8-2013

Chemoenzymatic synthesis of an analogue of the potent antifungal mycosubtilin

Mayank Srivastava

Chemoenzymatic Synthesis of an Analogue of the Potent Antifungal Mycosubtilin

by

Mayank Srivastava

Thesis

Submitted to the Department of Chemistry

Eastern Michigan University

In partial fulfillment for the degree of

MASTER OF SCIENCE

in

Chemistry

Thesis Committee:

Deborah Heyl-Clegg, Ph.D., Chair

Jamie Scaglione, Ph.D., Chair

Hedeel Guy Evans, Ph.D.

August, 2013

Ypsilanti, Michigan

Acknowledgements

It gives me a great pleasure to acknowledge all who contributed in some way or the other towards the successful completion of my graduate study in Chemistry.

I would like to take this opportunity to thank my research mentors Dr. Deborah Heyl-Clegg and Dr. Jamie Scaglione for their excellent guidance, motivation and constant support during my graduate studies at Eastern. Thanks to Dr. Heyl-Clegg for showing confidence in me and helping me become an independent researcher. Thanks to Dr. Scaglione for this exciting project and all the Biochemistry skills I learned. My research advisors have been the motivation behind all my success throughout the Master's studies.

Special thanks to Dr. Ruth Ann Armitage for the Mass Spectral data of peptides. It was her tremendous support that made this project possible in such limited time duration.

I would like to express my deepest sense of gratitude to Dr. Timothy Brewer, our academic advisor, for the Graduate Assistantship position that helped me achieve this level of education.

I express my sincere thanks to my thesis committee member, Dr. Hedeel Evans for her endless support; our department head, Dr. Steven Pernecky and other faculty members for all the teachings and motivation. I really enjoyed my time being a graduate student at this esteemed university.

Abstract

Mycosubtilin is a naturally occurring antifungal obtained from *Bacillus subtilis* that also displays limited antibiotic activity. Structurally, mycosubtilin is a macrocyclic lipoheptapeptide of sequence Asn-Tyr-Asn-Gln-Pro-Ser-Asn, with the N-terminal Asn joined by a β-amino fatty acid. Besides the antifungal and antibiotic activities, these molecules are also hemolytic in nature. Hence, the purpose of this study is to synthesize a potent antifungal analogue of mycosubtilin, with a modified β-amino fatty acid, devoid of any hemolytic activity. A chemoenzymatic approach was used to synthesize the cyclic peptide, which involved the synthesis of the linear peptide chain of desired amino acid sequence, thiophenyl derivatization of the peptide at the C-terminus, followed by its enzymatic cyclization using the isolated thioesterase from *B. subtilis*. Thus far, we have successfully synthesized the analogue of mycosubtilin. Future work will focus on purifying the product and testing it for antifungal and hemolytic activity.

Table of Contents

List of Tables

List of Figures

1. Introduction

1.1. Pathogenic Resistance and Lipopeptides:

The development of resistance by bacteria and fungi to most of the clinically available antimicrobials has been an emerging concern to modern medicine. Though the resistance is more commonly reported in bacteria, the problem is equally critical in the management of fungal infections due to the availability of only a very limited number of antifungal agents for systemic infections. The use of several other antifungal agents is limited to the skin infections due to their harmful side effects (Vandeputte et al., 2012). The increasing emergence of drug-resistant pathogenic strains along with the lack of options to treat systemic fungal infections emphasizes the need for a new class of antifungals with a novel mechanism of action. Despite the technological advances in the areas of synthetic chemistry, nature is the major source of new drugs, and natural products still play a crucial role in the drug discovery process. Two promising classes of naturally occurring compounds that have gained enormous attention in past few decades are host-defense antimicrobial peptides (AMPs) and lipopeptides.

Host-defense antimicrobial peptides (AMPs) are produced by all kinds of living organisms and constitute an important element of the innate immune system of any organism. These molecules are broad spectrum in their activity, being active against bacteria, fungi, and most other pathogens (Boman, 1995; Hancock and Diamond, 2000; Zasloff, 2002). Structurally, they are composed of 12-50 amino acids, approximately 50% of which are hydrophobic in nature. The level of hydrophobicity is an important determinant of the activity. Most of them adopt an amphipathic structure in proximity to membranes, which is considered to be essential for antimicrobial activity (Shai and Oren, 2001; Tossi and Sandri,

2002). AMPs are quite diverse as more than 1700 natural compounds have been isolated to date, and many more analogues have been synthesized (Brahmachary et al., 2004). Studies on the mechanism of action of AMPs have revealed that they act by disrupting membranes of pathogens, unlike conventional antibiotics, which have DNA or enzymes as specific targets. Also, the action of AMPs is rapid and more damaging. Due to these reasons, it is comparatively difficult for the pathogens to develop resistance against AMPs (Hancock and Sahl, 2006; Jenssen et al., 2006; Peschel and Sahl, 2006; Zasloff, 2002). It is believed that these AMPs, owing to their positive charge, bind to negatively charged LPS (lipopolysaccharide) of gram negative bacteria or lipoteichoic acid of gram positive bacteria, which is followed by permeation and disruption of the phospholipid membrane. In fungi, AMPs cause lysis by initially binding to the membrane phosphatidylinositol (PI) or cell wall (rich in polybranched β-D-glucan) (Avrahami and Shai, 2004; Balkovec, 1994; De Lucca and Walsh, 1999; Powers and Hancock, 2003; Thevissen et al., 1999). Furthermore, it was found that these AMPs damage membranes by first accumulating at the cell surface at a concentration enough to penetrate the membrane, followed by membrane destruction in a detergent-like manner (Pouny et al., 1992; Shai, 2002). Hence, AMPs have demonstrated huge potential as lead molecules in the discovery of antimicrobials against multi-drug resistant pathogens.

Lipopeptides are amphipathic compounds produced only by bacteria and fungi (Balkovec, 1994; De Lucca and Walsh, 1999). They are characterized by a hydrophobic lipid tail attached to a peptide moiety. The fatty acid can have a varied number of carbon atoms, while the peptide portion may be either cationic or anionic and can be composed of both proteinaceous and non-proteinaceous amino acids. The length of lipid tail and the amino acid

sequence of peptide chain determine the cell specificity and spectrum of antimicrobial activity of the lipopeptide (Makovitzki et al., 2006). A few classical examples of lipopeptides with a cationic peptide motif are polymyxin B and colistin, while those with an anionic peptide motif are daptomycin and surfactin (Jerala, 2007; Strieker and Marahiel, 2009). The mechanism of action of most of these lipopeptides is still not completely understood. There has been a consensus that these lipopeptides, due to their hydrophobic nature, act on membranes to show their lytic activity. However, one of the biggest hurdles in the clinical use of these lipopeptides is their non-specific action. They damage both pathogenic and host cells. Recently, daptomycin was approved by the Food and Drug Administration for topical use only (Steenbergen et al., 2005). Table 1 shows some natural product-based lipopeptide antibiotics currently in preclinical and clinical studies (Pirri et al., 2009). Nevertheless, the use of lipopeptides for treating systemic infections is still limited.

Lipopeptides, especially the ones obtained from *Bacillus* species, also find their use as biocontrol agents to combat several phytopathogens, including bacteria, fungi, and oomycetes (Ongena and Jacques, 2008).

One of the most common bacterial strains that is known for producing a wide variety of antimicrobial lipopeptides is *Bacillus subtilis*. Approximately 4-5% of its genome encodes for more than two dozen antimicrobials (Stein, 2005).

Table 1: Natural product derived lipopeptides currently in pre-clinical and clinical studies.

Compound name (commercial name)	Therapeutic area	Method of Manufacture	Lead compound & producing organism	Company	Phase
Daptomycin (Cubicin)	Complicated infections of skin & skin structure/*S. aureus* bacteremia & right-sided endocarditis	Fermentation	Daptomycin & *Streptomyces roseosporus* NRRL11379	Cubist	Market
CB-182, 804	Gram-negative infections	Semi-synthetic	-----	Cubist	IND
WAP-8294A$_2$	MRSA infections	Fermentation	WAP-8294A$_2$ & *Lysobacter* sp.	aRigen	Phase I
NAB739 & NAB7061	MDR Gram-negative bacterial infections	Semi-synthetic	Polymyxin B/colistin & *Bacillus polymyxa*	Northern Antibiotics	Preclinical
MX-2401	Serious gram positive bacterial infections	Semi-synthetic	Amphomycin & *Streptomyces canus* ATCC 12237	Migenix	Preclinical
Lipohexapeptides HB1275 & HB1345	Acne, rosacea, MRSA & coutaneous mycoses	Synthetic	HB1275 & HB1345 None	Helix Biomedix	Preclinical
Telavancin	Complicated skin & skin structure infections (cSSSI) caused by Gram-positive bacteria	Semi-synthetic	Vancomyncin & *Amycolatopsis orientalis*	Theravance	NDA
Caspofungin (Cancidas)	Antifungal	Semi-synthetic	Echinocandins & *Glarea lozoyensis*	Merck and Co.	Market
Micafungin (Mycamine)	Antifungal	Semi-synthetic	Echinocandins & *Coleophoma empetri*	Astellas Pharmaceuticals	Market
Anidulafungin (Eraxis)	Antifungal	Semi-synthetic	Echinocandins & *Aspergillus nidulans*	Pfizer Pharmaceuticals	Market

1.2. *Bacillus* Lipopeptides:

Iturins, surfactins, and fengycins are the major classes of macrocyclic biologically active lipopeptides, among more than two dozen antibiotics isolated from different strains of *Bacillus* (Ongena and Jacques, 2008). Iturins are mainly produced by *B. subtilis* (Bonmatin et al., 2003) and *B. amyloliquefaciens* (Koumoutsi et al., 2004); surfactins and similar lipopeptides by *B. coagulans* (Huszcza and Burczyk, 2006), *B. pumilus*, and *B. licheniformis* (Peypoux et al., 1999); and fengycins by *B. cereus* (Tsuge et al., 1999), *B. thuringiensis* (Kim et al., 2004), *B. subtilis* (Jacques et al., 1999), and *B. amyloliquefaciens*. These compounds are macrocyclic in nature and possess antibacterial, antifungal, and surfactant properties. In a study by Chen et al. (2008), the antimicrobial activity of *B. subtilis* lipoheptapeptides was demonstrated against the species *F. graminearum*, *R. solani*, *P. irregulare*, *C. fulvum*, *B. cineria*, *A. niger*, and *P. expansum*. In general, surfactins possess broad spectrum antibacterial activity, while iturins and fengycins are fungicidal in nature. Due to these antimicrobial lipopeptides, *B. subtilis* finds its potential application as a biocontrol agent in the agriculture and food industries.

Surfactins have several variants, but all of them are heptapeptides cyclized by the hydroxyl group of a β-hydroxy fatty acid (Figure 1) (Peypoux et al., 1999). Surfactins are strong surfactants which endow them with powerful emulsifying properties. Furthermore, due to their amphiphilic nature, they have the ability to disrupt biological membranes. Studies have suggested that at concentrations of CMC (Critical Micellar Concentration), surfactins can completely dissolve and disrupt a membrane (Carrillo et al., 2003; Heerklotz and Seelig, 2007). However, the amount of cholesterol in the membrane can adversely affect the membrane perturbing ability of surfactins. High cholesterol content in membranes

diminishes the surfactin activity (Carrillo et al., 2003). This explains why surfactins possess strong antibacterial, antiviral, and hemolytic activities, but no marked fungicidal activity.

The antimicrobial activity of iturins against different strains like *M. luteus*, *S. cerevisiae*, and *Candida* species has been established (Besson et al., 1978; Besson et al., 1979; Fickers et al., 2009). Basically, iturins possess strong antifungal activity against different kinds of fungi and yeasts, and mild antibacterial activity against *Micrococcus* and *Sarcina* species (Delcambe and Devignat, 1957). However, these molecules are also strongly hemolytic, which limits their clinical use. Iturins are characterized by a heptapeptide of alpha amino acids of the LDDLLDL configuration cyclized with a beta amino fatty acid of the R configuration. Iturins (A-E), Bacillomycins L, D, F, Lc, and mycosubtilin are some of the variants of the iturin class which have some common structural properties (Figure 1) (Ongena and Jacques, 2008).

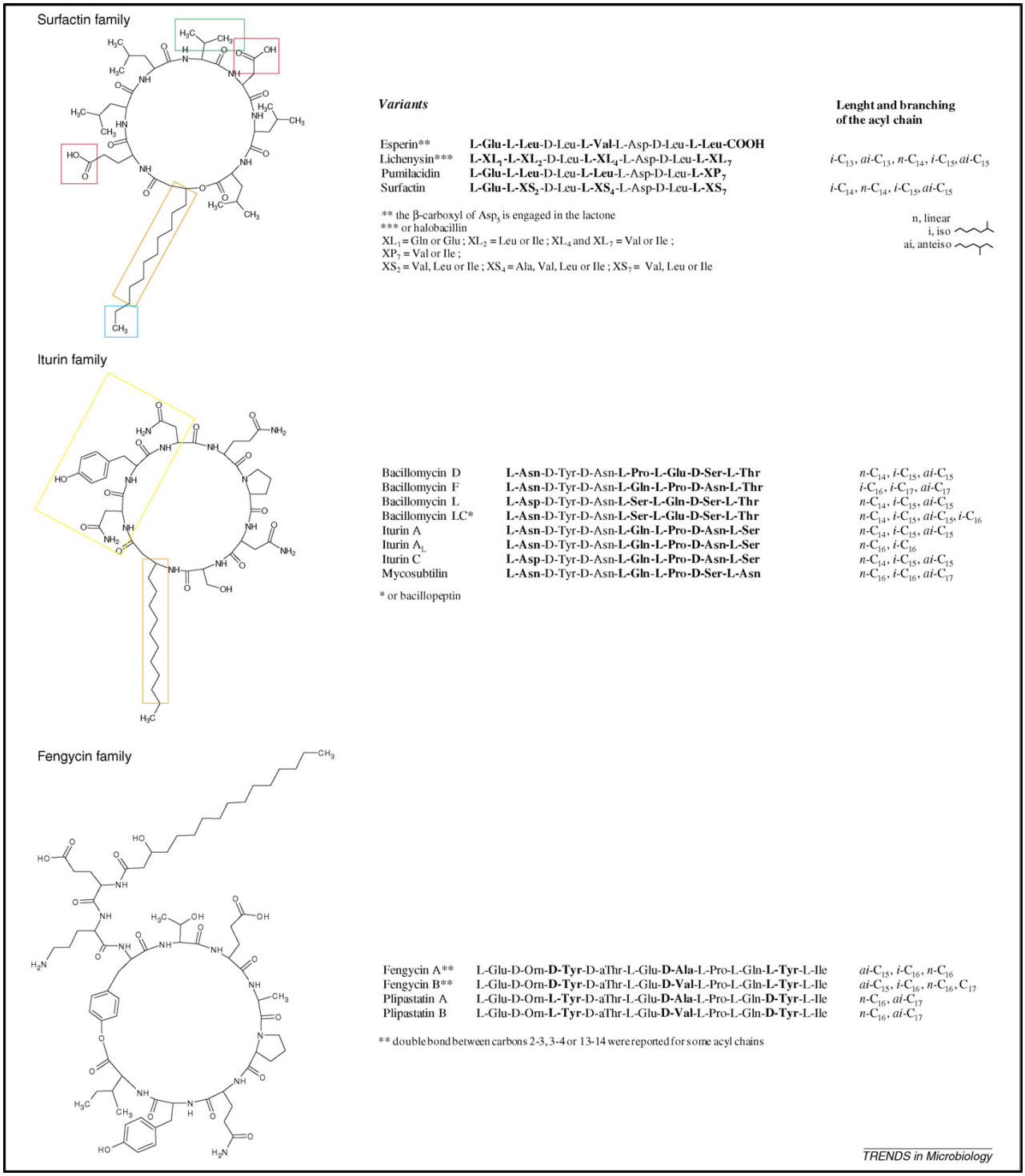


Figure 1: Structures of major classes of lipopetides from strains of *Bacillus* and the amino acid sequence of their structural variants. *Bacillus* lipopeptides are characterized by a macrocyclic peptide ring structure attached to a lipid tail.

1.3. Mycosubtilin:

Mycosubtilin is the most potent antifungal agent from the iturin class (Besson et al., 1979). It is composed of a heptapeptide of α-amino acids cyclized by a beta amino acid that has a long fatty acid chain attached to it. The amino acid sequence of mycosubtilin follows the order Asn-Tyr-Asn-Gln-Pro-Ser-Asn from N to C terminus, with the characteristic configuration LDDLLDL. The natural molecule has C_{14} to C_{17} carbon atoms in the β-amino acid (Peypoux et al., 1976).

Structurally, mycosubtilin differs from iturin A only in the inversion of the last two amino acids. Hence, iturin A contains D-Asn and L-Ser, while mycosubtilin contains D-Ser and L-Asn as the amino acids at C-terminus (Figure 2). This slight structural difference leads to a dramatic difference in their activities. Recent studies have shown the superiority of mycosubtilin over iturin A as antimicrobial agents (Maget-Dana and Peypoux, 1994). Structure-activity relationship (SAR) studies provide a better idea of the amino acids and groups required for the antifungal activity of iturins, which further help in optimizing their activities.

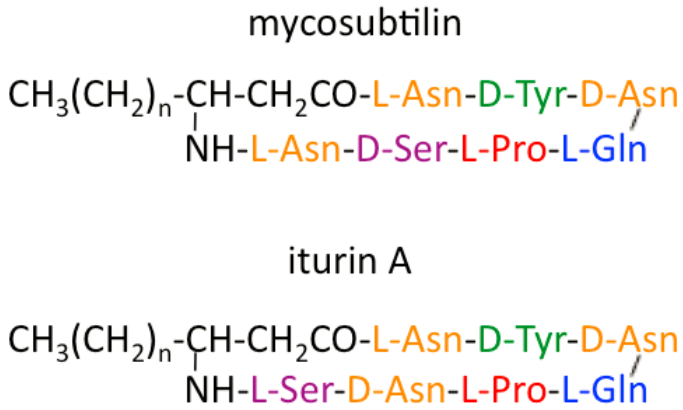


Figure 2: Structure of mycosubtilin and iturin A. α-amino acids are shown in color and the β-amino fatty acid is shown in black. Natural occurring molecule contains 14 to 17 carbon atoms in the β-amino acid.

1.3.1. Structure-Activity Relationship (SAR) studies:

Previous SAR studies have shown that the first three amino acids in iturins, L-Asx (x=p or n), D-Tyr, and D-Asn are important for the retention of antimicrobial activity. This is evident from the fact that the replacement of L-Asn in iturin A by L-Asp in iturin C leads to a loss of antimicrobial activity (Volpon et al., 2007). Also, the methylation of the phenolic group of D-Tyr causes substantial reduction in activity. This suggests that these three amino acids in iturins may be responsible for interaction with the target membranes.

SAR studies revealed the importance of a tyrosine residue at the 2nd position for the retention of antimicrobial activity. Methylation or acetylation of the phenolic group of tyrosine resulted in a substantial decrease in the antibacterial activity of mycosubtilin on *Micrococcus luteus*. Methylation caused an absolute loss of activity with 0% growth inhibition of *M. luteus* after 8 hours for 20 µg/ml concentration of antibiotic. Acetylation of the tyrosine phenolic group in mycosubtilin resulted in slightly higher activity than the methylated derivative, with 6% inhibition at 20 µg/ml and 22% inhibition at 60 µg/ml, compared to 0% and 3% with the methylated derivative, respectively. This indicates the importance of a polar hydroxyl group in the activity of mycosubtilin. A similar reduction in activity was observed with acetylation of the hydroxyl group of serine in mycosubtilin, again indicating the importance of polarity in retention of activity (Peypoux et al., 1979).

Besson et al. (1979) performed the antifungal activity testing of iturin A, mycosubtilin, bacillomycin L, and their derivatives on *S. cerevisiae* and found that methylation and acetylation of tyrosine and serine lead to a marked reduction in antifungal activity, similar to antibacterial activity on *M. luteus*. Hence, it was concluded that the polar hydroxyl groups play a crucial role in the antimicrobial activity of iturins.

The conformational studies on mycosubtilin in solution by 2-D NMR demonstrated that the cyclic peptide is highly rigid in the region of L-proline and more flexible in the region of D-tyrosine (Genest et al., 1987). This supports the fact that the flexible D-tyrosine may be responsible for interactions with lipid/biological membranes and possibly for their disruption.

1.3.2. Mechanism of Action:

The mechanism of action of mycosubtilin has not been fully elucidated. A number of studies have been reported on the interaction of mycosubtilin with target cellular membranes; however, the molecular mechanism behind its activity is not yet thoroughly explored (Nasir and Besson, 2011). It has been proposed that iturins act by inducing pore formation in cytoplasmic membranes, which leads to an increase in K^+ permeability (Maget-Dana and Peypoux, 1994). The change in permeability of plasma membranes also leads to the release of biomolecules like proteins, nucleotides, and lipids from cells, which ultimately causes cell death (Besson and Michel, 1989).

In a study by Maget-Dana et al. (1985), the ability of iturin A to form pores in the membranes was evaluated by the measurement of electrical conductance in bimolecular lipid membranes (BLM). The addition of low concentrations of iturin A to the bimolecular membrane showed an increase in the electrical conductance of the membrane in discrete patterns. This suggests the formation of pores in the membranes by iturins. Additionally, iturins in the presence of cholesterol showed a dramatic increase in the electrical conductance of the membrane. This is attributed to the simultaneous opening of pores, along with formation of new pores. Hence, the study suggests that iturins may induce pore formation in

the membranes, the extent of which depends on the concentration of cholesterol in the membrane.

The monolayer and multilamellar studies on the interaction of mycosubtilin with phosphatidylcholine phospholipid (DPMC) and cholesterol containing membranes suggest that mycosubtilin has a higher affinity for cholesterol than phosholipids. Nasir et al. (2010) demonstrated the ability of mycosubtilin to bind to DPMC phospholipid monolayer membranes and reported the constant exclusion pressure during the mycosubtilin-membrane interaction. This interaction involves mycosubtilin aggregate formation in the phospholipid monolayer, leading to a change in conformation and damage to the membrane. In another study by Nasir and Besson (2011), the higher exclusion pressure was demonstrated for the phospholipid/cholesterol (DMPC-cholesterol) model compared to phospholipid (DPMC) alone, indicating the higher affinity of mycosubtilin for cholesterol than for phospholipids. The monolayer studies on cholesterol containing membranes indicate the involvement of the hydroxyl group of cholesterol in the interaction with mycosubtilin, as lower exclusion pressure was observed on derivatization of hydroxyl to an acetyl group. The multilamellar studies have shown significant structural changes in the peptide backbone and the tyrosyl residue of mycosubtilin induced by cholesterol, indicating the involvement of the tyrosyl phenolic group of mycosubtilin in the cholesterol-mycosubtilin interaction. Hence, in conclusion, mycosubtilin possesses preferential affinity for cholesterol over other sterols and lipids, and its antifungal activity is possibly the result of the interaction between its polar residues and the hydroxyl group of cholesterol in biological membranes.

In the antifungal activity study of mycosubtilin and other iturins on *S. cerevisiae*, cholesterol was found to be a strong inhibitor of the activity (Besson et al., 1979).

Furthermore, no reduction in activity was observed with cholesterol derivatives like cholesterol methyl ether, cholesterol acetate, and cholesterol stearate. These results are in accordance with the other results suggesting mycosubtilin interaction with the hydroxyl group of cholesterol.

1.4. Mycosubtilin Production:

There are three major biosynthetic pathways by which *Bacillus* species synthesize secondary metabolites: Polyketide synthetases (PKSs), non-ribosomal peptide synthetases (NRPSs), and hybrid PKSs/NRPSs. These pathways, owing to their modular nature, produce both a structurally and functionally diverse group of biologically active molecules, which includes immunosuppressants, antimicrobials, and antitumor agents.

Type I PKSs in bacteria are the multifunctional proteins with multiple catalytic sites, termed as modules. Each module catalyzes one cycle of chain elongation and is composed of multiple domains. A typical module is composed minimally of an acyltransferase (AT) domain, acyl carrier protein (ACP), and a ketoacyl synthase (KS) domain. AT and ACP are involved in building block unit selection and loading, while the KS domain catalyzes the chain elongation or condensation reaction between the growing chain and the building blocks. These modules can have some additional domains like a ketoreductase (KR) for reducing a β-ketone to an alcohol, a dehydratase (DH) for dehydration of an alcohol to an alkene, and an enoyl reductase (ER) to yield the saturated product. An amino-transferase (AMT) domain is the link between PKSs and NRPSs in hybrid PKSs/NRPSs systems that converts a fatty acid to an amino acid (Figure 3) (Du et al., 2001).

Non-ribosomal peptides are short peptides of generally less than 20 amino acids. NRPs can be linear, cyclic, or branched and may contain both proteinaceous and non-proteinaceous amino acids. The ability of NRPSs to incorporate a wide variety of amino acids in the growing peptide chain, combined with its modular nature, explains the huge diversity in the products produced by this pathway. The order and number of modules in NRPSs determine the sequence and number of amino acids in the peptide chain. A typical module is composed of an adenylation (A) domain for activation of a specific amino acid as an aminoacyl adenylate, a thiolation (T) domain or peptidyl carrier protein (PCP) for thioesterification of the amino acid and its attachment to a PCP domain, and a condensation (C) domain for the transpeptidation reaction and chain elongation (Figure 3). Additional domains for epimerization (E) and N-methylation can modify the amino acids before their incorporation in the chain (De Crecy-Lagard et al., 1995; Marahiel et al., 1997).

PKSs and NRPSs share a similar modular strategy for the biosynthesis of two different types of natural products. Both of them have a carrier protein, which is tethered to the growing chain throughout the elongation process. Once the chain reaches the most C-terminal carrier protein and has grown completely in length, it is released by the thioesterase (TE) domain located at the distal C-terminus of the pathway (Figure 3).

The release of the product involves initial transfer of the chain from the terminal carrier protein (ACP or PCP) to the serine residue of the TE domain to form a product-O-TE intermediate. This is followed by either a hydrolysis reaction to give a linear product (the hydrolysis product) or cyclization of the chain to yield a cyclization product (Figure 3).

Mycosubtilin is produced by a PKSs/NRPSs hybrid system, which is composed of four open reading frames *fenF*, *mycA*, *mycB*, and *mycC* (Figure 3) (Duitman et al., 1999).

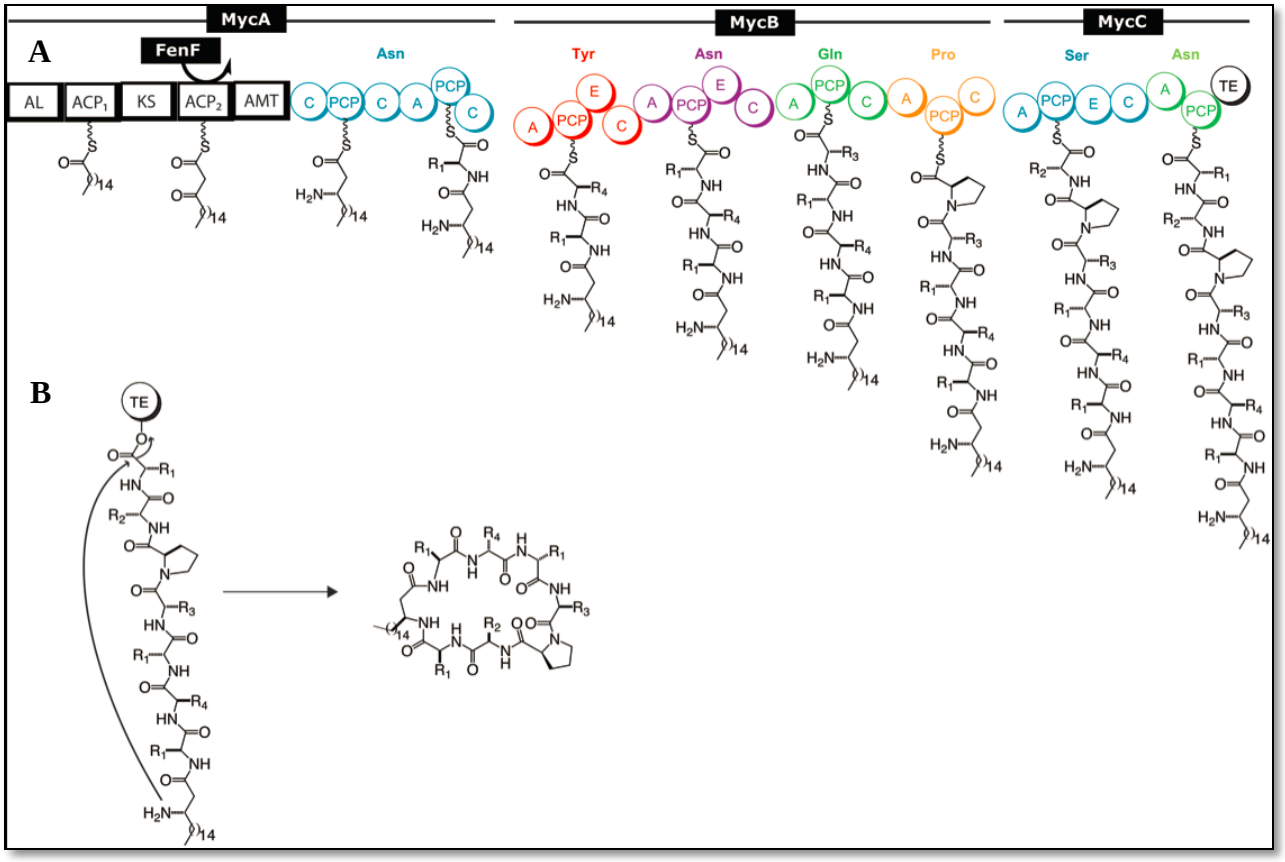


Figure 3: **A)** Diagram showing PKSs/NRPSs biosynthetic pathway for mycosubtilin production. PKS domains are depicted with squares and NRPSs domains are depicted with circles. Each module is shown in a different color. AL= acyl CoA ligase, ACP= acyl carrier protein, KS= ketosynthase, AMT= aminotransferase, C= condensation, PCP= peptidyl carrier protein, A= adenylation, E= epimerization, TE= thioesterase.

B) Cyclization of the linear peptide chain by TE (thioesterase) domain located at the distal C-terminus of the biosynthetic pathway.

The process starts when aminoacyl ligase (AL) domain loads palmitic acid onto the carrier protein, ACP1, and fenF loads malonyl-CoA onto ACP2. This is followed by their condensation by KS domain to give a β-keto thioester, which is readily aminated by AMT domain to produce a β-amino thioester tethered to PCP domain. At this point, the β-amino thioester enters the NRPSs pathway. The adenylation (A) domain then activates the first

amino acid, asparagine, which is incorporated into the growing chain by the condensation (C) domain. The chain then moves to the PCP domain of the next amino acid, tyrosine. The process continues until all the amino acids are sequentially added to the growing chain by each cluster of domains and the fully grown peptide chain reaches the terminal PCP domain (Duitman et al., 1999). The TE (thioesterase) domain, here, catalyzes the final step of cyclization to produce macrocyclic mycosubtilin.

1.5. Isolated thioesterase as a tool for cyclization:

TE domains can produce different cyclization products, depending on the type and location of the nucleophile in the linear chain. If the nucleophile is an amino group at the N-terminus of the peptide, head-to-tail cyclization takes place to yield the product. Similarly, a branched cyclic molecule results if the nucleophile is a group in the side chain.

Previous studies have demonstrated the ability of isolated thioesterase domains to cyclize lipopeptides of diverse families. This promiscuity of catalytic activity of thioesterase can be utilized to produce a pool of natural macrocyclic products and their variants. Trauger et al. (2000) demonstrated the ability of isolated TE from the Tyrocidine NRPS pathway to cyclize peptides that meet minimal requirements of a few conserved amino acids. This was followed by another study by Kohli et al. (2001), in which thioesterases isolated from different biosynthetic pathways of tyrocidine, gramicidin S, and surfactin were tested for their cyclization ability (Figure 4). All of the TEs were found to be liberal in their substrate selection for catalytic activity. Furthermore, a small pool of cyclic peptides was produced using isolated Tyc TE. This special ability of isolated TEs offers an excellent way to synthesize macrocyclic lipopeptides using a chemoenzymatic approach.

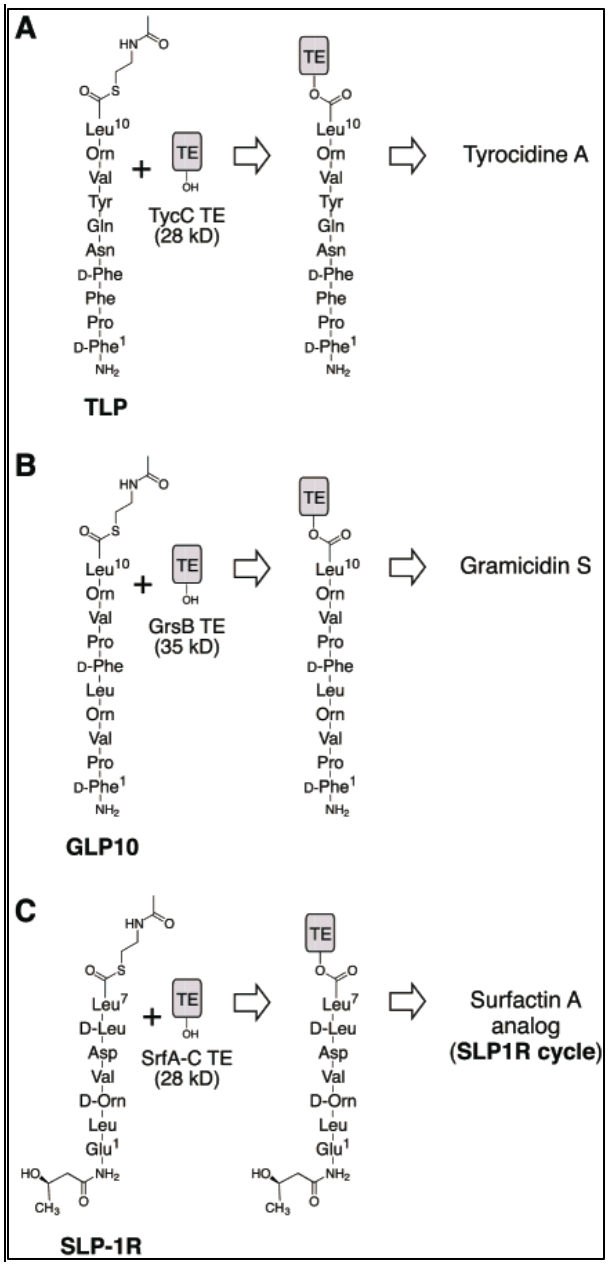


Figure 4: Diagram showing the cyclization of peptide thioester substrates using isolated TEs from biosynthetic pathways of tyrocidine A, gramicidin S and surfactin A to yield respective macrocyclic products.

1.6. Hypothesis:

With the identification of some mycoses-causing fungal strains resistant to the current therapy, molecules like mycosubtilin have gained enormous interest in the past few years.

Additionally, mycosubtilin has shown its potent activity against a few resistant fungal strains (Fickers et al., 2009). Clearly, mycosubtilin has a huge potential as an effective antifungal agent. However, its clinical use has been limited by its deleterious hemolytic activity (Besson et al., 1989).

This study aims at synthesizing an analogue of mycosubtilin, with strong antifungal but little or no hemolytic activity. Assuming that the hemolytic activity is partially or completely controlled by the lipid tail, more specifically, we are synthesizing a mycosubtilin analogue with a modified lipid tail. The synthetic analogue has the same α-amino acid sequence as the natural occurring molecule; however, it has just 7 carbon atoms in the β-amino acid compared to 14 to 17 carbon atoms in the natural mycosubtilin. Hence, the present study probes the effect of shortening the lipid tail on the antifungal and hemolytic activity of mycosubtilin.

A chemoenzymatic approach was used to synthesize the mycosubtilin analogue. The linear peptide chain was synthesized using solid phase peptide synthesis (Figure 5) and cyclized using the TE excised from the mycosubtilin biosynthetic pathway of *B. subtilis* ATCC 6633.

1.7. Solid Phase Peptide Synthesis (SPPS):

As the name suggests, in SPPS, the peptide is synthesized on a solid support followed by its cleavage to yield the desired peptide product. Different kinds of resins are used as solid support. The α-amino group and the reactive side chains of all the amino acids are protected to prevent any polymerization or side reactions. The process starts by attaching the C-terminus of the first amino acid to the resin. This is followed by sequential addition of the

amino acids to the resin to obtain the peptide chain of desired amino acid sequence (Merrifield, 1963).

Each amino acid addition involves a set of coupling-wash-deprotection-wash steps (Figure 5) (Protein Technologies, Inc.). For the amino acid addition to the growing peptide chain, initially the N-terminus of the amino acid (attached to resin) is deprotected using piperidine. Also, the C-terminus of the incoming amino acid is activated using an activating agent, HBTU or HOBt, to form a reactive ester which allows a better nucleophilic attack from nitrogen to form an amide bond. Each deprotection and coupling is followed by a wash step to remove any excess reagent left from the last reaction.

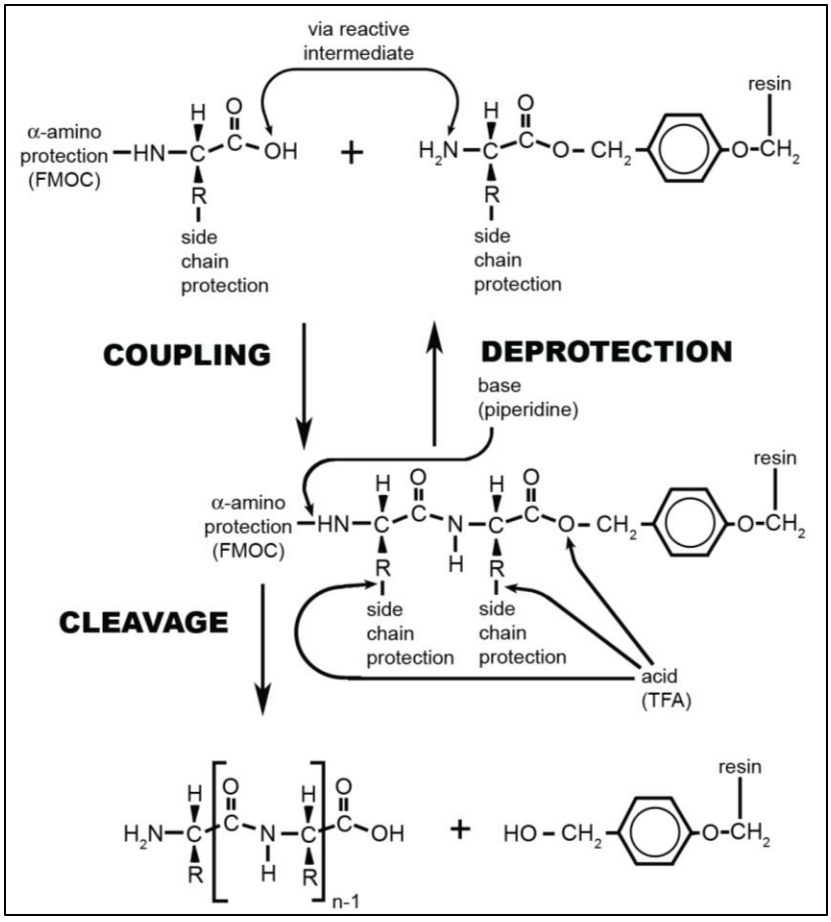


Figure 5: Scheme for Solid Phase Peptide Synthesis (SPPS).

Once the complete chain of amino acids is synthesized, the peptide is cleaved from the resin and the side chain protecting groups are removed to yield the desired product.

SPPS is the accepted method for synthesizing peptides on the laboratory scale. The extreme success of this method in the field of peptide synthesis lies in the high yield of product obtained compared to any other method, which is the result of repeated wash cycles and the addition of amino acids in excess to the resin.

One of the simple, reliable, and efficient synthesizers based on the principle of SPPS is the PS3 synthesizer (Figure 6) (Protein Technologies, Inc.). The resin is added to the reaction vessel and the Fmoc-amino acids, mixed with activating agent, are added to the vials. These vials are then loaded onto the carousel from C to N terminus. Piperidine is used as the deprotecting agent, and a base is used to aid in the activation of amino acids.

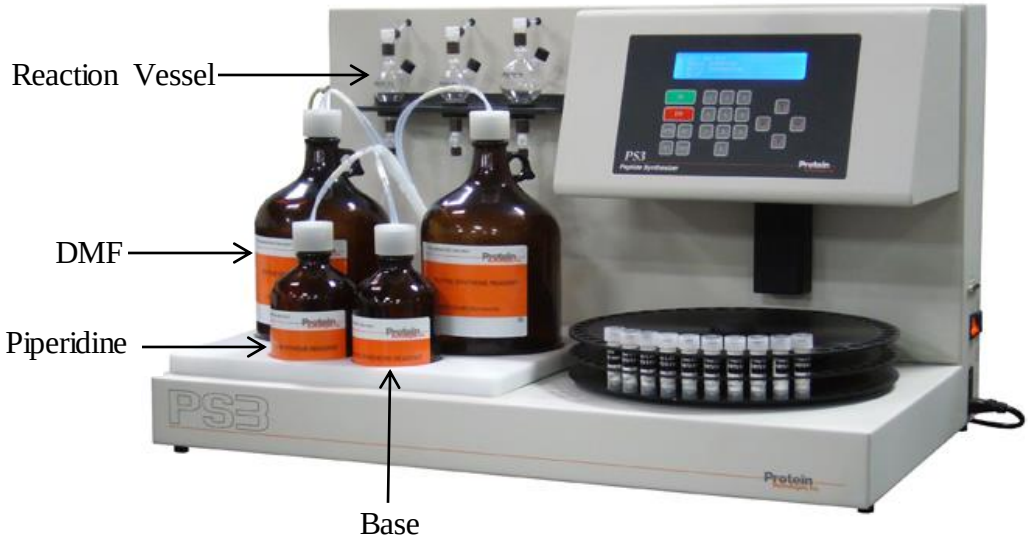


Figure 6: PS3 Peptide Synthesizer. Fmoc-amino acids mixed with activating agent are placed in the vials. Piperidine is the deprotecting agent and DMF (dimethylformamide) is the solvent.

2. Experimental Procedures

Mycosubtilin was synthesized on a laboratory scale using a three-step approach, which involved initial formation of the linear peptide chain of the desired amino acid sequence (by SPPS), followed by derivatization of the peptide at the C-terminus using thiophenol, and finally enzymatic cyclization of this modified peptide using an isolated thioesterase (TEase) domain from the mycosubtilin biosynthetic pathway in *Bacillus subtilis* ATCC 6633.

2.1. Synthesis of linear N-terminal protected peptide:

The peptide of the amino acid sequence 3-aminoheptanoic acid-Asn-Tyr-Asn-Gln-Pro-Ser-Asn was synthesized by solid phase peptide synthesis using Asn-derivatized 2-chlorotrityl resin, Fmoc protected amino acids (except for 3-aminoheptanoic acid which was Boc protected), the activating agent HBTU, and the deprotecting agent piperidine (Kohli et al., 2001). This N-terminal Boc protected peptide was then cleaved from the solid support to make the C-terminus free to react with thiophenol. The side chain protecting groups and N-terminal Boc protecting group ensure selective reaction of thiophenol with the carboxyl group at the C-terminus of the peptide. The details follow below.

2.1.1. Reagents:

H-Asn(Trt)-2-ClTrt resin, Fmoc protected α-amino acids, Boc protected β-3-amino-heptanoic acid, HBTU (O-benzotriazolyl-N,N,N',N'-tetramethyluronium-hexafluorophosphate), piperidine, DIEA (N,N-diisopropylethylamine), DMF (N,N-dimethylformamide), acetic acid, trifluoroethanol, methylene chloride, cold n-hexane, cold diethyl ether, and 35% acetonitrile/water solution.

2.1.2. Methods:

A) *Solid Phase Peptide Synthesis*:

The Fmoc-protected amino acids (0.4 mmol) mixed with activating agent HBTU (0.4 mmol) in separate vials were loaded on the peptide synthesizer in the desired sequence from C-terminus to N- terminus (D-Ser, Pro, Gln, D-Asn, D-Tyr, Asn and β-3-amino-heptanoic acid). The reactive side chains were protected with t-butyl (Tyr and Ser) and trityl (Gln and Asn) groups. Asn-derivatized 2-chlorotrityl resin (0.1 mmol) was added to the reaction vessel, and the peptide synthesis was initiated using DMF as solvent and piperidine as Fmoc-deprotecting agent. The synthesizer was programmed and the peptide synthesis was set to run overnight.

B) *Peptide Cleavage from Resin*:

The peptide was cleaved from the resin by reacting it with a mixture of 2 ml acetic acid, 2 ml trifluoroethanol, and 6 ml methylene chloride for 3 hours at 24˚C. The resin was then removed by filtration.

C) *Precipitation and Lyophilization*:

50 ml cold n-hexane was added to the peptide solution followed by the addition of 50 ml cold diethyl ether. No precipitation was observed. The solvents were then removed by rotary evaporation, and 2-3 ml of water was added to precipitate the peptide. The peptide was then dissolved in 35% acetonitrile solution in water and lyophilized to obtain a dried crystalline powder.

2.2. Thiophenyl derivatization of the peptide:

The peptide was derivatized at the C-terminus by first activating the carboxyl group with DCC and HOBt and then reacting it with thiophenol (Sieber et al., 2004). The side chain protecting groups, along with the Boc protecting group, were removed to get the deprotected peptidyl thioester. Details of the reaction follow below.

2.2.1. Reagents:

DCC (N,N'-dicyclohexylcarbodiimide), HOBt (1-hydroxybenzotriazole hydrate), thiophenol, potassium carbonate, THF (tetrahydrofuran), copper sulphate-treated silica, TFA (trifluoroacetic acid), trifluoroethanol, cold diethyl ether, 35% acetonitrile/water solution, and methanol.

2.2.2. Methods:

A) *Derivatization*:

The protected peptide (0.1 mmol) was reacted with DCC (0.2 mmol), HOBt (0.2 mmol), and thiophenol (1 mmol) in THF for 3 hours. Potassium carbonate (0.4 mmol) was added after 30 minutes. The reaction mixture was run through a filter packed with $CuSO_4$-treated silica to get rid of residual thiophenol.

B) *Deprotection*:

The solvent was removed in a rotary evaporator and the side chain protecting groups were removed by treating it with a mixture of 9.5 ml TFA, 0.25 ml trifluoroethanol, and 0.25 ml water for 2 hours.

C) *Precipitation, Filtration and Lyophilization*:

The deprotected peptide was precipitated using 50 ml cold diethyl ether, filtered, dissolved in 35% acetonitrile/water solution, and lyophilized to get the dried thiophenyl derivative of the peptide.

D) *Purification*:

The peptide was purified by Reverse Phase High Performance Liquid Chromatography (RP-HPLC) using an acetonitrile-water solvent system as the mobile phase. The C-18 highly hydrophobic column was employed as the stationary phase. After dissolving the peptide in a minimal amount of methanol, it was injected into the loop and was purified using a solvent gradient system of 10 to 50% acetonitrile over 2 hours at the flow rate of 10 ml/min. The absorbance was observed at 280 nm due to the presence of the tyrosine residue in the peptide. The eluent, corresponding to the peaks, was collected in test tubes and lyophilized. The purity of the peptide was tested by analytical RP-HPLC and found to be 95% by peak integration.

E) *Mass Spectrometric Analysis*:

The purified peptide was then characterized by electrospray ionization-Mass Spectrometry (ESI-MS) and time-of-flight (TOF) mass analyzer using ultramark 1621 (Lancaster Synthesis) as a calibration standard for the range 922-2021 amu. Results will be presented in the Results and Discussion section.

2.3. Cloning, Expression, and Purification of PCP-TE domain from *Bacillus subtilis*:

The thioesterase from *B. subtilis* ATCC 6633 was cloned into the ampicillin-resistant pET 21b plasmid vector using standard cloning procedure to give a C-terminal His-tag. It

was then overexpressed in *E. coli*, and proteins were purified using nickel affinity chromatography. Details are provided below.

2.3.1. Reagents:

B. subtilis ATCC 6633 strain, pET 21b plasmid vector, Plasmid Miniprep kit, Instagene matrix, Master mix, PCR clean up kit, primers, restriction enzymes (*XhoI* and *NdeI*), ligases, *E. coli* BL21 cells, isopropyl β-D-thiogalacto-pyranoside, lysozyme, resin, and imidazole solution.

2.3.2. Methods:

A) *Cloning*:

The cloning was done using an ampicillin-resistant pET 21b plasmid vector (Figure 7). *E. coli* cells containing plasmid DNA were plated on LB agar media. A small culture was then taken and inoculated into LB broth media (containing ampicillin), which was agitated overnight at 37˚C. It was centrifuged at 4000 rpm for 10 minutes; the supernatant was discarded and plasmid DNA was purified from the pellets using a Plasmid Miniprep kit. The concentration of DNA in solution was determined spectrophotometrically using water as the blank.

B. subtilis ATCC 6633 colonies were grown in agar media. A small amount of culture was scraped and suspended in 1 ml of autoclaved water. It was centrifuged (11000 rpm for 1 minute), and the supernatant was discarded. The genomic DNA was isolated by incubation with 200 µl of Instagene matrix at 56˚C for 30 minutes and 100˚C for 8 minutes. It was centrifuged (12000 rpm for 3 minutes), and the supernatant was used for amplification of genomic DNA using PCR (Polymerase Chain Reaction). A mixture of 25 µl of Master mix, 1 µl each of the two primers (20 µM concentration), and 3 µl of water was added to 20 µl of

genomic DNA solution, and PCR was set for DNA amplification. The amplified DNA was then subjected to gel electrophoresis and bands were compared to the standard ladder. A PCR clean-up kit was used to get rid of the remaining nucleotides, buffer, and dye. Two volumes (84 µl) of DNA binding buffer were added to the PCR product. The PCR product was transferred to the Zymo-spin column, and trapped DNA was washed with wash buffer and eluted using 8 µl of water.

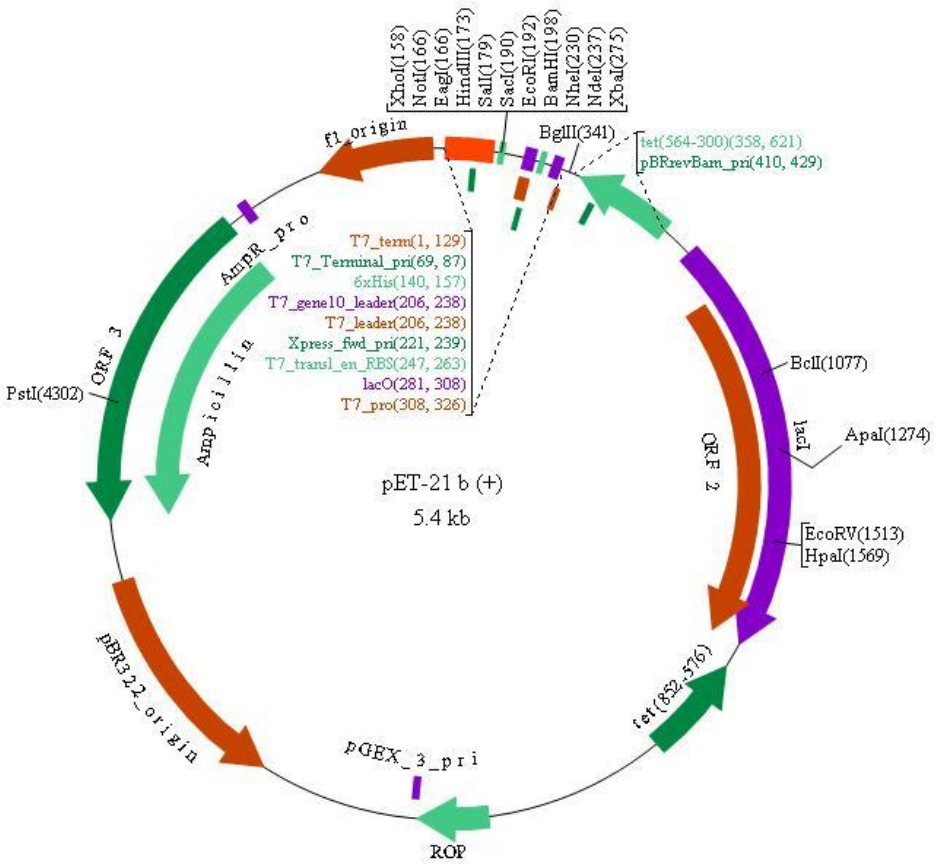


Figure 7: Diagram showing pET-21b plasmid DNA with a list of commonly used restriction enzymes. Ampicillin resistant gene is shown in green (BV Tech).

Both the plasmid DNA and insert (4 µl) were digested by incubating with 1 µl of *XhoI* and 1 µl of *NdeI* restriction enzymes at 37˚C for 1 hour (Figure 7). The enzymes were

deactivated by heating at 65˚C for 20 minutes. The digested insert and plasmid DNA were joined by incubating with DNA ligase in ligase buffer solution for 3 hours at room temperature. The construct was confirmed via DNA sequencing.

B) *Expression*:

The plasmid construct was transformed into *E. coli* BL21 competent cells for protein expression. The cells were grown in the TB media in a volume of 2 liters at 37˚C, which was then cooled to 18˚C. Isopropyl β-D-thiogalacto-pyranoside was then added at a final concentration of 0.2mM and agitated overnight. It was then centrifuged and pellets obtained were used to harvest proteins.

C) *Purification*:

The proteins were purified from pellets by lysing the cells in the presence of lysozyme and lysis buffer using sonication. The pellets were suspended in 25 ml of lysis buffer, cooled and then subjected to repeated cycles of sonication for 15 seconds with 45 second intervals. It was then centrifuged for 30 minutes and the supernatant was incubated with nickel beads to trap the desired proteins on the beads. The slurry was then poured on the column and washed with lysis buffer. Non-specific proteins were removed by 20 mM imidazole solution and finally, the desired proteins were eluted using 400 mM imidazole solution (Figure 8). The identity of proteins was confirmed by SDS-PAGE.

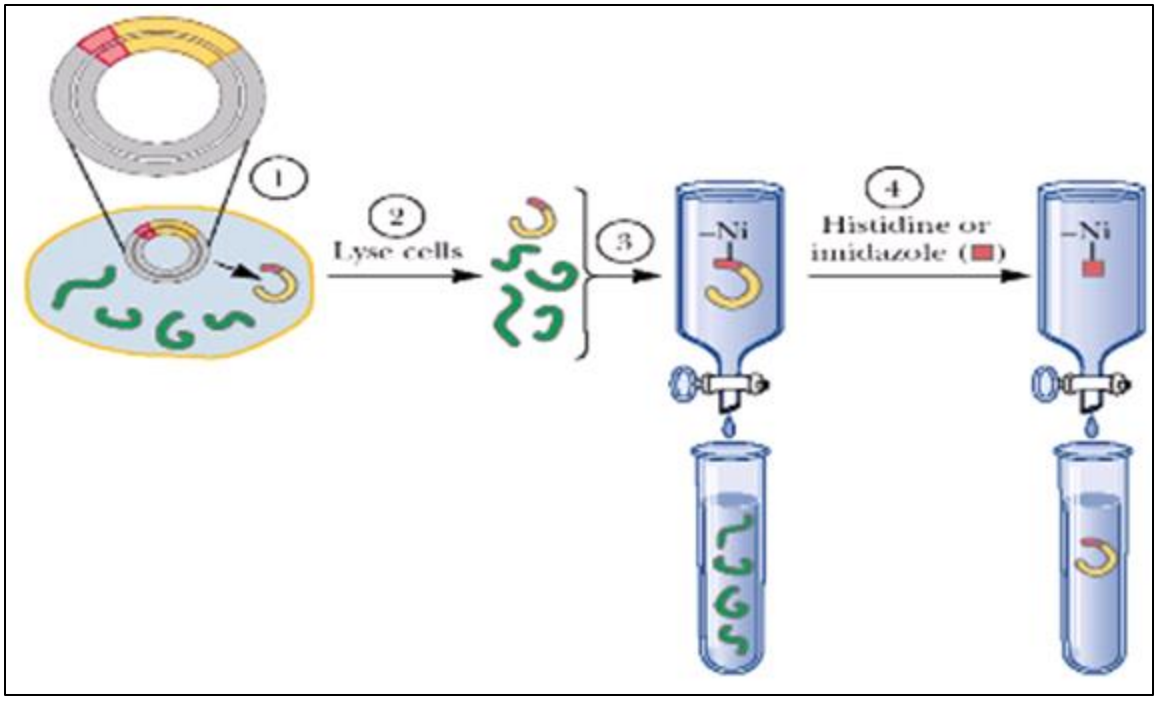


Figure 8: Peptide purification using Affinity Chromatography.

2.4. DTNB-Thiols Assay:

The catalytic activity of enzyme was tested by DTNB-Thiols Assay. The peptide was treated with the enzyme and DTNB was added to the reaction mixture. The observation of yellow color indicates a positive enzyme activity (Reaction 1) (Thermo Scientific). The details follow below.

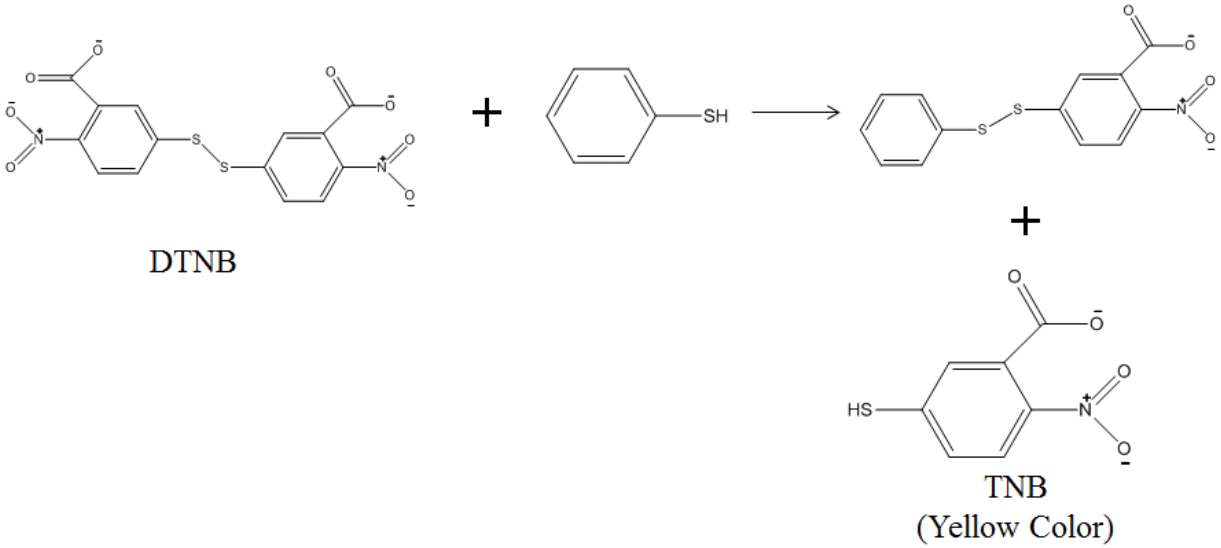
2.4.1. Reagents:

HEPES, sodium chloride, DMSO (Dimethyl Sulfoxide), peptidyl thiophenol, enzyme (thioesterase), and DTNB (5,5'-dithiobis-(2-nitrobenzoic acid))

2.4.2. Methods:

Buffer solution was prepared with a final concentration of 25mM HEPES and 50mM NaCl (pH 7.0). 5 µl of peptide solution (1mM) in DMSO was mixed with 37 µl of buffer solution and the reaction was initiated by adding 8 µl of enzyme. The reaction was set to go

for 1 hour. A saturated solution of DTNB in 50 mM phosphate buffer (pH 8.0) was added to the reaction mixture and the absorbance was measured at 412 nm (Reaction 1). A higher absorbance compared to the control indicates a positive enzyme activity.



Reaction 1: DTNB reacts with free thiophenol (produced by TE) to produce yellow color. The absorbance was observed at 412 nm.

2.5. Cyclization of thiophenyl peptide substrate using the isolated PCP-TE:

The thiophenyl peptide substrate was cyclized using the isolated thioesterase domain using a similar reaction as described in the DTNB-Thiols Assay, and analyzed by Mass Spectrometry (ESI-MS). Results will be presented in the Results and Discussion section.

3. Results and Discussion

3.1. Peptide synthesis and derivatization:

Mass Spectrometry (ESI-MS) of the thiophenyl derivatized peptide showed peaks at molecular weight 1055, 1056, and 1077 Da (Figure 9).

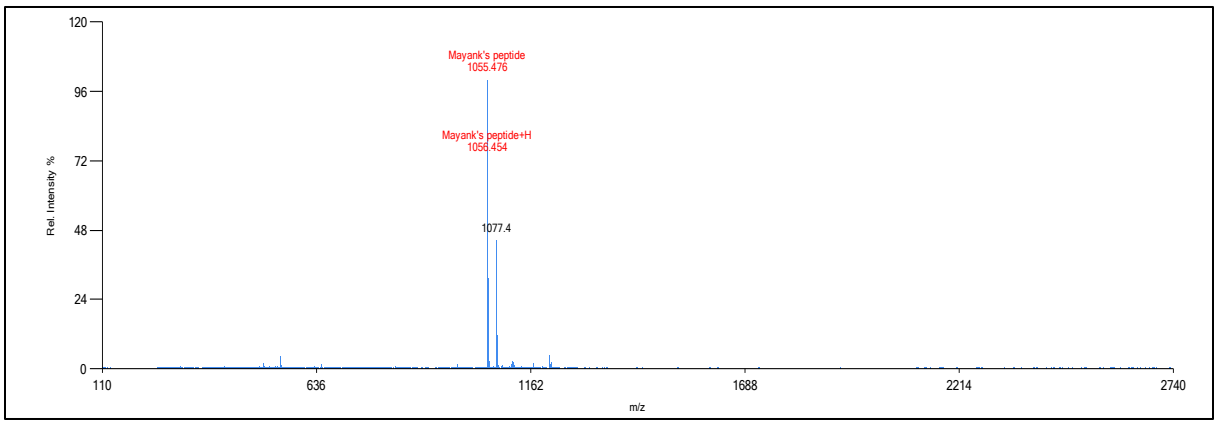


Figure 9: Mass Spectrometry (ESI-MS) of peptidyl thiophenol.

The expected molecular weight of the derivatized peptide was 1055 Da. Peaks at 1056 and 1077 Da correspond to Peptide + H^+ and sodium adduct of the peptide respectively. These results confirm the identity of the derivatized peptide and indicate that the synthesis protocol was successful. Sharp peaks affirm the purity of product.

3.2. Cloning, Expression, and Purification of PCP-TE domain:

The Gel electrophoresis of the PCR product showed a sharp band at 1000 bp, which matches with the expected base pairs of the PCP-TE domain from *Bacillus subtilis*. This PCP-TE domain was then cloned into the pET21b vector to give the C-terminal histidine tag. The identity of the construct was confirmed by DNA sequencing results. It was then

overexpressed in *E. coli* and proteins were purified using nickel affinity beads. SDS-PAGE of the purified proteins showed bands just below 37 kDa in accordance with the reported data (Figure 10).

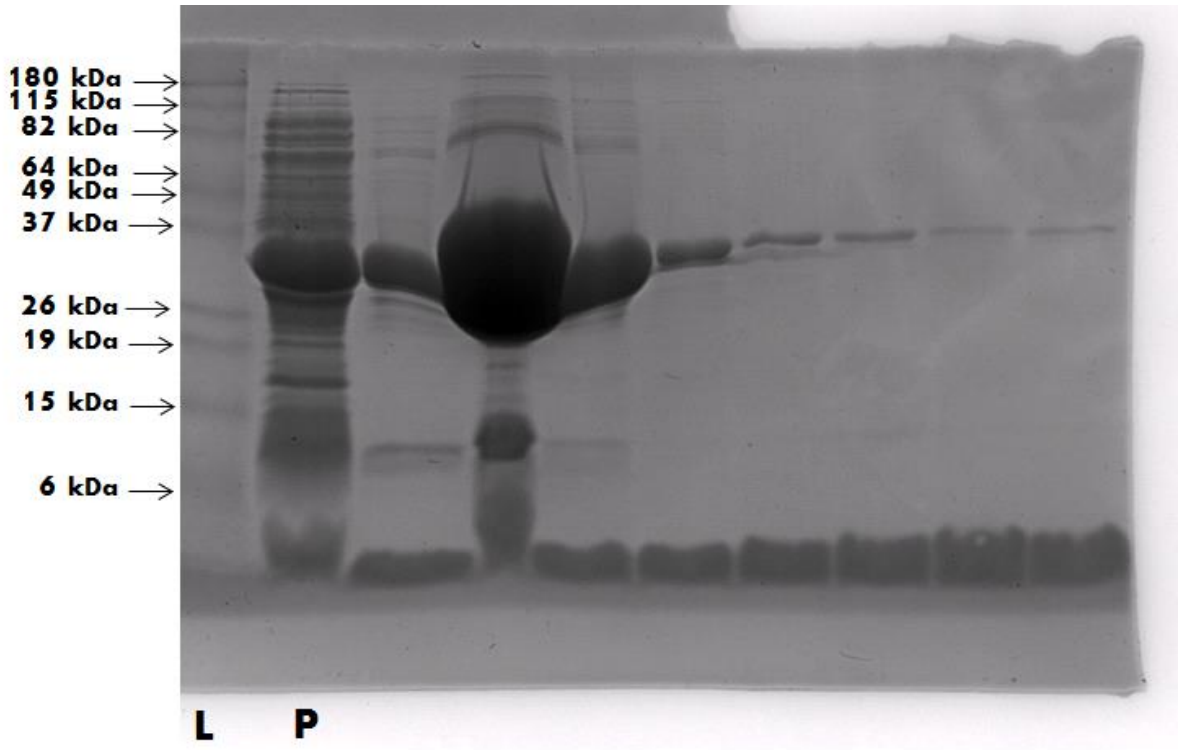


Figure 10: SDS-PAGE of the purified protein. L= Ladder and P= Protein.

3.3. DTNB-Thiols Assay:

In this assay, DTNB reacts with the free thiol to produce a yellow color, the absorbance of which is measured spectrophotometrically (Reaction 1). Hence, an active thioester, owing to its ability to yield a free thiophenol from thiophenyl peptide, will give a higher absorbance compared to the control. An absorbance of 0.232 was observed for the enzyme-treated peptide, which verified the enzyme activity of isolated thioesterase.

3.4. Cyclization of thiophenyl peptide substrate using the isolated PCP-TE:

The derivatized peptide was treated with isolated thioesterase in the presence of buffer and the reaction was run overnight. Mass spectrometry of the reaction mixture showed multiple peaks including the peak at 945 Da that corresponds to the cyclic product indicating that the cyclization by the enzyme was successful (Figure 11). The TE recognized the thiophenyl peptide as substrate despite it not being the natural substrate. This gives hope that the process can be utilized to cyclize a larger set of novel analogues. A very small peak at 963 Da, corresponding to the hydrolysis product, was also observed corroborating a positive thioesterase activity. However, the purification of peptide using HPLC remains a challenge.

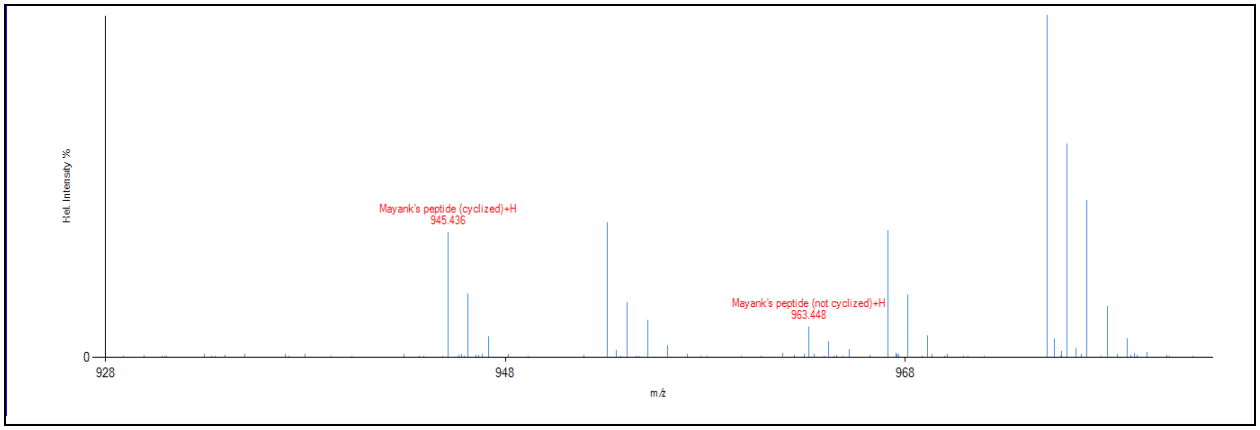


Figure 11: Mass Spectrometry (ESI-MS) of the cyclic peptide (mycosubtilin analogue).

One of the possible reasons behind the failure of peptide purification is the low yield of peptide. The thiophenyl peptide obtained after derivatization was just 7 mg. Even if assuming that all the substrate was converted to the cyclic peptide, it is a very small quantity for HPLC to purify. Furthermore, the final cyclic product, unlike other peptides, was insoluble or partially soluble in commonly used solvents like methanol and acetonitrile.

Hence, synthesizing the peptide in a considerably larger quantity and finding the right combination of solvents to solubilize it, is worth a try prior to peptide purification by HPLC.

4. Conclusion & Future Work

The analogue of mycosubtilin with a short lipid tail was successfully synthesized using the process of peptide synthesis and enzyme expression and catalysis. Though the desired product from the reaction mixture could not be isolated, the feasibility of synthesis of macrocyclic peptides on the laboratory scale using a chemoenzymatic approach was demonstrated. This approach can be further utilized in the synthesis of a wide array of naturally occurring macrocyclic compounds, which will not only help in Structure-Activity Relationship (SAR) studies of these molecules, but also understanding their mechanism of action. Furthermore, the catalytic activity of TE was confirmed by the DTNB-Thiols Assay.

Future work will be based on purifying the cyclic molecule followed by testing it for antifungal and hemolytic activity to probe the effect of shortening the lipid tail on the activity of mycosubtilin. A number of analogues will be synthesized by modifying the β-amino fatty acid using this approach. Future analogs will have modifications that vary the double bonds, branching, length, and substitution of the fatty acid side chain.

5. References

Avrahami, D.; Shai, Y. A New Group of Antifungal and Antibacterial Lipopeptides Derived from Non-membrane Active Peptides Conjugated to Palmitic Acid. *J. Biol. Chem.* **2004**, *279 (13)*, 12277-12285.

Balkovec, J. M. Section Review: Anti-infectives: Lipopeptide antifungal agents. *Expert Opin. Invest. Drugs* **1994**, *3 (2)*, 65-82.

Besson, F.; Michel, G. Action of mycosubtilin, an antifungal antibiotic of *Bacillus subtilis*, on the cell membrane of *Saccharomyces cerevisiae*. *Microbios* **1989**, *59 (239)*, 113-121.

Besson, F.; Peypoux, F.; Michel, G.; Delcambe, L. Mode of action of iturin A, an antibiotic isolated from *Bacillus subtilis*, on *Micrococcus luteus*. *Biochem. Biophys. Res. Commun.* **1978**, *81 (2)*, 297-304.

Besson, F.; Peypoux, F.; Michel, G.; Delcambe, L. ANTIFUNGAL ACTIVITY UPON *SACCHAROMYCES CEREVISIAE* OF ITURIN A, MYCOSUBTILIN, BACILLOMYCIN L AND OF THEIR DERIVATIVES; INHIBITION OF THIS ANTIFUNGAL ACTIVITY BY LIPID ANTAGONISTS. *J. Antibiot. (Tokyo)* **1979**, *32 (8)*, 828-833.

Besson, F.; Quentin, M. J.; Michel, G. Action of mycosubtilin on erythrocytes and artificial membranes. *Microbios* **1989**, *59 (240-241)*, 137-143.

Boman, H. G. PEPTIDE ANTIBIOTICS AND THEIR ROLE IN INNATE IMMUNITY. *Annu. Rev. Immunol.* **1995**, *13*, 61-92.

Bonmatin, J. M.; Laprévote, O.; Peypoux, F. Diversity among microbial cyclic lipopeptides: Iturins and surfactins. Activity-structure relationships to design new bioactive agents. *Comb. Chem. High Throughput Screening* **2003**, *6 (6)*, 541-556.

Brahmachary, M.; Krishnan S. P. T.; Koh, J. L. Y.; Khan, A. M.; Seah, S. H.; Tan, T. W.; Brusic, V.; Bajic, V. B. ANTIMIC: a database of antimicrobial sequences. *Nucleic Acids Res*. **2004**, *32*, D586-D589.

BV Tech. http://www.biovisualtech.com/bvplasmid/pET-21_b_%28+%29.jpg (accessed June 26, 2013).

Carrillo, C.; Teruel, J. A.; Aranda, F. J.; Ortiz, A. Molecular mechanism of membrane permeabilization by the peptide antibiotic surfactin. *Biochim. Biophys. Acta* **2003**, *1611 (1-2)*, 91-97.

Chen, H.; Wang, L.; Su, C. X.; Gong, G. H.; Wang, P.; Yu, Z. L. Isolation and characterization of lipopeptide antibiotics produced by *Bacillus subtilis*. *Lett. Appl. Microbiol*. **2008**, *47*, 180-186.

De Crecy-Lagard, V.; Marliere, P.; Saurin, W. Multienzymatic non ribosomal peptide biosynthesis: identification of the functional domains catalysing peptide elongation and epimerization. *C. R. Acad Sci. Ser. III* **1995**, *318 (9)*, 927–936.

De Lucca, A. J.; Walsh, T. J. Antifungal Peptides: Novel Therapeutic Compounds against Emerging Pathogens. *Antimicrob. Agents Chemother*. **1999**, *43 (1)*, 1-11.

Delcambe, L.; Devignat, R. L'Iturine, nouvel antibiotique d'origine congolaise. *Acad. Roy. Sci. Coloniales* **1957**, *6*, 1-77.

Du, L.; Sanchez, C.; Shen, B. Hybrid Peptide-Polyketide Natural Products: Biosynthesis and Prospects toward Engineering Novel Molecules. *Metab. Eng*. **2001**, *3*, 78-95.

Duitman, E. H.; Hamoen, L. W.; Rembold, M.; Venema, G.; Seitz, H.; Saenger, W.; Bernhard, F.; Reinhardt, R.; Schmidt, M.; Ullrich, C.; Stein, T.; Leenders, F.; Vater, J. The mycosubtilin synthetase of *Bacillus subtilis* ATCC6633: A multifunctional hybrid

between a peptide synthetase, an amino transferase, and a fatty acid synthase. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96 (23)*, 13294-13299.

Fickers, P.; Guez, J. S.; Damblon, C.; Leclere, V.; Bechet, M.; Jacques, P.; Joris, B. High-Level Biosynthesis of the Anteiso-C_{17} Isoform of the Antibiotic Mycosubtilin in *Bacillus subtilis* and Characterization of Its Candidacidal Activity. *Appl. Environ. Microbiol.* **2009**, *75 (13)*, 4636-4640.

Genest, M.; Marion, D.; Caille, A.; Ptak, M. Modelling and refinement of the conformation of mycosubtilin in solution from two-dimensional NMR data. *Eur. J. Biochem.* **1987**, *169*, 389-398.

Hancock, R. E. W.; Diamond, G. The role of cationic antimicrobial peptides in innate host defences. *Trends Microbiol.* **2000**, *8 (9)*, 402-410.

Hancock, R. E. W.; Sahl, H. G. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat. Biotechnol.* **2006**, *24 (12)*, 1551-1557.

Heerklotz, H.; Seelig, J. Leakage and lysis of lipid membranes induced by the lipopeptide surfactin. *Eur. Biophys. J.* **2007**, *36*, 305-314.

Huszcza, E.; Burczyk, B. Surfactin Isoforms from *Bacillus coagulans*. *Z. Naturforsch., C: J. Biosci.* **2006**, *61*, 727-733.

Jacques, P.; Hbid, C.; Destain, J.; Razafindralambo, H.; Paquot, M.; De Pauw, E.; Thonart, P. Optimization of Biosurfactant Lipopeptide Production from *Bacillus subtilis* S499 by Plackett-Burman Design. *Appl. Biochem. Biotechnol.* **1999**, *77-79*, 223-233.

Jenssen, H.; Hamill, P.; Hancock, R. E. W. Peptide Antimicrobial Agents. *Clin. Microbiol. Rev.* **2006**, *19 (3)*, 491-511.

Jerala, R. Synthetic lipopeptides: a novel class of anti-infectives. *Expert Opin. Invest. Drugs* **2007**, *16 (8)*, 1159-1169.

Kim, P. I.; Bai, H.; Bai, D.; Chae, H.; Chung, S.; Kim, Y.; Park, R.; Chi, Y. T. Purification and characterization of a lipopeptide produced by *Bacillus thuringiensis* CMB26. *J. Appl. Microbiol.* **2004**, *97*, 942-949.

Kohli, R. M.; Trauger, J. W.; Schwarzer, D.; Marahiel, M. A.; Walsh, C. T. Generality of Peptide Cyclization Catalyzed by Isolated Thioesterase Domains of Nonribosomal Peptide Synthetases. *Biochemistry* **2001**, *40 (24)*, 7099-7108.

Koumoutsi, A.; Chen, X-H.; Henne, A.; Liesegang, H.; Hitzeroth, G.; Franke, P.; Vater, J.; Borriss, R. Structural and Functional Characterization of Gene Clusters Directing Nonribosomal Synthesis of Bioactive Cyclic Lipopeptides in *Bacillus amyloliquefaciens* Strain FZB42. *J. Bacteriol.* **2004**, *186 (4)*, 1084-1096.

Maget-Dana, R.; Peypoux, F. Iturins, a special class of pore-forming lipopeptides: biological and physicochemical properties. *Toxicology* **1994**, *87 (1-3)*, 151-174.

Maget-Dana, R.; Ptak, M.; Peypoux, F.; Michel, G. Pore-forming properties of iturin A, a lipopeptide antibiotic. *Biochim. Biophys. Acta* **1985**, *815*, 405-409.

Makovitzki, A.; Avrahami, D.; Shai, Y. Ultrashort antibacterial and antifungal lipopeptides. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103 (43)*, 15997-16002.

Marahiel, M. A.; Stachelhaus, T.; Mootz, H. D. Modular Peptide Synthetases Involved in Nonribosomal Peptide Synthesis. *Chem. Rev. (Washington, DC, U.S.)* **1997**, *97 (7)*, 2651–2673.

Merrifield, R. B. Solid Phase Peptide Synthesis. I. The Synthesis of a Tetrapeptide. *J. Am. Chem. Soc.* **1963**, *85 (14)*, 2149–2154.

Nasir, M. N.; Besson, F. Specific Interactions of Mycosubtilin with Cholesterol-Containing Artificial Membranes. *Langmuir* **2011**, *27*, 10785-10792.

Nasir, M. N.; Thawani, A.; Kouzayha, A.; Besson, F. Interactions of the natural antimicrobial mycosubtilin with phospholipid membrane models. *Colloids Surf., B* **2010**, *78*, 17-23.

Ongena, M.; Jacques, P. *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. *Trends Microbiol.* **2008**, *16 (3)*, 115-125.

Peschel, A.; Sahl, H. G. The co-evolution of host cationic antimicrobial peptides and microbial resistance. *Nat. Rev. Microbiol.* **2006**, *4*, 529-536.

Peypoux, F.; Michel, G.; Delcambe, L. Structure de la mycosubtiline, antibiotique isole de *Bacillus subtilis*. *Eur. J. Biochem.* **1976**, *63*, 391-398.

Peypoux, F.; Besson, F.; Michel, G.; Delcambe, L. PREPARATION AND ANTIBACTERIAL ACTIVITY UPON *MICROCOCCUS LUTEUS* OF DERIVATIVES OF ITURIN A, MYCOSUBTILIN AND BACILLOMYCIN L, ANTIBIOTICS FROM *BACILLUS SUBTILIS*. *J. Antibiot. (Tokyo).* **1979**, *32 (2)*, 136-140.

Peypoux, F.; Bonmatin, J. M.; Wallach, J. Recent trends in the biochemistry of surfactin. *Appl. Microbiol. Biotechnol.* **1999**, *51*, 553-563.

Pirri, G.; Giuliani, A.; Nicoletto, S. F.; Pizzuto, L.; Rinaldi, A. C. Lipopeptides as anti-infectives: a practical perspective. *Cent. Eur. J. Biol.* **2009**, *4 (3)*, 258-273.

Pouny, Y.; Rapaport, D.; Mor, A.; Nicolas, P.; Shai, Y. Interaction of Antimicrobial Dermaseptin and Its Fluorescently Labeled Analogues with Phospholipid Membranes. *Biochemistry* **1992**, *31 (49)*, 12416-12423.

Powers, J-P. S.; Hancock, R. E. W. The relationship between peptide structure and antibacterial activity. *Peptides* **2003**, *24*, 1681-1691.

Protein Technologies, Inc. http://www.ptipep.com/rks_images/shopcart/item_pdf_21.pdf (accessed June 26, 2013).

Shai, Y. Mode of Action of Membrane Active Antimicrobial Peptides. *Biopolymers* **2002**, *66 (4)*, 236-248.

Shai, Y.; Oren, Z. From "carpet" mechanism to de-novo designed diastereomeric cell-selective antimicrobial peptides. *Peptides* **2001**, *22*, 1629-1641.

Sieber, S. A.; Tao, J.; Walsh, C. T.; Marahiel, M. A. Peptidyl Thiophenols as Substrates for Nonribosomal Peptide Cyclases. *Angew. Chem., Int. Ed.* **2004**, *43 (4)*, 493-498.

Steenbergen, J. N.; Alder, J.; Thorne, G. M.; Tally, F. P. Daptomycin: a lipopeptide antibiotic for the treatment of serious Gram-positive infections. *J. Antimicrob. Chemother.* **2005**, *55 (3)*, 283-288.

Stein, T. *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. *Mol. Microbiol.* **2005**, *56 (4)*, 845-857.

Strieker, M.; Marahiel, M. A. The Structural Diversity of Acidic Lipopeptide Antibiotics. *ChemBioChem* **2009**, *10 (4)*, 607-616.

Thermo Scientific. http://www.piercenet.com/browse.cfm?fldID=02040902 (assessed July 9, 2013).

Thevissen, K.; Terras, F. R. G.; Broekaert, W. F. Permeabilization of Fungal Membranes by Plant Defensins Inhibits Fungal Growth. *Appl. Environ. Microbiol.* **1999**, *65 (12)*, 5451-5458.

Tossi, A.; Sandri, L. Molecular Diversity in Gene-Encoded, Cationic Antimicrobial Polypeptides. *Curr. Pharm. Des.* **2002**, *8*, 743-761.

Trauger, J. W.; Kohli, R. M.; Mootz, H. D.; Marahiel, M. A.; Walsh, C. T. Peptide cyclization catalyzed by the thioesterase domain of tyrocidine synthetase. *Nature* **2000**, *407 (6801)*, 215-218.

Tsuge, K.; Ano, T.; Hirai, M.; Nakamura, Y.; Shoda, M. The Genes *degQ*, *pps*, and *lpa-8* (*sfp*) Are Responsible for Conversion of *Bacillus subtilis* 168 to Plipastatin Production. *Antimicrob. Agents Chemother.* **1999**, *43 (9)*, 2183-2192.

Vandeputte, P.; Ferrari, S.; Coste, A. T. Antifungal Resistance and New Strategies to Control Fungal Infections. *Int. J. Microbiol.* **2012**, 713687.

Volpon, L.; Tsan, P.; Majer, Z.; Vass, E.; Hollosi, M.; Noguera, V.; Lancelin, J. M.; Besson, F. NMR structure determination of a synthetic analogue of bacillomycin Lc reveals the strategic role of L-Asn1 in the natural iturinic antibiotics. *Spectrochim. Acta, Part A* **2007**, *67*, 1374-1381.

Zasloff, M. Antimicrobial peptides of multicellular organisms. *Nature* **2002**, *415*, 389-395.