Fmoc-Val-OH, Fmoc-D-Ala-OH, Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Val-OH, Fmoc-Pro-OH.

Leu-12 lactocin S (**181**) and Nle-12 lactocin S (**182**) were purified by a procedure similar to that used in the purification of lactocin S (**98**). Pure Leu-12 lactocin S (**181**) was obtained with an overall yield of 2.3%, corresponding to an average yield of 94% per step over 33 deprotections and couplings, 2 macrocyclizations, 2 allyl deprotections and 1 cleavage/hydrolysis reaction. Pure Nle-12 lactocin S (**182**) was obtained with an overall yield of 3.8 %, corresponding to an average yield of 95% per step over 33 deprotections and couplings, 2 macrocyclizations, 2 allyl deprotections and 1 cleavage/hydrolysis reaction. The formation of desired peptides **181** and **182** was confirmed by MALDI-TOF mass spectrometry.

3.3.11 Activity testing of analogues at methionine and the implications for the SAR of lactocin S

Spot-on-lawn activity assays were used to compare the antimicrobial activity of Leu-12 lactocin S (**181**) and Nle-12 lactocin S (**182**) to lactocin S (**98**). *P. acidilactici* was one of the organisms investigated. The experimental section lists others. Figure 3-4 shows they are both active against *P. acidilactici*. This dilution assay demonstrates that both Leu-12 lactocin S (**181**) and Nle-12 lactocin S (**182**) retain a similar level of biological activity against the indicator organism. These compounds also display the same behavior as lactocin S when tested against the other organisms.

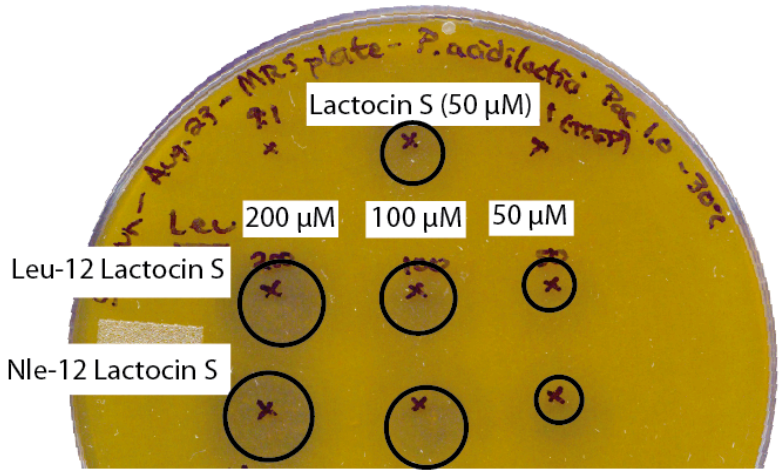


Figure 3-4 Activity testing of Leu-12 lactocin S (**181**) and Nle-12 lactocin S (**182**) against *P. acidilactici* Pac 1.0. The black circles indicate a zone of clearing where no bacterial growth is observed.

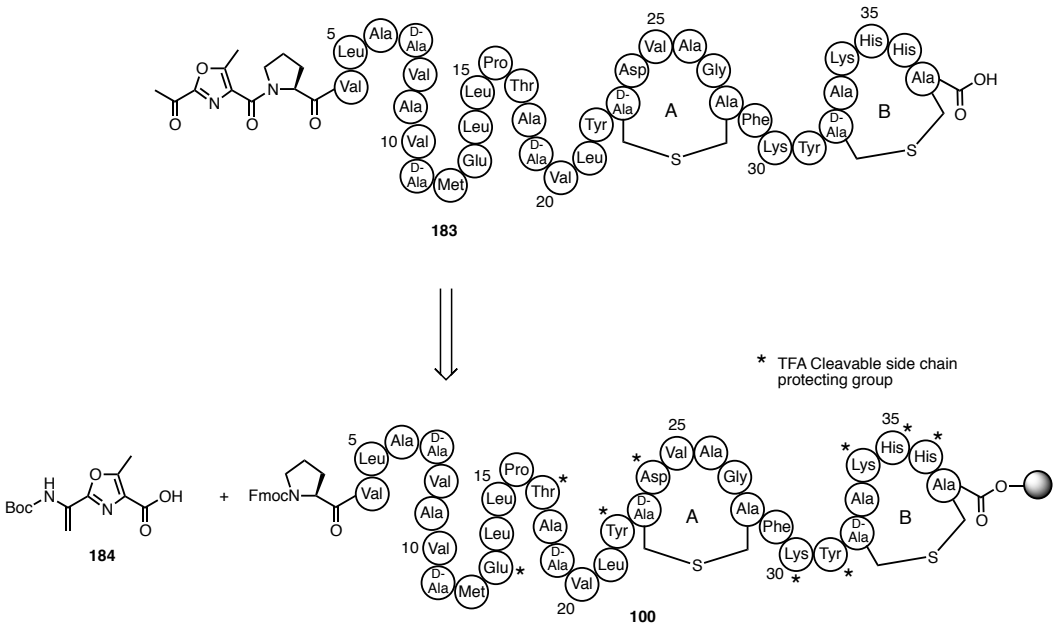
These results suggest that methionine 12 is not vital for the biological activity of lactocin S (**98**) and, much like the lanthionine of the A-ring, can be replaced by a suitable carbon mimic. Mass spectrometry evidence implies that the sulfur of methionine 12 is the first to oxidize in lactocin S, and so it is very advantageous to substitute this atom for one that is less reactive. Not only is the direct carbon analogue of methionine (norleucine) active but also a natural amino acid analogue (leucine).

It is very encouraging to identify suitable replacements for sulfur atoms in lantibiotics. Many of the clinically relevant lantibiotic peptides (such as lacticin 3147 A1 (**51**) and A2 (**52**)) have limitations to their application due to oxidative instability. These results obtained for lactocin S, strongly suggest that carbon analogues are a powerful tool for improving the utility of lantibiotics.

3.3.12 *N*-terminal analogue of lactocin S

The mode of action of lactocin S is unclear. Perhaps, like many other lantibiotics, lactocin S forms pores in the cell membrane of Gram-positive bacteria. The structure of the peptide has several features that should allow this to occur. The *N*-terminal region of lactocin S is very hydrophobic and this could allow the peptide to insert into the hydrophobic core of the cell membrane of a bacterium. As described previously, the cationic *C*-terminus of the peptide could interact with the polar cell surface of the membrane. The final analogue of lactocin S is designed to mimic the *N*-terminus of the peptide and simultaneously incorporate a planar moiety. This analogue should be equally hydrophobic and have a more rigid *N*-terminus that could improve insertion into a cell membrane.

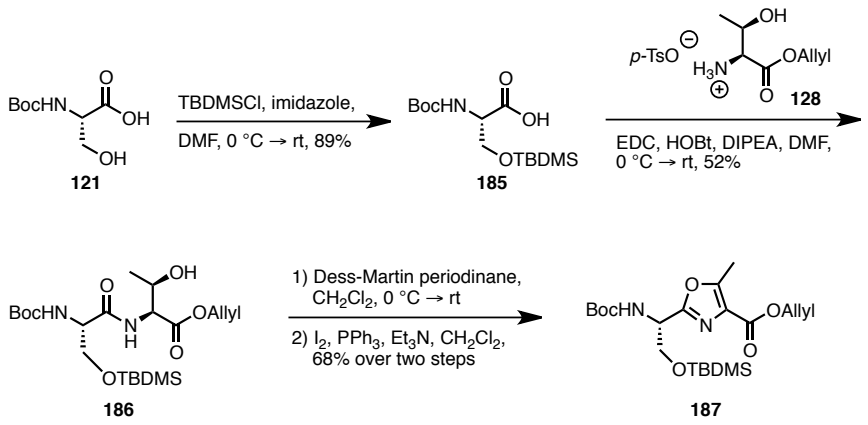
Oxazole lactocin S (**183**) could be synthesized by coupling cyclic dipeptide mimic **184** and the lactocin S precursor **100** on resin followed by acid cleavage. To complete this synthesis, oxazole **184** must first be made in solution.



Scheme 3-19 Retrosynthetic approach to oxazole lactocin S (**183**)

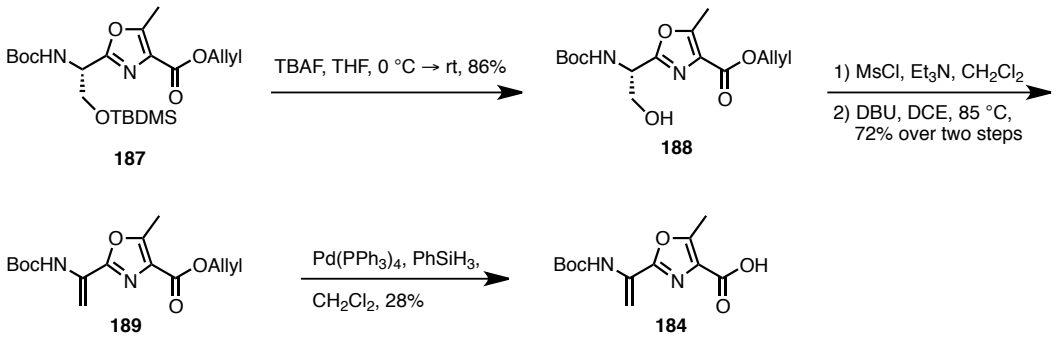
3.3.13 Synthesis of *N*-terminal oxazole

The synthesis of **184** commenced with the protection of the hydroxyl side chain of Boc-Ser-OH **121** as a *tert*-butyldimethylsilyl ether using TBDMS chloride and imidazole (Scheme 3-20).[167] The resulting protected serine **185** was coupled with allyl-threonine **128** using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and HOBt.



Scheme 3-20 Synthesis of protected oxazole **187**

Following methodology developed by Wipf et. al.,[168] the hydroxyl group of threonine in dipeptide **186** was oxidized to the corresponding ketone using Dess-Martin periodinane. Treatment with iodine, triphenylphosphine and triethylamine facilitates formation of the oxazole functionality in **187**. Silyl protection on the serine hydroxyl was then removed by tetra-*n*-butylammonium fluoride to give **188** (Scheme 3-21).



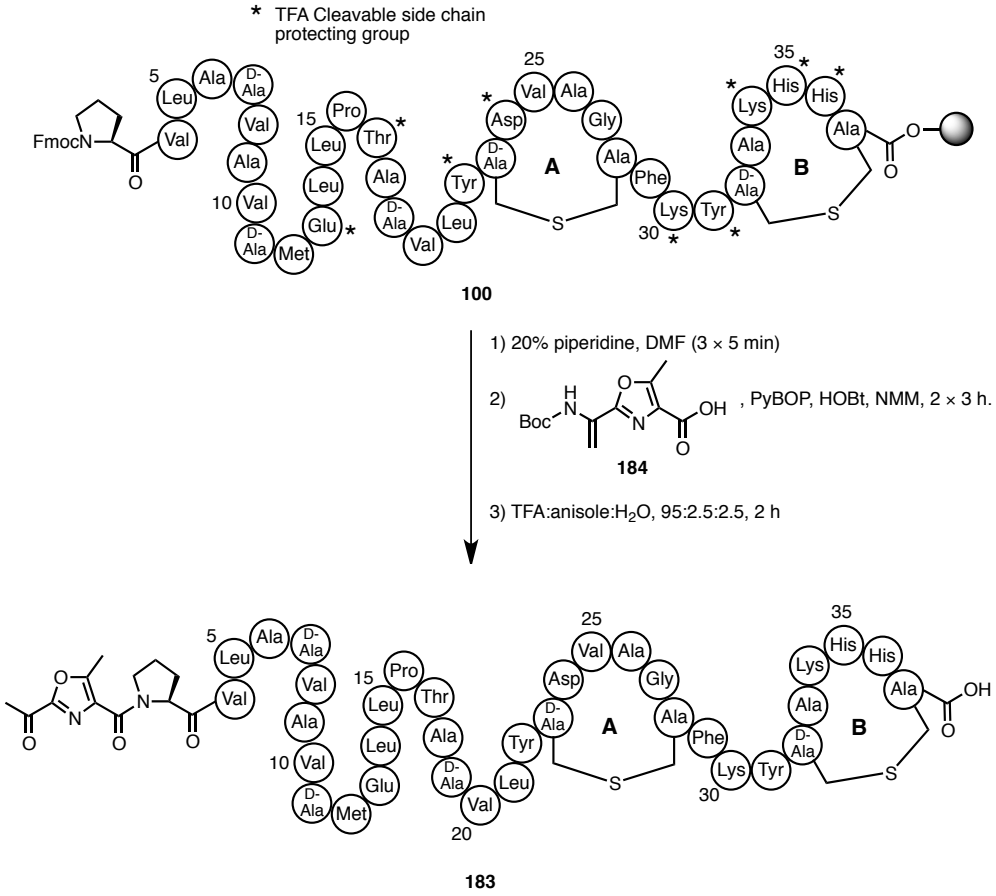
Scheme 3-21 Synthesis of *N*-terminal oxazole **184**

The dehydroalanine of **189** was generated by mesylation of **188** with methanesulfonyl chloride and triethylamine followed by elimination under basic conditions. Finally deprotection of the allyl ester in **188** with $Pd(PPh_3)_4$ in the presence of phenylsilane gave oxazole **184,** ready for use in the synthesis of oxazole lactocin S (**183**).

3.3.14 Synthesis of oxazole lactocin S

The synthesis of oxazole lactocin S (**183**) was completed by deprotection of the *N*-terminal Fmoc of **100** and coupling of the free amine to oxazole **184**. Cleavage under acidic conditions gives the fully deprotected peptide **183** with the desired α-ketoamide in place at the *N*-terminus.

Oxazole lactocin S (**183**) was purified following a procedure similar to that used in the purification of lactocin S (**98**). Pure oxazole lactocin S (**183**) was obtained in an overall yield of 5.3%, corresponding to an average yield of 96% per step over 33 deprotections and couplings, 2 macrocyclizations, 2 allyl deprotections and 1 cleavage/hydrolysis reaction. MALDI-TOF mass spectrometry was used to confirm that the desired peptide **183** was formed.



Scheme 3-22 Synthesis of oxazole-lactocin S (**183**)

3.3.15 Activity testing of oxazole lactocin S and implications for SAR understanding of lactocin S

Oxazole lactocin S (**183**) was tested for antibacterial activity against *P. acidilactici* and *L. sakei* using spot-on-lawn assays. No inhibition of bacterial growth was observed. It appears that the exact structure of the *N*-terminus of lactocin S is vital to the biological activity of this peptide and that the planar oxazole designed and tested in this project is not a suitable mimic for this functionality. This is an intriguing result, as it suggests that the *N*-terminus may

be involved in some sort of specific association and not just a general hydrophobic interaction. Formation of the oxazole replaces a hydrogen bond donor/acceptor (amide NH) with a hydrogen bond acceptor (oxazole N). This functionality could be involved in an important recognition interaction.

Another possibility is that the *N*-terminus is locked in the wrong conformation by this oxazole. For instance, the orientation of the methyl group on the dehydrobutyrine double bond is changed from the natural peptide. Finally, perhaps it is important that the *N*-terminus be flexible. Although activity was observed for lan-lacticin 3147 A2 (**53**) with Fmoc in place of the first five residues, this may not translate to lactocin S (**98**).

3.4 Conclusions and Future Direction

In this chapter the synthesis and biological testing of a series of analogues of lactocin S (**98**) have been described. A method for the synthesis of orthogonally protected DAP (**137**) has been developed, and this building block has been used to synthesize three different ring analogues of lactocin S. These peptides appear to be more oxidatively stable than lactocin S (**98**), and one of these, A-DAP lactocin S (**183**) is very active, displaying greater inhibitory activity than the parent **98**. This finding suggests that the B-ring of lactocin S is very important to the bioactivity of the peptide. These results show DAP is a very useful mimic of lanthionine and in the future its utility in the synthesis of analogues of other peptides should be investigated.

Analogues of lactocin S (**98**) in which methionine is replaced with carbon-containing amino acids were synthesized. Leu-12 lactocin S (**181**) and Nle-12 lactocin S (**182**) display similar activity compared to lactocin S (**98**) and appear to be more stable to atmospheric oxygen. Combining these results with those described above, synthesis of a lactocin S derivative with DAP for the A-ring and norleucine at residue 12 is currently underway in our group.

A final analogue of lactocin S in which the first two residues are replaced with an oxazole functionality has been synthesized. This analogue does not show any activity and may indicate that residues 1 and 2 are vital for the biological functioning of the peptide.

Table 3-1 A comparison of lactocin S and its analogues

Peptide	Number	Modification	Relative activity
lactocin S	**98**	N/A	++
A-DAP lactocin S	**151**	A-ring DAP	+++
B-DAP lactocin S	**152**	B-ring DAP	+
DAP lactocin S	**153**	A,B-rings DAP	+
Leu-12 lactocin S	**181**	residue 12 leucine	++
Nle-12 lactocin S	**182**	residue 12 norleucine	++
oxazole lactocin S	**151**	*N*-terminal oxazole	-

Taken together these analogues allow us to develop an understanding of what is important for the bioactivity of lactocin S. They also suggest that DAP

could be very useful as a replacement for lanthionine in other lantibiotic compounds with greater therapeutic potential.

Chapter 4: Conclusions and Future Directions

Lantibiotic peptides are potent antimicrobial compounds produced by bacteria to kill competing strains of bacteria. These compounds show promise as both food preservatives and clinical antibiotics. Because of the global challenge of drug resistant antibiotics, lantibiotics are appealing candidates for drug development due to the dual mode of action many possess.

The studies described in this dissertation have focused on the lantibiotic lactocin S (**98**). This peptide is produced in minute quantities by *Lactobacillus sakei* L45, and structural determination has been limited due availability of sufficient quantities from the natural source.

Using SPPS, lactocin S has been synthesized from individual amino acids. The unusual lanthionine residues were first made in solution with orthogonal protecting groups (allyl, Alloc and Fmoc). This building block (**58**) was then incorporated into the SPPS approach and the lanthionine rings were formed by removal of the allyl/Alloc protecting groups and subsequent on-resin cyclization. The highly modified *N*-terminus of the peptide was also prepared in solution. The α-ketoamide moiety was formed using a biomimetic approach, where it is introduced in SPPS as a Boc-protected dehydroalanine. When the full-length peptide is cleaved from the resin under acidic conditions, the protecting groups are simultaneously removed. The *N*-terminal enamine then tautomerizes to an iminium ion that is hydrolyzed to the desired α-ketoamide. This synthesis is only

the second total synthesis of a natural lantibiotic and the first achieved using SPPS.

A simplified purification of lactocin S (**98**) from *L. sakei* L45 was developed, and this allowed comparison of the natural and synthetic peptides. A variety of techniques were used to analyze these peptides. Biological activity, chiral GC/MS, MS/MS confirmed that the two peptides are identical and therefore the proposed structure of lactocin S (**98**) is correct.

In the second half of this thesis, a series of analogues of lactocin S (**98**) were synthesized. The lanthionine rings were systematically replaced using diaminopimelate to investigate the importance of the sulfur atom within these rings and as an attempt to improve the oxidative stability of the peptide. Orthogonally protected DAP (**137**) was synthesized in solution and then used in an SPPS approach in the same fashion as the lactocin S synthesis. The key step in the formation of the DAP building block **137**, was a photolysis reaction that converted a diacylperoxide (**170**) into the backbone of DAP by a radical decarboxylation/recombination mechanism.

A-DAP lactocin S (**151**) retained the full activity of the natural peptide and appeared to be even slightly more active. This is the first synthetic analogue of a lantibiotic to display activity as potent as the natural peptide. In constrast, B-DAP (**152**) and DAP lactocin S (**153**) are both half as active as the natural peptide. Considered together, these analogues provide considerable insight into the SAR of lactocin S (**98**). It would seem that ring B is vital for the bioactivity of the peptide as it is sensitive to structural changes. This may be because the cationic

B-ring is involved in a recognition interaction with the anionic cell surface of the bacterial cell membrane. Future studies should focus on these cationic residues and their effect on peptide activity.

The methionine residue at position 12 was replaced with norleucine and leucine to investigate if these more oxidatively stable amino acids would alter the activity of the peptide. Both analogues (**181** and **182**) retained full activity, which is a very promising result. Investigations are currently underway in our lab to produce an analogue with the sulfurs of both the A-ring and the methionine substituted with carbon.

An investigation of the *N*-terminus of lactocin S, replaced the first two "amino acids" with an oxazole mimic. This peptide displayed no biological activity. Further investigation into the importance of this portion of the molecule could reveal whether this is because of disruption of a specific interection or a conformational problem.

This thesis describes the synthesis of seven lantibiotic peptides and analogues, in total 327 individual solid phase reactions (excluding the preparation of complex amino acid starting materials) were done to provide these compounds. The modifications of lactocin S described include several beneficial alterations that could be applied to a large number of other lantibiotics. The use of diaminopimelate and leucine/norleucine to improve the stability of clinically relevant lantibiotics could drastically expand their application and utility.

Chapter 5: Experimental Procedures

5.1 General Information

5.1.1 Reagents, solvents and purifications

All commercially available reagents and protected amino acids were purchased from Sigma-Aldrich Canada Ltd., Fisher Scientific Ltd., Calbiochem-Novabiochem Corporation, Chem-Impex International Inc. or VWR International and used without further purification. All solvents used for anhydrous reactions were distilled prior to use: dichloromethane and 1,2-dichloroethane were distilled over calcium hydride, tetrahydrofuran was distilled over sodium with benzophenone as an indicator and ethyl acetate was distilled over potassium carbonate. HPLC grade acetonitrile, dimethylformamide, isopropyl alcohol and methanol were used without further purification. Commercially available ACS grade solvents (>99.0% purity) were used for column chromatography without any further purification. Air sensitive reactions were performed under an atmosphere of argon. All reactions and fractions from column chromatography were monitored by thin layer chromatography (TLC) using glass plates with a UV fluorescent indicator (normal SiO_2, Merck 60 F_{254}). One or more of the following methods were used for visualization: UV absorption by fluorescence quenching; staining with phosphomolybdic acid in ethanol (10 g/100 mL) or staining with ninhydrin (ninhydrin : acetic acid : *n*-butanol/ 0.6 g : 6 mL : 200 mL). Flash chromatography was performed using Merck type 60, 230-400 mesh silica gel.

High performance liquid chromatography (HPLC) was performed on a Beckman System Gold chromatograph equipped with a model 166 variable wavelength UV detector and a Rheodyne 7725i injector fitted with a 100 μL sample loop. The column used was a GE Healthcare stainless steel walled μRPC C2/C18 (3 μm, 4.6 × 100 mm). All HPLC solvents were filtered with a Millipore filtration system under vacuum before use.

5.1.2 Characterization

Nuclear Magnetic Resonance (NMR) spectra were recorded on a Varian Inova 600, Inova 500, Inova 400, Inova 300 or Unity 500 spectrometer at 27 °C. For ^{1}H (300, 400, 500 or 600 MHz) spectra, δ values were referenced to $CDCl_3$ (7.26 ppm) or CD_3OD (3.30 ppm), and for ^{13}C (75, 100, 125 or 150 MHz) spectra, δ values were referenced to $CDCl_3$ (77.0 ppm) or CD_3OD (49.0 ppm). Reported splitting patterns are abbreviated as s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. When appropriate, a signal is preceded by br, to indicate that it is broad.

Infrared spectra (IR) were recorded on either a Nicolet Magna 750 or a 20SX FT-IR spectrometer. Cast refers to the evaporation of a solution on a NaCl plate.

Mass spectra (MS) were recorded on a Kratos AEIMS-50, Bruker 9.4T Apex-Qe FTICR (high resolution, HRMS) or on a Perspective Biosystems Voyager™ Elite MALDI-TOF MS using either α-Cyano-4-hydroxy cinnamic acid

(CHCA) or 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) as a matrix. MS/MS was performed on a Bruker Ultraflextreme MALDI/TOF/TOF. For MALDI-TOF MS, a typical sample preparation is described below. A solution of sample peptide (0.1 μL) in 0.1% TFA (aq.) is mixed in a 1:1 ratio (vol/vol) with a stock solution of sinapinic acid (10 mg/mL) in 50% acetonitrile containing 0.1% TFA (aq.). To prepare the sample plate, a sinapinic acid layer (0.7 μL; 10mg/mL sinapinic acid in 3:2 acetone:methanol) is pipetted onto a stainless steel target plate. The solvent is allowed to evaporate, leaving a thin layer of sinapinic acid on the surface of the plate. The sample-matrix solution (0.4 μL) is then spotted onto the previous layer and allowed to dry.

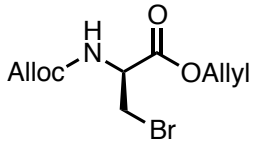
Optical rotations were measured on a Perkin Elmer 241 polarimeter with a microcell (10 cm, 1 mL) at ambient temperature and are reported in units of 10^{-1} deg cm^2 g^{-1}. All reported optical rotations were referenced against air and measured at the sodium D line (λ = 589.3 nm)

5.2 Synthesis and Characterization of Compounds

5.2.1 Synthesis of lactocin S (98)

5.2.1.a Synthesis of orthogonally protected lanthionine (58)

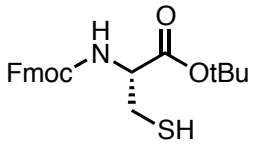
(*S*)-allyl 2-(((allyloxy)carbonyl)amino)-3-bromopropanoate (56)



This known compound was prepared according to literature procedure.[99] CBr_4 (18.3 g, 55.2 mmol) was added to a solution of **106** (11.5 g, 50.2 mmol) in CH_2Cl_2 (400 mL) at 0 °C, then triphenylphosphine (14.5 g, 55.2 mmol) was added portion-wise (~ 2 g each time) over 10 min. The reaction was left to stir at 0 °C for 1.5 h and then warmed to rt and allowed to stir a further 30 min. The reaction was washed with 10% $NaHCO_3$ (1 × 100 mL), then H_2O (2 × 50 mL) and dried over $MgSO_4$. The solvent was removed *in vacuo* and the resulting crude product was purified by flash chromatography (SiO_2, 9:1 hexanes:EtOAc) to yield **56** (4.8 g, 32.7%) as a colorless oil. (R_f 0.71 on SiO_2, 1:1 hexanes:EtOAc); $[\alpha]_D^{26}$ -12.1 (*c* 1.3, CH_2Cl_2); IR (CH_2Cl_2 cast) 3340, 2948, 1726, 1514, 1194 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 5.97-5.88 (m, 2H, -$OCH_2C\underline{H}$=CH_2), 5.65 (d, 1H, *J* = 6.5 Hz, -N$\underline{H}$), 5.39-5.22 (m, 4H, -OCH_2CH=$C\underline{H}_2$), 4.84-4.79 (m, 1H, -$C\underline{H}_\alpha$), 4.74-4.66 (m, 2H, --$OC\underline{H}_2$), 4.60 (d, 2H, *J* = 5.5 Hz, -$OC\underline{H}_2$), 3.85 (dd, 1H, *J* = 10.5, 3.0 Hz, -$C\underline{H}_\beta$), 3.75 (dd, 1H, *J* = 10.5, 3.5 Hz, -$C\underline{H}_\beta$); ^{13}C NMR ($CDCl_3$, 125

MHz): δ 168.6, 155.4, 132.3, 131.1, 119.4, 118.1, 66.8, 66.1, 54.3, 33.7; HRMS (ES) Calculated for $C_{10}H_{14}NO_4BrNa$ 313.9998, found 313.9995 $(M+Na)^+$.

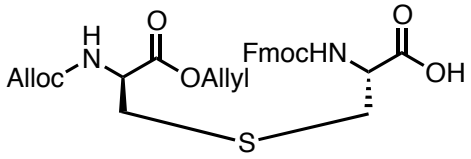
(*R*)-*tert*-butyl 2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-3-mercaptopropanoate (57)



This known compound was prepared according to literature procedure.[99,126] Tributylphosphine (1.90 mL, 7.53 mmol) was added to a solution of Fmoc-Cystine-OtBu (3.00 g, 3.76 mmol) in THF (300 mL). Water (30 mL) was added and the reaction was stirred for 3.5 h. Solvent was removed *in vacuo*, the residue dissolved in EtOAc (250 mL) and washed with 10% citric acid (3 × 100 mL), H_2O (3 × 100 mL) and brine (3 × 100 mL). The organic layer was dried over $MgSO_4$ and then concentrated *in vacuo*. The resulting crude product was purified by flash chromatography (SiO_2, 4:1 hexanes:EtOAc) to yield **57** (2.62 g, 87.2%) as a white foam. (R_f 0.53 on SiO_2, 4:1 hexanes:EtOAc); $[\alpha]_D^{26}$ -0.9 (*c* 2.2, CH_2Cl_2); IR (CH_2Cl_2 cast) 3335, 2979, 2570, 1719, 1509, 1450, 1248 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ 7.78 (d, 2H, *J* = 7.2 Hz, Ar-H), 7.62 (d, 2H, *J* = 7.2 Hz, Ar-H), 7.41 (t, 2H, *J* = 7.2 Hz, Ar-H), 7.78 (t, 2H, *J* = 7.6 Hz, Ar-H), 5.69 (d, 1H, *J* = 6.0 Hz, -NH), 4.58-4.53 (m, 1H, CH_α), 4.48-4.37 (m, 2H, Fmoc-CH_2), 4.24 (t, 1H, *J* = 6.8 Hz, Fmoc-CH), 3.04-2.97 (m, 1H, -CH_β), 1.51 (s, 9H, -$C(CH_3)_3$), 1.35 (t, 1H, *J* = 8.8 Hz, -SH); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 168.9,

153.6, 143.9, 143.7, 141.34, 141.32, 127.8, 127.1, 125.13, 125.06, 120.02, 120.0, 83.1, 67.1, 55.4, 47.2, 28.0, 27.4; HRMS (ES) Calculated for $C_{22}H_{25}NO_4SNa$ 422.1397, found 422.1397 $(M+Na)^+$.

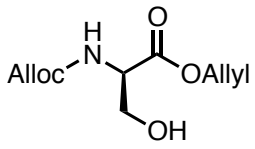
(*R*)-2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-3-(((*S*)-3-(allyloxy)-2-(((allyloxy)carbonyl)amino)-3-oxopropyl)thio)propanoic acid (58)



This known compound was prepared according to literature procedure.[99,126] Phenylsilane (0.58 mL, 4.73 mmol) and TFA (40 mL) were added to a solution of **107** (2.89 g, 4.73 mmol) in CH_2Cl_2 (40 mL). The reaction was stirred at rt for 2 h and then the solvent was removed *in vacuo*. The crude product was dissolved in toluene (30 mL) and left at 4 °C for 24 h. Toluene was decanted off and the resulting oil was dried under vacuum to yield **58** (0.87 g, 33.0 %) as a white foam. (R_f 0.01 on SiO_2, 1:1 hexanes:EtOAc); $[\alpha]_D^{26}$ -3.30 (*c* 1.0, MeOH); IR (CH_2Cl_2 cast) 3325, 3068, 2946, 1720, 1522, 1248, 1210 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz, *2 diastereomers in ~ 9:1 ratio*): δ 7.78 (d, 2H, *J* = 7.5 Hz, Ar-H), 7.67 (d, 2H, *J* = 7.0 Hz, Ar-H), 7.38 (t, 2H, *J* = 7.5 Hz, Ar-H), 7.30 (t, 2H, *J* = 7.5 Hz, Ar-H), 5.95-5.84 (m, 2H, $-OCH_2CH=CH_2$), 5.30 (dd, 1H, *J* = 17.0, 1.5 Hz, $-OCH_2CH=CH_2$), 5.29 (d, 1H, *J* = 17.0 Hz, $-OCH_2CH=CH_2$), 5.19 (d, 1H, *J* = 10.5 Hz, $-OCH_2CH=CH_2$), 5.15 (d, 1H, *J* = 11.0 Hz, $-OCH_2CH=CH_2$), 4.60 (d, 2H, *J* =

5.5 Hz, -OC$\underline{H}_2$), 4.53 (d, 2H, *J* = 5.0 Hz, -OC$\underline{H}_2$), 4.47-4.38 (m, 2H, 2 × -C$\underline{H}_\alpha$), 4.37-4.29 (m, 2H, Fmoc-C$\underline{H}_2$), 4.23 (t, 1H, *J* = 7.0 Hz, Fmoc-C$\underline{H}$), 3.11-3.02 (m, 2H, -C$\underline{H}_\beta$), 2.98-2.88 (m, 2H, -C$\underline{H}_\beta$); ^{13}C NMR (CD_3OD, 125 MHz): δ 173.8, 172.0, 158.5, 158.3, 145.29, 145.25, 142.6, 134.2, 133.1, 128.8, 128.2, 126.4, 120.9, 118.9, 117.7, 68.3, 67.1, 66.8, 55.7 (major), 55.5 (minor), 55.4 (major), 55.3 (minor), 48.4, 35.5, 35.2 HRMS (ES) Calculated for $C_{28}H_{30}N_2O_8S$ 553.1650, found 553.1654 $(M+H)^+$. The diastereomeric ratio (~ 9:1) could be determined by integrating the ^{13}C NMR α-carbon signal as described previously.[99,126]

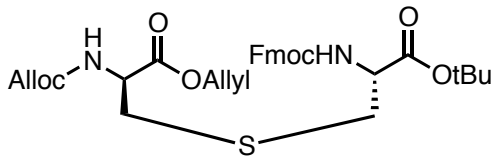
(*R*)-allyl 2-(((allyloxy)carbonyl)amino)-3-hydroxypropanoate (106)



This known compound was prepared according to literature procedure.[99,120] allyl alcohol (64.7 mL, 951.5 mmol) was added to a solution of D-serine (10 g, 95.2 mmol) and *p*-toluenesulfonic acid (21.7 g, 114.2 mmol) in toluene (300 mL). The reaction mixture was heated to reflux with a Dean-Stark apparatus for 16 h. Solvent was then removed *in vacuo* and the resulting residue was dissolved in EtOAc (200 mL). To this was added Et_3N (53 mL, 104.6 mmol) and the reaction was stirred for 10 min. The reaction mixture was cooled to 0 °C and a solution of allylchloroformate (112 mL, 112.1 mmol) in EtOAc (100 mL) was added dropwise over 1 h. The reaction was stirred for 18 h and then washed with H_2O (1 × 200 mL), 0.5 M HCl (1 × 200 mL), H_2O (1 × 200 mL) and brine (1 × 100 mL).

The organic layer was dried over $MgSO_4$ and then concentrated *in vacuo*. The resulting crude product was purified by flash chromatography (SiO_2, 7:3 hexanes:EtOAc) to yield **106** (11.5 g, 52.8%) as a colorless oil. (R_f 0.42 on SiO_2, 1:1 hexanes:EtOAc); $[\alpha]_D^{26}$ 0.9 (*c* 2.2, CH_2Cl_2); IR (CH_2Cl_2 cast) 3389, 2947, 1725, 1649, 1527, 1199 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ 5.91 (ddt, 1H, *J* = 17.2, 10.4, 5.6 Hz, -$OCH_2C\underline{H}=CH_2$), 5.91 (ddt, 1H, *J* = 17.2, 10.4, 5.6 Hz, -$OCH_2C\underline{H}=CH_2$), 5.80-5.72 (m, 1H, -N$\underline{H}$), 5.34 (dq, 1H, *J* = 16.4, 1.6 Hz, -$OCH_2CH=C\underline{H}_2$), 5.32 (d, 1H, *J* = 17.6 Hz, -$OCH_2CH=C\underline{H}_2$), 5.26 (dq, 1H, *J* = 10.4, 1.2 Hz, -$OCH_2CH=C\underline{H}_2$), 5.22 (dq, 1H, *J* = 10.4, 1.2 Hz, -$OCH_2CH=C\underline{H}_2$), 4.69-4.66 (m, 2H, -O$C\underline{H}_2$), 4.60-4.57 (m, 2H, -O$C\underline{H}_2$), 4.49-4.43 (m, 1H, -$C\underline{H}_\alpha$), 4.01 (dd, 1H, *J* = 11.2, 3.6 Hz, -$C\underline{H}_\beta$), 3.93 (dd, 1H, *J* = 11.2, 3.6 Hz, -$C\underline{H}_\beta$), 2.58-2.36 (br s, 1H, -O$\underline{H}$); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 170.3, 132.5, 131.4, 118.9, 118.0, 66.3, 66.1, 63.2, 56.1; HRMS (ES) Calculated for $C_{10}H_{15}NO_5Na$ 252.0842, found 252.0839 $(M+Na)^+$.

(*S*)-allyl 3-(((*R*)-2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-3-(*tert*-butoxy)-3-oxopropyl)thio)-2-(((allyloxy)carbonyl)amino)propanoate (107)

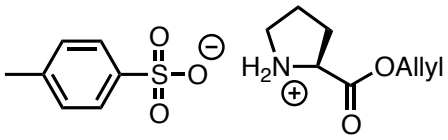


This known compound was prepared according to literature procedure.[99,126] Tetrabutylammonium bromide (8.39 g, 26.0 mmol) was dissolved in 0.5 M $NaHCO_3$ (100 mL, pH 8.5) and stirred for 5 min. A solution of **56** (1.90 g, 6.50

mmol) and **57** (2.60 g, 6.50 mmol) in EtOAc (100 mL) was then added and the biphasic reaction was stirred vigorously at rt for 24 h. After separation the organic layer was washed with H_2O (100 mL), dried over $MgSO_4$ and then the solvent was removed *in vacuo*. The resulting crude product was purified by flash chromatography (SiO_2, 4:1 hexanes:EtOAc) to yield **107** (2.89 g, 72.8%) as a colourless oil. (R_f 0.20 on SiO_2, 3:1 hexanes:EtOAc); $[\alpha]_D^{26}$ -6.8 (*c* 0.5, EtOAc); IR (EtOAc cast) 3338, 2979, 2935, 1727, 1515, 1250 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ 7.77 (d, 2H, *J* = 7.6 Hz, Ar-H), 7.65-7.59 (m, 2H, Ar-H), 7.41 (t, 2H, *J* = 7.6 Hz, Ar-H), 7.32 (td, 2H, *J* = 7.6, 1.2 Hz, Ar-H), 5.96-5.84 (m, 2H, -OCH_2CH=CH_2), 5.76 (d, 1H, *J* = 6.4 Hz, -NH), 5.68 (d, 1H, *J* = 7.2 Hz, -NH), 5.36-5.18 (m, 4H, -OCH_2CH=CH_2), 4.64 (dd, 2H, *J* = 5.6, 1.2 Hz, -OCH_2), 4.64-4.59 (m, 1H, -CH_α), 4.59 (d, 2H, *J* = 5.6 Hz, -OCH_2), 4.53-4.47 (m, 1H, -CH_α), 4.40 (d, 2H, *J* = 7.2 Hz, Fmoc-CH_2), 4.24 (t, 1H, *J* = 7.2 Hz, Fmoc-CH), 3.08-2.94 (m, 4H, -CH_β), 1.49 (s, 9H, -$C(CH_3)_3$); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 170.1, 169.3, 155.76, 155.74, 143.8, 143.78, 141.3, 132.5, 131.2, 127.7, 127.1, 125.1, 119.9, 119.2, 117.9, 83.1, 67.2, 66.4, 66.0, 54.3, 53.9, 47.1, 35.9, 35.8; HRMS (ES) Calculated for $C_{32}H_{39}N_2O_8S$ 611.2422, found 611.2413 $(M+H)^+$.

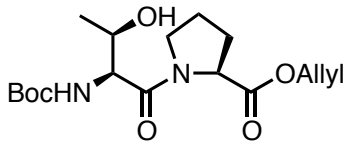
5.2.1.b Synthesis of *N*-terminal portion of lactocin S

(*S*)-2-((allyloxy)carbonyl)pyrrolidin-1-ium 4-methylbenzenesulfonate (115)



This known compound was prepared according to literature procedure.[169] allyl alcohol (29.5 mL, 434 mmol) and *p*-toluenesulfonic acid (9.90 g, 52.1 mmol) were added to a solution of L-proline (5.00 g, 43.4 mmol) in toluene (250 mL). The mixture was heated at reflux with a Dean-Stark apparatus for 16 h. The solvent was removed *in vacuo* to yield **115** (14.2 g, 99.0%) as a yellow oil. The crude product was carried through the next reaction without further purification. (R_f 0.21 on SiO_2, 90:10:1 EtOAc:MeOH:Acetic acid); $[\alpha]_D^{26}$ -21.3 (*c* 1.0, $CHCl_3$); IR ($CHCl_3$ cast) 3473, 2982, 2769, 1747, 1223, 1168 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 9.40-9.26 (br s, 1H, -NH), 8.64-8.51 (br s, 1H, -NH), 7.66 (d, 2H, *J* = 8.1 Hz, Ar-H), 7.08 (d, 2H, *J* = 8.1 Hz, Ar-H), 5.76 (ddt, 1H, *J* = 17.1, 10.5, 5.7 Hz, -OCH_2CH=CH_2), 5.21 (dq, 1H, *J* = 17.4, 1.5 Hz, -OCH_2CH=CH_2), 5.14 (dq, 1H, *J* = 10.4, 1.2 Hz, -OCH_2CH=CH_2), 4.60-4.42 (m, 3H, -CH_α, -OCH_2), 3.50-3.31 (d, 2H, -$CH_{2\delta}$), 2.36-2.21 (m, 4H, -CH_β, -CH_3), 2.07-1.79 (m, 3H, -CH_β, -$CH_{2\gamma}$); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 168.7, 141.1, 140.7, 131.0, 129.0, 128.9, 128.2, 126.1, 119.5, 67.1, 59.5, 46.4, 28.7, 23.5, 21.4; HRMS (ES) Calculated for $C_8H_{14}NO_2$ 156.1019, found 156.1017 $(M+H)^+$.

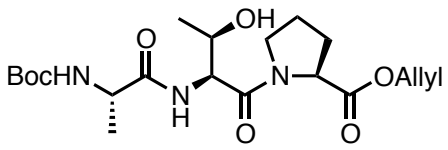
(*S*)-allyl 1-((2*S*,3*R*)-2-((*tert*-butoxycarbonyl)amino)-3-hydroxybutanoyl)pyrrolidine-2-carboxylate (116)



EDC (2.24 g, 11.7 mmol), HOBt (1.45 g, 10.7 mmol) and DIPEA (3.73 mL, 21.4 mmol) were added to a solution of **115** (3.18 g, 9.79 mmol) and Boc-Thr-OH (2.13 g, 9.79 mmol) in DMF (60 mL). The reaction was allowed to stir for 16 h and then H_2O (500 mL) was added. The reaction was extracted with EtOAc (3 × 100 mL) and the organic layer was washed with 1 M HCl (50 mL), 10% $NaHCO_3$ (50 mL), H_2O (50 mL) and brine (50 mL). The organic layer was dried over Na_2SO_4 and the solvent removed *in vacuo*. The resulting crude product was purified by flash chromatography (SiO_2, 2:1 hexanes:EtOAc) to yield **116** (1.29 g, 37.2%) as a colourless oil. $[\alpha]_D^{26}$ -63.5 (*c* 1.0, $CHCl_3$); IR ($CHCl_3$ cast) 3427, 2978, 2935, 2883, 1745, 1710, 1639, 1505, 1445, 1173 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 5.88 (ddt, 1H, *J* = 17.0, 10.5, 6.0 Hz, -$OCH_2C\underline{H}$=CH_2), 5.45 (d, 1H, *J* = 9.5 Hz, -N$\underline{H}$), 5.31 (dq, 1H, *J* = 17.5, 1.5 Hz, -OCH_2CH=C$\underline{H}_2$), 5.23 (dq, 1H, *J* = 10.5, 1.0 Hz, -OCH_2CH=C$\underline{H}_2$), 4.65-4.55 (m, 2H, -OC$\underline{H}_2$), 4.55-4.51 (m, 1H, Pro-C$\underline{H}_\alpha$), 4.38 (dd, 1H, *J* = 9.0, 2.0 Hz, Thr-C$\underline{H}_\alpha$), 4.17-4.11 (m, 1H, Thr-C$\underline{H}_\beta$), 3.82-3.75 (m, 1H, -C$\underline{H}_\delta$), 3.74-3.68 (m, 1H, -C$\underline{H}_\delta$), 3.39 (s, 1H, -O$\underline{H}$), 2.28-2.18 (m, 1H, -C$\underline{H}_\beta$), 2.07-1.94 (m, 3H, -C$\underline{H}_\beta$, -C$\underline{H}_{2\gamma}$), 1.41 (s, 9H, -C($C\underline{H}_3$)$_3$), 1.19 (d, 3H, *J* = 6.5 Hz, -C$\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 171.7, 170.9, 156.0,

131.6, 118.7, 79.9, 67.4, 65.9, 58.9, 55.6, 47.2, 29.0, 28.3, 24.9, 18.5; HRMS (ES) Calculated for $C_{17}H_{28}N_2O_6Na$ 379.1840, found 379.1841 $(M+Na)^+$.

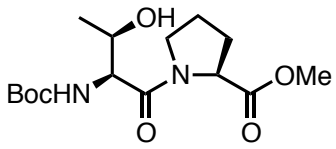
(*S*)-allyl 1-((2*S*,3*R*)-2-((*S*)-2-((*tert*-butoxycarbonyl)amino)propanamido)-3-hydroxybutanoyl)pyrrolidine-2-carboxylate (118)



TFA (20 mL) was added to a solution of **116** (3.00 g, 11.7 mmol) in CH_2Cl_2 (20 mL) and the reaction was stirred for 2 h. The solvent was removed *in vacuo* and the resulting residue was dissolved in DMF (60 mL). Boc-Ala-OH (2.40 g, 12.9 mmol), PyBOP (6.70 g, 12.9 mmol), HOBt (1.70 g, 12.9 mmol) and DIPEA (4.70 mL, 26.9 mmol) were added and the reaction was allowed to stir for 3 h. Additional DIPEA (3.00 mL, 17.2 mmol) was added and the reaction was left a further 2.5 h. H_2O (500 mL) was added and the reaction was extracted with EtOAc (3 × 100 mL). The organic layer was washed with 1 M HCl (50 mL), 10% $NaHCO_3$ (50 mL), H_2O (50 mL) and brine (50 mL). The organic layer was dried over Na_2SO_4 and the solvent removed *in vacuo*. The resulting crude product was purified by flash chromatography (SiO_2, 1:1 hexanes:EtOAc) to yield **118** (2.66 g, 53.2%) as a white foam. (R_f 0.35 on SiO_2, 100 % EtOAc); $[\alpha]_D^{26}$ -85.3 (*c* 1.0, MeOH); IR (MeOH cast) 3328, 2979, 2937, 2882, 1745, 1712, 1649, 1518, 1453, 1367, 1170 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz): δ 5.93 (ddt, 1H, *J* = 17.0, 10.5, 5.5 Hz, $-OCH_2C\underline{H}=CH_2$), 5.33 (dq, 1H, *J* = 17.0, 1.5 Hz, $-OCH_2CH=C\underline{H}_2$), 5.22

(dq, 1H, J = 10.5, 1.5 Hz, -OCH$_2$CH=C$\underline{H}_2$), 4.64-4.59 (m, 2H, -OC$\underline{H}_2$), 4.55 (d, 1H, J = 6.5 Hz, Thr-C$\underline{H}_\alpha$), 4.45 (dd, 1H, J = 9.0, 5.5 Hz, Pro-C$\underline{H}_\alpha$), 4.13-4.05 (m, 1H, Ala-C$\underline{H}_\alpha$), 4.03-3.96 (m, 1H, Thr-C$\underline{H}_\beta$), 3.94-3.86 (m, 1H, Pro-C$\underline{H}_\delta$), 3.79-3.72 (m, 1H, Pro-C$\underline{H}_\delta$), 2.33-2.24 (m, 1H, Pro-C$\underline{H}_\beta$), 2.09-1.92 (m, 3H, Pro-C$\underline{H}_\beta$, Pro-C$\underline{H}_{2\gamma}$), 1.43 (s, 9H, -C(C$\underline{H}_3$)$_3$), 1.29 (d, 3H, J = 7 Hz, Ala-C$\underline{H}_3$), 1.25 (d, 3H, J = 6.5 Hz, Thr-C$\underline{H}_3$); ^{13}C NMR (CD_3OD, 125 MHz): δ 178.5, 175.7, 173.7, 160.2, 135.9, 121.3, 83.3, 71.4, 69.4, 63.1, 60.7, 54.1, 52.1, 51.4, 32.7, 31.3, 28.5, 22.4, 20.7; HRMS (ES) Calculated for $C_{20}H_{33}N_3O_7Na$ 450.2211, found 450.2213 $(M+Na)^+$.

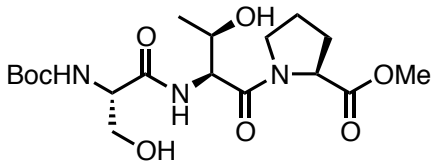
(*S*)-methyl 1-((2*S*,3*R*)-2-((*tert*-butoxycarbonyl)amino)-3-hydroxybutanoyl)pyrrolidine-2-carboxylate (122)



This known compound was prepared according to a modified literature procedure.[170] DIPEA (13.1 mL, 75.4 mmol) was added to a solution of H-Pro-OMe hydrochloride (5.00 g, 30.2 mmol), Boc-Thr-OH (7.30 g, 33.2 mmol), PyBOP (17.3 g, 33.2 mmol) and HOBt (4.50 g, 33.2 mmol) in DMF (80 mL) at 0 °C. The reaction was allowed to warm to rt and was stirred for a further 2 h. H_2O (500 mL) was added and the reaction was extracted with EtOAc (3 × 100 mL). The organic layer was washed with 1 M HCl (50 mL), 10% $NaHCO_3$ (50 mL), H_2O (50 mL) and brine (50 mL). The organic layer was dried over Na_2SO_4

and the solvent removed *in vacuo*. The resulting crude product was purified by flash chromatography (SiO_2, 1:1 hexanes:EtOAc) to yield **122** (5.20 g, 52.2%) as a white foam. (R_f 0.68 on SiO_2, 1:1 acetone:EtOAc); $[\alpha]_D^{26}$ -61.7 (*c* 1.0, CH_2Cl_2); IR (CH_2Cl_2 cast) 3425, 2978, 1747, 1710, 1639, 1510, 1440, 1171 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ 5.51 (d, 1H, *J* = 9.2 Hz, -NH), 4.41-4.36 (m, 1H, Pro-CH_α), 4.26 (dd, 1H, *J* = 8.8, 2.4 Hz, Thr-CH_α), 4.03-3.94 (m, 1H, Thr-CH_β), 3.71-3.63 (m, 1H, Pro-CH_δ), 3.59 (s, 3H, $-OCH_3$), 3.52-3.46 (m, 1H, Pro-CH_δ), 2.15-2.02 (m, 1H, Pro-CH_β), 1.96-1.78 (m, 3H, Pro-CH_β, Pro-$CH_{2\gamma}$), 1.30 (s, 9H, $-C(CH_3)_3$), 1.08 (d, 3H, *J* = 6.4 Hz, Thr-CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 172.3, 170.6, 155.9, 79.6, 67.3, 58.7, 55.9, 52.2, 47.1, 28.8, 28.2, 24.8, 18.5; HRMS (ES) Calculated for $C_{15}H_{26}N_2O_6Na$ 353.1683, found 353.1683 $(M+Na)^+$.

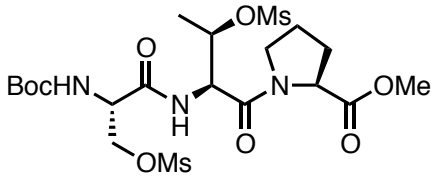
(*S*)-methyl 1-((2*S*,3*R*)-2-((*S*)-2-((*tert*-butoxycarbonyl)amino)-3-hydroxypropanamido)-3-hydroxybutanoyl)pyrrolidine-2-carboxylate (123)



TFA (10 mL) was added to a solution of **122** (2.50 g, 7.50 mmol) in CH_2Cl_2 (20 mL) and was stirred for 2 h. The solvent was removed *in vacuo* and the resulting residue was dissolved in DMF (25 mL). Boc-Ser-OH (1.71 g, 8.30 mmol), PyBOP (4.73 g, 9.10 mmol) and HOBt (1.23 g, 9.10 mmol) were added and the reaction was cooled to 0 °C. DIPEA (3.30 mL, 18.9 mmol) was added and the reaction was allowed to warm to rt over 16 h. Solvent was removed *in vacuo* and

the residue was purified by flash chromatography (SiO_2, 1:2 hexanes:EtOAc) to yield **123** (1.70 g, 53.8%) as a white foam. (R_f 0.39 on SiO_2, 100% EtOAc); $[\alpha]_D^{26}$ -65.2 (*c* 1.0, CH_2Cl_2); IR (CH_2Cl_2 cast) 3318, 2979, 1744, 1712, 1634, 1519, 1452, 1173 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 7.22 (d, 1H, *J* = 7.5 Hz, -NH), 5.53 (d, 1H, *J* = 7.0 Hz, -NH), 4.69 (dd, 1H, *J* = 8.5, 3.0 Hz, Thr-CH$_\alpha$), 4.54 (dd, 1H, *J* = 8.5, 4.5 Hz, Pro-CH$_\alpha$), 4.28-4.20 (m, 2H, Ser-CH$_\alpha$, Thr-CH$_\beta$), 4.06-3.99 (m, 1H, Ser-CH$_\beta$), 3.84-3.77 (m, 1H, Pro-CH$_\delta$), 3.74 (s, 3H, -OCH_3), 3.75-3.69 (m, 1H, Ser-CH$_\beta$), 3.69-3.63 (m, 1H, Pro-CH$_\delta$), 3.46-3.35 (br s, 2H, -OH), 2.32-2.21 (m, 1H, Pro-CH$_\beta$), 2.09-1.94 (m, 3H, Pro-CH$_\beta$, Pro-CH$_{2\gamma}$), 1.45 (s, 9H, -C(CH_3)$_3$), 1.25 (d, 3H, *J* = 6.5 Hz, Thr-CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 172.5, 171.8, 170.1, 155.8, 80.4, 67.4, 63.0, 59.0, 55.9, 55.6, 52.5, 47.4, 43.8, 28.9, 28.3, 24.9, 19.1; HRMS (ES) Calculated for $C_{18}H_{31}N_3O_8Na$ 440.2003, found 440.2004 $(M+Na)^+$.

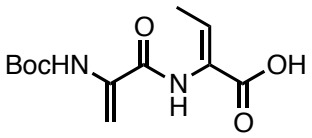
(*S*)-methyl 1-((2*S*,3*R*)-2-((*S*)-2-((*tert*-butoxycarbonyl)amino)-3-((methylsulfonyl)oxy)propanamido)-3-((methylsulfonyl)oxy)butanoyl)pyrrolidine-2-carboxylate (124)



Et_3N (1.05 mL, 7.50 mmol) was added to a solution of **123** (0.70 g, 1.70 mmol) in CH_2Cl_2 (30 mL) at 0 °C and was stirred for 10 min. Methanesulfonyl chloride (0.52 mL, 6.80 mmol) was added and the reaction was stirred for 1 h at 0 °C and

then allowed to warm to rt and stirred for a further 30 min. The reaction mixture was washed with H_2O (2 × 10 mL), brine (10 mL) and the organic layer was dried over Na_2SO_4. The solvent was removed *in vacuo* to yield **124** (0.82 g, 85.4%) as a white foam. The crude product was carried through the next reaction without further purification. (R_f 0.59 on SiO_2, 100% EtOAc); $[\alpha]_D^{26}$ -47.4 (*c* 1.0, CH_2Cl_2); IR (CH_2Cl_2 cast) 3287, 2981, 1745, 1697, 1640, 1357, 1176 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz): δ 7.15 (d, 1H, *J* = 9.0 Hz, -NH), 5.48 (d, 1H, *J* = 8.4 Hz, -NH), 5.05-4.99 (m, 1H, Thr-CH_β), 4.94-4.91 (m, 1H, Thr-CH_α), 4.68-4.64 (m, 1H, Ser-CH_β), 4.62-4.56 (m, 1H, Ser-CH_α), 4.50 (dd, 1H, *J* = 9.0, 5.4 Hz, Pro-CH_α), 4.40 (dd, 1H, *J* = 10.8, 4.2 Hz, Ser-CH_β), 3.86-3.81 (m, 1H, Pro-CH_δ), 3.77-3.72 (m, 4H, Pro-CH_δ, -OCH_3), 3.06 (s, 3H, S-CH_3), 3.05 (s, 3H, S-CH_3), 2.30-2.23 (m, 1H, Pro-CH_β), 2.10-1.98 (m, 3H, Pro-CH_β, Pro-$CH_{2\gamma}$), 1.52 (d, 3H, *J* = 6.6 Hz, Thr-CH_3), 1.47 (s, 9H, -$C(CH_3)_3$); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 171.9, 168.3, 166.7, 155.4, 81.2, 77.7, 68.7, 59.1, 54.4, 53.6, 52.4, 47.7, 38.5, 37.3, 29.1, 28.2, 24.8, 18.0; HRMS (ES) Calculated for $C_{20}H_{35}N_3O_{12}S_2Na$ 596.1554, found 596.1549 $(M+Na)^+$.

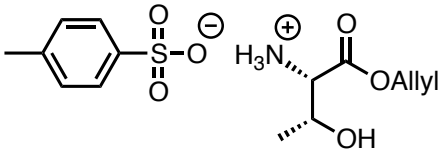
(*Z*)-2-(2-(*tert*-butoxycarbonylamino)acrylamido)but-2-enoic acid (126)



$PhSiH_3$ (0.40 mL, 3.20 mmol) was added to a stirred solution of **130** (0.50 g, 1.60 mmol) in degassed CH_2Cl_2 (25 mL). $Pd(PPh_3)_4$ (0.19 g, 0.16 mmol) was added to

the mixture and the reaction was stirred in the dark for 2 h. The solvent was removed *in vacuo* and the resulting crude product was purified using flash chromatography (SiO_2, 50:1:0.1 CH_2Cl_2:MeOH:Acetic acid) to yield **126** (0.13 g, 30.0%) as a pale pink solid. (R_f 0.06 on SiO_2, 50:1:0.1 CH_2Cl_2:MeOH:Acetic acid); IR ($CHCl_3$ cast) 3354, 2980, 2934, 1706, 1506 cm^{-1}; ^1H NMR (CD_3OD, 400 MHz): 6.90 (q, 1H, J = 7.2 Hz, -C=C<u>H</u>CH_3), 5.83 (s, 1H, -C=C<u>H</u>H), 5.47(s, 1H, -C=CH<u>H</u>), 1.77 (d, 3H, J = 7.2 Hz, -C<u>H</u>$_3$), 1.47 (s, 9H, -C(C<u>H</u>$_3$)$_3$); ^{13}C NMR (CD_3OD, 100 MHz): δ 167.3, 165.7, 154.7, 137.2, 136.9, 128.7, 102.8, 81.5, 28.3, 14.0; HRMS (ES) Calculated for $C_{12}H_{18}N_2NaO_5$ 293.1107, found 293.1106 $(M+Na)^+$.

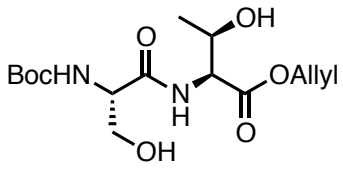
((2*S*,3*R*)-1-(allyloxy)-3-hydroxy-1-oxobutan-2-aminium 4-methylbenzenesulfonate (128)



This known compound was prepared according to literature procedure.[169] *p*-Toluenesulfonic acid monohydrate (19.2 g, 101 mmol) was added to a stirred suspension of L-threonine (10.0 g, 84.0 mmol) in toluene (250 mL). Following addition of allyl alcohol (57.1 mL, 840 mmol) the mixture was heated to reflux for 16 h using a condenser and Dean-Stark apparatus. Solvent was removed *in vacuo* to yield **128** (27.0 g, 97.1 %) as an orange oil. The crude product was carried through the next reaction without further purification. (R_f 0.07 on SiO_2,

100% EtOAc); $[\alpha]_D^{26}$ -6.7 (*c* 1.3, CH_2Cl_2); IR (CH_2Cl_2 cast) 3336, 3029, 2981, 1748, 1600, 1516, 1498, 1452, 1216, 1176 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz): δ 7.93 (d, 3H, *J* = 4.8 Hz, -NH_3), 7.69 (d, 2H, *J* = 8.4 Hz, Ar-H), 7.08 (d, 2H, *J* = 8.4 Hz, Ar-H), 5.72 (ddt, 1H, *J* = 16.8, 10.2, 6.0 Hz, -OCH_2CH=CH_2), 5.20 (dd, 1H, *J* = 17.4, 1.2 Hz, -OCH_2CH=CH_2), 5.12 (dq, 1H, *J* = 10.2, 1.2 Hz, -OCH_2CH=CH_2), 4.56-4.44 (m, 2H, -OCH_2), 4.19-4.14 (m, 1H, -CH_α), 3.96-3.90 (m, 1H, -CH_β), 2.31 (s, 3H, -OCH_3), 1.23 (d, 3H, *J* = 3.3 Hz, -CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 167.6, 140.9, 140.4, 131.0, 129.0, 126.1, 119.2, 67.1, 65.9, 59.2, 21.3, 19.9; HRMS (ES) Calculated for $C_7H_{14}NO_3$ 160.0968, found 160.0968 $(M+H)^+$.

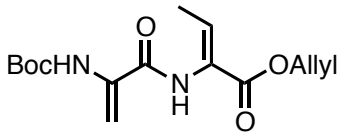
(2*S*,3*R*)-allyl 2-((*S*)-2-((*tert*-butoxycarbonyl)amino)-3-hydroxypropanamido)-3-hydroxybutanoate (129)



This known compound was prepared according to a modified literature procedure.[169] **128** (27.0 g, 82.0 mmol) was dissolved in 20:1 CH_2Cl_2:DMF (250 mL), then PyBOP (46.7 g, 90.0 mmol), HOBt (12.1 g, 90.0 mmol) and Boc-Ser-OH (18.4 g, 90.0 mmol) were added to the solution and the mixture was cooled to 0 °C. After addition of DIPEA (49.7 mL, 285 mmol) the reaction was warmed to rt and left to stir for 22 h. The solvent was removed *in vacuo* and the residue dissolved in EtOAc (700 mL), the EtOAc layer was washed with 10% citric acid

solution (100 mL), 10% $NaHCO_3$ (100 mL), H_2O (100 mL) and then brine (100 mL). The organic layer was dried over Na_2SO_4 and then concentrated *in vacuo*. The resulting crude product was purified using flash chromatography (SiO_2, 1:1 hexanes: EtOAc) to yield **129** (17.6 g, 60%) as a colorless oil. (R_f 0.45 on SiO_2, 100% EtOAc); $[\alpha]_D^{26}$ -0.33 (*c* 0.6, EtOAc); IR ($CHCl_3$ cast) 3345, 2979, 2936, 1668, 1525 cm^{-1}; ^1H NMR (CD_3OD, 600 MHz): δ 5.97-5.90 (m, 1H, -$OCH_2C\underline{H}=CH_2$), 5.36-5.21(m, 2H, -$OCH_2CH=C\underline{H_2}$), 4.65-4.64 (m, 2H, -$OC\underline{H_2}CH=CH_2$), 4.50 (d, 1H, *J* = 2.4 Hz, Thr-C$\underline{H}_\alpha$), 4.32-430 (m, 1H, Thr-C$\underline{H}_\beta$), 4.21-4.20 (m, 1H, Ser-C$\underline{H}_\alpha$), 3.79-3.77 (dd, 2H, *J* = 8.5, 4.0 Hz, Ser-C$\underline{H}_\beta$), 1.45 (s, 9H, -$C(C\underline{H_3})_3$), 1.18 (d, 3H, *J* = 2.4 Hz, -$C\underline{H_3}$); 13C NMR (CD_3OD, 150 MHz): δ 173.7, 171.6, 157.9, 133.2, 118.7, 81.1, 68.6, 67.1, 63.2, 59.3, 58.1, 28.7, 20.4; HRMS (ES) Calculated for $C_{15}H_{26}N_2NaO_7$ 369.1632, found 369.1637 $(M+Na)^+$.

(*Z*)-allyl 2-(2-(*tert*-butoxycarbonylamino)acrylamido)but-2-enoate (130)



Et_3N (1.90 mL, 13.6 mmol) was added to a stirred solution of **129** (1.05 g, 3.03 mmol) in CH_2Cl_2 (50 mL) at 0 °C. After the reaction had been stirred for 10 min methanesulfonyl chloride (0.95 mL, 12.2 mmol) was added and the reaction was allowed to warm to rt with stirring. After 1 h the solvent was removed *in vacuo* and the residue redissolved in DCE (50 mL). Following the addition of DBU (2.00 mL, 13.6 mmol) the reaction was heated to reflux for 17 h. The reaction

mixture was concentrated *in vacuo* and the residue was redissolved in CH_2Cl_2 (100 mL). The organic solution was washed with 10% citric acid solution (50 mL), 10% $NaHCO_3$ (50 mL), H_2O (50 mL) and then brine (50 mL). The organic layer was dried over Na_2SO_4 and then concentrated *in vacuo*. The resulting crude product was purified using flash chromatography (SiO_2, 5:1 hexanes:EtOAc) to yield **130** (0.60 g, 64.0%) as a pale yellow oil. (R_f 0.71 on SiO_2, 1:1 hexanes:EtOAc); IR ($CHCl_3$ cast): 3395, 3342, 2980, 2935, 1729, 1670, 1632, 1492 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 7.57 (br s, 1H, -NH), 7.22 (br s, 1H, -NH), 6.88 (q, 1H, J = 7.5 Hz, -C=C<u>H</u>CH_3), 6.09 (s, 1H, -C=C<u>H</u>H), 5.94-5.86 (m, 1H, -OCH_2C<u>H</u>=CH_2), 5.33-5.22 (m, 3H, -OCH_2CH=C<u>H_2</u> + -C=CH<u>H</u>), 4.66-4.64 (m, 2H, -OC<u>H_2</u>CH=CH_2), 1.75 (d, 3H, J = 7.5 Hz, -C<u>H_3</u>), 1.45 (s, 9H, -C(C<u>H_3</u>)$_3$); ^{13}C NMR (CD_3OD, 150 MHz): δ 163.8, 162.2, 152.6, 134.6 ,134.5, 131.6, 125.5, 118.6, 99.2, 80.6, 66.0, 28.1, 14.7; HRMS (ES) Calculated for $C_{15}H_{22}N_2NaO_5$ 333.1421, found 333.1423 $(M+Na)^+$.

5.2.1.c Solid phase synthesis of lactocin S (98)

General procedure for peptide elongation using Fmoc solid phase peptide synthesis (SPPS)

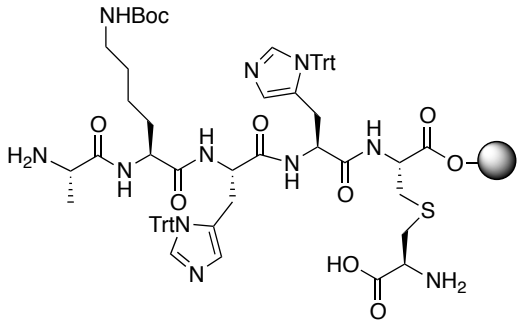
N-Methylmorpholine (NMM, 6 equiv) was added to a solution of Fmoc protected amino acid (5.0 equiv to resin loading), HOBt (5.0 equiv) and PyBOP (4.9 equiv) in DMF (10 mL) and the solution was allowed to pre-activate for 5 min. The solution was transferred to the reaction vessel containing pre-swelled resin and was bubbled with argon for 3 h. A small sample of the peptide was cleaved from the resin (by treatment with 95:2.5:2.5, TFA:TIPS:H_2O or 95:2.5:2.5, TFA:anisole:H_2O for 2 h) and the completion of the reaction was determined by MALDI-TOF analysis. Resin was washed with DMF (3 × 10 mL), then 20% Acetic anhydride in DMF (10 mL) was added to the resin for 10 min to effect end capping. Resin was again washed with DMF (3 × 10 mL). Then, 20% piperidine in DMF (3 × 10 mL) was added to remove the *N*-terminal Fmoc protecting group. This reaction was monitored by UV-Vis spectroscopy, observing the dibenzofulvene-piperidine adduct at λ = 301 nm.

5.2.1.d General procedure for the Fmoc solid phase peptide synthesis (SPPS) of lactocin S (98) and its analogues (151, 152, 153, 181, 182, 183)

The SPPS approach to the synthesis of lactocin S and its analogues was the same in all cases. This general procedure provides a detailed description of the steps

taken. The synthesis of lactocin S (**98**) is used as the example. Protocols A-E were then applied to the synthesis of the analogues as described later in this chapter.

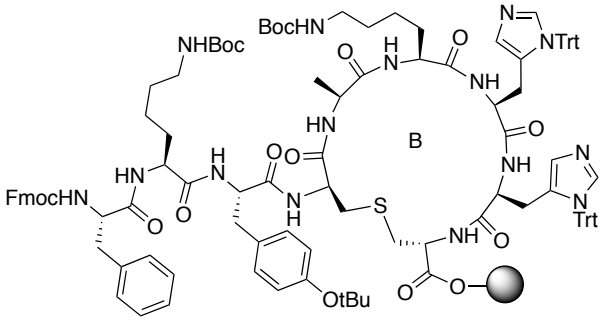
5.2.1.e Protocol A - Synthesis of the linear precursor to the B-ring of lactocin S (102)



2-Chlorotrityl chloride resin (2.0 g, 2.6 mmol) was pre-swelled in CH_2Cl_2 (20 mL) without stirring for 30 min. A solution of protected lanthionine (**58**) (0.18 g, 0.32 mmol) and DIPEA (0.28 mL, 1.60 mmol) in CH_2Cl_2 (20 mL) was added and the mixture stirred very gently for 2.5 h. The slurry was transferred to a peptide reaction vessel and the resin washed with CH_2Cl_2 (2 × 20 mL). To cap the remaining reactive sites on the resin, a solution of acetic acid (0.11 mL, 2.0 mmol) and DIPEA (1.39 mL, 8.0 mmol) in CH_2Cl_2 (20 mL) was added to the resin and the reaction mixture was bubbled with argon for 2 h. The resin was then washed with CH_2Cl_2 (2 × 20 mL). This yielded a resin with a reduced loading (0.16 mmol/g) of reactive sites functionalized with the *C*-terminal amino acid of lactocin S (**90**), ready for SPPS. The *N*-terminal Fmoc protecting group was removed using a solution of 20% piperidine in DMF. The resin bound peptide was

coupled with Fmoc-His(Trt)-OH (res 36 and 35), Fmoc-Lys(Boc)-OH (res 34) and Fmoc-Ala-OH (res 33) in the presence of PyBOP and HOBt. To form the B ring of lactocin S, the orthogonal protecting groups of lanthionine were removed to allow cyclization. The allyl/Alloc groups were removed by treatment of the resin with a solution of $Pd(PPh_3)_4$ (0.74 g, 0.64 mmol) and $PhSiH_3$ (0.40 mL, 3.20 mmol) in 1:1 DMF CH_2Cl_2 (40 mL) in the dark for 2 h. The resin was then washed with CH_2Cl_2 (40 mL) until the dark brown color was removed. The resin was then washed with 0.5% sodium diethyldithiocarbamate in DMF (3 × 20 mL) and then DMF (2 × 20 mL). This procedure allowed the removal of any remaining palladium from the resin. The *N*-terminal Fmoc protecting group was removed using the standard protocol of 20% piperidine in DMF. The resin was washed with CH_2Cl_2 (3 × 20 mL) and then DMF (3 × 20 mL). Resin-bound peptide **102** was then ready for cyclization to form ring B.

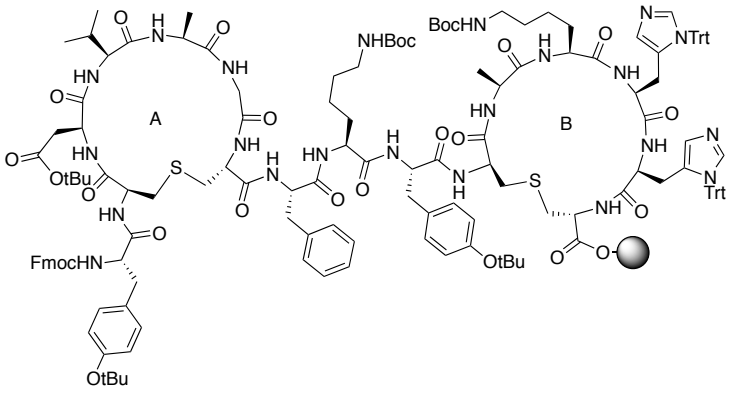
5.2.1.f Protocol B - Synthesis of the B-ring of lactocin S (108)



To cyclize the B ring of lactocin S (**108**) a solution of PyBOP (0.83 g, 1.60 mmol), HOBt (0.22 g, 1.60 mmol) and NMM (0.35 mL, 3.20 mmol) in DMF (20

mL) was added to the resin and the reaction mixture was bubbled with argon for 2 h. The resin was washed with DMF (3 × 10 mL) and then a second solution of PyBOP (0.83 g, 1.60 mmol), HOBt (0.22 g, 1.60 mmol) and NMM (0.35 mL, 3.20 mmol) in DMF (20 mL) was added to the resin and the reaction mixture was bubbled with argon for 2 h. The *N*-terminus of the peptide was elongated by Fmoc-SPPS with PyBOP and HOBt to introduce residues 31-29 (Fmoc-Tyr(OtBu)-OH, Fmoc-Lys(Boc)-OH and Fmoc-Phe-OH). A small sample of the resin-bound peptide **108** was treated with 95:2.5:2.5, TFA:TIPS:H_2O for 2 h to remove the peptide from the solid support. Following filtration, the filtrate was concentrated *in vacuo* and the peptide was precipitated by addition of Et_2O at 0 °C. The resulting white solid was analyzed using MALDI-TOF MS: Calculated for $C_{66}H_{82}N_{15}O_{13}S$ 1324.5, found 1324.7 $(M+H)^+$.

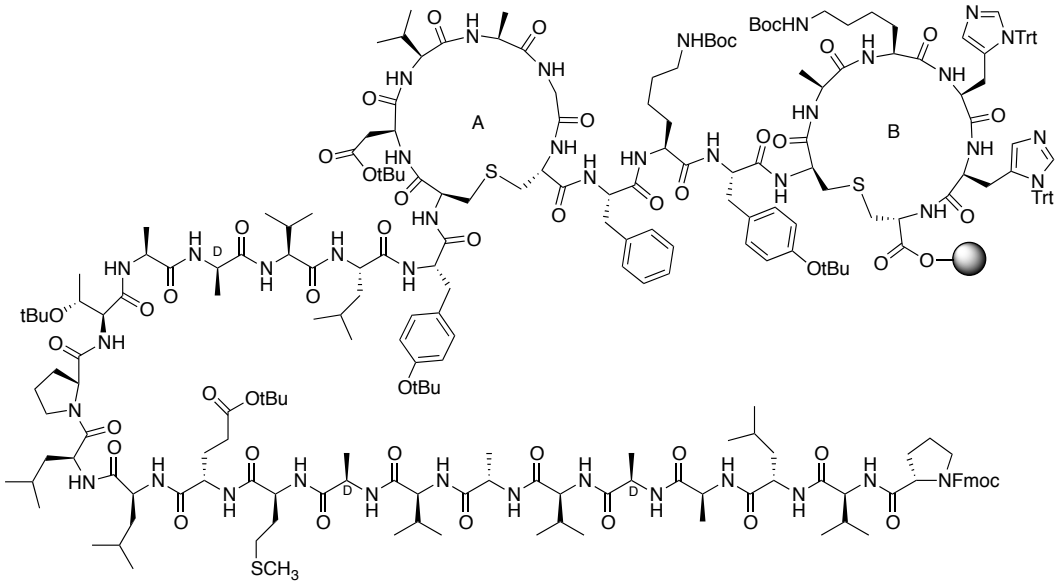
5.2.1.g Protocol C - Synthesis of the A-ring of lactocin S (109)



The amino acids of the A-ring (res 28-24) of lactocin S (**98**) were introduced using Fmoc-SPPS in the following order: Fmoc-Alloc/allyl-lanthionine (**58**),

Fmoc-Gly-OH, Fmoc-Ala-OH, Fmoc-Val-OH, Fmoc-Asp(OtBu)-OH. Removal of the allyl/Alloc groups was done in an analogous fashion to that described for the B-ring. The *N*-terminal Fmoc was deprotected using 20% piperidine in DMF and after the resin had been washed with CH_2Cl_2 and DMF the cyclization of ring A was effected using PyBOP and HOBt as previously described. To aid identification by MALDI-TOF MS, the next amino acid Fmoc-Tyr(OtBu)-OH was added using PyBOP and HOBt. A small sample of **109** was then cleaved from the resin using 95:2.5:2.5 TFA:TIPS:H_2O as described for ring B and analyzed by MALDI-TOF MS: Calculated for $C_{95}H_{121}N_{22}O_{23}S_2$ 2002.2, found 2002.4 $(M+H)^+$.

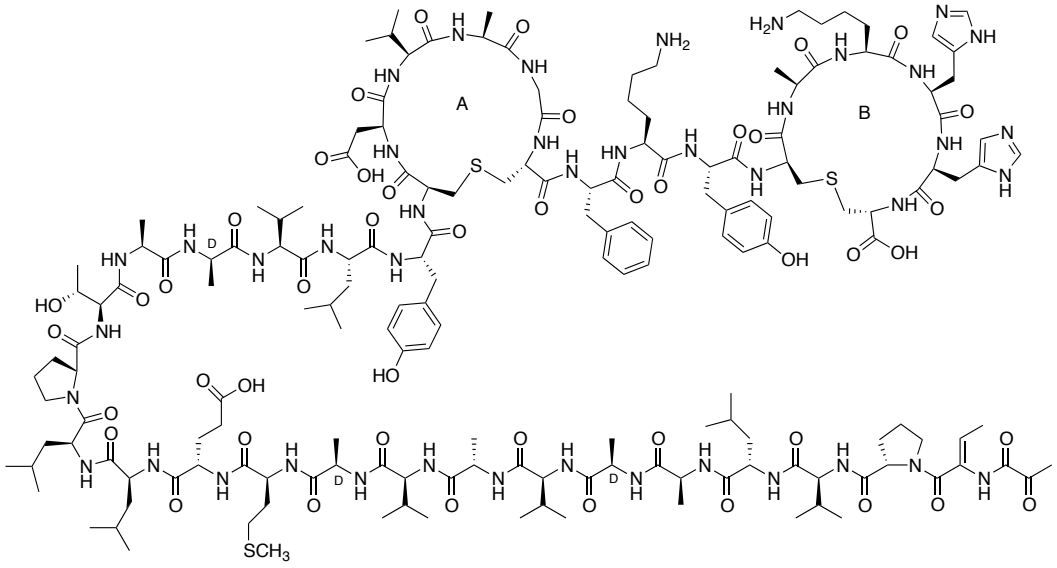
5.2.1.h Protocol D - Addition of residues 3-37 of lactocin S (100)



The amino acids corresponding to residues 3-21 were introduced via Fmoc-SPPS with PyBOP and HOBt as coupling reagents in the following order: Fmoc-Leu-

OH, Fmoc-Val-OH, Fmoc-D-Ala-OH, Fmoc-Ala-OH, Fmoc-Thr(OtBu)-OH, Fmoc-Pro-OH, Fmoc-Leu-OH, Fmoc-Leu-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Met-OH, Fmoc-D-Ala-OH, Fmoc-Val-OH, Fmoc-Ala-OH, Fmoc-Val-OH, Fmoc-D-Ala-OH, Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Val-OH, Fmoc-Pro-OH. This sample (**100**) was then cleaved from the resin using 95:2.5:2.5 TFA:TIPS:H_2O as described for rings A and B and analyzed by MALDI-TOF MS: Calculated for $C_{181}H_{268}N_{41}O_{45}S_3$ 3830.9, found 3831.3 $(M+H)^+$.

5.2.1.i Protocol E - Synthesis and purification of lactocin S (98)



Following standard Fmoc deprotection of **100** (0.02 mmol) using piperidine in DMF, the resulting resin-bound peptide was treated with a solution of dipeptide **126** (0.05 g, 0.20 mmol), PyBOP (0.10 g, 0.20 mmol), HOBt (0.03 g, 0.20 mmol) and NMM (0.05 mL, 0.40 mmol) in DMF (10 mL) for 3 h. The resin was washed with DMF (3 × 10 mL) and then treated with a second solution of dipeptide **126**

(0.05 g, 0.20 mmol), PyBOP (0.104 g, 0.20 mmol), HOBt (0.027 g, 0.20 mmol) and NMM (0.05 mL, 0.40 mmol) in DMF (10 mL) for 3 h to ensure the reaction goes to completion. To remove the protecting groups on the side chains, cleave the peptide from resin and to unmask the α-keto-amide functionality at the *N*-terminus of lactocin S, the peptide immobilized on resin was transferred into a vial and was gently shaken with a solution of 95:2.5:2.5 TFA:anisole:H_2O for 2 h. The resin beads were removed by filtration through glass wool and the filtrate was concentrated *in vacuo*. Crude lactocin S (**98**) was obtained as a pale yellow solid (25 mg) by precipitation with cold Et_2O. The cleavage and precipitation were done under an atmosphere of argon to minimize oxidation of the sulfur containing methionine and lanthionine residues. A portion of the crude peptide (5 mg) was dissolved in (1:1) H_2O:methanol (with 0.1% TFA) and injected onto a C_2/C_{18} RP-HPLC column (GE Healthcare, 4.6 x 100 mm, 3 μm) using methanol (0.1% TFA) and H_2O (0.1% TFA) as eluents. The HPLC method followed was: Gradient starting at 5% methanol for 1 min, climb to 60% over 4 min, then climb to 100% over 8 min, remain at 100% for 2 min, return to 5% over 2 min and remain at 5% for 8 min (flow rate 1 mL/min, UV detection at 220 nm). Note: TCEP (1.5 mg/L) was added to the HPLC solvents and argon was continuously bubbled through the solvents during HPLC to minimize oxidation of the sulfurs in methionine and lanthionine. In addition, the product was collected under a stream of argon and during concentration the pressure in the rotary evaporator was restored to 1 atm using an argon balloon. Without these precautions, considerable oxidation of the peptide was observed by mass spectrometry. The sulfur in methionine was

particularly susceptible and the peptide oxidation increased when in dilute solution, and also as the purity increased (ie. after HPLC). The use of TCEP and argon substantially decreased the oxidation of sulfurs within lactocin S (**98**) during HPLC purification.

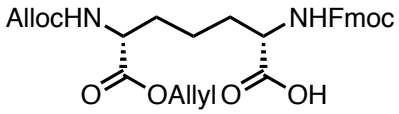
5.2.1.j Isolation of synthetic lactocin S (98)

The peptide was collected off the HPLC as a broad peak at 13 min and solvent was removed *in vacuo*. The residue was then redissolved in approximately 900 μL H_2O and 100 μL methanol (both with 0.1% TFA) and lyophilized to give **98** (1.51 mg, overall yield = 10% [33 deprotections and couplings, 2 macrocyclizations, 2 allyl deprotections, 1 cleavage/hydrolysis]) as a fluffy white solid. A high resolution MALDI-FT-ICR-MS spectrum of the peptide was obtained using an FT-ICR Bruker 9.4T Apex-Qe FTICR (Bruker Daltonics, Billerica, MA, USA). For synthetic lactocin S (**98**) an elemental composition of $C_{173}H_{268}N_{42}O_{46}S_3$ for the $(M+H)^+$ ion was obtained with a mass accuracy of 0.23 ppm.

5.2.2 Synthesis of A-DAP (151), B-DAP (152) and DAP lactocin S (153)

5.2.2.a Synthesis of orthogonally protected diaminopimelate (137)

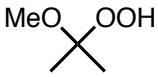
(2*S*,6*R*)-2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-7-(allyloxy)-6-(((allyloxy)carbonyl)amino)-7-oxoheptanoic acid (137)



171 (0.35 g, 0.82 mmol) was dissolved in a mixture of dioxane (15 mL) and H_2O (10 mL) and was cooled to 0 °C. $NaHCO_3$ (0.22 g, 2.64 mmol) was added, followed by addition of Fmoc-OSu (0.30 g, 0.90 mmol) and the reaction was left to stir for 2 h at 0 °C. The reaction was allowed to warm to rt and stirred for a further 1 h. Solvent was removed *in vacuo* and the residue was dissolved in H_2O (10 mL). This solution was extracted with EtOAc (2 × 50 mL) and then the aqueous layer was acidified with 10% citric acid (~ 10 mL). The aqueous layer was then further extracted with EtOAc (5 × 25 mL). The organic layers were combined and dried over Na_2SO_4 and the solvent was removed *in vacuo*. The resulting crude product was purified using flash chromatography (SiO_2, 100 % EtOAc) to yield **137** (0.37 g, 83.0%) as a white foam. (R_f 0.31 on SiO_2, 1:1:0.01 hexanes:EtOAc:Acetic acid); $[\alpha]_D^{26}$ 3.3 (*c* 0.25, CH_2Cl_2); IR (CH_2Cl_2 cast) 3327, 3067, 2951, 1722, 1528, 1210 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz): δ 7.76 (d, 2H, *J* = 8 Hz, Ar-H), 7.65 (t, 2H, *J* = 8 Hz, Ar-H), 7.36 (t, 2H, *J* = 8 Hz, Ar-H), 7.29 (t, 2H, *J* = 8 Hz, Ar-H), 5.94-5.85 (m, 2H, $-OCH_2CH=CH_2$), 5.29 (d, 1H, *J* = 17.0 Hz, $-OCH_2CH=CH_2$), 5.28 (d, 1H, *J* = 17.0 Hz, $-OCH_2CH=CH_2$), 5.18 (d, 1H, *J* =

11.0 Hz, -OCH$_2$CH=C$\underline{H}_2$), 5.15 (d, 1H, *J* = 10.5 Hz, -OCH$_2$CH=C$\underline{H}_2$), 4.58 (m, 2H, -OC$\underline{H}_2$), 4.52 (m, 2H, -OC$\underline{H}_2$), 4.33 (d, 2H, *J* = 7.0 Hz, Fmoc-C$\underline{H}_2$), 4.19 (t, 1H, *J* = 7.0 Hz, Fmoc-C$\underline{H}$), 4.17-4.12 (m, 2H, 2 × -C$\underline{H}_\alpha$), 1.90-1.80 (m, 2H, -C$\underline{H}_\beta$), 1.74-1.62 (m, 2H, -C$\underline{H}_\beta$), 1.58-1.49 (m, 1H, -C$\underline{H}_{2\gamma}$). 1.49-1.41 (m, 1H, -C$\underline{H}_{2\gamma}$); ^{13}C NMR (CD$_3$OD, 125 MHz): δ 173.7, 158.7, 158.5, 145.4, 145.2, 142.6, 134.3, 133.3, 128.8, 128.2, 126.3, 121.0, 118.7, 117.7, 68.0, 66.7, 66.6, 55.5, 55.4, 48.4, 32.2, 32.2, 23.4; HRMS (ES) Calculated for $C_{29}H_{32}N_2NaO_8$ 559.2051, found 559.2047 $(M+Na)^+$.

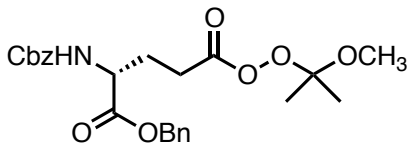
2-hydroperoxy-2-methoxypropane (156)



Compound **156** was synthesized according to literature procedure.[171] **154** (2.50 mL, 21.0 mmol) was dissolved in 60 mL of a mixture of MeOH and CH_2Cl_2 (9:51), and then $NaHCO_3$ (10.0 mg, 0.010 mmol) was added. The mixture was cooled to -78 °C and ozone was bubbled through it for 25 min. The solution was purged with oxygen for 45 min and then butylated hydroxytoluene (4.6 mg, 0.02 mmol) was added and the mixture was allowed to warm to rt. Solvent was removed *in vacuo* (temperature maintained ≤ 30 °C) to yield **156** (1.90 g, 86.0 %) as a pale yellow oil. The crude product was carried through to the next reaction without further purification. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.99 (s, 1H, -OO$\underline{H}$), 3.27 (s, 3H, -OC$\underline{H}_3$), 1.35 (s, 6H, -C(C$\underline{H}_3$)$_2$); ^{13}C NMR ($CDCl_3$, 100 MHz): δ

105.4, 50.6, 49.3, 23.0, 22.3; HRMS (ES) Calculated for $C_4H_{10}NaO_3$ 129.0522, found 129.0522 $(M+Na)^+$.

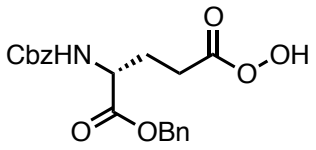
(*R*)-benzyl 2-(((benzyloxy)carbonyl)amino)-5-((2-methoxypropan-2-yl)peroxy)-5-oxopentanoate (157)



Cbz-D-Glu-OBn (3.00 g, 8.00 mmol), DCC (2.70 g, 12.9 mmol) and DMAP (100 g, 0.81 mmol) were added to a solution of **156** (1.30 g, 12.0 mmol) in CH_2Cl_2 (20 mL) at -20 °C. The reaction was allowed to warm to rt over 2 h with stirring and was then filtered through a plug of celite. The celite was washed with CH_2Cl_2 (50 mL) and the organic solutions were combined. Solvent was removed *in vacuo* and the resulting crude product was purified using flash chromatography (SiO_2, 4:1 hexanes:EtOAc) to yield **157** (3.40 g, 91.5%) as a yellow oil. (R_f 0.38 on SiO_2, 2:1 hexanes:EtOAc); $[\alpha]_D^{26}$ -1.9 (*c* 0.7, CH_2Cl_2); IR (CH_2Cl_2 cast) 3351, 3065, 3034, 2996, 2948, 1776, 1727, 1525, 1455, 1216 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 7.32-7.24 (m, 10H, Ar-H), 5.67 (d, 1H, J = 8.0 Hz, -NH), 5.12 (s, 2H, -OCH_2), 5.05 (s, 2H, -OCH_2), 4.46-4.37 (m, 1H, Glu-CH_α), 3.26 (s, 3H, -OCH_3), 2.42-2.26 (m, 2H, Glu-$CH_{2\gamma}$), 2.26-2.13 (m, 1H, Glu-CH_β), 2.03-1.93 (m, 1H, Glu-CH_β), 1.39 (s, 6H, -$C(CH_3)_2$); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 171.7, 169.6, 156.0, 136.2, 135.1, 128.6, 128.5, 128.3, 128.1, 128.0, 107.0, 67.3, 67.0, 53.3,

49.7, 27.4, 27.1, 22.4; HRMS (ES) Calculated for $C_{24}H_{29}NNaO_8$ 482.1785, found 482.1780 $(M+Na)^+$.

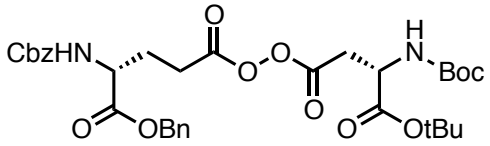
(*R*)-5-(benzyloxy)-4-(((benzyloxy)carbonyl)amino)-5-oxopentaneperoxoic acid (158)



50% TFA (aq.) (40.0 mL) was added to **157** (3.30 g, 7.20 mmol) in $CHCl_3$ (100 mL). The reaction was stirred vigorously for 15 min and then was cooled to 0 °C and quenched by addition of 10% $NaHCO_3$ (200 mL). Once the solution reached pH 8 it was separated and the aqueous layer was extracted with diethyl ether (2 × 100 mL). The organic layers were combined and washed with H_2O (100 mL) and brine (100 mL) and then dried over Na_2SO_4. The solvent was removed *in vacuo* to yield **158** (2.36 g, 84.9%) as a colourless oil. The crude product was carried through to the next reaction without further purification. (R_f 0.38 on SiO_2, 2:1 hexanes:EtOAc); $[\alpha]_D^{26}$ -32.1 (*c* 0.7, CH_2Cl_2); IR (CH_2Cl_2 cast) 3333, 3066, 3035, 2939, 1720, 1527, 1264, 1215 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 7.40-7.28 (m, 10H, Ar-H), 5.51 (d, 1H, J = 8.0 Hz, -NH), 5.18 (s, 2H, -OCH_2), 5.10 (s, 2H, -OCH_2), 4.51-4.43 (m, 1H, Glu-CH_α), 2.53-2.35 (m, 2H, Glu-$CH_{2\gamma}$), 2.34-2.25 (m, 1H, Glu-CH_β), 2.09-1.98 (m, 1H, Glu-CH_β); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 173.1, 171.2, 156.0, 136.0, 134.9, 128.7, 128.6, 128.4, 128.3, 128.2,

67.7, 67.3, 53.1, 51.8, 27.5, 26.6; HRMS (ES) Calculated for $C_{20}H_{20}NNaO_7$ 386.1245, found 386.1243 $(M+Na)^+$.

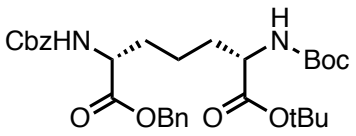
(*R*)-5-(benzyloxy)-4-(((benzyloxy)carbonyl)amino)-5-oxopentanoic (*S*)-4-(*tert*-butoxy)-3-((*tert*-butoxycarbonyl)amino)-4-oxobutanoic peroxyanhydride (159)



Boc-Asp-OtBu (0.61 g, 2.10 mmol) and DCC (0.49 g, 2.40 mmol) were added to a solution of **158** (0.84 g, 2.20 mmol) in CH_2Cl_2 (25 mL) at 0 °C. After 30 min the reaction was allowed to warm to rt and was stirred for a further 2 h. The mixture was then filtered through a celite plug and this was washed with CH_2Cl_2 (50 mL). The solutions were concentrated *in vacuo* and the resulting crude product was purified using flash chromatography (SiO_2, 7:3 hexanes:EtOAc) to yield **159** (1.20 g, 83.8%) as a colourless oil. (R_f 0.45 on SiO_2, 2:1 hexanes:EtOAc); $[\alpha]_D^{26}$ 14.0 (*c* 1.0, CH_2Cl_2); IR (CH_2Cl_2 cast) 3353, 3066, 3035, 2979, 2935, 1813, 1783, 1720, 1501, 1369, 1254, 1156 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ 7.38-7.27 (m, 10H, Ar-H), 5.54 (d, 1H, J = 8.0 Hz, -NH), 5.47 (d, 1H, J = 7.6 Hz, -NH), 5.16 (s, 2H, -OCH_2), 5.09 (s, 2H, -OCH_2), 4.53-4.41 (m, 2H, Asp-CH_α, Glu-CH_α), 3.07-2.89 (m, 2H, Asp-$CH_{2\beta}$), 2.56-2.38 (m, 2H, Glu-$CH_{2\gamma}$), 2.36-2.23 (m, 1H, Glu-CH_β), 2.11-1.98 (m, 1H, Glu-CH_β), 1.46 (s, 9H, -$(CH_3)_3$), 1.45 (s, 9H, -$(CH_3)_3$); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 171.1, 168.8,

167.9, 166.7, 155.9, 155.2 136.0, 134.9, 128.6, 128.5, 128.46, 128.3, 128.0, 83.0, 80.1, 67.5, 67.1, 53.1, 50.3, 33.0, 28.2, 27.7, 27.5, 26.0; HRMS (ES) Calculated for $C_{33}H_{42}N_2NaO_{12}$ 681.2630, found 681.2627 $(M+Na)^+$.

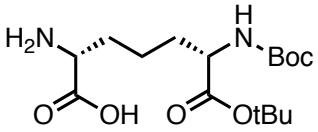
(2*R*,6*S*)-1-benzyl 7-*tert*-butyl 2-(((benzyloxy)carbonyl)amino)-6-((*tert*-butoxycarbonyl)amino)heptanedioate (160)



A solution of **159** (0.50 g, 0.76 mmol) in CH_2Cl_2 (5 ml) was deposited on the bottom of the reaction vessel (shaped like a 15 cm diameter recrystallization dish with a quartz lid and two adaptors to allow a flow of argon through the vessel). Solvent was evaporated under a stream of Ar and the reaction vessel was cooled to -78 °C with a Thermo Neslab Cryotrol/CC-100 cryo-cooler and maintained at this temperature throughout the reaction. The vessel was filled with argon and then sealed. The reaction was irradiated with a 0.9 Amp UV lamp (254 nm) for two days. The residue was then dissolved in CH_2Cl_2 (5 mL) and re-deposited on the bottom of the vessel. The material was subjected to the reaction conditions for a further two days. The resulting crude product was purified using flash chromatography (SiO_2, 4:1 hexanes:EtOAc) to yield **160** (0.08 g, 18.8 %) as a colourless oil. (R_f 0.60 on SiO_2, 2:1 hexanes:EtOAc); $[\alpha]_D^{26}$ 1.7 (*c* 0.22, CH_2Cl_2); IR (CH_2Cl_2 cast) 3347, 3065, 3034, 2977, 2935, 2870, 1719, 1519, 1456, 1367, 1250, 1156 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 7.35-7.29 (m, 10H, Ar-H), 5.53

(d, 1H, J = 7.5 Hz, -NH), 5. 16-5.13 (m, 2H, -OCH_2), 5.10-5.07 (m, 2H, -OCH_2), 5.06-5.01 (m, 1H, -NH), 4.41-4.35 (m, 1H, -CH_α), 4.14-4.08 (m, 1H, -CH_α), 1.92-1.81 (m, 1H, -CH_β), 1.78-1.62 (m, 2H, -C$H_{2\gamma}$), 1.58-1.49 (m, 1H, -CH_β), 1.46-1.35 (m, 19H, -CH_β, -(CH_3)$_3$), 1.35-1.26 (m, 1H, -CH_β); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 172.1, 171.7, 156.0, 155.5, 136.3, 135.3, 128.6, 128.5, 128.4, 128.2, 128.1, 81.8, 79.7, 67.1, 66.9, 53.8, 53.5, 32.5, 31.9, 28.3, 27.9, 20.9; HRMS (ES) Calculated for $C_{31}H_{42}N_2NaO_8$ 593.2833, found 593.2827 $(M+Na)^+$.

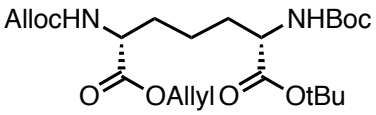
(2*R*,6*S*)-2-amino-7-(*tert*-butoxy)-6-((*tert*-butoxycarbonyl)amino)-7-oxoheptanoic acid (161)



10% Pd/C (0.016 g) was added to a solution of **160** (0.16 g, 0.28 mmol) in EtOH (5.0 mL). The reaction was placed under 1 atmosphere of H_2 and stirred vigorously for 18 h. The reaction was filtered through celite and washed with EtOAc (30 mL), CH_2Cl_2 (30 mL) and EtOH (30 mL). The extracts were combined and concentrated *in vacuo* to yield **161** (0.072 g, 74.2%) as a colourless gum. $[\alpha]_D^{26}$ -11.6 (*c* 0.3, MeOH); IR (MeOH cast) 3237, 2977, 2931, 2863, 1713, 1641, 1367, 1154 cm^{-1}; ^{1}H NMR (CD_3OD, 400 MHz): δ 3.96-3.90 (m, 1H, -CH_α), 3.61-3.56 (m, 1H, -CH_α), 1.95-1.72 (m, 3H, -CH_β, -C$H_{2\gamma}$), 1.69-1.57 (m, 1H, -CH_β), 1.56-1.49 (m, 1H, -CH_β), 1.46 (s, 9H, -(CH_3)$_3$), 1.43 (s, 9H, -(CH_3)$_3$), 1.36-1.27 (m, 1H, -CH_β); ^{13}C NMR (CD_3OD, 125 MHz): δ 176.2, 175.5, 160.7, 85.1,

83.0, 64.1, 58.4, 34.9, 34.5, 31.3, 30.8, 23.4; HRMS (ES) Calculated for $C_{16}H_{31}N_2O_6$ 347.2177, found 347.2176 $(M+H)^+$.

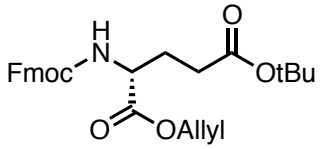
(2*R*,6*S*)-1-allyl 7-*tert*-butyl 2-(((allyloxy)carbonyl)amino)-6-((*tert*-butoxycarbonyl)amino)heptanedioate (162)



A solution of **170** (0.50 g, 0.90 mmol) dissolved in CH_2Cl_2 (4 mL) was deposited into the reaction vessel (shaped like a 15 cm diameter recrystallization dish with a quartz lid and two adaptors to allow a flow of argon through the vessel). The solvent was evaporated under a stream of argon to give a thin film of starting material coated on the bottom of the vessel. The reaction was then cooled to -78 °C with a Thermo Neslab Cryotrol/CC-100 cryo-cooler and maintained at this temperature throughout the reaction. The vessel was filled with argon and then sealed. The reaction was irradiated with a 0.9 Amp UV lamp (254 nm) for two days. The residue was then dissolved in CH_2Cl_2 (4 mL) and re-deposited on the bottom of the vessel. The material was subjected to the reaction conditions for a further two days. The resulting crude product was purified using flash chromatography (SiO_2, 4:1 hexanes:EtOAc) to yield **162** (0.14 g, 31.0%) as a colourless oil. (R_f 0.55 on SiO_2, 2:1 hexanes:EtOAc); $[\alpha]_D^{26}$ 1.2 (*c* 1.0, CH_2Cl_2); IR (CH_2Cl_2 cast) 3344, 2989, 2934, 1718, 1521, 1368, 1156 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 5.95-5.87 (m, 2H, -$OCH_2C\underline{H}$=CH_2), 5.39 (d, 1H, *J* = 7.5 Hz , -N<u>H</u>), 5.36-5.21 (m, 4H, -OCH_2CH=$C\underline{H}_2$), 5.07 (d, 1H, *J* = 7.0 Hz, -N<u>H</u>), 4.64

(d, 2H, *J* = 6.0 Hz, -OC$\underline{H}_2$), 4.57 (d, 2H, *J* = 5.0 Hz, -OC$\underline{H}_2$), 4.40-4.34 (m, 1H, Asp-C$\underline{H}_\alpha$), 4.19-4.12 (m, 1H, Glu-C$\underline{H}_\alpha$), 1.95-1.84 (m, 1H, Asp-C$\underline{H}_\beta$), 1.84-1.76 (m, 1H, Glu-C$\underline{H}_\beta$), 1.76-1.66 (m, 1H, Asp-C$\underline{H}_\beta$), 1.66-1.55 (m, 1H, Glu-C$\underline{H}_\beta$), 1.50-1.32 (m, 2H, Glu-C$\underline{H}_{2\gamma}$), 1.46 (s, 9H, -C(C$\underline{H}_3$)$_3$), 1.45 (s, 9H, -C(C$\underline{H}_3$)$_3$); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 171.9, 171.7, 155.8, 155.5, 132.6, 131.5, 119.0, 117.8, 82.0, 79.8, 66.0, 65.8, 53.7, 53.6, 32.6, 32.1, 28.4, 28.0, 20.9; HRMS (ES) Calculated for $C_{23}H_{39}N_2O_8$ 471.2701, found 471.2705 $(M+H)^+$.

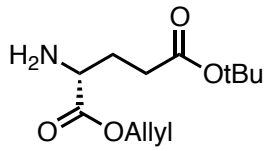
(*R*)-1-allyl 5-*tert*-butyl 2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino) pentanedioate (164)



This known compound was prepared according to a modified literature procedure.[172] Fmoc-D-Glu(O^{t}Bu)-OH (30.0 g, 71.0 mmol) was dissolved in DMF (500 mL). Following addition of Na_2CO_3 (9.00 g, 85.0 mmol) and allyl bromide (7.30 mL, 85.0 mmol) the reaction was allowed to stir at room temperature for 16 h. The solvent was removed *in vacuo* and the resulting residue was dissolved in EtOAc (250 mL). The EtOAc layer was washed with H_2O (100 mL) and brine (100 mL). The organic layer was dried over Na_2SO_4 and then concentrated *in vacuo* to yield **164** (31.0 g, 95.0%) as a white solid. The crude product was carried through the next reaction without further purification. (R_f 0.82 on SiO_2, 1:1 hexanes:EtOAc); $[\alpha]_D^{26}$ 5.6 (*c* 1.1, EtOAc); IR (EtOAc cast) 3337, 3067,

2979, 1728, 1529, 1252 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ 7.78 (d, 2H, J = 7.6 Hz, Ar-H), 7.64-7.61 (m, 2H, Ar-H), 7.42 (t, 2H, *J* = 7.6 Hz, Ar-H), 7.34 (t, 2H, J = 7.6 Hz, Ar-H), 5.97-5.90 (m, 2H, -OCH_2CH=CH_2), 5.60 (d, 1H, *J* = 8 Hz, -NH), 5.36 (dd, 2H, *J* = 1.2 Hz, 17.6 Hz -OCH_2CH=CH_2), 5.28 (dd, 2H, *J* = 1.2 Hz, 10.8 Hz -OCH_2CH=CH_2), 4.68 (d, 2H, *J* = 5.6 Hz, -OCH_2CH=CH_2), 4.48-4.37 (m, 3H, Fmoc-CH_2, Glu-CH_α), 4.25 (t, 1H, *J* = 6.8 Hz, Fmoc-CH), 2.41-2.34 (m, 2H, Glu-$CH_{2\gamma}$), 2.25-2.20 (m, 1H, Glu-CH_β), 2.07-2.01 (m, 1H, Glu-CH_β), 1.49 (s, 9H, -$C(CH_3)_3$); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 172.3, 172.0, 156.3, 144.2, 144.0, 141.6, 141.6, 131.8, 128.0, 127.4, 125.4, 125.4, 120.2, 119.2, 81.1, 67.3, 66.3, 53.9, 47.4, 31.7, 28.3, 27.8; HRMS (ES) Calculated for $C_{27}H_{31}NNaO_6$ 488.2044, found 488.2047 $(M+Na)^+$.

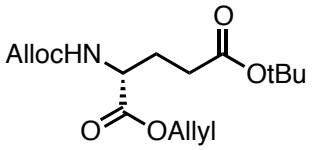
(*R*)-1-allyl 5-*tert*-butyl 2-aminopentanedioate (165)



This known compound was prepared according to literature procedure.[172] **164** (19.0 g, 41.0 mmol) was stirred in Et_2NH (150 mL) and CH_2Cl_2 (150 mL) at room temperature for 3.5 h. Solvent was removed *in vacuo*, the resulting residue was dissolved in EtOAc (600 mL) and then extracted with 10% citric acid (100 mL), H_2O (100 mL) and brine (100 mL). K_2CO_3 was added to the aqueous layer until pH 8 was reached and then the aqueous layer was re-extracted with EtOAc (3 × 150 mL). All organic layers were combined, dried over Na_2SO_4 and concentrated

in vacuo. The resulting crude product was purified using flash chromatography (SiO_2, 3:1 hexanes:EtOAc) to yield **165** (6.80 g, 69.0%) as a colourless oil. (R_f 0.33 on SiO_2, 1:1 hexanes:EtOAc); $[\alpha]_D^{26}$ -3.1 (*c* 1.5, EtOAc); IR (EtOAc cast): 3388, 3320, 2979, 2935, 1730, 1368, 1157 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ 5.87 (ddt, 1H, *J* = 16.4, 10.4, 5.6 Hz, -$OCH_2C\underline{H}=CH_2$), 5.28 (dq, 1H, *J* = 17.2, 1.2 Hz, -$OCH_2CH=C\underline{H_2}$), 5.20 (dq, 1H, *J* = 10.4, 1.2 Hz, -$OCH_2CH=C\underline{H_2}$), 4.57 (dt, 2H, *J* = 6.0, 1.2 Hz, -$OC\underline{H_2}CH=CH_2$), 3.44 (dd, 1H, *J* = 8.4, 5.2 Hz, Glu-$C\underline{H}_\alpha$), 2.32 (t, 2H, *J* = 7.6 Hz, Glu-$C\underline{H_2}_\gamma$), 2.04-1.96 (m, 1H, Glu-$C\underline{H}_\beta$), 1.80-1.71 (m, 1H, Glu-$C\underline{H}_\beta$), 1.45 (br s, 2H, $N\underline{H_2}$), 1.39 (s, 9H, -$C(C\underline{H_3})_3$); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 175.5, 172.5, 132.1, 118.8, 80.5, 65.7, 54.0, 32.0, 30.1, 28.1; HRMS (ES) Calculated for $C_{12}H_{22}NO_4$ 244.1543, found 244.1544 $(M+H)^+$.

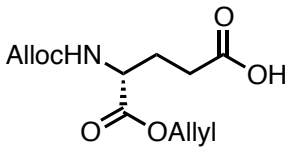
(*R*)-1-allyl 5-*tert*-butyl 2-(((allyloxy)carbonyl)amino)pentanedioate (166)



165 (6.80 g, 28.0 mmol) was dissolved in EtOAc (75 mL) and Et_3N (4.50 mL, 31.0 mmol) was added. Reaction was allowed to stir for 10 min and then was cooled to 0 °C. A solution of allylchloroformate (3.60 mL, 34.0 mmol) in EtOAc (75 mL) was added drop-wise over 30 min and the reaction was left to stir for 22 h at room temperature. The reaction was washed with 10% citric acid (50 mL), H_2O (50 mL) and brine (50 mL). The organic solvent was dried over Na_2SO_4 and then concentrated *in vacuo.* The resulting crude product was purified using flash

chromatography (SiO_2, 3:1 hexanes:EtOAc) to yield **166** (5.30 g, 58.0%) as a pale yellow oil. (R_f 0.69 on SiO_2, 2:1 hexanes:EtOAc); $[\alpha]_D^{26}$ 8.9 (*c* 1.4, EtOAc); IR (EtOAc cast) 3349, 2979, 2934, 1728, 1527, 1368, 1257 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz): δ 5.82-5.75 (m, 2H, -$OCH_2C\underline{H}=CH_2$), 5.67 (d, 1H, *J* = 7.8 Hz, -N<u>H</u>), 5.23-5.06 (m, 4H, -$OCH_2CH=C\underline{H_2}$), 4.52 (d, 2H, *J* = 5.4 Hz, -$OC\underline{H_2}$), 4.44 (d, 2H, J = 4.8 Hz, -$OC\underline{H_2}$), 4.30-4.24 (m, 1H, Glu-$C\underline{H}_\alpha$), 2.28-2.16 (m, 2H, Glu-$C\underline{H_2}_\gamma$), 2.07-2.02 (m, 1H, Glu-$C\underline{H}_\beta$), 1.88-1.80 (m, 1H, Glu-$C\underline{H}_\beta$), 1.32 (s, 9H, -$C(C\underline{H_3})_3$); ^{13}C NMR ($CDCl_3$, 150 MHz): δ 171.8, 171.7, 155.8, 132.6, 131.5, 118.6, 117.5, 80.5, 65.8, 65.6, 53.4, 31.4, 27.9, 27.3; HRMS (ES) Calculated for $C_{16}H_{26}NO_6$ 328.1755, found 328.1757 $(M+H)^+$.

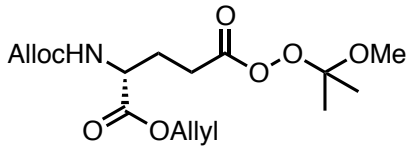
(*R*)-5-(allyloxy)-4-(((allyloxy)carbonyl)amino)-5-oxopentanoic acid (167)



166 (5.30 g, 16.0 mmol) was dissolved in CH_2Cl_2 (100 mL) and phenylsilane (2.00 mL, 16.0 mmol) was added followed by TFA (100 mL). The reaction was allowed to stir for 3 h at room temperature and then solvent was removed *in vacuo*. The resulting crude product was purified using flash chromatography (SiO_2, 100% EtOAc) to yield **167** (4.20 g, 96.0%) as a pale yellow oil. (R_f 0.2 on SiO_2, 1:1 hexanes:EtOAc); $[\alpha]_D^{26}$ 11.6 (*c* 1.3, EtOAc); IR (EtOAc cast) 3329, 3088, 2950, 1720, 1533, 1413, 1270, 1208 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz): δ 5.96-5.87 (m, 2H, -$OCH_2C\underline{H}=CH_2$), 5.65-5.57 (m, 1H, -N<u>H</u>), 5.37-5.20 (m, 4H, -

$OCH_2CH=C\underline{H}_2$), 4.66-4.63 (m, 2H, $-OC\underline{H}_2$), 4.59-4.56 (m, 2H, $-OC\underline{H}_2$), 4.48-4.41(m, 1H, Glu-$C\underline{H}_\alpha$), 2.54-2.41 (m, 2H, Glu-$C\underline{H}_{2\gamma}$), 2.27-2.19 (m, 1H, Glu-$C\underline{H}_\beta$), 2.04-1.95 (m, 1H, Glu-$C\underline{H}_\beta$); ^{13}C NMR ($CDCl_3$, 150 MHz): δ 177.4, 171.8, 156.1, 132.4, 131.3, 119.0, 117.9, 66.2, 66.0, 53.3, 29.9, 27.2; HRMS (ES) Calculated for $C_{12}H_{16}NO_6$ 270.0983, found 270.0981 $(M-H)^-$.

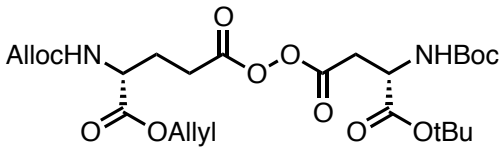
(*R*)-allyl 2-(((allyloxy)carbonyl)amino)-5-((2-methoxypropan-2-yl)peroxy)-5-oxopentanoate (168)



To a solution of **156** (1.90 g, 18.0 mmol) in CH_2Cl_2 (30 mL) at -20 °C was added a solution of **167** (3.20 g, 12.0 mmol) in CH_2Cl_2 (60 mL), followed by DCC (3.90 g, 19.0 mmol) and DMAP (catalytic). The reaction was allowed to warm to room temperature over 1.5 h and then allowed to stir a further 30 min. The reaction was filtered through a plug of celite, and the celite was washed with CH_2Cl_2 (100 mL). The CH_2Cl_2 fractions were combined and concentrated *in vacuo*. The resulting crude product was purified using flash chromatography (SiO_2, 3:1 hexanes:EtOAc) to yield **168** (3.80 g, 90.0%) as a pale yellow oil. (R_f 0.41 on SiO_2, 2:1 hexanes:EtOAc); $[\alpha]_D^{26}$ -6.3 (*c* 0.6, CH_2Cl_2); IR (EtOAc cast) 3350, 2996, 2946, 1778, 1727, 1528, 1215 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ 5.96-5.86 (m, 4H, $-OCH_2C\underline{H}=CH_2$), 5.43 (d, 1H, *J* = 7.6 Hz, $-N\underline{H}$), 5.37-5.21 (m, 4H, $-OCH_2CH=C\underline{H}_2$), 4.66 (d, 2H, *J* = 6.0 Hz, $-OC\underline{H}_2$), 4.58 (d, 2H, *J* = 5.6 Hz, -

OC$\underline{H}_2$), 4.47-4.39 (m, 1H, Glu-C$\underline{H}_\alpha$), 3.34 (s, 3H, -OC$\underline{H}_3$), 2.54-2.38 (m, 2H, Glu-C$\underline{H}_{2\gamma}$), 2.37-2.24 (m, 1H, Glu-C$\underline{H}_\beta$), 2.10-2.00 (m, 1H, Glu-C$\underline{H}_\beta$), 1.46 (s, 6H, -C(C$\underline{H}_3$)$_2$); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 171.5, 170.0, 156.1, 132.7, 131.5, 119.5, 118.2, 107.3, 66.5, 66.2, 53.5, 50.1, 27.9, 27.4, 22.7; HRMS (ES) Calculated for $C_{16}H_{25}NNaO_8$ 382.1472, found 382.1479 $(M+Na)^+$.

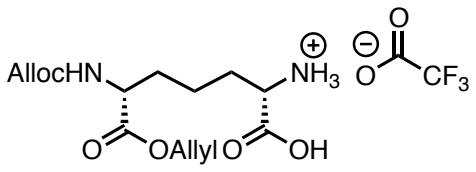
(*R*)-5-(allyloxy)-4-(((allyloxy)carbonyl)amino)-5-oxopentanoic (*S*)-4-(*tert*-butoxy)-3-((*tert*-butoxycarbonyl)amino)-4-oxobutanoic peroxyanhydride (170)



To a solution of **169** (3.50 g, 9.70 mmol) in $CHCl_3$ (100 mL) was added TFA (15 mL) and H_2O (15 mL). The reaction was stirred vigorously for 15 min. Then it was cooled to 0 °C and saturated $NaHCO_3$ (~300 mL) was added until pH 8 was reached. The two layers were separated and then the aqueous layer was extracted with Et_2O (3 × 100 mL). The organic layers were combined and washed with H_2O (100 mL) and brine (100 mL). The organic solvent was dried over Na_2SO_4 and concentrated *in vacuo*. The resulting residue was dissolved in CH_2Cl_2 (100 mL) and cooled to 0 °C. Boc-Asp-O^{t}Bu (2.80 g, 9.60 mmol) and DCC (2.20 g, 11.0 mmol) were added and the reaction was allowed to slowly warm to rt over 1 h. After the reaction had stirred for a further 2 h, it was filtered through a plug of celite. The celite was washed with CH_2Cl_2 (100 mL), the two fractions were

combined and the solvent was removed *in vacuo*. The resulting crude product was purified using flash chromatography (SiO_2, 3:1 hexanes:EtOAc) to yield **170** (4.90 g, 92.0%) as a colourless oil. (R_f 0.42 on SiO_2, 2:1 hexanes:EtOAc); $[\alpha]_D^{26}$ 9.9 (*c* 1.0, EtOAc); IR (EtOAc cast) 3347, 2979, 2937, 1722, 1515, 1369, 1253, 1155 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 5.98-5.89 (m, 2H, -$OCH_2C\underline{H}$=CH_2), 5.47 (d, 1H, *J* = 7.5, -N$\underline{H}$), 5.39-5.24 (m, 5H, -OCH_2CH=$C\underline{H_2}$, -N$\underline{H}$), 4.68 (dd, 2H, *J* = 1.5 Hz, 6.0 Hz, -$OC\underline{H_2}$), 4.60 (d, 2H, J = 5.5 Hz, -$OC\underline{H_2}$), 4.56-4.50 (m, 1H, Asp-C$\underline{H}_\alpha$), 4.50-4.43 (m, 1H, Glu-C$\underline{H}_\alpha$), 3.08 (dd, 1H, *J* = 4 Hz, 16.5 Hz Asp-C$\underline{H}_\beta$), 2.99 (dd, 1H, *J* = 5 Hz, 17 Hz Asp-C$\underline{H}_\beta$), 2.64-2.51 (m, 2H, Glu-$C\underline{H_2}_\gamma$), 2.39-2.32 (m, 1H, Glu-C$\underline{H}_\beta$), 2.14-2.06 (m, 1H, Glu-C$\underline{H}_\beta$), 1.49 (s, 9H, -$C(C\underline{H_3})_3$), 1.47 (s, 9H, -$C(C\underline{H_3})_3$); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 171.0, 168.8, 168.0, 166.8, 155.8, 155.3, 132.4, 131.2, 119.4, 118.1, 83.2, 80.2, 66.4, 66.1, 53.1, 50.4, 33.2, 28.3, 27.8, 27.77, 26.2; HRMS (ES) Calculated for $C_{25}H_{38}N_2NaO_{12}$ 581.2317, found 581.2312 $(M+Na)^+$.

(2*S*,6*R*)-7-(allyloxy)-6-(((allyloxy)carbonyl)amino)-2-amino-7-oxoheptanoic acid (171)

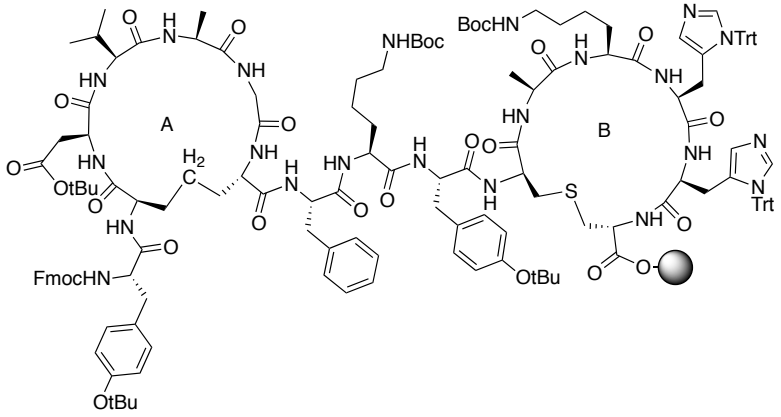


To a solution of **162** (0.40 g, 0.85 mmol), dissolved in CH_2Cl_2 (10 mL) was added TFA (10 mL). The reaction was stirred vigorously for 7 h. Solvent was removed *in vacuo* to yield **171** (0.36 g, 99.0 %) as a yellow wax. The crude product was

carried through the next reaction without further purification. $[\alpha]_D^{26}$ -21.2 (*c* 0.1, MeOH); IR (MeOH cast) 3306, 3089, 2945, 1696, 1532, 1203 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz): δ 5.97-5.88 (m, 2H, -$OCH_2C\underline{H}=CH_2$), 5.33 (dq, 1H, *J* = 17, 1.5 Hz, -$OCH_2CH=C\underline{H}_2$), 5.30 (dd, 1H, *J* = 17 Hz, -$OCH_2CH=C\underline{H}_2$), 5.22 (dq, 1H, *J* = 10.5, 1.5 Hz -$OCH_2CH=C\underline{H}_2$), 5.18 (dq, 1H, *J* = 11, 1.5 Hz -$OCH_2CH=C\underline{H}_2$), 4.62 (dt, 2H, *J* = 6.0, 1.5 Hz, -$OC\underline{H}_2$), 4.55-4.52 (m, 2H, -$OC\underline{H}_2$), 4.21 (q, 1H, *J* = 5 Hz, -$C\underline{H}_\alpha$), 3.96 (t, 1H, *J* = 6 Hz, -$C\underline{H}_\alpha$), 2.02-1.93 (m, 1H, -$C\underline{H}_\beta$), 1.92-1.84 (m, 2H, -$C\underline{H}_\beta$), 1.77-1.68 (m, 1H, -$C\underline{H}_\beta$), 1.61-1.53 (m, 2H, -$C\underline{H}_\gamma$); ^{13}C NMR (CD_3OD, 125 MHz): δ 173.4, 171.6, 158.6, 134.2, 133.3, 118.7, 117.6, 66.8, 66.7, 55.1, 53.7, 32.1, 31.0, 22.5; HRMS (ES) Calculated for $C_{14}H_{23}N_2O_6$ 315.1551, found 315.1550 $(M+H)^+$.

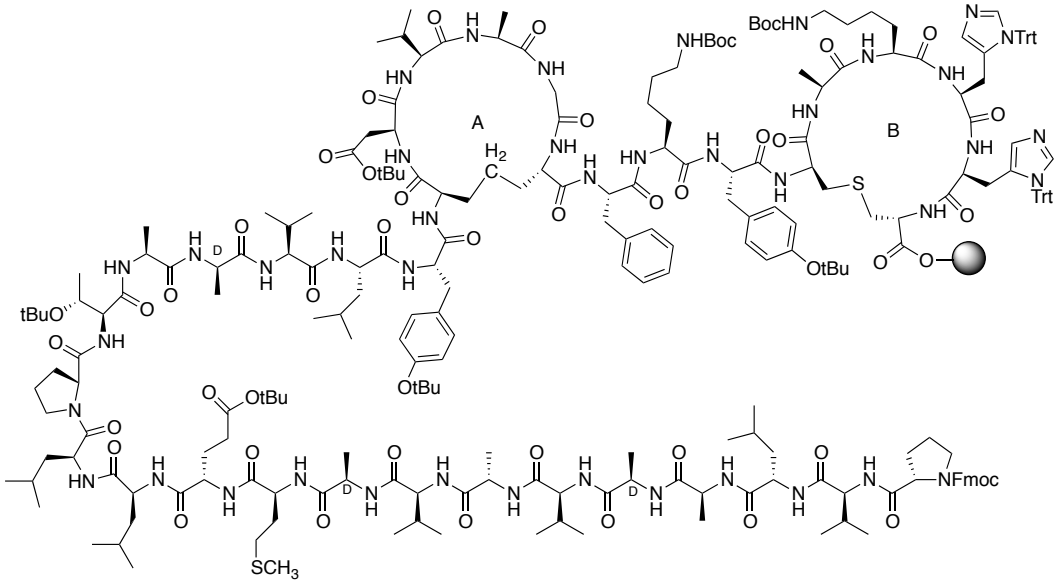
5.2.2.b Synthesis of A-DAP Lactocin S (151)

Synthesis of A-ring of A-DAP lactocin S (172)



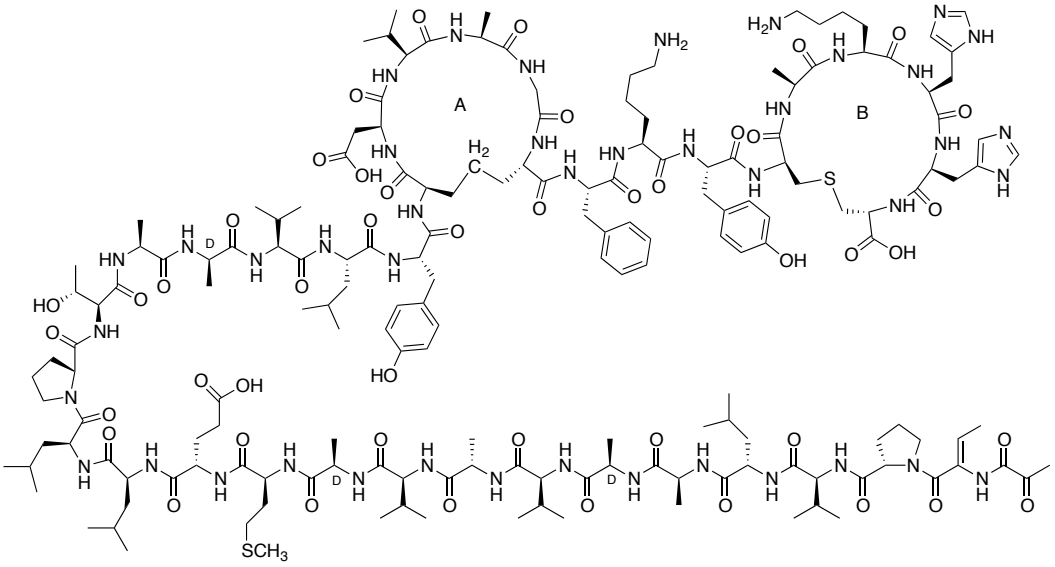
This synthesis begins with intermediate **108** (0.048 mmol) from the synthesis of lactocin S. Following protocol C, the A-ring intermediate (**172**) of A-DAP lactocin S was synthesized. Briefly, the amino acids of the A-ring were installed (orthogonally protected diaminopimelate **137** is incorporated in place of the orthogonally protected lanthionine **58** from lactocin S) and then after deprotection of the orthogonal protecting groups, the ring was cyclized. Fmoc-Tyr(OtBu)-OH was introduced and following cleavage of a small sample from the resin the peptide was analyzed by MALDI-TOF MS: Calculated for $C_{96}H_{123}N_{22}O_{23}S$ 1984.2, found 1984.7 $(M+H)^+$.

Synthesis of residues 3-37 of A-DAP lactocin S (173)



Following protocol D, residues 37-3 (**173**) of A-DAP lactocin S were synthesized. Briefly, the amino acids corresponding to residues 21-3 were introduced to **172** via Fmoc-SPPS and then a small sample was cleaved from the resin and analyzed by MALDI-TOF MS: Calculated for $C_{182}H_{270}N_{41}O_{45}S_2$ 3815.5, found 3816.0 $(M+H)^+$.

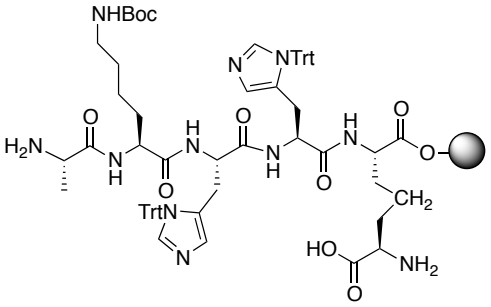
Synthesis of A-DAP lactocin S (151)



A-DAP lactocin S (**151**) was synthesized following protocol E. Briefly, **173** (0.04 mmol) was deprotected and then coupled with dipeptide **126** (0.062 g, 0.2 mmol) twice. The full length peptide (0.016 mmol) was then cleaved from the resin and crude A-DAP lactocin S (**151**) (5 mg) was obtained as a pale brown solid. The peptide was purified by HPLC and was collected as a broad peak at 13.5 min. Pure **151** (1.5 mg, overall yield = 2.5 % [33 deprotections and couplings, 2 macrocyclizations, 2 allyl deprotections, 1 cleavage/hydrolysis]) was obtained as a fluffy white solid. A high resolution MALDI-FT-ICR-MS spectrum of the peptide obtained using an FT-ICR Bruker 9.4T Apex-Qe FTICR (Bruker Daltonics, Billerica, MA, USA) gives an elemental composition of $C_{174}H_{270}N_{42}O_{46}S_2$ for the $(M+H)^+$ ion of A-DAP lactocin S (**151**) with a mass accuracy of 1.04 ppm.

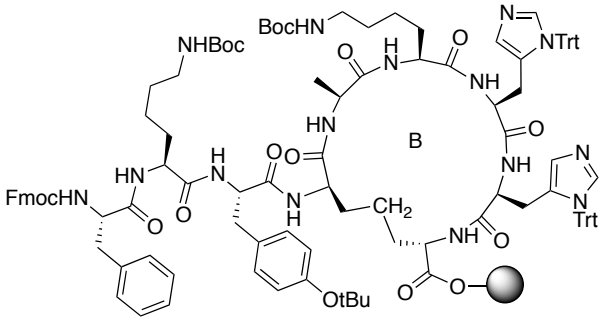
5.2.2.c Synthesis of B-DAP Lactocin S (152)

Synthesis of the linear precursor to B-ring of B-DAP Lactocin S (175)



The linear portion of the B-ring (**175**) of B-DAP lactocin S was synthesized following protocol A. Briefly, orthogonally protected-DAP **137** (0.043 g, 0.080 mmol) was loaded onto 2-chlorotrityl resin with a loading of 0.16 mmol/g. The amino acids of the ring were incorporated and then the *N*-terminal and orthogonal protecting groups were removed in preparation for cyclization.

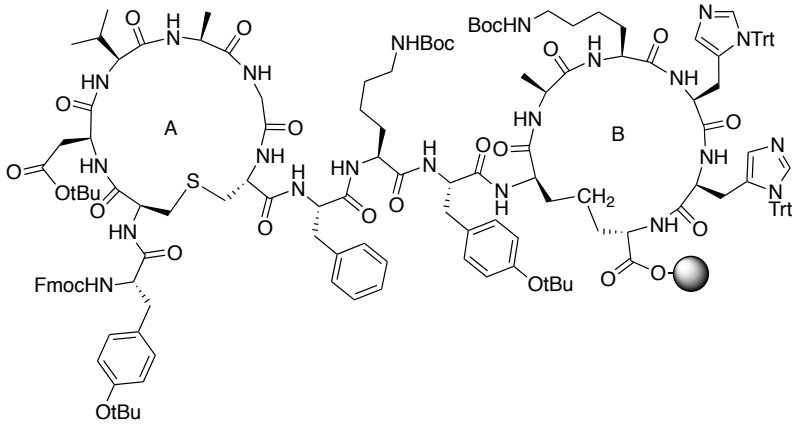
Synthesis of B-ring of B-DAP lactocin S (176)



Following protocol B, the cyclized B-ring intermediate (**176**) of B-DAP lactocin S was synthesized. Briefly, the peptide was exposed to coupling conditions to effect cyclization and then residues 31-29 were introduced via Fmoc-SPPS. A small sample of the resin-bound peptide was cleaved from the resin and analyzed using

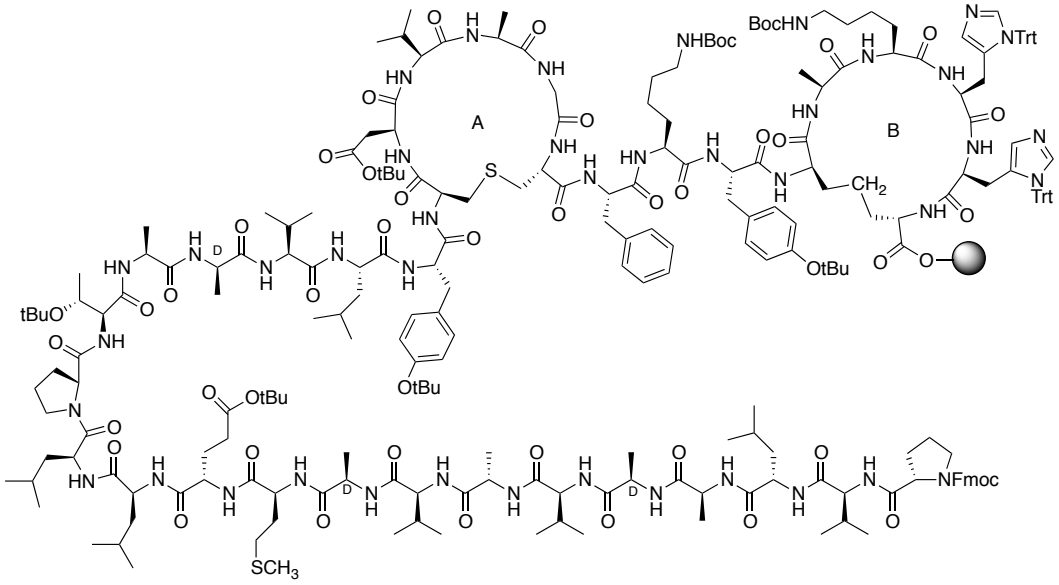
MALDI-TOF MS: Calculated for $C_{67}H_{84}N_{15}O_{13}$ 1306.5, found 1307.3 $(M+H)^+$. At this point the resin was divided into two portions. 1/2 (0.04 mmol) was saved for use in the synthesis of DAP lactocin S (**153**) and 1/2 (0.04 mmol) was carried through the rest of the synthesis for B-DAP lactocin S (**152**).

Synthesis of A-ring of B-DAP lactocin S (177a)



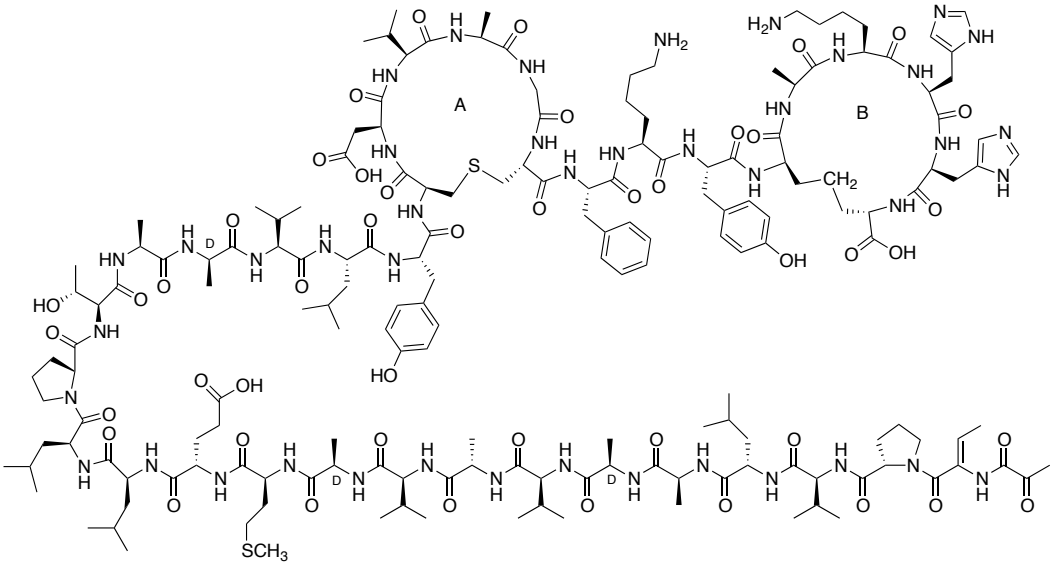
Following protocol C, the A-ring intermediate (**177a**) of B-DAP lactocin S was synthesized. Briefly, the amino acids of the A-ring were installed (orthogonally protected lanthionine **58** is used as in lactocin S) and then after deprotection of the orthogonal protecting groups, the ring was cyclized. Fmoc-Tyr(OtBu)-OH was introduced and following cleavage of a small sample from the resin, the peptide was analyzed by MALDI-TOF MS: Calculated for $C_{96}H_{123}N_{22}O_{23}S$ 1984.2, found 1984.6 $(M+H)^+$.

Synthesis of residues 3-37 of B-DAP lactocin S (177)



Following protocol D, residues 37-3 (**177**) of B-DAP lactocin S were synthesized. Briefly, the amino acids corresponding to residues 21-3 were introduced to **177a** via Fmoc-SPPS and then a small sample was cleaved from the resin and analyzed by MALDI-TOF MS: Calculated for $C_{182}H_{270}N_{41}O_{45}S_2$ 3815.5, found 3815.8 $(M+H)^+$.

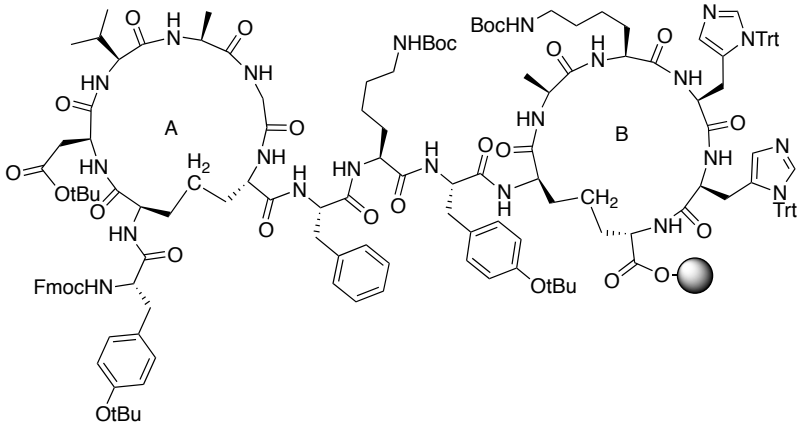
Synthesis of B-DAP lactocin S (152)



B-DAP lactocin S (**152**) was synthesized following protocol E. Briefly, **177** (0.03 mmol) was deprotected and then coupled with dipeptide **126** (0.050 g, 0.15 mmol) twice. The full length peptide (0.014 mmol) was then cleaved from the resin and crude B-DAP lactocin S (**152**) (4 mg) was obtained as a yellow solid. The peptide was purified by HPLC and was collected as a broad peak at 13.5 min. Pure **152** (1.0 mg, overall yield = 1.9% [33 deprotections and couplings, 2 macrocyclizations, 2 allyl deprotections, 1 cleavage/hydrolysis]) was obtained as a fluffy white solid. A high resolution MALDI-FT-ICR-MS spectrum of the peptide obtained using an FT-ICR Bruker 9.4T Apex-Qe FTICR (Bruker Daltonics, Billerica, MA, USA) gives an elemental composition of $C_{174}H_{270}N_{42}O_{46}S_2$ for the $(M+H)^+$ ion of B-DAP lactocin S (**152**) with a mass accuracy of -0.10 ppm.

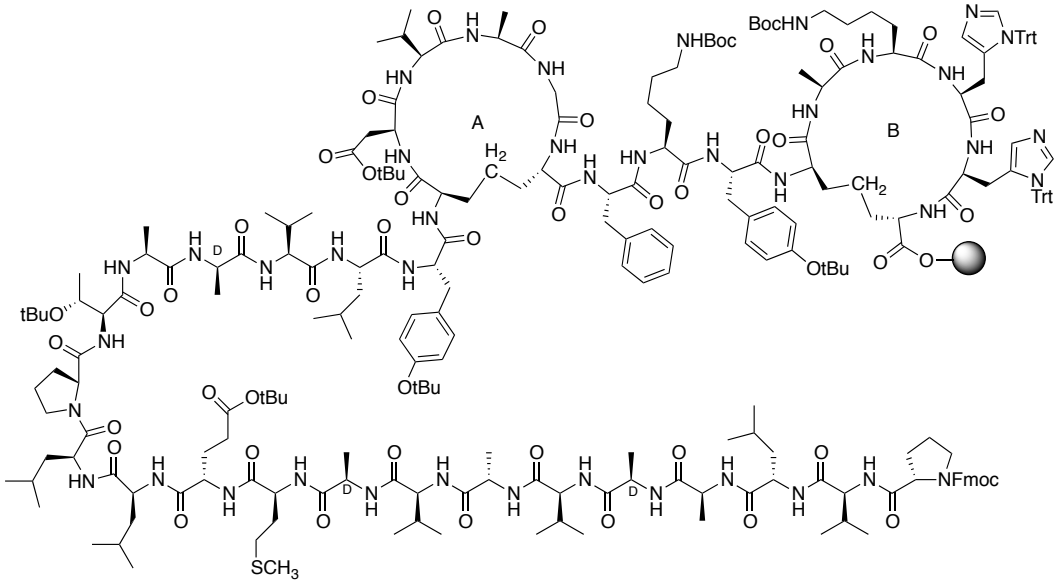
5.2.2.d Synthesis of DAP Lactocin S (153)

Synthesis of A-ring of DAP lactocin S (178a)



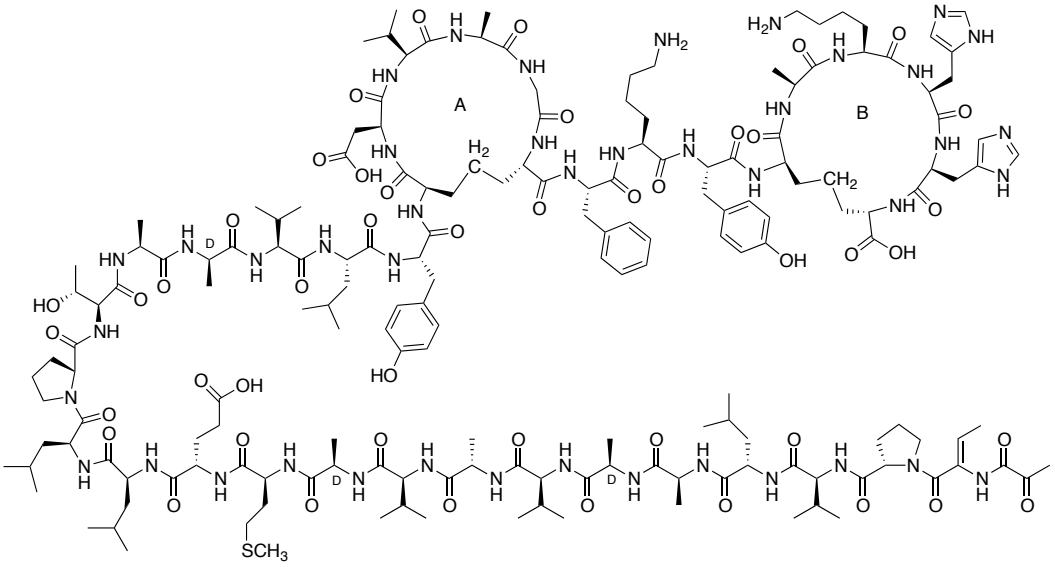
This approach utilizes intermediate **176** (0.04 mmol) from the synthesis of B-DAP lactocin S as starting material. Following protocol C, the A-ring intermediate (**178a**) of DAP lactocin S was synthesized. Briefly, the amino acids of the A-ring were installed (orthogonally protected diaminopimelate **137** is incorporated in place of the orthogonally protected lanthionine **58** from lactocin S) and then after deprotection of the orthogonal protecting groups, the ring was cyclized. Fmoc-Tyr(OtBu)-OH was introduced and following cleavage of a small sample from the resin, the peptide was analyzed by MALDI-TOF MS: Calculated for $C_{97}H_{125}N_{22}O_{23}$ 1966.2, found 1967.5 $(M+H)^+$.

Synthesis of residues 3-37 of DAP lactocin S (178)



Following protocol D, residues 37-3 (**178**) of DAP lactocin S were synthesized. Briefly, the amino acids corresponding to residues 21-3 were introduced to **178a** via Fmoc-SPPS and then a small sample was cleaved from the resin and analyzed by MALDI-TOF MS: Calculated for $C_{183}H_{272}N_{41}O_{45}S$ 3797.4, found 3799.0 $(M+H)^+$.

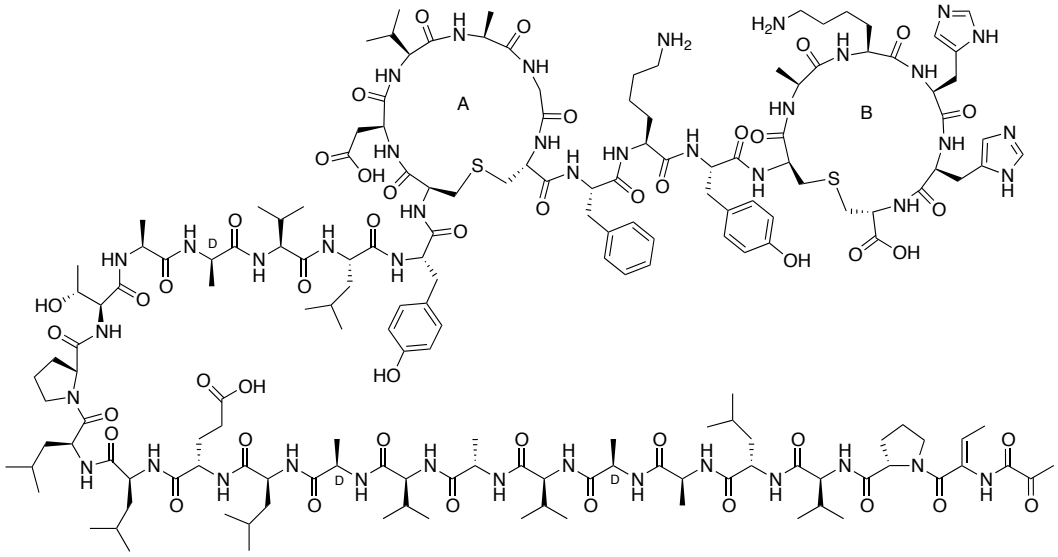
Synthesis of DAP lactocin S (153)



DAP lactocin S (**153**) was synthesized following protocol E. Briefly, **178** (0.03 mmol) was deprotected and then coupled to dipeptide **126** (0.050 g, 0.15 mmol). The full length peptide (0.017 mmol) was then cleaved from the resin and crude DAP lactocin S (**153**) (5 mg) was obtained as a brown solid. The peptide was purified by HPLC and was collected as a broad peak at 13 min. Pure **153** (1.0 mg, overall yield = 1.6 % [33 deprotections and couplings, 2 macrocyclizations, 2 allyl deprotections, 1 cleavage/hydrolysis]) was obtained as a fluffy white solid. A high resolution MALDI-FT-ICR-MS spectrum of the peptide obtained using an FT-ICR Bruker 9.4T Apex-Qe FTICR (Bruker Daltonics, Billerica, MA, USA) gives an elemental composition of $C_{175}H_{272}N_{42}O_{46}S$ for the $(M+H)^+$ ion of DAP lactocin S (**153**) with a mass accuracy of -0.58 ppm.

5.2.3 Methionine 12 analogues of lactocin S

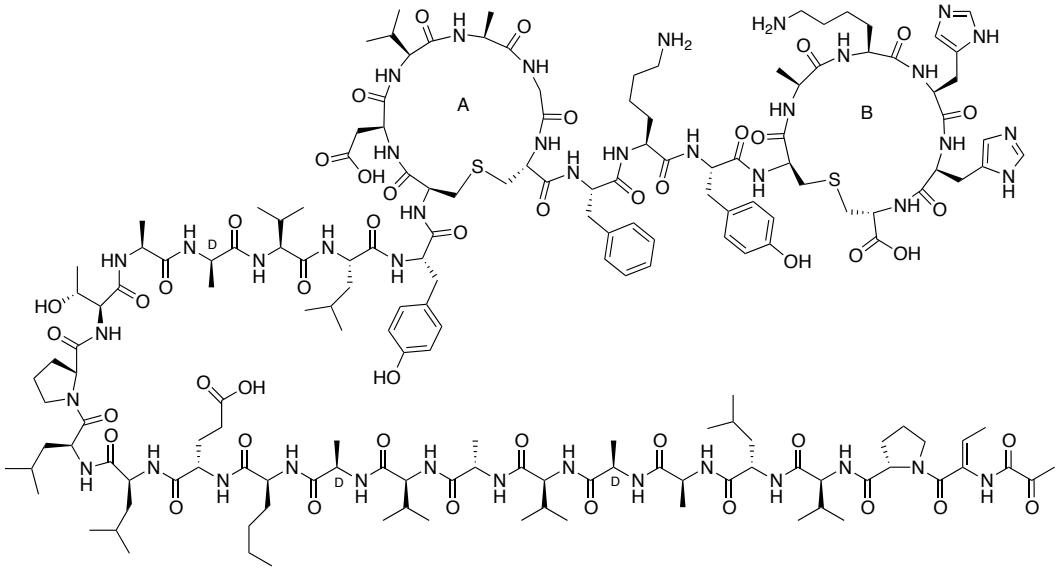
5.2.3.a Synthesis of Leu-12 lactocin S (181)



With the assistance of Mr. Michael Little, an analogue of lactocin S (**98**) in which methionine 12 was replaced with leucine (**181**) was synthesized. Following protocols A-E, the peptide was produced in an identical manner to lactocin S (**98**), except that Fmoc-Leu-OH was incorporated at residue 12. To obtain the desired peptide, the full length peptide (0.027 mmol) was then cleaved from the resin and crude Leu-12 lactocin S (**181**) (23 mg) was obtained as a yellow solid. A portion of the crude peptide (3 mg) was purified by HPLC and was collected as a broad peak at 13 min. Pure **181** (0.3 mg, overall yield = 2.3 % [33 deprotections and couplings, 2 macrocyclizations, 2 allyl deprotections, 1 cleavage/hydrolysis]) was obtained as a fluffy white solid. A high resolution MALDI-FT-ICR-MS spectrum of the peptide obtained using an FT-ICR Bruker 9.4T Apex-Qe FTICR (Bruker Daltonics, Billerica, MA, USA) gives an elemental composition of

$C_{174}H_{267}N_{42}O_{46}S_2$ for the $(M+H)^+$ ion of Leu-12 lactocin S (**181**) with a mass accuracy of 0.26 ppm.

5.2.3.b Synthesis of Nle-12 lactocin S (182)



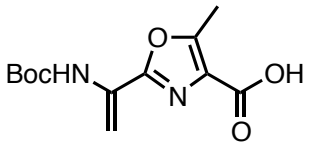
With the assistance of Mr. Michael Little, an analogue (**182**) of lactocin S (**98**) in which methionine 12 was replaced with norleucine was synthesized. Following protocols A-E, the peptide was produced in an identical manner to lactocin S (**98**), except that Fmoc-Nle-OH was incorporated at residue 12. To obtain the desired peptide, the full length peptide (0.027 mmol) was cleaved from the resin and crude Nle-12 lactocin S (**182**) (14 mg) was obtained as a yellow solid. A portion of the crude peptide (3 mg) was purified by HPLC and was collected as a broad peak at 13 min. Pure **182** (0.5 mg, overall yield = 3.8 % [33 deprotections and couplings, 2 macrocyclizations, 2 allyl deprotections, 1 cleavage/hydrolysis]) was obtained as a fluffy white solid. A high resolution MALDI-FT-ICR-MS spectrum

of the peptide obtained using an FT-ICR Bruker 9.4T Apex-Qe FTICR (Bruker Daltonics, Billerica, MA, USA) gives an elemental composition of $C_{174}H_{267}N_{42}O_{46}S_2$ for the $(M+H)^+$ ion of Nle-12 lactocin S (**182**) with a mass accuracy of -0.60 ppm.

5.2.4 Synthesis of oxazole lactocin S (183)

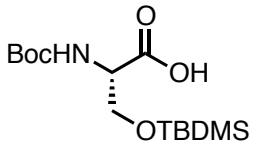
5.2.4.a Synthesis of oxazole (184)

2-(1-((*tert*-butoxycarbonyl)amino)vinyl)-5-methyloxazole-4-carboxylic acid (184)



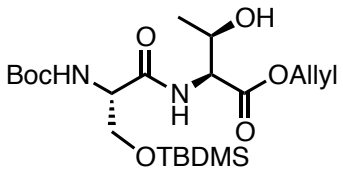
$PhSiH_3$ (0.20 mL, 1.60 mmol) was added to a stirred solution of **189** (0.25 g, 0.82 mmol) in degassed CH_2Cl_2 (100 mL). $Pd(PPh_3)_4$ (0.100 g, 0.080 mmol) was added to the mixture and the reaction was stirred in the absence of light for 1.5 h. The solvent was removed *in vacuo* and the resulting crude product was purified using flash chromatography (SiO_2, 40:1:0.1 CH_2Cl_2:MeOH:Acetic acid) to yield **184** (0.60 g, 27.5%) as a pale orange solid. (R_f 0.13 on SiO_2, 2:1 hexanes:EtOAc); IR (CH_2Cl_2 cast) 3395, 3058, 2979, 2930, 2852, 1781, 1724, 1504, 1160 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 10.25-9.47 (br s, 1H, -O<u>H</u>), 7.30 (s, 1H, -N<u>H</u>), 6.03 (s, 1H, -C=C<u>H</u>H), 5.48 (s, 1H, -C=CH<u>H</u>), 2.62 (s, 3H, -C<u>H</u>$_3$), 1.46 (s, 9H, -C(C<u>H</u>$_3$)$_3$); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 164.9, 157.7, 155.1, 151.2, 126.7, 126.0, 98.8, 79.4, 26.7, 10.9; HRMS (ES) Calculated for $C_{12}H_{16}N_2O_5Na$ 291.0951, found 291.0951 $(M+Na)^+$.

(*S*)-2-((*tert*-butoxycarbonyl)amino)-3-((*tert*-butyldimethylsilyl)oxy)propanoic acid (185)



This known compound was prepared according to literature procedure.[167] Imidazole (15.1 g, 244 mmol) was added to a solution of Boc-Ser-OH (20.0 g, 97.5 mmol) and TBDMS-Cl (16.2 g, 107 mmol) in DMF (200 mL) at 0 °C. Reaction was allowed to warm to rt and was stirred for 16 h, then the solvent was removed *in vacuo*. The residue was re-dissolved in EtOAc:MeOH (95 mL:5 mL), washed with H_2O and dried over Na_2SO_4. The solvent was removed *in vacuo* to yield **185** (27.7 g, 89.1 %) as a pale yellow oil. The crude product was carried through to the next reaction without further purification. (R_f 0.83 on SiO_2, 1:1 hexanes:EtOAc); $[\alpha]_D^{26}$ 5.96 (*c* 1.0, MeOH); IR (MeOH cast) 3329, 3148, 2978, 2935, 2880, 2646, 1708, 1519, 1167 cm^{-1}; ^1H NMR (CD_3OD, 300 MHz): δ 4.14 (m, 1H, -C$\underline{H}_\alpha$), 3.96 (dd, 1H, *J* = 10.2, 4.2 Hz, -C$\underline{H}_\beta$), 3.88 (dd, 1H, *J* = 10.2, 3.9 Hz, -C$\underline{H}_\beta$), 1.44 (s, 9H, -C(C$\underline{H}_3$)$_3$), 0.88 (s, 9H, -C(C$\underline{H}_3$)$_3$), 0.06 (s, 3H, -C$\underline{H}_3$), 0.05 (s, 3H, -C$\underline{H}_3$); ^{13}C NMR (CD_3OD, 125 MHz): δ 175.5, 157.5, 80.4, 65.1, 58. 1, 28.8, 26.4, -5.2; HRMS (ES) Calculated for $C_{14}H_{29}NO_5SiNa$ 342.1707, found 342.1712 $(M+Na)^+$.

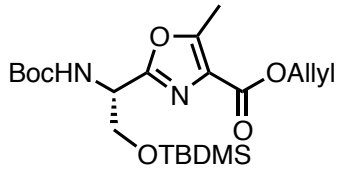
(2*S*,3*R*)-allyl 2-((*S*)-2-((*tert*-butoxycarbonyl)amino)-3-((*tert*-butyldimethylsilyl)oxy)propanamido)-3-hydroxybutanoate (186)



128 (6.84 g, 20.7 mmol) and **185** (6.00 g, 18.8 mmol) were dissolved in DMF (200 mL) and EDC (4.32 g, 23.0 mmol) and HOBt (3.04 g, 23.0 mmol) were added. The reaction was cooled to 0 °C and DIPEA (8.17 mL, 47.0 mmol) was added. The reaction was allowed to warm to rt and was left to stir for 18 h. Solvent was removed *in vacuo* and the resulting residue was re-dissolved in EtOAc (400 mL) then washed with 10% citric acid (100 mL), 10% $NaHCO_3$ (100 mL) H_2O (100 mL) and brine (100 mL). The organic layer was dried over Na_2SO_4 and then concentrated *in vacuo*. The resulting crude product was purified using flash chromatography (SiO_2, 3:1 hexanes:EtOAc) to yield **186** (4.47 g, 51.7%) as a pale yellow oil. (R_f 0.31 on SiO_2, 3:1 hexanes:EtOAc); $[\alpha]_D^{26}$ 12.54 (*c* 1.0, EtOAc); IR (EtOAc cast) 3356, 2977, 2955, 2931, 2885, 2858, 1745, 1720, 1672, 1523, 1502, 1255, 1171 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 7.36-7.18 (m, 1H, -N<u>H</u>), 5.92 (ddt, 1H, *J* = 6.0, 10.5, 17.0 Hz ,-$OCH_2C\underline{H}=CH_2$), 5.42-5.35 (m, 1H, -N<u>H</u>), 5.35 (dd, 1H, *J* = 17.0, 1.5 Hz, -$OCH_2CH=C\underline{H}_2$), 5.27 (dd, 1H, *J* = 10.5, 1.5 Hz, -$OCH_2CH=C\underline{H}_2$), 4.69-4.66 (m, 2H, -$OC\underline{H}_2CH=CH_2$), 4.64 (dd, 1H, *J* = 9.0, 3.0 Hz, Thr-C<u>H</u>$_\alpha$), 4.39-4.33 (m, 1H, Thr-C<u>H</u>$_\beta$), 4.27-4.20 (m, 1H, Ser-C<u>H</u>$_\alpha$), 4.06 (dd, 1H, *J* = 10.0, 4.0 Hz, Ser-C<u>H</u>$_\beta$), 3.73 (dd, 1H, *J* = 10.0, 6.5 Hz, Ser-C<u>H</u>$_\beta$), 2.38-2.32 (br s, 1H, -OH), 1.48 (s, 9H, -C(C<u>H</u>$_3$)$_3$), 1.22 (d, 3H, *J* = 6.0 Hz, Thr-C<u>H</u>$_3$), 0.91 (s, 9H, Si-C(C<u>H</u>$_3$)$_3$), 0.11 (s, 6H, Si-C<u>H</u>$_3$); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 171.2, 170.2, 155.5, 131.5, 118.9, 80.2, 68.1, 66.1, 63.2, 57.5, 55.8, 28.3,

25.9, 19.9, 18.3, -5.4, -5.5; HRMS (ES) Calculated for $C_{21}H_{40}N_2O_7SiNa$ 483.2497, found 483.2496 $(M+Na)^+$.

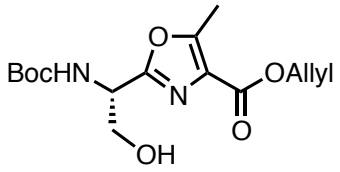
(*S*)-allyl 2-(2,2,3,3,10,10-hexamethyl-8-oxo-4,9-dioxa-7-aza-3-silaundecan-6-yl)-5-methyloxazole-4-carboxylate (187)



Dess-Martin periodinane (4.20 g, 9.80 mmol) was added to a solution of **186** (4.30 g, 9.30 mmol) in CH_2Cl_2 (100 mL) at 0 °C. After 15 min the reaction was warmed to rt and left to stir for a further 1 h. Et_2O (50 mL) was added and the solvent was removed *in vacuo*. The residue was re-dissolved in Et_2O (200 mL) and washed with a solution of 10% $Na_2S_2O_3$ /10% $NaHCO_3$ (100 mL) followed by H_2O (100 mL) and brine (100 mL). The aqueous layers were back extracted into Et_2O (3 × 75 mL) and then the organic layer was washed with H_2O (50 mL) and brine (50 mL). The organic layers were combined and dried over Na_2SO_4. Solvent was removed *in vacuo* and the residue was dissolved in CH_2Cl_2 (75 mL) and was added drop-wise to a solution of triphenylphosphine (4.90 g, 18.7 mmol), I_2 (4.74 g, 18.7 mmol) and Et_3N (5.20 mL, 37.4 mmol) in CH_2Cl_2 (75 mL). The reaction was stirred for 30 min and was then concentrated *in vacuo*. The resulting residue was dissolved in diethyl ether (250 mL) and was washed with 10% $Na_2S_2O_3$ (100 mL), 10% $NaHCO_3$ (100 mL), H_2O (100 mL) and brine (100 mL). The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The resulting crude

product was purified using flash chromatography (SiO_2, 4:1 hexanes:EtOAc) to yield **187** (2.80 g, 68.3%) as a pale yellow oil. (R_f 0.67 on SiO_2, 3:1 hexanes:EtOAc); $[\alpha]_D^{26}$ -15.46 (*c* 0.45, CH_2Cl_2); IR (CH_2Cl_2 cast) 3446, 3356, 2955, 2931, 2885, 2858, 1719, 1502, 1254, 1175, 1099 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ 5.99 (ddt, 1H, *J* = 17.2, 10.4, 5.6 Hz, -$OCH_2C\underline{H}$=CH_2), 5.49 (d, 1H, *J* = 7.6 Hz, -N$\underline{H}$), 5.36 (dq, 1H, *J* = 17.2, 1.6 Hz, -OCH_2CH=C$\underline{H}_2$), 5.26 (dq, 1H, *J* = 10.4, 1.6 Hz, -OCH_2CH=C$\underline{H}_2$), 4.96-4.88 (m, 1H, -C$\underline{H}_\alpha$), 4.83-4.78 (m, 2H, -O$\underline{CH}_2CH$=CH_2), 4.00 (dd, 1H, *J* = 10.4, 4.0 Hz, -C$\underline{H}_\beta$), 3.38 (dd, 1H, J = 10.4, 4.4 Hz, -C$\underline{H}_\beta$), 2.58 (s, 3H, -C$\underline{H}_3$), 1.43 (s, 9H, -C(C$\underline{H}_3$)$_3$), 0.80 (s, 9H, Si-C(C$\underline{H}_3$)$_3$), -0.03 (s, 3H, Si-C$\underline{H}_3$), -0.05 (s, 3H, Si-C$\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 161.9, 160.8, 156.5, 155.1, 132.0, 127.5, 118.7, 80.1, 65.5, 64.5, 51.0, 28.3, 25.6, 18.1, 12.0, -5.6; HRMS (ES) Calculated for $C_{21}H_{37}N_2O_6Si$ 441.2415, found 441.2414 $(M+H)^+$.

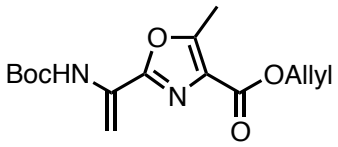
(*S*)-allyl 2-(1-((*tert*-butoxycarbonyl)amino)-2-hydroxyethyl)-5-methyloxazole-4-carboxylate (188)



1 M Tetra-*n*-butylammonium fluoride in THF (14.5 mL, 14.5 mmol) was added to a solution of **187** (2.13 g, 4.80 mmol) in THF (200 mL) at 0 °C. The reaction was left to stir for 2.5 h whilst slowly warming to rt. Solvent was removed *in vacuo* and the resulting crude product was purified using flash chromatography

(SiO_2, 1:1 hexanes:EtOAc) to yield **188** (1.36 g, 86.2%) as a colourless oil. (R_f 0.37 on SiO_2, 1:1 hexanes:EtOAc); $[\alpha]_D^{26}$ -36.9 (*c* 1.0, CH_2Cl_2); IR (CH_2Cl_2 cast) 3449, 2978, 2934, 1718, 1620, 1518, 1171 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ 5.99 (ddt, 1H, *J* = 17.2, 10.4, 6.0 Hz, -$OCH_2C\underline{H}=CH_2$), 5.72 (d, 1H, *J* = 7.6 Hz, -$N\underline{H}$), 5.38 (dq, 1H, *J* = 16.8, 1.2 Hz, -$OCH_2CH=C\underline{H}_2$), 5.28 (dq, 1H, *J* = 10.4, 1.2 Hz, -$OCH_2CH=C\underline{H}_2$), 4.99-4.88 (m, 1H, -$C\underline{H}_\alpha$), 4.82-4.77 (m, 2H, -$OC\underline{H}_2CH=CH_2$), 4.07 (dd, 1H, *J* = 11.2, 4.0 Hz, -$C\underline{H}_\beta$), 3.93 (dd, 1H, *J* = 11.2, 4.0 Hz, -$C\underline{H}_\beta$), 3.41-3.01 (br s, 1H, -$O\underline{H}$), 2.60 (s, 3H, -$C\underline{H}_3$), 1.43 (s, 9H, -$C(C\underline{H}_3)_3$); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 161.9, 160.9, 157.1, 155.7, 132.0, 127.5, 119.2, 80.6, 65.9, 63.9, 50.8, 28.5, 20.7, 12.4; HRMS (ES) Calculated for $C_{15}H_{22}N_2O_6Na$ 349.1370, found 349.1364 $(M+Na)^+$.

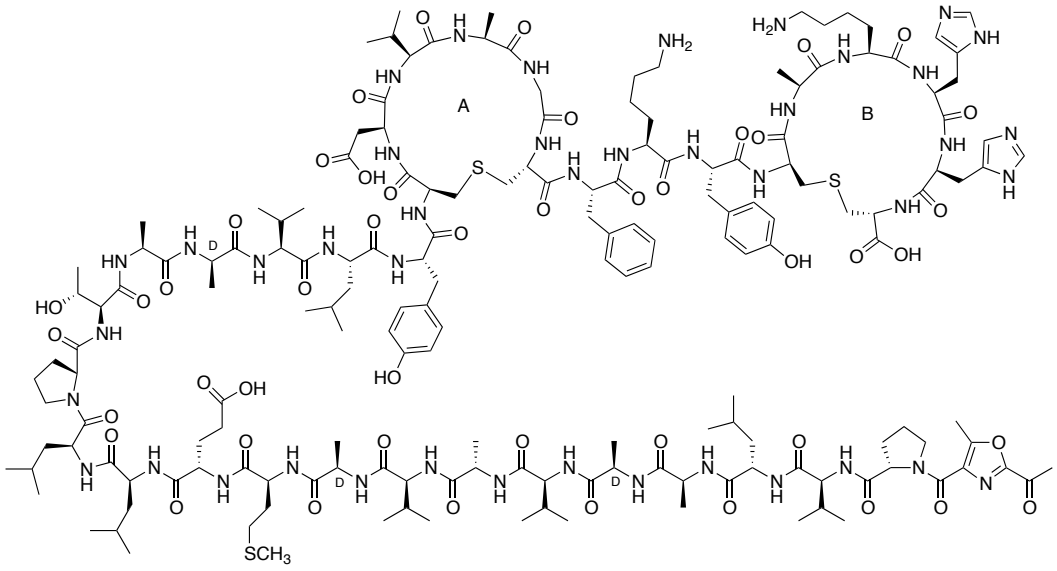
allyl 2-(1-((*tert*-butoxycarbonyl)amino)vinyl)-5-methyloxazole-4-carboxylate (189)



Et_3N (0.69 mL, 4.96 mmol) was added to a solution of **188** (0.62 g, 1.99 mmol) in CH_2Cl_2 (50 mL) at 0 °C. After 10 min, methanesulfonyl chloride (0.31 mL, 3.97 mmol) was added and after a further 30 min it was allowed to warm to rt. Solvent was removed *in vacuo* after 1 h and the resulting residue was dissolved in DCE (50 mL). DBU (0.89 mL, 5.95 mmol) was added and the reaction was heated to reflux for 18 h. Solvent was removed *in vacuo* and the resulting crude product

was purified using flash chromatography (SiO_2, 5:1 hexanes:EtOAc) to yield **189** (0.42 g, 72.2%) as a yellow oil. (R_f 0.94 on SiO_2, 2:1 hexanes:EtOAc); IR (CH_2Cl_2 cast) 3411, 2980, 2934, 1785, 1723, 1616, 1510, 1160 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ 7.25 (s, 1H, -C=C<u>H</u>H), 6.07 (s, 1H, -N<u>H</u>), 6.03 (ddt, 1H, *J* = 17.2, 10.8, 6.0 Hz, -OCH$_2$C<u>H</u>=CH$_2$), 5.53-5.50 (m, 1H, -C=CH<u>H</u>), 5.41 (dq, 1H, *J* = 17.2, 1.6 Hz, -OCH$_2$CH=C<u>H</u>$_2$), 5.31 (dd, 1H, *J* = 10.4, 1.2 Hz, -OCH$_2$CH=C<u>H</u>$_2$), 4.87-4.82 (m, 2H, -OC<u>H</u>$_2$CH=CH$_2$), 2.65 (s, 3H, -C<u>H</u>$_3$), 1.49 (s, 9H, -C(C<u>H</u>$_3$)$_3$); 13C NMR ($CDCl_3$, 100 MHz): δ 161.9, 157.5, 156.7, 152.9, 132.0, 128.6, 128.3, 119.4, 100.0, 81.1, 66.1, 28.5, 12.6; HRMS (ES) Calculated for $C_{15}H_{21}N_2O_5$ 309.1445, found 309.1443 $(M+H)^+$.

5.2.4.b Synthesis and purification of oxazole lactocin S (183)



Oxazole lactocin S (**183**) was synthesized from lactocin S intermediate **100**, following procedure E. Briefly, **100** (0.005 mmol) was deprotected and then coupled to oxazole **184** (0.01 g, 0.05 mmol). The full length peptide (0.002 mmol) was then cleaved from the resin and crude oxazole lactocin S (**183**) (2 mg) was obtained as a yellow solid. The peptide was purified by HPLC and was collected as a broad peak at 13 min. Pure **183** (0.41 mg, overall yield = 5.3% [33 deprotections and couplings, 2 macrocyclizations, 2 allyl deprotections, 1 cleavage/hydrolysis]) as a fluffy white solid. A high resolution MALDI-FT-ICR-MS spectrum of the peptide obtained using an FT-ICR Bruker 9.4T Apex-Qe FTICR (Bruker Daltonics, Billerica, MA, USA) gives an elemental composition of $C_{173}H_{263}N_{42}O_{46}S_3$ for the $(M+H)^+$ ion of oxazole lactocin S (**183**) with a mass accuracy of 0.97 ppm.

5.3 Purification of Natural Lactocin S

5.3.1 Growth of *Lactobacillus sakei* L45 in MRS media without Tween 80

Media was prepared from individual ingredients, as listed on the package of standard MRS broth (Difco), without the addition of Tween 80. The adjusted media contained (per liter):

10.0 g proteose peptone No. 3 (Difco)

10.0 g beef extract (Difco)

5.0 g yeast extract (Difco)

20.0 g dextrose

2.0 g ammonium citrate

5.0 g sodium acetate

0.1 g magnesium sulfate

0.05 g manganese sulfate

2.0 g dipotassium phosphate

Tween 80-free MRS broth (4 × 1L) was inoculated with an overnight culture of *L. sakei* L45 (10 mL/1 L of broth, 1% inoculum). The cultures were incubated for 18-20 h at 30 °C under an atmosphere of argon in two 2 L flasks to minimize the air space above the media. Initial overnight cultures were also grown in Tween 80-free MRS broth (10 mL/tube) to correspond with the broth used for purification cultures. The strain was maintained as a frozen stock culture at -80 °C in standard MRS broth (Difco) with 20% glycerol (vol/vol).)

5.3.2 Purification of lactocin S from *L. sakei* grown in MRS media without Tween 80

Note. This purification was monitored using activity testing (see Section 5.5) and MALDI-TOF mass spectrometry.

Following incubation, ammonium sulfate was added (200 g/L) to precipitate proteins and the culture was centrifuged (8000 rpm, JA-10 rotor, 20 min, 4 °C) to pellet the insoluble matter. The pellets containing cell debris and precipitated protein were re-suspended in 70% IPA (400 mL, acidified to pH 2 with HCl), and then stirred at 4 °C for 90 minutes. The cell debris was then removed by centrifugation (8000 rpm, JA-10, 20 min, 4 °C) and the supernatant concentrated *in vacuo* to a final volume of approximately 120 mL. The supernatant was then diluted with H_2O (30 mL), loaded onto three parallel, prepacked 60 mL columns containing C_{18} silica (Varian C_{18} Mega Bond Elut®) and washed with H_2O (60 mL). Approximately 50 mL, or one-third of the supernatant, was loaded onto each column. Each column was pre-equilibrated with methanol (60 mL) followed by H_2O (120 mL). The columns were then washed successively with 30% ethanol (50 mL), 30% acetonitrile (50 mL), 40% IPA (50 mL) and 70% IPA at pH 2 (100 mL). The solvent was removed *in vacuo* from the active fraction (70% IPA at pH 2) and the residue (10 mg) redissolved in 0.1% TFA (aq.) (0.5 mL) and methanol (0.1% TFA) (0.5 mL) to a final volume of 1 mL. The solution was centrifuged (13000 rpm, 20 min, 4°C) and 10 × 100 μL of the supernatant were injected onto a C_2/C_{18} RP-HPLC column (GE Healthcare, 4.6 x 100 mm, 3 μm) using methanol

(0.1% TFA) and H_2O (0.1% TFA) as eluents. The HPLC method followed was: Gradient starting at 5% methanol for 1 min, climb to 60% over 4 min, then climb to 100% over 8 min, remain at 100% for 2 min, return to 5% over 2 min and remain at 5% for 8 min (flow rate 1 mL/min, UV detection at 220 nm). The peptide was collected as a broad peak at 13 min and solvent was removed *in vacuo*. The residue was then re-dissolved in approximately 0.1% TFA (aq.) (900 μL) and methanol (0.1% TFA) (100 μL) and lyophilized to yield natural lactocin S (**98**) as a white solid (100 μg). Monoisotopic MW Calculated for $C_{173}H_{265}N_{42}O_{46}S_3$ 3762.9, found 3762.6 $(M+H)^+$. TCEP (1.5 mg/L) was added to the HPLC solvents and argon was continuously bubbled through the solvents during HPLC to minimize oxidation of the sulfurs in methionine and lanthionine. In addition, the product was collected under a stream of argon, and during concentration the pressure in the rotary evaporator was restored to 1 atm using an argon balloon.

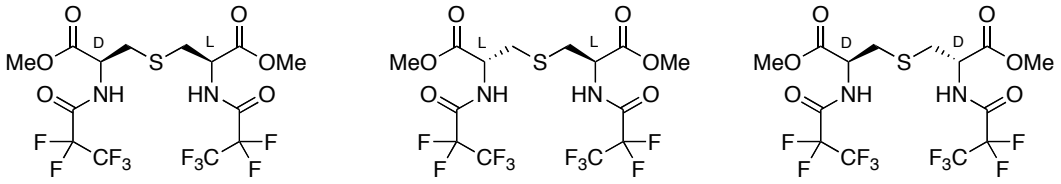
5.4 Chiral GC/MS Analysis of Natural and Synthetic Lactocin S

Natural and synthetic lactocin S samples were acid hydrolyzed and derivatized for analysis by chiral Gas Chromatography Mass Spectrometry (GC/MS). The identity of the lanthionine residue within each peptide was confirmed by comparison with standard lanthionine samples.

5.4.1 Hydrolysis and derivatization of lactocin S

6 M HCl (3 mL) was added to a sample of lactocin S (0.2 mg). The mixture was heated with stirring at 110 °C in a sealed tube for 24 h. The reaction was cooled and solvent removed *in vacuo*. Methanol (5 mL) was cooled in an ice-H_2O bath and acetyl chloride (1.5 mL) was added dropwise. This solution was added to the hydrolysate residue and the mixture was heated at 110 °C for 45 min, then allowed to cool. The solvent was removed by flowing a stream of argon over the solution until dry. CH_2Cl_2 (3 mL) and pentafluoropropionic anhydride (1 mL) were added to the vessel at 0 °C. The mixture was then heated at 110 °C for 15 min, then allowed to cool and dried under a stream of argon. The residue was dissolved in methanol (1 mL), transferred into a clean vial and the methanol was removed under a stream of argon.

5.4.2 Synthesis of pentafluoropropanamide methyl esters of lanthionine (133, 134, 135)



Lanthionine (20 mg) was dissolved in 0.2 M HCl (0.24 mL). Part of the solution (50 μL) was transferred into a reaction vessel and diluted with 0.2 M HCl to 3 mL. The vessel was sealed and heated to 110 °C for 5 min. The reaction was cooled and solvent removed *in vacuo*. Methanol (5 mL) was cooled in an ice-H_2O bath and acetyl chloride (1.5 mL) was added dropwise. This solution was added to the hydrolysate residue and the mixture was heated at 110 °C for 45 min, then allowed to cool. The solvent was removed by flowing a stream of argon over the solution until dry. CH_2Cl_2 (3 mL) and pentafluoropropionic anhydride (1 mL) were added to the vessel at 0 °C. The mixture was then heated at 110 °C for 15 min, then allowed to cool and dried under a stream of argon. The residue was dissolved in methanol (1 mL), transferred into a clean vial and methanol was removed under a stream of argon to give derivatized lanthionine standard.

5.4.3 Chiral GC/MS analysis

The hydrolyzed and derivatized natural and synthetic lactocin S samples were analyzed individually using GC/MS. The three lanthionine standards (D,D, D,L and L,L) were also added individually to the lactocin S samples and analyzed by

GC/MS. The synthetic lactocin S was analyzed using a Varian 25 m × 0.22 mm fused silica wall coated open tubular Chirasil-L-Val (0.12 μm) column on a Waters (Micromass) VG7070E with Agilent 5890 Series II GC. The sample was dissolved in MeOH and introduced into the machine via a splitless injection. The temperature method used was: 160 °C (10 min) raised to 180 °C by 3 °C/min, hold at 180 °C (10 min). All three stereoisomers of lanthionine elute as distinct peaks at 17-19 min (D,D then D,L then L,L).

Natural lactocin S was analyzed using the same Chirasil-L-Val column on an Agilent Technologies 7890 GC with 5975C MSD. The sample was dissolved in MeOH and introduced into the machine via a pulsed, splitless injection. The MS was operated in simultaneous Scan/SIM mode (monitoring at 365, 234 and 202 Da). The temperature method used was: 100 °C raised to 180 °C at 2 °C/min. For both the synthetic and natural lactocin S samples it was found that the predominant stereoisomer was the D,L-lanthionine. When either the D,D or L,L-lanthionine standards were added to the lactocin S samples two major peaks were observed in the trace. However when the D,L-lanthionine standard was added only one major peak was observed. The presence of small amounts of the D,D and L,L-lanthionine in the natural sample is believed to result from epimerization of the amino acid during acid hydrolysis of the peptide.

5.5 Biological Testing of Lactocin S (98) and Analogues (151, 152, 153, 181, 182, 183)

5.5.1 General procedure for spot-on-lawn activity assays

Anti-bacterial activity was measured using spot-on-lawn activity assays. Aliquots (1 × 10 μL or 5 × 2 μL) of the sample being tested for activity were spotted onto hard agar plates (10 mL) and allowed to dry. Molten soft agar (10 mL) was inoculated with an overnight culture of the organism of interest (100 μL, 1% inoculation), and then poured over each hard agar plate and allowed to solidify. Plates were sealed with Parafilm and incubated overnight (30 °C or 37 °C). Activity was measured as zones of inhibited growth.

Depending on the organism being tested, plates containing either MRS agar (10 mL, 1.5% agar, Becton Dickinson: Difco) or APT agar (10 mL, 1.5% agar) were used for spot-on-lawn assays. MRS agar was prepared using MRS agar (70 g/L, Becton Dickinson: Difco). APT agar was prepared by combining APT broth (46.2 g/L, Becton Dickinson: Difco) with Bacto Agar (15.0 g/L, Becton Dickinson: Difco). MRS soft agar (0.75% agar) was prepared by combining MRS broth (27.5 g/L, Becton Dickinson: Difco) with MRS Agar (35.0 g/L, Becton Dickinson: Difco). APT soft agar (0.75% agar) was prepared by combining APT broth (46.2 g/L, Becton Dickinson: Difco) with Bacto Agar (7.5 g/L, Becton Dickinson: Difco).

5.5.2 Activity testing of synthetic lactocin S

Using the spot-on-lawn procedure described above, synthetic lactocin S was compared to natural lactocin S. The peptide solutions (250 μM concentration each) were tested against both an organism sensitive to natural lactocin S (*P. acidilactici* Pac 1.0) and an organism immune to natural lactocin S (*L. sakei* L45) (Figure 5-1). This testing was done on MRS plates.

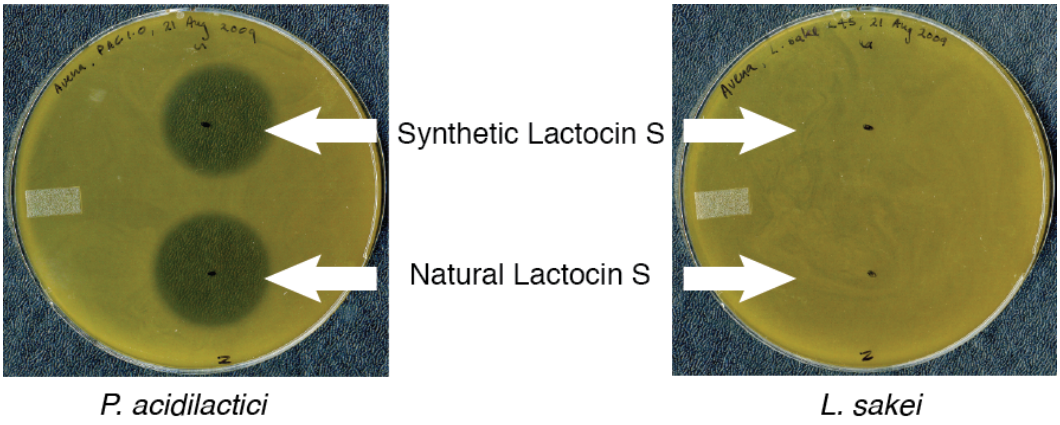


Figure 5-1 Comparative spot-on-lawn activity test for synthetic and natural lactocin S

5.5.3 Biological testing of analogues – General procedure

The anti-bacterial activity was again assayed using spot-on-lawn activity assays as described above. Following an initial test at high concentration (200 μM), active analogues were also tested in a dilution assay.

General procedure for the serial dilution assay

Each peptide was dissolved in 9:1 MQ-H_2O/MeOH (0.1% TFA) to yield an initial concentration of 100 μM. Serial dilutions with MQ-H_2O (0.1% TFA) gave 50 μM, 10 μM, 1 μM solutions. Using a spot-on-lawn activity assay, as described above, the serial dilutions of each peptide were tested. The zones of inhibited growth identified activity. This was meant as a purely qualitative assay and not a quantitative assessment. (Note, an equivalent concentration of 200 μM was obtained by spotting 10 × 2 μL of the 100 μM solution. This is compared to 5 × 2 μL of the 100 μM for 100 μM)

5.5.3.a Activity testing of A-DAP (151), B-DAP (152) and DAP lactocin S (153)

The panel of organisms used for testing these analogues is documented in Table 5-1.

Table 5-1 Organisms used for the testing of A-DAP (**151**), B-DAP (**152**) and DAP lactocin S (**153**)

Indicator species	Strain [Source]	Growth medium	Growth temperature
Lactobacillus acidophilus	4356 [ATCC]	MRS	37 °C
Lactobacillus delbrueckii subsp. *bulgaricus*	11842 [ATCC]	MRS	37 °C
Lactobacillus sakei (Producer)	L45 [*]	MRS	30 °C
Pediococcus acidilactici	Pac 1.0 [*]	MRS	30 °C
Pediococcus pentosaceus	FBB63 [TNO]	MRS	37 °C
Listeria monocytogenes	ScottA [FVM]	APT	30 °C

ATCC, American Type Culture Collection (Rockville, USA), FVM, Facultad de Veterinaria (Madrid, Spain), TNO, Nutrition and Food Research (Zeist, The Netherlands), * Provided by Prof. Ingolf Nes (Norwegian University of Life Sciences, Norway)

Activity was seen against *P. acidilactici* and *L. delbrueckii* and dilution assays for these organisms are shown in Figure 5-2 and Figure 5-3. No activity was observed for either the natural peptide or the analogues against the other strains tested.

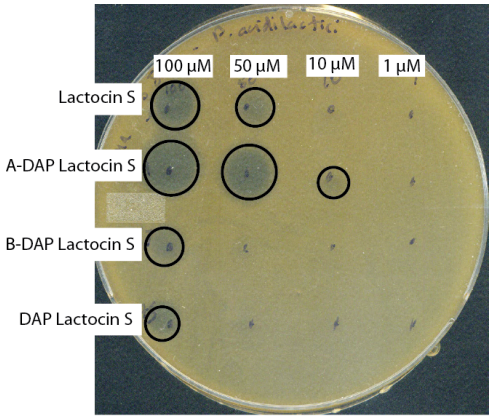


Figure 5-2 Activity test against *P. acidilactici* Pac 1.0. Black circles indicate the zones of inhibited growth.

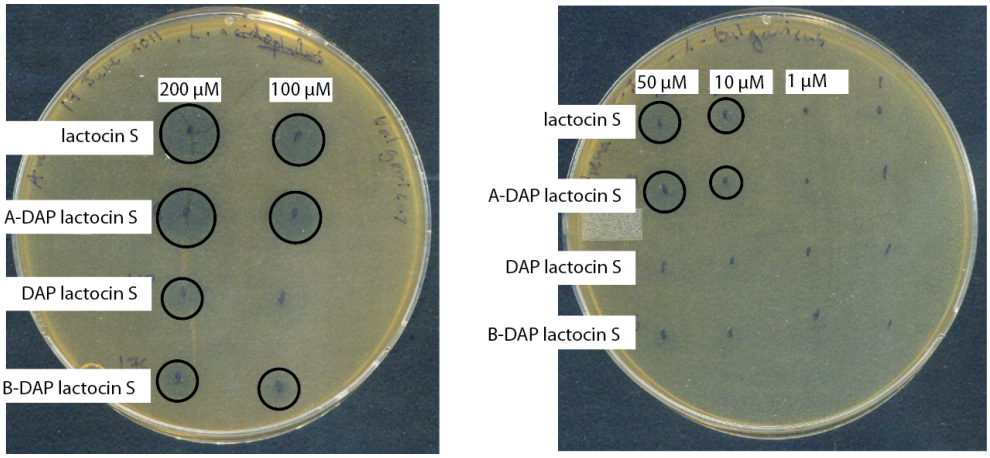


Figure 5-3 Activity test against *L. delbrueckii* 11842. Black circles indicate the zones of inhibited growth.

5.5.3.b Activity testing of Leu-12 lactocin S (181) and Nle-12 lactocin S (182)

This work was done with the assistance of Mr. Michael Little and Mr. Shaun McKinnie. The panel of organisms used for testing these analogues is documented in Table 5-2.

Table 5-2 Organisms used for the testing of Leu-12 (**181**) and Nle-12 (**182**) lactocin S

Indicator species	Strain	Growth medium	Growth temperature
Lactobacillus delbrueckii subsp. *bulgaricus*	11842 [ATCC]	MRS	37 °C
Lactobacillus helveticus	15009 [ATCC]	MRS	37 °C
Lactobacillus sakei (Producer)	L45 [*]	MRS	30 °C
Pediococcus acidilactici	Pac 1.0 [*]	MRS	30 °C
Pediococcus pentosaceus	FBB63 [TNO]	MRS	37 °C

ATCC, American Type Culture Collection (Rockville, USA), TNO, Nutrition and Food Research (Zeist, The Netherlands), * Provided by Prof. Ingolf Nes (Norwegian University of Life Sciences, Norway)

Activity was seen against *P. acidilactici* and *L. delbrueckii* and dilution assays for these organisms are shown in Figure 5-4. No activity was observed for either the natural peptide or the analogues against the other strains tested.

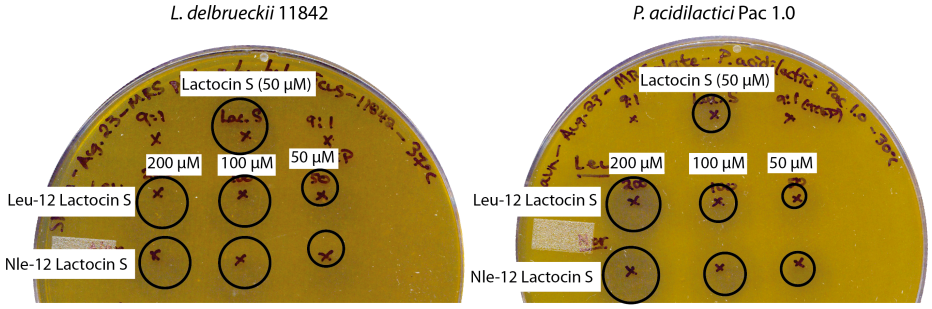


Figure 5-4 Activity results for Leu-12 (**181**) and Nle-12 lactocin S (**182**) against *L. delbrueckii* and *P. acidilactici.* Black circles indicate the zones of inhibited growth.

5.5.3.c Activity testing of oxazole lactocin S (183)

The organisms used for testing this analogue are documented in Table 5-3.

Table 5-3 Organisms used for the testing of oxazole lactocin S (**183**)

Indicator species	Strain	Growth medium	Growth temperature
Lactobacillus sakei (Producer)	L45 [*]	MRS	30 °C
Pediococcus acidilactici	Pac 1.0 [*]	MRS	30 °C

* Provided by Prof. Ingolf Nes (Norwegian University of Life Sciences, Norway)

Oxazole lactocin S (**183**) showed no inhibitory effect on the growth of either organism at concentrations up to 1 mM.

Chapter 6: References

(1) Arias, E.; Rostron, B. L.; Tejada-Vera, B. United States Life Tables 2005. In *National Vital Statistics Reports*, 2010; Vol. 58; pp 1-132.

(2) Armstrong, G. L.; Conn, L. A.; Pinner, R. W. Trends in infectious disease mortality in the United States during the 20th century. *J. Am. Med. Assoc.* **1999**, *281*, 61-66.

(3) Barer, M.; Lond, M. B. Typhoid bacilluria treated with sulphanilamide. *Lancet* **1937**, *230*, 964-965.

(4) Anonymous. The discovery of Penicillin. *Br. Med. J.* **1955**, *1*, 711.

(5) Fleming, A. On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of *B. influenzae*. *Br. J. Ex. Pathol.* **1929**, *10*, 226-236.

(6) Fischbach, M. A.; Walsh, C. T. Antibiotics for emerging pathogens. *Science* **2009**, *325*, 1089-1093.

(7) Abraham, E. Selective reminiscences of β-lactam antibiotics: Early research on penicillin and cephalosporins. *Bioessays* **1990**, *12*, 601-606.

(8) Boniece, W. S.; Wick, W. E.; Holmes, D. H.; Redman, C. E. *In vitro* and *in vivo* laboratory evaluation of cephalothin, a new broad spectrum antibiotic. *J. Bacteriol.* **1962**, *84*, 1292-1296.

(9) Schatz, A.; Bugie, E.; Waksman, S. A. Streptomycin, a substance exhibiting antibiotic activity against Gram-positive and Gram-negative bacteria. *Proc. Soc. Exp. Biol. Med.* **1944**, *55*, 66-69.

(10) Atlas, E.; Turck, M. Laboratory and clinical evaluation of rifampicin. *Am. J. Med. Sci.* **1968**, *256*, 247-254.

(11) Fairbrother, R. W.; Williams, B. L. Two new antibiotics. Antibacterial activity of novobiocin and vancomycin. *Lancet* **1956**, *268*, 1177-1179.

(12) Oppenheimer, S.; Turck, M. Laboratory and clinical evaluation of 7-chloro-7-deoxylincomycin. *Am. J. Med. Sci.* **1968**, *256*, 314-321.

(13) Boothe, J. H.; Morton, J.; Petisi, J. P.; Wilkinson, R. G.; Williams, J. H. Tetracycline. *J. Am. Chem. Soc.* **1953**, *75*, 4621.

(14) Brownlee, G.; Jones, T. S. G. The polymyxins- A related series of antibiotics derived from *B. polymyxa*. *Biochem. J.* **1948**, *43*, R25-R26.

(15) Henderson, F. G.; Powell, C. E.; Rose, C. L.; Robbins, E. B. Pharmacodynamics of ilotycin (erythromycin, Lilly). *J. Pharmacol. Exp. Ther.* **1952**, *106*, 395.

(16) Roy, C.; Foz, A.; Segura, C.; Tirado, M.; Teixell, M.; Teruel, D. Activity of ciprofloxacin (BAYo 9867) against *Pseudomonas aeruginosa* and ampicillin-resistant *Enterobacteriacea*. *Infection* **1983**, *11*, 326-328.

(17) Brötz-Oesterhelt, H.; Brunner, N. A. How many modes of action should an antibiotic have? *Curr. Opin. Pharm.* **2008**, *8*, 564-573.

(18) Lange, R. P.; Locher, H. H.; Wyss, P. C.; Then, R. L. The targets of currently used antibacterial agents: Lessons for drug discovery. *Curr. Pharm. Des.* **2007**, *13*, 3140-3154.

(19) Overbye, K. M.; Barrett, J. F. Antibiotics: where did we go wrong? *Drug Discov. Today* **2005**, *10*, 45-52.

(20) Gootz, T. D. Discovery and development of new antimicrobial agents. *Clin. Microbiol. Rev.* **1990**, *3*, 13-31.

(21) Palumbi, S. R. Humans as the world's greatest evolutionary force. *Science* **2011**, *293*, 1786-1790.

(22) Arias, C. A.; Murray, B. E. Antibiotic-resistant bugs in the 21st century- A clinical super-challenge. *New Engl. J. Med.* **2009**, *360*, 439-443.

(23) Walsh, C. Where will new antibiotics come from? *Nat. Rev. Microbiol.* **2003**, *1*, 65-70.

(24) Kahne, D.; Leimkuhler, C.; Lu, W.; Walsh, C. Glycopeptide and lipoglycopeptide antibiotics. *Chem. Rev.* **2005**, *105*, 425-448.

(25) Nyquist, A.-C.; Ganzales, R.; Steiner, J. F.; Sande, M. A. Antibiotic prescribing for children with colds, upper respiratory tract infections, and bronchitis. *J. Am. Med. Assoc.* **1998**, *279*, 875-877.

(26) Eliopoulos, G. M.; Willey, S.; Reiszner, E.; Spitzer, P. G.; Caputo, G.; Moellering Jr, R. C. In vitro and in vivo activity of LY 146032, a new

cyclic lipopeptide antibiotic. *Antimicrob. Agents Chemother.* **1986**, *30*, 532-535.

(27) Kesado, T.; Hashizume, T.; Asahi, Y. Antibacterial activities of a new stabilized thienamycin, *N*-formimidoyl thienamycin, in comparison with other antibiotics. *Antimicrob. Agents Chemother.* **1980**, *17*, 912-917.

(28) Zurenko, G. E.; Yagi, B. H.; Schaadt, R. D.; Allison, J. W.; Kilburn, J. O.; Glickman, S. E.; Hutchinson, D. K.; Barbachyn, M. R.; Brickner, S. J. *In vitro* activities of U-100592 and U-100766, novel oxazolidinone antibacterial agents. *Antimicrob. Agents Chemother.* **1996**, *40*, 839-845.

(29) Baltz, R. H. Renaissance in antibacterial discovery from actinomycetes. *Curr. Opin. Pharm.* **2008**, *8*, 557-563.

(30) Li, J. W.-H.; Vederas, J. C. Drug discovery and natural products: End of an era or an endless frontier? *Science* **2009**, *325*, 161-165.

(31) Bister, B.; Bischoff, D.; Ströbele, M.; Riedlinger, J.; Reicke, A.; Wolter, F.; Bull, A. T.; Zähner, H.; Fiedler, H.-P.; Süssmuth, R. D. Abyssomicin C- A polycyclic antibiotic from a marine *Verrucosispora* strain as an inhibitor of the *p*-aminobenzoic acid/tetrahydrofolate biosynthesis pathway. *Angew. Chem. Int. Ed.* **2004**, *43*, 2574-2576.

(32) Piel, J. Metabolites from symbiotic bacteria. *Nat. Prod. Rep.* **2009**, *26*, 338-362.

(33) Scott, J. J.; Oh, D.-C.; Yuceer, M. C.; Klepzig, K. D.; Clardy, J.; Currie, C. R. Bacterial protection of beetle-fungus mutualism. *Science* **2008**, *322*, 63.

(34) Banskota, A. H.; McAlpine, J. B.; Sørensen, D.; Ibrahim, A.; Auidate, M.; Piraee, M.; Alarco, A.-M.; Farnet, C. M.; Zazopoulos, E. Genomic analyses lead to novel secondary metabolites. Part 3, ECO-0501 a novel antibacterial of a new class. *J. Antibiot.* **2006**, *59*, 533-542.

(35) Brötz-Oesterhelt, H.; Beyer, D.; Kroll, H.-P.; Endermann, R.; Ladel, C.; Schroeder, W.; Hinzen, B.; Raddatz, S.; Paulsen, H.; Henninger, K.; Bandow, J. E.; Sahl, H.-G.; Labischinski, H. Dysregulation of bacterial proteolytic machinery by a new class of antibiotics. *Nat. Med.* **2005**, *11*, 1082-1087.

(36) Cotter, P. D.; Hill, C.; Ross, R. P. Bacteriocins: Developing innate immunity for food. *Nat. Rev. Microbiol.* **2005**, *3*, 777-788.

(37) Nes, I. F.; Yoon, S.-S.; Diep, D. B. Ribosomally synthesized antimicrobial peptides (bacteriocins) in lactic acid bacteria: a review. *Food Sci. Biotechnol.* **2007**, *16*, 675-690.

(38) Draper, L. A.; Ross, R. P.; Hill, C.; Cotter, P. D. Lantibiotic Immunity. *Curr. Protein Peptide Sci.* **2008**, *9*, 39-49.

(39) van Belkum, M. J.; Stiles, M. E. Nonlantibiotic antibacterial peptides from lactic acid bacteria. *Nat. Prod. Rep.* **2000**, *17*, 323-335.

(40) Gillor, O.; Kirkup, B. C.; Riley, M. A. Colicins and microcins: The next generation antimicrobials. *Adv. Appl. Microbiol.* **2004**, *54*, 129-146.

(41) Gratia, A. Sur un remarquable exemple d'antagonisme entre deux souches de colibacille. *C. R. Soc. Biol.* **1925**, *93*, 1040-1042.

(42) Klaenhammer, T. R. Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiol. Rev.* **1993**, *12*, 39-86.

(43) Mattick, A. T. R.; Hirsch, A. A powerful inhibitory substance produced by group *N* streptococci. *Nature* **1944**, *154*, 551-551.

(44) Chatterjee, S.; Chatterjee, D. K.; Jani, R. H.; Blumbach, J.; Ganguli, B. N. Mersacidin, a new antibiotic from *Bacillus in vitro* and *in vivo* antibacterial activity. *J. Antibiot.* **1992**, *45*, 839-845.

(45) Henderson, J. T.; Chopko, A. L.; van Wassenaar, P. D. Purification and primary structure of pediocin PA-1 produced by *Pediococcus acidilactici* PAC-1.0. *Arch. Biochem. Biophys.* **1992**, *295*, 5-12.

(46) Nissen-Meyer, J.; Holo, H.; Håvarstein, L. S.; Sletten, K.; Nes, I. F. A novel lactococcal bacteriocin whose activity depends on the complementary action of two peptides. *J. Bacteriol.* **1992**, *174*, 5686-5692.

(47) Holo, H.; Nilssen, Ø.; Nes, I. F. Lactococcin A, a new bacteriocin from *Lactococcus lactis* subsp. *cremoris*: isolation and characterization of the protein and its gene. *J. Bacteriol.* **1991**, *173*, 3879-3887.

(48) Floriano, B.; Ruiz-Barba, J. L.; Jiménez-Díaz, R. Purification and genetic characterization of enterocin I from *Enterococcus faecium* 6T1a, a novel antilisterial plasmid-encoded bacteriocin which does not belong to the pediocin family of bacteriocins. *Appl. Environ. Microbiol.* **1998**, *64*, 4883-4890.

(49) Cintas, L. M.; Casaus, P.; Holo, H.; Hernandez, P. E.; Nes, I. F.; Håvarstein, L. S. Enterocins L50A and L50B, two novel bacteriocins from

Enterococcus faecium L50, are related to stapylococcal hemolysins. *J. Bacteriol.* **1998**, *180*, 1988-1994.

(50) Brede, D. A.; Faye, T.; Johnsborg, O.; Ødegård, I.; Nes, I. F.; Holo, H. Molecular and genetic characterization of propionicin F, a bacteriocin from *Propionibacterium freudenreichii*. *Appl. Environ. Microbiol.* **2004**, *70*, 7303-7310.

(51) Joerger, M. C.; Klaenhammer, T. R. Characterization and purification of helveticin J and evidence for a chromosomally determined bacteriocin produced by *Lactobacillus helveticus* 481. *J. Bacteriol.* **1986**, *167*, 439-446.

(52) Martin-Visscher, L. A.; van Belkum, M. J.; Garneau-Tsodikova, S.; Whittal, R. M.; Zheng, J.; McMullen, L. M.; Vederas, J. C. Isolation and characterization of carnocyclin A, a novel circular bacteriocin produced by *Carnobacterium maltaromaticum* UAL307. *Appl. Environ. Microbiol.* **2008**, *74*, 4756-4763.

(53) Rodríguez, J. M.; Martínez, M.; Kok, J. Pediocin PA-1, a wide-spectrum bacteriocin from lactic acid bacteria. *Crit. Rev. Food Sci. Nutr.* **2002**, *42*, 91-121.

(54) Delves-Broughton, J.; Blackburn, P.; Evans, R. J.; Hugenholtz, J. Application of the bacteriocin nisin. *Antonie Leeuwenhoek* **1996**, *69*, 193-202.

(55) Rogers, L. A.; Whittier, E. O. Limiting factors in the lactic fermentation. *J. Bacteriol.* **1928**, *16*, 211-229.

(56) Rogers, L. A. The inhibiting effect of *Streptococcus lactis* on *Lactobacillus bulgaricus*. *J. Bacteriol.* **1928**, *16*, 321-325.

(57) Bierbaum, G.; Sahl, H.-G. Lantibiotics: Mode of action, biosynthesis and bioengineering. *Curr. Pharm. Biotechnol.* **2009**, *10*, 2-18.

(58) Chatterjee, C.; Paul, M.; Xie, L.; van der Donk, W. A. Biosynthesis and mode of action of lantibiotics. *Chem. Rev.* **2005**, *105*, 633-683.

(59) Willey, J. M.; van der Donk, W. A. Lantibiotics: Peptides of diverse structure and function. *Annu. Rev. Microbiol.* **2007**, *61*, 477-501.

(60) Cotter, P. D.; O'Connor, P. M.; Draper, L. A.; Lawton, E. M.; Deegan, L. H.; Hill, C.; Ross, R. P. Posttranslational conversion of L-serines to D-alanines is vital for optimal production and activity of the lantibiotic lacticin 3147. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 18584-18589.

(61) Skaugen, M.; Nissen-Meyer, J.; Jung, G.; Stevanovic, S.; Sletten, K.; Abildgaard, C. I. M.; Nes, I. F. *In vivo* conversion of L-serine to D-alanine in a ribosomally synthesized polypeptide. *J. Biol. Chem.* **1994**, *269*, 27183-27185.

(62) Goto, Y.; Ökesli, A.; van der Donk, W. A. Mechanistic studies of Ser/Thr dehydration catalyzed by a member of the LanL lanthionine synthetase family. *Biochemistry* **2011**, *50*, 891-898.

(63) Goto, Y.; Li, B.; Claesen, J.; Shi, Y.; Bibb, M. J.; van der Donk, W. A. Discovery of unique lanthionine synthetases reveals new mechanistic and evolutionary insights. *PLoS Biol.* **2010**, *8*, e1000339.

(64) You, Y. O.; Levengood, M. R.; Ihnken, L. A. F.; Knowlton, A. K.; van der Donk, W. A. Lacticin 481 synthetase as a general serine/threonine kinase. *ACS Chem. Biol.* **2009**, *4*, 379-385.

(65) You, Y. O.; van der Donk, W. A. Mechanistic investigation of the dehydration reaction of lacticin 481 synthetase using site-directed mutagenesis. *Biochemistry* **2007**, *46*, 5991-6000.

(66) Chatterjee, C.; Miller, L. M.; Leung, Y. L.; Xie, L.; Yi, M.; Kelleher, N. L.; van der Donk, W. A. Lacticin 481 synthetase phosphorylates its substrate during lantibiotic production. *J. Am. Chem. Soc.* **2005**, *127*, 15332-15333.

(67) Li, B.; van der Donk, W. A. Identification of essential catalytic residues of the cyclase NisC involved in the biosynthesis of nisin. *J. Biol. Chem.* **2007**, *282*, 21169-21175.

(68) Li, B.; Yu, J. P. J.; Brunzelle, J. S.; Moll, G. N.; van der Donk, W. A.; Nair, S. K. Structure and mechanism of the lantibiotic cyclase involved in nisin biosynthesis. *Science* **2006**, *311*, 1464-1467.

(69) Rozema, D. B.; Poulter, C. D. Yeast protein farnesyltransferase. pKas of peptide substrates bound as zinc thiolates. *Biochemistry* **1999**, *38*, 13138-13146.

(70) Hasper, H. E.; Kramer, N. E.; Smith, J. L.; Hillman, J. D.; Zachariah, C.; Kuipers, O. P.; de Kruijff, B.; Breukink, E. An alternative bactericidal mechanism of action for lantibiotic peptides that target lipid II. *Science* **2006**, *313*, 1636-1637.

(71) Breukink, E.; de Kruijff, B. Lipid II as a target for antibiotics. *Nat. Rev. Drug Discov.* **2006**, *5*, 321-332.

(72) Lubelski, J.; Rink, R.; Khusainov, R.; Moll, G. N.; Kuipers, O. P. Biosynthesis, immunity, regulation, mode of action and engineering of the model lantibiotic nisin. *Cell. Mol. Life Sci.* **2008**, *65*, 455-476.

(73) Moll, G. N.; Roberts, G. C. K.; Konings, R. N. H.; Driessen, A. J. M. Mechanism of lantibiotic-induced pore-formation. *Antonie Leeuwenhoek* **1996**, *69*, 185-191.

(74) Breukink, E.; Wiedemann, I.; van Kraaij, C.; Kuipers, O. P.; Sahl, H.-G.; de Kruijff, B. Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. *Science* **1999**, *286*, 2361-2364.

(75) Hsu, S.-T.; Breukink, E.; Tischenko, E.; Lutters, M. A. G.; de Kruijff, B.; Kaptein, R.; Bonvin, A. M. J. J.; van Nuland, N. A. J. The nisin-lipid II complex reveals a pyrophosphate cage that provides a blueprint for novel antibiotics. *Nat. Struct. Mol. Biol.* **2004**, *11*, 963-967.

(76) Wiedemann, I.; Böttiger, T.; Bonelli, R. R.; Wiese, A.; Hagge, S. O.; Gutsmann, T.; Seydel, U.; Deegan, L.; Hill, C.; Ross, R. P.; Sahl, H.-G. The mode of action of the lantibiotic lacticin 3147- A complex mechanism involving specific interaction of two peptides and the cell wall precursor lipid II. *Mol. Microbiol.* **2006**, *61*, 285-296.

(77) Cotter, P. D.; Hill, C.; Ross, R. P. Bacterial lantibiotics : Strategies to improve therapeutic potential. *Curr. Protein Peptide Sci.* **2005**, *6*, 61-75.

(78) Deegan, L. H.; Cotter, P. D.; Hill, C.; Ross, P. Bacteriocins: Biological tools for bio-preservation and shelf-life extension. *Int. Dairy J.* **2006**, *16*, 1058-1071.

(79) Gálves, A.; Abriouel, H.; López, R. L.; Omar, N. B. Bacteriocin-based strategies for food biopreservation. *Int. J. Food Microbiol.* **2007**, *120*, 51-70.

(80) Parada, J. L.; Caron, C. R.; Medeiros, A. B. P.; Soccol, C. R. Bacteriocins from lactic acid bacteria: Purification, properties and use as biopreservatives. *Braz. Arch. Biol. Technol.* **2007**, *50*, 521-542.

(81) Burton, J. P.; Chilcott, C. N.; Moore, C. J.; Speiser, G.; Tagg, J. R. A preliminary study of the effect of probiotic *Streptococcus salivarius* K12 on oral malodour parameters. *J. Appl. Microbiol.* **2006**, *100*, 754-764.

(82) Hillman, J. D.; Mo, J.; McDonell, E.; Cvitkovitch, D.; Hillman, C. H. Modification of an effector strain for replacement therapy of dental caries to enable clinical safety trials. *J. Appl. Microbiol.* **2007**, *102*, 1209-1219.

(83) Burton, J. P.; Wescombe, P. A.; Moore, C. J.; Chilcott, C. N.; Tagg, J. R. Safety assessment of the oral cavity probiotic *Streptococcus salivarius* K12. *Appl. Environ. Microbiol.* **2006**, *72*, 3050-3053.

(84) Jung, G. Lantibiotics - Ribosomally synthesized biologically active polypeptides containing sulfide bridges and α,β-didehydroamino acids. *Angew. Chem. Int. Ed.* **1991**, *30*, 1051-1068.

(85) Fernández, L.; Delgado, S.; Herrero, H.; Maldonado, A.; Rodríguez, J. M. The bacteriocin nisin, an effective agent for the treatment of staphylococcal mastitis during lactation. *J. Hum. Lact.* **2008**, *24*, 311-316.

(86) Silkin, L.; Hamza, S.; Kaufman, S.; Cobb, S. L.; Vederas, J. C. Spermicidal bacteriocins: Lacticin 3147 and subtilosin A. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 3103-3106.

(87) Kruszewska, D.; Sahl, H.-G.; Bierbaum, G.; Pag, U.; Hynes, S. O.; Ljungh, Å. Mersacidin eradicates methicillin-resistant *Staphylococcus aureus* (MRSA) in a mouse rhinitic model. *J. Antimicrob. Chemother.* **2004**, *54*, 648-653.

(88) Rea, M. C.; Clayton, E.; O'Connor, P. M.; Shanahan, F.; Kiely, B.; Ross, R. P.; Hill, C. Antimicrobial activity of lacticin 3147 against clinical *Clostridium difficile* strains. *J. Med. Microbiol.* **2007**, *56*, 940-946.

(89) van Heel, A. J.; Montalban-Lopez, M.; Kuipers, O. P. Evaluating the feasibility of lantibiotics as an alternative therapy against bacterial infections in humans. *Expert Opin. Drug Metab. Toxicol.* **2011**, *7*, 675-680.

(90) Liu, W.; Hansen, J. N. Some chemical and physical properties of nisin, a small-protein antibiotic produced by *Lactococcus lactis*. *Appl. Environ. Microbiol.* **1990**, *56*, 2551-2558.

(91) Wilson-Stanford, S.; Kalli, A.; Håkansson, K.; Kastrantas, J.; Orugunty, R. S.; Smith, L. Oxidation of lanthionines renders the lantibiotic nisin inactive. *Appl. Environ. Microbiol.* **2009**, *75*, 1381-1387.

(92) McClerren, A. L.; Cooper, L. E.; Quan, C.; Thomas, P. M.; Kelleher, N. L.; van der Donk, W. A. Discovery and *in vitro* biosynthesis of haloduracin, a two-component lantibiotic. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 17243-17248.

(93) Cooper, L. E.; McClerren, A. L.; Chary, A.; van der Donk, W. A. Structure-activity relationship studies of the two-component lantibiotic haloduracin. *Chem. Biol.* **2008**, *15*, 1035-1045.

(94) Oman, T. J.; van der Donk, W. A. Insights into the mode of action of the two-component lantibiotic haloduracin. *ACS Chem. Biol.* **2009**, *4*, 865-874.

(95) Ross, A. C.; Vederas, J. C. Fundamental functionality: recent developments in understanding the structure-activity relationships of lantibiotic peptides. *J. Antibiot.* **2011**, *64*, 27-34.

(96) Asaduzzaman, S. M.; Sonomoto, K. Lantibiotics: Diverse activities and unique modes of action. *J. Biosci. Bioeng.* **2009**, *107*, 475-487.

(97) Cotter, P. D.; Deegan, L. H.; Lawton, E. M.; Draper, L. A.; O'Connor, P. M.; Hill, C.; Ross, R. P.; Complete alanine scanning of the two-component lantibiotic lacticin 3147: generating a blueprint for rational drug design. *Mol. Microbiol.* **2006**, *62*, 735-747.

(98) Levengood, M. R.; Knerr, P. J.; Oman, T. J.; van der Donk, W. A. *In vitro* mutasynthesis of lantibiotic analogues containing nonproteinogenic amino acids. *J. Am. Chem. Soc.* **2009**, *131*, 12024-12025.

(99) Pattabiraman, V. R.; McKinnie, S. M. K.; Vederas, J. C. Solid-supported synthesis and biological evaluation of the lantibiotic peptide bis(desmethyl) lacticin 3147 A2. *Angew. Chem. Int. Ed.* **2008**, *47*, 9472-9475.

(100) Pattabiraman, V. R.; Stymiest, J. L.; Derksen, D. J.; Martin, N. I.; Vederas, J. C. Multiple on-resin olefin metathesis to form ring-expanded analogues of the lantibiotic peptide, lacticin 3147 A2. *Org. Lett.* **2007**, *9*, 699-702.

(101) Liu, H.; Pattabiraman, V. R.; Vederas, J. C. Synthesis and biological activity of oxa-lacticin A2, a lantibiotic analogue with sulfur replaced by oxygen. *Org. Lett.* **2009**, *11*, 5574-5577.

(102) Kent, S. B. H. Total chemical synthesis of proteins. *Chem. Soc. Rev.* **2009**, *38*, 338-351.

(103) Fukase, K.; Kitazawa, M.; Sano, A.; Shimbo, K.; Fujita, H.; Horimoto, S.; Wakamiya, T.; Shiba, T. Total synthesis of peptide antibiotic nisin. *Tetrahedron Lett.* **1988**, *29*, 795-798.

(104) Fukase, K.; Kitazawa, M.; Sano, A.; Shimbo, K.; Horimoto, S.; Fujita, H.; Kubo, A.; Wakamiya, T.; Shiba, T. Synthetic study on peptide antibiotic nisin. 5. Total synthesis of nisin. *Bull. Chem. Soc. Jpn.* **1992**, *65*, 2227-2240.

(105) Wakamiya, T.; Shimbo, K.; Sano, A.; Fukase, K.; Shiba, T. Synthetic study on peptide antibiotic nisin. I. The synthesis of ring A. *Bull. Chem. Soc. Jpn.* **1983**, *56*, 2044-2049.

(106) Fukase, K.; Wakamiya, T.; Shiba, T. Synthetic study on peptide antibiotic nisin. II. The synthesis of ring B. *Bull. Chem. Soc. Jpn.* **1986**, *59*, 2505-2508.

(107) Fukase, K.; Kitazawa, M.; Wakamiya, T.; Shiba, T. Synthetic study on peptide antibiotic nisin. III. Synthesis of ring C. *Bull. Chem. Soc. Jpn.* **1990**, *63*, 1838-1840.

(108) Fukase, K.; Oda, Y.; Kubo, A.; Wakamiya, T.; Shiba, T. Synthetic study on peptide antibiotic nisin. IV. Synthesis of ring D/E. *Bull. Chem. Soc. Jpn.* **1990**, *63*, 1758-1763.

(109) Harpp, D. N.; Gleason, J. G. Preparation and mass spectral properties of cystine and lanthionine derivatives. A novel synthesis of L-lanthionine by selective desulfurization. *J. Org. Chem.* **1971**, *36*, 73-80.

(110) Miller, M. J. Isourea-mediated preparation of dehydro amino acids. *J. Org. Chem.* **1980**, *45*, 3131-3132.

(111) Kamber, B.; Hartmann, A.; Eisler, K.; Riniker, B.; Rink, H.; Sieber, P.; Rittel, W. The synthesis of cystine peptides by iodine oxidation of *S*-trityl-cysteine and *S*-acetamidomethyl-cysteine peptides. *Helv. Chim. Acta* **1980**, *63*, 899-915.

(112) Merrifield, R. B. Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. *J. Am. Chem. Soc.* **1963**, *85*, 2149-2154.

(113) Muttenthaler, M.; Alewood, P. F. Selenopeptide chemistry. *J. Pept. Sci.* **2008**, *14*, 1223-1239.

(114) Amblard, M.; Fehrentz, J.-A.; Martinez, J.; Subra, G. Methods and protocols of modern solid phase peptide synthesis. *Mol. Biotechnol.* **2006**, *33*, 239-254.

(115) Ghalit, N.; Kemmink, J.; Hilbers, H. W.; Versluis, C.; Rijkers, D. T. S.; Liskamp, R. M. J. Step-wise and pre-organization induced synthesis of a crossed alkene-bridged nisin Z DE-ring mimic by ring-closing metathesis. *Org. Biomol. Chem.* **2007**, *5*, 924-934.

(116) Ghalit, N.; Poot, A. J.; Fürstner, A.; Rijkers, D. T. S.; Liskamp, R. M. J. Ring-closing alkyne metathesis approach toward the synthesis of alkyne

mimics of thioether A-, B-, C-, and DE-ring systems of the lantibiotic nisin Z. *Org. Lett.* **2005**, *7*, 2961-2964.

(117) Ghalit, N.; Reichwein, J. F.; Hilbers, H. W.; Breukink, E.; Rijkers, D. T. S.; Liskamp, R. M. J. Synthesis of bicyclic alkene-/alkane-bridged nisin mimics by ring-closing metathesis and their biochemical evaluation as lipid II binders: toward the design of potential novel antibiotics. *ChemBioChem* **2007**, *8*, 1540-1554.

(118) Ghalit, N.; Rijkers, D. T. S.; Kemmink, J.; Versluis, C.; Liskamp, R. M. J. Pre-organization induced synthesis of a crossed alkene-bridged nisin Z DE-ring mimic by ring-closing metathesis. *Chem. Commun.* **2005**, 192-194.

(119) Ghalit, N.; Rijkers, D. T. S.; Liskamp, R. M. J. Alkene- and alkyne-bridged mimics of nisin as potential peptide-based antibiotics. *J. Mol. Catal. A: Chem.* **2006**, *254*, 68-77.

(120) Bregant, S.; Tabor, A. B. Orthogonally protected lanthionines: Synthesis and use for the solid-phase synthesis of an analogue of nisin ring C. *J. Org. Chem.* **2005**, *70*, 2430-2438.

(121) Mothia, B.; Appleyard, A. N.; Wadman, S.; Tabor, A. B. Synthesis of peptides containing overlapping lanthionine bridges on the solid phase: An analogue of rings D and E of the lantibiotic nisin. *Org. Lett.* **2011**, *13*, 4216-4219.

(122) Mustapa, M. F. M.; Harris, R.; Esposito, D.; Chubb, N. A. L.; Mould, J.; Schultz, D.; Driscoll, P. C.; Tabor, A. B. Synthesis of a cyclic peptide containing norlanthionine: Effect of the thioether bridge on peptide conformation. *J. Org. Chem.* **2003**, *68*, 8193-8198.

(123) Cobb, S. L.; Vederas, J. C. A concise stereoselective synthesis of orthogonally protected lanthionine and β-methyllanthionine. *Org. Biomol. Chem.* **2007**, *5*, 1031-1038.

(124) Martin, N. I. Concise preparation of tetra-orthogonally protected (2*S*,6*R*)-lanthionines. *J. Org. Chem.* **2009**, *74*, 946-949.

(125) Matteucci, M.; Bhalay, G.; Bradley, M. Cystine mimetics-solid phase lanthionine synthesis. *Tetrahedron Lett.* **2004**, *45*, 1399-1401.

(126) Mustapa, M. F. M.; Harris, R.; Bulic-Subanovic, N.; Elliot, S. L.; Bregant, S.; Groussier, M. F. A.; Mould, J.; Schultz, D.; Chubb, N. A. L.; Gaffney, P. R. J.; Driscoll, P. C.; Tabor, A. B. Synthesis of orthogonally protected lanthionines. *J. Org. Chem.* **2003**, *68*, 8185-8192.

(127) Narayan, R. S.; VanNieuwenhze, M. S. Versatile and stereoselective syntheses of orthogonally protected β-methylcysteine and β-methyllanthionine. *Org. Lett.* **2005**, *7*, 2655-2658.

(128) Paul, M.; van der Donk, W. A. Chemical and enzymatic synthesis of lanthionines. *Mini-Rev. Org. Chem.* **2005**, *2*, 23-37.

(129) Zhu, X.; Schmidt, R. R. Efficient synthesis of differently protected lanthionines via β-bromoalanine derivatives. *Eur. J. Org. Chem.* **2003**, 4069-4072.

(130) Nakajima, K.; Oda, H.; Okawa, K. Studies on 2-aziridinecarboxylic acid. IX. Convenient synthesis of optically active *S*-alkylcysteine, *threo-S*-alkyl-*β*-methylcysteine, and lanthionine derivatives via the ring-opening reaction of aziridine by several thiols. *Bull. Chem. Soc. Jpn.* **1983**, *56*, 520-522.

(131) Liu, H.; Pattabiraman, V. R.; Vederas, J. C. Stereoselective syntheses of 4-oxadiaminopimelic acid and its protected derivatives via aziridine ring opening. *Org. Lett.* **2007**, *9*, 4211-4214.

(132) Corey, E. J.; Gin, D. Y.; Kania, R., S. Enantioselective total synthesis of ecteinascidin 743. *J. Am. Chem. Soc.* **1996**, *118*, 9202-9203.

(133) Ohta, S.; Okamoto, M. Synthesis of carbonyl compounds via biogenetic-type transamination reaction. *Synthesis* **1982**, *1982*, 756-758.

(134) Liu, W.; Chan, A. S. H.; Liu, H.; Cochrane, S. A.; Vederas, J. C. Solid supported chemical syntheses of both components of the lantibiotic lacticin 3147. *J. Am. Chem. Soc.* **2011**, *133*, 14216-14219.

(135) Ross, A. C.; Liu, H.; Pattabiraman, V. R.; Vederas, J. C. Synthesis of the lantibiotic lactocin S using peptide cyclizations on solid phase. *J. Am. Chem. Soc.* **2010**, *132*, 462-463.

(136) Trnka, T. M.; Grubbs, R. H. The development of $L_2X_2Ru{=}CHR$ olefin metathesis catalysts: An organometallic success story. *Acc. Chem. Res.* **2001**, *34*, 18-29.

(137) Mørtvedt, C. I.; Nes, I. F. Plasmid-associated bacteriocin production by a *Lactobacillus sake* strain. *J. Gen. Microbiol.* **1990**, *136*, 1601-1607.

(138) Cintas, L. M.; Casaus, P.; Fernandez, M. F.; Hernandez, P. E. Comparative antimicrobial activity of enterocin L50, pediocin PA-1, nisin A and lactocin S against spoilage and foodborne pathogenic bacteria. *Food Microbiol.* **1998**, *15*, 289-298.

(139) Mørtvedt, C. I.; Nissen-Meyer, J.; Sletten, K.; Nes, I. F. Purification and amino acid sequence of lactocin S, a bacteriocin produced by *Lactobacillus sake* L45. *Appl. Environ. Microbiol.* **1991**, *57*, 1829-1834.

(140) Mørtvedt-Abildgaard, C. I.; NIssen-Meyer, J.; Jelle, B.; Grenov, B.; Skaugen, M.; Nes, I. F. Production and pH-dependent bactericidal activity of lactocin S, a lantibiotic from *Lactobacillus sake* L45. *Appl. Environ. Microbiol.* **1995**, *61*, 175-179.

(141) Rodríguez, J. M.; Sobrino, O. J.; Moreira, W. L.; Cintas, L. M.; Casaus, P.; Fernández, M. F.; Sanz, B.; Hernández, P. E. Inhibition of *Yersinia enterocolitica* by *Lactobacillus sake* strains of meat origin. *Meat Sci.* **1994**, *37*, 305-313.

(142) Rodríguez, J. M.; Sobrino, O. J.; Moreira, W. L.; Fernández, M. F.; Cintas, L. M.; Casaus, P.; Sanz, B.; Hernández, P. E. Inhibition of *Listeria monocytogenes* by *Lactobacillus sake* strains of meat origin. *Meat Sci.* **1994**, *38*, 17-26.

(143) Sobrino, O. J.; Rodríguez, J. M.; Moreira, W. L.; Cintas, L. M.; Fernández, M. F.; Sanz, B.; Hernández, P. E. Sakacin M, a bacteriocin-like substance from *Lactobacillus sake* 148. *Int. J. Food Microbiol.* **1992**, *16*, 215-225.

(144) Sobrino, O. J.; Rodríguez, J. M.; Moreira, W. L.; Fernández, M. F.; Sanz, B.; Hernández, P. E. Antibacterial activity of *Lactobacillus sake* isolated from dry fermented sausages. *Int. J. Food Microbiol.* **1991**, *13*, 1-10.

(145) Meyer, H. E.; Heber, M.; Eisermann, B.; Korte, H.; Metzger, J.; Jung, G. Sequence analysis of lantibiotics: Chemical derivatization procedures allow a fast access to complete Edman degradation. *Anal. Biochem.* **1994**, *223*, 185-190.

(146) Skaugen, M.; Abildgaard, C. I. M.; Nes, I. F. Organization and expression of a gene cluster involved in the biosynthesis of the lantibiotic lactocin S. *Mol. Gen. Genet.* **1997**, *253*, 674-686.

(147) Skaugen, M.; Nes, I. F. Transposition in *Lactobacillus sakei*: inactivation of a second lactocin S operon by the insertion of IS*1520*, a new member of the IS*3* family of insertion sequences. *Microbiology* **2000**, *146*, 1163-1169.

(148) Rawlinson, E. L. A.; Nes, I. F.; Skaugen, M. LasX, a transcriptional regulator of the lactocin S biosynthetic genes in *Lactobacillus sakei* L45, acts both as an activator and a repressor. *Biochimie* **2002**, *84*, 559-567.

(149) Skaugen, M.; Andersen, E. L.; Christie, V. H.; Nes, I. F. Identification, characterization, and expression of a second, bicistronic, operon involved in the production of lactocin S in *Lactobacillus sakei* L45. *Appl. Environ. Microbiol.* **2002**, *68*, 720-727.

(150) Küsters, E.; Allgaier, H.; Jung, G.; Bayer, E. Resolution of sulphur-containing amino acids by chiral phase gas chromatography. *Chromatographia* **1984**, *18*, 287-293.

(151) Allgaier, H.; Jung, G.; Werner, R. G.; Schneider, U.; Zähner, H. Epidermin: sequencing of a heterodet tetracyclic 21- peptide amide antibiotic. *Eur. J. Biochem.* **1986**, *160*, 9-22.

(152) Kellner, R.; Jung, G.; Hörner, T.; Zähner, H.; Schnell, N.; Entian, K. D.; Götz, F. Gallidermin: a new lanthionine-containing polypeptide antibiotic. *Eur. J. Biochem.* **1988**, *177*, 53-59.

(153) Kellner, R.; Jung, G.; Josten, M.; Kaletta, C.; Entian, K. D.; Sahl, H. G. Pep5 - structure elucidation of a large lantibiotic. *Angew. Chem. Int. Ed.* **1989**, *28*, 616-619.

(154) Pattabiraman, V. R. PhD Thesis: Chemical synthesis of lantibiotics and their analogues. University of Alberta, 2007.

(155) Gross, E.; Morell, J. L. The structure of nisin. *J. Am. Chem. Soc.* **1971**, *93*, 4634-4635.

(156) Liu, H.; Boudreau, M. A.; Zheng, J.; Whittal, R. M.; Austin, P.; Roskelley, C. D.; Roberge, M.; Anderson, R. J.; Vederas, J. C. Chemical synthesis and biological activity of the neopetrosiamides and their analogues: Revision of disulfide bond connectivity. *J. Am. Chem. Soc.* **2010**, *132*, 1486-1487.

(157) Cox, R. J.; Sutherland, A.; Vederas, J. C. Bacterial diaminopimelate metabolism as a target for antibiotic design. *Biorg. Med. Chem.* **2000**, *8*, 843-871.

(158) Born, T. L.; Blanchard, J. S. Structure/function studies on enzymes in the diaminopimelate pathway of bacterial cell wall biosynthesis. *Curr. Opin. Chem. Biol.* **1999**, *3*, 607-613.

(159) Cox, R. J. The DAP pathway to lysine as a target for antimicrobial agents. *Nat. Prod. Rep.* **1996**, *13*, 29-43.

(160) Arakawa, Y.; Goto, T.; Kawase, K.; Yoshifuji, S. Stereospecific synthesis of cyclic hydrazoacetic acids and *meso*-diaminodicarboxylic acids. *Chem. Pharm. Bull.* **1998**, *46*, 674-680.

(161) Garner, P.; Park, J. M. 1,1,-Dimethylethyl (*S*)- or (*R*)-4-formyl-2,2-dimethyl-3-oxazolidinecaboxylate: a useful serinal derivative. *Org. Synth.* **1992**, *70*, 18-24.

(162) Jurgens, A. R. Asymmetric synthesis of differentially protected meso-2,6-diaminopimelic acid. *Tetrahedron Lett.* **1992**, *33*, 4727-4730.

(163) Spantulescu, M. D.; Jain, R. P.; Derksen, D. J.; Vederas, J. C. Photolysis of diacyl peroxides: A radical-based approach for the synthesis of functionalized amino acids. *Org. Lett.* **2003**, *5*, 2963-2965.

(164) Hiebl, J.; Kollmann, H.; Rovenszky, F.; Winkler, K. One step synthesis of orthogonally protected diaminodicarboxylic acids by mixed kolbe electrolysis. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 2963-2966.

(165) Twomey, D.; Ross, R. P.; Ryan, M.; Meaney, B.; Hill, C. Lantibiotics produced by lactic acid bacteria: structure, function and applications. *Antonie Leeuwenhoek* **2002**, *82*, 165-185.

(166) Derksen, D. J.; Boudreau, M. A.; Vederas, J. C. Hydrophobic interactions as substitutes for a conserved disulfide linkage in the type IIa bacteriocins, leucocin A and pediocin PA-1. *ChemBioChem* **2008**, *9*, 1898-1901.

(167) Chen, J.; Fu, X.-G.; Zhou, L.; Zhang, J.-T.; Qi, X.-L.; Cao, X.-P. A convergent route for the total synthesis of malyngamides O, P, Q, and R. *J. Org. Chem.* **2009**, *74*, 4149-4157.

(168) Wipf, P.; Miller, C. P. A new synthesis of highly functionalized oxazoles. *J. Org. Chem.* **1993**, *58*, 3604-3606.

(169) Waldmann, H.; Kunz, H. Allylester als selektiv abspaltbare carboxyschutzgruppen in der peptid- und *N*-glycopeptidsynthese. *Liebigs Ann. Chem.* **1983**, 1712-1725.

(170) Thajudeen, H.; Park, K.; Moon, S.-S.; Hong, I. S. An efficient green synthesis of proline-based cyclic dipeptides under water-mediated catalyst-free conditions. *Tetrahedron Lett.* **2009**, *51*, 1303-1305.

(171) Dussault, P.; Sahli, A. 2-Methoxyprop-2-yl hydroperoxide: A convenient reagent for the synthesis of hydroperoxides and peracids. *J. Org. Chem.* **1992**, *57*, 1009-1012.

(172) O'Brien, D. P.; Entress, R. M. H.; Cooper, M. A.; O'Brien, S. W.; Hopkinson, A.; Williams, D. H. High affinity surface binding of a strongly dimerizing vancomycin-group antibiotic to a model of resistant bacteria. *J. Am. Chem. Soc.* **1999**, *121*, 5259-5265.

Appendix A: Isolation and Structure Determination of D-DAB$_3$ Polymyxin B$_1$ and B$_2$

A.1 Introduction to Polymyxin Peptides

A.1.1 Structure of polymyxin B

Antimicrobial peptides called lantibiotics have been the major focus of this thesis, but it is another group of antimicrobial peptides called the polymyxins that will be described in this Appendix. The polymyxins are small cationic peptides produced by Gram-positive bacteria (e.g. *Bacillus polymyxa*) that are broadly active against Gram-negative bacteria (e.g. *Pseudomonas aeruginosa*).[1] Most commonly, the molecules in this family have ten amino acids (including the unusual amino acid 2,4-diaminobutyric acid ((DAB), Figure A-1) and form a peptide bond between the *C*-terminal carboxylate and the side chain of residue 4. Additionally, the *N*-terminus is acylated by a fatty acid.[2-9] Polymyxin B$_1$ (**A-1**) and B$_2$ (**A-2**) are produced by the same organism and differ only in the identity of the *N*-terminal lipid (Figure A-1).

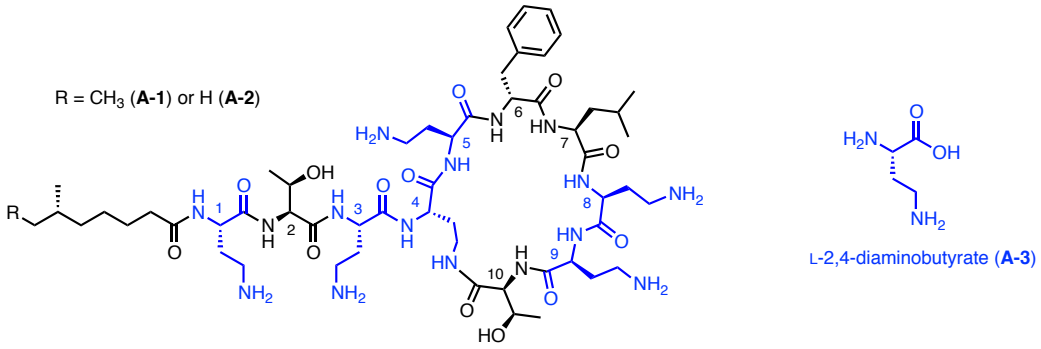


Figure A-1 Structure of polymyxin B_1 (**A-1**), B_2 (**A-2**), the uncommon amino acid 2,4-diaminobutyrate (**A-3**)

A.1.2 Applications

Polymyxin B (**A-1** and **A-2**) and polymyxin E (also called colistin) have both been used clinically for the treatment of bacterial infections.[9] The well-known antibacterial ointment POLYSPORIN®, contains polymyxin B (**A-1** and **A-2**) in addition to a second antibiotic peptide called bacitracin (**A-4**) (see Figure A-2).

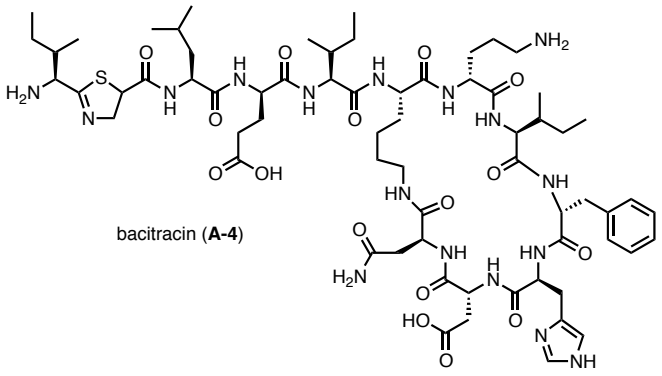


Figure A-2 Structure of bacitracin (**A-4**)

Despite being useful in topical applications, the polymyxins have seen limited use in the treatment of systemic infection due to considerable nephro- and neurotoxicity.[9,10] Multidrug resistant bacteria have necessitated a re-assessment of the polymyxin peptides. Recent clinical trials have found somewhat improved toxicity profiles for these compounds.[10] Falagas *et. al.* believe this may be a result of administration of lower and/or more accurately calculated dosages to patients.[11]

A.1.3 Biosynthesis

Unlike the lantibiotics (or more generally the bacteriocins), polymyxins are produced in nature by non-ribosomal peptide synthases (NRPS). Therefore, these peptides are not gene encoded and synthesized as a linear prepeptide by the ribosome, followed by enzyme catalyzed post-translational modification. Rather, a suite of dedicated enzymes converts the constituent building blocks (amino acids) into the peptide in a manner similar to polyketide and fatty acid biosynthesis.[12] Generally, a single suite of enzymes is dedicated to producing one final product. Each biosynthetic module is responsible for the introduction and modification of a single amino acid in the final peptide. Despite extensive research into the peptides of the polymyxin family, little was known about their biosynthesis until recently when the gene cluster for polymyxin A was published.[13] The authors found the modules responsible for the incorporation of the amino acids of the peptide were encoded on three genes and surprisingly the

order of the modules was not co-linear with the sequence of the amino acids in polymyxin A as is frequently seen in NRPS systems.[13]

A.1.4 New polymyxin B variant

Our collaborator, Prof. Susan Jensen (Biological Sciences, University of Alberta) recently identified a polymyxin biosynthetic gene cluster whilst characterizing another gene cluster responsible for the biosynthesis of the antifungal metabolite fusaricidin in *Paenibacillus polymyxa* PKB1.[14] Figure A-3 shows the biosynthetic gene cluster for the proposed polymyxin.

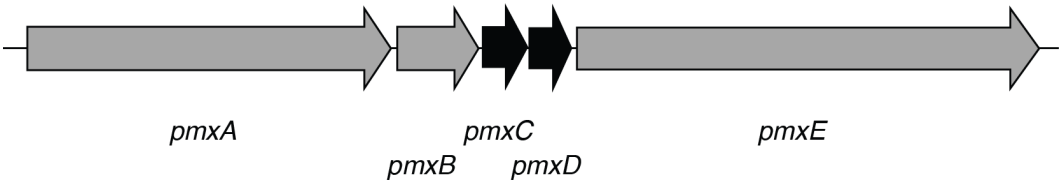


Figure A-3 Polymyxin B variant gene cluster. Grey genes are part of an NRPS system, black genes are membrane permease portions of ABC transporters. Adapted from Shaheen *et. al.*[14]

Analysis of the genetic sequence of the gene clusters, with NRPS enzyme prediction software, suggested the module designations shown in Figure A-4. *pmxE* contains the synthetic machinery for residues 1-5 of a polymyxin, *pmxA*

encodes for the next 4 amino acid modules and *pmxB* contains the final amino acid module.

To confirm that this cluster was involved in producing a polymyxin, the gene *pmxE* in *P. polymyxa* PKB1 was disrupted by deleting approximately 2.0 kb from the 5' end of the gene, and replacing it with an antibiotic resistance cassette (Apra^{R}Cm^R*oriT* cassette). Mutagenesis was accomplished using a PCR-targeted gene disruption protocol originally developed for mutagenesis of the fusaricidin gene cluster in the same species.[15] The resultant *pmxE* mutant was then grown, and activity testing of the supernatant showed complete abolition of the antibacterial activity observed for the wildtype strain. This result, in combination with the bioinformatic analysis of the cluster, suggested that this is the gene cluster responsible for producing a polymyxin-like product. The predicted amino acid sequence is the same as polymyxin B (**A-1**, **A-2**), however, an additional epimerization domain in the biosynthetic module for the DAB (**A-3**) at residue 3 suggests that DAB-3 may be present with D-stereochemistry. Following isolation of the active components from bacterial cell culture, analysis by LC/MS found the two peptides had masses consistent with polymyxin B (**A-1**, **A-2**) but different retention times from B$_1$ (**A-1**) or B$_2$ (**A-2**).

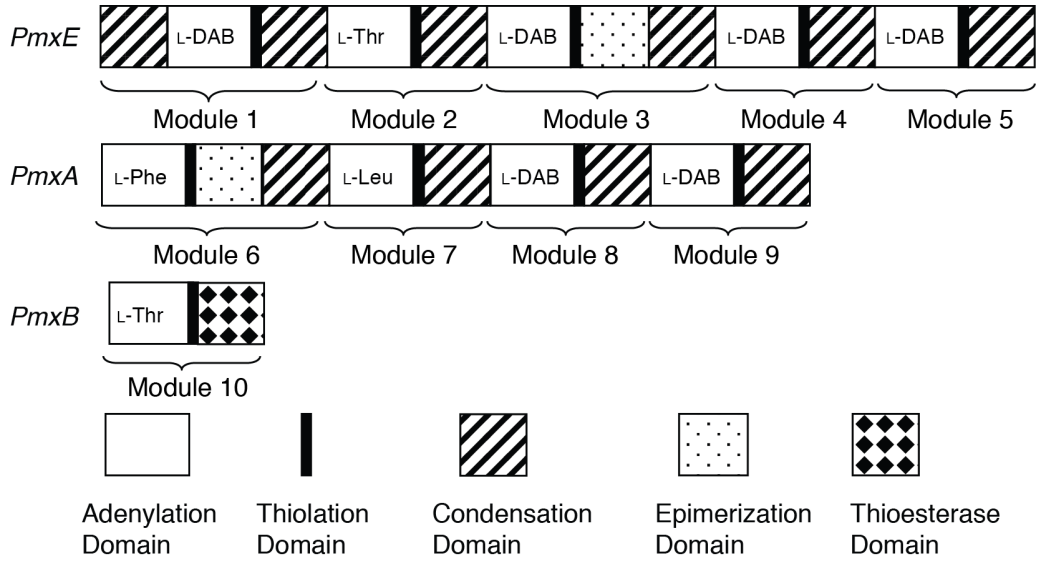


Figure A-4 The biosynthetic modules for each amino acid in D-DAB$_3$ polymyxin B (**A-5** and **A-6**) are spread across three genes. Modules 3 and 6 contain epimerization domains that may convert the amino acids to their D-isoforms. Figure adapted from Shaheen *et. al.*[14]

Based upon the differing physical properties of the newly identified polymyxins, our collaborators proposed that they had isolated a unique variant of polymyxin B (**A-1**, **A-2**) in which the third residue was D-DAB. The new peptides were called D-DAB$_3$ polymyxin B$_1$ (**A-5**) and B$_2$ (**A-6**).

A.1.5 Project objectives

The objective of this project was to confirm the structure and stereochemistry of D-DAB$_3$ Polymyxin B$_1$ (**A-5**) and B$_2$ (**A-6**). Several types of

mass spectral analysis could be utilized to determine the overall mass, sequence and amino acid stereochemistries.

A.2 Results and Discussion

A.2.1 Determination of sequence for D-DAB$_3$ polymyxin B

To obtain an accurate molecular formula for **A-5** and **A-6**, each peptide was analyzed by high-resolution mass spectrometry. An elemental composition of $C_{56}H_{99}N_{16}O_{13}$ is obtained for D-DAB$_3$-polymyxin B$_1$ (**A-5**) and an elemental composition of $C_{55}H_{97}N_{16}O_{13}$ is obtained for D-DAB$_3$-polymyxin B$_2$ (**A-6**). These molecular formulas are the same as reported for polymyxin B1 (**A-1**) and B2 (**A-2**).

To confirm the proposed sequence of **A-5** and **A-6,** MS/MS sequencing was done. MS/MS sequencing of D-DAB polymyxin B$_1$ (**A-5**) and B$_2$ (**A-6**) is made more complex by the cyclic nature of the peptides, as the *C*-terminus of each peptide is connected to the amino side chain of residue 4 by an amide bond. This means that the formation of smaller ions is often the product of two fragmentations; one to break open the ring and a second to shorten the resulting linear peptide. When the peptide breaks apart in the spectrometer and the ring is opened, it is either the bond between the *C*-terminus and the side chain of residue 4, or the backbone amide bond between residues 4 and 5 that is broken (Figure A-5). When the side chain to *C*-terminal bond breaks, the resulting linear peptide has the sequence, residues 1-10. However, if the backbone bond between residues 4 and 5 breaks, the resulting linear peptide has the sequence 1, 2, 3, 4, 10, 9, 8, 7,

6, 5. Using both sets of linear fragments along with larger fragments containing an intact ring the sequence of **A-5** and **A-6** is confirmed.

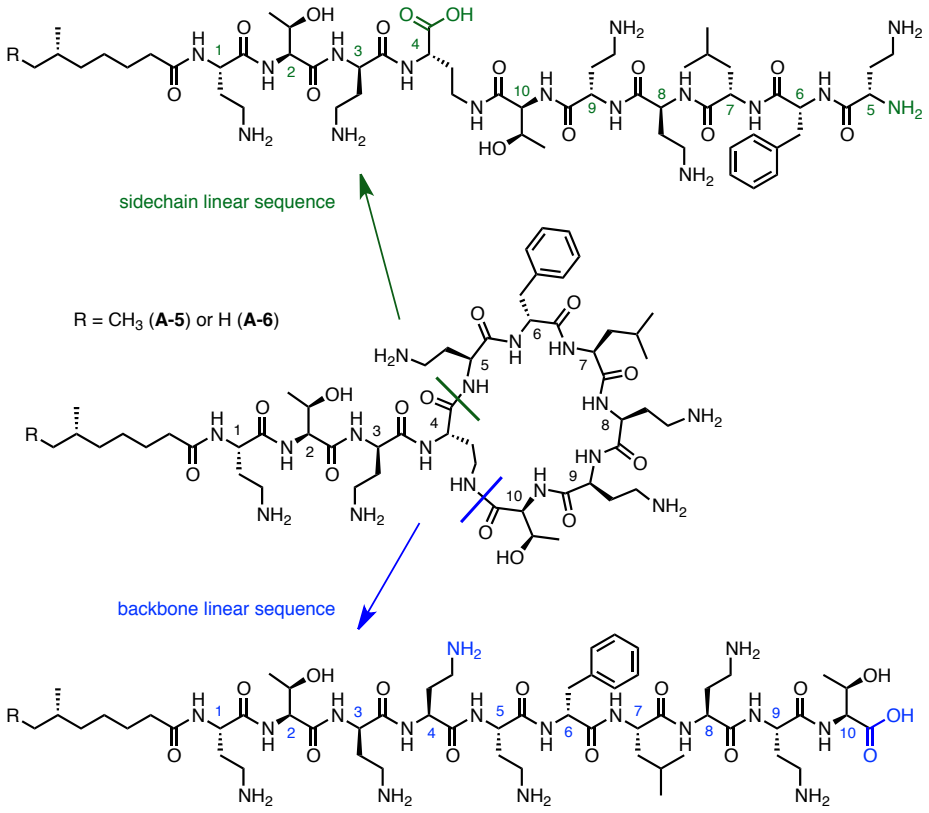
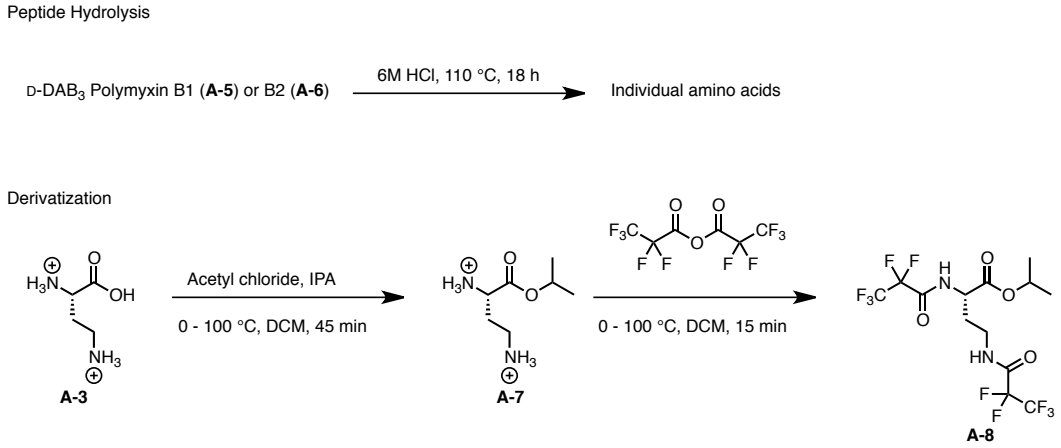


Figure A-5 Structure of D-DAB$_3$ polymyxin B$_1$ (**A-5**) and B$_2$ (**A-6**) with two full length linear fragments. Blue line indicates the bond that breaks when the backbone linear sequence of the peptide is obtained in MS/MS. Green line indicates the bond that breaks when the sidechain linear sequence of the peptide is obtained in MS/MS.

Both high resolution MS and MS/MS sequencing confirm that **A-5** and **A-6** contain the same amino acids, in the same order, as polymyxin B (**A-1** and **A-2**). To determine the stereochemistry of the amino acids, chiral GC/MS was utilized.

A.2.2 Stereochemical analysis of D-DAB$_3$ polymyxin B

In the biosynthetic modules identified by our collaborators there was an epimerization domain present for the DAB residue at position 3. This could mean that the DAB residue is present in the molecule as D-DAB rather than the more common L-DAB. To determine if this is the case, chiral gas chromatography mass spectrometry (GC/MS) was used to identify the stereochemical configuration of the residues within D-DAB Polymyxin B1 (**A-5**) and B2 (**A-6**).[16] To do this, each peptide was hydrolyzed under acidic conditions to yield individual amino acids. To improve the volatility of the amino acids within the GC, they were derivatized to pentafluoropropanamide isopropyl esters (Scheme A-1). Briefly, each peptide was refluxed in 6M HCl for 18 h and then treated with acetyl chloride in neat isopropyl alcohol to generate isopropyl esters of the amino acids (**A-7**). Reaction of the amino acid esters with pentafluoropropanoic anhydride gave the fully derivatized amino acids (**A-8**). No purification was undertaken during any of these synthetic steps, the solvent/reagents were simply removed *in vacuo* between each reaction.



Scheme A-1 Hydrolysis of D-DAB$_3$ polymyxin B$_1$ (**A-5**) and B$_2$ (**A-6**) and an example of the derivatization procedure with one standard (L-DAB **A-3**).

In addition to derivatizing the hydrolysates of each peptide, L and D amino acid standards for each proposed amino acid were also converted to their pentafluoropropanamide isopropyl esters for use in the GC/MS analysis. The derivatized hydrolysates were injected individually onto a 25 m long Chirasil-L-Val column within a GC, coupled to an MS detector. **A-5** (Figure A-6) and **A-6** (Figure A-7-A) give the same trace, showing the presence of five different amino acids. When the two samples are mixed together, the same trace is observed, confirming that the amino acid composition of the two peptides is the same.

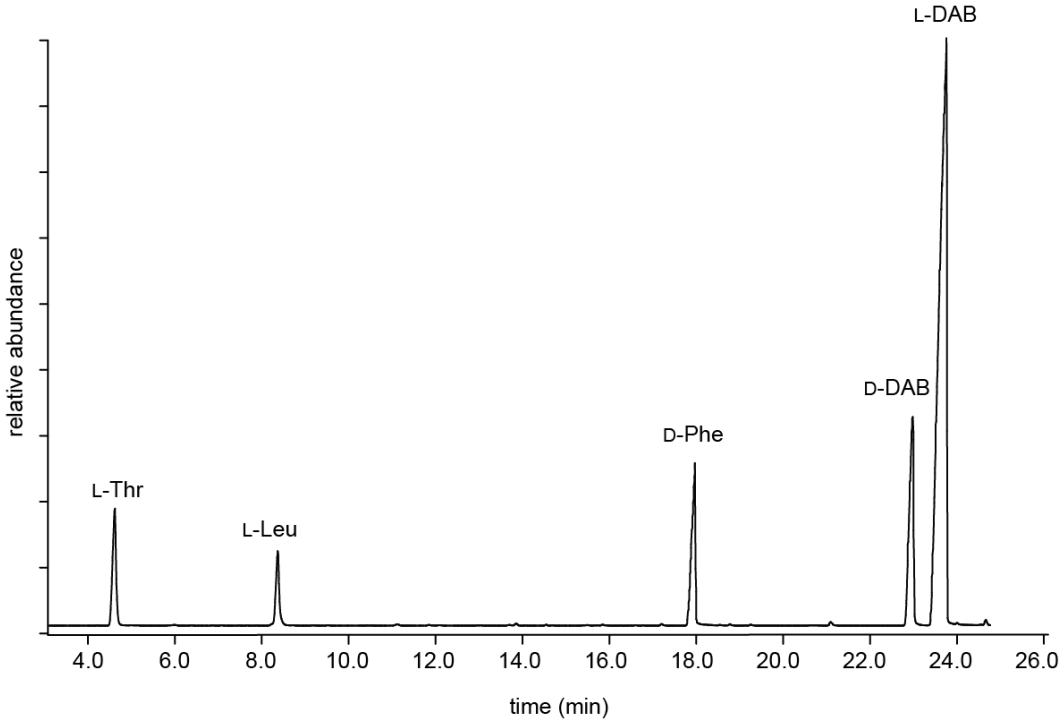


Figure A-6 Chiral GC/MS trace showing the amino acid composition of D-DAB$_3$ polymyxin B1 (**A-5**), determined using co-injection with standards (see **Figure A-7**)

Independently adding different standards to each hydrolysate sample and running the chiral GC/MS analysis, confirmed the identity of each amino acid. Figure A-7 shows the supplementation experiments. There are six DAB residues in these peptides. The chiral GC/MS analysis identifies that there are both D-DAB and L-DAB present and integration of the peak areas for the D-DAB and L-DAB residues found a relative ratio of 1:5 for the D:L amino acids. This evidence strongly supports the conclusion that D-DAB$_3$ polymyxin B$_1$ (**A-5**) and B$_2$ (**A-6**)

contain a D-DAB residue (likely at position 3) and therefore that these peptides are novel variants of polymyxin B_1 (**A-1**) and B_2 (**A-2**).

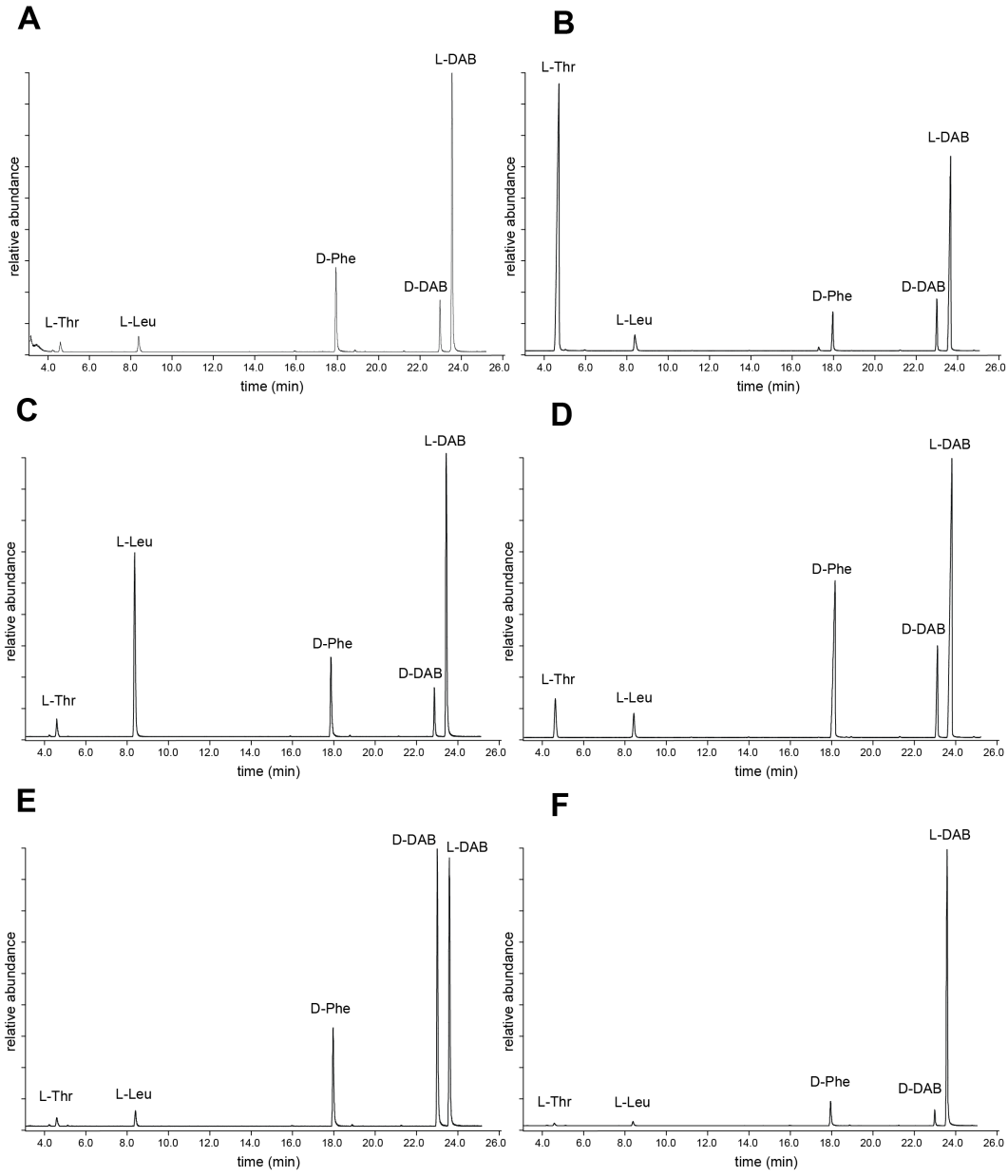


Figure A-7 GC/MS traces for the constituent amino acids of D-DAB$_3$ polymyxin B2 (**A-6**) after acid hydrolysis and derivatization as pentafluoropropanamide isopropyl esters and **A-5**/**A-6** hydrolysates spiked with each standard. **A**. **A-6**

alone **B**. spiked with L-Thr **C**. spiked with L-Leu **D**. spiked with D-Phe **E**. spiked with D-DAB **F**. spiked with L-DAB

A.2.3 Conclusion

Using a combination of high resolution mass spectrometry and MS/MS sequencing, the mass and sequence of two novel polymyxin compounds referred to as D-DAB$_3$ polymyxin B$_1$ (**A-5**) and B$_2$ (**A-6**) was confirmed. Following acid hydrolysis and derivatization as pentafluoropropanamide isopropyl esters, the stereochemistry of the constituent amino acids of each peptide were confirmed via analysis by chiral-GC/MS. Taken together with the results obtained by our collaborators, it is possible to state that a new variant of polymyxin B has been identified and its peptide structure is that of D-DAB$_3$ polymyxin B$_1$ (**A-5**) and B$_2$ (**A-6**). One facet of the structure of these peptides that remains tentative is the exact structure of the *N*-terminal lipids. So far based on overall mass, it has been assumed that the structures are analogous to **A-1** and **A-2**, however, future investigations of these peptides could be done to confirm this assumption.

A.3 Experimental Procedures

The same general procedures as documented in Chapter 5 apply.

A.3.1 High resolution mass spectrometry for A-5 and A-6

After HPLC purification, the high resolution matrix-assisted laser desorption ionization-fourier transform ion cyclotron resonance-mass spectrometry (MALDI-FTICR-MS) spectrum of the peptides (**A-5** and **A-6**) was obtained using a Bruker 9.4T Apex-Qe FTICR (Bruker Daltonics, Billerica, MA, USA). For D-DAB$_3$-polymyxin B$_1$ (**A-5**) an elemental composition of $C_{56}H_{99}N_{16}O_{13}$ for the $(M+H)^+$ ion was obtained with a mass accuracy of 0.63 ppm. For D-DAB$_3$-polymyxin B$_2$ (**A-6**) an elemental composition of $C_{55}H_{97}N_{16}O_{13}$ for the $(M+H)^+$ ion was obtained with a mass accuracy of 0.81 ppm.

A.3.2 MS/MS sequencing of A-5 and A-6

HPLC purified and concentrated peptide solutions were directly spotted onto a Bruker Daltonics MTP AnchorChip^{TM} 800/384 target and air dried. α-Cyano-4-hydroxy cinnamic acid matrix solution (0.42 µL, CHCA) was spotted on top and air dried. The matrix solution was prepared as follows. A 36 µL aliquot of a saturated matrix solution prepared in 0.1% TFA in 90:10 ACN:H_2O was diluted to a final volume of 800 µL using 0.1% TFA in 85:15 ACN:H_2O, containing 1mM ammonium phosphate. Mass spectra were obtained in the positive reflectron mode of ionization using a Bruker Daltonics (Bremen, Germany) ultrafleXtreme MALDI TOF/TOF mass spectrometer. The MS/MS spectra were obtained manually with CID (collision-induced dissociation) gas turned off. DataAnalysis,

BioTools and Sequence Editor software packages provided by the manufacturer were used for analysis of the mass spectra.

A.3.3 Hydrolysis and derivatization of polymyxin samples

Each peptide sample (1 mg) was heated to 110 °C in a sealed tube in 6M HCl (3 mL) for 18 h. The solution was allowed to cool to room temperature and the solvent was removed *in vacuo*. Acetyl chloride (1.5 mL) was added drop-wise to isopropyl alcohol (5 mL) at 0 °C. This solution was added to the peptide residue and the mixture was heated to 110 °C in a sealed tube for 45 min. The solution was allowed to cool and the solvent was removed *in vacuo*. A solution of pentafluoropropionic anhydride (1 mL) in dichloromethane (3 mL) was added to the peptide residue and the mixture was heated to 110 °C in a sealed tube for 15 min. The mixture was allowed to cool to room temperature and then the solvent was evaporated under a stream of argon. The resulting residue was dissolved in dichloromethane (1 mL) and transferred into a vial for storage.

Derivatization of standard amino acids was carried out in a similar manner, each amino acid sample (4 mg) was heated to 110 °C in a sealed tube in 0.2 M HCl (3 mL) for 5 min. The solution was allowed to cool to room temperature and the solvent was removed *in vacuo*. Acetyl chloride (1.5 mL) was added drop-wise to isopropyl alcohol (5 mL) at 0 °C. This solution was added to the amino acid residue and the mixture was heated to 110 °C in a sealed tube for 45 min. The solution was allowed to cool and the solvent was removed *in vacuo*.

A solution of pentafluoropropionic anhydride (1 mL) in dichloromethane (3 mL) was added to the amino acid residue and the mixture was heated to 110 °C in a sealed tube for 15 min. The mixture was allowed to cool to room temperature and then the solvent was evaporated under a stream of argon. The resulting residue was dissolved in dichloromethane (1 mL) and transferred into a vial for storage.

A.3.4 Chiral GC/MS analysis of polymyxin samples

The hydrolyzed and derivatized samples of **A-5** and **A-6** were analyzed using chiral GC/MS. All samples were analyzed using a Varian 25 m × 0.22 mm fused silica wall coated open tubular Chirasil-L-Val (0.12 μm) column on an Agilent Technologies 7890 GC with 5975C MSD and 7683B series injector. Each sample was introduced into the machine dissolved in dichloromethane via a pulsed splitless injection. The temperature method used was 90 °C (5 min) raised to 180 °C by 3 °C/min.

Each peptide was analyzed independently and then the two peptides were mixed together and analyzed. It was found that **A-5** and **A-6** contained the same amino acids. The identity of each amino acid in the peptide was confirmed by sequential addition of derivatized standard amino acids. This confirmed that the amino acids present were indeed L-threonine, L-leucine, D-phenylalanine, L-diaminobutyric acid and D-diaminobutyric acid. The integration supports that the ratio of L:D diaminobutyric acid is 5:1 respectively.

A.4 References

(1) Storm, D. R.; Rosenthal, K. S.; Swanson, P. E. Polymyxin and related peptide antibiotics. *Annu. Rev. Biochem.* **1977**, *46*, 723-763.

(2) Vaara, M. Polymyxins and their novel derivatives. *Curr. Opin. Microbiol.* **2010**, *13*, 574-581.

(3) Velkov, T.; Thompson, P. E.; Nation, R. L.; Li, J. Structure-activity relationships of polymyxin antibiotics. *J. Med. Chem.* **2010**, *53*, 1898-1916.

(4) Govaerts, C.; Orwa, J.; van Schepdael, A.; Roets, E.; Hoogmartens, J. Characterization of polypeptide antibiotics of the polymyxin series by liquid chromatography electrospray ionization ion trap tandem mass spectrometry. *J. Pept. Sci.* **2002**, *7*, 45-55.

(5) Govaerts, C.; Orwa, J.; van Schepdael, A.; Roets, E.; Hoogmartens, J. Liquid chromatography-ion trap tandem mass spectrometry for the characterization of polypeptide antibiotics of the colistin series in commercial samples. *J. Chromatogr. A* **2002**, *976*, 65-78.

(6) Martin, N. I.; Hu, H.; Moake, M. M.; Churey, J. J.; Whittal, R. M.; Worobo, R. W.; Vederas, J. C. Isolation, structural characterization, and properties of Mattacin (polymyxin M), a cyclic peptide antibiotic produced by *Paenibacillus kobensis* M. *J. Biol. Chem.* **2003**, *278*, 13124-13132.

(7) Orwa, J. A.; Govaerts, C.; Busson, R.; Roets, E.; van Schepdael, A.; Hoogmartens, J. Isolation and structural characterization of polymyxin B components. *J. Chromatogr. A* **2001**, *912*, 369-373.

(8) Vogler, K.; Studer, R. O. The chemistry of the polymyxin antibiotics. *Experientia* **1966**, *22*, 345-416.

(9) Evans, M. E.; Feola, D. J.; Rapp, R. P. Polymyxin B sulfate and colistin: Old antibiotics for emerging multiresistant Gram-negative bacteria. *Ann. Pharmacother.* **1999**, *33*, 960-967.

(10) Falagas, M. E.; Kasiakou, S. K. Toxicity of polymyxins: a systematic review of the evidence from old and recent studies. *Crit. Care* **2006**, *10*, R27.

(11) Li, J.; Nation, R. L.; Turnidge, J. D.; Milne, R. W.; Coulthard, K.; Rayner, C. R.; Paterson, D. L. Colistin: the re-emerging antibiotic for multidrug-resistant Gram-negative bacterial infections. *Lancet Infect. Dis.* **2006**, *6*, 589-601.

(12) von Döhren, H.; Keller, U.; Vater, J.; Zocher, R. Multifunctional peptide synthetases. *Chem. Rev.* **1997**, *97*, 2675-2705.

(13) Choi, S.-K.; Park, S.-Y.; Kim, R.; Kim, S.-B.; Lee, C.-H.; Kim, J. F.; Park, S.-H. Identification of a polymyxin synthetase gene cluster of *Paenibacillus polymyxa* and heterologous expression of the gene in *Bacillus subtilis*. *J. Bacteriol.* **2009**, *191*, 3350-3358.

(14) Shaheen, M.; Li, J.; Ross, A. C.; Vederas, J. C.; Jensen, S. E. *Paenibacillus polymyxa* PKBI produces novel variants of polymyxin B-type antibiotics. *Chem. Biol.*, *Accepted*.

(15) Li, J.; Beatty, P. K.; Shah, S.; Jensen, S. E. Use of PCR-targeted mutagenesis to disrupt production of fusaricidin-type antifungal antibiotics in *Paenibacillus polymyxa*. *Appl. Environ. Microbiol.* **2007**, *73*, 3480-3489.

(16) Küsters, E.; Allgaier, H.; Jung, G.; Bayer, E. Resolution of sulphur-containing amino acids by chiral phase gas chromatography. *Chromatographia* **1984**, *18*, 287-293.