Hybrid Lantibiotics: Combining Synthesis and Biosynthesis

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Dedicated to my parents with gratitude for giving me their love and having faith in me

Without the Mercy and Blessings of our Sustainer we can achieve nothing.

I thank the Almighty Allah for having granted me the health, strength, and time to complete this Eng.D.

Declaration

I, Begum Mothia, hereby state that the following is entirely my own work and has not been submitted for any other degree or examination.

Begum Mothia

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Abstract

The synthesis of two sets of different orthogonally protected lanthionine ready for incorporation into solid phase peptide synthesis to form cyclised peptides is described in this thesis, along with the cyclisation of individual rings D and E and the overlapping rings D and E.

Previously developed orthogonally protected lanthionine containing Aloc, allyl, Fmoc and *t*Bu protecting groups was synthesised using published synthetic route developed by Tabor's group. A novel orthogonally protected lanthionine containing Teoc, TMSE, Fmoc and Tce group derivative has also been synthesised, after carrying several synthetic pathways. Both lanthionine residues contain protecting groups which are orthogonal to each other, which are also orthogonal to the transient Fmoc and permanent Boc/*t*Bu protecting groups which are used in Fmoc based solid phase peptide synthesis.

Incorporation of the previously developed lanthionine with Aloc/allyl protecting groups was carried out to form an analogue of ring E of nisin for the first time. Deprotection of the Aloc/allyl protecting groups were carried out with $Ph(PPh_3)_4$ using *N',N*-dimethyl-barbituric acid (NDMBA).

The second orthogonally protected lanthionine was also incorporated into solid phase peptide synthesis to synthesise an analogue of ring D of nisin. This was also to see whether this can be used to synthesise lanthionine-containing thio-ether bridged cyclic peptide by solid phase peptide synthesis. Teoc and TMSE deprotection was carried out in the presence of TBAF without effecting the other side chain and Fmoc protecting groups. Full characterisation of individual rings D and E were obtained.

Quadruply orthogonal protecting group strategy was used to synthesise bicyclic peptide with two overlapping lanthionine bridges rings D and E. An effective methodology has been developed for the synthesis of the overlapping rings D and E of nisin by solid phase peptide synthesis.

Contents

Abbreviation

δ	- Chemical shift in a NMR spectrum
A_2pr	- 2,3-diaminopropionic acid
AgCN	- Silver cyanide
Ala, A	- Alanine
Aloc	- Allyloxycarbonyl
AOP	- (7-Azabenzotriazole-1-yloxy)-tris(dimethylamino)-phosphonium hexafluorophosphate
Arg, R	- Arginine
Asn, N	- Asparagine
Asp, D	- Aspartic acid
ATP	- Adenosine 5'-triphosphate
AviCys	- 2-Aminovinylcysteine
AviMeCys	- methyl-2-aminovinylcysteine
B	- Base
BDP	- benzotriazole-l-yl diethyl phosphate
Boc	- *tert*-Butoxycarbonyl
BOP	- (Benzotriazol-1-yloxy)-tris (dimethylamino)-phosphonium hexafluorophosphate
t-Bu	- *tert*-Butyl
bd	- Broad doublet
BrCN	- Cyanogen bromide
bs	- Broad singlet
bm	- Broad multiplet
Bn	- Benzyl
bt	- Broad triplet
Bzl, Bn	- Benzyl
Cbz	- Benzyloxycarbonyl
CD	- Circular dichoroism
CF	- Anionic Carboxyfluorescein
COSY	- Correlated spectroscopy
CPK	- Creatine phosphokinase
Cys, C	- Cysteine

d - Doublet

Da - Dalton

dd - Doublet of doublet

DCC - *N,N'*-Dicyclohexylcarbodiimide

DCM - Dichloromethane

DCU - Dicyclohexyl urea

Dde - 1-(4,4-Dimethyl-2,6-dioxocyclohex-1-ylidine)-ethyl

Ddz - α, α,-dimethyl-3,5-dimethoxybenyloxycarbonyl

DEAD - Diethyl azodicarboxylate

DEPT - Distortionless Enhancement by Polarisation Transfer

Dha - 2,3-dehydroalanine

Dhb - 2,3-dehydrobutyrine

DIC - *N,N'*-Diisopropylcarbodiimide

DIPEA - *N,N'*-Diisopropylethylamine

DMAP - Dimethylamino pyridine

Dmb - 2,4-dimethoxybenzyl

DMF - *N,N*-dimethylformamide

DMSO - Dimethylsulphoxide

DNA - Deoxyribonucleic acid

DOPC - Dioleoylphosphatidylcholine

DOPG - Dioleoylphosphatidylglycerol

DPC - Dodecylphosphocholine

Dpr - Diaminopropionic acid

DQ - Design qualification

EDC -1,2-Dichloroethane

EDCI - 3-[Cyano(ethyl)amino]propyl-dimethyl azanium chloride

EDT -1,2-Ethanediol

EDTA - Ethylenediaminetetraacetic acid

EI - electron impact

EtOAc - Ethyl acetate

EtOH - Ethanol

ESP - Electrospray

ESP^+ - Electrospray (positive) ionisation

Equiv - Equivalence

FAB - Fast atom bombardment

Fmoc - 9-fluorenylmethoxycarbonyl

Gln, Q - Glutamine

Glu, E - Glutamic acid

Gly, G - Glycine

HBr - Hydrogen bromide

HBTU - *N*-[1 *H*-benzotriazole-1-yl)-(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophophate *N*-oxide

HF - Hydrofluoric acid

His, H - Histidine

HMBC - Heteronulear Multiple-Bond Correlation

HSQC - Heteronulear Single-Quantum Correlation

^{1}H NMR - Proton nuclear magnetic resonance

HOAt - 1- Hydroxy-7-azabenzotriazole

HOBt - 1-Hydroxybenzotriazole

HPLC - High performance liquid chromatography

HyAsp - 3-hydroxyaspartic acid

Ile, I - Isoleucine

IQ - Installation qualification

IR - Infra-red

ivDde - 1-(4,4-Dimethyl-2,6-dioxocyclo-hexylidene)-3-methylbutyl

J - Coupling constant

Lan - Lanthionine

Lan*B* - Lantibiotic gene cluster } A(I) lantibiotic

Lan*C* - Lantibiotic gene cluster } A(I) lantibiotic } Modification enzymes

Lan*M* - Lantibiotic gene cluster A(II) lantibiotic } Modification enzymes

LC-MS - Liquid Chromatography-Mass Spectrometry

Leu, L - Leucine

Lys, K - Lysine

LysN-Ala - Lysinoalanine

m - Multiplet

MeLan - Methyllanthionine

Mesyl - Methanesulfonyl

Met, M	- Methionine
MHP	- Molecular hydrophobicity potential
MIC	- Minimum inhibitory concentration
Ms	- Methanesulfonyl
mmol	- Millimole
MRSA	- Methicillin resistant *Staphylococcus aureus*
NDMBA	- 1,3-dimethylbarbituric acid
NMM	- *N*-Methylmorpholine
NMR	- Nuclear Magnetic Resonance
NOE	- Nuclear Overhauser Effect
OPOE	- *n*-octyl-polyoxyethylene
OQ	- Operational qualification
PC	- Phosphatidylcholine
PCOR	- Peptide cyclisation on oxime resin
PCS	- Plastic composite support discs
PD	- Process design
$(Pd(PPh_3)_4$	- Tetrakis(triphenylphosphine)Palladium (0) catalyst
PEG	- Polyethylene glycol
PG	- Phosphatidylglycerol
Phe, F	- Phenylalanine
Pro, P	- Proline
PQ	- Performance Qualification
PQ	- Process Qualification
PTC	- Phase transfer catalysis
PyAOP	- (7-Azabenzotriazol-1-yloxy)-tris(pyrrolidino)-phosphonium hexafluorophosphate
PyBOP	- (Benzotriazole-1-yloxy)-tris(pyrrolidino)-phosphonium hexafluorophosphate
RP	- Reverse phase
R_T	- Retention time
s	- Singlet
SAR	- Structure activity relationship
SDS	- Sodium dodecyl sulphate
Ser, S	- Serine
SES	- 2-Trimethylsilanyl-ethanesulfonic acid amide

SES-Cl	- 2-Trimethylsilanyl-ethanesulfonyl chloride
SPE	- Solid Phase Extraction
SPPS	- Solid phase peptide synthesis
t	- Triplet
*t*Bu	- *tert*-Butyl
TBAB	- Tetrabutyl ammonium bromide
TBAF	- *tetra*-*N*-Butyl ammonium fluoride
TBAHS	- Tetrabytul ammonium hydrogen sulfate
Tce	- 2,2,2-Trichloroethyl ester
Tce-OH	- 2,2,2-Trichloroethanol
TEA	- Triethylamine
Teoc	- β (trimethylsilyl)ethoxycarbonyl
Teoc-ONp	- 4-nitrophenyl 2-(trimethylsilyl)ethyl carbonate
Teoc-ONSu	- 1-[2-(Trimethylsilyl)ethoxycarbonyloxy]pyrrolidin-2,5-dione
TES	- Triethylsilane
TFA	- Trifluroacetic acid
THF	- Tetrahydrofuran
Thr, T	- Threonine
TIPS, TIS	- Triisopropylsilane
TLC	- Thin layer chromatography
TMS	- Trimethylsilyl
TMSCl	- Trimethylsilyl chloride
TMSE	- 2-trimethylsilanyl-ethyl
TOCSY	- Total correlation Spectroscopy
Tosyl	- toluenesulfonyl
Trityl	- Triphenylmethyl
Trp, W	- Tryptophan
Trt	- Triphenylmethyl
Tyr, Y	- Tyrosine
UV	- Ultra-violet
Val, V	- Valine

List of Figures

List of Tables

List of Schemes

List of Appendices

1. Introduction

Diseases caused by bacterial infection have always played a major part in human history, and continue to be a threat even in the 21st century. Mortality due to bacterial infections has been decreased dramatically by improved hygiene and the use of antibiotics. However, bacteria evolving resistance to antibiotics through mutations is a major problem, making many drugs rapidly obsolete and this makes the search for new antibiotics with novel mechanisms a constant challenge.

There are large numbers of antibiotics known; an important class of antibiotics are peptidic in nature. These include: vancomycin, which is used in serious and life-threatening infections by Gram-positive bacteria; [1] polymyxin, used for serious Gram-negative infections; and the lantibiotic nisin, which is one of the few used as a food preservative. [2]

The lantibiotics are a family of thio-ether bridged antimicrobial peptides produced by Gram positive bacteria. Their name is indicative of the fact that they contain the amino acid lanthionine. [3, 4] Many of these peptides show antibiotic activity against Gram-positive bacteria and anaerobic prokaryotes, [5] believed to be mediated through a variety of mechanisms.

1.1. Overview of lantibiotics

1.1.1. Unusual amino acids

All lantibiotics contain one or more of the unusual, double headed amino acid lanthionine (Lan) **1**. Incorporation of a lanthionine residue into a peptide sequence results in a peptide with a thio-ether linkage between the side-chain of two alanine residues. In lantibiotics a cyclic peptide structure with a side-chain to side-chain thio-ether bridge is formed (Figure 1 shows the cyclised ring C of nisin). The unique properties of lantibiotics, such as conformational constraints and stability to degradation by proteases result from these thio-ether bridges. In most lantibiotics the lanthionine residues have the *meso*-stereochemistry (Figure 1).

Most lantibiotics also contain *threo*-β-methyl lanthionine (MeLan) **2**. They also typically but not always contain the modified amino acids 2, 3-didehydroalanine **3** (Dha), 2, 3-didehydrobutyrine **4** (Dhb), 2-aminovinylcysteine **5** (AviCys), methyl-2-aminovinylcysteine **6** (AviMeCys), lysinoalanine **7** (LysN-Ala) and 3-hydroxyaspartic acid **8** (HyAsp) (Figure 1).

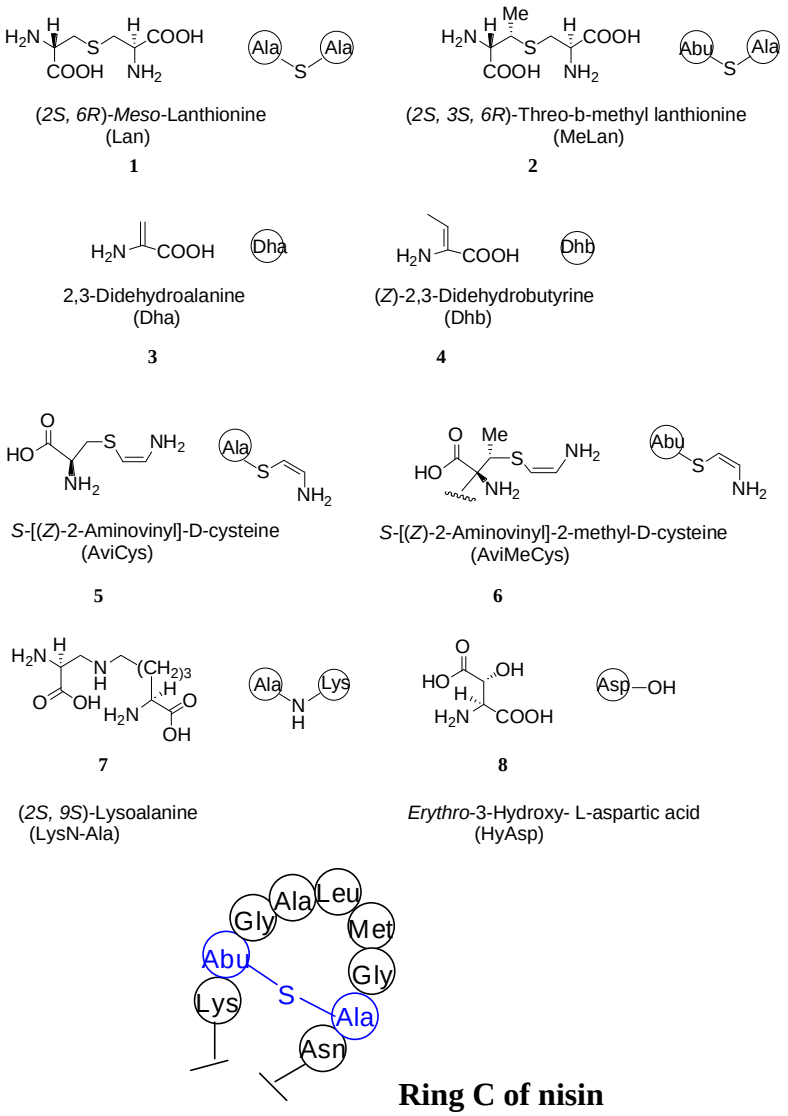


Figure 1: Structures of unusual amino acids Lan, MeLan, Dha, Dhb, AviCys, AviMeCys, LysN-Ala, HyAsp and a cyclic peptide.

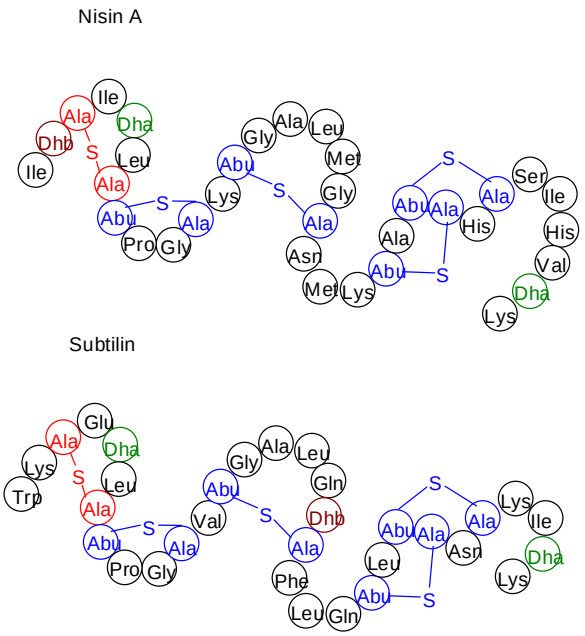
Studies of a range of lantibiotics confirm that the unusual amino acids play a key role in the stability and activity of these antibiotic peptides. It has been demonstrated that by mutagenesis of the lantibiotic encoding genes the structures of lantibiotics can be modified and that this may lead to profound changes in activity. For example, the replacement of the dehydroamino acids with the corresponding saturated amino acids can lead to a significant reduction of the antibacterial activity. [6] Thus the antibacterial activity of these lantibiotics are at least in part due to the electrophilic centres of the dehydroamino acids which can readily react with nucleophilic groups present in bacterial DNA and enzymes, and without these unusual amino acids these lantibiotics do not possess significant activity. [7, 8]

1.1.2. Structure and mode of action

To date more than 50 lantibiotics have been isolated from Gram positive bacteria and they show a variety of structures, sizes, and, modes of action. Based on the topology of lantibiotic ring structures and the biological activity, Jung [9] classified the lantibiotics as Type A and Type B.

Type A lantibiotics (Figure 2) such as nisin, subtilin, nukacin, Pep5, Epidermin and lacticin are flexible, elongated amphipathic screw-shaped cationic peptides, and their lanthionine bridges have similar arrangements. [3, 10] They are primarily active against Gram-positive bacteria strains, however when ion chelaters such as EDTA or citrate [11, 12] are used to disrupt the outer membrane Gram-negative bacteria are also affected. [13] Type A lantibiotics such as nisin form pores in the biological membrane and have a positive charge overall.

Type A lantibiotics



Type A lantibiotics continued

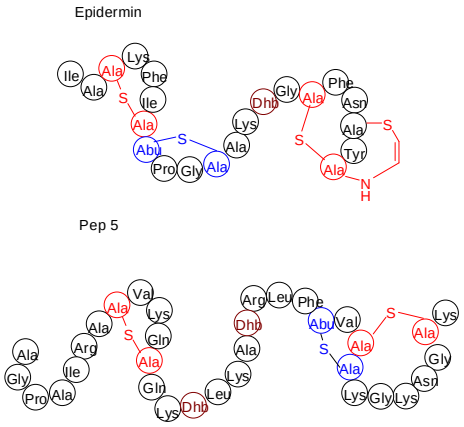


Figure 2: [3] **Simplified representative structures of Type A lantibiotics.**

The Type B lantibiotics include mersacidin, actagardine, lacticin 481 and cinnamycin (Figure 3). These lantibiotics are more globular and compact in structure, with a greater number of overlapping thio-ether bridges. At pH 7 they have no net charge or negative charge. [3] Like Type A lantibiotics, they are also active against a range of Gram-positive bacteria. They are not active against Gram negative bacteria, due to these peptides being unable to pass the outer membrane of bacteria and unable to penetrate the cell membrane. [13]

Type B lantibiotics

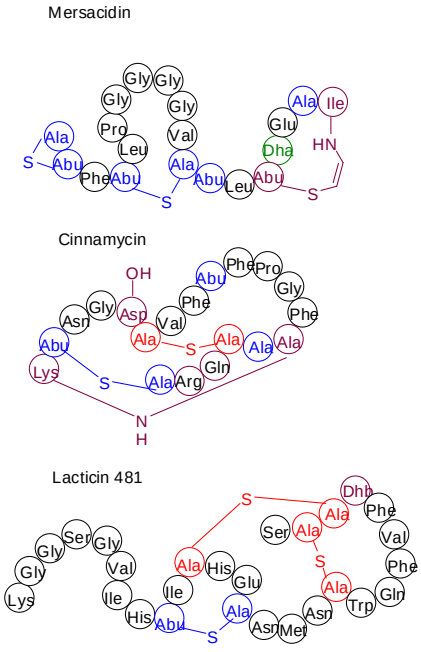


Figure 3: [3] **Simplified representative structures of Type B lantibiotics.**

In total, there are 5 broad subclasses of lantibiotics depending on the differences in the chemistry and biosynthesis. Based on the structures, Type A lantibiotic are further divided into three subtypes, namely Type A (I), Type A (II) and A (III), whilst Type B consists of two component lantibiotics, lantibiotics that are globular and compact in structure and lantibiotics of unknown structures. [14]

1.1.3. Type A (I) and Type A (II) lantibiotics

Type A (I) lantibiotic leader sequences have a high percentage of charged amino acids which are generally hydrophilic. They have a net negative or a slightly positive charge. [15] The formation of Lan and MeLan residues occurs by the action of two distinct enzymes (*LanB* and *LanC*) which lead to the dehydration of Ser and Thr residues and cyclisation to form the thioether bridges (Section 1.1.4). The presence of *LanB* and *LanC* enzymes classifies the lantibiotic as Type A (I) lantibiotic and an example of Type A (I) lantibiotic is nisin. [3]

Type A (II) lantibiotic leader sequences tend to comprise highly negative net charges and unlike Type A (I) they possess consensus sequences. [15] Formation of Lan and MeLan are catalysed by a single enzyme (*LanM*) in Type A (II) lantibiotics. An example of a Type A (II) lantibiotic is Lactocin. [3]

Table 1 shows a list of Gram positive bacteria and the lantibiotics they produce. It can be seen that similarities can occur between different lantibiotics; for example nisin and epidermin have ring sizes which are similar and located near the amino terminal, whereas ring B is completely conserved. This sequence conservation can be used to distinguish further sub-categorisations. Thus conservation of rings, high charges and the *N*-terminal blocked with hydroxyl-, butyryl-, or propionyl groups categorises Pep 5 and Epidicin 280 within the same subgroup.

Lantibiotic	Producer strain	Lan	MeLan	Dha	Dhb	Other	Amino acids	Type	Ref
Nisin A	*Lactococcus lactis*	1	4	2	1	-	34	**A (I)**	16
Nisin Z	*Lactococcus lactis*	1	4	2	1	-	34	**A (I)**	17
Subtilin	*Bacillus subtilis*	1	4	2	1	-	32	**A (I)**	18
Epidermin	*Staphylococcus epidermis*	2	1	0	1	AviCys	22	**A (I)**	19
Gallidermin	*Staphylococcus gallinarium*	2	1	0	1	AviCys	22	**A (I)**	20
Mutacin 1140	*Streptococcus mutans*	2	1	1	1	AviCys	22	**A (I)**	21
Mutacin B-Ny266	*Streptococcus mutans*	2	1	1	1	AviCys	22	**A (I)**	22
Mutacin III	*Streptococcus mutans*	2	1	1	1	AviCys	22	**A (I)**	23
Mutacin I	*Streptococcus mutans*	3	0	2	0	AviCys	24	**A (I)**	24
Pep 5	*Staphylococcus epidermis*	2	1	0	2	D-Ala, 2-Oxobutyryl	34	**A (I)**	25
Epicidin 280	*Staphylococcus epidermis*	1	2	0	1	2-Hydroxypropionyl	30	**A (I)**	26
Epilancin K7	*Staphylococcus epidermis*	2	1	2	2	2-Hydroxypropionyl	31	**A (I)**	27
Lacticin 481	*Lactococcus lactis*	2	1	0	1	-	27	**A (II)**	28
Lactocin S	*Lactobacillus sakei*	2	0	0	1	2-oxopropionyl	37	**A (II)**	29
Mutacin II	*Streptococcus mutans*	2	1	0	1	-	27	**A (II)**	30
Variacin	*Micrococcus varians*	2	1	0	1	-	25	**A (III)**	31
Salivaricin A	*Streptococcus salivarius*	1	2	0	0	-	22	**A (II)**	32
Streptococcin A-FF22	*Streptococcus pyogenes*	1	2	0	1	-	26	**A (II)**	33
Cypemycin	*Streptomyces*	0	0	0	4	AviCys, $AlaMe_2$	22	**A (II)**	34
Plantaricin C	*Lactobacillus plantarum*	1	3	1	0	-	27	**A (II)**	35
Butyrivibriocin OR79A	*Butyrivibrio fibriosolvens*	1	2	0	1	-	25	**A (II)**	36
Cinnamycin	*Streptomyces cinnamoneus*	1	2	0	0	Asp-OH, LysNAla	19	**B**	37
Duramycin	*Streptoverticillium hachijoense*	1	2	0	0	Asp-OH, LysNAla	19	**B**	38
Duramycin B	*Streptoverticillium*	1	2	0	0	Asp-OH, LysNAla	19	**B**	39
Duramycin C	*Streptomyces griseoluteus*	1	2	0	0	Asp-OH, LysNAla	19	**B**	39
Ancovenin	*Streptomyces sp.*	1	2	1	0		19	**B**	40
Mersacidin	*Bacillus sp. Strain*	0	3	1	0	AviMeCys	20	**B**	41
Actagardine	*Actinoplanes linguriae*	1	2	0	0	MeLan- Sulfoxide	19	**B**	42
Ala-(0)-actagardine	*Actinoplanes linguriae*	1	2	0	0	MeLan- Sulfoxide	20	**B**	5
Lacticin 3147A1	*Lactococcus lactis*	2	2	0	2	D-Ala	30	**Two-component lantibiotics**	43
Lacticin 3147A2	*Lactococcus lactis*	1	2	0	2	D-Ala x2, 2-oxoproionyl	29	**Two-component**	43

Table 1: [3] **Examples of Gram positive bacteria with the lantibiotics produced, the number of amino acids, number of Lan and MeLan and other amino acids produced and the category.**

1.1.4. Lantibiotic biosynthesis

The structural genes for the lantibiotics encode linear prepeptides that consist of two parts: a leader peptide and a prolantibiotic. The biosynthetic pathway of a lantibiotic involves the ribosomal synthesis of the prepeptide consisting of the *N*-terminal leader sequence followed by a prolantibiotic part. [42] The prolantibiotic contains only the 20 proteinogenic amino acids, which may then undergo extensive posttranslational modification by specific enzymes.

Dehydration of serine and threonine residues introduces α,β-unsaturated amino acid residues that may participate in intramolecular Michael-type additions with cysteine to form thio-ether cross links. [44] Dha and Dhb are vulnerable to nucleophilic addition and addition of the thiol groups of cysteine [45] generates Lan **1** and MeLan **2** respectively (Figure 4). There are occasions where the cysteine residues located at the *C*-terminus of the lantibiotic may oxidise and decarboxylate followed by the nucleophilic addition of its thiol group to Dha and Dhb. This leads to forming 2- aminovinyl-D-cysteine (AviCys) as in the case of epidermin. [45] The active form is generated upon cleavage of a leader peptide. [46]

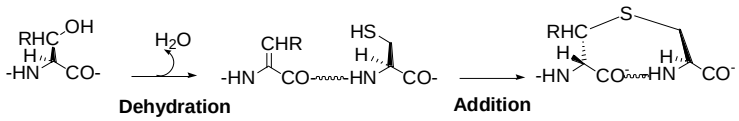


Figure 4: Dehydration of serine and threonine followed by addition with the thiol during biosynthesis. Serine is transformed into Dha when R = H and threonine is transformed into Dhb when R = Me. [47]

Lan is the generic symbol given to lantibiotic genes; however a more specific name is given for some individual lantibiotics such as *Nis* for nisin. The 57 amino acid prepeptide for nisin is ribosomally synthesised, encoded for by the *NisA* gene (Figure 5). *NisB* catalyses the dehydration of the underlined Ser and Thr residues to Dha and Dhb. Thio-ether bonds are formed by Michael addition of the appropriate cysteine residue giving five of the Dha (green)

and Dhb (magenta) residues, producing the five characteristic lanthionine rings, one of which is cross-linked by lanthionine (red) and the other four methyllanthionines (blue). [2] The *NisC* enzyme catalyses the conjugate addition of Cys residues to Dha and Dhb in a regio- and stereo-specific manner. Once the dehydration and cyclisation is complete, the leader peptide is removed proteolytically by *NisP* protease (Figure 5). [48]

Please see: A. W. van der Donk; *J. Org. Chem.;* 2006, *71,* 9561 to see Figure 5.

Figure 5: Posttranslational maturation process of lantibiotic. Picture adapted from Van der Donk. [48]

Similarly to most other biosynthetic pathways in bacteria, the genes for nisin biosynthesis are clustered (Figure 6) and it has been found that this cluster consists of 11 genes. [49] During lantibiotic biosynthesis a leader peptide is produced that contains multi-residue extension known as the *N*-terminal, this is followed by a *C*-terminal propeptide moiety which undergoes enzymatic modifications to give the mature lantibiotic. The modifications involve the formation of unusual amino acids, dehydration of serine and threonine residues to Dha and Dhb, cyclisations of Dha and Dhb residues forming the thio-ether rings and cleavage of

the leader peptide. [3, 10] *NisA* genes encodes for the 57 amino acid prepeptide. *NisB* gene is involved in the dehydration of serine and threonine to Dha and Dhb whilst *NisC* gene catalyses the ring formation by conjugate addition of cysteine residues to these dehydro amino acids. *NisIFEG* is concerned with self immunity, protecting the organism producing nisin from its toxic effects. *NisK* and *NisR* genes are regulatory genes which consist of a two component sensory system which are important for regulation of lantibiotic production. *NisT* an ATP-binding cassette (ABC) transporter is involved in the secretion of the final product whilst protease *NisP* is responsible for the removal of the leader peptide. [3, 45]

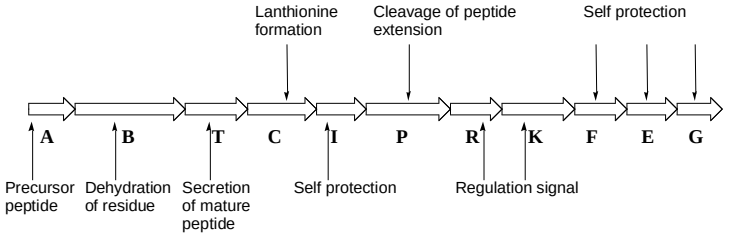


Figure 6: Roles of genes in lantibiotic biosynthesis.

1.1.4.1. Classifications of antimicrobial peptides

Classification of the antimicrobial peptides depends on the biosynthetic mechanism of ribosomally synthesised peptides or bacteriocins. Depending on the biochemical and genetic properties there are up to four Classes. [50, 51] Class I and Class II bacteriocins are membrane active peptides that are cationic and amphiphilic as small as 3-10 kDa. Unusual amino acids, lanthionine and methyllanthionine are contained within Class I lantibiotics or bacteriocins. Modification of Class I bacteriocin are engineered by two enzymes *LanB* and *LanC* and an example of a Class I bacteriocins is the most studied lantibiotic nisin. Class II bacteriocins only contain one enzyme *LanM* which catalyses dehydration and cyclisation. Class II bacteriocins are subdivided into further three groups: i) Class IIa which contains the conserved sequence Y-G-N-G-V-X-C near the *N* terminus; ii) Class IIb which consists of two lantibiotic peptides and both are required for antimicrobial activity; iii) Class IIc are peptides that are thiol activated. [50] Examples of Class II lantibiotics are mercasidin, actagardine and two component lantibiotics such as cytolysin. Class III lantibiotics are larger, with molecular masses > 30 kDa and have other functions rather than just antimicrobial activity such as

being heat-labile proteins. [3] Class IV lantibiotics are more complex peptides which contain carbohydrate or lipid moieties which are important for activity. [52]

1.1.4.2. *LanB* and *LanC* enzymes

LanA is present in all lantibiotics which encode the ribosomally synthesised precursor peptides known as the prepeptide. The prepeptide is biologically inactive and carry the leader sequence, containing 23-29 amino acids which are unaffected during post-translational modification. The leader sequences play a role in protection by keeping the antibiotic inactive within the cell and act as a recognition sequence for the biosynthetic enzymes.

LanB encode proteins as large as approximately 1000 residues that are hydrophilic by nature and have some hydrophobicity. *LanB* has been reported to play a key role; in nisin production, the secretion of nisin only takes place when *NisB* is detected. It was found that when nisin variants: [Trp30] nisin and [Lys27, Lys31] nisin were produced by strains, Ser33 was not transformed to Dha33 by dehydration in 50 % of the total peptide produced. [53] However fully modified nisin was produced when *NisB* was cloned and overexpressed in these strains indicating there was complete transformation of Ser to Dha in the mature peptide. [54] This shows the importance of *NisB* in dehydration of Ser residues.

Products of *LanC* gene range from 398 (PepC) to 455 (EpiC) amino acids. The key role of this enzyme is the formation of thio-ether bridges after the dehydration of the prelantibiotic. Koponen *et al*. [55] illustrated the first direct evidence of *NisC* being responsible for the cyclisation of thio-ether rings. They formed mutant strains of *L. lactis* lacking the *NisB* and *NisC* genes. They used a precursor peptide of nisin which was tagged with histidine to ensure simple purification of the final peptide. It was discovered that no dehydration or cyclisation took place when the tagged prepeptide was exposed to a strain lacking *NisB*. Whereas when the same tagged prepeptide was used in a strain lacking *NisC*, a dehydrated propeptide was engineered however cyclised peptide was not obtained.

1.1.4.3. *LanM* enzymes

LanB or *LanC* are not present in the gene clusters of class II lantibiotics such as lactosin S, mersacidin, lacticin 481, actagardine, and a two component lantibiotic cytolysin. *LanM* present in class II lantibiotics encodes proteins of 900 - 1000 amino acid. The *C*-terminal of *LanM* proteins shows similarity to the *C*-terminal of the *LanC* proteins. However there is no similarity in the *N*-terminal of *LanM* with that of the *N*-terminal of *LanB*. This indicated that the possibility for the origin of *LanM* existing from the fusion of *LanB* and *LanC* was unlikely. Due to other enzymes not being present in the gene clusters for catalysing the posttranslational modification, this showed that *LanM* was responsible for both dehydration and cyclisation reactions. [56] This was confirmed when the production of the mature lantibiotic was prevented when the *LctM* was disrupted in lacticin.

1.1.4.4. Two - component lantibiotics

Lacticin 3147 is a two component lantibiotic, containing two *LtnM* genes which is an unusual feature of the gene cluster. The presence of two *LtnM* genes is unique; it can be assumed that one *LtnM* protein is responsible for the dehydration while the second is responsible for the formation of thio-ether rings similar to *LanB* and *LanC*. This however is made unlikely due to the absence of the second enzyme in lactosin S, cytolysin and lacticin 481. Lacticin is composed of two small peptides 3-4 kDa, where *LtnA1* and *LtnA2* genes are proposed as candidates for the structural genes of lacticin 3147. McAuliffe *et al*. [57] demonstrated for activities each prepeptide require separate modification enzymes. They demonstrated that *LtnM1* is responsible for modifying *LtnA1* peptide, whereas *LtnM2* modifies *LtnA2* peptide. There is no similarity in the sequence of lacticin 3147 and cytolysin peptides however it can be seen from the protein sequence that *LtnA1* and *LtnA2* are closely related to staphylococcin C55 components. Also compared to lacticin 3147 and staphylococcin C55 the peptide components in cytolysin are closely related to each other. This may be why lacticin 3147 require two *LanM* modification enzymes whilst cytolysin requires one *LanM* proteins.

1.2. Nisin

Nisin is a lantibiotic produced by *Lactococcus lactis*. It is a 34 residue pentacyclic peptide with a molecular weight of 3353 Da, with 13 residues post-translationally modified (Figure 7). Nisin was discovered one year prior to penicillin [58] in 1928 [59, 60] although the structure was only elucidated in 1971. [16] It has been used as a food preservative for many years and is part of a Type A lantibiotic which is elongated and shares similar characteristics with other antimicrobial peptides. Effective inhibition of Gram-positive bacteria was carried out by inhibiting bacterial cell wall synthesis. This was due to nisin interfering with energy transduction at the cytoplasmic membrane. [15] Gram-negative bacteria inhibition also took place. Generally nisin does not have bactericidal effect on the growth of Gram negative organisms or intact Gram negative organisms such as *Salmonella*. This is due to the outer membrane preventing access of the hydrophobic substances to the petidoglycan layer. However chemical agents like EDTA [12] disrupt the outer lipopolysaccharide membrane which allows nisin to enter the membrane where lipid II is present. This leads to nisin inhibiting Gram negative bacteria. Nisin also inhibits the outgrowth of spores of *Bacilli* and *Clostridium*, this activity is believed to take place by nucleophilic attack from Cys residues on Dha5 which results in the modification of a target on the spore. [3, 61] During spore outgrowth, nisin targets the thiol groups on the exterior of spores from *Bacillus cereus* interfering with modifications of sulfhydryl groups. [62] Nisin has been used as a potential antibacterial agent in health care products, in the pharmaceutical and veterinary sectors. [63]

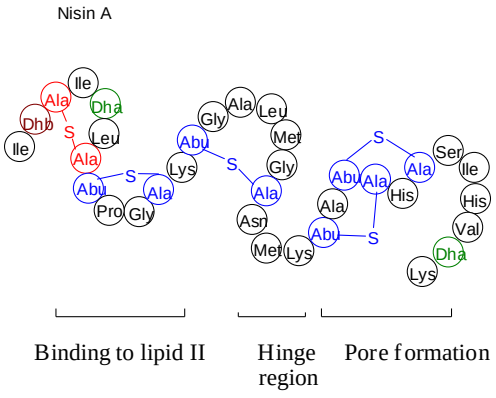


Figure 7: [3] **Structure of nisin.**

1.2.1. Nisin Isoforms

Nisin **A** and nisin **Z** (Figure 8) are two naturally occurring nisin isoforms [2, 17, 64] with similar activities. Nisin Z is produced from *Lactococcus lactis* strain NIZO 22186 with antimicrobial activity. It has been characterised with a molecular weight that is identical to nisin which is produced by a various *Lactococcus lactis* strains including NIZO R5.

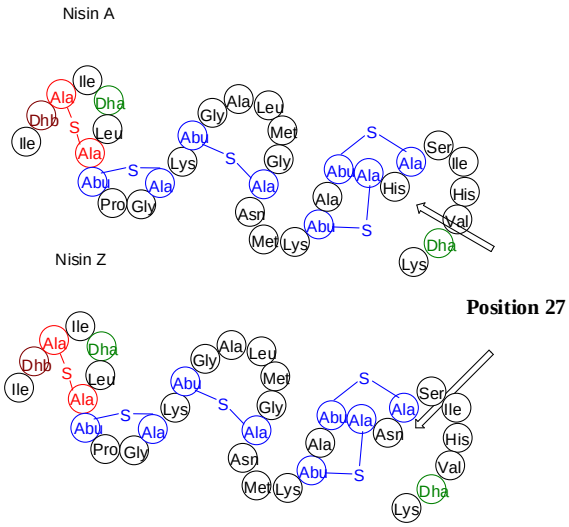


Figure 8: Structures of nisin isoforms A and Z.

1.2.2. Interaction of nisin with target membranes

Model systems have been used to determine molecular aspects of pore formation [2] by initial interaction of nisin with the membrane. Sahl *et al.* [65] was the first group to show that nisin can permeabilise membranes composed of phospholipids. They showed that membranes that are composed of zwitterionic phospholipid dioleoylphosphatidylcholine (DOPC which forms stable bilayer) could be permeabilised by nisin at a peptide/lipid molar ratio of 1:20. Nisin, pep-5 and subtilin were used to investigate their influence on non-energised membranes. It was found that nisin had considerable effect on the fluidity of DOPC vesicles containing 3 % pyrenedecanoic acid (PDA) but there was no effect by pep-5 and subtilin. Nisin and pep-5 had an increased effect on the fluidity of DOPC/DMPS (dimyristoylphosphatidylserine) vesicles but a decreased effect for pure phosphatidylserine (PS) membranes. This suggests that the affinity of the peptides were dependent on the electrostatic interactions and that the negative charged phospholipids are essential for pep-5 and nisin membrane interactions. [65]

Sahl *et al*. [65] found the hydrophobic central domain of subtilin partially penetrated into the membranes due to the tryptophan. Once DOPC vesicles at a peptide/lipid ratio of 1:400 was added the polarity of the tryptophan decreased. This shows the transfer of the indole ring to the surrounding area of the aliphatic chains. The indole ring of tryptophan in subtilin enters the hydrophobic core of the membrane slightly whilst the remaining of the tryptophan residue is located close to the water-lipid interface. [65]

1.2.3. Binding of nisin to model membranes

Binding to the target membrane is the first step in the antibacterial action of nisin. It is thought that the *C*-terminus of nisin is responsible for the initial interaction with the membrane surface, as the major part of the positive charge is concentrated within this part of the molecule. Breukink *et al*. [66] showed in detail the anionic lipid dependency of the membrane interaction of nisin. The process was mimicked using unilamellar vesicles which were composed of zwitterionic lipids DOPC and DOPG forming stable bilayer.

Nisin Z and negatively charged [Glu-32]-nisin Z were used to observe the membrane interaction. The study demonstrated that nisin Z has a higher affinity for membranes containing negatively charged lipid DOPG than membranes containing zwitterionic DOPC. For efficient binding, insertion and pore formation to take place the negatively charged lipid DOPG is essential compared to the zwitterionic DOPC. [66]

Mutations at the *C*-terminus of nisin affect the binding to model phosphatidylcholine/phosphatidylglycerol (PC/PG) lipid membranes particularly in lipid systems with greater than 40 % PG present. An increase in negative charge at position 32 opposed the attraction of positively charged residues to the negatively charged bacterial membrane leading to elimination of the anionic lipid-dependent binding of nisin (Figure 9). [67] The sensitivity of Gram-positive bacteria to nisin can be explained by the fact that generally the plasma membranes of Gram-positive bacteria are found to possess greater concentrations of anionic lipids than Gram-negative bacteria.

Gram-negative bacteria are not generally sensitive to nisin. This is due to the outer membrane of Gram-negative bacteria acting as a permeability barrier for the cell, preventing molecules

such as detergents, dyes and antibiotics from entering the cytoplasmic membrane. It is the magnesium ions which stabilize the lipopolysaccharide layer of the outer membrane. [12] As previously mentioned Gram-negative bacteria can be sensitised to nisin by combining nisin treatments with chelating agents such as EDTA. EDTA binds to the magnesium ions present in the lipopolysaccharide layer, this removes magnesium ions from the lipopolysaccharide layer of the outer membrane leading to loss of lipopolysaccharide which forms cells that are susceptible to antibiotics and detergents.

Please see: E. Breukink, B de Kruijff; *Biochim. Biophys. Acta*.; 1999, *1462*, 223 And L. Lins, P. Ducarme, E. Breukink, R. Brasseur; *Biochim. Biophys. Acta.;* 1999, *1420*, 111 to see Figure 9.

Figure 9: [2] Binding of nisin Z (□) and [Glu-32]-nisin Z (◯) to lipid vesicles with different DOPG/DOPC ratios at constant nisin: phospholipids molar ratio of 1:25. Graph adapted from R. Brasseur *et al.* [67]

1.2.4. Membrane insertion of nisin

Breukink *et al*. [66] injected nisin Z underneath monolayers made up of DOPG and DOPC mixtures. The changes in surface pressure were measured to analyze the ability of nisin to insert into the lipid part of the membrane. For efficient insertion of nisin Z into the monolayer, the presence of DOPG in the monolayer is essential.

After the initial binding of nisin to the membrane the insertion into the lipid phase is then facilitated by the amphiphilic properties of the peptide. Monolayer studies [2, 66] show anionic phospholipids are essential for effective insertion. Changes to the *C*-terminus do not affect the insertion, [2, 66] however changes to the *N*-terminus drastically reduced the ability of nisin to insert into the lipid monolayer. This shows that the *N*-terminal part of the nisin inserts into the lipid phase of the membrane. Molecular hydrophobicity potential calculation of nisin

shows that the *N*-terminus of nisin is most hydrophobic (Figure 10). [2, 67, 68] It is this hydrophobic interaction which plays a key role for the insertion of the *N*-terminus of nisin into the lipid phase of the membrane. [2, 67]

The hydrophobic side of nisin is inserted into the outer leaflet [69] of the bilayer by the amphiphilic properties of the peptide. Van de Hooven *et al*. [70] described the interaction between nisin and cellular membranes; they report that nisin is located at the surface of zwitterionic dodecylphosphocholine (Dod*P*Cho) and the anionic sodium dodecylsulphate (SDS) micelles. It was found that residues Ala3 – Ala19 except Ile4 of the *N*-terminal form the hydrophobic region which is immersed below the charged surface of the micelles. The *C*-terminal residues Lys22, Ala23, Ala26 and His27 are positioned on one side resulting in the hydrophilic part, residing at the charged surface of the micelle. Residues Met21, Ala24, Ala25 and Ala28 are on one side forming the hydrophobic part of the *C*-terminal which is immersed in the hydrophobic interior of the micelle. From these observations it was concluded that in these model membranes nisin is localised on the surface of the micelles with the hydrophobic residues immersed below the surface and the more polar or charged residues orientated outwards.

Nisin variants containing fluorescent tryptophan residues at three different positions, 1, 17 and 32, were used [2] to determine the positions of the three tryptophan residues in the membrane by measurement of the tryptophan fluorescence quenching by spin labelled lipids and to study the insertion step. The *C*-terminal tryptophan was located near the membrane surface whilst the *N*-terminal tryptophan had the deepest location within the membrane.

Please see: E. Breukink, B de Kruijff; *Biochim. Biophys. Acta*.; 1999, *1462*, 223
And L. Lins, P. Ducarme, E. Breukink, R. Brasseur; *Biochim. Biophys. Acta.;* 1999, *1420,* 111 to see Figure 10.

Figure 10: [2] Molecular hydrophobicity potential (MHP) around nisin which is represented in creatine phosphokinase (CPK). Green envelopes represent hydrophilic potentials and orange/brown envelopes, the hydrophobic ones. Picture adapted from R. Brasseur *et al.* [67]

1.2.5. Pore formation

The antimicrobial activity of nisin arises from pore formation in the cytoplasmic membrane of the target bacteria. Aggregation of peptides in the membrane results in pore formation and this has been demonstrated using model vesicles. [66]

1.2.6. Models for nisin membrane interactions

Two mechanisms for pore formation have been postulated: the "Barrel-stave'' model [71] and "wedge'' model. [72] Nisin has both water solubility and membrane binding properties which are required for both models.

When considering pore formation model according to the "barrel-stave" model a hydrophilic surface is required for the water soluble nisin monomer. A conformational change is postulated to take place after the initial binding of the monomer to the membrane exposing hydrophobic sites. Interactions then take place between the hydrophobic lipid cores of the membrane with the non-polar side chains of the peptide. A water-filled pore is then formed as several nisin molecules aggregate with their hydrophilic side-chains pointing inward.

The "wedge" [72] model suggests the pores are formed by the destabilisation of the lipid bilayer as a result of amphiphilic molecules adhering to the surface of the membrane.

Montville and Chen [73] proposed two models of barrel-stave and wedge model (Figure 11). Both models propose that through some degree of electrostatic interaction nisin initially binds to the target membrane where the model shows that *N*-terminus is initially coming into closer proximity to the inner leaf of the membrane. The two models B and C show the binding positions of nisin and the proton motive force enhancing (B) and mediating (C) nisin insertion and pore formation into the membrane.

Please see T. J. Montville, Y. Chen; *Appl. Microbiol. Biotechnol.;* 1998, *50,* 511 to see Figure 11.

Figure 11: Models for nisin pore formation. (A) Peptide showing *N* and *C* terminal domains with hydrophobic face shaded white and hydrophilic face shaded dark. (B) Barrel-stave model. (C) Wedge model. Model shows interaction of the *C* and *N* terminus of nisin with the lipid bilayer, insertion then aggregation to form pores. In both cases of the model the hydrophilic face of nisin molecule faces the lumen of the pore. Picture adapted from T. J. Montville and Y. Chen. [73]

1.3. Interaction of nisin with lipid II

An important step forward in understanding the mode of antibacterial action of nisin was the discovery that lipid II plays a key role (Figure 12). [74]

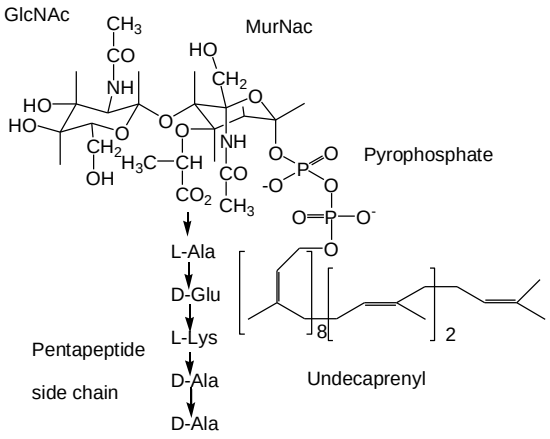


Figure 12: Structure of lipid II.

Lipid II is a component of the cytoplasmic membrane, and is necessary for the biosynthesis of the bacterial cell wall. It consists of an undecaprenyl lipid attached to a disaccharide of (MurNAc-GlcNAc). A pentapeptide, L-Ala-D-Glu-L-Lys-D-Ala-D-Ala is also attached at the MurNAc (Figure 12).

1.3.1. Lipid II-Nisin interaction

Somner and Reynolds [75] showed that nisin sensitivity of intact cells decreased when the biosynthesis of lipid II was blocked with ramoplanin (Figure 14 step B). The activity of nisin was measured by monitoring CF leakage from a model membrane composed of DOPC to which increasing amounts of pure lipid II were added. Nisin-induced CF leakage was detected at concentration of 1μM in the absence of lipid II, whereas in the presence of lipid II the CF leakage was detected at 1nM. [76]

This indicates that nisin has a high affinity for lipid II, and nisin activity is dependent on lipid II concentrations ranging from 0.001 to 0.1 mol %. [76] This suggests that different concentrations of lipid II in the membrane of different bacteria cause nisin to display diverse activity. In addition, accessibility of lipid II for nisin in intact cells should also be considered. [76]

1.3.2. Mechanism of nisin-lipid II pore formation

Hasper *et al*. [74] used pyrene fluorescence spectroscopy (performed with *Large Unilamellar Vesicles* of DOPC) and CD (performed with *Small Unilamellar Vesicles*) to analyse the assembly and stability of nisin pores. The assembly of pores were formed from the interaction between nisin and lipid II, these pores contained a consistent structure. Maximum intensity of the excimer fluorescence was observed which show there are distinct amounts of nisin and lipid II in the pores regardless of the concentration of lipid II in the membrane and the distribution. Hasper *et al*. [74] also showed that there was an interaction between two pyrene-labelled lipid II molecules with one pore complex. There was no discrimination between the un-labelled and labelled lipid II during pore formation and that there were four molecules of lipid II for a pores complex. Due to a 2:1 nisin/lipid II ratio occurring at the excimer fluorescence it suggested that there were 8 nisin molecules and 4 lipid II molecules in the pores.

The mechanism of pore formation by nisin can be divided into steps where binding to the target membrane is the first step, this is followed by insertion into the lipid phase of the membrane which leads to the pore formation. [2]

Hasper *et al*. [74] modelled formation of nisin-lipid II pores in lipid bilayer where complex **A** (Figure 13) [74] is formed, by the *N*-terminal rings A and B binding to the pyrophosphate and saccharide regions of lipid II. Meetings of multiple nisin and lipid II molecules at the interface of the bilayer form complex **B**. This is followed by insertion of nisin molecules into an orientation with respect to the membrane surface which then forms the stable pore complex **C** (Figure 13). [74]

Please see: H. E. Hasper, B. De Kruijff, E. Breukink; *Biochemistry.;* 2004, *43*, 11567 to see Figure 13.

Figure 13: Model for the formation of nisin-lipid II pores in the lipid bilayer. (A) Initial binding complex between nisin and lipid II. (B) Multiple meeting on surface of bilayer. (C) Nisin-lipid II pore (8 nisin grey oval and 4 lipid II white triangle). Picture adapted from Hasper *et al*. [74]

Stability of the nisin-lipid II pores was investigated, pores containing pyrene-labeled lipid II and nisin were challenged with unlabeled lipid II. There was minor decrease in the pyrene excimer fluorescence which indicated that the nisin-lipid II pore complexes are very stable and the pore complexes stayed intact. Also addition of detergents such as *n*-octyl-polyoxyethylene (OPOE) [74] which dissolve the membrane did not affect the pore complex. Circular dichoroism (CD) measurements confirmed this; upon solubilisation the conformation of the pore complex did not change. However addition of a stronger detergent sodium dodecylsulfate (SDS) encourages the dissociation of the nisin-lipid II pore complexes.

I. Wiedemann [77] suggested that for the nisin-lipid II interaction a negative surface charge is not required. Instead in a 1:1 stoichiometry the *N*-terminal of nisin bind to the carbohydrate moiety of lipid II. After binding, conformational rearrangement of rings A and B in the *N*-terminal of nisin takes place due to the aggregation of nisin-lipid II complex. This is followed by the insertion into the membrane of the *C*-terminal of nisin by the flexible hinge region containing ring C. Translocation across the membrane of the *C*-terminal takes place allowing the interaction between nisin and the moiety of lipid II.

1.3.3. Peptidoglycan biosynthesis cycle

Lipid I and lipid II are two intermediates in the peptidoglycan biosynthesis cycle. Figure 14 shows how the cell wall subunits are transported from within the cell to the outside of the cell. Lipid I is synthesised by the UDP-activated amino sugar MurNAc pentapeptide binding to the undecaprenylphosphate releasing UMP (Step A). Lipid II is then formed by the addition of the second UDP-activated amino sugar GlcNAc to the MurNAc group of lipid I and releasing UDP (Step B). Lipid II is then flipped from within the inner leaf of the membrane to the outer leaf of the membrane via an unknown mechanism (Step C). Step D then couples the two amino sugars and the pentapeptide to the cell wall peptidoglycan. The remaining undecaprenylpyrophosphate is transported back to the inner leaf of the membrane (Step E), followed by dephosphorylation to undecaprenylphosphate (Step F) where the cycle is complete and ready to start again.

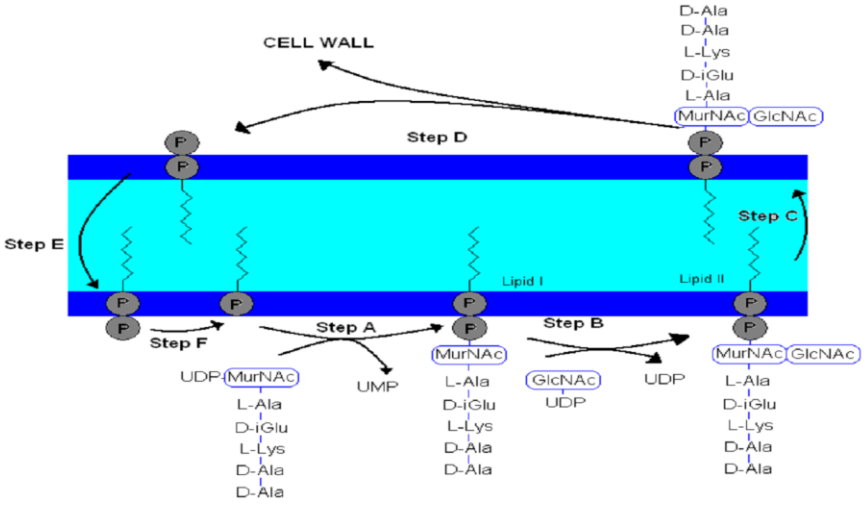


Figure 14: Schematic presentation of the role of lipid II in the cell wall biosynthesis cycle. Picture adapted from D. Nicolau. [2, 78]

Nisin not only forms highly specific pores when it interacts with lipid II but it also inhibits peptidoglycan biosynthesis. Lipid II is thought to be used as a docking target by several other antibiotics with antimicrobial activity.

Studies have shown that nisin inhibits cell wall synthesis via Lipid II binding [79] (Figure 15).[80] The cross-linked peptidoglycan network in the bacterial cell wall is prevented from forming by the interruption of biosynthesis when lantibiotics bind to lipid I or lipid II precursors. Binding prevents access to the lipid precursors by transpeptidase and transglycosylase. Another example of an antibiotic that kills bacteria by targeting lipid II is vancomycin which binds to the D-Ala-D-Ala moiety of lipid II. [80] Like other lantibiotics nisin competes with vancomycin lipid II binding. Nisin is effective against some strains resistant to vancomycin because it binds to a different region of lipid II. [6, 81]

Please see: B. Ostash, S. Walker; *Curr. Opin. Chem. Biol.;* 2005, *9*, 459 to see Figure 15.

Figure 15: Lipid II a specific target for a range of antimicrobial agents and their binding points. Picture adapted from B. Ostash and S. Walker. [80]

1.4. Nuclear magnetic resonance studies

Spectrums of nisin A and nisin Z were very similar containing the same three unsaturated amino acids dehydroalanine at positions 5 and 33 and dehydrobutyrine at position 2 however comparison of the low-field part of the H-NMR shows lack of His 27 resonances and additional of an Asn resonance (Figure 16). [2, 82] The presence of Asn in nisin Z results in a more polar side chain increasing solubility in aqueous solutions.

Please see: J. W. M. Mulders, I. J. Boerrigter, H. S. Rollema, R. J. Siezen, W. M. de VOS; *Eur. J. Biochem.;* 1991, *201*, 581 to see Figure 16.

Figure 16: NMR analysis of nisin A and nisin Z. (A) ^{1}H NMR of nisin A at pH 3.6 (B) ^{1}H NMR of nisin Z at pH 3.3. Spectra adapted from W. M. De Vos *et al.* [17]

The solution structure of nisin has been determined (Figure 17) [3] using NMR spectroscopy [3] in the presence of dodecylphosphocholine (DPC) and sodium dodecyl sulphate (SDS) micelles which mimic the cellular membrane. [77, 83, 84] The studies have shown that the structure of nisin has two well-defined domains. The *N*-terminal domain consists of residues 1-19 and the three lanthionine rings A, B and C. Residues 23-28 form the second domain consists of ring D and E and the *C*-terminal residues. [66] The *N*-terminus of nisin recognises and specifically binds to lipid II, which is followed by pore formation by nisin and this is mediated by the *C*-terminus. [2] Nisin appears to have a double mode of action where both termini appear to participate in antibacterial action. Residues 20-22 are known as the flexible

hinge region that allows rotational movement (Figure 7) and separates the two domains. Hinge regions are often found in antimicrobial peptides and affect their activity, though it is not clear how.

Please see: C. Chatterjee, M. Paul, L. Xie, W. A. van der Donk; *Chem. Rev.*; 2005, *105*, 633 to see Figure 17.

Figure 17: (A) A NMR structure of nisin in the presence of DPC micelles. [84] **Here the molecule adopts an extended conformation with the *N* and *C* termini curling back toward each other. β turn conformation is enforced by the four amino acid rings B, C, D and E, which is also adopted by the residues 21-24 in a non-covalent manner. (B) Same view points as in part A with the A ring in blue, B ring in yellow, C ring in cyan and the fused D and E rings in orange. Picture adapted from Chatterjee *et al.*** [3]

Hsu *et al.* [81] used a lipid II variant 3LII, an analogue with a short prenyl tail of three isoprene units instead of 11 to determine the structure of the complex of nisin with lipid II in DMSO. This analogue was used to eliminate multiple of alkyl peaks and make lipid II more water

soluble. DMSO was used as a membrane mimic due to the dielectric constant ε = 47.2 lying between water ε = 80 and that of the interior of the membrane ε = 2-4. From NOE spectral data two intramolecular hydrogen bonds were identified which involved the amide groups of Dhb2 and Abu8 of nisin and the pyrophosphate moiety of 3LII. It was also observed a cage like structure was formed due to the *N*-terminal of nisin folding back onto the first two lanthionine rings A and B during binding to 3LII (Figure 18 A). [81] All side chains, except the part of the lanthionine ring linkage Ala3 and Ala7, reside on the rim of the binding cleft. Five intramolecular hydrogen bonds were allowed to be formed between the backbone amides of nisin and the pyrophosphate group (Figure 18 B). [81]

Please see: S. T. D. Hsu, E. Breukink, E. Tischenko, M. A. G. Lutters, B. De Kruijff, R. Kaptein, A. M. J. J. Bonvin, N. A. J. Van Nuland; *Nat. Struct. Mol. Biol.;* 2004*, 11,* 963 to see Figure 18.

Figure 18: Structure of nisin 3LII complex. Picture adapted from Hsu *et al.* [81]

From the structure it can be seen that nisin-lipid II recognition is determined by interaction of the *N*-terminal of nisin with MurNAc, pyrophosphate and the first isoprene in lipid II. This explains earlier observations such as:

i) Kuipers [49] generated nisin mutants with additional residues at the *N*-terminus and found that this reduces the antimicrobial activity. ii) Lipid II binding affinity of nisin was reduced when at the side chain of Ala3, S3T-nisin a extra methyl group was introduced whereas there was little effect when a similar addition was carried in position 2, Dhb2. [77] iii) Ala5-nisin was biologically inactive when ring A was opened by hydrolytic cleavage.

The interface model of the complex explains the 3 observations above. i) Interfacial complimentarity may be affected by the extension of the *N*-terminal as Ile1 side chain of the *N*-terminal is part of the interface (Figure 18 A). ii) Intermolecular hydrogen bond formation

is sterically hindered by the additional methyl group in S3T-nisin (Figure 18 B, green arrow). iii) Due to ring opening the rigidity of the structure is lost and the intermolecular hydrogen bond between Dha5 and the pyrophosphate moiety is destabilised. [81]

Lipid II is a specific target which nisin uses for its antimicrobial activity and this is found in all eubacterial membranes, [74, 76, 79] where it transports peptidoglycan subunits. Nisin permeabilise the membrane and forms pores when it binds to lipid II, resulting in the collapse of the vital ion gradient across the membrane. Lipid II binding also interferes with cell wall synthesis as the peptidoglycan transport is disrupted. Thus in order for nisin to act as an antibiotic the formation of the pores is not essential but increases the efficiency of bacterial killing. [74]

1.5. Nisin fermentation and isolation

The nisin producing organism *Lactococcus lactis* subsp. *Lactis* is cultured in milk or whey, [63] in pH-controlled batch fermentation.

Demirci *et al*. [86] cultured *Lactococcus lactis* subsp. *Lactis* in a medium containing glucose, yeast extract, peptone, KH_2PO_4, NaCl and $MgSO_4.7H_2O$, per litre of deionised water which was adjusted to pH 6.8 with 4 M NaOH. This is then stored at 4 °C and subculturing is carried out monthly to maintain viability.

Ardine Z, Sensient Bionutrients and peptone which are less expensive industrial yeast extracts were used and the glucose concentration was increased to 80 g/L for the fermentation medium. The indicator microorganism *Micrococcus luteus* (ATCC 10240) was used for analysing fermentation samples of nisin. *M. luteus* was grown in a *Micrococcus luteus* medium containing peptone, yeast extract, glucose and NaCl. *M. luteus* was maintained in Agar slants containing Bacto agar and *Micrococcus luteus* medium. The stock slants are then stored at 4 ºC and subculturing is carried out weekly.

They used Celligen Cell Culture fermenter for batch fermentation. Plastic composite support discs (PCS) containing defatted soybean flour, polypropylene, yeast extract, soybean hulls and bovine albumin was added to the reactor vessel along with PCS rings both different in

size in 430 cm^3. Everything was sterilised, different media concentrations were used to test, glucose and nitrogenous components, mineral salts were added to the reactor after being sterilised separately and then inoculation with an overnight culture of *L. lactis* was carried out. The broths from fermentations were agitated without aeration at constant 30 °C and controlled pH. Samples were taken and analysed for nisin activity. [86]

1.6. Actagardine

Actagardine (formerly called gardimycin) is a Type B Class II lantibiotic produced by fermentation of *Actinoplanes* strains ATCC 31048 and ATCC 31049 and has good activity in bacterial infections in mice coupled with low acute toxicity. It contains 19 amino acids and has a molecular mass of 1873 Daltons. Actagardine has a globular structure which consists of two domains that are joined between residues 6 and 7 (Figure 19 and 20).

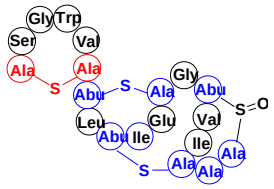


Figure 19: Structure of actagardine.

Three small stranded β-sheets are formed by residues 7-8, 9-12 and 17-19, with two parallel and one anti parallel strand. One Lan ring is found in the *N*-terminal domain whilst the *C*-terminal domain contains three MeLan rings. The thio-ether bridges impart a large degree of rigidity and the two domains are in an L-shaped planar arrangement due to van der Waals interaction. [3] The thio-ether bridges, Glu11 and Ser2, form a hydrophilic pocket. [3] The thio-ether bridge between residues 14-19 and the backbone amide loop form a second pocket (Figure 20).

Please see: N. Zimmermann, G. Jung; *Eur. J. Biochem.;* 1997*, 246,* 809 and C. Chatterjee, M. Paul, L. Xie, W. A. van der Donk; *Chem. Rev.*; 2005*, 105,* 633 to see Figure 20

Figure 20: (A) Stereoview NMR structure of actagardine reported by Jung and Zimmermann. [87] **(B) View of actagardine in the same orientation as A with rings highlighted and side chains removed. Ring A in blue making up the first domain, domain two is highlighted by magenta formed by ring B (green), ring C (red), and ring D (purple). Adapted from Chatterjee *et al*.** [3]

1.6.1. Lipid II binding

Actagardine also forms a complex with lipid II. This does not result in pore formation but results in slow cell lysis by blocking the incorporation of lipid II into peptidoglycan. [69]

It is believed that actagardine interferes either during transfer of the pentapeptide to the polysaccharide chain after synthesis of the disaccharide or the cross linking stage of the cell wall synthesis, as it draws out the growth of UDP-MurNAc-pentapeptide. [88]

1.6.2. Synthetic modification of actagardine

Malabarba *et al.* [89] synthesised chemical derivatives of actagardine (Figure 21). Selected amines were reacted with actagardine in DMF in the presence of diphenylphosphorylazide and triethylamine (Table 2). [89]

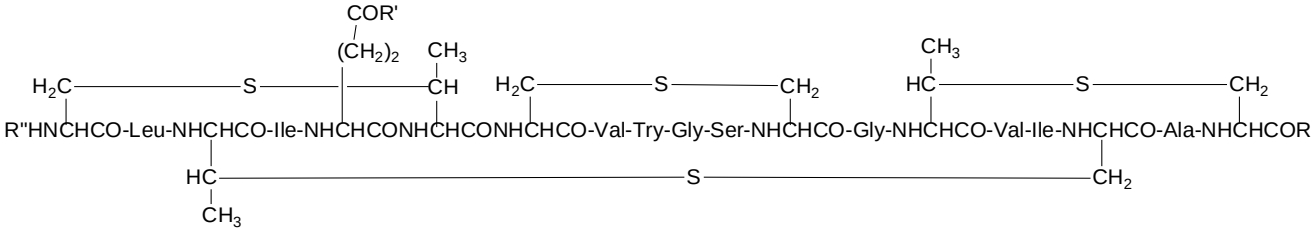


Figure 21: Chemical structure of actagardine.

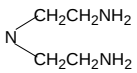
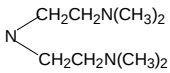
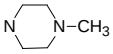
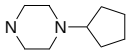
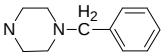
Compound	R	R'	R"	Yield (%)
9	$NHCH_2CH_2NH_2$	H	H	10
10	$NH(CH_2)_4NH_2$	H	H	10
11	$NH(CH_2)_3N(CH_3)_2.HCl$	H	H	52
12		H	H	15
13		H	H	10
14		H	H	15
15		H	H	43
16		H	H	20
17		H	H	44
18	NHC_2H_5	NHC_2H_5	H	88
19	$NHCH_2COOC_2H_5$	$NHCH_2COOC_2H_5$	H	76
20	H	H	$CO(CH_2)_{14}CH_3$	60

Table 2: [89] Derivatives of actagardine.

Similar antibacterial activity to that of actagardine was obtained for all compounds apart from the *N*-acyl derivative **20**, which showed negligible activity.

1.6.3. Actagardine fermentation and isolation

Actinoplanes liguriae ATCC 31048 strain produce actagardine, [90] *Actinoplanes liguriae* ATCC 31048 strains was used to inoculate a seed medium containing agar. The seed medium solution contained starch; peptone, glucose, yeast extract and corn steep liquor in water. The medium was first sterilised by autoclaving this was followed by inoculation of the seed stage medium with plugs from a culture plate. This was then used to inoculate a nutrient medium containing glycerol, casein peptone, K_2HPO_4, NaCl and $MgSO_4.7H_2O$. These cultures were incubated followed by isolation of pure actagardine. [5] The purification involved passing the filtered fermentation broth through a column and then combining the active fractions, concentrating *in vacuo* and then freeze drying.

Actagardine can be extracted as a free acid or as a sodium salt from the culture filtrate due to it being an amphoteric peptide with overall negative charge. [91] The isolation is carried by an extraction of the culture broth with butanol at pH 8. Dialysis of a 10 % aqueous solution of the crude material led to purification. [91]

When the same fermentation process was used with soya meal/mannitol production medium instead of the nutrient culture an additional lantibiotic, Ala(0)-actagardine was formed. [87] The new compound was isolated by solid phase extraction, molecular sieve chromatography and preparative HPLC. [5] The molecular mass for the new isolated compound was 1960 Dalton (Figure 22). It has greater antimicrobial activity than the parent actagardine. [5]

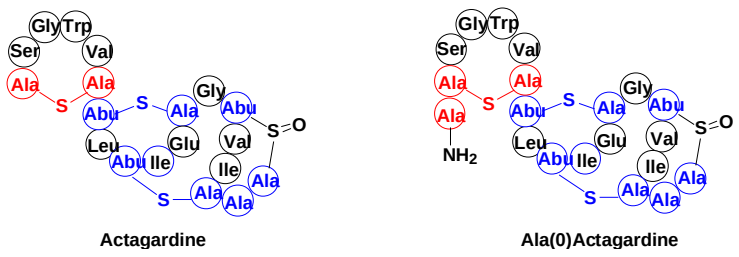


Figure 22: Structure of actagardine and the new isolated product Ala(0)-actagardine.

1.7. Mersacidin

Mersacidin (Figure 23) (formerly named M87-1551) is isolated from *Bacillus HIL Y-85-54728*. [92] Mersacidin has significant *in vivo* efficiency against methicillin-resistant *Staphylococcus aureus* MRSA, [93] curing infections in mice and abscesses in rats and also removes MRSA in a mouse rhinitis model. This makes it a potential candidate as a therapeutic agent. It is a tetracyclic Type B lantibiotic with broad *in vitro* activity against Gram positive bacteria. It consists of 20 amino acid residues with a molecular weight of 1825 Dalton. It contains three methyllanthionines, one dehydroalanine and one S-aminovinyl-2-methylcysteine residue **6** (Figure 1). It carries no overall charge, does not depolarise membranes and has hydrophobic properties overall. [92] There are three domains formed by the thio-ether bridges residues 1-3, 4-12 and 13-20 (Figure 24). [3] There are only two charged groups, namely Glu17 and the *N*-terminus, and the sequence consists mainly of neutral side chains. In the second domain residues 4-12 contain glycine-rich sequences which gives some conformational flexibility whilst the third domain residues 13-20 results in backfolding and rigidity of the structure. There are intramolecular hydrogen bonds between domain one residues 1-3 and domain three residues 13-20. [3, 94]

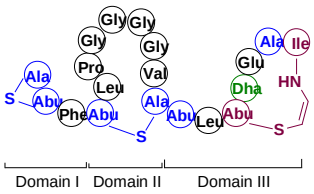


Figure 23: Structure of mersacidin.

Please see: S. T. Hsu, E. Breukink, G. Bierbaum, H.-G. Sahl, B. De Kruijff, R. Kaptein, N. A. Van Nuland, A. M. Bonvin; *J. Biol. Chem.;* 2003, *278*, 13110 and C. Chatterjee, M. Paul, L. Xie, W. A. van der Donk; *Chem. Rev.*; 2005, *105*, 633 to see Figure 24.

Figure 24: (A) Stereoview structure of mersacidin. (B) View of mersacidin in the same orientation of A with rings highlighted and side chains removed. [95] **Ring A in blue, ring B in red, ring C in green and ring D in magenta. Picture adapted from Chatterjee *et al*.** [3]

1.7.1. Lipid II binding

Like actagardine, mersacidin also inhibits peptidoglycan biosynthesis by binding to lipid II blocking access by transglycosylase and transpeptidase enzymes. [96, 97] Residues 13-18 of domain III of mersacidin, and the disaccharide region and maybe the pyrophosphate moiety of lipid II appear to be involved during mersacidin lipid II binding. [96] From an NMR study it was found that residues 12 and 13 Ala and Abu between rings B and C act as a hinge region which allows the charged side chains of mersacidin to interact with lipid II. [95] Mersacidin does not form pores in the bacterial membrane. [3]

1.7.2. Synthetic modification of mersacidin

A mutagenesis approach was used to investigate the structure activity relationship. Mutants F3L, S16I and E17A were developed by Szekat [98] and were either found to be weakly active or inactive. Appleyard *et al*. [99] investigated a large number of mutants consisting of three libraries where amino acids were inserted, substituted or deleted (Figure 25 and 26). [99]

Please see: A. N. Appleyard, S. Choi, D. M. Read, A. Lightfoot, S. Boakes, A. Hoffmann, I. Chopra, G. Bierbaum, B. A. M. Rudd, M. J. Dawson, J. Cortes; *Chem. Biol.;* 2009, *16,* 490 to see Figure 25.

Figure 25: Mersacidin substitution library, circles represents variants. Pink circles represent mersacidin, purple circles are variants produced at high yields and blue circles represent variants produced at trace levels. Picture adapted from Appleyard *et al.* [99]

Please see: A. N. Appleyard, S. Choi, D. M. Read, A. Lightfoot, S. Boakes, A. Hoffmann, I. Chopra, G. Bierbaum, B. A. M. Rudd, M. J. Dawson, J. Cortes; *Chem. Biol.;* 2009, *16,* 490 to see Figure 26.

Figure 26: Mersacidin insertion library, arrows show where amino acids were inserted, circles are the variants inserted. Pink circles represent mersacidin, purple circles are variants produced at high yields and blue circles represent variants produced at trace levels. Picture adapted from Appleyard *et al.* [99]

From the study it was discovered that ring A cannot be extended or mutated. However ring B is open to both insertion and substitution but it is not flexible at positions near the ring forming residues. It was found that mutants with some activity were associated with those of ring B but mutants with activity were spread throughout the molecule. Deletion were not accepted as products were not formed, suggesting that the biosynthetic enzymes cannot process substrates which contain fewer amino acid residues. It was found that changes to Glu17, Leu5 and Ile19 abolished all activity indicating that these residues were vital for activity (Figure 27). [100] Most variants formed were less active than the parent mersacidin.

Please see: A. C. Ross, J. C. Vederas; *J. Antibiotics.;* 2011, *64*, 27 to see Figure 27.

Figure 27: Mutants and structure activity relationship of mersacidin. Active mutants are placed above mersacidin structure with inactive below. Insertion of amino acids are shown by arrows and residues vital to activity are shown in red bordered lines. Picture adapted from Ross and Vederas. [100]

1.7.3. Mersacidin fermentation and isolation

Bacillus sp. was isolated from a soil sample. The first step involved sterilising the seed medium containing casamino acid, corn steep liquor, galactose and glycerol in demineralised water by autoclaving. The seed stage medium was inoculated with plugs from a culture plate with *Bacillus sp*. This was followed by preparation of the seed culture. [41] The second step involved sterilising the medium containing sucrose, KH_2PO_4, Na_2HPO_4, $(NH_4)_2SO_4$, Na-

glutamate, $MgSO_4.7H_2O$, $MnSO_4.H_2O$, $(NH_4)_6Mo_7O_{24}.4H_2O$, $ZnSO_4.7H_2O$ and $FeSO_4.7H_2O$ in a fermenter. This was then inoculated with the seed medium from step 1.

More medium was sterilised in step 3 same as in step 2 and then this was used to inoculate with the seed culture from step 2. [41]

Centrifugation separated the culture broth from the mycelial cake after 45 hr of harvesting. Mersacidin was found in the culture filtrate which was passed through a resin column. Activity against *S. aureus* 209P was monitored with the fractions collected, fractions with mersacidin was reduced to give crude mersacidin. [41]

Medium pressure liquid chromatography was carried out on crude mersacidin. The eluant with mersacidin was reduced and columned again as above giving semi pure sample which was purified further by precipitation giving pure mersacidin.

2. Synthesis of lantibiotics

There are advantages in carrying out chemical synthesis of lantibiotics; as this enable changes to be made to the peptide sequence of nisin. These changes can include the incorporation of additional amino acids, unusual amino acids such as cystathione and changes in the stereocentres of the lanthionine. Fluorophores and isotopic labels can be introduced which will enable additional NMR studies of lantibiotics such as nisin and give more information on the mode of action.

Incorporation of lanthionine as a novel conformational constraint in other biologically active peptides leads to peptides with one or more thio-ether bridges. These confer stability to proteolytic cleavage, and cannot be reductively cleaved, whereas conformationally constrained peptides containing Cys-S-S-Cys bridges are generally not stable in vivo. Due to these properties many medicinally related peptides have had lanthionine analogues incorporated in them to obtain biologically active thio-ether bridged peptidomimetics. [101, 102]

Synthesis of lanthionine residues with the correct stereochemistry at the α positions (and β positions in the case of the β-methyl lanthionine) is important and essential. It is also important for regioselective cyclisation of the peptide to take place.

There are two approaches which can be considered for the synthesis of lantibiotics, the first approach involves making the linear precursor first and then cyclise to form lanthionine or methyllanthionine generating the thio-ether bridges. Whilst the second approach involve making the key bis-amino acids, lanthionine and methyl lanthionine first and then incorporating them into the peptide.

2.1. Solution phase synthesis

Shiba *et al*. [103] synthesised nisin which was identical to the naturally occurring peptide giving 10 % overall yields. Shiba's group used solution phase synthesis to assemble the Cys-Cys bridged analogues of the individual segments: ring A, ring B, ring C, and rings D and E by condensation of the four main segments. Desulfurisation was carried out to convert these

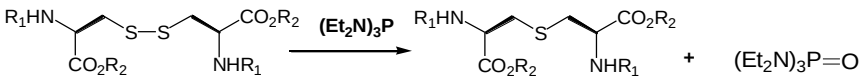
lanthionine bridged segments which was followed by dehydration, forming the Dha and Dhb residues. Segment couplings of the protected fragments were than carried out in solution to give the desired peptide.

2.1.2. Total synthesis of nisin via desulfurisation

T. Shiba *et al.* [103] first carried out desulfurisation with hexaethylphosphorus triamide to form all the thio-ether bridges. This allowed the formation of the optically pure lanthionine by selective removal of the sulphur atom from the appropriate cystine. This was an approach used for the preparation of lanthionine by Harpp and Gleason. [104]

2.1.2.1. Desulfurisation of the cystine to form the lanthionine

Harpp and Gleason [104] found that aminophosphines can effect selective desulfurisation in simple disulfide systems giving monosulfide compounds. However methyl or ethyl ester protection was required on cystine derivatives as carboxylic groups react with the aminophosphine (Scheme 1). [104]



Scheme 1: [104] **Desulfurisation of cystine to form the lanthionine. R_1 = $COCF_3$ or $CO_2CH_2C_6H_5$ and R_2 = CH_3 or C_2H_5.**

When this method was used to desulfurise unsymmetrical substituted disulfides, byproducts were obtained (Figure 28).

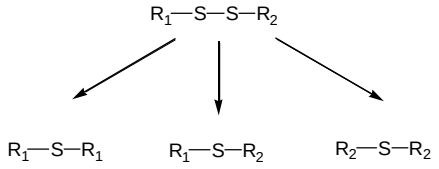
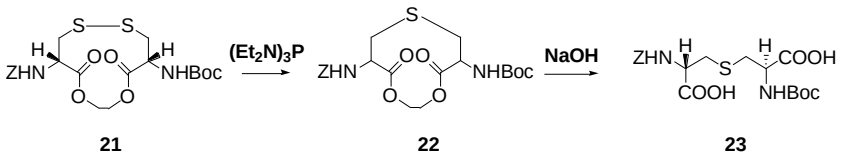


Figure 28: Three lanthionine products synthesised from desulfurisation of unsymmetrically substituted cystine.

This method was adapted by Cavalier-Frontin *et al.* [105] who modified the method in order to synthesise the unsymmetrical lanthionine **23** from cyclic cystine **21** (Scheme 2). [105] Desulfurisation was carried out on the cyclic cystine where the two amino groups were protected with different protecting groups. Saponification of **22** led to the unsymmetrical meso-lanthionine **23**.

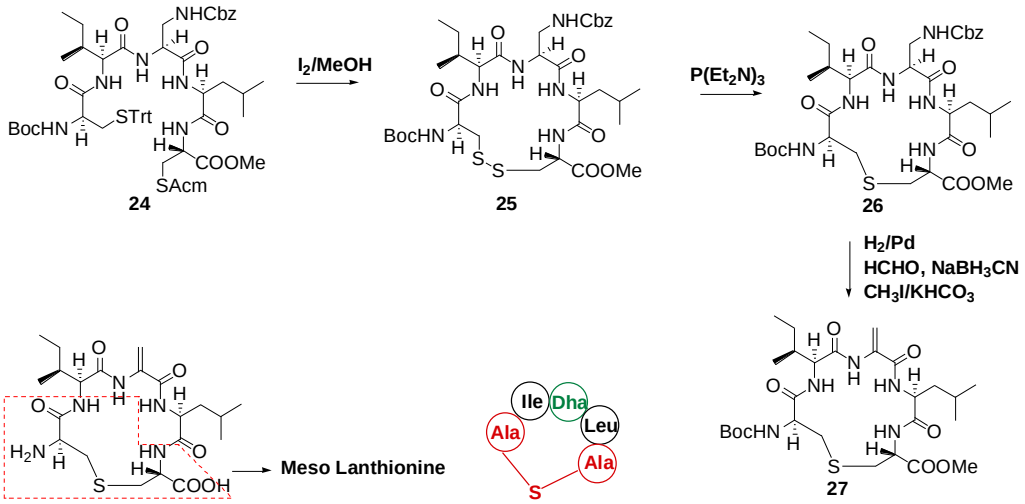


Scheme 2: [105] **Desulfurisation of an unsymmetrical substituted cystine.**

2.1.2.2. Synthesis of ring A fragment

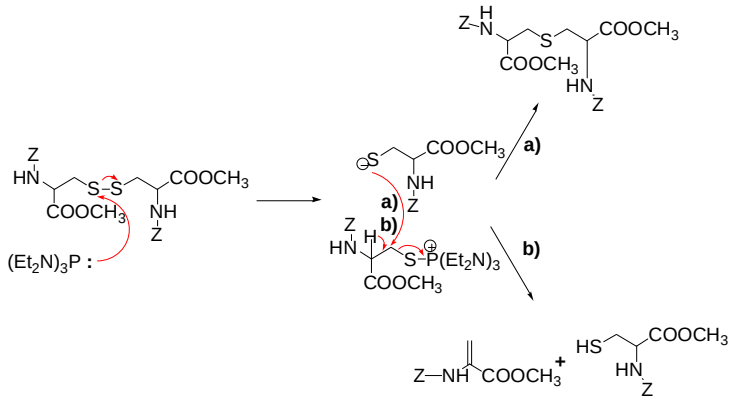
Shiba *et al.* [103] had to resolve two problems during the synthesis of nisin; this involved the introduction of the dehydramino acids and the formation of the sulfide ring. These problems were solved by carrying out desulfurisation of cyclic disulfide peptides as they judged that it was easier to form the S-S bond than to carry out cyclisation via the amide bond.

Shiba *et al.* [106] investigated the methodology for preparing the lanthionine moiety in a cyclic peptide. Linear peptide **24** was prepared containing two cysteine residues. [106]



Scheme 3: Synthesis of protected ring A of nisin via desulfurisation.

The peptide was then cyclised to give peptide **25**; the oxidation proceeded smoothly to give the disulfide product. Tris(diethylamino)phosphine in DMF was used for desulfurisation of **25** to give peptide **26** without the formation of any dehydroalanyl peptide. The desulfurisation step was attempted in other solvents such as $CHCl_3$, THF, dioxane, and benzene however these solvents resulted in forming the dehydroalanine derivative as the major product due to intramolecular β–elimination (Scheme 4 b). [106] Desulfurisation was followed by removal of the Cbz group of the diaminopropionic acid (Dpr) sidechain. *N*-methylation was then carried out immediately. Conversion of the A_2pr residue to dehydroalanine was achieved by the addition of 1-1.5 equivalent of MeI in DMF in the presence of $KHCO_3$. This gave the desired dehydroalanyl peptide **27**, ring A of nisin (Scheme 3). [106]



Scheme 4: [106] **Mechanism for desulfurisation.**

It was important to make sure that dry solvents were used during desulfurisation as otherwise side reactions occurred (Figure 29). Solvents used for the desulfurisation of each ring depended on the substrate, where DMF was used for the formation of Boc-(ring A)-OMe, [106] benzene for Boc-(ring B)-OMe [107] and Boc-(ring D and E)-OMe, [108] and THF for Boc-(ring C)-OMe. [109]

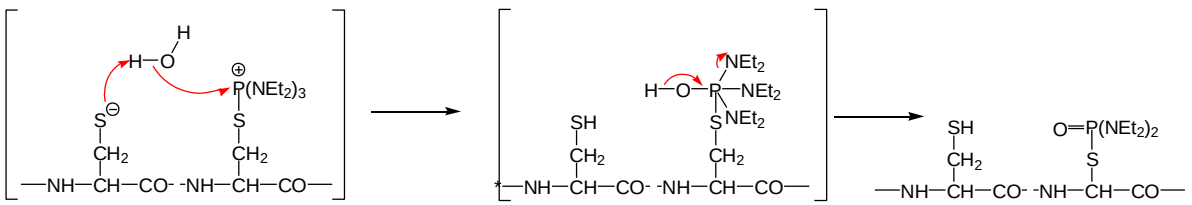
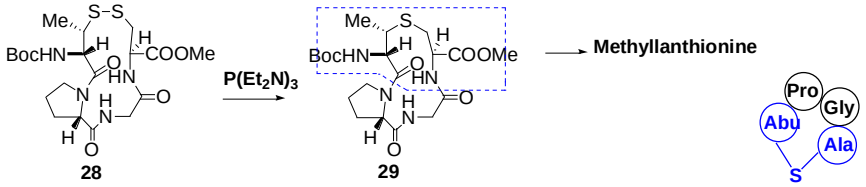


Figure 29: Mechanism for the competing formation of a phosphoramidate byproduct.

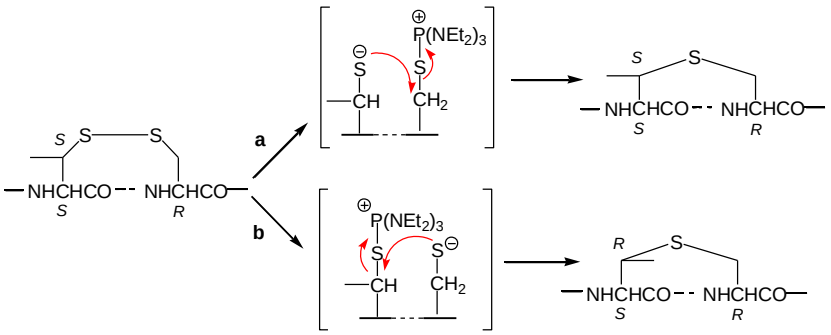
2.1.2.3. Synthesis of ring B fragment

Ring B of nisin was synthesised using the above method, by elimination of one sulphur atom from the cysteine and *threo*-3-methyl-D-cysteine sulphide bonds. [107] The reaction of ring B of nisin (Scheme 5) was carried out in benzene which gave a better yield than it did in DMF.



Scheme 5: [107] **Formation of protected ring B of nisin via desulfurisation.**

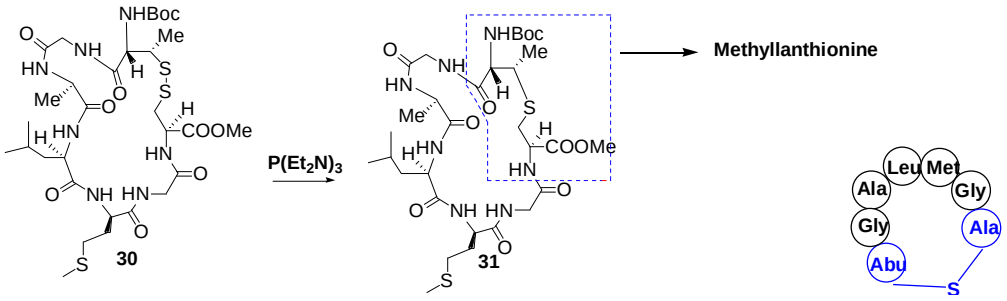
In order for the desulfurisation of peptide **28** to take place to form ring B (peptide **29**); it was important that the *threo* form of methyllanthionine found in wild type nisin was obtained. The *threo* form of methyllanthionine was achieved by keeping the configuration on the β-carbon of *threo*-3-Methyl-D-cysteine (Scheme 6 a). [107] If the mechanism of the reaction proceeded via path **b**, *erythro*-methyllanthionine will be obtained as a result of the inversion of configuration on the β-carbon of 3-methylcysteine (Scheme 6 b). [107] It was found that path **a** of Scheme 6 takes place, this could possibly be due to a selective nucleophilic attack taking place at the sulphur atom of D-cysteine residue because of the steric hindrance of the 3-methyl group in the *threo*-3-methyl-D-cysteine. Another reason could be due to the thiolate anion of cysteine residue being less stable than the thiolate anion of 3-methylcysteine residue. Shiba *et al*. [110] confirmed the formation of *threo*-methyllanthionine by carrying out amino acid analysis of the hydrolyzate of **29**. [110]



Scheme 6: [107] **Desulfurisation pathways of ring B.**

2.1.2.4. Synthesis of ring C fragment

The same methodology was used to synthesise ring C of nisin (Scheme 7). The naturally occurring threo-β-methyl lanthionine residue was again synthesised from threo-3-methyl-D-cysteine, during desulfurisation the reaction proceeded in the configuration-retaining route. [109]



Scheme 7: [109] **Formation of protected ring C of nisin via desulfurisation.**

2.1.2.5. Synthesis of rings D and E fragment

Rings D and E of nisin are the most complicated rings to synthesise, due to the two overlapping disulfide bridges of the unique bicyclic structure. Two methods were used to synthesise rings D and E. The first method involved a one step desulfurisation, this facilitated the formation of the two sulphide rings from the bis-disulfide peptide. The advantage of this method is it requires less reaction steps however there is the possibility for rearrangements of the rings to take place during the desulfurisation step (Figure 30 method A). [108] The second method involved a stepwise construction of the two rings (Figure 30 method B). [108]

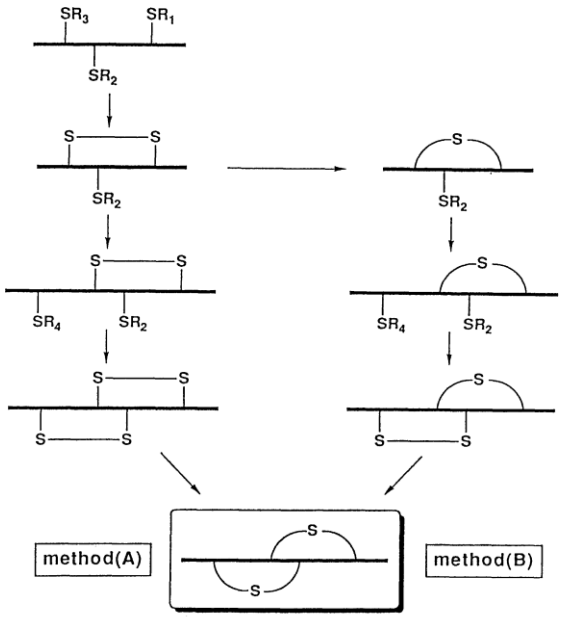
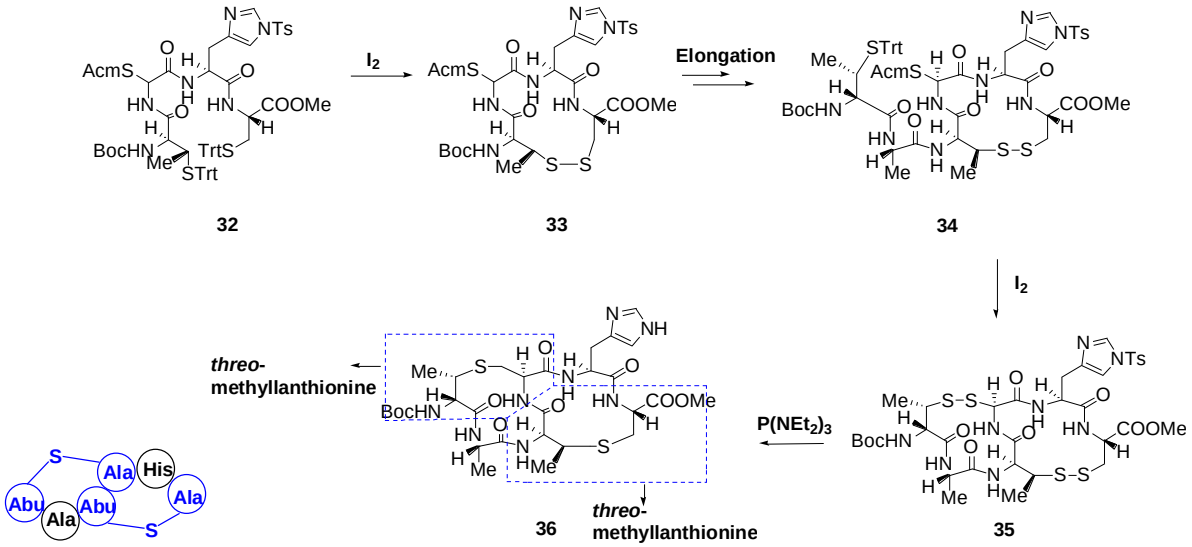


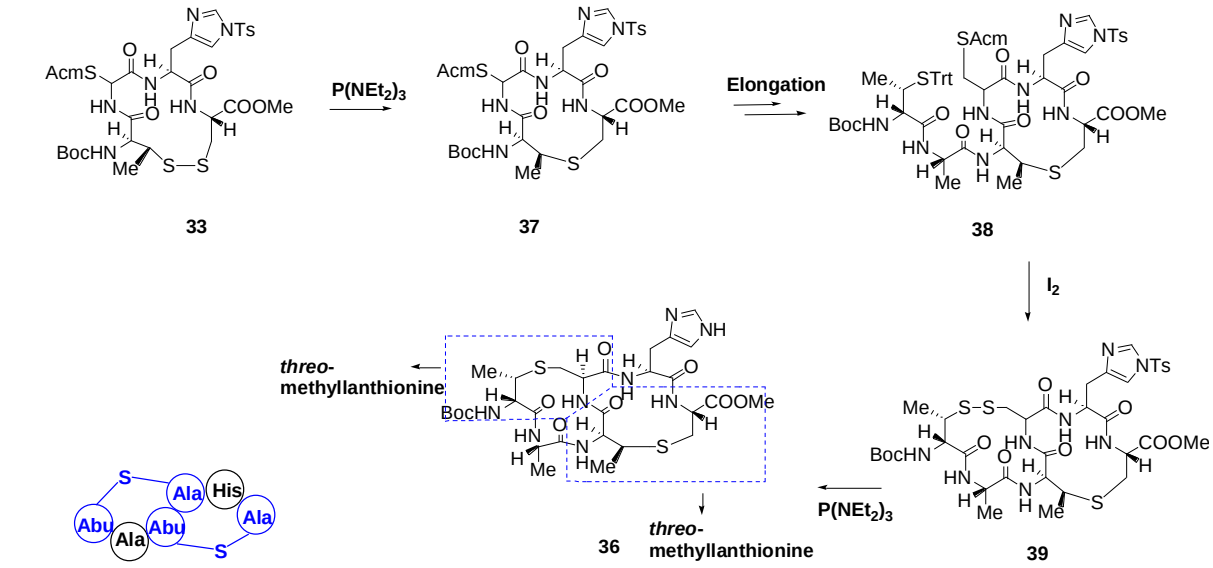
Figure 30: Two possible methods for the synthesis of rings D and E. Picture adapted from Shiba *et al*. [108]

The synthesis started with the preparation of linear peptide **32**. Iodine oxidation of **32** gave the disulfide peptide **33**. Deprotection of the S-Trt group was carried out by iodine oxidation in the presence of CF_3CH_2OH in CH_2Cl_2. [108] Dipeptide **33** was then elongated to give hexapeptide **34**. During this coupling step an active reagent benzotriazole-l-yl diethyl phosphate (BDP) was used, this was to avoid the steric influence in cyclic sulfide or disulfide peptide which will cause a slow rate in the coupling reaction. During this step the Ts protecting group is removed, which has to be reintroduced with tosyl chloride and triethylamine once the coupling reaction is complete. [108] The second disulfide ring was formed by a second iodine oxidation, simultaneously removing the Trt and Acm groups and cycling to form peptide **35**. Then using $P(NEt_2)_3$ in a one step desulfurisation reaction the bicyclic sulfide rings **36** were obtained in benzene (Scheme 8). [108]



Scheme 8: Protected rings D and E of nisin synthesised by desulfurisation via synthetic strategy path A (Figure 30).

For comparison of the two routes, the stepwise construction of the two rings D and E were carried out. The cyclic sulfide peptide ring E **37** was obtained after the first desulfurisation of disulfide peptide **33** in anhydrous benzene. Elongation was carried out on the cyclic peptide to give the hexapeptide **38** this was followed by iodine oxidation of **38**, removing the S-Trt and S-Acm group and simultaneously forming the bicyclic peptide **39**. The desired peptide **36** ring D was then formed by the second desulfurisation (Scheme 9). [108] Both desulfurisation steps resulted in very low yields, due to formation of multiple oligomers.



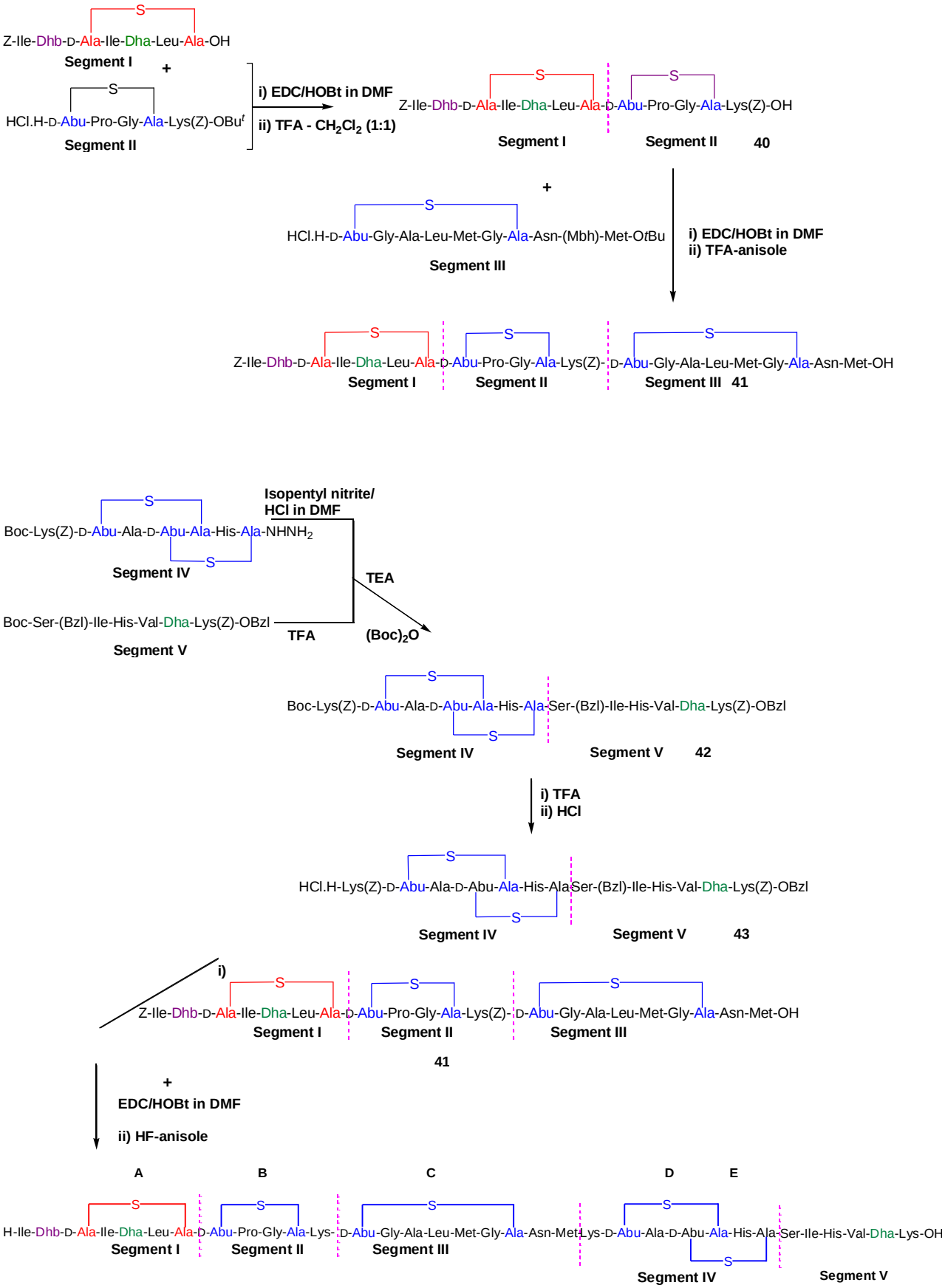
Scheme 9: Protected rings D and E of nisin synthesised by desulfurisation via synthetic strategy path B (Figure 30).

From the two routes it was found that the first route (Scheme 8) [108] gave better results. This can be concluded from the fact that less reaction steps were required, the yields obtained from the desulfurisation were much higher and it was much easier to carry out elongation of the peptide. Low yields were obtained by the second route (Scheme 9) due to oligomers being produced. [108]

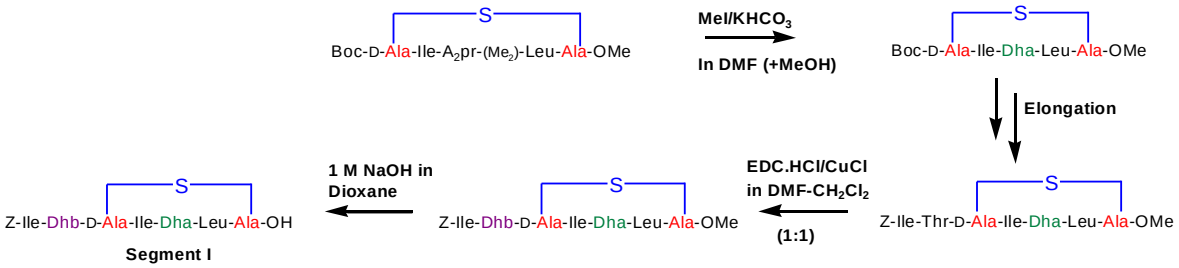
2.1.2.6. Fragment Coupling

A segment condensation method was used by Shiba *et al*. [103] in the total synthesis of nisin (Scheme 10). The five cyclic segments were synthesised separately as described above, carbodiimide and copper (I) chloride (II) [111] were used to dehydrate the threonine to Dhb of segment I (Scheme 11). The segments were then coupled together as shown in Scheme 10.

In the previous study a methyl ester derivative [107] was used for the synthesis of ring B (Scheme 5), it was found that this decomposes very easily under saponification conditions due to which benzyl ester derivatives were preferred. [103] The structures of the synthetic intermediates were confirmed by comparing with enzymatic (a part of trypsin digestive fragment) and BrCN degradation products of natural nisin. [103] ^{1}H NMR, FAB mass spec and retention time in HPLC confirmed that synthetic nisin was identical to natural nisin.



Scheme 10: Total synthesis of nisin by coupling the five fragments of nisin.



Scheme 11: Dehydration of serine and threonine to Dha and Dhb.

2.2. Biomimetic synthesis of lantibiotics

Individual rings of lantibiotics have been synthesised by different groups using a biomimetic approach (Figure 31, Scheme 12). This synthetic approach mimics the lantibiotic biosynthetic pathway that takes place by extensive posttranslational modification by specific enzymes which catalyses the dehydration of Ser and Thr residues by genes *NisB* and *NisC* discussed in Chapter 1, Section 1.1.4. Michael addition of cysteine residues to dehydroamino acids lead to the formation of thio-ether bridges. Studies on biomimetic additions of cysteine residues to Dha and Dhb have shown formation of diastereoisomers as the reaction is not stereospecific. [112] Thus the regio- or stereoselectivity of the cyclisation step is difficult to control in the absence of the biosynthetic enzymes.

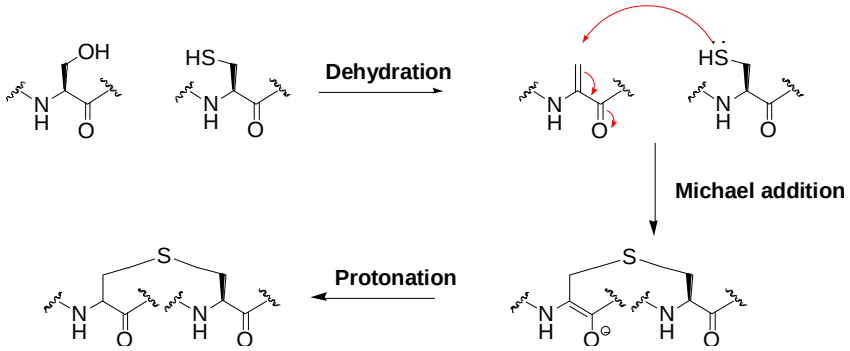
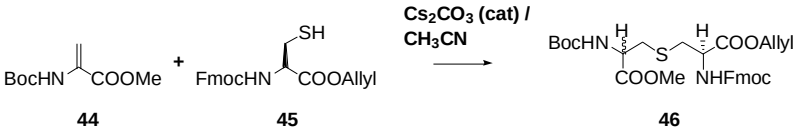


Figure 31: Biomimetic synthesis of lanthionine-bridged peptides.

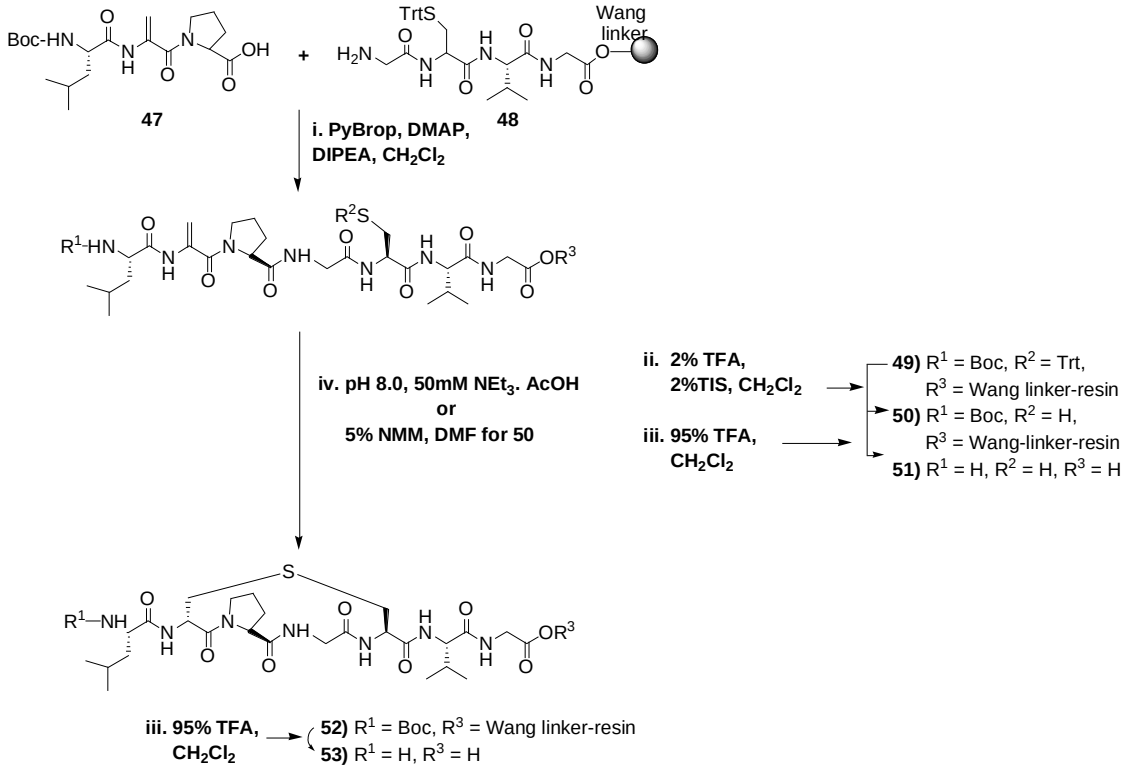
Bradley *et al.* [112] isolated mixtures of diastereoisomers resulting in no stereocontrol demonstrating this approach is not stereospecific (Scheme 12).



Scheme 12: [112] **Lanthionine synthesis via Michael addition.**

Bradley *et al*. [113] developed a synthetic methodology to allow synthesis of peptides containing multiple dehydro amino acids, which can be used for the biomimetic synthesis of lantibiotic subunits. Incorporation of multiple dehydro amino acids were achieved by synthesising peptides with *S*-methylcysteine which undergoes oxidation/elimination reaction. Dehydroalanine residues were generated within a linear peptide this was followed by a Michael addition of cysteine sulfhydryl to form the thio-ether bridge which produced the lanthionine bridged cyclic peptides (Scheme 13).

They undertook the synthesis of subunits of subtilin. For example ring B was synthesised from peptide **51** (Figure 32). The cyclisation proceeded in 50mM triethylammonium acetate (pH 8) (Scheme 13) [113] to give the single stereoisomer which was confirmed by NMR and HPLC analysis. There was complete consumption of starting material under 10 min shown by UV/VIS analysis. The same stereoselectivity was observed for ring E.



Scheme 13: [113] **Biomimetic synthesis of ring B of subtilin.**

H–Leu-**Dha**-Pro-Gly-**Cys**-Val–Gly-OR **51 = B - ring** precursor

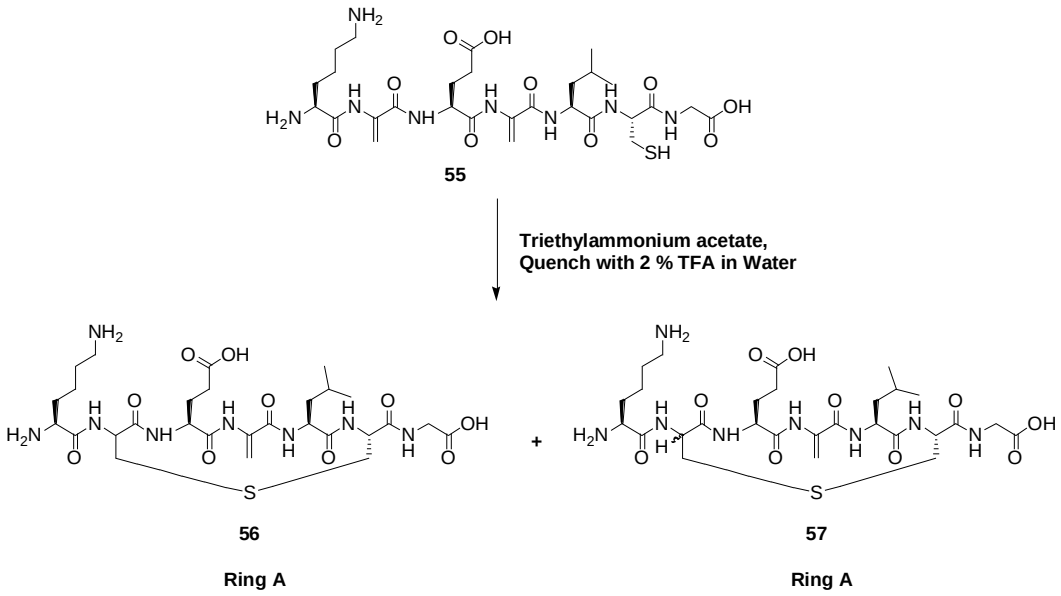
H–Leu-**Dha**-Ala–Asn-**Cys**-Lys–Ile–OH **54 = E - ring** precursor

H–Lys–**Dha**-Glu-**Dha**-Leu-**Cys**-Ala-OH **55 = A - ring** precursor

Figure 32: Precursors of rings A, B and E.

In the same way, ring A (Figure 32) was synthesised. Cyclisation was carried out on the crude peptide due to the problems of stability of the linear precursor peptide. However, two products in a ratio of 3:1 were produced. NMR studies showed the structures to be stereo-isomers rather than regio-isomers. TOCSY experiments confirmed that each product was a single diastereoisomer, and that regioselective Michael addition has occurred on Dha2, giving natural regioisomer of the subtilin ring A (Scheme 14) as a mixture of diastereoisomers. [113]

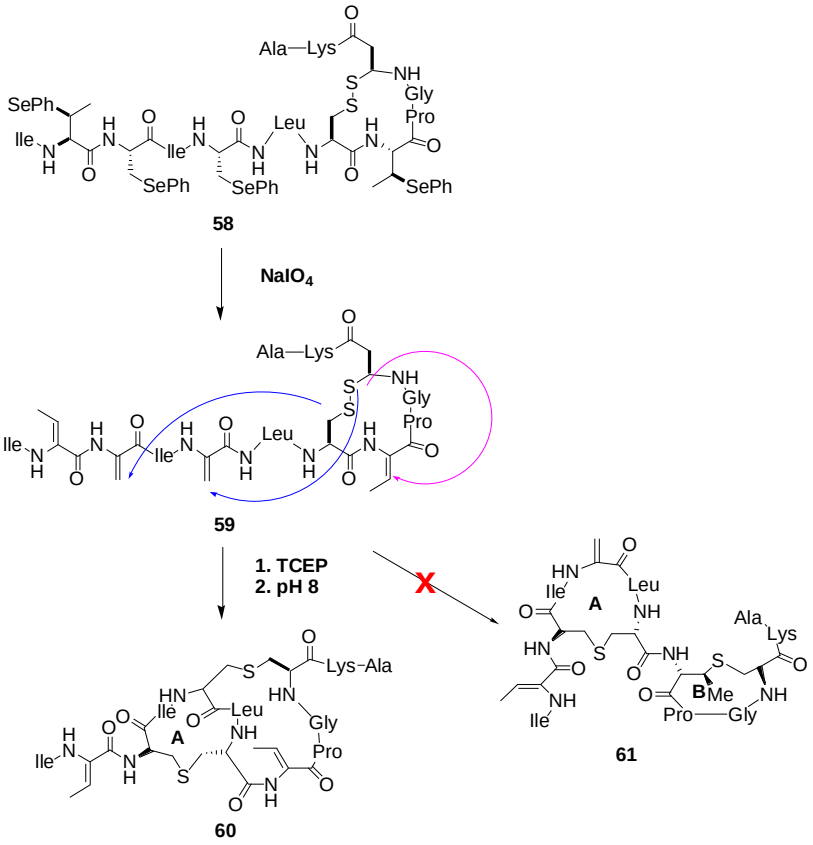
There was a possibility of the cyclisation taking place at residue 4 however addition to this dehydroamino acid was not detected.



Scheme 14 [113] **Subtilin ring A.**

Zhu *et al.* [114] carried out a biomimetic approach investigating the stereochemistry of (non-enzymatic) intramolecular Michael additions producing lanthionines and methyllanthionines. From the previous studies it has been seen that biomimetic intramolecular conjugate additions from cyclic thio-ethers often have the same stereochemistry as those found in lantibiotics.

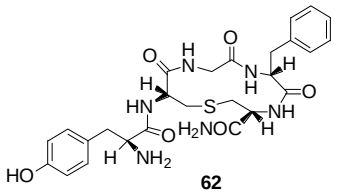
They attempted to explore the regiochemistry of the formation of rings A and B of nisin by the use of solid phase reaction conditions. Dehydroamino acids were incorporated by oxidative elimination methodology from selenocysteine derivatives. Zhu *et al.* [114] found that the attempt to synthesise rings A and B of nisin did not give the desired product. This was due to Cys11 reacting with Dha5 forming a six residue lanthionine ring, forming nisin with different rings (Scheme 15). [114] Diastereoisomers and constitutional isomers were formed which was shown by LC-MS analysis. Resonances of the Dhb vinyl protons were present in the product whilst the vinyl protons of Dha were absent. Enzymatic control [114] is thus required for regioselectivity of the cyclisation reactions. This approach is therefore an unsuitable method for multiple thio-ether bridged ring synthesis.



Scheme 15: [114] **Synthesis of rings via biomimetic approach.**

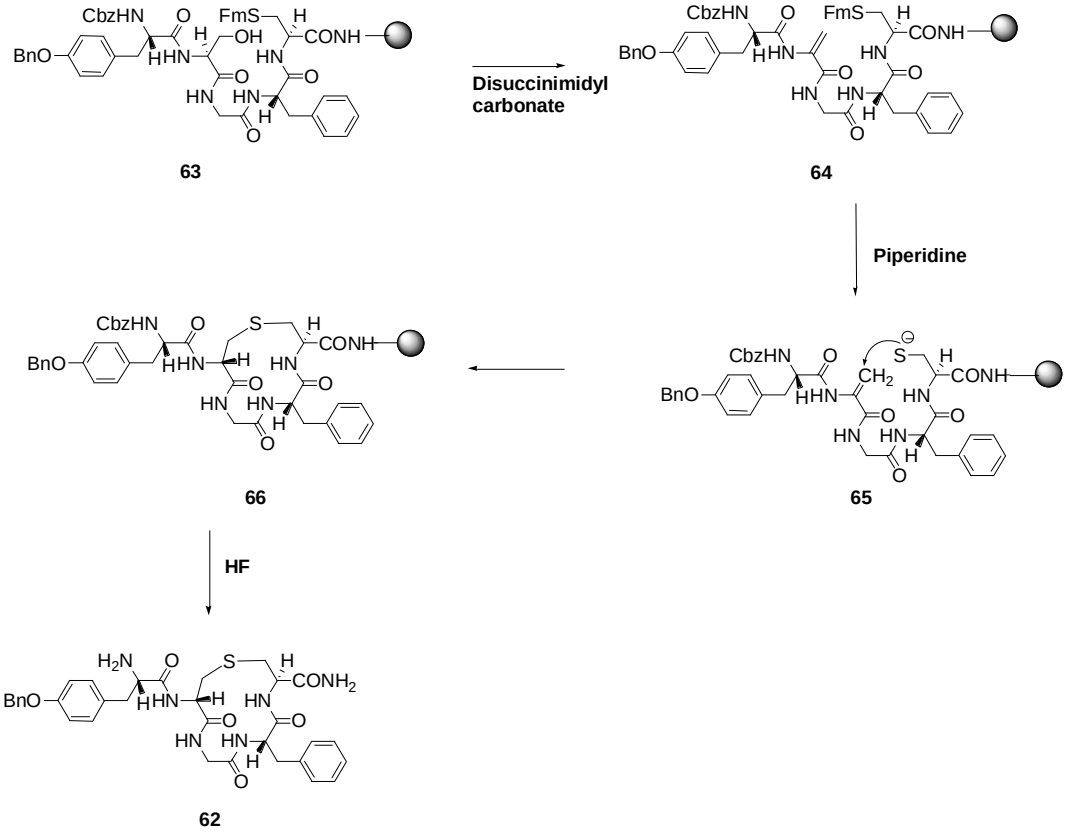
In order to probe the receptor binding selectivity of enkephalin mimics, Goodman *et al.* [115] synthesised a conformationally constrained analogue of enkephalin **62** with a lanthionine ring between residues 2 and 5 (Figure 33, Scheme 16) using a biomimetic approach. This peptide contains one ring containing one dehydroamino acid and only one diastereoisomer was

obtained which meant the peptide was stereospecific. Michael additions are not normally stereoselective however the steric hindrance provided by the solid support adjacent to the SH is responsible for the diastereoselectivity of this reaction.



62

Figure 33: Structure of an analogue of enkephalin.



Scheme 16: [115] **Synthesis of enkephalin analogue 62.**

2.3. Solid phase synthesis

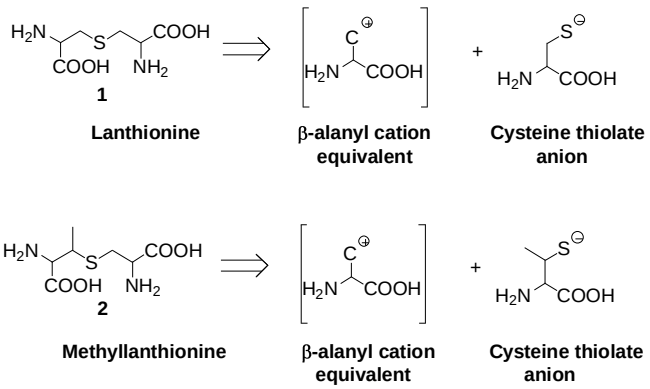
An alternative approach for the synthesis of both naturally occurring lantibiotics and analogues with different sequences and ring sizes is to incorporate suitably protected lanthionine derivatives into a precursor linear peptide, using solid phase peptide synthesis. If suitable protecting groups are chosen, the lanthionine residue can be selectively deprotected

and the peptide cyclised as required, leading to the synthesis of lanthionine containing peptides with complete regio- and stereocontrol.

2.4. Synthesis of lanthionine

2.4.1. β-Alanyl cation equivalent

Incorporation of lanthionine building blocks that are orthogonally protected was an alternative strategy in synthesising these lantibiotic peptides. Retrosynthetic analysis of lanthionine suggests that reaction of a β-alanyl cation equivalent and a cysteine thiolate anion could be used to form both lanthionine and methyllanthionine (Scheme 17).



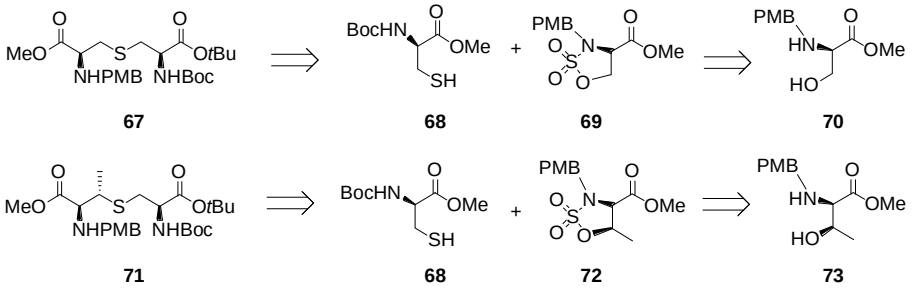
Scheme 17: Reterosynthesis of lanthionine and methyllanthionine.

A good leaving group at the β position would allow S_N2 nucleophilic substitution by cysteine to form lanthionine. This includes ring opening of serine β-lactones, [116, 117, 118] aziridines, [119, 120] cyclic sulfamidates [121] and β iodoalanine. [122, 123, 124]

2.4.2. Sulfamidates as β-alanyl cation equivalent

Nucleophiles can stereo- and regioselectively open sulfamidates, which are useful synthetic building blocks for the synthesis of lanthionines. [121, 125] Vederas [121] obtained stereochemically pure orthogonally protected lanthionine **67** and methyllanthionine **71** (Scheme 18), by using protected cysteine **68** as a thiol nucleophile which reacts with the cyclic sulfamidate **69** or **72**.

Competing elimination reactions can take place by the attack on the sulphur nucleophiles on the sulfamidates; this can be prevented by blocking the α positions. [125]



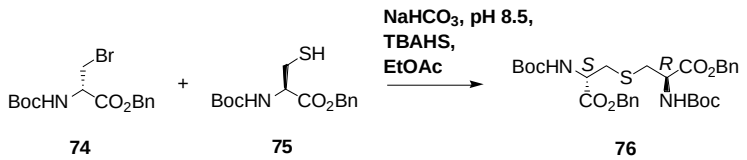
Scheme 18: Synthesis of lanthionine and methyllanthionine using cyclic sulfamidate.

Vederas [121] first attempted these reactions using Fmoc-Cys-O*t*Bu as a thiol nucleophile which gave mixtures of products leading to unsuccessful reactions. This could possibly be due to the Fmoc group being unstable under the basic conditions used. Smith and Goodman [117] observed similar problems when they attempted ring opening of α-methyl-D-serine β-lactone with Fmoc-Cys-OMe and Cs_2CO_3.

2.4.3. β-Bromoalanine derivatives as β-alanyl cation equivalent

Zhu and Schmidt's [102] strategy involved using β-bromoalanine derivatives and phase transfer catalysis (PTC) to synthesise protected lanthionine. β-bromoalanine derivatives have high reactivity towards nucleophiles under mild basic conditions.

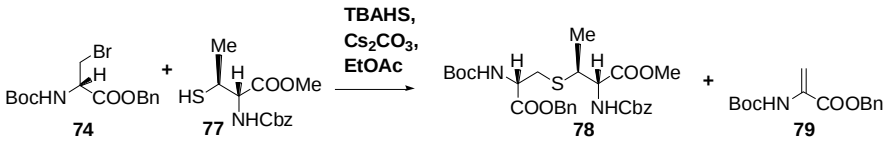
A quantitative yield of (2*S*, 6*R*) *meso* lanthionine **76** was obtained by coupling bromide **74** with Boc-Cys-OBn **75**. The coupling was carried out in the presence of tetrabutyl ammonium hydrogensulfate in ethyl acetate and aqueous solution of $NaHCO_3$ at pH 8.5 (Scheme 19).



Scheme 19: Synthesis of lanthionine using β-bromoalanine.

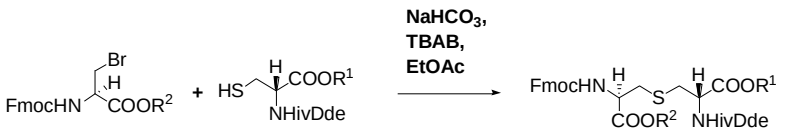
This strategy did not lead to elimination followed by Michael addition and therefore no trace of the opposite diastereoisomer was detected.

Other groups had problems using the above methodology. When VanNieuwenhze [118] attempted synthesising β-methyllanthionine via this strategy, byproduct **79** was produced (Scheme 20). Protected methyl cysteine **77** was *S*-alkylated with carbamates similar to **74** to give lanthionine **78**. The competing elimination reaction was observed due to the reduced nucleophilicity of the methyl cysteine which is more sterically hindered. VanNieuwenhze [118] optimised the conditions including the use of aqueous Cs_2CO_3 however the dehydroalanine byproduct was still produced.



Scheme 20: Synthesis of lanthionine using bromoalanine.

The nature of the protecting groups present on the cysteine is very important for this reaction. N. I. Martin [126] used ivDde protecting group to synthesise a tetra orthogonally protected lanthionine. An Fmoc protected β-bromoalanine was reacted with ivDde-cysteine this produced the desired product (Scheme 21). It was found that the nucleophile TMSE protected cysteine **83** gave better yields than the allyl protected cysteine **82**.

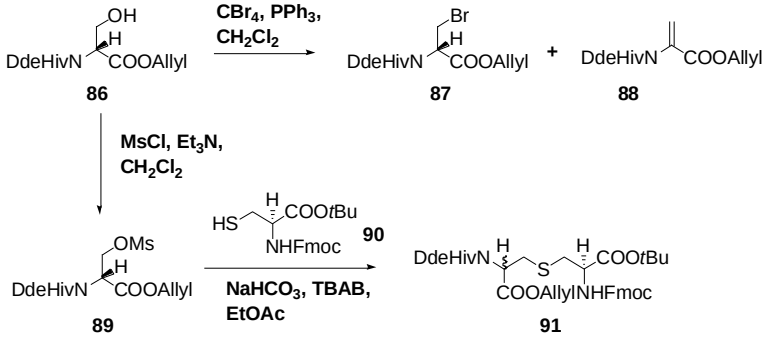


80 R^2 = *t*Bu **82** R^1 = allyl
81 R^2 = allyl **83** R^1 = TMSE

84 R^1 = allyl, R^2 = *t*Bu
85 R^1 = TMSE, R^2 = allyl

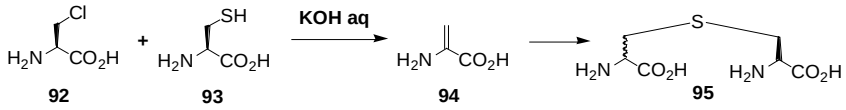
Scheme 21: Lanthionine formation via bromoalanine.

Bromination of **86** to synthesise bromoalanine **87** formed the undesired dehydroalanine product due to the competing elimination reaction occurring. When mesylation of **86** was carried out followed by *S*-alkylation this resulted in racemisation at the C_2 position of the lanthionine to give **91** (Scheme 22).



Scheme 22: Formation of lanthionine via bromoalanine.

β-chloroalanine was also used as a β-alanyl cation equivalent this was reacted with cysteine thiolate by Vigneaud *et al*. [127] to synthesise lanthionine. Racemisation of the lanthionine took place due to strong base and harsh conditions being used. Dha was formed via the elimination reaction followed by Michael addition with cysteine (Scheme 23). [128]

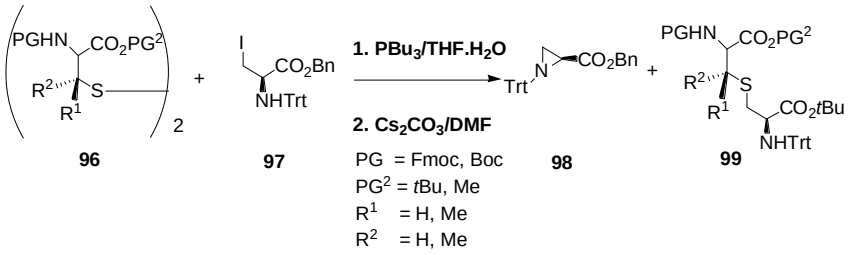


Scheme 23: Racemisation of lanthionine.

2.4.4. Iodoalanine derivatives as β-alanyl cation equivalent

Dugave and Menez [122] have shown that trityl protection of β-iodoalanine prevents α-proton abstraction. It can be removed selectively in the presence of other protecting groups and it protects the α-ester from saponification. The α proton abstraction is prevented due to the trityl group being electron donating compared to protecting groups such as Boc. This makes the αC less acidic preventing dehydration.

The study focused on *N*-trityl-3-iodoalanine benzyl ester **97** that have good reactivity towards heteroatom nucleophiles. Iodide **97** is substituted by L-cysteine to give the lanthionine in good yields in the presence of some aziridine (Scheme 24).

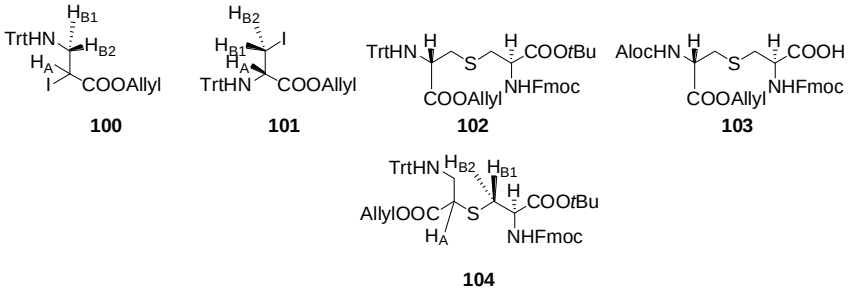


96 **97** **98** **99**

Scheme 24: Synthesis of lanthionine using iodoalanine.

Iodine substitution with thiolates is a good yielding strategy where the trityl group prevent epimerisation of the stereogenic centre, giving the orthogonally protected lanthionine stereoselectively. They reported that the ^{1}H NMR of the *N*-trityl iodoalanine **97** and the lanthionine **99** had two sets of signals, but attributed this to the presence of two rotamers.

However Tabor *et al*. [129] have shown that the strategy used by Dugave and Menez [122] reacting alanyl β-cation equivalent and cysteine is problematic. Synthesis of iodoalanine using this strategy produced two products. The major product synthesised was the undesired regioisomer α-iodo-β-alanine **100** (Figure 34) which may have been formed by the aziridine intermediate, and the minor product being the desired β iodoalanine.



100 **101** **102** **103** **104**

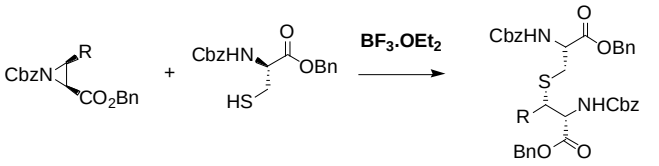
Figure 34: Structures of desired iodoalanine, lanthionine and undesired iodoalanine and lanthionine.

Two isomers were also seen by Dugave and Menez [122] when synthesising iodoalanine **101** but this again was attributed to the presence of rotameric forms of iodoalanine. Tabor *et al*. [129] used the published route, [121] they reacted the iodoalanine with Fmoc-Cys-O*t*Bu in the presence of Cs_2CO_3 to give lanthionine **102**. This was followed by trityl deprotection then Aloc protection which were then followed by *tert*-butyl ester deprotection to give the orthogonally protected lanthionine **103**. Two sets of signals were observed from the ^{1}H NMR

for **102** which was also seen by Dugave and Menez [122] however multiple isomers were seen after removal of the bulky trityl group and replacement of the Aloc group. After preparative HPLC purification two distinct isomers were obtained. The major product was identified as being the nor-lanthionine **104** formed from the α-iodo-β-alanine and the minor product being lanthionine **102** (Scheme 31).

2.4.5. Aziridine as β-alanyl cation equivalent

Nakajima *et al.* [119] reacted cysteine derivative with aziridine **105 a** or methyl substituted aziridine **105 b** to synthesise lanthionine **107 a** and Methyllanthionine **107 b** in low yields (Scheme 25).



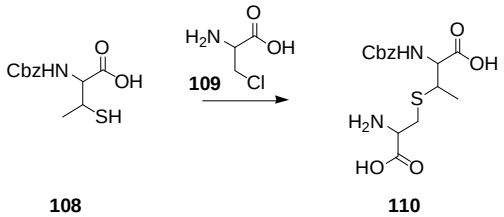
105
105 a R = H
105 b R = CH_3

106

107
107 a R = H
107 b R = CH_3

Scheme 25: Synthesis of lanthionine from aziridine.

Shiba *et al.* [110] used (2*S*, 3*S*) and (2*S*, 3*R*)-3-methyl-D-cysteine protected derivatives **108 a** and **108 b** with β-chloroalanine **109** in D or L form, to form the *meso* lanthionine. The lanthionine was then treated with hydrogen bromide in glacial acetic acid to synthesise methyllanthionine **110** which gave four diastereoisomers (Scheme 26).



108

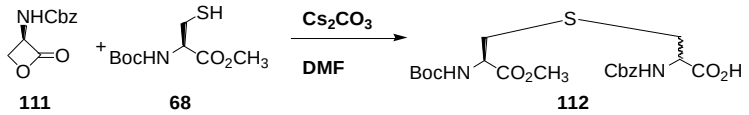
110

108 a = 2*S*,3*S*
108 b = 2*S*, 3*R*
109 a L-isomer
109 b D-isomer
110 a = 2*S*,3*S*,6*R*
110 b = 2*S*,3*S*,6*S*
110 c = 2*S*,3*R*,6*R*
110 d = 2*S*,3*R*,6*S*

Scheme 26: Synthesis of methyllanthionine using β-chloroalanine.

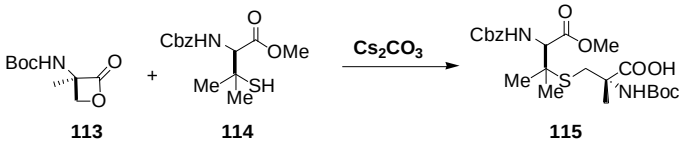
2.4.6. β-Lactone as β-alanyl cation equivalent

Vederas *et al.* [130] and Goodman *et al.* [116] used protected cysteine residues to open serine β-lactones (Scheme 27) to give lanthionine derivatives in moderate yields.



Scheme 27: Synthesis of lanthionine using β-lactone.

The advantage of this method is that the opening of the β-lactones leads to stereochemically pure compounds however the yield obtained was low. Better yields were obtained in the synthesis of 3,3-dimethyllanthionine using the same methodology but in the presence of α-methyl-D-serine β-lactone [117] (Scheme 28).



Scheme 28: Synthesis of methyllanthionine using β-lactone.

2.5. Solid phase peptide synthesis of lantibiotics

Solid phase peptide synthesis (SPPS) involves the synthesis of a peptide on an insoluble support. The advantage of this method is that any unreacted reagents left at the end of each synthetic step can be removed by a simple wash procedure. The method relies on having the α-amino group protected with an Fmoc group, and any reactive side chains orthogonally protected using Boc or *t*Bu protecting groups. The first amino acid is coupled to a linker resin by the carboxylic acid. The Fmoc group is then deprotected leaving a free amino group. This free amino group is then coupled to a second protected amino acid and this repeats to form a sequence. After the desired sequence is produced the peptide is cleaved off the resin (Figure 35).

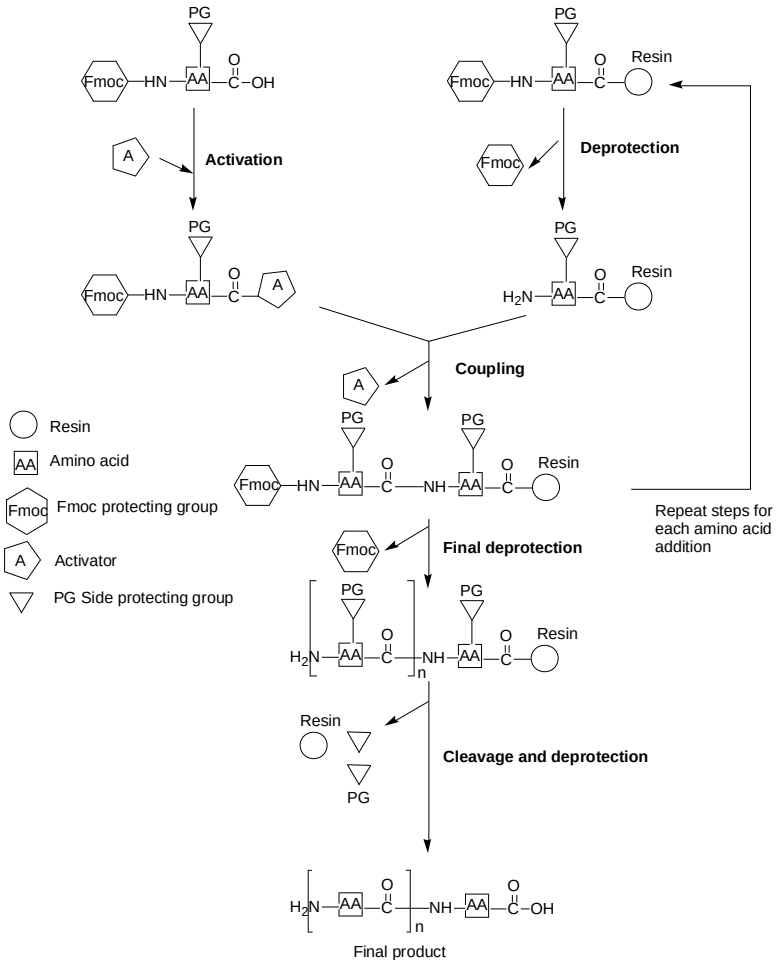


Figure 35: Solid phase peptide synthesis.

2.5.1. On resin cyclisation

On resin cyclisation is advantageous due to the "pseudodilution" effect. The risks of intermolecular reactions are reduced as there are large distances between the growing chains on the solid support. [131] Usually it is good to use low or medium loading as this increases the distance between the growing chains.

2.5.2. Peptide cyclisation on an oxime resin (PCOR)

Polystyrene-bound oxime esters used as supports in SPPS were first developed by DeGrado and Kaiser. [132] Oxime resins can only be used for Boc methodology as the secondary amines used in Fmoc methodology to cleave the Fmoc group can also cleave the growing peptide from the oxime resin.

After the synthesis of the linear peptide TFA is used to cleave protecting groups such as Boc and *t*Bu, any remaining TFA is neutralised by DIPEA. [133] Acetic acid is then used to simultaneously catalyse cyclisation followed by cleavage off the peptide from the resin.

Goodman *et al*. [101] synthesised thio-ether bridged analogues of somatostatin using a cyclisation method on an oxime resin followed by condensation reaction. They used an orthogonally protected lanthionine **116**. The peptide synthesis was carried out using Boc chemistry and BOP activation. The lanthionine was coupled using BOP and DIPEA. Acetic acid catalysis was used to carry out cyclisation after the Boc group was deprotected from the lanthionine and then simultaneously release of the peptide from the resin was carried out.

Scheme 29: [101] **Synthesis of sandostatin an analogue of somatostatin.**

Deprotection reactions were then carried out in solution, HBr/acetic acid was used to deprotect the benzyloxycarbonyl groups from the *N*-terminus and the bzl groups. Elongation of the peptide was carried out by coupling Cbz-DPhe-OH. The methyl ester group was hydrolysed and then the remaining protecting groups were deprotected to give **121** (Scheme 29). [101]

2.5.3. Orthogonally protected lanthionines

Mustapa and Tabor [82, 129] required orthogonally protected lanthionine **103** (Figure 36) for the solid phase peptide synthesis of lanthionine containing peptides. They chose to use Dugave and Menez [122] approach based on the generation of an alanyl β-cation. This route appeared to be advantageous as it was direct and high yielding.

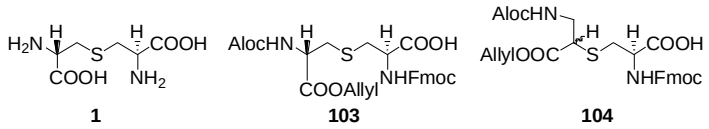
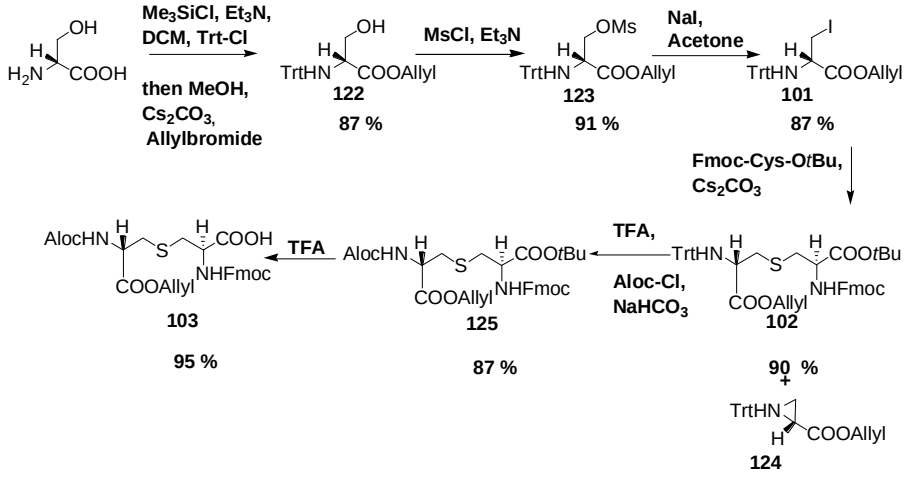


Figure 36: Structures of lanthionine.

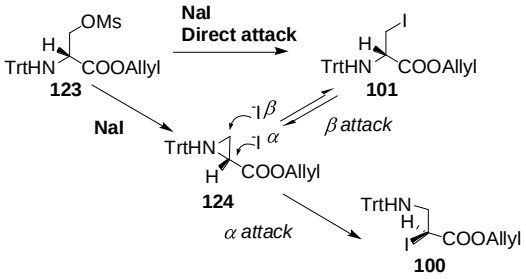
During the synthesis problems occurred due to which a full investigation was carried out on the regio and stereo chemical outcome of the reaction between cysteine and iodoalanine.



Scheme 30: Orthogonally protected lanthionine.

Nucleophilic reaction is known to be most successful at the β-position of serine when the amino group is trityl protected. Serine was therefore converted to **122**. This was mesylated to give **123** and reacted with NaI to give **101** (Scheme 30). [82] From this methodology two distinct peaks were observed in the proton NMR of the purified product **101**. This was attributed by Dugave and Menez [122] to two rotameric forms of the iodoalanines being present. VT experiments of **102** at temperatures up to 100 ^{o}C showed no coalescence of the signals, which indicated that the two sets of signals seen in the ^{1}H NMR spectrum of **102** did not arise from the presence of two rotamers.

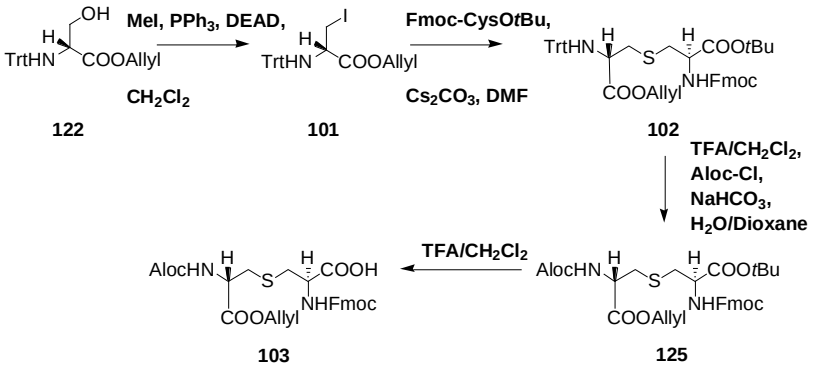
However, it was established that, in fact an intermediate aziridine **124** is formed during the synthesis of **101**, and under these reaction conditions it reacts further to form **100** (Scheme 31). [82]



Scheme 31: Aziridine formation.

2.5.3.1. Lanthionine synthesis via Mitsunobu reaction

It was found that the reaction of aziridine **124** with Fmoc-Cys-O*t*Bu does not lead to either the correct or the incorrect ring-opened product. Therefore it was important to find a way to make compound **101** with minimal aziridine formation with no excess I^- present to ring-open **124** if it is formed. This was done by an alternative synthesis of **101** using the Mitsunobu reaction. The reaction is carried out at low temperature making trityl-β-iodoalanine without forming the undesired major isomer. This is then used for the synthesis of the protected lanthionine (Scheme 32). [124]



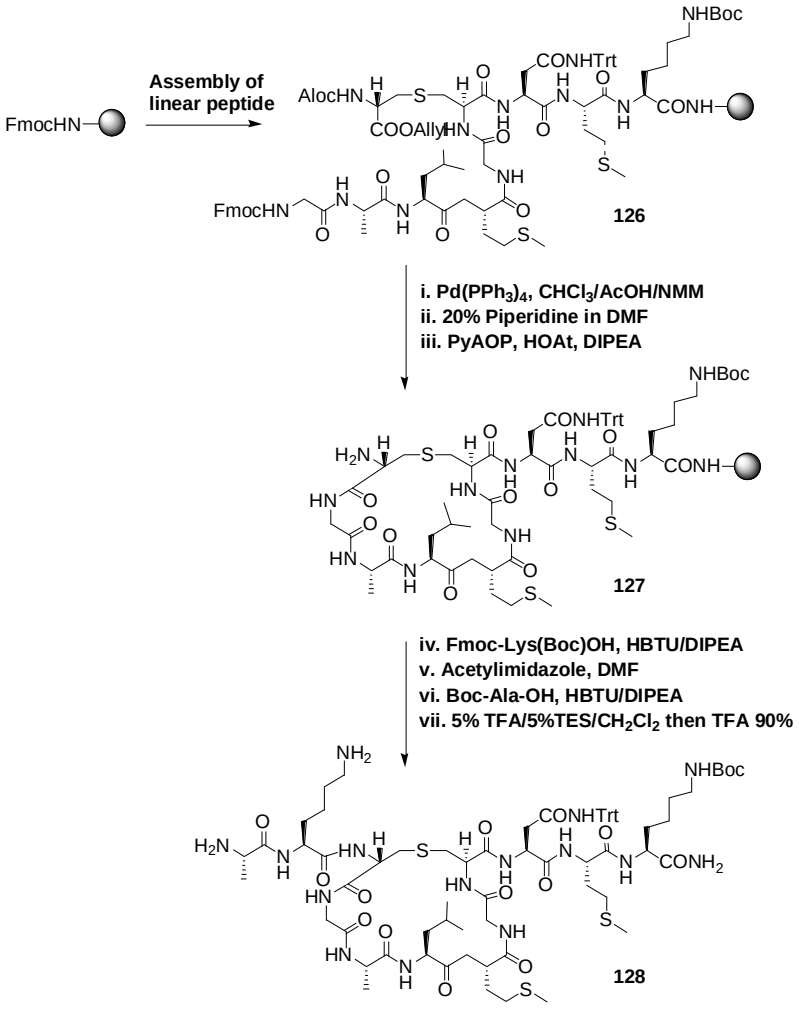
Scheme 32: [124] Orthogonally protected lanthionine.

In this route the production of the undesired regio-isomeric α-iodo-β-alanine was avoided. It reduced the formation of aziridine dramatically however there were traces of it during purification. Lanthionine **102** was obtained by reaction of **101** with Fmoc-Cys-O*t*Bu in the presence of Cs_2CO_3 giving **102**. Removal of the trityl group and replacement with the Aloc

group afforded **125** which were followed by removal of *t*Bu ester to give the desired lanthionine **103**.

2.5.4. Solid phase peptide synthesis of lantibiotics

Bregant and Tabor [124] used Fmoc chemistry to incorporate the orthogonally protected lanthionine **103** into an analogue of ring C of nisin. The linear peptide **126** was first assembled manually on the resin using HBTU and DIPEA to pre-activate and couple the Fmoc protected amino acid. Each amino acid coupling was followed by capping with acetylimidazole and deprotected by 20% piperidine in DMF (Scheme 33). [124]



Scheme 33: Analogue of ring C of nisin.

With palladium (0) the Aloc and allyl protecting groups were removed. Intramolecular cyclisation was carried out by using PyAOP/HOAt after the removal of Fmoc group. The peptide was cleaved off the resin and purified by HPLC.

2.6. Lacticin 3147

Lacticin 3147 is a two-component lantibiotic consisting of two posttranslationally modified peptides A1 and A2 (Figure 37). [134] The lantibiotics individually are very weak antimicrobial peptides, however they have strong antibacterial action synergistically. [3]

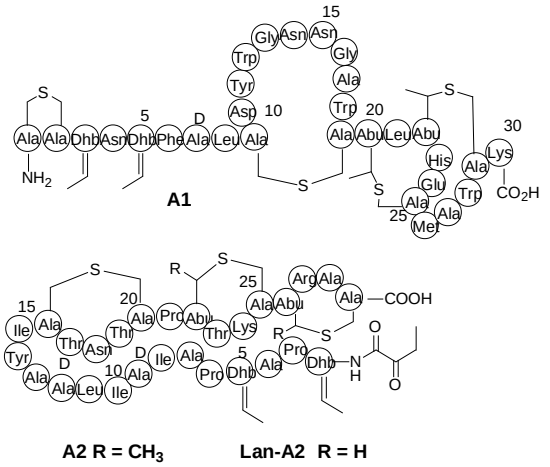


Figure 37: [134] **Structures of Lacticin 3147 components A1 and A2. The bis(desmethyl) lanthionine analogue (R=H) of lacticin A2 is Lan-A2.**

A three component complex is formed, as follows: Lacticin 3147 A1 first binds to lipid II [135] followed by recognition of the complex by A2 resulting in the formation of pores in the cell membrane.

Vederas *et al*. [134] used solid phase methods to synthesise an analogue of A2 where the two β-methyllanthionine rings were replaced with lanthionine rings forming bis(desmethyl) lacticin 3147 Lan-A2 (Figure 37), using orthogonally protected lanthionine **103**.

103 was first attached to chlorotrityl chloride resin by reacting partially capped resin with lanthionine and DIPEA to give **129**. A low loading resin (0.16 mmol g^{-1}) was used to prevent any intermolecular coupling and crosslinking complications which could otherwise take place after the deprotection of the carboxyl and amino group of the lanthionine. Fmoc peptide

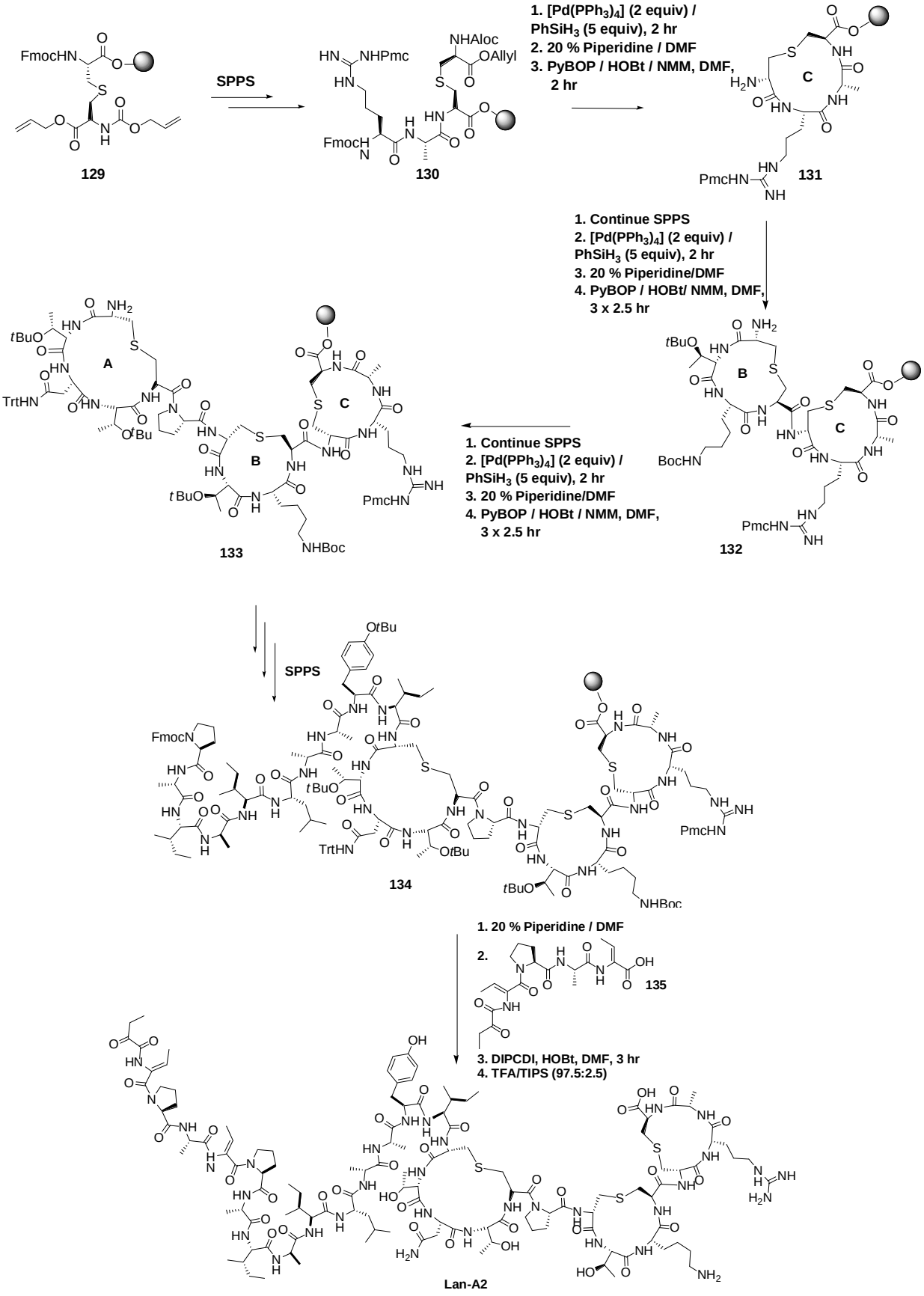
synthesis was carried out for the elongation of the peptide to obtain **130** using PyBOP coupling reagent. Deprotection of Aloc/allyl protecting groups were carried out using Tabor *et al*'s [86] methodology using $[Pd(PPh_3)_4]$ and $PhSiH_3$. Once the Aloc and allyl groups of **130** were deprotected, Fmoc deprotection followed by cyclisation using PyBOP/HOBt was carried out to give ring C of **131**. The linear sequence was continued followed by another Aloc/allyl deprotection to give ring B **132**. The same methodology was used to synthesise ring A giving **133** and then further elongation was carried out by SPPS. Residues 1-5 **135** was synthesised in solution which was then coupled to the peptide followed by cleavage of the peptide of the resin to give Lan-A2 (Scheme 34). [110]

Removal of the methyl groups prevented biological activity thus losing its independent antimicrobial activity.

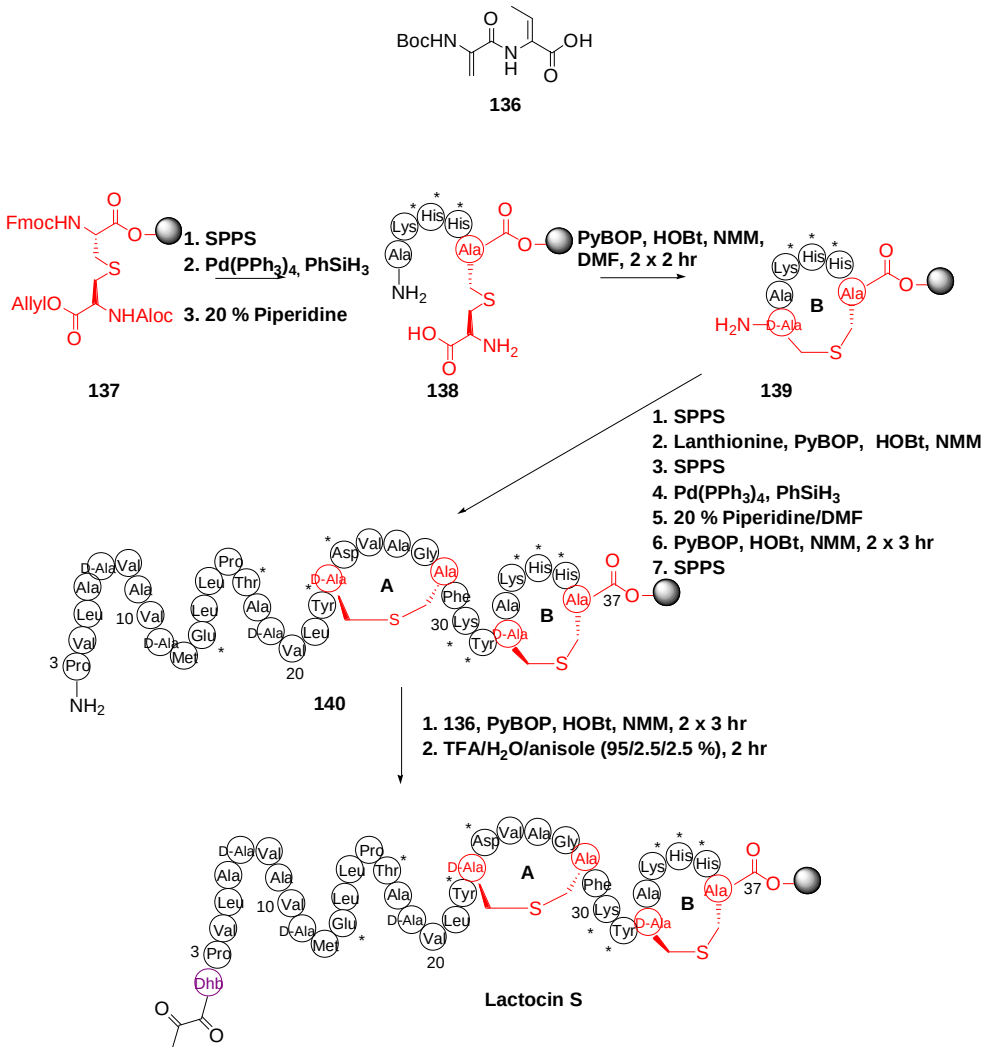
2.7. Lactocin S

Lactocin, a 37 residue lantibiotic, [136] is isolated from *Lactobacillus Sakei*. Vederas *et al*. [136] recently presented the synthesis of the natural lantibiotic. Amino acid **136** was synthesised in solution, whilst the rest of the residues 3-37 were synthesised on solid support using Fmoc SPPS. They coupled the orthogonally protected lanthionine to the 2-chlorotrityl chloride resin. The linear sequence was carried out followed by Fmoc deprotection, Aloc/allyl deprotection using $[Pd(PPh_3)_4]$ and $PhSiH_3$, and then cyclisation with PyBOP and HOBt to give ring B. Elongation between rings A and B were completed by SPPS which included coupling a second Aloc/allyl lanthionine which was deprotected after Fmoc deprotection and then cyclised to give ring A. Further elongation was carried out giving peptide **140** to which **136** was coupled. The peptide was then cleaved to give lactocin S (Scheme 35). [136]

The inhibition growth of *Pediococcus acidlactici* was similar for both the natural and synthetic peptide lactocin S however there was no antibacterial activity against *L. Sakei* L45 the producer organism by either peptide.



Scheme 34: [110] **Synthesis of lacticin.**



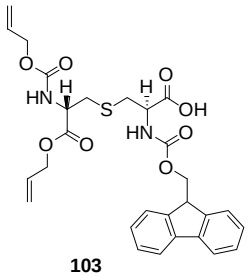
Scheme 35: [136] **Synthesis of lactocin S.**

2.8. Project outline

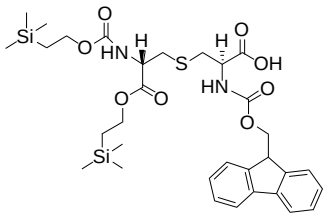
The initial aim of this project was to prepare hybrid lantibiotics by a combination of novel peptide synthesis techniques and bioengineering. Complementary techniques of solid phase peptide synthesis and biosynthesis would have been used to produce hybrid lantibiotics. The *C*-terminal of nisin (the pore forming region) produced by solid phase synthesis, would have been coupled with the lipid II binding lantibiotic mercasidin or actagardine, produced by fermentation.

This would give further insights of the role of each subsection of the original molecule; it may also lead to discovery of more potent and selective antibacterials, and may lead to hybrid lantibiotics.

Therefore it was important to synthesise an analogue of rings D and E of nisin **141** residues 22-29 (Figure 38, 39) using protected lanthionine building blocks. The synthesis of orthogonally protected lanthionines, **103** and **169** was initially required.

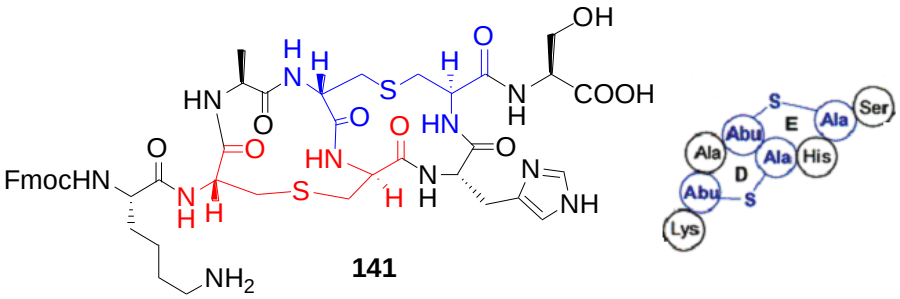


103



169

The aim was then to incorporate both protected lanthionines in a linear peptide precursor by solid phase synthesis. Deprotection of the two protecting groups of the first lanthionine, followed by on-resin cyclisation would then be carried out selectively with the protecting groups on the second lanthionine unaffected. In addition, the protecting groups for both lanthionines had to be stable to both acid and base (Figure 39).



141

Figure 38: Analogue of rings D and E of nisin.

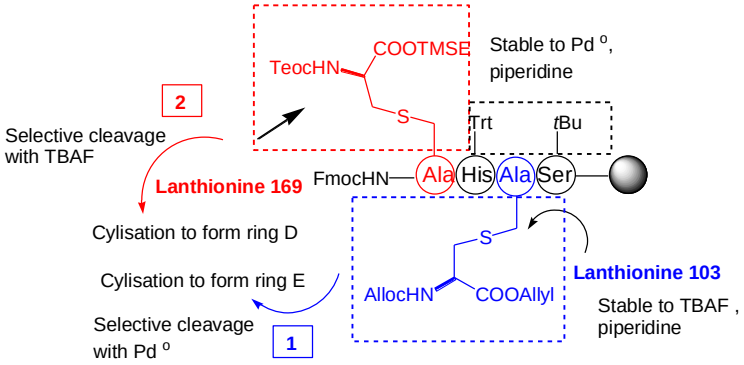
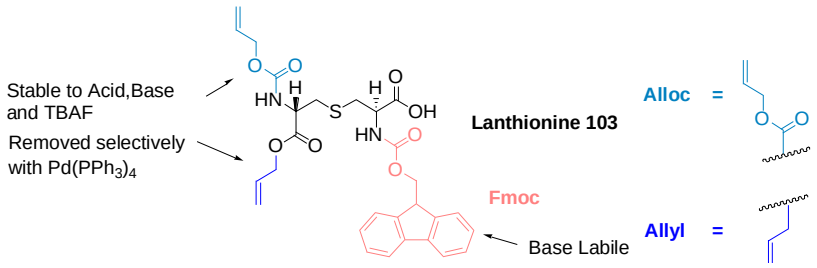


Figure 39: Strategy for synthesising rings D and E.

3. Synthesis of orthogonally protected lanthionine residues

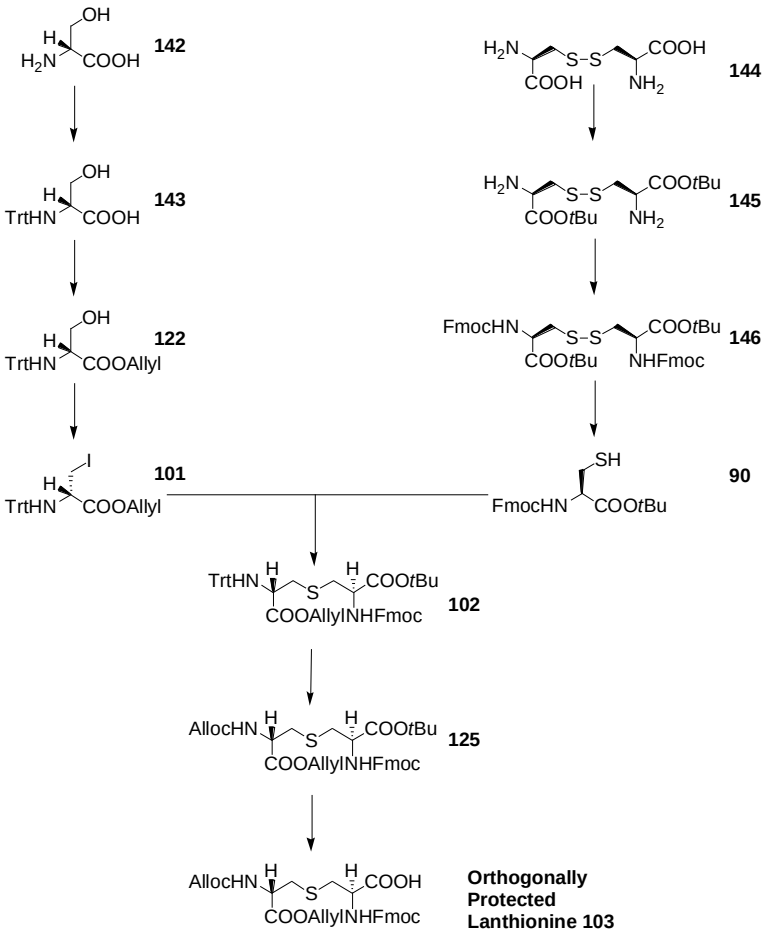
This chapter focuses on the synthesis of the orthogonally protected lanthionine **103** containing Aloc/allyl protecting groups and the orthogonally protected lanthionine **169** containing Teoc/TMSE protecting groups. The synthesis of an analogue of the overlapping rings D and E of nisin, using these lanthionine building blocks, will be discussed in chapter 4.

3.1. (Aloc, allyl) / (Fmoc, OH) lanthionine 103



3.1.1. Aloc/allyl lanthionine residue

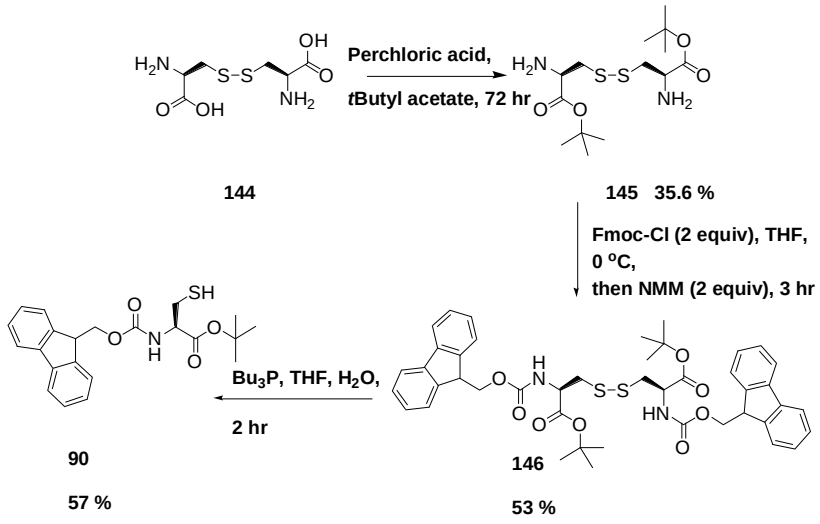
To synthesise (Aloc, allyl) / (Fmoc, OH) lanthionine **103**, the route developed by Bregant and Tabor, [124] via β-iodoalanine **101**, was used. Scheme 36 shows the planned route for the synthesis of (Aloc, allyl) / (Fmoc, OH) lanthionine which will be discussed in this section.



Scheme 36: Planned route for the (Aloc, allyl) / (Fmoc, OH) lanthionine.

3.1.1.1. Synthesis of protected cysteine

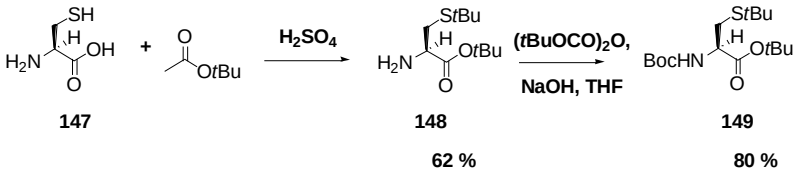
To synthesise the protected cysteine **90** three different reactions were carried out. The reactions involved the synthesis of *tert*-butyl protected cysteine which was followed by Fmoc protection and then cleavage of the disulfide bond.



Scheme 37: Synthesis of Fmoc-Cys-O*t*Bu.

L-cystine was converted to the bis-*tert*-butyl ester using *tert*-butyl acetate (Scheme 37). Purification was not required at this stage as ^{1}H NMR showed that the product **145** obtained was pure. It was important to make sure that the next reaction was carried out quickly to avoid degradation of the compound which was visible by the change of colour.

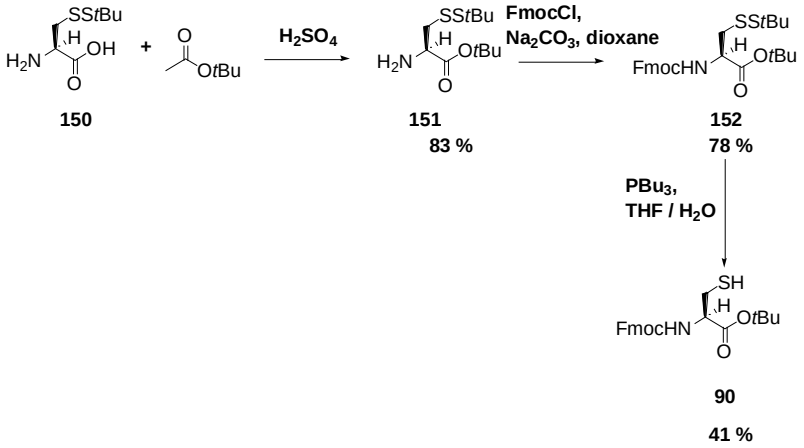
Perchloric acid has a pKa of -7.0 and is therefore highly corrosive, furthermore there is a high risk of explosion of metal perchlorates. For a safer approach the protection of the carboxylic acids were first attempted using sulphuric acid. [137] However previous work [137] in the group had shown that the thiol group was also protected in the reaction giving **149** (Scheme 38).



Scheme 38: Synthesis of thiol protected cysteine.

M. F. A. Groussier [137] then attempted the reaction with *S-tert*-butylthiocysteine. Here the thiol group was first protected to avoid *t*Bu protection of the thiol. *t*Bu protection of the acid was then carried out this was followed by Fmoc protection of the free amine. These steps proceeded in high yields; however the final step involving the disulfide cleavage to give the

free thiol gave low yields. One of the major problems in this step was the formation of *tert*-butyl thiol as a byproduct which was difficult to separate during purification (Scheme 39).



Scheme 39: Synthesis of Fmoc-Cys-O*t*Bu.

The free amine of *t*-butyl protected cysteine was Fmoc protected with Fmoc-Cl in the presence of *N*-methylmorpholine (Scheme 37). Recrystallisation from CH_2Cl_2: methanol did not give the pure product **146**. Instead ^{1}H NMR spectroscopy showed the presence of Fm-OH which recrystallised from the quenching of the excess reagent. Due to this purification by flash column chromatography was carried out in hexane and ether.

The final reaction involved the cleavage of the disulfide bond of cystine with tributyl phosphine (Scheme 37). It was found that addition of excess water after the addition of tributyl phosphine made the cleavage step more effective. The reaction was described as "wet THF" by Dugave and Menez [122] however it was found that higher yields were produced with a large excess of water in a ratio of 9:1 THF: water. The mechanism below shows how water plays a key role in this reaction (Figure 40). Product **90** was obtained as a white solid after purification by flash column chromatography.

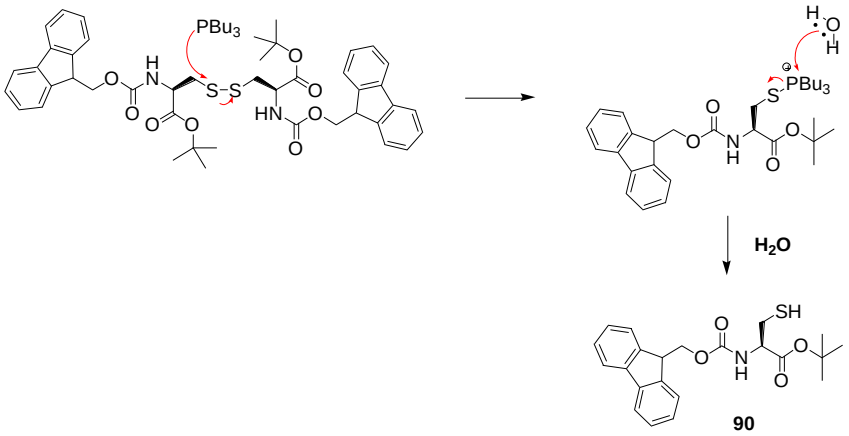
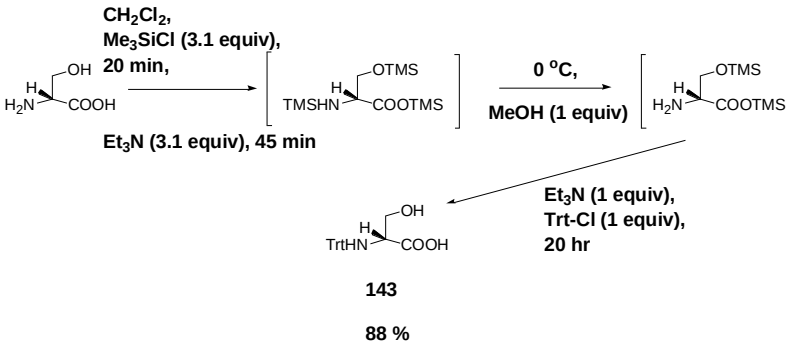


Figure 40: Mechanism for the disulfide cleavage.

3.1.1.2. Synthesis of iodoalanine

In order to synthesise the protected lanthionine, β-iodoalanine **101** was also required. The iodoalanine was synthesised from *D*-serine using experimental procedures developed previously. [82, 124, 129] Synthesis of the β-iodoalanine also involved 3 reactions, trityl protection of the amino group, allyl protection of the carboxylic group and the synthesis of the desired iodoalanine via Mitsunobu reaction.

3.1.1.2.1. Synthesis of trityl serine

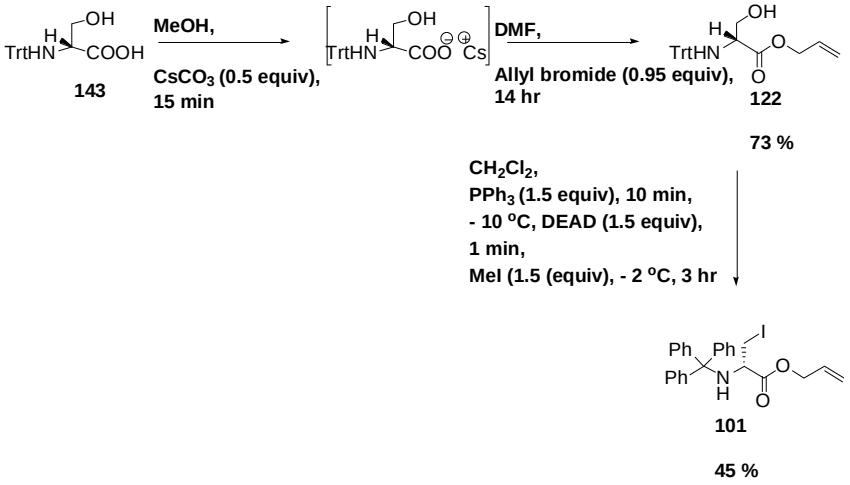


Scheme 40: Synthesis of trityl serine.

The synthetic route to the iodoalanine required the carboxylic acid and the amino group to be differentially protected. The 'one pot' approach of Barlos *et al.* [138] was used to selectively protect the amine with a trityl group in the presence of the -OH and carboxylic groups. In this reaction all functional groups are initially protected with trimethylsilyl chloride. This was followed by selective deprotection of the amine using 1 equiv of methanol, followed by

addition of the trityl group to the free amine. The final step of the reaction involved addition of excess triethylamine and methanol. This was to remove the trimethylsilyl group from the carboxylic acid and alcohol.

3.1.1.2.2. Synthesis of allyl iodoalanine



Scheme 41: Synthesis of iodoalanine 101.

The next step involved esterification of **143** to give **122**. Addition of allyl bromide to the salt resulted in allyl protection of the carboxylic acid (Scheme 41) in good yield. [139] ^{1}H NMR spectroscopy showed the product to be pure and therefore no further purification was carried out. Other methods such as using triethylamine as a base were previously explored however these only give yields less than 50 %. [140]

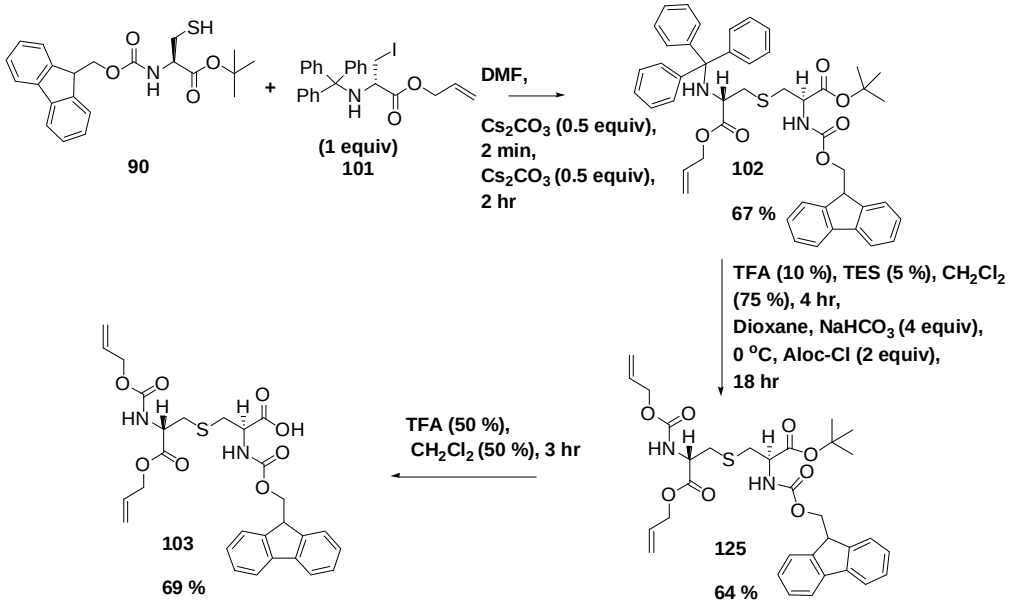
The key intermediate *N*-trityl-β-iodo alanine **101** was formed by carrying out a Mitsunobu reaction from the alcohol **122** directly from the protected serine. In order to avoid synthesis of the undesired α-iodo-β alanine [82] Mitsunobu reaction was carried out (section 2.5.3, Scheme 31).

The reaction proceeded under inert conditions and at low temperature. The temperature was kept stable using an ethanol bath refrigerated by a cryostat. Purification was carried out immediately to avoid aziridine formation, where the whole reaction mixture was directly filtered by flash column chromatography in hexane and ethyl acetate. Traces of aziridine

were seen in tiny amounts in the ^{1}H NMR spectra. This was simply due to difficulty in separating iodoalanine and aziridine during purification.

The reaction was found to be very low yielding, after several attempts 45 % yield was obtained compared to the literature yield 72 %. [124] This may possibly be due to the reaction not going to completion. Starting material **122** (Scheme 41) was easily separated by column chromatography which can be reused. Long reaction times and the use of polar solvents during purification led to the formation of the undesired aziridine. In order to minimise this undesirable effect, the reaction was stopped after 3 hr and a high ratio of 20: 1 hexane: ethyl acetate was used for the flash column chromatography.

3.1.1.3. Synthesis of (Aloc, allyl / Fmoc, OH) Lanthionine 103



Scheme 42: (Aloc, allyl) / (Fmoc, OH) lanthionine.

Using the published method [124] coupling of the protected cysteine and iodoalanine was carried out under inert atmosphere which led to the synthesis of lanthionine **102**. Intermediates **90** and **101** (Scheme 42) were dissolved in DMF, followed by addition of dry Cs_2CO_3. It was very important to use very dry Cs_2CO_3 to avoid any Fmoc deprotection from the cysteine. [116] It was also important to carry out an extraction once the reaction was complete, again to avoid Fmoc deprotection.

An Aloc group was then required in the place of the trityl group. The synthetic pathway required the cleavage of the trityl group and replacement with an Aloc group. The substitution was carried out in a 'one pot' reaction.

The trityl deprotection was carried out using TFA and triethylsilane (TES) in CH_2Cl_2. Here the TES traps the triphenylmethyl carbocation by acting as a scavenger and preventing any reverse reactions. 10 % TFA was used for the trityl removal which was not sufficient for the *t*-butyl deprotection. After an extraction, the reaction was carried out in dioxane and sodium bicarbonate; this was followed by addition of allyl chloroformate to protect the free amine (Scheme 42). After purification 64 % yield was obtained. TLC and ^{1}H NMR spectroscopy showed that the reaction did not go to completion which explains the yield obtained. One reason could have been due to the pH of the solution being slightly acidic and not basic before the addition of allyl chloroformate.

Many problems have been encountered in obtaining good yields. The solutions to the problems were carried out and the yield has been improved from initial 18 % to a reproducible 64 %. This involved carrying out the reaction under inert atmosphere, under argon and ensuring that the trityl group has been removed completely. It was also important that the mixture was between pH 7 and pH 8 before the addition of allyl chloroformate and cold temperature was essential for the reaction. Strict repetition of these precautions led to reproducible yields.

The final step to synthesising the orthogonally protected lanthionine involved removing the *tert*-butyl group. Cleavage of the *tert*-butyl group was carried out using 50 % TFA in CH_2Cl_2 over 3 hr and the product purified using reverse phase column chromatography, overall giving 69 % product. The material was then ready for incorporation into solid phase peptide synthesis with the desired orthogonal protecting groups in place.

In summary (Aloc, allyl / Fmoc, OH) lanthionine **103** has been synthesised using the published procedure, [124] ready for incorporation into cyclic peptides using solid phase peptide synthesis.

3.2. Second lanthionine

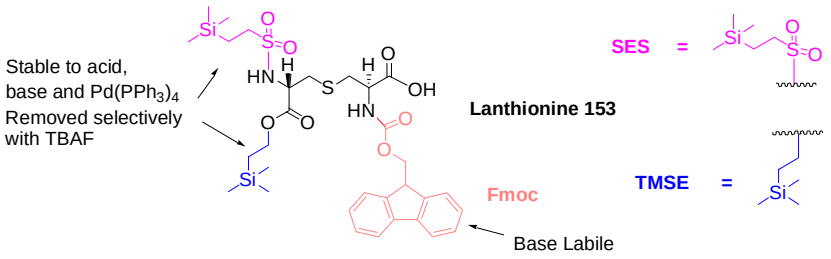
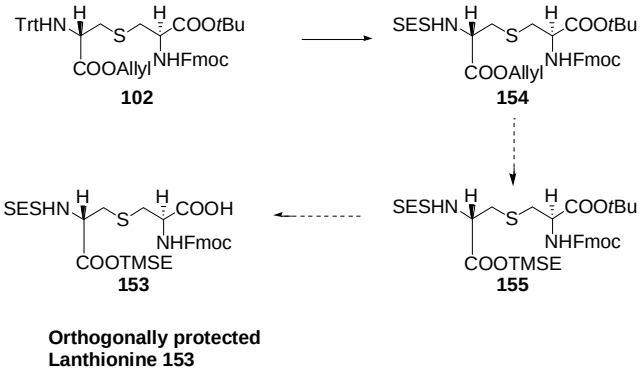


Figure 41: Structure of initial second lanthionine.

The original aim of the project involved synthesising the second orthogonally protected lanthionine **153** containing 2-trimethylsilanyl-ethanesulfonic acid amide (SES) and 2-trimethylsilanyl-ethyl ester (TMSE) protecting groups (Figure 41, Scheme 43). Silyl-based protecting groups are orthogonal to Aloc and allyl protecting groups. The purpose of choosing these protecting groups was to avoid deprotection of the protecting groups of the second lanthionine when Aloc and allyl deprotection was carried out. These silyl groups are stable to acid, base and $Pd(PPh_3)_4$, and they are selectively removed by F^-, for example using tetrabutyl ammonium fluoride (TBAF).

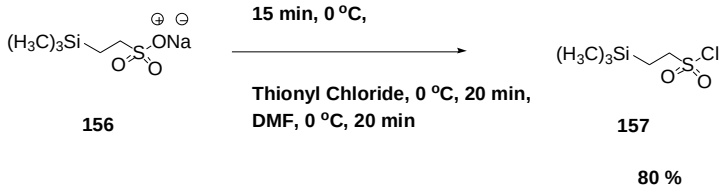
Synthesis of lanthionine **153** was planned to follow the same reaction sequence as the synthesis of lanthionine **103**. It was envisaged that Iodoalanine **101** and cysteine **90** would be coupled, as before to give lanthionine **102**. This would be followed by the replacement of the trityl group with an SES protecting group. Scheme 43 shows the planned route to the synthesis of the second lanthionine.



Scheme 43: Planned route for synthesis of (SES, TMSE) / (Fmoc, OH) lanthionine 153.

3.2.1. Synthesis of 2-trimethylsilanyl-ethanesulfonyl chloride

In order to synthesise the second orthogonally protected lanthionine containing the SES and TMSE protecting groups, it was necessary to synthesise the SES-Cl reagent, as this was not commercially available at the start of the project. The procedure of Weinreb [141] and Bregant, [142] using 2-(trimethylsilyl)ethane sulfonic acid sodium salt, was used to synthesise SES-Cl (Scheme 44).



Scheme 44: Synthesis of SES-Cl.

Purification was attempted via distillation using a hot oil bath with the aid of a heat gun to separate the product and any remaining $SOCl_2$. This was a problem for small scale reactions due to charring of the small amount of crude **157**. Distillation was therefore carried out using a hot water bath and vacuum to give **157**. Confirmation of the product was obtained by mass spectrometry, a peak was seen at m/z 257 $[M + K]^+$. ^1H NMR indicated the presence of residual DMF in the product. Attempts to remove this *in vacuo* led to problems, as during the removal of DMF an impurity was formed. Longer exposure to vacuum increased the intensity of the impurity peaks.

The mechanism for the formation of SES-Cl by DMF catalysed reaction of the sulfonate with thionyl chloride is shown below (Figure 42).

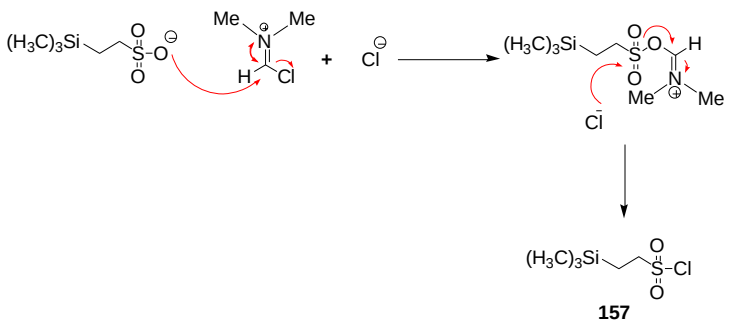
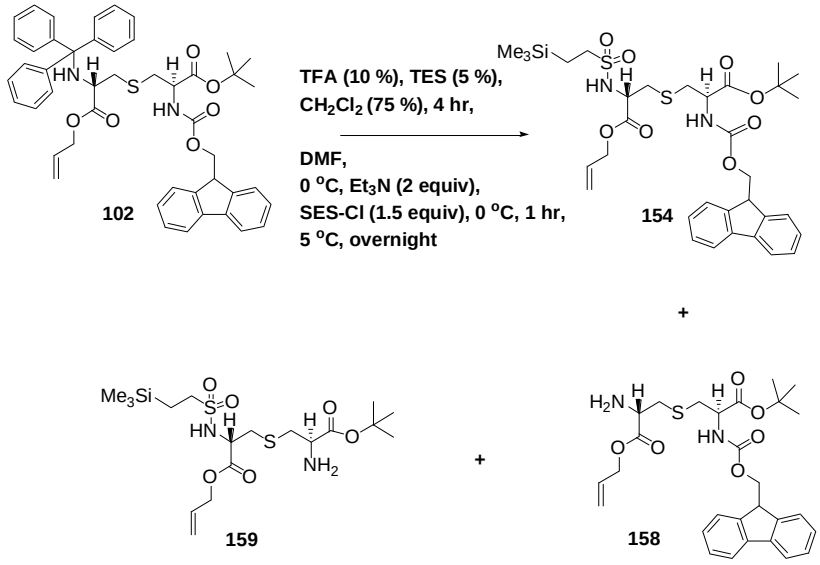


Figure 42: Mechanism for the formation of SES-Cl.

3.2.2. Synthesis of (SES, allyl) / (Fmoc, *t*Bu) lanthionine



Scheme 45: Synthesis of (SES, allyl) / (Fmoc, *t*Bu) lanthionine.

With SES-Cl in hand, the synthesis of the second orthogonally protected lanthionine was attempted. This involved replacing the trityl group of the common intermediate **102** with the SES group. Several attempts were carried out to synthesise **154** using SES-Cl **157** (Table 3).

In the first attempt on (Table 3, entry 1) the desired product was not synthesised, instead from ^{1}H NMR spectroscopy, peaks correlating to the free amine **158** were observed.

The second attempt involved reversing the order of addition, adding 2 equivalents of triethylamine followed by the addition of SES-Cl **157** in DMF (Table 3, entry 2). The desired product **154** was synthesised in 30 % yield along with the intermediate **158** in 44 % yields. The procedure was repeated a couple of times but the yields were variable and low. The reaction was attempted using 5 equivalents of SES-Cl rather than 1.5 equivalents (Table 3, entry 3), which gave 38 % yield, this was the best yield obtained by far however it was not reproducible.

Entry	Trityl deprotection / Time	Reaction solvents	Base (equiv)	SES-Cl, 157 (equiv)	Reaction time / temperature	Product Isolated 154 (%)	Byproduct Isolated 158 (%)	159 (%)
1	TFA/TES/ CH_2Cl_2, 4 hr	DMF	Triethylamine, (2)	(1.5)	1 hr, 0 °C, 24 hr, 5 °C	-	38 %	-
2	TFA/TES/ CH_2Cl_2, 4 hr	DMF	Triethylamine, (2)	(1.5)	1 hr, 0 °C, 24 hr, 5 °C	30 %	44 %	-
3	TFA/TES/ CH_2Cl_2, 4 hr	DMF	Triethylamine, (2)	(5)	1 hr, , 0 °C, 24 hr, 5 °C	38 %	-	-
4	TFA/TES/ CH_2Cl_2, 4 hr	DMF	Triethylamine, (2)	(2.6)	1 hr 30 min, 0 °C, 1 hr 30 min, 5 °C, 40 min, RT	27 %	45 %	-
5	TFA/TES/ CH_2Cl_2, 4 hr	DMF	Triethylamine, (3)	(1.5)	24 hr, 5 °C	-	-	35 %
6	TFA/TES/ CH_2Cl_2, 4 hr	DMF	Triethylamine, (3)	(1.5)	24 hr, 5 °C	NR *	NR *	NR *
7	TFA/TES/ CH_2Cl_2, 4 hr	1,4-Dioxane	Sodium bicarbonate, (4)	(3)	24 hr, 4 °C	26 %	-	-
8	TFA/TES/ CH_2Cl_2, 4 hr	DMF	Triethylamine, (2) Triethylamine, (2)	(5) (5)	3 hr, RT 1 hr 30 min, RT	35 %	-	-
9	TFA/TES/ CH_2Cl_2, 4 hr	DMF	Triethylamine, (1.5)	(1.6)	5 hr, 0 °C – RT	12 %	-	-
10	TFA/TES/ CH_2Cl_2, 4 hr	Benzene	AgCN, (1.5)	(1.5)	22 hr, 60 °C	-	30 %	-
11	TFA/TES/ CH_2Cl_2, 4 hr	Acetonitrile	4-methoxy pyridine-N-oxide hydrate, (6)	(2)	24 hr, RT	19 %	-	-

* NR : No Reaction

Table 3: Conditions used for the synthesis of (SES, allyl) / (Fmoc, *t*Bu) lanthionine.

When 2.6 equivalents of SES-Cl were used (Table 3, entry 4), mass spectrometry and COSY NMR confirmed that the product was obtained in 27 % and ^{1}H NMR indicated that intermediate **158** was isolated. Increasing the equivalents of triethylamine (Table 3, entry 5) resulted in base-catalysed cleavage of the Fmoc group giving **159**. Hence the number of equivalents of the triethylamine could not be increased**.**

There was a possibility that the low yield could be due to traces of TFA being present from the deprotection of the trityl group. Once excess toluene was added and removed *in vacuo* 3 times followed by CH_2Cl_2. The residue was dissolved in CH_2Cl_2 and stirred with sodium bicarbonate for 1 hr 30 min (Table 3, entry 6). This was to remove any traces of TFA which could be present. The procedure for placing the SES group was the same as (Table 3, entry 5), after purification it was discovered only starting material **102** was isolated in 90 % yield.

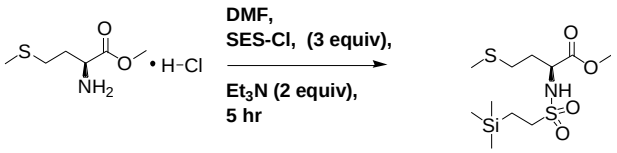
The reaction was also attempted using identical conditions to those used for the synthesis of (Aloc, allyl) / (Fmoc, *t*Bu) lanthionine **125** (Table 3, entry 7). However this did not improve the yield, with a maximum of 26 % yield obtained.

Conditions from Table 3, entry 8 were then attempted this gave 35 % yield however this was not reproducible. Only 12 % yield was obtained when the reaction was carried out using conditions from Table 3, entry 9.

After several attempts of using previously prepared SES-Cl, SES-Cl became commercially available. All previous attempts were then repeated using the commercially available material. It was envisaged that the impurities that may appear due to removing DMF might have caused the poor yields. However this was not the case, as the reactions using the commercially available SES-Cl also gave very poor yields. Moreover, the ^{1}H NMR spectrum of the commercially available reagent also showed several impurities.

3.2.3. Trial protection of Met-OMe with SES-Cl

SES protection of (H, allyl) / (Fmoc, *t*Bu) lanthionine **158** does occur however in poor yields. To optimise protection of the free amines with SES-Cl, reactions were carried out on L-methionine methyl ester **160** using both the commercially available SES-Cl and **157**.



Scheme 46: Synthesis of SES-Met-OMe.

Initially, L-methionine methyl ester hydrochloride **160** was dissolved in DMF; SES-Cl was added, followed by 2 equiv triethylamine (Scheme 46, Table 4, entry 1). The desired product **161** was isolated in 71 % yield. Better yields were obtained when only 2 equiv of triethylamine was added to the reaction to synthesise **161** (Table 4, entry 1) compared to the synthesis of **154** (Table 3, Entry 1, 2, 3 and 4).

After concluding that triethylamine was not the best condition to use, attempts were carried out to install the SES protecting group using other conditions. Hale [143] found that the use of AgCN to introduce the SES group gave higher yields compared to the traditional amine catalysed protocols which lead to slower and more problematic issues of side reactions.

Entry	Reaction solvent	Base (equiv)	SES-Cl, 157 (equiv)	Reaction time / temperature	Product Isolated 161 (%)
1	DMF	Triethylamine, (2)	(3)	5 hr, RT	71 %
2	Benzene	AgCN, (1)	(1.5)	22 hr, 75 °C	42 %
3	Acetonitrile	4-methoxy pyridine-N-oxide hydrate, (6)	(1.5)	24 hr, RT	45 %

Table 4: Conditions used for the synthesis of SES-Met-OMe

Therefore these conditions were tested on Met-OMe.HCl (Table 4, entry 2); however the product was isolated in only 42 % yield.

The reaction was then attempted on lanthionine **102** (Table 3, entry 10). Two different fractions collected from chromatography were analysed. From ^{1}H NMR it was observed that starting material **102** was isolated where the trityl group did not deprotect after exposure to TFA, TES and CH_2Cl_2. The second fraction showed intermediate **158** was synthesised. The two observations indicated that the reaction between the free amine and SES-Cl did not take place. The failure of this reaction could be due to steric hindrance or due to partial deprotection of the trityl group taking place.

160 and 6 equivalents of 4-methoxy pyridine-N-oxide hydrate **162** were reacted in acetonitrile. [144] 2 equivalents of SES-Cl were added (Table 4, entry 3) and the product **161** was isolated by flash column chromatography in 45 % yield.

The protocol was then attempted on lanthionine **102**. After trityl deprotection of **102**, the residual amine **158** and 4-methoxy pyridine-N-oxide hydrate **162** were dissolved in dry acetonitrile, SES-Cl was added and stirred for 24 hr. The reaction was quenched with saturated sodium bicarbonate which was extracted with CH_2Cl_2. The product was isolated via

flash column chromatography giving **154** in 19 % yield (Table 3, entry 11). The mechanism of the reaction can be seen in Figure 43.

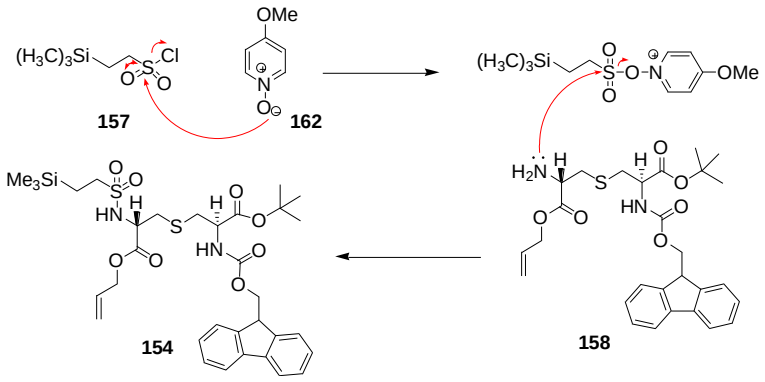


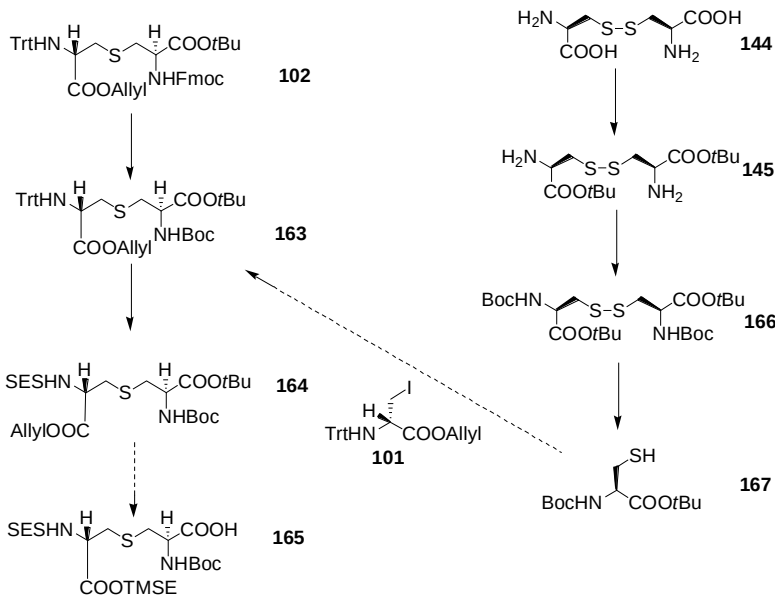
Figure 43: Mechanism of SES protection.

Van der Laan *et al*. [144] obtained 75 % yield for the protection of their compound. When the same conditions were used on methionine 45 % yield was obtained and only 19 % for the lanthionine. The reaction did not proceed very well possibly due to steric hindrance.

From the reactions above it can be concluded that maybe the SES-Cl reagent is not reactive enough to be used as a protecting group for this particular amine. Also increasing the number of equivalents of triethylamine was problematic as this caused deprotection of the Fmoc group forming **159**. Various reactions with different conditions were carried out, however all yields obtained were very poor.

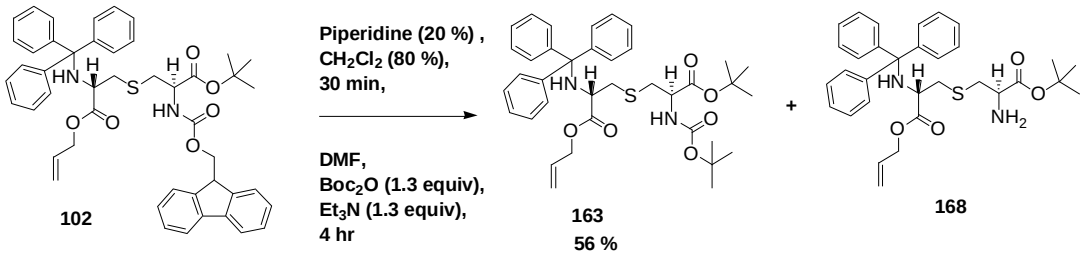
3.2.4. Boc Lanthionine

Changes in the protecting groups were considered to avoid deprotection of the Fmoc group and low yield reactions. The amino group was Boc protected instead of Fmoc protection (Scheme 47).



Scheme 47: Second planned route for the (SES, TMSE) / (Fmoc, OH) lanthionine.

3.2.4.1. Synthesis of (Trt, allyl) / (Boc, *t*Bu) lanthionine



Scheme 48: Removal of the Fmoc group and protection with Boc group.

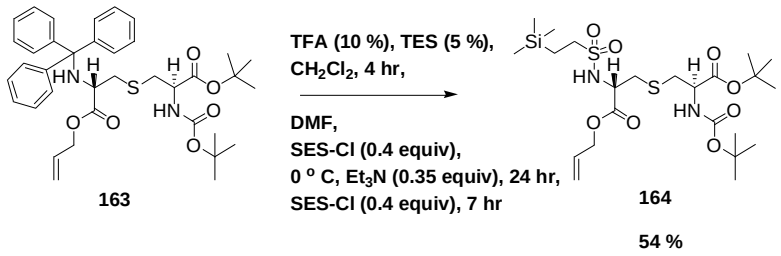
Entry	Fmoc deprotection solvents / Time	Reaction solvent	Base (equiv)	Boc anhydride (equiv)	Reaction time / temperature	Product Isolated 163 (%)	Byproduct Isolated 168 (%)
1	20% piperidine in CH_2Cl_2, 30 min	CH_2Cl_2	Sodium bicarbonate, (1.3)	(1.3)	30 min, RT 4 hr, RT	3 %	65 %
2	20% piperidine in CH_2Cl_2, 30 min	DMF	Triethylamine, (1.3)	(1.3)	4 hr, RT	56 %	-

Table 5: Conditions used for the synthesis of (Trt, allyl) / (Boc, *t*Bu) lanthionine.

Fmoc deprotection was carried out followed by Boc protection to give product **163** in only 3 % which meant very little Boc protection took place, starting material **102** was also isolated in 20 % and the free amine **168** (Table 5, entry 1).

Boc protection was attempted again but in DMF instead of CH_2Cl_2, and triethylamine was used rather than sodium bicarbonate (Scheme 48, Table 5, entry 2). It was found that the reaction works better in DMF then it does in CH_2Cl_2; the product was isolated in 56 % yield. Next SES protection on the amino group of the lanthionine **163** was carried out.

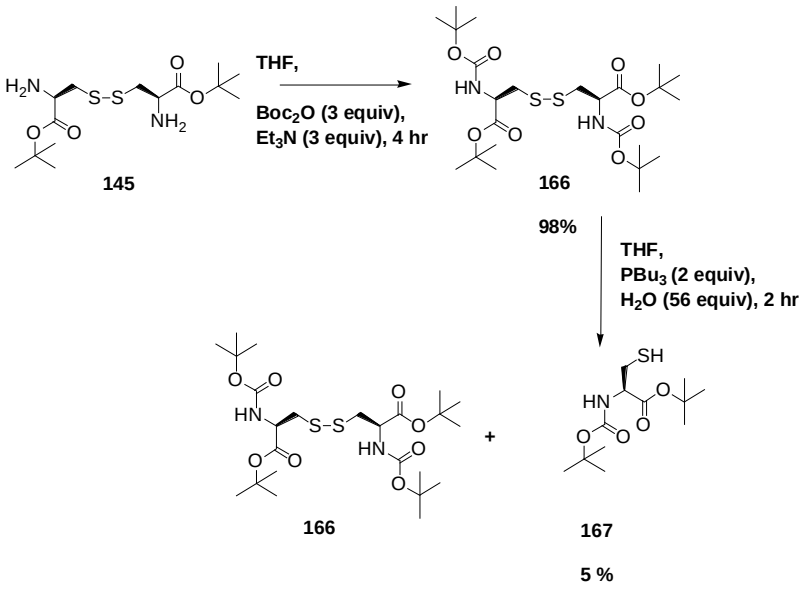
3.2.4.2. Synthesis of (SES, allyl) / (Boc, *t*Bu) lanthionine



Scheme 49: SES protection.

After trityl deprotection, the residue was suspended in DMF to which SES-Cl was added and cooled to 0 ºC. Triethylamine was added and the reaction was stirred for 24 hr; further SES-Cl and triethylamine was then added and the reaction was stirred for a further 7 hr. After purification 54 % yield of the desired product was obtained (Scheme 49). Comparison of the yields obtained by Boc and Fmoc protection, indicated that Boc protection increased the yield of the desired product.

3.2.5. Synthesis of Boc and *t*Bu protected cysteine



Scheme 50: *t*Bu and Boc protected cystine.

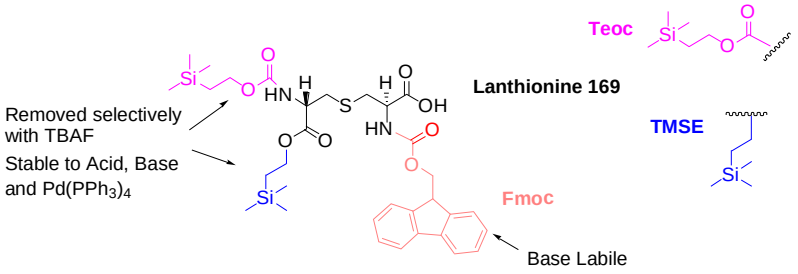
Large amounts of Fmoc-Cl were used for the protection of the amino groups of the cysteine (Scheme 37). Fmoc deprotection of lanthionine **163** is required followed by protection with Boc group (Scheme 48), before SES protection can be carried out. In order to avoid using Fmoc-Cl which would be a waste during scale up and time consuming, synthesis of the Boc and *t*Bu protected cysteine was carried out (Scheme 50). Compound **145** was synthesised from **144** using previous experimental conditions (Scheme 37). **145** were then dissolved in THF to which Boc anhydride was added followed by triethylamine (Scheme 50). A yield of 98 % was obtained.

Trial reactions were carried out to cleave the disulfide bond, the same conditions used for the formation of **90** was used (Scheme 37). From mass spectrometry and ^{1}H NMR it was seen that mainly the starting material was recovered. The product was isolated but at a very low yield along with oxidised tributylphosphine (Appendix 1).

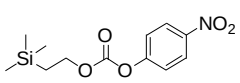
At this point the SES-Cl became unavailable from the commercial suppliers and due to the difficulties encountered in either synthesising pure SES-Cl or in finding a reliable supplier of

pure SES-Cl it was decided to investigate the use of alternative silyl protecting groups for the α-NH_2.

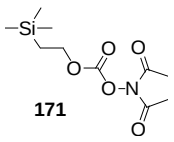
3.2.6. New silyl protecting groups



For protection of the amino and carboxylic acid moieties, β (trimethylsilyl)ethoxycarbonyl (Teoc) and trimethylsilylethyl (TMSE) groups were used repectively, because TBAF can easily remove these silyl-based protecting groups under mild conditions and at a neutral pH. Two reagents, 4-nitrophenyl 2-(trimethylsilyl)ethyl carbonate (Teoc-ONp) **170** and 1-[2-(Trimethylsilyl)ethoxycarbonyloxy]pyrrolidin-2,5-dione (Teoc-OSu) **171** were used to install the Teoc protecting group.

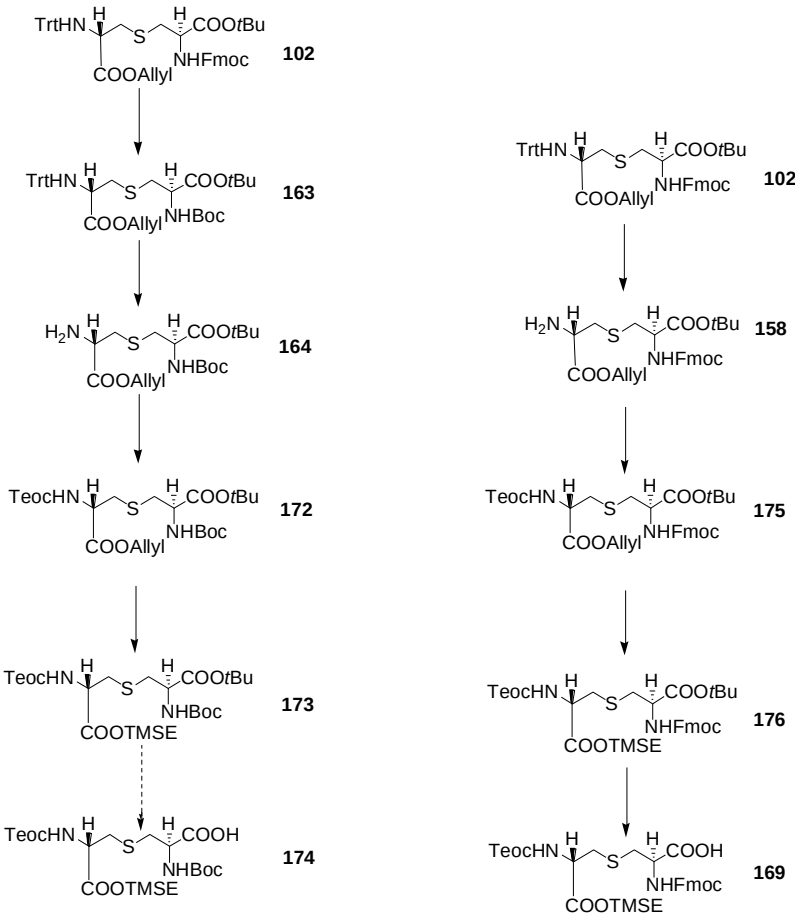


170



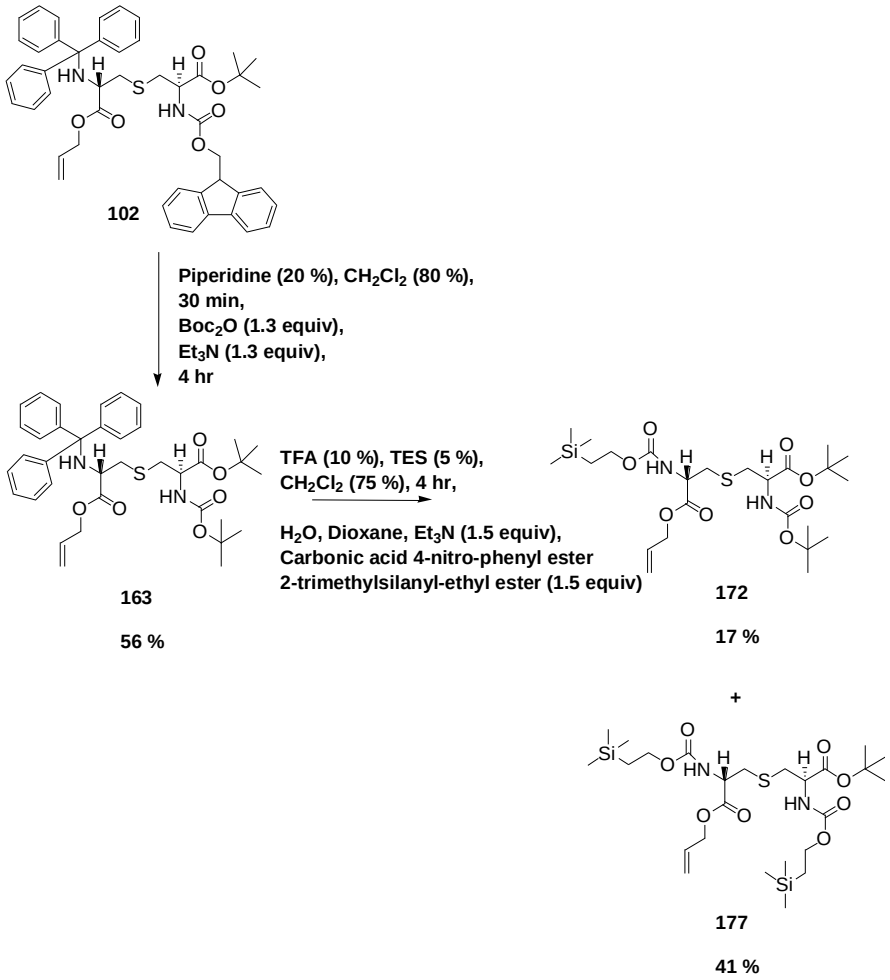
171

The protections were attempted on both (Trt, allyl) / (Boc, *t*Bu) lanthionine **163** and (Trt, allyl) / (Fmoc, *t*Bu) lanthionine **102** as outlined on the planned route (Scheme 51). Each reaction will be discussed separately in this section.



Scheme 51: Planned route for the (Teoc, TMSE) / (Fmoc, OH) and (Teoc, TMSE) / (Boc, OH) lanthionines.

3.2.6.1. Synthesis of (Teoc, allyl) / (Boc, *t*Bu) lanthionine



Scheme 52: Removal of the trityl group and protection with Teoc group of Boc lanthionine.

Lanthionine **102** was used as a common intermediate, from which different protecting groups were put in place. The Fmoc group of **102** was deprotected giving a free amino group; this was then protected using Boc anhydride to give **163** (Scheme 52). The reaction was carried out in distilled water and Teoc-ONp **170** was used for Teoc protection (Table 6, entry 1). 17 % yield was obtained for the synthesis of the desired product, it was also noticed that a second compound was formed. The Boc group deprotected giving a free amino group; this was then protected by ester **170** giving **177** in 41 % yields. The same results were obtained when the reaction was repeated.

Entry	Trityl deprotection / Time	Reaction solvents	Base (equiv)	Protecting group Teoc-ONp 170 (equiv)	Teoc-ONSu 171 (equiv)	Reaction time / temperature	Product Isolated 172 (%)	Byproduct Isolated 177 (%)
1	TFA/TES/ CH_2Cl_2, 4 hr	H_2O / 1,4-dioxane	aq. sodium bicarbonate, (4)	(2)	-	Cool to 0 °C, 18 hr, ≤ 4 °C	17 %	41 %
2	TFA/TES/ CH_2Cl_2, 4 hr	DMF	Triethylamine, (2 drops)	(2)	-	24 hr, RT	NR *	NR *
3	TFA/TES/ CH_2Cl_2, 4 hr	H_2O	Triethylamine, (2), dioxane	(2)	-	24 hr, RT	-	< 10 % **
4	TFA/TES/ CH_2Cl_2, 4 hr	DMF	Triethylamine, (2), dioxane	(2)	-	24 hr, RT	-	< 10 % **
5	TFA/TES/ CH_2Cl_2, 4 hr	H_2O	Triethylamine, (1.5), dioxane	(1.5)	-	24 hr, RT	17 %	-
6	TFA/TES/ CH_2Cl_2, 4 hr	H_2O	Triethylamine, (2), dioxane	-	(2)	18 hr ≤ 4 °C	-	< 10 % **
7	TFA/TES/ CH_2Cl_2, 4 hr	DMF	Triethylamine, (2), dioxane	-	(2)	18 h at ≤ 4 °C	14 %	-
8	TFA/TES/ CH_2Cl_2, 4 hr	H_2O	Triethylamine, (1.5), dioxane	-	(1.5)	24 hr, RT	10 %	-
9	TFA/TES/ CH_2Cl_2, 4 hr	H_2O / 1,4-dioxane	aq. sodium bicarbonate, (4)	-	(2)	Cool to 0 °C, 18 hr, ≤ 4 °C	14 %	< 10 % **

* NR: No Reaction

** < 10 %: Identified by mass spec but not isolated

Table 6: Conditions used for the synthesis of (Teoc, allyl) / (Boc, *t*Bu) lanthionine.

The reaction was attempted in DMF with 2 drops of triethylamine and Teoc-ONp (Table 6, entry 2). From NMR and mass spectrometry it was found that the reaction did not occur. This was obviously due to lack of base used.

The approach of Shute and Rich [145] was then attempted. The reaction was carried out in water, with 2 equivalents of triethylamine diluted in 1,4-dioxane. The increased equivalents of triethylamine deprotected the Boc group followed by protection with the carbonate **170**, giving **177** (Table 6, entry 3). The reaction was repeated in DMF (Table 6, entry 4); this also led to the formation of compound **177**. When the reaction was attempted with 1.5 equivalents of triethylamine a very low yield of the desired product **172** was obtained (Table 6, entry 5).

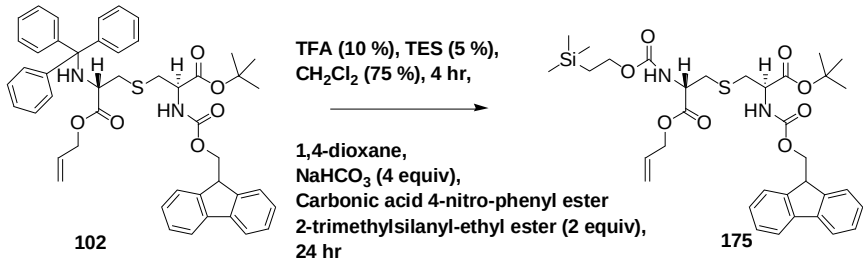
Byproduct **177** was also obtained when the reaction was carried out in water, with 2 equivalents of triethylamine in dioxane and 2 equivalents of Teoc-ONSu **171** (Table 6, entry 6). The reaction was repeated (Table 6, entry 7) by suspending the residue in DMF; again **177** was formed and the desired product **172** was formed in only 14% yield.

Use of low equivalents of triethylamine and protecting group **171** led to very low yields (Table 6, entry 8).

Table 6, entry 1 shows that when sodium bicarbonate was used some reaction occurred forming the desired product **172** in 17 %. The reaction was attempted again using the same conditions however in the presence of the second protecting reagent **171** (Table 6, entry 9). From NMR and mass spectrometry it was seen that the product was obtained in only 14 % yield however the same problem arose where the Boc group deprotected and then protection with the Teoc group took place giving **177**.

The conditions developed earlier for Aloc protection did not deprotect the Fmoc group unless excess sodium bicarbonate is used. It was therefore decided to carry out attempts on Fmoc protected lanthionine **102** to find a suitable route.

3.2.6.2. Synthesis of (Teoc, allyl) / (Fmoc, *t*Bu) lanthionine



Scheme 53: Removal of the trityl group and protection with Teoc group of Fmoc lanthionine.

The reaction was first attempted using sodium bicarbonate (Scheme 53, Table 7, entry 1). After trityl deprotection the residue was dissolved in dioxane, aqueous sodium bicarbonate was added followed by the protecting reagent. This did not show any improvement in the yields. However there was no Fmoc deprotection.

Entry	Trityl deprotection / Time	Reaction solvents	Base (equiv)	Protecting group Teoc-ONp 170 (%)	Teoc-ONSu 171 (%)	Reaction time / temperature	Product Isolated 175 (%)
1	TFA/TES/ CH_2Cl_2, 4 hr	1,4-dioxane	aq. sodium bicarbonate, (4)	(2)	-	24 hr, RT	23 %
2	TFA/TES/ CH_2Cl_2, 4 hr	1,4-dioxane	aq. sodium bicarbonate, (4)	(2)	-	Cool to 0 °C, 24 hr at RT	14 %
3	TFA/TES/ CH_2Cl_2, 4 hr	1,4-dioxane	Triethylamine, (0.5), dioxane, Triethylamine, (0.5), dioxane	(2)	-	2 hr, RT 24 hr, RT	21 %
4	TFA/TES/ CH_2Cl_2, 4 hr	H_2O	Triethylamine, (0.5), dioxane, Triethylamine, (0.5), dioxane	(2)	-	2 hr, RT 24 hr RT	21 %
5	TFA/TES/ CH_2Cl_2, 4 hr	H_2O	Triethylamine, (0.5), dioxane Triethylamine, (0.5), dioxane	-	(2)	2 hr, RT, 24 hr, RT	21 %
6	TFA/TES/ CH_2Cl_2, 4 hr	1,4-dioxane	aq. sodium bicarbonate, (4)	-	(1)	24 hr, RT	23 %
7	TFA/TES/ CH_2Cl_2, 4 hr	1,4-dioxane	aq. sodium bicarbonate, (4), Triethylamine, (couple of drops)	-	(2)	Cool to 0 °C, 24 hr, RT	2 %
8	TFA/TES/ CH_2Cl_2, 4 hr	1,4-dioxane	aq. sodium bicarbonate, (4)	-	(2)	Cool to 0 °C, 24 hr, RT	31 %
9	TFA/TES/ CH_2Cl_2, 4 hr	H_2O	Triethylamine, (0.5), dioxane, Triethylamine, (0.5), dioxane	-	(2) (2)	3 hr 30 min, RT, 24 hr, RT	25 %
10	TFA/TES/ CH_2Cl_2, 4 hr	1,4-dioxane	aq. sodium bicarbonate, (4.6)	-	(2)	Cool to 0 °C, 24 hr in freezer, 5 hr, 0 °C	50 %
11	TFA/TES/ CH_2Cl_2, 4 hr	1,4-dioxane	aq. sodium bicarbonate, (5)	-	(2.5)	Cool to 0 °C, 24 hr in freezer, 5 hr, 0 °C – RT	83 %

Table 7: Conditions used for the synthesis of (Teoc, allyl) / (Fmoc, *t*Bu) lanthionine.

Table 7, entry 1 and Table 7, entry 2 show attempts where the conditions used were the same apart from the temperature of the reaction mixture before the addition of the ester. The reaction mixture of Table 7, entry 2 was cooled to 0 °C before the addition of the ester. Comparison of the two reactions showed that there was a reduction in the yield, indicating that lower temperature may not be appropriate.

The reaction in 1,4-dioxane, with triethylamine and the protecting reagent **170** (Table 7, Entry 3) gave a yield of only 21 %. Similar yields were obtained when the reaction was repeated. When the reaction was carried out in water with diluted triethylamine in 1,4 dioxane (Table 7, entry 4) there was no difference in the yield.

Conditions similar to Table 7, entry 4 were used, and instead of ester **170**, ester **171** was used (Table 7, entry 5). The yield obtained was similar to that of Table 7, entry 4.

Table 7, entry 6 gave the product in 23 % yield when the reaction was carried out in 1,4-dioxane using 1 equivalents of **171**. Only 2 % of the product was obtained when the reaction was carried out using similar conditions to Table 7, entry 7, however the reaction mixture was cooled to 0 $^{\circ}$C before **171** and triethylamine were added. The low yield obtained may have been due to traces of TFA being present in the residue after the removal of the trityl group preventing the reaction from proceeding.

To observe whether there was a difference in using triethylamine or not, a reaction was carried out using the similar conditions to Table 7, entry 7 but without any use of triethylamine (Table 7, entry 8). The results showed there was an increase in the yield to 31 %, when the reaction was repeated 38 % yield was obtained.

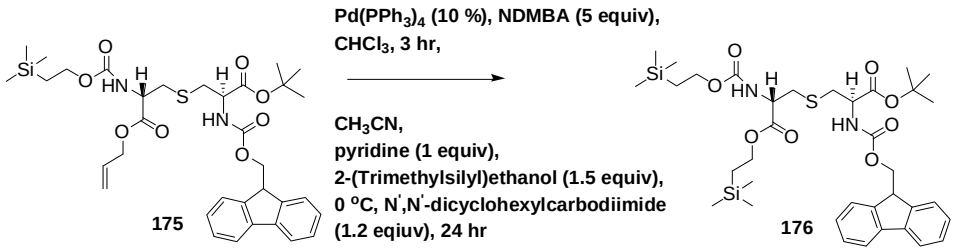
The protecting reagent was added twice (Table 7, entry 9) to see how this affects the reaction. The reaction was carried out in H_2O after trityl deprotection, 0.5 equiv triethylamine in 1,4-dioxane was added followed by Teoc-ONSu **171**. Further 0.5 equiv triethylamine in 1,4-dioxane and Teoc-ONSu were added after 3 hr 30 min. The yield obtained was 25 % which was still very low.

Better yields were obtained using Teoc-ONSu **171** and therefore it was decided **171** would be a better reagent to use compared to Teoc-ONp **170**.

Comparing all the yields, the best yield obtained was 38 % from carrying out the Teoc protection using the conditions from Table 7, entry 8. The reaction was repeated again with slight modifications, once **171** was added the reaction was left in the freezer overnight. The reaction was then stirred at 0 ^{o}C for 5 hr (Table 7, entry 10). 50 % yield was obtained which was promising compared to the yields which were previously obtained.

The reaction was repeated again using similar conditions where the reaction was stirred at 0 °C to room temperature for 5 hr instead of maintaining the reaction at 0 °C. This gave 83 % yield, and these optimised conditions were therefore used (Table 7, entry 11).

3.2.6.3. Removal of the allyl group and protection with TMSE



Scheme 54: Synthesis of (Teoc, TMSE) / (Fmoc, *t*Bu) lanthionine.

The next step involved deprotecting the allyl group and then protecting with a TMSE group (Scheme 54). Lanthionine **175** was dissolved in a solution of tetrakis(triphenyl phosphine)Palladium(0) catalyst ($Pd(PPh_3)_4$) and 1,3-dimethylbarbituric acid (NDMBA) [78, 146, 147] in chloroform which was stirred for 3 hr.

From mass spectrometry a clean peak was observed at 653 $[M + Na]^+$ (Appendix 2) this indicates that allyl deprotection had taken place. However from the crude ^1H NMR a broad H peak was not observed for the free OH, also the allyl peaks were still present (Appendix 3).

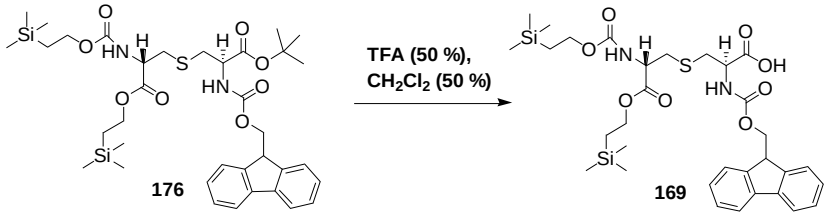
Without any purification DCC coupling was then carried out to protect the free acid with a TMSE group.

Mass spectrometry showed a clean peak at m/z 753 $[M + Na]^+$ confirming that the reaction has proceeded (Appendix 4). From ^1H NMR peaks corresponding to the hydrogen of the desired product were observed. However it was noticed that the desired compound was co-eluting with a compound containing peaks corresponding to an allyl group (Appendix 5). Mass spectrometry did not detect any other molecular weight that may refer to the allyl group other than the molecular weight of the product. Also only one spot was seen by TLC. It was concluded that the allyl group was deprotected and the lanthionine was successfully TMSE

protected. It was assumed the allyl group that was deprotected, had further reacted to give a different compound that co-elutes with the desired product (Appendix 6).

When the reaction was attempted again using the same conditions, purification was carried out after the allyl deprotection. The allyl peaks were still co-eluting with the intermediate. Once TMSE protection was carried out and the reaction was complete, after purification it was noticed the same purification issue occurred. The next step involved *tert*-butyl deprotection and then purification by reverse phase chromatography. It was hoped that all allyl impurities present which were not removed by flash column chromatography would be removed by reverse phase chromatography in the subsequent step.

3.2.6.4. Synthesis of (Teoc, TMSE) / (Fmoc, OH) lanthionine



Scheme 55: Removal of the *t*Bu group to give (Teoc, TMSE) / (Fmoc, OH) lanthionine.

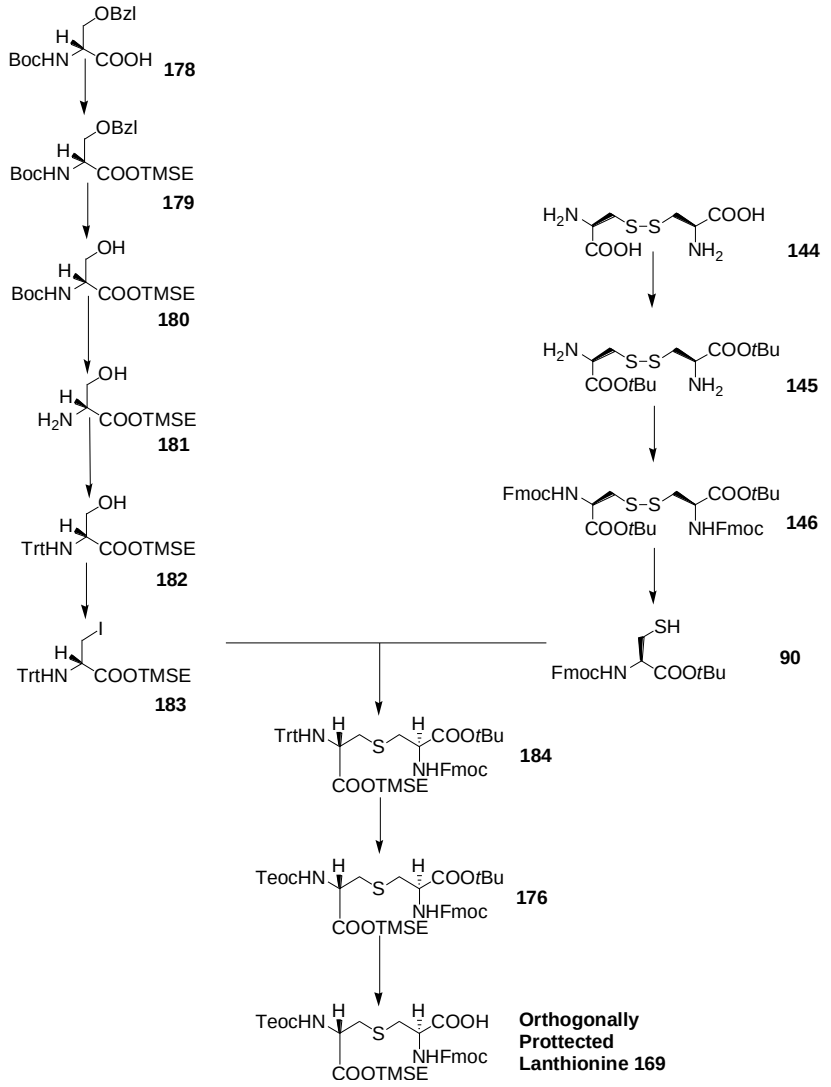
The final step to synthesising the second orthogonally protected lanthionine was by removing the *tert*-butyl group. The *tert*-butyl group was removed by 50 % TFA in CH_2Cl_2 over 3 hr and purified using reverse phase chromatography. Mass spectrometry confirmed that the reaction had taken place, also from the mass spectrum it was observed that there was TMSE deprotection giving another byproduct. ^{1}H NMR showed the presence of the allyl peaks co-eluting with the product.

Further purification was carried out, analytical HPLC was carried out (Appendix 7) to find the right method to separate the compounds. 10- 60 % acetonitrile over 35 min separated out 1 major peak at 23.5 min along with 14 other small peaks. From preparative HPLC a big peak was observed at 29 min and a broad peak at 34 min representing the product (Appendix 8). ^{1}H NMR showed that the product was not pure, separation between the compounds co-eluting with the product were separated however the product picked up other impurities. Due to the

small quantity of the sample and the number of peaks present, further purifications by HPLC were not carried out.

3.2.7. New synthetic route to (Teoc, TMSE) / (Fmoc, OH) lanthionine

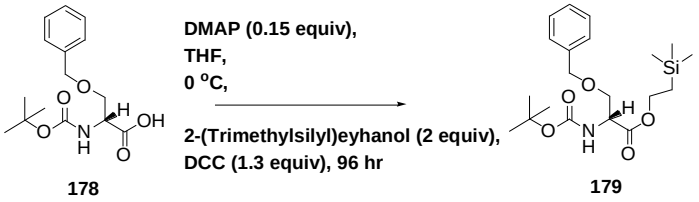
Since there were issues with the purification above the synthetic route was changed (Scheme 56). This route overcomes the problem of the compound co-eluting with the final product as the allyl protecting group was eliminated from the synthetic route. Scheme 56 shows the planned route for the synthesis of the second orthogonally protected lanthionine.



Scheme 56: Planned route for the second orthogonally protected lanthionine.

The original synthetic route was used to synthesise the protected cysteine **90** ready for the coupling with the iodoalanine (Scheme 56, 36, conditions Scheme 37). The route to synthesise the iodoalanine was completely changed. The new route started from the commercially available Boc-(*D*)Ser(Bn)-OH **178** (Scheme 56).

3.2.7.1. TMSE protection of Boc-(*D*)-Ser(Bn)-OH

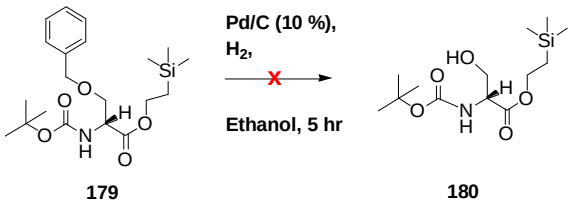


Scheme 57: TMSE protection of Boc-(*D*)Ser(Bn)-OH.

The first step involved the protection of the carboxylic functional group of the serine. Instead of TMSE protecting the carboxylic group at the end of the synthetic route just before the *tert*-butyl deprotection as in Scheme 54, TMSE protection was carried out in the first reaction of the synthetic route. The reason behind this decision was to avoid the purification issue which had been faced with the conditions previously used (Section 3.2.6.3, Scheme 54).

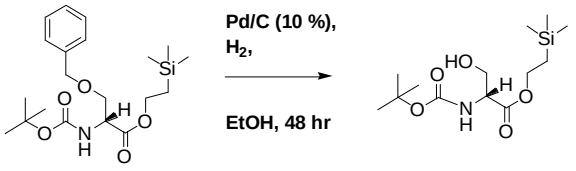
Samy *et al*. [148] used DCC for a similar coupling, with DMAP as a nucleophilic catalyst. 3-Benzyloxy-2-*tert*-butoxycarbonylamino-propionic acid **178** was used and DCC coupling was used to give **179** (Scheme 57). After purification 80 % yield was achieved which was promising as literature [148] showed yield obtained for TMSE protection of carboxylic group of Boc, methyl and benzyl protected threonine was 56 %.

3.2.7.2. Benzyl deprotection of Boc-(*D*)Ser(Bn)-TMSE



Scheme 58: Benzyl deprotection of Boc-(*D*)Ser(Bn)-TMSE.

The next step was to deprotect the benzyl group by hydrogenation, Compound **179** was dissolved and 10 % Palladium catalyst on carbon was suspended in ethanol and stirred under hydrogen (Scheme 58). Literature precedent suggests placing hydrogen under atmospheric pressure for 5 hr and then filter through a pad of Celite. [148] However these conditions did not work for deprotecting the benzyl group of **179**.

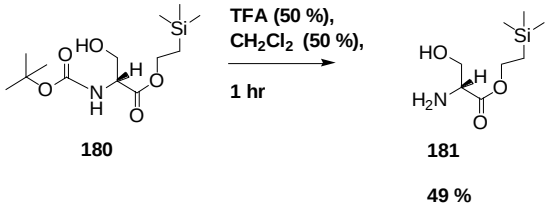


Scheme 59: Benzyl deprotection of Boc-(*D*)Ser(Bn)-TMSE.

When the reaction was repeated again for 24 hr under hydrogen at atmospheric pressure, with refill of hydrogen and addition of more catalyst 53 % yield was obtained. This proved the hypothesis to be correct where longer exposure to hydrogen leads to the reaction proceeding. The reaction was repeated again for 48 hr (Scheme 59), where the hydrogen was refilled and two further portions of the catalyst were added; this increased the yield to 87 %.

3.2.7.3. Boc deprotection of Boc-(*D*)Ser-TMSE

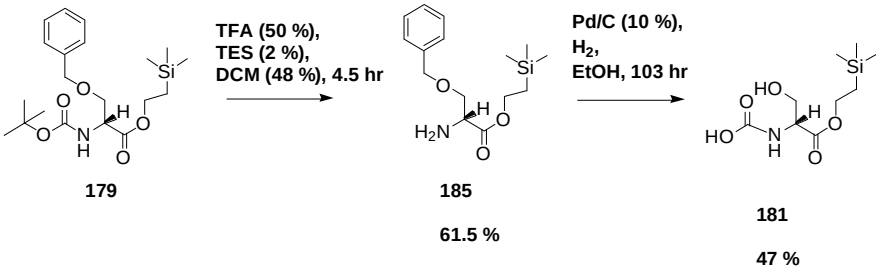
The next step was to deprotect the Boc group. Boc protected serine **180** was stirred in a solution of trifluroacetic acid (95 %), triisopropylsilane (2.5 %) and water (2.5 %) for 2 hr. TIPS was used as a scavenger in the reaction. From mass spectrometry it was observed that the desired product was not obtained.



Scheme 60: Boc deprotection of Boc-(*D*)Ser-TMSE.

The reaction was repeated again, this time starting material **180** was stirred in a solution of 50 % TFA and 50 % CH_2Cl_2 for 1 hr. After purification by flash column chromatography the

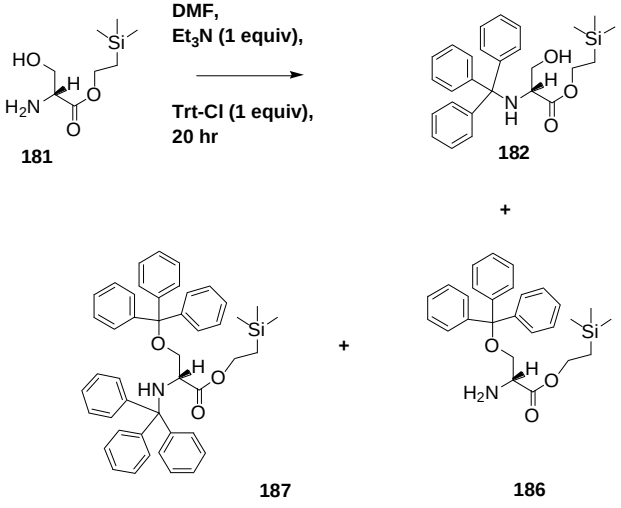
product was isolated in 49 % yield (Scheme 60). The yield was slightly low, and a higher yield would be preferred however it was acceptable at this stage.



Scheme 61: Synthesis of TMSE serine.

It was later found that a higher yield was obtained when the Boc group was deprotected first followed by hydrogenation to deprotect the benzyl group (Scheme 61). This made the purification step much easier as aqueous work up was not required, flash column chromatography gave the pure product. Previously aqueous work up was carried out after Boc deprotection, due to the presence of free amine and OH this made the work up difficult (Scheme 60).

3.2.7.4. Trityl protection of TMSE serine



Scheme 62: Synthesis of the trityl, TMSE serine and the byproducts.

In order to prepare the iodoalanine from serine both the carboxylic acid and the amino groups had to be protected. Boc deprotection gives a free amine, which must then be protected with a trityl group. Amino acid **181** was dissolved in DMF, triethylamine was added followed by

triphenylmethylchloride and then stirred for 20 hr (Scheme 62). Due to the NH_2 being more reactive then the OH group it was assumed that the reaction will take place at NH_2 and therefore trityl protection will take place. Mass spectrometry showed a peak at m/z 470 $[M + Na]^+$ which indicated the product was isolated which was also indicated by ^1H NMR.

When HMBC NMR was carried out it was observed that both the OH and the NH functional groups were protected with the trityl group. This was also confirmed when the mass spectrum was analysed again, a peak at m/z 722 (**187** + H) was observed which corresponds to both OH and NH group being protected with the trityl group. From the HMBC NMR a 3 bond correlation is observed where carbon 5 (5C) is coupling to the 2 hydrogens on carbon 4 ($4CH_2$). This indicates that the O is protected with the trityl group. Another 3 bond correlation is also observed where the carbon 1 (1C) is coupling to the hydrogen on carbon 3 (3CH) (Figure 44).

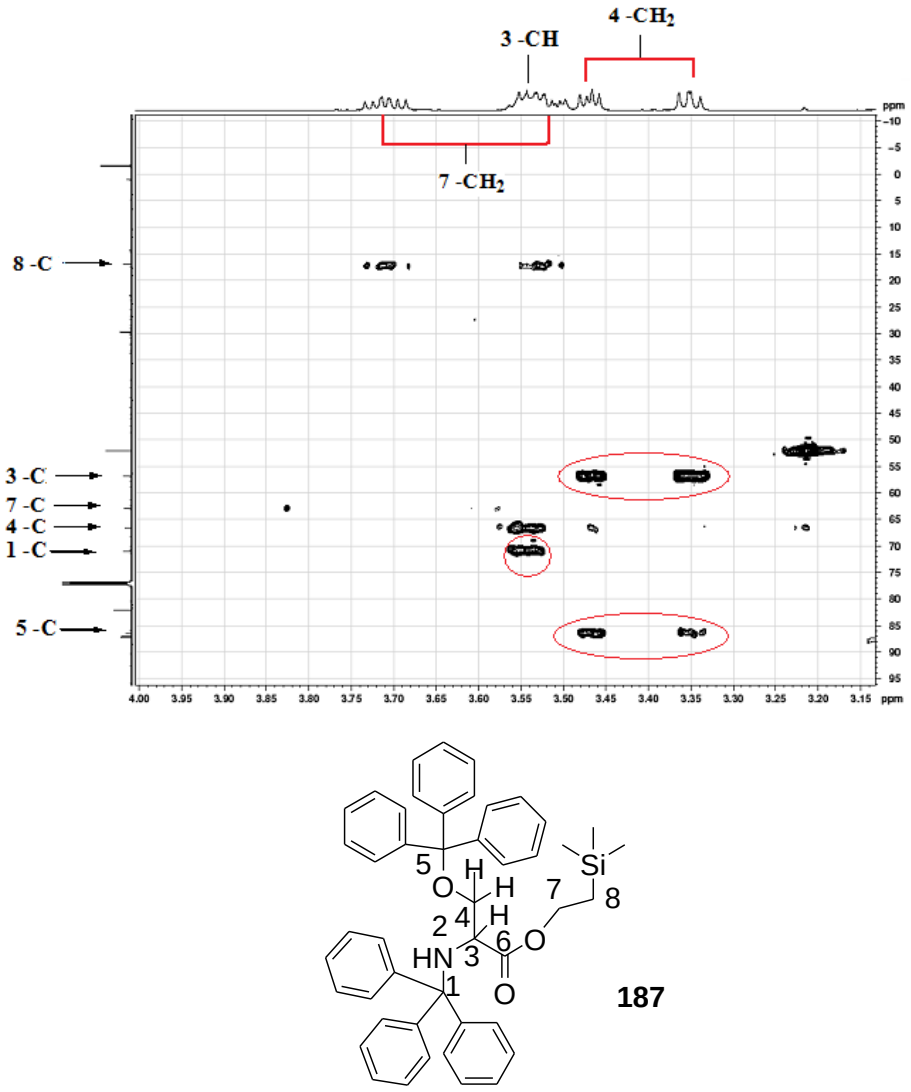
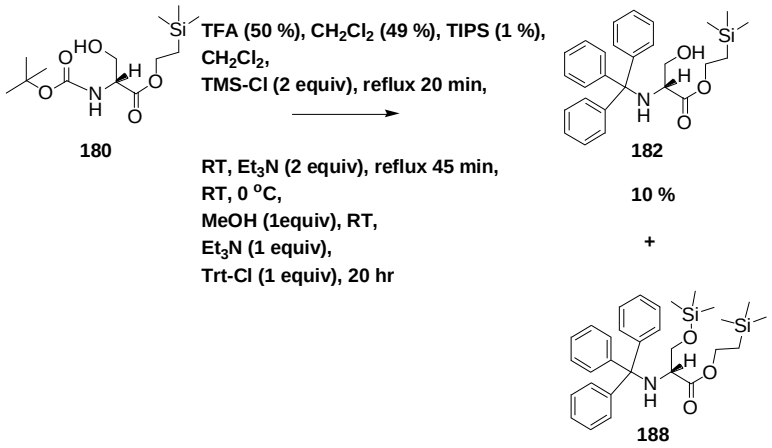


Figure 44: HMBC NMR showing the protection of OH and NH with trityl group.



Scheme 63: Trityl protection of TMSE serine.

A second attempt was carried out, the original procedure for the synthesis of **143** (Scheme 40) was used. The two functional groups were protected first and then selective deprotection of the TMS group on the amine was carried out which was then protected with the trityl group. This was to avoid protecting both functional groups with trityl group as Scheme 62.

The Boc group was deprotected in 50 % TFA, 49 % CH_2Cl_2 and 1% TIPS. Protection of both functional groups OH and NH_2 were carried out by trimethylsilyl chloride (TMS). Selective deprotection of the amine was carried out by adding anhydrous methanol. This was followed by triethylamine then triphenylmethyl chloride for the protection of the amine with the trityl group (Scheme 63). The removal of the TMS group on the alcohol was achieved with excess methanol and triethylamine. From the mass spectrum a peak at *m/z* 470 corresponding to **182** $[M + Na]^+$ and peaks at *m/z* 526 and 542 corresponding to **188** $[M + Na]^+$ and **188** $[M + K]^+$ were observed. This indicated that a mixture of the product **182** and the intermediate **188** containing the TMS group on the alcohol was obtained. Proton NMR confirmed the presence of the mixture of both compounds.

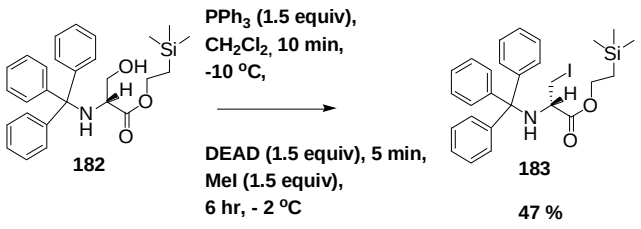
The oily residue containing a mixture of the two compounds was redissolved in excess methanol and triethylamine at 0 °C and stirred at room temperature for 1 hr to remove the TMS group. Compound isolated after purification still contained a mixture of both the product and the intermediate. The reaction was repeated again and the same was observed. On the third attempt after purification the product was isolated in only 12 % yield.

Another attempt involved purification of the crude material after Boc deprotection by flash column chromatography. This was followed by protection of the OH and NH_2 groups with TMS then selective deprotection was carried out followed by trityl protection using conditions in Scheme 63. After workup and purification product **182** was isolated in 43 %.

It was therefore important to purify the intermediate after Boc deprotection and then to protect both alcohol and amino functional groups then carry out selective deprotection followed by trityl protection to give the desired product.

The intermediate **188** was observed repeatedly during this reaction. The problem was solved by carrying out a deprotection of the TMS group. Evans *et al*. [149] used $NaHCO_3$ in methanol at room temperature to deprotect TMS group, this was attempted on compound **188** which gave product **182**. An attempt was carried out where after trityl protection the crude containing a mixture of **182** and **188** was stirred in methanol with $NaHCO_3$. It was discovered that purification was required before deprotection of the TMS group in order to ensure the reaction proceeds to give **182**.

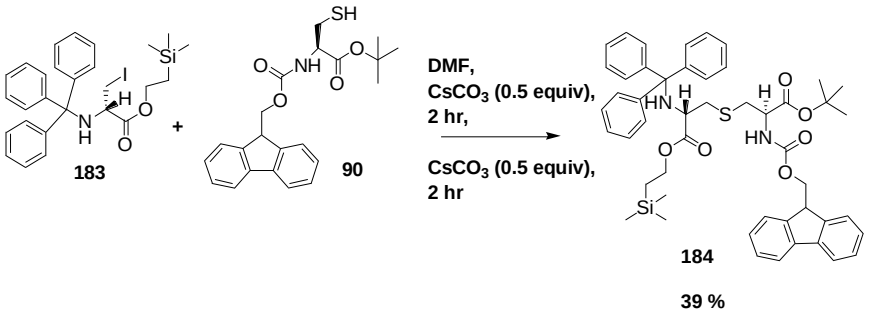
3.2.7.5. Synthesis of TMSE iodoalanine



Scheme 64: Synthesis of TMSE, trityl iodoalanine.

Trityl protected serine **182** and triphenylphosphine were dissolved in dry CH_2Cl_2 and cooled to -10 °C. DEAD was added followed by iodomethane and the reaction was stirred for 3 hr. 7 % yield was obtained however when the reaction was repeated again and left stirring for 6 hr the yield increased to 47 % (Scheme 64).

3.2.7.6. Synthesis of (Trt, TMSE) / (Fmoc, *t*Bu) lanthionine



Scheme 65: Synthesis of (Trt, TMSE) / (Fmoc, *t*Bu) lanthionine.

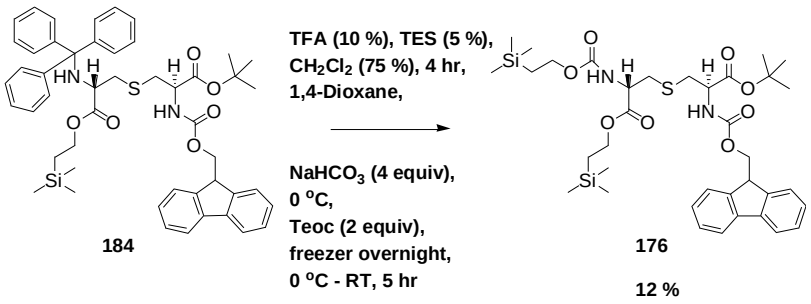
The next step was to synthesise the lanthionine by coupling the iodoalanine and cysteine. **183** and **90** were dissolved in DMF; caesium carbonate was added and the reaction mixture was stirred for 2 hr. Further caesium carbonate was added and stirring contained for further 2 hr (Scheme 65).

It was important to ensure that the caesium carbonate was dry to avoid any Fmoc deprotection. It was also found that 0.5 equivalents of caesium carbonate followed by another portion of 0.5 equivalents of caesium carbonate after 2 hr of first addition gave higher yields. Splitting caesium carbonate into two portions also prevented Fmoc deprotection compared to the addition of 1 equivalent of caesium carbonate all at once. After purification a mixture of two compounds were observed from proton NMR and TLC. It contained the desired product **184** but it was difficult to work out what the second compound was. It was thought that mass spectrometry may help to find out what the second compound was, however there was no observations of peaks corresponding to another compound.

On the second attempt after purification by flash column chromatography the product was separated from the impurities in the crude giving a yield of 39 %.

In the presence of a non-nucleophilic base there was no dehydroalanine elimination by product formation. This was due to the trityl group on the free amine which prevented α-proton abstraction.

3.2.7.7. Synthesis of (Teoc, TMSE) / (Fmoc, *t*Bu) lanthionine

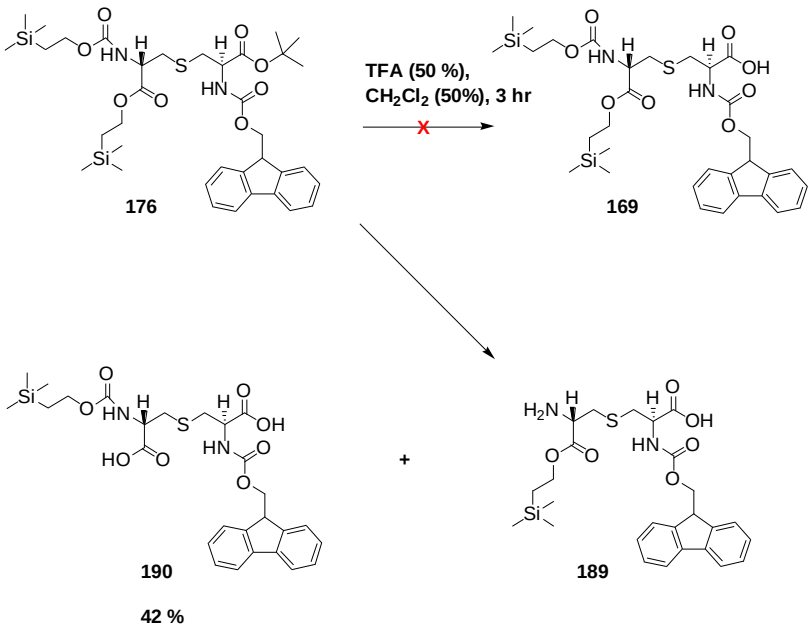


Scheme 66: Removal of the trityl group and protection with Teoc group

In order to synthesise the desired second orthogonally protected lanthionine, replacement of the trityl group with Teoc group was required. This was one step before the end of the synthesis. The optimised conditions from Table 7, entry 11 were used (Scheme 66). After purification by flash column chromatography a mixture of two compounds were obtained, one of which was the desired product.

Due to only 30 mg being obtained purification was carried out by preparative HPLC. An analytical HPLC (Appendix 9) was carried out to see the number of peaks present. There was one major peak at 26.7 min along with 7 other small peaks. The product separated at 27.51 min on the preparative HPLC (Appendix 10). Mass spectrometry showed peaks at *m/z* 753 and 769 corresponding to **176**, $[M + Na]^+$ and $[M + K]^+$ confirmed by a proton NMR (Appendix 11) giving 12 % yield. Third peak at *m/z* 702 represents the second compound, possibly $[M + Na]^+$ or $[M + K]^+$.

3.2.7.8. Formation of byproducts during synthesis of (Teoc, TMSE) / (Fmoc, OH) lanthionine



Scheme 67: Synthesis of byproducts during *t*Bu deprotection.

The last step involved the deprotection of the *tert*-butyl group in TFA and CH_2Cl_2, for 3 hr to give **169** ready for incorporation into solid phase peptide synthesis (Scheme 67). Reverse phase chromatography was then carried out to obtain pure product **169**.

During this reaction unexpected results were obtained. The desired product **169** was not synthesised. ^{1}H NMR and mass spectrometry showed that byproducts **189** and **190** were formed.

It was possible that during purification deprotection of the Teoc and TMSE groups may have taken place, due to the use of saturated sodium bicarbonate in the reverse phase chromatography. The reaction was repeated, and the purification was carried out in water and acetonitrile. Unfortunately the same two byproducts were observed.

It was also possible that the deprotection of the Teoc and TMSE groups were taking place after the reaction when the TFA and CH_2Cl_2 solution were removed, *in vacuo*. As more

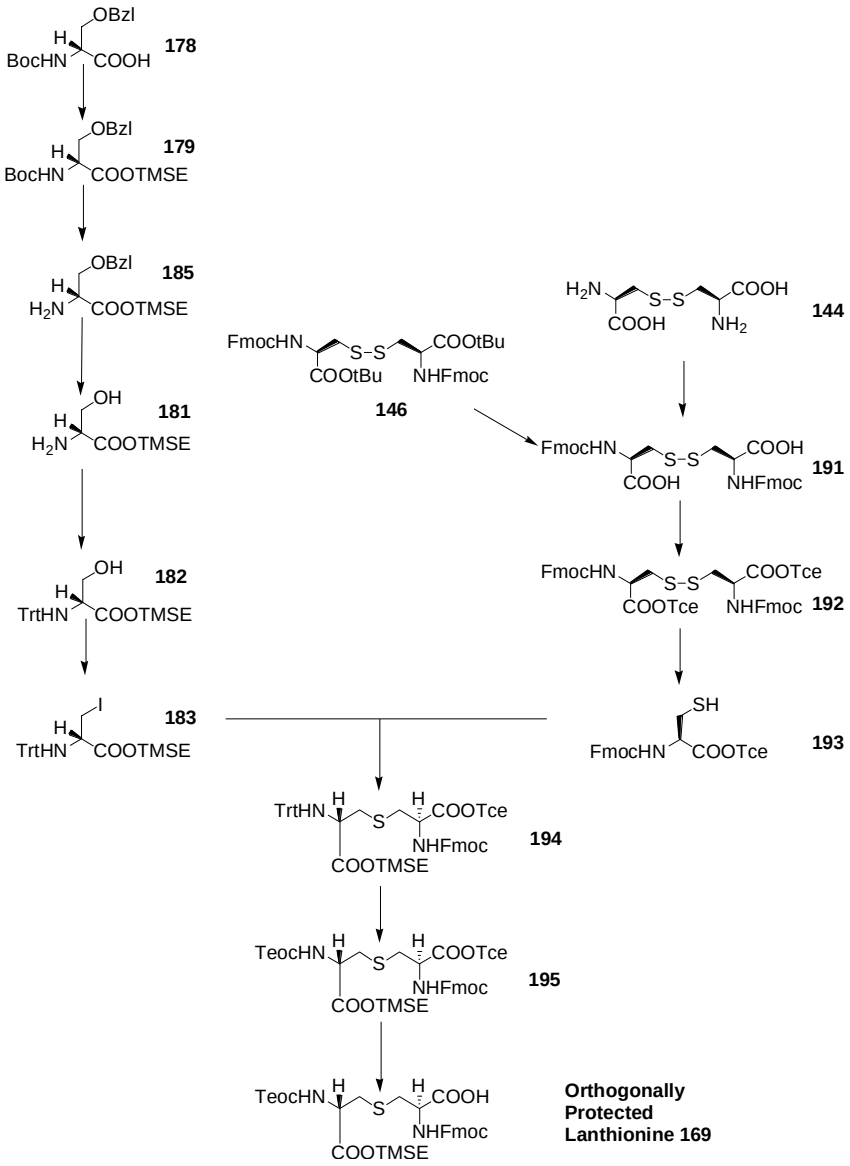
volatile CH_2Cl_2 is removed first, the concentration of TFA increases. In order to confirm this, the reaction was then attempted again.

The reaction was repeated in deuterated TFA and deuterated CH_2Cl_2, and the reaction was monitored by ^{1}H NMR. ^{1}H NMR of the reaction samples were taken at 10 min, 30 min, 1 hr, 2 hr and 3 hr. From the NMR it was seen that the deprotection was taking place during the reaction itself (Appendices 12-16). This was unexpected because Teoc and TMSE protecting groups theoretically should be stable to acid and base. However Wood *et al*. [150] used excess TFA to deprotect (trimethylsilyl)ethyl ester to afford the free carboxylic functional group.

This represented a major problem, if the *tert*-butyl group could not be selectively deprotected in the presence of Teoc and TMSE protecting groups. In order to solve this problem there were two options.

The first was to find an alternative way of deprotecting the *tert*-butyl group using different conditions. This could be done by using thermitase catalysed deprotection of the *tert*-butyl moiety using esterases biocatalyst enzymes which Schmidt *et al*. used, [151] esterase are an advantage to use because they are stable in organic solvents. Schultz *et al*. [152] also carried out enzymatic cleavage of the *tert*-butyl group in a thermitase-catalysed reaction. Gmeiner *et al*. [153] used HOAc/iPrOH/H_2O to hydrolyze the *tert*-butyl ester. This method was not selected due to the particular enzymes not being readily available.

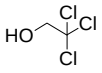
The second option was to change the protecting groups. Having spent a lot of time putting the Teoc and TMSE groups in place and finding good conditions it was felt to be wise to change the *tert*-butyl protecting group. This required finding different protecting groups which can be selectively deprotected in the presence of Teoc and TMSE protecting groups. Scheme 68 shows the planned route to the second orthogonally protected lanthionine with Teoc, TMSE, Fmoc, Tce groups.



Scheme 68: Planned route for the (Teoc, TMSE) / (Fmoc, OH) lanthionine.

The synthesis of **183** from **178** was carried out as before with one modification the order of one reaction was changed. Problems were encountered when **181** was synthesised yields produced previously were not reproducible. This was due to the work up of the reaction. Due to the free carboxylic acid and a free amine during aqueous workup it was difficult to extract the product out of the aqueous phase. Changing the solvents and pH did not have an effect. Purification was carried out straight after the reaction solvent was reduced *in vacuo* without any extraction this also did not make any difference, the compound isolated was still low in yield with the presence of impurities. Due to this the order of the reaction was slightly changed. After TMSE protection, the Boc group of **179** was deprotected and then purified to

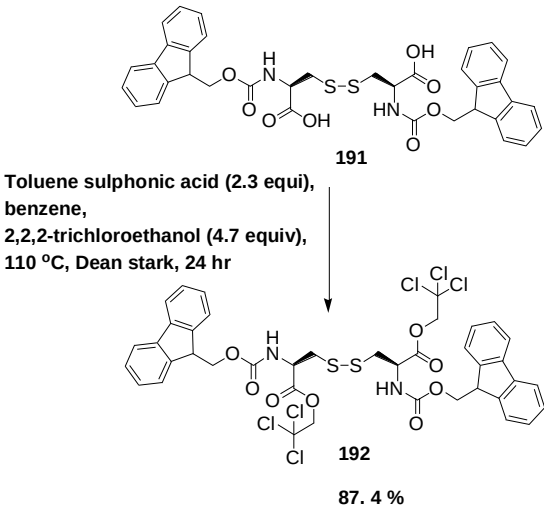
give **185**. Hydrogenation was then carried out to remove the benzyl group giving **181**, this eliminated any aqueous workup as no TFA was used at this stage. The reaction was filtered and reduced followed by purification.



196

Instead of *tert*-butyl ester, 2,2,2-trichloroethyl ester **196** was used for the carboxy protecting group. Out of many other protecting groups available for amino acids Tce was selected because of its facile removal using mild conditions with zinc dust whereas base hydrolysis is required for alkyl esters. Woodward *et al*. [154] used trichloroethyl ester in the synthesis of cephalosporin C as a protecting group.

3.2.7.9. (Fmoc-Cys-OTce)$_2$



Scheme 69: Tce protection of cystine.

The *tert*-butyl group of **146** was deprotected in TFA and CH_2Cl_2. Then Jou *et al*'s approach [155] was used where the residue was dissolved in CH_2Cl_2 to which TceOH was added followed by DMAP and DCC (Table 8, entry 1). 18 % yield was obtained the product was not pure after purification due to urea being present.

Bogers *et al*'s approach [156] was used where they synthesised the opposite isomer. The free amine of Cystine **144** was first Fmoc protected using Fmoc-Cl in THF-Water 1:1 in the presence of NaOH. HOBt, TceOH and DCC were used (Table 8, entry 2). Only 2.7 % was obtained compared to 63 % yield obtained by Boger *et al*. [156] It was very difficult to remove urea, after two flash column chromatography the urea was still present.

Entry	Compound 144	146	191	Tertbutyl deprotection / Time	Fmoc protection reagents, (equiv) / Time	Reaction solvent	Reagents (equiv)	TceOH 196 (equiv)	Reaction time / temperature	Product Isolated 192 (%)
1	-	X	-	TFA/ CH_2Cl_2, 4 hr	-	CH_2Cl_2	DMAP (0.4), DCC (2.2), DMAP (0.4)	(2.2) (2.2)	0 °C - RT, 24 hr, RT, 24 hr, RT	18 %
2	X	-	-	-	NaOH (4.2), THF – H_2O, Fmoc-Cl (2.1), RT, 14 hr	Pyridine	HOBt (2.9), DCC (2.3)	(3.45)	- 20 °C, 24 hr	2.7 %
3	-	X	-	TFA/ CH_2Cl_2, 4 hr	-	CH_2Cl_2	DMAP (0.4), DCC (6), DMAP (0.4)	(3) (3)	0 °C - RT 24 hr, RT 24 hr, RT	25.3 %
4	-	X	-	TFA/ CH_2Cl_2, 4 hr	-	Pyridine	HOBt (9), DCC (9.9)	(11.6)	- 20 °C, 24 hr	44.4 %
5	-	-	X	-	-	Pyridine	HOBt (8.4), DCC (8.4), 2 x HOBt (8.4), DCC (8.4)	(9.35) (2 x 9.35)	- 20 °C, 24 hr - 20 °C 2 x 24 hr	17.7 %
6	-	-	X	-	-	CH_2Cl_2	DMAP (0.47), DCC (8.4), 2 x DMAP (0.47), DCC (8.4)	(9.35) (2 x 9.35)	0 °C - RT 24 hr, RT 2 x 24 hr, RT	26 %
7	-	-	X	-	-	CH_2Cl_2	DMAP (0.47), EDCI-polymer (2), DMAP (0.24), EDCI-polymer (2)	(9.35) (9.35)	0 °C 48 hr, RT 72 hr, RT	3.9 %
8	-	-	X	-	-	CH_2Cl_2	DMAP (0.4), EDCI (2.2), DMAP (0.4)	(4) (4)	0 °C 24 hr, RT 24 h at RT	NR *
9	-	-	X	-	-	DMF	*N*-hydroxy Succinimide (2), EDCI.HCl (2), *N*-hydroxy Succinimide (2), EDCI.HCl (2)	(4) (4)	24 hr, RT 24 h at RT	32.7 %
10	-	-	X	-	-	Toluene	Toluene-4-Sulphonic acid (2.4)	(2.4) (2.4)	140 °C, 5 hr 140 °C, 24 hr	60 %
11	-	-	X	-	-	Benzene	Toluene-4-Sulphonic acid (2.4)	(2.4) (2.4)	80 - 105 °C, 5 hr 80 - 105 °C, 5 hr	87.4 %

* NR: No Reaction

Table 8: Conditions used for Tce protection.

Due to the poor yield, Jou *et al*'s approach [155] was carried out again (Table 8, entry 3) using different number of equivalents. Increasing the number of equivalence of DCC and TceOH increased the yield by only 7 %.

Table 8, entry 4 shows an increase of the yield to 44.4 % when Bogers *et al*'s [156] approach was attempted. Having seen this increase commercially available **191** was used. Here 9.35 equivalence of TceOH, 8.4 equivalence of HOBt and 8.4 equivalence of DCC was used. Surprisingly this did not increase the yield (Table 8, entry 5). The reaction was repeated using Jou *et al*'s approach [155] (Table 8, entry 6), this also did not have much effect on the yield. There may have been increase on the yield if an increased DMAP equivalence was used, however to avoid Fmoc deprotection only catalytic amounts were used.

The best yield obtained was 44.4 % (Table 8, entry 4) which was acceptable at this stage in order to continue with the next reaction. The problem encountered in this reaction was the presence of urea, different solvent systems were used however it was unsuccessful to separate the product and the urea by purification.

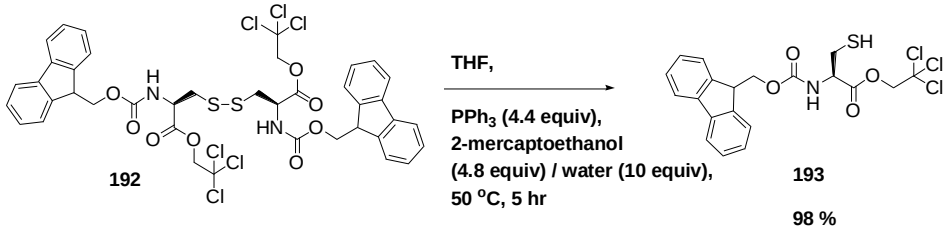
To avoid impure **192** polymer-bound EDC was used for the protection in the presence of DMAP and TceOH. A mass spectrum of the reaction showed the protection of only one acid. Further TceOH, DMAP and EDC-polymer was added however after purification only 3.9 % of **192** were obtained. This low yield was expected, due to the resin bound EDC which was likely to decrease the reaction. When the reaction was attempted with EDCI using Rademann *et al* [157] and Vallinayagam *et al*'s approach [158] to protect the carboxylic functional groups (Table 8, entry 8), no products were formed.

EDCI.HCl was then used in order to overcome the problem of urea. It was believed since the compound was a hydrochloride the urea would remain in the aqueous layer during aqueous workup (Table 8, entry 9). However after purification from ^{1}H NMR it was observed that the urea was still present.

Hoyem *et al*. [159] used Dean-Stark conditions to couple Tce protecting groups to their diacid. **191** were dissolved in toluene in the presence of toluene-4-sulphonic acid, TceOH was added and the reaction was refluxed at 140 °C with a Dean-Stark trap (Table 8, entry 10). After work up and purification 60 % yield of the pure product was obtained which was good

considering the low yields and impure products previously obtained. This approach solved the problem of urea being present in the product as DCC or EDCI was not used. To improve the yield the reaction was attempted in Benzene which increased the yield to 87.4 %.

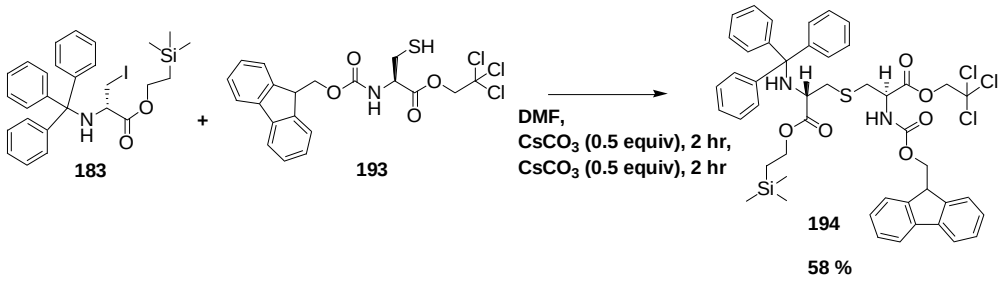
3.2.7.10. Fmoc-Cys-OTce



Scheme 70: Disulfide cleavage.

Reduction of the disulfide precursor was carried out in THF with mercaptoethanol. It was found that the addition of excess water after the addition of triphenylphosphine made the cleavage step more effective. [156]

3.2.7.11. Synthesis of (Trt, TMSE) / (Fmoc, Tce) lanthionine



Scheme 71: Synthesis of (Trt, TMSE) / (Fmoc, Tce) lanthionine.

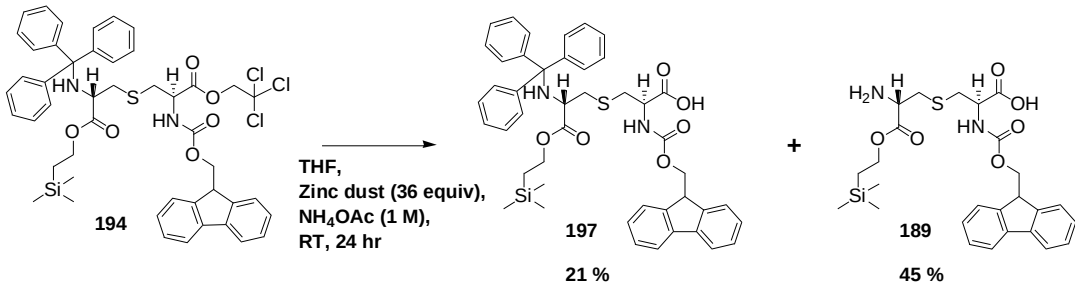
A similar approach to **102** was used to prepare **194**. Intermediates **183** and **193** (Scheme 71) were dissolved in DMF this was followed by addition of dry Cs_2CO_3. The lanthionine was obtained in 58 % yield.

The next steps were to replace the trityl group with the Teoc group then deprotect the Tce protecting group to give the free carboxylic acid. Before these reactions were carried out, a

trial reaction of the deprotection of the Tce group was attempted to ensure it can be selectively deprotected in the presence of TMSE.

Due to the problems previously described while deprotecting *tert*-butyl ester when the Teoc/TMSE protecting groups were also deprotected, trial reactions were carried with Tce protection to see if this can be selectively deprotected in the presence of Teoc and TMSE groups.

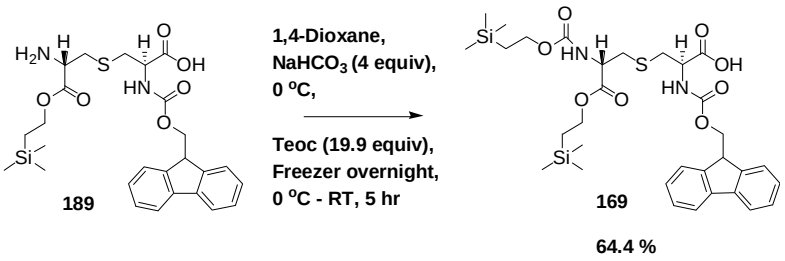
3.2.7.12. Synthesis of (Trt, TMSE) / (Fmoc, OH) lanthionine



Scheme 72: Deprotection of the Tce group.

Reductive removal of the 2,2,2-trichloroethyl group with zinc dust in ammonium acetate gave the acid **197**. Jou *et al*. [155] used zinc dust for Tce deprotection, this approach was adapted. **194** were dissolved in THF to which zinc dust was added followed by 1 M ammonium acetate. After purification via flash column chromatography **197** was obtained in 21 % yield. From ^{1}H NMR and mass spectroscopy it was discovered that compound **189** was also synthesised in 45 % yield (Scheme 72). This showed that Zinc dust with ammonium acetate deprotected the trityl protecting group along with the trichloroethyl group. At this stage this was not a problem, as the trityl group would be deprotected and protected with a Teoc group. The concern was whether the TMSE group was affected during Tce deprotection. Mass spec and ^{1}H NMR confirmed that the Tce protecting group deprotected selectively in the presence of the TMSE group.

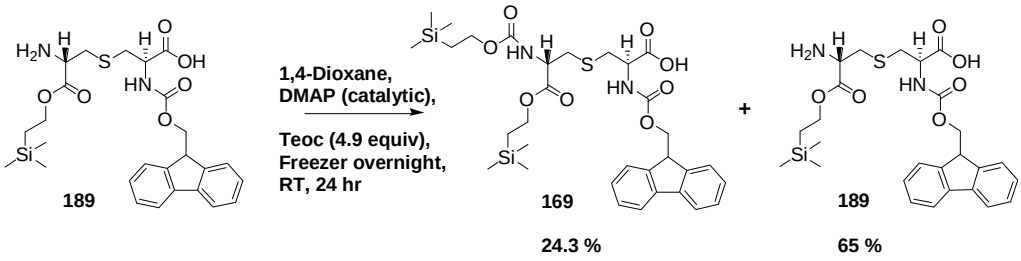
3.2.7.13. Synthesis of (Teoc, TMSE) / Fmoc, OH) lanthionine



Scheme 73: Protection of the free amine of lanthionine with Teoc.

Deprotection of the trityl group saves a synthetic step giving the intermediate **189**. Conditions from Table 7, entry 11 were used on **189**, after purification product **169** was obtained in 64 % yield (Scheme 73).

At this point the second orthogonally protected lanthionine was obtained with a free carboxylic group ready for incorporation into solid phase peptide synthesis. When the reaction was repeated the yield was not reproducible.

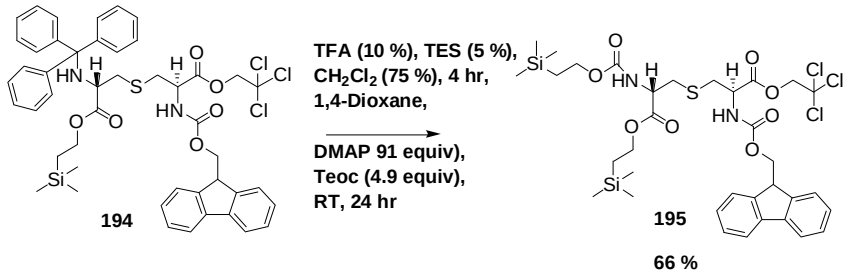


Scheme 74: Teoc protection to give the second orthogonally protected lanthionine.

The coupling of Teoc was then attempted using DMAP as catalytic amount. **189** were dissolved in 1,4-dioxane, DMAP was added in catalytic amount followed by Teoc. After purification it was found that the reaction did not go to completion giving 65 % starting material. Longer reaction time did not increase the yield. Increasing the DMAP increased Teoc protection however it resulted in Fmoc deprotection.

The reaction was then attempted on **194** where Teoc protection was carried out first and then Tce deprotection.

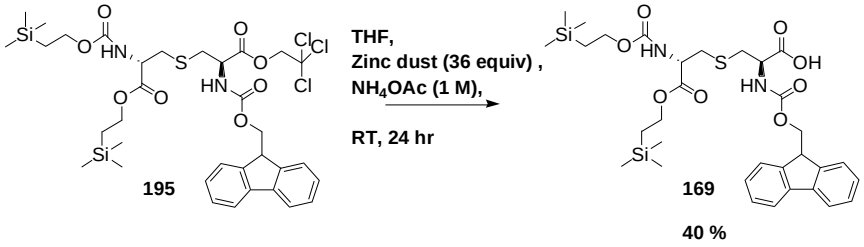
3.2.7.14. (Teoc, TMSE) / (Fmoc, OH) lanthionine



Scheme 75: Removal of the trityl group and protection with Teoc group.

R. Shute and D. Rich's [145] used triethylamine as their base in their reaction for Teoc protection. Scheme 75 shows the use of DMAP as the nucleophilic catalyst. Product **195** was obtained as clear oil in 66 % yield.

The Tce protecting group was then deprotected to give **169** when the reaction was stirred in THF with zinc dust and 1 M ammonium acetate for 24 hr (Scheme 76), proton and carbon NMR can be seen in the Appendix 17 and 18. This second orthogonally protected lanthionine was ready for incorporation into solid phase peptide synthesis.



Scheme 76: Deprotection of the Tce group to give the second orthogonally protected lanthionine.

3.3. Summary

(Aloc, allyl / Fmoc, OH) lanthionine **103** has been synthesised using the published procedure.[124]

Due to low yielding reactions from SES protected lanthionine **154** an alternative protecting group Teoc was chosen. New synthetic routes were adapted where the TMSE group was protected to give **179** during the first synthetic step of lanthionine formation. This eliminated the use of allyl protected serine **122** which otherwise led to issues on purification when the allyl group was replaced with a TMSE group from **175** to **176** at the end of the synthetic route.

Tce protecting group was used as an alternative to *tert*-butyl protecting group. This was to ensure that the Teoc and TMSE groups do not fall apart during the selective deprotection of Tce to give the free carboxylic acid **169** from **195**. This however was the problem when the *tert*-butyl protecting group was deprotected from **176**.

An efficient and good yielding pathway has been developed to synthesise the Teoc, TMSE orthogonally protected lanthionine **169** which was produced in 300 mg routinely in 40 % yield.

4. Solid phase peptide synthesis of rings D and E of nisin

To synthesise an analogue of rings D and E of nisin two orthogonally protected lanthionine residues **103** and **169** were required. The first, **103**, has one amine and one carboxylic acid protected with Aloc and allyl groups respectively and the other amine with an Fmoc group. The second orthogonally protected lanthionine **169** has one amine and one carboxylic acid protected with Teoc and TMSE groups respectively and the other amine with an Fmoc group (Figure 45). The synthesis of these protected lanthionines was discussed in Chapter 3.

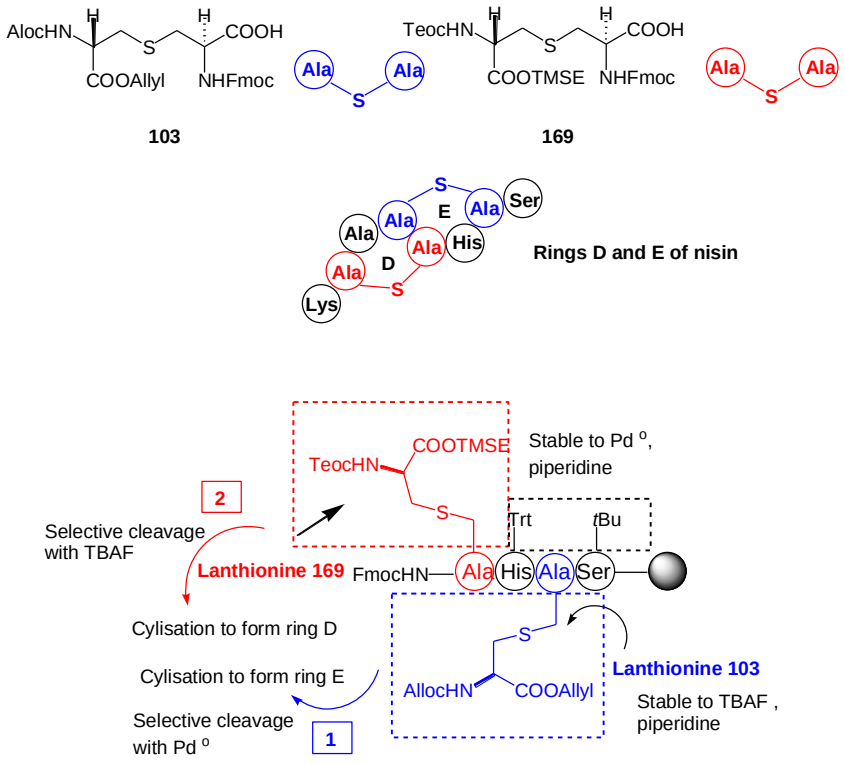


Figure 45: Strategy to synthesising an analogue of nisin rings D and E.

4.1. SPPS methodologies

There are two commonly used methodologies for SPPS, Merrifield SPPS and Fmoc/*t*Bu SPPS. *tert*-Butoxycarbonyl (Boc) is used as a temporary protecting group in Merrifield SPPS. The Boc group is on the *N*-terminus which is cleaved using neat TFA. Benzyl - derived

protecting groups are used as the permanent protecting groups on reactive side chains, these are removed using HF. The disadvantage of this approach was the use of HF, which is very corrosive and capable of dissolving many materials including glass, for this reason special glassware was required. The second approach uses Fmoc and *tert*-butyl groups as the temporary and permanent protecting groups respectively. The Fmoc group is used to protect the α amino group and is removed using a mild base, usually piperidine. [160] Acid labile protecting groups are used as the side chain protecting groups and these are cleaved with TFA during the end of the peptide synthesis. As these milder conditions are used compared to the Merrifield approach the second approach is found to be more convenient and easier to handle (Figure 35). This explains why lanthionine **103** and **169** are Fmoc protected.

4.2. Coupling reagents

There are a large number of coupling reagents for peptide synthesis. In this work HBTU has been used for amino acid coupling and PyAOP/HOAt has been used for the cyclisation steps.

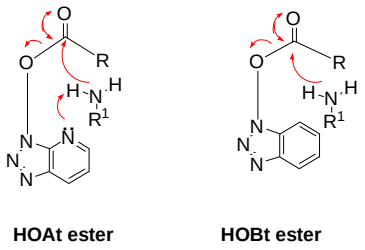
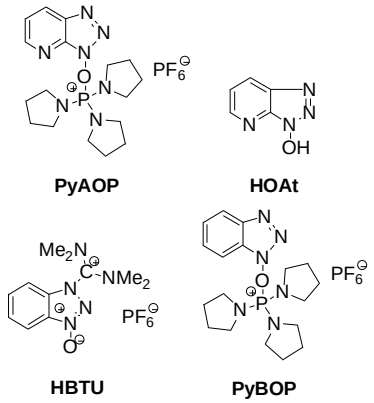


Figure 46: Mechanism for coupling using HOAt and HOBT esters.

1-Hydroxy-7-azabenzotriazole (HOAt) [161, 162] and PyAOP [161, 163] are better cyclisation reagents compared with DCC giving high yielding reactions and reducing racemisation during coupling and cyclisation. Racemisation takes place during cyclisation when HBTU [164] is used; however HBTU is a very efficient cyclisation reagent. The decrease in racemisation by the use of HOAt and PyAOP is due to hydrogen bonding of the nitrogen from the fused pyridine ring with the incoming amine, which allows for greater stabilisation for the overall negative charge of the leaving group (Figure 46). PyAOP side products are also known to be unreactive and relatively harmless, as compared to carcinogenic byproducts of BOP or AOP.



4.3. Model Peptide

4.3.1. Met-His-Met-Ser

Synthesis of a model peptide containing residues H-Met-His-Met-Ser-OH **198** was chosen, this was to be familiar with SPPS. The residues were chosen according to the *C*-terminus that would be synthesised however the lanthionine residues were replaced with the methionine residues.

Highly acid labile NovaSyn TGT alcohol resin was chosen, preloaded with Ser(*t*Bu) with a loading of 0.19 mmol g^{-1}. The peptide synthesis was carried out on a Merrifield Bubbler using standard solid phase peptide synthesis techniques.

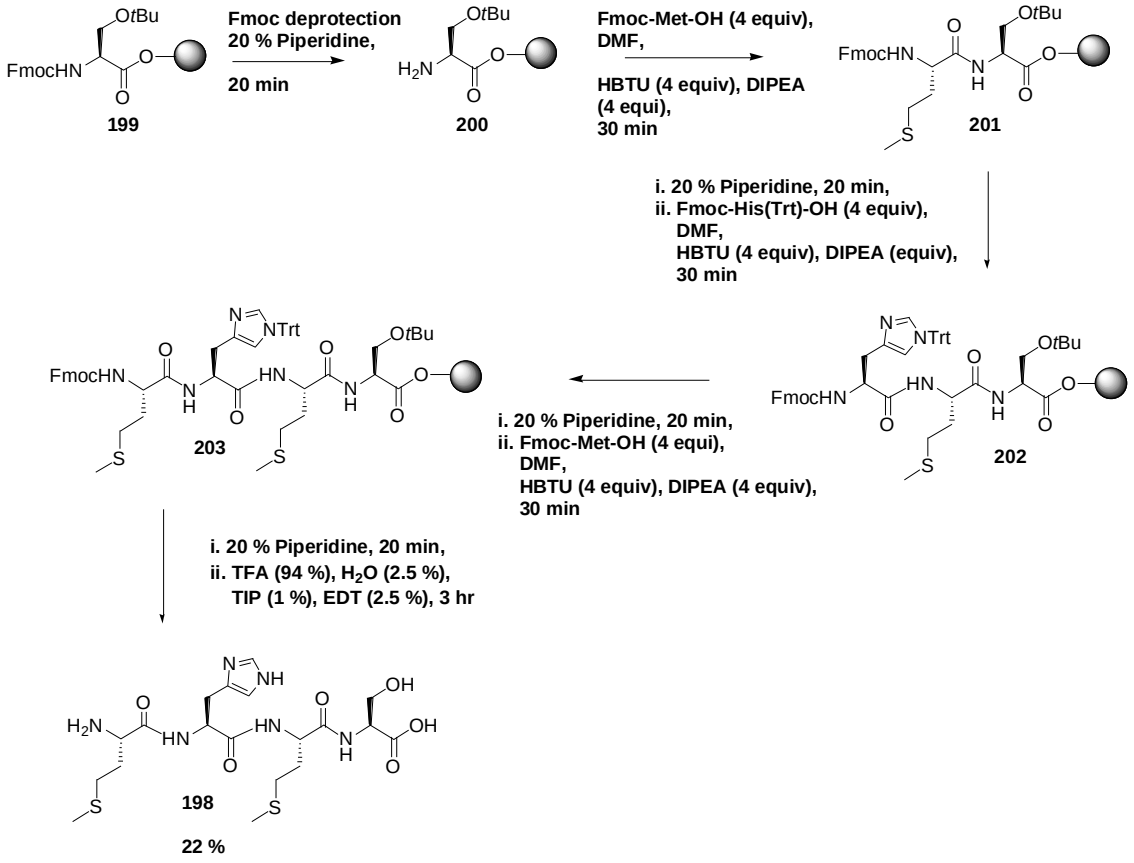
Fmoc deprotections of all the coupled amino acids were completed using 20 % piperidine in DMF this leads to comprehensive cleavage of the Fmoc group, whilst all the coupling of the amino acids were carried out using standard coupling reagents HBTU and DIPEA.

The peptide synthesis was carried out using a Merrifield Bubbler (Experimental, Figure 74). Fmoc deprotection was carried out using 20 % piperidine in DMF bubbling with argon for 20 min, the solution was then removed. The resin was washed 6 times with DMF, during which time the amino acid was activated for 20 min. The activating solution was made up with 4 equivalents of the amino acid, 4 equivalents of HBTU in 1 mL of DMF and 4 equivalents of DIPEA. After The solution was added to the resin and bubbled for 30 min, the solution was then removed, followed by washings 6 times with DMF. The procedure was repeated to

couple Fmoc-Met-OH, Fmoc-His(Trt)-OH and Fmoc-Met-OH each coupling was followed by Fmoc deprotection. The final Fmoc deprotection was carried out to give **198** (Scheme 77).

Kaiser tests [165] were carried out after each Fmoc deprotection steps and after each coupling steps, which showed that each reaction proceeded well. The final step involved the deprotection of side chain protecting groups and the cleavage of the peptide from the resin using a cocktail solution of TFA (94 %), H_2O (2.5 %), TIPS (1 %) and EDT (2.5 %). The cocktail was added to the resin and bubbled for 3 hr, the solution was collected by flushing out and then the resin was washed twice with TFA. The TFA cocktail was reduced *in vacuo* and the peptide precipitated using cold ether, the solvent was removed *in vacuo* and the residue was redissolved in water and freeze dried to give the crude product **198** as small brown crystals.

The crude peptide was characterised by LCMS which gave a peak at *m/z* 505 corresponding to $[M + H]^+$, along with with the presence of other peaks. Analytical HPLC showed that there were traces of several peaks (Appendix 19 a). Purification via preparative HPLC confirmed the presence of the same peptide in two fractions affording the desired linear peptide **198** with a yield of 22% (Scheme 77). After purification a peak corresponding to the desired product was observed by mass spectroscopy using electrospray positive mode (ESI+) (Appendix 19 b). ^1H NMR experiments were carried out in CD_3OD, however the compound was observed to still contain impurities.

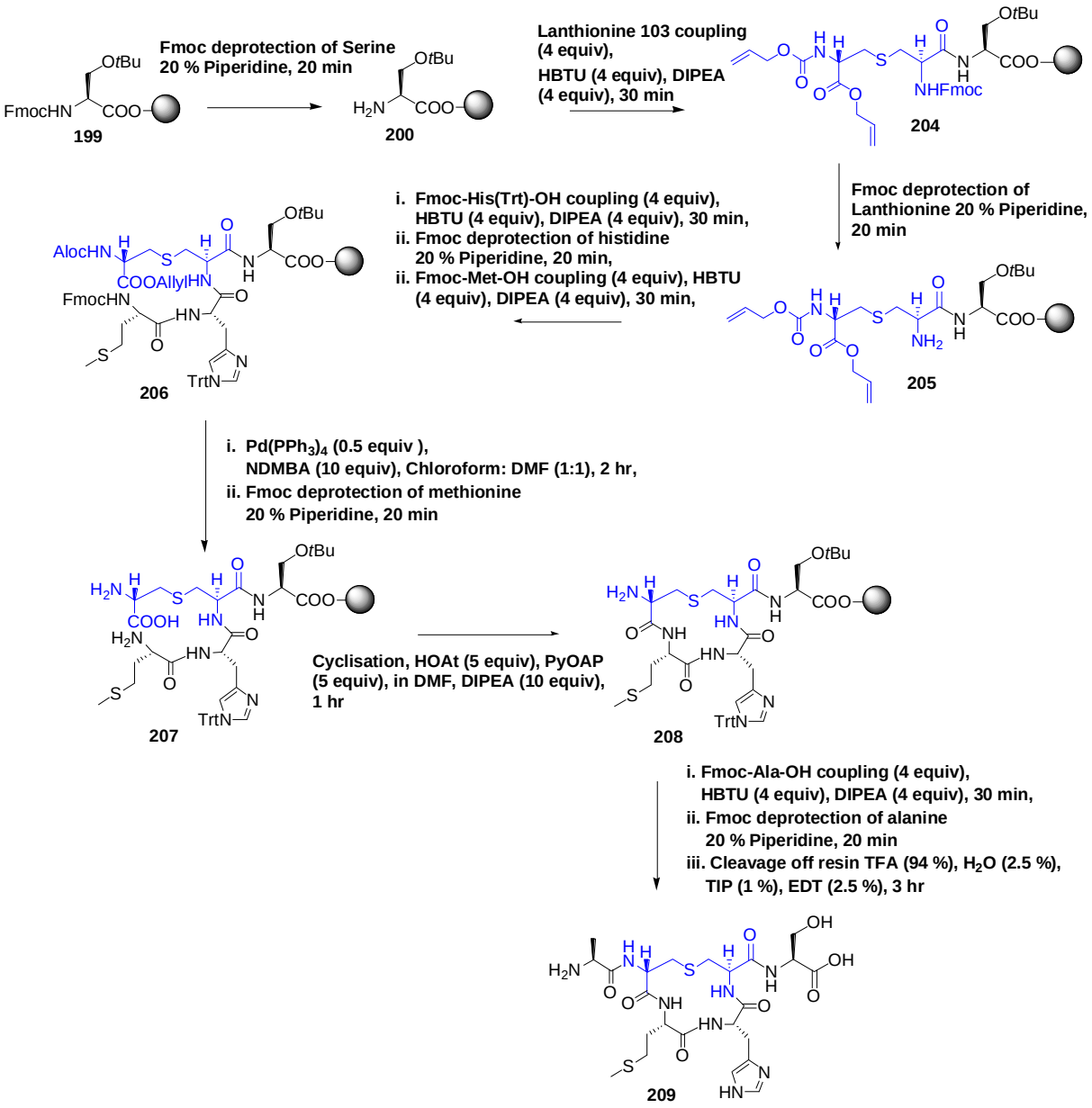


Scheme 77: Solid phase peptide synthesis of the model peptide.

After preliminary investigation of the model peptide methodology, analogue of ring D and ring E of nisin were each synthesised separately containing a single lanthionine bridge.

4.4. Synthesis of an analogue of ring E of nisin

An analogue of ring E of nisin was synthesised, containing a single lanthionine bridge, and with a methionine residue substituted for the second lanthionine at the fourth amino acid (Scheme 78). As previously NovaSyn TGT resin was used, preloaded with Ser(*t*Bu). (Aloc, allyl / Fmoc) protected lanthionine **103** was used to prepare the analogue of ring E of nisin, and it was envisaged that standard coupling conditions would be used to incorporate **103** into a linear resin-bound precursor **206**.



Scheme 78: Synthesis of an analogue of ring E of nisin.

The first attempt in synthesising analogue of ring E of nisin was carried out using a Merrifield bubbler (Experimental, Figure 74). The resin was placed in a bubbler in which argon was bubbled vigorously to make sure the resin is constantly moving. All addition of reagents for Fmoc deprotection, coupling of amino acids and washes after each synthetic step were carried out manually. All standard amino acids were activated for 20 min before being added to the resin in the Merrifield bubbler, Kaiser tests were carried out after each synthetic step. The detailed procedure is described in the Experimental chapter.

The first step involved the deprotection of the Fmoc group of serine using 20 % piperidine over 20 min followed by washes with DMF to give **200**, Kaiser test showed purple colour a positive result indicating Fmoc deprotection has taken place. Lanthionine **103** activated over 20 min was added for coupling, the resin was bubbled for 30 min, followed by washing in DMF to give **204**. A yellow colour was obtained from the Kaiser test indicating coupling has taken place. Fmoc deprotection was carried out to give **205** followed by coupling with activated Fmoc-His(Trt)-OH, Fmoc deprotection of Histidine and then coupling of activated Fmoc-Met-OH to give **206**.

Selective deprotection of the Aloc and allyl protecting groups of the lanthionine were carried out using tetrakis(triphenylphosphine)Palladium (0) catalyst $Pd(PPh_3)_4$ and 1,3-dimethyl barbituric acid (NDMBA). [78, 146, 147, 166] A solution of $Pd(PPh_3)_4$ 0.5 equivalents and 10 equivalents of barbituric acid were dissolved in DMF: chloroform which were gently heated. Since $Pd(PPh_3)_4$ was both light and heat sensitive, fume hood lights were switched off, flask containing the solution was covered in foil, and when the flask was heated it was ensured that the colour did not change from yellow to orange. The solution was added to the resin in the Merrifield bubbler, the bubbler was wrapped in foil to avoid any light entering and the reaction was carried out for 2 hr, it was made sure that the solution added was a nice yellow colour which indicates that the palladium has not gone off.

Figure 47 shows the mechanism of the deprotection of the Aloc and allyl protecting groups using palladium catalyst and NMM, where the resonance stabilisation of the carboxylate and carbamate activates the π-allyl palladium complex formed. This is then trapped by a known mild nucleophile *N*-Methylmorpholine (NMM). [167] It was found that barbituric acid gives a better result than NMM due to which barbituric acid was used. [78]

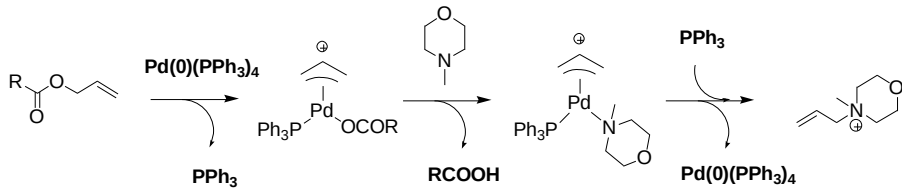


Figure 47 Mechanism for allyl deprotection. [167]

The deprotection was followed by washes by DMF, DIPEA 0.5 % v/v in DMF, DMF, sodium diethyldithiocarbamate trihydrate 0.5 % w/v in DMF, followed by the final washes with DMF. This was to ensure that the palladium catalyst was scavenged off the resin, where the DIPEA acts as a base to the barbituric acid and cleans everything whilst sodium diethyldithiocarbamate trihydrate acts as a ligand to the palladium. The qualitative Kaiser test was observed to be purple.

The Fmoc group on the methionine was deprotected and this was followed by cyclisation of ring E (Scheme 78) using PyAOP, HOAt and DIPEA for 1 hr to give the intermediate **208** (Scheme 78). Coupling of the activated Fmoc-Ala-OH was carried out followed by deprotection of the Fmoc group of alanine, followed by the cleavage of the peptide from the resin to give **209**.

From mass spectrometry the molecular weight for the peptide was visible at *m/z* 639 $[M + Na]^+$ along with a peak corresponding to an oxidised peptide. The oxidation could have been possibly due to contact with air as the bubbler was not sealed. The peptide was not visible when analytical HPLC was carried out this was due to the absence of aromatic groups therefore purification was not carried out on the preparative HPLC.

A second attempt was carried out using a Syro peptide synthesiser using a 2 mL syringe. In this attempt the Fmoc group from the alanine was not deprotected (Figure 48) to ensure easy purification via HPLC. Modifications in the reaction conditions during this attempt involved using 40 % piperidine for Fmoc deprotection and HOAt and PyAOP reagents during the lanthionine coupling instead of HBTU. The oxidised peptide was synthesised again. It was difficult to separate the oxidised peptide from the desired product which led to two peptides being isolated. From the structure it can be seen there was a possibility of oxidation of either the Met or Lan residues. However in this case only one sulphur atom had been oxidised and this was confirmed from mass spectrometry. Further NMR experiments were not carried out to distinguish which sulphur had been oxidised so it was difficult to predict from a ^{1}H NMR at this stage which sulphur had oxidised.

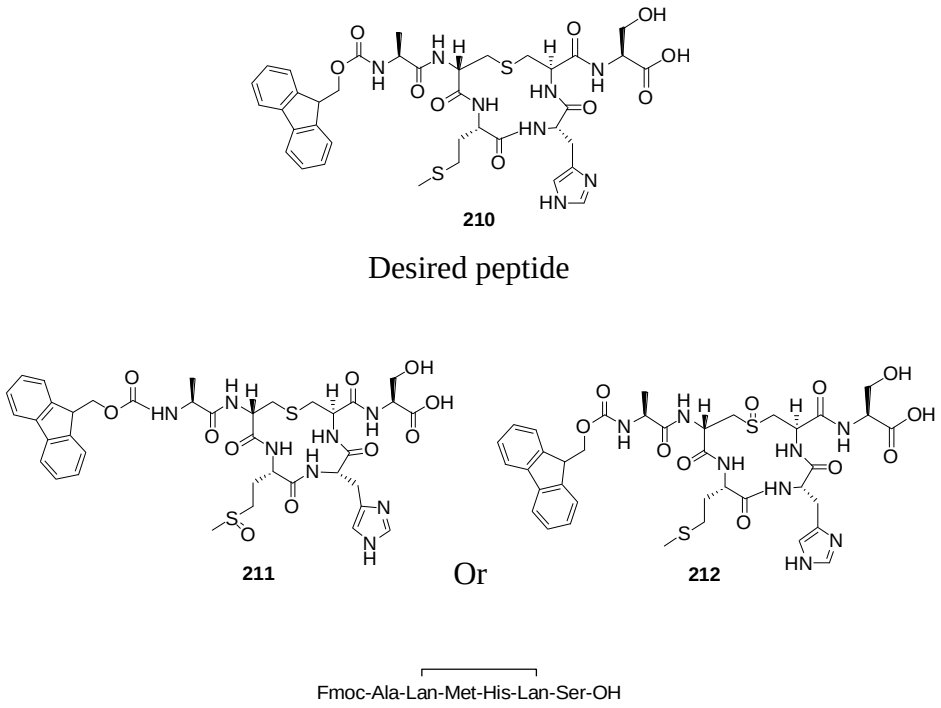


Figure 48: Structures of the desired peptide and the oxidised peptides.

The synthesis was repeated again using the MultiSyroTech peptide synthesiser. Highly acid labile NovaSyn TGT alcohol resin were used, this was to ensure easy cleavage of the peptide off the resin when using 94 % TFA. The resin was preloaded with Serine which was *tert*-butyl protected with a loading of 0.19 mmol g^{-1}. This low loading was used to prevent any cross linking between functional groups on the same resin taking place at **207** between the free amine of the lanthionine and the free carboxylic group of the lanthionine. This low loading also helps to prevent any aggregation of the peptide during the synthesis, which might interfere with any subsequent reactions. These aggregations that may take place with the solid support or with itself could be due to the hydrophobic effects or hydrogen bonding.

The first step in the solid phase peptide synthesis involved Fmoc deprotection of serine using 40 % piperidine in DMF for 30 min. In order to ensure good coupling of the lanthionine Fmoc deprotection was carried out twice on serine followed by washes with DMF after each deprotection. After each synthetic reaction the resin was washed 6 times with DMF to remove any unreacted reagents. Amino acids were not required to be activated in advance when the peptide synthesiser was used. Once the amino acid was added to the resin followed by the coupling reagents the amino acid was activated on the resin, hence the reaction time

was longer. All additions of standard amino acids, reagents and washes were carried out automatically.

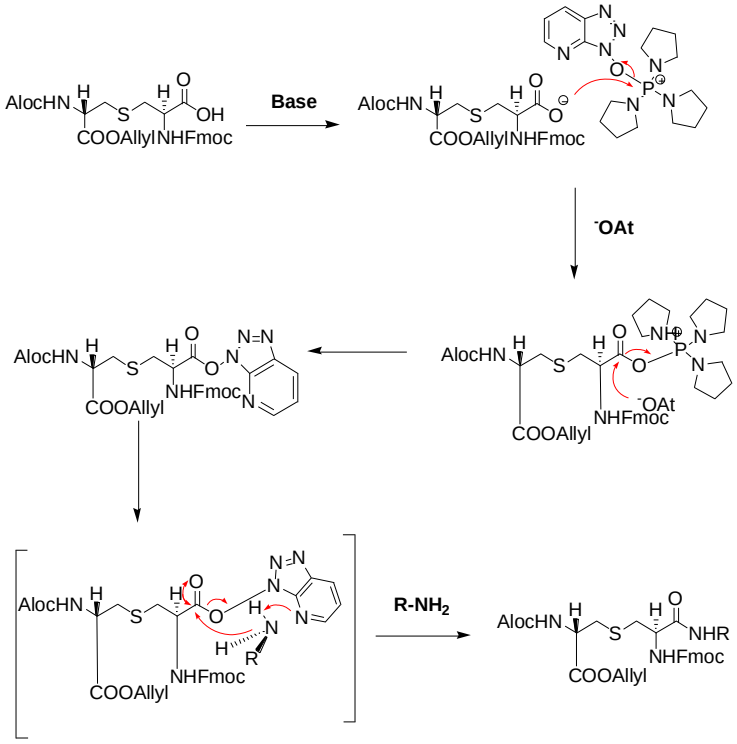


Figure 49: Mechanism of peptide coupling using PyAOP and HOAt.

Lanthionine coupling was carried out manually in the presence of PyAOP, HOAt (Scheme 79) and DIPEA for 1 hr 15 min. The syringe was sealed with an argon balloon attached, the sealing's were to make sure the amino acid in solution was not drained or leaked out. Once the reaction time was complete all sealing were removed and the syringe was placed back on the synthesiser.

The Fmoc group of the lanthionine was then deprotected with 40 % piperidine over 30 min followed by washes with DMF, this was followed by coupling of Fmoc-His(Trt)-OH using less reactive coupling reagents HBTU and DIPEA over 1 hr 15 min. Fmoc deprotection was carried out on the histidine followed by coupling of Fmoc-Met-OH to give **206** (Scheme 79).

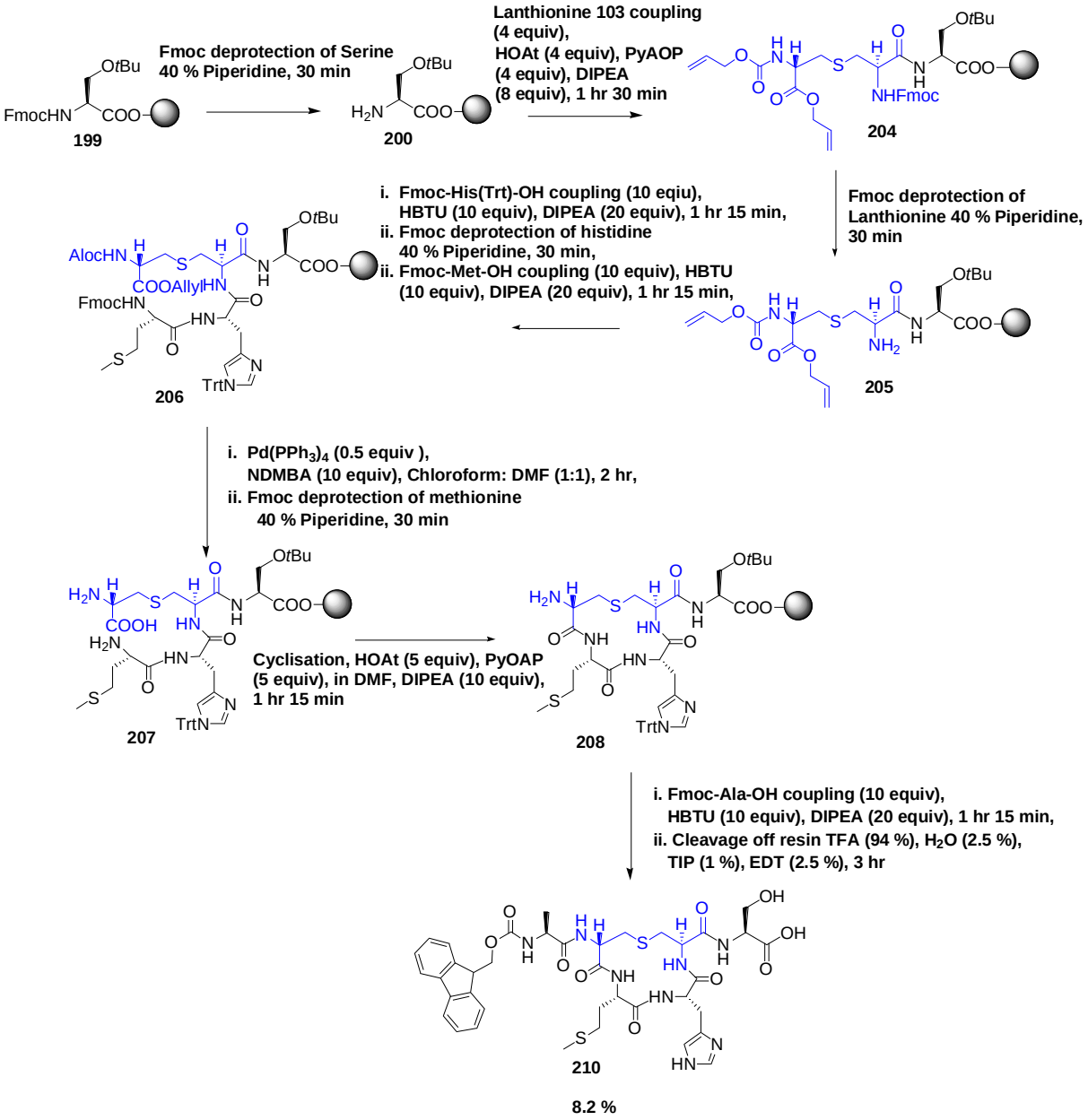
In order to check Fmoc deprotection and coupling of amino acids were taking place, Kaiser tests were carried out after each synthetic step.

The next step involved selective deprotection of the Aloc and allyl protecting groups from the lanthionine using ($Pd(PPh_3)_4$ and 1,3-dimethyl barbituric acid. [78, 146, 147, 166] The solution was prepared as previously mentioned. Precautions involved carrying out the reaction manually; the syringe was sealed from the bottom with a cap and top with a bung, and covered with foil to prevent light entering. Balloon filled with argon attached to a needle was pierced into the syringe. The solution was added manually to the resin in the syringe and the syringe was then placed back onto the synthesiser and reacted for 2 hr.

The deprotection was followed by DMF washes 6 times, DIPEA 0.5 % in v/v in DMF 6 times, DMF 6 times followed by sodium diethyldithiocarbamate trihydrate 0.5 % w/v in DMF 8 times and the final washes with 6 times DMF. This was to ensure that the palladium catalyst was scavenged off the resin, where the DIPEA acts as a base to the barbituric acid and cleans everything whilst sodium diethyldithiocarbamate trihydrate acts as a ligand to the palladium.

The Fmoc group on the methionine was then deprotected and this was followed by cyclisation of ring E (Scheme 79) using PyAOP, HOAt and DIPEA for 1 hr and 30 min to give the intermediate **208** (Scheme 79). Additions of reagents during the cyclisation step were carried out manually and the syringe was again sealed to avoid the loss of any solution. Kaiser tests were negative after each coupling and positive after each deprotection.

Using standard amino acid coupling conditions alanine was coupled, after which the peptide was cleaved off the resin using a cocktail of TFA (94 %), ethanedithiol (2.5 %), water (2.5 %) and triisopropylsilane (1 %). The resin was left in solution for 3 hr, after which the solution was collected and the resin was washed twice with TFA. The procedure not only cleaves the peptide off the resin but also leads to the cleavage of the side chain permanent protecting groups that are orthogonally protected to the Fmoc, Aloc and allyl protecting groups. TFA cocktail were collected in a Falcon tube, resin washed with neat TFA was also collected in the same Falcon tube, the peptide was precipitated using cold ether, centrifuged and then the ether layer was decanted. The procedure was repeated five times, the peptide was then redissolved in water which was freeze dried.



Scheme 79: Synthesis of an analogue of ring E of nisin.

The crude product obtained from freeze drying was a cream solid, 12.3 mg of the crude peptide was obtained from a theoretical yield of 22 mg. Mass spectrometry confirmed the molecular weight of the peptide at *m/z* 839 [M + H]$^{+}$. Analytical HPLC showed 3 major peaks along with minor peaks using a gradient of 10 – 60 % acetonitrile over 30 min with the product appearing at R_T 16.80. An assumption of where the product appears was assisted by LCMS which showed the first peak to be the actual peptide. When a purity test was carried out on the crude on the LCMS it was found to be 26.3 % pure (Figure 50). It was possible to

get better purer sample (Appendix 20) where 68 % purity was obtained during a third attempt using the same procedure discussed however this was not reproducible.

Synthesis of oxidised peptides was avoided during this and the third attempt. In these two attempts strict precautions were carried out compared to the second attempt. It was ensured that the syringe was sealed with argon balloon during lanthionine coupling, Aloc / allyl deprotection and cyclisation steps.

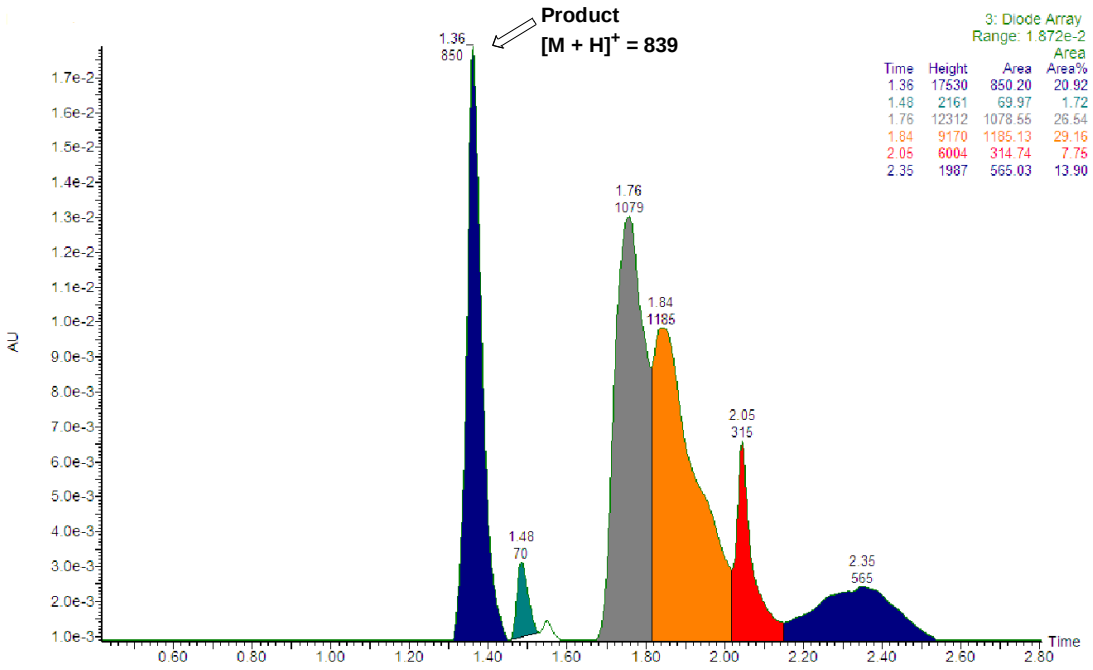


Figure 50: Crude LCMS of an analogue of ring E of nisin before purification

Purification via preparative HPLC using a linear gradient of 10 % – 60 % acetonitrile in water over 40 min isolated the pure peptide in 1.7 mg at 22.3 min (Figure 51). Mass spectrometry and ^{1}H NMR (Figure 52) showed a clean peptide, this was confirmed by analytical HPLC which showed one peak at R_T 16.80 corresponding to the peptide (Figure 53).

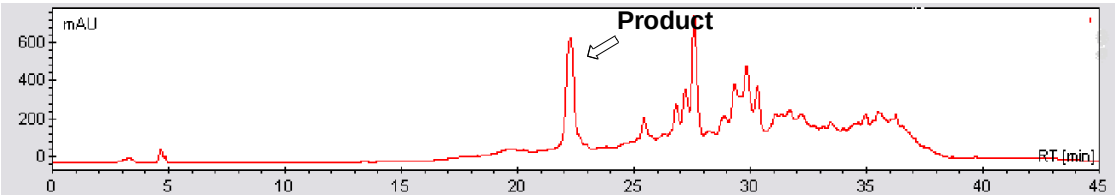


Figure 51: Preparative HPLC trace of an analogue of ring E of nisin , R_T 22.3 min; H_2O/0.5 % TFA and acetonitrile/0.5 % TFA at a gradient of 10 % - 60 % acetonitrile over 40 min.

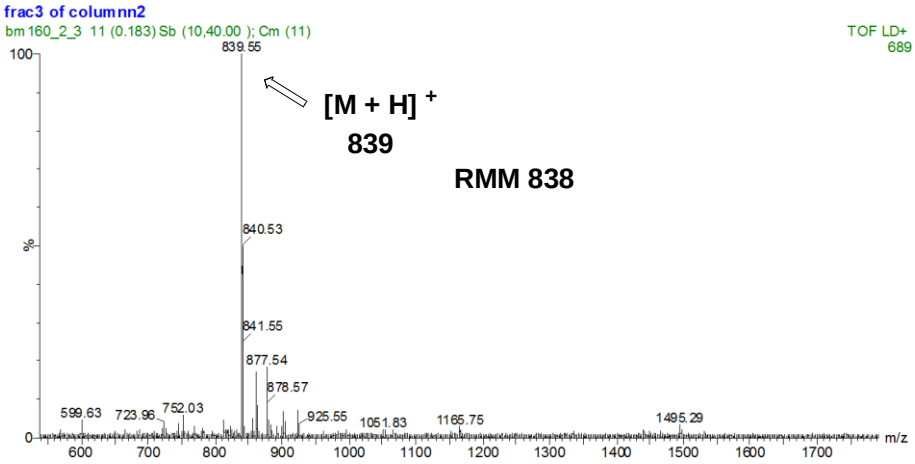


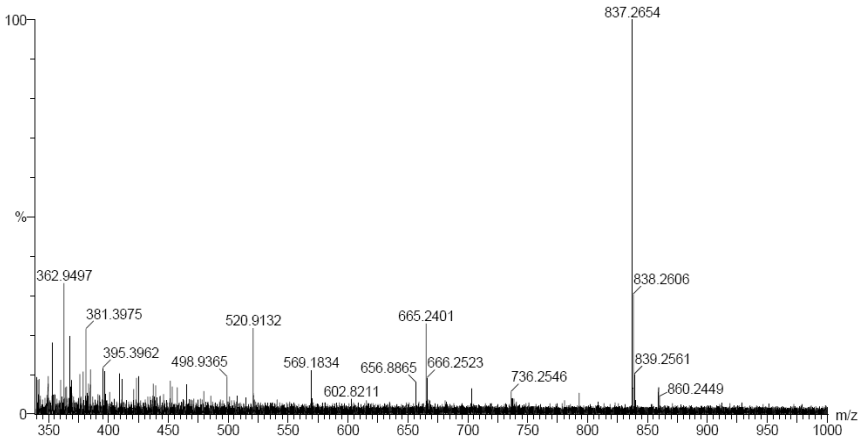
Figure 52: (a)

Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 10.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off

Monoisotopic Mass, Even Electron Ions
4 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 38-38 H: 4-200 N: 8-8 O: 10-10 Na: 0-1 S: 2-2 I: 0-1

26-Jan-2011
bm_ringe3 18 (0.411) Cm (13:24)
1: TOF MS ES-
4.19e+002



Minimum:				-1.5	
Maximum:		20.0	10.0	50.0	
Mass	Calc. Mass	mDa	PPM	DBE	Formula
837.2654	837.2700	-4.6	-5.5	20.5	C38 H45 N8 O10 S2

Figure 52: (b)

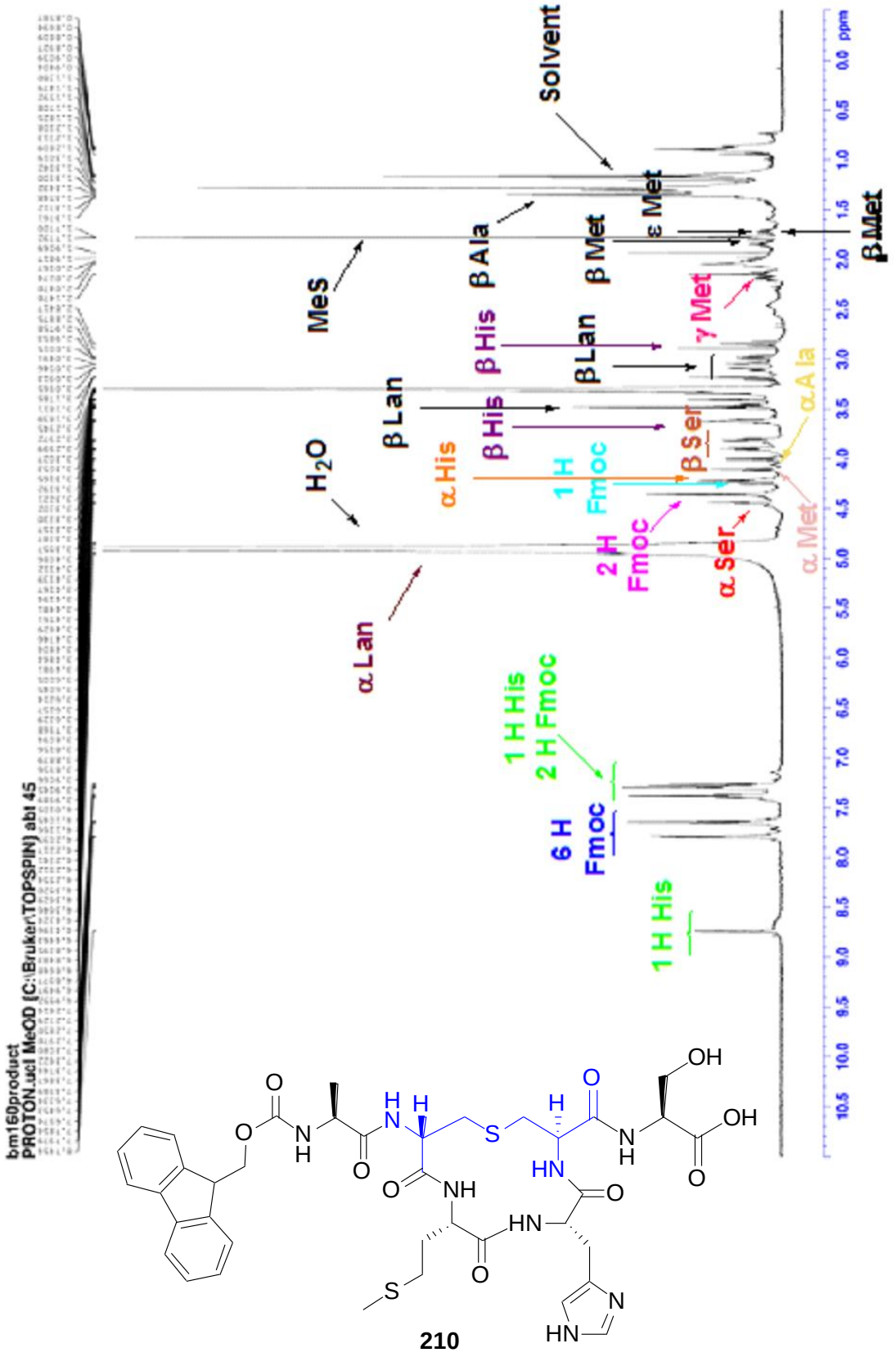
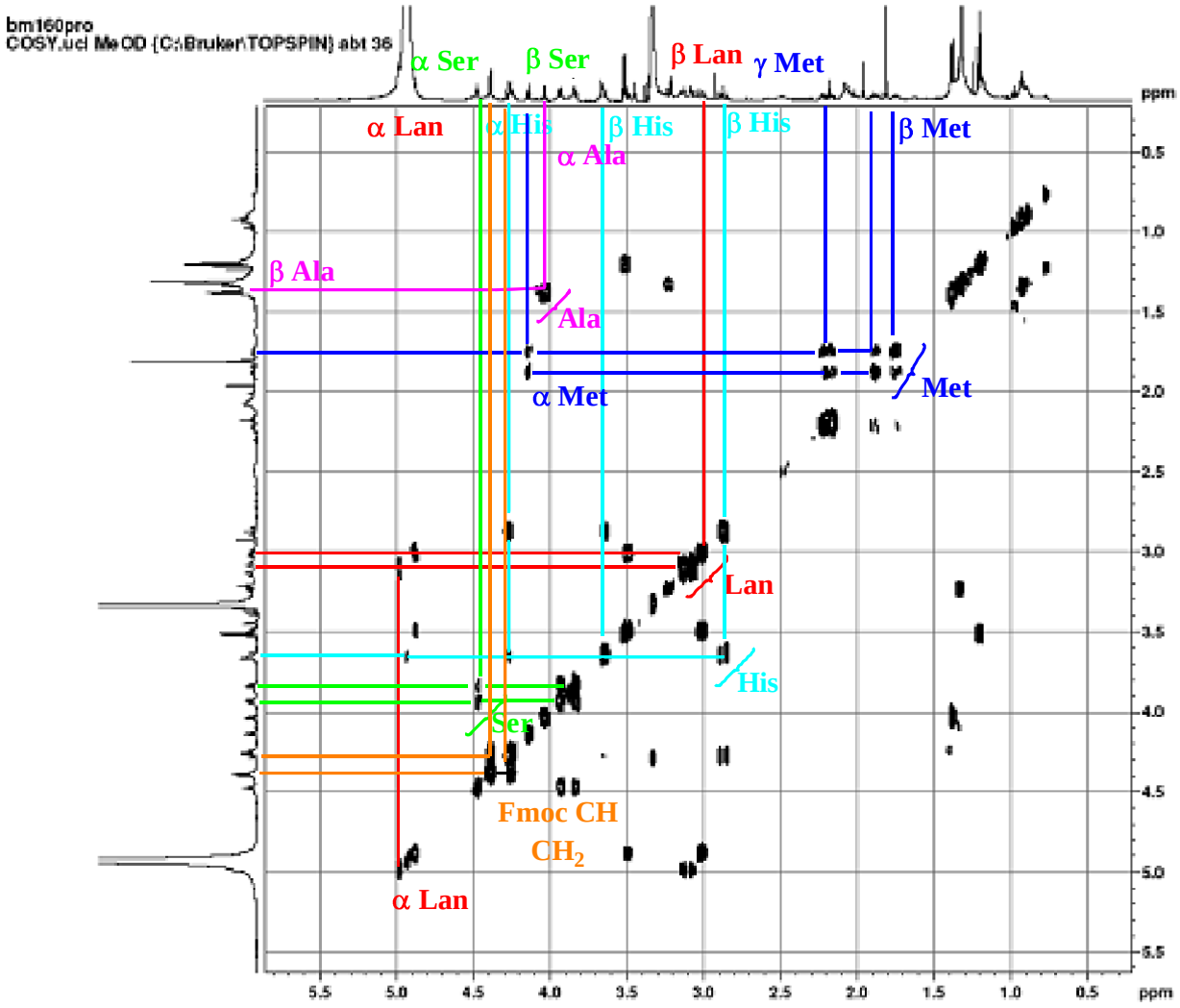
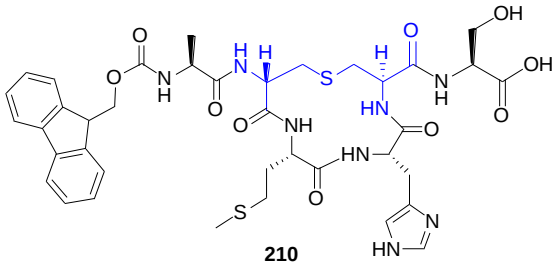

bm160product
PROTON.ucl MeOD (C:\Bruker\TOPSPIN) abt 45
Solvent
Mes
βAla
βMet
εMet
βMet
H2O
βLan
βHis
βLan
γMet
αHis
βHis
βLan
αAla
1H Fmoc
βSer
2H Fmoc
αMet
αSer
αLan
1H His
2H Fmoc
6H Fmoc
1H His
210

Figure 52: (c)





210

Figure 52: (d)

Figure 52: (a) MALDI mass spec, (b) accurate mass spec, (c) proton NMR, (d) COSY NMR of an analogue of ring E of nisin.

The ^{1}H NMR was assigned with the help of a 2D NMR spectra, chemical shifts were compared with a list of chemical shifts for protons of standard amino acids. [168] α, β protons

of lanthionine were the interesting assignments belonging to this peptide. Assignments of standard amino acids in the peptide were carried out first, chemical shifts for the α proton of lanthionine was difficult to visualise from a 1D NMR. When cross coupling were assigned in a 2D NMR, coupling peaks were noticed hiding under the water residual peak, which cross coupled between itself and the β protons of lanthionine (Figure 52, (d)). Full characterisation was carried out on **210** and all the NMR experiments can be found in the Appendices 21-24.

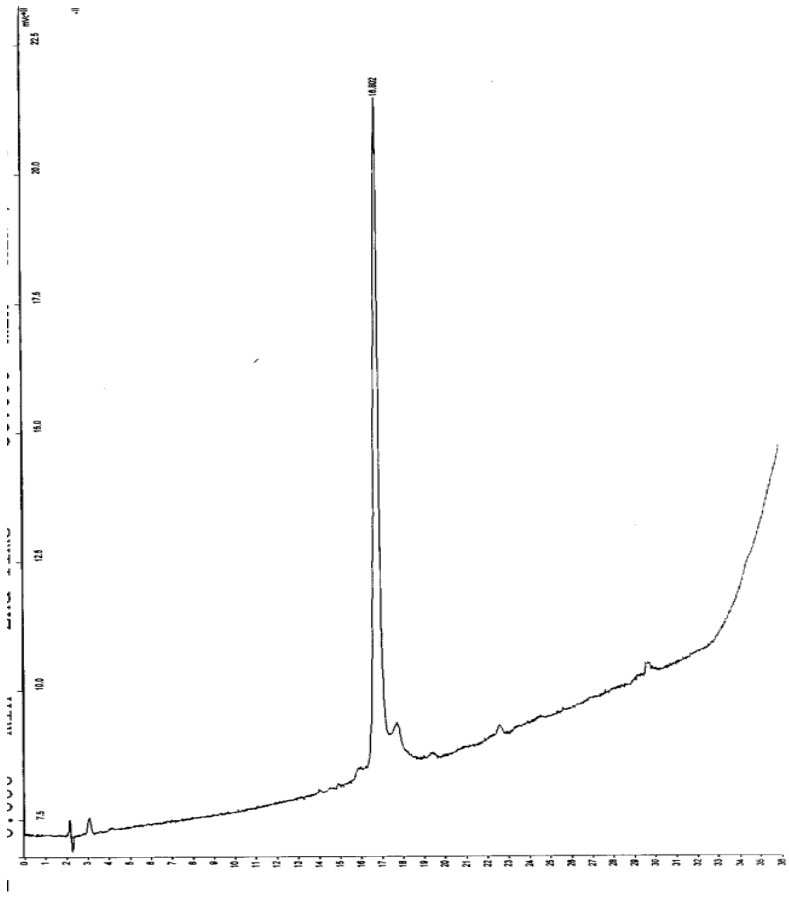


Figure 53: Analytical HPLC trace of an analogue of ring E of nisin, R_T 16.8 min; A: H_2O/0.5 % TFA; B: acetonitrile/0.5% TFA; B gradient 10 % - 60 % B over 30 min.

It has also been found that once the peptide was cleaved off the resin it requires to be purified straight away otherwise the peptide starts degrading. Figure 54 shows an analytical HPLC trace after cleavage and Figure 55 shows an analytical HPLC when the peptide started degrading.

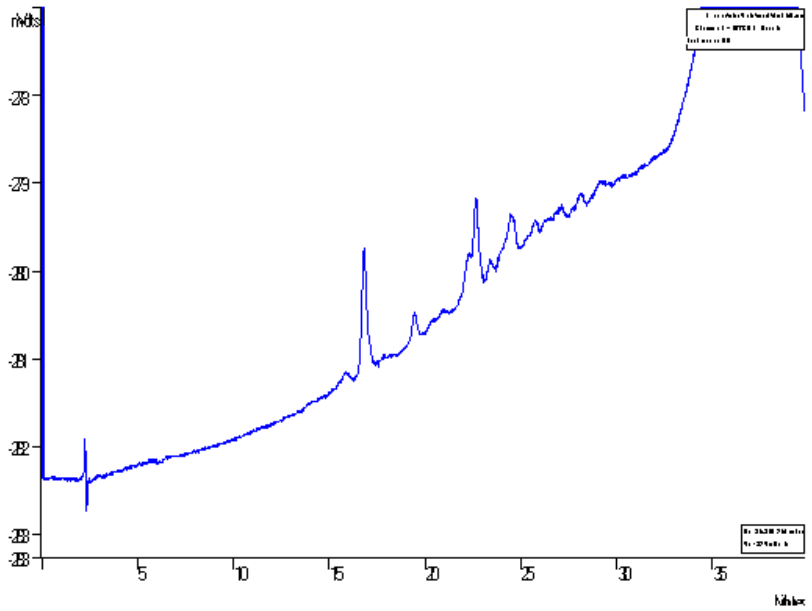


Figure 54: Analytical HPLC trace after cleavage of the analogue ring E of nisin. A: H_2O/0.5 % TFA; B: acetonitrile/0.5% TFA; B gradient 10 % - 60 % B over 30 min.

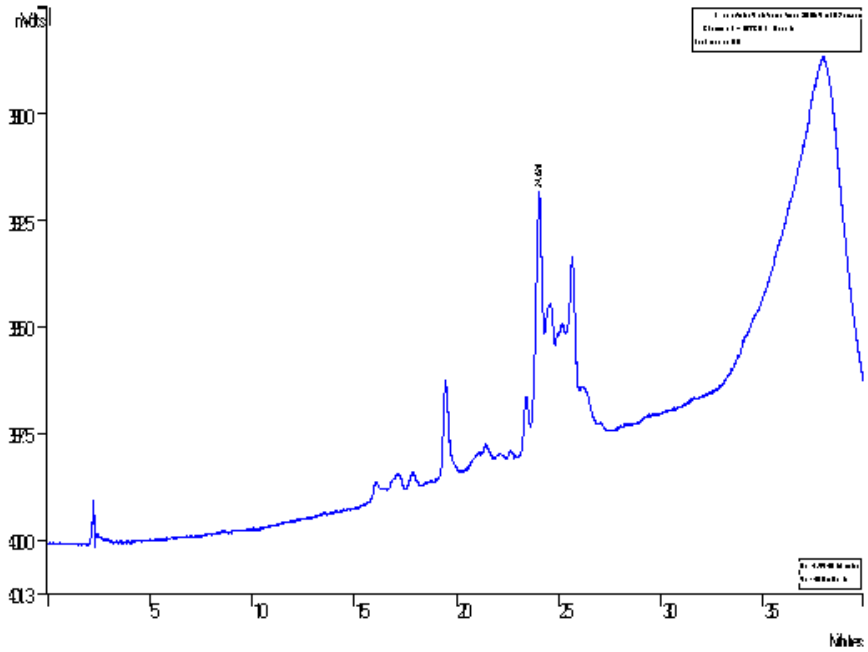


Figure 55: Analytical HPLC trace of an analogue of ring E of nisin when the peptide started degrading. Analytical was carried out 7 days after cleaving. A: H_2O/0.5 % TFA; B: acetonitrile/0.5% TFA; B gradient 10 % - 60 % B over 30 min.

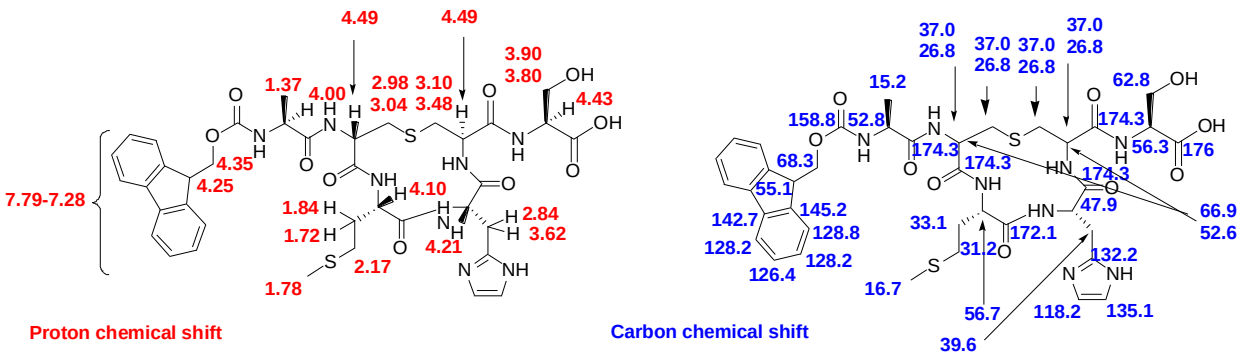
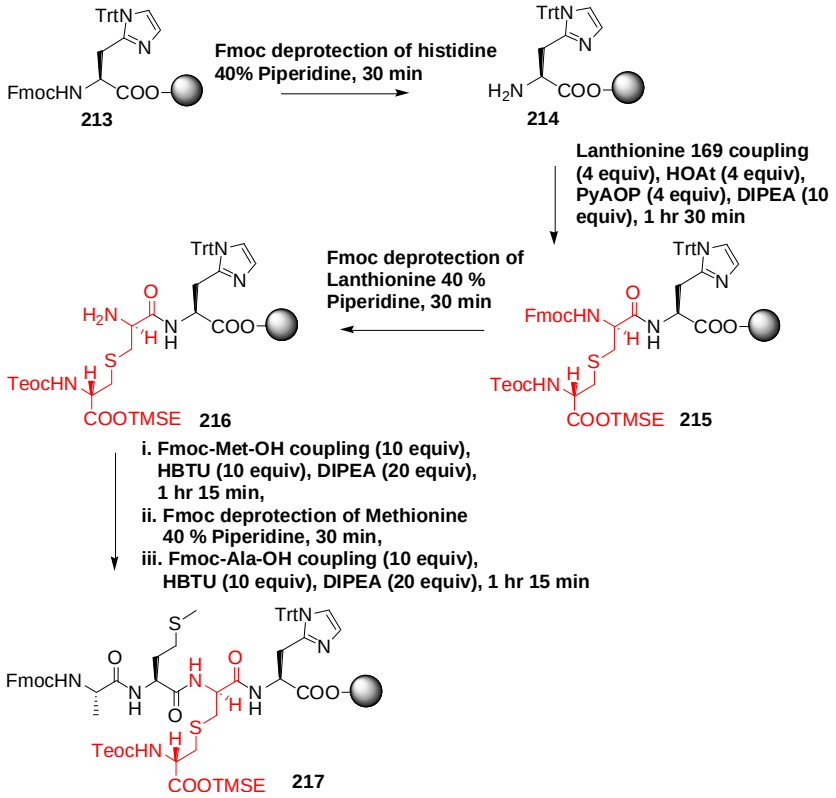


Figure 56: Structures of an analogue of ring E of nisin showing proton and carbon chemical shifts.

4.5. Synthesis of an analogue of ring D of nisin



Scheme 80: Synthesis of a linear peptide of an analogue of ring D of nisin.

The synthesis of an analogue of ring D of nisin was then attempted with the second orthogonally protected lanthionine **169**. **169** was incorporated as the second amino acid, and substituting a methionine residue as the third amino acid in place for the other lanthionine (Scheme 80). An analogue of ring D of nisin was synthesised with a single lanthionine bridge

using NovaSyn TGT resin, preloaded with His(Trt) with the α amino group Fmoc protected. (Teoc, TMSE / Fmoc) protected lanthionine **169** was used to prepare the analogue of ring D of nisin.

Synthesis of ring D was carried out using a Syro peptide synthesiser using a 2 mL syringe. As previously described all additions of amino acids, reagents and washes were carried out automatically, however the lanthionine coupling, Teoc and TMSE deprotection and the cyclisation step were carried out manually.

As ring E the first step involved Fmoc deprotection of histidine using 40 % piperidine in DMF for 30 min to give **214**. The procedure was repeated twice to ensure good coupling of the lanthionine. After each Fmoc deprotection the resin was washed 6 times with DMF. Throughout the synthesis, after each reaction the resin was washed 6 times with DMF to remove any unreacted reagents. Teoc and TMSE lanthionine coupling was carried out in the presence of PyAOP, HOAt and DIPEA for 1 hr 15 min giving **215**.

The Fmoc group of the lanthionine was then deprotected followed by washing the resin with DMF, this was followed by coupling of Fmoc-Met-OH using HBTU and DIPEA. Fmoc deprotection was carried out on the methionine followed by coupling of Fmoc-Ala-OH to give the linear resin-bound precursor **217**.

The next step involved selective deprotection of the Teoc and TMSE protecting groups from the lanthionine using tetrabutylammonium fluoride (TBAF). Sieber [169] has previously used 2.1 M TBAF to deprotect the TMSE groups. Marlowe [170] used 1.0 M TBAF in DMF to deprotect the TMSE group of a peptide on resin, the method was adapted from Ukei and Amemiya [171] where they used 0.1 M TBAF in DMF to deprotect the Fmoc group of their peptide. This meant during Teoc and TMSE deprotection it was probable that the Fmoc was also deprotected. 100 equivalents of TBAF was dissolved in DMF, the solution was added to the resin in the syringe and reacted for 1 hr. After the reaction the resin was washed 10 times with DMF to remove any unreacted reagents. However, to ensure that Fmoc deprotection was complete, the usual deprotection with 40 % piperidine was carried out.

Figure 57 shows the mechanism of the deprotection of the Teoc and TMSE protecting groups using TBAF. Nucleophiles that are strongly electronegative are effective nucleophiles for silicon which form strong bonds with the silicon upon reaction. The reaction of TBAF occurs via a pentacovalent silicon centre led by a nucleophilic attack of fluoride anion; this is permitted due to hybridisation with the vacant d-orbitals of silicon. Figure 57 shows the mechanism of the deprotection of the Fmoc group.

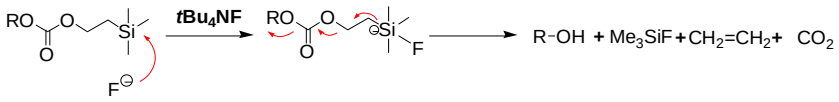


Figure 57: Mechanism for TMSE deprotection.

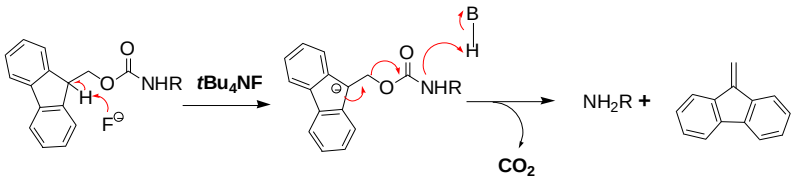
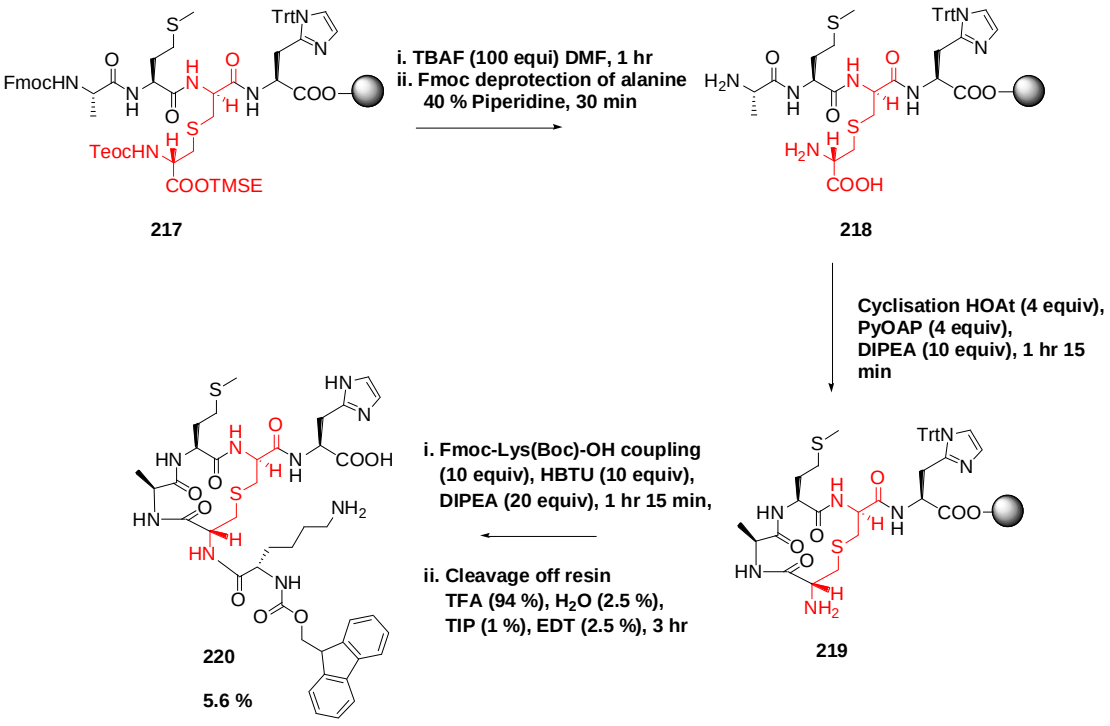


Figure 58: Mechanism for Fmoc deprotection.

After Fmoc deprotection of the alanine residue, cyclisation of **217**, to give ring D was carried out using PyAOP, HOAt and DIPEA for 1 hr and 30 min giving intermediate **219** (Scheme 81). Again PyAOP and HOAt were used due to it being more powerful coupling reagent (Figure 49).



Scheme 81: Synthesis of an analogue of ring D of nisin.

Using standard amino acid coupling conditions Fmoc-Lys(Boc)-OH was coupled, after which the permanent acid labile side chain protecting groups (Trityl and Boc) were cleaved from the cyclic peptide by TFA in the presence of a scavenger. The cleavage of the peptide off the resin and the deprotection of the side chain protecting groups were carried out in a one pot reaction using a cocktail solution containing TFA, ethanedithiol, water and triisopropylsilane. Once the TFA cocktail was reduced to a minimum volume *in vacuo*, the peptide was precipitated using cold ether, centrifuged and then the ether layer was decanted, this procedure was repeated 5 times. The peptide residue was then redissolved in water and freeze dried.

18.0 mg of the crude peptide was obtained from a theoretical yield of 17.5 mg as a cream solid. Mass spectrometry showed the molecular weight of the peptide at *m/z* 880 $[M + H]^+$ (Figure 59). Analytical HPLC showed 2 major peaks along with minor peaks when a gradient of 15 – 40 % acetonitrile over 35 min was used with the product appearing at R_T 21.55 (Figure 60).

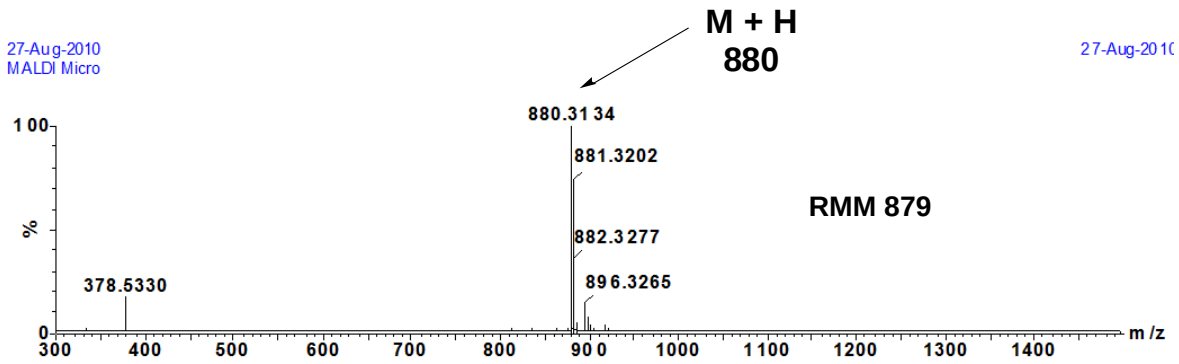


Figure 59: Mass spectrum of an analogue of ring D of nisin.

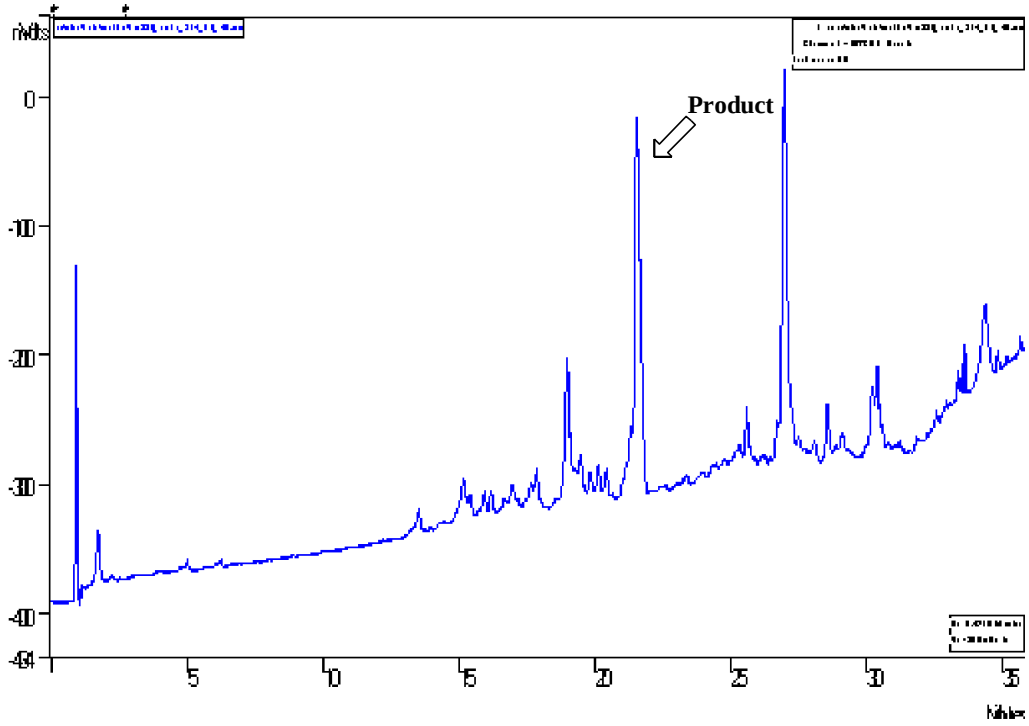


Figure 60: Analytical trace of the crude sample of an analogue of ring D of nisin. A: H_2O/0.5 % TFA; B: acetonitrile/0.5 % TFA; B gradient 15 % - 40 % over 30 min.

Purification via preparative HPLC using a linear gradient of 15 % – 40 % acetonitrile in water over 40 min isolated the pure peptide at 14.4 min giving 1.0 mg (Figure 61). Mass spectrometry and proton NMR (Figure 62) showed a clean peptide, this was confirmed by analytical HPLC which showed a single peak at R_T 16.60 corresponding to the peptide when a gradient of 10 - 60 % acetonitrile over 30 min was used (Figure 63).

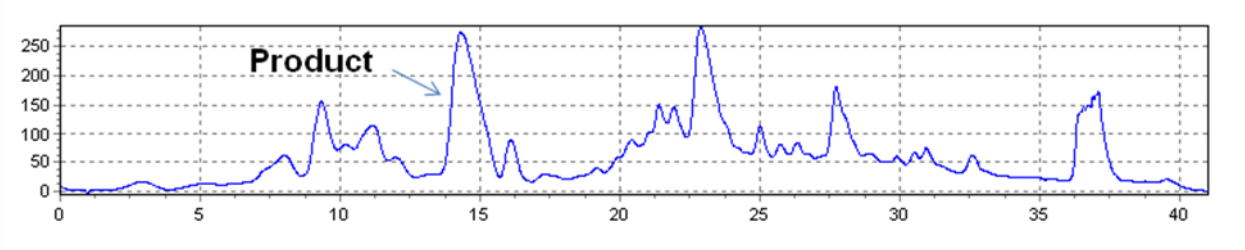


Figure 61: Preparative HPLC trace of an analogue of ring D of nisin. R_T 14.1 min; A: H_2O/0.5 % TFA; B: acetonitrile/0.5 % TFA; B gradient 15 % - 40 % over 30 min.

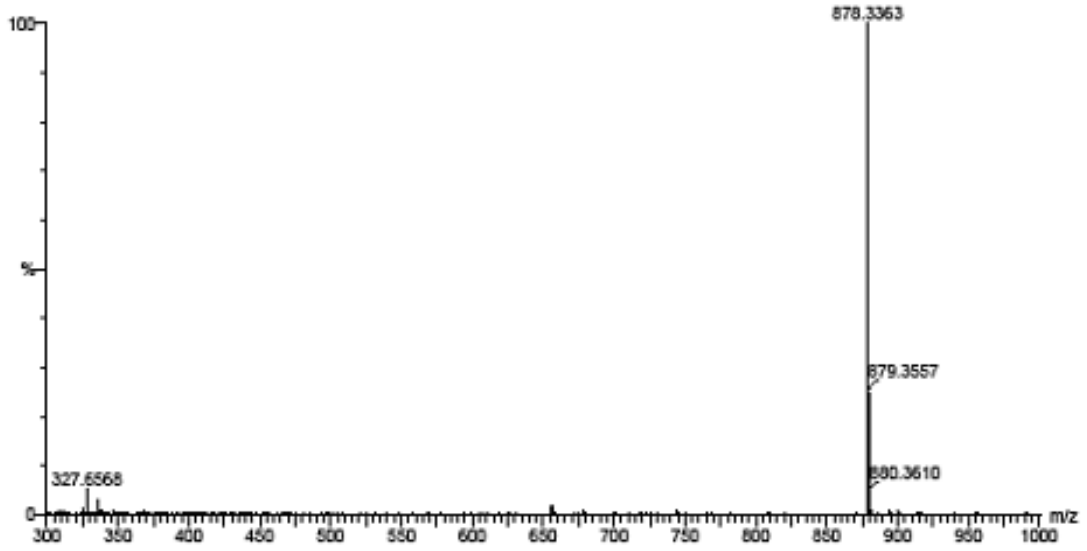
Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 100.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off

Monoisotopic Mass, Even Electron Ions
4 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 41-41 H: 4-200 N: 9-9 O: 9-9 Na: 0-1 S: 2-2 I: 0-1

26-Jan-2011
bm_ringd_AA 14 (0.784) Cm (14:17)

2: TOF MS ES-
3.60e+003



Minimum:				-1.5	
Maximum:		20.0	100.0	50.0	
Mass	**Calc. Mass**	**mDa**	**PPM**	**DBE**	**Formula**
878.3363	878.3329	3.4	3.9	20.5	C41 H52 N9 O9 S2

Figure 62 (a)

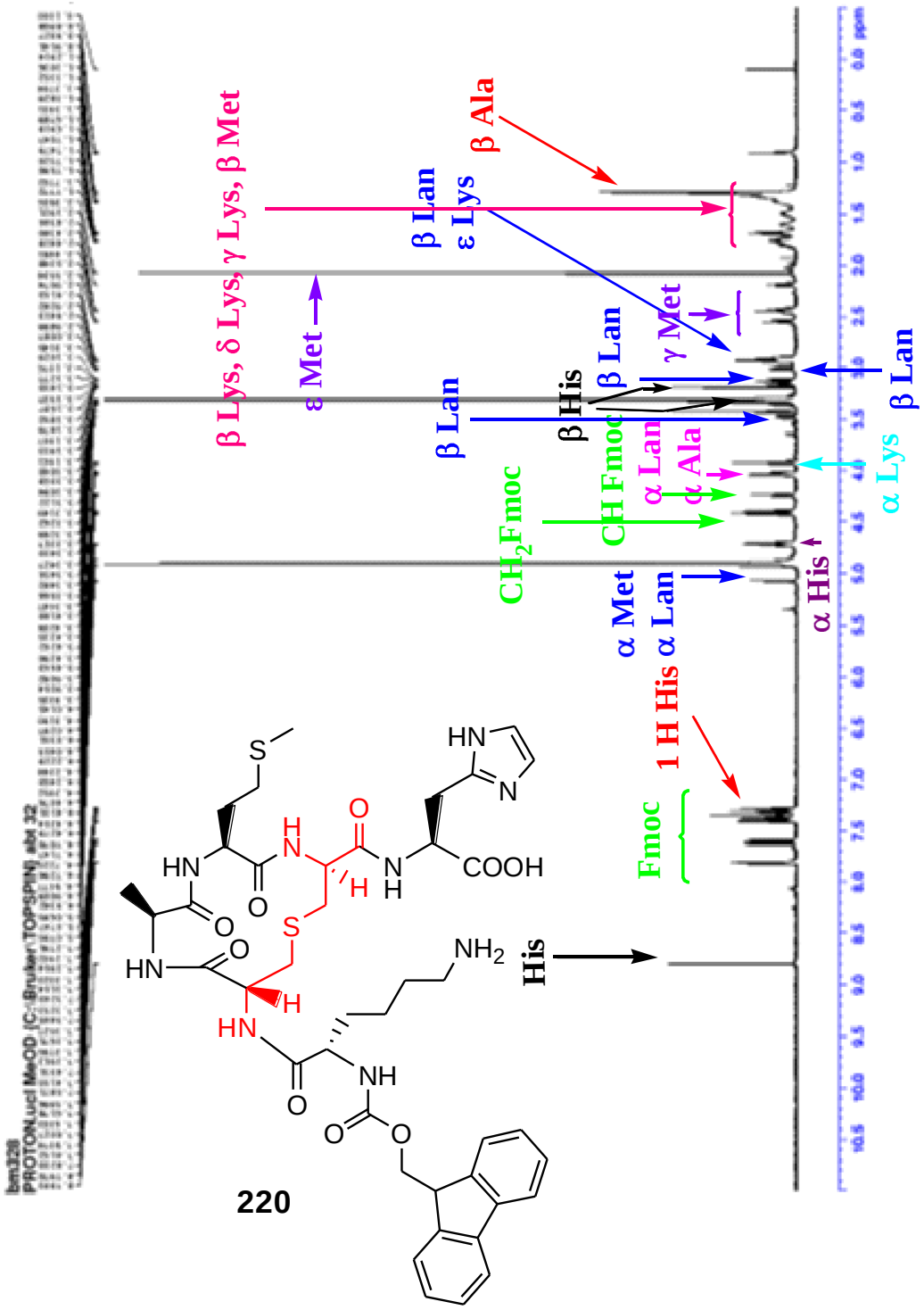
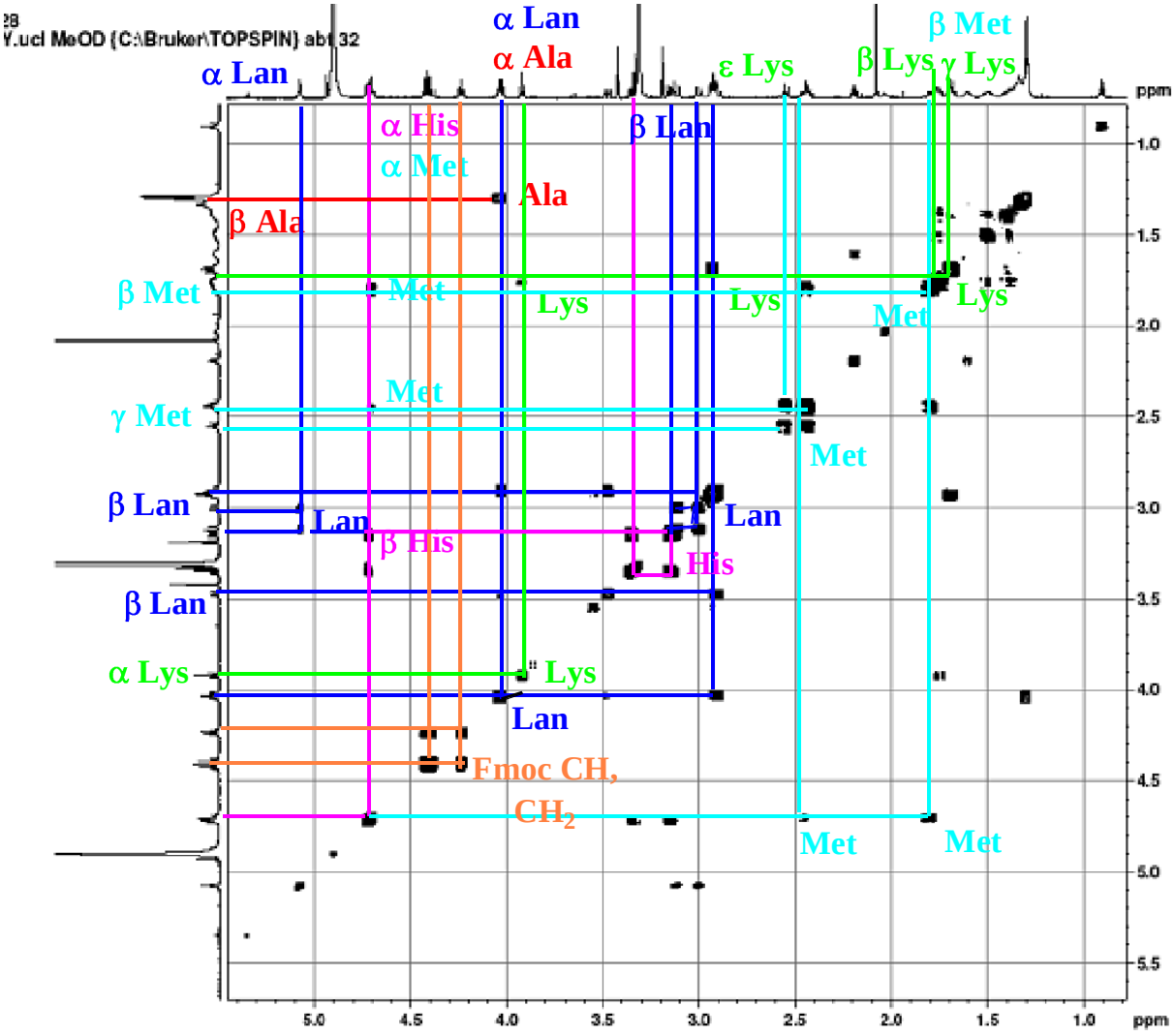


Figure 62 (b)

A 2D NMR was carried out to help characterise the proton NMR this was followed by full characterisation of **220** (Appendices 25-29).



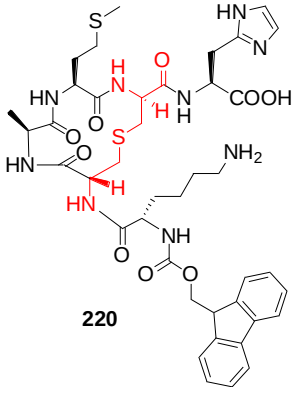


Figure 62 (c)

Figure 62: (a) Accurate mass, (b) proton NMR (c) COSY NMR of an analogue of ring D of nisin.

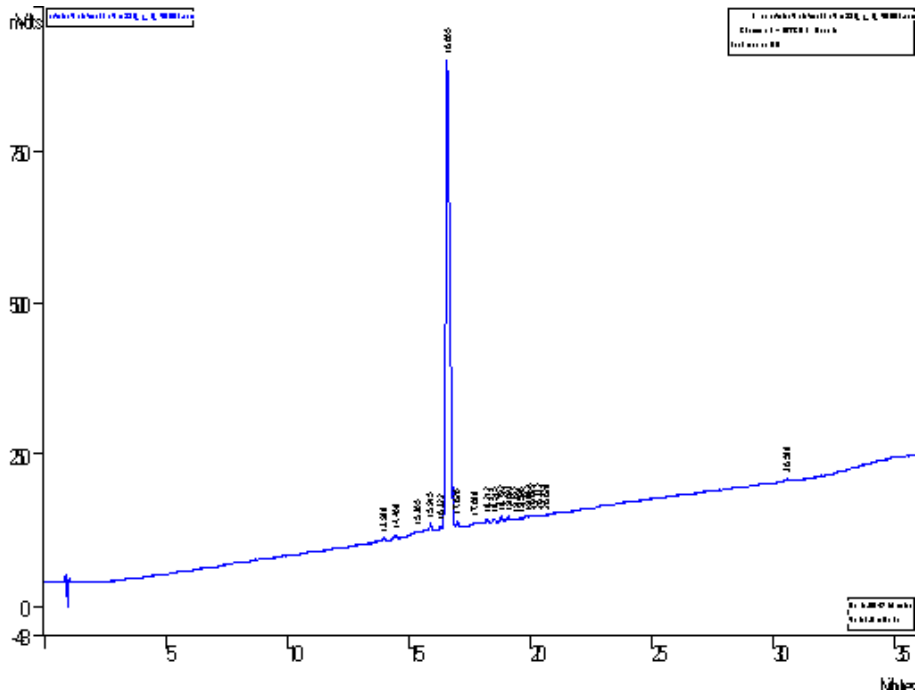


Figure 63: Analytical HPLC trace of an analogue of ring D of nisin. A: H_2O/0.5 % TFA; B: acetonitrile/0.5 % TFA; B gradient 10 % - 60 % over 30 min.

Purification of the peptide was carried out straight away to avoid any decomposition and oxidation. The peptide was easily separated from the impurities.

The Fmoc group from the lysine was not deprotected to ensure easy purification via preparative HPLC and to correlate the ^{1}H NMR spectra of an analogue of ring D of nisin with the ^{1}H NMR spectra of an analogue of ring E of nisin.

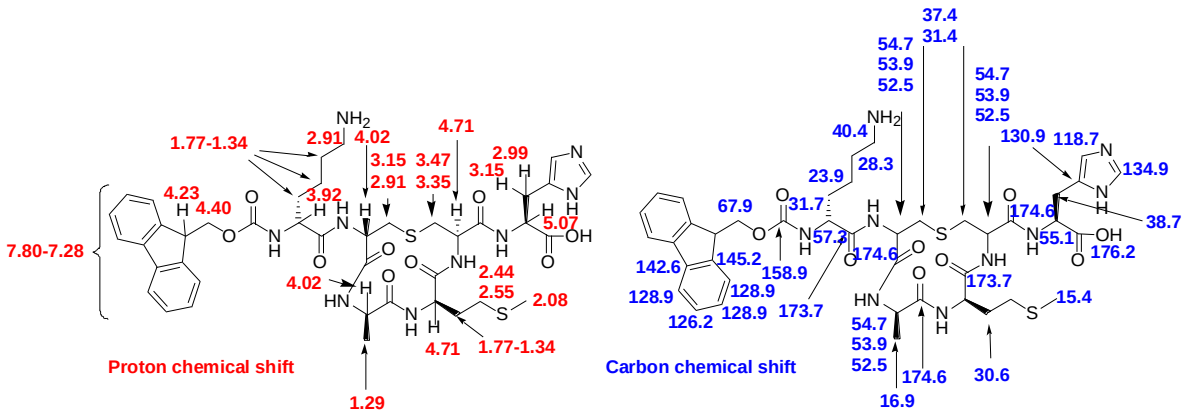
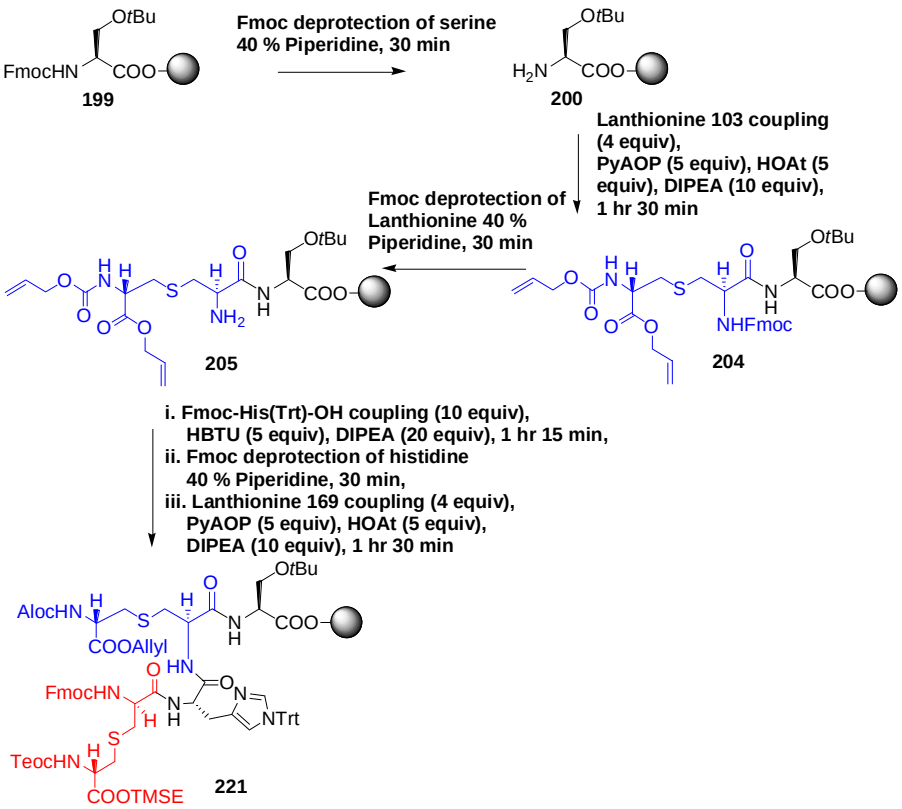


Figure 64: Structures of an analogue of ring D of nisin showing proton and carbon chemical shifts.

4.6. Synthesis of an analogue of rings D and E of nisin

After preliminary investigation of the methodology for the synthesis of the two individual rings, ring D and ring E, bicyclic peptide was then attempted. An analogue of ring D and E of nisin was synthesised containing two overlapping lanthionine bridges. The two distinct lanthionine residues with protecting groups orthogonal to each other were used: (Aloc, allyl) / (Fmoc) protected lanthionine **103** was used to prepare analogue of ring E of nisin and (Teoc, TMSE) / (Fmoc) protected lanthionine **169** was used to prepare analogue of ring D of nisin.



Scheme 82: Synthesis of the linear peptide of an analogue of the overlapping rings D and E of nisin.

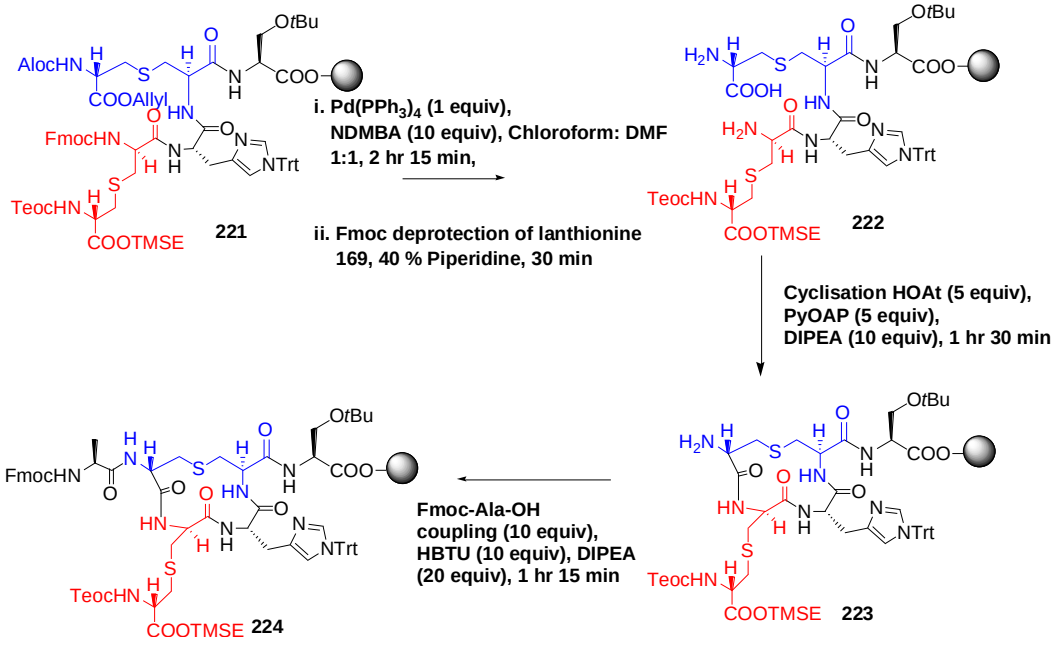
NovaSyn TGT resin, preloaded with Fmoc-Ser(*t*Bu), was used. Again the low loading resin was used to prevent any cross linking between functional groups on the same resin and this approach had been successful when synthesising the individual rings D and E.

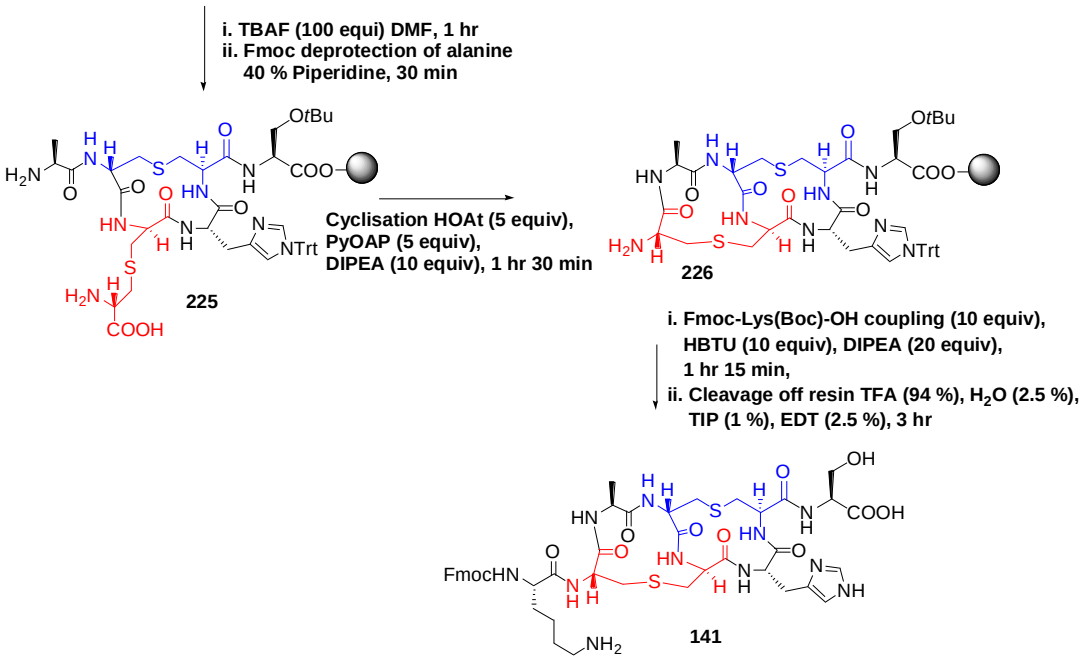
The synthesis of the linear peptide **221** was carried on the peptide synthesiser. The resin was washed 6 times with DMF after each synthetic steps. The Fmoc group of serine was

deprotected using piperidine, this was repeated twice to ensure complete deprotection. The coupling of **103** was carried out in the presence of PyAOP, HOAt and DIPEA over 1 hr 30 min. The reagents were added manually, making sure the syringe was well sealed to avoid losing any of the lanthionine amino acid solution. Addition of the second lanthionine **169**, deprotection of the protecting groups of both lanthionine and cyclisations were also carried out manually. Additions of all other standard amino acids, reagents and washes were done automatically.

The Fmoc group of lanthionine **103** was deprotected over 30 min followed by washings with DMF, this was followed by coupling of Fmoc-His(Trt)-OH using HBTU and DIPEA over 1 hr 15 min. Fmoc deprotection was carried out on the histidine followed by coupling of Fmoc protected second lanthionine **169** to give **221** (Scheme 82).

The next step involved selective deprotection of Aloc and allyl protecting groups from lanthionine **103** using ($Pd(PPh_3)_4$ and 1,3-dimethyl barbituric acid, using the same method which was previously used for the synthesis of the analogue of ring E **210**. After all the washes the Fmoc group of the second lanthionine **169** was deprotected which was followed by cyclisation forming ring E (Scheme 83) using PyAOP, HOAt and DIPEA over 1 hr and 30 min to give the resin-bound intermediate **223**. Standard amino acid coupling conditions were used to couple Fmoc-Ala-OH to give **224**.





Scheme 83: Synthesis of the analogue of the overlapping rings D and E of nisin.

The next step involved selective deprotection of the Teoc and TMSE protecting groups from the lanthionine using TBAF. The solution was added to the syringe and reacted for 1 hr. After washes with DMF the base labile N^{α}-Fmoc protecting group of alanine was deprotected by piperidine, and cyclisation of ring D (Scheme 83) was carried out using PyAOP, HOAt and DIPEA for 1 hr and 30 min to give the intermediate **226**. Fmoc-Lys-OH was then coupled followed by cleavage of the permanent protecting groups on the peptide and cleavage from the resin to give **141** (Scheme 83).

The crude peptide obtained from freeze drying was a cream solid, 26.4 mg of crude peptide was obtained from a theoretical yield of 21.15 mg. Mass spectrometry showed the molecular weight of the peptide at *m/z* 1008 $[M + H]^{+}$ (Figure 65).

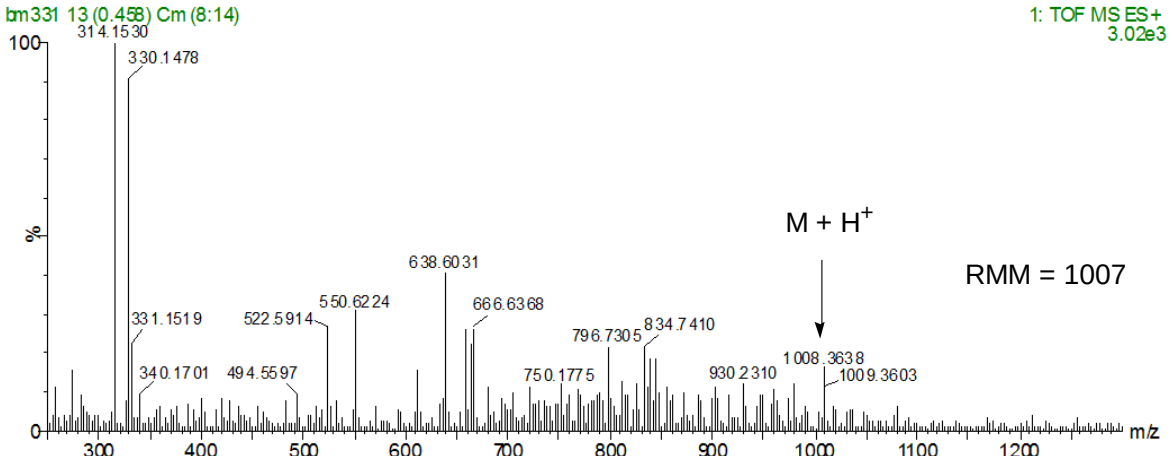


Figure 65: Crude mass spectrum of the analogue of rings D and E of nisin.

Analytical HPLC of crude peptide showed a hump with two slightly distinguished peaks along with many small peaks when a gradient of 15 % - 50 % acetonitrile was used (Figure 66). Different gradients were used in an attempt to obtain a good separation however this was not successful.

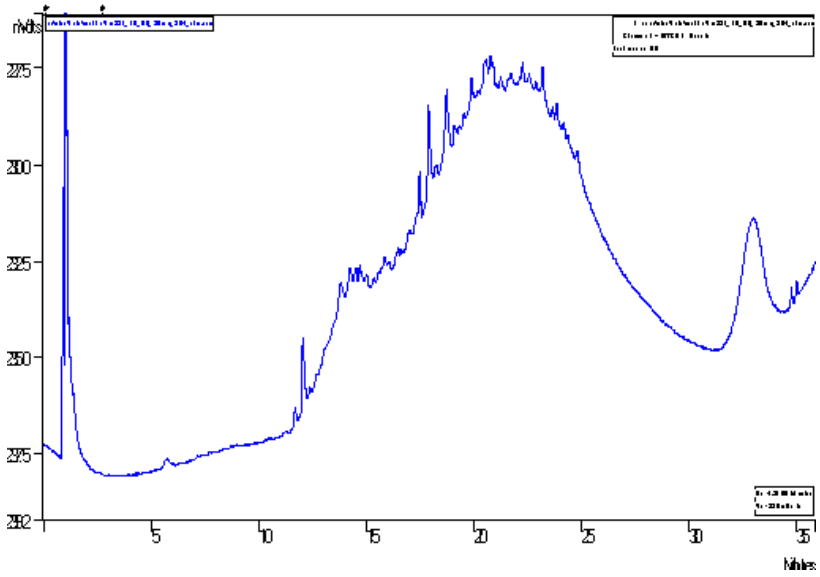


Figure 66: Crude analytical HPLC trace of the analogue of rings D and E of nisin. A: H_2O/0.5 % TFA; B: acetonitrile/0.5 % TFA; B gradient 15 % - 50 % over 30 min.

A test preparative HPLC was carried out to see whether a separation could be observed during purification. Fractions were collected every 3 to 5 min, and mass spec was carried out on each fraction to identify where the product was. From the mass spectrums it was discovered that the product was in fraction 22.33 – 27.58 min (Figure 67) however no peaks could be distinguished on the HPLC spectra.

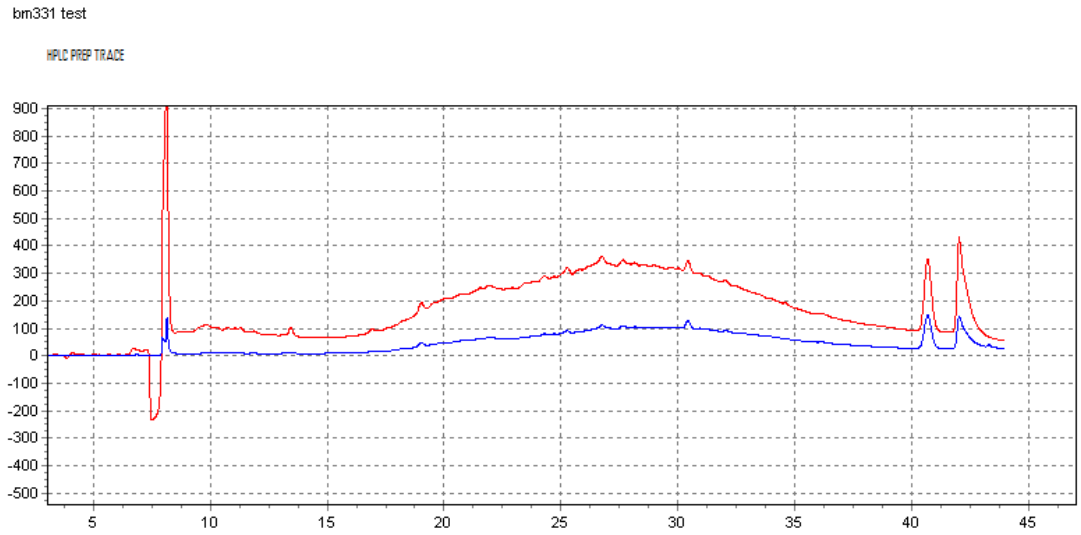


Figure 67: Test preparative HPLC trace of an analogue of rings D and E of nisin. A: H_2O/0.5 % TFA; B: acetonitrile/0.5 % TFA; B gradient 15 % - 50 % over 35 min. Trace in red shows absorption at 214 and trace in blue show absorption at 254.

The crude sample was put through preparative HPLC in couple of runs, when the time reached 22 min the fractions were collected every min until 28 min to find the retention time of the product. From these runs mass spectrum (Appendix 30) showed that the product comes between 26.1 – 27.0 min. A proton NMR was carried out. From ^{1}H NMR it was hard to assign peaks however it was observed that the product was present, the Fmoc group was clearly visible. Also where the α and β protons of the amino acid residues should be present, peaks were obtained however they were broad and overlapping which made the assignments impossible.

From analytical HPLC it was seen that the sample obtained was much cleaner compared to that of the crude. A sharp peak was seen along with a slightly broad peak which was very promising (Appendix 31).

An attempt was carried out to separate the above two peaks, the peptide was dissolved in 50 % EtOH and 50 % H_2O which was then centrifuged. The solvent was decanted and the procedure repeated. LCMS was carried out using a gradient of 10 % - 90 % acetnoitrile with 0.1 % TFA to see whether the product was present in the supernatant. A nice peak of the product was present along with another unknown peak, LCMS of the pellet sample also showed the product however there was less of a hump in the supernatant sample compared to the pellet sample (Appendix 32).

Analytical HPLC trace of the pure sample after the third purification showed a sharper peak however the hump was still present (Figure 68). A ^{1}H NMR was attempted with this sample, however there was insufficient material to obtain an assignable spectra.

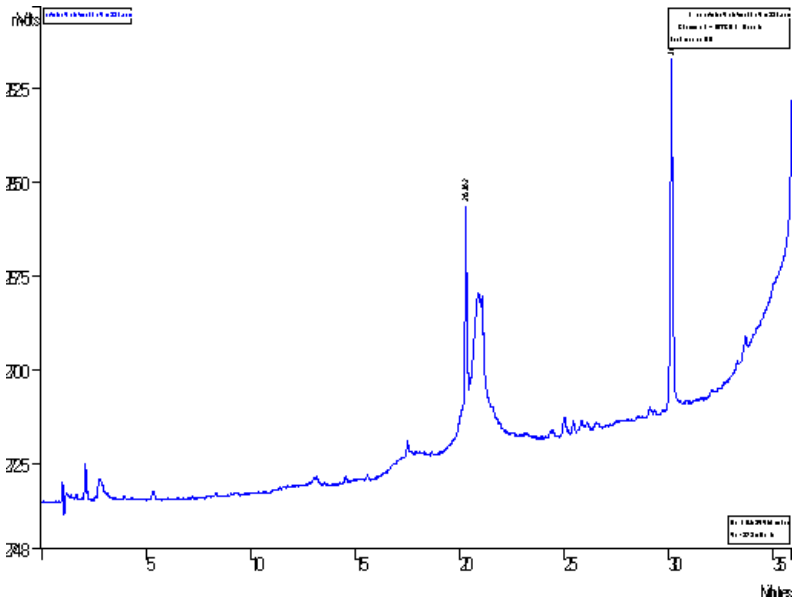


Figure 68: Analytical HPLC trace after the third purification. A: H_2O/0.5 % TFA; B: acetonitrile/0.5 % TFA; B gradient 10 % - 90 % over 30 min.

Synthesis of the analogue **141** rings D and E of nisin was attempted again using the same procedure as previously described. The mass spectrum obtained from the crude peptide showed a single product (Figure 69), however HPLC revealed that this was not the case. Analytical HPLC proved to be much better, two peaks could be distinguished and were better resolved (Appendix 33).

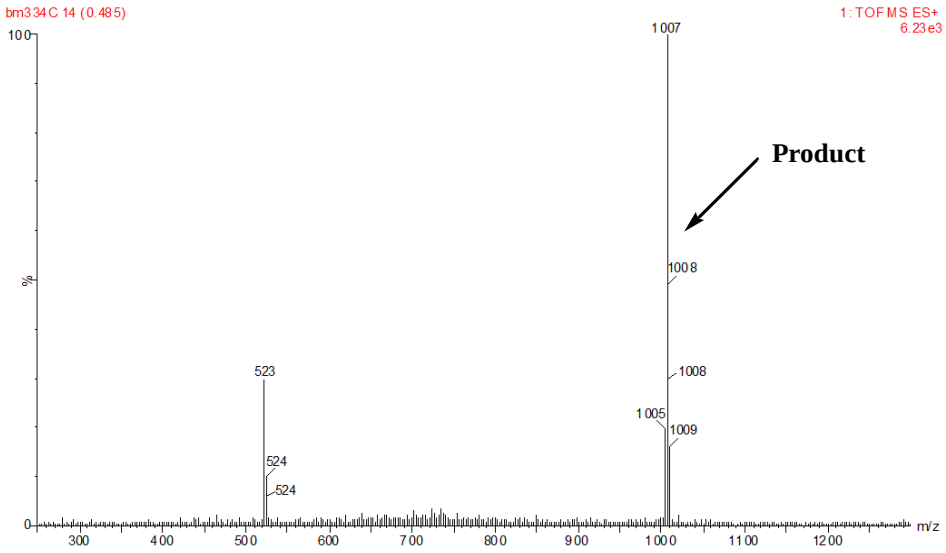


Figure 69: Mass spectrum of rings D and E of nisin obtained from the second attempt.

During the first attempt when, purification was carried out, 80 % of the material was lost during preparative HPLC. To avoid this huge loss a Varian Bond-Elute C18 column was used. Here the column was first washed with 100 % methanol then with 100 % water to saturate the column. The crude peptide was dissolved in minimum amount of water and loaded onto the column, the solvent was collected as flow through and then the column was loaded with 100 % water. Table 9 shows the eluant conditions for the fractions collected during the column. 0.1 % TFA in acetonitrile was used to wash the column. Mass spectrometry showed that the product was removed from the column and collected in fraction 16 (Table 9, Fraction 16), an analytical trace can be seen in the appendices (Appendix 34).

Fraction	Water (%)	Methanol (%)	Acetonitrile (%)	TFA (%)
Flow through	-	-	-	-
1	100	0	-	-
2	95	5	-	-
3	90	10	-	-
4	85	15	-	-
5	80	20	-	-
6	75	25	-	-
7	70	30	-	-
8	65	35	-	-
9	60	40	-	-
10	50	50	-	-
11	40	60	-	-
12	30	70	-	-
13	20	80	-	-
14	10	90	-	-
15	0	100	-	-
16	-	-	99.9	0.1

Table 9: Eluant conditions for overlapping rings D and E peptide purification.

Fraction 16 obtained from the C18 column was then analysed by LCMS. From mass spectrum it was observed the product was present in the sample so purifcation of this sample was attempted through LCMS where fractions were collected. The trace showed two peaks, a sharp peak and a slightly broad peak which were partially seperated (Figure 70).

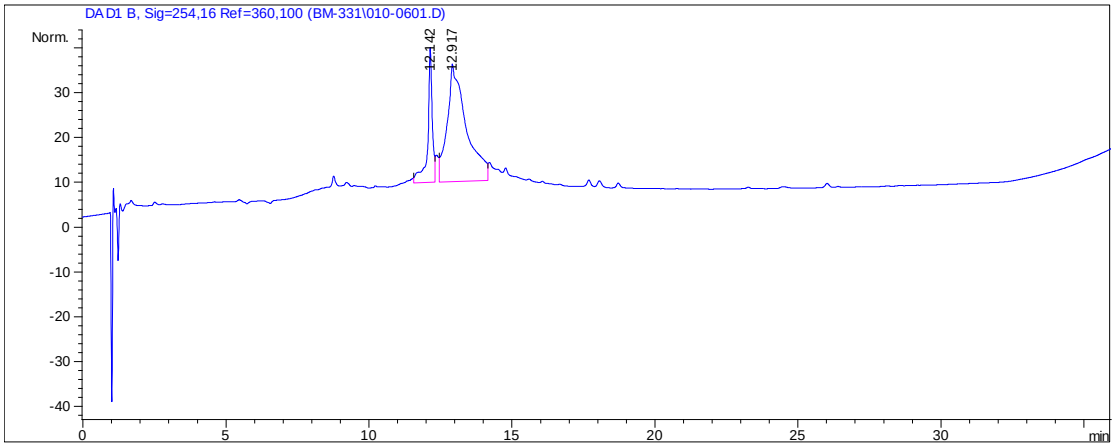


Figure 70: Purification by LCMS, trace observed during purification.

The fractions collected during LCMS purifcation were freeze dried to give a cream fluffy solid in low yield. However, the spectra observed were not clean due to which it was difficult to assign peaks. Due to the lack of material further purification was not carried out.

Third synthesis of the analogue **141** rings D and E of nisin was attempted using the same procedure as previously described.

Purifications via preparative HPLC were problematic because of the tailing of impurities which led to a hump this was observed from the first attempt, C18 SPE columns were therfore used in the second attempt. C18 SPE columns were also used for purification from the third synthesis. This attempt involved carrying out a two stage purification procedure using C18 SPE column. It was discovered in the previous purification attempt that the product was collected when acetonitrile was used instead of methanol. The column therefore was saturated with acetonitrile followed by water. Once the crude sample solution was loaded onto the 100 mg column the eluant was collected as a flow through, Table 10 shows the eluant used for the purification.

Fraction	Water (%), 0.15 (%) TFA	Acetonitrile (%), 0.15 (%) TFA	Water (%)
Flow through	-	-	-
1	-	-	100
2	95	5	-
3	90	10	-
4	85	15	-
5	80	20	-
6	75	25	-
7	70	30	-
8	65	35	-
9	60	40	-
10	50	50	-
11	40	60	-
12	30	70	-
13	20	80	-
14	10	90	-
15	0	100	-

Table 10: Eluant conditions for overlapping rings D and E purification.

After freeze drying mass spectrometry were carried out on all the fractions that were collected. The peptide eluted in fraction 6 (Table 10, Fraction 6), analytical trace showed a sharp peak with a much smaller hump compared to previous spectras (Figure 71), however NMR obtained showed broad peaks. Analytical HPLC were carried out on all the fractions that were collected, mass spectrometry clearly tied in with the analytical. Most of the fractions showed a hump with the same peak which was representing an impurity.

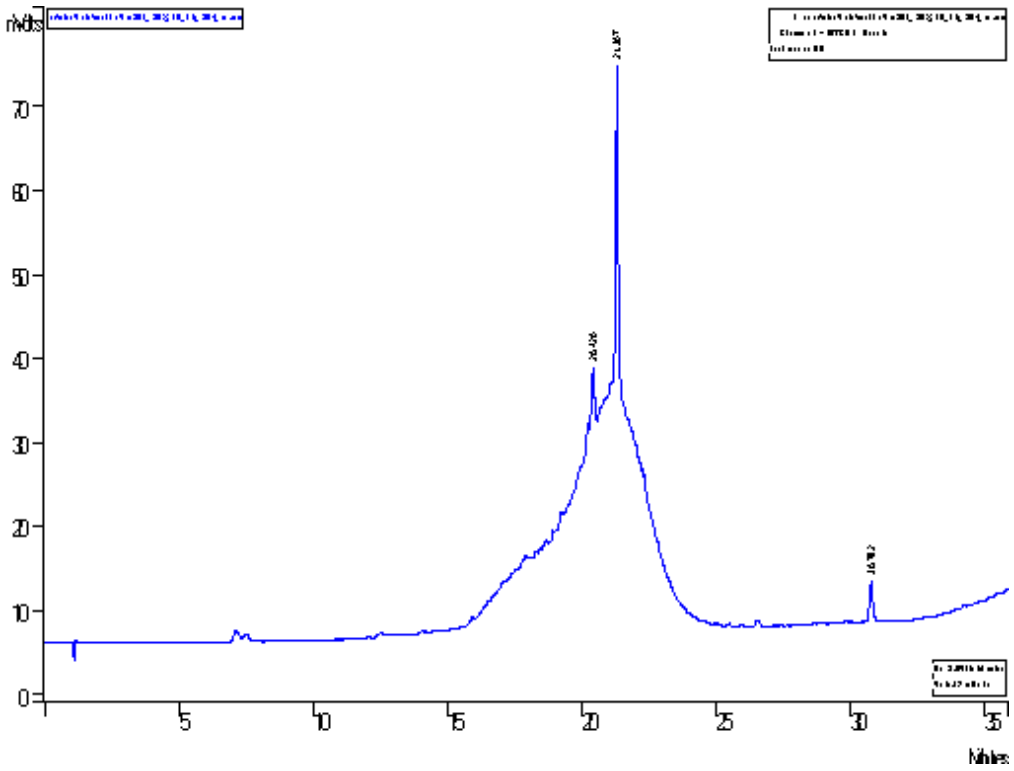


Figure 71: Analytical trace of fraction 6 collected at 25 % acetonitrile. A: H_2O/0.5 % TFA; B: acetonitrile/0.5 % TFA; B gradient 10 % - 60 % over 30 min.

A second purification was carried out on the fraction 6 (Table 10) collected at 25 % acetonitrile, Bond-Elute C18 column with a capacity of 50 mgs was used. The elutant used were both deuterated water and acetonitrile in 0.15 % TFA (Table 11), fractions were directly collected into NMR tubes, this was to avoid transferring samples which may have led to sample loss.

Fraction	Water (%), 0.15 (%) TFA	Acetonitrile (%), 0.15 % TFA
Flow through	-	-
1	83	17
2	81	19
3	79	21
4	77	23
5	75	25
6	73	27
7	71	29
8	60	40
9	50	50
10	40	60
11	30	70
12	20	80
13	10	90
14	0	100
15	0	100

Table 11: Eluant conditions for purifications of overlapping rings D and E.

Mass spectrum analysis indicated majority of the product was obtained in fraction 4 (Table 11) in less than 0.1 mg, and a slightly better analytical trace was obtained (Figure 72).

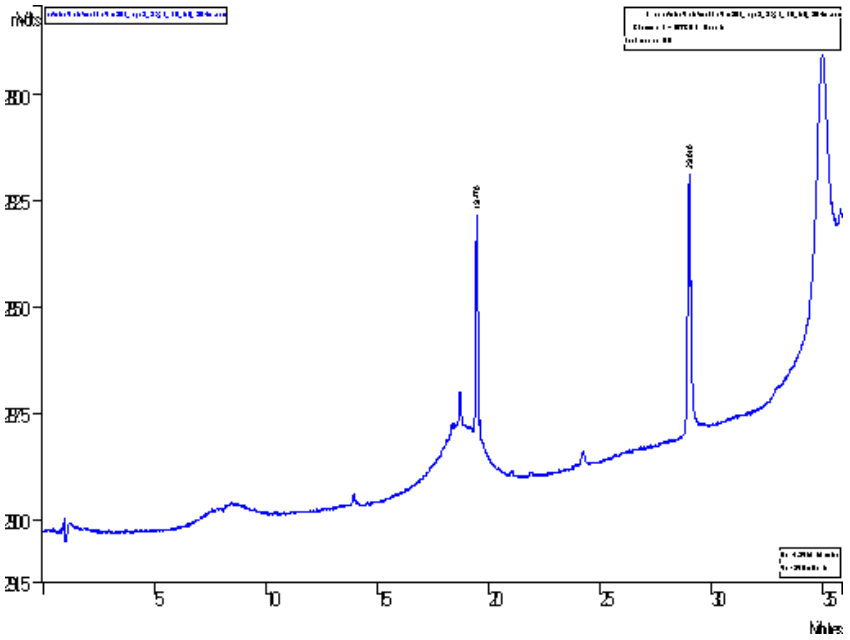


Figure 72: Analytical trace of product 141 after second SPE column collected in fraction 4 (23 % acetonitrile). A: H_2O/0.5 % TFA; B: acetonitrile/0.5 % TFA; B gradient 10 % - 60 % over 30 min.

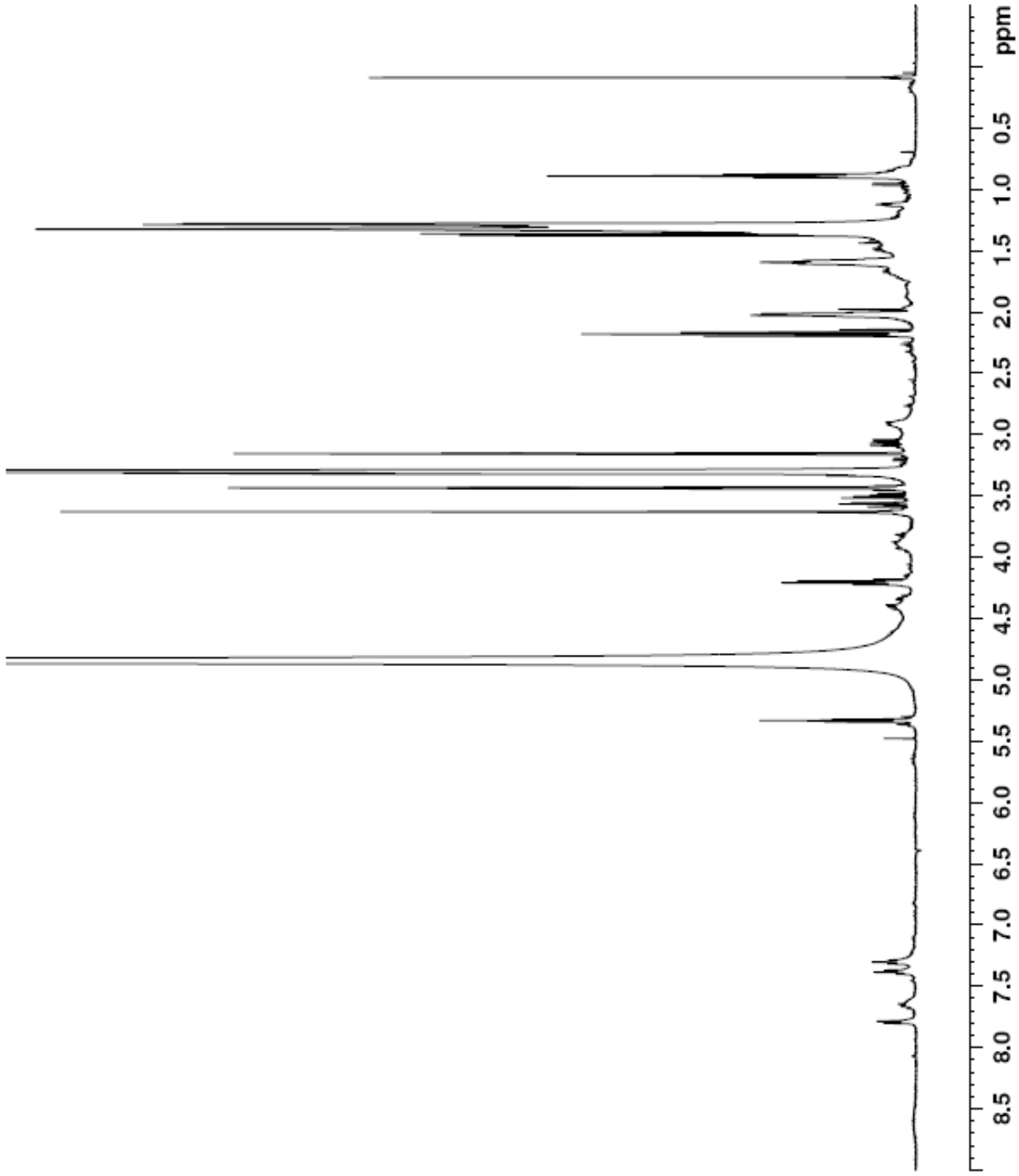


Figure 73: Proton NMR of an analogue of rings D and E of nisin.

NMR experiments were carried out on the sample, different solvents such as D_2O, deuterated acetonitrile, deuterated TFA, and deuterated methanol were used. NMR's obtained for the peptide from different solvents were all similar with very broad peaks. The best ^1H NMR obtained was in D_4-Methanol (Figure 73) however this itself was very difficult to assign. There was still presence of impurities this possibly could be PEG which has also come through during the cleavage step which is causing the hump in the analytical HPLC trace and also causing the peaks of the proton NMR to be broad. Another possibility could be due to the aggregation of the peptide or conformational constrainsts of the peptide however this should not broaden the Fmoc group.

The sample had very poor solubility properties which made it very difficut to get all samples in solution. There was very little sample with traces of impurities which was large compared with the sample. Also there was not enough sample for a 2D NMR due to these reason it was difficult to assign peaks.

4.7. Conclusion

The orthogonally protected bis-amino acid (Teoc, TMSE) / Fmoc lanthionine **169** has been successfully synthesised. The synthetic route has been optimised, permitting the necessary scale up required for ensuing solid phase peptide synthesis has been successful (Chapter 3).

Using a quadruply orthogonal protecting group strategy, analogue of the overlapping rings D and E of nisin has been synthesised. An analogue of the ring E has been synthesised for the first time using previous developed (Aloc, allyl) / Fmoc lanthionine **103** (Chapter 3). An analogue of the ring D has also been successfully synthesised for the first time using newly developed (Teoc, TMSE) / Fmoc lanthionine **169 (**Chapter 3).

Having the two lanthionines **103** and **169** with protecting groups orthogonal to each other and also to the transient Fmoc and permanent *t*Bu/Boc protecting groups enabled first solid phase peptide synthesis of an analogue of rings D and E of nisin **141**.

Purification of individual rings D and E were carried out without any problems and full characterisation were obtained. In the case of the overlapping rings the mass recovery was good however the purification was poor. Possible reasons could be as a result of aggregation of the peptide, slowly interconverting conformers or possibly due to isolating PEG from the resin/linker during the cleavage step, and another main issue was poor solublity.

Rings D and E of nisin both contain methyl lanthionine, however due to time constraints it was not possible to further develop this chemistry to give the required orthogonally protected methyl lanthionine building blocks.

In conclusion an effective methodology for the solid phase peptide synthesis of peptides containing overlapping lanthionine rings has been developed.

4.8. Future work

Although the analogue **141** of the overlapping rings D and E has been synthesised, further work is necessary to optimise the purification conditions. It will be necessary to repeat the synthesis using different resins and linkers, as it is possible that impurities from the final cleavage steps are causing the problem in purification. Removal of the Fmoc group prior to purification to give a more hydrophilic peptide will also be attempted.

Further work on SPE column will be carried out to optimise conditions for the purification of the overlapping rings, to get better results other techniques such as gel filtration may be carried out along with SPE columns.

Finally, with larger quantities of pure peptide in hand, a full structural analysis by NMR will be possible, followed by bioconjugation to mersacidin and actagardine to afford hybrid lantibiotics.

5. Validation *

Validation involves establishing documented evidence of a specific process which provide assurance that the process will reliably and constantly produce a product that meets the determined properties, specifications and quality attributes when manufacturing. This will include health and safety, quality and efficacy.

5.1. Introduction

The work presented in this thesis show that Aloc, allyl / Fmoc lanthionine **103** has been synthesised using the published procedure, [124] and an efficient and good yielding pathway has been developed to synthesise the Teoc, TMSE / Fmoc lanthionine **169** in a small scale. These amino acids were incorporated into cyclic peptides using solid phase peptide synthesis and therefore an effective methodology has been developed for the solid phase peptide synthesis of peptides containing the overlapping lanthionine rings in a small scale.

Prior to the synthesis of these two final amino acids there are multiple reaction steps which have their own regulatory requirements for performance qualification, process design, process performance qualification, installation qualification, operational qualification and process operation. The regulatory requirements concerned to biopharmaceutical manufacture are very strict and the requirements for the synthesis of the two amino acids and the peptide in the biopharmaceuticals will be discussed.

It is important to satisfy regulatory requirements on health and safety and produce efficient and economically viable products where the process step operates correctly within the process limits, and the performance objectives are met reliably and consistently during manufacturing. Analysis will be carried out to confirm product quality is achieved under the regulatory of FDA (USA) and MCA (EU/UK).

This chapter will discuss two scenarios:

1. Batch synthesis of the two orthogonally protected lanthionines
2. Automated synthesis of the overlapping rings D and E peptide

in an industrial context and suggestions in marketing of the peptide. The specific objectives include:

* This chapter is included as part of the UCL requirement for the award of the EngD degree.

- Validation required to be carried out on design, installation, operation, process, systems, performance qualification, cleaning and purification for the synthesis of the two amino acids and the peptide.
- Validation and evaluation of industrial implementations

5.2. Validation of lanthionines 103 and 169

5.2.1. Design qualification

The synthetic route to the two unusual amino acids **103** and **169** requires to be checked to ensure the protocols are successful and meet the process design requirements for the desired compounds. This then leads to the lanthionines ready for incorporation into solid phase peptide synthesis eliminating the formation of by-products such as **189** and **190** (Chapter 3) during manufacturing.

At the design stage the synthetic process specifications include the chemical reagents selected for the reactions, mass, volumes and equivalence of the reagents, where the reagents are purchased from, reaction conditions (i.e. inert atmosphere), temperature, time and pH, reaction work up and type of purification method. Operating equipment such as the column size, diameter, flasks size, glassware (such as round bottom flasks, syringes, measuring cylinders, reflux condenser, chromatography columns), magnetic stirrer, hot plates, ice baths, thermometer, and Syro peptide synthesiser and a centrifuge (Chapter 3 and 6). It is important to ensure that during this stage that all critical reactions are monitored and characterised, and that the critical parameters affecting the process and its key quality attributes are identified and considered.

Before operating at the large manufacturing scale, a pilot plant scale process would be carried out for the synthesis of the two lanthionines to ensure that the process is robust and likely to be successful at the larger scale. Small scale reactions (Chapter 3) have enabled an understanding of how to control undesired compounds, understand the process and discover the product impurities. The product qualities of the lanthionines were primarily determined by NMR, mass spectrometry and optical rotation. The effectiveness of the process designs has been confirmed from the small scale reactions carried out during the synthesis of lanthionine **103** and **169**.

5.2.2. Installation qualification

Performing an installation qualification verifies that all equipment are built and installed per design specifications. For small scale reactions the assembling of the equipment were very simple, installation qualification was not required at this stage. An argon cylinder was already connected to a dual manifold schlenk line containing several ports located in the fume hood. This dual manifold schlenk line has the second manifold connected to a high vacuum pump. The magnetic stirrer and hot plate employs a rotating magnetic field and means for heating the reaction, which also did not require any installation.

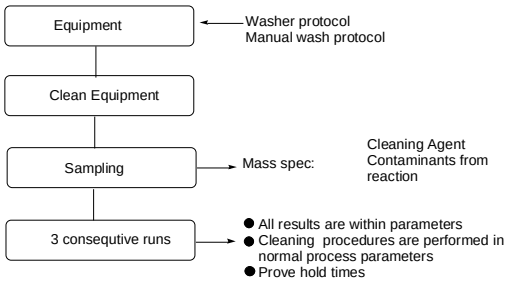
At large scale, reactors with impellers will be required for efficient mixing. Therefore, overhead-stirring with screw impellers may be essential to obtain satisfactory results at pilot-plant scale and large scale, where agitation rates of 220 - 500 rpm will be more than adequate. It will also be important to ensure that large argon cylinders or tanks are directly connected to large scale reactors to avoid exposure of oxygen to reactions which require inert conditions. In-process control requirements such as temperature and pH will require installing for critical Mitsunobu and Aloc protection reactions. At larger scale more complicated valves will be required for the addition of reagents and solvents and clean-in-phase.

5.2.3. Operation / Cleaning validation

Thorough cleaning will be carried out on all glassware to ensure all remnants of prior contaminating materials are removed. Glassware/reactors will be cleaned manually to remove contaminants and then rinsed with de-ionised water. This will be followed by the process which includes cleaning-in-place. All manually cleaned glassware will be placed in an automated dishwasher at 80 ºC. Once the cleaning-in-place process is complete, glassware/reactors will then be rinsed with acetone and placed in an oven at 100 ºC for 24 hr.

Glassware that are not clean from the cleaning-in-place process; a second process will be carried out involving cleaning-out-of-place process, where manual cleaning will be supplemented, followed by cleaning-in-place process.

Inspection of glassware will be carried out to ensure the cleaning method is correct, mass spectrum of clean glassware can be carried out; if impurities are present in the spectrum then contaminants are present on the glassware. If this problem occurs then the temperature set for cleaning-in-place process will be raised higher, and the washing detergent will be changed.



5.2.4. Process and factors affecting product quality

The use of the orthogonally protected lanthionine as crucial building-blocks for solid phase peptide synthesis (Chapter 4) may be of benefit to industrial applications. These orthogonally protected lanthionines show potential for the production of biopharmaceuticals, such as for the synthesis of other overlapping peptides such as cinnamycin. The qualities of the lanthionines were confirmed by the yield, mass spectrometry and NMR (Chapter 3 and 6).

Synthesis of the two lanthionines and the procedure for the synthesis can be seen in Chapter 3 and 6 (Scheme 36 and 68). It is important to ensure that the starting materials are pure. Sampling of the starting materials was carried out by NMR to confirm the purity of the commercially purchased reagents.

tert-Butyl protected cystine **145** and Tce protected cystine **192** are both key starting materials in the synthesis of the orthogonally protected lanthionines **103** and **169**, and the ability to obtain kilogram quantities of **145** and **192** was important for the total synthesis. It is important to ensure that perchloric acid is added dropwise in the presence of a glass shield for safety precautions; due to perchloric acid being explosive this may be a safety issue for the operation of large scale reaction processes. Experimental technique during perchloric acid reaction involves the reaction to be carried out in Pyrex glass but in the case of large-scale, reactor vessels made of chemically pure titanium or 316 stainless steel is required with efficient impellers for liquid reaction. A well-ventilated room, with forced draft and non-combustible construction is also required. Tce protection of the carboxylic acids for the synthesis of **192** may also be problematic during large-scale reactions due to the

carcinogenicity and highly flammable properties of benzene. Due to perchlorate and benzene these reaction steps may need modifying when large-scale reactions are carried out. An alternative safer approach may need to be investigated for the protection of the carboxylic acids as in both cases, the solvent and acid will be utilised as industrially-unfriendly chemicals.

The Mitsunobu reaction is a critical reaction and critical process parameters needs to be considered. This is considered as the first part of performance qualification as this will have an impact on the product quality. The temperature requires to be maintained at -2 ºC to make sure high yields of the product are obtained. Good temperature monitoring is required. In the case of small scale reactions a cryostat was used to maintain the temperature. During large scale it will have to be ensured that temperature sensors are installed which will alert if there are any changes to the temperature.

Another critical reaction is the Aloc protection of lanthionine **103** it is important to ensure the pH is between pH 7 and pH 8 before addition of the allyl chloroformate. During the small-scale reaction the pH was easily monitored using pH paper. A pH sensor will be required for large-scale reaction processes where the sensor will need to be directly connected to the large-scale reactor.

The final critical reaction involves the hydrogenation reaction to deprotect the benzyl group of **185** to give **181**. Safety aspects of using hydrogen gas would likely lead to scale-up safety issues and therefore might cause difficulties for industrial synthesis making this protocol unsuitable for larger scale use, which means an alternative may be required. However the requirements for large scale would include using a pressure reactor vessel with efficient impellers for gas-liquid reaction. The selection of the pressure vessel will have to be made keeping in mind the cleanability & product impurity profile. Degree of agitation, pressure, temperature ratings and space availability. The vessel will be required to have features such as optimum utilisation of gas, reliable containment of gas and the product of the reaction. The vessel must have high vacuum capability via dry seal pumps or utility vacuum via liquid ring pumps. Once the reaction is complete gases are vented through local scrubbers to ensure complete destruction of any hazardous or flammable vapours.

The remaining protocols (typically 10- 13 steps) are industrially amenable since the reagents used are of minimal toxicity risk and the reaction proceeds in benign solvents and at 0 ºC to

ambient temperatures. The ease of the chemistry involved in these reactions makes these protocols suitable for further scale up activities. The protocols designed achieve the desired and intended results reliably and reproducibly meeting critical quality acceptance criteria. Large scale reactions will include carrying out reactions in 500 - 700 g scale in order to obtain large amounts of lanthionines, which are ready for incorporation into solid phase peptide synthesis.

From the small scale reactions design qualification (DQ), installation qualification (IQ), operational qualification (OQ) and performance qualification (PQ) have been checked and proved to be successful.

Product: Products synthesised will be kept in vials under inert conditions and stored in fridges to avoid any by-products from forming and any contamination taking place.

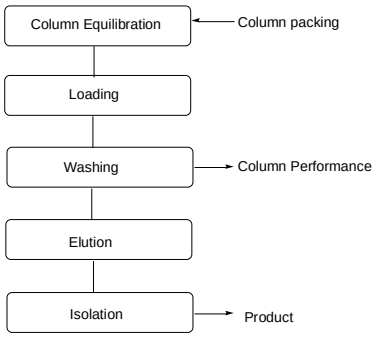
Process environment: Reactions will be carried out at room temperature or as stated in the protocol e.g. Mitsunobu. Light will not affect the reaction process, temperature will not affect reaction rates other than those reaction stated to be kept at certain temperatures e.g. the Mitsunobu reaction.

5.2.5. Purification validation

Validation is required on purification to ensure that it is capable to remove impurities. This is the third part of performance qualification which reduces impurities and contaminants. Purification will be performed by column chromatography.

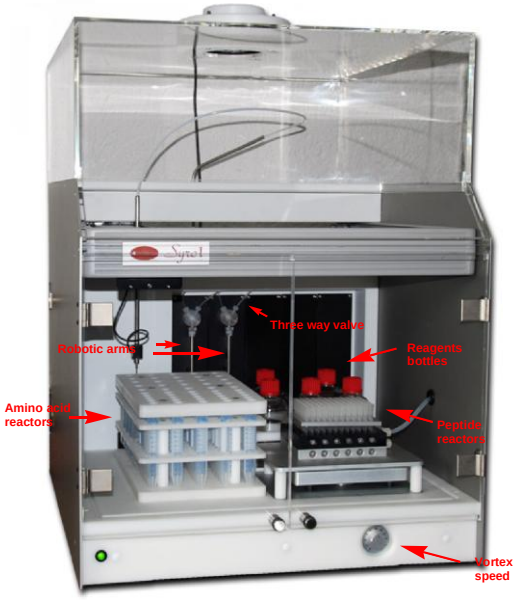
Depending on the quantity of materials column dimension will vary. Standard flash column chromatography and reverse-phase chromatography will be carried out for purification. After each chromatography silica waste will be placed in a container which will be disposed to a contractor and clean in-place will be used. Purification methods have been optimised during small scale reactions, solvents and gradient has been selected for each reaction to provide pure sample, Rf values for each compound has been confirmed (Chapter 6).

Columns will be packed with silica gel with a length of 15 cm however the diameter will depend on the column dimension. It will be ensured that no air bubbles are formed inside the pack to avoid dead space or change in the flow rate thus leading to changes in the output of the compounds. Air pressure will be used which will be maintained manually.



Purification by chromatography was easily handled during small scale it may be an issue when large scale products need to be purified. Modification of purification by column chromatography may be investigated to find alternative methods for purifications such as recrystallisation to save time, cost and volume of solvents during large scale. However modification will be avoided on purification on lanthionines **103** and **169** by reverse phase chromatography.

5.3. Validation of an analogue of the overlapping rings D and E of nisin 141



5.3.1. Design qualification, installation qualification, operational qualification and purification

Syro I an automated, computer controlled peptide synthesiser will be used to synthesise the overlapping rings D and E of nisin which is based on a one arm pipetting robot. The robotic arm contains a pipette tip that is connected to a pump with a tube and a three way valve. The

robotic arms can sample reagents in bottles or reactors and deliver to its chosen reactor. There is one Type U reactor block accepting 2, 5 and 10 mL reactors (syringes) and the vortex will guarantee optimal mixing of the reactants. All containers, reactors, and bottles are equipped with inert gas. Digital injection pumps allow injection of samples, and solution into the reactor. The Syro peptide synthesiser and the centrifuge do not require the facility design aspect as both equipments are already up and running. This is to confirm that the equipments are operating accurately to suit the specifications of the process. This includes the vortex of the peptide synthesiser, speed of the centrifuge and the speed of the stirrer plate therefore installation qualification is not required at this stage as the equipment is already set up and running. Operational qualification was carried out to ensure that the correct speed was used for the agitation during peptide synthesis.

A small-scale reaction was carried out to obtain the specifications for the process. The program was set up in order to synthesise the desired peptide. Calculation for all amino acids, solvents and peptide sequence were carried out using Syro xp software. From this small-scale reaction it was confirmed all parameters were accomplished to give the desired peptide **141**.

The second part of performance qualification involves demonstrating that the manufacturing process is consistent. Reactions will be carried out at manufacturing scale using the protocols of small-scale peptide synthesis which confirm DQ, IQ, OQ and PQ where the objectives were met and all of these were analysed. Commercially available amino acids will be purchased to save time.

Batch production will be carried out to synthesise the overlapping rings on the automated peptide synthesiser. This requires a simple process validation, where there is no temperature mapping, 10 peptides will be synthesised in one go, disposable reactor vessel (syringe) will be used to place the resin linker. The use of disposable reactor vessel reduces the cost load, allows process and validation development prototyping, reduce validation loads and allow scale down. It is a single use throw away, which is chemically clean and sterile.

All reagents and amino acids will be weighed out when the resin is being soaked in DMF. Solvents will be prepared and delivered into the bottles, and the amino acids will be placed in the vials and placed in the rack. The synthesis will be carried out under inert conditions.

The critical step during the peptide synthesis is the first Fmoc deprotection of the amino acid loaded on the resin. In order to ensure that deprotection takes place, Fmoc deprotection will be carried out twice. The other critical parameters are the lanthionine coupling, deprotection of the protecting groups of the lanthionine and cyclisation of the rings. These steps will be carried out manually to ensure that extra care is given.

Purification of the peptide will be carried out using in house Preparative HPLC which again will not require facility design qualification as the equipment is already installed and running. Another purification method will include using Bond-Elute SPE columns which will be purchased commercially, this will also reduce validation. Issues on purification (Chapter 4) were encountered during small-scale reactions and so an alternative method may be required during manufacturing scale.

5.4. Industrial implementation

The purpose to synthesising an analogue of rings D and E of nisin was to develop a methodology to cyclise complicated overlapping rings in a peptide. It is assumed that this peptide may have biological activity however it is unknown. It will be a benefit to industries if this peptide has antibacterial activity and therefore manufacturing in a large scale will be necessary before marketing the analogue of rings D and E of nisin.

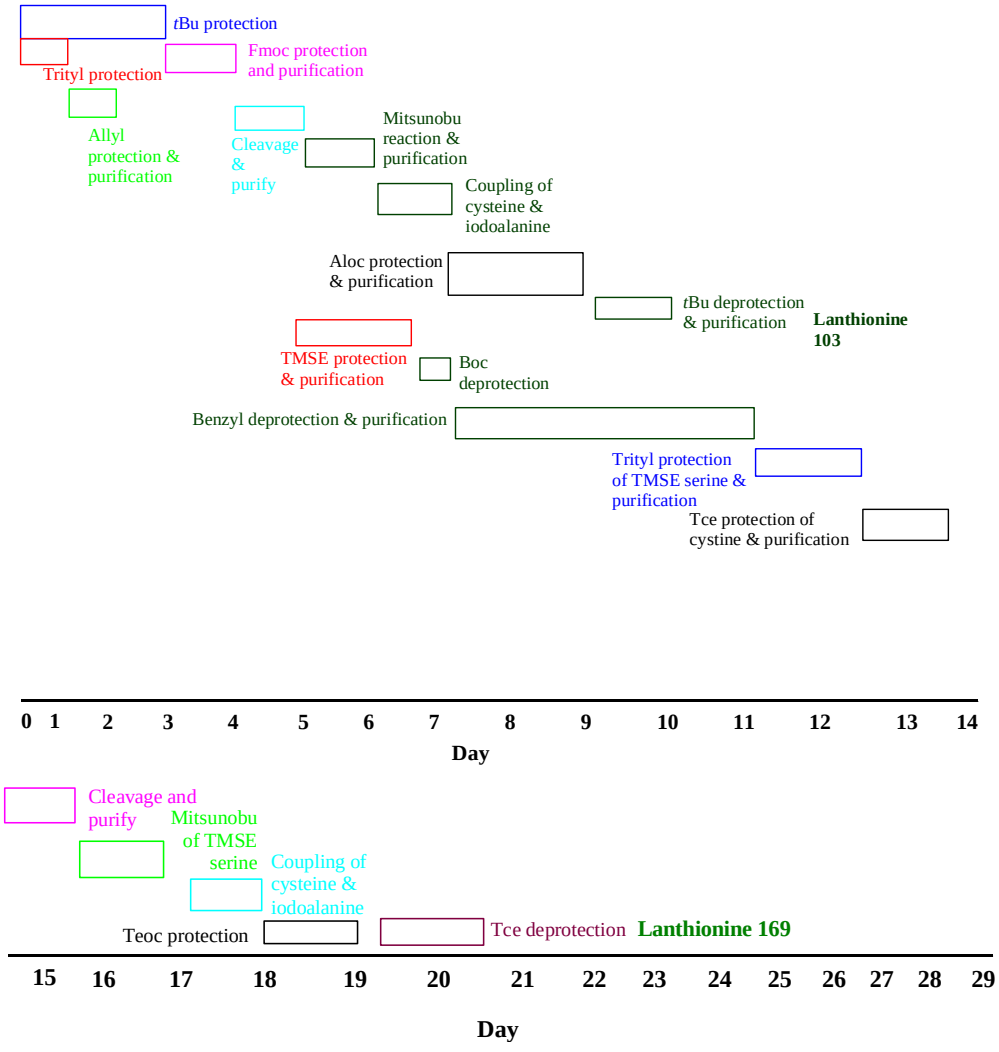
It is important to demonstrate to the industrial companies the biological activity of peptide **141** therefore scaled down pilot plant is required to be carried out which allows the company to quickly evaluate the potential benefits of the product to their company. Industrial manufacture is essential once the approval by the management team is confirmed for the capital investment of an analogue of rings D and E of nisin **141**. Planned time scale for the manufacturing synthesis can be seen below for the two lanthionines and the final peptide.

10 or more years ago manufacturing of peptide therapeutic containing >30 amino acids were stated as being difficult and expensive. [172] High production cost of US $75-100 per gram per amino acid residue [173] associated with synthetic peptides in the pharmaceutical industry is correct however only when the production is carried out in a few kilo grams. When production is carried out in a pilot plant capacity of 100 kg per year the cost should drop to $7.5-10 and to $1 per gram per amino acid residue when the production is carried out in a multi-tonne per year.

The industrial manufacture will include requirements of:

- 2 large reactors with sensors allowing automated monitoring of pH and temperatures, direct connection of gas cylinders for inert conditions.
- Rotary evaporators which can efficiently and gently remove large volumes of solvents from samples by evaporation.
- Cost-effective production scale chromatography
- Automated peptide synthesiser, power supply, all reagents, solvents, amino acids
- Electricity, water and finally the resin.

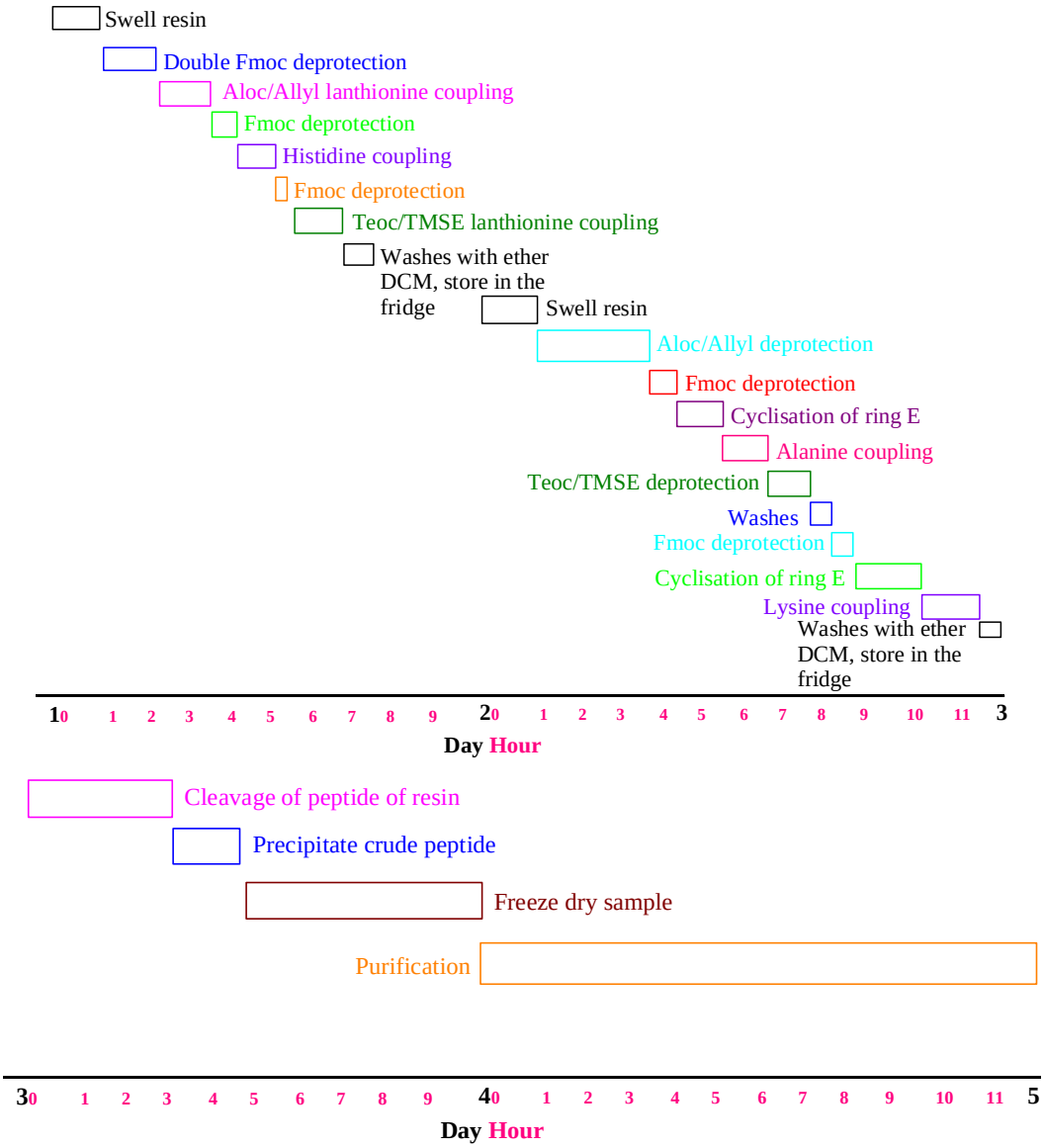
The set up cost depending on all equipments and reagents at 400-700 kg ranges from $238350 - $588004 (£150000 - £370000).



Planned timeline for the synthesis of the two orthogonally protected lanthionine

A number of issues need to be considered and developments of alternative methods are required which may otherwise cause a barrier to manufacturing at industrial scale. These include the change in reaction solvent benzene, perchloric acid, hydrogenation reaction (Chapter 3 and 6) and the issues of purification of the final peptide (Chapter 4 and 6).

In summary this chapter has evaluated and justified manufacturing of analogue of rings D and E of nisin **141** at industrial scale. A review on design, installation, operation, process, systems, performance qualification, cleaning and purification for the synthesis of the two amino acids and the peptide were carried out.



Planned timeline for the synthesis of the overlapping rings D and E of nisin

6. Experimental

6.1. Characterisation procedures and instrumentation

Unless specified all reagents were obtained from chemical suppliers with no further purification. THF and CH_2Cl_2 were dried over anhydrous alumina columns, [174] moisture levels were usually <15 ppm by Karl Fischer titration. Anhydrous methanol, anhydrous benzene and anhydrous triethylamine were purchased from Sigma-Aldrich in Sure/Seal™ bottles. Water was HPLC grade or distilled. Ether refers to diethyl ether and brine to saturated sodium chloride solution in water. Ethanol refers to ethanol 96 % v/v unless otherwise stated. Petroleum ether refers to petroleum ether 40- 60 %.

^{1}H and ^{13}C NMR spectra were recorded on Bruker AVANCE III 600 (600 MHz), Avance 500 (500 MHz), Varian VXR 400 (400 MHz) and Bruker AC300 (300 MHz) spectrometers, using $CDCl_3$ and CD_3OD solvents. The chemical shifts (δ) were given in ppm units relative to tetramethylsilane, and coupling constants (*J*) are measured in Hertz. Proton ^{1}H NMR multiplicities are shown as s (singlet), d (doublet), t (triplet), m (multiplet), dd (doublet of doublet), bs (broad singlet), bd (broad doublet), bt (broad triplet) and bm (broad multiplet). ^{13}C signals were assigned from DEPT, HSQC and HMBC spectra.

Electrospray mass spectra were recorded on a Thermo Finnegan MAT 900XP and high resolution electrospray mass spectra on a Waters LCT Premier XE spectrometer. MALDI-TOF mass spectra were recorded on a Waters Micromass MX instrument. LCMS on a Waters Aquity UPLC – SQD instrument. FAB mass spectra were recorded using a VG70-SE mass spectrometer.

Melting points were determined using a Gallenkamp instrument.

The cryostat used was a Radleys Huber TC50E. Optical rotation measurements were all measured at 25 °C on a Perkin-Elmer model 343 polarimeter. IR spectra were recorded on a Perkin-Elmer Spectrum 100 FT-IR spectrometer in chloroform and methanol solutions.

Thin layer chromatography was performed on Fluka silica gel with fluorescent indicator (254 nm); layer thickness 0.2 mm. The spots were visualised with U.V. light, and also by dipping in: ninhydrin in ethanol; bromocresol green; potassium permanganate; and PMA, as appropriate. Normal flash column chromatography was carried out using silica gel with particle size < 60 μm. Reverse phase TLC was carried out on Merck RP-F_{254} plates. Silanized $silica_{60}$ gel purchased from BDH was used for reverse phase chromatography. Reverse phase chromatography was carried out as follows the compound was dissolved in the minimum amount of acetonitrile. This solution was then diluted approximately 10-fold with saturated aqueous sodium bicarbonate solution 20 % to give an emulsion. The column was loaded with the emulsion and collected as flow through. 20 % bicarbonate solution was used as the eluant for one column width followed by one column width of 10 % acetonitrile in 20 % bicarbonate solution, then 20 % acetonitrile followed by two column width of 30 % acetonitrile followed by 40 %. TLC was carried out in an eluant made up of 40 % acetonitrile in 20 % bicarbonate, and observed using U.V. light. Acetonitrile was removed *in vacuo*, concentrated HCl was added to the collected fraction containing the product to reach pH 3 or below followed by addition of NaCl and CH_2Cl_2. Extraction was carried out using CH_2Cl_2 or chloroform.

All amino acids, coupling reagents and resins were purchased from Novabiochem or Perseptive Biosystems. Peptides were synthesized using a Syro I multiple peptide synthesiser (MultiSynTech GmbH) using solid phase peptide Fmoc strategy. Most steps were carried out using automated synthesiser protocols, however lanthionine coupling steps, orthogonal deprotection steps with $Pd(PPh_3)_4$ or TBAF, and on-resin cyclisation steps with PyAOP and HOAt were carried out manually.

A qualitative Kaiser test [165, 175] was carried out to check for the presence of free amines as follows. The resin was manually washed with CH_2Cl_2 to remove traces of DMF, eliminating any effects from possible interfering impurities. Several resin beads were removed and placed in an Eppendorf vial, and 4 drops of each Kaiser test solution were added, in order from solution 1 to 3, and heated at 110 °C for 5 min. A purple/red/blue colour indicates a positive Kaiser test, indicating the presence of free amines, while a clear yellow solution indicated a negative result.

Solution 1: KCN (2 ml, 0.001 M) in pyridine (98 mL)

Solution 2: Ninhydrin (5 % w/v) in t-butanol

Solution 3: Phenol (80 % v/v) in *t*-butanol

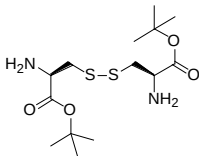
Peptides were purified by preparative reverse phase HPLC and analysed by analytical reverse phase HPLC, with aqueous TFA (0.1 % v/v) and acetonitrile (with 0.1 % TFA, v/v) as solvents. Preparative HPLC was carried out with Varian Prostar 210 instrument with a Galaxy chromatography workstation. A Wide Pore C18, 25 cm x 21.2 mm, 10 μm column was used. Analytical HPLC was carried out on Varian Prostar 410 instrument with a Star chromatography workstation. A Wide Pore C18, 25 cm x 4.6 mm, 10 μm and Onyx Monolithic C18, 100 x 3.0 mm columns were used.

Bond Elute C18 Solid Phase Extraction (SPE) columns (Agilent) were used for peptide purification (of rings D and E).

Thermo Finnigan Surveyor LCMS LCQ DECA XP plus HPLC C18 column Agilent technologies was used for mass spectrometry and separation.

6.2. Experimental section

(*R*,*R*)-Cystine bis-*tert*-butyl ester 145 [176]



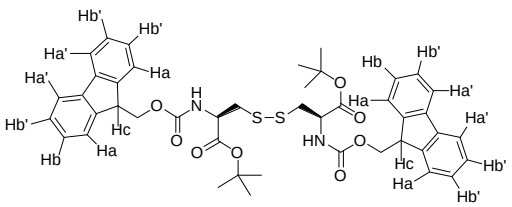
Hazard

Metal perchlorates are explosive, and as a safety precaution this reaction was carried out behind a blast shield.

L-cystine **144** (10 g, 82 mmol) was suspended in perchloric acid (70 % v/v, 15.6 mL, 259 mmol, 4 equiv) and *tert*-butyl acetate (100 mL) was added to the slurry. Two immiscible layers were formed which disappeared on stirring. The reaction was carried out at room temperature, after 2 hr a white precipitate appeared in solution. The reaction mixture was stirred for a further 72 hr after which it was cooled in an ice bath for 30 min. Sodium hydroxide (2 M, aq) was slowly added until the solution reached pH 11. The product was extracted with ether (6 x 100 mL) and ethyl acetate (6 x 100 mL) the organic layers were combined and dried over anhydrous sodium sulphate. Removal of solvents *in vacuo* yielded the title product **145** as pale yellow oil, identical by NMR to the literature. [123]

Yield: 5.22 g, 14. 8 mmol, 36 %;
NMR: δ_H (300 MHz, $CDCl_3$), 3.66 (2 H, dd, *J* = 7.8, 4.5 Hz, 2 x C*H*CH_2S), 3.10 (2 H, dd, *J* = 13.4, 4.5 Hz, CHC*H*HS), 2.84 (2 H, dd, *J* = 13.4, 7.8 Hz, 2 x CHCH*H*S), 1.44 (18 H, s 2 x $CO_2C(CH_3)_3$).

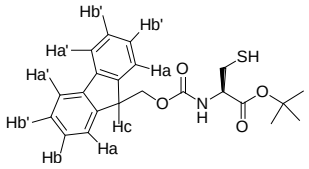
***NN*'-Bis(9-fluorenylmethoxycarbonyl)-(*R*,*R*)-cystine bis-*tert*-butyl ester 146** [177]



Diamine **145** (5.22 g, 14 mmol) and 9-fluorenylmethoxycarbonyl chloride (7.67 g, 29.6 mmol, 2 equiv) were dissolved in THF (152 mL). The reaction was cooled in an ice bath, and NMM (3.26 mL, 29.6 mmol, 2 equiv) was added dropwise. The reaction went cloudy yellow and the resulting mixture was stirred for 3 hr. Ethyl acetate (400 mL) was added and the mixture was washed with potassium hydrogen sulphate (5 %, aq. w/v, 4 x 100 mL) and water (3 x 100 mL). The organic layer was dried over anhydrous sodium sulphate and the solvent removed *in vacuo* yielding yellow oil. Purification via flash column chromatography (hexane: ether, 2:1) gave the title product **146** as white foam, identical by NMR and mp to the literature. [123]

Yield: 6.1 g, 7.66 mmol, 53 %;
R_f: 0.1 (Hexane:Ether; 2:1);
Mp: 148 - 152 º C (lit. [123] 151.5 – 152 ºC);
NMR: δ_H (300 MHz, $CDCl_3$), 7.74 (4 H, dd, *J* = 7.6 Hz, Ar), 7.57 (4 H, dd, *J* = 7.3 Hz, Ar), 7.40 (4 H, bt, *J* = 7.5 Hz, Ar), 7.29 (4 H, dd, *J* = 7.6 Hz, Ar), 5.73 (4 H, bd, *J* = 7.7 Hz, N*H*), 4.57 (2 H, m, C*H*CH_2S), 4.35 (4 H, bm, $CH_cC\mathit{H}_2O$), 4.22 (2 H, bm, H_c), 3.28 (2 H, bm, CHCH_2S), 3.13 (2 H, bm, CHCH_2S), 1.47 (18 H, s, $CO_2C(C\mathit{H}_3)_3$);
MS: (ES^+), $C_{44}H_{48}N_2O_8S_2$, *m/z*: 819.4 $[M + Na]^+$.

***N*-9-fluorenylmethoxycarbonyl-(*R*)-cystine bis-*tert*-butyl ester 90** [122]

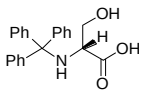


A solution of *N*,*N*'-bis(9-fluorenylmethoxycarbonyl)-(*R*,*R*)-cystine bis-*tert*-butyl ester **146** (6.1 g, 7.8 mmol) in dry THF (150 mL) was treated with tributylphosphine (3.8 mL, 15 mmol). The mixture was stirred for 2 min after which water (distilled, 7.7 mL, 428 mmol, 56 equiv) was added. The reaction was stirred for a further 2 hr. The mixture was concentrated *in vacuo* and dissolved in ethyl acetate (300 mL). The solution was washed with citric acid (10 % aq. w/v, 300 mL) and then brine (300 mL). The organic layer was dried over anhydrous sodium sulphate and the solvent removed *in vacuo* to yield pale yellow oil. Purification via flash column chromatography (hexane: ether, 2:1) yielded the title product **90**

as an extremely viscous oil which solidified to give a white solid **90**, identical by NMR to the literature. [123]

Yield: 3.5 g, 8.77 mmol, 57 %;
R_f: 0.3 (Hexane: Ether; 2:1);
NMR: δ_H (300 MHz, $CDCl_3$), 7.76 (2 H, d, *J* = 7.4 Hz, Ar), 7.61 (2 H, d, *J* = 7.3 Hz, Ar), 7.41 (2 H, t, *J* = 7.3 Hz, Ar), 7.3 (2 H, t, *J* = 7.4 Hz, Ar), 5.68 (1 H, d, *J* = 7.1 Hz, N*H*), 4.52-4.61 (1 H, bm, C*H*CH_2S), 4.41 (2 H, bm, $CH_cC\mathit{H}_2O$), 4.23 (1 H, t, *J* = 6.8 Hz, H_c), 2.99 (2 H, m, CHCH_2S), 1.50 (9 H, s, $CO_2C(C\mathit{H}_3)_3$).

***N*-Triphenylmethyl-(*R*)-serine 143** [124]



Trimethylsilylchloride (37.4 mL, 292 mmol, 3.1 equiv) was added to a suspension of *R*-serine (10 g, 95 mmol) in CH_2Cl_2 (133 mL) under argon. The mixture was heated under reflux for 20 min and then cooled to room temperature. Triethylamine (41.1 mL, 294 mmol, 3.1 equiv) was then added after which the reaction was heated under reflux for 45 min. The mixture was then allowed to cool to room temperature and then cooled in an ice bath to 0 ºC. Methanol (anhydrous, 3.85 mL, 92 mmol, 1 equiv) was then added, and the mixture was allowed to reach room temperature. Triethylamine (13.2 mL, 95 mmol, 1 equiv) was added, followed by triphenylmethylchloride (26.5 g, 95.2 mmol, 1 equiv) and the reaction was stirred for 20 hr.

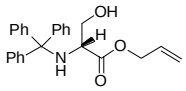
Excess triethylamine (30 mL) and methanol (150 mL) were then added until the white solids dissolved. The solvents were removed *in vacuo* yielding a mixture of yellow and white crystals which was partitioned between ethyl acetate (400 mL) and citric acid (5 % aq. w/v, 4 x 50 mL) pre-cooled to 4 ºC. The organic layer was washed with sodium hydroxide (2 M, 5 x 50 mL) and water (3 x 100 mL). The aqueous layers were combined, washed with ethyl acetate (300 mL) and neutralised with glacial acetic acid at 0 ºC. The precipitated product was extracted with ethyl acetate (4 x 100 mL) and the organic layer was dried over anhydrous sodium sulphate. Removal of solvent under pressure yielded the title product **143** as a white solid identified by mass spectrometry, identical by NMR to the literature. [124]

Yield: 29.2 g, 84.1 mmol, 88 %;

NMR: δ_H (300 MHz, $CDCl_3$), 7.48 (6 H, m, Trt), 7.25 (9 H, m, Trt), 3.17 (1 H, dd, *J* = 11.0, 6.8 Hz, CHC*H*$_2$OH), 3.09 (1 H, m, C*H*CH$_2$OH), 2.81 (1 H, dd, *J* = 11.0, 4.5 Hz, CHC*H*$_2$OH);

MS: (ES^+), $C_{22}H_{21}NO_3$, *m/z*: 370 $[M + Na]^+$.

_N_-Triphenylmethyl-(_S_)-serine allyl ester 122 [123]



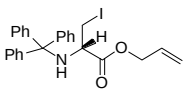
N-Triphenylmethyl-(*R*)-serine **143** (6 g, 17 mmol) was dissolved in methanol (170 mL). Caesium carbonate (2.81 g, 8.6 mmol, 0.5 equiv) was added and the solution was stirred for 15 min before being concentrated *in vacuo*. The resulting caesium salt was then dissolved in DMF (170 mL), allyl bromide (1.98 mL, 16 mmol, 0.95 equiv) was added dropwise at room temperature and the reaction was stirred for 14 hr. Ethyl acetate (44 mL) was then added to the mixture. The mixture was washed with aqueous citric acid (5 % w/v, 5 x 100 mL) precooled to 4 ºC. The organic layer was dried over anhydrous magnesium sulphate, concentrated *in vacuo*, and purified by flash column chromatography (CH_2Cl_2: MeOH, 20:1) to give the title product **122** as cream oil, identical by NMR to the literature. [123]

Yield: 4.91 g, 12.6 mmol, 73 %;

R_f: 0.59 (CH_2Cl_2: MeOH; 20:1);

NMR: δ_H (500 MHz, $CDCl_3$), 7.47 (6 H, m, Trt), 7.25 (6 H, m, Trt), 7.16 (3 H, m, Trt), 5.68 (1 H, m, CH$_2$C*H*=CH$_2$), 5.16 (1 H, dd, *J* = 10.5, 1.4 Hz, CH$_2$CH=C*H*$_2$), 5.11 (1 H, m, CH$_2$CH=C*H*$_2$), 4.2 (1 H, dd, *J* = 10.2, 5.8 Hz, C*H*$_2$CH=CH$_2$), 4.09 (1 H, dd, *J* = 10.2, 4.3 Hz, C*H*$_2$CH=CH$_2$), 3.71 (1 H, m, H_α), 3.57 (2 H, m, C*H*$_2\beta$).

_N_-Triphenylmethyl-β-iodo-(_S_)-serine allyl ester 101 [124]



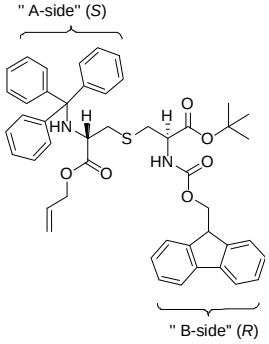
N-Triphenylmethyl-(*R*)-alanine allyl ester **122** (3.68 g, 9.4 mmol) was dissolved in dry CH_2Cl_2 (9.5 mL) at room temperature in the presence of triphenylphosphine (3.75 g, 14.2

mmol, 1.5 equiv). After 10 min, the reaction mixture was cooled to -10 ºC using an acetone/ice bath. Diethylazodicarboxylate (2.25 mL, 14.4 mmol, 1.5 equiv) was then added dropwise to the reaction over 1 min. After further 5 min, this was followed by the careful addition of iodomethane (8.89 mL, 14.2 mmol, 1.5 equiv). The reaction was kept at -2 ºC over 3 hr using an ethanol bath refrigerated by a cryostat.

The reaction mixture was directly filtered by flash column chromatography, beginning with 20:1 hexane: ethyl actate reducing to 10:1 until the removal of the title product **101** obtained as a clear oil, identical by NMR to the literature. [124] A 4:1 eluant was then used to collect the starting material **122**.

Yield: 1.11 g, 2.23 mmol, 23 %;
R_f: 0.31 (Hexane: Ethyl acetate; 10:1);
NMR: δ_H (300 MHz, $CDCl_3$), 7.51 (6 H, m, Trt), 7.24 (9 H, m, Trt), 5.71 (1 H, m, CH_2CH=CH_2), 5.23 (1 H, 2 dd, *J* = 12.4, 1.4 Hz, CH_2CH=CH_2), 5.17 (1 H, m, CH_2CH=CH_2), 4.21 (1 H, dd, *J* = 13.0, 5.9 Hz, CH_2CH=CH_2), 4.11 (1 H, dd, *J* = 4.7, 1.2 Hz, CH_2CH=CH_2), 3.49 (1 H, bm, H_α), 3.32 (1 H, dd, *J* = 9.8, 3.5 Hz CH_2I), 3.18 (1 H, dd, *J* = 9.8, 2.9 Hz CH_2I).

3-(*R*)-2-*tert*-Butoxycarbonyl-2-(fluoren-9-ylmethoxcarbonylamino)ethylsulfanyl)-(*S*)-(allyloxycarbonylamino)propionic acid allyl ester 102 [123]



N-9-fluorenylmethoxycarbonyl-(*R*)-cystine bis-*tert*-butyl ester **90** (1.67 g, 4.18 mmol) and *N*-triphenylmethyl-β-iodo-(*S*)-alanine allyl ester **101** (2.08 g, 4.18 mmol, 1 equiv) were dissolved in DMF (68 mL) under inert conditions. Cs_2CO_3 (0.68 g, 2.09 mmol, 0.5 equiv) dried in a dessicator overnight was added over 2 min and the reaction was stirred for 2 hr. Further dry Cs_2CO_3 (0.68 g, 2.09 mmol, 0.5 equiv) was added over 1 min and the reaction

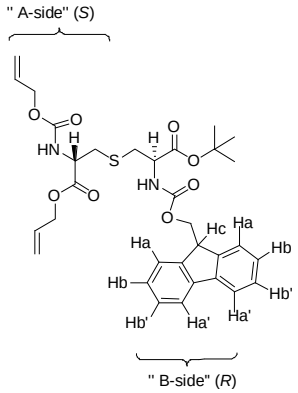
was stirred for a further 2 hr. Excess ethyl acetate (600 mL) was added and the mixture was washed with cold citric acid (5 % w/v aq, 300 mL) followed by distilled water (10 x 250 mL). The ethyl acetate layer was dried over anhydrous sodium sulphate and concentrated *in vacuo*. Purification was carried out by flash column chromatography (hexane: EtOAc, 4:1) to give the title product **102** as a white fluffy solid, identical by NMR to the literature. [123, 124]

Yield: 1.12 g, 1.45 mmol, 67 %;

R_f: 2.5 (Hexane: Ethyl acetate; 4:1);

NMR: δ_H (600 MHz, $CDCl_3$), 7.77-7.16 (23 H, m, Ar), 5.63 (1 H, m, CH_2CH=CH_2), 5.62 (1 H, d, *J* = 7.9 Hz, N*H*Fmoc), 5.18 (1 H, dd, *J* = 17.3, 1.4 Hz, CH_2CH=CH_2), 5.14 (1 H, m, CH_2CH=CH_2), 4.49 (1 H, m, H_α B side), 4.35 (2 H, m, CH_2Fmoc), 4.22 (1 H, t, *J* = 7.1 Hz, C*H*Fmoc), 4.15 (1 H, dd, *J* = 13.1, 7.3, 5.8 Hz, CH_2CH=CH_2), 3.97 (1 H, dd, *J* = 13.1, 7.2, 5.9 Hz, CH_2CH=CH_2), 3.54 (1 H, m, H_α A side), 2.88 (4 H, m, CH_2 β A and B sides), 1.56 (1 H, s, N*H*Trt), 1.47 (9 H, s, *t*Bu).

3-(*R*)-2-*tert*-Butoxycarbonyl-2-(fluoren-9-ylmethoxcarbonylamino)ethylsulfanyl)-(*S*)-(allyloxycarbonylamino)propionic acid allyl ester 125 [123]



3-(*R*)-2-*tert*-Butoxycarbonyl-2-(fluoren-9-ylmethoxcarbonylamino)ethylsulfanyl)-(*S*)-(allyloxycarbonylamino)propionic acid allyl ester **102** (0.2 g, 0.26 mmol) was treated with a solution of TFA (10 %, 0.4 mL), triethylsilane (TES) (5 %, 0.2 mL) and CH_2Cl_2 (3.2 mL) under argon for 4 hr. Excess CH_2Cl_2 (350 mL) was then added. The organic layer was washed with saturated sodium bicarbonate (300 mL) and brine (300 mL), dried over anhydrous sodium sulphate, and concentrated *in vacuo*. The residue was dissolved in 1,4-dioxane (3.2 mL) and a solution of sodium bicarbonate (0.1 g, 1.19 mmol, 4.5 equiv) in water

was added (3.2 mL). The resulting mixture was cooled to 0 ºC and allyl chloroformate (0.065 mL, 0.61 mmol, 2.3 equiv) was added. The mixture was left for 18 hr at ≤ 4 ºC. Excess CH_2Cl_2 (500 mL) was added and the organic layer was washed with saturated sodium bicarbonate (300 mL) and brine (300 mL). The organic layer was dried over anhydrous sodium sulphate, concentrated *in vacuo*, and purified by flash column chromatography (hexane: EtOAc, 4:1) to give the title product **125** as a clear viscous oil, identical by NMR and mass spec to the literature. [124]

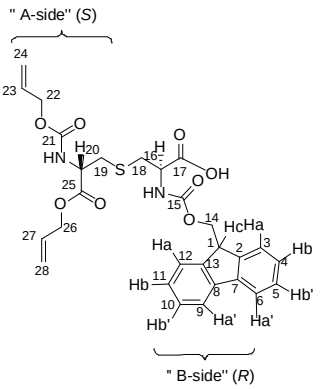
Yield: 0.094 g, 0.15 mmol, 64 %;

R_f: 0.18 (Hexane: Ethyl acetate; 4:1);

NMR: δ_H (300 MHz, $CDCl_3$), 7.76 (2 H, d, *J* = 7.4 Hz, Ar), 7.61 (2 H, d, *J* = 7.3 Hz, Ar), 7.39 (2 H, t, *J* = 7.4 Hz, Ar), 7.32 (2 H, t, *J* = 6.7 Hz, Ar), 5.91 (2 H, m, CH_2CH=CH_2 Aloc and allyl), 5.88 (1 H, d, *J* = 5.7 Hz, N*H*Fmoc), 5.84 (1 H, d, *J* = 5.5 Hz, N*H* Aloc), 5.33 (2 H, m, CH_2CH=CH_2 allyl), 5.26 (2 H, m, CH_2CH=CH_2 Aloc), 4.63 (3 H, m, CH_2CH=CH_2 allyl and H_α A side), 4.58 (2 H, m, CH_2CH=CH_2 Aloc), 4.51 (1 H, m, H_α B side), 4.39 (2 H, m, CH_2Fmoc), 4.24 (1 H, t, *J* = 7.0 Hz, H_cFmoc), 3.03 (4 H, m, CH_2 β A and B sides), 1.48 (9 H, s, *t*Bu);

MS: (ES^+), $C_{32}H_{38}N_2O_8S$, *m/z*: 633 $[M + Na]^+$.

3-(*S*)-(2-Allyloxycarbonyl-2-allyloxycarbonylamino-ethylsulfanyl)-(*R*)-2-(9H-fluoren-9-ylmethoxycarbonylamino)-propionic acid 103 [124]



3-(*R*)-2-*tert*-Butoxycarbonyl-2-(fluoren-9-ylmethoxcarbonylamino)ethylsulfanyl)-(*S*)-(allyloxycarbonylamino)propionic acid allyl ester **125** (0.96 g, 1.57 mmol) was treated with a mixture of trifluoroacetic acid (TFA) (1.92 mL) and CH_2Cl_2 (1.92 mL) (1:1), the resulting

mixture was stirred for 3 hr. The solvents were then removed under high vacuum. Removal of TFA was helped by several additions of toluene (3 x 100 mL) followed by evaporation. Traces of TFA were removed by purifying the crude mixture by reverse phase chromatography. Acetonitrile was removed under high vacuum. Concentrated sulphuric acid was added to bring the aqueous layer to pH 2 and sodium chloride was added until saturation was reached. The product was extracted from the aqueous layer with chloroform. The organic layer was then dried over anhydrous sodium sulphate and concentrated *in vacuo* to give the title product **103** as a white fluffy solid, identical by NMR, ^{13}C NMR and mass spec to the literature. [124]

Yield: 0.60 g, 1.05 mmol, 69 %;

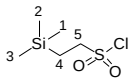
R_f: 0.35 [AcCN (40 %): Sat $NaHCO_3$] reverse phase TLC;

NMR: δ_H (600 MHz, CD_3OD), 7.79 (2 H, d, *J* = 7.6 Hz, Ar), 7.70 (2 H, d, *J* = 7.3 Hz, Ar), 7.38 (2 H, t, *J* = 7.4 Hz, Ar), 7.31 (2 H, t, *J* = 7.4 Hz, Ar), 5.91 (2 H, m, CH_2CH=CH_2 allyl and Aloc), 5.29 (2 H, m, CH_2CH=CH_2 allyl), 5.18 (2 H, m, CH_2CH=CH_2 Aloc), 4.61 (2 H, m, CH_2CH=CH_2 allyl), 4.60 (2 H, m, CH_2CH=CH_2 Aloc), 4.44 (1 H, m, H_α A side), 4.40 (1 H, m, H_α B side), 4.35 (2 H, m, CH_2Fmoc), 4.24 (1 H, t, *J* = 7.2 Hz, H_cFmoc), 3.31 - 2.93 (4 H, m, CH_2 β A and B sides);

NMR: δ_C (150 MHz, CD_3OD), 173.8 (C_{17}), 171.9 (C_{25}), 158.5 (C_{15}), 158.3 (C_{21}), 145.2 (C_{Ar}), 142.5 (C_{Ar}), 134.1 (C_{23}), 133.1 (C_{27}), 128.7 (C_{Ar}), 128.2 (C_{Ar}), 126.3 (C_{Ar}), 120.9 (C_{Ar}), 118.8 (C_{24}), 117.6 (C_{28}), 68.2 (C_{14}), 67.4 (C_{22} or C_{26}), 67.0 (C_{26} or C_{22}), 56.0 (C_{16} or C_{20}), 55.6 (C_{20} or C_{16}), 48.3 (C_1), 35.3 (C_{19} or C_{18}), 35.1 (C_{19} or C_{18});

MS: (ES^+), $C_{28}H_{30}N_2O_8S$, *m/z*: 577.58 $[M + Na]^+$.

2-(Trimethylsilanyl)-ethanesulfonyl chloride 157 [141, 142]



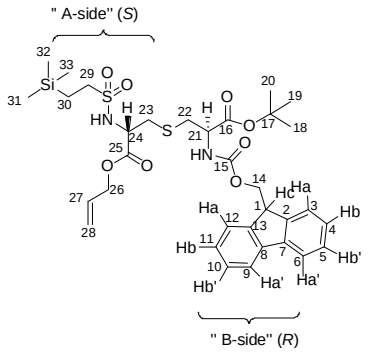
2-(Trimethylsilyl)ethane sulfonic acid sodium salt **156** (1 g, 4.9 mmol) was stirred and cooled down to 0 ºC for 15 min without any solvents under inert conditions. At this temperature, thionyl chloride (2.75 mL, 37.6 mmol, 7.7 equiv) was added dropwise with continuous stirring over 20 min. The SO_2 generated was allowed to evacuate through a Pasteur pipette. DMF (0.013 mL) was slowly added via a syringe resulting in a substantial increase in the

evolution of SO_2. The solution was stirred for an additional 20 min at 0 ºC during which time evolution of SO_2 ceased. The reaction mixture was warmed to room temperature and stirred overnight, resulting in a white precipitate. The reaction mixture was then concentrated *in vacuo*. The resulting white paste was diluted with hexane (2 x 25 mL) and the removal of residual $SOCl_2$ and hexane were carried out *in vacuo*. The semi solid obtained was then mixed to hexane and the slurry was filtered through a pad of Celite. Several hexane washings of the solid were carried out and the combined filtrate was then concentrated affording 2-(trimethylsilanyl)-ethanesulfonyl chloride **157** as a brown liquid. The ^{1}H NMR showed peaks for **157**, identical to the literature [142] however signals for an unidentified impurity (impurities) were observed.

Kugelrohr distillation was attempted at 85 - 100 ºC at 0.3 - 0.5 mm however at this temperature the compound charred.

Yield: 0.85 g, 3.93 mmol, 80 %;
NMR: δ_H (300 MHz, $CDCl_3$), 3.58 (2 H, m, CH_2SO$_2$), 1.27 (2 H, m, CH_2TMS), 0.04 (9 H, s, Si(CH_3)$_3$), **Impurities**: 3.36 (2 H, s, DMF), 3.12 (2 H, s, DMF), 2.98 (4 H, m), 1.05 (4 H, m);
NMR: δ_C (150 Mz, $CDCl_3$), 63.50 (C_5), 12.01 (C_4), -1.87 ($C_{1,\,2,\,3}$);
MS: (ES^+), $C_5H_{13}ClO_2SSi$, *m/z*: 255 $[M + K]^+$;
IR: ν_{max} 2955, 1366, 1252, 1173 cm^{-1}.

(S)-3-[2-Allyloxycarbonyl-2-(2-trimethylsilanyl-ethanesulfonylamino)-ethylsulfanyl]-(R)-2-(fluoren-9-ylmethoxcarbonylamino)-propionic acid tert-butyl ester 154



Entry	Trityl deprotection / Time	Reaction solvents	Base (equiv), / Temperature	SES-Cl, 157 (equiv), °C	Reaction time / temperature	Product Isolated 154 (%)	Byproduct Isolated 158 (%)	159 (%)
1	TFA/TES/ CH_2Cl_2, 4 hr	DMF	Triethylamine, (2), 0 °C	(1.5), 0 °C	1 hr, 0 °C, 24 hr, 5 °C	-	38 %	-
2	TFA/TES/ CH_2Cl_2, 4 hr	DMF	Triethylamine, (2), 0 °C	(1.5), 0 °C	1 hr, 0 °C, 24 hr, 5 °C	30 %	44 %	-
3	TFA/TES/ CH_2Cl_2, 4 hr	DMF	Triethylamine, (2), 0 °C	(5), 0 °C	1 hr, , 0 °C, 24 hr, 5 °C	38 %	-	-
4	TFA/TES/ CH_2Cl_2, 4 hr	DMF	Triethylamine, (2), 0 °C	(2.6), 0 °C	1 hr 30 min, 0 °C, 1 hr 30 min, 5 °C, 40 min, RT	27 %	45 %	-
5	TFA/TES/ CH_2Cl_2, 4 hr	DMF	Triethylamine, (3), 0 °C	(1.5), 0 °C	24 hr, 5 °C	-	-	35 %
6	TFA/TES/ CH_2Cl_2, 4 hr	DMF	Triethylamine, (3), 0 °C	(1.5), 0 °C	24 hr, 5 °C	NR *	NR *	NR *
7	TFA/TES/ CH_2Cl_2, 4 hr	1,4-Dioxane	Sodium bicarbonate, (4), RT	(3), 0 °C	24 hr, 4 °C	26 %	-	-
8	TFA/TES/ CH_2Cl_2, 4 hr	DMF	Triethylamine, (2), 0 °C Triethylamine, (2), RT	(5), 0 °C, (5), RT	3 hr, RT 1 hr 30 min, RT	35 %	-	-
9	TFA/TES/ CH_2Cl_2, 4 hr	DMF	Triethylamine, (1.5), 0 °C	(1.6), RT	5 hr, 0 °C – RT	12 %	-	-
10	TFA/TES/ CH_2Cl_2, 4 hr	Benzene	AgCN, (1.5), RT	(1.5), RT	22 hr, 60 °C	-	30 %	-
11	TFA/TES/ CH_2Cl_2, 4 hr	Acetonitrile	4-methoxy pyridine-N-oxide hydrate, (6), RT	(2), RT	24 hr, RT	19 %	-	-

* NR: No Reaction

Optimised experiments

General procedure

3-(*R*)-2-*tert*-Butoxycarbonyl-2-(fluoren-9-ylmethoxcarbonylamino)ethylsulfanyl)-(*S*)-(allyloxycarbonylamino)propionic acid allyl ester **102** was treated with a solution of TFA (10 %, 0.4 mL), triethylsilane (TES) (5 %, 0.2 mL) and CH_2Cl_2 (3.2 mL) under argon for 4 hr. Excess toluene was added and removed *in vacuo* 3 times, followed by addition of CH_2Cl_2 and then concentration *in vacuo*. The residue was then suspended in the appropriate solvent and the base was added followed by the addition of the SES-Cl at the required temperature. The equivalent number of reagents, the reaction time, and temperature of the reaction and the addition of further reagents were carried out and maintained according to the optimised experiments table above.

- Entry 9 involved the addition of SES-Cl first, followed by the addition of the base at 0 ºC. All reaction mixtures were diluted in excess ethyl acetate (except entry 11 which was diluted in CH_2Cl_2), the layer was washed 3 times with aqueous citric acid (5 %) and then with brine. The organic layer was dried over anhydrous magnesium sulphate and concentrated *in vacuo*. This afforded a crude oil which was purified via flash column chromatography on silica gel (hexane: EtOAc, 4:1) to give the title product **154** as a clear viscous oil.

R_f: 0.2 (Hexane: Ethyl acetate; 4:1);

NMR: δ_H (300 MHz, $CDCl_3$), 7.76 (2 H, d, *J* = 7.4 Hz, Ar), 7.62 (2 H, d, *J* = 7.1 Hz, Ar), 7.40 (2 H, t, *J* = 7.4 Hz, Ar), 7.32 (2 H, t, *J* = 7.4 Hz, Ar), 5.87 (1 H, m, CH_2CH=CH_2), 5.70 (1 H, d, *J* = 7.4 Hz N*H*Fmoc), 5.45 (1 H, d, *J* = 8.7 Hz, N*H*SES), 5.29 (2 H, m, CH_2CH=CH_2), 4.64 (2 H, m, CH_2CH=CH_2), 4.50 (1 H, m, H_α B side), 4.39 (3 H, m, CH_2Fmoc and H_α A side), 4.23 (1 H, t, *J* = 7.0 Hz, C*H*Fmoc), 3.08 (2 H, m, CH_2 β A side), 2.98 (2 H, m, CH_2SO_2), 2.91 (2 H, m, CH_2 β B sides), 1.48 (9 H, s, CH_3 *t*Bu), 1.12 (2 H, m, CH_2TMS), 0.02 (9 H, s, Si(CH_3)$_3$);

NMR: δ_C (125 MHz, $CDCl_3$), 173.1 (C_{25}), 158.3 (C_{15}), 146.2 (C_{Ar}), 143.7 (C_{27}), 133.6 (C_{Ar}), 130.1 (C_{Ar}), 129.5 (C_{Ar}), 127.6 (C_{Ar}), 122.4 (C_{28}), 85.6 (C_{17}), 69.7 (C_{14}), 68.7 (C_{26}), 58.5 (C_{21}), 57.2 (C_{24}), 52.8 (C_{29}), 49.5 (C_{30}), 39.5 (C_1), 32.1 ($C_{23, 22}$), 30.4 ($C_{18, 19, 20}$), 12.8 (Si), 0.4 ($C_{31, 32, 33}$);

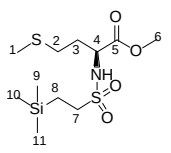
IR ν_{max}: 3400, 3010, 2999, 2810, 1700, 1510 cm^{-1};

HRMS: cald (ES^-), *m/z*: Found $[M + H]^+$, 691.25293; $C_{33}H_{47}N_2O_8SiS_2$, requires 691.25430; (ES^+), $C_{33}H_{46}N_2O_8SiS_2$, *m/z*: 713 $[M + Na]^+$;

$[\alpha]^{20}{}_D$ = - 7.5 (*c* 6.5 mg/mL, $CHCl_3$);

(ES^+), **158** $C_{28}H_{34}O_6N_2S$, *m/z*: 549 $[M + Na]^+$; (ES^+), **159** $C_{18}H_{36}O_6N_2S$, *m/z*: 491 $[M + Na]^+$, 178 [Fmoc].

4-Methylsulfanyl-2-(2-trimethylsilanyl-ethanesulfonylamino)-butyric acid methyl ester 161



Entry	Reaction solvent	Base (equiv), Temperature	SES-Cl, 157 (equiv), °C	Reaction time / temperature	Product Isolated 161 (%)
1	DMF	Triethylamine, (2), RT	(3), RT	5 hr, RT	71 %
2	Benzene	AgCN, (1), RT	(1.5), RT	22 hr, 75 °C	42 %
3	Acetonitrile	4-methoxy pyridine-N-oxide hydrate, (6), RT	(1.5), RT	24 hr, RT	45 %

Optimised experiments

General procedure

SES-Cl was added to a stirring solution containing methionine hydrochloride ester **160** and the selected base in the appropriate solvent as mentioned in the table above.

- The base in Entry 1 was added after the addition of SES-Cl.

The equivalent number of reagents, the reaction time and the temperature of the reaction were carried out and maintained according to the table above. The mixture was diluted in excess organic solvent (Entry 1: ethyl acetate and Entry 3: CH_2Cl_2). The organic layer was washed with saturated $NaHCO_3$ then with brine. The organic layer was dried over anhydrous magnesium sulphate and concentrated *in vacuo* this afforded a crude oil.

The reaction mixture of Entry 2 was filtered through Celite and then washed with ethyl acetate, the filtrate was then concentrated *in vacuo*.

The residues of all Entries were purified via flash column chromatography (hexane: EtOAc, 4:1) on silica gel to give the title product **161** as a clear oil.

R_f: 0.62 (Hexane: Ethyl acetate; 4:1);

NMR: δ_H (500 MHz, $CDCl_3$), 4.21 (1 H, m, NHC*H*CH_2), 3.74 (3 H, s, OC*H*$_3$), 2.91 (2 H, m, CH_2C*H*$_2$SCH_3), 2.58 (2 H, m, C*H*$_2SO_2$), 2.06 (4 H, m, CH_2SC*H*$_3$ and C*H*$_2CH_2SCH_3$), 1.92 (1 H, m, C*H*$_2CH_2SCH_3$), 1.00 (2 H, m, $(H_3C)_3$SiC*H*$_2CH_2$), 0.00 (9H, s, Si(C*H*$_3)_3$);

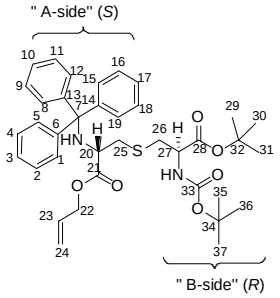
NMR: δ_C (125 MHz, $CDCl_3$), 172.8 (C_5), 54.9 (C_4), 52.9 (C_6), 49.9 (C_7), 32.3 (C_3), 29.9 (C_2), 15.3 (C_1), 10.4 (C_8), -1.87 ($C_{9, 10, 11}$);

HRMS: cald (ES^-), *m/z*: Found $[M - H]^-$, 326.0932; $C_{11}H_{24}NO_4SiS_2$, requires 326.0916. (ES^+), $C_{11}H_{25}NO_4SiS_2$, *m/z*: 350 $[M + Na]^+$;

IR ν_{max}: 3276, 2953, 1744, 1435, 1323 cm^{-1};

$[\alpha]^{20}_{D}$ = -1.6 (*c* 11.2 mg/mL, $CHCl_3$).

(S)-2-[Allyloxycarbonyl-2-(trityl-amino)-ethylsulfanyl]-(R)-2-tert-butoxycarbonylamino-propionic acid tert-butyl ester 163



Entry	Fmoc deprotection solvents / Time	Reaction solvent	Base (equiv), Temperature	Boc anhydride (equiv), °C	Reaction time / temperature	Product Isolated 163 (%)	Byproduct Isolated 168 (%)
1	20% piperidine in CH_2Cl_2, 30 min	CH_2Cl_2	Sodium bicarbonate, (1.3), RT	(1.3), RT	30 min, RT 4 hr, RT	3 %	65 %
2	20% piperidine in CH_2Cl_2, 30 min	DMF	Triethylamine, (1.3), RT	(1.3), RT	4 hr, RT	56 %	-

Optimised experiments

General procedure

3-(*R*)-2-*tert*-Butoxycarbonyl-2-(fluoren-9-ylmethoxcarbonylamino)ethylsulfanyl)-(*S*)-(allyloxycarbonylamino)propionic acid allyl ester **102** was added to a solution of piperidine (20 %) in CH_2Cl_2. The solution was removed *in vacuo* and the residue was redissolved in the appropriate. Di-*tert*-butyl dicarbonate (1.3 equiv) was added to the solution and (stirred for 30 min, Entry 1) this was followed by addition of the base (1.3 equiv). The mixture was stirred for 4 hr at room temperature. The reaction mixture of Entry 1 was concentrated *in vacuo*. Whilst the mixture of Entry 2 was diluted in excess CH_2Cl_2 and the layer was washed with saturated sodium bicarbonate followed by brine. The organic layer was dried over anhydrous magnesium sulphate and concentrated *in vacuo*. The crude of both entries were purified by flash column chromatography (hexane: EtOAC, 9:1) on silica gel to give the title product **163**.

R_f: 0.53 (Hexane: Ethyl acetate; 4:1);

: 0.19 (Hexane: Ethyl acetate; 9:1);

NMR: δ_H (500 MHz, $CDCl_3$), 7.54 (6 H, d, *J* = 8.6 Hz, Trt), 7.27 (6 H, m, Trt), 7.18 (3 H, m, Trt), 5.71 (1 H, m, CH_2CH=CH_2), 5.36 (1 H, d, *J* = 7.6 Hz, N*H*Boc), 5.17 (2 H, m, CH_2CH=CH_2), 4.40 (1H ,m, H_α B side), 4.13 (1 H, dd, *J* = 13.0, 6.0 Hz, CH_2CH=CH_2), 3.99 (1 H, dd, *J* = 13.1, 6.0 Hz, CH_2CH =CH_2), 3.56 (1 H, bm, H_α A side), 2.9 (3 H, m, H_β), 2.79 (1 H, m, H_β), 1.56 (1 H, s, N*H*Trt), 1.47 (18 H, s, *t*Bu);

NMR: δ_C (125 MHz, $CDCl_3$), 173.1 (C_{21}), 170.0 (C_{28}), 155.3 (C_{33}), 145.8 (C_{Ar}), 131.9 (C_{23}), 128.8 (C_{Ar}), 127.8 (C_{Ar}), 126.8 (C_{Ar}), 118.6 (C_{24}), 82.5 (C_{32}), 79.9 (C_{34}), 71.3 (C_{22}), 65.7 (C_7), 56.4 (C_{27}), 54.0 (C_{20}), 38.3 (C_{25}), 35.7 (C_{26}), 28.4 ($C_{29, 30, 31}$), 28.1 ($C_{35, 36, 37}$);

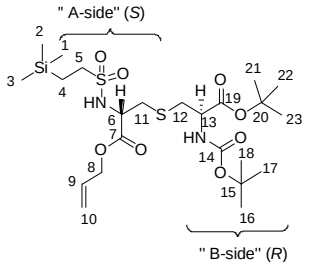
HRMS: cald (ES^+), *m/z*: Found $[M + Na]^+$, 669.2983; $C_{37}H_{46}N_2O_6NaS$, requires 669.2974; (ES^+), $C_{37}H_{46}N_2O_6S$, *m/z*: 669 $[M + Na]^+$;

IR ν_{max}: 2977, 1714, 1492, 1367 cm^{-1};

$[\alpha]^{20}{}_D$ = - 6.0 (*c* 10.6 mg/mL, $CHCl_3$);

^{1}H NMR was not carried out on the byproduct 168, byproduct was identified by mass spec (ES^+), 161 $C_{32}H_{38}N_2O_4S$, 569 $[M + Na]^+$.

(S)-3-[2-Allyloxycarbonyl-2-(2-trimethylsilanyl-ethanesulfonylamino)-ethylsulfanyl]-(R)-2-tert-butoxycarbonylamino-propionic acid tert-butyl ester 164

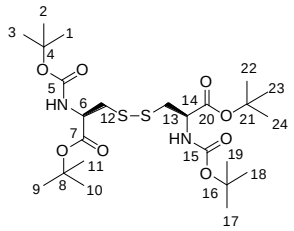


A solution of TFA (0.05 mL, 5 %), triethylsilane (0.025 mL, 2.5 %) and CH_2Cl_2 (0.93 mL, 92.5 %) was added to (*S*)-2-[Allyloxycarbonyl-2-(trityl-amino)-ethylsulfanyl]-(*R*)-2-*tert*-butoxycarbonylamino-propionic acid *tert*-butyl ester **163** (0.47 g, 0.72 mmol) and stirred for 4 hr. The mixture was concentrated *in vacuo*, removal of TFA was helped by several additions of toluene (3 x 100 mL) and CH_2Cl_2 evaporation. The residue was dissolved in DMF (2 mL), SES-Cl **157** (0.058 mL, 0.31 mmol, 0.4 equiv) was added and then cooled to 0 ºC. Triethylamine (0.032 mL, 0.25 mmol, 0.35 equiv) was added and stirred for 24 hr. Further SES-Cl **157** (0.058 mL, 0.31 mmol, 0.4 equiv) was added and triethylamine (0.032

mL, 0.25 mmol, 0.35 equiv) was added and stirred for 7 hr. The mixture was then diluted in excess CH_2Cl_2 and the organic layer was washed with citric acid (5 %) and then with brine. The organic layer was dried over anhydrous magnesium sulphate and concentrated *in vacuo*. Purification was carried out by flash column chromatography (hexane: EtOAc, 9:1) to give the title product **164** as a clear oil.

Yield: 0.023 g, 0.040 mmol, 54 %;
R_f: 0.13 (Hexane: Ethyl acetate; 4:1);
NMR: δ_H (500 MHz, $CDCl_3$), 5.92 (1 H, m, CH_2CH=CH_2), 5.72 (1 H, d, *J* = 7.5 Hz, N*H*SES), 5.56 (1 H, d, *J* = 7.0 Hz, N*H*Boc), 5.29 (2 H, m, CH_2CH=CH_2), 4.72 (1 H, d, *J* = 5.8 Hz, H_α B side), 4.68 (2 H, m, CH_2CH=CH_2), 4.45 (2 H, m, CH_2SO_2), 3.41 (1 H, m, H_α A side), 3.02 (4 H, m, 2 C$H_2\beta$ A and B side), 1.46 (18 H, d, *J* = 6.6 Hz, *t*Bu), 1.09 (2 H, m, CH_2Si), 0.03 (9 H, s, Si(CH_3)$_3$);
NMR: δ_C (150 MHz, $CDCl_3$), 169.4 ($C_{7, 19}$), 155.1 (C_{14}), 129.8 (C_9), 128.0 (C_{10}), 78.8 (C_{20}), 68.9 (C_{15}), 63.3 (C_8), 53.5 (C_{13}), 51.6 (C_6), 46.2 (C_5), 29.8 ($C_{11, 12}$), 28.4 ($C_{21, 22, 23}$), 27.7 ($C_{16, 17, 18}$), 9.83 (C_4), -1.70 ($C_{1, 2, 3}$);
HRMS: cald (ES^+), *m/z*: Found $[M + Na]^+$, 591.2178; $C_{23}H_{44}N_2O_8NaSiS_2$, requires 591.2206; ($ES^+$), $C_{23}H_{44}N_2O_8NaSiS_2$, *m/z*: 591 $[M + Na]^+$;
IR ν_{max}: 2954, 1718, 1329 cm^{-1};
$[\alpha]^{20}{}_D$ = - 3.1 (*c* 5.8 mg/mL, $CHCl_3$).

2-*tert*-Butoxycarbonylamino-3-(2-*tert*-butoxycarbonyl-2-*tert*-butoxycarbonylamino-ethyldisulfanyl)-propionic acid *tert*-butyl ester 166 [178]

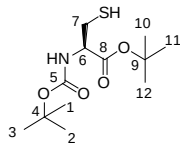


(*R*,*R*)-cystine bis-*tert*-butyl ester **145** (0.88 g, 2.51 mmol) was dissolved in THF. Di-*tert*-butyl dicarbonate (1.65 g, 7.56 mmol, 3 equiv) was added to the solution, this was followed by triethylamine (1.05 mL, 7.55 mmol, 3 equiv) and the reaction was left stirring for 4 hr. The mixture was diluted in excess CH_2Cl_2 and washed with saturated $NaHCO_3$ and brine, the organic layer was then dried over anhydrous magnesium sulphate and concentrated *in vacuo*.

The crude was purified by flash column chromatography (hexane: EtOAc, 9:1) to give the title product **166** as a yellow viscous oil, identical by NMR to the literature. [178]

Yield: 1.37 g, 2.48 mmol, 98 %;
R_f: 0.39 (Hexane: Ethyl acetate; 4:1);
NMR: δ_H (600 MHz, $CDCl_3$), 5.35 (2 H, d, *J* = 6.9 Hz, N*H*), 4.45 (2 H, m, 2 x C*H*CH_2S), 3.20 (2 H, dd, *J* = 13.8, 4.6 Hz, CHC*H*$_2$S), 3.12 (2 H, dd, *J* = 13.7, 5.5 Hz, CHC*H*$_2$S), 1.47 (18 H, s, 2 x (C*H*$_3$)$_3$), 1.44 (18 H, s, 2 x (C*H*$_3$)$_3$);
NMR: δ_C (150 MHz, $CDCl_3$), 169.0 ($C_{7, 20}$), 155.1 ($C_{5, 15}$), 82.8 ($C_{8, 21}$), 80.1 ($C_{4, 16}$), 53.7 ($C_{6, 14}$), 42.2 ($C_{12, 13}$), 28.4 ($C_{9, 10, 11, 22, 23, 24}$), 28.1 ($C_{1, 2, 3, 17, 18, 19}$);
HRMS: cald (ES^+), *m/z*: Found $[M + Na]^+$, 575.24437; $C_{24}H_{44}N_2O_8NaS$, requires 575.24366; (ES^+), $C_{24}H_{44}N_2O_8S$, *m/z*: 575 $[M + Na]^+$;
IR ν_{max}: 2980, 1710, 1498, 1368, 1216 cm^{-1}.

2-*tert*-Butoxycarbonylamino-3-(2-*tert*-butoxycarbonyl-2-*tert*-butoxycarbonylamino-ethyldisulfanyl)-propionic acid *tert*-butyl ester 167 [122]



2-*tert*-Butoxycarbonylamino-3-(2-*tert*-butoxycarbonyl-2-*tert*-butoxycarbonylamino-ethyldisulfanyl)-propionic acid *tert*-butyl ester **166** (0.39 g, 0.71 mmol) in dry THF (20 mL) was treated with tributylphosphine (0.31 mL, 1.55 mmol, 2 equiv). The mixture was stirred for 2 min after which water (distilled, 0.62 mL, 34.4 mmol, 56 equiv) was added. The reaction mixture was stirred for further 2 hr. The mixture was then concentrated *in vacuo* and dissolved in ethyl acetate (150 mL). The solution was washed with citric acid (10 % aq. w/v, 100 mL) and then brine (100 mL). The organic layer was dried over anhydrous sodium sulphate and the solvent was removed under reduced pressure to yield pale yellow oil. Purification via flash column chromatography (Petroleum ether: Diethyl ether, 9:1 then to 5:1) yielded the starting material, oxidised tributylphosphine O=PBu_3 (Appendix 1) and the title product **167** as pale yellow oil with, identical by NMR to the literature. [178]

Yield: 0.01 g, 0.036 mmol, 5 %;
R_f: 0.46 (Petroleum ether: Diethyl ether; 2:1);

NMR: δ_H (600 MHz, $CDCl_3$), 5.39 (1 H, d, *J* = 6.5 Hz, N*H*), 4.46 (1 H, m, C*H*CH_2S), 2.95 (2 H, m, CHC*H*$_2$S), 1.48 (9 H, s, (C*H*$_3$)$_3$), 1.44 (9 H, s, (C*H*$_3$)$_3$), 1.35 (1 H, t, *J* = 8.8 Hz, CH_2S*H*);

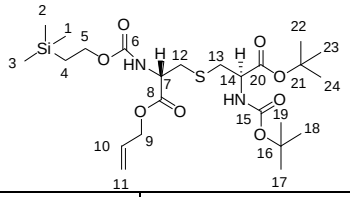
NMR: δ_C (150 MHz, $CDCl_3$), 169.4 (C_8), 155.2 (C_5), 82.9 (C_9), 80.6 (C_4), 55.1 (C_6), 28.2 ($C_{10, 11, 12}$), 28.1 ($C_{1, 2, 3}$), 27.9 (C_7);

HRMS: cald (ES^+), *m/z*: Found $[M + H]^+$, 278.14288; $C_{12}H_{24}NO_4NS$, requires 278.14260; (ES^+), $C_{12}H_{24}NO_4S$, *m/z*: 278 $[M + H]^+$;

IR ν_{max}: 2979, 1715, 1498, 1367, 1249 cm^{-1};

$[\alpha]^{20}{}_D$ = + 8.2 (*c* 8.0 mg/mL, $CHCl_3$).

(R)-3-(2-tert-Butoxycarbonyl-2-tert-butoxycarbonylamino-ethylsulfanyl)-(S)-2-(2-trimethylsilanyl-ethoxycarbonylamino)-propionic acid allyl ester 172



Entry	Trityl deprotection / Time	Reaction solvents	Base (equiv), Temperature	Protecting group Teoc-ONp 170 (equiv) °C	Teoc-ONSu 171 (equiv) °C	Reaction time / temperature	Product Isolated 172 (%)	Byproduct Isolated 177 (%)
1	TFA/TES/ CH_2Cl_2, 4 hr	H_2O / 1,4-dioxane	aq. sodium bicarbonate, (4), RT	(2), 0 °C	-	18 hr, ≤ 4 °C	17 %	41 %
2	TFA/TES/ CH_2Cl_2, 4 hr	DMF	Triethylamine, (2 drops), RT	(2), RT	-	24 hr, RT	NR *	NR *
3	TFA/TES/ CH_2Cl_2, 4 hr	H_2O	Triethylamine, (2), dioxane, RT	(2), RT	-	24 hr, RT	-	< 10 % **
4	TFA/TES/ CH_2Cl_2, 4 hr	DMF	Triethylamine, (2), dioxane, RT	(2), RT	-	24 hr, RT	-	< 10 % **
5	TFA/TES/ CH_2Cl_2, 4 hr	H_2O	Triethylamine, (1.5), dioxane, RT	(1.5), RT	-	24 hr, RT	17 %	-
6	TFA/TES/ CH_2Cl_2, 4 hr	H_2O	Triethylamine, (2), dioxane, RT	-	(2), 0 °C	18 hr ≤ 4 °C	-	< 10 % **
7	TFA/TES/ CH_2Cl_2, 4 hr	DMF	Triethylamine, (2), dioxane, RT	-	(2), 0 °C	18 h at ≤ 4 °C	14 %	-
8	TFA/TES/ CH_2Cl_2, 4 hr	H_2O	Triethylamine, (1.5), dioxane, RT	-	(1.5), RT	24 hr, RT	10 %	-
9	TFA/TES/ CH_2Cl_2, 4 hr	H_2O / 1,4-dioxane	aq. sodium bicarbonate, (4), RT	-	(2), 0 °C	18 hr, ≤ 4 °C	14 %	< 10 % **

* NR: No Reaction

** < 10 %: Identified by mass spec but not isolated

Optimised experiments

General procedure

(*S*)-2-[Allyloxycarbonyl-2-(trityl-amino)-ethylsulfanyl]-(*R*)-2-*tert*-butoxycarbonylamino-propionic acid *tert*-butyl ester **163** was treated with a solution of TFA (10 %, 0.4 mL), triethylsilane (TES) (5 %, 0.2 mL) and CH_2Cl_2 (3.2 mL) under argon for 4 hr. Excess CH_2Cl_2 (100 mL) was then added. The organic layer was washed with saturated sodium bicarbonate (50 mL) and brine (50 mL), dried over anhydrous sodium sulphate, and then concentrated *in vacuo*. The residue was suspended in the appropriate solvent and then the base was added followed by the addition of the protecting reagent at the required temperature. The equivalents of reagents, the reaction time and the temperature of the reaction were carried out and maintained according to the optimised experiments table above. Excess CH_2Cl_2 (150 mL) was added and the mixture was washed with saturated sodium bicarbonate (80 mL) and brine (80 mL). The organic layer was dried over anhydrous sodium sulphate, concentrated *in vacuo*, and purified by flash column chromatography (hexane: EtOAC, 9:1) to give the title product **172** and undesired product **177** identified by mass spec.

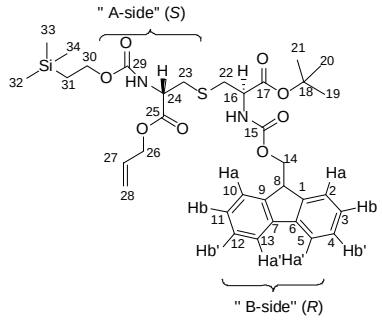
Desired product

R_f: 0.39 (Hexane: Ethyl acetate; 4:1);
HRMS: cald (ES^+), *m/z*: Found $[M + H]^+$, 548.2590; $C_{24}H_{44}N_2O_8SiS$, requires 548.2588;
IR ν_{max}: 2925, 1592, 1499, 1320 cm^{-1};
$[\alpha]^{20}{}_D$ = + 12.4 (*c* 2.7 mg/mL, $CHCl_3$);

Byproduct

R_f: 0.13 (Hexane: Ethyl acetate; 4:1);
HRMS: 177 cald (ES^+), *m/z*: Found $[M + Na]^+$ 615.2570; $C_{25}H_{48}N_2O_8NaSi_2S$, requires 615.2568; ($ES^+$), $C_{25}H_{48}N_2O_8Si_2S$, *m/z*: 615 $[M + Na]^+$.

(S)-3-[2-Allyloxycarbonyl-2-(2-trimethylsilanyl-ethoxycarbonylamino)-ethylsulfanyl]-(R)-2-(9H-fluoren-9-ylmethoxycarbonylamino)-propionic acid tert-butyl ester 175



Entry	Trityl deprotection / Time	Reaction solvents	Base (equiv), Temperature	Protecting group Teoc-ONp 170 (%) °C	Teoc-ONSu 171 (%) °C	Reaction time / temperature	Product Isolated 175 (%)
1	TFA/TES/ CH_2Cl_2, 4 hr	1,4-dioxane	aq. sodium bicarbonate, (4), RT	(2), RT	-	24 hr, RT	23 %
2	TFA/TES/ CH_2Cl_2, 4 hr	1,4-dioxane	aq. sodium bicarbonate, (4), RT	(2), 0 °C	-	24 hr, RT	14 %
3	TFA/TES/ CH_2Cl_2, 4 hr	1,4-dioxane	Triethylamine, (0.5), dioxane, RT, Triethylamine, (0.5), dioxane, RT	(2), RT	-	2 hr, RT 24 hr, RT	21 %
4	TFA/TES/ CH_2Cl_2, 4 hr	H_2O	Triethylamine, (0.5), dioxane, RT, Triethylamine, (0.5), dioxane, RT	(2), RT	-	2 hr, RT 24 hr RT	21 %
5	TFA/TES/ CH_2Cl_2, 4 hr	H_2O	Triethylamine, (0.5), dioxane, RT, Triethylamine, (0.5), dioxane, RT	-	(2), RT	2 hr, RT, 24 hr, RT	21 %
6	TFA/TES/ CH_2Cl_2, 4 hr	1,4-dioxane	aq. sodium bicarbonate, (4), RT	-	(1), RT	24 hr, RT	23 %
7	TFA/TES/ CH_2Cl_2, 4 hr	1,4-dioxane	aq. sodium bicarbonate, (4), RT, Triethylamine, (couple of drops), 0 °C	-	(2), 0 °C	24 hr, RT	2 %
8	TFA/TES/ CH_2Cl_2, 4 hr	1,4-dioxane	aq. sodium bicarbonate, (4), RT	-	(2), 0 °C	24 hr, RT	31 %
9	TFA/TES/ CH_2Cl_2, 4 hr	H_2O	Triethylamine, (0.5), dioxane,	-	(2), RT	3 hr 30 min, RT,	25 %

			RT, Triethylamine, (0.5), dioxane, RT		(2), RT	24 hr, RT	
10	TFA/TES/ CH_2Cl_2, 4 hr	1,4-dioxane	aq. sodium bicarbonate, (4.6), RT	-	(2), 0 °C	24 hr in freezer, 5 hr, 0 °C	50 %
11	TFA/TES/ CH_2Cl_2, 4 hr	1,4-dioxane	aq. sodium bicarbonate, (5), RT	-	(2.5), 0 °C	24 hr in freezer, 5 hr, 0 °C – RT	83 %

Optimised experiments

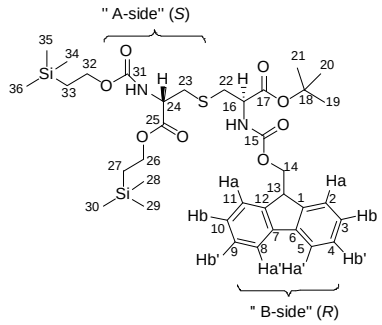
General procedure

3-(*R*)-2-*tert*-Butoxycarbonyl-2-(fluoren-9-ylmethoxcarbonylamino)ethylsulfanyl)-(*S*)-(allyloxycarbonylamino)propionic acid allyl ester **102** was treated with a solution of TFA (10 %, 0.4 mL), triethylsilane (TES) (5 %, 0.2 mL) and CH_2Cl_2 (3.2 mL) under argon for 4 hr. Excess CH_2Cl_2 (100 mL) was then added. The organic layer was washed with saturated sodium bicarbonate (50 mL) and brine (50 mL), dried over anhydrous sodium sulphate, and then concentrated *in vacuo*. The residue was suspended in the appropriate solvent and then the base was added followed by the addition of the protecting reagent at the required temperature. The equivalents of reagents, the reaction time, and temperature of the reaction and the addition of further reagents were carried out and maintained according to the optimised experiments table above. Excess CH_2Cl_2 (150 mL) was added and the mixture was washed with saturated sodium bicarbonate (80 mL) and brine (80 mL). The organic layer was dried over anhydrous sodium sulphate, concentrated *in vacuo*, and purified by flash column chromatography (hexane: EtOAC, 9:1) to give the title product **175** as a clear oil.

R_f: 0.45 (Hexane: Ethyl acetate; 9:1);
NMR: δ_H (500 MHz, $CDCl_3$), 7.76 (2 H, d, *J* = 7.5 Hz, Ar), 7.62 (2 H, d, *J* = 5.4 Hz, Ar), 7.39 (2 H, t, *J* = 7.4 Hz, Ar), 7.38 (2 H, t, *J* = 7.4 Hz, Ar), 5.86 (1 H, m, CH_2CH=CH_2), 5.81 (1 H, d, *J* = 7.1 Hz, N*H*Fmoc), 5.58 (1 H, d, *J* = 5.2 Hz, N*H*Teoc), 5.28 (2 H, m, CH_2CH=CH_2), 4.63 (3 H, m, *J* = 5.8 Hz, CH_2CH=CH_2 and H_α A side), 4.49 (1 H, m, H_α B side), 4.38 (2 H, m, CH_2Fmoc), 4.24 (1 H, t, *J* = 7.3 Hz, C*H*Fmoc), 4.16 (2 H, t, *J* = 8.6 Hz, CH_2OCO), 3.02 (4 H, m, CH_2 β A and B side), 1.48 (9 H, s, *t*Bu), 0.97 (2 H, bt, *J* = 8.6 Hz, CH_2TMS), 0.01 (9 H, s, Si(CH_3)$_3$);

NMR: δ_C (125 MHz, $CDCl_3$), 170.0 (C_{25}), 169.4 (C_{17}), 156.0 (C_{15}), 155.9 (C_{29}), 143.9 (C_{Ar}), 143.8 (C_{Ar}), 141.4 (C_{27}), 131.4 (C_{Ar}), 127.8 (C_{Ar}), 127.2 (C_{Ar}), 125.6 (C_{Ar}), 120.1 (C_{28}), 67.3 (C_{18}), 66.4 (C_{14}), 63.7 (C_{26}), 60.5 (C_{16}), 54.4 (C_{24}), 53.9 (C_{30}), 47.2 (C_8), 35.9 ($C_{22,\ 23}$), 28.1 ($C_{19,\ 20,\ 21}$), 14.2 (C_{31}), -1.23 ($C_{32,\ 33,\ 34}$);
HRMS: cald (ES^+), *m/z*: Found $[M + Na]^+$, 693.2605; $C_{34}H_{46}N_2O_8NaSiS$, requires 693.2642. (ES^+), $C_{34}H_{46}N_2O_8SiS$, *m/z*: 693 $[M + Na]^+$;
IR ν_{max}: 2952, 1715, 1519 cm^{-1};
$[\alpha]^{20}{}_D$ = - 5.2 (*c* 8.5 mg/mL, $CHCl_3$).

(R)-2-(9H-Fluoren-9-ylmethoxycarbonylamino)-3-[2-(2-trimethylsilanyl-ethoxycarbonyl)-(S)-2-(2-trimethylsilanyl-ethoxycarbonylamino)-ethylsulfanyl]-propionic acid tert-butyl ester 176



(*S*)-3-[2-Allyloxycarbonyl-2-(2-trimethylsilanyl-ethoxycarbonylamino)-ethylsulfanyl]-(*R*)-2-(9H-fluoren-9-ylmethoxycarbonylamino)-propionic acid *tert*-butyl ester **175** (0.17 g, 0.25 mmol) was dissolved in a solution of tetrakis-triphenyl phosphine, $Pd(PPh_3)_4$ (10 % w/v, 0.017 g, 0.014 mmol), 1,3-dimethylbirbaturic acid (0.020 g, 0.128 mmol, 5 equiv) in $CHCl_3$ (1 mL) and the reaction was stirred for 3 hr. The mixture was diluted in excess ethyl acetate which was washed with brine and then with distilled water. The organic layer was dried over anhydrous sodium sulphate, filtered and concentrated *in vacuo* (mass spec and NMR in Appendix 2 and 3). The residue was then dissolved in acetonitrile (2 mL). To the solution dry pyridine (0.02 mL, 1 equiv) and 2-(trimethylsilanyl)ethanol (0.054 mL, 0.37 mmol, 1.5 equiv) was added. The mixture was cooled to 0 ºC then *N',N'*-dicyclohexylcarbodiimide (0.06 g, 0.29 mmol, 1.2 equiv) was added and the reaction mixture was stirred overnight at room temperature under inert conditions. The DCU precipitate was removed by filtration and washed with ethyl acetate, the filtrate was then removed *in vacuo*. The resulting oily residue was redissolved in ethyl acetate and washed with saturated sodium bicarbonate and water. The organic layer was dried over anhydrous sodium sulphate, concentrated *in vacuo*, and

purified by flash column chromatography (hexane: EtOAc, 9:1) to give the title product **176** as a clear viscous oil.

Yield: 0.095 g, 0.130 mmol, 51 %;

R_f: 0.34 (Hexane: Ethyl acetate; 4:1);

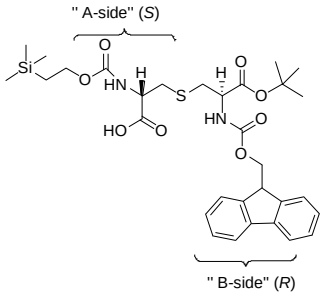
NMR: δ_H (500 MHz, $CDCl_3$), 7.75 (2 H, d, *J* = 7.5 Hz, Ar), 7.62 (2 H, bt, *J* = 6.4 Hz, Ar), 7.39 (2 H, t, *J* = 7.5 Hz, Ar), 7.31 (2 H, t, *J* = 7.5 Hz, Ar), 5.82 (1 H, d, *J* = 7.4 Hz, N*H*Fmoc), 4.58 (1 H, m, N*H*Teoc), 4.50 (1 H, m, H_α B side), 4.36 (2 H, m, CH_2Fmoc), 4.24 (2 H, m, C*H*Fmoc and H_α A side), 4.16 (4 H, m, 2 x $Si(CH_3)_3CH_2CH_2$), 3.01 (4 H, m, CH_2 β A and B side), 1.45 (9 H, s, *t*Bu), 0.98 (4 H, m, 2 x $Si(CH_3)_3CH_2CH_2$), 0.03 (18 H, d, *J* = 4.4 Hz, 2 x $Si(CH_3)_3$), co-eluting with 5.50 (1 H, m, allyl impurity), 5.12 (4 H, m, allyl impurity), 2.71 (2 H, d, *J* = 7.5 Hz, allyl impurity), (Appendix 5);

NMR: δ_C (125 MHz, $CDCl_3$), 168 ($C_{17,\ 25}$), 156.0 ($C_{15,\ 31}$), 143.8 (C_{Ar}), 141.0 (C_{Ar}), 127.8 (C_{Ar}), 127.2 (C_{Ar}), 125.3 (C_{Ar}), 83.2 (C_{18}), 67.4 (C_{14}), 64.5 (C_{26}), 54.5 (C_{16}), 54.0 ($C_{24,\ 32}$), 47.2 (C_{13}), 36.1 ($C_{22,\ 23}$), 28.1 ($C_{19,\ 20,\ 21}$), 17.7 (C_{27}), 17.5 (C_{33}) -1.44 ($C_{28,\ 29,\ 30,\ 34,\ 35,\ 36}$);

HRMS: cald (ES^+), *m/z*: Found $[M + Na]^+$, 753.3058; $C_{36}H_{54}N_2O_8NaSi_2S$, requires 753.3037; ($ES^+$), $C_{36}H_{54}N_2O_8Si_2S$, *m/z*: 753 $[M + Na]^+$ (Appendix 4).

*Sample not pure.

3-[2-<u>tert</u>-Butoxycarbonyl-2-(9H-fluoren-9-ylmethoxycarbonylamino)-ethylsulfanyl]-2-(2-trimethylsilanyl-ethoxycarbonylamino)-propionic acid

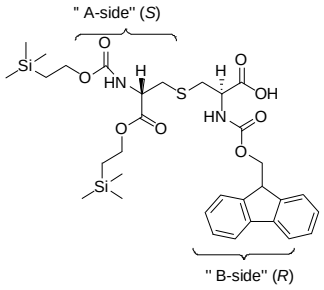


(*S*)-3-[2-Allyloxycarbonyl-2-(2-trimethylsilanyl-ethoxycarbonylamino)-ethylsulfanyl]-(*R*)-2-(9H-fluoren-9-ylmethoxycarbonylamino)-propionic acid *tert*-butyl ester **175** (0.17 g, 0.25 mmol) was dissolved in a solution of tetrakis-triphenyl phosphine, $Pd(PPh_3)_4$ (10 % w/v, 0.017 g, 0.014 mmol), 1,3-dimethylbarbituric acid (0.020 g, 0.128 mmol, 5 equiv) in $CHCl_3$ (1 mL) and stirred for 3 hr. Excess ethyl acetate was added to the reaction mixture, and the

resulting organic layer was washed with brine, followed by distilled water. The organic layer was dried over anhydrous sodium sulphate, filtered and concentrated *in vacuo* to give 3-[2-*tert*-butoxycarbonyl-2-(9H-fluoren-9-ylmethoxycarbonylamino)-ethylsulfanyl]-2-(2-trimethylsilanyl-ethoxycarbonylamino)-propionic acid.

NMR: δ_H (500 MHz, $CDCl_3$), 7.87 (2 H, d, *J* = 7.4 Hz, Ar), 7.72 (2 H, bd, *J* = 7.5 Hz, Ar), 7.36 (2 H, t, *J* = 7.3 Hz, Ar), 7.28 (2 H, t, *J* = 7.5 Hz, Ar), 6.01 (1 H, bd, *J* = 7.3 Hz, N*H*Fmoc), 5.72 (1 H, bd, *J* = 7.3 Hz, N*H*Teoc), 4.42 (1 H, m, H_α B side), 4.34 (2 H, m, C*H*Fmoc and H_α A side), 4.20 (2 H, t, *J* = 7.2 Hz, CH_2Fmoc), 4.12 (2 H, m, CH_2OCO), 3.00 (4 H, m, CH_2 β A and B side), 1.46 (9 H, s, *t*Bu), 0.94 (2 H, t, CH_2TMS), 0.00 (9 H, s, Si(CH_3)$_3$), co-eluting with 5.49 (1 H, m, allyl impurity), 5.27 (4 H, m, allyl impurity), 2.71 (2 H, d, *J* = 7.5 Hz, allyl impurity), (Appendix 3);
MS: (ES^+), $C_{31}H_{42}N_2O_8SiS$, *m/z*: 653 $[M + Na]^+$ (Appendix 2).
*Sample not pure.

(R)-2-(9H-Fluoren-9-ylmethoxycarbonylamino)-3-[2-(2-trimethylsilanyl-ethoxycarbonyl)-(S)-2-(2-trimethylsilanyl-ethoxycarbonylamino)-ethylsulfanyl]-propionic acid 169



(*R*)-2-(9H-Fluoren-9-ylmethoxycarbonylamino)-3-[2-(2-trimethylsilanyl-ethoxycarbonyl)-(*S*)-2-(2-trimethylsilanyl-ethoxycarbonylamino)-ethylsulfanyl]-propionic acid *tert*-butyl ester **176** (0.27 g, 0.36 mmol) was treated with a solution of TFA (1 mL) and CH_2Cl_2 (1 mL) and the mixture was stirred for 3 hr. The reaction mixture was concentrated *in vacuo* with toluene (100 mL), this was added to aid the removal of TFA. Purification was completed by reverse-phase column chromatography in acetonitrile and saturated $NaHCO_3$, gradient 0 % - 40 % actonitrile in 100 % - 60 % (20 % $NaHCO_3$) saturated $NaHCO_3$. The acetonitrile was removed under vacuum. Concentrated sulphuric acid was added to bring the aqueous layer to pH 2 and sodium chloride was added until saturation was reached. The product was extracted from the aqueous layer with chloroform. The organic layer was then dried over anhydrous

sodium sulphate and concentrated *in vacuo* to give the title product **169** followed by preparative HPLC.

Yield: 0.071 g, 0.105 mmol, 28 %;

R_f: 0.13 (AgCN ((40 %): Sat. $NaHCO_3$) Reverse phase TLC;

NMR: δ_H (500 MHz, $CDCl_3$), 7.73 (2 H, d, *J* = 7.2 Hz, Ar), 7.69 (2 H, m, Ar), 7.32 (2 H, bt, *J* = 7.35 Hz, Ar), 7.24 (2 H, t, *J* = 7.7 Hz, Ar), 6.48 (1 H, bs, N*H*Fmoc), 4.61 (1 H, bs, N*H*Teoc), 4.39 (2 H, m, H_α B side and C*H*Fmoc), 4.40 - 4.11 (7 H, m, CH_2Fmoc, H_α A side and 2 x $Si(CH_3)_3CH_2CH_2$), 3.27 (4 H, m, CH_2 β A and B side), 0.86 (4 H, m, 2 x $Si(CH_3)_3CH_2CH_2$), 0.04 (9 H, s, $Si(CH_3)_3$), -0.07 (9 H, s, $Si(CH_3)_3$), co-eluting with 5.29 (1 H, m, allyl), 5.06 (2 H, m, allyl), 2.71 (2 H, d, *J* = 7.5 Hz, allyl), (Appendix 6);

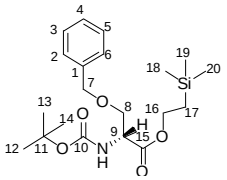
MS: (ES^+), $C_{32}H_{46}N_2O_8Si_2S$, *m/z*: 675 $[M + H]^+$;

Analytical HPLC: (Wide Pore C18); **R_T** 23.35; A: H_2O/0.5 % TFA; B: acetonitrile/0.5% TFA; gradient 10 % - 60 % B over 35 min (Appendix 7);

Preparative HPLC: (Wide Pore C18) in H_2O/0.5 % TFA and acetonitrile/0.5 % TFA at a gradient of 10 % - 60 % acetonitrile over 45 min to give **162**, which eluted at 34 min (Appendix 8).

*Sample not pure.

(R)-3-Benzyloxy-2-tert-butoxycarbonylamino-propionic acid (2-trimethylsilanyl)ethyl ester 179



Boc-D-Serine(Bzl)-OH **178** (2.30 g, 7.79 mmol) and 4-(dimethylamino)pyridine (DMAP) (0.155 g, 1.27 mmol, 0.15 equiv) were dissolved in dry THF (32 mL) and cooled to 0 ºC under nitrogen. 2-Trimethylsilylethanol (2.43 mL, 16.9 mmol, 2 equiv) was added dropwise, followed by 1,3-dicyclohexylcarbodiimide (2.27 g, 11.0 mmol, 1.3 equiv) in one portion, the solution was allowed to reach room temperature and stirred for 36 hr. After 36 hr the mixture was filtered, washed with ethyl acetate and the filtrate was concentrated *in vacuo*. The residue was redissolved in ethyl acetate. The organic layer was washed with saturated sodium bicarbonate (200 mL) and brine (200 mL), and then dried over anhydrous magnesium sulphate and the solvent was removed *in vacuo*. Flash column chromatography (hexane: EtOAc, 9:1) was carried out to give the title product **179** as a clear oil.

Yield: 2.7 g, 6.83 mmol, 80 %;

R_f: 0.5 (Hexane: Ethyl acetate; 4:1);

NMR: δ_H (500 MHz, $CDCl_3$), 7.73-7.25 (5 H, m, Ar), 5.40 (1 H, d, *J* = 8.6 Hz, Boc*H*NCH), 4.53 (1 H, d, *J* = 12.2 Hz, ArC*H*$_2$OCH$_2$CH), 4.46 (1 H, d, *J* = 12.2 Hz, ArC*H*$_2$OCH$_2$CH), 4.39 (1 H, m, ArCH$_2$OCH$_2$C*H*), 4.22 (2 H, m, CH$_2$CO$_2$C*H*$_2$CH$_2$Si(CH$_3$)$_3$), 3.86 (1 H, dd, *J* = 9.4, 3.0 Hz, ArCH$_2$OC*H*$_2$CH), 3.68 (1 H, dd, *J* = 9.4, 3.0 Hz, ArCH$_2$OC*H*$_2$CH), 1.44 (9 H, s, Boc), 0.96 (2 H, m, *J* = 8.9, 8.3 Hz, C*H*$_2$SiCH$_3$)$_3$), 0.02 (9 H, s, Si(C*H*$_3$)$_3$);

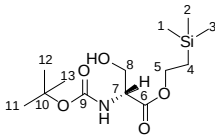
NMR: δ_C (125 MHz, $CDCl_3$), 170.8 (C_{15}), 155.6 (C_{10}), 137.7 (C_{Ar}), 129.7 (C_{Ar}), 128.4 (C_{Ar}), 127.6 (C_{Ar}), 79.8 (C_{11}), 73.3 (C_7), 70.1 (C_8), 63.9 (C_{16}), 54.2 (C_9), 28.3 ($C_{12, 13, 14}$), 17.4 (C_{17}), -1.45 ($C_{18, 19, 20}$);

HRMS: cald (ES^+), *m/z*: Found $[M + Na]^+$, 418.1855; $C_{20}H_{33}NO_3NaSi$, requires 418.1848; (ES^+), $C_{20}H_{33}NO_3Si$, *m/z*: 418 $[M + Na]^+$;

IR ν_{max}: 3447, 2953, 2119, 1721, 1717, 1497 cm^{-1};

$[\alpha]^{20}{}_D$ = - 7.6 (*c* 10 mg/mL, $CHCl_3$).

(R)-2-tert-Butoxycarbonylamino-3-hydroxy-propionic acid (2-trimethylsilanyl)ethyl ester 180



(*R*)-3-Benzyloxy-2-*tert*-butoxycarbonylamino-propionic acid (2-trimethylsilanyl)ethyl ester **179** (2.47 g, 6.25 mmol) was dissolved in ethanol (80 mL), Pd/C (10 %, 0.5 g) was added to the mixture. The suspension was placed under a hydrogen atmosphere and stirred at atmospheric pressure for 24 hr. Further Pd/C (10 %, 0.5 g) was added and the hydrogen re-charged; the suspension was stirred for a further 7 hr. A further Pd/C (10 %, 0.5 g) was added and the suspension was stirred for 24 hr. A final portion of Pd/C (10 %, 0.5 g) was added, the hydrogen re-charged, and the suspension was stirred for a further 48 hr. The mixture was filtered through a pad of Celite, rinsing several times with ethyl acetate. The combined filtrates were concentrated *in vacuo* and the title product **180** was isolated as a clear oil by flash column chromatography (hexane: EtOAc, 9:1) on silica gel.

Yield: 1.76 g, 5.77 mmol, 87 %;

R_f: 0.17 (Hexane: Ethyl acetate; 9:1);

0.71 (Hexane: Ethyl acetate; 4:1);

NMR: δ_H (500 MHz, $CDCl_3$), 5.47 (1 H, d, *J* = 4.4 Hz, BocN*H*CH), 4.32 (1 H, m, BocNHC*H*OH), 4.24 (2 H, m, $Si(CH_3)_3CH_2CH_2CO_2$), 3.90 (2 H, m, BocNHCHCH_2OH), 2.56 (1 H, bs, O*H*), 1.43 (9 H, s, Boc), 1.00 (2 H, t, *J* = 5.1 Hz, $Si(CH_3)_3CH_2CH_2TMS$), 0.02 (9 H, s, $Si(CH_3)_3$);

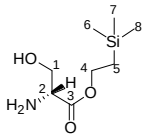
NMR: δ_C (125 MHz, $CDCl_3$), 170.9 (C_6), 155.8 (C_9), 80.2 (C_{10}), 64.3 (C_8), 60.5 (C_5), 55.9 (C_7), 28.3 ($C_{11, 12, 13}$), 14.2 (C_4), -1.49 ($C_{1, 2, 3}$);

HRMS: cald (ES^+), *m/z*: Found $[M + H]^+$, 306.1739, $C_{13}H_{28}NO_5Si$, requires 306.1736; (ES^+), $C_{13}H_{27}NO_5Si$, *m/z*: 306 $[M + H]^+$;

IR ν_{max}: 2954, 1717, 1497, 1366 cm^{-1};

$[\alpha]^{20}{}_D$ = - 8.3 (*c* 9.9 mg/mL, $CHCl_3$).

(R)-2-Amino-3-hydroxy-propionic acid (2-trimethylsilanyl)ethyl ester 181



Condition 1

(*R*)-*2-tert*-Butoxycarbonylamino-3-hydroxy-propionic acid (2-trimethylsilanyl)ethyl ester **180** (0.2 g, 0.65 mmol) was treated with a solution of TFA (1 mL) and CH_2Cl_2 (1 mL) and the reaction mixture was stirred for 1 hr. The reaction mixture was then concentrated *in vacuo*, excess toluene was added and removed *in vacuo*, this was repeated 2 times followed by CH_2Cl_2. The title product **181** was isolated by flash column chromatography on silica gel as colourless oil. Column was loaded with (MeOH: CH_2Cl_2, 2:98) followed by (MeOH: CH_2Cl_2, 5:95).

Yield: 0.066 g, 0.32 mmol, 49 %;

R_f: 0.25 (MeOH: CH_2Cl_2; 10:90).

Condition 2

(*R*)-2-Amino-3-benzyloxy-propionic acid (2-trimethylsilanyl)ethyl ester **185** (0.26 g, 0.90 mmol) was dissolved in ethanol, Pd/C (10 %, 0.06 g) was then added to the mixture. The suspension was placed under a hydrogen atmosphere and stirred at atmospheric pressure for 24 hr. Further Pd/C (10 %, 0.06 g) was added and the hydrogen re-charged; the suspension

was stirred for a further 7 hr. A further portion of Pd/C (10 %, 0.06 g) was added and the suspension was stirred for 24 hr. A final portion of Pd/C (10 %, 0.06 g) was added, the hydrogen re-charged, and the suspension was stirred for a further 48 hr. The mixture was filtered through a pad of Celite, rinsing several times with ethyl acetate. The combined filtrates were concentrated *in vacuo* and the title product **181** was isolated by flash column chromatography on silica gel (CH_2Cl_2: MeOH, 95:5) as a colourless oil.

Yield: 0.057 g, 0.27 mmol, 47 %;

R_f: 0.6 (CH_2Cl_2: MeOH; 20:80);

NMR: δ_H (500 MHz, CD_3OD), 4.29 (2 H, m, $CO_2CH_2CH_2Si(CH_3)_3$), 3.92 (3 H, m, $NHCHCH_2OH$), 1.05 (2 H, m, $CH_2Si(CH_3)_3$), 0.04 (9 H, s, $Si(CH_3)_3$);

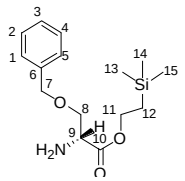
NMR: δ_C (125 MHz, CD_3OD), 170.9 (C_3), 65.6 (C_1), 62.0 (C_4), 56.5 (C_2), 18.2 (C_5), -1.40 ($C_{6, 7, 8}$);

HRMS: cald (ES^+), *m/z*: Found $[M + Na]^+$, 228.1040; $C_8H_{19}NO_3NaSi$, requires 228.1032; (ES^+), $C_8H_{19}NO_3Si$, *m/z*: 228 $[M + Na]^+$;

IR ν_{max}: 3235, 2954, 1736, 1640, cm^{-1};

$[\alpha]^{20}{}_D$ = - 41.0 (*c* 7.3 mg/mL, MeOH).

(R)-2-Amino-3-benzyloxy-propionic acid (2-trimethylsilanyl)ethyl ester 185



(*R*)-3-Benzyloxy-2-*tert*-butoxycarbonylamino-propionic acid (2-trimethylsilanyl)ethyl ester **179** (0.58 g, 1.47 mmol) was treated with a solution of TFA (1 mL), TIPS (0.04 mL) and CH_2Cl_2 (0.98 mL), and the reaction was stirred for 4.5 hr under argon. Excess CH_2Cl_2 (200 mL) was added and the organic layer was washed with saturated $NaHCO_3$ (200 mL) and brine (200 mL). The organic layer was dried over anhydrous magnesium sulphate and concentrated *in vacuo*. The title product **185** was isolated as colourless oil by flash column chromatography on silica gel (CH_2Cl_2: MeOH, 95:5).

Yield: 0.266 g, 0.91 mmol, 62 %;

R_f: 0.37 (MeOH: CH_2Cl_2; 10:90);

NMR: δ_H (500 MHz, $CDCl_3$), 7.31 (5 H, m, Ar), 4.55 (1 H, d, *J* = 12.1 Hz, ArC*H*$_2$OCH$_2$), 4.51 (1 H, d, *J* = 12.1 Hz, ArC*H*$_2$OCH$_2$), 4.21 (2 H, m, C*H*$_2$CH$_2$Si(CH$_3$)$_3$), 3.72 (1 H, dd, *J* = 9.0, 5.2 Hz, ArCH$_2$OC*H*$_2$CH), 3.69 (1 H, dd, *J* = 9.0, 3.8 Hz, ArCH$_2$OC*H*$_2$CH), 3.61 (1 H, dd, *J* = 5.0, 3.8 Hz, ArCH$_2$OCH$_2$C*H*) 0.97 (2 H, m, CH$_2$C*H*$_2$Si(CH$_3$)$_3$), 0.03 (9 H, s, Si(C*H*$_3$)$_3$);

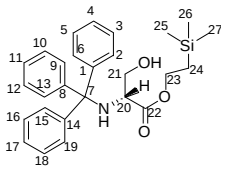
NMR: δ_C (150 MHz, $CDCl_3$), 173.9 (C_{10}), 137.9 (C_{Ar}), 128.7 (C_{Ar}), 127.9 (C_{Ar}), 127.6 (C_{Ar}), 72.70 (C_7), 71.98 (C_8), 64.02 (C_{11}), 55.24 ($C_{9,}$), 17.50 (C_{12}), -1.39 ($C_{13, 14, 15}$);

HRMS: cald (ES^+), *m/z*: Found $[M + Na]^+$, 318.15092; $C_{15}H_{25}NO_3NaSi$, requires 318.15013; (ES^+), $C_{15}H_{25}NO_3Si$, *m/z*: 318 $[M + Na]^+$;

IR ν_{max}: 3384, 2952, 1735, 1454, 1250 cm^{-1};

$[\alpha]^{20}{}_D$ = + 1.3 (*c* 15.0 mg/mL, $CHCl_3$).

(R)-3-Hydroxy-2-(triphenylmethylamino)-propionic acid (2-trimethylsilanyl)ethyl ester 182



Entry	SM	Boc deprotection / Time	Reaction solvents	Base (equiv), Temperature	TMS-Cl, (equiv), °C	Trt-Cl (equiv), °C	Reaction time, / °C	Product Isolated 182 (%)	Byproduct Isolated % 186 187 188		
1	180	TFA / TIPS / CH_2Cl_2, 4 hr	DMF	Triethylamine (1), RT	-	(1), RT	24 hr, RT	-	Observed by MS		-
2	180	TFA / TIPS / CH_2Cl_2, 2 hr	CH_2Cl_2	Triethylamine, (2), RT, MeOH, (1), 0 °C, Triethylamine, (1), RT,	(2), RT	(1), RT	Reflux, 20 min, Reflux, 45 min, 24 hr, RT	10 %	-	-	20 %
3	180	TFA / TIPS / CH_2Cl_2, 1 hr 30 min	THF	Triethylamine, (2), RT, MeOH, (1), 0 °C, Triethylamine, (1), RT,	(2), RT	(1), RT	Reflux, 20 min, Reflux, 45 min, 24 hr, RT	12 %	-	-	15 %
4	181	-	CH_2Cl_2	Triethylamine, (2), RT, MeOH, (1), 0 °C, Triethylamine, (1), RT,	(2), RT	-	Reflux, 20 min, Reflux, 45 min, 24 hr, RT	43 %	-	-	20 %

Optimised experiments

General procedure

(*R*)-2-*tert*-Butoxycarbonylamino-3-hydroxy-propionic acid (2-trimethylsilanyl)ethyl ester **180** (0.1 g, 0.32 mmol) was treated with TFA (50 %, 0.5 mL), CH_2Cl_2 (49 %, 0.49 mL) and TIPS (1 %, 0.01 mL), for the time specified in the table above. Excess toluene was added and removed *in vacuo* 3 times, followed by addition of CH_2Cl_2 and then concentration *in vacuo* (Entries 1, 2 and 3). Residue (Entry 1, 2, and 3), -Amino-3-hydroxy-propionic acid 2-trimethylsilanyl-ethyl ester **181** (Entry 4) were suspended in the appropriate solvent (Table above).

- Entry 1: triphenylmethyl chloride (1 equiv) was added followed by triethylamine (1 equiv) and the mixture was stirred for 20 hr, the reaction solvent was removed under vacuum.
- Entry 2, 3 and 4: Trimethylsilylchloride (2 equiv) was added to the suspension under argon. The mixture was refluxed for 20 min and then cooled to room temperature. Triethylamine (2 equiv) was then added after which the reaction was refluxed for 45 min. The mixture was then allowed to cool to room temperature and then cooled in an ice bath to 0 ºC. Methanol (anhydrous, 1 equiv) was then added and the mixture was allowed to reach room temperature. Triethylamine (1 equiv) was added followed by triphenylmethylchloride (1 equiv), and the reaction was stirred for a further 24 hr at room temperature.

Excess triethylamine (10 mL) and methanol (60 mL) were then added until white solid dissolved. The solvent was removed *in vacuo* yielding a mixture of yellow and white crystals which was partitioned between ethyl acetate (100 mL) and citric acid (5 % aq, w/v, 50 mL) precooled to 0 ºC. The organic layer was dried over anhydrous sodium sulphate. The product **182** was isolated as colourless oil by flash column chromatography in (CH_2Cl_2: MeOH, 98:2).

R_f: 0.28 (MeOH: CH_2Cl_2; 1:9);

NMR: δ_H (500 MHz, $CDCl_3$), 7.50 (6 H, m, Trt), 7.28 (6 H, m, Trt), 7.20 (3 H, m, Trt), 3.80 (1 H, m, TrtNHC*H*CH_2OH), 3.70 (2 H, m, $CO_2CH_2CH_2Si(CH_3)_3$), 3.51 (2 H, m, TrtNHCHCH_2OH), 0.79 (2 H, m, $CH_2Si(CH_3)_3$), 0.01 (9 H, s, $Si(CH_3)_3$);

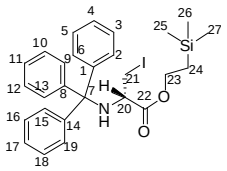
NMR: δ_C (125 MHz, $CDCl_3$), 173.7 (C_{22}), 145.8 (C_{Ar}), 128.7 (C_{Ar}), 128.0 (C_{Ar}), 126.4 (C_{Ar}), 71.1 (C_7), 64.9 (C_{21} or C_{23}), 63.5 (C_{21} or C_{23}), 57.9 (C_{20}), 17.3 (C_{24}), -1.49 ($C_{25, 26, 27}$);

HRMS: cald (ES^+), *m/z*: Found $[M + Na]^+$, 470.2115; $C_{27}H_{33}NO_3NaSi$, requires 470.2127; (ES^+), $C_{27}H_{33}NO_3NaSi$, *m/z*: 470 M + Na;

IR ν_{max}: 3465, 2952, 1726, 1597 cm^{-1};

$[\alpha]^{20}_{D}$ = - 0.6 (*c* 8.6 mg/mL, $CHCl_3$).

(S)-3-Iodo-2-(triphenylmethylamino)-propionic acid (2-trimethylsilanyl)ethyl ester 183



Entry	Reaction solvent	PPh_3 (equiv), Temperature	DEAD (equiv), / °C	MeI (equiv), / °C	Reaction time, / °C	Product Isolated 183 (%)
1	CH_2Cl_2	(1.5), RT	(1.5), -10 °C	(1.5), -10 °C	3 hr, -2 °C	8.6 %
2	CH_2Cl_2	(1.5), RT	(1.5), -10 °C	(1.5), -10 °C	6 hr, -2 °C	47 %

Optimised experiments

General procedure

(*R*)-3-Hydroxy-2-(triphenylmethylamino)-propionic acid (2-trimethylsilanyl)ethyl ester **182** was dissolved in dry CH_2Cl_2 (4 mL) at room temperature in the presence of triphenylphosphine (1.5 equiv). After 10 min, the reaction mixture was cooled to -10 ºC using an acetone/ice bath. Diethylazodicarboxylate (1.5 equiv) was then added dropwise to the reaction over 1 min. After further 5 min, this was followed by the careful addition of iodomethane (1.5 equiv). The reaction was kept at -2 ºC for the time above using an ethanol bath refrigerated by a cryostat.

The reaction mixture was directly filtered by flash column chromatography, beginning with 20: 1 hexane: EtOAc reducing to 10:1 until the removal of the title product **183** was collected as a colourless oil. A 4:1 eluant was then used to collect the starting material **182** in 50 % yield.

R_f: 0.15 (Hexane: Ethyl acetate; 15:1);
NMR: δ_H (600 MHz, $CDCl_3$), 7.50 (6 H, m, Trt), 7.33 (6 H, m, Trt), 7.18 (3 H, m, Trt), 3.78 (1 H, td, *J* = 5.8 Hz, $CO_2CH_2CH_2Si(CH_3)_3$), 3.68 (1 H, td, *J* = 5.8 Hz, $CO_2CH_2CH_2Si(CH_3)_3$),

3.43 (1 H, m, TrtNHC*H*CH$_2$I), 3.31 (1 H, dd, *J* = 9.8, 3.4 Hz, TrtNHCHC*H$_2$*I), 3.18 (1 H, dd, *J* = 9.8, 6.8 Hz, TrtNHCHC*H$_2$*I), 2.90 (1 H, d, *J* = 9.4 Hz, N*H*), 0.85 (2 H, m, C*H$_2$*Si(CH$_3$)$_3$), 0.01 (9 H, s, Si(C*H$_3$*)$_3$);

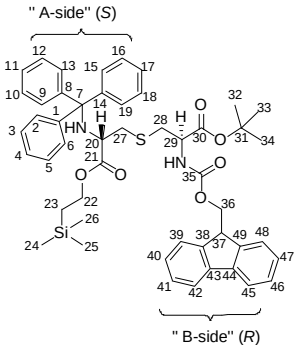
NMR: δ_C (125 MHz, $CDCl_3$), 172.4 (C_{22}), 145.7 (C_{Ar}), 128.7 (C_{Ar}), 128.1 (C_{Ar}), 126.7 (C_{Ar}), 71.2 (C_7), 63.7 (C_{23}), 56.2 (C_{20}), 14.2 (C_{24}), 10.3 (C_{21}), -1.40 ($C_{25, 26, 27}$);

HRMS: cald (ES^+), *m/z*: Found $[M + Na]^+$, 580.113105; $C_{27}H_{32}INO_2NaSi$, requires 580.11447; (ES^+), $C_{27}H_{32}INO_2Si$, *m/z*: 580 [M + Na];

IR ν_{max}: 3058, 2952, 1730, 1596, 1490, 1448 cm^{-1};

$[\alpha]^{20}{}_D$ = - 8.1 (*c* 5.2 mg/mL, $CHCl_3$).

(<u>R</u>)-2-(9H-Fluoren-9-ylmethoxycarbonylamino)-3-[(S)-2-(2-trimethylsilanyl-ethoxycarbonyl)-2-(triphenylmethylamino)-ethylsulfanyl]-propionic acid <u>tert</u>-butyl ester 184



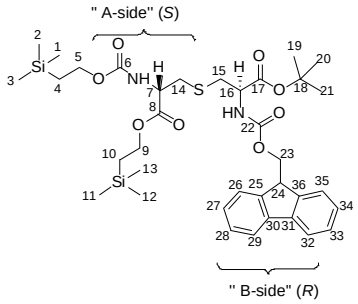
N-9-fluorenylmethoxycarbonyl-(*R*)-cystine bis-*tert*-butyl ester **90** (0.09 g, 0.24 mmol) and (*S*)-3-Iodo-2-(triphenylmethylamino)-propionic acid (2-trimethylsilanyl)ethyl ester **183** (0.13 g, 0.24 mmol, 1 equiv) were dissolved in DMF (10 mL) under argon. Dry Cs_2CO_3 (0.038 g, 0.12 mmol, 0.5 equiv) was added over 2 min and the reaction was stirred for 2 hr. Further dry Cs_2CO_3 (0.038 g, 0.12 mmol, 0.5 equiv) was added over 1 min and the reaction was stirred for a further 2 hr. Excess ethyl acetate (200 mL) was added and the mixture was washed with cold citric acid (5 % w/v aq, 100 mL) and distilled water (5 x 50 mL). The ethyl acetate layer was dried over anhydrous sodium sulphate and concentrated *in vacuo*. Purification was carried out by flash column chromatography (hexane: EtOAc, 50:1 followed by 25:1) to give the title product **184** as a white foam.

Yield: 0.078 g, 0.094 mmol, 39 %;

R_f: 0.37 (Hexane: Ethyl acetate; 4:1);

NMR: δ_H (500 MHz, $CDCl_3$), 7.77-7.15 (23 H, m, Ar), 5.66 (1 H, d, *J* = 7.9 Hz, N*H*Fmoc), 5.60 (1 H, d, *J* = 7.4 Hz, N*H*Trt), 4.50 (1 H, m, H_α B side), 4.36 (2 H, m, CH_2Fmoc), 4.24 (1 H, t, *J* = 7.0 Hz, C*H*Fmoc), 3.72 (2 H, m, C$H_2$$CH_2$Si), 3.61 (2 H, m, C$H_2$$CH_2$Si), 3.51 (1 H, m, H_α A side), 3.00 (4 H, CH_2 β A and B side), 1.48 (9 H, s, *t*Bu), 0.77 (2 H, m, CH_2CH_2Si), 0.00 (9 H, s, Si(CH_3)$_3$;
HRMS: cald (ES^+), *m/z*: Found $[M + Na]^+$, 851.3513; $C_{49}H_{56}N_2O_6NaSiS$, requires 851.3526; (ES^+), $C_{49}H_{56}N_2O_6NaSiS$, *m/z*: 851 $[M + Na]^+$.

(<u>R</u>)-2-(9H-Fluoren-9-ylmethoxycarbonylamino)-3-[2-(2-trimethylsilanyl-ethoxycarbonyl)-(<u>S</u>)-2-(2-trimethylsilanyl-ethoxycarbonylamino)-ethylsulfanyl]-propionic acid <u>tert</u>-butyl ester 176



(*R*)-2-(9H-Fluoren-9-ylmethoxycarbonylamino)-3-[(*S*)-2-(2-trimethylsilanyl-ethoxycarbonyl)-2-(triphenylmethylamino)-ethylsulfanyl]-propionic acid *tert*-butyl ester **184** (0.08 g, 0.09 mmol) was treated with TFA (10 %, 0.2 mL), triethylsilane (TES) (5 %, 0.1 mL and CH_2Cl_2 (85 %, 1.7 mL), the mixture was stirred under argon for 4 hr. Excess CH_2Cl_2 (100 mL) was added and the organic layer was washed with saturated sodium bicarbonate (50 mL) and brine (50 mL). The organic layer was dried over anhydrous sodium sulphate, filtered and concentrated *in vacuo*. The residue was dissolved in 1,4-dioxane (2 mL) and then a solution of sodium bicarbonate (0.04 g, 0.47 mmol, 4 equiv) in water (2 mL) was added to the mixture. The resulting mixture was cooled to 0 ºC, Carbonic acid 2,5-dioxo-pyrrolidin-1-yl ester 2-trimethylsilanyl-ethyl ester **171** (0.07 g, 0.026 mmol, 2 equiv) was added. The reaction was left in the freezer for 24 hr and then stirred at 0 ºC to room temperature for 5 hr. Excess CH_2Cl_2 (100 mL) was added and the organic layer was washed with saturated sodium bicarbonate (50 mL) and brine (50 mL). The organic layer was dried over anhydrous sodium sulphate, concentrated *in vacuo*, and purified by flash column chromatography (hexane: EtOAc, 10:1 and then 3:1). This was followed by purification by preparative HPLC to give **176** as colourless oil.

Yield: 0.008 g, 0.011 mmol, 12 %;

R_f: 0.18 (Hexane: Ethyl acetate; 4:1);

NMR: δ_H (500 MHz, $CDCl_3$), 7.91-7.28 (8 H, m, Ar), 5.81 (1 H, d, *J* = 7.3 Hz, N*H*Fmoc), 5.54 (1 H, d, *J* = 7.8 Hz, N*H*Teoc), 4.54 (2 H, m, H_α B side and C*H*Fmoc), 4.35 (2 H, d, *J* = 7.0 Hz, CH_2Fmoc), 4.24 (1 H, m, H_α A side), 4.20 (4 H, m, 2 x C$H_2$$CH_2Si(CH_3)_3$), 3.12 (4 H, m, C$H_2$ β A and B side), 1.47 (9 H, s, *t*Bu), 0.99 (4 H, m, 2 x CH_2TMS), 0.09 (18 H, s, 2 x $Si(CH_3)_3$), (Appendix 11);

NMR: δ_C (150 MHz, $CDCl_3$), 170.8 (C_{17}), 168 (C_8), 156.3 (C_6), 156.0 (C_{22}), 143.8 (C_{Ar}), 141.0 (C_{Ar}), 127.8 (C_{Ar}), 127.2 (C_{Ar}), 125.3 (C_{Ar}), 83.2 (C_{18}), 67.4 (C_{23}), 64.5 (C_5 or $_9$), 163.7 C_5 or $_9$), 54.5 (C_{16}), 54.0 (C_7), 47.2 (C_{24}), 37.4 (C_{14} or $_{15}$), 36.1 (C_{14} or $_{15}$), 28.1 ($C_{19,\ 20,\ 21}$), 17.7 (C_4 or $_{10}$), 17.5 (C_4 or $_{10}$), -1.44 ($C_{1,\ 2,\ 3,\ 11,\ 12,\ 13}$);

HRMS: cald (ES^+), *m/z*: Found $[M + Na]^+$, 753.3052; $C_{36}H_{54}N_2O_8NaSi_2S$, requires 753.3037; ($ES^+$), $C_{36}H_{54}N_2O_8Si_2S$, *m/z*: 769 $[M + K]^+$;

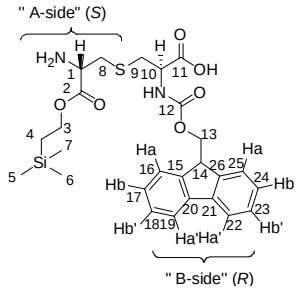
IR ν_{max}: 2953, 1720, 1511, 1340 cm^{-1};

$[\alpha]^{20}{}_D$ = - 9.3 (*c* 6.0 mg/mL, $CHCl_3$);

Analytical HPLC: (Wide Pore C18), **R_T** 26.7; A: H_2O/0.5 % TFA; B: isopropanol /0.5% TFA; gradient 10 % - 90 % B over 35 min (Appendix 9);

Preparative HPLC: (Wide Pore C18), in H_2O/0.5 % TFA and isopropanol/0.5 % TFA at a gradient of 10 % - 90 % isopropanol over 35 min to give the title product which eluted at 27.5 min (Appendix 10).

(S)-3-[2-Amino-2-(2-trimethylsilanyl-ethoxycarbonyl)-ethylsulfanyl]-(R)-2-(9H-fluoren-9-ylmethoxycarbonylamino)-propionic acid 189



Condition 1

(*R*)-2-(9H-Fluoren-9-ylmethoxycarbonylamino)-3-[2-(2-trimethylsilanyl-ethoxycarbonyl)-(*S*)-2-(2-trimethylsilanyl-ethoxycarbonylamino)-ethylsulfanyl]-propionic acid *tert*-butyl ester

176 (0.26 g, 0.36 mmol) was treated with a solution of TFA (1 mL) and CH_2Cl_2 (1 mL), the resulting mixture was stirred for 3 hr at room temperature. The mixture was concentrated *in vacuo*, removal of TFA was helped by several additions of toluene (3 x 100 mL) followed by evaporation. Purification was completed by reverse-phase column chromatography in acetonitrile and saturated $NaHCO_3$, gradient 0 % - 40 % actonitrile in 100 % - 60 % (20 % $NaHCO_3$) saturated $NaHCO_3$. Acetonitrile was removed under vacuum. Concentrated sulphuric acid was added until saturation was reached and the product was extracted from the aqueous layer with chloroform. The organic layer was then dried over anhydrous sodium sulphate and concentrated *in vacuo* to give the title product **189** as colourless oil.

Yield: 0.087 g, 0.16 mmol, 42 %;

R_f : 0.01 [AcCN (40 %): Sat $NaHCO_3$] reverse phase TLC;

NMR: δ_H (600 MHz, CD_3OD), 7.79 (2 H, d, *J* = 7.6 Hz, Ar), 7.67 (2 H, d, *J* = 7.4 Hz, Ar), 7.39 (2 H, t, *J* = 7.4 Hz, Ar*)*, 7.31 (2 H, t, *J* = 7.4, Hz, Ar), 4.42 (1 H, m, C*H*$_2$Fmoc), 4.29 (1 H, m, C*H*$_2$Fmoc), 4.23 (4 H, m, C*H*Fmoc, H_α B side and C*H*$_2$$CH_2$Si), 3.92 (1 H, m, H_α A side), 3.08 (2 H, m, C*H*$_2$ *β*), 2.93 (2 H, m, C*H*$_2$ *β*), 1.00 (2 H, m, CH_2C*H*$_2$Si$(CH_3)_3$), 0.02 (9 H, s, Si(C*H*$_3$)$_3$);

NMR: δ_C (150 MHz, CD_3OD), 180 (C_{11}), 163.3 (C_2), 158.4 (C_{12}), 145.6 (C_{Ar}), 142.5 (C_{Ar}), 128.8 (C_{Ar}), 128.2 (C_{Ar}), 126.2 (C_{Ar}), 68.0 (C_{13}), 65.6 (C_3), 57.3 (C_{14}), 54.5 (C_1), 47.9 (C_{10}), 39.0 ($C_{8, 9}$), 18.3 (C_4), -1.42 ($C_{5, 6, 7}$);

HRMS: cald (ES^+), *m/z*: Found $[M + Na]^+$, 553.1805; $C_{26}H_{34}N_2O_6NaSiS$, requires 553.1805; (ES^+), $C_{26}H_{34}N_2O_6NaSiS$, *m/z*: 553 $[M + Na]^+$;

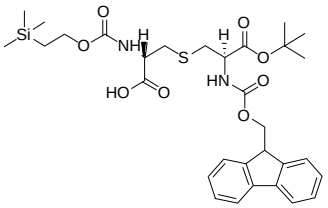
IR ν_{max}: 3355, 1690, 1410 cm^{-1};

$[\alpha]^{20}{}_D$ = - 4.4 (*c* 5.5, MeOH).

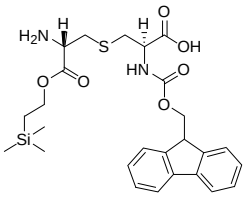
Condition 2

(*R*)-2-(9H-Fluoren-9-ylmethoxycarbonylamino)-3-[2-(2-trimethylsilanyl-ethoxycarbonyl)-(*S*)-2-(2-trimethylsilanyl-ethoxycarbonylamino)-ethylsulfanyl]-propionic acid *tert*-butyl ester **176** (0.029 g, 0.040 mmol) was treated with a solution of TFA (0.3 mL) and deuteriated CH_2Cl_2 (0.7 mL), the mixture was stirred for 3 hr at room temperature. Proton NMRs were taken when the solution was added to the starting material (Appendix 12), then at 10 min (Appendix 13), 1 hr (Appendix 14), 2 hr (Appendix 15) and 3 hr (Appendix 16). The product

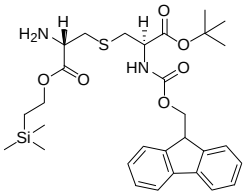
was extracted from the aqueous layer with chloroform (50 mL) washed with water (8 x 25 mL) followed by brine (25 mL), isolating 3 products as crude in 4.1 mgs.



HRMS: cald (ES^+), *m/z*: Found $[M + Na]^+$, 653.2329; $C_{31}H_{42}N_2O_8NaSiS$, requires 653.2329; (ES^+), $C_{31}H_{42}N_2O_8SiS$, *m/z*: 653 $[M + Na]^+$.

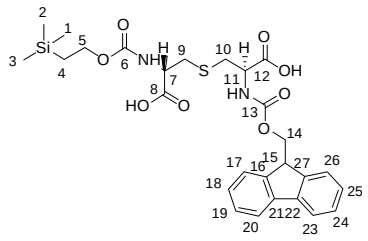


(ES^+), $C_{26}H_{34}N_2O_6SiS$, *m/z*: 553 $[M + Na]^+$.



NMR: δ_H (500 MHz, CD_3OD), 7.93 (2 H, m, Ar), 7.71 (2 H, d, *J* = 7.5 Hz, Ar), 7.37 (2 H, t, *J* = 7.4 Hz, Ar*)*, 7.30 (2 H, t, *J* = 7.3 Hz, Ar), 4.36 (1 H, m, H_α B side), 4.23 (5 H, m, CH_2Fmoc, C*H*Fmoc and CH_2CH$_2$Si), 3.82 (1 H, m, H_α A side), 3.11-2.82 (4 H, m, CH_2 β A and B sides), 1.35 (9 H, s, *t*Bu), 1.03 (2 H, m, CH$_2$CH_2Si(CH$_3$)$_3$), 0.00 (9 H, s, Si(CH_3)$_3$); (ES^+), $C_{30}H_{42}N_2O_6SiS$, *m/z*: 609 $[M + Na]^+$.

(S)-3-[2-Carboxy-2-(2-trimethylsilanyl-ethoxycarbonylamino)-ethylsulfanyl]-(R)-2-(9H-fluoren-9-ylmethoxycarbonylamino)-propionic acid 190



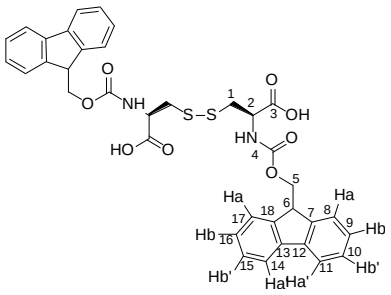
(*R*)-2-(9H-Fluoren-9-ylmethoxycarbonylamino)-3-[2-(2-trimethylsilanyl-ethoxycarbonyl)-(*S*)-2-(2-trimethylsilanyl-ethoxycarbonylamino)-ethylsulfanyl]-propionic acid *tert*-butyl ester **176** (0.26 g, 0.36 mmol) was treated with a solution of TFA (1 mL) and CH_2Cl_2 (1 mL), the resulting mixture was stirred for 3 hr. The mixture was concentrated *in vacuo*, removal of TFA was helped by several additions of toluene (3 x 100 mL) followed by evaporation. Purification was completed by reverse-phase column chromatography in acetonitrile and saturated $NaHCO_3$, gradient 0 % - 40 % actonitrile in 100 % - 60 % (20 % $NaHCO_3$) saturated $NaHCO_3$. Acetonitrile was removed under vacuum. Concentrated sulphuric acid was added until saturation was reached and the product was extracted from the aqueous layer with chloroform. The organic layer was then dried over anhydrous sodium sulphate and concentrated *in vacuo* to give the title product **190** as yellow foam.

NMR: δ_H (600 MHz, CD_3OD), 7.83 (2 H, m, Ar), 7.67 (3 H, m, Ar), 7.42 (2 H, m, Ar*)*, 7.35 (1 H, m, Ar), 4.21-4.42 (7 H, m, C*H*$_2$Fmoc, C*H*Fmoc and H_α B side, H_α A side and C*H*$_2$CH$_2$Si), 3.15 (2 H, m, C*H*$_2$ β), 2.94 (2 H, m, C*H*$_2$ β), 1.09 (2 H, m, $CH_2CH_2Si(CH_3)_3$), 0.02 (9 H, s, $Si(CH_3)_3$);

NMR: δ_C (150 MHz, CD_3OD), 169.2 ($C_{8,\ 12}$), 158.7 ($C_{6,\ 13}$), 145.2 (C_{Ar}), 142.3 (C_{Ar}), 128.8 (C_{Ar}), 128.7 (C_{Ar}), 126.2 (C_{Ar}), 68.2 (C_{14}), 66.5 ($C_{7,\ 11}$), 53.6 (C_5), 37.7 (C_{15}), 33.3 ($C_{9,\ 10}$), 18.2 (C_4), -1.65 ($C_{1,\ 2,\ 3}$);

HRMS: cald (ES^+), *m/z*: Found $[M + H]^+$, 575.1883; $C_{27}H_{35}N_2O_8SiS$, requires 575.1862; (ES^+), $C_{27}H_{34}N_2O_8SiS$, *m/z*: 575 $[M + H]^+$.

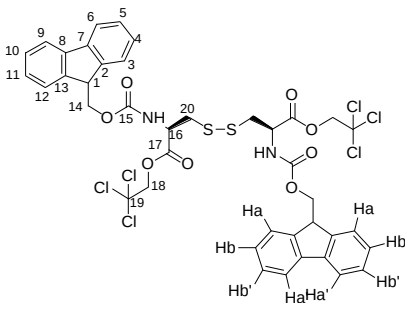
(Fmoc-Cys-OH)$_2$ 191



N',*N*'-Bis(9-fluorenylmethoxycarbonyl)-(*R*,*R*)-cystine bis-*tert*-butyl ester **146** (3.00 g, 3.7 mmol) was treated with a solution of TFA (6 mL) and CH_2Cl_2 (1 mL), the mixture was stirred for 3 hr. The product was concentrated *in vacuo* and left in a freeze dryer overnight. Yellow fluffy foam was isolated.

R_f: 0.11 (CH_2Cl_2: MeOH; 15:1);
NMR: δ_H (600 MHz, CD_3OD), 7.76 (4 H, m, Ar), 7.65 (4 H, d, *J* = 7.5 Hz, Ar), 7.35 (4 H, m Ar), 7.28 (4 H, t, *J* = 7.4 Hz, Ar), 4.54 (2 H, m, H_α A and B side) 4.29 (4 H, m, CHCH_2Fmoc), 4.19 (2 H, t, *J* = 7.2 Hz, C*H*CH_2Fmoc), 3.28 (2 H, d, *J* = 4.2 Hz, CH_2 β), 3.02 (2 H, dd, *J* = 13.8, 4.4 Hz, CH_2 β);
NMR: δ_C (150 MHz, CD_3OD), 174.0 (C_3), 158.5 (C_4), 145.2 (C_{Ar}), 142.5 (C_{Ar}), 128.7 (C_{Ar}), 128.2 (C_{Ar}), 126.3 (C_{Ar}), 68.2 (C_5), 54.6 (C_2), 48.3 (C_6), 41.0 (C_1);
HRMS: cald (ES^-), *m/z*: Found $[M - H]^-$, 683.1476; $C_{36}H_{31}N_2O_8S_2$, requires 683.1522;
(ES^-), $C_{36}H_{32}N_2O_8S_2$, *m/z*: 683 $[M - H]^-$;
IR ν_{max}: 3390, 3010, 2159, 1720, 1524 cm^{-1};
$[\alpha]^{25}{}_D$ = + -79.6 (*c* 8.9 mg/mL, MeOH).

(Fmoc-Cys-OTce)$_2$ 192



Entry	Compound 144	146	191	Tertbutyl deprotection / Time	Fmoc protection reagents, (equiv) / Time	Reaction solvent	Reagents (equiv), Temperature	TceOH 196 (equiv), °C	Reaction time / temperature	Product Isolated 192 (%)
1	-	X	-	TFA/ CH_2Cl_2, 4 hr	-	CH_2Cl_2	DMAP (0.4), RT, DCC (2.2), 0 °C, DMAP (0.4), 0 °C	(2.2), RT, (2.2), RT	0 °C - RT, 24 hr, RT, 24 hr, RT	18 %
2	X	-	-	-	NaOH (4.2), THF – H_2O, Fmoc-Cl (2.1), RT, 14 hr	Pyridine	HOBt (2.9), RT, DCC (2.3), -20 °C	(3.45), RT	- 20 °C, 24 hr	2.7 %
3	-	X	-	TFA/ CH_2Cl_2, 4 hr	-	CH_2Cl_2	DMAP (0.4), RT, DCC (6), 0 °C, DMAP (0.4), RT	(3), RT, (3), RT	0 °C – RT, 24 hr, RT, 24 hr, RT	25.3 %
4	-	X	-	TFA/ CH_2Cl_2, 4 hr	-	Pyridine	HOBt (9), RT, DCC (9.9), -20 °C	(11.6), RT	- 20 °C, 24 hr	44.4 %
5	-	-	X	-	-	Pyridine	HOBt (8.4), RT, DCC (8.4), -20 °C, 2 x HOBt (8.4), - 20 °C, DCC (8.4), -20 °C	(9.35), RT, (2 x 9.35), -20 °C,	- 20 °C, 24 hr, - 20 °C, 2 x 24 hr	17.7 %
6	-	-	X	-	-	CH_2Cl_2	DMAP (0.47), RT DCC (8.4), 0 °C, 2 x DMAP (0.47), RT, DCC (8.4)	(9.35), RT, (2 x 9.35), RT	0 °C – RT, 24 hr, RT, 2 x 24 hr, RT	26 %
7	-	-	X	-	-	CH_2Cl_2	DMAP (0.47), RT, EDCI-polymer (2), 0 °C, DMAP (0.24), RT, EDCI-polymer (2), RT	(9.35), RT, (9.35), RT	0 °C, 48 hr, RT 72 hr, RT	3.9 %
8	-	-	X	-	-	CH_2Cl_2	DMAP (0.4), 0 °C, EDCI (2.2), 0 °C, DMAP (0.4), RT	(4), RT, (4), RT	0 °C, 24 hr, RT, 24 h at RT	NR *
9	-	-	X	-	-	DMF	*N*-hydroxy Succinimide (2), RT, EDCI.HCl (2), RT,	(4), RT, (4), RT	24 hr, RT, 24 h at RT	32.7 %

							N-hydroxy Succinimide (2), RT, EDCI.HCl (2), RT			
10	-	-	X	-	-	Toluene	Toluene-4-Sulphonic acid (2.4), RT	(2.4), RT, (2.4), 140 °C	140 °C, 5 hr, 140 °C, 24 hr	60 %
11	-	-	X	-	-	Benzene	Toluene-4-Sulphonic acid (2.4), RT	(2.4), RT, (2.4), 80 - 105 °C	80 - 105 °C, 5 hr, 80 - 105 °C, 5 hr	87.4 %

* NR: No reaction

Optimised experiments

General procedure

Entry 1, 3, 4, 5, 6, 7, 8 and 9

N',*N'*-bis(fluorenylmethoxycarbonyl)-(R,R)-cystine-bis-*tert*-butyl ester **146** (Entry 1, 3 and 4) was treated with a solution of trifluroacetic acid (TFA) (1 mL) and CH_2Cl_2 (1 mL), the mixture was stirred for 4 hr at room temperature. The solvent was reduced *in vacuo* to give fluffy cream solid. Residue (Entry 1, 3, 4), 3-[2-carboxy-2-(9H-fluoren-9-ylmethoxycarbonylamino)-ethyldisulfanyl]-2-(9H-fluoren-9-ylmethoxycarbonylamino) propionic acid $(Fmoc\text{-}Cys\text{-}OH)_2$ **191** (Entry 5, 6, 7, 8 and 9) was dissolved at room temperature in the appropriate solution (Table above) containing 2,2,2-trichloroethanol **196** and the correct reagent (DMAP or HOBt) in the appropriate solvent. The temperature of the reaction was attained according to the table above this was followed by the addition of the coupling reagent (DCC, EDC-polymer bound, EDCI or EDCI.HCl). The number of equivalents of the reagents, time and temperature of the reaction, and the addition of further reagents were maintained and carried out as stated in the table above. After the reaction time was complete the white precipitate of DCU was removed by filtration and the filtrate was concentrated *in vacuo* (Entry 1, 3, 4, 5, 6 and 7). An extraction was then carried out.

Entry 1, 3, 6

The residue was taken up in organic solvent (200 mL) Entry 1 and 3 in chloroform, Entry 6 in EtOAc, the organic layer was washed with 10 % citric acid (2 x 50 mL) and then brine (2 x 50 mL).

Entry 4 and 5

The residue was diluted with Ethyl acetate (100 mL) and the organic phase was washed with 1 M aqueous HCl (100 mL) and then brine (50 mL).

Entry 7, 8 and 9

Excess CH_2Cl_2 (100 mL) was added and the organic layer was washed with H_2O (5 x 50 mL) and then brine (50 mL).

Drying and filtration of each entries followed by solvent removal gave white solid, this was purified by flash column chromatography (hexane: EtOAc 9:1) giving the title product **192** as white solid.

Entry 2

A solution of *L*-cystine **144** (2.5 g, 10.4 mmols) and NaOH (1.76 g, 44 mmol) in 300 mL of THF – H_2O (1: 1) was treated with FmocCl (5.7 g, 22.1 mmol, 2.1 equiv) and the reaction mixture was stirred at 25 ºC for 14 hr. The reaction mixture was diluted with water (250 mL) and washed with $CHCl_3$ (3 x 250 mL). The aqueous phase was acidified with the addition of 6 M aqueous HCl (250 mL) and extracted with $CHCl_3$. The combined organic phases were dried over anhydrous sodium sulphate, filtered and concentrated *in vacuo*. The residue was dissolved in pyridine (100 mL), HOBt (4.2 g, 31.07 mmols, 2.9 equiv) and 2,2,2-trichloroethanol (3.45 mL, 35.9 mmols, 3.45 equiv) were added. The mixture was cooled to -20 ºC and treated with DCC (5 g, 24.2 mmols, 2.3 equiv). The resulting mixture was stirred at - 20 ºC under argon for 24 hr. The white precipitate of DCU was removed by filtration and the filtrate was concentrated *in vacuo*. The residue was diluted with ethyl acetate (500 mL) and the organic phase was washed with 1 M aqueous HCl (500 mL), saturated aqueous $NaHCO_3$ (500 mL) and brine (250 mL). The organic phase was dried, filtered and concentrated *in vacuo*. Purification by flash column chromatography on silica gel (hexane: EtOAc, 80:20) afforded the title product **192** as a white solid.

Entry 10 and 11 [156]

2,2,2-trichloroethanol **196** was added to a solution containing the commercially available $(Fmoc\text{-}Cys\text{-}OH)_2$ **191** and toluene-4-sulphonic acid (2.4 equiv) in the correct solvent (315 mL). The reaction was heated under reflux with a Dean-Stark trap attached for 24 hr. [157] The temperature of the reaction and the equivalents of 2,2,2-trichloroethanol **196** were maintained and used as reported in the table above. After 24 hr the reaction was cooled to 0 ºC and any unreacted acid was removed by filtration. The reaction solvent was removed *in vacuo* and the

residue was dissolved in chloroform (500 mL) and washed with H_2O (4 x 150 mL) and brine (150 mL). The crude product was purified by flash column chromatography (hexane: EtOAc 9:1 followed by CH_2Cl_2: MeOH 98:2) to give the title product **192** as a white powder, identical by NMR with the literature. [156]

R_f: 0.6 (Hexane: Ethyl acetate; 2:1);

NMR: δ_H (600 MHz, $CDCl_3$), 7.75 (4 H, d, *J* = 7.4 Hz, Ar), 7.58 (4 H, d, *J* = 7.2 Hz, Ar), 7.39 (4 H, t, *J* = 7.3 Hz, Ar*)*, 7.29 (4 H, t, *J* = 7.3 Hz, Ar), 5.72 (2 H, d, *J* = 8.0 Hz, N*H*), 4.83 (2 H, m, C*H*CH_2S), 4.79 (2 H, d, *J* = 11.8 Hz, CH_2Cl_3), 4.74 (2 H, d, *J* = 11.8 Hz, CH_2Cl_3), 4.41 (4 H, m, CHCH_2Fmoc), 4.21 (2 H, t, *J* = 6.8 Hz, C*H*CH_2Fmoc), 3.26 (4 H, m, CHCH_2S);

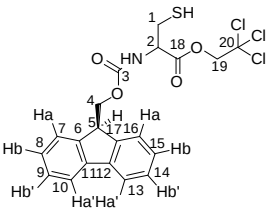
NMR: δ_C (150 MHz, $CDCl_3$), 169.1 (C_{17}), 155.7 (C_{15}), 143.7 (C_{Ar}), 141.4 (C_{Ar}), 127.9 (C_{Ar}), 127.2 (C_{Ar}), 125.2 (C_{Ar}), 120.2 (C_{Ar}), 94.3 (C_{19}), 74.9 (C_{18}), 67.5 (C_{14}), 53.5 (C_{16}), 47.1 (C_1), 40.8 (C_{20});

HRMS: cald (ES^+), *m/z*: Found $[M + Na]^+$, 966.9785; $C_{40}H_{34}N_2O_8NaS_2Cl_6$, requires 966.9745; ($ES^+$), $C_{40}H_{34}N_2O_8S_2Cl_6$, *m/z*: 969 $[M + Na]^+$;

IR ν_{max}: 2970, 1770, 1580, 1447 cm^{-1};

$[\alpha]^{20}{}_D$ = - 10.7 (*c* 1.4 mg/mL, $CHCl_3$).

Fmoc-Cys-OTce 193 [156]



$(Fmoc\text{-}Cys\text{-}OTce)_2$ **192** (0.061 g, 0.064 mmol) in THF was treated with triphenylphosphine (0.075 g, 0.28 mmol, 4.4 equiv), 2-mercaptoethanol (0.022 mL, 0.31 mmol, 4.8 equiv) and water (0.011 mL, 0.6 mmol, 10 equiv), the reaction mixture was stirred at 50 ºC for 5 hr before being concentrated *in vacuo*. Purification by flash column chromatography (hexane: EtOAc, 9:1) afforded the title product **193** as a white solid, identical by NMR with the literature. [156]

Yield: 0.060 g, 0.12 mmol, 98 %;

R_f: 0.23 (Hexane: Ethyl acetate; 9:1);

NMR: δ_H (600 MHz, $CDCl_3$), 7.77 (2 H, d, *J* = 7.6 Hz, Ar), 7.61 (2 H, d, *J* = 7.4 Hz, Ar), 7.41 (2 H, t, *J* = 7.4 Hz, Ar*)*, 7.33 (2 H, m, Ar), 5.72 (1 H, d, *J* = 8.0 Hz, N*H*), 4.92 (1 H, d, *J* = 11.9 Hz, CH_2Cl_3), 4.83 (1 H, m, C*H*CH_2S), 4.72 (1 H, d, *J* = 11.9 Hz, CH_2CCl_3), 4.45 (2 H, d, *J* = 6.9 Hz, CHCH_2OFmoc), 4.24 (1 H, t, *J* = 6.9 Hz, C*H*CH_2OFmoc), 3.14 (1 H, m, CHCH_2S), 3.03 (1 H, m, CHCH_2S), 1.46 (1 H, t, *J* = 9.1 Hz, S*H*);

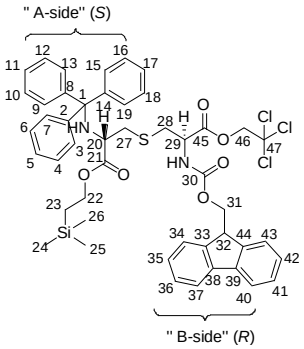
NMR: δ_C (150 MHz, $CDCl_3$), 168.8 (C_{18}), 155.7 (C_3), 143.8 (C_{Ar}), 141.4 (C_{Ar}), 127.9 (C_{Ar}), 127.2 (C_{Ar}), 125.2 (C_{Ar}), 120.2 (C_{Ar}), 94.3 (C_{20}), 74.7 (C_{19}), 67.2 (C_4), 55.5 (C_2), 47.2 (C_5), 27.5 (C_1);

HRMS: cald (ES^+), *m/z*: Found $[M + Na]^+$, 495.99270; $C_{20}H_{18}NO_4NaSCl_3$, requires 495.99198; (ES^+), $C_{20}H_{18}NO_4SCl_3$, *m/z*: 496 $[M + Na]^+$;

IR ν_{max}: 2953, 1733, 1454 cm^{-1};

$[\alpha]^{20}{}_D$ = - 2.4 (*c* 17.3 mg/mL, $CHCl_3$).

(<u>R</u>)-2-(9H-Fluoren-9-ylmethoxycarbonylamino)-3-(<u>S</u>)-[2-(2-trimethylsilanyl-ethoxycarbonyl)-2-(trityl-amino)-ethylsulfanyl]-propionic acid 2,2,2-trichloro-ethyl ester 194



Fmoc-Cys-OTce **193** (0.077 g, 0.16 mmol) and (*S*)-3-iodo-2-(triphenylmethylamino)-propionic acid (2-trimethylsilanyl)ethyl ester **183** (0.091 g, 0.16 mmol, 1 equiv) were dissolved in DMF 93 mL) under argon. Dry Cs_2CO_3 (0.003 g, 0.079 mmol, 0.5 equiv) was added over 2 min and the reaction was stirred for 2 hr. Further dry Cs_2CO_3 (0.003 g, 0.079 mmol, 0.5 equiv) was added over 1 min and the reaction was stirred for a further 2 hr. Excess ethyl acetate (200 mL) was added and the mixture was washed with cold citric acid (5 %, 200 mL) and distilled water (8 x 100 mL). The organic layer was dried over anhydrous sodium sulphate and concentrated *in vacuo*. Purification was carried out by flash column chromatography (hexane: EtOAc, 15:1) to give the title product **194** as a white fluffy solid.

M. pt. 54 ºC;

Yield: 0.086 g, 0.095 mmol, 58 %;

R_f: 0.5 (Hexane: Ethyl acetate; 4:1);

NMR:δ_H (600 MHz, $CDCl_3$), 7.77 (2 H, d, *J* = 7.5 Hz, Ar), 7.62 (2 H, t, *J* = 8.1 Hz, Ar), 7.49 (6 H, m, Ar), 7.40 (2 H, m, Ar), 7.32-7.21 (8 H, m, Ar), 7.17 (3 H, m, Ar), 5.72 (1 H, d, *J* = 8.3 Hz, N*H*Fmoc), 4.83 (1 H, d, *J* = 11.9 Hz, CH_2CCl$_3$), 4.75 (1 H, m, H_α B side), 4.71 (1 H, d, *J* = 11.9 Hz, CH_2CCl$_3$), 4.39 (2 H, m, CH_2Fmoc), 4.23 (2 H, t, *J* = 7.2 Hz, C*H*Fmoc), 3.74 (1 H, m, CH_2CH$_2$Si(CH$_3$)$_3$), 3.59 (1 H, m, CH_2CH$_2$Si(CH$_3$)$_3$), 3.50 (1 H, m, H_α A side), 3.12 (1 H, dd, *J* = 13.9, 4.6 Hz, H_β), 3.00 (1 H, dd, *J* = 13.9, 6.4 Hz, H_β), 2.91 (1 H, dd, *J* = 13.4, 4.6 Hz, H_β), 2.8 (1 H, m, N*H*Trt), 2.74 (1 H, dd, *J* = 13.9, 7.7 Hz, H_β), 0.76 (2 H, m, CH$_2$CH_2Si(CH$_3$)$_3$, -0.01 (9 H, s, Si(CH_3)$_3$;

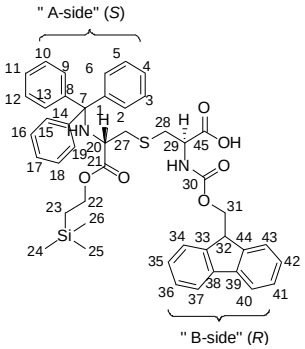
NMR: δ_C (150 MHz, $CDCl_3$), 173.6 (C_{21} or $_{45}$), 169.4 (C_{21} or $_{45}$), 155.9 (C_{30}), 145.7 (C_{Ar}), 143.8 (C_{Ar}), 141.4 (C_{Ar}), 128.9 (C_{Ar}), 128.2 (C_{Ar}), 128.1 (C_{Ar}), 127.2 (C_{Ar}), 126.7 (C_{Ar}), 125.3 (C_{Ar}), 120.1 (C_{Ar}), 94.4 (C_{47}), 74.7 (C_{46}), 71.3 (C_1), 67.6 (C_{31}), 63.5 (C_{22}), 56.4 (C_{20}), 53.7 (C_{29}), 47.2 (C_{32}), 37.74 (C_{27}), 34.69 (C_{28}), 17.5 (C_{23}), -1.4 ($C_{24, 25, 26}$);

HRMS: cald (ES^+), *m/z*: Found $[M + Na]^+$, 925.2056; $C_{47}H_{49}N_2O_6NaSiSCl_3$, requires 925.2044; ($ES^+$), $C_{47}H_{49}N_2O_6SiSCl_3$, *m/z*: 925 $[M + Na]^+$;

IR ν_{max}: 3325, 2953, 1764, 1718, 1600, 1512, 1448 cm^{-1};

$[\alpha]^{20}{}_D$ = - 32.9 (*c* 10.0 mg/mL, $CHCl_3$).

2-(9H-Fluoren-9-ylmethoxycarbonylamino)-3-[2-(2-trimethylsilanyl-ethoxycarbonyl)-2-(trityl-amino)-ethylsulfanyl]-propionic acid 197



Zinc dust (0.086 g, 0.504 mmol, 36 equiv) was added portionwise to a solution of (*R*)-2-(9H-Fluoren-9-ylmethoxycarbonylamino)-3-(*S*)-[2-(2-trimethylsilanyl-ethoxycarbonyl)-2-(trityl-amino)-ethylsulfanyl]-propionic acid 2,2,2-trichloro-ethyl ester **194** (0.033 g, 0.037 mmol) in

THF (1.6 mL), followed by the addition of 1 M aqueous NH_4OAc solution (0.28 mL). The mixture was stirred vigorously under nitrogen for 24 hr at room temperature. After filtration and solvent removal the residue was taken up in chloroform (50 mL) and washed with saturated aqueous NaCl solution (2 x 20 mL). The organic layer was dried over anhydrous magnesium sulphate, after drying and filtration followed by solvent removal, the crude was obtained as oil. This was purified by flash column chromatography (gradient, CH_2Cl_2: MeOH, 98:2 to 95:5) giving the title product **197** as oil.

Yield: 0.006 g, 7.7^{-03} mmol, 21 %;

R_f: 0.61 (CH_2Cl_2: MeOH; 90:10);

NMR: δ_H (600 MHz, CD_3OD), 7.77 (2 H, d, *J* = 7.4 Hz, Ar), 7.64 (2 H, dd, *J* = 12.0, 6.4 Hz, Ar), 7.47 (5 H, d, *J* = 7.6 Hz, Ar), 7.38 (3 H, m, Ar), 7.30-7.21 (8 H, m, Ar), 7.15 (3 H, t, *J* = 7.26 Hz, Ar), 5.15 (1 H, bs, N*H*Fmoc), 4.63 (1 H, bs, N*H*Trt), 4.35 (2 H, m, C*H*$_2$Fmoc), 4.24 (2 H, m, C*H*Fmoc and H_α B side), 3.70 (1 H, m, C*H*$_2$$CH_2Si(CH_3)_3$), 3.59 (1 H, m, C*H*$_2$$CH_2Si(CH_3)_3$), 3.45 (1 H, m, H_α A side), 3.05 (1 H, dd, *J* = 13.2, 4.8 Hz, H_β), 2.91 (1 H, dd, *J* = 14.4, 6.0 Hz, H_β), 2.82 (1 H, dd, *J* = 15.0, 9.0 Hz, H_β), 2.74 (1 H, dd, *J* = 13.8, 7.2 Hz, H_β), 0.75 (2 H, m, CH_2C*H*$_2$$Si(CH_3)_3$, -0.03 (9 H, s, $Si(CH_3)_3$;

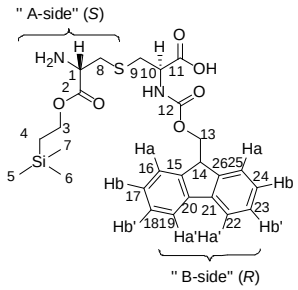
NMR: δ_C (150 MHz, CD_3OD), 171.6 (C_{45}), 172.9 (C_{21}), 158.3 (C_{30}), 147.2 (C_{Ar}), 145.3 (C_{Ar}), 142.5 (C_{Ar}), 129.9 (C_{Ar}), 128.9 (C_{Ar}), 128.7 (C_{Ar}), 128.2 (C_{Ar}), 127.5 (C_{Ar}), 126.4 (C_{Ar}), 72.3 (C_7), 68.2 (C_{31}), 64.3 (C_{22}), 61.4 (C_{20}), 54.8 (C_{29}), 30.6 (C_{32}), 23.7 (C_{27}), 20.8 (C_{28}), 14.4 (C_{23}), -1.54 ($C_{24, 25, 26}$);

HRMS: cald (ES^+), *m/z*: Found $[M + Na]^+$, 795.2861; $C_{45}H_{48}N_2O_6NaSiS$, requires 795.2900; (ES^+), $C_{45}H_{48}N_2O_6SiS$, *m/z*: 795 $[M + Na]^+$;

IR ν_{max}: 3292, 2800, 1611, 1400 cm^{-1};

$[\alpha]^{22}{}_D$ = - 18.2 (*c* 3.1 mg/mL, MeOH).

(S)-3-[2-Amino-2-(2-trimethylsilanyl-ethoxycarbonyl)-ethylsulfanyl]-(R)-2-(9H-fluoren-9-ylmethoxycarbonylamino)-propionic acid 189



Zinc dust (0.086 g, 0.504 mmol, 36 equiv) was added portionwise to a solution of (*R*)-2-(9H-Fluoren-9-ylmethoxycarbonylamino)-3-(*S*)-[2-(2-trimethylsilanyl-ethoxycarbonyl)-2-(trityl-amino)-ethylsulfanyl]-propionic acid 2,2,2-trichloro-ethyl ester **194** (0.033 g, 0.037 mmol) in THF (1.6 mL), followed by the addition of 1 M aqueous NH_4OAc solution (0.28 mL). The mixture was stirred vigorously under nitrogen for 24 hr at room temperature. After filtration and solvent removal the residue was taken up in chloroform (50 mL) and washed with saturated aqueous NaCl solution (2 x 20 mL). The organic layer was dried over anhydrous magnesium sulphate, after drying and filtration followed by solvent removal, the crude was obtained as oil. This was purified by flash column chromatography (gradient, CH_2Cl_2: MeOH, 98:2 to 95:5) giving the title product **189** as oil.

Yield: 0.0089 g, 0.016 mmol, 45 %;

R_f: 0.08 (CH_2Cl_2: MeOH: 85:15);

NMR: δ_H (600 MHz, CD_3OD), 7.79 (2 H, d, *J* = 7.6 Hz, Ar), 7.67 (2 H, d, *J* = 7.4 Hz, Ar), 7.39 (2 H, t, *J* = 7.4 Hz, Ar), 7.31 (2 H, t, *J* = 7.4 Hz, Ar), 4.42 (1 H, m, C*H*$_2$Fmoc), 4.29 (1 H, m, C*H*$_2$Fmoc), 4.23 (4 H, m, C*H*Fmoc, H_α B side and C*H*$_2$CH$_2$Si), 3.92 (1 H, m, H_α A side), 3.08 (2 H, m, C*H*$_2$ β), 2.93 (2 H, m, C*H*$_2$ β), 1.00 (2 H, m, CH$_2$C*H*$_2$Si(CH$_3$)$_3$), 0.02 (9 H, s, Si(C*H*$_3$)$_3$);

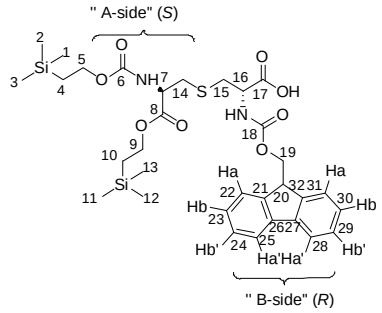
NMR: δ_C (150 MHz, CD_3OD), 180 (C_{11}), 163.3 (C_2), 158.4 (C_{12}), 145.6 (C_{Ar}), 142.5 (C_{Ar}), 128.8 (C_{Ar}), 128.2 (C_{Ar}), 126.2 (C_{Ar}), 68.0 (C_{13}), 65.6 (C_3), 57.3 (C_{14}), 54.5 (C_1), 47.9 (C_{10}), 39.0 ($C_{8,9}$), 18.3 (C_4), -1.42 ($C_{5,6,7}$);

HRMS: cald (ES^+), *m/z*: Found $[M + Na]^+$, 553.1805; $C_{26}H_{34}N_2O_6NaSiS$, requires 553.1805; (ES^+), $C_{26}H_{34}N_2O_6NaSiS$, *m/z*: 553 $[M + Na]^+$;

IR ν_{max}: 3355, 1690, 1410 cm^{-1};

$[\alpha]^{20}_{D}$ = - 4.4 (*c* 5.5, MeOH).

(R)-2-(9H-Fluoren-9-ylmethoxycarbonylamino)-3-[(S)-2-(2-trimethylsilanyl-ethoxycarbonyl)-2-(2-trimethylsilanyl-ethoxycarbonylamino)-ethylsulfanyl]-propionic acid 169



Entry	Reaction solvents	Base (equiv), Temperature	Protecting group 171 (%), °C	Reaction time / temperature	Product Isolated 169 (%)
1	1,4-dioxane	aq. sodium bicarbonate, (4), RT	(19.9), 0 °C	24 hr in freezer 5 hr, 0 °C - RT	64 %
2	1,4-dioxane	DMAP, (cat), RT	(4.9), RT	24 hr, RT	24 %

Optimised experiments

General procedure

(*S*)-3-[2-Amino-2-(2-trimethylsilanyl-ethoxycarbonyl)-ethylsulfanyl]-(*R*)-2-(9H-fluoren-9-ylmethoxycarbonylamino)-propionic acid **189** was dissolved in 1,4-dioxane. The correct base was added followed by the addition of carbonic acid 2,5-dioxo-pyrrolidin-1-yl ester 2-trimethylsilanyl-ethyl ester **171** at the required temperature. The equivalents of the protecting reagent, reaction time and the temperature of the reaction were carried out and maintained according to the table above. After the reaction time was complete excess chloroform (50 mL) was added and the organic layer was washed with water (2 x 30 mL). The organic layer was dried over anhydrous magnesium sulphate, concentrated *in vacuo*, and purified by flash column chromatography (CH_2Cl_2: MeOH, 98:2) to give the title product **169** as white foam.

R_f: 0.37 (CH_2Cl_2: MeOH; 90:10);

NMR: δ_H (600 MHz, CD_3OD), 7.77 (2 H, d, *J* = 7.6 Hz, Ar), 7.67 (2 H, d, *J* = 7.3 Hz, Ar), 7.37 (2 H, t, *J* = 7.4 Hz, Ar), 7.29 (2 H, t, *J* = 7.4 Hz, Ar), 4.40 (1 H, m, C*H*Fmoc), 4.36 (1 H, m, H_α A side), 4.26 (3 H, m, CH_2Fmoc and H_α B side), 4.15 (4 H, m, 2 x CH_2CH$_2$Si(CH$_3$)$_3$), 3.11 (1 H, dd, *J* = 13.8, 4.3 Hz, H_β), 3.02 (1 H, dd, *J* = 13.8, 5.2 Hz, H_β), 2.93 (2 H, m, H_β), 0.94 (4 H, m, 2 x CH$_2$CH_2Si(CH$_3$)$_3$), 0.00 (18 H, d, *J* = 2.4 Hz, Si(CH_3)$_3$);

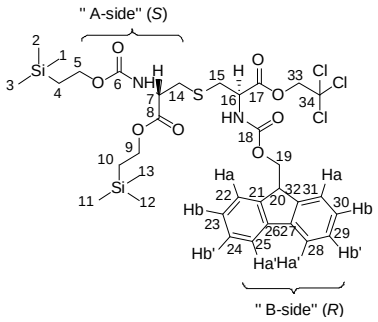
NMR: δ_C (150 MHz, CD_3OD), 178.3 (C_{17}), 172.6 (C_8), 158.7 (C_6), 158.4 (C_{18}), 145.3 (C_{Ar}), 142.5 (C_{Ar}), 128.7 (C_{Ar}), 128.2 (C_{Ar}), 126.4 (C_{Ar}), 121.0 (C_{Ar}), 68.2 (C_{19}), 64.8 (C_5 or $_9$), 64.4 (C_5 or $_9$), 57.1 (C_7 or $_{16}$), 55.7 (C_7 or $_{16}$), 48.4 (C_{20} signal obscured by CD_3OD), 36.7 (C_{14} or $_{15}$), 35.3 (C_{14} or $_{15}$), 18.5 (C_4 or $_{10}$), 18.2 (C_4 or $_{10}$), -1.40 ($C_{1, 2, 3, 11, 12, 13}$);

HRMS: cald (ES^+), *m/z*: Found $[M + Na]^+$, 697.2405; $C_{32}H_{46}N_2O_8NaSi_2S$, requires 697.2411; ($ES^+$), $C_{32}H_{46}N_2O_8Si_2S$, *m/z*: 697 $[M + Na]^+$;

IR ν_{max}: 3300 (broad), 2954, 1716, 1593, 1418 cm^{-1};

$[\alpha]^{25}{}_D$ = + 2.2 (*c* 10.0 mg/mL, MeOH).

(R)-2-(9H-Fluoren-9-ylmethoxycarbonylamino)-3-[(S)-2-(2-trimethylsilanyl-ethoxycarbonyl)-2-(2-trimethylsilanyl-ethoxycarbonylamino)-ethylsulfanyl]-propionic acid 2,2,2-trichloro-ethyl ester 195



2-(9H-Fluoren-9-ylmethoxycarbonylamino)-3-[2-(2-trimethylsilanyl-ethoxycarbonyl)-2-(trityl-amino)-ethylsulfanyl]-propionic acid 2,2,2-trichloro-ethyl ester **194** (0.102 g, 0.112 mmol) was treated with TFA (0.4 mL), TES (0.2 mL) and CH_2Cl_2 (3.4 mL), the mixture was stirred under argon for 24 hr. Excess chloroform (100 mL) was added and the organic layer was washed with sodium bicarbonate (50 mL) and brine (50 mL). The organic layer was dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo*. The residue was dissolved in 1,4-dioxane (3.4 mL), DMAP (0.013 g, 0.112 mmol, 1 equiv) was added followed by carbonic acid 2,5-dioxo-pyrrolidin-1-yl ester 2-trimethylsilanyl-ethyl ester **171** (0.143 g, 0.551 mmol, 4.9 equiv). The reaction was left to stir at room temperature for 24 hr. Excess Chloroform (50 mL) was added and the organic layer was washed with brine (2 x 30

mL). The organic layer was dried over anhydrous magnesium sulphate, concentrated *in vacuo*, and purified by flash column chromatography (hexane: EtOAc, 9:1 then 4:1) to give the title product **195** as a clear oil.

Yield; 0.06 g, 0.07 mmol, 66 %;

R_f: 0.36 (Hexane: Ethyl acetate; 4:1);

NMR: δ_H (600 MHz, $CDCl_3$), 7.76 (2 H, d, *J* = 7.5 Hz, Ar), 7.63(2 H, m, Ar), 7.40 (2 H, m, Ar*)*, 7.31 (2 H, m, Ar), 5.95 (1 H, d, *J* = 7.9 Hz, N*H*Teoc), 5.48 (1 H, d, *J* = 7.4 Hz, N*H*Fmoc), 4.86 (1 H, d, *J* =11.82 Hz, CH_2CCl$_3$), 4.76 (2 H, m, CH_2CCl$_3$ and H_α B side), 4.55 (1 H, m, H_α A side), 4.41 (2 H, m, CH_2Fmoc), 4.19 (5 H, m, C*H*Fmoc and 2 x CH_2CH$_2$Si(CH$_3$)$_3$), 3.11 (3 H, m, H_β), 2.96 (1 H, dd, *J* = 13.9, 5.8 Hz, H_β), 0.98 (4 H, m, 2 x CH$_2$CH_2Si(CH$_3$)$_3$), 0.02 (18 H, d, *J* = 7.7 Hz, Si(CH_3)$_3$);

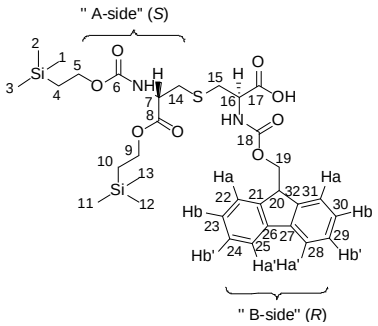
NMR: δ_C (150 MHz, $CDCl_3$), 170.6 (C_{17}), 169.2 (C_8), 156.3 (C_6), 155.9 (C_{18}), 143.8 (C_{Ar}), 141.4 (C_{Ar}), 127.9 (C_{Ar}), 127.2 (C_{Ar}), 125.3 (C_{Ar}), 120.1 (C_{Ar}), 94.3 (C_{34}), 74.7 (C_{33}), 67.5 (C_{19}), 64.6 ($C_{5,}$ or $_9$), 63.9 (C_5 or $_9$), 53.9 (C_{16}), 47.1 (C_{20}), 36.2 (C_7), 35.8 (C_{14} or $_{15}$), 35.4 (C_{14} or $_{15}$), 17.7 ($C_{4,}$ or $_{10}$), 17.5 ($C_{4,}$ or $_{10}$), -1.37 ($C_{1, 2, 3, 11, 12, 13}$);

HRMS: cald (ES^+), *m/z*: Found $[M + Na]^+$, 829.1566; $C_{34}H_{48}N_2O_8Cl_3NaSi_2S$, requires 829.1553; ($ES^+$), $C_{34}H_{48}N_2O_8Cl_3Si_2S$, *m/z*: $[M + Na]^+$;

IR ν_{max}: 2954, 1709, 1509, 1337 cm^{-1};

$[\alpha]^{25}_D$ = - 8.8 (*c* 4.9 mg/mL, $CHCl_3$).

(R)-2-(9H-Fluoren-9-ylmethoxycarbonylamino)-3-[(S)-2-(2-trimethylsilanyl-ethoxycarbonyl)-2-(2-trimethylsilanyl-ethoxycarbonylamino)-ethylsulfanyl]-propionic acid 169



Zinc dust (0.017 g, 2.7 mmol, 36 equiv) was added portionwise to a solution of (*R*)-2-(9H-Fluoren-9-ylmethoxycarbonylamino)-3-[(*S*)-2-(2-trimethylsilanyl-ethoxycarbonyl)-2-(2-

trimethylsilanyl-ethoxycarbonylamino)-ethylsulfanyl]-propionic acid 2,2,2-trichloro-ethyl ester **195** (0.06 g, 0.075 mmol) in THF (6 mL), followed by the addition of 1 M aqueous NH_4OAc solution (0.56 mL). The mixture was stirred vigorously under nitrogen for 24 hr at room temperature. After filtration and solvent removal the residue was taken up in chloroform (10 mL) and washed with saturated aqueous NaCl solution (2 x 50 mL). The organic layer was dried over anhydrous magnesium sulphate, after drying and filtration followed by solvent removal, the crude was obtained as oil. This was purified by flash column chromatography (gradient, CH_2Cl_2: MeOH, 98:2 to 95:5) giving the title product **169** as white foam.

M. pt. 59 º C;

Yield: 0.02 g, 0.029 mmol, 40 %;

R_f: 0.37 (CH_2Cl_2: MeOH; 90:10);

NMR: δ_H (600 MHz, CD_3OD), 7.77 (2 H, d, *J* = 7.6 Hz, Ar), 7.67 (2 H, d, *J* = 7.3 Hz, Ar), 7.37 (2 H, t, *J* = 7.4 Hz, Ar), 7.29 (2 H, t, *J* = 7.4 Hz, Ar), 4.40 (1 H, m, C*H*Fmoc), 4.36 (1 H, m, H_α A side), 4.26 (3 H, m, CH_2Fmoc and H_α B side), 4.15 (4 H, m, 2 x C$H_2$$CH_2Si(CH_3)_3$), 3.11 (1 H, dd, *J* = 13.8, 4.3 Hz, H_β), 3.02 (1 H, dd, *J* = 13.8, 5.2 Hz, H_β), 2.93 (2 H, m, H_β), 0.94 (4 H, m, 2 x CH_2C$H_2Si(CH_3)_3$), 0.00 (18 H, d, *J* = 2.4 Hz, Si(CH_3)$_3$), (Appendix 17);

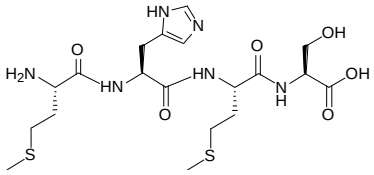
NMR: δ_C (150 MHz, CD_3OD), 178.3 (C_{17}), 172.6 (C_8), 158.7 (C_6), 158.4 (C_{18}), 145.3 (C_{Ar}), 142.5 (C_{Ar}), 128.7 (C_{Ar}), 128.2 (C_{Ar}), 126.4 (C_{Ar}), 121.0 (C_{Ar}), 68.2 (C_{19}), 64.8 (C_5 or $_9$), 64.4 (C_5 or $_9$), 57.1 (C_7 or $_{16}$), 55.7 (C_7 or $_{16}$), 48.4 (C_{20} signal obscured by CD_3OD), 36.7 (C_{14} or $_{15}$), 35.3 (C_{14} or $_{15}$), 18.5 (C_4 or $_{10}$), 18.2 (C_4 or $_{10}$), -1.40 ($C_{1, 2, 3, 11, 12, 13}$), (Appendix 18);

HRMS: cald (ES^+), *m/z*: Found $[M + Na]^+$, 697.2405; $C_{32}H_{46}N_2O_8NaSi_2S$, requires 697.2411; ($ES^+$), $C_{32}H_{46}N_2O_8Si_2S$, *m/z*: 697 $[M + Na]^+$;

IR ν_{max}: 3300 (broad), 2954, 1716, 1593, 1418 cm^{-1};

$[\alpha]^{25}{}_D$ = + 2.2 (*c* 10.0 mg/mL, MeOH).

Synthesis of Model Peptide 198



Molecular Weight : 504

Peptide **198** was synthesised manually on a Merrifield bubbler using solid phase peptide Fmoc strategy. Fmoc-Ser(*t*Bu)-NovaSyn TGT resin (0.2 g 0.038 mmol) was suspended in DMF (5 mL) for 1 hr on a Merrifield bubbler. The terminal Fmoc group was removed by treatment with piperidine (20 % piperidine in DMF, 2 mL) with Ar bubbling for 20 min, followed by thorough washing with DMF (6 x 4 mL). A qualitative Kaiser test showed a positive result.

Fmoc-Met-OH (0.056 g, 0.15 mmol, 4 equiv) in DMF (2 mL) was then activated under inert conditions, in a separate flask, with a solution of HBTU in DMF (0.058 g, 0.15 mmol, 4 equiv, 1 mL of DMF) and DIPEA (0.026 mL, 4 equiv) for 20 min. The activated amino acid solution was then added to the resin and Ar was bubbled for 30 min. The coupling solution was removed and the resin was washed with DMF (6 x 4 mL). A qualitative Kaiser test showed a negative result. The terminal Fmoc group was removed with piperidine (20 % piperidine in DMF, 2 mL) with Ar bubbling for 20 min, followed by thorough washing with DMF (6 x 4 mL). A qualitative Kaiser test showed a positive result.

Fmoc-His(Trt)-OH (0.094 g, 0.15 mmol, 4 equiv) was then activated under inert conditions, in a separate flask, with a solution of HBTU in DMF (0.058 g, 0.15 mmol, 4 equiv, 1 mL of DMF) and DIPEA (0.026 mL, 4 equiv) for 20 min. The activated amino acid solution was then added to the resin with Ar bubbling for 30 min. The coupling solution was removed and the resin was washed with DMF (6 x 4 mL). A qualitative Kaiser test showed a negative result. The terminal Fmoc group was removed with piperidine (20 % Piperidine in DMF, 2 mL) with Ar bubbling for 20 min, followed by thorough washing with DMF (6 x 4 mL). A qualitative Kaiser test showed a positive result.

Fmoc-Met-OH (0.056 g, 0.15 mmol, 4 equiv) in DMF (2 mL) was then activated under inert conditions, in a separate flask, with a solution of HBTU in DMF (0.058 g, 0.15 mmol, 4 equiv, 1 mL of DMF) and DIPEA (0.026 mL, 4 equiv) for 20 min. The activated amino acid

solution was then added to the resin with Ar bubbling for 30 min. The coupling solution was removed and the resin was washed with DMF (6 x 4 mL). A qualitative Kaiser test showed a negative result.

Removal of the terminal Fmoc group with 20 % piperidine in DMF (2 mL) with Ar bubbling for 20 min was followed by washing with DMF (6 x 4 mL). A qualitative Kaiser test showed a positive result. Cleavage of the peptide from the solid support was carried out by successively bubbling the resin with a solution of TFA (94 %), H_2O (2.5 %), TIPS (1 %) and EDT (2.5 %) for 3 hr. The cleavage cocktail were collected in a flask, the resin was washed with pure TFA and collected in the cleaved cocktail flask. TFA was removed under high vacuum to a minimum amount after which ether was added to precipitate the product, the solvent was removed under high vacuum. This was repeated twice. The precipitate was redissolved in water and the solution freeze dried overnight which yielded the crude peptide (42 mg).

The resulting mixture was then purified via preparative reverse-phase HPLC monitoring at 214 nm. Purification was performed at a flow rate 15 mL min^{-1} using a linear gradient in H_2O and CH_3CN (90 %: 10 % for 10 min then increased to 10 %: 90 % over 30 min), the peptide eluted at 17.8 min. The CH_3CN was removed under vacuum, and the peptide solution was freeze dried. Freeze drying yielded the peptide **198** as clear oil (4 mg, 7.9 x 10^{-3} mmol, 21 %).

$(ES+)^+$, $C_{19}H_{32}N_6O_6S_2$, *m/z*: $[M+H]^+ = 505$.

Analytical HPLC (Wide Pore C18) $\mathbf{R_T}$ 18.8 min; A: H_2O/0.5 % TFA; B: acetonitrile/0.5% TFA; gradient 10 % - 60 % B over 30 min.

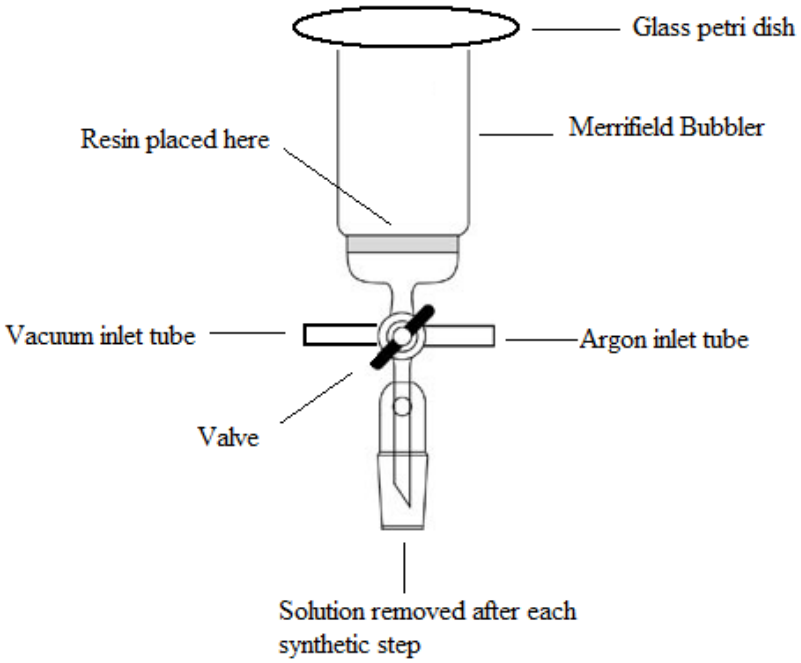
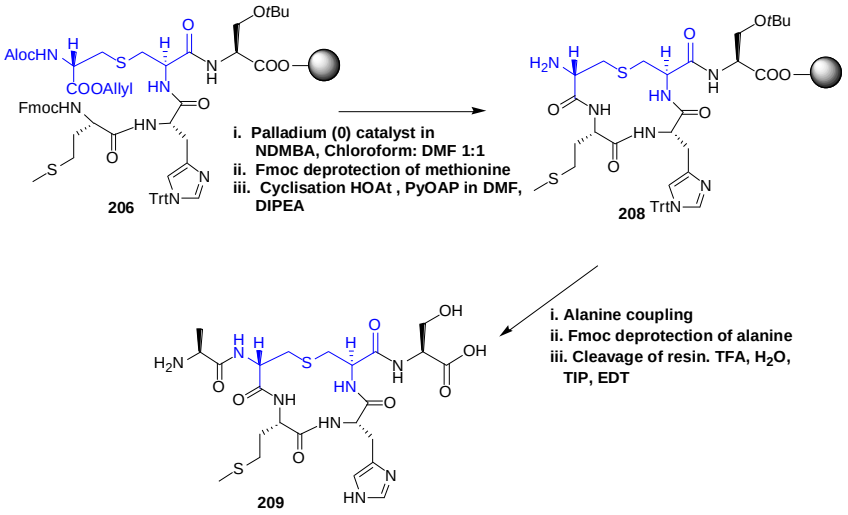


Figure 74: Merrifield bubbler.

Synthesis of an analogue of ring E of nisin 203



Peptide **209** was synthesised manually on a Merrifield bubbler using solid phase peptide Fmoc strategy. Fmoc-Ser(*t*Bu)-NovaSyn TGT resin (0.2 g 0.038 mmol) was suspended in DMF (5 mL) for 1 hr on a Merrifield bubbler. The terminal Fmoc group was removed by treatment with piperidine (20 % piperidine in DMF, 2 mL) with Ar bubbling for 20 min,

followed by thorough washing with DMF (6 x 4 mL). A qualitative Kaiser test showed a positive result.

Lanthionine **103** (0.084 g, 0.13 mmol, 4 equiv) in DMF (2 mL) was then activated under inert conditions, in a separate flask, with a solution of HBTU in DMF (0.058 g, 0.15 mmol, 4 equiv, 1 mL of DMF) and DIPEA (0.026 mL, 4 equiv) for 20 min. The activated amino acid solution was then added to the resin with Ar bubbling for 30 min. The coupling solution was removed and the resin was washed with DMF (6 x 4 mL). A qualitative Kaiser test showed a negative result. The terminal Fmoc group of **103** was removed with piperidine (20 % piperidine in DMF, 2 mL) with Ar bubbling for 20 min, followed by thorough washing with DMF (6 x 4 mL). A qualitative Kaiser test showed a positive result.

Fmoc-His(Trt)-OH (0.094 g, 0.15 mmol, 4 equiv) was then activated under inert conditions, in a separate flask, with a solution of HBTU in DMF (0.058 g, 0.15 mmol, 4 equiv, 1 mL of DMF) and DIPEA (0.026 mL, 4 equiv) for 20 min. The activated amino acid solution was then added to the resin with Ar bubbling for 30 min. The coupling solution was removed and the resin was washed with DMF (6 x 4 mL). A qualitative Kaiser test showed a negative result. The terminal Fmoc group of histidine was removed with piperidine (20 % piperidine in DMF, 2 mL) with Ar bubbling for 20 min, followed by thorough washing with DMF (6 x 4 mL). A qualitative Kaiser test showed a positive result.

Fmoc-Met-OH (0.056 g, 0.15 mmol, 4 equiv) in DMF (2 mL) was then activated under inert conditions, in a separate flask, with a solution of HBTU in DMF (0.058 g, 0.15 mmol, 4 equiv, 1 mL of DMF) and DIPEA (0.026 mL, 4 equiv) for 20 min. The activated amino acid solution was then added to the resin with Ar bubbling for 30 min. The coupling solution was removed and the resin was washed with DMF (6 x 4 mL). A qualitative Kaiser test showed a negative result.

Before the cyclisation step, the fully protected, resin bound peptide **206** was treated with tetrakis(triphenylphosphine)Palladium (0) (0.043 g, 0.037 mmol, 0.5 equiv) which was dissolved in previously dissolved 1,3-dimethyl barbituric acid (0.059 g, 0.37 mmol, 10 equiv) in a mixture of DMF/$CHCl_3$ (1:1, volume 1.5/1.5 mL, total volume 3 mL) by gentle heating. The mixture was gently bubbled with Ar for 2 hr in the dark. The resin was then washed successively with DMF (6 x 4 mL) followed by a solution of DIPEA in DMF (0.5%

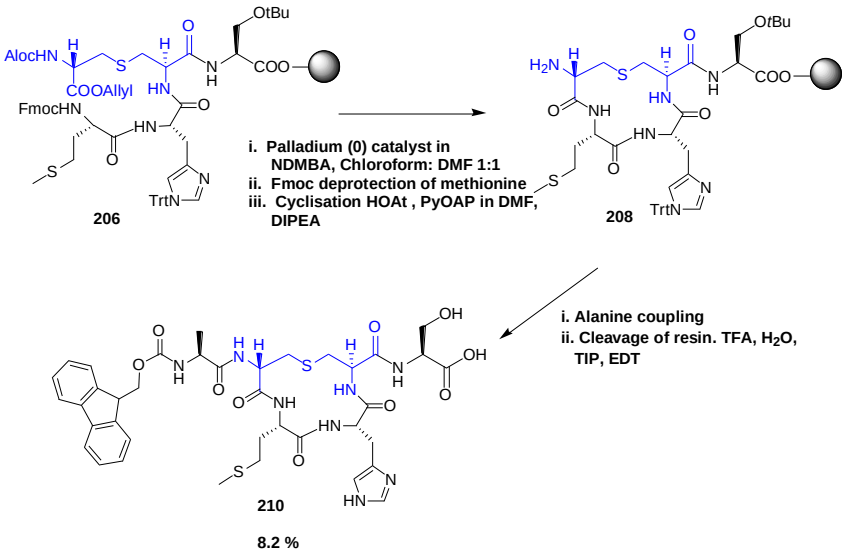
v/v, 5 x 4 mL) followed by further washing with DMF (6 x 4 mL). It was then washed with sodium diethyldithiocarbamate trihydrate in DMF (0.5% w/v, 5 x 4 mL) followed by a final washing with DMF (6 x 4 mL). The qualitative Kaiser test was purple. Removal of the terminal Fmoc group of methionine with 20 % piperidine in DMF (2 mL) with Ar bubbling for 20 min was followed by washing with DMF (6 x 4 mL), leading to the cyclization substrate. During Fmoc deprotection the cyclization reagents were prepared. HOAt (0.026 g, 0.18 mmol, 5 equiv) was dissolved in DMF (1 mL) and mixed with a solution of PyAOP (0.099 g, 0.18 mmol, 5 equiv) dissolved in DMF (1 mL). The mixture was added to the Merrifield bubbler containing the resin, this was followed by addition of DIPEA (0.066 mL, 0.38 mmol, 10 equiv) and left bubbling under Ar for 1 hr. The resin was then washed with DMF (6 x 4 mL), Kaiser test showed a dark purple colour.

Fmoc-Ala-OH (0.047 g, 0.15 mmol, 4 equiv) in DMF (2 mL) was then activated under inert conditions, in a separate flask, with a solution of HBTU in DMF (0.058 g, 0.15 mmol, 4 equiv, 1 mL of DMF) and DIPEA (0.026 mL, 4 equiv) for 20 min. The activated amino acid solution was then added to the resin with Ar bubbling for 30 min. The coupling solution was removed and the resin was washed with DMF (6 x 4 mL). A qualitative Kaiser test showed a negative result.

Removal of the terminal Fmoc group of alanine with 20 % piperidine in DMF (2 mL) with Ar bubbling for 20 min was followed by washing with DMF (6 x 4 mL). A qualitative Kaiser test showed a positive result. Cleavage of the peptide from the solid support was carried out by successively bubbling the resin with a solution of TFA (94 %), H_2O (2.5 %), TIPS (1 %) and EDT (2.5 %) for 3 hr. The cleavage cocktail were collected in a flask, the resin was washed with pure TFA and collected in the cleaved cocktail flask. TFA was removed under high vacuum to a minimum amount after which ether was added until the peptide precipitated, the solvent was then removed under high vacuum this was repeated twice. The precipitate was redissolved in water and the solution freeze dried overnight, yielding the crude peptide **209** (8.2 mg).

(ES+), $C_{23}H_{36}N_8O_8S_2$, *m/z*: $[M+Na]^+$ = 639, [M oxidised] = 634.

Synthesis of an Fmoc protected analogue of ring E of nisin 210



Linear resin-bound peptide 206

Peptide **210** was synthesised on a MultiSynTech Syro Peptide Synthesiser (Model MP-60) using solid phase peptide Fmoc strategy. Fmoc-Ser(*t*Bu)-NovaSyn TGT resin (0.120 g, 0.22 mmol/g, 0.026 mmol) was suspended in DMF (5 mL) for 1 hr in a 2 mL syringe in order to pre-swell the resin followed by thorough washing with DMF (6 x 1.2 mL). The terminal Fmoc group was removed by treatment with piperidine (40 % in DMF, 2 mL) with Ar bubbling for 30 min, this was repeated again followed by thorough washing with DMF (6 x 0.9 mL). A qualitative Kaiser test showed a positive result.

3-(2-Allyloxycarbonyl-2-allyloxycarbonylamino-ethylsulfanyl)-2-(9H-fluoren-9-ylmethoxycarbonylamino)-propionic acid **103** (0.0585 g, 0.12 mmol, 4 equiv), HOAt (0.017 g, 0.13 mmol, 4 equiv) and PyAOP (0.068 g, 0.13 mmol, 4 equiv) were dissolved in DMF (1 mL). The mixture was added to the syringe containing the resin, followed by DIPEA (0.045 mL, 0.25 mmol, 8 equiv) and the mixture was bubbled with Ar for 1 hr 30 min. The coupling solution was removed and the resin was washed with DMF (6 x 0.9 mL). A qualitative Kaiser test showed a negative result. The terminal Fmoc group was removed with piperidine (40 % in DMF, 2 mL) with Ar bubbling for 30 min, followed by thorough washing with DMF (6 x 0.9 mL). A qualitative Kaiser test showed a positive result.

Fmoc-His(Trt)-OH (0.16 g, 0.26 mmol, 10 equiv) and HBTU (0.10 g, 0.26 mmol, 10 equiv) were dissolved in DMF (1 mL). The mixture was added to the resin followed by DIPEA (0.091 mL, 0.53 mmol, 20 equiv) and bubbled with Ar for 1 hr 15. The coupling solution was removed and the resin was washed with DMF (6 x 0.9 mL). A qualitative Kaiser test showed a negative result. The terminal Fmoc group was removed with piperidine (40 % in DMF, 2 mL) with Ar bubbling for 30 min, followed by thorough washing with DMF (6 x 0.9 mL). A qualitative Kaiser test showed a positive result.

Fmoc-Met-OH (0.09 g, 0.26 mmol, 10 equiv) and HBTU (0.10 g, 0.26 mmol, 10 equiv) were dissolved in DMF (1 mL). The mixture was added to the resin followed by DIPEA (0.091 mL, 0.53 mmol, 20 equiv) and bubbled with Ar for 1 hr 15 min. The coupling solution was removed and the resin was washed with DMF (6 x 0.9 mL) to give the resin-bound peptide **206**. A qualitative Kaiser test showed a negative result.

Deprotection and cyclisation to give cyclic resin-bound peptide 208

Before the cyclisation step, the fully protected, resin bound peptide was treated with a solution of 1,3-Dimethylbarbituric acid (NDMBA) (0.041 g, 0.26 mmol, 10 equiv) dissolved in a mixture of DMF/$CHCl_3$ (1:1, volume 0.5/0.5 mL, total volume 1 mL). Tetrakis(triphenylphosphine)palladium (0) (0.013 g, 0.011 mmol, 0.5 equiv) was then added and the mixture heated gently to completely dissolve the palladium reagent. The solution was added to the fully protected, resin bound peptide **206**, and the mixture was gently bubbled with Ar for 2 hr in the dark. The resin was then washed successively with DMF (6 x 0.9 mL) followed by a solution of DIPEA in DMF (0.5 % v/v, 6 x 1 mL) followed by further washing with DMF (6 x 0.9 mL). It was then washed with sodium diethyldithiocarbamate trihydrate in DMF (0.5 % w/v, 8 x 1 mL) followed by final washing with DMF (6 x 0.9 mL). The qualitative Kaiser test was purple. Removal of the terminal Fmoc group with piperidine (40 % in DMF, 2 mL) with Ar bubbling for 30 min was followed by washing with DMF (6 x 0.9 mL).

During Fmoc deprotection the cyclization reagents were prepared. HOAt (0.018 g, 0.13 mmol, 5 equiv) was dissolved in DMF (1 mL) and mixed with a solution of PyAOP (0.069 g, 0.13 mmol, 5 equiv) dissolved in DMF (1 mL). The mixture was added to the syringe containing the resin peptide, this was followed by addition of DIPEA (0.044 mL, 0.25 mmol, 10 equiv). The mixture was bubbled with Ar for 1 hr 15 min. The resin was then washed with

DMF (6 x 0.9 mL), giving the cyclised resin-bound peptide **208**. The Kaiser test showed a dark purple colour.

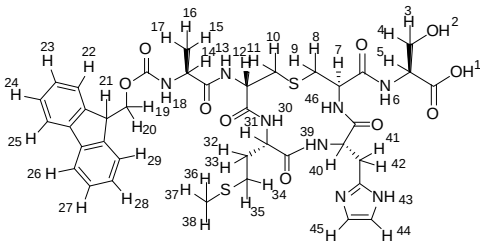
Chain elongation to give ring E analogue 210

Fmoc-Ala-OH (0.081 g, 0.20 mmol, 10 equiv) and HBTU (0.10 g, 0.26 mmol, 10 equiv) were dissolved in DMF (1 mL). The mixture was added to the resin, followed by DIPEA (0.091 mL, 0.53 mmol, 20 equiv) and gently bubbled with Ar for 1 hr 15 min. The coupling solution was removed and the resin was washed with DMF (6 x 0.9 mL). A qualitative Kaiser test showed a negative result.

In order to cleave the peptide from the solid support and remove the side-chain protecting groups, a solution of TFA (94 %), H_2O (2.5 %), TIPS (1 %) and EDT (2.5 %) was added to the resin and gently bubbled with Ar for 3 hr. The cleavage cocktail solution was collected in a Falcon tube. The resin was washed again with TFA and the washings were added to the cleavage cocktail. Diethyl ether was added until the crude peptide precipitated. The solution was centrifuged at 4000 rpm for 7 min at 4 ºC after which the ether layer was decanted. The centrifuging and decanting process was repeated, washing on each occasion with diethyl ether (5 x 13 mL). The precipitate was redissolved in water and the mixture freeze dried overnight, yielding the crude peptide (16.7 mg). The peptide was purified by preparative HPLC (Wide Pore C18) in H_2O/0.5 % TFA and acetonitrile/0.5 % TFA at a gradient of 10 % - 60 % acetonitrile over 30 min to give peptide **210**, which eluted at 22.3 min.

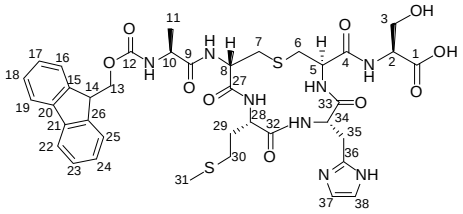
Yield: 1.8 mg, 2.14^{-03} mmol, 8.2%;

Analytical HPLC (Wide Pore C18) $\mathbf{R_T}$ 16.8 min; A: H_2O/0.5 % TFA; B: acetonitrile/0.5% TFA; gradient 10 % - 60 % B over 30 min.



NMR: δ_H (500MHz, CD_3OD), 8.75 (1 H, s, Histidine *2H*), 7.79 (2 H, d, *J* = 7.5 Hz, Fmoc Ar), 7.64 (2 H, t, *J* = 7.2 Hz, Fmoc Ar), 7.38 (2 H, dd, *J* = 14.7, 7.3 Hz, Fmoc Ar), 7.28 (3 H,

m, Fmoc Ar + His *4H*), αLan C$H_{7, 12}$ peaks obscured by residual H_2O (but visible in COSY) 4.44 (1 H, t, *J* = 4.0 Hz, α Ser H_5), 4.35 (2 H, d, *J* = 6.5 Hz, CH_2Fmoc $H_{19, 20}$), 4.25 (1 H, m, C*H*Fmoc H_{21}), 4.21 (1 H, m, α His H_{40}), 4.10 (1 H, t, *J* = 7.3 Hz, α Met H_{31}), 4.00 (1 H, q, *J* = 7.0 Hz, α Ala H_{14}), 3.90 (1 H, dd, *J* = 11.3, 4.7 Hz, *β* Ser H_3 or H_4), 3.80 (1 H, dd, *J* = 11.3, 3.7 Hz, β Ser H_4 or H_4), 3.62 (1 H, m, β His H_{41} or H_{42}), 3.48 (1 H, dd, *J* = 7.0, 14.1 Hz, β Lan H_8 or H_9 or H_{10} or H_{11}), 3.10 (1 H, dd, *J* = 14.4, 3.3 Hz, β Lan H_8 or H_9 or H_{10} or H_{11}), 3.04 (1 H, dd, *J* = 14.4, 3.3 Hz, β Lan H_8 or H_9 or H_{10} or H_{11}), 2.98 (1 H, dd, *J* = 15.3, 9.8 Hz, β Lan H_8 or H_9 or H_{10} or H_{11}), 2.84 (1 H, t, *J* = 12.6 Hz, β His H_{41} or H_{42}), 2.17 (2 H, m, γ Met $H_{34, 35}$), 1.84 (1 H, m, β Met H_{32} or H_{33}), 1.78 (3 H, s, ε Met $H_{36, 37, 38}$), 1.72 (1 H, m, β Met H_{32} or H_{33}), 1.37 (2 H, d, *J* = 7.0 Hz, β Ala $H_{15, 16, 17}$), Assignments are carried out on 1-d and 2-d spectras (Figure 52 c and d, Chapter 4, Appendix 21);

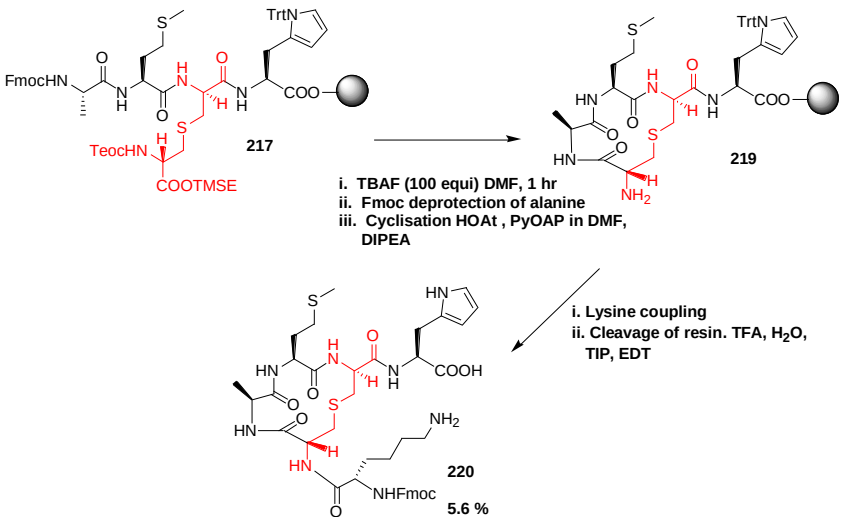


NMR: δ_C (150 MHz, CD_3OD), 176.0 (C_1), 174.3 (CONH), 174.1 (CONH), 172.4 (CONH), 172.1 (CONH), 163.1 (CONH), 158.8 (C_{12}), 145.2 (C_{Ar}), 142.7 (C_{Ar}), 135.1 (C_{38}), 132.2 (C_{36}), 128.8 (C_{Ar}), 128.2 (C_{Ar}), 126.4 (C_{Ar}), 120.9 (C_{Ar}), 118.2 (C_{37}), 68.3 (C_{13}), 66.9 (C_5 or $_8$), 62.8 (C_3), 56.7 (C_{28}), 56.3 (C_2), 55.1 (C_{14}), 52.8 (C_{10}), 52.6 (C_5 or C_8), 47.9 (C_{34}), 39.6 (C_{35}), 37.0 (C_6 or $_7$), 33.1 (C_{29}), 31.2 (C_{30}), 26.8 (C_6, or $_7$), 16.7 (C_{31}), 15.2 (C_{11}), (Appendices 21-24);

HRMS: (ES^-), *m/z*: Found: 837.2654 $[M-H]^-$. $C_{38}H_{45}N_8O_{10}S_2$ requires 837.2700 (Figure 52 b, Chapter 4);

MALDI-TOF, $C_{38}H_{46}N_8O_{10}S_2$, *m/z*: 839.55 $[M + H]^+$, (Figure 52 b, Chapter 4).

Synthesis of an analogue of ring D of nisin 220



Linear resin-bound peptide 217

Peptide **220** was synthesised on a MultiSynTech Syro Peptide Synthesiser (Model MP-60) using solid phase peptide Fmoc strategy. Fmoc-His(Trt)-NovaSyn TGT resin (0.100 g 0.20 mmol/g, 0.020 mmol) was suspended in DMF (5 mL) for 1 hr in a 2 mL syringe in order to pre-swell the resin followed by thorough washing with DMF (6 x 1.2 mL). The terminal Fmoc group was removed by treatment with piperidine (40 % in DMF, 2 mL) with Ar bubbling for 30 min, followed by thorough washing with DMF (6 x 1.2 mL).

2-(9H-Fluoren-9-ylmethoxycarbonylamino)-3-[2-(2-trimethylsilanyl-ethoxycarbonyl)-2-(2-trimethylsilanyl-ethoxycarbonylamino)-ethylsulfanyl]-propionic acid **169** (0.0539 g, 0.079 mmol, 4 equiv), HOAt (0.013 g, 0.099 mmol, 4 equiv) and PyAOP (0.051 g, 0.098 mmol, 4 equiv) were dissolved in DMF (1 mL) and added to the syringe containing the resin. DIPEA (0.034 mL, 0.20 mmol, 10 equiv) was then added to the resin and the mixture was bubbled with Ar for 1 hr 30 min. The coupling solution was removed and the resin was washed with DMF (6 x 1.2 mL). The terminal Fmoc group was removed with piperidine (40 % in DMF, 2 mL) with Ar bubbling for 30 min, followed by thorough washing with DMF (6 x 1.2 mL).

Fmoc-Met-OH (0.074 g, 0.20 mmol, 10 equiv), HBTU (0.075 g, 0.20 mmol, 10 equiv) were dissolved in DMF (1 mL) and added to the resin. DIPEA (0.069 mL, 0.40 mmol, 20 equiv) was then added, and the mixture was bubbled with Ar for 1 hr 15 min. The coupling solution

was removed and the resin was washed with DMF (6 x 1.2 mL). The terminal Fmoc group was removed with piperidine (40 % in DMF, 2 mL) with Ar bubbling for 30 min, followed by thorough washing with DMF (6 x 1.2 mL).

Fmoc-Ala-OH (0.062 g, 0.20 mmol, 10 equiv), HBTU (0.075 g, 0.20 mmol, 10 equiv) were dissolved in DMF (1 mL) and added to the resin. DIPEA (0.069 mL, 0.40 mmol, 20 equiv) was then added, and the mixture bubbled with Ar for 1 hr 15 min. The coupling solution was removed and the resin was washed with DMF (6 x 1.2 mL) to give the linear resin-bound peptide **217**.

Deprotection and cyclisation to give cyclic resin-bound peptide 219

Before the cyclisation step, the fully protected, resin-bound peptide **217** was treated with tetrabutylammonium fluoride (TBAF) (0.522 g, 2.00 mmol, 100 equiv) which was dissolved in DMF (2 mL). The mixture was gently bubbled with Ar for 1 hr. The resin was then washed successively with DMF (10 x 1.2 mL). Removal of the terminal Fmoc group with piperidine (40 % in DMF, 2 mL) with Ar bubbling for 30 min was followed by washing with DMF (6 x 1.2 mL).

During Fmoc deprotection the cyclization reagents were prepared. HOAt **(**0.013 g, 0.099 mmol, 4 equiv**)** was dissolved in DMF (1 mL) and mixed with a solution of PyAOP (0.051 g, 0.098 mmol, 4 equiv**)** dissolved in DMF (1 mL). The cyclisation reagents mixture was then added to the syringe containing the resin-bound peptide. This was followed by addition of DIPEA (0.034 mL, 0.20 mmol, 10 equiv). The mixture was gently bubbled with Ar for 1 hr 15 min, giving the cyclised resin-bound peptide **219**. The resin was then washed with DMF (6 x 1.2 mL).

Chain elongation to give ring D analogue 220

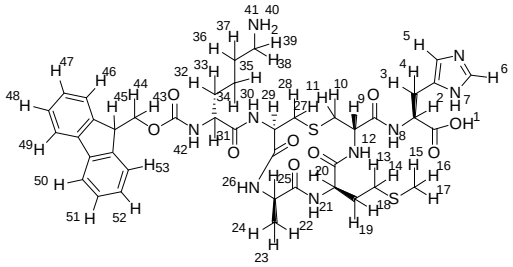
Fmoc-Lys(Boc)-OH (0.094 g, 0.19 mmol, 10 equiv) and HBTU (0.075 g, 0.20 mmol, 10 equiv) were dissolved in DMF (1 mL). The mixture was added to the resin followed by DIPEA (0.069 mL, 0.40 mmol, 20 equiv) and gently bubbled with Ar for 1 hr 15 min. The coupling solution was removed and the resin was washed with DMF (6 x 1.2 mL).

In order to cleave the peptide from the solid support and remove the side-chain protecting groups, a solution of TFA (94 %), H_2O (2.5 %), TIPS (1 %) and EDT (2.5 %) was added to

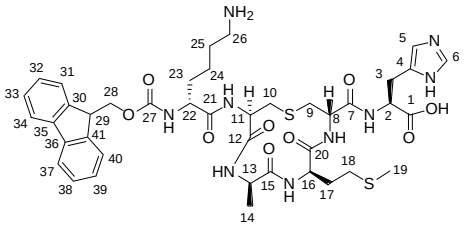
the resin and gently bubbled with Ar for 3 hr. The cleavage cocktail solution was collected in a Falcon tube. Diethyl ether was added until the crude peptide precipitated. The solution was centrifuged at 4000 rpm for 7 min at 4 ºC after which the ether layer was decanted. The centrifuging and decanting process was repeated, washing on each occasion with diethyl ether (5 x 13 mL). The precipitate was dissolved in water, and the solution freeze dried overnight, yielding the crude peptide (18 mg). The peptide was purified by preparative HPLC (Onyx Monolithic C18) in H_2O/0.5 % TFA and acetonitrile/0.5 % TFA at a gradient of 15 % - 60 % acetonitrile over 30 min to give peptide **220**, which eluted at 14.4 min.

Yield: 1.0 mg, 1.13 x 10^{-3} mmol, 5.6 %;

Analytical HPLC: (Onyx Monolithic C18) $\mathbf{R_T}$ 16.6 min; A: H_2O/0.5 % TFA; B: acetonitrile/0.5 % TFA; gradient 10 % - 60 % over 30 min.



NMR: δ_H (600 MHz, CD_3OD), 8.79 (1 H, s, His *2H*), 7.80 (2 H, dd, *J* = 7.6, 2.9 Hz, Fmoc Ar), 7.62 (1 H, d *J* = 7.5 Hz, Fmoc Ar), 7.59 (1 H, d, *J* = 7.5 Hz, Fmoc Ar), 7.42-7.28 (5 H, m, Fmoc Ar + His *4H*), 5.07 (1 H, t, *J* = 2.9 Hz, α Lan H_9 or H_{29}), 4.71 (2 H, m, α His H_2 + α Met H_{20}) 4.40 (2 H, m, CH_2Fmoc $H_{43, 44}$), 4.23 (1 H, t, *J* = 7.0 Hz, C*H*Fmoc H_{45}), 4.02 (2 H, m, α Lan H_9 or H_{29} + α Ala H_{25}), 3.92 (1 H, t, *J* = 7.3 Hz, α Lys H_{31}), 3.47 (1 H, dd, *J* = 12.8, 2.8 Hz, β Lan H_{10} or $_{11}$ or $_{27}$ or $_{28}$), 3.35 (1 H, m, β His H_3 or $_4$), 3.15 (2 H, m, β Lan H_{10} or $_{11}$ or $_{27}$ or $_{28}$ + β His H_3 or $_4$), 2.99 (1 H, dd, *J* = 14.8, 3.5 Hz, β Lan H_{10} or $_{11}$ or $_{27}$ or $_{28}$), 2.91 (3 H, m, β Lan H_{10} or $_{11}$ or $_{27}$ or $_{28}$ + ε Lys $H_{38, 39}$), 2.55 (1 H, m, γ Met H_{13} or $_{14}$), 2.44 (1 H, m, γ Met H_{13} or $_{14}$), 2.08 (3 H, s, ε Met, $H_{15, 16, 17}$), 1.77-1.34 (8 H, m, β Lys, δ Lys, γ Lys, β Met, $H_{32, 33, 36, 37, 34, 35, 18, 19}$), 1.29 (3 H, d, *J* = 7.3 Hz, β Ala $H_{22, 23, 24}$), Assignments are carried out on 1-d and 2-d spectras (Figure 62 b and c, Chapter 4, Appendix 25);



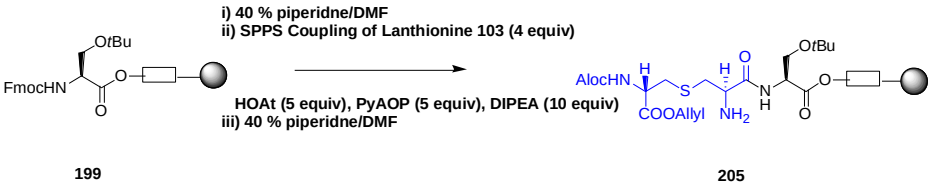
NMR: δ_C (150 MHz, CD_3OD), 176.2 (C_1), 174.6 (CONH), 174.2 (CONH), 173.7 (CONH), 172.5 (2 signals, CONH), 158.9 (C_{27}), 145.2 (C_{Ar}), 142.6 (C_{Ar}), 134.9 (C_6), 130.9 (C_4), 128.9 (C_{Ar}), 128.2 (C_{Ar}), 126.2 (C_{Ar}), 121.1 (C_{Ar}), 118.7 (C_5), 67.9 (C_{28}), 57.3 (C_{22}), 55.1 (C_8 or C_{11}), 54.7 (C_2 or $_{13}$), 53.9 (C_8 or $_{11}$ or $_{13}$), 52.5 (2 signals, C_8 or $_{11}$ or $_{13}$), C_{29} missing, 40.4 (C_{26}), 38.7 (C_9 or $_{10}$), 37.4 (C_9 or $_{10}$), 31.7 (2 signals C_{18} or $_{23}$), 31.4 (C_3), 30.6 (C_{17}), 28.3 (C_{25}), 23.9 (C_{24}), 16.9 (C_{14}), 15.4 (C_{19}), (Appendices 26-31);

HRMS: (ES^-), *m/z*: Found: 878.3363 $[M-H]^-$, $C_{41}H_{52}N_9O_9S_2$ requires 878.3329, (Figure 62 a, Chapter 4);

MALDI-TOF, $C_{41}H_{53}N_9O_9S_2$, *m/z*: 879 $[M + H]^+$, (Figure 59, Chapter 4).

Synthesis of an analogue of rings D and E of nisin 141

Coupling of Aloc/allyl lanthionine to Fmoc-Ser(tBu)-NovaSyn TGT resin



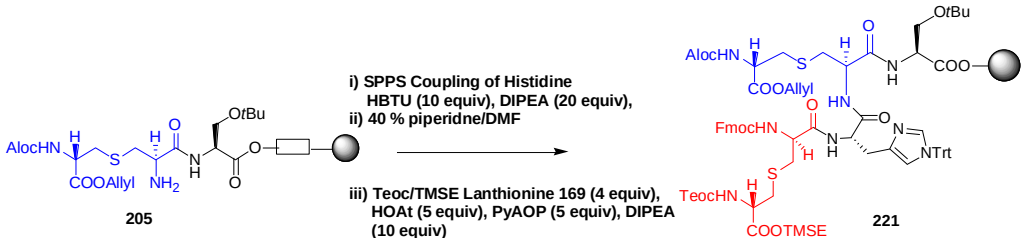
199 **205**

Fmoc-Ser(*t*Bu)-NovaSyn TGT resin **199** (0.100 g 0.021 mmol) was suspended in DMF (2 mL) for 1 hr in a 2 mL syringe in order to pre-swell the resin. The terminal Fmoc group was removed by treatment with piperidine (40% piperidine in DMF, 2 mL) with Ar bubbling for 30 min followed by thorough washing with DMF (6 x 0.9 mL).

3-(2-Allyloxycarbonyl-2-allyloxycarbonylamino-ethylsulfanyl)-2-(9H-fluoren-9-ylmethoxycarbonylamino)-propionic acid **103** (0.047 g, 0.084 mmol, 4 equiv), HOAt (0.014 g, 0.10 mmol, 5 equiv) and PyAOP (0.055 g, 0.10 mmol, 5 equiv) were dissolved in DMF (1 mL). The mixture was added to the syringe containing the resin, followed by DIPEA (0.036

mL, 0.21 mmol, 10 equiv) and the mixture was bubbled with Ar for 1 hr 30 min. The coupling solution was removed and the resin was washed with DMF (6 x 0.9 mL). The terminal Fmoc group was removed with piperidine (40 % in DMF, 2 mL) with Ar bubbling for 30 min, followed by thorough washing with DMF (6 x 0.9 mL).

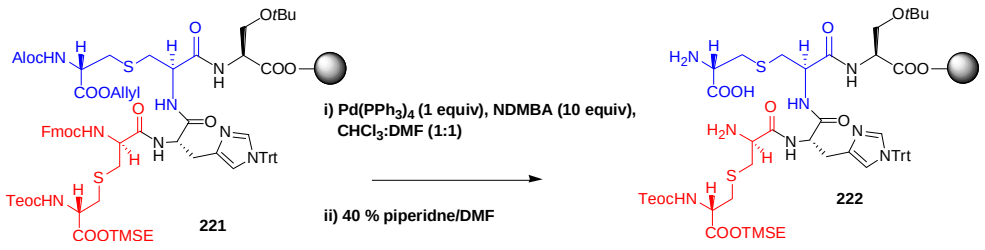
Elongation by SPPS to give linear resin-bound peptide 221



Fmoc-His(Trt)-OH (0.13 g, 0.21 mmol, 10 equiv) and HBTU (0.079 g, 0.21 mmol, 10 equiv) were dissolved in DMF (1 mL). The mixture was added to the resin followed by DIPEA (0.073 mL, 0.42 mmol, 20 equiv) and bubbled with Ar for 1 hr 15 min. The coupling solution was removed and the resin was washed with DMF (6 x 0.9 mL). The terminal Fmoc group was removed with piperidine (40 % in DMF, 2 mL) with Ar bubbling for 30 min, followed by thorough washing with DMF (6 x 0.9 mL).

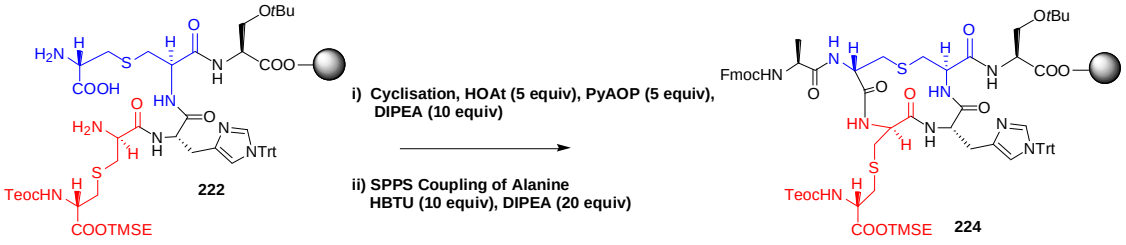
2-(9H-Fluoren-9-ylmethoxycarbonylamino)-3-[2-(2-trimethylsilanyl-ethoxycarbonyl)-2-(2-trimethylsilanyl-ethoxycarbonylamino)-ethylsulfanyl]-propionic acid **169** (0.0566 g, 0.084 mmol, 4 equiv), HOAt (0.014 g, 0.10 mmol, 5 equiv) and PyAOP (0.055 g, 0.10 mmol, 5 equiv) were dissolved in DMF (1 mL). The mixture was added to the syringe containing the resin followed by DIPEA (0.036 mL, 0.21 mmol, 10 equiv) and bubbled with Ar for 1 hr 30 min. The coupling solution was removed and the resin was washed with DMF (6 x 1.2 mL) to give resin-bound peptide **221**.

Removal of the Aloc/allyl and Fmoc protecting groups



1,3-Dimethyl barbituric acid (0.033 g, 0.21 mmol, 10 equiv) was dissolved in a mixture of DMF/$CHCl_3$ (1:1, volume 1.0/1.0 mL, total volume 2 mL). Tetrakis(triphenylphosphine)Palladium (0) (0.024 g, 0.020 mmol, 1.0 equiv) was then added and the mixture was gently heated to completely dissolve the Palladium reagent ensuring the colour remains close to yellow. (It is important that the tetrakis(triphenylphosphine)Palladium (0) reagent used is yellow: if the reagent is dark or discoloured recrystallisation, or the use of a freshly synthesised batch, is recommended). The solution was added to the fully protected, resin-bound linear peptide **221**, and the mixture was gently bubbled with Ar for 2 hr 15 min in the dark. The resin was then washed successively with DMF (6 x 0.9 mL) followed by a solution of DIPEA in DMF (0.5 % v/v, 6 x 1 mL) followed by further washing with DMF (6 x 0.9 mL). It was then washed with sodium diethyldithiocarbamate trihydrate in DMF (0.5 % w/v, 8 x 1 mL) followed by a final wash with DMF (6 x 0.9 mL). Removal of the terminal Fmoc group with piperidine (40 % in DMF, 2 mL) with Ar bubbling for 30 min was followed by washing with DMF (6 x 0.9 mL).

Cyclisation to form ring E of nisin and SPPS elongation 224

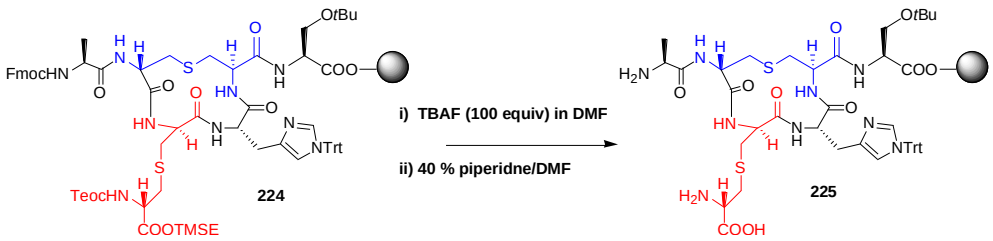


During the preceding Fmoc deprotection step, the cyclization reagents were prepared. HOAt (0.014 g, 0.10 mmol, 5 equiv) and PyAOP (0.055 g, 0.10 mmol, 5 equiv) were dissolved in DMF (1 mL). The mixture was added to the syringe containing the resin, this was followed

by addition of DIPEA (0.036 mL, 0.21 mmol, 10 equiv) and the mixture was left bubbling under Ar for 1 hr 30 min. The coupling solution was removed and resin was then washed with DMF (6 x 0.9 mL).

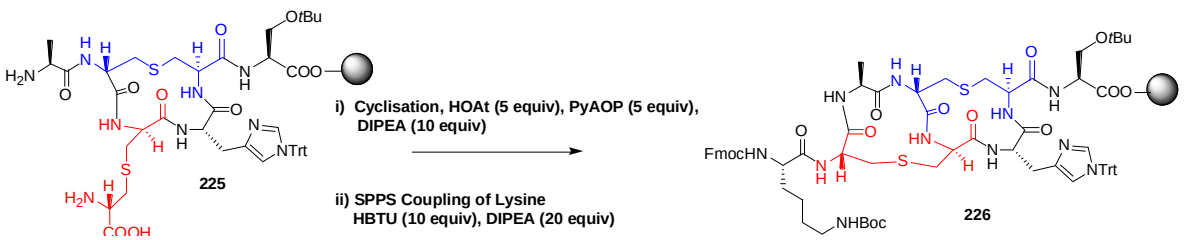
Fmoc-Ala-OH (0.065 g, 0.21 mmol, 10 equiv) and HBTU (0.079 g, 0.21 mmol, 10 equiv) were dissolved in DMF (1 mL). The mixture was added to the resin followed by DIPEA (0.073 mL, 0.42 mmol, 20 equiv) and bubbled with Ar for 1 hr 15 min. The coupling solution was removed and the resin was washed with DMF (6 x 0.9 mL) to give cyclised and elongated resin-bound peptide **224**.

Removal of the Teoc/TMSE and Fmoc protecting groups



The fully protected, resin-bound peptide **224** was treated with tetrabutylammonium fluoride (TBAF) (0.522 g, 2.00 mmol, 100 equiv) dissolved in DMF (2 mL). The mixture was gently bubbled with Ar for 1 hr. The resin was then washed successively with DMF (10 x 1.2 mL). Removal of the terminal Fmoc group with piperidine (40 % in DMF, 2 mL) with Ar bubbling for 30 min was followed by washing with DMF (6 x 1.2 mL), leading to the cyclization substrate **225**.

Cyclisation to form ring D of nisin and SPPS elongation

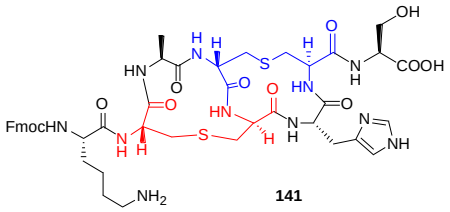


During Fmoc deprotection the cyclization reagents were prepared. HOAt (0.014 g, 0.10 mmol, 5 equiv) and PyAOP (0.055 g, 0.10 mmol, 5 equiv) were dissolved in DMF (1 mL).

The mixture was added to the syringe containing the resin. This was followed by addition of DIPEA (0.036 mL, 0.21 mmol, 10 equiv) and the mixture was bubbled with Ar for 1 hr 30 min. The coupling solution was removed and resin was then washed with DMF (6 x 0.9 mL).

Fmoc-Lys(Boc)-OH (0.098 g, 0.21 mmol, 10 equiv) and HBTU (0.079 g, 0.21 mmol, 10 equiv) were dissolved in DMF (1 mL). The mixture was added to the resin, followed by DIPEA (0.073 mL, 0.42 mmol, 20 equiv) and bubbled with Ar for 1 hr 15 min. The coupling solution was removed and the resin was washed with DMF (6 x 0.9 mL).

Deprotection, cleavage from the resin and purification to give bicyclic analogue of nisin rings D and E 141



141

A solution of cleavage cocktail (TFA (94 %), H_2O (2.5 %), TIPS (1 %) and EDT (2.5 %), 2mL) was added to the resin-bound peptide and gently bubbled with Ar for 3 hr. The cleavage cocktail was collected in a Falcon tube. Diethyl ether was added until the crude peptide precipitated. The solution was centrifuged at 4000 rpm for 7 min, after which the ether layer was decanted. The centrifuging and decanting procedure was repeated, washing on each occasion with diethyl ether (5 x 13 mL). The precipitate was dissolved in water, and the solution was freeze dried overnight, yielding the crude peptide as a yellow fluffy solid (20 mg, 0.019 mmol, 94.5 % crude yield).

The peptide proved difficult to purify by HPLC due to tailing of impurities, and an alternative strategy involving two SPE columns was therefore developed. The first column was carried out using a Bond Elute C18 column with a capacity of 100 mg in 1 mL. The column was loaded with the crude peptide dissolved in water (1 mL) and the eluant collected as a flow through fraction. The column was then washed with water (3 x 1 mL) and the eluant collected as a second fraction. The column was then eluted with a water: acetonitrile: TFA mixture: Solvent A, H_2O/0.5% TFA; Solvent B, acetonitrile/0.5 % TFA. A mixture of 95 % A and 5 % B (1 mL) was applied to the column and the eluant collected as the third fraction. This process was repeated with successive batches (1 mL) of eluant in which the fraction of

Solvent B was increased by 5 % each time until a fraction containing 60 % Solvent B and 40 % Solvent A was reached. The percentage of Solvent B was then increased by 10 % in each successive batch of eluant, and the eluant was collected until 100 %. Each of the 1 mL fractions were collected and freeze-dried separately to give cream fluffy solids. MS analysis indicated that the majority of the desired peptide was found in the fraction collected at 25 % acetonitrile, 75 % water.

The second purification was carried out using a Bond Elute C18 column with a capacity of 50 mg in 1 mL to further purify the fraction collected at 25 % of the first column. The column was loaded with the 25 % fraction dissolved in D_2O (1 mL). The column was first eluted with a mixture of 17 % CD_3CN, 83% D_2O and 0.15 % TFA (2 x 0.5 mL) and the eluant collected. This process was repeated with successive batches of eluant (2 x 0.5 mL) in which the fraction of CD_3CN was increased by 2 % each time until a fraction of 29 % CD_3CN, 71% D_2O, (0.15% TFA) was reached. The fraction of CD_3CN was then increased by 10 % each time until 100 % CD_3CN (0.15% TFA) was reached. MS analysis indicated that the majority of the desired peptide was found in the fraction collected at 23 % acetonitrile, 77% water. A yield of less than 0.1 mg of the peptide was obtained, which was not sufficient for detailed NMR analysis.

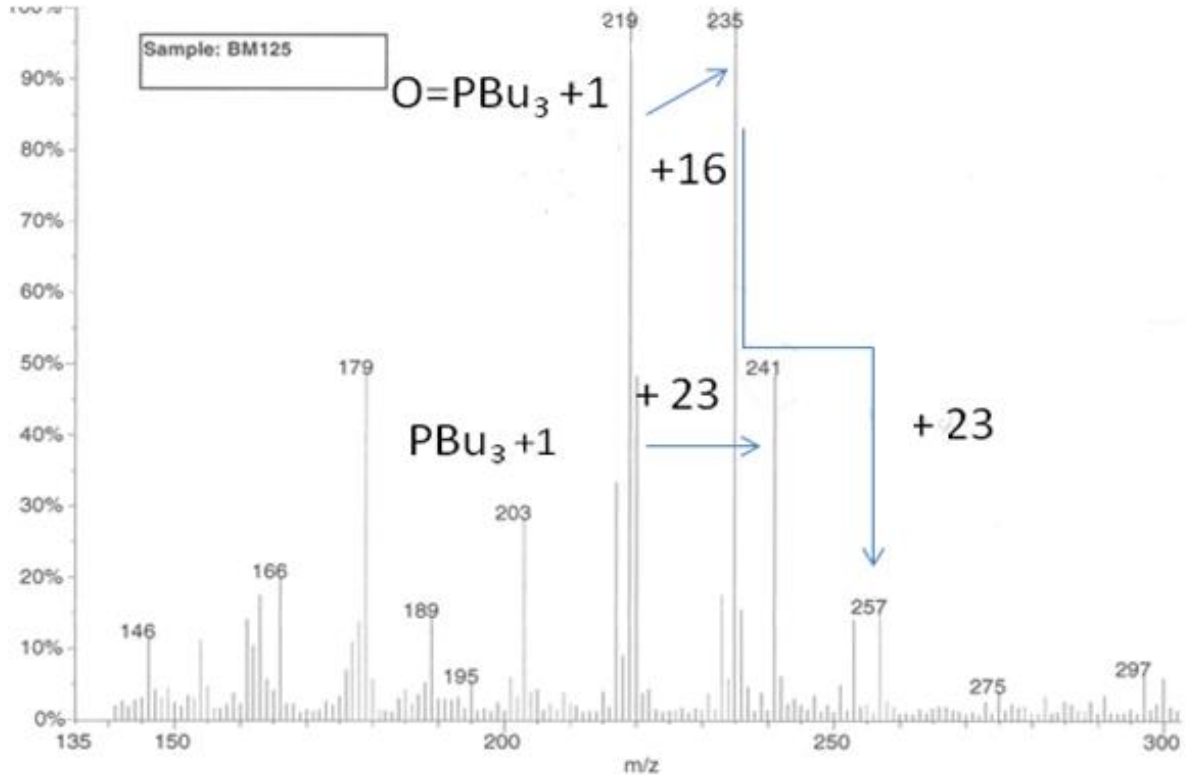
Analytical HPLC (Onyx Monolithic C18) $\mathbf{R_T}$ 19.4 min; A: H_2O/0.5 % TFA; B: acetonitrile/0.5 % TFA; gradient 10 % - 60 % B over 30 min.

HRMS (ES^+), *m/z*: Found: 1008.3724 $[M+H]^+$. $C_{45}H_{56}N_{11}O_{12}S_2$ requires 1008.3708.

7. Appendices

Appendix 1

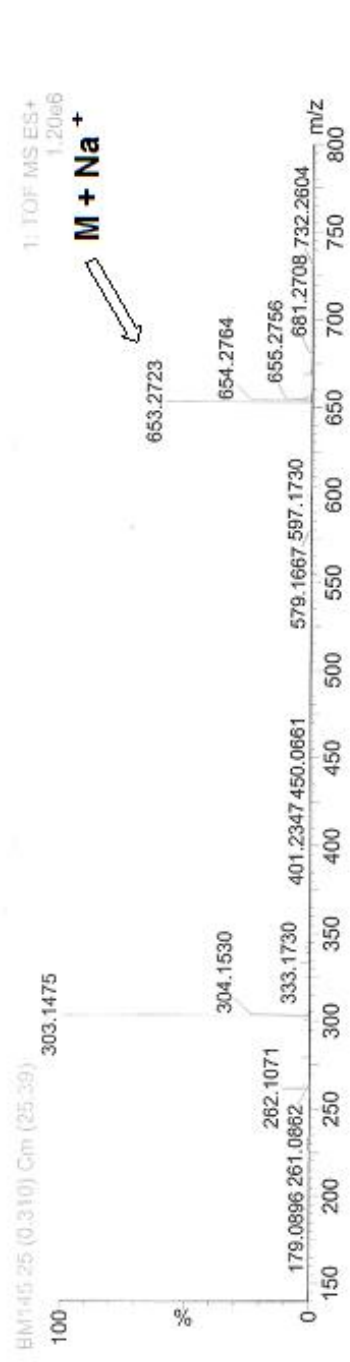
Mass spectrum for **167** showing the presence of oxidised tributylphosphine.

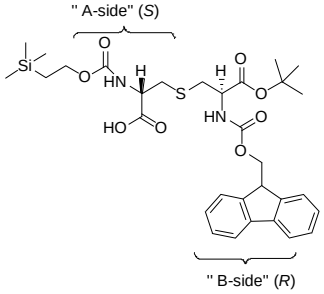


Appendix 2

Mass spectrum of (Teoc, OH / Fmoc, *t*Bu) lanthionine after removal of allyl group and protected with TMSE group:

(3-[2-<u>tert</u>-Butoxycarbonyl-2-(9H-fluoren-9-ylmethoxycarbonylamino)-ethylsulfanyl]-2-(2-trimethylsilanyl-ethoxycarbonylamino)-propionic acid).

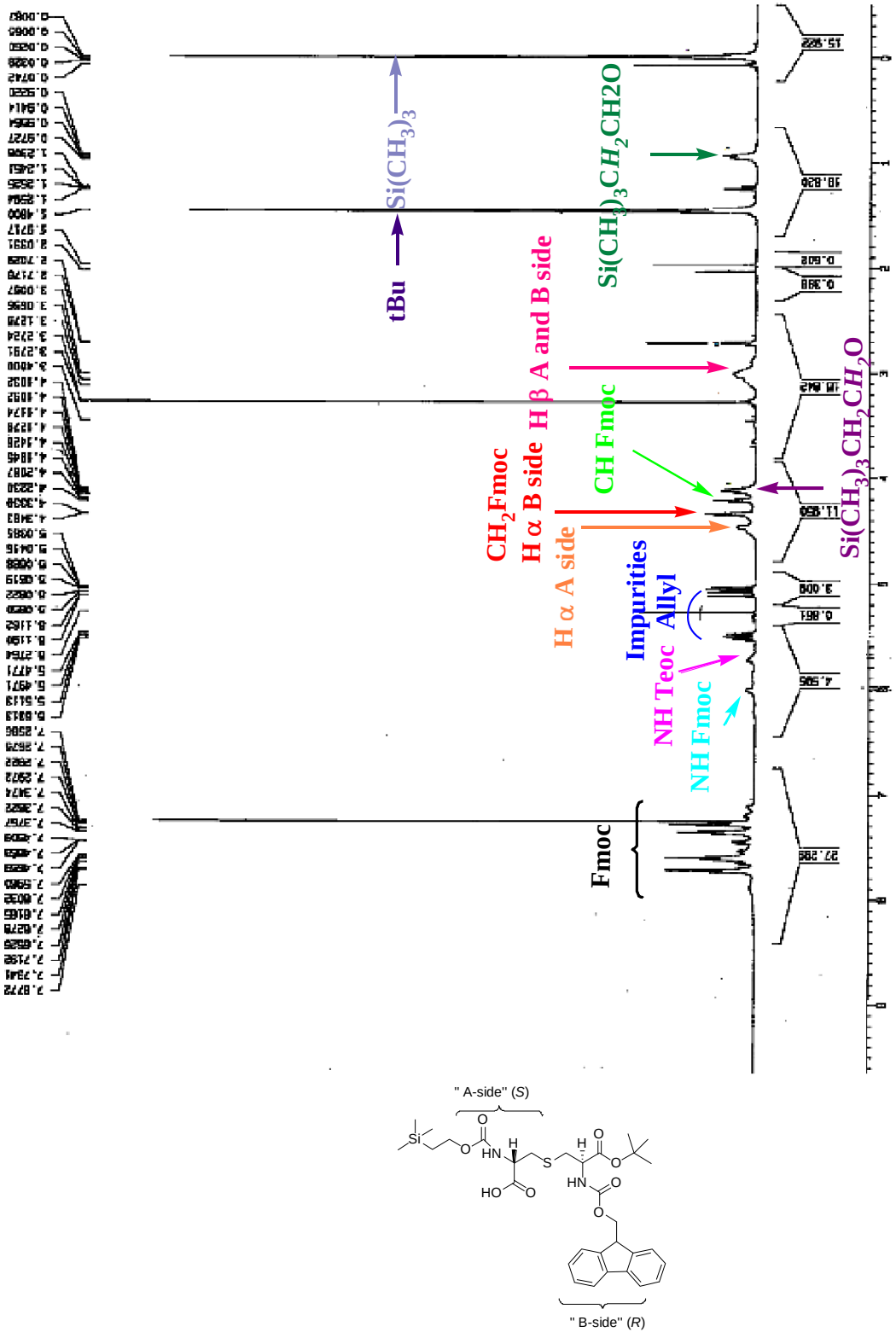




Appendix 3

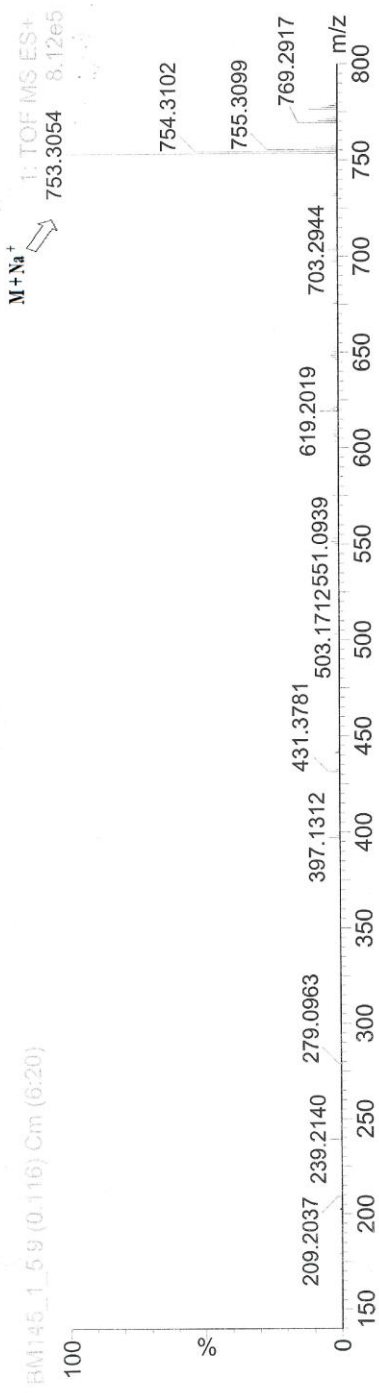
^{1}H NMR of (Teoc, OH / Fmoc, *t*Bu) lanthionine, co-eluting with an allylic impurity:

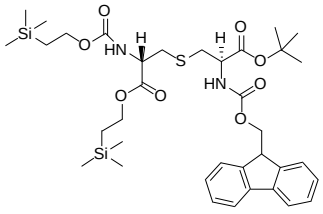
(3-[2-tert-Butoxycarbonyl-2-(9H-fluoren-9-ylmethoxycarbonylamino)-ethylsulfanyl]-2-(2-trimethylsilanyl-ethoxycarbonylamino)-propionic acid).



Appendix 4

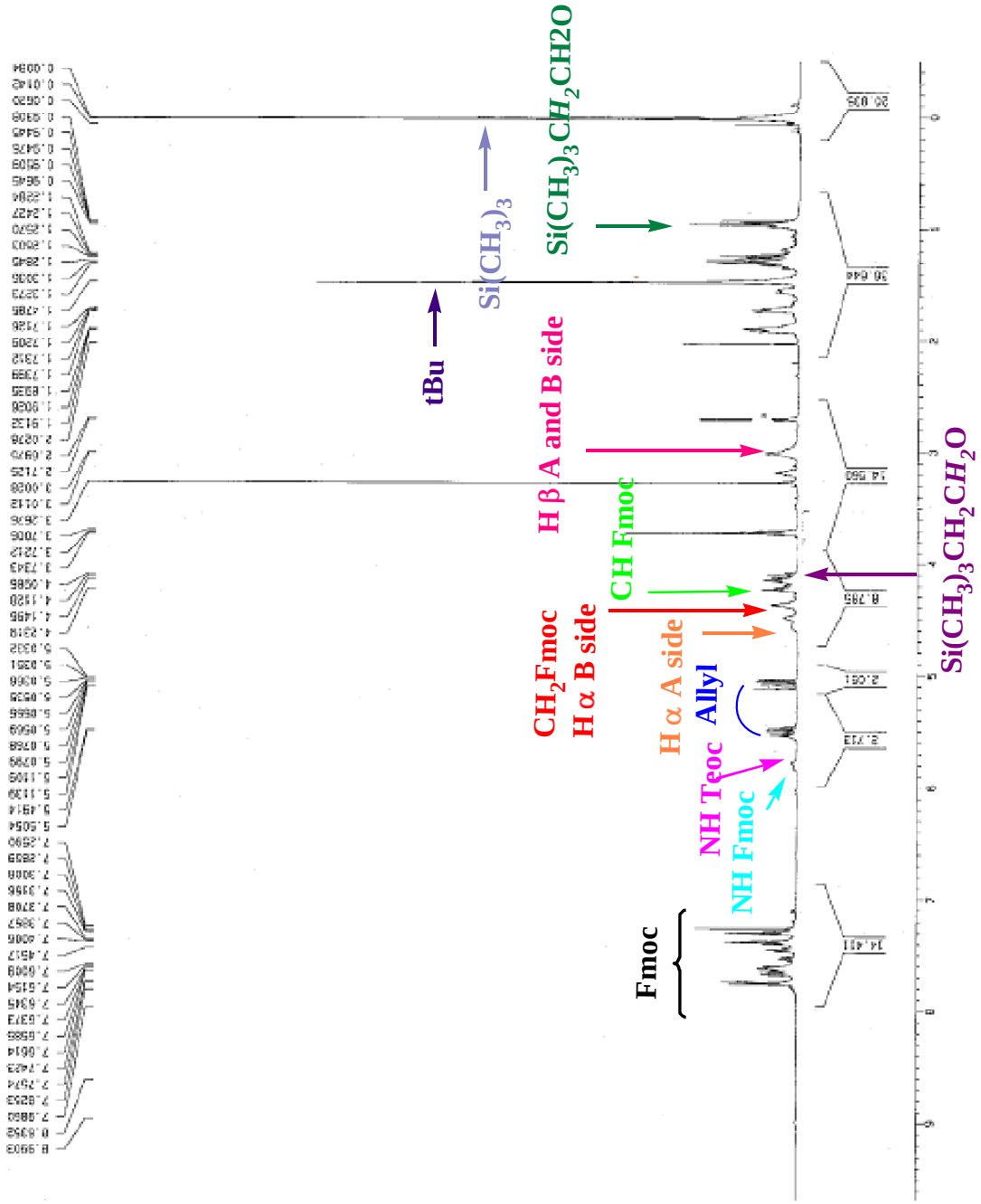
Mass spectrum of (Teoc, TMSE / Fmoc, *t*Bu) lanthionine **176**.

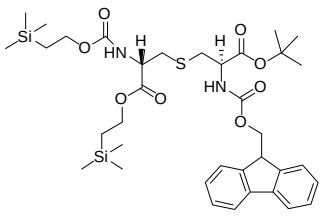




Appendix 5

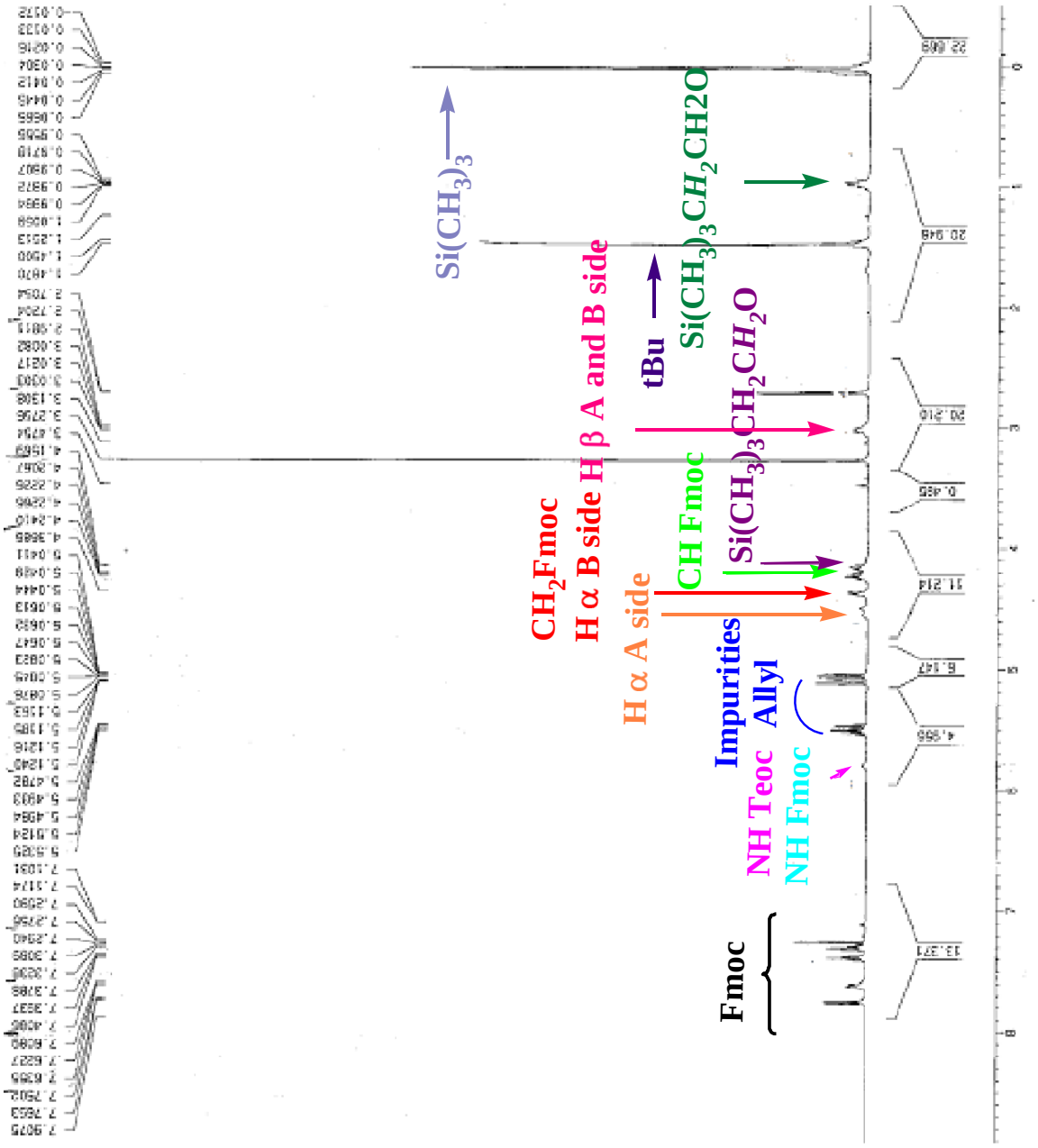
^{1}H NMR of (Teoc, TMSE / Fmoc, *t*Bu) lanthionine **176** before purification.

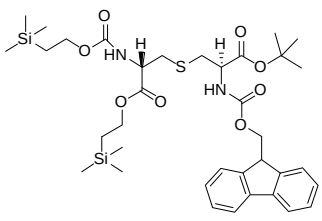




Appendix 6

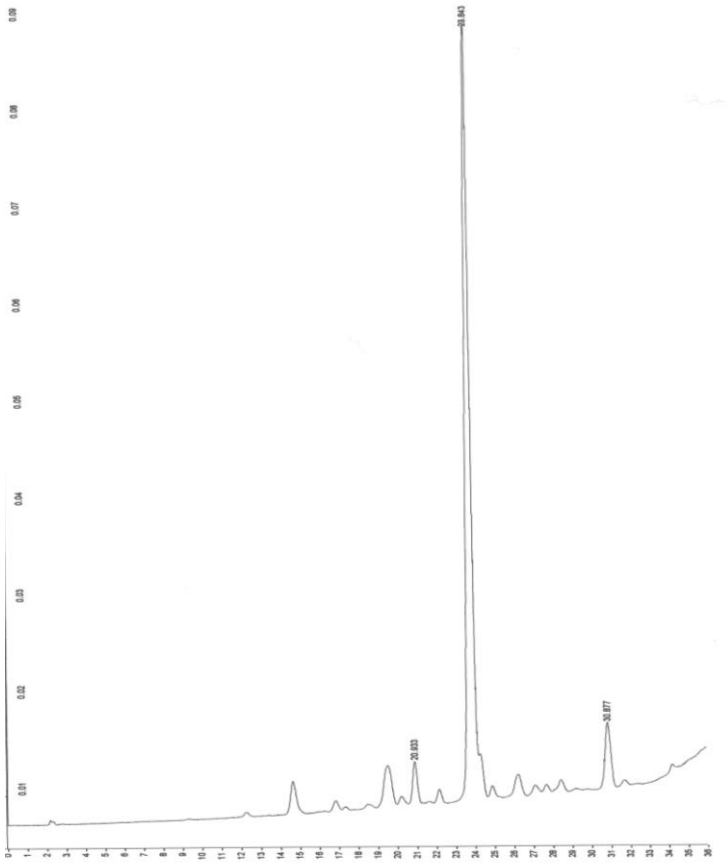
^{1}H NMR of (Teoc, TMSE / Fmoc, *t*Bu) lanthionine **176** after purification by flash column chromatography.

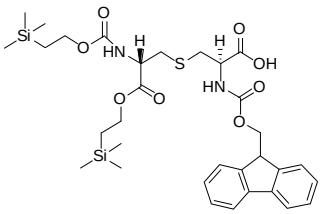




Appendix 7

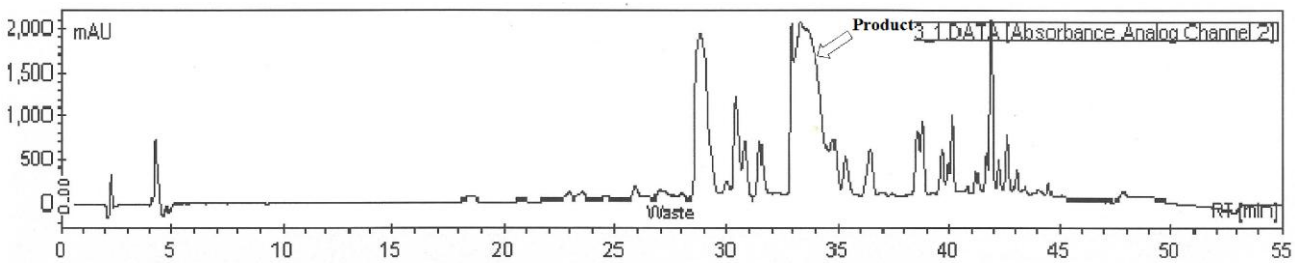
Analytical HPLC of crude (Teoc, TMSE / Fmoc, OH) lanthionine **169**.





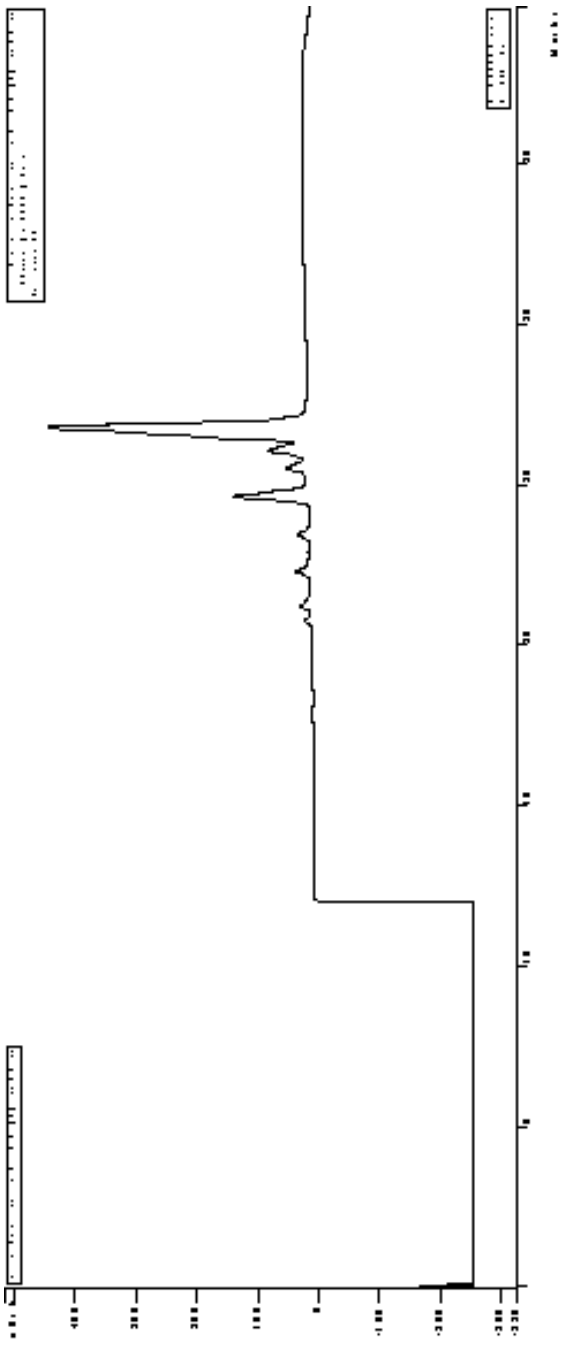
Appendix 8

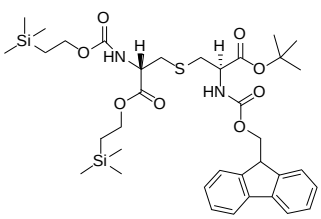
Preparative HPLC of (Teoc, TMSE / Fmoc, OH) lanthionine **169**.



Appendix 9

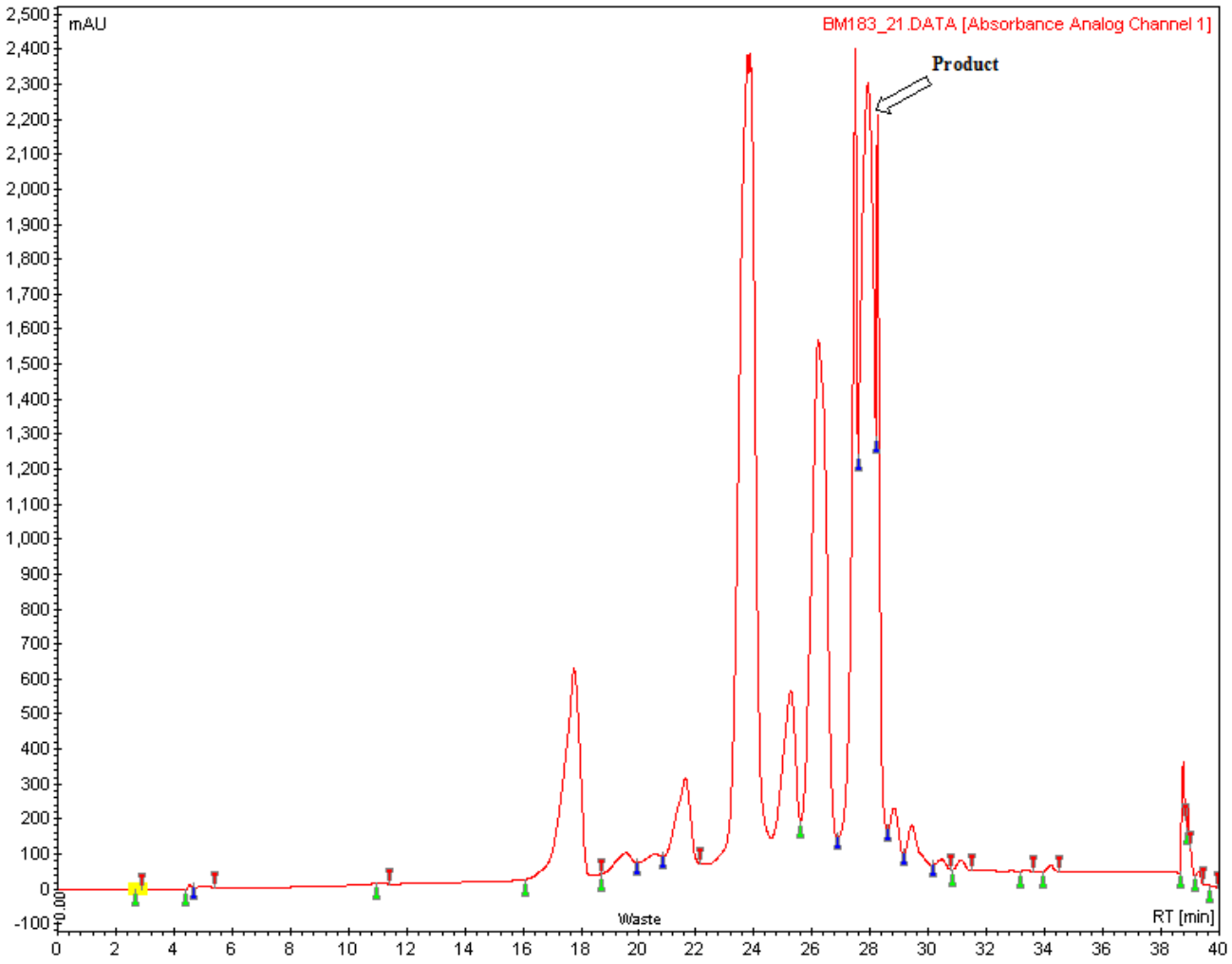
Analytical HPLC of (Teoc, TMSE / Fmoc, *t*Bu) lanthionine **176**.

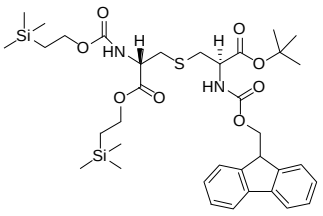




Appendix 10

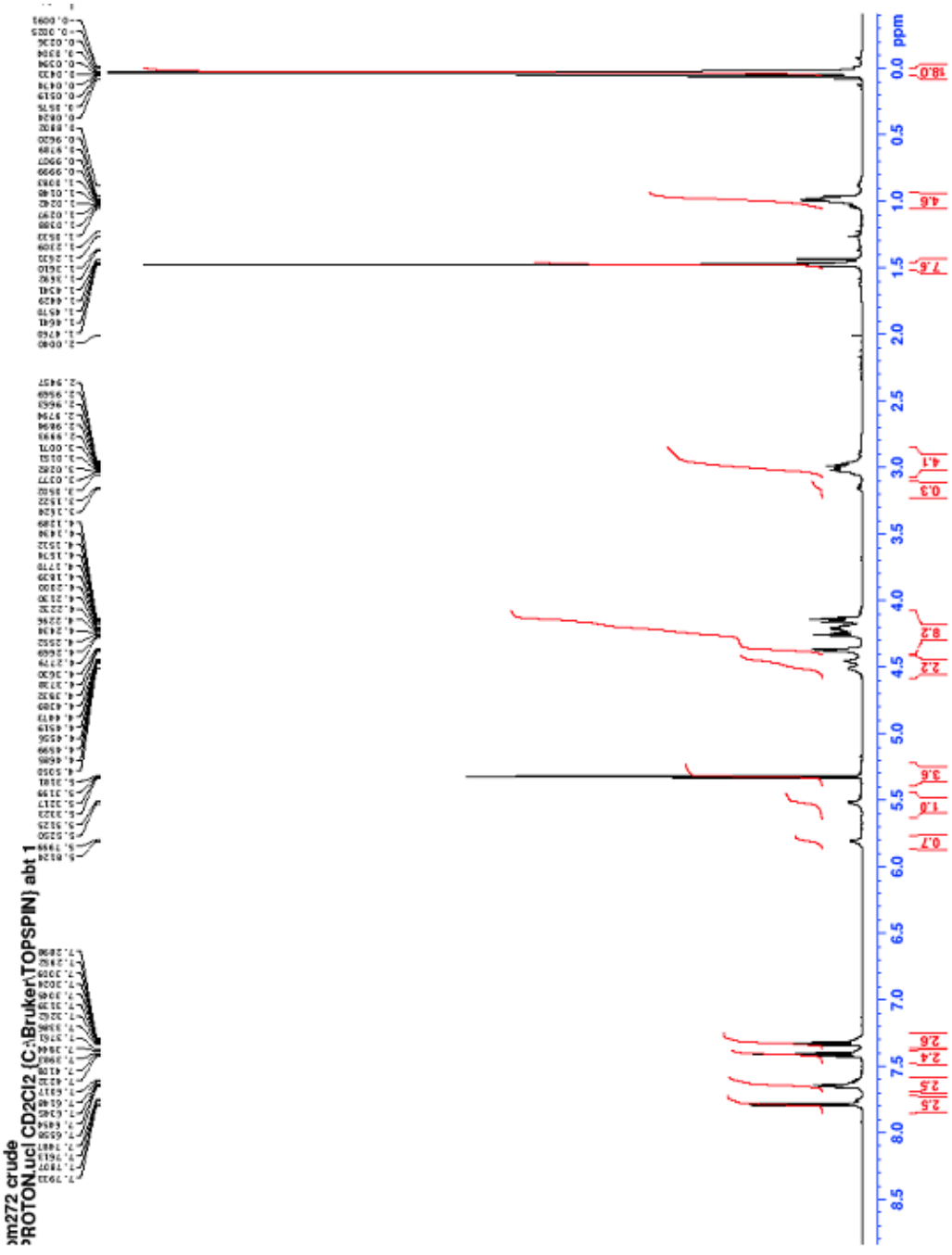
Preparative HPLC of (Teoc, TMSE / Fmoc, *t*Bu) lanthionine **176**.

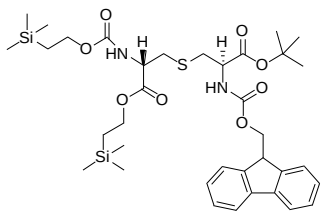




Appendix 11

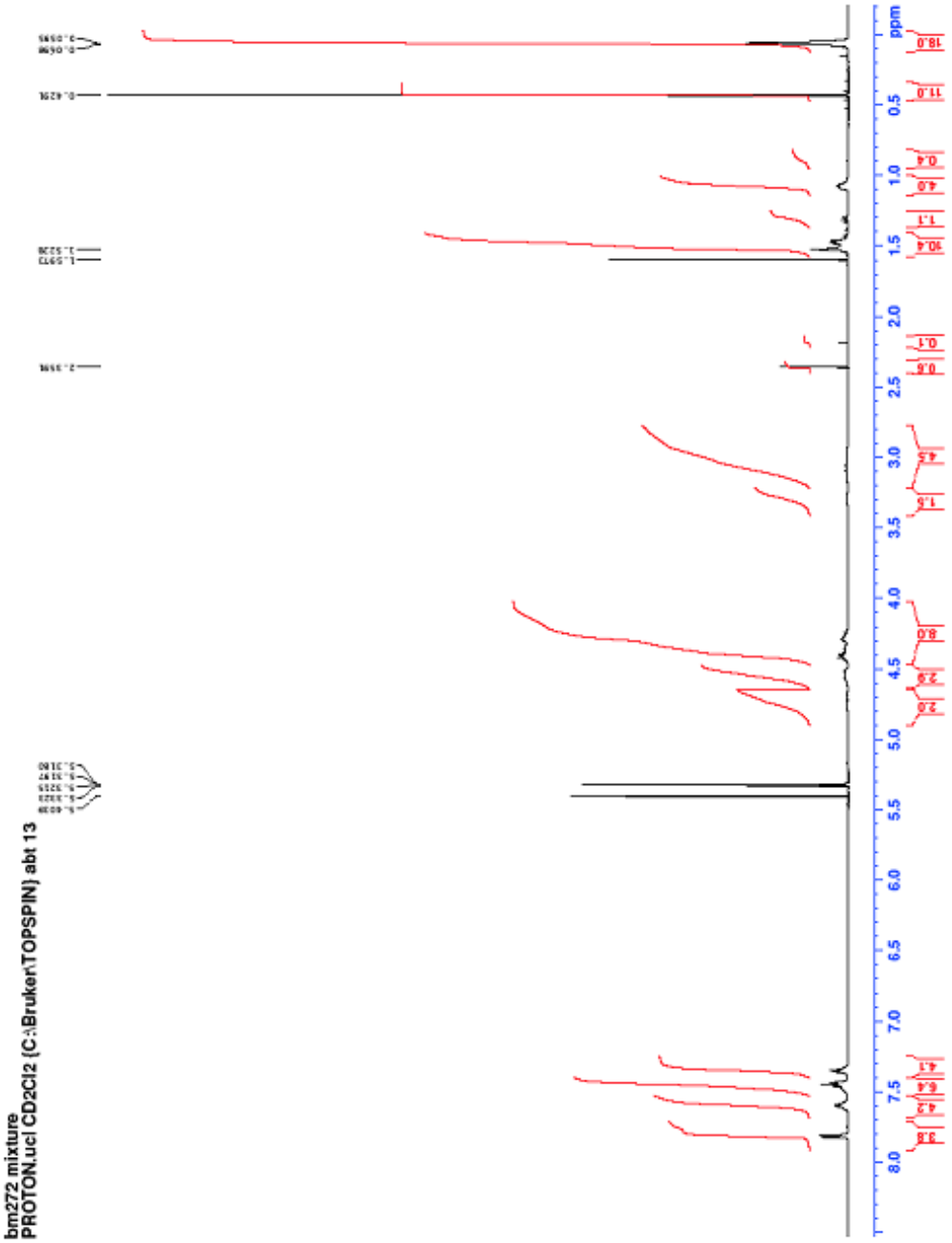
Proton NMR of (Teoc, TMSE / Fmoc, *t*Bu) lanthionine **176**.

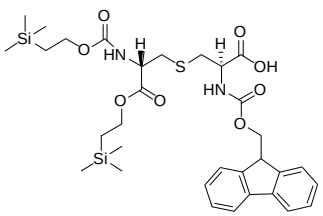




Appendix 12

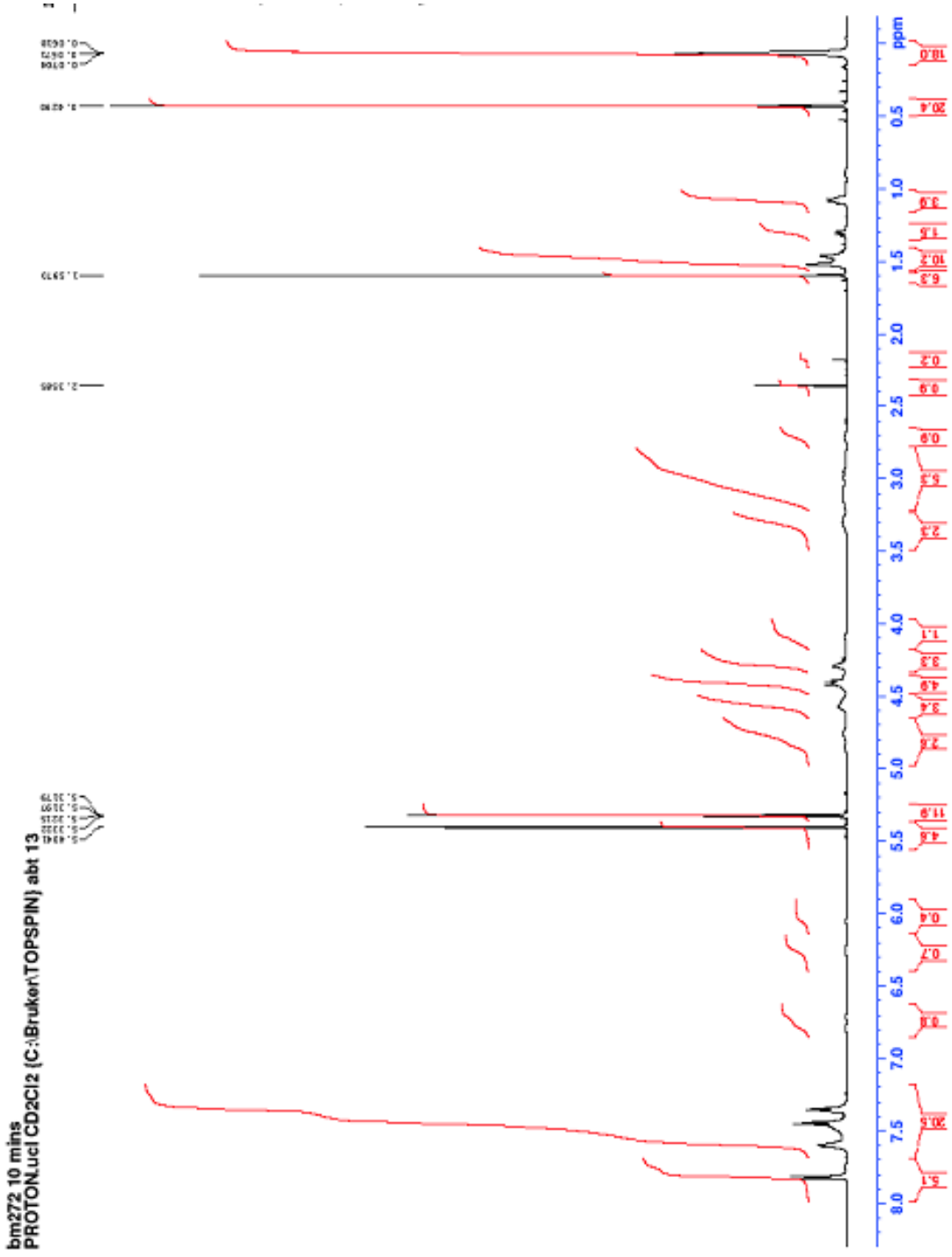
Proton NMR of the reaction mixture: during the synthesis of the (Teoc, TMSE / Fmoc, OH) lanthionine **169**.

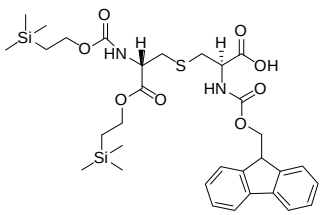




Appendix 13

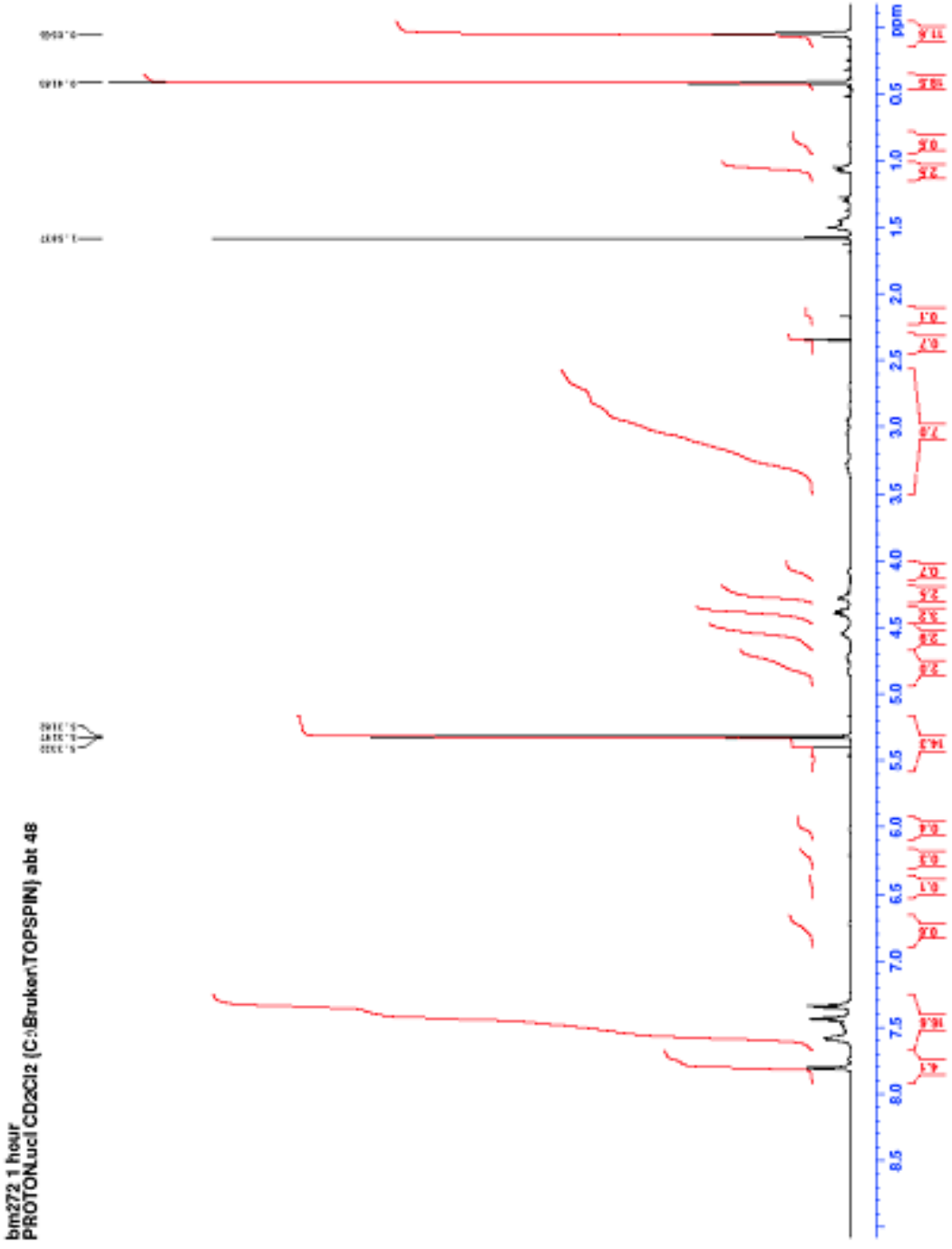
Proton NMR at 10 min: during the synthesis of the (Teoc, TMSE / Fmoc, OH) lanthionine **169**.

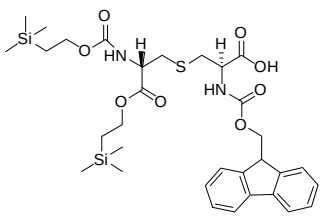




Appendix 14

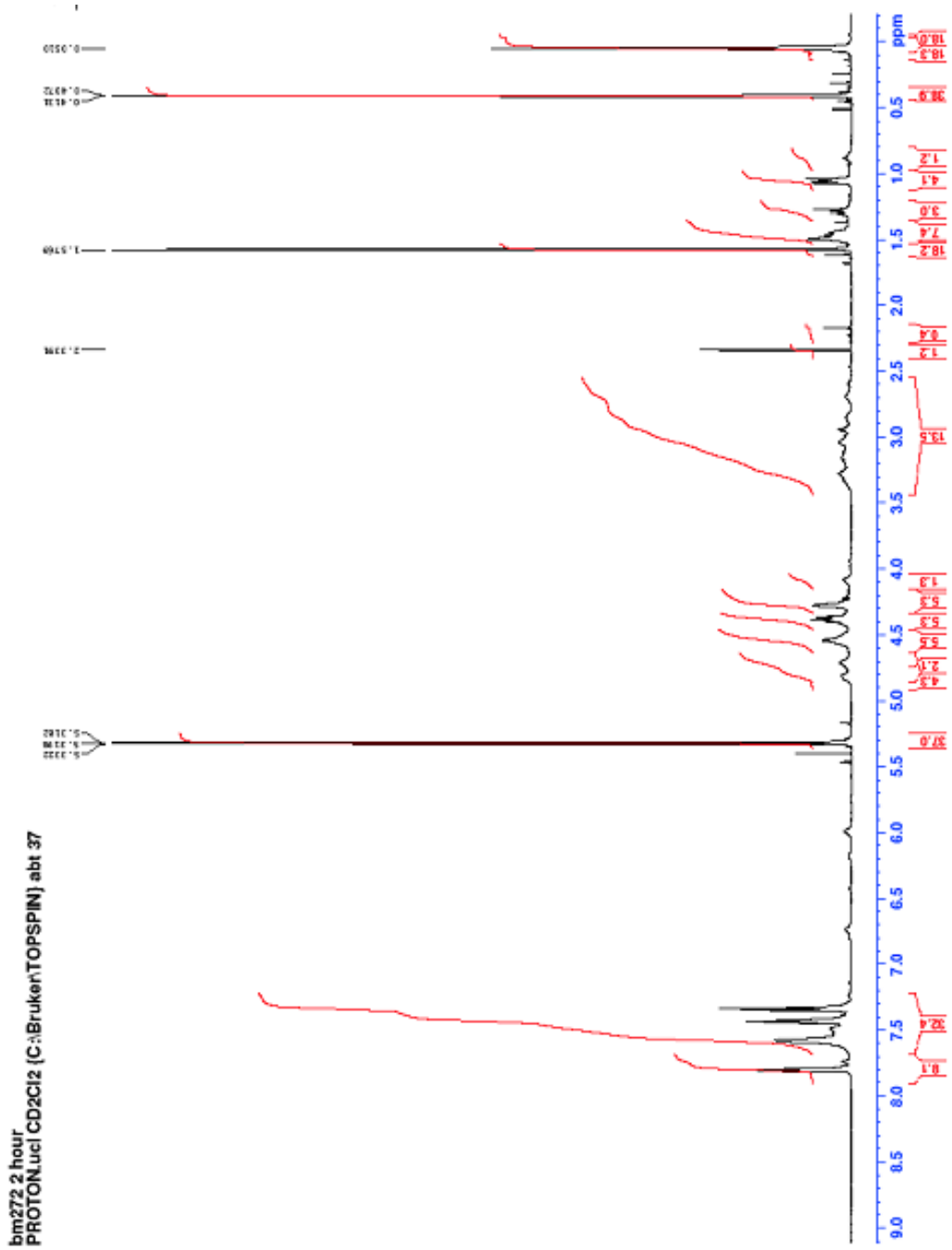
Proton NMR at 1 hour: during the synthesis of the (Teoc, TMSE / Fmoc, OH) lanthionine **169**.

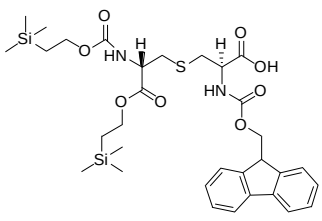




Appendix 15

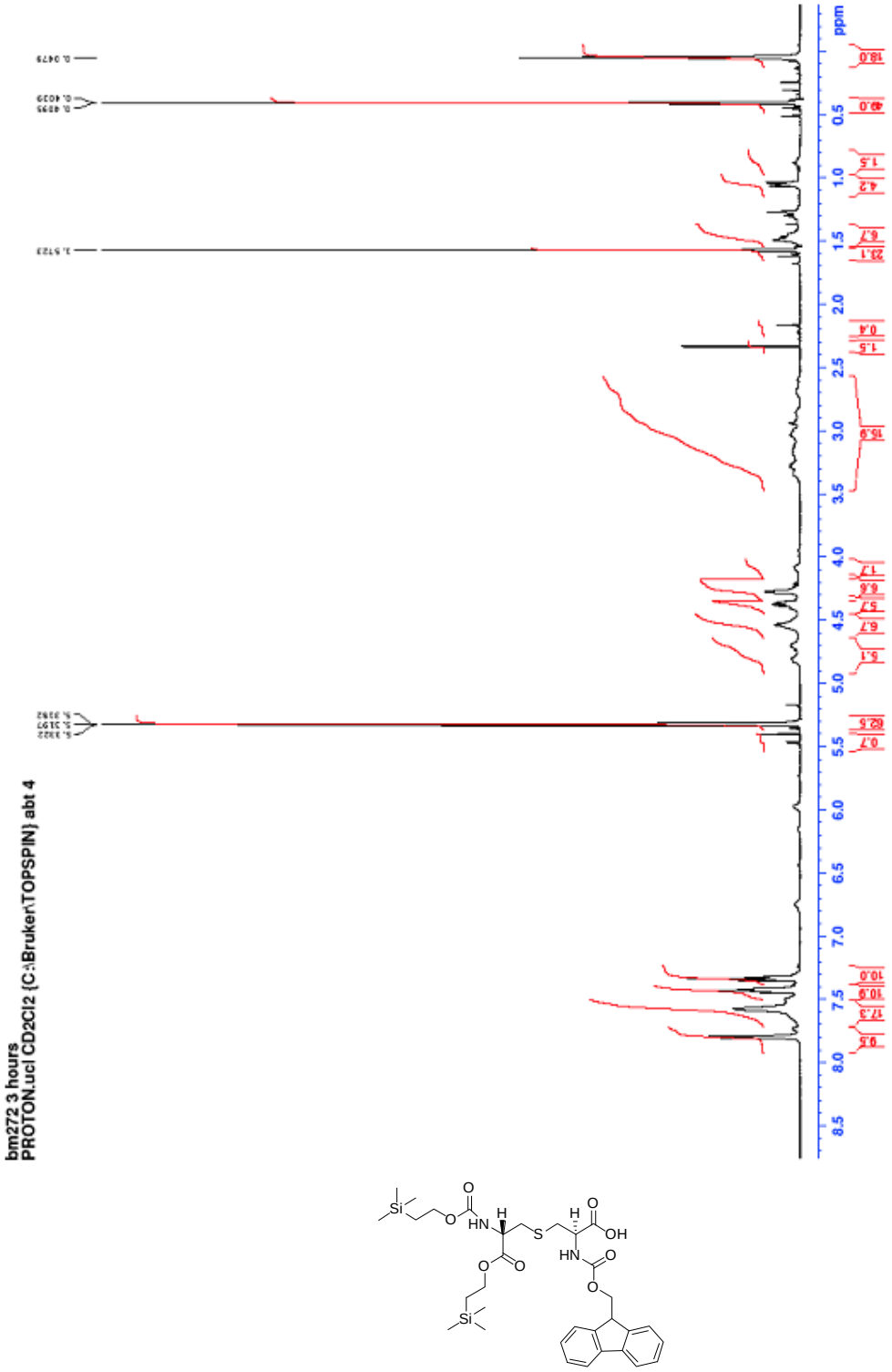
Proton NMR at 2 hour: during the synthesis of the (Teoc, TMSE / Fmoc, OH) lanthionine **169**.





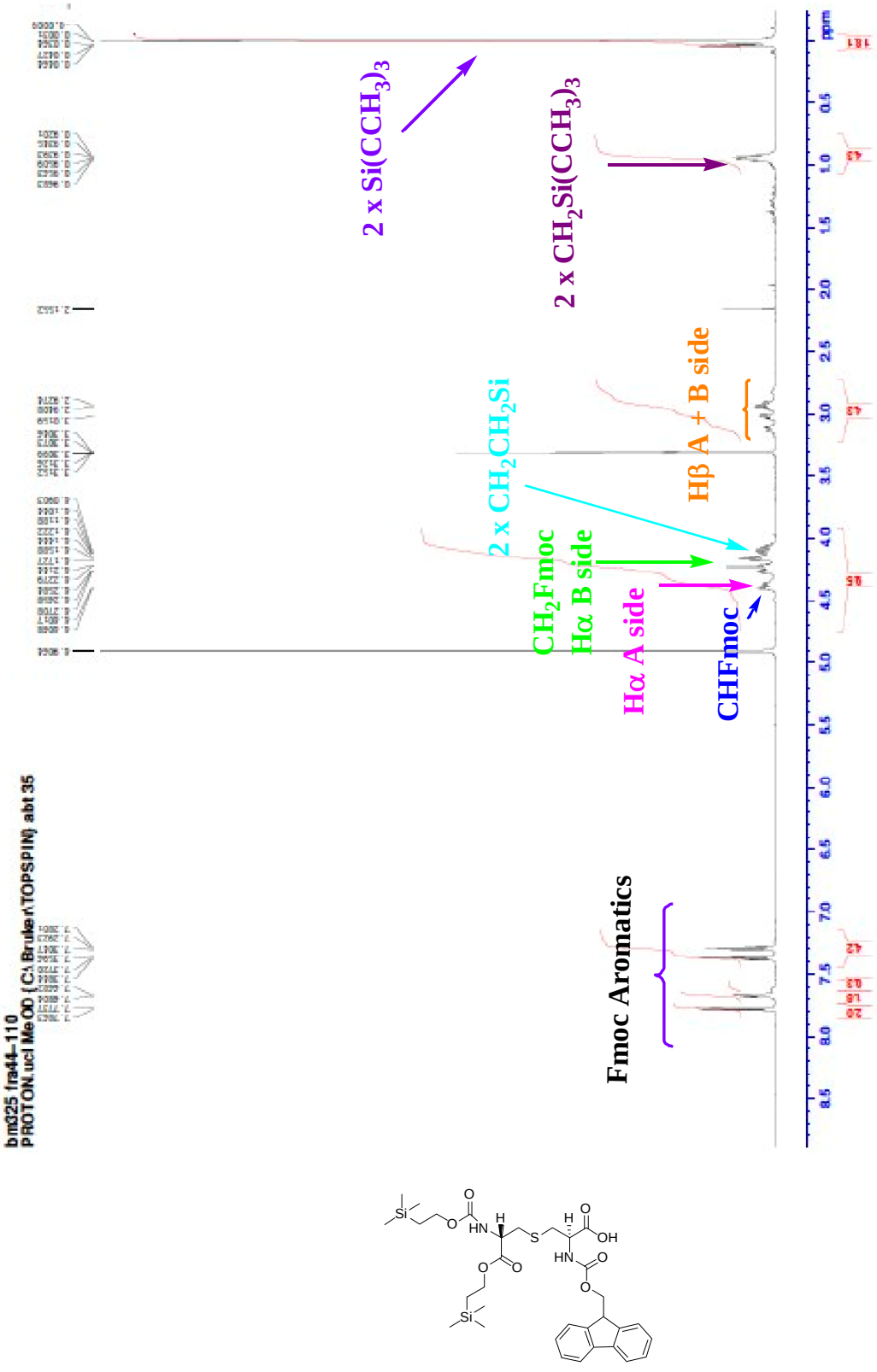
Appendix 16

Proton NMR at 3 hour: during the synthesis of the (Teoc, TMSE / Fmoc, OH) lanthionine **169**.



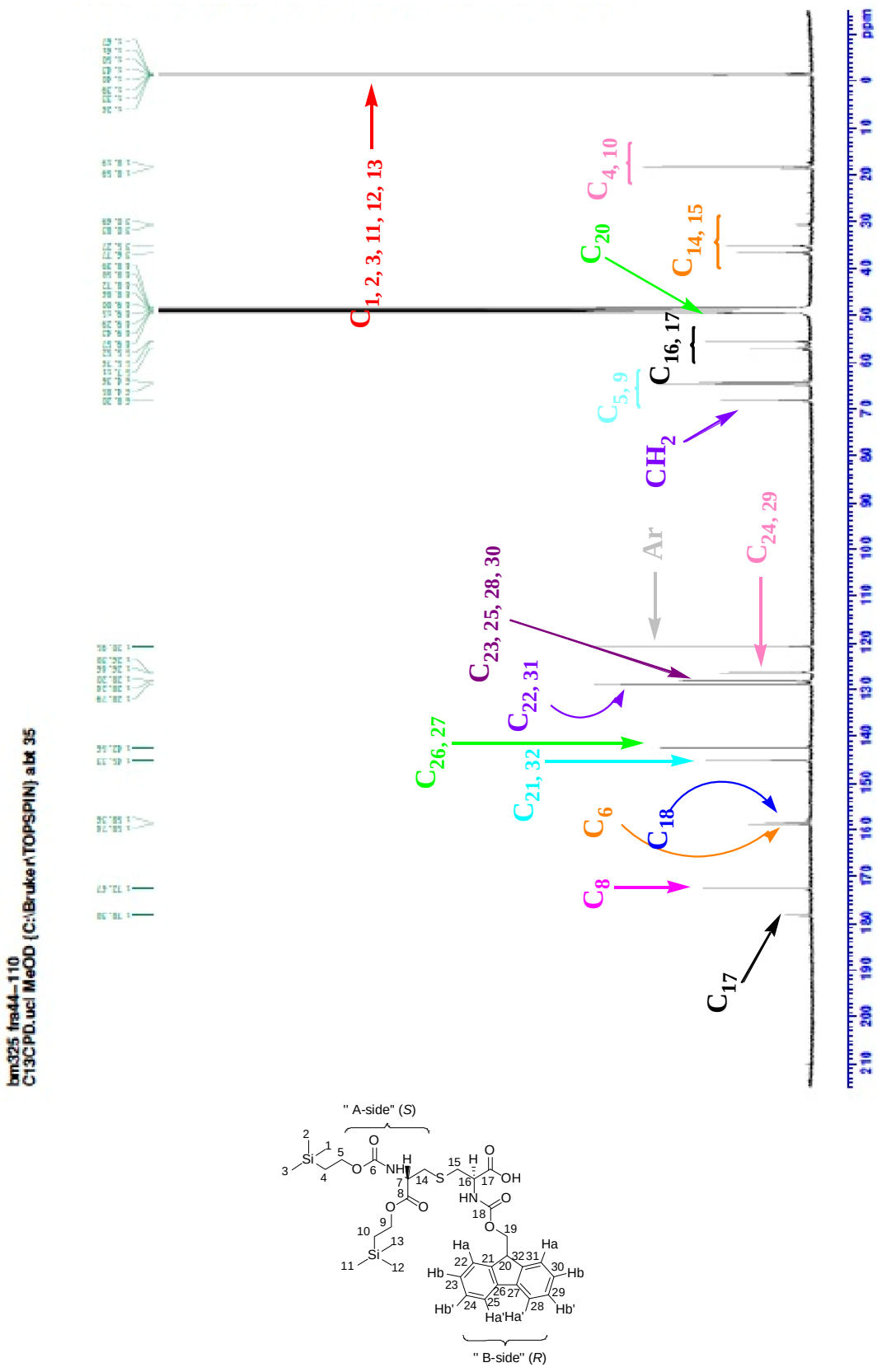
Appendix 17

Proton NMR of (Teoc, TMSE / Fmoc, OH) lanthionine **169**.

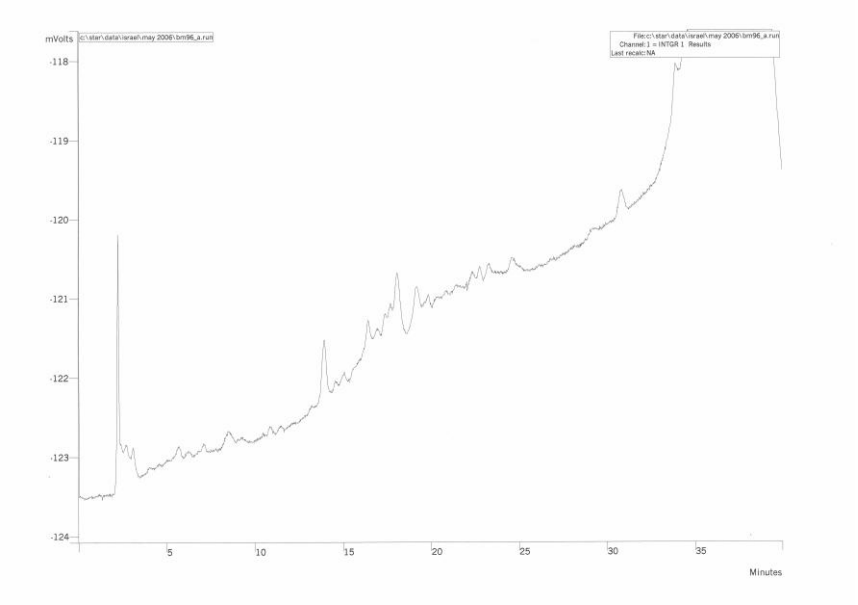


Appendix 18

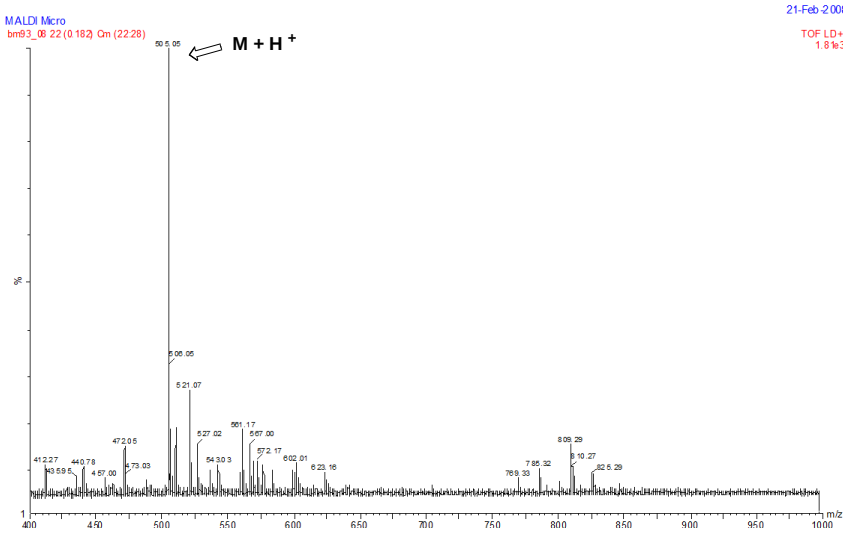
Carbon NMR of (Teoc, TMSE / Fmoc, OH) lanthionine **169**.



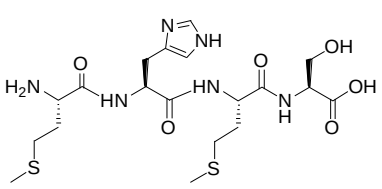
Appendix 19



(a) Analytical HPLC of model peptide **198**: Met-His-Met-Ser.

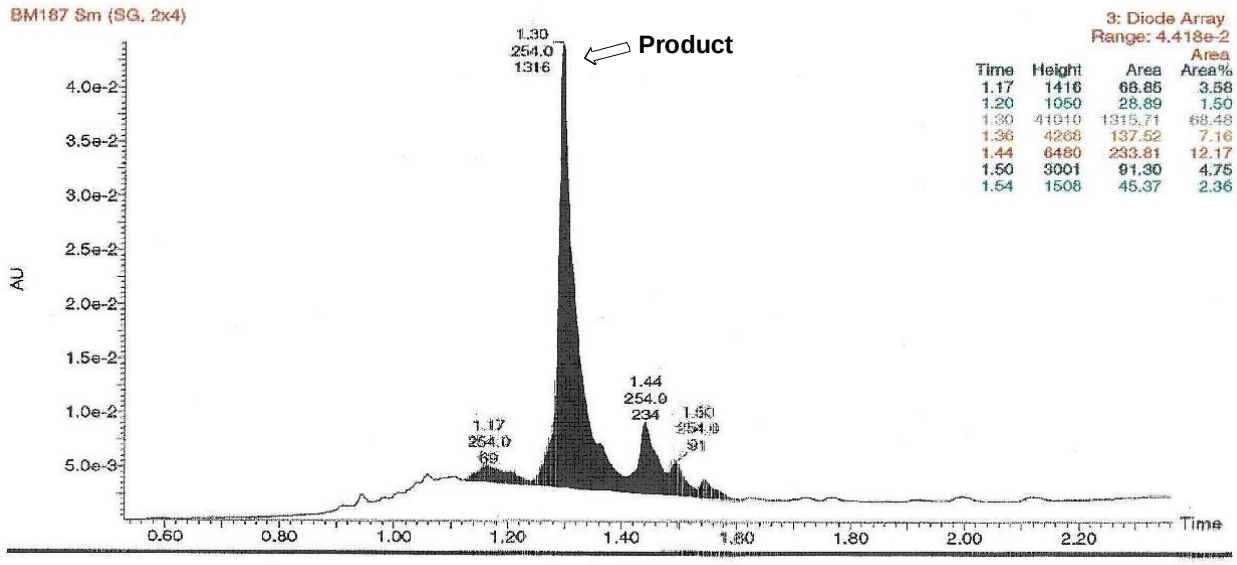


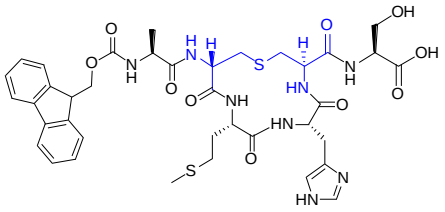
(b) Mass spectrum of model peptide **198**: Met-His-Met-Ser.



Appendix 20

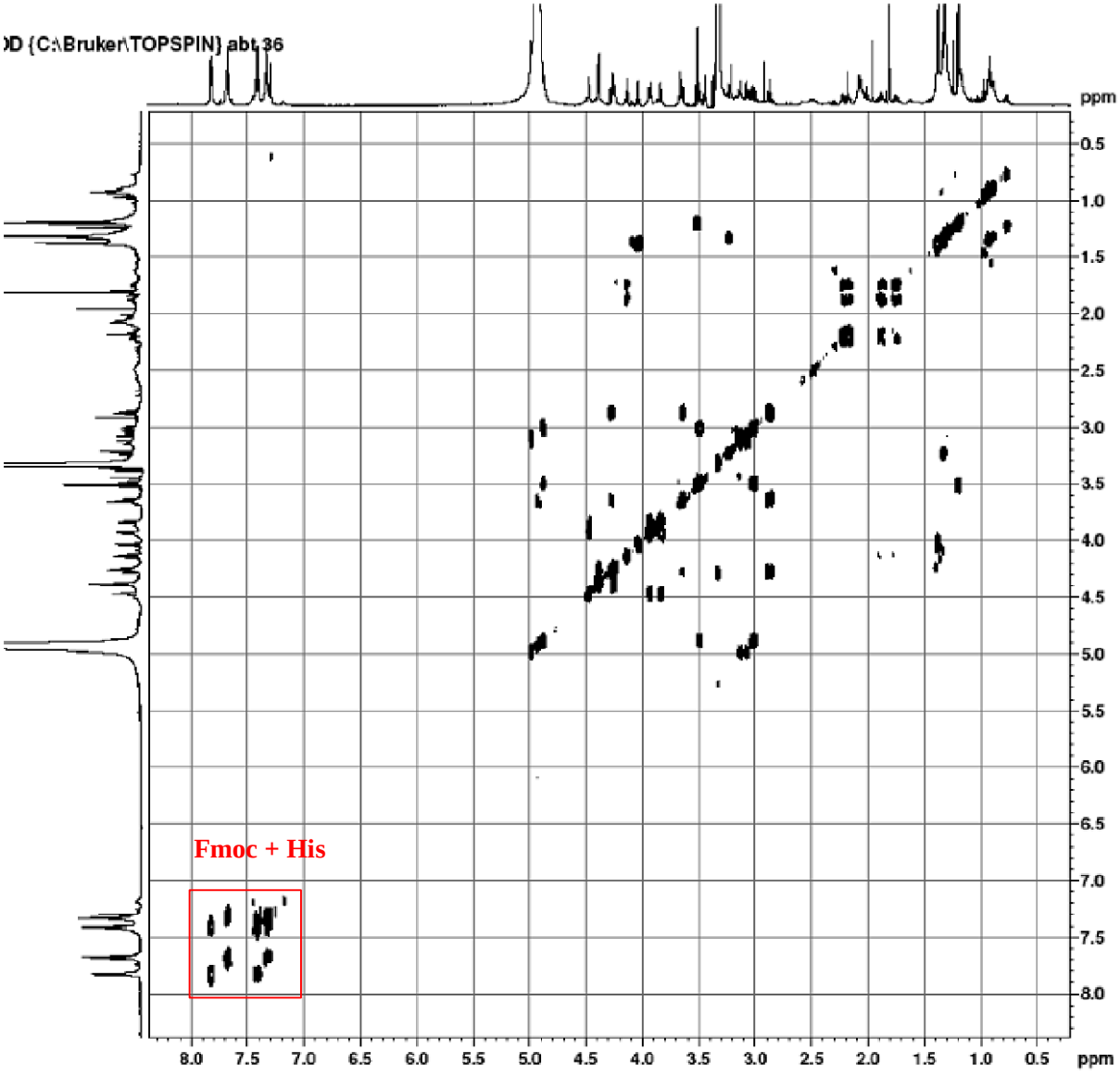
Purity test by LCMS on a third attempt on sample of analogue of ring E of nisin **210**.

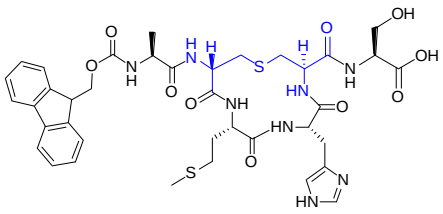




Appendix 21

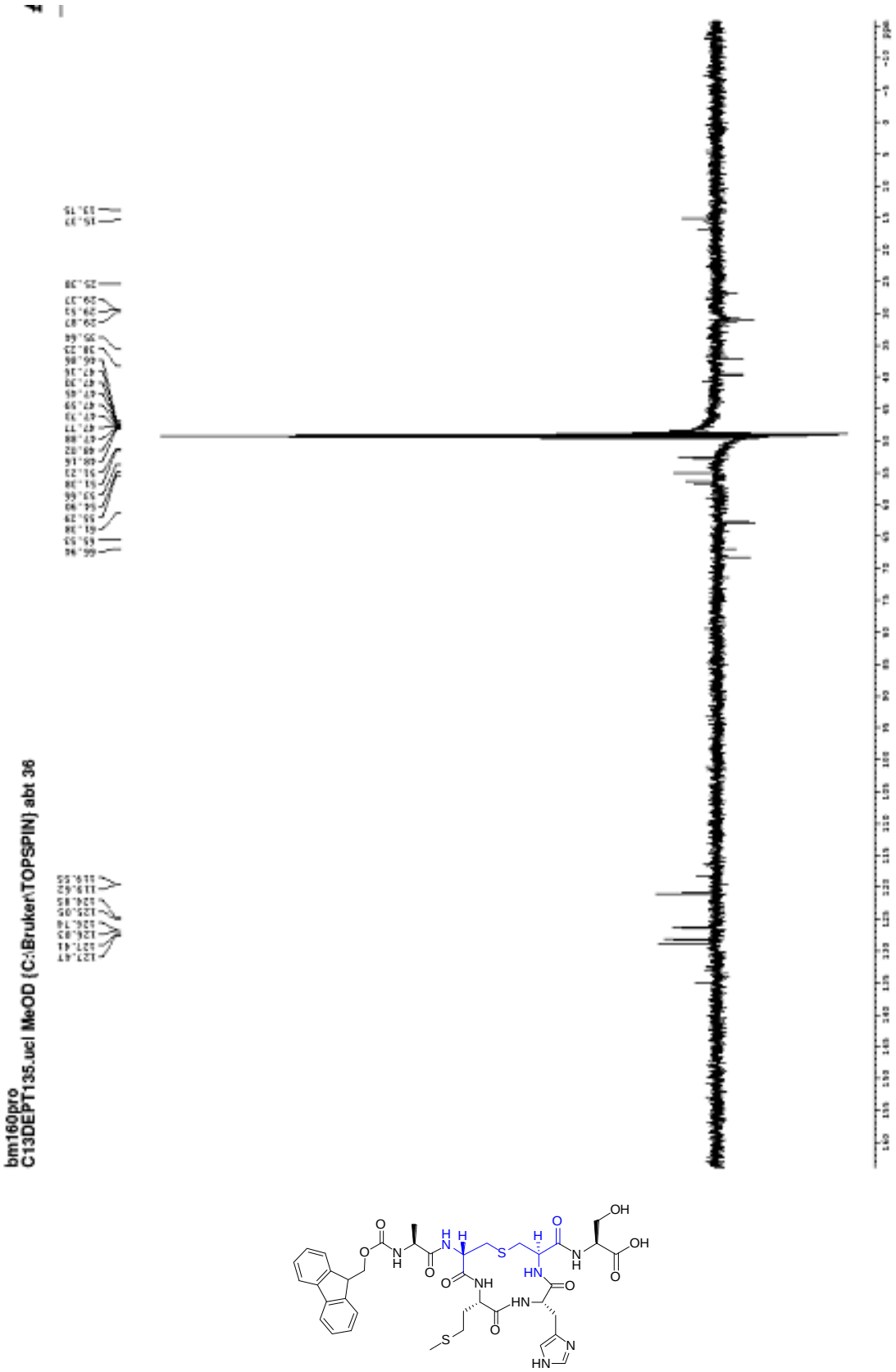
COSY NMR of an analogue of ring E of nisin **210**.





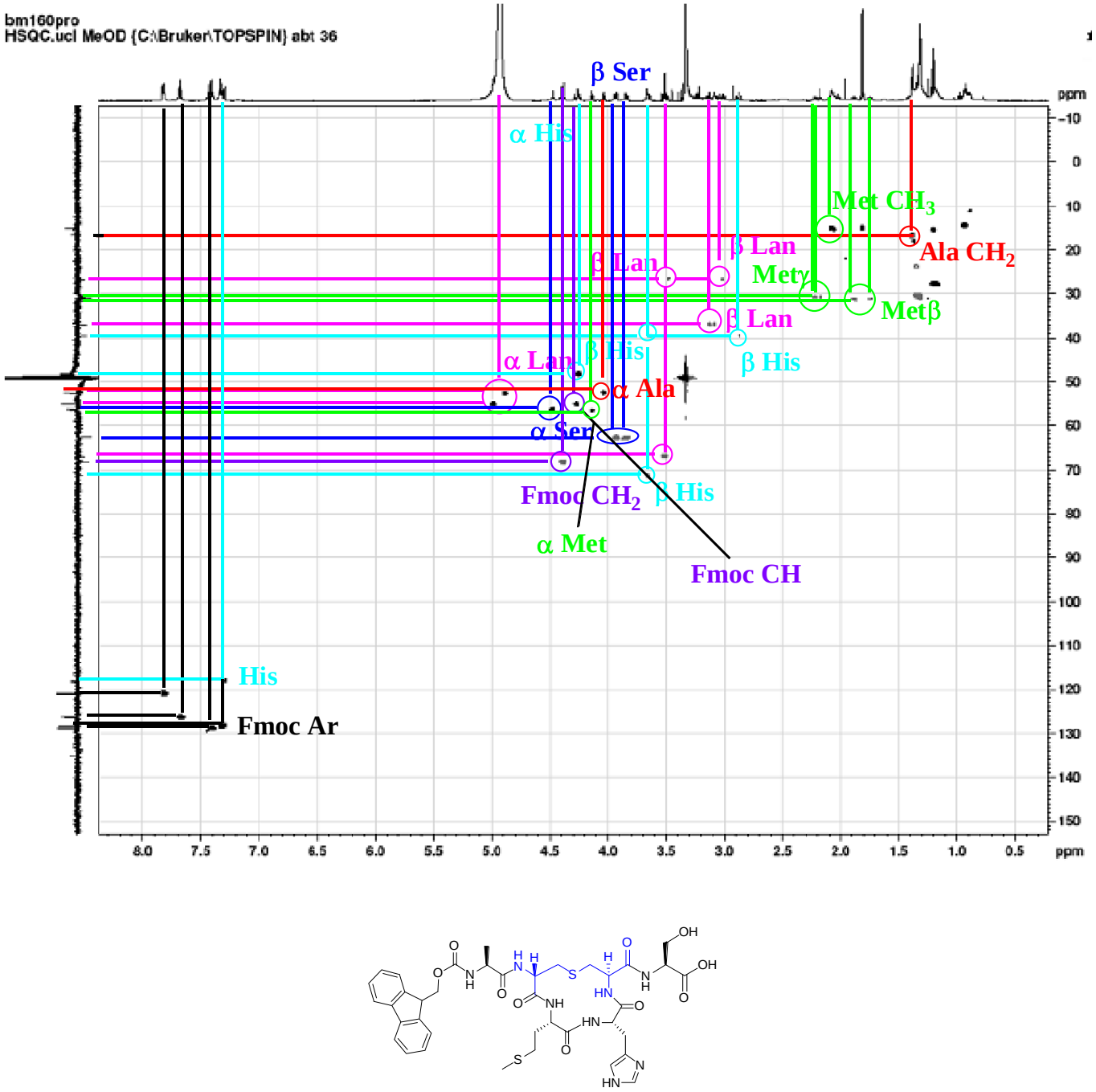
Appendix 22

Carbon DEPT NMR of an analogue of ring E of nisin **210**.



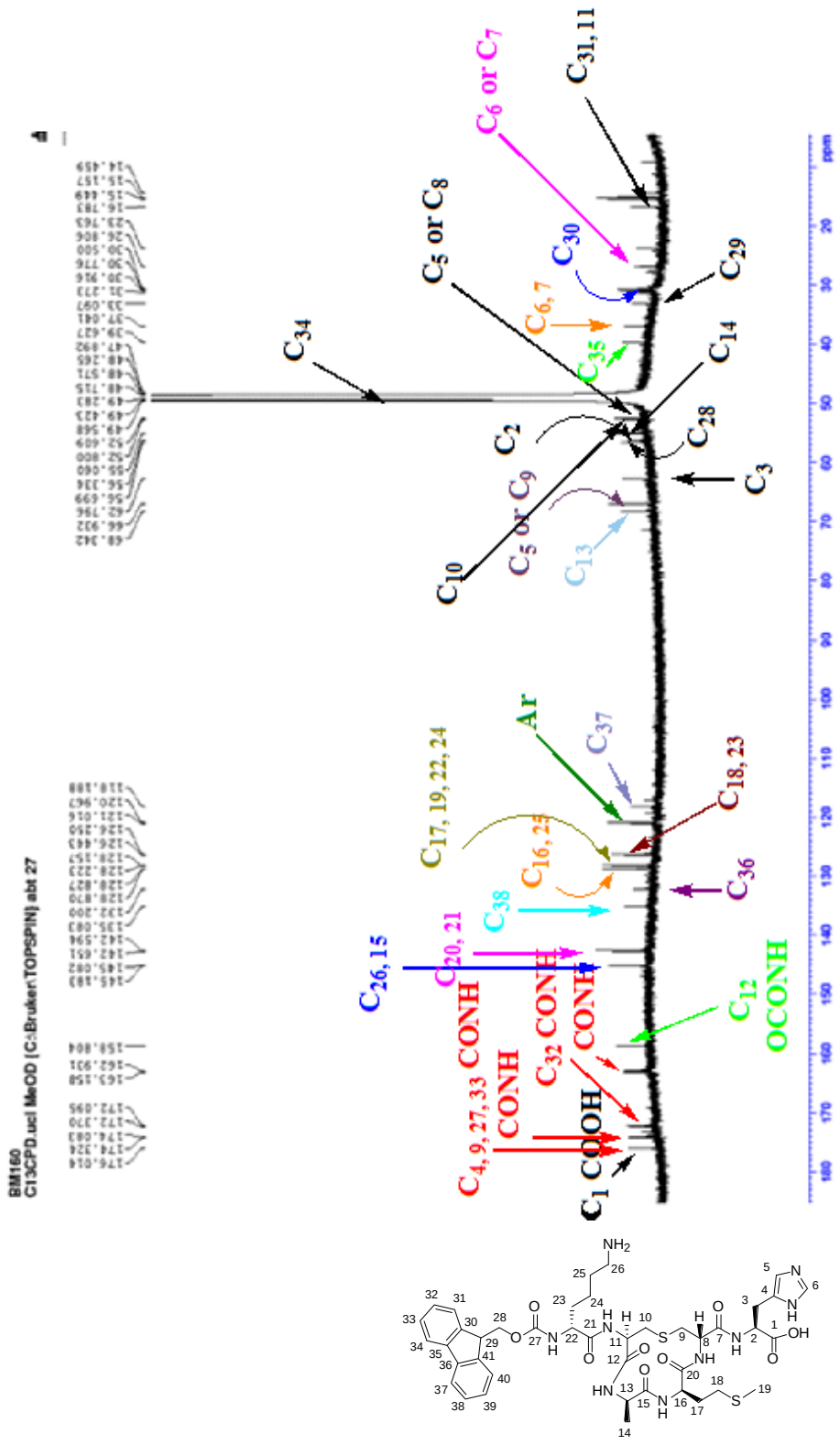
Appendix 23

HSQC NMR of an analogue of ring E of nisin **210**.



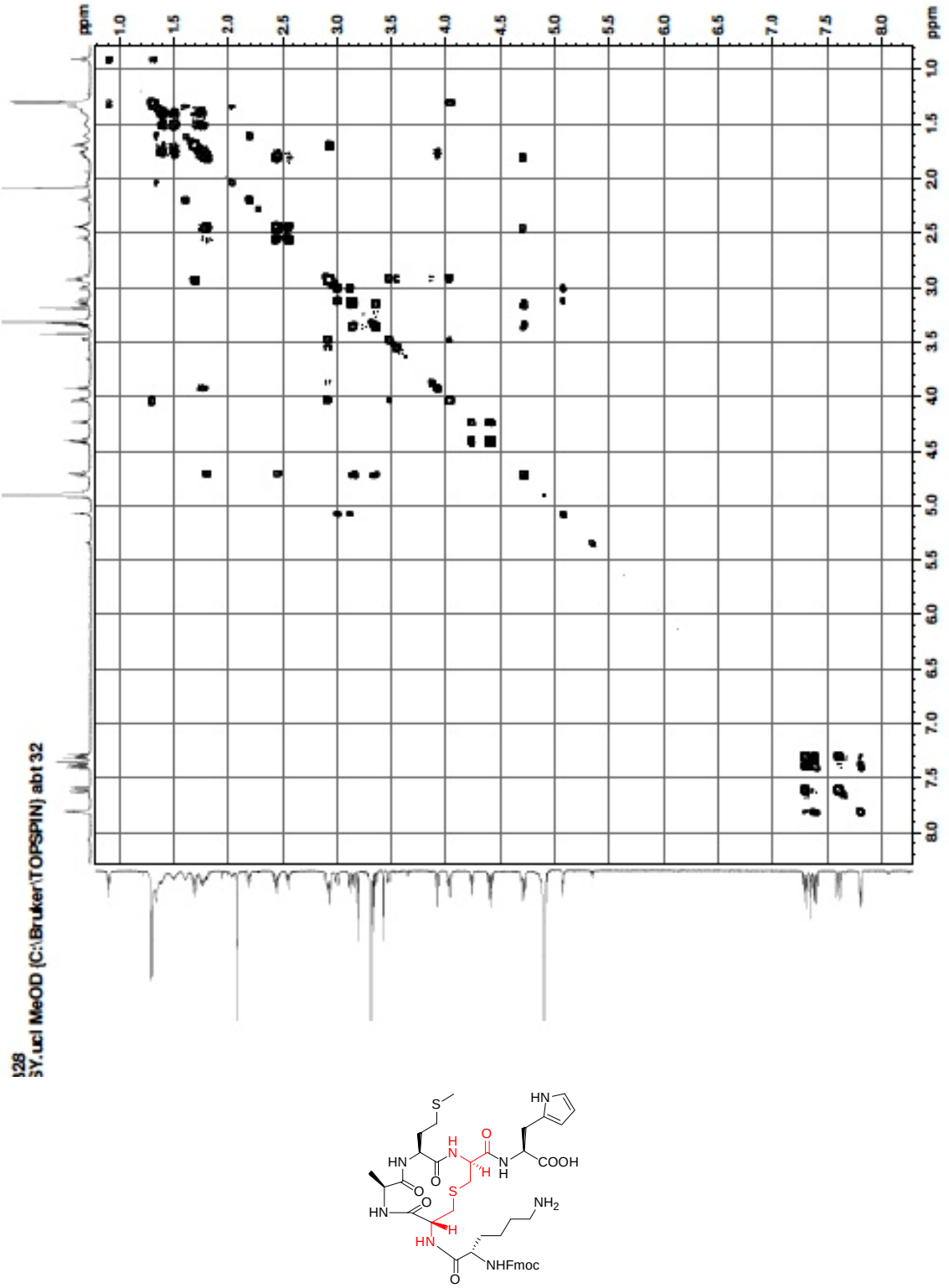
Appendix 24

Carbon NMR of an analogue of ring E of nisin **210**.



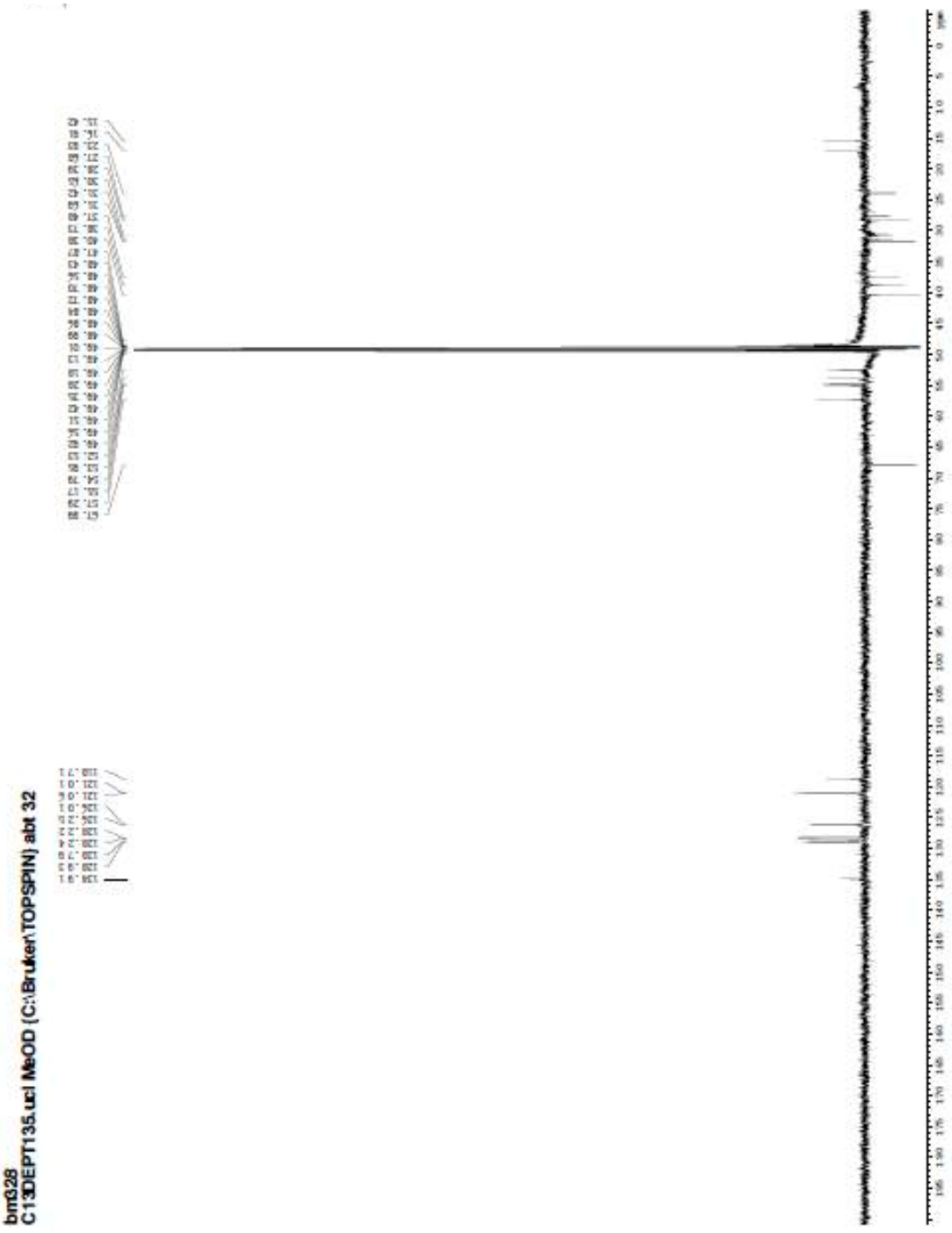
Appendix 25

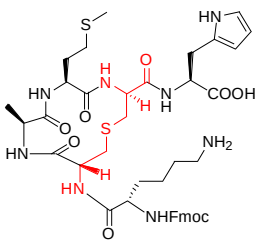
COSY NMR of an analogue of ring D of nisin **220**.



Appendix 26

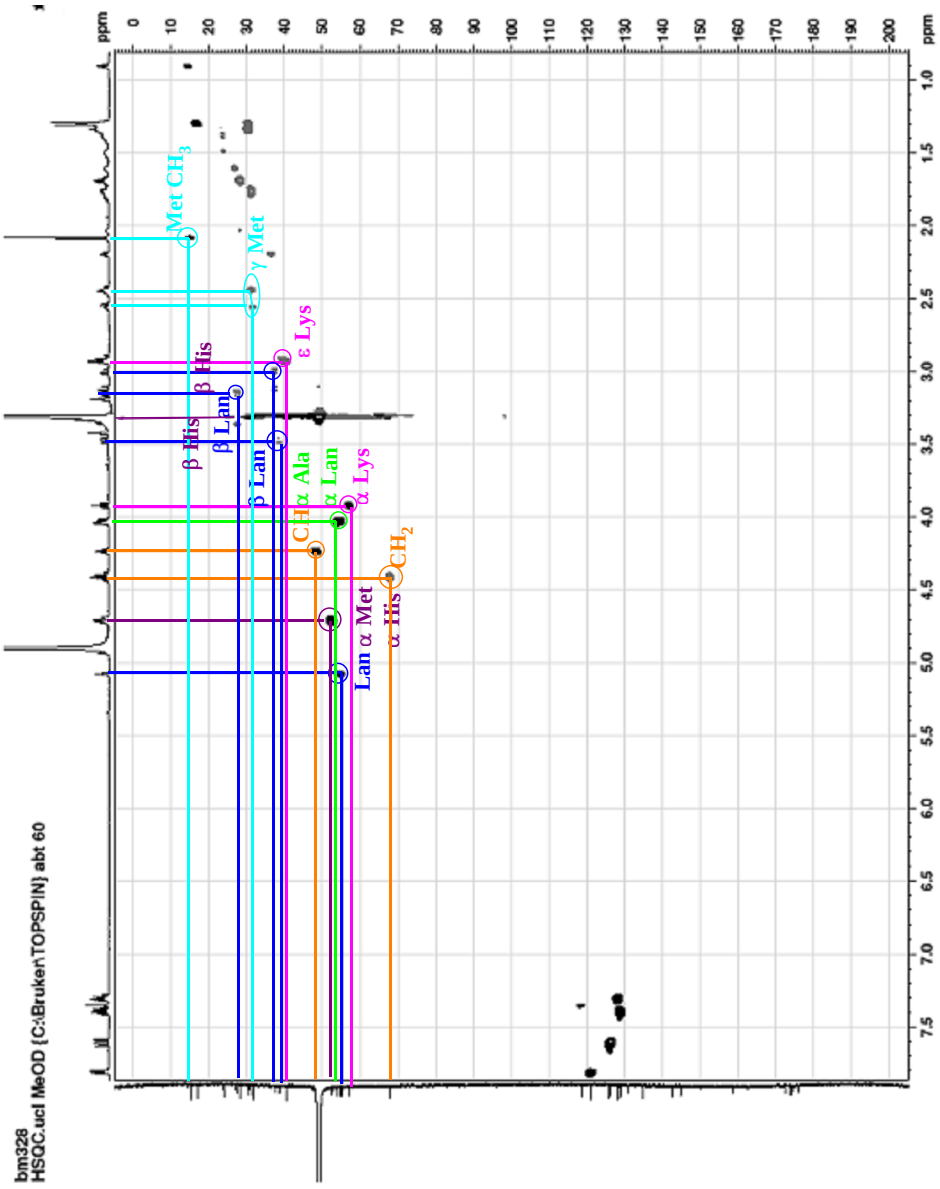
Carbon DEPT NMR of an analogue of ring D of nisin **220**.

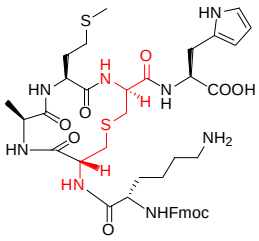




Appendix 27

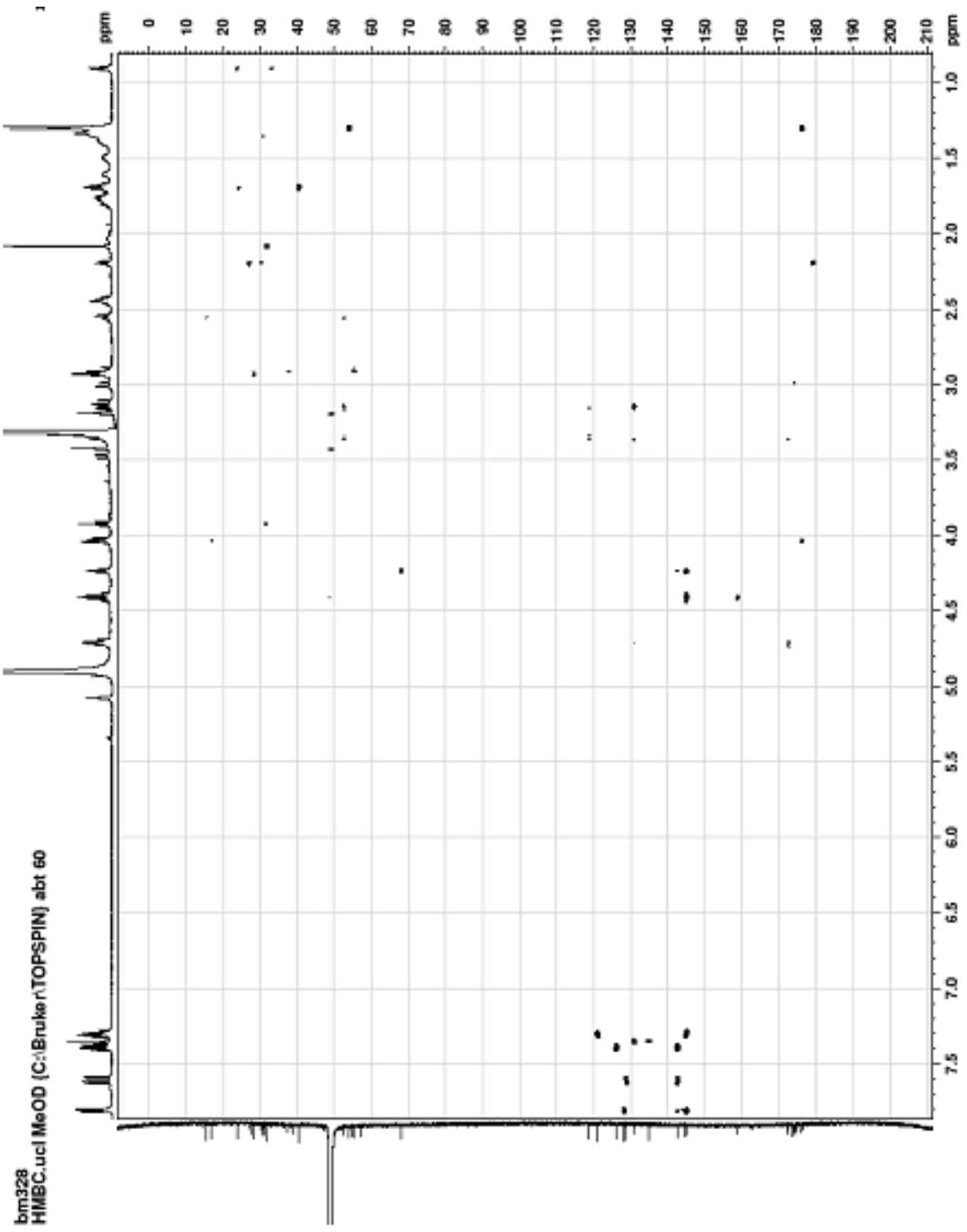
HSQC NMR of an analogue of ring D of nisin **220**.





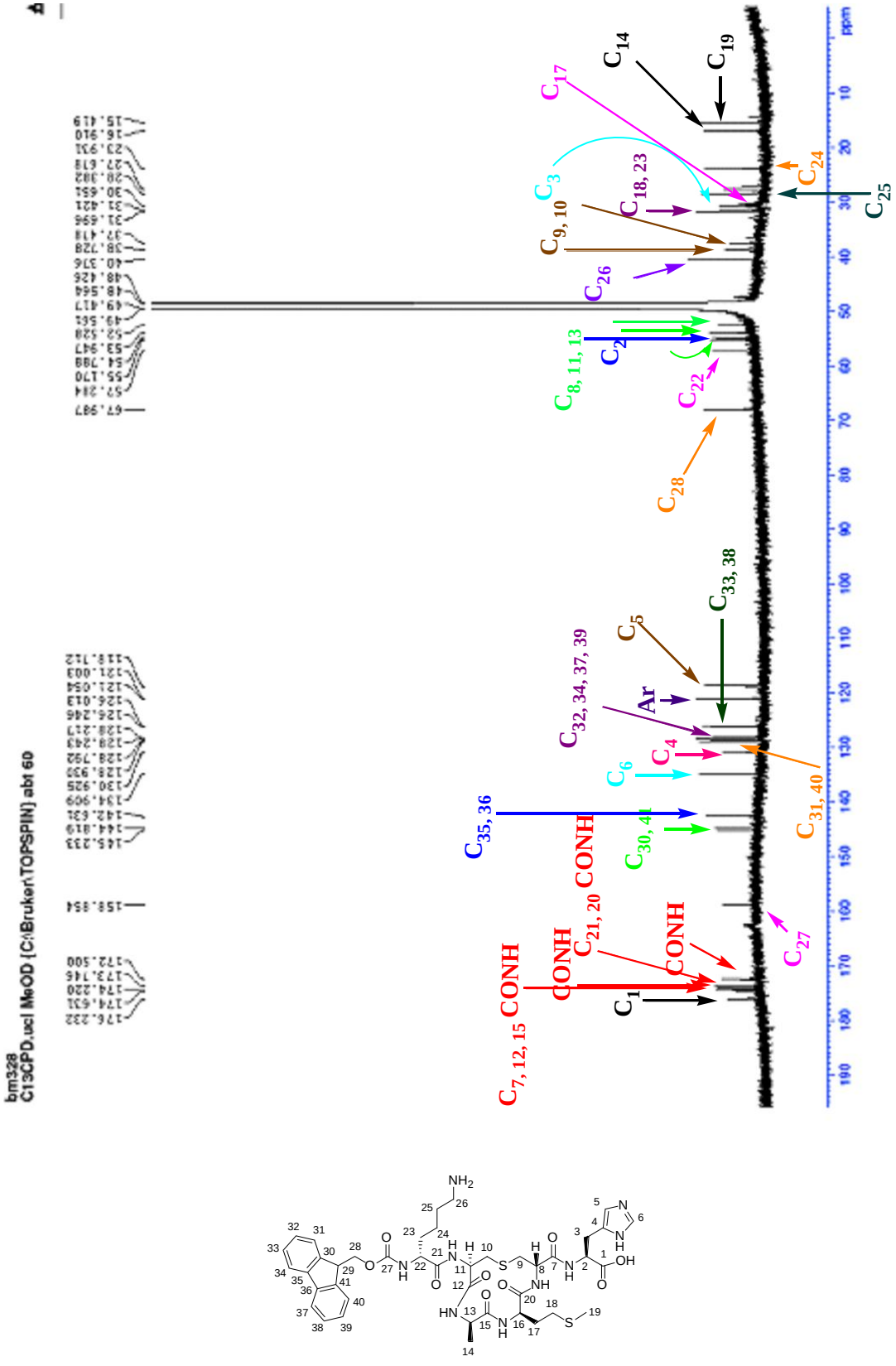
Appendix 28

HMBC NMR of an analogue of ring D of nisin **220**.



Appendix 29

Carbon NMR of an analogue of ring D of nisin **220**.



Appendix 30

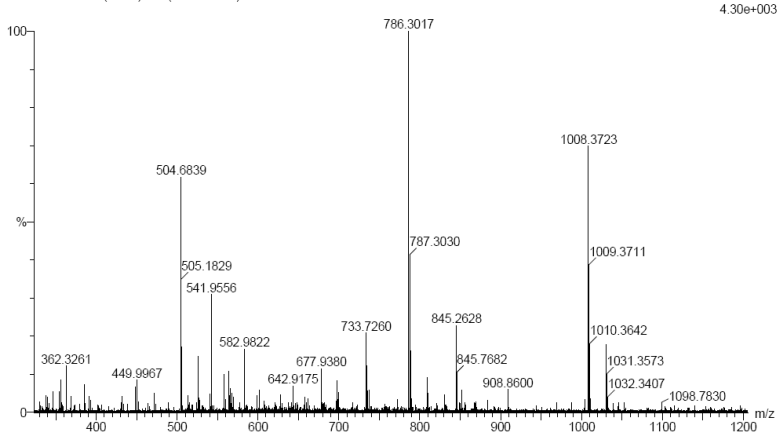
Accurate mass spectrum of an analogue of rings D and E of nisin **141**: HPLC fraction eluting at 26-27 min.

Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 100.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
2 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 45-45 H: 4-250 N: 11-11 O: 12-12 Na: 0-1 S: 2-2

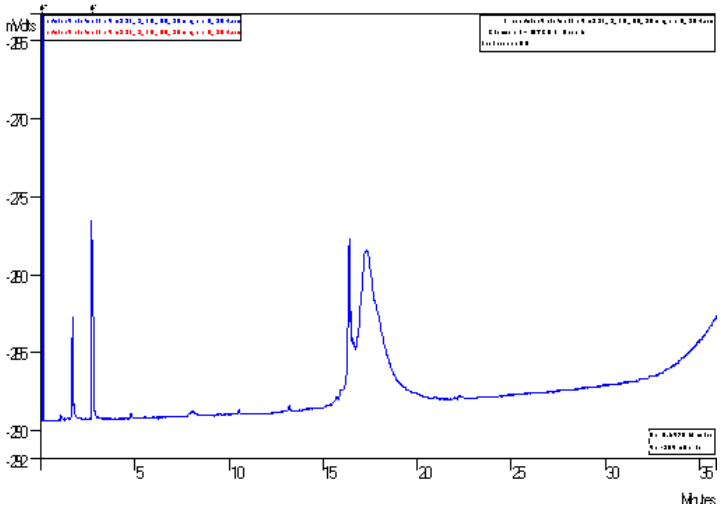
BM331RUN25 19 (0.653) Cm (17:22-41:47) 1: TOF MS ES+ 4.30e+003



Minimum:				-1.5		
Maximum:		20.0	100.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
1008.3723	1008.3708	1.5	1.5	22.5	1.4	C45 H58 N11 O12 S2

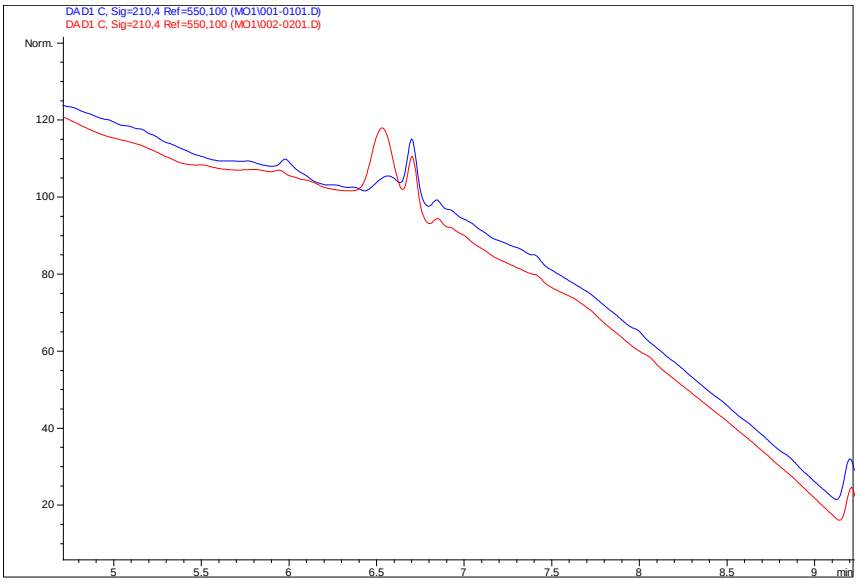
Appendix 31

Analytical HPLC trace of an analogue of rings D and E of nisin **141** collected at 26-27 min. A: H_2O/0.5 % TFA; B: acetonitrile/0.5 % TFA; B gradient 15 % - 50 % over 30 min.



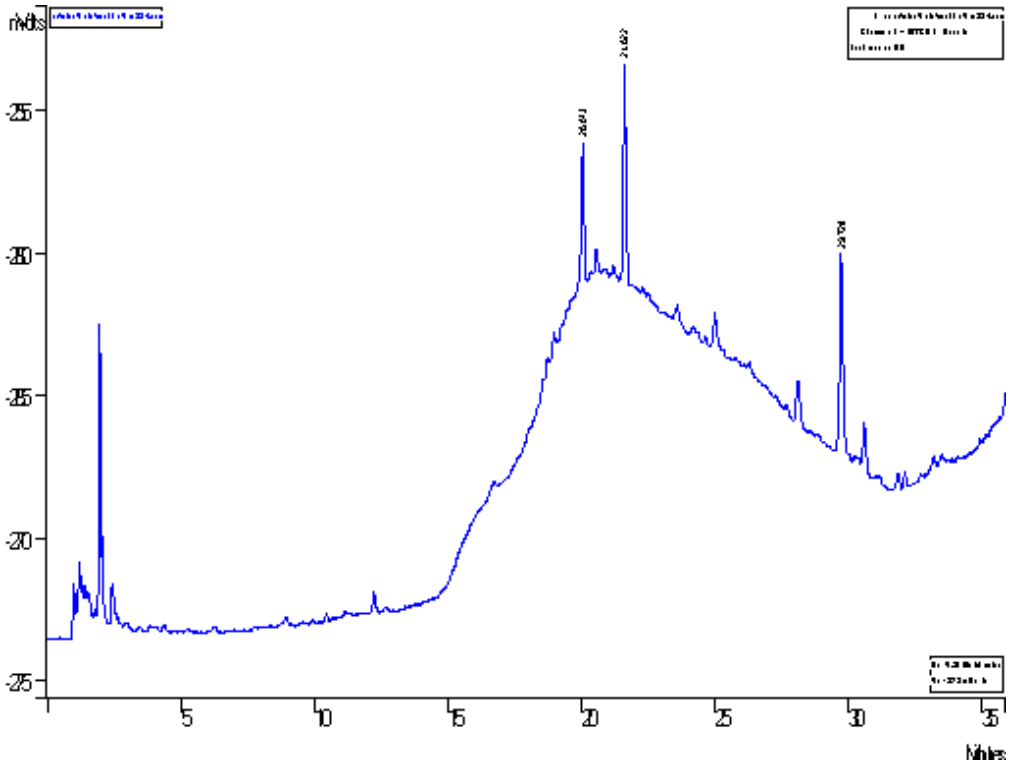
Appendix 32

LCMS of the supernatant sample in blue and the pellet sample in red. A: H_2O/0.5 % TFA; B: acetonitrile/0.5 % TFA; B gradient 10 % - 90 % over 35 min.



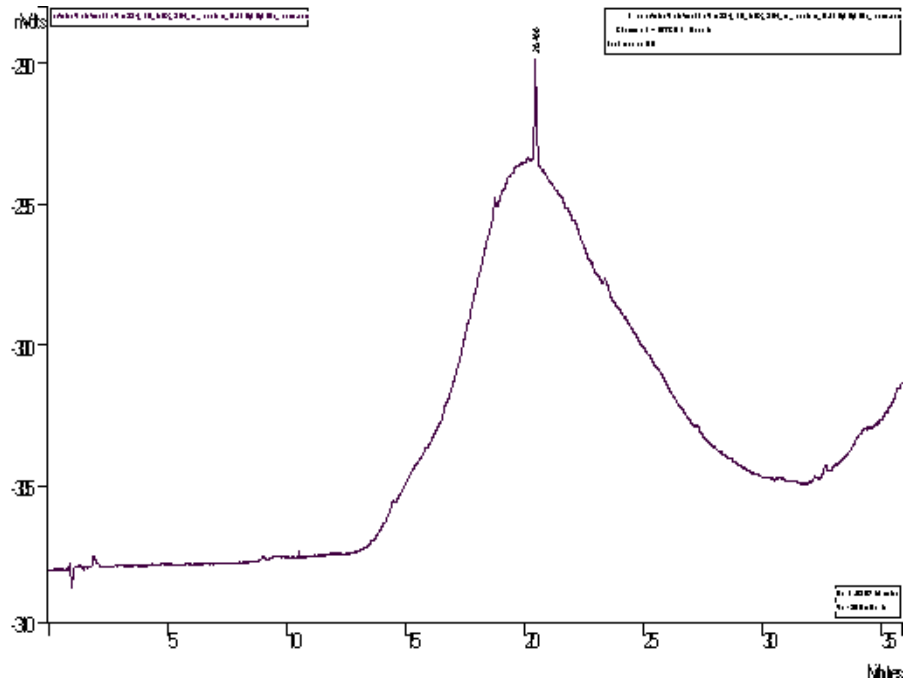
Appendix 33

Crude analytical HPLC trace of the analogue of rings D and E of nisin **141** obtained from the second attempt. A: H_2O/0.5 % TFA; B: acetonitrile/0.5 % TFA; B gradient 10 % - 60 % over 30 min.



Appendix 34

Analytical trace of fraction 16 collected after the first purifcation by C18 column. A: H_2O/0.5 % TFA; B: acetonitrile/0.5 % TFA; B gradient 10 % - 90 % over 30 min.



Appendix 35

Synthesis of Peptides Containing Overlapping Lanthionine Bridges on the Solid Phase: An Analogue of Rings D and E of the Lantibiotic Nisin

ORGANIC LETTERS

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Synthesis of Peptides Containing Overlapping Lanthionine Bridges on the Solid Phase: An Analogue of Rings D and E of the Lantibiotic Nisin

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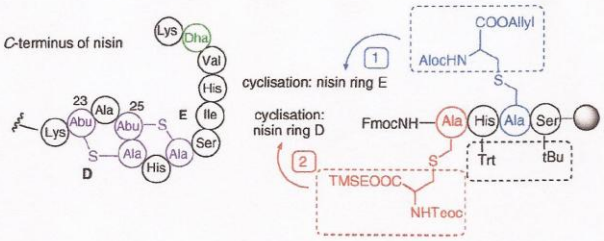
ABSTRACT



A methodology for the solid-phase synthesis of the overlapping lanthionine bridges found in many lantibiotics has been developed. A novel Teoc/TMSE-protected lanthionine derivative has been synthesized, and this lanthionine, and an Aloc/allyl-protected lanthionine derivative, have been incorporated into a linear peptide using solid-phase peptide synthesis. Selective deprotection of the silyl protecting groups, followed by sequential cyclization, deprotection of the allyl protecting groups, and further cyclization, enabled the regioselective formation of an analogue of rings D and E of nisin.

The rapid rise of antibiotic resistant bacteria makes it imperative that chemical biologists explore the synthesis and mode of action of natural products which exert antibiotic activity through novel pathways. Lantibiotics such as nisin have a unique mode of antibiotic action, which involves binding to lipid II, a key intermediate in the biosynthesis of the cell walls of Gram-positive bacteria.[1,2] They may therefore represent important new leads in the search for new antibacterial agents to treat antibiotic-resistant infections. These peptides have very complex structures, with multiple thioether bridges between side chains, making them a formidable challenge for synthetic chemists.[3,4]

We have previously developed a flexible approach to the solid-phase synthesis of lanthionine-containing peptides, using orthogonally protected lanthionine derivative **1**.[5] This approach has been used by ourselves and others to synthesize fragments and analogues[6–8] of lantibiotics. The

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(1) Chatterjee, C.; Paul, M.; Xie, L.; van der Donk, W. A. *Chem. Rev.* **2005**, *105*, 633.

(2) (a) Breukink, E.; de Kruijff, B. *Biochim. Biophys. Acta* **1999**, *1462*, 223. (b) Hsu, S.-T. D.; Breukink, E.; Tischenko, E.; Lutters, M. A. G.; de Kruijff, B.; Kaptein, R.; Bonvin, A. M. J. J.; van Nuland, N. A. J. *Nature Struct. Mol. Biol.* **2004**, *11*, 963. (c) Hasper, H. E.; Kramer, N. E.; Smith, J. L.; Hillman, J. D.; Zachariah, C.; Kuipers, O. P.; de Kruijff, B.; Breukink, E. *Science* **2006**, *313*, 1636.

(3) (a) Polinsky, A.; Cooney, M. G.; Toy-Palmer, A.; Ösapay, G.; Goodman, M. *J. Med. Chem.* **1992**, *35*, 4185. (b) Toogood, P. L. *Tetrahedron Lett.* **1993**, *34*, 7833. (c) Burrage, S.; Raynham, T.; Williams, G.; Essex, J. W.; Allen, C.; Cardno, M.; Swali, V.; Bradley, M. *Chem.—Eur. J.* **2000**, *6*, 1455. (d) Okeley, N. M.; Y. Zhu, Y.; van der Donk, W. A. *Org. Lett.* **2002**, *4*, 1335.

(4) (a) Ösapay, G.; Goodman, M. *J. Chem. Soc., Chem. Commun.* **1993**, 1599. (b) Ösapay, G.; Prokai, L.; Kim, H.-S.; Medzihradszky, K. F.; Coy, D. H.; Liapakis, G.; Reisine, T.; Melacini, G.; Zhu, Q.; Wang, S. H.-H.; Mattern, R.-H.; Goodman, M. *J. Med. Chem.* **1997**, *40*, 2241.

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approach allows both single rings and peptide sequences with multiple thioether bridges in sequence to be prepared, most recently demonstrated by Vederas and co-workers in the total synthesis, on-resin, of lactocin S.[9] However, until now it has not been possible to synthesize lantibiotic sequences having two overlapping thioether bridges using solid-phase techniques. This motif occurs very frequently in lantibiotics, for example in the C-terminus of nisin **2**, rings D and E, which is thought to be the pore-forming region of this lantibiotic.

In this paper, we report the first solid-phase synthesis of a bicyclic peptide containing two overlapping thioether bridges, using a quadruply orthogonal protecting group strategy to prepare an analogue of rings D and E of nisin. We envisaged that the overlapping bridges of rings D and E of nisin could be prepared from a linear resin-bound peptide intermediate **3** (Figure 1). This contains two distinct lanthionine residues with protecting groups orthogonal to each other and also to the transient (Fmoc) and permanent (Boc/tBu) protecting groups used in Fmoc-based solid-phase peptide synthesis. We had already demonstrated that the allyl ester and Aloc protecting groups of **1** could be selectively removed with $Pd(PPh_3)_4$ without loss of either Fmoc or Boc protecting groups.[6,7] Of the many other protecting groups available for amino acids, we selected the β-(trimethylsilyl)ethoxycarbonyl (Teoc)[10] and trimethylsilylethyl (TMSE)[11] groups for the amino and carboxylic acid, respectively, as these silyl-based protecting groups could be easily removed using TBAF under mild conditions at neutral pH.

As a first step, it was necessary to synthesize the (Teoc, TMSE)/Fmoc-protected lanthionine **4**. Trt-D-Ser-OTMSE **5** was prepared from commercially available Boc-D-Ser-(Bzl)-OH and then converted to iodoalanine **6** via a Mitsunobu reaction (Scheme 1).[7] Coupling of **6** to Fmoc-Cys-OTce[12] gave lanthionine **7**, which was converted to the Teoc-protected amine **8** and then successfully deprotected under neutral conditions[13] to afford the desired protected lanthionine **4**.

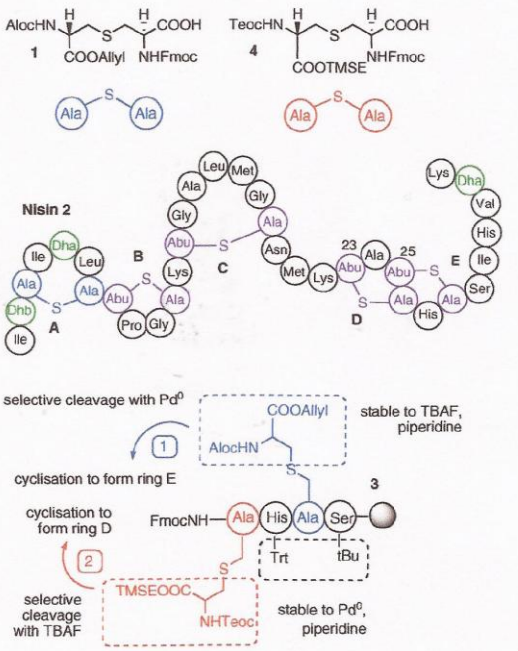
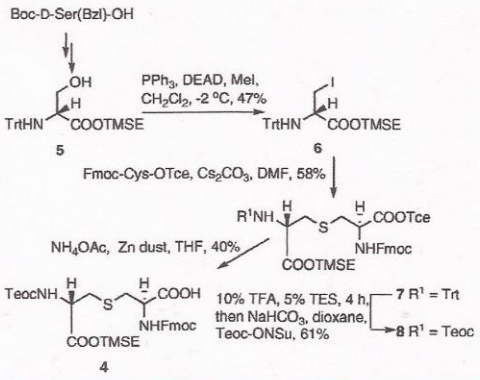


Figure 1. Strategy for synthesizing nisin rings D and E.

Scheme 1. Synthesis of (Teoc, TMSE/Fmoc) Lanthionine **4**



In order to test whether **4** could be used in the solid-phase synthesis of lanthionine-containing thioether-bridged cyclic peptides, we first prepared an analogue of ring D of nisin. The linear resin-bound peptide **9** was first prepared by standard solid-phase peptide synthesis methods, incorporating **4** as the second amino acid, and substituting a Met residue as the third amino acid in place of the other lanthionine (Scheme 2).

(5) (a) Mohd Mustapa, M. F.; Harris, R.; Bulic-Subanovic, N.; Elliott, S. L.; Bregant, S.; Groussier, M. F. A.; Mould, J.; Schultz, D.; Chubb, N. A. L.; Gaffney, P. R. J.; Driscoll, P. C.; Tabor, A. B. *J. Org. Chem.* **2003**, *68*, 8185. For other recent syntheses of orthogonally protected lanthionine and nor-lanthionine derivatives, see: (b) Zhu, X.; Schmidt, R. R. *Eur. J. Org. Chem.* **2003**, 4069. (c) Narayan, R. S.; VanNieuwenhze, M. S. *Org. Lett.* **2005**, *7*, 2655. (d) Martin, N. I. *J. Org. Chem.* **2009**, *74*, 946. (e) Avenoza, A.; Busto, J. H.; Jiménez-Osés, G.; Peregrina, J. M. *Org. Lett.* **2006**, *8*, 2855. (f) Cobb, S. L.; Vederas, J. C. *Org. Biomol. Chem.* **2007**, *5*, 1031.

(6) Mohd Mustapa, M. F.; Harris, R.; Esposito, D.; Chubb, N. A. L.; Mould, J.; Schultz, D.; Driscoll, P. C.; Tabor, A. B. *J. Org. Chem.* **2003**, *68*, 8193.

(7) Bregant, S.; Tabor, A. B. *J. Org. Chem.* **2005**, *70*, 2430.

(8) Pattabiraman, V. R.; McKinnie, S. M. K.; Vederas, J. C. *Angew. Chem., Int. Ed.* **2008**, *47*, 9472.

(9) Ross, A. C.; Liu, H.; Pattabiraman, V. R.; Vederas, J. C. *J. Am. Chem. Soc.* **2010**, *132*, 462.

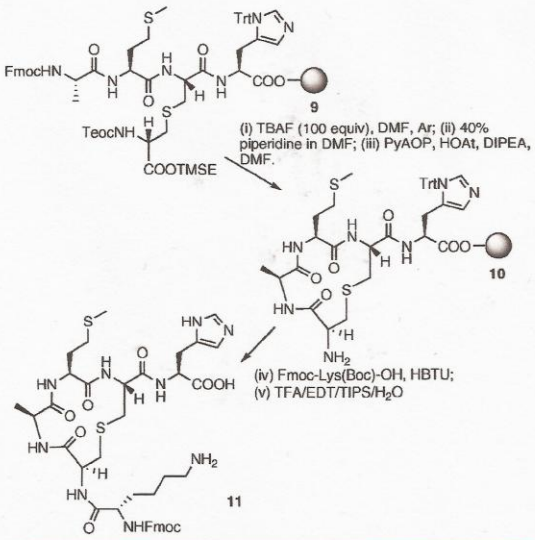
(10) Carpino, L. A.; Tsao, J.-H.; Ringsdorf, H.; Fell, E.; Hettrich, G. *J. Chem. Soc., Chem. Commun.* **1978**, 358.

(11) (a) Sieber, P. *Helv. Chim. Acta* **1977**, *60*, 2711. (b) Marlowe, C. K. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 437.

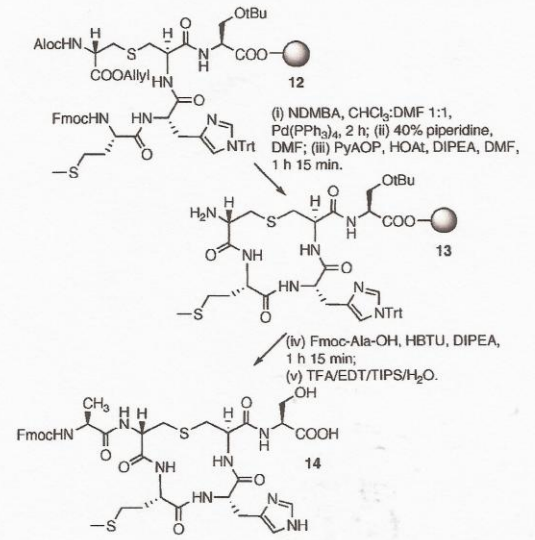
(12) Boger, D. L.; Ichikawa, S.; Tse, W. C.; Hedrick, M. P.; Jin, Q. *J. Am. Chem. Soc.* **2001**, *123*, 561.

(13) Jou, G.; González, I.; Albericio, F.; Lloyd-Williams, P.; Giralt, E. *J. Org. Chem.* **1997**, *62*, 354.

Scheme 2. Synthesis of an Analogue of Ring D of Nisin



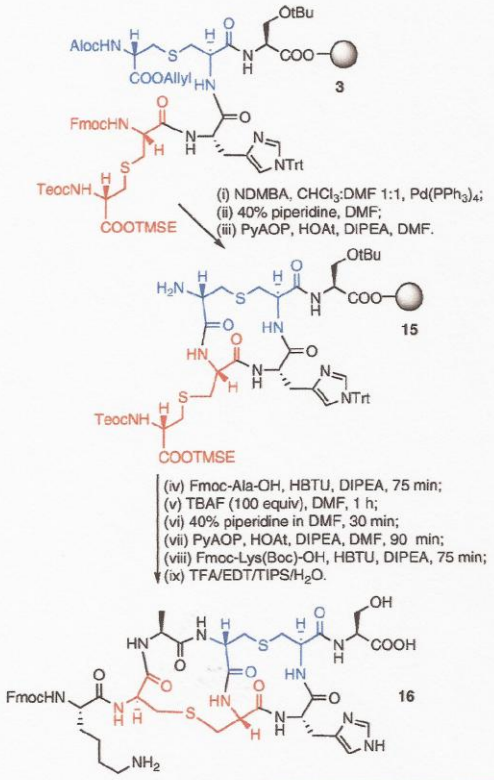
Scheme 3. Synthesis of an Analogue of Ring E of Nisin



Treatment with TBAF successfully removed the Teoc and TMSE groups;[10,11] although the Fmoc group was probably also removed during the reaction,[14] the resin-bound peptide was then treated with piperidine to ensure complete deprotection of the N-terminus. Cyclization on-resin[15] then gave the resin-bound peptide **10**. Finally, Fmoc-Lys(Boc)-OH was added to the free N-terminus and the cyclic peptide **11** cleaved from the resin. Purification by HPLC gave **11**, which was characterized by NMR.

We also used the (Aloc, allyl)/Fmoc-protected lanthionine **1**[7] to prepare an analogue of ring E of nisin. Incorporation of **1** into a linear resin-bound precursor **12** was carried out using standard coupling conditions, again substituting a Met residue for the lanthionine at the fourth amino acid (Scheme 3). Deprotection of the allyl and Aloc groups was carried out with $Ph(PPh_3)_4$ using *N*′,*N*-dimethylbarbituric acid (NDMBA) as an allyl group scavenger.[16] Removal of the Fmoc group was again followed by on-resin cyclization to give **13**; the sequence was then extended with Fmoc-Ala-OH before cleavage from the resin. Purification by reversed-phase HPLC gave **14**, which was also characterized by NMR.

Scheme 4. Synthesis of an Analogue of Rings D and E of Nisin



With the two individual rings successfully synthesized and characterized, we then tackled the synthesis of the bicyclic peptide with two overlapping lanthionine bridges.

(14) Ueki, M.; Amemiya, M. *Tetrahedron Lett.* **1987**, *28*, 6617.
(15) Albericio, F.; Cases, M.; Alsina, J.; Triolo, S. A.; Carpino, L. A.; Kates, S. A. *Tetrahedron Lett.* **1997**, *38*, 4853.
(16) (a) Kunz, H.; März, J. *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 1375. (b) Machauer, R.; Waldmann, H. *Chem.—Eur. J.* **2001**, *7*, 2940.

Linear resin-bound peptide **3** was first synthesized, incorporating both of the protected lanthionines **1** and **4** (Scheme 4).

Deprotection of the allyl and Aloc groups proceeded smoothly using the same conditions as before, without removal of the Teoc and TMSE groups. Removal of the Fmoc group from the (Teoc, TMSE/Fmoc) lanthionine residue then allowed ring E to be formed on-resin, giving **15** (Scheme 4). In order to install ring D, chain extension with Fmoc-Ala-OH was then followed by removal of the Teoc and TMSE groups and a second cyclization reaction. Finally, Fmoc-Lys(Boc)-OH was again added to the free N-terminus, and the cyclic peptide **16** was cleaved from the resin. A two-stage purification procedure using C18 SPE columns gave the pure peptide **16**. High-resolution mass spectrometry showed that the desired peptide had been synthesized, with a mass of 1008.37 Da.[17]

In conclusion, we have developed an effective methodology for the solid-phase synthesis of peptides containing overlapping lanthionine bridges. We have demonstrated the applicability of this approach in the solid-phase synthesis of rings D and E of nisin. The D and E segment of nisin has been synthesized previously in solution by Shiba and co-workers using a solution-phase desulfurization approach[18] and has been coupled to other segments of nisin.[19] However the solid-phase synthesis approach described here avoids the pitfalls of segment synthesis[20] and will allow for the rapid preparation of all types of lanthionine containing peptides. This quadruply orthogonal protecting group approach will be a powerful additional tool for the chemical synthesis of highly structurally complex lantibiotics, with the potential to prepare more potent and bioavailable synthetic analogues, unrestricted in amino acid sequence and composition, that could not be accessed via a biosynthetic approach.[21]

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Supporting Information Available. Experimental procedures and characterization of all new amino acid and lanthionine derivatives and of peptides **11**, **14**, and **16**. This material is available free of charge via the Internet at http://pubs.acs.org

(17) Sequence characterisation by MS/MS of these bicyclic peptides is not possible because of the crossed lanthionine bridges which do not give a unique fragmentation pattern: (a) Kleinnijenhuis, A. J.; Duursma, M. C.; Breukink, E.; Heeren, R. M. A.; Heck, A. J. R. *Anal. Chem.* **2003**, *75*, 3219. (b) Lavanant, H.; Derrick, P. J.; Heck, A. R.; Mellon, F. A. *Anal. Biochem.* **1998**, *255*, 74. (c) Ghalit, N.; Kemmink, J.; Hilbers, H. W.; Versluis, C.; Rijkers, D. T. S.; Liskamp, R. M. J. *Org. Biomol. Chem.* **2007**, *5*, 924.

(18) Fukase, K.; Oda, Y.; Kubo, A.; Wakamiya, T.; Shiba, T. *Bull. Chem. Soc. Jpn.* **1990**, *63*, 1758.

(19) Fukase, K.; Kitazawa, M.; Sano, A.; Shimbo, K.; Horimoto, S.; Fujita, H.; Kubo, A.; Wakamiya, T.; Shiba, T. *Bull. Chem. Soc. Jpn.* **1992**, *65*, 2227.

(20) Kent, S. B. H. *Chem. Soc. Rev.* **2009**, *38*, 338.

(21) Ross, A. C.; Vederas, J. C. *J. Antibiot.* **2011**, *64*, 27.

8. References

1. P. M. Small, H. F. Chambers; *Antimicrob. Agents. Chemother*.; **1990**, *34*, 1227.

2. E. Breukink, B de Kruijff; *Biochim. Biophys. Acta*.; **1999**, *1462*, 223.

3. C. Chatterjee, M. Paul, L. Xie, W. A. van der Donk; *Chem. Rev.*; **200**5, *105*, 633.

4. N. Schrell, K. D. Entian, U. Schreider, F. Götz, H. Zahner, R. Kellner, G. Jong; *Nature.;* **1988**, *333*, 276.

5. L. Vertesy, W. Aretz, A. Bonnefoy, E. Ehlers, M. Kurz, A. Markus, M. Schiell, M. Vogel, J. Wink, H. Kogler; *J. Antibiotics.;* **1999**, *52*, 730.

6. S. M. Asaduzzaman, K. Sonomoto; *J. Biosci. Bioeng.;* **2009**, *107*, 475.

7. C. K. Bower, M. K. Bothwell, J. McGuire; *Colloids. Surfaces. B.;* **2001**, *22*, 259.

8. W. Liu, J. N. Hansen; *Appl. Environ. Microbiol.;* **1990**, *56*, 2551.

9. G. Jung; *Lantibiotics*.; a survey, p. 1-34, *In*: G. Jung, H.-G. Sahl (Eds), Nisin and novel lantibiotics. ESCOM, Leidon, The Netherlands, **1991**.

10. J. I. Nagao, S. M. Asaduzzaman, Y. Aso, K. I. Okuda, J. Nakayama, K. Sonomoto; *J. Biosci. Bioeng.;* **2006**, *102*, 139.

11. M. Kordel, R. Benz, H.-G. Sahl; *J. Bacteriol.;* **1988**, *170*, 84.

12. K. A. Stevens, B. W. Sheldon, N. A. Klapes, T. R. Klaenhammer; *Appl. Environ. Microbiol.;* **1991**, *57*, 3613.

13. A. Hoffmann, U. Pag, I. Weidemann, H.-G. Sahl; *II. Farmaco.;* **2002**, *57*, 685.

14. L. Smith, J.D. Hillman; *Curr. Opin. Microbiol.;* **2008**, *11*, 401.

15. O. McAuliffe, R. P. Ross, C. Hill; *FEMS Microbiol. Rev.;* **2001**, *25*, 285.

16. E. Gross, L. J. Morell; *J. Am. Chem. Soc.;* **1971**, *93*, 4634.

17. J. W. M. Mulders, I. J. Boerrigter, H. S. Rollema, R. J. Siezen, W. M. de VOS; *Eur. J. Biochem.;* **1991**, *201*, 581.

18. E. Gross, H. H. Kiltz, E. Nebelin; *Hoppe-Seyler's Z. Physiol. Chem.;* **1973**, *354*, 810.

19. H. Allgaier, G. Jung, G. R. Werner, U. Schneider; *Angew. Chem. Int. Ed. Engl.;* **1985**, *24*, 1051.

20. R. Kellner, G. Jung, T. Hoerner, H. Zaehner, N. Schnell, K.-D. Entian, F. Goetz; *Eur. J. Biochem.;* **1988**, *177*, 53.

21. L. Smith, J. Novak, J. Rocca, S. McClung, J. D. Hillman, A. S. Edison; *Eur. J. Biochem.;* **2000**, *267*, 6810.

22. M. Mota-Meira, C. Lacroix, G. LaPointe, M. C. Lavoie; *FEBS Lett*.; **1997**, *410*, 275.

23. F. Qi, P. Chen, P. W. Caufield; *Appl. Environ. Microbiol.;* **1999**, *65*, 3880.

24. F. Qi, P. Chen, P. W. Caufield; *Appl. Environ. Microbiol.;* **2000**, *66*, 3221.

25. R. Kellner, G. Jung, M. Josten, C. Kaletta, K. D. Entian, H.-G. Sahl; *Angew. Chem.;* **1989**, *101*, 618.

26. C. Heidrich, U. Pag, M. Josten, J. Metzger, R. W. Jack, G. Bierbaum, G. Jung, H.-G. Sahl; *Appl. Environ. Microbiol.;* **1998**, *64*, 3140.

27. M. van de Kamp, L. M. Horstink, H. W. Van den Hooven, R. N. H. Konings, C. W. Hilbers, A. Frey, H. –G. Sahl, J. W. Metzger, F. J. M. Van de ven; *Eur. J. Biochem.;* **1995**, *227*, 757.

28. H. W. Van den Hooven, F. M. Lagerwerf, W. Heerma, J. Haverkamp, J. C. Piard, C. W. Hilbers, R. J. Siezen, O. P. Kuipers, H. S. Rollema; *FEBS Lett.;* **1996**, *391*, 317.

29. M. Skaugen, J. Nissenmeyer, G. Jung, S. Stevanovic, K. Sletten, C. I. M. Abildgaard, I. F. Nes; *J. Biol. Chem.;* **1994**, *269*, 27183.

30. R. E. Krull, P. Chen, J. Novak, M. Kirk, S. Barnes, J. Baker, N. R. Krshna, P. W. Caufield; *J. Biol. Chem.;* **2000**, *275*, 15845.

31. D. Pridmore, N. Rekhif, A. C. Pittet, B. Suri, B. Mollet; *Appl. Environ, Microbiol.;* **1996**, *62*, 1799.

32. K. F. Ross, C. W. Ronson, J. R. Tagg; *Appl. Environ. Microbiol.;* **1993**, *59*, 2014.

33. R. W. Jack, A. Carne, J. Metzger, S. Stefanovic, H.-G. Sahl, G. Jung, J. Tagg; *Eur. J. Biochem.;* **1994**, *220*, 455.

34. Y. Minami, K. Yoshida, R. Azuma, A. Urakawa, T. Kawauchi, T. Otani, K. Komiyama, S. Omura; *Tetrahedron. Lett.;* **1994**, *35*, 8001.

35. D. L. Turner, L. Brennan, H. E. Meyer, C. Lohaus, C. Siethoff, H. S. Costa, B. Gonzalez, H. Santos, J. E. Suarez; *Eur. J. Biochem.*; **1999**, *264*, 833.

36. M. L. Kalmokoff, D. Lu, M. F. Whitford, R. M. Teather; *Appl. Environ. Microbiol.;* **1999**, *65*, 2128.

37. H. Kessler, S. Steuernagel, M. Will, G. Jung, R. Kellner, D. Gillessen, T. Kamiyama; *Helv. Chim. Acta.;* **1988**, *71*, 1924.

38. F. Hayashi, K. Nagashima, Y. Terui, Y. Kawamura, K. Matsumoto, H. Itazaki; *J. Antibiotics.;* **1990**, *43*, 1421.

39. N. Zimmermann, S. Freund, A. Fredenhagen, G. Jung; *Eur. J. Biochem.;* **1993**, *216*, 419.

40. T. Wakamiya, Y. Ueki, T. Shiba, Y. Kido, Y. Motoki; *Bull. Chem. Soc. Jpn.;* **1990**, *63*, 1032.

41. S. Chatterjee, S. Chatterjee, S. J. Lad, M. S. Phansalkar, R. H. Rupp, B. N. Ganguli; *J. Antibiotics.;* **1992**, *45*, 832.

42. N. Zimmermann, J. W. Metzger, G. Jung; *Eur. J. Biochem.;* **1995**, *228*, 786.

43. N. I. Martin, T. Sprules, M. R. Carpenter, P. D. Cotter, C. Hill, R. P. Ross, J. C. Vederas; *Biochem.;* **2004**, *43*, 3049.

44. L. Xie, W. A. Van der Donk; *Curr. Opin. Chem. Biol.;* **2004**, *8*, 498.

45. H. G. Sahl, G. Bierbaum; *Annu. Rev. Microbiol.;* **1998**, *52*, 41.

46. S. Yoganathan, J. C. Vederas; *Chem. Biol.;* **2008**, *15*, 999.

47. C. Van Kraaij, W. M. de Vos, R. J. Siezen, O. P. Kuipers; *Nat. Prod. Rep.;* **1999**, *16*, 575.

48. A. W. van der Donk; *J. Org. Chem.;* **2006**, *71*, 9561.

49. O. P. Kuipers, M. M. Beerthuyzen, W. M de Vos; *Eur. J. Biochem.;* **1993**, *216*, 281.

50. L. Hyungjae, H. Y. Kim; *J. Microbiol. Biotechnol.;* **2011**, *2*, 229.

51. T. R. Klaenhammer; *FEMS Microbiol. Rev.;* **1993**, *12*, 39.

52. J. A. Majchrzykiewicz, J. Lubelski, G. N. Moll, A. Kuipers, J. J. E. Bijlsma, O. P. Kuipers, R. Rink; *Antimicrob. Agents. Chemother.;* **2010**, 1498.

53. H. Lavanant, P. J. Derrick, A. J. Heck, F. A. Mellon; *Anal. Biochem.;* **1998**, *255*, 74.

54. A. S. Karakas, A. Narbad, N. Horn, H. M. Dodd, A. J. Parr; *Eur. J. Biochem.;* **1999**, *261*, 524.

55. O. Koponen, M. Tolonen, M. Qiao, G. Wahlstrom, J. Helin, E. J. Saris Per; *Microbiol.;* **2002**, *148*, 3561.

56. A. Rince, A. Dufour, T. S. Le Pogam, D. Thuault, C. M. Bourgeois, J. P. Le Pennec; *Appl. Environ. Microbiol.;* **1994**, *60*, 1652.

57. O. McAuliffe, C. Hill, R. P. Ross; *Microbiol.;* **2000**, *146*, 2147.

58. A. Fleming; *Br. J. Exp. Pathol.;* **1929**, *10*, 226.

59. L. A. Rogers; *J. Bacteriol.;* **1928**, *16*, 321.

60. L. A. Rogers, E. O. Whittier; *J. Bacteriol.;* **1928**, *16*, 211.

61. W. Liu, J. N. Hansen; *Appl. Environ. Microbiol.;* **1993**, *59*, 648.

62. S. L. Morris, R. C. Walsh, J. N. Hansen; *J. Biol. Chem.;* **1984**, *259*, 13590.

63. L. J. De Arauz, A. F. Jozala, P. G. Mazzola, T. C. V. Penna; *Trends in Food Science & Technology.;* **2009**, *20*, 146.

64. G. W. Buchman, S. Banerjee, J. N. Hansen; *J. Biol. Chem.*; **1988**, *263*, 16260.

65. M. Kordel, F. Schueller, H.-G. Sahl; *FEBS Lett.;* **1989**, *244*, 99.

66. E. Breukink, C. van Kraaij, R. A. Demel, R. J. Siezen, O. P. Kuipers, B de Kruijff; *Biochemistry.;* **1997**, *36*, 6968.

67. L. Lins, P. Ducarme, E. Breukink, R. Brasseur; *Biochim. Biophys. Acta.;* **1999**, *1420*, 111.

68. R. Brasseur.; *J. Biol. Chem.;* **1991**, *266*, 16120.

69. R. Bauer, L. M. T. Dicks; *Int. J. Food. Microbiol.;* **2005**, *101*, 201.

70. H. W. Van Den Hooven, C. A. E. M. Spronk, M. Van De Kamp, R. N. H. Konings; *Eur. J. Biochem.;* **1996**, *235*, 394.

71. D. M. Ojcius, J. D. E. Young; *Trends. Biochem. Sci.;* **1991**, *16*, 225.

72. A. J. M. Driessen, H. W. van den Hooven, W. Kuiper, M. van de Kamp M, H.-G. Sahl, R. N. H. Konings, W. N. Konings; *Biochemistry.;* **1995**, *34*, 1606.

73. T. J. Montville, Y. Chen; *Appl. Microbiol. Biotechnol.;* **1998**, *50*, 511.

74. H. E. Hasper, B. De Kruijff, E. Breukink; *Biochemistry.;* **2004**, *43*, 11567.

75. E. A. Somner, P. E. Reynolds; *Antimicrob. Agents. Chemother.;* **1990**, 413.

76. E. Breukink, I. Wiedemann, C. Van Kraaij, O. P. Kuipers, H.-G. Sahl, B. de Kruijff; *Science*.; **1999**, *286*, 2361.

77. I. Wiedemann, E. Breukink, C. van Kraaij, O. P. Kuipers, G. Bierbaum, B. de Kruijff, H.-G. Sahl; *J. Biol. Chem.;* **2001**, *276*, 1772.

78. D. Nicolau PhD 'Synthesis of orthogonally protected lanthionine, and the solid phase peptide synthesis of an analogue of nisin rings A and B.

79. H. Brotz, M. Josten, I. Wiedemann, U. Schneider, F. Gotz, G. Bierbaum, H.-G. Sahl; *Mol. Microbiol.;* **1998**, *30*, 317.

80. B. Ostash, S. Walker; *Curr. Opin. Chem. Biol.;* **2005**, *9*, 459.

81. S. T. D. Hsu, E. Breukink, E. Tischenko, M. A. G. Lutters, B. De Kruijff, R. Kaptein, A. M. J. J. Bonvin, N. A. J. Van Nuland; *Nat. Struct. Mol. Biol.;* **2004**, *11*, 963.

82. M. F. M. 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, A. B. Tabor; *J. Org. Chem.;* **2003**, *68*, 8185.

83. F. J. Van de Van, H. W. Van den Hoovan, R. N. Konings, C. W. Hilbers; *Eur. J. Biochem.;* **1991**, *202*, 1181.

84. H. W. Van den Hooven, C. C. Doeland, M. Van de Kamp, R. N. Konings, C. W. Hilbers, F. J. M. Van de Van; *Eur. J. Biochem.;* **1996**, *235*, 382.

85. O. P. Kuipers, H. S. Rollema, W. M. de Vos, R. J. Siezen; *FEBS Lett.;* **1993**, *330*, 23.

86. J. A. Bober, A. Demirci; *CICR. J. Sci. Res. Devel*.; **2004**, Manuscript FP 04-001, *4*, 15.

87. N. Zimmermann, G. Jung; *Eur. J. Biochem.;* **1997**, *246*, 809.

88. S. Somma, W. Merati, F. Parenti; *Antimicrobiol. Agents.Chemother.;* **1977**, *11*, 396.

89. A. Malabarba, R. Pallanza, M. Berti, B. Cavalleri; *J. Antibiotics.;* **1990**, *43*, 1089.

90. F. Parenti, H. Pagani, G. Bertta; *J. Antibiotics.;* **1976**, *29*, 501.

91. C. Coronelli, G. Tamoni, G. C. Lancini; *J. Antibiotics.;* **1976**, *29*, 507.

92. G. Bierbaum, H. Brotz, K. P. Koller, H.-G. Sahl; *Microbiol. Lett.;* **1995**, *127*, 121.

93. S. Chatterjee, D. K. Chatterjee, R. H. Jani, J. Blumbach, B. N. Ganguli, N. Klesel, M. Limbert, G. Seibert; *J. Antibiotics.;* **1992**, *45*, 839.

94. T. Prasch, T. Naumann, R. L. M. Marker, M. Sattler, W. Schubert, S. Schaal, M. Bauch, H. Kogler, C. Griesinger; *Eur. J. Biochem.;* **1997**, *244*, 501.

95. S. T. Hsu, E. Breukink, G. Bierbaum, H.-G. Sahl, B. De Kruijff, R. Kaptein, N. A. Van Nuland, A. M. Bonvin; *J. Biol. Chem.;* **2003**, *278*, 13110.

96. H. Brötz, G. Bierbaum, K. Leopold, P. Reynolds, H.-G. Sahl; *Antimicrobial. Agents. Chemother.;* **1998**, *42*, 154.

97. H. Brötz, G. Bierbaum, A. Markus, E. Molitor, H.-G. Sahl; *Antimicrobial. Agents. Chemother.;* **1995**, *39*, 714.

98. C. Szekat, R. Jack, D. Skutlarek, H. Färber, G. Bierbaum; *Appl. Environ. Microbiol.;* **2003**, *69*, 3777.

99. A. N. Appleyard, S. Choi, D. M. Read, A. Lightfoot, S. Boakes, A. Hoffmann, I. Chopra, G. Bierbaum, B. A. M. Rudd, M. J. Dawson, J. Cortes; *Chem. Biol.;* **2009**, *16*, 490.

100. A. C. Ross, J. C. Vederas; *J. Antibiotics.;* **2011**, *64*, 27.

101. G. Ösapay, L. Prokai, H. S. Kim, K. F. Medzihradszky, D. H. Coy, G. Liapakis, T. Reisine, G. Melacini, Q. Zhu, S. H.-H. Wang, R.-H. Mattern, M. Goodman; *J. Med. Chem.;* **1997**, *40*, 2241.

102. X. Zhu, R. R. Schmidt; *Eur. J. Org. Chem.;* **2003**, 4069.

103. K. Fukase, M. Kitazawa, A. Sano, K. Shimbo, S. Horimoto, H. Fujita, A. Kubo, T. Wakamiya, T. Shiba; *Bull. Chem. Soc. Jpn.;* **1992**, *65*, 2227.

104. D. N. Harpp, J. G. Gleason; *J. Org. Chem.;* **1971**, *36*, 73.

105. F. Cavelier-Frontin, J. Daunis, R. Jacquier; *Tetrahedron. Asymmetry.;* **1992**, *3*, 85.

106. T. Wakamiya, K. Shimbo, A. Sano, K. Fukase, T. Shiba; *Bull. Chem. Soc. Jpn.;* **1983**, *56*, 2044.

107. K. Fukase, T. Wakamiya, T. Shiba; *Bull. Chem. Soc. Jpn.;* **1986**, *59*, 2505.

108. K. Fukase, Y. Oda, A. Kubo, T. Wakamiya, T. Shiba; *Bull. Chem. Soc. Jpn.;* **1990**, *63*, 1758.

109. K. Fukase, M. Kitazawa, T. Wakamiya, T. Shiba; *Bull. Chem. Soc. Jpn.;* **1990**, *63*, 1838.

110. T. Wakamiya, Y. Oda, K. Fukase, T. Shiba; *Bull. Chem. Soc. Jpn.;* **1985**, *58*, 536.

111. M. J. Miller; *J. Org. Chem.;* **1980**, *45*, 3131.

112. J. M. Probert, D. Rennex, M. Bradley; *Tetrahedron. Lett.;* **1996**, *37*, 1101.

113. S. Burrage, T. Raynham, G. Williams, J. W. Essex, C. Allen, M. Cardno, V. Swali, M. Bradley; *Eur. J. Chem.;* **2000**, *6*, 1455.

114. Y. Zhu, M. D. Gieselman, H. Zhou, O. Averin, W. A. V der Donk; *Org. Biomol. Chem.;* **2003**, *1*, 3304.

115. A. Polinsky, M. G. Cooney, A. T. Palmer, G. Osapay, M. Goodman; *J. Med. Chem.;* **1992**, *35*, 4185.

116. H. Shao, S. H. H. Wang, C. W. Lee, G. Osapay, M. Goodman; *J. Org. Chem.;* **1995**, *60*, 2956.

117. N. D. Smith, M. Goodman; *Org. Lett.;* **2003**, *5*, 1035.

118. R. S. Narayan, M. S. Van Nieuwenhze; *Org. Lett.;* **2005**, *7*, 2655.

119. K. Nakajima, H. Oda, K. Okawa; *Bull. Chem. Soc. Jpn.;* **1983**, *56*, 520.

120. Y. Hata, M. Watanabe; *Tetrahedron.;* **1987**, *43*, 3881.

121. S. L. Cobb, J. C. Vederas; *Org. Biomol. Chem.;* **2007**, *5*, 1031.

122. C. Dugave, A. Menez; *Tetrahedron. Asymmetry.;* **1997**, *8*, 1453.

123. V. Swali, M. Matteucci, R. Elliot, M. Bradley; *Tetrahedron.;* **2002**, *58*, 9101.

124. S. Bregant, A. B.Tabor; *J. Org. Chem.;* **2005**, *70*, 2430.

125. A. Avenoza, J. H. Busto, G.-J. Oses, J. M. Peregrina; *J. Org. Chem.;* **2006**, *71*, 1692.

126. N. I. Martin; *J. Org. Chem.;* **2009**, *74*, 946.

127. V. Du Vigneaud, G. Brown; *J. Biol. Chem.;* **1941**, *140*, 767.

128. I. Photaki, I. Samouilidis, S. Caranikas, L. Zervas; *J. Chem. Soc. Perkin Trans.;* **1979**, *1*, 2599.

129. M. F. M. Mustapa, R. Harris, J. Mould, N. A. L. Chubb, D. Schultz, P. C. Driscoll, A. B. Tabor; *Tetrahedron Lett.;* **2002**, *43*, 8359.

130. L. D. Arnold, T. H. Kalantar, J. C. Vederas; *J. Am. Chem. Soc.;* **1985**, *107*, 7105.

131. S. Mazur, P. Jayalekshmy; *J. Am. Chem. Soc.;* **1979**, *101*, 677.

132. W. F. DeGrado, E. T. Kaiser; *J. Org. Chem.;* **1980**, *45*, 1295.

133. G. Ösapay, A. Profit, J. W. Taylor; *Tetrahedron. Lett.;* **1990**, *31*, 6121.

134. R. Pattabiraman, S. M. K. McKinnie, J. C. Vederas; *Angew. Chem. Int. Ed.;* **2008**, *47*, 9472.

135. I. Wiedemann, T. Bottiger, R. R. Bonelli, A. Wiese, S. O. Hagge, T. Gutsmann, U. Seydel, L. Deegan, C. Hill, P. Ross, H.-G. Sahl; *Mol. Microbiol.;* **2006**, *61*, 285.

136. A. C. Ross, H. Liu, V. R. Pattabiraman, J. C. Vederas; *J. Am. Chem. Soc.;* **2010**, *132*, 462.

137. M. F. A. Groussier; "A new orthogonal protecting group strategy for lanthionine containing peptides" *Ph.D*. University College London, **2000**.

138. K. Barlos, D. Papaioannou, D. Theodoropoulos; *J. Org. Chem.;* **1982**, *47*, 1324.

139. H. Kunz, H. Waldman, C. Unverzagt; *Int. J. Peptide. Protein. Res.;* **1985**, *26*, 493.

140. M. F. M. Mustapa; "Synthesis of lanthionine-containing peptides on solid phase via an orthogonal protecting group strategy" *Ph.D.* University College London, **2002**.

141. S. M. Weinreb, C. E. Clase, P. Wipf, S. Venkatraman; *Org. Syn.;* **1997**, *75*, 161.

142. S. Bregant; Postdoctoral report, Department of Chemistry, UCL, **2005**.

143. K. J. Hale, M. M. Domostoj, D. A. Tocher, Ed Irving, F. Scheinmann; *Org. Lett.*; **2003**, *5*, 2927.

144. A. C. van der Laan, R. Stromberg, J. H. Van Boom, E. Kuyl-Yeheskiely, V. A. Efimov, O. G. Chakhmakhcheva; *Tetrahedron. Lett.;* **1996**, *37*, 7857.

145. R. E. Shute, D. H. Rich; *Synthesis.;* **1987**, 346.

146. H. Kunz, J. Marz; *Angew. Chem., Int. Ed. Engl.;* **1988**, *27*, 1375.

147. R. Machauer, H. Waldmann; *Eur. J. Chem.*; **2001**, *7*, 2940.

148. R. Samy, H. K. Kim, M. Brady, P. L. Toogood; *J. Org. Chem.;* **1999**, *64*, 2711.

149. D. A. Evans, V. J. Cee, T. E. Smith, D. M. Fitch, P. S. Chio.; *Angew. Chem. Int. Ed.;* **2000**, *39*, 2533.

150. J. L. Wood, B. D. Thompson, N. Yusuff, D. A. Pflum, M. S. P. Mattaus; *J. Am. Chem. Soc.;* **2001**, *123*, 2097.

151. M. Schmidt, E. Barbayianni, I. Fotakopoulou, M. Hohne, V. C. Kokotou, U. T. Bornscheuer, G. Kokotos; *J. Org. Chem.;* **2005**, *70*, 3737.

152. M. Schultz, P. Hermann, H. Kunz; *Synlett.;* **1992**, 37.

153. P. Gmeiner, P. L. Feldman, M. Y. Chu-Moyer, H. Rapoport; *J. Org. Chem.;* **1990**, *55*, 3068.

154. R. B. Woodward, K. Heusler, J. Gosteli, P. Naegeli, W. Oppolzer, R. Ramage, S. Ranganathan, H. Vorbuggen; *J. Am. Chem. Soc.;* **1966**, *88*, 852.

155. G. Jou, I. Gonzalez, F. Albericio, P. L. Williams, E. Giralt; *J. Org. Chem.;* **1997**, *62*, 354.

156. D. L. Boger, S. Ichikawa, W. C. Tse, M. P. Hedrick, Q. Jin; *J. Am. Chem. Soc.;* **2001**, *123*, 561.

157. J. Bauer, K. Brandenburg, U. Zahringer, J. Rademann; *Eur. J. Chem.;* **2006**, *12*, 7116.

158. R. Vallinayagam, F. Schmitt, J. Barge, G. Wagnieres, V. Wenger, R. Neier, L. J. Jeanneret; *Bioconjugate. Chem.;* **2008**, *19*, 821.

159. S. Hoyem, S. Bruheim, G. Maelandsmo, M. Standal, D. E. Olberg, B. Brudeli, A. Asberg, J. Klaveness, P. Rongved; *Eur. J. Med. Chem.;* **2009**, *44*, 3874.

160. W. Chan, P. White; Fmoc Solid Phase Peptide Synthesis: A Practical Approach.; Oxford Press, New York, **2000**.

161. L. A. Carpino; *J. Am. Chem. Soc.;* **1993**, *115*, 4397.

162. L. A. Carpino, A. El-Faham, F. Albericio; *Tetrahedron. Lett.;* **1994**, *35*, 2279.

163. F. Albericio, M. Cases, J. Alsina, S. A. Triolo, L. A. Carpino, S. A. Kates; *Tetrahedron. Lett.;* **1997**, *38*, 4853.

164. D. Delforge, M. Dieu, E. Delaive, M. Art, B. Gillon, B. Devreese, M. Raes, J. Van Beeumen, J. Remacle; *Lett. Pept. Sci.;* **1996**, *3*, 89.

165. E. T. Kaiser, R. L. Colescott, C. D. Bossinger, P. I. Cook; *Anal. Biochem.;* **1970**, *84*, 595.

166. F. Guibe; *Tetrahedron.;* **1998**, *54*, 2967.

167. P. J. Kocienski; *Protecting Groups*.; 3rd edition, Thieme, New York.; **2005**, Chapter 1, 12.

168. K. Wüthrich; NMR of proteins and nucleic acid.; Wiley interscience, United States of America, **1986**.

169. P. Sieber; *Helv. Chim. Act.;* **1977**, *60*, 2711.

170. C. K. Marlowe; *Biorg. Med. Chem. Lett.;* **1993**, *3*, 437.

171. M. Ueki, M. Amemiya; *Tetrahedron. Lett.;* **1987**, *28*, 6617.

172. B. L. Bray; *Nat. Rev. Drug. Disc.;* **2003**, *2*, 587.

173. W. S. Kelly; *Biotechnol.;* **1996**, *14*, 28.

174. A. B. Pangborn, M. A. Giardello, R. H. Grubbs, R. K. Rosen, F. J. Timmers; *Organometallics.;* **1996**, *15*, 1518.

175. G. Ösapay, M. Goodman; *J. Chem. Soc. Chem. Commun.;* **1993**, 1599.

176. M. J. S. A. Amaral, M. A. Macedo, M. I. A. Oliveira; *J. Chem. Soc. Perkin. Trans. 1.;* **1977**, 205.

177. J. Metzger, K. -H. Weismuller. G. Jung; *Int. J. Peptide. Protein. Res.;* **1991**, *38*, 545.

178. R. K. Olsen, G. D. Kini, W. J. Hennen; *J. Org. Chem.;* **1985**, *50*, 4332.