Pseudomycoicidin, a novel lantibiotic produced by *Bacillus pseudomycoides*

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ABBREBIATIONS

°C	Degree Celsius
µl	Microliter
µM	Micromolar
aa	Amino acid(s)
ACN	Acetonitrile
APS	Ammoniumperoxodisulfate
ATCC	American type culture collection
ATP	Adenosine triphosphate
bp	Base pair
BSA	Bovine serum albumin
cm	Centimeter
D/W	Distilled water
Da	Dalton
Dha	Didehydroalanine
Dhb	Didehydrobutyrine
DMSO	Dimethylsulfoxide
DNA	Deoxy ribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
DSMZ	German research centre for microorganisms and cell cultures
DTT	Dithiothreitol
e.g.	Exempli gratia (Latin) means for “example”
EDTA	Ethylenediaminetetraacetic acid
et al.	et alii, and others
g	Gram
GlcNAc	N-acetylglucosamine
GTP	Guanosine triphosphate
h	Hour
HPLC	High performance liquid chromatography
IAA	Iodo acetamide
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	Kilobase pair
kDa	Kilo dalton
l	Liter
Lan	Lanthionine
LCMS	Liquid chromatography mass spectrometry
Lipid II	Glcnac-murnac-pentapeptide-pyrophosphoryl-undecaprenol
m/z	Mass-to-charge ratio
MALDI TOF	Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight
MeLan	Methyllanthionine

mg	Milligram
MIC	Minimal inhibitory concentration
MilliQ	Ultrapure water
min	Minute(s)
ml	Milliliter
mM	Millimolar
mRNA	Messenger RNA
MRSA	Methicillin- resistant *Staphylococcus aureus*
MS	Mass spectrometry
MurNAc	N-acetlymuramic acid
MW	Molecular weight
NADH	Nicotinamide adenine dinucleotide
NCS	German Reference Centre for Staphylococci, Werningerode, Germany
NCTC	Health protection agency culture collections, Salisbury,UK
Ni	Nickel ion
nm	Nanometer
NMR	Nuclear magnetic resonance
NRRL	Agricultural research service (ARS) culture collection
O.D. $_{600}$	Optical density at a wavelength of 600 nm
ORF	Open reading frame
PCR	Polymerase chain reaction
pH	Pondus hydrogenii, hydrogen ion concentration
PMA	Phosphomolybdic acid
PMSF	Phenylmethanesulfonylfluoride
RNA	Ribonucleic acid
rPCR	Reverse polymerase chain reaction
rpm	Revolutions per minute
RT	Room temperature
s	Second(s)
SDS -PAGE	Sodium dodecyl sulfate - poly acrylamide gel electrophoresis
TAE	Tris – acetate- EDTA
TCEP	Tris-(2-carboxyethyl) phosphine
TEMED	Tetramethylethylenediamine
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
Tris	Tris (hydroxymethyl)aminomethane
U	Units
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume
X-gal	5-bromo-4-chloro-3-indolyl beta- D-galactopyranoside
βME	Beta mercaptoethanol

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Abstract

The continuous increase of antibiotic resistant pathogens necessitates a search for new antibiotics. In this study a novel lantibiotic gene cluster was identified using the publicly available genome database. Blast searches employing the biosynthetic enzyme of the lantibiotic mersacidin (MrsM) in the NCBI database revealed a putative class II lantibiotic gene cluster in *Bacillus pseudomycoides* DSM 12442.

Lantibiotics are lanthionine and methyllanthionine containing antimicrobial peptides. They are ribosomally synthesized antimicrobial peptides with extensive posttranslational modifications. During the posttranslational modification, lanthionine and methyllanthionine are introduced to the peptide by means of dehydration of Ser/Thr residues, followed by the reaction of the didehydro amino acids with cysteines to form the thioether linkages. The structural (*lanA*), modification (*lanM, lanB, lanC, lanP, labKC* and *lanL*), regulation (*lanR, lanK*), export (*lanT* (*P*)) and immunity genes (*lanEFG*) are organized in the biosynthetic gene clusters.

The aim of the project was to characterize the putative lantibiotic gene cluster from *B. pseudomycoides*. In production assays, the cell wash extract of *B. pseudomycoides* showed the presence of an antimicrobial substance. The antimicrobial substance exhibited an activity against Gram-positive bacteria. The substance was partially purified by RP-HPLC, and the subsequent MALDI TOF MS predicted a mass of 2786.0 Da for an active peptide. The putative *pseA* and *pseM* genes from the predicted lantibiotic gene cluster of *B. pseudomycoides* were heterologously expressed in *E. coli*. The coexpression of the prepeptide (PseA) along with its corresponding modification enzyme (PseM) resulted in the production of a modified peptide, with four out of eight possible dehydrations. After the proteolytic removal of the leader peptide *in vitro* from the heterologously produced peptide, the core peptide showed an antimicrobial activity against the indicator strain *M. luteus*. The mass of the heterologously produced peptide was 2785.6 Da, which corresponded to the mass of the antimicrobial substance produced by *B. pseudomycoides*. This antimicrobial peptide is a novel class II lantibiotic and was named pseudomycoicidin. The chemical assays (IAA and βME) demonstrated the presence of four Lan/MeLan rings and one additional disulfide bond in the peptide. Further experiments are required to confirm the structure of the peptide and its mode of action.

1. Introduction

An antimicrobial substance is an agent that kills microorganisms or inhibits their growth. The toxic effect of antimicrobial substance against microorganism led it to be used as an important agent in controlling diseases. Antimicrobials that kill microbes are called microbicidal, while those that merely inhibit their growth are called microbiostatic. Antimicrobial substances can be grouped according to the target microorganisms they act upon; such as antibacterials (or antibiotics) against bacteria and antifungals against fungi. Several microbes, higher plants and animals produce antimicrobial substances (Thompson and Wright, 1998). A large number of antimicrobials currently used in the clinical therapy are derived from the natural products of microbial origin.

The discovery of penicillin by Alexander Fleming more than 90 years ago, made a revolution in medicine (Fleming, 1929). This invention saved millions of people from the death caused by bacterial infections. However, the wide spread use of antibiotics has caused a strong selective pressure resulting in the emergence of resistant strains. For example, a high percentage of resistance is acquired in hospitals and farming, where antibiotics are intensively used. In the 1960's, *Staphylococcus aureus,* the most common cause of hospital acquired infections, developed resistance to methicillin and several other antibiotics. Consequently, vancomycin was only a reliable agent for treating infections due to methicillin resistant *S. aureus* (MRSA) (Rubin et al., 1999). However, due to the excessive use of vancomycin to treat MRSA, vancomycin resistant *Enterococcus faecium* (VRE) appeared. In addition, there are numbers of Gram-negative pathogens such as *Pseudomonas aeruginosa*, *Acinetobacter baumanii* and Enterobacteriaceae which are resistant to many antibiotics (Livermore, 2004). Asuduzzaman et al. (2010), further conclude that today there are no antibiotics in clinical use, to which the resistance has not developed. Bacterial resistances to antibiotics will lead to treatment failure, unless new drugs are discovered. Thus, the search and the characterization of new drugs have become a primary concern for public health.

In search for alternatives to antibiotics, antimicrobial agents such as antimicrobial peptides, bacteriophages and probiotic bacteria are now being considered. Bacteria represent an inexhaustible source for the discovery of new antibiotics (Newman and Cragg, 2007; Davies, 2011). Even those bacteria, which once thought useless, are now reconsidered for the production of antibiotics such as *Burkholderia*, *Lysobacter*, plant and insect associated bacteria like *Pantoea* and *Photorhabdus*, *Janthinobacterium*, and anaerobic bacteria like clostridia (Pidot et al., 2014).

1.1. Antimicrobial peptides from bacteria

Many bacteria of different taxonomic branches and from various environments produce antimicrobial substances that are active against other bacteria. Both Gram-positive and Gram-negative bacteria produce bacteriocins. The bacteriocins form a heterogenous subgroup of ribosomally synthesized antimicrobial peptides. Members of the *Bacillus* group are considered as the good producers of antimicrobial substances, including, peptides, lipopeptides antibiotics, and polyketides (Stein, 2005). Some of its members, such as *B. subtilis*, devote more than 4% of its genome for the synthesis of polyketides (PKs), non-ribosomal peptides (NRPs), bacteriocins as well as other unusual antibiotics (Patrick, 2012).

1.1.1. Non-ribosomal peptides

Non-ribosomally synthesized peptides are produced by bacteria, fungi, and streptomycetes. Such peptides are synthesized in an ordered fashion by the very large multifunctional peptide synthetases. A single peptide synthetase gene (e.g., *grsB* of the gramicidin S biosynthetic operon) can be as large as 13 kb (4,300 amino acids) and contain four to six modules (resulting in the addition of four to six residues) (Krätzschmar et al., 1989). Each module recognizes a residue, activates it, modifies it as necessary, and adds it to the growing peptide chain (Felnagle et al., 2008). These modules consist of three main core domains - i) the adenylation domain (A), which selects and activates the cognate amino acid as an amino acyladenylate, ii) the thiolation domain (T), which catalyses the binding of the activated monomer to the synthetase through a phosphopantetheinyl arm, and iii) the condensation domain (C), which is responsible for the formation of the peptide linkage between the activated amino acids from the two adjacent T modules. The cyclic lipopeptide daptomycin and the glycopeptide vancomycin are examples for clinically established non-ribosomal peptides (Hancock and Chapple, 1999).

1.1.2. Ribosomally synthesized peptides

Ribosomally produced peptides are commonly known as bacteriocins. These peptides form a heterogenous group of proteinaceous antimicrobial substances. They display a high degree of target specificity against related bacteria, although many have a wider spectrum of activity (Jack et al., 1995). Characteristically, bacteriocins are gene-encoded and synthesized as precursors consisting of an N-terminal leader sequence and a C-terminal propeptide. The leader peptide has several functions: 1. it assists in the transport of the peptide through the cytoplasmic membrane, 2. it serves as a recognition signal for the modification machinery, 3.

it acts as an intramolecular chaperone, 4. it keeps the modified prepeptide inactive prior to export, and 5. it enhances the stability against proteolytic degradation resulting in a prolonged half-life of the precursor (Jack et al., 1995). The bacteriocins are classified differently at many instances by different authors.

Klaenhammer (1993) defined four distinct classes of bacteriocins: a. Class I, lanthionine containing peptides (lantibiotics); b. Class II, non-lanthionine-containing relatively heat-stable small (10 kDa) membrane-active peptides; c. Class III, large (30 kDa), heat-labile proteins; and d. Class IV, complex bacteriocins containing essential lipid or carbohydrate moieties in addition to the protein. The class II bacteriocins were further divided into three subclasses: i. Class IIa, *Listeria*-active peptides with the N-terminal consensus sequence -Tyr-Gly-Asn-Gly-Val-Xaa-Cys-; ii. Class IIb, poration complexes requiring two different peptides for their activity; and iii. Class IIc, thiol-activated peptides requiring reduced Cys residues for their activity.

Nes et al. (1996) and Moll et al. (1999) described three classes of bacteriocins: a. Class I, the small heat-stable polycyclic peptides (<5 kDa) containing unusual and posttranslationally modified amino acids also known as lantibiotics; b. Class II, the small (<10 kDa) heat-stable non-lantibiotics; and c. Class III, the large (>30 kDa) heat-labile bacteriocins. The class II bacteriocins were further divided into four subgroups – i. Class IIa, the so-called pediocin-like bacteriocins with a strong anti-*Listeria* activity; ii. Class IIb, the two-peptide bacteriocins; iii. Class IIc, the sec-dependent bacteriocins; and iv. Class IId, the bacteriocins not included in the previous class II subgroups.

1.1.2.1. Thiazole oxazole modified microcins (TOMMs)

The TOMMs are ribosomally produced peptides with posttranslationally installed heterocycles derived from Cys, Ser and Thr residues (Melby et al., 2011). The TOMM precursor peptide has an N-terminal region and a C-terminal region. The N-terminal region of the precursor peptide is known as the leader peptide, which contains basic recognition motifs for the biosynthetic machinery. The C-terminal region is known as the core peptide. The core peptide is rich in heterocyclizable residues and can be the site for numerous other posttranslational modifications (Oman and van der Donk, 2010). The TOMMs are encoded by the structural genes that code for peptides of 50 - 70 amino acids, and are rich in Cys, Ser, Thr and Gly residues. These amino acids are often found in repetitive motifs and serve as a precursor amino acids for the class-specific thiazoles and oxazoles (Haft, 2009; Melby et al., 2011) (Figure 1). Microcin B17 (Li et al., 1996), thiopeptides (Bagley et al., 2005),

cyanobactins (Sivonen et al., 2010) and goadsporins (Onaka et al., 2005) are few examples of TOMMs. Lee et al. (2008) described a wide spread distribution of biosynthetic gene clusters for producing a subclass of toxins with thiazole and oxazole rings. During TOMM biosynthesis, the BCD synthetase complex binds to the precursor peptide through the specific motifs within the N-terminal leader sequence (Roy et al., 1999; Mitchell et al., 2009). This synthesis is completed in two enzymatic steps (Scholz et al., 2011). At first, Cys and Ser/Thr residues are converted into the corresponding thiazolines and (methyl) oxazolines. This step is catalyzed by a cyclodehydratase enzyme. In the second step, the thiazoline and oxazoline rings are oxidised to yield thiazole and (methyl) oxazole rings, with a net loss of 20 Da. This step is catalyzed by a dehydrogenase enzyme. Finally, the prepeptide is proteolytically cleaved and thus the leader peptide is removed from the modified peptide. The fully matured TOMMs are then actively exported from the cell by an ABC transport system. In addition to cyclodehydratase and dehydrogenase, TOMMs have a docking protein (Haft, 2009; Melby et al., 2011). The docking protein is often fused to C-terminus of cyclodehydratase. Such docking proteins regulate the cyclodehydratase and assemble the active synthetic complex. Furthermore, TOMM like biosynthetic gene clusters may contain additional modification enzymes (Lee et al., 2008). For example, the cluster of goadsporin contains acyltranferases and lantibiotic dehydratases (Onaka et al., 2005).

Recently, a new family of TOMMs was identified (Haft et al., 2010). The precursor peptides of this family possess very long leader sequences and share homology either to the alpha subunit of the enzyme nitrile hydratase (NHase) (Nitrile hydratase related leader peptides, NHLP) or to the nitrogen fixing proteins from cyanobacteria (NiF11). NHLPs are found adjacent to cyclodehydratase scaffold docking proteins.

Likewise, thiopeptides are another group of TOMMs having thiazole-containing compounds (Bagley et al., 2005). Most thiopeptides are characterized by a macrocyclic core consisting of thiazoles / thiazolines. These thiazoles are joined together by a six-membered heterocycle such as pyridine, hydroxypyridine or dehydropiperidine (for example in thiostrepton). Examples of such thiopeptides are thiostrepton (Kelly et al., 2009), thiocillin (Brown et al., 2009), siomycin (Liao et al., 2009), thiomuracin (Morris et al., 2009) and nosiheptide (Yu et al., 2009). The leader sequences (34–48 residues) of thiopeptides are rich in aspartate and glutamate and have some highly conserved hydrophobic residues. Several leader peptides have the typical double glycine motif (Oman and van der Donk, 2010). The modification of thiopeptides occurs in three main steps: (i) Ser and Thr are dehydrated to dehydroamino acids by the enzymes that are similar to the lantibiotic dehydratases, (ii)

thiazoles and thiazolines are formed by enzymes that have homology to the cyanobactin, microcin B17 and SLS biosynthetic enzymes, and (iii) two dehydroalanines are cyclised to produce the central six-membered heterocycle compound. Additionally, in some cases compound-specific modifications also occur, which complete the maturation process.

The TOMM-like compounds have different activities such as antifungal, antibacterial, antimalarial, antitumor, etc. The activities of these compounds depend upon membrane depolarization, inhibition of DNA gyrase and inhibition of the protein biosynthesis (Lee et al., 2008; Melby et al., 2011).

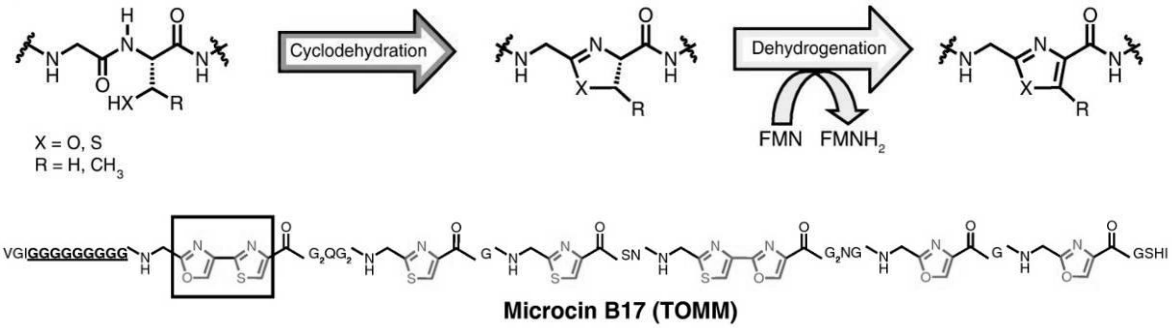

Figure 1: The biosynthesis and structure of thiazole/oxazole containing Microcin B17 compound. Figure adapted from Melby et al. (2011).

1.1.2.2. Lantibiotics

Lantibiotics are small, heat-stable, ribosomally synthesized, and posttranslationally modified antimicrobial peptides (bacteriocins) produced by Gram-positive bacteria (Schnell et al., 1988; Arnison et al., 2012). The lantibiotics, unlike other bacteriocins, are characterized by the presence of thioether amino acids, lanthionine (Lan) and 3-methyllanthionine (MeLan) and various modified amino acids, such as didehydroalanine (Dha) and didehydrobutyrine (Dhb) (Schnell et al., 1988). The structures of some representative groups of lantibiotics are shown in Figure 2. Lantibiotics are initially synthesized as the inactive linear prepeptides that later undergo subsequent extensive modifications to be biologically active. The modifications include the dehydration of serine and threonine residues to form the didehydro amino acids Dha and Dhb, respectively. Later, the Dha and Dhb react with the nearby C-terminally located Cys residues to form a thioether linkage forming Lan and MeLan, respectively. Finally, in order to be active, the modified peptide is exported and cleaved from its leader.

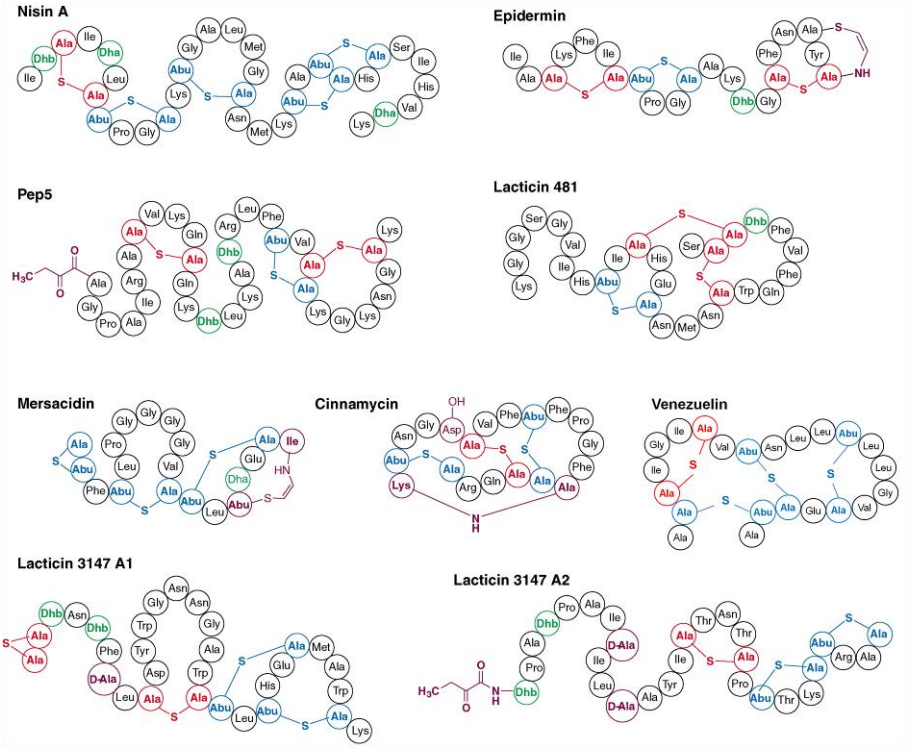

Figure 2: Structures of representative members of the lantibiotics. Post translational modifications are represented by its abbreviations: Abu - 2-aminobutyric acid; Ala-S-Ala - lanthionine (red); Abu-S-Ala - 3-methyllanthionine (blue); Dha - dehydroalanine and Dhb - dehydrobutyrine (green). All other post-translational modifications including Asp-OH, b-hydroxy aspartate, are shown in purple. Figure adapted from Xie and van der Donk (2004) and Goto et al. (2010).

Although, the first characterized lantibiotic, nisin, was discovered over eighty years ago (Rogers and Whittier, 1928), the ribosomal origin of such modified peptides were revealed only after 60 years, when epidermin biosynthetic gene cluster from *Staphylococcus epidermidis* Tü 3298 was sequenced (Schnell et al., 1988). Since then, around 100 lantibiotics have been described, and most of which are produced by Gram-positive bacteria (Emma et al., 2013). In addition, numbers of actinomycete lanthipeptide biosynthetic gene clusters have been characterized - cinnamycin from *Streptomyces cinnamoneus* DSM 40005 (Widdick et al., 2003), SapB from *Streptomyces coelicolor* (Kodani et al., 2004), SapT from *Streptomyces tendae* (Kodani et al., 2005), actagardine from *Actinoplanes garbadinensis* (Boakes et al., 2009), deoxyactagardine B (DAB) from *Actinoplanes liguriae* (Boakes et al., 2010), venezuelin from *Streptomyces venezuelae* (Goto et al., 2010), and microbisporicin from *Microbispora corallina* (Foulston et al., 2010). Furthermore, lanthipeptide gene clusters are

not limited to the firmicutes and actinomycetes, but are also found in proteobacteria, chlamydiae, bacteroidetes and cyanobacteria (Li et al., 2010; Marsh et al., 2010).

1.1.2.2.1. Lantibiotic gene cluster

The genes for lantibiotic biosynthesis are generally found in clusters. The generic locus is designated by the symbol '*lan'*, with a more specific designation for each lantibiotic member e.g. *nis* for nisin, *gdm* for gallidermin, and *cin* for cinnamycin. Gene clusters can be found on conjugative transposable elements (e.g., nisin), on the chromosome of the host (e.g., subtilin), or on the plasmids (e.g., epidermin, lacticin 481) (Chatterjee et al., 2005b). A biosynthetic gene cluster consists of several genes encoding the prepeptide (LanA), the modification enzymes (LanB, LanC, LanM, LanL and LabKC) that introduce the thioethers, an ABC transporter (LanT) that exports the modified prepetide and removes the leader, and / or extracellular protease (LanP) that removes the leader, and finally immunity proteins [LanI (H) and/or LanFEG] that protect the producer. Usually, parts of the cluster are organized in operons that allow the co-expression of proteins that are involved in the similar functions. The structural gene (LanA) is often clustered with the genes of modification enzymes in a biosynthetic operon, and it is usually the first ORF. Besides this, there is no uniform gene order in the individual gene clusters (Siezen et al., 1996; Willey and van der Donk, 2007). The genes that are necessary for modification, transport, processing, self protection, and regulation are found in close proximity to the structural genes. Some representative biosynthetic gene clusters are shown in Figure 3.

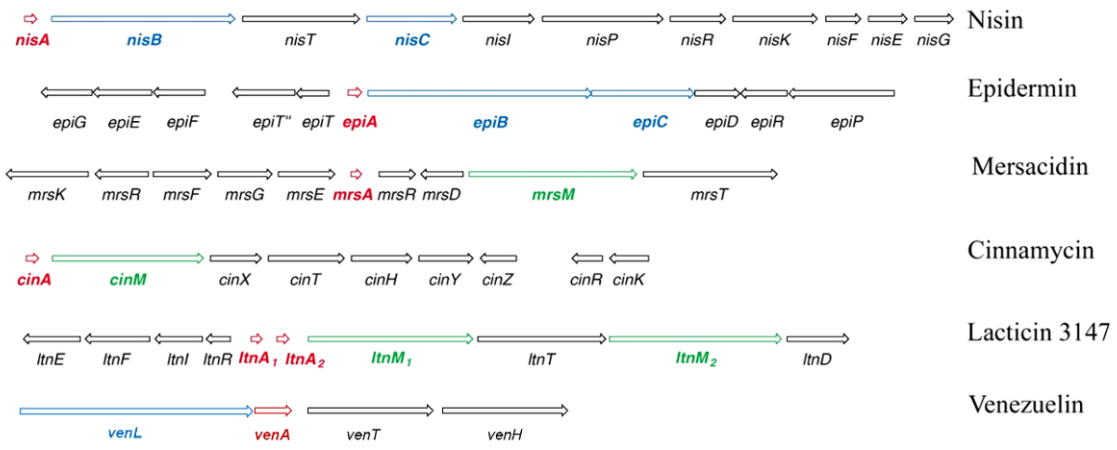

Figure 3: Representative biosynthetic gene clusters of the lantibiotics nisin (Kuipers et al., 1993), epidermin (Schnell et al., 1992), mersacidin (Altena et al., 2000), cinnamycin (Widdick et al., 2003), lacticin 3147 (McAuliffe et al., 2001), venezuelin (Goto et al., 2010). Figure adapted from Xie and van der Donk (2004).

1.1.2.2.3. Biosynthesis and modification

Post translational modifications and proteolytic removal of the leader peptide converts the inactive propeptide to their active forms. In general modification occurs in a two-step reaction – a dehydration step and a cyclization step. In dehydration step, Ser and Thr residues dehydrate to form didehydroamino acids: dehydroalanines (Dha) (from Ser) and dehydrobutyrines (Dhb) (from Thr) (Figure 4). This step is catalysed by a dehydratase. Later, in a cyclization step, following the regio- and stereoselective Michael addition reaction, a sulfhydryl-group of a neighboring Cys residue is linked to the unsaturated double bond of the didehydro amino acid, resulting in the generation of lanthionine (Dha + Cys) and methyllanthionine (Dhb + Cys) (Ingram, 1969; 1970; Toogood, 1993). This step is catalyzed by a cyclase. Although, the dehydration and cyclization steps are catalyzed by different enzymes, in some cases, a single enzyme carries out both steps. So far, five different modification enzymes are identified to be involved in the lanthionine formation - LanB, LanC, LanM, RamC/LabKC and LanL. Recently, an *in vitro* study in nisin showed that the dehydratase enzyme dehydrates its substrate in the presence of glutamate, ATP, Mg^{2+} and the ribosomal / membrane fraction of the bacterial cell extract (Garg et al., 2013). Likewise, the lantibiotic cyclase utilizes zinc for activation of a thiol of its substrate (Mathews et al., 1997; Okeley et al., 2003).

In addition to the dehydration and cyclization steps, some lantibiotics undergo other additional modifications. For example, in labyrinthopeptins, labionin (Lab) is formed through a subsequent 2-fold Micheal type addition cyclization, which is induced by the nucleophilic attack of a C-terminal cysteine side chain (Meindl et al., 2010). Likewise, in epidermin and mersacidin, the oxidative decarboxylases (EpiD and MrsD) catalyze the oxidative decarboxylation of the C-terminal cysteine to form S-Aminovinyl-D-cysteine (AviCys) and S-Aminovinyl-3-Methyl-D-cysteine (MeAviCys) (Figure 4) (Kupke and Götz, 1996). In cinnamycin, a lysinoalanine bridge is formed by addition of the ε-amino group of Lys to Dha (Kaletta et al., 1991; Zimmermann et al., 1993). Apart from these modifications in the peptides that result in the cyclic structures, many peptides undergo large number of structural modifications that do not necessarily result in the cyclic structures. Among such modifications are the hydroxylations of Asp in cinnamycin and duramycins (Kaletta et al., 1991), hydroxylation of Pro and chlorination of Trp in microbisporicin (Knerr and van der Donk, 2012), and *allo*-Ile in cypemycin, and D-Ala in lacticin 3147 and lactocin S. Furthermore, Dha and Dhb residues, that are N-terminally exposed after leader peptide processing, spontaneously hydrolyze to yield a 2-oxopropionyl (OPr) moiety in lactocin S, and a 2-

oxobutyryl (OBu) group in Pep5. OPr may be further modified by its reduction to a 2-hydroxypropionyl (Hop) residue in epilancin 15X, epilancin K7, and epicidin 280 (Kellner et al., 1989; van de Kamp et al., 1995; Ekkelenkamp et al., 2005). Such structural modifications improve the peptide stability and protect the peptide from proteolysis (Knerr and van der Donk, 2012).

The removal of the leader peptide from the modified peptide activates the lantibiotic. In nisin like peptides and lactocin S, the removal of the leader peptide from the modified peptide is catalysed by the subtilisin like serine protease (LanP). This event can occur, both before and after the export from the cell by the ABC transporter LanT. Those lantibiotics, which contain the double Gly cleavage site in their prepeptides, possess LanT transporters. These LanT transpoters perform both the cleavage and the export reactions (Sahl and Bierbaum, 1998). In contrast, with the cytolysins, this processing by the transporter, does not lead to the activation of the peptides. After secretion, the prepeptides are cleaved for the second time by the specialized serine protease CylA (Booth et al., 1996).

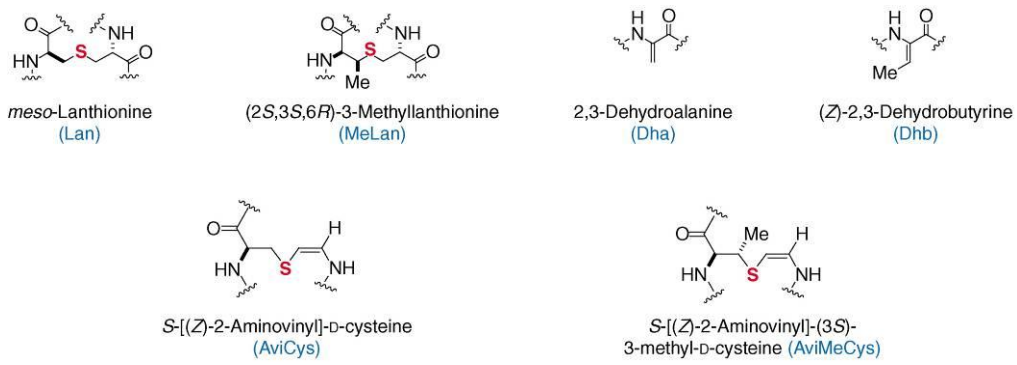

Figure 4: Structural motifs found in lantibiotics that are introduced by post translational modification. Figure adapted from Xie and van der Donk (2004).

1.1.2.2.2. Regulation of lantibiotic biosynthesis

Production of a lantibiotic is energy–intensive, and therefore, this process should be tightly regulated. Regulation of lantibiotic production is important for the producer strain to maintain the bacteriocin production and immunity to its product. Production of many lantibiotics e.g. nisin, subtilin, mersacidin, streptococcin AFF22 and salivaricin A is regulated either by two component regulatory systems or by growth phase dependent mechanisms (Chatterjee et al., 2005b). Many regulatory mechanisms belong to the quorum-sensing systems. Quorum-sensing is an intraspecies communication process that allows cells to sense other organisms in

their surroundings in a cell-density-dependent manner. The typical two component regulatory system consists of a receptor-histidine kinase (LanK) and a transcriptional response regulator (LanR) (McAuliffe et al., 2001; Bierbaum and Sahl, 2009). The receptor histidine kinases possess extracellular loops and are involved in detecting extracellular changes, leading to a signal cascade initiated by autophosphorylation of a histidine residue. Subsequently, the phosphate group is transferred to the appropriate response regulator, that often functions as a transcriptional activator (van Kraaij et al., 1999), and regulates transcription of the target genes (Yonezawa and Kuramitsu, 2005; Willey and van der Donk, 2007).

In most of the cases, the extracellular lantibiotic functions as an auto inducer for its production. Only in a few exceptions, such as in epidermin production by *Staphylococcus epidermis*, the regulation is not autoinduced (Kies et al., 2003). In case of the nisin regulatory system, the phosphoryl group from NisK is transferred to an aspartate in NisR, which initiates its binding to the NisA and NisF operators (Chatterjee et al., 2005b). This in turn, activates transcription of the *nisABTCIP* and the *nisEFG* operon which are involved in nisin biosynthesis and self protection respectively.

Some lantibiotic gene clusters (e.g. mersacidin and cinnamycin) contain an extra regulatory gene apart from the two component regulatory gene. In the mersacidin gene cluster, in addition to the two component regulatory system, MrsR2 and MrsK2, which activate the transcription of the immunity genes, the MrsR1 is also present, which activates biosynthesis (Guder et al., 2002). Likewise, in the cinnamycin gene cluster, apart from the two component regulatory system, CinK/CinR, the extra regulator CinR1 is also present. At first CinK and CinR transcriptionally activate CinR1 which in turn induces the transcription of CinA (Widdick et al., 2003).

Many lantibiotics contain orphan regulators in their gene cluster e.g. MutR in mutacin II, EpiQ in epidermin, LtnR in lacticin 3147 and RamR in SapB. These genes positively regulate the production of these lantibiotics (Bierbaum and Sahl, 2009; Li et al., 2012). Other regulators can also influence the production of lantibiotics, such as the quorum sensing regulator accessory gene regulator (Agr), which is involved in the regulation of the epidermin protease (EpiP) (Kies et al., 2003). Likewise, the two-component lantibiotic cytolysin is also regulated by a cell-density-dependent mechanism. In the absence of target cells, the cytolysin production is repressed by CylR1. When the target cells are present, CylLL binds to cholesterol lipid bilayers (phospatidylcholine), leading to its inability to titrate any free CylLS. This accumulation of the free CylLS results in a high level of cytolysin expression (Coburn et al., 2004).

The gene cluster of lacticin 481 lacks any regulatory genes. Lacticin 481 transcription is regulated by pH mediated control of P1 and P3 promoters located upstream of lctA. During growth of *L. lactis*, it produces lactic acid, which leads to a decrease in the pH of the growth medium from 7 to 5.8. This natural acidification correlates with the amount of lacticin 481 produced by *L. lactis* (Chatterjee et al., 2005a). Since the lacticin 481 does not contain a dedicated regulation system, transcription from the P1 and P3 promoters is probably governed by a general regulatory system such as a broad specificity quorum sensing system (Hindre et al., 2004).

In some cases, lantibiotic production can also be regulated by the other environmental conditions. For example, normally nisin biosynthesis by *Lactococcus lactis* is autoregulated by signal transduction *via* the two component regulatory system. However, during the growth in the presence of galactose, transcription of NisA is also induced by galactose, even in the absence of NisRK (Chandrapati and O'Sullivan 1999, 2002).

1.1.2.2.4. Self protection/ immunity

Any bacterial strain that produces lantibiotics, which are active against closely related bacteria, must protect itself from the inhibitory action of its own product (Chatterjee et al., 2005b). Immunity may be mediated by: i) the immunity genes (*lanI*) and ii) the ABC transport protein consisting of two or three subunits (LanEFG).

The immunity gene *lanI* is found in the gene clusters of lantibiotics like cytolysin, epicidin 280, Pep5 and lactocin S. The LanI proteins or peptides are highly specific and do not show similarity to each other (McAuliffe et al., 2001; Bierbaum and Sahl, 2009). PepI was the first LanI protein described for lantibiotics. PepI is a short peptide (57 - 69 amino acids) attached to the outer side of the membrane and is thought to shield the membrane from the pore forming activity of Pep5. The gene clusters of nisin, subtilin and cytolysin contain larger LanI proteins known as NisI, SpaI and CylI respectively. NisI and SpaI carry an N-terminal lipoprotein sequence. These proteins become peripheral membrane proteins, and are attached to the membrane by lipids (McAuliffe et al., 2001). It is known that the C-terminal domain of NisI is involved in the binding of nisin and most probably it interacts with NisFEG *via* its N-terminal part (Bierbaum and Sahl, 2009). Studies on nisin and lacticin 3147 showed that their immunity peptides directly interact with the corresponding lantibiotic, resulting in the formation of an insoluble complex (Stein et al., 2003; Draper et al., 2009).

Some gene clusters possess an additional LanH protein for immunity. In epidermin the LanH protein acts as an ancillary peptide for the transport (Peschel et al., 1997). In contrast, in

the nukacin ISK-1 immunity system, the membrane protein NukH recognizes the C-terminus of nukacin. Nukacin is bound by NukH and subsequently removed from the membrane by NukFEG (Okuda et al., 2008).

The ABC transporter proteins LanEFG are found in the gene clusters of nisin, epidermin, mersacidin, lacticin, etc. In the subtilin system, the ABC transporter involved in immunity is encoded by the two genes, *spaF* and *spaG* (Klein et al., 1994). Similar to the HisP family of translocators (Blight et al., 1990), SpaF contains both an ATP-binding domain at N-terminus and a membrane – spanning domain at the C-terminus (Klein et al., 1994). The lacticin 3147 gene cluster harbours genes encoding an ATP-binding domain (LtnF) and membrane-spanning domain (LtnE) of an ABC transporter (McAuliffe et al., 2000). Peschel and Götz (1996) proposed that the LanEFG proteins could mediate immunity, either by active extrusion of the respective peptide, which would keep the lantibiotic concentration in the membrane below a critical level, or by the uptake and intracellular degradation.

1.1.2.2.5. Classification of lantibiotics

Previously, based on structural similarities, charges and sizes of the active peptide, lantibiotics were classified into two groups - type A and type B. The members of type A were strongly cationic peptides with 2 - 7 positive charges and their molecular mass was greater than 2.1 kDa. In contrast the members of type B were weakly cationic peptides and their molecular mass was smaller than 2.1 kDa with complex ring structures (Jung, 1991). The type A lantibiotics were further divided into two subgroups - type A-I and type A-II, based on the presence or absence of the FNLDV motif in their leader sequences respectively (de Vos et al., 1995).

Recently, the lantibiotics have been categorized into four new classes, on the basis of their biosynthetic enzymes (Figure 5), mature peptide structure, leader peptide sequence, and antimicrobial activity (Willey and van der Donk, 2007). The detailed classification of lantibiotics is presented in Table 1, page 15.

In class I lantibiotics, the peptide is dehydrated by LanB followed by its cyclization by LanC. The LanB dehydratases (Figure 1) convert Ser and Thr to didehydroalanine (Dha) and Z-didehydrobutyrine (Dhb), respectively. A subsequent intramolecular addition of Cys thiols to Dha / Dhb is catalyzed by LanC cyclases and form the characteristic lanthionine (Lan from Ser) and methyllanthionine (MeLan, from Thr) thioether crosslinks. Then the mature prepeptide is transported by an ABC transporter LanT. The leader is cleaved intracellularly or extracellularly by the protease LanP (van der Meer et al., 1993; Meyer et al., 1995) or by

another protease of the producers. The leader peptide of class I lantibiotics contains a characteristic F (N/DL (E/N/D) motif at -20 to -15 towards the cleavage site (van der Meer et al., 1993; Oman and van der Donk, 2010). The conserved FNLD box of the leader peptide is an important component for efficient *in vivo* production of the lantibiotic (Knerr and van der Donk, 2012).

In class II lantibiotics, a bi-functional LanM modifying enzyme carries the function of both dehydratase and cyclase. The C-terminal cyclase domain of LanM proteins has sequence homology to the LanC enzymes, but the N-terminal dehydratase domain of LanM proteins has no homology with LanB enzymes (Siezen et al., 1996) (Figure 5). According to Paul et al. (2007), ATP hydrolysis is required for the dehydration reactions. LanM protein consists of three zinc binding ligands, which are essential for the cyclase activity (Willey and van der Donk, 2007). Here, the zinc ions are involved in the activation of the Cys derived SH-group into an ethiolate, which subsequently reacts with the didehydro-amino acid to form the thioether-amino acid (methyl-) lanthionine (Zhu et al., 2003; Li et al., 2006). Also, the secretion and leader processing is performed by a single, multifunctional protein with a conserved N-terminal cysteine protease domain. The LanT of class II lantibiotics is a member of the ABC transporter maturation and secretion (AMS) protein family. Members of ABC transporter sub family are involved in the secretion of the peptides, and contain an additional N-terminal domain involved in the processing of their substrates at the GG site at the C-terminal end of the leader peptide (Nishie et al., 2011). The class II peptides, usually have a double glycine cleavage site (GG) and a leader motif ELXX(V/L/I)X (Patton et al., 2008). In some cases GG cleavage site is altered to GA or GS (Chatterjee et al., 2005b). Within the class II lantibiotics, there are the two-component lantibiotics; here two peptides assemble to form the mature antibiotic. Each peptide is encoded by its own structural gene and is modified by separate LanM enzyme. However, a single LanT protein removes the leader peptide and secretes both products (Willey and van der Donk, 2007).

In class III lantibiotics, the lanthionine containing peptides lack antimicrobial activity; instead they perform morphogenic and signalling functions for the producer cells. These peptides are modified by RamC whose C-terminal cyclase domain bears homology to LanM but lacks zinc ligands (Figure 5). This indicates that their maturation mechanism may be different (Kodani et al., 2005; Willey and van der Donk, 2007). The leader peptides often have GG protease sites and additionally a conserved helical region at their N-termini (Müller et al., 2011). Likewise, the labyrinthopeptins are also classified as the class III lantibiotics, because they are also posttranslationally processed by the RamC-like kinase-cyclase LabKC

enzyme (Meindl et al., 2010). LabKC shows little sequence similarity to a LanC-like domain and lacks active sites like zinc motifs. It catalyzes all necessary modification reactions such as serine phosphorylation, formation of didehydroalanines, and subsequent 2-fold Micheale-type addition cyclizations. It requires GTP for its kinase activity (Müller et al., 2010). The gene clusters of the labyrinthopeptins also possess two LanT like transporters without a dedicated protease (Müller et al., 2011, Knerr and van der Donk, 2012).

In class IV lantibiotics, the peptides are modified by the lanthionine synthetase LanL (Goto et al., 2010). The LanL contains a LanC-like cyclase domain in its C-terminal part with the conserved zinc binding sites. In its N-terminal part, it contains an OspF-like lyase domain and a central serine/threonine kinase domain, both of which align well with the class III synthetase RamC. But unlike RamC, the C-terminal cyclase domain of LanL possesses the zinc-binding motif (Figure 5). Goto et al. (2010) postulated that the Thr/Ser residues are phosphorylated followed by phosphate elimination to generate dehydrated amino acids. LanL requires ATP and $MgCl_2$ for the dehydration reaction. Similar to the enzymes of the class III lantipeptides, it also has a LanT-like transporter, but no dedicated protease or protease domain (Goto et al., 2010, Knerr and van der Donk, 2012).

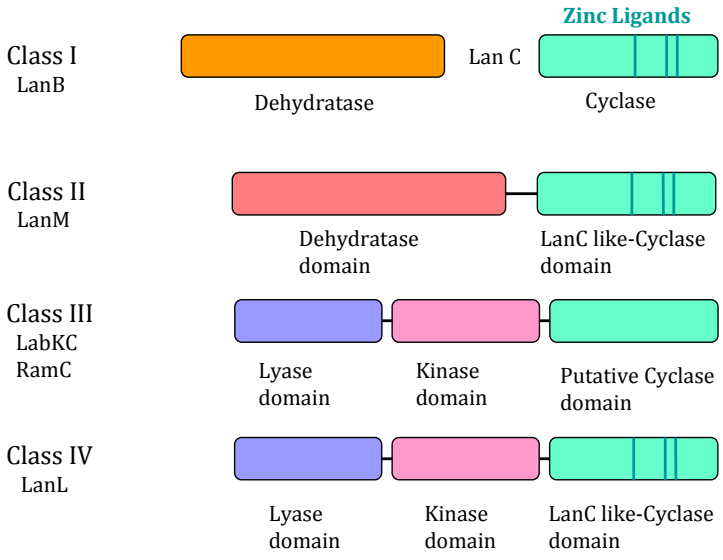

Figure 5: Schematic representation of four classes of biosynthetic enzymes. Zinc ligands are highlighted. Abbrebiations: LanB- lanthipeptide dehydratase; LanC- lanthipeptide cyclase; LanM- class II lanthipeptide synthetase; LabKC- labionin synthetase; RamC, SapB - modifying synthetase; LanL- class IV lanthipeptide synthetase. Figure adapted from Knerr and van der Donk (2012).

Table 1: Classification of lantibiotics.

Class	Sub Class	Name	Producer	MW (Da)	aa	Charge	Total rings	Other residues	Reference
Class I, LanBC modified peptides	Nisin like lantibiotics	NisinA	*Lactococcus lactis* ATCC 11454	3353	34	+3	5	Dha, Dhb	Gross and Morell, 1971
		Nisin Z	*Lactococcus lactis* n8, NIZO22186	3330	34	+3	5	Dha, Dhb	Mulders et al., 1991
		Nisin Q	*Lactococcus lactis* 61-14	3327	34	+3	5	Dha, Dhb	Zendo et al., 2003
		Nisin U	*Streptococcus uberis* 42	3029	34	+3	5	Dha, Dhb	Wirawan et al., 2006 and 2007
		Nisin F	*Lactococcus lactis* F10	3457	34	+3	5	Dha, Dhb	de Kwaadsteniet et al., 2008
		Subtilin	*Bacillus subtilis*	3317	32	+2	5	Dha, Dhb	Gross et al., 1973
		Ericin S	*Bacillus subtilis* A1/3	3442	32	+1	5	Dha	Stein et al., 2002
		Ericin A	*Bacillus subtilis* A1/3	2986	29	+2	5	Dha	Stein et al., 2002
		Planosporicin (LAB97518)	*Planomonospora sps.*	2194	24	-1	5	Dha, Dhb	Maffioli et al., 2009 Castiglione et al., 2007
		Streptin 1	*Streptococcus pyogens* M25	2424	23	+3	3	Dhb	Karaya et al., 2001
		Streptin 2	*Streptococcus pyogens* M25	2821	23	+3	3	Dha	Wescombe and Tagg, 2003
		Entianin	*Bacillus subtilis* DSM 15029T	3348	32	+2	5	Dha, Dhb	Fuchs et al., 2011
		Salivaricin D	*Streptococcus salivarius* 5M6c	3475	34	+1	4	Dha, Dhb	Birri et al., 2012
		Geobacillin I	*Geobacillus thermodenitrificans* NG80-2		34		7	Dhb	Garg et al., 2012
	Epidermin like lantibiotics	Epidermin	*Staphylococcus epidermidis*	2167	22	+3	4	Dhb, AviCys	Allgaier et al., 1986
		Epidermin (Val1,Leu6)	*Staphylococcus epidermidis*	2151	22	+3	4	Dhb, AviCys	Israil et al., 1996
		Gallidermin	*Staphylococcus gallinarum* Tü3928	2165	22	+3	4	Dhb	Kellner et al., 1988
		Staphylococcin T	*Staphylococcus cohnii*						Furmanek et al., 1999
		BsaA2, Staphylococcin Au-26	Community acquired MRSA	2089	22	+2	4	Dha, Dhb, AviCys	Daly et al., 2010
		Mutacin B-Ny266	*Streptococcus mutans*	2270	22	+3	4	Dhb, AviCys	Mota-meira et al., 1997
		Mutacin 1140 Mutacin II	*Streptococcus mutans*	2263	22	+3	4	AviCys	Hillman et al., 1998; Qi et al., 1999
		Mutacin I	*Streptococcus mutans*	2364	24	+2	4	AviCys	Qi et al., 2000
		Clausin	*Bacillus clausii* OC		22	0	4	Dha,Dhb, AviCys	Bouhss et al., 2009
		Microbisporocin	*Microbispora sps. (corallina)*	2246	24	+1	5	AviCys	Castiglione et al., 2008
		(Different variants)	*Microbispora sps. (corallina)*	2230	24	+1			

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Class	Sub Class	Name	Producer	MW (Da)	aa	Charge	Total rings	Other residues	Reference
Class I, LanBC modified peptides	Pep5 like lantibiotics	Pep5	*Staphylococcus epidermidis*	3488	34	+7	3	Dha, 2-oxobutyryl	Kaletta et al., 1989
		Epilancin K7	*Streptococcus epidermidis* K7	3032	31	+5	3	Dha, Dhb, 2-hydroxypropionyl	van de Kamp et al., 1995
		Epilancin 280	*Streptococcus epidermidis*	3133	30	+4	3	2-hydroxypropionyl	Heidrich et al., 1998
		Epilancin 15X	*Streptococcus epidermidis*	3173	30	+7	3	Lactate	Ekkelenkamp et al., 2005
		Epicidin 280	*Staphylococcus epidermis* BN280	3133	30	+4	3	Dhb, 2-hydroxypropionyl	Heidrich et al., 1998
		Elgicin AI	*Paenibacillus elgii* B69	4536					Teng et al 2012
		Elgicin AII	*Paenibacillus elgii* B69	4593					
		Elgicin B	*Paenibacillus elgii* B69	4706					
		Elgicin C	*Paenibacillus elgii* B69	4820					
Class II, LanM modified peptides	Lacticin 481like	Lacticin 481	*Lactococcus lactis* CNRZ 481	2901	27	0	3	Dhb	Piard et al., 1993
		Mutacin II, JT-8, H-29B	*Streptococcus mutans* T8	3245	27	+1	3	none	Novak et al., 1994
		Nukacin ISK-1	*Staphylococcus warneri*	2960	27	+3	3	Dhb	Sashihara et al., 2000
		Nukacin KQU131	*Staphylococcus hominis* KQU-131	3004	27	+3	3	Dhb	Wilaipun et al., 2008
		Mutacin K8	*Streptococcus mutans* K8	2734					Robson et al., 2007
		Streptococcin SA-FF22	*Streptococcus pyogene* SA-FF22	2795	26	+2	3	Dhb	Jack et al., 1994
		Streptococcin AM 49	*Streptococcus pyogenes*						Hynes et al., 1994
		Macedocin	*Streptococcus macedonicus* ACA-DC 198						Georgalaki et al., 2002
		Macedocin A1	*Streptococcus macedonicus* ACA-DC 198		23	0	3	Dha	Papadelli et al., 2007
		Macedovicin	*Streptococcus macedonicus* ACA-DC 198	3428	33	+2	2	Disulfide bridge	Georgalaki et al., 2013
		Variacin	*Kocuria varians*	2658	25	-1	3	Dhb	Pridmore et al., 1996
		Plantaricin C	*Lactobacillus plantarum*	2880	27	+1	4	Dha	Gonzalez et al., 1994
		Ruminococcin A	*Ruminococcus gnavus*	2675	24	0	2	none	Dabard et al., 2001
		Butyrivibriocin OR79A	*Butyrivibrio fibrisolvens*	2910	25	0	3	Dhb	Kalmokoff et al., 1999
		Butyrivibriocin OR36	*Butyrivibrio fibrisolvens*		25	0	3	Dhb	Whitford et al., 2001
		Butyrivibriocin OB247	*Butyrivibrio fibrisolvens*		25	0	3	Dhb	Dufour et al., 2007
		Salivaricin A	*Streptococcus salivarius* 20P3	2315	22	0	3	None	Ross et al., 1993
		Salivaricin B	*Streptococcus salivarius* K12	2740	25	0	3	Putative lanA; structure predicted based on similarity to lacticin 481	Hyink et al., 2007

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Class	Sub Class	Name	Producer	MW (Da)	aa	Charge	Total rings	Other residues	Reference
Class II, LanM modified peptides	Lacticin 481like	Salivaricin A1	*Streptococcus agalactiae* 120		22	0		Unknown structure	Nakagawa et al., 2003
			Streptococcus pyogenes MGAS315		22	0		Unknown structure	
			S. pyogenes M1		22	0		Unknown structure	
			S. pyogenes MGAS10394		22	0		Unknown structure	Banks et al., 2004
		Salivaricin A2	*Streptococcus salivarius*		22	0		Unknown structure	Wescombe et al., 2006
		Salivaricin A3	*Streptococcus salivarius*		22	0		Unknown structure	
		Salivaricin A4	*Streptococcus salivarius*		22	0		Unknown structure	
		Salivaricin A5	*Streptococcus salivarius*		22	0		Unknown structure	
		Salivaricin 9	*Streptococcus salivarius strain* 9	2560	24	+2	3	Dhb	Wescombe et al., 2011
		Plantaricin C	*Lactobacillus plantarum*	2880	27	+1	4	Dha	Gonzalez et al., 1994
		Bovicin HJ50	*Streptococcus bovis* HJ50	3428	33	+2	2	Disulfide bridge	Xiao et al., 2004
		Thermophilin 1277	*Streptococcus thermophilus*				2	Disulfide bridge	Kabuki et al., 2007
		Perecin	*Clostridium perfringens* D str. JGS1721	3514	33		2	Disulfide bridge	Wang et al., 2014
		Cerecin	*Bacillus cereus* As 1.348		34		2	Disulfide bridge	
		Thuricin	*Bacillus thuringiensis* As 1.013	3728	34		2	Disulfide bridge	
	Mersacidin like lantibiotics	Mersacidin	*Bacillus amyloliquefaciens*	1825	20	0	4	Dha, MeAviCys	Chatterjee et al., 1992
		Actagardine	*Actinoplanes liguriae*	1890	19	0	4	LanO (lanthionine sulphoxide)	Zimmermann et al., 1995
		Ala(O)-Actagardine	*Actinoplanes liguriae*	1961	20	0	4	LanO (lanthionine sulphoxide)	Vertesy et al., 1999
		Deoxy-actagardine B (DAB)	*Actinoplanes liguriae* NCIMB 41362		19	0	4	LanO (lanthionine sulphoxide)	Boakes et al., 2010
		Michiganin A	*Clavibacter michiganesis*	2145	21	0	3	Dhb	Holtsmark et al., 2006
		Nai-802	*Actinoplanes sps.* 104802/104771		20	+1	4	Structure predicted based on similarity to actagardine	Simone et al., 2013
	Cinnamycin like lantibiotics	Cinnamycin; lantibiotic Ro 09-198	*Streptomyces Cinnamoneus/ griseoverticillatus*	2042	19	+1	4	Asp-OH,LysAla	Marki et al., 1991; Fredenhagen et al., 1990
		Duramycin	*Streptomyces cinnamoneus*	2014	19	0	3	Asp-OH,LysAla	
		Duramycin B	*Streptomyces verticillium sps.*	1951	19	0	3	Asp-OH,LysAla	
		Duramycin C	*Streptomyces griseoluteus*	2008	19	-1	3	Asp-OH,LysAla	
		Ancovenin	*Streptomyces sps.*	1959	19	0	3	Dha	Kido et al., 1983

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Class	Sub Class	Name	Producer	MW (Da)	aa	Charge	Total rings	Other residues	Reference
Class II, LanM modified peptides	Two- peptide lantibiotics	Lacticin 3147	*Lactococcus lactis subsp.lactis* DPC3147	3322	30	0	4	Dhb, D-Ala	Ryan et al., 1996
				2847	29	+1	3	2-Oxobuturyl; D-Ala	
		Staphylococcin C55	*Staphylococcus aureus* C55	3339	30	0	3	Dha, Dhb, D-Ala	Navaratna et al., 1998
				2993	37	-1	3	Dha, Dhb	
		Plantaricin W	*Lactobacillus plantarum*	3223	29	0	3	Disulfide bridge	Holo et al., 2001
				3099	32	+6	4	Dhb	
		Haloduracin	*Bacillus halodurans* C-125	3043	28	+1	3	Disulfide bridge	McClerren et al., 2006
				2330	24	+1	4	Dha, Dhb	
		Smb	*Streptococcus mutans* GS5		30	+2	3	Dha, Dhb	Yonezewa et al., 2005
					32	+2	3	Dha, Dhb	
		BHT	*Streptococcus ratti* BHT	3375	32	+1	3	Dha, Dhb	Hyink et al., 2005
				2802	30	+2	3	Dhb	
		Lichenicidin	*Bacillus licheniformis* DSM 13/VK 21/ATCC14580	3250	32	+1	4	Dha, Dhb, 2-oxobuturyl	Begley et al., 2009; Dischinger et al., 2009; Shenkarev et al., 2010; Caetano et al., 2011
				3021	32	+2	4	Dha, Dhb, 2-oxobuturyl	
		Cytolysin	*Enterococcus faecalis*	4164	21	+1	2	Dhb	Booth et al., 1996
				2631	38	+1	1	Unknown structure, 1 thioether bridge confirmed	
		Pneumococcin	*Streptococcus pneumonia* R6		38	+1		2 thioether bridge confirmed of a NisBC modified chimeric peptide	Majchrzykiewicz et al., 2010
					30	+4			
		Enterocin W	*Enterococcus faecalis* NKR-4-1	3256	30	0	3	Disulfide bridge	Sawa et al., 2012
				2728	29	+2	4	Dha, Dhb	
Class III lantibiotics, lack of antibiotic activity, but shows morphogenetic functions		SapB	*Streptomyces coelicolor*	2026	21	0	2	Dha	Kodani et al., 2004
		SapT	*Streptococcus tendae*	2032	21	0	4	none	Kodani et al., 2005
		AmfS	*Streptococcus griseus*		19	-1		Unknown structure	Ueda et al., 2002
		Prochlorosin	*Prochlorococcus* MIT9313		17				Li et al., 2010
		Geobacillin II	*Geobacillus thermodenitrificans*		35		4	Dhb	Garg et al., 2012

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Class	Sub Class	Name	Producer	MW (Da)	aa	Charge	Total rings	Other residues	Reference
Class III lantibiotics, lack of antibiotic activity, but shows morphogenetic functions	Labyrinthopeptins, LabKC modified, labionin containing peptide	Labyrinthopeptin A1	*Actinomadura namibiensis* DSM 6313		20	-1	2	Labionin, disulfide bridge, Dhb	Müller et al., 2010
		Labyrinthopeptin A2	*Actinomadura namibiensis* DSM 6313	1923	18	-1	2	Labionin, disulfide bridge,	Müller et al., 2010
		Labyrinthopeptin A3	*Actinomadura namibiensis* DSM 6313		21	-1		Labionin, disulfide bridge, Dhb	Müller et al., 2010
		Avermipeptin	*Streptococcus avermitilis*		22	0	4 (2 lan, 2 labionin)	Dha	Völler et al., 2012
		Erythreapeptin	*Saccharopolyspora erythreae* NRRL 2328		27	0	4 (2 lan, 2 labionin)	Dha	Völler et al., 2012
		Griseopeptin	*Streptomyces griseus* DSM GO236		22	-1	4 (2 lan, 2 labionin)	none	Völler et al., 2012
		Curvopeptin	*Thermomospora curvata*				2	Dha	Krawczyk et al., 2012
		Catenulipeptin	*Catenulispora acidiphila* DSM 44928		27		2	labionin	Wang and van der Donk, 2012
Lantibiotic without classification/ unknown structure and biosynthesis		Lactosin S	*Lactobacillus sake* L-45	3764	37	+1	2	Dha, Dhb, D-Ala	Mortvedt and Nes, 1990; Skaugen and Nes, 1994
		Pediocin PD-1	*Pediococcus damnosus*	2866	27			Only partially identified, no structure	Bauer et al., 2005
		Carnocin UI 49	*Carnibacterium pisciola*	4635	35			Only partially identified (N-terminus), no structure	Stoffels et al., 1992
					37				
		Paenibacillin	*Paenibacillus sps.*	2983	30	+4	5	Dha, Dhb, N-acetylalanine	He et al., 2007
		Unnamed	*Bifidobacterium longum* DJO10A					Predicted LanD- AviCys or AviMeCys, unknown structure	Lee et al., 2011
		Venezuelin	*Streptomyces venezuelae*				4		Goto et al., 2010
		Subtilomycin	*Bacillus subtilis* MMA7	3234	32			5 thioether rings proposed	Phelan et al., 2013
		Amylolysin	*Bacillus amyloliquefaciens* GA1	3318	31				Arguelles Arias et al., 2013
Lantibiotic related peptide (formerly falsely classified as lantibiotic)		Cypemycin	*Streptomyces sps.*	2094	22	+1		AviCys; Linearidin	Komiyama et al., 1993; Claesen and Bibb, 2010
		Sublancin 168	*Bacillus subtilis* 3876	3876	37	+3		Disulfide bridge; Glycosylation; S-linked glycopeptides	Paik et al., 1998, Oman et al., 2011

1.1.2.2.6. Role of the leader peptide

The role of the leader peptide is not yet fully elucidated. The leader peptides are important to increase the dehydration activity of the LanM enzymes and also for processivity and possibly directionality, as in lacticin 481 (Levengood et al., 2007). Furthermore, in some lantibiotics e.g. nisin, the leader peptides keep the lantibiotics inactive inside the cell, thereby protecting the producer cell. There are some evidences that, the leader peptides could interact with the propeptides and stabilize its conformation (Sahl et al., 1998). Likewise, for modification of the core peptide, the N-terminal residues are critical in class I (Plat et al., 2011) and class III lantibiotics (Müller et al., 2011), whereas the C-terminus seems more important for class II lantibiotics (Xie et al., 2004). Recently it was shown for the labyrinthopeptins, a class III lantibiotic, that the substrate modifications do not occur if the leader peptide is not directly attached to the core peptide (Müller et al., 2011).

1.1.2.2.7. Mode of action of lantibiotic

The activities of lantibiotics depend on different killing mechanisms (Asaduzzaman and Sonomoto, 2009). While the lantibiotics provide a good protection against Gram-positive bacteria, they are relatively ineffective against Gram-negative bacteria (Castiglione et al., 2008). Gram-negatives are generally not affected by lantibiotics due to the prevention of access to the cytoplasmic membrane by the protective outer membrane. But some lantibiotics e.g. nisin Z at higher concentrations can affect *E. coli* and other Gram-negatives like *Neisseria* or *Helicobacter pylori*. This inhibitory effect of nisin Z is probably due to self-promoted uptake or a lantibiotic based destabilization of the outer membrane of the bacteria by binding to their lipopolysaccharides (Nagao et al., 2009). In this context, lantibiotics from Actinobacteria such as microbisporicin are promising, because they exhibit an antimicrobial activity also against some Gram-negative bacteria such as *Moraxella catarrhalis*, *Neisseria* sp., and *Haemophilus influenza* (O'Sullivan and Lee, 2011). Likewise, lantibiotic produced by *Bifidobacterium longum* DJO10A has a potential to inhibit members of the Enterobacteriaceae. The antibacterial activities of lantibiotics are primarily based on two mechanisms: pore formation and inhibition of peptidoglycan synthesis (Brötz et al., 1998b).

a. Pore formation

Pore formation is an important mechanism for the mode of action of many lantibiotics. The pore formed by the lantibiotics may have the life time of few to several hundred milliseconds, and a diameter up to 2 nm. Many lantibiotics use lipid II as a docking molecule, which

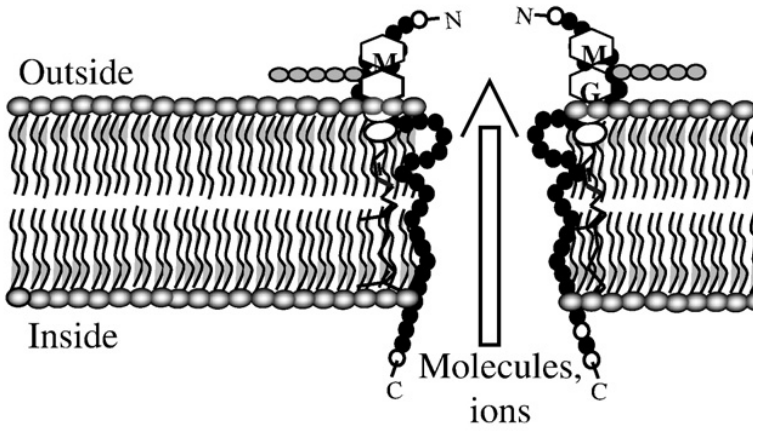
mediates a targeted pore formation (Brötz et al., 1998). The binding of lipid II appears to enable the peptides to integrate into the membrane (van Heusden et al., 2002). Furthermore, lipid II stabilizes the resulting pores (Breukink et al., 1999; Wiedemann et al., 2004).

The mechanism of pore formation has been extensively studied for the nisin (Figure 6A). The first evidence of pore formation due to nisin was described by Ramseier (1960). While treating Clostridial cells with nisin he observed a leakage of intracellular compounds. Later, Brötz et al. (1998b) and Breukink et al. (2003) determined that nisin binds to lipid II, using it as a docking molecule to form a stable and highly efficient pore. Nisin binds to the pyrophosphate moiety of lipid II using five hydrogen bonds. This interaction occurs specifically with its two N-terminal lanthionine rings (Hsu et al., 2003) and results in the insertion of the elongated C-terminus of nisin into the membrane (van Heusden et al., 2002; Hasper et al., 2004). This insertion of nisin causes an efflux of ions and small cytoplasmic compounds, leading to the dissipation of the membrane potential. Although, nisin causes both the pore formation and inhibition of the peptidoglycan biosynthesis, pore formation is likely the primary cause for its antimicrobial activity. This is based on the fact that the pore formation results in a rapid killing, before the inhibition of peptidoglycan biosynthesis can take effect (Brötz et al., 1998b; Wiedemann et al., 2001).

The two peptide lantibiotics, lacticin 3147 (Morgan et al., 2005) and haloduracin (Oman et al., 2011) also form pores by binding to lipid II. In lacticin 3147, the A1 peptide binds to lipid II to form a complex. Later, the A2 peptide binds to the complex of peptide A1 and lipid II to form a trivalent complex. This results in the deeper insertion of the complex into the membrane (Figure 6 B) (Deegan et al., 2006; Wiedemann et al., 2006; Oman and van der Donk 2009). This complex arrangement allows the A2 peptide to adopt a transbilayer orientation, thereby resulting in the pore formation. These pores were found to be significantly smaller (0.6 nm) than those formed by the nisin (Bonelli et al., 2006).

Other lantibiotics, such as streptococcin A-FF22 and Pep5 can form unstable pores. These pores then dissipate the membrane potential but do not release the large molecules from the cell (Kordel et al., 1988; Jack et al., 1994). Likewise, the ability of small peptides such as gallidermin and epidermin to form pores depends on the thickness of the membrane (Bonelli et al., 2006). Thus, the bactericidal effect of epidermin and gallidermin may not always be due to pore formation but could also be due to inhibition of peptidoglycan synthesis (Xiulan et al., 2012).

A)



B)

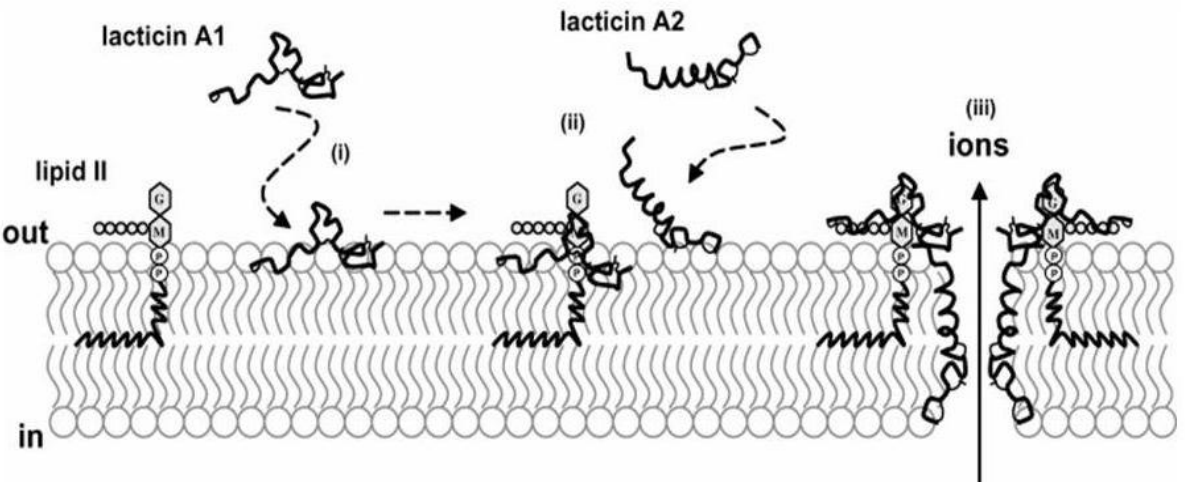


Figure 6: Pore formation by lantibiotics: (A) Nisin using lipid II as a docking molecule and (B) The two peptide lantibiotic lacticin 3147. Figures taken from Ryan et al. (2002), and Asaduzzaman and Sonomoto (2009).

b. Inhibition of peptidoglycan synthesis

The inhibition of peptidoglycan biosynthesis is another mode of action of lantibiotics. Lipid I and lipid II are the essential precursors for cell wall biosynthesis (Linett and Strominger, 1973). Lantibiotics bind to these precursors, in consequence, the transpeptidase and transglycosylase will not be able to utilize lipid II, which ultimately inhibits the peptidoglycan biosynthesis.

Nisin inhibits the synthesis of peptidoglycan by accumulation of lipid I (Reisinger et al., 1980). Brötz et al. (1998b) suggested that the nisin and epidermin may also inhibit the conversion of lipid I to lipid II. Likewise, in the nisin-like lantibiotics with the conserved two N-terminal lanthionine rings dissipate the lipid II from its functional location in the cell wall biosynthesis complex (Hasper et al., 2006). This dissipation of lipid II results in the accumulation of UDP-linked peptidoglycan precursors in the cytoplasm, ultimately inhibiting peptidoglycan synthesis (Castiglione et al., 2007, 2008).

Other lantibiotics such as mersacidin, actagardine, and cinnamycin also inhibit cell wall biosynthesis by binding to lipid II, but they do not form pores (Brötz et al., 1997; Hsu et al., 2003). Specifically, these compounds inhibit the activity of transglycosylases (Brötz et al., 1997). It is believed that the lantibiotics of the mersacidin group target the acetylglucosamine moiety and most probably the sugar and phosphate residues of the lipid II. All mersacidin-like lantibiotics possess a conserved TxS/TxEC motif (Hsu et al., 2006; Böttiger et al., 2009). Especially, the Glu residue in the motif is essential for the antibacterial action of mersacidin (Szekat et al., 2003). This motif is found within its essential C-ring, which is involved in lipid II binding although it differs from lipid II binding pocket of nisin. This essential C-ring is similar to the A-ring of the lacticin 481 subfamily peptides (Zimmermann and Jung, 1997). Moreover, the bioactivity of mersacidin is ion dependent, since, the presence of calcium ions increases its activity *in vivo*. Since lipid II is mostly negatively charged, calcium ions can transfer a net positive charge to the neutral peptides like mersacidin, which might result in enhanced membrane interaction and consequently in deeper membrane insertion (Böttiger et al., 2009). For positively charged peptides like plantaricin C, calcium might stabilize the conformation that promotes the membrane insertion. An ion independent mode of action was observed for an uncharged lacticin 481. It contains a positively charged amino acid on its N-terminus that might be sufficient for membrane interaction and formation of lipid II complexes (Böttiger et al., 2009).

1.1.2.2.8. Other biological functions

Many lantibiotics have other biological activites apart from pore formation and inhibition of cell wall biosynthesis. Class III lantibiotics like SapT and SapB exhibit a morphogenetic rather than an antibacterial effect. Because of their amphiphilic nature, both peptides serve as the biosurfactants, facilitating the emergence of aerial hyphae (Kodani et al., 2004, 2005).

Nisin and Pep5 also induce autolysis of certain staphylococcal strains, leading to a break down of the cell wall at the septa of dividing cells (Bierbaum and Sahl, 1985, 1987).

Likewise, nisin, subtilin and sublancin inhibit the outgrowth of spores from *Bacillus* and *Clostridium* species (Hurst, 1981; Paik et al., 1998).

The lantibiotics of cinnamycin subgroup (duramycin, duraycin B, duramycin C, cinnamycin and ancovenin) have a restricted antibactericidal activity. They target only a few bacterial strains and *Bacillus* sp. In particular, duramycin impairs ATP dependent protein translocation (Chen and Tai, 1987) and interferes with calcium uptake (Navarro et al., 1985). In addition, duramycin blocks the transport of chloride, sodium and potassium (Xie et al., 1983; Stone et al., 1984) and inhibits the proton pump of clathrin coated vesicles (Nakamura et al., 1984). However cinnamycin is also known as immunopotentiator peptide (Hosoda et al., 1996). Cinnamycin-like peptides inhibit phospholipase A2 by binding the phosphatidylethanolamine (PE) or lysophosphatidylethanolamine (lysoPE) in the cell membrane (Choung et al., 1988; Marki et al., 1991). Both cinnamycin and duramycin promote their binding to PE-containing membrane by deforming membrane curvature (Iwamoto et al., 2007).

Likewise, nukacin ISK-1 has a bacteriostatic mode of action rather than a bactericidal effect. It does not affect the membrane potential or form pores, but it reduces the width of the cell wall, causing incomplete formation of the septum, and thus preventing active growth (Assaduzzaman and Sonomoto, 2009).

1.1.2.2.9. Applications of lantibiotics

Lantibiotics have notable characteristics such as low MW, a broad range of antimicrobial activity, lack of toxicity and low immunogenicity (Asaduzzaman and Sonomoto, 2009; Dischinger et al., 2014). These characteristics make them suitable for applications in multiple areas such as food industries, health care, biomedical and medical applications. Nevertheless, lantibiotics have to overcome some obstacles before they may be used in the medical applications. Nisin for instance has drawbacks such as a low stablility at the physiological pH of the gastrointestinal tract, a low solubility, and has the tendency to interact with the blood components (Dischinger et al., 2014).

At present, nisin is the only lantibiotic used commercially. Since it has no known toxicity to humans, it has been used as a powerful and safe food preservative in processed dairy products, canned fruits and vegetables (Delves-Broughton, 1990). Since its first introduction as food preservative fifty years ago, many new applications have been investigated. Nisin exhibits an antimicrobial activity against food spoilage bacteria like *Listeria monocytogenes* (Cotter et al., 2005). It is applied in veterinary medicine for treatment

of bovine mastitis (Broadbent et al., 1989). In addition, as nisin is effective against clinically relevant human pathogens, like *Helicobacter pylori*, it is effective in peptic ulcer treatment too. Likewise, it is also used in treatment of oral decay, enterococcal infections and treatment of enterocolitis (Ryan et al., 2002; Nascimento et al., 2006). Other applications of nisin include the inhibition of experimental vascular graft infections caused by methicillin-resistant *Staphylococcus epidermidis* (Ghiselli et al., 2004). In addition to its antibiotic effect, nisin also inhibits sperm motility, showing its potential as a contraceptive agent (Aranha et al., 2004).

Beside nisin, other lantibiotics have also been investigated for their possible applications as antimicrobials. Mersacidin and actagardine show a remarkable activity against MRSA, bacterial mastitis, oral decay, acne, etc (Limbert et al., 1991). Likewise, gallidermin and epidermin are affective against acne, eczema, follicultis, and impetigo, thus, they might be used for personal care products. Pep5 and epidermin prevent the adhesion of coagulase-negative staphylococci, specifically *S. epidermidis*, to siliconisated catheters (Fontana et al., 2006). Likewise, cinnamycin might be used against inflammation and viral infections, and for blood pressure regulation (Ryan et al., 2002). Similarly, mutacin 1140 may prevent dental cavities. Duramycin and ancovenin can be used for inflammation and blood pressure regulation respectively. Lacticin 3147 prevents *Propionibacterium acnes* from causing acne, thus these substance may also be used as additives in cosmetics and personal care products (Kellner et al., 1988; Lawton et al., 2007). Salivaricins are effective against *Streptococcus pyogenes* strains. Therefore, the salivaricin A producer was supplemented as a probiotic to milk drinks. The strain was demonstrated to persist in the oral cavity, and resulted in the prevention of *S. pyogenes* infections (Dierksen et al., 2007). Moreover, chewing gums and lozenges containing salivaricin-producing strains have been developed (BLIS Technologies). Recently, several actinomycete lanthipeptides of clinical interest have been described. NVB302 (an actagardine derivative) and Moli1901 (also known as lancovutide or duramycin, a structural analogue of cinnamycin) successfully completed Phase I and Phase II clinical trails for the treatment of *Clostridium difficle* infections (Crowther et al., 2013) and cystic fibrosis (Grasemann et al., 2007; Oliynyk et al., 2010). Likewise, NAI-107 (also known as microbisporin) is in a late stage of preclinical development for the treatment of multi drug resistant Gram-positive pathogens (Jabes et al., 2011). Besides medical applications, lantibiotics could also be used in the food products. Lacticin 3147 or lacticin 481 producing *Lactococcus lactis*-starter cultures could be used to inhibit *Listeria monocytogenes* in cheese production (McAuliffe et al., 1999; Rodriguez et al., 2001).

Furthermore, the use of biosynthetic enzymes of lantibiotics *in vivo* and *in vitro* to design novel lantibiotics could be another promising application. The lantibiotic biosynthetic enzymes are able to modify the peptides also *in vitro*. This allows the introduction of didehydro amino acids and thioether rings even into chemically synthesized substrates (Chatterjee et al., 2005b). Several experiments have shown that the novel antibiotics could be designed, by introduction of novel modified residues into peptides or existing lantibiotics (Sahl and Bierbaum, 1998; Kluskens et al., 2005; Rink et al., 2005). The optimization of lantibiotics is the most important goal for possible biotechnological and medical applications. In this context, it has been shown that the stability and specific activity of subtilin was increased by exchanging Glu4 for Ile (Liu et al., 1992). Nisin peptides were engineered for an improved stability by exchanging Dha5 for Dhb in nisin Z (Rollema et al., 1995). Likewise, the solubility of nisin was improved by replacing Asn27 and His31 with Lys in nisin Z. Similarly, Leevengood et al. (2007) established an *in vitro* mutasynthesis method to introduce unnatural amino acid side chains to improve the class II lantibiotic lacticin 481.

Hence, lantibiotics can be promising candidates as alternatives for traditional antibiotics, preservatives, probiotics, and additives in food or cosmetic industry as well as in human and veterinary medicine.

1.2. *Bacillus pseudomycoides*

Bacillus pseudomycoides is a Gram-positive bacterium that shows a false fungus-like growth (Nakamura, 1998). It is mainly isolated from soil. Vegetative cells are non-motile, 1 µm wide and 3 - 5 µm long and occur singly or in short chains. The cells produce ellipsoidal spores that do not distend the mother cell. On an agar plate, colonies are white to cream, opaque, and usually rhizoidal. The species is facultatively anaerobic. The optimum temperature for its growth is 28 °C, with a maximum at 40 °C, and a minimum at 15 °C.

1.3. Aim of the study

The intensive use of antibiotics led to the emergence of resistant bacterial strains such as methicillin resistant *Staphylococcus aureus* and vancomycin resistant enterococci (VRE) (Novak et al., 1999; Weigel et al., 2003). The constant rise in multi drug resistant microorganisms has increased the need to investigate and develop new antibacterial compounds. There are a few antimicrobial compounds, such as antimicrobial peptides, probiotic bacteria, and bacteriophages which are considered as alternatives to antibiotics. Among them, lantibiotics have become promising candidates for the future antibiotics. Lantibiotics have a high potency to inhibit diverse pathogenic bacteria and are less likely to select for resistance (Fickers et al., 2012). About 100 lantibiotics have been discovered from bacterial sources today; however there are many bacterial strains that remain to be explored for the production of lantibiotics. Thus, the objective of this study was to investigate a novel lantibiotic.

The specific aim of this project was to investigate a novel putative lantibiotic gene cluster from *Bacillus pseudomycoides.* This was addressed using the following approaches:

1. Prediction of lantibiotic gene cluster from the genomic data mining.
2. Production of the antimicrobial substance by *B. pseudomycoides*. Purification and characterization of the antimicrobial substance by activity assay, stability assay and MALDI TOF MS.
3. Heterologous expression of the predicted structural gene and corresponding modification enzyme as His-tagged protiens in *E. coli*.
4. Purification and characterization of the heterologously produced peptide by MALDI TOF MS.
5. *In vitro* leader processing by factor Xa to produce the biologically active peptide.
6. Prediction of the ring topology of the heterologously produced peptide by creating LanA analogs by mutagenesis.
7. Initial approaches to elucidate the mode of action of the peptide by lipid II interaction assays.

2. Materials and Methods

2.1. Chemicals, solvents and enzymes

Table 2: Chemicals, solvents and enzymes used in this study.

Chemicals	**Manufacturer**
Acrylamid : Bisacrylamid (37,5:1), 40% (w/v)	Serva Electrophoresis, Heidelberg, Germany
Agar, BactoTM Becton	Dickinson & Company (BD), Heidelberg, Germany
Agarose, Top VisionTM,	LE GQ Fermentas, St. Leon-Rot, Germany
Ammoniumphosphate, #75568	Sigma-Aldrich, Taufkirchen, Germany
Ampuwa®-Water, "PCR-H_2O"	Fresenius Kabi, Bad Homburg, Germany
APS	Bio-Rad, München, Germany
DMSO	Amersham Biosciences, Freiburg, Germany
DNase I, from bovine pancreas	Sigma-Aldrich, Taufkirchen, Germany
DTT (DL-), #43819	Sigma-Aldrich, Taufkirchen, Germany
Ethidiumbromide	Sigma-Aldrich, Taufkirchen, Germany
Factor Xa	NEB, Frankfurt am Main, Germany
Formaldehyde	Sigma-Aldrich, Taufkirchen, Germany
Urea	Sigma-Aldrich, Taufkirchen, Germany
IAA	Sigma-Aldrich, Taufkirchen, Germany
TCEP	Sigma-Aldrich, Taufkirchen, Germany
IPTG	Fermentas, St. Leon-Rot, Germany
Lysozyme, #62971	Sigma-Aldrich, Taufkirchen, Germany
PMSF	Merck, Dramstadt, Germany
EDTA	Merck, Dramstadt, Germany
Triton X-100	Sigma Aldrich, Taufkirchen, Germany
Chymotrysin	Sigma Aldrich, Taufkirchen, Germany
Trypsin	Serva Electrophoresis, Heidelberg, Germany
Pronase E	Merck, Dramstadt, Germany
Proteinase K	Sigma Aldrich, Taufkirchen, Germany
Ni-NTA Agarose	Qiagen, Hilden, Germany
Nucleotide (dNTPs)	Fermentas, St. Leon-Rot, Germany
Primer, DNA-Oligonucleotide	Metabion, Martinsried, Germany and Microsynth, Lindau, Switzerland
PhusionTM High Fidelity DNA Polymerase	Finnzymes, sales: NEB, Frankfurt am Main, Germany
Restrictions enzyme buffers	Roche, Mannheim / Fermentas, St. Leon-Rot, Germany
RNase A, from bovine pancreas	Sigma-Aldrich, Taufkirchen, Germany
TEMED	Bio-Rad, München, Germany
β Mercaptoethanol	Sigma-Aldrich, Taufkirchen, Germany
TLC aluminium sheet	E. Merck, Dramstadt, Germany
Phosphomolybdic acid	Fulka chemika, Sigma-Aldrich, Germany
Quick change site directed mutagenesis kit	Agilent, USA

2.2. Antibiotics and antimicrobial peptides

Table 3: Antibiotics and antimicrobial peptides used in this study.

Antibiotic/peptide	Solvents	Source
Ampicillin	MilliQ water	Sigma-Aldrich, Taufkirchen, Germany
Kanamycin	MilliQ water	Sigma-Aldrich, Taufkirchen, Germany
Nisin	0.05% Acetic acid	Michaele Josten, University of Bonn, Germany
Mersacidin	MilliQ water	Aventis, Frankfurt, Germany

2.3. Instruments / Equipments

Table 4: Instruments and equipments used in this study.

Equipments	Manufacturer
Agarose gel electrophoresis chamber (40 ml)	Peqlab Biotechnology, Erlangen, Germany
Agarose gel electrophoresis chamber, Model B3 (60 ml)	OWL, Sales: Peqlab Biotechnology, Erlangen, Germany
Gene PulserTM (Electroporation)	Bio-Rad, München, Germany
Mini-PROTEAN® II Electrophoresis Cell	Bio-Rad, München, Germany
Multifuge 1 S-R (Rotor #75003348 & FA12.94)	Heraeus / Thermo Fisher Scientific, Waltham, MA, USA
NanoDrop® ND-1000 Spectrophotometer (220-750 nm)	NanoDrop Technologies Inc., DE, Wilmington, USA
Photometer, UV-160	Shimadzu Deutschland GmbH, Duisburg, Germany
Power Pac 200 (Gel electrophoresis Power supply)	Bio-Rad, München, Germany
Pulse Controller (Electroporation)	Bio-Rad, München, Germany
Ultrapure water installation, MilliQ Biocel System A10	Millipore GmbH, Schwalbach/Ts., Germany
Shaking water bath SW20 und SW22	Julabo GmbH, Seelbach, Germany
Shaker, Certomat U B.	Braun, Melsungen, Germany
Shaking, Titertek	Flow Laboratories, Meckenheim, Germany
SensoQuest Thermocycler (labcycler)	SensoQuest Biomed. Electronic GmbH, Göttingen, Germany
Sonicator Sonoplus HD2200	Bandelin, Berlin, Germany
Sorvall Evolution (Rotor SLC-4000)	Thermo Fisher Scientific, Waltham, MA, USA
Sorvall Discovery M120SE (Rotor S80 AT-3 & S120 AT-2)	Thermo Fisher Scientific, Waltham, MA, USA
UV-Imager, ImageMaster VDS	Amersham Biosciences, Freiburg, Germany
UV-Board, Chroma 43 (302 nm)	Vetter GmbH, Wiesloch, Germany
Vortexer, MS 2 Minishaker IKA -	Werke GmbH & Co. KG, Staufen, Germany

Contd...

Equipments	Manufacturer
XCell SureLock® Mini-Cell (Electrophoresis chamber)	Invitrogen, Karlsruhe, Germany
Centrifuge RVC 2-18, Typ 100218	Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany
HPLC	Gilson BV, Germany
MALDI TOF Bruker Biflex,	Bruker Daltonics, Bremen, Germany
Rotory evaporator	Rotavapor Re11, Essen, Germany
LCMS	Bruker Daltonics, Bremen, Germany
Tandem MSMS	Bruker Daltonics, Bremen, Germany

2.4. Microbiological methods

2.4.1. Bacterial strains, culture media, growth conditions

The Trypticase Soy Broth (TSB) was from Difco (Detroit, MI, USA), and Luria Bertani (LB), Müller Hinton Broth (MHB), Brain Heart Infusion (BHI) and Nutrient Broth (NB) were from Oxoid Basingstoke, UK. Trypsin, chymotrypsin, proteinase K and pronase E were from Sigma (St. Louis, MO, USA). All other media and reagents were from Merck (Darmstadt, Germany). All the media used in this study are listed in Table 5.

The producer strain *B. pseudomycoides* DSM 12442 was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). *B. pseudomycoides* strain was grown aerobically in NB or on nutrient agar. All the bacterial strains used in this study are listed in Table 7. For stock preparation, the cells were cultivated overnight, mixed with sterile glycerol (final concentration of 50% v/v) and stored at -70 °C. Strains were recovered from frozen samples by streaking out on appropriate agar plates. Liquid cultures were incubated in a shaking water bath (200 rpm for *Bacillus* strains, 100-160 rpm for all other strains) or on an orbital shaker (170 rpm). For preparation of solid culture media, 14 g agar (Oxoid, Wesel, Germany) were added to 1 L of liquid medium prior to autoclaving. Agar plates were cultivated in incubators. *Bacillus* strains were grown in TSB, or on tryptic soy agar (TSA, Oxoid). For aerobic cultivation, Erlenmeyer flasks filled with liquid medium (1/100 of the total volume) were used and were closed by a silicon vent containing an ultra-fine glass microfiber filter (BugStopper™, Whatman/GE Healthcare, USA) or cotton plugs. Streptococci, enterococci and staphylococci were grown on Columbia blood agar plates. For antimicrobial activity testing, indicator strains were grown in MH broth and on MH agar plates. *Escherichia coli* strains were cultivated on LB agar.

To examine the antimicrobial activity, the following indicator strains were used: Gram-positive bacteria - *Bacillus cereus* DSM 31, *B. halodurans* DSM 18197 (producer of the two peptide lantibiotic haloduracin; DSMZ), *B. licheniformis* MW3 (Waschkau et al., 2008), *B. megaterium* KM3 (ATCC 13632), *B. subtilis* 186 (DSM 402), *B. amyloliquefaciens* FH1856, *Enterococcus faecium* L4001 (clinical isolates), *E. faecalis* 017940 (clinical isolate), *Lactobacillus sake* 790 E2 (laboratory stock), *Lactococcus lactis* NCTC 497, *Micrococcus flavus* DSM 1790, *M. luteus* ATCC 4698 (used as standard indicator strain), *Staphylococcus aureus* 825/96 (MRSA), *S. aureus* 635/93 (MRSA), *S. aureus* Cowan (ATCC 12598), *S. aureus* 1000/93 (MRSA), *S. aureus* 2747/97, *S. aureus* SG511 (Sass et al., 2009), *S. aureus* Wood 46 (ATCC10832), *S. carnosus* TM300 (Schleifer et al., 2002), *S. aureus* COL, *S. aureus* Mu50, *S. aureus* Sanger, *S. saprophyticus* DSM 20229, *S. simulans* 22 (Bierbaum et al., 1987), *S. epidermidis* 5, *Streptococcus* G 3645"10", *S. pyogenes* O-19310 (clinical isolate). Gram-negative bacteria – *Citrobacter freundii*, *E. coli* JM109 (Yanisch-Perron et al., 1985), *E. coli* 7118, *E. coli* BHH71-18, *Klebsiella pneumonia*, *Proteus mirabilis* and *Pseudomonas aeroginosa*. Yeast - *Candida albicans*.

Table 5: Media used in this study.

Medium	**Ingredients / Source**
Nutient broth (NB)	Peptone 5.0 g/l , meat extract 3.0 g/l
Luria-Bertani broth (LB)	10.0 g/l BactoTM Tryptone (BD); 5.0 g/l Yeast extract powder LP0021 (Oxoid); 10.0 g/l NaCl; pH adjusted to 7.5
Müller Hinton broth (MH)	Beef, dehydrated infusion from 300 g/l, Casein hydrolysate 17.5 g/l, Starch 1.5 g/l; pH 7.3 ± 0.1
Trypticase soy broth (Soybean-Casein Digest Medium) (Oxoid; CM0129) (TSB)	Pancreatic digest of casein 17.0 g/l, Enzymatic digest of soybean meal 3.0 g/l, NaCl 5.0 g/l, Dipotassium phosphate 2.5 g/l, Glucose 2.5 g/l; pH 7.3 ± 0.2
Brain heart infusion (BHI)	Calf brain (Infusion solids) 12.5 g/l, Beef heart (Infusion solids) 5.0 g, Proteose peptone 10.0 g/l, Glucose 2.0 g/l, NaCl 5.0 g/l, Disodium phosphate 2.5 g/l; pH 7,4 ± 0.2
Müller Hinton II agar	Becton Dickinson GmbH, Germany
Columbia agar with 5% sheep blood	Becton Dickinson GmbH, Germany

Table 6: Preparation of 2× BPM (*Bacillus* production medium).

Medium	Ingredients
Wolfe's mineral salts	Titriplex I (500 mg/l); $ZnSO_4 \cdot 7\ H_2O$ (180 mg/l); $CuSO_4 \cdot 5\ H_2O$ (50 mg/l); $CoCl_2 \cdot 6\ H_2O$ (170 mg/l); $CaCl_2 \cdot 2\ H_2O$ (130 mg/l); $NaMoO_4 \cdot 1\ H_20$ (11 mg/l); $Al_2\ (SO_4)_3$ (26 mg/l); H_3BO_3 (10 mg/l); 1 M NaOH (5 ml/l)
Mineral salt	10 mM $CaCl_2$; 2 mM $FeSO_4$; 1 mM $MnSO_4$
2× BPM	0.25 M $(NH_4)_2SO_4$ (20 ml); 0.1 M $MgSO_4$ (2 ml); 2 M K-P-buffer pH 7.0 (2 ml); 0.5 M Tris-maleate pH 7.0 (20 ml); Wolfe's mineral salts (2 ml); D/W (27.5 ml); after autoclaving 2.5 M glucose (16 ml); mineral salts (10 ml) were added to a total volume of 100 ml.

Source: Bierbaum et al. (1995)

Table 7: Bacterial strains used in this study.

Strain	Characteristics	Source/References
B. pseudomycoides	Antimicrobial substance producer	DSMZ, Germany
E.coli SCS110	Intermediate cloning host for recombinant plasmids	Yanisch-Perron et al., 1985
E. coli JM109/ *E. coli* JM83	Intermediate cloning host for recombinant plasmids	Yanisch-Perron et al., 1985
E. coli BL21	Strain for recombinant protein expression, λDE3-lysogen, T7 RNA polymerase under control of the l*acUV* promotor	Studier and Moffatt, 1986
E. coli C41/ *E. coli* C43	BL21(DE3) derivatives for expression of toxic proteins	Miroux and Walker, 1996
E. coli XL10 Gold	Cloning host for mutagenic recombinant plasmid construction	Agilent, Germany
B. subtilis TMB016	Reporter strain containing peptide-sensing and detoxification module (LiaRS)	Burkhard and Stein, 2005
B. subtilis TMB299	Reporter strain containing peptide-sensing and detoxification module (YxdJK-LM)	Starón *et al*., 2011
B. subtilis TMB588	Reporter strain containing peptide-sensing and detoxification module (PsdRS-AB)	Starón *et al*., 2011

2.4.2. Sterilization of media, equipment and bacterial cultures

Media, plastic wares and chemicals were autoclaved at 121 °C for 20 min (Varioklav 75S, H+P Labortechnik AG, Oberschleißheim, Germany). Heat labile solutions were sterilized by filtering through sterile syringe filters of 0.2 or 0.4 µm pore size (Supor®Membrane; Pall Life Science Corporation, Ann Arbor, USA). Glasswares and metallicwares were heat sterilized for 4 h at 200 °C (Kelvitron®, Heraeus). Bacterial cultures, disposables substances and contaminated labwares were autoclaved at 134 °C for 30 min.

2.4.3. Determination of optical density of bacterial culture

The optical density of bacterial culture was determined by an UV-160 spectral photometer at a wavelength of 600 nm (O.D. $_{600}$). Cultures with higher optical density were diluted in an appropriate medium before measurement.

2.4.4. Detection of protease activity

Protease activity in cell-free culture supernatants was detected on skim milk agar plates (3 g/l yeast extract, 5 g/l peptone, 2.5 g/l skim milk powder and 15 g/l agar, at pH 7.2). The well was introduced into the agar surface with a sterile cork borer and 50 µl of fresh filter-sterilized supernatant was loaded into it. Protease activity was determined by the appearance of clear zones around the well after incubation at 37 °C overnight.

2.4.5. Detection of haemolytic activity

The haemolytic activity was tested on Columbia blood agar (CA) plate. *B. pseudomycoides* was streaked on the CA plate. Likewise, 10 µl of each, isopropanol cell wash extract and pseudomycoicidin were spotted on the CA plate. Then the plates were incubated at 37 °C overnight. After overnight incubation, the plates were observed for possible zones of haemolysis.

2.4.6. Reporter gene assay

For the assay, the reporter strains *B. subtilis* TMB016, *B. subtilis* TMB299 and *B. subtilis* TMB588 were selected. Three TSA plates supplemented with X-gal were seeded with each of the 3 reporter strains. The isopropanol cell wash extract (50 µl) was loaded into wells and the plates were incubated at 37 °C for 24 h. Similarly, 5 - 10 µl of pseudomycoicidin was spotted on an agar plate. After 24 h of incubation, the plates were inspected for a blue coloration around the zone of inhibition.

2.4.7. Growth of the test microorganism in the presence of antimicrobial agent

M. luteus and *S. aureus* SG 511 were grown in MH broth at 37 °C. After 5 h, 500 µl of the partially purified antimicrobial compound was added to 20 ml of culture and the culture was further incubated. As a control, 20 ml of the culture was incubated without addition of the antimicrobial compound. Aliquots were taken at 1 h intervals and O.D. was measured.

2.5. Methods in molecular genetics

2.5.1. Purification of nucleic acids

Genomic DNA was prepared using PrestospinD Bug mini column kit (Molzym, Bremen, Germany). This kit works on the principle of binding the nucleic acid to clay minerals. Clay minerals reversibly bind DNA, so that the cell constituents and salts can be removed by washing with specially developed buffers and 70% ethanol. Finally, bound DNA is eluted with a small volume of TE buffer. Genomic DNA from *Bacillus* sp. was prepared according to the protocol of the supplier with some modifications - 1. for lysis, cells were incubated at 37 °C for 30 min in buffer 1 that had been supplemented with 10 µl lysozyme (100 mg/ml) and 2. DNA was eluted in 100 µl pre-heated (70 °C) MilliQ water and was stored at -20 °C.

Plasmid DNA was isolated using the Gene-Jet™ Plasmid Miniprep Kit (Fermentas) according to the protocol of the supplier. The kit utilizes a silica based membrane technology for the isolation. Cell lysis of *Bacillus* was achieved by adding 10 µl of lysozyme (100 mg/ml) in the resuspension buffer and incubating it at 37 °C for 30 min. The plasmid DNA was eluted in 50 µl MilliQ water. For elution of plasmids larger than 5 kb, MilliQ water was pre-heated up to 80 °C. All plasmids used in this study are listed in Table 8. Quality and quantity of the nucleic acids were analysed by agarose gel electrophoresis and spectrophotometry.

Table 8: Plasmids used in this study.

Plasmid	Properties	Source
pUC 19	shuttle vector; AMPr, CMr	Yanisch-Perron et al., 1985
pET22bΔpelB	pET22 derivative, expression vector with C-terminal 6XHis-tag, pelB leader sequence deleted, T7 *lac*-Promotor, AmpR	Sass and Bierbaum, 2007
pET22bΔpelBlanM	pET22 derivative harbouring C-terminally His-tagged *lanM*	This study
pET28b	His-tag expression vector, kanaR	Novagen
pET28blanA	pET22 derivative harbouring N-terminally His-tagged *lanA*	This study
pET28blanAXa	pET22 derivative harboring the N-terminally his-tagged *lanA* including a factor Xa cleavage site	This study
pET28blanAXaC52A	pET22 derivative harboring the N-terminally His-tagged *lanA* including a factor Xa cleavage site with C52A	This study
pET28blanAXaC56A	pET22 derivative harboring the N-terminally His-tagged *lanA* including a factor Xa cleavage site with C56A	This study

Contd...

Plasmid	Properties	Source
pET28blanAXaC62A	pET22 derivative harboring the N-terminally His-tagged *lanA* including a factor Xa cleavage site with C62A	This study
pET28blanAXaC65A	pET22 derivative harboring the N-terminally His-tagged *lanA* including a factor Xa cleavage site with C65A	This study
pET28blanAXaC70A	pET22 derivative harboring the N-terminally His-tagged *lanA* including a factor Xa cleavage site with C70A	This study
pET28blanAXaC75A	pET22 derivative harboring the N-terminally His-tagged *lanA* including a factor Xa cleavage site with C75A	This study

2.5.2. Determination of the concentration and purity of nucleic acids

The concentration and purity of nucleic acids were determined by the nanodrop spectrometer at 260 nm. The purity was determined by the ratio of the spectrometric absorbance of the sample at 260 nm and 280 nm.

2.5.3. DNA sequencing

DNA sequencing was performed by Sequiserve (Vaterstetten, Germany), Seqlab (Göttingen, Germany) and Microsynth (Balgach, Switzerland) by the Sanger chain determination method (Sanger et al., 1977).

2.5.4. Agarose gel electrophoresis

DNA fragments were analysed by standard agarose gel electrophoresis in a horizontal electrophoresis apparatus. DNA was loaded on agarose gel (0.8 - 1% in 1× TAE buffer) and run in 1× TAE buffer applying a voltage of 80 - 120 volt. DNA was stained with ethidium bromide and visualized under the UV-imager.

2.5.5. Polymerase chain reaction (PCR)

PCR is a method of amplifying the DNA of a certain length and sequence. The PCR amplification was performed in 50 µl reaction mixtures in a PCR express Thermal cycler (Hybaid, ThermoLifeScience, Engelsbach, Germany) or the SensoQuest Labcycler (SensoQuest, Göttingen, Germany). DNA was amplified using the Phusion™ HF polymerase (NEB, Frankfurt/ Main, Germany) and deoxynucleotide triphosphates (premixed solutions, 25 mM each, Fermentas). All the primers were synthesized by Microsynth (Balgach,

Switzerland) or Metabion (Martinsried, Germany). Primers were designed using the Primer 3 tool: http://biotools.umassmed.edu/bioapps/primer3_www.cgi, and are listed in Table 9.

2.5.6. Enzymatic digestion of DNA

For ligation or examination of clones, PCR products and plasmid DNA were digested by the Fast digest® restriction enzymes or conventional restriction enzymes (Fermentas or NEB). Digestions were performed as explained in the manual.

2.5.7. Purification of DNA fragments and gel extraction

To purify the PCR product, the Gene Jet PCR purification kit was used. This kit utilizes a silica based membrane technology for the removal of the impurities from the PCR product. The purification was executed as described in the manual. The PCR product was eluted from the column by MilliQ or Elution buffer.

2.5.8. DNA ligation

The ligation was carried out to ligate a DNA fragment into a plasmid vector. The reaction was performed either by T4 ligase (5 U/µl) (Fermentas) in 20 µl total volume for 1 h or by T4-ligase (6 U/µl) (Roche) in 10 µl total volume overnight.

2.5.9. Site directed mutagenesis

In order to introduce a point mutation or to replace an amino acid, the Quick Change Lightning Site-Directed Mutagenesis Kit (Agilent) was used. This kit utilizes the double stranded DNA vector with the insert of interest and two oligonucleotide primers containing the desired mutation. The recombinant vectors were used as templates and the PCR was performed using both mutagenic primers employing *Pfu*Ultra HF DNA polymerase (Agilent) without primer displacement. Mutagenesis was performed following the supplier's instructions. Primers containing the desired exchanges were designed by the following tool: http://www.genomics.agilent.com/ CollectionSubpage.aspx? PageType=Tool&SubPageType= ToolQCPD&PageID=2296. Primers used for mutagenesis are listed in Table 9. Parental plasmids, that lacked the desired mutations, were digested by *DpnI*, specific for (hemi) methylated DNA. Subsequently, batches were transformed into super-competent *E. coli* XL10-Gold cells (Agilent). The medium used in the transformation is listed in Annex XVI.

Table 9: Primers used in this study.

Primers	Template	Restriction enzyme	Primer sequence (5'-3')
PseAfor	lanA	*BamHI*	AAAGGATCCAAGTGTCAAATACATATATAACGGATAAA
PseArev		*HindIII*	AAAAAGCTTTCTGATCATGATTCGAACAGC
PseAforI	lanA	*NdeI*	AAACATATGAATGATAAAATTATCCAATACTGGAA
PseArevI		*XhoI*	AAACTCGAGTTAGCAAGACCAGCTCCAACAA
PseMfor	lanM	*NcoI*	AAACCATGGGGATGCTTGCAA ATCAAGCCTT AAAA
PseMrev		*XhoI*	AAACTCGAGTTTTAGTGCTGTTATAGACTCCAA
PseMfor3	Internal lanM primers for sequencing lanM	-	GGA CGA GGC CTC AAC ATA AG
PseMrev3			GAG CCA GCT GAA CCG TCT AT
PseAXafor	pET28blanA	-	GCTTTCAGACGCTGATCTGGATAAAATAGAGGGTCGTGGTGATTGCGGT
PseAXarev			ACCGCAATCACCACGACCCTCTATTTTATCCAGATCAGCGTCTGAAAGC
LanAXaC52Afor	pET28blanAXa	-	TAAAATAGAGGGTCGTGGTGATGCCGGTGGTACTTGTACA
LanAXaC52Arev			TGTACAAGTACCACCGGCATCACCACGACCCTCTATTTTA
LanAXaC56Afor	pET28blanAXa	-	TGGTGATTGCGGTGGTACTGCTACATGGACAAAAGATTGC
LanAXaC56Arevr			GCAATCTTTTGTCCATGTAGCAGTACCACCGCAATCACCA
LanAXaC62Afor	pET28blanAXa	-	TGGTACTTGTACATGGACAAAAGATGCCTCAATTTGTCCATCATGG
LanAXaC62Arev			CCATGATGGACAAATTGAGGCATCTTTTGTCCATGTACAAGTACCA
LanAXaC65Afor	pET28blanAXa	-	CTTGTACATGGACAAAAGATTGCTCAATTGCTCCATCATGGTCTTGT
LanAXaC65Arev			ACAAGACCATGATGGAGCAATTGAGCAATCTTTTGTCCATGTACAAG
LanAXaC70Afor	pET28blanAXa	-	GTTAGCAAGACCAGCTCCAAGCAGACCATGATGGACAAATTG
LanAXaC70Arev			CTTGTTGGAGCTGGTCTGCCTAACTCGAGCACCACC
LanAXaC75Afor	pET28blanAXa	-	CTTGTTGGAGCTGGTCTGCCTAACTCGAGCACCACC
LanAXaC75Arev			GGTGGTGCTCGAGTTAGGCAGACCAGCTCCAACAAG
PseAgap	G. DNA from *B. pseudomycoides*		ATCGTTCACCGTCGTGATGC
Gapfor	G. DNA from *B. pseudomycoides*	-	TGCACAGCATTATGTAGAAGAGTT
Gaprev	G. DNA from *B. pseudomycoides*	-	GCTGGGGCATTCCAGTATT
Gapfor2	Internal primer for sequencing	-	CCT TTT CGA AGA AGA AAG CTA CC
Gaprev2	Internal primer for sequencing	-	TCA TGC GTG AAA TTA CCT CTT T
Gapfor3	Internal primer for sequencing	-	GGT CGT TTT CTC CTA ATA ATC GC
Gapfor4	Internal primer for sequencing	-	AAA ATC CCC TCC ATA GTA AAC AGA
GapIIfor	G. DNA from *B. pseudomycoides*	-	ACA GCA ACT GGC GAT GGT AG
GapIrev	G. DNA from *B. pseudomycoides*	-	ATT TCC TGC AGG GAT TAC TGG
GapIIfor1	Internal primer for sequencing	-	GGG CGA AAG ATT TGG AAG AGC
GapIIfor2	Internal primer for sequencing	-	TGG TAT TGT AAA TTA CTC GTC ATC AA
GapIIfor3	Internal primer for sequencing	-	GGT ATT GTA AAT TAC TCG TCA
GapIIfor4	Internal primer for sequencing	-	TGG TGC ACG GAA ACC TCT TT
FPgap1	G. DNA from *B. pseudomycoides*	-	GAC CAG CTC CAA CAA GAC CA
RPgap1	G. DNA from *B. pseudomycoides*	-	CAG CAA CTG GCG ATG GTA GT
rPCRFP1	G. DNA from *B. pseudomycoides*	*PsiI*	CAT CTG ACC TAC CAA CAG CT
rPCRRP1	G. DNA from *B. pseudomycoides*	*PsiI/ BstBI*	AGT ATT GGA TGA TTT TAT CAT GC
rPCRFP2	G. DNA from *B. pseudomycoides*	*BstBI*	CTT TCA GCA GCA GAA CTT AGC

2.5.10. Transformation of *E. coli*

2.5.10.1. Preparation of electrocompetent *E. coli* cells

For generation of electro-competent *E. coli* strains, the method described by Dower et al. (1988) was used. The desired strain of *E. coli* was freshly streaked on an LB plate. A single colony was picked to inoculate 5 ml of LB broth and incubated at 37 °C overnight. 500 ml LB, inoculated with 500 µl of the overnight culture, was grown to 0.5 $O.D._{600}$ units. After incubation on ice for 30 min, the cells were harvested by centrifugation (5,000 × g, 4 °C, 10 min) followed by three washing steps with 500 ml and 250 ml pre-chilled MilliQ water and with 20 ml pre-chilled 10% glycerol solution. Finally, the cells were resuspended in 0.8 ml glycerol (10%) and frozen for long time storage at -75 °C in 50 µl aliquots.

2.5.10.2. Preparation of chemocompetent *E. coli* cells

For the preparation of chemocompetent cells, the method described by Morrison (1977) was used. The desired strain of *E. coli* was freshly streaked on an LB plate. A single colony was picked to inoculate 5 ml of LB broth and incubated at 37 °C overnight. The *E. coli* culture (100 µl) was inoculated into 50 ml of TYM broth and grown at 37 °C with shaking to 0.6 $O.D._{600}$ units. Subsequently, the flask was kept on ice and shaken to cool the culture. The cells were harvested (5,000 × g, 4 °C, 5 min, Acc. 9, Br. 7) and the resulting cell pellets were resuspended in 5 ml of pre-chilled and filter-sterilized TFBI. After centrifugation, the cells were resuspended in 2.5 ml pre-chilled TFBII medium and aliquoted into 100 µl samples. Cells were shock-frozen in liquid nitrogen and stored at -75 °C until further use. Media used during the preparation of competent cells are listed in Table 10.

Table 10: Media for preparation of chemocompetent cells.

Medium	Volume	Ingredients
TYM broth	100 ml	2 g Bacto-Trypton (Difco); 0.5 g yeast extract (Difco); 584.4 mg NaCl; 246.48 mg $MgSO_4$
TFBI	50 ml	5 M potassium acetate (0.3 ml); $MnCl_2$ (495 mg); 2 M KCl (2.5 ml); 1 M $CaCl_2$ (0.5 ml) and glycerol (7.5 ml)
TFBII	50 ml	2 M MOPS pH 7 adjusted with 5 M KOH (2.5 ml); 2 M KCl (0.25 ml); 1 M $CaCl_2$ (3.7 ml) and glycerol (7.5 ml)

2.5.10.3. Transformation of electrocompetent *E. coli*

The electroporation apparatus was set to the appropriate program settings. Frozen cells were thawed on ice and immediately mixed with 3 µl of ligation batches or 100 ng of plasmid

DNA. The mixture was electroporated employing a pre-chilled electro cuvette (1 mm) in a gene pulser (program EC2, Bio-Rad, Munich, Germany). The cells were resuspended in 1 ml LB and incubated at 37 °C under shaking for up to 1 h for regeneration. Finally, 100 µl of each, undiluted, 10 fold diluted and 100 fold diluted cell suspension, were plated on three selective agar plates respectively. The rest of cell suspension was centrifuged (15000 rpm, 1 min, RT, Br. 9), and the recovered pellet was resuspended in about 100 µl LB and plated on selective agar plate.

2.5.10.4. Transformation of chemocompetent *E. coli*

Frozen chemo-competent *E. coli* cells were thawed on ice before use. 10 - 20 µl of a ligation batch or 100 - 200 ng of plasmid DNA was mixed with 50 µl competent cells and incubated on ice for 30 min. For transformation, the cells were exposed to a heat shock treatment at 42 °C for 30 s followed by incubation on ice for 3 min. Transformed cells were resuspended in 1 ml pre-warmed LB and incubated at 37 °C for 45 - 60 min with shaking. Finally, 100 µl of each, undiluted, 10 fold diluted and 100 fold diluted cell suspension, were plated on selective agar plates respectively. The rest of cell suspension was centrifuged (15000 rpm, 1 min, RT, Br. 9), and the recovered pellet was resuspended in about 100 µl LB and plated on selective agar plate.

2.5.11. Analysis of biosynthetic gene cluster

Information about the lantibiotic gene cluster of *B. pseudomycoides* was accessed *via* National centre for Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi) with the accession number CM000745 ACMX01000000.

2.5.12. Bioinformatic tools

Table 11: Bioinformatic tools used in this study.

Tools	Sources
Blast	NCBI: http://blast.ncbi.nlm.nih.gov/Blast.cgi.
GC content	Genomatix: http://www.genomatix.de/cgi-bin/tools/tools.pl
Protease cleavage site on the peptide	ExPASY peptide cutter: http://www.expasy.ch/tools/peptidecutter/
Multi sequence alignments	ClustalW: http://www.ebi.ac.uk/Tools/msa/clustalw2/MultiAlin: http://multalin.toulouse.inra.fr/multalin/
Restriction analyses	NEBcutter: http://tools.neb.com/NEBcutter2/index.php

Contd...

Tools	Sources
Transmembrane sequences	TMHMM web server v. 2.0.: http://www.cbs.dtu.dk/services/TMHMM/.
Subcellular localizations of the proteins	CELLO v.2.5: http://cello.life.nctu.edu.tw/.
Molecular weight of the peptide	ExPASY ProtParam: http://web.expasy.org/protparam/.ExPASY Peptide mass: http://web.expasy.org/peptide_mass/
Vector cards	pDRAW32 version 1.1.114

2.5.13. Cloning of the *pseA* and *pseM* genes.

Genomic DNA was isolated from an overnight culture of *B. pseudomycoides* using a Mini Spin Column Kit according to the supplier's manual (Molzym, Bremen, Germany). The *pseA* (*lanA*) was PCR amplified by 30 cycles of denaturing (98 °C for 3 min), annealing (64.4 °C for 30 s), and extending stages (72 °C for 1 min). The genomic DNA isolated from *B. pseudomycoides* was used as a template and PseAforI and PseArevI were used as primers (Table 9). Amplifications were confirmed by 1% agarose gel electrophoresis, and the PCR products were purified using a PCR Purification Kit (Fermentas). The insert DNA fragment and pUC19 vector (Table 8) were double digested in separate reactions containing 1× fast digest green buffer (Fermentas) with *BamHI* and *HindIII* for 1 h at 37 °C. The resulting DNA products were ligated at 25 °C for 10 min in 1× T4 DNA ligase buffer with T4 DNA ligase (0.7 U / µl). Again, the *lanA* gene was amplified from the genomic DNA by PCR using PseAfor and PseArev as primer pair (Table 9). The obtained insert DNA fragment and the pET28b vector (Table 8) were double digested in separate reactions containing 1× fast digest green buffer (Fermentas) with *NdeI* and *XhoI* for 1 h at 37 °C. The resulting DNA products were ligated at 25 °C for 10 min in 1× T4 DNA ligase buffer with T4 DNA ligase (0.7 U / µl). *E. coli* JM109 cells were transformed with 6 µl of the ligation product by heat shock, and the cells were plated on LB-kanamycin agar plates and grown overnight at 37 °C. Several colonies were picked and used to inoculate separate 5 ml cultures of LB-kanamycin medium. The cultures were grown at 37 °C for 12 h, and plasmids were isolated using a Spin Miniprep Kit (Fermentas). The sequences of the inserts of the plasmids were confirmed by DNA sequencing.

The *lanM* gene was PCR amplified by using the genomic DNA isolated from *B. pseudomycoides* as template and PseMfor and PseMrev as primers (Table 9). The cycler

program for amplification is presented in Annex XVII. Amplifications were confirmed by 1% agarose gel electrophoresis, and the PCR products were purified using a PCR Purification Kit (Fermentas). The insert DNA fragment and the pET22bΔpelb vector (Table 8) were double digested in separate reactions containing 1× fast digest green buffer (Fermentas) with *NcoI* and *XhoI* for 30 min at 37 °C. The resulting DNA products were ligated at 25 °C for 10 min in 1× T4 DNA ligase buffer with T4 DNA ligase (0.7 U / µl). *E. coli* JM109 cells were transformed with 6 µl of the ligation product by heat shock and the cells were plated on LB-ampicillin agar plates and grown at 37 °C for 15 h. Several colonies were picked and used to inoculate separate 5 ml cultures of LB-ampicillin medium. The cultures were grown at 37 °C for 12 h, and plasmids were isolated using a Gene Jet Plasmid Miniprep Kit (Fermentas). The sequences of the inserts of the plasmids were confirmed by DNA sequencing.

2.5.14. Molecular engineering of a factor Xa cleavage site into the *pseA* gene.

A site-directed mutagenesis approach was used to install a proteolytic cleavage site directly N-terminal to the core peptide of LanA. The primers were designed to contain the nucleotide sequences necessary to encode the amino acids IEGR in place of four wild-type peptide residues (VVGA). The plasmid pET28b_ lanAXa (Table 8) was PCR amplified according to the Quick Change Lightining Site-Directed Mutagenesis Kit using PseAXafor and PseAXarev (Table 9) as primers and pET28b_ lanA (Table 8) as template. The amplification was confirmed by 1% agarose gel electrophoresis. To digest the methylated template prior to transformation, 2 µl of *DpnI* was added to the PCR product and the digest was incubated at 37 °C for 5 min. XL10-Gold ultracompetent cells were transformed with 2 µl of the digest product by heat shock, and cells were plated on LB-kanamycin agar plates and grown overnight at 37 °C. Several colonies were picked and used to inoculate separate 5 ml cultures of LB-kanamycin medium. The cultures were grown at 37 °C overnight, and the plasmids were isolated using a Gene Jet Plasmid Miniprep Kit (Fermentas). The sequences of the resulting plasmid products were confirmed by DNA sequencing.

2.5.15. Attempts to sequence the gap upstream of the gene cluster

Since the structural gene is encoded in the 5’ end of contig 00045, the upstream sequences of the gene cluster are not yet clear. According to NCBI, contig 00074 (bpmyx0001_45890-thioredoxine reductase) is located adjacent to contig 00045 with a gap of approximately 100 bp. To fill the gap upstream of *pseA*, PCR and rPCR were performed. The primers used for PCR and rPCR are listed in Table 9. The genomic DNA extracted from *B. pseudomycoides*

was used as a template for the PCR. The PCR products were gel extracted and purified. Purified PCR products were sent for DNA sequencing.

2.6. Protein and biochemical methods

2.6.1. Production of antimicrobial substance from *B. pseudomycoides*

To detect the production of an antimicrobial substance, *B. pseudomycoides* was cultured in TSB, LB, MHB, BHI, NB and 2× BPM (Table 6) media at different incubation temperatures (28 °C, 30 °C, 37 °C and 40 °C) and incubation periods (24, 45, 48, 68 and 71 h). After incubation the cells were pelleted by centrifugation at 10,000 × g and 4 °C for 30 min. For further analysis, the culture supernatant was sterilized by filtration and stored at -20 °C. The cell pellet was resuspended in 35 ml, 70% isopropanol (adjusted to pH 2 with HCl) and incubated at 4 °C for 4 h with continuous stirring. The cells were removed by centrifugation and the supernatant was sterilized by filtration and stored at -20 °C. Antimicrobial activity from the supernatant and isopropanol cell wash extract was examined by an agar well diffusion assay against *M. luteus* and several other indicator strains. After incubation at 37 °C overnight, inhibition zones were measured.

2.6.2. Purification of the antimicrobial substance from *B. pseudomycoides*

Prior to purification, isopropanol from the cell wash extract was removed by rotary evaporation (Rotavapor Re11, Essen, Germany). Various chromatographic purification methods were tested for the purification of the cell wash extract from *B. pseudomycoides* as explained below.

2.6.2.1. SerdolitR PAD I adsorption

The 10 g of PAD I resin was soaked in 50% of methanol and stored at 4 °C. Before use, methanol was removed from resin by washing with deionised distilled water for several times. Then the resin was poured into the column. The antimicrobial substance (10 ml) was loaded onto the column with the PAD resin. Then the column was washed with water and eluted with different percentages of methanol, acetronitrile and isopropanol (Annex X). The collected fractions were evaporated to remove the solvents. Then each fraction was tested for antimicrobial activity by an agar well diffusion assay.

2.6.2.2. Cation and anion exchange chromatography

Both the cation exchanger (HI Trap SP column) and the anion exchanger (HI Trap Q HP column) were equilibrated by start buffer with a flow rate of 1 ml/min. In both chromatographic methods, 10 ml of isopropanol cell wash extract was loaded onto the respective columns. Both the columns were washed with 25 ml of start buffer to remove the unbound compounds. The samples were then eluted with 30 ml elution buffer. Buffers used for cation and anion exchange chromatography are listed in Annex VIII and IX respectively. Fractions of 1 ml were collected and tested for an antimicrobial activity.

2.6.2.3. High performance liquid chromatography (HPLC)

For the purification of the antimicrobial substance, Poros (10R2, 100 x 4.6 mm, Perseptive Biosystems, Freiburg, Germany) and C18 (3C18, 250 x 4.6 mm, Schambeck SFD GmbH, Rheindorfer, Germany) HPLC columns were used. The gradient was generated by water with 0.1% TFA and acetonitrile with 0.1% TFA. The empty column was first washed with acetonitrile with 0.1% TFA followed by equilibration with water supplemented with 0.1% TFA. Then, 2 ml cell wash extract was loaded onto the poros column. Elution was performed by using the linear gradient of 0 - 100% acetonitrile with 0.1% TFA at a flow rate of 1 ml/min for 30 min. The peaks were detected at the absorbance of 220 nm. Fractions were collected, and assayed for their antimicrobial activity against *M. luteus* by an agar well diffusion assays. The active fractions were then analysed by MALDI TOF MS. All the active fractions from the Poros column were then pooled and further purified by the C18 HPLC column. Again, the peaks were detected at an absorbance of 220 nm and 266 nm. The fractions were collected and assayed for an antimicrobial activity against *M. luteus* by an agar well diffusion assays and analysed by MALDI TOF MS.

For the purification of the heterologously produced peptide, a C4 RP-HPLC column was used. The peptide was loaded onto the column and was eluted using a gradient of 0 - 100% of ACN in 0.1% TFA for 30 min. The peaks were detected at an absorbance of 220 nm and 266 nm. The fractions were collected and assayed for an antimicrobial activity against *M. luteus* by an agar well diffusion assay and further analysed by MALDI TOF MS.

2.6.3. Mass spectrometry analysis

The mass spectrometric analysis of the peptide preparations was performed using a MALDI TOF mass spectrometer (Bruker Biflex, Bruker Daltonics, Bremen, Germany). Aliquots of 1 µl of the isopropanol cell wash extracts were mixed with 2 µl matrix (α-cyano-4-

hydroxycinnamic acid in ACN: 0.1% TFA in water (1:3) or sinnapinic acid in ACN: 0.1% TFA in water (1:3)). For MALDI TOF analysis of the active HPLC fractions, 20 µl of each fraction was concentrated at the ratio of 1:10 using a Rotational Vacuum Concentrator (RVC 2-18, Christ, Osterode, Germany). The samples were then spotted onto the MALDI target and dried in air. Mass spectra were measured in the positive ion mode in the range of 1500 to 4000 Da for the cell wash extract or at a range of 1000 to 20,000 Da for the heterologously produced peptide. The peaks were determined using Flexanalysis 2.0 (Bruker Daltonics).

The heterologously produced PseAXa and its mutant variants were analysed by LC-MS and MS-MS. LC-MS was performed using Agilent 1200 series HPLC with a reverse-phase column (KNAUER Eurospher II 100-5 RP C-18, 5 um, 4.0 mm x 250 mm) coupled with an ESI-micrOTOF-Q (Bruker Daltonics) mass spectrometer and were operated at a flow rate of 1.0 ml/min, for 50 min using 75% ACN and 25% water supplemented with 0.1% TFA (v/v). The wavelength for detection was set at 273 nm. The spectra were recorded by infusion into the ESI source using methanol as the solvent. The instrument was first autotuned on the *m/z* value of the ion to be fragmented. Then, the $[M+H]^+$ ion of each compound was isolated in the linear ion trap and fragmented by CID. Sets of consecutive, high-resolution, full MS/MS scans were acquired. Mass spectra were measured in the range of 200 - 3000 Da and finally analysed by Bruker Compass DataAnalysis 4.0 (Bruker Daltonics).

2.6.4. Agar well diffusion assay

The antimicrobial activity of culture supernatants, cell wash extracts and HPLC fractions was determined by an agar well diffusion assay. One colony of the indicator strain was suspended in 5 ml MH broth. One ml of this diluted cell suspension was used to inoculate a second 5 ml MH broth, and was used for seeding MH agar plates with the indicator strains. For seeding, the surface of the agar plates was overlaid with the cell suspension. The cell suspension was then removed by pipetting, followed by incubation for 15 min at RT to air-dry the plates. Wells (7 mm diameter) were introduced into the surface of the agar plate using a sterile cork borer. 50 µl of each sample were loaded into a well, and dried for 15 min at RT. The plates were then incubated at 37 °C overnight. The activity was expressed in the diameter (cm) of the inhibition zones formed around the wells.

For the determination of the activity of heterologously produced peptides, each peptide (5 - 10 µl) was directly pipetted on the surface of agar plates seeded with indicator strain (also known as spot on the lawn method). *M. luteus* was used as a standard indicator strain as it is

known to be one of the most sensitive indicator strains for lantibiotics. Buffer used during factor Xa cleavage was used as a control.

2.6.5. Stability assay

To determine the sensitivity of the antimicrobial substance to different temperatures, pH, enzymes and organic solvents, a stability assay was performed. The thermal stability of the antimicrobial compound was tested by incubating 2 ml of the isopropanol cell wash extract at 25 °C, 37 °C, 45 °C, 65 °C, 80 °C and 100 °C for 4 h. The stability of the antimicrobial activity against enzymes were determined by adding several enzymes - 10 mg/ml of proteinase K (pH 7), pronase E (pH 7), α-chymotrypsin (pH 7.6), and trypsin (pH 7.6) to the cell wash extract. The samples were incubated at 37 °C for 1 h. The influence of pH on the antimicrobial activity was examined by adjusting the pH to 3, 4, 5, 6, 7, 8, 9 and 11 by sodium phosphate buffer and incubating them for 4 h at RT. The stability of the antimicrobial compound against organic solvents was determined by adding organic solvents - ethanol, methanol, acetone, acetonitrile and 2-propanol to the cell wash extract and left for incubation up to 4 h at RT. The residual antimicrobial activity was then determined by an agar well diffusion assay against *M. luteus*.

2.6.6. SDS-PAGE and the NuPAGE

The protein and peptide profile of fractions was determined by standard SDS-PAGE (Laemmli, 1970) in a Mini Protean III vertical electrophoresis apparatus (BioRad) or the NuPAGE electrophoresis system (XCell SureLock® Mini-Cell, Invitrogen). The Tris glycine buffer system or NuPAGE® MES running buffer was used for the separation of proteins (Annex XII). For small peptides like LanA, 20 - 24% SDS-gels or commercially available 4 - 12% gradient Novex Bis-Tris gels were used. LanM proteins were analysed on 10% SDS-gels. 10× LDS sample buffer (Invitrogen) was added to the samples, which were heated (70 °C) for 10 min prior to electrophoresis. Prestained (10-250 kDa) or unstained protein (5- 200 kDa) ladders (Fermentas) were used as a protein markers. Gels were stained with the PageBlue™ Protein Stain (Fermentas) or Coomassie staining (Annex XIII) or silver staining (Annex XIV) according to Heukeshoven and Dernick (1988).

Aliquots of the partially purified cell wash extract were verified on polyacrylamide gel 20% SDS-gels. After electrophoresis, the gels were washed with sterile distilled water for the removal of SDS. Then the gels were cut into two parts. One part of the gel was used for the *in situ* overlay assay and the other part for staining. For the *in situ* overlay, the gels were placed

on the MH agar plate seeded with *M. luteus*. The plates were incubated at 37 °C for 24 h and the presence of inhibitory zones was observed. Later, the other part of gel was stained with page blue staining solution to detect the peptide bands. Low molecular weight standard (Sigma) was used as a protein marker.

2.6.7. Dialysis of peptides

After Ni-NTA chromatography, the peptide eluate contained a high amount of salts. Thus, it was necessary to remove salts from the peptide before conducting further experiments with the peptide. Removal of the salt from the peptide was achieved by a dialysis in a semi permeable membrane. The dialysis cassettes (slide-A-Lyzer ® system, Thermo Fisher Scientific, MWCO 2,000; 0.1 - 0.5 ml and MWCO 10,000; 0.1-0.5 ml) were used for the removal of the salt. For maldi analysis, dialysis was performed in 0.05% HCl, and for the Factror Xa cleavage assay, dialysis was done in buffer solution containing 20 mM Tris-HCl (pH 8), 100 mM NaCl and 2 mM $CaCl_2$. Dialysis was performed in two steps: (i) the eluate buffer was exchanged with a dialysis buffer at 4 °C for 2 h with stirring and (ii) it was further dialysed in fresh buffer at 4 °C overnight with stirring. Finally, all peptides were stored at -20 °C for further experiments.

2.6.8. Determination of peptide concentration

The concentration of the peptide was determined by Bradford assay according to Bradford (1976). In this method, the pigment (Coomassie Brilliant Blue G-250) binds to the protein, allowing the detection of the peptide concentration, indirectly from its emission spectra. The binding of the dye to the protein causes a shift in the absorption maximum of the dye from 365 to 595 nm. This increase in absorption at 595 nm is monitored to quantify the protein concentration.

The working solution of the Coomassie Brilliant Blue G-250) (BioRad) was prepared by diluting it to 1:5 ratio using MilliQ. The calibration curve was prepared from different concentrations, mg/ml (0.125, 0.25, 0.5, 0.75, 1, 1.5, and 2) of Bovine Serum Albumin (BSA). 10 µl of each BSA standard solution was mixed with 90 µl of MilliQ to make 100 µl solution in the cuvette. The peptide eluate of 5 µl was mixed with 95 µl MilliQ to make 100 µl solution in the cuvette. 3 ml of reagent was added to both the standards and the peptide sample, and was incubated at room temperature for 5 min. The absorbance of standards and samples were measured against a blank (100 µl MilliQ + 900 µl reagent) at 595 nm in the spectrophotometer. The absorbance of each standard was plotted to create a standard

calibration curve. The concentration of the peptide was determined by comparing its absorbance value to the standard curve.

2.6.9. Peptide desalting by ZipTip

After factor Xa cleavage and chemical modification assays, the peptides were desalted by ZipTip (Millipore protocol). The solutions used for the purification were freshly prepared before purification (Table 12). The final pH of the sample was maintained below 4 as required for the ZipTip purification. The ZipTip column was equilibrated with the wetting solution before loading the sample. To bind the sample to the tip, the sample was aspirated and dispensed for 7-10 times. The tip was washed twice with washing solution to remove impurities and then the peptide sample was eluted using the elution solution.

Table 12: Solutions used for the ZipTip purification.

Solution	Composition
Wetting solution	100% acetonitrile
Equilibration solution	0.1% trifluoroacetic acid (TFA) in MilliQ water
Washing solution	0.1% trifluoroacetic acid (TFA) in MilliQ water + 5% methanol
Elution solution	0.1% TFA/90% ACN + 0.1% formic acid

2.6.10. Overexpression and purification of PseM

The chemocompetent *E. coli* BL21 strain was transformed with the pET22bΔpelb_lanM (Table 8) construct containing C-terminal hexa-histidine *pseM* fusion. The cultures were inoculated from a single colony of transformants and were grown overnight at 37 °C in LB broth supplemented with 80 µg/ml ampicillin. The overnight grown culture was used to inoculate 1 L of LB broth, and the cells were grown at 37 °C to 0.5 - 0.6 $O.D._{600}$. Expression was induced by the addition of 1 mM IPTG, and the culture was incubated at 18 °C for additional 20 h. The cells were harvested by centrifugation at 6500 × g for 20 min at 4 °C. The pellet was resuspended in 10 ml lysis buffer and lyzed by sonication at 65% intensity for 15 min. DNase and RNase were added to the sample, vortexed carefully and incubated on ice for 30 min. The sample was centrifuged at 15,000 × g for 20 min at 4 °C. The cell free supernatant was filtered through a 0.45 µm filter. It was purified by IMAC using a 2 ml Ni^{2+} column. After the sample had been applied to the column, it was washed with two column volumes of wash buffer 1, 2 and 3 consecutively. The protein was eluted with two column volumes of elution buffer 1 and 2 (Table 13). The fractions were analysed by SDS-PAGE;

those containing PseM protein were pooled and desalted *via* dialysis. The protein was stored in storing buffer at -80 °C.

Table 13: Buffers used in the purification of PseM.

Buffer	Chemical Ingredients
Lysis Buffer	20 mM Tris pH 7.6, 500 mM NaCl, 10% Glycerol
Wash Buffer 1	20 mM Tris pH 7.6, 500 mM NaCl, 10% Glycerol, 25 mM Imidazole
Wash Buffer 2	20 mM Tris pH 7.6, 500 mM NaCl, 10% Glycerol, 50 mM Imidazole
Wash Buffer 3	20 mM Tris pH 7.6, 500 mM NaCl, 10% Glycerol, 75 mM Imidazole
Elution Buffer 1	20 mM Tris pH 7.6, 500 mM NaCl, 10% Glycerol, 200 mM Imidazole
Elution Buffer 2	20 mM Tris pH 7.6, 500 mM NaCl, 10% Glycerol, 500 mM Imidazole
Storing Buffer	20 mM Tris pH 7.6, 100 mM KCl, 10% Glycerol

2.6.11. Overexpression and purification of PseA and PseAXa

The chemocompetent *E. coli* BL21 strains were transformed with pET28b_lanA and pET28b_lanAXa (Table 8) constructs respectively. The construct contained N-terminal hexa-histidine *pseA* and *pseAXa* fusions. The cultures were inoculated from a single colony of transformants and grown overnight at 37 °C in LB broth supplemented with 50 µg/ml kanamycin. The overnight culture was used to inoculate 1 L of LB broth, and the cells were grown at 37 °C to 0.6 - 0.8 $O.D._{600}$. Expression was induced by the addition of 1 mM IPTG, and the culture was incubated at 37 °C for additional 4 h. The cells were harvested by centrifugation at 6500 × g for 20 min at 4 °C. The pellet was resuspended in 10 ml start buffer and lysed by sonication at 65% intensity for 15 min. DNase and RNase were added to the sample, vortexed and incubated on ice for 30 min. Later, the sample was centrifuged at 15,000 × g for 20 min at 4 °C. The supernatant was discarded and the pellet was resuspended in 10 ml start buffer (Table 14). The sonication and centrifugation steps were repeated and the pellet was resuspended in 10 ml of buffer 1. The sample was sonicated and centrifuged and the supernantant was filtered through a 0.45 µl filter. It was purified by IMAC using a 2 ml Ni^{2+} column. The extracted sample was loaded onto the column, and the column was washed with two column volumes of buffer 1 followed by two column volumes of buffer 2 (Table 14). Then the sample was eluted with two column volumes of elution buffer. Fractions were analysed by SDS-PAGE; those eluates containing the peptide were pooled and desalted *via* dialysis. Reverse phase HPLC was performed on a C4 column using a gradient of 0 - 100% of ACN in 0.1% TFA.

Table 14: Buffers used in the purification of PseA.

Buffer	Chemical Ingredients
Start Buffer	20 mM Sodium phosphate pH 7.5, 500 mM NaCl, 20% Glycerol, 0.5 mM Imidazole
Buffer 1	6 M Guanidine hydrochloride, 20 mM Sodium phosphate pH 7.5, 500 mM NaCl, 0.5 mM Imidazole
Buffer 2	4 M Guanidine hydrochloride, 20 mM Sodium phosphate pH 7.5, 300 mM NaCl, 30 mM Imidazole
Elution Buffer	4 M Guanidine hydrochloride, 20 mM Sodium phosphate pH 7.5, 100 mM NaCl, 1M Imidazole

2.6.12. Purification of PseA from inclusion bodies

The chemocompetent *E. coli* BL21 strain was transformed with the pET28b_lanAXa or pET28b_lanA construct containing the N-terminal hexa-histidine *pseAXa* or *pseA* fusions. Cultures were inoculated from a single colony of the clones and were grown overnight at 37 °C in LB broth supplemented with 50 µg/ml kanamycin. The overnight culture was used to inoculate 1 L of LB broth, and cells were grown at 37 °C to 0.6 - 0.8 $O.D._{600}$. Expression was induced by the addition of 1 mM IPTG, and the culture was incubated at 37 °C for additional 3 h. The centrifuge bottle was weighed before adding the culture. The cells were harvested by centrifugation at 6000 × g for 20 min at 4 °C. Again the bottle with pellet was weighed and the weight of pellet was calculated. 3 ml of buffer A (Annex XV) per gram of pellet was added and the pellet was resuspended. The suspended pellet was transfered into 50 ml falcon tubes and 100 mM PMSF was added to make 1 mM final concentration. 16 µl of lysozyme (50 mg/ml) was added to the cell suspension and mixed thoroughly. It was then placed in a water bath at 37 °C until the solution consistency became viscous. The sample was sonicated and then centrifuged at 18000 rpm for 30 min. The supernatant was collected and saved for SDS-PAGE and the recovered pellet was resuspended in buffer B (Annex XV) (3 ml/g of cell). The same amount of PMSF as in the previous step and 10 µl of TritonX-100 per ml of solution were added to the resuspended pellet. It was centrifuged at highest speed for 20 min and the supernantant was collected. The remaining pellet (inclusion bodies) was resuspended in 20 ml of 8 mM urea with DTT and was heated at 37 - 50 °C to dissolve the pellet. Urea was removed by dialysis in 4 L of 50 mM Tris-HCl buffer solution at pH 8.5 using 2 kDa MWCO dialysis cassettes for 2 days. The sample was recovered and centrifuged at 20000 rpm for 30 min and the supernantant was collected and stored in the freezer at -20 °C. After that, the extracted protein was purified by HPLC as explained elsewhere.

2.6.13. Cotransformation and coexpression

Equal amounts of pET28b_lanAXa and pET22bΔpelb_lanM, pET28b_lanA and pET22bΔpelb_lanM (Table 8) were used to cotransform chemocompetent *E. coli* BL21 and E. coli C41/C43 strains. The transformed competent cells were plated on LB agar plates containing kanamycin and ampicillin.

The cells carrying pET28b_lanAXa and pET22bΔpelb_lanM, and pET28b_lanA and pET22bΔpelb_lanM were grown overnight at 37 °C in LB broth supplemented with kanamycin and ampicillin. The overnight culture was used to inoculate 1 L of LB broth, and the cells were grown at 37 °C to 0.5 - 0.8 O.D. $_{600}$. Expression was induced by the addition of 0.8 mM IPTG, and the culture was incubated at 37 °C for additional 4 h. 2 ml of the culture was employed for plasmid extraction and to verify whether both plasmids were maintained for 20 h after induction. Cells were harvested by centrifugation at 6500 × g for 20 min at 4 °C. The pellet was resuspended in 10 ml start buffer and stored at -20 °C.

2.6.14. Purification of modified PseAXa and PseA peptides

The pellet resuspended in 10 ml start buffer was lysed by sonication at 65% intensity for 15 min. The sample was centrifuged at 15,000 × g for 20 min at 4 °C. The supernatant was discarded and the pellet was resuspended in 10 ml of start buffer. The sonication and centrifugation steps were repeated and the pellet was resuspended in 10 ml of buffer 1. Then the sample was sonicated and centrifuged. The supernantant was filtered through a 0.45 µl filter, and was purified by IMAC using a 2 ml Ni^{2+} column. After loading the sample onto the column, it was washed with two column volumes of buffer 1 followed by two column volumes of buffer 2. The protein was then eluted with two column volumes of elution buffer. Fractions were analysed by SDS-PAGE; those eluates containing the protein were pooled and desalted *via* dialysis. Dialysis was performed using 2,000 MWCO dialysis cassettes, in which the peptide sample buffer was exchanged for 0.05% HCl. The sample was further purified using a C4 RP-HPLC column and was analysed by MALDI TOF MS. The concentration of the peptide was estimated in the Bradford assay.

2.6.15. Factor Xa cleavage of peptide leader sequence

The protein sample buffer was exchanged for 20 mM Tris-HCl (pH 8) with 100 mM NaCl and 2 mM $CaCl_2$. Factor Xa (New England Biolabs) was used to remove the leader sequence from the PseAXa peptide. Factor Xa was added directly to the PseAXa at a final concentration of 0.075 mg/ml. The sample was incubated at 4 °C overnight to fully proteolyze the peptide. The

reaction was stopped with 0.1% TFA. Then the peptide was desalted by $ZipTip_{C18}$ and was analysed by MALDI TOF MS. The peptide mixture contains both the core peptide and the leader peptide. Thus, to separate the core peptide from the leader peptide, a C18 HPLC column was employed. The peptide mixture containing the core and the leader was centrifuged at 15000 rpm for 2 min, and the resulting supernatant was loaded to the C18 HPLC column. Finally, all the fractions were analysed by MALDI TOF MS.

2.6.16. Construction of PseAXa mutants

To elucidate the presence of lanthionine rings in PseAXa, after its modification by PseM, a series of PseAXa analogs were made. A single Cys residue on PseAXa was replaced with an Ala residue yielding several analogs *viz.* PseAXaC52A, PseAXaC56A, PseAXaC62A, PseAXaC65A, PseAXaC70A and PseAXaC75A (Table 8). Each PseAXa analog was coexpressed with the PseM. Then, the modified peptides were purified by IMAC using a 2 ml Ni^{2+} column. The fractions were analysed by SDS-PAGE; those eluates containing the protein were pooled and desalted *via* dialysis. Dialysis was performed using 2,000 MWCO dialysis cassettes in which the peptide sample buffer was exchanged for 0.05% HCl. Finally, the dialysed peptide sample was analysed using MALDI TOF MS.

2.6.17. Iodoacetamide modification assay for detection of free cysteines

To detect the presence of free cysteine thiols in modified PseAXa, an iodoacetamide assay was performed. The modified peptide was incubated with IAA (10 mM) (Sigma Aldrich Chemie GmbH, Taufkirchen, Germany) and Tris buffer (50 mm, pH 8.3), in the presence and in absence of reducing agent TCEP (1 mM) (Sigma Aldrich Chemie GmbH, Taufkirchen, Germany). The reaction mixture was incubated at 25 °C / RT for 45 min in dark. Then, the samples were purified by $ZipTip_{C18}$ and subjected to MALDI TOF MS. Similarly, the IAA assay was performed for all the PseAXa mutants, and their masses were analysed by MALDI TOF MS.

2.6.18. Trypsin digestion of the peptide

The modified PseAXa peptide and its mutants were incubated with trypsin (pH 7.6) at 37 °C up to 4 h. The samples were drawn after 1 h and 4 h of incubation for MALDI TOF MS.

2.6.19. Determination of free didehydro amino acids in the modified peptide

Mercaptoethanol (βME) was employed to determine the presence of free Dha residues in the peptide following Wang et al., (2012). The modified peptide was incubated with 5 mM βME in Tris buffer (pH 8.5) for 2 h at 30 °C. The reaction mixture was purified by $ZipTip_{C18}$ and then subjected to MALDI TOF MS.

2.6.20. Determination of minimal inhibition concentration (MIC)

MIC determinations were carried out in 96 well polypropylene microtiter plates (NuncTM; Thermo Fischer Scientific, Schwerte, Germany) employing the standard broth dilution method in MH broth with 1.25 mM $CaCl_2$. Test strains were grown to 1 $O.D._{600}$ and subsequently diluted to 2×10^5 cells/ml. The peptide was diluted in the range of 12 µM to 0.005 µM by two-fold dilution steps. 50 µl of the bacterial suspension was mixed with 50 µl of the peptide solution, and the inoculated microtiter plate was incubated for 10 min at RT on a microtiter shaker (Titertek; Flow laboratories, Mechenheim, Germany). The MIC was read after 24 h of incubation at 37 °C without agitation. The results presented are the mean values of at least two independent experiments.

Later the effect of lipid II on the MIC of the peptide was determined. To this end, concentrations ranging from 0.25 fold to 3 fold MIC were tested. The peptide and the lipid II were mixed in an estimated ratio of 1:1 and 1:2 (peptide: lipid II) with 1.25 mM $CaCl_2$. The effect of lipid II on the antibacterial activity of the peptide was observed after 24 h of incubation at 37 °C without agitation.

2.6.21. Phosphate determination

The phosphate determination was performed according to Rouser (1970). To this end, the lipid samples were transferred to the phosphate free glass tubes (prepared by rinsing the glass tubes with chloroform) and dried at 140 °C for 20 min. Afterwards, 0.3 ml $HClO_4$ (70%, v/v) was added, and the tubes were incubated in a block heater at 180 °C for 1 h. After cooling to RT, released inorganic phosphate was reduced by adding 1 ml MilliQ water, 0.4 ml ammonium molybdate (1.25%, w/v) and 0.4 ml freshly prepared ascorbic acid (5%, w/v) to the tube. Then, the tube was incubated in a boiling water for 5 min. The absorption spectrum of the blue-coloured complex was read at 795 nm and the phosphate concentrations of the samples were calculated by using a standard curve of 0 - 30 nmol inorganic phosphate (stock solution: 1 mM KH_2PO_4).

2.6.22. Lipid II binding assay

In order to detect the binding ability of the active peptide to the lipid II, a lipid II binding assay was performed on a TLC sheet. For the binding assay, the substrate lipid II and the peptides (at an estimated molar ratio of 1:1, 1:2, 1:3) were incubated at RT for 30 min. The assay was performed in presence and in absence of $CaCl_2$ (final concentration, 1.25 mM). The reaction mixture was analysed by TLC using chloroform-methanol-water-ammoniumhydroxide (88:48:10:1, v/v) as a solvent. Spots or lanes were visualized using PMA (2.5%, w/v) staining reagent. Nisin and mersacidin were used as controls.

3. Results

3.1. The novel lantibiotic gene cluster from *Bacillus pseudomycoides* DSM 12442

The genomic data mining found a putative class II lantibiotic gene cluster in *Bacillus pseudomycoides* DSM 12442. Thus, a detailed bioinformatic analysis of the gene cluster was performed. The putative structural and modification genes from the cluster were heterologously expressed in *E. coli*. Thus produced proteins were purified and characterized by stability assays and MALDI TOF analysis.

3.1.1. Bioinformatic analysis

A novel putative lantibiotic gene cluster was found in *Bacillus pseudomycoides* DSM 12442 (Gene bank accession no NZ_CM000745 NZ_ACMX01000000) (Figure 7) (Zwick et al., 2012). *B. pseudomycoides* DSM 12442 is also known as NRRL B-617 (T), and is deposited in the Agricultural Research Service Culture Collection, Peoria, IL, USA (Nakamura, 1998). The hypothesis proposed for this study is that the putative gene cluster found in *B. pseudomycoides* would result in the production of a novel lantibiotic. Applying the common nomenclature for lantibiotic biosynthetic genes, the genes of a putative biosynthetic gene cluster in *B. pseudomycoides* were referred to by '*pse*'.

The putative lantibiotic structural gene *pseA* (bpmyx0001_45460) had 40% identity to the *mrsA* gene, which encodes the mersacidin precursor peptide (Bierbaum et al., 1995; Chatterjee et al., 1992). Similarly, *pseA* had 40% identity to *amlA*, which encodes the amylolysin peptide (Arguelles Arias et al., 2013). The downstream ORF (bpmyx0001_45470) was predicted to encode the enzyme that performs the post translational modification resulting in thioether formation, and was designated *pseM*. It showed 36% identity to the mersacidin modifiying enzyme MrsM of *Bacillus amyloliquefaciens* (Altena et al., 2000), 37% identity to the haloduracin modifying enzyme (HalM1, BH0455) of *Bacillus halodurans* C-125 (McClerren et al., 2006; Takami et al., 2000) and 36% identity to the lichenicidin modification enzyme LicM1 (Begley et al., 2009; Dischinger et al., 2009; Caetano et al., 2011). As in other class II LanMs, PseM contains the conserved Zn-binding motives (Figure 8) GXXHGXXG, WCXG and CHG/CCG (Paul et al., 2007). Located immediately downstream of the *pseA* and *pseM*, *pseT* (bpmyx0001_45480) was predicted to encode a lantibiotic ABC exporter. PseT contains an N-terminal double-glycine peptidase domain and the ATP binding site and shares 42% identity with the mersacidin exporter (MrsT) (Altena et al., 2000). This ABC exporter

may be responsible for the removal of the leader peptide from the modified prepeptide and for the export of the modified core peptide. The ORFs bpmyx0001_45490, bpmyx0001_45500 and bpmyx0001_45510 were predicted to encode the subunits of another ABC transporter and may be responsible for self-immunity of the producer strain. They showed 59%, 33% and 29% similarity to the mersacidin immunity transporter MrsFEG respectively (Altena et al, 2000 Altena et al., 2000). In addition, there were other ORFs downstream of the gene cluster that cannot be directly linked to the lantibiotic gene cluster. Bpmyx0001_45520 is a hypothetical protein which has some similarity (27%) to a hypothetical protein BH0831 of *B. halodurans* C-125 (Takami et al., 2000). Likewise, bpmyx0001_45530 is a hypothetical small multidrug export protein that has 49% similarity to a hypothetical protein BH1312 of *B. halodurans* C-125. However, both of these proteins were not located within the haloduracin gene cluster (McClerren et al, 2006), therefore the corresponding genes of *B. pseudomycoides* were also assumed not to be part of the pseudomycoicidin gene cluster. Bpmyx0001_45540 is a hypothetical protein that has similarity to the putative lipoprotein YgaO of *B. amyloliquefaciens* (Blom et al., 2012; Hao et al., 2012). Further downstream bpmyx0001_45550 constitutes a helix-turn-helix XRE family like protein that is similar to a transcriptional regulator found in some bacilli and might be involved in regulation. In conclusion, the biosynthetic gene cluster in *B. pseudomycoides* represented at least 6 ORFs, comprising one of each lantibiotic structural gene (*pseA*), modification gene (*pseM*), ABC transporter (*pseT*) and immunity genes (*pseFEG*) (Figure 7, Table 15).

In addition to this lantibiotic gene cluster, a further search for proteins that might be involved in antibiotic biosyntheses was performed and indicated the presence of several ORFs with similarity to parts of non-ribosomal peptide synthetases, encoded e. g. by bpmyx0001__50260 to bpmyx0001_50280 on contig00057 (acc. no. ACMX01000094). Contig00076 (acc. no. ACMX01000261) and contig00431 (acc. no. ACMX01000147) also represent parts of non-ribosomal peptide biosynthesis operons. Likewise, bpmyx0001_45430 is similar to the gramicidin S biosynthesis protein GrsT of *Aneurinibacillus megulanus* (Kraetzschmar et al., 2004). Bpmyx0001_52750 constitutes a nonribosomal peptide synthetase C that has 48% similarity to the surfactin synthetase subunit 2 of *Brevibacillus laterosporus* GI-9 (NCBI ref seq: WP_003339100.1), 45% similarity to a linear gramicidin synthase subunit C of *Brevibacillus laterosporus* LMG 15441 (NCBI ref seq: WP_003337597.1).

Just upstream of *pseA*, a short ORF is present which might encode a peptide. This sequence (NHYISHYKRGNFTHDKIIQYWNAPARCSTLSTAELSKIPVIPAGNALTKLSSDLPTA

LKCQIHIRINFKNFKKYKIYIAFLYKMLIYFRGGI) has 71% identity to the leader peptide of PseA. The genome of the producer strain has been deposited in NCBI as a scaffold of 305 contigs (Zwick et al., 2012). According to the NCBI entry, this sequence is located right at the 5' end of contig00045. Since the structural gene is encoded at the 5' end of contig 00045, the upstream sequences of the gene cluster are not yet clear. In the PubMed graphic assembly of the genome contigs, contig 00074 is located adjacent to contig 00045. The adjacent gene on contig00074 is annotated as a thioredoxin reductase gene (bpmyx0001_45890). The sequence in the gap between these two contigs is unknown, however the gap was predicted to have 100 bp (URL: http://tinyurl.com/k7hhj66). In order to fill this gap, PCR and sequencing were performed. Genomic DNA from *B. pseudomycoides* was used as template for the PCR. However, it was not possible to obtain a PCR product that harboured the overlapping sequences with contig 00045 and contig 00074. Attempts to use reverse PCR employing the primer pairs- rPCRFP1 and rPCRRP1, and rPCRFP2 and rPCRRP1 were also unsuccessful.

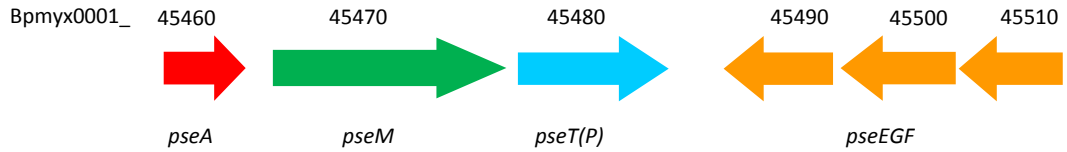


Figure 7: Hypothetical lantibiotic gene cluster in *Bacillus pseudomycoides.* The numbers indicate the locus tags of genes, *pseA* – structural gene, *pseM* – modification gene, *pseT (P)* – transporter gene, and *pseEGF* – immunity genes. The arrows indicate the relative direction of transcription.

Table 15: Comparision of the putative lantibiotic proteins from *B. pseudomycoides* with the known mersacidin (Mrs) proteins.

Putative protein from *B. pseudomycoides* (no. of aa)	**Mersacidin (Mrs) protein**				**% Identity**
	Name (no. of aa)	**Producer strain**	**Function**	**Reference**	
PseA (75)	MrsA (68)	*Bacillus species* HIL-Y85/54728	Mersacidin precursor	CAB60258	40
PseM (833)	MrsM (822)	*B. sp* HIL-Y85/54728	Modification	CAB60261	36
PseT (729)	MrsT (730)	*B. sp* HIL-Y85/54728	ABC transporter	CAB60262	42
PseG (258)	MrsG (252)	*B. sp* HIL-Y85/54728		CAB60256	29
PseE (248)	MrsE (244)	*B. sp* HIL-Y85/54728	Permease	CAB60257	34
PseF (304)	MrsF (303)	*B. sp* HIL-Y85/54728	ABC transpoter	CAB60255	58

```
      901                                                  950
PseM  FGEHILQKAI PQATGI.GWK ISVSED.... .....A...L P.GFSHGTSG
HalM  CSEHLMKNAI KTDQGI.GWK PPWEVT.... .....P...L T.GFSHGVSG
MrsM  CGNRLIQNIN VMEKGV.GWK VPANPT.... .....P...A S.GFAHGASG
CinM  CAEHLLRHAE DDGTTL.SWP PSAADE.... .....TYGNL T.GFSHGSGG
NisC  FLSNLTK... .ENNGLISLY IKSENQMSQS ESEMYPLGCL NMGLAHGLAG
SpaC  YLVRLTEDII VDGEKVPGWH IPSQHQFTDI EKKAYPYGNF NMGLAHGIPG
EpiC  HILNYFKTIH YSKDN...WL VSNEHQFLDI DKQNFPSGNI NLGLAHGILG

      951                                                 1000
PseM  IVWAL.HELY QLTGEQMLYD ALKQGLAYER SLYMEEKRNW A.........
HalM  VMASF.IELY QQTGDERLLS YIDQSLAYER SFFSEQEENW L.........
MrsM  IIWAL.YEIY AITKQTVFKE VAEKALEFER TLFIPEKNNW ADI......K
CinM  IGWAL.IQLG RHTGRSDYIE AGRKAFAYED RHVDEQEKDW YDL......R
NisC  VGCILAYAHI KGYSNEASLS ALQKIIFIYE KFELERKKQF LWKDGLVADE
SpaC  PICVLSSALI QGIKVKGQEA AIEKMANFLL EFSEKEQDSL FWKGIISFEE
EpiC  PLSLTALSKM NGIEIEGHEE FLQDFTSFLL KPEFKNNNEW FDRYDI.LEN

      1001                                                1050
PseM  LPSAAELP.. .QLPCAWCHG AAGVVLSRLL LKKA.GYSDS LIDIEIRVGL
HalM  TPNK.ETP.. .VV..AWCHG APGILVSRLL LKKC.GYLDE KVEKEIEVAL
MrsM  LENGQFRN.. .DNFVAWCNG AAGIGLSRIL ILPH.NQ.NE LIKDEAHVAI
CinM  INNGSAVKGA RHFSNAWCNG AAGIGLARIS SWAALDRSDE QLLRDAQQAL
NisC  LKKEKVIREA SFIRDAWCYG GPGISLLYLY GGLALDN.DY FVDKAEKILE
SpaC  YQYGSPPNAV NFSRDAWCYG RPGVCLALVK AGKALQN.TE LINIGVQNLR
EpiC  Y...IPNYSV ...RNGWCYG DTGIMNTLLL SGKALNN.EG LIKMSKNILI

      1051                                                1100
PseM  ETIIK.EGFG RDHSLCHGDT GNSAVLLLAS KVLKEDLWKQ YSYAVGEHVL
HalM  STTIR.KGLG NNRSLCHGDF GQLEILRFAA EVLGDSYLQE VVNNLSGELY
MrsM  NTTLK.YGFE HDQSLCHGDL GNLDILMYAA ENFNKKLSVN VT.ELSHKIL
CinM  SATLRNFPRL KNHTLCHGTS GNAELLLRFA RLSDEPAFQL EANVQVQALW
NisC  SAMQRKLG.I DSYMICHGYS GLIEICSLFK RLLNTKKFDS YMEEFNVNSE
SpaC  YTISDIRG.I FSPTICHGYS GIGQILLAVN LLTGQEYFKE ELQEI...KQ
EpiC  NIIDKNNDDL ISPTFCHGLA SHLTIIHQAN KFFNLSQVST YIDTI...VR
```

Figure 8: Partial sequence alignment of putative LanM proteins. The aa sequence of modification enzyme PseM is compared with MrsM (mersacidin), CinM (cinnamycin), NisC (nisin), EpiC (epidermin), HalM (haloduracin), SpaC (subtilin) (Gross et al., 1973; Allgaier et al., 1986; McClerren et al., 2006; Marki et al., 1991; Chatterjee et al., 1992; Ryan et al., 1996). The conserved zinc binding motifs (Zhu et al., 2003; Li et al., 2006; Paul et al., 2007) are highlighted in grey.

3.1.2 Bioinformatic analysis of putative lantibiotic prepeptide PseA

The prepetide PseA encodes a peptide of 75 amino acid residues, with a predicted 48-residue leader sequence and 27–residue core peptide (Figure 9 A). The leader sequence shows highest (37%) similarity to the leader sequence of mersacidin (Bierbaum et al., 1995).The core peptide of PseA is rich in Ser (5 residues) and Thr (3 residues). Those residues may dehydrate to the corresponding didehydro amino acids. Likewise, the Cys (6 residues) present in the core peptide of PseA might form rings with the dehydrated amino acids. The amino acid sequence of the predicted prepeptide PseA was compared with the previously known class II lantibiotics, e.g. mersacidin, actagardine and haloduracin alpha peptide. (Figure 9 B). As other class II prepeptides, PseA contains a GA/GG type of cleavage site. The N-terminus of the core peptide contains the conserved lipid II binding motif of class II lantibiotics (CTxS/TxE/DC). This motif might be involved in binding of lipid II. In contrast to the leader sequence, the aa sequence of the core peptide of PseA does not show significant similarities to any known lantibiotic other than the lipid II binding motif. In addition, the core peptide is characterized by a repeating 'SWSCWSWSC' motif (Figure 9), which is not common in class II peptides. Such motives are also found in the heterocycloanthracins (Haft et al., 2009) and have not yet been observed in lantibiotics; the first member of the heterocycloanthracin group, sonorensin from *Bacillus sonorensis* MT93, contains five repeated SCWSC motives and has recently been purified (Chopra et al, 2014).

(A)

MNDKIIQYWNDPAKRSTLSAAELSKMPVNPAGDILAELSDADLDKVVGAGDCGGTCTWTKDCSICPSWSCWSWSC

(B)

```
PseA    MNDK-IIQYWNDPAKRSTLSAAELSKMPVNPAGDILAEL-----SDAD-LDKVVGA----------GDCGGTCTWTKDCSICPSWSCWSWSC
HalA1   MTN--LLKEWKMPLERTHNNS--------NPAGDIFQEL-----EDQDILAGVNGACAWYNISCRLGNKGAYCTLTVECMPSCN--------
MrsA    MSQEAIIRSWKDPFSRENSTQ--------NPAGNPFSELKEAQMDKLVGAGDMEAA-----CTFTLPGGGGVCTLTSECIC-----------
ActA    MSALAIEKSWKDVDLRDGATS--------HPAGLGFGEL---TFEDLREDRTIYAA----------SSGWVCTLTIECGTVICAC------
                                                                                                        
                                                  Leader Region                  Core Region
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Figure 9: A) Amino acid sequence of PseA. The Cys residues and possible dehydration sites are indicated in grey letters. The cleavage site is highlighted inside the box and the putative core region is underlined. B) Sequence alignment of PseA by Clustal W. The amino acid sequence of PseA is compared to class II lantibiotics: haloduracin alpha peptide (HalA1), mersacidin (MrsA) and actagardine (ActA). AA similarities are highlighted in light grey, while the lipid II binding motif (CTxS/TxE/DC) is highlighted in dark grey. (Note: aa alignment of the PseA core peptide with other additional peptides is provided in Figure 36, page no. 97).

3.2. Characterization of *B. pseudomycoides* DSM 12442

Bacillus pseudomycoides DSM 12442 is a Gram-positive, rod shaped and spore forming bacterium. It was sensitive to chloramphenicol and erythromycin (data not shown). It grows as rapidly spreading rhizoidal colonies on agar plates (Figure 10), and does not produce a zone of haemolysis on blood agar plates.

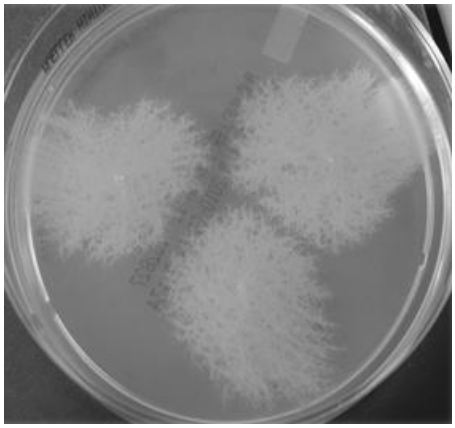


Figure 10: Rapidly growing rhizoidal colonies of *B. pseudomycoides* on an agar plate incubated at 37 °C overninght.

3.3. Production of an antimicrobially active substance by *B. pseudomycoides*

B. pseudomycoides DSM 12442 produced an antimicrobial substance, when it was grown in 50 ml TSB, LB or NB in 500 ml flasks, at 30 °C or 37 °C with agitation after 21, 24, 45 or 48 h; although the highest yield was observed in TSB broth incubated at 30 °C after 24 h. Cells were removed by centrifugation and the supernatant was collected. The pellet was resuspended in 70% isopropanol and incubated at 4 °C for 4 h under continuous stirring. The cells were removed by centrifugation and the cell wash extract was collected. Finally, the isopropanol was removed from the extract by evaporation.

The crude culture supernatant and the isopropanol cell wash extract were assayed for their antimicrobial activity in an agar well diffusion assay against different indicator strains. The crude culture supernatant did not show antimicrobial activity (Figure 11). However, the isopropanol cell wash extract exhibited antimicrobial activity against most of the Gram-positive bacteria, such as *Micrococcus luteus*, *Staphylococcus aureus*, *S. simulans*, *Bacillus sp*., *Enterococcus sp.*, *Lactococcus sp.*, *Streptococcus sp*., etc, but not against Gram-negative bacteria and yeasts (Figure 12). An analysis of the isopropanol cell wash extract by MALDI TOF MS showed a distinct mass signal at *m/z* 2786.5 that might correspond to the active compound.

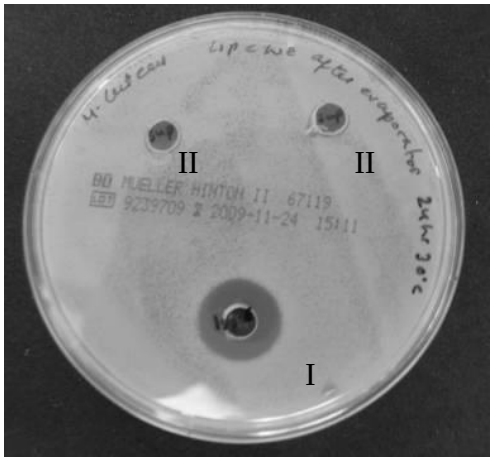


Figure 11: Agar well diffusion assay. The isopropanol cell wash extract (I) showed antimicrobial activity against *M. luteus,* but not the supernatant (II).

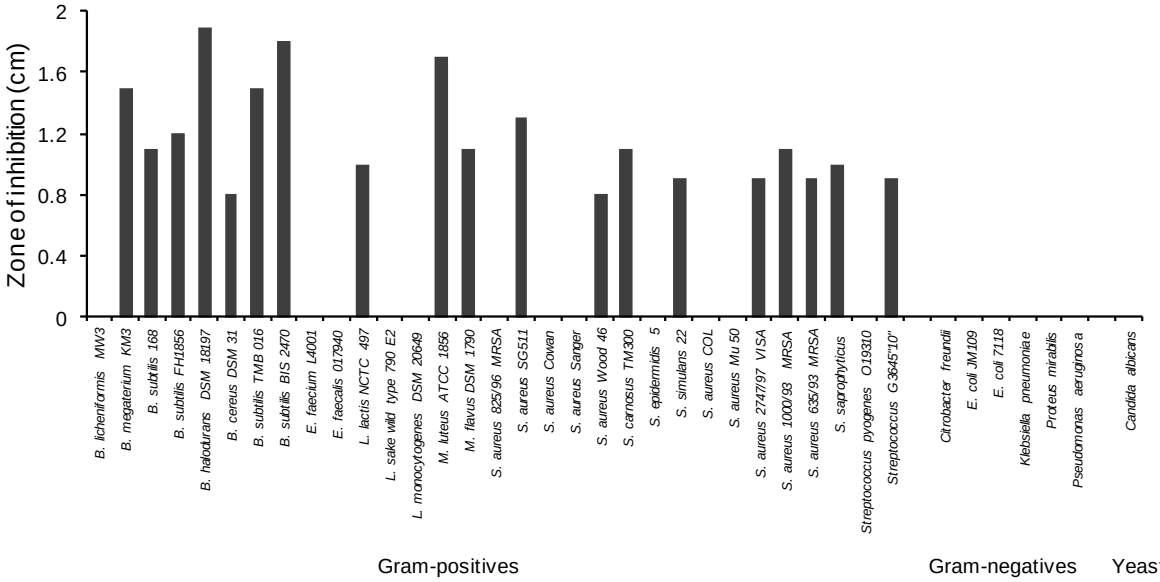


Figure 12: Antimicrobial spectrum of the isopropanol cell wash extract produced by *B. pseudomycoides* against selected bacteria (Gram-positive and Gram-negative) and the yeast *C. albicans*. Antimicrobial activity was determined by an agar well diffusion assay.

3.4. Purification of the antimicrobially active substance from *B. pseudomycoides*

The first attempts to purify the antimicrobially active substance by $(NH_4)_2SO_4$ precipitation failed. Hence, a commercially available anion exchange column, a cation exchange column and an XAD column were tested for the purification. The antimicrobial substance was loaded onto these columns and eluted with the appropriate buffer solutions. On all of three columns, the active fraction was either eluted in the flowthrough fraction or the active fraction was not

eluted from the column at all. Thus, the purification of the active compound using these columns was unsatisfactory.

HPLC purification and MALDI TOF analysis

A Poros RP-HPLC column and a C18 HPLC column were employed for further purification. 2 ml cell wash extract, containing 0.1% trifluoroacetic acid (TFA, Sigma-Aldrich, Taufkirchen, Germany) was loaded onto a Poros column and eluted in a gradient of 20% to 100% ACN (containing 0.1% TFA). The peaks were detected at an absorbance of 220 nm (Figure 13). The fractions were collected and assayed for the antimicrobial activity against *M. luteus ATCC* 4698 in an agar well diffusion assay. The fraction collected at 20 - 30% of ACN showed a zone of inhibition. The active fraction was analysed by MALDI TOF MS in the range of 1500 to 4000 Da, which showed the mass signal at *m/z* 2786.5. The fraction was further purified by rechromatography on a C18 column. The peaks were detected by an absorbance at 220 nm and 266 nm (Figure 14). Different fractions were collected and assayed for the antimicrobial activity against *M. luteus* in an agar well diffusion assay. The active fraction was analysed by MALDI TOF MS. Again, the MALDI TOF analysis yielded a mass signal at *m/z* 2786.026 for the active fraction (Figure 15). The minor signals *m/z* 1731.3 and 2818.6 (two fold dehydrated) were also observed in MALDI TOF MS. The observed mass (2786.026 Da) of the active fraction matched to the calculated mass of the unmodified corepeptide with possible 1, 4 or 8 putative dehydrations (Table 16).

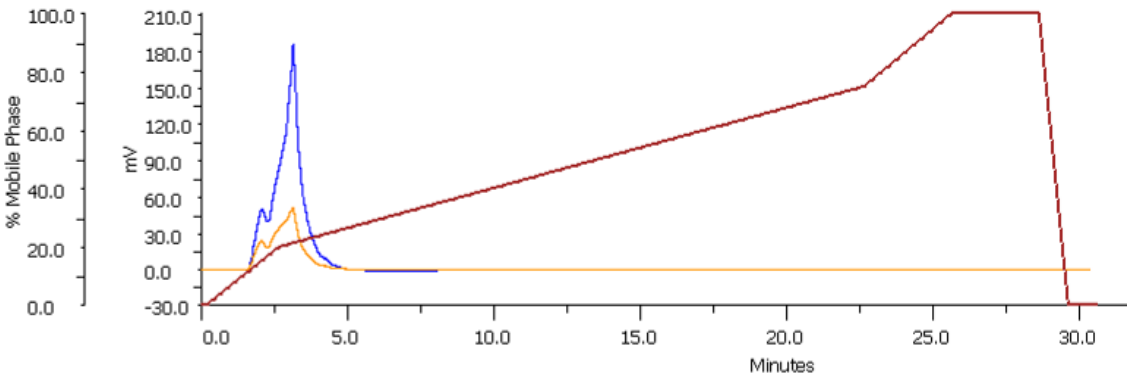


Figure 13: HPLC chromatogram of the cell wash extract from *B. pseudomycoides*. The cell wash extract was applied to a Poros RP-HPLC column and eluted in a gradient of 20% to 100% acetronitrile (containing 0.1% TFA). The active fraction was eluted after 4 min by 20 - 30% acetronitrile.

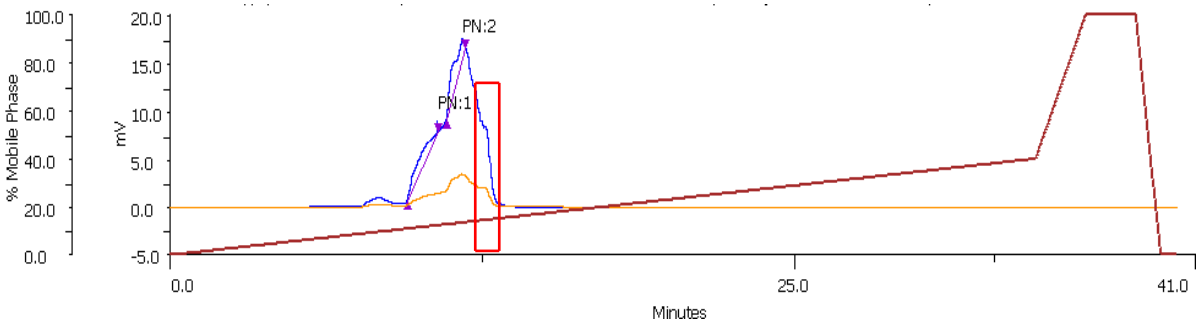


Figure 14: HPLC chromatogram of the cell wash extract from *B. pseudomycoides*. The cell wash extract was applied to a C18 RP-HPLC column and eluted in a gradient of 20% to 100% acetronitrile (containing 0.1% TFA). The active fraction was eluted by 20 - 30% acetonitrile at around 10 min as indicated by the box.

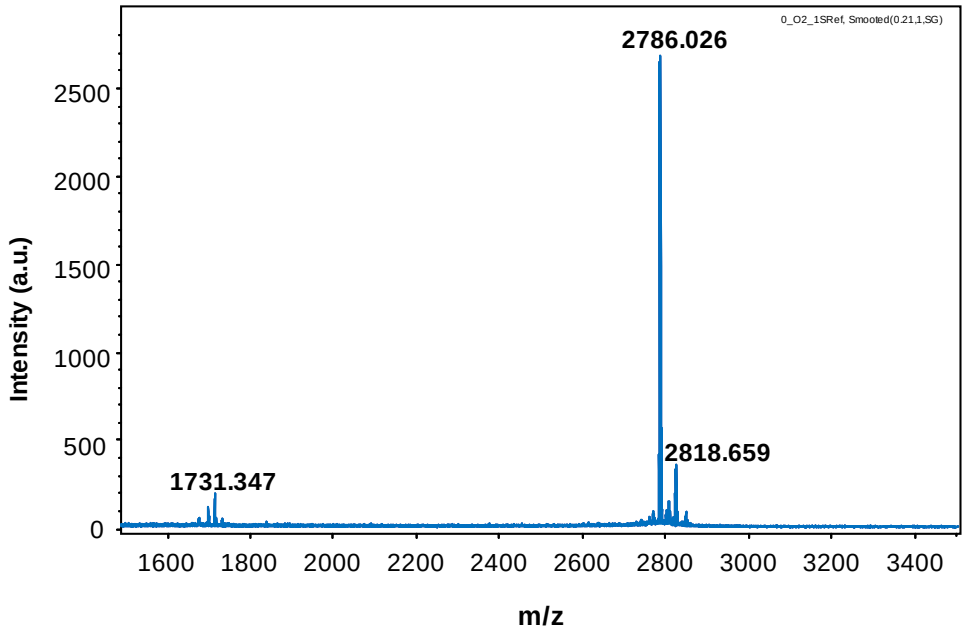


Figure 15: MALDI TOF MS of the active cell wash extract from *B. pseudomycoides* after two consecutive HPLC runs showing the distinct mass of 2786.026 Da.

3.5. Stability assay of the antimicrobial substance from *B. pseudomycoides*

For a further characterization of the antimicrobial compound, its stability against heat, pH and organic solvents, as well as resistance against proteases were determined. The antimicrobial activity of the cell wash extract was fully stable at 20 - 80 °C, even up to the observed period of 4 h. But at 100 °C, the cell wash extract was stable only up to 1 h and further incubation reduced its stability (Figure 16 A). Likewise, at low pH (3 - 5) the compound was stable even up to 4 h; however at moderate pH (6 - 8), it was stable only up to 1 h; and at very high pH (9

- 11), the activity was completely lost (Figure 16 B). Likewise, it was relatively stable against various organic solvents - ethanol, methanol, acetone, acetonitrile and isopropanol (Figure 16 C). Finally, in the protease susceptibility test, the compound was sensitive to proteinase K and pronase E indicating that the substance might be a peptide, but resistant to trypsin (data not shown).

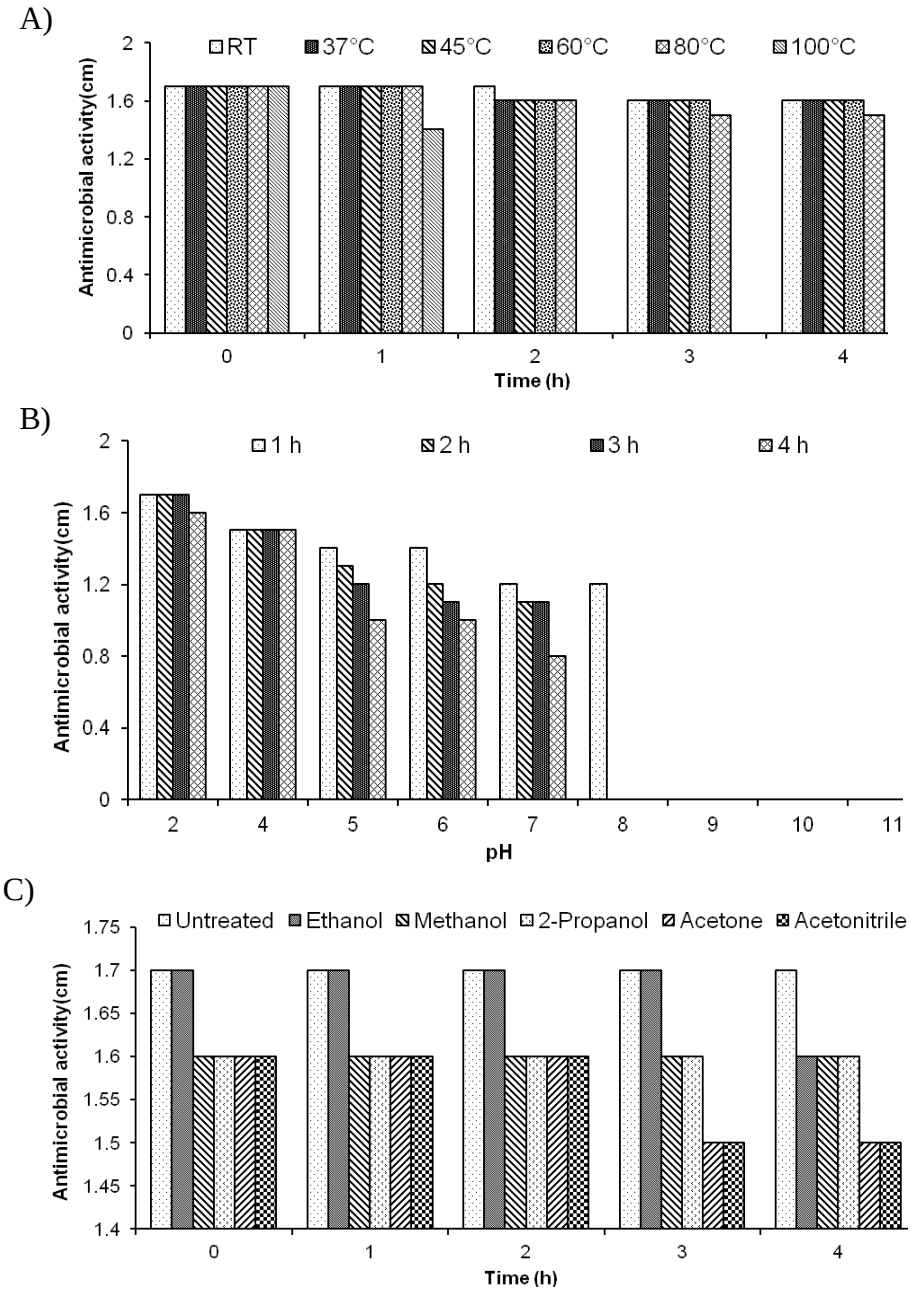


Figure 16: Stability assays: A) Effect of heat treatment on the antimicrobial activity of the cell wash extract. B) Effect of pH on the antimicrobial activity of the cell wash extract. Bars at pH 2 represent the control. C) Effect of different organic solvents on the antimicrobial activity of the cell wash extract. Antimicrobial activity was observed against *M. luteus* by an agar well diffusion assay.

In order to verify the ability of the producer strain to excrete proteases during the antibiotic production, a protease production test was performed. In the test, the producer strain produced a protease in the culture supernatant (as observed on skim milk agar plates). Although, the culture supernatant exhibited the protease activity, the activity of the cell wash extract (against *M. luteus*) was not lost even after its incubation with the culture supernatant up to 2 h (Figure 17). This indicates that the compound was resistant against the proteases excreted by the producer.

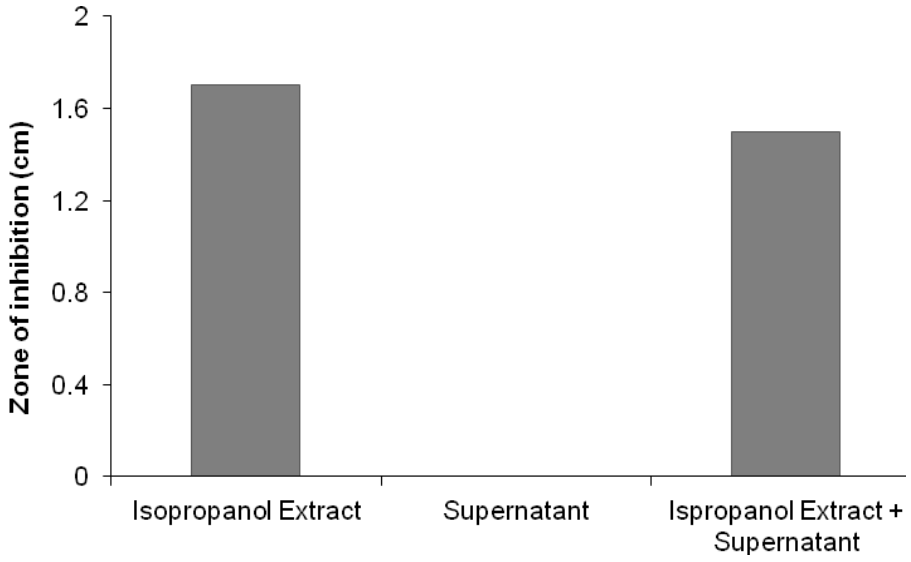


Figure 17: Antimicrobial activity of the isopropanol extract, the supernatant and the coincubation of isopropanol extract with the supernatant. The indicator strain used was *M. luteus*.

3.6. SDS-PAGE analysis of the antimicrobial substance from *B. pseudomycoides*

In an SDS-PAGE, the peptide bands could not be visualized (data not shown). But, when the polyacrylamide gel was placed onto an agar plate seeded with *M. luteus*, a zone of inhibition was observed. This observed inhibition zone might be due to the presence of the peptide in the SDS-PAGE. In addition, as inhibition zone was observed at the lower end of the gel, it could be speculated that the peptide is of low molecular weight (less than 10 kDa).

3.7. Is the active substance encoded by the lantibiotic gene cluster?

To verify the relation between the bioinformatically predicted lantibiotic gene cluster and the antimicrobial substance produced by *B. pseudomycoides*, the following experiments were conducted. Owing to its rhizoidal colony morophology, a knock out strategy for inactivation

of putative modification gene (*pseM*), and subsequent test of the mutant for its ability to produce the antimicrobial substance was difficult. Hence, as an alternate strategy, the putative structural (*pseA*) and modification (*pseM*) genes from the predicted gene cluster of *B. pseudomycoides* were heterologously expressed in *E. coli* and the clone was checked for its ability to produce an antimicrobial substance.

3.7.1. Cloning of *pseM* in pET22bΔpelb

The gene encoding the putative modification enzyme (PseM) of the lantibiotic gene cluster of *B. pseudomycoides* was introduced into the pET22bΔpelb vector to express a C-terminally His-tagged PseM. The complete sequence of *pseM* was amplified by PCR, using the PseMfor and PseMrev primer pair, and the genomic DNA of *B. pseudomycoides* DSM 12442 as a template. The resulting PCR fragment was 3223 bp long and lacked the terminal stop codon. This constructed expression vector was named pET22bΔpelb_lanM.

3.7.2. Cloning of *pseA* in pUC19 and pET28b

Using the genomic DNA isolated from *B. pseudomycoides* as a template and the PseAfor and PseArev primer pair, *pseA* was amplified by PCR. The resulting PCR fragment was subsequently digested by *BamH*I and *HindIII*, and was ligated into the corresponding restriction site of pUC19. In addition, to obtain the N-terminally His-tagged prepetide, *pseA* was cloned into the pET28b vector. The gene was amplified using the PseAforI and PseArevI primer pair. The resulting PCR fragment was 229 bp. It was subsequently digested by *NdeI* and *XhoI* and ligated into the corresponding restriction sites of pET28b. The resulting expression vector was named pET28b_lanA, and was confirmed by sequencing. Finally the constructed recombinant vectors were introduced into the expression strain *E. coli* BL21.

3.8. Introduction of factor Xa cleavage sequence into precursor peptide PseA

In order to investigate the biological activity of the peptides, the leader peptide must be removed. Thus, to facilitate the removal of leader sequence *in vitro*, an engineered factor Xa protease cleavage site was introduced into *pseA*. There were two possible cleavage sites in the PseA peptide *viz.* GA and GG. If ‘GG’ was chosen as a cleavage site, the calculated mass of the core peptide after cleavage would be much smaller than the observed mass of the active extract (i.e. 2786 Da) (Table 16). Additionally, if GG was the possible cleavage site, there would be a Cys residue within the leader sequence, which is unusual for lantibiotics. But if the GA motif was chosen for the cleavage (Figure 9 A), the calculated mass of the core peptide

would match the observed mass of the active extract with four dehydrations. In a first attempt, the GA motif was chosen to introduce the factor Xa protease cleavage site into *pseA*. The pET28b_lanA recombinant vector was used as a template to introduce the factor Xa recognition sequence by a mutagenesis approach. Immediately N-terminal to the putative GA cleavage site of the PseA peptide, the last four amino acid residues 'VVGA' were replaced with the factor Xa recognition sequence 'IEGR' (Figure 18) using the PseAXafor and PseAXarev primer pair. Finally, the N-terminal hexa-histidine lanAXa_pET28b construct was transformed into the expression strain *E. coli* BL21.

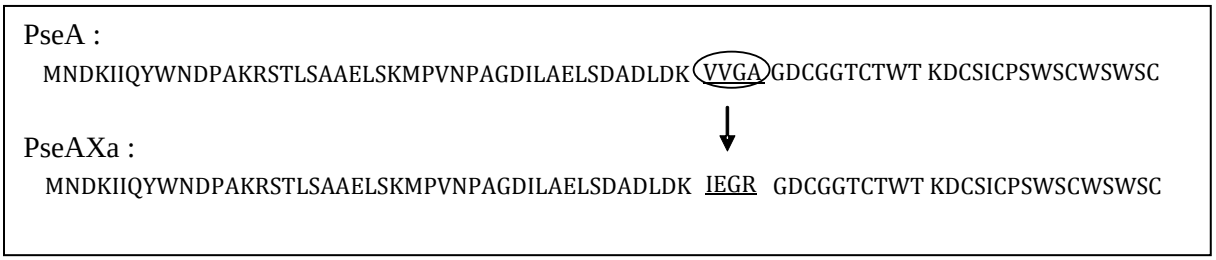


Figure 18: Introduction of the factor Xa cleavage site into PseA. The four aa 'VVGA' were replaced by the factor Xa cleavage site 'IEGR' by site directed mutagenensis and are indicated by underline.

Table 16: Calculated molecular weights of PseA peptides depending on the dehydration states of the active peptide. Masses predicted to represent the mass of the active peptides as indicated by MALDI TOF MS are highlighted in grey. The possible cleavage sites (GA / GG) in aa- sequences are shown in bold letters.

AA- Sequence	Number of putative dehydrations and corresponding molecular weight (Da)								
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
GAGDC**GG**TCTWTKDCSICPSWSCWSWSC	2987.10	2969.1	2951.1	2933.1	2915.1	2897.1	2879.1	2861.1	2843.1
AGDC**GG**TCTWTKDCSICPSWSCWSWSC	2931.2	2913.2	2895.2	2877.2	2859.2	2841.2	2823.2	2805.2	2787.2
GDC**GG**TCTWTKDCSICPSWSCWSWSC	2859.04	2841.04	2823.04	2805.04	2787.04	2768.04	2751.04	2733.04	2715.04
DC**GG**TCTWTKDCSICPSWSCWSWSC	2803.1	2785.1	2767.1	2749.1	2731.1	2713.1	2695.1	2677.1	2659.1
C**GG**TCTWTKDCSICPSWSCWSWSC	2688	2670	2652	2634	2616	2598	2580	2562	2544
GGTCTWTKDCSICPSWSCWSWSC	2584.9	2566.9	2548.9	2530.9	2512.9	2494.9	2476.9	2458.9	2440.9
GTCTWTKDCSICPSWSCWSWSC	2527.8	2509.8	2491.8	2473.8	2455.8	2437.8	2419.8	2401.8	2383.8
TCTWTKDCSICPSWSCWSWSC	2470.8	2452.8	2434.8	2416.8	2398.8	2380.8	2362.8	2344.8	2326.8
CTWTKDCSICPSWSCWSWSC	2369.7	2351.7	2333.7	2315.7	2297.7	2279.7	2261.7	2243.7	
TWTKDCSICPSWSCWSWSC	2266.5	2248.5	2230.5	2212.5	2194.5	2176.5	2158.5	2140.5	
WTKDCSICPSWSCWSWSC	2165.4	2147.4	2129.4	2111.4	2093.4	2075.4	2057.4		
TKDCSICPSWSCWSWSC	1979.2	1961.2	1943.2	1925.2	1907.2	1889.2	1871.2		
KDCSICPSWSCWSWSC	1878.1	1860.1	1842.1	1824.1	1806.1	1788.1			
DCSICPSWSCWSWSC	1749.9	1731.9	1713.9	1695.9	1677.9	1659.9			
CSICPSWSCWSWSC	1634.8	1616.8	1598.8	1580.8	1562.8	1544.8			
SICPSWSCWSWSC	1531.7	1513.7	1495.7	1477.7	1459.7	1441.7			
ICPSWSCWSWSC	1444.6	1426.6	1408.6	1390.6	1372.6				
CPSWSCWSWSC	1331.5	1313.5	1295.5	1277.5	1259.5				
PSWSCWSWSC	1228.3	1210.3	1192.3	1174.3	1156.3				
SWSCWSWSC	1131.2	1113.2	1095.2	1077.2	1059.2				
WSCWSWSC	1044.1	1026.1	1008.1	990.1					
SCWSWSC	857.9	839.9	821.9	803.9					
CWSWSC	770.8	752.8	734.8						
WSWSC	667.7	649.7	631.7						

3.9. Heterologous expression and purification of PseM

After overexpressing the pET22Bpelb_lanM recombinant vector in *E. coli* BL21, the purified His-tagged PseM was successfully obtained. The presence of PseM was verified in the SDS-PAGE. Those eluates (lanes) that had a protein band with a MW equivalent to the calculated mass of His-tagged PseM (124 kDa) were confirmed to have the PseM (Figure 19).

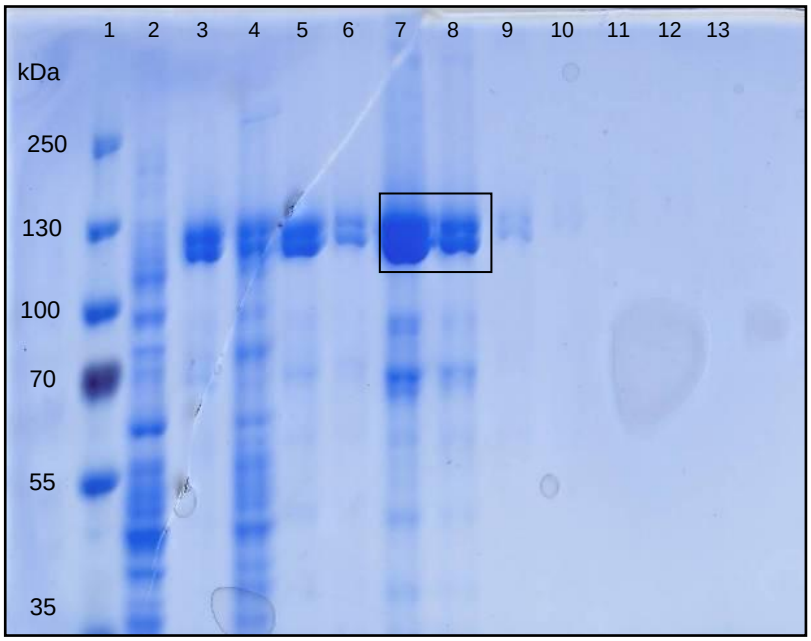


Figure 19: SDS-PAGE analysis of His-tagged PseM purified by Ni-NTA affinity chromatography. Purified His-tagged PseM protein was analysed by SDS-PAGE (15%) and stained with Page Blue protein staining solution. Lane 1 = Prestained protein marker (kDa), Lane 2 = flowthrough, Lanes 3 - 5 = wash fractions and Lanes 6 - 13= eluates. The 124 kDa PseM protein was frequently observed in the wash fractions and in the eluates 7 - 8 (indicated inside the box).

3.10. Heterologous expression and purification of the PseA and PseAXa peptides

Heterologous expression of the recombinant vectors - pET28b_lanA and pET28b_lanAXa in *E. coli* BL21 was performed, followed by Ni-NTA purification (McClerren et al., 2006). However, the His-tagged PseA and the His-tagged PseAXa could not be purified under the previously established expression and purification conditions. Additional attempts to purify PseA and PseAXa from the inclusion bodies were also not successful. Based on these results, it was assumed that the recombinant PseA and PseAXa might be toxic to *E. coli* BL21. Therefore, pET28b_lanA and pET28b_lanAXa were transformed into *E. coli* C41 and *E. coli*

C43 (which are optimized for overexpression of toxic proteins). However, overexpression of PseA and PseAXa in *E. coli* C41 or *E. coli* C43 strains was not successful either, indicating that the unmodified PseA / PseAXa might be unstable in *E. coli*.

3.11. Cotransformation and coexpression

Since PseA and PseAXa purification was not successful, a cotransformation and coexpression strategy was adopted. The chemocompetent *E. coli* BL21 and *E. coli* C43 were transformed with pET28b_lanAXa and pET22bΔpelb_lanM, or pET28b_lanA and pET22bΔpelb_lanM vectors. The transformed *E. coli* strains were grown and induced to coexpress PseA and PseM or PseAXa and PseM. Both the plasmids were stable after induction, as verified by the presence of full pET28b_lanAXa and pET22bΔpelb_lanM plasmids (Figure 20 A), or pET28b_lanA and pET22bΔpelb_lanM plasmids (data not shown) in the agarose gel electrophoresis. Growth curves of transformed *E. coli* BL21 containing PseA, PseAXa, PseA+PseM and PseAXa+PseM were compared. All the transformed bacteria grew well in the liquid media. However, the optical densities of the bacterial cultures harbouring a single expression of PseA or PseAXa had a relatively lower O.D. $_{600}$ (0.67) as compared to the coexpressed PseA+PseM or PseAXa+PseM (1.4) (Figure 20 B). This indicates that the single expression of PseA or PseAXa were toxic to the bacteria, while in the presence of PseM *i.e.* in PseA+PseM or PseAXa+PseM, the toxicity of of PseA or PseAXa was reduced.

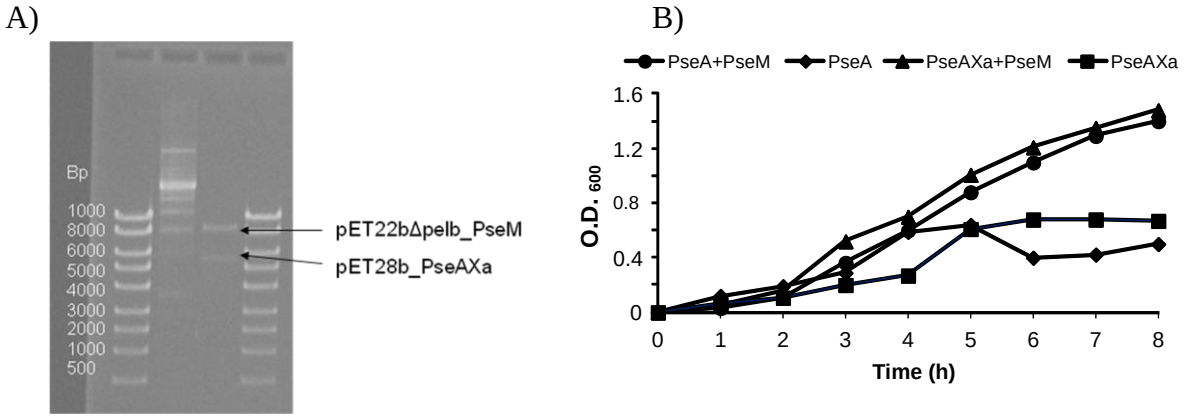


Figure 20: A) Agarose gel electrophoresis of plasmid preparations containing both the recombinant vectors pET22bΔpelb_PseM and pET28b_PseAXa as shown by the arrows. B) Growth rate of *E. coli* BL21 harbouring PseA, PseAXa, PseA+PseM and PseAXa+PseM after induction of protein expression.

3.12. Purification of modified PseA and PseAXa

The His-tagged PseA and PseAXa were purified by Ni-NTA affinity chromatography according to McClerren et al. (2006). All the eluates were analysed by SDS-PAGE. Successful production of modified PseA and PseAXa was verified by the presence of protein bands of 10.20 kDa in SDS-PAGE (Figure 21), which matched the predicted mass of the peptide calculated from its aa sequence. The additional bands (approximately 24 kDa) observed in lanes 5 and 6 might correspond to the dimers of the peptide. The eluates containing PseA or PseAXa were dialysed using a 2,000 MWCO dialysis cassette. The peptide sample buffer was exchanged with 0.05% HCl for MALDI TOF MS. The concentrations of the peptides were measured using the Bradford assay.

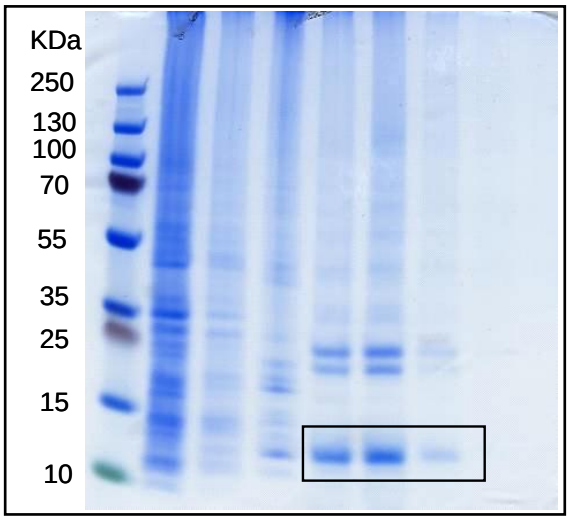


Figure 21: SDS-PAGE analysis of His-tagged PseAXa purified by Ni-NTA affinity chromatography. Purified His-tagged PseAXa protein was analysed by SDS-PAGE (20%) and stained with Page Blue protein staining solution. Lane 1 = Prestained protein marker (kDa), Lane 2 = flowthrough, Lanes 3 - 4 = wash fractions and Lanes 5 - 9 = eluates. A 10.2 kDa peptide that matched the calculated MW of PseAXa was observed in the eluates 5 - 7 as indicated inside the box.

The peptide was further purified using HPLC. The peptide was loaded on a C4 column and the active fraction was eluted with 100% ACN with 0.1% TFA after 21 - 23 min (Figure 22). All the fractions were analysed by MALDI TOF MS. The analysis showed a major mass signal at *m/z* 10203 and minor mass signals (*m/z* 10236, 10253 and 10271 which might represent the incompletely modified peptides with two-, one-fold dehydrated and unmodified peptide) (Figure 23). The observed molecular weight (10203 Da) of the peptide was 74 Da

smaller than the calculated MW of the ion of the unmodified propeptide (*m/z* 10277.4). This reduction in molecular weight of the peptide might be due to the loss of four water molecules during posttranslational modification indicating that the peptide obtained by coexpression was the modified product and that out of 8 Ser/Thr residues in putative core peptide; only 4 had been dehydrated during modification. Thus, 4 Ser/Thr escaped the dehydration step; a phenomenon that is not uncommon in lanthionine containing peptides.

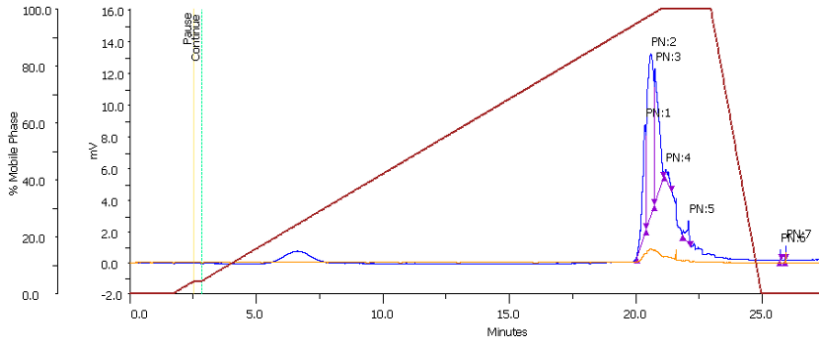


Figure 22: HPLC chromatogram of the modified PseAXa. The modified PseAXa was obtained in the fraction eluted by 100% acetronitrile with 0.1% TFA at 21 - 23 min.

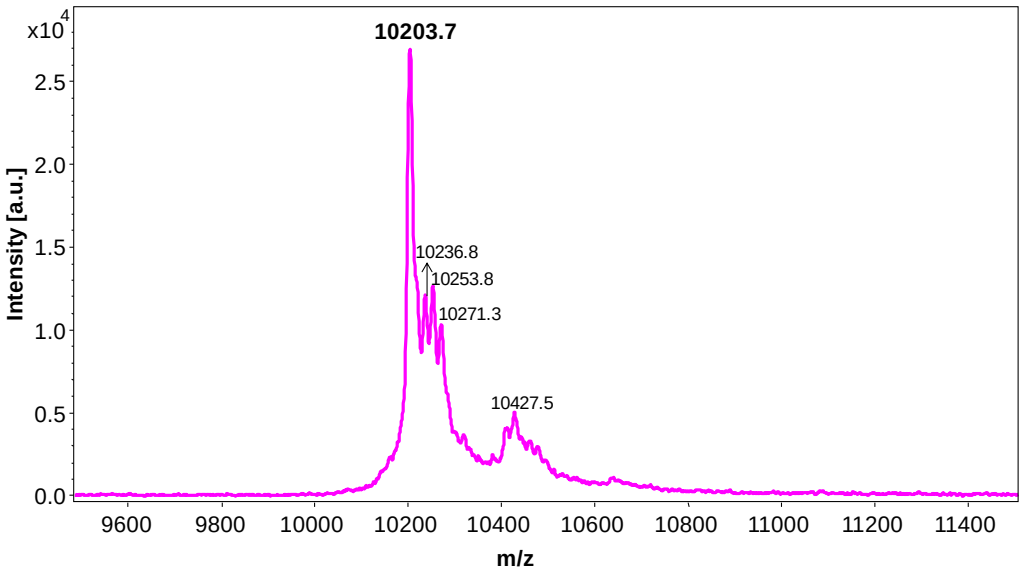


Figure 23: MALDI TOF MS of the modified PseAXa peptide (10203.7 Da) produced by coexpression of PseAXa with PseM in *E. coli*. The minor signals (*m/z* 10236, 10253 and 10271) might represent peptides with incomplete modification.

The PseAXa protein sample buffer was exchanged for buffer containing 20 mM Tris-HCl (pH 8), 100 mM NaCl and 2 mM $CaCl_2$ through dialysis prior to digestion by factor Xa. Then the peptide was incubated with factor Xa protease at 4 °C overnight. The reaction was

stopped by 0.1% TFA. An aliquot of the reaction was purified using a ZipTip$_{C18}$, and was analysed by MALDI TOF MS. The analysis revealed a mass of 2785.6 Da (Figure 24 A) that matched the calculated MW of the core peptide with four fold dehydration (Table 16, page 67) and as well as minor mass signals representing traces of the one-, two- and three fold dehydrated core peptides were observed. MALDI TOF MS also revealed the three other masses (2171.3 Da, 3526.4 Da and 5679.8 Da), which might be attributed to the fragments of the leader peptide (Figure 24 A and Table 17). The mass of the core peptide was consistent with the mass obtained from the cell wash extract of *B. pseudomycoides* with four dehydrations. An attempt to separate the core peptide from the leader peptide fragments using HPLC was not successful so far.

In addition, the modified prepeptide was incubated with trypsin for 4 h and yielded a mass signal at *m/z* 2785.7 (Figure 24 B), corresponding to the mass of the core peptide. Additionally, a mass signal was observed at *m/z* 2538.6, which corresponded to calculated mass of the N-terminal tryptic fragment (GSSHHHHHHSSGLVPRGSHMNDK) of the leader sequence.

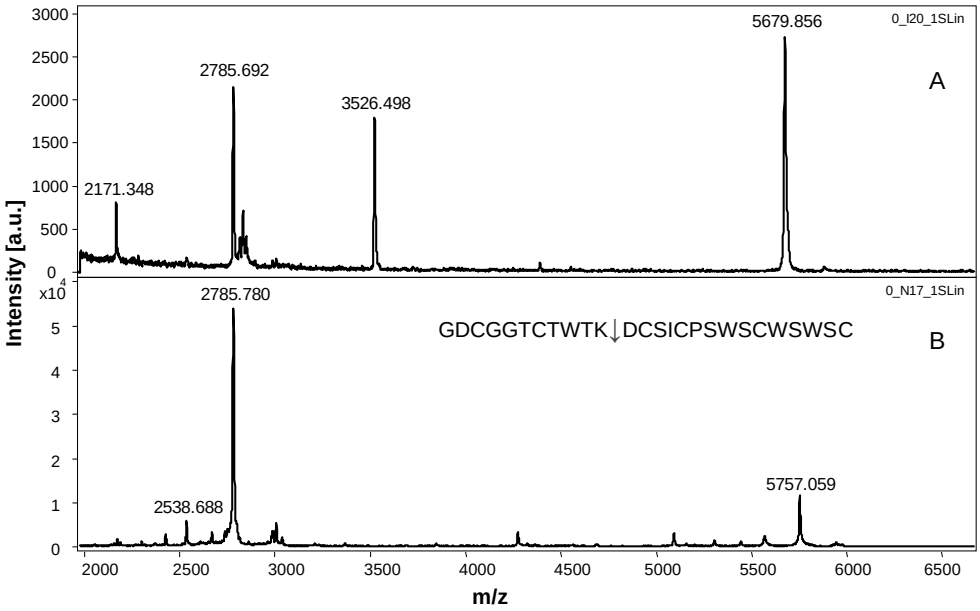


Figure 24: MALDI TOF MS of PseAXa after digestion by (A) factor Xa and (B) trypsin. Inset: Amino acid sequence of PseA showing a possible cleavage site (↓) for trypsin. The peptide was not cleaved which indicates that a ring structure might prevent activity of the enzyme.

Furthermore, the heterologously produced peptide showed antimicrobial activity after the proteolytic removal of the leader sequence from the modified lantibiotic precursor (Figure 25). This heterologously produced active peptide was named pseudomycoicidin.

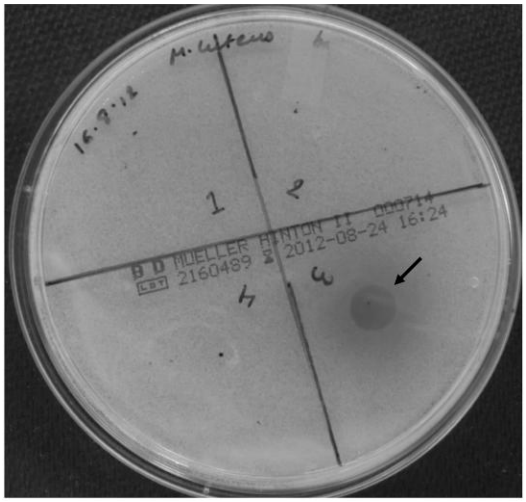


Figure 25: Detection of antimicrobial activity of the peptide after cleavage of the leader peptide by spot on the lawn method. Spot 1 = factor Xa cleavage buffer, spot 2 = peptide before removal of the leader, spot 3 = peptide after removal of the leader, inhibition zone is indicated by arrow, and spot 4 = not used.

Table 17: The calculated masses of peptide fragments of PseAXa that could be generated (with predicted molecular weight before and after dehydration), when digested by factor Xa (after every Arg residue). Observed masses are indicated in bold (core peptide) or italics (leader sequences).

PseAXa fragments	**Molecular weight (Da) with possible dehydrations**				
	-0 H_2O	**-1 H_2O**	**-2 H_2O**	**-3 H_2O**	**-4 H_2O**
GSSHHHHHHSSGLVPRGSHMNDKIIQYWNDPAKR STLSAAELSKMPVNPAGDILAELSDADLDKIEGRG DCGGTCTWTKDCSICPSWSCWSWSC	10276.4	10258.4	10240.4	10222.4	10204.4
GSSHHHHHHSSGLVPR	1768.8				
GSHMNDKIIQYWNDPAKRSTLSAAELSKMPVNPA GDILAELSDADLDKIEGRGDCGGTCTWTKDCSICP SWSCWSWSC	8525.5	8507.5	8489.5	8471.5	8453.5
GSSHHHHHHSSGLVPRGSHMNDKIIQYWNDPAKR	3924.2				
GSSHHHHHHSSGLVPRGSHMNDKIIQYWNDPAKR STLSAAELSKMPVNPAGDILAELSDADLDKIEGR	7430.2				
GDCGGTCTWTKDCSICPSWSCWSWSC	2859.04	2841.04	2823.04	2805.04	**2787.04**
STLSAAELSKMPVNPAGDILAELSDADLDKIEGRG DCGGTCTWTKDCSICPSWSCWSWSC	6370.1	6352.1	6334.1	6316.1	6298.1
STLSAAELSKMPVNPAGDILAELSDADLDKIEGR	*3526.79*				
GSHMNDKIIQYWNDPAKRSTLSAAELSKMPVNPA GDILAELSDADLDKIEGR	*5683.3*				
GSHMNDKIIQYWNDPAKR	*2173.4*				

3.13. Determination of free Cys and free Dha residues in the peptide

To determine the number of lanthionine rings in the peptide, the following experiments were performed. When a peptide contains a free Cys residue, IAA reacts with the Cys residue leading to an increase in its mass by 57 Da. This increase in the peptide mass ultimately proves the absence of a lanthionine ring (McClerren et al., 2006). In this study, the presence of free Cys was determined by alkylation with IAA together with and without the reducing agent TCEP. When the PseAXa peptide was treated with IAA in the presence of TCEP, its mass increase indicated the presence of two IAA adducts (approx. 114 Da). This increase in mass corresponded to the alkylation of two Cys in the core peptide (Figure 26). In contrast, in absence of TCEP, the IAA assay did not result in any increase in the mass of the peptide (Figure 26). This indicates that the two SH-groups might be tied up by a disulfide linkage. On the other hand, the other four Cys residues might be involved in the Lan or the MeLan ring formation. When the peptide was treated with TCEP, there was an increase in the mass of the peptide by 2 Da (10205 Da) (Figure 26). This increase in the peptide mass also indicated the presence of a disulfide bond in the peptide.

Likewise, addition of βME will result in an increase of 79 Da if a free Dha residue is present. However, when the peptide was incubated with βME, a βME adduct was not detected (Figure 26). This indicates the absence of free Dha residues in the PseAXa peptide. However, the treatment with βME led to an addition of 2 Da in the mass of the peptide. Altogether, above results suggest the presence of four lanthionine rings and one disulfide linkage in the PseA peptide. Thus, the heterologously produced peptide was confirmed to be a modified lantibiotic.

In addition to the lanthionine rings, the disulfide bond might also be essential for the activity of the peptide. To evaluate the importance of the disulfide bond in the peptide, the PseA peptide was incubated with TCEP. The breaking of disulfide link following TCEP treatment abolished the antibacterial activity of the peptide compared to control peptide without TCEP (Figure 27). This indicates that the disulfide bond is essential for the activity of the peptide.

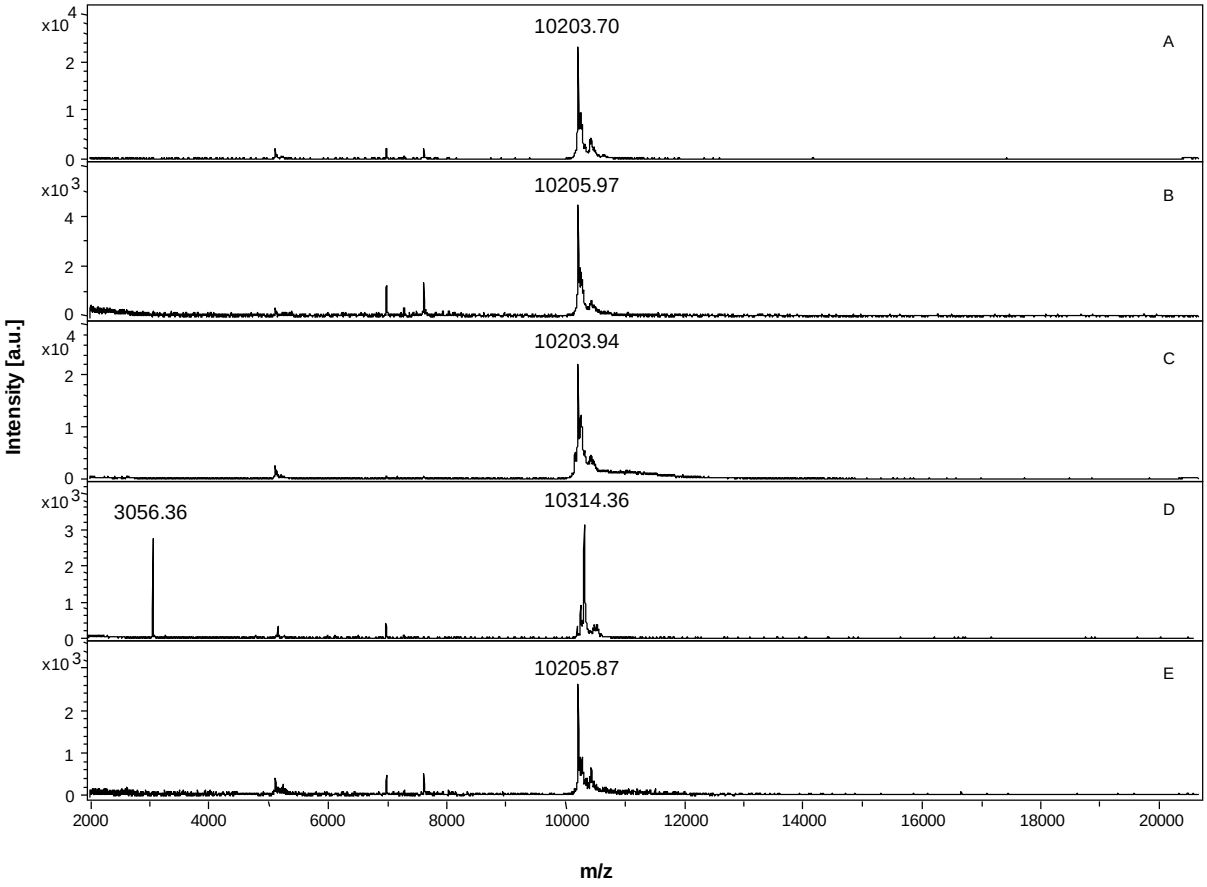


Figure 26: MALDI TOF MS of (A) heterologously produced peptide (B) peptide after TCEP treatment (C) peptide after incubation with IAA in absence of TCEP (D) peptide after IAA treatment in presence of TCEP and (E) peptide after incubation with βME.

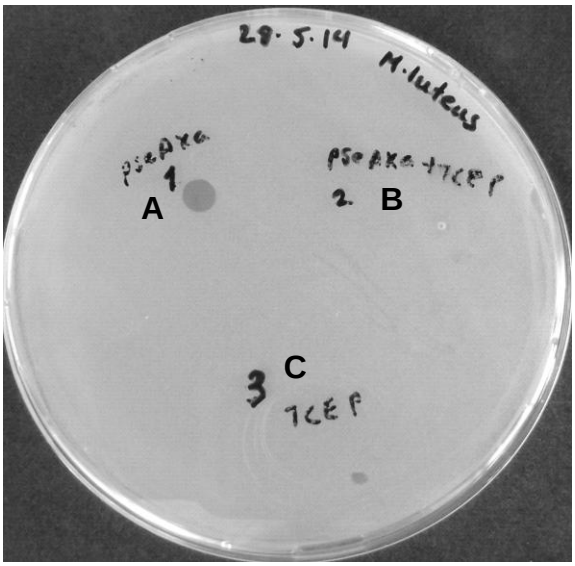


Figure 27: Activity assay of (A) PseAXa (B) PseAXa peptide treated with TCEP and (C) TCEP control, against *M. luteus* using the spot on the lawn method.

3.14. Determination of the ring topology of the modified PseA

To obtain the preliminary informations on the ring topology of the modified PseA, six PseAXa analogs were created. Those analogs were created by replacing single Cys by Ala residues as shown in Figure 28. Each PseAXa mutant was coexpressed along with PseM in *E. coli* C41 and was purified. The purified mutant peptides were named PseAXaC52A, PseAXaC56A, PseAxaC62A, PseAxaC65A, PseAXaC70A and PseAXaC75A.

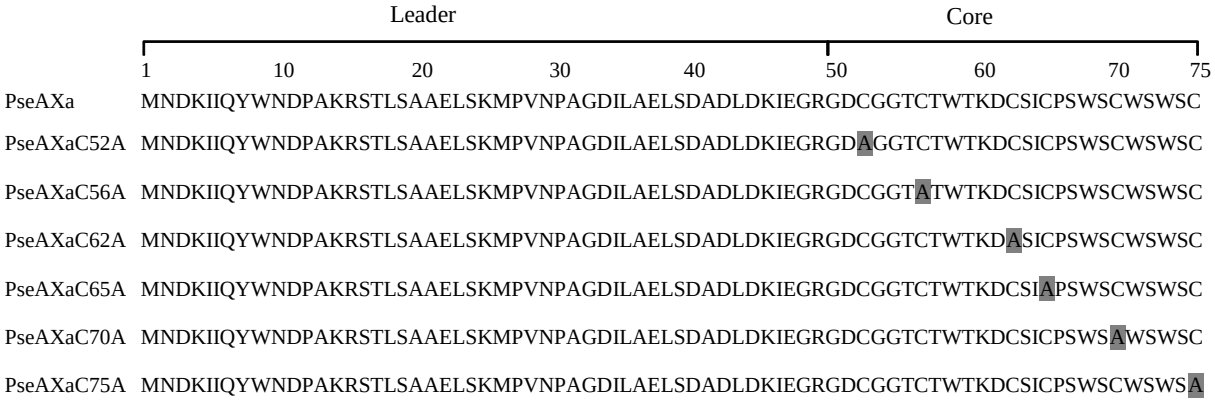


Figure 28: The aa sequences of PseAXa and its mutant peptides. The mutant peptides were constructed by replacing single Cys with Ala residue in the core peptide region of PseAXa. The replaced aa are highlighted.

Mutant peptides were successfully purified by Ni-NTA chromatography as verified by the presence of a protein band of approximately 10.2 kDa in SDS-PAGE for each mutant (data not shown). After dialysis they were analysed by MALDI TOF MS. The analysis of the mutated peptides showed a mixture of prepeptides with different dehydration stages (Figure 29).

MALDI TOF MS of PseAXaC52A showed a main peak (10227 Da), which seemed to correspond to the one fold dehydrated mass (Figure 29 A). After incubation with TCEP, the fully modified peptide was observed as the main peak (10173 Da) (Figure 29 a, Annex XXII). The IAA assay showed that after incubation with TCEP, one IAA adduct was coupled to the peptide, indicating that PseAXaC52A had one free Cys residue (Annexes XX and XXI). When PseAXaC52A was incubated with trypsin, a weak signal with the mass of the core peptide (2755 Da) was retained. This indicates the presence of the thioether ring(s) that protected the PseAXaC52A from trypsin digestion at least in a small fraction. In addition, there were other mass signals observed for N-terminal leader fragment (*m/z* 2540) and fragments generated by trypsin self digest (*m/z* 1676, 2195).

MALDI TOF of PseAXaC56A showed a main peak at 10225 Da that again resembled to a one fold dehydrated mass (Figure 29 B). After incubation of PseAXaC56A with TCEP, the main peak (10172 Da) indicating the four fold dehydrated stage was observed (Figure 29 b). The IAA and βME assays predicted the presence of one free Cys residue and one free Dha/Dhb residue in PseXaC56A (Annexes XX, XXI and XXII). The mass of the core peptide (2756 Da) was retained after incubation with trypsin, indicating the presence of at least one thioether ring that protected the peptide from trypsin digestion (Figure 30 c). Additionally, mass signals for leader fragments (*m/z* 2178, 2398) and trypsin fragments (*m/z* 1112.5, 1434.7, 3154.1) were observed in MALDI TOF MS of the digest.

MALDI TOF MS of PseAXaC62A showed one main peak at 10225.6 Da that represented the one fold dehydrated mass (Figure 29 C). When the peptide was incubated with TCEP, a main peak with a mass at 10228 Da appeared that again corresponded to a one fold dehydrated state (Figure 29 c, Annex XXIII). When PseAXaC62A was incubated with trypsin, it was completely digested by trypsin as indicated by the absence of the core peptide signal in MALDI TOF MS (Figure 30 d). However, a mass signal for N-terminal leader fragment (*m/z* 1768) was observed. These imply that all the thioether rings spanning the trypsin cleavage site were not closed in PseAXaC62A.

MALDI TOF MS of PseAXaC65A showed, a major peak at 10206.1 Da that matched to the two fold dehydrated mass (Figure 29 D). When incubated with TCEP, the peptide displayed one main peak at 10207 Da that fitted to the two fold dehydrated state (Figure 29 d, Annex XXIII). PseAXaC62A was completely digested by trypsin as the core peptide signal did not appear in MALDI TOF MS (Figure 30 e). However, a mass signal for N-terminal leader fragment (*m/z* 1768) was observed. These findings indicate that not all the thioether rings were closed in PseAXaC65A.

MALDI TOF MS of PseAXaC70A showed a major signal at *m/z* 10190.2 and a minor signal at *m/z* 10412 Da (Figure 29 E). The major signal corresponded to three fold dehydrated mass. After TCEP addition, this peptide showed one main peak with mass of 10190 Da that resembled a three fold dehydrated stage. The other peak with the observed mass of 10494 Da (10190 + 304 Da) was indicative of a glutathione adduct (GSH) (Figure 29 e, Annex XXIII). The IAA and βME assays predicted the presence of two free Cys residues and absence of Dha/Dhb residues in PseAXaC70A (Annex XX, XXI and XXII). The mass of the core peptide (2773 Da) was retained even after incubation with trypsin (Figure 30 f). In addition, there was a mass signal for N-terminal fragment of the leader (*m/z* 2539.9) in MALDI TOF MS. These

results indicated that the PseAXaC70A might have three thioether rings and one disulfide link.

MALDI TOF MS of PseAXaC75A showed one major peak of 10224.4 Da, that resembled to its one fold dehydrated state and the minor peaks at *m/z* 10174, 10190, 10207 and 10241, that corresponded to four, three, two fold dehydrated states and unmodified state respectively (Figure 29 F). After incubation with TCEP, one major peak with a mass of 10174 Da (4 fold dehydrated state) was observed. Additionally, another peak with a mass of 306 Da larger than the main peak; a representative of the glutathione adduct (expected increase mass =305.1Da), was observed. The IAA and βME assays of PseAXaC75A demonstrated the presence of one free Cys residue in it (Annex XXIII). The mass signal of PseAXaC75A core peptide was absent in MALDI TOF MS of the trypsin digest, explaining that the protease had cut the peptide. In addtion, several other mass signals of the leader fragment (*m/z* 1767) and the other fragments yielded by trypsin self digest (*m/z* 1432.5, 2161.4, 3151.1) were observed (Figure 30 g). Thus, these results suggest that the thioether rings that stabilize the cleavage site were not closed in this peptide and a disulfide bond was not formed as well.

All the PseAXa mutants were inactive against *M. luteus* even after the removal of the leader by factor Xa (data not shown). The MALDI TOF MS of all the mutants after factor Xa digestion showed a mixture of different peaks, indicating the different dehydration states of the core peptide as well as the presence of adducts with some peptides (Figure 31).

The core peptide of PseA has a few similarities to the known lantibiotics (see Figure 37). Hence, the lanthionine bridging pattern can only be predicted by homology (see discussion) but clear cut experimental evidence will have to be obtained by further studies. This is due to the fact that all mutant peptides were not fully modified; rather they were mixture of peptides having different dehydration states. Tandem MS-MS resulted in an unsatisfactory fragmentation of PseAXa and its mutant analogs and only little results were obtained (Annex XVIII).

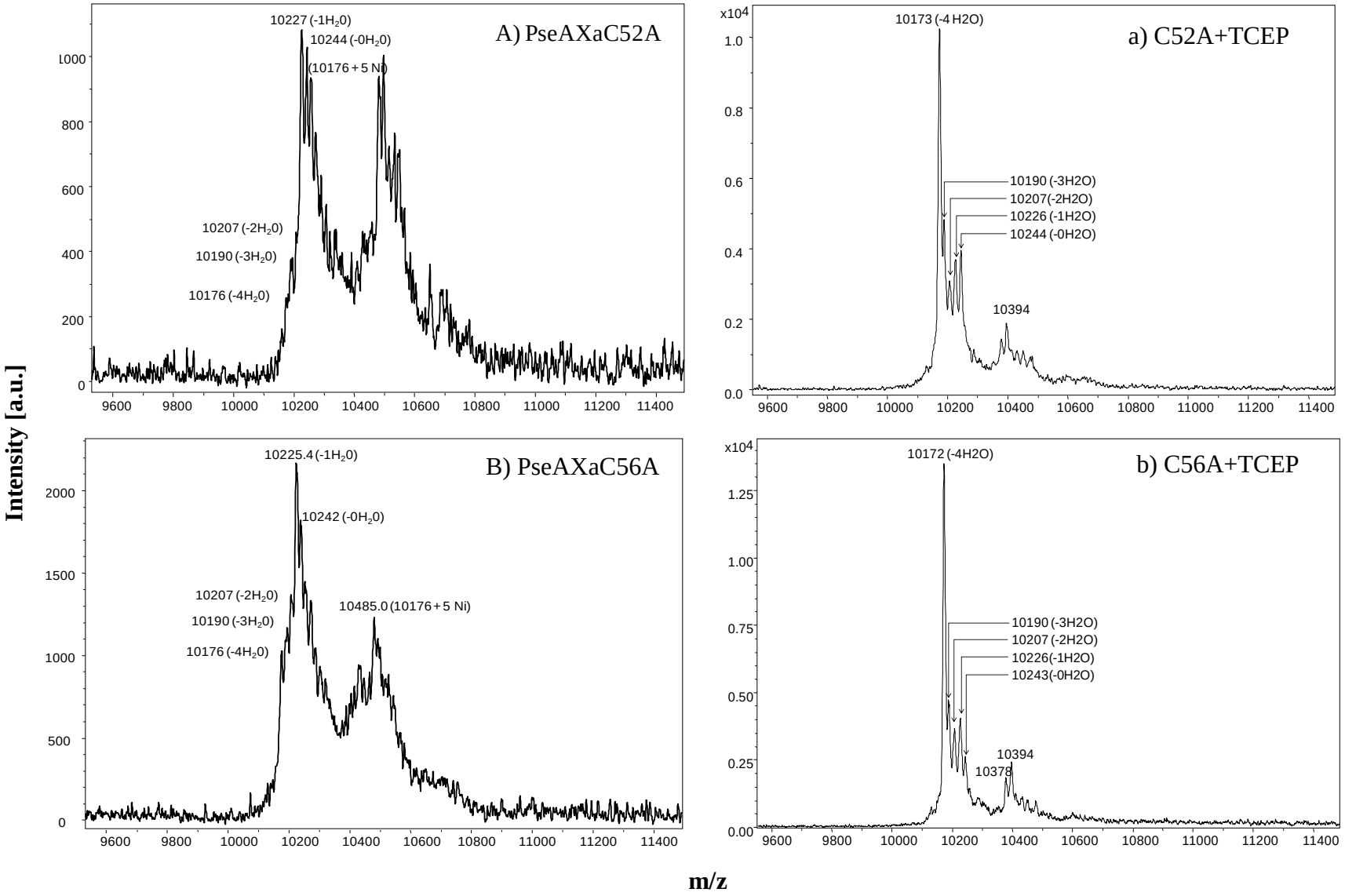
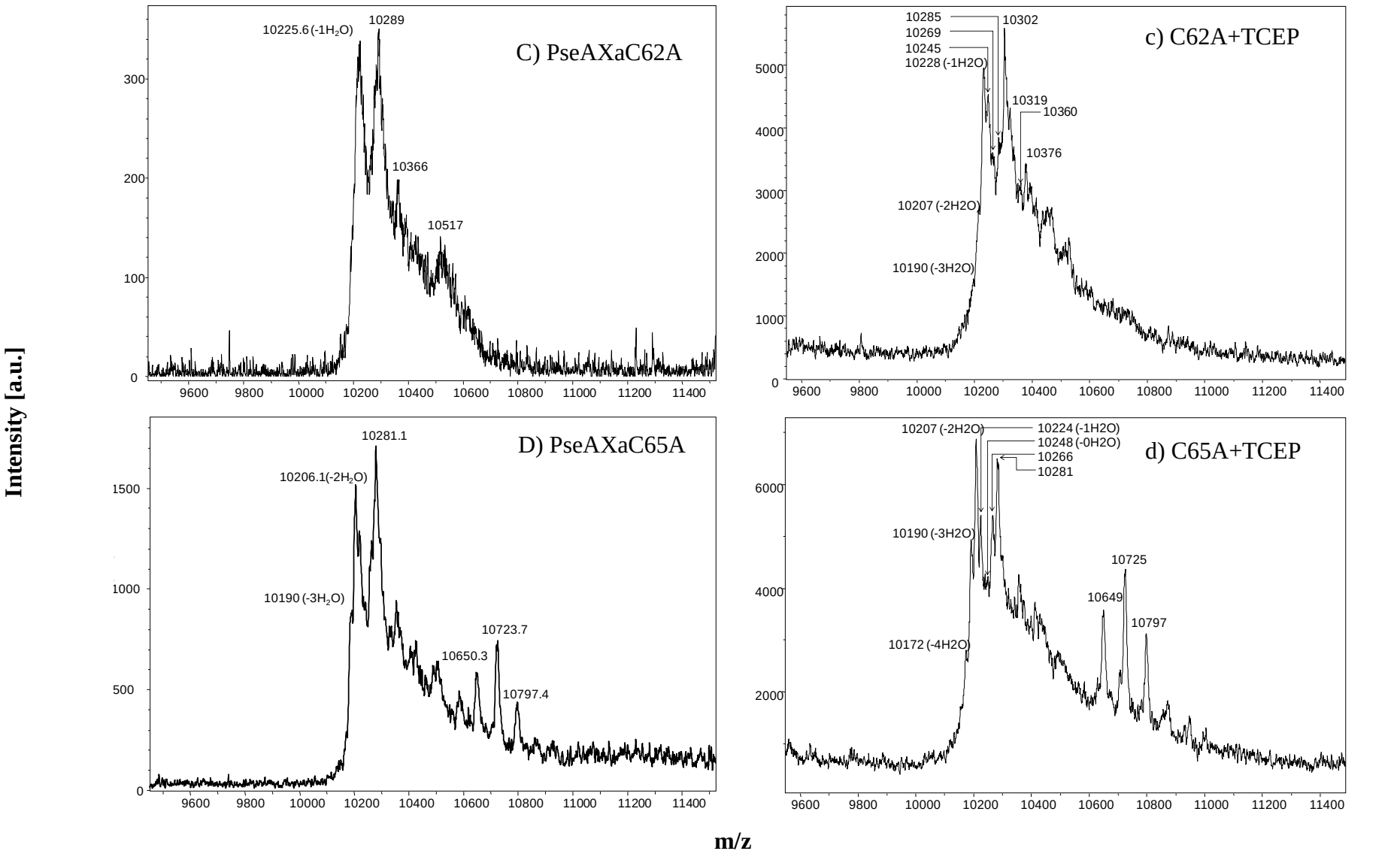
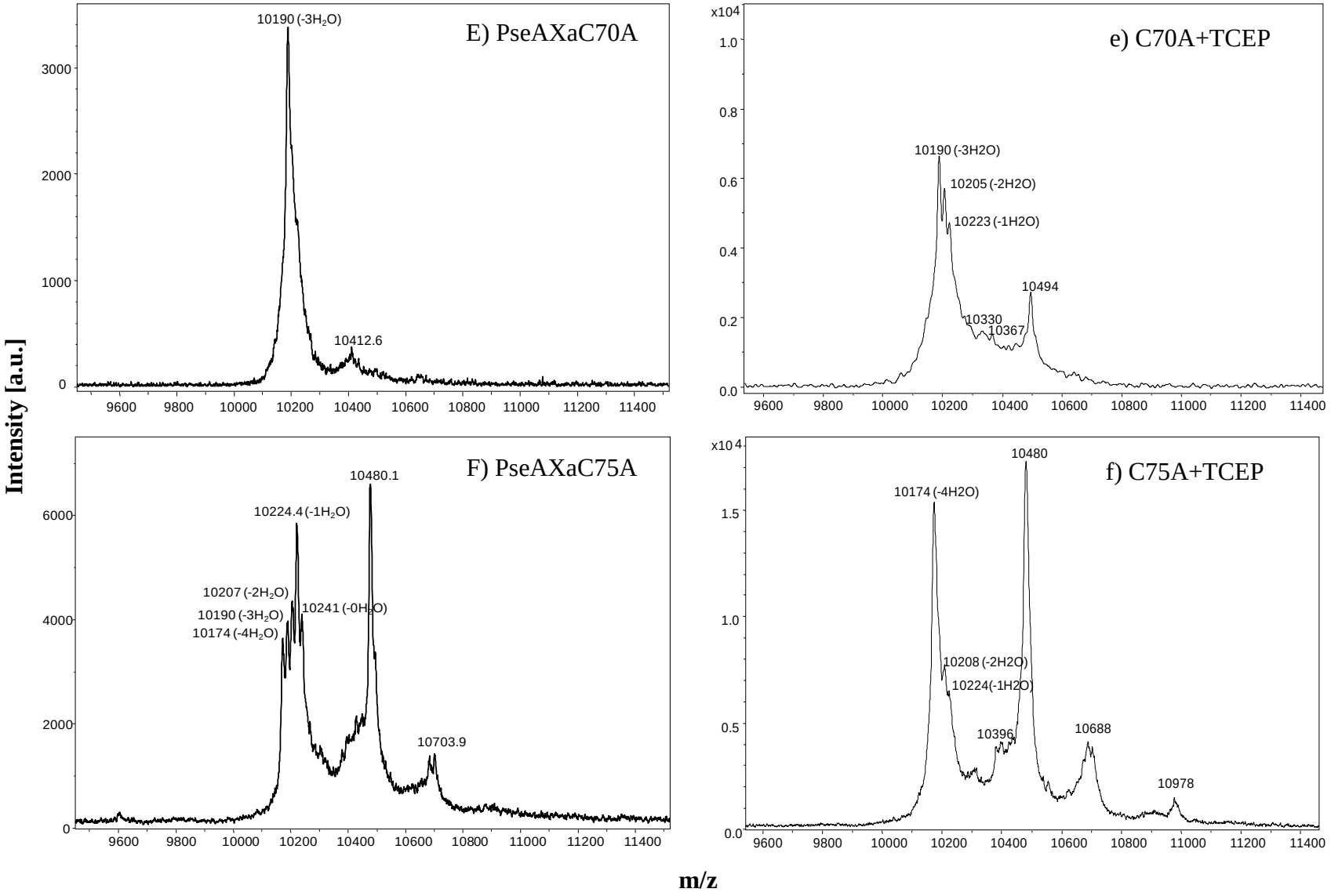


Figure 29: MALDI TOF MS of PseAXa mutant peptides (A-F) with its respective TCEP treated peptides (a-f).

Contd...


C) PseAXaC62A
10225.6 (-1H2O)
10289
10366
10517
c) C62A+TCEP
10285
10269
10245
10228 (-1H2O)
10302
10319
10360
10376
10207 (-2H2O)
10190 (-3H2O)
D) PseAXaC65A
10281.1
10206.1(-2H2O)
10190 (-3H2O)
10723.7
10650.3
10797.4
d) C65A+TCEP
10207 (-2H2O)
10224 (-1H2O)
10248 (-0H2O)
10266
10281
10190 (-3H2O)
10725
10649
10797
10172 (-4H2O)
Intensity [a.u.]
m/z

Contd...


E) PseAXaC70A
10190 (-3H2O)
10412.6
e) C70A+TCEP
10190 (-3H2O)
10205 (-2H2O)
10223 (-1H2O)
10494
10330
10367
F) PseAXaC75A
10480.1
10224.4 (-1H2O)
10207 (-2H2O)
10190 (-3H2O)
10174 (-4H2O)
10241 (-0H2O)
10703.9
f) C75A+TCEP
10480
10174 (-4H2O)
10208 (-2H2O)
10224(-1H2O)
10396
10688
10978
Intensity [a.u.]
m/z

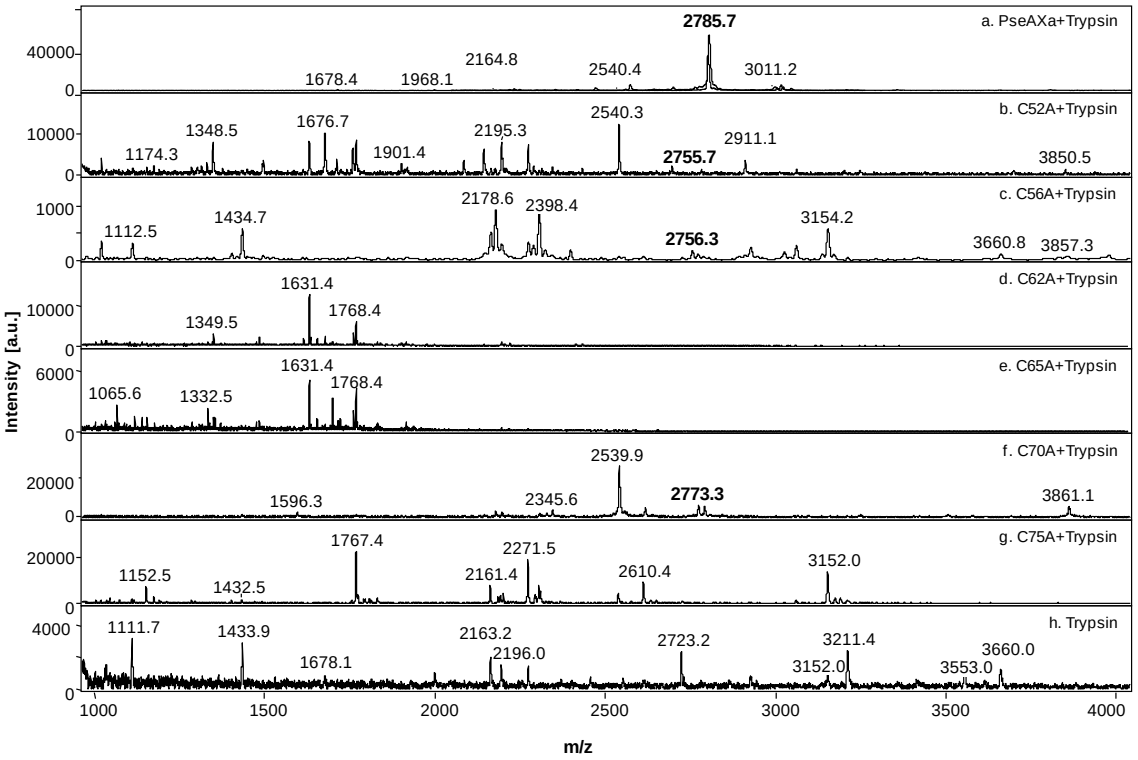


Figure 30: MALDI TOF MS of PseAXa peptide (a) and its mutants (b-g) after treatment with trypsin. The mass of the core peptide is represented in bold letters.

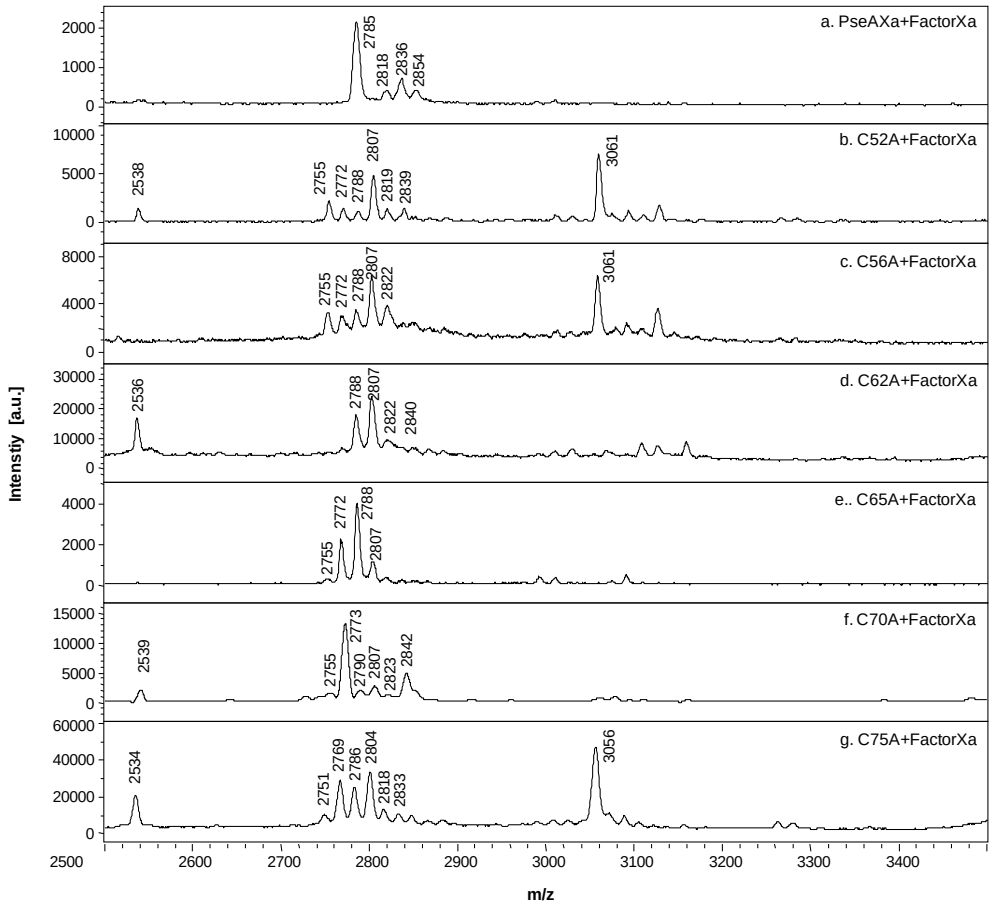


Figure 31: MALDI TOF MS of PseAXa (a) and its mutants (b-g) after cleavage by factor Xa.

3.15. Mode of action

3.15.1. Growth of microorganisms in the presence of the antimicrobial substance

In order to elucidate the effect of the antimicrobial substance from *B. pseudomycoides* on the growth of microorganisms, the following experiment was conducted. *M. luteus* and *S. aureus* SG511 were grown separately in MH broth. After 5 h (exponential phase) of incubation, the antimicrobial substance that had been partially purified from cell pellet was added to the cultures. This addition prevented further growth and reduced their optical density, indicating a lytic effect of the antimicrobial substance (Figure 32).

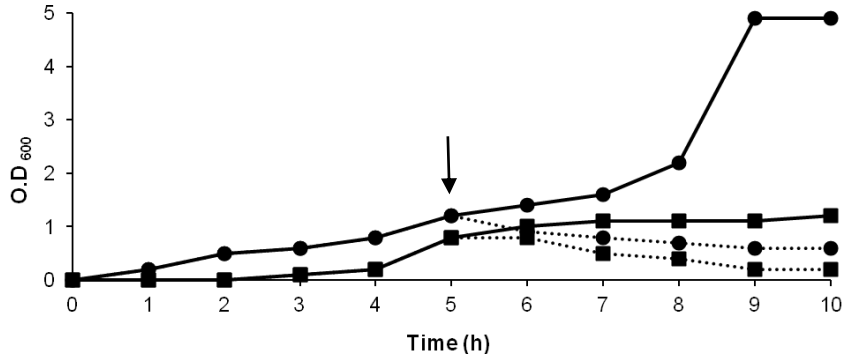


Figure 32: Effect of the antimicrobial substance on the growing cells of *M. luteus* (■) and *S. aureus* (●) in MH broth. The bacterial batches treated with the antimicrobial substance after 5 h are indicated by a dashed line (⋯) while the untreated controls are indicated by a solid line (—).

3.15.2. Minimum inhibition concentration (MIC)

To determine the MIC of pseudomycoicidin, its inhibitory effect against the indicator strain *M. luteus* was tested. The concentration of the peptide was estimated by Bradford assay. Here, the heterologously produced pseudomycoicidin showed an MIC of 3 µM in the presence of Ca^{2+} (Figure 33).

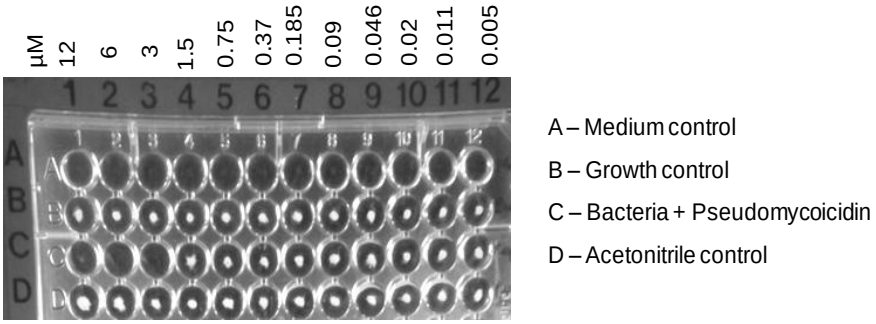


Figure 33: Determination of the MIC of pseudomycoicidin against *M. luteus* in a microtiter plate. The peptide is pipetted in 2-fold dilution from left to right (row C1-C12, 12µM to 0.005µM).

3.15.3. Reporter gene assay

In order to investigate the binding ability of the antimicrobial substance purified from cell wash extract of *B. pseudomycoides* and heterologously produced pseudomycoicidin with lipid II, the following experiments were conducted. Both preparations were screened for their ability to induce the expression of LacZ promoter fusions of the detoxification systems LiaRS (TMB016), Psd RSA-AB (TMB299) and Yxdjk-LM (TMB588), in three different reporter strains *viz. B. subtilis* TMB016, *B. subtilis* TMB 299 and *B. subtilis* TMB 588. In this system, LacZ is expressed in response to the antibiotics that stimulate sensing and detoxification systems in *Bacillus* strains (Burkhard and Stein, 2008). PsdRSA-AB system is induced by lantibiotics like nisin and actagardine, whereas, LiaRS system is induced by cinnamycin. The substances were pipetted on a TSA plate containing X-gal seeded with the three different reporter strains. Although, all three *Bacillus* strains were affected by the antimicrobial substance purified from cell wash extract and the heterologously produced pseudomycoicidin, the absence of blue rings around the zones of inhibition of both preparations (Figure 34) indicated that both, the antimicrobial substance and the pseudomycoicidin, did not stimulate the detoxification systems of *B. subtilis*.

A) B)

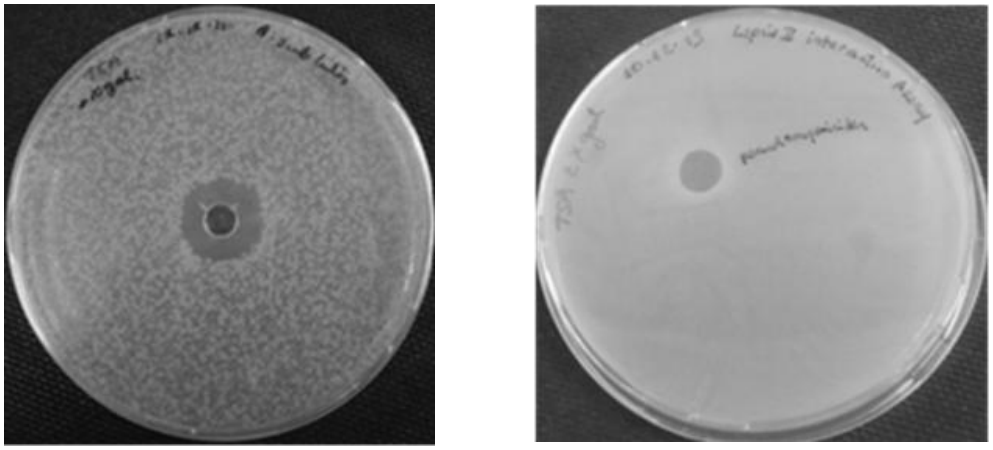


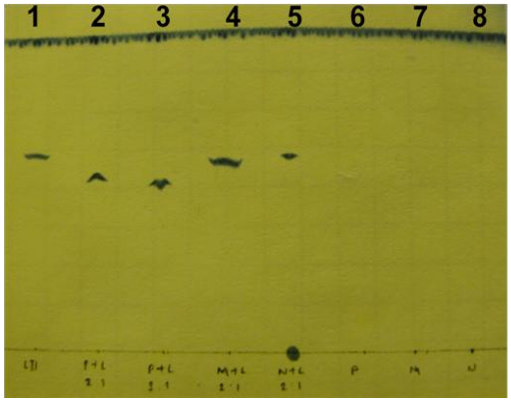
Figure 34: Reporter gene assay: A) a partially purified antimicrobial substance from cell wash extract of *B. pseudomycoides* B) the heterologously produced pseudomycoicidin. Agar well diffusion method was used with *B. subtilis* TMB 299 seeded TSA plate containing X-gal.

3.15.4. Lipid II interaction assay

Additionally, a possible interaction of lipid II with pseudomycoicidin was also determined by TLC. Lipid II was incubated with pseudomycoicidin in estimated ratios of 1:1, 1:2 and 1:3 in the presence of Ca^{2+}. Nisin and mersacidin were used as controls. The plates were stained

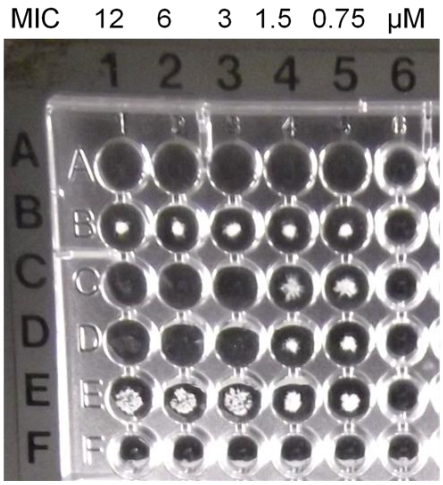
with phosphomolybdic acid stain. Nisin formed a complex at the origin with the lipid II. In contrast, both mersacidin and pseudomycoicidin did not retain lipid II at the origin. However, both peptides - mersacidin and pseudomycoicidin delayed the migration of lipid II compared to the lipid II control (Figure 35 A). Furthermore, the antibacterial activity of pseudomycoicidin seemed to be inhibited when lipid II was incubated with pseudomycoicidin (data not shown). However, MIC of pseudomycoicidin against *M. luteus* was not affected by the addition of lipid II in a molar ratio of 1:1 (data not shown) and 1:2 (Figure 35 B) in the presence of Ca^{2+}.

A)



1- Lipid II
2- Pseudomycoicidin + Lipid II (2:1)
3- Pseudomycoicidin + Lipid II (3:1)
4- Mersacidin + Lipid II (2:1)
5- Nisin + Lipid II (2:1)
6- Pseudomycoicidin
7- Mersacidin
8- Nisin

B)



A – Medium control
B – Growth control
C – *M. luteus* + Peptide
D – *M. luteus* + Peptide+Lipid II (1:2)
E – Acetonitrile control

Figure 35: Lipid II interaction assay: A) TLC of reaction mixture of lipid II incubated with different peptides. B) MIC of the heterologously produced pseudomycoicidin in the presence of lipid II against *M. luteus*.

4. Discussion

The identification and production of wide variety of antibiotics have revolutionized medical approaches. Unfortunately, the intensive use and misuse of clinical antibiotics have led to the emergence of resistant strains. For example, *Staphylococcus aureus* strains have been found to be resistant to methicillin; even vancomycin resistant enterococci (VRE) have now become quite common (Novak et al., 1999; Weigel et al., 2003). The constant increase in multi drug resistant microorganisms necessitates the need to investigate and develop new antibacterial compounds. Lantibiotics are considered as promising candidates for the future antibiotics. They are highly effective against diverse pathogenic bacteria (Fickers et al., 2012).

The genus *Bacillus* (*B. subtilis, B. polymyxa, B. brevis, B. licheniformis, B. circulans* and *B. cereus*) is well known as a producer of polypeptide antibiotics such as bacitracin, gramicidin S, polymyxin, tyrocidine, etc (Mittenhuber et al., 1989; Morikawa et al., 1992; Perez et al., 1992; Drablos et al., 1999).

The possibility that *B. pseudomycoides* DSM 12442 might also produce a lantibiotic was recognized during the database search for new lantibiotic gene clusters in different bacteria, when genomic data mining revealed a putative class II lantibiotic gene cluster.

Putative lantibiotic gene cluster of *B. pseudomycoides*

In silico analysis of *B. pseudomycoides* showed the presence of gene cluster that might encode a class II type single peptide lantibiotic. The gene cluster consists of one structural gene (*pseA*), a single modification gene (*pseM*), a transpoter gene (*pseT*) and the immunity genes (*pseEFG*). However, the gene cluster lacks the regulatory genes. The typical two component regulatory system (*lanR, lanK*) is present in the gene cluster of nisin (Kuipers et al., 1993) and class II lantibiotics like mersacidin (Altena et al., 2000) or cinnamycin (Widdick et al., 2003). Some lantibiotic gene clusters contain an extra regulatory gene such as *mrsR1* in mersacidin (Guder et al., 2002) and *cinR1* in cinnamycin (Widdick et al., 2003). Likewise, some lantibiotics contain orphan regulators in their gene cluster *viz.* MutR in mutacin II, EpiQ in epidermin, and LtnR in lacticin 3147 (Bierbaum et al., 2009; Li et al., 2012). Lantibiotics like Pep5 (Kaletta et al., 1989), lacticin 481(Piard et al., 1993), lactocin S (Mortvedt et al., 1991) and lanthipeptides like catenulipeptin (Wang et al., 2012) and venezuelin (Goto et al., 2010) do not have regulatory genes.

The gene cluster from *B. pseudomycoides* has a single bifunctional modification enzyme (PseM). PseM contains the conserved 'CHG', 'GXXHGXXG' and 'WCXG' domains that are generally present in LanM proteins (Paul et al., 2007). So far, several class II type lantibiotics - mersacidin (Chatterjee et al., 1992), lacticin 481 (Piard et al., 1993), nukacin ISK-1 (Sashihara et al., 2000), mutacin II (Novak et al., 1994), actagardine (Zimmerman et al., 1995), cinnamycin, etc (Marki et al., 1991) have been shown to have the bifunctional modification enzyme (LanM), performing both the dehydration and cyclization step (Chatterjee et al., 2005b).

The PseA prepetide has 75 amino acids and two putative protease cleavage motifs (GA/GG) (Figure 9). Such a 'GG' or 'GA' cleavage motif is found in most of the class II lantibiotics (Sahl and Bierbaum, 1998). Interestingly, the amino acid sequence of the core peptide of PseA is different from the known lantibiotics. However, it possesses a classical lantibiotic leader (Figure 9) that shows similarities to the known lantibiotics e.g. mersacidin (Bierbaum et al., 1995). In similarity to all mersacidin-like lantibiotics (except duramycin), the core peptide possess a conseved motif (TxS/TxE/DC), which might be involved in binding of lipid II. The similarity of the PseA amino acid sequence to the known lantibiotics and the presence of the LanM modification enzyme suggest that it could be a novel class II lantibiotic. In addition, the core peptide has a unique amino acid sequences 'SWSCWSWSC' at the C-terminal end (Figure 9). Such repetitive amino acid motifs have been described for TOMM like peptides. Here the S and C residues serve as precursors for thiazoles or oxazoles (Haft, 2009; Melby et al., 2011). A similar "CWSC" motif is present in the class IIa bacteriocin thuricin 17, thuricin H and cerein MRX1 produced by *B. thuringiensis* (Chehimi et al., 2007; Abriouel et al., 2011; Haft, 2009). Sonorensin, a member of heterocycloanthracins, purified from *Bacillus sonorensis* MT93, contains five repeated SCWSC motifs (Chopra et al., 2014). Since the core peptide of PseA has a repetitive motif similar to TOMMs, the formation of thiazole or oxazole rings might have been possible in PseA. Additionally, possible thiazole and oxazole modification enzymes are encoded in the genome, however, not in the lantibiotic gene cluster. These unique features made it an attractive compound for investigations. The fate of ribosomally synthesized, posttranslationally modified peptides seems to be determined by the sequences of their leader peptides, which direct the prepeptides to their respective modification machineries (Oman and van der Donk, 2010). This has been demonstrated for some lantibiotics as well as microcin B17 and streptolysin S, which both contain thiazole and oxazole residues. The leader sequence of pseudomycoicidin shows high similarity to that of MrsA and ActA and therefore would direct the peptide to the LanM enzyme (Fig. 1).

PseA is rich in Trp residues. The Trp-rich peptides preferentially disrupt large unilammelar vesicles with a net negative charge following their insertion into interfacial region of phospholipid bilayer (Schibli et al., 2002). Such a mode of action has been already reported for Trp-rich antimicrobial peptides such as tritrpticin (Lawyer et al., 1996), indolicidin (Selsted et al., 1992), puroindoline (Haney et al., 2013), and the antibacterial fragments of lactoferrin and lactoferricine (Schibli et al., 2002; Chan et al, 2006). In contrast the Trp residues stabilise the conformation of pediocin-like bacteriocins (Fimlandet al., 2002).

The PseA core peptide is anionic in nature having a net -1 charge. There are also few other existing lanthipeptides which exhibit a net -1 charge, such as duramycin C (Fredenhagen et al., 1990; Märki et al., 1991), variacin (Pridmore et al., 1996), Amfs (Ueda et al., 2002) and the labyrinthopeptins (Müller et al., 2010). In this study, the PseA peptide was identified by bioinformatic analysis, rather than by screening of producer strains. Similarly, several lantibiotics and lanthipeptides such as haloduracin (McClerren et al., 2006), lichenicidin (Begley et al., 2009; Dischinger et al., 2009), Bsa (Daly et al., 2010), venezuelin (Goto et al., 2010) and the prochlorosins (Li et al., 2010) were also identified by genomic data mining.

Upstream of *pseA*, a short ORF having 71% identity to the PseA leader sequence is present. Since this sequence does not have start codon and cleavage motive, it is not clear that the sequence might encode a peptide. Its structural gene is encoded at the 5' end of the contig 00045 and the upstream sequences of the gene cluster are not yet clear. From the PubMed graphic assembly of the genome contigs, it seems that contig 00074 is adjacent to contig 00045. However, attempts to fill the gap in this study using PCR and sequencing were unsuccessful.

The antimicrobial substance from *B. pseudomycoides*

The antimicrobial substance produced by *B. pseudomycoides* had a good antibacterial spectrum against wide range of Gram-positive bacteria including some MRSA strains and a few other human pathogens (Figure 12). However, Gram-negative bacteria and yeasts were not affected by the antimicrobial substance. A similar antimicrobial spectrum was shown for lichenicidin from *B. licheniformis*. Among indicator strains tested, lichenicidin inhibited many Gram-positive bacteria including MRSA strains but Gram-negative bacteria and yeasts were not inhibited (Dischinger et al., 2009) and this has been shown for all lantibiotics tested so far since they are not able to penetrate the outer membrane.

Ammonium sulphate precipitation, ion exchange columns and reverse phase chromatography were successfully used in the past to purify the peptides from bacteria such as streptin (Wescombe and Tagg, 2003), Pep 5 (Sahl and Brandis, 1981), epicidin 280 (Heidrich et al., 1998), etc. However, in this study the attempts to purify the antimicrobial substance by means of $(NH_4)_2SO_4$ precipitation, an anion exchange column, a cation exchange column and an XAD column were unsatisfactory. The active fraction either eluted in the flowthrough or the active fraction did not elute at all. There could be many possible reasons (single or in combination) for the problems encountered during the purification of antimicrobial substance, like, limited solubility, its high affinity to the column materials, its composition with multiple W and C residues which are prone to oxidation and / or hydrogen bonding.

Many antimicrobial substances are produced as a defense of the producer strain and are secreted into the medium. But, contrastingly the antimicrobial substance of *B. pseudomycoides* was associated with the cell wall. Thus, the antimicrobial substance might be related to other functions such as morphogenesis or metabolism of the producer strain, rather than representing only a defense mechanism of the bacterium. There are a few other lantibiotics functioning as morphogenetic peptides, such as those from *Streptomyces* (Willey and van der Donk, 2007). A location of the peptide in the cell wall of the producer strain was also described for lichenicidin (Dischinger et al., 2009), haloduracin (Lawton et al., 2007) and lacticin (Cotter et al., 2006). For the lichenicidin peptide, the authors discussed that the peptides have a net positive charge, thus, the negatively charged capsule of the producer strain might have restricted the diffusion of the cationic peptides from the cell wall (Dischinger et al., 2009). Likewise, for the lacticin peptides, it was hypothesised that the LtnA2 peptide might adhere to the cell wall *via* the LtnA1 peptide (Cotter et al., 2006).

The use of lantibiotic peptides in medicine is limited due to their susceptibility towards proteases, and in case of nisin low stability at physiological pH level (Breukink and de Kruijff, 2006). Thus, the stability of a peptide towards the proteases and physiological pH would be an advantage for its application. In this study, the antimicrobial substance produced by *B. pseudomycoides* was stable to heat and acidic pH (3 – 5) but was unstable at higher pH values. Similarly, though there is one possible cleavage site for trypsin (WTK ↓ DCS) the antimicrobial substance was resistant to trypsin. This indicates the presence of the ring structures in the peptide which protect it from the proteolytic cleavage by the enzyme. Likewise, the addition of pronase E and proteinase K inactivated the antimicrobial substance indicating that it has a proteinaceous nature. A similar result was observed for the activity

from isopropanol cell wash extract of *B. licheniformis* with proteases. It was resistant to trypsin and chymotrypsin, while sensitive to proteinase K and pronase E (Dischinger et al., 2009). The extract from *B. licheniformis* was highly heat stable and moderately stable to ranges of pH (1.5 – 9). Likewise, a streptin produced by *S. pyogenes* was stable to high heat (100 °C) for 10 min and acidic pH (3), while it was sensitive to trypsin and moderately stable at alkaline pH (Wescombe and Tagg, 2003). Similarly, mutacin from *S. mutans* (Novak et al., 1994) was also found to be sensitive to trypsin.

Additionally, the antimicrobial substance from *B. pseudomycoides* was resistant to the protease produced by the producer itself since activity of the antimicrobial substance was retained, after incubation with supernantant containing protease. A similar result was shown for lichenicidin, where, the cell wash extract was active against *S. aureus* ATCC 33592 even after incubation with the supernatant containing protease (Dischinger et al., 2009). In contrast, subtilomycin a new lantibiotic from *S. griseus* was sensitive to the protease produced by the producer itself; however subtilomycin was resistant to proteinase K and chymotrypsin (Phelan et al., 2013).

Heterologous expression of lantibiotic genes

The production assays indeed confirmed the expression of an antimicrobially active substance by *B. pseudomycoides*, however, BLASTs identified three proteins with high similarity to the putative heterocycloanthracin biosynthesis genes described by Haft (2009) on contig00260 (acc. no. ACMX01000028) and comprise a protein carrying the conserved "ocin_THiF_like" domain (bpmyx0001_11050), a SagD/YcaO like cyclodehydratase (bpmyx0001_11040) and a MbcC like oxidoreductase (bpmyx0001_11030). A MbcC like oxidoreductase shows 71% similarity to SagB-type dehydrogenase domain of B. *cereus* (WP_016119836.1) and shows similarity to the part of SagB of *S. pyogenes* (acc. no. TIGR03605) that is responsible for the maturation of streptolysin S from ribosomally produced precursor peptide (Lee et al., 2008). In contrast to *B. licheniformis* ATCC14580/DSM13, no precursor peptide is encoded upstream of these genes, but bpmyx0001_22790 on contig00122 (acc. no. ACMX01000049) (MKMNEFQQE LQALSLNDYQSGNVMYWDQQNQYPYYYIEDDARRCGGCGRCGGFRCGGFRCGGFR CFGCFGCFSCFGCGGCGGCSNCFDGFNGATWWVI) shows similarity to the heterocycloanthracin precursor genes described in (Haft, 2009). In consequence, it could not be excluded that the antibacterial activity in the cell wash extract might represent another substance. It is not unusual for *Bacillus* species to produce an array of different antibiotics, for

example *B. amyloliquefaciens* FZB42 harbours gene clusters that enable production of ten different antimicrobial substances (Chen et al., 2009; Scholz et al., 2011) and even *B. subtilis* 168 is still able to excrete several antimicrobial compounds (Stein, 2005). Generally a knock out strategy is adopted to verify the role of a lantibiotic gene cluster in the production of an antimicrobially active substance. In this strategy, the modification enzyme LanM from the gene cluster is knocked out and subsequently the production of the antimicrobially active substance from the mutant is determined (Dischinger et al., 2009). However, the genetic manipulation of *B. pseudomycoides* was difficult because of its colony morphology (Figure 10). Therefore a heterologous expression system was established. The heterologous expression of the lantibiotic modification enzyme PseM and its corresponding lantibiotic precursor PseA were tested separately, where the overexpression and purification of the PseM protein were successful. However, the overexpression of PseA alone was not successful. The PseA peptide may be unstable, thus it might have degraded during the harvest, lysis or even during the growth after induction. Thus, *in vitro* modification of PseA by PseM could not be performed. In contrast successful heterologous production and *in vitro* modification have been reported for other lantibiotics such as haloduracin, nisin and prochlorosin (McClerren et al., 2006; Li et al., 2010).

Besides *in vitro* modification assays, the heterologous expression of complete gene clusters were successfully performed for several lantibiotics in bacteria too, such as nisin and subtilin in *B. subtilis* (Yuksel et al., 2007), lacticin 3147 in *Enterococcus faecalis* (Ryan et al 2001) and lichenicidin in *E. coli* (Caetano et al., 2011) and gallidermin by *Lactococcus lactis* (van Heel, 2013). In contrast, Nagao et al. (2011), Shi et al. (2011) and Okesli et al. (2011) described another novel methodology for conducting post translational modifications. This method generates lanthipeptides in the heterologous host *E. coli* yielding fully modified prepeptides by coexpression of His-tagged LanA with LanM. With this method, modified prepeptides can be obtained by one step purification. Following a similar strategy, the modified precursor peptide was produced in this study after coexpression of PseM and PseA in *E. coli*. The produced preptide was named pseudomycoicidin. The observed MW of the prepeptide was 74 Da less than the calculated MW of the unmodified prepeptide. This reduction in the mass could be attributed to the loss of four water molecules during maturation, indicating that the PseM dehydrated 4 serine or threonine residues in the core region of PseA and catalyzed the subsequent cyclization of cysteine thiols to form the thioether rings. Since there are eight Ser / Thr residues in the putative core peptide, only four of those residues were dehydrated, the remaining four residues escaped from dehydration. The

phenomenon of free hydroxyamino acid escaping from dehydration was also noted in some other lantibiotics such as nisin A, lacticin 481, haloduracin and some prochlorosins (Chatterjee et al., 2005a; Li et al., 2010).

All lantibiotics, characterized so far, require the removal of the N-terminal leader sequence of the modified peptide to attain their activity (van der Meer et al., 1993). Since the sequence of the PseA contains two protease cleavage motifs *viz.* GA and GG, there are two possible cleavage sites in the peptide. MALDI TOF MS showed the active cell wash extract harbours a molecule with a mass of 2786 Da and that is consistent with a peptide mass if cleaved after GA cleavage site (Table 16, page 67). Therefore, the GA motif was chosen as the cleavage site. In plantaricin W (PlwAβ) (Holo et al., 2001) and haloduracin (HalA2) (McClerren et al., 2006), 'Gly-Asp' is present after putative double glycine cleavage site. In both cases, after cleavage of the prepetide at Gly-Gly processing site, an additional proteolysis occurs resulting in a removal of six N- terminal residues from the core peptide. Similarly, in cytolysin an additional cleavage by CylA was shown to be necessary for activity of cytolysin (Cox et al., 2005). Here, PseA also has 'Gly-Asp' after Gly-Gly cleavage site but the additional cleavage is not possible because Cys residue is present immediately after 'GD'. Thus, to facilitate the *in vitro* cleavage of the N-terminal leader sequence, a factor Xa cleavage site was placed at 'GA' cleavage site of PseA. The core peptide obtained after cleavage of the leader sequence showed an antimicrobial activity against *M. luteus* (Figure 25, spot 3). This strategy of *in vitro* removal of leader after the *in vivo* modification of peptide is known as semi-*in vitro* biosynthesis (Lin et al., 2011). Bovicin HJ50 was produced in the same way by coexpressing BovA with BovM in *E. coli* followed by the removal of the leader peptide *in vitro* using BovT.

Additionally, the PseAXa peptide cleaved by the factor Xa was analysed by MALDI TOF MS for the determination of the masses of the modified core and the leader peptide. The core peptide had a molecular mass of 2785 Da, which matched the calculated core peptide mass of 2787 Da with four dehydrations. This MW of the core peptide also agreed with the detected mass of 2786 Da for the active cell wash extract of *B. pseudomycoides*. The decrease in the mass of 2 Da between the observed and the calculated mass of the core peptide might be attributed to the formation of one disulfide bond in the peptide during modification. Likewise, there were 3 other mass signals observed in MALDI TOF MS *viz. m/z* 5679, 3526 and 2171. These signals corresponded to the calculated masses of fragments of the leader peptide formed by the cleavage by factor Xa, which obviously processed the leader after every Arg 'R' residue. Such unspecific cleavage after Arg residues has been observed before

(Hall, I., Riggs, P., unpublished observations, c.f. https://www.neb.com/products/p8010-factor-xa-protease).

When the pseudomycoicidin was incubated with trypsin, although a trypsin cleavage site is present in the peptide, the cleavage products were not observed; rather the mass signal for the entire core peptide (*m/z* 2785) was still present after the digest (Figure 24 B). This indicates that the heterologously produced pseudomycoicidin is fully modified and that the lanthionine ring/s stabilized the peptide against the digest. Lantibiotics such as nisin, epidermin and Pep5 are elongated peptides and posses a hinge region. This hinge region is essential for pore formation but it is also susceptible to the activity of proteases like trypsin or chymotrypsin (Wilimowska-Pelc et al., 1976; Allgaier et al., 1986; Bierbaum et al., 1996). As shown for Pep 5, by introducing additional rings the hinge region can be stabilized and protected against the attack by such proteases (Bierbaum et al., 1996). Such a stability against proteases is an important property when considering for their application in different fields such as food industry, and human and veterinary medicine (Ryan et al., 2002).

Presence of ring and disulfide linkage in PseAXa

The cyclized and uncyclized peptides have identical masses, so they are indistinguishable by MALDI TOF MS (Paul et al., 2007). There are eight Ser/Thr residues and six Cys residues in the pseudomycoicidin prepeptide. Hence, it was possible that all Cys could be involved in ring formation. However, since pseudomycoicidin has four dehydrated amino acids, only four lanthionine rings were possible in this peptide. McClerren et al. (2006), Paul et al. (2007) and Goto et al. (2011) have performed chemical modifications with a thiol specific reagents (IAA or N-ethylmaleimide or p-hydroxymercuribenzoic acid) to determine the presence of free cysteines in the peptide. Similarly, in this study, the addition of IAA was performed to verify the presence of free Cys residues in the modified PseAXa. Individual Cys residues react with IAA to form an adduct which results in a mass shift of + 57 Da (McClerren et al., 2006). Thus, when the modified PseAXa was incubated with IAA in presence of the reducing agent TCEP, a product (10314 Da) with an increase in mass due to formation of two IAA adducts (~114 Da) was obtained. This indicates that there are two free Cys residues in the peptide which could react with IAA and that the remaining four Cys residues are involved in the formation of Lan/MeLan rings. However in absence of TCEP, there was no formation of adducts after treatment with IAA. Similarly the haloduracin α-peptide showed addition of two adducts after treatment with reductant, suggesting that the two free Cys residues are tied up in a disulfide linkage (McClerren et al., 2006). Thus, it may be speculated that the two free Cys

residues of PseAXa are involved in the formation of a disulfide linkage as similar to Halα, sublancin or Plwα (Paik et al., 1998; Holo et al., 2001; McClerren et al., 2006).

Wang and van der Donk (2012) determined the location of free Dha residues in catenulipeptin by using βME. They found the presence of one extra dehydroalanine residue in addition to four Dha residues required for labionin formation. Likewise, the presence of 4 rings in pseudomycoicidin was further confirmed by the βME assay. When the peptide was incubated with βME, the peptide did not form a βME adduct, demonstrating the absence of free Dha residues in the peptide and confirming that all four dehydrated amino acid residues are involved in Lan / MeLan formation.

Likewise, when the pseudomycoicidin was treated with the reducing agents TCEP and βME, its mass was increased by 2 Da. This increase in the mass could be attributed to the reduction of a disulfide bond in the PseAXa. Most lantibiotics do not possess disulfide bridges (Bulaj et al., 2005). But the disulfide linkages are generally thought to enhance the stability of a protein, and sublancin 168 (Paik et al., 1998), Plwα (Holo et al. 2001), thermophilin1277 (Kabuki et al. 2009), bovicin HJ50 (Xiao et al., 2004), and Halα (McClerren et al., 2006) are lantibiotics that contain disulfide linkages. When PseAXa was incubated with TCEP, the antimicrobial activity of the peptide was completely lost (Figure 27), showing the importance of the disulfide bond for its activity. Similarly, the disulfide bridge was indispensable for full antimicrobial activity of bovicin HJ50 (Lin et al., 2011). In contrast, the reduced haloduracin peptides exhibited activity against the indicator strain (McClerren et al., 2006).

Prediction of ring topology in PseA

Although the peptide has four Lan / MeLan rings and additional one disulfide bond, the pattern of rings is still unknown. The ring topology of some lanthionine-containing peptides has been determined by the spectrometric analysis of LanA analogs (Caetano et al., 2011; Goto et al., 2011; Garg et al., 2012). The analogs are prepared by replacing Cys with Ala residues in the peptide, followed by a spectrometric analysis using MALDI TOF and tandem mass spectrometry. For some of these peptides, the topology determined by tandem MS studies was confirmed by NMR spectroscopy (Shenkarev et al., 2010). In this work, to gain insight into the topology of the lanthionine rings in pseudomycoicidin, six PseAXa analogs were constructed by replacing the single Cys with Ala residues at different positions in the peptide. The constructs were designed to disrupt only one ring at a time. Subsequently, each mutant peptide was subjected to MALDI TOF MS and tandem MS-MS after coexpression and purification. The MALDI TOF MS showed that the mutant peptides were a mixture of fully

and partially modified peptides. Before addition of TCEP none of the mutants showed a single peak (Figure 29) (with four fold dehydration) as shown by the wild type peptide. Hence, all Cys residues seem to be essential for full modification of PseAXa. Since the modification of lantibiotics is thought to be completed before the secretion through the membrane (Wescombe and Tagg, 2003), it is possible that such incompletely modified forms of peptide are present in the cell lysates as used here. For example, incompletely dehydrated forms of Pep5 were found in lysates of the producer cell, while the fully dehydrated form was only detected in the culture supernatant (Sahl et al., 1991). Similarly, the cell wash extract from *B. pseudomycoides* also exhibited incompletely dehydrated forms of peptide in MALDI TOF MS.

The mechanisms by which the LanM enzymes carry out the dehydration and cyclization reactions are little known (Lee et al., 2009). Studies conducted *in vitro* on the bifunctional lacticin 481 and haloduracin synthetase provided the first clues into the mechanism of dehydration (Xie et al., 2004; Chatterjee et al., 2005a; Miller et al., 2006; McClerren et al., 2006). In nisin and lacticin 481, dehydration takes place independently from the cyclization activity (Li et al., 2006; Paul et al., 2008). Likewise, Chatterjee et al. (2006) showed that the dehydration activity of LctM is not coupled to the cyclization activity. In contrast to our expectations, the replacement of Cys residues in the PseAXa prepeptide, not only prevented the ring formation but also interfered with the dehydration step. These findings show that, with PseM, the dehydration and cyclization reactions of PseAXa might occur simultaneously. A similar result was observed by Lee et al. (2009) when they tested the dehydration and cyclization of HalA2 by HalM2. However, with lacticin 481, removal of one or more rings by mutation of Cys residues did not affect the dehydration of Ser / Thr residues (Chatterjee et al., 2006). Additionally, in PseAXa, when the N-terminal Cys and the C-terminal Cys had been replaced by Ala, four fold dehydrated peptides were observed. In contrast, the replacement of the more centrally located Cys residues by Ala resulted in one to three fold dehydrated peptides, and in the case of Cys 56 a free Dha or Dhb residue was observed. The modification by PseM was disturbed by the removal of Cys residues, indicating that the substrate specificity of PseM is much narrower in contrast to that of NisB (Majchrzykiewicz et al., 2010) or LctM (Patton et al., 2008). This was also confirmed when a trial to co-express another lantibiotic structural gene from *Caldicellulosiruptor bescii* (Kataeva et al., 2009) fused to the pseudomycoicidin leader along with PseM in *E. coli*, did not result in modification of the core peptide.

All PseA mutants were inactive after the removal of the leader suggesting that all the thioether rings and the disulfide link are essential for its antimicrobial activity. In contrast, not all rings were essential for the bioactivity of haloduracin as shown by the mutation studies of Halα and Halβ (Cooper et al., 2008). Chen et al. (1998) showed that the two of the mutacin II variants (C15A and C26A), constructed by replacing Cys with Ala failed to exhibit antimicrobial activity; however one of the variant (C27A) was able to retain some residual antimicrobial activity.

Similarly, some mutants of PseAXa - PseAXaC52A, PseAXaC56A and PseAXaC70A were resistant against trypsin (Figure 30). This shows that the ring(s) that protected the trypsin cleavage site are present in all of these three mutant peptides. However, the other mutant peptides *viz.* PseAXaC62A, PseAXaC65A and PseAXaC75A were completely digested by trypsin, showing the absence of ring(s) that could have protected them from cleavage in these peptides.

The analysis of the MALDI TOF MS and MS-MS fragmentation data from the mutant peptides was difficult because i) several internal fragments were produced during MS-MS and ii) many mutant peptides were not fully modified showing different dehydration patterns.

The subsequent analyses of PseAXaC52A and PseAXaC75A showed formation of one IAA adduct and the absence of βME adducts, indicating the presence of one free Cys in each peptide. From these data, it could be predicted that the Cys residues at positions 52 and 75 might be involved in the disulfide bonding. Such a head-to-tail disulfide bond was observed also in the S-linked glycopeptide sublancin 168 (Paik et al., 1998; Oman et al., 2011). Furthermore, this indicated that the Cys residues at positions 56, 62, 65 and 70 might be involved in Lan/MeLan ring formation. The lanthionine bridging pattern of mersacidin (Figure 36) is conserved in other lantibiotics of this family, such as lichenicidin (Rey et al., 2004), actagardine (Zimmermann et al., 1995), haloduracin (McClerren et al., 2006), plantaricin W α (Holo et al., 2001) and michiganin A (Holtsmark et al., 2006). In these lantibiotics, ring C of mersacidin is conserved and is considered to be involved in binding to lipid II (Zimmermann and Jung, 1997; Appleyard et al., 2009). PseA also has the conserved lipid II binding motif. Thus, PseA might also contain the conserved double ring system involving Thr 57 – Cys 62 and Thr 59 – Cys 65 (Figure 37). Likewise, following back-to-front rule of cyclisation, MeLan ring involving Thr 55 – Cys 56 might also be formed. Additionally, there might be a ring structure between Ser 63 – Cys 70, since a mass indicating fragmentation between Pro 66 and Ser 67 was observed in the MS-MS of PseAXaC70A (Annex XIX). The bridging pattern proposed here is also confirmed by the trypsin digests, as

the mutant peptides PseAXaC62A, PseAXaC65A and PseAXaC75A were completely digested by trypsin. In contrast the mutant peptides PseAXaC52A, PseAXaC56A and PseAXaC70A were resistant to the tryptic digest.

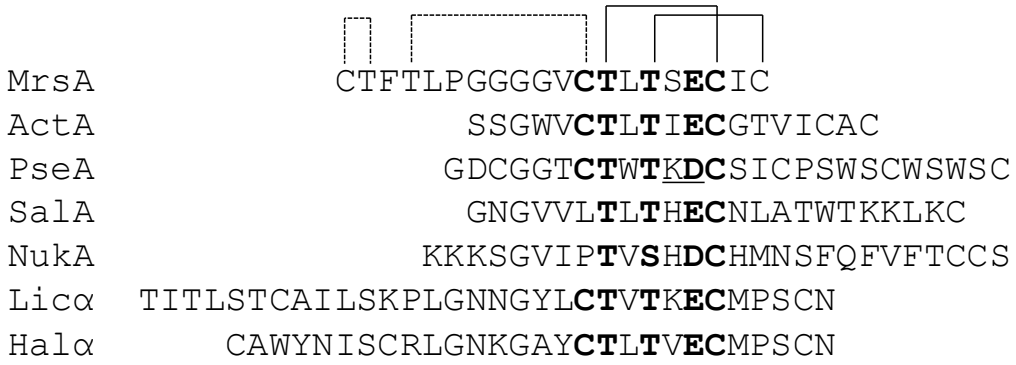


Figure 36: Amino acid sequence alignment of MrsA (Bierbaum et al., 1995), ActA (Zimmermann et al., 1995), PseA, SalA (Wescombe et al., 2006), NukA (Sashihara et al., 2000), Licα (Dischinger et al., 2009) and Halα (McClerren et al., 2006). The conserved lipid II binding motif is given in bold letters. The thioether ring pattern represents that of MrsA in which the conserved double ring pattern is presented by a solid line. Underline indicates the trypsin cleavage site in PseA.

Based on the experimental data and on the structure of mersacidin, the predicted structure for pseudomycoicidin is presented in Figure 37.

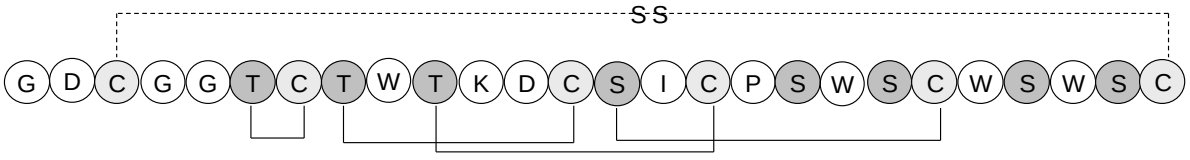


Figure 37: A possible bridging pattern in pseudomycoicidin. Solid lines show the possible thioether ring pattern. Disulfide bridge is indicated by '---S-S---'.

The class II lantibiotics, such as mersacidin (Chatterjee et al., 1992), actagardine (Zimmermann et al., 1995), cinnamycin and duramycin (Fredenhagen et al., 1990; Märki et al., 1991), are highly globular peptides with over lapping rings. Here, pseudomycoicidin also seems to be a globular peptide because of the disulfide bridge and overlapping rings. However, lacticin 481 and related peptides are globular with a linear N-terminus (Dufor et al., 2006). The class I lantibiotics – nisin (Gross and Morell, 1971), epidermin (Allgaier et al., 1986), Pep5 (Kaletta et al., 1989), etc. are elongated peptides. Furthermore, for confirmation

of the structure of pseudomycoicidin, mutant peptides could be created by replacing Ala in place of Ser or Thr residues and could determine the residues which have been dehydrated. Moreover, the complete elucidation and determination of the ring topology requires further structural confirmation by NMR spectroscopy.

Mode of action

Lantibiotics kill bacterial cells either by pore formation or by inhibition of the cell wall biosynthesis or by a combination of both mechanisms (Breukink et al., 1999). Lantibiotics like nisin and other structurally related lantibiotics (gallidermin, epidermin) use lipid II as a docking molecule to form a pore (Brötz et al., 1998b; Breukink et al., 1999; Wiedemann et al., 2001), whereas mersacidin like-peptides inhibit the transglycosylation step of cell wall biosynthesis, thereby blocking the precursor from incorporation into the cell wall (Brötz et al., 1997). *In silico* analysis highlighted the sequence TxS/TxDC as a putative lipid II interaction site. The lipid II binding motif of pseudomycoicidin resembles to that of mersacidin. Therefore, it was assumed that the interaction of PseA with lipid II would be similar to that of mersacidin. Furthermore, the lipid II binding lantibiotics e.g. nisin, epidermin, subtilin, mersacidin, lacticin 481, and haloduracin possess an ABC transport protein LanFEG (Brötz et al., 1997; Breukink et al., 1999; Hsu et al., 2003; Bonelli et al., 2006; Oman and van der Donk, 2009). Since the pseudomycoicidin gene cluster also encodes a putative LanFEG transporter that might play a role in immunity, the ability of pseudomycoicidin to bind lipid II was tested.

In order to evaluate the lipid II interaction ability of a lantibiotic, different experiments were performed in the past. Burkhard and Stein (2008) and Starón et al. (2011) established an assay for identification of peptide antibiotics that interfere with the lipid II cycle. They constructed four *B. subtilis* biosensors that can sense the induction of detoxification systems (LiaRS, PsdRS-AB, Yxdjk-LM and BceRS-AB) by different lipid II interfering peptide antibiotics. These indicator strains can sense bacitracin, vancomycin and lantibiotics like mersacidin, actagardine, gallidermin, duramycin, subtilin and nisin. Nisin and actagardine can induce PsdRS-AB expression. Likewise, the BceRS-AB system is induced by mersacidin and actagardine. However, in this study, pseudomycoicidin did not show induction of any of the biosensors.

Additionally, Brötz et al. (1998a) showed the antagonization of the activity of mersacidin by purified lipid II and a strong inhibition of nukacin ISK-I activity in the presence of lipid I and lipid II (Islam et al., 2012). However, the MIC of pseudomycoicidin

was not affected by the addition of lipid II. The estimated molar concentrations (2:1 and 1:1) of lipid II over pseudomycoicidin did not affect the MIC, indicating that the amount of lipid II added was not sufficient for competing with the cell bound target for available pseudomycoicidin. Furthermore, it was presumed that the affinity of pseudomycoicidin for the purified lipid II in solution might be weaker than for the membrane bound lipid II. The N-terminal part of PseA harbours the conserved mersacidin-like lipid II binding motif (CTxT/SxE/DC) and the C-terminal part contains three Trp residues and another Trp is in the N-terminus. Thus, in contrast to mersacidin, it can be speculated that PseA might exhibit its mode of action by inserting and integrating into the membrane and as well as by binding to the lipid II.

In TLC, lipid II showed different migration patterns after incubation with pseudomycoicidin, nisin and mersacidin. The nisin-lipid II complex was retained at the origin, while lipid II that had been incubated with the pseudomycoicidin and mersacidin migrated more slowly compared to free lipid II. This finding indicates that there might be a weak interaction between lipid II and the peptides (pseudomycoicidin and mersacidin). Wiedemann et al. (2006) observed that nisin and plantaricin C form a stable complex with lipid II and remain at the origin, whereas lipid II showed migration in the TLC system after incubation with mersacidin. The authors predicted that the presence of a highly positively charged N terminus may have facilitated the strong interaction of plantaricin C with lipid II. The binding of pseudomycoicidin to lipid II can be tentatively attributed to the same binding motif found in mersacidin. The binding motif in pseudomycoicidin differs from that of mersacidin in only one amino acid (Asp ‘D’ instead of Glu ‘E’), and an exchange of Asp 13 for Glu in nukacin ISK-1 yielded a higher activity (Islam et al., 2009). Therefore, the molecular interaction between PseA and lipid II might differ from that between the mersacidin and lipid II. This difference in the interaction pattern between the peptide and lipid II might have led to the slight retardation of lipid II incubated with pseudomycoicidin and mersacidin. So far, there is only little evidence for an interaction of pseudomycoicidin and lipid II, i.e. the retardation in thin layer chromatography. To confirm the importance of TxS/TxDC motif in pseudomycoicidin, a mutant could be made by replacing D by E residue and test for higher activity. Moreover, it would be interesting to monitor microscopically the influence of pseudomycoicidin on cell wall integrity of bacteria. Wenzel et al. (2012) investigated the influence of lantibiotics such as nisin, mersacidin, gallidermin on cell wall integrity of *B. subtilis* using fluorescent microscopy. Therefore, further experiments are required to elucidate the full understanding of the mode of action of pseudomycoicidin.

5. Summary and Conclusions

Lantibiotics are antibiotic peptides produced by Gram-positive bacteria. They are ribosomally synthesized and posttranslationally modified. The presence of thioether amino acids lanthionine and methyllanthionine is the characteristic feature of the lantibiotics. Posttranslational modification includes- a. dehydration of Ser or Thr residues, b. formation of thioether linkages between dehydro amino acids and Cys residues and c. proteolytic removal of the leader. All the genes involved in the biosynthesis of lantibiotics such as the structural (*lanA*), modification (*lan BC*/*M*/*lab KC*/*lanP*/*lanL*), regulation (*lanR, lank*), export (*lanT*/*P*) and immunity (*lanI*/*EFG*/*H*) genes are normally organized in a gene cluster.

In this work, genomic data mining was performed to identify a new lantibiotic gene cluster by blasting the biosynthetic enzyme MrsM. The database search found a putative class II type of lantibiotic gene cluster in *Bacillus pseudomycoides* DSM 12442.

B. pseudomycoides produced an antimicrobially active substance in the cell wash extract. The extract was active against many Gram-positive bacteria but not against Gram-negative bacteria and yeast. Moreover, the substance exerted a lytic effect on the bacterial cells as for example the sensitive indicator strains *Micrococcus luteus* and *Staphylococcus aureus* SG511. Although the antimicrobial substance was partially purified by RP HPLC, its complete purification failed so far. The antimicrobial substance had a mass of 2786.0 Da, which corresponds to the calculated mass of the LanA core peptide with four fold dehydration. It was relatively stable to heat, acidic pH and organic solvents. Likewise, it was resistant to enzymes like trypsin while sensitive to pronase E and proteinase K. In addition, it was also resistant against the proteases excreted by the producer strain itself.

Secondly, to test whether the antimicrobial activity in the cell wash extract was associated with the putative lantibiotic gene cluster, heterologous expression of the structural (PseA) and the modification enzyme (PseM) was attempted in *E. coli*. To this end, PseA and PseM were successfully cloned as N-terminally His-tagged PseA and C-terminally His-tagged PseM respectively. A factor Xa cleavage site was introduced into the precursor peptide by site directed mutagenesis for *in vitro* leader processing. Purified PseM was successfully obtained by overexpression. The modified PseAXa was obtained by coexpression of PseAXa with PseM. In addition, after *in vitro* cleavage of the leader, the peptide exhibited activity against *M. luteus*. In MALDI TOF MS analyses, the peptide had a mass of 2785 Da, which was equivalent to its calculated mass with four fold dehydration. The peptide thus produced was named pseudomycoicidin.

Chemical modification assays with IAA, TCEP and βME demonstrated the presence of four thioether rings and an additional disulfide linkage in pseudomycoicidin. Similarly, MALDI TOF MS of the trypsin digested peptide did not lead to loss of the signal for the core peptide. As the peptide was not cleaved by trypsin, it was confirmed that the cleavage site was protected by thioether rings.

For the prediction of the ring pattern in pseudomycoicidin, six PseAXa mutant analogs were created by replacing single Cys with Ala residues. After coexpression with PseM, all the mutant peptides were partially modified and showed mixtures of different dehydration states. This result shows that the removal of Cys residues is unfavourable for modification and that it affected not only the cyclization process but also the dehydration step. Here, the bridging patterns of these peptides could not be elucidated. Therefore, the structural prediction of the ring pattern of pseudomycoicidin will need additional NMR data.

To gain a first insight on the mode of action of pseudomycoicidin, the interaction of peptide with the lipid II was studied. When the peptide was tested for its ability to induce the lacZ promoters in the *Bacillus* reporter strains, positive induction was not observed. Additionally, the MIC of the peptide was not affected by the addition of lipid II. After thin layer chromatography of a mixture of the peptide and lipid II, the peptide-lipid II complex was not retained at the origin, however the lipid II band seemed to be retarded. Although, the lipid II binding motif (CTxS/TxDC) is present in the peptide, the interaction with lipid II was not confirmed so far by first experiments thus further experiments are required to determine the mode of action of the peptide in detail.

In conclusion, genomic data mining identified a new class II lantibiotic gene cluster in *B. pseudomycoides*. The strain produces an antimicrobially active compound with a mass of 2786 Da that is active against Gram-positive bacteria. This antimicrobial substance is fairly stable to heat, acidic pH and organic solvents, and is resistant to trypsin. The coexpression of the structural and modification gene, *pseA* and *pseM* from the gene cluster produced a modified prepeptide. After *in vitro* cleavage of the leader, the peptide was active and had a molecular mass of 2785 Da with four thioether rings and one disulfide ring. All the four thioether rings and disulfide linkage were indispensable for the activity of the peptide. Thus, the production of antimicrobially active peptide by coexpression of PseA with PseM indicates that the active substance in cell wash extract is indeed a lantibiotic. This antimicrobial peptide is a class II lantibiotic and was named “pseudomycoicidin”.

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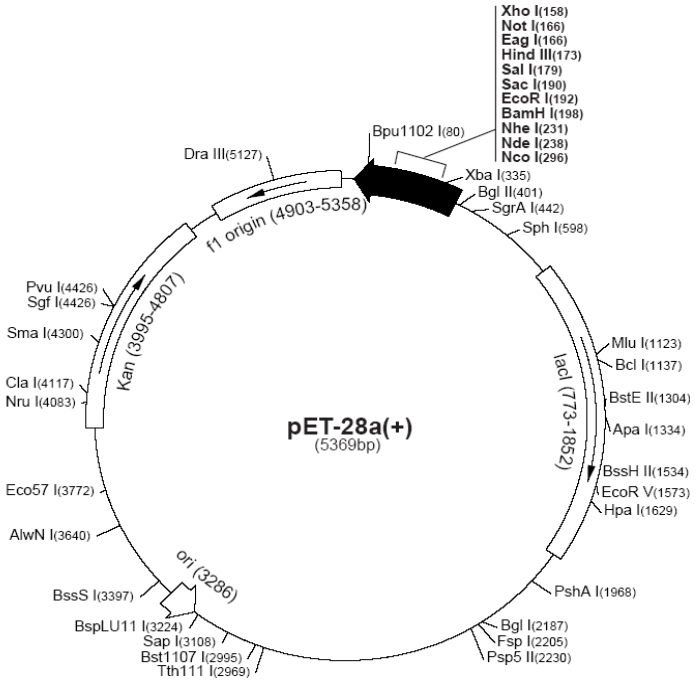
7. Annexes

Annex I: Physical map of expression vector pET28a (Novagen/Merck chemicals). Plasmids confer resistance against kanamycin (Kan). His-tag coding sequences are located next to the mcs and allow cloning of N- or C-terminal His-tagged proteins.



Annex II: Gene sequence of respective inserts and vectors

A) PseA sequence having 229 bp

ATGAATGATAAAATTATCCAATACTGGAACGATCCAGCTAAACGTTCTACTCTTTCAGCAGCAGAACTTAGCAAGAT
GCCAGTAAATCCTGCAGGAGATATTCTTGCAGAGCTTTCAGACGCTGATCTGGATAAAGTAGTGGGTGCTGGTGATT
GCGGTGGTACTTGTACATGGACAAAAGATTGCTCAATTTGTCCATCATGGTCTTGTTGGAGCTGGTCTTGCTAA

B) PseAXa sequence having 229 bp. The replaced bases for factor Xa are highlighted.

ATGAATGATAAAATTATCCAATACTGGAACGATCCAGCTAAACGTTCTACTCTTTCAGCAGCAGAACTTAGCAAGAT
GCCAGTAAATCCTGCAGGAGATATTCTTGCAGAGCTTTCAGACGCTGATCTGGATAAAATAGAGGGTCGTGGTGATT
GCGGTGGTACTTGTACATGGACAAAAGATTGCTCAATTTGTCCATCATGGTCTTGTTGGAGCTGGTCTTGCTAA

C) pET28b sequence with the insert PseAXa (represented by lowercase letters)

TGGCGAATGGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACAC
TTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAA
GCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGG
TGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATA
GTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGATTTTGCCG

ATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAAATATTAACGTTTAC
AATTTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGT
ATCCGCTCATGAATTAATTCTTAGAAAAACTCATCGAGCATCAAATGAAACTGCAATTTATTCATATCAGGATTATC
AATACCATATTTTTGAAAAAGCCGTTTCTGTAATGAAGGAGAAAACTCACCGAGGCAGTTCCATAGGATGGCAAGAT
CCTGGTATCGGTCTGCGATTCCGACTCGTCCAACATCAATACAACCTATTAATTTCCCCTCGTCAAAAATAAGGTTA
TCAAGTGAGAAATCACCATGAGTGACGACTGAATCCGGTGAGAATGGCAAAAGTTTATGCATTTCTTTCCAGACTTG
TTCAACAGGCCAGCCATTACGCTCGTCATCAAAATCACTCGCATCAACCAAACCGTTATTCATTCGTGATTGCGCCT
GAGCGAGACGAAATACGCGATCGCTGTTAAAAGGACAATTACAAACAGGAATCGAATGCAACCGGCGCAGGAACACT
GCCAGCGCATCAACAATATTTTCACCTGAATCAGGATATTCTTCTAATACCTGGAATGCTGTTTTCCCGGGGATCGC
AGTGGTGAGTAACCATGCATCATCAGGAGTACGGATAAAATGCTTGATGGTCGGAAGAGGCATAAATTCCGTCAGCC
AGTTTAGTCTGACCATCTCATCTGTAACATCATTGGCAACGCTACCTTTGCCATGTTTCAGAAACAACTCTGGCGCA
TCGGGCTTCCCATACAATCGATAGATTGTCGCACCTGATTGCCCGACATTATCGCGAGCCCATTTATACCCATATAA
ATCAGCATCCATGTTGGAATTTAATCGCGGCCTAGAGCAAGACGTTTCCCGTTGAATATGGCTCATAACACCCCTTG
TATTACTGTTTATGTAAGCAGACAGTTTTATTGTTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGC
GTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAA
AAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTC
AGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACC
GCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGG
ACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAG
CGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGC
GGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATC
TTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTA
TGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGC
GTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCG
AGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATT
TCACACCGCATATATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGTATACACTCCGCTAT
CGCTACGTGACTGGGTCATGGCTGCGCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTC
CCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTCATCACCGAA
ACGCGCGAGGCAGCTGCGGTAAAGCTCATCAGCGTGGTCGTGAAGCGATTCACAGATGTCTGCCTGTTCATCCGCGT
CCAGCTCGTTGAGTTTCTCCAGAAGCGTTAATGTCTGGCTTCTGATAAAGCGGGCCATGTTAAGGGCGGTTTTTTCC
TGTTTGGTCACTGATGCCTCCGTGTAAGGGGGATTTCTGTTCATGGGGGTAATGATACCGATGAAACGAGAGAGGAT
GCTCACGATACGGGTTACTGATGATGAACATGCCCGGTTACTGGAACGTTGTGAGGGTAAACAACTGGCGGTATGGA
TGCGGCGGGACCAGAGAAAAATCACTCAGGGTCAATGCCAGCGCTTCGTTAATACAGATGTAGGTGTTCCACAGGGT
AGCCAGCAGCATCCTGCGATGCAGATCCGGAACATAATGGTGCAGGGCGCTGACTTCCGCGTTTCCAGACTTTACGA
AACACGGAAACCGAAGACCATTCATGTTGTTGCTCAGGTCGCAGACGTTTTGCAGCAGCAGTCGCTTCACGTTCGCT
CGCGTATCGGTGATTCATTCTGCTAACCAGTAAGGCAACCCCGCCAGCCTAGCCGGGTCCTCAACGACAGGAGCACG
ATCATGCGCACCCGTGGGGCCGCCATGCCGGCGATAATGGCCTGCTTCTCGCCGAAACGTTTGGTGGCGGGACCAGT
GACGAAGGCTTGAGCGAGGGCGTGCAAGATTCCGAATACCGCAAGCGACAGGCCGATCATCGTCGCGCTCCAGCGAA
AGCGGTCCTCGCCGAAAATGACCCAGAGCGCTGCCGGCACCTGTCCTACGAGTTGCATGATAAAGAAGACAGTCATA
AGTGCGGCGACGATAGTCATGCCCCGCGCCCACCGGAAGGAGCTGACTGGGTTGAAGGCTCTCAAGGGCATCGGTCG
AGATCCCGGTGCCTAATGAGTGAGCTAACTTACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACC
TGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCCAGGGTGGTTTT
TCTTTTCACCAGTGAGACGGGCAACAGCTGATTGCCCTTCACCGCCTGGCCCTGAGAGAGTTGCAGCAAGCGGTCCA
CGCTGGTTTGCCCCAGCAGGCGAAAATCCTGTTTGATGGTGGTTAACGGCGGGATATAACATGAGCTGTCTTCGGTA
TCGTCGTATCCCACTACCGAGATATCCGCACCAACGCGCAGCCCGGACTCGGTAATGGCGCGCATTGCGCCCAGCGC
CATCTGATCGTTGGCAACCAGCATCGCAGTGGGAACGATGCCCTCATTCAGCATTTGCATGGTTTGTTGAAAACCGG
ACATGGCACTCCAGTCGCCTTCCCGTTCCGCTATCGGCTGAATTTGATTGCGAGTGAGATATTTATGCCAGCCAGCC
AGACGCAGACGCGCCGAGACAGAACTTAATGGGCCCGCTAACAGCGCGATTTGCTGGTGACCCAATGCGACCAGATG

CTCCACGCCCAGTCGCGTACCGTCTTCATGGGAGAAAATAATACTGTTGATGGGTGTCTGGTCAGAGACATCAAGAA
ATAACGCCGGAACATTAGTGCAGGCAGCTTCCACAGCAATGGCATCCTGGTCATCCAGCGGATAGTTAATGATCAGC
CCACTGACGCGTTGCGCGAGAAGATTGTGCACCGCCGCTTTACAGGCTTCGACGCCGCTTCGTTCTACCATCGACAC
CACCACGCTGGCACCCAGTTGATCGGCGCGAGATTTAATCGCCGCGACAATTTGCGACGGCGCGTGCAGGGCCAGAC
TGGAGGTGGCAACGCCAATCAGCAACGACTGTTTGCCCGCCAGTTGTTGTGCCACGCGGTTGGGAATGTAATTCAGC
TCCGCCATCGCCGCTTCCACTTTTTCCCGCGTTTTCGCAGAAACGTGGCTGGCCTGGTTCACCACGCGGGAAACGGT
CTGATAAGAGACACCGGCATACTCTGCGACATCGTATAACGTTACTGGTTTCACATTCACCACCCTGAATTGACTCT
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CCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCCGGAT

Annex III: Amino acid sequence of N terminally His-tagged PseA and PseAXa having 10.2 kDa. Additional amino acids are represented by bold letters and replaced aa are underlined.

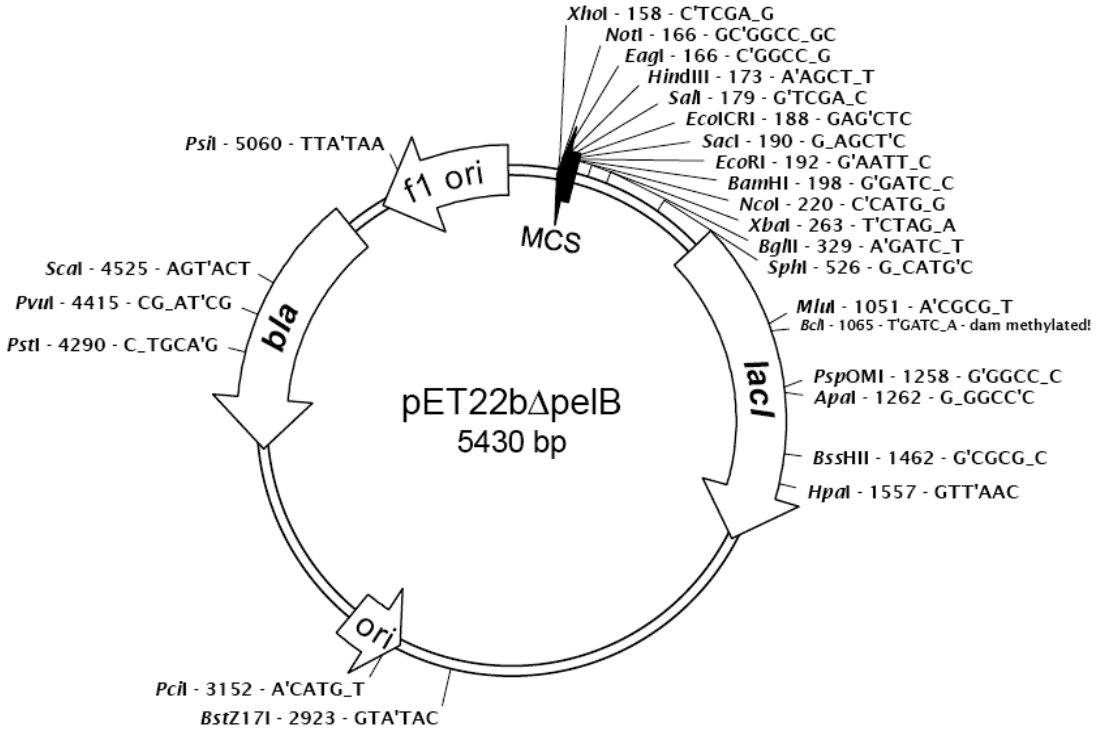
A) PseA

GSSHHHHHHSSGLVPRGSHMNDKIIQYWNDPAKRSTLSAAELSKMPVNPAGDILAELSDADLDKVVGAGDCGGTCTW
TKDCSICPSWSCWSWSC

B) PseAXa

GSSHHHHHHSSGLVPRGSHMNDKIIQYWNDPAKRSTLSAAELSKMPVNPAGDILAELSDADLDKIEGRGDCGGTCTW
TKDCSICPSWSCWSWSC

Annex IV: Physical map of expression vectors pET22b Δpelb (Novagen/Merck chemicals). Plasmids confer resistance against ampicillin (Ap). His-tag coding sequences are located next to the mcs and allow cloning of N- or C-terminal His-tagged proteins.



Annex V: Amino acid sequence of C terminally His-tagged PseM having 123.4 kDa. Additional amino acids are given in bold letter.

MGMLANQALKKAFFIEERVQKSTTSNLAQEMQPDDYSNLRISNWIDGMGNNEDLFLKNLHLHNIEHKDLHKIMISNN
YDSDSSAEWLSIIGELERFNYQEPELPRILQKIIALNPNEPIAFFNFLLPFLKIGVERIEKAISLSEKKHGISLSKE
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WALPSAAELPQLPCAWCHGAAGVVLSRLLLKKAGYSDSLIDIEIRVGLETIIKEGFGRDHSLCHGDTGNSAVLLLAS
KVLKEDLWKQYSYAVGEHVLDEIQQGGWKSGLPQYLETYGAMVGISGVGLGLLKLYDINGVPSITHLESITALK**LEH**
HHHHH.

Annex VI: Sequence of pET22bΔpelB with insert *pseM* (represented by lowercase letters).

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Annex VII: Column materials for protein purification

Column	Material
Anion exchange	Hi Trap Q, 5 ml
Cation exchange	Hi Trap SP, 5 ml
XAD	Serdolit PAD I
Affinity chromatography	Ni-NTA

Annex VIII: Buffers used for anion exchange chromatography

Solutions	Ingredients
Start buffer	20 mm N-Methylpeperazine pH 4.75
Elution buffer	Start buffer + 1 M NaCl (1:10)

Annex IX: Buffer for cation exchange chromatography

Solutions	Ingredients
Start buffer	10 mM citric acid pH 3
Elution buffer / Regeneration buffer	10 mM citric acid , 1 M NaCl, 1:10 ratio of citric acid to NaCl

Annex X: Solvents for XAD column chromatography

Isopropanol (100 %),

Methanol (50 %, 60 %, 70 %, 100 %)

Acetonitrile (25 %, 30 %, 45 %, 100 %)

Annex XI: Buffers and solutions for agarose gel electrophoresis

Solution	Ingredients
50X TAE Buffer	121.14 g Tris Base , 9.31 g EDTA-Na, 27 ml Acetic acid (100 %), D/W up to 500 ml
DNA loading buffer	0.05 ml 0.05 % Bromophenol blue, 0.5 ml 0.1 % SDS, 41.015 mg 5 mM Na_Acetate, 25 mg 25 % sucrose, D/W up to 100 ml

Annex XII: Buffers and solutions for SDS gel electrophoresis

1. Separating Gel

Stock solutions	Final concentration		
	10 %	15 %	20 %
40 % Acrylamid/Bisacrylamid (37.5:1)	2.25 ml	3.3756 ml	4.5 ml
Solution A:2M Tris, pH 8.5	2.25 ml	2.25 ml	2.25 ml
Solution D: 1M Tris + 0.8 %SDS	0	0	0
D/W	3.126 ml	2.109 ml	0.984 ml
20 % SDS	0.06 ml	0.06 ml	0.06 ml
APS	1.2 ml	1.2 ml	1.2 ml
Temed	0.006 ml	0.006 ml	0.006 ml

2. Stacking Gel

Stock solutions	5 % acrylamide concentration
40 % Acrylamid/Bisacrylamid (37.5:1)	0.5 ml
Solution A:2M Tris, pH 8.5	0
Solution D: 1M Tris + 0.8 %SDS	0.6 ml
Distilled water (D/W)	3.09 ml
20 % SDS	0
APS	0.8 ml
Temed	0.01 ml

3. Running buffer: 10 X Tris-Glycine-buffer (pH 8.5), 25 mM Tris, 192 mM Glycine, 0.1 % SDS

Annex XIII: Solutions used in Coomassie staining

Solutions	Ingredients
Staining solution (100 ml)	0.25 % (w/v) coomassie brilliant blue R250, 45 % Methanol, 10 % Glacial acetic acid
Destaining solution (1 L)	45 % Methanol, 10 % Glacial acetic acid

Annex XIV: Solutions for silver staining (Heukeschoven and Dernick; 1998)

Solutions	Ingredients
Fixing solution (1L)	400 ml Ethanol, 100 ml Glacial acetic acid, 500 ml D/W
Sensitizing solution	75 ml Ethanol, 17 g Na- acetate.$3H_2O$, 1.3 ml 25 % Glutaraldehyde (add fresh), 0.5 g sodium thiosuphate.$5H_2O$, D/W up to 250 ml
Silver nitrate solution	0.125 g silver nitrate, 25 µl Formaldehyde (add fresh), D/W up to 125 ml
Developing solution	4.125 g sodium carbonate, 12.5 µl Formaldehyde, D/W up to 125 ml
Stop solution	14.6 g EDTA-Na.$2H_2O$, D/W up to 1000 ml
Preservation solution	100 ml Glycerol, D/W up to 1000 ml

Annex XV: Buffers for protein purification from inclusion bodies

Solution	Ingredients
Buffer A	50 mM Tris-HCl pH 8, 5 mM EDTA, 10 mM NaCl, D/W up to 200 ml
Buffer B	20 mM Na_2HPO_4, pH 7.2, 20 mM NaCl, 5 mM EDTA, 25 % sucrose, D/W up to 200 ml

Annex XVI: Preparation of NZY broth

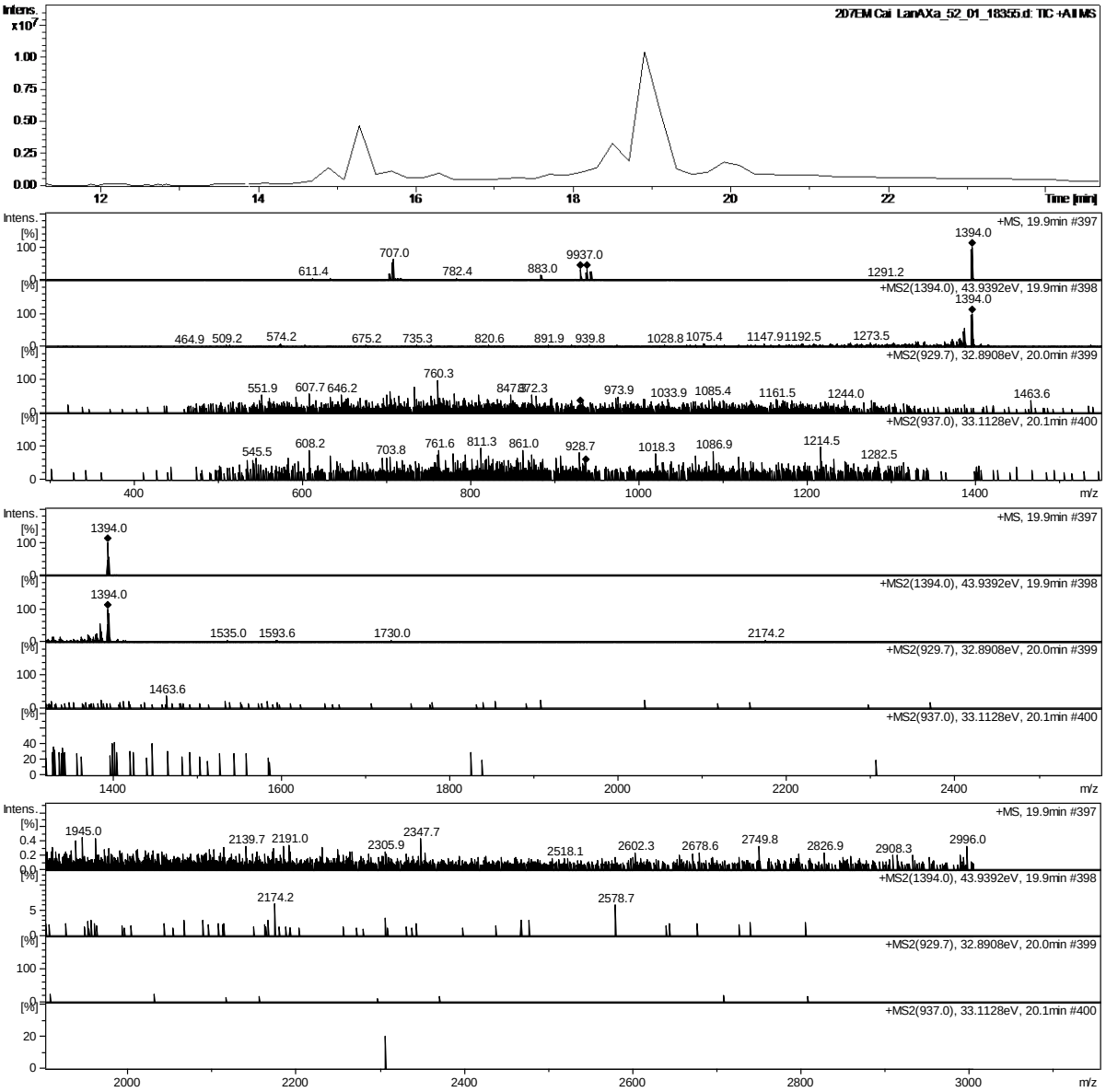
Medium	Components/composition
NZY^+ medium	10 g of NZ amine (casein hydrolysate) 5 g of yeast extract 5 g of NaCl Add deionised H_2O to final volume of 1 liter Adjust pH 7.5 using NaOH Autoclave and add the following 12.5 ml of 1M $MgCl_2$ 12.5 ml of 1M $MgSO_4$ 20 ml of 20 % (w/v) glucose

Annex XVII: Cycler program for amplification of LanM

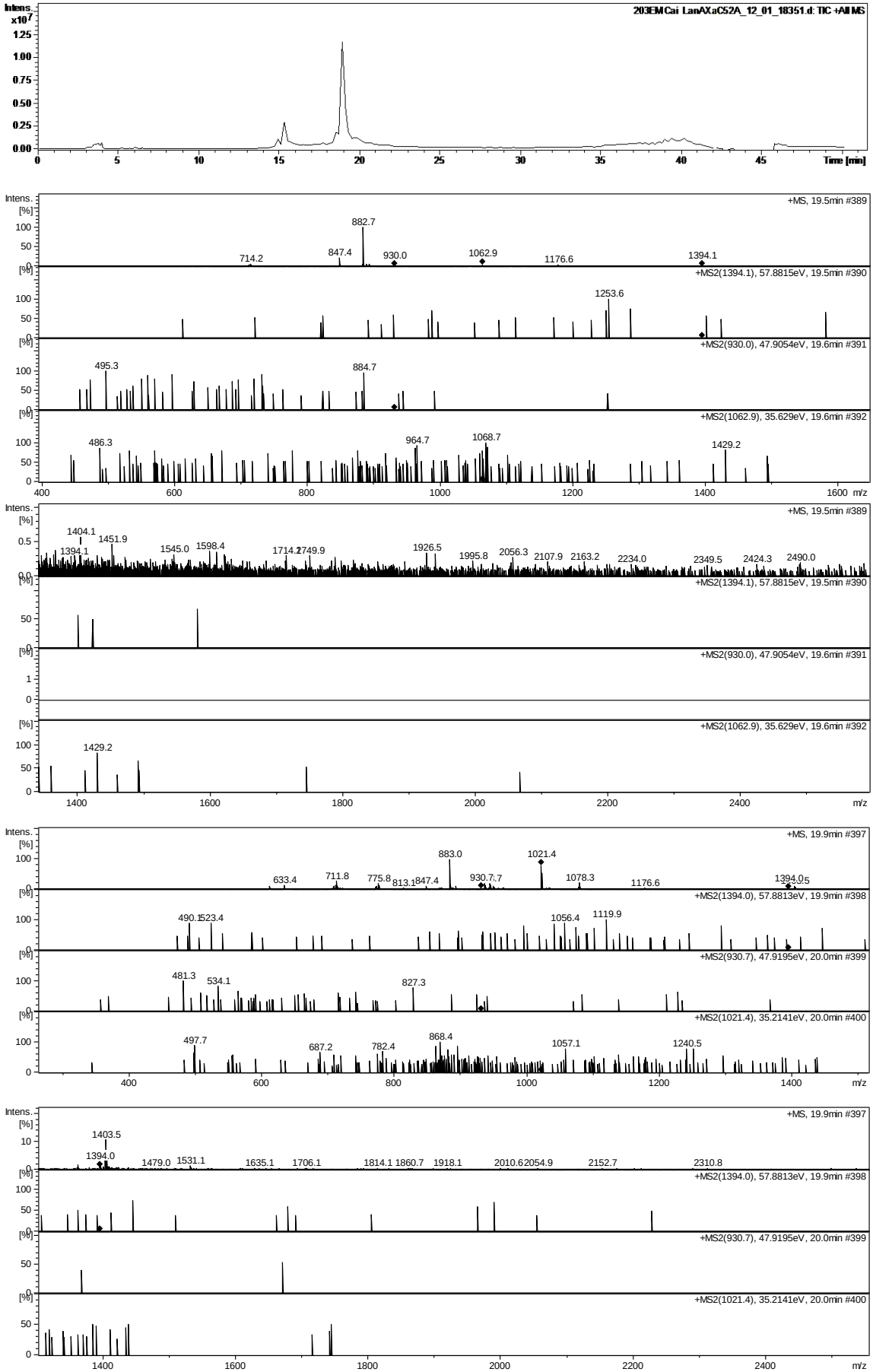
Step	Temperature (°C)	Time (s)	No. of cycles
Initial denaturation	97	180	1
Denaturation	96	20	35
Annealing	62 ± 6	30	35
Extension	72	240	35
Final extension	72	600	2
Hold	4	∞	1

Annex XVIII: LC- MS and MS-MS fragmentation of the PseAXa and its mutant peptides

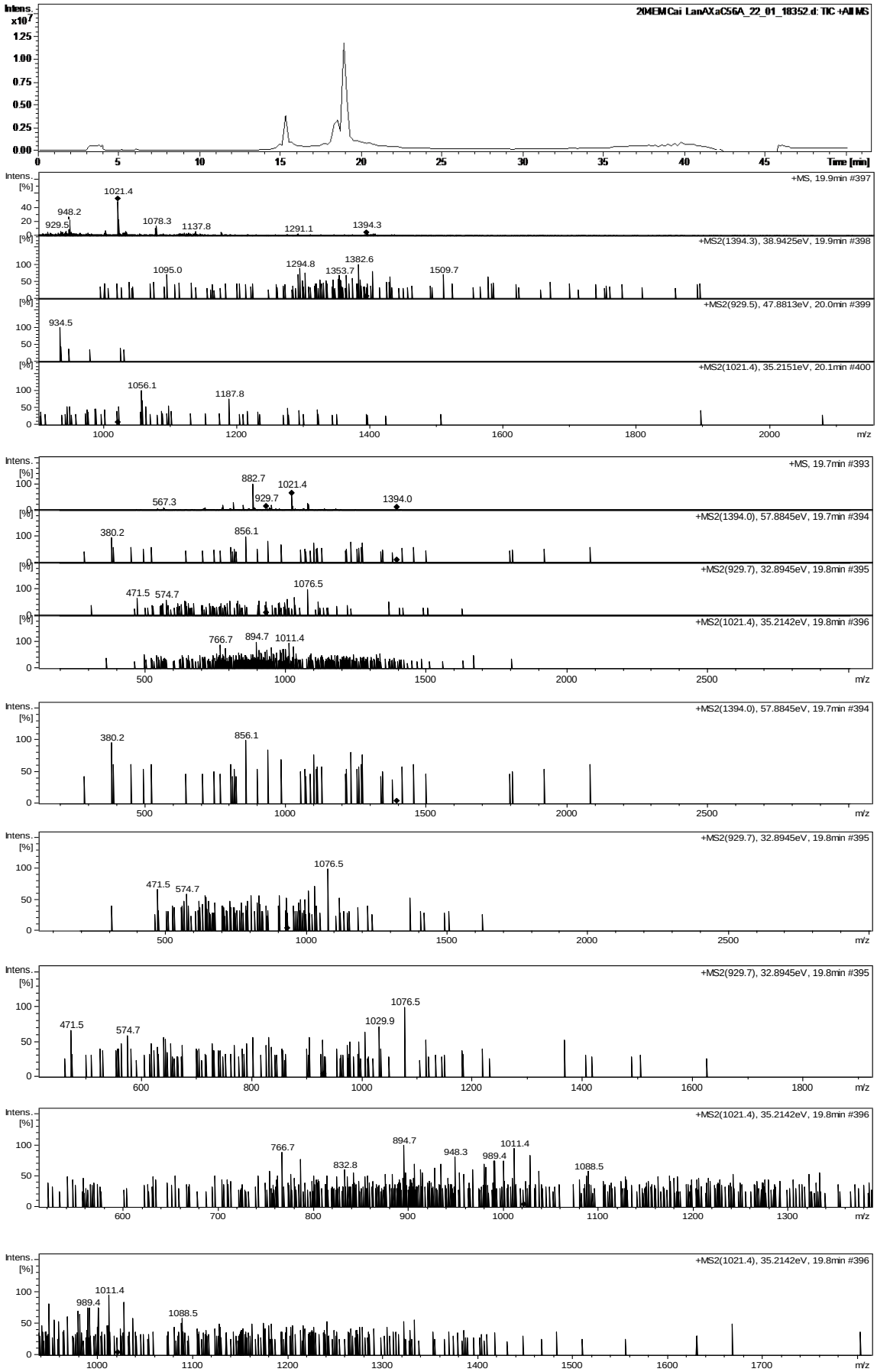
1. PseAXa



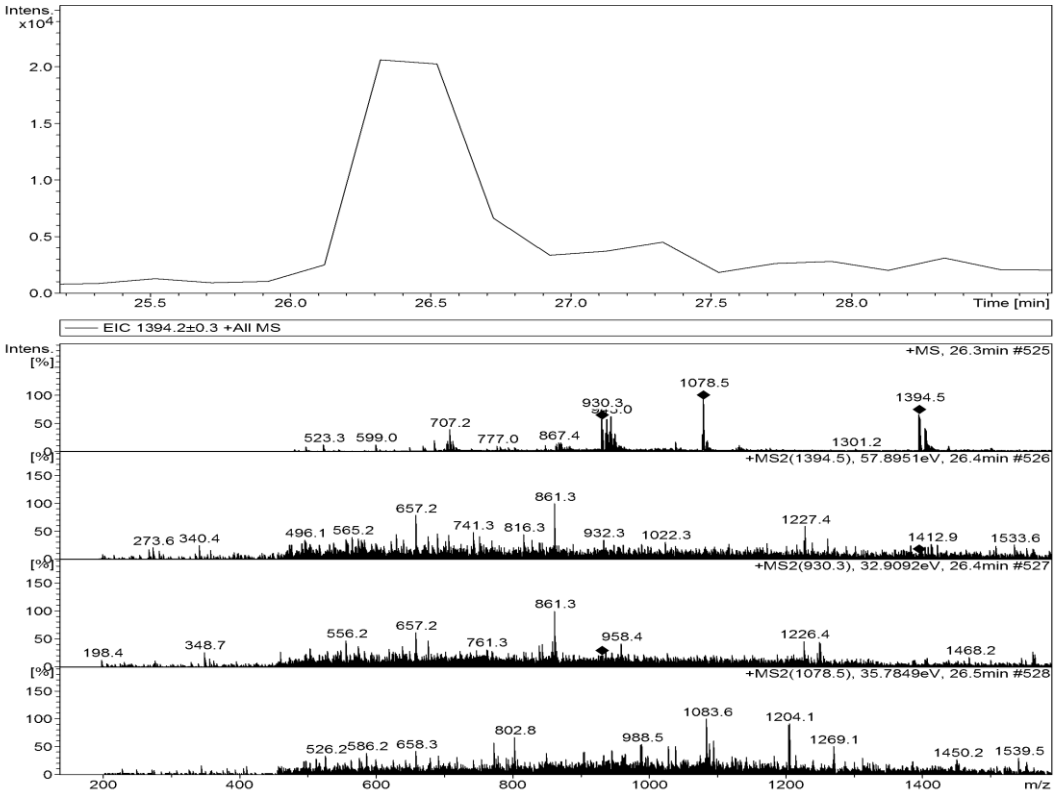
2. LanAXaC52A



3. LanAXaC56A



4. PseAXaC62A



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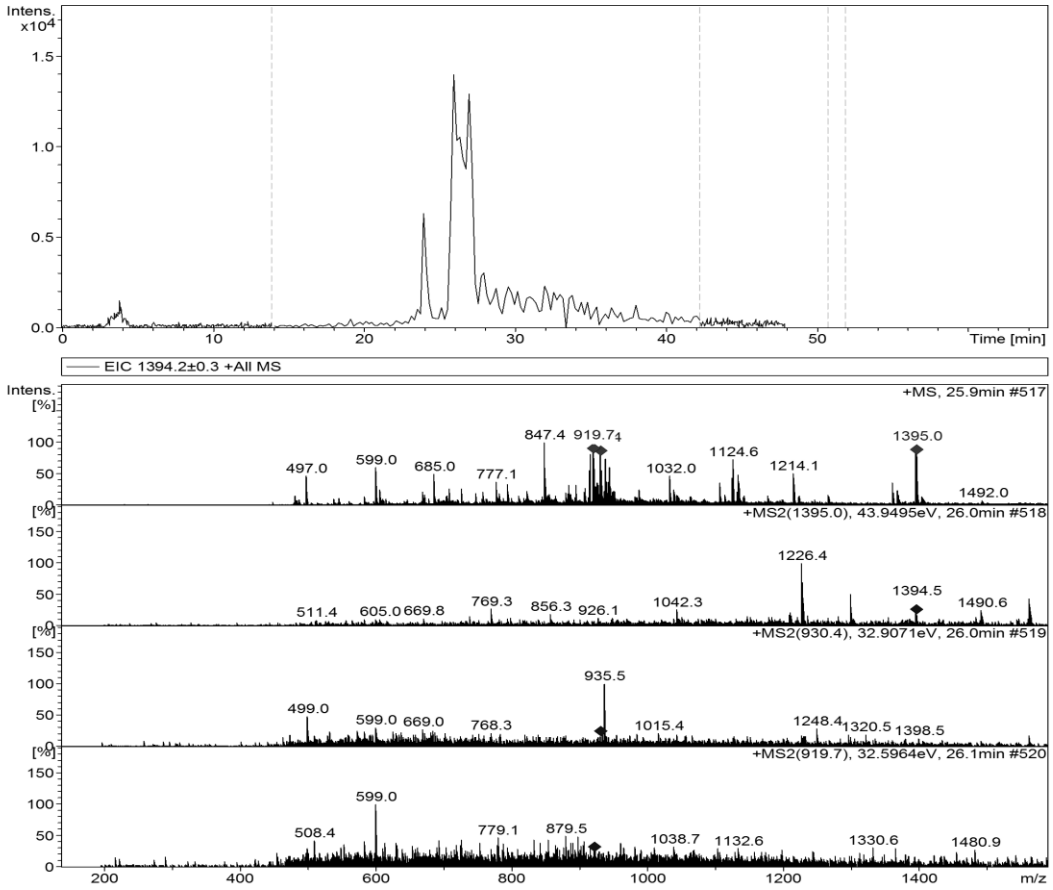
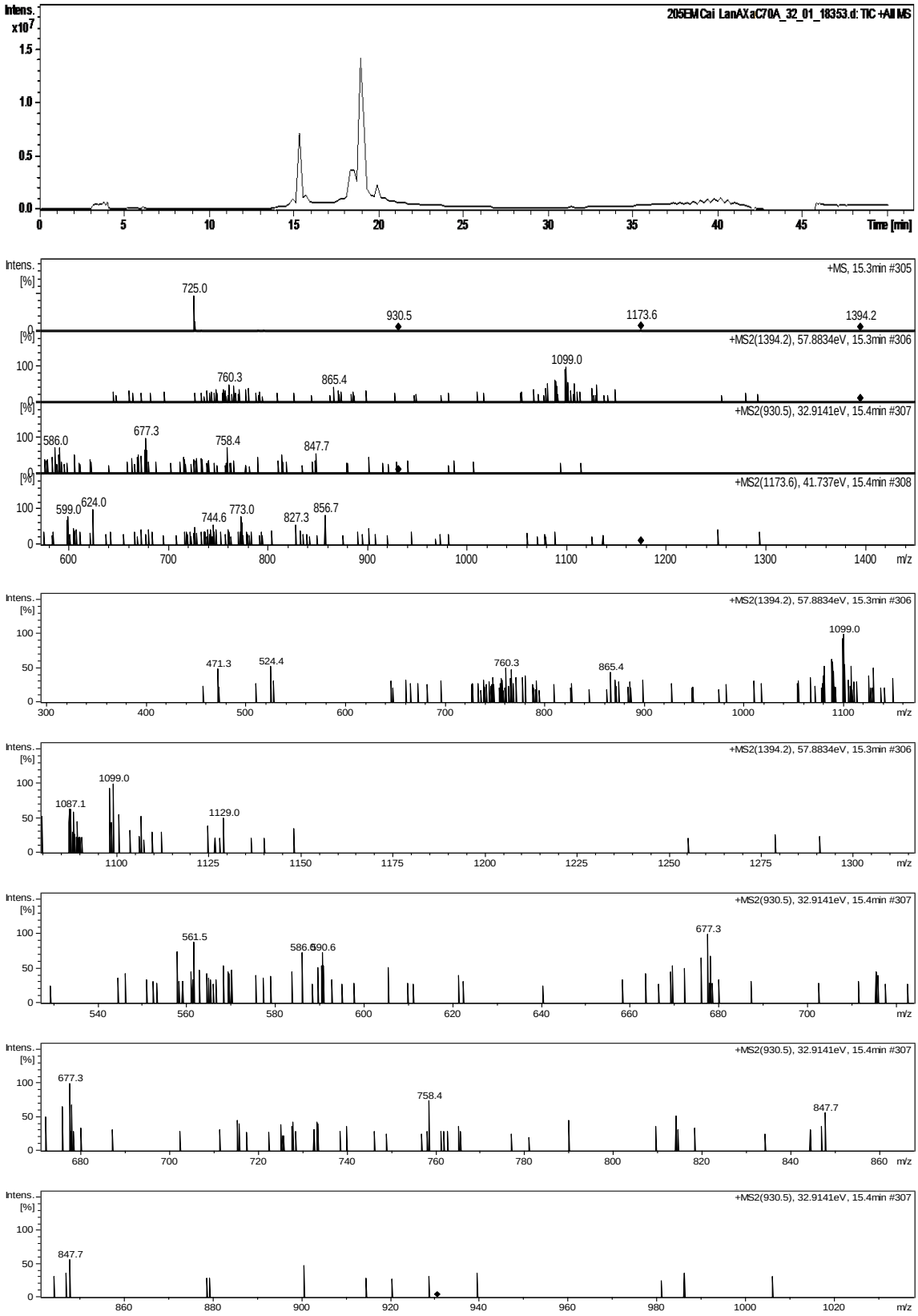
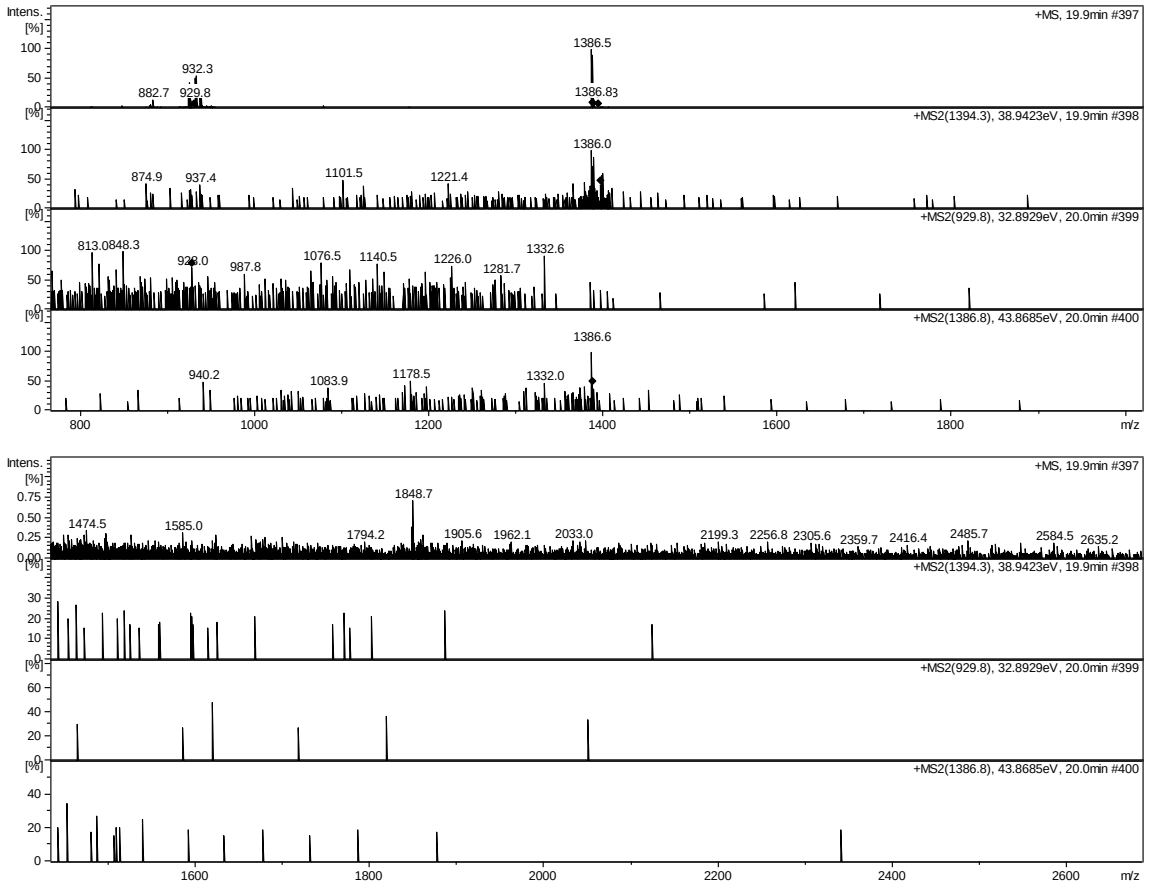
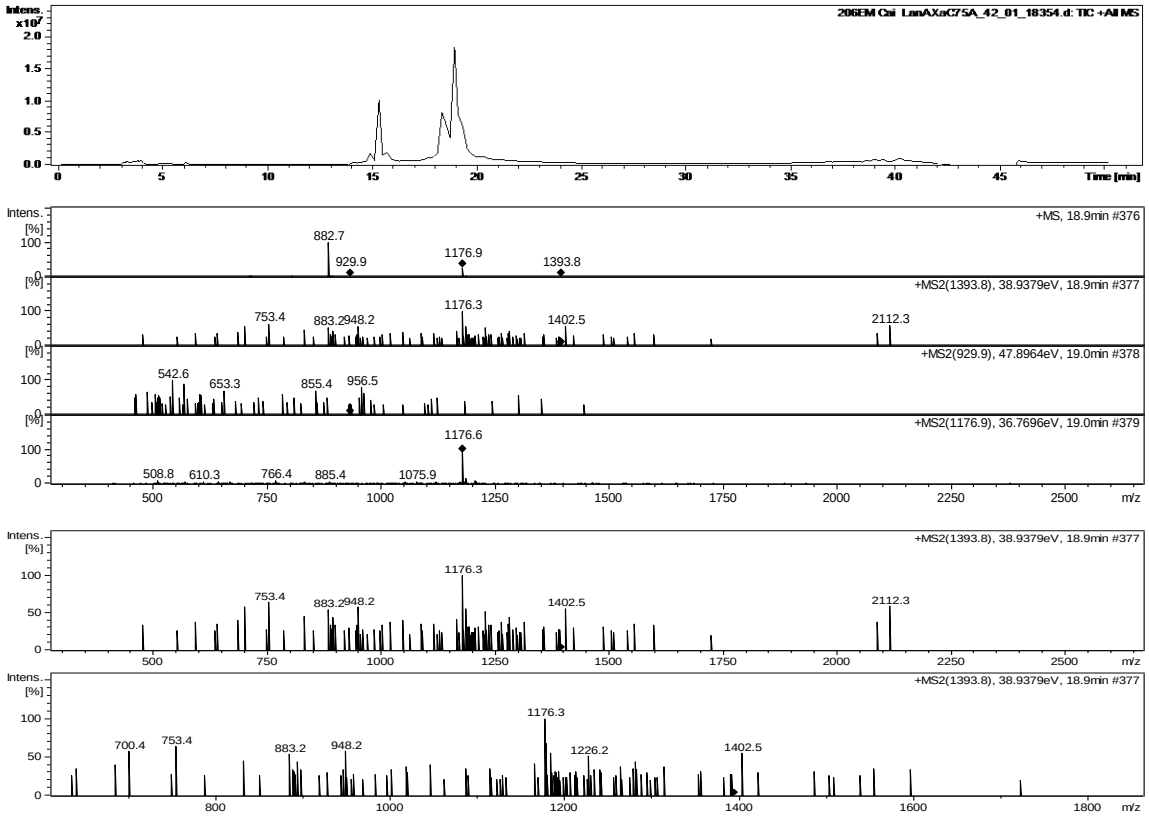


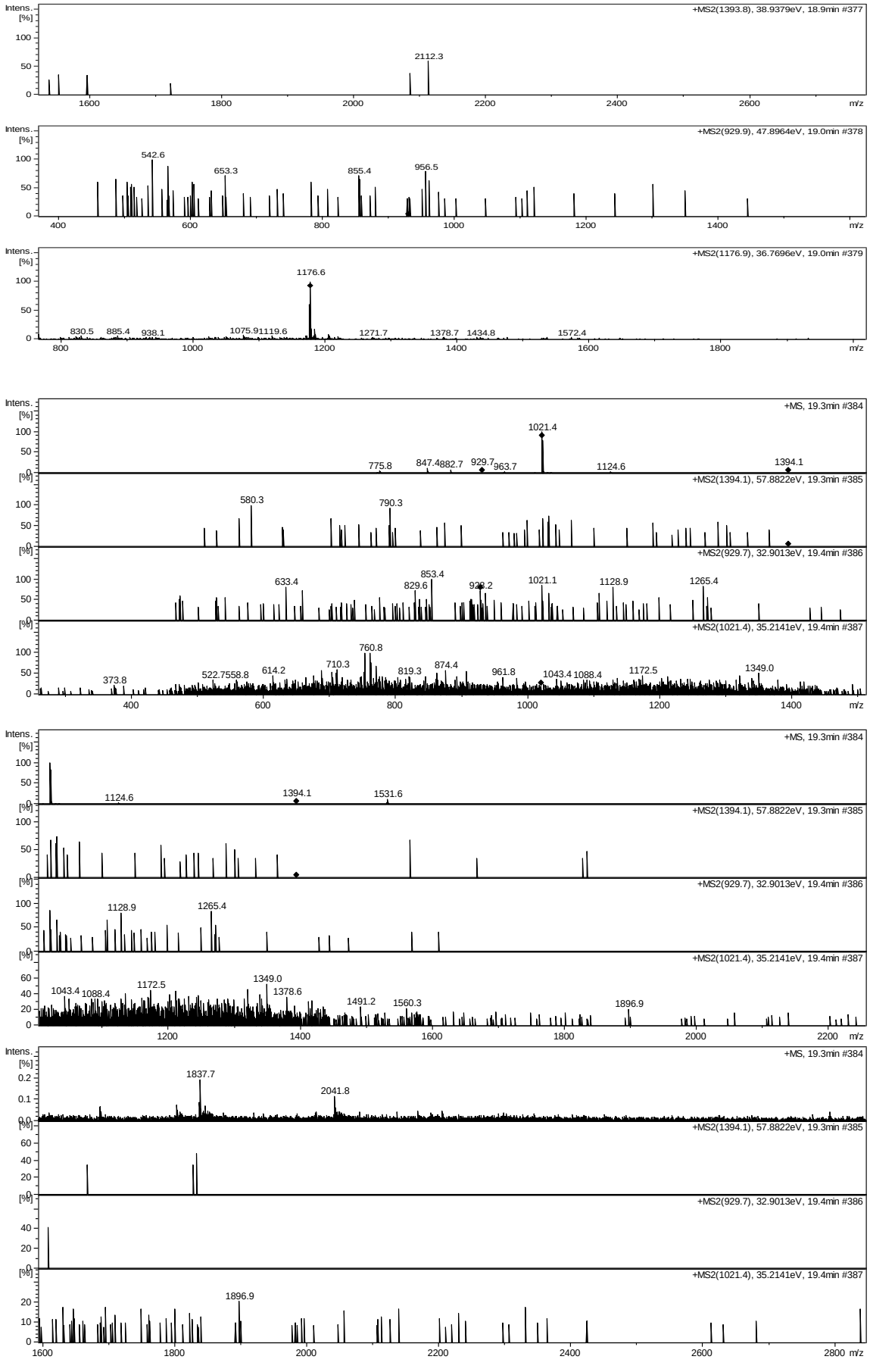
6. LanAXaC70A



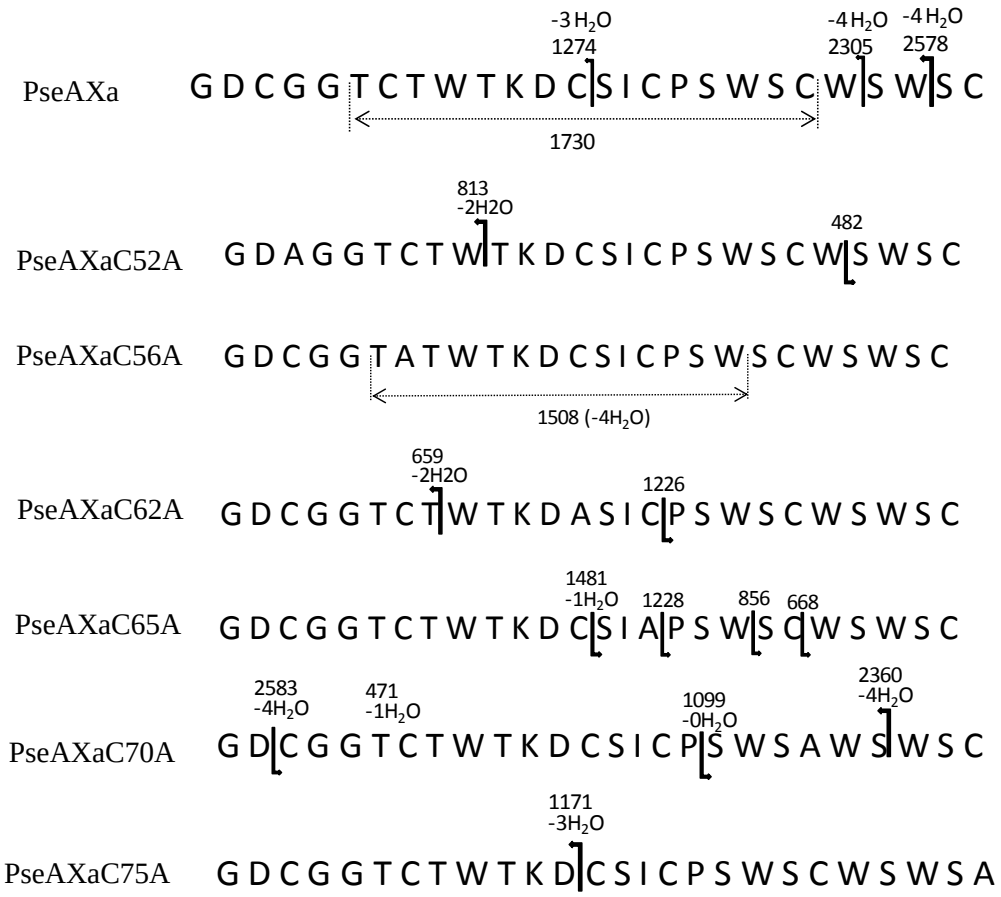


7. LanAXaC75A

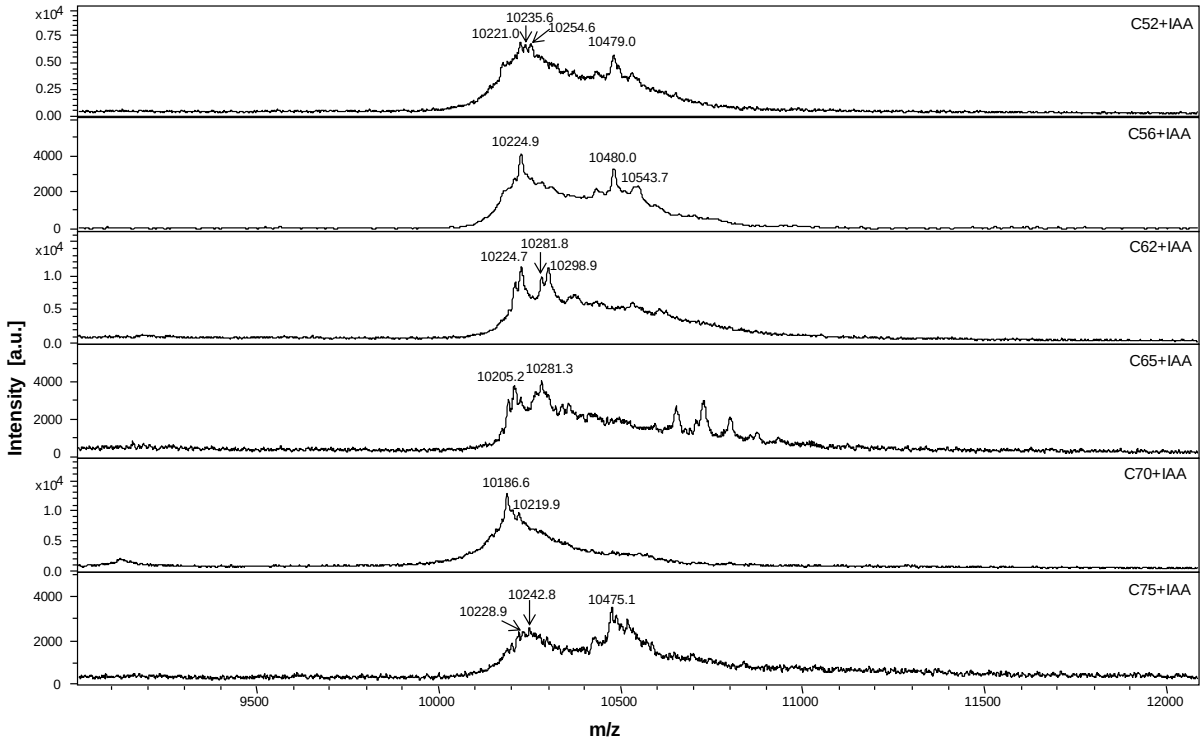



Intens.
[%]
+MS2(1393.8), 38.9379eV, 18.9min #377
2112.3
m/z
+MS2(929.9), 47.8964eV, 19.0min #378
542.6
653.3
855.4
956.5
+MS2(1176.9), 36.7696eV, 19.0min #379
1176.6
830.5
885.4
938.1
1075.9
1119.6
1271.7
1378.7
1434.8
1572.4
+MS, 19.3min #384
1021.4
775.8
847.4
882.7
929.7
963.7
1124.6
1394.1
+MS2(1394.1), 57.8822eV, 19.3min #385
580.3
790.3
+MS2(929.7), 32.9013eV, 19.4min #386
633.4
829.6
853.4
928.2
1021.1
1128.9
1265.4
+MS2(1021.4), 35.2141eV, 19.4min #387
373.8
522.7
558.8
614.2
710.3
760.8
819.3
874.4
961.8
1043.4
1088.4
1172.5
1349.0
1531.6
1491.2
1560.3
1837.7
2041.8
1896.9

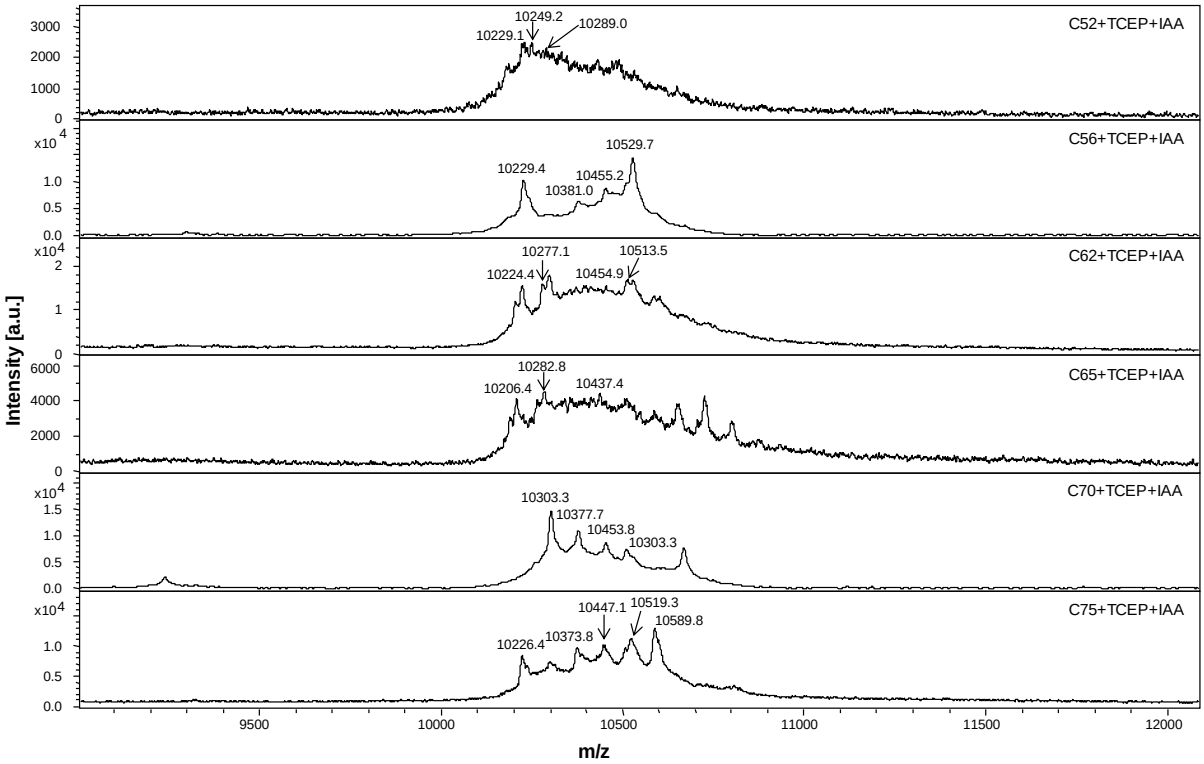
Annex XIX: Fragmentation pattern of PseAXa and its mutants



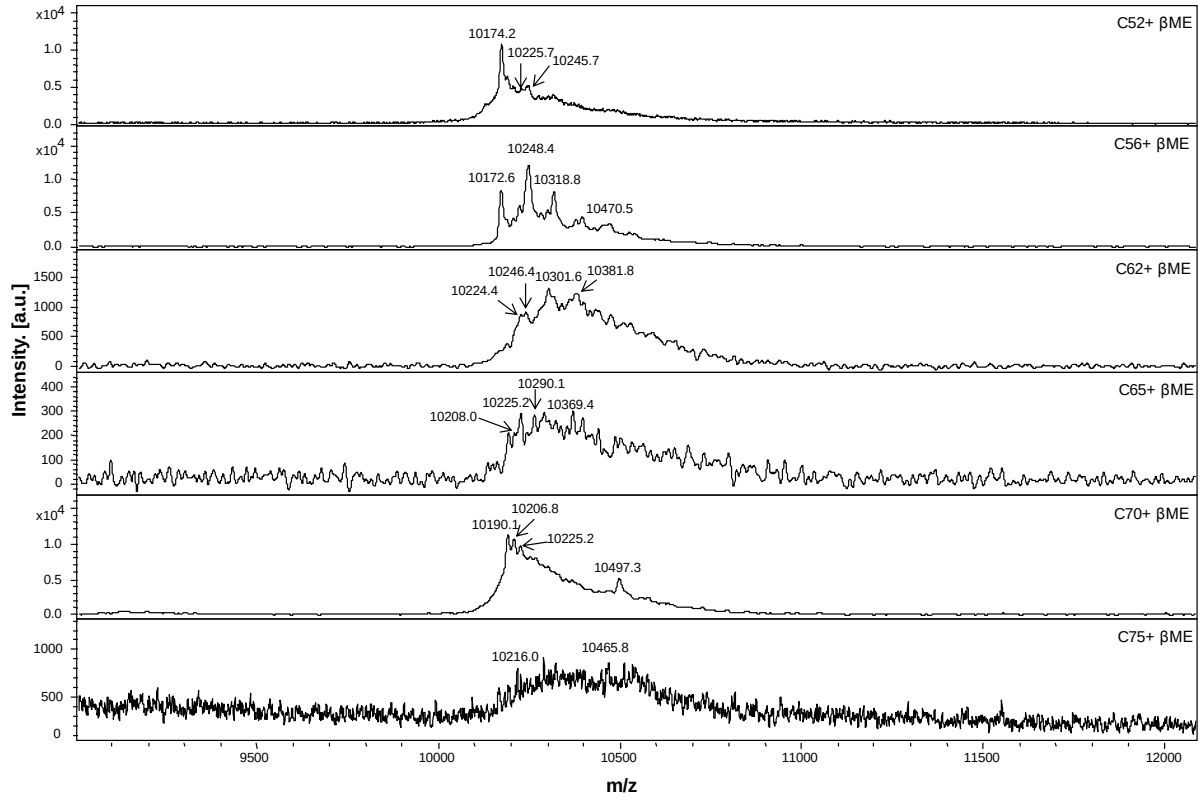
Annex XX: MALDI TOF MS of PseAXa mutant peptides after incubation with IAA



Annex XXI: MALDI TOF MS of PseAXa mutant peptides after incubation with IAA in presence of TCEP



Annex XXII: MALDI TOF MS of PseAXa mutant peptides after incubation with βME



Annex XXIII: Masses of the PseAXa and its mutant peptides as observed in MALDI TOF MS before and after incubation with different chemicals. The possible addition (+) or removal (-) of no. of H_2O/ IAA adduct to yield the respective masses are given in an adjacent column 'Prediction'. The overall predictions from the respective reactions are provided in the column 'Inferences'

Peptides	Control		+TCEP		+IAA		+TCEP+IAA		+βME		Inferences
	Observed mass (Da)	Prediction	Observed mass (Da)	Prediction	Observed mass (Da)	Prediction	Observed mass (Da)	Prediction	Observed mass (Da)	Prediction	
PseAXa	10204.3	-4 H_2O	10207.1	-4 H_2O	10203.9	-4 H_2O	10314.3	+ 2 IAA	10207.1	-4 H_2O	4 thioether, 1 disulfide
PseXaC52A	10227.4	-1 H_2O	10173	-4 H_2O	10221		10229	+1 IAA	10174	-4 H_2O	
	10207		10190	-3 H_2O	10254	-0 H_2O	10249		10189	-1 H_2O	
	10190		10207	-2 H_2O	10235		10289		10225		1 free Cys
	10176		10226	-1 H_2O	10479				10245		
	10484	+ 5 Ni	10244	-0 H_2O					10480		
PseXaC56A	10225.3	-1 H_2O	10172	-4 H_2O	10224		10229	+1 IAA	10172	-4 H_2O	
	10207		10190	-3 H_2O	10480	-2 H_2O	10455		10247	-2 H_2O	1 free Cys,
	10190		10207	-2 H_2O	10543	-1 H_2O	10529		10252	+1 βME	1 free Dha
	10176		10226				10381		10318	-1 H_2O	residue
	10485	+ 5 Ni	10243	-0 H_2O					10470		
PseXaC62A	10225.6	-1 H_2O	10228	-1 H_2O	10224	-1 H_2O	10224	-1 H_2O	10224	-1 H_2O	
	10289		10245	-0 H_2O	10281		10277		10246	-0 H_2O	
	10336		10302		10298		10455		10301		free Cys
	10517		10319				10513		10363		
			10367						10381		
PseXaC65A	10206	-2 H_2O	10206	-2 H_2O	10205	-2 H_2O	10206	-2 H_2O	10208	-2 H_2O	
	10281		10281		10281		10282		10225	-1 H_2O	
	10723		10725				10437		10290		free Cys
	10797		10797						10369		
PseXaC70A	10190.2	-3 H_2O	10190	-3 H_2O	10186	-3 H_2O	10303	+2 IAA	10190	-3 H_2O	
	10412		10205	-2 H_2O	10219		10377		10206	-2 H_2O	2 free Cys,
			10223	-1 H_2O			10453		10225	-1 H_2O	3 rings,
			10494	+ GSH			10510		10497		1disulfide
PseXaC75A	10224	-1 H_2O	10174	-4 H_2O	10228		10226	+1 IAA	10216		
	10207	-2 H_2O	10208	-2 H_2O	10242	-0 H_2O	10373		10465		
	10190	-3 H_2O	10480	+GSH	10475		10447				1 free Cys
	10174	-4 H_2O					10589				
	10480	+5 Ni					10519				

Publications

Peer reviewed articles

Basi-Chipalu, S., Dischinger, J., Josten, M., Szekat C., Sahl, H.-G., Bierbaum, G. (*Submitted*) Pseudomycoicidin, a novel class II lantibiotic produced by *Bacillus pseudomycoides.* Appl Environ Microbiol.

Dischinger, J., **Basi-Chipalu**, S., Bierbaum, G. (2014). Lantibiotics: promising candidates for future applications in health care. Int J Med Microbial. 304 (1): 51-62.

Oral presentations

Basi-Chipalu, S., Dischinger, J., Reif, M., Braun, K., Schuster, D.,Josten, M., Sahl, H.-G., Bierbaum, G. (2013). Pseudomycoicidin a new lantibiotic from *Bacillus pseudomycoides*. In oral presentation: Minisymposium Novel Developments in Lantibiotic Research, 16-17 June 2013, Verona, Italy.

Chipalu, S. (2008). Microbiological study on gastroenteritis of children from Kanti Children's Hospital with reference to Cyclospora. In Oral presentation: Fifth National Conference on Science and Technology, National Academy of Science and Technology, Kathmandu, Nepal.

Posters

Basi-Chipalu, S., Dischinger, J., Reif, M., Zweynert, A., Braun, K., Schuster, D., Josten, M., Toma, R A., Süssmuth, R D., Sahl, H.-G., Bierbaum, G. (2013). Novel lantibiotics from microbial genomes. In poster presentation: 2nd International symposium of DFG Research unit 854 on the current trends in Antibacterial research, Bonn Röttgen, Germany.

Dischinger, J., **Basi-Chipalu**, S., Reif, M., Braun, K., Schulster, D., Josten, M., Szekat, C., Sahl, H.-G., Bierbaum, G. (2013). Identification and characterization of novel lantibiotics by heterologous expression in *E. coli*. In poster presentation: Gordon Research Conference on "Antimicrobial Peptides", Ventura, CA, USA.

Basi-Chipalu, S., Dischinger, J., Reif, M., Braun, K., Schuster, D.,Josten, M., Sahl, H.-G., Bierbaum, G. (2013). Heterologous production of novel class II lantibiotics in *E. coli*. In Poster presentation: Annual Conference of the Association for General and Applied Microbiology (VAAM) in collaboration with the Royal Netherlands Society for Microbiology (KNVM), Bremen, Germany.

Basi-Chipalu, S., Dischinger, J., Oedenkoven, M., Braun, K., Josten, M., Sahl H.-G. and Bierbaum G. (2012). Heterologous production of novel class II lantibiotics in *E. coli.* In poster presentation: 7 joint PhD students meeting, Freudenstadt-Lauterbad, Germany.

Basi-Chipalu, S., Dischinger J., Josten, M., Szekat, C., Sahl, H.-G. and Bierbaum, G. (2012). Characterization of an antimicrobial substance produced by *Bacillus pseudomycoides* DSM 12442.In Poster presentation: Annual Conference of the Association for General and Applied Microbiology (VAAM), Tübingen, Germany.

Basi-Chipalu, S., Dischinger, J., Josten, M., Szekat, C., Sahl, H.-G. and Bierbaum, G. (2010). Characterization of an antimicrobial substance produced by *Bacillus pseudomycoides* DSM 12442.In Poster presentation: International symposium of DFG Research unit 854 on the current trends on Antibacterial research, Königswinter, Germany.

Basi-Chipalu, S., Dischinger, J., Josten, M., Szeka,t C., Sahl, H.-G. and Bierbaum, G. (2010). Characterization of an antimicrobial substance produced by *Bacillus pseudomycoides* DSM 12442.In Poster presentation: Annual Conference of the Association for General and Applied Microbiology (VAAM), Hannover, Germany.

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Declaration (Eidesstattliche Erklärung)

Hiermit erkläre ich an Eides statt, dass ich für die Anfertigung der vorliegenden Dissertationsschrift nur die angegebenen Hilfsmittel verwendet habe und dass die Stellen und Zitate, die anderen Werken dem Wortlaut oder dem Sinn nach entnommen wurden, unter Angabe der Quelle als Entlehnung kenntlich gemacht sind. Diese Arbeit ist weder identisch noch teilidentisch mit einer Arbeit, die an der Rheinischen Friedrich-Wilhelms-Universität Bonn oder einer anderen Hochschule zur Erlangung eines akademischen Grades oder als Prüfungsleistung vorgelegt worden ist. Teile der Dissertation sind vorab an den unter „Publications“ aufgeführten Stellen auszugsweise veröffentlicht worden.

Bonn, 2014

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