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Synthesis and Conformational Studies of the Lipid II-Binding Rings of Nisin and Mutacin I

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Declaration

I, Rachael Dickman, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed:

Dated: 29-06-2018

Abstract

Antibiotic resistance is a huge global health threat, and there is urgent need for new classes of antimicrobials to combat the spread of resistant organisms. In recent years, a class of antimicrobial peptides called the lantibiotics has emerged as a promising potential source of new antibiotics. The first discovered lantibiotic, nisin, has become a model compound for the class. It displays extremely potent antibacterial activity, but its poor pharmacokinetic properties mean that it is currently unable to be used therapeutically. This thesis describes the synthesis and structural analysis of the target-binding region of nisin and its close structural relative mutacin I.

The lantibiotics are characterised by their complex cyclic structures formed by the unusual bis-amino acids lanthionine and methyllanthionine, and frequently contain the dehydrated amino acids dehydroalanine and dehydrobutyrine. To synthesise these peptides, methods to introduce each of these unusual residues are required. Therefore, the first aim of this research was to synthesise two orthogonally protected (methyl)lanthionines, as well as various precursors to the dehydrated residues.

With these in hand, several novel truncated analogues of nisin and mutacin I were synthesised. Simpler analogues with fewer unusual amino acids were prepared, as well as peptides with the wild-type sequence. Finally, the solution state structure of each of the peptides was calculated from NMR data.

From these studies, key conformational differences were observed between the synthesised truncated analogues in comparison to both full length and truncated wild-type nisin. In addition, significant differences were seen between the wild-type peptides and the simplified analogues. Taken together, these results indicate which of the unusual amino acids it may be possible to substitute whilst still maintaining native solution structure. It is hoped that this will guide future analogue design, and aid in the development of new semisynthetic antibiotics based on the structure of nisin.

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Acknowledgements

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Abbreviations

$[\alpha]_D$	Specific rotation
β-Me-Cys	β-methyl-cysteine
δ_C	chemical shift of ^{13}C resonances
δ_H	chemical shift of ^{1}H resonances
μm	Micromole(s)
μW	Microwave
aa	Amino acid
Abu	α-Aminobutyric acid
AcCl	Acetyl chloride
AcOH	Acetic acid
Ala (A)	Alanine
Alloc	Allyloxycarbonyl
Ar	Aryl
Arg (R)	Arginine
Asn (N)	Asparagine
Asp (D)	Aspartic acid
AspN	Endoproteinase AspN
Bn	Benzyl
Boc	*tert*-Butoxycarbonyl
Cbz	Carboxybenzyl
COSY	Correlation spectroscopy
CPDY	Carboxypeptidase Y
Cys (C)	Cysteine
d	Day(s)
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCC	*N*,*N*′-Dicyclohexylcarbodiimide
DEAD	Diethylazodicarboxylate
Dha	Dehydroalanine
Dhb	Dehydrobutyrine
DIC	*N*,*N*′-Diisopropylcarbodiimide
DIPEA	Diisopropylethylamine
DMAP	4-(Dimethylamino)pyridine
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNs	2,4-dinitrobenzenesulfonyl
DOPC	Dioleoyl phosphatidylcholine
DOPG	Dioleoyl phosphatidylglycerol
DPC	Dodecylphosphocholine
DTT	D,L-Dithiothreitol
EDCI	*N*-Ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride
EtOAc	Ethyl acetate
eq	Equivalent(s)
Fmoc	9-Fluorenylmethoxycarbonyl
Fmoc-OSu	9-Fluorenylmethyl-succinimidyl carbonate
GlcNAc	*N*-acetylglucosamine
Gln (Q)	Glutamine
Glu (E)	Glutamic acid

Gly (G)	Glycine
h	Hour(s)
HBTU	O-(Benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate
His (H)	Histidine
Hmb	2-hydroxy-4-methoxybenzyl
HMBA	4-Hydroxymethylbenzoic acid
HMBC	Heteronuclear multiple bond coherence
HOAt	1-Hydroxy-7-azabenzotriazole
HPLC	High pressure liquid chromatography
HRMS	High resolution mass spectrometry
HSQC	Heteronuclear single quantum correlation
Ile (I)	Isoleucine
Lan	Lanthionine
LCMS	Liquid chromatography-mass spectrometry
Leu (L)	Leucine
lit.	Literature
Lys (K)	Lysine
m/z	Mass to charge ratio
M	Molar
MD	Molecular dynamics
MeCys	β-methyl-cysteine
MeIm	*N*-methylimidazole
MeLan	Methyllanthionine
Mesyl	Methanesulfonyl
Met (M)	Methionine
MHz	Megahertz
MIC	Minimum inhibitory concentration
min	Minute(s)
mL	Millilitre(s)
mmol	Millimole(s)
mol	Mole(s)
mp	Melting point
MsCl	Mesyl chloride
MSNT	1-(Mesitylene-2-sulfonyl)-3-nitro-1H-1,2,4-triazole
MurNAc	*N*-acetylmuramic acid
NMM	*N*-methylmorpholine
NMP	*N*-methylpyrrolidone
NMR	Nuclear magnetic resonance
NOE(SY)	Nuclear Overhauser effect (spectroscopy)
pep	Peptide
pet. ether	Petroleum ether 40-60 °C
PG	Protecting group
Phe (F)	Phenylalanine
Pht	Phthalimide
*p*NZ	*p*-nitro-benzyloxycarbonyl
PP	Polypropylene
Pro (P)	Proline
psf	Protein structure file
*p*TsCl	*para*-Toluenesulfonyl chloride

*p*TsOH	*para*-Toluenesulfonic acid
PyAOP	(7-Azabenzotriazol-1-yloxy)trispyrrolidinophosphonium hexafluorophosphate
ROESY	Rotational nuclear Overhauser effect spectroscopy
rt	Room temperature
s	Second(s)
SAR	Structure-activity relationship
SDS	Sodium dodecyl sulfate
Sec	Selenocysteine
SecPh	Phenylselenocysteine
Ser (S)	Serine
SPPS	Solid-phase peptide synthesis
STmp	Trimethoxyphenylthio
TBAF	Tetrabutylammonium fluoride
^{t}Bu	*tert*-Butyl
Tce	2,2,2-Trichloroethyl
TCEP	Tris(2-carboxyethyl)phosphine hydrochloride
Teoc	(Trimethylsilyl)ethoxycarbonyl
Teoc-OSu	(Trimethylsilyl)ethoxycarbonyl- succinimidyl carbonate
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
Thr (T)	Threonine
TIPS	Triisopropylsilane
TLC	Thin layer chromatography
TMSCl	Trimethylsilyl chloride
TMSE	Trimethylsilylethyl
TOCSY	Total correlated spectroscopy
Tosyl	*para*-Toluenesulfonyl
Triflic acid	Trifluoromethanesulfonic acid
TRIS	2-Amino-2-(hydroxymethyl)-1,3-propanediol
Trityl	Triphenylmethyl
Trp (W)	Tryptophan
Trt	Trityl
TrtCl	Trityl chloride
TsCl	Tosyl chloride
Tyr (Y)	Tyrosine
Val (V)	Valine
v/v	Volume by volume
WT	Wild type
w/v	Weight by volume
w/w	Weight by weight
Xaa	Any amino acid

1. INTRODUCTION

1.1. The Need for New Antimicrobials

Since the first clinical use of penicillin in the 1940s, antibacterial resistance has become an increasing threat to global public health.[1] Infections caused by resistant bacteria are harder to control and cure, leading to longer and more expensive hospital stays,[1] and in turn, an increased risk of the spread of infection. The reality of a 'post-antibiotic era', in which common infections, minor surgeries and small injuries are once again life-threatening, is rapidly approaching.[2]

The development of resistance towards each new antibacterial agent introduced to the clinic can be attributed to a number of factors, including but not limited to overuse of antibiotics, inappropriate prescribing, and extensive use in livestock.[3,4] A number of ways of managing antibacterial resistance have been recommended, such as more responsible prescribing and stewardship practices, and improvement of diagnostic tools to ensure that appropriate antibiotics are used for treatment.[5] Ultimately though, these strategies alone are insufficient. To plug the gap in the dwindling antibiotic pipeline, and fight the rise of resistant infections, the introduction of new agents is necessary.[6] However, no new classes of broad-spectrum antibiotics have been introduced since the first wave of discovery in the 1940s-1960s, which has prompted a renewed interest in natural products as a rich source of potent antibacterial drugs.[7]

1.2. Antimicrobial Peptides

One such class of natural products is antimicrobial peptides, which play a vital role in the innate immunity of a wide variety of organisms including bacteria, fungi, plants, insects, animals,[8–10] and even humans.[11] Antimicrobial peptides are produced by these organisms in a variety of ways: some, such as mammalian defensins,[11] are gene-encoded,[10,12] some, such as the lantibiotics, are gene-encoded and then entensively enzymatically modified,[13] and others, including glycopeptide antibiotics such as vancomycin, are synthesised entirely by large multifunctional enzyme complexes called non-ribosomal peptide synthetases.[14] Many antimicrobial peptides have broad spectrum antibacterial activity, and are active against a range of Gram-positive and Gram-negative organisms,[15] as well as against other pathogens, such as fungi[16] and protozoans.[17] These desirable activities have meant that despite common obstacles to the development of peptide-based drugs,

such as poor absorption, low solubility and rapid proteolysis and blood clearance,[18] the number of peptides in clinical trials is growing.[19] Between the years 2000 and 2016, three new structurally-related glycopeptide-based antibiotics were approved, telavancin (**1**) (**Figure 1.1**), dalbavancin and oritavancin.[5,20]

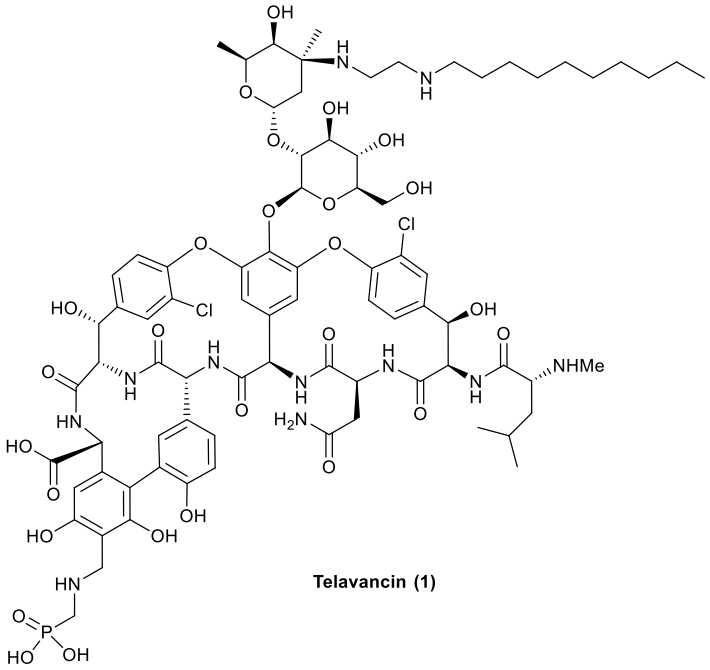


Figure 1.1: Structure of recently approved glycopeptide antibiotic telavancin.

There is huge structural diversity among antimicrobial peptides, though most are cationic, amphiphilic, and exert their effect on the target bacteria by attaching and inserting into the membrane bilayer, forming transmembrane pores which lead to cell death.[12] The rapid way in which these peptides disrupt cell membranes also means that the generation of resistant mutants is often prevented.[15] However, mechanisms of naturally-occurring resistance have been reported, which involve proteolysis of the antimicrobial peptide,[21] active transport of the peptide out of the cell by efflux pumps,[22] and differences in composition of membrane lipid or surface charge which decrease peptide insertion.[9] In addition to pore-formation, some antimicrobial peptides also have more specific intracellular activities which contribute to their activity,[9] such as inhibition of cell wall biosynthesis,[23] DNA synthesis[24] or enzyme activity.[25]

Antimicrobial peptides which undergo extensive post-translational modification (PTM) can be categorised as ribosomally-synthesised and post-translationally modified peptides (RiPPs). The range of potential modification is vast, and peptides are grouped into families based on their structural features (**Figure 1.2**).[26] For example, the cyclotides are

defined by a head-to-tail cyclisation and a conserved disulfide knot bridging pattern (**2**),[27] glycocins are *S*-linked to monosaccharides *via* Cys side chains (**3**),[28] cyanobactins (**4**)[29] and thiopeptides (**5**)[30] contain thiazole heterocycles, and the lasso peptides have novel threaded architectures formed by head-to-side chain cyclisation (**6**).[31,32] These modifications, and many more not discussed here, often improve metabolic stability of the peptides by conferring protease resistance, as well as reducing flexibility and improving target recognition.[33]

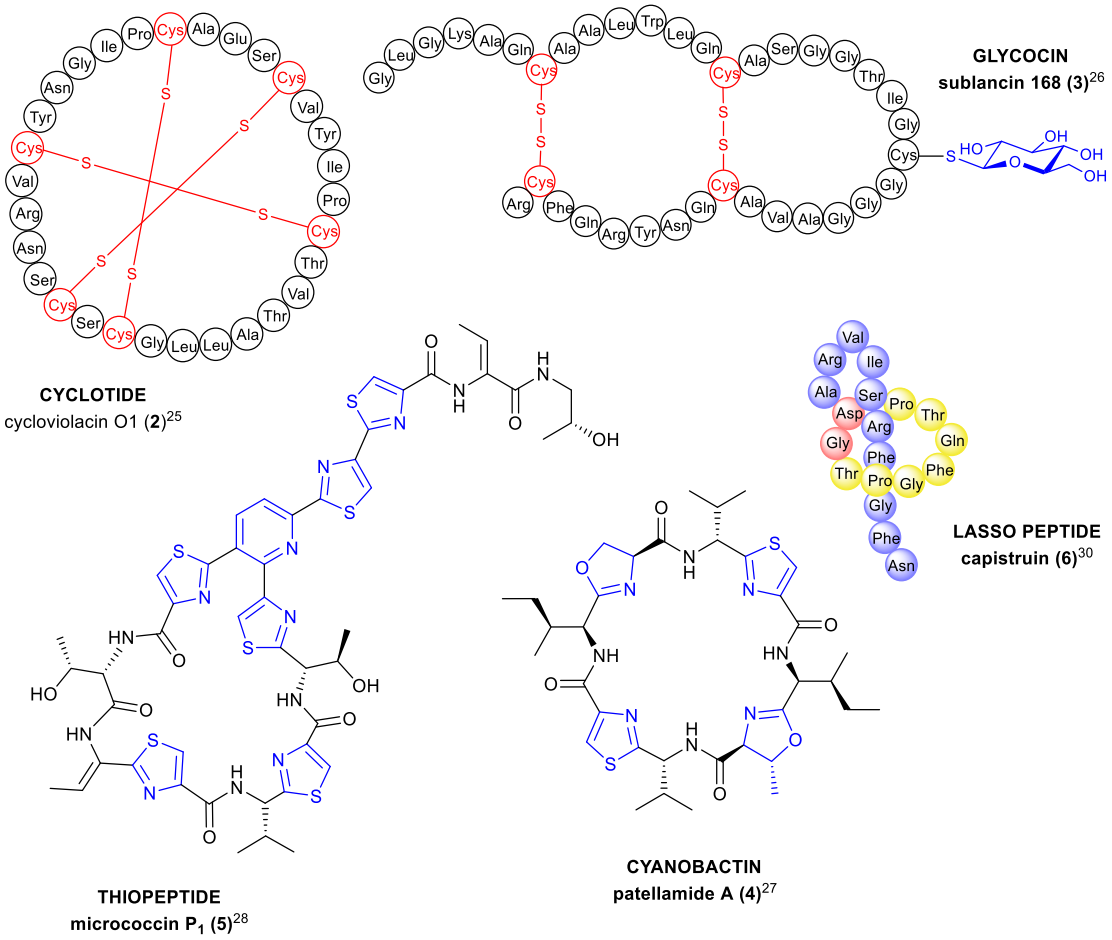


Figure 1.2: Examples of the extensive range of PTMs undergone by different families of RiPPs. Unusual features of cyclotides, glycocins, thiopeptides and cyanobactins are coloured red and blue. Residues of the lasso peptide are coloured to highlight the topology: yellow = ring, red = location of head-side chain cyclisation, blue = loop and tail which form the thread.

1.2.1. The Lantibiotics

One of the first discovered families of RiPPs is defined by the presence of thioether-bridged amino acids called lanthionines, which are introduced by

post-translational modification (**Figure 1.3**). Peptides belonging to this family are called the lantipeptides (lanthionine-containing peptides), and lantipeptides which display antibacterial activity are specifically termed lantibiotics.[33,34] Two lanthionines are routinely found in the lantibiotics, (2*S*, 6*R*)-lanthionine (Lan, **7**) and (2*S*, 3*S*, 6*R*)-methyllanthionine (MeLan, **8**), although more recently Tang *et al.* reported that the lantibiotic cytolysin also contains a more unusual (2*R*, 6*R*)-lanthionine.[35] The α,β-unsaturated amino acids dehydroalanine (Dha, **9**) and dehydrobutyrine (Dhb, **10**) are also commonly found within the lantibiotics. Less common are the lysinoalanine (**11**) and aminovinylcysteine (**12**) residues, which form additional bridges in some peptides of the family.

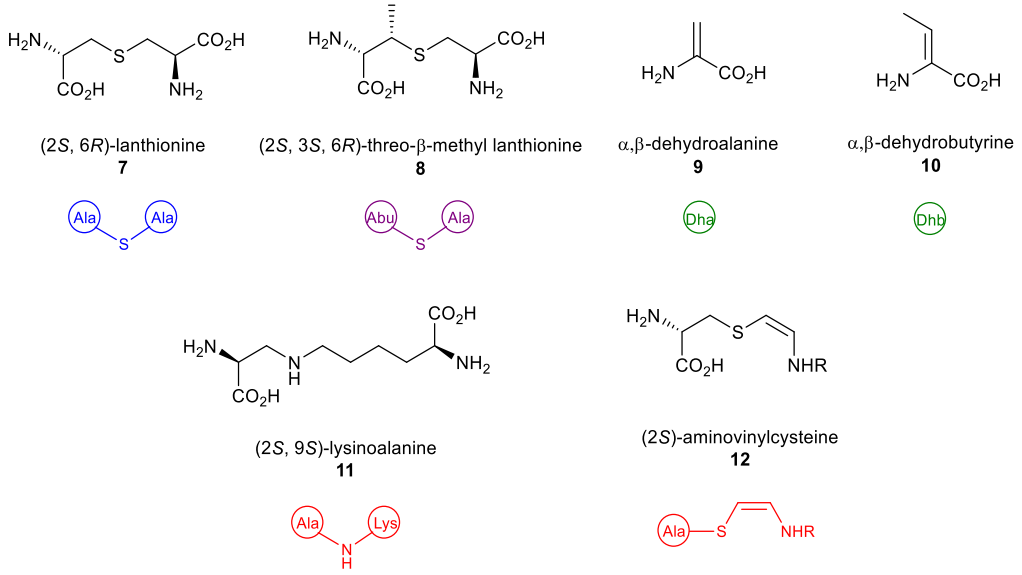


Figure 1.3: Structures of the key unusual amino acids present in the lantibiotics.

Within the lantibiotic family there is great structural diversity, though all of the peptides are small (19-38 residues) and are both dehydrated and cyclised to some extent.[34] This causes them to have complex characteristic polycyclic structures, generated by the bridging amino acids.[36] In the past, attempts have been made to classify the peptides based on their shape and structure,[37] but recently it has become more useful to categorise this ever-expanding class of peptides based on their biosynthetic machinery (**Figure 1.4**).[38] The biosynthesis of class I lantibiotics such as nisin (**13**), subtilin (**14**) and epidermin (**15**) involves two separate modification enzymes, whereas class II lantibiotics, for example cinnamycin (**16**), lacticin 481 (**17**) and the two-component lantibiotic lacticin 3147 (**18**), are produced by a single bifunctional modification enzyme. The most recently discovered

classes, III and IV, use trifunctional enzymes and produce peptides such as SapB (**19**) and venezuelin (**20**) (see **Chapter 1.3.1** for discussion of lantibiotic biosynthesis).[39]

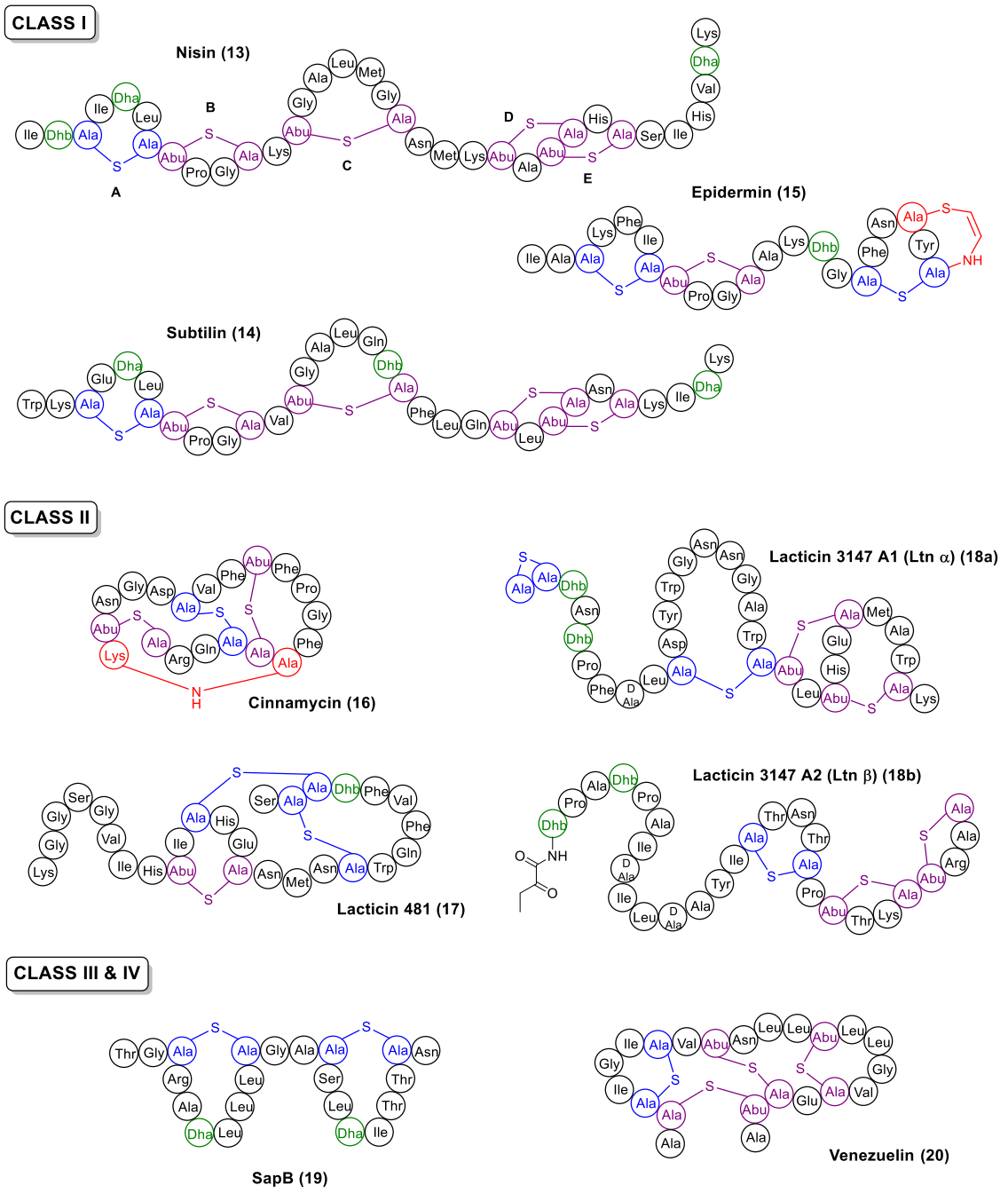


Figure 1.4: Examples of peptides in each class of lantibiotics. Unusual amino acids are coloured as described in **Figure 1.3**.

Lantibiotics are bacteriocins: they are both produced by, and exert their antibacterial effect on, Gram-positive bacteria.[36] The need for potent new antibiotics has revived interest in this class of peptides, particularly as they have a well-characterised activity, and low MIC (μM or nM) against a number of clinically relevant species (**Table 1.1**).[20,40]

Despite these advantages, currently no lantibiotics are used clinically, although some are in the early stages of testing.[41] Mutacin 1140 (MU1140, **21**) and microbisporicin (NAI-107, **22**) are in preclinical studies for *C. difficile* infection[40] and intravenous treatment of serious Gram-positive infections,[42,43] respectively (**Figure 1.10**). The semisynthetic lantibiotic NVB302 (**23**), developed by Novacta Biosystems, also to treat *C. difficile* infection, was the first lantibiotic to enter clinical trials and completed phase I in 2011 (**Figure 1.5**).[44]

Strain	Lantibiotic	MIC (μg mL^{-1})
MRSA	Nisin	62.5
	Mutacin III	3.1
	Microbisporicin	< 0.2
VRE	Nisin	62.5
	Mutacin III	6.2
Lactococcus lactis	Subtilin	1.1
Staphylococcus sp.	Nisin	4.2
	Mutacin 1140	< 1.0
	Mutacin B-Ny266	1.6
Micrococcus sp.	Nisin	1.1
	Mutacin B-Ny266	0.05
Bacillus sp.	Nisin	4.2
	Lacticin 3147	2.4
	Mutacin B-Ny266	0.8
Listeria sp.	Nisin	4.2
	Lacticin 3147	15.4
	Mutacin B-Ny266	0.8
Enterococcus sp.	Nisin	16.7
	Mutacin B-Ny266	12.8
	Microbisporicin	1.0

Table 1.1: Antimicrobial activities of selected lantibiotics.[42,45–49]

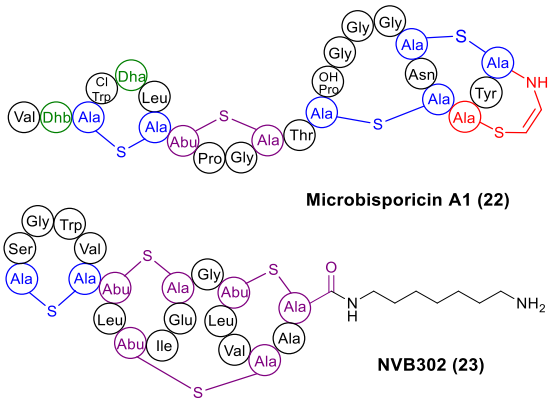


Figure 1.5: Structures of lantibiotics currently undergoing clinical trials. (Microbisporicin A1 'OH Pro' = 3,4-dihydroxyPro, 'Cl Trp' = 4-Cl-Trp).

1.3. Nisin

Nisin (**13**) (**Figure 1.4**), first discovered in 1928 by Rogers, is the most studied member of the lantibiotic family,[50] although its structure was not fully determined until the early 1970s.[51,52] It is 34 amino acids in length, 13 residues of which are post-translationally modified to give one Lan residue, four MeLan residues (two of which form overlapping rings), and three dehydro residues: one Dhb and two Dha.

Eight natural nisin variants have been discovered to date, and are isolated from a range of *Lactococcus* and *Streptococcus* species. Nisin A and nisin Z are by far the most common variants, the only difference between the two peptides being a one amino acid mutation at position 27, from His in nisin A to Asn in nisin Z, resulting in the increased solubility of nisin Z at neutral pH.[53,54] The six other variants, named F,[55] Q,[56] U, U2,[57] P,[58] and H,[53] have between two and ten mutations from nisin A (**Figure 1.6**). Nisins U, U2 and P are also *C*-terminally truncated.

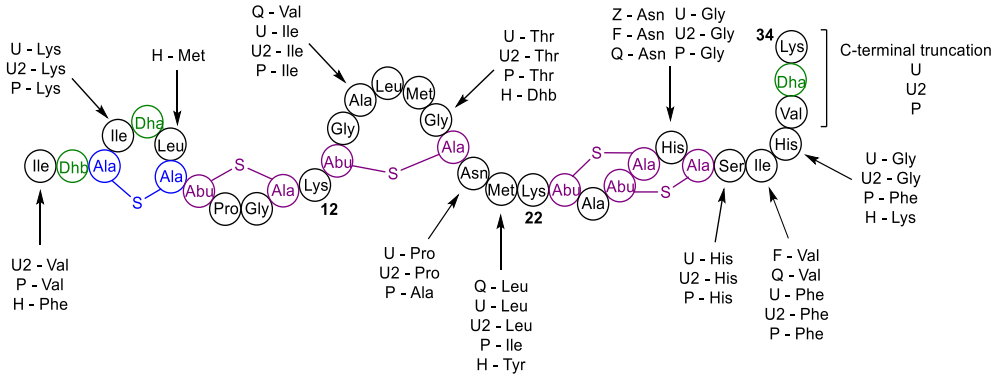


Figure 1.6: The differences between the naturally-occurring nisin variants. The drawn peptide sequence is of nisin A, positions where the variants differ are indicated at each residue.

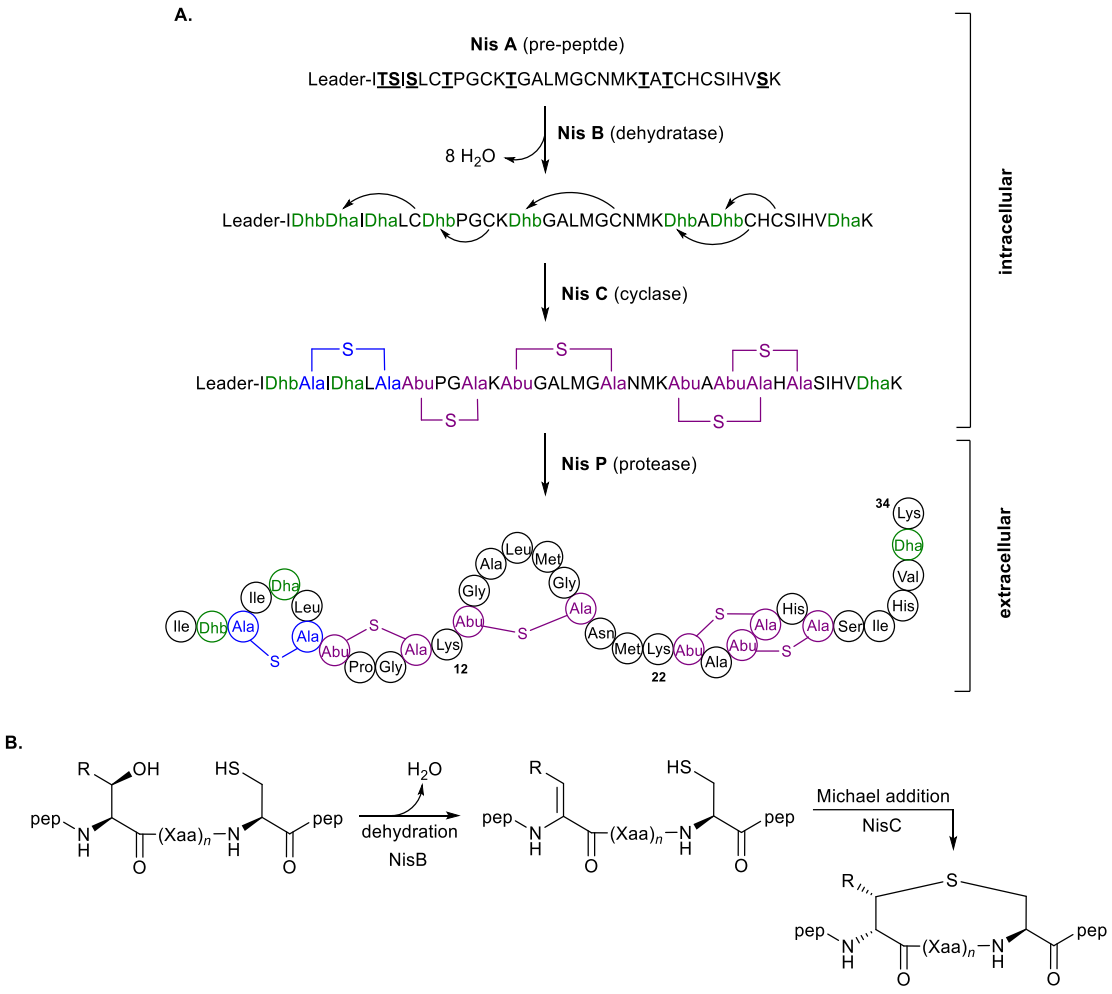
The first *in vivo* studies on the antibacterial activity of nisin were conducted in the 1940s.[59] More recent studies have shown activity against *Listeria monocytogenes*, *Bacilus cereus*, *Enterococcus faecalis* and several *S. aureus* species,[49] including MRSA.[60] Synergistic effects have also been observed between nisin and ramoplanin, vancomycin, ciprofloxacin and daptomycin, in *in vitro* activity assays with MRSA and vancomycin-resistant *enterococcus* (VRE) species.[60–62] However, in general, nisin has poor activity against Gram-negative organisms because the outer membrane acts as a permeability barrier, blocking access to the cytoplasmic membrane,[63,64]

Nisin is approved for use in food production worldwide (E234), where its antimicrobial activity is utilised most commonly as a preservative in milk products, particularly cheese, but also in other processed foods.[65,66] It is mass produced for commercial applications by fermentation of the producing strain: *Lactococcus lactis*.[65]

1.3.1. Biosynthesis of Nisin

The genes encoding nisin A and its production enzymes are found in a cluster in the genome of certain strains of *L. lactis*, a lactic acid bacteria.[37] NisA is the first gene of the cluster and encodes the 57 amino acid straight chain pre-peptide, consisting of the 34 amino acid nisin propeptide and an *N*-terminal leader sequence. This leader peptide is important for recognition of the substrate by the modification enzymes, and all class I lantibiotics contain a conserved 'FNLD' recognition motif in the leader peptide sequence.[67–69] Class I lantibiotics also have separate modification enzymes for dehydration and cyclisation, in nisin these are NisB and NisC respectively. Together with the transporter NisT, these form a membrane-bound lanthionine synthetase complex, responsible for producing the PTMs found in mature nisin.[70]

An overview of the nisin biosynthetic sequence is given in **Scheme 1.1**. After the ribosomal synthesis of NisA, the dehydratase NisB produces the dehydro residues Dha and Dhb from eight serine and threonine residues of the propeptide, followed by the regio- and stereoselective Michael addition of Cys residues onto the dehydro residues to form the thioether bridges of Lan and MeLan, catalysed by the cyclase NisC. The peptide is then translocated across the cell membrane by NisT,[71] and the leader sequence cleaved by the extracellular serine protease NisP, giving WT nisin (**13**).[72]



Scheme 1.1: A. Overview of the biosynthesis of WT nisin A. **B.** Formation of lanthionine (R = H) and methyllanthionine (R = CH_3) by dehydration of Ser or Thr, followed by nucleophilic attack by the Cys thiol.

The nisin gene cluster also encodes a number of other proteins relating to immunity and gene regulation. Four of these, NisI, a lipoprotein, and NisF, NisE and NisG, which together form an ATP-dependant transporter,[73] are responsible for immunity of the producing cell to nisin.[13] NisI is located outside the cytoplasmic membrane, and binds to incoming nisin to prevent it accessing the cytoplasmic membrane, whilst NisFEG transports nisin that has already reached the membrane back into the extracellular space. Stein *et al.* have shown that these proteins work independently but cooperatively, with full nisin immunity only seen when both genes are expressed. Interestingly, immunity is specific to nisin and non-transferrable, even to other very similar lantibiotics in the same class, such as subtilin.[74] Production of the immunity proteins, as well as nisin itself, is regulated by a two-component system formed of the NisR and NisK proteins. In response

to extracellular signals, NisK phosphorylates NisR, activating the transcription of nisin production and immunity proteins.[37,75]

1.3.2. Nisin Mode of Action

Antimicrobial peptides such as nisin carry a net positive charge at neutral pH due to the high number of lysine and arginine residues they contain. Thus, they are attracted to the surface of Gram-positive bacteria, which are covered in negatively charged teichoic acids, over the neutral cell membranes of mammalian cells.[15,76] Underneath the teichoic acids, Gram-positive bacteria have a layer of peptidoglycan up to 80 nm thick: a matrix of sugar backbones crosslinked by peptide chains which provides rigidity and strength to the cell, preventing cell swelling and protecting the plasma membrane of the bacterium from lysis (**Figure 1.7**).[77,78]

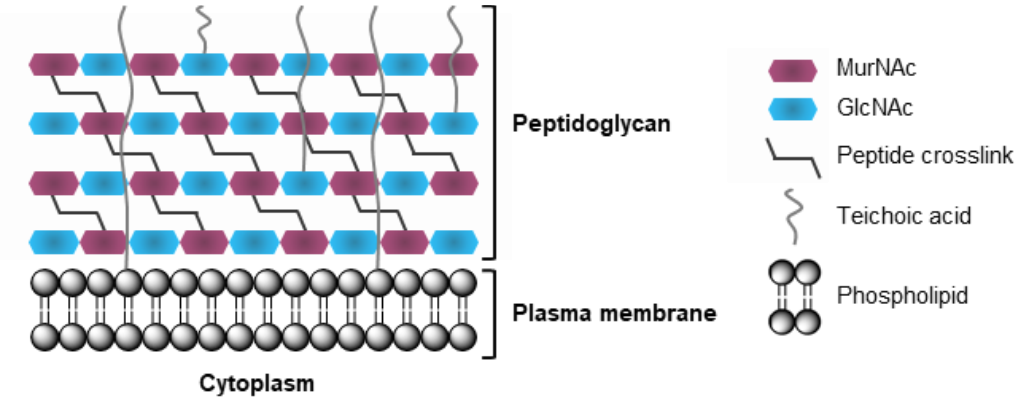


Figure 1.7: The makeup of the Gram-positive cell envelope.

It has been known for many years that the treatment of Gram-positive bacteria with nisin causes disruption of the plasma membrane, leading to rapid efflux of compounds such as amino acids from the cytosol and complete collapse of membrane potential in less than one minute.[79] Another long-established activity of nisin is inhibition of peptidoglycan biosynthesis.[80] Peptidoglycan synthesis starts in the cytosol with the assembly of the disaccharide and pentapeptide peptidoglycan subunit (**24**), which is linked *via* a pyrophosphate to a membrane-bound polyisoprenoid carrier (**25**) (**Figure 1.8**). Firstly, the pentapeptide-MurNAc unit (**26**), activated by uridine diphosphate (UDP), is added to the isoprenoid, creating intermediate lipid I (**27**). A second activated sugar, UDP-GlcNAc, is then added, creating the lipid II intermediate (**28**) (see **Figure 1.8 B** for chemical structure), which is translocated to the extracellular side of the lipid membrane where the disaccharide is incorporated into the growing peptidoglycan chain.[81] In 1980,

Reisinger *et al.* were able to show that the mechanism of nisin inhibition on this process was by complex formation with both lipid I and lipid II.[82]

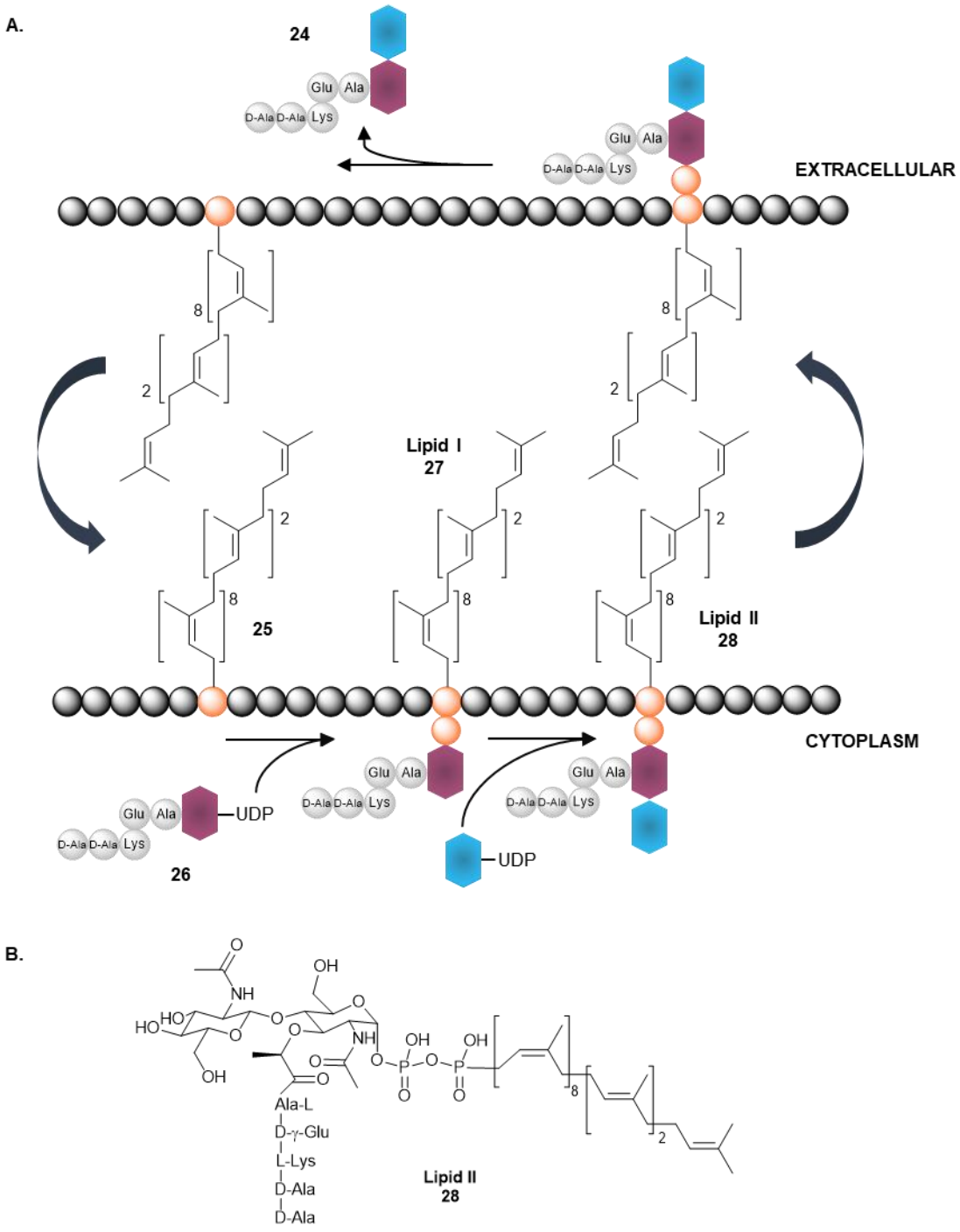


Figure 1.8: A. Peptidoglycan biosynthesis. Orange spheres correspond to phosphate, other fragments are colour-coded as described in **Figure 1.7**. **B.** Chemical structure of lipid II.

A more complete picture of the mode of action of nisin was provided by Breukink *et al.*, who showed by competition experiments with vancomycin that the 'mediator' of the

nisin-membrane interaction was lipid II.[83] This was confirmed by treatment of lipid-II-enriched vesicles with nisin, in which a strong positive correlation was observed between the concentration of lipid II in the membrane, and the leakage of an encapsulated fluorescent dye (carboxyfluorescein). This work also accounted for the discrepancy in the activity of nisin in whole cells (low nM) compared to the activity observed in artificial membranes or vesicles composed only of lipid extracts (μM range).[84]

Thus, in the absence of lipid II, nisin exerts its antimicrobial effect in a similar manner to many other antimicrobial peptides: the peptide aggregates in the membrane of the bacteria and forms a transient pore.[76] In the presence of lipid II however, nisin has a two-fold effect.[85] The primary mechanism is the rapid and efficient formation of specific pores, of which lipid II is an intrinsic component.[85,86] Nisin first docks to lipid II, followed by insertion of the pore-forming tail region into the plasma membrane (**Figure 1.9 A**). Four of these 1:1 nisin:lipid II complexes then associate with four additional nisin molecules to form a stable 8:4 pore structure (**Figure 1.9 B**).[87,88] Interestingly, two other lipid II binding lantibiotics, haloduracin and lacticin 3147, have also recently been shown to bind lipid II in this 2:1 ratio.[89,90] The second antimicrobial effect of nisin is a sequestration mechanism, in which the A and B rings of nisin bind to lipid II in the membrane and prevent it from being localised where it is required for cell wall biosynthesis, hence killing the bacterium. This sequestration is the main mechanism of action for lipid II-binding lantibiotics which are too small to span the lipid membrane, and hence are unable to form pores, such as mutacin 1140 (**21**) (see **Figure 1.10** for structure of mutacin 1140).[91]

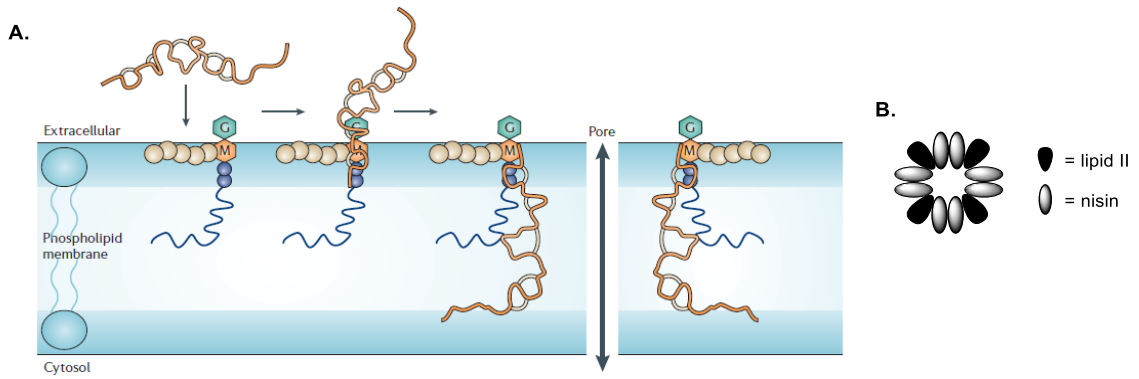


Figure 1.9: A. Formation of the nisin:lipid II pore,[92] and **B.** schematic of the structure of the pore formed by nisin and lipid II.

Unlike other non-lantibiotic antimicrobial peptides known to bind lipid II, NMR studies of nisin and lipid II conducted by Hsu *et al.* showed that nisin binds to lipid II at the

pyrophosphate moiety (see **Chapter 1.7.2** for a discussion of this work). The binding of nisin to the lipid II pyrophosphate has more recently been confirmed by 't Hart *et al.*, who studied the binding interaction of nisin with lipid II analogues using isothermal calorimetry (ITC) experiments. This study also revealed that the pentapeptide portion of lipid II experiences a reduction in mobility following pore formation, suggesting that although not directly involved in binding nisin, the pentapeptide may confer a degree of stability to the pore complex.[93] Support for this theory is provided by MD simulations conducted by Mulholland *et al.*, who showed that the *N*-terminus of a second nisin molecule docked onto the nisin:lipid II complex interacts with lipid II at the *C*-terminus of the pentapeptide. Additionally, in half of the trajectories calculated, the second nisin is embedded in the plasma membrane, leading the authors to hypothesise that this may be the first step in pore formation.[94] Other MD simulations conducted by Koch *et al.* have revealed that on binding to nisin, the isoprenyl tail of lipid II extends further into the membrane towards the cytosol, forming a 'scaffold-like structure' which the pore complex may assemble around.[95]

It has been suggested that lipid II is an ideal drug target, as it is essential for cell wall biosynthesis and present in low concentration in the cell relative to the amount of peptidoglycan: there are only 2000 copies of lipid II per bacterial cell.[92] In addition, as mammalian cells have no cell wall and hence no requirement for lipid II, it is likely that any drugs developed to specifically bind lipid II will have fewer off-target effects in humans.[92] The pyrophosphate-binding mechanism of nisin is especially advantageous and decreases the likelihood of development of nisin resistance,[96] as mutation of the pyrophosphate configuration by the target bacteria is unlikely.[93,97,98] Indeed, naturally-occurring nisin resistance is low, and generation of resistance is difficult for the target cell due to nisin's two-fold mechanism of action.[99] However, experiments attempting to induce resistance by repeated exposure to sub-MIC concentrations of nisin have shown that bacteria can develop a tolerance to it, usually either by changing the thickness and charge of the cell wall to prevent nisin reaching the membrane, or by alteration of the membrane lipid composition.[99,100] General bacterial resistance mechanisms, such as biofilm formation, have also been shown to confer nisin resistance,[62,99] as well as more specific innate mechanisms such as proteolytic degradation of nisin by nisin resistance protein,[101] reduction of the *C*-terminal Dha to Ala by nisinase,[99] and removal of nisin from the membrane by a transporter, as occurs in nisin-producing strains as part of their

host immunity.[73] A more detailed discussion of lantibiotic resistance mechanisms can be found in the review published by Draper *et al.*.[99]

1.3.3. Lantibiotics with Nisin-Like AB Ring Bridging Patterns

Several other naturally-occurring lantibiotics bear the same *N*-terminal AB ring bridging pattern as nisin (**Table 1.2**), and it is believed that they are all able to bind lipid II in the same manner.[91] Despite this common *N*-terminus, there are large *C*-terminal differences between the peptides. The same *C*-terminal bridging pattern is observed between subtilin (**14**),[47] ericin S[102] and the nisin variants,[53–58] whereas epidermin (**15**), gallidermin,[103] Bsa (**29**)[104] and the mutacins[105–107] have a *C*-terminal aminovinylcysteine (**12**). Ericin A[102] and microbisporicin (**22**)[42] have unique *C*-terminal bridging patterns.

	Residue											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Nisin A, Z, F and Q	I	Dhb	Lan	I	Dha	L	Lan	MeLan	P	G	MeLan	K
Nisin U	I	Dhb	Lan	K	Dha	L	Lan	MeLan	P	G	MeLan	K
Nisin U2	N	Dhb	Lan	K	Dha	L	Lan	MeLan	P	G	MeLan	K
Nisin P	V	Dhb	Lan	K	Dha	L	Lan	MeLan	P	G	MeLan	K
Nisin H	F	Dhb	Lan	I	Dha	M	Lan	MeLan	P	G	MeLan	K
Subtilin	W	K	Lan	E	Dha	L	Lan	MeLan	P	G	MeLan	V
Epidermin	I	A	Lan	K	F	I	Lan	MeLan	P	G	MeLan	A
Gallidermin	I	A	Lan	K	F	L	Lan	MeLan	P	G	MeLan	A
Mutacin I	F	Dha	Lan	L	Dha	L	Lan	Lan	L	G	Lan	T
Mutacin III	F	K	Lan	W	Dha	L	Lan	MeLan	P	G	MeLan	A
Mutacin 1140	F	K	Lan	W	Dha	L	Lan	MeLan	P	G	MeLan	A
Mutacin B-Ny266	F	K	Lan	W	Dha	F	Lan	MeLan	P	G	MeLan	A
Ericin A	V	L	Lan	K	Dha	L	Lan	MeLan	P	G	MeLan	I
Ericin S	W	K	Lan	E	Dha	V	Lan	MeLan	P	G	MeLan	V
Bsa	I	Dhb	Lan	H	Dha	L	Lan	MeLan	P	G	MeLan	A
Microbisporicin	V	Dhb	Lan	Cl-W	Dha	L	Lan	MeLan	P	G	MeLan	T

Table 1.2: Comparison of the AB rings of naturally-occurring nisin-like lantibiotics.

These differences in *C*-terminal bridging result in distinct mechanisms of action. The longer peptides which have a nisin-like *C*-terminus are able to form specific pores with lipid II,[47] whereas the shorter peptides with an epidermin-like structure do not cause pore formation, but instead prevent cell wall biosynthesis by sequestration of lipid II as previously discussed.[91] Studies with mutacin 1140 (**21**) by Smith *et al.* have shown that these shorter peptides have the same affinity for lipid II as nisin. However,

pyrene-labelled lipid II experiments indicate that the resulting complexes are different with each peptide, with the lipid II molecules further apart in the mutacin 1140 complex.[46]

1.3.3.1. Mutacins

The largest sub-family of peptides with a nisin-like lipid II binding region are the mutacins (**Figure 1.10**). These are a family of class I lantibiotics produced by *Streptococcus mutans* species, a primary causative agent of tooth decay in humans owing to their ability to form biofilms on the surface of tooth enamel.[107–110]

Mutacin B-Ny266 (**30**) was the first lantibiotic mutacin to be isolated, and was named after the producing strain: *S. mutans* Ny266.[105] Excellent antimicrobial activity has been reported for the peptide across a broad range of Gram-positive pathogens, and *in vivo* experiments have shown it to be as active as vancomycin against MRSA.[111] Mutacin 1140 (**21**), first isolated from *S. mutans* strain JH1000,[106] has also shown good activity against a range of Gram-positive organisms *in vivo*, and is currently undergoing preclinical testing.[40] Mature mutacin III, isolated from *S. mutans* strain UA787, has the same structure as mutacin 1140 (**21**), though is classified as a different lantibiotic as its dehydration enzyme is drastically different to that which produces mutacin 1140.[45]

Mutacins B-Ny266 (**30**), 1140 (**21**) and III all have the epidermin-like *C*-terminal bridging pattern, with four residues forming the flexible region between the B and C rings (**Figure 1.10** and **Figure 1.4**). Although it shares the same bridging pattern, mutacin I (**31**), produced by *S. mutans* strains CH43 and UA140 and characterised by Qi *et al.*, has two more residues in the central flexible region than the other mutacins.[107,109] Additionally, unlike the other nisin-like lantibiotics which have a Lan forming ring A and a MeLan forming ring B, ring B of mutacin I (**31**) is also formed by a Lan residue. This may potentially be explained by the fact that the mutacin I dehydratase *mutB* appears not to produce Dhb residues from Thr in the propeptide, leaving Thr12 in mutacin I (**31**) undehydrated.[107] If so, this would mean that formation of MeLan bridges with the mutacin I biosynthetic machinery may not be possible. Further differences are that in mutacin I (**31**) the two dehydro residues are both Dha, unlike in other nisin-like peptides with two dehydro residues which have one Dha and one Dhb, and that it bears a Leu rather than a Pro residue at position 9 (**Table 1.2**). These small structural simplifications to the AB rings are interesting from a synthetic perspective, as the presence of only one kind of

thioether bridge and one kind of dehydro residue implies that mutacin I (**31**) may be easier to produce using SPPS methods than many of the other peptides listed.

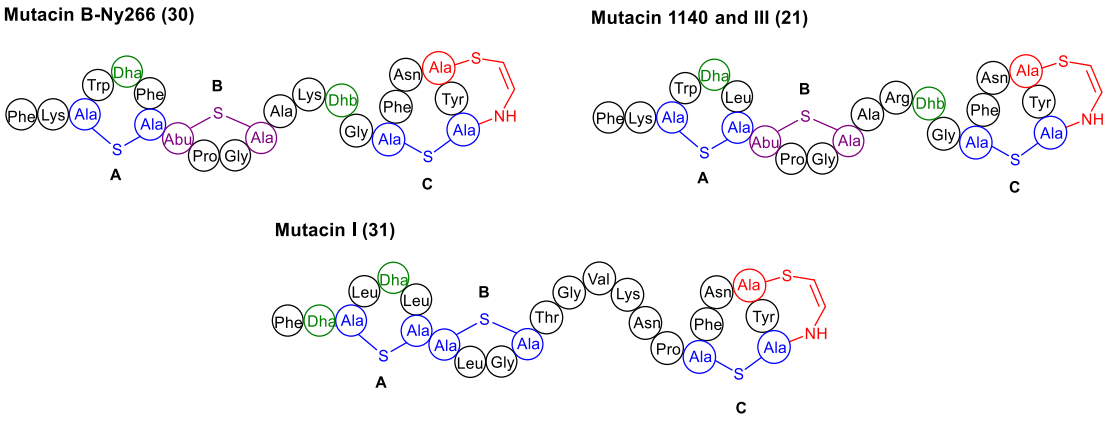


Figure 1.10: Structures of mutacin B-Ny266 (**30**), mutacin 1140/III (**21**), and mutacin I (**31**).

1.4. Synthesis of Lantibiotic Peptides

The production of nisin and other lantibiotics poses a significant challenge due to their highly modified structure and oxidative instability. To date, only a limited number of effective approaches have been established.[112] What follows is a brief overview of the most commonly utilised biosynthetic and chemical methods for production of native and analogue lantibiotic sequences; for more extensive reviews the reader is referred to reviews by Tabor,[112,113] Knerr *et al.*,[38] and Field *et al.*.[114,115]

1.4.1. Biosynthesis of Lantibiotics

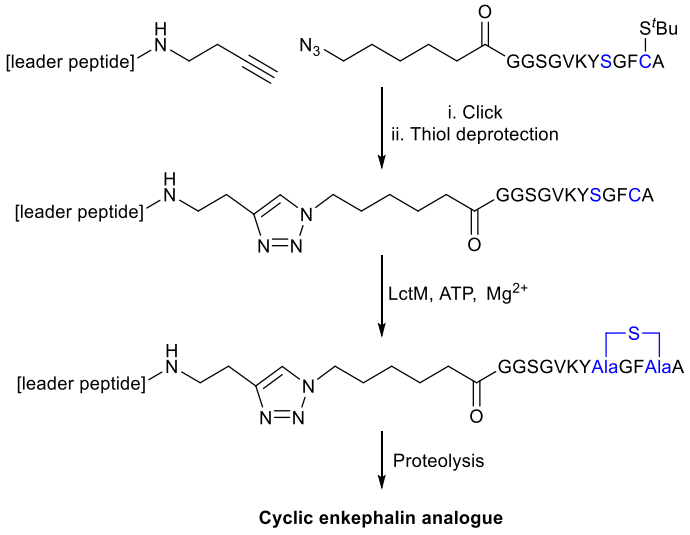
There are two key biosynthetic approaches to lantibiotic production: i) *in vitro* engineering, which uses isolated lantibiotic modification enzymes in biotransformations with linear peptides, and ii) *in vivo* engineering, in which mutagenesis of the lantibiotic pre-peptide gene leads to the production of mutant lantibiotics in whole cell systems.

Both of these techniques offer the capability to produce a huge variety of novel lanthipeptides, as well as lantibiotic analogues with improved physiochemical properties, such as increased stability and solubility at physiological pH, and lower susceptibility to proteolytic digestion.[114] However, one drawback of the biosynthetic approach is that it remains difficult to predict which PTMs will occur in a given system,[116] despite the fact that much more is now understood regarding how the pre-peptide sequence and modification enzymes determine ring topology (**Chapter 1.3.1**).[58]

1.4.1.1. In vitro Engineering

The use of class II lantibiotic synthetases for *in vitro* engineering is common, as one enzyme is able to conduct both the dehydration and cyclisation of the peptide substrate.[39] Additionally, a number of these synthetases, for example the prochlorosin synthetase ProcM and the lacticin 481 synthetase LctM, have been shown to be naturally promiscuous, and are able to turn over diverse propeptide sequences.[117,118]

Leader recognition is important for the function of lantibiotic modification enzymes, so the propeptide must be linked to the leader peptide before the biotransformation can occur.[119] One method of achieving this is to create a natural peptidic linkage between the two peptides, such as results from the expression of mutant linear peptides in *E. coli*.[120,121] Another method used to fuse the propetide to the leader peptide is *via* a non-peptidic linkage, commonly a triazole, formed from the Cu^{I}-catalysed click reaction of an azide and alkyne.[122] This is a particularly useful strategy if the propeptide is synthesised by SPPS, as the necessary azide or alkyne moieties can be easily incorporated using commercially-available reagents. Levengood *et al.* employed this technique to produce cyclic and dehydro-containing analogues of naturally-occurring bioactive peptides using LctM (**Scheme 1.2**).[123] In addition, synthesising the desired propeptide by SPPS allows the introduction of non-proteinogenic amino acids such as β-amino acids and *N*-alkylated residues, hence expanding the number of lantibiotic peptides accessible by biosynthetic methods.[124]



Scheme 1.2: Attachment of the leader sequence to the propeptide *via* a triazole linkage.

The semi-synthesis of hybrid lantibiotics also utilises biotransformation to produce novel peptides. However, in this approach digestion enzymes such as trypsin and chymotrypsin are used to produce fragments of the lantibiotic peptide for subsequent chemical modification or ligation (**Figure 1.11**). There are many examples of nisin semi-synthesis in the literature which have produced lantibiotics with improved physiochemical properties, whilst maintaining antibacterial activity. For example, Slootweg *et al.* have produced a hybrid nisin analogue by coupling the native ABC ring fragment to a synthetic analogue of the DE rings,[125] and Koopmans *et al.* produced semi-synthetic lipopeptides by coupling the WT AB rings of nisin to a variety of lipid tails.[126]

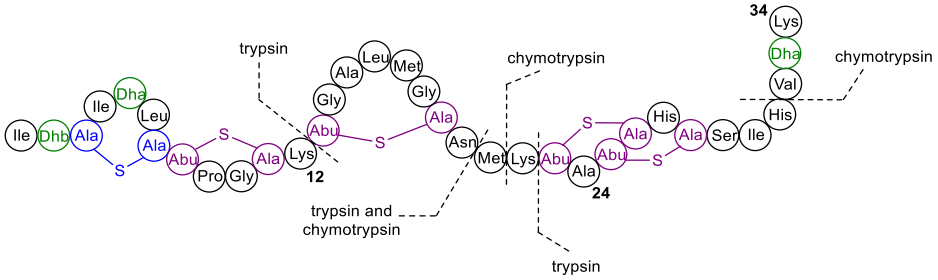


Figure 1.11: Sites at which nisin may be cleaved by digestion enzymes.

1.4.1.2. In vivo Engineering

The gene-encoded nature of lantibiotics means that they are amenable to bioengineering.[115] Numerous biosynthetic studies on nisin have been reported, in which the desired changes are made to the nisin propeptide by site-directed mutagenesis, and the resultant pre-peptide is expressed, modified and exported by an *L. lactis* host strain. Several of the produced mutants even display an improved bioactivity over native nisin. For example, Field *et al.* produced three nisin analogues with a single mutation at position 29 (S29A, S29D and S29E) which have enhanced activity against both Gram-positive and Gram-negative pathogens,[127] and two hinge region mutations, M21V and K22T, which displayed increased activity against a range of Gram-positive bacteria, including MRSA.[128] Further mutagenesis studies on the hinge region by Healy *et al.* and Zhou *et al.* have shown that analogues where residues 20-22 are simultaneously mutated can display increased activity, but that changing the number of residues in this region is detrimental to bioactivity.[129,130] Rink *et al.* have also conducted SAR studies on the lipid II binding region of nisin, finding that changing the identity of ring A residues was well tolerated, and could even enhance activity, but that bulky residues in ring B abolished all activity.[131]

In addition to producing nisin mutants, NisB and NisC have been successfully used *in vivo* to produce the PTMs in class II lantibiotic peptides with no sequence homology to nisin,[132] and to introduce dehydro residues or lanthionines into non-lantibiotic peptides, including bioactive peptides such as enkephalin (**32**), vasopressin (**33**),[133,134] and angiotensin 1-7 (**34**), leading to the production of analogues with increased stability and bioactivity (**Figure 1.12**).[135] Zambaldo *et al.* have also shown that the nisin modification enzymes will turn over mutants of the propeptide containing unnatural amino acids, enabling the production of lantibiotics with novel ring topologies.[136] Further diversity and an expanded range of possible PTMs can also be generated in the mature peptide by using combinations of lantibiotic modification enzymes.[116] This was demonstrated in work by van Heel *et al.*, who showed that using a combination of NisBCT and either the lacticin 3147 reductase LctJ or gallidermin decarboxylase GdmD, it is possible to generate new-to-nature lanthipeptides.[137]

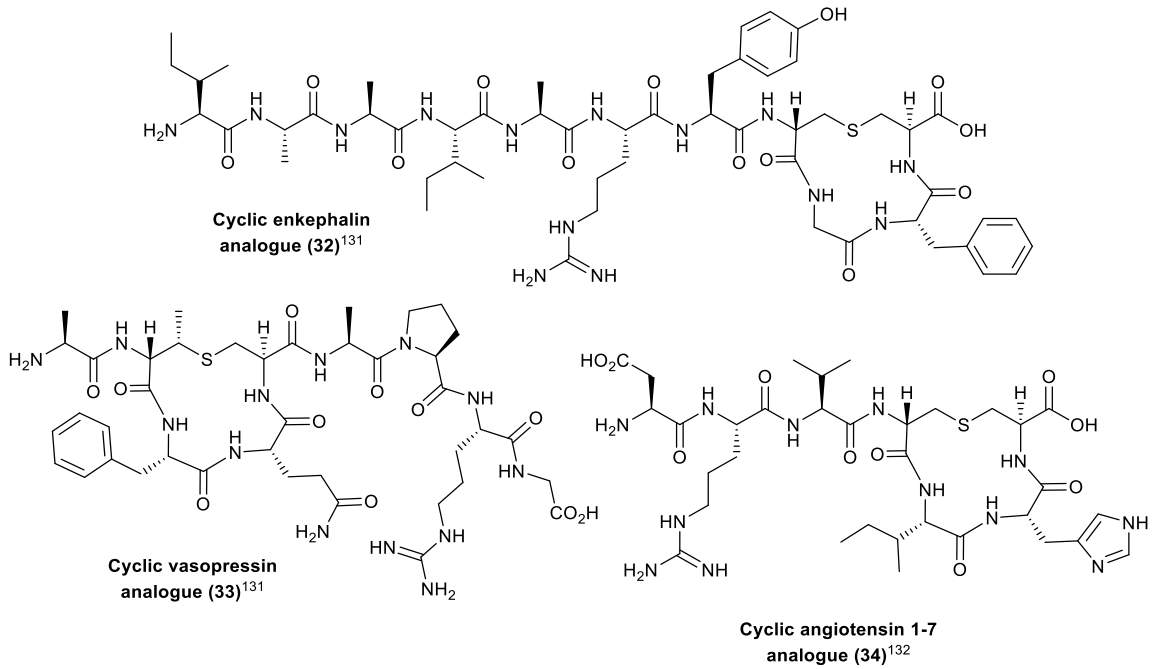


Figure 1.12: Cyclic variants of bioactive non-lantibiotic peptides.

Another use for *in vivo* engineering is the expression of new lantibiotics found from genome mining. In this technique, the genomes of prokaryotic organisms are scanned for conserved regions of lantibiotic synthetases, such as the common leader peptide recognition motif,[119] in an attempt to find novel lantibiotic propeptides.[138] Once an expected lantibiotic gene has been identified, the desired peptide can either be isolated from the producing strain, or the propeptide gene can be fused to the nisin leader peptide

and cloned into a production host expressing NisBCT, leading to the production of a novel mature lantibiotic.[116,139] Examples of lantibiotics identified in this fashion are Bsa (bacteriocin of Staphylococcus aureus, **29**), which is produced by many community acquired *S. aureus* strains,[104] lichenicidin (**35**), a two-component lantibiotic which was isolated following a screen for LanM-type synthetases,[140] and venezuelin (**20**), produced by the class IV synthetase LanL (**Figure 1.13**).[141]

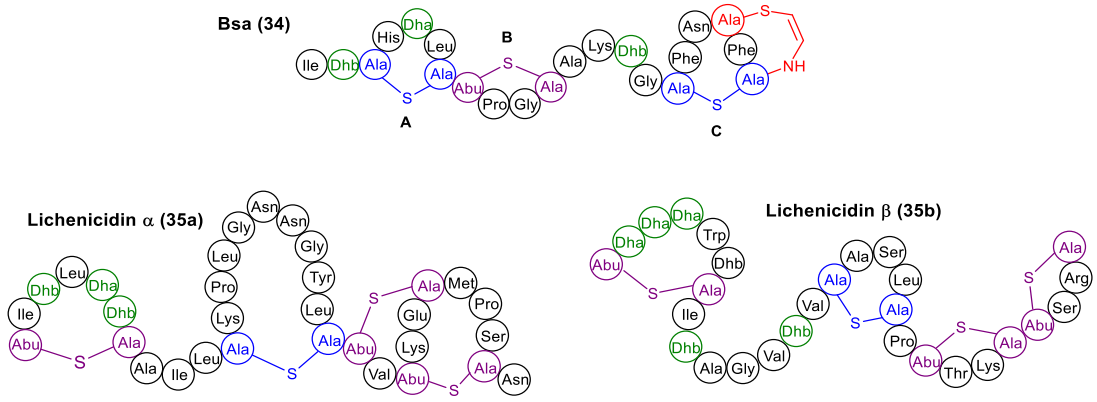


Figure 1.13: Lantibiotics identified from genome mining. Venezuelin (**20**) shown in **Figure 1.4**.

One of the major problems with the biosynthesis of lantibiotics *in vivo* is that host strains often produce very low levels of peptide, and the triggers for lantibiotic production are still not fully understood.[7,142] However, better methods for forcing overproduction have now been established,[143] and fermentation of the producing organism remains the most viable method for the large-scale production of lantibiotics necessary for commercial applications.[40]

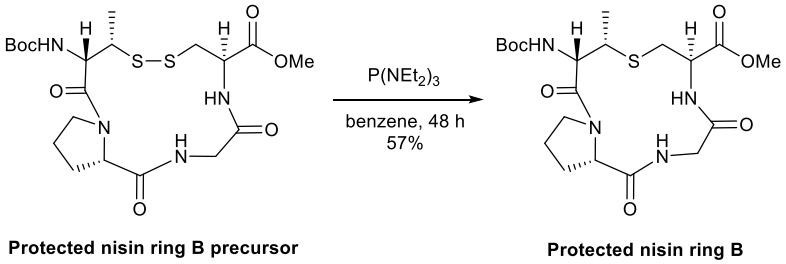
1.4.2. Chemical Synthesis of Lantibiotics

Chemical synthesis of lantibiotics, although currently much more expensive than biosynthesis due to the high cost of the reagents, is a convenient and well-established method for the synthesis of both native sequences and analogues containing unnatural amino acids and novel bridging architectures.[144] Additionally, molecular labels such as fluorophores and radioisotopes can be readily incorporated into peptides using solid-phase strategies, whilst allowing the facile production of smaller subunits for use in SAR studies.[112] Many different approaches to lantibiotic synthesis have been reported in the literature, which differ mainly in the method of lanthionine bridge formation. Three of the most important and frequently used methods will be briefly discussed here.

1.4.2.1. Lanthionine Formation by Desulfurisation

In 1988, Fukase *et al.* completed the first total synthesis of a lantibiotic peptide with their synthesis of WT nisin. The synthetic route involved the individual formation of each of the lantibiotic rings, except the overlapping D and E rings which were synthesised as a single peptide. This was followed by the stepwise solution state coupling of the fragments, forming full-length nisin.[145,146]

The synthesis of the lanthionine residues in the work of Fukase *et al.* was achieved by the desulfurisation of disulfide bridged cyclic peptides with $P(NEt_2)_3$ (**Scheme 1.3**),[147] as first described by Harpp and Gleason in their preparation of an optically pure L,L-lanthionine.[148]



Scheme 1.3: Fukase *et al.* synthesis of nisin ring B by desulfurisation.

1.4.2.2. Biomimetic Lanthionine Formation

Another reasonably well explored method of lantibiotic synthesis is a biomimetic approach, in which the thioether bridges of the Lan and MeLan residues are formed by the Michael addition of a Cys residue onto a dehydro residue (**Scheme 1.1 B**). However, a lack of selectivity is observed in the spontaneous non-enzymatic cyclisation of some lantibiotic propeptides, where mixtures of stereoisomers result for all rings with three or more residues separating the reacting Cys and Dha residues (e.g. nisin rings A and C).[133] On the other hand, if only two residues separate the reacting Cys and Dha (e.g. nisin ring B), the cyclisation has been shown to proceed in a stereoselective manner, producing exclusively the native Lan and MeLan (2*S*, 6*R*) configuration. This is perhaps unexpected due to the absence of any external chiral influence on the reaction, though a possible explanation is that a small and constrained peptide may be pre-organised in such a way that only one stereochemical outcome is possible.[149]

For example, in the first reported use of a biomimetic method for the preparation of a lanthionine-containing peptide, Polinsky *et al.* described the synthesis of a

thioether-bridged analogue of enkephalin (**36**), an opioid peptide, in which the new stereocentre was generated with exclusively DL chirality (**Figure 1.14**).[150] This surprising stereoselectivity in the Michael addition step has since also been demonstrated in the biomimetic formation of several small class I lantibiotic rings, including Lan-containing analogues of epidermin ring B,[149] nisin ring B,[151] and subtilin rings B and E,[152,153] and even in the biomimetic reaction of a Cys and Dhb to form WT subtilin ring B with the native (2*S*, 3*S*, 6*R*) MeLan geometry.[154]

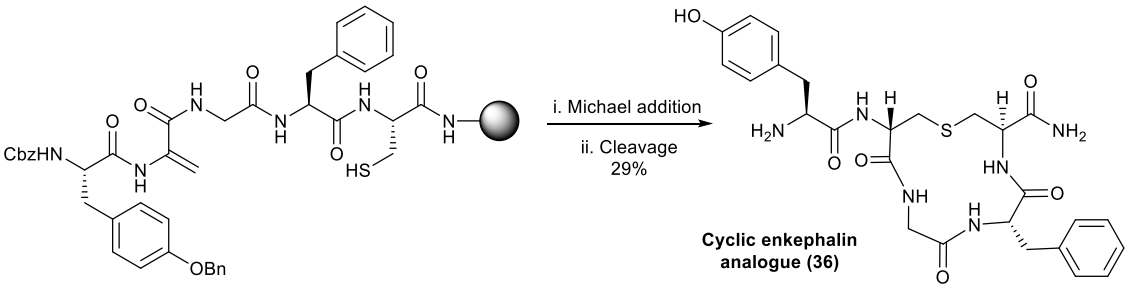


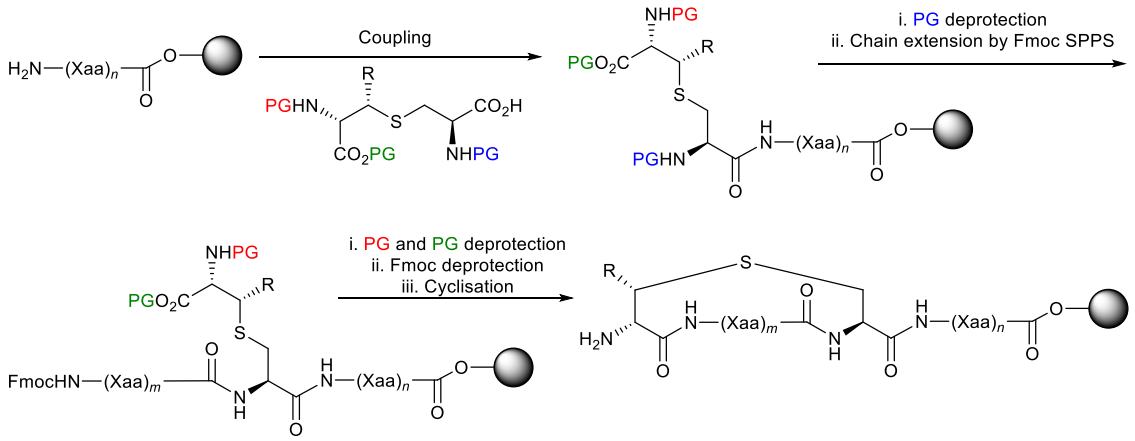
Figure 1.14: Chemical synthesis of Lan-containing enkephalin by Michael addition.

1.4.2.3. Incorporation of Orthogonally Protected Lanthionines into SPPS

A widely used synthetic approach for the preparation of WT and analogue lantibiotics is SPPS, where the thioether bridges of Lan and MeLan are installed into the growing linear peptide chain as pre-formed bis-amino acids. Orthogonal protection of these residues ensures the correct regioselectivity in cyclisation (**Scheme 1.4**). For use with Fmoc SPPS, one side of the bis-amino acid must be Fmoc protected, and the other must have protecting groups which are both stable to the piperidine Fmoc deprotection conditions, and which are not acid labile so that premature cleavage of the peptide from the resin is avoided. One of the key advantages of this strategy over the biomimetic method is that the stereochemistry of (methyl)lanthionine chiral centres is pre-set.

The first example of a lanthionine-containing peptide synthesis using this method was reported by Ösapay and Goodman, in which five thioether bridged analogues of bioactive compounds such as enkephalin and somatostatin were prepared using Boc SPPS.[155] Following this, several syntheses of lantibiotic peptides have been reported using an (Alloc, Allyl/Fmoc) protected Lan (**37**) (**Figure 1.15**) in conjunction with Fmoc SPPS.[153-157] Mitchell later employed a (Teoc, TMSE/Fmoc) Lan (**38**) (**Figure 1.15**) in the synthesis of two novel analogues of nisin rings AB (**Figure 1.15 B**), in which both rings were formed by a lanthionine bridge, and the dehydro residues replaced with either

hydrated (**39**) or saturated analogues (**40**) (a discussion of the synthesis of these peptides can be found in **Chapter 4.3**).[161]



Scheme 1.4: Synthesis of lantibiotic rings using orthogonally protected (methyl)lanthionines. Lan R = H, MeLan R = CH_3, PG = protecting group.

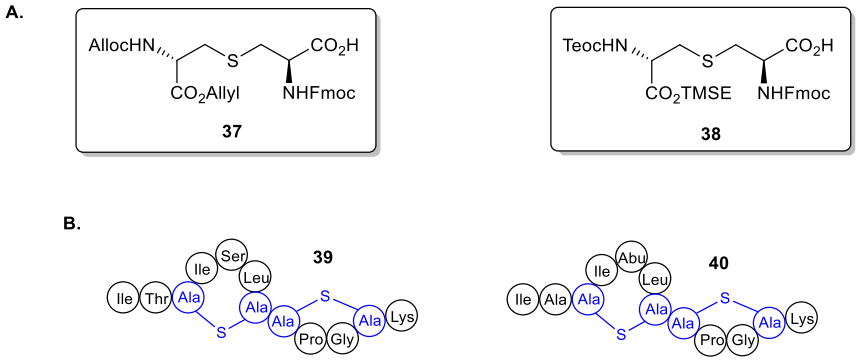
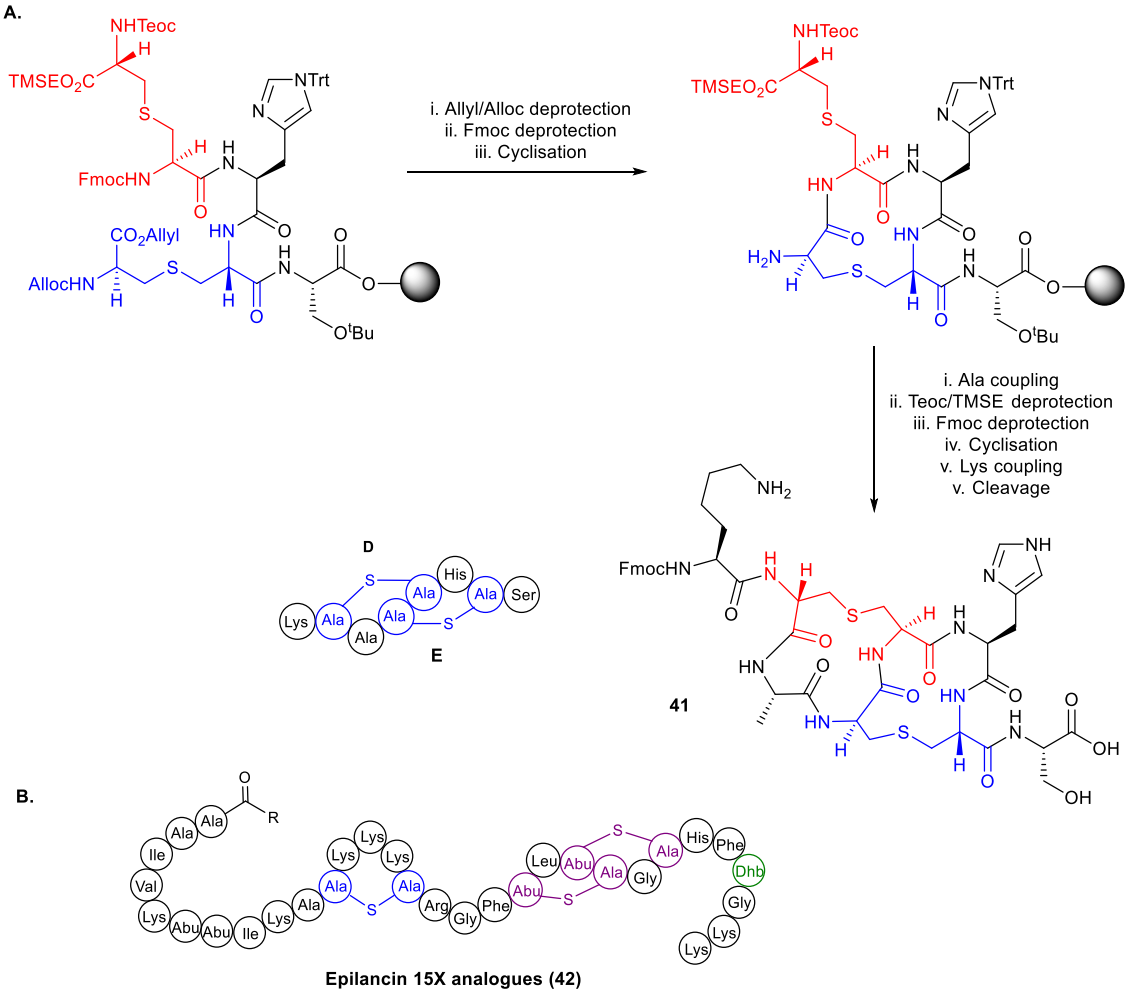


Figure 1.15: A. Structures of commonly used orthogonally protected lanthionines, and **B.** Mitchell *et al.* nisin ring AB analogues **39** and **40**.

Solid-phase techniques with a second orthogonally protected (methyl)lanthionine have also been applied to the more complex synthesis of overlapping lantibiotic rings. In this case, the sequential removal of two different sets of orthogonal protection is required in order to facilitate the regioselective cyclisation of the two interlocking lanthionines. For example, Mothia *et al.* used Alloc/Allyl Lan (**37**) in conjunction with Teoc/TMSE Lan (**38**) to synthesise a nisin ring DE analogue (**41**) (**Scheme 1.5 A**).[162] This technique has also been employed by Knerr *et al.* to prepare WT and analogues of lacticin 481 (**17**), as well as analogues of epilancin 15X (**42**) (**Scheme 1.5 B**),[163,164] while Liu *et al.* have prepared both components of WT lacticin 3147 (**18**).[165]



Scheme 1.5: A. Synthesis of nisin rings DE (**41**) using two orthogonally protected lanthionines. **B.** Structure of epilancin 15X analogues. R = COMe or CH(OH)Me.

1.4.2.4. Use of Lanthionine Mimics in SPPS

SPPS with orthogonally protected bis-amino acids is also a popular method for the synthesis of lantibiotic peptide analogues with more reductively stable bridges. For example, 4-oxa-2,6-diaminopimelic acid (**43**), in which the Lan sulfur atoms are replaced by oxygen atoms, has been used by Liu *et al.* in the synthesis of oxa-lacticin 3147 A2,[166] and diaminopimelic acid (**44**), the all-carbon analogue of Lan, has been used by Ross *et al.* in the synthesis of a lactocin S analogue (**Figure 1.16**).[167]

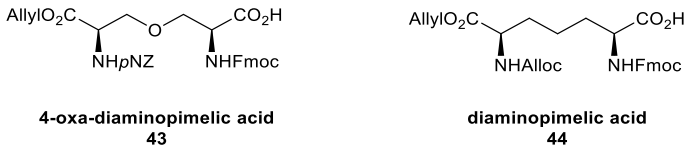
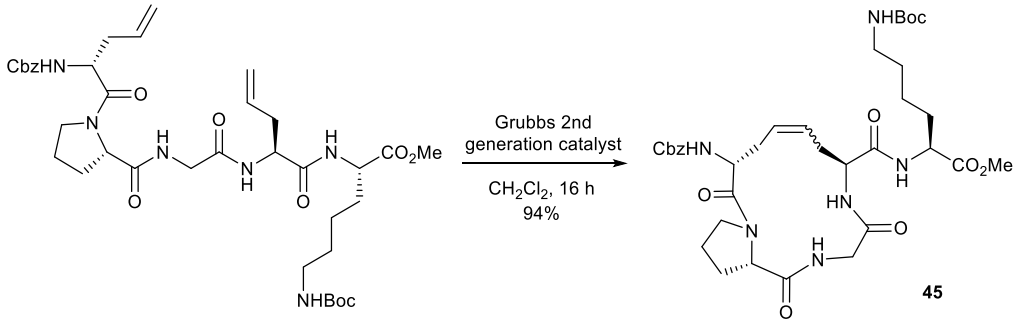


Figure 1.16: Structure of orthogonally protected Lan alternatives.

Extensive work has also been conducted on the generation of unsaturated carbocyclic bridges using ring-closing metathesis (RCM). Contrary to the previously described methods, in this strategy it is necessary to install the two sides of the bridge separately, before treatment with Grubbs second-generation catalyst to generate ring-expanded analogues of the lanthionine-containing peptide. Pattabiraman *et al.* employed this methodology on-resin in order to synthesise a carbocyclic lacticin 3147 A2 analogue,[168] and Liskamp *et al.* have synthesised several alkene (**45**) and alkyne bridged nisin fragments (**Scheme 1.6**).[169–172] Reduction of the alkene bridged ring AB analogue to the corresponding saturated peptide by treatment with H_2 and Pd/C also revealed that carba-bridged analogues are able to retain their lipid II binding affinity, demonstrating their utility as lanthionine isosteres.[173,174]



Scheme 1.6: Synthesis of ring-expanded nisin ring B analogue (**45**) by RCM.

1.5. Syntheses of Orthogonally Protected Lanthionine and Methyllanthionine

Many different approaches towards the synthesis of protected (methyl)lanthionines have been reported, some of the earliest examples of which involve the desulfurisation of disulfide bonds using an aminophosphine. For example, $P(NEt_2)_3$ has been used by Cavelier-Frontin *et al.* to produce a protected D,L-lanthionine,[175] and by Olsen *et al.* to produce an L,L-lanthionine with four-fold orthogonal protection (**46**) from the corresponding disulfide (**47**) in 52% yield (**Figure 1.17**).[176]

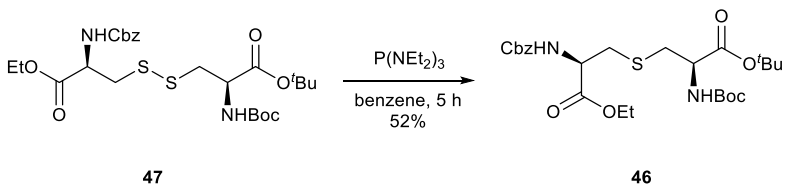


Figure 1.17: Olsen *et al.* preparation of lanthionines by desulfurisation with $P(NEt_2)_3$.

Biomimetic approaches to the synthesis of orthogonally protected lanthionines have also been described. This approach is not stereospecific when conducted on isolated dehydro and Cys residues, and results in mixtures of diastereomers, as demonstrated by Probert *et al.* in their synthesis of an orthogonally protected lanthionine (**48**) from Boc-Dha-OMe and Fmoc-Cys-OAllyl (**Figure 1.18**).[177] More recently however, Aydillo *et al.* developed a method for the asymmetric Michael addition of Cys onto dehydro residues using chiral auxillaries.[178]

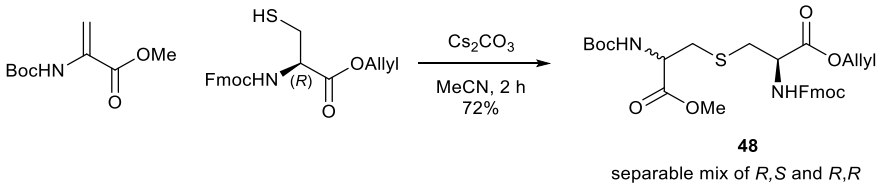
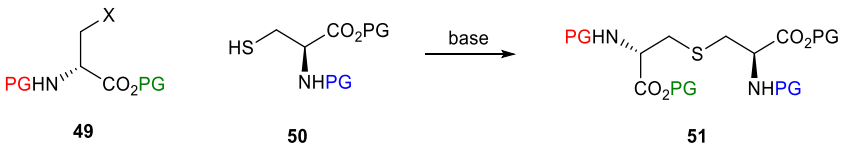


Figure 1.18: Lanthionine formation by Michael addition of Cys onto Dha residues.

Two of the most commonly utilised synthetic approaches, and the methods utilised for (methyl)lanthionine synthesis in this work, involve the reaction of a cysteine with either a β-haloalanine, or a cyclised D-Ala precursor such as an aziridine, lactone or sulfamidate. A brief overview of these two main methods follows, for a more detailed discussion the reader is referred to reviews by Tabor.[112,113]

1.5.1. Synthesis of (Methyl)Lanthionines *via* β-Haloalanines

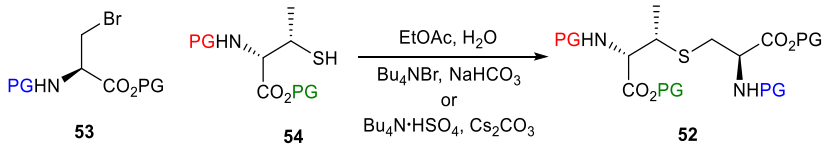
The coupling of β-haloalanine (**49**) and cysteine (**50**) residues under basic conditions has been widely explored as a route to lanthionines (**51**) (**Scheme 1.7**). This method was first reported in 1941 by Brown and du Vigneaud using β-chloroalanine, and gave a mixture of diastereomers as a result of a competing elimination/conjugate addition sequence.[179] Since then, a number of groups have reported stereoselective (methyl)lanthionine syntheses with both β-bromo and β-iodo analogues.[156,180,181]



Scheme 1.7: Lanthionine formation by reaction of β-haloalanines with cysteines (X = Cl, Br, I).

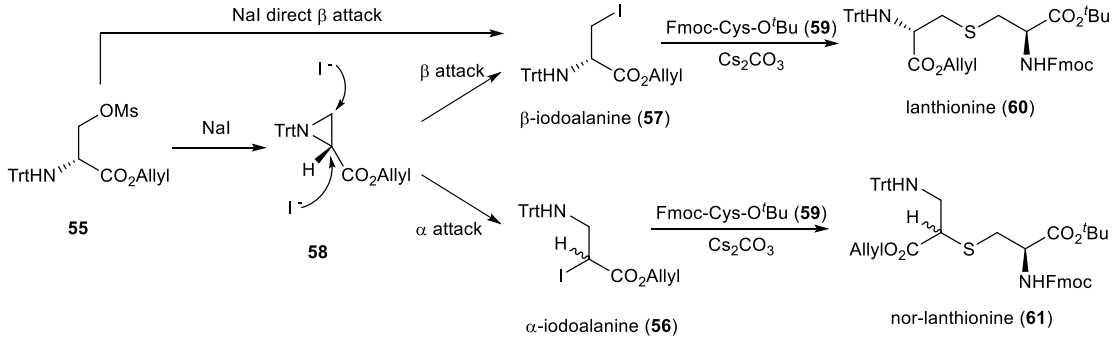
For example, protected β-bromoalanines were used by Zhu and Schmidt to produce *R,R* and *S,R* lanthionines under phase-transfer conditions.[180] These conditions have also been used by both Narayan *et al.* and Knerr *et al.* to synthesise protected methyllanthionines

(**52**) by the reaction of β-bromoalanines (**53**) with β-methyl-cysteines (**54**) (**Scheme 1.8**).[164,182]



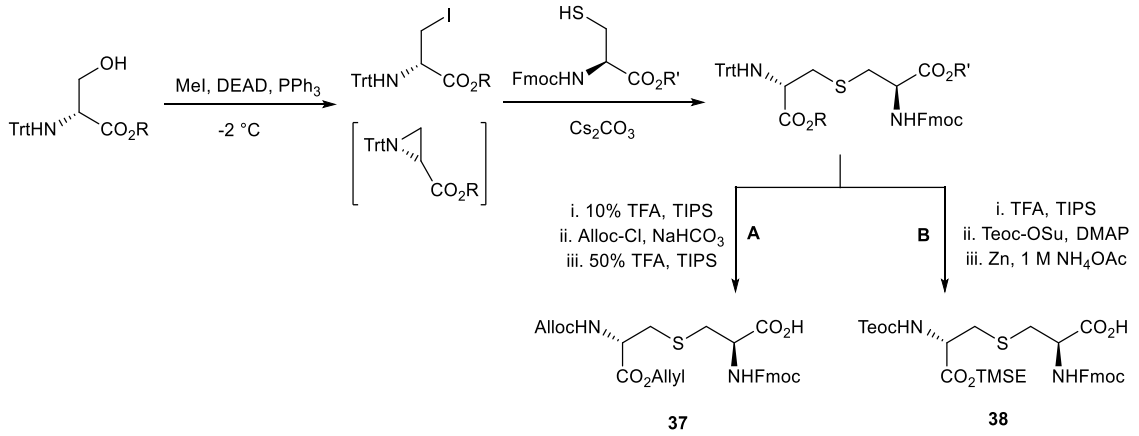
Scheme 1.8: Synthesis of MeLan using β-bromoalanines under phase-transfer conditions.

Tabor *et al.* have described the synthesis of protected lanthionines by the coupling of cysteine and β-iodoalanine residues under basic conditions. Initial work investigating the synthetic route reported by Dugave and Ménez[181] revealed that reaction of mesylate (**55**) with NaI resulted in the production of both α- (**56**) and β-iodoalanines (**57**), *via* an aziridine intermediate (**58**) (**Scheme 1.9**). Coupling with Fmoc-Cys-O*t*Bu (**59**) under basic conditions then resulted in the formation of a mixture of lanthionine (**60**) and norlanthionine diastereomers (**61**).[183,184]



Scheme 1.9: Mixture of lanthionine (**60**) and nor-lanthionines (**61**) produced using the procedure reported by Dugave and Ménez.

As an alternative, Bregant and Tabor developed a synthesis of β-iodoalanine (**57**) using Mitsunobu conditions at low temperature to decrease aziridine formation, enabling the production of (Alloc, Allyl/Fmoc) Lan (**37**) as a single diastereomer.[156] Later, Mothia *et al.* reported the synthesis of (Teoc, TMSE/Fmoc) Lan (**38**) using the same method (**Scheme 1.10**).[162]

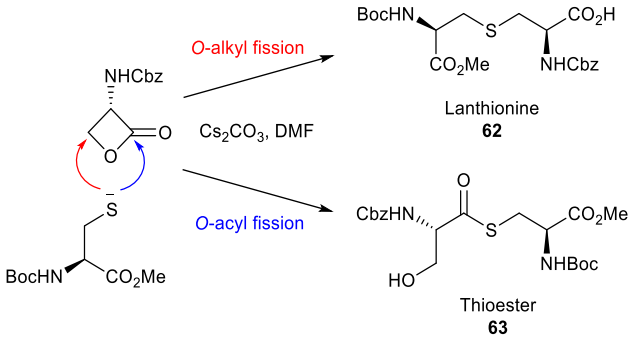


Scheme 1.10: Tabor *et al.* syntheses of orthogonally protected lanthionines. **A**: (Alloc, Allyl/Fmoc) Lan (**37**), R = Allyl, R' = *t*Bu; **B**: (Teoc, TMSE/Fmoc) Lan (**38**), R = TMSE, R' = Tce.

1.5.2. Synthesis of (Methyl)Lanthionines *via* Ring-Opening

An alternative method for the synthesis of (methyl)lanthionines is the ring-opening of either lactones, sulfamidates or aziridines with a protected cysteine.

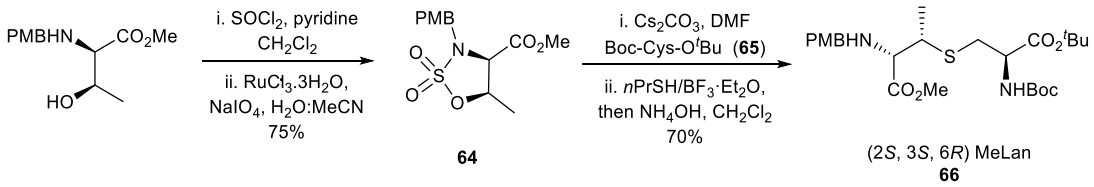
The first Lan synthesis by ring-opening of a lactone was reported by Arnold *et al.* in 1988.[185] Goodman *et al.* later adapted this procedure to synthesise protected lanthionines (**62**) using Cs_2CO_3, though the competing reaction to form thioester (**63**) by *O*-acyl fission was frequently observed (**Scheme 1.11**).[155,186]



Scheme 1.11: Synthesis of lanthionines by ring-opening of lactones derived from serine.

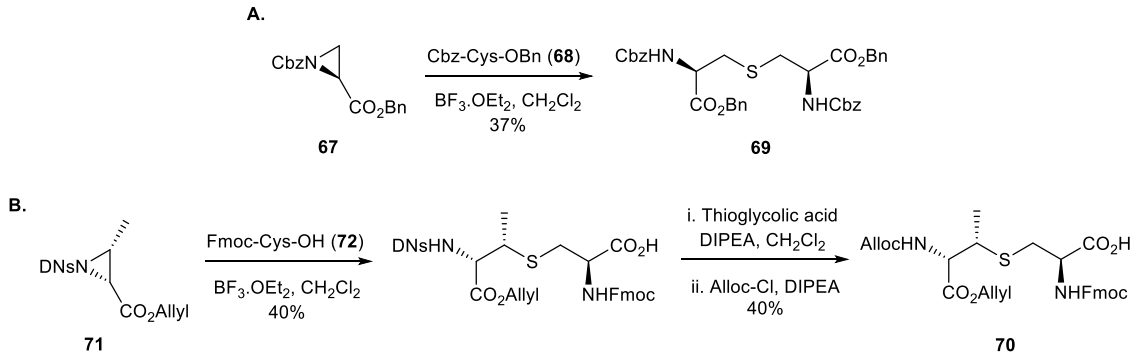
Cobb and Vederas have shown that the ring-opening of sulfamidates is an effective method for the preparation of stereochemically pure (methyl)lanthionines.[187] Beginning from Ser, Thr or *allo*-Thr, sulfamidates (**64**) were formed by reaction with $SOCl_2$ and oxidation with $RuCl_3$. Ring-opening with Boc-Cys-O*t*Bu (**65**) then gave the corresponding thioethers (**66**) in good yield (**Scheme 1.12**). A similar route has also been

reported by Peregrina *et al.*, using the ring-opening of sulfamidates, derived from α-methylisoserine, with Boc-Cys-OMe to prepare both (*R*,*R*) and (*S*,*R*) α-methyl nor-lanthionines.[188]



Scheme 1.12: Representative Cobb and Vederas lanthionine synthesis by sulfamidate ring-opening.

The production of (methyl)lanthionines by aziridine ring-opening with cysteines was first reported by Nakajima *et al.* in 1983.[189] The aziridines (**67**) were prepared from the corresponding Ser or Thr derivatives, followed by ring opening with Cbz-Cys-OBn (**68**) and $BF_3.OEt_2$ to produce the (Me)Lan (**69**), though in low yield (**Scheme 1.13 A**). More recently, Vederas *et al.* used a similar method to synthesise stereochemically pure (Alloc, Allyl/Fmoc) MeLan (**70**), by the ring-opening of aziridine (**71**) with Fmoc-Cys-OH (**72**) (**Scheme 1.13 B**).[165]

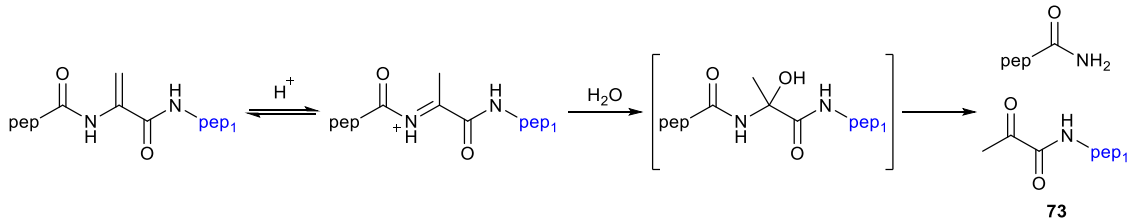


Scheme 1.13: Representative (Me)Lan syntheses by aziridine ring-opening. **A.** Nakajima *et al.* Lan synthesis, **B.** Vederas *et al.* MeLan synthesis.

1.6. Incorporation of Dehydro Residues in SPPS

A wide range of α,β-unsaturated amino acids are found in bioactive peptides,[190] the simplest of which are Dha (**9**) and Dhb (**10**). As these residues are frequently found in the lantibiotics, reliable methods for their incorporation on the solid-phase are required. Production of peptides containing these residues is challenging due to their instability as they are known to undergo rapid Michael addition with thiols and amines.[191] They are also prone to hydrolysis to give the corresponding pyruvate (**73**), particularly in strongly

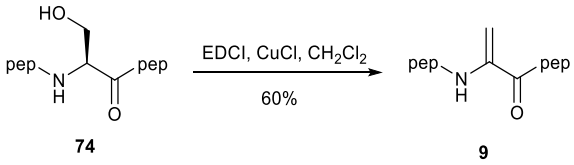
acidic conditions (**Scheme 1.14**).[192] Despite these difficulties, a variety of synthetic methods have been developed for the synthesis of dehydro residues, and the reader is referred to a range of extensive reviews for a comprehensive discussion of this subject.[190,191,193,194] Key methods used for the synthesis of dehydro-containing lantibiotics in the literature, including the strategies employed in this work, are discussed below.



Scheme 1.14: Hydrolysis of dehydro residues in acid, exemplified with Dha.

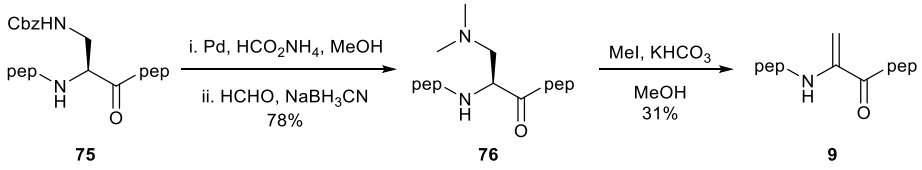
1.6.1. Dehydroalanine (Dha) Incorporation

Due to the reactive nature of the Dha residue, its direct incorporation into a growing peptide chain by SPPS is not feasible. Instead, it is necessary to incorporate a precursor residue into the peptide, often bearing a hydroxyl or thiol, which can be transformed at a later stage to reveal the Dha. For example, in their recent synthesis of dicarba analogues of nisin ring A, Slootweg *et al.* generated Dha residues (**9**) by elimination from Ser (**74**) under basic conditions, finding that EDCI and CuCl gave the best results in the dehydration step. (**Scheme 1.15**).[173]



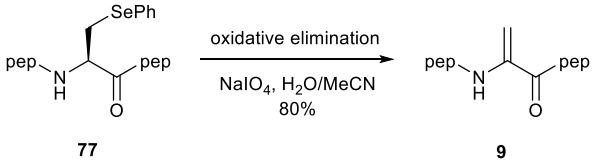
Scheme 1.15: Production of Dha residues from Ser precursors using EDCI and CuCl.

The total synthesis of nisin reported by Fukase *et al.* was the first chemical synthesis of a lantibiotic peptide containing dehydro residues. In this work, a 2,3-diaminopropionic acid residue (**75**) was incorporated into the peptide at the desired position of dehydration, followed by Cbz deprotection and β-*N*-methylation to give intermediate (**76**). Hofmann elimination with MeI and $KHCO_3$ then produced the Dha residue (**9**) (**Scheme 1.16**).[146]



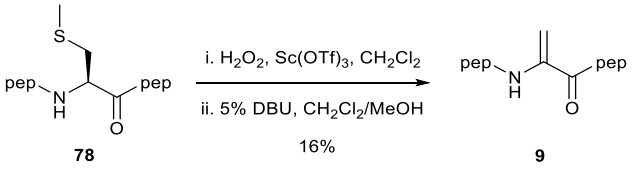
Scheme 1.16: Fukase *et al.* incorporation of Dha residues.

Oxidative elimination of phenylselenocysteine (Sec(Ph)) residues has also been used to incorporate Dha into peptides. This approach was initially reported by Hashimoto *et al.*,[195] and was later developed by van der Donk *et al.* to produce a Dha-containing peptide used in a biomimetic synthesis of nisin ring B.[153] In this strategy, a Sec(Ph) residue (**77**), synthesised from L-Ser, was incorporated at the desired position using regular SPPS techniques, and on treatment with either $NaIO_4$ or H_2O_2 underwent oxidative elimination to give the Dha (**9**) (**Scheme 1.17**).[196]



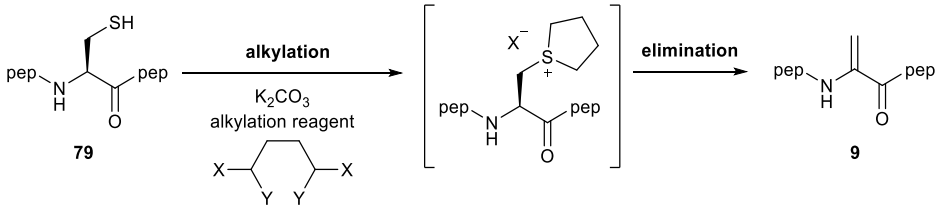
Scheme 1.17: Introduction of Dha into peptides by oxidative elimination of a Sec(Ph) residue.

A number of different methods have been reported for the generation of Dha from Cys residues. For example, Matteucci *et al.* reported the oxidation-elimination of *S*-methyl-cysteine (**78**) as part of their biomimetic synthesis of nisin ring B (**Scheme 1.18**).[151] One downside to this approach is that it is incompatible with lantibiotic syntheses requiring orthogonally protected (methyl)lanthionines, as the strongly oxidising conditions needed to produce the intermediate sulfoxide would cause undesired oxidation of the thioether bridge.



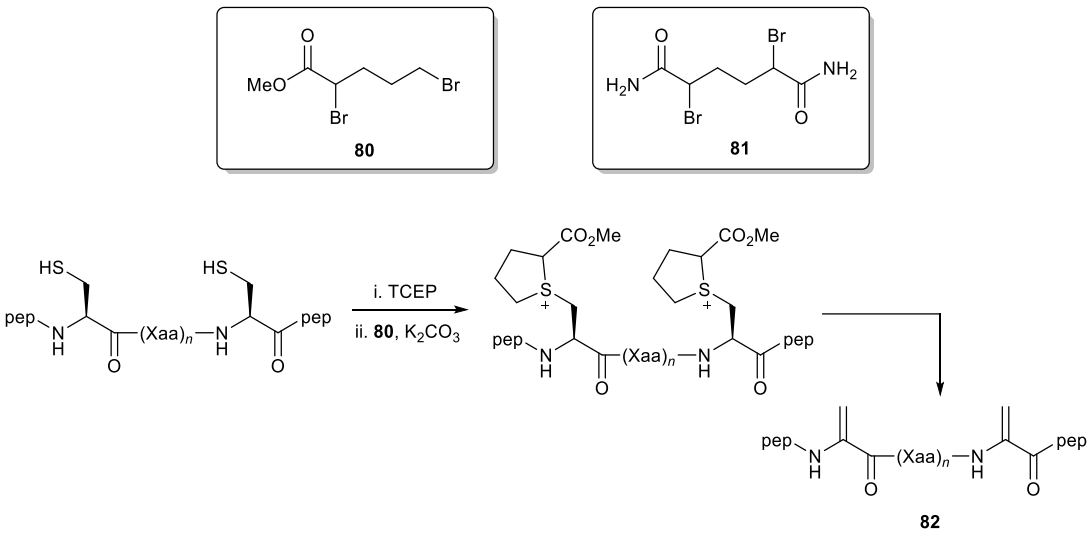
Scheme 1.18: Matteucci *et al.* production of Dha residues from Cys residues.

Although not yet applied to lantibiotic synthesis, the introduction of Dha residues (**9**) in peptides and proteins by the bis-*S*-alkylation and β-elimination of Cys residues (**79**), pioneered by Davis *et al.*, has been widely explored (**Scheme 1.19**).[197]



Scheme 1.19: Dha incorporation by alkylation and β-elimination of Cys residues. X = Br, Cl, I, OMs; Y = $CONH_2$.

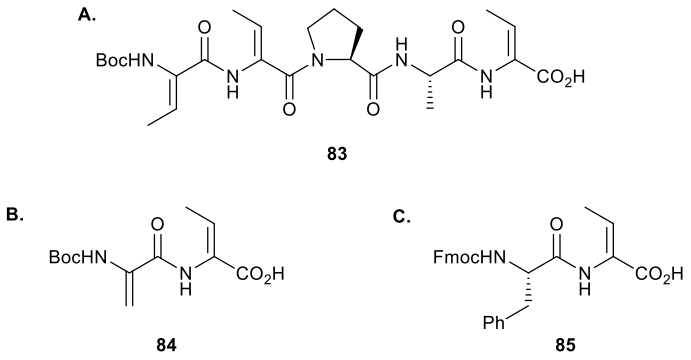
Morrison *et al.* have recently reported an improvement this methodology using a new alkylation reagent, methyl 2,5-dibromopentanoate (**80**) (**Scheme 1.20**). This reagent enabled the simultaneous introduction of multiple Dha residues into peptides, whilst avoiding the undesired crosslinking between Cys residues which can occur when using other common bis-alkylation reagents such as dibromoadipamide (**81**). In the adapted strategy, Cys residues are first incorporated into the peptide at every position in which a Dha is required (**Scheme 1.20**). Following treatment with TCEP to ensure that all cysteines are in the reduced form, dibromopentanoate (**80**) and K_2CO_3 are added to effect the alkylation-elimination, producing the Dha-containing peptide (**82**).[198]



Scheme 1.20: Morrison *et al.* strategy for multiple Dha incorporation.

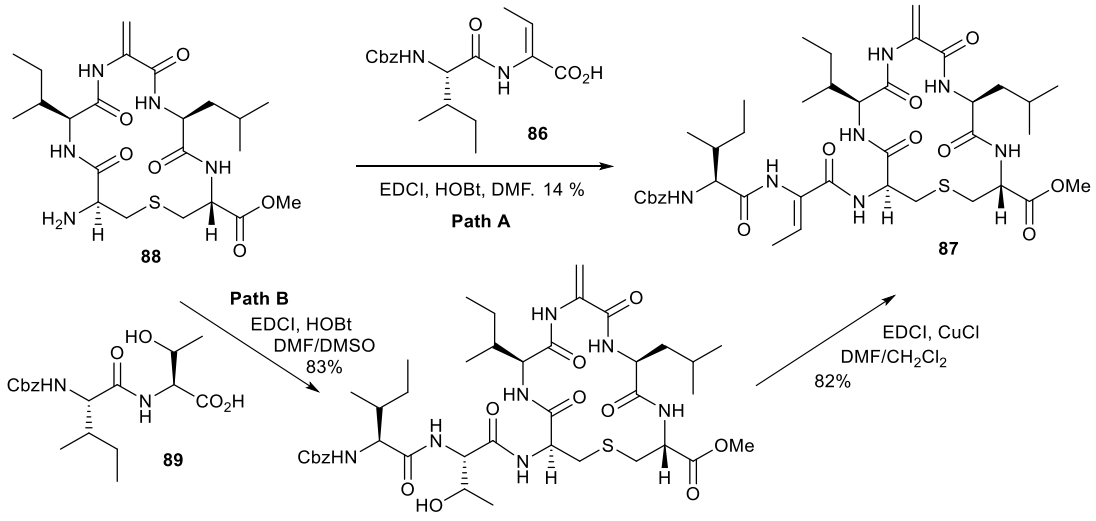
1.6.2. Dehydrobutyrine (Dhb) Incorporation

Dhb tends to be more resistant to unwanted Michael addition than Dha due to the presence of the β-methyl group. Therefore, successful incorporation of this residue into lantibiotic peptides with the alkene group already installed has been reported in a number of syntheses. For example, Vederas *et al.* have employed a Dhb-containing pentapeptide (**83**) to introduce the highly modified *N*-terminal sequence in their synthesis of lacticin 3147 (**18**) and analogues (**Scheme 1.21 A**),[165,168] as well as a Dha-Dhb dipeptide (**84**) in their synthesis of WT lactocin S (**Scheme 1.21 B**).[160] Knerr and van der Donk have also utilised this approach, employing a Phe-Dhb dipeptide (**85**) to incorporate the Dhb residue into both lacticin 481 (**17**) and analogues of epilancin 15X (**42**) (**Scheme 1.21 C**).[163,164]



Scheme 1.21: Direct incorporation of Dhb as part of an oligopeptide. **A.** and **B.** Vederas *et al.* syntheses, **C.** Dipeptide employed by Knerr and van der Donk.

Both Fukase *et al.* and Slootweg *et al.* have reported low yields when attempting to incorporate a Dhb residue as part of an Ile-Dhb dipeptide (**86**) in their syntheses of nisin ring A WT (**87**) or analogues (**Scheme 1.22**, **Path A**).[146,173] This may be due to a combination of the more hindered terminal amine of nisin ring A (**88**) compared to that of a linear peptide, and the lower reactivity of the Dhb residue due to conjugation of the carboxylic acid.[146] As an alternative, both groups investigated the coupling of a protected Ile-Thr dipeptide (**89**) to the ring. Subsequent dehydration with EDCI and CuCl following completion of the synthesis then gave the Dhb-containing peptide (**87**) in good yield (**Scheme 1.22**, **Path B**).[145,173]



Scheme 1.22: Synthesis of nisin ring A by addition of Ile-Dhb or Ile-Thr dipeptides, exemplified by the Fukase *et al.* syntheses.

1.7. Structural Studies of Nisin by NMR

One of the most widely-used tools for studying the structure of lantibiotics is NMR. This technique has played an important role in elucidating the sequence and bridging pattern of many lantibiotic peptides, as well as enabling their three-dimensional structures to be determined. To conduct these structure determinations, experimental NMR data, such as interproton distances from NOE intensities and torsion angles from ^{3}J coupling constants, is required. This data is used as an input for programs such as CYANA[199] or XPLOR-NIH,[200] that calculate conformations of the peptide which satisfy the experimental restraints, ultimately producing an ensemble of likely 3D structures. Using this method, the structures of several lantibiotics have been elucidated.[201]

1.7.1. Solution State Conformation of Nisin

The solution state conformation of several class I lantibiotics have been reported, including those with nisin-like AB ring systems such as subtilin (**14**)[202] and mutacin 1140 (**21**),[203] as well as more globular class II peptides such as cinnamycin (**16**)[204] and gallidermin.[205] Nisin is by far the most well-studied lantibiotic, and a number of investigations of the solution state structure of both the full-length peptide and fragments have been conducted.

The first NMR assignment of WT nisin and identification of the inter-residue NOEs was published by Slijper *et al.* in 1989.[206] Subsequently, van de Ven *et al.* calculated the structure of the peptide in aqueous solution, revealing the presence of well-defined ring structures linked by the central flexible hinge region. In particular, the structure of the smaller B, D and E rings could be determined with good precision, whilst the lower resolution of the A and C rings was attributed to the higher degree of flexibility within these larger structures.[207] Later work conducted by Lian *et al.* confirmed the flexibility of the full-length peptide, and suggested that it does not adopt any preferred overall conformation in aqueous solution. The structure of two common nisin degradation products, **90** and **91**, were also studied as part of this work, both of which result from Dha hydrolysis (**Figure 1.19**). This showed that *C*-terminal truncation has no effect on overall conformation, and that hydrolysis of ring A causes a large increase in the flexibility of residues 1-8. However, at 5 °C the hydrolysed peptide adopts a similar conformation to that of WT nisin, indicating that one backbone conformation within ring A is preferred.[208]

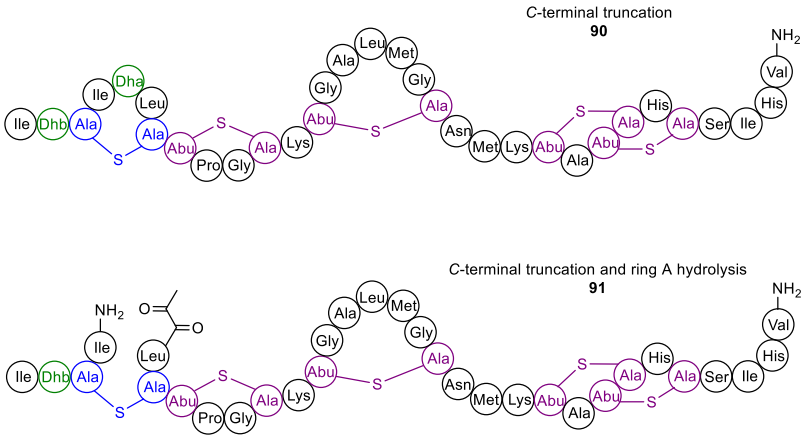


Figure 1.19: Structures of the nisin degradation products studied by NMR.

Additionally, Palmer *et al.* synthesised analogues of nisin ring A (**92**) and ring B (**93**) using the desulfurisation method reported by Shiba *et al.*, and reported their structures in d_6-DMSO (**Chapter 1.4.2.1**). Ensembles of the lowest energy structures for these isolated peptides were remarkably similar to the ring conformations adopted by the WT peptide as reported by Lian *et al.*,[208] except that mixtures of cis and trans Pro were observed in the isolated ring B peptide (**93**) (**Figure 1.20**).[209]

1.7.2. Conformation of Nisin in Lipidic Environments

Structures of several lantibiotics have been studied in lipidic environments. The conformation of these peptides in the presence of their cellular target lipid II (**28**), or in membrane-mimicking systems such as micelles, is more relevant to the mechanism of action and may indicate active conformations. For example, Hsu *et al.* reported the structure of mersacidin (**94**) in DPC micelles containing lipid II, and suggested that electrostatic interactions govern the binding of the peptide and lipid (**Figure 1.21 A** and **B**).[210] Recently, Bakhtiary *et al.* solved the structure of lacticin 3147 (**18**) bound to lipid II, revealing that the *C*-terminus of the peptide forms a cage around the pyrophosphate of the lipid (**Figure 1.21 C**).[90]

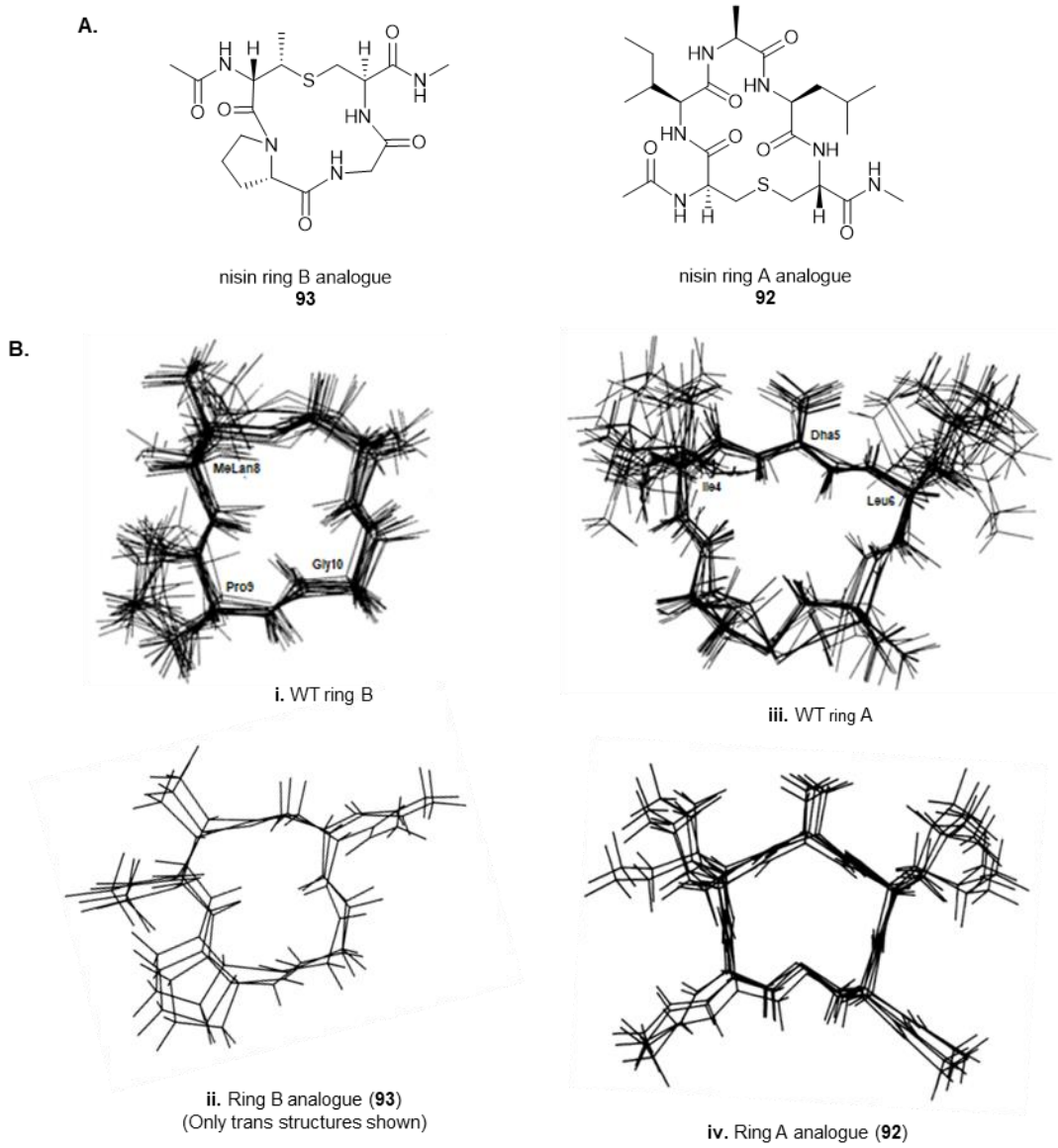


Figure 1.20: A. Structure of A and B ring analogues, **B.** Comparison of solution state structures of **92** and **93**[209] with the same section of the full length WT peptide reported by Lian *et al.*.[208]

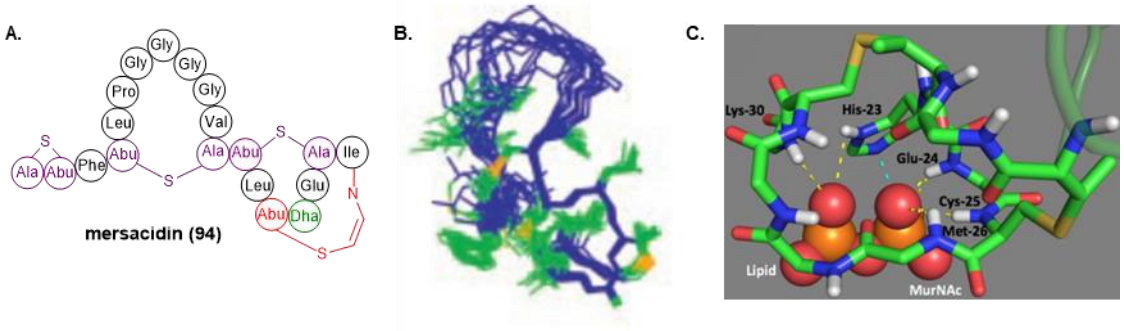


Figure 1.21: Structures of lantibiotics in lipidic environments. **A.** Structure of mersacidin (**94**). **B.** Conformation of mersacidin in lipid II-containing DPC micelles. Peptide backbone: blue, side chains: green, MeLan sulfur: yellow.[210] **C.** Structure of lacticin 3147 A1 bound to lipid II.[90]

The structure of nisin in model membranes has also been extensively studied. Initial work reported by van den Hooven *et al.* compared the chemical shifts of nisin in TFE/water mixtures to that in micelles composed of DPC and SDS, finding that significant conformational change occurs in the *N*-terminus of the peptide in a membrane environment.[211] Two further studies by these workers, again in DPC and SDS micelles, showed that the structure of nisin in these environments differs from the structure in aqueous solution, particularly in ring A (**Figure 1.22**), and that nisin binds at the surface of the micelle with the hydrophobic residues inserted into the membrane.[212,213]

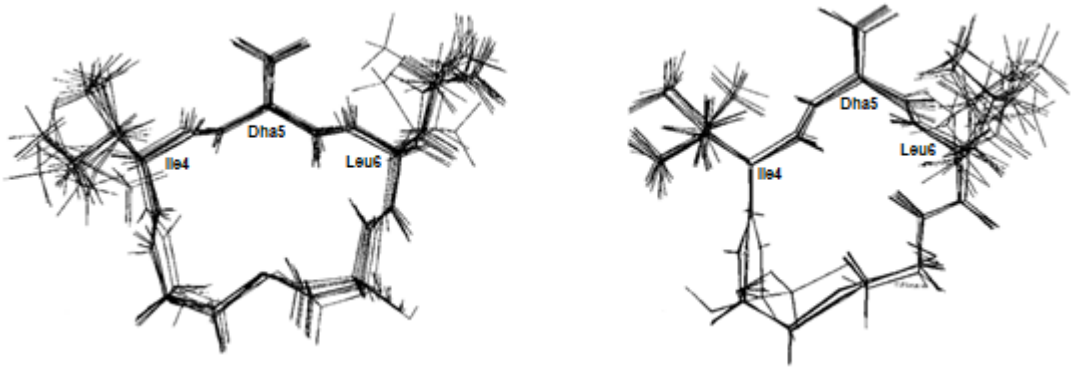


Figure 1.22: Structure of nisin ring A (partial display of full length WT nisin). Left: in micelles, right: in aqueous solution.[213]

Following the discovery that nisin binds to lipid II, Hsu *et al.* studied the nisin-lipid II interaction in SDS micelles. Large chemical shift perturbations in the *N*-terminus, particularly of Ile4, Leu6 and Gly10, led the authors to suggest that the first step of pore formation involves the binding of nisin rings AB to lipid II.[214] Additionally, Bonev *et al.* reported a solid-state NMR study of nisin and lipid II in DOPC/DOPG bilayers, which indicated that the interaction of Ile1 and the lipid II pyrophosphate is important for target recognition.[215]

The most important NMR study of nisin to date was published by Hsu *et al.* in 2004, and described the binding mode of nisin to lipid II. Using 3D NMR experiments in d_6-DMSO solution, intermolecular NOEs and hydrogen bonds were identified at the interface of a 1:1 complex between WT nisin and a lipid II analogue with a shorter prenyl tail. Structure calculation using these distance restraints revealed a pyrophosphate cage formed by a hydrogen bond network between the backbone amides of nisin rings AB with the lipid II pyrophosphate, causing a reduction in flexibility in the *N*-terminus of the peptide (**Figure 1.23**).[96]

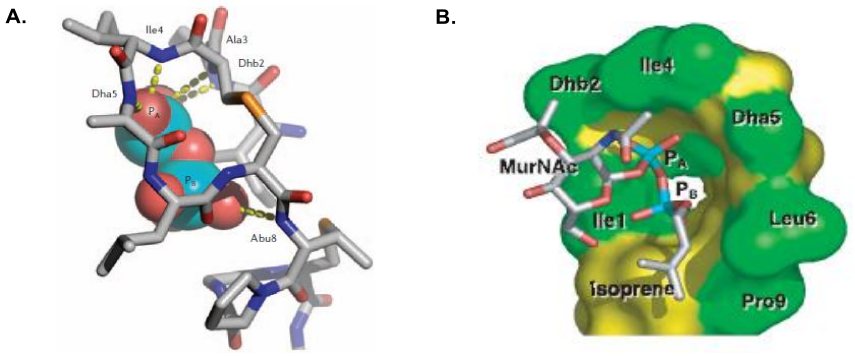


Figure 1.23: A. NMR studies revealed a pyrophosphate cage formed by hydrogen-bonding of the nisin backbone to the pyrophosphate of lipid II (hydrogen bonds shown in yellow), and **B.** van der Waals surface of nisin N-terminus with a truncated analogue of lipid II.[96]

1.8. Project Aims

The vision of this project was to develop a more detailed understanding of the conformational effect of residue mutation within the lipid II-binding region of nisin and mutacin I, in order to aid the rational design of semisynthetic lantibiotic peptides.[161] These peptides would contain a nisin-like lipid II-binding region, used to efficiently deliver the peptide to the bacterial membrane, coupled to a simpler linear pore-forming peptide. Ultimately, it is hoped that this would result in antimicrobial peptides which are both more stable than nisin and easier to access by SPPS.

Three aims were laid out in order to achieve this vision. The first aim was to synthesise fragments of the lipid II binding regions of nisin (**13**) and mutacin I (**31**) by SPPS, using the method of Mothia *et al.*,[162] and study their solution state structures by NMR. Mutacin I (**31**) was selected as the second lantibiotic for study as it has the same bridging pattern as nisin but contains fewer unusual amino acids, hence simplifying the synthesis. Although no binding studies have yet been reported for mutacin I, the high degree of similarity between the *N*-terminal regions of nisin and mutacin I indicates that their lipid II binding mechanisms are likely to be similar, if not identical.

As described above (**Chapter 1.4.2.3**), the synthesis of lantibiotic rings using the method of Mothia *et al.* involves the incorporation of orthogonally protected (methyl)lanthionines into the peptide. The first aim of this work was therefore the preparation of one orthogonally protected Lan, and one orthogonally protected MeLan. The (Teoc, TMSE/Fmoc) Lan (**38**) described by Mothia *et al.*[162] and the (Alloc, Allyl/Fmoc)

MeLan (**70**) described by Vederas *et al.*[165] were selected for their compatibility with SPPS and ease of synthesis (**Figure 1.24**). The synthesis of several precursors to the dehydro residues were also required to enable an investigation of the incorporation of Dha and Dhb residues into WT lantibiotic fragments.

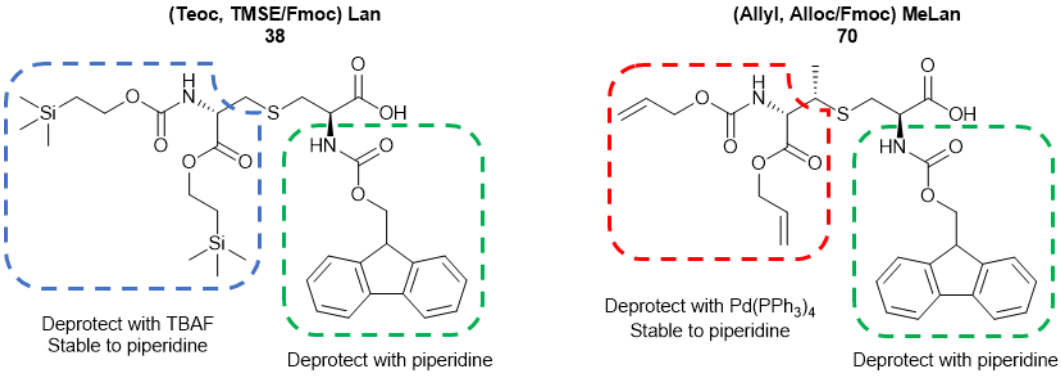


Figure 1.24: Structures of the orthogonally protected (methyl)lanthionines used in this work.

The second aim of this work was the synthesis of the lipid II binding rings of nisin and mutacin I, using the SPPS strategy outlined above (**Scheme 1.4**). WT and analogue sequences were desired, of both single ring systems (ring A and ring B individually) and double ring systems (rings AB together). A discussion of the peptides selected for synthesis can be found below (**Chapter 1.8.1**).

Finally, each of the prepared peptides were analysed by NMR and their solution state structures determined in XPLOR-NIH.[200] In contrast to many previously described NMR studies, the synthesis of single and double ring fragments of nisin in this work enabled a study of the conformation of the lipid II binding rings without the influence of the tail of the peptide. It was hypothesised that comparison of the structures of the analogue and WT peptides would enable the effect of structural simplification on solution state conformation to be examined. Additionally, comparison of structures of the individual rings with both WT nisin rings AB isolated from a commercial preparation,[216] and the published structure of full length nisin in complex with lipid II (PDB ID: 1WCO),[96] may indicate to what degree rings A and B are preorganized for lipid II binding, and reveal whether the presence of a second ring has an effect on the solution state structure.

1.8.1. Analogues Chosen for Synthesis in this Work

A combination of WT and analogue peptides were selected for synthesis to enable a detailed investigation of the conformation of the lipid II binding regions of nisin and mutacin I. Initially, individual rings were synthesised, each of which would be compared to the structures of the corresponding rings in both isolated WT nisin rings AB (**95**), and in full length WT nisin in complex with lipid II as reported by Hsu *et al.* (PDB ID: 1WCO).[96]

The desired ring B peptides are shown below (**Figure 1.25**). The WT sequences of both mutacin I (**96**) and nisin (**97**) were synthesised, as well as a Lan analogue of nisin ring B (**98**) to enable the structural effect of a Lan-MeLan substitution to be evaluated. As the only difference between the WT (**97**) and analogue (**98**) peptides is a methyl group, it was expected that their solution state structures would differ little from each other. Additionally, this mutation was expected to be well tolerated as Pattabiraman *et al.* have shown that a Lan analogue of lacticin 3147 A2 (**18b**) retains its synergistic activity with the A1 peptide.[158]

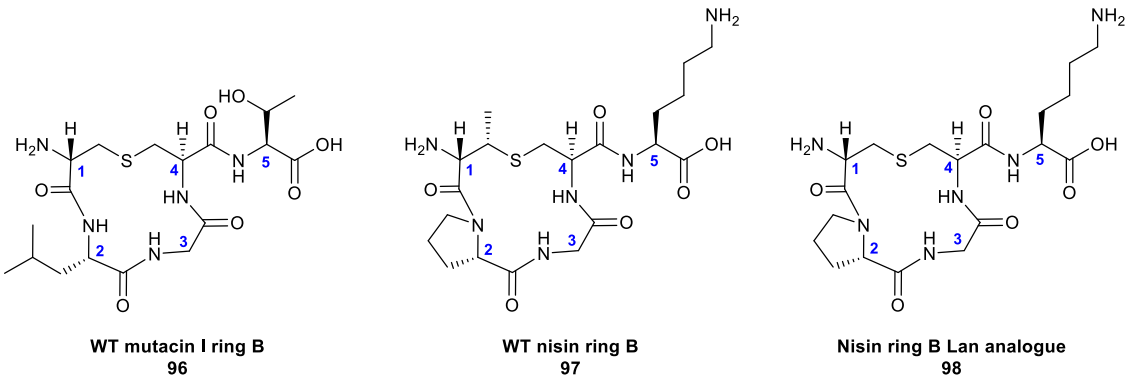


Figure 1.25: Structures of the desired ring B peptides.

A variety of ring A peptides were also prepared (**Figure 1.26**). WT mutacin I ring A (**99**) and nisin ring A (**100**) were synthesised, as well as two analogues of mutacin I ring A: Ser/Ala analogue (**101**) and truncated Ser analogue (**102**). Although Palmer *et al.* have shown that swapping the Dha for Ala in nisin ring A does lead to conformational change,[217] there is evidence in the literature which suggests that substitution of either of the dehydro residues in WT nisin and mutacin I would be tolerated. For example, retention of bioactivity against *M. luteus* is observed for both nisin and mutacin 1140 (**21**) with Dha5 replaced by Ala,[218,219] and Wiedemann *et al.* have shown that the replacement of Dhb2 in nisin with either Ser, Ala or Val has little effect on MIC.[85] Comparison of the

solution state structures of the synthesised peptides would therefore help to build a clearer picture of the conformational effect of replacing the dehydro residues in ring A. In addition, production of Ser analogue (**102**) would allow an investigation of the effect of the tail residues (residues one and two) on ring conformation.

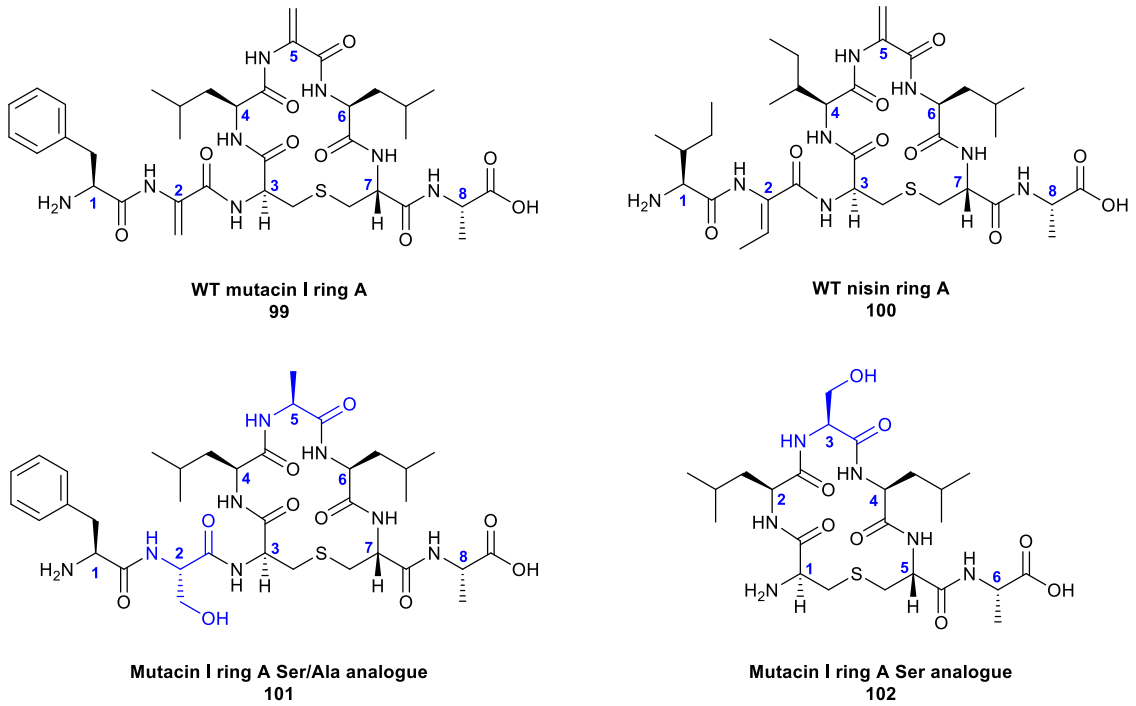


Figure 1.26: Structures of the desired ring A peptides. Residues which differ from those in the corresponding WT peptide are highlighted in blue.

Finally, WT and analogue double ring peptides were desired (**Figure 1.27**). Isolation of WT nisin rings AB (**95**), prepared by enzymatic digestion of the full-length peptide, was required to enable a comparison with the reported structure of WT nisin bound to lipid II (PDB ID: 1WCO),[96] indicating whether the conformation of the AB rings differs when in complex with lipid II. Two simplified nisin analogues, previously described by Mitchell (**Chapter 1.4.2.3**),[161] were also prepared. Both peptides contain only one thioether bridge type, and the dehydro residues were replaced with less reactive amino acids: Thr and Ser to create hydrated analogue (**39**), and Abu and Ala to create saturated analogue (**40**). Comparison of these analogues to WT nisin rings AB (**95**) would provide further evidence for the structural effect of Lan-MeLan substitution and Dha replacement. WT mutacin I rings AB (**103**) and analogues were also desired to enable a comparison of the structures of the AB rings of nisin and mutacin I. Similarities in solution conformation of the WT peptides could confirm that mutacin I is likely to bind lipid II in the same manner as nisin,

whereas analysis of the analogues would provide further data on the effects of Dha substitution.

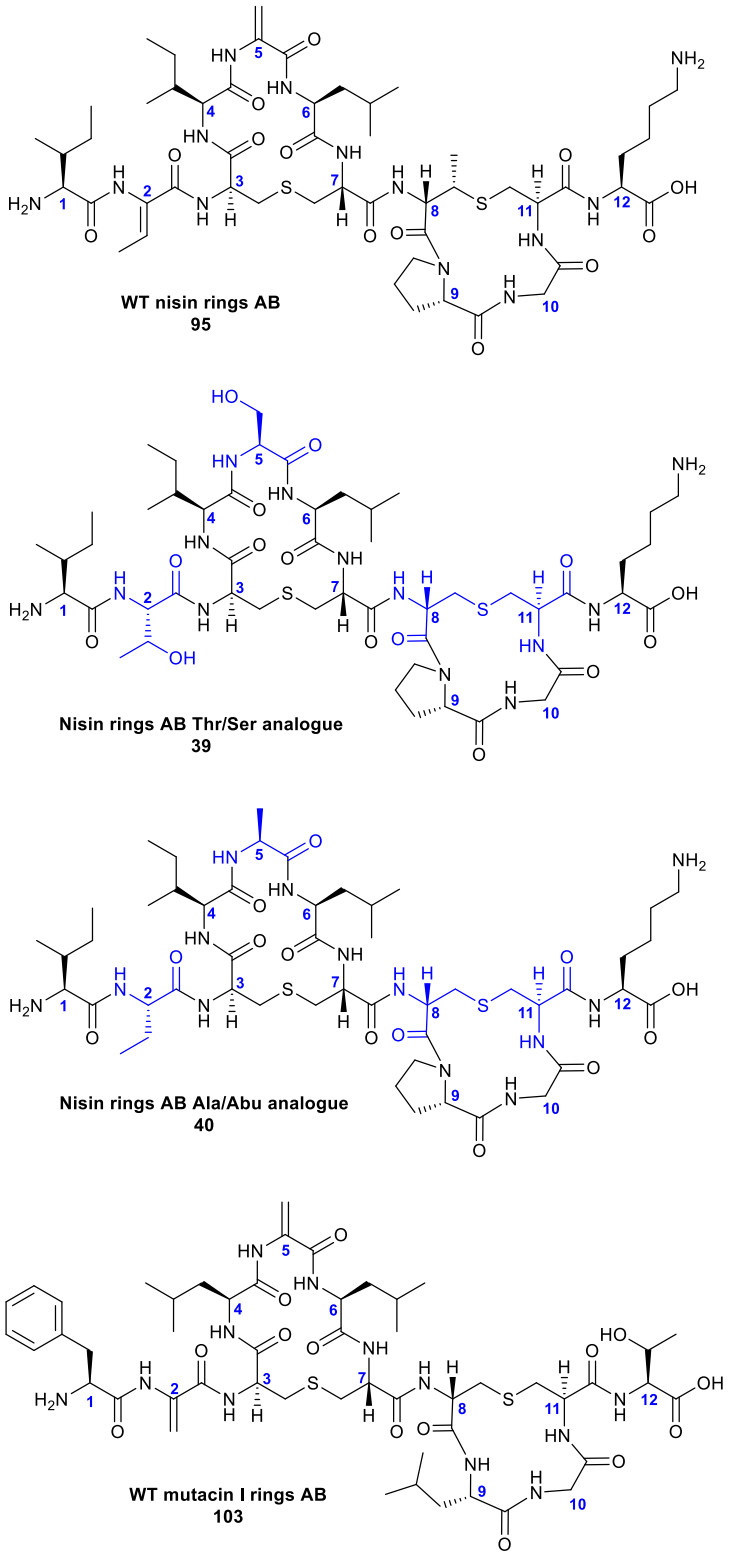


Figure 1.27: Structures of the desired ring AB peptides. Residues which differ from those in the corresponding WT peptide are highlighted in blue.

2. SYNTHESIS OF LANTHIONINES & DEHYDRO PRECURSORS

2.1. Introduction

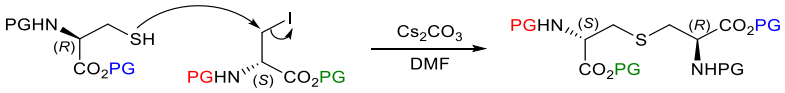
This chapter details the synthesis of unusual amino acids and precursors to dehydro residues which were required for the synthesis of the lipid II binding regions of nisin and mutacin I.

The syntheses of both of the orthogonally protected (methyl)lanthionines used in this work were based on published procedures, and involved an S_N2 reaction between the thiol of a protected Cys and the β-carbon of a second protected amino acid in order to produce the thioether.[162,165] These were designed to be incorporated into peptides according to the methods outlined in **Chapters 3.1.1** and **4.1.1**.

In order to synthesise WT fragments of nisin and mutacin I, reliable methods to introduce both Dha and Dhb are essential. Several different routes for Dha introduction have been investigated as part of this work, starting from Ser, Cys or *Se*-phenylselenocysteine.[173,196,198] As Dhb is less reactive as a Michael acceptor than Dha, routes involving its direct incorporation could be investigated in addition to its generation from precursor residues.[146,165] The use of these precursors and reagents in SPPS is discussed in **Chapters 3** and **4**.

2.2. Lanthionine Synthesis

Lanthionine (**38**) was synthesised following the route published by Mothia *et al.*, in which a protected β-iodo-Ala and a protected Cys produce the thioether bridge *via* an S_N2 reaction at the Ala β-carbon (**Scheme 2.1**).[162] In order to obtain the native (2*S*, 6*R*) lanthionine stereochemistry of nisin and mutacin I, the β-iodoalanine portion was synthesised from a D-amino acid, and the Cys portion from an L-amino acid.[51,107]



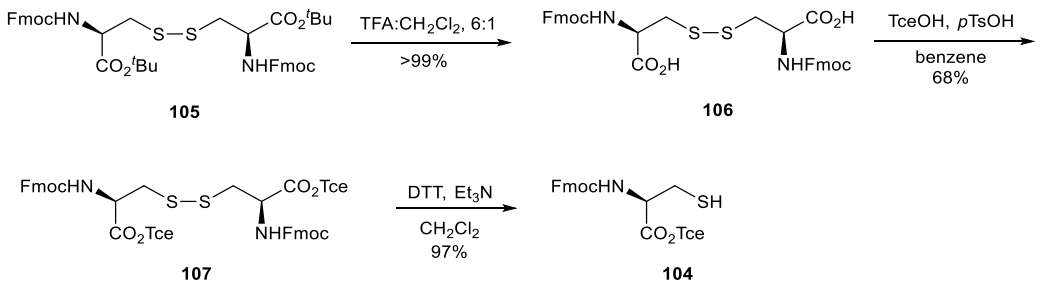
Scheme 2.1: General scheme showing the key thioether forming step in the Mothia *et al.* lanthionine synthesis.[162]

In addition to the Fmoc protecting group, temporary protection of the Cys acid is needed during the synthesis of the lanthionine and is removed in the final step. As reported by

Mothia *et al.*, a Tce group was used. Again following the literature procedure, the orthogonal protecting groups used on the β-iodoalanine were Teoc and TMSE: silyl groups which can be removed with TBAF on resin.[162]

2.2.1. Synthesis of Fmoc-Cys-OTce (**104**)

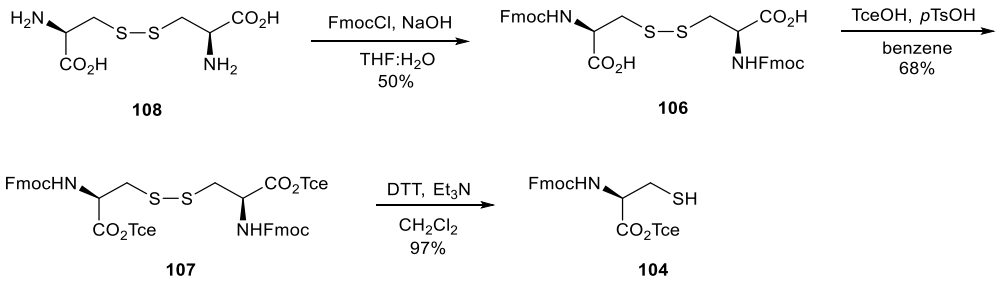
The first half of the lanthionine to be synthesised was Fmoc-Cys-OTce (**104**) (see **Scheme 2.2**). Starting from (Fmoc-Cys-O*t*Bu)$_2$ (**105**), the *t*Bu groups were first removed by treatment with TFA, followed by reprotection of diacid **106** by esterification with 2,2,2-trichloroethanol to give cystine **107**. Benzene was found to be the best solvent for this reaction, and gave a 16% higher yield than when toluene was used. Additionally, in contrast to the lengthy column chromatography reported by Mothia *et al.*,[162] it was found that this cystine could be more easily and rapidly purified by precipitation from EtOAc. The final step in the synthesis was reduction of the disulfide with DTT according to the method of Wright *et al.* to give the desired Fmoc-Cys-OTce (**104**) in good yield and purity without chromatography.[220]



Scheme 2.2: Synthesis of Fmoc-Cys-OTce (**104**) from (Fmoc-Cys-OTce)$_2$ (**105**). Overall yield: 65%.

Due to the expense of the (Fmoc-Cys-O*t*Bu)$_2$ (**105**) starting material, an alternative route to Fmoc-Cys-OTce (**104**) was then investigated in an attempt to both decrease the cost of the synthesis and increase atom efficiency. Unprotected L-cystine **108** was chosen as a more cost-effective starting point (**Scheme 2.3**). It was hypothesised that following the introduction of Fmoc protecting groups, the Tce protection and DTT reduction steps could then be carried out in the same way as described in **Scheme 2.2**. The procedure reported by Boger *et al.* was used for the Fmoc protection, in which NaOH and FmocCl are added to the cystine in a biphasic solution of THF and water to give (Fmoc-Cys-OH)$_2$ (**106**) in reasonable yield.[221] This was subsequently Tce protected and reduced, using the

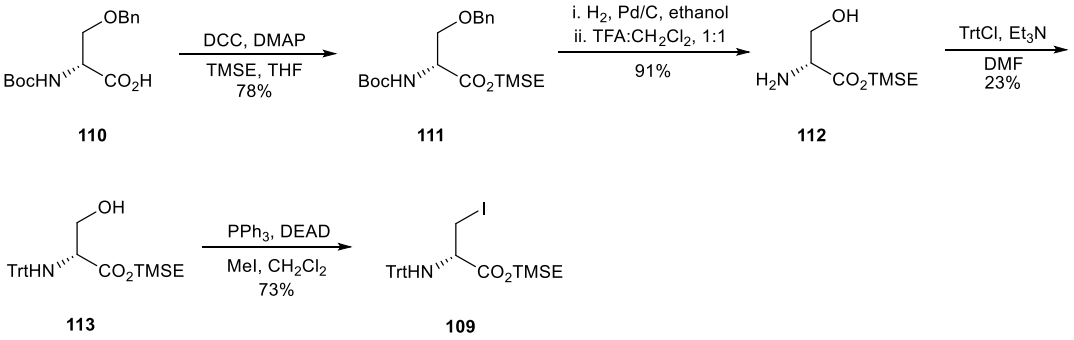
same methods as in the previously described synthesis, to give Cys **104** in the same number of steps beginning from a markedly cheaper starting material.



Scheme 2.3: Synthesis of Fmoc-Cys-OTce (**104**) from L-cystine (**108**). Overall yield: 33%.

2.2.2. Synthesis of Trt-β-Iodo-Ala-OTMSE (**109**)

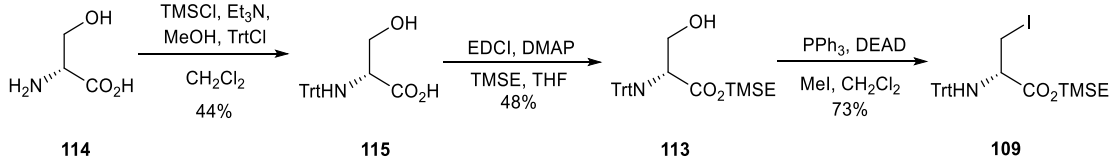
The second half of the lanthionine to be synthesised was Trt-β-iodo-Ala-OTMSE (**109**). Initially, the route of Mothia *et al.* was followed exactly (see **Scheme 2.4**).[162] Starting from Boc-D-Ser(OBn)-OH (**110**), the acid was first protected by DCC/DMAP catalysed esterification with trimethylsilylethanol giving **111**, followed by deprotection of the benzyl and Boc groups with H_2 over Pd/C and TFA respectively. The resulting amine (**112**) was then reprotected with TrtCl and Et_3N to give Trt-Ser-OTMSE (**113**). The final step in the sequence was a Mitsunobu reaction of the protected Ser (**113**) with PPh_3, DEAD and MeI. In order to minimise undesired aziridine formation,[184] the Mitsunobu reaction was conducted at 0 °C and purified directly by column chromatography to avoid an aqueous work-up. This ensured that β-iodo-Ala (**109**) could be isolated in good yield and as a single isomer.



Scheme 2.4: Synthesis of Trt-β-iodo-Ala-OTMSE (**109**) from Boc-Ser(OBn)-OH (**110**). Overall yield: 12%.

In a further attempt to decrease the cost of the synthesis, an investigation into the production of Trt-β-iodo-Ala-OTMSE (**109**) from D-Ser (**114**) was conducted (**Scheme**

2.5). The *N*-tritylation method reported by Barlos *et al.*[222] had previously been used with success in lanthionine syntheses by both Wright *et al.*[223] and Mothia *et al.*,[224] and was therefore chosen as the first protection step. However, this reaction proved to be capricious and the desired Trt-D-Ser (**115**) could only be isolated in a maximum yield of 44%. An EDCI/DMAP catalysed esterification with trimethylsilylethanol then gave the Trt-D-Ser-OTMSE (**113**) which could be used in the same Mitsunobu reaction as previously described to give the protected β-iodo-Ala (**109**) in fewer steps and in comparable yield to the synthesis from Boc-Ser(OBn)-OH (**110**).

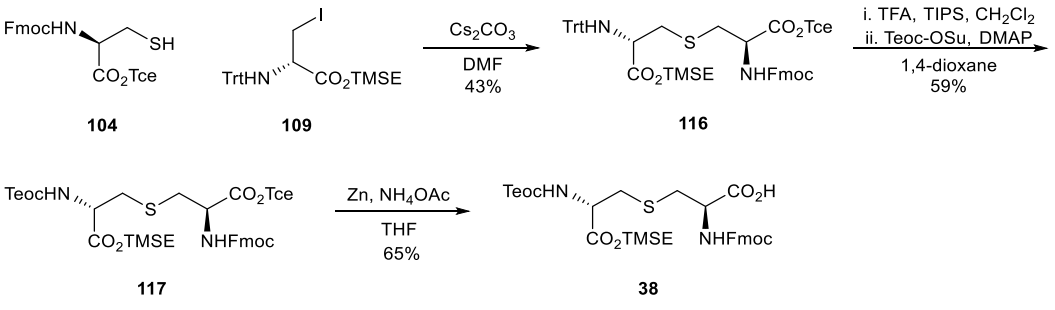


114 **115** **113** **109**

Scheme 2.5: Synthesis of Trt-β-iodo-Ala-OTMSE (**109**) from D-Ser (**114**). Overall yield: 15%.

2.2.3. Synthesis of (Teoc, TMSE/Fmoc) Lanthionine (**38**)

With the protected β-iodo-Ala (**109**) and Cys **104** in hand, the thioether bridge of the lanthionine was formed according to the method of Mothia *et al.*, using Cs_2CO_3 in DMF (**Scheme 2.6**).[162] In this reaction there is again a risk of forming the unwanted *N*-trityl aziridine, which could undergo S_N2 ring-opening by the Cys thiol to give nor-lanthionines (see **Chapter 1.5.1**).[183,184] It was hoped that conducting the reaction at 0 °C would minimise this, and although some aziridine formation was observed, no nor-lanthionine could be detected. Further contributing to the poor yield of this reaction was oxidation of thiol **104** back to the cystine, which was recovered from the reaction and re-reduced.



104 **109** **116** **117** **38**

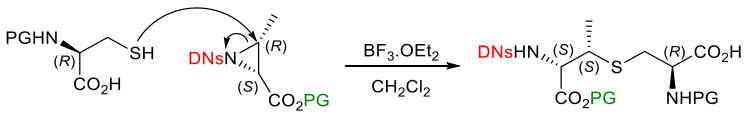
Scheme 2.6: Synthesis of (Teoc, TMSE/Fmoc) Lan (**38**) from Fmoc-Cys-OTce (**104**) and Trt-β-iodo-Ala-OTMSE (**109**).

Following the formation of the first lanthionine (**116**), the desired protecting groups could be installed following the method of Mothia *et al.*.[162] Firstly, the Trt group was removed

with TFA and replaced with a Teoc group in a DMAP catalysed reaction with Teoc succinimide, giving penultimate lanthionine **117**. Finally, the Tce group was removed with zinc dust and 1 M aqueous NH_4OAc according to the method of Jou *et al.*, to give the desired (Teoc, TMSE/Fmoc) lanthionine (**38**), ready for use in SPPS, in 13% overall yield from (Fmoc-Cys-O*t*Bu)$_2$ (**105**).[225]

2.3. Methyllanthionine Synthesis

Methyllanthionine **70** was necessary for the synthesis of nisin ring B WT. The method of Liu *et al.* was followed, in which the thioether bridge is formed by ring opening of a DNs-protected aziridine with a protected Cys (**Scheme 2.7**).[165,226] In order to generate the required stereochemistry, the aziridine was derived from Thr, and coupled to a protected Cys to give the native (2*S*, 3*S*, 6*R*) MeLan.

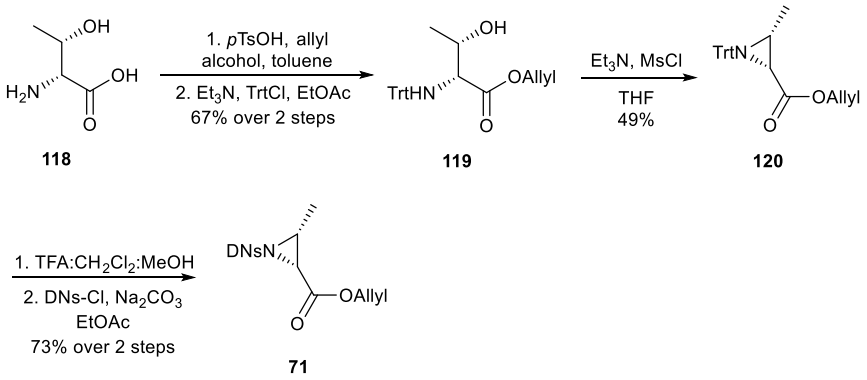


Scheme 2.7: General scheme showing the key thioether forming step in the Liu *et al.* lanthionine synthesis.[165]

Following formation of the thioether, the DNs group was replaced to give the Alloc/Allyl orthogonal protection which can be removed on resin with $Pd(PPh_3)_4$. In contrast to the Mothia *et al.* Lan synthesis,[162] Liu *et al.* conducted the S_N2 reaction with no temporary Cys carboxyl protection.[165]

2.3.1. Synthesis of *N*-DNs/Allyl Aziridine (**71**)

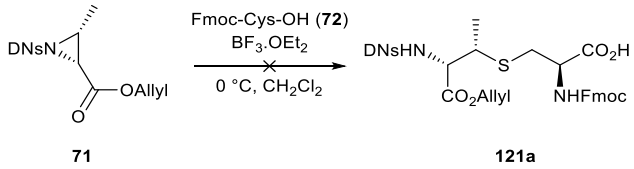
The synthesis of the *N*-DNs/CO_2Allyl aziridine (**71**) began from D-Thr (**D-118**), according to the procedure reported by Liu *et al.*.[165] Using a two-step procedure, the acid moiety was first protected with allyl alcohol in a *p*TsOH catalysed esterification, followed by trityl protection of the amine with trityl chloride and Et_3N (**Scheme 2.8**). The resulting protected Thr (**D-119**) was then treated with Et_3N and MsCl to produce the *N*-Trt aziridine (**D-120**) in an intramolecular S_N2 reaction. In order to install the necessary electron-withdrawing DNs protection on the aziridine nitrogen, the *N*-Trt group was then removed with TFA before reprotection using Na_2CO_3 and DNs-Cl to give the desired aziridine **71** in good yield.



Scheme 2.8: Synthesis of *N*-DNs/CO_2Allyl aziridine (**71**) from D-Thr (**D-118**).

2.3.2. Synthesis of (Alloc, Allyl/Fmoc) Methyllanthionine (**70**)

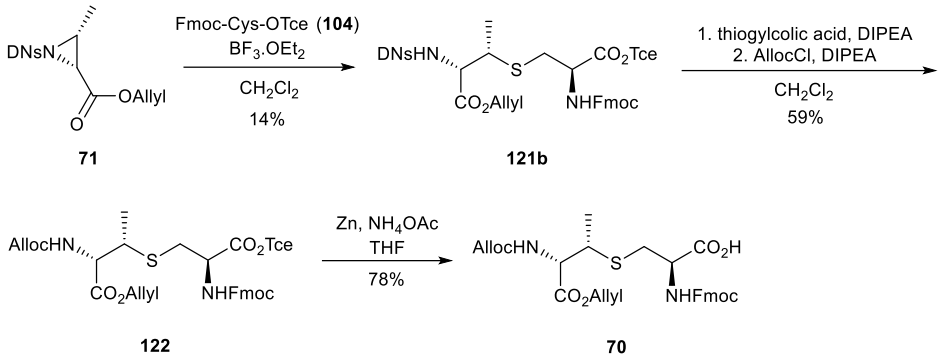
Initially, reaction of *N*-DNs aziridine (**71**) with Fmoc-Cys-OH (**72**), generated by reduction of Fmoc-cystine (**106**) with TCEP, was investigated for formation of MeLan **121a** using the method of Liu *et al.* (**Scheme 2.9**).[165]



Scheme 2.9: Attempted synthesis of DNs/Allyl, Fmoc/OH MeLan (**121a**) with Fmoc-Cys-OH (**72**).

Although the mass of the desired lanthionine **121a** could be detected by LCMS analysis, none could be isolated following purification by flash column. Attempts to improve the yield by cooling the reaction to -78 °C, and adding Cys **72** and $BF_3.OEt_2$ as solutions in CH_2Cl_2, were also unsuccessful. It was hypothesised that the lack of Cys acid protection may have been a significant contributing factor to the production of by-products and difficulties in purification. Thus, ring-opening of aziridine **71** with the previously synthesised Fmoc-Cys-OTce (**104**) was examined.

Addition of Cys **104** and $BF_3.OEt_2$ to *N*-DNs aziridine (**71**) at -78 °C gave the desired (DNs, Allyl/Fmoc, Tce) MeLan (**121b**) in low yield, though it could be more readily purified from the reaction mixture (**Scheme 2.10**). Subsequent protecting group manipulations, removal of the *N*-DNs group, reprotection with Alloc-Cl,[165] and removal of the temporary Tce protection, gave the (Alloc, Allyl/Fmoc) methyllanthionine (**70**) ready for use in SPPS, in an overall yield of 2% from D-Thr.



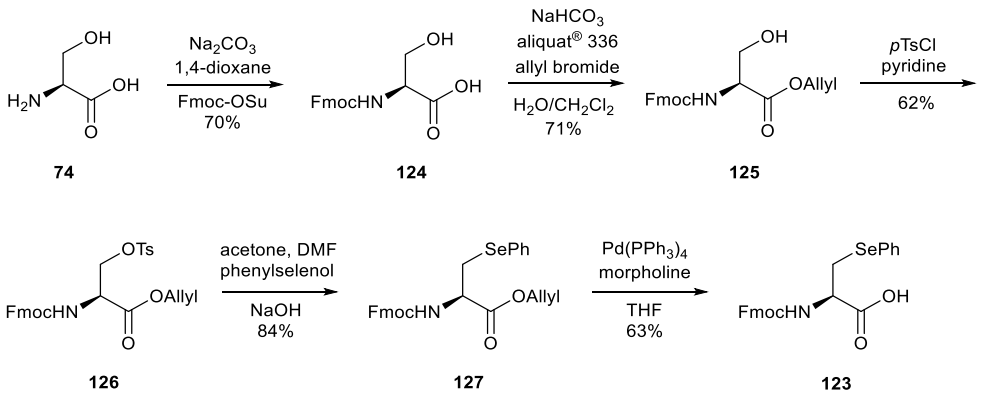
Scheme 2.10: Synthesis of (Alloc, Allyl/Fmoc) MeLan (**70**) from Fmoc-Cys-OTce (**104**).

2.4. Dehydroalanine Precursor Synthesis

In order to access WT nisin and mutacin I, both of which contain Dha residues, a robust method for introduction of Dha into lanthionine-containing peptides was required. Two of the methods of Dha incorporation studied as part of this work required the synthesis of either an unusual amino acid or a double-alkylation reagent. In the former, the dehydration is generated by the oxidative elimination of an Fmoc-*Se*-phenylselenocysteine residue,[196] and in the latter a Cys residue is alkylated with a synthesised dibromo reagent, before undergoing a β-elimination to give the Dha.[198]

2.4.1. Synthesis of *Se*-phenylselenocysteine (**123**)

Synthesis of phenylselenocysteine (**123**) began from L-Ser (**74**) (**Scheme 2.11**). Fmoc protection using Fmoc succinimide, followed by protection of the *C*-terminus with allyl bromide, gave Fmoc-Ser-OAllyl (**125**), according to the method of Brimble *et al.*.[227]



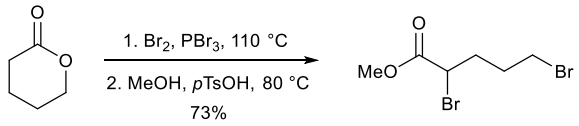
Scheme 2.11: Synthesis of Fmoc-*Se*-phenylselenocysteine-OH (**123**) from L-Ser (**74**).

The remainder of the synthesis was then conducted as described by Levengood *et al.*.[196] Alcohol **125** was first tosylated with *p*TsCl to give tosylate **126**, followed by reaction with phenylselenol to give phenylselenocystine **127** in good yield. Finally, removal of the allyl

group with $Pd(PPh_3)_4$ gave the Fmoc-*Se*-phenylselenocysteine (**123**) in 16% yield from L-Ser (**74**). The use of this residue in the synthesis of WT mutacin I ring A is discussed in **Chapter 3.7.1**.

2.4.2. Synthesis of Methyl 2,5-dibromopentanoate (**80**)

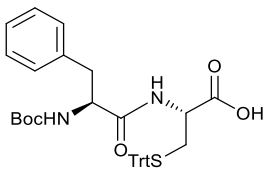
The alkylation reagent chosen for the generation of Dha residues from Cys was the methyl 2,5-dibromopentanoate (**80**) used by the Webb lab (**Scheme 2.12**). This was easily synthesised in two steps from δ-valerolactone (**128**) following the published procedure, in which the lactone is first halogenated with Br_2 and PBr_3 before methylation of the acid by refluxing with *p*TsOH in MeOH.[198] Use of this method in the synthesis of nisin and mutacin I ring A WT peptides is discussed in **Chapters 3.3.3.2.** and **3.3.4.2**.



128 **80**

Scheme 2.12: Synthesis of methyl 2,5-dibromopentanoate (**80**) from δ-valerolactone (**128**).

It was hypothesised that this reagent could also be utilised in combination with a Boc-Phe-Cys(Trt)-OH dipeptide (**129**) in the synthesis of mutacin I rings AB (**Figure 2.1**), reducing the number of required SPPS steps by simultaneously incorporating both tail residues (see **Chapter 4.4.2.3** for dipeptide synthesis and synthetic strategy).



129

Figure 2.1: Structure of the Phe-Cys dipeptide (**129**) used in combination with dibromopentanoate (**80**) in the synthesis of mutacin I rings AB.

2.5. Dehydrobutyrine Precursor Synthesis

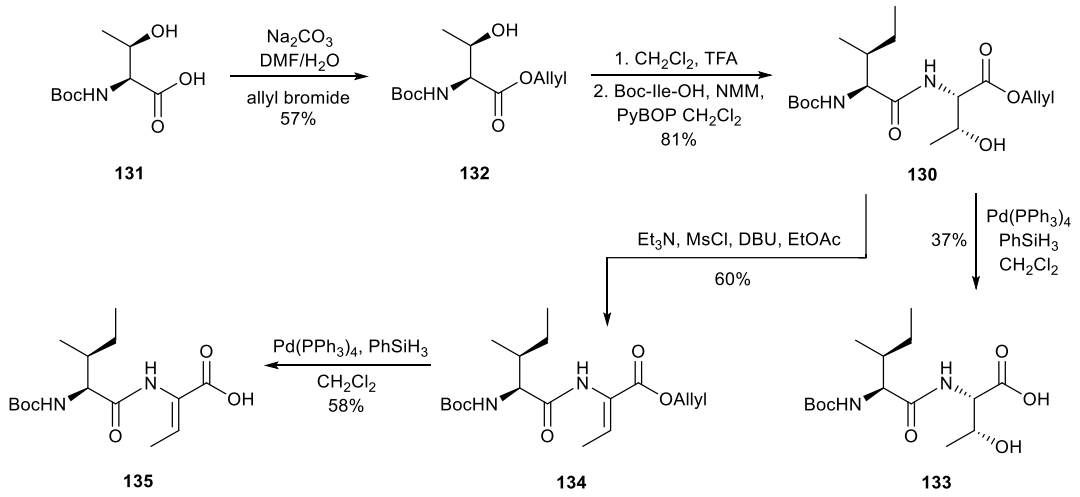
In addition to dehydroalanine, methods to access dehydrobutyrine are required for the synthesis of WT nisin ring A, which contains a Dhb in the tail of the peptide. One commonly used method is the introduction of the Dhb as part of an oligopeptide which can be coupled directly onto the growing peptide chain.[146,160,163–165,168] An alternative approach uses the transformation of a precursor amino acid to install the alkene, in a

similar manner as previously described for Dha synthesis. The two methods used as part of this work were dehydration of a Thr residue,[173] and β-elimination of a Cys derivative using the Morrison *et al.* dibromopentanoate methodology.[198]

2.5.1. Synthesis of Ile-Dhb and Ile-Thr Dipeptides

To synthesise the tail of WT nisin A by the addition of a Dhb-containing peptide or by dehydration of Thr, two different Ile-containing dipeptides were necessary (see **Chapter 3.3.4.1** for synthetic strategies). Both peptides could be obtained starting from the same Boc-Ile-Thr dipeptide (**130**) (**Scheme 2.13**).

Thus, Boc-Thr (**131**) was treated with allyl bromide to give Boc-Thr-OAllyl (**132**), followed by Boc deprotection with TFA, and a PyBOP catalysed coupling with Boc-Ile-OH to give the dipeptide (**130**). To give Boc-Ile-Thr-OH (**133**) ready for use in SPPS, the intermediate dipeptide **130** was allyl deprotected using $Pd(PPh_3)_4$ to give the free acid in 17% overall yield from Boc-Thr-OH.



Scheme 2.13: Synthesis of Boc-Ile-Dhb-OH (**135**) and Boc-Ile-Thr-OH (**133**).

Conversion of Thr-containing peptide **130** to Dhb-containing peptide **134** was achieved by dehydration using the method of Liu *et al.*.[165] Subsequent allyl deprotection, again using $Pd(PPh_3)_4$, gave free acid **135** in 16% overall yield from Boc-Thr-OH. The use of both of these dipeptides in the synthesis of nisin A is discussed in **Chapter 3.3.4.1**.

2.5.2. Synthesis and Use of Fmoc-β-methyl-cysteine (**136**)

Based on the work of Morrison *et al.*, it was hypothesised that a similar alkylation-elimination approach used to generate dehydroalanines could be employed for the generation of Dhb in peptides.[198] To this end, an L-Cys bearing a β-methyl group

would be required, which on treatment with base and dibromopentanoate (**80**) would give the WT *Z*-dehydrobutryine stereochemistry (**Figure 2.2**).

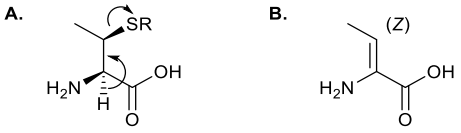
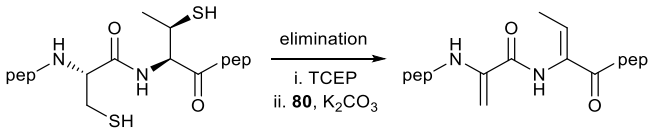


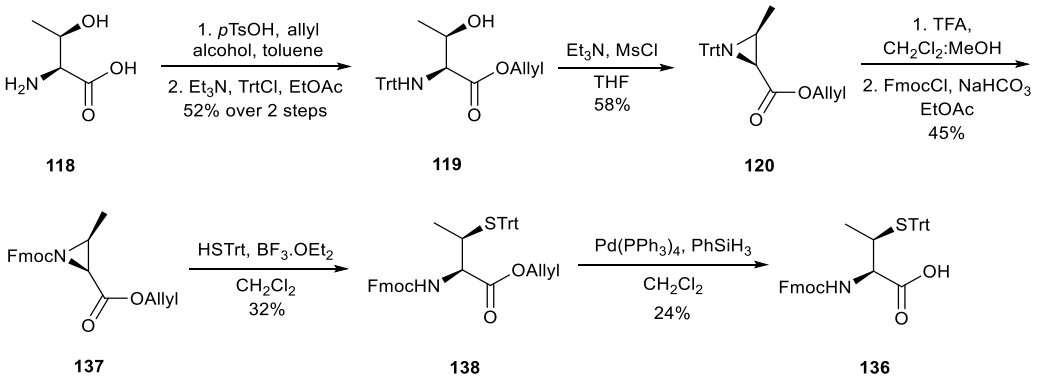
Figure 2.2: A. Proposed E2 elimination mechanism of the β-methyl-cysteine. **B.** The native *Z*-dehydrobutyrine stereochemistry found in nisin.

It was hypothesised that by using an Fmoc-β-methyl-cysteine, it would be possible to place a Dhb at any position in a peptide utilising the same procedure reported by Morrison *et al.* for multiple Dha incorporation.[198] Additionally, it was proposed that the simultaneous introduction of both Dha and Dhb into a peptide would be possible without alteration of the reaction conditions (**Scheme 2.14**).



Scheme 2.14: Proposed method for the simultaneous introduction of Dha and Dhb into peptides by alkylation and β-elimination of Cys and β-Me-Cys residues.

The preparation of several protected β-methyl-cysteines have been previously reported in the literature,[182,187,228] and the synthesis of the protected β-Me-Cys (**136**) used in this work was based on the method of Liu *et al.* (**Scheme 2.15**).[226] Protection of L-Thr (**L-118**) with allyl alcohol and trityl chloride produced Trt-Thr-OAllyl (**L-119**) in a two-step procedure, followed by reaction with MsCl and Et_3N to give *N*-trityl aziridine (**L-120**). Protecting group manipulation then installed the Fmoc group necessary for SPPS, giving Fmoc-aziridine (**137**), and ring-opening with triphenylmethanethiol and $BF_3.OEt_2$, based on the method of Maynard *et al.*, generated the β-Me-Cys (**138**).[229] Finally, removal of the allyl protecting group with $Pd(PPh_3)_4$ gave the Fmoc-β-Me-Cys(Trt)-OH (**136**) in 1% overall yield from L-Thr. The use of this residue in alkylation-elimination reactions to form WT nisin ring A is discussed in **Chapter 3.3.4.2**.



Scheme 2.15: Synthesis of Fmoc-β-Me-Cys(Trt)-OH (**136**) from L-Thr (**L-118**).

2.6. Summary

In summary, an orthogonally protected lanthionine was synthesised for use in the SPPS of lantibiotic fragments, based on the method of Mothia *et al.*.[162] Two different routes were investigated for the synthesis of each half of the lanthionine: one using the published literature procedure, and the other utilising cheaper starting materials. In the case of the β-iodo-Ala (**109**) fragment, the new route described here allowed access to the product in fewer steps. Beginning from either Boc-D-Ser(Bzl)-OH or D-Ser and either $(Fmoc\text{-}Cys\text{-}OtBu)_2$ or L-cystine, the desired (Teoc, TMSE/Fmoc) lanthionine (**38**) could be synthesised in gram-scale quantities. An orthogonally protected methyllanthionine (**70**) was also synthesised using an adapted version of the Liu *et al.* protocol, where the carboxyl group of the Cys fragment was Tce protected.[165] With this method, sufficient quantities of MeLan could be produced for use in the SPPS of WT nisin peptides.

Two different routes for the incorporation of dehydroalanines into peptides were enabled by the synthesis of an Fmoc-Sec(Ph) residue (**123**) according to the method of Levengood *et al.*,[196] and the dibromopentanoate alkylation reagent (**80**) reported by Morrison *et al.*.[198] A Phe-Cys dipeptide (**129**) was also synthesised for use in conjunction with the alkylation-elimination methodology, which was expected to expedite the synthesis of mutacin I rings AB.

Finally, three different precursors for the incorporation of Dhb residues were synthesised. Two were dipeptides: one containing a Dhb (**135**), and one containing a Thr (**133**) to be dehydrated to reveal the Dhb. A protected β-Me-Cys (**136**) was also synthesised to investigate whether the alkylation-elimination strategy could be used to generate both Dha and Dhb residues.[198]

3. SYNTHESIS OF SINGLE RINGS

3.1. Introduction

This chapter discusses the SPPS of single lanthionine-containing rings from the lipid II binding regions of nisin and mutacin I, both WT peptides and analogues.

Firstly, WT and analogue sequences of the smaller ring B peptides were synthesised. As these contain only one unusual amino acid, a (methyl)lanthionine which forms the thioether bridge, and no dehydro residues, they were expected to be a good initial test of the synthetic methodology.

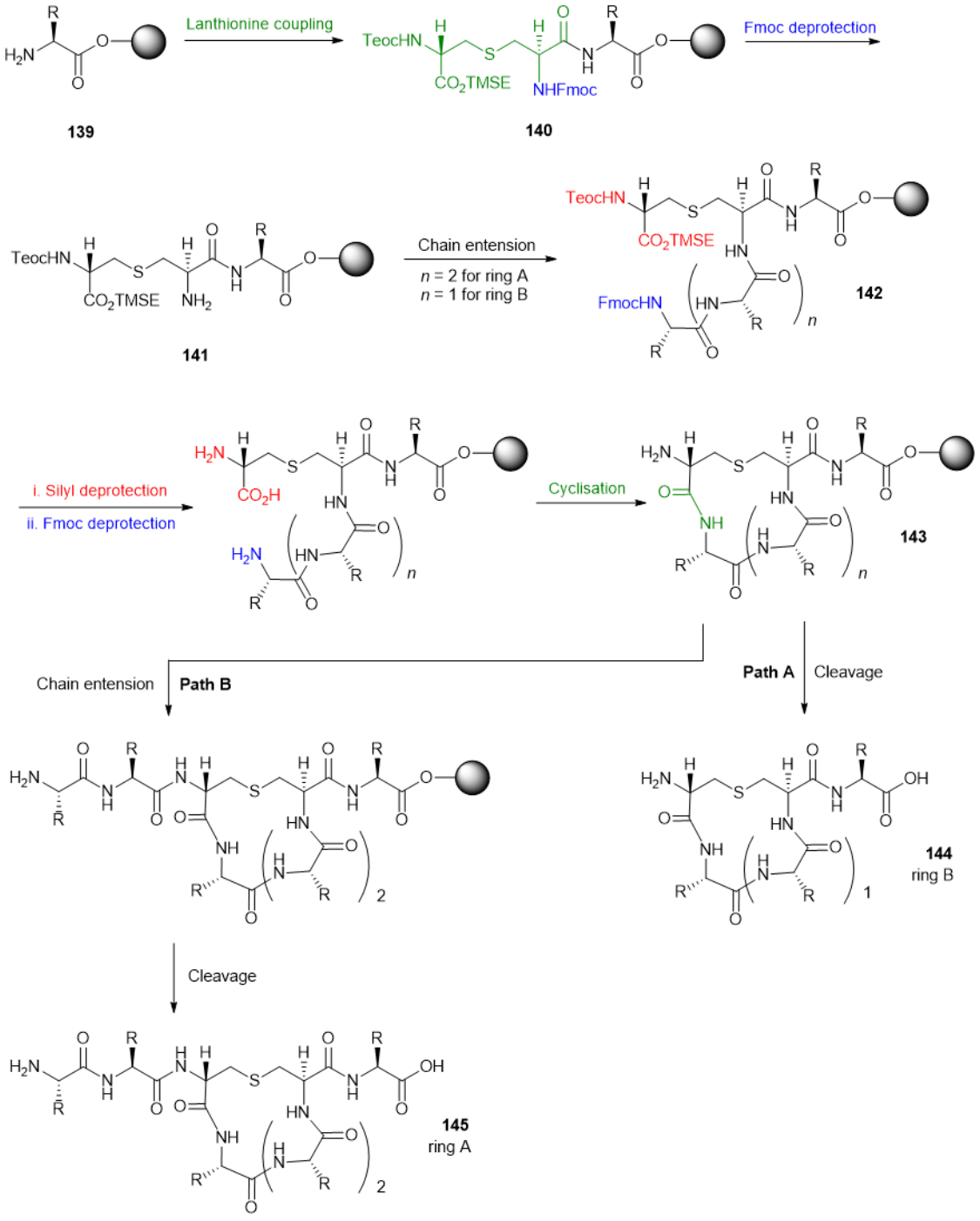
Secondly, ring A peptides were synthesised. As both WT nisin and mutacin I ring A contain dehydro residues in addition to the Lan, the methods for Dha and Dhb incorporation described in **Chapter 2** were investigated during the syntheses of these peptides. Analogues of the A rings were also produced in which the Dha residues were replaced with either Ser or Ala, acting as hydrated or saturated analogues respectively.

3.1.1. General Strategy for Single Lantibiotic Ring Synthesis

The strategy used for the synthesis of all of the single ring peptides in this work was based on methods previously reported by the Tabor lab, shown in **Scheme 3.1**.[156,162] The first step of each synthesis was the addition of an orthogonally protected lanthionine to an acid-sensitive resin which was pre-loaded with the first amino acid (**139**) to give **140**. In the case of the B rings this pre-loaded residue was the native residue 12 (Thr for mutacin I and Lys for nisin), but for the A rings an Ala residue was used as a non-oxidisable mimetic for the (methyl)lanthionine bridge, found next to the ring A lanthionine in the native peptides. The use of low-loading Novasyn TGT resins was essential to avoid crosslinking between different peptides during intramolecular cyclisation steps.[158,165]

Following addition of the lanthionine, the Fmoc group was removed to give **141**, and normal SPPS techniques were used to add the rest of the residues in the ring (two residues for ring B syntheses and three for ring A), resulting in peptide **142**. Removal of the orthogonal protecting groups, using 50% TBAF in DMF for the Teoc/TMSE Lan (**38**) or 2 eq $Pd(PPh_3)_4$ for the Allyl/Alloc MeLan (**70**), followed by Fmoc deprotection and cyclisation, gave the lantibiotic ring (**143**). For ring B syntheses (Path A, **Scheme 3.1**), the peptide was cleaved from the resin to give free peptide **144**. For ring A syntheses

(Path B, **Scheme 3.1**), two additional amino acids could be added if necessary to form the tail of the peptide, using normal Fmoc SPPS techniques, followed by cleavage of the peptide from the resin to give free peptide **145**.



Scheme 3.1: General synthetic strategy for the formation of single lantibiotic rings, exemplified here by the synthesis of a peptide using the (Teoc, TMSE/Fmoc) Lan (**38**).

Standard Fmoc SPPS protocols were used throughout: PyAOP and HOAt were used for coupling and cyclisation steps in order to reduce racemisation of the lanthionine, and Fmoc deprotections were carried out with 40% piperidine in DMF.[230,231] All non-lanthionine residues were added using a double coupling procedure to increase efficiency. Following completion of the synthesis, peptides were cleaved from the resin with high percentages of TFA (≥ 94%), using TIPS as a cation scavenger and EDT as a reducing agent for cysteine-containing peptides.

Due to the bulky nature of protected (Me)Lan residues, and the difficulty often reported in the cyclisation of small peptide rings, special measures were taken to ensure that all (Me)Lan coupling and cyclisation reactions went to completion.[232] Microwave irradiation was chosen as an appropriate method for this purpose, as it has been shown to decrease reaction time and concurrently increase crude peptide purity.[233] In their work on the SPPS of ProTx-II analogues, Wright *et al.* recently reported that the optimal conditions for microwave-assisted incorporation of orthogonally protected lanthionines was to conduct the reaction with irradiation at 60 °C and 300 W for 5 min.[220] This strategy was therefore employed in this work, for both coupling of (methyl)lanthionine residues and cyclisation reactions.

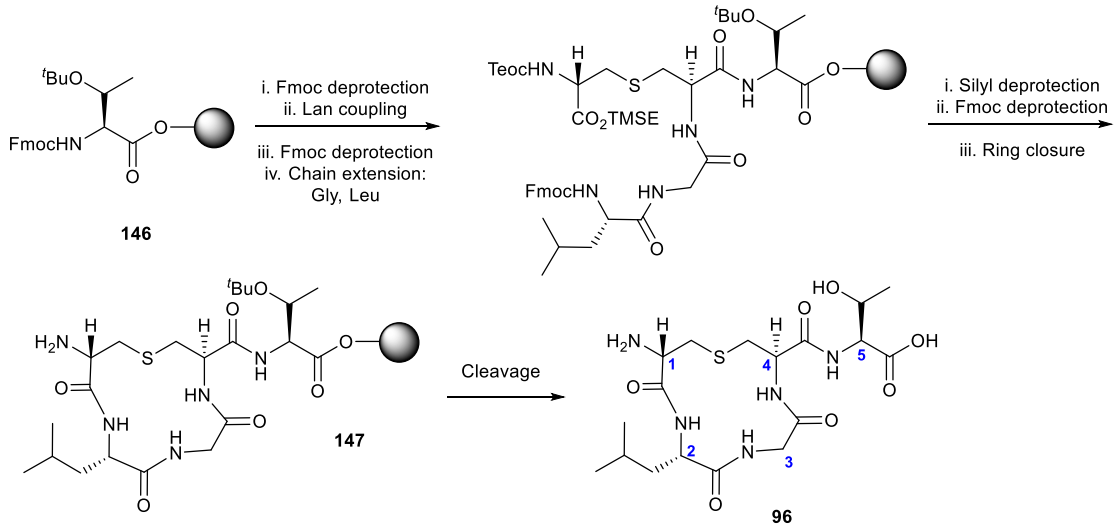
3.2. Synthesis of Ring B Peptides

To verify whether the proposed synthetic strategy would be effective for the production of nisin and mutacin I rings A and B, the smaller ring B peptides were first synthesised. Two Lan-containing peptides were prepared, followed by one MeLan-containing peptide.

3.2.1. Mutacin I Ring B WT (**96**)

To synthesise WT mutacin I ring B (**96**), an Fmoc-Thr(*t*Bu)-Novasyn TGT resin (**146**) was used, and (Teoc, TMSE/Fmoc) Lan (**38**) was incorporated using the microwave protocol. The two non-lanthionine residues in mutacin I ring B are Gly and Leu, which were coupled using standard Fmoc SPPS procedures. The orthogonal silyl protecting groups were then removed with TBAF, and although it was expected that this would also remove the Fmoc protecting groups, the peptide was then treated with 40% piperidine to ensure complete Fmoc deprotection. The cyclisation reaction to form **147** was effected by double coupling for 2 h with PyAOP, HOAt and DIPEA, with 5 min of microwave

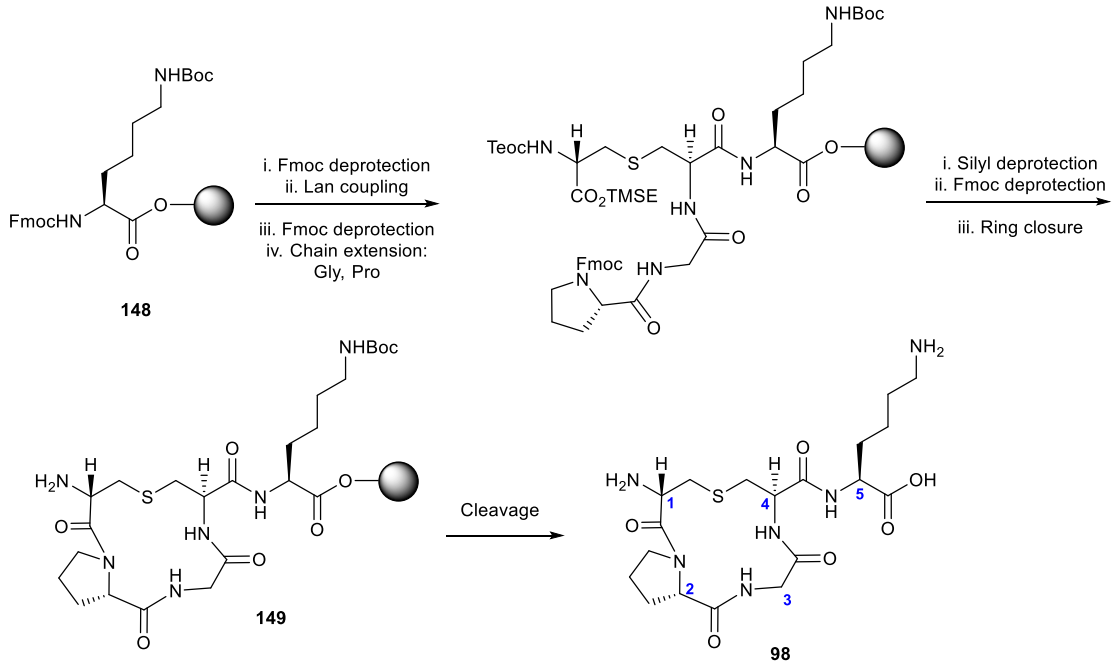
irradiation. After completion of the synthesis, the peptide was cleaved from the resin and purified by reverse phase HPLC to give the ring B peptide (**96**) in 34% yield.



Scheme 3.2: Synthesis of mutacin I ring B WT (**96**).

3.2.2. Nisin Ring B Lan Analogue (**98**)

Following the successful synthesis of mutacin I ring B (**96**), attention was turned to the synthesis of a nisin ring B analogue (**98**), in which the thioether bridge was formed by Lan rather than the native MeLan.

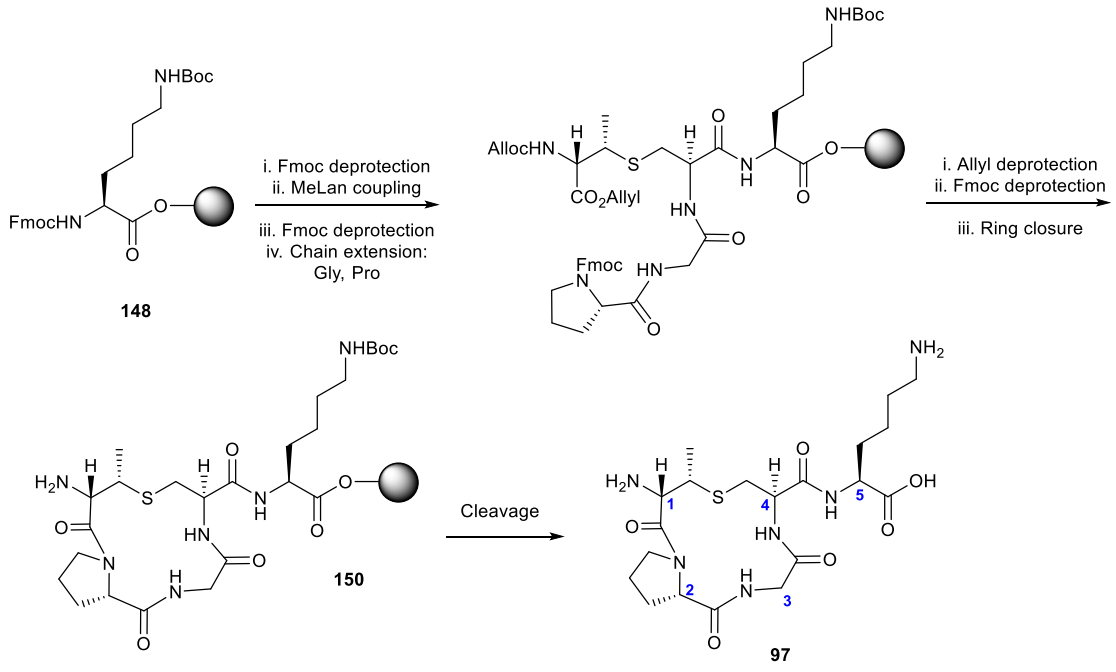


Scheme 3.3: Synthesis of nisin ring B Lan analogue (**98**), with the thioether bridge formed by Lan rather than MeLan.

Starting with an Fmoc-Lys(Boc)-Novasyn TGT resin (**148**), (Teoc, TMSE/Fmoc) Lan (**38**) was first incorporated, followed by the addition of Gly and Pro using standard Fmoc SPPS. Deprotection of the Teoc/TMSE and Fmoc groups, followed by cyclisation with PyAOP, HOAt and DIPEA and cleavage of the peptide from the resin, gave the desired nisin analogue (**98**) in 7% yield after HPLC purification.

3.2.3. Nisin Ring B WT (**97**)

The first step in the synthesis of WT nisin ring B (**97**) was attachment of (Alloc, Allyl/Fmoc) MeLan (**70**) to an Fmoc-Lys(Boc)-Novasyn TGT resin (**148**), using PyBOP, HOBt and NMM according to the method of Liu *et al.*.[165] The Gly and Pro residues were then added using standard Fmoc SPPS procedures before removal of the Alloc/Allyl groups with $Pd(PPh_3)_4$, again following the method of Liu *et al.*.[165] The final Fmoc group was then removed, and the cyclisation conducted using PyBOP, HOBt and NMM with microwave irradiation, giving **150**. After cleavage and purification, WT nisin ring B (**97**) was isolated in 37% yield.



Scheme 3.4: Synthesis of nisin ring B WT (**97**).

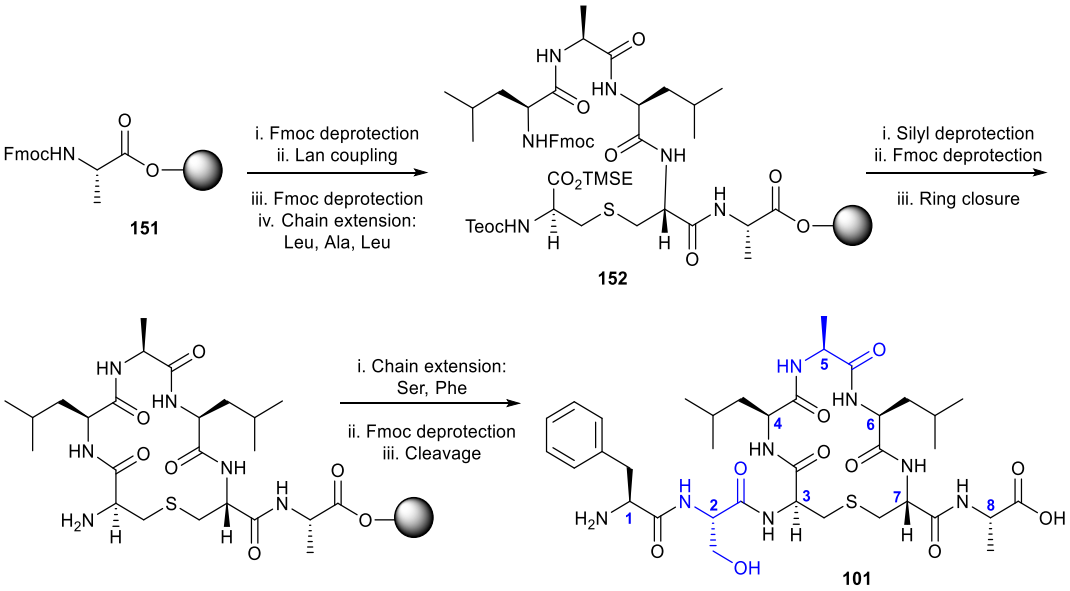
3.3. Synthesis of Ring A Peptides

The same procedure used to synthesise the ring B peptides was utilised in the synthesis of the ring A peptides, except that three extra residues were required overall: one in the ring itself and two adjacent to it to form the tail of the peptide. In order to ensure that the

existing methodology was still effective with a slightly larger ring size, for the first ring A it was decided that the synthesis should be simplified as much as possible. Following this, investigations were conducted into whether the reagents for dehydro residue incorporation described in **Chapter 2** could be utilised in syntheses of the WT peptides.

3.3.1. Mutacin I Ring A Ser/Ala Analogue (**101**)

To synthesise the initial simplified peptide (**101**), the two Dha residues present in mutacin I WT ring A were replaced: one with Ser, the biosynthetic precursor to Dha, and one with Ala, which would act as a saturated analogue for Dha (**Scheme 3.5**).



Scheme 3.5: Synthesis of mutacin I ring A Ser/Ala analogue (**101**). Dha replacements highlighted in blue.

An Fmoc-Ala-Novasyn TGT resin (**151**) was used for the synthesis of Ser/Ala analogue (**101**). After Fmoc deprotection, (Teoc, TMSE/Fmoc) Lan (**38**) was added using PyAOP, HOAt and DIPEA, and the chain extended by addition of Leu, Ala, and a second Leu, giving **152**. After silyl deprotection and cyclisation, the final Ser and Phe residues were added, again using PyAOP and HOAt, before cleavage of the peptide from the resin (Path B in **Scheme 3.1**).

Although the desired peptide (**101**) could be detected by LCMS analysis of the crude sample, a truncated peptide (**153**), with no addition of the final two amino acids, was also observed (**Figure 3.1**). This suggested that following the cyclisation, the terminal amine

may be less accessible, leading to inefficient coupling of the final two residues. This prompted a brief investigation into alternate coupling methods for the addition of these two amino acids.

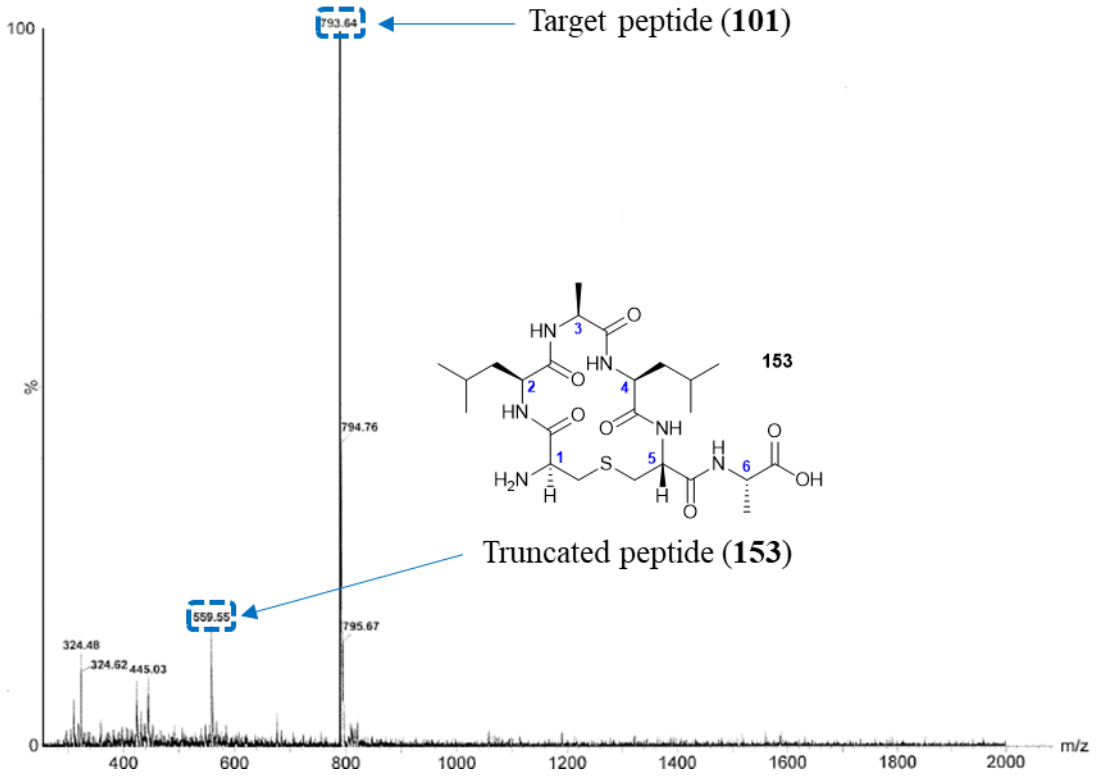
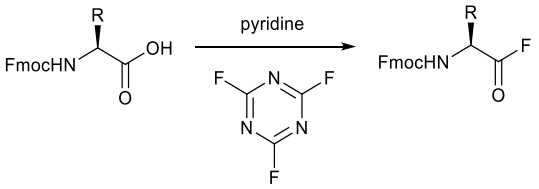


Figure 3.1: Crude LCMS spectrum showing the presence of both the target peptide (**101**) and a truncated peptide (**153**).

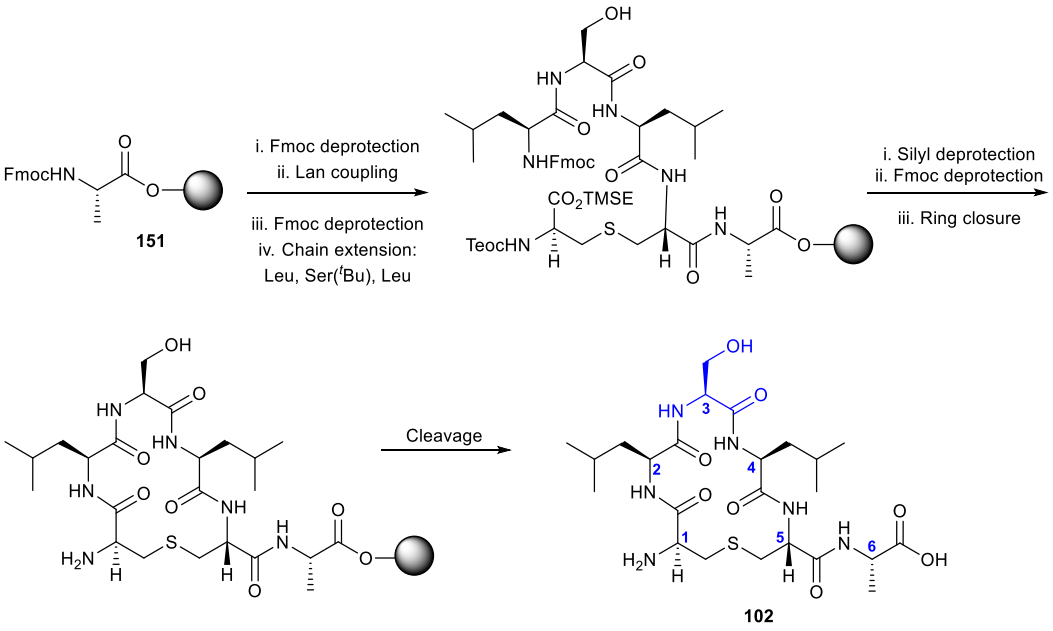
HATU has been reported to give good results with sterically hindered couplings, such as in the coupling of two adjacent Aib residues.[231,234,235] However, in this case only a small increase in the ratio of desired product (**101**) to truncated peptide (**153**) was observed with this reagent. Best results were achieved using amino acid fluorides, synthesised from the Fmoc amino acids with cyanuric fluoride (**Scheme 3.6**),[236,237] and incorporated into the peptide by coupling with DIPEA in CH_2Cl_2.[238] This further increased the ratio of **101** to **153**, although the reaction could not be pushed to completion, and truncated peptide **153** was still observed by LCMS. Despite this, sufficient quantities of the desired Ser/Ala analogue (**101**) could be produced to enable purification. Purification by HPLC gave the mutacin I ring A analogue (**101**) in 3% yield, and truncated peptide **153** in 2% yield.



Scheme 3.6: General scheme for the synthesis of amino acid fluorides.

3.3.2. Mutacin I Ring A Ser Analogue (**102**)

After ensuring that the proposed strategy was effective for the synthesis of ring A peptides, attentions turned towards the production of WT mutacin I ring A. Initially, truncated peptide **102** was synthesised to investigate Dha introduction by β-elimination of serine-containing precursor peptide (**Scheme 3.7**).[239] Additionally, peptide **102** would provide access to a further analogue peptide for structural studies (see **Chapter 5.3.2**).



Scheme 3.7: Synthesis of mutacin I ring A Ser analogue (**102**). Dha precursor residue highlighted in blue.

The synthesis of peptide **102** began from an Fmoc-Ala-Novasyn TGT resin (**151**). The synthesis proceeded in an analogous manner to that described for Ser/Ala analogue (**101**), with the chain instead extended by the addition of Leu, Ser, and a second Leu. After ring closure, the peptide was cleaved from the resin and purified by reverse phase HPLC. Interestingly, three distinct peaks containing the mass of the target peptide were observed during purification (**Figure 3.2**). As the peaks were so well separated, it was proposed

that these would most likely correspond to three diastereomers formed by scrambling of the lanthionine stereocentres during the cyclisation reaction, rather than three different conformations, though in-depth studies by NMR would be necessary to confirm this. The peptide producing the main peak (eluting at 8.78 min) was fully characterised, and was isolated with a yield of 5%.

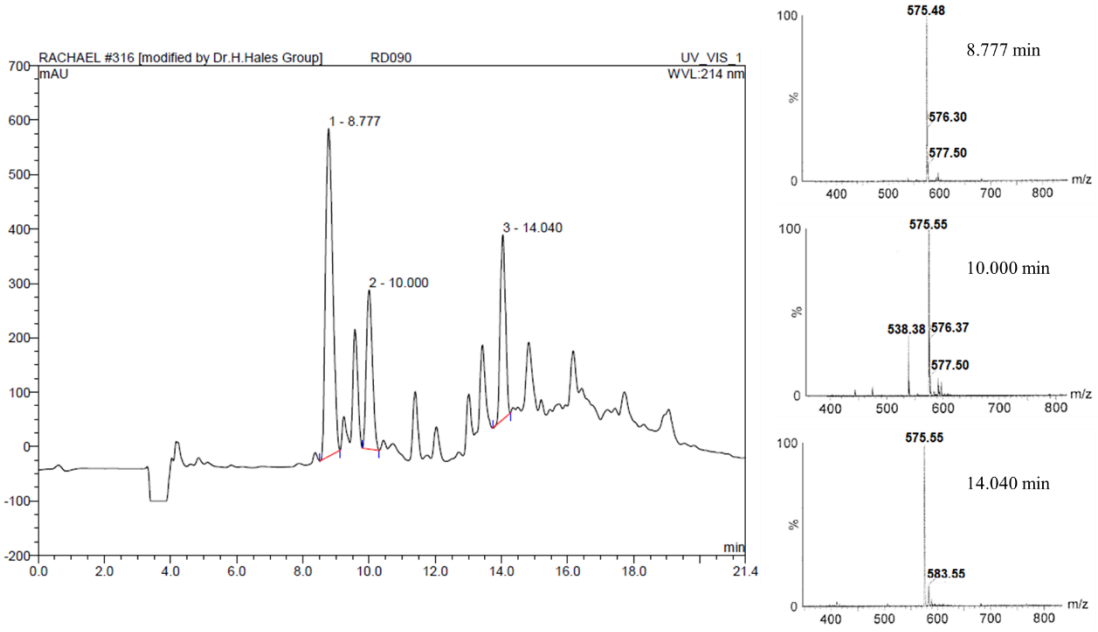
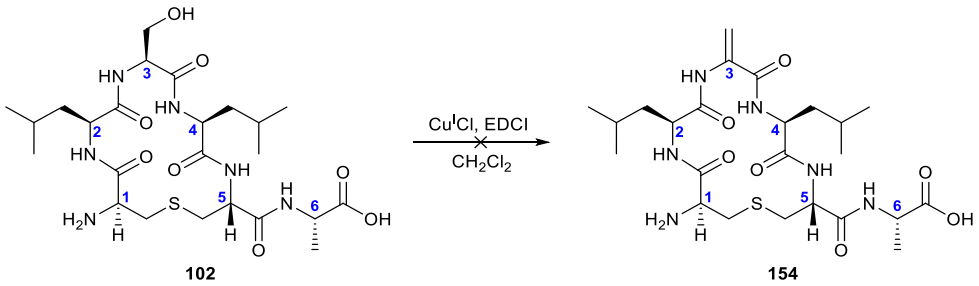


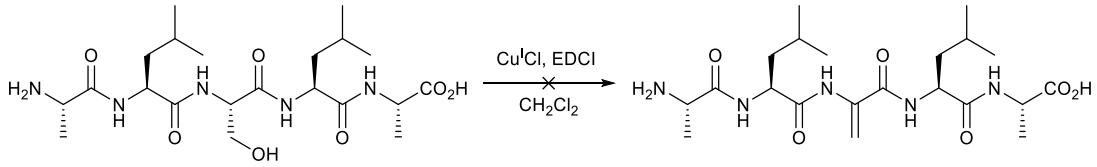
Figure 3.2: Semi-preparative HPLC trace of peptide (**102**). The three labelled fractions contained a peptide of mass 574, and the corresponding LCMS spectra for each peak are shown on the right.

Following isolation of Ser peptide **102**, attempts were made to produce the Dha by β-elimination of the Ser residue according to the method of Slootweg *et al.* (**Scheme 3.8**).[173] Unfortunately, after work up neither the Ser precursor (**102**) or the target peptide (**154**) could be detected by LCMS.



Scheme 3.8: Attempted dehydration of serine precursor (**102**) to give truncated WT mutacin ring A (**154**).

To investigate whether the problem with this reaction was due to the procedure itself or related to the complexity of the lantibiotic peptide, the dehydration reaction was also attempted on a simpler system. A pentapeptide with sequence ALSLA (**155**) was chosen to act as a linear mimic of mutacin I ring A (**Scheme 3.9**), and was synthesised on an Fmoc-Ala-Novasyn TGT resin using standard SPPS protocols. After cleavage of the peptide from the resin and purification by HPLC, the dehydration was conducted, again using the method of Slootweg *et al.*.[173] The reaction was also unsuccessful in this instance, and none of the dehydrated peptide (**156**) could be detected by LCMS after work up. It was reasoned that this was most likely caused by oligomerisation of the unprotected peptide due to the presence of coupling agent, as previously this reaction has only been reported using peptides with protected *N*- and *C*-termini.[145,173] As this method of Dha introduction by Ser dehydration had proven ineffective with cleaved peptides, efforts were instead concentrated on the generation of Dha using alternative methodologies.



155 156

Scheme 3.9: Attempted dehydration of mutacin I ring A mimic (**155**) to give **156**.

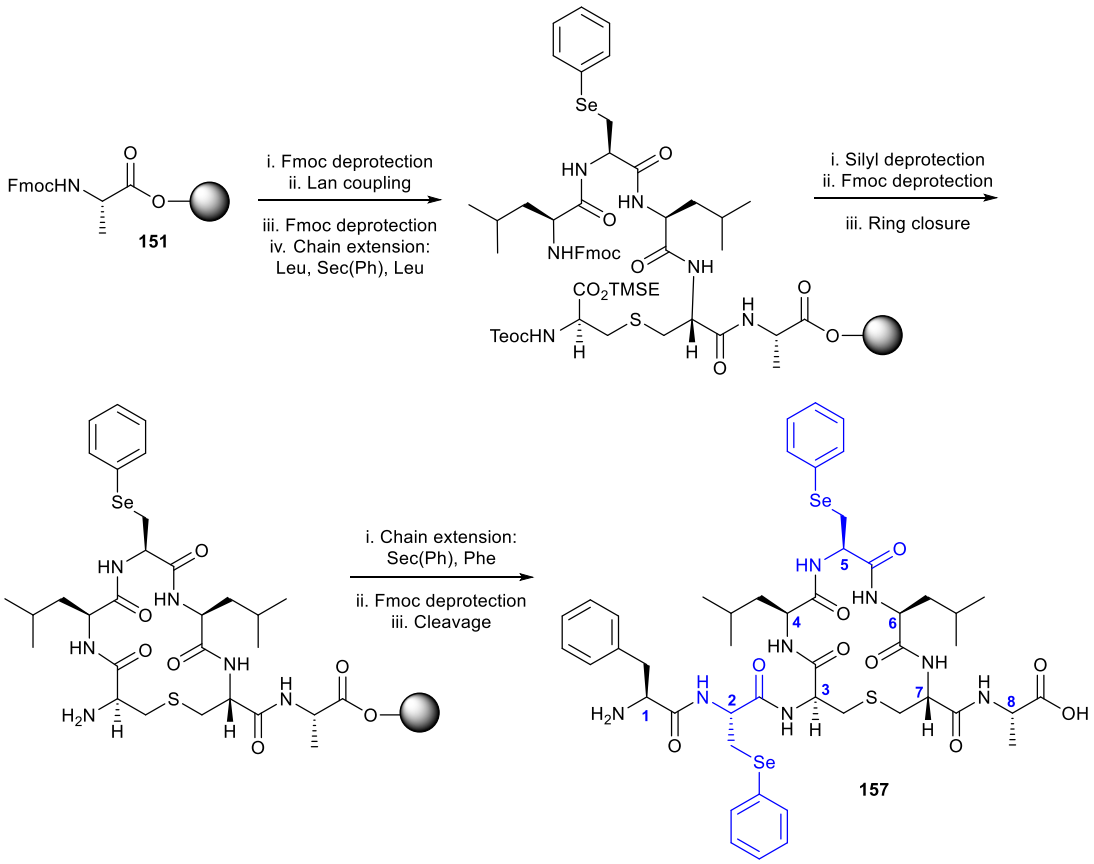
3.3.3. Mutacin I Ring A WT (**99**)

In contrast to the analogues **101** and **102** (**Scheme 3.5** and **Scheme 3.7**), in the following syntheses of WT mutacin I ring A, attempts were made to include the two Dha residues and both tail residues in the peptide. Two different routes for incorporation of Dha were attempted: i) *via* the oxidative elimination of Sec(Ph) residues with $NaIO_4$, according to the procedure of Levengood *et al.*,[196] and ii) by the alkylation and β-elimination of a Cys residue with a dibromopentanoate, according to the method of Morrison *et al.*.[198]

3.3.3.1. Synthesis Using Sec(Ph) as a Dha Precursor

To make WT mutacin I ring A by oxidation of selenocysteines, the precursor peptide **157** (**Scheme 3.10**) is required, in which Sec(Ph) residues are installed in the positions where dehydroalanines occur in the WT peptide (positions 2 and 5). Thus, the (Teoc, TMSE/Fmoc) Lan (**38**) was first added to an Fmoc-Ala-Novasyn TGT resin before

coupling of Fmoc-Leu using standard SPPS protocols. The first Fmoc-Sec(Ph)-OH residue (**123**) was then added according to the procedure reported by Levengood *et al.*, in which HOBt and DIC coupling reagents are used in order to minimise racemisation at the chiral centre.[196] Also important to note at this point is that following incorporation of a Sec(Ph) residue, all subsequent Fmoc deprotections must be carried out with a 20% piperidine solution in three cycles of 1 min in order to minimise premature elimination to the Dha. A further Fmoc-Leu was then added, followed by cyclisation and addition of the second Sec(Ph), again using HOBt and DIC. Finally, after the addition of Fmoc-Phe and a final Fmoc deprotection, the peptide was cleaved from the resin.



Scheme 3.10: Proposed synthesis of Sec(Ph)-containing precursor peptide (**157**). Dha precursors highlighted in blue.

Analysis of the crude sample by LCMS indicated that none of the target peptide (**157**) was produced. Instead, three different truncated peptides, **158**, **154** and **159**, were the major products of the reaction, all of which were missing the two tail residues (**Figure 3.3**). One peptide (**158**) contained the desired Sec(Ph) residue in the ring (product **A**), in the second peptide the Sec(Ph) residue had already undergone elimination to give the Dha

(**154**) (product **B**), and in the third peptide the cyclisation reaction had failed and the Sec(Ph) had undergone elimination (**159**) (product **C**).

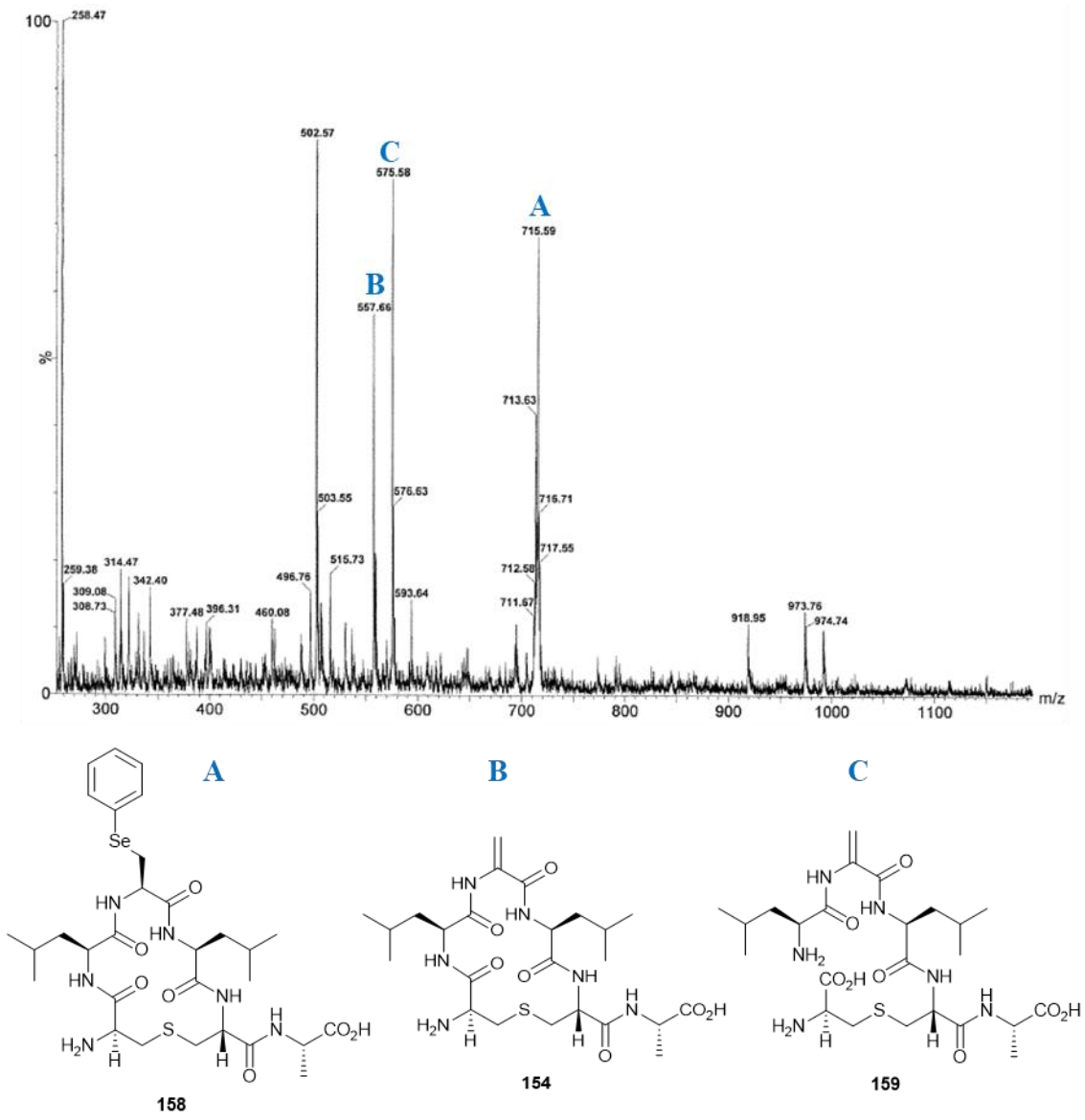


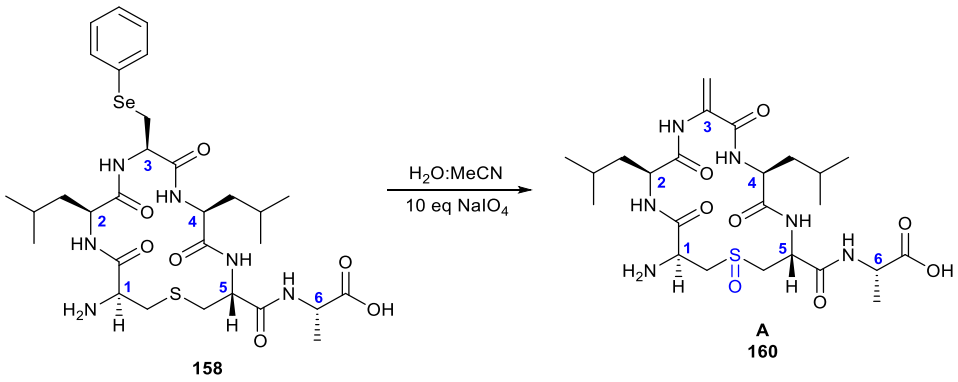
Figure 3.3: Crude LCMS spectrum showing the presence of three different peptides. Peaks labelled **A**, **B** & **C** correspond to the three peptides below the spectrum.

Due to the complexity of this product mixture, it was necessary to repeat the synthesis so that sufficient quantities of a Sec(Ph)-containing, cyclised species could be obtained to enable a trial of the oxidative elimination procedure. To minimise the number of possible peptides which could be produced, addition of the tail residues was not attempted in this second synthesis. One further difference between the procedures was that COMU was used as the coupling reagent for the cyclisation reaction in place of PyAOP and HOAt, as

it has been shown to lead to high coupling efficiency, especially when used in combination with microwave irradiation.[240,241] Aside from these two factors, the modified synthesis was conducted in the same manner as described for the production of **157**, with truncated peptide **158** as the target peptide.

Although some of the uncyclised peptide (**159**) was still observed in the crude LCMS spectra of this reaction, the modified protocol produced an increased ratio of the desired peptide (**158**) over the by-products. Following the synthesis, peptide **158** was purified by HPLC and isolated in 3% yield.

With Sec(Ph)-containing peptide **158** now in hand, the most effective conditions for the oxidative elimination could be explored. Use of conditions reported by Levengood *et al.*, 10 eq of $NaIO_4$ in a water:acetonitrile mixture,[196] led to oxidation of the Lan bridge, in addition to elimination of the selenocysteine, to give peptide **160** as the sole product of the reaction (**Scheme 3.11**). This over-oxidation was undesirable as it has been shown that oxidised nisin is unable to bind lipid II, rendering the peptide inactive.[242] To decrease the incidence of over-oxidation and maximise production of the desired peptide (**154**), the reaction was repeated at 0 °C with stoichiometric amounts of $NaIO_4$. Despite these measures, a small amount of the over-oxidised peptide **A** (**160**) was still observed; though target peptide **B** (**154**) could be isolated following HPLC in 2% yield (see **Figure 3.4**).



Scheme 3.11: Oxidative elimination of **158** with 10 eq $NaIO_4$ gave only over-oxidised peptide **160**.

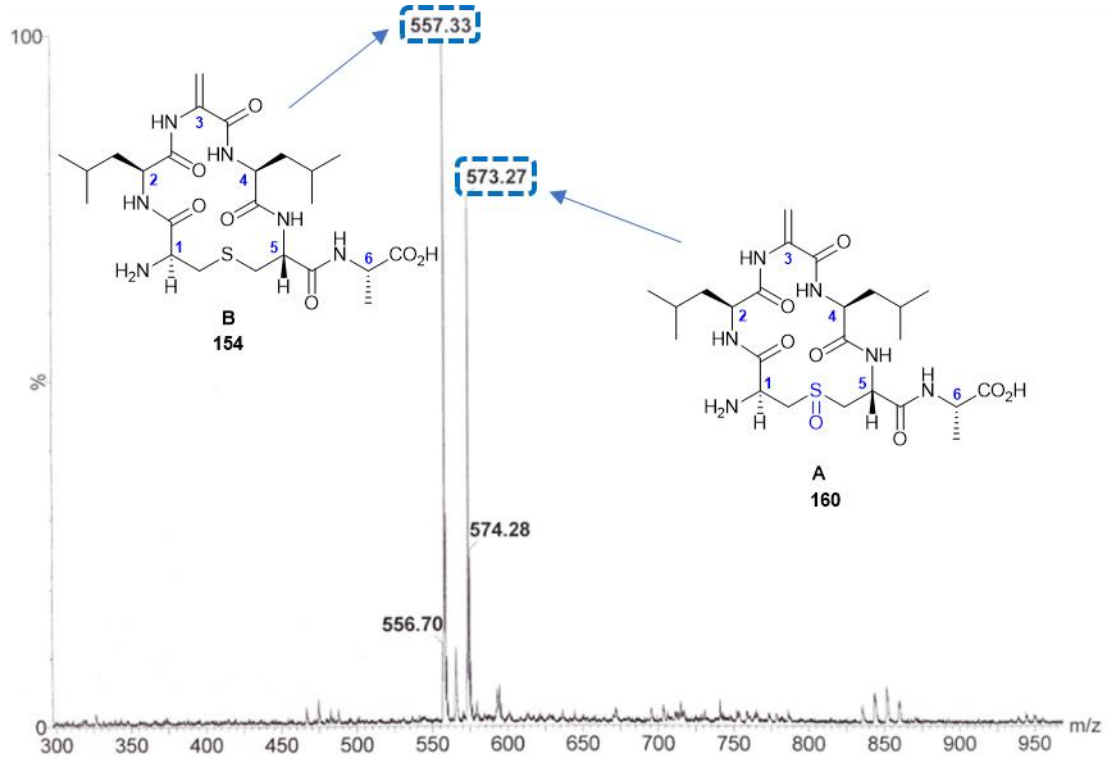
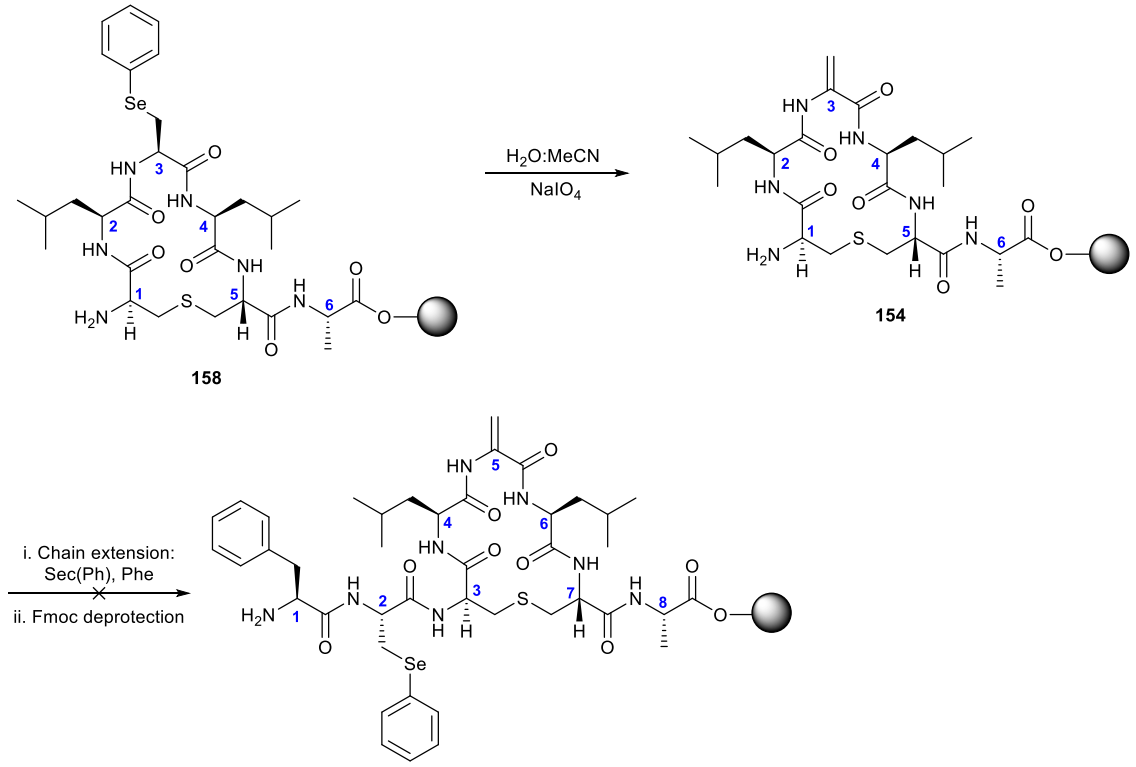


Figure 3.4: LCMS spectrum showing mixtures of over-oxidised peptide **A** (**160**) and target peptide **B** (**154**) from oxidative elimination of **158** with $NaIO_4$.

Having established that Dha-containing lantibiotic rings could be synthesised from Sec(Ph)-containing precursors, attention turned towards addition of the tail residues to the peptide ring, given that double coupling with HOBt/DIC had proven ineffective (see **Figure 3.3**). As in the case of mutacin I ring A Ser/Ala analogue (**101**), it was hypothesised that difficulties in coupling the tail residues to resin-bound **158** could be caused by inaccessibility of the terminal amine. As Dha is known to exert a strong conformational bias,[217] introduction of the alkene *via* on-resin oxidative elimination of the Sec(Ph) residue, after cyclisation but prior to coupling of the tail residues, was investigated. It was hoped that this would cause a change in conformation of the peptide, making the amine more accessible. Thus, Sec(Ph) peptide **158** was synthesised as previously described, but not cleaved from the resin (**Scheme 3.12**). A suspension of the resin-bound peptide (**158**) in water:acetonitrile was cooled to 0 °C, and 2 eq $NaIO_4$ were added. After chain extension with Fmoc-Sec(Ph) and Fmoc-Phe, and cleavage from the resin, LCMS analysis of the crude peptide showed that the on-resin elimination to give resin-bound **154** had been successful, with no over-oxidation. However, no addition of the final two amino acids was observed. This strongly suggested that the change from

Sec(Ph) to Dha at position three of ring A did not sufficiently alter the conformation of the ring to affect the accessibility of the amine.

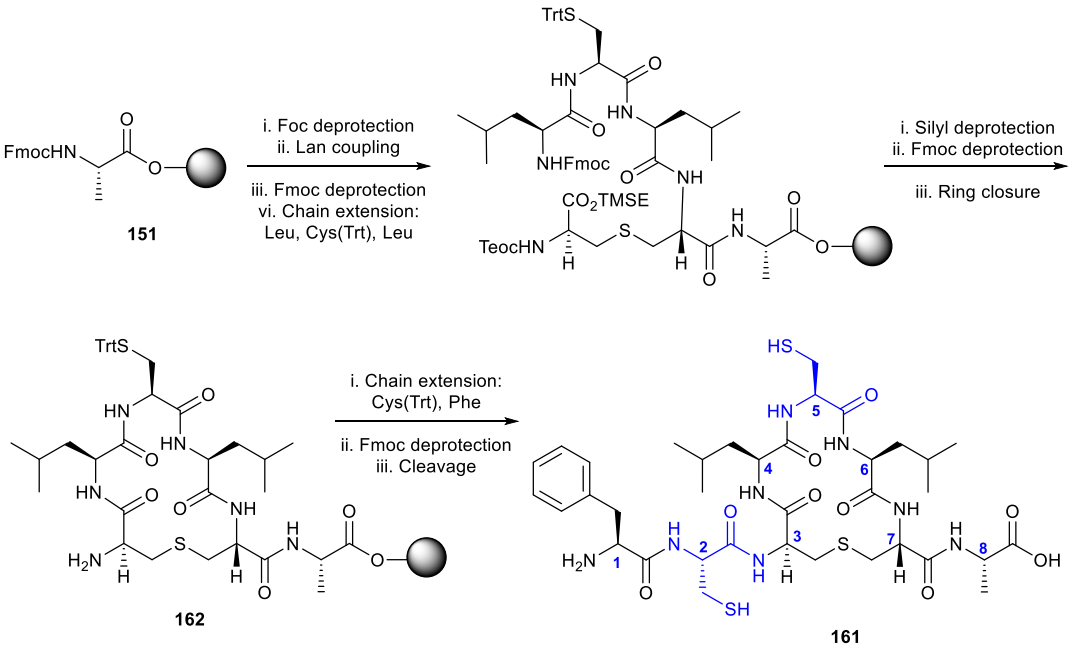


Scheme 3.12: Oxidative elimination of Sec(Ph)-containing peptide **158** on resin, and attempted coupling of tail residues.

As the synthesis of WT mutacin I ring A including the tail region *via* a Sec(Ph) precursor had proved unsuccessful, alternative approaches were investigated.

3.3.3.2. Synthesis Using Cys as a Dha Precursor

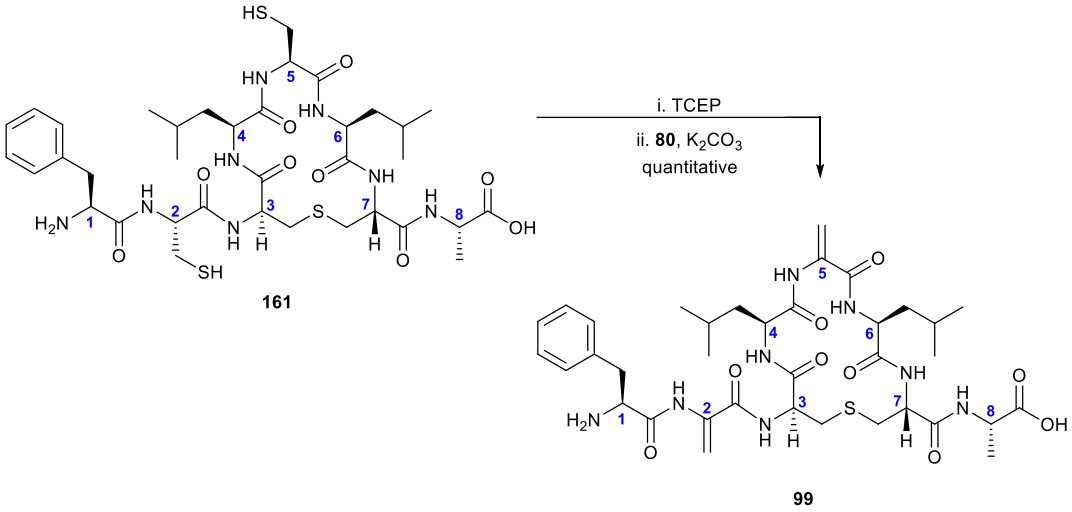
Morrison *et al.* have recently described an alkylation-elimination methodology to install Dha residues from Cys.[198] In order to investigate the use of this approach in the synthesis of mutacin I ring A, a precursor peptide bearing Cys residues in place of the native dehydroalanines was required (**161**) (**Scheme 3.13**).



Scheme 3.13: Synthesis of the desired Cys-containing precursor peptide (**161**). Dha precursors highlighted in blue.

The synthesis began with the attachment of the (Teoc, TMSE/Fmoc) Lan (**38**) to an Fmoc-Ala-Novasyn TGT resin (**151**). Standard SPPS techniques were then used to add the Leu, Cys and Leu residues, before cyclisation to give **162**. The Cys and Phe tail residues were then also added, and the peptide cleaved from the resin. Without any optimisation of the standard procedure described in **Scheme 3.1**, precursor peptide **161** was obtained in 15% yield following purification by reverse phase HPLC. Following the difficulties encountered in coupling of the tail residues in both Ser/Ala analogue **101** (**Figure 3.1**) and Sec(Ph) peptide **154** (**Scheme 3.12**), the success in this case was surprising. It was speculated that this may have been caused by a different ring conformation, necessitated by the bulkiness of the Cys(Trt) group.

After isolation of precursor peptide **161**, the alkylation-elimination reaction to form the Dha residues, as described by Morrison *et al.*, was investigated (**Scheme 3.14**).[198] Treatment of **161** with TCEP, followed by the addition of a large excess of dibromopentanoate (**80**) and K_2CO_3 at 37 °C for 2 h, gave WT mutacin I ring A (**99**) in 11% yield (compared to resin loading) after purification by HPLC.



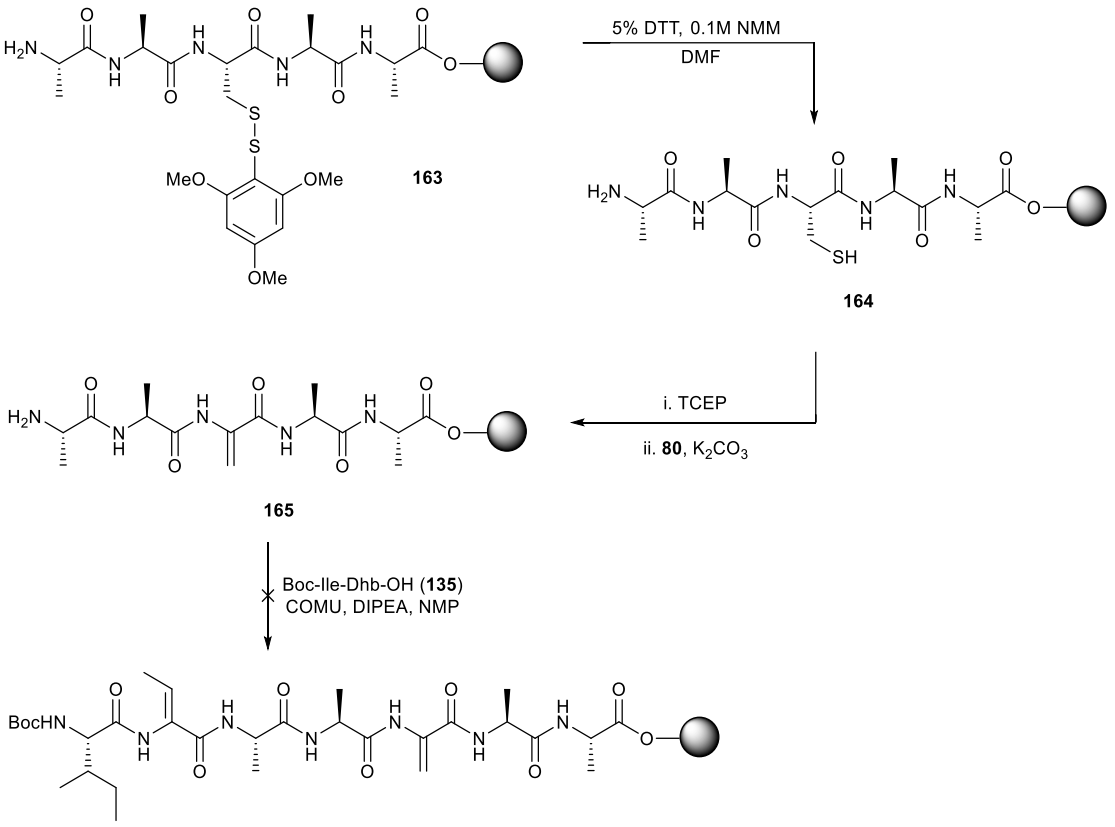
Scheme 3.14: Synthesis of WT mutacin I ring A (**99**) from a Cys-containing precursor (**161**).

3.3.4. Nisin Ring A WT (**100**)

Following the successful synthesis of WT mutacin I ring A, efforts turned to the synthesis of WT nisin ring A (**100**), which contains both dehydro residues: Dha in the ring and Dhb in the tail. Three different routes for Dhb incorporation were attempted: i) addition of Ile-Dhb dipeptide (**135**), ii) dehydration of an incorporated Ile-Thr dipeptide (**133**) according to the method of Slootweg *et al.*,[173] and iii) alkylation and β-elimination of a β-Me-Cys residue, in an adaptation of the method of Morrison *et al.*.[198] It was envisaged that each of these strategies could be used in conjunction with the alkylation-elimination methodology of Cys to produce the Dha residue in WT nisin ring A (**100**).

3.3.4.1. Synthesis Using Ile-Dhb and Ile-Thr Dipeptides

The first method investigated for Dhb incorporation was the addition of an Ile-Dhb dipeptide (**135**). However, as the Ile-Dhb peptide was known to be deactivated due to the conjugated nature of the carboxyl group, the coupling conditions were first tested on a linear peptide.[146] Simultaneously, an investigation was conducted into the feasibility of an on-resin β-elimination of Cys to Dha, with the aim of reducing the number of purification steps required during the WT nisin ring A synthesis. The proposed synthetic strategy is shown in **Scheme 3.15**.



Scheme 3.15: Synthetic scheme for the proposed elimination of Cys to Dha on resin, and attempted attachment of Boc-Ile-Dhb dipeptide (**135**).

A starting linear peptide of sequence AAC(STmp)AA (**163**) was synthesised on an Fmoc-Ala-Novasyn TGT resin, using standard SPPS techniques, followed by STmp deprotection by mild thiolysis with a 5% DTT solution containing 0.1 M NMM for a total of 15 minutes, to give resin-bound free thiol **164**.[243,244] To produce the Dha, an altered alkylation-elimination procedure was employed, in which the resin was suspended in a water:DMF mixture, and a full equivalent of TCEP was added to ensure complete reduction. Excess dibromopentanoate (**80**) and K_2CO_3 were then added in DMF, rather than DMSO, and the resin was incubated at 40 °C for 2 h. After this time, LCMS analysis of a small portion of cleaved resin revealed two major products: Dha-containing peptide **165**, and the alkylated intermediate (**166**) (**Figure 3.5**). This indicated that the β-elimination reaction was incomplete, and incubation with additional dibromopentanoate and K_2CO_3 for a further 1.5 h was necessary to obtain complete conversion to Dha-containing peptide (**165**). Following elimination, attachment of the Boc-Ile-Dhb dipeptide (**135**) was attempted by double coupling with COMU and DIPEA in NMP (**Scheme 3.15**). Unfortunately, after cleavage of the peptide from the resin, only

pentapeptide **165** was observed by LCMS, indicating that addition of dipeptide **135** had been unsuccessful.

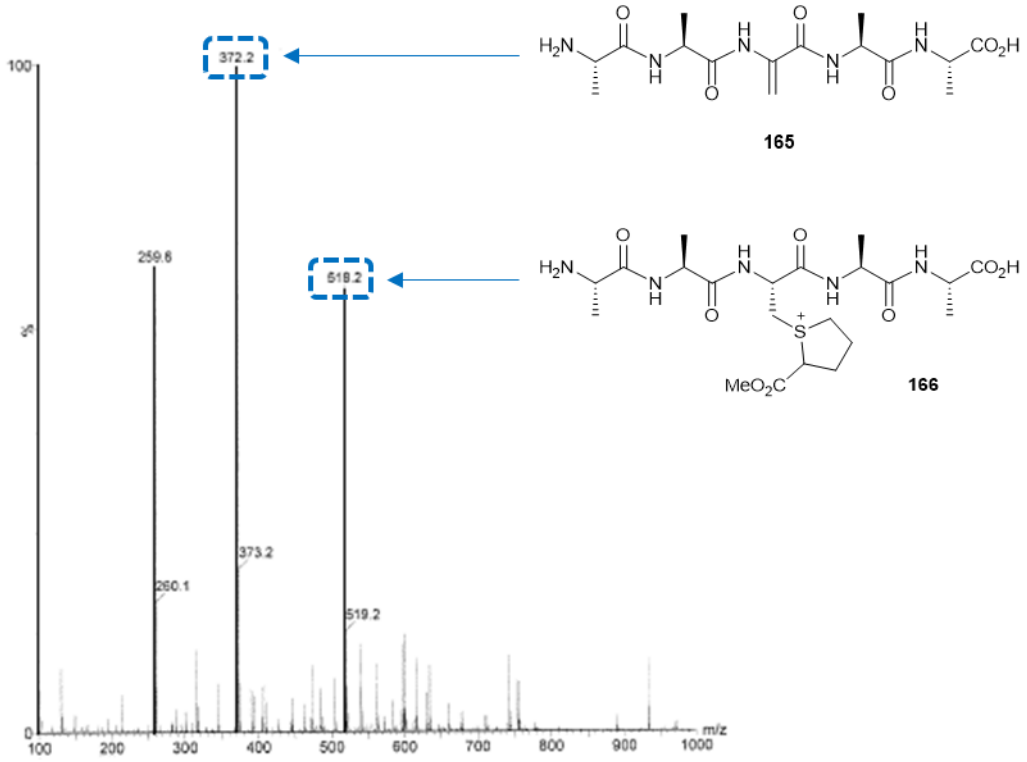
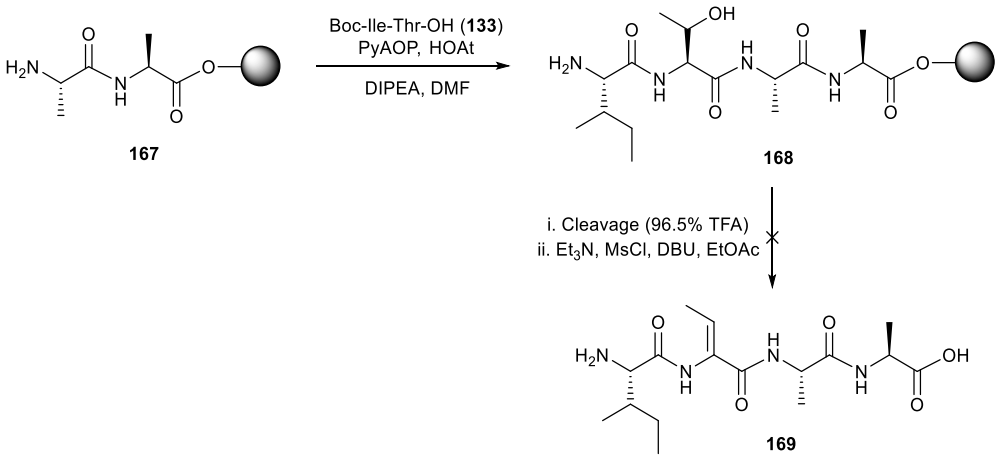


Figure 3.5: LCMS spectrum of a mini-cleave showing product (**165**) and intermediate **166** of the alkylation-elimination reaction on AACAA (**163**).

These tests suggested that although the Morrison *et al.* alkylation-elimination methodology could be adapted for use with a resin-bound peptide, the reaction was sluggish in comparison to its solution-phase counterpart. Consequently, in all subsequent cases this reaction was conducted with cleaved peptide. Addition of the Ile-Dhb dipeptide (**135**) also proved to be a problematic step, and no addition was observed even though coupling to an unhindered linear peptide. Investigations therefore instead turned to the use of a Boc-Ile-Thr-OH dipeptide (**133**), which would be dehydrated after incorporation to generate the Dhb, as reported by Slootweg *et al.* in their synthesis of nisin A analogues.[173]

Conditions for the incorporation and dehydration of dipeptide **133** were first investigated in a linear test peptide (**Scheme 3.16**). Thus, Ala-Ala dipeptide **167** was synthesised by the coupling of Fmoc-Ala to an Fmoc-Ala-Novasyn TGT resin with HBTU, followed by double coupling of dipeptide **133** with PyAOP and HOAt to give tetrapeptide **168**.

Successful coupling was confirmed by LCMS. Finally, the peptide was cleaved from the resin, lyophilised, and the dehydration reaction carried out as described by Slootweg *et al.*.[173] Unfortunately, only Thr-containing peptide **168**, and none of the desired Dhb peptide **169**, was observed by LCMS analysis after work-up.



Scheme 3.16: Test reactions for attachment and dehydration of Ile-Thr dipeptide (**133**).

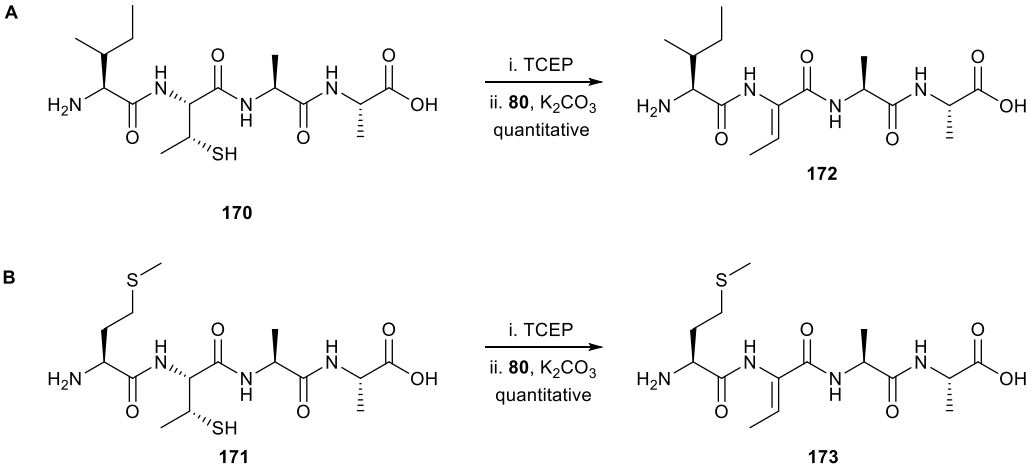
As the synthesis of a simple Dhb-containing peptide *via* incorporation of Ile-Dhb dipeptide **135** or dehydration of Ile-Thr dipeptide **133** had both proved unsuccessful, an alternative method for the synthesis of WT nisin ring A (**100**) was explored.

3.3.4.2. Synthesis Using β-Me-Cys as a Dhb Precursor

As demonstrated in the synthesis of WT mutacin I ring A (**99**), Dha residues could be reliably produced from Cys(Trt) using the Morrison *et al.* alkylation-elimination methodology (see **Chapter 3.7.2**),[198] with no oxidation of the Lan thioether and facile incorporation of the required precursor residues. In view of this success, investigations were conducted into the introduction of Dhb using an analogous approach, *via* the elimination of β-methyl-cysteine residues (see **Chapter 2.5.2** and **Chapter 1.6.1**).

Before attempting this transformation in a complex lantibiotic peptide, the incorporation method and elimination reaction were trialled on two linear test peptides. Ile-MeCys-Ala-Ala (**170**) was synthesised to mimic the tail region of nisin ring A, and Met-MeCys-Ala-Ala (**171**) was synthesised to determine whether thioether moieties, for example a lanthionine bridge, were likely to be affected by the elimination reaction conditions (**Scheme 3.17**). The peptides were synthesised on an Fmoc-Ala-Novasyn TGT resin, using standard SPPS protocols. The first amino acid to be added was Fmoc-Ala,

followed by the addition of the Fmoc-β-Me-Cys(Trt)-OH (**136**). This residue was double coupled with PyAOP and HOAt, and its incorporation was monitored by LCMS analysis of a small portion of cleaved resin following the reaction. Pleasingly, after just 2 x 1 h couplings, none of the Ala-Ala starting material remained. Finally, either Fmoc-Ile or Fmoc-Met were added before cleaving the peptides from the resin. The alkylation-elimination was then conducted on the crude peptides according to the method of Morrison *et al.*.[198]



Scheme 3.17: Elimination of the β-Me-Cys test peptides to form their Dhb-containing counterparts: **A** elimination of Ile-MeCys-Ala-Ala (**170**) and **B** elimination of Met-MeCys-Ala-Ala (**171**).

LCMS analysis of aliquots taken from each reaction mixture after 2 h incubation at 37 °C showed that in both cases the elimination of β-Me-Cys to Dhb had been successful, with no evidence of any remaining starting peptides or thioether alkylation. Following purification by HPLC, Ile-containing peptide (**172**) was isolated in 78% yield and Met-containing peptide (**173**) was isolated in 84% yield (compared to resin loading).

An additional benefit of the synthesis of these test peptides was that it enabled an examination of the Dhb geometry by analysis of the NOESY spectra of each peptide (expected NOEs for each geometry in **Figure 3.6**, **A**). A cross peak was observed between the Ala NH and Dhb βH in both peptides, indicating that an anti-elimination from the β-Me-Cys had occurred, producing the *Z*-Dhb. (**Figure 3.6 B** and **C**).

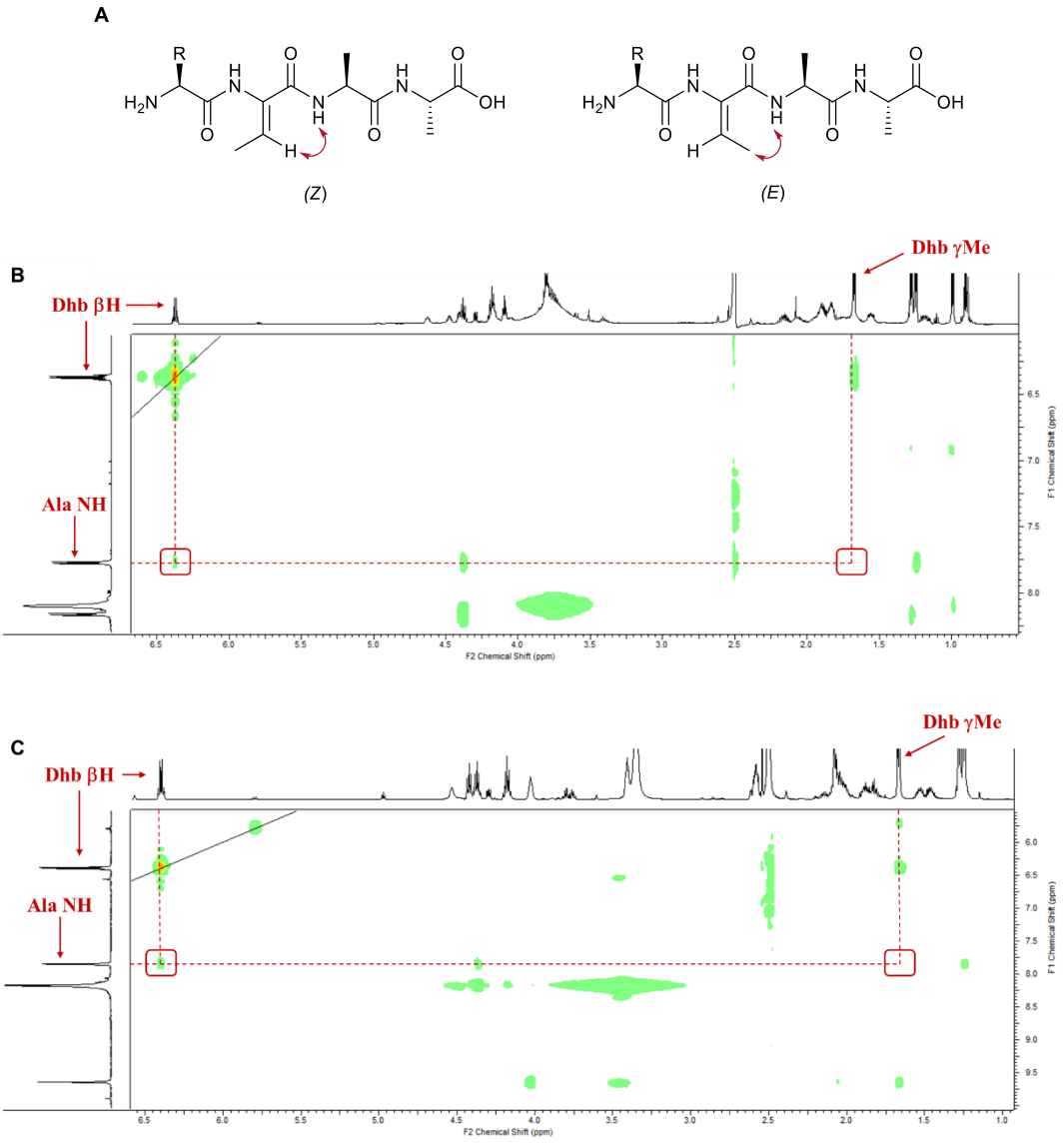
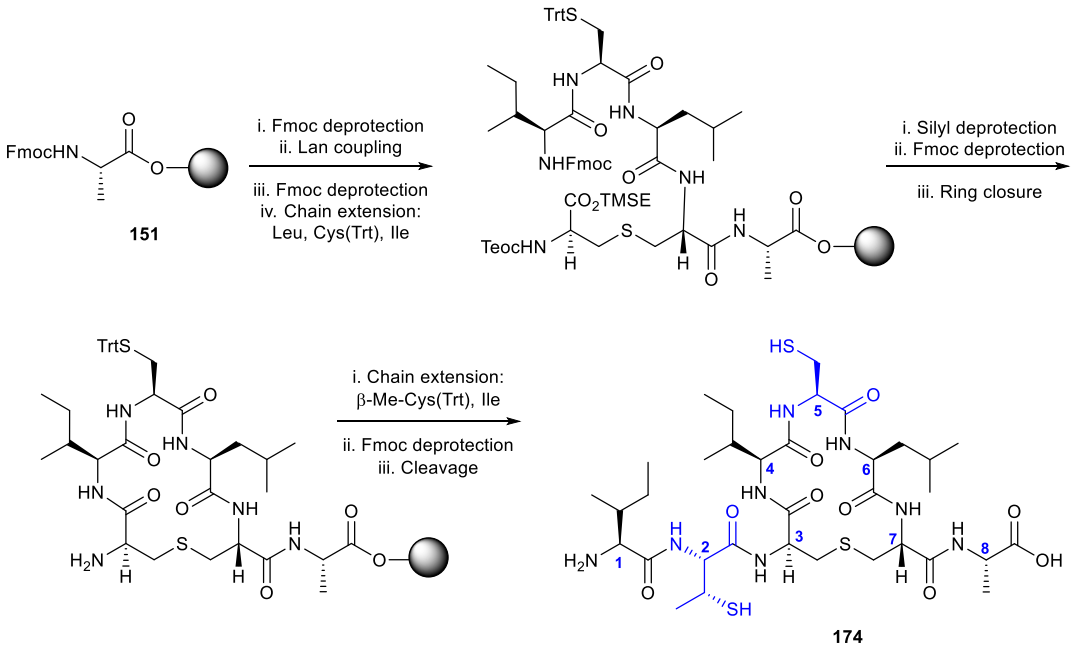


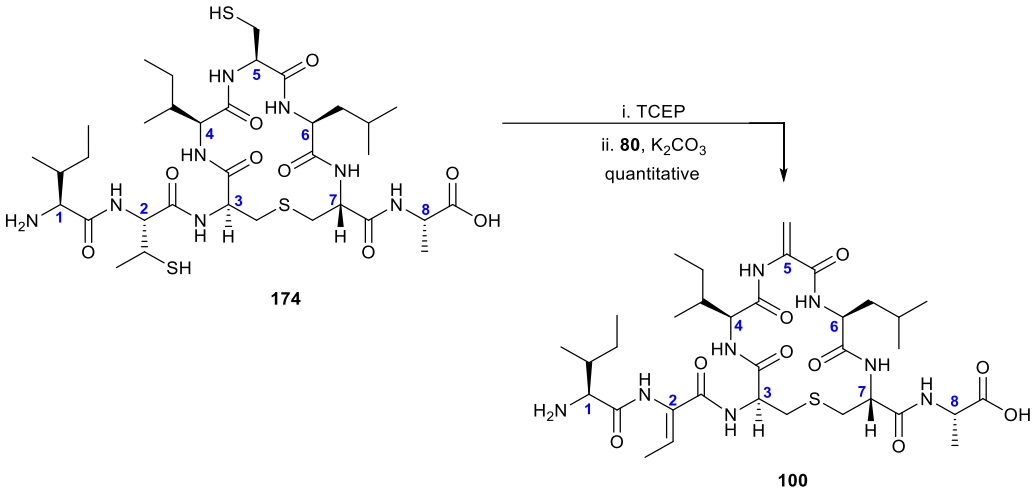
Figure 3.6: A. Expected NOEs for each geometry of Dhb. **B.** NOE spectra of Ile-Dhb-Ala-Ala (**172**), **C.** NOE spectra of Met-Dhb-Ala-Ala (**173**). Positions of expected cross peaks with the Ala NH are shown in red boxes; only the Ala NH-Dhb βH cross peaks are seen.

Following the success of the test reaction, the alkylation-elimination of β-Me-Cys to form the Dhb in WT nisin ring A (**100**) was investigated. Synthesis of the required precursor peptide (**174**) (**Scheme 3.18**) began with the attachment of the (Teoc, TMSE/Fmoc) Lan (**38**) to an Fmoc-Ala-Novasyn TGT resin. Leu, Cys(Trt) and Ile were then sequentially added using standard SPPS techniques, before cyclisation to form the lantibiotic ring and coupling of the β-Me-Cys(Trt) and Ile residues. Cleavage of the peptide from the resin gave precursor peptide (**174**) in 11% yield after purification by HPLC.



Scheme 3.18: Synthesis of Cys-containing precursor (**174**). Dha precursors shown in blue.

The alkylation-elimination reaction was conducted by treating precursor peptide **174** with TCEP, dibromopentanoate (**80**) and K_2CO_3, according to the method of Morrison *et al.* (**Scheme 3.19**).[198] Gratifyingly, LCMS analysis of an aliquot showed complete conversion to WT nisin ring A (**100**) had occurred after just 2 h at 37 °C. Purification by HPLC gave **100** in 1% yield (compared to resin loading).



Scheme 3.19: Synthesis of WT nisin ring A (**100**) from Cys-containing precursor **174**.

3.4. Summary

In summary, a total of seven single lantibiotic rings from the lipid II binding regions of nisin and mutacin I have been synthesised. Although several groups have previously reported the synthesis of A and B ring fragments of nisin, this is the first chemical synthesis of rings A and B of mutacin I.[145,146,149,157,209]

Three ring B peptides and four ring A peptides were synthesised in total (**Figure 3.7**). Four of these peptides are the WT sequences of nisin and mutacin I: two ring A peptides **99** and **100**, and two ring B peptides **96** and **97**. The remaining three are analogue sequences: one Lan analogue of nisin ring B (**98**), and two analogues of mutacin I ring A in which the dehydro residues were replaced with simpler amino acids, **101** and **102**. All of these peptides were isolated and purified, and their solution state structures were analysed by NMR.

The synthesis of these peptides also enabled a study of the different methods of dehydro generation in lantibiotic peptides. A number of methods were investigated, including the incorporation of Dhb/Thr dipeptides and oxidative elimination of Sec(Ph) residues. Both of these methods however produced little success, either proving impossible to incorporate efficiently or leading to overoxidation of the target peptide. Eventually, elimination from Cys or β-Me-Cys using the method of Morrison *et al.* was found to be the simplest and most effective way to install Dha and Dhb residues, and is now the method of choice for introducing dehydro residues in this work.[198]

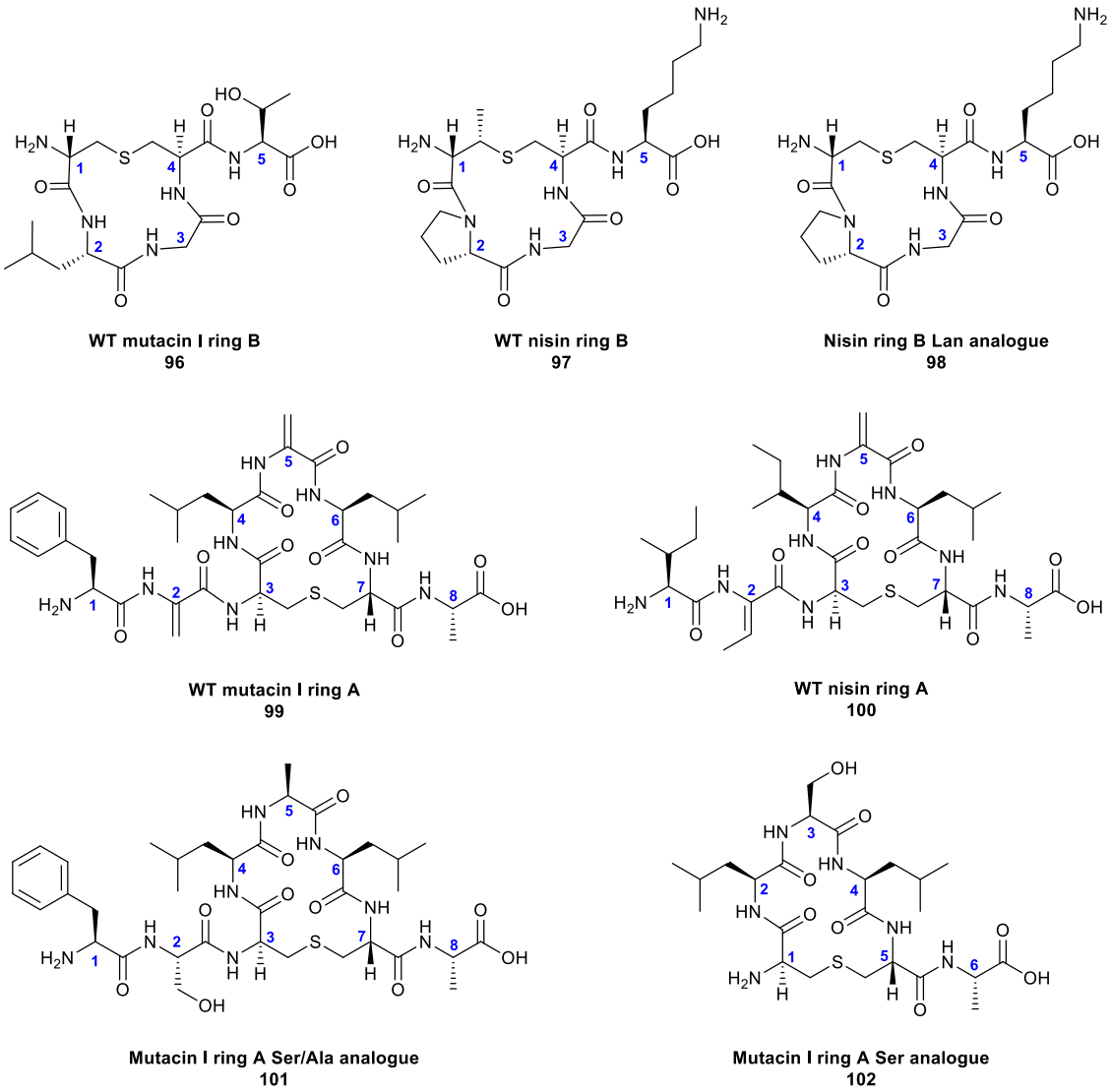


Figure 3.7: Structures of the synthesised single ring peptides.

4. SYNTHESIS OF DOUBLE RINGS

4.1. Introduction

This chapter discusses the production of WT and analogues of nisin rings AB, and attempts towards the synthesis of WT and analogue sequences of the same region of mutacin I.

Firstly, WT and analogue sequences of nisin rings AB were prepared. The WT peptide, which has several PTMs including one lanthionine, one methyllanthionine and both dehydro residues, was accessed by enzymatic digestion of the full-length, commercially available, peptide. Two simplified nisin analogues were synthesised by SPPS.

Secondly, the synthesis of WT and analogue sequences of mutacin I rings AB was attempted. Two of the target peptides were synthetic precursors to the WT ring AB peptide, which would enable the introduction of Dha residues using the methods described in **Chapter 2**. The other target peptides were all simplified analogues, in which the Dha residues were replaced with either Ser or Ala.

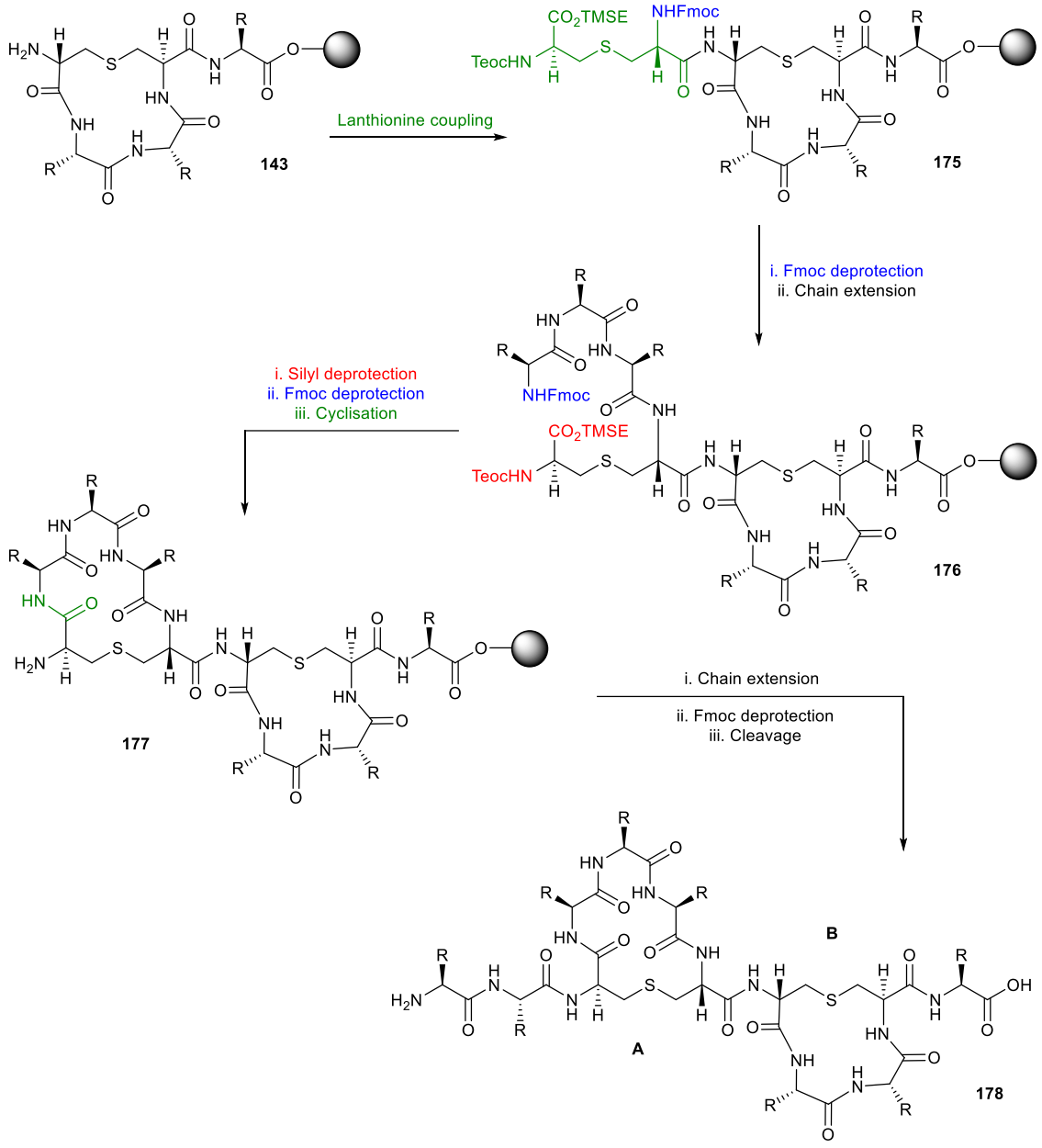
4.1.1. General Strategy for Double Lantibiotic Ring Synthesis

All coupling, deprotection and cleavage steps in the SPPS of the AB rings were carried out as previously described for single ring syntheses, again based on the general strategy reported by the Tabor lab (**Chapter 3.1.1**).[162,224] However, contrary to this published method, which was used to make the nisin ring DE fragment, to make the AB double ring system of nisin and mutacin I the two lanthionines must be adjacent to each other rather than overlapping. Two main approaches were envisaged to achieve this topology: i) sequential on-resin SPPS, in which the second lanthionine would be coupled directly onto the first following the cyclisation of ring B (**Scheme 4.1**), or ii) separate synthesis of each ring by SPPS, and subsequent coupling of the two (**Scheme 4.3**).

4.1.1.1. Synthesis by Sequential On-Resin SPPS

The synthesis of double ring systems by sequential on-resin SPPS (**Scheme 4.1**) continued from the end of the single ring synthesis shown in **Scheme 3.1** with the resin-bound ring B peptide (**143**). A second Teoc/TMSE lanthionine (**38**) would first be coupled with the free amine of the B ring to give **175**. The Fmoc group would then be removed and the three non-lanthionine residues of ring A added using normal SPPS techniques, giving **176**. Following silyl deprotection with TBAF and another Fmoc

deprotection, the A ring would be cyclised using PyAOP and HOAt with microwave irradiation to give double ring peptide **177**. The final two amino acids could then be added forming the tail of the peptide, again using standard SPPS techniques, which after Fmoc deprotection and cleavage from the resin would yield the desired AB ring system (**178**).



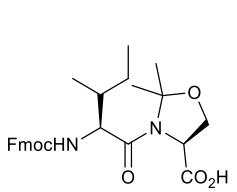
Scheme 4.1: General synthetic strategy for the formation of double lantibiotic rings by sequential on-resin synthesis, exemplified here by the synthesis of a peptide containing two Teoc/TMSE lanthionines (**38**).

4.1.1.2. Pseudoproline Dipeptides and Hmb-Protected Residues

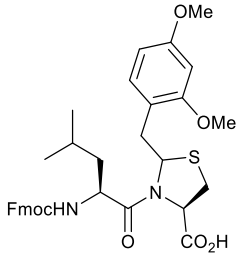
The incorporation of pseudoproline dipeptides and Hmb-protected amino acids is a common method for the improvement of synthetic efficiency in SPPS, particularly in long

or cyclic peptides. Both methods were employed in the synthesis of lanthionine-containing peptides by Mitchell[161] and Wright,[223] who found that incorporation of these residues increased cyclisation efficiency, enabling isolation of the target peptides. These residues were therefore also used in this work in the sequential on-resin synthesis of nisin and mutacin I ring AB sequences.

Pseudoproline dipeptides, first developed by Wöhr and Mutter *et al.* in 1995, contain either a Ser, Thr or Cys which is temporarily protected as an oxazolidine or thiazolidine (**Figure 4.1**).[245,246] This protection is removed following the synthesis by treatment with acid to reveal the native peptide structure. As well as allowing two residues to be incorporated simultaneously into the peptide, hence decreasing the number of required coupling and deprotection steps, the proline-like oxazolidine or thiazolidine rings act as turn inducers. This means that they are able to both disrupt secondary structure formation and increase cyclisation efficiency in small peptides.[247–249]



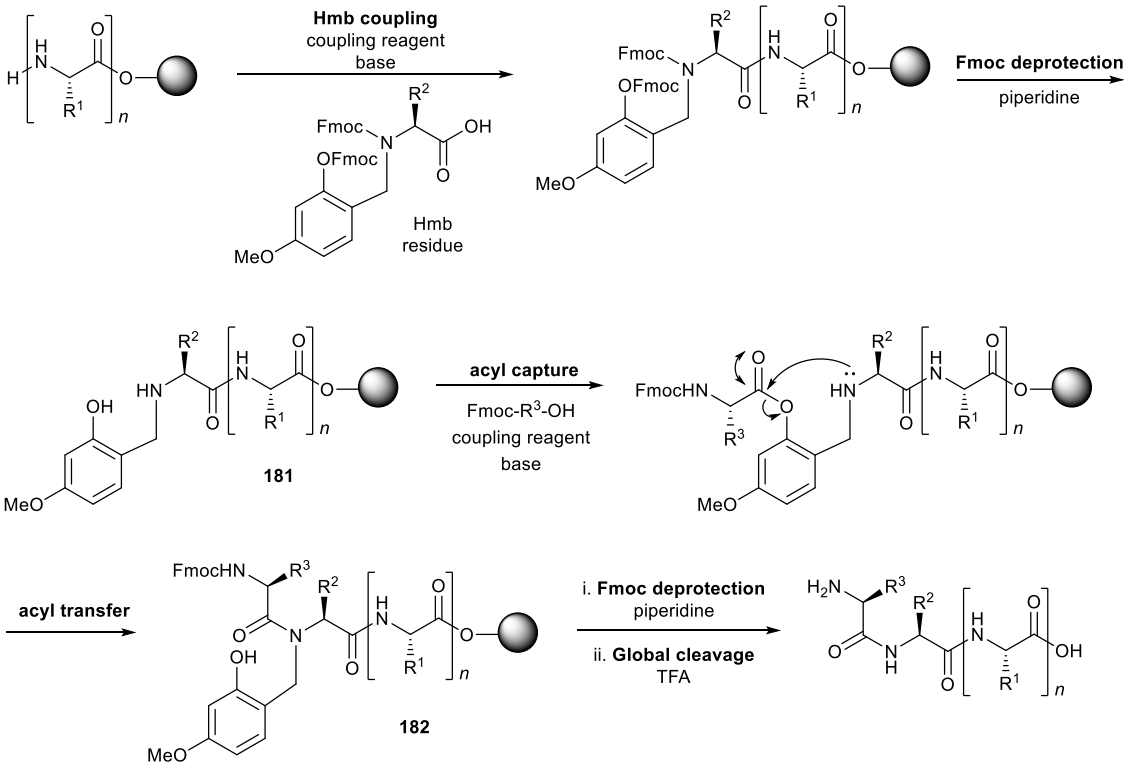
179



180

Figure 4.1: Structures of the pseudoproline dipeptides used in this work: Fmoc-Ile-Ser($\psi^{Me,Me}$pro)-OH (**179**) and Fmoc-Leu-Cys($\psi^{Dmp,H}$pro)-OH (**180**).

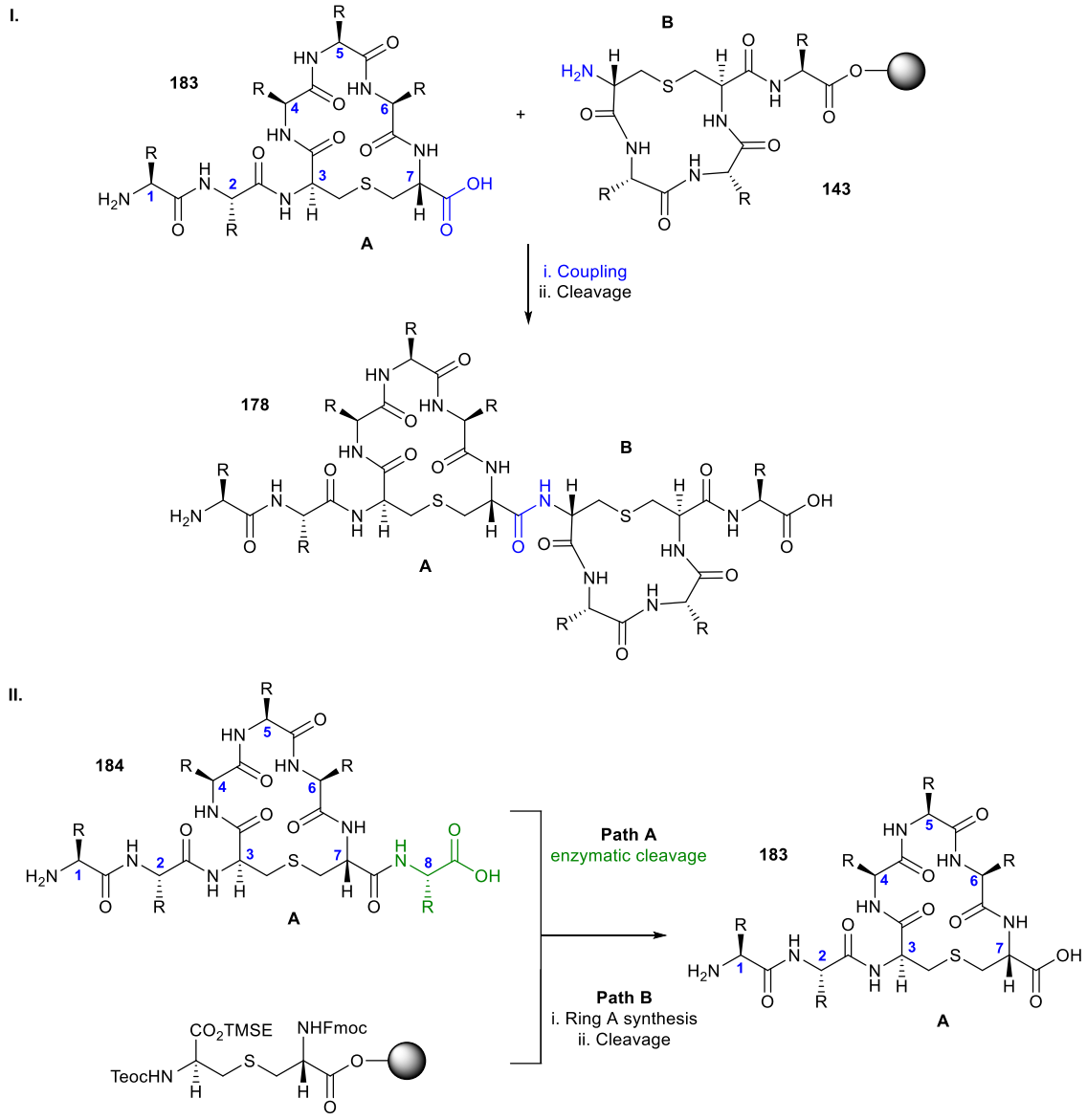
Hmb protection, first introduced in 1993, is used to temporarily protect the backbone amide bond of the peptide, helping to disrupt hydrogen bonding and prevent aggregation, therefore increasing the efficiency of the synthesis.[250,251] It has also been reported that the use of *N*-alkylated residues, such as those bearing Hmb protection, are able to promote the cyclisation of small peptides which bear no other turn-inducing moieties.[252] Following the synthesis, the Hmb group is removed during the global cleavage of the peptide from the resin, liberating the native backbone amide. Although the Hmb group increases the steric bulk around the terminal amine, it has been suggested that the coupling of the next residue is accelerated by base-catalysed acyl capture at the Hmb phenol (**181**), followed by intramolecular acyl transfer to give the native peptide bond (**182**) (**Scheme 4.2**).[251]



Scheme 4.2: Mechanism of acyl capture and transfer by Hmb-protected amino acids.

4.1.1.3. Synthesis by Coupling of Individual Rings

To synthesise the AB rings by coupling of two pre-formed peptides, pure ring A fragment **183** and resin-bound ring B peptide **143** would be required (**Scheme 4.3**, **I**). In the previously described ring A syntheses (**Chapter 3**), pre-loaded resins were used, resulting in peptides with an eighth residue on the *C*-terminal side of the lanthionine. For the purpose of AB ring syntheses however, the *C*-terminal of the ring A lanthionine (residue 7) must be coupled directly to the B ring. It would therefore be necessary to either remove residue 8 from a previously synthesised ring A peptide (**184**), for example by enzymatic cleavage with a protease (Path A, **II**, **Scheme 4.3**), or to load the ring A lanthionine directly onto the resin and synthesise the peptide as described in **Scheme 3.1** (Path B, **II**, **Scheme 4.3**). Ring A fragment **183** could then be coupled to the resin-bound ring B before cleavage of the peptide from the resin, giving the AB ring system (**178**) (**II**, **Scheme 4.3**).



Scheme 4.3: General synthetic strategy for the formation of double lantibiotic rings by the coupling of separately synthesised A and B rings. **I.** Coupling of the A and B rings. **II.** Synthesis of ring A fragment (**183**).

4.2. Digestion and Purification of Nisin Rings AB WT

The first double ring peptide to be isolated was WT nisin rings AB (**95**). Digestion of commercially available WT nisin to give the AB rings has been extensively described in the literature, and has been used by a number of groups to produce fragments of the WT peptide for various applications.[125,173,253–255] However, commercially available nisin from *L. lactis* contains only ~2.5% nisin, with the rest of the sample being composed of salts and denatured milk solids. Thus, Slootweg *et al.* developed a method for the tenfold enrichment of nisin before reaction with trypsin, in order to maximise the yield of the

digested peptide.[216] However, efforts to isolate nisin rings AB (**95**) using this approach, involving a water/CH_2Cl_2 enrichment followed by trypsin digest in TRIS-acetate buffer at pH 7.0, were low-yielding, and gave < 5 mg of **95** from 20 g of commercial sample. Use of the modified digestion procedure described by Koopmans *et al.*, in which higher concentrations of nisin and trypsin were used, was found to require shorter reaction times, hence decreasing the risk of lanthionine oxidation caused by extended periods of incubation in buffer.[126]

Following the digestion, the reaction mixture was lyophilised before purification by semi-preparative HPLC. In addition to the desired AB ring fragment (**95**), nisin ring C (**185**) was also detected by LCMS, as described by Slootweg *et al.* (**Figure 4.2**).[216] With the WT nisin ring AB peptide in hand, efforts then moved to the SPPS of simplified analogues of the same region.

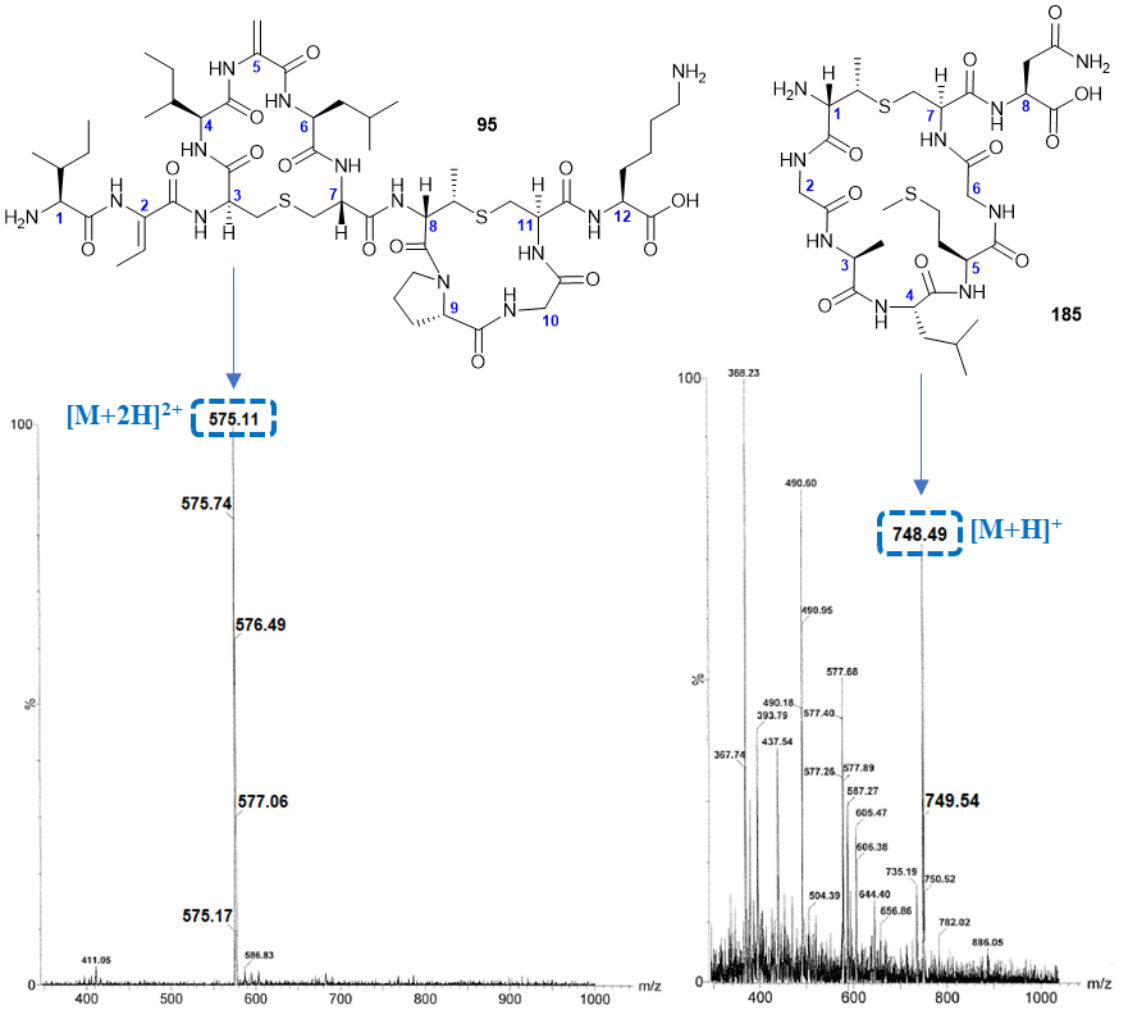
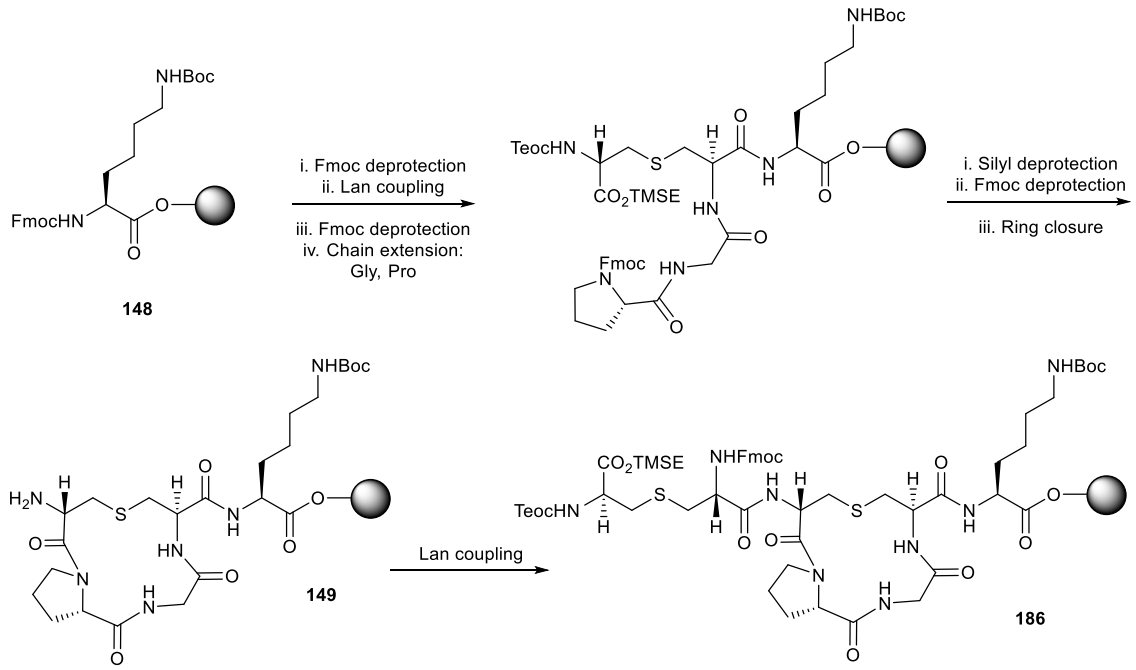


Figure 4.2: LCMS spectra of fragments of WT nisin obtained following trypsin digestion of the full-length peptide.

4.3. Nisin Ring AB Analogues

The two required nisin ring AB analogues were synthesised according to the method of Mitchell (**Chapter 1.4.2.3**), in which Teoc/TMSE Lan (**38**) was used to form the A and B rings of both peptides.[161] In one analogue (**39**), Thr and Ser were used as analogues for Dhb and Dha, and in the second analogue (**40**), Abu and Ala were used (**Scheme 4.5** and **Scheme 4.6**, dehydro substitutions are shown in blue). As described above, it was anticipated that these simplifications would be well tolerated, as nisin analogues bearing these alterations have been shown to maintain their biological activity (**Chapter 1.8.1**).

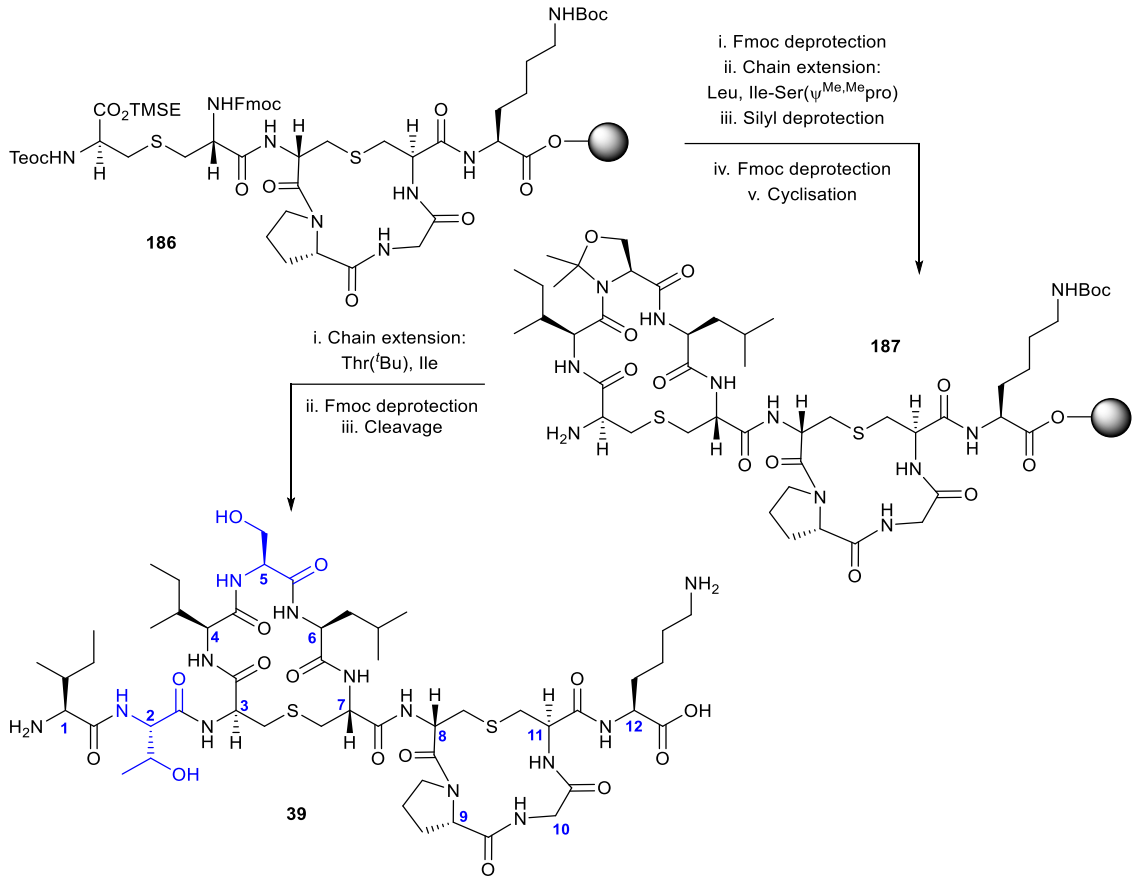
Both peptides were synthesised according to the general strategy described in **Scheme 4.1**. Teoc/TMSE Lan (**38**) was added to an Fmoc-Lys(Boc)-Novasyn TGT resin, before the addition of Gly and Pro using standard Fmoc SPPS. The silyl and Fmoc groups were then removed using TBAF and piperidine respectively, and the cyclisation conducted by double coupling with PyAOP and HOAt, giving resin-bound ring B (**149**). Addition of a second Teoc/TMSE Lan (**38**), followed by Fmoc deprotection, gave resin-bound peptide (**186**), ready for addition of the ring A residues (**Scheme 4.4**). Here the syntheses of the two analogues diverged.



Scheme 4.4: Synthesis of nisin ring AB analogue intermediate (**186**).

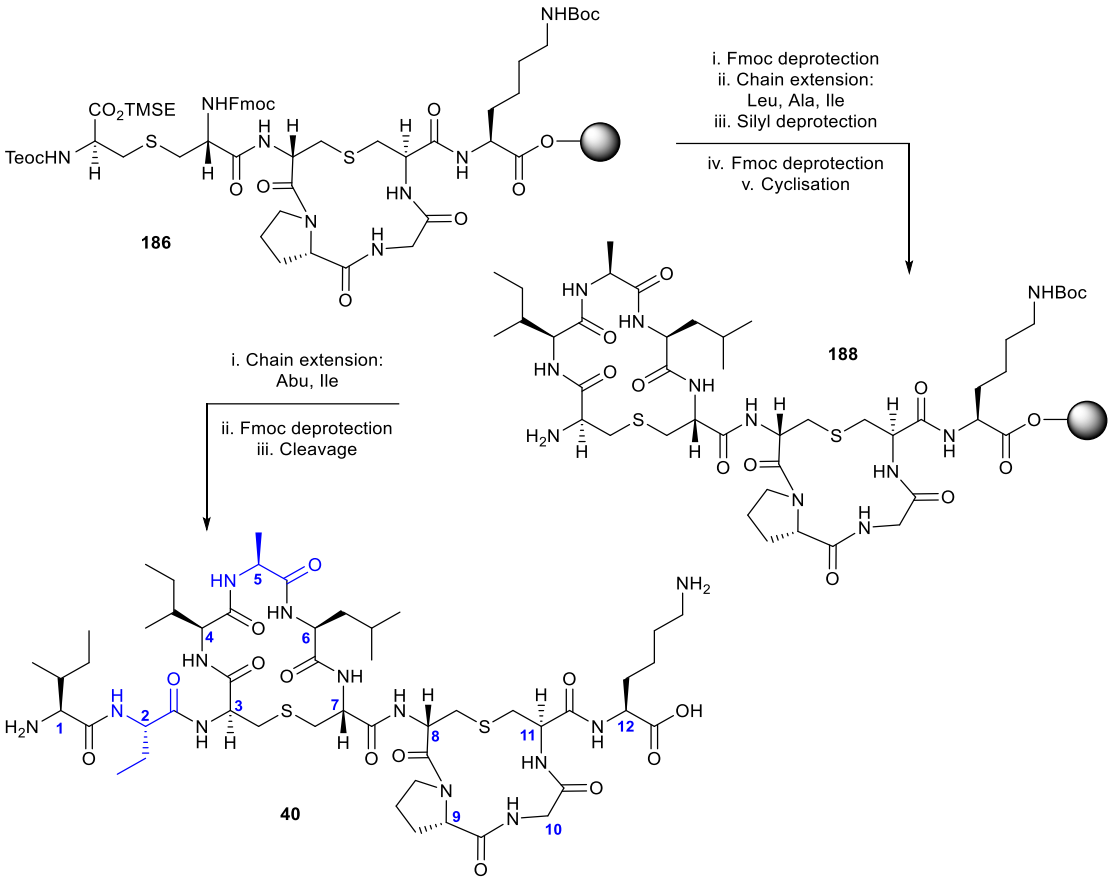
To complete the synthesis of Thr/Ser analogue (**39**), an Fmoc-Leu residue was added, followed by an Fmoc-Ile-Ser($\psi^{Me,Me}$pro)-OH pseudoproline dipeptide (**179**) (**Figure 4.1**).

Silyl and Fmoc deprotection, followed by cyclisation, gave resin-bound AB ring peptide **187**. Finally, the two tail residues, Fmoc-Thr(*t*Bu) and Fmoc-Ile, were added using standard SPPS procedures, before removal of the Fmoc group and cleavage from the resin to give Thr/Ser analogue **39** (**Scheme 4.5**).



Scheme 4.5: Synthesis of nisin rings AB Thr/Ser analogue (**39**).

To complete the synthesis of Abu/Ala analogue (**40**), the three ring A residues were added using Fmoc SPPS techniques. Standard Fmoc-Leu, Fmoc-Ala and Fmoc-Ile residues were used as this analogue contains no Ser, Thr or Cys to enable the use of a pseudoproline dipeptide. The silyl and Fmoc groups were then removed, followed by cyclisation of the A ring to give resin-bound intermediate **188**. Finally, Fmoc-Abu and Fmoc-Ile were added, again with standard SPPS techniques, to form the tail of the peptide. After removal of the Fmoc group, the peptide was cleaved from the resin giving Abu/Ala analogue **40** (**Scheme 4.6**).



Scheme 4.6: Synthesis of nisin rings AB Ala/Abu analogue (**40**).

Analysis of the crude samples by LCMS showed that though the synthesis of the Abu/Ala analogue (**40**) had been successful, the global TFA cleavage had failed to remove the Ser($\psi^{Me,Me}$pro) group from Thr/Ser analogue **39**. However, as previously reported, treatment of the crude material with triflic acid gave complete deprotection, as observed by LCMS, after 5 min at 0 °C (**Figure 4.3**).[161]

Both Thr/Ser analogue **39** and Abu/Ala analogue **40** were then purified by semi-preparative and analytical HPLC. Abu/Ala analogue **40** was isolated in 0.5% yield and 76% purity (estimated by HPLC), while the Thr/Ser analogue (**39**) was isolated in 3% yield. However, two distinct peaks were present in the analytical HPLC trace of Thr/Ser analogue (**39**), which were identical by LCMS (**Figure 4.4**). It was hypothesised that this phenomenon could be caused by one of two possibilities: the presence of diastereomers, most probably by scrambling of a lanthionine stereocentre in basic conditions during a coupling or deprotection step, or the presence of two slowly interconverting conformers.

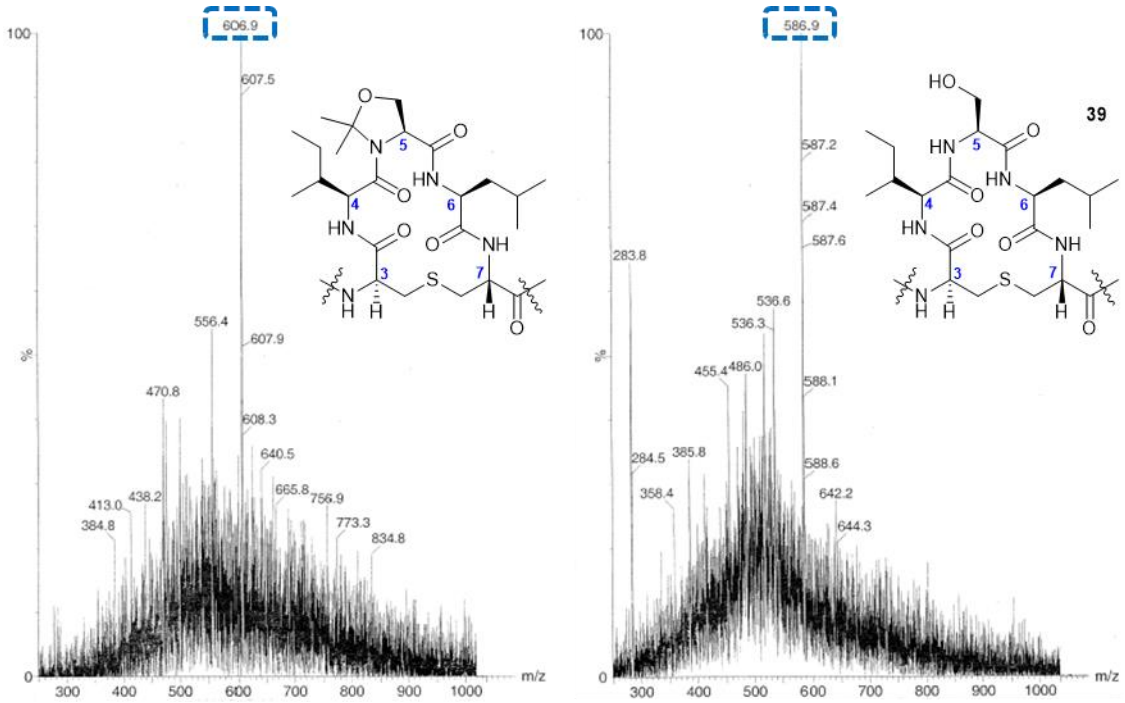


Figure 4.3: LCMS spectra showing before and after triflic acid deprotection of Thr/Ser analogue **39**. Only ring A of the peptide is shown for clarity.

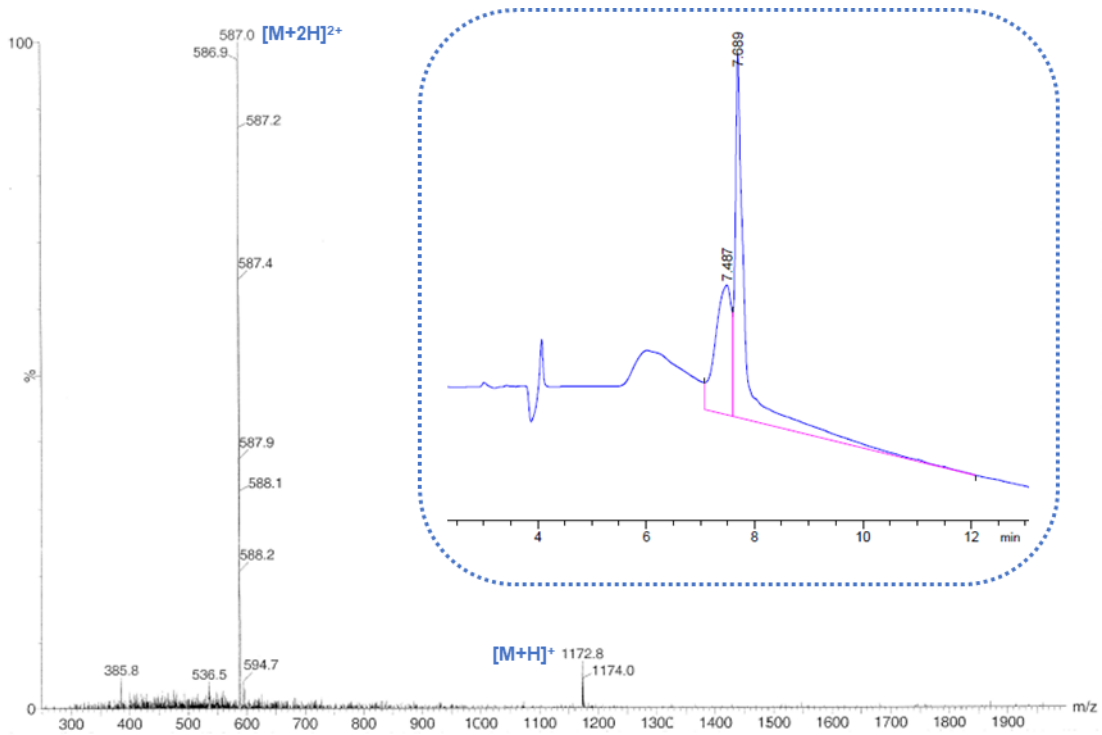


Figure 4.4: LCMS spectrum and analytical HPLC trace (inset) of Thr/Ser analogue **39**.

Comparison of the 1D ^{1}H NMR spectra of analogues **39** and **40** showed significant peak broadening in the spectrum of the Thr/Ser analogue **39** compared to the Abu/Ala analogue (**40**), particularly in the NH region (**Figure 4.5**). This, coupled with the presence of a similar number of peaks in both spectra, suggested that the two peaks in the analytical HPLC of Thr/Ser analogue **39** were caused by interconverting conformers.

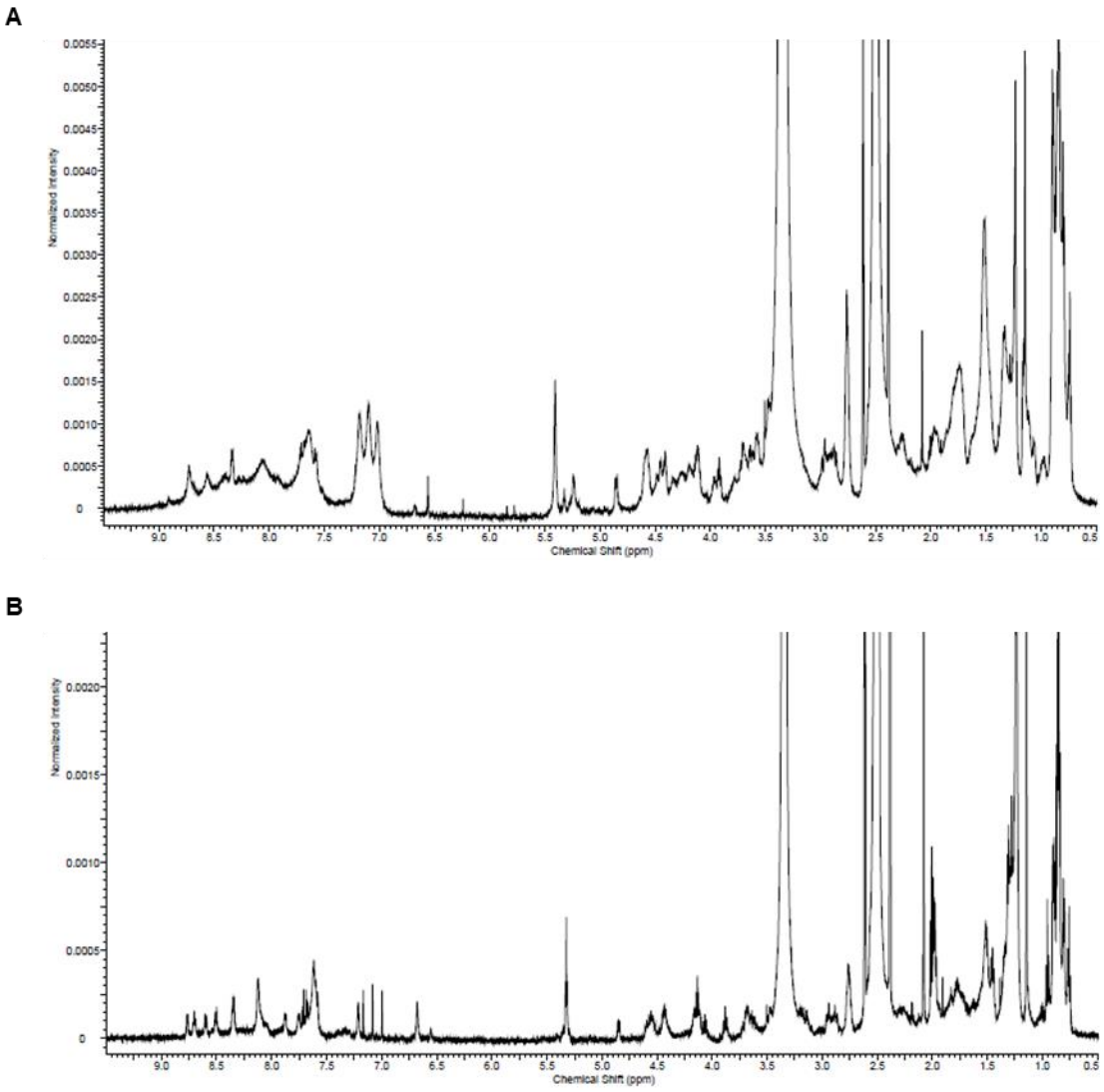


Figure 4.5: 1D ^{1}H NMR spectra of nisin ring AB analogues. **A.** Thr/Ser analogue (**39**), showing broadening in NH region, and **B.** Abu/Ala analogue (**40**).

4.4. Mutacin I Ring AB Analogues

Following isolation of the nisin ring AB sequences, the syntheses of WT (**103**) and analogues of the same region of mutacin I were investigated (**Figure 4.6**). Both the sequential on-resin SPPS strategy (**Scheme 4.1**) and separate synthesis and coupling of the A and B rings (**Scheme 4.3**) were attempted.

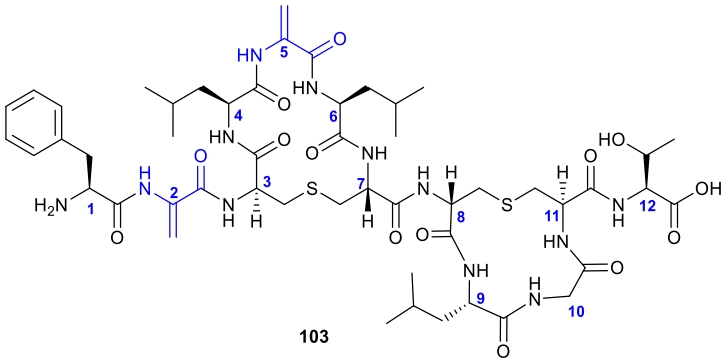
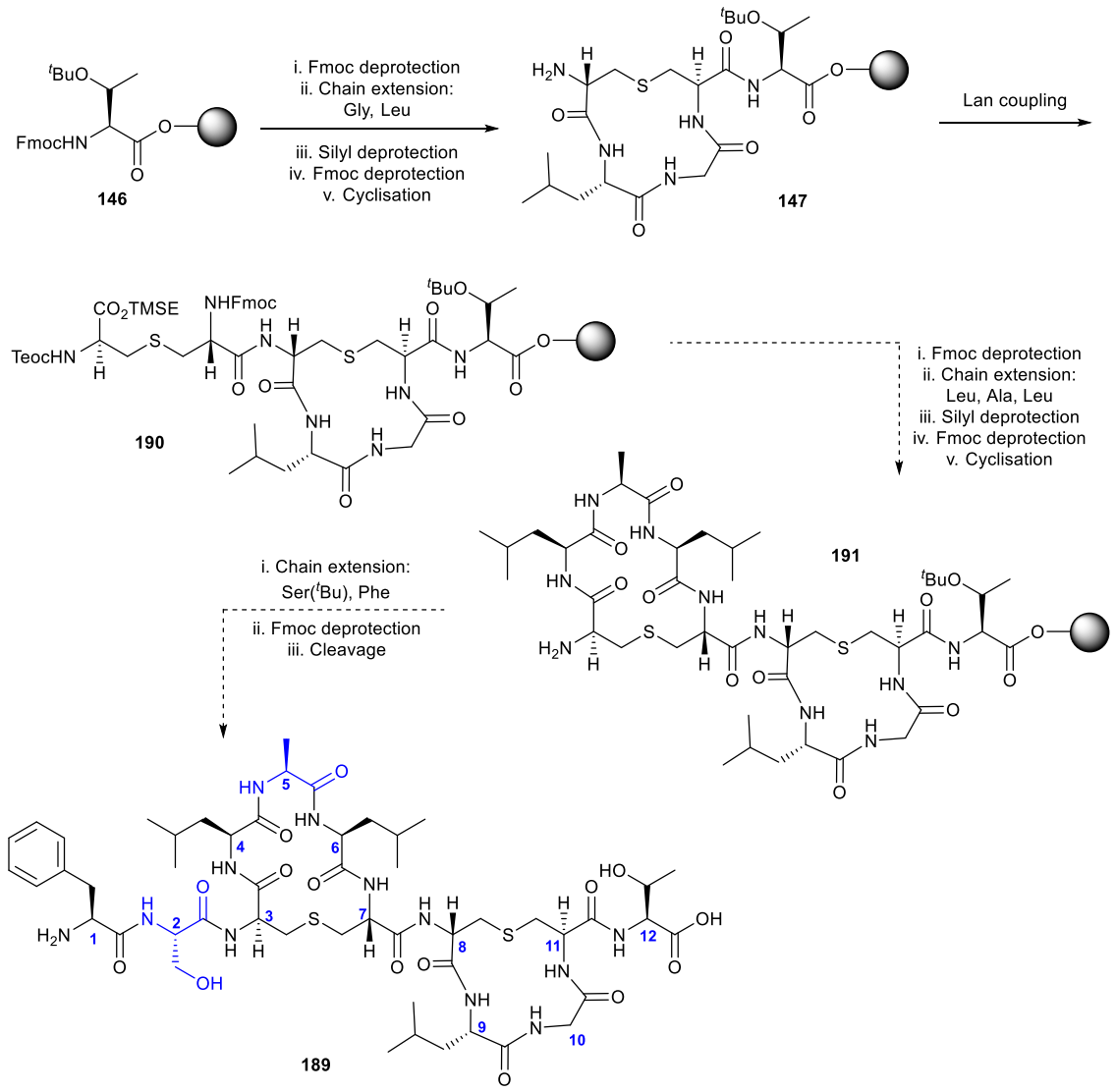


Figure 4.6: Structure of WT mutacin I rings AB (**103**). Residues to be changed in analogue sequences are highlighted in blue.

4.4.1. Synthesis of Ser/Ala Analogue (**189**) by Sequential On-Resin SPPS

Initially, the synthesis of a simplified mutacin I ring AB analogue (**189**) was investigated, with the Dha residues replaced by Ser and Ala (**Scheme 4.7**). Single Fmoc amino acids and standard SPPS techniques were initially employed in combination with microwave irradiation for lanthionine incorporation and cyclisation. Firstly, the WT mutacin I ring B (**147**) was formed as previously described (**Chapter 3.2.1**). A second Teoc/TMSE Lan (**38**) was then added and the A ring was formed by the addition of Leu, Ala and a second Leu, according to the approach described in **Scheme 4.1**, giving truncated peptide (**191**). Following addition of the Ser and Phe tail residues, the peptide was cleaved from the resin. In contrast to the nisin analogues, none of the desired Ser/Ala analogue (**189**), or any truncation or deletion sequences, could be detected by LCMS analysis of the crude sample.



Scheme 4.7: Attempted synthesis of the target mutacin I Ser/Ala analogue (**189**). Dha replacements shown in blue.

In an effort to enable some of the peptide to be isolated, the synthesis was repeated using Hmb-protected amino acids (**Scheme 4.2**). Two different positions in mutacin I ring A were deemed appropriate for Hmb protection - positions five and six (**Figure 4.7**). Incorporation of an Hmb-protected residue at position four was not attempted as it was hypothesised that the bulky Hmb group would hinder the cyclisation of ring A. The synthesis of two new Ser/Ala analogues was therefore attempted, one with Hmb-Ala at position five (**192**), and one with Hmb-Leu at position six (**193**), both of which would yield Ser/Ala analogue (**189**) after cleavage from the resin (**Figure 4.7**). Both peptides were prepared in the same way as described for Ser/Ala analogue (**189**) (**Scheme 4.7**), except that residues following the Hmb-protected amino acids were incorporated as the

symmetric anhydrides,[250,256] prepared according to the method of Montero *et al.*,[257] and incorporated into the peptides using 10 eq in CH_2Cl_2. In each case, completion of the anhydride coupling reaction was confirmed by LCMS analysis of a small portion of cleaved resin.

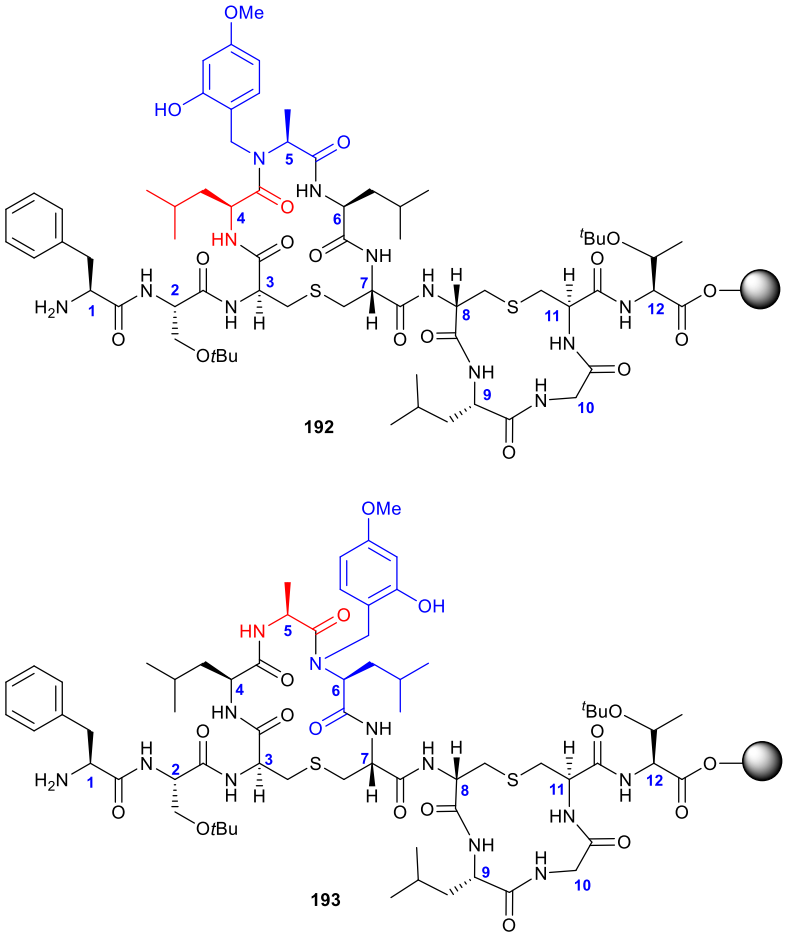


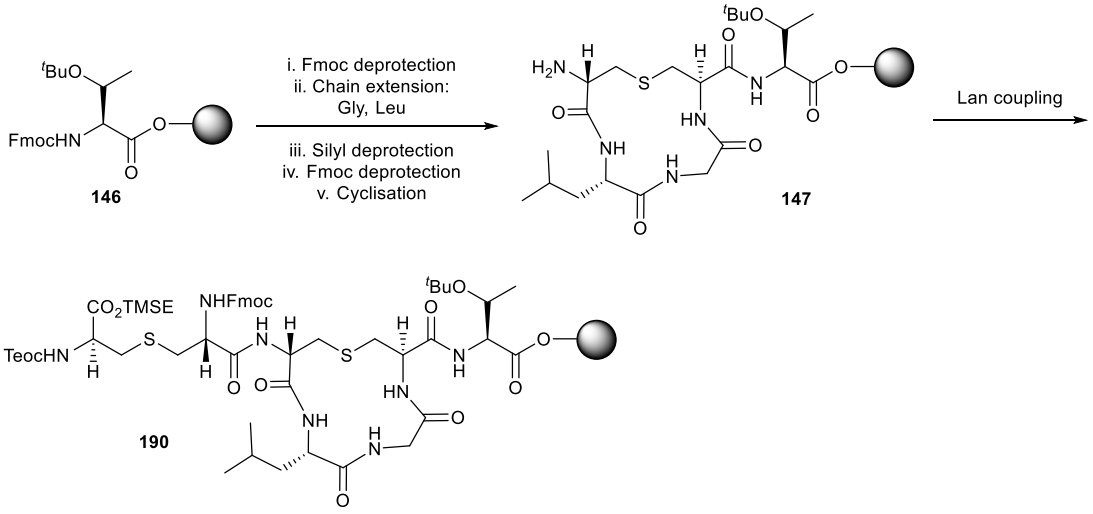
Figure 4.7: Structures of Ser/Ala analogues containing Hmb-protected residues. Hmb residues are shown in blue, residues coupled using symmetric anhydrides are shown in red.

After completion of the synthetic sequence, peptides **192** and **193** were cleaved from the resin and the crude samples were analysed by LCMS. Disappointingly, none of the target Ser/Ala analogue (**189**) was observed in either sample.

4.4.2. Synthesis of Other Mutacin I Analogues by Sequential On-Resin SPPS

As the synthesis of Ser/Ala analogue (**189**) had been unsuccessful, the syntheses of three alternative mutacin I analogues were investigated, employing a range of techniques which had previously enabled the isolation of lantibiotic peptides in this work.

All three of these analogues were synthesised according to the general procedure described above (**Scheme 4.1**). The beginning of each synthesis was identical: the B ring (**147**) was first formed on an Fmoc-Thr(*t*Bu)-Novasyn TGT resin (**146**) according to the method described in **Chapter 3.2.1**, before the addition of a second Teoc/TMSE lanthionine (**38**) to form intermediate **190** (**Scheme 4.8**). The formation of ring A and addition of the tail residues then proceeded differently for each peptide.

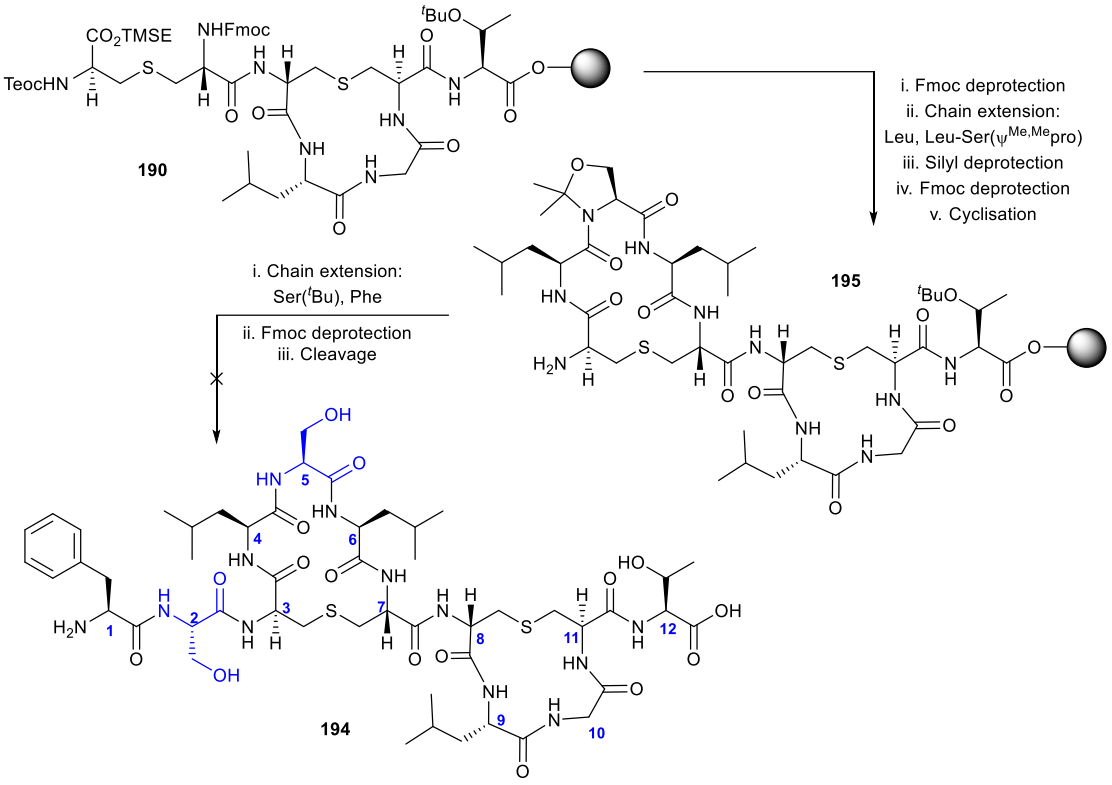


Scheme 4.8: Synthesis of mutacin I rings AB intermediate (**190**).

*4.4.2.1. Mutacin I Ring AB Ser/Ser Analogue (**194**)*

Firstly, the synthesis of mutacin I Ser/Ser analogue **194** was investigated, prepared using a pseudoproline dipeptide at positions four and five, in an analogous manner to nisin Thr/Ser analogue **39** (**Scheme 4.9**).

To form ring A of Ser/Ser analogue (**194**), Fmoc-Leu and Fmoc-Leu-Ser($\psi^{Me,Me}$pro)-OH were added to intermediate **190** using standard Fmoc SPPS techniques, and the ring closure reaction conducted by double coupling for 2 h with PyAOP and HOAt to give ring AB peptide (**195**). A small portion of cleaved resin was then analysed by LCMS to confirm the presence of cyclised peptide (**195**) before completion of the synthesis. Although many unknown peptide impurities were present, the mass corresponding to cyclised ring A peptide **195** was observed (**Figure 4.8**).



Scheme 4.9: Attempted synthesis of the mutacin I Ser/Ser analogue (**194**). Dha replacements shown in blue.

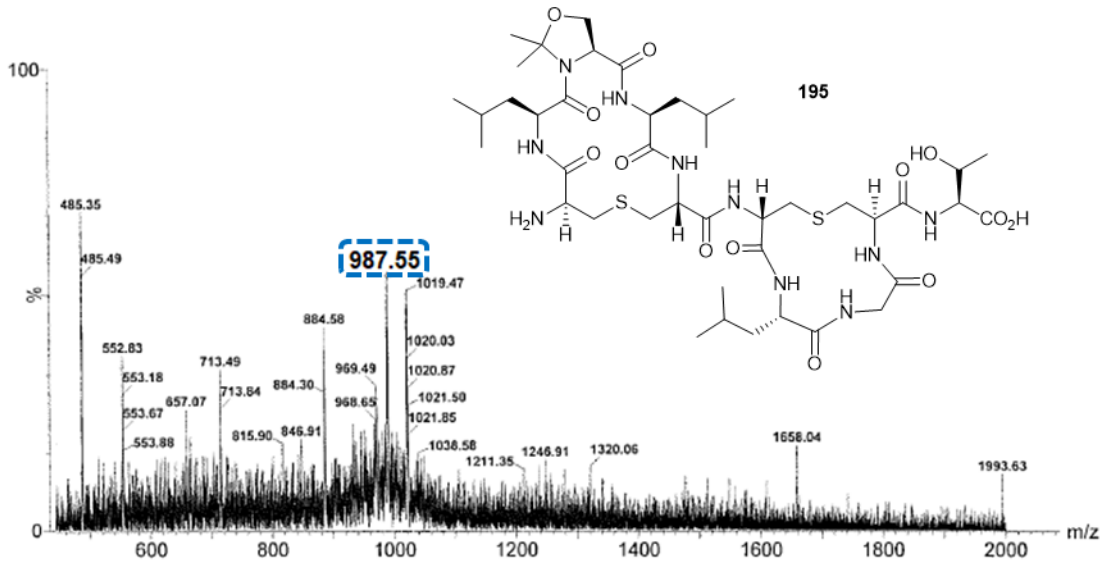


Figure 4.8: LCMS spectrum showing cyclisation of **195** after 2 x 2 h double coupling.

The two tail residues, Fmoc-Ser(*t*Bu) and Fmoc-Phe, were then coupled using standard SPPS techniques, and the peptide cleaved from the resin. Unfortunately, the only peptide which could be identified by LCMS analysis of the crude sample was (**196**), which corresponds to the addition of Ser(*t*Bu) to one side of the uncyclised A ring (**Figure 4.9**). Again, many unknown peptide impurities were present, and no evidence of any cyclised peptides, with or without the addition of the tail residues, were observed.

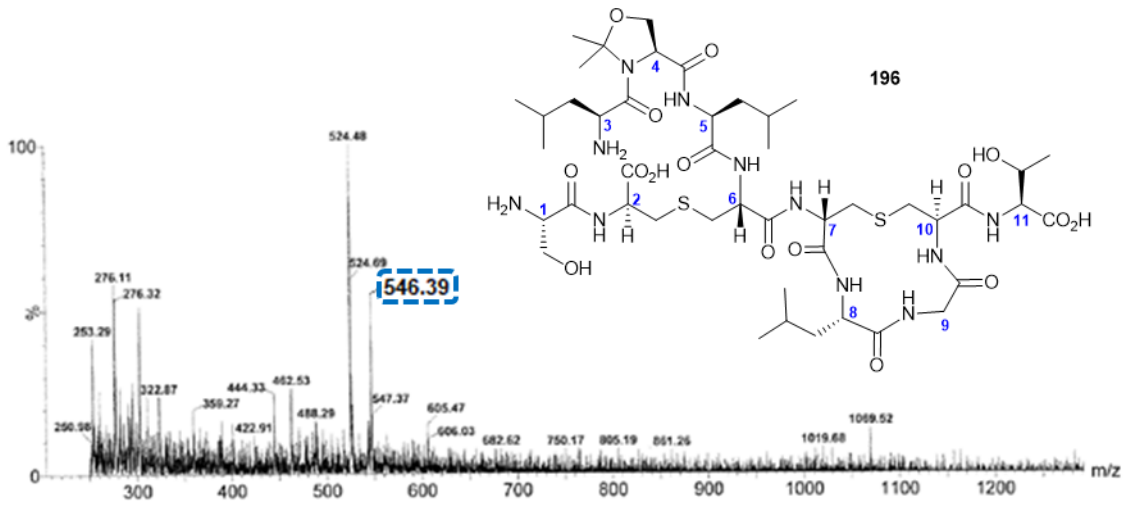
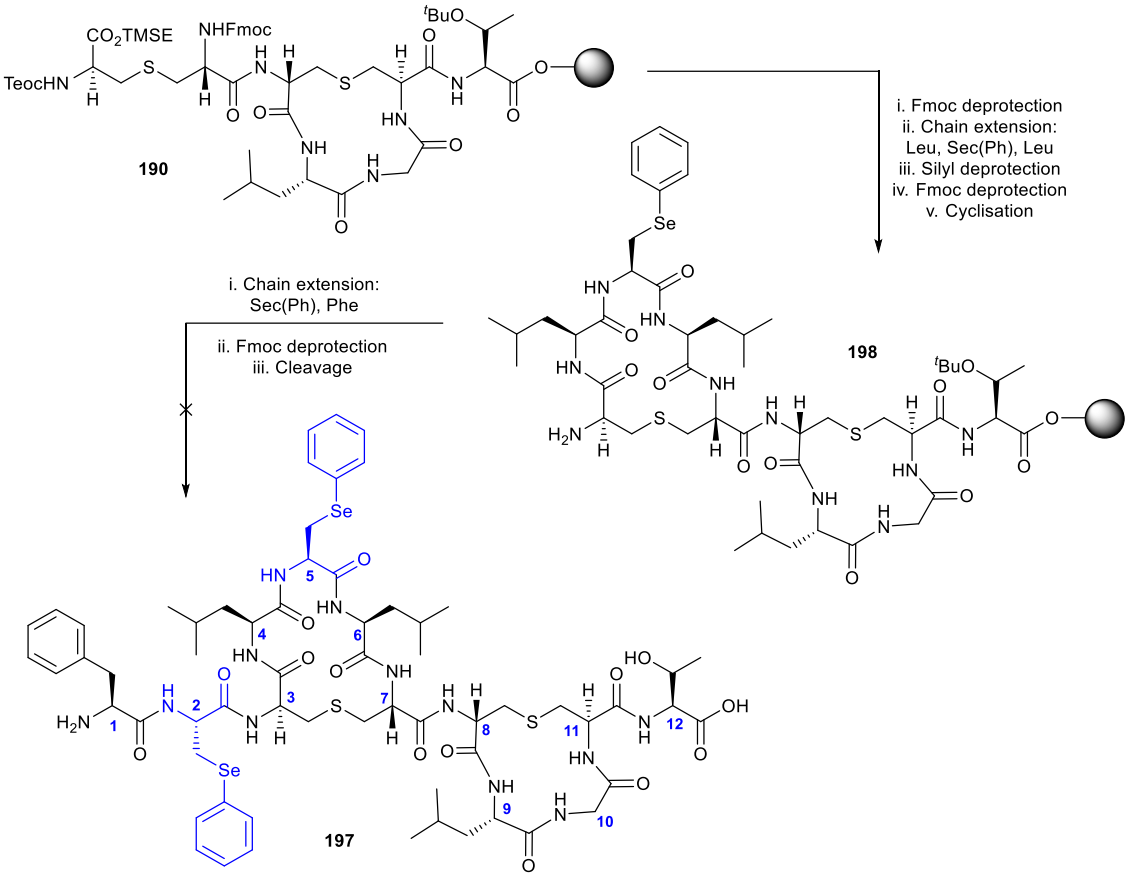


Figure 4.9: Crude LCMS spectrum of Ser/Ser analogue (**194**), with the peak corresponding to uncyclised peptide (**196**) indicated.

*4.4.2.2. Mutacin I Ring AB Sec(Ph)/Sec(Ph) Analogue (**197**)*

Secondly, the synthesis of mutacin I Sec(Ph)/Sec(Ph) analogue (**197**) was investigated, prepared using the optimised cyclisation conditions developed during the synthesis of mutacin I ring A analogue **158** (**Scheme 4.10**). If successful, this analogue would also provide access to WT mutacin I rings AB (**103**) by oxidative elimination of the Sec(Ph) residues.

To form ring A of the Sec(Ph)/Sec(Ph) analogue (**197**), Fmoc-Leu, Fmoc-Sec(Ph) and Fmoc-Leu were sequentially added to the resin. The Fmoc-Sec(Ph) residue was coupled using HOBt and DIC, and all Fmoc deprotections subsequent to Sec(Ph) incorporation were carried out with 20% piperidine solutions for three cycles of 1 min, to minimise premature elimination to Dha (**Chapter 3.3.3.1**). Cyclisation to give ring AB peptide **198** was conducted with COMU and DIPEA in DMF, before addition of another Fmoc-Sec(Ph) and an Fmoc-Phe to form the tail of the peptide. Following cleavage of the peptide from the resin, none of the desired peptide (**197**) could be detected by LCMS.



Scheme 4.10: Attempted synthesis of the mutacin I Sec(Ph)/Sec(Ph) analogue (**197**). Dha replacements shown in blue.

To discern whether truncation of the peptide had occurred before the formation of the A ring, the synthesis was repeated with each amino acid addition confirmed by LCMS analysis. Successful coupling of all of the ring A residues was observed. Cyclisation of ring A was conducted by double coupling with COMU, and a small portion of cleaved peptide was analysed by ESI to confirm the presence of cyclised ring AB peptide (**198**) (**Figure 4.10**). Although a large number of peptide impurities were already present in the sample, cyclised peptide (**198**) was observed and none of the uncyclised peptide could be detected. Addition of the two tail residues, Fmoc-Sec(Ph) and Fmoc-Phe, was therefore attempted before cleavage of the peptide from the resin. However, analysis of the crude sample by LCMS indicated that no addition of either of these residues had occurred. Moreover, purification by semi-preparative HPLC proved unsuccessful, and none of the truncated ring AB peptide (**198**) could be isolated.

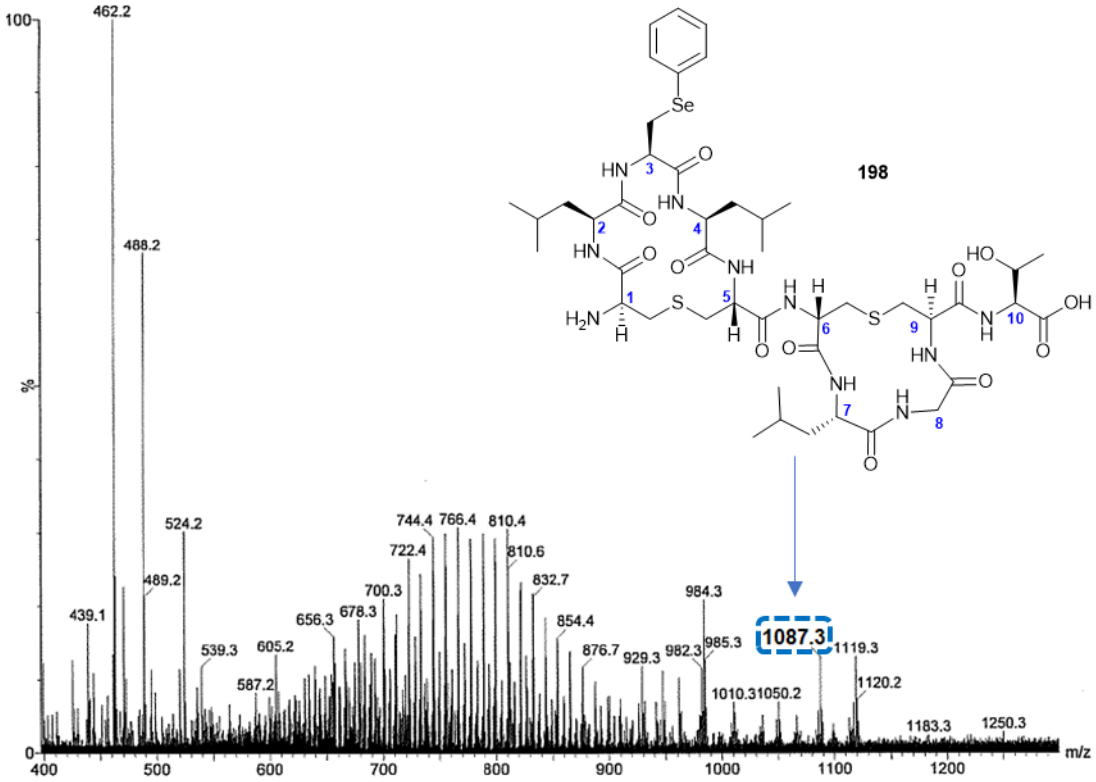
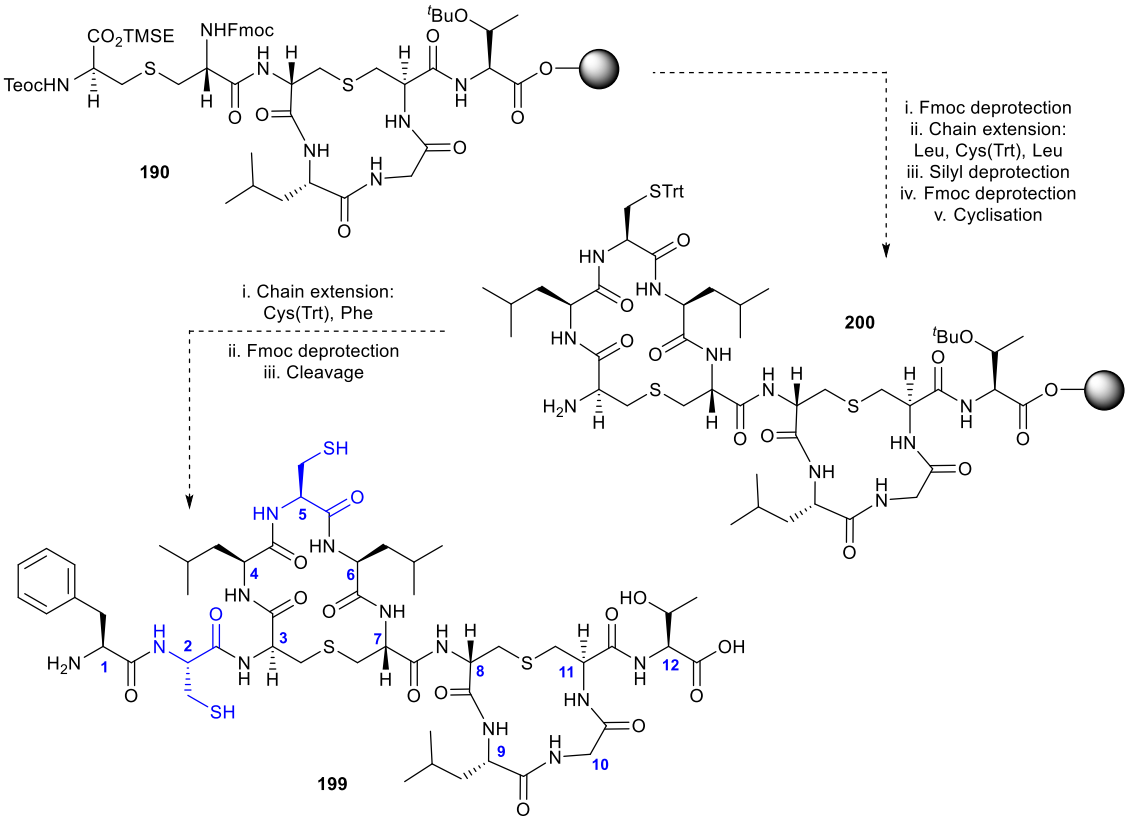


Figure 4.10: Mass spectrum following ring A cyclisation of Sec(Ph)/Sec(Ph) analogue. Peak corresponding to the closed ring A peptide (**198**) is indicated.

*4.4.2.3. Mutacin I Ring AB Cys/Cys Analogue (**199**)*

Thirdly, the synthesis of a mutacin I Cys/Cys analogue (**199**) was investigated, which if successful would also provide access to WT mutacin I rings AB (**103**) by alkylation-elimination of the Cys residues.

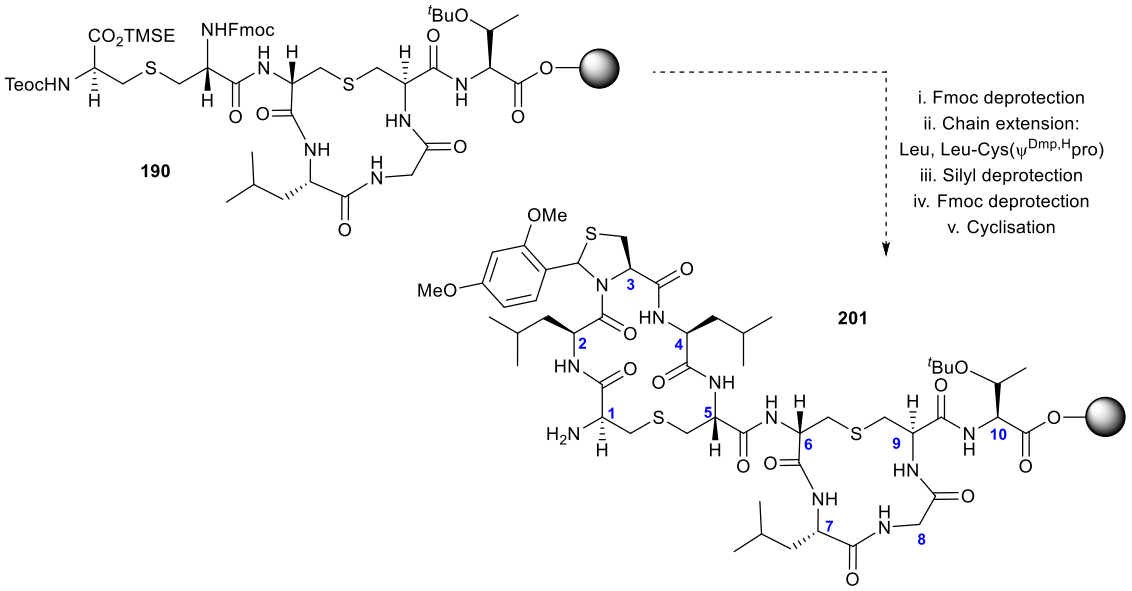
To form ring A of the Cys/Cys analogue (**199**), Fmoc-Leu, Fmoc-Cys(Trt) and a second Fmoc-Leu were added to intermediate **190** using standard SPPS protocols (**Scheme 4.11**). Cyclisation to form ring AB peptide (**200**) was conducted by double coupling with PyAOP and HOAt, before addition of another Fmoc-Cys(Trt) and Fmoc-Phe to form the tail of the peptide. After completion of the synthesis, the peptide was cleaved from the resin and the crude sample analysed by LCMS. None of the desired peptide (**199**), or any truncation or deletion sequences, could be detected.



Scheme 4.11: Attempted synthesis of the mutacin I Cys/Cys analogue (**199**). Dha replacements shown in blue.

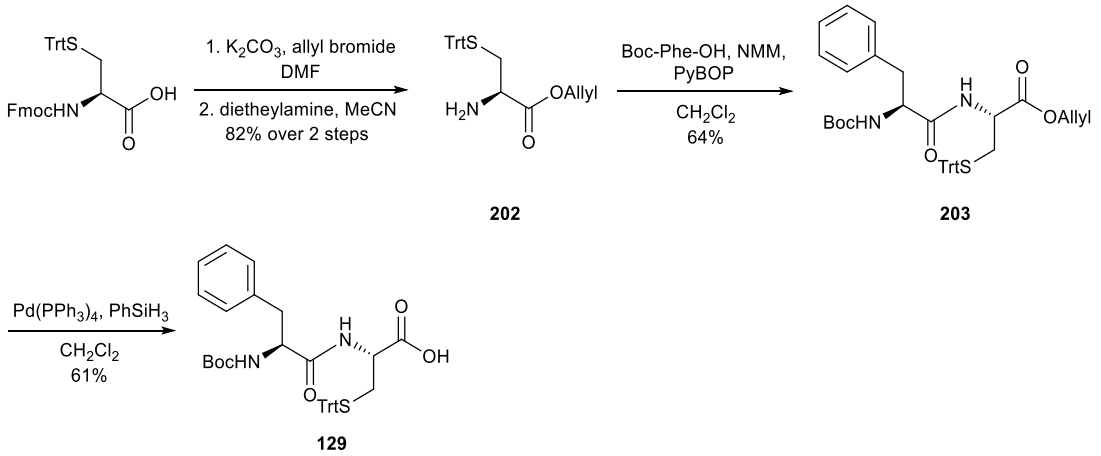
The synthesis was then repeated using a Leu-Cys pseudoproline dipeptide in ring A (**Scheme 4.12**). Thus, Fmoc-Leu and Fmoc-Leu-Cys($\psi^{Dmp,H}$pro)-OH (**180**) were first coupled to resin-bound Teoc/TMSE lanthionine (**190**), and successful incorporation of both amino acids was confirmed by LCMS analysis of a small portion of cleaved peptide. In contrast to the Ile-Ser pseudoproline dipeptide, which required treatment with triflic acid to produce the native sequence, some deprotection of the Leu-Cys pseudoproline was observed with TFA. The cyclisation to form ring AB peptide (**201**) was then effected by double coupling with PyAOP and HOAt for 2 h.

Two different methods were then simultaneously investigated for the addition of the tail residues to ring AB peptide **201**: i) coupling of a Boc-Phe-Cys(Trt) dipeptide (**129**), and ii) coupling of individual Fmoc-Cys(Trt) and Fmoc-Phe residues (**Scheme 4.14**).

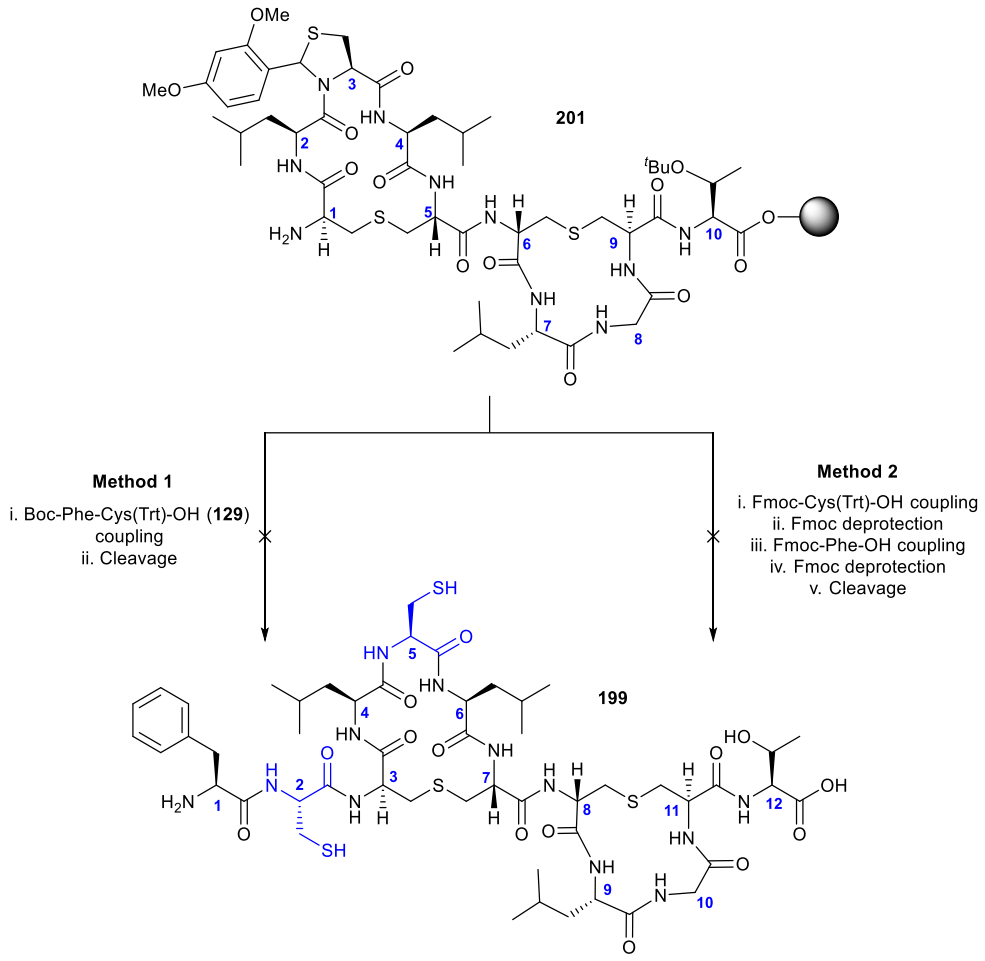


Scheme 4.12: Synthesis of ring A of the Cys/Cys analogue **201** using a Leu-Cys pseudoproline dipeptide.

The required Phe-Cys dipeptide (**129**) was synthesised from L-Phe and Fmoc-Cys(Trt)-OH (**Scheme 4.13**). Allyl protection of Fmoc-Cys(Trt)-OH with allyl bromide and subsequent Fmoc deprotection with diethylamine, according to the method of You *et al.*, gave H_2N-Cys(Trt)-OAllyl (**202**).[258] Boc-Phe-OH, synthesised from L-Phe with NaOH and Boc anhydride,[259] was then coupled with Cys **202** using PyBOP to give dipeptide (**203**). Finally, allyl deprotection with $Pd(PPh_3)_4$ gave the desired Boc-Phe-Cys(Trt)-OH dipeptide (**129**) in 32% overall yield from Fmoc-Cys(Trt)-OH.



Scheme 4.13: Synthesis of the Boc-Phe-Cys(Trt)-OH dipeptide (**129**).

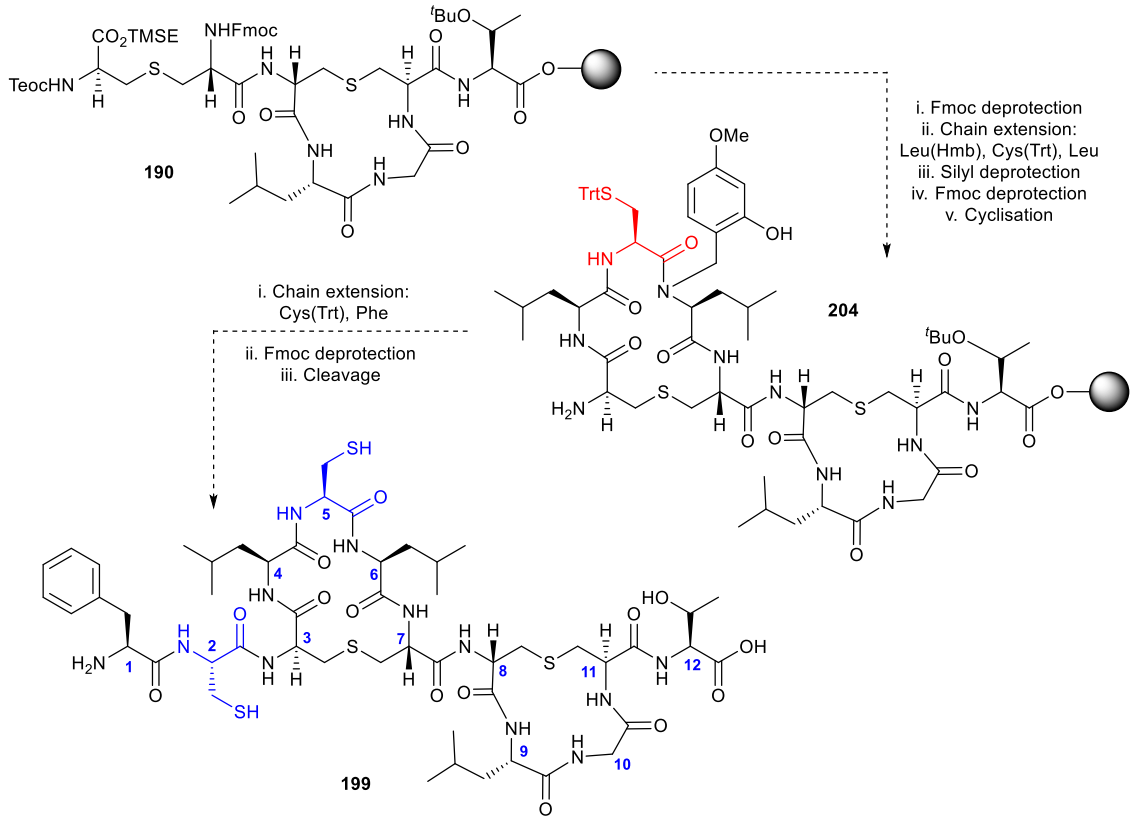


Scheme 4.14: Two attempted methods for the attachment of the tail residues to form mutacin I Cys/Cys analogue (**199**).

Standard Fmoc synthesis techniques were applied for coupling of the tail residues in both of the above described approaches, before cleavage of the peptide from the resin (**Scheme 4.14**). No addition of dipeptide (**129**) or the individual amino acids could be detected by LCMS analysis of the crude cleavage mixture.

A third approach was then investigated for the synthesis of mutacin I Cys/Cys analogue (**199**), using an Hmb-protected Leu at position six (**Scheme 4.15**). To form ring A, Hmb-Leu was first coupled to resin-bound Teoc/TMSE lanthionine (**190**), followed by coupling of Fmoc-Cys(Trt) using the symmetric anhydride.[256] Complete addition of both the Hmb-Leu and Cys(Trt) was confirmed by LCMS. A final Fmoc-Leu was added to the ring, before cyclisation by double coupling with HOAt and PyAOP to give ring AB peptide **204**. Fmoc-Cys(Trt) and Fmoc-Phe were then added to form the tail of the peptide

before cleavage from the resin. None of the desired Cys/Cys peptide (**199**) could be detected in the crude LCMS, or isolated by semi-preparative HPLC.



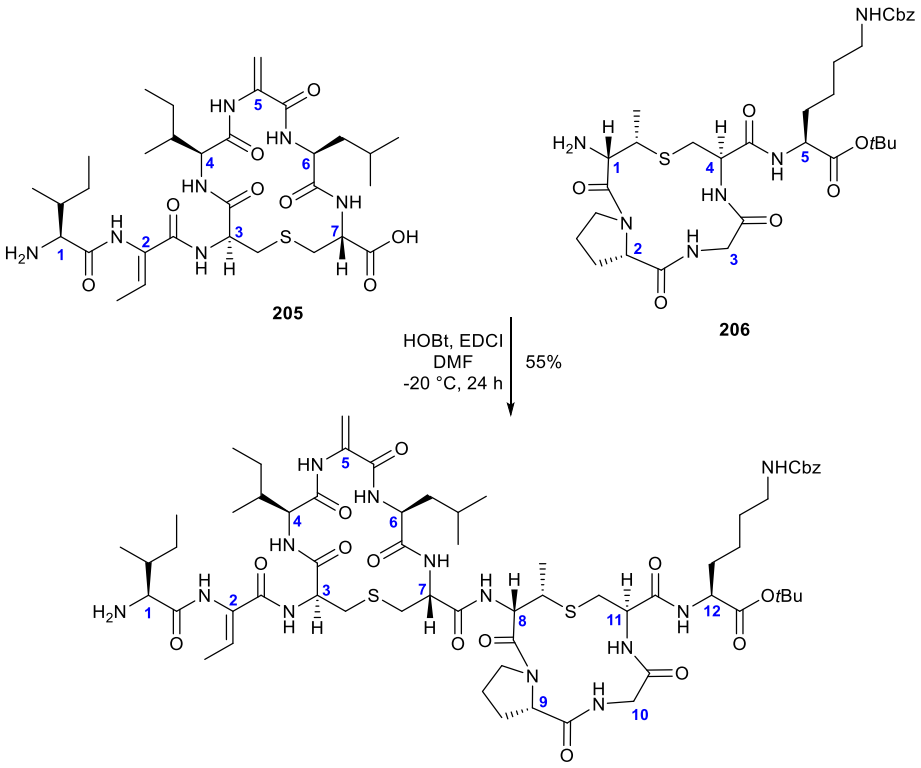
Scheme 4.15: Attempted synthesis of mutacin I Cys/Cys analogue (**199**) with Hmb-Leu at position six. Residues coupled using symmetric anhydrides shown in red, Dha replacements shown in blue.

As the synthesis of mutacin I ring AB analogues **194**, **197** and **199** using this strategy had proved impossible, efforts were instead concentrated on the coupling of separately synthesised A and B rings.

4.4.3. Synthesis by Joining Separately Synthesised A & B Rings

The second strategy investigated for generation of the AB ring topology of nisin and mutacin I in this work was the attachment of two separately synthesised A and B rings, according to the method outlined in **Scheme 4.3**. Although in more recent years chemical synthesis of lantibiotics has been conducted on the solid-phase, in the first total synthesis of nisin the ring AB fragment was formed by the solution-phase coupling of an unprotected ring A (**205**) to a *C*-terminally protected ring B (**206**) (**Scheme 4.16**).[145,146] It was therefore hypothesised that this methodology could also be applied to the synthesis

of mutacin I rings AB, with the carboxy terminus of the B ring attached to a resin rather than *t*Bu protected.



Scheme 4.16: Solution-phase formation of rings AB in the Shiba *et al.* total synthesis of nisin.

From the previous synthesis of mutacin I ring B (**Chapter 3.2.1**), it was known that the necessary resin-bound ring B fragment (**147**) could be easily accessed (**Figure 4.11**). However, investigations needed to be conducted into methods of producing mutacin I ring A which did not bear an eighth residue on *C*-terminal side of the Lan. Two different approaches were used as part of this work (**II**, **Scheme 4.3**). Firstly, attempts were made to enzymatically cleave the additional residues from ring A peptides using either carboxypeptidase Y (CPDY) or endoproteinase AspN (AspN). Secondly, routes involving the direct attachment of the Teoc/TMSE lanthionine (**38**) to the resin or to a chemically labile linker were explored.

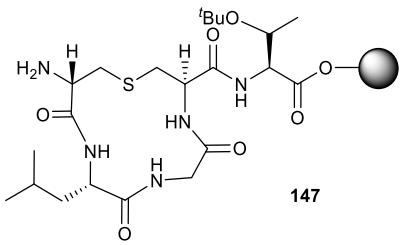
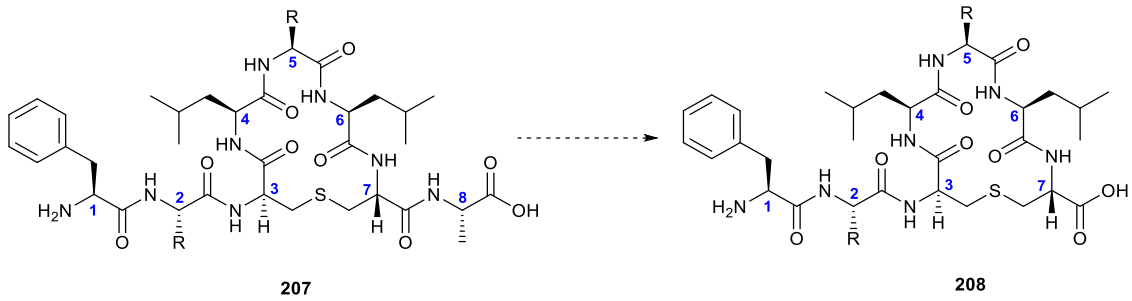


Figure 4.11: Resin-bound mutacin I ring B (**147**) for use in the synthesis of mutacin I rings AB according to the procedure outlined in **Scheme 4.3**.

4.4.3.1. Production of Mutacin I Ring A by Proteolysis

To begin, the enzymatic cleavage of the *C*-terminal Ala from three previously synthesised mutacin I ring A sequences, Ser/Ala analogue (**101**) (**Chapter 3.3.1**), truncated Ser analogue (**102**) (**Chapter 3.3.2**), and Cys-containing WT ring A precursor (**161**) (**Chapter 3.3.3.2**), was studied (**Scheme 4.17**). CPDY was used as it has broad specificity, and has been shown to be effective in the cleavage of *C*-terminal residues from Lan-containing peptides.[260,261] To conduct these reactions, each peptide was first dissolved in 0.1 M PyOAc at pH 5.5 before the addition of the CPDY, according to the method of Breddam *et al.*.[262] After incubation at 37 °C for 4 h, the reactions were quenched and the crude samples analysed by LCMS.



Scheme 4.17: General scheme for the attempted cleavage of the *C*-terminal Ala from mutacin I ring A peptides with CPDY. *Reagents & Conditions:* 0.1 M PyOAc buffer pH 5.5, 1:100 w/w CPDY:peptide, 37 °C. R = CH_3, CH_2OH or CH_2SH.

No conversion was observed for the Ser/Ala analogue (**101**) or Cys/Cys analogue (**161**), but mixtures of starting material and the target peptide were seen for truncated Ser analogue **102** (**Figure 4.12**). Unfortunately, even with the addition of more enzyme and incubation for 16 h, the reaction could not be pushed to completion, and desired peptide **209** could not be isolated by semi-preparative HPLC.

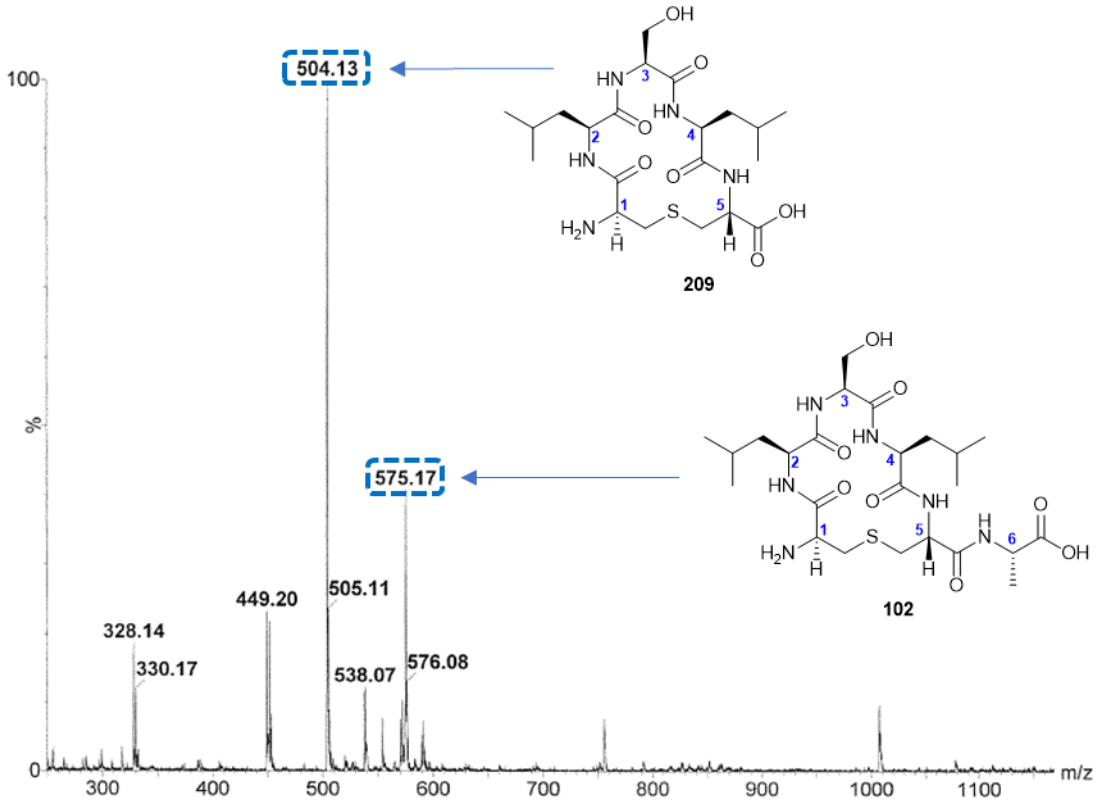
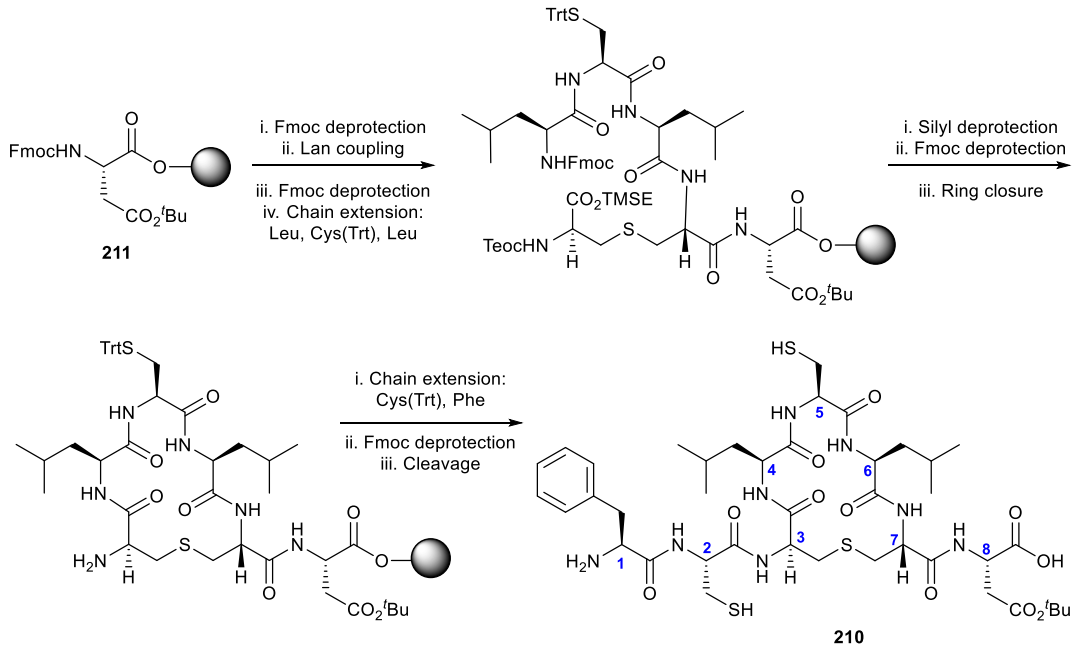


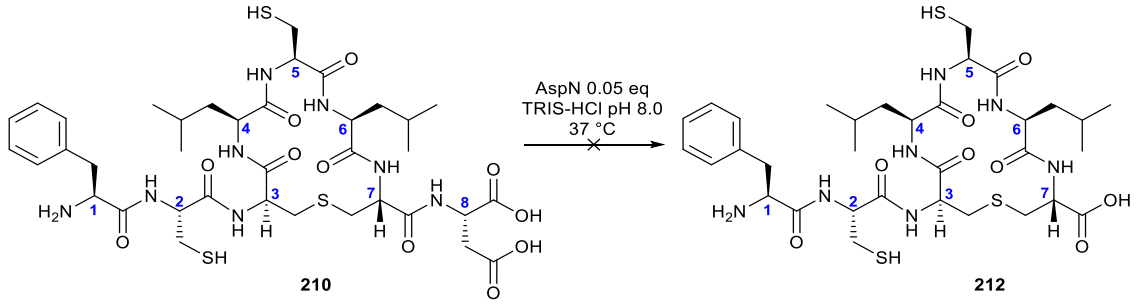
Figure 4.12: Crude LCMS analysis of CDPY cleavage with Ser analogue (**102**), showing partial conversion to the target peptide (**209**).

Despite reports that CDPY can efficiently cleave at Ala residues, this method could not be effectively applied here.[262] However, endoproteinase AspN has been used by Tang *et al.* to cleave cytolysin leader peptides, indicating that this enzyme would be tolerant to the presence of lantibiotic ring systems.[35] As AspN cleaves peptides at the *N*-terminal side of aspartic acid residues, the synthesis of a new mutacin I ring A was required. Cys analogue (**210**) was chosen as the target sequence, as it could be used to generate the WT ring A if the cleavage reaction proved successful (**Scheme 4.18**). The synthesis of this peptide was identical to that used in the production of WT mutacin I ring A Cys precursor (**161**), except that an Fmoc-Asp(O*t*Bu)-Novasyn TGT resin (**211**) was used in place of the Ala resin (**Chapter 3.3.3.2**). Following purification by semi-preparative HPLC, the peptide was isolated in 6% yield.



Scheme 4.18: Synthesis of mutacin I ring A Cys analogue (**210**) on Fmoc-Asp(OtBu)-Novasyn TGT resin (**211**).

Proteolysis of Cys analogue **210** was then conducted according to the method of Tarentino *et al.* (**Scheme 4.19**).[263] Peptide **210** was dissolved in 50 mM TRIS-HCl buffer containing zinc sulfate, and a solution of AspN was added. After adjustment of the pH to 8.0, the reaction was incubated at 37 °C for 16 h. Analysis by LCMS revealed only the presence of the starting peptide. Addition of more enzyme and incubation for a further 24 h had no effect, and no conversion to peptide (**212**) was observed by LCMS. As AspN is an endopeptidase, this lack of proteolysis was perhaps unsurprising due to the *C*-terminal position of the Asp residue in **210**, though no further experiments with exopeptidases were conducted.

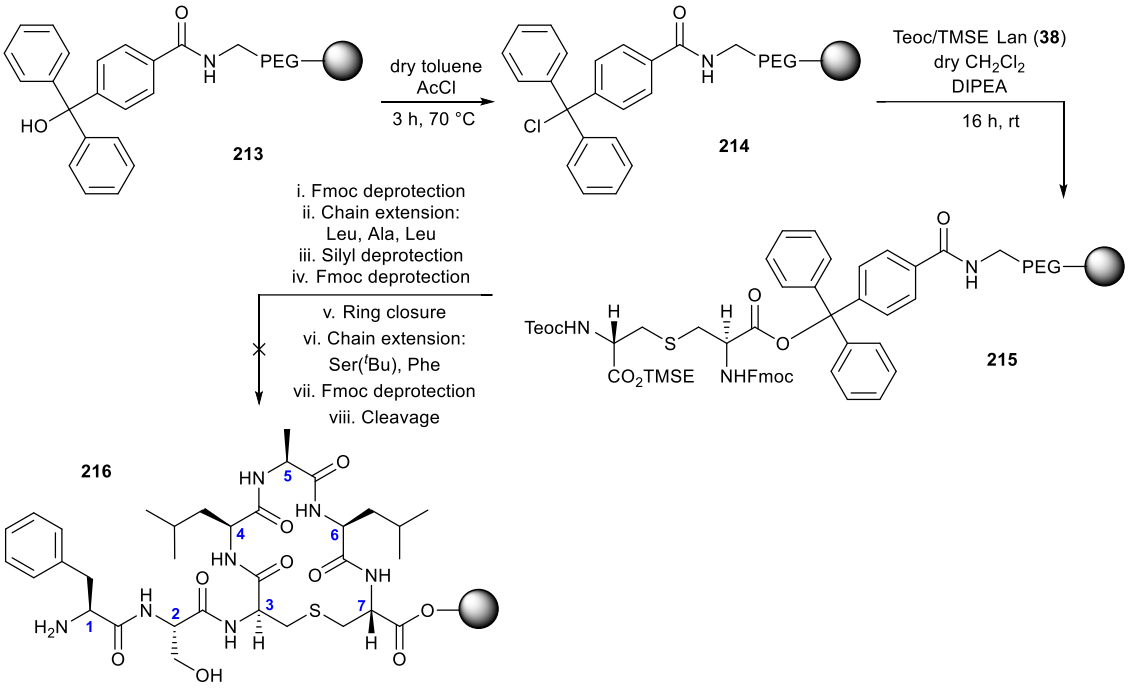


Scheme 4.19: Attempted cleavage of Asp from mutacin I ring A Cys analogue (**212**).

*4.4.3.2. Production of Mutacin I Ring A by Direct Attachment of Teoc/TMSE Lan (**38**) to the Resin*

Efforts were next concentrated on the synthesis of mutacin I ring A with Teoc/TMSE Lan (**38**) attached directly either to the resin itself or to an extended labile linker. Direct attachment of Lan to the resin was deemed a viable method for the production of mutacin I ring A peptides, as this is prevalent in the literature and the first step of many lantibiotic syntheses by Vederas *et al.* involve the coupling of an orthogonally protected (methyl)lanthionine to the solid support before formation of the ring.[158–160,165–167] Although the Vederas syntheses often utilise 2-chlorotrityl resins, Novasyn TGT resins, which also have a trityl linker, continued to be used in this work as the properties are similar, and both prevent racemisation during addition of the first amino acid.[264,265]

To enable attachment of Teoc/TMSE Lan (**38**), the unsubstituted alcohol form of the TGT resin (**213**) was first converted to the chloride form (**214**) according to the method of Fyles *et al.* (**Scheme 4.20**).[264,266] The chlorinated resin was then immediately coupled to the Teoc/TMSE Lan to give intermediate **215** using the method of Liu *et al.*, in which a solution of Lan (**38**) and resin was treated with DIPEA.[165] After overnight shaking at rt, the resin was washed with a solution of CH_2Cl_2, MeOH and DIPEA to cap any unreacted trityl alcohol. The final resin loading, determined by cleavage of the Fmoc group with DBU, was 0.11 mmol g^{-1}.[267]



Scheme 4.20: Synthetic scheme for the production of mutacin I ring A peptides directly on TGT alcohol resin.

With the resin-bound lanthionine in hand, the synthesis of mutacin I ring A analogue **216** was then conducted as previously described in **Chapter 3.3.1**. Following completion of the synthetic sequence, the peptide was cleaved from the resin with TFA and the sample analysed by LCMS. Disappointingly, none of the target peptide, or any truncation or deletion sequences, could be detected.

As the synthesis of a mutacin I ring A peptide had not been possible with the Teoc/TMSE Lan (**38**) directly next to the resin, its attachment *via* a longer cleavable linker was then investigated. A strategy based on the method of Hossain *et al.* was used in this work, in which a "solublising" linker terminating with 4-hydroxymethylbenzoic acid (HMBA) is placed between the resin and the peptide of interest.[268] In addition to enabling the production of the native sequence at the end of the synthesis by mild hydrolysis with 0.1 M NaOH or aqueous TFA, it was thought that the presence of a linker between the trityl resin and the Lan would remove the potential risk of low coupling efficiency due to steric hindrance.

To synthesise the linker, two Leu residues were first added to an Fmoc-Thr(*t*Bu)-Novasyn TGT resin using standard Fmoc SPPS techniques, giving tripeptide **217**, before coupling of HMBA with HBTU and DIPEA to give **218** (**Scheme 4.21**). The HMBA was then esterified with Teoc/TMSE Lan (**38**) using MSNT and MeIm, giving peptide (**219**).[269] Loading was judged to be complete due to a negative result from a colourimetric test for resin-bound hydroxyl groups, and the presence of the desired mass in the LCMS spectrum of a small sample of resin cleaved with 1% TFA (**Figure 4.13**).

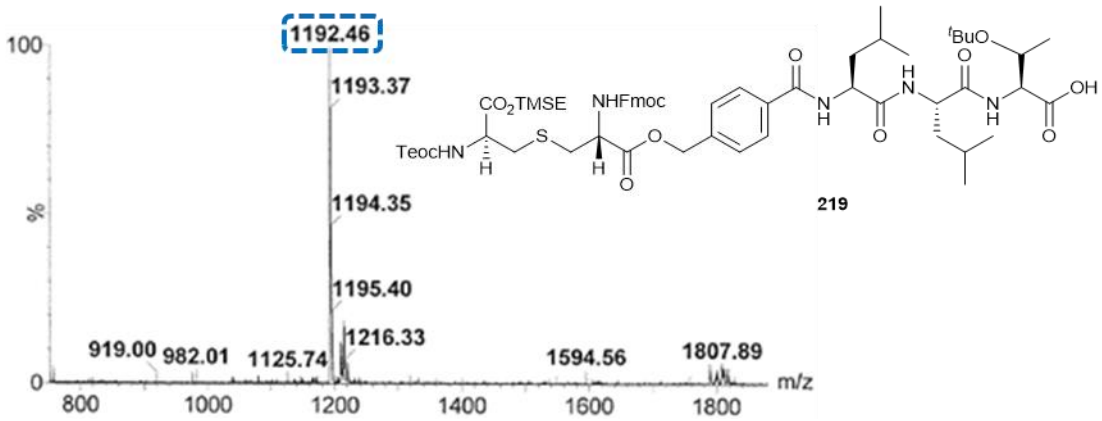
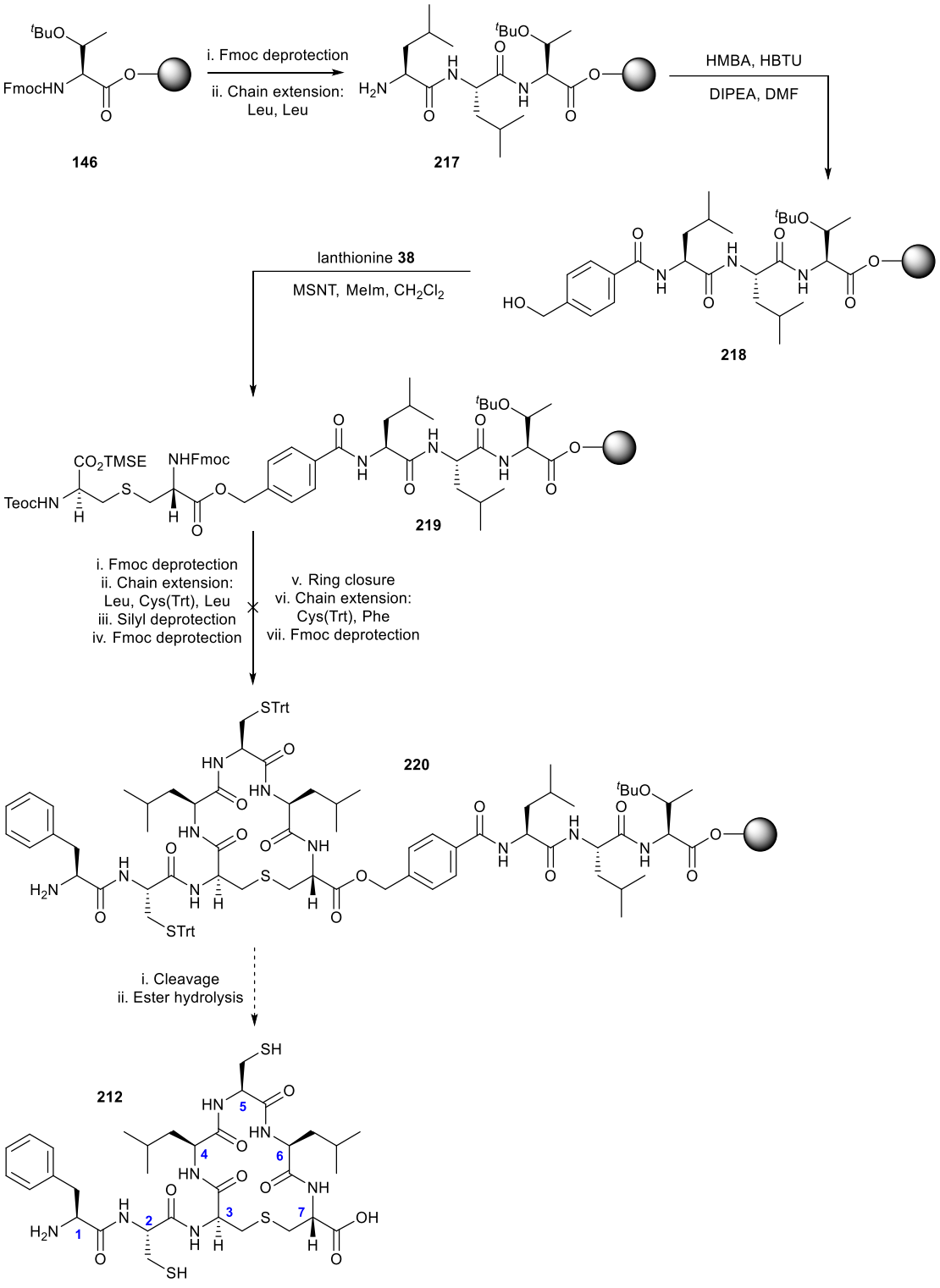


Figure 4.13: LCMS analysis of the HMBA linker following cleavage with 1% TFA, showing attachment of the Teoc/TMSE lanthionine (**38**).



Scheme 4.21: Synthetic scheme for the production of mutacin I ring A peptide **212** on a cleavable HMBA linker.

With the lanthionine successfully attached to the linker, the synthesis of mutacin I ring A analogue **212** was attempted as previously described (**Chapter 3.3.3.2**). Following completion of the synthesis and cleavage of the peptide from the resin, the sample was

analysed by LCMS. Again, none of the target peptide (**212**), or any truncation or deletion sequences, could be detected.

As all investigations into the generation of mutacin I ring A without the surplus *C*-terminal residue had failed, production of mutacin I rings AB by the solution-phase coupling of separately synthesised A and B rings could not be attempted. Unfortunately, research into alternative methods for the synthesis of this peptide was beyond the scope of this work.

4.5. Summary

In summary, WT nisin was enriched from a commercial sample according to the method of Slootweg *et al.*, before trypsin digestion and purification to yield the ring AB fragment (**95**), ready for NMR analysis (**Figure 4.14**).[216]

Two nisin ring AB analogues were also synthesised, Thr/Ser analogue **39** and Ala/Abu analogue **40**, according to the method of Mitchell (**Figure 4.14**).[161] Preliminary NMR experiments on Thr/Ser analogue **39** revealed that it may exist as conformers in solution, but further experiments at elevated temperature are required to confirm this. Full NMR analysis of these peptides has yet to be conducted however, and both will need to be re-synthesised in order to obtain sufficient quantities of high purity peptide for full characterisation and structural analysis. Once calculated, the solution state structures of these analogues will be compared to the WT nisin ring AB fragment (**95**), providing further information on the effect of the dehydro residues on ring conformation.

Extensive investigations into the synthesis of WT and analogues of mutacin I rings AB all proved unsuccessful. Disappointingly, none of the target analogues could be accessed by sequential on-resin SPPS, as had proved successful for nisin analogues **39** and **40**, even with the use of pseudoproline dipeptides or Hmb-protected amino acids. The reason for this was unclear, though one hypothesis was that the presence of the Pro residue in nisin ring B may alter the conformation of the peptide significantly enough to enable more efficient coupling reactions and completion of the synthesis. Synthetic strategies involving the separate synthesis of the A and B rings were also ineffective due to the inability to produce the ring A peptide without the additional *C*-terminal residue, either by enzymatic cleavage or direct attachment of lanthionine to the resin. Further optimisation of the synthetic method would therefore be necessary to enable the isolation of a mutacin I ring AB peptide for comparison with WT nisin rings AB (**95**).

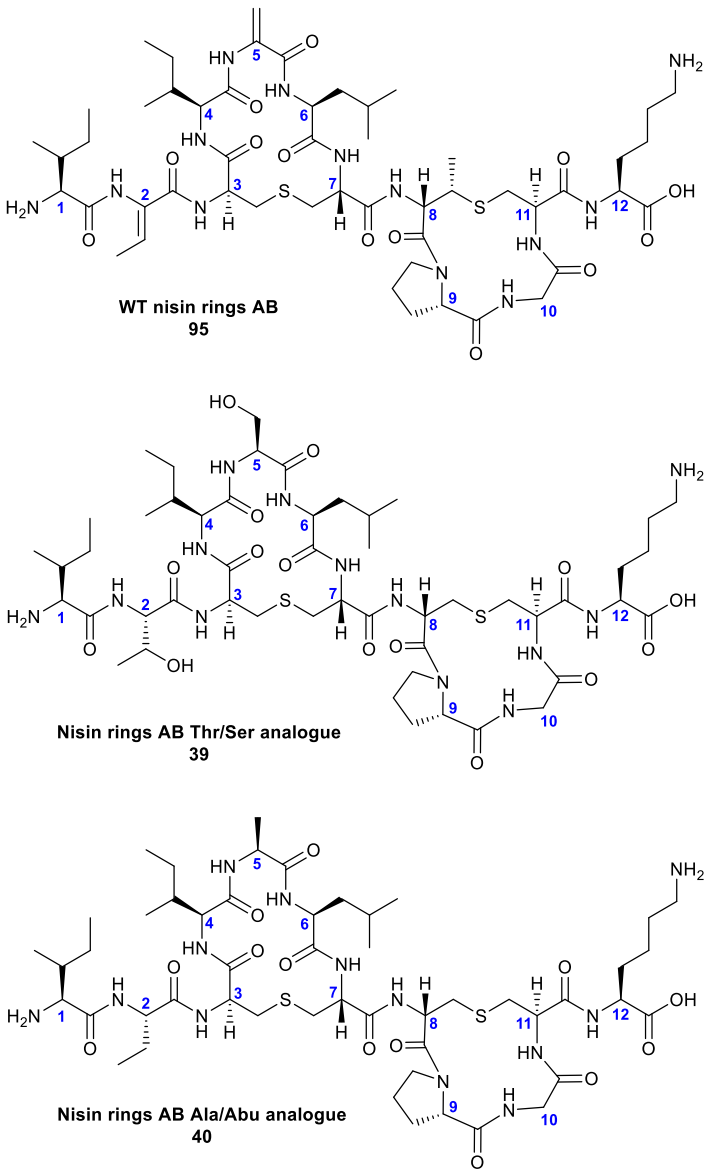


Figure 4.14: Structures of the isolated double ring peptides.

5. SOLUTION STATE NMR STRUCTURE DETERMINATION

5.1. Introduction

This chapter discusses the NMR assignment and solution state structure determination of eight of the lantibiotic peptides which were isolated in this work (**Table 5.1**).

Ring A Peptides	Ring B Peptides	Ring AB Peptides
Mutacin I Ser/Ala analogue (**101**)	WT mutacin I (**96**)	WT nisin (**95**)
Mutacin I Ser analogue (**102**)	WT nisin (**97**)	
WT mutacin I (**99**)	Nisin Lan analogue (**98**)	
WT nisin (**100**)		

Table 5.1: Lantibiotic peptides which were analysed by NMR.

A variety of different methods have previously been used to evaluate the interaction of peptides with lipid II. The most direct of these is the use of isothermal titration calorimetry (ITC)[270] to measure the binding affinity of the peptide and lipid, as reported by Cochrane *et al.* for tridecaptin A1 and Gram-negative lipid II.[271] Alternatively, antagonization assays with WT nisin in carboxyfluorescein-loaded vesicles can be conducted, allowing an evaluation of the activity of the peptides expressed as their affinity for lipid II, as reported by Slootweg *et al.* for dicarba-bridged nisin analogues.[173] A third possibility is solution state NMR of the peptide in the presence of lipid II, as reported by Bakhtiary *et al.* in studies on the mechanism of lacticin 3147.[90] The advantage of this method is that it accounts for the induced fit model, which states that the peptides may only adopt the correct binding conformation in the presence of lipid II.[272] However, each of these methods requires access to lipid II, or its analogues, which are currently still difficult and time-consuming to access by synthetic methods.[271] For this reason, in this work the pre-organisation for lipid II binding was evaluated by examination of the solution state structures of the peptides in the absence of lipid II.

Following purification, each of the peptides were fully characterised by NMR to enable distance and torsion angle restraint generation. These restraints were then applied in simulated annealing molecular dynamics simulations using the structure determination program XPLOR-NIH.[200,273] After refinement and validation, the solution state structures were compared to each other and to NMR structures of nisin available in the literature. Although many NMR studies have been published of full length and fragments of

nisin,[207,209,213,214] only one structure of the peptide in complex with lipid II exists (PDB ID: 1WCO).[96] It was envisaged that comparison of each of the isolated WT fragments to this structure may indicate whether or not the rings were pre-organised for target binding, or if they were dynamic in solution. By comparing the WT and analogue fragments to each other, the effect of structural simplification on solution conformation could be analysed, helping to guide future design of nisin ring AB analogues.

5.2. NMR Chemical Shift Assignment and Structure Determination

All NMR spectra were run in d_6-DMSO to enable direct comparisons to be drawn with the published structure of nisin in complex with lipid II.[96] Firstly, proton and carbon resonances for each peptide were assigned using a range of two-dimensional NMR techniques. COSY, TOCSY and NOESY were used to assign the ^{1}H shifts, though methylene groups were not stereospecifically assigned, while HSQC and HMBC were used to assign ^{13}C shifts. Following full characterisation, experimentally-derived distance and angle restraints were used to inform the structure calculation. Distance restraints were generated from homonuclear ^{1}H-^{1}H NOESY spectra, and angle restraints from ^{3}J coupling constants (see **Appendix I** for tables of restraint data for each of the synthesised peptides).

Secondly, in order to perform the structure calculation, new topologies had to be created for the dehydro residues which accounted for the significant charge redistribution compared to the 20 proteinogenic amino acids. Additionally, an Abu residue was needed to form one side of the MeLan bridge, and two new 'patch residues' (patches) were necessary for the generation of lanthionine and methyllanthionine. With all of these added to the XPLOR topology file, solution state structures could then be determined in XPLOR-NIH (see **Appendix I** for new topologies and parameters).

5.2.1. Restraint Generation

Distance restraints are the most important source of structural information from NMR data, and are derived from ^{1}H-^{1}H NOESY or ROESY spectra. NOE/ROE crosspeaks result from through-space magnetisation transfer, and are typically observed between protons which are less than 5 Å apart. The closer the protons are, the higher the intensity of the crosspeak, producing the inverse relationship shown in **Equation 1**. The 'make distance restraints' facility in the CCPN software suite, which was used to generate distance restraints for all the peptides analysed in this work, utilises this relationship to

produce a list of interproton distances for each set of assigned NOE/ROE crosspeak intensities.[274]

$$I \propto \frac{1}{r^6}$$

Equation 1: Relationship between NOE intensity and interproton distance; where I = NOE intensity and r = interproton distance.

NOEs are usually placed into three distinct bands and classified as either strong (short range interactions, 1.8 - 2.8 Å), medium (2.9 - 3.5 Å) or weak (long range interactions, 3.6 - 5.0 Å). However, CCPN creates a list of continuous interproton distances, and these values were used initially in structure calculations. If these continuous values were then found to result in many violations during the first round of simulated annealing, the NOEs were instead grouped into bands and the error values loosened accordingly, with an additional 1 Å added to the upper bound of NOEs involving methyl groups to account for the increased intensity of these crosspeaks.[275] Although intra- and inter-residue crosspeaks are observed in peptide and protein NMR, typically only the inter-residue signals are used in calculations because intra-residue distances are constrained by the known bond lengths and angles of each amino acid. Therefore, in this work only inter-residue distances were used as restraints, and crosspeaks possibly resulting from an intra-residue interaction were not included. Where crosspeaks overlapped, ambiguous assignments were incorporated into the restraint list.

Dihedral angle restraints are another important source of structural information from NMR, and are derived from scalar coupling constants produced by through-bond magnetisation transfer. The ^3J coupling constant is commonly measured for protein structure calculation, and is related to the backbone dihedral angle (ϕ) by the Karplus equation (**Equation 2**).[276,277]

$$J = \mathrm{A} + \mathrm{B}\cos\phi + \mathrm{C}\cos 2\phi$$

Equation 2: The Karplus equation, where J = coupling constant, ϕ = dihedral angle and A, B and C are constants.

The values of the three constants in the Karplus equation depend on which ^3J value is being measured. In this case only homonuclear couplings were measured, so the constants derived by Vögeli *et al.* for backbone H_N-H_α coupling were used (A = 7.97 Hz, B = -1.26 Hz and C = 0.63 Hz).[278] These were then put into the Karplus equation with ^3J

couplings measured from ^{1}H NMR spectra, and solved for the dihedral angle ϕ. These calculations were verified and error margins determined for each value by comparison with the relationship between $^{3}J_{HN\text{-}H\alpha}$ and ϕ described by Lubecka *et al.* (**Table 5.2**).[279] If the ^{3}J coupling could not be directly measured from the spectra, the Bax group TALOS+ or TALOS-N servers were used to make predictions of torsion based on chemical shift, which in proteins is highly dependent on local backbone structure.[280,281] By comparing input chemical shifts to a database of proteins with known shift and dihedral angle, the programs can predict the value of an unknown dihedral, and rate it as either "good", "bad", or "ambiguous" relative to the known structures. Only "good" predictions were considered for use in structure calculations in this work. An additional advantage of using these servers is that they are able to predict backbone ψ angles and sidechain χ^{1} rotameric states, though again these were only used in calculation if the prediction was judged to be "good".

$^{3}J_{HN\text{-}H\alpha}$ Coupling Constant (Hz)	ϕ Angle
< 6	-60° ± 30°
6 - 8	-90° ± 30°
8 - 9	-120° ± 40°
> 9	-120° ± 20°

Table 5.2: Relationship between $^{3}J_{HN\text{-}H\alpha}$ and ϕ used in structure determination by Lubecka *et al.*.[279]

A third important factor in structure calculation is whether the amide bonds to any proline residues in the peptide are cis or trans. As 5% of all Xaa-Pro amide bonds in proteins are in cis conformation, the geometry must be correctly identified in order to obtain an accurate structure. The conformation can sometimes be determined by NOEs. For example, in the work by Falcomer *et al.* on the conformation of cyclic tetrapeptides with sequence Cys-Pro-Xaa-Cys, which are similar to nisin ring B but with a disulfide rather than a thioether bridge, the Cys-Pro bond was judged to be trans by the presence of a strong NOE between Cys1 H_{α} and the Pro2 H_{δ} (**Figure 5.1**).[282] In cases where NOEs are ambiguous, the likelihood that the Xaa-Pro bond is cis can instead be predicted by examination of the proline β and γ ^{13}C signals according to the method of Shen and Bax, who found that there is a significant difference between the chemical shift of these carbons when in cis or trans conformation. The Bax group have also developed the

Promega server for the purpose of this prediction, which produces a probability score that the unknown Xaa-Pro bond is cis based on comparison of input chemical shifts to proteins of known shift and Xaa-Pro conformation.[283] Using this method, the Lan-Pro bond in WT nisin ring AB fragment (**95**) was found to be 100% likely to be trans, while in nisin ring B fragments **97** and **98** these bonds were >91% likely to be cis.

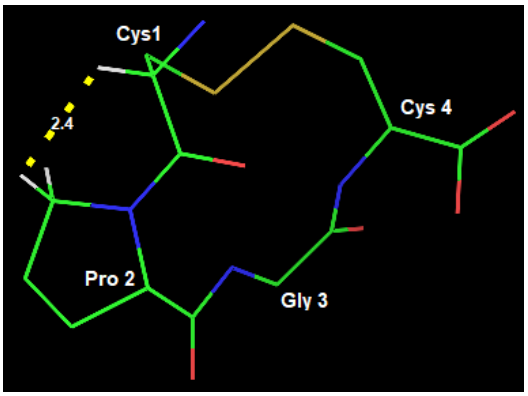


Figure 5.1: Strong NOE in the cyclic tetrapeptide Cys-Pro-Xaa-Cys, indicating that the Cys-Pro amide bond is trans (measurement in Å). Structure generated with Chem3D 15.1 and visualised in PyMOL (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC).

5.2.2. Generating New Topology

After all the necessary restraints for the structure calculations had been determined, topologies had to be defined in XPLOR-NIH for the unusual residues found in the lantibiotics.

Firstly a patch residue was created for the Lan bridge from two cysteines, based on the existing patch for a disulfide bond and the lanthionine patch created by Earl.[284] The γH from one cystine and the SH group from the second cystine were both deleted, followed by an 'add bond' command to join the remaining sulfur to the βC of the second Cys (see **A**, **Figure 5.2**, for an example of how this patch works with a ring B peptide). The same principle was used to generate the methyllanthionine patch, but instead of two cystines an Abu residue and a cysteine were used, creating the new bond between the Abu βC and the sulfur atom (**B**, **Figure 5.2**). However, as Abu itself is an unusual amino acid, new topology to define this residue also had to be built. This was simply done by basing the topology on that of a Thr residue, with the OH group replaced with an additional βH.[210] Finally, to fully define the topology of the thioethers and ensure that the required stereochemistry was generated, the desired bonds, angles, impropers and dihedrals were

specified for each patch and added to the XPLOR topology files. Pre-existing XPLOR force constants were used for these parameters as new atom types were not required.

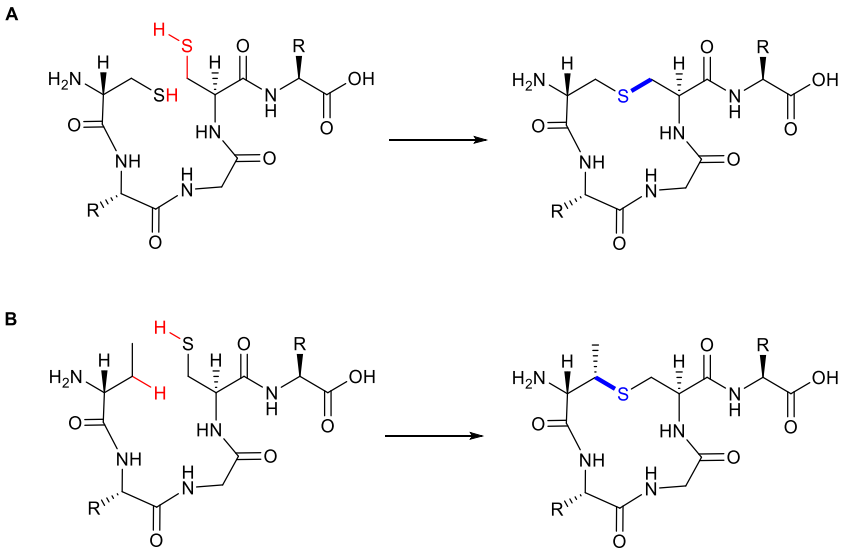


Figure 5.2: Generic ring B peptide used to exemplify the creation of **A.** the lanthionine patch residue and **B.** the methyllanthionine patch residue. Red bonds and atoms are those which need to be deleted, blue bonds are those which need to be created.

A second set of topologies then needed to be built for the dehydro residues. As none of the proteinogenic amino acids have α,β-unsaturation, completely new atom types with different partial charges were necessary in order to account for the different charge distribution within Dha and Dhb (**Figure 5.3**). Parameters for all new bond lengths, angles, impropers and dihedrals therefore also had to be defined in order for XPLOR to build these residues with the correct geometry; all of which were based on the CHARMM force field parameters for Dha and Dhb developed by Turpin *et al.*.[285]

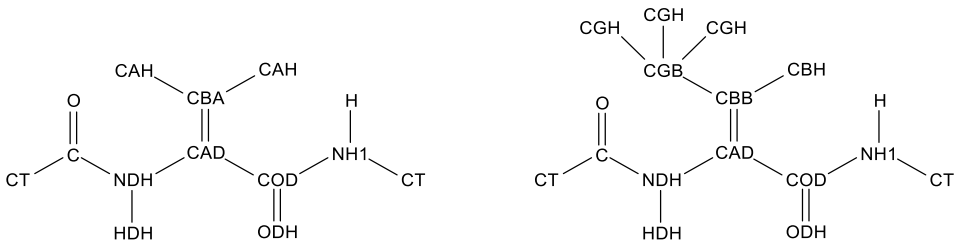


Figure 5.3: Key to the new atom types which needed to be defined for the dehydro residues.

5.2.3. Structure Determination

With all the necessary parameters for the unusual amino acids added to the topology files, structure determination in XPLOR-NIH could begin.[200,273] XPLOR-NIH is a

biomolecular structure determination package, based on the older XPLOR and CNS software,[286] which uses the CHARMM energy function for molecular dynamics (MD) and minimisation.[287] In particular, simulated annealing molecular dynamics is used, in which the molecule is computationally heated and then slowly cooled in order to find the global minimum. By making incremental changes to the structure, the conformational space can be more efficiently explored.[288]

The workflow for determination of protein structures in XPLOR-NIH is shown in **Figure 5.4**. Firstly, a protein structure file (psf) is generated from the peptide sequence, and an initial template structure is calculated. A large group of structures are then generated from this initial structure by simulated annealing, and are refined over multiple rounds before selection of the 15 lowest energy structures for validation. Each of these steps was run using an edited XPLOR tutorial script, developed for structure determination of small protein domains. Although these are now deemed insufficient for whole protein structure calculation, they were judged to be adequate in this case as all of the peptides synthesised in this work were of low molecular weight. Example scripts for each stage of the calculation are given in **Appendix I**.

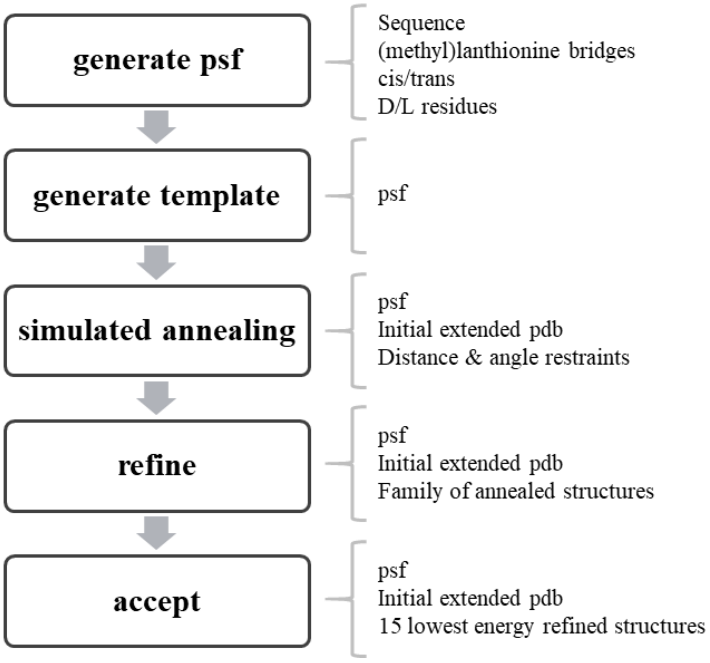


Figure 5.4: XPLOR-NIH workflow used to calculate NMR structures. Each box indicates a new script that is run for a stage of the calculation, with required inputs shown on the right.

A psf generation script first had to be run in order for XPLOR to accurately reproduce the topology of the peptide. A total of four inputs were necessary: the amino acid sequence,

location of the (methyl)lanthionine bridges, whether any of the amide bonds exist in cis conformation and whether any residues are D rather than L. In this work, the sequence was entered linearly, with each side of the (methyl)lanthionine residue replaced by either Cys or Abu, and the appropriate patch residue invoked to generate the thioether. For example, mutacin I ring B was input as 'CYS LEU GLY CYS THR', and the lanthionine patch was used to create the correct connectivity as described in **Figure 5.2 A**. As the left-hand side of all (methyl)lanthionines in nisin and mutacin I are D, the LTOD patch included in the XPLOR topology file was also invoked to ensure that the correct stereochemistry was generated. Finally, the XPLOR CIPP patch was invoked for peptides containing cis prolines. Once the psf file had been created, it was then used in the template generation script to calculate an initial extended structure of the peptide, output as a pdb file.

This extended structure file was used in the simulated annealing script to produce a family of 100-250 structures using simulated annealing molecular dynamics, with both distance and angle restraints included in the calculations. Each of the structures were equilibrated at 1000 K for 6000 timesteps of 0.003 or 0.005 ps, before cooling slowly over 3000 timesteps. The produced pdb files were then checked for large violations of NOEs and dihedral angles which may have indicated an assignment error. Using the refine scripts, multiple rounds of refinement were then run on the family of structures until no further improvements in energy were observed. A similar simulated annealing protocol was used at this stage, except that cooling was instead conducted over 2000 timesteps to a final temperature of 100 K. The 15 structures with lowest energy were then selected for analysis.

As an initial test of structure quality, the accept script was run to check for structures stuck in local minima. To assess structure quality more thoroughly, external validation was also conducted with the Protein Structure Validation Server (PSVS), which uses root-mean square deviation (RMSD), calculated using **Equation 3**, to compare the structures on an atom-by-atom basis.[289] PSVS also utilises PROCHECK to analyse where dihedral angles fall in the Ramachandran plot, indicating what percentage of residues lie in 'disallowed' regions.[290,291] Although potentially large violations of dihedral angle were expected from this analysis due to the unusual amino acids, all of the measured and predicted dihedral angles for regular L-residues in the synthesised peptides appeared in

'allowed' regions in the updated Ramachandran plots published by Hovmöller *et al.*.[96,292] The full table of structure quality statistics can be found in **Appendix II**.

$$RMSD = \sqrt{\frac{1}{N}\sum_{i=1}^{N}\delta_i^2}$$

Equation 3: Root-mean square deviation equation, where N = number of pairs of equivalent atoms and δ = distance between pairs of atoms.

5.3. Solution State NMR Structures

The structure calculation process described above was applied to each of the isolated peptides. Ensembles of the lowest energy structures were produced in PyMOL (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC), by alignment of the peptide backbone within the rings. Side chains (excluding proline) have been omitted from all of the following structures for clarity. Representative structures (closest to the average) were used for comparative purposes.

5.3.1. Structure of B Rings

The ^{1}H NMR spectrum of mutacin I ring B (**96**) revealed the presence of two sets of resonances in a 3:1 ratio, which could be separately assigned (**Figure 5.5**, **A**). Re-running the NMR at elevated temperature confirmed that these corresponded to conformers rather than two different peptides or diastereomers, as the peaks coalesced into one average structure (**Figure 5.5**, **B**). Interestingly, this was also observed by Toogood in his work on the structural analysis of an epidermin ring B lanthionine analogue.[149]

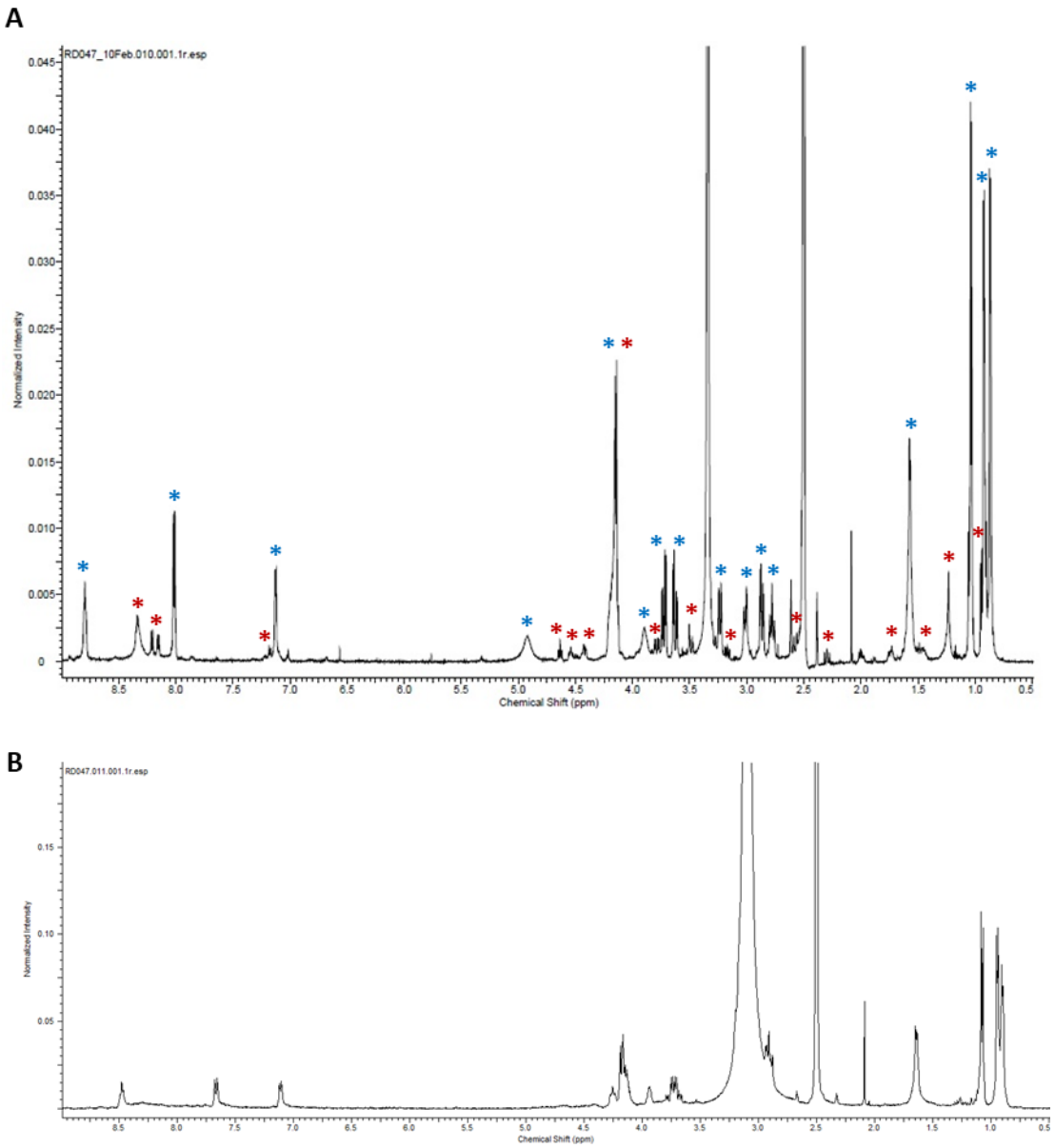


Figure 5.5: ^{1}H NMR spectra of mutacin I ring B (**96**). **A.** Taken at rt. * = major conformer, * = minor conformer. **B.** Taken at 80 °C.

Structure calculation indicated that the two conformers were very similar (**Figure 5.6**). Although below 1 Å for both, the backbone RMSD was much better for the major than minor conformer, perhaps because the minor conformer was calculated from fewer restraints due difficulties in assigning the overlapping signals. On comparison of the two, the main difference between the conformers seemed to be a peptide plane flip along the Leu-Gly amide bond (**Figure 5.6**). Peptide plane flipping is a conformational change defined by large changes in the ϕ and ψ angle of two neighbouring residues, with little effect on the surrounding residues or side chain orientation.[293,294] Numerous examples have been reported in the literature, particularly as a key part of protein folding or

function, but most notably it has been observed in the Pro-Gly bond of mutacin 1140.[203,295,296] In the case of mutacin I ring B WT (**96**), it appeared that at rt this flip occurs slowly enough to enable both states of ϕ and ψ to be resolved.

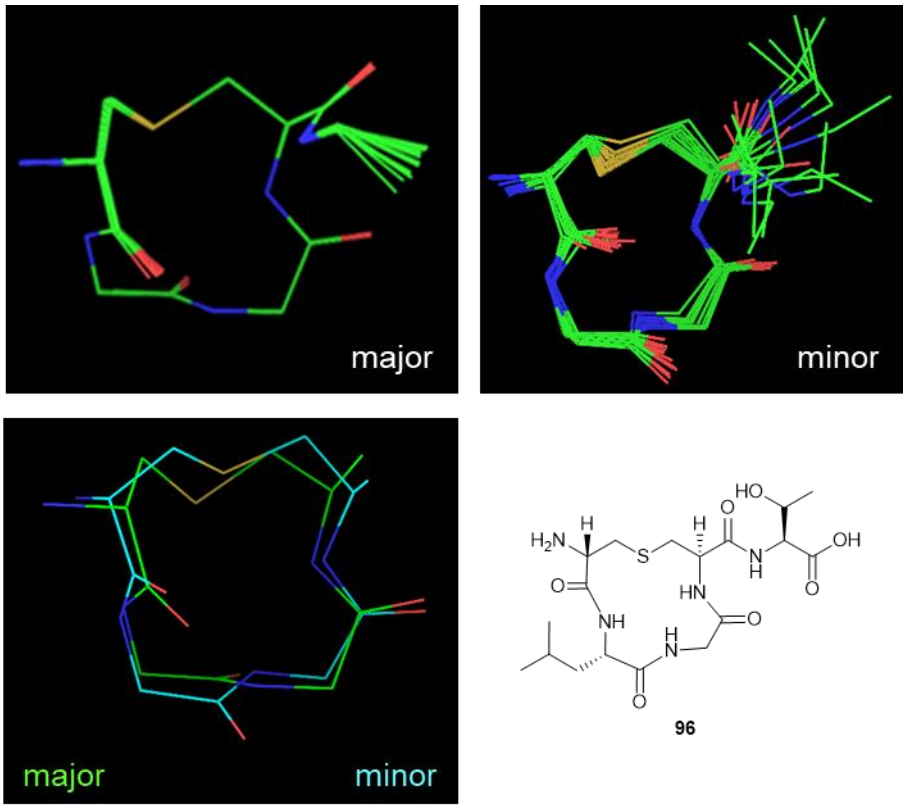


Figure 5.6: Ensembles of the 15 lowest energy structures of the major and minor conformers of **96**, and overlay of both showing the 180° flip around the Leu-Gly amide bond (Thr omitted for clarity).

Contrary to mutacin I ring B (**96**), the spectra of nisin ring B peptides **97** and **98** indicated the presence of only one conformer, and determination of the geometry of the Lan-Pro bonds using the Promega server as previously described (**Chapter 5.2.1**) indicated that the likelihood of this bond to exist in cis conformation in the Lan peptide (**98**) was 91%, and 99.4% in the MeLan peptide (**97**). This was unexpected, as previously reported structural analysis of a nisin ring B analogue (**93**) by Palmer *et al.* showed mixtures of cis and trans Pro.[209] It was hypothesised that this difference may have been caused by the alternative synthetic route taken in this work, and perhaps the presence of a preformed thioether bridge necessitated the cis Pro in order to bring the two sides of the ring close enough to form the new amide bond.

Ensembles of the 15 lowest energy structures of nisin ring B WT (**97**) and nisin ring B Lan analogue (**98**) are shown in **Figure 5.7**. Backbone RMSD in both peptides was excellent (0.2 Å), and as expected, the replacement of Lan for MeLan did not significantly change the backbone conformation of the peptide (comparison of the two peptides shown in **Figure 5.7**, **C**). In fact, the only noteworthy difference between the two structures is a slight change in the thioether bridge orientation. This indicated that MeLan could successfully be replaced by Lan in the synthesis of future analogues, providing faster access to peptides as only one orthogonally protected lanthionine would be necessary. Additional evidence for the fact that this substitution would be tolerated comes from the reported NMR structure of nisin and lipid II, which showed that it is the hydrogen bonding network between the backbone amides and the pyrophosphate which is important for binding.[96]

Finally, both WT B rings **96** and **97** were compared to the ring B segment from the published NMR structure of full length nisin bound to lipid II (taken from the PDB, ID 1WCO). Of the mutacin I ring B (**96**) conformers, the minor conformer was most similar to the published sructure as the Leu-Gly amide bond is in the right configuration (**Figure 5.8**, **A**). The largest difference between the two peptides was observed in the (Me)Lan-Leu/Pro section of the backbone, though all of the amides were in broadly the same positions and orientations. This suggested that mutacin I may bind lipid II in the same manner as nisin, though is perhaps more flexible in solution due to the absence of the Pro residue. Comparison of synthesised nisin ring B WT (**97**) to 1WCO again revealed that the two were most dissimilar along the MeLan-Pro backbone section. This was to be expected, as in the synthesised peptide (**97**) the MeLan-Pro amide bond is cis, whereas in the full length peptide it is trans. This difference suggests that when synthesised as described in this work, nisin ring B does not adopt the correct lipid II binding conformation in solution.

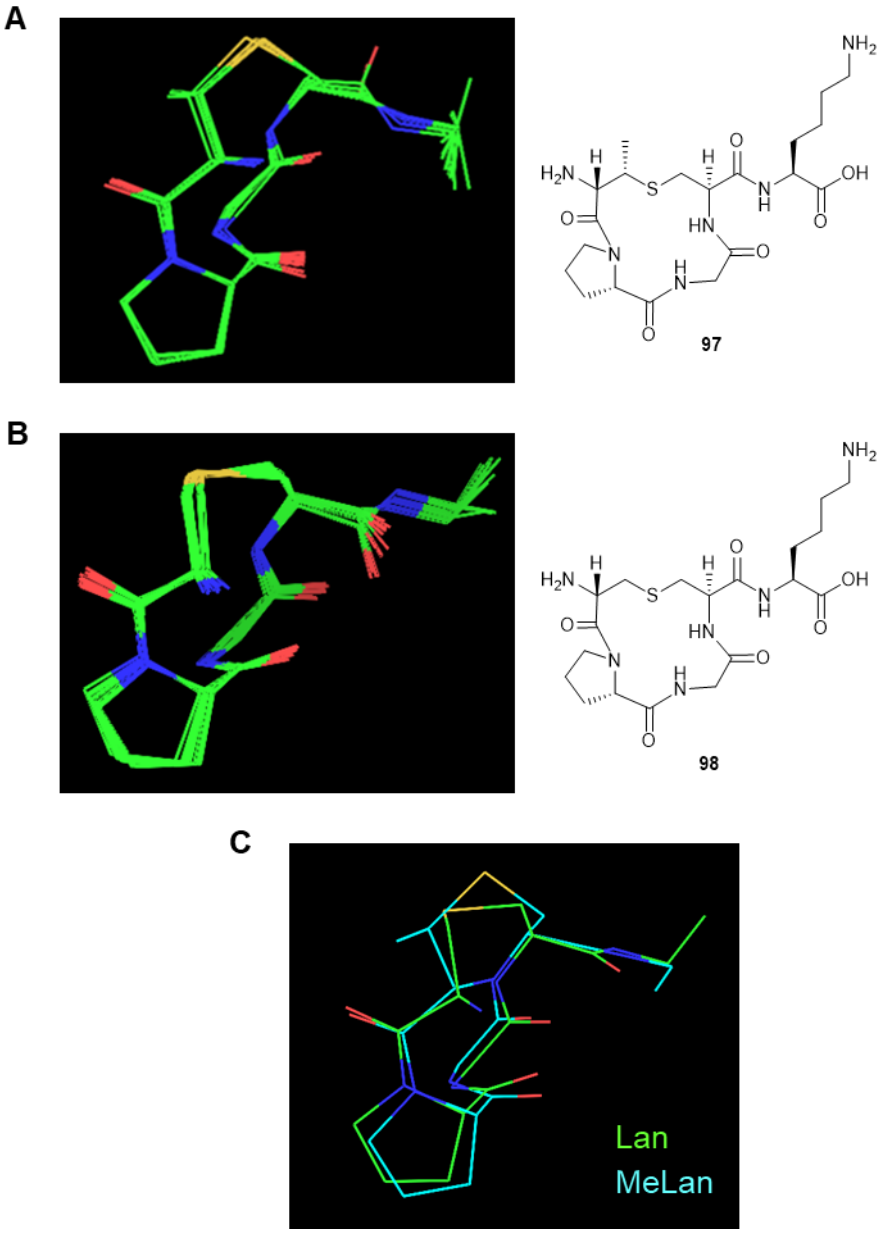


Figure 5.7: Ensembles of the 15 lowest energy structures of **A.** WT peptide (**97**) and **B.** Lan analogue (**98**). **C.** Overlay of **97** and **98** showing their similarity.

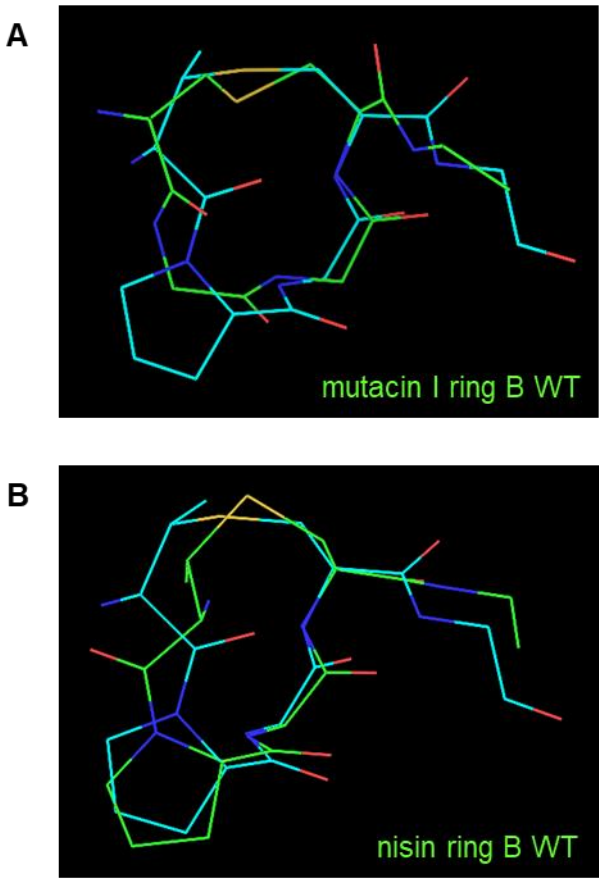


Figure 5.8: Comparison of the ring B segment of PDB ID 1WCO (full length nisin in complex with lipid II, shown in cyan) to **A.** mutacin I ring B WT and **B.** nisin ring B WT.

5.3.2. Structure of A Rings

Ensembles of the 15 lowest energy structures for all four of the synthesised A rings are shown in **Figure 5.9**. Backbone RMSDs were best for mutacin I WT (**99**) (0.5 Å) and Ser analogue **102** (0.3 Å). Higher RMSDs were observed for both mutacin I Ser/Ala analogue (**101**) (1.2 Å) and nisin ring A WT (**100**) (1.0 Å), which was attributed to the increased flexibility of the tail region without a dehydro residue, and calculation from fewer inter-residue NOEs respectively.

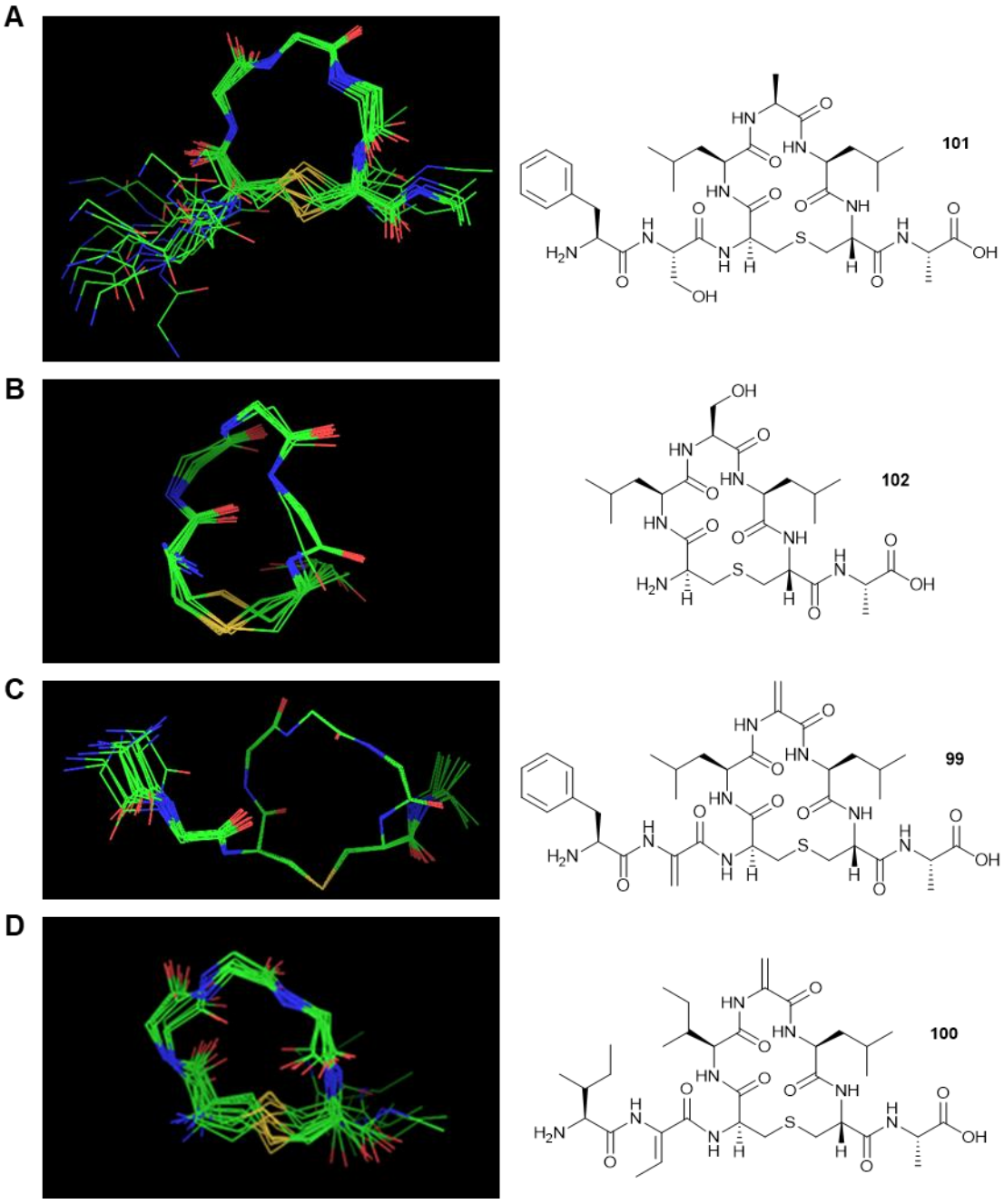


Figure 5.9: Ensembles of the 15 lowest energy structures for the isolated ring A peptides. **A.** mutacin I ring A Ser/Ala analogue (**101**), **B.** mutacin I Ser analogue (**102**), **C.** mutacin I ring A WT (**99**), and **D.** nisin ring A WT (**100**), with *N*-terminal Ile and Dhb omitted for clarity.

To examine the effect of the dehydro residues on solution conformation, the structures of the mutacin I ring A peptides were compared to each other (**Figure 5.10**, **A**). The most noticeable difference was that the absence of the two tail residues caused Ser analogue **102** to have a less rounded, more elongated shape than the other mutacin peptides (**99** and **101**), indicating that these residues play an important role in restricting the accessible

solution conformations. Ser/Ala analogue (**101**) and the WT peptide (**99**) were fairly similar to each other however, with most flexibility observed in the thioether bridge, as was also described by Lian *et al.* in nisin ring A.[208] Comparison of all three mutacin peptides showed that the replacement of Dha5 in the WT peptide (**99**) for either Ser or Ala did not have a large impact on the overall conformation of the Leu4-Xaa5-Leu6 portion of ring A, although the central amide bond was more planar in WT peptide (**99**) due to the presence of the Dha.[217] Overall, the fact that Ser/Ala analogue (**101**) and the WT peptide (**99**) could be overlaid with low RMSD indicated that the solution state structure of ring A was not significantly affected by the replacement of Dha5, and suggested that analogues without a dehydro residue in this position could be used in future studies.

Secondly, the structures of the two WT ring A peptides (**99** and **100**) were compared (**Figure 5.10**, **B**). Most flexibility was also observed around the thioether bridge in this case, though there was little difference between the two peptides in the rest of the ring. This was perhaps to be expected as the only change is Leu→Ile at position four. In particular, the backbone amides adopt the same relative positions and orientations in both peptides. As the ring A backbone NH groups form the majority of the hydrogen bonding network to lipid II, this suggests that mutacin I may bind the pyrophosphate of lipid II in the same fashion as nisin.

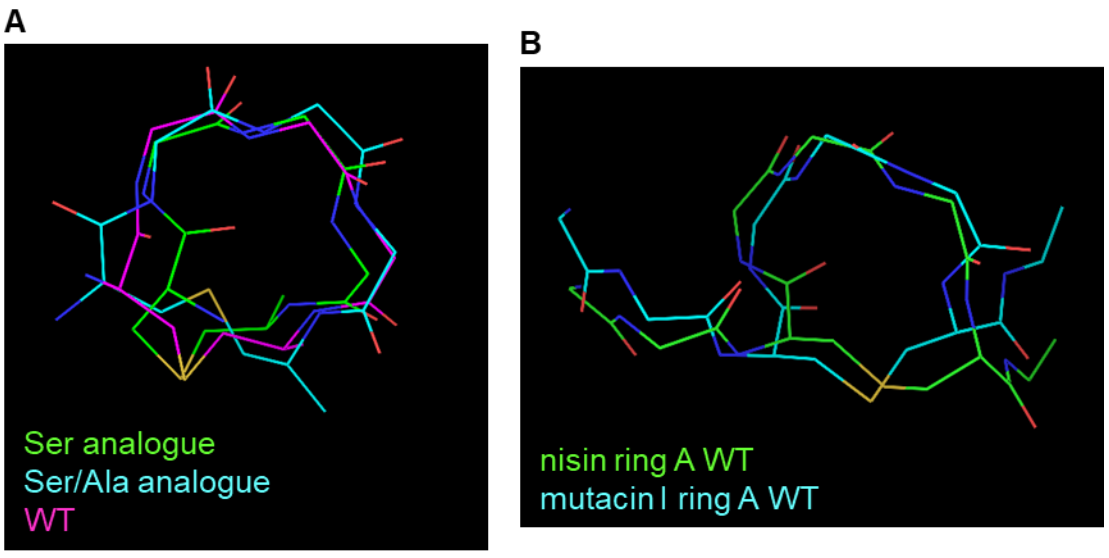


Figure 5.10: Comparisons of **A.** the mutacin I ring A peptides mutacin I Ser analogue (**102**), mutacin I Ser/Ala analogue (**101**) and WT mutacin I (**99**) (non-ring residues omitted for clarity), and **B.** WT nisin (**100**) and WT mutacin I (**99**) ring A peptides.

Finally, WT ring A peptides **99** and **100** were compared to the ring A segment from the published NMR structure of full length nisin bound to lipid II (PDB ID: 1WCO) (**Figure 5.11**). Both could be overlaid with the published structure with low RMSD, implying that the WT A ring peptides are pre-organised for target binding, and adopt the necessary conformation in solution.

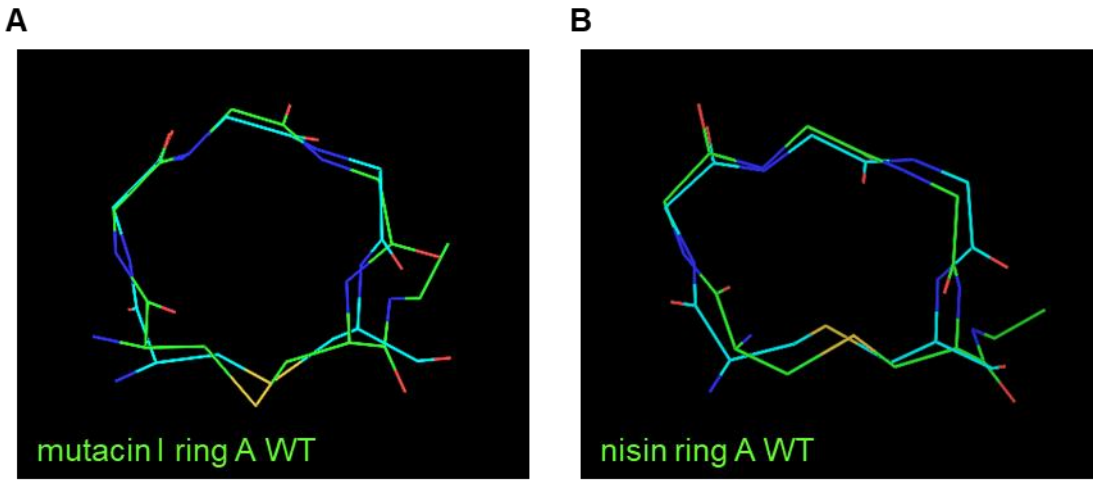
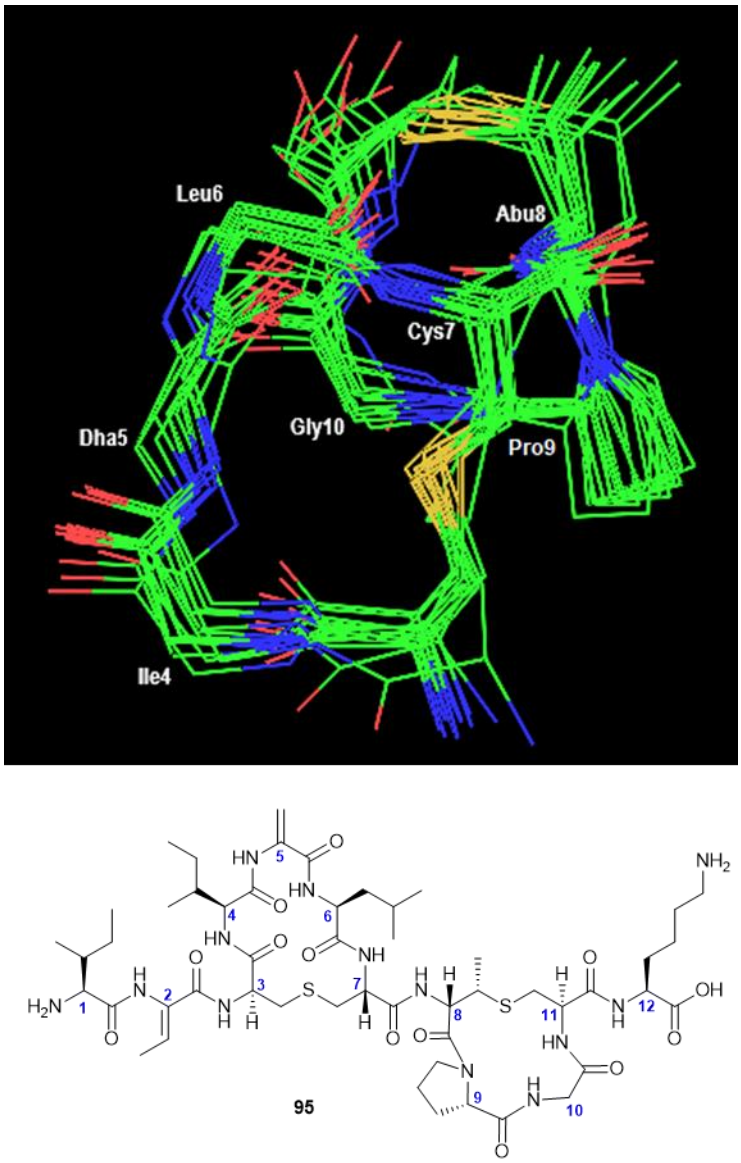


Figure 5.11: Comparison of the ring A segment of PDB ID 1WCO (full length nisin in complex with lipid II, shown in cyan) to **A.** mutacin I ring A WT and **B.** nisin ring A WT.

5.3.3. Structure of Truncated Nisin rings AB WT (**95**)

Before structure determination of isolated nisin rings AB WT (**95**) could begin, determination of the proline geometry was necessary. Promega predicted that in this case, the likelihood of the MeLan-Pro bond to be in cis conformation was 0%.[283] The ensemble of 15 lowest energy structures of **95** calculated in XPLOR is shown in **Figure 5.12**.

Two main comparisons were made to this double ring structure: firstly to the WT single ring peptides, and secondly to the published NMR structure of full length nisin in complex with lipid II.



95

Figure 5.12: Ensemble of the 15 lowest energy structures of nisin rings AB WT (**95**). For clarity, key residues are labelled at the αC.

Firstly, to investigate whether the presence of a second ring influenced the conformation of the peptide, the pertinent section of the WT nisin double ring system (**95**) (ring A: **95A**, ring B: **95B**) was compared to the nisin WT single ring peptides: WT nisin ring A (**100**) and WT nisin ring B (**97**). The main distinction between the isolated B ring (**97**) and **95B** was the structure of the MeLan-Pro section, caused by the different proline geometries in the peptides (**Figure 5.13**, **A**). Further comparisons with synthetic lantibiotic AB rings would be required to determine whether the observed difference in solution conformation was solely due to the presence of the A ring, or whether the cis geometry is necessitated by the synthetic method employed in this work. A much lower backbone RMSD was

observed between the isolated A ring (**100**) and **95A** however, with most flexibility observed in the lanthionine bridge (**Figure 5.13**, **B**). This indicated that the overall solution conformation of WT nisin ring A was relatively unaffected by the presence of a second lantibiotic ring.

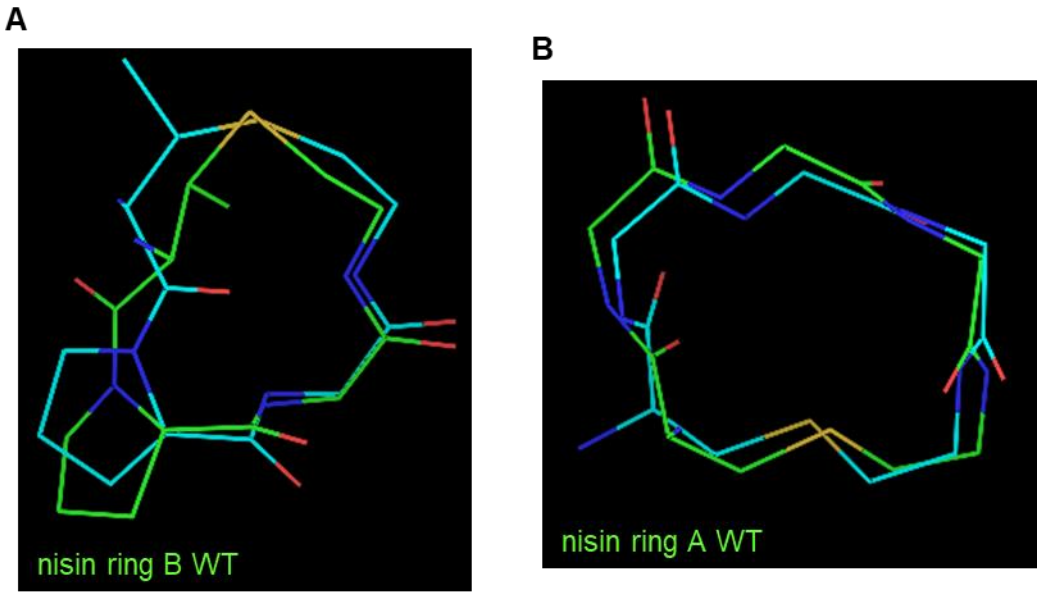


Figure 5.13: Comparisons of the synthesised single ring nisin WT peptides with the relevant fragment of isolated WT nisin rings AB (**95**) (shown in cyan). **A.** Comparison with nisin ring B WT (**97**). **B.** Comparison with nisin ring A WT (**100**).

Secondly, to study whether the structures of the nisin A and B rings undergo a change of conformation on target binding or if there is a degree of pre-organisation in both rings, the isolated double ring peptide (**95**) was compared to the AB ring fragment of the published structure of full-length nisin in complex with lipid II. It became apparent when attempting to overlay the two peptides that the MeLan in the published structure did not bear the native (2*S*, 3*S*, 6*R*) stereochemistry, first determined by Gross and Morell.[52] Instead, the MeLan had (2*S*, 3*R*, 6*R*) stereochemistry (**Figure 5.14**).[96] Depending on how many NOEs were observed to the MeLan methyl group and βH in the original work, re-calculation of this structure with corrected stereochemistry may lead to a different lowest energy solution state conformation. For the purposes of this work it was envisaged that the stereochemistry would not severely affect the validity of any comparisons drawn, whilst noting that it may contribute to poor structural similarity of the peptides in the MeLan bridge region.

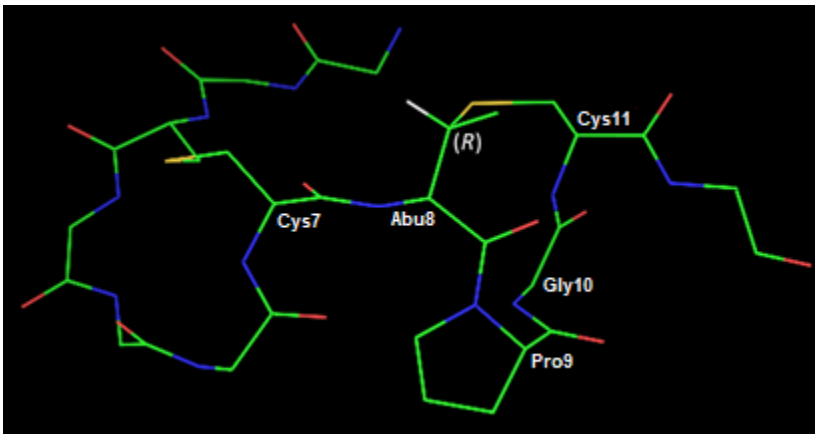


Figure 5.14: Snapshot of the AB ring fragment from the published structure of full length nisin bound to lipid II (PDB ID: 1WCO), showing the (2*S*, 3*R*, 6*R*) MeLan stereochemistry. Lipid II is omitted from the diagram for clarity, and key residues are labelled at the αC.

On overlaying the structures, it was immediately obvious that **95** and 1WCO were dissimilar (**Figure 5.15**). The backbone alignment within each individual ring was reasonable despite the stereochemical discrepancy, though the orientation of the A and B rings with respect to each other was very different, suggesting that they are not pre-organised in the correct conformation for lipid II binding. In a similar fashion to the orientation described in other published NMR studies of full length nisin, in the published structure the thioether bridges are aligned and adjacent to each other; whereas in the isolated double ring system (**95**), there is a twist in the central amide bond between the rings, which results in the A ring Lan pointing towards the B ring.[207,213,214] Interestingly, this rotation of the A and B rings around the central amide bond was also observed in structural studies of mutacin 1140.[203] It was hypothesised that this difference could be caused by one of two possibilities: i) the rest of the peptide could potentially play a vital role in templating the orientation of the two rings, causing a large change in conformation when the AB rings are isolated, ii) as the NMR spectra were run directly following HPLC and lyophilisation, the different conformation could perhaps be caused by the AB rings in truncated WT nisin (**95**) binding tightly around, for example, a trifluoroacetate counterion.

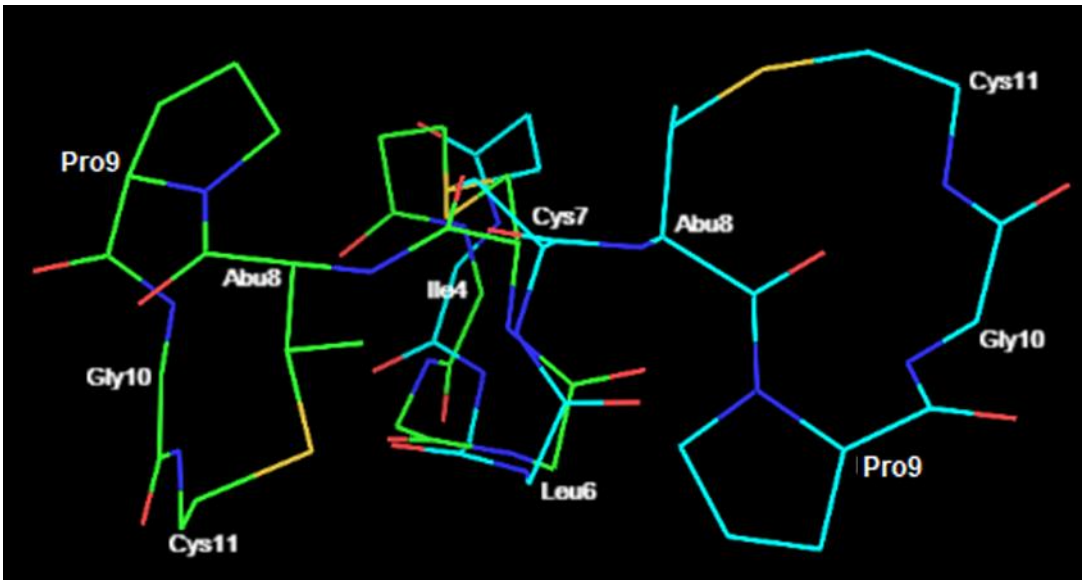


Figure 5.15: Comparison of isolated WT nisin rings AB (**95**) (green) with the ring AB fragment from the published structure of full length nisin bound to lipid II (PDB ID: 1WCO) (cyan). Peptides are aligned to the A ring backbone, and key residues are labelled at the αC.

5.4. Summary

In summary, eight lantibiotic peptides have been fully characterised by NMR and their solution state structures calculated in XPLOR-NIH. This allowed comparisons to be drawn between the single and double ring peptides, as well as to previously published NMR structures.

Structure calculation of the B ring peptides revealed that mutacin I ring B (**96**) exists as two interconverting conformers in solution, both of which could be assigned. Both nisin ring B peptides, WT (**97**) and Lan analogue (**98**), contain a cis (Me)Lan-Pro bond and adopt similar solution state conformations. This suggested that MeLan could be effectively swapped for Lan in lantibiotic analogues synthesised in future work, though biological testing of peptides bearing this substitution would be necessary to confirm this.

Comparison of the mutacin I A ring analogues **101** and **102** to the WT ring A peptide (**99**) indicated that removal of the two *N*-terminal residues causes a change in the shape of the ring, but that substitution of Dha for either Ser or Ala does not have a large impact on solution conformation. The WT mutacin I and WT nisin A rings, **99** and **100**, also have similar solution state conformations, indicating that mutacin I may bind to lipid II in a similar fashion to nisin. Additionally, an overlay of both WT peptides with the A ring

fragment from the published structure of nisin bound to lipid II indicated that they exhibit a degree of pre-organisation for target binding.

Conversely, an overlay of WT nisin rings AB (**95**) with the AB ring fragment from the published structure of nisin bound to lipid II showed that the two adopt very different conformations in solution. Further experiments will be required to distinguish if this is due to a templating effect by the rest of the peptide, or if the different structure of the isolated rings (**95**) was due to coordination of a counterion. An initial investigation could entail running the NMR in a range of different solvents to observe whether the same peptide orientation is observed, or re-running the d_6-DMSO NMR after ion exchange chromatography to remove any possible counterions.

6. CONCLUSIONS & FUTURE WORK

In conclusion, this thesis has discussed the synthesis and structural analysis of the lipid II binding region of nisin and mutacin I, with the intent of guiding future analogue design.

The first aim was to synthesise each of the unusual amino acids required for the SPPS of lantibiotic ring fragments. One orthogonally protected lanthionine and one orthogonally protected methyllanthionine were synthesised using modified literature protocols. The (Teoc,TMSE/Fmoc) Lan (**38**) previously synthesised within the Tabor group was first synthesised according to the literature procedure from (Fmoc-Cys-O*t*Bu)$_2$ (**105**) and Boc-D-Ser(OBn)-OH (**110**), and then using a modified procedure with cheaper starting materials.[162] Using these methods, the desired Lan could be synthesised on the gram scale in 13% overall yield from (Fmoc-Cys-O*t*Bu)$_2$ (**105**). The (Alloc, Allyl/Fmoc) MeLan (**70**) was also synthesised according to the method of Vederas, and could be isolated in sufficient quantities for SPPS, in 2% overall yield from D-Thr.[165] The yield of the thioether forming step in particular was poor, and a more in-depth investigation into the reaction conditions would be necessary in order to increase the scale of the synthesis in the future.

Several precursors for the incorporation of dehydro residues were also synthesised. These included Fmoc-Sec(Ph)-OH (**123**) for Dha introduction, Boc-Ile-Dhb-OH (**135**) and Boc-Ile-Thr-OH (**133**) dipeptides for Dhb introduction, and a dibromopentanoate reagent (**80**) to generate Dha by alkylation and β-elimination of cysteine residues. For use in conjunction with this latter reagent, a novel Fmoc-β-Me-Cys(Trt)-OH (**136**) was also designed to enable more efficient Dhb introduction. Although sufficient Fmoc-β-Me-Cys(Trt)-OH (**136**) could be synthesised for use in these studies using standard literature protocols, yields throughout were low, leading to an overall yield of only 1% over seven steps. Whilst an optimisation of the reaction conditions would be required to enable the synthesis of this residue on an increased scale, this regent provided access to a faster and cleaner method for Dhb generation.

The second aim was to use the synthesised amino acids and precursors in the SPPS of fragments of the lipid II binding regions of nisin and mutacin I, including both WT peptides and analogues. A total of seven novel single ring peptides were synthesised, four WT sequences and three analogues. Three nisin double ring fragments were also obtained:

the WT peptide was produced by digestion of the full length peptide with trypsin, and two analogues were synthesised by SPPS. Unfortunately, all attempts toward the synthesis of WT or analogues of mutacin I rings AB were unsuccessful, and development of the SPPS technique used in this work would be required to enable the production of this peptide.

An investigation into the incorporation of dehydro amino acids was conducted during the syntheses of the WT ring A peptides. Generation of Dha by dehydration of Ser proved ineffective in this case, as did incorporation of Dhb using either Boc-Ile-Dhb-OH (**135**) or dehydration from Boc-Ile-Thr-OH (**133**). Fmoc-Sec(Ph)-OH (**123**) could be incorporated into the peptides and eliminated to give the Dha, though the oxidative elimination conditions were difficult to control, and resulted in the undesired oxidation of the lanthionine thioether. The final method to be tested was an adaptation of the Morrison *et al.* dibromopentanoate alkylation-elimination methodology using cysteine and β-methyl cysteine.[198] This proved to be remarkably successful, allowing for the first time the simultaneous incorporation of multiple Dha and Dhb residues into a lantibiotic peptide in a single step, and is now the method of choice for the introduction of dehydro residues in this work.

The third aim of this work was to analyse the solution state structure of each of the synthesised lantibiotic peptides by NMR. Using distance restraints derived from NOE intensities and dihedral angle restraints derived from ^{3}J values, the structures were calculated in XPLOR-NIH. The purpose of this was to gauge whether the A and B rings exhibited a degree of pre-organisation for lipid II binding, and to ascertain if structural simplifications would have an impact on solution conformation. From the work conducted in this thesis, it can be concluded that the swap of MeLan for Lan, or Ser and Ala for Dha, do not significantly affect the structure of the single lantibiotic rings. This suggests that these are valid substitutions which could be considered in the design of further analogues of nisin and mutacin rings AB. Additionally, comparison of the structures of the isolated ring A WT peptides **99** and **100** to that of full length nisin in complex with lipid II (PDB ID 1WCO) indicated that these portions of the peptides were pre-organised for target binding, and when free in solution they adopt the same conformation as when bound to lipid II. Conversely, the isolated WT nisin ring AB fragment (**95**) adopts a very different solution conformation to the full length peptide in

complex with lipid II, possibly due to counterion binding or a templating effect by the rest of the peptide. Further NMR experiments with this analogue would be necessary to determine which is the case.

The first avenue for future work would be to synthesise and purify enough of the nisin ring AB analogues **39** and **40** to allow a detailed NMR study and structure determination to be conducted, enabling further examination of the effect of Dha on solution conformation by comparison of these structures to that of WT nisin rings AB (**95**). Promega analysis of the Pro ^{13}C NMR shifts would also reveal whether the Lan-Pro amide bond is cis when part of a synthesised double ring system, or if this geometry was the conformational preference of the isolated ring B peptides. Another NMR experiment which could be conducted is a binding study of WT nisin rings AB (**95**) to an isolated pyrophosphate, to investigate whether the solution conformation would be the same as that of the full length peptide bound to lipid II. If so, this would indicate that though the individual rings display a degree of pre-organisation, the AB rings do not and will only adopt the binding conformation when in the presence of the biological target.

Another potential avenue for future work would be to measure the binding affinity of the double ring analogues **39** and **40** for lipid II, either using isothermal titration calorimetry (ITC)[271] or carboxyfluorescein leakage experiments in large unilamellar vesicles (LUVs) spiked with lipid II.[85,271] If the affinity of the peptides for lipid II was similar to that of WT nisin rings AB (**95**), this would confirm that the MeLan to Lan substitution was well tolerated. Additionally, as rings A and B of nisin or mutacin I have never previously been tested for activity individually, it will be interesting to evaluate the binding affinity of these fragments by ITC. The results of this would also help to determine which fragments of the AB region are important for maintaining high affinity lipid II binding. Furthermore, NMR binding studies with lipid II could be conducted with both the single and double ring peptides, which would confirm whether the same binding mode is observed with WT nisin and the shorter peptides.

As the synthesis of WT mutacin I rings AB (**103**) was not possible by any of the methods reported in this thesis, one avenue for future work would be to develop a route to access this peptide. Several potential methods for this could be explored. For example, the synthesis could be conducted on a different resin, such as 2-chlorotrityl, to examine whether this would increase solubility of the peptide and prevent truncation due to

precipitation. Alternatively, synthesis *via* the coupling of separate A and B rings could be further explored, either by cleavage of *C*-terminal residues from synthesised ring A peptides with other carboxypeptidases, or by synthesis of the ring A peptide with the Lan residue attached directly to the resin. Again, switching to a different resin for the synthesis may increase success in this area. Following the synthesis of **103**, it would be interesting to conduct binding affinity experiments with this peptide, to establish whether it binds lipid II with a similar affinity to WT nisin rings AB (**95**). An NMR binding study of WT mutacin I rings AB (**103**) with lipid II could then also be conducted to determine whether a similar hydrogen bonding network exists between the peptide backbone and the pyrophosphate in mutacin I as it does in nisin.

Further NMR experiments of all peptides could also be conducted using NMR analysis of molecular flexibility in solution (NAMFIS analysis).[297] In this technique, averaged NMR variables are deconvoluted by varying the molar fractions of a computational ensemble calculated by unrestrained Monte Carlo molecular mechanics, until the best possible fit of the experimental NMR data is obtained. The result of this is an ensemble of all conformations which are present in solution, and their probabilities, hence providing a more complete picture of the shape of the compound in solution than is possible by average structure calculation. For bioactive peptides, typically it is expected that the bound bioactive conformation will be populated to ~15% in solution, even in absence of the binding partner. By comparison of NAMFIS results to the published structure of WT nisin in complex with lipid II (PDB ID 1WCO), the likelihood of any synthesised ring AB analogues to efficiently bind lipid II could be evaluated.

Finally, further synthetic efforts could also be conducted towards creating a larger library of nisin and mutacin I AB analogues, in order to build a more complete picture of the effect of residue replacement on conformation and lipid II binding. Although dehydro replacement with both hydrated and saturated analogues has been explored in this work (peptides **39** and **40**), replacement of the (Me)Lan bridges with carba or oxa bridges was not examined. For example, if diaminopimelic acid was used in place of Lan, analogues which are more oxidatively stable than the parent lantibiotics could be produced. Any analogues which are determined to be good lipid II binders could then be incorporated into semisynthetic lantibiotics, bearing a nisin-like lipid II binding group coupled to a simple, linear pore-forming peptide. These hybrid peptides would then be assayed for

antimicrobial activity against clinically relevant Gram-positive bacterial starins, such as MRSA.

In conclusion, whilst there are still many questions to answer, the work presented in this thesis represents not only an important step forward in the total chemical synthesis of WT lantibiotic peptides, by providing a method for the incorporation of multiple dehydro residues, but has also contributed valuable insights into the structural nature of the lipid II binding region of these peptides. Taken together, these findings are expected to aid the future design and synthesis of biologically active semisynthetic peptides based on the structures of nisin and mutacin I.

7. EXPERIMENTAL

7.1. Synthesis of Diaminodiacids, Dehydro Precursors and Dipeptides

7.1.1. General Experimental

All chemicals were used as ordered unless otherwise stated, with reagents being purchased from Sigma-Aldrich Co. Ltd., Acros Organics or Alfa Aesar. $(Fmoc\text{-}Cys\text{-}O^tBu)_2$ was produced from a custom synthesis carried out by Oxygen Healthcare, Cambridge, UK. Boc-D-Ser(Bn)-OH was purchased from Santa Cruz Biotechnology. $Pd(PPh_3)_4$ was used as purchased from Sigma-Aldrich Co. Ltd.. Caesium carbonate was dried *in vacuo* before use. *p*TsCl was recrystallised from warm hexane before use. Trypsin (from bovine pancreas) was obtained from Sigma-Aldrich Co. Ltd., carboxypeptidase Y (from yeast) from Alfa Aesar, endoproteinase AspN from New England BioLabs Inc. and crude nisin from MP Biomedicals. Anhydrous THF, CH_2Cl_2 and DMF (dried over molecular sieves) were used as purchased from Acros Organics. Pet. ether refers to petroleum ether 40-60 °C fractions. Ether refers to diethyl ether. All water used was distilled with an Elga Purelab Option R7 purifier. Solvent used for HPLC was all HPLC-grade and used directly from the bottle.

Reactions requiring anhydrous conditions were carried out under an argon atmosphere, using oven-dried glassware. TLC analysis was on aluminium backed Sigma-Aldrich TLC plates with F_{254} fluorescent indicator, with UV visualisation at 254 nm or visualisation by staining with $KMnO_4$, ninhydrin or Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid)). Flash column chromatography was carried out using Merck silica gel 60 (40-60 μm).

LCMS spectra were recorded on a Waters Acquity UPLC SQD using a linear gradient of 5 - 95% B over 5 min (A = water, B = acetonitrile, 0.1% formic acid) with a C8 column, flow rate 0.6 mL min^{-1}. Analysis of the chromatograms was conducted using MassLynx software. HRMS spectra were recorded on a Waters LCT Premier XE instrument with data analysed using MassLynx software. HRMS spectra of **39** and **40** were run by King's College Mass Spectrometry Service, Britannia House, on a Waters Xevo G2-XS QTof instrument with ESI source, attached to a Waters Acquity UPLC system (I class) with an Acquity UPLC BEH C18, 1.7 μm column. Optical rotations were measured at 25 °C unless otherwise stated, on a Perkin-Elmer Model 343 Polarimeter. Specific rotations are

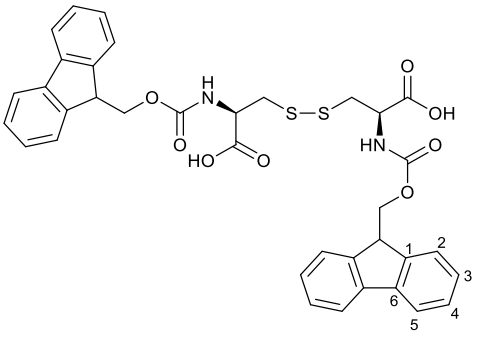
given in 10^{-1} deg cm^2 g^{-1}. Melting points are non-corrected and were recorded using Stuart SMP11 Analogue melting point apparatus. Infrared spectra were recorded on a Perkin Elmer 100 FT-IR spectrometer.

^1H, ^{13}C and all 2D NMR spectra were recorded on either a Bruker Avance-300, -500 or -600 spectrometer, with chemical shifts (δ) given in ppm relative to the solvent signal, and coupling constants (*J*) given in Hz. Carbon signals were assigned from HSQC and HMBC crosspeaks. Where they could be distinguished, terminal protons of allyl groups ($CH_2CH=C\underline{H_2}$) are labelled cis and trans with respect to the central CH ($CH_2C\underline{H}=CH_2$). Where the resonances for symmetric carbon atoms in the Fmoc group could be distinguished, the shift of both is given. Deuterated solvents used were purchased from Sigma-Aldrich Co. Ltd.. Abbreviations used in ^1H NMR assignment are as follows: Ar = aromatic, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br s = broad singlet, br d = broad doublet, dd = doublet of doublets, dt = doublet of triplets, dq = doublet of quartets, ddt = doublet of doublet of triplets, td = triplet of doublets, tt = triplet of triplets, ttd = triplet of triplet of doublets, qd = quartet of doublets. Data processing was carried out using ACD/NMR Processor Academic Edition, version 12.01 (Advanced Chemistry Development Inc.).

7.1.2. Lanthionine Synthesis

(Fmoc-Cys-OH)$_2$ (106)[224]

(2*R*,2'*R*)-3,3'-Disulfanediylbis(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino) propanoic acid)



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Method 1:

A 6:1 solution of TFA:CH_2Cl_2 (28 mL) was added to (Fmoc-Cys-O*t*Bu)$_2$ (**105**) (10.0 g, 12.6 mmol) under N_2 at rt. After 4 h, the solvent was removed *in vacuo* to give title compound **106** as a white solid (8.50 g, 12.4 mmol, 99%).

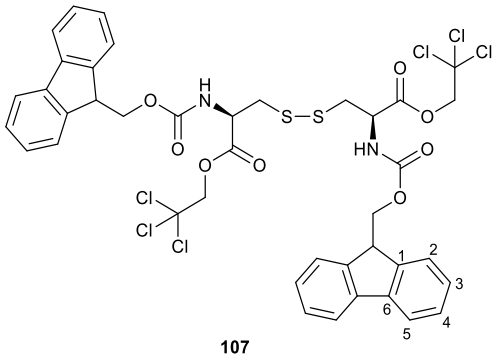
Method 2:

To a stirred solution of L-cystine (**108**) (5.00 g, 20.8 mmol) in H_2O:THF (1:1, 200 mL), NaOH (3.33 g, 83.2 mmol) was added. This solution was then cooled to 0 °C before adding Fmoc chloride (11.3 g, 43.7 mmol) portionwise. The reaction was then warmed to rt for 12 h before removing the THF *in vacuo*. The remaining solution was then acidified with HCl (6 M, 150 mL), filtered, and the solid dried *in vacuo*. The residue was then redissolved in EtOAc, and cooled to 5 °C for 8 h before collection of the precipitated product by filtration. This dissolving and precipitation was repeated once more to give title compound **106** as a white solid (7.11 g, 10.4 mmol, 50%).

R_f 0.45 (CH_2Cl_2:MeOH, 15:1); mp 119-122 °C; $[\alpha]_D^{25}$ -58.3 (*c* 9.3 mg mL^{-1}, MeOH), [lit.[224] $[\alpha]_D^{25}$ -79.6 (*c* 8.9 mg mL^{-1}, MeOH)]; δ_H (300 MHz, $CDCl_3$) 3.02 (2H, dd, *J* 13.8, 9.3, SC*H*$_2$CH), 3.28 (2H, m, SC*H*$_2$CH), 4.17-4.22 (2H, m, 2 x ArC*H*CH$_2$), 4.29-4.32 (4H, m, 2 x ArCHC*H*$_2$), 4.54 (2H, m, 2 x SCH$_2$C*H*), 7.27 (4H, t, *J* 6.9, Ar*H*(3)), 7.36 (4H, t, *J* 7.5, Ar*H*(4)), 7.65 (4H, d, *J* 7.2, Ar*H*(2)), 7.76 (4H, d, *J* 7.3, Ar*H*(5)); δ_C (150 MHz, $CDCl_3$) 41.2 (S*C*H$_2$CH), 48.5 (Ar*C*HCH$_2$), 54.8 (SCH$_2$*C*H), 68.4 (ArCH*C*H$_2$), 121.0 (*C*5), 126.5 (*C*2), 128.3 (*C*3), 128.9 (*C*4), 142.7 (*C*6), 145.4 (*C*1), 158.7 (*C*ONH), 174.1 (*C*O$_2$H); *m/z* (LCMS, ES-) 683.5 [M-H]$^-$, (LCMS, ES+) 685.3 [M+H]$^+$, 707.3 [M+Na]$^+$. Data is in agreement with the literature.[298]

(Fmoc-Cys-OTce)$_2$ (107)[162]

Bis(2,2,2-trichloroethyl)3,3'-disulfanediyl(2*R*,2'*R*)-bis(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)propanoate)



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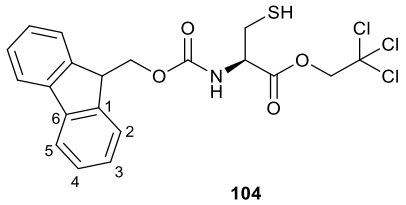
To a stirred solution of (Fmoc-Cys-OH)$_2$ (**106**) (8.98 g, 13.1 mmol) in benzene (250 mL), was added *p*-toluenesulfonic acid (5.99 g, 31.5 mmol) and 2,2,2-trichloroethanol (3.02 mL, 31.5 mmol). The solution was heated at reflux with a Dean and Stark trap for 48 h, before cooling to 0 °C and filtering to remove excess acid. The solvent was then removed *in vacuo* and the residue washed with EtOAc (150 mL) and dried *in vacuo* to give title compound **107** as a white solid (8.42 g, 8.89 mmol, 68%). R_f 0.35 (CH_2Cl_2); mp 189-201 °C; $[\alpha]_D^{25}$ -8.57 (*c* 1.4 mg mL^{-1}, $CHCl_3$), [lit.[162] $[\alpha]_D^{25}$ -10.7 (*c* 1.4 mg mL^{-1}, $CHCl_3$)]; δ_H (600 MHz, $CDCl_3$) 3.27 (4H, d, *J* 5.3, SC*H*$_2$CH), 4.22 (2H, t, *J* 6.3, ArC*H*CH$_2$), 4.39-4.46 (4H, m, ArCHC*H*$_2$), 4.75-4.87 (6H, m, SCH$_2$C*H* and C*H*$_2$CCl$_3$), 5.75 (2H, d, *J* 7.8, N*H*), 7.31 (4H, t, *J* 7.5, ArH(3)), 7.40 (4H, t, *J* 7.5, ArH(4)), 7.59 (4H, d, *J* 7.2, ArH(2)), 7.76 (4H, d, *J* 7.5, ArH(5)); δ_C (150 MHz, $CDCl_3$) 40.8 (S*C*H$_2$CH), 47.0 (Ar*C*HCH$_2$), 53.3 (SCH$_2$*C*H), 67.4 (ArCH*C*H$_2$), 74.8 (*C*H$_2$CCl$_3$), 94.1 (CH$_2$*C*Cl$_3$), 120.0 (*C*5), 125.0 (*C*2), 127.1 (*C*3), 127.8 (*C*4), 141.3 (*C*6), 143.5 (*C*1), 155.6 (*C*ONH), 168.9 (*C*O$_2$Tce); *m/z* (LCMS, ES+) 945.4 [M(6x^{35}Cl)+H]$^+$, 947.4 [M(5x^{35}Cl)+H]$^+$, 969.3 [M(5x^{35}Cl)+Na]$^+$. Data is in agreement with the literature.[162]

Fmoc-Cys-OTce (104)[162]

2,2,2-Trichloroethyl (((9H-fluoren-9-yl)methoxy)carbonyl)-L-cysteinate



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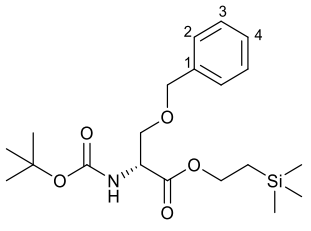
To a stirred solution of (Fmoc-Cys-OTce)$_2$ (**107**) (2.50 g, 2.64 mmol) in CH_2Cl_2 (50 mL) was added DTT (489 mg, 3.17 mmol) and Et_3N (0.44 mL, 3.17 mmol). After 1 h the reaction mixture was washed with saturated sodium bicarbonate (3 x 50 mL), brine (1 x 50 mL) and H_2O (1 x 50 mL). The organic layer was then dried ($MgSO_4$) and the solvent removed *in vacuo* to give title compound **104** as an off-white solid (2.42 g, 5.10 mmol, 96%). R_f 0.52 (CH_2Cl_2); mp 67-70 °C; $[\alpha]_D^{25}$ -2.5 (*c* 17.5 mg mL^{-1}, $CHCl_3$), [lit.[162] $[\alpha]_D^{25}$ -2.4 (*c* 17.3 mg mL^{-1}, $CHCl_3$)]; δ_H (600 MHz, $CDCl_3$) 1.47 (1H, t, *J* 9.0, $CHCH_2SH$), 3.02-3.07 (1H, m, $CHCH_2SH$), 3.14-3.18 (1H, m, $CHCH_2SH$), 4.25 (1H, t, *J* 6.8, $ArCHCH_2$), 4.46 (2H, d, *J* 6.8, $ArCHCH_2$), 4.74 (1H, d, *J* 11.7, $CH_AH_BCCl_3$), 4.83-4.86 (1H, m, $CHCH_2SH$), 4.94 (1H, d, *J* 12.0, $CH_AH_BCCl_3$), 5.70 (1H, d, *J* 8.3, NH), 7.34 (2H, tdd, *J* 7.4, 2.4, 0.9, ArH(3)), 7.43 (2H, t, *J* 7.5, ArH(4)), 7.62 (2H, d, *J* 7.5, ArH(2)), 7.79 (2H, d, *J* 7.5, ArH(5)); δ_C (150 MHz, $CDCl_3$) 26.9 ($CHCH_2SH$), 47.1 ($ArCHCH_2$), 55.1 ($CHCH_2SH$), 67.2 ($ArCHCH_2$), 74.6 (CH_2CCl_3), 94.2 (CH_2CCl_3), 120.0 (C5), 125.0 (C2), 127.1 (C3), 127.8 (C4), 141.3 (C6), 143.5 (C1), 143.8 (C1), 155.6 (CONH), 168.7 (CO_2Tce); *m/z* (LCMS, ES+) 474.3 $[M(3x^{35}Cl)+H]^+$, 476.2 $[M(2x^{35}Cl)+H]^+$, 496.2 $[M(3x^{35}Cl)+Na]^+$, 498.3 $[M(2x^{35}Cl)+Na]^+$, 296.1 $[M+H-Fmoc]^+$. Data is in agreement with the literature.[162]

Boc-D-Ser(Bn)-OTMSE (111)[162]

2-(Trimethylsilyl)ethyl *O*-benzyl-*N*-(*tert*-butoxycarbonyl)-D-serinate

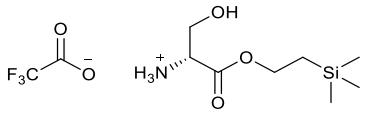


111

A solution of Boc-D-Ser(Bn)-OH (**110**) (10.0 g, 33.9 mmol) and 4-(dimethylamino)pyridine (621 mg, 5.08 mmol) in anhydrous THF (170 mL) was cooled to 0 °C before adding 2-(trimethylsilyl)ethanol (9.7 mL, 67.7 mmol) dropwise. To this, DCC (9.08 g, 44.1 mmol) was added and the solution was warmed to rt. After stirring for 2.5 d, the solution was filtered and the residue washed with EtOAc before removal of the solvent *in vacuo*. The residue was redissolved in EtOAc (200 mL) and washed with aqueous $NaHCO_3$ (2 x 70 mL) and brine (70 mL), then dried ($MgSO_4$) and the solvent removed *in vacuo*. Purification by flash column chromatography (pet. ether → 20:1, pet. ether:EtOAc) gave title compound **111** as a clear viscous oil (10.4 g, 26.3 mmol, 78%). R_f 0.79 (pet. ether:EtOAc, 5:1); $[\alpha]_D^{25}$ -4.43 (*c* 11.8 mg mL^{-1}, $CHCl_3$), [lit.[162] $[\alpha]_D^{25}$ -7.6 (*c* 10.0 mg mL^{-1}, $CHCl_3$)]; δ_H (500 MHz, $CDCl_3$) 0.05 (9H, s, $Si(CH_3)_3$), 0.96-1.00 (2H, m, $CH_2CH_2Si(CH_3)_3$), 1.46 (9H, s, $OC(CH_3)_3$), 3.69 (1H, dd, *J* 9.5, 3.0, $ArCH_2OCH_AH_BCH$), 3.88 (1H, dd, *J* 9.5, 3.0, $ArCH_2OCH_AH_BCH$), 4.22-4.26 (2H, m, $CH_2CH_2Si(CH_3)_3$), 4.40-4.42 (1H, m, $ArCH_2OCH_2CH$), 4.49 (1H, d, *J* 12.0, $ArCH_AH_BOCH_2CH$), 4.56 (1H, d, *J* 12.0, $ArCH_AH_BOCH_2CH$), 5.40 (1H, d, *J* 8.5, NH), 7.27-7.35 (5H, m, Ar*H*); δ_C (100 MHz, $CDCl_3$) -1.6 ($Si(CH_3)_3$), 17.3 ($CH_2CH_2Si(CH_3)_3$), 28.3 ($OC(CH_3)_3$), 54.1 ($ArCH_2OCH_2CH$), 63.8 ($CH_2CH_2Si(CH_3)_3$), 70.0 ($ArCH_2OCH_2CH$), 73.2 ($ArCH_2OCH_2CH$), 79.8 ($OC(CH_3)_3$), 127.6 (*C*2), 127.7 (*C*4), 128.3(*C*3), 137.6 (*C*1), 155.4 (CO_2NH), 170.7 (CO_2TMSE); *m/z* (LCMS, ES+) 396.4 $[M+H]^+$, 418.4 $[M+Na]^+$. Data is in agreement with the literature.[162]

D-Ser-OTMSE (112)[162]

2-(Trimethylsilyl)ethyl D-serinate



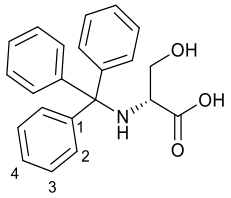
112

A stirred solution of Boc-D-Ser(Bn)-OTMSE (**111**) (10.0 g, 25.3 mmol) and Pd/C (10wt %, 1.35 g, 1.26 mmol) in ethanol (250 mL) was degassed before pump-filling with H_2. The solution was stirred at rt for 24 h before filtering through Celite® and removal of the solvent *in vacuo*. The residue was redissolved in a 5:2 solution of CH_2Cl_2:TFA (30 mL) and stirred for 18 h before removal of the solvent *in vacuo*. This yielded the TFA salt of title compound **112** as a white solid which was used without further purification

(7.36 g, 23.0 mmol, 91%). R_f 0.49 (CH_2Cl_2:MeOH, 9:1); $[\alpha]_D^{25}$ -40.4 (*c* 12.0 mg mL^{-1}, MeOH), [lit.[162] $[\alpha]_D^{25}$ -41.0 (*c* 7.3 mg mL^{-1}, MeOH)]; δ_H (500 MHz, CD_3OD) 0.08 (9H, s, $Si(CH_3)_3$), 1.06-1.12 (2H, m, $CH_2CH_2Si(CH_3)_3$), 3.90-4.09 (3H, m, $CHCH_2OH$), 4.32-4.39 (2H, m, $CH_2CH_2Si(CH_3)_3$); δ_C (100 MHz, CD_3OD) -1.47 ($Si(CH_3)_3$), 18.4 ($CH_2CH_2Si(CH_3)_3$), 56.3 ($CHCH_2OH$), 60.8 ($CH_2CH_2Si(CH_3)_3$), 66.2 ($CHCH_2OH$), 169.2 (CO_2TMSE); *m/z* (LCMS, ES+) 206.2 $[M+H]^+$. Data is in agreement with the literature.[162]

Trt-D-Ser-OH (115)[224]

Trityl-D-serine



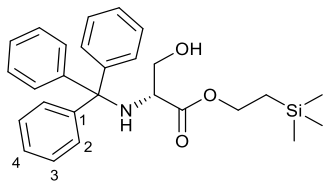
115

D-serine (10.0 g, 95.2 mmol) was stirred in CH_2Cl_2 (140 mL) before adding TMSCl (37.4 mL, 300 mmol) and heating at reflux for 20 min. The solution was then cooled to rt and Et_3N (41 mL, 300 mmol) and CH_2Cl_2 (140 mL) were added and the solution heated at reflux for 45 min. After cooling to 0 °C, dry MeOH (3.8 mL, 95.2 mmol) was added dropwise. The reaction was then warmed to room temperature and Et_3N (13.28 mL, 300 mmol) and trityl chloride (26.5 g, 300 mmol) were added before stirring for 12 h. Excess Et_3N (66 mL, 480 mmol) and MeOH (400 mL) were then added in order to dissolve the solids. The solvent was then removed *in vacuo* before redissolving the residue in EtOAc (600 mL) and washing with a cold aqueous citric acid solution (5% w/v, 600 mL). The organic layer was collected and the solvent removed *in vacuo*. Purification by flash column chromatography (CH_2Cl_2 → CH_2Cl_2:MeOH, 9:1) gave title compound **115** as a fluffy white solid (14.4 g, 41.4 mmol, 44%). R_f 0.53 (CH_2Cl_2:MeOH, 9:1); mp 147-150 °C (lit.[299] 154-157 °C); $[\alpha]_D^{25}$ -10.9 (*c* 3.4 mg mL^{-1}, EtOH); δ_H (600 MHz, CD_3OD) 3.03-3.06 (1H, m, $CHCH_AH_BOH$), 3.26-3.31 (2H, m, $CHCH_2OH$ and $CHCH_AH_BOH$), 3.55 (1H, br s, $CHCH_2OH$), 7.20 (3H, t, *J* 7.2, Ar*H*(4)), 7.27 (6H, t, *J* 7.8, Ar*H*(3)), 7.50 (6H, d, *J* 7.2, Ar*H*(2)); δ_C (150 MHz, CD_3OD) 60.2 ($CHCH_2OH$), 65.4 ($CHCH_2OH$), 72.8 (CPh_3), 127.8 (*C*4), 129.0 (*C*3), 130.3 (*C*2), 147.7 (*C*1), 180.3 (CO_2H);

m/z (LCMS, ES-) 346.4 $[M-H]^-$, 693.7 $[2M-H]^-$. Data is in agreement with the literature.[224]

Trt-D-Ser-OTMSE (113)[162]

2-(Trimethylsilyl)ethyl trityl-D-serinate



113

Method 1:

To a stirred solution of D-Ser-OTMSE (**112**) (8.12 g, 39.5 mmol) in DMF (300 mL) at 0 °C was added Et_3N (11.0 mL, 79.1 mmol) followed by trityl chloride (9.92 g, 35.6 mmol). The solution was warmed to rt and stirred for 24 h before removal of the solvent *in vacuo*. The residue was redissolved in CH_2Cl_2 (200 mL) and washed with water (300 mL). The aqueous layer was extracted with CH_2Cl_2 (2 x 200 mL), and the organic layers were combined, dried ($MgSO_4$) and the solvent removed *in vacuo*. Purification by flash column chromatography (CH_2Cl_2) gave title compound **113** as white crystals (4.00 g, 8.94 mmol, 23%).

Method 2:

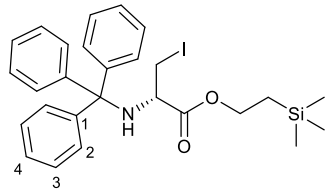
A solution of Trt-D-Ser-OH (**115**) (5.40 g, 15.5 mmol) and 2-(trimethylsilyl)ethanol (16 mL, 109 mmol) in CH_2Cl_2 (5 mL) under Ar was cooled to 0 °C before the addition of DMAP (7.59 g, 62.2 mmol) followed by EDCI (2.89 g, 18.7 mmol). The reaction was allowed to warm to rt and stirred for 36 h before the addition of CH_2Cl_2 (100 mL). The solution was washed with a cold aqueous citric acid solution (5% w/v, 150 mL) and brine (150 mL), dried ($MgSO_4$) and the solvent removed *in vacuo*. Purification by flash column chromatography (pet. ether:EtOAc, 19:1 → 9:1) gave title compound **113** as a colourless oil (2.97 g, 7.50 mmol, 48%).

R_f 0.39 (CH_2Cl_2); $[\alpha]_D^{25}$ -2.1 (*c* 9.7 mg mL^{-1}, $CHCl_3$), [lit.[162] $[\alpha]_D^{25}$ -0.6 (*c* 8.6 mg mL^{-1}, $CHCl_3$)]; δ_H (500 MHz, $CDCl_3$) -0.01 (9H, s, $Si(CH_3)_3$), 0.72-0.83 (2H, m, $CH_2CH_2Si(CH_3)_3$), 3.48-3.54 (2H, m, $CHCH_2OH$), 3.66-3.71 (2H, m, $CH_2CH_2Si(CH_3)_3$),

3.76-3.82 (1H, m, C*H*CH_2OH), 7.19 (3H, tt, *J* 7.3, 1.4, Ar*H*(4)), 7.25-7.29 (6H, m, Ar*H*(3)), 7.48-7.49 (6H, m, Ar*H*(2)); δ_C (100 MHz, $CDCl_3$) -1.7 (Si(*C*H_3)$_3$), 16.9 (CH_2*C*H_2Si$(CH_3)_3$), 57.8 (*C*HCH_2OH), 63.2 (*C*H_2CH_2Si$(CH_3)_3$), 64.8 (CH*C*H_2OH), 70.9 (NH*C*Ph_3), 126.4 (*C*4), 127.8 (*C*3), 128.6 (*C*2), 145.6 (*C*1), 173.6 (*C*O_2TMSE); *m/z* (LCMS, ES+) 448.4 $[M+H]^+$. Data is in agreement with the literature.[162]

Trt-β-Iodo-Ala-TMSE (109)[162]

2-(Trimethylsilyl)ethyl (*S*)-3-iodo-2-(tritylamino)propanoate

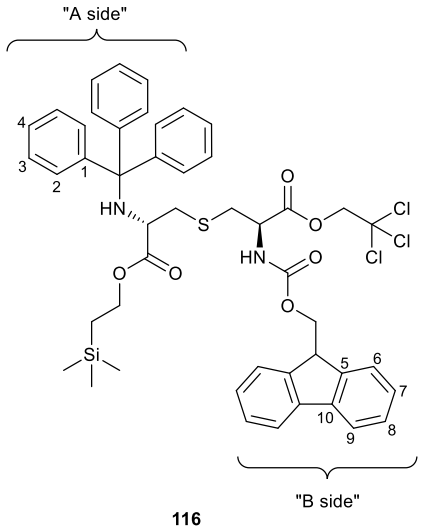


109

A minimum amount of dry CH_2Cl_2 (5 mL) was used to dissolve Trt-D-Ser-OTMSE (**113**) (1.00 g, 2.23 mmol) and PPh_3 (0.88 g, 3.36 mmol) at rt, before cooling the solution in an ice bath. To this, DEAD (0.53 mL, 3.36 mmol) was added dropwise and the solution stirred for 2 min before adding iodomethane (0.17 mL, 3.36 mmol) dropwise. The solution was then stirred for 6 h, ensuring that that temperature remained below 5 °C. Direct purification of the reaction mixture by flash column chromatography (pet. ether → pet. ether:EtOAc, 9:1) gave title compound **109** as a colourless oil (0.91 g, 1.63 mmol, 73%). R_f 0.91 (pet. ether:EtOAc, 9:1); $[\alpha]_D^{25}$ -7.4 (*c* 5.1 mg mL^{-1}, $CHCl_3$), [lit.[162] $[\alpha]_D^{25}$ -8.1 (*c* 5.2 mg mL^{-1}, $CHCl_3$)]; δ_H (600 MHz, $CDCl_3$) 0.01 (9H, s, Si(C*H*$_3$)$_3$), 0.76-0.86 (2H, m, CH_2C*H*$_2$Si$(CH_3)_3$), 2.91 (1H, d, *J* 9.6, N*H*), 3.20 (1H, dd, *J* 9.9, 7.2, CHC*H*$_2$I), 3.32 (1H, dd, *J* 9.6, 3.0, CHC*H*$_2$I), 3.43-3.46 (1H, m, C*H*CH_2I), 3.67-3.72 (1H, m, C*H*$_2$$CH_2Si(CH_3)_3$), 3.79-3.83 (1H, m, C*H*$_2$$CH_2Si(CH_3)_3$), 7.20 (3H, t, *J* 7.2, Ar*H*(4)), 7.28 (6H, t, *J* 7.2, Ar*H*(3)), 7.51 (6H, d, *J* 7.2, Ar*H*(2)); δ_C (125 MHz, $CDCl_3$); -1.6 (Si(*C*H_3)$_3$), 10.2 (CH*C*H_2I), 17.2 (CH_2*C*H_2Si$(CH_3)_3$), 56.2 (*C*HCH_2I), 63.6 (*C*H_2CH_2Si$(CH_3)_3$), 71.1 (NH*C*Ph_3), 126.6 (*C*4), 128.0 (*C*3), 128.7 (*C*2), 145.7 (*C*1), 172.4 (*C*O_2TMSE); *m/z* (LCMS, ES+) 558.3 $[M+H]^+$, 430.4 [M-I]. Data is in agreement with the literature.[162]

(Trt, TMSE/Fmoc, Tce) Lanthionine (116)[162]

(*R*)-2-(9H-Fluoren-9-ylmethoxycarbonylamino)-3-[(*S*)-2-(2-trimethylsilylethoxycarbonyl)-2-(triphenylmethylamino)-ethylsulfanyl-propionic acid 2,2,2-trichloroethyl ester



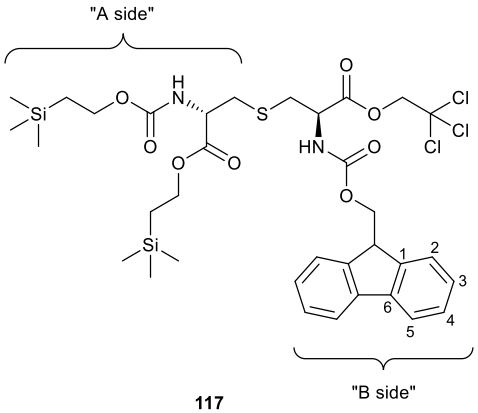
116

A stirred solution of Trt-1-iodo-L-Ala-OTMSE (**109**) (1.74 g, 3.12 mmol) and Fmoc-Cys-OTce (**104**) (1.48 g, 3.12 mmol) in anhydrous DMF (30 mL) was cooled to 0 °C before adding Cs_2CO_3 (1.02 g, 3.12 mmol). After 6 h, EtOAc (150 mL) was added and the solution washed with cold aqueous citric acid (5% w/v, 300 mL) and water (2 x 100 mL). The organic layer was then dried ($MgSO_4$) and the solvent removed *in vacuo*. Purification by flash column chromatography (pet. ether → pet. ether:EtOAc, 9:1) gave title compound **116** as a clear oil (1.22 g, 1.35 mmol, 43%). R_f 0.45 (pet. ether:EtOAc, 9:1); $[\alpha]_D^{25}$ -22.0 (*c* 10.7 mg mL^{-1}, $CHCl_3$), [lit.[162] $[\alpha]_D^{25}$ -32.9 (*c* 10.0 mg mL^{-1}, $CHCl_3$)]; δ_H (600 MHz, $CDCl_3$) 0.00 (9H, s, Si(C*H*$_3$)$_3$), 0.72-0.82 (2H, m, $CH_2CH_2Si(CH_3)_3$), 2.74 (1H, dd, *J* 13.2, 7.8, CHC*H*$_2$S, A side), 2.90-2.93 (1H, m, CHC*H*$_2$S, A side), 3.01 (1H, dd, *J* 13.8, 6.6, CHC*H*$_2$S, B side), 3.15 (1H, dd, *J* 13.8, 4.2, CHC*H*$_2$S, B side), 3.50-3.53 (1H, m, C*H*CH$_2$S, A side), 3.58-3.63 (1H, m, C*H*$_2$CH$_2$Si(CH$_3$)$_3$), 3.73-3.78 (1H, m, C*H*$_2$CH$_2$Si(CH$_3$)$_3$), 4.24 (1H, t, *J* 7.2, FmocC*H*CH$_2$), 4.38-4.43 (2H, m, FmocCHC*H*$_2$), 4.72 (1H, d, *J* 12.6, C*H*$_2$CCl$_3$), 4.75-4.78 (1H, m, C*H*CH$_2$S, B side), 4.85 (1H, d, *J* 11.4, C*H*$_2$CCl$_3$), 5.71 (1H, d, *J* 7.8, N*H*Fmoc), 7.18 (3H, t, *J* 7.5, Ar*H*(4)), 7.27 (6H, t, *J* 8.1, Ar*H*(3)), 7.32 (2H, t, *J* 7.2, Ar*H*(7)), 7.41 (2H, t, *J* 7.8, Ar*H*(8)), 7.50 (6H, d, *J* 6.6, Ar*H*(2)), 7.63 (2H, t, *J* 7.8, Ar*H*(6)), 7.78 (2H, d, *J* 7.8, Ar*H*(9)); δ_C (150 MHz, $CDCl_3$) -1.6 (Si(*C*H$_3$)$_3$), 17.0

($CH_2CH_2Si(CH_3)_3$), 34.5 ($CHCH_2S$, B side), 37.6 ($CHCH_2S$, A side), 47.0 (Fmoc$CHCH_2$), 53.6 ($CHCH_2S$, B side), 56.3 ($CHCH_2S$, A side), 63.4 ($CH_2CH_2Si(CH_3)_3$), 67.4 (FmocCHCH_2), 71.2 ($NHCPh_3$), 74.5 (CH_2CCl_3), 94.3 (CH_2CCl_3), 120.0 (*C*9), 125.1 (*C*6), 126.6 (*C*4), 127.1 (*C*7), 127.7 (*C*8), 127.9 (*C*2), 128.7 (*C*3), 141.3 (*C*10), 143.7 (*C*5), 145.6 (*C*1), 155.8 ($NHCO_2$Fmoc), 169.2 (CO_2Tce), 173.5 (CO_2TMSE); *m/z* (LCMS, ES+) 907.6 $[M(2x^{37}Cl)+H]^+$. Data is in agreement with the literature.[162]

(Teoc, TMSE/Fmoc, Tce) Lanthionine (117)[162]

(*R*)-2-(9H-Fluoren-9-ylmethoxycarbonylamino)-3-[(*S*)-2-(2-trimethylsilylethoxycarbonyl)-2-(trimethylsilanyl-ethoxycarbonylamino)-ethylsulfanyl-propionic acid 2,2,2-trichloroethyl ester



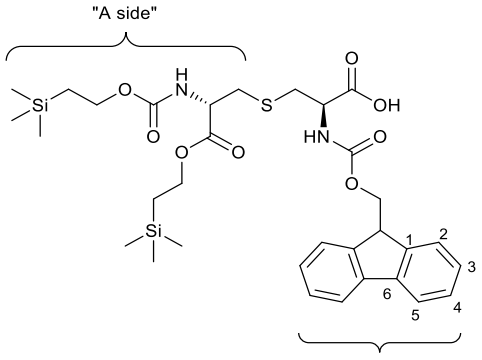
117

To a stirred solution of lanthionine **116** (1.22 g, 1.35 mmol) in a 10:1 mixture of CH_2Cl_2:TFA (28 mL:2.8 mL) was added TIPS (0.8 mL). After 1.5 h, $CHCl_3$ (100 mL) was added and the solution was washed with aqueous $NaHCO_3$ (2 x 200 mL) and brine (200 mL), dried ($MgSO_4$) and the solvent removed *in vacuo*. The residue was redissolved in 1,4-dioxane (25 mL), and 4-(dimethylamino)pyridine (202 mg, 1.62 mmol) and 1-[2-(trimethylsilyl)ethoxycarbonyloxy]pyrrolidone-2,5-dione (1.79 g, 6.75 mmol) were added. After 18 h, $CHCl_3$ (200 mL) was added and the solution was washed with brine (150 mL), dried ($MgSO_4$) and the solvent removed *in vacuo*. Purification by flash column chromatography (pet. ether:EtOAc, 9:1 → 17:3) gave title compound **117** as a clear oil (641 mg, 0.79 mmol, 59%). R_f 0.30 (pet. ether:EtOAc, 9:1); $[\alpha]_D^{25}$ -7.2 (*c* 5.0 mg mL^{-1}, $CHCl_3$), [lit.[162] $[\alpha]_D^{25}$ -8.8 (*c* 4.9 mg mL^{-1}, $CHCl_3$)]; δ_H (600 MHz, $CDCl_3$) 0.03 (18H, m, 2 x $Si(CH_3)_3$), 0.98-1.02 (4H, m, $CH_2CH_2Si(CH_3)_3$), 2.98 (1H, dd, *J* 13.8, 6.0, $CHCH_2S$,

B side), 3.06-3.15 (3H, m, CHC*H*$_2$S, B side and CHC*H*$_2$S, A side), 4.18 (1H, t, *J* 8.7, FmocC*H*CH$_2$) 4.21-4.28 (4H, m, 2 x *C*H$_2$CH$_2$Si(CH$_3$)$_3$), 4.38-4.45 (2H, m, FmocCHC*H*$_2$), 4.57 (1H, m, C*H*CH$_2$S, A side), 4.77-4.81 (2H, m, C*H*$_A$H$_B$CCl$_3$ and C*H*CH$_2$S, B side), 4.86 (1H, d, *J* 12.0, CH$_A$*H*$_B$CCl$_3$), 5.49 (1H, d, *J* 7.2, N*H*Fmoc), 5.95 (1H, d, *J* 7.2, N*H*Teoc), 7.33 (2H, t, *J* 7.2, Ar*H*(3)), 7.42 (2H, t, *J* 7.2, Ar*H*(4)), 7.64 (2H, t, *J* 8.4, ArH(2)), 7.78 (2H, d, *J* 7.8, Ar*H*(5)); δ_C (125 MHz, CDCl$_3$) -1.7 (Si(*C*H$_3$)$_3$), -1.6 (Si(*C*H$_3$)$_3$), 17.2 (CH$_2$*C*H$_2$Si(CH$_3$)$_3$), 17.5 (CH$_2$*C*H$_2$Si(CH$_3$)$_3$), 35.0 (CH*C*H$_2$S, B side), 35.8 (CH*C*H$_2$S, A side), 46.9 (Fmoc*C*HCH$_2$), 53.7 (*C*HCH$_2$S, B side), 60.2 (*C*HCH$_2$S, A side), 63.5 (*C*H$_2$*C*H$_2$Si(CH$_3$)$_3$), 64.3 (*C*H$_2$*C*H$_2$Si(CH$_3$)$_3$), 67.3 (FmocCH*C*H$_2$), 74.4 (*C*H$_2$CCl$_3$), 94.2 (CH$_2$*C*Cl$_3$), 119.8 (*C*5), 125.0 (*C*2), 126.9 (*C*3), 127.6 (*C*4), 141.1 (*C*6), 143.5 (*C*1), 155.7 (NH*C*O$_2$Fmoc), 156.1 (NH*C*O$_2$TMSE), 168.9 (*C*O$_2$TMSE), 170.4 (*C*O$_2$Tce); *m/z* (LCMS, ES+) 805.4 [M(3x^{35}Cl)+H]$^+$, 807.4 [M(2x^{35}Cl)+H]$^+$. Data is in agreement with the literature.[162]

(Teoc, TMSE/Fmoc) Lanthionine (38)[162]

(*R*)-2-(9H-Fluoren-9-ylmethoxycarbonylamino)-3-[(*S*)-2-(2-trimethylsilylethoxycarbonyl)-2-(trimethylsilanyl-ethoxycarbonylamino)-ethylsulfanyl-propionic acid



38

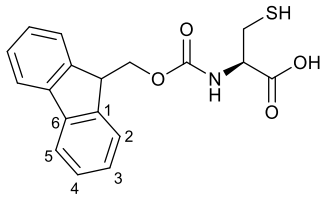
To a stirred solution of lanthionine **117** (640 mg, 0.794 mmol) in THF (130 mL) was slowly added zinc dust (519 mg, 7.94 mmol). An aqueous solution of NH$_4$OAc was added dropwise (1.0 M, 6.4 mL), and the solution stirred at rt for 8 h. The solution was filtered and the solvent removed *in vacuo*, before redissolving the residue in CHCl$_3$ (150 mL) and washing with brine (150 mL). The organic layer was dried (MgSO$_4$) and the solvent removed *in vacuo*. Purification by flash column chromatography (CH$_2$Cl$_2$ → CH$_2$Cl$_2$:MeOH, 9:1) yielded title compound **38** as a white solid (347 mg,

0.514 mmol, 65%). R_f 0.55 (CH_2Cl_2:MeOH, 19:1); mp 60-62 °C (lit.[162] 59 °C); $[\alpha]_D^{25}$ +2.8 (*c* 10.0 mg mL^{-1}, MeOH), [lit.[162] $[\alpha]_D^{25}$ +2.2 (*c* 10.0 mg mL^{-1}, MeOH)]; δ_H (600 MHz, CD_3OD) 0.012-0.015 (18H, m, 2 x Si(CH_3)$_3$), 0.94-0.99 (4H, m, $CH_2CH_2Si(CH_3)_3$), 2.91-2.95 (2H, m, $CHCH_AH_BS$, A side and $CHCH_AH_BS$, B side), 3.02 (1H, dd, *J* 13.2, 4.8, $CHCH_AH_BS$, B side), 3.11 (1H, dd, *J* 13.8, 4.2, $CHCH_AH_BS$, A side), 4.08-4.19 (4H, m, $CH_2CH_2Si(CH_3)_3$), 4.23-4.29 (3H, m, FmocC*H*CH_2, FmocCHCH_AH_B and C*H*CH_2S, A side), 4.36 (1H, dd, *J* 8.1, 5.1, FmocCHCH_AH_B), 4.44 (1H, m, C*H*CH_2S, B side), 7.31 (2H, t, *J* 7.8, Ar*H*(3)), 7.39 (2H, t, *J* 7.2, Ar*H*(4)), 7.69 (2H, d, *J* 7.2, Ar*H*(2)), 7.79 (2H, d, *J* 7.2, Ar*H*(5)); δ_C (150 MHz, CD_3OD) -1.36 (Si(CH_3)$_3$), -1.30 (Si(CH_3)$_3$), 18.3 ($CH_2CH_2Si(CH_3)_3$), 18.7 ($CH_2CH_2Si(CH_3)_3$), 35.4 ($CHCH_2S$, side B), 36.7 ($CHCH_2S$, A side), 48.5 (Fmoc*C*HCH_2), 55.9 (*C*HCH_2S, B side), 56.9 (*C*HCH_2S, A side), 64.5 ($CH_2CH_2Si(CH_3)_3$), 65.0 ($CH_2CH_2Si(CH_3)_3$), 68.3 (FmocCH*C*H_2), 121.1 (*C*5), 126.5 (*C*2), 126.6 (*C*2), 128.3 (*C*3), 128.4 (*C*3), 128.9 (*C*4), 142.7 (*C*6), 145.5 (*C*1), 158.5 (NH*C*O_2Fmoc), 158.9 (NH*C*O_2TMSE), 172.8 (*C*O_2TMSE), 177.6 (*C*O_2H); *m/z* (LCMS, ES+) 675.5 $[M+H]^+$, 697.5 $[M+Na]^+$, 575.4 $[M+H\text{-TMSE}]^+$. Data is in agreement with the literature.[162]

7.1.3. Methyllanthionine Synthesis

Fmoc-Cys-OH (72)[300]

(((9H-Fluoren-9-yl)methoxy)carbonyl)-L-cysteine



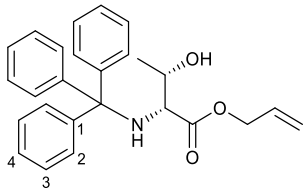
72

To a solution of (Fmoc-Cys-OH)$_2$ (**106**) (4.00 g, 5.84 mmol) in DMF (20 mL) under Ar, TCEP (2.19 g, 8.76 mmol) was added. The solution was stirred at rt for 2 h before adding CH_2Cl_2 (50 mL) and brine (50 mL), and washing with 0.1 M HCl (500 mL). The organic layer was then washed with a further portion of 0.1 M HCl (500 mL), dried ($MgSO_4$) and the solvent removed *in vacuo* to yield crude title compound **72** as a white solid that was used without further purification (2.80 g, 8.15 mmol, 70%). R_f 0.53 (CH_2Cl_2:MeOH, 9:1); δ_H (600 MHz, $CDCl_3$) 1.44 (1H, t, *J* 9.0, $CHCH_2SH$), 3.00-3.11 (2H, m, $CHCH_2SH$), 4.24

(1H, t, *J* 6.9, FmocC*H*CH_2), 4.41-4.47 (2H, m, FmocCHC*H*$_2$), 4.68-4.71 (1H, m, C*H*CH_2SH), 5.75 (1H, d, *J* 7.8, N*H*), 7.33 (2H, m, Ar*H*(3)), 7.41 (2H, t, *J* 7.2, Ar*H*(4)), 7.61 (2H, d, *J* 7.2, Ar*H*(2)), 7.77 (2H, d, *J* 7.8, Ar*H*(5)); δ_C (150 MHz, $CDCl_3$) 27.1 (CH*C*H_2SH), 47.3 (Ar*C*HCH_2), 55.1 (*C*HCH_2SH), 67.3 (ArCH*C*H_2), 120.2 (*C*5), 125.2 (*C*2), 127.2 (*C*3), 127.9 (*C*4), 141.5 (*C*6), 143.7 (*C*1), 143.9 (*C*1), 155.9 (*C*ONH), 172.9 (CO_2H). *m/z* (LCMS, ES+) 344.0 $[M+H]^+$, 366.2 $[M+Na]^+$, 687.1 $[2M+H]^+$, 709.2 $[2M+Na]^+$. Data is in agreement with the literature.[300]

Trt-D-Thr-OAllyl (D-119)[226]

Allyl trityl-D-threoninate



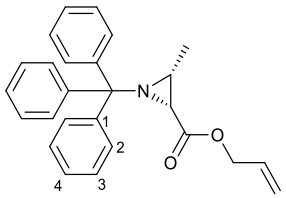
D-119

To a solution of D-threonine (5.00 g, 42.0 mmol) in toluene (100 mL) was added *p*-toluenesulfonic acid monohydrate (9.58 g, 42.0 mmol) and allyl alcohol (28.6 mL, 0.42 mol). The reaction was heated at reflux with a Dean & Stark trap for 24 h before removal of the solvent *in vacuo*. The residue was redissolved in EtOAc (100 mL) and cooled in ice. To this, Et_3N (21.1 mL, 0.15 mol) was added dropwise and the solution stirred for 10 minutes before the addition of trityl chloride (11.7 g, 42.0 mmol) in EtOAc (50 mL) *via* addition funnel over 45 min. The solution was allowed to warm to rt and was stirred for 16 h before washing with water (2 x 150 mL) and brine (100 mL). The organic layer was dried ($MgSO_4$) and the solvent removed *in vacuo*. Purification by flash column chromatography (pet. ether:EtOAc, 5:1 → 2:1) yielded title compound **119** as a pale yellow oil (11.3 g, 28.1 mmol, 67% over 2 steps); R_f 0.54 (pet. ether:EtOAc, 2:1); $[\alpha]_D^{25}$ -9.0 (*c* 23 mg mL^{-1}, $CHCl_3$), [lit.[226] $[\alpha]_D^{25}$ -9.02 (*c* 23 mg mL^{-1}, $CHCl_3$)]; δ_H (600 MHz, $CDCl_3$) 1.24 (3H, d, *J* 6.0, CHC*H*$_3$), 2.87 (1H, br s, N*H*), 3.42 (1H, d, *J* 7.2, C*H*CO_2Allyl), 3.60 (1H, br s, O*H*), 3.80-3.84 (1H, m, C*H*CH_3), 3.86 (1H, ddt, *J* 13.2, 6.0, 1.2, $CO_2CH_AH_BCH=CH_2$), 4.09 (1H, ddt, *J* 13.2, 6.0, 1.2, $CO_2CH_AH_BCH=CH_2$), 5.16-5.21 (2H, m, $CO_2CH_2CH=CH_2$), 5.65-5.71 (1H, m, $CO_2CH_2CH=CH_2$), 7.21 (3H, tt, *J* 7.2, 1.2, Ar*H*(4)), 7.28 (6H, t, *J* 7.2, Ar*H*(3)), 7.50 (6H, d, *J* 7.2, Ar*H*(2)); δ_C (150 MHz, $CDCl_3$) 18.9 (CH*C*H_3), 62.5 (*C*HCO_2Allyl), 65.6 ($CO_2CH_2CH=CH_2$), 69.7 (*C*HCH_3), 70.7

(N*C*Ph$_3$), 118.9 ($CO_2CH_2CH=CH_2$), 126.6 (*C*4), 127.9 (*C*3), 128.9 (*C*2), 131.4 ($CO_2CH_2CH=CH_2$), 145.4 (*C*1), 172.9 (CO_2Allyl); *m/z* (LCMS, ES+) 402.3 $[M+H]^+$. Data is in agreement with the literature.[226]

***N*-Trt/CO_2Allyl aziridine (D-120)**[226]

Allyl (2*R*,3*R*)-3-methyl-1-tritylaziridine-2-carboxylate

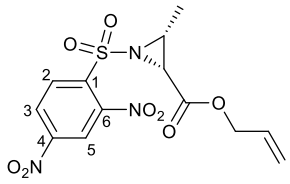


D-120

A stirred solution of allyl trityl-D-threoninate (**119**) (2.00 g, 4.98 mmol) in THF (50 mL) under Ar was cooled in ice before the addition of Et_3N (1.53 mL, 10.96 mmol) dropwise over 10 min. Methanesulfonyl chloride (0.43 mL, 5.50 mmol) was added dropwise and the solution stirred in ice for 30 min before heating at reflux for 48 h. The solvent was removed *in vacuo* and the residue redissolved in EtOAc (60 mL). The organic layer was washed with 10% aqueous citric acid (3 x 20 mL), water (2 x 20 mL), aqueous $NaHCO_3$ (2 x 30 mL), water (2 x 20 mL) and brine (20 mL). The organic layer was dried (Na_2SO_4) and the solvent removed *in vacuo*. Purification by flash column chromatography (pet. ether:EtOAc, 9:1) yielded title compound **120** as a clear oil (0.938 g, 2.45 mmol, 49%). R_f 0.71 (pet. ether:EtOAc, 9:1); $[\alpha]_D^{25}$ +98.4 (*c* 12 mg mL^{-1}, $CHCl_3$), [lit.[226] $[\alpha]_D^{25}$ +98.4 (*c* 12 mg mL^{-1}, $CHCl_3$)]; δ_H (600 MHz, $CDCl_3$) 1.39 (3H, d, *J* 5.4, CHC*H*$_3$), 1.65 (1H, quintet, *J* 6.0, C*H*CH_3), 1.92 (1H, d, *H* 6.6, C*H*CO_2Allyl), 4.62 (1H, ddt, *J* 13.2, 5.4, 1.8, $CO_2CH_AH_BCH=CH_2$), 4.71 (1H, ddt, *J* 13.2, 5.4, 1.8, $CO_2CH_AH_BCH=CH_2$), 5.25 (1H, dq, *J* 10.2, 1.2, $CO_2CH_2CH=CH_2$cis), 5.33 (1H, dq, *J* 17.4, 1.2, $CO_2CH_2CH=CH_2$trans), 5.90-5.97 (1H, m, $CO_2CH_2CH=CH_2$), 7.22 (3H, tt, *J* 7.5, 1.2, Ar*H*(4)), 7.28 (6H, t, *J* 7.5, Ar*H*(3)), 7.53 (6H, d, *J* 7.8, Ar*H*(2)); δ_C (150 MHz, $CDCl_3$) 13.3 ($CHCH_3$), 34.9 (*C*HCH_3), 35.9 (*C*HCO_2Allyl), 65.4 ($CO_2CH_2CH=CH_2$), 74.9 (N*C*Ph$_3$), 118.5 ($CO_2CH_2CH=CH_2$), 126.8 (*C*4), 127.6 (*C*3), 129.4 (*C*2), 132.1 ($CO_2CH_2CH=CH_2$), 143.9 (*C*1), 169.9 (CO_2Allyl); *m/z* (LCMS, ES+) 384.28 $[M+H]^+$. Data is in agreement with the literature.[226]

***N*-DNs/CO_2Allyl aziridine (71)**[165]

Allyl (2*R*,3*R*)-1-((2,4-dinitrophenyl)sulfonyl)-3-methylaziridine-2-carboxylate

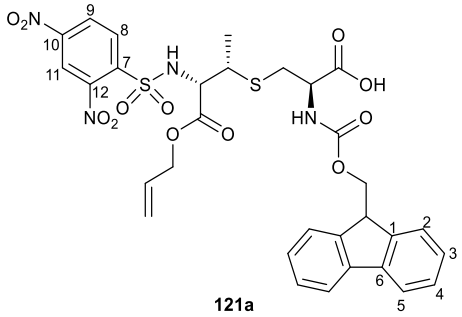


71

A solution of *N*-Trt/CO_2Allyl aziridine **120** (0.94 g, 2.45 mmol) in CH_2Cl_2:MeOH (3:1, 1.7 mL) under Ar was cooled in ice and TFA (1.25 mL) added dropwise. The solution was stirred in ice for 2 h before pouring into ether (2 mL) and washing with water (2 mL). The organic layer was further washed with water (3 x 2 mL) and the aqueous layers combined and cooled in ice before being basified to pH 9 with Na_2CO_3. To this, EtOAc (8 mL) and DNs-Cl (0.72 g, 2.70 mmol) were added. The biphasic mixture was then warmed to room temperature and stirred vigorously for 24 h. After this time the layers were separated and the aqueous layer extracted with EtOAc (3 x 10 mL). The organic layers were combined, dried (Na_2SO_4) and the solvent removed *in vacuo*. Purification by flash column chromatography (pet. ether:EtOAc, 5:1) yielded title compound **71** as a yellow oil (0.66 g, 1.78 mmol, 73%). R_f 0.34 (pet. ether:EtOAc, 5:1); $[\alpha]_D^{25}$ +21.9 (*c* 8.6 mg mL^{-1}, $CHCl_3$), [lit.[165] $[\alpha]_D^{25}$ +21.3 (*c* 8.5 mg mL^{-1}, $CHCl_3$)]; δ_H (600 MHz, $CDCl_3$) 1.47 (3H, d, *J* 6.0, CHC*H*$_3$), 3.48-3.52 (1H, m, C*H*CH_3), 3.81 (1H, d, *J* 7.2, C*H*CO_2Allyl), 4.62-4.74 (2H, m, CO_2CH_2CH=CH_2), 5.30 (1H, dq, *J* 7.2, 1.2, CO_2CH_2CH=C*H*$_2$cis), 5.37 (1H, dq, *J* 16.8, 1.4, CO_2CH_2CH=C*H*$_2$trans), 5.90-5.96 (1H, m, CO_2CH_2C*H*=CH_2), 8.55 (1H, dd, *J* 8.4, 0.6, Ar*H*(2)), 8.60 (1H, dd, *J* 9.0, 2.4, Ar*H*(3)), 8.67 (1H, d, *J* 1.8, Ar*H*(5)); δ_C (150 MHz, $CDCl_3$) 12.4 (CH*C*H$_3$), 43.4 (*C*HCH_3), 43.9 (*C*HCO_2Allyl), 66.6 (CO_2*C*H$_2$CH=CH_2), 119.5 (CO_2CH_2CH=*C*H$_2$), 120.3 (*C*5), 127.1 (*C*3), 131.0 (CO_2CH_2*C*H=CH_2), 133.4 (*C*2), 137.6 (*C*1), 148.6 (*C*6), 150.3 (*C*4), 164.7 (*C*O_2Allyl); *m/z* (LCMS, ES+) 372.12 $[M+H]^+$, 743.13 $[2M+H]^+$, 513.17 $[2M\text{-DNs}+H]^+$. Data is in agreement with the literature.[165]

Attempted Synthesis of (DNs, Allyl/Fmoc) Methyllanthionine (121a)[165]

***N*-(((9H-Fluoren-9-yl)methoxy)carbonyl)-*S*-((2*S*,3*R*)-4-(allyloxy)-3-((2,4-dinitrophenyl)sulfonamido)-4-oxobutan-2-yl)-L-cysteine**

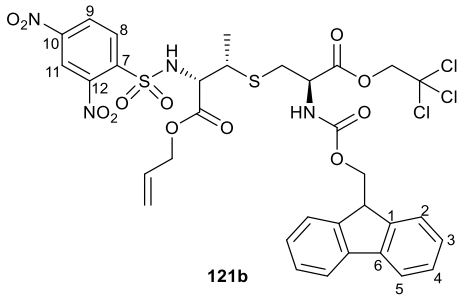


121a

A stirred solution of *N*-DNs/CO_2Allyl aziridine (**71**) (41 mg, 0.11 mmol) in dry CH_2Cl_2 (1 mL) was cooled to -78 °C under Ar. Separate solutions of Fmoc-Cys-OH (**72**) (150 mg, 0.44 mmol) in dry CH_2Cl_2 (0.5 mL) and $BF_3.OEt_2$ (108 µL, 0.87 mmol) in dry CH_2Cl_2 (0.5 mL) were also prepared and cooled to -78 °C. The Fmoc-Cys-OH (**72**) solution was added dropwise to the aziridine, followed by the dropwise addition of the $BF_3.OEt_2$ solution. The reaction was then stirred at -78 °C for 15 min before being allowed to warm to rt and stirring for 48 h. The solvent was then removed *in vacuo* before attempted purification by flash column chromatography (pet. ether:EtOAc, 7:3 → 7:1 with 1% AcOH). None of the desired compound could be isolated.

(DNs, Allyl/Fmoc, Tce) Methyllanthionine (121b)

Allyl (2*R*,3*S*)-3-(((*R*)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-oxo-3-(2,2,2-trichloroethoxy)propyl)thio)-2-((2,4-dinitrophenyl)sulfonamido)butanoate



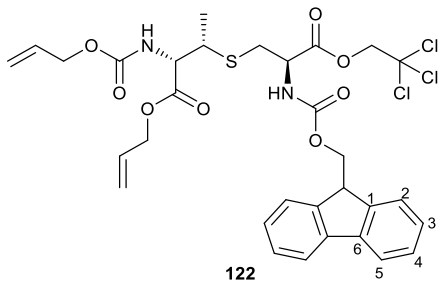
121b

A stirred solution of *N*-DNs/CO_2Allyl aziridine (**71**) (100 mg, 0.25 mmol) in dry CH_2Cl_2 (1 mL) was cooled to -78 °C under Ar. Separate solutions of Fmoc-Cys-OTce (**104**) (475 mg, 1.00 mmol) in dry CH_2Cl_2 (1 mL) and $BF_3.OEt_2$ (0.25 mL, 2.0 mmol) in dry

CH_2Cl_2 (1 mL) were also prepared and cooled to -78 °C. The Fmoc-Cys-OTce (**104**) solution was added dropwise to the aziridine, followed by the dropwise addition of the $BF_3.OEt_2$ solution. The reaction was then stirred at -78 °C for 15 min before being allowed to warm to rt and stirred for 48 h. The solvent was removed *in vacuo* before purification by flash column chromatography (pet. ether:EtOAc, 5:1) which yielded title compound **121b** as a yellow oil (30.0 mg, 0.035 mmol, 14%). R_f 0.55 (pet. ether:EtOAc, 7:3); $[\alpha]_D^{25}$ +43.6 (*c* 5.0 mg mL^{-1}, $CHCl_3$); δ_H (600 MHz, $CDCl_3$) 1.45 (3H, d, *J* 7.2, CHCH(C*H*$_3$)S), 2.94 (1H, dd, *J* 15.0, 7.2, CHC*H*$_A$H$_B$S), 3.11 (1H, dd, *J* 14.0, 4.5, CHCH$_A$*H*$_B$S), 3.52-3.57 (1H, m, CHC*H*(CH_3)S), 4.25 (1H, t, *J* 6.9, ArC*H*CH_2), 4.36 (1H, dd, *J* 9.6, 3.0, C*H*CH(CH_3)S), 4.41-4.50 (4H, m, ArCHC*H*$_2$ and CO_2C*H*$_2$CH=CH_2), 4.71-4.77 (2H, m, C*H*CH_2S and C*H*$_A$H$_B$$CCl_3$), 4.86 (1H, d, *J* 12.0, CH_A*H*$_B$$CCl_3$), 5.19 (1H, dd, *J* 10.2, 1.8, CO_2CH_2CH=C*H*$_2$cis), 5.21 (1H, dd, *J* 16.8, 1.2, CO_2CH_2CH=C*H*$_2$trans), 5.58 (1H, d, *J* 7.8, N*H*Fmoc), 5.68-5.74 (1H, m, CO_2CH_2C*H*=CH_2), 6.63 (1H, d, *J* 9.6, N*H*DNs), 7.33 (2H, t, *J* 7.5, Ar*H*(3)), 7.43 (2H, t, *J* 7.2, Ar*H*(4)), 7.61 (2H, d, *J* 6.0, Ar*H*(2)), 7.79 (2H, d, *J* 7.2, Ar*H*(5)), 8.27 (1H, d, *J* 8.4, Ar*H*(8)), 8.49 (1H, dd, *J* 8.4, 2.4, Ar*H*(9)), 8.72 (1H, d, *J* 1.8, Ar*H*(11)); δ_C (150 MHz, $CDCl_3$) 19.7 (*C*H_3), 29.7 (CH*C*H_2S), 43.5 (CH*C*H(CH_3)S), 47.0 (Ar*C*HCH_2), 53.7 (*C*HCH_2S), 61.7 (*C*HCH(CH_3)S), 66.9 (CO_2*C*H_2CH=CH_2), 67.4 (ArCH*C*H_2), 74.7 (*C*H_2CCl_3), 94.1 (CH_2*C*Cl_3), 120.1 (CO_2CH_2CH=*C*H_2 and *C*5), 120.9 (*C*11), 125.0 (*C*2), 127.1 (*C*3 and *C*9), 127.8 (*C*4), 130.6 (CO_2CH_2*C*H=CH_2), 131.9 (*C*8), 139.7 (*C*7), 141.3 (*C*6), 143.5 (*C*1), 147.7 (*C*12), 149.7 (*C*10), 155.7 (*C*ONH), 168.7 (*C*O_2Tce), 168.9 (*C*O_2Allyl); *m/z* (HRMS, ES-) required for $[C_{33}H_{31}N_4O_{12}S_2Cl_3$-H]$^-$ 843.0367, found $[C_{33}H_{31}N_4O_{12}S_2Cl_3$-H]$^-$ 843.0362.

(Alloc, Allyl/Fmoc, Tce) Methyllanthionine (122)

Allyl (2*R*,3*S*)-3-(((*R*)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-oxo-3-(2,2,2-trichloroethoxy)propyl)thio)-2-(((allyloxy)carbonyl)amino)butanoate

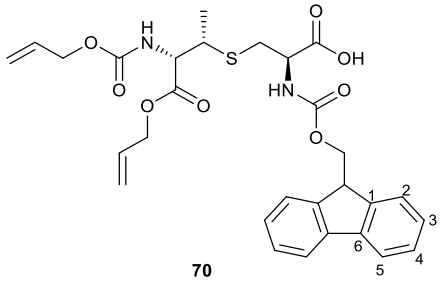


122

To a solution of methyllanthionine **121b** (22.5 mg, 0.027 mmol) in dry CH_2Cl_2 (300 µL) at 0 °C was added DIPEA (14 µL) and thioglycolic acid (2.3 µL), and the solution was stirred at rt for 2 h. After this time, the reaction was cooled to 0 °C and DIPEA (14 µL) and Alloc-Cl (4.5 µL) were added. The solution was allowed to warm to rt and stirred for 15 h before removal of the solvent *in vacuo*. Purification by flash column chromatography (pet. ether:EtOAc, 4:1) yielded title compound **122** as a clear oil (11.0 mg, 0.016 mmol, 59%). R_f 0.71 (pet. ether:EtOAc, 7:3); $[\alpha]_D^{25}$ -23.2 (*c* 5.0 mg mL^{-1}, $CHCl_3$); δ_H (500 MHz, $CDCl_3$) 1.36 (3H, d, *J* 7.0, CHCH(C*H*$_3$)S), 3.02 (1H, dd, *J* 13.5, 6.0, CHC*H*$_A$H$_B$S), 3.14 (1H, dd, *J* 14.0, 4.5, CHCH$_A$*H*$_B$S), 3.42-3.48 (1H, m, CHC*H*(CH$_3$)S), 4.26 (1H, t, *J* 7.0, ArC*H*CH$_2$), 4.45 (2H, d, *J* 7.0, ArCHC*H*$_2$), 4.57-4.75 (6H, m, C*H*CH$_2$S, C*H*CH(CH$_3$)S, CO$_2$C*H*$_2$CH=CH$_2$ and NHCO$_2$C*H*$_2$CH=CH$_2$), 4.77 (1H, d, *J* 12.0, C*H*$_A$H$_B$CCl$_3$), 4.87 (1H, d, *J* 12.0, CH$_A$*H*$_B$CCl$_3$), 5.22-5.39 (4H, m, CO$_2$CH$_2$CH=C*H*$_2$ and NHCO$_2$CH$_2$CH=C*H*$_2$), 5.57 (1H, d, N*H*Alloc), 5.69 (1H, d, N*H*Fmoc), 5.88-5.97 (2H, m, CO$_2$CH$_2$C*H*=CH$_2$ and NHCO$_2$CH$_2$C*H*=CH$_2$), 7.33 (2H, td, *J* 7.5, 0.9, Ar*H*(3)), 7.42 (2H, t, *J* 7.4, Ar*H*(4)), 7.62 (2H, d, *J* 6.1, Ar*H*(2)), 7.78 (2H, d, *J* 7.4, Ar*H*(5)); δ_C (125 MHz, $CDCl_3$) 19.4 (*C*H$_3$), 33.5 (CH*C*H$_2$S), 44.0 (CH*C*H(CH$_3$)S), 47.0 (Ar*C*HCH$_2$), 53.7 (*C*HCH$_2$S), 58.4 (*C*HCH(CH$_3$)S), 66.1 (one of CO$_2$*C*H$_2$CH=CH$_2$), 66.5 (one of CO$_2$*C*H$_2$CH=CH$_2$), 67.4 (ArCH*C*H$_2$), 74.7 (*C*H$_2$CCl$_3$), 94.2 (CH$_2$*C*Cl$_3$), 118.0 (one of CO$_2$CH$_2$CH=*C*H$_2$), 119.5 (one of CO$_2$CH$_2$CH=*C*H$_2$), 120.0 (*C*5), 125.0 (*C*2), 127.1 (*C*3), 127.8 (*C*4), 131.2 (one of CO$_2$CH$_2$*C*H=CH$_2$), 132.4 (one of CO$_2$CH$_2$*C*H=CH$_2$), 141.3 (*C*6), 143.6 (*C*1), 143.7 (*C*1), 155.7 (NH*C*O$_2$Fmoc), 156.2 (NH*C*O$_2$Alloc), 168.9 (*C*O$_2$Tce), 170.1 (*C*O$_2$Allyl); *m/z* (HRMS, ES+) required for $[C_{31}H_{33}N_2O_8SCl_3+H]^+$ 699.1102, found $[C_{31}H_{33}N_2O_8SCl_3+H]^+$ 699.1100.

(Alloc, Allyl/Fmoc) Methyllanthionine (70)[165]

***N*-(((9H-Fluoren-9-yl)methoxy)carbonyl)-*S*-((2*S*,3*R*)-4-(allyloxy)-3-(((allyloxy)carbonyl)amino)-4-oxobutan-2-yl)-L-cysteine**



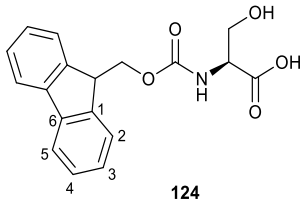
70

To a stirred solution of methyllanthionine **122** (48.0 mg, 0.069 mmol) in THF (9.6 mL) was slowly added zinc dust (44.8 mg, 0.69 mmol) followed by aqueous NH_4OAc (1 M solution, 0.51 mL). The solution was stirred at rt for 5 h, filtered and the solvent removed *in vacuo*. The residue was redissolved in CH_2Cl_2 (25 mL), washed with brine (50 mL), dried ($MgSO_4$), and the solvent removed *in vacuo*. Purification by flash column chromatography (CH_2Cl_2:MeOH, 9:1) yielded title compound **70** as an off-white solid (30.9 mg, 0.054 mmol, 78%). R_f 0.59 (CH_2Cl_2:MeOH, 9:1); $[\alpha]_D^{25}$ -1.38 (*c* 2.7 mg mL^{-1}, CH_2Cl_2), [lit.[165] $[\alpha]_D^{25}$ -0.03 (*c* 4.0 mg mL^{-1}, CH_2Cl_2)]; δ_H (600 MHz, $CDCl_3$) 1.29 (3H, d, *J* 7.2, CHCH(C*H*$_3$)S), 2.88 (1H, dd, *J* 13.2, 7.2, CHC*H*$_A$H$_B$S), 3.11 (1H, dd, *J* 15.0, 4.8, CHCH$_A$*H*$_B$S), 3.36-3.38 (1H, m, CHC*H*(CH_3)S), 4.19-4.30 (3H, m, C*H*CH_2S, ArC*H*CH_2, ArCHC*H*$_A$H$_B$), 4.42-4.44 (2H, m, ArCHCH$_A$*H*$_B$, C*H*CH(CH_3)S), 4.54 (2H, m, CO_2C*H*$_2$CH=CH_2), 4.63-4.65 (2H, m, $NHCO_2C$*H*$_2$CH=CH_2), 5.17 (1H, d, *J* 10.2, $CO_2CH_2CH=C$*H*$_2$cis), 5.21 (1H, d, *J* 10.2, $NHCO_2CH_2CH=C$*H*$_2$cis), 5.31 (1H, dd, *J* 17.4, 1.2, $CO_2CH_2CH=C$*H*$_2$trans), 5.35 (1H, dd, *J* 17.4, 1.8, $NHCO_2CH_2CH=C$*H*$_2$trans), 5.89-5.97 (2H, m, CO_2CH_2C*H*=CH_2, $NHCO_2CH_2C$*H*=CH_2), 7.31 (2H, t, *J* 7.2, Ar*H*(3)), 7.39 (2H, t, *J* 7.4, Ar*H*(4)), 7.68 (2H, d, *J* 7.2, Ar*H*(2)), 7.80 (2H, d, *J* 7.5, Ar*H*(5)); δ_C (150 MHz, $CDCl_3$) 19.9 (*C*H_3), 30.9 (CH*C*H_2S), 44.0 (CH*C*H(CH_3)S), 48.5 (Ar*C*HCH_2), 57.3 (*C*HCH_2S), 60.6 (*C*HCH(CH_3)S), 67.0 (one of CO_2*C*H_2CH=CH_2), 67.2 (one of CO_2*C*H_2CH=CH_2) 68.1 (ArCH*C*H_2), 117.9 (one of $CO_2CH_2CH=$*C*H_2), 119.3 (one of $CO_2CH_2CH=$*C*H_2), 121.1 (*C*5), 126.4 (*C*2), 126.5 (*C*2), 128.4 (*C*3), 128.9 (*C*4), 133.3 (one of CO_2CH_2*C*H=CH_2), 134.3 (one of CO_2CH_2*C*H=CH_2), 142.7 (*C*6), 145.4 (*C*1), 145.5 (*C*1), 155.5 (NH*C*O_2Alloc), 155.9 (NH*C*O_2Fmoc), 169.1 (*C*O_2Allyl), 171.9 (*C*O_2H); *m/z* (LCMS, ES+) 569.24 $[M+H]^+$. Data is in agreement with the literature.[165]

7.1.4. *Se*-phenylselenocysteine Synthesis

Fmoc-L-Ser-OH (124)[227]

(((9H-Fluoren-9-yl)methoxy)carbonyl)-L-serine

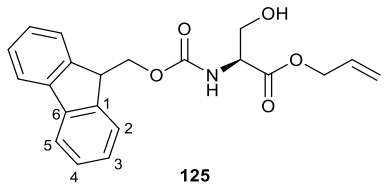


124

To a suspension of L-serine (2.00 g, 19.0 mmol) in 1,4-dioxane (15 mL) was added 10% aqueous Na_2CO_3 (35 mL) and the solution was cooled in ice. Fmoc succinimide (6.74 g, 20.0 mmol) was then dissolved in 1,4-dioxane (25 mL) with gentle heating, and the solution added dropwise to the stirring L-serine suspension *via* a dropping funnel over 15 min. The reaction was left to stir at rt for 18 h before removal of the solvent *in vacuo*. The residue was then redissolved in water (150 mL) and washed twice with ether (100 mL). The aqueous layer was acidified to pH 3.5 using 6 M HCl, and then extracted with EtOAc (3 x 150 mL). The organic layers were combined, dried ($MgSO_4$) and the solvent removed *in vacuo*. The solid was recrystallised, first from CH_2Cl_2/pet. ether and then from hot 2-propanol to give the title compound **124** as a white solid which was used without further purification (3.18 g, 9.71 mmol, 51%); δ_H (500 MHz, CD_3OD) 3.85 (1H, dd, *J* 11.5, 6.0, CHC*H*$_A$H$_B$OH), 3.90 (1H, dd, *J* 11.5, 4.8, CHCH$_A$*H*$_B$OH), 4.23 (1H, t, *J* 7.0, ArC*H*CH$_2$), 4.29 (1H, t, *J* 4.5, C*H*CH$_2$OH), 4.32-4.40 (2H, m, ArCHC*H*$_2$), 7.30 (2H, td, *J* 7.5, 0.8, Ar*H*(3)), 7.38 (2H, t, *J* 7.5, Ar*H*(4)), 7.67 (2H, t, *J* 7.0, Ar*H*(2)), 7.78 (2H, d, *J* 7.5, Ar*H*(5)); δ_C (125 MHz, CD_3OD) 48.5 (Ar*C*HCH$_2$), 57.9 (*C*HCH$_2$OH), 63.2 (CH*C*H$_2$OH), 68.3 (ArCH*C*H$_2$), 121.0 (*C*5), 126.4 (*C*2), 128.3 (*C*3), 128.9 (*C*4), 142.7 (*C*6), 145.4 (*C*1), 145.5 (*C*1), 158.7 (*C*ONH), 173.9 (*C*O$_2$H); *m/z* (LCMS, ES+) 328.2 $[M+H]^+$, (LCMS, ES-) 326.3 $[M-H]^-$, 653.5 $[2M-H]^-$. Data is in agreement with the literature.[227]

Fmoc-L-Ser-OAllyl (125)[196]

Allyl (((9H-fluoren-9-yl)methoxy)carbonyl)-L-serinate

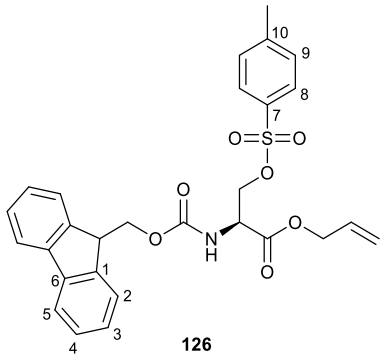


125

To a vigorously stirred solution of Fmoc-L-serine (**124**) (3.00 g, 9.17 mmol) and $NaHCO_3$ (0.77 g, 9.17 mmol) in water (23 mL) was added a solution of aliquat 336 (3.7 g, 9.17 mmol) and allyl bromide (0.87 mL, 10.1 mmol) in CH_2Cl_2 (23 mL). The solution was stirred at rt for 30 h before the addition of a further portion of allyl bromide (1.2 mL, 13.9 mmol) and stirring for a further 18 h. The layers were then separated and the aqueous layer extracted with CH_2Cl_2 (3 x 20 mL). The organic layers were combined, dried ($MgSO_4$) and the solvent removed *in vacuo*. Purification by flash column chromatography (pet. ether:EtOAc, 2:1) yielded title compound **125** as a white solid (2.39 g, 6.51 mmol, 71%). R_f 0.39 (pet. ether:EtOAc, 2:1); mp 77-79 °C (lit.[227] 76-78 °C); $[\alpha]_D^{25}$ +0.6 (*c* 75 mg mL^{-1}, EtOAc), [lit.[227] $[\alpha]_D^{25}$ +0.4 (*c* 75 mg mL^{-1}, EtOAc)]; δ_H (600 MHz, $CDCl_3$) 3.96 (1H, dd, *J* 10.8, 3.0, CHCH_AH$_B$OH), 4.05 (1H, dd, *J* 10.8, 3.0, CHCH$_A$$H_B$OH), 4.25 (1H, t, *J* 6.6, ArC*H*CH_2), 4.44-4.45 (2H, m, ArCHCH_2), 4.47-4.50 (1H, m, C*H*CH_2OH), 4.71 (2H, d, *J* 5.4, OCH_2CH=CH_2), 5.28 (1H, d, *J* 10.2, OCH$_2$CH=CH_2cis), 5.36 (1H, d, *J* 16.8, OCH$_2$CH=CH_2trans), 5.72 (1H, d, *J* 7.2, N*H*), 5.90-5.96 (1H, m, OCH$_2$C*H*=CH_2), 7.33 (2H, tt, *J* 7.2, 1.5, Ar*H*(3)), 7.42 (2H, td, *J* 7.2, 0.6, Ar*H*(4)), 7.61-7.63 (2H, m, Ar*H*(2)), 7.78 (2H, dd, *J* 7.3, 0.6, Ar*H*(5)); δ_C (150 MHz, $CDCl_3$) 47.1 (Ar*C*HCH_2), 56.0 (*C*HCH$_2$OH), 63.3 (CH*C*H$_2$OH), 66.4 (O*C*H$_2$CH=CH_2), 67.2 (ArCH*C*H$_2$), 119.1 (OCH$_2$CH=*C*H$_2$), 120.0 (*C*5), 125.1 (*C*2), 127.1 (*C*3), 127.7 (*C*4), 131.2 (OCH$_2$*C*H=CH_2), 141.3 (*C*6), 143.6 (*C*1), 143.8 (*C*1), 156.2 (*C*O$_2$NH), 170.1 (*C*O$_2$Allyl); *m/z* (LCMS, ES+) 368.3 $[M+H]^+$, 735.5 $[2M+H]^+$. Data is in agreement with the literature.[196]

Fmoc-L-Ser(OTs)-OAllyl (126)[196]

Allyl *N*-(((9H-fluoren-9-yl)methoxy)carbonyl)-*O*-tosyl-L-serinate

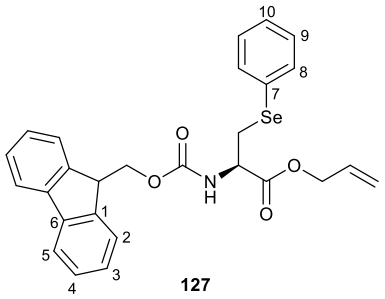


126

To a flask containing Fmoc-L-Ser-OAllyl (**125**) (2.00 g, 5.44 mmol) and *p*TsCl (5.19 g, 27.2 mmol) under Ar was added anhydrous pyridine (8.7 mL). The solution was stirred at 0 °C for 17 h before the addition of ether (100 mL). This was then washed successively with water (50 mL), 10% aqueous $KHSO_4$ (4 x 40 mL), saturated sodium bicarbonate (50 mL) and brine (50 mL). The organic layer was dried ($MgSO_4$) and the solvent removed *in vacuo*. Purification by flash column chromatography (pet. ether:EtOAc, 2:1) yielded title compound **126** as a clear oil (1.75 g, 3.36 mmol, 62%). R_f 0.73 (pet. ether:EtOAc, 2:1); $[\alpha]_D^{25}$ +0.3 (*c* 75 mg mL^{-1}, EtOAc), [lit.[196] $[\alpha]_D^{25}$ +0.3 (*c* 75 mg mL^{-1}, EtOAc)]; δ_H (600 MHz, $CDCl_3$) 2.36 (3H, s, SO_2ArCH_3), 4.20 (1H, t, *J* 7.2, $ArCHCH_2$), 4.27 (1H, dd, *J* 10.5, 7.5, $ArCHCH_AH_B$), 4.35-4.38 (2H, m, $CHCH_AH_BOTs$ and $ArCHCH_AH_B$), 4.46 (1H, dd, *J* 10.2, 3.0, $CHCH_AH_BOTs$), 4.59-4.62 (2H, m, $OCH_AH_BCH=CH_2$, $CHCH_2OTs$), 4.67 (1H, ddt, *J* 13.2, 6.0, 1.2, $OCH_AH_BCH=CH_2$), 5.27 (1H, dd, *J* 10.2, 1.2, $OCH_2CH=CH_2$cis), 5.32 (1H, dd, *J* 16.8, 1.2, $OCH_2CH=CH_2$trans), 5.65 (1H, d, *J* 8.4, N*H*), 5.82-5.89 (1H, m, $OCH_2CH=CH_2$), 7.28 (2H, d, *J* 8.4, Ar*H*(8)), 7.34 (2H, dd, 7.8, 6.6, Ar*H*(3)), 7.42 (2H, td, *J* 7.2, 3.6, Ar*H*(4)), 7.60 (2H, dd, *J* 12.0, 7.2, Ar*H*(2)), 7.75-7.91 (4H, m, Ar*H*(9) and Ar*H*(5)); δ_C (150 MHz, $CDCl_3$) 21.6 (SO_2ArCH_3), 46.9 ($ArCHCH_2$), 53.4 ($CHCH_2OTs$), 66.9 ($OCH_2CH=CH_2$), 67.5 ($ArCHCH_2$), 69.0 ($CHCH_2OTs$), 119.4 ($OCH_2CH=CH_2$), 120.0 (*C*5), 125.1 (*C*2), 125.2 (*C*2), 127.1 (*C*3), 127.8 (*C*4), 128.0 (*C*9), 129.4 (*C*8), 130.9 ($OCH_2CH=CH_2$), 132.1 (*C*10), 141.2 (*C*6), 143.5 (*C*1), 143.8 (*C*1), 145.3 (*C*7), 155.5 (*C*ONH), 167.9 (CO_2Allyl); *m/z* (LCMS, ES+) 522.4 $[M+H]^+$. Data is in agreement with the literature.[196]

Fmoc-L-Sec(Ph)-OAllyl (127)[196]

Allyl (*R*)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(phenylselanyl) propanoate



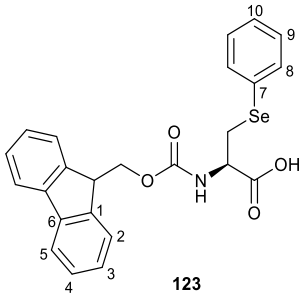
127

A flask containing a 1 M solution of NaOH (5 mL) and one containing acetone (20 mL) were sparged with Ar for 30 min. Fmoc-L-Ser(OTs)-OAllyl (**126**) (1.00 g, 1.91 mmol) was added to a separate flask under Ar and dissolved in the sparged acetone (10 mL). A solution of phenylselenol (0.23 mL, 2.10 mmol) in anhydrous DMF (5 mL) was then cooled in ice before the addition of the sparged NaOH solution (1.9 mL). This solution was left to stir for a further 5 min before the addition of the Fmoc-L-Ser(OTs)-OAllyl (**126**) solution. The reaction was stirred at 0 °C for 3.5 h before the addition of EtOAc (100 mL). The solution was then washed with saturated aqueous NH_4Cl (3 x 40 mL) and brine (50 mL) before drying ($MgSO_4$) and removal of the solvent *in vacuo*. Purification by flash column chromatography (pet. ether:EtOAc, 3:1) yielded title compound **127** as a clear oil (626 mg, 1.24 mmol, 70%). R_f 0.65 (pet. ether:EtOAc, 3:1); $[\alpha]_D^{25}$ +22.2 (*c* 36 mg mL^{-1}, $CHCl_3$), [lit.[196] $[\alpha]_D^{25}$ +10.4 (*c* 36 mg mL^{-1}, $CHCl_3$)]; δ_H (600 MHz, $CDCl_3$) 3.38 (1H, dd, *J* 13.2, 4.8, CHCH_AH$_B$SePh), 3.43 (1H, dd, *J* 13.2, 4.8, CHCH$_A$$H_B$SePh), 4.20 (1H, t, *J* 7.2, ArC*H*CH_2), 4.28-4.36 (3H, m, ArCHCH_2 and OCH_AH$_B$CH=CH_2), 4.52 (1H, dd, *J* 12.6, 6.0, OCH$_A$$H_B$CH=$CH_2$), 4.76-4.79 (1H, m, C*H*CH_2SePh), 5.23 (1H, dd, *J* 10.2, 1.2, OCH_2CH=CH_2cis), 5.28 (1H, dd, *J* 16.8, 1.2, OCH_2CH=CH_2trans), 5.63 (1H, d, *J* 5.63, N*H*), 5.76-5.83 (1H, m, OCH_2C*H*=CH_2), 7.25-7.27 (3H, m, Ar*H*(8), Ar*H*(10)), 7.31-7.34 (2H, m, Ar*H*(3)), 7.42 (2H, td, *J* 7.2, 2.4, Ar*H*(4)), 7.57-7.59 (4H, m, Ar*H*(2), Ar*H*(9)), 7.78 (2H, d, *J* 7.8, Ar*H*(5)); δ_C (150 MHz, $CDCl_3$) 30.4 (CH*C*H_2SePh), 47.0 (Ar*C*HCH_2), 53.9 (*C*HCH_2SePh), 66.3 (OCH_2CH=CH_2), 67.2 (ArCH*C*H_2), 119.1 (OCH_2CH=*C*H_2), 120.0 (*C*5), 125.2 (*C*2), 127.1 (*C*3), 127.7 (*C*4 and *C*7), 128.7 (*C*10), 129.2 (*C*8), 131.2 (OCH_2*C*H=CH_2), 133.8 (*C*9), 141.3 (*C*6), 143.7 (*C*1), 143.8 (*C*1),

155.5 (*C*ONH), 170.0 (*C*O_2Allyl); *m/z* (LCMS, ES+) 504.3 [M(^{76}Se)+H]$^+$, 505.4 [M(^{77}Se)+H]$^+$, 506.4 [M(^{78}Se)+H]$^+$. Data is in agreement with the literature.[196]

Fmoc-L-Sec(Ph)-OH (123)[196]

(*R*)-2-((((9H-Fluoren-9-yl)methoxy)carbonyl)amino)-3-(phenylselanyl)propanoic acid



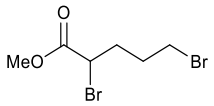
123

A flask containing Fmoc-L-Sec(Ph)-OAllyl (**127**) (600 mg, 1.18 mmol) and $Pd(PPh_3)_4$ (69 mg, 59.0 μmol) was covered with foil and purged with Ar for 10 min before dissolving in anhydrous THF (6.2 mL). A solution of morpholine (0.11 mL, 1.24 mmol) in THF (5 mL) was then added dropwise by addition funnel over 10 min and the reaction stirred for 30 min. After this time EtOAc (50 mL) was added and the solution washed with 2 M HCl (3 x 30 mL). The organic layer was then dried ($MgSO_4$) and the solvent removed *in vacuo*. Purification by flash column chromatography (CH_2Cl_2:MeOH, 15:1 with 0.1% v/v AcOH) yielded title compound **123** as a pale yellow solid (344 mg, 0.74 mmol, 63%). R_f 0.52 (CH_2Cl_2:MeOH, 9:1); mp 118-122 °C (lit.[196] 121-124 °C); $[\alpha]_D^{25}$ -26.6 (*c* 70 mg mL^{-1}, EtOAc, 25 °C), [lit.[196] $[\alpha]_D^{22}$ -32.4 (*c* 70 mg mL^{-1}, EtOAc, 22 °C)]; δ_H (400 MHz, $CDCl_3$) 3.30 (1H, br s, CHCH_AH$_B$SePh) 3.38 (1H, br s, CHCH$_A$$H_B$SePh), 4.06 (1H, br s, ArC*H*CH$_2$), 4.21 (1H, br s, ArCHCH_AH$_B$), 4.31 (1H, br s, ArCHCH$_A$$H_B$), 4.53 (1H, br s, C*H*CH$_2$SePh), 5.83 (1H, br s, N*H*), 7.08 (3H, br s, SeAr*H*(8) and SeAr*H*(10)), 7.16 (2H, m, Ar*H*(3)), 7.29 (2H, m, Ar*H*(4)), 7.42-7.46 (4H, m, SeAr*H*(9)and Ar*H*(2)), 7.67 (2H, d, *J* 7.6, Ar*H*(5)); δ_C (150 MHz, $CDCl_3$) 29.7 (CH*C*H$_2$SePh), 47.0 (Ar*C*HCH$_2$), 53.9 (*C*HCH$_2$SePh), 67.3 (ArCH*C*H$_2$), 120.0 (*C*5), 125.1 (*C*2), 127.1 (*C*3), 127.7 (*C*4 and *C*7), 128.6 (*C*10), 129.3 (*C*8), 133.7 (*C*9), 141.3 (6), 143.6 (*C*1), 143.7 (*C*1), 155.7 (*C*ONH), 174.2 (*C*O_2H); *m/z* (LCMS, ES+) 465.4 [M(^{77}Se)+H]$^+$, 466.3 [M(^{78}Se)+H]$^+$, 930.4 [2M(1x^{77}Se,1x^{78}Se)+H]$^+$, 931.5

$[2M(^{78}Se)+H]^+$, (LCMS, ES-) 464.4 $[M(^{78}Se)-H]^-$, 929.6 $[2M(^{78}Se)-H]^-$. Data is in agreement with the literature.[196]

7.1.5. Methyl 2,5-dibromopentanoate Synthesis

Methyl 2,5-dibromopentanoate (80)[198]



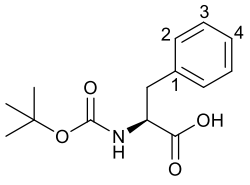
80

To a stirred solution of δ-valerolactone (1.00 g, 9.99 mmol) in Br_2 (0.77 mL, 15.0 mmol) was added PBr_3 (18.1 μL, 0.19 mmol). This solution was heated at 110 °C for 2 h before cooling in ice. MeOH (2 mL, 49.9 mmol) and *p*-toluenesulfonic acid monohydrate (18.3 mg, 0.096 mmol) were then added and the solution heated for a further 3 h at 80 °C before removal of the solvent *in vacuo*. The residue was diluted with CH_2Cl_2 (30 mL) and then washed with water (10 mL), 10% NaOH (10 mL) and water (10 mL), dried ($MgSO_4$) and the solvent removed *in vacuo*. Purification by flash column chromatography (pet. ether:EtOAc, 100:1) yielded title compound **80** as a clear oil (1.99 g, 7.26 mmol, 73%). R_f 0.85 (pet. ether:EtOAc, 4:1); δ_H (600 MHz, $CDCl_3$) 1.92-1.99 (1H, m, CH_AH$_B$CH$_2$Br), 2.04-2.19 (2H, m, CH$_A$$H_BCH_2$Br, CHBrC$H_AH_B$), 2.21-2.29 (1H, m, CHBrCH$_A$$H_B$), 3.43 (2H, t, *J* 7.8, CH$_2$CH_2Br), 3.79 (3H, s, CH_3), 4.26 (1H, dd, *J* 10.2, 7.2, C*H*BrCH$_2$); δ_C (150 MHz, $CDCl_3$) 30.2 (*C*H$_2$CH$_2$Br), 32.1 (CH$_2$*C*H$_2$Br), 33.3 (CHBr*C*H$_2$), 44.6 (*C*HBrCH$_2$), 53.2 (*C*H$_3$), 170.0 (*C*O$_2$CH$_3$); *m/z* (EI+) 272.0 $[M(2x^{79}Br)]^+$, 274.0 $[M(^{79}Br+^{81}Br)]^+$, 276.0 $[M(2x^{81}Br)]^+$. Data is in agreement with the literature.[198]

7.1.6. Phe-Cys Dipeptide Synthesis

Boc-Phe-OH (224)[259,301]

(*Tert*-butoxycarbonyl)-L-phenylalanine

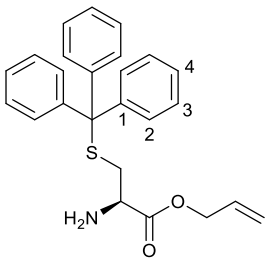


224

A stirred solution of L-phenylalanine (1.65 g, 10 mmol) in 1 M NaOH (10 mL), water (10 mL) and dioxane (20 mL) was cooled to 0 °C before adding di-*tert*-butyl dicarbonate (2.2 g, 10.1 mmol). The solution was stirred at rt for 20 h before removal of the dioxane *in vacuo*. The remaining aqueous solution was acidified with saturated aqueous $KHSO_4$ before extraction with EtOAc (3 x 40 mL). The organic layers were combined, dried ($MgSO_4$) and the solvent removed *in vacuo* to yield title compound **224** as a colourless oil which was used without further purification (2.60 g, 9.8 mmol, 98%). R_f 0.64 (CH_2Cl_2:MeOH, 9:1); $[\alpha]_D^{20}$ +20.4 (*c* 10.3 mg mL^{-1}, EtOH, 20 °C), [lit.[301] $[\alpha]_D^{20}$ +24.0 (*c* 10.3 mg mL^{-1}, EtOH, 20 °C)]; δ_H (600 MHz, $CDCl_3$) 1.43 (9H, s, C(C*H*$_3$)$_3$), 3.09 (1H, dd, *J* 13.8, 6.6, CHC*H*$_A$H$_B$Ph), 3.21 (1H, dd, *J* 13.8, 5.4, CHCH$_A$*H*$_B$Ph), 4.61-4.64 (1H, m, C*H*CH$_2$Ph), 4.95 (1H, d, *J* 7.2, N*H*), 7.19-7.21 (2H, m, Ar*H*(2)), 7.31-7.33 (3H, m, Ar*H*(3) and Ar*H*(4)); δ_C (150 MHz, $CDCl_3$) 28.3 (C(*C*H$_3$)$_3$), 37.7 (CH*C*H$_2$Ph), 54.2 (*C*HCH$_2$Ph), 80.3 (*C*(CH$_3$)$_3$), 128.6 (*C*3 and *C*4), 129.4 (*C*2), 135.8 (*C*1), 155.4 (*C*ONH), 176.3 (*C*O$_2$H); *m/z* (LCMS, ES+) 266.3 $[M+H]^+$, 166.3 $[M+H\text{-}Boc]^+$, 531.4 $[2M+H]^+$, 796.6 $[3M+H]^+$, (LCMS, ES-) 264.3 $[M-H]^-$, 529.5 $[2M-H]^-$. Data is in agreement with the literature.[301]

H-Cys(Trt)-OAllyl (202)[258,302]

Allyl *S*-trityl-L-cysteinate



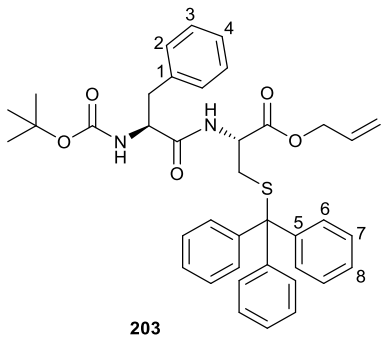
202

To a solution of Fmoc-Cys(Trt)-OH (1.17 g, 2.00 mmol) in DMF (20 mL) under Ar was added K_2CO_3 (304 mg, 2.20 mmol) and allyl bromide (260 µL, 3.00 mmol). The reaction was stirred at rt for 2 h before removal of the solvent *in vacuo*. The residue was redissolved in EtOAc (50 mL) and water added (75 mL). The layers were separated and the aqueous layer was then extracted further with EtOAc (2 x 25 mL). The organic layers were combined, dried ($MgSO_4$) and the solvent removed *in vacuo*. The residue was redissolved in MeCN (6 mL) and diethylamine (6 mL) added. The reaction was stirred for 30 min at rt before removal of the solvent *in vacuo*. Purification by flash column

chromatography (pet. ether → EtOAc) yielded title compound **202** as a yellow oil (658 mg, 1.63 mmol, 82%). R_f 0.32 (CH_2Cl_2); $[\alpha]_D^{20}$ +37.1 (*c* 10.0 mg mL^{-1}, EtOH, 20 °C), [lit.[302] $[\alpha]_D^{27}$ +13.1 (*c* 10.0 mg mL^{-1}, EtOH, 27 °C)]; δ_H (600 MHz, $CDCl_3$) 2.62-2.69 (2H, m, CHC*H*$_2$STrt), 3.25 (1H, dd, *J* 7.8, 4.8, C*H*CH$_2$STrt), 4.56 (2H, dq, *J* 5.4, 1.8, OC*H*$_2$CH=CH$_2$), 5.23 (1H, dq, *J* 10.8, 1.2, OCH$_2$CH=C*H*$_2$cis), 5.27 (1H, dq, *J* 17.4, 1.2, OCH$_2$CH=C*H*$_2$trans), 5.82-5.89 (1H, m, OCH$_2$C*H*=CH$_2$), 7.22 (3H, tt, *J* 7.2, 1.2, Ar*H*(4)), 7.29 (6H, m, Ar*H*(3)), 7.43 (6H, m, Ar*H*(2)); δ_C (150 MHz, $CDCl_3$) 35.1 (CH*C*H$_2$STrt), 53.2 (*C*HCH$_2$STrt), 66.2 (O*C*H$_2$CH=CH$_2$), 67.1 (S*C*Ph$_3$), 118.8 (OCH$_2$CH=*C*H$_2$), 126.9 (*C*4), 128.1 (*C*3), 129.5 (*C*2), 131.4 (OCH$_2$*C*H=CH$_2$), 144.2 (*C*1), 171.2 (*C*O$_2$Allyl); *m/z* (LCMS, ES+) 404.3 $[M+H]^+$, 807.6 $[2M+H]^+$. Data is in agreement with the literature.[302]

Boc-Phe-Cys(Trt)-OAllyl (203)

Allyl *N*-((*tert*-butoxycarbonyl)-L-phenylalanyl)-*S*-trityl-L-cysteinate



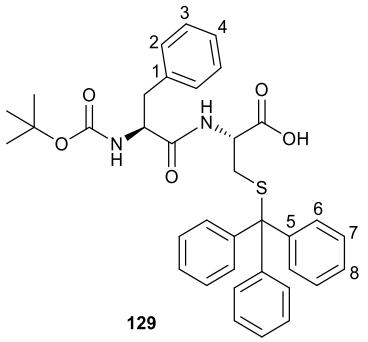
203

To a stirred solution of cysteine **202** (640 mg, 1.59 mmol) in CH_2Cl_2 (17 mL) under Ar was added *N*-methylmorpholine (0.52 mL, 4.77 mmol), Boc-Phe-OH (**224**) (421 mg, 1.59 mmol) and PyBOP (825 mg, 1.59 mmol). The solution was left to stir at rt for 4 h before removal of the solvent *in vacuo*. Purification by flash column chromatography (pet. ether → pet. ether:EtOAc, 5:1) yielded title compound **203** as a white solid (662 mg, 1.02 mmol, 64%). R_f 0.93 (pet. ether:EtOAc, 3:1); mp 52-54 °C; $[\alpha]_D^{20}$ -4.1 (*c* 10.0 mg mL^{-1}, MeOH, 20 °C); δ_H (600 MHz, $CDCl_3$) 1.41 (9H, s, C(C*H*$_3$)$_3$), 2.57 (1H, dd, *J* 12.0, 4.8, CHC*H*$_A$H$_B$STrt), 2.64 (1H, dd, *J* 12.6, 6.0, CHCH$_A$*H*$_B$STrt), 3.05 (2H, d, *J* 6.6, CHC*H*$_2$Ph), 4.48 (1H, m, C*H*CH$_2$STrt), 4.56-4.65 (3H, m, C*H*CH$_2$Ph and CO$_2$C*H*$_2$CH=CH$_2$), 5.27 (1H, dq, *J* 10.2, 1.2, CO$_2$CH$_2$CH=C*H*$_2$cis), 5.31 (1H, dq, *J* 17.4, 1.2, CO$_2$CH$_2$CH=C*H*$_2$trans), 5.84-5.91 (1H, m, CO$_2$CH$_2$C*H*=CH$_2$), 6.28 (1H, d, *J* 7.2, CON*H*) 7.21-7.30 (14H, m, Ar*H*(2, 3, 4, 7, 8)), 7.35 (6H, d, *J* 7.8, Ar*H*(6)); δ_C (150 MHz,

$CDCl_3$) 28.2 ($C(CH_3)_3$), 33.5 ($CHCH_2STrt$), 38.5 ($CHCH_2Ph$), 51.3 ($CHCH_2STrt$), 55.4 ($CHCH_2Ph$), 66.2 ($CO_2CH_2CH=CH_2$), 66.9 ($SCPh_3$), 80.1 ($C(CH_3)_3$), 118.8 ($CO_2CH_2CH=CH_2$), 126.9 (*C*8), 127.7 (*C*3), 128.0 (*C*7), 128.7 (*C*4), 129.5 (*C*6), 129.7 (*C*2), 131.4 ($CO_2CH_2CH=CH_2$), 136.6 (*C*1), 144.2 (*C*5), 154.2 (CO_2tBu), 169.6 (CO_2Allyl), 170.8 (*C*ONH); ν_{max} (cm^{-1}) 3307 (amide N-H), 3058, 3029 (Ar C-H), 2976, 2929 (alkyl C-H), 1741 (C=O), 1664 (C=O); *m/z* (HRMS, ES+) required for $[C_{39}H_{42}N_2O_5S+H]^+$ 651.2892, found $[C_{39}H_{42}N_2O_5S+H]^+$ 651.2890.

Boc-Phe-Cys(Trt)-OH (129)

***N*-((*Tert*-butoxycarbonyl)-L-phenylalanyl)-*S*-trityl-L-cysteine**



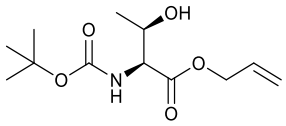
129

To a solution of Boc-Phe-Cys(Trt)-OAllyl **203** (500 mg, 0.768 mmol) in degassed CH_2Cl_2 (32 mL) under Ar was added $PhSiH_3$ (0.19 mL, 1.54 mmol) and $Pd(PPh_3)_4$ (89 mg, 0.077 mmol). The solution was stirred in the dark for 2 h before removal of the solvent *in vacuo*. Purification by flash column chromatography (CH_2Cl_2:MeOH, 9:1) yielded title compound **129** as a brown solid (287 mg, 0.470 mmol, 61%). R_f 0.59 (CH_2Cl_2:MeOH, 9:1); mp 124-126 °C; $[\alpha]_D^{20}$ -3.1 (*c* 13.0 mg mL^{-1}, MeOH, 20 °C); δ_H (600 MHz, CD_3OD) 1.33 (9H, s, $C(CH_3)_3$), 2.58-2.65 (2H, m, $CHCH_2STrt$), 2.81 (1H, dd, *J* 13.8, 9.6,$CHCH_AH_BPh$), 3.15 (1H, dd, *J* 13.8, 4.2, $CHCH_AH_BPh$), 4.28-4.34 (2H, m, $CHCH_2STrt$ and $CHCH_2Ph$), 6.78 (1H, d, 8.4), 7.16-7.24 (8H, m, Ar*H*(8, 2, 3, 4)), 7.28 (6H, t, *J* 7.2, Ar*H*(7)), 7.37 (6H, d, *J* 8.4, Ar*H*(6)); δ_C (150 MHz, CD_3OD) 28.8 ($C(CH_3)_3$), 35.5 ($CHCH_2STrt$), 39.2 ($CHCH_2Ph$), 54.3 ($CHCH_2STrt$), 57.5 ($CHCH_2Ph$), 67.6 ($SCPh_3$), 80.8 ($C(CH_3)_3$), 127.7 (*C*4), 128.0 (*C*8), 129.1 (*C*7), 129.5 (*C*3), 130.5 (*C*2), 130.9 (*C*6), 138.9 (*C*1), 146.2 (*C*5), 157.8 (*C*ONH), 173.9 (CO_2H); ν_{max} (cm^{-1}) 3307 (amide N-H), 3056, 3029 (Ar C-H), 2976, 2930 (alkyl C-H), 1656 (C=O), 1596 (C=O); *m/z* (HRMS, ES-) required for $[C_{36}H_{38}N_2O_5S-H]^-$ 609.2423, found $[C_{36}H_{38}N_2O_5S-H]^-$ 609.2412.

7.1.7. Ile-Dhb and Ile-Thr Dipeptide Synthesis

Boc-Thr-OAllyl (132)[303]

Allyl (*tert*-butoxycarbonyl)-L-threoninate

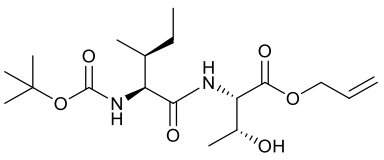


132

To a solution of Boc-L-Thr-OH (1.5 g, 6.8 mmol) in DMF (40 mL) under Ar was added Na_2CO_3 (1.44 g, 13.6 mmol), allyl bromide (0.71 mL, 8.16 mmol) and water (1 mL). The solution was stirred at for 18 h before removal of the solvent *in vacuo*. The residue was then redissolved in EtOAc (50 mL) and water added (75 mL). The layers were separated and the aqueous layer was then extracted further with EtOAc (2 x 25 mL). The organic layers were combined, dried ($MgSO_4$) and the solvent removed *in vacuo* to yield title compound **132** as a pale yellow oil which was used without further purification (1.00 g, 3.86 mmol, 57%). R_f 0.95 (pet. ether:EtOAc, 1:1); $[\alpha]_D^{25}$ -14.2 (*c* 6.5 mg mL^{-1}, $CHCl_3$), [lit.[303] $[\alpha]_D^{25}$ -13.2 (*c* 10.6 mg mL^{-1}, $CHCl_3$)]; δ_H (600 MHz, $CDCl_3$) 1.26 (3H, d, *J* 6.6, $CHCH_3$), 1.49 (9H, s, $C(CH_3)_3$), 4.27-4.34 (2H, m, $CHCO_2$Allyl and $CHCH_3$), 4.64-4.70 (2H, m, $CO_2CH_2CH=CH_2$), 5.26 (1H, dd, *J* 10.2, 1.2, $CO_2CH_2CH=CH_AH_B$), 5.34-5.37 (2H, m, $CO_2CH_2CH=CH_AH_B$ and N*H*), 5.89-5.95 (1H, m, $CO_2CH_2CH=CH_2$); δ_C (150 MHz, $CDCl_3$) 19.9 ($CHCH_3$), 28.3 ($C(CH_3)_3$), 58.7 ($CHCO_2$Allyl), 66.0 ($CO_2CH_2CH=CH_2$), 68.2 ($CHCH_3$), 80.1 ($C(CH_3)_3$), 118.8 ($CO_2CH_2CH=CH_2$), 131.5 ($CO_2CH_2CH=CH_2$), 156.1 ($NHCO_2t$Bu), 171.2 (CO_2Allyl); *m/z* (LCMS, ES+) 260.3 $[M+H]^+$, 519.5 $[2M+H]^+$. Data is in agreement with the literature.[303]

Boc-Ile-Thr-OAllyl (130)[304]

Allyl (*tert*-butoxycarbonyl)-L-isoleucyl-L-threoninate

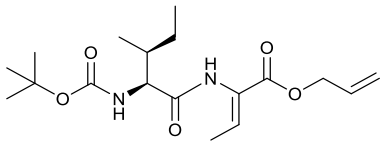


130

To a solution of protected threonine **132** (1.00 g, 3.86 mmol) in CH_2Cl_2 (23 mL), TFA (23 mL) was added. The solution was stirred at rt for 1 h before removal of the solvent *in vacuo*. The residue was redissolved in CH_2Cl_2 (58 mL) and stirred under Ar. To this solution was added *N*-methylmorpholine (1.27 mL, 11.6 mmol), Boc-Ile-OH (0.89 g, 3.86 mmol) and PyBOP (2.00 g, 3.86 mmol). The solution was stirred at rt for 18 h before removal of the solvent *in vacuo*. Purification by flash column chromatography (pet. ether:EtOAc, 2:1) yielded title compound **130** as a white solid (1.16 g, 3.11 mmol, 81%). R_f 0.70 (pet. ether:EtOAc, 1:1); $[\alpha]_D^{25}$ -19.2 (*c* 9.7 mg mL^{-1}, $CHCl_3$); δ_H (600 MHz, $CDCl_3$) 0.93 (3H, t, *J* 7.2, Ile δC*H*$_3$), 0.97 (3H, d, *J* 6.6, Ile γC*H*$_3$), 1.50-1.21 (1H, m, Ile γC*H*$_A$H$_B$), 1.23 (3H, d, *J* 6.0, Thr γC*H*$_3$), 1.44 (9H, s, C(C*H*$_3$)$_3$), 1.58 (1H, br s, Ile γCH$_A$*H*$_B$), 1.86 (1H, br s, Thr βC*H*), 2.14 (1H, br s, O*H*), 3.97 (1H, dd, *J* 9.0, 6.9, Ile αC*H*), 4.39 (1H, qd, *J* 6.0, 2.4, Thr βC*H*), 4.63-4.68 (3H, m, CO_2CH_2CH=CH_2 and Thr αC*H*), 5.11 (1H, d, *J* 8.4, N*H*Boc), 5.27 (1H, dq, *J* 10.2, 1.4, CO_2CH_2CH=C*H*$_2$cis), 5.35 (1H, dq, *J* 17.2, 1.4, CO_2CH_2CH=C*H*$_2$trans), 5.88-5.95 (1H, m, CO_2CH_2C*H*=CH_2), 6.68 (1H, d, *J* 8.7, CON*H*); δ_C (150 MHz, $CDCl_3$) 11.3 (Ile δ*C*H$_3$), 15.4 (Ile γ*C*H$_3$), 20.0 (Thr γ*C*H$_3$), 24.9 (Thr γ*C*H$_2$), 28.3 (C(*C*H$_3$)$_3$), 37.0 (Ile βCH), 57.2 (Thr α*C*H), 59.5 (Ile α*C*H), 66.2 (CO_2*C*H_2CH=CH_2), 68.2 (Thr β*C*H), 80.1 (*C*(CH$_3$)$_3$), 119.0 (CO_2CH_2CH=*C*H$_2$), 131.4 (CO_2CH_2*C*H=CH_2), 155.9 (NH*C*O$_2$*t*Bu), 170.3 (*C*ONH), 172.2 (*C*O$_2$Allyl); *m/z* (LCMS, ES+) 373.3 $[M+H]^+$, 745.6 $[2M+H]^+$. Data is in agreement with the literature.[304]

Boc-Ile-Dhb-OAllyl (134)[304]

Allyl (*Z*)-2-((2*S*,3*S*)-2-((*tert*-butoxycarbonyl)amino)-3-methylpentanamido)but-2-enoate



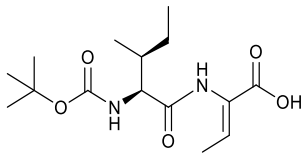
134

To a stirred solution of Boc-Ile-Thr-OAllyl **130** (1.14 g, 3.06 mmol) in EtOAc (58 mL) under Ar, Et_3N (0.88 mL, 6.43 mmol), MsCl (0.50 mL, 0.643 mmol) and DBU (0.96 mL, 6.43 mmol) were added slowly over 5 min. The reaction was then stirred at reflux for 4 h. After this time the mixture was extracted with water (2 x 75 mL) and brine (75 mL), dried (Na_2SO_4) and the solvent removed *in vacuo*. Purification by flash column

chromatography (pet. ether:EtOAc, 5:1) yielded title compound **134** as a white solid (0.620 g, 1.75 mmol, 60%). R_f 0.71 (pet. ether:EtOAc, 2:1); mp 78-80 °C (lit.[304] 107-109 °C); $[\alpha]_D^{25}$ -14.3 (*c* 10.4 mg mL^{-1}, $CHCl_3$); δ_H (500 MHz, $CDCl_3$) 0.93 (3H, t, *J* 7.5, Ile δC*H*$_3$), 0.99 (3H, d, *J* 7.0, Ile γC*H*$_3$), 1.13-1.22 (1H, m, Ile γC*H*$_A$H$_B$), 1.45 (9H, s, C(C*H*$_3$)$_3$), 1.52-1.58 (1H, m, Ile γCH$_A$*H*$_B$), 1.78 (3H, d, *J* 7.0, Dhb γC*H*$_3$), 1.94-1.99 (1H, m, Ile βC*H*), 4.06-4.09 (1H, m, Ile αC*H*), 4.64 (2H, dt, *J* 6.0, 1.5, CO_2CH_2CH=CH_2) 5.03-5.04 (1H, m, N*H*Boc), 5.24 (1H, dq, *J* 10.5, 1.5, CO_2CH_2CH=C*H*$_2$cis), 5.33 (1H, dq, *J* 17.0, 1.5, CO_2CH_2CH=C*H*$_2$trans), 5.88-5.96 (1H, m, CO_2CH_2C*H*=CH_2), 6.86 (1H, q, *J* 7.0, Dhb βC*H*), 7.32 (1H, br s, CON*H*); δ_C (125 MHz, $CDCl_3$) 11.6 (Ile δ*C*H$_3$), 14.8 (Dhb γ*C*H$_3$), 15.7 (Ile γ*C*H$_3$), 24.8 (Ile γ*C*H$_2$), 28.4 (C(*C*H$_3$)$_3$), 37.1 (Ile β*C*H), 59.7 (Ile α*C*H), 66.0 (CO_2*C*H_2CH=CH_2), 79.4 (*C*(CH$_3$)$_3$), 118.7 (CO_2CH_2CH=*C*H$_2$), 125.8 (Dhb α*C*), 131.9 (CO_2CH_2*C*H=CH_2), 134.8 (Dhb β*C*H), 155.9 (NH*C*O$_2$*t*Bu), 164.0 (*C*O$_2$Allyl), 170.1 (*C*ONH); *m/z* (LCMS, ES+) 355.1 $[M+H]^+$, 709.4 $[2M+H]^+$, 731.5 $[2M+Na]^+$. Data is in agreement with the literature.[304]

Boc-Ile-Dhb-OH (135)[173]

(*Z*)-2-((2*S*,3*S*)-2-((*Tert*-butoxycarbonyl)amino)-3-methylpentanamido)but-2-enoic acid



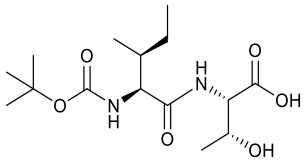
135

To a solution of Boc-Ile-Dhb-OAllyl **134** (560 mg, 1.58 mmol) in degassed CH_2Cl_2 (66 mL) under Ar, $PhSiH_3$ (0.39 mL, 3.16 mmol) and $Pd(PPh_3)_4$ (183 mg, 0.158 mmol) were added. The solution was stirred in the dark for 2 h before removal of the solvent *in vacuo*. Purification by flash column chromatography (CH_2Cl_2:MeOH, 9:1) yielded title compound **135** as a white solid (286 mg, 0.91 mmol, 58%). R_f 0.56 (CH_2Cl_2:MeOH, 9:1); mp 148-150 °C (lit.[146] 150-152 °C); $[\alpha]_D^{25}$ -38.5 (*c* 10.4 mg mL^{-1}, MeOH), [lit.[146] $[\alpha]_D^{25}$ -33.6 (*c* 10.4 mg mL^{-1}, MeOH)]; δ_H (600 MHz, CD_3OD) 0.92 (3H, t, *J* 7.2, Ile δC*H*$_3$), 1.00 (3H, d, *J* 6.6, Ile γC*H*$_3$), 1.17-1.22 (1H, m, Ile γC*H*$_A$H$_B$), 1.45 (9H, s, C(C*H*$_3$)$_3$), 1.57-1.61 (1H, m, Ile γCH$_A$*H*$_B$), 1.70 (3H, d, *J* 7.2, Dhb γC*H*$_3$), 1.84-1.88 (1H, m, Ile

βC*H*), 4.04 (1H, d, *J* 7.2, Ile αC*H*), 6.67 (1H, q, *J* 7.2, Dhb βC*H*); δ_C (150 MHz, CD_3OD) 11.6 (Ile δ*C*H_3), 14.2 (Dhb γ*C*H_3), 16.1 (Ile γ*C*H_3), 25.8 (Ile γ*C*H_2), 28.7 (C(*C*H_3)$_3$), 38.4 (Ile β*C*H), 61.0 (Ile α*C*H), 80.7 (*C*(CH$_3$)$_3$), 131.2 (Dhb α*C*), 132.0 (Dhb β*C*H),), 158.0 (NH*C*O$_2$*t*Bu), 171.4 (*C*O$_2$H), 173.2 (*C*ONH); *m/z* (LCMS, ES+) 315.3 $[M+H]^+$, 629.6 $[2M+H]^+$, 651.5 $[2M+Na]^+$, 943.8 $[3M+H]^+$. Data is in agreement with the literature.[173]

Boc-Ile-Thr-OH (133)[173]

(*Tert*-butoxycarbonyl)-L-isoleucyl-L-threonine



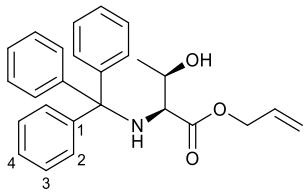
133

To a solution of Boc-Ile-Thr-OAllyl (**130**) (700 mg, 1.88 mmol) in degassed CH_2Cl_2 (70 mL) under Ar, $PhSiH_3$ (0.46 mL, 3.76 mmol) and $Pd(PPh_3)_4$ (217 mg, 0.188 mmol) were added. The solution was stirred in the dark for 2 h before removal of the solvent *in vacuo*. Purification by flash column chromatography (CH_2Cl_2:MeOH, 9:1) yielded title compound **133** as a white solid (232 mg, 0.70 mmol, 37%). R_f 0.26 (CH_2Cl_2:MeOH, 9:1); $[\alpha]_D^{25}$ -13.7 (*c* 10.0 mg mL^{-1}, MeOH); δ_H (600 MHz, CD_3OD) 0.91 (3H, t, *J* 7.2, Ile δC*H*$_3$), 0.94 (3H, d, *J* 7.2, Ile γC*H*$_3$), 1.15-1.21 (4H, m, Thr γC*H*$_3$ and Ile γC*H*$_A$H$_B$), 1.45 (9H, s, C(C*H*$_3$)$_3$), 1.48-1.56 (1H, m, Ile γCH$_A$*H*$_B$), 1.82-1.89 (1H, m, Ile βC*H*), 3.96 (1H, d, *J* 7.2, Ile αC*H*), 4.20-4.24 (1H, m, Thr βC*H*), 4.27-4.29 (1H, m, Thr αC*H*); δ_C (150 MHz, CD_3OD) 11.7 (Ile δ*C*H$_3$), 16.3 (Ile γ*C*H$_3$), 19.9 (Thr γ*C*H$_3$), 26.0 (Thr γ*C*H$_2$), 28.9 (C(*C*H$_3$)$_3$), 38.1 (Ile βCH), 60.2 (Thr α*C*H), 61.3 (Ile α*C*H), 69.3 (Thr β*C*H), 80.8 (*C*(CH$_3$)$_3$), 158.3 (NH*C*O$_2$*t*Bu), 174.4 (*C*ONH), 177.4 (*C*O$_2$H); *m/z* (LCMS, ES+) 333.1 $[M+H]^+$, 665.6 $[2M+H]^+$, 997.9 $[3M+H]^+$, (LCMS, ES-) 331.4 $[M-H]^-$, 663.7 $[2M-H]^-$. Data is in agreement with the literature.[173]

7.1.8. β-methyl-cysteine Synthesis

Trt-L-Thr-OAllyl (L-119)[226]

Allyl trityl-L-threoninate

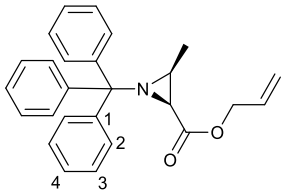


L-119

To a solution of L-threonine (2.50 g, 21.0 mmol) in toluene (50 mL) was added *p*-toluenesulfonic acid monohydrate (4.79 g, 21.0 mmol) and allyl alcohol (14.3 mL, 0.21 mol). The reaction was then heated at reflux with a Dean & Stark trap for 24 h before removal of the solvent *in vacuo*. The residue was then redissolved in EtOAc (50 mL) and cooled in ice. To this was added Et_3N (10.6 mL, 0.075 mol) dropwise and the solution stirred for 10 min before the addition of trityl chloride (5.85 g, 21.0 mmol) in EtOAc (25 mL) *via* addition funnel over 45 min. The solution was then allowed to warm to rt and stirred for 16 h before washing with water (2 x 150 mL) and brine (100 mL). The organic layer was dried ($MgSO_4$) and the solvent removed *in vacuo*. Purification by flash column chromatography (pet. ether:EtOAc, 5:1→2:1) yielded title compound **119** as a pale yellow oil (4.41 g, 11.0 mmol, 52% over 2 steps); R_f 0.32 (pet. ether:EtOAc, 5:1); $[\alpha]_D^{20}$ +8.6 (*c* 23 mg mL^{-1}, $CHCl_3$), [lit.[226] $[\alpha]_D^{25}$ -9.02 (*c* 23.0 mg mL^{-1}, $CHCl_3$, data for D isomer)]; δ_H (600 MHz, $CDCl_3$) 1.22 (3H, d, *J* 6.6, CHC*H*$_3$), 3.40 (1H, d, *J* 7.8, C*H*CO_2Allyl), 3.78-3.84 (1H, m, C*H*CH_3), 3.86 (1H, ddt, *J* 12.6, 6.0, 1.5, $CO_2CH_AH_BCH=CH_2$), 4.08 (1H, ddt, *J* 13.2, 6.0, 1.2, $CO_2CH_AH_BCH=CH_2$), 5.15-5.20 (2H, m, $CO_2CH_2CH=CH_2$), 5.63-5.70 (1H, m, $CO_2CH_2CH=CH_2$), 7.20 (3H, tt, *J* 7.2, 1.2, Ar*H*(4)), 7.27 (6H, m, Ar*H*(3)), 7.48 (6H, m, Ar*H*(2)); δ_C (150 MHz, $CDCl_3$) 18.9 ($CHCH_3$), 62.5 ($CHCO_2$Allyl), 65.6 ($CO_2CH_2CH=CH_2$), 69.8 ($CHCH_3$), 70.8 ($NHCPh_3$), 118.9 ($CO_2CH_2CH=CH_2$), 126.6 (*C*4), 127.9 (*C*3), 128.9 (*C*2), 131.5 ($CO_2CH_2CH=CH_2$), 145.4 (*C*1), 172.9 (CO_2H); *m/z* (LCMS, ES+) 402.4 $[M+H]^+$, 424.3 $[M+Na]^+$, 803.6 $[2M+H]^+$, 825.7 $[2M+Na]^+$. Data is in agreement with the literature.[226]

***N*-Trt/CO_2Allyl Aziridine (L-120)**[226]

Allyl (2*S*,3*S*)-3-methyl-1-tritylaziridine-2-carboxylate

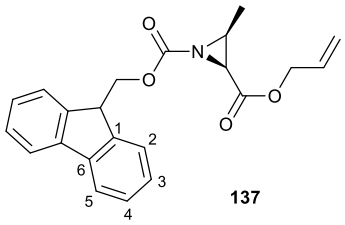


L-120

A stirred solution of allyl trityl-L-threoninate (**119**) (4.00 g, 9.96 mmol) in THF (100 mL) under Ar was cooled in ice before the addition of Et_3N (3.06 mL, 21.9 mmol) dropwise over 10 min. Methanesulfonyl chloride (0.85 mL, 11.0 mmol) was then added dropwise and the solution stirred in ice for 30 min before heating at reflux for 48 h. The solvent was removed *in vacuo* before being redissolved in EtOAc (60 mL) and washed with 10% aqueous citric acid (3 x 20 mL), water (2 x 20 mL), aqueous $NaHCO_3$ (2 x 30 mL), water (2 x 20 mL) and brine (20 mL). The organic layer was dried ($MgSO_4$) and the solvent removed *in vacuo*. Purification by flash column chromatography (pet. ether:EtOAc, 95:5 → 9:1) yielded title compound **120** as a clear oil (2.21 g, 5.76 mmol, 58%). R_f 0.70 (pet. ether:EtOAc, 5:1); $[\alpha]_D^{25}$ -105.4 (*c* 12.5 mg mL^{-1}, $CHCl_3$), [lit.[226] $[\alpha]_D^{25}$ +98.4 (*c* 12 mg mL^{-1}, $CHCl_3$, data for D isomer)]; δ_H (600 MHz, $CDCl_3$) 1.39 (3H, d, *J* 5.4, CHC*H*$_3$), 1.65 (1H, quintet, *J* 6.0, C*H*CH_3), 1.92 (1H, d *J* 6.6, C*H*CO_2Allyl), 4.63 (1H, ddt, *J* 13.2, 6.0, 1.2, $CO_2CH_AH_BCH{=}CH_2$), 4.72 (1H, ddt, *J* 13.2, 6.0, 1.2, $CO_2CH_AH_BCH{=}CH_2$), 5.25 (1H, dq, *J* 10.2, 1.5, $CO_2CH_2CH{=}CH_2$cis), 5.34 (1H, dq, *J* 17.4, 1.5, $CO_2CH_2CH{=}CH_2$trans), 5.91-5.97 (1H, m, $CO_2CH_2CH{=}CH_2$), 7.22 (3H, tt, *J* 7.2, 1.5, Ar*H*(4)), 7.27-7.30 (6H, m, Ar*H*(3)), 7.54 (6H, d, *J* 7.8, Ar*H*(2)); δ_C (150 MHz, $CDCl_3$) 13.3 (CH*C*H_3), 34.9 (*C*HCH_3), 36.0 (*C*HCO_2Allyl), 65.4 ($CO_2CH_2CH{=}CH_2$), 75.0 (NH*C*Ph_3), 118.5 ($CO_2CH_2CH{=}CH_2$), 126.8 (*C*4), 127.6 (*C*3), 129.4 (*C*2), 132.1 ($CO_2CH_2CH{=}CH_2$), 143.9 (*C*1), 169.9 (CO_2Allyl); *m/z* (LCMS, ES+) 384.3 $[M+H]^+$, 406.3 $[M+Na]^+$. Data is in agreement with the literature.[226]

***N*-Fmoc/CO_2Allyl Aziridine (137)**[229]

1-((9H-Fluoren-9-yl)methyl) 2-allyl (2*S*,3*S*)-3-methylaziridine-1,2-dicarboxylate

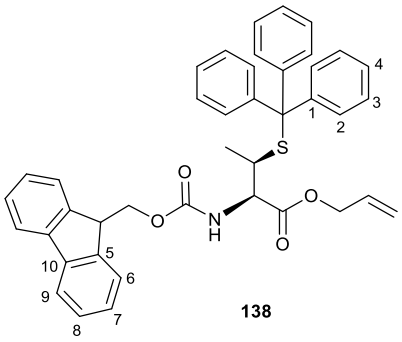


137

A solution of aziridine **L-120** (2.05 g, 5.35 mmol) in CH_2Cl_2:MeOH (3:1, 4 mL) under Ar was cooled in ice before the dropwise addition of TFA (3 mL). The solution was stirred in ice for 2 h before pouring into ether (4 mL) and washing with water (4 mL). The organic layer was further washed with water (3 x 4 mL) and the aqueous layers combined and cooled in ice before basifying to pH 9 with Na_2CO_3. To this solution was then added EtOAc (16 mL) and FmocCl (1.73 g, 6.69 mmol). The biphasic mixture was then warmed to room temperature and stirred vigorously for 24 h. After this time, the layers were separated and the aqueous layer extracted with EtOAc (3 x 20 mL). The organic layers were combined, dried ($MgSO_4$) and the solvent removed *in vacuo*. Purification by flash column chromatography (pet. ether:EtOAc, 9:1) yielded title compound **137** as a clear oil (1.50 g, 4.13 mmol, 45%). R_f 0.32 (pet. ether:EtOAc, 5:1); $[\alpha]_D^{20}$ -59.5 (*c* 13.2 mg mL^{-1}, $CHCl_3$); δ_H (600 MHz, $CDCl_3$) 1.38 (3H, d, *J* 5.4, CHC*H*$_3$), 2.69-2.73 (1H, m, C*H*CH_3), 3.11 (1H, d, *J* 6.6, C*H*CO_2Allyl), 4.25 (1H, t, *J* 7.2, ArC*H*CH_2), 4.44 (2H, d, *J* 6.6, ArCHC*H*$_2$), 4.71 (2H, tt, *J* 5.7, 1.2, CO_2C*H*$_2$CH=CH_2), 5.30 (1H, dq, *J* 10.2, 1.2, CO_2CH_2CH=C*H*$_2$cis), 5.38 (1H, dq, *J* 16.8, 1.2, CO_2CH_2CH=C*H*$_2$trans), 5.93-5.99 (1H, m, CO_2CH_2C*H*=CH_2), 7.31-7.34 (2H, m, Ar*H*(3)), 7.42 (2H, tt, *J* 7.8, 0.9, Ar*H*(4)), 7.59-7.61 (2H, m, Ar*H*(2)), 7.77 (2H, dt, *J* 7.8, 0.6, Ar*H*(5)); δ_C (150 MHz, $CDCl_3$) 12.9 (CH*C*H_3), 39.0 (*C*HCH_3), 39.8 (*C*HCO_2Allyl), 46.9 (Ar*C*HCH_2), 66.1 (CO_2*C*H_2CH=CH_2), 68.5 (ArCH*C*H_2), 119.1 (CO_2CH_2CH=*C*H_2), 120.0 (*C*3), 125.1 (*C*4), 127.1 (*C*2), 127.9 (*C*5), 131.5 (CO_2CH_2*C*H=CH_2), 141.3 (*C*6), 143.4 (*C*1), 161.6 (N*C*O_2Fmoc), 166.8 (*C*O_2Allyl); *m/z* (LCMS, ES+) 142.2 $[M\text{-Fmoc+H}]^+$, 364.3 $[M+H]^+$, 386.2 $[M+Na]^+$, 727.5 $[2M+H]^+$. Data is in agreement with the literature.[229]

Fmoc-β-Me-Cys(Trt)-OAllyl (138)[229]

Allyl (2*R*,3*R*)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(tritylthio) butanoate



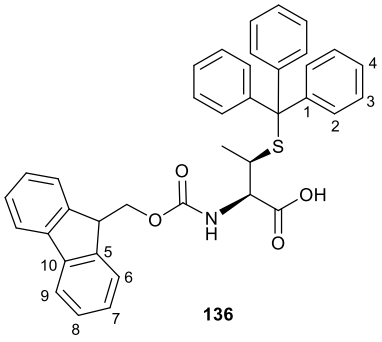
138

To a stirred solution of aziridine **137** (1.29 g, 3.55 mmol) in dry CH_2Cl_2 (30 mL) under Ar at 0 °C was added triphenylmethanethiol (3.43 g, 12.4 mmol) followed by the dropwise addition of a solution of $BF_3.OEt_2$ (0.92 mL, 7.46 mmol) in dry CH_2Cl_2 (5.7 mL). The reaction was stirred for 3 h before quenching with saturated aqueous $NaHCO_3$. The layers were separated and the aqueous layer further extracted with CH_2Cl_2 (2 x 20 mL). The organic layers were then combined, dried ($MgSO_4$) and the solvent removed *in vacuo*. Purification by flash column chromatography (pet. ether:EtOAc, 9:1 → 7:1) yielded title compound **138** as a white solid (730 mg, 1.14 mmol, 32%). R_f 0.29 (pet. ether:EtOAc, 5:1); mp 44-46 °C; $[\alpha]_D^{20}$ +22.0 (*c* 12.0 mg mL^{-1}, $CHCl_3$); δ_H (600 MHz, $CDCl_3$) 0.88 (3H, d, 7.2, CHC*H*$_3$), 2.77-2.81 (1H, m, C*H*CH$_3$), 4.26 (1H, t, *J* 6.9, ArC*H*CH$_2$), 4.41 (2H, d, *J* 6.6, ArCHC*H*$_2$), 4.42-4.48 (2H, m, C*H*CO$_2$Allyl and CO$_2$C*H*$_A$H$_B$CH=CH$_2$), 4.66 (1H, dd, *J* 13.2, 6.0, CO$_2$CH$_A$*H*$_B$CH=CH$_2$), 5.24 (1H, dd, *J* 10.2, 1.2, CO$_2$CH$_2$CH=C*H*$_2$cis), 5.31 (1H, dd, *J* 17.4, 1.2, CO$_2$CH$_2$CH=C*H*$_2$trans), 5.40 (1H, d, *J* 9.0, N*H*Fmoc), 5.80-5.86 (1H, m, CO$_2$CH$_2$C*H*=CH$_2$), 7.22 (3H, t, *J* 7.2, Ar*H*(4)), 7.28-7.35 (8H, m, Ar*H*(3) and Ar*H*(7)), 7.40-7.44 (2H, m, Ar*H*(8)), 7.52 (6H, d, *J* 7.8, Ar*H*(2)), 7.63 (2H, t, *J* 7.2, Ar*H*(6)), 7.78 (2H, t, *J* 7.2, Ar*H*(9)); δ_C (150 MHz, $CDCl_3$) 20.1 (CH*C*H$_3$), 42.9 (*C*HCH$_3$), 47.1 (Ar*C*HCH$_2$), 59.4 (*C*HCO$_2$Allyl), 66.2 (CO$_2$*C*H$_2$CH=CH$_2$), 67.2 (ArCH*C*H$_2$), 67.7 (S*C*Ph$_3$), 119.0 (CO$_2$CH$_2$CH=*C*H$_2$), 120.0 (*C*9), 125.1 (*C*6), 126.7 (*C*4), 127.1 (*C*7), 127.7 (*C*8), 127.9 (*C*3), 129.6 (*C*2), 131.4 (CO$_2$CH$_2$*C*H=CH$_2$), 141.3 (*C*10), 143.7 (*C*5), 144.6 (*C*1), 156.1 (*C*ONH), 170.1 (*C*O$_2$Allyl); ν_{max} (cm^{-1}) 3402 (amide N-H), 3056 (Ar C-H), 2948 (alkyl C-H), 1720

(C=O); *m/z* (HRMS, ES+) required for $[C_{41}H_{37}NO_4S+Na]^+$ 662.2341, found $[C_{41}H_{37}NO_4S+Na]^+$ 662.2345.

Fmoc-β-Me-Cys(Trt)-OH (136)

(2*R*,3*R*)-2-((((9H-Fluoren-9-yl)methoxy)carbonyl)amino)-3-(tritylthio)butanoic acid



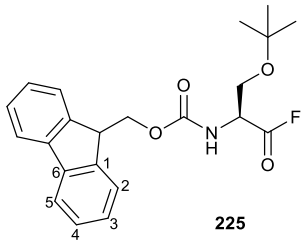
136

To a solution of Fmoc-β−Me-Cys(Trt)-OAllyl **138** (730 mg, 1.14 mmol) in degassed CH_2Cl_2 (47 mL) under Ar was added $PhSiH_3$ (0.282 mL, 2.28 mmol) and $Pd(PPh_3)_4$ (132 mg, 0.114 mmol). The solution was stirred in the dark for 2 h before removal of the solvent *in vacuo*. Purification by flash column chromatography (CH_2Cl_2:MeOH, 9:1) yielded title compound **136** as a brown solid (162 mg, 0.270 mmol, 24%). R_f 0.61 (CH_2Cl_2:MeOH, 9:1); mp 113-116 °C; $[\alpha]_D^{20}$ +7.2 (*c* 13.2 mg mL^{-1}, $CHCl_3$); δ_H (600 MHz, CD_3OD) 0.78 (3H, d, *J* 7.2, CHC*H*$_3$), 2.66-2.70 (1H, m, C*H*CH$_3$), 4.22 (1H, t, *J* 6.9, ArC*H*CH$_2$), 4.29-4.37 (2H, m, C*H*CO$_2$H and ArCHC*H*$_A$H$_B$), 4.41 (1H, dd, *J* 10.8, 6.9, ArCHCH$_A$*H*$_B$), 7.19 (3H, t, *J* 7.2, Ar*H*(4)), 7.25-7.30 (8H, m, Ar*H*(3) and Ar*H*(7)), 7.38 (2H, q, *J* 7.2, Ar*H*(8)), 7.49 (6H, dd, *J* 8.1, 0.9, Ar*H*(2)), 7.65 (2H, dd, *J* 10.8, 8.1, Ar*H*(6)), 7.78 (2H, dd, *J* 7.2, 2.7, Ar*H*(9)); δ_C (150 MHz, CD_3OD) 19.3 (CH*C*H$_3$), 43.8 (*C*HCH$_3$), 48.6 (Ar*C*HCH$_2$), 61.1 (*C*HCO$_2$H), 68.1 (ArCH*C*H$_2$), 69.0 (S*C*Ph$_3$), 121.1 (*C*9), 126.5 (*C*6), 127.9 (*C*4), 128.3 (*C*7), 128.9 (*C*8), 129.0 (*C*3), 131.0 (*C*2), 142.7 (*C*10), 145.3 (*C*5), 146.4 (*C*1), 158.4 (*C*ONH), 173.7 (*C*O$_2$H); ν_{max} (cm^{-1}) 3059 (Ar C-H), 2948 (alkyl C-H), 1722 (C=O); *m/z* (HRMS, ES-) required for $[C_{38}H_{33}NO_4S-H]^-$ 598.2051, found $[C_{38}H_{33}NO_4S-H]^-$ 598.2052.

7.1.9. Amino Acid Fluoride Synthesis

Fmoc-Ser(O*t*Bu)-F (225)[237]

(9H-Fluoren-9-yl)methyl(*S*)-(3-(*tert*-butoxy)-1-fluoro-1-oxopropan-2-yl)carbamate

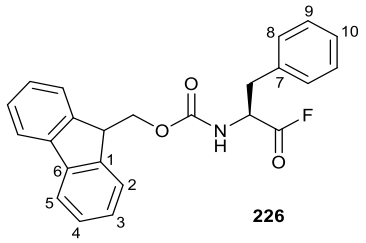


225

To a stirred solution of Fmoc-Ser(*t*Bu)-OH (250 mg, 0.65 mmol) in dry CH_2Cl_2 (2.5 mL) was added dropwise dry pyridine (52.7 µL, 0.65 mmol) followed by cyanuric fluoride (111.5 µL, 1.30 mmol). The solution was heated at reflux for 3 h, after which time a pale yellow precipitate formed. The reaction mixture was then cooled to room temperature and poured into CH_2Cl_2 (10 mL), and washed with ice water (3 x 10 mL) and brine (15 mL). The organic layer was dried ($MgSO_4$) and the solvent removed *in vacuo* to yield the crude title compound **225** as an orange oil which was used without further purification (219 mg, 0.57 mmol, 87%). $[\alpha]_D^{20}$ +21.4 (*c* 7.5 mg mL^{-1}, EtOAc, 20 °C), [lit.[305] $[\alpha]_D^{26}$ +28.8 (*c* 7.5 mg mL^{-1}, EtOAc, 26 °C)]; δ_H (600 MHz, $CDCl_3$) 1.20 (9H, s, OC(CH_3)$_3$), 3.63 (1H, dd, *J* 9.3, 2.9, CHCH_AH$_B$O*t*Bu), 3.91 (1H, dd, *J* 9.3, 2.6, CHCH$_A$$H_B$O*t*Bu), 4.27 (1H, t, *J* 7.2, ArC*H*CH$_2$), 4.41 (1H, dd, *J* 10.8, 7.2, ArCHCH_AH$_B$), 4.48 (1H, dd, *J* 10.8, 7.2, ArCHCH$_A$$H_B$), 4.70-4.72 (1H, m, C*H*CH$_2$O*t*Bu), 5.67 (1H, d, *J* 8.8, N*H*), 7.34 (2H, td, *J* 7.4, 0.9, Ar*H*(3)), 7.42 (2H, t, *J* 7.5, Ar*H*(4)), 7.62 (2H, t, *J* 6.9, Ar*H*(2)), 7.78 (2H, d, *J* 7.2, Ar*H*(5)); δ_C (150 MHz, $CDCl_3$) 27.2 (OC(*C*H$_3$)$_3$), 47.0 (Ar*C*HCH$_2$), 53.9 (J_{CF} 60.2 Hz, *C*HCH$_2$O*t*Bu), 61.1 (CH*C*H$_2$O*t*Bu), 67.4 (ArCH*C*H$_2$), 74.2 (O*C*(CH$_3$)$_3$), 120.0 (*C*5), 125.0 (*C*2), 127.1 (*C*3), 127.8 (*C*4), 141.3 (*C*6), 143.5 (*C*1), 143.7 (*C*1), 155.9 (*C*ONH), 161.2 (J_{CF} 367.5 Hz, *C*OF); ν_{max} (cm^{-1}) 3439 (amide N-H), 3313, 3066 (aryl C-H), 2974 (alkyl C-H), 1851, 1717 (C=O), 1507 (COF); *m/z* (LCMS, ES+, converted to the methyl ester during analysis) 398.4 $[M+H]^+$, 795.7 $[2M+H]^+$. Data is in agreement with the literature.[237]

Fmoc-Phe-F (226)[306]

(9H-Fluoren-9-yl)methyl (*S*)-(1-fluoro-1-oxo-3-phenylpropan-2-yl)carbamate



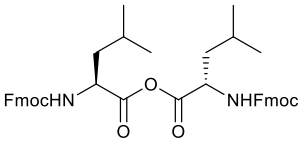
226

To a stirred solution of Fmoc-Phe-OH (258 mg, 0.67 mmol) in dry CH_2Cl_2 (2.5 mL) was added dropwise dry pyridine (54.0 µL, 0.67 mmol) followed by cyanuric fluoride (114 µL, 1.33 mmol). The solution was heated at reflux for 3 h, after which time a white precipitate formed. The reaction mixture was then cooled to room temperature and poured into CH_2Cl_2 (10 mL), and washed with ice water (3 x 10 mL) and brine (15 mL). The organic layer was dried ($MgSO_4$) and the solvent removed *in vacuo*. Recrystallisation from CH_2Cl_2/hexane gave title compound **226** as a fluffy white solid (194.5 mg, 0.50 mmol, 75%). mp 121-124 °C (lit.[305] 118-120 °C); $[\alpha]_D^{20}$ +25.0 (*c* 7.5 mg mL^{-1}, CH_2Cl_2, 20 °C), [lit.[305] $[\alpha]_D^{24}$ +35.5 (*c* 7.5 mg mL^{-1}, CH_2Cl_2, 24 °C)]; δ_H (600 MHz, $CDCl_3$) 3.17-3.24 (2H, m, CHC*H*$_2$Ph), 4.21 (1H, t, *J* 6.6, ArC*H*CH$_2$), 4.41-4.49 (2H, m, ArCHC*H*$_2$), 4.70 (1H, dd, *J* 13.8, 6.0, C*H*CH$_2$Ph), 5.24 (1H, d, *J* 7.8, N*H*), 7.15 (1H, t, *J* 7.2, Ar*H*(9)), 7.28-7.36 (5H, m, Ar*H*(3), Ar*H*(8) and Ar*H*(10)), 7.42 (2H, dd, *J* 13.2, 6.6, Ar*H*(4)), 7.55 (2H, dd, *J* 14.4, 7.2, Ar*H*(2)), 7.78 (2H, d, *J* 7.2, Ar*H*(5)); δ_C (150 MHz, $CDCl_3$) 37.7 (CH*C*H$_2$Ph), 47.1 (Ar*C*HCH$_2$), 53.7 (J_{CF} 61.2 Hz, *C*HCH$_2$Ph), 67.0 (ArCH*C*H$_2$), 120.0 (*C*5), 125.0 (*C*2), 127.1 (*C*3), 127.3 (*C*10), 127.8 (*C*4), 128.7 (*C*8), 129.2 (*C*9), 135.5 (*C*7), 141.3 (*C*6), 143.6 (*C*1), 143.7 (*C*1), 155.7 (*C*ONH), 174.5 (*C*OF); ν_{max} (cm^{-1}) 3315 (amide N-H), 3064 (aryl C-H), 3031, 2955 (alkyl C-H), 1840, 1695 (C=O), 1536 (COF); *m/z* (LCMS, ES+, converted to the methyl ester during analysis) 402.4 $[M+H]^+$, 803.6 $[2M+H]^+$. Data is in agreement with the literature.[306]

7.1.10. Amino Acid Anhydride Synthesis

Fmoc-Leu anhydride (227)

(*S*)-2-((((9H-Fluoren-9-yl)methoxy)carbonyl)amino)-4-methylpentanoic anhydride

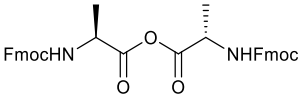


227

To a solution of Fmoc-Leu-OH (250 mg, 707 µmol) in CH_2Cl_2 (10 mL) was added DCC (73 mg, 350 µmol, 0.5 eq). After 3 h the solution was filtered and the solvent removed *in vacuo*. The title compound **227** was used directly in SPPS without purification.

Fmoc-Ala anhydride (228)

(*S*)-2-((((9H-Fluoren-9-yl)methoxy)carbonyl)amino)propanoic anhydride

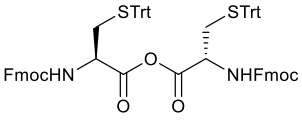


228

To a solution of Fmoc-Ala-OH (250 mg, 803 µmol) in CH_2Cl_2 (10 mL) was added DCC (83 mg, 401 µmol, 0.5 eq). After 3 h the solution was filtered and the solvent removed *in vacuo*. The title compound **228** was used directly in SPPS without purification.

Fmoc-Cys(Trt) anhydride (229)

(*R*)-2-((((9H-Fluoren-9-yl)methoxy)carbonyl)amino)-3-(tritylthio)propanoic anhydride



229

To a solution of Fmoc-Cys(Trt)-OH (250 mg, 427 µmol) in dry CH_2Cl_2 (2.5 mL) was added DCC (42 mg, 203 µmol, 0.48 eq). After 30 min the solution was filtered and the

solvent removed *in vacuo*. The title compound **229** was used directly in SPPS without purification.

7.2. Synthesis of Peptides

7.2.1. General Peptide Experimental

Peptides were synthesised by hand using the Fmoc solid-phase synthesis strategy. All residues were added to the peptides manually. The resin was continually agitated throughout coupling, deprotection and cleavage steps by shaking at 480 rpm on an IKA KS130 basic platform shaker. For reactions requiring heating as well as shaking, a Bioer Mixing Block MB-102 was used. Microwave reactions were conducted using a Personal Chemistry Smith Creator microwave-assisted organic synthesizer system in 5 mL reaction vials, with maximum 300 W power. An Eppendorf centrifuge model 5810R was used for centrifugation of peptide products before freeze drying by a SP Scientific VirTis BenchTop Pro. All steps not conducted in the microwave were performed whilst shaking at room temperature in a 5 mL PP reaction syringe with a frit. The resin was washed copiously with DMF following each coupling and deprotection. Washing the resin refers to the addition of solvent to the resin followed by immediate evacuation. Following cleavage from the resin, crude peptides were analysed by LCMS to check for the presence of intermediate and by-product sequences, such as truncation and deletion sequences, and uncyclised peptide. Where these were found, the LCMS traces are shown in **Chapter 3** and **Chapter 4**.

Peptides were purified by preparative reverse phase HPLC on a Dionex 580 HPLC System with PDA-100 photodiode array detector, P580 Pump and a model ASI-100 automated sample injector. A Phenomenex Onyx C18 100 x 10 mm column, a Dr Maisch GmbH Reprosil Gold 200 C8 5µm 150 x 10 mm column or an Agilent Zorbax 300SB-C18 5µm 250 x 9.4 mm column were used (as stated), with detection at 214 and 254 nm. Water (0.1% TFA) and acetonitrile (0.1% TFA) were used as solvents. Chromatograms were analysed using Chromeleon Software version 2.0. Analytical HPLC of peptides was performed on the above described machine or an Agilent Technologies 1260 Infinity system using either a Fluka Analytical Discovery BIO C18-10 25 x 4.6 mm column, an ACE5 C18-300 150 x 4.6 mm column, or a Dr Maisch GmbH Reprosil Gold 200 C8 5µm 250 x 4.6 mm column, with detection at 214 and 254 nm. A linear solvent gradient of

2-98% MeCN (0.1% TFA) in H_2O (0.1% TFA) over 15 min was used, at a flow rate of 1 mL min^{-1}.

All 1D and 2D NMR spectra were recorded on a Bruker Avance-600 MHz spectrometer, with chemical shifts (δ) given in ppm relative to the solvent signal, and coupling constants (*J*) given in Hz. To prepare peptide samples for NMR, the lyophilised powders were dissolved in 0.3 mL anhydrous deuterated d_6-DMSO which was purchased from Sigma-Aldrich Co. Ltd.. Abbreviations used in ^{1}H NMR assignment are the same as those described in **Chapter 7.1.1.** Although not stereospecifically assigned, in peptides containing Leu, the two δ methyl groups and their corresponding carbon shifts could be distinguished from each other, and the coupling pairs are denoted 'a' and 'b'. Data processing was carried out using ACD/NMR Processor Academic Edition, version 12.01 (Advanced Chemistry Development Inc.), CCPN Analysis version 2.4 and CCPN Chembuild.[274]

General Methods for Peptide Synthesis

The exact masses and volumes of amino acid, coupling reagent and base used per coupling step are tabulated under the entry for each peptide synthesised.

1. Swelling the resin:
   DMF (2 mL) was added to the resin in a syringe and shaken for 30 min. The DMF was then evacuated and the resin washed with DMF (2 x 2 mL).
2. Fmoc Deprotection:
   A solution of piperidine in DMF (40% v/v, 1.5 mL) was added to the resin and left to shake for 3 min. After this time the syringe was evacuated. Another portion of piperidine in DMF (20% v/v, 1.5 mL) was then added to the resin and left to stir for 10 min. This was evacuated and the resin washed with DMF (6 x 2 mL).
   For Fmoc deprotections following Fmoc-Sec(Ph)-OH incorporation.
   A solution of piperidine in DMF (20% v/v, 1.5 mL) was added to the resin and left to shake for 1 min. After this time the syringe was evacuated. Another portion of piperidine in DMF (20% v/v, 1.5 mL) was then added to the resin and left to shake for 1 min. This was evacuated and a final portion of piperidine in DMF (20% v/v, 1.5 mL) added to the resin, shaken for 1 min and then the solution evacuated. The resin was then washed with DMF (6 x 2 mL).

3. (Methyl)lanthionine coupling steps:

   For lanthionine-containing peptides.

   The protected lanthionine **38** (3 eq), HOAt (5 eq) and PyAOP (5 eq) were dissolved in DMF (2 mL) in a glass vial and DIPEA (10 eq) was added. This solution was left to preactivate for 2 min and then, along with the resin, was transferred to a microwave vial and coupled in the microwave at 60 °C for 5 min followed by a further 1 h stirring at rt. The resin and coupling solution were then transferred back to the reaction syringe, the coupling solution was removed and the resin washed thoroughly with DMF (4 x 2 mL).

   For methyllanthionine-containing peptides.

   The same procedure was followed exactly for methyllanthionine-containing peptides, except that protected methyllanthionine **70** (2.5 eq), HOBt (5 eq), PyBOP (5 eq) and NMM (10 eq) were used in place of Lan **38**, HOAt, PyAOP and DIPEA.

4. Elongation steps:

   Double coupling of normal Fmoc protected amino acids.

   The desired Fmoc protected amino acid (5 eq), HOAt (5 eq) and PyAOP (5 eq) were dissolved in DMF (1.5 mL for 50 mg scale reactions, 2 mL for 100 and 150 mg scale reactions) and DIPEA (10 eq) was added. This solution was left to preactivate for 2 min and then added to the syringe containing the resin. The suspension was stirred at rt for 2 h before removal of the coupling solution. A fresh sample of the same preactivated coupling solution was then added to the resin and left to stir for 2 h before evacuation. The resin was then washed with DMF (4 x 2 mL).

   Double coupling with amino acid anhydrides.

   The desired amino acid anhydride (10 eq) was dissolved in CH_2Cl_2 (1.5 mL for 50 mg scale reactions, 2 mL for 100 and 150 mg scale reactions) and added to the syringe containing the resin. The suspension was stirred at rt for 2 h before removal of the coupling solution. A fresh sample of the same coupling solution was then added to the resin and left to stir for 2 h before evacuation. The resin was then washed with DMF (4 x 2 mL).

5. Ring closing steps:

   a. The silyl or allyl protecting groups were first removed. To remove silyl groups, a solution of TBAF (1M in THF, 1 mL) in DMF (1 mL) was added to the resin and left to stir at rt under Ar for 1 h. After this time the TBAF solution was removed and the resin washed with DMF (6 x 2 mL). To remove allyl groups, a solution of

$Pd(PPh_3)_4$ (2 eq) and $PhSiH_3$ (10 eq) in CH_2Cl_2:DMF (1:1, 2 mL) was added to the resin and stirred in the dark for 2 h. The deprotection solution was then removed and the resin washed with CH_2Cl_2 (5 x 2 mL), sodium diethyldithiocarbamate (0.5 % w/v in DMF, 5 x 3 mL) and DMF (5 x 2 mL).

b. The terminal Fmoc group was then removed as described above.

c. *For lanthionine-containing peptides.*

A solution of HOAt (5 eq), PyAOP (5 eq) and DIPEA (10 eq) in DMF (1.5 mL for 50 mg scale reactions, 2 mL for 100 and 150 mg scale reactions) was preactivated and then, along with the resin, was transferred to a microwave vial and coupled in the microwave at 60 °C for 5 min followed by a further 1 h stirring at rt. The resin and coupling solution were then transferred back to the reaction syringe, and the coupling solution evacuated before the addition of a fresh solution of activated coupling reagents to the resin and leaving to stir for 2 h. The coupling solution was then removed and the resin washed with DMF (4 x 2 mL).

For methyllanthionine-containing peptides.

A solution of HOBt (5 eq), PyAOP (5 eq) and NMM (10 eq) in DMF (2 mL) was preactivated and then, along with the resin, was transferred to a microwave vial and coupled in the microwave at 60 °C for 5 min followed by a further 1 h stirring at rt. The resin and coupling solution were then transferred back to the reaction syringe, and the coupling solution evacuated before the addition of a fresh sample of the same preactivated coupling solution to the resin and leaving to stir for 2 h. The solution was then evacuated and the same 2 h rt coupling was repeated one further time before evacuating the solution and washing the resin with DMF (4 x 2 mL).

6. Cleavage:

Cleavage cocktail 1 (for cysteine-containing peptides):

TFA (940 µL), EDT (25 µL), water (25 µL), TIPS (10 µL)

Cleavage cocktail 2 (for peptides containing no cysteine):

TFA (965 µL), water (25 µL), TIPS (10 µL)

The resin was washed with CH_2Cl_2 (3 x 2 mL), MeOH (2 x 2 mL) and ether (2 x 2 mL) and then dried *in vacuo* for 30 min before cleavage. The cleavage cocktail (1 mL) was pre-mixed in a glass vial before addition to the resin. This was left to shake for 40 min before evacuating directly into a 15 mL Falcon tube containing cold ether (7 mL). A further portion of cleavage cocktail (1 mL) was then also added to the resin and left

to shake for 30 min before adding to the Falcon tube. The volume was then made up to 14 mL with more cold ether.

This was centrifuged at 4000 rpm at 5 °C for 15 min, after which time a precipitate formed. The ether was poured off and fresh ether added to resuspend the pellet, before a further round of centrifugation (4000 rpm, 5 °C, 10 min). The resuspension and centrifugation process was repeated once more. The resultant pellet was dissolved in water (3 mL) and lyophilised to yield the crude peptide.

7. Elimination of cysteine and β-methylcysteine in solution to form Dha and Dhb:

   TCEP (0.44 eq) was added to a solution of the purified cysteine-containing peptide in water (2.3 mg mL^{-1}) and this was shaken on a shaker plate at 480 rpm at rt for 45 min. A solution of methyl 2,5-dibromopentanoate (**80**) (60 eq) in DMSO (2.3 mg mL^{-1}) was then added to the peptide, followed by K_2CO_3 (150 eq) and this was incubated at 37 °C whilst shaking at 500 rpm for 2 h. Following the reaction, the solution was filtered with a syringe filter, diluted with water and purified directly by HPLC.

8. *C*-terminal residue cleavage with CPDY:

   The peptide was dissolved in 0.1 M PyOAc and the pH adjusted to 5.5 before the addition of a solution of CPDY in water (1:100 w/w CPDY:peptide). The solution was incubated at 37 °C with shaking for 4 h before adjusting to pH 2.0 with HCl to denature the enzyme, diluting in water and filtering with a syringe filter.

9. Elimination of serine to form Dha:

   a. A fresh sample of CuCl was first prepared. Three solutions were required:

      1. Na_2SO_3 (2.5 g) in water (13 mL)
      2. $CuCl_2.2H_2O$ (3.3 g) in water (7 mL)
      3. Na_2SO_3 (0.3 g) and 2 M HCl (3 mL) in water (250 mL)

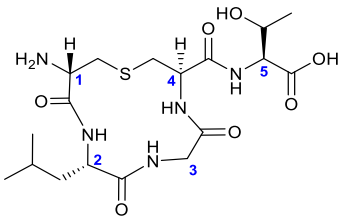
      Solution 1 was added to solution 2 with stirring. Solution 3 (125 mL) was then added and the stirring turned off to allow the precipitate to settle. Most of the supernatant was then decanted and the remaining solution filtered and washed with more of solution 3 (125 mL). Then, ensuring that a layer of liquid remains above the solid at all times, the filter cake was washed with glacial AcOH (2 x 5 mL), ethanol (2 x 10 mL) and ether (2 x 10 mL) before drying the solid *in vacuo*.

   b. To form the dehydroalanine, the peptide was dissolved in dry CH_2Cl_2 (0.06 M) before the addition of CuCl (0.3 eq) and EDCI (1.1 eq). The solution was stirred at rt for 5 h before pouring the reaction mixture into a separating funnel and

washing with water (2 x 2 mL). The organic layer was then dried (Na_2SO_4) and the solvent removed *in vacuo*.

7.2.2. Single Ring Synthesis

Mutacin I Ring B WT (96)



96

Reagents		Mass/Volume	µmol
Amino Acids	Teoc/TMSE lanthionine	55 mg	81
	Fmoc-Gly-OH	40 mg	135
	Fmoc-Leu-OH	48 mg	135
Coupling Reagents	HOAt	19 mg	135
	PyAOP	70 mg	135
	DIPEA	47 µL	270

Fmoc-Thr(tBu)-NovaSyn TGT resin (150 mg, 27.0 µmol) was swollen in DMF and the Fmoc group removed as described in General Methods Sections 1 and 2.

The protected Teoc/TMSE lanthionine was coupled using the microwave procedure described in General Methods Section 3. The Fmoc group was then removed.

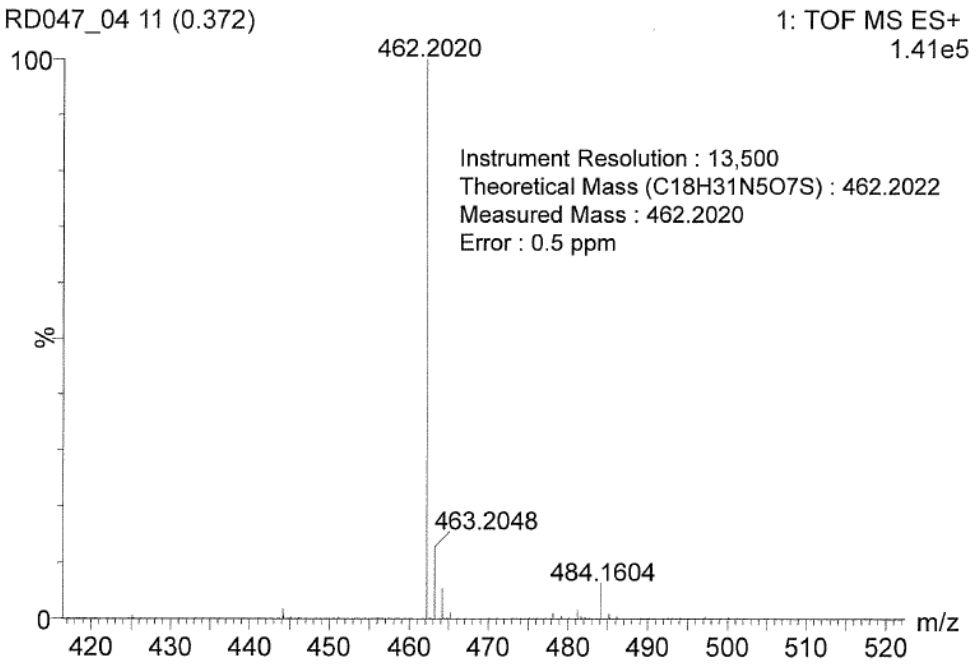
The peptide chain was elongated using the double coupling procedure described in General Methods Section 4. Fmoc-Gly-OH was added first, the Fmoc group was removed, followed by the addition of Fmoc-Leu-OH.

The cyclisation reaction was then carried out as described in General Methods Section 5. The silyl protecting groups were removed (General Methods Section 5a), followed by removal of the final Fmoc group (General Methods Section 5b). The cyclisation reaction was then carried out as described in Section 5c for lanthionine containing peptides.

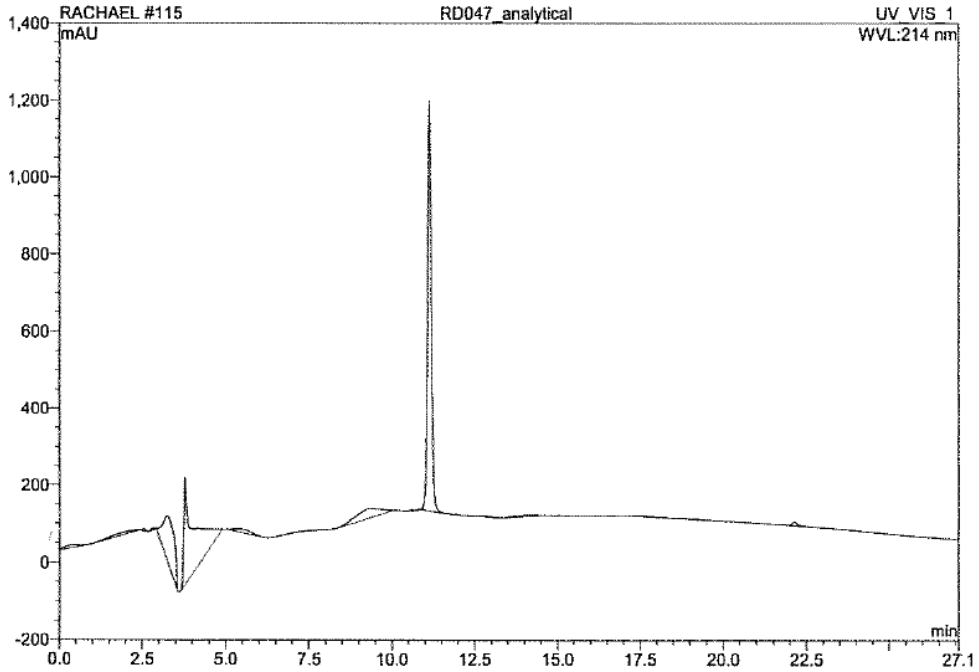
Following completion of the synthesis, the peptide was cleaved from the resin using the procedure described in General Methods Section 6 and cleavage cocktail 2.

The crude peptide was purified by preparative reverse phase HPLC using a semi-prep Phenomenex Onyx C18 100 x 10 mm column. A linear solvent gradient of 5-30% MeCN (0.1% TFA) in H_2O (0.1% TFA) over 40 min, at a flow rate of 1 mL min^{-1} was used. The fractions containing the target peptide were collected (retention time 11 min) and lyophilised to give the pure sample as a fluffy white powder (4.2 mg, 34%).

m/z (HRMS, ES+) required for $[C_{18}H_{31}N_5O_7S]$ 462.2022, found $[C_{18}H_{31}N_5O_7S]$ 462.2020.



analytical HPLC (Fluka Analytical Discovery BIO C18-10 25 x 4.6 mm column on Dionex HPLC system) retention time 11.13 min.



NMR δ_H (600 MHz, $(CD_3)_2SO$), δ_c (150 MHz, $(CD_3)_2SO$)

Major Conformer

^1H						
Residue Number	**NH**	**α**	**β**	**γ**	**δ**	**Exchangeable**
1 - Lan (Dha)		3.90	2.87 3.05			NH_2 – 8.33
2 - Leu	8.20	4.14	1.58	1.58	0.86 0.92	
3 - Gly	8.77	3.64 3.73				
4 - Lan (Cys)	7.16	4.24	3.21 2.81			
5 - Thr	8.02	4.14	4.14	1.03		OH – 4.93

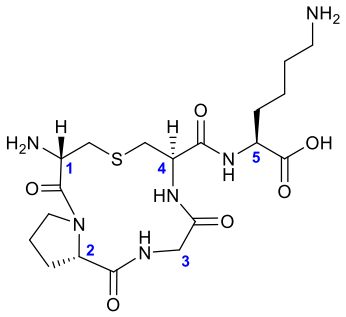
^{13}C					
Residue Number	**CO**	**α**	**β**	**γ**	**δ**
1 – Lan (Dha)	173.96	57.25	40.02		
2 - Leu	175.23	56.11	41.68	27.37	25.31 25.73
3 - Gly	172.20	45.74			
4 - Lan (Cys)	173.57	55.23	39.92		
5 - Thr		60.75	69.36	23.57	

Minor Conformer

^{1}H						
Residue Number	**NH**	**α**	**β**	**γ**	**δ**	**Exchangeable**
1 - Lan (Dha)		4.63	2.58 3.17			NH_2 - 8.00
2 - Leu	8.12	4.55	1.61	1.74	0.91 0.95	
3 - Gly	8.34	3.50 3.78				
4 - Lan (Cys)	7.18	4.41	2.30 3.29			
5 - Thr	7.88	4.19	4.16	1.06		

^{13}C					
Residue Number	**CO**	**α**	**β**	**γ**	**δ**
1 - Lan (Dha)		59.28	32.91		
2 - Leu	176.63	56.47	43.24	27.46	23.80 26.81
3 - Gly	173.21	46.40			
4 - Lan (Cys)		54.02	34.38		
5 - Thr		60.74	70.47	23.57	

Nisin Ring B Lan analogue (98)



98

Reagents		Mass/Volume	µmol
Amino Acids	Teoc/TMSE lanthionine	37 mg	54
	Fmoc-Gly-OH	27 mg	90
	Fmoc-Pro-OH	30 mg	90
Coupling Reagents	HOAt	12 mg	90
	PyAOP	47 mg	90
	DIPEA	31 µL	180

Fmoc-Lys(Boc)-NovaSyn TGT resin (100 mg, 18.0 µmol) was swollen in DMF and the Fmoc group removed as described in General Methods Sections 1 and 2.

The protected Teoc/TMSE lanthionine was coupled using the microwave procedure described in General Methods Section 3. The Fmoc group was then removed.

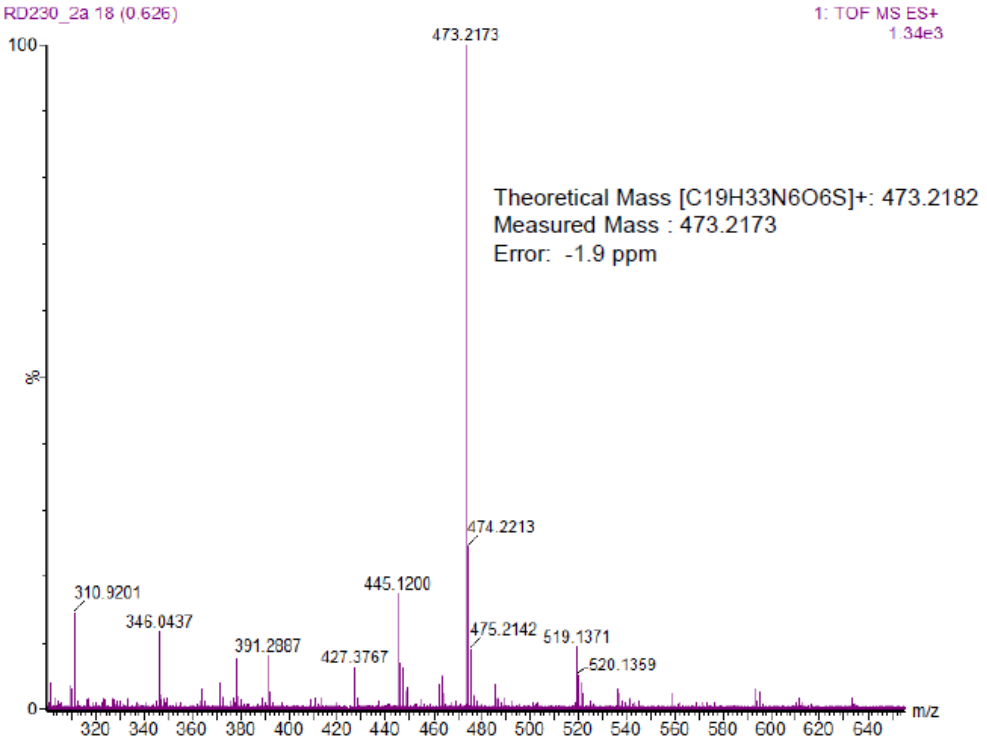
The peptide chain was elongated using the double coupling procedure described in General Methods Section 4. Fmoc-Gly-OH was added first, the Fmoc group was removed, followed by the addition of Fmoc-Pro-OH.

The cyclisation reaction was then carried out as described in Section 5. The silyl protecting groups were removed (General Methods Section 5a), followed by removal of the final Fmoc group (General Methods Section 5b). The cyclisation reaction was then carried out as described in Section 5c for lanthionine containing peptides.

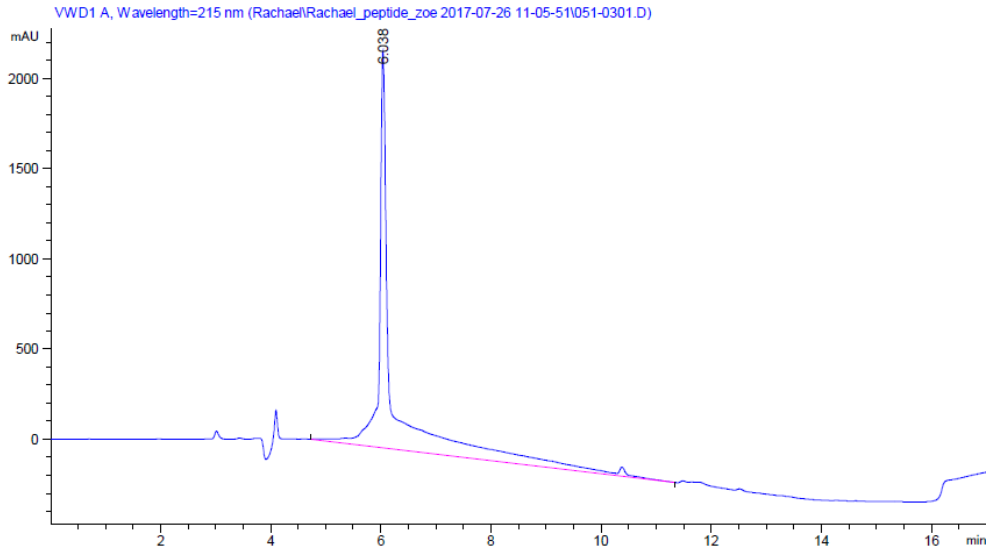
Following completion of the synthesis, the peptide was cleaved from the resin using the procedure described in General Methods Section 6 and cleavage cocktail 2.

The crude peptide was purified by preparative reverse phase HPLC using a semi-prep Agilent Zorbax 300SB-C18 5µm 250 x 9.4 mm column. A linear solvent gradient of 5-40% MeCN (0.1% TFA) in H_2O (0.1% TFA) over 25 min, at a flow rate of 2 mL min^{-1} was used. The fractions containing the target peptide were collected (retention time 9 min) and lyophilised to give the pure sample as a fluffy white powder (600 µg, 7%).

m/z (HRMS, ES+) required for $[C_{19}H_{33}N_6O_6S]^+$ 473.2182, found $[C_{19}H_{33}N_6O_6S]^+$ 473.2173.



analytical HPLC (Dr Maisch GmbH Reprosil Gold 200 C8 5µm 250 x 4.6 mm column on Agilent HPLC system) retention time 6.04 min.

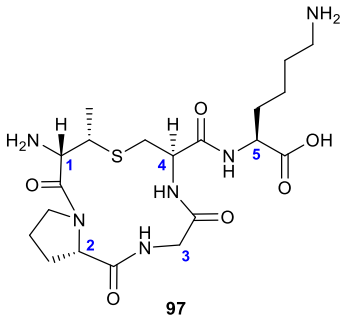


NMR δ_H (600 MHz, $(CD_3)_2SO$), δ_c (150 MHz, $(CD_3)_2SO$)

^1H							
Residue Number	**NH**	**α**	**β**	**γ**	**δ**	**ε**	**Exchangeable**
1 - Lan (Dha)		4.45	2.28 3.32				NH_2 - 8.23
2 - Pro		4.89	2.04 2.28	1.71 1.82	3.42 3.50		
3 - Gly	8.58	3.54 3.72					
4 - Lan (Cys)	8.12	4.44	2.52 3.18				
5 - Lys	8.47	4.09	1.57 1.73	1.35	1.52	2.77	NH_2 - 7.63 CO_2H - 12.66

^{13}C						
Residue Number	**CO**	**α**	**β**	**γ**	**δ**	**ε**
1 - Lan (Dha)	174.63	52.19				
2 - Pro	175.24	62.15	34.96	25.21	50.56	
3 - Gly	172.98	46.76				
4 - Lan (Cys)	173.85	59.65	32.68			
5 - Lys	176.70	55.21	33.55	25.75	29.85	41.88

Nisin Ring B WT (97)



97

Reagents		Mass/Volume	µmol
Amino Acids	Alloc/Allyl methyllanthionine	15 mg	23
	Fmoc-Gly-OH	14 mg	45
	Fmoc-Pro-OH	15 mg	45
Coupling Reagents	HOAt	6 mg	45
	PyAOP	24 mg	45
	DIPEA	16 µL	90
	HOBt	6 mg	45
	PyBOP	24 mg	45
	NMM	10 µL	90
Deprotection Reagents	$Pd(PPh_3)_4$	21 mg	18
	$PhSiH_3$	11 µL	90

Fmoc-Lys(Boc)-NovaSyn TGT resin (50 mg, 9.0 µmol) was swollen in DMF and the Fmoc group removed as described in General Methods Sections 1 and 2.

The protected Alloc/Allyl methyllanthionine was coupled using the microwave procedure described in General Methods Section 3. The Fmoc group was then removed.

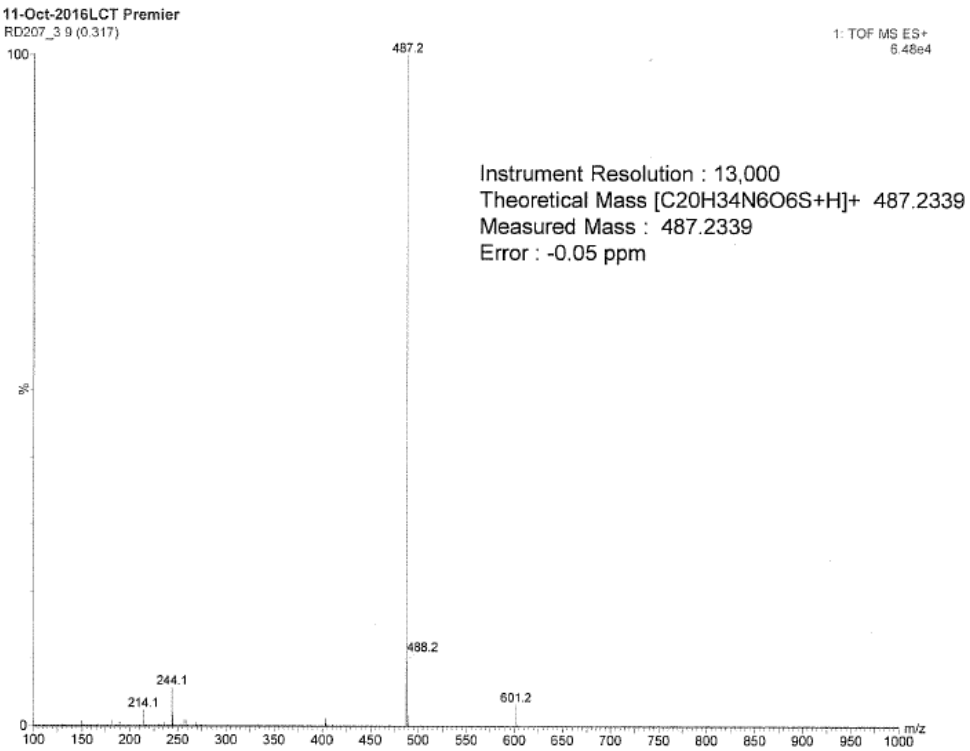
The peptide chain was elongated using the double coupling procedure described in General Methods Section 4. Fmoc-Gly-OH was added first, the Fmoc group was removed, followed by the addition of Fmoc-Pro-OH.

The cyclisation reaction was then carried out as described in Section 5. The allyl protecting groups were removed (General Methods Section 5a), followed by removal of the final Fmoc group (General Methods Section 5b). The cyclisation reaction was then carried out as described in Section 5c for methyllanthionine containing peptides.

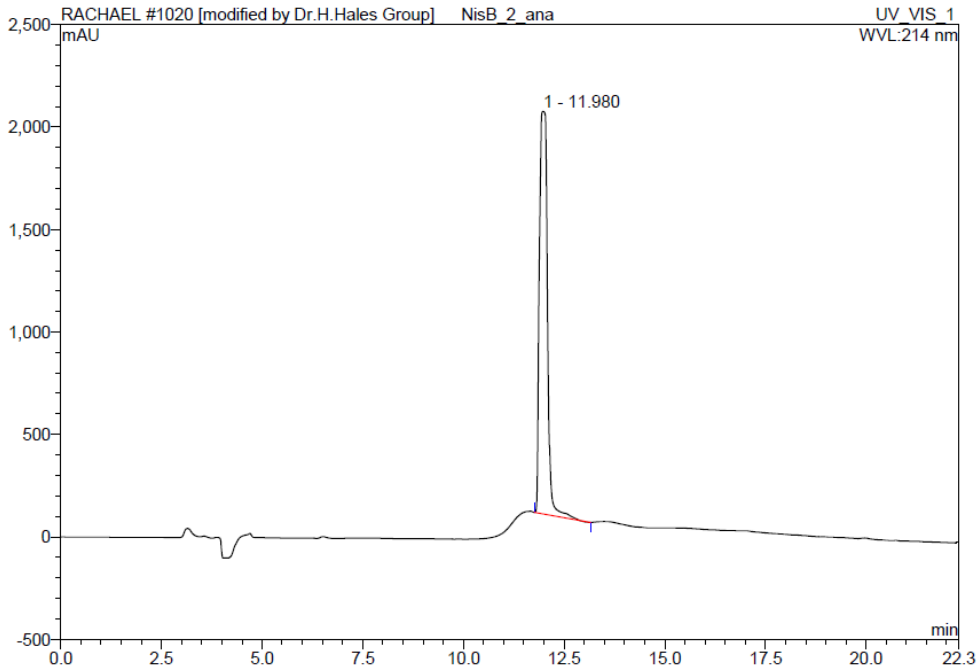
Following completion of the synthesis, the peptide was cleaved from the resin using the procedure described in General Methods Section 6 and cleavage cocktail 2.

The crude peptide was purified by preparative reverse phase HPLC using a semi-prep Agilent Zorbax 300SB-C18 5µm 250 x 9.4 mm column. A linear solvent gradient of 5-40% MeCN (0.1% TFA) in H_2O (0.1% TFA) over 30 min, at a flow rate of 2 mL min^{-1} was used. The fractions containing the target peptide were collected (retention time 10 min) and lyophilised to give the pure sample as a fluffy white powder (1.6 mg, 37%).

m/z (HRMS, ES+) required for $[C_{20}H_{34}N_6O_6S+H]^+$ 487.2339, found $[C_{20}H_{34}N_6O_6S+H]^+$ 487.2339.



analytical HPLC (Dr Maisch GmbH Reprosil Gold 200 C8 5µm 250 x 4.6 mm column on Dionex HPLC system) retention time 11.98 min.

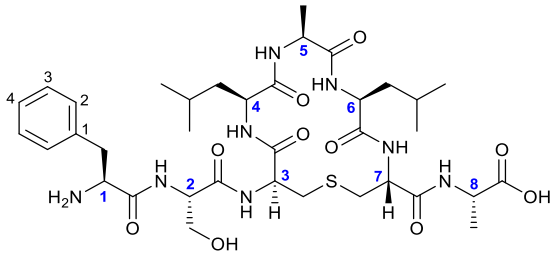


NMR δ_H (600 MHz, $(CD_3)_2SO$), δ_c (150 MHz, $(CD_3)_2SO$)

^1H							
Residue Number	**NH**	**α**	**β**	**γ**	**δ**	**ε**	**Exchangeable**
1 - MeLan (Dhb)		3.76	3.05	1.11			NH_2 - 8.26
2 - Pro		4.66	1.97 2.22	1.87	3.48		
3 - Gly	8.63	3.60 3.83					
4 - MeLan (Cys)	7.81	4.43	2.98 2.93				
5 - Lys	8.65	4.22	1.74 1.59	1.34	1.52	2.77	NH_2 - 7.64 CO_2H - 12.73

^{13}C						
Residue Number	**CO**	**α**	**β**	**γ**	**δ**	**ε**
1 - MeLan (Dhb)	172.18	57.41	43.80	20.91		
2 - Pro	175.14	62.87	34.72	25.61	50.70	
3 - Gly	173.34	46.65				
4 - MeLan (Cys)	173.41	56.62	34.42			
5 - Lys	176.04	54.97	33.73	25.68	29.84	41.78

Mutacin I Ring A Ser/Ala Analogue (101)



101

Reagents		**Mass/Volume**	**µmol**
Amino Acids	Teoc/TMSE lanthionine	17 mg	26
	Fmoc-Leu-OH	15 mg	43
	Fmoc-Ala-OH	14 mg	43
	Fmoc-Ser(O*t*Bu)-F	10 mg	26
	Fmoc-Phe-F	10 mg	26
Coupling Reagents	HOAt	6 mg	43
	PyAOP	22 mg	43
	DIPEA	15 µL	85

Fmoc-Ala-NovaSyn TGT resin (50 mg, 8.5 µmol) was swollen in DMF and the Fmoc group removed as described in General Methods Sections 1 and 2.

The protected Teoc/TMSE lanthionine was coupled using the microwave procedure described in General Methods Section 3. The Fmoc group was then removed.

The peptide chain was elongated using the double coupling procedure described in General Methods Section 4. Fmoc-Leu-OH was added first, the Fmoc group was removed, followed by the addition of Fmoc-Ala-OH, removal of the Fmoc group and coupling of a second Fmoc-Leu-OH.

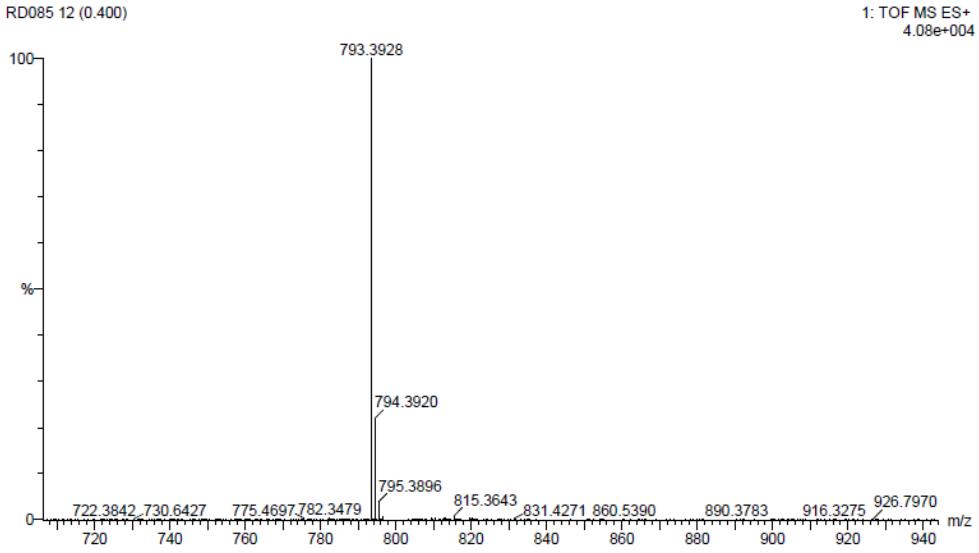
The cyclisation reaction was then carried out as described in Section 5. The silyl protecting groups were removed (General Methods Section 5a), followed by removal of the final Fmoc group (General Methods Section 5b). The cyclisation reaction was then carried out as described in Section 5c for lanthionine containing peptides.

Best results for coupling the final two amino acids of this peptide were achieved by double coupling with amino acid fluorides. To a solution of Fmoc-Ser(O*t*Bu)-F (10 mg, 25.5 μmol, 3eq) in dry CH_2Cl_2 (2 mL), DIPEA (4 μL, 25.5 μmol, 3 eq) was added. This solution was then added to the resin and stirred at rt for 1 h. The solution was then evacuated and the same coupling reaction was repeated. This solution was then evacuated and the resin washed with CH_2Cl_2 (2 x 2 mL) and DMF (4 x 2 mL). The Fmoc group was then removed. The same double coupling procedure was employed to add the phenylalanine, using solutions of Fmoc-Phe-F (10 mg, 25.5 μmol, 3eq) and DIPEA (4 μL, 25.5 μmol, 3 eq) in dry CH_2Cl_2 (2 mL). The final Fmoc group was then removed.

Following completion of the synthesis, the peptide was cleaved from the resin using the procedure described in General Methods Section 6 and cleavage cocktail 2.

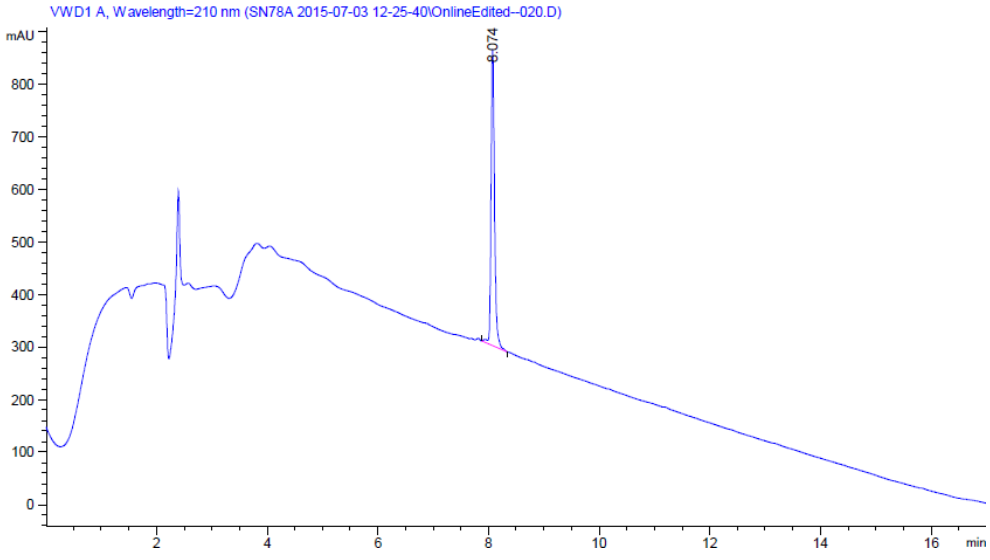
The crude peptide was purified by preparative reverse phase HPLC using a semi-prep Phenomenex Onyx C18 100 x 10 mm column. A linear solvent gradient of 5-40% MeCN (0.1% TFA) in H_2O (0.1% TFA) over 40 min, at a flow rate of 2 mL min^{-1} was used. The fractions containing the target peptide were collected (retention time 22 min) and lyophilised to give the pure sample as a fluffy white powder (200 μg, 3%).

m/z (HRMS, ES+) required for [$C_{36}H_{57}N_8O_{10}S$] 793.3918, found [$C_{36}H_{57}N_8O_{10}S$] 793.3928.



Minimum:				-1.5		
Maximum:		5.0	20.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
793.3928	793.3918	1.0	1.3	12.5	5573.5	C36 H57 N8 O10 S

analytical HPLC (ACE 300Å C18-300 150 x 4.6 mm column on Agilent HPLC system) retention time 8.07 min.

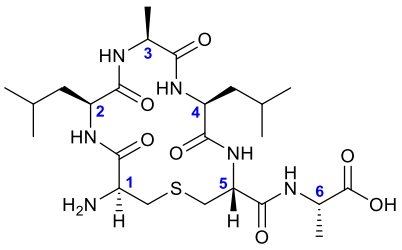


NMR δ_H (600 MHz, $(CD_3)_2SO$), δ_c (150 MHz, $(CD_3)_2SO$)

^1H							
Residue Number	**NH**	**α**	**β**	**γ**	**δ**	**Ar**	**Exchangeable**
1 - Phe		4.13	2.88 3.12			2 - 7.32 3 - 7.29 4 - 7.26	NH_2 - 8.09
2 - Ser	8.85	4.48	3.62				
3 - Lan (Dha)	8.33	4.39	2.92 2.83				
4 - Leu	8.34	3.93	1.49 1.74	1.54	a - 0.80 b - 0.83		
5 - Ala	7.94	4.15	1.27				
6 - Leu	8.00	4.05	1.60 1.69	1.57	a - 0.85 b - 0.90		
7 - Lan (Cys)	7.78	4.47	2.79 2.92				
8 - Ala	7.93	4.07	1.30				CO_2H - 12.63

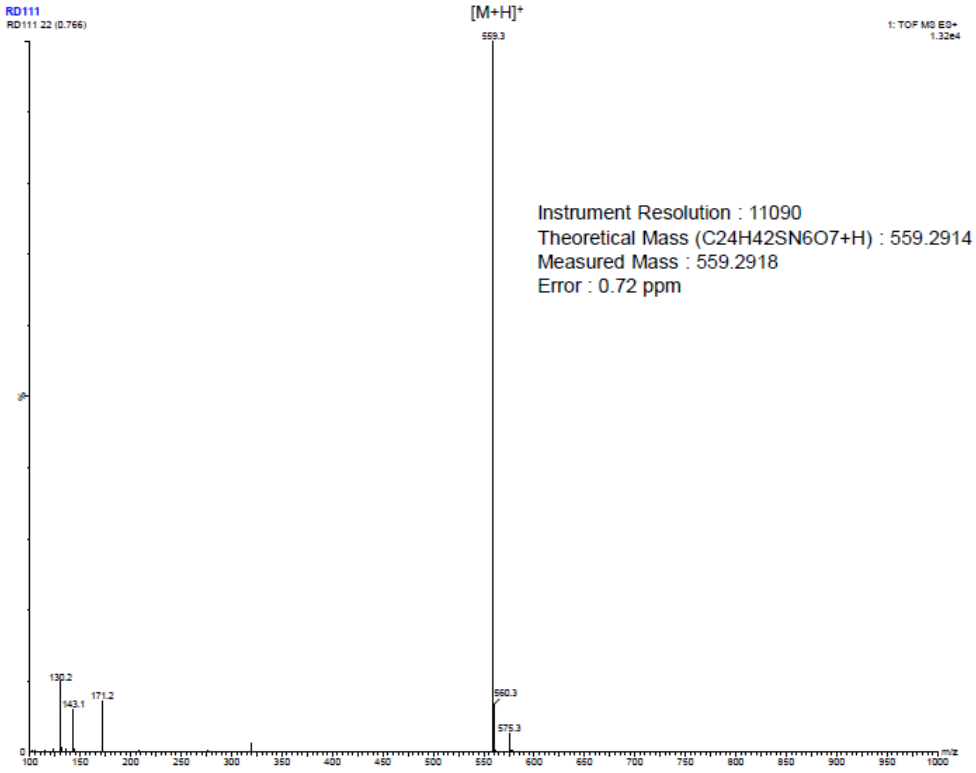
^{13}C						
Residue Number	**CO**	**α**	**β**	**γ**	**δ**	**Ar**
1 - Phe	171.34	56.36	40.04			1 - 138.00 2 - 131.83 3 - 132.67 4 - 130.43
2 - Ser	171.88	57.64	64.83			
3 - Lan (Dha)	173.03	56.58	40.51			
4 - Leu	174.50	55.77	25.77	41.71	a - 24.71 b - 26.39	
5 - Ala	177.01	50.79	20.14			
6 - Leu	170.11	54.33	27.54	42.12	a - 24.76 b - 26.34	
7 - Lan (Cys)	174.34	56.07	37.60			
8 - Ala	175.24	54.33	20.61			

Side product also isolated during purification (retention time 11 min) (100 µg, 2%):

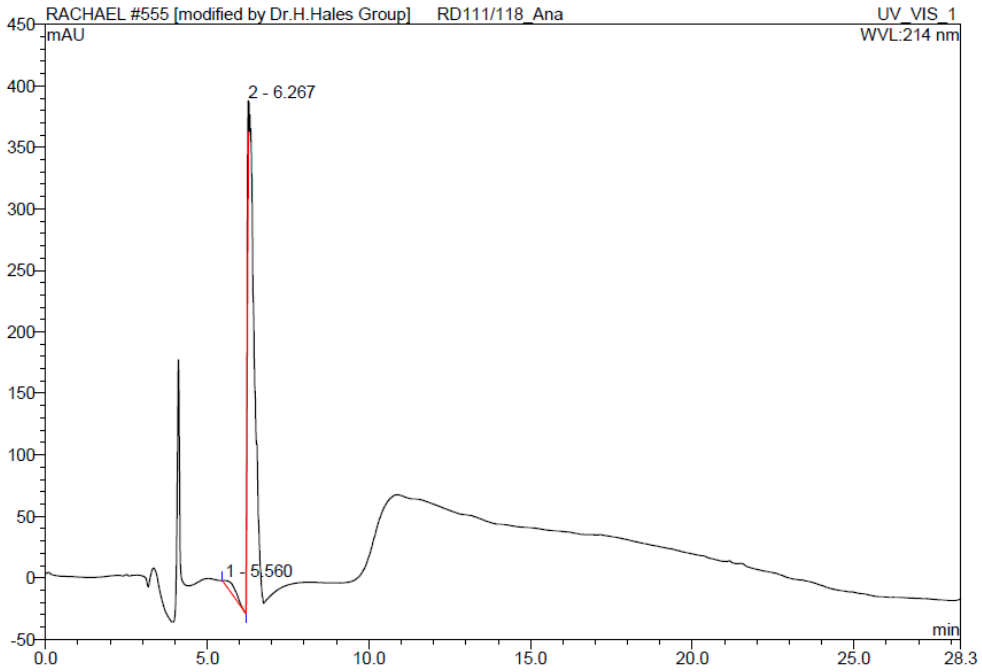


153

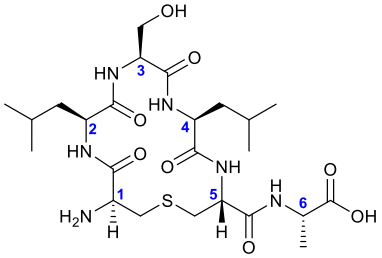
m/z (HRMS, ES+) required for $[C_{24}H_{42}N_6O_7S+H]^+$ 559.2914, found $[C_{24}H_{42}N_6O_7S+H]^+$ 559.2918.



analytical HPLC (Fluka Analytical Discovery BIO C18-10 250 x 4.6 mm column on Dionex HPLC system) retention time 6.27 min.



Mutacin I Ring A Ser Analogue (102)



102

Reagents		Mass/Volume	µmol
Amino Acids	Teoc/TMSE lanthionine	52 mg	77
	Fmoc-Leu-OH	45 mg	128
	Fmoc-Ser(*t*Bu)-OH	49 mg	128
Coupling Reagents	HOAt	17 mg	128
	PyAOP	67 mg	128
	DIPEA	44 µL	255

Fmoc-Ala-NovaSyn TGT resin (150 mg, 25.5 μmol) was swollen in DMF and the Fmoc group removed as described in General Methods Sections 1 and 2.

The protected Teoc/TMSE lanthionine was coupled using the microwave procedure described in General Methods Section 3. The Fmoc group was then removed.

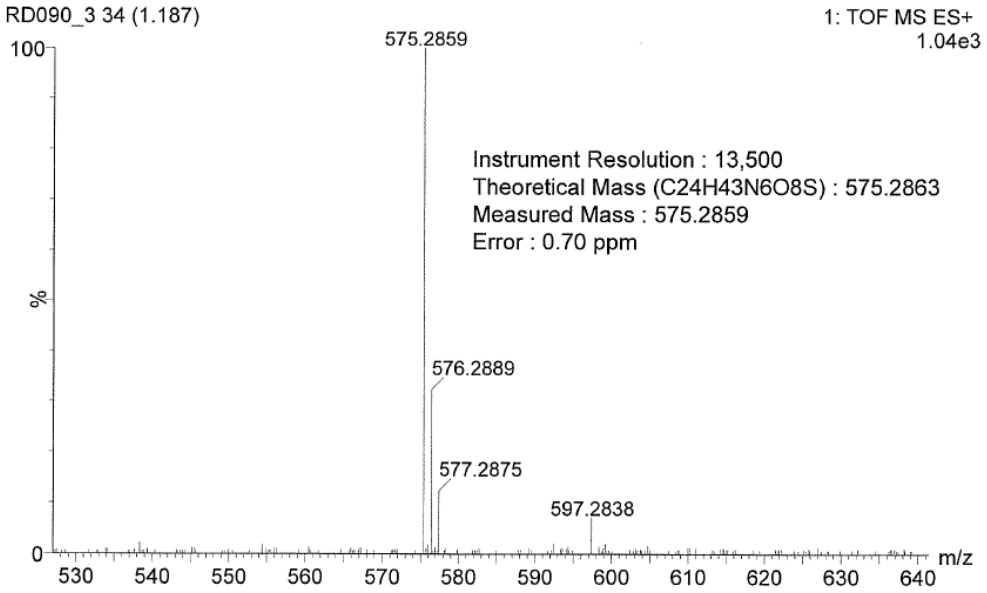
The peptide chain was elongated using the double coupling procedure described in General Methods Section 4. Fmoc-Leu-OH was added first, the Fmoc group was removed, followed by the addition of Fmoc-Ser(*t*Bu)-OH, removal of the Fmoc group and coupling of a second Fmoc-Leu-OH.

The cyclisation reaction was then carried out as described in Section 5. The silyl protecting groups were removed (General Methods Section 5a), followed by removal of the final Fmoc group (General Methods Section 5b). The cyclisation reaction was then carried out as described in Section 5c for lanthionine containing peptides.

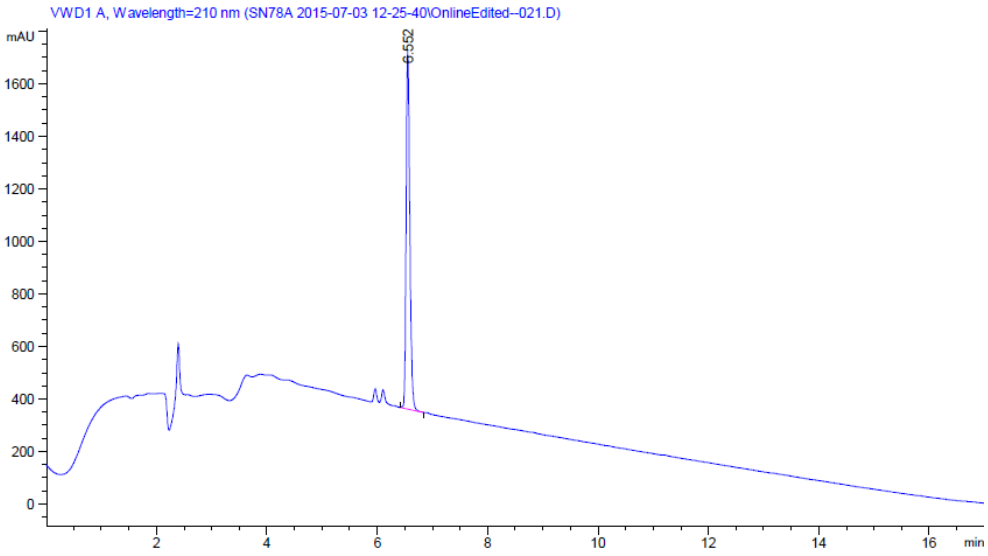
Following completion of the synthesis, the peptide was cleaved from the resin using the procedure described in General Methods Section 6 and cleavage cocktail 2.

The crude peptide was purified by preparative reverse phase HPLC using a semi-prep Phenomenex Onyx C18 100 x 10 mm column. A linear solvent gradient of 5-50% MeCN (0.1% TFA) in H_2O (0.1% TFA) over 20 min, at a flow rate of 2 mL min^{-1} was used. The fractions containing the target peptide were collected (retention time 9 min) and lyophilised to give the pure sample as a fluffy white powder (800 μg, 5%).

m/z (HRMS, ES+) required for [$C_{24}H_{43}N_6O_8S$] 575.2863, found [$C_{24}H_{43}N_6O_8S$] 575.2859.



analytical HPLC (Fluka Analytical Discovery BIO C18-10 25 x 4.6 mm column on Agilent HPLC system) retention time 6.55 min.

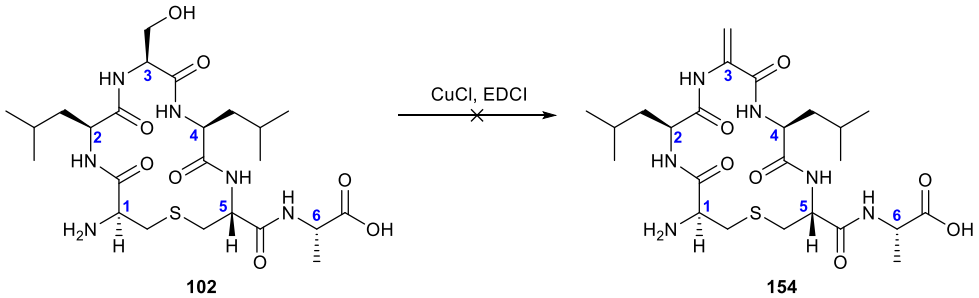


NMR δ_H (600 MHz, $(CD_3)_2SO$), δ_c (150 MHz, $(CD_3)_2SO$)

^1H						
Residue Number	**NH**	**α**	**β**	**γ**	**δ**	**Exchangeable**
1 - Lan (Dha)		3.93	2.92 3.02			NH_2 - 8.19
2 - Leu	9.02	3.84	1.55 1.99	1.65	a - 0.95 b - 0.89	
3 - Ser	7.94	4.09	3.69			
4 - Leu	7.51	4.23	1.67	1.70	a - 0.93 b - 0.84	
5 - Lan (Cys)	7.79	4.38	2.93			
6 - Ala	7.79	4.14	1.27			CO_2H - 12.63

^{13}C					
Residue Number	**CO**	**α**	**β**	**γ**	**δ**
1 - Lan (Dha)	170.14	55.05	35.99		
2 - Leu	174.84	57.03	40.37	27.37	a - 26.37 b - 26.11
3 - Ser	173.32	61.05	63.63		
4 - Leu	174.92	54.66	42.29	28.30	a - 24.13 b - 24.57
5 - Lan (Cys)	172.27	55.45	37.54		
6 - Ala	176.80	50.83	20.23		

Attempted Dehydration of Mutacin I Ring A Ser Analogue (102) to form Dha-Containing Peptide (154)

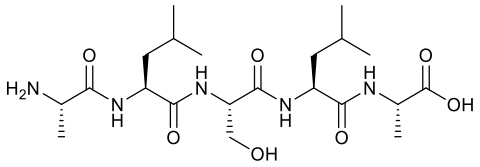


102 154

Reagents		Mass/Volume	µmol
Dehydration Reagents	CH_2Cl_2	30 µL	
	CuCl	8.4 µL of a 5 mg mL^{-1} stock solution in CH_2Cl_2	0.42
	EDCI	15 µL of a 20 mg mL^{-1} stock solution in CH_2Cl_2	1.5

The reaction to form the dehydroalanine was attempted as described in General Methods Section 9, on 0.8 mg of peptide (**102**) (1.4 µmol). None of the target peptide could be detected by LCMS.

H-Ala-Leu-Ser-Leu-Ala-OH (155)



155

Reagents		Mass/Volume	µmol
Amino Acids	Fmoc-Leu-OH	30 mg	85
	Fmoc-Ser(*t*Bu)-OH	33 mg	85
	Fmoc-Ala-OH	26 mg	85
Coupling Reagents	HOAt	12 mg	85
	PyAOP	44 mg	85
	DIPEA	30 µL	170

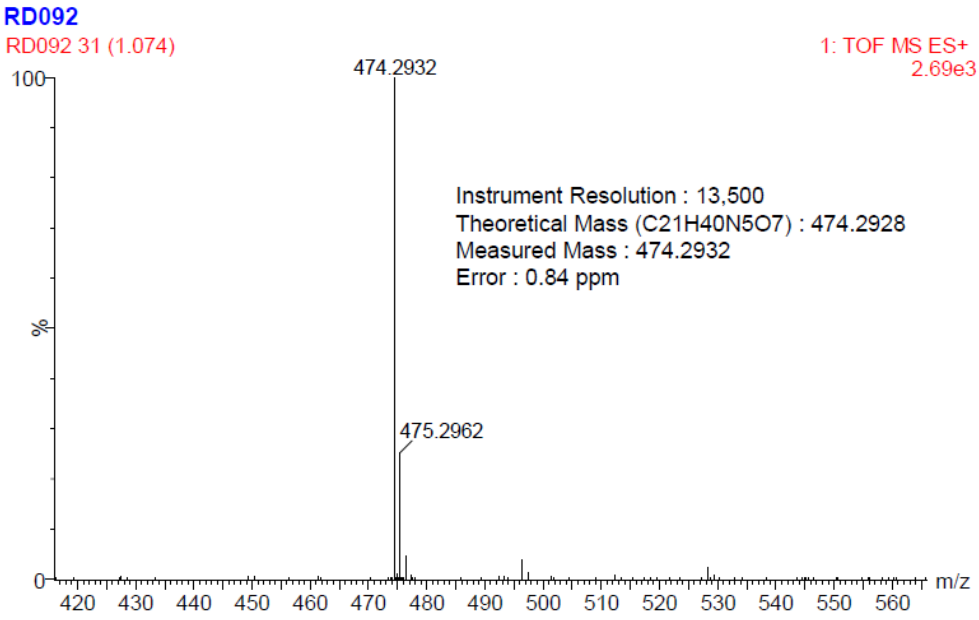
Fmoc-Ala-NovaSyn TGT resin (100 mg, 17 µmol) was swollen in DMF and the Fmoc group removed as described in General Methods Sections 1 and 2.

The peptide chain was then further elongated using the double coupling procedure described in General Methods Section 4, with an Fmoc deprotection between additions of each new amino acid. Firstly Fmoc-Leu-OH was added, followed by Fmoc-Ser(*t*Bu)-OH, a second Fmoc-Leu-OH and finally Fmoc-Ala-OH. The terminal Fmoc group was then removed.

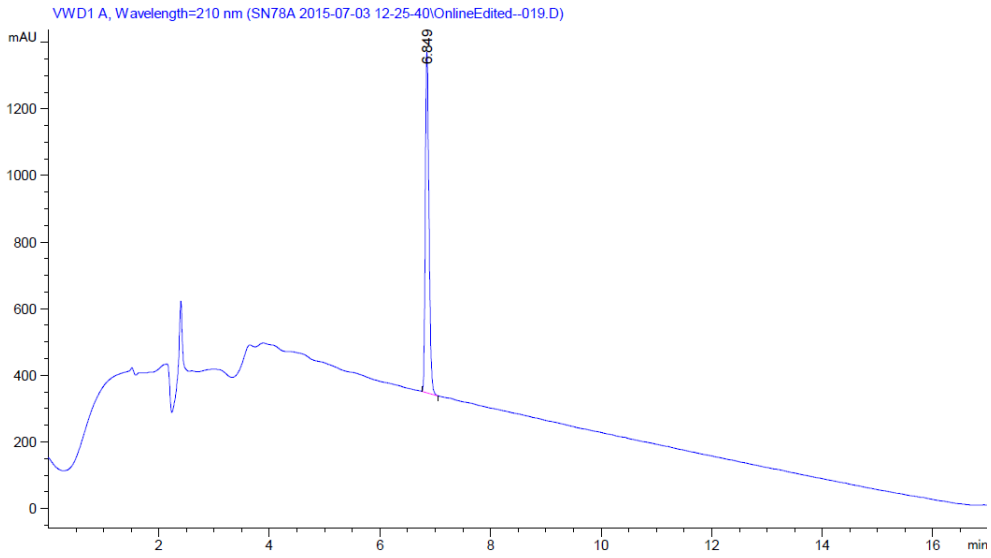
Following completion of the synthesis, the peptide was cleaved from the resin using the procedure described in General Methods Section 6 and cleavage cocktail 2.

The crude peptide was purified by preparative reverse phase HPLC using a semi-prep Phenomenex Onyx C18 100 x 10 mm column. A linear solvent gradient of 2-70% MeCN (0.1% TFA) in H_2O (0.1% TFA) over 20 min, at a flow rate of 2 mL min^{-1} was used. The fractions containing the target peptide were collected (retention time 9 min) and lyophilised to give the sample as a fluffy white powder (4.6 mg, 57%).

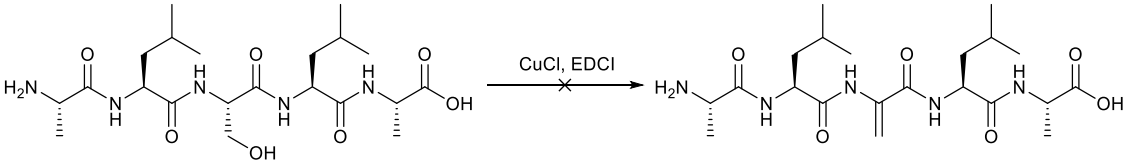
m/z (HRMS, ES+) required for $[C_{21}H_{40}N_5O_7]$ 474.2928, found $[C_{21}H_{40}N_5O_7]$ 474.2932.



analytical HPLC (ACE5 C8-300 150 x 4.6 mm column on Agilent HPLC system) retention time 6.85 min.



Attempted Dehydration of H-Ala-Leu-Ser-Leu-Ala-OH (155) to form Dha-Containing Peptide (156)

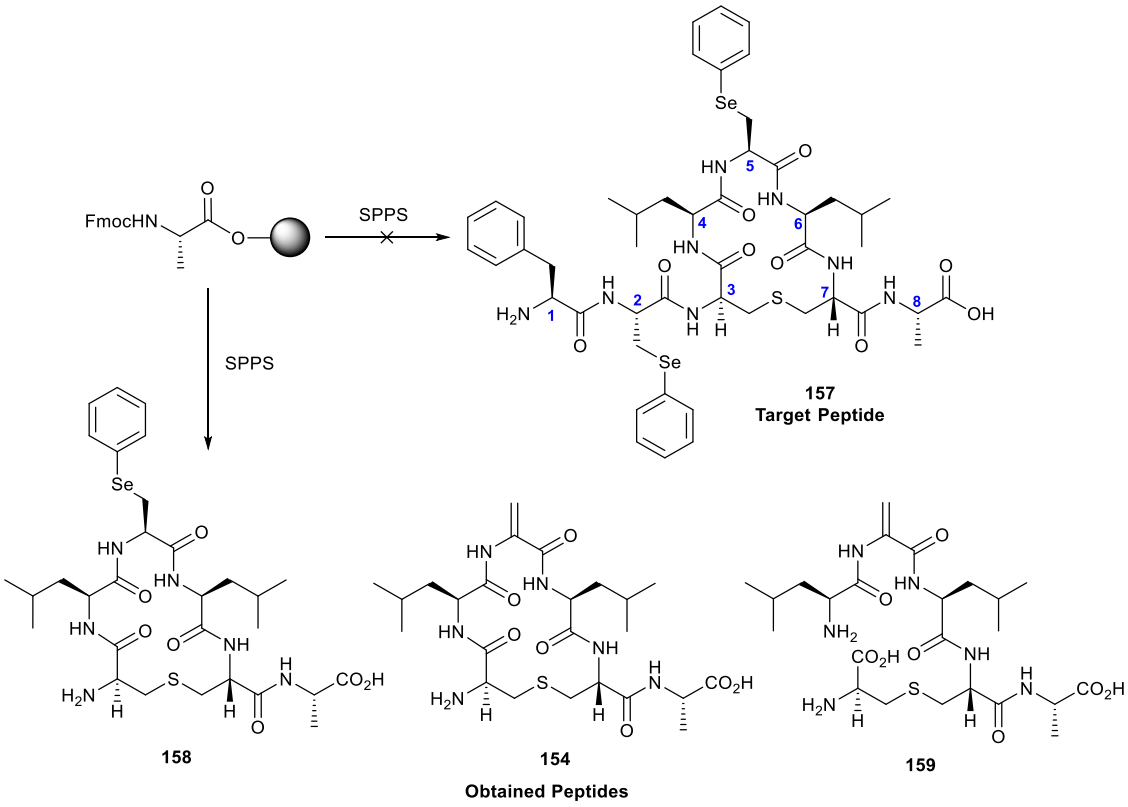


155 **156**

Reagents		Mass/Volume	µmol
Dehydration Reagents	CH_2Cl_2	260 µL	
	CuCl	150 µL of a 2 mg mL^{-1} stock solution in CH_2Cl_2	3
	EDCI	2 mg	11

The reaction to form the dehydroalanine was then attempted as described in General Methods Section 9, on 4.6 mg of peptide (**155**) (9.7 µmol). None of the target peptide could be detected by LCMS.

Attempted Synthesis of Mutacin I Ring A WT (99) *via* Sec(Ph)-containing Precursor (157)



Reagents		Mass/Volume	µmol
Amino Acids	Teoc/TMSE lanthionine	17 mg	26
	Fmoc-Leu-OH	15 mg	43
	Fmoc-Sec(Ph)-OH	16 mg	34
	Fmoc-Phe-OH	17 mg	43
Coupling Reagents	HOAt	6 mg	43
	PyAOP	22 mg	43
	DIPEA	15 µL	85
	HOBt	5 mg	34
	DIC	5 µL	34

Fmoc-Ala-NovaSyn TGT resin (50 mg, 8.5 μmol) was swollen in DMF and the Fmoc group removed as described in General Methods Sections 1 and 2.

The protected Teoc/TMSE lanthionine was coupled using the microwave procedure described in General Methods Section 3. The Fmoc group was then removed.

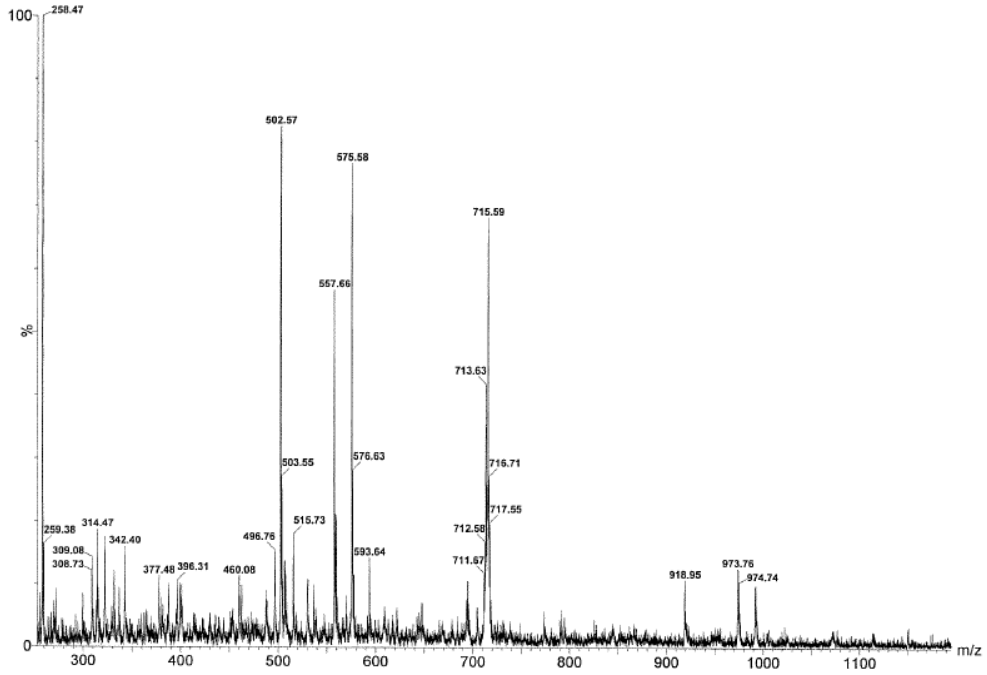
The peptide chain was then elongated. Fmoc-Leu-OH was added first using the double coupling procedure described in General Methods Section 4. The Fmoc group was removed, followed by the addition of Fmoc-Sec(Ph)-OH (4 eq). This residue was coupled at rt for 2 h using HOBt (4 eq) and DIC (4 eq) in DMF (1.5 mL). After evacuation of the coupling solution the resin was washed with DMF (2 x 4 mL). The Fmoc group was then removed and a second Fmoc-Leu-OH added using the double coupling procedure described in General Methods Section 4.

The cyclisation reaction was then carried out as described in Section 5. The silyl protecting groups were removed (General Methods Section 5a), followed by removal of the final Fmoc group (General Methods Section 5b). The cyclisation reaction was then carried out as described in Section 5c for lanthionine containing peptides.

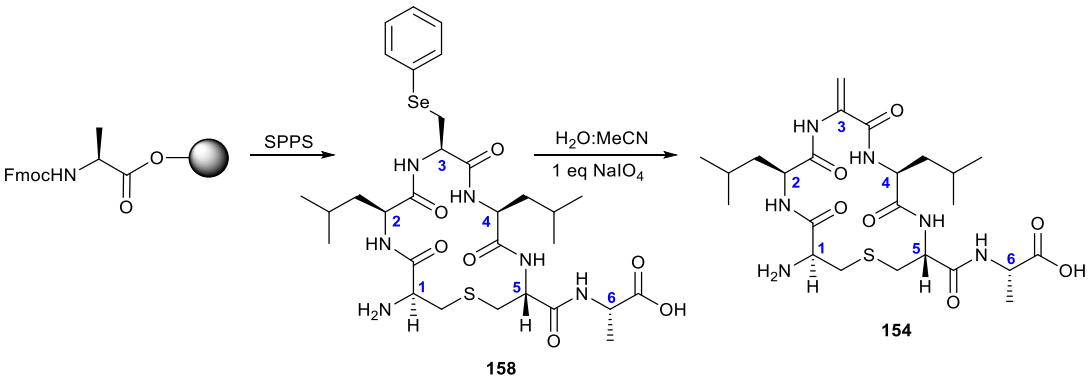
The peptide chain was then further elongated using the double coupling procedure described in General Methods Section 4. Firstly, a second Fmoc-Sec(Ph)-OH residue was added using the same procedure described for first Sec(Ph), followed by removal of the Fmoc group removed, addition of Fmoc-Phe-OH, and removal of the final Fmoc group.

Following completion of the synthesis, the peptide was cleaved from the resin using the procedure described in General Methods Section 6 and cleavage cocktail 2. None of the desired intermediate peptide (**157**) could be detected by crude LCMS so the peptide was not purified.

m/z (LCMS, ES+) **154** $[M+H]^+$ 557.7, **159** $[M+H]^+$ 575.6, **158** $[M+H]^+$ 715.6.



Mutacin I Ring A Truncated WT (154)



Reagents		Mass/Volume	µmol
Amino Acids	Teoc/TMSE lanthionine	17 mg	26
	Fmoc-Leu-OH	15 mg	43
	Fmoc-Sec(Ph)-OH	16 mg	34
Coupling Reagents	HOAt	6 mg	43
	PyAOP	22 mg	43
	DIPEA	15 µL	85
	HOBt	5 mg	34
	DIC	5 µL	34
	COMU	18 mg	43
SecPh Elimination Reagents	H_2O:MeCN	50 µL:30 µL	
	$NaIO_4$	40 µL of a 1.25 mg mL^{-1} stock solution in water	0.3

Peptide Synthesis

Fmoc-Ala-NovaSyn TGT resin (50 mg, 8.5 µmol) was swollen in DMF and the Fmoc group removed as described in General Methods Sections 1 and 2.

The protected Teoc/TMSE lanthionine was coupled using the microwave procedure described in General Methods Section 3. The Fmoc group was then removed.

The peptide chain was then elongated. Fmoc-Leu-OH was added first using the double coupling procedure described in General Methods Section 4. The Fmoc group was removed, followed by the addition of Fmoc-Sec(Ph)-OH (4 eq). This residue was coupled at rt for 2 h using HOBt (4 eq) and DIC (4 eq) in DMF (1.5 mL). After evacuation of the coupling solution the resin was washed with DMF (2 x 4 mL). The Fmoc group was then

removed and a second Fmoc-Leu-OH added using the double coupling procedure described in General Methods Section 4.

The cyclisation reaction was then carried out as described in Section 5. The silyl protecting groups were removed (General Methods Section 5a), followed by removal of the final Fmoc group (General Methods Section 5b). The cyclisation reaction was then carried out as described in Section 5c for lanthionine containing peptides, except that COMU (5 eq) was used in place of PyAOP and HOAt.

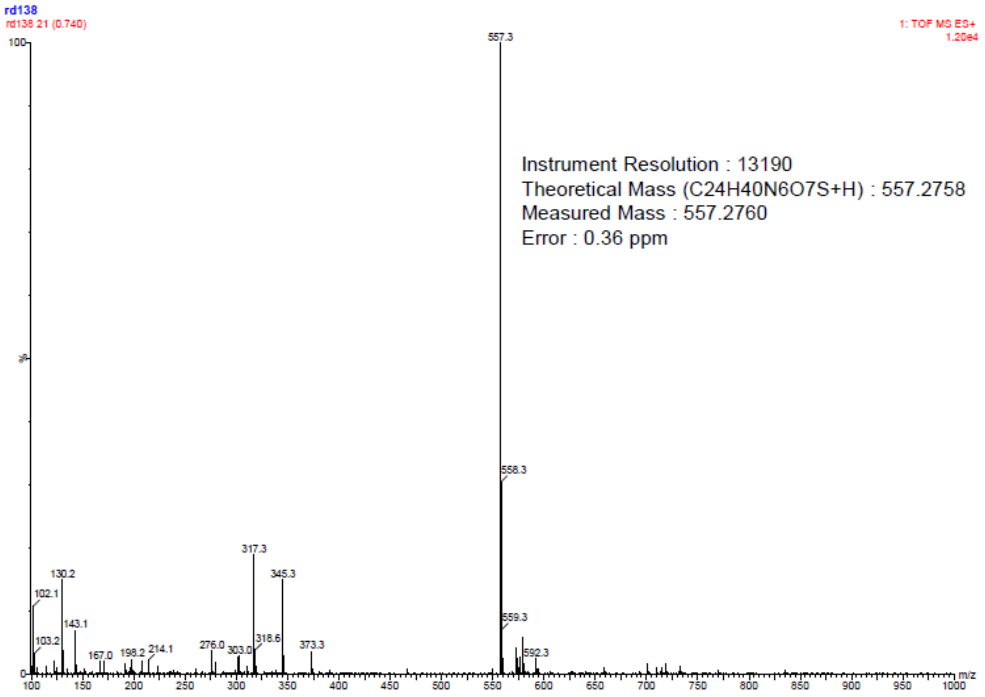
Following completion of the synthesis, the peptide was cleaved from the resin using the procedure described in General Methods Section 6 and cleavage cocktail 2.

The crude peptide was purified by preparative reverse phase HPLC using a semi-prep Phenomenex Onyx C18 100 x 10 mm column. A linear solvent gradient of 20-60% MeCN (0.1% TFA) in H_2O (0.1% TFA) over 30 min, at a flow rate of 2 mL min^{-1} was used. The fractions containing the target peptide were collected (retention time 13 min) and lyophilised yielding a fluffy white solid (200 μg, 0.3 μmol).

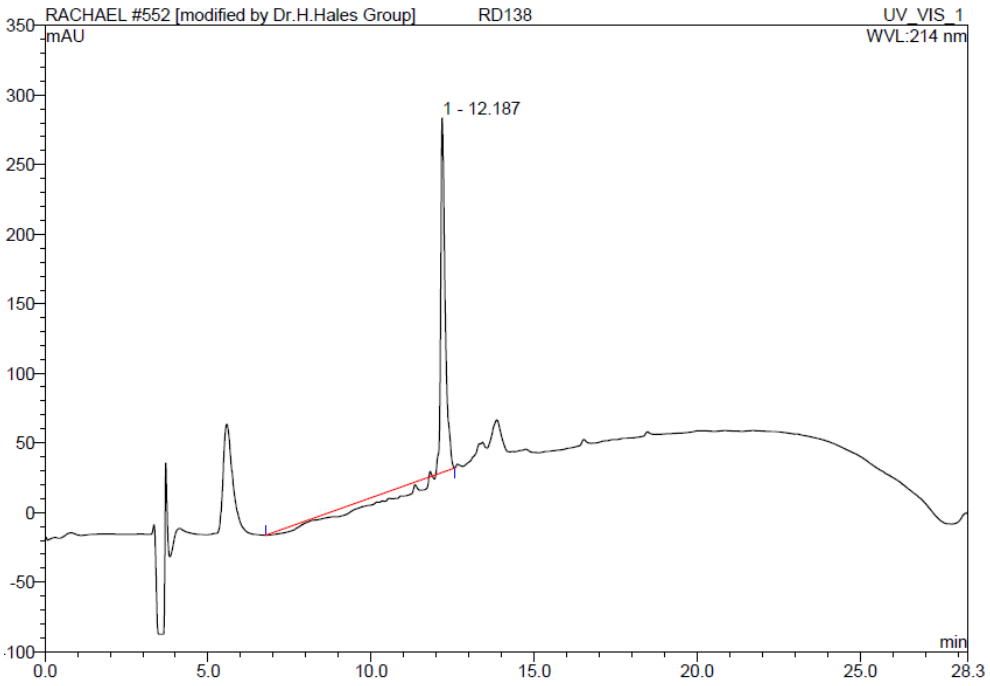
Dehydration to form Dha

To form the dehydroalanine residue, a solution of the peptide in water:MeCN was first cooled to 0 °C in an ice bath before the addition of $NaIO_4$. This was stirred for 1 h, before filtering the reaction mixture with a syringe filter and purifying directly by preparative reverse phase HPLC. A semi-prep Phenomenex Onyx C18 100 x 10 mm column was used, with a linear solvent gradient of 2-98% MeCN (0.1% TFA) in H_2O (0.1% TFA) over 20 min, at a flow rate of 2 mL min^{-1}. The fractions containing the peptide were collected and lyophilised to give the pure sample as a white powder (100 μg, 2%).

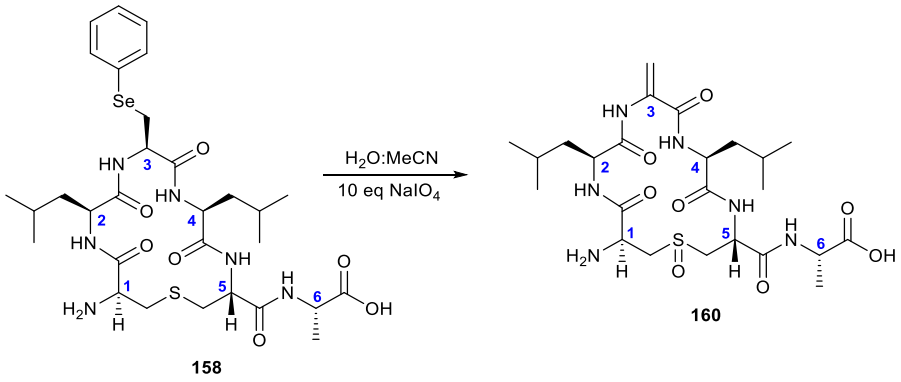
m/z (HRMS, ES+) required for $[C_{24}H_{40}N_6O_7S+H]^+$ 557.2758, found $[C_{24}H_{40}N_6O_7S+H]^+$ 557.2760.



analytical HPLC (Dr Maisch GmbH Reprosil Gold 200 C8 5µm 250 x 4.6 mm column on Dionex HPLC system) retention time 12.19 min.



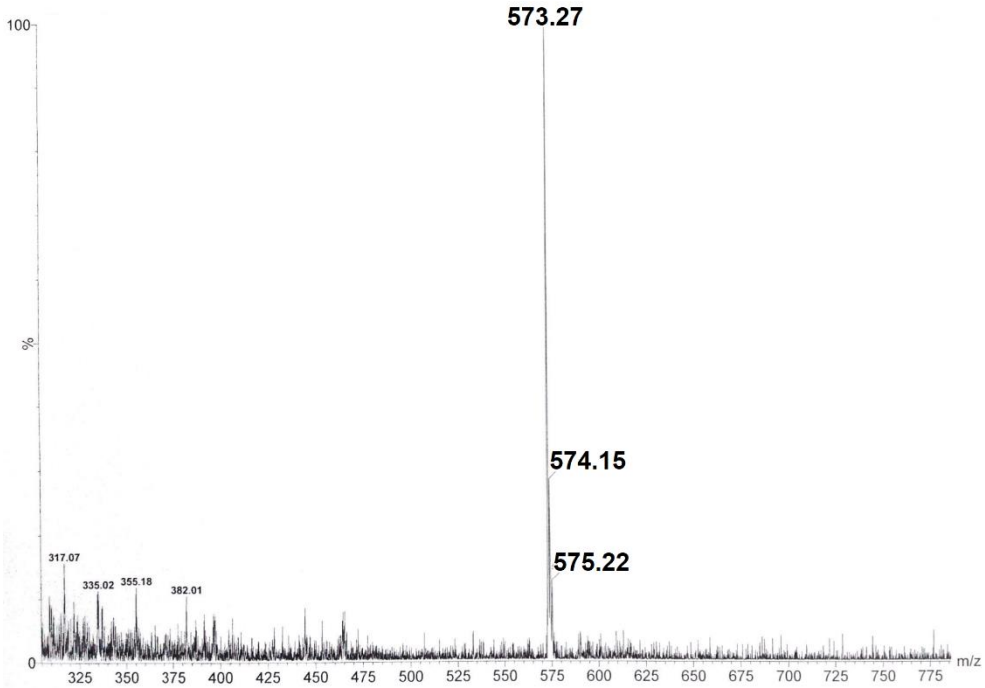
Attempted Oxidative Elimination of Sec(Ph)-Containing Peptide (158)



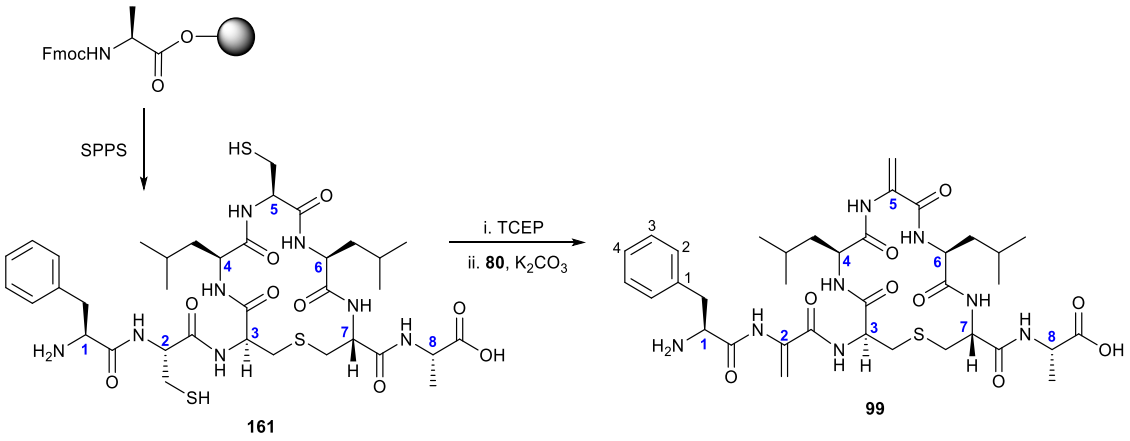
Reagents		Mass/Volume	µmol
SecPh Elimination Reagents	H_2O:MeCN	500 µL:300 µL	
	$NaIO_4$	5 mg	23

To form the dehydroalanine residue, a solution of peptide (**158**) (1.6 mg, 2.3 µmol) in water:MeCN was first cooled to 0 °C in an ice bath before the addition of $NaIO_4$. This was stirred for 1 h, before filtering the reaction mixture with a syringe filter. Analysis of the crude peptide by LCMS showed only over-oxidised peptide (**160**) and none of the target peptide (**154**), so the peptide was not purified.

m/z (LCMS, ES+) **160** $[M+H]^+$ 573.3.



Mutacin I Ring A WT (99)



Reagents		Mass/Volume	µmol
Amino Acids	Teoc/TMSE lanthionine	34 mg	51
	Fmoc-Leu-OH	30 mg	85
	Fmoc-Cys(Trt)-OH	50 mg	85
	Fmoc-Phe-OH	33 mg	85
Coupling Reagents	HOAt	12 mg	85
	PyAOP	44 mg	85
	DIPEA	30 µL	170
Cys Elimination Reagents	H_2O	270 µL	
	TCEP	615 µL of a 0.5 mg mL^{-1} stock solution in water	1.1
	methyl 2,5-dibromopentanoate	39 mg	144
	DMSO	880 µL	
	K_2CO_3	49 mg	360

Peptide Synthesis

Fmoc-Ala-NovaSyn TGT resin (100 mg, 17 μmol) was swollen in DMF and the Fmoc group removed as described in General Methods Sections 1 and 2.

The protected Teoc/TMSE lanthionine was coupled using the microwave procedure described in General Methods Section 3. The Fmoc group was then removed.

The peptide chain was elongated using the double coupling procedure described in General Methods Section 4. Fmoc-Leu-OH was added first, the Fmoc group was removed, followed by the addition of Fmoc-Cys(Trt)-OH, removal of the Fmoc group and coupling of a second Fmoc-Leu-OH.

The cyclisation reaction was then carried out as described in Section 5. The silyl protecting groups were removed (General Methods Section 5a), followed by removal of the final Fmoc group (General Methods Section 5b). The cyclisation reaction was then carried out as described in Section 5c for lanthionine containing peptides.

The peptide chain was then further elongated using the double coupling procedure described in General Methods Section 4. Firstly Fmoc-Cys(Trt)-OH was added, the Fmoc group removed, followed by the addition of Fmoc-Phe-OH, and removal of the final Fmoc group.

Following completion of the synthesis, the peptide was cleaved from the resin using the procedure described in General Methods Section 6 and cleavage cocktail 1.

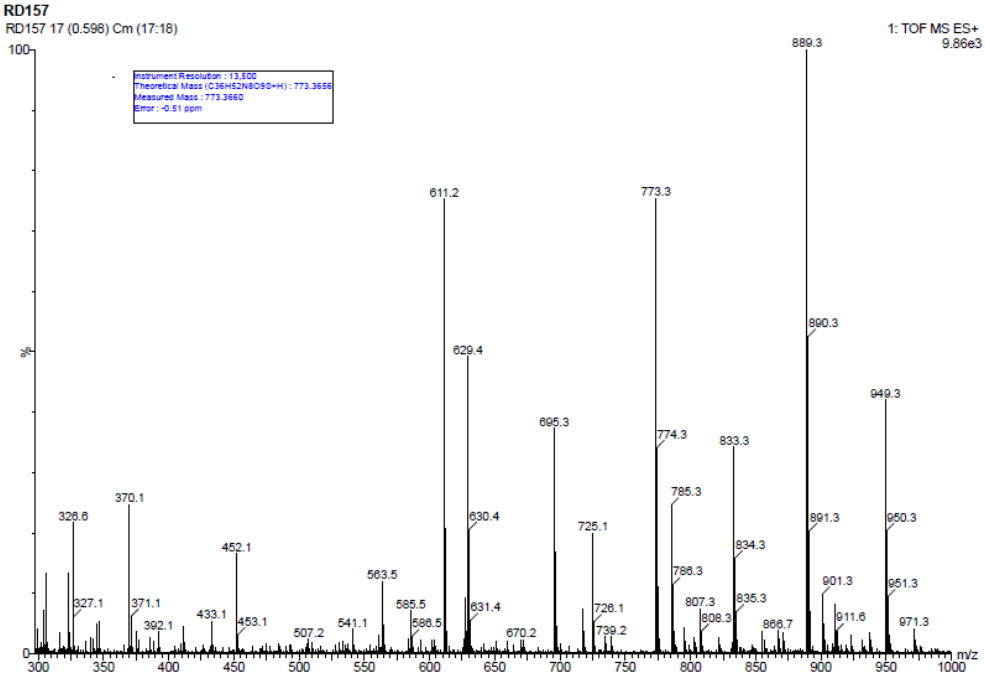
The crude peptide was purified by preparative reverse phase HPLC using a semi-prep Phenomenex Onyx C18 100 x 10 mm column. A linear solvent gradient of 20-80% MeCN (0.1% TFA) in H_2O (0.1% TFA) over 30 min, at a flow rate of 2 mL min^{-1} was used. The fractions containing the target peptide were collected (retention time 11 min) and lyophilised to give a fluffy white powder (2 mg, 2.4 μmol).

Formation of Dha

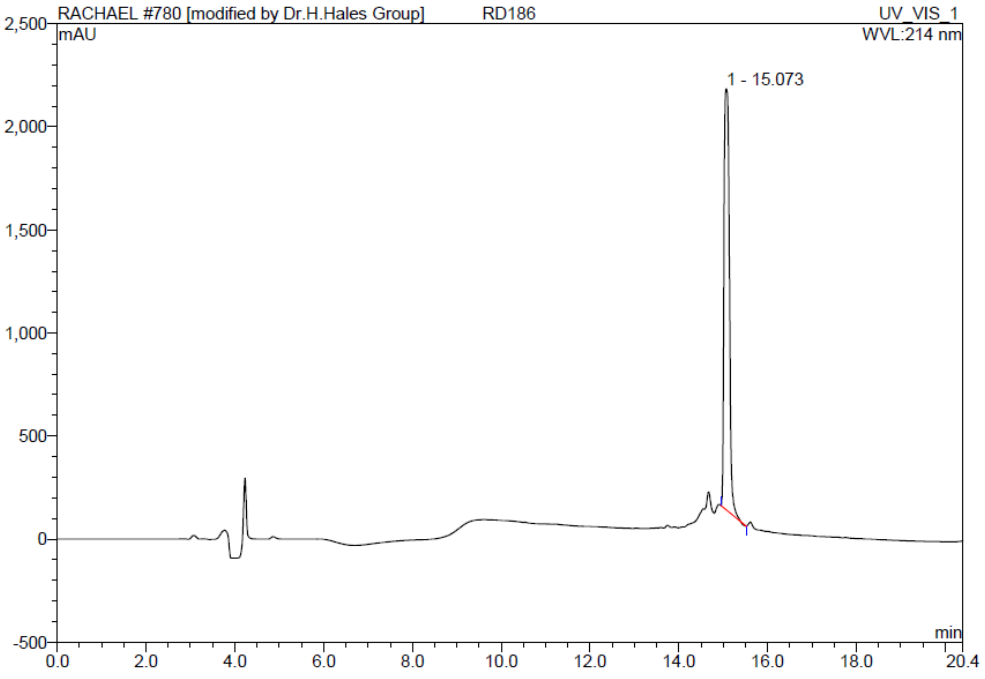
The dehydroalanine residues were formed according to the procedure reported in General Methods Section 7. The peptide was then purified by preparative reverse phase HPLC using a semi-prep Phenomenex Onyx C18 100 x 10 mm column. A linear solvent gradient

of 20-55% MeCN (0.1% TFA) in H_2O (0.1% TFA) over 30 min, at a flow rate of 2 mL min^{-1} was used. The fractions containing the peptide were collected (retention time 13 min) and lyophilised to give the pure sample as a fluffy white powder (1.5 mg, 11%).

m/z (HRMS, ES+) required for $[C_{36}H_{52}N_8O_9S+H]^+$ 773.3656, found $[C_{36}H_{52}N_8O_9S+H]^+$ 773.3660.



analytical HPLC (Dr Maisch GmbH Reprosil Gold 200 C8 5µm 250 x 4.6 mm column on Dionex HPLC system) retention time 15.07 min.

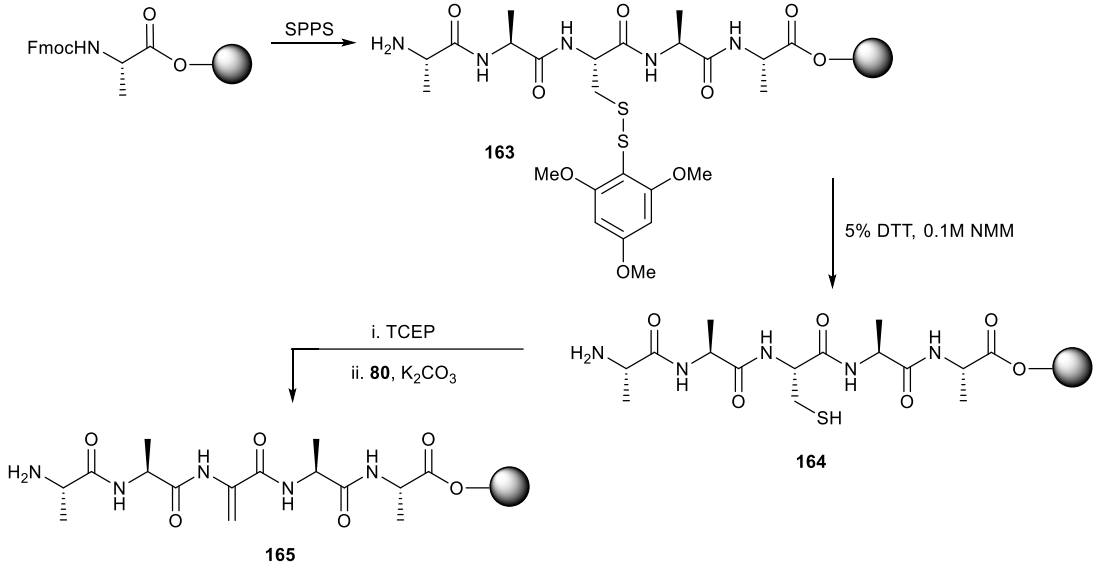


NMR δ_H (600 MHz, $(CD_3)_2SO$), δ_c (150 MHz, $(CD_3)_2SO$)

^1H							
Residue Number	**NH**	**α**	**β**	**γ**	**δ**	**Ar**	**Exchangeable**
1 - Phe		4.42	3.11			2 - 7.28 3 - 7.33 4 - 7.27	NH_2 - 8.19
2 - Dha	-		5.63 6.23				
3 - Lan (Dha)	8.57	4.59	2.75 3.19				
4 - Leu	8.40	4.18	1.67	1.58	a - 0.86 b - 0.81		
5 - Dha	8.81		5.54 5.97				
6 - Leu	8.43	4.36	1.65	1.52	a - 0.89 b - 0.85		
7 - Lan (Cys)	7.94	4.53	2.77 3.02				
8 - Ala	8.30	4.18	1.27				CO_2H - 12.56

^{13}C						
Residue Number	**CO**	**α**	**β**	**γ**	**δ**	**Ar**
1 - Phe		55.35	53.27			1 - 132.57 2 - 132.45 3 - 131.61 4 - 130.23
2 - Dha	166.70	137.87	107.67			
3 - Lan (Dha)	173.46	56.07	36.35			
4 - Leu	173.72	55.11	40.82	27.51	a - 26.90 b - 24.69	
5 - Dha	167.85	138.09	108.92			
6 - Leu	174.75	54.29	41.60	27.23	a - 26.27 b - 24.30	
7 - Lan (Cys)	173.02	57.30	36.37			
8 - Ala	176.92	50.84	20.48			

H-Ala-Ala-Dha-Ala-Ala-OH (165)



Reagents		Mass/Volume	µmol
Amino Acids	Fmoc-Ala-OH	26 mg	85
	Fmoc-Cys(STmp)-OH	46 mg	85
Coupling Reagents	HOAt	12 mg	85
	PyAOP	44 mg	85
	DIPEA	30 µL	170
	HBTU	32 mg	85
STmp Deprotection Reagents	DTT	300 mg	1945
	NMM in DMF	66 µL in 6 mL	
Cys Elimination Reagents	DMF:H_2O	1 mL:0.5 mL	
	TCEP	2.2 mg	7.7
	methyl 2,5-dibromopentanoate in DMF	280 mg in 0.5 mL	1020
	K_2CO_3 in H_2O	470 mg in 1 mL	3400

Peptide Synthesis

Fmoc-Ala-NovaSyn TGT resin (100 mg, 17 μmol) was swollen in DMF and the Fmoc group removed as described in General Methods Sections 1 and 2.

The next amino acid to be coupled was Fmoc-Ala-OH. This residue was preactivated for 2 min in a glass vial with HBTU and DIPEA in DMF (1.5 mL) before being added to the syringe containing the resin. This was left to shake at rt for 1 h before evacuation and washing the resin with DMF (4 x 2 mL). The Fmoc group was then removed.

Fmoc-Cys(STmp)-OH was then added using the double coupling procedure described in General Methods Section 4. The Fmoc group was then removed, followed by the addition of Fmoc-Ala-OH, using the HBTU coupling procedure described for the first Ala residue. The Fmoc group was then removed and the final Fmoc-Ala-OH coupled, again using the HBTU coupling procedure. The final Fmoc group was then removed. The resin was then washed with CH_2Cl_2 (5 x 2 mL) and DMF (5 x 2 mL).

STmp Deprotection and Dehydration to form Dha on Resin

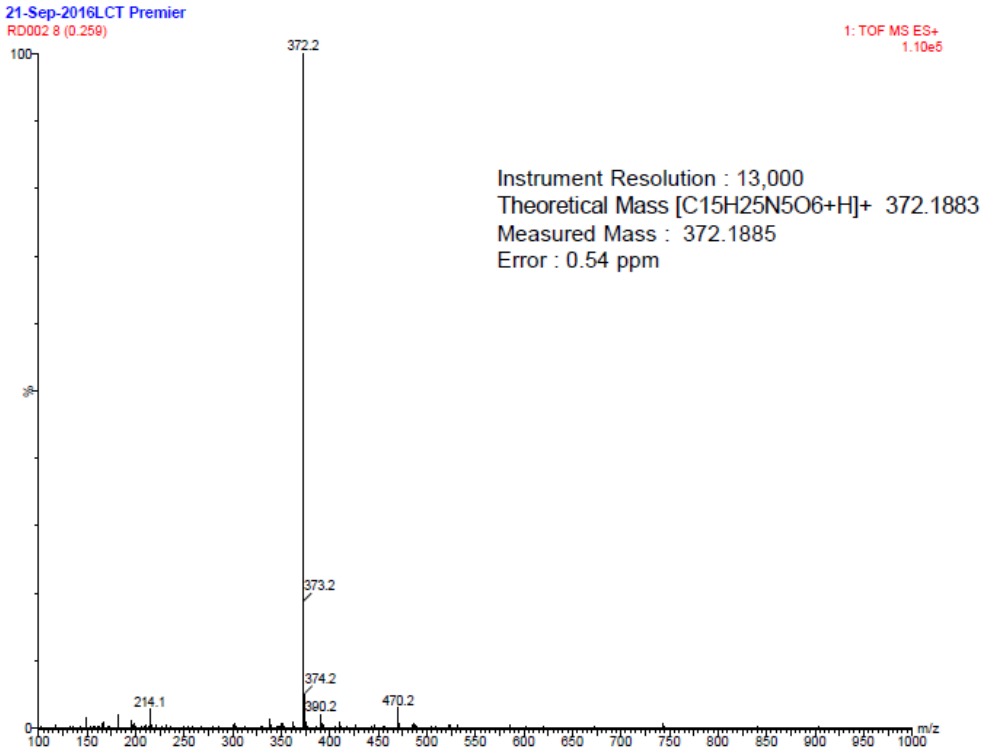
To form the dehydroalanine residue on resin, the STmp protecting group was first removed. A solution of DTT in DMF (5% w/v with 0.1 M NMM, 2 mL) was added to the resin and shaken for 5 min before removal of the solution. The addition and removal of the DTT solution was repeated twice more, each time leaving the deprotection for 5 min, before washing the resin with DMF (5 x 5 mL), CH_2Cl_2 (5 x 2 mL) and DMF (5 x 2 mL). Successful deprotection was confirmed by performing Ellman's test on a small portion of resin.[307] To perform the elimination, the resin was suspended in a DMF:H_2O mixture and TCEP was added. The resin was shaken at rt for 1 h before the addition of methyl 2,5-dibromopentanoate in DMF and K_2CO_3 in H_2O. This was shaken at 40 °C for 3.5 h before removal of the solution and washing the resin with DMF (6 x 2 mL).

Following completion of the synthesis, the peptide was cleaved from the resin using the procedure described in General Methods Section 6 and cleavage cocktail 2.

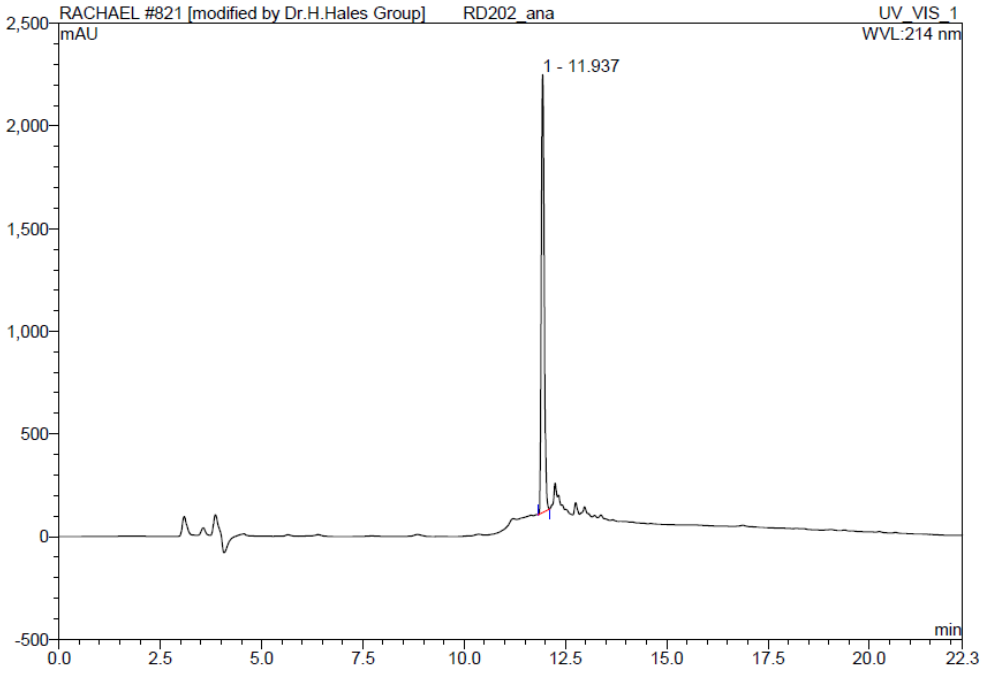
The crude peptide was purified by preparative reverse phase HPLC using a semi-prep Dr Maisch GmbH Reprosil Gold 200 C8 5μm 150 x 10 mm column. A linear solvent gradient of 5-75% MeCN (0.1% TFA) in H_2O (0.1% TFA) over 30 min, at a flow rate of

2 mL min^{-1} was used. The fractions containing the target peptide were collected (retention time 5 min) and lyophilised to give the sample as a fluffy white powder (4.7 mg, 74%).

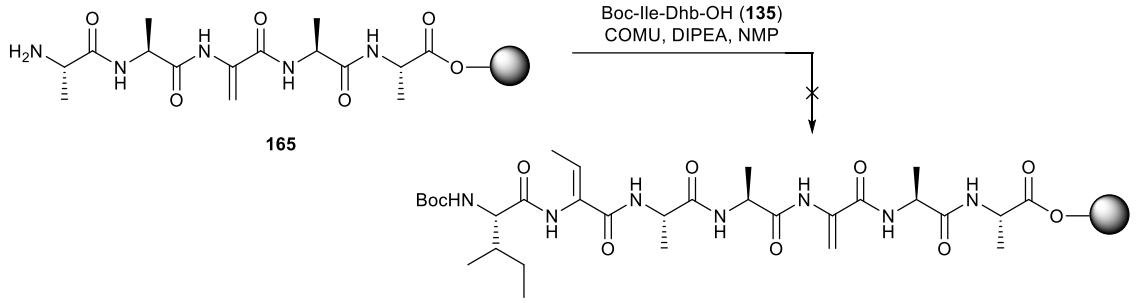
m/z (HRMS, ES+) required for $[C_{15}H_{25}N_5O_6+H]^+$ 372.1883, found $[C_{15}H_{25}N_5O_6+H]^+$ 372.1885.



analytical HPLC (Dr Maisch GmbH Reprosil Gold 200 C8 5µm 250 x 4.6 mm column on Dionex HPLC system) retention time 11.94 min.



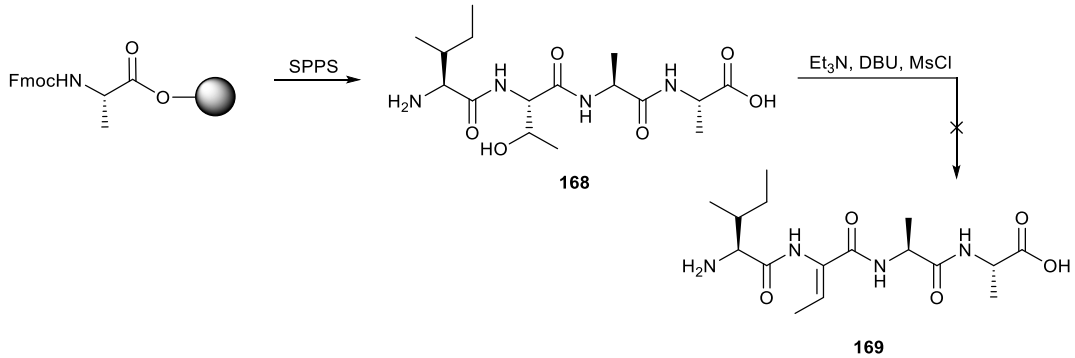
Attempted Coupling of Ile-Dhb Dipeptide (135) to H-Ala-Ala-Dha-Ala-Ala-OH (165)



Reagents		Mass/Volume	µmol
Amino Acids	Boc-Ile-Dhb-OH	13 mg	85
Coupling Reagents	DIPEA	30 µL	170
	COMU	18 mg	85

Boc-Ile-Dhb-OH, COMU and DIPEA (30 µL) were dissolved in NMP (1 mL) before being added to the resin-bound (**165**) (17 µmol of resin) and shaken at rt for 2 h. The solution was then evacuated and a fresh sample of the same coupling solution added to the resin and shaken for a further 2 h. This solution was then also evacuated and the resin washed with DMF (4 x 2 mL). No dipeptide addition could be detected by LCMS.

H-Ile-Thr-Ala-Ala-OH (168)



Reagents		Mass/Volume	µmol
Amino Acids	Fmoc-Ala-OH	13 mg	43
	Boc-Ile-Thr-OH	14 mg	43
Coupling Reagents	HBTU	16 mg	43
	HOAt	6 mg	43
	PyAOP	11 mg	43
	DIPEA	15 µL	85
Dehydration Reagents	Et_3N	3 µL	22
	MsCl	2 µL	22
	DBU	3 µL	22
	MeCN	200 µL	

Peptide Synthesis

Fmoc-Ala-NovaSyn TGT resin (50 mg, 8.5 µmol) was swollen in DMF and the Fmoc group removed as described in General Methods Sections 1 and 2.

The next amino acid to be coupled was Fmoc-Ala-OH. This residue was preactivated for 2 min in a glass vial with HBTU and DIPEA in DMF (1.5 mL) before being added to the syringe containing the resin. This was left to shake at rt for 1 h before evacuation and washing the resin with DMF (4 x 2 mL). The Fmoc group was then removed.

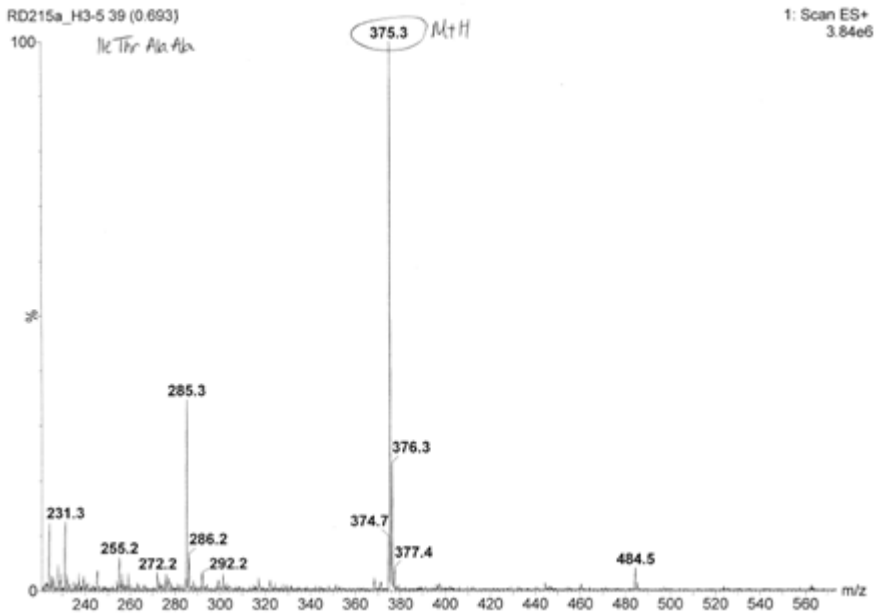
The double coupling procedure described in General Methods Section 4 was then used to couple the Boc-Ile-Thr-OH dipeptide.

Following completion of the synthesis, the peptide was cleaved from the resin using the procedure described in General Methods Section 6 and cleavage cocktail 2.

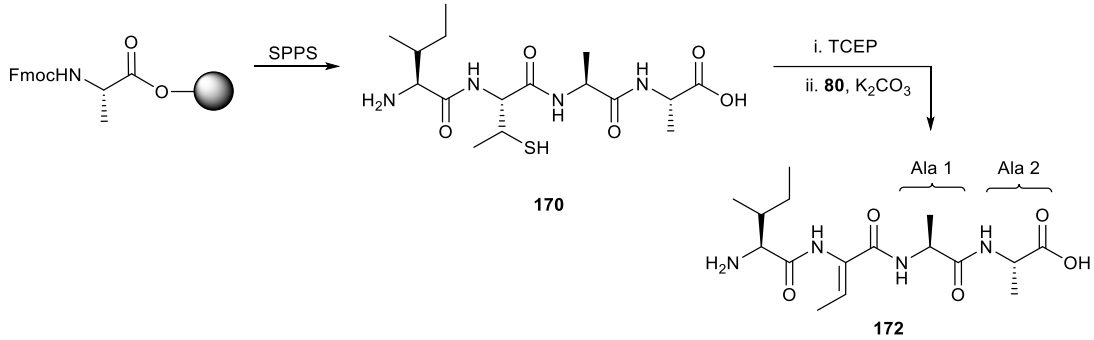
Attempted Dehydration to form Dhb

The cleaved peptide (4 mg, 10.7 μmol) was dissolved in MeCN in dry glassware and stirred under Ar. Et_3N, MsCl and DBU were added slowly and the solution heated at reflux for 3 h. After this time water (3 mL) was added and the solution lyophilised. No Thr elimination could be detected by LCMS so the peptide was not purified.

m/z (LCMS, ES+) found $[M+H]^+$ 375.3.



H-Ile-Dhb-Ala-Ala-OH (172)



Reagents		Mass/Volume	µmol
Amino Acids	Fmoc-Ala-OH	16 mg	53
	Fmoc-β-Me-Cys(Trt)-OH	19 mg	32
	Fmoc-Ile-OH	19 mg	53
Coupling Reagents	HOAt	7 mg	53
	PyAOP	27 mg	53
	DIPEA	18 µL	105
	HBTU	20 mg	53
Cys Elimination Reagents	H_2O	4 mL	
	TCEP	3 mg	11
	methyl 2,5-dibromopentanoate	408 mg	1490
	DMSO	4 mL	
	K_2CO_3	518 mg	3750

Peptide Synthesis

Fmoc-Ala-NovaSyn TGT resin (50 mg, 10.5 µmol) was swollen in DMF and the Fmoc group removed as described in General Methods Sections 1 and 2.

The next amino acid to be coupled was Fmoc-Ala-OH. This residue was preactivated for 2 min in a glass vial with HBTU and DIPEA in DMF (1.5 mL) before being added to the syringe containing the resin. This was left to shake at rt for 1 h before evacuation and washing the resin with DMF (4 x 2 mL). The Fmoc group was then removed.

Fmoc-β-Me-Cys(Trt)-OH was then added using the double coupling procedure described in General Methods Section 4. The Fmoc group was then removed, followed by the

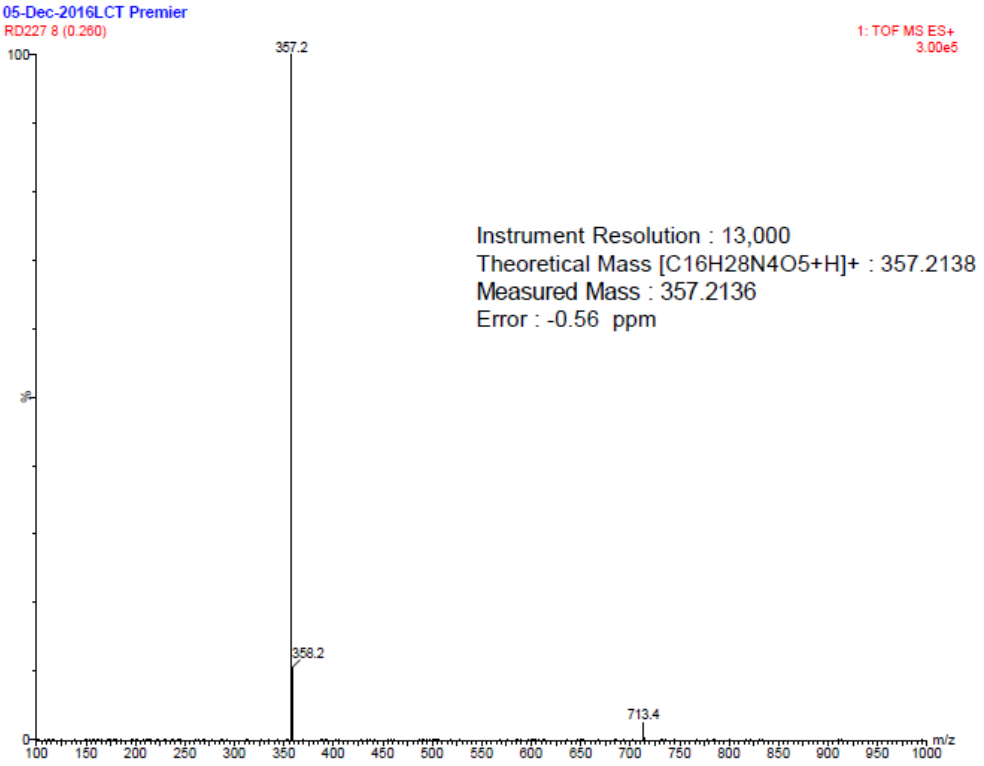
addition of Fmoc-Ile-OH, using the same procedure as described for the Ala residue, with the same masses of coupling reagents and volumes of base and DMF. The final Fmoc group was then removed.

Following completion of the synthesis, the peptide was cleaved from the resin using the procedure described in General Methods Section 6 and cleavage cocktail 1.

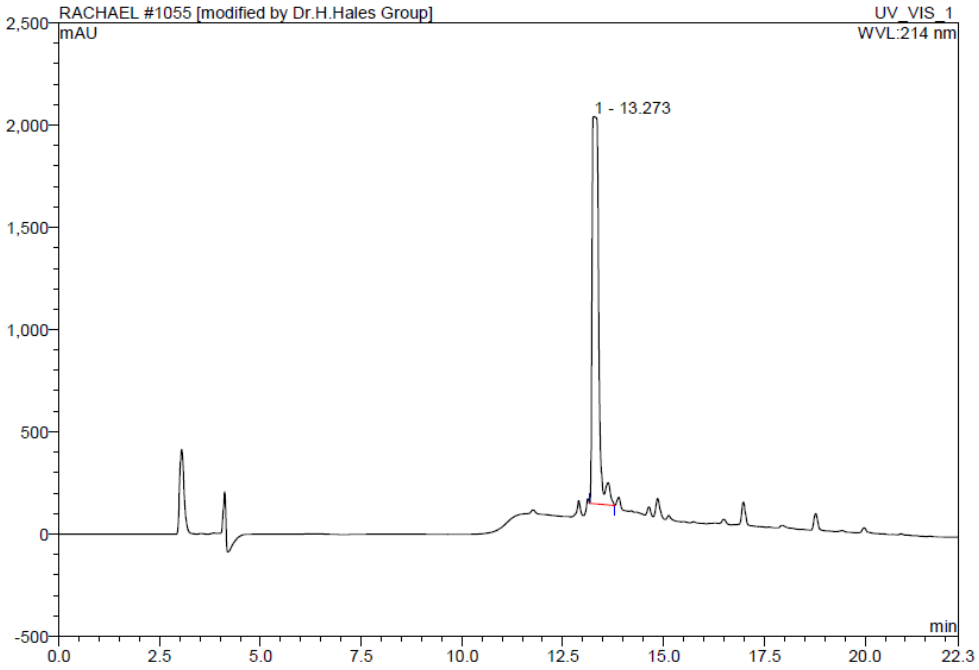
Elimination to form Dhb

The dehydrobutyrine residue was then formed on the crude peptide (9.8 mg, 25 µmol) according to the procedure reported in General Methods Section 7. The peptide was then purified by preparative reverse phase HPLC using a semi-prep Agilent Zorbax 300SB-C18 5µm 250 x 9.4 mm column. A linear solvent gradient of 2-98% MeCN (0.1% TFA) in H_2O (0.1% TFA) over 20 min, at a flow rate of 2 mL min^{-1} was used. The fractions containing the peptide were collected (retention time 13 min) and lyophilised to give the pure sample as a fluffy white powder (2.9 mg, 78%).

m/z (HRMS, ES+) required for $[C_{16}H_{28}N_4O_5+H]^+$ 357.2138, found $[C_{16}H_{28}N_4O_5+H]^+$ 357.2136.



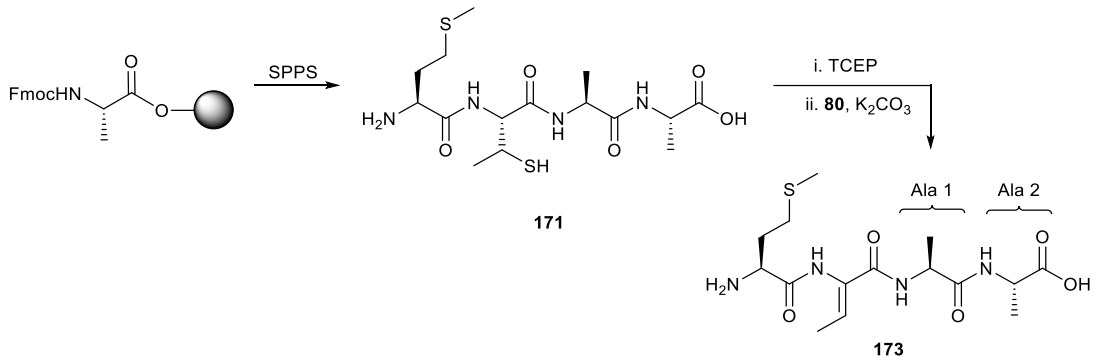
analytical HPLC (Dr Maisch GmbH Reprosil Gold 200 C8 5µm 250 x 4.6 mm column on Dionex HPLC system) retention time 13.27 min.



NMR

δ_H (600 MHz, $(CD_3)_2SO$) 0.89 (3H, t, *J* 7.2, Ile δC*H*$_3$), 0.99 (3H, d, *J* 7.2, Ile γC*H*$_3$), 1.15-1.20 (1H, m, Ile γC*H*$_A$H$_B$), 1.24 (3H, d, *J* 6.6, Ala 1 βC*H*$_3$), 1.27 (3H, d, *J* 7.8, Ala 2 βC*H*$_3$), 1.53-1.57 (1H, m, Ile γCH$_A$*H*$_B$), 1.67 (3H, d, *J* 6.6, Dhb γC*H*$_3$), 1.87-1.91 (1H, m, Ile βC*H*), 3.79-3.81 (1H, m, Ile αC*H*), 4.17-4.20 (1H, m, Ala 2 αC*H*), 4.35-4.40 (1H, m, Ala 1 αC*H*), 6.37 (1H, q, *J* 7.2, Dhb βC*H*), 7.78 (1H, d, *J* 7.2, Ala 1 N*H*), 8.10 (2H, br d, *J* 4.2, N*H*$_2$), 8.17 (1H, d, *J* 7.2, Ala 2 N*H*), 9.58 (1H, s, Dhb N*H*); δ_c (150 MHz, $(CD_3)_2SO$) 11.3 (Ile δ*C*H$_3$), 13.2 (Dhb γ*C*H$_3$), 14.6 (Ile γ*C*H$_3$), 17.0 (Ala 2 β*C*H$_3$), 18.0 (Ala 1 β*C*H$_3$), 23.8 (Ile γ*C*H$_2$), 24.8 (Ile β*C*H), 47.0 (Ala 2 α*C*H), 48.0 (Ala 1 α*C*H), 56.7 (Ile α*C*H), 127.4 (Dhb β*C*H), 130.0 (Dhb α*C*), 163.4 (Dhb *C*O), 166.9 (Ile *C*O), 172.0 (Ala 1 *C*O), 174.0 (CO_2H).

H-Met-Dhb-Ala-Ala-OH (173)



Reagents		Mass/Volume	µmol
Amino Acids	Fmoc-Ala-OH	16 mg	53
	Fmoc-β-Me-Cys(Trt)-OH	19 mg	32
	Fmoc-Met-OH	20 mg	53
Coupling Reagents	HOAt	7 mg	53
	PyAOP	27 mg	53
	DIPEA	18 µL	105
	HBTU	20 mg	53
Cys Elimination Reagents	H_2O	3.3 mL	
	TCEP	2 mg	7
	methyl 2,5-dibromopentanoate	266 mg	970
	DMSO	3.3 mL	
	K_2CO_3	335 mg	2430

Peptide Synthesis

Fmoc-Ala-NovaSyn TGT resin (50 mg, 10.5 μmol) was swollen in DMF and the Fmoc group removed as described in General Methods Sections 1 and 2.

The next amino acid to be coupled was Fmoc-Ala-OH. This residue was preactivated for 2 min in a glass vial with HBTU and DIPEA in DMF (1.5 mL) before being added to the syringe containing the resin. This was left to shake at rt for 1 h before evacuation and washing the resin with DMF (4 x 2 mL). The Fmoc group was then removed.

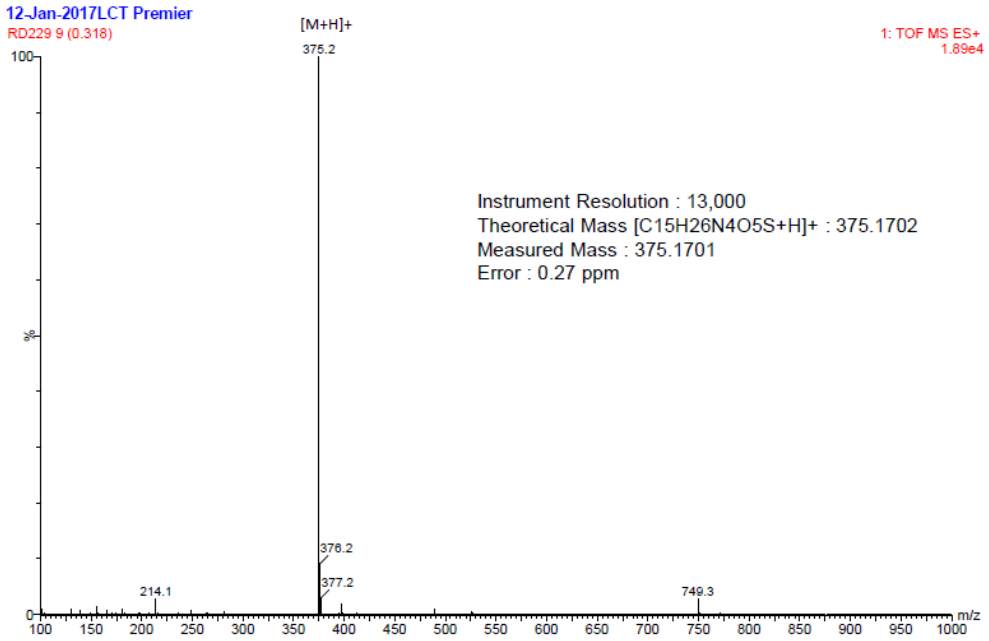
Fmoc-β-Me-Cys(Trt)-OH was then added using the double coupling procedure described in General Methods Section 4. The Fmoc group was then removed, followed by the addition of Fmoc-Met-OH, using the same procedure as described for the Ala residue, with the same masses of coupling reagents and volumes of base and DMF. The final Fmoc group was then removed.

Following completion of the synthesis, the peptide was cleaved from the resin using the procedure described in General Methods Section 6 and cleavage cocktail 1.

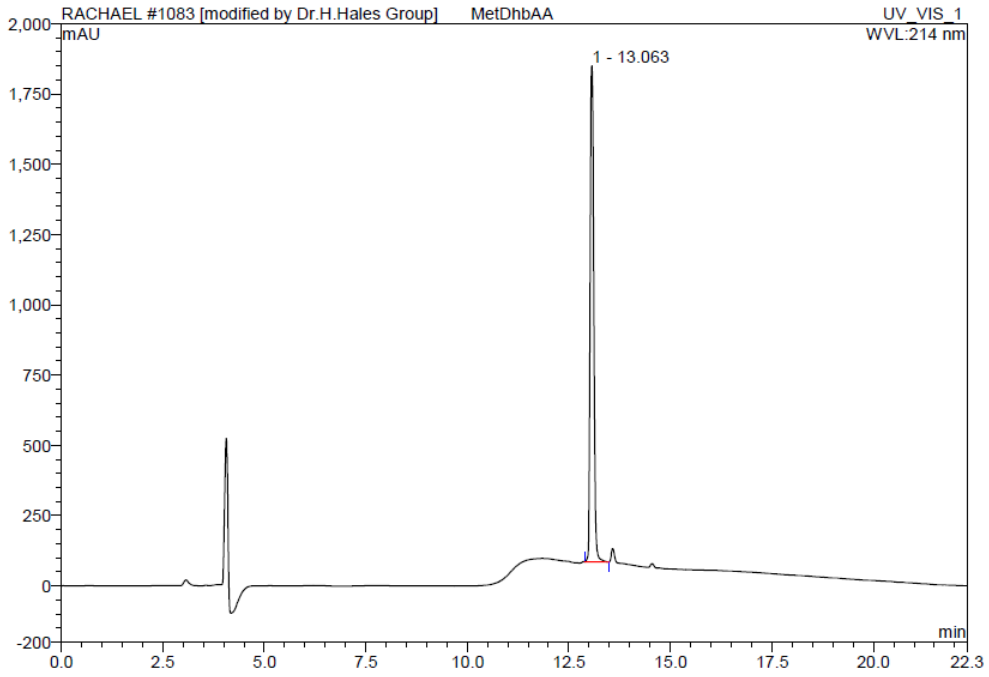
Elimination to form Dhb

The dehydrobutyrine residue was then formed on the crude peptide (6.6 mg, 16 μmol) according to the procedure reported in General Methods Section 7. The peptide was then purified by preparative reverse phase HPLC using a semi-prep Agilent Zorbax 300SB-C18 5μm 250 x 9.4 mm column. A linear solvent gradient of 2-98% MeCN (0.1% TFA) in H_2O (0.1% TFA) over 20 min, at a flow rate of 2 mL min^{-1} was used. The fractions containing the peptide were collected (retention time 12 min) and lyophilised to give the pure sample as a fluffy white powder (3.3 mg, 84%).

m/z (HRMS, ES+) required for $[C_{15}H_{26}N_4O_5S+H]^+$ 375.1702, found $[C_{15}H_{26}N_4O_5S+H]^+$ 375.1701.



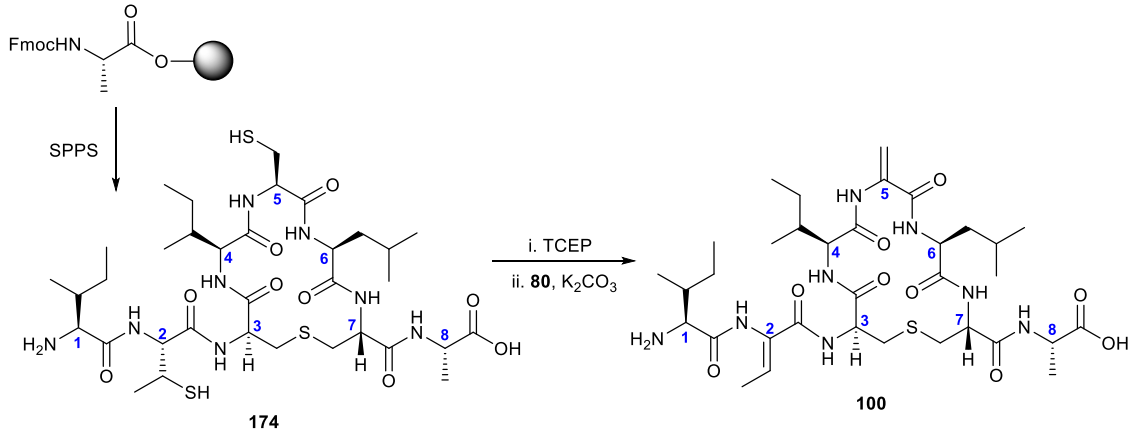
analytical HPLC (Dr Maisch GmbH Reprosil Gold 200 C8 5µm 250 x 4.6 mm column on Dionex HPLC system) retention time 8.07 min.



NMR

δ_H (600 MHz, $(CD_3)_2SO$) 1.24 (3H, d, *J* 7.2, Ala 1 βC*H*$_3$), 1.27 (3H, d, *J* 7.8, Ala 2 βC*H*$_3$), 1.66 (3H, d, *J* 7.2, Dhb γC*H*$_3$), 1.99-2.06 (2H, m, Met βC*H*$_2$), 2.08 (3H, s, SC*H*$_3$), 2.56-2.60 (2H, m, Met γC*H*$_2$), 4.02 (1H, t, *J* 6.0, Met αC*H*), 4.18 (1H, quintet, *J* 7.2, Ala 2 αC*H*), 4.37 (1H, quintet, *J* 7.2, Ala 1 αC*H*), 6.40 (1H, q, *J* 7.2, Dhb βC*H*), 7.86 (1H, d, *J* 7.8, Ala 1 N*H*), 8.19 (3H, br d, *J* 7.2, Ala 2 N*H* and N*H*$_2$), 9.66 (1H, s, Dhb N*H*), 12.54 (1H, br s, CO_2H); δ_c (150 MHz, $(CD_3)_2SO$) 13.2 (Dhb γ*C*H$_3$), 14.3 (S*C*H$_3$), 17.1 (Ala 2 β*C*H$_3$), 18.2 (Ala 1 β*C*H$_3$), 28.1 (Met γ*C*H$_2$), 30.8 (Met β*C*H$_2$), 47.5 (Ala 2 α*C*H), 48.1 (Ala 1 α*C*H), 51.8 (Met α*C*H), 127.9 (Dhb β*C*H), 129.9 (Dhb α*C*), 163.4 (Dhb *C*O), 167.4 (Met *C*O), 172.0 (Ala 1 *C*O), 174.5 (*C*O$_2$H).

Nisin Ring A WT (100)



Reagents		Mass/Volume	µmol
Amino Acids	Teoc/TMSE lanthionine	43 mg	63
	Fmoc-Leu-OH	37 mg	105
	Fmoc-Cys(Trt)-OH	62 mg	105
	Fmoc-Ile-OH	37 mg	105
	Fmoc-β-Me-Cys(Trt)-OH	12 mg	42
Coupling Reagents	HOAt	14 mg	105
	PyAOP	55 mg	105
	DIPEA	37 µL	210
Cys Elimination Reagents	H_2O	270 µL	
	TCEP	615 µL of a 0.5 mg mL^{-1} stock solution in water	1.1
	methyl 2,5-dibromopentanoate	40 mg	147
	DMSO	880 µL	
	K_2CO_3	51 mg	370

Peptide Synthesis

Fmoc-Ala-NovaSyn TGT resin (100 mg, 21 µmol) was swollen in DMF and the Fmoc group removed as described in General Methods Sections 1 and 2.

The protected Teoc/TMSE lanthionine was coupled using the microwave procedure described in General Methods Section 3. The Fmoc group was then removed.

The peptide chain was elongated using the double coupling procedure described in General Methods Section 4. Fmoc-Leu-OH was added first, the Fmoc group was removed, followed by the addition of Fmoc-Cys(Trt)-OH, removal of the Fmoc group and coupling of Fmoc-Ile-OH.

The cyclisation reaction was then carried out as described in Section 5. The silyl protecting groups were removed (General Methods Section 5a), followed by removal of the final Fmoc group (General Methods Section 5b). The cyclisation reaction was then carried out as described in Section 5c for lanthionine containing peptides.

The peptide chain was then further elongated using the double coupling procedure described in General Methods Section 4. Firstly Fmoc-β-Me-Cys(Trt)-OH (2 eq per coupling) was added, the Fmoc group removed, followed by the addition of Fmoc-Ile-OH, and removal of the final Fmoc group.

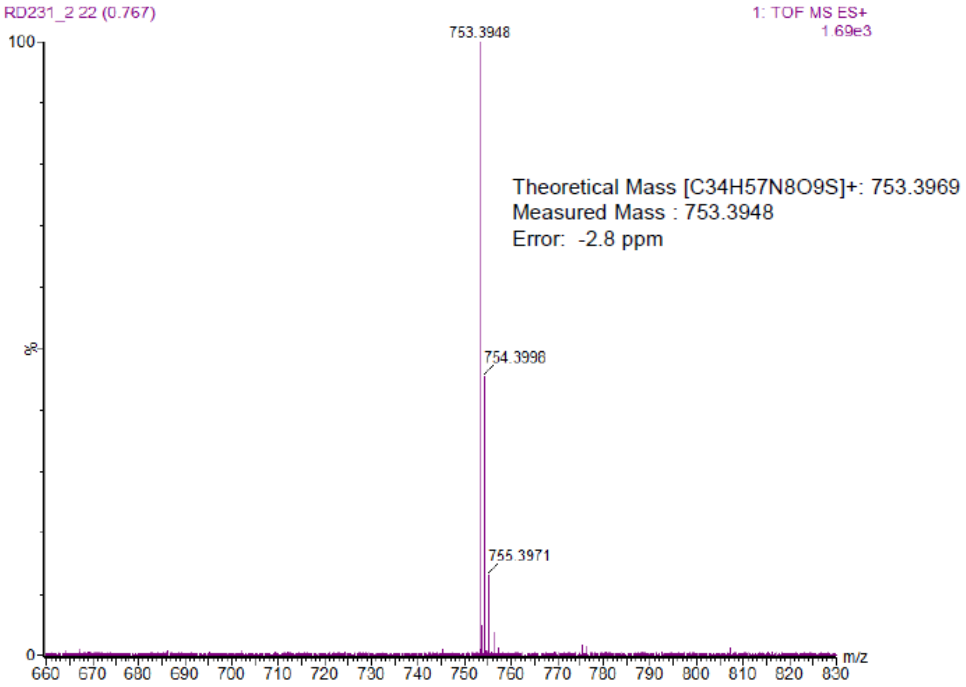
Following completion of the synthesis, the peptide was cleaved from the resin using the procedure described in General Methods Section 6 and cleavage cocktail 1.

The crude peptide was purified by preparative reverse phase HPLC using a semi-prep Phenomenex Onyx C18 100 x 10 mm column. A linear solvent gradient of 20-80% MeCN (0.1% TFA) in H_2O (0.1% TFA) over 30 min, at a flow rate of 2 mL min^{-1} was used. The fractions containing the target peptide were collected (retention time 11 min) and lyophilised to give a fluffy white powder (2 mg, 2.4 μmol).

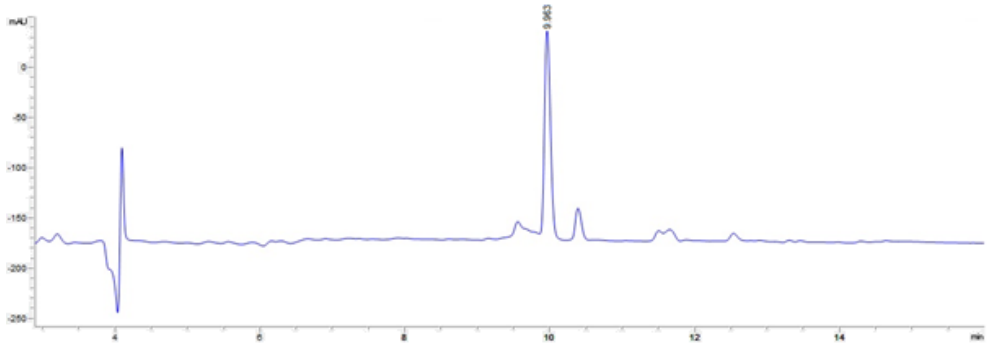
Elimination to form Dehydro Residues

The dehydro residues were formed according to the procedure reported in General Methods Section 7. The peptide was then purified by preparative reverse phase HPLC using a semi-prep Agilent Zorbax 300SB-C18 5μm 250 x 9.4 mm column. A linear solvent gradient of 20-70% MeCN (0.1% TFA) in H_2O (0.1% TFA) over 28 min, at a flow rate of 2 mL min^{-1} was used. The fractions containing the peptide were collected (retention time 22 min) and lyophilised to give the pure sample as a fluffy white powder (200 μg, 1%).

m/z (HRMS, ES+) required for $[C_{34}H_{57}N_8O_9S]^+$ 753.3969, found $[C_{34}H_{57}N_8O_9S]^+$ 753.3948.



analytical HPLC (Dr Maisch GmbH Reprosil Gold 200 C8 5µm 250 x 4.6 mm column on Agilent HPLC system) retention time 9.96 min.



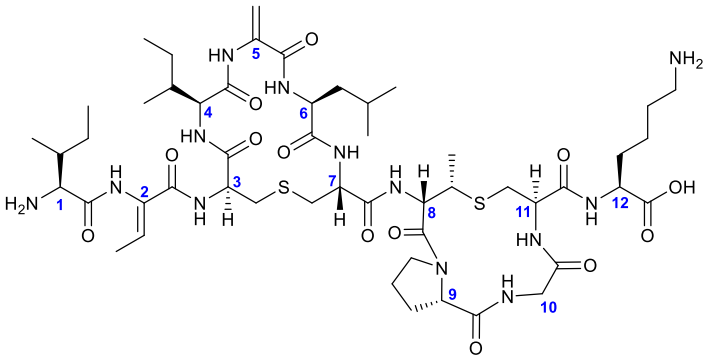
NMR δ_H (600 MHz, $(CD_3)_2SO$), δ_c (150 MHz, $(CD_3)_2SO$)

^1H						
Residue Number	**NH**	**α**	**β**	**γ**	**δ**	**Exchangeable**
1 - Ile		3.79	1.91	CH_2 - 1.18, 1.53 CH_3 - 0.97	0.89	NH_2 - 8.09
2 - Dhb	9.78		6.30	1.71		
3 - Lan (Dha)	8.15	4.38	2.94			
4 - Ile	7.94	4.09	2.00	CH_2 - 1.08, 1.38 CH_3 - 0.88	0.77	
5 - Dha	8.82		5.53 6.04			
6 - Leu	8.70	4.25	1.63	1.55	a - 0.86 b - 0.90	
7 - Lan (Cys)	7.94	4.47	2.81 2.95			
8 - Ala	8.21	4.17	1.27			CO_2H - 12.61

^{13}C					
Residue Number	**CO**	**α**	**β**	**γ**	**δ**
1 - Ile		59.89	39.33	CH_2 - 26.88 CH_3 - 14.61	17.82
2 - Dhb		133.38	129.68	16.56	
3 - Lan (Dha)	173.95	57.18	37.83		
4 - Ile	177.48	60.79	39.25	CH_2 - 27.63 CH_3 - 13.91	18.98
5 - Dha		132.79	107.03		
6 - Leu		55.18	41.04	40.86	a - 25.02 b - 26.36
7 - Lan (Cys)		56.93	43.26		
8 - Ala	176.99	50.72	20.35		

7.2.3. Double Ring Synthesis

Nisin Rings AB WT (95)[216]



95

Enrichment

A commercially available nisin preparation (20 g) was stirred vigorously in deionised water (500 mL) for 15 min before the addition of CH_2Cl_2 (400 mL). The resulting suspension was transferred to Falcon tubes and centrifuged (2500 rpm, 15 min). The liquid was then decanted and the pellets dried *in vacuo*, before redissolving in deionised water (250 mL) and filtering through Celite®. The solution was then concentrated *in vacuo* to a volume of 50 mL before lyophilisation to give the enriched nisin as a pale brown solid (726 mg).

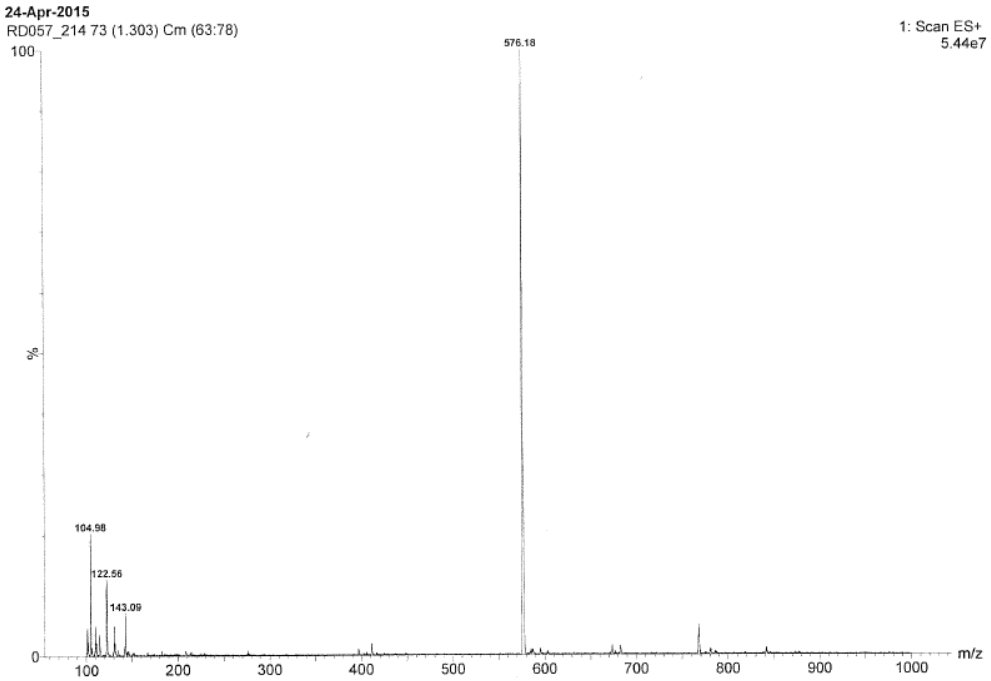
Trypsin Digestion

A sample of enriched nisin (60 mg) was added to a 50 mL Falcon tube and dissolved in acetate buffer (50 mL; 25 mM NaOAc, 5 mM TRIS base, 5 mM $CaCl_2$, adjusted to pH 7 with acetic acid). This was repeated in 5 additional tubes (total volume of dissolved nisin was 360 mg). The solutions were cooled in an ice-water bath before the addition of trypsin (5 mg) to each, and then were incubated at 30 °C for 24 h. An additional portion of trypsin was then added (5 mg) to each tube and the solutions stirred at 30 °C for another 24 h. The addition-incubation cycle was repeated once more (total incubation time 72 h) before combining the solutions and acidifying to pH 4 with HCl. The solvent was then removed *in vacuo*, with the addition of acetonitrile where necessary to prevent excessive foaming. The residue was then redissolved in water and lyophilised.

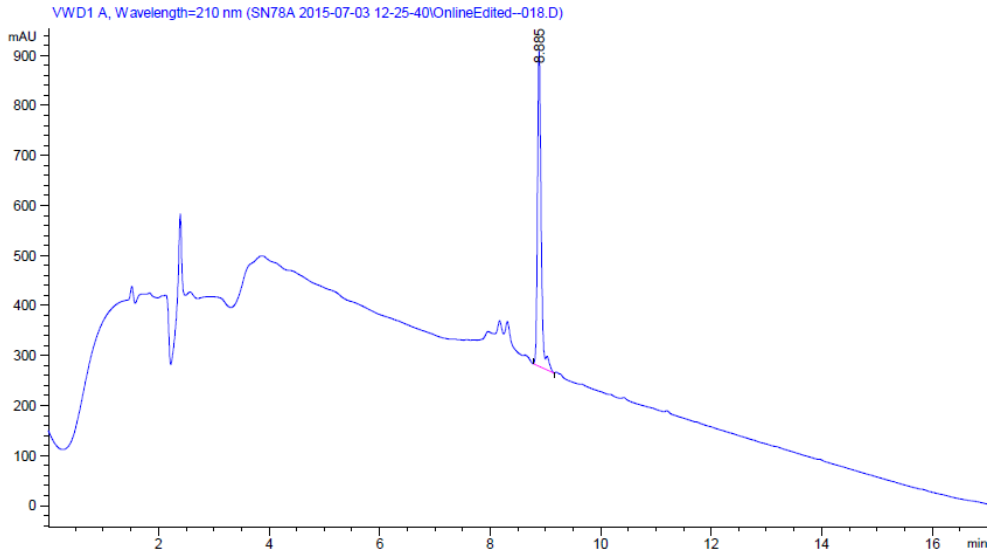
The crude peptide was purified by preparative reverse phase HPLC using a semi-prep Dr Maisch GmbH Reprosil Gold 200 C8 5µm 150 x 10 mm column. Initially, a linear solvent

gradient of 5-40% MeCN (0.1% TFA) in H_2O (0.1% TFA) over 40 min, at a flow rate of 2 mL min^{-1} was used (retention time 34 min). This was followed by a second purification on a semi-prep Phenomenex Onyx C18 100 x 10 mm column, with a linear solvent gradient of 25-40% MeCN (0.1% TFA) in H_2O (0.1% TFA) over 23 min, at a flow rate of 2 mL min^{-1}. The fractions containing the target peptide (retention time 10 min) were collected and lyophilised to give the sample as a fluffy white powder (2.3 mg). Data is in agreement with the literature.[206,216]

m/z (LCMS, ES+) found $[M+2H]^{2+}$ 576.2.



analytical HPLC (ACE5 C8-300 150 x 4.6 mm column on Agilent HPLC system) retention time 8.89 min.

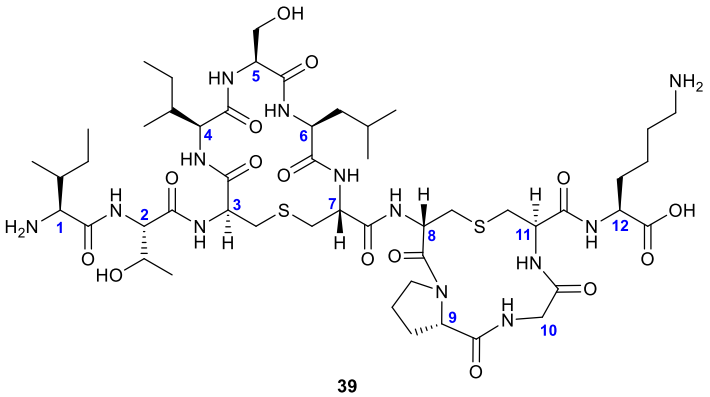


NMR δ_H (900 MHz, $(CD_3)_2SO$), δ_c (150 MHz, $(CD_3)_2SO$)

^1H							
Residue Number	**NH**	**α**	**β**	**γ**	**δ**	**ε**	**Exchangeable**
1 - Ile		3.79	1.90	CH_2 - 1.19, 1.56 CH_3 - 0.98	0.88		NH_2 - 8.13
2 - Dhb	9.72		6.28	1.71			
3 - Lan (Dha)	8.15	4.39	2.94 3.03				
4 - Ile	7.91	4.09	2.02	CH_2 - 1.07, 1.38 CH_3 - 0.89	0.78		
5 - Dha	8.85		5.50 5.99				
6 - Leu	8.77	4.23	1.55 1.64	1.55	a - 0.85 b - 0.89		
7 - Lan (Cys)	7.95	4.64	2.79 3.08				
8 - MeLan (Dhb)	8.16	4.17	3.45	1.18			
9 - Pro		4.19	1.65 2.25	1.89 1.76	3.27 3.38		
10 - Gly	8.25	4.97					
11 - MeLan (Cys)	7.59	3.84	2.97 3.39				
12 - Lys	8.21	4.18	1.75	1.34	1.55	2.76	NH_2 - 7.64 CO_2H - 12.70

^{13}C						
Residue Number	**CO**	**α**	**β**	**γ**	**δ**	**ε**
1 - Ile	175.92	59.60	39.19	CH_2 - 27.33 CH_3 - 17.84	14.26	
2 - Dhb	167.39	133.13	129.70	16.23		
3 - Lan (Dha)	175.42	57.01	37.87			
4 - Ile	172.83	61.02	36.62	CH_2 - 27.54 CH_3 - 18.87	13.80	
5 - Dha	167.83	137.79	107.23			
6 - Leu	175.69	54.57	33.61	27.62	a - 24.80 b - 25.96	
7 - Lan (Cys)	173.58	57.39	42.77			
8 - MeLan (Dhb)	176.28	60.24	51.43	25.01		
9 - Pro	173.94	66.08	31.81	29.38	50.90	
10 - Gly	174.43	59.89				
11 - MeLan (Cys)	172.25	56.63	39.45			
12 - Lys	176.87	54.52	41.05	25.54	29.55	41.76

Nisin Rings AB Thr/Ser Analogue (39)



39

Reagents		Mass/Volume	µmol
Amino Acids	Teoc/TMSE lanthionine	37 mg	54
	Fmoc-Gly-OH	27 mg	90
	Fmoc-Pro-OH	30 mg	90
	Fmoc-Leu-OH	32 mg	90
	Fmoc-Ile-Ser($\psi^{Me,Me}$pro)-OH	43 mg	90
	Fmoc-Thr(*t*Bu)-OH	36 mg	90
	Fmoc-Ile-OH	32 mg	90
Coupling Reagents	HOAt	12 mg	90
	PyAOP	47 mg	90
	DIPEA	31 µL	180

Fmoc-Lys(Boc)-NovaSyn TGT resin (100 mg, 18.0 µmol) was swollen in DMF and the Fmoc group removed as described in General Methods Sections 1 and 2.

The protected Teoc/TMSE lanthionine was coupled using the microwave procedure described in General Methods Section 3. The Fmoc group was then removed.

The peptide chain was elongated using the double coupling procedure described in General Methods Section 4. Fmoc-Gly-OH was added first, the Fmoc group was removed, followed by the addition of Fmoc-Pro-OH.

The cyclisation reaction was then carried out as described in Section 5. The silyl protecting groups were removed (General Methods Section 5a), followed by removal of the Fmoc group (General Methods Section 5b). The cyclisation reaction was then carried out as described in Section 5c for lanthionine containing peptides.

A second Teoc/TMSE lanthionine was then coupled onto the B ring using the microwave procedure described in General Methods Section 3. The Fmoc group was then removed and the peptide chain further elongated using the double coupling procedure described in General Methods Section 4. Fmoc-Leu-OH was added first and the Fmoc group was removed, followed by the addition of Fmoc-Ile-Ser($\psi^{Me,Me}$pro)-OH. The second cyclisation reaction was then carried out in the same way as the first, as described in General Methods Section 5.

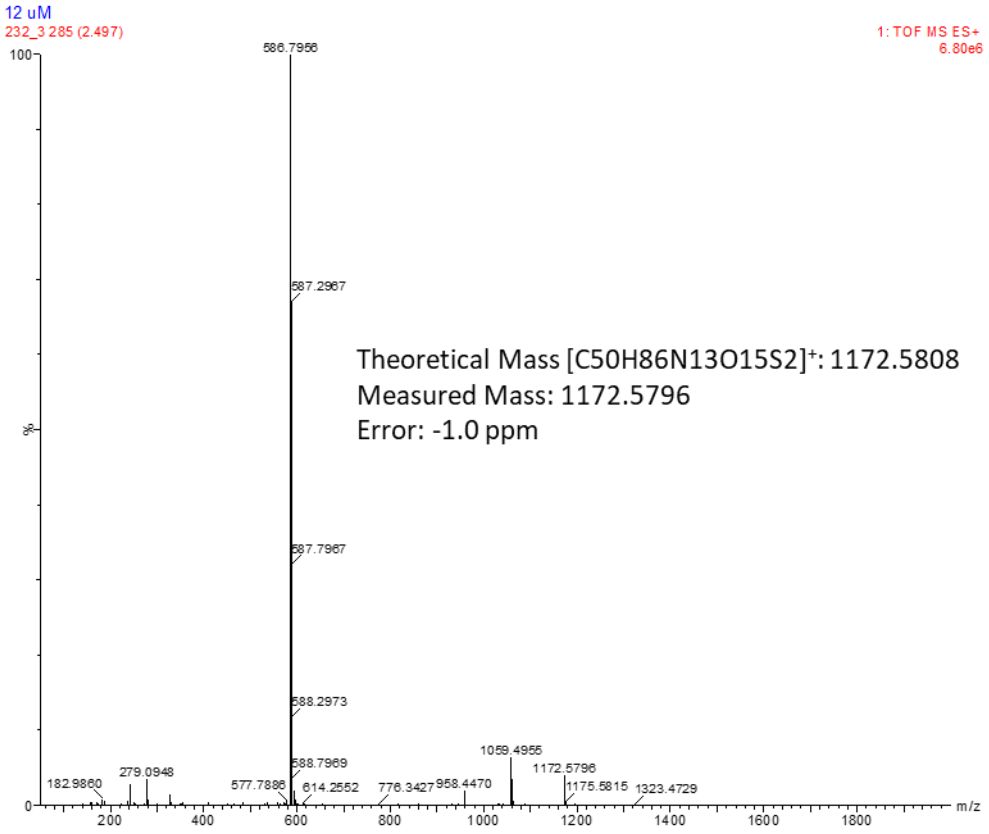
The peptide chain was then further elongated using the double coupling procedure described in General Methods Section 4. Firstly Fmoc-Thr(*t*Bu)-OH was added and the Fmoc group removed, followed by the addition of Fmoc-Ile-OH, and removal of the final Fmoc group.

Following completion of the synthesis, the peptide was cleaved from the resin using the procedure described in General Methods Section 6 and cleavage cocktail 2.

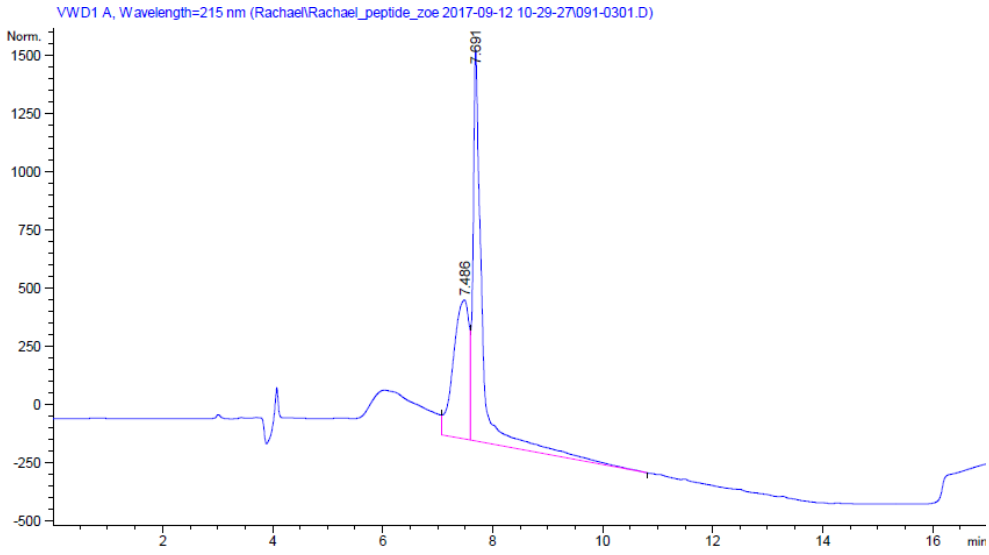
The crude peptide (9.5 mg) was then transferred to a glass vial and cooled in ice. Triflic acid (200 µL) was added dropwise and the solution agitated until the peptide was dissolved. The solution was left in ice for 5 min before transferring the whole reaction mixture to a Falcon tube containing cold ether (10 mL) and centrifuging at 4000 rpm at 5 °C for 15 min, after which time a precipitate formed. The ether was poured off and fresh ether added to resuspend the pellet, before a further round of centrifugation (4000 rpm, 5 °C, 10 min). The resuspension/centrifugation process was repeated once more. The resultant pellet was resuspended in water (3 mL) and lyophilised.

The crude peptide was purified by preparative reverse phase HPLC using a semi-prep Phenomenex Onyx C18 100 x 10 mm column with a linear solvent gradient of 20-60% MeCN (0.1% TFA) in H_2O (0.1% TFA) over 28 min. A second round of purification was then carried out on the Agilent HPLC system using a Dr Maisch GmbH Reprosil Gold 200 C8 5µm 250 x 4.6 mm column and a gradient of 15-25% MeCN (0.1% TFA) in H_2O (0.1% TFA) over 35 min. The fractions containing the target peptide (retention time 23-24 min) were collected and lyophilised to give the pure sample as a white powder (600 µg, 3%).

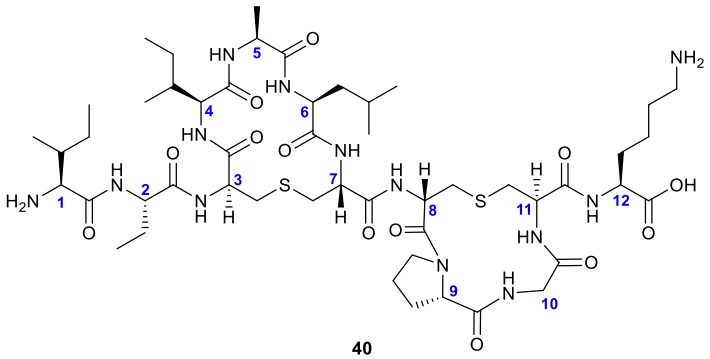
m/z (HRMS, ES+) required for $[C_{50}H_{86}N_{13}O_{15}S_2]^+$ 1172.5808, found $[C_{50}H_{86}N_{13}O_{15}S_2]^+$ 1172.5796.



analytical HPLC (Dr Maisch GmbH Reprosil Gold 200 C8 5µm 250 x 4.6 mm column on Agilent HPLC system) retention time 7.69 min.



Nisin Rings AB Abu/Ala Analogue (40)



40

Reagents		Mass/Volume	µmol
Amino Acids	Teoc/TMSE lanthionine	37 mg	54
	Fmoc-Gly-OH	27 mg	90
	Fmoc-Pro-OH	30 mg	90
	Fmoc-Leu-OH	32 mg	90
	Fmoc-Ala-OH	28 mg	90
	Fmoc-Ile-OH	32 mg	90
	Fmoc-Abu -OH	29 mg	90
Coupling Reagents	HOAt	12 mg	90
	PyAOP	47 mg	90
	DIPEA	31 µL	180

Fmoc-Lys(Boc)-NovaSyn TGT resin (100 mg, 18.0 µmol) was swollen in DMF and the Fmoc group removed as described in General Methods Sections 1 and 2.

The protected Teoc/TMSE lanthionine was coupled using the microwave procedure described in General Methods Section 3. The Fmoc group was then removed.

The peptide chain was elongated using the double coupling procedure described in General Methods Section 4. Fmoc-Gly-OH was added first, the Fmoc group was removed, followed by the addition of Fmoc-Pro-OH.

The cyclisation reaction was then carried out as described in Section 5. The silyl protecting groups were removed (General Methods Section 5a), followed by removal of the Fmoc group (General Methods Section 5b). The cyclisation reaction was then carried out as described in Section 5c for lanthionine containing peptides.

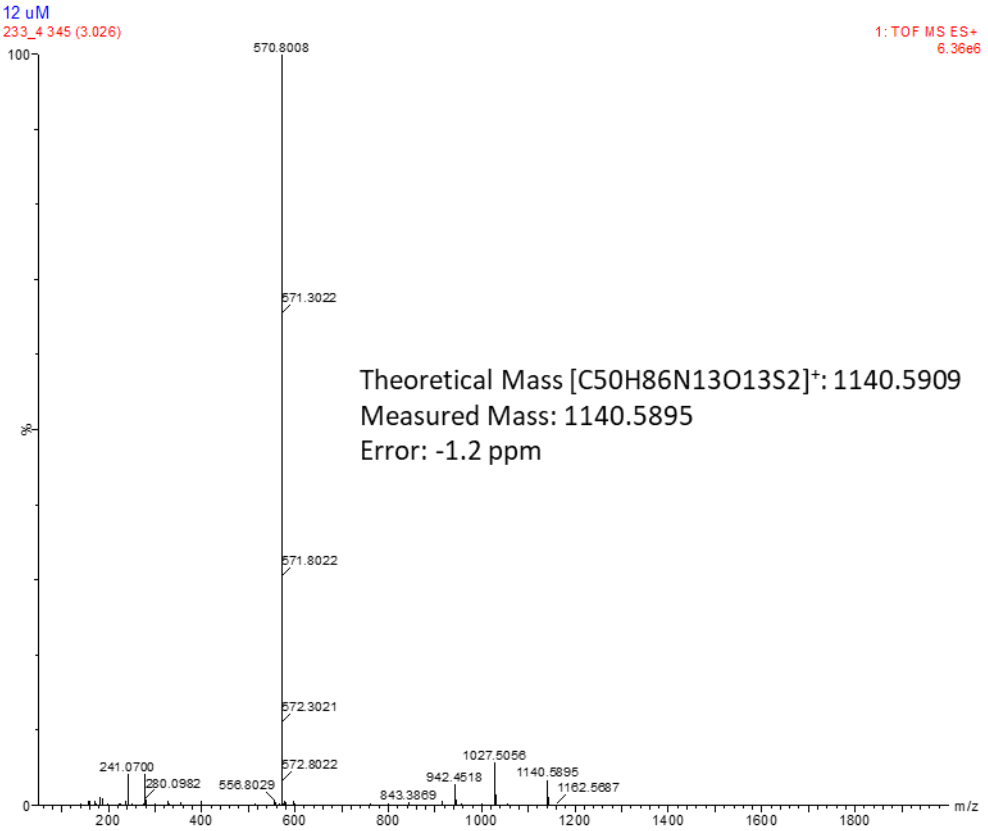
A second Teoc/TMSE lanthionine was then coupled onto the B ring using the microwave procedure described in General Methods Section 3. The Fmoc group was then removed and the peptide chain further elongated using the double coupling procedure described in General Methods Section 4. Fmoc-Leu-OH was added first, the Fmoc group was removed, followed by the addition of Fmoc-Ala-OH, removal of the Fmoc group and coupling of Fmoc-Ile-OH. The second cyclisation reaction was then carried out in the same way as the first, as described in General Methods Section 5.

The peptide chain was then further elongated using the double coupling procedure described in General Methods Section 4. Firstly Fmoc-Abu-OH was added and the Fmoc group removed, followed by the addition of Fmoc-Ile-OH, and removal of the final Fmoc group.

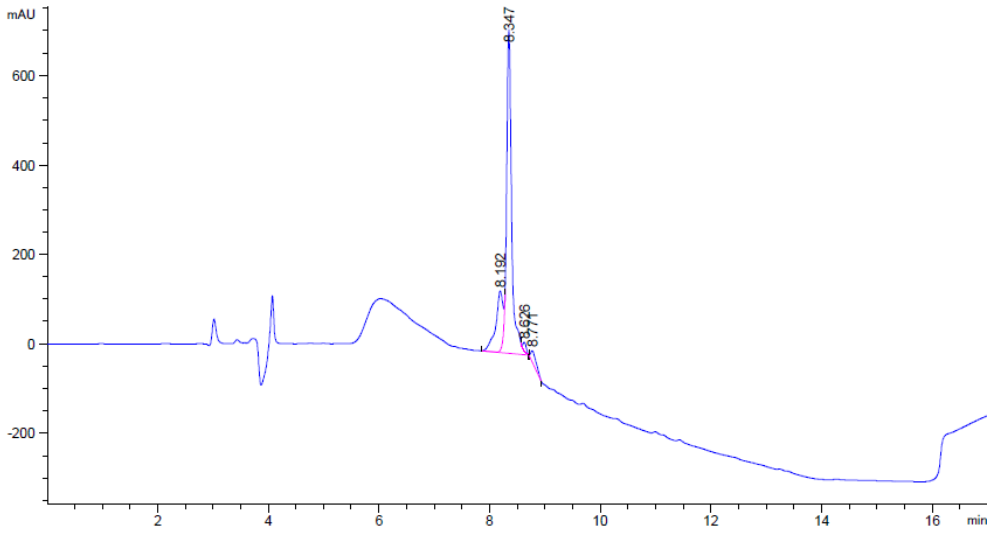
Following completion of the synthesis, the peptide was cleaved from the resin using the procedure described in General Methods Section 6 and cleavage cocktail 2.

The crude peptide was partially purified (76% by HPLC) by preparative reverse phase HPLC using a semi-prep Phenomenex Onyx C18 100 x 10 mm column with a linear solvent gradient of 5-40% MeCN (0.1% TFA) in H_2O (0.1% TFA) over 27 min. A second round of purification was then carried out on the Agilent HPLC system using a Dr Maisch GmbH Reprosil Gold 200 C8 5µm 250 x 4.6 mm column and a gradient of 15-35% MeCN (0.1% TFA) in H_2O (0.1% TFA) over 35 min. The fractions containing the target peptide were collected (retention time 29 min) and lyophilised to give the sample as a white powder (100 µg, 0.5%).

m/z (HRMS, ES+) required for $[C_{50}H_{86}N_{13}O_{13}S_2]^+$ 1140.5909, found $[C_{50}H_{86}N_{13}O_{13}S_2]^+$ 1140.5895.



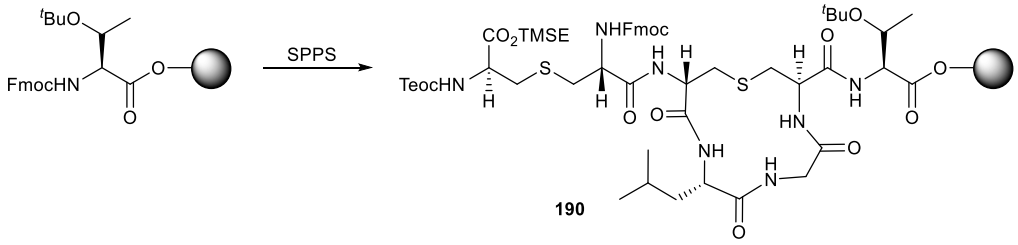
analytical HPLC (Dr Maisch GmbH Reprosil Gold 200 C8 5µm 250 x 4.6 mm column on Agilent HPLC system) retention time 8.35 min.



Attempts Towards Mutacin I Rings AB WT and Analogues

N.B. The synthesis of each peptide began identically and is described below, with the exact masses and volumes of amino acid, coupling reagent and base used tabulated under the entry for each analogue. Procedures used to complete the syntheses are described under the entry for each analogue. In all cases, none of the desired peptide could be isolated.

Synthesis of Ring B and Addition of 2nd Lanthionine



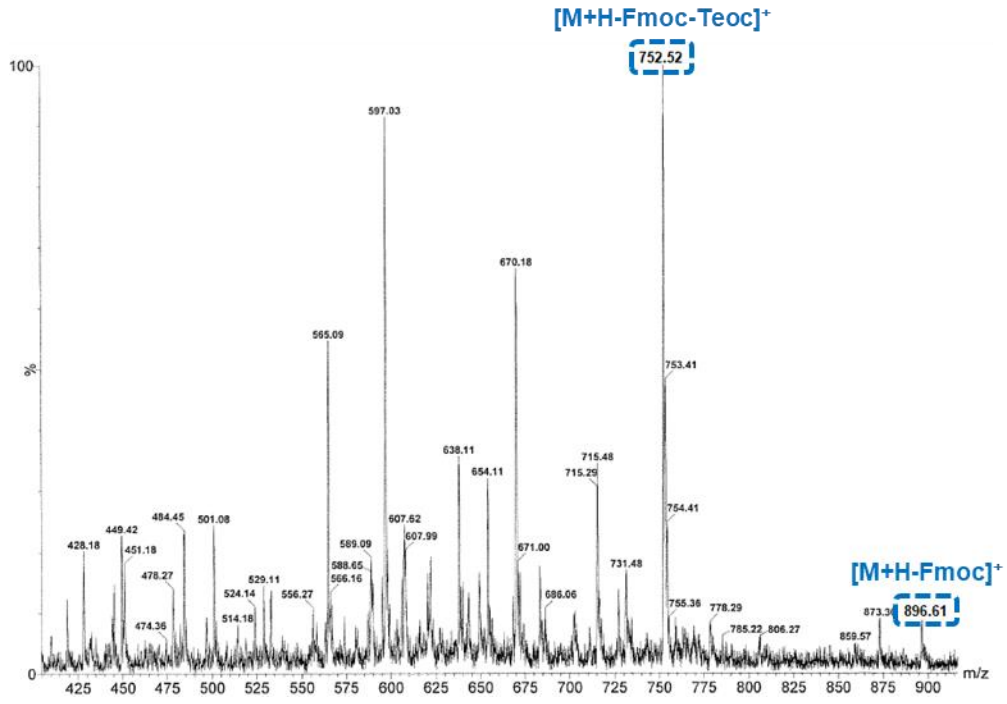
Fmoc-Thr(tBu)-NovaSyn TGT resin (50 mg, 9.0 μmol) was swollen in DMF and the Fmoc group removed as described in General Methods Sections 1 and 2.

The protected Teoc/TMSE lanthionine was coupled using the microwave procedure described in General Methods Section 3. The Fmoc group was then removed.

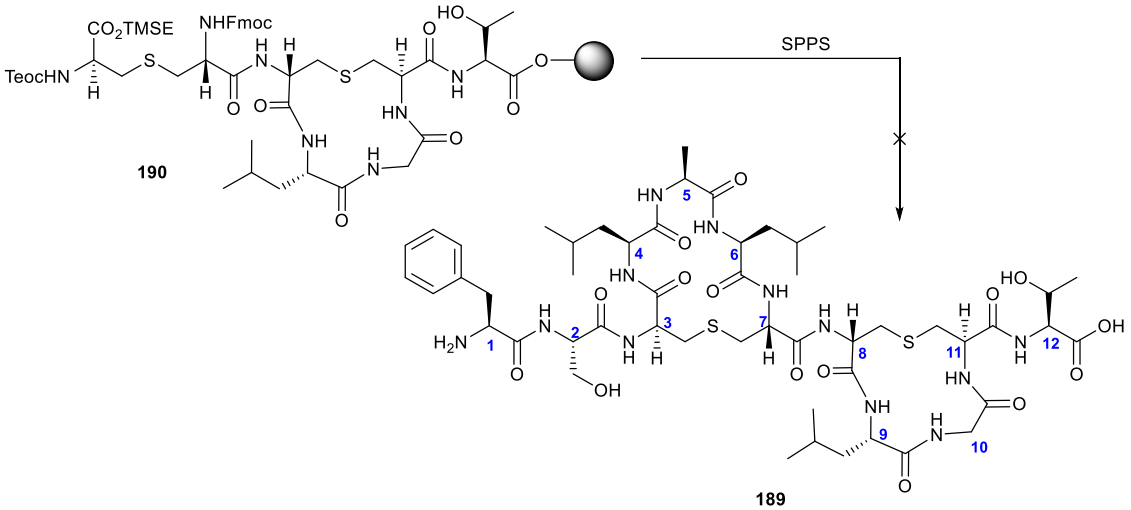
The peptide chain was elongated using the double coupling procedure described in General Methods Section 4. Fmoc-Gly-OH was added first, the Fmoc group was removed, followed by the addition of Fmoc-Leu-OH.

The cyclisation reaction was then carried out as described in Section 5. The silyl protecting groups were removed (General Methods Section 5a), followed by removal of the final Fmoc group (General Methods Section 5b). The cyclisation reaction was then carried out as described in Section 5c for lanthionine containing peptides.

A second Teoc/TMSE lanthionine was then coupled onto the B ring using the microwave procedure described in General Methods Section 3, and the Fmoc group was removed. Synthesis of intermediate **190** was confirmed by LCMS analysis of a small portion of cleaved resin.



Ser/Ala Analogue (**189**)



Reagents		Mass/Volume	µmol
Amino Acids	Teoc/TMSE lanthionine	18 mg	27
	Fmoc-Gly-OH	13 mg	45
	Fmoc-Leu-OH	16 mg	45
	Fmoc-Ala-OH	14 mg	45
	Fmoc-Ser(*t*Bu)-OH	17 mg	45
	Fmoc-Phe-OH	17 mg	45
	Fmoc-(FmocHmb)Leu-OH	32 mg	45
	Fmoc-Ala anhydride	54 mg	90
	Fmoc-(FmocHmb)Ala-OH	30 mg	45
	Fmoc-Leu anhydride	62 mg	90
Coupling Reagents	HOAt	6 mg	45
	PyAOP	24 mg	45
	DIPEA	16 µL	90

1. Regular Fmoc amino acids

Using the double coupling procedure described in General Methods Section 4, Fmoc-Leu-OH, Fmoc-Ala-OH and then Fmoc-Leu-OH were added, with Fmoc deprotections between each subsequent addition. The second cyclisation reaction was then carried out in the same way as the first, as described in General Methods Section 5.

The peptide chain was then further elongated using the double coupling procedure described in General Methods Section 4. Firstly Fmoc-Ser(*t*Bu)-OH was added and the Fmoc group removed, followed by the addition of Fmoc-Phe-OH, and removal of the final Fmoc group.

Following completion of the synthesis, the peptide was cleaved from the resin using the procedure described in General Methods Section 6 and cleavage cocktail 2.

2. Fmoc-(FmocHmb)Leu at position 6

Using the double coupling procedures described in General Methods Section 4, Fmoc-(FmocHmb)Leu-OH, Fmoc-Ala anhydride and then Fmoc-Leu-OH were added, with Fmoc deprotections between each subsequent addition. The second cyclisation reaction was then carried out in the same way as the first, as described in General Methods Section 5.

The peptide chain was then further elongated using the double coupling procedure described in General Methods Section 4. Firstly Fmoc-Ser(*t*Bu)-OH was added and the Fmoc group removed, followed by the addition of Fmoc-Phe-OH, and removal of the final Fmoc group.

Following completion of the synthesis, the peptide was cleaved from the resin using the procedure described in General Methods Section 6 and cleavage cocktail 2.

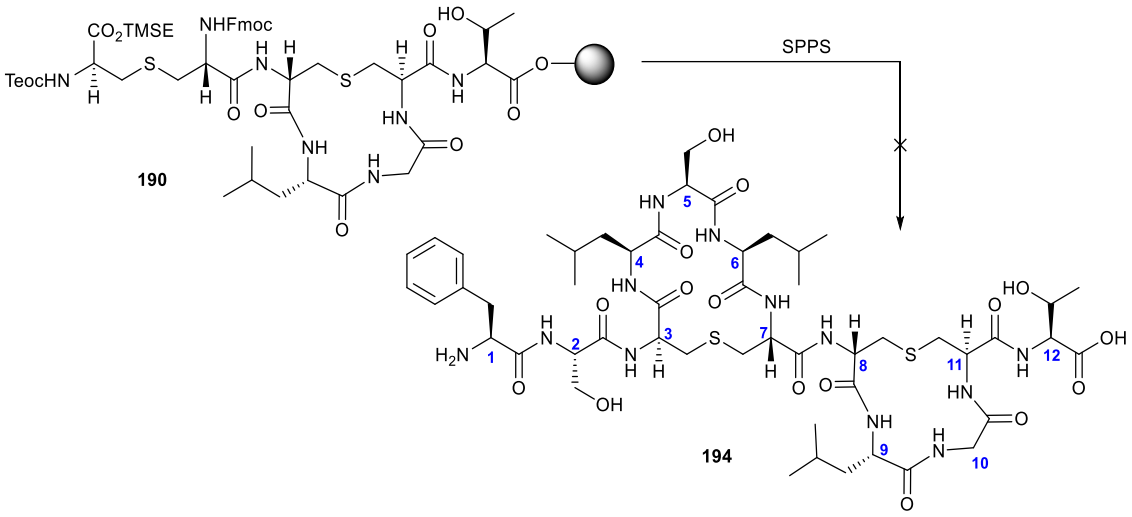
3. Fmoc-(FmocHmb)Ala at position 5

Using the double coupling procedures described in General Methods Section 4, Fmoc-Leu-OH, Fmoc-(FmocHmb)Ala-OH and then Fmoc-Leu anhydride were added, with Fmoc deprotections between each subsequent addition. The second cyclisation reaction was then carried out in the same way as the first, as described in General Methods Section 5.

The peptide chain was then further elongated using the double coupling procedure described in General Methods Section 4. Firstly Fmoc-Ser(*t*Bu)-OH was added and the Fmoc group removed, followed by the addition of Fmoc-Phe-OH, and removal of the final Fmoc group.

Following completion of the synthesis, the peptide was cleaved from the resin using the procedure described in General Methods Section 6 and cleavage cocktail 2.

Ser/Ser Analogue (**194**)



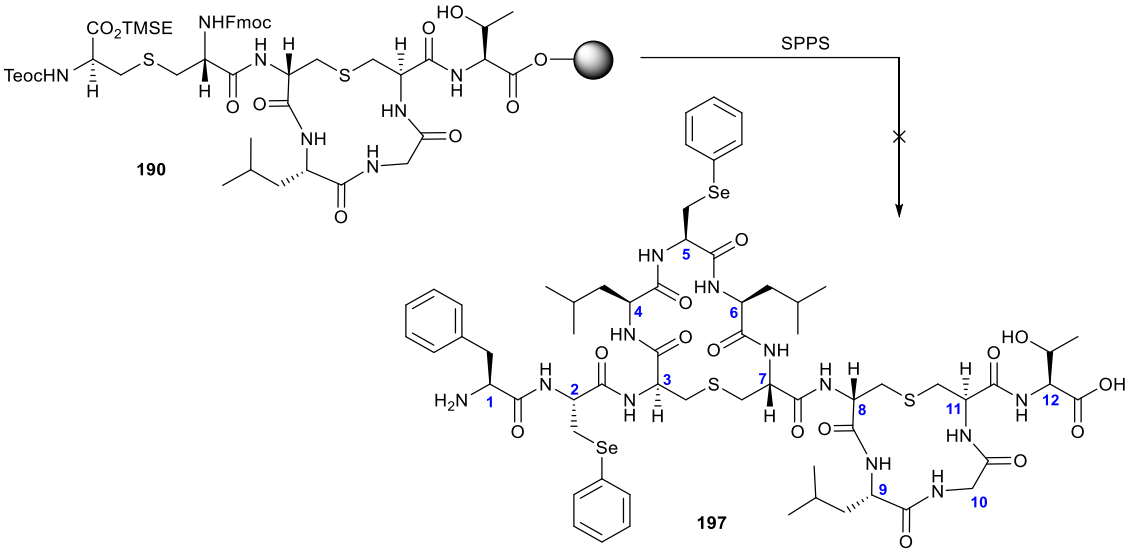
Reagents		Mass/Volume	µmol
Amino Acids	Teoc/TMSE lanthionine	18 mg	27
	Fmoc-Gly-OH	13 mg	45
	Fmoc-Leu-OH	16 mg	45
	Fmoc-Leu-Ser($\psi^{Me,Me}$pro)-OH	22 mg	45
	Fmoc-Ser(*t*Bu)-OH	17 mg	45
	Fmoc-Phe-OH	17 mg	45
Coupling Reagents	HOAt	6 mg	45
	PyAOP	24 mg	45
	DIPEA	16 µL	90

Using the double coupling procedure described in General Methods Section 4, Fmoc-Leu-OH and then Fmoc-Leu-Ser($\psi^{Me,Me}$pro)-OH were added, with an Fmoc deprotection between each addition. The second cyclisation reaction was then carried out in the same way as the first, as described in General Methods Section 5.

The peptide chain was then further elongated using the double coupling procedure described in General Methods Section 4. Firstly Fmoc-Ser(*t*Bu)-OH was added and the Fmoc group removed, followed by the addition of Fmoc-Phe-OH, and removal of the final Fmoc group.

Following completion of the synthesis, the peptide was cleaved from the resin using the procedure described in General Methods Section 6 and cleavage cocktail 2.

SecPh/SecPh Analogue (**197**)



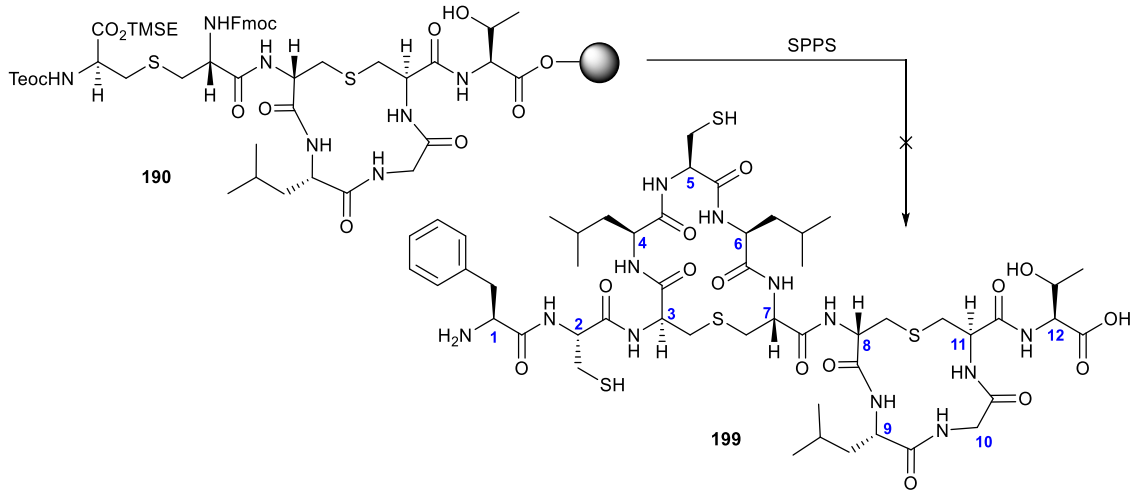
Reagents		Mass/Volume	µmol
Amino Acids	Teoc/TMSE lanthionine	18 mg	27
	Fmoc-Gly-OH	13 mg	45
	Fmoc-Leu-OH	16 mg	45
	Fmoc-Sec(Ph)-OH	17 mg	36
	Fmoc-Phe-OH	17 mg	45
Coupling Reagents	HOAt	7 mg	45
	PyAOP	27 mg	45
	DIPEA	18 µL	90
	HOBt	6 mg	36
	DIC	6 µL	36
	COMU	19 mg	45

The peptide chain was then elongated. Fmoc-Leu-OH was added first using the double coupling procedure described in General Methods Section 4. The Fmoc group was removed, followed by the addition of Fmoc-Sec(Ph)-OH (4 eq). This residue was coupled at rt for 2 h using HOBt (4 eq) and DIC (4 eq) in DMF (1.5 mL). After evacuation of the coupling solution the resin was washed with DMF (2 x 4 mL). The Fmoc group was then removed and a second Fmoc-Leu-OH added using the double coupling procedure described in General Methods Section 4. The second cyclisation reaction was then carried out in the same way as the first, as described in General Methods Section 5, except that COMU was used in place of PyAOP and HOAt.

The peptide chain was then further elongated with Fmoc-Sec(Ph)-OH (4 eq), coupled at rt for 2 h using HOBt (4 eq) and DIC (4 eq) in DMF (1.5 mL). The Fmoc group was then removed and the double coupling procedure described in General Methods Section 4 used to add Fmoc-Phe-OH, followed by removal of the final Fmoc group.

Following completion of the synthesis, the peptide was cleaved from the resin using the procedure described in General Methods Section 6 and cleavage cocktail 2.

Cys/Cys Analogue (**199**)



Reagents		Mass/Volume	µmol
Amino Acids	Teoc/TMSE lanthionine	18 mg	27
	Fmoc-Gly-OH	13 mg	45
	Fmoc-Leu-OH	16 mg	45
	Fmoc-Cys(Trt)-OH	26 mg	45
	Fmoc-Phe-OH	17 mg	45
	Fmoc-Leu-Cys($\psi^{Dmp,H}$pro)-OH	28 mg	45
	Boc-Phe-Cys(Trt)-OH	28 mg	45
	Fmoc-(FmocHmb)Leu-OH	32 mg	45
	Fmoc-Cys(Trt) anhydride	104 mg	90
Coupling Reagents	HOAt	6 mg	45
	PyAOP	24 mg	45
	DIPEA	16 µL	90

1. Regular Fmoc amino acids

Using the double coupling procedure described in General Methods Section 4, Fmoc-Leu-OH, Fmoc-Cys(Trt)-OH and then Fmoc-Leu-OH were added, with Fmoc deprotections between each subsequent addition. The second cyclisation reaction was then carried out in the same way as the first, as described in General Methods Section 5.

The peptide chain was then further elongated using the double coupling procedure described in General Methods Section 4. Firstly Fmoc-Cys(Trt)-OH was added and the Fmoc group removed, followed by the addition of Fmoc-Phe-OH, and removal of the final Fmoc group.

Following completion of the synthesis, the peptide was cleaved from the resin using the procedure described in General Methods Section 6 and cleavage cocktail 1.

2. Leu-Cys pseudoproline

Using the double coupling procedure described in General Methods Section 4, Fmoc-Leu-OH and then Fmoc-Leu-Cys($\psi^{Dmp,H}$pro)-OH were added, with an Fmoc deprotection between each addition. The second cyclisation reaction was then carried out in the same way as the first, as described in General Methods Section 5.

The peptide chain was then further elongated using one of two methods, each of which utilised the double coupling procedure described in General Methods Section 4. In the first, Fmoc-Cys(Trt)-OH was added and the Fmoc group removed, followed by the addition of Fmoc-Phe-OH, and removal of the final Fmoc group. In the second, Boc-Phe-Cys(Trt)-OH (**129**) was added.

Following completion of the synthesis, the peptide was cleaved from the resin using the procedure described in General Methods Section 6 and cleavage cocktail 1.

3. Fmoc-(FmocHmb)Leu at position 6

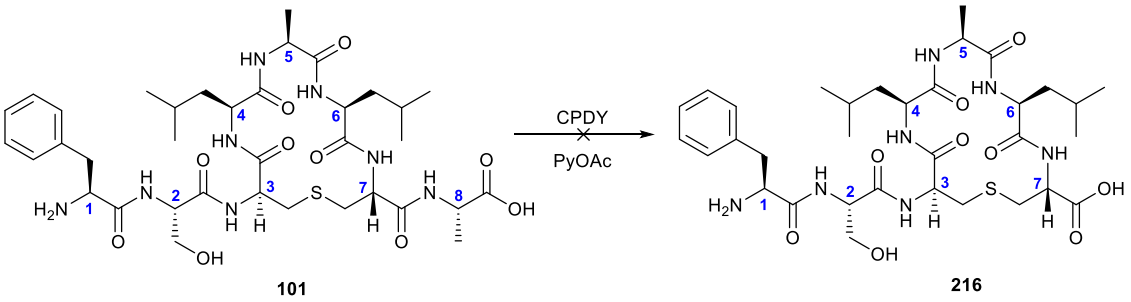
Using the double coupling procedures described in General Methods Section 4, Fmoc-(FmocHmb)Leu-OH, Fmoc-Cys(Trt) anhydride and then Fmoc-Leu-OH were added, with Fmoc deprotections between each subsequent addition. The second cyclisation reaction was then carried out in the same way as the first, as described in General Methods Section 5.

The peptide chain was then further elongated using the double coupling procedure described in General Methods Section 4. Firstly Fmoc-Cys(Trt)-OH was added and the Fmoc group removed, followed by the addition of Fmoc-Phe-OH, and removal of the final Fmoc group.

Following completion of the synthesis, the peptide was cleaved from the resin using the procedure described in General Methods Section 6 and cleavage cocktail 1.

Attempted cleavage of residue 8 from ring A peptides with CPDY

Ser/Ala Analogue (**101**)

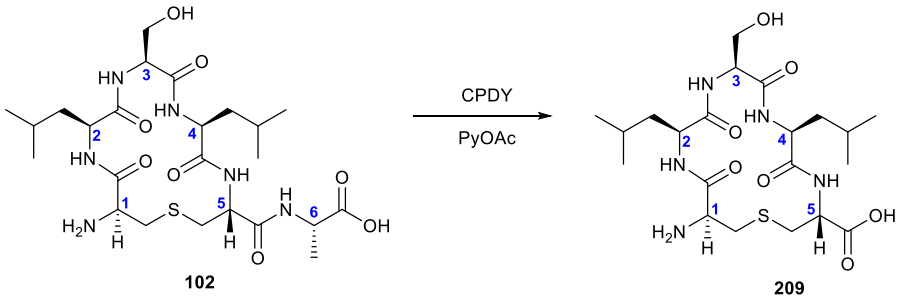


101 **216**

Reagents		Mass/Volume
CPDY Reaction Reagents	0.1 M aqueous PyOAc	0.75 mL
	CPDY	50 µL of a 2.5 mg mL^{-1} stock solution in water

The cleavage of the *C*-terminal Ala residue was attempted as described in General Methods Section 8, with 1.5 mg of peptide (**101**) (1.9 µmol). No conversion was detected by LCMS.

Ser Analogue (**102**)

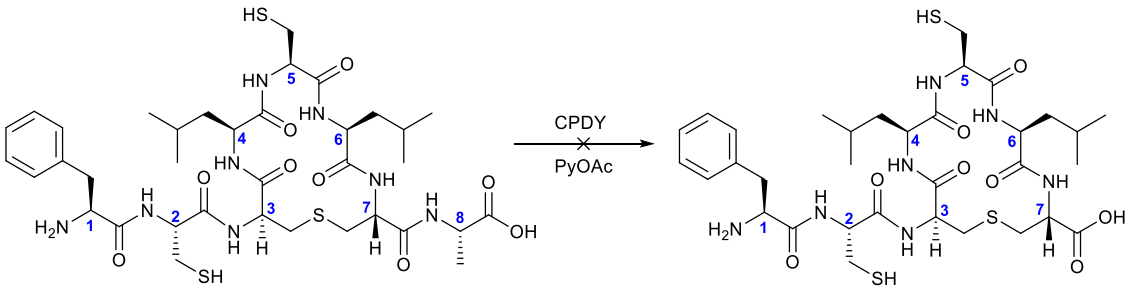


102 **209**

Reagents		**Mass/Volume**
CPDY Reaction Reagents	0.1 M PyOAc	1 mL
	CPDY	20 µL of a 1 mg mL^{-1} stock solution in water

The cleavage of the *C*-terminal Ala residue was attempted as described in General Methods Section 8, with 1.5 mg of peptide (**102**) (2.6 µmol). Some conversion could be seen by LCMS but insufficient peptide could be purified for further reaction.

Mutacin I ring A Cys-containing precursor (**161**)

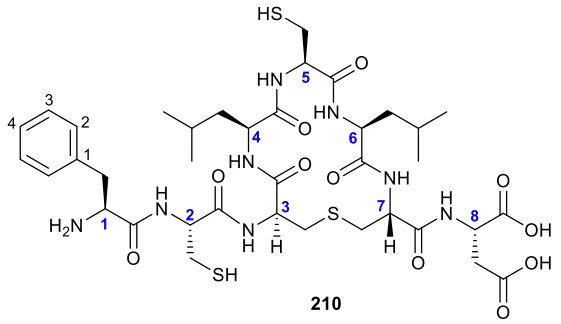


161 **212**

Reagents		**Mass/Volume**
CPDY Reaction Reagents	0.1 M PyOAc	0.5 mL
	CPDY	10 µL of a 1 mg mL^{-1} stock solution in water

The cleavage of the *C*-terminal Ala residue was attempted as described in General Methods Section 8, with 0.5 mg of peptide (**161**) (0.6 µmol). No conversion could be detected by LCMS.

Mutacin I Ring A Cys/Cys Analogue (210)



Reagents		Mass/Volume	µmol
Amino Acids	Teoc/TMSE lanthionine	21 mg	32
	Fmoc-Leu-OH	19 mg	53
	Fmoc-Cys(Trt)-OH	31 mg	53
	Fmoc-Phe-OH	20 mg	53
Coupling Reagents	HOAt	7 mg	53
	PyAOP	27 mg	53
	DIPEA	18 µL	105

Fmoc-Asp(O*t*Bu)-NovaSyn TGT resin (50 mg, 10.5 µmol) was swollen in DMF and the Fmoc group removed as described in General Methods Sections 1 and 2.

The protected Teoc/TMSE lanthionine was coupled using the microwave procedure described in General Methods Section 3. The Fmoc group was then removed.

The peptide chain was elongated using the double coupling procedure described in General Methods Section 4. Fmoc-Leu-OH was added first, the Fmoc group was removed, followed by the addition of Fmoc-Cys(Trt)-OH, removal of the Fmoc group and coupling of a second Fmoc-Leu-OH.

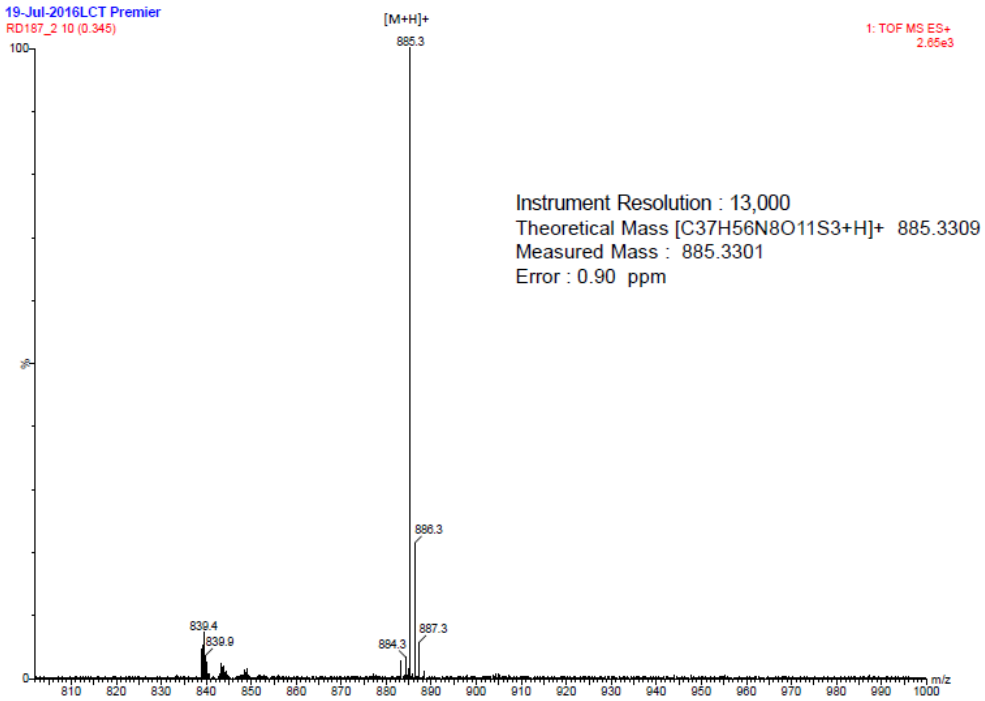
The cyclisation reaction was then carried out as described in Section 5. The silyl protecting groups were removed (General Methods Section 5a), followed by removal of the Fmoc group (General Methods Section 5b). The cyclisation reaction was then carried out as described in Section 5c for lanthionine containing peptides.

The peptide chain was then further elongated using the double coupling procedure described in General Methods Section 4. Firstly Fmoc-Cys(Trt)-OH was added, the Fmoc group removed, followed by the addition of Fmoc-Phe-OH, and removal of the final Fmoc group.

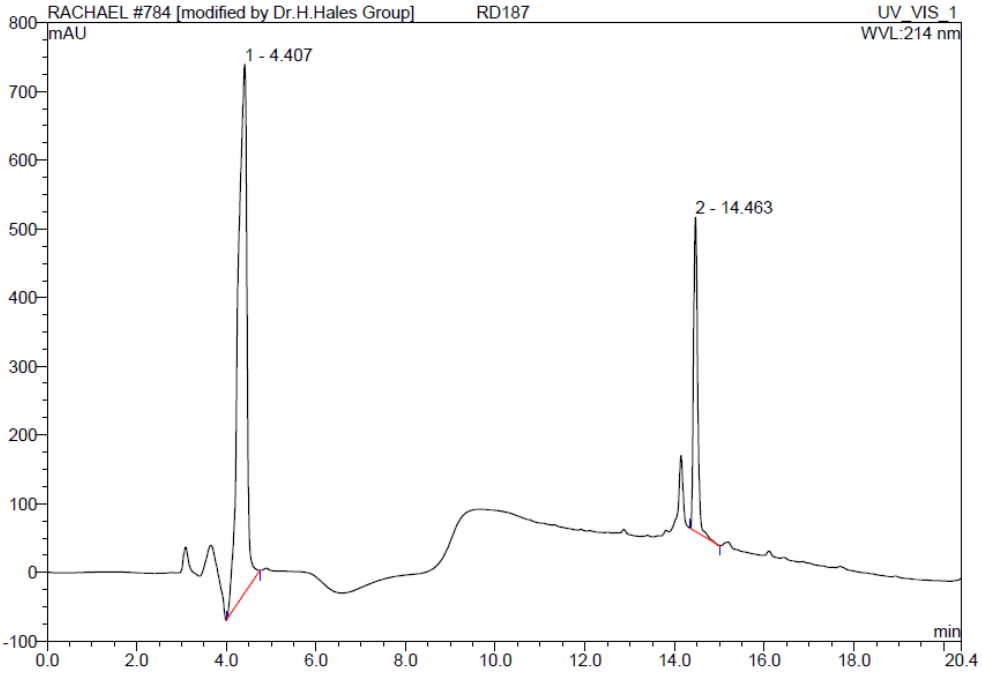
Following completion of the synthesis, the peptide was cleaved from the resin using the procedure described in General Methods Section 6 and cleavage cocktail 1.

The crude peptide was partially purified by preparative reverse phase HPLC using a semi-prep Phenomenex Onyx C18 100 x 10 mm column. A linear solvent gradient of 20-80% MeCN (0.1% TFA) in H_2O (0.1% TFA) over 30 min, at a flow rate of 2 mL min^{-1} was used. The fractions containing the target peptide were collected (retention time 11 min) and lyophilised to give the sample as a fluffy white powder (600 µg, 6%).

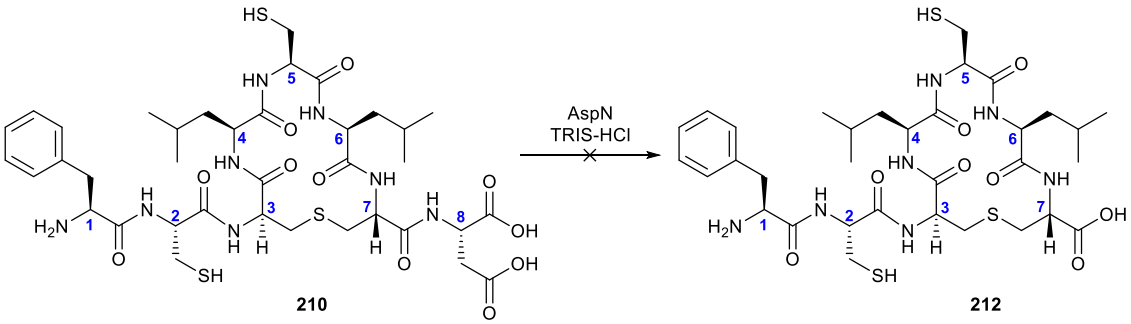
m/z (HRMS, ES+) required for $[C_{37}H_{56}N_8O_{11}S_3+H]^+$ 885.3309, found $[C_{37}H_{56}N_8O_{11}S_3+H]^+$ 885.3301.



analytical HPLC (ACE 300Å C18-300 150 x 4.6 mm column on Dionex HPLC system) retention time 14.42 min.



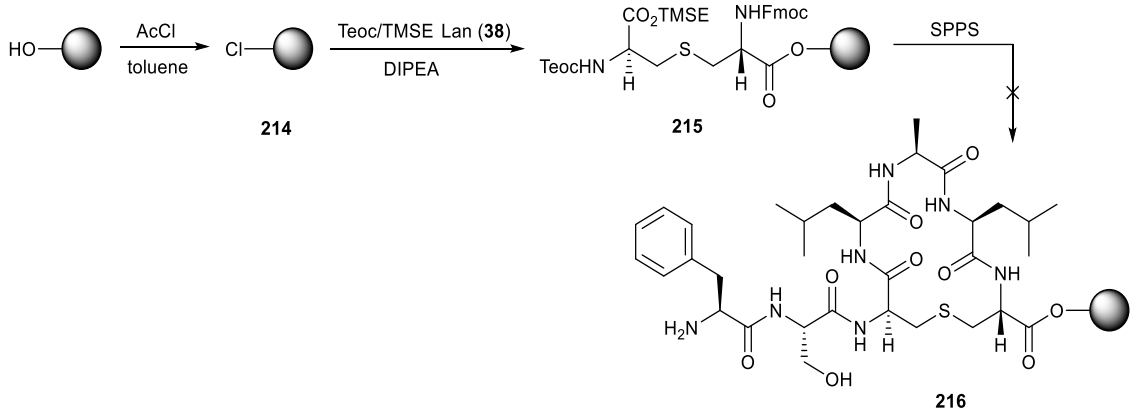
Attempted cleavage of Asp8 from (210) with AspN



Endoproteinase AspN (30 µg, 0.05 eq) was dissolved in water (150 µL) and added to a solution of the peptide (0.6 mg, 0.7 µmol) dissolved in a buffer of 50 mM Tris-HCl and 2.5 mM $ZnSO_4$ at pH 8.0 (200 µL). The solution was incubated at 37 °C with shaking for 16 h before adjusting to pH 2.0 with HCl to denature the enzyme, diluting in water and filtering with a syringe filter. Analysis by LCMS showed only starting material.

Attaching Lanthionine to Resin

1. Direct Attachment



Reagents		Mass/Volume	µmol
Amino Acids	Fmoc-Leu-OH	29 mg	83
	Fmoc-Ala-OH	26 mg	83
	Fmoc-Ser(*t*Bu)-OH	32mg	83
	Fmoc-Phe-OH	32 mg	83
Coupling Reagents	HOAt	11 mg	83
	PyAOP	43 mg	83
	DIPEA	29 µL	165

Chloridation of Alcohol Resin

Novasyn TGT alcohol resin (200 mg, 56 µmol) was placed in a syringe with a frit and washed with DMF (3 x 2 mL), dry CH_2Cl_2 (3 x 2 mL) and dry toluene (3 x 2 mL). The resin was then immediately transferred to a dry flask and enough dry toluene added to cover the resin (1 mL). Freshly distilled acetyl chloride (200 µL, 2.81 mmol) was added and the solution heated under reflux at 65 °C for 3 h. The solution was then filtered to collect the resin, and the resin washed with dry toluene (3 x 2 mL) and dry CH_2Cl_2 (3 x 2 mL).

Loading Lanthionine

A solution of Teoc/TMSE Lan (**38**) (75.6 mg, 112 µmol, 2 eq) and DIPEA (78 µL, 448 µmol, 8 eq) in dry CH_2Cl_2 (2 mL) was added to a reaction syringe containing the resin and shaken at rt for 16 h. The coupling solution was then evacuated and the resin washed with CH_2Cl_2:MeOH:DIPEA (17:2:1, 3 x 3 mL), CH_2Cl_2 (3 x 2 mL), DMF (2 x 2 mL) and CH_2Cl_2 (2 x 2 mL) before drying *in vacuo* over KOH. The resin was then washed with dioxane (3 x 2 mL) and dried *in vacuo*. Loading was determined to be 0.11 mmol g^{-1} using the method reported by Gude *et al*, in which the concentration of dibenzofluvene liberated from Fmoc deprotection is measured by UV spectroscopy.[267,308]

Synthesis of Ring A

A portion of the lanthionine resin (150 mg, 16.5 µmol) was swollen in DMF and the Fmoc group removed as described in General Methods Sections 1 and 2.

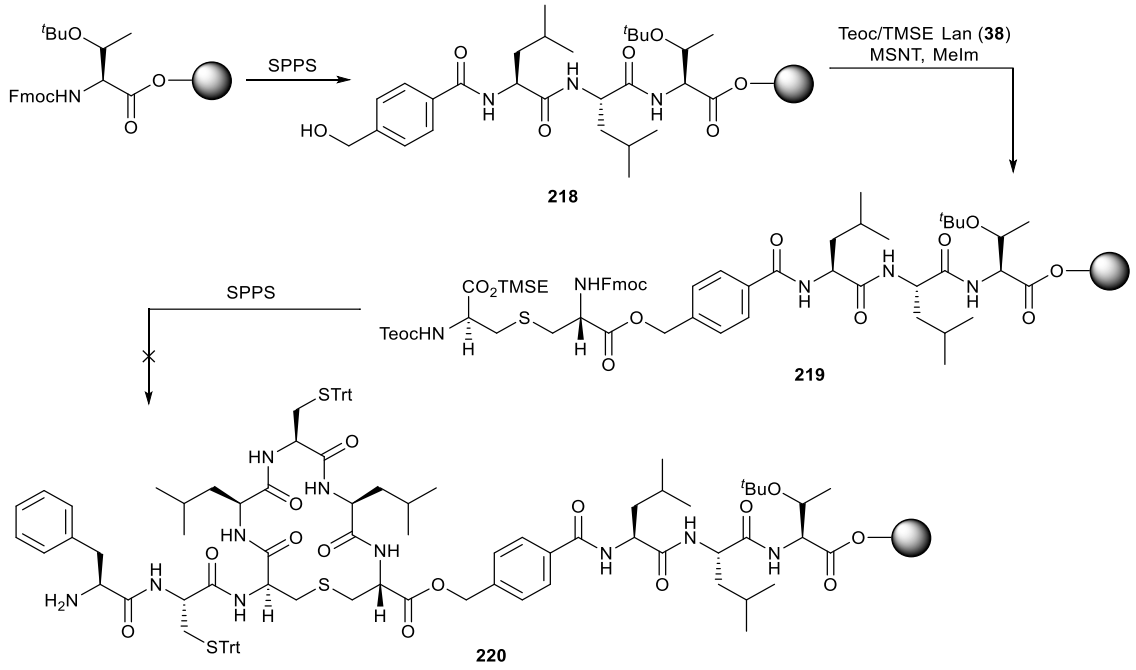
The peptide chain was elongated using the double coupling procedure described in General Methods Section 4. Fmoc-Leu-OH was added first, the Fmoc group was removed, followed by the addition of Fmoc-Ala-OH, removal of the Fmoc group and coupling of a second Fmoc-Leu-OH.

The cyclisation reaction was then carried out as described in Section 5. The silyl protecting groups were removed (General Methods Section 5a), followed by removal of the Fmoc group (General Methods Section 5b). The cyclisation reaction was then carried out as described in Section 5c for lanthionine containing peptides.

The peptide chain was then further elongated using the double coupling procedure described in General Methods Section 4. Firstly Fmoc-Ser(*t*Bu)-OH was added, the Fmoc group removed, followed by the addition of Fmoc-Phe-OH, and removal of the final Fmoc group.

Following completion of the synthesis, the peptide was cleaved from the resin using the procedure described in General Methods Section 6 and cleavage cocktail 2. None of the target peptide could be detected by LCMS.

2. *via* HMBA Linker



Reagents		Mass/Volume	µmol
Amino Acids	Fmoc-Leu-OH	16 mg	45
	HMBA	7 mg	45
	Teoc/TMSE lanthionine	18 mg	27
	Fmoc-Cys(Trt)-OH	26 mg	45
	Fmoc-Phe-OH	17 mg	45
Coupling Reagents	HBTU	17 mg	45
	DIPEA	16 µL	90
	MSNT	8 mg	27
	MeIm	2 mg	20
	HOAt	6 mg	45
	PyAOP	24 mg	45

Synthesising Linker

Fmoc-Thr(*t*Bu)-NovaSyn TGT resin (50 mg, 9.0 μmol) was swollen in DMF and the Fmoc group removed as described in General Methods Sections 1 and 2.

The next amino acid to be coupled was Fmoc-Leu-OH. This residue was preactivated for 2 min in a glass vial with HBTU and DIPEA in DMF (1.5 mL) before being added to the syringe containing the resin. This was left to shake at rt for 1 h before evacuation and washing the resin with DMF (4 x 2 mL). The Fmoc group was then removed and the same coupling procedure repeated to add the second Fmoc-Leu-OH. The Fmoc group was then removed and HMBA was coupled using the same HBTU protocol.

Loading Lanthionine

To a solution of Teoc/TMSE Lan (**38**) (3 eq) in dry CH_2Cl_2 (0.5 mL) was added MeIm followed by MSNT. This solution was then added to the syringe containing the resin and shaken under Ar for 1 h. The coupling solution was then evacuated and the resin washed with CH_2Cl_2 (5 x 2 mL) and DMF (5 x 2 mL). Loading was judged to be complete due to a negative result from the resin-bound hydroxyl colourimetric test described by Burkett *et al*,[309] as well as the presence of the desired mass in the LCMS spectrum of a small sample of resin (~2 mg) cleaved with 1% TFA in CH_2Cl_2.

Synthesis of Ring A

Following lanthionine addition the peptide chain was elongated using the double coupling procedure described in General Methods Section 4. The Lan Fmoc group was first removed, followed by the addition of Fmoc-Leu-OH, removal of the Fmoc group, addition of Fmoc-Cys(Trt)-OH, removal of the Fmoc group and coupling of a second Fmoc-Leu-OH.

The cyclisation reaction was then carried out as described in Section 5. The silyl protecting groups were removed (General Methods Section 5a), followed by removal of the Fmoc group (General Methods Section 5b). The cyclisation reaction was then carried out as described in Section 5c for lanthionine containing peptides.

The peptide chain was then further elongated using the double coupling procedure described in General Methods Section 4. Firstly Fmoc-Cys(Trt)-OH was added, the Fmoc

group removed, followed by the addition of Fmoc-Phe-OH, and removal of the final Fmoc group.

Following completion of the synthesis, the peptide was cleaved from the resin using the procedure described in General Methods Section 6 and cleavage cocktail 1. None of the target peptide could be detected by LCMS.

7.3. Peptide Structure Calculation

Restraint Generation

Distance restraints for structure calculation were determined from 2D ^{1}H-^{1}H NOESY spectra (mixing time 0.6 s) using the 'Make Distance Restraints' tool in CCPN Analysis.[274] Only inter-residue restraints were used for structure calculation. Backbone ϕ and ψ angle restraints were either calculated from $^{3}J_{HN\text{-}H\alpha}$ coupling constants measured directly from 1D ^{1}H spectra, using constants for the Karplus equation reported by Vögeli *et al.*,[278] or predicted using the TALOS-N server from the Bax group at NIH.[280,281] Geometry of Xaa-Pro peptide bonds was determined by examination of the Pro β and γ ^{13}C shifts and prediction using the Promega server from the Bax group at NIH.[283]

Structure Calculation

The additional topology and parameters required for the unusual amino acids (lanthionine, methyllanthionine, dehydroalanine and dehydrobutyrine) were based on the parameterisation used by Earl[284] and Turpin *et al.*,[285] and were added to the XPLOR .top and .par files.

The angle and distance restraints were then used in structure calculation in XPLOR-NIH version 2.45.[200,273] The calculation procedure was as follows: a psf file was created from the peptide sequence, followed by calculation of an initial extended structure. A family of 100-250 structures were then generated with simulated annealing using NOE and dihedral restraints (heating to 1000 K over 6000 timesteps of 0.005 or 0.003 ps, followed by cooling over 3000 timesteps). This family of structures was then refined using a similar simulated annealing protocol (cooling was conducted over 2000 timesteps to a final temperature of 100 K) over 2-5 rounds of refinement, followed by selection of the lowest 15 energy structures for analysis. Figures were generated using PyMOL (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC). Ensembles of the lowest

energy structures were validated using the Protein Structure Validation Software Suite (PSVS) version 1.5 (http://psvs-1_5-dev.nesg.org/).[289] Edited sections of the XPLOR parameter and topology files, example scripts and NOE and dihedral restraint tables for all peptides can be found in **Appendix 1**.

8. REFERENCES

1 R. Smith and J. Coast, *Br. Med. J.*, 2013, **346**, 1–5.

2 World Health Organisation, *Antimicrobial Resistance: Global Report on Surveillance*, 2014.

3 C. L. Ventola, *Pharm. Ther.*, 2015, **40**, 277–83.

4 A. E. Clatworthy, E. Pierson and D. T. Hung, *Nat. Chem. Biol.*, 2007, **3**, 541–548.

5 C. L. Ventola, *Pharm. Ther.*, 2015, **40**, 344–352.

6 K. H. Luepke, K. J. Suda, H. Boucher, R. L. Russo, M. W. Bonney, T. D. Hunt and J. F. Mohr, *Pharmacotherapy*, 2017, **37**, 71–84.

7 K. Lewis, *Nat. Rev. Drug Discov.*, 2013, **12**, 371–87.

8 M. Zasloff, *Nature*, 2002, **415**, 389–395.

9 K. A. Brogden, *Nat. Rev. Microbiol.*, 2005, **3**, 238–250.

10 N. Singh and J. Abraham, *J. Antibiot. (Tokyo).*, 2014, **67**, 277–289.

11 T. Ganz, *Nat. Rev. Immunol.*, 2003, **3**, 710–720.

12 M. Papagianni, *Biotechnol. Adv.*, 2003, **21**, 465–499.

13 C. Van Kraaij, W. M. De Vos, J. Siezen and O. P. Kuipers, *Nat. Prod. Rep.*, 1999, **16**, 575–587.

14 R. Finking and M. A. Marahiel, *Annu. Rev. Microbiol.*, 2004, **58**, 453–488.

15 R. E. W. Hancock, *Lancet*, 1997, **349**, 418–422.

16 P. Fehlbaum, P. Bulet, L. Michaut, M. Lagueux, W. F. Broekaert, C. Hetru and J. A. Hoffmann, *J. Biol. Chem.*, 1994, **269**, 33159–33163.

17 M. Zasloff, *Proc. Natl. Acad. Sci. U. S. A.*, 1987, **84**, 5449–5453.

18 L. Di, *AAPS J.*, 2015, **17**, 134–43.

19 D. J. Craik, D. P. Fairlie, S. Liras and D. Price, *Chem. Biol. Drug Des.*, 2013, **81**, 136–147.

20 A. Henninot, J. C. Collins and J. M. Nuss, *J. Med. Chem.*, 2018, **61**, 1382–1414.

21 M. Sieprawska-Lupa, P. Mydel, K. Krawczyk, K. Wójcik, M. Puklo, B. Lupa, P. Suder, J. Silberring, M. Reed, J. Pohl, W. Shafer, F. McAleese, T. Foster, J. Travis and J. Potempa, *Antimicrob. Agents Chemother.*, 2004, **48**, 4673–4679.

22 K. E. Hagman, W. Pan, B. G. Spratt, J. T. Balthazar, R. C. Judd and W. M. Shafer, *Microbiology*, 1995, **141**, 611–622.

23 H. Brötz, G. Bierbaum, K. Leopold, P. E. Reynolds and H. Sahl, *Antimicrob. Agents Chemother.*, 1998, **42**, 154–160.

24 C. Subbalakshmi and N. Sitaram, *FEMS Microbiol. Lett.*, 1998, **160**, 91–96.

25 G. Kragol, S. Lovas, G. Varadi, B. A. Condie, R. Hoffmann and L. Otvos Jr.,

Biochemistry, 2001, **40**, 3016–3026.

26 M. A. Ortega and W. A. van der Donk, *Cell Chem. Biol.*, 2016, **23**, 31–44.

27 D. J. Craik, N. L. Daly, T. Bond and C. Waine, *J. Mol. Biol.*, 1999, **294**, 1327–1336.

28 G. E. Norris and M. L. Patchett, *Curr. Opin. Struct. Biol.*, 2016, **40**, 112–119.

29 C. Ireland and P. J. Scheuer, *J. Am. Chem. Soc.*, 1980, **102**, 5688–5691.

30 M. C. Bagley, J. W. Dale, E. A. Merritt and X. Xiong, *Chem. Rev.*, 2005, **105**, 685–714.

31 J. D. Hegemann, M. Zimmermann, X. Xie and M. A. Marahiel, *Acc. Chem. Res.*, 2015, **48**, 1909–1919.

32 M. O. Maksimov, S. J. Pan and A. J. Link, *Nat. Prod. Rep.*, 2012, **29**, 996–1006.

33 P. G. Arnison, M. J. Bibb, G. Bierbaum, A. A. Bowers, T. S. Bugni, G. Bulaj, J. A. Camarero, D. J. Campopiano, G. L. Challis, J. Clardy, P. D. Cotter, D. J. Craik, M. Dawson, E. Dittmann, S. Donadio, P. C. Dorrestein, K.-D. Entian, M. A. Fischbach, J. S. Garavelli, U. Göransson, C. W. Gruber, D. H. Haft, T. K. Hemscheidt, C. Hertweck, C. Hill, A. R. Horswill, M. Jaspars, W. L. Kelly, J. P. Klinman, O. P. Kuipers, A. J. Link, W. Liu, M. A. Marahiel, D. A. Mitchell, G. N. Moll, B. S. Moore, R. Müller, S. K. Nair, I. F. Nes, G. E. Norris, B. M. Olivera, H. Onaka, M. L. Patchett, J. Piel, M. J. T. Reaney, S. Rebuffat, R. P. Ross, H.-G. Sahl, E. W. Schmidt, M. E. Selsted, K. Severinov, B. Shen, K. Sivonen, L. Smith, T. Stein, R. D. Süssmuth, J. R. Tagg, G.-L. Tang, A. W. Truman, J. C. Vederas, C. T. Walsh, J. D. Walton, S. C. Wenzel, J. M. Willey and W. A. van der Donk, *Nat. Prod. Rep.*, 2013, **30**, 108–160.

34 N. Schnell, K.-D. Entian, U. Schneider, F. Götz, H. Zähner, R. Kellner and G. Jung, *Nature*, 1988, **333**, 276–278.

35 W. Tang and W. A. van der Donk, *Nat. Chem. Biol.*, 2013, **9**, 157–159.

36 J. M. Willey and W. A. van der Donk, *Annu. Rev. Microbiol.*, 2007, **61**, 477–501.

37 C. Chatterjee, M. Paul, L. Xie and W. A. van der Donk, *Chem. Rev.*, 2005, **105**, 633–683.

38 P. J. Knerr and W. A. van der Donk, *Annu. Rev. Biochem.*, 2012, **81**, 479–505.

39 L. M. Repka, J. R. Chekan, S. K. Nair and W. A. van der Donk, *Chem. Rev.*, 2017, **117**, 5457–5520.

40 L. Smith and J. D. Hillman, *Curr. Opin. Microbiol.*, 2008, **11**, 401–408.

41 J. L. Fox, *Nat. Biotechnol.*, 2013, **31**, 379–382.

42 F. Castiglione, A. Lazzarini, L. Carrano, E. Corti, I. Ciciliato, L. Gastaldo, P. Candiani, D. Losi, F. Marinelli, E. Selva and F. Parenti, *Chem. Biol.*, 2008, **15**, 22–31.

43 D. Jabés, C. Brunati, G. Candiani, S. Riva, G. Romanó and S. Donadio, *Antimicrob. Agents Chemother.*, 2011, **55**, 1671–1676.

44 S. Boakes and M. J. Dawson, in *Natural Products: Discourse, Diversity, and Design*, 2014, vol. 24, pp. 455–468.

45 F. Qi, P. Chen and P. W. Caufield, *Appl. Environ. Microbiol.*, 1999, **65**, 3880–3887.

46 L. Smith, H. Hasper, E. Breukink, J. Novak, J. Cerkasov, J. D. Hillman, S. Wilson-Stanford and R. S. Orugunty, *Biochemistry*, 2008, **47**, 3308–14.

47 J. Parisot, S. Carey, E. Breukink, W. C. Chan, A. Narbad and B. Bonev, *Antimicrob. Agents Chemother.*, 2008, **52**, 612–618.

48 M. Mota-Meira, G. LaPointe, C. Lacroix and M. C. Lavoie, *Antimicrob. Agents Chemother.*, 2000, **44**, 24–29.

49 C. Iancu, A. Grainger, D. Field, P. D. Cotter, C. Hill and R. P. Ross, *Probiotics Antimicrob. Proteins*, 2012, **4**, 108–115.

50 L. A. Rogers, *J. Bacteriol.*, 1928, **16**, 321–325.

51 E. Gross and J. L. Morell, *J. Am. Chem. Soc.*, 1971, **93**, 4634–4635.

52 J. L. Morell and E. Gross, *J. Am. Chem. Soc.*, 1973, **247**, 1966–1967.

53 P. M. O'Connor, E. F. O'Shea, C. M. Guinane, O. O'Sullivan, P. D. Cotter, R. P. Ross and C. Hill, *Appl. Environ. Microbiol.*, 2015, **81**, 3953–3960.

54 J. W. M. Mulders, I. J. Boerrigter, H. S. Rollema, R. J. Siezen and W. M. de Vos, *Eur. J. Biochem.*, 1991, **201**, 581–584.

55 M. de Kwaadsteniet, K. ten Doeschate and L. M. T. Dicks, *Appl. Environ. Microbiol.*, 2008, **74**, 547–549.

56 T. Zendo, M. Fukao, K. Ueda, T. Higuchi, J. Nakayama and K. Sonomoto, *Biosci. Biotechnol. Biochem.*, 2003, **67**, 1616–1619.

57 R. E. Wirawan, N. A. Klesse, R. W. Jack and J. R. Tagg, *Appl. Environ. Microbiol.*, 2006, **72**, 1148–1156.

58 Q. Zhang, Y. Yu, J. E. Vélasquez and W. A. van der Donk, *Proc. Natl. Acad. Sci.*, 2012, **109**, 18361–18366.

59 A. T. R. Mattick and A. Hirsch, *Lancet*, 1947, **253**, 5–8.

60 S. Dosler and A. A. Gerceker, *Chemotherapy*, 2012, **57**, 511–516.

61 W. Brumfitt, M. R. J. Salton and J. M. T. Hamilton-Miller, *J. Antimicrob. Chemother.*, 2002, **50**, 731–734.

62 S. Dosler and E. Mataraci, *Peptides*, 2013, **49**, 53–58.

63 H. Nikaido and M. Vaara, *Microbiol. Rev.*, 1985, **49**, 1–32.

64 L. Zhou, A. J. van Heel, M. Montalbán-López and O. P. Kuipers, *Front. Cell Dev. Biol.*, 2016, **4**, 1–9.

65 L. J. de Arauz, A. F. Jozala, P. G. Mazzola and T. C. Vessoni Penna, *Trends Food Sci. Technol.*, 2009, **20**, 146–154.

66 J. Cleveland, T. J. Montville, I. F. Nes and M. L. Chikindas, *Int. J. Food Microbiol.*, 2001, **71**, 1–20.

67 X. Yang and W. A. van der Donk, *Chem. A Eur. J.*, 2013, **19**, 7662–7677.

68 W. A. van der Donk and S. K. Nair, *Curr. Opin. Struct. Biol.*, 2014, **29**, 58–66.

69 R. Khusainov, R. Heils, J. Lubelski, G. N. Moll and O. P. Kuipers, *Mol. Microbiol.*, 2011, **82**, 706–718.

70 K. Sieger, S. Heinzmann and K. Entian, *J. Biol. Chem.*, 1996, **271**, 12294–12301.

71 A. Kuipers, E. de Boef, R. Rink, S. Fekken, L. D. Kluskens, A. J. M. Driessen, K. Leenhouts, O. P. Kuipers and G. N. Moll, *J. Biol. Chem.*, 2004, **279**, 22176–22182.

72 J. Lubelski, R. Rink, R. Khusainov, G. N. Moll and O. P. Kuipers, *Cell. Mol. Life Sci.*, 2008, **65**, 455–476.

73 Z. AlKhatib, M. Lagedroste, J. Zaschke, M. Wagner, A. Abts, I. Fey, D. Kleinschrodt and S. H. J. Smits, *Microbiol. Open*, 2014, **3**, 752–63.

74 T. Stein, S. Heinzmann, I. Solovieva and K.-D. Entian, *J. Biol. Chem.*, 2003, **278**, 89–94.

75 G. Engelke, Z. Gutowski-Eckel, P. Kiesau, K. Siegers, M. Hammelmann and K.-D. Entian, *Appl. Environ. Microbiol.*, 1994, **60**, 814–825.

76 E. Breukink, C. Van Kraaij, R. A. Demel, R. J. Siezen, O. P. Kuipers and B. de Kruijff, *Biochemistry*, 1997, **36**, 6968–6976.

77 N. Malanovic and K. Lohner, *Pharmaceuticals*, 2016, **9**, 1–33.

78 N. Malanovic and K. Lohner, *Biochim. Biophys. Acta - Biomembr.*, 2016, **1858**, 936–946.

79 H. R. Ramseier, *Arch. Microbiol.*, 1960, **37**, 57–94.

80 P. E. Linnett and J. L. Strominger, *Antimicrob. Agents Chemother.*, 1973, **4**, 231–236.

81 N. Y. Yount and M. R. Yeaman, *Ann. N. Y. Acad. Sci.*, 2013, **1277**, 127–138.

82 P. Reisinger, H. Seidel, H. Tschesche and W. P. Hammes, *Arch. Microbiol.*, 1980, **127**, 187–193.

83 E. Ruhr and H.-G. Sahl, *Antimicrob. Agents Chemother.*, 1985, **27**, 841–845.

84 E. Breukink, I. Wiedemann, C. van Kraaij, O. P. Kuipers, H.-G. Sahl and B. de Kruijff, *Science*, 1999, **286**, 2361–2365.

85 I. Wiedemann, E. Breukink, C. van Kraaij, O. P. Kuipers, G. Bierbaum, B. de Kruijff and H. G. Sahl, *J. Biol. Chem.*, 2001, **276**, 1772–1779.

86 H. Brötz, M. Josten, I. Wiedemann, U. Schneider, F. Götz, G. Bierbaum and H.-G. Sahl, *Mol. Microbiol.*, 1998, **30**, 317–327.

87 E. Breukink, H. E. van Heusden, P. J. Vollmerhaus, E. Swiezewska, L. Brunner, S. Walker, A. J. R. Heck and B. de Kruijff, *J. Biol. Chem.*, 2003, **278**, 19898–19903.

88 H. E. Hasper, B. de Kruijff and E. Breukink, *Biochemistry*, 2004, **43**, 11567–11575.

89 T. J. Oman, T. J. Lupoli, T.-S. A. Wang, D. Kahne, S. Walker and W. A. van der Donk, *J. Am. Chem. Soc.*, 2011, **133**, 17544–17547.

90 A. Bakhtiary, S. A. Cochrane, P. Mercier, R. T. McKay, M. Miskolzie, C. S. Sit and J. C. Vederas, *J. Am. Chem. Soc.*, 2017, **139**, 17803–17810.

91 H. E. Hasper, N. E. Kramer, J. L. Smith, J. D. Hillman, C. Zachariah, O. P. Kuipers, B. de Kruijff and E. Breukink, *Science*, 2006, **313**, 1636–1637.

92 E. Breukink and B. de Kruijff, *Nat. Rev. Drug Discov.*, 2006, **5**, 321–32.

93 P. 't Hart, S. F. Oppedijk, E. Breukink and N. I. Martin, *Biochemistry*, 2015, **55**, 232–237.

94 S. Mulholland, E. R. Turpin, B. B. Bonev and J. D. Hirst, *Sci. Rep.*, 2016, 1–11.

95 D. C. Koch, T. H. Schmidt, H. G. Sahl, U. Kubitscheck and C. Kandt, *Biochim. Biophys. Acta - Biomembr.*, 2014, **1838**, 3061–3068.

96 S.-T. D. Hsu, E. Breukink, E. Tischenko, M. A. G. Lutters, B. de Kruijff, R. Kaptein, A. M. J. J. Bonvin and N. A. J. van Nuland, *Nat. Struct. Mol. Biol.*, 2004, **11**, 963–967.

97 B. de Kruijff, V. van Dam and E. Breukink, *Prostaglandins. Leukot. Essent. Fatty Acids*, 2008, **79**, 117–21.

98 V. Ng and W. C. Chan, *Chem. A Eur. J.*, 2016, **22**, 12606–12616.

99 L. A. Draper, P. D. Cotter, C. Hill and R. P. Ross, *Microbiol. Mol. Biol. Rev.*, 2015, **79**, 171–191.

100 N. E. Kramer, E. J. Smid, J. Kok, B. De Kruijff, O. P. Kuipers and E. Breukink, *FEMS Microbiol. Lett.*, 2004, **239**, 157–161.

101 Z. Sun, J. Zhong, X. Liang, J. Liu, X. Chen and L. Huan, *Antimicrob. Agents Chemother.*, 2009, **53**, 1964–1973.

102 T. Stein, S. Borchert, B. Conrad, J. Feesche, B. Hofemeister, J. Hofemeister and K. D. Entian, *J. Bacteriol.*, 2002, **184**, 1703–1711.

103 R. R. Bonelli, T. Schneider, H.-G. Sahl and I. Wiedemann, *Antimicrob. Agents Chemother.*, 2006, **50**, 1449–1457.

104 K. M. Daly, M. Upton, S. K. Sandiford, L. A. Draper, P. A. Wescombe, R. W. Jack, P. M. O'Connor, A. Rossney, F. Götz, C. Hill, P. D. Cotter, R. P. Ross and J. R. Tagg, *J. Bacteriol.*, 2010, **192**, 1131–1142.

105 M. Mota-Meira, C. Lacroix, G. LaPointe and M. C. Lavoie, *FEBS Lett.*, 1997, **410**, 275–279.

106 J. D. Hillman, J. Novák, E. Sagura, J. A. Gutierrez, T. A. Brooks, P. J. Crowley, M. Hess, A. Azizi, K.-P. Leung, D. Cvitkovitch and A. S. Bleiweis, *Infect. Immun.*, 1998, **66**, 2743–2749.

107 F. Qi, P. Chen and P. W. Caufield, *Appl. Environ. Microbiol.*, 2000, **66**, 3221–

3229.

108 T. Nguyen, Z. Zhang, I.-H. Huang, C. Wu, J. Merritt, W. Shi and F. Qi, *Microbiology*, 2009, **155**, 551–556.

109 F. Qi, P. Chen and P. W. Caufield, *Appl. Environ. Microbiol.*, 2001, **67**, 15–21.

110 W. J. Loesche, *Microbiol. Rev.*, 1986, **50**, 353–380.

111 M. Mota-Meira, H. Morency and M. C. Lavoie, *J. Antimicrob. Chemother.*, 2005, **56**, 869–871.

112 A. B. Tabor, *Org. Biomol. Chem.*, 2011, **9**, 7606–28.

113 A. B. Tabor, *Bioorg. Chem.*, 2014, **55**, 39–50.

114 D. Field, P. D. Cotter, C. Hill and R. P. Ross, *Front. Microbiol.*, 2015, **6**, 1–8.

115 D. Field, P. D. Cotter, R. P. Ross and C. Hill, *Bioengineered*, 2015, **5979**, 37–41.

116 M. Montalbán-López, A. J. van Heel and O. P. Kuipers, *FEMS Microbiol. Rev.*, 2016, **41**, 5–18.

117 Q. Zhang, X. Yang, H. Wang and W. A. van der Donk, *ACS Chem. Biol.*, 2014, **9**, 2686–2694.

118 X. Zhang and W. A. Van Der Donk, *J. Am. Chem. Soc.*, 2007, **129**, 2212–2213.

119 B. J. Burkhart, G. A. Hudson, K. L. Dunbar and D. A. Mitchell, *Nat. Chem. Biol.*, 2015, **11**, 564–570.

120 C. Chatterjee, G. C. Patton, L. Cooper, M. Paul and W. A. van der Donk, *Chem. Biol.*, 2006, **13**, 1109–1117.

121 L. Xie, L. M. Miller, C. Chatterjee, O. Averin, N. L. Kelleher and W. A. van der Donk, *Science*, 2004, **303**, 679–681.

122 Q. Wang, T. R. Chan, R. Hilgraf, V. V. Fokin, K. B. Sharpless and M. G. Finn, *J. Am. Chem. Soc.*, 2003, **125**, 3192–3193.

123 M. R. Levengood and W. A. van der Donk, *Bioorganic Med. Chem. Lett.*, 2008, **18**, 3025–3028.

124 M. R. Levengood, P. J. Knerr, T. J. Oman and W. A. Van Der Donk, *J. Am. Chem. Soc.*, 2009, **131**, 12024–12025.

125 J. C. Slootweg, N. Peters, H. C. Quarles Van Ufford, E. Breukink, R. M. J. Liskamp and D. T. S. Rijkers, *Bioorganic Med. Chem.*, 2014, **22**, 5345–5353.

126 T. Koopmans, T. M. Wood, P. 't Hart, L. H. J. Kleijn, A. P. A. Hendrickx, R. J. L. Willems, E. Breukink and N. I. Martin, *J. Am. Chem. Soc.*, 2015, **137**, 9382–9389.

127 D. Field, M. Begley, P. M. O'Connor, K. M. Daly, F. Hugenholtz, P. D. Cotter, C. Hill and R. P. Ross, *PLoS One*, 2012, **7**, 1–12.

128 D. Field, L. Quigley, P. M. O'Connor, M. C. Rea, K. Daly, P. D. Cotter, C. Hill and R. P. Ross, *Microb. Biotechnol.*, 2010, **3**, 473–486.

129 L. Zhou, A. J. van Heel and O. P. Kuipers, *Front. Microbiol.*, 2015, **6**, 1–8.

130 B. Healy, D. Field, P. M. O'Connor, C. Hill, P. D. Cotter and R. P. Ross, *PLoS One*, 2013, **8**, 1–8.

131 R. Rink, J. Wierenga, A. Kuipers, L. D. Kluskens, A. J. M. Driessen, O. P. Kuipers and G. N. Moll, *Appl. Environ. Microbiol.*, 2007, **73**, 5809–5816.

132 J. A. Majchrzykiewicz, J. Lubelski, G. N. Moll, A. Kuipers, J. J. E. Bijlsma, O. P. Kuipers and R. Rink, *Antimicrob. Agents Chemother.*, 2010, **54**, 1498–1505.

133 R. Rink, L. D. Kluskens, A. Kuipers, A. J. M. Driessen, O. P. Kuipers and G. N. Moll, *Biochemistry*, 2007, **46**, 13179–13189.

134 L. D. Kluskens, A. Kuipers, R. Rink, E. de Boef, S. Fekken, A. J. M. Driessen, O. P. Kuipers and G. N. Moll, *Biochemistry*, 2005, **44**, 12827–12834.

135 L. D. Kluskens, S. A. Nelemans, R. Rink, L. de Vries, A. Meter-Arkema, Y. Wang, T. Walther, A. Kuipers, G. N. Moll and M. Haas, *J. Pharmacol. Exp. Ther.*, 2009, **328**, 849–854.

136 C. Zambaldo, X. Luo, A. P. Mehta and P. G. Schultz, *J. Am. Chem. Soc.*, 2017, **139**, 11646–11649.

137 A. J. van Heel, D. Mu, M. Montalbán-López, D. Hendriks and O. P. Kuipers, *ACS Synth. Biol.*, 2013, **2**, 397–404.

138 A. J. van Heel, A. de Jong, M. Montalbán-López, J. Kok and O. P. Kuipers, *Nucleic Acids Res.*, 2013, **41**, 448–453.

139 A. J. van Heel, T. G. Kloosterman, M. Montalbán-López, J. Deng, A. Plat, B. Baudu, D. Hendriks, G. N. Moll and O. P. Kuipers, *ACS Synth. Biol.*, 2016, **5**, 1146–1154.

140 M. Begley, P. D. Cotter, C. Hill and R. P. Ross, *Appl. Environ. Microbiol.*, 2009, **75**, 5451–5460.

141 Y. Goto, B. Li, J. Claesen, Y. Shi, M. J. Bibb and W. A. van der Donk, *PLoS Biol.*, 2010, **8**, 4–13.

142 S. K. Sandiford, *Expert Opin. Drug Discov.*, 2015, **10**, 315–320.

143 W. Kong and T. Lu, *ACS Synth. Biol.*, 2014, **3**, 439–445.

144 E. L. Ongey and P. Neubauer, *Microb. Cell Fact.*, 2016, **15**, 1–16.

145 K. Fukase, M. Kitazawa, A. Sano, K. Shimbo, H. Fujita, S. Horimoto, T. Wakamiya and T. Shiba, *Tetrahedron Lett.*, 1988, **29**, 795–798.

146 K. Fukase, M. Kitazawa, A. Sano, K. Shimbo, S. Horimoto, H. Fujita, A. Kubo, T. Wakamiya and T. Shiba, *Bull. Chem. Soc. Jpn.*, 1992, **65**, 2227–2240.

147 F. Fukase, T. Wakamiya and T. Shiba, *Bull. Chem. Soc. Jpn.*, 1986, **59**, 2505–2508.

148 D. N. Harpp and G. Gleason, *J. Org. Chem.*, 1971, **36**, 73–80.

149 P. L. Toogood, *Tetrahedron Lett.*, 1993, **34**, 7833–7836.

150 A. Polinsky, M. G. Cooney, A. Toy-Palmer, G. Ösapay and M. Goodman, *J. Med.*

Chem., 1992, **35**, 4185–4194.

151 M. Matteucci, G. Bhalay and M. Bradley, *Tetrahedron Lett.*, 2004, **45**, 1399–1401.

152 S. Burrage, T. Raynham, G. Williams, J. W. Essex, C. Allen, M. Cardno, V. Swali and M. Bradley, *Chem. A Eur. J.*, 2000, **6**, 1455–66.

153 N. M. Okeley, Y. Zhu and W. A. van Der Donk, *Org. Lett.*, 2000, **2**, 3603–3606.

154 H. Zhou and W. A. van der Donk, *Org. Lett.*, 2002, **4**, 1335–1338.

155 G. Ösapay and M. Goodman, *J. Chem. Soc. Chem. Commun.*, 1993, 1599–1600.

156 S. Bregant and A. B. Tabor, *J. Org. Chem.*, 2005, **70**, 2430–2438.

157 K. Manzor, K. ó Proinsias and F. Kelleher, *Tetrahedron Lett.*, 2017, **58**, 2959–2963.

158 V. R. Pattabiraman, S. M. K. McKinnie and J. C. Vederas, *Angew. Chem. Int. Ed. Engl.*, 2008, **47**, 9472–9475.

159 S. M. K. McKinnie, A. C. Ross, M. J. Little and J. C. Vederas, *Medchemcomm*, 2012, **3**, 971–975.

160 A. C. Ross, H. Liu, V. R. Pattabiraman and J. C. Vederas, *J. Am. Chem. Soc.*, 2010, **132**, 462–463.

161 S. Mitchell, PhD Thesis, UCL, 2017.

162 B. Mothia, A. N. Appleyard, S. Wadman and A. B. Tabor, *Org. Lett.*, 2011, **13**, 4216–4219.

163 P. J. Knerr and W. A. van der Donk, *J. Am. Chem. Soc.*, 2013, **135**, 7094–7097.

164 P. J. Knerr and W. A. van der Donk, *J. Am. Chem. Soc.*, 2012, **134**, 7648–7651.

165 W. Liu, A. S. H. Chan, H. Liu, S. A. Cochrane and J. C. Vederas, *J. Am. Chem. Soc.*, 2011, **133**, 14216–14219.

166 H. Liu, V. R. Pattabiraman and J. C. Vederas, *Org. Lett.*, 2009, **11**, 5574–7.

167 A. C. Ross, S. M. K. McKinnie and J. C. Vederas, *J. Am. Chem. Soc.*, 2012, **134**, 2008–2011.

168 V. R. Pattabiraman, J. L. Stymiest, D. J. Derksen, N. I. Martin and J. C. Vederas, *Org. Lett.*, 2007, **9**, 699–702.

169 N. Ghalit, J. Kemmink, H. W. Hilbers, C. Versluis, D. T. S. Rijkers and R. M. J. Liskamp, *Org. Biomol. Chem.*, 2007, **5**, 924–34.

170 N. Ghalit, D. T. S. Rijkers, J. Kemmink, C. Versluis and R. M. J. Liskamp, *Chem. Commun.*, 2005, 192–4.

171 J. C. Slootweg, J. Kemmink, R. M. J. Liskamp and D. T. S. Rijkers, *Org. Biomol. Chem.*, 2013, **11**, 7486–96.

172 N. Ghalit, A. J. Poot, A. Fürstner, D. T. S. Rijkers and R. M. J. Liskamp, *Org. Lett.*, 2005, **7**, 2961–2964.

173 J. C. Slootweg, E. F. van Herwerden, M. F. M. van Doremalen, E. Breukink, R. M.

J. Liskamp and D. T. S. Rijkers, *Org. Biomol. Chem.*, 2015, **13**, 5997–6009.

174 N. Ghalit, J. F. Reichwein, H. W. Hilbers, E. Breukink, D. T. S. Rijkers and R. M. J. Liskamp, *Chembiochem*, 2007, **8**, 1540–54.

175 F. Cavelier-Frontin, J. Daunis and R. Jacquier, *Tetrahedron: Asymmetry*, 1992, **3**, 85–94.

176 R. K. Olsen, G. D. Kini and W. J. Hennen, *J. Org. Chem.*, 1985, **50**, 4332–4336.

177 J. M. Probert, D. Rennex and M. Bradley, *Tetrahedron Lett.*, 1996, **37**, 1101–1104.

178 C. Aydillo, A. Avenoza, J. H. Busto, G. Jiménez-Osés, J. M. Peregrina and M. M. Zurbano, *Org. Lett.*, 2012, **14**, 334–337.

179 G. B. Brown and V. du Vigneaud, *J. Biol. Chem.*, 1941, **140**, 767–771.

180 X. Zhu and R. R. Schmidt, *European J. Org. Chem.*, 2003, **2003**, 4069–4072.

181 C. Dugave and A. Ménez, *Tetrahedron: Asymmetry*, 1997, **8**, 1453–1465.

182 R. S. Narayan and M. S. VanNieuwenhze, *Org. Lett.*, 2005, **7**, 2655–2658.

183 M. F. M. Mustapa, R. Harris, J. Mould, N. A. L. Chubb, D. Schultz, P. C. Driscoll and A. B. Tabor, *Tetrahedron Lett.*, 2002, **43**, 8359–8362.

184 M. F. Mohd Mustapa, R. Harris, N. Bulic-Subanovic, S. L. Elliott, S. Bregant, M. F. A. Groussier, J. Mould, D. Schultz, N. A. L. Chubb, P. R. J. Gaffney, P. C. Driscoll and A. B. Tabor, *J. Org. Chem.*, 2003, **68**, 8185–8192.

185 L. D. Arnold, R. G. May and J. C. Vederas, *J. Am. Chem. Soc.*, 1988, **110**, 2237–2241.

186 H. Shao, S. H. H. Wang, C. Lee, G. Ösapay and M. Goodman, *J. Org. Chem.*, 1995, **60**, 2956–2957.

187 S. L. Cobb and J. C. Vederas, *Org. Biomol. Chem.*, 2007, **5**, 1031–1038.

188 A. Avenoza, J. H. Busto, G. Jiménez-Osés and J. M. Peregrina, *Org. Lett.*, 2006, **8**, 2855–2858.

189 K. Nakajima, H. Oda and K. Okawa, *Bull. Chem. Soc. Jpn.*, 1983, **56**, 520–522.

190 C. Bonauer, T. Walenzyk and B. König, *Synthesis (Stuttg).*, 2006, **1**, 1–20.

191 J. M. Humphrey and A. R. Chamberlin, *Chem. Rev.*, 1997, **97**, 2243–2266.

192 W. C. Chan, B. W. Bycroft, L. Y. Lian and G. C. K. Roberts, *FEBS Lett.*, 1989, **252**, 29–36.

193 U. Schmidt, A. Lieberknecht and J. Wild, *Synthesis (Stuttg).*, 1988, 159–172.

194 J. Jiang, Z. Ma and S. L. Castle, *Tetrahedron*, 2015, **71**, 5431–5451.

195 K. Hashimoto, M. Sakai, T. Okuno and H. Shirahama, *Chem. Commun.*, 1996, 1139–1140.

196 M. R. Levengood and W. A. van der Donk, *Nat. Protoc.*, 2006, **1**, 3001–3010.

197 J. M. Chalker, S. B. Gunnoo, O. Boutureira, S. C. Gerstberger, M. Fernández-

González, G. J. L. Bernardes, L. Griffin, H. Hailu, C. J. Schofield and B. G. Davis, *Chem. Sci.*, 2011, **2**, 1666–1676.

198 P. M. Morrison, P. J. Foley, S. L. Warriner and M. E. Webb, *Chem. Commun.*, 2015, **51**, 13470–13473.

199 P. Güntert and L. Buchner, *J. Biomol. NMR*, 2015, **62**, 453–471.

200 C. D. Schwieters, J. J. Kuszewski, N. Tjandra and G. M. Clore, *J. Magn. Reson.*, 2003, **160**, 65–73.

201 F. J. M. van de Ven and G. Jung, *Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol.*, 1996, **69**, 99–107.

202 W. C. Chan, B. W. Bycroft, M. L. Leyland, L.-Y. Lian, J. C. Yang and G. C. K. Roberts, *FEBS Lett.*, 1992, **300**, 56–62.

203 L. Smith, C. Zachariah, R. Thirumoorthy, J. Rocca, J. Novák, J. D. Hillman and A. S. Edison, *Biochemistry*, 2003, **42**, 10372–10384.

204 H. Kessler, S. Steuernagel, M. Will, G. Jung, R. Kellner, D. Gillessen and T. Kamiyama, *Helv. Chim. Acta*, 1988, **71**, 1924–1929.

205 S. Freund, G. Jung, O. Gutbrod, G. Folkers, W. A. Gibbons, H. Allgaier and R. Werner, *Biopolymers*, 1990, **31**, 803–811.

206 M. Slijper, C. W. Hilbers, R. N. H. Konings and F. J. M. van de Ven, *FEBS Lett.*, 1989, **252**, 22–28.

207 F. J. van de Ven, H. W. van den Hooven, R. N. Konings and C. W. Hilbers, *Eur. J. Biochem.*, 1991, **202**, 1181–1188.

208 L. Lian, W. C. Chan, S. D. Morley, G. C. K. Roberts, B. W. Bycroft and D. Jackson, *Biochem. J.*, 1992, **283**, 413–420.

209 D. E. Palmer, D. F. Mierke, C. Pattaroni, M. Goodman, T. Wakamiya, K. Fukase, M. Kitazawa, H. Fujita and T. Shiba, *Biopolymers*, 1989, **28**, 397–408.

210 S. T. D. Hsu, E. Breukink, G. Bierbaum, H. G. Sahl, B. De Kruijff, R. Kaptein, N. A. J. Van Nuland and A. M. J. J. Bonvin, *J. Biol. Chem.*, 2003, **278**, 13110–13117.

211 H. W. van den Hooven, F. Fogolari, H. S. Rollema, R. N. H. Konings, C. W. Hilbers and F. J. M. van de Ven, *FEBS Lett.*, 1993, **319**, 189–194.

212 H. W. van den Hooven, C. E. A. M. Spronk, M. van de Kamp, R. N. H. Konings, C. W. Hilbers and F. J. M. van de Ven, *Eur. J. Biochem.*, 1996, **235**, 394–403.

213 H. W. van den Hooven, C. C. M. Doeland, M. Kamp, R. N. H. Konings, C. W. Hilbers and F. J. M. Ven, *Eur. J. Biochem.*, 1996, **235**, 382–393.

214 S.-T. Hsu, E. Breukink, B. de Kruijff, R. Kaptein, A. M. J. J. Bonvin and N. a. J. van Nuland, *Biochemistry*, 2002, **41**, 7670–7676.

215 B. B. Bonev, E. Breukink, E. Swiezewska, B. de Kruijff and A. Watts, *FASEB J.*, 2004, **18**, 1862–1869.

216 J. C. Slootweg, R. M. J. Liskamp and D. T. S. Rijkers, *J. Pept. Sci.*, 2013, **19**, 692–699.

217 D. E. Palmer, C. Pattaroni, K. Nunami, R. K. Chadha, M. Goodman, T. Wakamiya, K. Fukase, S. Horimoto, M. Kitazawa, H. Fujita, A. Kubo and T. Shiba, *J. Am. Chem. Soc.*, 1992, **114**, 5634–5642.

218 W. C. Chan, H. M. Dodd, N. Horn, K. Maclean, L. Y. Lian, B. W. Bycroft and L. Lian, *Appl. Environ. Microbiol.*, 1996, **62**, 2966–9.

219 S. Chen, S. Wilson-Stanford, W. Cromwell, J. D. Hillman, A. Guerrero, C. A. Allen, J. A. Sorg and L. Smith, *Appl. Environ. Microbiol.*, 2013, **79**, 4015–4023.

220 Z. V. F. Wright, S. McCarthy, R. Dickman, F. E. Reyes, S. Sanchez-Martinez, A. Cryar, I. Kilford, A. Hall, A. K. Takle, M. Topf, T. Gonen, K. Thalassinos and A. B. Tabor, *J. Am. Chem. Soc.*, 2017, **139**, 13063–13075.

221 D. L. Boger, S. Ichikawa, W. C. Tse, M. P. Hedrick and Q. Jin, *J. Am. Chem. Soc.*, 2001, **123**, 561–568.

222 K. Barlos, D. Papaioannou and D. Theodoropoulos, *J. Org. Chem.*, 1982, **47**, 1324–1326.

223 Z. V. F. Wright, PhD Thesis, UCL, 2015.

224 B. Mothia, PhD Thesis, UCL, 2012.

225 G. Jou, I. Gonzalez, F. Albericio, P. Lloyd-Williams and E. Giralt, *J. Org. Chem.*, 1997, **62**, 354–366.

226 H. Liu, V. R. Pattabiraman and J. C. Vederas, *Org. Lett.*, 2007, **9**, 4211–4214.

227 M. A. Brimble, R. Kowalczyk, P. W. R. Harris, P. R. Dunbar and V. J. Muir, *Org. Biomol. Chem.*, 2008, **6**, 112–121.

228 Y. Zhu, M. D. Gieselman, H. Zhou, O. Averin and W. A. van der Donk, *Org. Biomol. Chem.*, 2003, **1**, 3304–3315.

229 S. J. Maynard, A. M. Almeida, Y. Yoshimi and S. H. Gellman, *J. Am. Chem. Soc.*, 2014, **136**, 16683–16688.

230 F. Albericio, M. Cases, J. Alsina, S. A. Triolo, L. A. Carpino and S. A. Kates, *Tetrahedron Lett.*, 1997, **38**, 4853–4856.

231 L. A. Carpino, *J. Am. Chem. Soc.*, 1993, **115**, 4397–4398.

232 A. Thakkar, T. B. Trinh and D. Pei, *ACS Comb. Sci.*, 2013, **15**, 120–129.

233 S. L. Pedersen, A. P. Tofteng, L. Malik and K. J. Jensen, *Chem. Soc. Rev.*, 2012, **41**, 1826–1844.

234 C. A. G. N. Montalbetti and V. Falque, *Tetrahedron*, 2005, **61**, 10827–10852.

235 F. Albericio, J. M. Bofill, A. El-Faham and S. A. Kates, *J. Org. Chem.*, 1998, **63**, 9678–9683.

236 R. Raz, *Org. Lett.*, 2011, **13**, 1606–1609.

237 B. J. van Lierop, W. R. Jackson and A. J. Robinson, *Tetrahedron*, 2010, **66**, 5357–5366.

238 R. Ingenito, D. Dreznjak, S. Guffler and H. Wenschuh, *Org. Lett.*, 2002, **4**, 1187–

1188.

239 M. J. Miller, *J. Org. Chem.*, 1980, **63**, 3132–3135.

240 A. El-Faham and F. Albericio, *J. Pept. Sci.*, 2010, **16**, 6–9.

241 R. Subiros-Funosas, G. A. Acosta, A. El-Faham and F. Albericio, *Tetrahedron Lett.*, 2009, **50**, 6200–6202.

242 S. Wilson-Stanford, A. Kalli, K. Håkansson, J. Kastrantas, R. S. Orugunty and L. Smith, *Appl. Environ. Microbiol.*, 2009, **75**, 1381–1387.

243 T. M. Postma, M. Giraud and F. Albericio, *Org. Lett.*, 2012, **14**, 5468–5471.

244 T. M. Postma and F. Albericio, *Org. Lett.*, 2013, **15**, 616–619.

245 T. Wöhr and M. Mutter, *Tetrahedron Lett.*, 1995, **36**, 3847–3848.

246 M. Mutter, A. Nefzi, T. Sato, X. Sun, F. Wahl and T. Wöhr, *Pept. Res.*, 1995, **8**, 145–153.

247 M. S. Y. Wong and K. A. Jolliffe, *Aust. J. Chem.*, 2010, **63**, 797–801.

248 T. M. Postma and F. Albericio, *Org. Lett.*, 2014, **16**, 1772–1775.

249 K. A. Fairweather, N. Sayyadi, I. J. Luck, J. K. Clegg and K. A. Jolliffe, *Org. Lett.*, 2010, **12**, 3136–3139.

250 T. Johnson, M. Quibell, D. Owen and R. C. Sheppard, *J. Chem. Soc. Chem. Commun.*, 1993, 369–372.

251 C. Hyde, T. Johnson, D. Owen, M. Quibell and R. C. Sheppard, *Int. J. Pept. Protein Res.*, 1994, **43**, 431–440.

252 A. Ehrlich, H. U. Heyne, R. Winter, M. Beyermann, H. Haber, L. A. Carpino and M. Bienert, *J. Org. Chem.*, 1996, **61**, 8831–8838.

253 C. J. Arnusch, A. M. J. J. Bonvin, A. M. Verel, W. T. M. Jansen, R. M. J. Liskamp, B. De Kruijff, R. J. Pieters and E. Breukink, *Biochemistry*, 2008, **47**, 12661–12663.

254 W. C. Chan, M. Leyland, J. Clark, H. M. Dodd, L. Lian, M. J. Gasson, B. W. Bycroft and G. C. K. Roberts, *FEBS Lett.*, 1996, **390**, 129–132.

255 J. C. Slootweg, S. van der Wal, H. C. Q. van Ufford, E. Breukink, R. M. J. Liskamp and D. T. S. Rijkers, *Bioconjug. Chem.*, 2013, **24**, 2058–2066.

256 W. R. Sampson, H. Patsiouras and N. J. Ede, *J. Pept. Sci.*, 1999, **5**, 403–409.

257 A. Montero, F. Albericio, M. Royo and B. Herradón, *Org. Lett.*, 2004, **6**, 4089–4092.

258 S. You and J. W. Kelly, *J. Org. Chem.*, 2003, **68**, 9506–9509.

259 A. Banerjee, K. Basu, A. Baral, S. Basak, A. Dehsorkhi, J. Nanda, D. Bhunia, S. Ghosh, V. Castelletto and I. Hamley, *Chem. Commun.*, 2016, **1**, 5–8.

260 R. Hayashi, S. Moore and W. H. Stein, *J. Biol. Chem.*, 1973, **248**, 2296–2302.

261 R. Rink, A. Arkema-Meter, I. Baudoin, E. Post, A. Kuipers, S. A. Nelemans, H. H. Akanbi and G. N. Moll, *J. Pharmacol. Toxicol. Methods*, 2010, **61**, 210–218.

262 K. Breddam and M. Ottesen, *Carlsberg Res. Commun.*, 1987, **52**, 55–63.

263 A. L. Tarentino, G. Quinones, B. G. Grimwood, C. R. Hauer and T. H. Plummer Jr, *Arch. Biochem. Biophys.*, 1995, **319**, 281–285.

264 P. White, Ed., in *Novabiochem Peptide Synthesis, 30 Years of Innovation*, 2014, p. 2.19.

265 K. Barlos, O. Chatzi, D. Gatos and G. Stavropoulos, *Int. J. Pept. Protein Res.*, 1991, **37**, 513–520.

266 T. M. Fyles and C. C. Leznoff, *Can. J. Chem.*, 1976, **54**, 935–942.

267 M. Gude, J. Ryf and P. D. White, *Lett. Pept. Sci.*, 2002, **9**, 203–206.

268 M. A. Hossain, A. Belgi, F. Lin, S. Zhang, F. Shabanpoor, L. Chan, C. Belyea, H. T. Truong, A. R. Blair, S. Andrikopoulos, G. W. Tregear and J. D. Wade, *Bioconjug. Chem.*, 2009, **20**, 1390–1396.

269 B. Blankemeyer-Menge, M. Nimtz and R. Frank, *Tetrahedron Lett.*, 1990, **31**, 1701–1704.

270 T. Wiseman, S. Williston, J. F. Brandts and L.-N. Lin, *Anal. Biochem.*, 1989, **179**, 131–137.

271 S. A. Cochrane, B. Findlay, A. Bakhtiary, J. Z. Acedo, E. M. Rodriguez-Lopez, P. Mercier and J. C. Vederas, *Proc. Natl. Acad. Sci.*, 2016, **113**, 11561–11566.

272 E. Koshland, *Proc. Natl. Acad. Sci. U. S. A.*, 1958, **44**, 98–104.

273 C. D. Schwieters, J. J. Kuszewski and G. M. Clore, *Prog. Nucl. Magn. Reson. Spectrosc.*, 2006, **48**, 47–62.

274 W. F. Vranken, W. Boucher, T. J. Stevens, R. H. Fogh, A. Pajon, M. Llinas, E. L. Ulrich, J. L. Markley, J. Ionides and E. D. Laue, *Proteins Struct. Funct. Bioinforma.*, 2005, **59**, 687–696.

275 J. J. Kuszewski, R. A. Thottungal, G. M. Clore and C. D. Schwieters, *J. Biomol. NMR*, 2008, **41**, 221–239.

276 M. Karplus, *J. Chem. Phys.*, 1959, **30**, 11–15.

277 M. Karplus, *J. Am. Chem. Soc.*, 1963, **85**, 2870–2871.

278 B. Vögeli, J. Ying, A. Grishaev and A. Bax, *J. Am. Chem. Soc.*, 2007, **129**, 2483–2494.

279 E. A. Lubecka, E. Sikorska, A. Marcinkowska and J. Ciarkowski, *J. Pept. Sci.*, 2014, **20**, 406–414.

280 Y. Shen and A. Bax, *J. Biomol. NMR*, 2013, **56**, 227–241.

281 Y. Shen, F. Delaglio, G. Cornilescu and A. Bax, *J. Biomol. NMR*, 2009, **44**, 213–223.

282 C. M. Falcomer, Y. C. Meinwald, I. Choudhary, S. Talluri, P. J. Milburn, J. Clardy and H. A. Scheraga, *J. Am. Chem. Soc.*, 1992, **114**, 4036–4042.

283 Y. Shen and A. Bax, *J. Biomol. NMR*, 2010, **46**, 199–204.

284 C. Earl, PhD Rotation Report, UCL, 2013.

285 E. R. Turpin, S. Mulholland, A. M. Teale, B. B. Bonev and J. D. Hirst, *RSC Adv.*, 2014, **4**, 48621–48631.

286 A. T. Brünger, P. D. Adams, G. M. Clore, W. L. DeLano, P. Gros, R. W. Grosse-Kunstleve, J. S. Jiang, J. Kuszewski, M. Nilges, N. S. Pannu, R. J. Read, L. M. Rice, T. Simonson and G. L. Warren, *Acta Crystallogr. Sect. D Biol. Crystallogr.*, 1998, **54**, 905–921.

287 B. R. Brooks, R. E. Bruccoleri, B. D. Olafson, D. J. States, S. Swaminathan and M. Karplus, *J. Comp. Chem.*, 1983, **4**, 187–217.

288 M. Nilges, G. M. Clore and A. M. Gronenborn, *FEBS Lett.*, 1988, **229**, 317–324.

289 A. Bhattacharya, R. Tejero and G. T. Montelione, *Proteins Struct. Funct. Bioinforma.*, 2007, **66**, 778–795.

290 C. Ramakrishnan and G. N. Ramachandran, *Proc. Indian Acad. Sci. - Sect. A*, 1964, **59**, 327–343.

291 R. A. Laskowski, M. W. MacArthur, D. S. Moss and J. M. Thornton, *J. Appl. Crystallogr.*, 1993, **26**, 283–291.

292 S. Hovmöller, T. Zhou and T. Ohlson, *Acta Crystallogr. Sect. D Struct. Biol.*, 2002, **58**, 768–76.

293 S. Hayward, *Protein Sci.*, 2001, **10**, 2219–2227.

294 W. G. Touw, R. P. Joosten and G. Vriend, *Acta Crystallogr. Sect. D Biol. Crystallogr.*, 2015, **71**, 1604–1614.

295 J. E. Milner-White, J. D. Watson, G. Qi and S. Hayward, *Structure*, 2006, **14**, 1369–1376.

296 L. Moynié, M. F. Giraud, A. Breton, F. Boissier, B. Daignan-Fornier and A. Dautant, *Protein Sci.*, 2012, **21**, 1185–1196.

297 D. O. Cicero, G. Barbato and R. Bazzo, *J. Am. Chem. Soc.*, 1995, **117**, 1027–1033.

298 S.-I. Murahashi, D. Zhang, H. Iida, T. Miyawaki, M. Uenaka, K. Murano and K. Meguro, *Chem. Commun.*, 2014, **50**, 10295–10298.

299 *Soc. Farm. Ital. BE 623243*, 1963.

300 C. Douat-Casassus, N. Marchand-Geneste, E. Diez, C. Aznar, P. Picard, S. Geoffre, A. Huet, M.-L. Bourguet-Kondracki, N. Gervois, F. Jotereau and S. Quideau, *Mol. Biosyst.*, 2006, **2**, 240–249.

301 C. D. Campbell, C. Concellón and A. D. Smith, *Tetrahedron Asymmetry*, 2011, **22**, 797–811.

302 A. Miyazaki, M. Asanuma, K. Dodo, H. Egami and M. Sodeoka, *Chem. A Eur. J.*, 2014, **20**, 8116–8128.

303 F. Corzana, J. H. Busto, M. G. De Luis, J. Jiménez-Barbero, A. Avenoza and J. M. Peregrina, *Chem. A Eur. J.*, 2009, **15**, 3863–3874.

304 X. Tian, L. Li, J. Han, X. Zhen and S. Liu, *Springerplus*, 2016, **5**, 1–8.

305 L. A. Carpino, D. Sadat-Aalaee, H. G. Chao and R. H. DeSelms, *J. Am. Chem. Soc.*, 1990, **112**, 9651–9652.

306 R. Raz and J. Rademann, *Org. Lett.*, 2011, **13**, 1606–1609.

307 G. L. Ellman, *Arch. Biochem. Biophys.*, 1959, **82**, 70–77.

308 P. White, Ed., in *Novabiochem Peptide Synthesis, 30 Years of Innovation*, 2014, p. 3.6-3.7.

309 B. A. Burkett, R. C. D. Brown and M. M. Meloni, *Tetrahedron Lett.*, 2001, **42**, 5773–5775.

APPENDIX I - XPLOR-NIH FILES

NOE and Dihedral Restraint Data

Mutacin I Ring B WT – Major Conformer

```
set echo=false end

set wrnlev=0 end
!Interresidue
!i-i+1
assign (resid 2 and name HA  )(resid 3 and name HN  ) 3.3
0.7 0.7
assign (resid 4 and name HN  )(resid 3 and name HN  ) 3.2
0.6 0.6
assign (resid 5 and name HN  )(resid 4 and name HN  ) 3.6
0.7 0.7
assign (resid 4 and name HB1 )(resid 5 and name HN  ) 3.5
0.7 0.7
assign (resid 5 and name HN  )(resid 4 and name HB2 ) 4.2
0.8 0.8
assign (resid 4 and name HA  )(resid 5 and name HN  ) 2.9
0.6 0.6
assign (resid 2 and name HA  )(resid 3 and name HA1 ) 4.4
0.9 0.9
assign (resid 3 and name HA2 )(resid 4 and name HN  ) 4.1
0.8 0.8
assign (resid 4 and name HN  )(resid 3 and name HA1 ) 4.3
0.9 0.9
assign (resid 5 and name HN  )(resid 4 and name HN  ) 3.7
0.7 0.7
assign (resid 3 and name HN  )(resid 2 and name HB# ) 4.3
0.9 0.9
!i-i+2
assign (resid 1 and name HT# )(resid 3 and name HN  ) 4.3
0.9 0.9
assign (resid 2 and name HA  )(resid 4 and name HN  ) 4.3
0.9 0.9
!i-i+3/4
assign (resid 5 and name HN  )(resid 2 and name HN  ) 3.8
0.8 0.8
assign (resid 1 and name HA  )(resid 5 and name HA  ) 3.5
0.7 0.7
!across Lan
assign (resid 1 and name HB2 )(resid 4 and name HB2 ) 3.7
0.7 0.7
assign (resid 4 and name HB1 )(resid 1 and name HB2 ) 4.2
0.8 0.8
assign (resid 4 and name HA  )(resid 1 and name HA  ) 4.3
0.9 0.9
set echo=true end
```

```
set wrnlev=5 end


! REMARK  error margins are set to conservative default
values of
! double the standard deviation observed in TALOS+, capped
! at a minimum of +/-20 degree.
!phi
!from 3J coupling
assign (resid    2 and name C   )(resid    3 and name N    )
       (resid    3 and name CA  )(resid    3 and name C    )
1.0   78.2    40.0 2
assign (resid    3 and name C   )(resid    4 and name N    )
       (resid    4 and name CA  )(resid    4 and name C    )
1.0  -82.2    40.0 2
assign (resid    1 and name C   )(resid    2 and name N    )
       (resid    2 and name CA  )(resid    2 and name C    )
1.0  -95.7    40.0 2
assign (resid    4 and name C   )(resid    5 and name N    )
       (resid    5 and name CA  )(resid    5 and name C    )
1.0  -101.6   40.0 2
!psi
!predicted by talos
assign (resid    3 and name N   )(resid    3 and name CA   )
       (resid    3 and name C   )(resid    4 and name N    )
1.0   11.9    37.9 2
```

Mutacin I Ring B WT – Minor Conformer

```
set echo=false end

set wrnlev=0 end
!Interresidue
!i-i+1
assign (resid 2 and name HN )(resid 1 and name HA  ) 5.2 3.0
1.0
assign (resid 2 and name HA )(resid 3 and name HN  ) 3.1 0.6
0.6
assign (resid 3 and name HN )(resid 4 and name HA  ) 4.0 0.8
0.8
assign (resid 2 and name HA )(resid 3 and name HN  ) 3.1 0.6
0.6
!i-i+3
assign (resid 1 and name HB1)(resid 4 and name HA  ) 3.5 0.7
0.7
set echo=true end

set wrnlev=5 end
```

```
! REMARK  error margins are set to conservative default
values of double the
! standard deviation observed in TALOS+, capped at a minimum
of +/-20 degree.
!phi
!predictions in agreement with calculated values
assign (resid    2 and name C   )(resid    3 and name N    )
       (resid    3 and name CA  )(resid    3 and name C    )
1.0  -74.3    30.0 2
assign (resid    3 and name C   )(resid    4 and name N    )
       (resid    4 and name CA  )(resid    4 and name C    )
1.0  -90.8    30.0 2
!calc
assign (resid    1 and name C   )(resid    2 and name N    )
       (resid    2 and name CA  )(resid    2 and name C    )
1.0  -90.8    60.0 2
!psi
!predictions
assign (resid    3 and name N   )(resid    3 and name CA   )
       (resid    3 and name C   )(resid    4 and name N    )
1.0  -21.8    35.0 2
```

Nisin Ring B Lan Analogue

```
set echo=false end

set wrnlev=0 end

!Interresidue
!i-i+1
assign (resid 3 and name HN  )(resid 4 and name HN  ) 3.6
0.7 0.7
assign (resid 3 and name HN  )(resid 2 and name HA  ) 3.4
0.7 0.7
assign (resid 2 and name HB2 )(resid 3 and name HN  ) 3.9
0.8 0.8
assign (resid 5 and name HG# )(resid 4 and name HA  ) 5.3
1.1 1.1
assign (resid 5 and name HB2 )(resid 4 and name HA  ) 5.3
1.1 1.1
assign (resid 5 and name HN  )(resid 4 and name HB2 ) 4.2
0.8 0.8
assign (resid 4 and name HN  )(resid 3 and name HA2 ) 4.7
0.9 0.9
assign (resid 4 and name HB1 )(resid 5 and name HN  ) 3.7
0.7 0.7
!i-i+2
assign (resid 3 and name HN  )((resid 1 and name HA) or
(resid 4 and name HA)) 4.5 0.9 0.9
```

```
assign (resid 4 and name HN  )(resid 2 and name HA  ) 4.5
0.9 0.9
assign (resid 2 and name HA  )((resid 1 and name HA) or
(resid 4 and name HA)) 3.2 0.6 0.6
assign ((resid 2 and name HB1) or (resid 1 and name
HB2))(resid 3 and name HN  ) 4.4 0.9 0.9
!i-i+3
assign (resid 1 and name HA)(resid 4 and name HB1 ) 3.6 0.7
0.7
assign ((resid 2 and name HB1) or (resid 1 and name
HB2))(resid 4 and name HN  ) 5.0 1.0 1.0
!i-i+4
assign ((resid 1 and name HA) or (resid 4 and name HA))(resid
5 and name HN  ) 3.2 0.6 0.6

set echo=true end

set wrnlev=5 end


! REMARK  error margins are set to conservative default
! values of double the standard deviation observed in TALOS+
! capped at a minimum of +/-20 degree.

!phi
!predictions
assign (resid    1 and name C   )(resid    2 and name N    )
       (resid    2 and name CA  )(resid    2 and name C    )
1.0  -68.9    60.0 2
!calc
assign (resid    2 and name C   )(resid    3 and name N    )
       (resid    3 and name CA  )(resid    3 and name C    )
1.0  -78.2    60.0 2
assign (resid    4 and name C   )(resid    5 and name N    )
       (resid    5 and name CA  )(resid    5 and name C    )
1.0  -90.8    60.0 2
```

Nisin Ring B WT

```
set echo=false end

set wrnlev=0 end

!Interresidue
!i-i+1
assign (resid 2 and name HB1 )((resid 3 and name HN) or
(resid 5 and name HN)) 3.6 1.8 1.4
assign (resid 2 and name HB2 )((resid 3 and name HN) or
(resid 5 and name HN)) 3.6 1.8 1.4
```

```
assign (resid 4 and name HN  )((resid 3 and name HN) or
(resid 5 and name HN)) 2.9 1.1 0.7
assign ((resid 3 and name HN) or (resid 5 and name HN))(resid
4 and name HB# ) 2.9 1.1 0.7
assign (resid 2 and name HA  )((resid 3 and name HN) or
(resid 5 and name HN)) 2.3 0.5 0.9
assign ((resid 3 and name HN) or (resid 5 and name HN))(resid
4 and name HA  ) 2.3 0.5 0.8
assign (resid 4 and name HN  )(resid 3 and name HA2 ) 3.6
1.8 1.4
assign (resid 4 and name HN  )(resid 3 and name HA1 ) 3.6
1.8 1.4
assign (resid 5 and name HG# )(resid 4 and name HA  ) 3.6
1.8 2.8
assign (resid 5 and name HB1 )(resid 4 and name HA  ) 3.6
1.8 2.9
assign (resid 1 and name HG# )(resid 2 and name HA  ) 3.6
1.8 2.9
assign (resid 1 and name HG# )(resid 2 and name HD# ) 3.6
1.8 1.4
assign (resid 2 and name HA  )(resid 1 and name HB1 ) 3.6
1.8 2.6
assign (resid 2 and name HA  )(resid 1 and name HA  ) 2.3
0.5 2.7
assign (resid 4 and name HA  )(resid 3 and name HA1 ) 3.6
1.8 1.4
!i-i+2
assign (resid 1 and name HT# )(resid 3 and name HA1 ) 3.6
1.8 2.4
assign ((resid 3 and name HN) or (resid 5 and name HN))(resid
1 and name HA  ) 3.6 1.8 1.4
assign (resid 2 and name HA  )(resid 4 and name HN  ) 3.6
1.8 1.4
!i-i+3
assign (resid 1 and name HG# )(resid 4 and name HN  ) 3.6
1.8 1.4
assign (resid 4 and name HN  )(resid 1 and name HA  ) 2.9
1.1 0.8
assign (resid 1 and name HG# )(resid 4 and name HA  ) 2.9
1.1 1.3
assign (resid 1 and name HG# )(resid 4 and name HB1 ) 3.6
1.8 1.4
!i-i+4
assign (resid 1 and name HG# )((resid 3 and name HN) or
(resid 5 and name HN)) 3.6 1.8 1.7
assign ((resid 3 and name HN) or (resid 5 and name HN))(resid
1 and name HT# ) 3.6 1.8 1.4
assign (resid 5 and name HE# )(resid 1 and name HT# ) 3.6
1.8 1.7

set echo=true end
```

```
set wrnlev=5 end


! REMARK  error margins are set to conservative default
values of double
! the standard deviation observed in TALOS+, capped at a
minimum of +/-20 degree.
!phi
!predictions
assign (resid    1 and name C   )(resid    2 and name N    )
       (resid    2 and name CA  )(resid    2 and name C    )
1.0  -62.7    30.0 2
!calc
assign (resid    2 and name C   )(resid    3 and name N    )
       (resid    3 and name CA  )(resid    3 and name C    )
1.0  -74.3    60.0 2
assign (resid    3 and name C   )(resid    4 and name N    )
       (resid    4 and name CA  )(resid    4 and name C    )
1.0  -82.2    60.0 2
```

Mutacin I Ring A Ser/Ala Analogue

```
set echo=false end

set wrnlev=0 end
!interresidue
!i-i+1
assign (resid 2 and name HN  )(resid 1 and name HA  ) 2.7
0.5 0.5
assign (resid 4 and name HN  )(resid 5 and name HN  ) 3.3
0.7 0.7
assign (resid 3 and name HN  )(resid 2 and name HB# ) 3.5
0.7 0.7
assign (resid 3 and name HN  )(resid 2 and name HA  ) 2.7
0.5 0.5
assign (resid 6 and name HN  )(resid 7 and name HN  ) 3.3
0.7 0.7
assign (resid 8 and name HN  )(resid 7 and name HN  ) 3.1
0.6 0.6
assign (resid 8 and name HN  )(resid 7 and name HA  ) 3.0
0.6 0.6
assign (resid 5 and name HN  )(resid 4 and name HA  ) 3.3
0.7 0.7
assign (resid 7 and name HN  )(resid 6 and name HA  ) 3.1
0.6 0.6
assign (resid 8 and name HN  )(resid 7 and name HB1 ) 3.5
0.7 0.7
assign (resid 8 and name HN  )(resid 7 and name HB2 ) 3.4
0.7 0.7
```

```
assign (resid 4 and name HB1 )(resid 5 and name HN  ) 3.2
0.6 0.6
assign (resid 4 and name HB2 )(resid 5 and name HN  ) 3.2
0.6 0.6
assign (resid 6 and name HN  )(resid 5 and name HB# ) 3.4
0.7 0.7
assign (resid 5 and name HB# )(resid 4 and name HA  ) 4.3
0.9 0.9
assign (resid 2 and name HN  )(resid 1 and name HB1 ) 3.7
0.7 0.7
assign (resid 2 and name HN  )(resid 1 and name HB2 ) 3.9
0.8 0.8
assign (resid 2 and name HN  )(resid 3 and name HN  ) 4.0
0.8 0.8
assign (resid 2 and name HN  )(resid 1 and name HT# ) 4.5
0.9 0.9
assign (resid 4 and name HD* )(resid 5 and name HN  ) 4.3
0.9 0.9
assign (resid 2 and name HB# )(resid 3 and name HA  ) 4.4
0.9 0.9
assign (resid 2 and name HN  )((resid 1 and name HZ) or
(resid 1 and name HD#)) 4.6 0.9 0.9
!i-i+2
assign (resid 4 and name HN  )(resid 6 and name HA  ) 4.5
0.9 0.9
!i-i+3
assign (resid 4 and name HN  )(resid 7 and name HN  ) 4.4
0.9 0.9
assign (resid 4 and name HD* )((resid 1 and name HZ) or
(resid 1 and name HD#)) 4.4 0.9 0.9
set echo=true end

set wrnlev=5 end


! REMARK  error margins are set to conservative default
values of double the
! standard deviation observed in TALOS+, capped at a minimum
of +/-20 degree.
!phi
!calculated
assign (resid    1 and name C   )(resid    2 and name N    )
       (resid    2 and name CA  )(resid    2 and name C    )
1.0  -90.8    80.0 2
assign (resid    6 and name C   )(resid    7 and name N    )
       (resid    7 and name CA  )(resid    7 and name C    )
1.0 -101.6    60.0 2
!phi
!calc
assign (resid    4 and name C   )(resid    5 and name N    )
```

```
       (resid    5 and name CA  )(resid    5 and name C    )
1.0  -82.2    60.0 2
assign (resid    5 and name C   )(resid    6 and name N    )
       (resid    6 and name CA  )(resid    6 and name C    )
1.0  -90.8    60.0 2
assign (resid    7 and name C   )(resid    8 and name N    )
       (resid    8 and name CA  )(resid    8 and name C    )
1.0  -82.2    60.0 2
```

Mutacin I Ring A Ser Analogue

```
set echo=false end

set wrnlev=0 end
!Interresidue
!i-i+1
assign (resid 4 and name HA  )(resid 5 and name HN  ) 3.1
0.6 0.6
assign (resid 4 and name HN  )(resid 3 and name HA  ) 3.5
0.7 0.7
assign (resid 3 and name HN  )(resid 2 and name HA  ) 3.7
0.7 0.7
assign (resid 2 and name HN  )(resid 3 and name HN  ) 3.6
0.7 0.7
assign (resid 4 and name HN  )(resid 3 and name HN  ) 3.0
0.6 0.6
assign (resid 4 and name HN  )(resid 5 and name HN  ) 3.0
0.6 0.6
assign (resid 2 and name HB1 )(resid 3 and name HN  ) 3.0
0.6 0.6
assign (resid 2 and name HB2 )(resid 3 and name HN  ) 3.2
0.6 0.6
assign (resid 2 and name HN  )(resid 1 and name HB1 ) 3.2
0.6 0.6
assign (resid 4 and name HN  )(resid 3 and name HB2 ) 3.7
0.7 0.7
assign (resid 4 and name HN  )(resid 3 and name HA  ) 3.6
0.7 0.7
assign (resid 2 and name HD1#)(resid 3 and name HN  ) 4.3
0.9 0.9
assign (resid 2 and name HB2 )(resid 3 and name HN  ) 3.0
0.6 0.6
assign (resid 2 and name HB1 )(resid 3 and name HN  ) 2.9
0.6 0.6
assign (resid 4 and name HB# )(resid 5 and name HN  ) 3.9
0.8 0.8
assign (resid 2 and name HN  )(resid 1 and name HT# ) 4.0
0.8 0.8
assign (resid 4 and name HN  )(resid 3 and name HN  ) 3.0
0.6 0.6
```

```
assign (resid 4 and name HN  )(resid 5 and name HN  ) 3.0
0.6 0.6
assign (resid 2 and name HB1 )(resid 3 and name HA  ) 4.4
0.9 0.9
!i-i+2
assign (resid 5 and name HN  )(resid 3 and name HN  ) 3.3
0.7 0.7
!i-i+3
assign (resid 1 and name HB1 )(resid 4 and name HN  ) 4.2
0.8 0.8
assign (resid 2 and name HN  )(resid 5 and name HN  ) 4.4
0.9 0.9
assign (resid 6 and name HB# )(resid 3 and name HA  ) 4.2
0.8 0.8
assign (resid 4 and name HB# )(resid 1 and name HA  ) 4.4
0.9 0.9
set echo=true end

set wrnlev=5 end


! REMARK  error margins are set to conservative default
values of double
! remark the standard deviation observed in TALOS+, capped
at a minimum of +/-20 degree.
!phi
!calculated
assign (resid    1 and name C   )(resid    2 and name N    )
       (resid    2 and name CA  )(resid    2 and name C    )
1.0  -82.2    60.0 2
assign (resid    2 and name C   )(resid    3 and name N    )
       (resid    3 and name CA  )(resid    3 and name C    )
1.0  -82.2    60.0 2
assign (resid    3 and name C   )(resid    4 and name N    )
       (resid    4 and name CA  )(resid    4 and name C    )
1.0  -90.8    60.0 2
!phi
!calc
assign (resid    4 and name C   )(resid    5 and name N    )
       (resid    5 and name CA  )(resid    5 and name C    )
1.0  -58.2    60.0 2
assign (resid    5 and name C   )(resid    6 and name N    )
       (resid    6 and name CA  )(resid    6 and name C    )
1.0  -86.3    60.0 2
```

Mutacin I Ring A WT

```
set echo=false end

set wrnlev=0 end
```

```
!Interresidue
!i-i+1
assign (resid 4 and name HD2#)(resid 5 and name HN  ) 4.5
0.9 0.9
assign (resid 5 and name HN  )(resid 4 and name HB1 ) 3.7
0.7 0.7
assign (resid 6 and name HD2#)(resid 7 and name HN  ) 4.5
0.9 0.9
assign (resid 4 and name HN  )(resid 3 and name HB2 ) 3.4
0.7 0.7
assign (resid 3 and name HB1 )(resid 4 and name HN  ) 3.3
0.7 0.7
assign (resid 8 and name HN  )(resid 7 and name HB2 ) 3.4
0.7 0.7
assign (resid 8 and name HN  )(resid 7 and name HB1 ) 3.7
0.7 0.7
assign (resid 6 and name HA  )(resid 7 and name HN  ) 2.8
0.6 0.6
assign (resid 4 and name HA  )(resid 5 and name HN  ) 3.0
0.6 0.6
assign (resid 6 and name HA  )(resid 5 and name HN  ) 4.5
0.9 0.9
assign (resid 8 and name HN  )(resid 7 and name HN  ) 3.6
0.7 0.7
assign (resid 6 and name HN  )(resid 7 and name HN  ) 3.4
0.7 0.7
assign (resid 3 and name HN  )(resid 4 and name HN  ) 3.5
0.7 0.7
assign (resid 4 and name HN  )(resid 5 and name HN  ) 3.2
0.6 0.6
assign (resid 6 and name HN  )(resid 5 and name HN  ) 3.2
0.6 0.6
assign (resid 5 and name HB1 )(resid 6 and name HA  ) 4.6
0.9 0.9
assign (resid 3 and name HA  )(resid 2 and name HB1 ) 4.6
0.9 0.9
assign (resid 6 and name HG  )(resid 5 and name HB1 ) 4.5
0.9 0.9
assign (resid 6 and name HB1 )(resid 5 and name HB1 ) 4.6
0.9 0.9
assign (resid 6 and name HD2#)(resid 5 and name HB1 ) 4.9
1.0 1.0
assign (resid 2 and name HB1 )(resid 3 and name HB2 ) 4.8
1.0 1.0
assign (resid 8 and name HN  )(resid 7 and name HA  ) 2.8
0.6 0.6
assign (resid 3 and name HA  )(resid 4 and name HN  ) 3.3
0.7 0.7
assign (resid 6 and name HN  )(resid 7 and name HA  ) 4.4
0.9 0.9
```

```
assign (resid 6 and name HN  )(resid 5 and name HB1 ) 2.9
0.6 0.6
assign (resid 6 and name HN  )(resid 5 and name HB2 ) 3.5
0.7 0.7
assign (resid 3 and name HN  )(resid 2 and name HB1 ) 2.9
0.6 0.6
assign (resid 3 and name HN  )(resid 2 and name HB2 ) 3.4
0.7 0.7
assign (resid 3 and name HB1 )(resid 2 and name HB1 ) 4.7
0.9 0.9
assign (resid 6 and name HB# )(resid 7 and name HN  ) 4.3
0.9 0.9
assign (resid 8 and name HB# )(resid 7 and name HA  ) 4.6
0.9 0.9
assign (resid 6 and name HB# )(resid 7 and name HB2 ) 4.4
0.9 0.9
!i-i+2
assign (resid 5 and name HN  )(resid 3 and name HB2 ) 3.8
0.8 0.8
assign (resid 3 and name HB1 )(resid 5 and name HN  ) 3.7
0.7 0.7
assign (resid 3 and name HA  )(resid 5 and name HN  ) 3.9
0.8 0.8
assign (resid 6 and name HN  )(resid 4 and name HA  ) 4.1
0.8 0.8
assign (resid 7 and name HN  )(resid 5 and name HN  ) 3.8
0.8 0.8
!i-i+5
assign (resid 3 and name HB1 )(resid 7 and name HN  ) 3.5
0.7 0.7
assign (resid 3 and name HA  )(resid 7 and name HB1 ) 3.0
0.6 0.6
set echo=true end

set wrnlev=5 end


! REMARK  error margins are set to conservative default
values of double the
! standard deviation observed in TALOS+, capped at a minimum
of +/-20 degree.
!phi
!calc
assign (resid    2 and name C   )(resid    3 and name N    )
       (resid    3 and name CA  )(resid    3 and name C    )
1.0  -95.7    80.0 2
assign (resid    6 and name C   )(resid    7 and name N    )
       (resid    7 and name CA  )(resid    7 and name C    )
1.0  -95.7    80.0 2
assign (resid    7 and name C   )(resid    8 and name N    )
```

```
       (resid    8 and name CA  )(resid    8 and name C    )
1.0  -86.3    60.0 2
```

Nisin Ring A WT

```
set echo=false end

set wrnlev=0 end
!interresidue
!i-i+1
assign (resid 6 and name HN  )(resid 5 and name HB2) 3.1 0.6
0.6
assign (resid 6 and name HN  )(resid 5 and name HB1) 3.7 0.7
0.7
assign (resid 6 and name HN  )((resid 7 and name HN) or
(resid 4 and name HN)) 4.0 0.8 0.8
assign (resid 8 and name HN  )((resid 7 and name HN) or
(resid 4 and name HN)) 3.8 0.8 0.8
assign (resid 3 and name HN  )((resid 7 and name HN) or
(resid 4 and name HN)) 3.3 0.7 0.7
assign (resid 2 and name HB  )(resid 3 and name HN  ) 3.4
0.7 0.7
assign (resid 8 and name HN  )(resid 7 and name HA  ) 3.2
0.6 0.6
assign ((resid 7 and name HN) or (resid 4 and name HN))(resid
6 and name HA  ) 3.1 0.6 0.6
assign  ((resid  3  and  name  HB#)  or  (resid  7  and  name
HB2))(resid 8 and name HN  ) 3.9 0.8 0.8
assign (resid 8 and name HN  )(resid 7 and name HB1 ) 3.9
0.8 0.8
assign (resid 5 and name HB2 )(resid 6 and name HB# ) 4.8
1.0 1.0
!i-i+3
assign (resid 4 and name HG11)(resid 1 and name HT# ) 3.6
0.7 0.7
assign (resid 4 and name HG11)(resid 1 and name HA  ) 3.4
0.7 0.7
!i-i+4
assign (resid 7 and name HB1 )(resid 3 and name HA  ) 3.9
0.8 0.8
set echo=true end

set wrnlev=5 end


! REMARK  error margins are set to conservative default
values of double the
! standard deviation observed in TALOS+, capped at a minimum
of +/-20 degree.
!phi
```

```
!calc
assign (resid    2 and name C   )(resid    3 and name N    )
       (resid    3 and name CA  )(resid    3 and name C    )
1.0  -86.3    60.0 2
assign (resid    5 and name C   )(resid    6 and name N    )
       (resid    6 and name CA  )(resid    6 and name C    )
1.0  -95.7    80.0 2
assign (resid    7 and name C   )(resid    8 and name N    )
       (resid    8 and name CA  )(resid    8 and name C    )
1.0  -86.3    60.0 2
!pred
assign (resid    3 and name C   ) resid    4 and name N    )
       (resid    4 and name CA  )(resid    4 and name C    )
1.0 -105.6    60.0 2
```

Nisin Rings AB WT

```
set echo=false end

set wrnlev=0 end
!Interresidue
!i-i+1
assign (resid  4 and name HD1#)((resid  5 and name HN) or
(resid  6 and name HN)) 3.6 1.8 2.4
assign ((resid   5 and name HN) or (resid   6 and name
HN))(resid  4 and name HG11) 3.6 1.8 2.4
assign ((resid   5 and name HN) or (resid   6 and name
HN))(resid  4 and name HG12) 3.6 1.8 2.4
assign ((resid   5 and name HN) or (resid   6 and name
HN))(resid  4 and name HB  ) 2.9 1.1 1.2
!*assign ((resid   5 and name HN) or (resid   6 and name
HN))((resid  4 and name HG2#) or (resid  6 and name HD2#) or
(resid  1 and name HD1#)) 2.9 1.1 0.9
assign (resid  5 and name HB2 )(resid  6 and name HD1#) 3.6
1.8 2.4
assign (resid  5 and name HB1 )(resid  6 and name HD1#) 3.6
1.8 2.4
assign ((resid   3 and name HB1) or (resid   7 and name
HB1))(resid  2 and name HB  ) 3.6 1.8 2.4
assign ((resid  3 and name HN) or (resid 12 and name HN) or
(resid  8 and name HN))((resid  4 and name HG2#) or (resid
6 and name HD2#) or (resid  1 and name HD1#)) 3.6 1.8 2.4
!assign (resid  5 and name HB2 )((resid  6 and name HG) or
(resid  6 and name HB2) or (resid 12 and name HD#) or (resid
1 and name HG12)) 3.6 1.8 3.0
```

```
!assign (resid  5 and name HB1 )((resid  6 and name HG) or
(resid  6 and name HB2) or (resid 12 and name HD#) or (resid
1 and name HG12)) 3.6 1.8 2.4
assign ((resid  6 and name HD*) or (resid  4 and name HG2#)
or (resid  1 and name HD1#))(resid  3 and name HA  ) 3.6 1.8
2.4
assign (resid  7 and name HN)((resid  5 and name HN) or
(resid  6 and name HN)) 2.9 1.1 0.8
assign ((resid 12 and name HG1) or (resid  4 and name
HG12))((resid  1 and name HA) or (resid 11 and name HA)) 3.6
1.8 1.4
!assign ((resid  1 and name HA) or (resid 11 and name
HA))((resid  6 and name HG) or (resid  6 and name HB2) or
(resid 12 and name HD#) or (resid  1 and name HG12)) 3.6 1.8
1.4
!i-i+2
assign ((resid  7 and name HB1) or (resid  3 and name
HB1))((resid  5 and name HN) or (resid  6 and name HN)) 3.6
1.8 1.4
assign ((resid  3 and name HB2) or (resid 11 and name
HB1))((resid  5 and name HN) or (resid  6 and name HN)) 3.6
1.8 1.4
assign ((resid  3 and name HN) or (resid 12 and name HN) or
(resid  8 and name HN))((resid  5 and name HN) or (resid  6
and name HN)) 3.6 1.8 1.4
assign (resid  3 and name HA  )((resid  5 and name HN) or
(resid  6 and name HN)) 3.6 1.8 1.4
!assign ((resid  6 and name HG) or (resid  6 and name HB2)
or (resid 12 and name HD#) or (resid  1 and name HG12))((resid
4 and name HG12) or (resid 12 and name HG1)) 3.6 1.8 1.4
!i-i+3
!*assign ((resid  3 and name HN) or (resid 12 and name HN)
or (resid  8 and name HN))((resid  4 and name HG12) or (resid
12 and name HG1)) 2.9 1.1 1.2
assign ((resid  8 and name HG#) or (resid  1 and name
HG11))((resid 11 and name HB1) or (resid  3 and name HB2))
3.6 1.8 1.4
assign (resid 11 and name HN  )((resid  3 and name HN) or
(resid 12 and name HN) or (resid  8 and name HN)) 2.3 0.5
1.1
assign ((resid  3 and name HN) or (resid 12 and name HN) or
(resid  8 and name HN))((resid  7 and name HN) or (resid  4
and name HN)) 2.9 1.1 0.7
```

```
assign ((resid   7 and name HN) or (resid   4 and name
HN))(resid  3 and name HA  ) 3.6 1.8 1.4
!*assign ((resid   7 and name HN) or (resid   4 and name
HN))((resid  7 and name HB1) or (resid  3 and name HB1)) 2.9
1.1 0.8
!i-i+4
assign (resid  5 and name HB2 )((resid  9 and name HB1) or
(resid  6 and name HB#)) 3.6 1.8 1.4
assign (resid  3 and name HA  )((resid  7 and name HB2) or
(resid 12 and name HE#)) 3.6 1.8 1.4
!i-i+5
assign ((resid   8 and name HG#) or (resid   1 and name
HG11))(resid  7 and name HB2 ) 3.6 1.8 2.4
assign ((resid   8 and name HG#) or (resid   1 and name
HG11))(resid  7 and name HB1 ) 3.6 1.8 1.4
!i-i+6
assign ((resid   7 and name HN) or (resid   4 and name
HN))((resid  9 and name HB1) or (resid  6 and name HB#) or
(resid  2 and name HG#)) 3.6 1.8 1.4
assign (resid  7 and name HA  )(resid  9 and name HD2 ) 3.6
1.8 1.4
!*assign (resid  6 and name HA  )((resid  6 and name HD*) or
(resid  4 and name HG2#) or (resid  1 and name HD1#)) 2.9
1.1 0.8
!*assign ((resid  6 and name HB2) or (resid  2 and name HG#)
or (resid  9 and name HB1))((resid  6 and name HD*) or (resid
4 and name HG2#) or (resid  1 and name HD1#)) 2.9 1.1 0.9
!i-i+7
assign (resid  5 and name HB1 )(resid  6 and name HA  ) 3.6
1.8 2.0
assign (resid  5 and name HB2 )(resid  6 and name HA  ) 3.6
1.8 2.0
!i-i+9
assign (resid  4 and name HN)((resid  5 and name HN) or
(resid  6 and name HN)) 3.6 1.8 1.4

set echo=true end

set wrnlev=5 end


! REMARK  error margins are set to conservative default
values of double the
! standard deviation observed in TALOS+, capped at a minimum
of +/-20 degree.
! REMARK - all in allowed regions of Ramachandran plots.
```

```
! REMARK Leu phi borderline - may need to be removed
! REMARK Gly psi prediction borderline - have removed
!phi
!calc. gly has bigger error margin to ensure overlap with
prediction
assign (resid    3 and name C    ) (resid    4 and name N
)
       (resid    4 and name CA   ) (resid    4 and name C
)    1.0  -90.8    80.0 2
assign (resid    5 and name C    ) (resid    6 and name N
)
       (resid    6 and name CA   ) (resid    6 and name C
)    1.0  -86.3    80.0 2
assign (resid    9 and name C    ) (resid   10 and name N
)
       (resid   10 and name CA   ) (resid   10 and name C
)    1.0 -110.0    80.0 2
assign (resid   11 and name C    ) (resid   12 and name N
)
       (resid   12 and name CA   ) (resid   12 and name C
)    1.0  -95.7    80.0 2
```

Parameterisation of Unusual Amino Acids

New topology required for Dha, Dhb and α-aminobutyric acid, as well as patches to create the lanthionine and methyllanthionine bridges, were added to the XPLOR protein.top file:

```
!
! xplor-nih protein topology file, version 1.0
!

residue DHA
  group
    atom N   type=NDH charge=-0.56 end
    atom HN  type=HDH charge= 0.30 end
  group
    atom CA  type=CAD charge= 0.26 end
  group
    atom CB  type=CBA charge=-0.46 end
    atom HB1 type=CAH charge= 0.23 end
    atom HB2 type=CAH charge= 0.23 end
  group
    atom C   type=COD charge= 0.60 end
    atom O   type=ODH charge=-0.60 end

  bond N  HN
  bond N  CA
  bond CA  CB   bond CB  HB1   bond CB  HB2
  bond CA  C
```

```
  bond C   O

  improper CA CB C  N
  improper CB CA HB1 HB2
  improper HB1 CB CA C
  improper HB2 CB CA N

end

residue DHB
  group
    atom N    type=NDH charge=-0.56 end
    atom HN   type=HDH charge= 0.30 end
 group
    atom CA   type=CAD charge= 0.26 end
 group
    atom CB   type=CBB charge=-0.17 end
    atom HB   type=CBH charge= 0.17 end
 group
    atom CG   type=CGB charge=-0.30 end
    atom HG1  type=CGH charge= 0.10 end
    atom HG2  type=CGH charge= 0.10 end
    atom HG3  type=CGH charge= 0.10 end
 group
    atom C    type=COD charge= 0.60 end
    atom O    type=ODH charge=-0.60 end

  bond N  HN
  bond N  CA
  bond CA CB     bond CB HB
  bond CB CG     bond CG HG1    bond CG HG2    bond CG HG3
  bond CA C
  bond C  O

  improper CA    CB     C     N
  improper CB    CA     HB    CG
  improper HB    CB     CA    N
  improper CG    CB     CA    C

  improper HG1 HG2 CB HG3  !stereo methyl
  dihedral CG CB  CA  N

end

residue MEL    !! Abu for MeLan
  group
    atom N    type=NH1 charge=-0.36 end
    atom HN   type=H   charge= 0.26 end
 group
    atom CA   type=CT  charge= 0.00 end
    atom HA   type=HA  charge= 0.10 end
```

```
 group
    atom CB   type=CT  charge=-0.20 end
    atom HB1  type=HA  charge= 0.10 end
    atom HB2  type=HA  charge= 0.10 end
 group
    atom CG   type=CT  charge=-0.30 end
    atom HG1  type=HA  charge= 0.10 end
    atom HG2  type=HA  charge= 0.10 end
    atom HG3  type=HA  charge= 0.10 end
 group
    atom C    type=C   charge= 0.48 end
    atom O    type=O   charge=-0.48 end

  bond N  HN
  bond N  CA     bond CA HA
  bond CA CB     bond CB HB1      bond CB HB2
  bond CB CG     bond CG HG1     bond CG HG2     bond CG HG3
  bond CA C
  bond C  O

  improper HA N  C   CB        !stereo CA
  improper HB1 HB2 CA CG       !stereo CB
  improper HG1 HG2 CB HG3      !stereo methyl

  dihedral CG  CB  CA  N

end

presidue DISU      ! lanthionine bridge  ...CYS - S - CYS...
  group
    delete    atom 1HG               end
    modify    atom 1CB  charge=-0.15  type=CT      end
    modify    atom 1SG  charge=-0.10  type=S       end
  group
    delete    atom 2HG               end
    delete    atom 2SG               end
    modify    atom 2CB  charge=-0.15  type=CT      end

  add bond 1SG 2CB

  add angle 1CB 1SG 2CB
  add angle 1SG 2CB 2CA
  add angle 1SG 2CB 2HB1
  add angle 1SG 2CB 2HB2

  add improper 2HB1 2HB2 2CA 1SG

  ADD DIHEdral 1SG 2CB 2CA 2N

end
```

```
presidue MDIS  ! methyllanthionine bridge ...MEL - S - CYS...
  group
    delete    atom 1HB2              end
    modify    atom 1CB   charge=-0.10  type=CT      end
  group
    delete    atom 2HG             end
    modify    atom 2SG  charge=-0.10  type=S       end
    modify    atom 2CB  charge=-0.10  type=CT      end

  add bond 1CB 2SG

  add angle 1CB 2SG 2CB
  add angle 1CA 1CB 2SG
  add angle 2SG 1CB 1HB1
  add angle 2SG 1CB 1CG

  add improper 1HB1 1CA 2SG 1CG

  ADD DIHEdral 1CB 2SG 2CB 2CA

end
```

The required bond lengths, angles, impropers and nonbonded approximations for Dha and Dhb were added to the XPLOR protein.par file under the relevant sections:

```
!
! xplor-nih protein parameter file, version 1.0
!

! BONDS
!
! Bonds for Dha/Dhb
bond      CAD  CBA                $kbon     1.34
bond      CAD  CBB                $kbon     1.34
bond      CAD  COD                $kbon     1.489
bond      CAD  NDH                $kbon     1.39
bond      COD  NH1                $kbon     1.345
bond      C    NDH                $kbon     1.345
bond      NDH  HDH                $kbon     0.98
bond      COD  ODH                $kbon     1.231
bond      CBA  CAH                $kbon     1.08
bond      CBB  CBH                $kbon     1.08
bond      CBB  CGB                $kbon     1.53
bond      CGB  CGH                $kbon     1.08

! ANGLES
!
! Angles for Dha/Dhb
```

```
angle     CBA  CAD  COD          $kang     120.0
angle     CBB  CAD  COD          $kang     120.0
angle     NDH  CAD  CBA          $kang     128.0
angle     NDH  CAD  CBB          $kang     128.0
angle     NDH  CAD  COD          $kang     110.0
angle     CAD  CBA  CAH          $kang     120.5
angle     CAD  COD  ODH          $kang     122.5
angle     HDH  NDH  CAD          $kang     117.0
angle     CAD  CBB  CGB          $kang     126.5
angle     CT   C    NDH          $kang     116.5
angle     O    C    NDH          $kang     122.5
angle     C    NDH  HDH          $kang     123.0
angle     C    NDH  CAD          $kang     120.0
angle     CAD  COD  NH1          $kang     116.5
angle     ODH  COD  NH1          $kang     122.5
angle     COD  NH1  H            $kang     123.0
angle     COD  NH1  CT           $kang     120.0
angle     CAH  CBA  CAH          $kang     120.0
angle     CGH  CGB  CGH          $kang     109.5
angle     CBH  CBB  CGB          $kang     120.0
angle     CAD  CBB  CBH          $kang     120.0
angle     CBB  CGB  CGH          $kang     109.5

! IMPROPERS
!
! Impropers for Dha/Dhb
improper  CGH  CGH  CGB  CGH      $kchi    0   -66.514 ! Dhb
methyl
improper  HDH  NDH  CAD  COD      $kpla    0    0.0
improper  CBA  CAD  NDH  HDH      $kpla    0  180.0
improper  NDH  CAD  CBB  CBH      $kpla    0  180.0
improper  NDH  CAD  CBB  CGB      $kpla    0    0.0
improper  HDH  NDH  CAD  CBB      $kpla    0  180.0
improper  CBH  CBB  CAD  NDH      $kpla    0  180.0
improper  CT   C    NDH  HDH      $kpx     0    0.0
improper  CAD  COD  NH1  H        $kpx     0    0.0
improper  O    C    NDH  HDH      $kpla    0  180.0
improper  O    C    NDH  CAD      $kpx     0    0.0
improper  ODH  COD  NH1  H        $kpla    0  180.0
improper  ODH  COD  NH1  CT       $kpx     0    0.0
improper  CAD  COD  NH1  CT       $kpx     0  180.0
improper  COD  CAD  NH1  CT       $kback   0  180.0  !  cis
pep bond
improper  COD  CAD  CBA  CAH      $kpla    0  180.0
improper  NDH  CAD  CBA  CAH      $kpla    0  180.0
improper  C    NDH  CAD  COD      $kpla    0  180.0
improper  CBB  CAD  COD  ODH      $kpla    0  180.0
improper  CBA  CAD  COD  ODH      $kpla    0  180.0
improper  NH1  CT   C    NDH      $kpla    0  180.0
improper  NH3  CT   C    NDH      $kpla    0  180.0
improper  CT   C    NDH  CAD      $kpx     0  180.0
```

```
improper  C    CT   NDH  CAD      $kback   0  180.0  !  cis
pep bond
improper  CGB  CBB  CAD  COD      $kpla    0  180.0
improper  NDH  CAD  COD  ODH      $kpla    0    0.0
improper  C    NDH  CAD  CBA      $kpla    0    0.0
improper  C    NDH  CAD  CBB      $kpla    0    0.0
improper  NDH  CAD  COD  NH1      $kpla    0  180.0
improper  COD  NH1  CT   C        $kpla    0  180.0
improper  CBB  CAD  COD  NH1      $kpla    0    0.0
improper  CBA  CAD  COD  NH1      $kpla    0    0.0
improper  CAD  CBB  CGB  CGH      $kpla    0    0.0  !  dhb
C(O)-gammaH eclipsed
improper  COD  CAD  NDH  ODH      $kpla    0    0.0     !
planar around CO
improper  C    CT   O    NDH      $kpla    0    0.0     !
planar around CO
improper  NDH  C    HDH  CAD      $kpla    0    0.0     !
planar around NH
improper  CAD  NDH  COD  CBA      $kpla    0    0.0
improper  CBA  CAH  CAH  CAD      $kpla    0    0.0
improper  COD  NH1  ODH  CAD      $kpla    0    0.0
improper  NH1  COD  H    CT       $kpla    0    0.0
improper  CAD  NDH  CBA  COD      $kpla    0    0.0
improper  COD  CAD  ODH  NH1      $kpla    0      0.0
improper  COD  CAD  NH1  ODH      $kpla    0    0.0
improper  CAD  CBA  COD  NDH      $kpla    0    0.0
improper  CAD  CBB  COD  NDH      $kpla    0    0.0
improper  CGH  CGH  CBB  CGH      $kchi    0  -66.514    !
betaC-gammaH
improper  CBA  CAD  CAH  CAH      $kpla    0    0.0
improper  CAD  CBA  COD  NH1      $kpla    0    0.0
improper  CAD  CBB  COD  NH1      $kpla    0    0.0
improper  CBB  CAD  CBH  CGB      $kpla    0    0.0
improper  COD  NH1  CBA  CAH      $kpla    0    0.0

!NONBONDED
!
!nonbonded  approximations  based  on  existing  types  in
protein.par (C=C of Dh approximated as CA)
nonbonded  NDH     0.1592   2.7618      0.1592   2.7618
nonbonded  ODH     0.2342   2.6406      0.2342   2.6406
nonbonded  COD     0.0903   3.2072      0.0903   3.2072
nonbonded  HDH     0.0498   1.4254      0.0498   1.4254
nonbonded  CGH     0.0045   2.6157      0.0045   2.6157
nonbonded  CAH     0.0045   2.6157      0.0045   2.6157
nonbonded  CBH     0.0045   2.6157      0.0045   2.6157
nonbonded  CAD     0.120    3.2072      0.120    3.2072
nonbonded  CBA     0.120    3.2072      0.120    3.2072
nonbonded  CBB     0.120    3.2072      0.120    3.2072
nonbonded  CGB     0.0903   3.2072      0.0903   3.2072
```

Example Scripts Run in XPLOR-NIH

The following example scripts were run for the structure calculation of mutacin I ring B.

1. generate_psf.inp

```
remarks  file generate/csa_generate.inp
 remarks  Generate structure file and hydrogens for MutIB
 remarks  based on csa_generate file

 topology
    @TOPPAR:protein_edit.top          {*Read standard all-h
topology*}
                                             {*file.
*}
 end

parameter
   @TOPPAR:protein.par                      {*Read all-h
parameters, append.*}

 end

 segment
   name="    "
   chain                                   {*Definition for
peptide linkage.*}
     @TOPPAR:toph19.pep
     sequence  CYS LEU GLY CYS THR end
   end
 end

 patch LTOD                                {*Make D-cys.*}
    reference=nil=( resid 1 )
 end

 patch DISU
    reference=1=( residue 1  ) reference=2=( residue 4 )
end
 write structure output=Mut1B_new1.psf end   {*This writes
the structure file.*}

 stop
```

2. generate_template.inp

```
remarks  file  nmr/generate_template.inp
remarks  Generates a "template" coordinate set.  This
produces
```

```
remarks  an arbitrary extended conformation with ideal
geometry.
remarks  Author: Axel T. Brunger

{====>}
structure @Mut1B_new1.psf end
{*Read structure file.*}

parameter
{====>}
   @TOPPAR:protein.par
{*Read parameters.*}
end

topology
   @TOPPAR:protein_edit.top
end

{====>}
                {*If protein contains S-S bridges,
appropriately modify and*}
                {*then uncomment the following lines.
*}
          {*Make D-cys.*}
!patch DISU reference=1=( residue 1  ) reference=2=(
residue 4  ) end

!patch LTOD reference=nil=( resid 1 ) end

vector do (x = random() * 10) (all)
vector do (y = random() * 10) (all)
vector do (z = random() * 10) (all)

vector do (fbeta=50) (all)                 {*Friction
coefficient, in 1/ps.*}
vector do (mass=100) (all)                          {*Heavy
masses, in amus.*}

parameter
   nbonds
      cutnb=5.5 rcon=20. nbxmod=-2 repel=0.9  wmin=0.1
tolerance=1.
      rexp=2 irexp=2 inhibit=0.25
   end
end

flags exclude * include bond end
mini powell nstep 100 nprint 10 end

flags exclude * include bond angle end
mini powell nstep 500 nprint 10 end
```

```
flags exclude * include bond angle impr end
mini powell nstep 500 nprint 10 end

param nbonds repel 0.8 rcon 0.01 wmin 0.0 end end
flags exclude * include bond angle impr vdw end
mini powell nstep 500 nprint 10 end

dynamics  verlet
   nstep=50  timestep=0.001 iasvel=maxwell  firsttemp= 300.
   tcoupling = true  tbath = 300.   nprint=50  iprfrq=0
end

parameter
   nbonds
      rcon=2. nbxmod=-3 repel=0.75
   end
end

minimize powell nstep=100 nprint=25 end

dynamics  verlet
   nstep=500  timestep=0.005 iasvel=maxwell  firsttemp=
300.
   tcoupling = true  tbath = 300.   nprint=100  iprfrq=0
end

flags exclude vdw elec end
vector do (mass=1.) ( name h* )
hbuild selection=( name h* ) phistep=360 end
flags include vdw elec end

minimize powell nstep=200 nprint=50 end

{*Write coordinates.*}
remarks produced by nmr/generate_template.inp
write coordinates output=generatetemplate.pdb end

stop
```

3. sa.inp

```
remarks file  nmr/sa.inp
remarks  Simulated annealing protocol for NMR structure
determination.
remarks  The starting structure for this protocol can be
any structure with
remarks  a reasonable geometry, such as randomly assigned
torsion angles or
remarks  extended strands.
remarks  Author: Michael Nilges
```

```
{====>}
evaluate ($init_t = 1000 )       {*Initial simulated
annealing temperature.*}
{====>}
evaluate ($high_steps= 6000 )         {*Total number of
steps at high temp.*}
{====>}
evaluate ($cool_steps = 3000 )      {*Total number of steps
during cooling.*}

parameter                                          {*Read the
parameter file.*}
{====>}
   @TOPPAR:protein.par
end

topology
   @TOPPAR:protein_edit.top
end

{====>}
structure @Mut1B_new1.psf end                      {*Read
the structure file.*}

{====>}
coordinates @generatetemplate.pdb                   {*Read
the coordinates.*}

noe
{====>}
   nres=1000             {*Estimate greater than the actual
number of NOEs.*}
   class all
{====>}
   @noe_nointra.tbl                            {*Read NOE
distance ranges.*}
end

{====>}
restraints

   dihedral

   reset
   @dihe_final.tbl                         {*Read dihedral
angle restraints.*}
end

{====>}
```

```
               {*If protein contains S-S bridges,
appropriately modify and *}
               {*then uncomment the following lines. The S-
S covalent bonds*}
               {*will be represented as fake NOE distances.
*}
!noe
!   assign (resid  51 and name sg) (resid 54 and name sg)
2.02 0.1 0.1
!   assign (resid  14 and name sg) (resid 38 and name sg)
2.02 0.1 0.1
!   assign (resid 30 and name sg) (resid 51 and name sg)
2.02 0.1 0.1
!end

flags exclude * include bonds angle impr vdw noe cdih end

                          {*Friction coefficient for MD
heatbath, in 1/ps.   *}
vector do (fbeta=10) (all)
                          {*Uniform heavy masses to speed
molecular dynamics.*}
vector do (mass=100) (all)

noe                               {*Parameters for NOE
effective energy term.*}
  ceiling=1000
  averaging  * cent
  potential  * soft
  scale      * 50.
  sqoffset   * 0.0
  sqconstant * 1.0
  sqexponent * 2
  soexponent * 1
  asymptote  * 0.1                          {*Initial value-
-modified later.*}
  rswitch    * 0.5
end

parameter                         {*Parameters for the
repulsive energy term.*}
    nbonds
      repel=1.                     {*Initial value for repel-
-modified later.*}
      rexp=2 irexp=2 rcon=1.
      nbxmod=3
      wmin=0.01
      cutnb=4.5 ctonnb=2.99 ctofnb=3.
      tolerance=0.5
   end
end
```

```
restraints dihedral
      scale=5.
end

{====>}
evaluate ($end_count=100)          {*Loop through a family
of 10 structures.*}

coor copy end

evaluate ($count = 0)
while ($count < $end_count ) loop main

   evaluate ($count=$count+1)

   coor swap end
   coor copy end

     {* ============================================
Initial minimization.*}
   restraints dihedral   scale=5.   end
   noe asymptote * 0.1  end
   parameter  nbonds repel=1.   end end
   constraints interaction
           (not name SG) (all) weights * 1  vdw 0.002 end
end
   minimize powell nstep=50 drop=10.  nprint=25 end

     {* ========================================= High-
temperature dynamics.*}
   constraints interaction (not name SG) (all)
               weights * 1  angl 0.4  impr 0.1 vdw 0.002
end end

   evaluate ($nstep1=int($high_steps * 2. / 3. ) )
   evaluate ($nstep2=int($high_steps * 1. / 3. ) )

   dynamics  verlet
      nstep=$nstep1   timestep=0.005   iasvel=maxwell
firstt=$init_t
      tcoupling=true  tbath=$init_t  nprint=50  iprfrq=0
   end

     {* ============== Tilt the asymptote and increase
weights on geometry.*}
   noe asymptote * 1.0  end

   constraints interaction
              (not name SG) (all) weights * 1  vdw 0.002
end end
```

```
   dynamics  verlet
      nstep=$nstep2   timestep=0.005    iasvel=current
tcoupling=true
      tbath=$init_t  nprint=50  iprfrq=0
   end

    {* ================================================
Cool the system.*}

   restraints dihedral   scale=200.   end

   evaluate ($final_t = 100)      { K }
   evaluate ($tempstep = 50)      { K }

   evaluate ($ncycle = ($init_t-$final_t)/$tempstep)
   evaluate ($nstep = int($cool_steps/$ncycle))

   evaluate ($ini_rad  = 0.9)        evaluate ($fin_rad  =
0.75)
   evaluate ($ini_con=  0.003)       evaluate ($fin_con=
4.0)

   evaluate ($bath  = $init_t)
   evaluate ($k_vdw = $ini_con)
   evaluate ($k_vdwfact = ($fin_con/$ini_con)^(1/$ncycle))
   evaluate ($radius=    $ini_rad)
   evaluate ($radfact = ($fin_rad/$ini_rad)^(1/$ncycle))

   evaluate ($i_cool = 0)
   while ($i_cool < $ncycle) loop cool
      evaluate ($i_cool=$i_cool+1)

      evaluate ($bath  = $bath  - $tempstep)
      evaluate ($k_vdw=min($fin_con,$k_vdw*$k_vdwfact))
      evaluate ($radius=max($fin_rad,$radius*$radfact))

      parameter  nbonds repel=$radius   end end
      constraints interaction (not name SG) (all)
                     weights * 1. vdw $k_vdw end end

      dynamics  verlet
         nstep=$nstep time=0.005 iasvel=current
firstt=$bath
         tcoup=true tbath=$bath nprint=$nstep iprfrq=0
      end

{====>}
{*Abort condition.*}
      evaluate ($critical=$temp/$bath)
      if ($critical >  10. ) then
```

```
         display  ****&&&& rerun job with smaller timestep
(i.e., 0.003)
         stop
      end if

   end loop cool

   {* ================================================
Final minimization.*}

   constraints interaction (all) (all) weights * 1. vdw 1.
end end
   parameter
      nbonds
         repel=0.80
         rexp=2 irexp=2 rcon=1.
         nbxmod=3
         wmin=0.01
         cutnb=6.0 ctonnb=2.99 ctofnb=3.
         tolerance=1.5
      end
   end

   minimize powell nstep=1000 drop=10.0 nprint=25 end

   {* ================================== Write out the
final structure(s).*}
   print threshold=0.5 noe
   evaluate ($rms_noe=$result)
   evaluate ($violations_noe=$violations)
   print threshold=5. cdih
   evaluate ($rms_cdih=$result)
   evaluate ($violations_cdih=$violations)
   print thres=0.05 bonds
   evaluate ($rms_bonds=$result)
   print thres=5. angles
   evaluate ($rms_angles=$result)
   print thres=5. impropers
   evaluate ($rms_impropers=$result)
   remarks
============================================================
====
   remarks
overall,bonds,angles,improper,vdw,noe,cdih
   remarks energies: $ener, $bond, $angl, $impr, $vdw,
$noe, $cdih
   remarks
============================================================
====
   remarks            bonds,angles,impropers,noe,cdih
```

```
   remarks rms-d:
$rms_bonds,$rms_angles,$rms_impropers,$rms_noe,$rms_cdih
   remarks
=============================================================
====
   remarks               noe,  cdih
   remarks violations.: $violations_noe, $violations_cdih
   remarks
=============================================================
====

{====>}                         {*Name(s) of the family of
final structures.*}
   evaluate ($filename="sa_"+encode($count)+".pdb")

   write coordinates output =$filename end

end loop main

stop
```

4. refine_1.inp

```
remarks file nmr/refine.inp -- Simulated annealing
refinement
remarks           for NMR structure determination
remarks  Authors: Michael Nilges, John Kuszewski, and Axel
T. Brunger

{====>}
evaluate ($init_t = 1000)            {*Initial annealing
temperature, in K.*}
{====>}
evaluate ($cool_steps = 2000 )      {*Total number of steps
during cooling.*}

parameter                                          {*Read the
parameter file.*}
{====>}
   @TOPPAR:protein.par
end

{====>} structure @Mut1B_new1.psf end
{*The structure file.*}

noe
{====>}
   nres=1000             {*Estimate greater than the actual
number of NOEs.*}
   class all
{====>}
```

```
   @noe_nointra.tbl                          {*Read NOE
distance ranges.*}
end

{====>}
restraints

   dihedral

   reset
   @dihe_final.tbl    {*Read dihedral angle restraints.*}
end
                            {*Friction coefficient for MD
heatbath, in 1/ps.*}
vector do (fbeta=10) (all)
vector do (mass=100) (all)      {*Heavy masses to speed
molecular dynamics.*}

noe                             {*Parameters for NOE
effective energy term.*}
   ceiling=1000
   averaging  * cent
   potential  * square
   sqconstant * 1.
   sqexponent * 2
   scale      * 50.           {*Constant NOE scale
throughout the protocol.*}
end

parameter                       {*Parameters for the
repulsive energy term.*}
   nbonds
      repel=0.5                  {*Initial value for repel-
-modified later.*}
      rexp=2 irexp=2 rcon=1.
      nbxmod=3
      wmin=0.01
      cutnb=4.5 ctonnb=2.99 ctofnb=3.
      tolerance=0.5
   end
end

restraints dihedral
        scale=200.
end

{====>}
evaluate ($end_count=100)          {*Loop through a family
of 10 structures.*}

evaluate ($count = 0)
```

```
while ($count < $end_count ) loop main

   evaluate ($count=$count+1)

{====>}                              {*Filename(s) for
embedded coordinates.*}
   evaluate ($filename="sa_"+encode($count)+".pdb")

   coor @@$filename

   flags exclude * include bond angl impr vdw noe cdih end

   vector do (vx=maxwell($init_t)) ( all )
   vector do (vy=maxwell($init_t)) ( all )
   vector do (vz=maxwell($init_t)) ( all )

   evaluate ($final_t = 100)     { K }
   evaluate ($tempstep = 50)     { K }

   evaluate ($ncycle = ($init_t-$final_t)/$tempstep)
   evaluate ($nstep = int($cool_steps/$ncycle))

   evaluate ($ini_rad  = 0.9)        evaluate ($fin_rad  =
0.75)
   evaluate ($ini_con=  0.003)       evaluate ($fin_con=
4.0)

   evaluate ($bath  = $init_t)
   evaluate ($k_vdw = $ini_con)
   evaluate ($k_vdwfact = ($fin_con/$ini_con)^(1/$ncycle))
   evaluate ($radius=    $ini_rad)
   evaluate ($radfact = ($fin_rad/$ini_rad)^(1/$ncycle))

   evaluate ($i_cool = 0)
   while ($i_cool < $ncycle) loop cool
      evaluate ($i_cool=$i_cool+1)

      evaluate ($bath  = $bath  - $tempstep)
      evaluate ($k_vdw=min($fin_con,$k_vdw*$k_vdwfact))
      evaluate ($radius=max($fin_rad,$radius*$radfact))

      parameter  nbonds repel=$radius   end end
      constraints interaction (all) (all) weights * 1. vdw
$k_vdw end end

      dynamics  verlet
         nstep=$nstep time=0.005 iasvel=current
firstt=$bath
         tcoup=true tbath=$bath nprint=$nstep iprfrq=0
      end
```

```
{====>}
{*Abort condition.*}
      evaluate ($critical=$temp/$bath)
      if ($critical >  10. ) then
         display  ****&&&& rerun job with smaller timestep
(i.e., 0.003)
         stop
      end if
   end loop cool

   {* ================================================
Final minimization.*}
   minimize powell nstep= 200 nprint=25 end

   {* ================================== Write out the
final structure(s).*}
   print threshold=0.5 noe
   evaluate ($rms_noe=$result)
   evaluate ($violations_noe=$violations)
   print threshold=5. cdih
   evaluate ($rms_cdih=$result)
   evaluate ($violations_cdih=$violations)
   print thres=0.05 bonds
   evaluate ($rms_bonds=$result)
   print thres=5. angles
   evaluate ($rms_angles=$result)
   print thres=5. impropers
   evaluate ($rms_impropers=$result)
   remarks
============================================================
====
   remarks
overall,bonds,angles,improper,vdw,noe,cdih
   remarks energies: $ener, $bond, $angl, $impr, $vdw,
$noe, $cdih
   remarks
============================================================
====
   remarks            bonds,angles,impropers,noe,cdih
   remarks rms-d:
$rms_bonds,$rms_angles,$rms_impropers,$rms_noe,$rms_cdih
   remarks
============================================================
====
   remarks               noe,  cdih
   remarks violations.: $violations_noe, $violations_cdih
   remarks
============================================================
====

{====>}     {*Name(s) of the family of final structures.*}
```

```
   evaluate ($filename="refine_1_"+encode($count)+".pdb")

   write coordinates output =$filename end

end loop main

stop
```

5. refine_2.inp

```
remarks file nmr/refine.inp  -- Simulated annealing
refinement
remarks             for NMR structure determination
remarks  Authors: Michael Nilges, John Kuszewski, and Axel
T. Brunger

{====>}
evaluate ($init_t = 1000)            {*Initial annealing
temperature, in K.*}
{====>}
evaluate ($cool_steps = 2000 )      {*Total number of steps
during cooling.*}

parameter                                          {*Read the
parameter file.*}
{====>}
   @TOPPAR:protein.par
end

{====>} structure @Mut1B_new1.psf end
{*The structure file.*}

noe
{====>}
   nres=1000             {*Estimate greater than the actual
number of NOEs.*}
   class all
{====>}
   @noe_nointra.tbl                          {*Read NOE
distance ranges.*}
end
{====>}
restraints

   dihedral

   reset
   @dihe_final.tbl                       {*Read dihedral
angle restraints.*}
end
```

```
                              {*Friction coefficient for MD
heatbath, in 1/ps.*}
vector do (fbeta=10) (all)
vector do (mass=100) (all)      {*Heavy masses to speed
molecular dynamics.*}

noe                             {*Parameters for NOE
effective energy term.*}
   ceiling=1000
   averaging  * cent
   potential  * square
   sqconstant * 1.
   sqexponent * 2
   scale      * 50.           {*Constant NOE scale
throughout the protocol.*}
end

parameter                       {*Parameters for the
repulsive energy term.*}
   nbonds
      repel=0.5                  {*Initial value for repel-
-modified later.*}
      rexp=2 irexp=2 rcon=1.
      nbxmod=3
      wmin=0.01
      cutnb=4.5 ctonnb=2.99 ctofnb=3.
      tolerance=0.5
   end
end

restraints dihedral
        scale=200.
end

{====>}
evaluate ($end_count=100)          {*Loop through a family
of 10 structures.*}

evaluate ($count = 0)
while ($count < $end_count ) loop main

   evaluate ($count=$count+1)

{====>}                             {*Filename(s) for
embedded coordinates.*}
   evaluate ($filename="refine_1_"+encode($count)+".pdb")

   coor @@$filename

   flags exclude * include bond angl impr vdw noe cdih end
```

```
   vector do (vx=maxwell($init_t)) ( all )
   vector do (vy=maxwell($init_t)) ( all )
   vector do (vz=maxwell($init_t)) ( all )

   evaluate ($final_t = 100)     { K }
   evaluate ($tempstep = 50)     { K }

   evaluate ($ncycle = ($init_t-$final_t)/$tempstep)
   evaluate ($nstep = int($cool_steps/$ncycle))

   evaluate ($ini_rad  = 0.9)        evaluate ($fin_rad  =
0.75)
   evaluate ($ini_con=  0.003)       evaluate ($fin_con=
4.0)

   evaluate ($bath  = $init_t)
   evaluate ($k_vdw = $ini_con)
   evaluate ($k_vdwfact = ($fin_con/$ini_con)^(1/$ncycle))
   evaluate ($radius=    $ini_rad)
   evaluate ($radfact = ($fin_rad/$ini_rad)^(1/$ncycle))
   evaluate ($i_cool = 0)
   while ($i_cool < $ncycle) loop cool
      evaluate ($i_cool=$i_cool+1)

      evaluate ($bath  = $bath  - $tempstep)
      evaluate ($k_vdw=min($fin_con,$k_vdw*$k_vdwfact))
      evaluate ($radius=max($fin_rad,$radius*$radfact))

      parameter  nbonds repel=$radius   end end
      constraints interaction (all) (all) weights * 1. vdw
$k_vdw end end

      dynamics  verlet
         nstep=$nstep time=0.005 iasvel=current
firstt=$bath
         tcoup=true tbath=$bath nprint=$nstep iprfrq=0
      end

{====>}
{*Abort condition.*}
      evaluate ($critical=$temp/$bath)
      if ($critical >  10. ) then
         display  ****&&&& rerun job with smaller timestep
(i.e., 0.003)
         stop
      end if
   end loop cool

   {* ================================================
Final minimization.*}
   minimize powell nstep= 200 nprint=25 end
```

```
   {* ================================= Write out the
final structure(s).*}
   print threshold=0.5 noe
   evaluate ($rms_noe=$result)
   evaluate ($violations_noe=$violations)
   print threshold=5. cdih
   evaluate ($rms_cdih=$result)
   evaluate ($violations_cdih=$violations)
   print thres=0.05 bonds
   evaluate ($rms_bonds=$result)
   print thres=5. angles
   evaluate ($rms_angles=$result)
   print thres=5. impropers
   evaluate ($rms_impropers=$result)
   remarks
============================================================
====
   remarks
overall,bonds,angles,improper,vdw,noe,cdih
   remarks energies: $ener, $bond, $angl, $impr, $vdw,
$noe, $cdih
   remarks
============================================================
====
   remarks            bonds,angles,impropers,noe,cdih
   remarks rms-d:
$rms_bonds,$rms_angles,$rms_impropers,$rms_noe,$rms_cdih
   remarks
============================================================
====
   remarks               noe,  cdih
   remarks violations.: $violations_noe, $violations_cdih
   remarks
============================================================
====

{====>}                        {*Name(s) of the family of
final structures.*}
   evaluate ($filename="refine_2_"+encode($count)+".pdb")

   write coordinates output =$filename end

end loop main

stop
```

6. accept.inp

```
remarks file  nmr/accept.inp
remarks  Analysis of a family of NMR structures--
```

```
remarks  generation of a subfamily of "acceptable"
structures

parameter                    {*Read the parameter file.*}
{====>}
   @TOPPAR:protein.par
end

{====>}
    structure @Mut1B_new1.psf end                {*Read
the structure file.*}

noe
{====>}
   nres=1000             {*Estimate greater than the actual
number of NOEs.*}
   class all
{====>}
   @noe_nointra.tbl         {*Read NOE distance ranges.*}
end
{====>}
restraints

   dihedral

   reset
   @dihe_final.tbl                     {*Read dihedral
angle restraints.*}
end

noe                             {*Parameters for NOE
effective energy term.*}
   ceiling=1000
   averaging  * cent
   potential  * square
   sqconstant * 1.
   sqexponent * 2
   scale      * 50.
end

parameter                       {*Parameters for the
repulsive energy term.*}
   nbonds
      repel=0.75
      rexp=2 irexp=2 rcon=4.
      nbxmod=3
      wmin=0.01
      cutnb=4.5 ctonnb=2.99 ctofnb=3.
      tolerance=0.5
   end
end
```

```
restraints dihedral
      scale=200.
end

flags exclude * include bonds angle impr vdw noe cdih end

set precision=4 end

{====>}
evaluate ($end_count=15)          {*Loop through a family
of 10 structures.*}

evaluate ($accept_count = 0)
evaluate ($count = 0)
while ($count < $end_count ) loop main
   evaluate ($count=$count+1)
{====>}                              {*Filename(s) for
embedded coordinates.*}
   evaluate ($filename="refine_1_"+encode($count)+".pdb")

   coor @@$filename

   evaluate ($accept=0)
                                {*Print all NOE violations
larger than 0.3 A *}
                                {*and compute RMS difference
between observed*}
                                {*and model distances. *}
   print threshold=0.5 noe
   evaluate ($rms_noe=$result)
   evaluate ($violations_noe=$violations)
   if ($violations_noe > 0) then  evaluate (
$accept=$accept + 1)   end if

            {*Print all dihedral angle restraint*}
                              {*violations. *}
   print threshold=5. cdih
   evaluate ($rms_cdih=$result)
   evaluate ($violations_cdih=$violations)
   if ($violations_cdih > 0) then  evaluate (
$accept=$accept + 1)   end if

   print thres=0.05 bonds         {*Print deviations from
ideal geometry.*}
   evaluate ($rms_bonds=$result)
   if ($result > 0.01) then  evaluate ( $accept=$accept +
1)   end if

   print thres=5. angles
   evaluate ($rms_angles=$result)
```

```
   if ($result > 1) then  evaluate ( $accept=$accept + 1)
end if

   print thres=5. impropers
   evaluate ($rms_impropers=$result)

   distance from=( not hydrogen ) to=( not hydrogen )
cutoff=1.5 end

               {*Acceptance criteria: no NOE violations
greater than 0.5 A,*}
               {*no dihedral angle restraint violations > 5
deg,           *}
               {*rms difference for bond deviations from
ideality < 0.01 A,*}
               {*rms difference for angle deviations from
ideality < 2 deg.*}
   energy end

   if ($accept = 0 ) then
      evaluate ($accept_count=$accept_count+1)

{====>}
      evaluate
($filename="accept_"+encode($accept_count)+".pdb")
      remarks
============================================================
=
      remarks            overall,bonds,angles,vdw,noe,cdih
      remarks energies: $ener, $bond, $angl, $vdw, $noe,
$cdih, $impr
      remarks
============================================================
=
      remarks            bonds, angles, impropers, noe,
cdih
      remarks rms-d:
$rms_bonds,$rms_angles,$rms_impropers,$rms_noe,$rms_cdih
      remarks
============================================================
=
      remarks               noe,  cdih
      remarks violations.: $violations_noe,
$violations_cdih

      write coordinates output=$filename end
   end if

end loop main

stop
```

APPENDIX II – STRUCTURE STATISTICS TABLE

Statistics were generated by the Protein Structure Validation Suite (PSVS).[289]

Percentage of residues in Ramachandran disallowed regions is from Procheck analysis.[291]

	Mutacin I ring B WT		Nisin ring B WT (97)	Nisin ring B Lan analogue (98)	Mutacin I ring A WT (99)	Mutacin I ring A Ser analogue (102)	Mutacin I ring A Ser/Ala analogue (101)	Nisin ring A WT (100)	Nisin rings AB WT (95)
	Major (96)	Minor (96)							
Distance Restraints									
Inter-residue NOE									
i - i+1	11	3	15	8	32	19	22	11	11
i – i>1	7	1	10	7	7	5	3	3	17
Dihedral Angles									
Backbone	5	4	3	3	3	5	5	4	4
Structure Statistics									
Pairwise RMSD (Å)									
Heavy atoms	0.6	1.2	0.8	0.8	1.2	0.8	1.8	1.7	1.3
Backbone atoms	0.1	0.6	0.2	0.2	0.5	0.3	1.2	1.0	0.9
RMSD from ideality									
Bond length (Å)	0.007	0.005	0.010	0.010	0.010	0.004	0.007	0.005	0.005
Bond angle (°)	1.7	1.5	1.5	1.5	1.9	1.7	1.5	1.5	1.3
% of residues in Ramachandran disallowed regions	0	0	0	0	0	0	6.7	6.7	0