University of Alberta

Chemical Synthesis of Lantibiotics and their Analogues for Structure Elucidation and Structure-Activity Relationship (SAR) Studies

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

Edmonton, Alberta

Spring 2008

Your file Votre référence
ISBN: 978-0-494-45577-7
Our file Notre référence
ISBN: 978-0-494-45577-7

ABSTRACT

Lantibiotic peptides, such as lacticin 3147 A1 (**11**) and A2 (**12**) represent a promising class of antimicrobial peptides for application in food preservation, veterinary medicine and human therapeutics. The chemical syntheses of two analogues of lacticin 3147 A2 and the attempted total synthesis of lactocin S are first described. In an effort to overcome the oxidative instability of lantibiotics, a carbocyclic-larger ring analogue of lacticin 3147 A2 (**43**) in which carbon atoms replace the oxidatively unstable sulfur atoms in the lanthionine rings has been successfully synthesized by a combination of solid-phase and solution phase peptide synthesis. During the course of the synthesis of the carbocyclic analogue (**43**), a highly practical method for the large scale synthesis of the post-translationally modified residues (1→5) of lacticin A2 (**45**) was also developed. Unfortunately, the carbocyclic analogue did not display any significant antimicrobial activity when tested against the indicator organism *Lactococcus lactis* HP. In the second project, to study the effect of replacement of the β-methyllanthionine rings with lanthionine rings, a lanthionine analogue of lacticin A2 (**44**) has been synthesized on-resin. The key orthogonally protected lanthionine building block (**167**) was synthesized in solution phase and this fragment along with the previously synthesized residues (1→5) of lacticin A2 were used to assemble the lanthionine analogue of lacticin A2 (**44**) on solid-support. A preliminary biological evaluation against *Lactococcus lactis* HP indicates that the lanthionine analogue (**44**) displays a potent synergistic antimicrobial activity, when tested in combination with lacticin A1. A serial dilution assay showed that the lanthionine analogue is about 100 times less effective than natural lacticin A2 in exhibiting its synergistic activity. A lead compound, residues (6→29) of lanthionine analogue of

lacticin A2 with a Fmoc group (**173**), for potentially simplifying lacticin A2 structure has also been identified. In the third project, attempts towards the total synthesis of lactocin S (**48**) are described. The two lanthionine rings A and B of lactocin S have been successfully synthesized on solid-support using the preformed orthogonally protected lanthionine building block (**167**). However, attempts to introduce residues (3→22) of lactocin S, from the bicyclic lanthionine rings, on solid-support did not result in any of the desired product.

LtnJ has been identified as the enzyme responsible for the reduction of dehydroalanines to D-alanines in lacticin 3147 A1 and A2. In order to prepare a substrate for the *in vitro* reconstitution of LtnJ activity, a 38 amino acid precursor peptide containing a single cysteine (**206**) has been designed and successfully synthesized by solid-phase peptide synthesis. The transfer of the cysteine residue in the precursor peptide to a dehydroalanine by converting the thiol group of the cysteine to a thiocyanate followed by elimination under basic conditions provides the substrate peptide (**49**) along with secondary cleavage products.

In an effort to make lantibiotics active against Gram-negative bacteria, the hypothesized recognition sequence, residues (75→88) from Gram-negative bacteria active colicin V (**50**), as well as its analogues with an azide (**222**) and an alkyne linker (**225**) have been successfully synthesized on solid support. Efforts towards conjugation of the synthesized peptide to lantibiotics via cycloaddition chemistry are underway.

Dedicated to my mother and all my other family members for their constant encouragement and support

ACKNOWLEDGEMENTS

I would like to acknowledge my supervisor Professor John C. Vederas for his excellent guidance, encouragement and support. His intellectual quest, enthusiasm for science, his highest standards for research and teaching, his mentorship and ability to instill independent thinking merit utmost respect. The opportunity to work in such a rich scientific environment has been greatly instrumental in shaping my scientific career and has certainly prepared me for the journey ahead.

I would like to thank Dr. Matthew Clay and Dr. Vijayalakshmi Moorthie for their valuable insights and careful proofreading of this manuscript. I would also like to acknowledge all the Vederas group members, past and present, for making the lab an enjoyable place to work. I will cherish the numerous scientific and non-scientific discussions and the happy time spent in the lab for a long time to come. Acknowledgements are due to the dedicated support staff in analytical and spectral services for their technical assistance. I would also like to acknowledge my early mentors Professor Ezhil Christudoss, Professor Murugesan Velayuthum and Dr. Manojit Pal for nurturing my scientific interest. To all my friends, both in and out of the Chemistry Department, thank you for making my stay here memorable.

Finally, I would like to thank my family, for their encouragement, love and understanding. You are all responsible for instilling in me the qualities of perseverance, independence, hard work and kindness. Your constant motivation and support have

helped me a great deal in realizing my goals and aspirations. Without you all, I would not have been where I am in my life and none of this would have been possible.

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$[\alpha]_D^{25}$	specific rotation
A or Ala	alanine
Abu	aminobutyrine
Ac	acetyl
Ac_2O	acetic anhydride
AcOH	acetic acid
ADP	adenosine 5'-diphosphate
AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
AllGly	allylglycine
Aloc	allyloxycarbonyl
app	apparent
Ar	aryl
atm	atmosphere
ATP	adenosine 5'-triphosphate
Bn	benzyl
BnBr	benzyl bromide
Boc	*tert*-butoxycarbonyl
$(Boc)_2O$	di-*tert*-butyl dicarbonate
^{t}Bu	*tertiary* butyl
^{t}BuOH	*tertiary* butanol
br	broad

Bz	benzoyl
c	concentration
C or Cys	cysteine
calcd	calculated
Cbz	benzyloxycarbonyl
CM	cross metathesis
COSY	correlation spectroscopy
δ	chemical shift in parts per million downfield from tetramethylsilane
d	doublet
D or Asp	aspartic acid
Da	Daltons
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCC	1,3-dicyclohexylcarbodiimide
DCM	dichloromethane
DCE	dichloroethane
DEAD	diethyl azodicarboxylate
Dha	dehydroalanine
Dhb	dehydrobutyrine
DIPCDI	diisopropylcarbodiimide
DIPEA	*N*,*N*-diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMF	*N*,*N*-dimethylformamide
DMSO	dimethylsulfoxide

DNA	deoxyribonucleic acid
DTT	dithiothreitol
E or Glu	glutamic acid
EDCI	1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide
EDTA	ethylene diamine tetracetic acid
EI	electron impact
equiv.	equivalents
ES	electrospray
Et	ethyl
EtOCOCl	ethyl chloroformate
Et_3N	triethylamine
Et_2O	diethyl ether
EtOAc	ethyl acetate
EtOH	ethanol
F or Phe	phenylalanine
Fmoc	9*H*-fluorenylmethoxycarbonyl
FTICR-MS	Fourier-transform ion cyclotron resonance mass spectrometry
g	gram
G or Gly	glycine
gCOSY	gradient correlation spectroscopy
gHMBC	gradient heteronuclear multiple bond correlation spectroscopy
gHMQC	gradient heteronuclear multiple quantum correlation spectroscopy
GlcNAc	*N*-acetylglucosamine

h	hour
H or His	histidine
HBTU	2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HEW	Horner-Emmons-Wadsworth reaction
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
I or Ile	isoleucine
IBX	*o*-iodoxybenzoic acid
IPTG	isopropyl-β-D-thiogalactopyranoside
IR	infrared
J	coupling constant
K or Lys	lysine
kDa	kilodalton(s)
L or Leu	leucine
Lan	lanthionine
LC	liquid chromatography
LiOH	lithium hydroxide
lit.	literature reference
m	multiplet
M or Met	methionine
MALDI-TOF	matrix-assisted laser desorption ionization / time of flight

MBP	maltose-binding protein
Me	methyl
MeCN/ACN	acetonitrile
MeOH	methanol
MeLan	β-methyllanthionine
Mes	mesityl
MHz	megahertz
MIC	minimum inhibitory concentration
min	minute(s)
mL	milliliter
Mmt	4-methoxytriphenyl
mol	mole
mp	melting point
MS	mass spectrometry
MsCl	methanesulfonyl chloride
MW	molecular weight
μM	micromolar
N or Asn	asparagines
NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)
nm	nanometers
nM	nanomolar

NMM	*N*-methylmorpholine
NMP	*N*-methylpyrrolidinone
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
ORTEP	Oak Ridge (National Lab) Thermal Ellipsoid Program (Molecular Modeling)
P or Pro	proline
PBu_3	tributylphosphine
$P(Cy)_3$	tricyclohexylphosphine
$Pd(PPh_3)_4$	tetrakis(triphenylphosphine)palladium
Pent	pentet
PG	protecting group
Ph	phenyl
$PhSiH_3$	phenylsilane
Pmc	2,2,5,7,8-pentamethylchroman-6-ylsulfonyl
PP	protein phosphatase
PPh_3	triphenylphosphine
ppm	parts per million
^{i}Pr	*iso*-propyl
psi	pounds per square inch
PTM	post-translational modification
py	pyridine

PyAOP	7-azabenzotriazol-1-yloxy-tris-(pyrrolidino)phosphonium hexafluorophosphate
PyBOP	benzotriazole-1-yl-oxy-*tris*-pyrrolidino-phosphonium hexafluorophosphate
q	quartet
Q or Gln	glutamine
R or Arg	arginine
RCM	ring closing metathesis
R_f	retention factor
RNA	ribonucleic acid
RP	reverse phase
rt	room temperature
s	singlet
SAR	structure-activity relationship
S or Ser	serine
SPPS	solid phase peptide synthesis
t	triplet
T or Thr	threonine
TBA-Br	tetra-butyl ammonium bromide
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TIPS	triisopropylsilane
TLC	thin layer chromatography

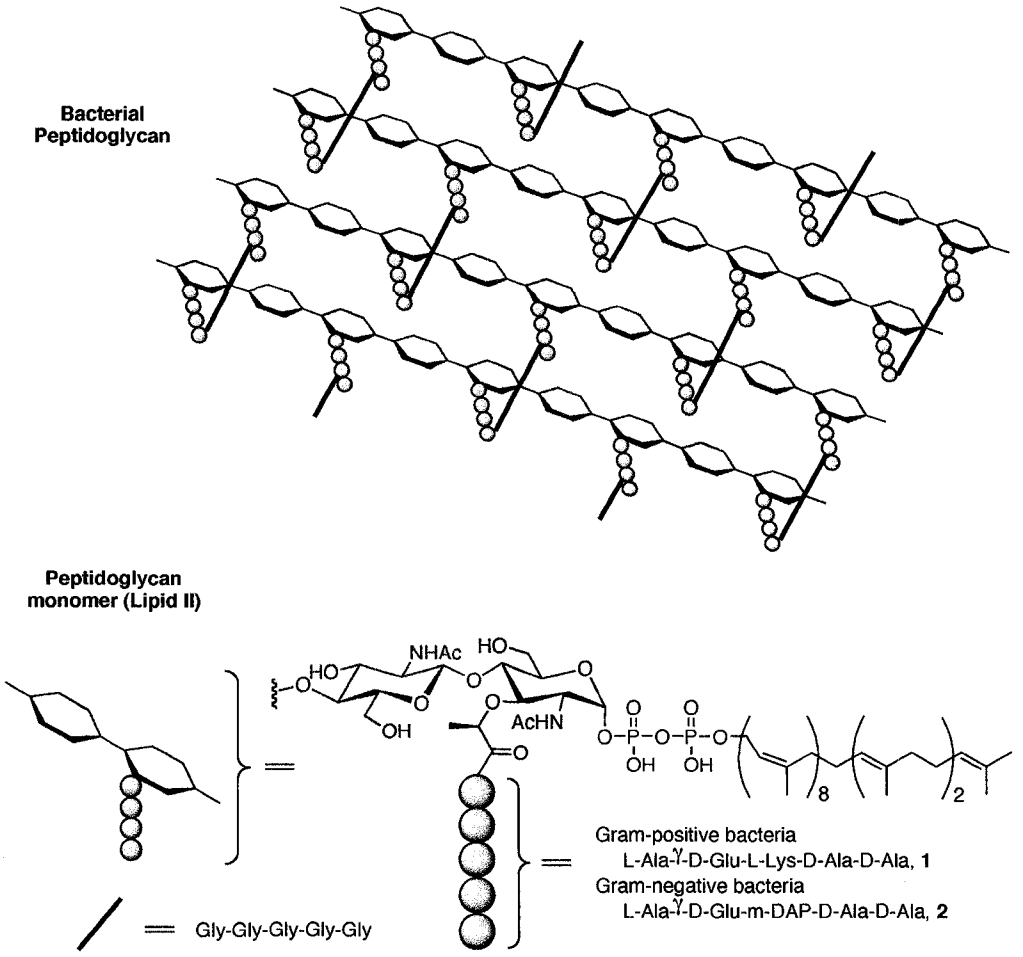
t_R	retention time
p-TSOH	*p*-toluene sulfonic acid
Trt	triphenylmethyl
UV	ultraviolet
V or Val	valine
W or Trp	tryptophan
Y or Tyr	tyrosine

CHAPTER 1. INTRODUCTION

1.1. Bacterial cell wall biosynthesis, conventional antibiotics and resistance

Almost all prokaryotes have a cell wall surrounding the cytoplasmic membrane that maintains the characteristic shape of the cell, offers mechanical protection and prevents the cell from bursting under osmotic pressure.[1, 2] The bacterial cell wall differs from many other organisms by the presence of a distinctive material called peptidoglycan. The precursor of peptidoglycan known as lipid II (Figure 1), are comprised of *N*-acetylglucosamine (NAG)-*N*-acetylmuramic (NAM) disaccharide with a pentapeptide chain attached to the D-lactyl moiety of each NAM. The monomers then undergo inter-strand cross-linking through the pentapeptide moiety to form the large 3-dimensional cell wall layer.[3-6] The thickness and composition of the peptidoglycan layer varies from organism to organism. There are over a 100 different peptidoglycan types, distinguished by the presence of different amino acids in the pentapeptide and the position of cross-linking.[7] In Gram-positive bacteria the cell wall is much thicker, generally comprising of around 20 peptidoglycan layers, in contrast to Gram-negative organisms where it is generally around 1.5 layers. However, Gram-negative bacteria possess an outer membrane enveloping the periplasmic space that acts as an extra layer of protection for the cell.[8]

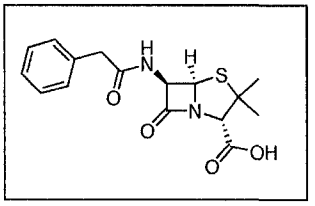
Figure 1. Simplified depiction of bacterial peptidoglycan and its monomer lipid II



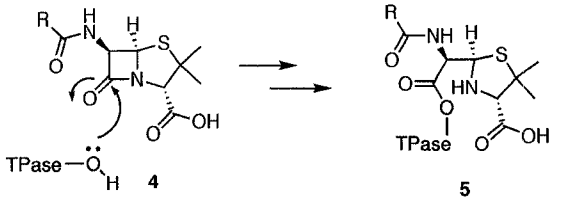
Historically, bacterial cell wall biosynthesis and protein biosynthesis on the ribosome has been the target for a vast number of antibiotics. One reason is the number of enzymatic steps involved in the biosynthetic process that provides ample targets for antibiotics. The other underlying reason is the distinct biochemical pathways in bacteria that do not have a mammalian equivalent and could be selectively targeted. The most successful antibiotic classes to date belong to the β-lactam and glycopeptide families that act on the peptidoglycan biosynthesis.[9] The first clinically useful antibiotic, penicillin, was

discovered by Alexander Fleming in 1929 from the mould of *Penicillium notatum.*[10] The β-lactams such as penicillins, cephalosporins, carbacephems and carbapenems elicit their antibacterial action by binding to one of the key enzymes involved in the transpeptidation step of the peptidoglycan biosynthesis. The strained four membered β-lactam ring in these antibiotics is opened by an active site serine residue in the transpeptidase that leads to a covalent linking of it to the antibiotic and inactivation of the enzyme (Figure 2).[11, 12]

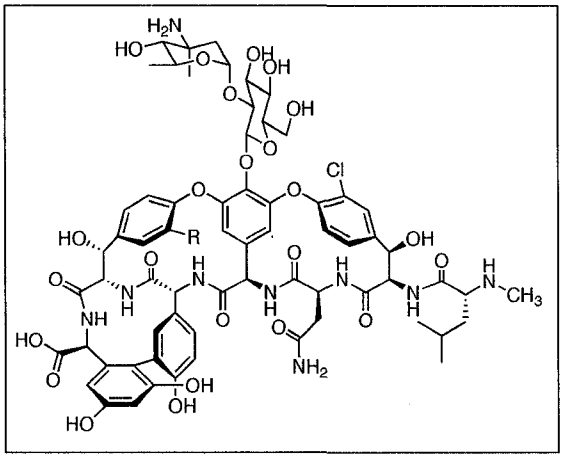
Figure 2. Front line antibiotics penicillin, vancomycin and their mode of action



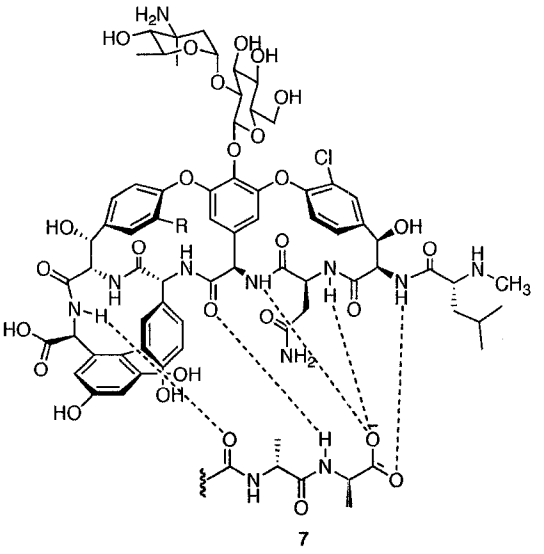
Penicillin G, 3



Inactivation of transpeptidase by penicilin



Vancomycin, 6



Sequestering of peptidoglycan D-Ala-D-Ala by vancomycin

Vancomycin is a glycopeptide antibiotic that is considered to be the last line of defense against clinically problematic Methicillin-resistant *Staphylococcus aureus* (MRSA). Vancomycin kills Gram-positive bacteria by sequestering the un-cross linked D-Ala-D-

Ala dipeptide termini of lipid II by forming five crucial hydrogen bonds, thereby inhibiting transpeptidation. Some other antibiotics that target the peptidoglycan biosynthesis process are shown in Table 1 along with their bacterial enzyme targets and mechanism of action.[9]

Table 1. Antibiotics that target cell wall biosynthesis

Antibiotic	Target	Action
Fosfomycin	MurA enzyme	Covalently traps an active site cys-115
Thiazolidinones	MurB enzyme	Mimics natural diphosphate substrate
Mureidomycins A to F liposidomycins tunicamycin	MraY enzyme	Competes with substrate for lipid I
Mersacidin Ramoplanin Nisin Z	Peptidoglycan	Binds to lipid II
Bacitracin	Peptidoglycan	Complexation of C_{55} lipid phosphate

The large-scale production and the wide spread use of penicillin in the early 1940's led to a monumental decrease in the number of deaths caused by infection. Soon after the discovery of penicillin, a surge of research activity started and by the 1960's most of the major classes of antibiotics used today were discovered. Around this period,

almost all infections were treatable and the then U.S. Surgeon General, William H. Stewart, was reported to have commented: '..... that we had essentially defeated infections and could close the book on them'.[13] The general consensus was that infections were no longer a threat and were of a lower public health priority. However, as early as one year after the introduction of antibiotics like penicillin, resistant bacterial species started emerging. For example, in 1946 first-generation penicillin resistance *Staphylococcus aureus* (*S. aureus*) started to appear. After a decade of research, a new β-lactam antibiotic methicillin was discovered and used to kill *Staphylococcus aureus*. By 1960's, methicillin-resistant *Staphylococcus aureus* (MRSA) became prevalent. After a splurge of research, vancomycin was introduced in 1986 and became the front-line antibiotic to treat MRSA infections. Soon after that vancomycin intermediate *S. aureus* (VRSA) evolved and linezolid was approved in the 1990's as a new alternative.[14]

Today, a plethora of antibiotic resistant bacterial species such as ceftazidime resistant *Escherichia coli* and *Klebsiella pneumoniae*, streptomycin and rifampicin resistant *Salmonella typhimurium*, and penicillin resistant *Streptococcus pneumoniae* are known.[15, 16] About 2 million people acquire bacterial infections in U.S. hospitals annually and 90,000 die as a result.[17, 18] The problem of antibiotic resistance also comes with a huge economic burden. According to the U.S. Institute of Medicine report in 1998, MRSA infections cost an average of $31,400 per case to treat compared to $27,700 per case for non-resistant infections. This translates to a cost of ~US $ 4-5 billion more annually to treat MRSA.[17, 19] Currently, there are no antibiotics known to which resistance of some form has not developed. Of the ~550 antibiotics currently in development, only

six are novel antibiotics.[13] This presents an immediate need for development of new classes of antibiotics to combat emerging resistance worldwide.

1.2. Lantibiotics

It is estimated that almost 99% of bacteria produce at least one bacterial toxin known as bacteriocins.[20, 21] Bacteriocins are ribosomally produced by both Gram-positive and Gram-negative bacteria to play a defensive role and act to prohibit the invasion of other strains. They also serve to enable the invasion of a strain into an established microbial community, and in some Gram-positive bacteria, they mediate cell-cell communication.[20] Some bacteriocins are effective in killing only closely related bacterial species (narrow spectrum) whereas others act on many (broad spectrum).[22] The producer organism is protected from its own bacteriocin by a dedicated immunity protein that ensures survival. Lactic acid bacteria (LAB) are prolific in bacteriocin production[23, 24] and have been used for centuries to ferment foods. Many bacteriocins are used worldwide for preservation of dairy products and meat. A classification of bacteriocins produced by LAB has been proposed by Klaenhammmer[21] (Table 2), in which lantibiotics constitutes a major sub-class.

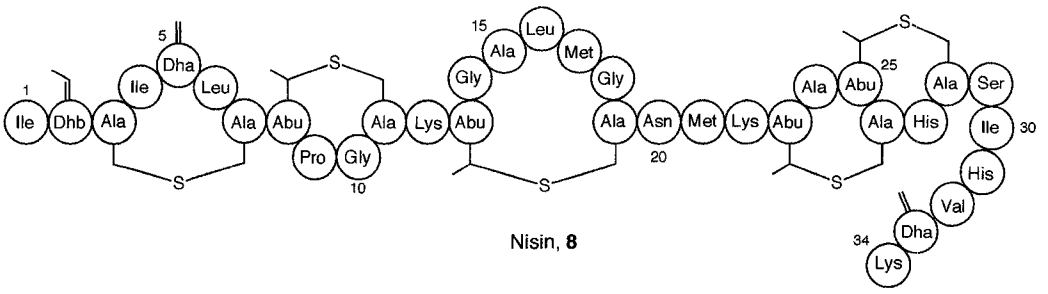
Table 2. Classification of bacteriocins produced by lactic acid bacteria

Class	Characteristics	Subclasses and Examples
I. Lantibiotics	- Ribosomally synthesized - Highly post-translationally modified - Presence of lanthionine and β-methyllanthionine	**Type A** (e.g., nisin A) Contains 21-38 amino acids, elongated, amphiphilic, cationic, act by pore-formation **Type B** (e.g., mersacidin) Size less than 19 amino acids, globular, anionic or neutral, kill by enzymatic inhibition
II. Non lantibiotics	- Ribosomally synthesized - Heat stable - Minimal post translational modification - Presence of double glycine cleavage site for release of the mature peptide from its pre-peptides - Cationic, presence of no lanthionine residues	**Type IIa** (e.g., pediocin PA-1, leucocin, sakacin A) Conserved YGNGVaaC amino terminus sequence, *Listeria* active, act by pore formation **Type IIb** (e.g., lacticin F, lactococcin G, plantaricin EF) Two-peptide bacteriocins, act by pore formation **Type IIc** (e.g., acidocin B, lactococcin 972) One-peptide bacteriocin with no YGNGVaaC conserved sequence, dependent on 'sec' pathway for export[a]
III. Non lantibiotics	- Large (>30 kDa), - Heat labile peptides	e.g., helveticin J
IV.[b]	- Bacteriocins requiring lipid or carbohydrate moieties	e.g., leuconosin S, lactocin 27

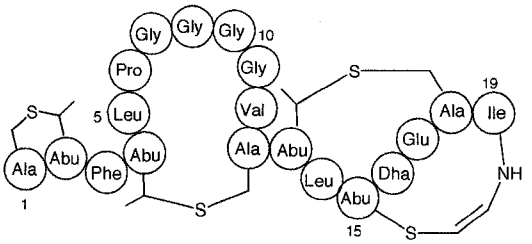
[a] Bacteriocins dependent on export using the translocase general secretory (sec) pathway (GSP)
[b] Currently this class is unnamed

Lantibiotics[25-28] are a sub-class of bacteriocins that are characterized by the presence of lanthionine (Lan) and β-methyllanthionine (MeLan) in their structures. The structures of lantibiotics exhibit extensively modified amino acid residues (Figure 3). It has been noted in some lantibiotics that up to 50% of the residues could be post-translationally modified. This unparalleled structural feature makes lantibiotics stand out among bacteriocins.

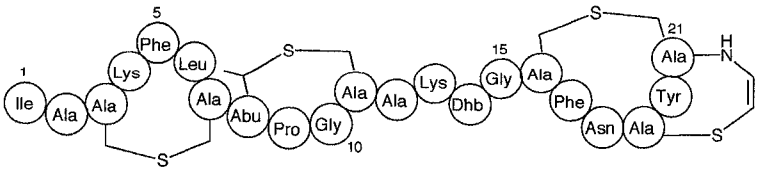
Figure 3. Structures of representative lantibiotics



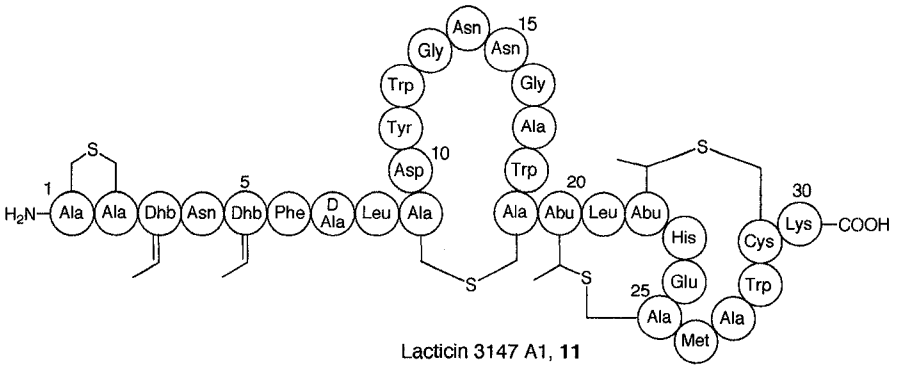
Nisin, **8**



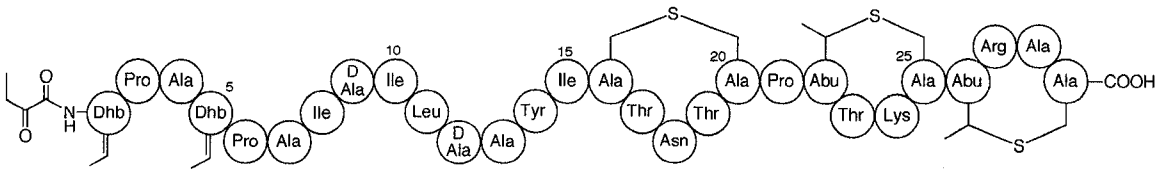
Mersacidin, **9**



Gallidermin, **10**



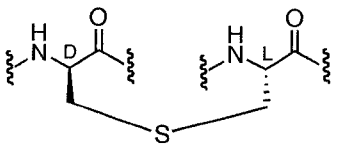
Lacticin 3147 A1, **11**



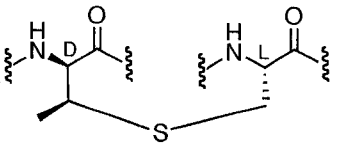
Lacticin 3147 A2, **12**

Lantibiotics (e.g., nisin A **8**, mersacidin **9**, gallidermin **10**, lacticin 3147 A1 **11**, lacticin 3147 A2 **12**) are ribosomally synthesized[29, 30] using the 20 natural amino acids with a leader peptide that is attached to the *N*-terminus of the mature sequence. Post-translational modification enzymes[31, 32] act on the pre-peptides to introduce the uncommon amino acid residues in lantibiotics, such as lanthionine **13**, β-methyllanthionine **14**, 2,3-didehydroalanine (Dha) **15**, (*Z*)-2,3-didehydrobutyrine (Dhb) **16**, D-alanine (D-Ala) **17**, 2-oxopropionyl **18** and 2-oxobutyryl **19** (Figure 4). Less frequently encountered modified amino acids include 2-hydroxypropionyl **20**, *S*-[(*Z*)-2-aminovinyl]-D-cysteine (AviCys) **21**, *S*-[(*Z*)-2-aminovinyl]-(3*S*)-3-methyl-D-cysteine (AviMeCys) **22**, *allo*-isoleucine **23**, (2*S*,8*S*)-lysinoalanine **24**, *erythro*-3-hydroxy-L-aspartic acid **25** and *N*, *N*-dimethylalanine **26**.[25]

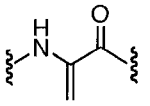
Figure 4. Structures of common post-translationally modified residues in lantibiotics



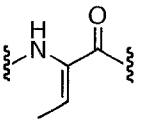
(2*S*,6*R*)-lanthionine
13



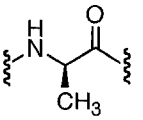
(2*S*,3*S*,6*R*)-3-methyllanthionine
14



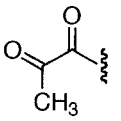
2,3-dehydroalanine
15



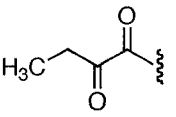
(*Z*)-2,3-dehydrobutyrine
16



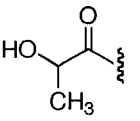
D-alanine
17



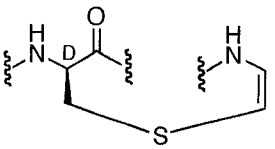
2-oxopropionyl
18



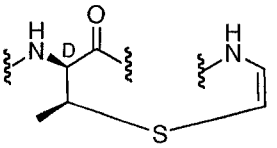
2-oxobutyryl
19



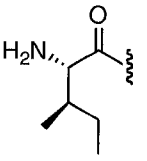
2-hydroxypropionyl
20



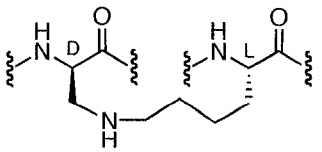
S-[(*Z*)-2-aminovinyl]-D-cysteine
(AviCys)
21



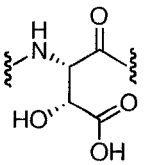
S-[(*Z*)-2-aminovinyl]-(3*S*)-
3-methyl-D-cysteine
(AviMeCys)
22



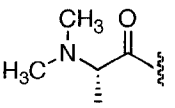
allo-Isoleucine
23



(2*S*,8*S*)-lysinoalanine
24



erythro-3-hydroxy-L-
aspartic acid
25



N,*N*-dimethylalanine
26

The structural genes and the genes necessary for post-translational modifications (PTM) and proteolytic processing as well as for accessory functions such as transport, producer self-protection and regulation are organized in biosynthetic gene clusters. [33, 34] The leader peptides generally range from 23 to 59 amino acids in length. The exact role of the leader peptides in lantibiotic biosynthesis is at present not clear. Several suggestions have been made regarding the possible function of the leader peptides, such as protection of the producer strain by keeping the lantibiotic inactive, signaling for export, and acting as a recognition scaffold for post-translational enzymes.[35-37] It is believed that the leader peptide is attached to the lantibiotic sequence during the post-translational modifications for most lantibiotics. However, in the case of at least one lantibiotic, lacticin 481, it has been shown that the leader peptide is not absolutely required for post-translational processing.[38] Once the lantibiotic is fully processed the leader peptide is removed by a specific protease. The dedicated proteases involved in the cleavage share a sequence homology with serine protease subtilisin, especially in the catalytic triad (Asp, His and Ser) and the Asn involved in the oxyanion hole formation.[39] Once the mature lantibiotic is produced it is generally transported to the extracellular medium by a family of ATP-dependent transporters. The transporters have about 500 to 600 amino acid residues and belong to the ATP-binding cassette (ABC) protein superfamily that shows similarity to the hemolysis transporter of *E. coli* HlyB.[40] Once the fully mature lantibiotic is produced it is potentially lethal to the producing organism. The producing strain avoids being killed by its own bacteriocin through production of highly specific immunity proteins / peptides[41] and / or type B ABC-transporters that are different from the ABC transporters involved in the lantibiotic biosynthesis.[42]

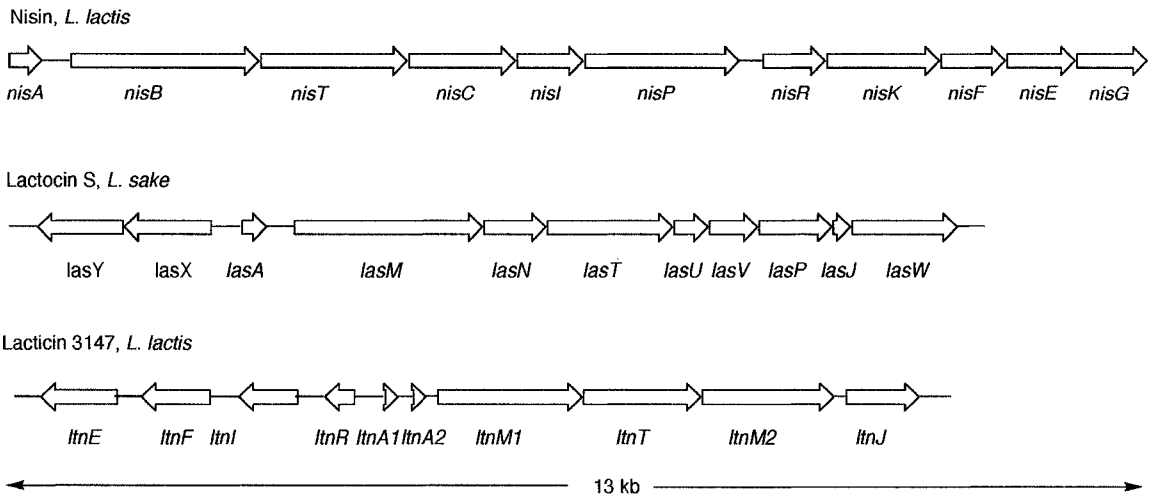
Lantibiotics have received a lot of attention recently due to the multiple mechanisms of killing they exhibit against many bacteria, including strains resistant to conventional antibiotics. Nisin, one of the most studied lantibiotic, was discovered in 1928 - a year prior to penicillin.[43] It has been used in over 80 countries for over 50 years without considerable development of resistance. Nisin, like all other lantibiotics, is proteinaceous, the feature that makes it suitable for applications in the food industry. Nisin is considered to be less toxic than table salt and was awarded the GRAS (generally regarded as safe) status in 1989 by the United States Food and Drug Administration (USFDA).[44] Many lantibiotics are being investigated for applications in the biomedical field and the agro-food industry.[45] For example, nisin Z is being investigated for treatment of bacterial mastitis in cattle caused by *Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus uberis*. It is also being studied for use with peptic ulcers in humans caused by *Helicobacter pylori*.[46, 47] Nisin is the active ingredient in two commercial products that are used in the prevention of mastitis, Consept® (a teat dip) and Wipe-out® (a teat wipe). Mersacidin and actagardine are promising lantibiotics that could become therapeutic agents to treat methicillin-resistant *Staphylococcus aureus* (MRSA).[48] Epidermin and gallidermin are used for the treatment of acne caused by *Propionibacterium acnes*.[49] Microcin B17 is a promising lead as a DNA gyrase inhibitor for pathogenic *Escherichia coli*.[50] Lacticin 3147 is effective against MRSA, vancomycin resistant *Enterococcol faecalis*, penicillin resistant *Pneumococcus*, *Propionibacterium acne* and *Streptococcus* mutants.[51] Because lacticin 3147 is active at physiological pH, it has also produced promising results when incorporated into teat seal products.[52] Lacticin

3147 is also being explored for applications in oral hygiene and bacterial mastitis. Lantibiotics are already successfully employed in the food industry. Nisin is used in the preservation of processed cheese, where the conditions are perfect for spore outgrowth of the toxin-producing bacterium *C. botulinum.* Only 6.25 μg of nisin per gram of cheese spread is necessary to kill the spores of the dangerous food pathogen *C. botulinum.*[53] Nisin has also been used in the preservation of canned vegetables, salad dressings, products containing pasteurized eggs and different varieties of cheese.[46, 53]

1.3. Biosynthesis

The lantibiotic biosynthesis genes are clustered and are localized on either bacterial chromosomes (e.g., mersacidin),[54, 55] plasmids (e.g., epidermin)[56] or transposons (e.g., nisin A).[57-59] They have been given the locus symbol *lan*, with a more specific genotypic designation for each lantibiotic, for example, *nis* for nisin, *gdm* for gallidermin and *ltn* for lacticin 3147. In the biosynthetic process, the structural gene *lanA* encodes for the lantibiotic precursor peptide that consists of a leader peptide with 23 to 59 amino acids attached to the *N*-terminus of the mature lantibiotic.[25] The genes *lanBC* (type A) and *lanM* (type B) code for post-translational modification enzymes involved in the formation of dehydroamino acids, lanthionine, and β-methyllanthionine. The *lanT* genes are found in most lantibiotic gene clusters and encode an ATP-binding cassette (ABC) transport system that exports the lantibiotic from the producer cell. LanP designates the protease coded by the *lanP* gene that cleaves the leader peptide. The *lanI* genes are responsible for production of LanI, an immunity protein or peptide, which is responsible for self-protection of the producer organism. The lantibiotic production is often regulated by a two-component regulatory gene *lanKR*. Additional genes such as *lanEFG* or *lanFG* function as a second transport system that confers additional self-immunity in lantibiotic producer strains. Biosynthetic gene clusters of representative lantibiotics nisin,[60, 61] lactocin S[62] and lacticin 3147[63] are shown in Figure 5.

Figure 5. Biosynthetic gene clusters of nisin, lactocin S and lacticin 3147 [60-63]



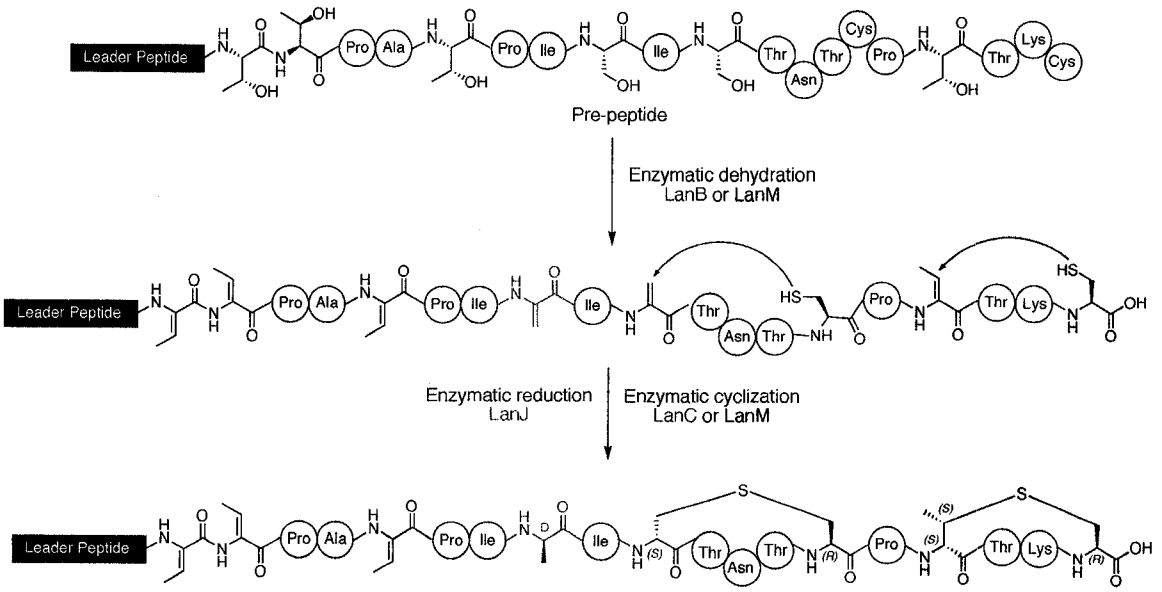
The production of lantibiotics is an energy-consuming process for the bacteria and is useful only when a sufficient concentration of bacteria has been reached that can participate in the biosynthesis. In most bacteriocins and lantibiotics the biosynthesis is regulated by a two-component regulatory system, suggesting a signal molecule might trigger the expression.[61] Nisin acts as a signaling molecule for its own production.[64]

1.3.1. Enzymatic formation of dehydroalanine, dehydrobutyrine, lanthionine, β-methyllanthionine and D-alanine

The uncommon amino acids found in lantibiotics such as dehydroalanine (Dha, **15**), dehydrobutyrine (Dhb, **16**), lanthionine (Lan, **13**), β-methyllanthionine (MeLan, **14**) and D-alanine (D-Ala, **17**) are a result of modifications by post-translational enzymes (Figure 4). The serine and threonine residues found in the *C*-terminal region of the pre-peptides

undergo a selective enzymatic dehydration reaction to form the dehydroalanine and dehydrobutyrine residues respectively. The serine and threonine residues found in the *N*-terminal leader peptide region remain untouched during the enzymatic reaction.[65] A stereo- and regioselective Michael addition of the cysteine thiol onto the Dha and Dhb takes place under enzymatic control to furnish (2*S*, 6*R*)-lanthionine **13** and (2*S*,3*S*,6*R*)-β-methyllanthionine **14**.[66] In the case of type A lantibiotics (e.g. nisin), the dehydratase enzyme LanB performs the dehydration and the cyclase enzyme LanC does the cyclization to form the thio-ether bridges (Figure 6).[67]

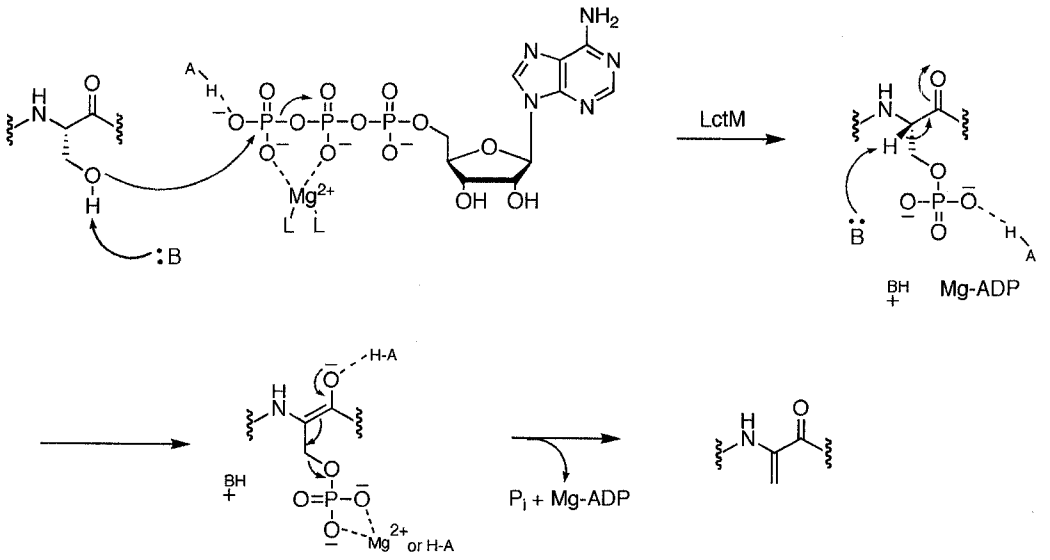
Figure 6. Enzymatic formation of dehydroalanine, dehydrobutyrine, lanthionine, β-methyllanthionine and D-alanine



In the case of type B lantibiotics (e.g., lacticin 481), a single enzyme LanM is found to perform both the cyclization and dehydration steps to form Lan and MeLan (Figure 6).[34]

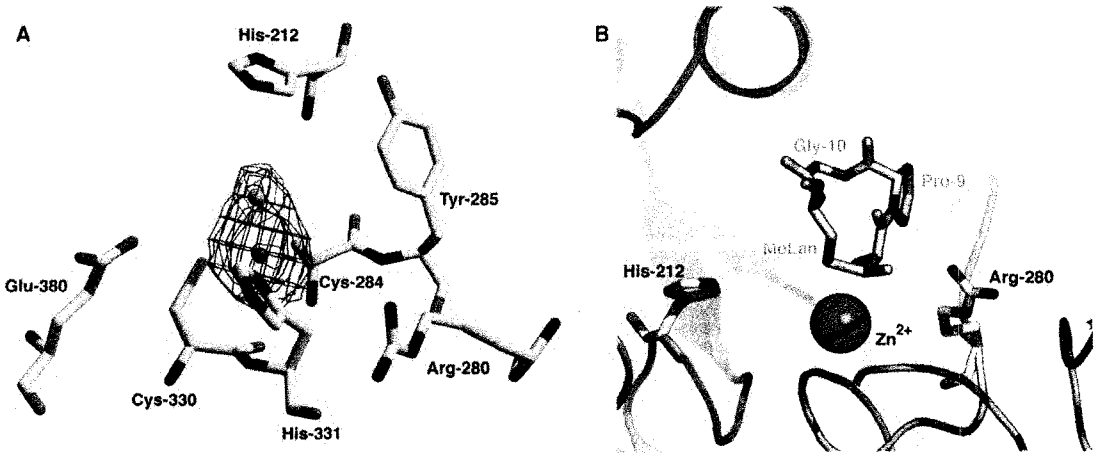
The first step in the post-translational processing of many lantibiotics is the dehydration of serine and threonine residues. With the successful isolation and *in vitro* reconstitution of LctM, the enzyme responsible for dehydration and cyclization to form lanthionine rings in lacticin 481, it has been possible to gain insight into the mechanism of this process (Figure 7).[68] It was found in the *in vitro* studies that lacticin 481 synthetase, LctM requires adenosine 5'-triphosphate (ATP) for activity. Further exploration with a designed substrate analogue indicated adducts with +80 mass units that corresponds to the mass of a phosphate group, suggesting the phosphorylation of serine and threonine residues before dehydration.[69] An active site Arg399 residue acts as a base in LctM and effects an anti-elimination to form Dha or *Z*-Dhb.[70] Also, it has been shown that LctM is a highly processive enzyme that performs multiple rounds of catalysis on the substrate prior to dissociation.[71]

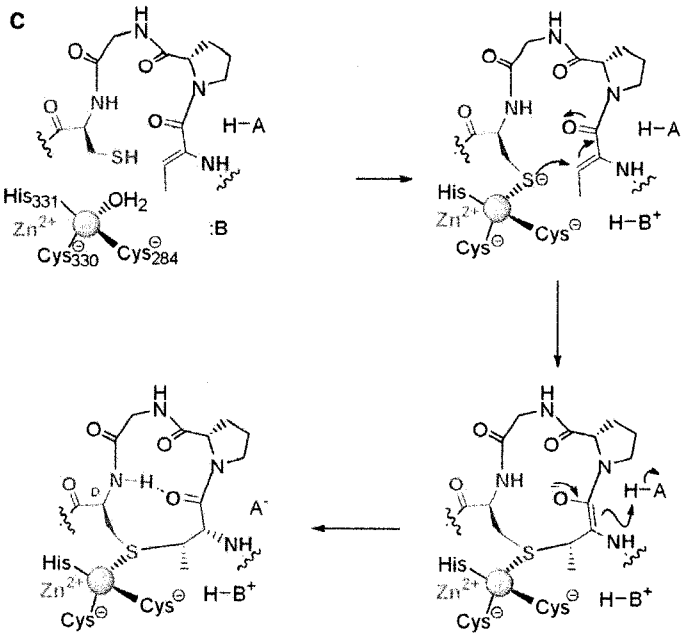
Figure 7. Proposed general mechanism for dehydration of serine residue by LctM



The second step in the biosynthesis of Lan or MeLan rings is believed to be the addition of cysteine residues to Dha or Dhb. The enzyme that is responsible for the formation of lanthionine rings in nisin, NisC, has been cloned and over-expressed in *E. coli*.[66] When an engineered, dehydrated nisin substrate was fed to NisC *in vitro*, the regio- and stereoselective formation of all five rings of nisin A was observed. A crystal structure of NisC has been obtained, which has shed light into the biosynthesis of lanthionine rings (Figure 8a).[66] A mechanism for formation of lanthionine ring B of nisin A has been proposed. Upon binding of the dehydrated substrate peptide, the thiol of the cysteine nucleophile ready for cyclization displaces a molecule of water from the active site Zn^{2+} ion and undergoes a deprotonation by a general base or water. The activated thiolate then attacks the β carbon of the dehydrobutyrine residue in an anti fashion to form an enolate intermediate that is protonated to provide the D configuration at the α-carbon (Figure 8c).[72]

Figure 8. (a) Crystal structure showing active site residues of NisC (b) Nisin ring B modeled into the active site (c) Proposed mechanism for lanthionine ring formation (Reproduced from Science, Li *et al.*, 2007)[66]

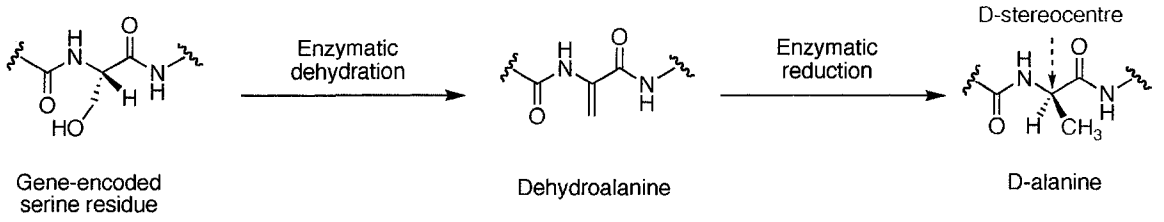




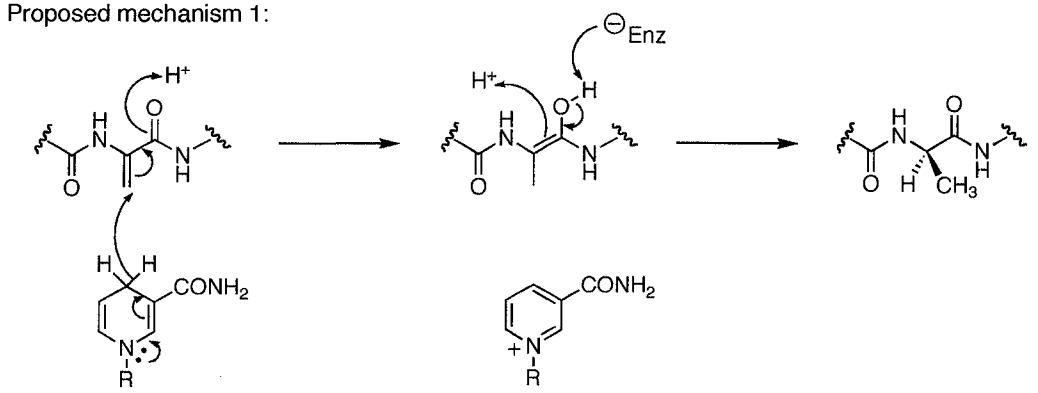
In two-component lantibiotics such as lacticin 3147 A1 & A2 and haloduracins α and β, two different processing enzymes are required for dehydration, cyclization and for the production of the mature lantibiotic of each component. In lacticin 3147, the enzymes LtnA1 and LtnA2 perform the dehydration and cyclization to produce the mature lacticin 3147 A1 and lacticin 3147 A2, respectively.[73] Double mutants have been created to confirm the requirement of two separate enzymes for processing each-component of lacticin 3147.[73] In haloduracins, the modifying enzymes HalM1 and HalM2 have been purified and their activity has been reconstituted *in vitro*.[74] The results further confirm that each component of haloduracin requires a different enzyme for post-translational processing. Moreover, in both lacticin 3147 and haloduracin incubation of the substrate of one component (e.g. LtnA1 or HalA1) with the processing enzyme for the other component (e.g. LtnM2 or HalM2) did not result in the dehydrated or cyclized product.

It is generally accepted that ribosomally synthesized polypeptides contain only the L-amino acid isomer in a sequence coded by the DNA / RNA. The D-isoform is only very rarely encountered in prokaryotic systems. Two ribosomally encoded lantibiotics, lacticin 3147 and lactocin S, contain D-alanine residues. In lactocin S, the positions that are occupied by three D-alanines were originally encoded for L-serine (Scheme 1). Based on this observation, Skaugen *et al*[75] proposed an enzymatic dehydration of gene-encoded serine residues followed by a stereospecific reduction. However, in the case of lactocin S the enzyme responsible for the stereoselective reduction has not been identified.

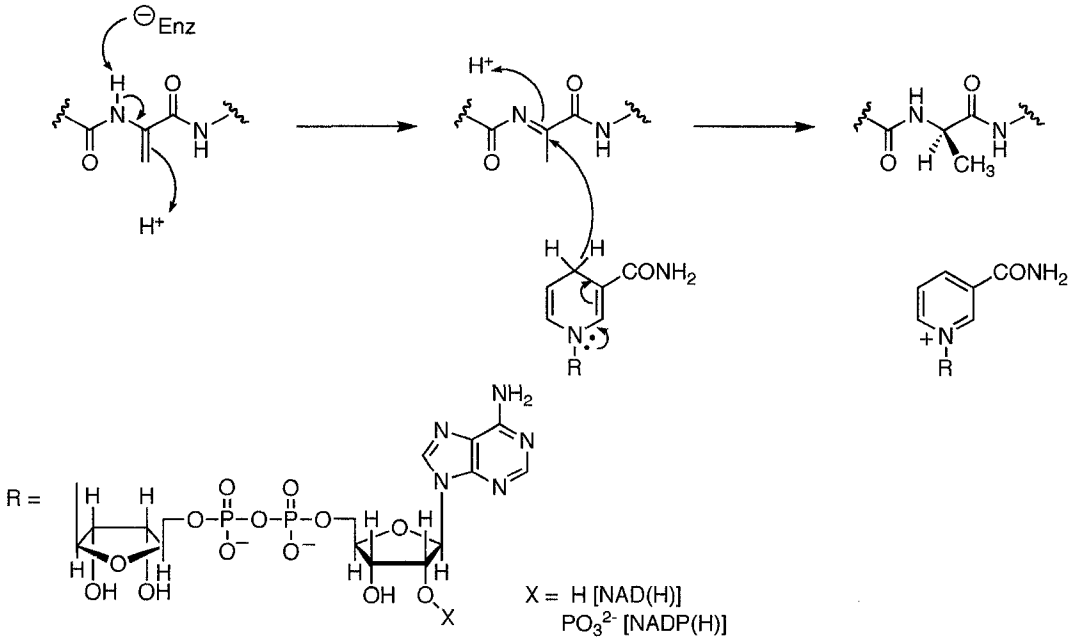
Scheme 1. Enzymatic formation of D-alanine from L-serine



Proposed mechanism 1:



Proposed mechanism 2:



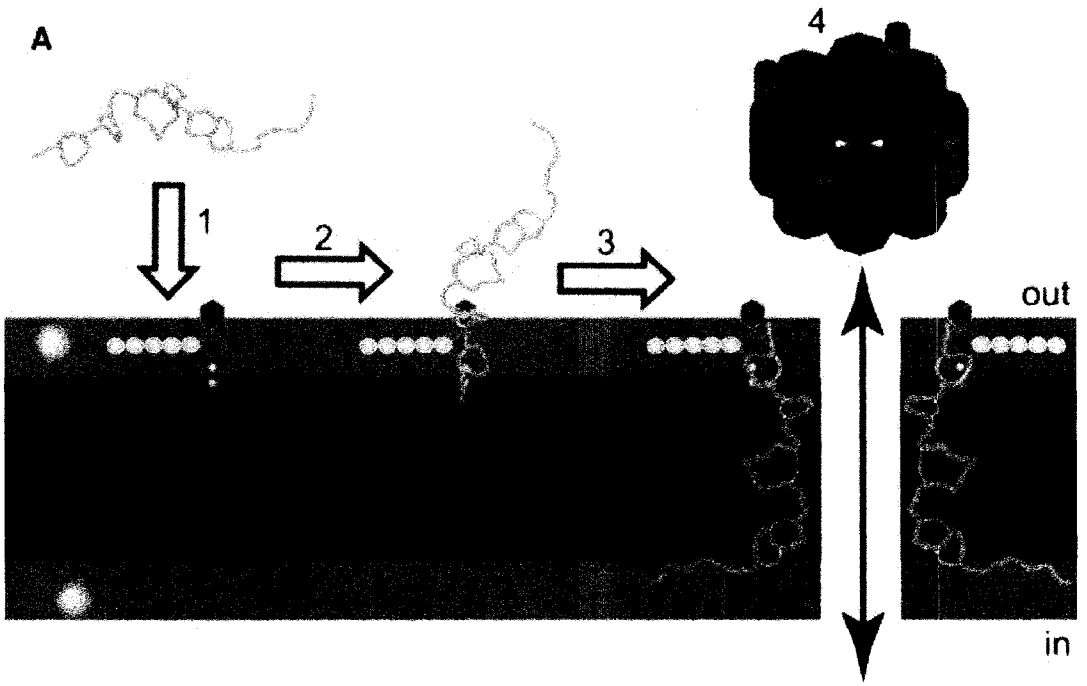
Lacticin 3147 A1 and A2 both contain D-Ala residues in the positions that are encoded for serine in the genetic code. An enzyme, LtnJ responsible for selective reduction of dehydroalanines in lacticin 3147 A1 & A2 to introduce D-Ala has been identified.[76] Deletion of the gene for LtnJ results in Dha intermediates with a concomitant reduction in biological activity. It has also been shown that replacement of D-Ala with L-Ala results in diminished peptide production and / or antimicrobial activity.

1.4. Mode of action of lantibiotics

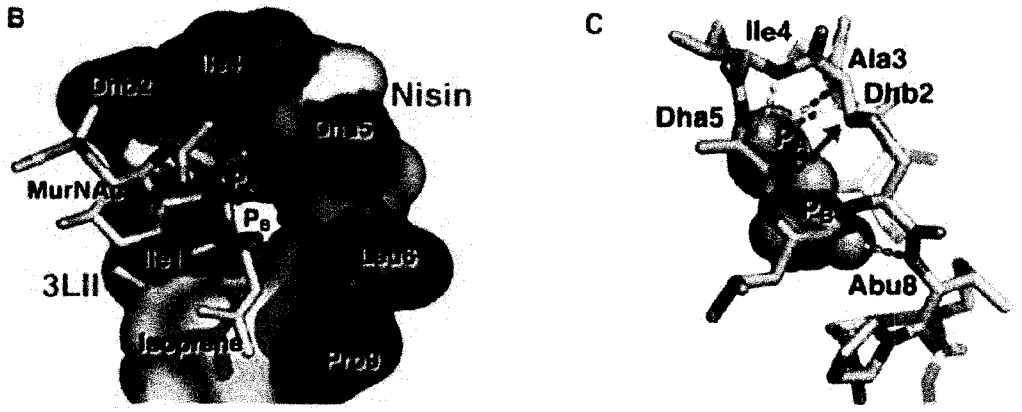
1.4.1. Mode of action of type A and type B lantibiotics

Most, if not all, lantibiotics are found to target the cell wall of bacteria, with more than one-third of lantibiotics targeting lipid II. Among Type A lantibiotics, nisin is one of the oldest known and has been well studied because of its multiple applications. It has been recently discovered that nisin exhibits a dual mode of action in a receptor-mediated process.[77] It specifically recognizes and binds with high affinity to lipid II in the cell wall of Gram-positive bacteria, thereby disrupting cell wall biosynthesis.[78] The binding event is followed by formation of stable pores[79, 80] that lead to rapid efflux of ions, consequently dissipating the membrane potential and resulting in cell death. A proposed model for the mode of action of nisin consistent with experimental evidence is shown in Figure 9. The first step is the binding of *N*-terminal (rings A and ring B) of nisin with lipid II. Recently a solution NMR structure of a 1:1 nisin-lipid II analog complex in DMSO,[81] revealed the specific interactions at the molecular level. The A and B rings of nisin interact with the pyrophosphate groups of lipid II via hydrogen bonding to Dhb-2, Ala-3, Ile-4, Dha-5 (ring A) and Abu-8 (ringB) to form a pyrophosphate cage. The flexible hinge region (residues 20→22)[82] and the *C*-terminus are involved in the pore formation.[83] Also, after anchoring of nisin onto lipid II in the membrane, conformational changes take place that lead to the assembly of a higher-order oligomeric complex.[84] This ultimately leads to a long-lived (several seconds), 2 nm diameter pore with 4:8 lipid II: nisin stoichiometry[79, 85] resulting in the leakage of cell contents.

Figure 9. (a) A proposed model for lipid II mediated pore formation by nisin (b) The *N*-terminal residues (1→12) of nisin encages the pyrophosphate moiety of lipid II analog (Reproduced from Molecular Microbiology, Breukink, E. 2006).[80] (c) Intermolecular hydrogen bond network of the pyrophosphate cage. (Reproduced from Nature Structural & Molecular Biology, Hsu *et al.*, 2004).[81]



(A) [1] Nisin interacts with the negatively charged bacterial plasma membrane due to its cationic nature [2] A nisin-lipid II complex is formed with the *N*-terminus of nisin [3] The flexible hinge region (residues 20→22) and the *C*-terminus insert into the membrane to form pores [4] During the pore-formation process an oligomeric complex with (4:8) stoichiometry of lipid II: nisin pore (2 nm diameter) is formed.



(B) Rings A and B (residues 1→12) of nisin with backbone and side chain atoms in green and yellow encages the pyrophosphate moiety of lipid II analog (C) Intermolecular hydrogen bonding of Dhb-2, Ala-3, Ile-4, Dha-5 and Abu-8 residues with pyrophosphate moiety.

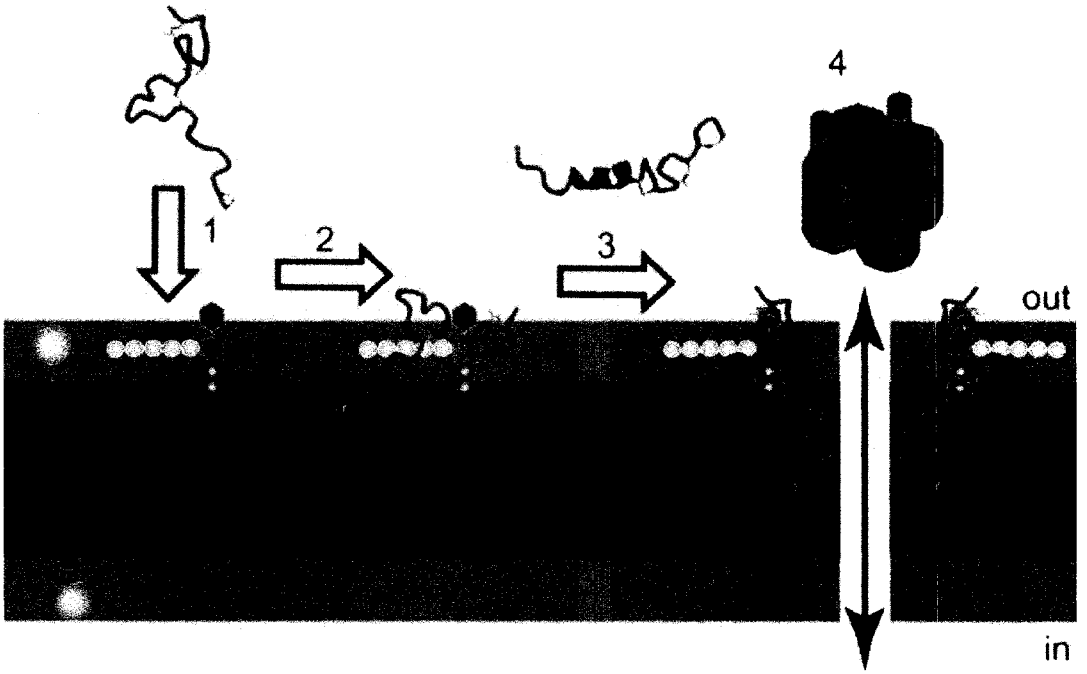
The type B lantibiotics (e.g. mersacidin) also trigger bacterial cell death by interaction with lipid II, thereby inhibiting cell wall biosynthesis.[86] However, due to the smaller size of type B lantibiotics the interaction with lipid II does not lead to pore formation. The minimum inhibitory concentration (MIC) values of mersacidin can increase in the presence of calcium (II) ions, suggesting that the divalent ion forms a bridge between the peptide and lipid II. The flexible hinge region comprising residues 12 and 13 is believed to be the site of electrostatic interaction for mersacidin. Recently, an alternative bactericidal mechanism for mersacidin-like peptides that cannot form pores has been described, which involves sequestering of lipid II from the cell division site and thus blocking cell wall biosynthesis.[77]

1.4.2. Mode of action of two-component lantibiotics

Two-peptide lantibiotics comprise a sub-class of lantibiotics where two individual components are required for full biological activity. To date, only seven such two-peptide lantibiotics have been identified and include lacticin 3147,[87] staphylococcin C55,[88] plantaricin W,[89] cytolysin,[90] haloduracin,[74] smb[91] and BHT-A.[92] Lacticin 3147 A1 & A2 are the only well-studied members of the two-peptide lantibiotics.[93] Similar to other two-peptide systems, the individual peptides lacticin A1 or lacticin A2, exhibit only moderate antimicrobial activity (μM concentration) by themselves. However, when both the A1 and A2 components are present together, a potent antimicrobial activity is observed at sub nanomolar concentration. As shown previously for nisin, lacticin 3147 also acts by interacting first with lipid II and later forming pores.[94] An intriguing feature of lacticin 3147 A1 & A2 with respect to the mode of action is that it appears that the individual peptides have split the roles of targeting lipid II and pore-formation among themselves. A working model for the antimicrobial activity of lacticin 3147 has been proposed,[94] involving lacticin A1, A2 and lipid II (Figure 10).

The mersacidin like lacticin A1 has been identified as the component that associates with the membrane and forms a complex with lipid II.[95] The complex that is formed in the first step induces or stabilizes a conformational change in lacticin A1. This leads to the generation of a high-affinity binding site for the A2 peptide and formation of a trimeric lacticin A1:A2:lipid II complex.

Figure 10. Mode of action of the two-peptide lantibiotic, lacticin 3147 A1 & A2 (Reproduced from Molecular Microbiology, Breukink, E. 2006).[80]



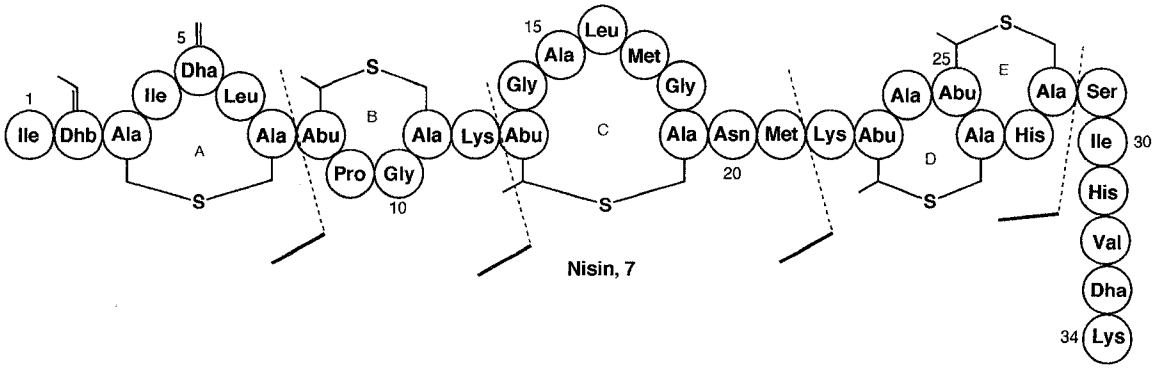
[1] Membrane association of A1 peptide due to its cationic nature [2] Formation of a A1 peptide:lipid II complex [3] Recruitment of A2 peptide by A1:lipid II complex giving a trimeric complex and pore-formation [4] An oligomeric complex with (1:1:1) stoichiometry of A1:A2:lipid II with 0.6 nm pore diameter is formed leading to cell death.

When bound to the dimeric A1:lipid II complex, the A2 peptide is able to adopt a transmembrane conformation which allows it to form defined pores that lead to cell death. The pores formed by A1:A2:lipid II (0.6 nm) are significantly smaller than those formed by nisin (2.0 nm). Based on the pore size it has been proposed that the lacticin 3147 pores represent just a one-fold or two-fold complex with (1:1:1) stoichiometry of A1:A2:lipid II.[94]

1.5. Chemical synthesis of lantibiotics and its analogues

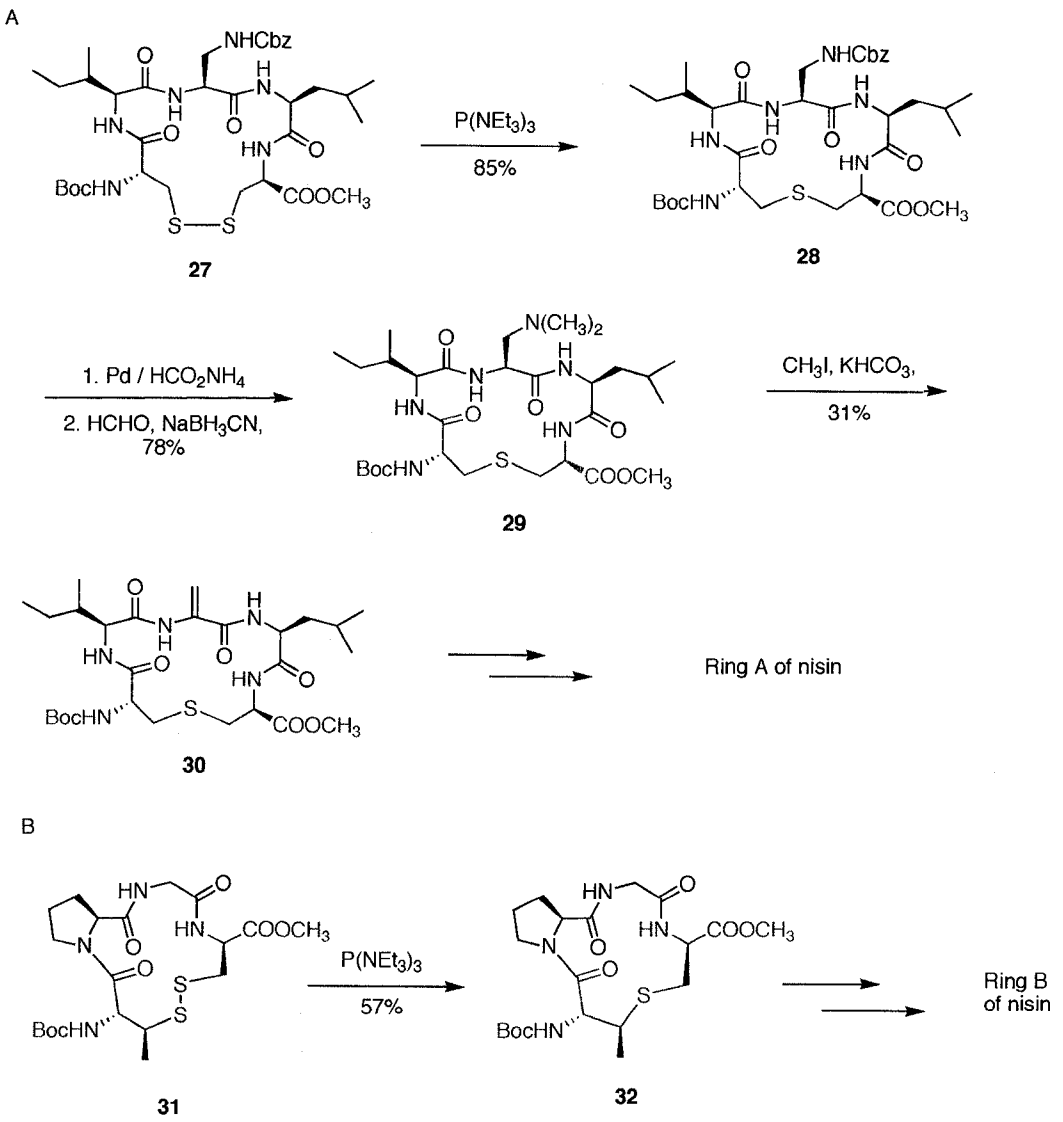
With the discovery and understanding of many new lantibiotics with therapeutic promise, the chemical synthesis of lantibiotics has gained interest in the past five years. To date, nisin is the only lantibiotic whose total synthesis has been completed. This was done in solution phase using a fragment condensation approach by Shiba and Wakamiya and their co-workers (Figure 11).[96-98]

Figure 11. Fragment condensation approach used for total synthesis of nisin



The highlights of the synthesis are the formation of lanthionine, and β-methyllanthionine rings and the overlapping D and E rings. All the Lan and MeLan rings precursors (e.g., compound **27** and **31**) were synthesized as the disulfide counterpart. A sulfur extrusion of the disulfide analog using the procedure of Harpp and Gleason[99, 100] was then performed to furnish the lanthionine and β-methyllanthionine containing rings A, B and C of nisin (Scheme 2).

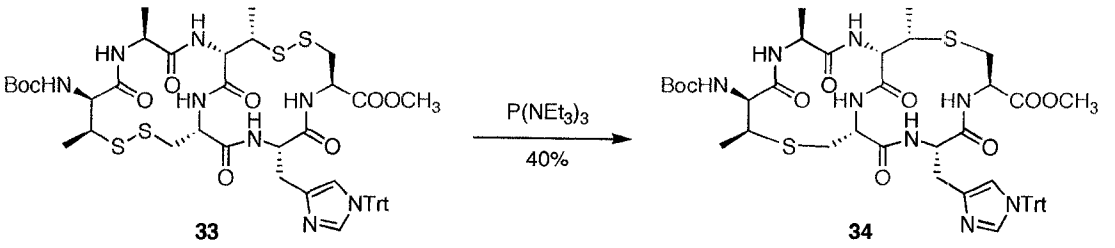
Scheme 2. Synthesis of lanthionine ring A and β-methyllanthionine ring B by sulfur extrusion in the total synthesis of nisin



The overlapping D and E MeLan rings were synthesized by a clever strategy again employing the sulfur extrusion procedure. The precursor for the extrusion step compound **33** containing two overlapping disulfide rings was synthesized by using an orthogonal cysteine protection strategy (Scheme 3). On treatment of **33** under extrusion

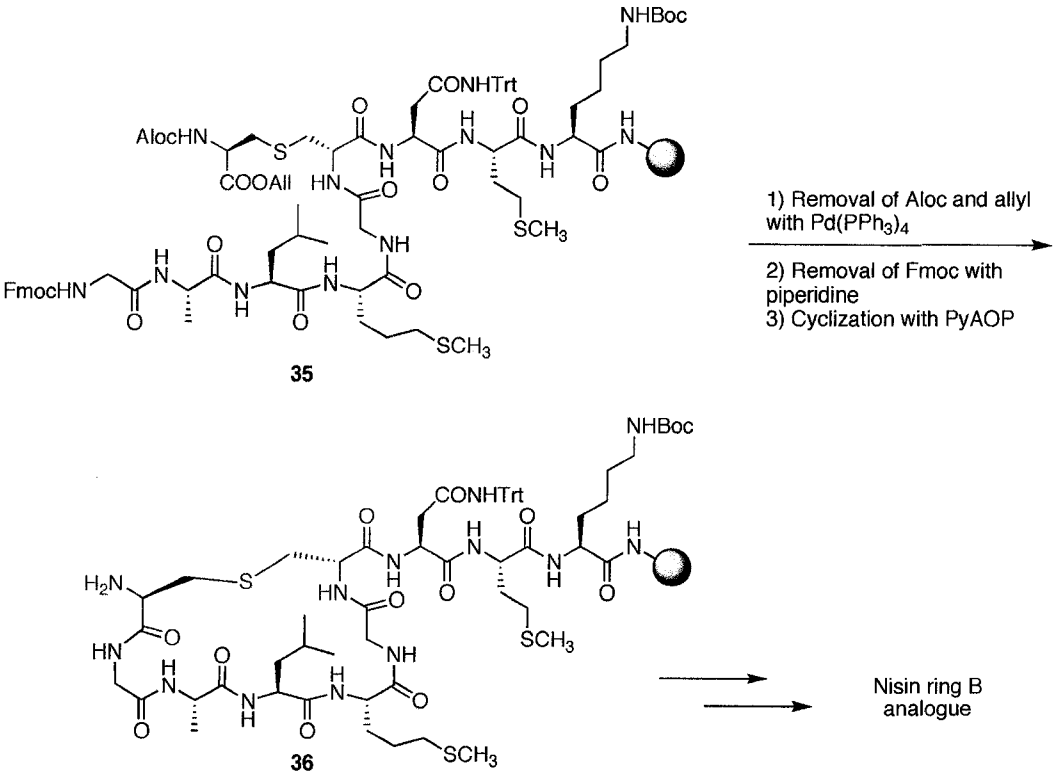
conditions, the D and E rings (**34**) were obtained in 40% yield. The ring fragments were then coupled together to complete the synthesis of nisin.

Scheme 3. Synthesis of overlapping D and E rings of nisin by Shiba and co-workers



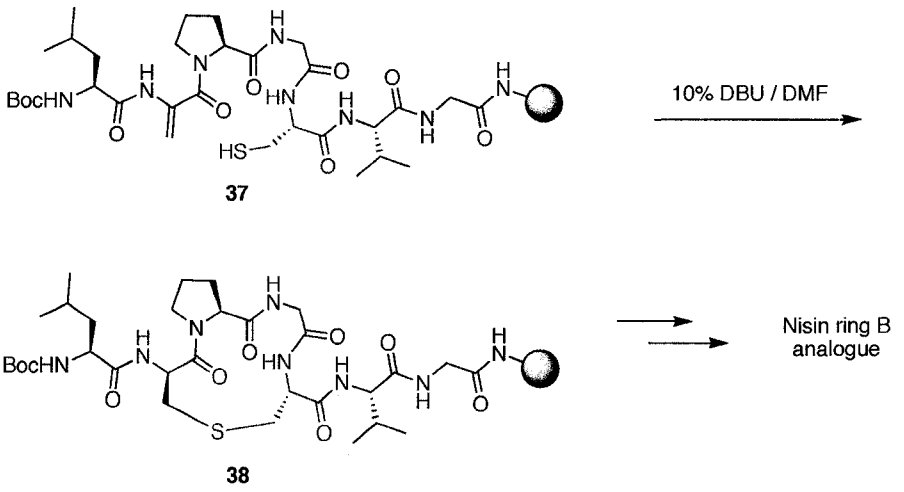
To date, methods for the synthesis of lantibiotics and their analogues on solid-support are limited. Prior to the work described in this thesis, only syntheses of fragments of lantibiotic analogues had been achieved on solid support. Notable among the solid-supported methods are the synthesis of an analogue of ring C of nisin by Tabor and co-workers[101] and a biomimetic synthesis of a ring B analogue of nisin by Bradley and co-workers.[102] In Tabor's synthesis, an orthogonally protected lanthionine building block with allyloxycarbonyl (Aloc), allyl and *N*-(9-Fluorenylmethoxycarbonyloxy) [Fmoc] protection (compound **35**) was prepared in solution and incorporated on solid support (Scheme 4). The Aloc, allyl and Fmoc protecting groups were removed on solid support using $Pd(PPh_3)_4$ with scavenger and 20% piperidine in DMF. The cyclization to form the lanthionine ring was performed on solid support using PyAOP as the coupling reagent. Introduction of two more residues by standard methods gave ring C analogue of nisin.

Scheme 4. Tabor and co-workers synthesis of an analogue of ring C of nisin



Bradley and co-workers utilized a biomimetic approach, specifically the Michael addition of a cysteine thiol onto a dehydroalanine residue to give ring B of nisin on solid support (Scheme 5). In general, the Michael addition of cysteine to dehydroalanine can give rise to diastereomeric products. However, the authors were able to obtain a single *meso*-diastereomer from the reaction. The stereochemistry was further confirmed by comparison with an authentic sample. The high diastereoselectivity obtained could presumably be due to pre-organization guided by the proline residue.

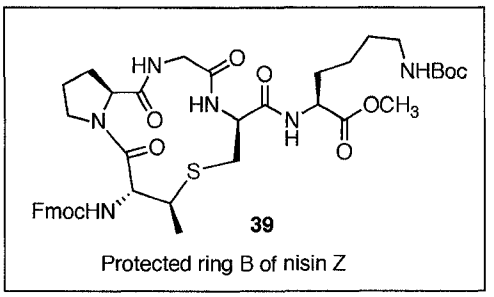
Scheme 5. Biomimetic formation of lanthionine on solid support to make ring B analogue of nisin

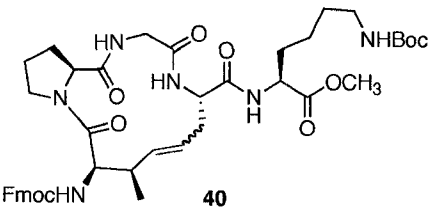


In addition to the synthesis of lanthionine containing fragments of lantibiotics some groups have been interested in the generation of analogues that would replace sulfur atoms with carbon atoms in the lanthionine rings. While the work described in this thesis was being done, Liskamp and co-workers[103-106] reported the syntheses of fragments of nisin Z in solution that contain carbon atoms instead of sulfur atoms. Their approach for the synthesis of the carbocyclic rings involves a solution phase ring-closing metathesis (RCM) between two suitably protected allyl glycine residues. Thus, an RCM reaction between two allylglycine residues in **40a** gives the ring B of nisin Z **40** (Scheme 6). For the synthesis of the carbocyclic analogue of the overlapping D and E rings of nisin Z (**42**) an interesting strategy was employed. It starts with a cross-metathesis reaction between two protected allyl glycine residues on solid-phase to form the ring E precursor **42a**. This was elaborated on-solid support to give the ring E derivative **42c**. Allylglycine residue was introduced to **42c** to obtain **42d** that is set up to undergo a RCM

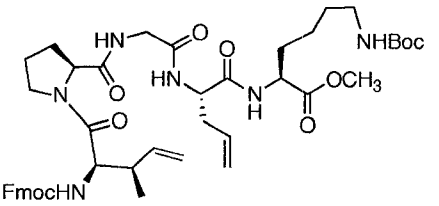
to give the carbocyclic analogue of ring D. The peptide was then cleaved from the solid-support and a RCM in solution led to the overlapping carbocyclic rings D and E **42**.

Scheme 6. Carbocyclic analogues of ring B and rings D/E of nisin Z prepared by Liskamp and co-workers

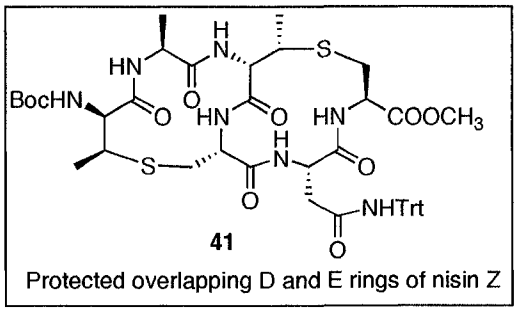


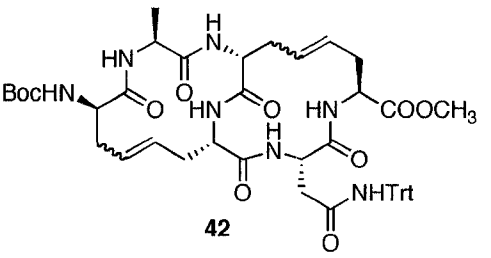


Protected carbocyclic analogue of ring B of nisin Z

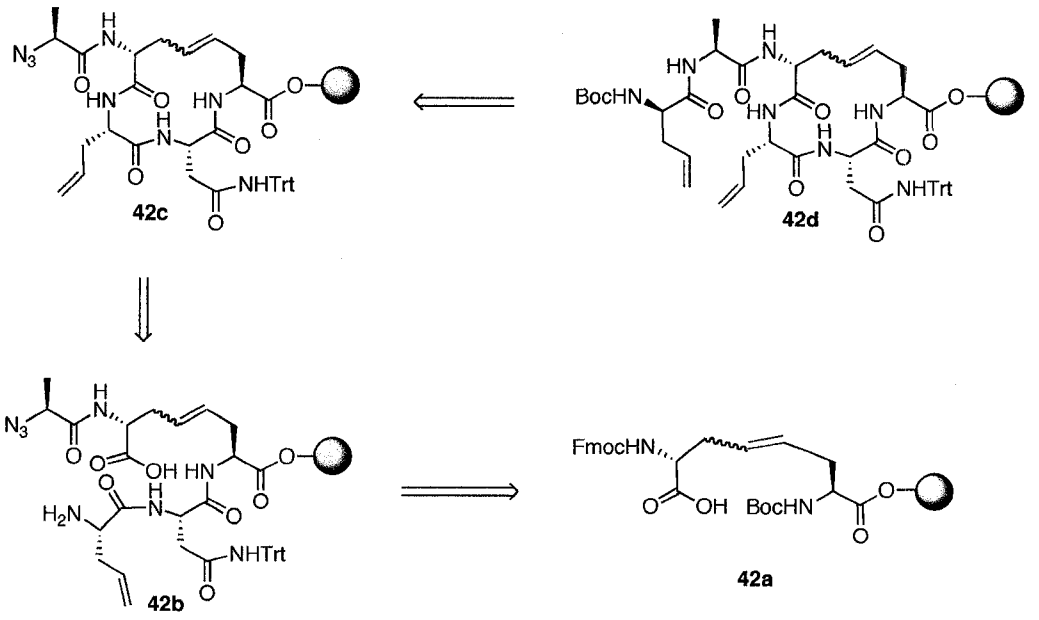


40a





Protected overlapping carbocyclic analogue of D and E rings of nisin Z



Although the strategies for the synthesis of fragments of the carbocyclic rings are notable, the disadvantage of the approach is that alanine residues have been used to replace the dehydroalanines and dehydrobutyrines of nisin Z. Also, for the ease of synthesis the L-leucine residue at position 16 in nisin Z has been replaced by D-leucine.[106] It has been shown by NMR studies that the dehydroalanine residue at position 5 of nisin is involved in the formation of pyrophosphate cage with lipid II.[81] Thus, substituting the dehydroamino acids along with the other additional replacements might affect the biological activity and could interfere in deducing the true effect of replacement of the lanthionine rings with carbocyclic rings when such an analog is completed.

1.6. Project objectives

This thesis is composed of three sections: 1) Development of chemical methods for the synthesis of lantibiotics and their analogues for structural confirmation and structure-activity relationship studies; 2) design and synthesis of a substrate for *in vitro* reconstitution of a post-translational enzyme that introduces D-alanines during biosynthesis of lantibiotics; and 3) development of approaches to modifying lantibiotics mode of action to make them active against Gram-negative organisms.

1.6.1. Chemical synthesis of lantibiotics and their analogues: syntheses of a carbocyclic-larger ring analogue, a lanthionine analogue, and analogues of residues (1→5) of lacticin 3147 A2 for structure-activity studies; the total synthesis of lactocin S for structure confirmation

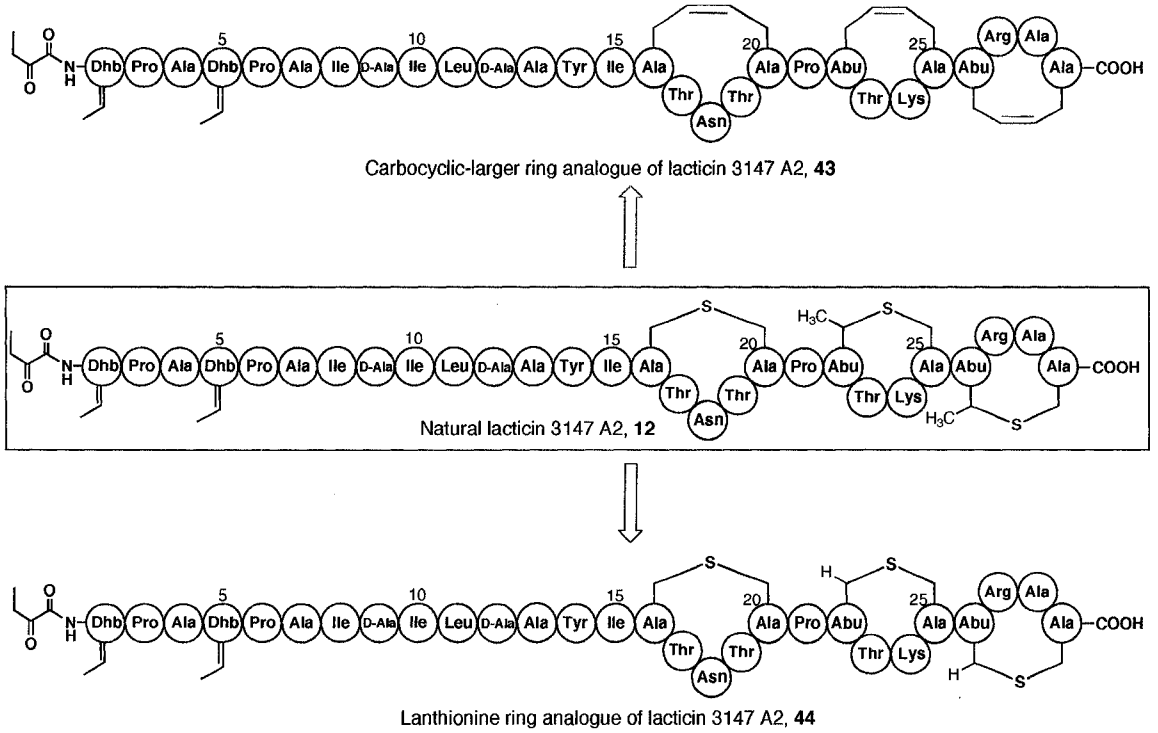
As additional lantibiotics with high potency are being identified for applications in food, veterinary medicine and human therapeutics, there is a growing interest in deducing the primary structure as well as understanding the exact roles of the uncommon amino acid residues. In this regard, a systematic structure-activity relationship (SAR) study on these highly potent lantibiotics could not only lead to simplified bioactive lantibiotic analogues, but would also help in deducing the principles for designing novel lantibiotics. However, the generation of lantibiotic analogues for SAR is partially hampered by the limited number of chemical methods available for the synthesis of

lantibiotics. Development of methods for the synthesis of lantibiotics, especially solid-supported techniques, would tremendously accelerate the SAR studies. It would also allow one to generate variants that are not readily accessible by biochemical methods.

The project objectives are to perform a structure-activity relationships study of lantibiotics using lacticin 3147 A2 as a model system, as it contains most of the common post-translational modifications found in lantibiotics and no overlapping lanthionine rings (Figure 12).

One of the common causes for instability of lantibiotics is due to the aerobic oxidation of the sulfur atoms in the lanthionine ring to sulfoxide. This leads to significant or complete loss of biological activity.[93] Substitution of the oxidatively unstable sulfur atom with a more robust carbon atom could avoid this problem and lead to an analogue with increased stability. The project goal is to synthesize a carbocyclic analogue of lacticin 3147 A2, **43**, with carbon atoms instead of sulfur atom on solid-support (Figure 12). This modification would help in deducing the importance of sulfur atoms in the lanthionine rings as well as the effect of the larger ring size on biological activity. Replacement of the disulfide bond with a dicarba bond in biologically active peptides has been reported in the literature using ring-closing metathesis. In many cases the carba analogues generated displayed increased stability. [107-110]

Figure 12. Natural lacticin 3147 A2, carbocyclic-larger ring analogue and lanthionine analogue of lacticin 3147 A2

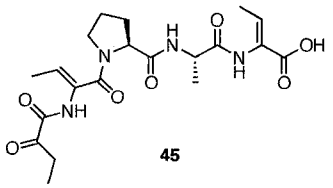


The second objective is to synthesize a lanthionine analogue of lacticin 3147 A2, **44**, wherein the β-methyllanthionine rings are replaced with lanthionine rings (Figure 12). The proposed analog would differ by two methyl groups from the natural product. For chemical synthesis of lantibiotics the key building blocks are orthogonally protected Lan and MeLan. However, accessing the orthogonally protected β-methyllanthionine fragment typically requires multiple steps and proceeds with low overall yield. There are very few reports in the literature for the stereoselective synthesis of β-methyllanthionine to date.[96, 111] Thus, synthesis of analogue **44** would shed light on the importance of the β-methyllanthionine in lantibiotics and could potentially lead to simplified structures where MeLan is replaced by the more accessible Lan.

For nisin (**8**), loss of antibacterial activity has been reported during prolonged storage of food[53] and freeze-dried products.[112] Among other processes, the primary cause has been identified as the chemical degradation of dehydroalanine-5 of nisin. In an attempt to stabilize nisin Z against this chemical degradation, dehydroalanine-5 was replaced with dehydrobutyrine with a slight decrease in biological activity.[113] However, Dha5Dhb nisin Z was found to be considerably more stable,[114] presumably because of the lower reactivity of Dhb towards Michael addition reactions. In natural lacticin A2 (**12**) the *N*-terminal residues (1→5) **45** are extensively post-translationally modified and contain two dehydrobutyrines and one α-ketoamide. As seen above with nisin, one mode of inactivation of lacticin A2 could be due to the instability of the dehydrobutyrines. Although Dhb are more stable than Dha, they are still prone to chemical degradation by Michael addition.

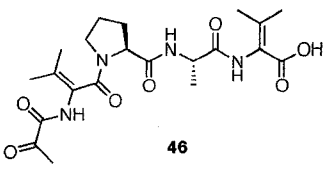
One objective is to synthesize analogues of **45** with the dehydrobutyrines replaced by dehydrovaline residues (**46**) and oxazole residues (**47**) (Figure 13). When residues (1→5) of natural lacticin A2 are replaced with **46** and **47**, it could render the newly generated analogues more resistant to Michael addition and confer further stability.

Figure 13. Residues (1→5) of lacticin A2, dehydrovaline and oxazole analogue



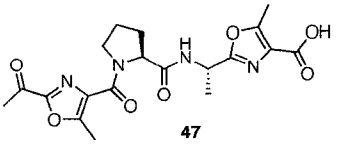
45

Residues (1-5) of lacticin A2



46

Dehydrovaline analogue of residues (1-5) of lacticin A2

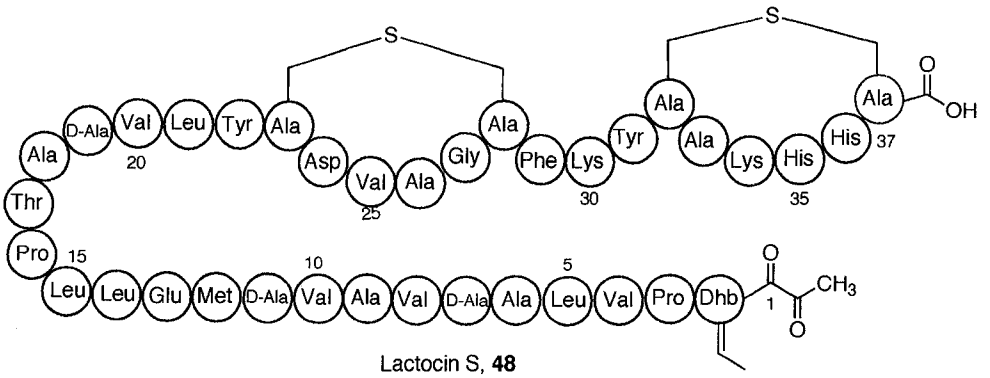


47

Oxazole analogue of residues (1-5) of lacticin A2

Lactocin S is a 37 amino acid lantibiotic peptide produced by *Lactobacillus sake* L45 isolated by Nes and co-workers.[115] It shows potent activity against *Listeria monocytogenes*, *Staphylococcus aureus* and *Clostridium botulinum.*[116] A primary structure of lactocin S (**48**), the longest lantibiotic known, has been proposed by Nes and co-workers, but has not been proven conclusively (Figure 14).[75]

Figure 14. Proposed primary structure of lactocin S



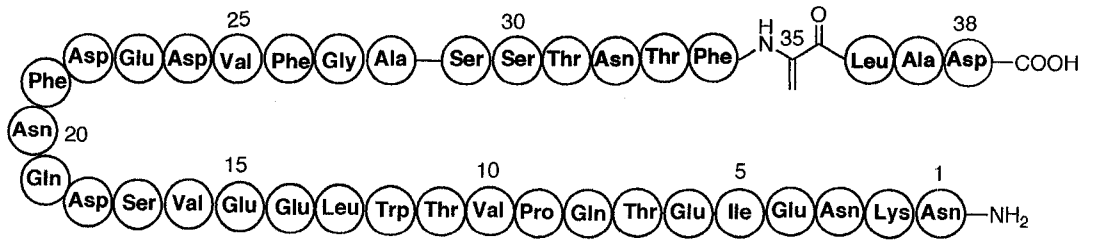
Lactocin S, **48**

At present, only 0.2 mg / L of lactocin S can be obtained by fermentation. This low fermentation yield limits the number of practical approaches to structure elucidation. Chemical synthesis of lactocin S would help to overcome this difficulty and could provide enough material for structure elucidation. One project goal is to execute a total synthesis of lactocin S on solid-support using Fmoc solid phase peptide synthesis (Fmoc-SPPS) and thus confirm the proposed structure.

1.6.2. Design and synthesis of a substrate for *in vitro* reconstitution of LtnJ

LtnJ has been identified as the enzyme responsible for reducing dehydroalanines to D-alanines in lacticin 3147 A1 and A2.[76] The D-alanines found in lacticin 3147 have been shown to be important for production as well as for biological activity. In addition, D-amino acids are generally resistant to proteolytic degradation and confer stability. Currently, chemical synthesis is the only convenient way to introduce D-amino acids into peptides. Development of a biochemical system, by imitating nature's design would prove advantageous for incorporation of D-amino acids into peptides. Previous work in the Vederas group has led to the isolation and purification of the LtnJ enzyme that is thought to be involved in the introduction of D-amino acids in peptides. Several dehydrodipeptides and tripeptides were synthesized and tested for enzymatic activity against LtnJ with little success (Lara Silkin, M. Sc. Thesis, University of Alberta, 2006).[117] One possible reason could be that the designed and tested dipeptides and tripeptides are missing some key recognition sequence. Recently, it has been shown in the literature that the leader peptide is essential for full activity of lantibiotic post-translational processing enzymes.[35-37] Based on these observations, we designed a substrate that consists of the leader peptide and part of the mature sequence of lacticin 3147 A1 (Figure 15).

Figure 15. Design of the substrate for the LtnJ enzyme with the leader sequence and part of the mature sequence of lacticin A1



Blue = Leader sequence of lacticin A1, Green = Part of the mature sequence of lacticin A1 with mutations

49

One project goal is to synthesize the designed LtnJ susbtrate **49**, which consists of a dehydroalanine in the position of the D-alanine found in the mature lacticin A1. Once such a peptide is synthesized it could be tested as a substrate for *in vitro* activity of LtnJ enzyme.

1.6.3. Modifying the mode of action of lantibiotics for activity against Gram-negative bacteria: synthesis of residues (75→88) of colicin V for attachment to lantibiotics

In general, lantibiotics exhibit a broad range of activity against Gram-positive bacteria. They are not usually active against Gram-negative bacteria. This is primarily because lantibiotics cannot cross the outer-membrane of the Gram-negative bacterial cell wall. It has been demonstrated that nisin can kill Gram-negative bacteria when chemical

agents such as EDTA,[118, 119] trisodium phosphate[120] or a temperature shock[121] disrupts the outer membrane. However, non-lantibiotic bacteriocin counterparts like colicins and microcins,[122] produced by Gram-negative bacteria, exhibit activity against other Gram-negative bacteria by taking advantage of a unique bacterial active transport machinery. One such system is the FhuA-tonB system,[123] which is involved in the active transport of iron into the bacteria via iron-chelated bacterial siderophores.[124] Colicins and microcins take advantage of this system by possibly mimicking an iron-chelator and cross the outer membrane of Gram-negative bacteria.[125, 126] When the *C*-terminal sequences of colicin V from *E. coli* and microcin L are compared, the *C*-terminal 32 amino acids show a sequence similarity of 87% and contain a disulfide bridge (Figure 16).[127] In a closely related bacteriocin microcin E492, the *C*-terminal 14 residues are post-translationally modified and are shown to be essential for antimicrobial activity and active transport by tonB system.[128]

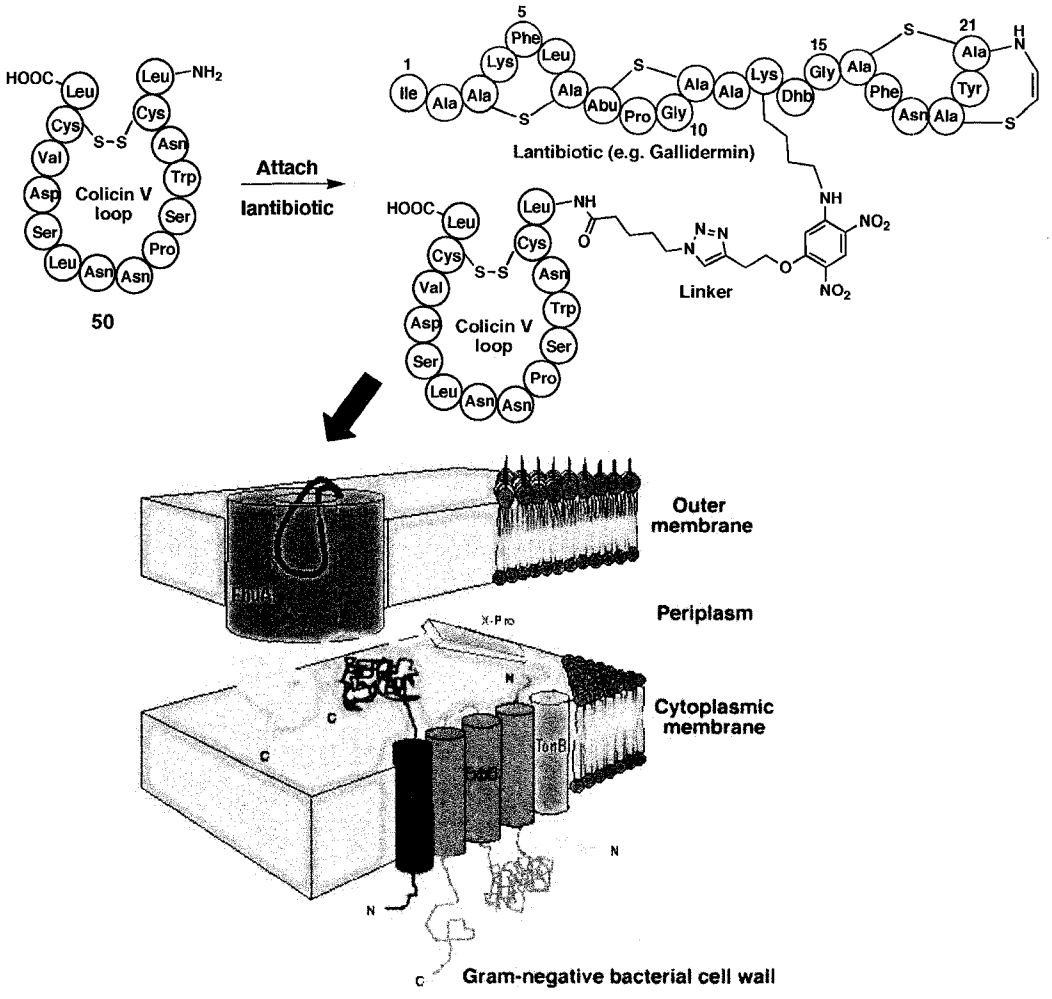
Figure 16. C-terminal sequence alignment of colicin V, microcin L and microcin E492

Colicin V	..PNPAMSPSGLGGTIKQKPEGIPSEAWNYAAGR**LCNWSPNNLSDVCL**
Microcin L	..VAALYDAAGNSNSAKQKPEGLPPEAWNYAEGR**MCNWSPNNLSDVCL**
Microcin E492	..PVNVPIPVLIGPSWNGSGSGYNSATSSSGSGS-Post-translationally modified

These observations suggest that the *C*-terminal 14 residues might be critical for recognition and active transport across the outer membrane of Gram-negative bacteria. Attachment of the 14 amino acid disulfide colicin V loop **50** to lantibiotics (e.g. gallidermin) via a short linker (Figure 17) could potentially lead to transport of

lantibiotics across the outer membrane of Gram-negative bacteria where the lantibiotic can interact with lipid II and exert its antimicrobial action.

Figure 17. Proposed mode of transport of lantibiotics across the outer-membrane of Gram-negative bacteria by exploiting FhuA-tonB active transport system



One goal of the project is to synthesize the colicin V loop **50** by solid phase peptide synthesis with various linkers that could be used for attachment to different lantibiotics.

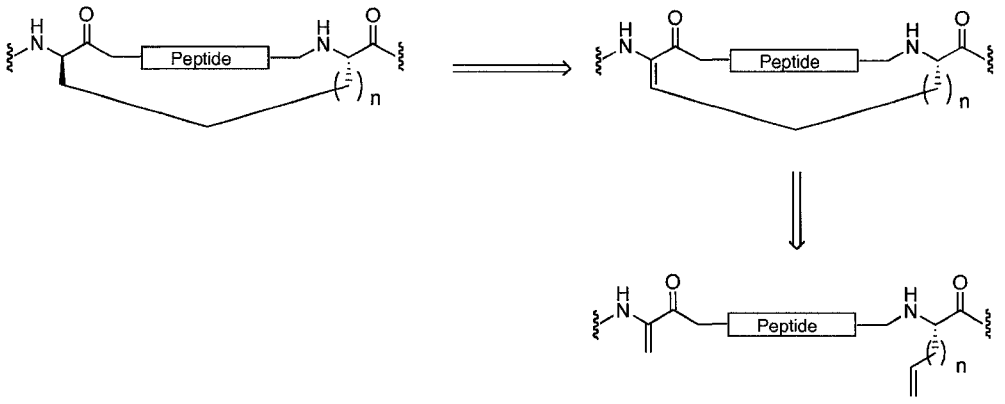
CHAPTER 2. RESULTS AND DISCUSSIONS

2.1. Methods for the chemical synthesis of lantibiotics and their analogues

2.1.1. Synthesis and testing of a carbocyclic-larger ring analogue of lacticin 3147 A2

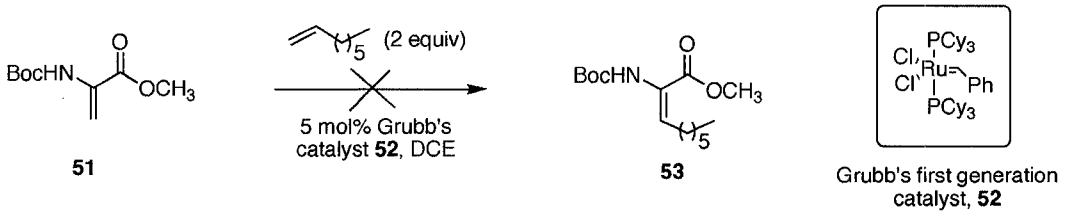
Ring-closing olefin metathesis (RCM) has emerged as one of the most powerful methods to make carbocyclic rings in natural products and peptides.[107, 108, 129] The discovery and development of olefin metathesis catalysts for practical applications led to the award of the Nobel Prize in chemistry for 2005 to Yves Chauvin, Robert H. Grubbs and Richard R. Schrock.[130, 131] The initial strategy envisioned for synthesis of carbocyclic rings A, B and C of lacticin A2 **44** was a ring-closing metathesis between an alkene side chain containing an amino acid and dehydroamino acid, followed by a stereoselective reduction of the resulting cyclic dehydroamino acid (Figure 18).

Figure 18. Construction of carbocyclic rings by ring-closing metathesis of dehydroamino acids.



Although there are a number of reports for the synthesis of carbocyclic rings using olefin-metathesis in peptides,[132] ring-closing metathesis of dehydroamino acids has always presented problems. For example, a cross-metathesis reaction between a dehydroamino acid **51** and an alkene attempted by Biagini *et al.* using Grubb's first generation catalyst **52** yields none of the desired product (Scheme 7).[133] This may be due to the electron-rich nature of the double bond in dehydroamino acids that may make it less reactive.

Scheme 7. Attempted cross-metathesis reaction of protected dehydroalanine by Biagini *et al.*



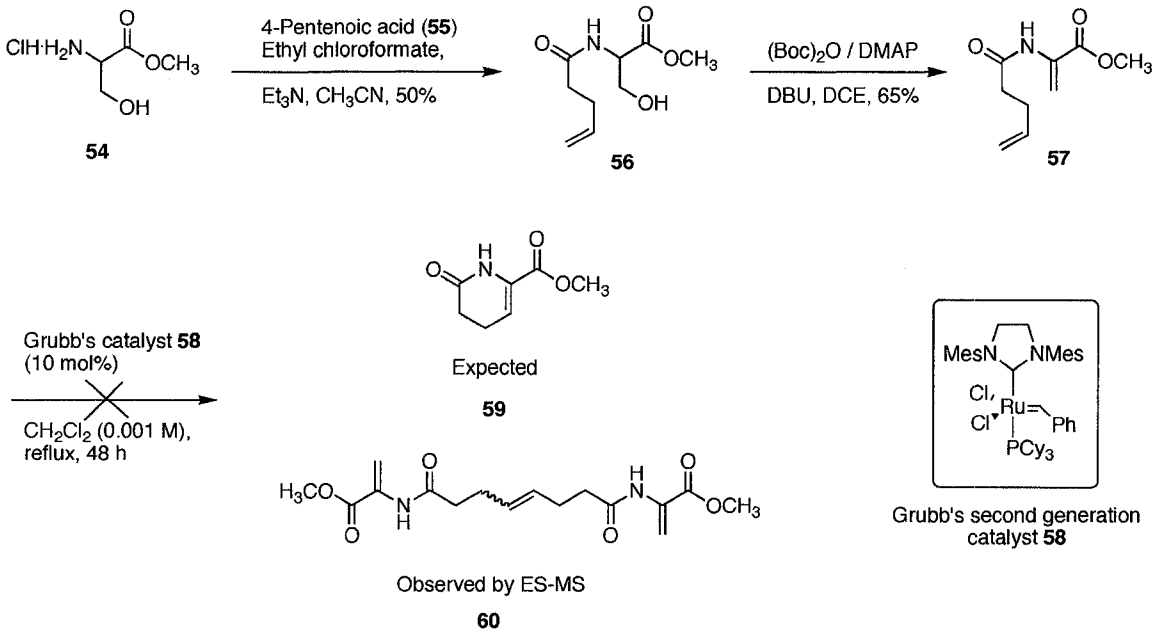
51 **53** Grubb's first generation catalyst, **52**

As there were no successful examples for ring closing olefin metathesis using dehydroamino acids, a methodology development was undertaken. Several substrates bearing an electron-withdrawing group on the amine of the amino acid with an alkene tether from either the *N*-terminal or the *C*-terminal side were designed.

To prepare the first substrate, commercially available D,L-serine methyl ester hydrochloride (**54**) is coupled with 4-pentenoic acid (**55**) by formation of a mixed anhydride using ethyl chloroformate to give **56** (Scheme 8). Activation of the serine hydroxyl of **56** with *di-tert*-butyl dicarbonate [$(Boc)_2O$] and 4-(*N*,*N*-

dimethylamino)pyridine (DMAP), according to the procedure by Ferreira and co-workers[134] followed by elimination with diazabicyclo[5.4.0]undec-7-ene (DBU) affords the dehydroalanine **57**. Attempted RCM reaction of dehydroalanine derivative **57** in the presence of Grubb's second-generation catalyst **58** yields none of the desired product **59**. Only the dimerized product **60**, formed via the alkene side chain, is detected by electrospray mass spectrometry (ES-MS). One possible reason for the result could be that the carbonyl group of the amide bond is not sufficiently electron withdrawing on the alkene of the dehydroamino acid to make it available for metathesis.

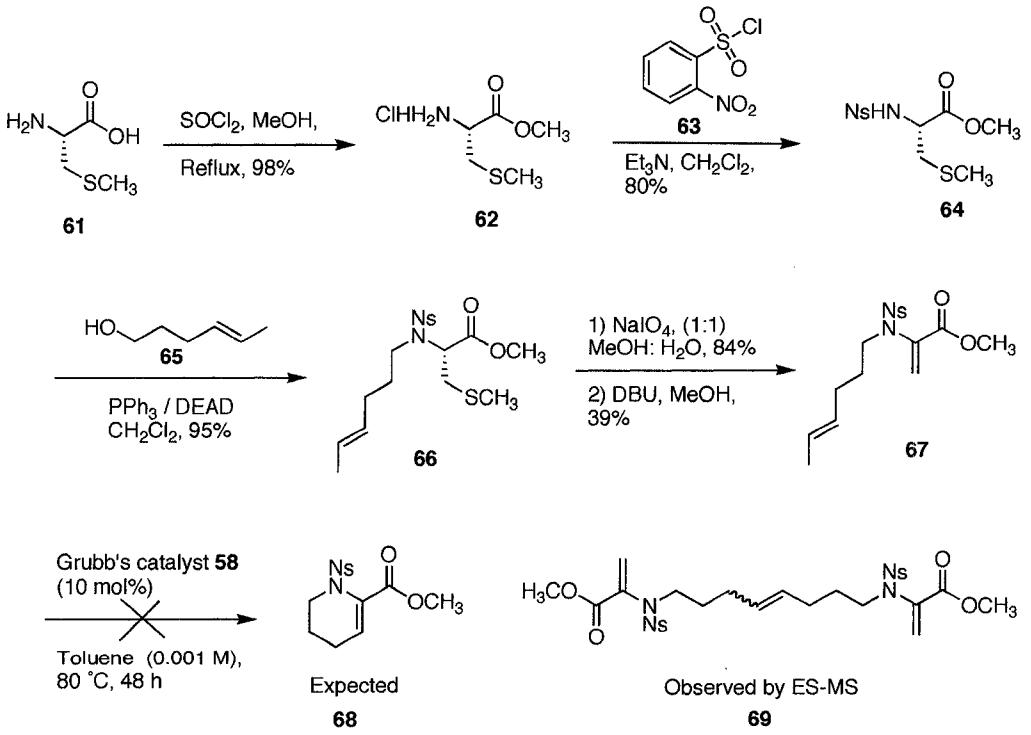
Scheme 8. Dehydroamino acid with a 5-pentenamide tether



To prepare a substrate with a stronger electron withdrawing 2-nitrobenzene-1-sulfonyl (*o*-nosyl) group, commercial *S*-methyl-L-cysteine (**61**) is converted to its methyl ester **62** with thionyl chloride followed by the reaction with 2-nitrobenzene-1-sulfonyl

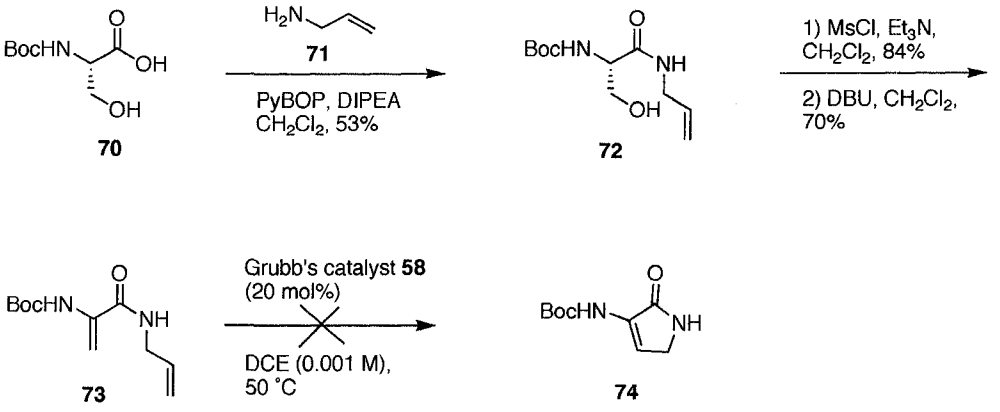
chloride (**63**) to provide **64** (Scheme 9). The NH of the sulfonamide of **64** is sufficiently acidic because of the *o*-nosyl group to undergo a Mitsunobu reaction with a primary alcohol.[135] Thus, reaction of commercially available (*E*)-hex-4-en-1-ol (**65**) with **64** in the presence of PPh_3 / DEAD gives **66**. For the formation of dehydroalanine, the sulfur atom in **66** is oxidized to the sulfoxide, and subsquent reaction with base affords the desired substrate **67**.[136] The RCM reaction attempted with Grubb's catalyst **58** under high dilution conditions in toluene affords none of the desired product **68**. Again, only the dimerized product **69** is observed by ES-MS.

Scheme 9. Dehydroamino acid with an electron-withdrawing *o*-nosyl group and a (*E*)-hex-4-en-1-ol tether



As a substrate with a highly electron-withdrawing *o*-nosyl group did not undergo RCM, the strategy was slightly revised. A substrate with an alkene tether attached to the *C*-terminal side of the amino acid was synthesized (Scheme 10). Thus, commercial Boc-Ser-OH (**70**) reacted with allylamine (**71**) to yield the amide **72**. The dehydroalanine derivative **73** is obtained by activation of the serine hydroxyl of **72** with methanesulfonyl chloride (MsCl) followed by elimination with DBU as the base. Attempted RCM reaction of compound **73** with Grubb's catalyst **58** results in none of the desired product **74**. However, in this case only the starting material is recovered. This could possibly be due to the deactivation of the catalyst **58** by co-ordination of **73** to the ruthenium centre in **58**.

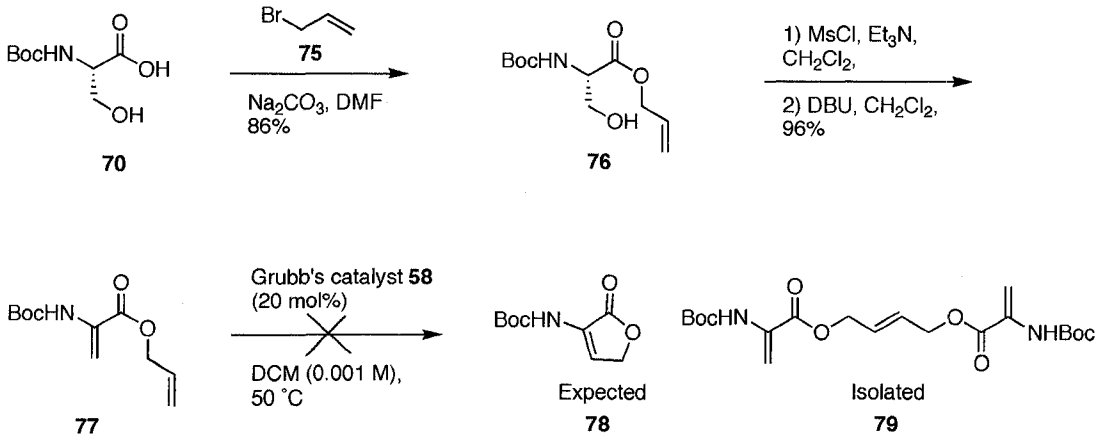
Scheme 10. Dehydroamino acid with an allylamide tether



Replacement of the NH group of the amide in **73** with an oxygen atom to give an ester could possibly overcome potential catalyst inactivation problems. The synthesis of such a substrate is initiated by protecting the free carboxylic acid of **70** as its allyl ester by reaction with allyl bromide (**75**). The fully protected dehydroalanine substrate **77** is

obtained as before by activation and elimination of the serine hydroxyl using MsCl and DBU. Reaction of **77** with Grubb's second-generation catalyst **58** yields only the dimerized starting material **79** (Scheme 11). The formation of the dimerized product with ester **77** supports the assumption that the catalyst might be deactivated in the metathesis reaction of secondary amide **73**. However RCM to yield the ring-closed product does not occur with an ester substrate either.

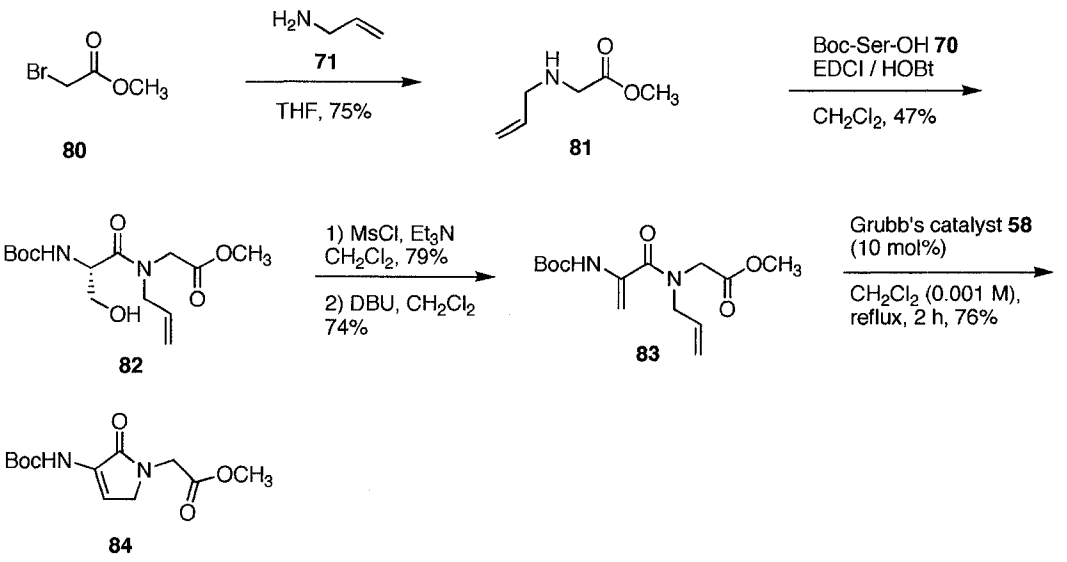
Scheme 11. Dehydroamino acid with an allylester tether



At this point, it seemed reasonable to reinvestigate the RCM reaction with a substrate similar to **73**. Possible deactivation of the catalyst by co-ordination could be avoided by having a tertiary amide instead of a secondary amide. The synthesis of a tertiary amide substrate begins by a nucleophilic displacement reaction of methyl bromoacetate (**80**) with allylamine (**71**) (Scheme 12). Coupling the resulting *N*-allylglycine methylester (**81**) to Boc-Ser-OH (**70**) with 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide (EDCI) and 1-hydroxybenzotriazole (HOBt) as the

coupling reagents yields the tertiary amide **82**. The dehydroalanine is introduced by the previously described activation and elimination protocol to afford **83**. The ring-closing reaction performed with Grubb's second-generation catalyst (**58**) in refluxing dichloromethane then successfully yields the desired ring-closed product **84**. This shows that the tertiary amide functionality could adopt a conformation favourable for ring closure and is likely less suitable for coordinating to the catalyst. The successful synthesis of **84** by ring closing metathesis on dehydroamino acids also uncovers a new route for accessing 'Freidinger lactam' type structures. Freidinger lactams are used as β-turn inducers in peptides and proteins for conformational studies.[137]

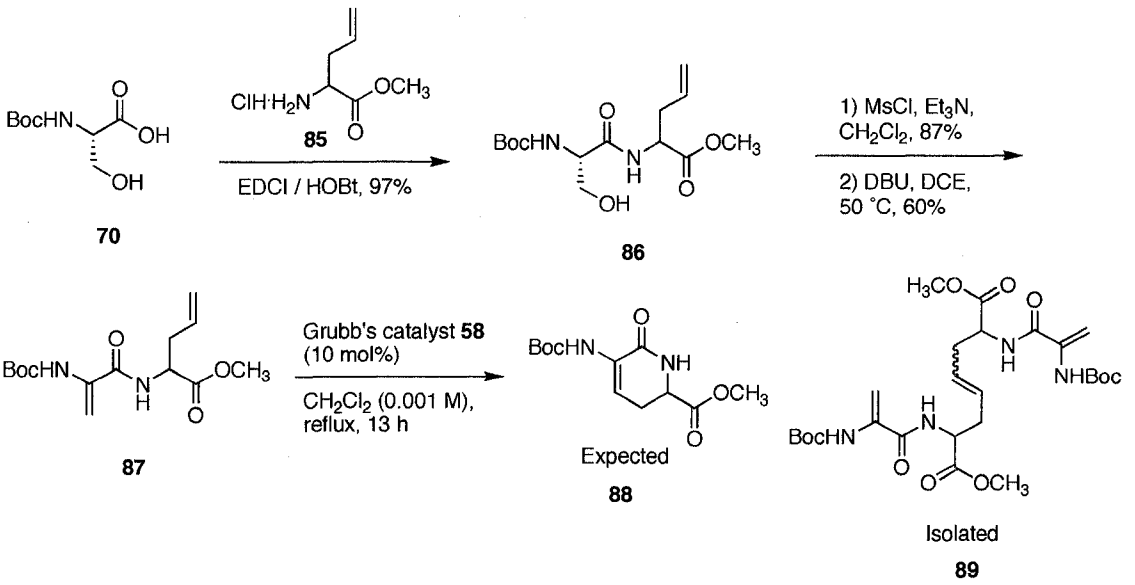
Scheme 12. Dehydroamino acid dipeptide with a tertiary allylamide tether



To further probe the importance of the tertiary amide functionality for ring-closure, a substrate wherein the allyl group is on the α-carbon of the *C*-terminal amino

acid of a dipeptide was designed. The synthesis commences with the coupling of Boc-Ser-OH (**70**) with D,L-allylglycine methyl ester hydrochloride (**85**) using EDCI and HOBt to provide the dipeptide **86** (Scheme 13). The dehydroalanine is produced by activation with MsCl, followed by elimination in the presence of DBU. A ring-closing reaction with catalyst **58** again yields only the dimerized product **89** and none of the desired cyclized 6-membered ring **88**. This result supports the hypothesis that a tertiary amide is required for successful metathesis of a dehydroamino acid.

Scheme 13. Dehydroamino acid-allylglycine dipeptide

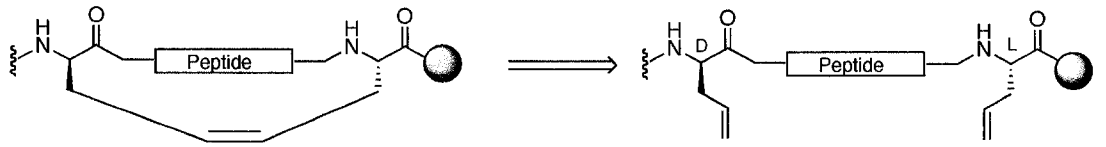


While further exploration of this hypothesis to make it amenable for synthesis of larger carbocyclic peptide rings was underway, an article was published by Chen *et al.* describing ring-closing methathesis on dehydroamino acids to make 3-amino-2-pyridone libraries.[138] As the results and conclusions drawn by the authors were very similar to the

results uncovered here, it left little room for expansion and any further exploration was abandoned.

An alternative approach was simultaneously investigated in the Vederas group for the synthesis of carbocyclic rings in peptides by using ring-closing metathesis of two allylglycine residues (Figure 19).[107, 139]

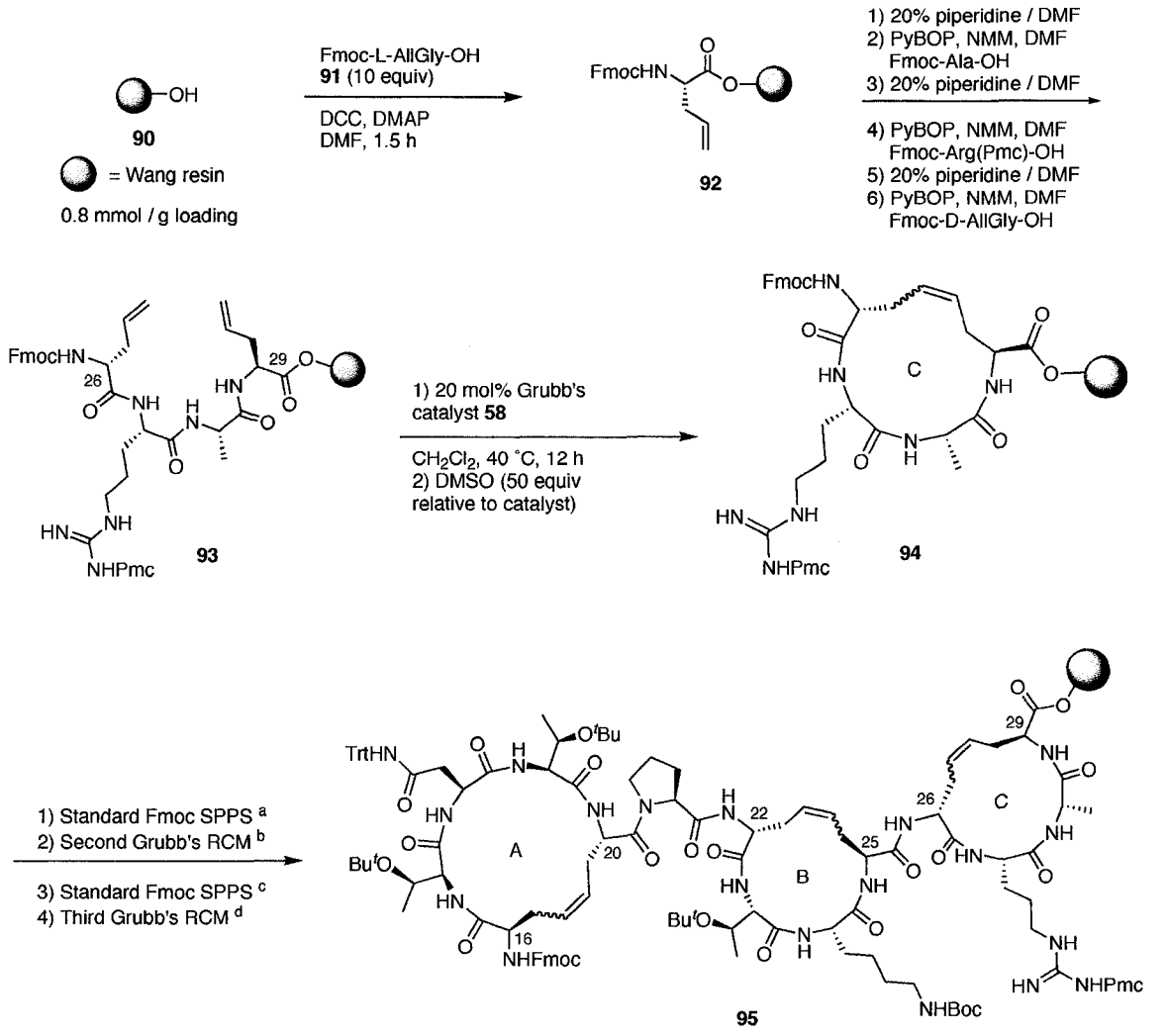
Figure 19. Ring-closing olefin metathesis of two allyl glycine residues



Based on the above strategy, a graduate student in the Vederas group, Jake L. Stymiest (Ph.D. thesis, 2005)[140] prepared carbocyclic rings A, B and C of **43**. A brief description of the synthesis has been included for clarity and completeness (Scheme 14).

The synthesis of carbocyclic rings begins with the loading of Fmoc-L-AllGly-OH onto Wang resin with a substitution capacity of 0.8 mmol / g. Standard Fmoc-SPPS is then performed to introduce residues (26→28). For the formation of the first carbocyclic ring C, reaction of **93** with 20 mol% Grubb's second-generation catalyst **58** on solid support affords the metathesized product **94**.

Scheme 14. Synthesis of carbocyclic rings A, B and C of **43**



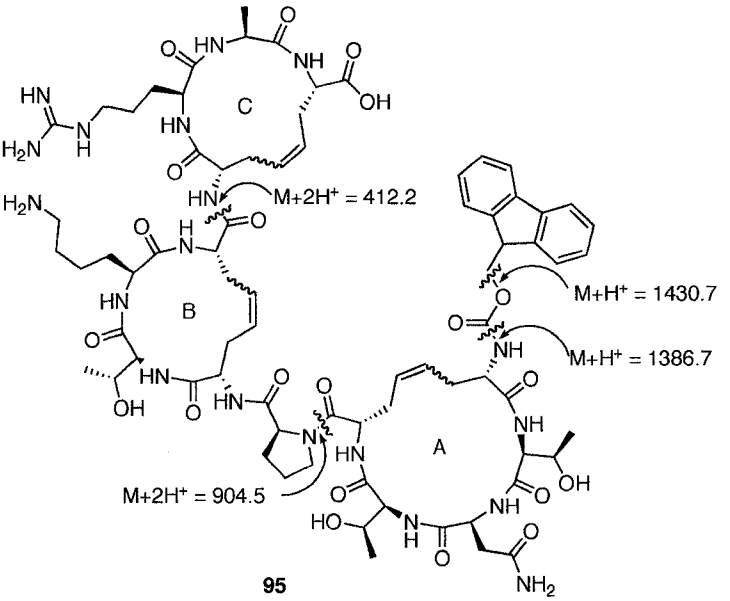
[a] Fmoc-amino acids were coupled in the following order with PyBOP: (i) Fmoc-L-AllGly-OH, (ii) Fmoc-Lys(Boc)-OH, (iii) Fmoc-Thr(ᵗBu)-OH, (iv) Fmoc-D-AllGly-OH [b] RCM with 20 mol% **58**, 12 h [c] Fmoc-amino acids were coupled in the following order with PyBOP: (i) Fmoc-Pro-OH (ii) Fmoc-L-AllGly-OH, (iii) Fmoc-Thr(ᵗBu)-OH, (iv) Fmoc-Asn(Trt)-OH, (v) Fmoc-Thr(ᵗBu)-OH, (vi) Fmoc-D-AllGly-OH [d] RCM with 50 mol% **58**, 48 h.

Fmoc-SPPS is continued to introduce residues (22→25) to give the linear precursor to ring B. RCM reaction performed on the linear precursor using 20 mol% catalyst **58** affords carbocyclic ring B cleanly. The linear precursor to ring A, residues (16→21), is introduced by Fmoc-SPPS. A third olefin metathesis with 50 mol% **58** and longer

reaction time yields the carbocyclic rings A, B and C (**95**) successfully on solid-support. Attempts to reduce the olefinic bonds in the rings did not yield any of the saturated product.

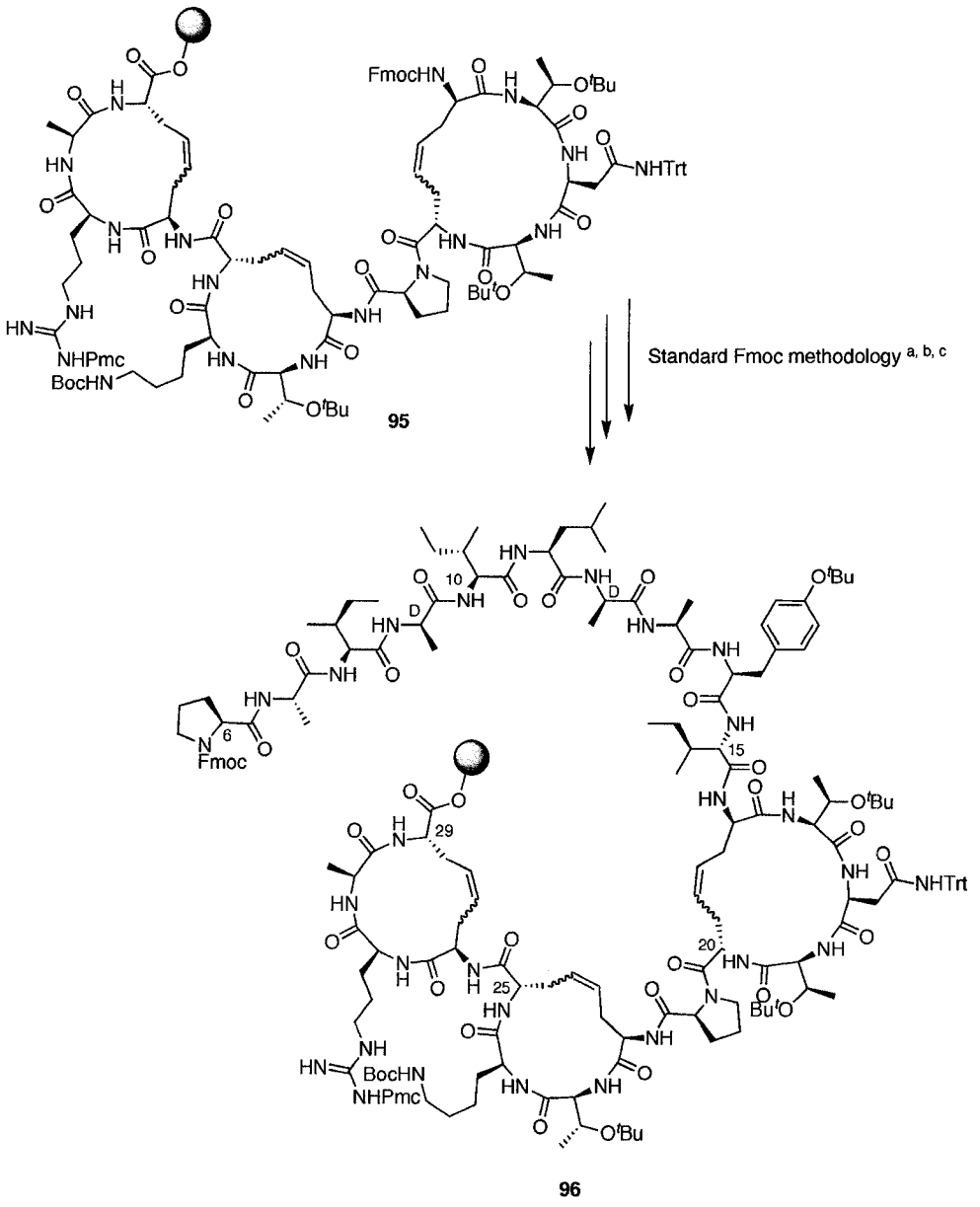
The carbotricyclic peptide **95** was then incorporated in the current project for further analysis and elaboration. A small sample of the tricyclic peptide was liberated from the resin using (95:2.5:2.5) TFA:TIPS:H_2O and used for analysis. To confirm that compound **95** had no unwanted cross-over products which can arise from ring-opening of one of the pre-formed carbocyclic rings followed by subsequent incorrect ring-closure, a MS / MS analysis was performed. Analysis of the peaks obtained indicated masses of fragments corresponding to ring C (412.2), ring CB (904.5) and ring CBA (1386.7). No peaks corresponding to the formation of crossover products were observed (Figure 20).

Figure 20. LC-MS / MS analysis of carbatricyclic rings A, B and C (**95**)



For further elaboration of the carbatricycle **95** to obtain **96** (Scheme 15), the Fmoc group is removed with 20% piperidine in DMF. The amino acids comprising residues (10→15) are introduced in the following sequence with benzotriazole-1-yl-oxy-trispyrrolidinophosphonium hexafluorophosphate (PyBOP) as the coupling reagent: Fmoc-Ile-OH, Fmoc-Tyr(^{t}Bu)-OH, Fmoc-Ala-OH, Fmoc-D-Ala-OH, Fmoc-Leu-OH, Fmoc-Ile-OH. To avoid any on-resin aggregation due to the presence of a continuous stretch of hydrophobic residues, the solvent is changed from DMF to *N*-methylpyrrolidinone (NMP). It has been shown that NMP prevents aggregation and helps in synthesizing difficult sequences.[141] Removal of the Fmoc group during the introduction of residues (6→9) is done with 2% DBU in NMP and coupling of amino acids are done with PyBOP in the following order: Fmoc-D-Ala-OH, Fmoc-Ile-OH, Fmoc-Ala-OH, Fmoc-Pro-OH. The use of piperidine was avoided for removing Fmoc from residues (6→9), as any traces of piperidine could lead to unwanted Michael addition reactions with the dehydroamino acids during introduction of residues (1→5) of **43**. Cleavage of a small sample with TFA and analysis by MALDI-TOF MS indicated a peak at 2605.8 (M+H) consistent with the calculated mass of **96**.

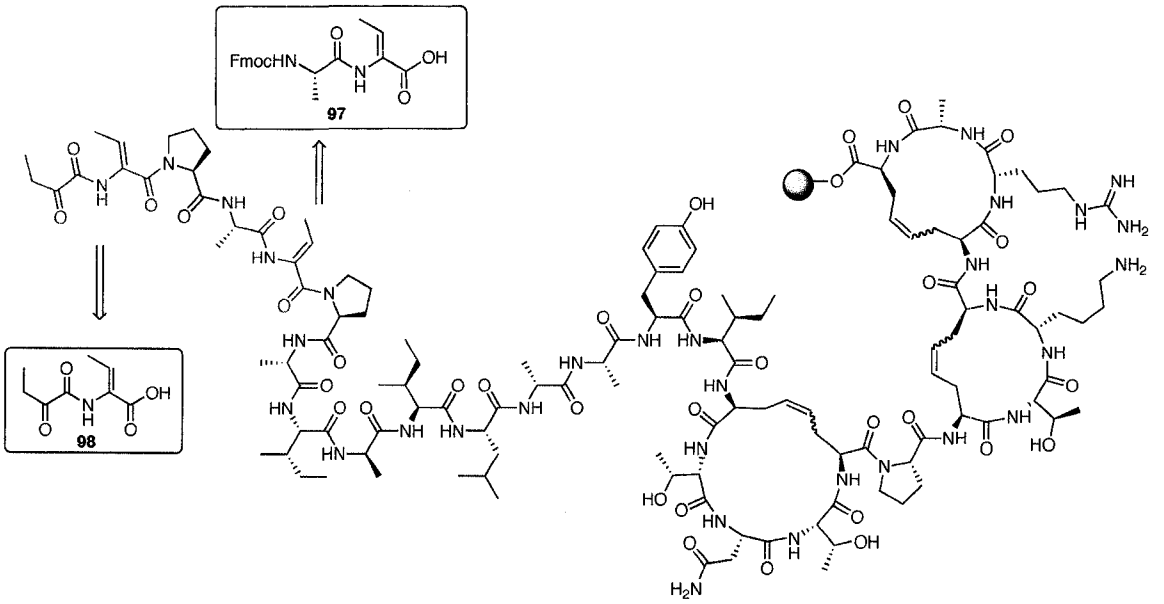
Scheme 15. Introduction of residues (6→15) of **43** by standard Fmoc methodology



[a] Conditions used for synthesis of **96** from **95**: (i) 20% piperidine in DMF, (ii) PyBOP, NMM, DMF, (iii) Fmoc-Ile-OH. [b] Repeat (i) and (ii) for amino acids: (iv) Fmoc-Tyr(ᵗBu)-OH, (v) Fmoc-Ala-OH, (vi) Fmoc-D-Ala-OH, (vii) Fmoc-Leu-OH, (viii) Fmoc-Ile-OH [c] Solvent was changed from DMF to NMP (ix) 2% DBU in NMP, (x) PyBOP, NMM, NMP, Repeat (ix) and (x) for amino acids (xi) Fmoc-D-Ala-OH, (xii) Fmoc-Ile-OH, (xii) Fmoc-Ala-OH (xiv) Fmoc-Pro-OH.

The strategy envisioned for the synthesis of highly modified residues (1→5) of **43** is outlined in Figure 21. The two dehydrobutyrines and the α-ketoamide could be synthesized in solution as dehydrobutyrine dipeptides **97**, **98** and coupled to peptide **96** on solid-support.

Figure 21. Dehydrodipeptide strategy for introducing residues (1→5) of **43**



The synthesis of the dipeptide **97** begins with coupling of Boc-Ala-OH (**99**) to L-threonine methyl ester hydrochloride (**100**) using PyBOP to give the dipeptide **111** (Scheme 16). To introduce the dehydrobutyrine, the hydroxyl group of threonine is activated with MsCl. Treatment with DBU to eliminate the mesylate yields **112**. The elimination proceeds stereospecifically in an *anti* fashion to give only the *Z*-isomer. A

further confirmation of the *Z*-geometry is provided by an X-ray crystallographic structure of **112** (Figure 22). Treatment of the dipeptide **112** with 1 M LiOH hydrolyses the methyl ester to give the free carboxylic acid **113**. The Boc group of **113** is exchanged for a Fmoc group suitable for solid phase synthesis, by a two-step procedure. The Boc group is cleaved under TFA conditions, and the salt is dissolved in 10% $NaHCO_3$, followed by reaction with *N*-(9-fluorenylmethoxycarbonyloxy) succinimide (Fmoc-OSu) **114** to afford the desired dehydrodipeptide **97**.

Scheme 16. Dehydrodipeptide strategy: synthesis of dipeptide **97**

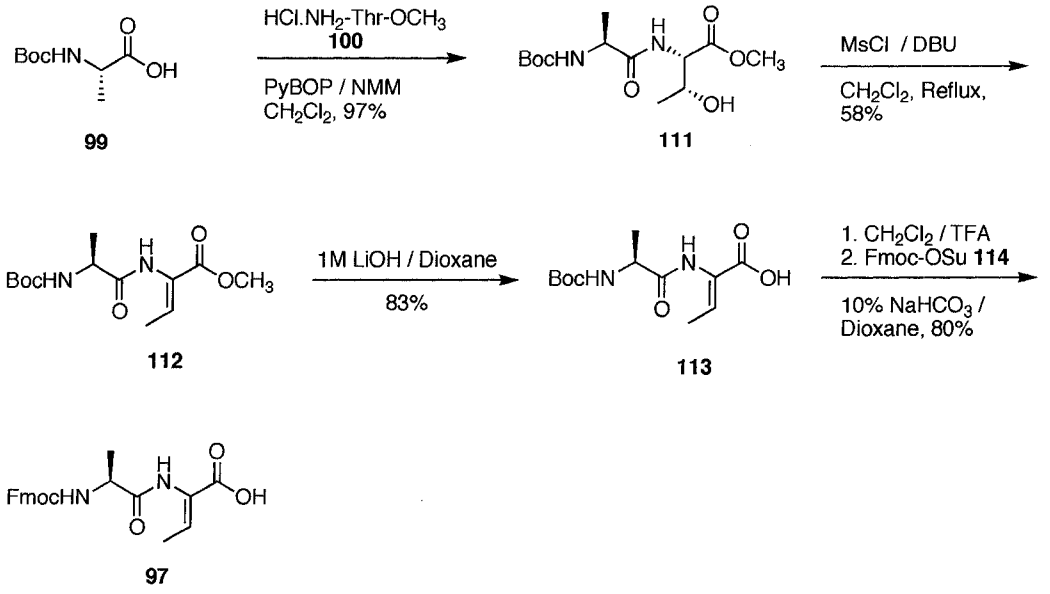
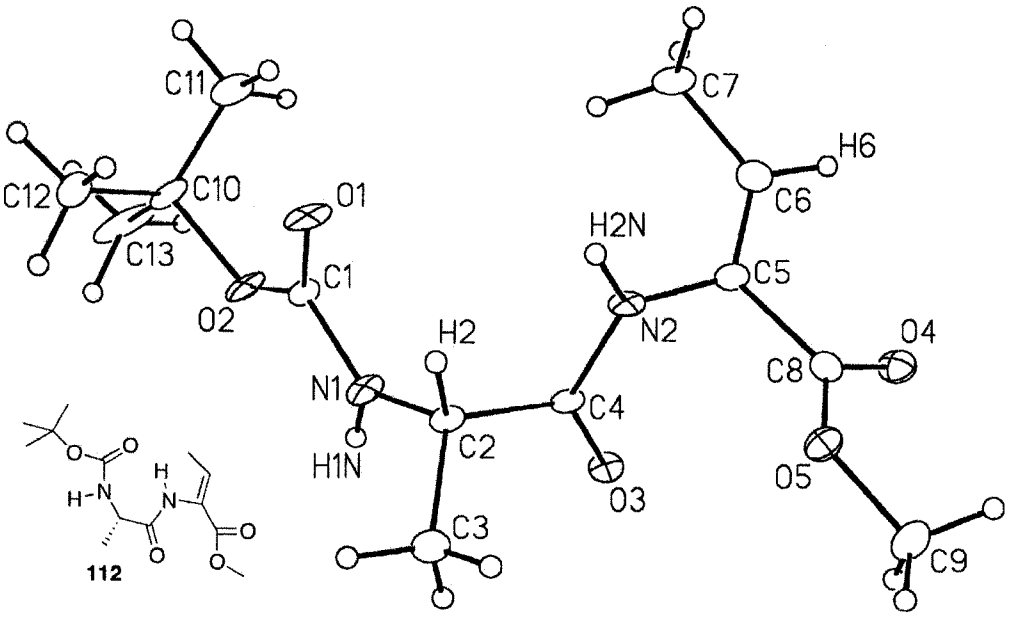
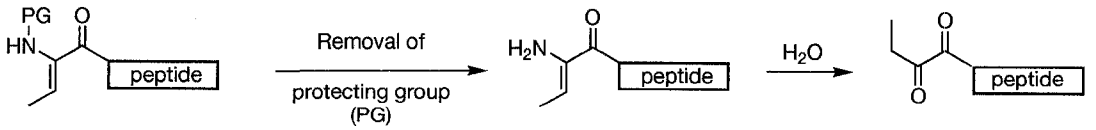


Figure 22. ORTEP diagram of the dehydrobutyrine **112**



For incorporating the α-ketoamide a strategy involving spontaneous deamination of a dehydrobutyrine residue was planned (Figure 23).[142]

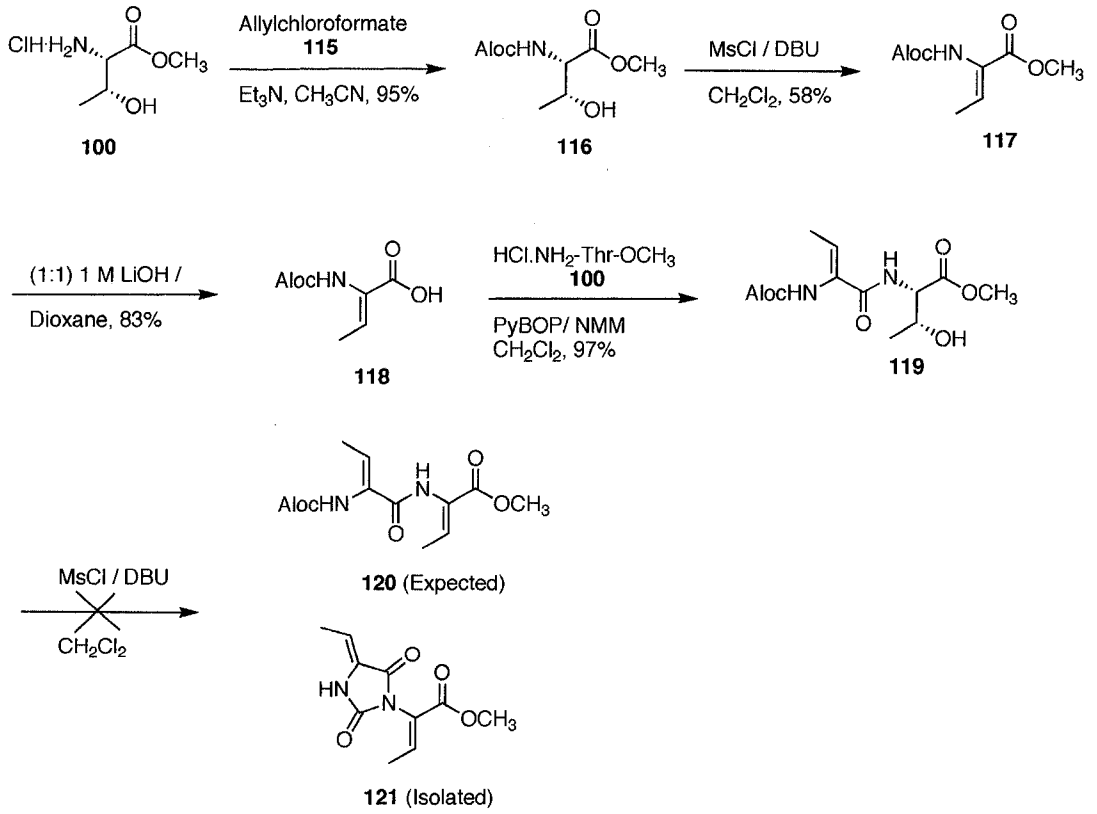
Figure 23. Strategy for formation of α-ketoamide



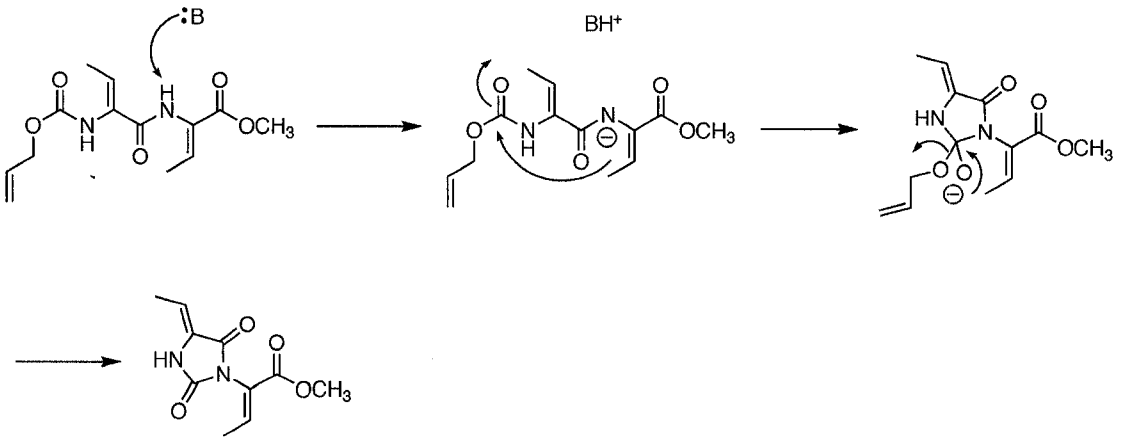
The attempted synthesis of precursor to **98** begins with allyloxycarbonyl (Aloc) protection of commercially available threonine ester **100** with allyl chloroformate (**115**)

to give **116** (Scheme 17). The dehydrobutyrine residue is introduced as before by the activation and elimination procedure to yield peptide **117**, which is then hydrolyzed with LiOH to form the carboxylic acid **118**. A PyBOP mediated coupling of **118** with **100** yields the dipeptide **119**. The attempt to introduce the second dehydrobutyrine by elimination with MsCl / DBU unexpectedly yields none of the desired product **120**, but only the corresponding hydantoin derivative **121**. It is possible that the second dehydrobutyrine places the dipeptide in a conformation suitable for formation of the hydantoin. A proposed mechanism for formation of **121** is shown in Scheme 18.

Scheme 17. Dehydrodipeptide strategy: attempted synthesis of precursor to dipeptide **98**

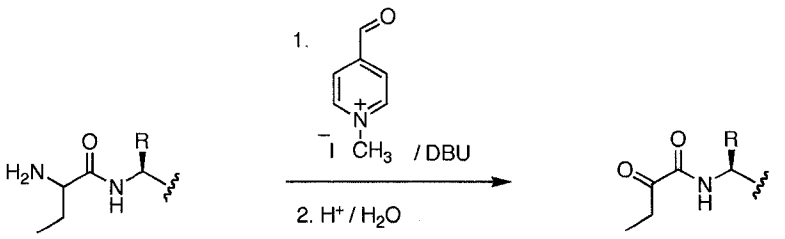


Scheme 18. Proposed mechanism for the formation of the hydantoin **120**



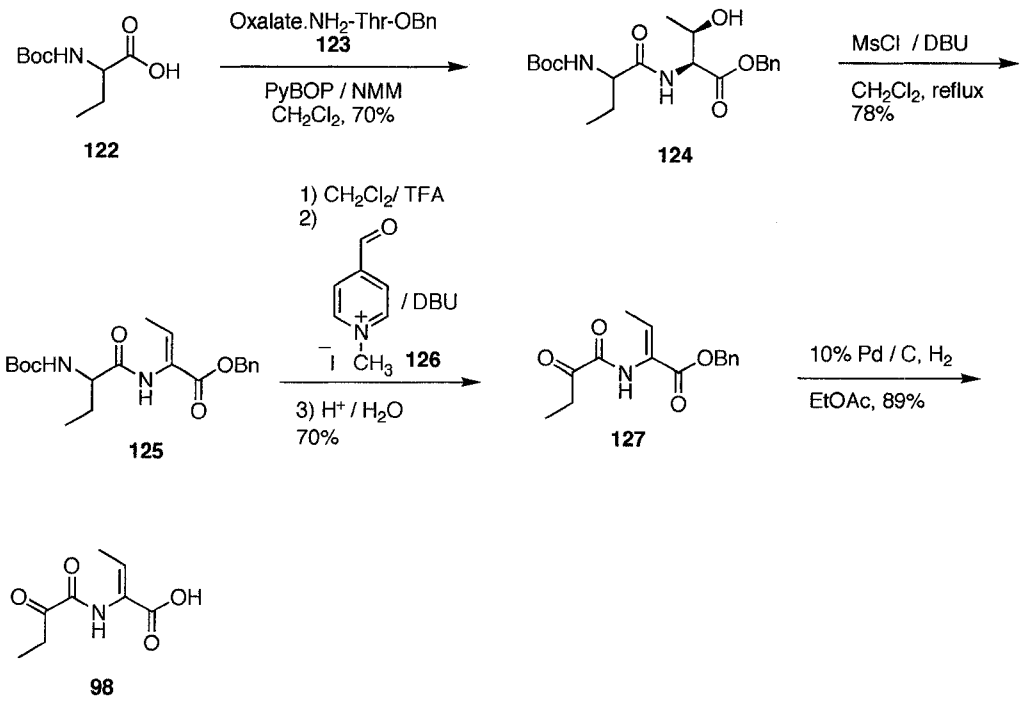
With the failure of the spontaneous deamination strategy, a new biomimetic strategy akin to pyridoxal phosphate chemistry[143] was devised for the introduction of α-ketoamide. It involves a transamination reaction with 4-pyridinium carboxaldehyde,[144, 145] a synthetic equivalent of pyridoxal phosphate (Figure 24).

Figure 24. Transamination strategy for formation of α-ketoamide



The synthesis of a dipeptide suitable for transamination starts with a coupling reaction between commercially available Boc-Abu-OH (**122**) and L-threonine benzyl ester oxalate (**123**) mediated by PyBOP to give the dipeptide **124** (Scheme 19). Activation and elimination of the side chain hydroxyl of **124** with MsCl and DBU yields the dehydrodipeptide **125** in good yield. To perform the transamination reaction, the Boc group of **125** is removed in the presence of TFA to yield the corresponding salt. The unprotected amine then reacts with 4-pyridinecarboxaldehyde-methyl iodide salt (**126**). The primary imine is then isomerized to a secondary imine with DBU and hydrolysis under aqueous acidic conditions yields the protected α-ketoamide dipeptide **127**. Dehydroamino acids are known to undergo selective reduction in the presence of Rh catalysts only under high hydrogen pressures.[146] Based on this observation, it seemed possible to remove the benzyl group selectively in the presence of the dehydroamino acid double bond with 10% Pd / C under 1 atm of hydrogen. Reaction of the α-ketoamide dipeptide **127** under the conditions described with ethyl acetate as the solvent yields the dipeptide **98** with ≥ 95% selectivity for the desired product. The success of the reaction depends on careful monitoring of its progress, followed by immediate quenching upon completion in order to avoid any over reduced product.

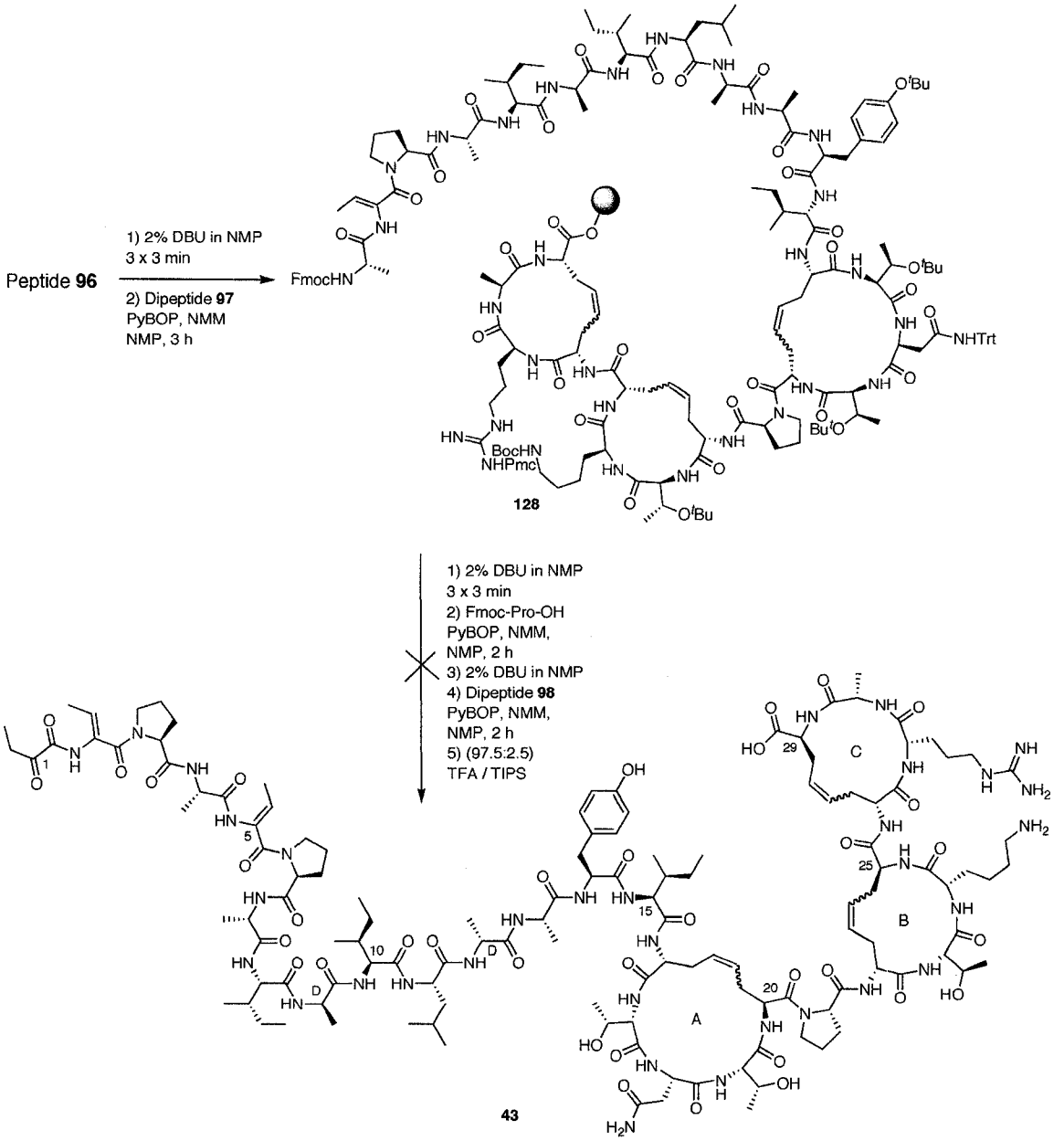
Scheme 19. Synthesis of dipeptide **98** by transamination strategy



Having obtained the dipeptides **97** and **98** by solution phase synthesis, the focus was shifted towards coupling the dehydrodipeptides to resin-bound **96**. The Fmoc group of the *N*-terminal amino acid residue of **96** is removed with freshly prepared 2% DBU in NMP. The dehydrodipeptide **97** is activated with PyBOP in the presence of *N*-methylmorpholine (NMM) and is reacted with the free amine on solid-support for 3 h (Scheme 20). Treatment of a 20 mg sample of the resin with TFA yields the crude peptide. Subsequent analysis by MALDI-TOF MS indicates the successful formation of **128**. Treatment of **128** with 2% DBU removes the *N*-terminal Fmoc group and the free amine couples to Fmoc-Pro-OH under standard coupling conditions. The Fmoc group of the coupled proline is then liberated with 2% DBU in NMP. Unfortunately, an attempt to

couple the α-ketoamide dipeptide **98** with PyBOP, disappointingly yields none of the final product **43**.

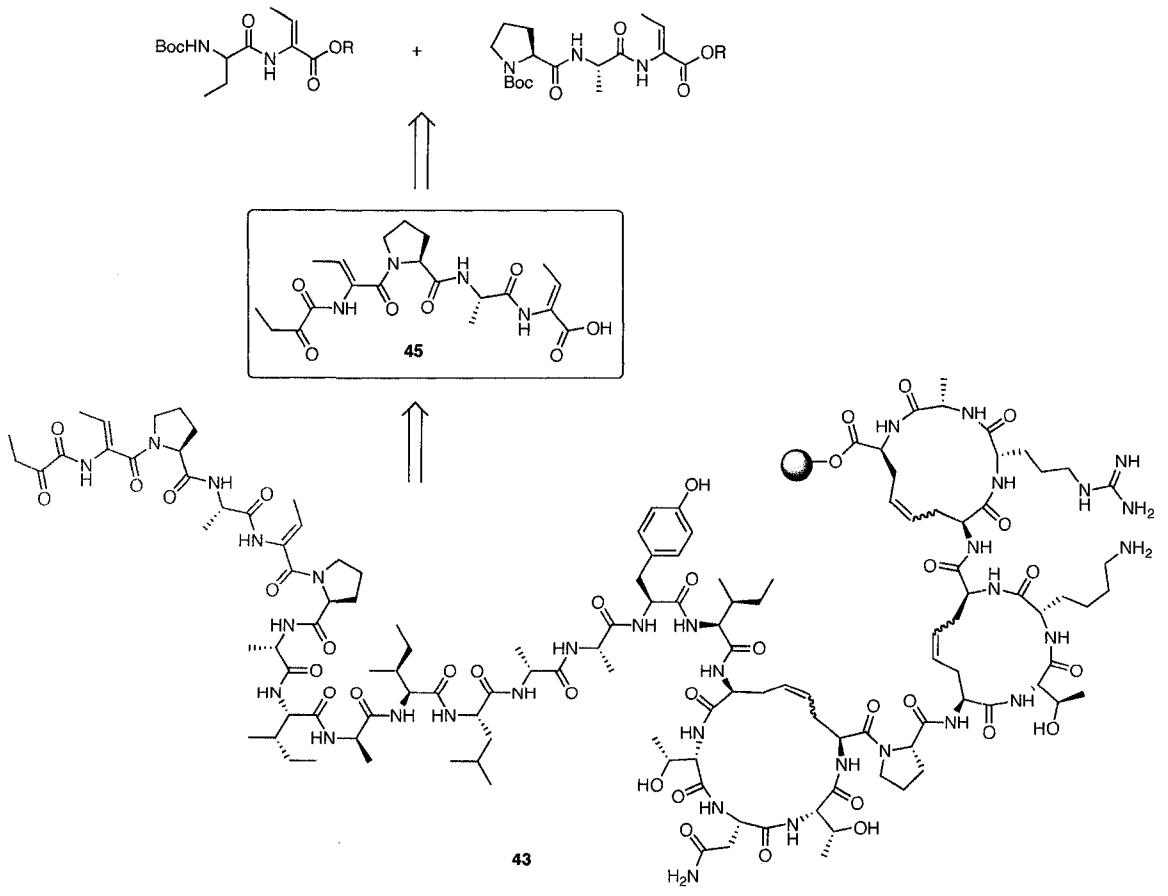
Scheme 20. Attempted coupling of dehydrodipeptides **97** & **98** towards synthesis of **43**



One reason for this result could be secondary reactions of the ketocarbonyl group upon activation of the *C*-terminus and decomposition making the dipeptide unavailable for coupling. An uncharacteristic color change was observed upon activation of the dipeptide **98** with PyBOP.

As the dehydrodipeptide approach was unsuccessful, a revised strategy was planned, wherein the residues (1→5) of **43** with all the modifications would be synthesized in solution phase as a pentapeptide and then coupled to the peptide on solid phase (Figure 25).

Figure 25. Dehydropentapeptide strategy for introduction of residues (1→5) **[45]** of **43**

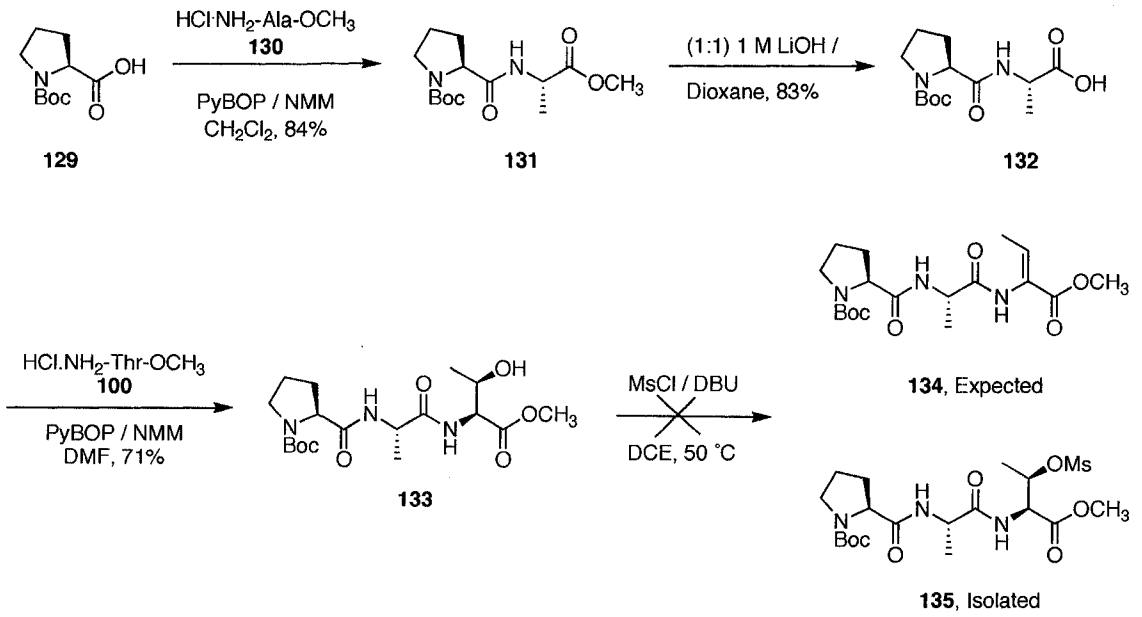


The synthesis of the dehydrotripeptide corresponding to residues (3→5) of the analogue **43** is initiated by coupling Boc-Pro-OH (**129**) with L-alanine methyl ester hydrochloride (**130**) to give the dipeptide **131** (Scheme 21, Strategy A). The methyl ester of the dipeptide undergoes hydrolysis under aqueous alkaline conditions to give the free carboxylic acid **132**, which then couples to the ester **100** to afford the tripeptide **133**. Treating the tripeptide with MsCl / DBU for formation of dehydrobutyrine surprisingly leads only to the mesylated intermediate **135** and none of the required product **134**. Attempted elimination of the mesylated intermediate with different bases such as NMM, pyridine, triethylamine (Et_3N) and *N*,*N*-diisopropylethyl amine (DIPEA), still does not yield the tripeptide **134**. This could be due to the conformation that the mesylate intermediate **134** adopts. The *N*-terminal proline residue with a sterically bulky Boc group can fold back on itself disallowing the approach of any base and thus hindering elimination.

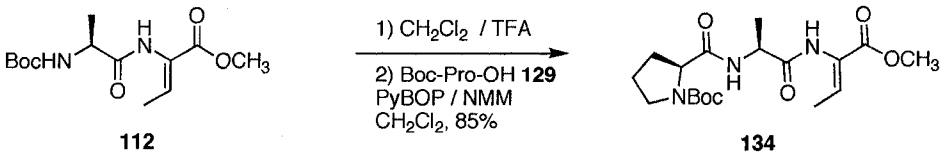
However, the tripeptide **134** is accessible by a different route using the previously synthesized dipeptide intermediate **112**. The Boc group of the dipeptide **112** is removed under acidic conditions and Boc-Pro-OH (**129**) couples to it in the presence of PyBOP to result in the desired tripeptide **134** (Scheme 21, Strategy B).

Scheme 21. Dehydropentapeptide strategy: synthesis of dehydrotripeptide **134**

Strategy A



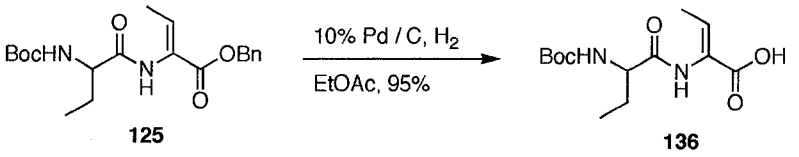
Strategy B



The dehydrodipeptide fragment **136** required for coupling to the dehydrotripeptide is obtained by removal of the benzyl group of previously synthesized dipeptide **125** with 10% Pd / C in ethyl acetate in excellent yield (Scheme 22). However, along with the product **136**, ~5% of the unwanted over reduced product is also observed

in ^{1}H NMR. Repeating the reaction did not result in any improvement, even when the reaction is strictly monitored and quenched. This could be a result of the differing activity of 10% Pd / C from different batches purchased. However, it was decided to use this material to test whether the dehydropentapeptide **45** could be obtained via the planned strategy.

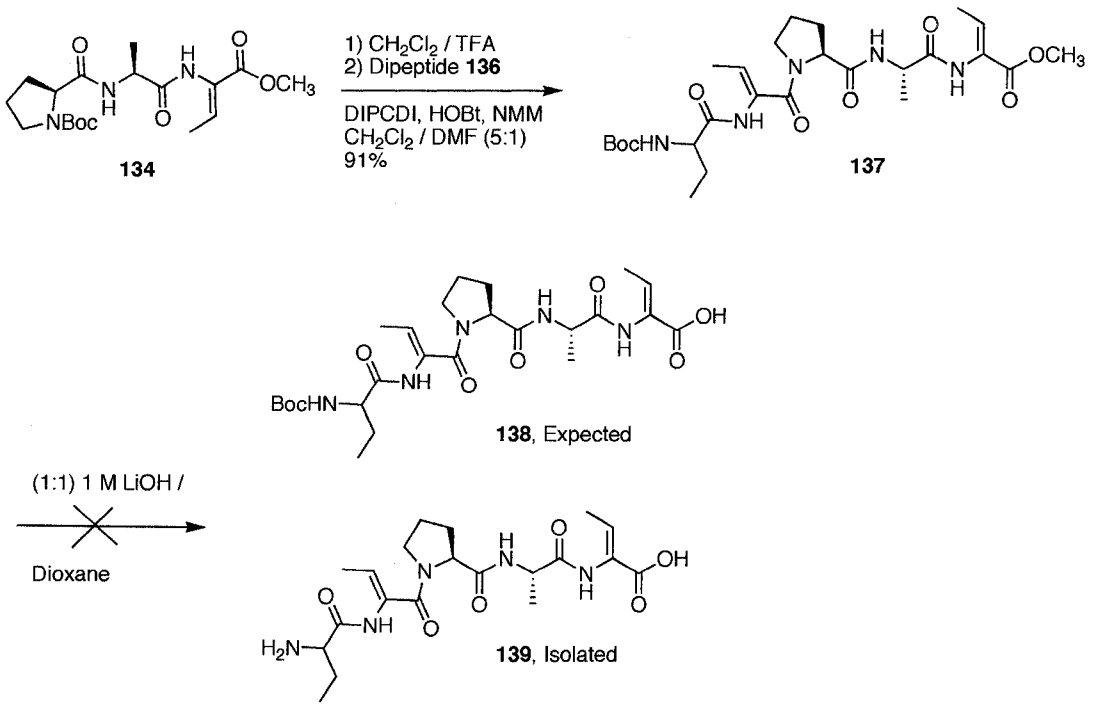
Scheme 22. Dehydropentapeptide strategy: synthesis of dehydrodipeptide **136**



125 **136**

The Boc group of the tripeptide **134** is cleaved with TFA in CH_2Cl_2 and the amine salt is neutralized with NMM. The dipeptide **136** reacts with it in the presence of diisopropylcarbodiimide (DIPCDI) and HOBt to yield the pentapeptide **137** in excellent yield (Scheme 23). At this point, it was decided to convert the methyl ester to an allyl ester, as removal of the methyl ester after introducing the α-ketoamide could possibly lead to undesired aldol type reactions involving the 1,2-diketone.[147] However, treating the pentapeptide with 1 M LiOH in 1,4-dioxane as the co-solvent leads to an unanticipated product **139**, which is missing the Boc group compared to the expected product **138**.

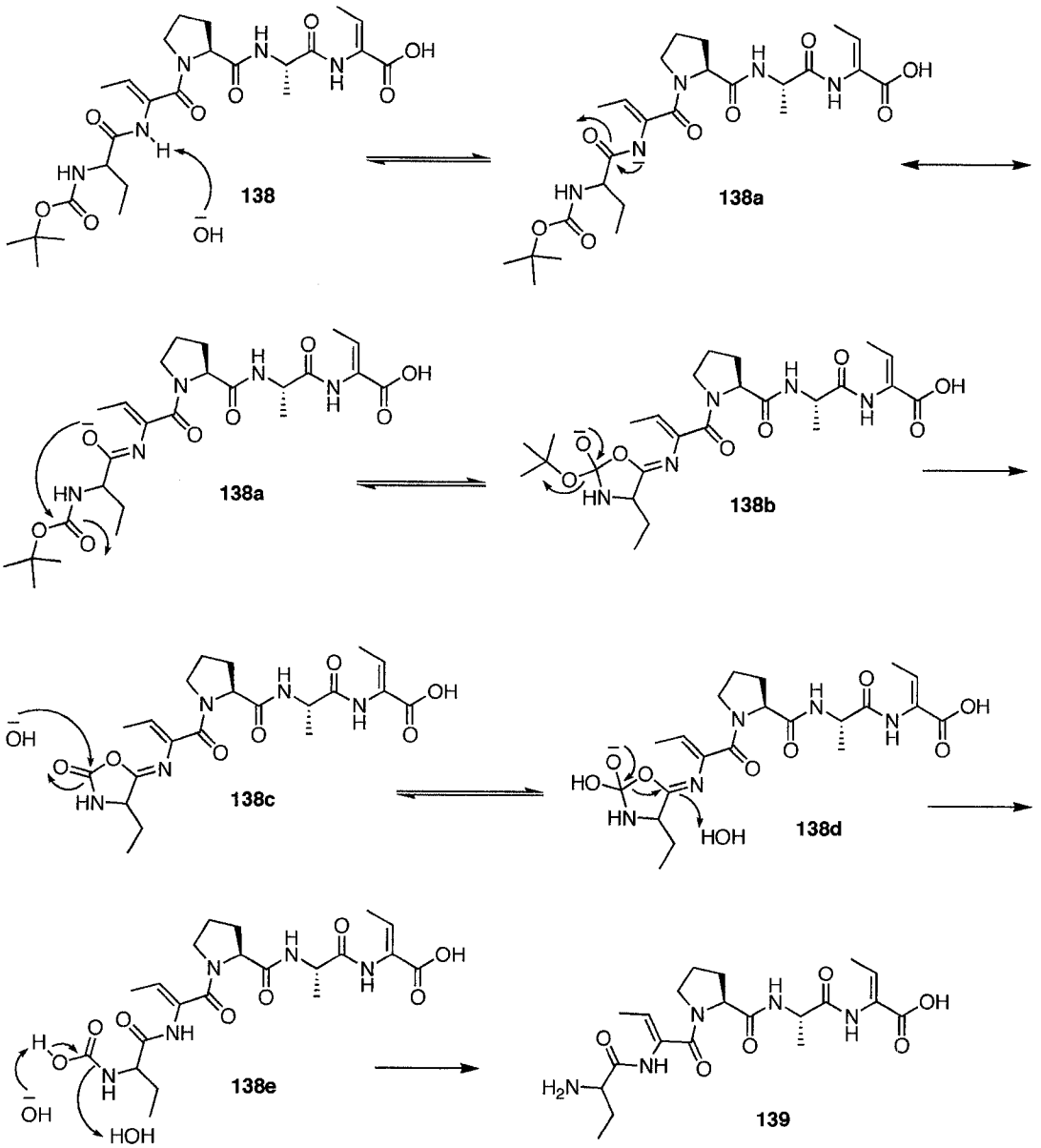
Scheme 23. Dehydropentapeptide strategy: coupling of dehydrodipeptide **136** to dehydrotripeptide **134**



Generally, cleavage of the Boc group occurs only under acidic conditions and it is rather unconventional for such a reaction to take place under basic conditions. On closer examination, an explanation could be offered for this anomalous result. Because of the lower acidity of the hydrogen of the NH group of the neighboring dehydroamino acid, it could play an assistive role in the cleavage of the Boc group. A proposed mechanism for the formation of **139** is shown in Scheme 24. The initial step is the deprotonation of **138** to form **138a**, followed by the attack on the carbonyl carbon of the carbamate group. The resulting tetrahedral intermediate **138b** could collapse with the liberation of *tert*-butoxide and form the hydantoin intermediate **138c**. Under strong aqueous alkaline conditions, the

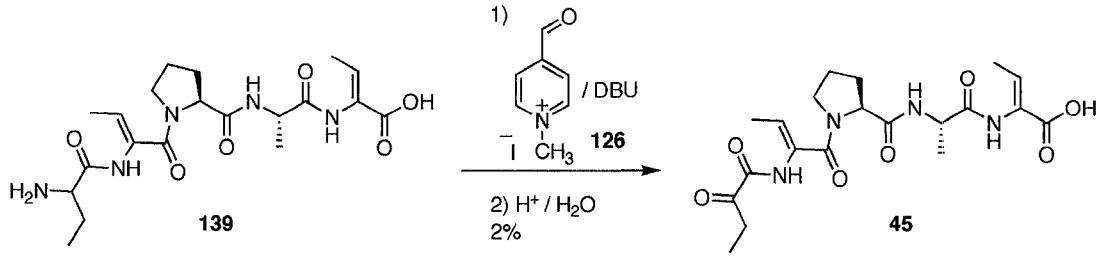
hydantion ring could be opened with hydroxide nucleophile leading to the formation of the carbamic acid **138e**. It could then undergo a spontaneous decarboxylation to form **139**.

Scheme 24. Proposed mechanism for formation of **139**



With pentapeptide **139** in hand, a transamination reaction was attempted to study the feasibility of using such an approach for the introduction of the α-ketoamide group with a highly modified pentapeptide. The pentapeptide **139** reacts with 4-pyridinecarboxaldehyde-methyl iodide salt (**126**) to form the imine; isomerization with DBU and hydrolysis under aqueous acidic conditions gives the desired pentapeptide **45**, albeit in an extremely poor yield of 2% (Scheme 25). However, this result clearly suggests that the transamination strategy could be successfully used to make **45**.

Scheme 25. Attempted transamination reaction with compound **139**

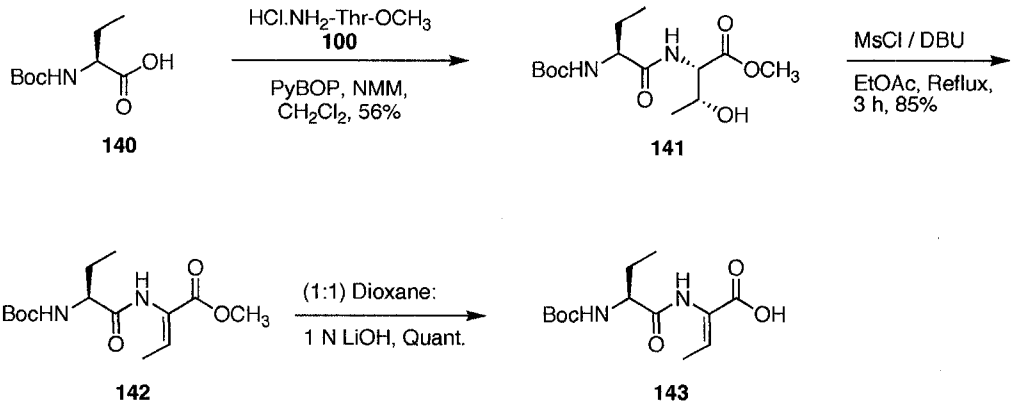


At this point, it was decided to use an allyl protection for the carboxyl terminus, as it would eliminate the problems encountered during the hydrolysis of the methyl ester of pentapeptide **137**. The allyl group can be removed under neutral conditions with tetrakis(triphenylphosphine)palladium(0) [$Pd(PPh_3)_4$] and is sufficiently orthogonal to the reaction conditions used in the formation of the intermediate tripeptide **134**.

The synthesis of the dehydrodipeptide **143** is initiated by coupling Boc-L-Abu-OH (**140**) with threonine methyl ester **100**. Dehydration of threonine with MsCl / DBU

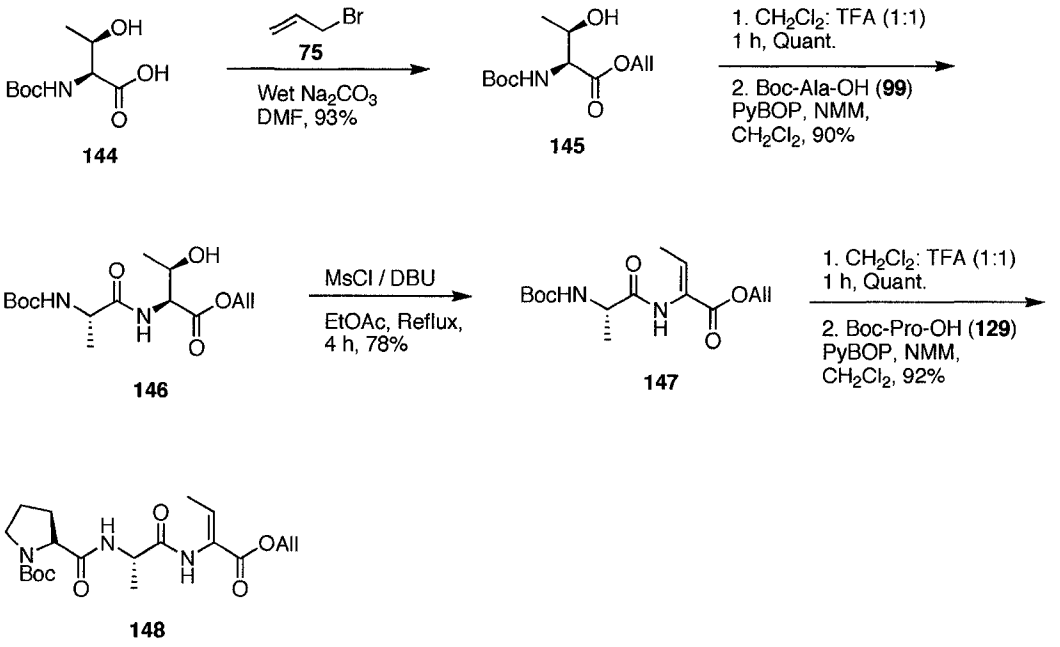
yields the dehydrodipeptide **142**. Hydrolysis of the methyl ester proceeds smoothly with 1 N LiOH and 1,4-dioxane as the co-solvent and affords the dipeptide **143** in an overall yield of 48% from **140** (Scheme 26).

Scheme 26. Dehydropentapeptide strategy: synthesis of dehydrodipeptide **143**



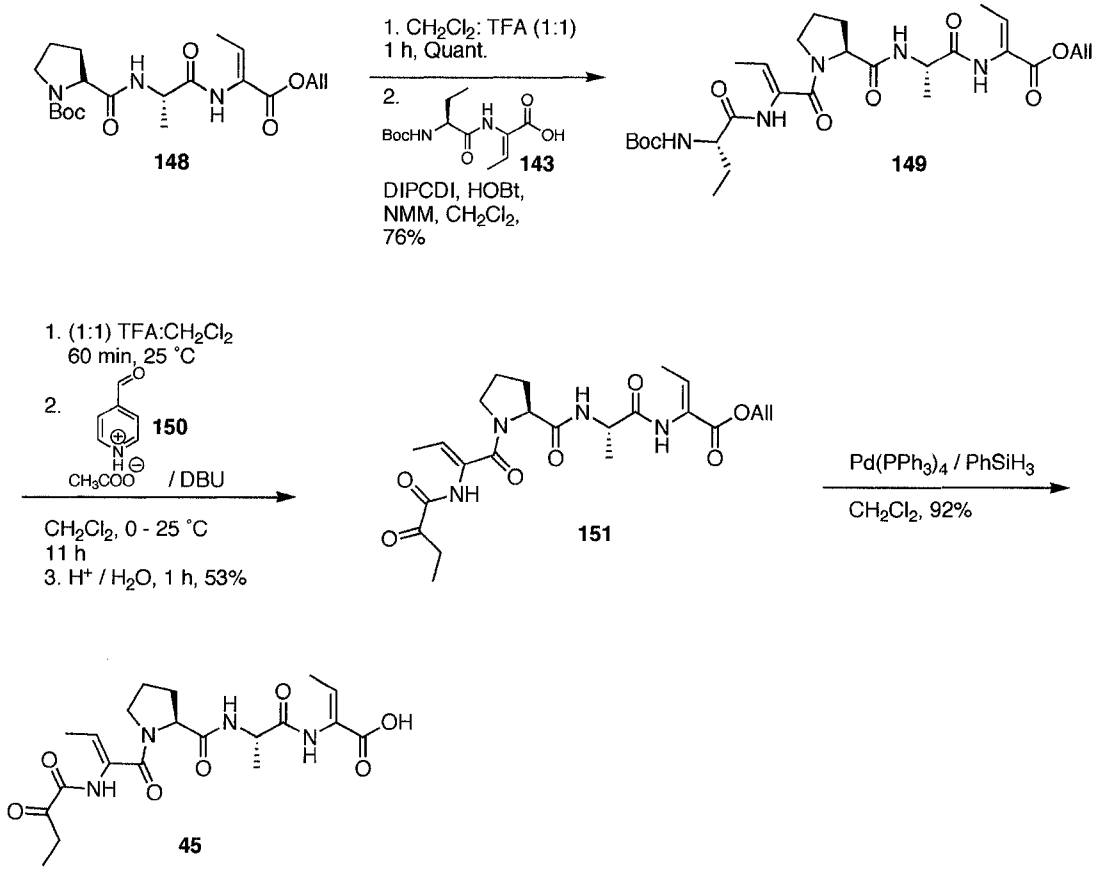
For the synthesis of dehydrotripeptide protected as an allyl ester, the carboxylic acid of commercial Boc-Thr-OH (**144**) reacted with allyl bromide under basic conditions to give allyl ester **145** (Scheme 27). TFA removed the Boc group protecting the nitrogen of **145** and Boc-Ala-OH (**99**) coupled with the resulting free amine to yield the dipeptide **146**. Dehydration of threonine as described before generated the required *Z*-dehydrobutyrine dipeptide **147**. Deprotection with TFA allows Boc-Pro-OH (**129**) to couple in the presence of PyBOP to successfully yield the dehydrotripeptide **148** with allyl protection. An overall yield of 65% was obtained over 5 steps.

Scheme 27. Dehydropentapeptide strategy: synthesis of dehydrotripeptide **148** containing an allyl ester



The Boc group of the dehydrotripeptide **148** is cleaved under acidic conditions and DIPCDI / HOBt mediates the coupling with the dehydrodipeptide **143** to yield the dehydropentapeptide **149** (Scheme 28). A transamination reaction with 4-pyridinecarboxaldehyde-acetic acid salt (**150**) followed by isomerization and hydrolysis of the secondary imine gives the α-ketoamide containing pentapeptide **151**. The removal of the allyl group occurs in the presence of $Pd(PPh_3)_4$ with $PhSiH_3$ as the scavenger to give the desired pentapeptide **45**.

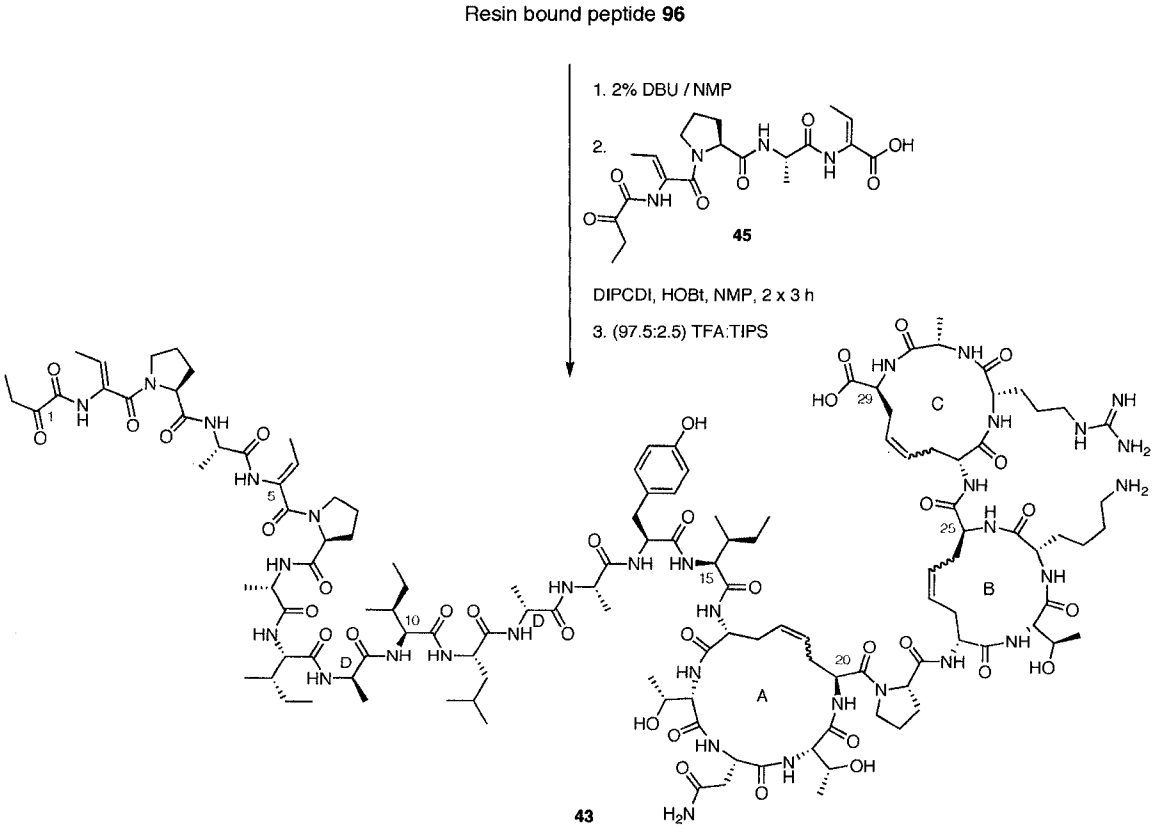
Scheme 28. Coupling of dehydrodipeptide **148** and dehydrotripeptide **143** fragments to obtain **45**



Having successfully obtained the pentapeptide **45**, efforts were focused on coupling it to **96**, the 24-mer on-resin. Treatment of resin-bound **96** with 2% DBU in NMP removes the *N*-terminal Fmoc group. The difficult coupling of a less reactive carboxylic acid of a dehydroamino acid with the secondary amino group of proline proceeded in high yield in solution phase with a combination of DIPCDI and HOBt as the coupling reagents (Scheme 28). The same coupling reagent combination is used to mediate the coupling of **45** to the free secondary amino terminus of solid-supported **96**

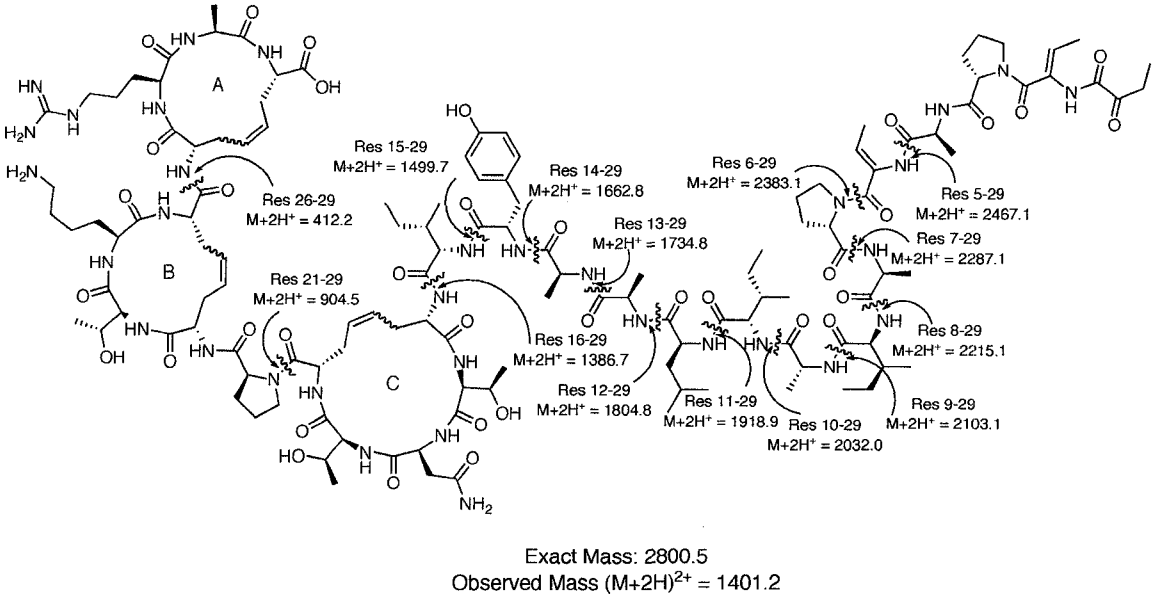
using NMP as the solvent (Scheme 29). The fully complete peptide is liberated from the solid-support with (97.5:2.5) TFA:TIPS. Water was omitted from the cleavage cocktail, as dehydroamino acids are prone to addition by water under strongly acidic conditions.[148] Analysis of the crude peptide obtained by MALDI-TOF MS revealed a major peak at 2801.6 (M+H), corresponding to the mass of the carbocyclic-larger ring analogue of lacticin 3147 A2, **43**. The final product was purified by reverse-phase HPLC to yield 1.8 mg of **43** with an overall yield of 0.5% for 25 coupling steps and three RCM reactions.

Scheme 29. Coupling of pentapeptide **45** to resin bound **96** to afford the carbocyclic-larger ring analogue of lacticin 3147 A2 **43**



A detailed LC-MS / MS analysis performed showed the fragments corresponding to masses of ring C (412.2 Da), rings CB (904.5 Da) and rings CBA (1386.7 Da) of **43**. The peptide sequenced excellently from residues (5-15) confirming the correct sequence as well as connectivity in the synthesized analogue **43** (Figure 26).

Figure 26. MS / MS analysis of carbocyclic-larger ring analogue of lacticin 3147 A2 (**43**)



Biological evaluation of carbocyclic-larger ring analogue of lacticin 3147 A2 (43)

The synthesized carbocyclic analogue of lacticin A2 **43** was tested for antimicrobial activity using spot on lawn assay. The indicator organism used was *Lactococcus lactis* HP. The natural lacticin A1 peptide was used in the testing to identify any synergistic biological activity with peptide **43**. Unfortunately, the carbocyclic

analogue **43** did not display any antimicrobial activity by itself or when tested along with natural lacticin A1 at concentrations up to 1 mM. It is plausible that the carbon atoms in the ring lead to a completely different conformation than in the natural substrate. Additionally, the sulfur atoms in the lanthionine and β-methyllanthionine rings could be involved in key recognition and binding interactions with the lacticin A1 counterpart or possibly with lipid II, the cell wall biosynthesis precursor. Thus, substituting the sulfur atom with carbon could impair the synergistic activity or the inherent antimicrobial activity.

Conclusions and future work

The synthesized carbocyclic-larger ring analogue **43** represents the first complete example of a chemical synthesis of a lantibiotic analogue on solid support. No lantibiotic other than this has been prepared thus far using solid-phase peptide synthesis. The methods described for the synthesis of **43** should find wider applications for the synthesis of peptidomimetics and conformationally constrained peptides.

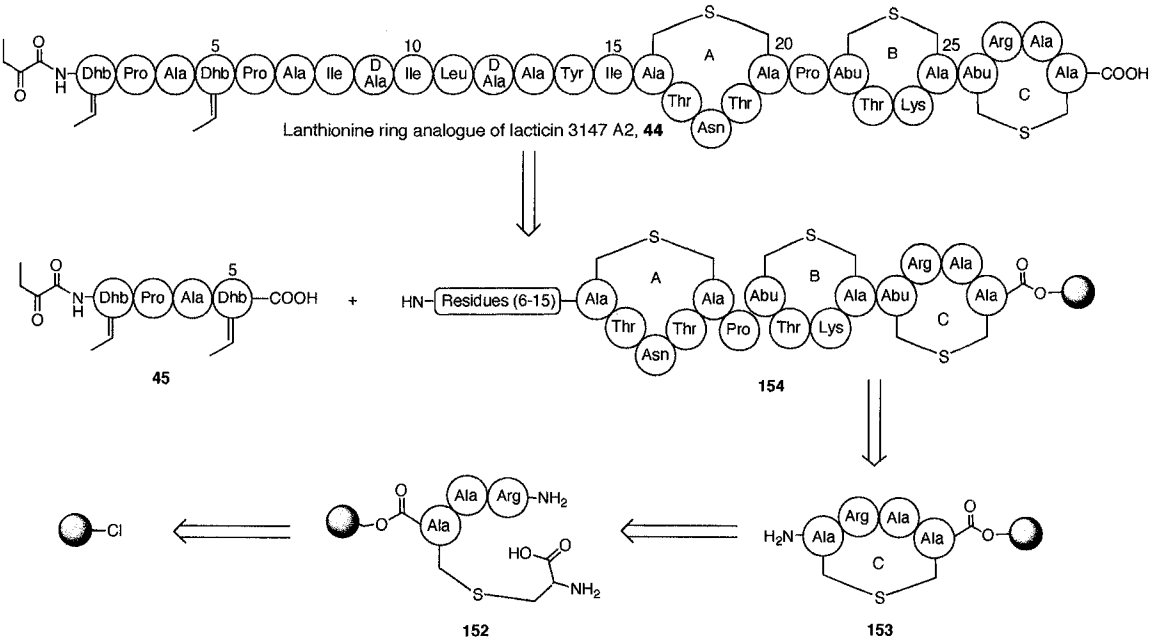
Future studies may be directed towards the synthesis of an analogue of lacticin A2 with an oxygen atom substituting the sulfur atom in the lanthionine bridges. The oxygen atom would be the closest mimic to a sulfur atom in terms of the bond angles and polarity. Also, an oxygen atom generally displays higher oxidative stability than a sulfur atom. Such a substitution could potentially mimic the lanthionine ring conformation, increase oxidative stability and retain biological activity.

To date, the details of molecular interaction between lacticin A1 and lacticin A2 and to lipid II are not known. However, it has been proposed that lacticin A2 binds to lacticin A1 and lipid II complex through its *C*-terminal residues that comprise the lanthionine rings. To understand the specific interaction, performing a carbocyclic ring scan, similar to an alanine scan in peptides, by systematically substituting the individual lanthionine rings of lacticin A2 (**12**) with carbocyclic rings might help in deducing the specific interaction between lacticin A1 and A2.

2.1.2. Synthesis and testing of lanthionine analogue of lacticin 3147 A2

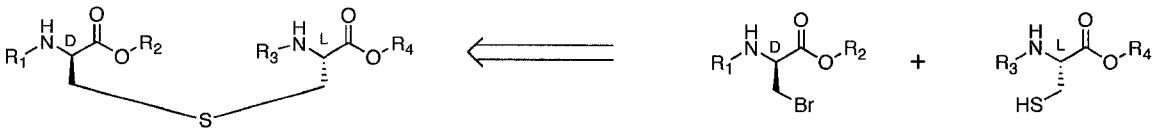
The strategy envisioned for the synthesis of the lanthionine analogue of lacticin 3147 A2 (**44**) utilizes a combination of solid and solution phase peptide synthesis as shown in Scheme 30. It employs a strategy very similar to Tabor and co-workers used for the synthesis of the lanthionine ring C of nisin via a triply orthogonally protected lanthionine **157** (Scheme 31).[101, 149] Loading of the lanthionine **157** to an acid labile resin followed by regular Fmoc-SPPS could lead to the linear precursor to ring C of **44**. Removal of the protecting groups could lead to **152** that could be cyclized on-resin to form **153**.

Scheme 30. Retrosynthesis of lanthionine analogue of lacticin 3147 A2 (**44**)



The lanthionine rings B and A could arise from a procedure similar to the one proposed for ring C. Regular Fmoc-SPPS could introduce residues (6→15). Fragment **45** [modified residues (1→5)], could be prepared in solution using the procedure described in section 2.1.1. The protected lanthionine could arise from a nucleophilic displacement of a bromide in bromoalanine **155** by the thiol group of cysteine **156** (Scheme 31).

Scheme 31. Strategy for synthesis of orthogonally protected lanthionine

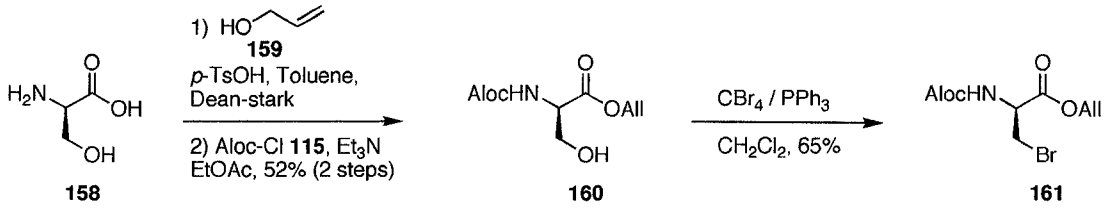


A number of groups are interested in methods for the synthesis of orthogonally protected lanthionine and β-methyllanthionines.[102, 111, 150-161] In order to have facile access to multigram quantities of the orthogonally protected lanthionine building block with minimal synthetic steps, we wanted to explore the possibility of combining the phase transfer reaction conditions to make protected lanthionines reported by Schmidt and co-workers,[160] with the triply orthogonal protecting group scheme investigated by Tabor and co-workers.[155]

Thus, the synthesis of lanthionine analogue **44** begins with the synthesis of the fragments required for making orthogonally protected lanthionine. The synthesis of protected bromo alanine **161** starts with the condensation of the carboxyl group of D-

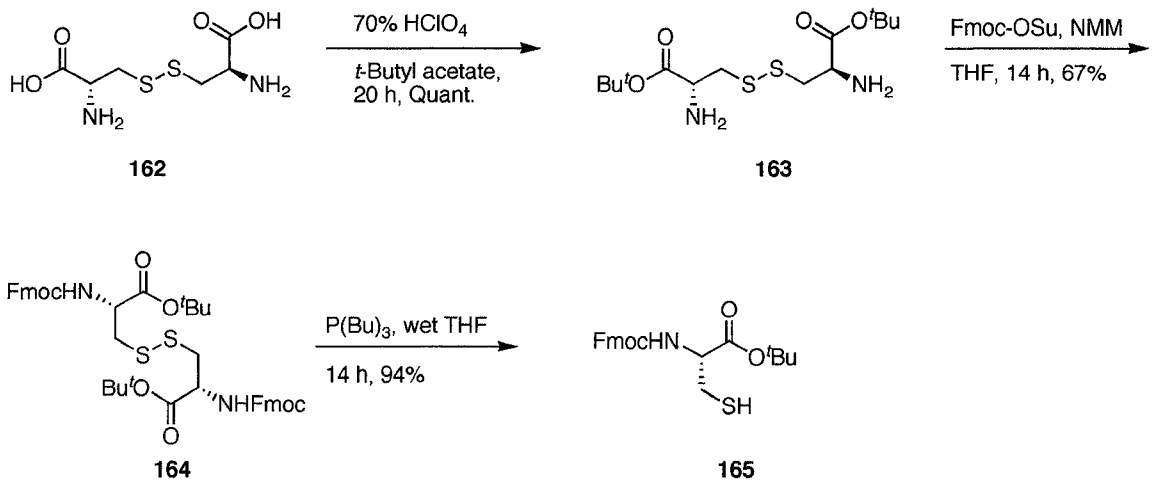
serine (**158**) with allyl alcohol (**159**) under Dean-Stark conditions catalyzed by *p*-toluenesulfonic acid (*p*-TsOH) to yield the allyl ester of D-serine as the *p*-toluenesulfonate salt (Scheme 32). Addition of triethylamine to neutralize the acid followed by reaction with allyl chloroformate (**115**) affords the Aloc / allyl protected D-serine **160**. The hydroxyl group of the side chain is converted to the bromide through an Appel reaction with CBr_4/ PPh_3, to give the required bromoalanine **161**.[162]

Scheme 32. Synthesis of Aloc-D-BrAla-OAll



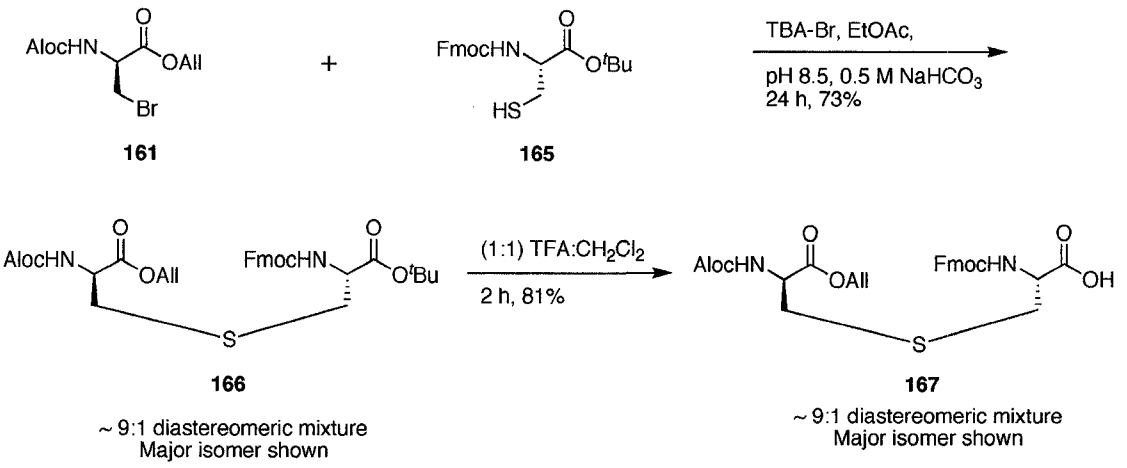
For the synthesis of the second fragment Fmoc-L-Cys-O^{t}Bu, the procedure reported by Bregant *et al.*[101] was followed. The protection of the two carboxylic acid groups of L-cystine (**162**) with *tert*-butyl groups occurs in the presence of *tert*-butyl acetate and 70% $HClO_4$ to yield the diester **163** (Scheme 33). Reaction of Fmoc-OSu with **163** in the presence of NMM protects the two primary amines with Fmoc groups to generate **164**. The disulfide bond is reduced via reaction with tributylphosphine in wet THF to give the desired Fmoc-L-Cys-O^{t}Bu (**165**).

Scheme 33. Synthesis of Fmoc-L-Cys-O^{t}Bu



The nucleophilic displacement reaction between the bromide and the thiol is performed according to the conditions reported by Schmidt and co-workers.[160] The Aloc / allyl protected D-bromoalanine **161** and the Fmoc / ^{t}Bu protected L-cysteine **165** react under basic (pH 8.5) biphasic conditions with *tetra*-butylammonium bromide as the phase transfer agent to yield the fully protected lanthionine **166** in very good yield (Scheme 34). Treatment with TFA removes the *tert*-butyl protection of **166** to afford the triply orthogonally protected lanthionine **167**.

Scheme 34. Coupling of **161** and **165** to form lanthionine **167**

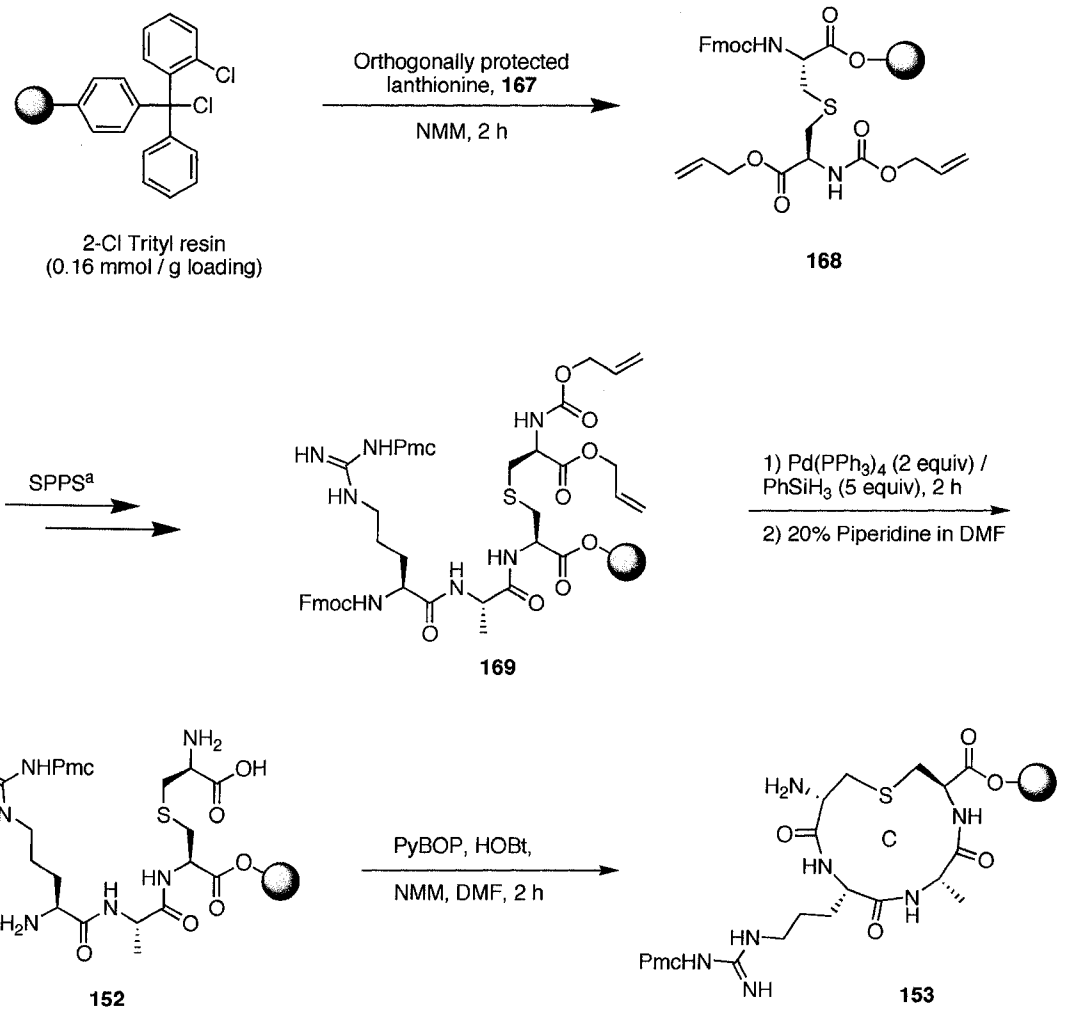


Upon closer examination of the ^{13}C NMR spectrum of **167**, two sets of peaks for the α-carbons were observed, indicating the formation of diastereomeric lanthionine in ~9:1 ratio. The diastereomeric ratio was derived by integration of the α-carbon peaks in the ^{13}C NMR spectra following the method reported by Tabor and co-workers.[155] By comparing the chemical shifts observed with the literature values, the major diastereomer was determined to be the desired *meso*-lanthionine isomer. The diastereomers presumably arise under the basic conditions used for the formation of the lanthionine. The bromoalanine **161** can undergo an elimination to form the corresponding dehydroalanine and an ensuing non-stereospecific Michael addition reaction could lead to two different isomers. With multigram quantities of orthogonally protected lanthionine in hand, we decided to proceed towards the synthesis of **44**. Further on in this thesis, whenever orthogonally protected lanthionine is mentioned it refers to the ~9:1 diastereomeric mixture of lanthionines **167**.

The first step towards the solid phase synthesis part of the synthesis is the loading of lanthionine **167** onto 2-chlorotrityl chloride resin. A low loading of 0.16 mmol / g was preferred for the synthesis, as higher resin substitution increases the possibility of growing peptide strands to be in close proximity to each other. This could potentially lead to the formation of the undesired interstrand diketopiperazine dimers during the cyclization step to make the lanthionine rings on-resin.

The initial substiution capacity of commercially available 2-chlorotrityl chloride resin (1.6 mmol / g) is first reduced to 0.6 mmol / g by reaction with acetic acid and NMM. Then, 0.16 mmol / g of lanthionine **167** reacts with the rest of the functionalizable sites. The remaining sites are capped with acetic acid and base to furnish **168** (Scheme 35). Fmoc-SPPS is performed using PyBOP to couple Fmoc-Ala-OH and Fmoc-Arg(Pmc)-OH to give the linear precursor to ring C **169**. The Aloc / allyl groups on the lanthionine residues in **169** are removed on solid support by reaction with $Pd(PPh_3)_4$ using $PhSiH_3$ as the scavenger. Treatment with 20% piperidine in DMF removes the Fmoc group on the arginine residue to yield **152** suited for cyclization. The amino group of the arginine residue and the carboxylic acid group of the lanthionine residue undergoes cyclization in the presence of PyBOP / HOBt / NMM for 2 h to furnish the first lanthionine ring C **153**. The mass of **153** as determined by ES-MS after cleavage and global deprotection with TFA is in agreement with the calculated mass for **153** [418.2 (M+H)].

Scheme 35. Loading and Fmoc solid phase synthesis of lanthionine ring C of **44**



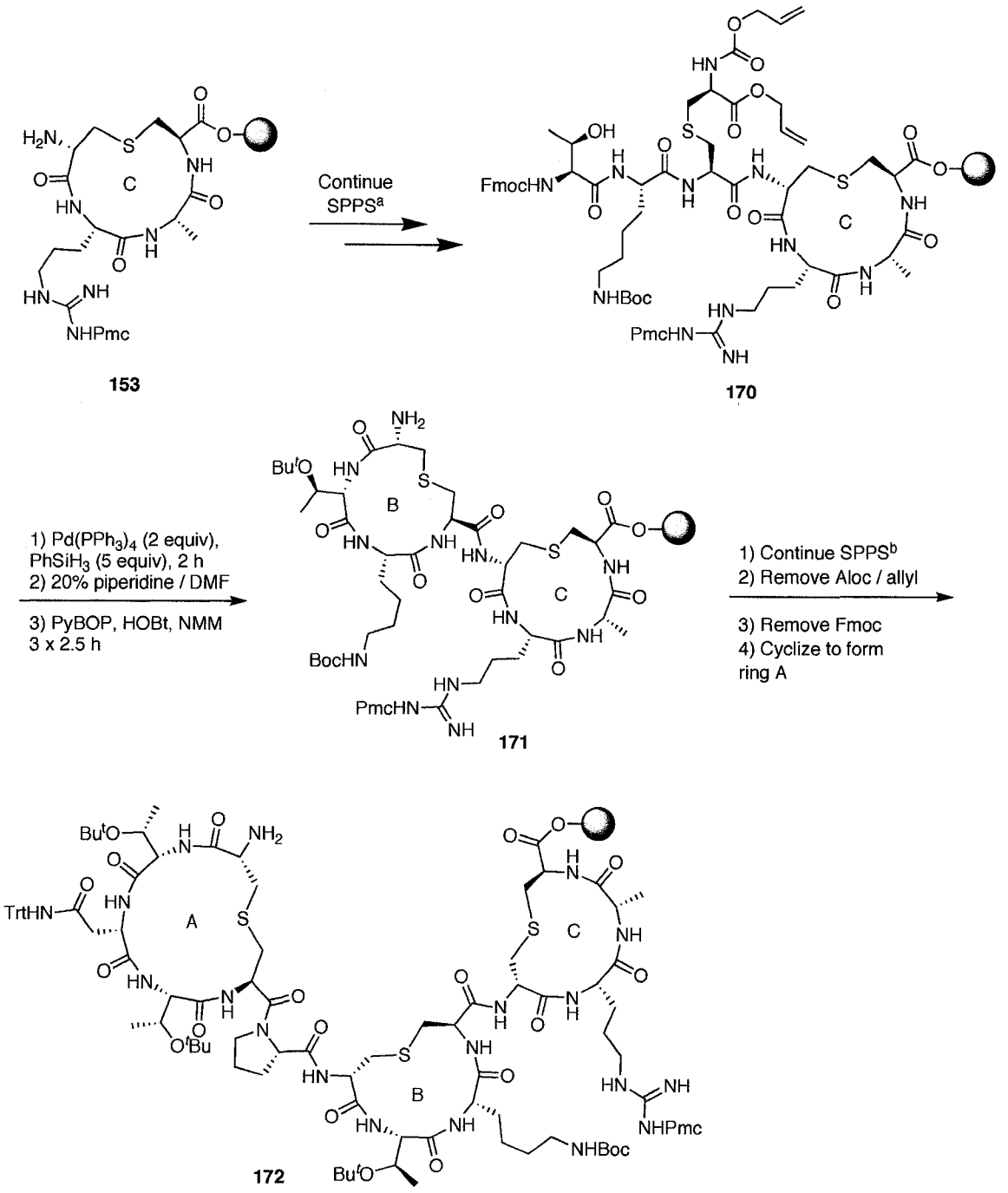
[a] Conditions used for synthesis of **169** from **168**: (i) 20% piperidine in DMF (ii) PyBOP, HOBt, NMM, DMF (iii) Fmoc-Ala-OH, (iv) repeat steps (i) and (ii) with Fmoc-Arg(Pmc)-OH.

The lanthionine rings B and A of **44** are introduced by a procedure similar to that used for formation of ring C. Fmoc-SPPS from **153** with PyBOP introduces the residues required for the linear precursor to the lanthionine ring B **170** (Scheme 36). The

following amino acids are coupled in the order: orthogonal lanthionine **167**, Fmoc-Lys(Boc)-OH, Fmoc-Thr(ᵗBu)-OH. The Aloc, allyl and Fmoc groups are removed as before and PyBOP is used for cyclization to form lanthionine ring B. The cyclization does not proceed to completion even after reaction with PyBOP for 5 h. Treatment of the resin for another 2.5 h with additional PyBOP forces the reaction to completion and affords **171**. Cleavage of a sample of peptide **171** from the solid-support (~11 mg) under acidic conditions followed by analysis by MALDI-TOF MS showed only a very weak peak for the desired product. Hence, a small sample of the resin (~10 mg) was coupled to Fmoc-Pro-OH, cleaved with TFA and subjected to MALDI-TOF MS analysis. A peak for the expected product in agreement with the calculated mass was observed [1138.4 (M+H)].

Fmoc-SPPS is continued from the free amino terminus of **171** using PyBOP to couple the following amino acids in the sequence: Fmoc-Pro-OH, orthogonal lanthionine **167**, Fmoc-Thr(ᵗBu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Thr(ᵗBu)-OH. The Aloc / allyl and Fmoc groups are removed under the previously described conditions to give the linear precursor to ring A. Employing an extended reaction time with PyBOP as the coupling reagent then yields lanthionine rings A, B and C (**172**) on solid-support (Scheme 36). A small sample of the resin (~15 mg) was coupled with the next residue Fmoc-Ile-OH, cleavage with TFA and analysis by MALDI-TOF MS, showed a peak that was in agreement with the calculated mass [1739.0 (M+H)].

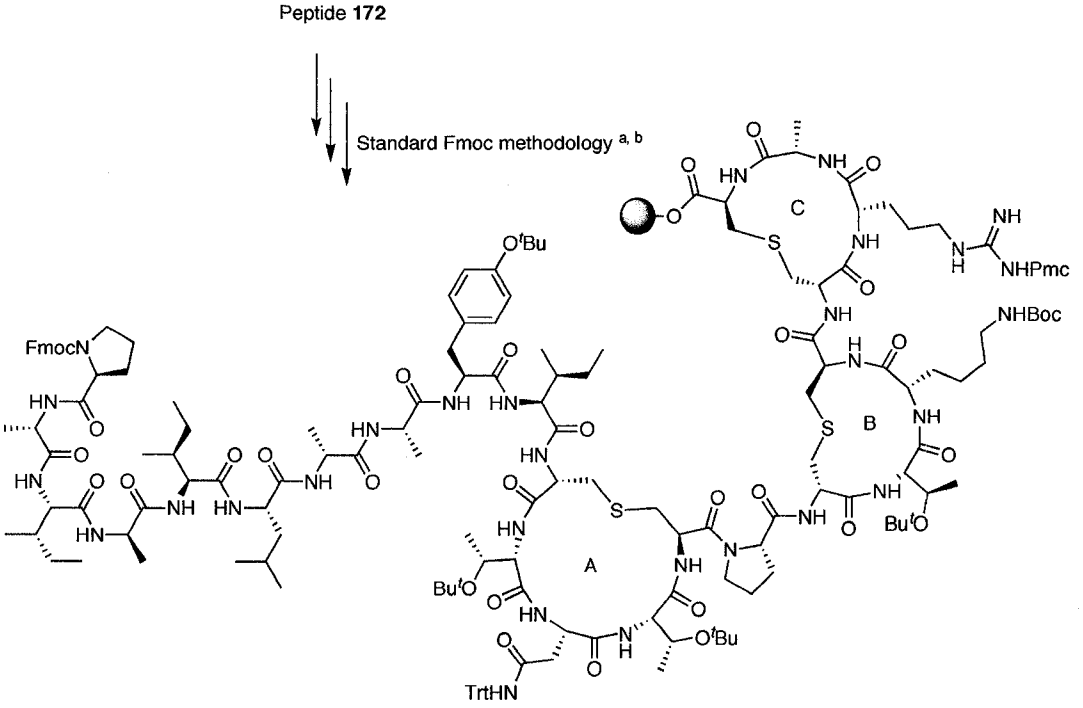
Scheme 36. Synthesis of lanthionine rings B and A of **44**



Fmoc-SPPS conditions: [a] (i) PyBOP, HOBt, NMM, DMF (ii) Orthogonal lanthionine **167** (iii) 20% piperidine in DMF (iv) Repeat steps (iii) and (i) for amino acids: Fmoc-Lys(Boc)-OH, Fmoc-Thr(ᵗBu)-OH [b] Perform step (i) with Fmoc-Pro-OH, repeat steps (iii) and (i) with orthogonal lanthionine **167**, Fmoc-Thr(ᵗBu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Thr(ᵗBu)-OH.

Having successfully formed lanthionine rings A, B and C (peptide **172**) on solid-support, the amino acids comprising residues (6→15) are introduced in the following sequence with PyBOP as the coupling reagent: Fmoc-Ile-OH, Fmoc-Tyr(ᵗBu)-OH, Fmoc-Ala-OH, Fmoc-D-Ala-OH, Fmoc-Leu-OH, Fmoc-Ile-OH, Fmoc-D-Ala-OH, Fmoc-Ile-OH, Fmoc-Ala-OH, Fmoc-Pro-OH. A small portion of the peptide is liberated from the resin with TFA and analysis by MALDI-TOF MS shows a peak at 2622.8 (M+H) indicating the formation of the desired product **173** on-resin (Scheme 37).

Scheme 37. Introduction of residues (6→15) of **44** by standard Fmoc methodology

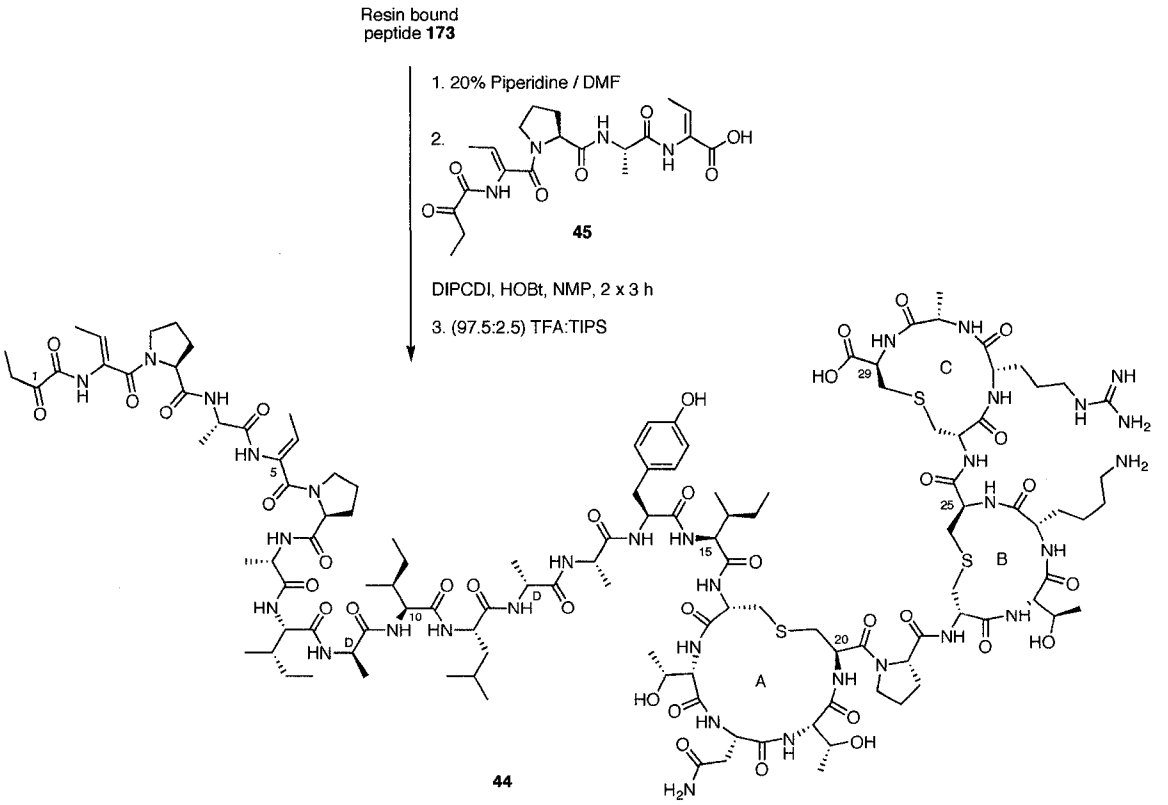


173

[a] Conditions used for synthesis of **173** from **172**: (i) Fmoc-Ile-OH, PyBOP, HOBt, NMM (ii) 20% piperidine in DMF, (iii) PyBOP, HOBt, NMM, DMF, [b] Repeat (ii) and (iii) for amino acids: (iv) Fmoc-Tyr(ᵗBu)-OH, (v) Fmoc-Ala-OH, (vi) Fmoc-D-Ala-OH, (vii) Fmoc-Leu-OH, (viii) Fmoc-Ile-OH (ix) Fmoc-D-Ala-OH, (x) Fmoc-Ile-OH: (xi) Fmoc-Ala-OH (xii) Fmoc-Pro-OH.

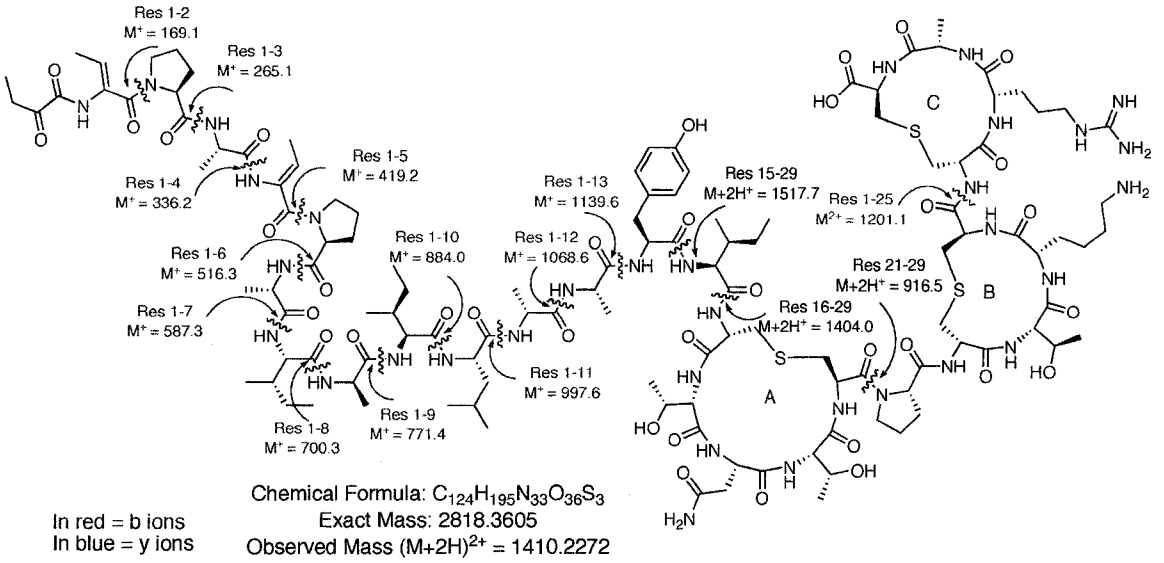
To complete the synthesis of lanthionine lacticin A2 analogue **44**, treatment of resin-bound peptide **173** with 20% piperidine removes the Fmoc group of proline (residue 6). Compound **45** comprising residues (1→5) and consisting two dehydrobutyrines and an *N*-terminal α-ketoamide, is prepared according to procedure described in section 2.1.1. For coupling of the pentapeptide, DIPCDI / HOBt proved to be the best coupling reagent combination based on previous experiences. Thus, the pentapeptide **45** is pre-activated with DIPCDI / HOBt / NMM and then reacted with the secondary amine of proline-6 on-resin. This step is repeated to ensure completion of the coupling reaction (Scheme 38).

Scheme 38. Coupling of **45** to resin bound **173** to complete the synthesis of a lanthionine analogue of lacticin A2 (**44**)



Treatment of the resin-bound peptide **44** with (97.5:2.5) TFA:TIPS cleaves the product from the solid support. Analysis of the crude peptide obtained by MALDI-TOF MS revealed a peak at 2819.6 (M+H), corresponding to the mass of the lanthionine analogue of lacticin 3147 A2, **44**. The final product was purified by reverse-phase HPLC to yield 1.0 mg of **44** with an overall yield of 1.3% for 22 couplings and three cyclization steps. A high resolution MALDI-FTICR-MS was also obtained for **44** to further confirm the mass of the expected product.

Figure 27. LC-MS / MS analysis of lanthionine analogue of lacticin A2, **44**



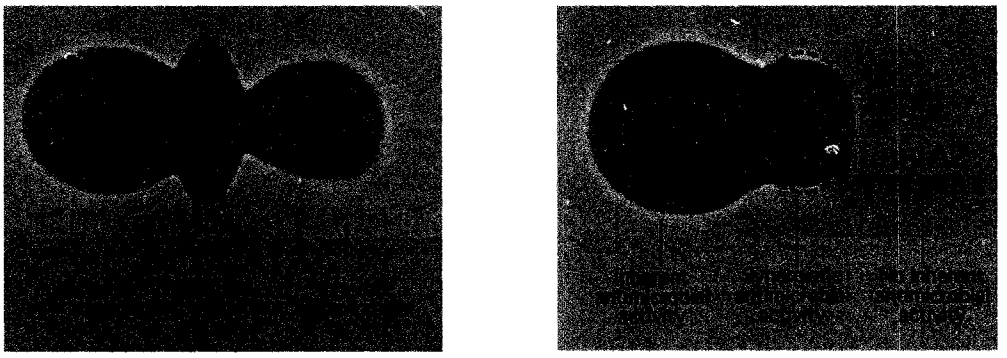
A detailed LC-MS / MS analysis performed gave fragments corresponding to the masses of rings CB (916.5 Da) and rings CBA (1404.0 Da) of **43** (Figure 27). The

peptide sequenced excellently from the *N*-terminus confirming the correct sequence as well as connectivity in the synthesized analogue **44**.

Biological evaluation of the lanthionine analogue of lacticin 3147 A2 (44)

A preliminary biological evaluation of **44** was done in combination with natural lacticin A1 against the Gram-positive indicator organism, *Lactococcus lactis* subspecies *cremoris* HP. The lanthionine analogue **44**, when spotted next to natural lacticin A1 **11**, exhibits synergistic biological activity similar to natural lacticin A2 **12**. However, in contrast to natural lacticin A2, the lanthionine analogue **44** did not exhibit any independent antimicrobial activity at comparable concentrations against the indicator organism (Figure 28)

Figure 28. Synergistic biological activity: (Left) Natural lacticin A1 and natural lacticin A2, (Right) Natural lacticin A1 and lanthionine analogue **44**



A serial dilution assay performed by mixing **44** with lacticin A1 showed that the lanthionine analogue is approximately 100 times less effective than natural lacticin A2 in its ability to exert synergistic antimicrobial activity. The decrease in the magnitude of synergy as well as the loss of independent antimicrobial activity of lanthionine analogue **44** when compared to natural lacticin A2, could perhaps be a result of a decreased ability of **44** to interact with the lacticin A1:lipid II complex. This also suggests that the synergistic activity of **44** could be arising from an alternative mode of action compared to natural lacticin A2.

Interestingly, peptide **173** having an Fmoc group instead of residues (1→5) also exhibited synergistic activity with lacticin A1, albeit at higher concentrations. The synergistic activity of **173** is lost when the Fmoc group on residue 6 is removed. This observation suggests that the Fmoc group acts as a mimic for residues (1→5), probably due to the similar planarity and hydrophobicity of the two structures. Further optimization could potentially lead to a structurally simpler analogue with fewer amino acids than natural lacticin A2. The chemical synthesis of lanthionine lacticin A2 analogue **44** has now provided a tool to distinguish and study the molecular mechanisms responsible for synergistic activity and the inherent independent antimicrobial activity of lacticin 3147.

Conclusions and future direction

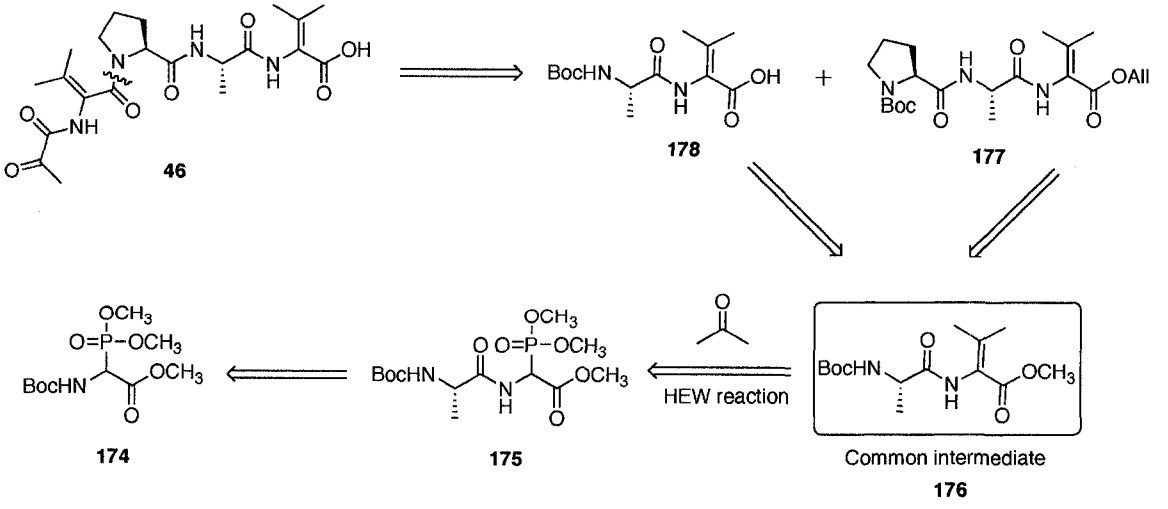
The lanthionine analogue of lacticin A2 **44** has been successfully synthesized by a combination of solid phase and solution phase peptide synthesis. To the best of our

knowledge, this is the first complete synthesis of a lantibiotic analogue containing multiple lanthionine rings on solid support. The synthesized lanthionine analogue **44** exhibits synergistic biological activity in combination with lacticin A1 against Gram-positive bacteria. A lead compound **173** for generation of a structurally simpler lacticin A2 analogue has also been successfully identified. The chemical method developed for the synthesis of the lanthionine analogue **44** opens the door for the synthesis of lantibiotics and their analogues on solid support for SAR, structure elucidation and to study their mode of action.

2.1.3. Synthesis of dehydrovaline and oxazole analogues of residues (1→5) of lacticin 3147 A2

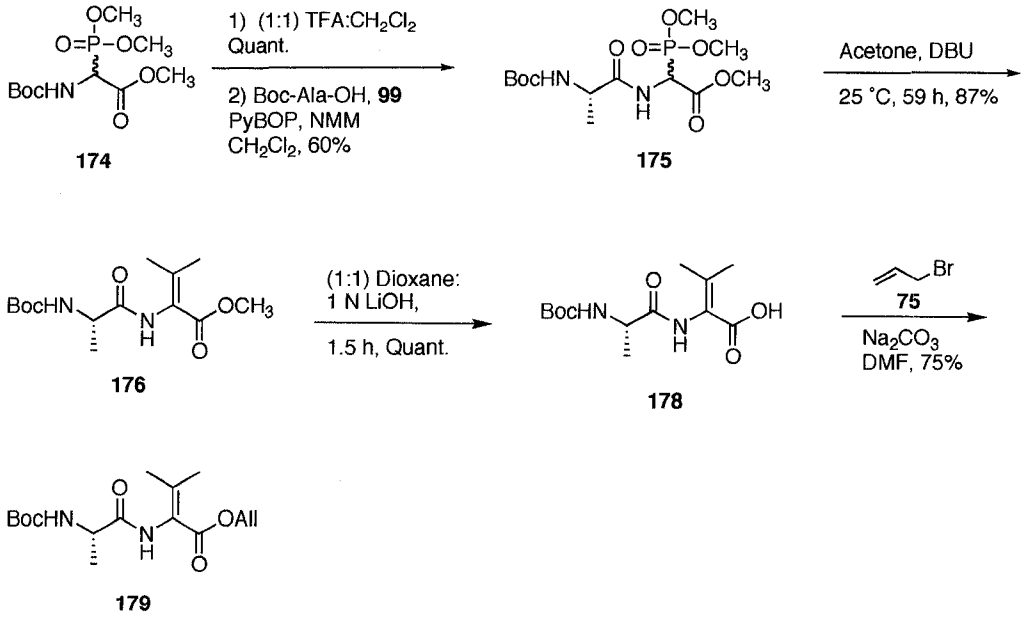
For the synthesis of the dehydrovaline analogue of residues (1→5) of lacticin 3147 A2 (**46**) the retrosynthetic strategy envisioned is shown in Scheme 39. The dehydrovaline pentapeptide could arise from a coupling between a dehydrodipeptide **178** and a dehydrotripeptide **177**. The dehydrovaline residue in the dipeptide **178** could arise from a Horner-Emmonds-Wadsworth (HEW) reaction between the phosphonate group of **175** and acetone to furnish **176**. This key dipeptide intermediate could be used as the starting material to make the tripeptide **177** as well as the coupling partner **178** to obtain **46**. The work described in this section was done by Shaun McKinnie (Summer 2006, Vederas group).

Scheme 39. Retrosynthetic plan for the synthesis of **46**



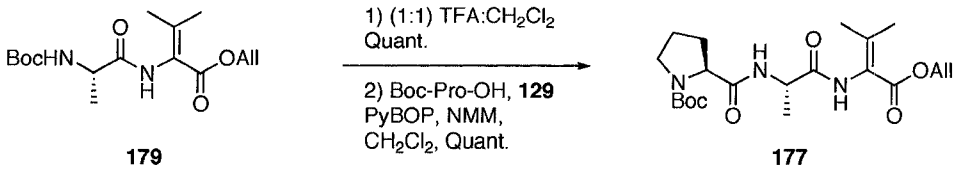
The synthesis of the dehydrovaline pentapeptide is initiated by removal of the Boc group of commercially available *tert*-butyloxycarbonylphosphonoglycine methyl ester (**174**). Boc-Ala-OH (**99**) is then coupled to **174** in the presence of PyBOP to afford **175** in moderate yield (Scheme 40). Compound **175** undergoes a Horner-Emmonds-Wadsworth reaction with acetone as the reagent and solvent and with DBU acting as the base to give the key dehydrovaline dipeptide intermediate **176**.[163] The methyl ester of **176** is then transesterified to an allyl ester via a two-step procedure. The first step is the hydrolysis of the methyl ester with 1 N LiOH to give the free carboxylic acid **178**, which in a second-step undergoes esterification with allyl bromide to arrive at **179**.

Scheme 40. Synthesis of dehydrovaline dipeptide **179**



For the synthesis of the dehydrovaline tripeptide **177**, the dipeptide **179** is reacted with Boc-Pro-OH under standard conditions to yield **177** in quantitative yield (Scheme 41).

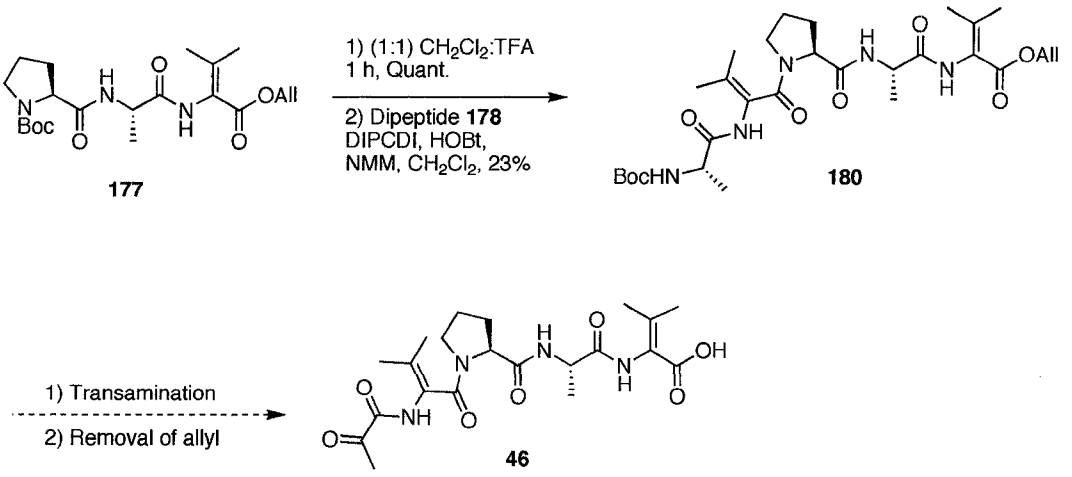
Scheme 41. Synthesis of dehydrovaline tripeptide **177**



Coupling of the fragments begins with the removal of the Boc group of the tripeptide **177** with TFA, followed by reaction with the dipeptide acid **178** mediated by DIPCDI to give the dehydrovaline pentapeptide **180** (Scheme 42). However, a poor yield of 23% is obtained for this difficult coupling. This could be because of the lower reactivity of the dehydroamino acid with the secondary amine of a proline. However, in the case of dehydrobutyrine congener **149** (section 2.1.1), the coupling proceeds in good yield. The extra steric hindrance introduced by the methyl groups of dehydrovaline compared to dehydrobutyrine could be an additional factor causing the low yield.

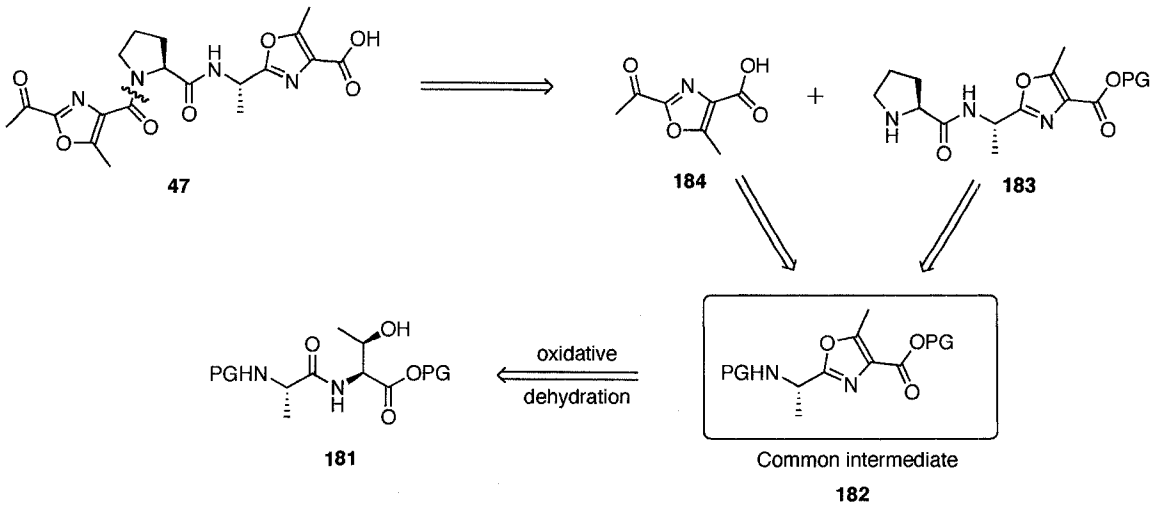
The final two steps towards **46** (i.e. transamination to introduce the α-ketoamide and the removal of the allyl group) could be readily performed according to previously developed procedures. However, it was decided to perform the final steps right before incorporation of **46** into any lacticin A2 analogue.

Scheme 42. Coupling of the dipeptide **178** to the tripeptide **177**



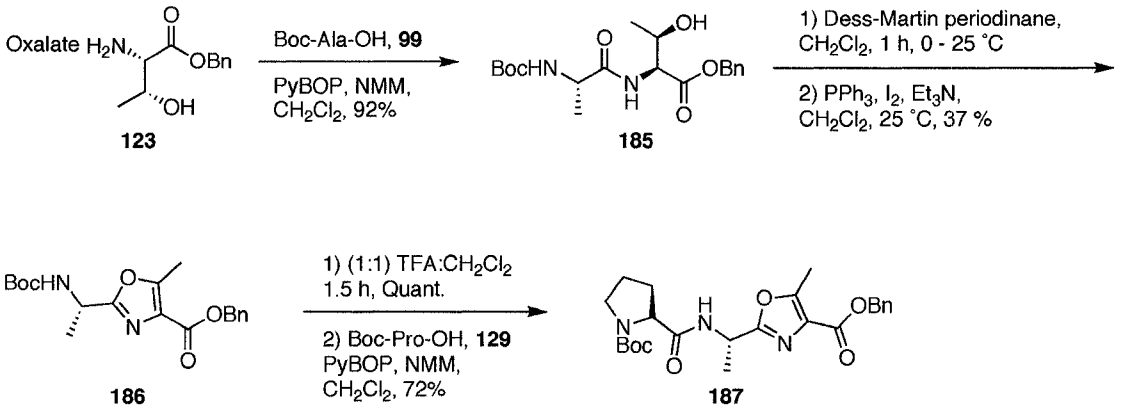
For the synthesis of the oxazole analogue of residues (1→5) of lacticin A2 (**47**), a strategy similar to the one used for the dehydrovaline analogue could be employed. The key reaction in this case would be the formation of the oxazole ring. This could arise from an oxidation of the side chain hydroxyl of a serine or threonine followed by a mild Robinson-Gabriel cyclodehydration of the resulting β-ketoamide.[164] A synthetic strategy is shown in Scheme 43.

Scheme 43. Retrosynthetic plan for synthesis of **47**



To initiate the synthesis, L-threonine benzyl ester oxalate (**123**) is coupled to Boc-Ala-OH (**99**) to obtain the dipeptide **185** (Scheme 44). For the formation of the oxazole the procedure described by Wipf *et al*[164] was followed. The first step is the Dess-Martin periodinane oxidation of the secondary hydroxyl group of threonine dipeptide **185** to yield a highly unstable β-ketone intermediate. This is immediately reacted with PPh_3, iodine and Et_3N to undergo a dehydrative cyclization with the neighboring amide group of the dipeptide **185** to yield **186** in a moderate yield of 37% for the two steps. TFA mediated removal of the Boc group of this key intermediate followed by coupling to Boc-Pro-OH then yields the oxazole tripeptide **187**.

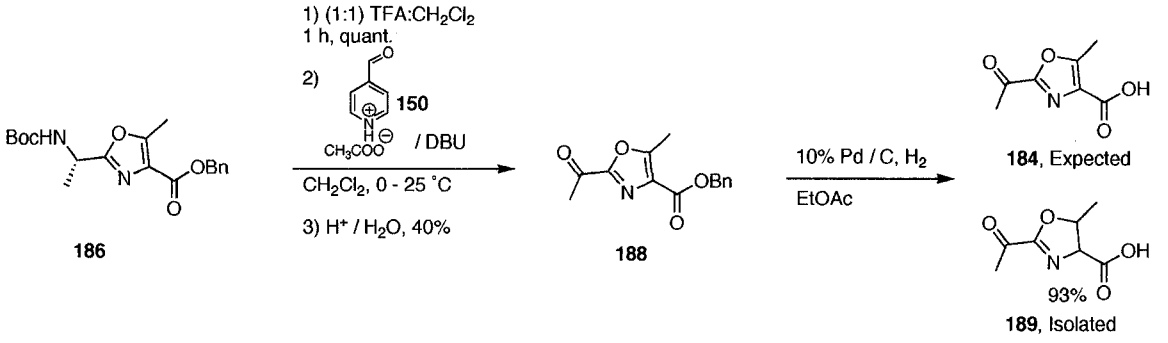
Scheme 44. Synthesis of oxazole tripeptide **187**



For the synthesis of the oxazole dipeptide **184**, the common intermediate **186** is deprotected and subjected to a transamination reaction with the carboxaldehyde **150** (Scheme 45). Aqueous acidic workup forms the desired oxazole dipeptide **188** with a methyl ketone substituting the 2-oxobutryl group in the natural *N*-terminus of lacticin A2.

In order to obtain the oxazole pentapeptide **47**, the benzyl protecting group of the oxazole dipeptide **188** must be removed and coupled to the oxazole tripeptide **187**. A reaction of the dipeptide **188** under standard hydrogenation conditions with 10% Pd / C unexpectedly yields only the unwanted over-reduced product **189** and none of the desired dipeptide **184**.

Scheme 45. Synthesis of residues (1→2) of oxazole analog **47**

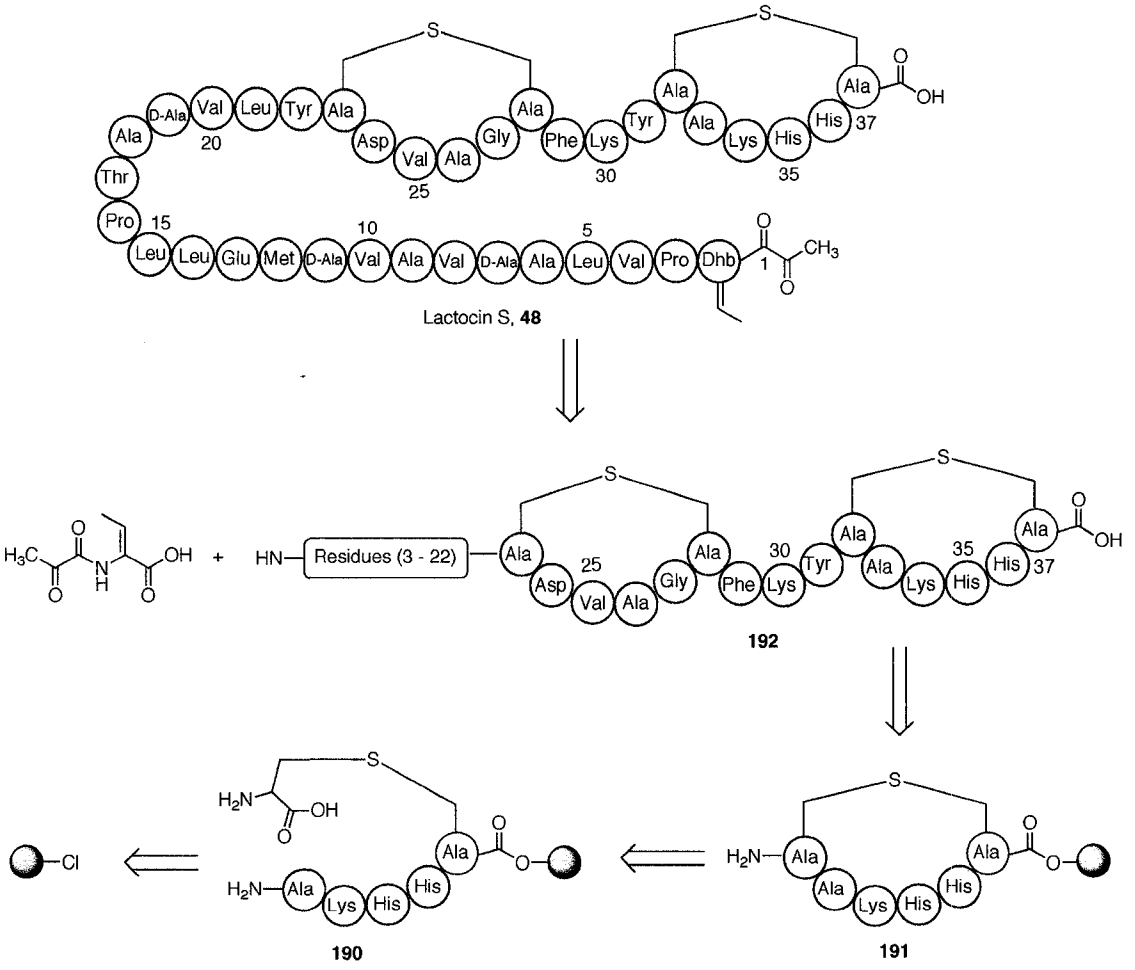


Further work had to be stopped at this stage due to lack of time. However the oxazoline **189** could be oxidized back to the oxazole utilizing the procedure reported by Williams and co-workers with bromotrichloromethane and DBU.[165] Alternatively, Lewis acid conditions could be employed for the removal of the benzyl group of **188** to potentially yield **184**. Coupling of **184** under standard conditions with the tripeptide **187** would complete the synthesis of **47**.

2.1.4. Studies towards total synthesis of lactocin S

The synthetic strategy envisioned for the synthesis of lactocin S **48** (Scheme 46) is identical to the one utilized for the synthesis of the lanthionine analogue of lacticin A2 (Scheme 30).

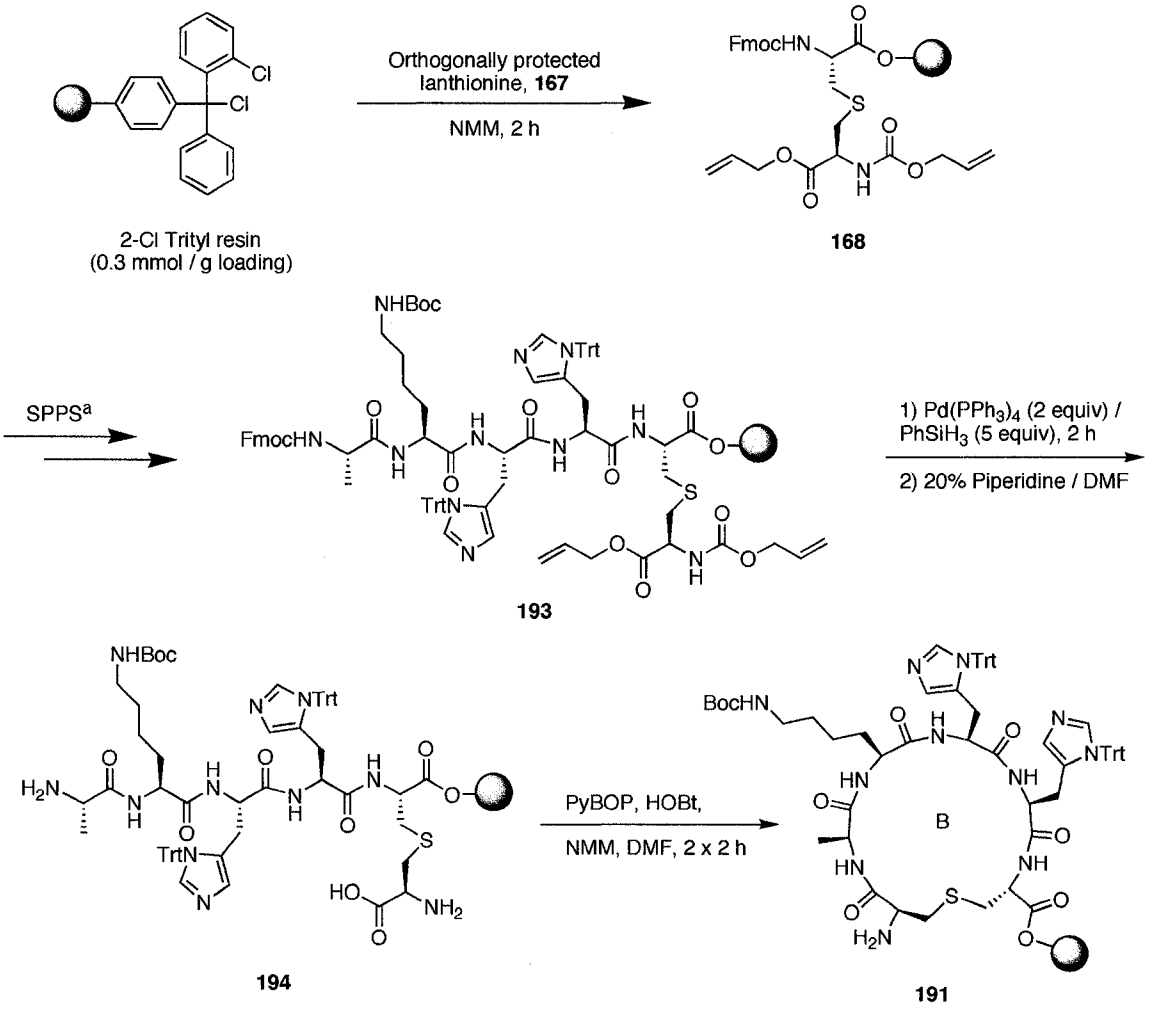
Scheme 46. Synthetic strategy for lactocin S (**48**)



The synthesis begins with the loading of orthogonally protected lanthionine **167** to 2-chlorotrityl chloride resin (Scheme 47). Only 0.30 mmol / g of the resin is

substituted with lanthionine. The rest of the functionalizable sites are capped with acetic acid to yield **168**. Standard Fmoc-SPPS with Fmoc-His(Trt)-OH, Fmoc-His(Trt)-OH, Fmoc-Lys(Boc)-OH and Fmoc-Ala-OH then yields **193**. Removal of the Aloc, allyl and Fmoc groups as before with a palladium catalyst and piperidine, respectively, yields **194**, which then undergoes cyclization mediated by PyBOP to give **191**.

Scheme 47. Attempted synthesis on 0.30 mmol / g scale

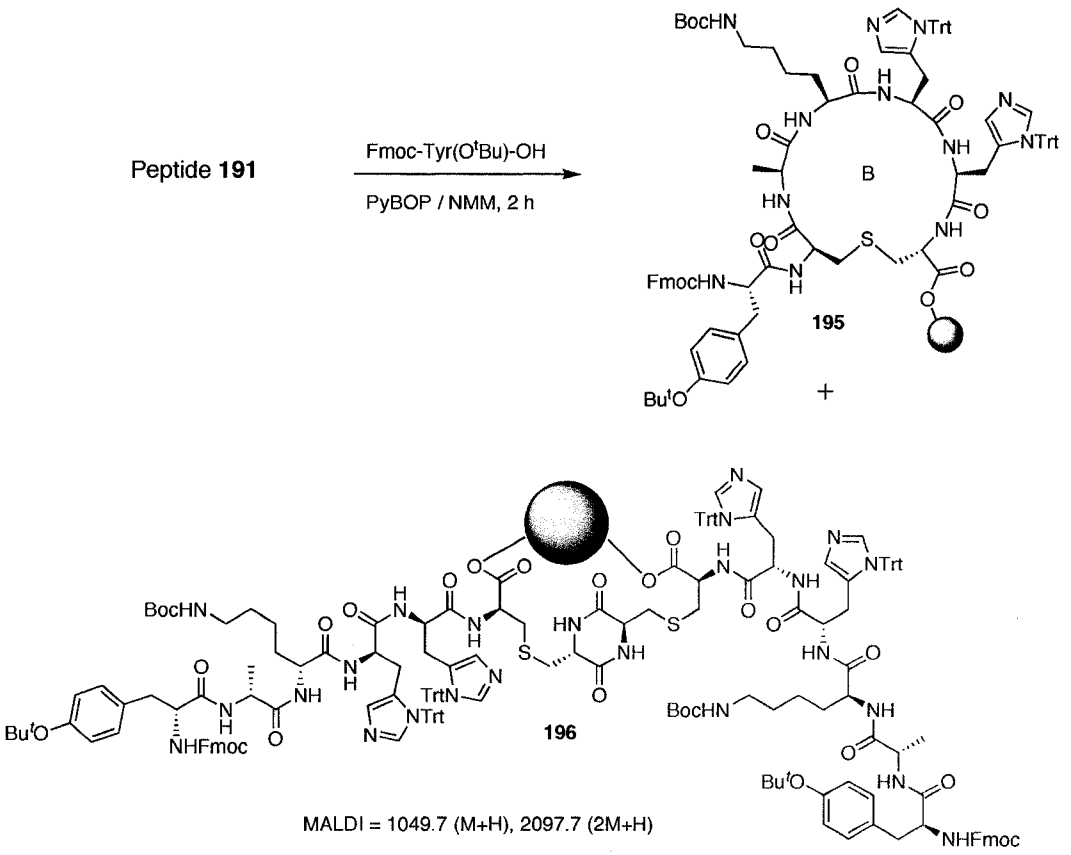


[a] Conditions used for synthesis of **193** from **168**: (i) 20% piperidine in DMF (ii) PyBOP, HOBt, NMM, DMF (iii) Fmoc-His(Trt)-OH, (iv) Repeat steps (i) and (ii) with Fmoc-His(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ala-OH.

Coupling of the next residue, Fmoc-Tyr(ᵗBu)-OH to **191**, followed by cleavage of a small sample of the resin with TFA and analysis by MALDI-TOF MS, indicated the presence of the desired product **195** [1049.7 (M+H)] as well as a product having exactly twice the mass of the desired product [2097.7 (M+H)] (Scheme 48). This was initially thought to be the 2M+H peak of the desired product. However, the intensity of the peak was almost equal to the desired product peak. As there is a possibility for formation of a chemical dimer that would be exactly the same mass as the 2M+H peak further investigation was undertaken. A MS / MS analysis clearly indicated that the peak with twice the mass of the desired product is the covalent dimer **196**. The inter-strand dimerization to give the diketopiperazine dimer **196** likely happens on-resin during the cyclization step to form the lanthionine ring when the growing peptide strands are closer to each other. This could be avoided by lowering the resin loading, which would lower the probability of two peptide strands being closer to each other. (This work was done before the work described in section 2.1.2 for the synthesis of lanthionine analogue of lacticin A2).

Based on the above results a new synthesis of lactocin S with a lower resin loading of 0.16 mmol / g was undertaken. Repeating the same synthetic steps as described above with a lower resin loading successfully yields only the lanthionine ring B **195** and none of the diketopiperazine dimer.

Scheme 48. Diketopiperazine dimer formation during synthesis of ring B of lactocin S



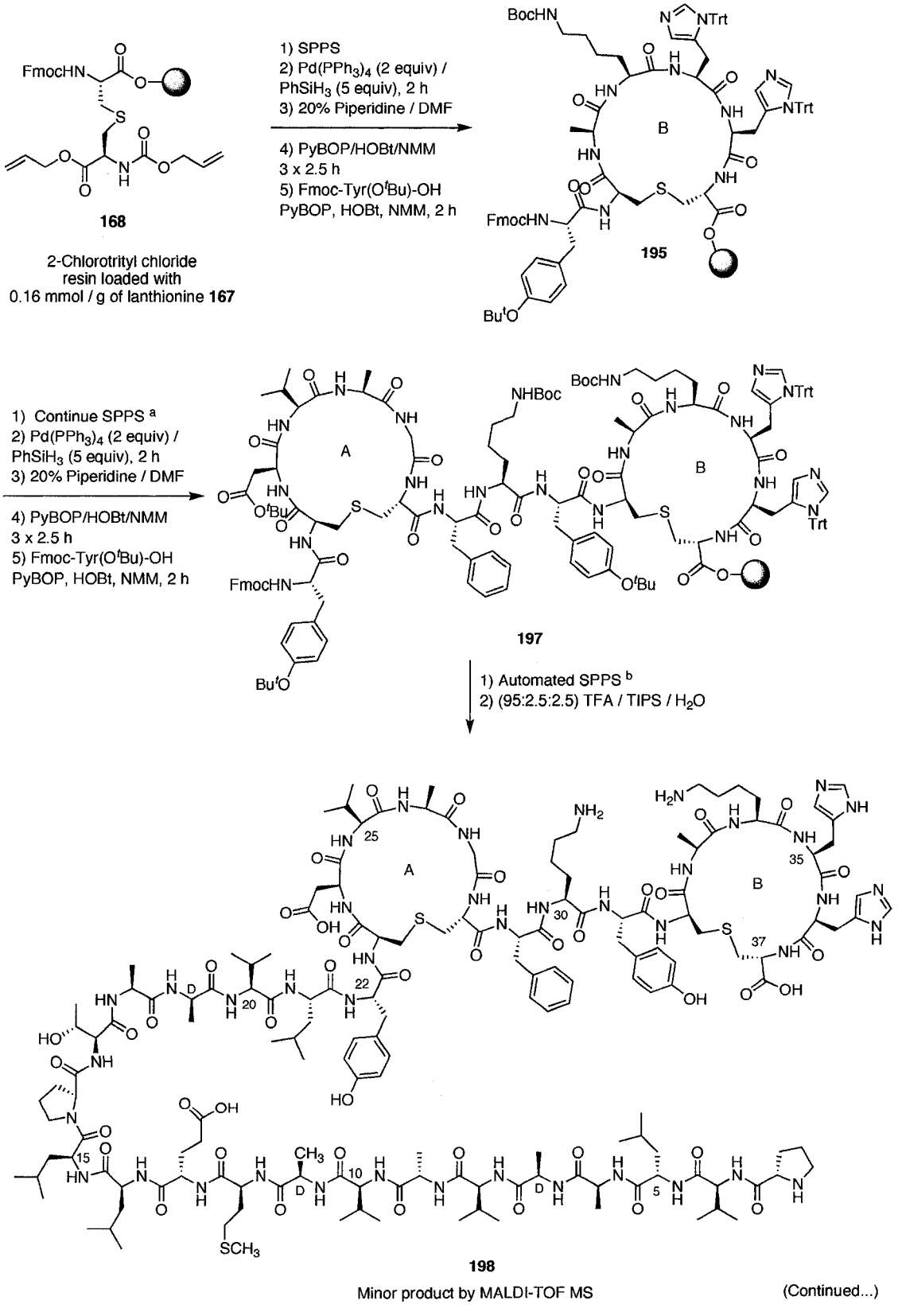
Fmoc-SPPS then introduces the following amino acids: Fmoc-Lys(Boc)-OH, Fmoc-Phe-OH, orthogonal lanthionine **167**, Fmoc-Gly-OH, Fmoc-Ala-OH, Fmoc-Val-OH, Fmoc-Asp(O^{t}Bu)-OH. Removal of Aloc, allyl and Fmoc groups followed by cyclization affords the lanthionine ring A of lactocin S. Fmoc-Tyr(^{t}Bu)-OH is then coupled to the free NH_2 of the lanthionine ring A to afford **197** (Scheme 49). Cleavage of a small sample and analysis of the peptide obtained revealed a peak at 2001.1 (M+H) corresponding to the mass of the expected product.

Having successfully obtained the lanthionine rings A and B of lactocin S **48**, we shifted our focus towards introducing the linear sequence, residues (3→22), of lactocin S. It was decided to do perform an automated Fmoc-SPPS using an ABI 433A peptide synthesizer to introduce the linear sequence of **48**. Thus, subjecting 0.10 mmol of resin bound **197** to automated synthesis to couple residues (3→22) yields the desired product **198** as a minor product by MALDI-TOF analysis [3631.8 (M+Na)]. The major peak observed arises from the lanthionine rings A and B, with an acetyl group at the *N*-terminus (**199**) [1658.9 (M+H)]. Furthermore, the peak for **198** was observed only once. All efforts to detect the required product **198** by MALDI-TOF MS failed.

This highly unexpected result could perhaps be the result of a mechanical error during the automated synthesis. Also, the coupling reagent and other reagent cocktails used for synthesis on a peptide synthesizer are slightly different from those used during manual synthesis. In order to eliminate the possibility of any error involving the peptide synthesizer or the conditions used for the automated synthesis, it was decided to introduce residues (3→22) to resin-bound **197** by manual solid phase peptide synthesis.

The third attempt to synthesize lactocin S **48** begins with the removal of the Fmoc group of resin-bound **197**. Regular Fmoc-SPPS introduces residues (15→21) with PyBOP as the coupling reagent (Scheme 50). Treatment of a small portion of the resin with TFA then cleaves the peptide from the resin. Analysis by MALDI-TOF MS revealed the desired peptide **200** [2667.3 (M+H)] as a minor peak along with other impurities.

Scheme 49. Synthesis of lactocin S with a resin loading of 0.16 mmol / g



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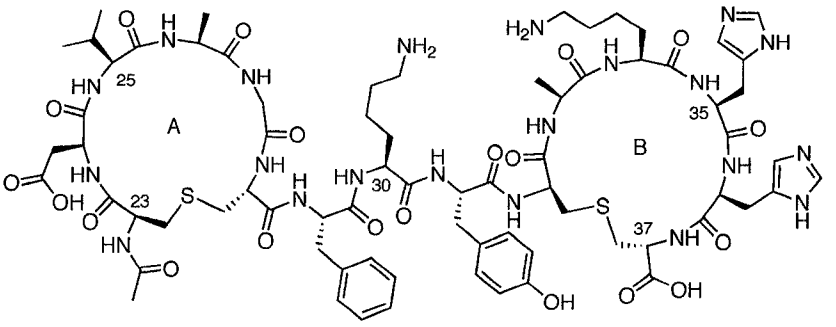
+



199

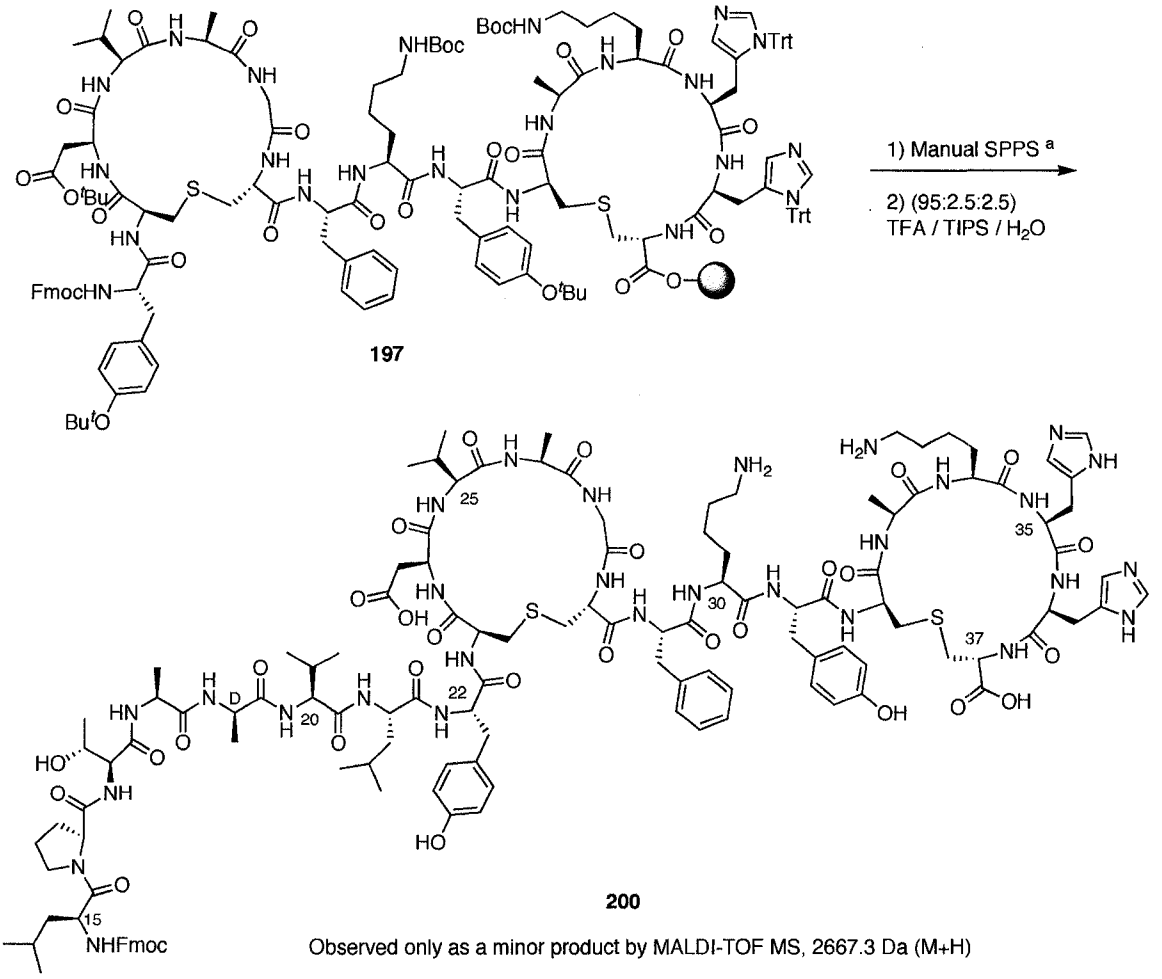
Major product detected by MALDI

[a] Fmoc-SPPS conditions: (i) 20% piperidine in DMF (ii) PyBOP, HOBt, NMM, DMF (iii) Fmoc-Lys(Boc)-OH, (iv) Repeat steps (i) and (ii) with Fmoc-Phe-OH, orthogonal lanthionine **167**, Fmoc-Ala-OH, Fmoc-Val-OH, Fmoc-Asp(O^{t}Bu)-OH. [b] Automated SPPS conditions: (v) 22% piperidine in NMP (vi) HBTU, HOBt, NMM, NMP, Fmoc-Leu-OH, (vii) Repeat steps (v) and (vi) with Fmoc-Val-OH, Fmoc-D-Ala-OH, Fmoc-Ala-OH, Fmoc-Thr(^{t}Bu)-OH, Fmoc-Pro-OH, Fmoc-Leu-OH, Fmoc-Leu-OH, Fmoc-Glu(O^{t}Bu)-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.

Although this was not a positive indication, Fmoc-SPPS was continued to introduce residues (9→14). Again, cleavage of a small sample of the peptide from resin with TFA followed by MALDI-TOF MS analysis showed none of the desired product **201**. Surprisingly, the major product formed is the previously observed bicyclic product **199** [1658.9 (M+H)]. An MS / MS analysis of **199** further confirms the proposed structure of **199**. This indicates that this unwanted end-capped product must arise during the course of the peptide chain extension. The mechanism for the formation of this product is not clear at this point. However, it is tempting to suggest that the growing peptide chain could adopt a conformation that is suited for cyclization upon itself. This could possibly be triggered by acetylation of the amide at the lanthionine ring junction (residue 23). On the other hand, the *N*-acetylation could occur after the cyclization-

cleavage of the growing peptide chain. It is also not very clear during exactly which coupling step this product starts forming. An effort to address this issue is currently under investigation in the Vederas group.

Scheme 50. Synthesis of lactocin S linear portion from ring A by manual SPPS



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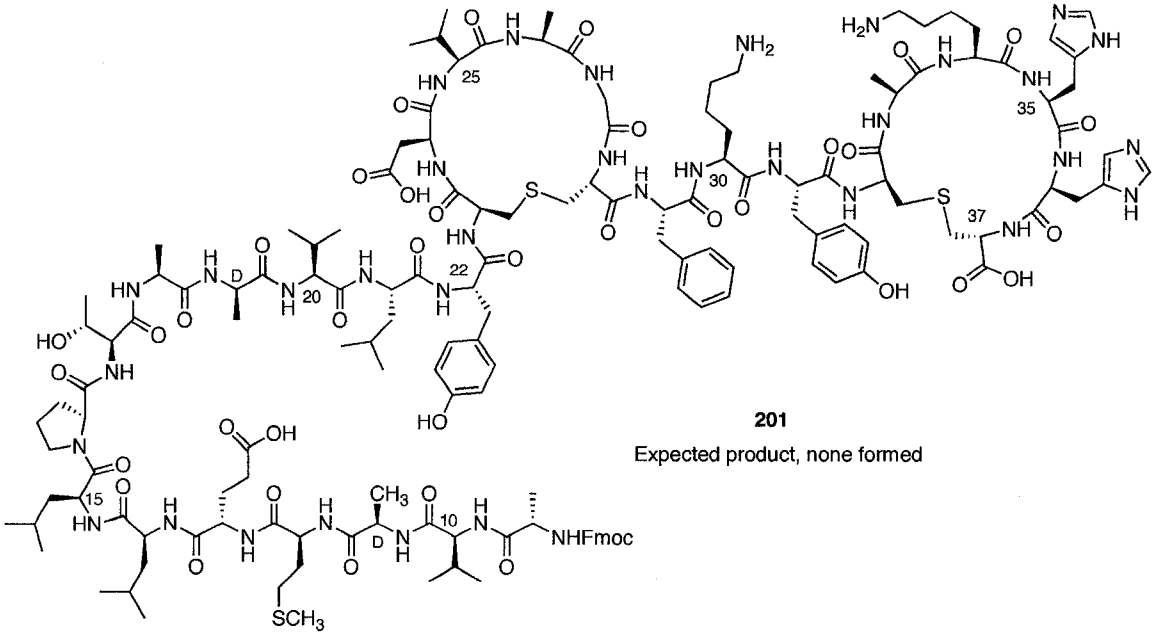
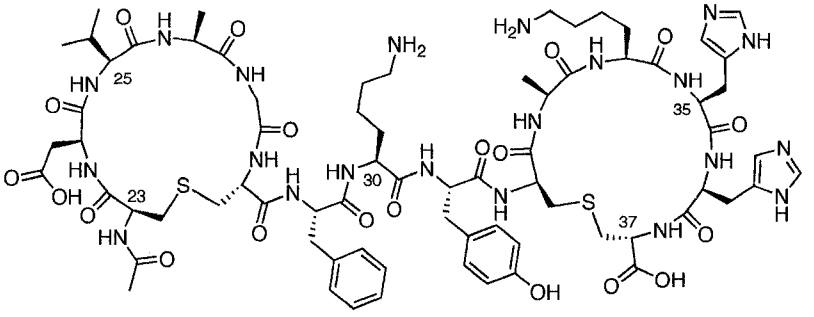


201

Expected product, none formed



199

Major product observed

[a] Conditions to obtain **200** from **197**: (i) 20% piperidine in DMF (ii) PyBOP, HOBt, NMM, DMF (iii) Fmoc-Leu-OH (iv) Repeat steps (i) and (ii) with Fmoc-Val-OH, Fmoc-D-Ala-OH, Fmoc-Ala-OH, Fmoc-Thr(ᵗBu)-OH, Fmoc-Pro-OH, Fmoc-Leu-OH

Biological evaluation of lanthionine rings A and B of lactocin S (199)

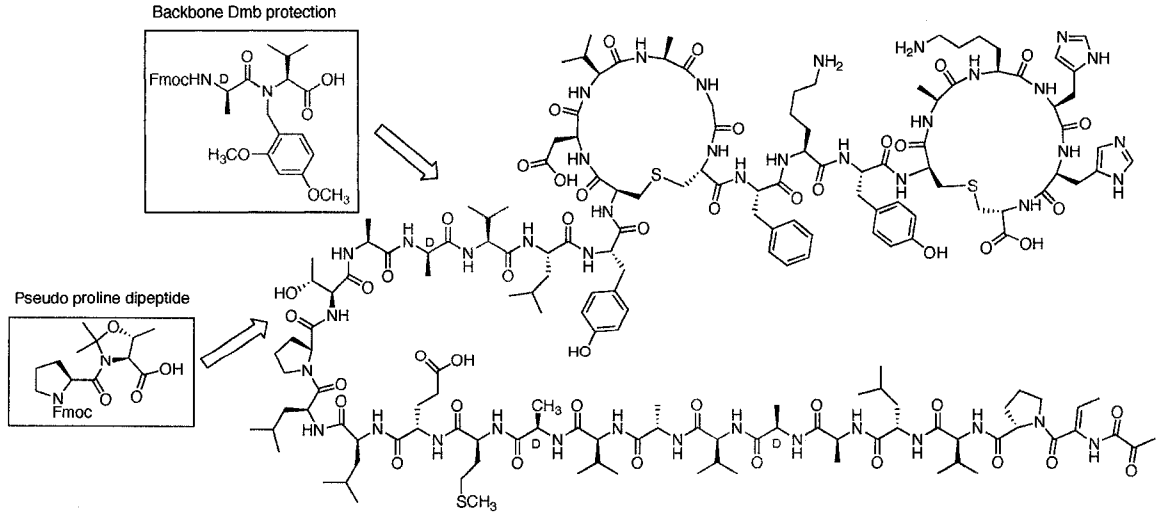
The crude bicyclic lanthionine fragment **199** of lactocin S was tested for antimicrobial activity using the spot on lawn assay with the indicator organism for lactocin S, *Pediococcus acidilactici*. No biological activity for the crude peptide **199** was observed.

Conclusions and future work

The bicyclic lanthionine rings A and B of lactocin S, have been successfully synthesized on solid-support by using preformed orthogonally protected lanthionine precursors. Unfortunately, efforts to introduce the linear sequence comprising of residues (3→22) have not been successful either by manual or automated SPPS.

To overcome the on-resin fragmentation problem during the peptide elongation to introduce residues (3→22), future studies could be directed towards changing the conformation of the growing peptide chain. This could be achieved by incorporation of backbone protection using the 2,4-dimethoxybenzyl (Dmb) group[166] at residue 20 and residue 10. Alternately, proline-pseudo proline dipeptides[167] could be introduced instead of residues 16 and 17 (Figure 29).

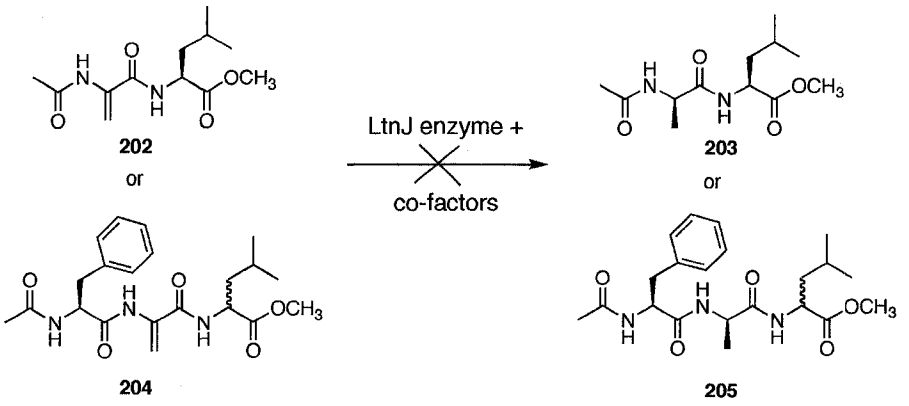
Figure 29. Suggested backbone protection to overcome on-resin fragmentation during lactocin S synthesis



2.2. Design and synthesis of a substrate for the *in vitro* reconstitution of posttranslational enzyme LtnJ, responsible for the introduction of D-alanines in lacticin 3147

LtnJ has been identified as the post-translational enzyme responsible for introduction of D-alanines in lacticin 3147.[76] LtnJ has been isolated and purified in the Vederas group. Previous attempts to demonstrate the enzymatic activity with a dehydrodipeptide and dehydrotripeptide substrate were unsuccessful (Scheme 51).

Scheme 51. Previous attempt to demonstrate LtnJ enzyme activity *in vitro* with dipeptide and tripeptide substrates



Recent literature reports[35-37] have shown that the leader peptide might be required for activity of post-translational enzymes. Based on this, a substrate with the leader peptide and part mature sequence of lacticin 3147 A1 was designed (Figure 30).

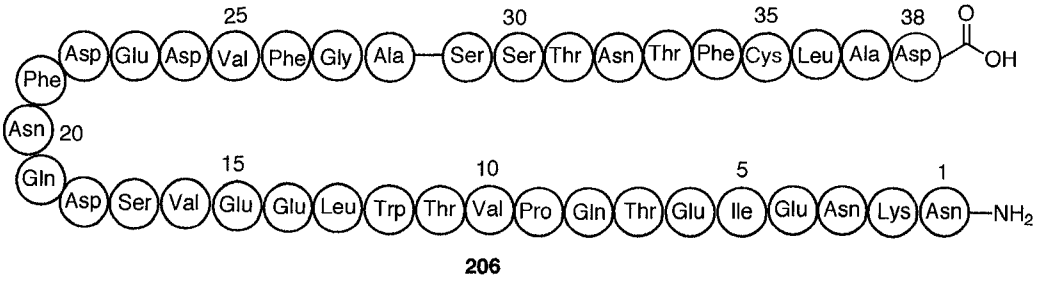
Figure 30. Design of substrate for LtnJ with leader peptide and part mature sequence of lacticin A1

Natural lacticin A1 with the leader sequence

MNKNEIETQPVTWLEEVSDQNFDEDVFGA - CSTNTFSLSDYWGNNGAWCTLTHECMAWCK

Designed LtnJ substrate with the leader sequence

-NKNEIETQPVTWLEEVSDQNFDEDVFGA – SSTNTFCLAD



206

Lacticin 3147 A1 peptide consists of a D-alanine at residue 7 in the mature sequence. Thus, in the designed substrate it is sought to have a dehydroalanine residue at position 7 that could be later tested for conversion to D-Ala by LtnJ.

To make a peptidic substrate with a dehydroalanine, we intended to use biochemical methods to produce the peptide consisting of natural amino acids. This would be followed by a chemical reaction to introduce the dehydroalanine residue. The synthetic challenge for such an approach is the selective manipulation of a specific functionality in the peptide in the presence of all other unprotected side chains. Based on literature reports,[168, 169] cysteine was chosen as the residue for conversion to dehydroalanine in an unprotected peptide. Thus, in our designed substrate peptide **206** the

serine at position 7 is replaced with cysteine and the cysteine at position 1 is replaced with serine. The serine at residue 9 is part of a lanthionine ring in the mature sequence. Thus in order to have a residue that could mimic part of the lanthionine at that position, serine 9 is replaced with alanine (Figure 30).

Our investigation began with model studies for introducing a dehydroalanine residue from a cysteine residue in an unprotected peptide. Byford[169] has reported the formation of a dehydroalanine from a cysteine residue as a side reaction during treatment with 0.1 M $Ba(OH)_2$. Further optimization of this reaction could provide a route to access dehydroalanine from a cysteine residue. Model studies were conducted with commercially available Insulin-like Growth Factor I (57-70) [ILGF] and GnRH Associated peptide (25-53), and human [GAP] peptides (Scheme 52 and Scheme 53). The progress of the model reactions was monitored by MALDI-TOF MS. Although mass spectrometry is not ideal for quantitation purposes, it can provide a rough approximation of the ratios of different products. All the ratios suggested for the model reactions are derived from MALDI-TOF MS. A concentration of 1 mg / mL of ILGF and GAP were used in the studies with 0.1 M $Ba(OH)_2$.

The results for the reaction of ILGF (**207**) with 0.1 M $Ba(OH)_2$ in a 1:1 ratio at different time intervals are shown in Table 3. The highest conversion to the product **208** is observed at 72 h along with unreacted starting material and the disulfide bridged product (Table 3, Entry 5).

Scheme 52. Model reaction for formation of dehydroalanine from cysteine with commercially available unprotected Insulin-like Growth Factor I (57-70) [ILGF] and $Ba(OH)_2$

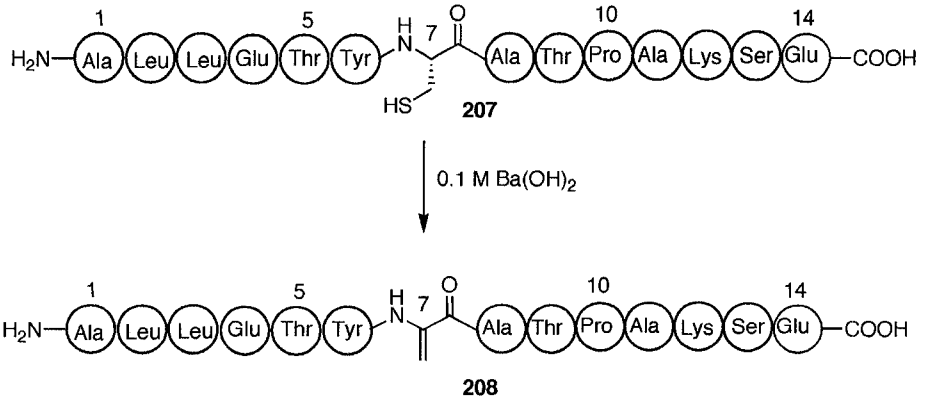


Table 3. Reaction of ILGF with 0.1 M $Ba(OH)_2$ in a 1:1 ratio to form dehydro-ILGF

No.	Temperature (°C)	Time (h)	Estimated ratio of **207**:**208**		Other products and secondary cleavage
1	25	0	1.0	-	-
2	25	1	1.0	0.25	Disulfide formation
3	25	3	1.0	0.4	Disulfide formation
4	25	24	1.0	1.0	-
5	25	72	0.3	1.0	-

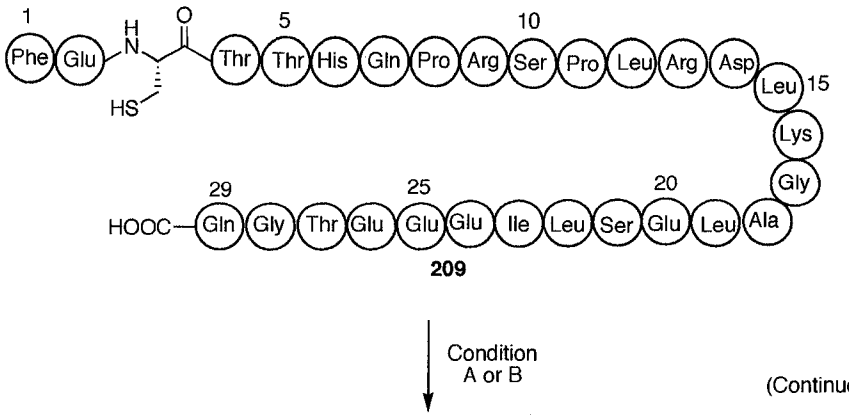
In an attempt to force the reaction to completion the ratio of ILGF: 0.1 M $Ba(OH)_2$ was changed to 1:2 and the reaction is performed at different time intervals and temperature. Although the quantitative conversion to the product is observed at longer reaction times at 25 °C or a shorter time at 45 °C, a number of side products are also observed (Table 4, Entry 4 and 5).

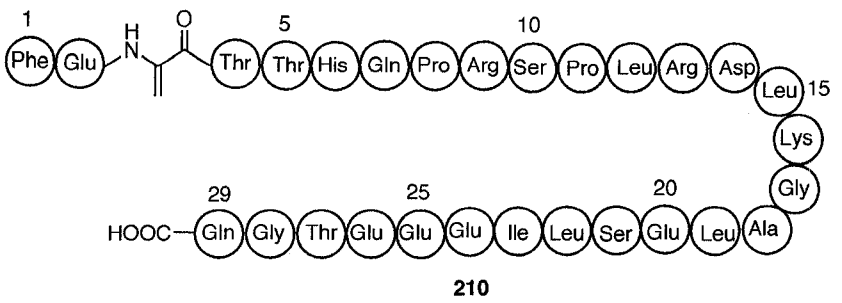
Table 4. Reaction of ILGF with 0.1 M $Ba(OH)_2$ in a 1:2 ratio to form dehydro-ILGF

No	Temperature (°C)	Time (h)	Estimated ratio of **207**:**208**		Other products and secondary cleavage
1	25	24	1.0	1.0	-
2	25	72	1.0	0.3	-
3	25	90	1.0	0.3	-
4	25	144	0.0	1.0	Secondary cleavage
5	45	15	0.0	1.0	Secondary cleavage

Commercially available GAP peptide **209** contains 29 amino acid residues with a single cysteine at residue 3. When reacted with 0.1 M $Ba(OH)_2$ for a longer reaction time based on the previous studies, conversion to the desired product **210** is observed along with the starting material and a product 18 Da higher in mass than **210** (Scheme 53, Condition A). This presumably arises from the addition of water to the dehydroalanine moiety in **210** (Table 5).

Scheme 53. Model reaction for formation of dehydroalanine from cysteine with commercially available unprotected GnRH Associated peptide (25-53), human [GAP] and $Ba(OH)_2$





Condition A: 0.1 M $Ba(OH)_2$, Condition B: 1-cyano-4-dimethylaminopyridinium tetrafluoroborate, basic pH 7 – 14 with different buffers.

Table 5. Reaction of GAP with 0.1 M $Ba(OH)_2$ in a 1:1 ratio to form dehydro-GAP

No	Temperature (°C)	Time (h)	Estimated ratio of **209:210**		Other products and secondary cleavage
1	25	168	0.3	1.0	Desired product + H_2O

The results so far obtained for the formation of dehydroalanine with $Ba(OH)_2$ are not satisfactory because of the incomplete conversions and side reactions. At this point, it was decided to use an alternative approach developed by Tam and co-workers[168] for introduction of a dehydroalanine from cysteine using 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP). Thus, reaction of GAP (**209**) with a solution of CDAP at various temperatures, pH and reaction times were examined (Table 6). The clean and complete conversion of GAP (**209**) to dehydro-GAP (**210**) occurs at room temperature in phosphate buffer (pH 7.5) when reacted for 23 h (Table 6, Entry 7).

This optimized condition will be used for the introduction of dehydroalanine in the designed substrate for LtnJ.

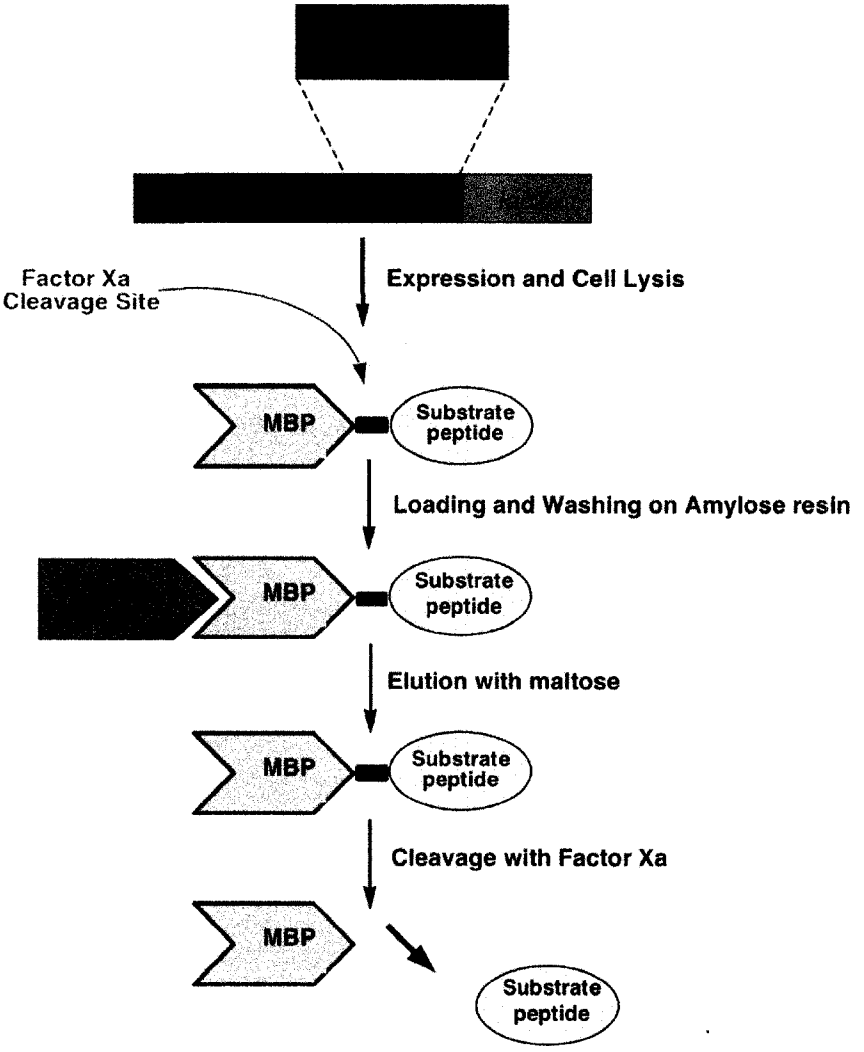
Table 6. Reaction of GAP with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate in a 1:10 ratio under basic pH to form dehydro-GAP

No	Temperature (°C)	pH	Time (h)	Estimated ratio of thiocyano intermediate : **210**		Other products and secondary cleavage
1	25	7.0	0.25	1.0	0.4	-
2	25	7.0	15	1.0	1.0	-
3	4	7.0	15	1.0	0.2	-
4	4	7.0	36	1.0	0.4	-
5	4	7.5	62	0.7	1.0	secondary cleavage
6	25	7.5	1.25	1.0	0.6	-
7	25	7.5	23	0.0	1.0	-
8	25	9.0	2	0.9	1.0	major – secondary cleavage
9	25	9.0	16	0.0	1.0	major – secondary cleavage
10	25	14.0	0.5	0.0	1.0	7 other secondary cleavage products

To obtain the pre-substrate peptide **206** for chemical transformation to **49**, an *E. coli* transformant containing the plasmid pQE60-lacticin which encodes for the pre-substrate peptide **206** was prepared and provided by Dr. Marco van Belkum (Research Associate, Vederas group) using the pMAL™ expression system.[170, 171] In this system, the recombinant protein contains a *N*-terminal maltose binding protein (MBP) followed by a Factor Xa cleavage site (Ile-Glu/Asp-Gly-Arg↓) and the pre-substrate peptide. The overexpression of the fusion protein is induced by isopropyl β-D-thiogalactopyranoside (IPTG). Typical yields of the fusion protein are between 10-40 mg per liter of culture. Purification of the MBP-tagged peptide is achieved by affinity chromatography (Figure 31).

A starter culture containing the *E. coli* transformant with the pQE60-lacticin is grown at 37 °C for 16 h at 225 rpm in Luria Broth base (LB broth). IPTG is added before the log-phase growth for inducing the over-expression of the recombinant protein. The cells containing the over-expressed MBP fusion are collected by centrifugation. Cell lysis releases the cytoplasmic MBP fusion into buffer. The cell debris is removed by centrifugation. The cell lysate is then diluted with the amylose column buffer and loaded onto the amylose resin, which selectively binds the MBP fusion. Elution of the MBP fusion from the column occurs in the presence of maltose, because of the high affinity of the maltose binding protein to maltose than the amylose resin. The product fractions are then dialyzed and lyophilized to give the substrate peptide **206** attached to MBP in 18-23 mg / L yield.

Figure 31. Schematic representation of the pMAL system

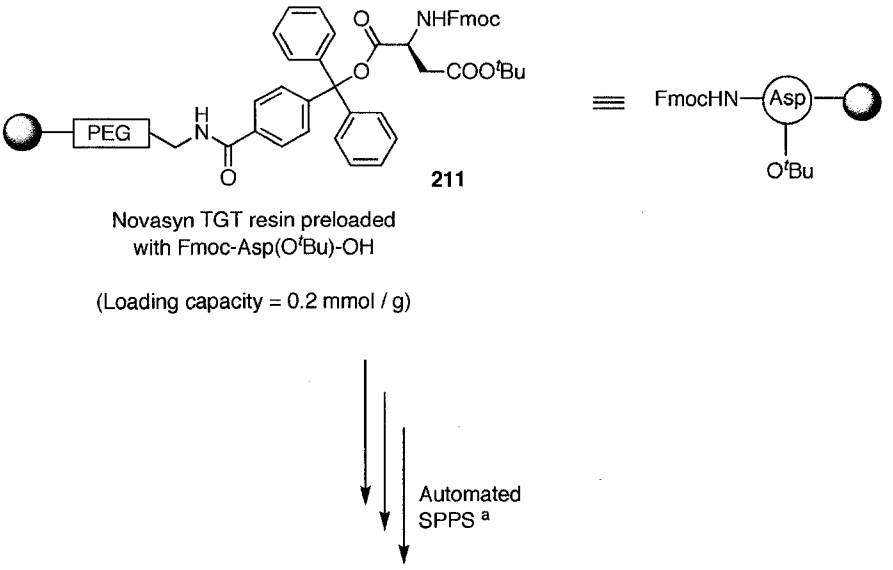


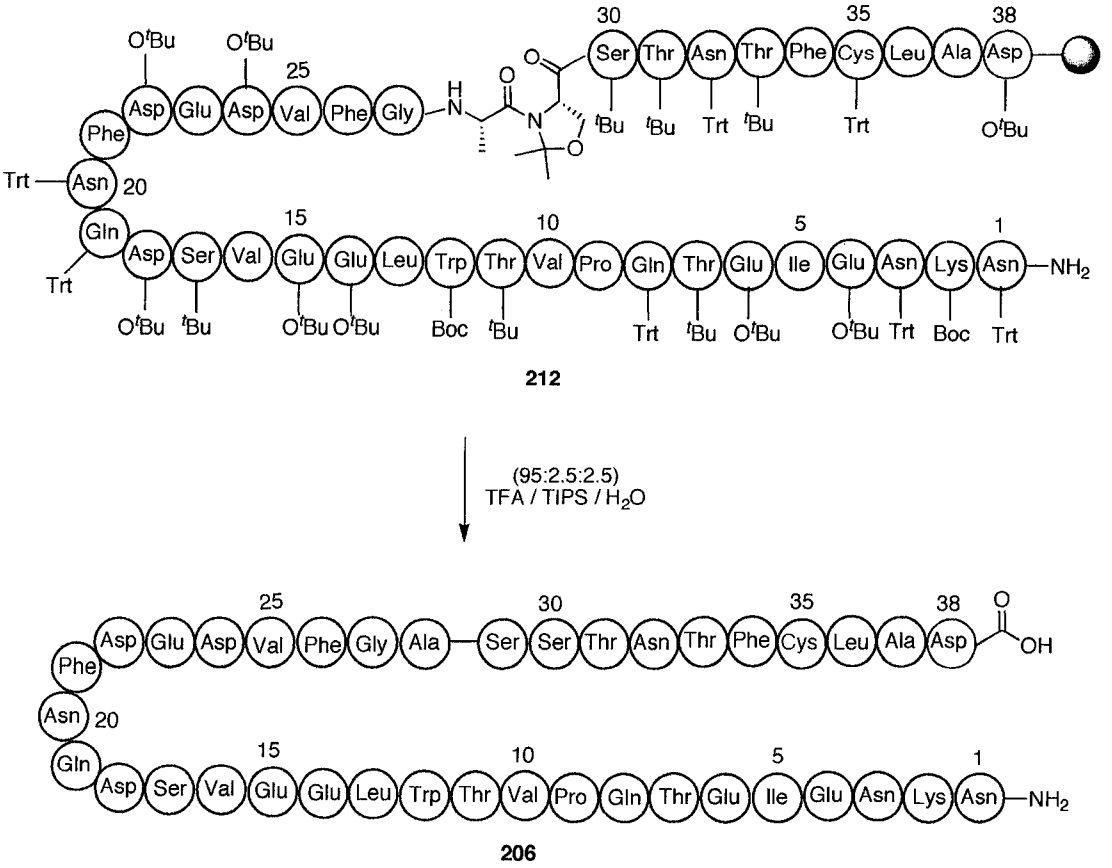
Pilot experiments were performed to optimize the conditions for cleavage of the peptide **206** from the maltose fusion at the engineered Factor Xa cleavage site. The progress of the cleavage reaction is monitored by MALDI-TOF MS. Completion of the cleavage by Factor Xa is ascertained by the disappearance of the peak for the fusion protein at ~ 46,800 Da. The best results for the cleavage reaction are obtained when the cutting experiment is done for 3 h at 25 °C with 1 weight% of Factor Xa corresponding to the MBP fusion. Secondary cleavage products were observed even under the optimized

conditions. A scale-up of the cutting experiment with 15 mg of the MBP fusion and Factor Xa followed by purification disappointingly yields only sub mg quantities of partially pure peptide **206**. Solubility problems with the cleaved peptide further complicate the isolation of **206**.

At this point, it was decided to resort to chemical synthesis for obtaining the precursor peptide **206**. The automated solid phase synthesis of peptide **206** was done on an ABI 433A peptide synthesizer with NovaSyn™ TGT resin preloaded with Fmoc-Asp(O^{t}Bu)-OH **211**, substitution capacity = 0.2 mmol/g. In order to facilitate a smooth synthesis and avoid any on-resin aggregation problems, the threonine at position 28 and alanine at position 29 of **206** were replaced with Fmoc-Ala-Thr($\psi^{Me,Me}$pro)-OH pseudo proline dipeptide.

Scheme 54. Chemical synthesis of substrate for LtnJ enzyme with the leader sequence and part mature sequence of lacticin A1





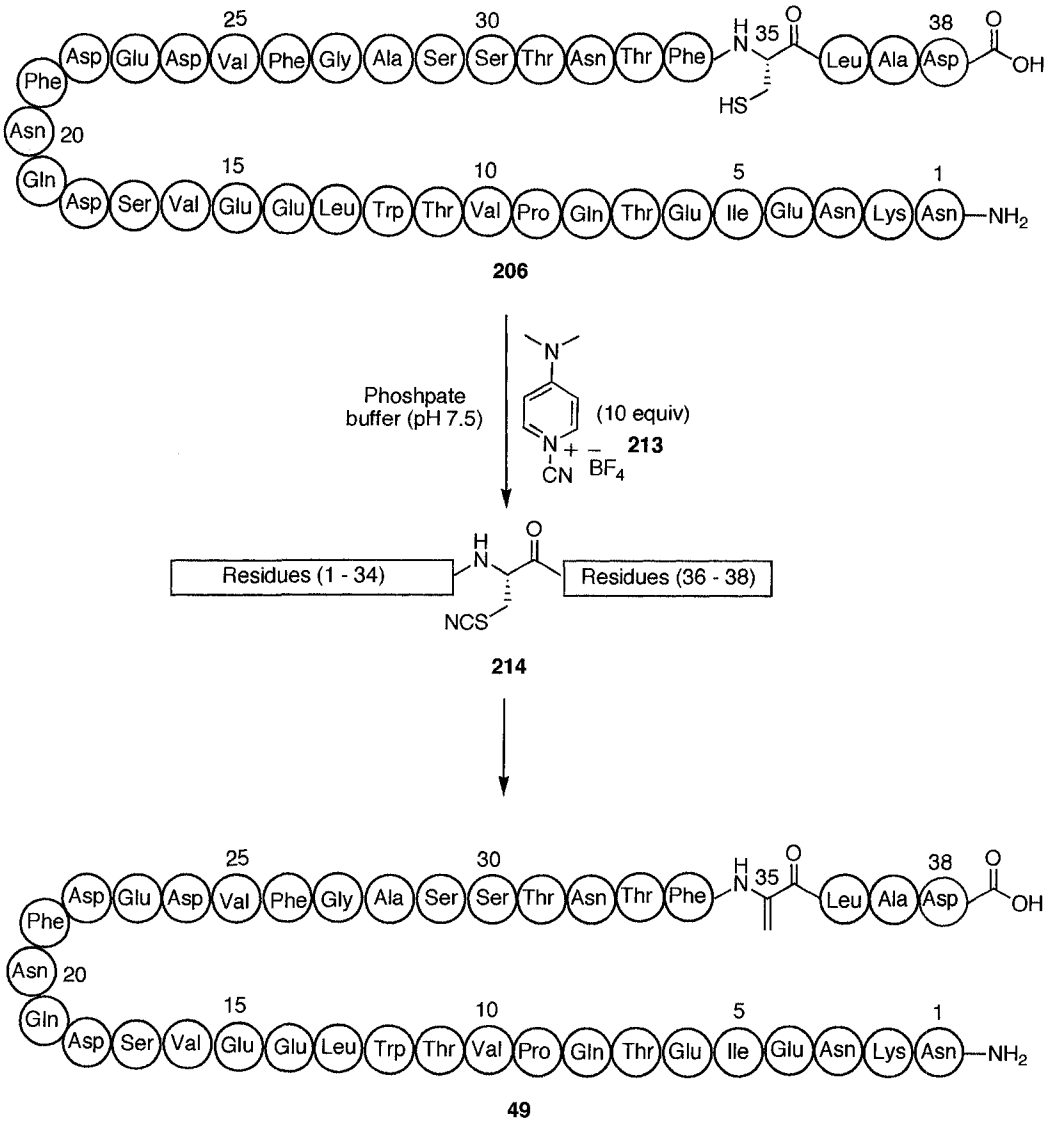
[a] Automated SPPS conditions to obtain **212** from **211**: (i) 22% piperidine in NMP (ii) HBTU, HOBt, NMM, NMP (iii) Fmoc-Ala-OH (iv) Repeat steps (i) and (ii) with Fmoc-Leu-OH, Fmoc-Cys(Trt)-OH, Fmoc-Phe-OH, Fmoc-Thr(ᵗBu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Thr(ᵗBu)-OH, Fmoc-Ser(ᵗBu)-OH, Fmoc-Ala-Ser($\psi^{Me,Me}$pro)-OH, Fmoc-Gly-OH, Fmoc-Phe-OH, Fmoc-Val-OH, Fmoc-Asp(OᵗBu)-OH, Fmoc-Glu(OᵗBu)-OH, Fmoc-Asp(OᵗBu)-OH, Fmoc-Phe-OH, Fmoc-Asn(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Asp(OᵗBu)-OH, Fmoc-Ser(ᵗBu)-OH, Fmoc-Val-OH, Fmoc-Glu(OᵗBu)-OH, Fmoc-Glu(OᵗBu)-OH, Fmoc-Leu-OH, Fmoc-Trp(Boc)-OH, Fmoc-Thr(ᵗBu)-OH, Fmoc-Val-OH, Fmoc-Pro-OH, Fmoc-Gln(Trt)-OH, Fmoc-Thr(OᵗBu)-OH, Fmoc-Glu(OᵗBu)-OH, Fmoc-Ile-OH, Fmoc-Glu(OᵗBu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Asn(Trt)-OH.

The coupling of residues (1→37) proceeds readily on solid-support to yield **212** (Scheme 54). The peptide is then cleaved from the resin with global deprotection using (95:2.5:2.5) TFA / TIPS / H_2O to obtain the desired peptide **206**. Analysis of the product by MALDI-TOF MS revealed a major peak for the desired product **49** [4293.3 (M+H)]

along with a minor peak 18 Da lower than the desired product. This impurity could arise from an on-resin aspartimide formation during the course of the synthesis. Aspartimide formation is a documented side reaction in solid-phase peptide synthesis and is known to arise when a *C*-terminal aspartic acid is coupled to a *N*-terminal alanine or glycine residue.[172] However, the automated synthesis proceeded with an excellent efficiency and about 30 mg of crude **206** is obtained from 175 mg of the resin. The crude product is then purified by reverse phase HPLC to yield 2.40 mg of **206**. The rather poor yield for the purification is due to the poor solubility of the peptide **206**.

With the desired precursor peptide **206** in hand, the reaction for introduction of the dehydroalanine at position 3 is undertaken. Thus, reaction of **206** with CDAP (**213**) in phosphate buffer (pH 7.5) cleanly converts **206** to the thiocyanate intermediate **214** in 30 minutes. Analysis of an aliquot of the reaction mixture by MALDI-TOF MS after 5 h showed a peak for the formation of the desired product **49** along with the thiocyanate intermediate **214** and other secondary cleavage products. Allowing the reaction to proceed for a much longer time only led to the disappearance of the desired product and only secondary cleavage product being observed. The structures of the secondary cleavage products have not yet been identified. Based on the mass differences there are indications that they could be a result of the cleavage of the *C*-terminal residues (35→38).

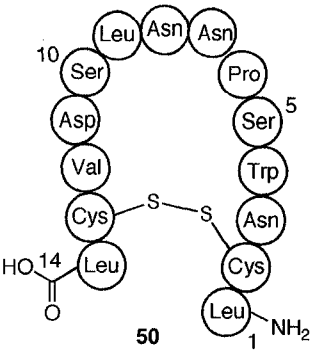
Scheme 55. Formation of a dehydroalanine by activation and elimination of cysteine (residue 35)



Further optimization of the reaction with respect to temperature and time could potentially solve the secondary cleavage problems and provide enough material for studies with the LtnJ enzyme. Efforts in this direction are ongoing in the Vederas group.

2.3. Modifying lantibiotics to be agents active against Gram-negative organisms: Synthesis of residues (75→88) of colicin V for attachment to lantibiotics

Lantibiotics are active only against Gram-positive organisms as they do not have the ability to penetrate the outer membrane of Gram-negative organisms. However, bacteriocins such as colicins and microcins are active against Gram-negative bacteria.[122, 125] Based on the consensus sequence in the *C*-terminus between colicin V and microcin L, it is hypothesized that the 14 residues (75→88) colicin V loop **50** might act as the recognition sequence for active transport by FhuA-tonB system.

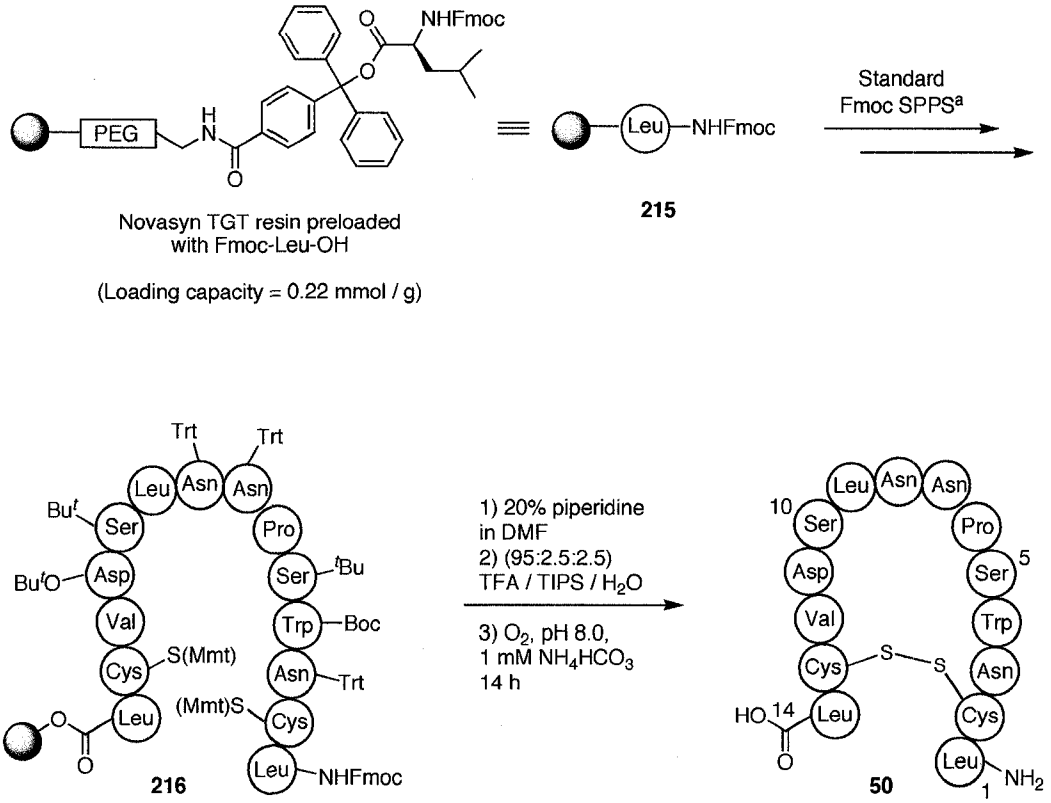


50

The synthesis of the colicin V loop **50** is initiated on solid support using NovaSyn™ TGT resin pre-loaded with Fmoc-Leu-OH **215**, substitution capacity = 0.22 mmol / g. Manual SPPS introduces residues (1→13) to afford **216** on solid support (Scheme 56). The final Fmoc removal is accomplished with 20% piperidine in DMF, and the peptide is then cleaved from the resin with concomitant removal of all the protecting groups using (95:2.5:2.5) TFA / TIPS / H_2O (Scheme 56). The 2,13-disulfide bond is formed by bubbling oxygen through a solution of the crude peptide in 1 mM NH_4HCO_3 buffered to pH 8.0. Purification by reverse phase HPLC yields the colicin V loop **50**. The

mass of **50** as determined by MALDI-TOF MS is in agreement with the calculated mass for the 14-mer colicin V loop [1575.6 (M+H)].

Scheme 56. Synthesis of residues (75→88) of colicin V (**50**) on NovaSyn TGT resin using Fmoc SPPS



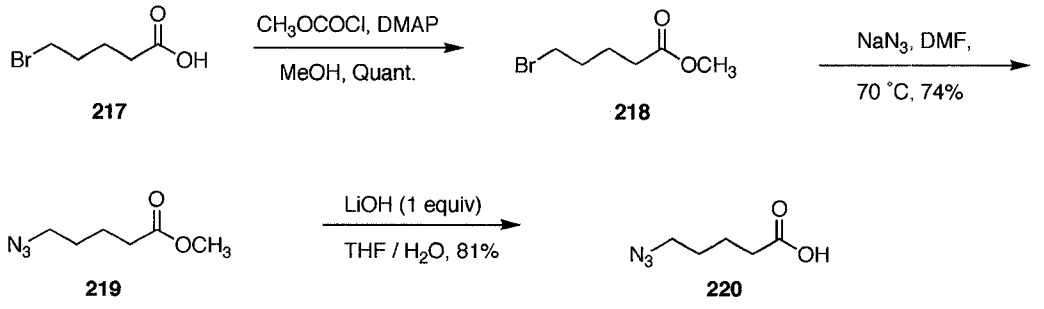
[a] Conditions used for synthesis of **216** from **215**: (i) 20% piperidine in DMF (ii) PyBOP, HOBt, NMM, DMF (iii) Fmoc-Cys(Mmt)-OH, (iv) repeat steps (i) and (ii) with Fmoc-Val-OH, Fmoc-Asp(O'Bu), Fmoc-Ser('Bu)-OH, Fmoc-Leu-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Pro-OH, Fmoc-Ser('Bu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Cys(Mmt)-OH, Fmoc-Leu-OH.

The conjugation of the colicin V loop to lantibiotics could be accomplished via a short linker attached to the *N*-terminus of **50**. The alkyne-azide cycloaddition[173] reaction

has emerged as one of the most powerful methods for ligation of fragments of biomolecules. We envision using the cycloaddition chemistry for attachment of the colicin V loop to lantibiotics such as nisin and gallidermin via an azide or an alkyne linker attached to **50**.

The synthesis of an azido carboxylate linker **220** begins with the esterification of the carboxylic acid functionality of commercially available 5-bromovaleric acid (**217**) to give **218** (Scheme 57). The displacement of the bromo group of **218** with sodium azide in DMF yields **219**. Hydrolysis of the ester with LiOH gives 5-azidopentanoic acid (**220**).

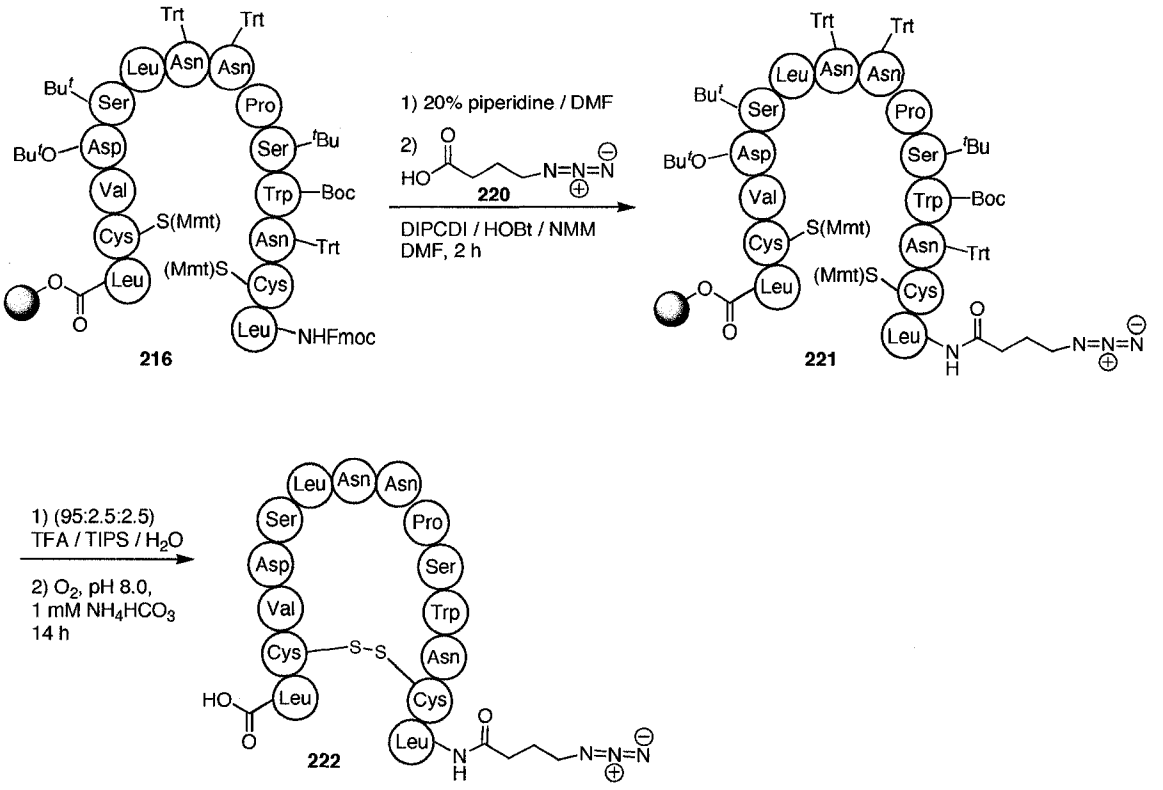
Scheme 57. Synthesis of an azido carboxylate linker **220**



The attachment of the azido carboxylate to the colicin V loop starts with the removal of the *N*-terminal Fmoc group using 20% piperidine in DMF. Activation of the azido carboxylate **220** with DIPCDI / HOBt / NMM followed by reaction with the free amine on-resin yields **221** (Scheme 58). Treatment of the resin with TFA along with scavenger cleaves the peptide from the resin. The 2,13-disulfide bond is formed by bubbling oxygen through a solution of the crude peptide dissolved in 1 mM NH_4HCO_3,

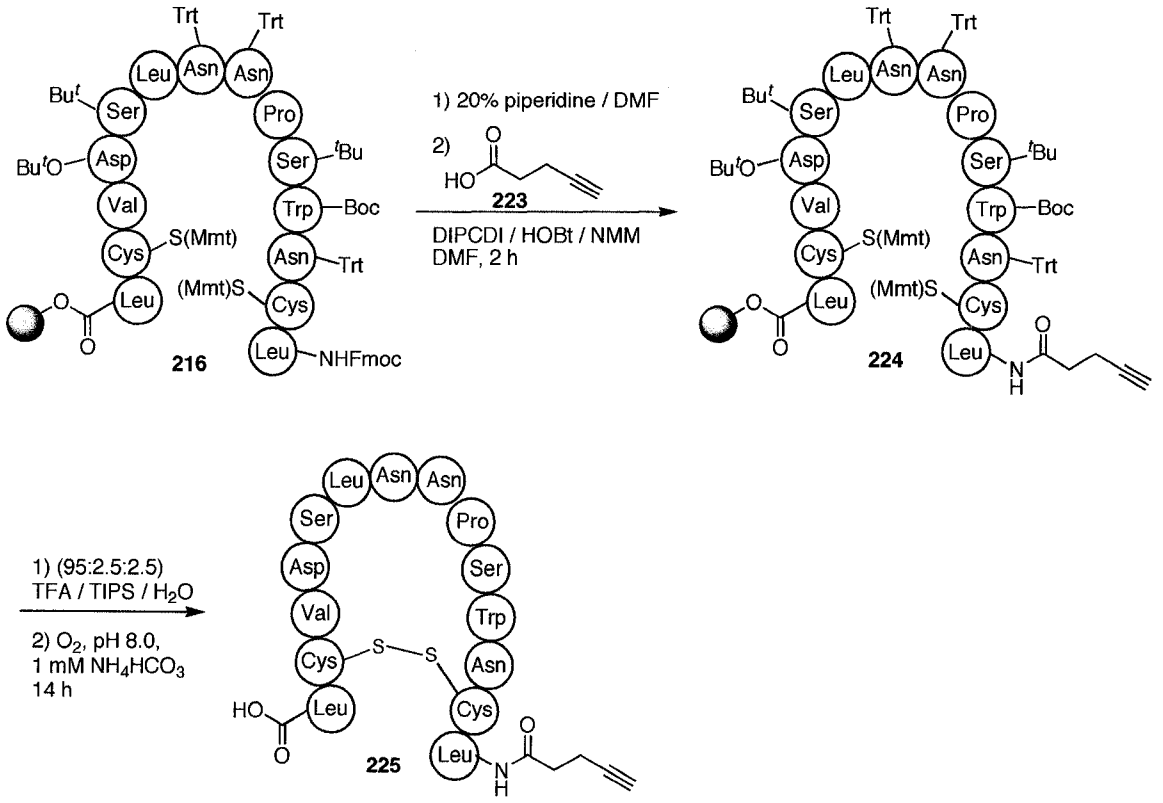
buffered to pH 8.0. Removal of the buffer and lyophilization yields an azide linked colicin V loop **222**. The mass of **222** determined by MALDI-TOF MS is in agreement with the calculated mass [1723.4 (M+Na)].

Scheme 58. Synthesis of residues (75→88) of colicin V with an azide linker **222**



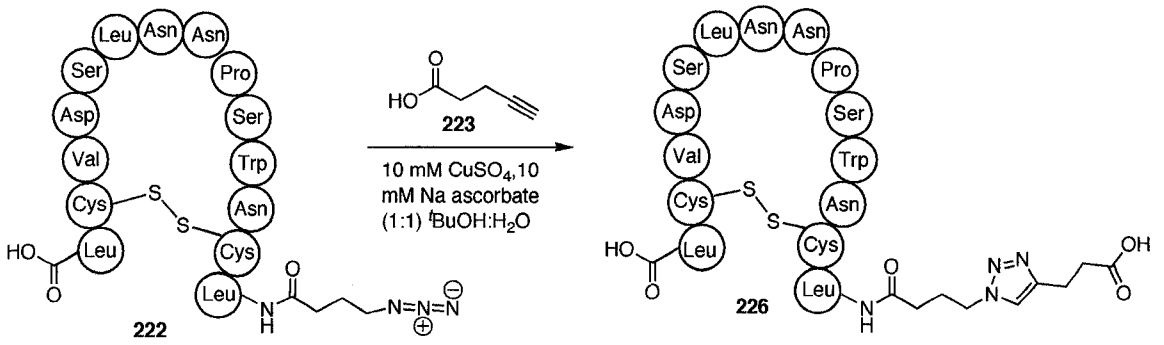
For the synthesis of the colicin V loop with an alkyne linker, a procedure similar to the attachment of the azide linker is followed. Thus, removal of the Fmoc group of **216** followed by reaction with the commercially available 4-pentynoic acid (**223**) yields **224** on solid support (Scheme 59). Cleavage of the peptide from solid support with TFA followed by formation of the 2,13-disulfide bond under the conditions used before yields **225**.

Scheme 59. Synthesis of residues (75→88) of colicin V with an alkyne linker **225**



With the azide and alkyne linked colicin V loop (**222** and **225**) in hand, a model azide-alkyne cycloaddition reaction was performed. Thus, reaction of the azide linked colicin V loop **222** with commercially available alkyne **223** under high concentrations of $CuSO_4$ and sodium ascorbate in 1:1 ^{t}BuOH: H_2O cleanly yields the crude **226**. The calculated mass of **226** is in agreement with the mass observed by MALDI-TOF MS: 1677.7 (M+Na).

Scheme 60. Model azide-alkyne cycloaddition reaction with peptide **222**



In conclusion, residues (75→88) of colicin V **50** have been successfully synthesized by solid phase peptide synthesis with an azide and an alkyne linker. The conjugation of the synthesized colicin V loop with an azide and alkyne linker (**222** and **225**) to lantibiotics is currently under investigation in the Vederas group.

CHAPTER 3. SUMMARY AND FUTURE DIRECTIONS

With the tremendous potential of lantibiotics for applications in food, veterinary medicine and human therapeutics being realized, there is a considerable interest in understanding the chemistry and biology of lantibiotic production. In this thesis studies related to the chemical synthesis, biosynthesis and mode of action of lantibiotics have been discussed.

Development of methods for the chemical synthesis of lantibiotics and their analogues, especially solid-supported techniques, would allow facile access to lantibiotic structures for structure-activity relationship studies and for structural confirmation. In order to gain insight into the specific roles of the post-translational modifications, two analogues of lantibiotic peptide lacticin 3147 A2, **43** and **44** have been successfully completed on solid-support. In an effort to overcome the oxidative instability of lantibiotic peptides, a carbocyclic analogue of lacticin 3147 A2 (**43**) in which carbon atoms replace the sulfur atoms of the lanthionine rings has been successfully synthesized by a combination of solid phase and solution phase peptide synthesis. The synthesis of a carbocyclic-larger ring analogue **43** represents the first complete synthesis of a lantibiotic analogue on solid-support. Nisin is the only other lantibiotic for which a chemical synthesis has been completed. Unfortunately, the carbocyclic analogue **43** did not exhibit antimicrobial activity when tested against the indicator organism *Lactococcus lactic* HP. To shed light on the importance of β-methyllanthionine rings in lantibiotics, a lanthionine analogue of lacticin 3147 A2 (**44**) wherein lanthionine rings replace the β-

methyllanthionine rings has been successfully synthesized on solid-support. The synthesized lanthionine analogue of lacticin A2 (**44**) displays potent synergistic antimicrobial activity against Gram-positive indicator organism *Lactococcus lactis* HP. Interestingly, the analogue **44** that differs only by two methyl groups from the natural lacticin A2 (**12**) displays no independent antimicrobial activity. Also, a potent lead compound, peptide **173** that could lead to a simplified analogue of lacticin A2 has been identified. The synthesis of analogue **44**, is the first example of a successful complete synthesis of a lanthionine containing lantibiotic analogue on solid-support. The facile and scalable solution phase method developed for the synthesis of the highly post-translationally modified residues (1→5) **[45]** as well as the chemistry developed for the synthesis of multiple lanthionine rings on solid-support are being applied towards the total synthesis of lantibiotic peptides lacticin 3147 A1 and A2.

In an effort to confirm the proposed structure of lactocin S (**48**), a total synthesis was undertaken. The insight gained from the synthesis of **44**, has led to the successful synthesis of the lanthionine rings A and B of lactocin S (**197**). Attempted elaboration from the bicyclic lanthionine rings has been less fruitful so far. Nevertheless, with the plethora of methods for overcoming difficult peptide sequences, the total synthesis of lactocin S could be readily completed. In summary, the syntheses of lantibiotic analogues **44**, **45** and part of the structure of **48** constitutes a significant development in the chemical synthesis of lantibiotics and their analogues, after the landmark total synthesis of nisin. The successful exploration now opens the door for the synthesis of lantibiotic

structures on solid-support for SAR studies that could lead to the generation of a blueprint for designing novel lantibiotic structures.

LtnJ has been identified as the enzyme responsible for the reduction of dehydroalanines to D-alanines in lacticin 3147 A1 & A2. Successful *in vitro* assay of LtnJ enzyme could provide insight into this biosynthetic process. In order to understand the substrate specificity of LtnJ, a substrate containing the leader peptide and part of the mature sequence of lacticin 3147 A1 with a dehydroalanine has been designed and synthesized (**49**). A scale up and optimization of the dehydroalanine formation reaction would readily provide access to **49** for studies with LtnJ enzyme. In the long-term, successful reconstitution of LtnJ would provide a way for the formation of D-amino acid containing peptides.

Lantibiotics are inactive against Gram-negative bacteria because of their inability to cross the outer membrane. Bacteriocins such as colicins and microcins cross the outer-membrane of Gram-negative bacteria by utilizing the active transport machinery in bacteria. The hypothesized recognition sequence consisting of residues (75→88) of colicin V (**50**) as well as its analogues with an azide (**222**) and an alkyne linker (**225**) have been successfully synthesized on solid-support. Attachment of the colicin V loop with an azide (**222**) or an alkyne (**225**) functionality to lantibiotics such as nisin, gallidermin through a cycloaddition reaction could lead to a lantibiotic conjugate with broad spectrum of activity. Efforts in this direction are underway.

CHAPTER 4: EXPERIMENTAL PROCEDURES

4.1. General Information

4.1.1. Instruments for compound characterization

Nuclear Magnetic Resonance (NMR) spectra were recorded on a Varian Inova 600, Inova 500, Inova 400, Inova 300 or Unity 500 spectrometer. Chemical shift values for proton and carbon NMR are reported in parts per million (ppm) downfield relative to tetramethylsilane (TMS). For ^{1}H NMR (300, 400, 500 or 600 MHz) spectra, δ values were referenced to $CDCl_3$ (7.26 ppm), CD_3OD (3.30 ppm), $(CD_3)_2CO$ (2.04 ppm) or DMSO-d_6 (3.53 ppm), and for ^{13}C (75, 100, 125 or 150 MHz) spectra, δ values were referenced to $CDCl_3$ (77.0 ppm), CD_3OD (49.0 ppm), $(CH_3)_2CO$ (29.8 ppm) or DMSO-d_6 (39.7 ppm) as the solvents. Additional assignments were made using pulsed field gradient versions of shift correlation spectroscopy (gCOSY), heteronuclear multiple quantum coherence spectroscopy (gHMQC) and Transverse Rotating-frame Overhauser Enhancement Spectroscopy (TROESY). ^{1}H NMR data are reported in the following order: multiplicity (app, apparent, s, singlet; d, doublet; t, triplet; q, quartet; pent, pentet; sext, sextet and m, multiplet), number of protons, coupling constant (*J*) in Hertz (Hz) and assignment. When appropriate, a signal is preceeded by br, indicating the signal was broad. The coupling constants reported are within an error range of 0.2-0.4 Hz, and have been rounded to the nearest 0.1 Hz. All literature compounds had IR, ^{1}H NMR and mass spectra consistent with the assigned structures.

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

Mass spectra (MS) were recorded on Kratos AEIMS-50 high resolution mass spectrometer (HRMS) or Micromass ZabSpec Hybrid Sector-TOF positive mode electrospray ionization ((ES), 0.5% solution of formic acid in MeCN:H_2O/1:1) instruments or on a Perspective Biosystems Voyager™ Elite MALDI-TOF using either 4-hydroxy-α-cyanocinnamic acid (HCCA), 2,5-dihydroxybenzoic acid (DHB) or 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) as matrices. LC-MS/MS or MS/MS were performed on a Waters (Micromass) Q-TOF-Premier Nano-Acquity nano flow UPLC system with a flow rate ranging from 0.5 to 3 μL/min. High resolution MALDI MS were recorded on a Bruker 9.4T Apex-Qe FTICR equipped with a Apollo II Dual ESI/MALDI source using DHB as the matrix.

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 were measured at the sodium D line (λ = 589.3 nm).

4.1.2. Reagents and solvents

All commercially available reagents and solvents were purchased from the Aldrich Chemical Company Inc. (Madison, WI), Sigma Chemical Company (St. Louis, MO), or Fisher Scientific Ltd. (Ottawa, ON). All protected amino acids and SPPS resins were purchased from the Calbiochem-Novabiochem Corporation (San Diego, CA), Sigma-Aldrich Canada Ltd. (Oakville, ON) or Bachem California Inc. (Torrance, CA). All reagents and solvents were of American Chemical Society (ACS) grade and used without further purification. All solvents used for anhydrous reactions were dried according to Perrin *et al*[174] and Vogel.[175] Tetrahydrofuran and diethyl ether were freshly distilled over sodium and benzophenone under dry argon prior to use. Dichloromethane, pyridine, and triethylamine were distilled over calcium hydride. Ethyl acetate was distilled over potassium carbonate. HPLC grade methanol, acetonitrile and dimethylformamide were used without purification.

4.1.3. Reactions and Purifications

Commercially available ACS grade solvents (>99.0% purity) were used for column chromatography without any further purification. Flash chromatography was performed according to the method of Still *et al.*[176] 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

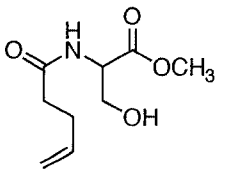
quenching; iodine staining; by dipping the TLC plates in a solutions of Ninhydrin:acetic acid:n-butanol (0.6 g:6 mL:200 mL); $Ce(SO_4)•4H_2O/(NH_4)MoO_{24}•4H_2O/H_2SO_4/H_2O$ (5 g:12.5 g:28 mL:472 mL) spray. Flash chromatography was performed using Merck type 60, 230-400 mesh silica gel. The removal of solvent *in vacuo* refers to evaporation under reduced pressure below 40 °C using a Buchi rotary evaporator followed by drying (<0.1 mm Hg) to a constant sample mass. Unless otherwise specified, solutions of NH_4Cl, $NaHCO_3$, HCl, citric acid, lithium hydroxide and sodium thiosulfate refer to aqueous solutions. Brine refers to a saturated aqueous solution of sodium chloride. In reactions 'rt' refers to room temperature.

High performance liquid chromatography (HPLC) was performed on a Varian Prostar chromatograph equipped with a model 325 variable wavelengths UV detector and a Rheodyne 7725i injector fitted with a 20 to 2000 μL sample loop. The columns used were Vydac Protein C_4 (214TP) steel walled column (reverse phase, C_4 column, 10 μm, 300 Å, 22 x 250 mm), Alltech ProSphere™ HP C_4 steel walled column (reverse phase, C_4 column, 5 μm, 300 Å, 4.6 x 250 mm), a Vydac protein & peptide C_{18} (218TP510) steel walled column (reverse phase, C_{18} column, 5 μM, 10 x 250 mm) or a Vydac protein & peptide C_{18} (218TP1022) steel walled column (reverse phase, C_{18} column, 10 μM, 22 x 250 mm). All HPLC solvents were filtered using a Millipore filtration system under vacuum before use.

4.2. Experimental procedures and data for compounds

4.2.1. Synthesis and testing of a carbocyclic-larger ring analogue of lacticin 3147 A2

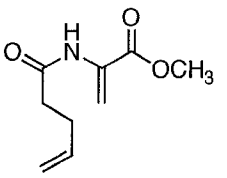
Methyl 3-hydroxy-2-pent-4-enamidopropanoate (56)



To a solution of 4-pentenoic acid (**55**) (2.00 g, 19.97 mmol) in CH_2Cl_2 (8.0 mL) Et_3N (5.56 mL, 39.94 mmol) was added at 0 °C, followed by ethyl chloroformate (2.160 g, 19.90 mmol) and stirred for 15 minutes. D,L-Serine methyl ester hydrochloride (**54**) (3.11 g, 19.98 mmol) was added and stirred for an additional 2 h at rt. The reaction mixture was washed sequentially with 1 M HCl (2 x 25 mL), $NaHCO_3$ (2 x 25 mL), water (2 x 25 mL) and brine (25 mL). The organic layer was separated, dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO_2, 6:94/MeOH:chloroform) to yield **56** (2.07 g, 52%) as a sticky white solid. (R_f 0.3 on SiO_2, 1:1/EtOAc:hexanes); IR ($CHCl_3$ cast) 3312, 3079, 2954, 1744, 1651, 1540 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 6.59 (br d, 1H, J = 5.5 Hz, NH), 5.87-5.79 (m, 1H, =CH), 5.08 (app dq, 1H, J = 17.0, 1.5 Hz, =CH*H*), 5.02 (app dq, 1H, J = 10.0, 1.5 Hz, =C*H*H), 4.67 (app pent, 1H, J = 3.5 Hz, H_α), 3.97 (dd, 1H, J = 11.5, 3.5 Hz, Ser-H_β), 3.89 (dd, 1H, J = 11.0, 3.5 Hz, Ser-$H_{\beta'}$), 3.78 (s, 3H, OCH_3), 3.03 (br s, 1H, OH), 2.43-2.35 (m, 4H, CH_2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.8 (C=O), 170.9 (C=O), 136.6 (CH), 115.6

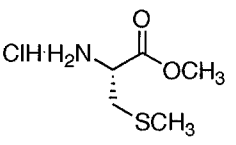
(CH_2), 63.2 (CH_2), 54.5 (CH), 52.6 (CH_3), 35.4 (CH_2), 29.3 (CH_2); HRMS (EI) Calcd for $C_9H_{15}NO_4$ 201.1001, found 201.0997.

Methyl 2-pent-4-enamidoacrylate (57)



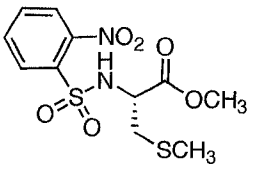
To a solution of compound **56** (0.10 g, 0.50 mmol) in DCE (10.0 mL), DMAP (6 mg, 0.05 mmol) was added followed by $(Boc)_2O$ (0.36 g, 1.64 mmol) and DBU (0.08 g, 0.50 mmol). The reaction was stirred for 3 h at 50 °C. The solvent was removed *in vacuo,* and the crude was purified by flash chromatography (SiO_2, 1.5:8.5/Et_2O:hexanes) to yield **57** (0.06 g, 52%) as a sticky white solid. (R_f 0.4 on SiO_2, 3:7/Et_2O:hexanes); IR ($CHCl_3$ cast) 3366, 2954, 1725, 1639, 1515 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.73 (br s, 1H, NH), 6.62 (s, 1H, Dha-CH), 5.91-5.78 (m, 2H, Dha-CH + =CH), 5.13-5.02 (m, 2H, $=CH_2$), 3.85 (s, 3H, OCH_3), 2.47-2.40 (m, 4H, $COCH_2$ + $=CHCH_2$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 171.0 (C=O), 164.6 (C=O), 136.5 (CH), 130.7 (C), 115.8 (CH_2), 108.6 (CH_2), 52.9 (CH_3), 36.7 (CH_2), 29.1 (CH_2); HRMS (EI) Calcd for $C_9H_{13}NO_3$ 183.0895, found 183.0888.

(*R*)-Methyl 2-amino-3-(methylthio)propanoate hydrochloride (62)



This known compound was prepared according to the procedure by Benoiton *et al.*[177] To a solution of commercially available *S*-methyl-L-cysteine (**61**) (5.00 g, 36.98 mmol) in MeOH (120.0 mL), thionyl chloride (10.79 mL, 147.93 mmol) was added dropwise using an addition funnel. The reaction mixture was refluxed for 2 h. Volatiles were removed from the reaction mixture *in vacuo* and the residue was washed with hexanes to yield **62** (6.76 g, 98%) as a foamy white solid. (R_f 0.1 on SiO_2, 1:9/MeOH:chloroform); $[\alpha]_D^{25}$ -4.02 (*c* 3.28, MeOH); IR ($CHCl_3$ cast) 2988, 2839, 2634, 1745 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 4.30 (dd, 1H, *J* = 7.5, 4.5 Hz, CH_α), 3.85 (s, 3H, OCH_3), 3.13 (dd, 1H, *J* = 15.0, 4.5 Hz, SCH*H*), 3.01 (dd, 1H, *J* = 15.0, 7.5 Hz, SC*H*H), 2.16 (s, 3H, SCH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 169.6 (C=O), 53.9 (CH), 53.1 (OCH_3), 34.9 (CH_2), 15.8 (CH_3); HRMS (ES) Calcd for $C_5H_{12}NO_2S$ 150.0583, found 150.0583.

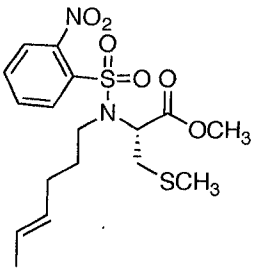
(*R*)-Methyl 3-(methylthio)-2-(2-nitrophenylsulfonamido)propanoate (64)



To a solution of compound **62** (1.00 g, 5.39 mmol) in CH_2Cl_2 (11.0 mL), Et_3N (1.09 g, 10.78 mmol) was added followed by *o*-nitrobenzenesulfonyl chloride (**63**) (1.19 g, 5.39 mmol) and the reaction was stirred for 12 h at rt. The reaction mixture was sequentially washed with water (2 x 25 mL) and brine (1 x 25 mL). The organic layer was separated, dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO_2, 3:7/EtOAc:hexanes) to yield **64** (1.43 g, 79%) as an off-white solid. (R_f 0.6 on SiO_2, 1:1/EtOAc:hexanes); $[\alpha]_D^{25}$ -155.5 (*c* 1.15, $CHCl_3$); IR ($CHCl_3$ cast) 3314, 2955, 1743, 1594 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 8.08-8.05 (m,

1H, Ar-CH=CNO_2), 7.93-7.90 (m, 1H, Ar-CH=CSO_2), 7.74-7.70 (m, 2H, Ar-CH), 6.37 (d, 1H, J = 8.5 Hz, NH), 4.42-4.38 (m, 1H, CH_α), 3.55 (s, 3H, OCH_3), 2.98 (dd, 1H, J = 14.0, 5.5 Hz, SCH*H*), 2.93 (dd, 1H, J = 14.0, 5.5 Hz, SC*H*H), 2.14 (s, 3H, SCH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 170.0 (C=O), 146.5 (C), 134.2 (C), 133.6 (CH), 132.8 (CH), 130.4 (CH), 125.6 (CH), 56.3 (CH), 52.7 (OCH_3), 37.4 (CH_2), 16.3 (CH_3); HRMS (EI) Calcd for $C_{11}H_{14}N_2O_6S_2$ 334.0293, found 334.0290.

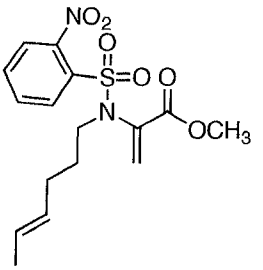
(*R*,*E*)-Methyl 2-(*N*-(hex-4-enyl)-2-nitrophenylsulfonamido)-3(methylthio)propanoate (66)



To a solution of compound **64** (0.60 g, 1.79 mmol) in CH_2Cl_2 (18.0 mL), PPh_3 (0.61 g, 2.33 mmol) was added followed by 4-hexen-1-ol (**65**) (0.27 mL, 2.33 mmol) and diethylazodicarboxylate (0.41 g, 2.33 mmol) dropwise at 0 °C. The reaction was stirred for 3 h, after which the solvents were removed *in vacuo* and the crude product was purified by flash chromatography (SiO_2, 3:7/EtOAc:hexanes) to yield **66** (0.70 g, 95%) as a colorless oil. (R_f 0.7 on SiO_2, 1:1/EtOAc:hexanes); $[\alpha]_D^{25}$ -0.54 (*c* 1.48, $CHCl_3$); IR ($CHCl_3$ cast) 2951, 1744, 1544, 1437 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 8.07-8.05 (m, 1H, Ar-CH=CNO_2), 7.67-7.66 (m, 2H, Ar-CH=CSO_2 + Ar-CH), 7.60-7.58 (m, 1H, Ar-CH), 5.60-5.30 (m, 2H, 2 x =CH), 4.72 (app t, 1H, J = 7.5 Hz, CH_α), 3.61 (s, 3H, OCH_3), 3.41-3.34 (m, 1H, NCH*H*), 3.18-3.09 (m, 2H, NC*H*H + SCH*H*), 2.74 (dd, 1H, J = 14.0,

7.5 Hz, SC*H*H), 2.14 (s, 3H, SCH_3), 1.93 (app q, 2H, J = 7.0 Hz, =CHCH_2), 1.80-1.71 (m, 1H, NCH_2CH*H*), 1.67-1.56 (m, 4H, CH_3 + NCH_2CH*H*); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 170.0 (C=O), 148.1 (C), 133.5 (CH), 133.2 (C), 131.4 (CH), 131.0 (CH), 129.6 (CH), 126.1 (CH), 124.1 (CH), 59.4 (CH), 52.5 (OCH_3), 46.3 (CH_2), 34.7 (CH_2), 30.2 (CH_2), 29.9 (CH_2), 17.8 (CH_3), 15.7 (CH_3); HRMS (EI) Calcd for $C_{17}H_{24}N_2O_6S_2$ 416.1075, found 416.1080.

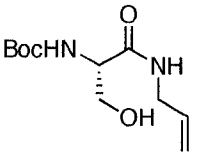
Methyl-(*E*)-2-(*N*-(hex-4-enyl)-2-nitrophenylsulfonamido)acrylate (67)



To a solution of compound **66** (0.10 g, 0.24 mmol) in (1:1) MeOH:water, $NaIO_4$ (0.06 g, 0.27 mmol) was added and stirred for 2 h. The solvents were removed under vacuum and the residue was dissolved in CH_2Cl_2 (20 mL) and washed with water (2 x 5 mL) and brine (5 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo* to yield the sulfoxide intermediate (0.089 g, 84%) as a light yellow oil. This material was redissolved in methanol (5 mL) and DBU (0.03 g, 0.19 mmol) was added and stirred for 1 h at rt. The solvent was removed under vacuum and the crude product was purified by flash chromatography (SiO_2, 4:6/EtOAc:hexanes) to yield **67** (26 mg, 37%) as a low melting solid. (R_f 0.8 on SiO_2, 100% EtOAc); IR ($CHCl_3$ cast) 3095, 2953, 1737, 1631, 1546 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.99-7.96 (m, 1H, Ar-CH=CNO_2), 7.71-7.56 (m, 3H, Ar-CH=CSO_2 + 2 x Ar-CH), 6.58 (s, 1H, Dha-CH), 6.02 (s, 1H, Dha-CH), 5.48-

5.33 (m, 2H, 2 x =CH), 3.62 (s, 3H, OCH_3), 3.56 (t, 2H, J = 7.2 Hz, NCH_2), 2.01 (q, 2H, J = 6.9 Hz, =CHCH_2), 1.64-1.52 (m, 5H, CH_3 + CH_2); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 169.5 (C=O), 148.1 (C), 134.3 (C), 133.5 (CH), 133.3 (C), 131.6 (CH_2), 131.5 (CH), 130.9 (CH), 130.0 (CH), 126.0 (CH), 124.0 (CH), 52.4 (OCH_3), 47.5 (CH_2), 30.3 (CH_2), 29.9 (CH_2), 17.8 (CH_3); HRMS (ES) Calcd for $C_{16}H_{20}N_2O_6SNa$ 391.0937, found 391.0934.

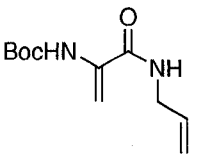
(*S*)-*tert*-Butyl 1-(allylamino)-3-hydroxy-1-oxopropan-2-ylcarbamate (72)



This known compound was prepared by a modified literature procedure.[178] To a solution of Boc-L-serine (**70**) (1.10 g, 5.35 mmol) in CH_2Cl_2 (20.0 mL), allylamine (**71**) (0.37 mL, 4.87 mmol) was added at 0 °C followed by DIPEA (2.54 mL, 14.61 mmol) and PyBOP (3.80 g, 7.30 mmol). The reaction was allowed to warm to rt and stirred for 20 h. The reaction mixture was diluted with CH_2Cl_2 (50 ml), washed sequentially with 5% $KHSO_4$ (2 x 10 mL), 5% $NaHCO_3$ (2 x 5 mL), water (1 x 5 mL) and brine (1 x 10 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO_2, 0.2:9.8/MeOH:chloroform) to yield **72** (0.69 g, 53%) as a foamy white solid. (R_f 0.4 on SiO_2, 1:9/MeOH:chloroform); $[\alpha]_D^{25}$ -35.99 (*c* 1.40, $CHCl_3$); IR ($CHCl_3$ cast) 3324, 3099, 2975, 1705, 1657, 1644 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.92 (br s, 1H, NH), 5.83-5.68 (m, 2H, =CH + NH), 5.22-5.05 (m, 2H, =CH_2), 4.22-4.10 (m, 1H, H_α), 4.08-3.72 (m, 4H, OCH_2 + NCH_2), 3.65 (br s, 1H, OH), 1.41 (s, 9H, ^{t}Bu); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.2 (C=O), 156.2 (C=O), 133.5

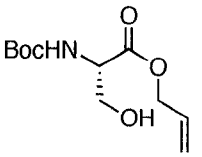
(CH), 116.2 (CH_2), 80.5 (C), 62.8 (CH_2), 55.0 (CH), 41.7 (CH_2), 28.2 (CH_3); HRMS (ES) Calcd for $C_{11}H_{20}N_2O_4Na$ 267.1313, found 267.1315.

***tert*-Butyl 3-(allylamino)-3-oxoprop-1-en-2-ylcarbamate (73)**



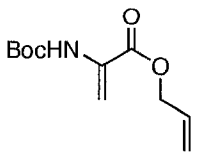
To a solution of compound **72** (0.30 g, 1.23 mmol) in CH_2Cl_2 (15.0 mL), Et_3N (0.34 mL, 2.46 mmol) was added followed by MsCl (95 μL, 1.23 mmol) at 0 °C and stirred for 2 h. The reaction was quenched by adding water (10 mL) and the organic layer was separated, washed with brine (10 mL), dried over Na_2SO_4, filtered and concentrated *in vacuo* to yield the mesylated intermediate (0.33 g, 84%) as a white solid. This intermediate was redissolved in DCE (10.0 mL) and treated with DBU (0.10 mL, 0.93 mmol) for 1 h at 50 °C. The solvent was removed under vacuum and the crude product was purified by flash chromatography (SiO_2, 2:8/EtOAc:hexanes) to yield **73** (0.15 g, 70%) as a white solid. (R_f 0.8 on SiO_2, 1:1/EtOAc:hexanes); IR ($CHCl_3$ cast) 3384, 2978, 1730, 1657, 1625 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.30 (br s, 1H, NH), 6.13 (br s, 1H, NH), 5.98 (d, 1H, *J* = 1.8 Hz, Dha-CH), 5.91-5.78 (m, 1H, =CH), 5.25-5.15 (m, 2H, $=CH_2$), 5.04 (app t, 1H, *J* = 1.5 Hz, Dha-CH), 3.98-3.93 (m, 2H, NCH_2), 1.47 (s, 9H, ^{t}Bu); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 163.8 (C=O), 152.7 (C=O), 134.9 (C), 133.4 (CH), 116.9 (CH_2), 97.2 (CH_2), 80.5 (C), 42.3 (CH_2), 28.2 (CH_3); HRMS (ES) Calcd for $C_{11}H_{18}N_2O_3Na$ 249.1212, found 267.1209.

(*S*)-Allyl 2-(*tert*-butoxycarbonylamino)-3-hydroxypropanoate (76)



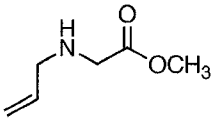
This known compound was prepared according to a modified literature procedure.[179] To a solution of Boc-L-serine (**70**) (2.00 g, 9.75 mmol) in DMF (20.0 mL), Na_2CO_3 (1.24 g, 11.70 mmol) was added followed by allyl bromide (**75**) (1.01 mL, 11.70 mmol) and stirred for 15 h at rt. DMF was removed from the reaction mixture and the residue was dissolved in EtOAc (20 mL) and washed with water (4 x 10 mL). The organic layer was separated, dried over Na_2SO_4, filtered and concentrated *in vacuo* to yield **76** (2.06 g, 86%) as a yellow oil. (R_f 0.50 on SiO_2, 1:9/MeOH:chloroform); $[\alpha]_D^{25}$ 2.53 (*c* 1.67, $CHCl_3$); IR ($CHCl_3$ cast) 3435, 2978, 1717, 1509 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 5.91 (ddt, 1H, *J* = 15.3, 9.7, 9.7 Hz, =CH), 5.50-5.24 (m, 3H, $=CH_2$ + NH), 4.69-4.67 (m, 2H, OCH_2), 4.48-4.34 (m, 1H, CH_α), 3.99 (dd, 1H, *J* = 11.1, 3.9 Hz, CH_β), 3.92 (dd, 1H, *J* = 11.1, 3.6 Hz, $CH_{\beta'}$), 2.05 (br s, 1H, OH), 1.47 (s, 9H, ^{t}Bu); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.5 (C=O), 155.7 (C=O), 131.3 (CH), 118.6 (CH_2), 80.2 (C), 66.0 (CH_2), 63.2 (CH_2), 55.7 (CH), 28.2 (CH_3); HRMS (ES) Calcd for $C_{11}H_{19}NO_5Na$ 268.1155, found 268.1157.

Allyl 2-(*tert*-butoxycarbonylamino)acrylate (77)



This known compound was prepared by a new procedure.[101] To a solution of compound **76** (0.50 g, 2.04 mmol) in CH_2Cl_2 (20.0 mL), MsCl (0.17 mL, 2.14 mmol) and Et_3N (0.57 mL, 4.08 mmol) were added and stirred for 40 min. DBU (0.31 mL, 2.04 mmol) was added to the reaction at rt and stirred for 2 h. The reaction mixture was washed with water (2 x 5 mL), dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO_2, 2:8/EtOAc:hexanes) to yield **77** (0.45 g, 96%) as colorless oil. (R_f 0.9 on SiO_2, 1:9/MeOH:chloroform); IR ($CHCl_3$ cast) 3417, 3085, 2978, 2933, 1719, 1652 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 6.93 (br s, 1H, NH), 6.06 (s, 1H, Dhb-C*H*H), 5.83 (dddd, 1H, J = 17.2, 10.5, 5.7, 5.7 Hz, =CH), 5.64 (d, 1H, J = 1.2 Hz, Dhb-CH*H*), 5.24 (app dq, 1H, J = 17.2, 1.4 Hz, =C*H*H), 5.24 (app dq, 1H, J = 10.5, 1.3 Hz, =CH*H*), 4.60 (dt, 2H, J = 5.8, 1.4 Hz, OCH_2), 1.37 (s, 9H, ^{t}Bu); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 163.4 (C=O), 152.7 (C=O), 131.3 (CH), 131.2 (C), 118.6 (CH_2), 105.0 (CH_2), 80.4 (C), 66.2 (CH_2), 28.2 (CH_3).

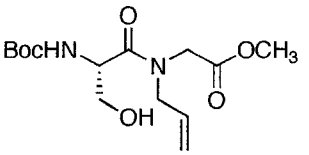
Methyl 2-(allylamino)acetate (81)



To a solution of allylamine (**71**) (7.50 mL, 100.00 mmol) in THF (100.0 mL), methylbromoacetate (**80**) (4.62 mL, 50.00 mmol) in THF (50.0 mL) was added via an addition funnel at 0 °C over 30 minutes. The reaction was allowed to proceed for an additional 2.5 h. The solvent was removed under vacuum and the oily residue was dissolved in Et_2O (100 mL). The precipitated salt was removed by filtration and the

filtrate was concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO_2, 100% Et_2O) to yield **81** (4.81 g, 75%) as colorless oil. (R_f 0.4 on SiO_2, 100% Et_2O); IR ($CHCl_3$ cast) 3338, 3081, 2952, 1742, 1672 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.85-5.75 (m, 1H, =CH), 5.16-5.04 (m, 2H, $=CH_2$), 3.67 (s, 3H, OCH_3), 3.35 (s, 2H, $COCH_2$), 3.22-3.19 (m, 2H, NCH_2), 1.75 (s, 1H, NH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.8 (C=O), 135.9 (CH), 116.5 (CH_2), 51.7 (CH_2), 51.6 (OCH_3), 49.7 (CH_2); HRMS (ES) Calcd for $C_6H_{11}NO_2$ 129.0862, found 130.0862 (M+H).

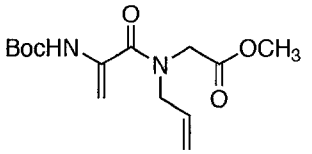
(*S*)-Methyl 2-(*N*-allyl-2-(*tert*-butoxycarbonylamino)-3-hydroxypropanamido)acetate (82)



To a solution of Boc-Serine-OH (**70**) (1.00 g, 4.87 mmol) in CH_2Cl_2 (20.0 mL), compound **81** (0.63 g, 4.87 mmol) was added at 0 °C followed by HOBt (0.66 g, 4.87 mmol) and EDCI (1.11 g, 5.36 mmol). The reaction was warmed to rt and stirred for 14 h. The reaction mixture was washed with water (2 x 30 mL), $NaHCO_3$ (10 mL), $KHSO_4$ (10 mL) and brine (15 mL). The organic layer was separated, dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO_2, 3.5:6.5/EtOAc:hexanes) to yield **82** (0.73 g, 47%) as a white solid. (R_f 0.55 on SiO_2, 1:1/EtOAc:hexanes); $[\alpha]_D^{25}$ -25.49 (*c* 1.12, $CHCl_3$); IR ($CHCl_3$ cast) 3338, 2979, 1751, 1710, 1651 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.85-5.64 (m, 1H, =CH), 5.6-5.5 (m, 1H, NH), 5.27-5.11 (m, 2H, $=CH_2$), 4.74-4.68 (m, 1H, H_α), 4.31-4.13 (m, 2H, OCH_2), 4.05-4.00 (m, 2H, $COCH_2$), 3.88-3.82 (m, 1H, NCH*H*), 3.74-3.67 (m, 4H, NC*H*H +

OCH_3), 2.75 (br s, 1H, OH), 1.41 (s, 9H, ^{t}Bu); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.9 (C=O), 169.5 (C=O), 155.4 (C=O), 132.1 (CH), 118.5 (CH_2), 80.1 (C), 64.3 (OCH_2), 52.3 (CH), 51.5 (OCH_3), 51.4 (CH_2), 47.1 (CH_2), 28.2 (CH_3); HRMS (ES) Calcd for $C_{14}H_{24}N_2O_6Na$ 339.1528, found 339.1526.

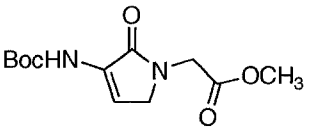
Methyl 2-(*N*-allyl-2-(*tert*-butoxycarbonylamino)acrylamido)acetate (83)



To a solution of compound **82** (0.56 g, 1.76 mmol) in CH_2Cl_2 (18.0 mL), Et_3N (0.36 g, 3.51 mmol) was added followed by MsCl (0.24 g, 2.11 mmol) at 0 °C and then the reaction was warmed to rt and stirred for 45 minutes. The reaction mixture was washed with 10% $NaHCO_3$ (15 mL) and water (15 mL), dried over Na_2SO_4, filtered and concentrated *in vacuo* to yield the mesylated intermdiate (0.55 g, 79%) as yellow oil. A portion of the mesylated compound (0.20 g, 0.51 mmol) was dissolved in DCE (5 mL) and to it DBU (0.076 mL, 0.51 mmol) was added and stirred for 4 h at rt. The reaction mixture was diluted with 10 mL of CH_2Cl_2 and washed with water (5 mL), dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO_2, 3.5:6.5/EtOAc:hexanes) to yield **83** (0.112 g, 74%) as a sticky white solid. (R_f 0.6 on SiO_2, 1:1/EtOAc:hexanes); IR ($CHCl_3$ cast) 3294, 2980, 1751, 1725, 1651, 1631, 1508 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.60 (s, 1H, NH), 5.85-5.56 (m, 2H, =CH + Dha-CH*H*), 5.30-5.10 (m, 2H, =CH_2), 4.84 (s, 1H, Dha-C*H*H), 4.24-3.90 (m, 4H, $COCH_2$ + NCH_2), 3.68 (s, 3H, OCH_3), 1.40 (s, 9H, ^{t}Bu); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 169.3 (C=O), 168.5 (C=O), 152.5 (C=O), 134.1 (C), 132.3 (CH), 118.3 (CH_2),

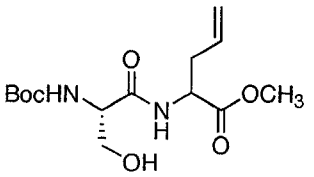
100.0 (CH_2), 80.6 (C), 53.1 (CH_2), 52.1 (OCH_3), 46.6 (CH_2), 28.1 (CH_3); HRMS (ES) Calcd for $C_{14}H_{22}N_2O_5Na$ 321.1421, found 321.1418.

Methyl 2-(3-(*tert*-butoxycarbonylamino)-2-oxo-2,5-dihydro-1*H*-pyrrol-1-yl)acetate (84)



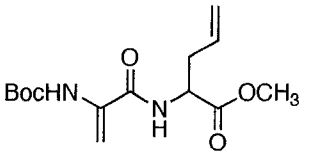
To a solution of compound **83** (39 mg, 0.13 mmol) in degassed CH_2Cl_2 (168.0 mL, 0.001 M), Grubb's second-generation catalyst (**58**) (22 mg, 0.026 mmol) was added and refluxed for 2 h. The reaction mixture was treated with DMSO (50 equiv to catalyst) and stirred for 12 h. The reaction mixture was concentrated under vacuum and the crude product was purified by preparative thin layer chromatography (SiO_2, 1:1/EtOAc:hexanes) to yield **84** (27 mg, 76%) as a white solid. (R_f 0.4 on SiO_2, 1:1/EtOAc:hexanes); IR ($CHCl_3$ cast) 3280, 2977, 1750, 1732, 1657, 1524 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 6.85 (s, 1H, NH), 6.70 (s, 1H, =CH), 4.22 (s, 2H, CH_2), 4.22 (d, 2H, J = 2.0 Hz, NCH_2), 3.71 (s, 3H, OCH_3), 1.47 (s, 9H, ^{t}Bu); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 169.0 (C=O), 167.0 (C=O), 152.5 (C=O), 130.3 (C), 113.8 (CH), 81.1 (C), 52.3 (OCH_3), 50.1 (CH_2), 43.7 (CH_2), 28.1 (CH_3); HRMS (ES) Calcd for $C_{12}H_{18}N_2O_5Na$ 293.1108, found 293.1107.

Methyl 2-((*S*)-2-(*tert*-butoxycarbonylamino)-3-hydroxypropanamido)pent-4-enoate (86)



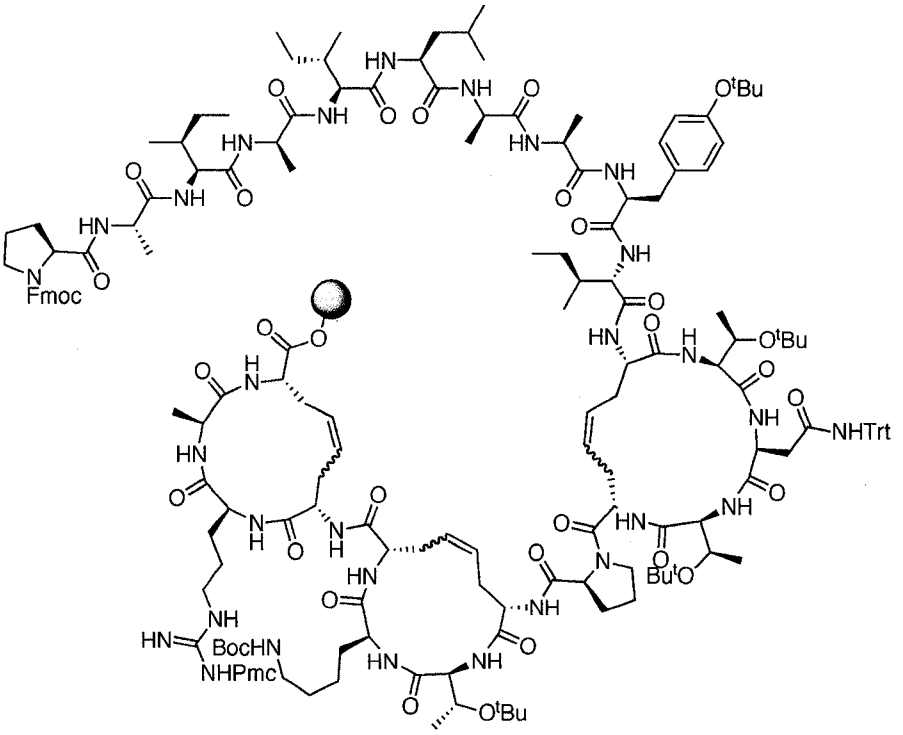
This known compound was prepared according to literature procedure.[180] To a solution of D,L-allylglycine methyl ester hydrochloride (**85**) (0.24 g, 1.46 mmol) in CH_2Cl_2 (10.0 mL), Et_3N (0.20 mL, 1.46 mmol) was added followed by Boc-Serine-OH (**70**) (0.30 g, 1.46 mmol), HOBt (0.20 g, 1.46 mmol) and EDCI (0.30 g, 1.46 mmol) at 0 °C and then warmed to rt and stirred for 14 h. The reaction mixture was washed with water (20 mL), dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO_2, 3:97/MeOH:chlorofom) to yield **86** (0.45 g, 97%) as colorless oil. (R_f 0.50 on SiO_2, 1:9/ MeOH:chlorofom); $[\alpha]_D^{25}$ -33.9 (*c* 0.74, $CHCl_3$); IR ($CHCl_3$ cast) 3319, 2979, 1744, 1666, 1525 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz, *diastereomeric mixture*) δ 7.20-6.95 (br d, 1H, NH), 5.75-5.60 (m, 1H, =CH), 5.50 (br s, 1H, NH), 5.19-5.11 (m, 2H, $=CH_2$), 4.68-4.60 (m, 1H, Ser-H_α), 4.22-4.04 (m, 2H, AllylGly-H_α + OCH*H*), 3.76 and 3.65 (s, 3H, OCH_3), 3.69-3.62 (m, 1H, OC*H*H), 2.65-2.46 (m, 2H, AllylGly -CH_2), 1.47 and 1.46 (s, 9H, ^{t}Bu); ^{13}C NMR ($CDCl_3$, 100 MHz, *diastereomeric mixture*) δ 171.8 (C=O), 171.1 (C=O), 155.9 (C=O), 131.8 (CH), 119.3 and 119.2 (CH_2), 80.3 (C), 62.8 (CH_2), 52.4 (CH), 51.8 (CH), 51.6 (OCH_3), 35.9 (CH_2), 28.1 (CH_3); HRMS (ES) Calcd for $C_{14}H_{24}N_2O_6Na$ 339.1526, found 339.1528.

Methyl 2-(2-(*tert*-butoxycarbonylamino)acrylamido)pent-4-enoate (87)



To a solution of compound **86** (100 mg, 0.32 mmol) in CH_2Cl_2 (6.0 mL), Et_3N (64 μL, 0.63 mmol) was added followed by MsCl (26 μL, 0.32 mmol) at 0 °C and then the reaction was warmed to rt and stirred for 1.5 h. The reaction mixture was washed with water (2 x 5 mL), dried over Na_2SO_4, filtered and concentrated *in vacuo* to yield the mesylated intermediate (109 mg, 87%) as a yellow oil. A portion of the mesylated compound (50 mg, 0.13 mmol) was dissolved in DCE (5 mL) and to it DBU (19 μL, 0.125 mmol) was added and stirred for 4 h at 50 °C. The solvent was removed *in vacuo* and the crude product was purified by flash chromatography (SiO_2, 3:7/EtOAc:hexanes) to yield **87** (22 mg, 58%) as a light yellow oil. (R_f 0.5 on SiO_2, 1:1/EtOAc:hexanes); IR ($CHCl_3$ cast) 3340, 2979, 1783, 1743, 1688 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 7.22 (s, 1H, NH), 7.20 (d, 1H, J = 7.0 Hz, AllylGly-NH), 6.00 (s, 1H, Dha-CH*H*), 5.65 (ddt, 1H, J = 17.0, 10.0, 7.1, =CH), 5.15-5.07 (m, 3H, $=CH_2$ + Dha-CH*H*), 4.69-4.65 (m, 1H, AllylGly-H_α), 3.75 (s, 3H, OCH_3), 2.64-2.51 (m, 2H, AllylGly -CH_2), 1.44 (s, 9H, ^{t}Bu); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.6 (C=O), 163.6 (C=O), 152.6 (C=O), 134.5 (C), 131.7 (CH), 119.6 (CH_2), 98.1 (CH_2), 80.5 (C), 52.5 (CH), 52.0 (OCH_3), 36.3 (CH_2), 28.1 (CH_3); HRMS (ES) Calcd for $C_{14}H_{22}N_2O_5Na$ 321.1426, found 321.1428.

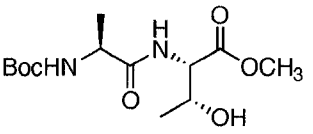
_N_-(9_H_-Fluorenylmethoxycarbonyl)-L-prolyl-L-alanyl-L-isoleucinyl-D-alanyl-L-isoleucinyl-L-leucinyl-D-alanyl-L-alanyl-L-tyrosinyl-L-isoleucinyl-D-(α-aminobutyrl)-L-threonyl-L-asparaginyl-L-threonyl-L-(α-aminobutyrl)-L-prolyl-D-(α-aminobutyrl)-L-threonyl-L-lysyl-L-(α-aminobutyrl)-D-(α-aminobutyrl)-L-arginyl-L-alanyl-L-(α-aminobutyrine) [(16→20), (22→25), (26→29)] (_E/Z_)-α,α'-D,L-diamino-γ,γ'-dehydrosuberic acid (96)



Fmoc-SPPS was carried out using PyBOP to couple amino acids (10→15) in the following order: Fmoc-Ile-OH, Fmoc-Tyr(ᵗBu)-OH, Fmoc-Ala-OH, Fmoc-D-Ala-OH, Fmoc-Leu-OH, Fmoc-Ile-OH. *N*-Methylpyrrolidinone was used as the solvent instead of DMF to avoid any potential aggregation due to the hydrophobic nature of the residues.[141] Fmoc-SPPS was continued with PyBOP to introduce amino acids (6→9) in this order: Fmoc-D-Ala-OH, Fmoc-Ile-OH, Fmoc-Ala-OH, Fmoc-Pro-OH. A small sample of the resin was treated with (95:2.5:2.5) TFA / TIPS / H_2O at rt for 3 h to liberate the peptide

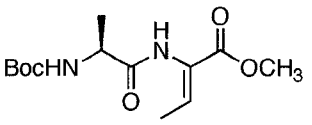
from the solid support. Concentration of the filtrate *in vacuo,* followed by precipitation with Et_2O gave **96** as an off-white solid; MALDI-TOF MS Calcd for $C_{125}H_{185}N_{29}O_{32}$ 2604.4, found 2605.8 (M+H).

(2*S*,3*R*)-Methyl 2-((*S*)-2-(*tert*-butoxycarbonylamino)propanamido)-3-hydroxy butanoate (111)



This known compound was prepared according to a modified literature procedure.[134] To a solution of L-threonine methyl ester hydrochloride (**100**) (1.35 g, 7.92 mmol) in CH_2Cl_2 (50.0 mL), NMM (1.92 mL, 17.44 mmol) was added followed by Boc-Ala-OH (**99**) (1.50 g, 7.93 mmol), PyBOP (4.33 g, 8.33 mmol) and stirred for 14 h at rt. The solvent was removed *in vacuo* and the crude product was purified by flash chromatography (SiO_2, 5.5:3.5/EtOAc:hexanes) to yield **111** (1.76 g, 73%) as a white solid. (R_f 0.50 on SiO_2, 100% EtOAc); $[\alpha]_D^{25}$ -17.33 (*c* 1.28, $CHCl_3$); IR ($CHCl_3$ cast) 3338, 2978, 2935, 1747, 1668, 1521 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.18 (d, 1H, *J* = 8.4 Hz, Thr-NH), 5.49 (d, 1H, *J* = 5.7 Hz, Ala-NH), 4.54 (dd, 1H, *J* = 8.7, 2.4 Hz, Thr-H_α), 4.12-4.02 (m, 2H, Thr-H_β + Ala-H_α), 3.70 (s, 3H, OCH_3), 1.38 (s, 9H, ^{t}Bu), 1.33 (d, 3H, *J* = 6.9 Hz, Ala-CH_3), 1.14 (d, 3H, *J* = 6.3 Hz, Thr-CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 173.4 (C=O), 171.3 (C=O), 155.6 (C=O), 80.6 (C), 68.2 (CH), 57.3 (CH), 52.5 (CH_3), 49.8 (CH), 28.4 (CH_3), 19.8 (CH_3), 18.1 (CH_3); HRMS (ES) Calcd for $C_{13}H_{24}N_2O_6Na$ 327.1526, found 327.1527.

(*S*,*Z*)-Methyl 2-(2-(*tert*-butoxycarbonylamino)propanamido)but-2-enoate (112)



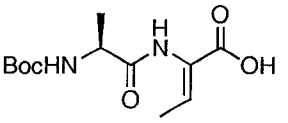
To a solution of compound **111** (1.30 g, 4.27 mmol) in CH_2Cl_2 (50.0 mL) MsCl (0.43 mL, 5.55 mmol) was added followed by DBU (1.66 mL, 11.11 mmol) and refluxed for 24 h. The solvent was removed *in vacuo* and the crude product was purified by flash chromatography (SiO_2, 3.5:5.5/EtOAc:hexanes) to yield **112** (0.71 g, 58%) as a white solid. (R_f 0.7 on SiO_2, 100% EtOAc); $[\alpha]_D^{25}$ -52.60 (*c* 1.02, $CHCl_3$); IR ($CHCl_3$ cast) 3302, 2979, 1722, 1678, 1513 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.50 (br s, 1H, Dhb-NH), 6.82 (q, 1H, J = 7.2 Hz, Dhb-CH), 5.00 (br s, 1H, Ala-NH), 4.34-4.24 (m, 1H, Ala-H_α), 3.76 (s, 3H, OCH_3), 1.77 (d, 3H, J = 7.2 Hz, Dhb-CH_3), 1.46 (s, 9H, ^{t}Bu), 1.43 (d, 3H, J = 7.2 Hz, Ala-CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 171.4 (C=O), 164.7 (C=O), 155.6 (C=O), 134.5 (CH), 126.1 (C), 80.0 (C), 52.1 (CH), 50.2 (CH_3), 28.3 (CH_3), 18.3 (CH_3), 14.3 (CH_3); HRMS (ES) Calcd for $C_{13}H_{22}N_2O_5Na$ 309.1420, found 309.1419.

Crystal structure data for (*S*,*Z*)-Methyl 2-(2-(*tert*-butoxycarbonylamino)propanamido)but-2-enoate (112)

This experiment was conducted by Dr. Robert McDonald of the University of Alberta X-ray crystallography laboratory. $C_{13}H_{22}N_2O_5$: M = 0.68 x 0.13 x 0.09 mm, monoclinic, a = 5.0742 (10), b = 24.674 (5), c = 6.0201 (12) Å, V = 753.6 (3) $Å^3$, T = 193 K, space group $P2_1$ (No. 4), Z = 2, μ = 0.097 mm^{-1}, 5110 reflections measured, 3058 unique (R_{int} = 0.0448) which were used in all least squares calculations, $R_1(F)$ = 0.0764 (for 2385

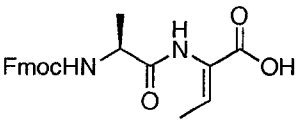
reflections with $F_o^2 \geq 2\sigma(F_o^2)$), wR_2 [$F_o^2 \geq -3\sigma(F_o^2)$] = 0.2019 (for all unique reflections).

(*S*,*Z*)-2-(2-(*tert*-Butoxycarbonylamino)propanamido)but-2-enoic acid (113)



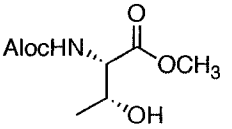
This known compound was prepared by a modified litertature procedure.[134] To a solution of compound **112** (0.87 g, 3.04 mmol) in 1,4-dioxane (20.0 mL), 1 N LiOH (20.0 mL) was added at 0 °C and stirred for 12 h. The solvents were removed *in vacuo* and the residue was dissolved in water (15 mL) and acidified to pH 2 at 0 °C with 10% citric acid. The free acid was immediately extracted with EtOAc (2 x 25 mL), dried over Na_2SO_4, filtered and concentrated *in vacuo* to yield **113** (0.69 g, 83%) as a white solid. (R_f 0.01 on SiO_2, 100% EtOAc); $[\alpha]_D^{25}$ -21.29 (*c* 0.34, $CHCl_3$); IR ($CHCl_3$ cast) 3305, 2981, 2937, 1706, 1521 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 8.32 (br s, 1H, COOH), 7.87 (br s, 1H, Dhb-NH), 6.92 (q, 1H, J = 7.2 Hz, Dhb-CH), 5.34 (br s, 1H, Ala-NH), 4.46-4.24 (m, 1H, Ala-H_α), 1.77 (d, 3H, J = 7.2 Hz, Dhb-CH_3), 1.44 (s, 9H, ^{t}Bu), 1.41 (d, 3H, J = 6.9 Hz, Ala-CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 173.6 (C=O), 167.3 (C=O), 157.6 (C=O), 136.6 (CH), 128.5 (C), 80.6 (C), 52.8 (CH), 28.7 (CH_3), 18.3 (CH_3), 14.1 (CH_3); HRMS (ES, negative mode) Calcd for $C_{12}H_{19}N_2O_5$ 271.1288, found 271.1288.

(*S*,*Z*)-2-(2-(((9*H*-Fluoren-9-yl)methoxy)carbonylamino)propanamido)but-2-enoic acid (97)



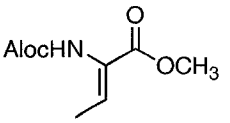
To a solution of compound **113** (0.44 g, 1.62 mmol) in CH_2Cl_2 (20.0 mL), TFA (20.0 mL) was added and stirred for 1.5 h. The solvents were removed under vacuum and the residue was dissolved in 10% $NaHCO_3$ (30 mL) and Fmoc-OSu (0.60 g, 1.78 mmol) in 1,4-dioxane (10 mL) was added at 0 °C and stirred for 14 h. The reaction mixture was diluted with 20 mL of water and acidified to pH 1 with 1 M HCl. The precipitated product was filtered off to give **97**. It was used without any further purification (0.51 g, 80%). (R_f 0.01 on SiO_2, 100% EtOAc); $[\alpha]_D^{25}$ -13.86 (*c* 0.22, $CHCl_3$); IR ($CHCl_3$, cast) 3283, 3040, 1692, 1669, 1524 cm^{-1}; ^1H NMR (CD_3OD, 300 MHz) δ 7.78 (d, 2H, *J* = 7.2 Hz, Ar-CH), 7.65 (q, 2H, *J* = 5.7 Hz, Ar-CH), 7.34-7.26 (m, 4H, Ar-CH), 6.84 (q, 1H, *J* = 7.2 Hz, Dhb-CH), 4.42-4.20 (m, 4H, Fmoc-CH_2 + Fmoc-CH + Ala-H_α), 1.73 (d, 3H, *J* = 7.2 Hz, Dhb-CH_3), 1.40 (d, 3H, *J* = 7.2 Hz, Ala-CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 174.6 (C=O), 167.2 (C=O), 158.2 (C=O), 145.3 (C), 145.2 (C), 142.6 (C), 136.8 (C), 128.8 (CH), 128.4 (CH), 128.1 (CH), 126.3 (CH), 126.1 (CH), 120.9 (CH), 67.9 (CH_2), 52.1 (CH), 48.4 (CH), 18.3 (CH_3), 14.1 (CH_3); HRMS (ES, negative mode) Calcd for $C_{22}H_{21}N_2O_5$ 393.1456, found 393.1458.

(2*S*,3*R*)-Methyl 2-(allyloxycarbonylamino)-3-hydroxybutanoate (116)



This known compound was prepared by a modified literature procedure.[181] To a solution of L-threonine methyl ester hydrochloride (**100**) (1.00 g, 5.90 mmol) in CH_3CN (50.0 mL), Et_3N (1.19 g, 11.79 mmol) was added followed by allylchloroformate (**115**) (0.71 g, 5.90 mmol) at 0 °C and stirred for 18 h. Acetonitrile was removed under vacuum and the crude was dissolved in EtOAc (50 mL), washed with water (30 mL), dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO_2, 2:8/EtOAc:hexanes) to yield **116** (1.22 g, 95%) as a white solid. (R_f 0.9 on SiO_2, 100% EtOAc); $[\alpha]_D^{25}$ -15.59 (*c* 0.87, $CHCl_3$); IR ($CHCl_3$, cast) 3401, 2980, 2955, 1725, 1649, 1527 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 5.93 (dddd, 1H, *J* = 15.5, 9.6, 9.6, 9.6 Hz, =CH), 5.50 (d, 1H, *J* = 8.7 Hz, NH), 5.38-5.30 (m, 1H, =CH*H*), 5.38-5.30 (app dq, 1H, *J* = 10.5, 1.4 Hz, =C*H*H), 4.60 (app dt, 2H, *J* = 5.6, 1.4 Hz, OCH_2), 4.38-4.28 (m, 2H, Thr-H_α + H_β), 3.79 (s, 3H, OCH_3), 1.82 (br s, 1H, OH), 1.27 (d, 3H, *J* = 6.6 Hz, Thr-CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 171.7 (C=O), 156.6 (C=O), 132.5 (CH), 117.8 (CH_2), 67.9 (CH), 66.0 (CH_2), 59.1 (CH), 52.6 (CH_3), 19.8 (CH_3); HRMS (ES) Calcd for $C_9H_{15}NO_5Na$ 240.0842, found 240.0844.

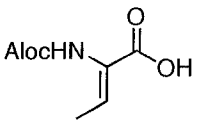
(*Z*)-Methyl 2-(allyloxycarbonylamino)but-2-enoate (117)



To a solution of compound **116** (0.10 g, 0.46 mmol) in CH_2Cl_2 (10 mL), MsCl (0.053 mL, 0.46 mmol) was added followed by DBU (0.21 mL, 1.38 mmol) and refluxed for 3 h. The solvent was removed *in vacuo* and the crude product was purified by flash chromatography (SiO_2, 3:7/EtOAc:hexanes) to yield **117** (0.066 g, 58%) as a colorless

oil. (R_f 0.6 on SiO_2, 1:1/EtOAc:hexanes); IR ($CHCl_3$, cast) 3320, 2953, 1724, 1661, 1508 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 6.73 (q, 1H, *J* = 7.2 Hz, Dhb-CH), 6.23 (br s, 1H, NH), 5.97-5.84 (m, 1H, =CH), 5.38-5.30 (app dq, 1H, *J* = 17.3, 1.5 Hz, =CH*H*), 5.38-5.30 (app dq, 1H, *J* = 10.5, 1.4 Hz, =C*H*H), 4.58 (app dt, 2H, *J* = 5.6, 1.4 Hz, OCH_2), 3.75 (s, 3H, OCH_3), 1.82 (dd, 3H, *J* = 7.2, 0.6 Hz, Dhb-CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 165.0 (C=O), 154.0 (C=O), 133.3 (CH), 132.4 (CH), 126.3 (C), 118.0 (CH_2), 66.2 (CH_2), 52.6 (CH_3), 14.2 (CH_3); HRMS (ES) Calcd for $C_9H_{13}NO_4Na$ 222.0737, found 222.0737.

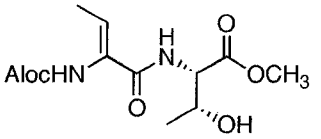
(Z)-2-(Allyloxycarbonylamino)but-2-enoic acid (118)



To a solution of compound **117** (1.91 g, 9.57 mmol) in 1,4-dioxane (20.0 mL), 1 N LiOH (20.0 mL) was added at 0 °C and stirred for 12 h. The solvents were removed *in vacuo* and the residue was dissolved in water (15 mL) and acidified to pH 1 at 0 °C with 1 M HCl. The free acid was extracted with EtOAc (2 x 25 mL), dried over Na_2SO_4, filtered and concentrated *in vacuo* to yield **118** (1.75 g, 98%) as a white foamy solid. (R_f 0.01 on SiO_2, 100% EtOAc); IR ($CHCl_3$, cast) 3288, 3018, 2888, 1693, 1656, 1520 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 6.91 (q, 1H, *J* = 7.4 Hz, Dhb-CH), 6.38 (br s, 1H, NH), 5.92 (ddt, 1H, *J* = 15.5, 9.8, 9.8 Hz, =CH), 5.35-5.29 (m, 1H, =CH*H*), 5.25-5.26 (m, 1H, =C*H*H), 4.61 (dt, 2H, *J* = 5.5, 1.5 Hz, OCH_2), 1.85 (d, 3H, *J* = 7.0 Hz, Dhb-CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 169.1 (C=O), 154.2 (C=O), 136.2 (CH), 132.1 (CH), 125.9 (C),

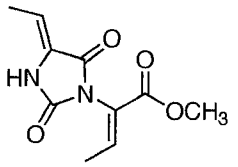
118.1 (CH_2), 66.3 (CH_2), 14.3 (CH_3); HRMS (ES, negative mode) Calcd for $C_8H_{10}NO_4$ 184.0604, found 184.0605.

(2*S*,3*R*)-Methyl 2-((*Z*)-2-(allyloxycarbonylamino)but-2-enamido)-3-hydroxy butanoate (119)



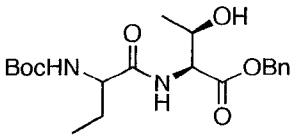
To a solution of L-threonine methyl ester hydrochloride (**100**) (0.28 g, 1.62 mmol) in CH_2Cl_2 (10.0 mL), NMM (0.39 g, 3.56 mmol) was added followed by compound **118** (0.30 g, 1.62 mmol), PyBOP (0.89 g, 1.70 mmol) and stirred for 12 h. The solvents were removed under vacuum and the crude product was purified by flash chromatography (SiO_2, 5.5:3.5/EtOAc:hexanes) to yield **119** (0.47 g, 97%) as a foamy white solid. (R_f 0.4 on SiO_2, 100% EtOAc); IR ($CHCl_3$, cast) 3305, 2978, 2953, 1733, 1673, 1639, 1518 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz) δ 6.72-6.78 (m, 1H, Dhb-CH), 6.56 (br d, 1H, J = 3.0 Hz, NH), 6.13 (br s, 1H, NH), 5.93 (dddd, 1H, J = 15.6, 9.6, 9.6, 9.6 Hz, =CH), 5.62-5.32 (m, 1H, =CH*H*), 5.26-5.24 (m, 1H, =C*H*H), 4.67 (dd, 1H, J = 9.0, 3.0 Hz, Thr-H_α), 4.64-4.62 (m, 2H, OCH_2), 4.34 (dq, 1H, J = 6.6, 2.8 Hz, Thr-H_β), 3.78 (s, 3H, OCH_3), 1.81 (dd, 3H, J = 6.6, 0.6 Hz, Dhb-CH_3), 1.26 (d, 3H, J = 6.6, 0.6 Hz, Thr-CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.2 (C=O), 164.7 (C=O), 154.2 (C=O), 132.0 (CH), 129.4 (CH), 126.3 (C), 118.3 (CH_2), 68.4 (CH), 66.4 (CH_2), 57.4 (CH), 52.5 (OCH_3), 20.0 (CH_3), 13.2 (CH_3); HRMS (ES) Calcd for $C_{13}H_{20}N_2O_6Na$ 323.1213, found 323.1214.

(Z)-Methyl 2-((Z)-4-ethylidene-2,5-dioxoimidazolidin-1-yl)but-2-enoate (121)



This literature compound was obtained according to the procedure by Shin *et al.*[182] To a solution of compound **119** (5 mg, 0.17 mmol) in CH_2Cl_2 (7.0 mL), MsCl (29 μL, 0.25 mmol) was added followed by DBU (100 μL, 0.67 mmol) and stirred at 25 °C for 8 h. The solvent was removed *in vacuo* and the crude product was purified by flash chromatography (SiO_2, 4:6/EtOAc:hexanes) to yield **121** (26 mg, 70%) as a fluffy white solid. (R_f 0.8 on SiO_2, 100% EtOAc); IR ($CHCl_3$, cast) 3274, 2954, 1781, 1727, 1687 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz) δ 8.68 (s, 1H, Dhb-NH), 7.34 (q, 1H, J = 7.2 Hz, Dhb-CH), 6.02 (q, 1H, J = 7.2 Hz, Dhb-CH), 3.78 (s, 3H, OCH_3), 1.86 (d, 3H, J = 7.2 Hz, Dhb-CH_3), 1.83 (d, 3H, J = 7.2 Hz, Dhb-CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 162.8 (C=O), 161.3 (C=O), 153.7 (C=O), 143.1 (CH), 128.9 (C), 122.7 (C), 112.4 (CH), 52.5 (CH_3), 14.3 (CH_3), 12.4 (CH_3); HRMS (ES) Calcd for $C_{10}H_{12}N_2O_4Na$ 247.0689, found 247.0688.

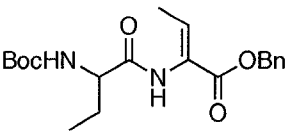
(2*S*,3*R*)-Benzyl 2-(2-(*tert*-butoxycarbonylamino)butanamido)-3-hydroxybutanoate (124)



To a solution of L-threonine benzyl ester oxalate (**123**) (1.40 g, 4.68 mmol) in CH_2Cl_2 (40.0 mL), NMM (1.54 mL, 14.03 mmol) was added followed by *tert*-butyloxycarbonyl-

D,L-2-aminobutyric acid (Boc-Abu-OH, **122**) (1.05 g, 5.14 mmol), PyBOP (2.68 g, 5.15 mmol) and stirred for 5 h. The reaction mixture was sequentially washed with water (2 x 25 mL), brine (25 mL), dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO_2, 4:6/EtOAc:hexanes) to yield **124** (1.30 g, 70%) as a fluffy white solid. (R_f 0.6 on SiO_2, 100% EtOAc); $[\alpha]_D^{25}$ -1.11 (*c* 1.65, $CHCl_3$); IR ($CHCl_3$, cast) 3337, 2975, 2934, 1661, 1520 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz, *diastereomeric mixture*) δ 7.35-7.30 (m, 5H, ArH), 7.19-7.06 (m, 1H, NH), 5.37-5.27 (m, 1H, NH), 5.20-5.13 (m, 2H, benzylic CH_2), 4.68-4.61 (m, 1H, Thr-H_α), 4.42-4.33 (m, 1H, Thr-H_β), 4.22-4.02 (m, 1H, Abu-H_α), 3.45 (br s, 1H, OH), 1.94-1.75 (m, 1H, Abu-CH*H*), 1.72-1.56 (m, 1H, Abu-C*H*H), 1.40 (s, 9H, ^{t}Bu), 1.18 (m, 3H, Thr-CH_3), 0.96-0.90 (m, 3H, Abu-CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz, *diastereomeric mixture*) δ 171.7 (C=O), 170.8, 170.7 (C=O), 155.9 and 155.7 (C=O), 135.2 (C), 128.6 (CH), 128.4 (CH), 128.2 (CH), 128.1 (CH), 80.1 (C), 68.2 and 67.9 (CH), 67.4 and 67.3 (CH_2), 57.4 (CH), 55.9 (CH), 28.3 (CH_3), 25.8 (CH_2), 20.0 and 19.9 (CH_3), 9.9 and 9.8 (CH_3); HRMS (ES) Calcd for $C_{20}H_{30}N_2O_6Na$ 417.1996, found 417.1995.

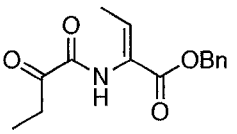
(*Z*)-Benzyl 2-(2-(*tert*-butoxycarbonylamino)butanamido)but-2-enoate (125)



To a solution of compound **124** (0.25 g, 0.64 mmol) in CH_2Cl_2 (20.0 mL), MsCl (74 μL, 0.96 mmol) was added followed by DBU (0.29 mL, 1.92 mmol) and refluxed for 14 h. The solvent was removed *in vacuo* and the crude product was purified by flash chromatography (SiO_2, 2:8/EtOAc:hexanes) to yield **125** (0.19 g, 78%) as foamy white

solid. (R_f 0.8 on SiO_2, 1:1/EtOAc:hexanes); IR ($CHCl_3$, cast) 3288, 2975, 2934, 1720, 1672, 1520 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.91 (s, 1H, Dhb-NH), 7.32-7.26 (m, 5H, ArH), 6.82 (q, 1H, J = 7.2 Hz, Dhb-CH), 5.35 (d, 1H, J = 5.2 Hz, NH), 5.14 (s, 2H, benzylic CH_2), 4.18-4.30 (m, 1H, Abu-H_α), 1.91-1.80 (m, 1H, Abu-CH*H*), 1.72 (d, 3H, J = 7.2 Hz, Dhb-CH_3), 1.69-1.60 (m, 1H, Abu-C*H*H), 1.40 (s, 9H, ^{t}Bu), 0.93 (t, 3H, J = 7.2 Hz, Abu-CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.8 (C=O), 164.1 (C=O), 155.8, (C=O), 135.6 (C), 134.8 (CH), 128.5 (CH), 128.2 (CH), 128.1 (CH), 126.2 (C), 79.9 (C), 66.9 (CH_2), 55.7 (CH), 28.3 (CH_3), 25.8 (CH_2), 14.3 (CH_3), 9.8 (CH_3); HRMS (ES) Calcd for $C_{20}H_{28}N_2O_5Na$ 399.1890, found 399.1888.

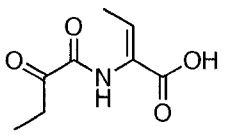
(*Z*)-Benzyl 2-(2-oxobutanamido)but-2-enoate (127)



To a solution of compound **125** (200 mg, 0.53 mmol) in CH_2Cl_2 (20.0 mL), TFA (20.0 mL) was added and stirred for 1.5 h at 25 °C. The solvents were removed *in vacuo* to give the TFA salt (208 mg, quant.) as a sticky solid. The TFA salt was dissolved in (1:3) DMF: CH_2Cl_2 (15 mL), followed by the addition of 4-pyridinecarboxaldehyde methyliodide salt (**126**) (1.74 g, 6.77 mmol) and stirred for 10 minutes. To the wine red solution, DBU (1.01 mL, 6.77 mmol) was added dropwise and stirred for 10 minutes. The reaction was completed by addition of saturated oxalic acid solution (20 mL) and stirring for 30 minutes. The organic layer was separated, washed with water (2 x 10 mL), dried over Na_2SO_4 filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO_2, 2:8/EtOAc:hexanes) to yield **127** (102 mg, 70%) as an off-white

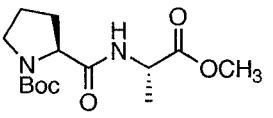
solid. (R_f 0.5 on SiO_2, 1:1/EtOAc:hexanes); IR ($CHCl_3$, cast) 3357, 3065, 2978, 2881, 1723, 1694, 1659, 1608, 1587 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz,) δ 8.33 (s, 1H, Dhb-NH), 7.38-7.33 (m, 5H, ArH), 6.94 (q, 1H, *J* = 7.2 Hz, Dhb-CH), 5.22 (s, 2H, benzylic CH_2), 2.98 (q, 2H, *J* = 7.2 Hz, $COCH_2$), 1.80 (d, 3H, *J* = 7.2 Hz, Dhb-CH_3), 1.14 (t, 3H, *J* = 7.2 Hz, CH_2CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 198.7 (C=O), 163.6 (C=O), 157.8 (C=O), 135.4 (C), 135.1 (CH), 128.6 (CH), 128.4 (CH), 128.2 (CH), 125.0 (C), 67.3 (CH_2), 30.3 (CH_2), 15.0 (CH_3), 7.1 (CH_3); HRMS (ES) Calcd for $C_{15}H_{17}NO_4Na$ 298.1050, found 298.1054.

(Z)-2-(2-Oxobutanamido)but-2-enoic acid (128)



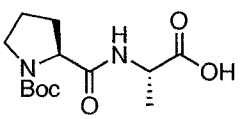
To a solution of compound **127** (50 mg, 0.18 mmol) in EtOAc (5.0 mL), 10% Pd/C (5 mg) was added and stirred under 1 atmosphere of hydrogen gas for 2 h. The reaction mixture was passed through a pad of celite to remove Pd / C and the solvent was removed *in vacuo* to yield **128** (30 mg, 78%) as a foamy white solid. (R_f 0.01 on SiO_2, 3:7/EtOAc:hexanes); IR ($CHCl_3$, cast) 3400-3100, 3094, 2979, 2883, 1725, 1694, 1659, 1608, 1505 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz,) δ 8.28 (s, 1H, Dhb-NH), 7.05 (q, 1H, *J* = 7.2 Hz, Dhb-CH), 2.99 (q, 2H, *J* = 7.2 Hz, Abu-H_β), 1.84 (d, 3H, *J* = 7.2 Hz, Dhb-CH_3), 1.15 (t, 3H, *J* = 7.2 Hz, Abu-CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 198.6 (C=O), 168.7 (C=O), 157.8 (C=O), 137.8 (CH), 124.5 (C), 30.3 (CH_2), 15.2 (CH_3), 7.0 (CH_3); HRMS (ES) Calcd for $C_8H_{11}NO_4Na$ 208.0580, found 208.0578.

(*S*)-*tert*-Butyl 2-((*S*)-1-methoxy-1-oxopropan-2-ylcarbamoyl)pyrrolidine-1-carboxylate (131)



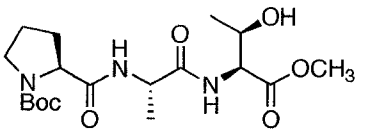
This known compound was prepared by a modified literature procedure.[183] To a solution of L-alanine methyl ester hydrochloride (**130**) (0.97 g, 6.97 mmol) in CH_2Cl_2 (50.0 mL), NMM (2.12 g, 20.90 mmol) was added followed by Boc-Pro-OH (**129**) (1.50 g, 6.97 mmol), PyBOP (3.63 g, 6.97 mmol) and stirred for 4 h at rt. The solvent was removed *in vacuo* and the crude product was purified by flash chromatography (SiO_2, 4:6/EtOAc:hexanes) to yield **131** (1.75 g, 84%) as a foamy white solid. (R_f 0.60 on SiO_2, 100% EtOAc); $[\alpha]_D^{25}$ -91.05 (*c* 0.90, $CHCl_3$); IR ($CHCl_3$ cast) 3306, 2977, 2878, 1747, 1701, 1666, 1544 cm^{-1}; ^1H NMR (DMSO-D_6, 500 MHz, 100 °C) δ 7.81 (d, 1H, J = 5.0 Hz, NH), 4.31 (app pent, 1H, J = 7.0 Hz, Ala-H_α), 4.13 (dd, 1H, J = 8.5, 3.5 Hz, Pro-H_α), 3.63 (s, 3H, OCH_3), 3.39-3.29 (m, 2H, Pro-H_δ), 2.11-2.03 (m, 1H, Pro-H_β), 1.85-1.75 (m, 3H, Pro-$H_{\beta'}$ + H_γ), 1.37 (s, 9H, ^{t}Bu), 1.29 (d, 3H, J = 7.0 Hz, Ala-CH_3); ^{13}C NMR (DMSO-D_6, 125 MHz, 100 °C) δ 172.2 (C=O), 171.5 (C=O), 153.0 (C=O), 78.0 (C), 58.9 (CH), 51.0 (CH_3), 47.0 (CH), 46.0 (CH_2), 29.8 (CH_2), 27.6 (CH_3), 22.7 (CH_2), 16.5 (CH_3); HRMS (ES) Calcd for $C_{14}H_{25}N_2O_5$ 300.1758, found 301.1756 (M+H).

(*S*)-2-((*S*)-1-(*tert*-Butoxycarbonyl)pyrrolidine-2-carboxamido)propanoic acid (132)



This known compound was prepared according to literature procedure.[183] To a solution of compound **131** (1.50 g, 5.00 mmol) in 1,4-dioxane (20.0 mL), 1 M LiOH (20.0 mL) was added at 0 °C and stirred for 2 h. The solvents were removed *in vacuo* and the residue was dissolved in water (20 mL) and acidified to pH 1 at 0 °C with 1 M HCl. The free acid was immediately extracted with EtOAc (2 x 30 mL), dried over Na_2SO_4, filtered and concentrated *in vacuo* to yield **132** (1.19 g, 83%) as a white solid. (R_f 0.01 on SiO_2, 100% EtOAc); $[\alpha]_D^{25}$ -93.68 (*c* 0.90, $CHCl_3$); IR ($CHCl_3$ cast) 3297, 2979, 1734, 1653, 1558 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz, 100 °C) δ 12.1 (br s, 1H, COOH), 7.64 (d, 1H, *J* = 6.5 Hz, NH), 4.26 (app pent, 1H, *J* = 7.0 Hz, Ala-H_α), 4.13 (dd, 1H, *J* = 8.0, 3.0 Hz, Pro-H_α), 3.38-3.28 (m, 2H, Pro-H_δ), 2.08-2.05 (m, 1H, Pro-H_β), 1.89-1.72 (m, 3H, Pro-$H_{\beta'}$ + H_γ), 1.37 (s, 9H, ^{t}Bu), 1.29 (d, 3H, *J* = 7.0 Hz, Ala-CH_3); ^{13}C NMR (DMSO-D_6, 125 MHz, 100 °C) δ 173.0 (C=O), 171.4 (C=O), 153.1 (C=O), 78.1 (C), 59.0 (CH), 46.9 (CH), 46.0 (CH_2), 29.8 (CH_2), 27.6 (CH_3), 22.7 (CH_2), 16.8 (CH_3); HRMS (ES) Calcd for $C_{13}H_{22}N_2O_5$ 286.1601, found 287.1600 (M+H).

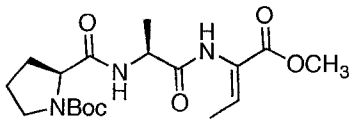
(*S*)-*tert*-Butyl 2-((*S*)-1-((2*S*,3*R*)-3-hydroxy-1-methoxy-1-oxobutan-2-ylamino)-1-oxopropan-2-ylcarbamoyl)pyrrolidine-1-carboxylate (133)



To a solution of L-threonine methyl ester hydrochloride (**100**) (0.27 g, 1.57 mmol) in DMF (10.0 mL), NMM (0.35 mL, 3.144 mmol) was added followed by compound **132** (0.45 g, 1.57 mmol), PyBOP (0.82 g, 1.57 mmol) and stirred for 4 h at rt. The solvent was removed *in vacuo* and the crude product was purified by flash chromatography (SiO_2,

1:9/acetone:EtOAc) to yield **133** (0.45 g, 71%) as a fluffy white solid. (R_f 0.70 on SiO_2, 3:7/acetone:EtOAc); $[\alpha]_D^{25}$ -73.99 (*c* 0.28, $CHCl_3$); IR ($CHCl_3$ cast) 3305, 2976, 1747, 1663, 1521 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 7.09 (br s, 1H, NH), 7.00 (br s, 1H, NH), 4.60-4.48 (m, 2H, Thr-H_α + Ala-H_α), 4.39-4.24 (m, 2H, Thr-H_β + Pro-H_α), 3.76 (s, 3H, OCH_3), 3.52-3.30 (m, 2H, Pro-H_δ), 2.43 (br s, 1H, OH), 2.28-2.02 (m, 2H, Pro-H_β), 1.89-1.94-1.80 (m, 2H, Pro-H_γ), 1.45 (s, 9H, ^{t}Bu), 1.42 (d, 3H, J = 7.5 Hz, Ala-CH_3), 1.19 (d, 3H, J = 6.5 Hz, Thr-CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 172.4 (C=O), 171.2 (C=O), 171.1 (C=O), 155.6 (C=O), 80.8 (C), 68.2 (CH), 60.4 (CH), 57.7 (CH), 52.5 (CH_3), 49.1 (CH), 47.3 (CH_2), 29.0 (CH_2), 28.3 (CH_3), 24.5 (CH_2), 20.0 (CH_3), 17.8 (CH_3); HRMS (ES) Calcd for $C_{18}H_{31}N_3O_7Na$ 424.2054, found 424.2057.

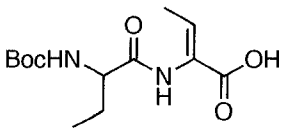
(*S*)-*tert*-butyl 2-((*S*)-1-((*Z*)-1-methoxy-1-oxobut-2-en-2-ylamino)-1-oxopropan-2-ylcarbamoyl)pyrrolidine-1-carboxylate (134)



To a solution of compound **112** (0.71 g, 2.48 mmol) in CH_2Cl_2 (20.0 mL), TFA (20.0 mL) was added and stirred for 30 minutes. The solvents were removed under vacuum and the residue was dissolved in CH_2Cl_2 (30 mL). To the solution, NMM (0.82 mL, 0.74 mmol), Boc-Pro-OH (**129**) (0.53 g, 2.48 mmol) and PyBOP (1.29 g, 2.48 mmol) were added and stirred for 4 h. The solvent was removed *in vacuo* and the crude product was purified by flash chromatography (SiO_2, 6.5:3.5/EtOAc:hexanes) to yield **134** (0.81 g, 85%) as a fluffy white solid. (R_f 0.40 on SiO_2, 100% EtOAc); $[\alpha]_D^{25}$ -114.69 (*c* 0.50, $CHCl_3$); IR ($CHCl_3$ cast) 3283, 2978, 1700, 1662, 1521 cm^{-1}; ^1H NMR (CD_3OD, 300

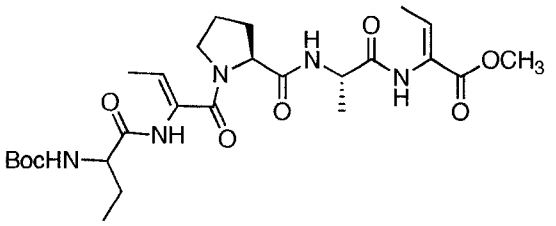
MHz) δ 6.79 (d, 1H, *J* = 7.2 Hz, Dhb-CH), 4.46 (app pent, 1H, *J* = 7.2 Hz, Ala-H_α), 4.20 (dd, 1H, *J* = 8.1, 4.2 Hz, Pro-H_α), 3.72 (s, 3H, OCH_3), 3.56-3.32 (m, 2H, Pro-H_δ), 2.30-2.15 (m, 1H, Pro-H_β), 2.02-1.80 (m, 3H, Pro-$H_{\beta'}$ + H_γ), 1.74 (d, 3H, *J* = 7.2 Hz, Dhb-CH_3), 1.44-1.42 (m, 12H, Ala-CH_3 + ^{t}Bu); ^{13}C NMR (CD_3OD, 125 MHz) δ 175.4 (C=O), 173.8 (C=O), 166.1 (C=O), 156.0 (C=O), 136.7 (CH), 128.2 (C), 81.4 (C), 61.7 (CH), 52.6 (CH_3), 50.5 (CH), 48.3 (CH_2), 32.3 (CH_2), 28.7 (CH_3), 24.7 (CH_2), 17.7 (CH_3), 14.0 (CH_3); HRMS (ES) Calcd for $C_{18}H_{29}N_3O_6Na$ 406.1948, found 406.1952.

(Z)-2-(2-(*tert*-Butoxycarbonylamino)butanamido)but-2-enoic acid (136)



To a solution of compound **125** (0.41 g, 1.08 mmol) in EtOAc (20.0 mL), 10% Pd on C (40 mg) was added and stirred under 1 atmosphere of hydrogen gas for 1 h. The reaction mixture was passed through a pad of celite to remove Pd on C and the solvent was removed *in vacuo* to yield **136** (0.30 g, 98%) as a foamy white solid. (R_f 0.01 on SiO_2, 100% EtOAc); IR ($CHCl_3$, cast) 3292, 2976, 2935, 1700, 1683, 1521 cm^{-1}; ^{1}H NMR (CD_3OD, 400 MHz) δ 6.83 (q, 1H, *J* = 7.2 Hz, Dhb-CH), 4.87 (s, 1H, Abu-NH), 4.05 (app t, 1H, *J* = 7.2 Hz, Abu-H_α), 1.91-1.80 (m, 1H, Abu-CH*H*), 1.74 (d, 3H, *J* = 7.2 Hz, Dhb-CH_3), 1.71-1.60 (m, 1H, Abu-C*H*H), 1.44 (s, 9H, ^{t}Bu), 1.00 (app t, 3H, *J* = 7.6 Hz, Abu-CH_3); ^{13}C NMR (CD_3OD, 125 MHz) δ 174.2 (C=O), 167.2 (C=O), 157.9, (C=O), 136.5 (CH), 128.5 (C), 80.6 (C), 57.7 (CH), 28.7 (CH_3), 26.7 (CH_2), 14.1 (CH_3), 10.7 (CH_3); HRMS (ES) Calcd for $C_{13}H_{22}N_2O_5$ 286.1601, found 287.1601 (M+H).

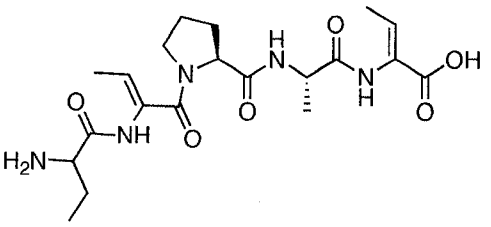
(*Z*)-Methyl 2-((2*S*)-2-((2*S*)-1-((*Z*)-2-(2-(*tert*-butoxycarbonylamino)butanamido)but-2-enoyl)pyrrolidine-2-carboxamido)propanamido)but-2-enoate (137)



To a solution of compound **134** (0.50 g, 1.30 mmol) in CH_2Cl_2 (20.0 mL), TFA (20.0 mL) was added and stirred for 2 h. The solvents were removed under vacuum and the residue was dissolved in 30 mL of (1:30) DMF: CH_2Cl_2. To the solution, NMM (0.13 mL, 1.30 mmol), HOBt (0.18 g, 1.30 mmol), compound **136** (0.37 g, 1.30 mmol) and DIPCDI (0.20 mL, 1.30 mmol) were added and stirred for 14 h. The solvents were removed *in vacuo* and the crude product was purified by flash chromatography (SiO_2, 2:8/Acetone:EtOAc) to yield **137** (0.56 g, 78%) as a fluffy white solid. (R_f 0.50 on SiO_2, 1:1/Acetone:EtOAc); $[\alpha]_D^{25}$ -27.42 (*c* 1.28, $CHCl_3$); IR ($CHCl_3$ cast) 3291, 2978, 2878, 1716, 1662, 1622, 1505 cm^{-1}; ^1H NMR (DMF-d_7, 500 MHz, *diastereomeric mixture,* 100 °C) δ 9.41, 9.18 (br s, 1H, Dhb-NH), 8.41, 8.36 (br s, 1H, Dhb-NH), 7.88-7.80 (m, 1H, Ala-NH), 6.56-6.50 (m, 1H, Dhb-CH, terminal), 6.42-6.34 (m, 1H, Abu-NH), 5.62-5.59 (m, 1H, Dhb-CH, internal), 4.45-4.36 (m, 2H, Ala-H_α + Abu-H_α), 4.24-4.16 (m, 1H, Pro-H_α), 3.70-3.55 (m, 5H, Pro-H_δ + OCH_3), 2.26-2.20 (m, 1H, Pro-H_β), 2.01-1.82 (m, 5H, Pro-$H_{\beta'}$ + H_γ + Abu-CH_2), 1.77-1.69 (m, 6H, Dhb-CH_3), 1.46-1.41 (m, 12H, Ala-CH_3 + ^{t}Bu), 0.99-0.96 (m, 3H, Abu-CH_3); ^{13}C NMR (Ac-D_6, 125 MHz, *diastereomeric mixture*) δ 173.6 & 173.0 (C=O), 172.5 & 172.4 (C=O), 171.8 (C=O), 167.8 (C=O), 165.6& 165.4 (C=O), 157.0 (C=O), 132.4, 132.3 (CH), 129.6, 129.4 (C), 120.5, 120.0 (C), 79.9, 79.5 (C), 62.4, 62.3 (CH), 56.7 (CH), 52.0 (CH_3), 50.3 (CH), 48.3 (CH_2), 36.1 (CH_2), 30.6,

30.5 (CH_2), 28.6, 28.5 (CH_3), 25.9, 25.6 (CH_2), 25.5, 25.3 (CH_2), 17.5,17.3 (CH_3), 13.6, 13.5 (CH_3), 12.0, 11.9 (CH_3), 10.9, 10.8 (CH_3); HRMS (ES) Calcd for $C_{26}H_{41}N_5O_8Na$ 574.2847, found 574.2848.

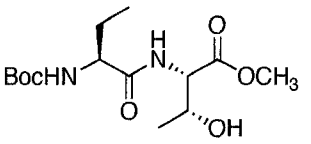
(*Z*)-2-((2*S*)-2-((2*S*)-1-((*Z*)-2-(2-Aminobutanamido)but-2-enoyl)pyrrolidine-2-carboxamido)propanamido)but-2-enoic acid (139)



To a solution of compound **137** (0.10 g, 1.81 mmol) in 1,4-dioxane (1.5 mL), 1 N LiOH (1.5 mL) was added and stirred for 2 h. The solvents were removed *in vacuo* to yield **139** along with LiCl as a highly hygroscopic solid. $[\alpha]_D^{25}$ -44.91 (*c* 0.81, MeOH); IR (MeOH cast) 3412, 2956, 2916, 1656, 1541 cm^{-1}; ^1H NMR (CD_3OD, 600 MHz, *diastereomeric mixture*) δ 6.89-6.79 (m, 1H, Dhb-CH, terminal), 5.87-5.79 (m, 1H, Dhb-CH, internal), 4.49-4.40 (m, 2H, Ala-H_α + Abu-H_α), 4.06-4.04 (m, 1H, Pro-H_α), 3.76-3.66 (m, 2H, Pro-H_δ), 2.35-2.30 (m, 1H, Pro-H_β), 2.08-1.88 (m, 5H, Pro-$H_{\beta'}$ + H_γ + Abu-CH_2), 1.83-1.81 (m, 3H, Dhb-CH_3, terminal), 1.75-1.73 (m, 3H, Dhb-CH_3, internal), 1.52-1.50 (m, 3H, Ala-CH_3), 1.11-1.04 (m, 3H, Abu-CH_3); ^{13}C NMR (CD_3OD, 125 MHz, *diastereomeric mixture*) δ 174.2 & 174.1 (C=O), 169.8 & 169.6 (C=O), 168.9 & 168.8 (C=O), 167.8 & 167.2 (C=O), 137.8 & 136.7 (CH), 131.3 & 130.9 (CH), 128.7 & 128.4 (C), 124.2 & 123.4 (C), 62.6 & 62.4 (CH), 55.4 & 55.2 (CH), 51.6 & 51.4 (CH), 51.1 & 50.7 (CH), 48.3 (CH_2), 31.0 & 30.9 (CH_2), 26.1 & 26.1 (CH_2), 25.9 & 25.7 (CH_2), 17.6 (CH_3), 14.1

& 14.0 (CH_3), 12.6 & 12.5 (CH_3), 9.7 & 9.6 (CH_3); HRMS (ES) Calcd for $C_{20}H_{31}N_5O_6$ 437.2347, found 438.2347 (M+H).

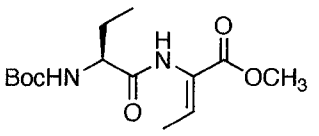
(2*S*,3*R*)-Methyl 2-((*S*)-2-(*tert*-butoxycarbonylamino)butanamido)-3-hydroxy butanoate (141)



N-Methylmorpholine (NMM) (0.54 mL, 4.94 mmol) was added to a solution of L-threonine methyl ester hydrochloride (**100**) (836 mg, 4.92 mmol), in CH_2Cl_2 (30.0 mL), followed by Boc-Abu-OH (**140**) (1.00 g, 4.92 mmol) and PyBOP (2.56 g, 4.92 mmol). The resulting mixture was stirred at 25 °C for 12 h. After the reaction was complete, the CH_2Cl_2 layer was washed with 15% $NaHCO_3$ (20 mL), 10% citric acid (20 mL) and water (2 x 20 mL). The separated organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO_2, 4:6/EtOAc:hexanes) to yield **141** (880 mg, 56%) as a white solid. (R_f 0.65 on SiO_2, 100% EtOAc); $[\alpha]_D^{25}$ -9.8 (*c* 2.09, $CHCl_3$); IR ($CHCl_3$ cast) 3336, 2977, 2936, 1746, 1665, 1523 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 6.93 (d, 1H, *J* = 9.2 Hz, Thr-NH), 5.20 (d, 1H, *J* = 8.0 Hz, Abu-NH), 4.62 (dd, 1H, *J* = 9.2, 2.4 Hz, Thr-H_α), 4.35 (dq, 1H, *J* = 6.4, 2.8 Hz, Thr-H_β), 4.12-4.02 (m, 1H, Abu-H_α), 3.77 (s, 3H, OCH_3), 2.85 (br s, 1H, Thr-OH), 1.87 (ddq, 1H, *J* = 13.9, 6.9, 6.9 Hz, Abu-H_β), 1.67 (ddq, 1H, *J* = 14.2, 7.1, 7.1 Hz, Abu-$H_{\beta'}$), 1.43 (s, 9H, ^{t}Bu), 1.20 (d, 3H, *J* = 6.4 Hz, Thr-CH_3), 0.97 (app t, 3H, *J* = 7.2 Hz, Abu-H_γ); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.5 (C=O), 171.1 (C=O), 155.7 (C=O), 80.0 (C), 68.1

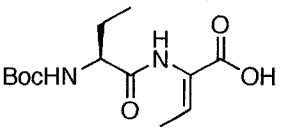
(CH), 57.2 (CH), 55.9 (CH), 52.4 (CH_3), 28.2 (CH_3), 25.5 (CH_2), 19.8 (CH_3), 9.8 (CH_3); HRMS (ES) Calcd for $C_{14}H_{26}N_2O_6Na$ 341.1683, found 341.1682.

(*S*,*Z*)-Methyl 2-(2-(*tert*-butoxycarbonylamino)butanamido)but-2-enoate (142)



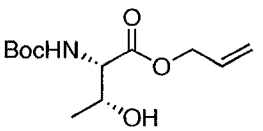
To a solution of **141** (995 mg, 3.13 mmol) in EtOAc (60.0 mL), MsCl (0.72 g, 6.25 mmol) was added and the resulting mixture was stirred for 20 min followed by addition of DBU (1.90 g, 12.51 mmol). The reaction mixture was then heated to reflux and stirred for 3 h. After completion of the reaction, the EtOAc layer was washed with water (2 x 50 mL) and brine (1 x 40 mL). The organic layer was then dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO_2, 2:8/EtOAc:hexanes) to yield **142** (795 mg, 85%) as a white solid. (R_f 0.55 on SiO_2, 1:1/EtOAc:hexanes); $[\alpha]_D^{25}$ -27.6 (*c* 1.49, $CHCl_3$); IR ($CHCl_3$ cast) 3299, 2976, 1725, 1674, 1518 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 7.45 (br s, 1H, Dhb-NH), 6.82 (q, 1H, *J* = 7.5 Hz, Dhb-H_β), 5.05 (br s, 1H, Abu-NH), 4.21-4.10 (m, 1H, Abu-H_α), 3.75 (s, 3H, OCH_3), 1.94 (ddq, 1H, *J* = 14.2, 7.2, 7.2 Hz, Abu-H_β), 1.76 (d, 3H, *J* = 7.5 Hz, Dhb-CH_3), 1.75-1.66 (m, 1H, Abu-$H_{\beta'}$) 1.45 (s, 9H, ^{t}Bu), 1.00 (app t, 3H, *J* = 7.5 Hz, Abu-CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 170.3 (C=O), 164.7 (C=O), 155.7 (C=O), 134.4 (C), 125.8 (CH_2), 80.2 (C), 56.0 (CH), 52.3 (CH_3), 28.3 (CH_3), 25.5 (CH_2), 14.5 (CH_3), 9.9 (CH_3); HRMS (ES) Calcd for $C_{14}H_{24}N_2O_5Na$ 323.1577, found 323.1576.

(*S*,*Z*)-2-(2-(*tert*-Butoxycarbonylamino)butanamido)but-2-enoic acid (143)



A solution of dipeptide **142** (665 mg, 2.21 mmol) in 1,4-dioxane (7.0 mL) and 1 N LiOH (7.0 mL) was stirred at 25 °C for 1.5 h. The reaction was then concentrated *in vacuo* and the resulting salt was dissolved in water (10 mL) and cooled to 0 °C. The aqueous layer was acidified to pH 1 with 6 M HCl and extracted with EtOAc (4 x 10 mL) The combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo* to yield the desired product **143** (632 mg, quant.) as a white solid. (R_f 0.01 on SiO_2, 1:1/EtOAc:hexanes); $[\alpha]_D^{25}$ -29.4 (*c* 2.29, $CHCl_3$); IR ($CHCl_3$ cast) 3360, 2978, 2936, 1704, 1519 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) δ 6.83 (q, 1H, *J* = 7.0 Hz, Dhb-CH), 4.85 (br s, 1H, Abu-NH), 4.04 (app t, 1H, *J* = 6.7 Hz, Abu-H_α), 1.85 (ddq, 1H, *J* = 14.2, 7.0, 7.0 Hz, Abu-H_β), 1.74 (d, 3H, *J* = 7.0 Hz, Dhb-CH_3), 1.72-1.63 (m, 1H, Abu-$H_{\beta'}$), 1.44 (s, 9H, ^{t}Bu), 1.01 (app t, 3H, *J* = 7.0 Hz, Abu-CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 174.2 (C=O), 167.2 (C=O), 157.9 (C=O), 136.5 (C), 128.5 (CH), 80.6 (C), 57.6 (CH), 28.7 (CH_3), 26.6 (CH_2), 14.1 (CH_3), 10.6 (CH_3); HRMS (ES) Calcd for $C_{13}H_{22}N_2O_5Na$ 309.1420, found 309.1420.

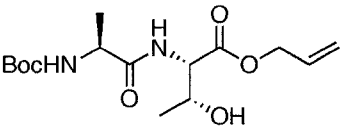
(2S,3R)-Allyl-2-(*tert*-butoxycarbonylamino)-3-hydroxybutanoate (145)



This compound was prepared according to the procedure by Bayo *et al.*[184] To a solution of Boc-Thr-OH (**144**) (3.00 g, 13.7 mmol) in DMF (75.0 mL), Na_2CO_3 (2.90 g, 28.4

mmol) was added followed by allyl bromide (**75**) (1.42 mL, 16.4 mmol) and water (2 mL). The resulting mixture was stirred for 12 h at 25 °C. The solvent was removed from the reaction mixture *in vacuo* and water (75 mL) was added. The aqueous layer was extracted with EtOAc (3 x 25 mL) and the combined organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo* to give compound **145** (3.30 g, 93%) as a colorless oil. (R_f 0.60 on SiO_2, 1:1/EtOAc:hexanes); $[\alpha]_D^{25}$ -114.1 (*c* 0.65, $CHCl_3$); IR ($CHCl_3$ cast) 3434, 2934, 1718, 1507, 1165 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz) δ 5.92 (ddt, 1H, *J* = 17.4, 10.8, 6.0 Hz, C*H*=CH_2), 5.38-5.25 (m, 3H, Thr-NH + CH=CH_2), 4.71-4.64 (m, 2H, OCH_2), 4.36-4.26 (m, 2H, Thr-H_α + Thr-H_β), 1.84 (br s, 1H, Thr-OH), 1.46 (s, 9H, ^{t}Bu), 1.27 (d, 3H, *J* = 6.6 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 171.1 (C=O), 156.1 (C=O), 131.5 (=CH), 118.8 (=CH_2), 80.1 (C), 68.2 (CH), 66.0 (CH_2), 58.7 (CH), 28.3 (CH_3), 19.9 (CH_3); HRMS (ES) Calcd for $C_{12}H_{21}NO_5Na$ 282.1312, found 282.1313.

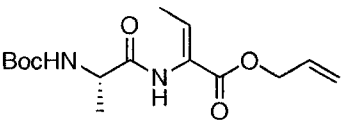
(2*S*,3*R*)-Allyl-2-((*R*)-2-(*tert*-butoxycarbonylamino)propanamido)-3-hydroxy butanoate (146)



This known compound was prepared by a modified literature procedure.[185] To a solution of compound **145** (1.76 g, 6.79 mmol) in CH_2Cl_2 (40.0 mL), TFA (40.0 mL) was added and the resulting mixture was stirred for 1 h at 25 °C. The solvents were removed *in vacuo* and the product dried to afford the TFA salt (1.74 g, 6.79 mmol, quant.). To a solution of the TFA salt in CH_2Cl_2 (100 mL), NMM (2.24 mL, 20.36 mmol) was added followed by Boc-Ala-OH (**99**) (1.28 g, 6.78 mmol) and PyBOP (3.53 g, 6.78 mmol). The

resulting mixture was stirred for 12 h at 25 °C. The solvent was removed *in vacuo* and the crude product was further purified by flash chromatography (SiO_2, 1:1.6/EtOAc:hexanes) to yield **146** (2.01 g, 90%) as a fluffy white solid. (R_f 0.80 on SiO_2, 100% EtOAc); $[\alpha]_D^{25}$ -17.8 (*c* 1.08, $CHCl_3$); IR ($CHCl_3$ cast) 3337, 2978, 2934, 1741, 1716, 1694, 1683, 1663, 1521 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.04 (d, 1H, *J* = 9.0 Hz, Thr-NH) 5.89 (ddt, 1H, *J* = 14.1, 11.4, 8.4 Hz, C*H*=CH_2), 5.36-5.21 (m, 3H, Ala-NH + CH=CH_2), 4.66-4.59 (m, 3H, OCH_2 + Ala- H_α), 4.34 (dq, 1H, *J* = 6.5, 2.7 Hz, Thr-H_β), 4.27-4.17 (m, 1H, Thr-H_α), 3.21 (br s, 1H, Thr-OH), 1.42 (s, 9H, ^{t}Bu), 1.36 (d, 3H, *J* = 7.2 Hz, Ala-CH_3), 1.19 (d, 3H, *J* = 6.5 Hz, Thr-CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.2 (C=O), 170.4 (C=O), 155.4 (C=O), 131.3 (=CH), 118.7 (=CH_2), 80.0 (C), 68.1 (CH), 66.0 (CH_2), 57.3 (CH), 50.0 (CH), 28.2 (CH_3), 19.8 (CH_3), 18.2 (CH_3); HRMS (ES) Calcd for $C_{15}H_{27}N_2O_6$ 331.1863, found 331.1860 (M+H).

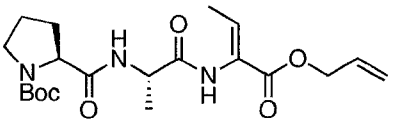
(*S*)-(*Z*)-Allyl 2-(2-(*tert*-butoxycarbonylamino)propanamido)but-2-enoate (147)



To a solution of **146** (1.95 g, 5.90 mmol) in EtOAc (100.0 mL), MsCl (0.96 mL, 12.40 mmol) was added followed by DBU (1.90 g, 12.51 mmol) over 10 min. The resulting mixture was refluxed for 4 h. The EtOAc layer was then washed with water (2 x 75 mL) and brine (75 mL). The organic layer was separated, dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO_2, 1:4/EtOAc:hexanes) to yield **147** (1.44 g, 78%) as a white solid. (R_f 0.50 on SiO_2, 1:1/EtOAc:hexanes); $[\alpha]_D^{25}$ -42.3 (*c* 1.03, $CHCl_3$); IR ($CHCl_3$ cast) 3292, 2978, 2934,

1717, 1675, 1506 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.50 (br s, 1H, Dhb-NH) 6.85 (q, 1H, *J* = 7.2 Hz, Dhb-CH), 5.92 (ddt, 1H, *J* = 17.2, 10.8, 5.5 Hz, C*H*=CH_2), 5.32 (app dq, 1H, *J* = 17.2, 1.4 Hz, CH=C*H*H), 5.24 (app dq, 1H, *J* = 10.4, 1.4 Hz, CH=CH*H*), 5.00 (br s, 1H, Ala-NH), 4.65 (dt, 2H, *J* = 5.8, 1.4 Hz, OCH_2), 4.34-4.22 (m, 1H, Ala-H_α), 1.77 (d, 3H, *J* = 7.2 Hz, Dhb-CH_3), 1.45 (s, 9H, ^{t}Bu), 1.42 (d, 3H, *J* = 7.2 Hz, Ala-CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 171.1 (C=O), 163.9 (C=O), 155.5 (C=O), 134.5 (=CH), 131.8 (=CH), 126.0 (C), 118.4 (=CH_2), 80.2 (C), 65.8 (CH_2), 50.2 (CH), 28.2 (CH_3), 18.2 (CH_3), 14.4 (CH_3); HRMS (ES) Calcd for $C_{15}H_{24}N_2O_5Na$ 335.1577, found 335.1578.

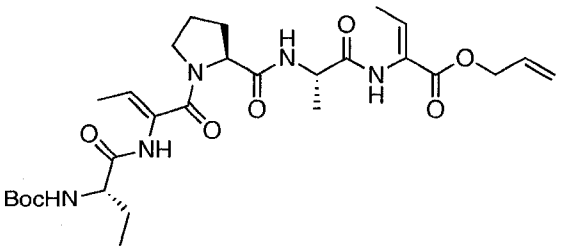
(*S*)-*tert*-Butyl 2-((*S*)-1-((*Z*)-1-(allyloxy)-1-oxobut-2-en-2-ylamino)-1-oxopropan -2-ylcarbamoyl)pyrrolidine-1-carboxylate (148)



To a solution of compound **147** (726 mg, 2.32 mmol) in CH_2Cl_2 (20 mL), TFA (20 mL) was added and stirred for 1 h at 25 °C. The solvents were removed *in vacuo* and the crude product was dried to give the TFA salt (758 mg, 2.32 mmol, quant.). It was dissolved in CH_2Cl_2 (60 mL) and NMM (0.77 mL, 6.97 mmol) was added followed by Boc-Pro-OH (**129**) (500 mg, 2.32 mmol) and PyBOP (1.21 g, 2.32 mmol). The resulting mixture was stirred for 14 h at 25 °C. The solvent was removed *in vacuo* and the crude product was purified by flash chromatography (SiO_2, 1.5:8.5/acetone:EtOAc) to yield **148** (878 mg, 92%) as a white solid. (R_f 0.70 on SiO_2, 3:7/acetone:EtOAc); $[\alpha]_D^{25}$ -56.0 (*c* 1.07, $CHCl_3$); IR ($CHCl_3$ cast) 3281, 2977, 2933, 1699, 1662, 1558 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz, 100 °C) δ 8.79 (br s, 1H, Dhb-NH), 7.63 (d, 1H, *J* = 6.8 Hz, Ala-NH), 6.61 (dq,

1H, J = 6.9, 0.8 Hz, Dhb-CH), 5.92 (dddd, 1H, J = 17.3, 10.6, 5.3, 5.3 Hz, C*H*=CH_2), 5.32 (app dq, 1H, J = 17.2, 1.6 Hz, CH=C*H*H), 5.24 (app dq, 1H, J = 10.8, 1.6 Hz, CH=CH*H*), 4.64 (dt, 2H, J = 5.2, 1.6 Hz, OCH_2), 4.44 (app pent 1H, J = 7.0 Hz, Ala-H_α), 4.16 (dd, 1H, J = 8.0, 3.2 Hz, Pro- H_α), 3.41-3.30 (m, 2H, Pro-H_δ), 2.14-2.04 (m, 1H, Pro-H_β), 1.92-1.73 (m, 3H, Pro-$H_{\beta'}$+ Pro-H_γ), 1.69 (dd, 3H, J = 6.8, 0.8 Hz, Dhb-CH_3), 1.39 (s, 9H, ^{t}Bu), 1.32 (d, 3H, J = 7.2 Hz, Ala-CH_3); ^{13}C NMR (DMSO-d_6, 100 MHz, *25 °C, rotameric mixture*) 172.0, 171.7 (C=O), 171.2 (C=O), 163.5 (C=O), 153.7, 153.1 (C=O), 132.7 (=CH), 132.4 (=CH), 127.5 (C), 117.4 (=CH_2), 78.6, 78.2 (C), 64.7 (CH_2), 59.1 (CH), 48.0, 47.8 (CH), 46.6, 46.4 (CH_2), 30.7, 29.7 (CH_2), 28.0, 27.8 (CH_3), 23.7, 23.0 (CH_2), 18.2, 17.8 (CH_3), 13.2 (CH_3); ^{13}C NMR (DMSO-d_6, 100 MHz, 100 °C, *rotamers coalesce*) δ 171.4 (C=O), 170.6 (C=O), 163.0 (C=O), 132.2 (=CH), 132.0 (=CH), 127.2 (C), 116.9 (=CH_2), 78.2 (C), 64.2 (CH_2), 59.2 (CH), 47.7 (CH), 46.1 (CH_2), 27.6 (CH_3), 22.8 (CH_2), 17.5 (CH_3), 12.6 (CH_3); HRMS (ES) Calcd for $C_{20}H_{31}N_3O_6Na$ 432.2107, found 432.2105.

(*Z*)-Allyl 2-((*S*)-2-((*S*)-1-((*Z*)-2-((*S*)-2-(*tert*-butoxycarbonylamino)butanamido) but-2-enoyl)pyrrolidine-2-carboxamido)propanamido)but-2-enoate (149)

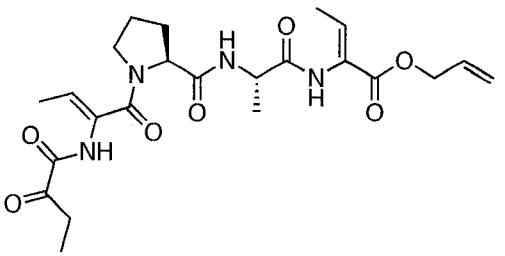


To a solution of compound **148** (0.83 g, 2.02 mmol) in CH_2Cl_2 (20.0 mL), TFA was added (20.0 mL) and the resulting mixture was stirred for 1 h at 25 °C. The solvents were

removed *in vacuo* and the crude product dried to afford the TFA salt (0.85 g, quant.). It was dissolved in CH_2Cl_2 (30 mL) and NMM was added (0.67 mL, 6.05 mmol) followed by dipeptide **143**, HOBt (0.27 g, 2.01 mmol) and DIPCDI (0.30 mL, 2.01 mmol). The resulting mixture was stirred for 12 h at 25 °C and the CH_2Cl_2 layer was washed with water (2 x 15 mL). The organic layer was separated, dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO_2, 1:9/acetone:EtOAc) to yield **149** (0.84 g, 72%) as a white solid. (R_f 0.60 on SiO_2, 3:7/acetone:EtOAc); $[\alpha]_D^{25}$ -159.8 (*c* 1.84, $CHCl_3$); IR ($CHCl_3$ cast) 3293, 2976, 1717, 1662, 1622, 1506 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz) δ 8.39 (br s, 1H, internal Dhb-NH), 8.30 (br s, 1H, terminal Dhb-NH), 7.83 (d, 1H, *J* = 8.4 Hz, Ala-NH), 6.06 (d, 1H, *J* = 8.4 Hz, Abu-NH), 6.80 (q, 1H, *J* = 7.2 Hz, terminal Dhb-CH), 5.91 (ddt, 1H, *J* = 17.3, 10.4, 5.8 Hz, C*H*=CH_2), 5.37-5.32 (m, 2H, CH=C*H*H + internal Dhb-CH), 5.23 (app dq, 1H, *J* = 10.8, 1.2 Hz, CH=CH*H*), 4.67 (ddt, 1H, *J* = 13.2, 5.6, 1.4 Hz, OC*H*H), 4.60 (ddt, 1H, *J* = 13.2, 5.7, 1.5 Hz, OCH*H*), 4.55 (app pent 1H, *J* = 7.8 Hz, Ala-H_α), 4.47 (dd, 1H, *J* = 8.4, 3.0 Hz, Pro- H_α), 4.14-4.05 (m, 1H, Abu-H_α), 3.89-3.85 (m, 1H, Pro- H_δ), 3.59-3.54 (m, 1H, Pro-$H_{\delta'}$), 2.30-2.22 (m, 2H, Pro-H_β), 2.00-1.93 (m, 3H, Pro-H_γ + Abu-H_β), 1.79 (d, 3H, *J* = 7.2 Hz, terminal Dhb-CH_3), 1.74 (d, 3H, *J* = 7.2 Hz, internal Dhb-CH_3), 1.65-1.58 (m, 1H, Abu-$H_{\beta'}$), 1.54 (d, 3H, *J* = 7.8, Hz, Ala-CH_3), 1.43 (s, 9H, ^{t}Bu), 0.95 (t, 3H, *J* = 7.2 Hz, Abu-CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.3 (C=O), 171.9 (C=O), 171.2 (C=O), 167.1 (C=O), 164.6 (C=O), 157.4 (C=O), 136.0 (=CH), 131.9 (=CH), 130.7 (C), 127.0 (C), 118.3 (=CH_2), 116.4 (=CH), 80.4 (C), 65.8 (CH_2), 61.3 (CH), 55.0 (CH), 50.2 (CH_2), 49.6 (CH), 30.0 (CH_2), 28.3 (CH_3), 24.4 (CH_2), 22.5 (CH_2), 16.7 (CH_3), 13.9

(CH_3), 11.6 (CH_3), 10.6 (CH_3); HRMS (ES) Calcd for $C_{28}H_{43}N_5O_8Na$ 600.3004, found 600.3007.

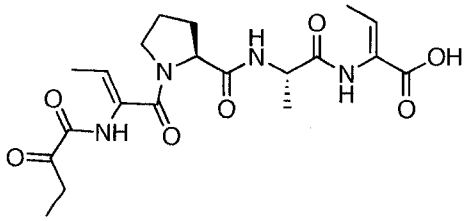
(*Z*)-Allyl 2-((*S*)-2-((*S*)-1-((*Z*)-2-(2-oxobutanamido)-but-2-enoyl)pyrrolidine-2-carboxamido)propanamido)but-2-enoate (151)



To a solution of compound **149** (705 mg, 1.35 mmol) in CH_2Cl_2 (20 mL) was added TFA (20 mL) and the resulting mixture was stirred for 1.5 h at 25 °C. The solvent was removed *in vacuo* and the crude product dried to give the TFA salt (723 mg, quant). To a solution of the TFA salt in CH_2Cl_2 (30.0 mL) 4-pyridine carboxaldehyde (650 μL, 6.77 mmol) and AcOH (390 μL, 6.77 mmol) were added and the resulting mixture was stirred for 3 h at 25 °C. The reaction mixture was cooled to 0 °C and then DBU (1.01 mL, 6.77 mmol) was added dropwise over 10 min. The resulting mixture was warmed to 25 °C and stirred for 11 h. A 1 M solution of HCl (10 mL) was added and stirring was continued for an additional 3 h. The organic layer was separated and washed with water (2 x 10 mL), dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude product (410 mg) was purified by flash chromatography (SiO_2, 1:9/acetone:EtOAc) to yield **151** (341 mg, 53%) as an off-white solid. (R_f 0.2 on SiO_2, 100% EtOAc); $[\alpha]_D^{25}$ -137.0 (*c* 1.73, $CHCl_3$); IR ($CHCl_3$ cast) 3322, 2980, 1724, 1668, 1617, 1506 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz,) δ 8.55 (br s, 1H, internal Dhb-NH), 8.15 (br s, 1H, terminal Dhb-NH), 7.77 (d, 1H, J = 8.8

Hz, Ala-NH), 6.82 (q, 1H, J = 7.2 Hz, terminal Dhb-CH), 5.85 (ddt, 1H, J = 17.2, 10.8, 5.2 Hz, C*H*=CH_2), 5.55 (dq, 1H, J = 7.2, 0.8 Hz, internal Dhb-CH), 5.27 (app dq, 1H, J = 17.2, 1.6 Hz, CH=C*H*H), 5.17 (app dq, 1H, J = 10.4, 1.3 Hz, CH=CH*H*), 4.62 (app pent 1H, J = 7.6 Hz, Ala-H_α), 4.54-4.49 (m, 3H, OCH_2 + Pro-H_α), 3.83 (ddd, 1H, J = 10.5, 5.3, 5.3 Hz, Pro-H_δ), 3.58 (ddd, 1H, J = 10.5, 8.3, 8.3 Hz, Pro-$H_{\delta'}$), 3.00 (dq, 1H, J = 20.0, 7.2 Hz, α-ketoamide-CH*H*), 2.84 (dq, 1H, 20.0, 7.2 Hz, α-ketoamide-C*H*H), 2.32-2.26 (m, 2H, Pro-H_β), 2.04-1.96 (m, 2H, Pro-H_γ), 1.81 (d, 3H, J = 7.2 Hz, internal Dhb-CH_3), 1.74 (dd, 3H, J = 7.2, 0.8 Hz, terminal Dhb-CH_3), 1.58 (d, 3H, J = 7.6, Hz, Ala-CH_3), 1.08 (t, 3H, J = 7.2 Hz, α-ketoamide-CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 197.8 (C=O), 171.4 (C=O), 170.8 (C=O), 166.1 (C=O), 163.6 (C=O), 158.4 (C=O), 136.0 (=CH), 132.2 (=CH), 129.2 (C), 126.5 (C), 118.9 (=CH), 117.9 (CH_2), 65.4 (CH_2), 61.4 (CH), 50.2 (CH_2), 49.4 (CH), 29.9 (Overlapping CH_2 + CH_2), 24.4 (CH_2), 17.1 (CH_3), 14.1 (CH_3), 11.7 (CH_3), 6.8 (CH_3); HRMS (ES) Calcd for $C_{23}H_{32}N_4O_7$ 476.2344, found 477.2345 (M+H).

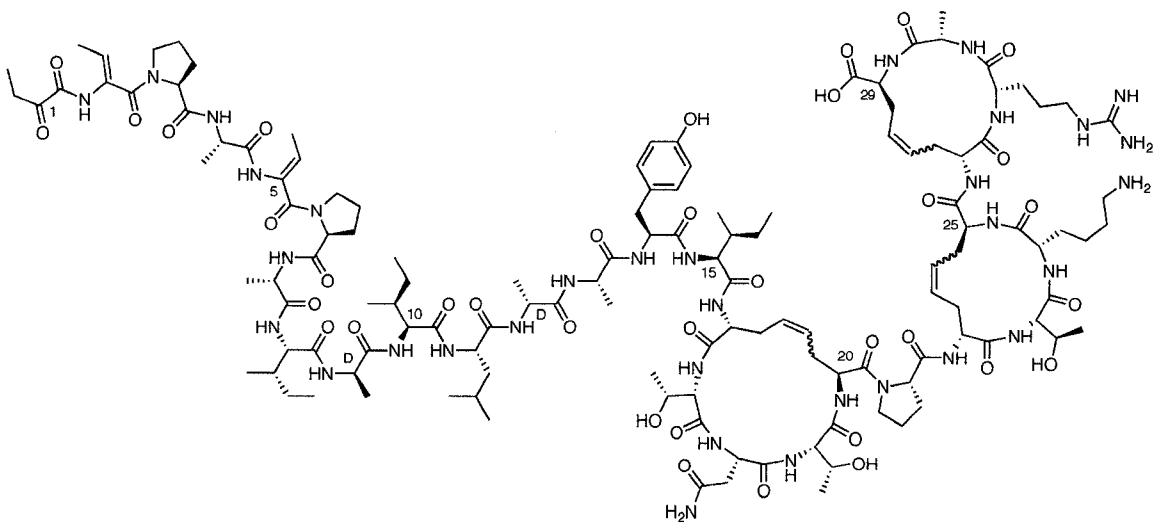
(*Z*)-2-((*S*)-2-((*S*)-1-((*Z*)-2-(2-Oxobutanamido) but-2-enoyl)pyrrolidine-2-carboxamido) propanamido)but-2-enoic acid (45)



To a solution of **151** (250 mg, 0.52 mmol) in degassed CH_2Cl_2 (30 mL) phenylsilane (113 μL, 1.04 mmol) was added followed by $Pd(PPh_3)_4$ (60 mg, 0.05 mmol). The resulting mixture was stirred under Ar for 1.5 h at 25 °C. The solvent was removed *in vacuo* and

the crude product was purified by flash chromatography (SiO_2, 94:4:2/DCM:MeOH:AcOH) to yield **45** (212 mg, 92%) as an off-white solid. (R_f 0.4 on SiO_2, (8:1:1) $CHCl_3$:MeOH:AcOH); $[\alpha]_D^{25}$ -63.8 (*c* 0.2, MeOH); IR ($CHCl_3$ cast) 3318, 2983, 2942, 1722, 1666, 1621, 1535, 1507 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz,) δ 9.16 (br s, 1H, internal Dhb-NH), 7.96 (br s, 1H, terminal Dhb-NH), 7.87 (d, 1H, *J* = 9.0 Hz, Ala-NH), 6.75 (q, 1H, *J* = 7.2 Hz, terminal Dhb-CH), 5.70 (q, 1H, *J* = 7.2 Hz, internal Dhb-CH), 4.67-4.61 (m, 2H, Ala-H_α + Pro-H_α), 3.93-3.90 (m, 1H, Pro-H_δ), 3.66-3.62 (m, 1H, Pro-$H_{\delta'}$), 2.85 (q, 2H, *J* = 7.2 Hz, α-ketoamide-CH_2), 2.28-2.25 (m, 2H, Pro-H_β), 2.06-1.98 (m, 2H, Pro-H_γ), 1.92 (d, 3H, *J* = 7.2 Hz, internal Dhb-CH_3), 1.69 (d, 3H, *J* = 7.2 Hz, terminal Dhb-CH_3), 1.59 (d, 3H, *J* = 7.8, Hz, Ala-CH_3), 1.01 (t, 3H, *J* = 7.2 Hz, α-ketoamide-CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 198.6 (C=O), 171.1 (C=O), 170.4 (C=O), 167.4 (C=O), 165.5 (C=O), 159.7 (C=O), 135.5 (=CH), 128.8 (C), 126.0 (C), 121.5 (=CH), 61.5 (CH), 50.4 (CH_2), 49.4 (CH), 30.4 (CH_2), 29.9 (CH_2), 24.3 (CH_2), 17.1 (CH_3), 14.8 (CH_3), 12.2 (CH_3), 7.0 (CH_3); HRMS (ES) Calcd for $C_{20}H_{28}N_4O_7Na$ 459.1850, found 459.1851.

2-Oxobutanyl-*Z*-didehydrobutyrl-L-prolyl-L-alanyl-*Z*-didehydrobutyrl-L-prolyl-L-alanyl-L-isoleucinyl-D-alanyl-L-isoleucinyl-L-leucinyl-D-alanyl-L-alanyl-L-tyrosinyl-L-isoleucinyl-D-(α-aminobutyrl)-L-threonyl-L-asparaginyl-L-threonyl-L-(α-aminobutyrl)-L-prolyl-D-(α-aminobutyrl)-L-threonyl-L-lysyl-L-(α-aminobutyrl)-D-(α-aminobutyrl)-L-arginyl-L-alanyl-L-(α-aminobutyrine) [(16→20), (22→25), (26→29)] (*E*/*Z*)-α,α'-D,L-diamino-γ,γ'-dehydrosuberic acid (43)



The Fmoc protecting group of resin-bound peptide **96** (210 mg) was removed by treatment with a solution of 2% DBU in NMP (5 mL, 3 x 3 min). The resin was washed with NMP (3 x 5 mL) to remove any traces of DBU. In a separate round bottom flask, NMM (30.0 μL, 0.27 mmol) was added to a solution of **45** (119 mg, 0.27 mmol) in NMP (5 mL) followed by the addition of HOBt (31.0 mg, 0.23 mmol) and DIPCDI (34.1 μL, 0.23 mmol). The reaction mixture was left shaking for 5 minutes after which the reaction contents were transferred to a peptide reaction vessel containing Fmoc-deprotected resin-bound **96**. The coupling was allowed to continue for 3 h under Ar bubbling. The above procedure was repeated to ensure complete coupling of **45** to resin-bound **96**. A sample of the resin-bound peptide (162 mg) was cleaved for analysis by treatment with a (97.5:2.5) TFA:TIPS solution for 3 h at rt. Concentration of the filtrate *in vacuo,* followed by precipitation with Et_2O gave the crude **43** (29.2 mg, 0.0104 mmol, overall yield = 8% as an off-white solid; The crude product was purified by reverse phase HPLC using a steel walled preparative C4 column (Vydac Protein C4 (214TP), 10 μm, 300 Å, 22 x 250 mm). The crude peptide was dissolved in (1:1) MeCN:0.1% HCOOH to give a

concentration of 1 mg / mL. In the method employed, 0.9 mL of the crude sample solution was injected and using water (containing 0.1% TFA) and acetonitrile (MeCN) as the eluents, the product peptide **43** was isolated. Method 1: Flow rate 10 mL/min, detection at 220 and 280 nm. Starting from 30% MeCN ramping up to 70% over 20 min, then 70% MeCN for 8 min followed by ramping down to 30% MeCN over 1 min, then 30% MeCN for 5 min. The desired product peptide **43** was isolated as a broad peak (t_R = 13.5 min). The combined HPLC fractions containing the product were concentrated *in vacuo* and were reinjected on an Alltech ProSphere™ HP C_4 steel walled column (reverse phase, C_4 column, 5 μm, 300 Å, 4.6 x 250 mm). The peptide was dissolved in (1:1) MeCN : 0.1% HCOOH to give a concentration of 1 mg / mL. In the method employed, 90 μL of the peptide sample was injected. The same method mentioned above was used except the flowrate was 1 mL/min. The desired product peak was collected as a broad peak (t_R = 12.0 min). The fractions containing the product were concentrated *in vacuo* and lyophilized to give **43** (1.8 mg, overall yield = 0.5%) as a fluffy white solid. MALDI-TOF MS Calcd for $C_{130}H_{201}N_{33}O_{36}$ 2800.5, found 2801.6 (M+H).

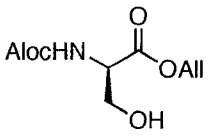
Biological evaluation of carbocyclic-larger ring analogue 43

Standard literature protocol[186] was followed for testing the antimicrobial activity of **43** and its parent natural peptides lacticin 3147 A1 and A2. Peptide analogue **43** (10 μL) and parent, lacticin 3147 peptides A1 & A2 (10 μL), were spotted on APT or LB agar plates and allowed to dry. The plates were overlaid with soft agar, seeded with the indicator organism *L. lactis* subsp. *cremoris* HP or the bacterium under investigation (100 μL fully

grown culture per 10 mL soft agar). The plates were then incubated at 25 °C overnight and the antibacterial activity was determined by a clear zone of inhibition. No zone of inhibition was observed when carbocyclic-larger ring analogue **43** was tested against the indicator organism upto 1 mM concentration.

4.2.2. Synthesis and testing of a lanthionine analogue of lacticin 3147 A2

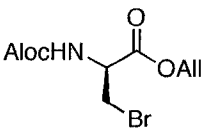
(*R*)-Allyl 2-(allyloxycarbonylamino)-3-hydroxypropanoate (160)



This known compound was prepared according to procedure by Bregant *et al.*[101] Allyl alcohol (**159**) (32.4 mL, 475.80 mmol) was added to a solution of *p*-toluenesulfonic acid (10.86 g, 57.10 mmol) and D-serine (**158**) (5.00 g, 47.57 mmol) in toluene (150.0 mL) and the mixture was refluxed for 5 h using a Dean-Stark apparatus. Toluene was removed under vacuum and the residue was redissolved in EtOAc (100 mL) followed by the addition of Et_3N (26.6 mL, 190.71 mmol) and stirred for 10 min. The reaction mixture was cooled on ice and allylchloroformate (5.6 mL, 52.32 mmol) in EtOAc (50 mL) was added via an addition funnel over 15 min and stirred for 14 h. The EtOAc layer was washed with 0.5 M HCl (1 x 100 mL), water (1 x 100 mL) and brine (1 x 100 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO_2, 3:7/EtOAc:hexanes) to yield **160** (5.50 g, 52%) as a colorless oil. (R_f 0.40 on SiO_2, 1:1/EtOAc:hexanes); $[\alpha]_D^{26}$ -3.2 (*c* 8.18, $CHCl_3$); IR ($CHCl_3$ cast) 3403, 2947, 2888, 1723, 1649, 1527 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.94-5.84 (m, 2H, 2 x =CH), 5.35-5.18 (m, 5H, $=CH_2$ + NH), 4.65 (app dt, 2H, *J* = 5.6, 1.4 Hz, allyl OCH_2), 4.57 (app d, 2H, *J* = 5.6 Hz, Aloc OCH_2), 4.47-4.39 (m, 1H, H_α), 4.01-3.86 (m, 2H, H_β), 2.92 (t, 1H, *J* = 5.6 Hz, OH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.3 (C=O), 156.2 (C=O), 132.4 (CH), 131.4 (CH), 118.8 (CH_2), 117.9 (CH_2), 66.3

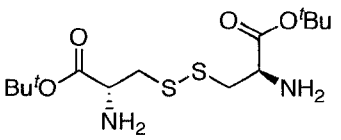
(CH_2), 66.0 (CH_2), 63.1 (CH_2), 56.1 (CH); HRMS (ES) Calcd for $C_{10}H_{15}NO_5Na$ 252.0844, found 252.0842.

(*S*)-Allyl 2-(allyloxycarbonylamino)-3-bromopropanoate (161)



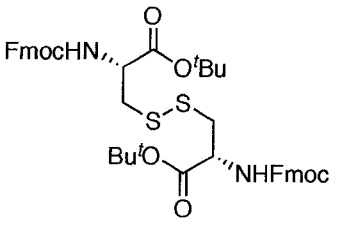
Triphenylphosphine (3.77 g, 14.40 mmol) was added portionwise to an ice-cold solution of **160** (3.00 g, 13.12 mmol) and CBr_4 (4.77 g, 14.40 mmol) in CH_2Cl_2 (100 mL) and the reaction mixture was warmed to 25 °C and stirred for 1.5 h. The reaction mixture was washed with water (1 x 100 mL), the organic layer was separated, dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO_2, 8:92/EtOAc:hexanes) to yield **161** (2.50 g, 65%) as a colorless oil. (R_f 0.80 on SiO_2, 1:1/EtOAc:hexanes); $[\alpha]_D^{26}$ -19.0 (*c* 6.23, $CHCl_3$); IR ($CHCl_3$ cast) 3332, 2947, 1725, 1649, 1514 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 5.98-5.85 (m, 2H, =CH), 5.67 (d, 1H, *J* = 7.2 Hz, NH), 5.39-5.20 (m, 4H, $=CH_2$), 4.83-4.78 (m, 1H, H_α), 4.75-4.62 (m, 2H, allyl OCH_2), 4.58 (app dt, 2H, *J* = 5.6, 1.3 Hz, Aloc OCH_2), 3.84 (dd, 1H, *J* = 10.5, 3.3 Hz, C*H*H), 3.73 (dd, 1H, *J* = 10.5, 3.6 Hz, CH*H*); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 168.5 (C=O), 155.4 (C=O), 132.3 (CH), 131.1 (CH), 119.3 (CH_2), 118.0 (CH_2), 66.7 (CH_2), 66.1 (CH_2), 54.3 (CH), 33.7 (CH_2); HRMS (ES) Calcd for $C_{10}H_{14}NO_4BrNa$ 314.0002, found 313.9998.

(2*R*,2'*R*)-*tert*-Butyl 3,3'-disulfanediylbis(2-aminopropanoate) (163)



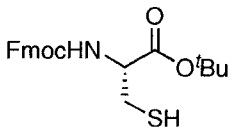
This known compound was prepared according to the procedure by Mustapa *et al.*[155] *tert*-Butyl acetate (100.0 mL) was added slowly to a stirred solution of L-cystine (**162**) (10 g, 41.61 mmol) in 70% v/v perchloric acid (16.6 mL) resulting in two immiscible layers that dispersed on stirring for 30 min. After 3 h, a white solid appeared and stirring was continued for another 16 h. The reaction mixture was cooled on ice for 15 min and water (100 mL) was added followed by EtOAc (100 mL). The pH was adjusted from 1 to 9 using 10 M NaOH with stirring. The organic layer was separated and the aqueous layer was extracted with EtOAc (2 x 70 mL). Combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo* to yield **163** (14.65 g, quant.) as light yellow oil. The crude product was used for further reaction without purification. $[\alpha]_D^{26}$ -41.00 (*c* 2.52, $CHCl_3$); IR ($CHCl_3$ cast) 3373, 2978, 2934, 1730, 1591, 1155 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 3.75 (dd, 2H, *J* = 7.6, 4.3 Hz, CH_α), 3.16 (dd, 2H, *J* = 14.0, 4.3 Hz, CH_β), 2.92 (dd, 2H, *J* = 13.6, 8.0 Hz, $CH_{\beta'}$), 2.83 (br s, 4H, NH_2), 1.47 (s, 18H, ^{t}Bu); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.2 (C=O), 82.2 (C), 54.2 (CH), 43.4 (CH), 28.0 (CH_3); HRMS (ES) Calcd for $C_{14}H_{28}N_2O_4S_2$ 352.1565, found 353.1563 (M+H).

(5*R*,10*R*)-di-*tert*-Butyl 1,14-di(9*H*-fluoren-9-yl)-3,12-dioxo-2,13-dioxa-7,8-dithia-4,11-diazatetradecane-5,10-dicarboxylate (164)



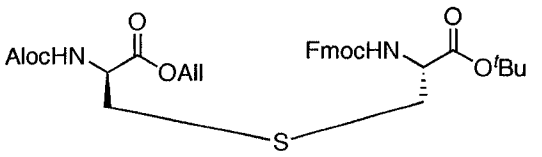
This known compound was prepared according to the procedure by Mustapa *et al.*[155] To a solution of **163** (14.60 g, 41.42 mmol) in THF (100.0 mL), NMM (9.1 mL) was added followed by Fmoc-OSu (27.94 g, 82.83 mmol) in THF (100 mL) via an addition funnel and the contents were stirred for 14 h. THF was removed *in vacuo* and the residue was redissolved in EtOAc (200 mL) and washed with water (2 x 100 mL) and brine (1 x 100 mL). Separated organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO_2, 1:9/EtOAc:hexanes) to yield **164** (22.20 g, 67%) as a white solid. (R_f 0.50 on SiO_2, 3:7/EtOAc:hexanes); $[\alpha]_D^{26}$ -5.3 (*c* 1.17, $CHCl_3$); IR ($CHCl_3$ cast) 3335, 3065, 2979, 1724, 1511 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.75 (d, 4H, *J* = 7.6 Hz, Ar-CH), 7.60 (d, 4H, *J* = 7.6 Hz, Ar-CH), 7.41-7.36 (m, 4H, Ar-CH), 7.30 (t, 4H, *J* = 7.2 Hz, Ar-CH), 5.75 (d, 2H, *J* = 7.6 Hz, NH), 4.59 (dd, 2H, *J* = 13.2, 5.6 Hz, H_α), 4.38 (dd, 4H, *J* = 18.0, 7.6 Hz, OCH_2), 4.21 (t, 2H, *J* = 7.2 Hz, CH), 3.27-3.17 (m, 4H, SCH_2), 1.50 (s, 18H, ^{t}Bu); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 169.2 (C=O), 155.6 (C=O), 143.7 (C), 141.2 (C), 127.6 (CH), 127.0 (CH), 125.0 (CH), 119.8 (CH), 83.0 (C), 67.1 (CH_2), 54.0 (CH), 47.0 (CH), 41.8 (CH_2), 27.9 (CH_3); HRMS (ES) Calcd for $C_{44}H_{48}N_2O_8S_2Na$ 819.2746, found 819.2744.

(*R*)-*tert*-Butyl 2-(((9*H*-fluoren-9-yl)methoxy)carbonylamino)-3-mercaptopropanoate (165)



This known compound was prepared according to the procedure by Mustapa *et al.*[155] To a solution of **164** (5.00 g, 6.27 mmol) in THF (75.0 mL), $P(Bu)_3$ (2.34 mL, 9.39 mmol) was added and stirred for 15 minutes, followed by the addition of water (7.5 mL) and stirred for an additional 14 h. THF was removed *in vacuo* and the residue was dissolved in EtOAc (100 mL) and washed with 10% citric acid (50 mL), water (50 mL) and brine (50 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO_2, 0.8:9.2/EtOAc:hexanes) to yield **165** (4.30 g, 86%) as a white solid. (R_f 0.50 on SiO_2, 2:8/EtOAc:hexanes); $[\alpha]_D^{26}$ 8.04 (*c* 3.86, $CHCl_3$); IR ($CHCl_3$ cast) 3340, 3066, 2978, 2568, 1723, 1510 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 7.77 (d, 2H, *J* = 7.5 Hz, Ar-CH), 7.62 (d, 2H, *J* = 7.0 Hz, Ar-CH), 7.41 (t, 2H, *J* = 7.5 Hz, Ar-CH), 7.35-7.31 (m, 2H, Ar-CH), 5.68 (d, 1H, *J* = 7.0 Hz, NH), 4.58-4.52 (m, 1H, H_α), 4.46-4.38 (m, 2H, OCH_2), 4.24 (t, 1H, *J* = 7.0 Hz, CH), 3.05-2.88 (m, 2H, SCH_2), 1.51 (s, 9H, ^{t}Bu), 1.35 (t, 1H, *J* = 9.0 Hz, SH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 168.9 (C=O), 155.6 (C=O), 143.9 (C), 143.7 (C), 141.34 (C), 141.32 (C), 127.7 (CH), 127.1 (CH), 125.1 (CH), 125.0 (CH), 120.01 (CH), 120.0 (CH), 83.1 (C), 67.1 (CH_2), 55.4 (CH), 47.2 (CH), 28.0 (CH_3), 27.4 (CH_2); HRMS (ES) Calcd for $C_{22}H_{25}NO_4SNa$ 422.1396, found 422.1396.

(5*R*,9*S*)-9-Allyl 5-*tert*-butyl 1-(9*H*-fluoren-9-yl)-3,11-dioxo-2,12-dioxa-7-thia-4,10-diazapentadec-14-ene-5,9-dicarboxylate (166)

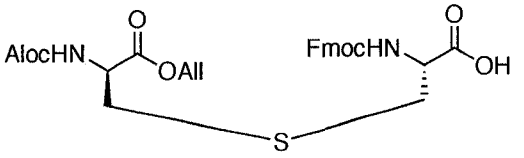


This known compound was prepared by a new procedure.[155] Tetrabutylammonium bromide (8.90 g, 27.60 mmol) was added to 75.0 mL of 0.5 M $NaHCO_3$ (pH was adjusted to 8.5 with 10% $NaHCO_3$) and stirred for 5 min. This solution was transferred to compound **161** (2.01 g, 6.90 mmol) and **165** (2.76 g, 6.90 mmol) dissolved in EtOAc (75.0 mL). The resulting biphasic mixture was stirred vigorously at 25 °C for 24 h. The organic layer was separated and washed with water (50 mL), dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO_2, 15:85/EtOAc:hexanes) to yield **166** (3.09 g, 73%) as a colorless sticky solid. (R_f 0.30 on SiO_2, 3:7/EtOAc:hexanes); $[\alpha]_D^{26}$ - 4.38 (*c* 1.03, $CHCl_3$); IR ($CHCl_3$ cast) 3336, 2979, 2952, 1723, 1648, 1522 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz, *diastereomers*) δ 7.77 (d, 2H, *J* = 7.5 Hz, Ar-CH), 7.65-7.60 (m, 2H, Ar-CH), 7.41 (t, 2H, *J* = 7.5 Hz, Ar-CH), 7.32 (dt, 2H, *J* = 7.5, 1.0 Hz, Ar-CH), 5.95-5.85 (m, 2H, =CH), 5.73 (d, 1H, *J* = 7.0 Hz, NH), 5.65 (d, 1H, *J* = 7.0 Hz, NH), 5.35-5.20 (m, 4H, $=CH_2$), 4.65-4.58 (m, 5H, OCH_2 + H_α), 4.53-4.46 (m, 1H, H_α), 4.44-4.36 (m, 2H, OCH_2), 4.25 (t, 1H, *J* = 7.0 Hz, CH), 3.15-2.94 (m, 4H, SCH_2), 1.49 (s, 9H, 'Bu); ^{13}C NMR ($CDCl_3$, 125 MHz, *diastereomeric mixture*) δ 170.1 (C=O), 169.3 (C=O), 155.7 (C=O), 155.6 (C=O), 143.9 (C), 143.8 (C), 141.3 (C), 132.5 (CH), 131.2 (CH), 127.7 (CH), 127.1 (CH), 125.1 (CH), 120.0 (CH), 119.3 (CH_2), 117.9 (CH_2), 83.1 (C), 67.2 (CH_2), 66.4 (CH_2), 66.0 (CH_2), 54.4 (CH), 53.9

(CH), 47.2 (CH), 35.9 (CH_2), 35.8 (CH_2), 28.0 (CH_3); HRMS (ES) Calcd for $C_{32}H_{38}N_2O_8SNa$ 633.2242, found 633.2241.

Note: The above reaction was also done using tetrabutylammonium hydrogen sulfate as the phase transfer catalyst with identical results.

(5*R*,9*S*)-9-(Allyloxycarbonyl)-1-(9*H*-fluoren-9-yl)-3,11-dioxo-2,12-dioxa-7-thia-4,10-diazapentadec-14-ene-5-carboxylic acid (167)



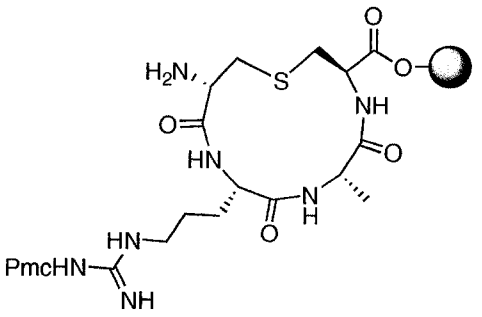
This known compound was prepared by a new procedure.[155] To a solution of **166** (2.08 g, 3.41 mmol) in CH_2Cl_2 (30.0 mL), TFA (30.0 mL) was added followed by $PhSiH_3$ (0.42 mL, 3.41 mmol) and stirred for 2 h. Volatiles were removed *in vacuo* to give a sticky white solid, which was dissolved in toluene with heating and left at 4 °C for 24 h. Toluene was carefully decanted off and the residue was dried under vacuum to yield **167** (1.54 g, 81%) as a white solid. (R_f 0.01 on SiO_2, 1:1/EtOAc:hexanes); $[\alpha]_D^{26}$ 13.3 (*c* 3.23, $CHCl_3$); IR ($CHCl_3$ cast) 3324, 3067, 2950, 1721, 1523 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz, *two diastereomers in 9:1 ratio*) δ 7.77 (d, 2H, *J* = 7.5 Hz, Ar-CH), 7.69-7.64 (m, 2H, Ar-CH), 7.37 (t, 2H, *J* = 7.5 Hz, Ar-CH), 7.29 (t, 2H, *J* = 7.5 Hz, Ar-CH), 5.89 (ddd, 2H, *J* = 19.4, 14.1, 8.9 Hz, =CH), 5.32-5.26 (m, 2H, $=CH_2$), 5.19-5.13 (m, 2H, $=CH_2$), 4.59 (d, 2H, *J* = 5.5 Hz, OCH_2), 4.52 (d, 2H, *J* = 5.0 Hz, OCH_2), 4.46-4.30 (m, 4H, OCH_2 + 2 x H_α), 4.22 (t, 1H, *J* = 7.0 Hz, CH), 3.11-3.00 (m, 2H, SCH_2), 2.99-2.87 (m, 2H, SCH_2); ^{13}C NMR (CD_3OD, 125 MHz) δ 173.8 (C=O), 172.0 (C=O), 158.5 (C=O), 158.3

(C=O), 145.3 (C), 145.2 (C), 142.6 (C), 134.2 (CH), 133.1 (CH), 128.8 (CH), 128.2 (CH), 126.4 (CH), 120.9 (CH), 118.9 (CH_2), 117.7 (CH_2), 68.3 (CH_2), 67.1 (CH_2), 66.8 (CH_2), 55.6 (major CH_α), 55.5 (minor CH_α), 55.4 (major CH_α), 55.3 (minor CH_α), 48.4 (CH), 35.5 (CH_2), 35.2 (CH_2); HRMS (ES) Calcd for $C_{28}H_{30}N_2O_8SNa$ 577.1618, found 577.1615.

The diastereomeric ratio (~9:1) was determined by integration of α-carbon on a ^{13}C NMR spectrum recorded at 125 MHz as shown by Tabor and co-workers.[101]

Solid Phase synthesis of the lanthionine analogue of lacticin 3147 A2 (44)

Synthesis of lanthionine ring C (153)



a) Fmoc Solid Phase Peptide Synthesis (SPPS) general conditions

Fmoc-amino acid (5.0 eq to resin loading) and HOBt (5.0 eq) were dissolved in DMF (10 mL) and to it, NMM (10.0 eq) was added followed by PyBOP (4.9 eq) and allowed to pre-activate for 5 min. The activated amino acid solution was then transferred to pre-swelled resin and reacted for 2 h. The completion of couplings were ascertained by negative Kaiser test[187] and end capping was performed with 20% Ac_2O in DMF for 10

min. The subsequent removal of the Fmoc group was done using 20% piperidine in DMF and monitored for completeness by the absorption of dibenzofulvene-piperidine adduct at $\lambda = 301$ nm on a UV-Vis spectrophotometer.

b) Loading of lanthionine **167** and SPPS to obtain **169**

2-Chlorotrityl chloride resin with a loading of 0.16 mmol/g was used for the synthesis. The initial loading of 1.6 mmol/g of the resin was reduced to 0.16 mmol/g to avoid any interstrand dimerization on-resin via a diketopiperazine.

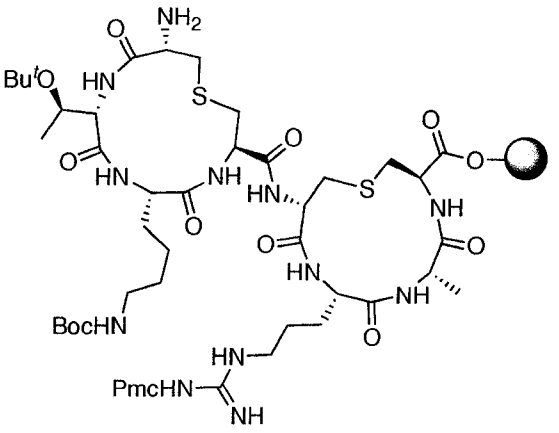
2-Chlorotrityl chloride resin (2.00 g, 3.20 mmol substitution capacity) was pre-swelled in CH_2Cl_2 (20 mL) for 20 min. To it, CH_3COOH (0.11 mL, 2.0 mmol) and DIPEA (1.394 mL, 8.00 mmol) in CH_2Cl_2 (20 mL) was added and reacted for 3 h. The resin was washed with CH_2Cl_2 (2 x 20 mL). To the partially capped resin, orthogonally protected lanthionine **167** (0.18 g, 0.32 mmol) and DIPEA (0.28 mL, 1.6 mmol) in CH_2Cl_2 (20 mL), was added and reacted for 2.5 h. The resin was again washed with CH_2Cl_2 (2 x 20 mL). The rest of the free sites on-resin after loading lanthionine were capped by reacting with CH_3COOH (2.00 mmol) and DIPEA (8.00 mmol) mixture for 2 h. Thus, the resin **168** obtained with a low loading of 0.16 mmol / g of **167** was used for further synthesis. Fmoc SPPS was continued using PyBOP to couple Fmoc-Ala-OH (residue 28) and Fmoc-Arg(Pmc)-OH (residue 27) to give linear fragment **169** on-resin.

c) Removal of Aloc/Allyl, Fmoc and cyclization on-resin to form ring C (**153**)

A solution of $Pd(PPh_3)_4$ (0.74 g, 0.64 mmol, 2 eq. to resin) and $PhSiH_3$ (0.39 mL, 3.2 mmol, 10 eq. to resin) in 1:1 DMF:CH_2Cl_2 (40 mL) was reacted with resin bound **169** protected from light for 2 h. The solution was drained and the resin was washed in the following sequence: a) CH_2Cl_2 (2 x 20 mL), b) 0.5% sodium diethyldithiocarbamate in DMF (3 x 20 mL) c) DMF (2 x 20 mL). The dark brown colored resin turned light yellow during the washings. The Fmoc group was removed with 20% piperidine in DMF to yield **152**. The resin was again carefully washed with DCM (3 x 10 mL) and DMF (3 x 10 mL), as residual piperidine could interfere in the subsequent cyclization step.

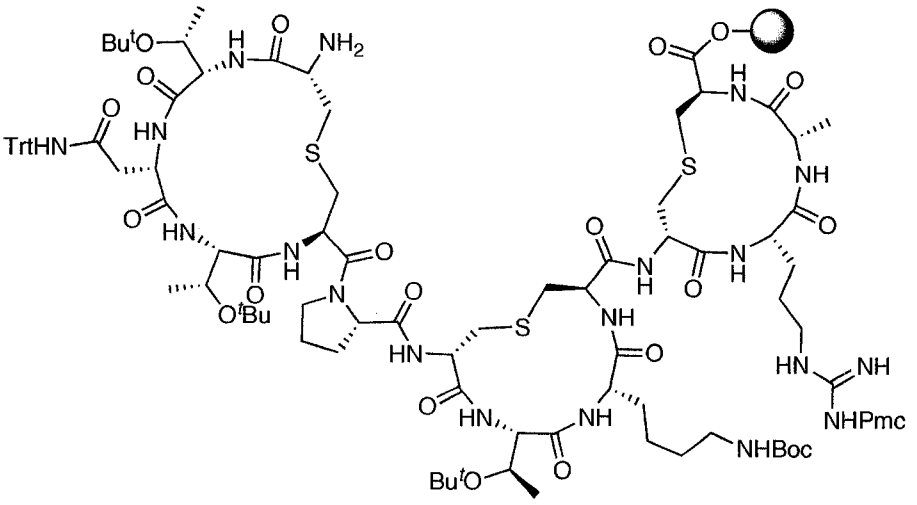
Cyclization to form lanthionine ring C **153** was done by adding a solution of PyBOP (0.83 g, 1.60 mmol, 5 eq. to resin), HOBt (0.22 g, 1.60 mmol, 5 eq. to resin) and NMM (0.35 mL, 1.92 mmol, 10 eq. to resin) in DMF (20 mL) to deprotected linear fragment and cyclized for 2 h. A small sample (~5 mg) of resin bound **153** was cleaved using (95:2.5:2.5) TFA:TIPS:H_2O for 2 h and filtered to remove the resin. The filtrate was concentrated *in vacuo* and precipitation with Et_2O yields the cleaved deprotected ring C **153** as an off-white solid; ES-MS Calcd for $C_{15}H_{27}N_7O_5S$ 417.2, found 418.2 (M+H). None of the diketopiperazine dimer or the linear precursor to ring C was detected by MS.

Synthesis of lanthionine ring B (171)



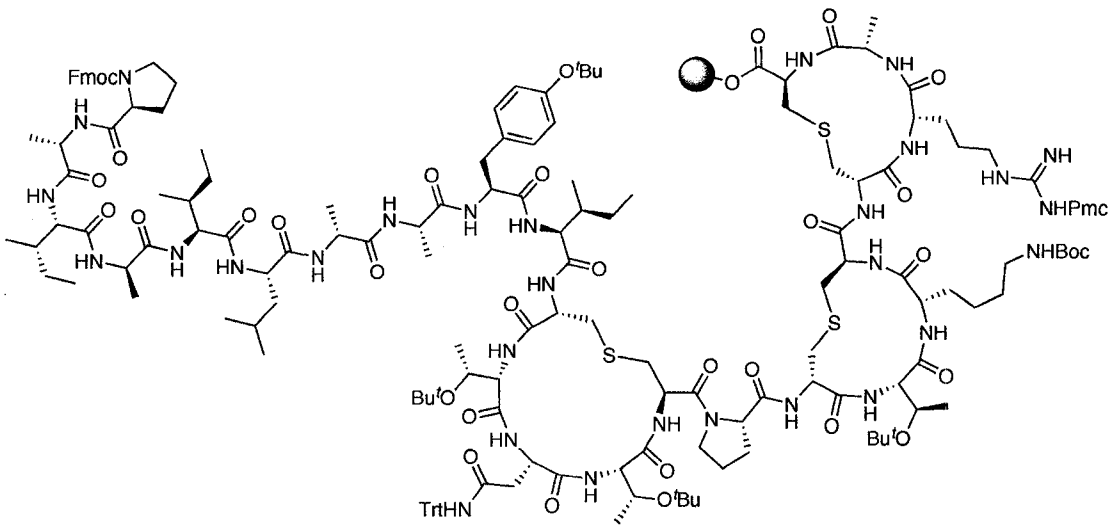
Fmoc SPPS was continued on resin bound **153** using PyBOP to couple protected amino acids (23→25) in the following order: Orthogonal lanthionine **167**, Fmoc-Lys(Boc)-OH, Fmoc-Thr(ᵗBu)-OH to give **170**. Aloc / allyl and Fmoc protecting groups of **170** were removed according to the procedure outlined to make ring C. Cyclization to give **171** was done with PyBOP similar to ring C, however, longer coupling times (3 x 2.5 h) were required to drive the reaction to completion. A small amount of resin (~11 mg) was cleaved with (95:2.5:2.5) TFA:TIPS:H_2O and analyzed by MALDI-TOF MS. Only a very weak signal was observed for the cleaved deprotected **171**. Hence, another small sample of the resin (~10 mg) was coupled with residue 21, Fmoc-Pro-OH using PyBOP, cleaved and analyzed; calcd $C_{51}H_{71}N_{13}O_{13}S_2$ 1137.5, found 1138.4 (M+H). None of the open form or the diketopiperazine dimer was observed by MS.

Synthesis of lanthionine ring A (172)



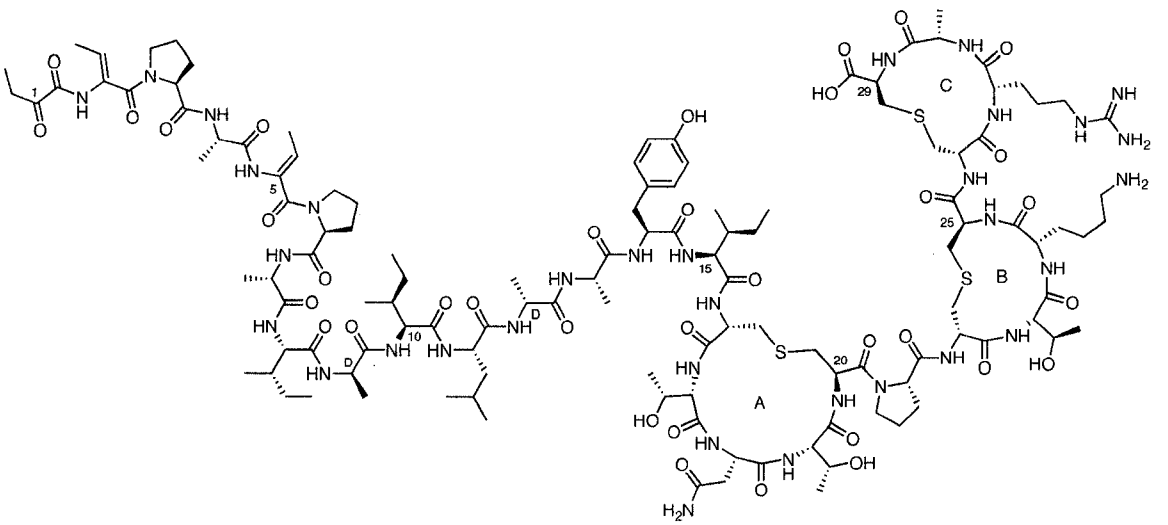
The residues (17→21) required for the linear portion of ring A were introduced under standard SPPS conditions using PyBOP in the order: Fmoc-Pro-OH, orthogonal lanthionine **167**, Fmoc-Thr(ᵗBu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Thr(ᵗBu)-OH. Removal of Aloc / allyl and Fmoc protecting groups and cyclization (3 x 2.5 h, PyBOP) was performed similar to ring C to give **172**. A small sample of the resin (~15 mg) was coupled with Fmoc-Ile-OH, cleaved using TFA and subjected to MALDI-TOF MS analysis: Calcd for $C_{75}H_{110}N_{20}O_{22}S_3$ 1738.7, found 1739.0 (M+H).

N-(9H-Fluorenylmethoxycarbonyl)-L-prolyl-L-alanyl-L-isoleucinyl-D-alanyl-L-isoleucinyl-L-leucinyl-D-alanyl-L-alanyl-L-tyrosinyl-L-isoleucinyl-D-(α-aminobutyrl)-L-threonyl-L-asparaginyl-L-threonyl-L-(α-aminobutyrl)-L-prolyl-D-(α-aminobutyrl)-L-threonyl-L-lysyl-L-(α-aminobutyrl)-D-(α-aminobutyrl)-L-arginyl-L-alanyl-L-(α-aminobutyrine) [(16→20), (22→25), (26→29)] D,L-lanthionine (173)



Fmoc-SPPS was done in DMF using PyBOP to couple amino acids (6→15) in the sequence: Fmoc-Ile-OH, Fmoc-Tyr(^{t}Bu)-OH, Fmoc-Ala-OH, Fmoc-D-Ala-OH, Fmoc-Leu-OH, Fmoc-Ile-OH, Fmoc-D-Ala-OH, Fmoc-Ile-OH, Fmoc-Ala-OH, Fmoc-Pro-OH. Cleavage of a small sample of the resin using (95:2.5:2.5) TFA:TIPS:H_2O at rt for 3 h, followed by precipitation with Et_2O gave **173** as an off-white solid. MALDI-TOF MS Calcd for $C_{119}H_{179}N_{29}O_{32}S_3$ 2622.2, found 2622.8 (M+H).

2-Oxobutanyl-*Z*-didehydrobutyrl-L-prolyl-L-alanyl-*Z*-didehydrobutyrl-L-prolyl-L-alanyl-L-isoleucinyl-D-alanyl-L-isoleucinyl-L-leucinyl-D-alanyl-L-alanyl-L-tyrosinyl-L-isoleucinyl-D-(α-aminobutyrl)-L-threonyl-L-asparaginyl-L-threonyl-L-(α-aminobutyrl)-L-prolyl-D-(α-aminobutyrl)-L-threonyl-L-lysyl-L-(α-aminobutyrl)-D-(α-aminobutyrl)-L-arginyl-L-alanyl-L-(α-aminobutyrine) [(16→20), (22→25), (26→29)] lanthionine (44)



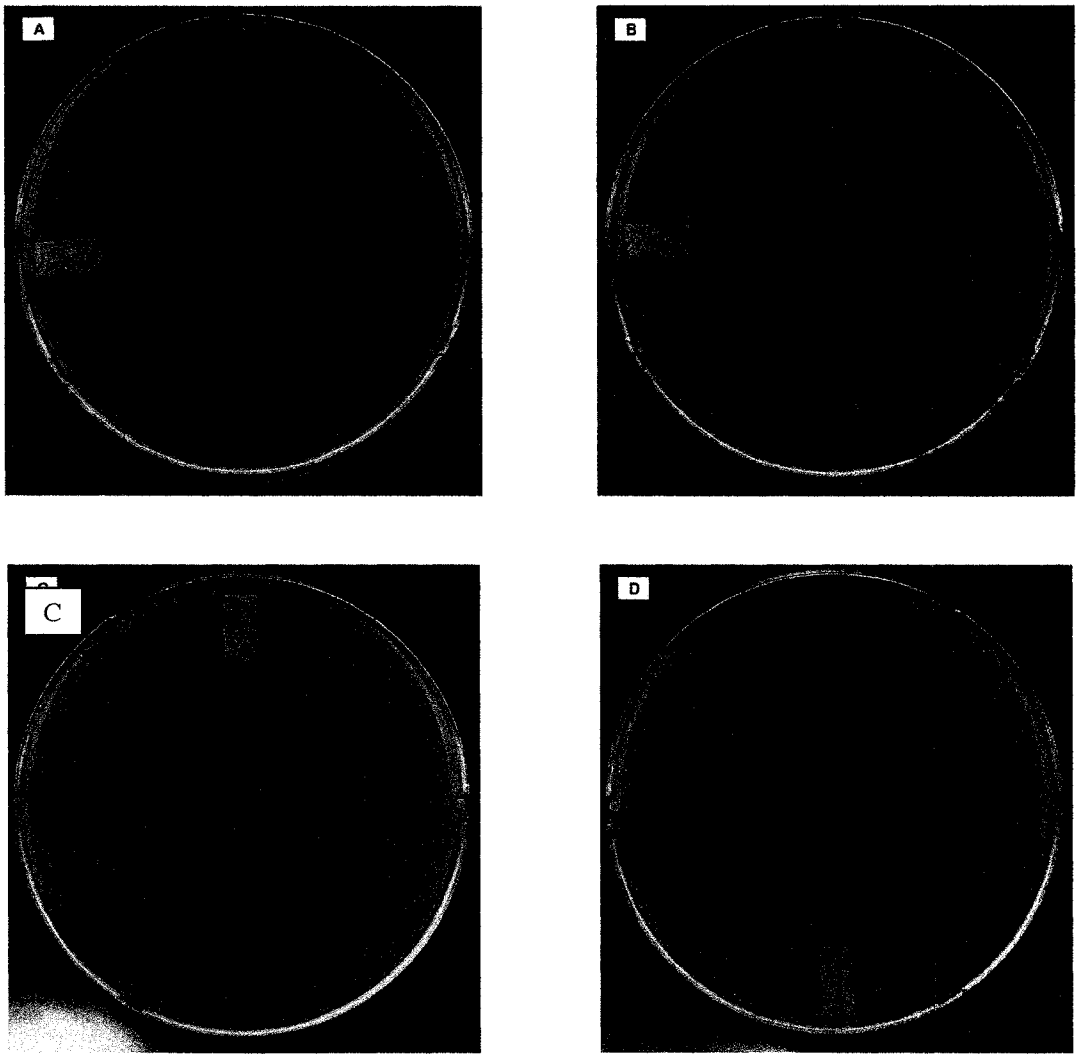
Fmoc protection of resin bound **173** (250 mg, 0.032 mmol) was removed using 20% piperidine in DMF and the resin was washed with DMF (3 x 15 mL) to remove any traces of piperidine. In a separate flask, the pentapeptide **45** (28 mg, 0.064 mmol) was dissolved in DMF (5 mL) and to it HOBt (8.6 mg, 0.064 mmol) and NMM (21 μL, 0.188 mmol) was added followed by DIPCDI (9.1 μL, 0.063 mmol) and reacted for 5 min. The pre-activated solution was transferred to peptide on resin and coupled for 3 h. A sample of resin (90 mg) was cleaved with (97.5:2.5) TFA:TIPS for 3 h. Concentration of the filtrate in vacuo, followed by precipitation with Et_2O gave crude **44** as an off-white solid. It was further purified by reverse phase HPLC using a steel walled semi-preparative C18 column (Vydac Protein & peptide C18 (218TP510), 5 μM, 10 x 250 mm). The crude peptide was dissolved in (2:8) MeCN:0.1% TFA to give a concentration of 1 mg / mL. In the method employed, 90 μL of the crude sample was injected with water (containing 0.1% TFA) and acetonitrile (MeCN) as the eluants. HPLC method: Flow rate 2 mL / min, detection at 220 and 254 nm. Gradient: Starting from 20% MeCN for 5 minutes and 1st ramp to 50% over 15 min, 2nd ramp to 70% MeCN over 3 minutes, followed by ramping down to 20% MeCN over 1 min, then 20% MeCN for 4 min. The desired product peak

44 was collected as a broad peak (t_R = 26.7 min). The fractions containing the product were concentrated *in vacuo* and lyophilized to give **44** (1.01 mg, overall yield = 1.3% [22 coupling steps and 3 cyclizations]) as a fluffy white solid. Monoisotopic MW Calcd for $C_{124}H_{195}N_{33}O_{36}S_3$ 2818.3605, found *high resolution* (FTICR-MALDI-MS) 2819.36774 (M+H).

Biological evaluation of lanthionine analogue of lacticin A2 (44)

(A) Synergistic activity assay: Standard literature protocol[93, 186] was followed for testing the antimicrobial activity of **44** and its parent natural peptides lacticin A1 & A2. M17 agar plates (supplemented with 10% lactose) were overlaid with soft agar, seeded with the indicator organism *L. lactis* subsp. *cremoris* HP or the bacterium under investigation (100 μL fully grown culture per 10 mL soft agar). 10 μL of lanthionine analogue of lacticin A2 **44**, the parent lacticin 3147 peptides A1 (**11**) and A2 (**12**) dissolved in MQ-H_2O / ACN and were spotted along with a negative control and allowed to dry. The plates were then incubated at 30 °C overnight and the antibacterial activity was determined by a zone of inhibition (Figure 32).

Figure 32. Synergistic biological activity of **44** and **173**

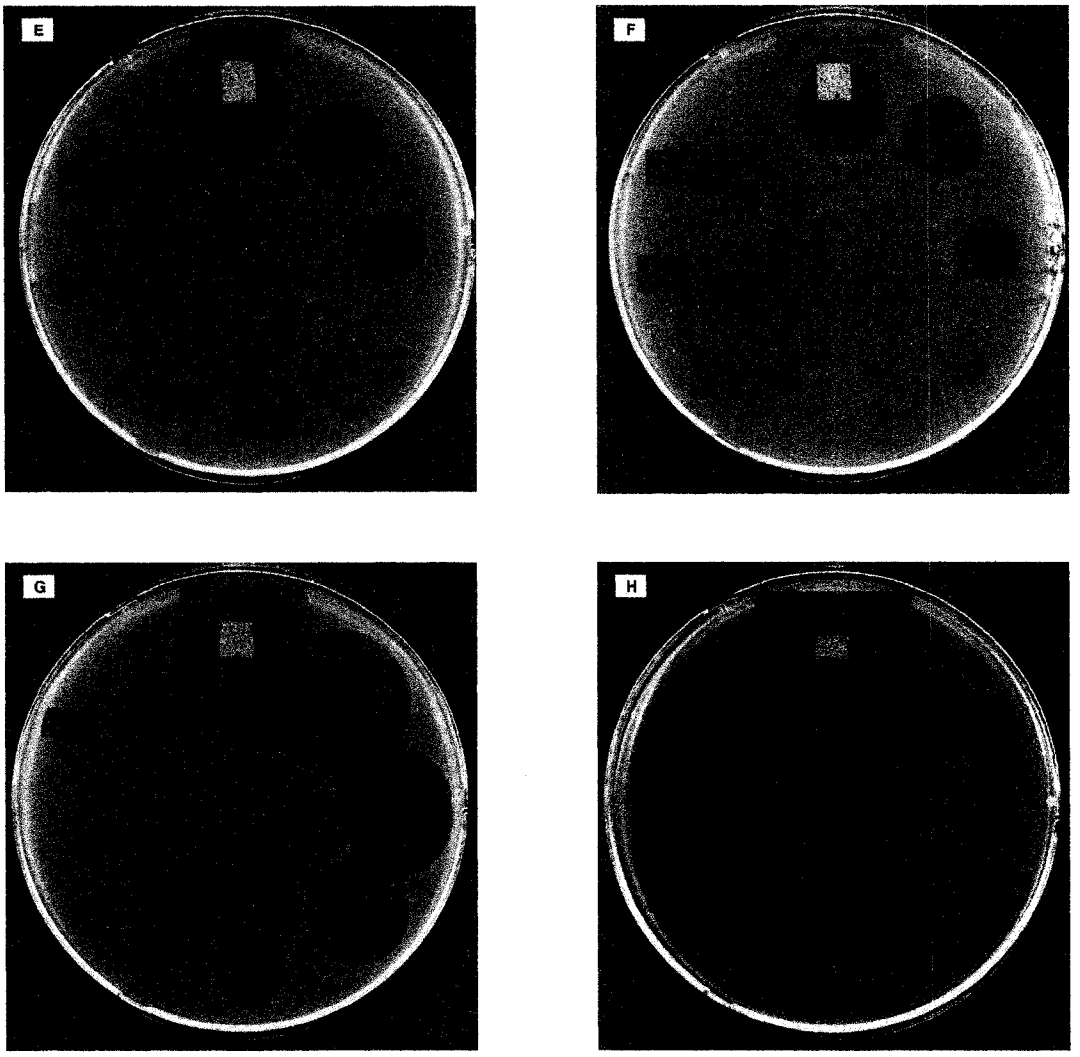


(**A**) Illustration of synergistic activity between (i) Natural lacticin A1 & A2 (ii) Lacticin A1 & analogue **44** (**B**) Synergistic activity between (i) Lacticin A1 & A2 (ii) Lacticin A1 & peptide **173** with Fmoc (iii) Lacticin A1 & peptide **173** without Fmoc (**C**) Synergistic activity between lacticin A1 & **44** at differing distances (**D**) Synergistic activity between lacticin A1 & peptide **173** with Fmoc (left) and without an Fmoc group right) at differing distance.

Synergistic biological activity of compound **173** with and without an Fmoc group (B) was done in a similar manner to that described for testing of **44**. C and D shows the synergistic biological activity of lanthionine analogue (**44**) and compound **173** with and without an Fmoc group at differing distances from natural lacticin A1.

(B) 10-fold serial dilution assay: A serial dilution assay was done by mixing natural lacticin A1 and lanthionine analogue of lacticin A2 (**44**) in a 1:1 ratio to give a concentration of 100 μM that was further diluted down to 1 nM with MQ-water. 10 μL of the mixture was spotted on M17 agar plates containing the indicator organism L. lactis subsp. cremoris HP (100 μL fully grown culture per 10 mL soft agar). The plates were then incubated at 30 °C overnight and the antibacterial activity was determined by a zone of inhibition (Figure 33)

Figure 33. Serial dilution assay for determining anti-bacterial activity of **44**



(**E**) 10-fold serial dilution of lacticin A1 starting at a concenration of 100 μM (**F**) 10-fold serial dilution of lacticin A2 starting at a concenration of 100 μM (**G**) 10-fold serial dilution of a (1:1) mixture of natural lacticin A1 & lacticin A2 starting at a concenration of 100 μM (**H**) 10-fold serial dilution of a (1:1) mixture of natural lacticin A1 & **44** starting at a concenration of 100 μM.

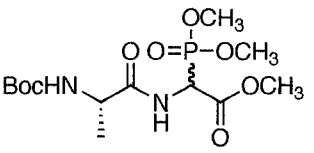
As a control, serial dilution assay of natural lacticin A1 by itself (Figure 33E), natural lacticin A2 by itself (Figure 33F) and a mixture of natural lacticin A1 and A2

(Figure 33G) was done in a similar manner to that described above for a mixture of lanthionine analogue 44 and natural lacticin A1.

4.2.3. Synthesis of dehydrovaline and oxazole analogues of residues 1 to 5 of lacticin 3147 A2

The experiments described in this section were done by Shaun McKinnie (Summer 2006, Vederas Group).

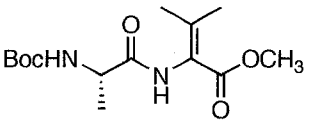
Boc-alanine-phosphonoglycine trimethyl ester (175)



This known compound was prepared by a modified literature procedure.[163] To a solution of D,L-Boc-α-phosphonoglycine trimethyl ester (Boc-Phg-OH, **174**) (0.50 g, 1.68 mmol) in DCM (10 mL), TFA (10 mL) was added and the resulting mixture was stirred for 1 h at 0 °C. The solvents were removed *in vacuo* and dried to give the TFA salt in quantitative yield. In a separate Erlenmayer flask, Boc-Ala-OH (**99**) (0.32 g, 1.68 mmol) was pre-activated by addition of PyBOP (0.87 g, 1.68 mmol) and NMM (1.85 mL, 16.82 mmol) in DCM (5 mL). The activated amino acid solution was then added to the TFA salt in CH_2Cl_2 (20 mL) and reacted for 24 h. The reaction mixture was washed with 10% $NaHCO_3$ (25 mL), 10% citric acid (25 mL) and water (25 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo* to obtain the crude product that was further purified by flash chromatography (SiO_2, 9:1/EtOAc:hexanes) to yield **175** (0.38 g, 60%) as a white solid. (R_f 0.40 on SiO_2, 100% EtOAc); IR ($CHCl_3$ cast) 3287, 2978, 1752, 1680, 1519 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 7.15 (br s, 1H, Phg-NH),

5.20 (ddd, 1H, J = 22.3 Hz, 9.0 Hz, 6.5 Hz, Phg-CH), 5.06-5.00 (m, 1H, Ala-NH), 4.30-4.18 (m, 1H, Ala-H_α), 3.84-3.79 (m, 9H, $COOCH_3$, $POCH_3$), 1.45 (s, 9H, ^{t}Bu), 1.37 (d, 3H, J = 7 Hz, Ala-CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.5 (C=O), 166.8 (C=O), 155.3 (C=O), 80.3 (C), 54.2 (OCH_3), 54.0 (OCH_3), 53.3 (OCH_3), 50.8 (CH), 50.1 & 49.3 (CH), 28.2 (CH_3), 18.0 (CH_3); HRMS (ES) Calcd for $C_{13}H_{25}N_2O_8PNa$ 391.1241, found 391.1243.

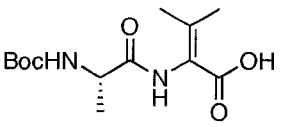
(*S*)-Methyl 2-(2-(*tert*-butoxycarbonylamino)propanamido)-3-methylbut-2-enoate (176)



This known compound was prepared by a modified literature procedure.[163] DBU (0.64 mL, 4.30 mmol) was added dropwise to a solution of **175** (1.67 g, 4.52 mmol) in acetone (50.0 mL) and stirred for 40 h at 25 °C. After this time, TLC indicated the presence of starting material. To force the reaction to completion more DBU (0.33 mL, 2.26 mmol) was added and stirred for an additional 19 h at 25 °C. The solvents were removed *in vacuo* and purified by flash chromatography (SiO_2, 1:1.5/EtOAc:hexanes) to yield **176** (1.19 g, 87%) as a white solid. (R_f 0.65 on SiO_2, 100% EtOAc); $[\alpha]_D^{26}$ -57.87° (*c* 0.5, $CHCl_3$); IR ($CHCl_3$ cast) 3301, 2980, 1721, 1513 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.48 (br s, 1H, Dhv-NH), 5.05 (d, 1H, J = 5.7 Hz, Ala-NH), 4.31-4.18 (m, 1H, Ala-H_α), 3.72 (s, 3H, OCH_3), 2.15 (s, 3H, *cis*-Dhv-CH_3), 1.83 (s, 3H, *trans*-Dhv-CH_3), 1.45 (s, 9H, ^{t}Bu), 1.39 (d, 3H, J = 7.2 Hz, Ala-CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 171.3 (C=O), 165.1 (C=O), 155.7 (C=O), 145.9 (C), 120.7 (C), 80.2 (C), 51.7 (OCH_3), 49.9 (CH), 28.3

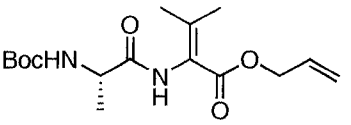
(CH_3), 22.3 (CH_3), 21.2 (CH_3), 17.8 (CH_3); HRMS (ES) Calcd for $C_{14}H_{24}N_2O_5Na$ 323.1577, found 323.1576.

(*S*)-2-(2-(*tert*-Butoxycarbonylamino)propanamido)-3-methylbut-2-enoic acid (178)



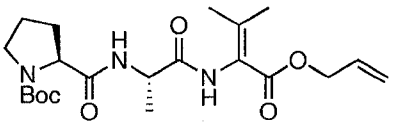
This known compound was prepared according to the procedure by Mahak *et al.*[188] A solution of **176** (1.22 g, 4.04 mmol) in dioxane (40.0 mL) and 1 N LiOH (40.0 mL) was stirred at 25 °C for 3 h. The solvents were removed under vacuum and the resulting salt was dissolved in water (50 mL) and acidified to pH 1 with 6 M HCl at 0 °C. The crude product was extracted from the aqueous layer with EtOAc (3 x 40 mL), dried over Na_2SO_4, filtered and concentrated *in vacuo*. The desired product **178** (1.20 g, quant. yield) was obtained as a white solid. (R_f 0.01 on SiO_2, 100% EtOAc); $[\alpha]_D^{26}$ -41.35° (*c* 0.99, $CHCl_3$); IR ($CHCl_3$ cast) 3296, 2980, 2934, 1700, 1517 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.61 (br s, 1H, Dhv-NH), 5.15-5.04 (m, 1H, Ala-NH), 4.33-4.22 (m, 1H, Ala-H_α), 2.21 (s, 3H, *cis*-Dhv-CH_3), 1.87 (s, 3H, *trans*-Dhv-CH_3), 1.46 (s, 9H, ^{t}Bu), 1.42 (d, 3H, J = 7.2 Hz, Ala-CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 172.3 (C=O), 168.1 (C=O), 155.9 (C=O), 149.2 (C), 120.5 (C), 80.4 (C), 49.9 (CH), 28.3 (CH_3), 22.8 (CH_3), 21.5 (CH_3), 17.9 (CH_3) ; HRMS (ES) Calcd for $C_{13}H_{22}N_2O_5Na$ 309.1421, found 309.1418.

(*S*)-Allyl 2-(2-(*tert*-butoxycarbonylamino)propanamido)-3-methylbut-2-enoate (179)



To a solution of the dipeptide **178** (0.70 g, 2.43 mmol) in DMF (100 mL), Na_2CO_3 (0.52 g, 4.87 mmol) was added followed by water (2.5 mL) and stirred for 5 minutes. Allyl bromide (**75**) (0.25 mL, 2.92 mmol) was then added to the reaction mixture and stirred for 4 h. The solvents were removed from the reaction mixture under vacuum and the crude product was dissolved in EtOAc (80 mL), washed with water (2 x 40 mL) and brine (40 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo* to obtain compound **179** (0.60 g, 75%) as a white solid. (R_f 0.65 on SiO_2, 100% EtOAc); $[\alpha]_D^{26}$ -44.92° (*c* 0.97, $CHCl_3$); IR ($CHCl_3$ cast) 3296, 2979, 2934, 1717, 1684, 1669, 1506 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 7.55 (br s, 1H, Dhv-NH), 5.91 (ddt, 1H, *J* = 12.0, 11.0, 5.5 Hz, =CH), 5.25 (m, 2H, $=CH_2$), 5.11 (d, 1H, *J* = 6.0 Hz, Ala-NH), 4.66-4.58 (m, 2H, OCH_2), 4.32-4.20 (m, 1H, Ala-H_α), 2.15 (s, 3H, *cis*-Dhv-CH_3), 1.83 (s, 3H, *trans*-Dhv-CH_3), 1.43 (s, 9H, ^{t}Bu), 1.38 (d, 3H, *J* = 7.5 Hz, Ala-CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 171.4 (C=O), 164.2 (C=O), 155.6 (C=O), 146.1 (C), 132.1 (CH), 120.8 (C), 118.3 (CH_2), 80.2 (C), 65.4 (CH_2), 49.9 (CH), 28.3 (C), 22.4 (CH_3), 21.2 (CH_3), 17.9 (CH_3); HRMS (ES) Calcd for $C_{16}H_{26}N_2O_5Na$ 349.1734, found 349.1733.

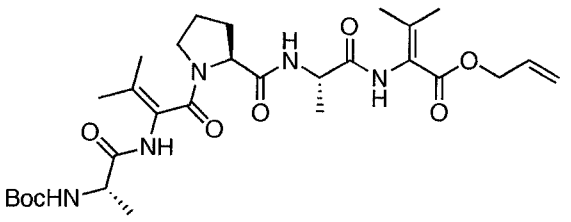
(*S*)-*tert*-Butyl 2-((*S*)-1-(1-(allyloxy)-3-methyl-1-oxobut-2-en-2-ylamino)-1-oxopropan-2-ylcarbamoyl)pyrrolidine-1-carboxylate (177)



To a solution of compound **179** (0.56 g, 1.73 mmol) in DCM (30.0 mL), TFA (30.0 mL) was added and stirred for 2 h at 0 °C. The solvents were removed *in vacuo* and dried to give the TFA salt in quantitative yield. In a separate Erlenmayer flask, Boc-Pro-OH (**129**)

(0.37 g, 1.73 mmol) was pre-activated by addition of PyBOP (0.90 g, 1.73 mmol) and NMM (1.90 mL, 17.28 mmol) in DCM (5 mL). The activated amino acid solution was then added to the TFA salt in DCM (90 mL) solution and stirred for 12 h. The solvent was removed *in vacuo* and the crude product was purified by flash chromatography (SiO_2, 4:1/EtOAc:hexanes) to yield **177** (0.73 g, quantitative yield) as a white solid. (R_f 0.40 on SiO_2, 100% EtOAc); $[\alpha]_D^{26}$ -97.53° (*c* 1.01, $CHCl_3$); IR ($CHCl_3$ cast) 3278, 2977, 1700, 1684, 1653, 1558, 1539 cm^{-1}; ^1H NMR (DMSO, 500 MHz, 100 °C) δ 8.74 (br s, 1H, Dhv-NH), 7.58 (br s, 1H, Ala-NH), 5.93-5.86 (m, 1H, =CH), 5.29, (dd, 1H, *J* = 17.0, 1.5 Hz, =CH*H*), 5.17 (dd, 1H, *J* = 10.5, 1.2 Hz, =C*H*H), 4.55 (d, 2H, *J* = 5.0, 1.2 Hz, OCH_2), 4.39 (app pent, 1H, *J* = 7.5 Hz, Ala-H_α), 4.14 (dd, 1H, *J* = 8.3, 3.3 Hz, Pro-H_α), 3.40-3.29 (m, 2H, Pro-H_δ) 2.11-2.05 (m, 2H, Pro-H_β), 2.03 (s, 3H, *cis*-Dhv-CH_3), 1.91-1.79 (m, 2H, Pro-H_γ), 1.77 (s, 3H, *trans*-Dhv-CH_3), 1.38 (s, 9H, ^{t}Bu), 1.28 (d, 3H, *J* = 7.0 Hz, Ala-CH_3); ^{13}C NMR (DMSO, 125 MHz, 100 °C) δ 172.6 (C=O), 171.8 (C=O), 164.7 (C=O), 154.5 (C=O), 142.6 (C), 133.5 (CH), 122.8 (C), 118.1 (CH_2), 79.4 (C), 65.1 (OCH_2), 60.4 (CH), 48.8 (CH), 47.3 (CH_2), 30.9 (CH_2), 28.9 (CH_3), 24.1 (CH_2), 22.0 (CH_3), 21.1 (CH_3), 18.7 (CH_3); HRMS (ES) Calcd for $C_{21}H_{33}N_3O_6Na$ 446.2262, found 446.2261.

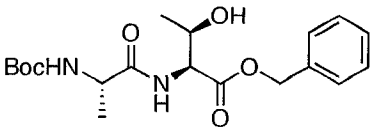
Allyl 2-((*S*)-2-((*S*)-1-(2-((*S*)-2-(*tert*-butoxycarbonylamino)propanamido)-3-methyl but-2-enoyl)pyrrolidine-2-carboxamido)propanamido)-3-methylbut-2-enoate (180)



To a solution of compound **177** (0.79 g, 1.86 mmol) in DCM (50.0 mL), TFA (50.0 mL) was added and stirred for 1 h. The solvents were removed *in vacuo* and dried, to give the TFA salt in quantitative yield. In a separate Erlenmayer flask, dipeptide **178** (0.50 g, 1.75 mmol) in CH_2Cl_2 (10 mL) was pre-activated with DIPCDI (0.26 mL, 1.75 mmol), HOBt (0.24 g, 1.75 mmol) and NMM (1.15 mL, 10.48 mmol). The activated amino acid solution was then added to the TFA salt solution (60 mL) and the reaction mixture was stirred for 16 h at 25 °C. The reaction mixture was filtered to remove the diisopropyl urea and the filtrate was concentrated *in vacuo* and purified by flash chromatography (SiO_2, 100% EtOAc) to yield **180** (0.26 g, 23%) as yellow oil. (R_f 0.7 on SiO_2, 3:7/acetone:EtOAc); $[\alpha]_D^{26}$ -185.43° (*c* 1.00, $CHCl_3$); IR ($CHCl_3$ cast) 3297, 2980, 2937, 1717, 1668, 1623, 1506 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 8.35-8.28 (m, 2H, 2 x Dhv-NH), 7.99 (d, 1H, J = 8.5 Hz, Ala-NH), 6.08 (d, 1H, J = 7.5 Hz, Ala-NH), 5.94 (ddt, 1H, J = 12.0, 11.0, 5.9 Hz, =CH), 5.37-5.31 (m, 1H, =CH*H*), 5.24-5.20 (m, 1H, =C*H*H), 4.71-4.61 (m, 2H, OCH_2), 4.56 (app pent, 1H, J = 7.5 Hz, Ala-H_α), 4.50 (dd, 1H, J = 9.0, 3.5 Hz, Pro-H_α), 4.32 (app pent, 1H, J = 7.5 Hz, Ala-H_α), 3.89 (ddd, 1H, J = 10.6, 7.4, 3.4 Hz, Pro-NCH*H*), 3.50-3.41 (m, 1H, Pro-NC*H*H), 2.32-2.19 (m, 2H, Pro-H_β), 2.09 (s, 3H, *cis*-Dhv-CH_3), 2.02-1.92 (m, 2H, Pro-CH_γ), 1.88 (s, 3H, *cis*-Dhv-CH_3), 1.74 (s, 6H, 2 x *trans*-Dhv-CH_3), 1.52 (d, 3H, J = 7.5 Hz, internal Ala-CH_3), 1.43 (s, 9H, ^{t}Bu), 1.34 (d, 3H, J = 7.0 Hz, terminal Ala-CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 172.3 (C=O), 172.0 (C=O), 171.2 (C=O), 167.3 (C=O), 165.5 (C=O), 157.0 (C=O), 146.1 (C), 132.2 (CH), 125.1 (C), 123.0 (C), 121.5 (C), 118.5 (CH_2), 80.5 (C), 65.8 (CH_2), 61.1 (CH), 49.3 (CH), 49.2 (CH), 48.6 (CH_2), 30.1 (CH_2), 28.3 (CH_3), 24.3 (CH_2), 22.5 (CH_3), 21.8 (CH_3), 19.6

(CH_3), 18.2 (CH_3), 16.7 (CH_3), 15.6 (CH_3); HRMS (ES) Calcd for $C_{29}H_{45}N_5O_8Na$ 614.3160, found 614.3164.

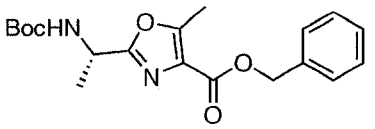
(2*S*,3*R*)-Benzyl 2-((*S*)-2-(*tert*-butoxycarbonylamino)propanamido)-3-hydroxy butanoate (185)



This known compound was prepared by a modified literature procedure.[189] To a solution of L-threonine benzyl ester oxalate (**123**) (8.81 g, 29.43 mmol) in DCM (250.0 mL), NMM (8.09 mL, 73.57 mmol) was added and stirred for 10 minutes. In a separate Erlenmayer flask, Boc-Ala-OH (**99**) (5.57 g, 29.43 mmol) in DCM (20 mL) was pre-activated by addition of PyBOP (15.31 g, 29.43 mmol) and NMM (3.88 mL, 35.31 mmol). The activated amino acid solution is added to the free amine in CH_2Cl_2 and stirred for 40 h. The reaction mixture was washed with 10% citric acid solution (20 mL), water (20 mL), and brine (20 mL). The separated organic layer was dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO_2, 1:4/EtOAc:hexanes) to yield **185** (11.00 g, 98%) as a sticky white solid. (R_f 0.85 on SiO_2, 100% EtOAc); $[\alpha]_D^{26}$ -14.42° (*c* 1.0, $CHCl_3$); IR ($CHCl_3$ cast) 3338, 2978, 2934, 1744, 1668, 1521 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.39-7.32 (m, 5H, Ar-CH), 6.81 (d, 1H, *J* = 8.7 Hz, Thr-NH), 5.23 (d, 1H, *J* = 12.3 Hz, CH*H*), 5.18 (d, 2H, *J* = 12.3 Hz, *CH*H), 5.04 (d, 1H, *J* = 6.6 Hz, Ala-NH), 4.64 (dd, 1H, *J* = 9.0, 2.4 Hz, Thr-H_α), 4.36 (dq, 1H, *J* = 6.5, 2.5 Hz, Thr-H_β), 4.24-4.14 (m, 1H, Ala-CH), 1.90 (br s, 1H, Thr-OH), 1.45 (s, 9H, ^{t}Bu), 1.37 (d, 3H, *J* = 7.2 Hz, Ala-CH_3), 1.20 (d, 3H, *J* = 6.6 Hz, Thr-CH_3); ^{13}C

NMR ($CDCl_3$, 100 MHz) δ 173.4 (C=O), 170.6 (C=O), 155.6 (C=O), 135.1 (C), 128.5 (C=H), 128.3 (C=H), 128.1 (C=H), 126.5 (C=H), 126.0 (C=H), 80.1 (C), 68.1 (CH_2), 67.2 (CH), 57.5 (CH), 50.1 (CH), 28.2 (CH_3), 19.8 (CH_3), 18.2 (CH_3); HRMS (ES) Calcd for $C_{19}H_{28}N_2O_6Na$ 403.1840, found 403.1844.

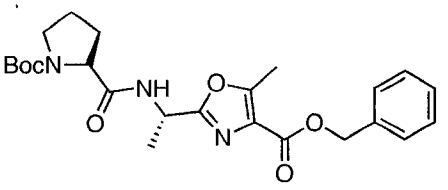
(*S*)-Benzyl 2-(1-(*tert*-butoxycarbonylamino)ethyl)-5-methyloxazole-4-carboxylate (186)



To a solution of dipeptide **185** (7.00 g, 18.40 mmol) in DCM (200.0 mL) at 0 °C, a 15 weight% Dess-Martin periodinane solution (54.63 mL, 19.32 mmol) was added dropwise via an addition funnel. The reaction mixture was warmed to rt and stirred for 2 h. It was then immediately loaded onto a basic alumina (Aluminum Oxide 150 basic Type T, 63-200 mesh, Merck) column and filtered through with DCM (10 x 50 mL). The filtrate was directly collected in a 1 L round bottomed flask containing a mixture of triphenylphosphine (9.65 g, 36.80 mmol), iodine (9.34 g, 36.80 mmol) and triethylamine (10.26 mL, 73.61 mmol) in DCM (20 mL). The reaction was stirred for 18 h and the solvent was removed *in vacuo* and the crude was purified by flash chromatography (SiO_2, 1:9/EtOAc:hexanes) to yield **186** (2.80 g, 37%) as an off-white solid. (R_f 0.6 on SiO_2, 3:7 EtOAc:hexanes); $[\alpha]_D^{26}$ -35.93° (*c* 1.0 , $CHCl_3$); IR ($CHCl_3$ cast) 3341, 2978, 1712, 1649, 1641, 1620, 1572, 1547, 1514, 1502 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.46-7.32 (m, 5H, Ar-CH), 5.36 (m, 2H, OCH_2), 5.19 (br s, 1H, Ala-NH), 4.98-4.87 (m, 1H, Ala-CH), 2.58 (s, 3H, oxazole-CH_3), 1.52 (d, 3H, J = 6.9 Hz, Ala-CH_3), 1.44 (s, 9H, ^{t}Bu); ^{13}C NMR

($CDCl_3$, 100 MHz) δ 162.9 (C=O), 161.9 (C=N), 156.4 (C=O), 154.8 (C=O), 135.6 (C), 128.7 (CH), 128.4 (CH), 128.3 (CH), 128.2 (CH), 127.2 (C), 80.0 (C), 66.4 (CH_2), 44.5 (CH), 28.2 (CH_3), 20.1 (CH_3), 12.1 (CH_3); HRMS (ES) Calcd for $C_{19}H_{24}N_2O_5Na$ 383.1577, found 383.1579.

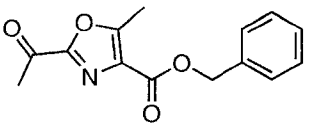
Benzyl 2-((*S*)-1-((*R*)-1-(*tert*-butoxycarbonyl)pyrrolidine-2-carboxamido)ethyl)-5-methyloxazole-4-carboxylate (187)



To a solution of compound **186** (1.20 g, 3.33 mmol) in DCM (20.0 mL), TFA (20.0 mL) was added and stirred for 1.5 h at 0 °C. The solvents were removed *in vacuo* and dried, to give the TFA salt in quantitative yield. In a separate Erlenmayer flask, Boc-Pro-OH (0.72 g, 3.33 mmol) in CH_2Cl_2 (5 mL) was pre-activated by the addition of PyBOP (1.73 g, 3.33 mmol) and NMM (0.44 mL, 4.00 mmol). The pre-activated amino acid solution was added to the TFA salt and stirred for 16 h. The solvent was removed *in vacuo* to obtain the crude product, which was further purified by flash chromatography (SiO_2, 6:4/EtOAc:hexanes) to yield **187** (1.10 g, 72%) as a sticky yellow solid. (R_f 0.5 on SiO_2, 100% EtOAc); $[\alpha]_D^{26}$ -78.66° (*c* 1.15, $CHCl_3$); IR ($CHCl_3$ cast) 3303, 2978, 2878, 1698, 1587, 1500 cm^{-1}; ^1H NMR (DMSO, 500 MHz, 100 °C) δ 8.09 (d, 1H, *J* = 6.0 Hz, Ala-NH), 7.42-7.32 (m, 5H, Ar-CH), 5.30 (app t, 2H, *J* = 13.0 Hz, OCH_2), 5.03 (app pent, 1H, *J* = 7.0 Hz, Ala-H_α), 4.12 (dd, 1H, *J* = 8.3, 3.3 Hz, Pro-H_α), 3.40-3.27 (m, 2H, Pro-H_δ), 2.53 (s, 3H, CH_3-oxazole), 2.50-2.48 (m, 1H, Pro-H_β), 2.14-2.03 (m, 1H, Pro-$H_{\beta'}$), 1.87-

1.71 (m, 2H, Pro-H_γ), 1.45 (d, 3H, J = 7.0 Hz, Ala-CH_3), 1.36 (s, 9H, ^{t}Bu); ^{13}C NMR (DMSO, 125 MHz, 100 °C) δ 171.4 (C=O), 162.0 (C=O), 160.9 (N=C), 155.5 (C=O), 153.0 (C), 135.6 (C), 127.9 (CH), 127.5 (CH), 127.4 (CH), 126.3 (CN), 78.0 (C), 65.2 (CH_2), 59.2 (CH), 46.0 (CH), 41.9 (CH_2), 29.9 (CH_2), 27.6 (CH_3), 22.8 (CH_2), 17.9 (CH_3), 11.1 (CH_3); HRMS (ES) Calcd for $C_{24}H_{31}N_3O_6Na$ 480.2105, found 480.2108.

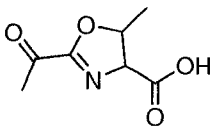
Benzyl 2-acetyl-5-methyloxazole-4-carboxylate (188)



To a solution of compound **186** (0.57 g, 1.58 mmol) in DCM (30.0 mL), TFA (30.0 mL) was added and stirred for 1 h. The solvent was removed *in vacuo* and dried, to give the TFA salt in quantitative yield. It was then taken up in CH_2Cl_2 (30 mL) and to it 4-pyridine carboxaldehyde (0.75 mL, 7.91 mmol) and AcOH (0.45 mL, 7.91 mmol) were added and stirred for 3 h. The reaction mixture was cooled to 0 °C and DBU (1.18 mL, 7.91 mmol) was added dropwise over 10 min. The resulting mixture was warmed to 25 °C and stirred for 16 h. To the reaction mixture 1 M HCl (50 mL) was added and further stirred for 3 h. The organic layer was separated and the aqueous layer was extracted with DCM (2 x 20 mL). The combined organic layers were washed with water (2 x 20 mL), dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO_2, 1.5:8.5/EtOAc:hexanes) to yield **188** (0.16 g, 40%) as a off-white solid (R_f 0.45 on SiO_2, 2:8 EtOAc:hexanes); IR ($CHCl_3$ cast) 2927, 1738, 1708, 1597, 1535 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.47-7.34 (m, 5H, Ar-CH), 5.41 (s, 2H, CH_2), 2.70 (s, 3H, CH_3), 2.69 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 185.5, (C=O),

161.2 (C=O), 159.5 (C), 153.0 (C), 135.3 (C), 128.6 (CH), 128.4 (CH + C), 128.4 (CH), 67.0 (OCH_2), 26.5 (CH_3), 12.5 (CH_3); HRMS (ES) Calcd for $C_{14}H_{13}NO_4Na$ 282.0737, found 282.0739.

2-Acetyl-5-methyl-4,5-dihydrooxazole-4-carboxylic acid (189)

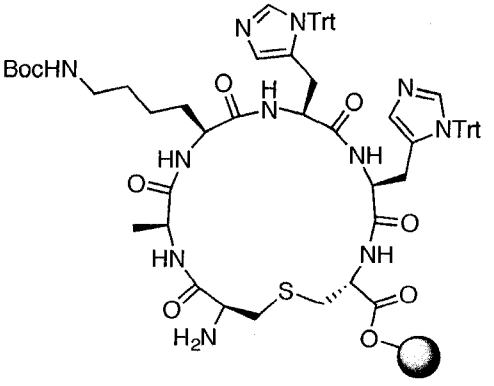


To a solution of **188** (0.36 g, 1.39 mmol) in MeOH (50.0 mL), 10% palladium on carbon (36 mg) was added and stirred under H_2 (1 atm) for 1 h at 25 °C. TLC indicated the presence of starting material and the reaction was continued for an additional 15 h. The reaction mixture was filtered through a pad of celite and the filtrate was evaporated *in vacuo* to yield oxazoline **189** (0.22 g, 93%) as an off-white solid. (R_f 0.01 on SiO_2, 100% EtOAc); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 4.97 (q, 1H, J = 6.6 Hz, C*H*CH_3), 3.21-3.00 (m, 1H, =NCH), 2.60 (s, 3H, $COCH_3$), 1.60 (d, 3H, J = 6.6 Hz, CH_3); LRMS (ES) Calcd for $C_7H_9NO_4Na$ 194.0, found 194.0.

4.2.4. Studies towards total synthesis of Lactocin S

(i) Attempted synthesis of ring B of lactocin S on 2-chlorotrityl chloride resin with 0.30 mmol/g resin loading

Attempted synthesis of lanthionine ring B of lactocin S (191)



a) Fmoc Solid Phase Peptide Synthesis (SPPS) conditions

General Procedure: Fmoc-amino acid (4.0 eq to resin loading) and HOBt (4.0 eq) were dissolved in DMF (10 mL) and to it, NMM (5.0 eq) was added followed by PyBOP (4.9 eq) and pre-activated for 5 min. The activated amino acid solution was then transferred to pre-swelled resin and reacted for 2 h. The completion of couplings were ascertained by negative Kaiser test[187] and end capping was performed with 20% Ac_2O in DMF for 10 min. The subsequent removal of the Fmoc group was done using 20% piperidine in DMF and monitored by the absorption of dibenzofulvene-piperidine adduct at $\lambda = 301$ nm on a UV-Vis spectrophotometer.

b) Loading of lanthionine **167** and SPPS to obtain **193**

2-Chlorotrityl chloride resin (1.00 g, 1.1 mmol/g) was pre-swelled in CH_2Cl_2 (20 mL) for 20 min. To it, orthogonally protected lanthionine **167** (0.166 g, 0.30 mmol) and DIPEA (0.26 mL, 1.5 mmol) in CH_2Cl_2 (20 mL) were added and reacted for 2 h. The resin was washed with CH_2Cl_2 (2 x 20 mL). The rest of the free sites on-resin after loading lanthionine were capped by reacting with a CH_3COOH (0.11 mL, 2 mmol) and DIPEA (1.40 mL, 8 mmol) mixture in CH_2Cl_2 (20 mL) for 2 h. The resin thus obtained with a low loading of 0.30 mmol/g of **167** was used for further synthesis. Fmoc SPPS was continued using PyBOP to couple Fmoc-His(Trt)-OH (residue 36) and Fmoc-His(Trt)-OH (residue 35), Fmoc-Lys(Boc)-OH (residue 34), Fmoc-Ala-OH (residue 33) to give linear fragment **193** on-resin.

c) Removal of Aloc/Allyl, Fmoc and cyclization on-resin to form ring B (**191**)

A solution of $Pd(PPh_3)_4$ (0.69 g, 0.60 mmol, 2 eq. to resin) and $PhSiH_3$ (0.74 mL, 3.0 mmol, 10 eq. to resin) in 1:1 DMF:CH_2Cl_2 (40 mL) was reacted with resin bound **193** protected from light for 2 h. The solution was drained and the resin was washed in the following sequence: a) CH_2Cl_2 (2 x 20 mL), b) 0.5% sodium diethyldithiocarbamate in DMF (3 x 20 mL) c) DMF (2 x 20 mL). The dark brown colored resin turned light yellow during the washings. The Fmoc group was removed with 20% piperidine in DMF to furnish **194**. The resin was again carefully washed with DCM (3 x 10 mL) and DMF (3 x 10 mL) to avoid the presence of any residual piperidine.

Cyclization to form lanthionine ring B **191** was done by adding a solution of PyBOP (0.78 g, 1.50 mmol, 5 eq. to resin), HOBt (0.20 g, 1.50 mmol, 5 eq. to resin) and NMM (0.33 mL, 3.00 mmol, 10 eq. to resin) in DMF (20 mL) to deprotected linear fragment **194** and cyclized for 2 h. A small sample (~5 mg) of resin bound **191** was cleaved using (95:2.5:2.5) TFA:TIPS:H_2O for 2 h and filtered to remove the resin. The filtrate was concentrated *in vacuo* and precipitation with Et_2O yields cleaved deprotected ring B **191** as an off-white solid; MALDI-TOF MS Calcd for $C_{27}H_{41}N_{11}O_7S$ 663.3, found 686.3 (M+Na). Fmoc SPPS was continued on resin bound **191** with PyBOP to couple Fmoc-Tyr(^{t}Bu)-OH to obtain **195**. Cleavage with (95:2.5.2.5) TFA:TIPS:H_2O followed by analysis by MALDI-TOF MS showed the presence of the desired product (1049.7, M+H) as well as the diketopiperazine dimer **196** (2097.9, M+H) formed by on-resin cyclization of the free amino and carboxyl functionalities of the lanthionine residue. The identity of the by-product **196** was further confirmed by LC-MS / MS analysis.

(ii) Attempted synthesis of lactocin S on 2-chlorotrityl chloride resin with 0.16 mmol/g loading

(a) General procedure: Manual Fmoc SPPS was done according to the general procedure above with the following modifications: Fmoc-amino acid (10.0 eq. to resin loading), HOBt (10.0 eq), NMM (10.0 eq) and PyBOP (9.8 eq). Automated Fmoc SPPS was performed on an ABI 433A peptide synthesizer using the method 'UV *Fastmoc*™ 0.1 mmol'. In the method employed, 10 equivalents of Fmoc-amino acids were used with

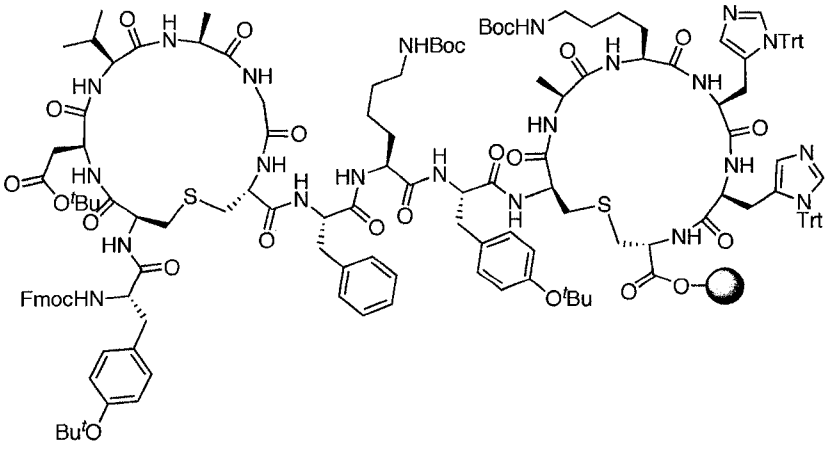
respect to the resin loading. Peptide couplings were done by mixing a solution of Fmoc-amino acid (in NMP) with HBTU/HOBt/DIPEA (in DMF) and pre-activated for 2.1 minutes. The activated solution is then transferred to the pre-swelled resin and allowed to react for 9.3 minutes. End capping is performed with a solution of Ac_2O/HOBt/DIPEA in NMP. Removal of Fmoc group is done by using 22% piperidine in NMP and monitored for completion based on the absorption of dibenzofulvene-piperidine adduct at λ = 301 nm on a UV-Vis spectrophotometer. The overall coupling cycle time for each amino acid is approximately 50 minutes.

(b) Loading of lanthionine **167** to 2-chlorotrityl chloride resin

2-Chlorotrityl chloride resin (2.00 g, 1.10 mmol / g) was pre-swelled in CH_2Cl_2 (20 mL) for 20 min. The resin was partially capped by reacting with CH_3COOH (86 μL, 1.50 mmol) and DIPEA (1.04 mL, 6.0 mmol) mixture in CH_2Cl_2 (20 mL) for 1.5 h. The resin was washed with CH_2Cl_2 (20 mL), followed by reaction with orthogonally protected lanthionine **167** (0.18 g, 0.32 mmol) and DIPEA (0.28 mL, 1.6 mmol) in CH_2Cl_2 (20 mL) for 2.5 h. The resin was again washed with CH_2Cl_2 (2 x 20 mL). The rest of the free sites on-resin after loading lanthionine were capped by reacting with CH_3COOH (0.26 mL, 4.50 mmol) and DIPEA (3.12 mL, 18.00 mmol) mixture in CH_2Cl_2 (20 mL) for 2.5 h. The resin, thus obtained with a low loading of 0.16 mmol / g of **167** was used for further synthesis. Fmoc SPPS was continued using PyBOP to couple Fmoc-His(Trt)-OH (residue 36) and Fmoc-His(Trt)-OH (residue 35), Fmoc-Lys(Boc)-OH (residue 34), Fmoc-Ala-OH (residue 33) to give the linear precursor. The Aloc / allyl groups of the lanthionine

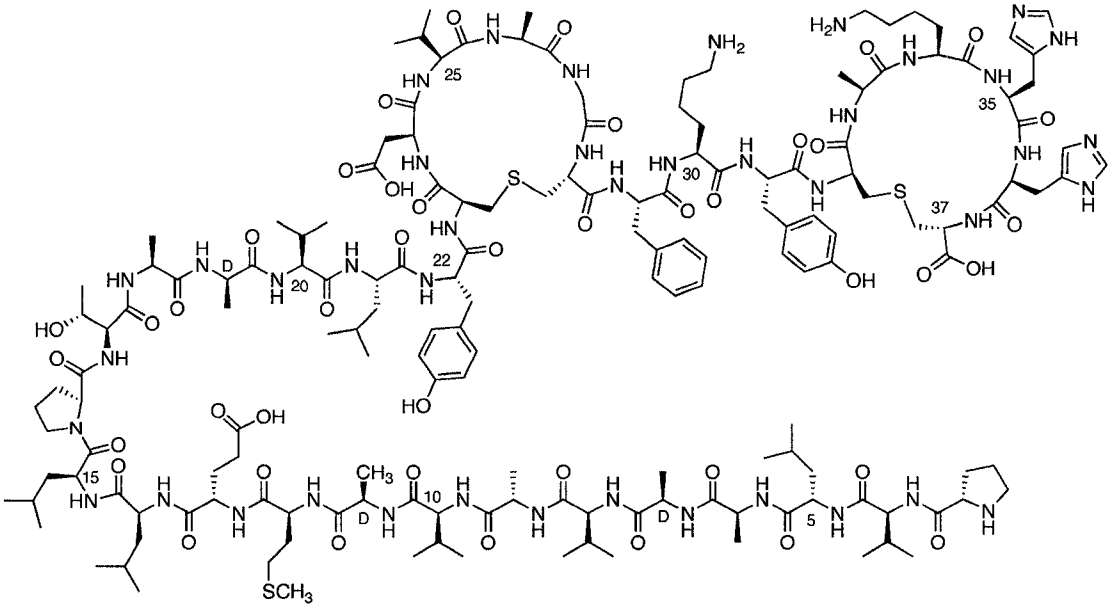
residue were removed by reacting the resin bound linear precursor with a solution of $Pd(PPh_3)_4$ (0.74 g, 0.64 mmol, 2 eq. to resin) and $PhSiH_3$ (0.39 mL, 3.2 mmol, 10 eq. to resin) in 1:1 DMF:CH_2Cl_2 (40 mL) for 2 h. The resin was washed with a) CH_2Cl_2 (2 x 20 mL), b) 0.5% sodium diethyldithiocarbamate in DMF (3 x 20 mL) c) DMF (2 x 20 mL). The Fmoc group was removed by treating with 20% piperidine in DMF. The lanthionine ring B was formed by treating the resin with 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) for 3 x 2.5 h. SPPS was continued on the resin bound lanthionine ring B of lactocin S to introduce Fmoc-Tyr(^{t}Bu)-OH (residue 31) and Fmoc-Lys(Boc)-OH (residue 30). Cleavage of a small sample (~15 mg) of the resin with (95:2.5.2.5) TFA:TIPS:H_2O and precipitation with Et_2O gave the desired product as an off-white solid: MALDI-TOF MS calcd for $C_{57}H_{72}N_{14}O_{12}S$ 1176.5, found 1177.1 (M+H). The resin was divided into 3 portions after washing with CH_2Cl_2 followed by drying under Ar for an hour and in a vacuum dessicator overnight. Only 2/3 of the resin was carried forward for further synthesis.

(c) Synthesis of lanthionine ring A **197** of lactocin S from **167**



Fmoc SPPS was continued to introduce the linear portion required for cyclization to form lanthionine ring A **197** using PyBOP as the coupling reagent to couple amino acids in the following order: Fmoc-Phe-OH, orthogonal lanthionine **167**, Fmoc-Gly-OH, Fmoc-Ala-OH, Fmoc-Val-OH, Fmoc-Asp(O^{t}Bu)-OH. The Aloc / allyl and the Fmoc group of lanthionine residue 28 was removed by treating with $Pd(PPh_3)_4$ and $PhSiH_3$ for 2 h and the cyclization reaction to form ring A was done by treating the resin with a mixture of PyBOP, HOBt and NMM for 3 x 2.5 h. Fmoc-Tyr(^{t}Bu)-OH, residue 22 was coupled under regular SPPS conditions and a small sample (~15 mg) of the resin was cleaved using (95:2.5:2.5) TFA:TIPS:H_2O for 2 h and filtered to remove the resin. The filtrate was concentrated *in vacuo* and precipitation with Et_2O yields deprotected lanthionine rings AB **197** as an off-white solid; MALDI-TOF MS Calcd for $C_{95}H_{120}N_{22}O_{23}S_2$ 2000.8, found 2001.1 (M+H).

(d) Attempted synthesis of residues (3→21) to obtain **198** from **197** by automated SPPS



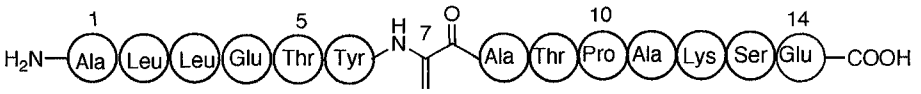
Automated Fmoc SPPS was performed on an ABI 433A peptide synthesizer using the method 'UV *Fastmoc*™ 0.1 mmol'. In the method employed, 10 equivalents of Fmoc-amino acids were used with respect to the resin loading. Peptide couplings were done by mixing a solution of Fmoc-amino acid (in NMP) with a solution of HBTU, HOBt and DIPEA in DMF and pre-activated for 2.1 minutes. The activated solution is then transferred to the pre-swelled resin and allowed to react for 9.3 minutes. End capping is performed with a solution of Ac_2O, HOBt and DIPEA in NMP. Removal of the Fmoc group is done by using 22% piperidine in NMP and monitored by the absorption of dibenzofulvene-piperidine adduct at λ = 301 nm on a UV-Vis spectrophotometer. The overall coupling cycle time for each amino acid is approximately 50 minutes. The following amino acids were coupled in the order: Fmoc-Leu-OH, Fmoc-Val-OH, Fmoc-D-Ala-OH, Fmoc-Ala-OH, Fmoc-Thr(ᵗBu)-OH, Fmoc-Pro-OH, Fmoc-Leu-OH, Fmoc-Leu-OH, Fmoc-Glu(*O*ᵗBu)-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. A small sample of the resin (~20 mg) was cleaved by reacting with (95:2.5:2.5) TFA:TIPS:H_2O and the resin was removed by filtration. Concentration of the filtrate under vacuum and precipitation with Et_2O gave an off-white solid that was analyzed by MALDI-TOF MS. The desired product (**198**) peak was observed only once as a very small peak: calcd for $C_{166}H_{257}N_{41}O_{43}S_3$ 3608.8 found 3631.8 (M+Na). A peak for an undesired product **199** was obtained as the major peak: calcd for $C_{73}H_{103}N_{21}O_{20}S_2$ 1657.7, found 1658.9 (M+H).

(iii) Attempted re-synthesis of residues (3→21) from 197 by manual SPPS

Manual Fmoc SPPS was done on resin bound **197** with PyBOP (10 eq.), HOBt (10 eq.) and NMM (10 eq.) as the coupling reagent to introduce amino acids in the following order: Fmoc-Leu-OH, Fmoc-Val-OH, Fmoc-D-Ala-OH, Fmoc-Ala-OH, Fmoc-Thr(^{t}Bu)-OH, Fmoc-Pro-OH, Fmoc-Leu-OH. Cleavage of a small sample of the resin [(95:2.5:2.5) TFA:TIPS:H_2O] and analysis by MALDI-TOF MS revealed a peak for the the desired product **200** as only a minor product: calcd for $C_{127}H_{175}N_{29}O_{31}S_2$ 2666.2, found 2667.3 (M+H). However, SPPS was continued to explore possibility of extending the minor desired product up to residue 3. Residues (9→14) were introduced using PyBOP in this sequence: Fmoc-Leu-OH, Fmoc-Glu(O^{t}Bu)-OH, Fmoc-Met-OH, Fmoc-D-Ala-OH, Fmoc-Val-OH, Fmoc-Ala-OH. Analysis of the peptide obtained by MS after cleavage of a small sample under acidic conditions showed none of the desired product peaks. The only product observed by MALDI-TOF MS was the undesired peptide **199**: calcd for $C_{73}H_{103}N_{21}O_{20}S_2$ 1657.7, found 1658.9 (M+H).

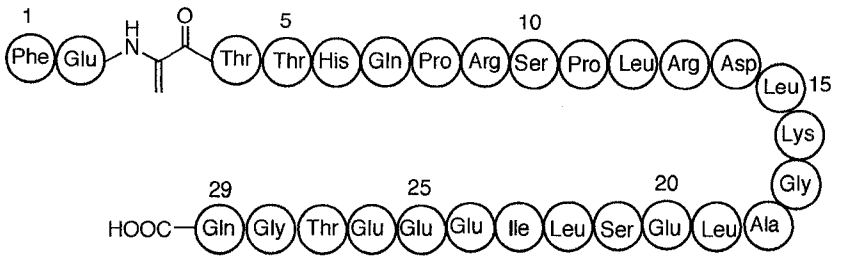
4.2.5. Design and synthesis of a substrate for *in vitro* reconstitution of posttranslational enzyme LtnJ, responsible for the introduction of D-alanines in lacticin 3147

L-Alanyl-L-leucinyl-L-leucinyl-L-glutamyl-L-threonyl-didehydroalanyl-L-threonyl-L-prolyl-L-alanyl-L-lysinyl-L-serinyl-L-glutamic acid (208)



Commercially available unprotected Insulin-like Growth Factor I (57-70) [ILGF] **207** was dissolved in (1:1) ACN:0.1% HCOOH to give a concentration of 1 mg / mL. 100 μL of the peptide solution was mixed with 100 μL and 200 μL of 0.1 M $Ba(OH)_2$ respectively to give two different solution with peptide:base in (1:1) and (1:2) ratio respectively. The reactions were performed either at 25 °C or at 45 °C. After 1 h, 3 h, 15 h, 24 h, 72 h, 90 h and 144 h, a 5 μL aliquot of the reaction mixture was acidified with 1% TFA solution and analysis by MALDI-TOF MS indicated the ratio of the starting material **207** to the desired product **208**. Monoisotopic mass calcd for $C_{65}H_{106}N_{15}O_{23}S_1$ (starting material) 1496.7, found 1497.6 (M+H); calcd for $C_{65}H_{104}N_{15}O_{23}$ (desired product) 1462.7, found 1463.6 (M+H).

L-Phenylalanyl-L-glutamyl-didehydroalanyl-L-threonyl-L-histidinyl-L-glutaminyl-L-prolyl-L-arginyl-L-serinyl-L-prolyl-L-leucinyl-L-arginyl-L-aspartyl-L-leucinyl-L-lysinyl-L-glycinyl-L-alanyl-L-leucinyl-L-glutamyl-L-serinyl-L-leucinyl-L-Isoleucinyl-L-glutamyl-L-glutamyl-L-glutamyl-L-threonyl-L-glycinyl-L-glutamine (210)



Condition A: Commercially available unprotected GnRH Associated Peptide (25-53), human [GAP] **209** was dissolved in (1:1) ACN:0.1% HCOOH to give a concentration of 1 mg / mL. 100 μL of the peptide solution was mixed with 100 μL of 0.1 M $Ba(OH)_2$ and reacted for 168 h at 25 °C. A 5 μL aliquot of the reaction mixture was acidified with 1% TFA and analysis by MALDI-TOF MS indicated the presence of the starting material **209**, the desired product **210** and a product 18 Da higher in mass than the desired product, likely corresponding to the addition of water across the dehydroamino acid residue. MS calcd for $C_{140}H_{227}N_{40}O_{49}S_1$ (starting material) 3284.6, found 3285.5 (M+H); calcd for $C_{140}H_{225}N_{40}O_{49}$ (desired product) 3250.6, found 3251.5 (M+H); calcd for $C_{140}H_{227}N_{40}O_{50}$ (desired product + H_2O adduct) 3268.6, found 3269.3 (M+H).

Condition B: Solutions of GAP at different pH were prepared by dissolved 0.1 mg of the peptide in 100 μL of pH 7.0 MQ-H_2O, pH 7.5 phoshpate buffer, pH 9.0 carbonate buffer and pH 14.0, 0.1 M $Ba(OH)_2$ respectively. A stock solution of 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) was prepared by dissolving 1.0 mg in 1.0 mL of MQ-H_2O. 20 μL of GAP solution at pH 7.0, 7.5, 9.0 and 14.0 was mixed with 20 μL of CDAP solution and reacted either at 4 °C or at 25 °C. A 5 μL aliquot of the reaction was taken at different time intervals, acidified with 1% TFA and analyzed by MALDI-TOF MS to obtain the product conversions. MS calcd for $C_{140}H_{227}N_{40}O_{49}S_1$ (starting material, **209**) 3284.6, found 3285.5 (M+H); calcd for $C_{141}H_{226}N_{41}O_{49}S_1$ (thiocyano intermediate) 3309.3, found 3310.6 (M+H); calcd for $C_{140}H_{225}N_{40}O_{49}$ (desired product, **210**) 3250.3, found 3251.5 (M+H).

Isolation of the maltose binding protein fused peptide 206 from *E. coli* TB1 containing pQE60-lacticin

Bacterial clone: An *E. coli* transformant containing the plasmid pQE60-lacticin, which encodes the designed substrate peptide with the lacticin A1 leader, was prepared and provided by Dr. Marco van Belkum (Vederas group, University of Alberta).

Isolation: 5 mL of sterile Luria Broth base (LB broth) containing 100 μg / mL of ampicillin was inoculated with *E. coli* TB1 containing pQE60-lacticin and incubated at 37 °C for 16 h at 225 rpm. Fermentations were done on a one-liter scale with 500 mL of sterile growth media per 2 L Erlenmeyer flask. Ampicillin was added to the sterile media

to give a final concentration of 100 μg/mL. Each 500 mL of LB media was inoculated with 5 mL E. coli TB1 culture and incubated at 37 °C with shaking at 225 rpm until the cultures reached an optical density of 0.5 at 600 nm. Production of the recombinant maltose binding protein-lacticin A1 leader substrate fusion was induced by addition of 1.5 mL of isopropyl β-D-thiogalactopyranoside (IPTG) to give a final concentration of 0.3 mM. The cultures were incubated for an additional 3 h at 25 °C and 225 rpm after induction before the cells were harvested by centrifugation (8000 rpm, 30 minutes, 4 °C). The cell pellets were collected and resuspended in ~60 mL of ice-cold amylose column buffer (20 mM Tris-Cl, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM NaN_3, pH 7.4 adjusted with 1 M HCl) containing one cφmplete™ EDTA-free protease inhibitor cocktail tablet. The centrifuge tubes containing the resuspended cells were then frozen at -78 °C. Lysozyme (tip of a spatula) was added to the frozen cells and warmed to 0 °C on ice. The freeze-thaw cycles were repeated two more times and the cells were lysed by sonication at 0 °C. The cell lysate was collected by centrifugation at 15,000 rpm for 30 minutes at 4 °C. The supernatent was collected, diluted to 300 mL with amylose column buffer and loaded (flow rate, 1 mL/min) onto 50 mL of pre-washed (5 column volumes, 250 mL) amylose resin. After loading, the column was further washed with 500-600 mL of column buffer with the flow through monitored at 218 nm. The maltose protein fusion was eluted out with the column buffer containing 10 mM maltose. The fractions (5 mL) were collected on a Bio-Rad fraction collector with UV monitoring at 218 nm. The fractions were tested for the presence of the desired MBP fusion by MALDI-TOF MS. The fractions containing the fusion protein were pooled together and dialyzed at 4 °C against MQ-H_2O using a 12,000 – 14,000 MWCO membrane (29 mm diameter, volume /

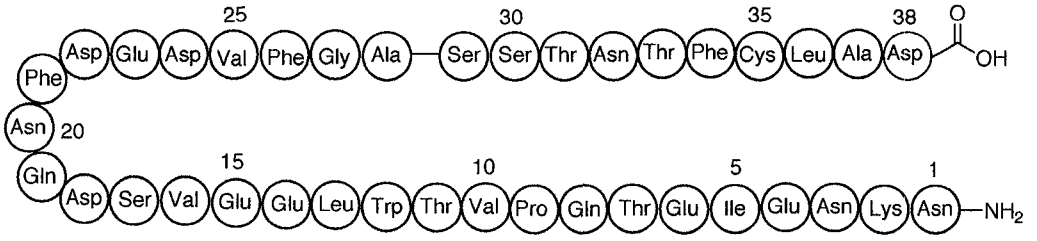
length = 6.4 mL / cm, flat width 45 +/- 2 mm, supplier Spectra/Por). The dialysis water was changed after 2 h, 6 h and 14 h. The dialyzed protein solution was collected, frozen and lyophilized to obtain 18 - 23 mg / L of the maltose binding protein - lacticin A1 leader substrate fusion as a fluffy white solid.

Cleavage of lacticin A1 leader substrate peptide 206 from the maltose binding protein fusion with Factor Xa

The cleavage of the lacticin A1 leader pre-substrate peptide from the maltose fusion was done by adding ~1 μg of Factor Xa enzyme per mg of the fusion protein in Factor Xa buffer (20 mM Tris-Cl, 100 mM NaCl, 2 mM $CaCl_2$, 1 mM DTT, pH adjusted to 8.0). Pilot experiments were performed to find out the time and temperature required for the cleavage reaction. The disappearance of the peak at ~42,600 Da corresponding to the MBP fusion was used to monitor the reaction by MALDI-TOF MS. The optimal time for cleavage was determined to be 3 h at 25 °C at a concentration of 1 mg / mL of the fusion protein. Based on the pilot experiments, cleavage of 15 mg of fusion protein with Factor Xa was done for 3 h at 25 °C. The Factor Xa in the reaction mixture is irreversibly inhibited by adding a 1.6 μM solution of 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF). The inhibited solution was directly injected onto a Vydac protein & peptide C18 reverse phase HPLC column. Method: Flow rate 10 mL / min, detection at 220 and 254 nm. Starting at 20% ACN for 5 minutes, ramping up to 50% ACN over 15 min, second ramp to 70% over 3 minutes, ramping down to 20% ACN in 1 min followed by 20% ACN for 5 min. The desired product peptide **206** eluted at t_R = 20.70 min. The

fractions containing the product were combined together, frozen and lyophilized to yield a white fluffy solid. However, the product peptide **206** was isolable only in sub milligram quantities along with secondary cleavage products.

Automated solid supported synthesis of the peptide 206

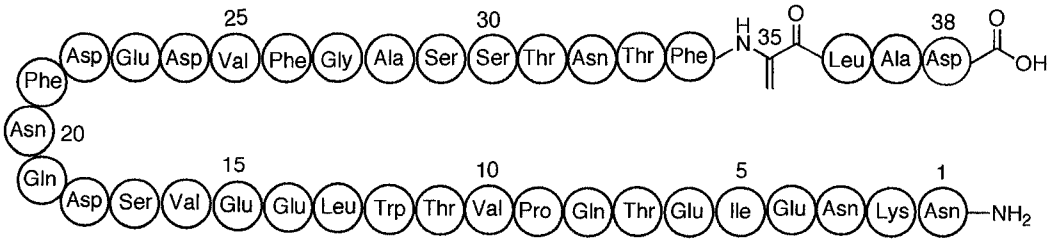


The synthesis of lacticin A1 leader peptide was done on a 0.1 mmol scale (500 mg resin) using NovaSyn TGT resin pre-loaded with Fmoc-Asp(O'Bu)-OH **211** with a substitution capacity of 0.2 mmol/g. Automated Fmoc SPPS was performed on an ABI 433A peptide synthesizer using the method 'UV *FastMoc*™ 0.1 mmol'. In the method employed, 10 equivalents of Fmoc-amino acids were used with respect to the resin loading. Peptide couplings were done by mixing a solution of Fmoc-amino acid (in NMP) with HBTU / HOBt / DIPEA (in DMF) and pre-activated for 2.1 minutes. The activated solution is then transferred to the pre-swelled resin and allowed to react for 9.3 minutes. End capping is performed with a solution of Ac_2O / HOBt / DIPEA in NMP. Removal of Fmoc group is done by using 22% piperidine in NMP and monitored by the absorption of dibenzofulvene-piperidine adduct at λ = 301 nm on a UV-Vis spectrophotometer. The overall coupling cycle time for each amino acid is approximately 50 minutes. Fmoc-Ala-Ser($\psi^{Me,Me}$pro)-OH pseudo-proline dipeptide was incorporated for residues 28 and 29 in

the sequence to disrupt on-resin peptide aggregation. The following amino acids were coupled in the order: Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Cys(Trt)-OH, Fmoc-Phe-OH, Fmoc-Thr(ᵗBu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Thr(ᵗBu)-OH, Fmoc-Ser(ᵗBu)-OH, Fmoc-Ala-Ser($\psi^{Me,Me}$pro)-OH, Fmoc-Gly-OH, Fmoc-Phe-OH, Fmoc-Val-OH, Fmoc-Asp(OᵗBu)-OH, Fmoc-Glu(OᵗBu)-OH, Fmoc-Asp(OᵗBu)-OH, Fmoc-Phe-OH, Fmoc-Asn(Trt)-OH, Fmoc-Gln(OᵗBu)-OH, Fmoc-Asp(OᵗBu)-OH, Fmoc-Ser(ᵗBu)-OH, Fmoc-Val-OH, Fmoc-Glu(OᵗBu)-OH, Fmoc-Glu(OᵗBu)-OH, Fmoc-Leu-OH, Fmoc-Trp(Boc)-OH, Fmoc-Thr(ᵗBu)-OH, Fmoc-Val-OH, Fmoc-Pro-OH, Fmoc-Gln(Trt)-OH, Fmoc-Thr(ᵗBu)-OH, Fmoc-Glu(OᵗBu)-OH, Fmoc-Ile-OH, Fmoc-Glu(OᵗBu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Asn(Trt)-OH. The resin (175 mg) containing the 38-mer **212** was treated with (95:2.5:2.5) TFA:TIPS:H_2O for 3 h and filtered to remove the resin. Concentration of the filtrate under vacuum and precipitation with Et_2O gave the crude 38-mer (100 mg) as an off-white solid. The crude peptide **206** (28 mg) was dissolved in 5 mL of 1:4 ACN: 0.1% TFA with few drops of DMF to aid solubility. The crude peptide solution was filtered to remove the insoluble solid and 0.9 mL of the soluble components were injected on a reverse-phase C_{18} HPLC column (Waters radial compression modules with Delta-Pak™ 15 μm, 100 Å, 25 x 100 mm C_{18}) and purified using the following method: Flow rate = 10 mL / min, dual wavelength detection at 220 and 254 nm. Starting at 20% ACN for 5 minutes, ramping up to 70% ACN in 14 min, second ramp to 90% over 1 minute, at 90% for 5 minutes, ramping down to 20% ACN in 0.2 min followed by 20% ACN for 5 min. The desired product peptide **206** was isolated at t_R = 14.16 min. The fractions containing the product as indicated by MALDI-TOF MS

were combined together, frozen and lyophilized to get a white fluffy solid (2.4 mg, 6% yield). MALDI-TOF MS: calcd for $C_{185}H_{274}N_{46}O_{70}S$ 4291.9, found 4293.3 (M+H).

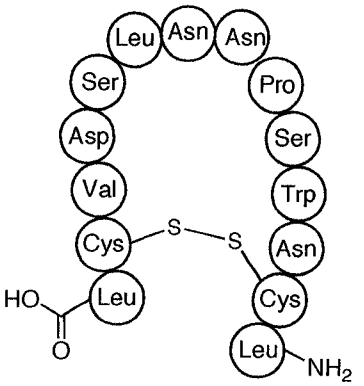
Dethiolation of 206 to obtain lacticin A1 leader - LtnJ substrate peptide 49



50 μL of a 1 mM solution of the 38-mer (2.14 mg in 0.5 mL, pH 7.5 phosphate buffer) was mixed with 50 μL of 10 mM solution of 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (1.2 mg in 0.5 mL MQ-H_2O) and reacted at 25 °C. After 30 minutes, a 5 μL aliquot of the reaction mixture was acidified with 1% TFA and analyzed by MALDI-TOF MS. Only a single peak for the mass corresponding to the cyanated cysteine was observed indicating the complete conversion of the starting material to the thiocyano intermediate. The reaction was continued for another 5 h and another 5 μL aliquot was taken and analyzed after acidification. A peak for the desired product **49** was seen along with the thiocyanate intermediate **214** and secondary cleavage products. The reaction was allowed to proceed further and analysis after 140 h indicated the absence of any of the desired product. The only products observed were secondary cleavage products.

4.2.6. Modifying the mode of action of lantibiotics for activity against Gram-negative bacteria - Synthesis of residues (75→88) of colicin V for attachment to lantibiotics

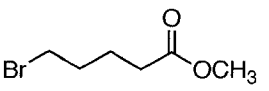
L-Leucinyl-L-cysteinyl-L-asparaginyl-L-tryptophanyl-L-serinyl-L-prolyl-L-asparaginyl-L-asparaginyl-L-leucinyl-L-serinyl-L-aspartyl-L-valinyl-L-cysteinyl-L-leucinyl- cyclic (2→14)- disulfide (50)



The synthesis of residues (75→88) of colicin V was done on 0.44 mmol scale (2.00 g of resin) using NovaSyn TGT resin pre-loaded with Fmoc-Leu-OH **215** with a substitution capacity of 0.22 mmol / g. Manual SPPS was done by adding a pre-activated solution of Fmoc-amino acid (2.0 eq. to resin loading), PyBOP (1.95 eq.) and NMM (2.0 eq.) in DMF (20 mL). The solution was then transferred to pre-swelled resin and reacted for 2 h. The completion of couplings were ascertained by negative Kaiser test[187] and end capping was performed with 20% Ac_2O in DMF for 10 min. An additional coupling step was done when required based on the Kaiser test. Subsequent removal of the Fmoc group was done using 20% piperidine in DMF and monitored by the absorption of dibenzofulvene-piperidine adduct at λ = 301 nm on a UV-Vis spectrophotometer. The Fmoc amino acids

were coupled in this sequence: Fmoc-Cys(Mmt)-OH, Fmoc-Val-OH, Fmoc-Asp(O^{t}Bu)-OH, Fmoc-Ser(^{t}Bu)-OH, Fmoc-Leu-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Pro-OH, Fmoc-Ser(^{t}Bu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Cys(Mmt)-OH, Fmoc-Leu-OH. A small portion of the resin (205 mg) was treated with (95:2.5:2.5) TFA:TIPS:H_2O for 2 h. The resin was separated from TFA solution by filtration and the filtrate was concentrated *in vacuo*. The crude peptide was obtained as an off-white solid after precipitated with cold Et_2O. The crude peptide was re-dissolved 1 mM NH_4HCO_3 and the pH was adjusted to 8.0 with NH_4OH. Oxygen was bubbled for 14 h after which the solution was freezed and lyophilized. The disulfide containing impure peptide was purified by reverse phase HPLC using Waters radial compression modules with Delta-Pak^{TM} 15 μm, 100 Å, 25 x 100 mm, C_{18} column. Method: Flow rate = 10 mL / min, dual wavelength detection at 220 and 254 nm. Starting at 5% ACN for 5 minutes, ramping up to 60% ACN in 12 min, second ramp to 90% over 0.5 minute, constant at 90% for 4 minutes, ramping down to 5% ACN in 0.5 min followed by 5% ACN for 5 min. The desired pure peptide **50** was isolated at t_R = 18.43 minutes. The fractions containing the desired peptide **50** were combined together, frozen and lyophilized to yield a white fluffy solid (16.3 mg, 23% yield). MALDI-TOF MS: calcd for $C_{67}H_{102}N_{18}O_{22}S_2$ 1574.7, found 1575.6 (M+H).

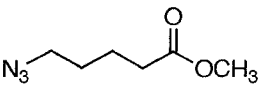
Methyl 5-bromopentanoate (218)



To a solution of 5-bromovaleric acid (**217**) (3.00 g, 16.57 mmol) in CH_2Cl_2 (30 mL), Et_3N (3.50 mL, 24.86 mmol) was added followed by DMAP (0.20 g, 1.65 mmol) and

methylchloroformate (1.41 mL, 1.22 mmol) dropwise at 0 °C and the reacted for 14 h. The reaction mixture was washed sequentially with 1 M HCl (20 mL), 10% $NaHCO_3$ (20 mL), water (2 x 20 mL) and brine (1 x 20 mL). The organic layer was separated, dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude product **218** (3.23 g, 16.56 mmol) obtained in quantitative yield and was used as such for further transformations without any purification. IR ($CHCl_3$ cast) 2952, 1738, 1437 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz) δ 3.68 (s, 3H, OCH_3), 3.41 (t, 2H, J = 6.6 Hz, -CH_2Br), 2.36 (t, 2H, J = 7.8 Hz, -$COCH_2$), 1.93-1.88 (m, 2H, -CH_2CH_2Br), 1.81-1.76 (m, 2H, -CH_2CH_2CO); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.4 (C=O), 51.5 (OCH_3), 33.0 (CH_2), 32.9 (CH_2), 31.9 (CH_2), 23.4 (CH_2); HRMS (ES) Calcd for $C_6H_{11}BrO_2Na$ 216.9835, found 216.9835.

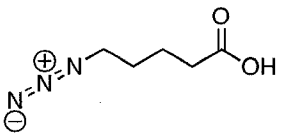
Methyl 5-azidopentanoate (219)



This known compound was prepared by a modified literature procedure.[190] To a solution of methyl 5-bromopentanoate **218** (0.50 g, 2.56 mmol) in DMF (5.0 mL), NaN_3 (0.17 g, 2.57 mmol) was added and reacted at 70 °C for 14 h. The reaction mixture was diluted with diethyl ether (50 mL) and washed with water (3 x 20 mL). The organic layer was separated, dried over Na_2SO_4, filtered and concentrated *invacuo*. The desired product **219** was obtained as colorless oil (0.30 g, 74% yield). It was used as such for further transformations without any purification. IR ($CHCl_3$ cast) 2934, 2873, 2098, 1736 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 3.68 (s, 3H, OCH_3), 3.30 (t, 2H, J = 6.8 Hz, -CH_2N_3), 2.36 (t, 2H, J = 6.8 Hz, -$COCH_2$), 1.76-1.58 (m, 4H, 2 x CH_2); ^{13}C NMR ($CDCl_3$, 125 MHz) δ

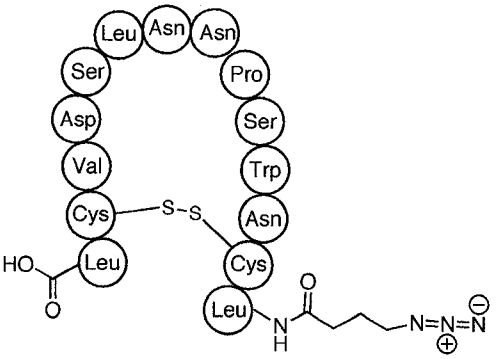
173.5 (C=O), 51.6 (OCH_3), 51.0 (CH_2), 33.4 (CH_2), 28.3 (CH_2), 22.1 (CH_2); HRMS (ES) Calcd for $C_6H_{11}N_3O_2Na$ 180.0741, found 180.0743.

5-Azidopentanoic acid (220)



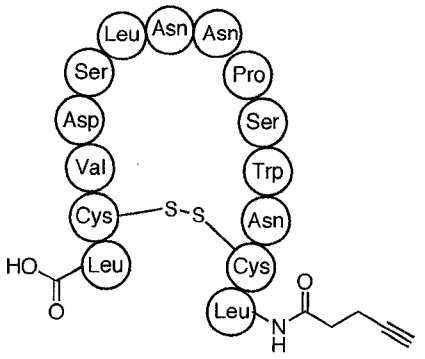
This known compound was prepared by a modified literature procedure.[191] To a solution of methyl 5-azidopentanoate **219** (270 mg, 1.72 mmol) in 5 mL of 1:1 THF:H_2O, $LiOH{\cdot}H_2O$ (79 mg, 1.88 mmol) was added and stirred for 3 h. The reaction mixture was diluted with $CHCl_3$ (30 mL) and acidified to pH 6 using 3 M HCl. The organic layer was separated and the aqueous layer was extracted twice with $CHCl_3$ (2 x 15 mL). The organic layers were combined, dried over Na_2SO_4, filtered and concentrated *in vacuo*. The desired product **220** was obtained as a yellow oil (0.20 g, 81% yield). The product was used as such for further transformations without any purification. (R_f 0.01 on SiO_2, 1:1/EtOAc:hexanes); IR ($CHCl_3$ cast) 2943, 2099, 1710 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 9.74 (br s, 1H, COOH), 3.31 (t, 2H, J = 6.8 Hz, $-CH_2N_3$), 2.40 (t, 2H, J = 7.2 Hz, $-COCH_2$), 1.77-1.62 (m, 4H, 2 x CH_2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 179.0 (C=O), 51.0 (CH_2), 33.4 (CH_2), 28.2 (CH_2), 21.8 (CH_2); HRMS (ES) Calcd for $C_5H_8N_3O_2$ 142.0611, found 142.0611.

Synthesis of residues (75→88) of colicin V with an azide linker (222)



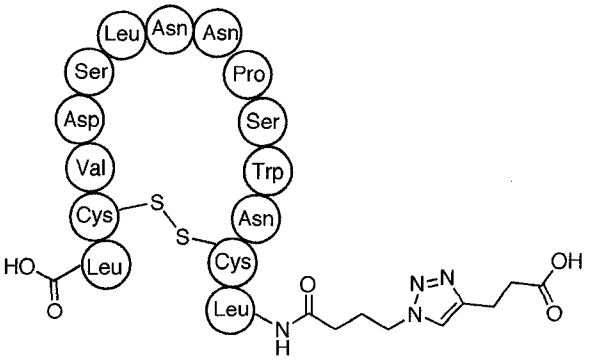
The Fmoc group of resin bound **216** (50 mg) was removed with 20% piperidine in DMF (3 x 3 min) and a solution of 5-azidovaleric acid **220** (25 mg, 0.17 mmol), DIPCDI (21 mg, 0.17 mmol), HOBt (23 mg, 0.17 mmol) and NMM (40 μL, 0.35 mmol) in DMF (1 mL) was added and reacted for 2 h. The resin with the azide linker **221** was washed with DMF (2 mL) and CH_2Cl_2 (2 x 2 mL), followed by treatment with (95:2.5:2.5) TFA:TIPS:H_2O for 2 h. After separating the resin from TFA solution by filtration, the filtrate was concentrated *in vacuo* to yield the open form of peptide **222** after precipitation with cold Et_2O. The crude peptide was redissolved in 1:4 ACN: 1 mM NH_4HCO_3 and the pH was adjusted to 8.0 with NH_4OH. Oxygen was bubbled through the solution for 14 h after which the solution was frozen and lyophilized to give peptide **222** as an off-white solid: MALDI-TOF MS calcd for $C_{72}H_{109}N_{21}O_{23}S_2$ 1699.7, found 1723.4 (M+Na).

Synthesis of residues (75→88) of colicin V with an alkyne linker (225)



The attachment of the alkyne linker was done using a similar procedure to that used for the azide linker with colicin V loop. The Fmoc group of resin bound **216** (50 mg) was removed with 20% piperidine in DMF (3 x 3 min) and a solution of commercially available 4-pentynoic acid (**223**) (11 mg, 0.11 mmol), DIPCDI (13 mg, 0.11 mmol), HOBt (15 mg, 0.11 mmol) and NMM (23 μL, 0.22 mmol) in DMF (1 mL) was added and reacted for 2 h. The resin with the alkyne linker **224** was washed with DMF (2 mL) and CH_2Cl_2 (2 x 2 mL), followed by treatment with (95:2.5:2.5) TFA:TIPS:H_2O for 2 h. After separating the resin from TFA solution by filtration, the filtrate was concentrated *in vacuo* to yield the open form of peptide **225** after precipitation with cold Et_2O. The peptide was redissolved 1:4 ACN: 1 mM NH_4HCO_3 and the pH was adjusted to 8.0 with NH_4OH. Oxygen was bubbled through the solution for 14 h after which the solution was frozen and lyophilized to give peptide **225** as an off-white solid: MALDI-TOF MS calcd for $C_{72}H_{106}N_{18}O_{23}S_2$ 1654.7, found 1677.7 (M+Na).

Model cycloaddition reaction of 4-pentynoic acid with residues (75→88) of colicin V with an azide linker (226)



To a 10 μL solution of peptide **222** (1.0 mg / mL concentration) in 1:1 ^{t}BuOH: H_2O, 10 μL of 4-pentynoic acid (10 mM) was added followed by 10 μL of $CuSO_4 \cdot 5H_2O$ (10 mM) and 10 μL of sodium ascorbate (10 mM). The reaction mixture was kept at 25 °C for 6 h. A 5 μL aliquot was taken, acidified with 1% TFA and analysis by MALDI-TOF MS indicated the formation of the triazole product **226**. Monoisotopic mass calcd for $C_{77}H_{115}N_{21}O_{25}S_2$ 1797.8, found 1820.7 (M+Na).

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