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THE BIOACTIVE PROPERTIES OF SYRINGOMYCIN E-RHAMNOLIPID  
MIXTURES AND SYRINGOPEPTINS

by

Mekki F. Bensaci

A dissertation submitted in partial fulfillment  
of the requirements for the degree  
of

DOCTOR OF PHILOSOPHY

in

Biology

Approved:

-----  
Jon Y. Takemoto  
Major Professor

-----  
Michelle Grilley  
Committee Member

-----  
Marie K. Walsh  
Committee Member

-----  
Anne Anderson  
Committee Member

-----  
Brad Kropp  
Committee Member

-----  
Byron Burnham  
Dean of Graduate studies

UTAH STATE UNIVERSITY  
Logan, Utah

2009

## ABSTRACT

The Bioactive Properties of Syringomycin E-Rhamnolipid  
Mixtures and Syringopeptins

by

Mekki F. Bensaci, Doctor of Philosophy

Utah State University, 2009

Major Professor: Dr. Jon Y. Takemoto  
Department: Biology

The need for new antimicrobial agents has become important in the last decade due to emerging resistance to a number of conventional antimicrobial agents. New approaches and sources are needed to generate novel and effective antimicrobials. For example, synergistic combinations between two or more agents may lead to new antimicrobial therapies. Furthermore, the increase in health problems caused by the exposure to agricultural crop pesticides and synthetic fungicides and the emerging development of organic farming has increased the necessity to develop natural products than can be used safely in controlling crop diseases.

In this work, I present the first studies on the bioactive properties, particularly fungicidal activities, of mixtures of SRE and rhamnolipids. The *in vitro* results clearly showed strong synergism between SRE and rhamnolipids against phytopathogenic fungi and yeast. However, no activity was observed against bacteria. The hemolytic activities and cytotoxicities of SRE and SYRA were dose dependent.

SRE acts on yeast and plant plasma membranes to cause numerous cellular effects. The effects are consistent with SRE's ability to form ion-conducting voltage sensitive channels in membrane bilayers. In addition, studies with yeast have revealed that sphingolipids and sterols modulate the fungicidal activity of SRE. *Saccharomyces cerevisiae* sphingolipid and sterol biosynthetic mutants were used to investigate the mechanism of action of SYRA against fungi. These results suggest that similar to SRE, SYRA antifungal action is promoted by sphingolipids and sterols of the plasma membrane and involves pore formation.

I further explored the antimicrobial spectrum of syringopeptin SP25A and show that it specifically inhibits Gram-positive bacteria and yeast. I also investigated its mechanism of action against yeast and bacteria. The results revealed the role for D-alanylation of teichoic acids in modulating the susceptibility of *B. subtilis* to SP25A and other syringopeptins. This is consistent with the charged nature of the cyclic peptide portions of the syringopeptins, and it provides an explanation for SP25A's higher degree of specificity for Gram-positive bacteria. In addition and similar to SRE, SP25A antifungal action is promoted by sphingolipids and sterols of the plasma membrane and involves pore formation.

Overall, the research shows that SRE and rhamnolipids are synergistically active against yeast and fungi and that the syringopeptins have antimicrobial activities against yeast and Gram-positive bacteria. Insights into the mechanisms of action of the SRE and rhamnolipid mixtures and the syringopeptins and their potential as novel antimicrobial agents are revealed.

## ACKNOWLEDGMENTS

“The only people with whom you should try to get even are those who have helped you.” John E. Southard

I would like to express my deepest appreciation to Dr. Jon Takemoto for the support and guidance he provided me while I was working in his laboratory. It was a wonderful experience!

I would like to convey my appreciation to all members of my committee, including Dr. Anne Anderson, Dr. Michelle Grilley, Dr. Marie Walsh, and Dr. Bradley R. Kropp, for their time, advice, support, and encouragement through all these years.

I am extremely grateful to all members of Dr. Takemoto’s laboratory. I owe special thanks to Lynnette Takemoto for all her support and kindness. Finally, I would like to thank my lab mates: Dr. Jolanta Idkowiak-Baldys, Camille Swasey, Yukie Kawasaki, Sanjib Shrestha, and my good friend and colleague Sitaram Harihar.

I would like to express my deep appreciation and my biggest thanks to my parents, my sisters, my brothers, and all my friends for all their support. I would like to dedicate this dissertation to my family, and especially to my father.

Mekki F. Bensaci

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## LIST OF ABBREVIATIONS

CAMP	cationic antimicrobial peptides
CM	cormycin
CP	corpeptin
HPLC	high performance liquid chromatography
IPC	inositolphosphorylceramide,
LTA	lipoteichoic acid
MIPC	mannosyl-inositolphosphorylceramide
M (IP <sub>2</sub> )C	mannosyl-diinositolphosphorylceramide
MIC	minimum inhibitory concentration
NCCLS	the National Committee on Clinical Laboratory Standards
PDB	potato dextrose broth
PDA	potato dextrose agar
RL	rhamnolipids
SP	syringopeptin
SRE	syringomycin E
SYRA	mixture of SRE and RL
TFA	trifluoroacetic acid
YPD	yeast extract-peptone dextrose
WTA	cell wall teichoic acid

## CHAPTER 1

### INTRODUCTION AND LITERATURE REVIEW

#### **Cyclic Lipodepsipeptides of *Pseudomonas syringae* pv *syringae***

The phytopathogenic strains of *Pseudomonas syringae* pv. *syringae* produce two major groups of cyclic lipodepsipeptides: the small cyclic lipodepsinonapeptides and the larger syringopeptins (2, 11, 83). Individual *P. syringae* pv. *syringae* isolates typically produce one of a variety of forms of the syringopeptins and one form of the smaller lipodepsinonapeptides (6, 74).

Four groups of small cyclic lipodepsinonapeptides are well known: syringomycins (Fig. 1.1), syringostatins, syringotoxins and pseudomycins (31). They all contain a long, unbranched-3-hydroxy fatty acid, with a positively charged and a hydrophilic cyclic ring of nine amino acids at the C terminus (31, 74). These small cyclic lipodepsipeptides are considered to be antifungal (31). They exhibit fungicidal activity against various yeast and filamentous fungi such as *Candida*, *Cryptococcus*, *Geotrichum*, and *Aspergillus* species (11, 23). Previous studies showed that these compounds tend to be more active against yeasts than against filamentous fungi (54, 76). Inhibitory activities against bacteria are not typically observed for the cyclic lipodepsinonapeptides (47). In addition to their antifungal activities, the cyclic lipodepsinonapeptides are hemolytic and cytotoxic to mammalian cells (22, 76).

The syringopeptins (SPs) are larger and more hydrophobic than the cyclic lipodepsinonapeptides. The best-studied syringopeptins are SP22 and SP25 (Fig. 1.2) (4). Structural analyses of these metabolites using  $^1\text{H}$  and  $^{13}\text{C}$  NMR (2) and tandem mass

spectrometry (2) revealed that SP22 and SP25 possess 22 and 25 amino acids, respectively (Fig. 1.2). The amino acids are predominantly hydrophobic with a prevalence of valines and alanines. About 70% of the chiral amino acids residues are of the D configuration, and there are four  $\alpha,\beta$ -unsaturated and two 2,4- diaminobutyric acid residues (2, 4, 32, 70). An N-terminal amino acid dehydroaminobutyric acid (Dhb) is N-acylated by a 3-hydroxylated fatty acid chain containing ten and twelve carbon atoms giving homologs designated A and B, respectively, and typically the more abundant and less abundant forms, respectively. The C-terminal carboxyl group is esterified by the hydroxyl group of the *allo*Thr residue positioned at the distance of seven residues, thus forming an eight-membered lactone macrocycle. Recent studies have shown that SPs have antimicrobial activity against Gram-positive bacteria *Bacillus megaterium* and *Micrococcus luteus* (54). However, the activities against other bacterial species have not been reported. The SPs are also antifungal but with less potency than the small cyclic lipodepsipeptides.

Other species of *Pseudomonas* such as *P. syringae* pv. *atofaciens* (87), *P. fuscovaginae* (5), *P. tolaasi* (66) and *P. corrugata* (28) also produce cyclic lipodepsipeptides (CLPs) that have antimicrobial properties. Studies by Vassilev et al. (87) showed that *P. syringae* pv. *atofaciens*, the causal agent of basal glume of cereals and grasses, also produces SRE and SP25A. *P. fuscovaginae*, the causal agent of bacterial brown sheath, produces syringotoxin and fuscopeptins (5, 30). Structure analysis revealed that the fuscopeptins structure differs slightly from SP22. The fuscopeptin lactone ring contains five amino acids rather than eight in the case of SP22. However, Like SPs, fuscopeptins have a high content of hydrophobic amino acids and

amphiphilic properties (5). The biological properties of the fuscopeptins more closely resemble those of the SPs than of the cyclic lipodepsinonapeptides (5, 30). The phytopathogenic bacterium from tomato, *P. corrugata* (69, 28) produces cyclic lipodepsipeptides known as corpeptin (MW 2121) and cormycin (MW1274). Structural analysis of corpeptin (CP) and cormycin (CM) revealed that overall these two are structurally similar to SPs and lipodepsinonapeptides, respectively. The main differences between CM and small lipodepsipeptides were attributed to the molecular net charge and the nature of the amino acids occurring at positions 2–6 (69). The CP peptide moiety and fatty acid moiety show very high homology to those of the SPs (28). Additionally, CP and CM display antimicrobial activities against bacteria and yeast (28, 69). The antimicrobial activities of CP and the SPs are similar. It was shown that CP was more active against Gram-positive bacteria than against yeast (28). Interestingly, Scaloni et al. (69) also showed that CM inhibits both bacteria and yeast. The antibacterial observation was not consistent with the antimicrobial activities of the other small lipodepsipeptides. CM also exhibited phytotoxic effects and hemolytic activity (69). Finally, Scaloni et al. (69) have shown that CM forms pores in planar lipid bilayers and its primary target is the plasma membrane.

Recent studies by De Bruijn et al. (21) using genomic analyses have revealed new CLPs produced by the bacterium strain *P. fluorescens* SBW25. Structure analyses revealed that the CLPs produced by SBW25 contain 9 amino acids linked to 3-hydroxydecanoic acid. The structural features of SBW25 CLP are closely similar to another CLP produced by *P. viscosa* called viscosin (55). In addition, the bioactivity studies showed that the SBW25 CLPs play roles in motility, biofilm formation and also

have antifungal activity against zoospores of *Phytophthora infestans* (21). This discovery shows that genome analyses are useful tools that complement structural analyses for finding new antibiotics produced by microorganisms.

### **Biosynthesis of Cyclic Lipodepsipeptides**

The biosynthesis of the cyclic lipodepsipeptides is a non-ribosomal, template-directed process. Certain enzymes – but not all - responsible for cyclic lipodepsipeptide production have been identified (11, 35). The biosynthesis of SRE has been the most thoroughly studied among the cyclic lipodepsipeptides.

SRE synthesis takes place on a multienzymatic thio-template. Analysis of the *syrB* and *syrC* genes of *P. syringae* pv. *syringae* indicates that syringomycin is synthesized by a thio-template mechanism (89). The syringomycin (*syr*) gene cluster contains a DNA region of approximately 37 kb on the chromosome of *P. syringae* pv. *syringae* B301D (36, 89). The gene cluster encodes four proteins (SyrB1, SyrB2, SyrC and SyrE) involved in biosynthesis (36, 89). The *syrE* gene encodes a 1,039 kDa synthetase containing eight amino acid activation modules (36). The *syrB* gene represents an operon that expresses two proteins (89). SyrB1 is a 68 kDa protein that carries adenylation and thiolation domains, and the 35 kDa SyrB2 protein (11). The *syrC* gene is located between the *syrB* operon and *syrE* (36). The *syrC* gene encodes a 48 kDa protein similar to several proteins containing thioesterase enzyme motifs (86). The *syrD* gene encodes a 63 kDa protein that is involved in secretion of syringomycin E and is a member of the ATP Binding Cassette transporter protein family (65).

The regulatory mechanisms for SRE production are complex. The regulation of syringomycin production by *P. syringae* pv. *syringae* is modulated by both nutritional

factors and plant signal molecules (34). For example, iron exerts a positive regulatory effect on SRE production, whereas inorganic phosphate represses the production (35). In addition, plant signal molecules such as arbutin also play a role in SRE synthesis. The *syr* gene cluster contains a regulatory gene called *syrP* that is responsible for the control of SRE production (91). SyrP is a 40 kDa protein that may function in a phosphorelay signal transduction pathway (65, 90), therefore, it is believed to be a component of a kinase cascade that regulates syringomycin production. Another gene, known as *syrA*, identified by Xu and Gross (88), encodes a regulatory protein required for both syringomycin synthesis and pathogenicity.

Global regulation of SRE production has been well studied (44, 68). The *lemA* and *gacA* genes encode members of a two-component sensory transduction system of *P. syringae* pv. *syringae* that regulates toxigenesis and the ability to cause necrotic lesions in plants (44). LemA is a transmembrane histidine protein kinase activated by an extracellular signal that phosphorylates GacA (44, 68). GacA is assumed to function as a transcriptional activator (68). The LemA-GacA protein pair appears to be at the top of the regulatory hierarchy controlling SRE production (11). Strains with mutations in either GacA or LemA do not produce syringomycin E. Kitten et al. identified *sala* as a member of the *lemA-gacA* regulon that can restore SRE production if it is overexpressed (52). Also, it was reported that the expression of *syrB-lacZ* was reduced to less than 3% in a *sala* mutant (52). The predicted SalA protein sequence exhibits an H-T-H DNA-binding motif with similarity to response regulators (3). It remains to be determined if SalA binds directly to the promoter region of *syrB* operon or activates the operon indirectly through intermediate regulators such as SyrP (52) (11).



SPs are also synthesized by non-ribosomal mechanisms and multienzymatic peptide synthetases (73). Mapping and sequence analysis have indicated that the *syp* gene cluster is located adjacent to the *syr* gene cluster (11, 37). Almost the entire *syp* and *syr* gene clusters are located on approximately 145 kb *DraI* fragment on the chromosome of *P. syringae* pv. *syringae* (73). The *syp* gene cluster is estimated to encompass 80 kb of DNA, and 70 kb of DNA from this region is dedicated to encoding peptide synthetases. Mutations located in regions encoding peptide synthetase disrupted either syringomycin or SPs production indicating that these two are synthesized by separate peptide synthetase systems (73). The *syr-syp* gene cluster contained genes of phytotoxin regulation (*syrP*, *sala*, *syrF*, and *syrG*) and secretion (*syrD*), in addition to syringomycin and SP biosynthesis genes (*syrE*, *syrB1*, *syrC*, and *sypA*) (72). Guenzi et al. (36) showed that SypA, SypB, and SypC exhibit 45.5, 45.6, and 45.9 % identity, respectively to SyrE, and 42.7, 44.3, and 43.3 % , respectively to SyrB1, both of which are syringomycin synthetases. SP is produced under the same conditions as syringomycin. It was suggested that the synthesis of SP is activated by the same regulatory system that regulates syringomycin (11).

### **Syringomycin E Mechanism of Action**

**Physiological studies.** SRE produced by *P. syringae* pv. *syringae* is the best studied of the small cyclic lipodepsipeptides. Recent studies with SRE have focused on the antifungal mechanism of action of the small cyclic lipodepsinonapeptides (40). The amphipathic lipopeptide structure of SRE promotes its interaction with the plasma membrane as the first site of action (11). Previous work by Zhang and Takemoto (92) on *Rhodotorula pilimanae* showed that SRE causes an increase in tetraphenylphosphonium

uptake indicating an increase in membrane potential (92). This results in a variety of effects such as  $K^+$  effluxes and  $H^+$  influx which eventually result in cell death (93). In plants, SRE induced stomata closing in *Xanthium strumarium* and *Vicia faba* by causing  $K^+$  efflux (59). A reversal effect was observed when fusicoccin was applied. This result leads to an argument that SRE does not permanently disrupt the plasma membrane (59). Other studies show that SRE collapses the pH gradient across the plasma membrane by inducing  $K^+$  efflux and  $H^+$  influx (11, 67, 92). Moreover, it was also shown that  $H^+$ -ATPase activities of *R. pilimanae* and red beet storage tissue were stimulated by SRE (13). However, no stimulation was observed when purified enzyme was used; this suggests that SRE's stimulation effect was not directly on the enzyme and that other components are involved. In addition to  $K^+$  and  $H^+$  exchange, SRE also stimulates  $Ca^{2+}$  influx (84, 85). Studies with syringomycin E-treated red beet storage tissue have shown an overall increase in  $Ca^{2+}$  uptake (84, 85). The influx of  $Ca^{2+}$  was followed by a cascade of events associated with cellular signaling in plants (84) such as the induction of kinase-mediated phosphorylation of membrane proteins ( $H^+$ -ATPase) (13) and the incorporation of 1,3-B-callose into plant cell walls (11, 51).

**Bilayer studies.** In recent years more studies were done to characterize SRE channel formation and properties using artificial lipid membranes. Studies by Hutchison and Gross (45) and Feigin et al. (29) revealed that SRE forms voltage-dependent channels in artificial planar bilayer membranes. These studies revealed that SRE forms non specific pores which are permeable to both monovalent and divalent cations. It was shown that at least six SRE molecules are required for channel formation and the pore radius was estimated to be 1 nm (29) (18) (46). In addition, Ostroumova et al. (62)

showed that SRE channels are larger at the *trans* side with radii of 0.5-0.9 nm versus 0.25-0.35 at the *cis* side, concluding that the SRE channel is asymmetrical. Additional studies showed that SRE forms two types of channels, “small” and “large” differing 6 to 7 times in their conductance (49, 71). The SRE channels cluster transiently and exhibit synchronous channel opening and closing resulting in the wide range of conductances (49). In addition, these channels display temperature-dependent pore inactivation (58, 71, 81).

### **The Roles of Yeast Sphingolipids and Sterols**

In yeast, sphingolipids and sterols play important roles in the action of SRE. Sphingolipids are major components of the eukaryotic plasma membrane, and they represent about 8 % of its total mass (42, 63). The sphingolipids are composed of a long chain base with an amide-linked fatty acid at the C-2 position and a polar constituent at the C-1 position. Sphingolipids are ubiquitous in eukaryotes, and they play roles in many cellular functions such as protein glycosylphosphatidylinositol (GPI) anchoring, heat stress responses, and modulation of membrane enzyme activities (27, 64).

The biosynthesis of *S. cerevisiae* sphingolipids has been well elucidated, and the major functional genes for sphingolipid biosynthesis have been identified (Fig. 1.3) (26). The first step of sphingolipid biosynthesis begins with condensation of palmitoyl-CoA and serine to form 3-ketodihydrosphingosine (14). The condensation reaction is catalyzed by a multi-subunit membrane enzyme serine palmitoyl transferase. In *S. cerevisiae* this enzyme is encoded by *LCB1* and *LCB2* (9, 15, 60). The next step of sphingolipid biosynthesis involves the reduction of 3-ketodihydrosphingosine to dihydrosphingosine by the NADPH-dependent reductase TSC10 (80). In yeast this is

followed by conversion of dihydrosphingosine into phytosphingosine by hydroxylation at the C4 position of the sphingoid base by the *SYR2/SUR2* gene product (17, 27, 33, 38). A special feature of sphingolipids is its very long fatty acyl side chain that contains 24 to 26 carbons atoms. The synthesis of these long chains is done by the *ELO2* and *ELO3* that convert C<sub>16</sub> and C<sub>18</sub> fatty acids to C<sub>20</sub> and C<sub>26</sub> sphingolipid molecules (Fig. 1.3).

In addition, the *FAH1* is responsible for  $\alpha$ -hydroxylation of the amide linked very long chain fatty acids.

Three main forms of sphingolipids are formed in *S. cerevisiae* inositolphosphorylceramide (IPC) (78), mannosyl-inositolphosphorylceramide (MIPC), and mannosyl-diinositolphosphorylceramide (M(IP)<sub>2</sub>C) (75) (Fig. 1.3). The formation of these three lipids requires successive additions of phosphorylinositol, mannose and a second phosphorylinositol to ceramide. First, the transfer of phosphorylinositol from phosphatidylinositol to ceramide is catalyzed by IPC synthase encoded by *AURI* (60). Then, a mannose group is added to IPC to form mannosylinositolphosphoceramide (MIPC). This step in yeast requires the genes *CSG1* and *CSG2* (9) (10). Finally, the last step in sphingolipids biosynthesis is the transfer of phosphorylinositol to M(IP)<sub>2</sub>C. This step is catalyzed by M(IP)<sub>2</sub>C synthase, the encoded protein of *SYR4/IPT1* (27, 56).

Findings with *S. cerevisiae* mutants resistant to SRE have shown that structural features of sphingolipids and sterols are involved in SRE's mechanism of action (Fig. 1.4). For instance, one of the genes that promotes SRE action is *SYR1/ERG3*, which encodes the sterol C5,6 desaturase for the biosynthesis of ergosterol, the primary sterol in yeast plasma membrane (82). The expression of specific sphingolipid biosynthetic genes *SYR2*, *FAH1*, *ELO2*, *ELO3*, *CSG1*, *CSG2* and *IPT1* also promote SRE action. A mutation

in any one of the genes causes no cell lethality, but each renders resistance to the killing effects of SRE (40, 79). In addition, the work of Kaulin et al. (50) revealed that the C4-OH sphingoid base is important for SRE channel forming ability and a mutation in the C4-hydroxylase gene *SYR2* causes a reduction in the number of pores formed by SRE.

### **Mechanism of Action of Syringopeptins**

Despite the structural differences between SRE and the SPs, both elicit similar physiological and membrane biophysical responses. Several studies have shown that the SPs are capable of forming ion conducting membrane pores (19, 45). The amphipathic nature of the SPs facilitates their insertion into lipid bilayers causing channel formation and disruption of the membrane. In plant systems, the SPs have been shown to cause electrolyte leakage in leaf tissues, which leads to the development of necrotic symptoms (47). In addition, Di Giorgio et al. (25) have shown that the SPs can alter the distribution of  $H^+$  across the plasma membrane of maize cells and promote stomatal closure in *Xanthium strumarium* leaves. The SPs also induce lysis and generate a rapid influx of  $^{45}Ca^{2+}$  across the plasma membrane of tobacco protoplasts (45). In yeast, SP action is promoted by sphingolipids and sterols of the plasma membrane (Bensaci et al., 2008 submitted and Chapter 4, this dissertation). Finally, the SPs interact with the bacterial cell surface teichoic acids (12) but the mechanism of interaction with bacterial membranes is still unknown.

## **Biosurfactants**

### **Diversity, Properties, and Roles**

Biosurfactants are a structurally diverse group of surface active molecules synthesized by a variety of microorganisms (bacteria, yeast and fungi) (24, 77). Biosurfactants are categorized mainly by their chemical composition and their microbial origin (7). There are six types of biosurfactants: lipopolysaccharides, glycolipids, lipopeptides-lipoproteins, phospholipids, and hydroxylated and cross-linked fatty acids (mycolic acids) (7, 24). The biosurfactants are amphiphatic molecules. As a consequence, these molecules have the ability to reduce surface and interfacial tensions in both aqueous solutions and hydrocarbon mixtures (8). In addition, biosurfactants are biodegradable, low in toxicity, ecologically acceptable, and can be produced from renewable substrates (57). In nature the functions of biosurfactants are not clear (77). However, it has been proposed that biosurfactants play a variety of roles that include: a) enhancement of bioavailability and biodegradation of slightly soluble organic carbon sources such as petroleum hydrocarbons (77) (b) attachment and detachment of bacterial cells to surfaces (77), and defense strategies against other microbes (c) (77). Biosurfactants are also used in cosmetics, health, pharmaceuticals, agriculture, and the food industry (57).

### **Rhamnolipids**

Among the glycolipid biosurfactants, rhamnolipids are the most studied. Rhamnolipids are produced by certain *Pseudomonads* and were first described in 1949 by Jarvis et al. (48). Their biosyntheses have been well studied by Hauser and Karnovsky and Burger et al. (16, 41). Rhamnolipids are secreted by *Pseudomonas aeruginosa*

during the stationary phase of growth under limiting conditions of nitrogen and iron (41, 61). The synthesis of rhamnolipids proceeds by sequential glycosyl transfer reactions, each catalyzed by specific rhamnosyltransferases with TDP-rhamnose acting as a rhamnosyl donor and

3-hydroxydecanoyl-3-hydroxydecanoate serving as the acceptor (53, 61). Structural analysis revealed that glycolipid biosurfactants are formed by the linking of rhamnose to fatty acids composed of saturated or unsaturated alkyl chains with lengths between C8 and C12 (Fig. 1.5) (39). Their molecular weights range from 504 to 650 (78, 94).

### **Antimicrobial Activities of Rhamnolipids**

Rhamnolipids possess antimicrobial activities (39). They are active against zoosporic plant pathogens such as *Phythium* and *Phytophthora* species (77). Rhamnolipids are also active against fungal species such as *Fusarium solani* and *Penicillium funiculosum* (1, 39). Haba et al. (39) have shown that rhamnolipids also have antibacterial activities against certain Gram-negative and Gram-positive bacteria. Among the Gram-negative and Gram-positive bacteria tested, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Bacillus subtilis* and *Micrococcus luteus* were the most sensitive to rhamnolipids (1, 39). However, no activity against yeast was observed (1, 39). The mechanism of action of rhamnolipids against fungi is still unknown. However, Stanghellini et al. (77) suggested that the rhamnolipids inhibit zoosporic fungi by disrupting the zoospore plasma membrane.

## Crop Fungicides

Fungicides are extensively used in agriculture for a number of purposes including protection of crops, seedlings, and storage. About 150 different fungicidal compounds are currently used with a global market value of about 6 billion U.S. dollars accounting for almost 20% of the agrochemicals market (43).

Fungicides can be classified in two groups based on their source: synthetic organic chemicals and naturally-derived fungicides (20). Organomercurials were the first synthetic organic chemicals used as agrofungicides in the early twentieth century (20). Later, varieties of organic fungicides were introduced such as: ferbam, ziram, and dinocap. These compounds are known to be multisite inhibitors. They disturb fungal physiology by interacting with functional groups of enzymes and other key constituents of fungal cells (20). Their non-selective action sometimes cause undesirable effects in plants, mammals, and beneficial organisms (20). Therefore, in the early 1960s, new measures and regulations were adopted to limit the use of these fungicides and to promote the search for novel fungicides with specific target sites (20). The search resulted in the discovery of specific-site fungicides such as the benzimidazoles, *N*-arylcarbamates, sterols and melanin biosynthesis inhibitors, phenylpyrroles, dicarboximides, and other fungicides such as aromatic hydrocarbons (20). However, due to the increase in resistance, side effects in mammals and wildlife with extensive use, and the low biodegradability of these compounds (20), a new effort was promoted to screen microbial products in order to discover new natural compounds that are highly biodegradable and low in toxicity. The search led to the first discovery by Takeuchi et al. in 1958 of blasticidin A (20). This fungicide is produced by *Streptomyces*



*griseochromogenes*. It was used for the control of rice blast disease caused by *Pyricularia oryzae*. Following this discovery more fungicides of microbial origin such as kasugamycin, polyoxins, validamycin A, and mildiomyacin were discovered and used in agriculture (20). Prior to 1970, most of the used fungicides were multisite inhibitors. Resistance to these compounds is rare. However, with the use of site specific fungicides the rate of resistance increased dramatically and the time between commercial introduction and the emergence of resistance was much shorter than with the multisite fungicides. A good example of resistance is the outbreak of benzimidazole resistance of *Cercospora beticola* in sugar beet and the loss of matalaxyl control of *Phytophthora infestans* (43). Fungicide resistance can have significant impacts on agriculture and crop protection that include economic loss. Awareness of these problems led researchers to define the factors which influenced the spread of resistance and to seek strategies to combat it (43). However, resistance is not the only factor to limit the use of particular fungicides. Selectivity and the distinct spectra of activity are a concern because most specific site fungicides have limited use against certain important plant pathogens (20). These problems raise the call for new and effective fungicides with broad spectra and no resistance.

### **Justification and objectives for the present research**

The need for novel antimicrobial agents has become important in the last decade due to emerging resistance to a number of conventional antimicrobial agents. New approaches and sources are needed to generate novel and effective antimicrobials. For example, synergistic combinations between two or more agents may lead to new antimicrobial therapies. Furthermore, the increase in health problems caused by the

exposure to agricultural crop pesticides and synthetic fungicides and the emerging development of organic farming has increased the necessity to develop natural products than can be used safely in controlling crop diseases.

The overall aim of the research is to investigate the bioactive and synergistic properties (particularly fungicidal activities) of mixtures of the cyclic lipodepsinonapeptide SRE and rhamnolipids. Being natural products, there is interest in the commercial applications of both compounds as fungicides. Both are physically and chemically similar with the ability to be extracted and processed using similar procedures from cultures of fluorescent pseudomonads. Rhamnolipids are now commercially available, and industrial production of SRE is under development. If synergistic, the mixtures of the two could have major applications in agricultural crop disease control as well as other important applications involving control of fungal growth. In the present research, the bioactive properties of SRE, rhamnolipids, and combination mixtures of the two were investigated.

The objectives of the research were to study:

- 1) the parameters of SRE-rhamnolipid mixtures (SYRAs) that give optimal antimicrobial activities against yeasts, fungi and bacteria,
- 2) the spectrum of fungal species that are inhibited by SYRAs,
- 3) the fungicidal mechanism of action of SYRAs,
- 4) the toxicities of SYRAs on mammalian cell lines, red blood cells, and honeybees.
- 5) the possibility of other pseudomonad cyclic lipodepsipeptides having synergistic antimicrobial activities when combined with rhamnolipids.

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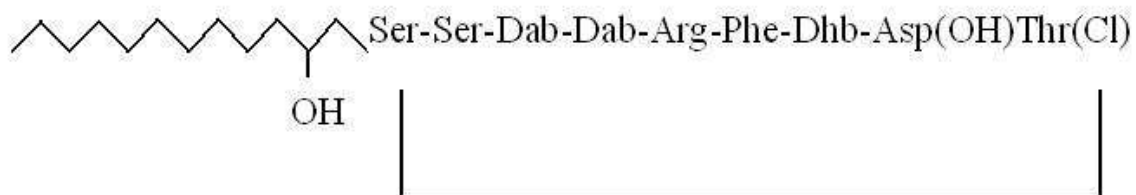
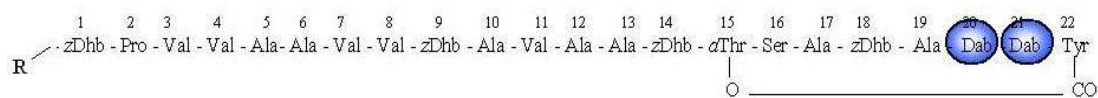


FIG. 1.1. Structure of the cyclic lipodepsinonapeptide syringomycin E. Dab:2,4 diaminobutyric acid; Dhb:dehydroaminobutanoic acid; Asp(OH):  $\beta$ -OH aspartic acid; Thr(Cl): 4-chlorothreonine.

SP22



SP25

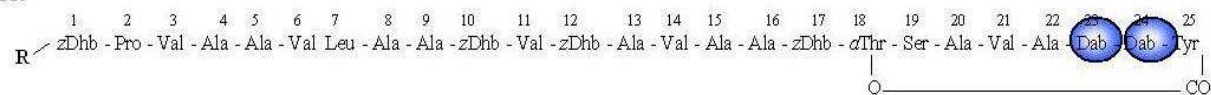


FIG. 1.2. Structure of syringopeptin. R =  $\text{CH}_3 - (\text{CH}_2)_n - \text{CH}(\text{OH}) - \text{CH}_2\text{CO}$ , SP22A ( $n=6$ ), SP25A( $n=6$ ) Abbreviation Dab=1,4-diaminobutyric acid; Dhb=2,3 dehydroaminobutyric acid; all other amino acids are identified by standard three letter biochemical notation.

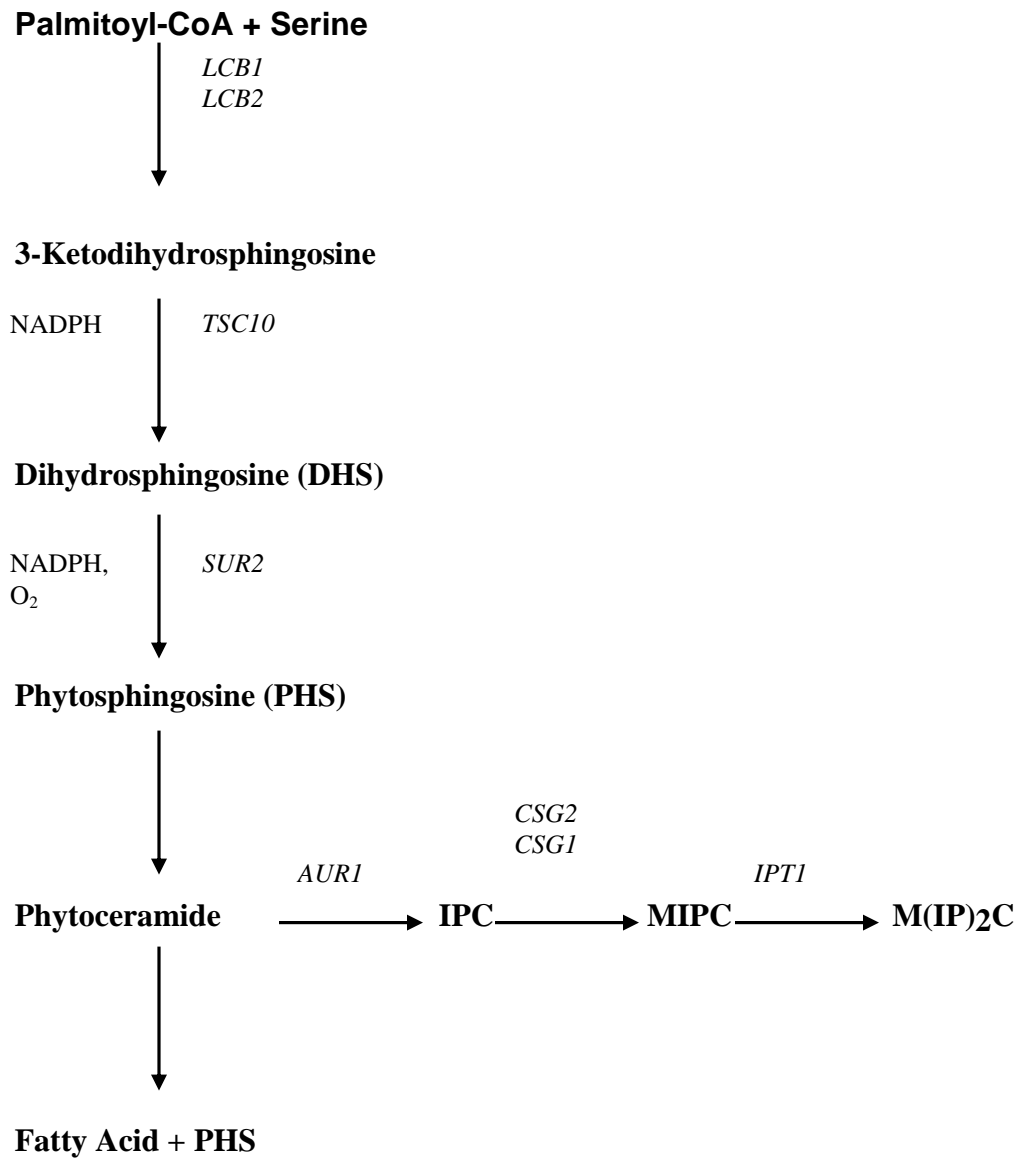


FIG. 1.3. Diagram of sphingolipid biosynthesis in *S. cerevisiae*

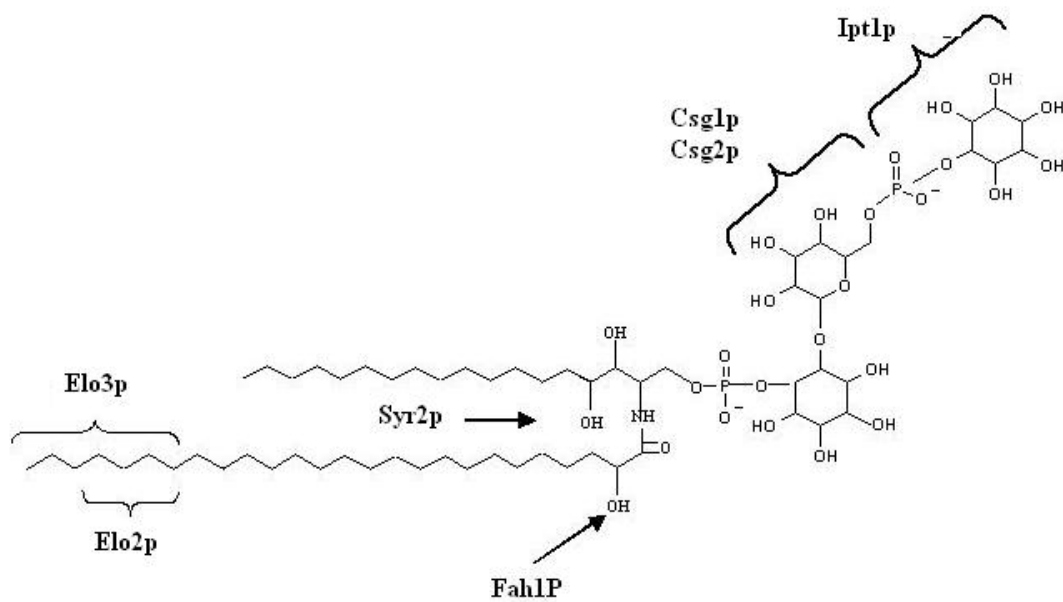


FIG. 1.4. The *S. cerevisiae* sphingolipid M(IP)<sub>2</sub>C. The enzymes that modify precursors of M(IP)<sub>2</sub>C during its biosynthesis are shown and the structural modification indicated

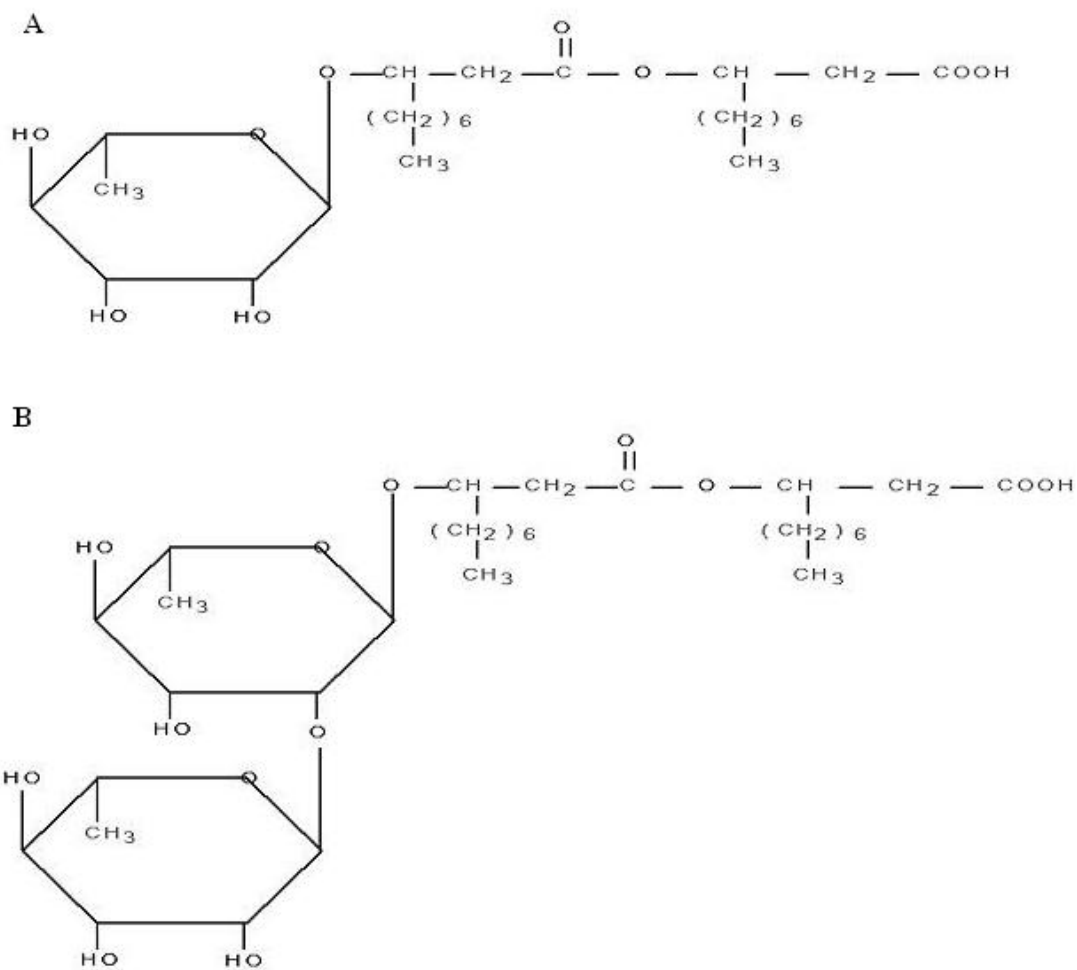


FIG. 1. 5. Chemical structures of rhamnolipids produced by *Pseudomonas aeruginosa*

CHAPTER 2  
THE BIOACTIVE PROPERTIES OF SRE AND  
RHAMNOLIPID MIXTURES

**Introduction**

The need for new antifungals and fungicidal biocontrol agents is growing due to the narrowing spectra of fungal targets and increasing resistance to existing antifungals such as amphotericin B and the azoles (30, 31). One approach to develop novel antifungals is to combine known antifungal compounds. Such combinations may have improved bioactivities and expanded target spectra due to the synergy between the compounds. Moreover, lower effective doses of the combination components may have less toxicity.

The plant bacterium *Pseudomonas syringae* pv. *syringae* produces an array of antimicrobial peptides known as the small cyclic lipodepsipeptides such as syringomycin, and the large cyclic lipodepsipeptides known as the syringopeptins (3, 38). The small cyclic lipodepsipeptides contain an unbranched-3-hydroxy fatty acid with a positively charged cyclic ring of nine amino acids at the C terminus (14, 32). Their molecular weights range from 1000 to 1300 (14, 32). The best studied is SRE. Previous studies showed that SRE has inhibitory activity against fungi and yeast such as *Botrytis cinerea*, *Geotrichum candidum* and *Rhodotorula pilimanae* (21, 38). It tends to be more active against yeasts than against filamentous fungi (21). Recent studies have focused on the antifungal mechanism of action of the small cyclic lipodepsinonapeptide SRE (16) was shown to form channels in phospholipid bilayers, and it is speculated that a similar

mechanism occurs in the target fungal membrane (7). Channel formation may cause the influx of ions such as  $K^+$  and  $Ca^{2+}$  (38).

Many strains of *Pseudomonas aeruginosa* produce several homologs of the biosurfactant known as rhamnolipid (1, 15). Rhamnolipids were first described in 1949 by Jarvis and Johnson (20) and their biosyntheses was well studied by Hauser and Karnovsky (17). Rhamnolipids are glycolipids secreted at the stationary phase of growth under limiting conditions of nitrogen and iron (17, 28). Structure analysis revealed that these compounds are formed by rhamnose linkage to fatty acids of saturated or unsaturated alkyl chain between C8 and C12 (15). Their molecular weights range from 504 to 650 (37, 41). The antimicrobial activities of rhamnolipids are well studied. They are active against zoosporic plant pathogens such as *Phythium* and *Phytophthora* species (35). Rhamnolipids are also active against fungal strains such as *Fusarium solani* and *Penicillium funiculosum* (1, 15). Moreover, Haba et al. (1, 15) have shown that rhamnolipids also have antibacterial activities against certain gram-negative and gram-positive bacteria. Among the gram-negative and Gram-positive bacteria tested, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Bacillus subtilis* and *Micrococcus luteus* were the most sensitive to rhamnolipids (15). However no activity against yeast was observed (1, 15).

The main objective of this study was to examine the *in vitro* interaction of SRE and RL against bacteria, yeast, and filamentous fungi. I investigated the antimicrobial properties and cytotoxicity of syringomycin E (SRE), rhamnolipids (RL<sub>Zonix</sub>) and the mixture SYRA (3:1 mixture of RL<sub>Zonix</sub> and SRE). Synergistic antifungal activities between SRE and RL<sub>Zonix</sub> in the mixtures (SYRAs) were observed. The SRE minimal

inhibitory concentrations (MICs) were lowered when combined with RL<sub>Zonix</sub>. However, no activity was observed against bacteria. In vitro cytotoxicity and erythrocyte lysis were also investigated. The hemolytic activity and cytotoxicity of SRE, RL<sub>Zonix</sub> and SYRA were dose dependent. However, the concentrations of SRE and SYRA that caused hemolysis and cytotoxicity were 3 to 5 times higher than the MICs. In addition, the honeybees toxicity effect of SRE, RL<sub>Zonix</sub>, and SYRA was also studied. The use of most pesticides in agriculture has often negative effects against bee populations and ecosystems. The ecological and agricultural importance of bees requires the use of safe and less toxic fungicides. The results showed no toxicity at concentrations higher than the MICs.

## Materials and Methods

### SRE and Rhamnolipids

SRE was purified from *P.syringae* pv.*syringae* strain B301D by the method of Bidwai et al. (4). *P.syringae* pv.*syringae* strain B301D was grown in non-commercial potato dextrose casamino acids medium in 4 or 8-liter cultures as described by Zhang et al. (40). The SRE was extracted from cultures using acidified acetone and purified using the chromatographic methods described by Bidwai et al. (4). HPLC column fractions were tested for bioactivity by applying 10 µl aliquots on a fresh lawn of *R. pilimanae* on potato-dextrose agar plates, incubation at 28°C, and observing cleared growth inhibition areas (40).

The rhamnolipids (RL<sub>Zonix</sub>) was obtained from Jeneil Biotech, Inc. Zonix contains 8.5 % solution of rhamnolipids in water. Two forms of rhamnolipids are in zonix ,

R1 (C<sub>26</sub>H<sub>48</sub>O<sub>9</sub>) has the molecular formula of α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate, R2(C<sub>32</sub>H<sub>58</sub>O<sub>13</sub>), 2-O-α-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoate.

### **Organism and Media**

The following organisms were obtained from the microbial culture collection of the Department of Biology, Utah State University: *R. pilimanae* ATCC9449, *Candida albicans* ATCC10231, *Aspergillus flavus*, *Aspergillus niger*, *Rhizopus stonifer*, *Fusarium oxysporum*, *Rhizoctonia solani*, *Botrytis cinerea*, *Cladosporium cladosporioides*, *Penicillium oxalicum*, *Penicillium* sp. All fungi were cultivated in potato dextrose medium (Difco) (PDA) at 28 to 30°C. *Saccharomyces cerevisiae* W303C, *S. cerevisiae* BY4741 and *S. cerevisiae* 8A-1B were obtained from Dr. Takemoto's laboratory collection. *S. cerevisiae* strains were grown on YPD at 28°C. *S. aureus* ATCC653, *B. subtilis* ATCC1965, *E. coli* ATCC25922, *Proteus vulgaris* ATCC13315. The bacteria strains were grown at 37°C for 24 h on Luria-Bertani medium except *S. aureus* was grown on Mueller-Hinton medium.

### **Antifungal Susceptibility**

**Agar disk diffusion method.** The disk diffusion assay was done according to National Committee for Clinical Laboratory Standards (NCCLS) protocols for antifungal testing with modification (26) (25). Fungal isolates were subcultured on PDA plates and incubated for 3 to 5 days at 35°C. The fungal plates were covered with 5 mL of distilled sterile water. Then, the fungal spores were harvested and suspended in sterile water. The suspension was adjusted to 10<sup>6</sup> spores/ml (36). A 100 µl of the suspension was



transferred and spread uniformly onto solid agar of the appropriate growth medium using cotton swabs (36).

To test the antifungal activity of SRE in combination with RL<sub>Zonix</sub>, 10 µl of SRE (1 mg/ml) was applied on disk and placed on plates containing different concentrations of RL<sub>Zonix</sub> (22). Another test was done by applying SRE and RL<sub>Zonix</sub> to a different disk. The distance between the disks was equal to the sum of radii of zones of inhibition of the drugs applied alone. The plates were incubated for 24 to 72 h at 35°C (5) and the zone of inhibition was measured. To examine the effect of varying the SRE and RL<sub>Zonix</sub> concentrations in SYRA, 4 mm diameter sterilized paper disks were deposited on the surface and 10 µl of different ratio of SRE and RL<sub>Zonix</sub> mixtures were applied.

Tested bacteria were grown in LB broth for 24 h and transferred onto solid agar medium of the appropriate growth medium. The cultures were spread over the surface as a thin film (39). Four mm diameter sterilized paper disk was deposited on the surface and 10 µl of SRE, RL<sub>Zonix</sub> and SYRA were applied. The plates were incubated for 24 h at 37°C and the zone of inhibition was measured.

**Hyphal extension assay.** The hyphal extension assay was done as described by Dash et al. (9) with modification. A 100 µl of  $1 \times 10^6$  spores/ml suspension was paper strip in a middle of the Petri dish and incubated for 48 h until the appearance of the growing hyphae. Sterile filter disks (4mm diameter) were deposited in the front of the growing hyphea and 10 µl of tested solutions were applied to each disk. The plates were further incubated at room temperature and pictures of the plate were taken at the designated time.

**Spore suspension assay.** The spore suspension assay was performed as described by Mauch et al. (23). The fungal spores were harvested as described previously. The spore suspension was adjusted to  $1 \times 10^6$  spores/ml. The freshly prepared suspension was spread on a Petri dish plate and incubated for 20 to 24 h at room temperature. After this period, four mm paper disks were deposited and 10  $\mu$ l of the test solutions were applied to the disks. The plates were further incubated and the zones of inhibitions were measured after 48 h.

**Microbroth dilution method.** The MICs were determined by the microbroth dilution assay according to method outlined by the NCCLS with modification (25, 26). The inocula suspensions were prepared as described previously (5). Fungal isolates were subcultured on PDA plate and incubated for 3 to 5 days at 35°C. The inoculum suspensions were prepared from fresh cultures. The fungal plates were covered with 5 ml of distilled sterile water. Then, the fungal spores were harvested and suspended in sterile water. After harvesting the spores, the conidia suspensions were diluted with RPMI medium (RPMI 1640 with L-glutamine buffered to pH 7 with 0.165 M morpholinepropanesulfonic acids (MOPS) and supplemented with 2% glucose) (18, 25, 29) (HyClone Laboratories) and adjusted to a final concentration of  $1 \times 10^5$  spores/ml using cell counting hemocytometer. A 50  $\mu$ l of the conidia suspensions were added to each well of 96-well polystyrene microtiter plates (Fisher Scientific catalog no. 21-377-203) to achieve a  $1 \times 10^4$  concentration with conidia/ml. Aliquots (50  $\mu$ l) of twofold serial dilutions of SRE and RL were added to each well (100  $\mu$ l total volume). The plates were incubated for 24 to 72 h at 35°C. The MIC was defined as the lowest concentration that

completely inhibited the growth. The tests were carried out in three replicates to confirm the MIC value.

For yeast, cells were grown to a final concentration of  $10^8$  CFU/ml and suspended at a final concentration of  $5 \times 10^5$  CFU/ml. Cell suspensions (25 ml) were added to 25-ml aliquots of two fold serial dilutions of SRE and RL<sub>Zonix</sub>, and appropriate broth media (27) were dispensed (100  $\mu$ l total volume) in wells of 96-well polystyrene microtiter plates (Fisher Scientific catalog no. 21-377-203). The plates were incubated for 24 h at 28°C.

The colony-forming unit (CFU) experiment was performed under the same experimental conditions as described for the microbroth dilution. In brief, starting inoculum of  $1-4 \times 10^5$  CFU/ml of *C. albicans* ATCC10231 were treated with different concentrations of SRE and SYRA. The CFU were counted by subculturing 100  $\mu$ l of serial dilution of each well on PDA. The experiment was performed in three triplicates, and the results were reported as mean values.

**Checkerboard microtiter plate testing.** To test the antifungal combinations of SRE and RL<sub>Zonix</sub>, a checkerboard microtiter assay was done according to the recommendations of the NCCLS for in vitro susceptibility testing (26). The testing was done in RPMI 1640 with L-glutamine buffered to pH 7 with 0.165 M of MOPS supplemented with 2% glucose (12). A 96-well plate was used to determine the fractional inhibitory concentration (FICs). Serial twofold dilutions of SRE and RL<sub>Zonix</sub> was prepared separately. Then, 25 $\mu$ l of different concentrations of SRE was added in a vertical orientation and 25  $\mu$ l of different concentrations of RL<sub>Zonix</sub> was added in horizontal orientation (33). The conidia and yeast cell suspensions were prepared as described previously (36) and 50  $\mu$ l of the suspensions were added to each well. The

final volume of the well contains 50  $\mu$ l of the drugs and 50  $\mu$ l of the tested culture. The plates were incubated for 24 to 72 h at 35°C. The fractional inhibitory concentrations (FICs) of each drug was defined as the MIC of the combination of SRE and RL<sub>Zonix</sub> divided by the MIC of the drug alone (12) (33). Then, the FIC index was calculated by adding up the FICs for each of the drugs (33). The interaction was defined as synergistic if the FIC index was <1.0, additive if the FIC index was 1.0, and antagonistic if the FIC index was >2.0 (12, 22).

**Time-kill curves.** The time-kill curve assay was done according to methods described by Marchetti et al. (22) with modification. An inoculum of  $5 \times 10^5$  CFU/ml of *C. albicans* ATCC10231 was used under the same experimental conditions as described for the microbroth dilution (27). The effects on viability of SRE (1.97 $\mu$ g/ml) and SYRA (0.97 SRE + RL<sub>Zonix</sub> 2.92  $\mu$ g /ml) after 3, 6, 9, 12, and 24 h of incubation were examined. A synergy effect is defined as  $\geq 2$  log<sub>10</sub> decrease in CFU per milliliter compared to the most active ingredient (6, 24).

### **Toxicity Assays**

**Hemolysis assay.** Hemolytic activity was determined by the method described by Dartois et al. (8) and Sorensen et al. (34) with modification. Sheep erythrocytes were used to test hemolytic activity of SRE, RL<sub>Zonix</sub>, and SYRA. Sheep red blood cells (RBC) were obtained by centrifuging whole blood at  $1,000 \times g$ , washed four times with PBS, and resuspended in 10 ml of PBS to a final concentration of 5% of RBC (8). The RBC suspension (80  $\mu$ l) was added to each well containing different concentrations of SRE or RL<sub>Zonix</sub> or SYRA (20  $\mu$ l). The plate was incubated at 37°C for 60 min. Deionized water

and triton X-100 1% (w/v) were used as positive controls. The percent of hemolysis was calculated using the following equation:

$$\% \text{ hemolysis} = \frac{(\text{absorbance of sample}) - (\text{absorbance of blank}) \times 100}{(\text{absorbance of positive control})}$$

Fifty percent hemolysis (HD<sub>50</sub>) values were calculated as the sample concentrations required to lyse 50 % of the RBC (8).

**In vitro Cytotoxicity.** The cytotoxicity assay against mammalian cells was determined by the method described by Dartois et al. (8). Cell viability was determined using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and the electron coupling reagent, phenazine methosulfate (PMS). This assay is based on the ability of the cells to convert a soluble tetrazolium salt to formazan product. The cell lines for the cytotoxicity tests were obtained from the Utah State University collection. The NIH-3T3 9 (mouse fibroblast), HEK293 and HeLa cells (American Type Culture Collection, Manassas, VA) were propagated in culture medium containing 90% (v/v) Dulbecco's modified Eagle's medium (D-MEM) (Invitrogen, Carlsbad, CA), 10 % defined fetal bovine serum (Hyclone Laboratories, Logan, UT). MDA-MB-435 cells were cultured in a 1:1 mixture of DMEM and Ham's F12 medium supplemented with 5% (v/v) fetal bovine serum (Hyclone Laboratories, Logan, UT), 1% non-essential amino acids, and 1mM sodium pyruvate. Cells were cultured in Corning tissue culture flasks at 37°C with 5 % CO<sub>2</sub>. The confluent cells were treated with trypsin-EDTA and diluted in serum free media. The cells were transferred into 96-well plates at a density of 2×10<sup>5</sup> cells per well and 20 µl of MTS reagent was added to each well containing 100 µl of cells in culture medium. The cells were incubated in a humidified 5 % CO<sub>2</sub> incubator at 37°C for 3 h (8). The plates were read with a microplate reader at

490 nm. Percent cytotoxicity was calculated as:  $(\text{control value} - \text{test value}) \times 100 / \text{control value}$ . Lethal concentrations that caused 50% mortality were calculated using Origin 7.0 (OriginLabCorporation, One Round House Plaza, Northampton, MA 01060).

**Honeybee toxicity.** The test was performed according to methods published by the U.S. Environmental Protection Agency (EPA) Office of Prevention, Pesticides, and Toxic Substances (OPPTS) with modification (13). As recommended by the OPPTS, *Apis mellifera* species were used. Worker honeybees were captured at the entrance of a single healthy hive the day of use. The bees were then brought to the laboratory and kept in a small cage at room temperature. Prior to use, all bees in each cage were anesthetized with CO<sub>2</sub> and transferred to three individual cups to have a total of 10 bees in each cup. Two tests were performed to test the toxicities of SRE, RL<sub>Zonix</sub> and SYRA. The first test was done by feeding the bees with 200  $\mu\text{l}$  of different concentrations of SRE, RL<sub>Zonix</sub> and SYRA (Table.2.4). For the second test, the bees in each cup were anesthetized again with CO<sub>2</sub> and different concentrations of SRE, RL<sub>Zonix</sub> and SYRA were applied in 1  $\mu\text{l}$  drops to the ventral side of the thorax of each bee. After treatments, the bees were fed with 3 ml of sucrose solution (50 % volume of sucrose and 50 % volume of water). The cups were kept at 28°C incubator for 48 h and checked every 4 h. The ratio of living to dead bees was recorded. The treatments with each concentration were replicated three times.

## Results

### Antifungal Activities

The combination of SRE with RL<sub>Zonix</sub> was tested by an agar disk diffusion assay in which *R. pilimanae* was plated onto PDA and PDA with RL<sub>Zonix</sub> (RL-PDA). Different concentrations of SRE were applied on disks and deposited on RL-PDA and PDA.

The results showed that the zones of inhibition of SRE in RL-PDB were slightly larger compared to the agar with no RL<sub>Zonix</sub> (Fig. 2.2). This indicates some degree of a positive interaction between SRE and RL<sub>Zonix</sub>. However, due to the lower concentrations of SRE the zone of inhibition did not increase noticeably compare to the control. In the experiments where SRE and RL<sub>Zonix</sub> were applied to a different disk, no change was observed in SRE zone of inhibition (data not shown).

In this experiment SRE and RL<sub>Zonix</sub> were mixed at different ratios prior to use. SRE, RL<sub>Zonix</sub> and SYRA were tested against yeast, fungi and bacteria. SRE and SYRA showed antifungal activities against all tested fungi and yeast. However, no activity was observed with RL<sub>Zonix</sub> except against *R.pilimanae*. A strong synergy between SRE and RL<sub>Zonix</sub> was observed (Fig. 2.1.). The SYRA activity was much stronger than SRE when against tested yeast and fungi. For example, against *C. albicans*, the zones of inhibition were 20 and 40 mm for SRE and SYRA, respectively (Fig. 2.1, Table 2.1). In addition, the concentration of SRE in SYRA (0.6mg/ml SRE) applied to the disks to give these zones of inhibition was 4-fold less than for SRE alone (2.4mg/ml). It is important to note that the highest zones of inhibition against yeast and fungi were obtained when SRE and RL<sub>Zonix</sub> were mixed at a ratio of 1:3 (as in SYRA). No activities were observed against tested bacteria.

To further investigate the antifungal activity of SRE, RL<sub>Zonix</sub> and SYRA, I tested their activity against *P. oxalicum*, *R. stonifer*, *A. flavus*, and *A. niger* both hyphal growth and germinated spores. In both experiments, SRE and SYRA were inhibitory against germinated spores and hyphal extension of all tested species (Fig. 2.3 and Fig. 2.4). However, no activity was observed with RL<sub>Zonix</sub>. The zones of inhibition were from 15 to 20 mm against germinated spores. However, the activity of SRE and SYRA were stronger when applied directly after inoculation of the plates (zones of inhibition were from 25 to 30mm). SRE and SYRA inhibited the hyphal growth as it was demonstrated by the crescent zone due to the retarded mycelia growth (Fig. 2.4).

To quantify the antifungal activity of SRE, RL<sub>Zonix</sub> and SYRA, the microbroth dilution and the checkerboard methods were used. SRE inhibited tested fungi and yeast with MIC's that ranged from 1.95 to 7.8 µg per ml (Table. 2.1). RL<sub>Zonix</sub> did not show activity against the tested organisms except *R. pilimanae* with an MIC of 93.75 µg per ml. However, no antibacterial activity was observed with SRE and RL<sub>Zonix</sub>.

The SYRA's MICs ranged from 0.5 to 3.9 µg /ml (Table 2.1). Based on the data obtained from the checkerboard assay, the FICs indices of SRE plus RL<sub>Zonix</sub> were ranging from 0.25 to 0.5 (FIC<1) indicating synergism between these two compounds. In addition, SRE and SYRA showed significant reduction in the viable counts against *C.albicans* (Fig. 2.5). However, SYRA was more fungicidal than SRE alone.

### **Time Kill Assay**

The synergisms observed between SRE and RL<sub>Zonix</sub> in the checkerboard microtiter assay were confirmed in time-kill curves experiments. Fig. 2.6. shows time-kill results for SRE and SYRA. SYRA was more active than SRE alone and displayed much more



rapid killing against *C.albicans*. The decreases in CFU with SYRA comply with a synergistic relationship between SRE and RL<sub>Zonix</sub>.

### **In vitro Toxicity Studies**

**Hemolysis assay.** SRE, RL<sub>Zonix</sub> and SYRA were found to be hemolytic and displayed dose dependent hemolytic activity against sheep RBCs (Fig. 2.5). RL<sub>Zonix</sub> was the least hemolytic among the three.

**Cytotoxicity studies.** The cytotoxicity effects of SRE, RL<sub>Zonix</sub> and SYRA on mammalian cell lines are shown in Fig. 2.6. SRE, RL<sub>Zonix</sub> and SYRA displayed a dose dependent cytotoxicity against NIH-3T3 (mouse fibroblast), HEK293, MDA-MB-435, and HeLa cells (Fig.2.6). However, the concentrations showing toxic effects on the cell lines were higher than those needed for hemolytic activity. Interestingly, SRE, RL<sub>Zonix</sub>, and SYRA displayed more toxicity against the MDA-MB-435 cell line. The MDA-MB-435 LC50 for SRE, RL<sub>Zonix</sub> and SYRA were almost three times lower comparing to the LC50 of NIH-3T3 (mouse fibroblast), HEK293, and HeLa cells (Table 2.3).

**Honeybee toxicity.** The honeybee toxicity studies of SRE, RL<sub>Zonix</sub> and SYRA were examined against *Apis mellifera*. The presented results were from three separate experiments. The no treatment control had no mortality and the positive control had 100% mortality after 48 h of observation. The lethal effect was observed when bees were on their back. Table2-4 shows the effects of SRE, RL<sub>Zonix</sub> and SYRA against honeybees. The SRE, RL<sub>Zonix</sub> and SYRA were slightly toxic when they were digested by the honeybees. The percentage of mortality was from 20 to 30% after 48 h observation. In contrast, the contact exposure test showed that at the same concentrations, the honeybees were indifferent.

## Discussion

In this work, I studied the antimicrobial activities and the toxicity of SRE, RL<sub>Zonix</sub> and the mixture (SYRA). As expected, SRE was active against a large array of fungi including yeasts and filamentous fungi such as *C. albicans*, *Aspergillus* spp., and *Penicillium* spp. The MIC's ranged from 1.95-7.8 µg/ml. However, no activity was observed against bacteria. RL<sub>Zonix</sub> did not show antimicrobial activities against most tested organisms. The present study is the first report on the antimicrobial activities of SRE and RL<sub>Zonix</sub> combinations. SRE showed strong synergism with RL<sub>Zonix</sub> for all tested yeast and fungi. Checkerboard analyses showed that RL<sub>Zonix</sub> reduced the SRE MICs two to four fold. The FIC indices ranged between 0.25-1 indicating strong synergism between SRE and RL<sub>Zonix</sub>. It is noteworthy that the strongest activity was observed when RL<sub>Zonix</sub> and SRE were combined at a 3:1 ratio, respectively. The mixture at this ratio is termed SYRA. In addition to spore germination inhibition, SRE and SYRA also displayed antifungal effects against germinated spores and hyphal growth.

Interestingly, RL<sub>Zonix</sub> alone did not exhibit antifungal activity against tested fungi. However, in the presence of SRE these two showed good synergism. Antifungal synergism was previously observed when amphotericin B was combined with gramicidin S or NF against *C. albicans* (19). In contrast, Marchetti et al. demonstrated that despite the antifungal activity of combined Fluconazole and cyclosporine (cy) (FLC) against *C.albicans* , the MIC of FLC remained unchanged (22). However, a fungicidal effect was observed after 48 h of exposure to this combination suggesting that perhaps Cy action was dependent on the slow effect of FLC on *C.albicans* cells , and Cy interaction with the multidrug transporters rendered *C.albicans* more susceptible to FLC (22). Work

by Arian et al. (2) showed favorable interaction between amphotericin B and caspofungin against *Aspergillus* and *Fusarium* spp (2). The combination resulted in a decrease in amphotericin B MICs (2), and this may be due to better penetration of amphotericin B following the effect of caspofungin on the cell wall (2).

Previous work by Stanghellini (35) showed that RL inhibits zoosporic plant pathogens by lysing zoospore membranes. Both SRE and RL<sub>Zonix</sub> act on the cell membrane but their mechanism of action are different. The nature of their interaction is still under investigation. One explanation could be that RL<sub>Zonix</sub> interaction with the plasma membrane facilitated SRE binding and interaction with the lipid membrane which resulted in enhancement of channel formation. Another explanation is that perhaps the anionic characteristic of RL<sub>Zonix</sub> at physiological pH and its interaction with cell surface provided more negative charges on the surface which allowed more binding and stronger interaction of SRE with the negatively charged membrane causing an increase in number of channels formed by SRE. The mechanism of interaction is still under investigation.

I also studied the effect of the addition of RL<sub>Zonix</sub> on the hemolytic activity and cytotoxicity of SRE. Previous studies by Sorensen et al. (34) and De Lucca et al. (10) have shown that SRE had hemolytic activity against sheep erythrocytes and toxicity on HeLa cells. However, De Lucca and Walsh (11) showed that SRE reduces fungal viability by 90 to 98% at concentrations that causes 20% release of hemoglobin from sheep erythrocytes. This result is consistent with my current findings, where SRE MICs were 3 to 5 times lower than the concentrations that cause 50% of hemolysis and cell toxicity. In addition, SRE, RL<sub>Zonix</sub> and SYRA effects on honeybees was studied. No

effects were observed against honeybees at the concentrations that showed hemolytic and cytotoxicity effects.

In this work, I present the first studies on the antimicrobial activities of SRE and RL<sub>Zonix</sub> combinations. The in vitro results showed clearly strong synergism between SRE and RL<sub>Zonix</sub> against phytopathogenic fungi and yeast. The antifungal spectrum and low toxicity suggests that SYRA is a potential candidate for fungicidal agricultural applications. However, more work is needed to better understand the mechanism of interaction in vivo.

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TABLE .2.1. Antimicrobial activities of SRE, RL and SYRA.

Organism	MIC ( $\mu\text{g/ml}$ )			ZI (mm)		
	SRE	RL	SYRA (SRE/RL)	SRE	SYRA	FIC index
<i>Aspergillus flavus</i>	7.8	>20	3.9/5.85	11	18	0.5
<i>Rhodotorula pilimanae</i>	3.9	93.75	0.97/2.92	22	35	0.24
<i>Candida albicans</i>	3.9	>250	1.95/5.85	21	40	0.5
<i>Rhizopus stonifer</i>	7.8	>250	3.9/11.7	11.5	20	0.5
<i>Fusarium oxysporum</i>	7.8	>250	3.9/11.7	10	17	0.5
<i>Penicillium sp1</i>	7.8	>250	7.8/23.43	10	16	1
<i>Botrytis cinerea</i>	3.9	93.75	0.975/2.92	13	25	0.24
<i>Rhizoctonia solani</i>	3.9	>250	1.95/5.85	10	19	0.5
<i>Cladosporium cladosporioides</i>	3.9	>250	1.95/5.85	15	20	0.5
<i>Penicillium oxalicum</i>	7.8	>250	1.95/5.85	13	22	0.25
<i>Saccharomyces cerevisiae W303C</i>	1.97	>250	0.48/1.44	14	25	0.25
<i>Saccharomyces cerevisiae BY4741</i>	1.97	>250	0.48/1.44	13	24	0.25
<i>Saccharomyces cerevisiae 8A-1B</i>	1.97	>250	0.48/1.44	15	24	0.25
<i>Aspergillus niger</i>	3.9	>250	1.95/5.85	11.5	20	0.5
<i>Staphylococcus aureus</i>	>250	>250	>250	>1	>1	ND
<i>Bacillus subtilis</i>	>250	>250	>250	>1	>1	ND
<i>Escherichia coli</i>	>250	>250	>250	>1	>1	ND
<i>Proteus vulgaris</i>	>250	>250	>250	>1	>1	ND

MICs values were obtained from triplicate determinations

The zone of inhibition: The error was  $\pm 2$  mm, as determined from triplicate determinations.

For FIC index calculations with RL, a value of 250 was used for those organisms with an MIC of >250.

ND, not determined

TABLE. 2.2. Hemolytic activity of SRE, RL<sub>Zonix</sub> and SYRA

	HD 50% (µg/ml)
SRE	26
RL <sub>Zonix</sub>	75.5
SYRA (SRE)	23

TABLE.2.3. Lethal concentration that caused mortality of 50% of the cells.

Cells		LC50 (µg/ml)
Hek292	SRE	96
	RL <sub>Zonix</sub>	101
	SYRA ([SRE])	28.78
3T3	SRE	100.63
	RL <sub>Zonix</sub>	95.85
	SYRA ([SRE])	25.40
Hela	SRE	95.85
	RL <sub>Zonix</sub>	96.13
	SYRA ([SRE])	26.25
MDA-MB-435	SRE	30.7
	RL <sub>Zonix</sub>	41.4
	SYRA ([SRE])	12

TABLE.2.4. Honeybee mortality

A- Contact exposure to SRE and RL<sub>Zonix</sub> .

Time (Hours)	4		12		24		48	
Concentration (mg/ml)	Dead	Live	Dead	Live	Dead	Live	Dead	Live
RL <sub>Zonix</sub> 12.5mg/ml	0	10	1	9	2	8	2	8
SRE 5 mg/ml	0	10	1	9	1	9	1	9

B- Feeding SRE and RL<sub>Zonix</sub>

Time (Hours)	4		12		24		48	
Concentration (mg/ml)	Dead	Live	Dead	Live	Dead	Live	Dead	Live
RL <sub>Zonix</sub> 12.5mg/ml	0	10	2	8	2	8	3	7
SRE 5 mg/ml	0	10	1	9	2	8	3	7

## D- Contact exposure to SYRA

Time (Hours)	4		12		24		48	
Concentration (mg/ml)	Dead	Live	Dead	Live	Dead	Live	Dead	Live
SYRA (SRE 4.5 mg/ml, RL <sub>Zonix</sub> 12.5 mg/ml)	0	10	0	10	0	10	0	10

## C- Feeding with SYRA

Time (Hours)	4		12		24		48	
Concentration (mg/ml)	Dead	Live	Dead	Live	Dead	Live	Dead	Live
SYRA (SRE 1.6 mg/ml, RL <sub>Zonix</sub> 5 mg/ml)	0	10	1	9	1	9	2	8

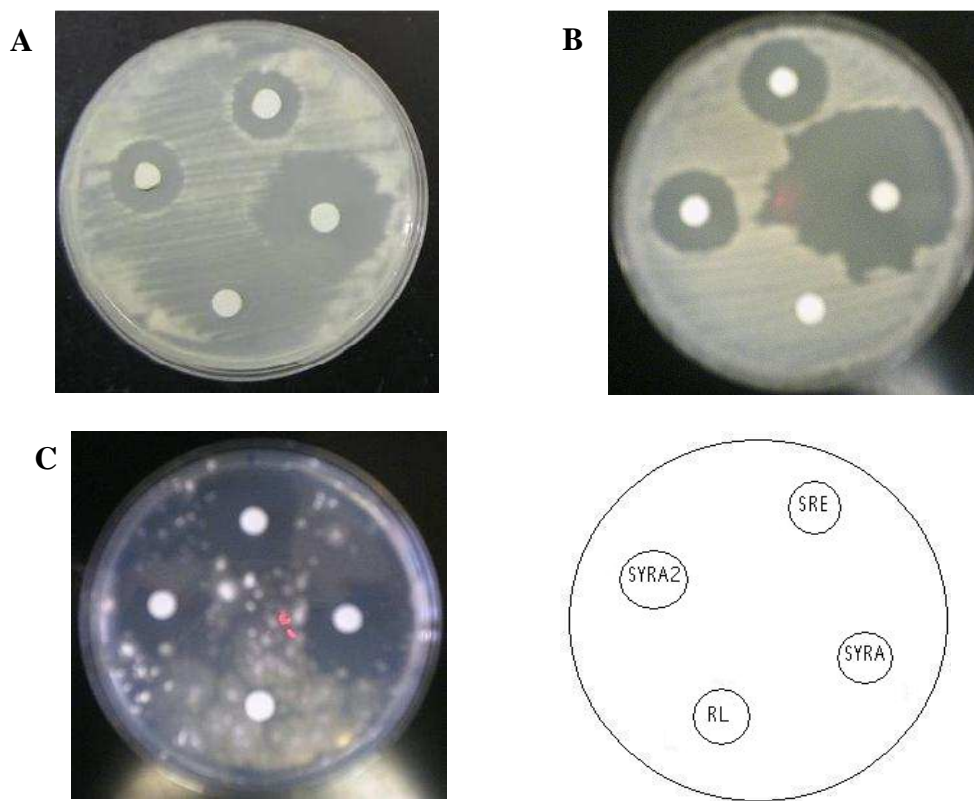


FIG.2.1. Disk diffusion assay testing the combination of SRE with RL<sub>Zonix</sub>. 10  $\mu$ l of SRE (2.5mg/ml), RL<sub>Zonix</sub> (2.5 mg/ml) and the combination SYRA (0.6mg/ml SRE and 1.95 mg/ml RL<sub>Zonix</sub>), SYRA2 (1.95 mg/ml SRE and 0.6 mg/ml RL<sub>Zonix</sub>) were applied on disks. A- *Botrytis cinerea*. B-*Candida albicans*. C-*Schizophyllum commun*.

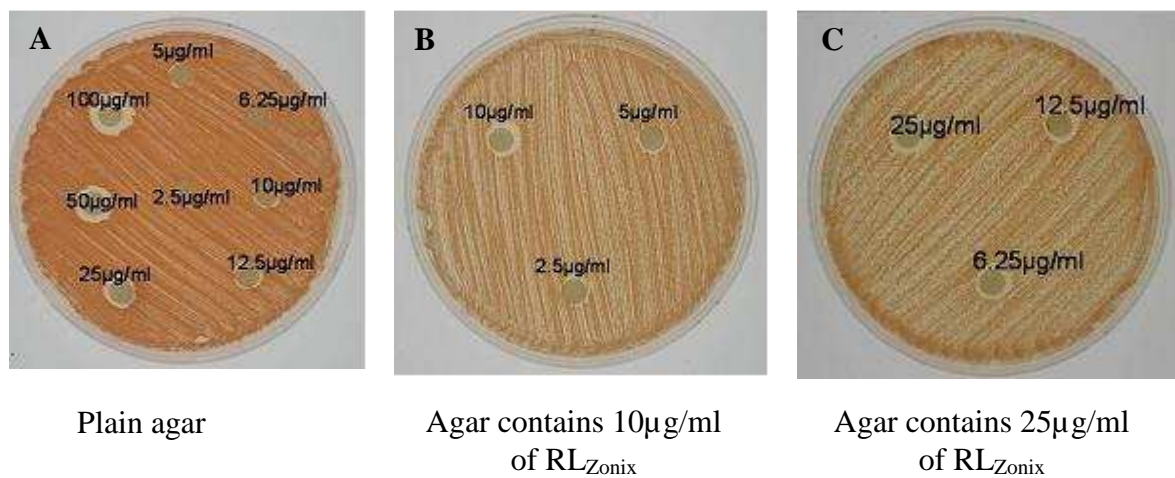


FIG. 2.2. Fungicidal activities of different amounts of SRE deposited on PDA containing RL<sub>Zonix</sub> against *R. pilimanae*. Different concentrations of SRE were applied to PDA (A) and Agar containing: 10 µg/ml (B), 25 µg/ml (C) of RL<sub>Zonix</sub> .

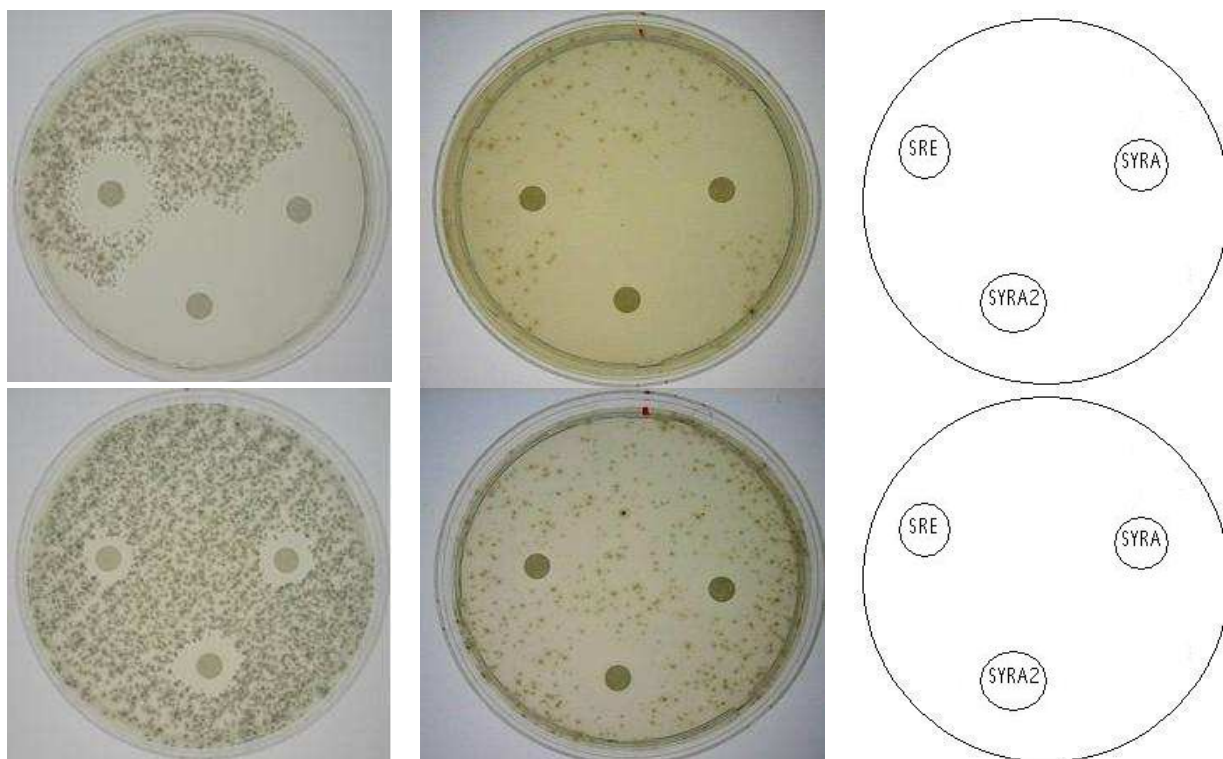


FIG.2.3. SRE and SYRA inhibition against fungal growth. The fungal spores (*P. oxalicum*, *R. stonifer*) were allowed to germinate on PDA plate and grow for 30 h before SRE (0.25mg/ml) and SYRA (0.25mg/ml SRE and 0.75mg/ml RL<sub>Zonix</sub>), SYRA (0.6mg /ml SRE and 1.95mg/ml RL<sub>Zonix</sub>) were applied on the disks (Column B). Then the plates were allowed to grow for 24 h. In column A, SRE and SYRA were applied directly after inoculating the plates with fungal spores. RL<sub>Zonix</sub> showed no inhibition.



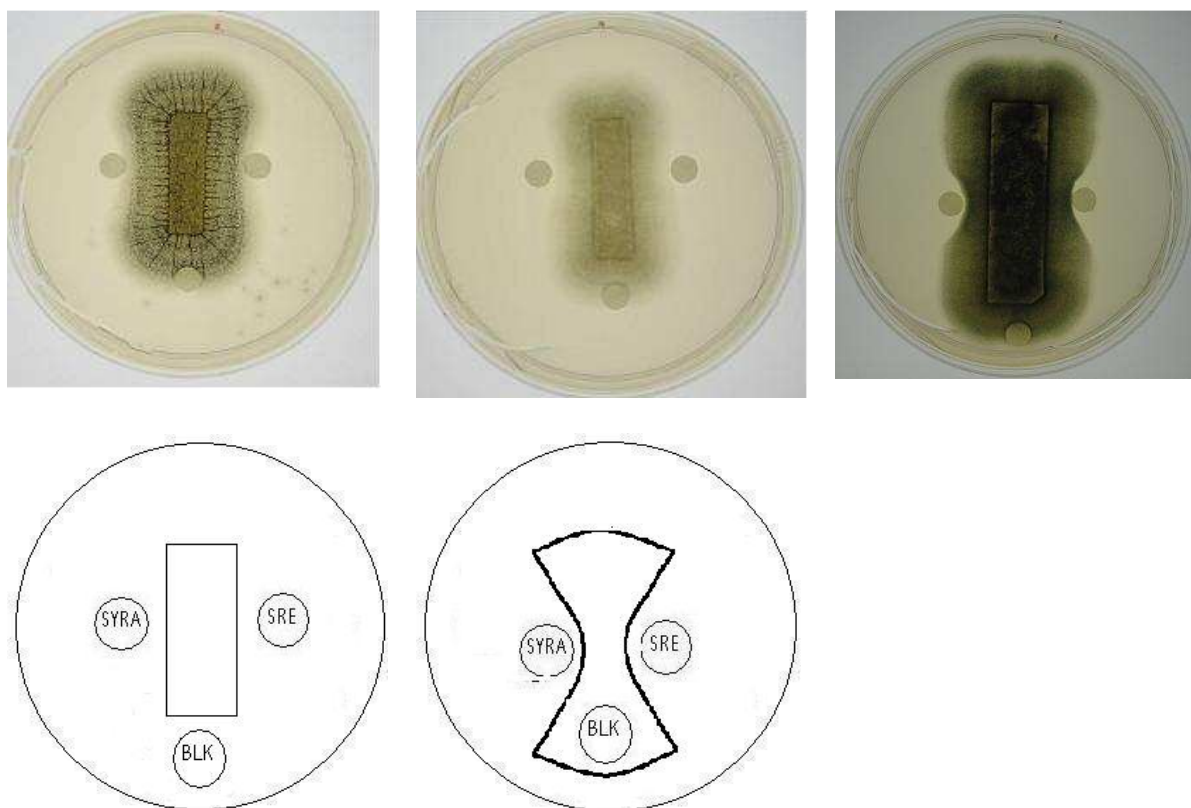


FIG. 2.4. Disk diffusion assay against hyphal extension. The fungal spores were applied to a filter strip and allowed to grow for 48 h until the appearance of hyphae. Then, the disks were deposited in front of the growing hyphae and 10  $\mu$ l of SRE (0.25mg/ml) and SYRA (0.25mg/ml SRE and 0.75mg/ml RL) were applied. The plates were incubated for another 48 h. RL showed no inhibition.

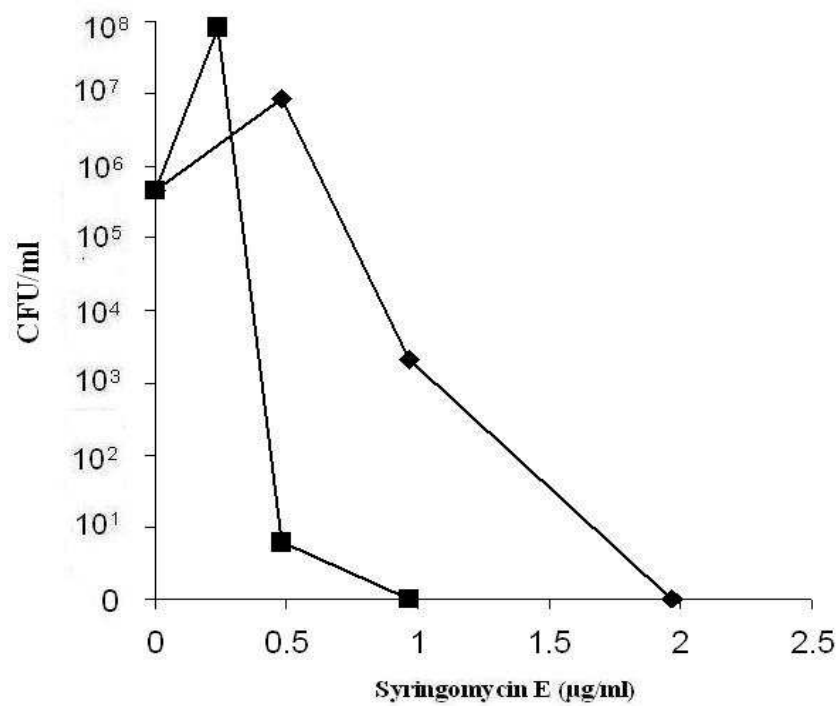


FIG. 2.5. Effects of SRE (♦) and SYRA(■) on the viability of *C. albicans*.

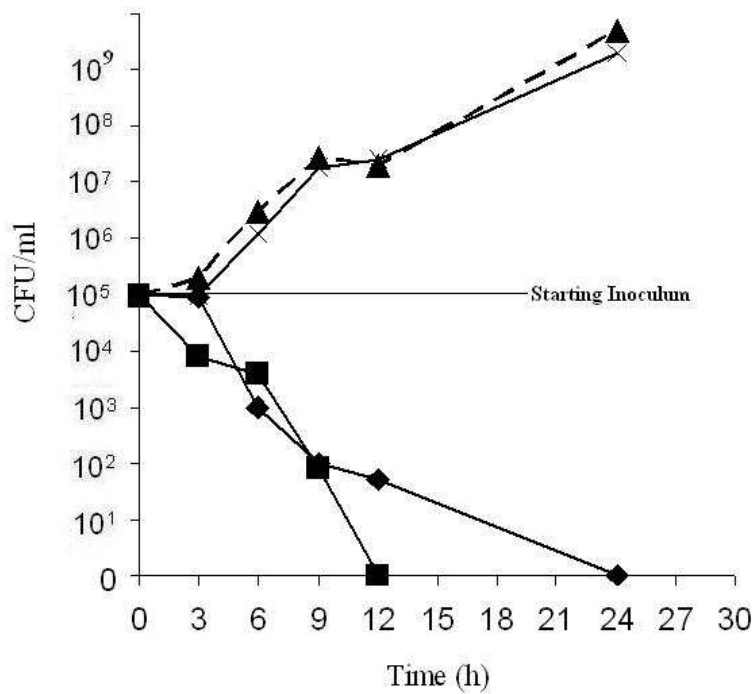


FIG. 2.6. Time-kill curves of *C. albicans*. CFU were determined after 3, 6, 9, 12, and 24 h of incubation. Mean CFU values of three separate experiments are shown. Symbols: SRE (◆), RL (×), SYRA(■), and control(▲)

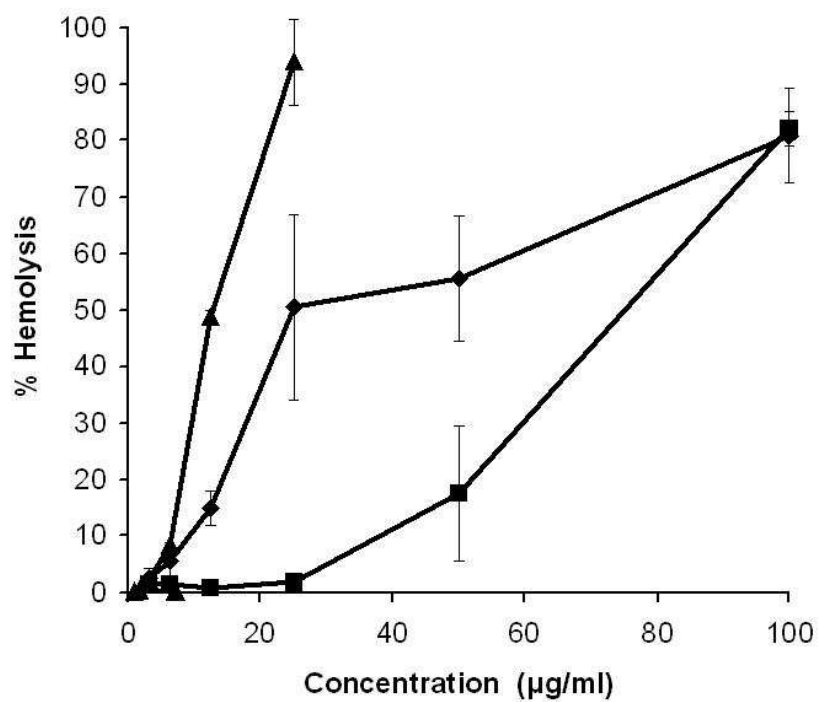


FIG. 2.7. Hemolytic activity of SRE (◆), RL<sub>Zonix</sub> (■), and SYRA (▲). The RBCs were incubated for 1 h at 37°C.

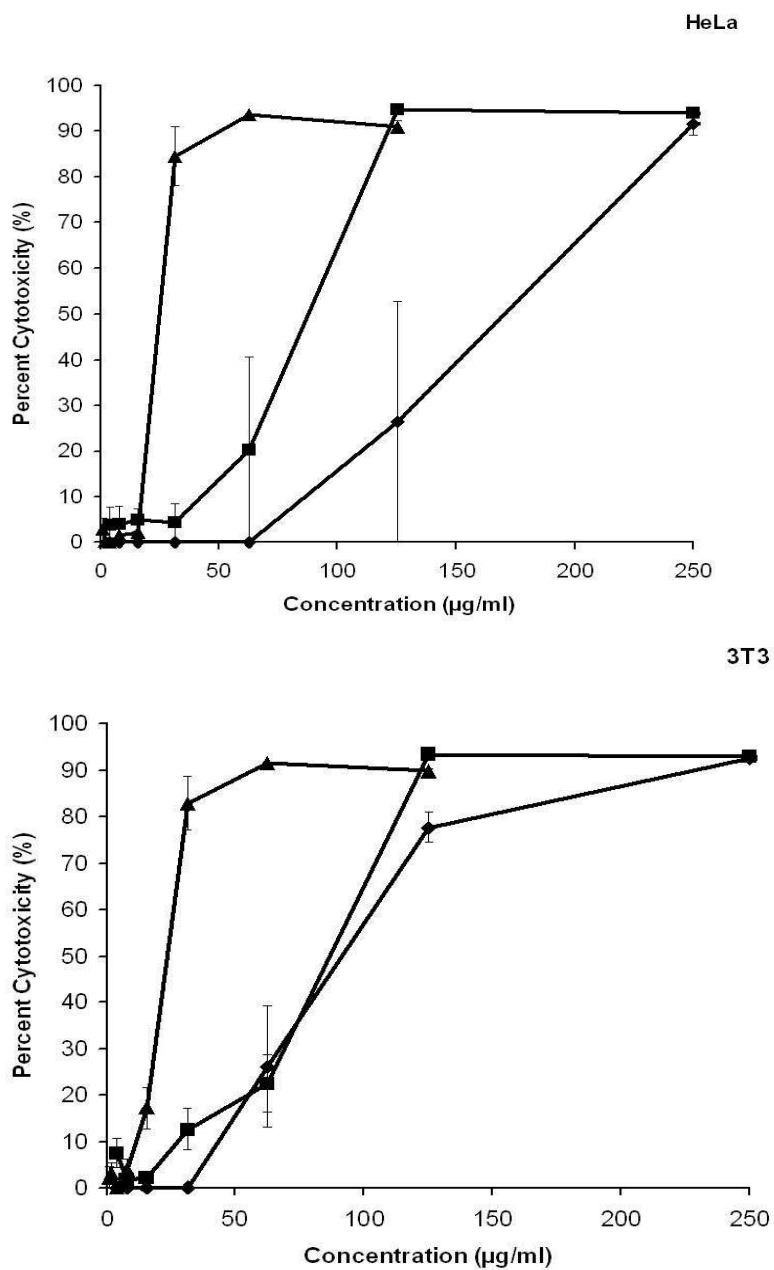
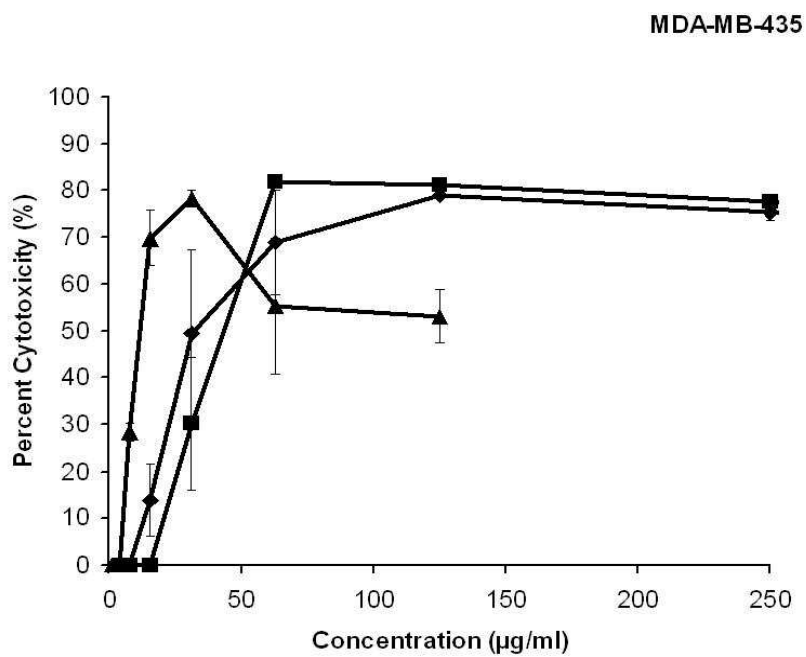
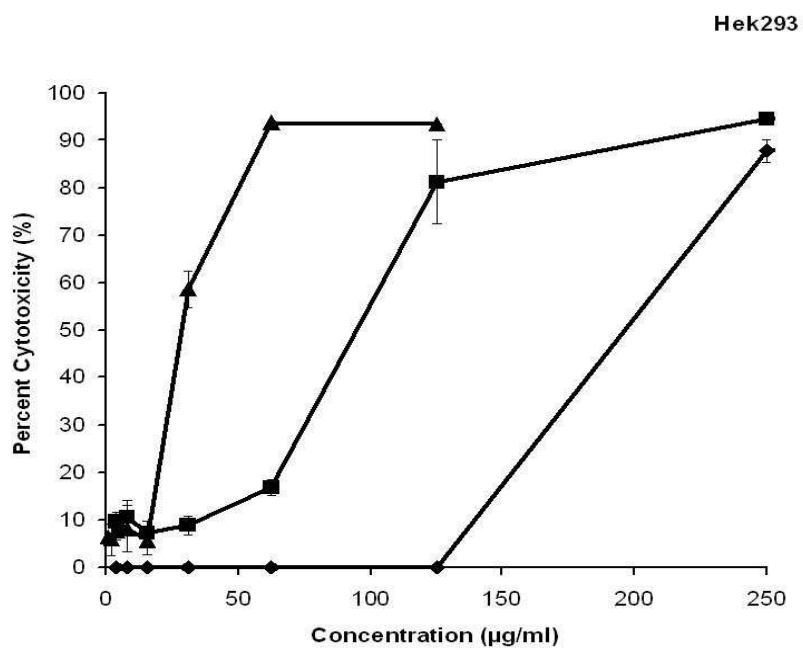


FIG. 2.8. Percent cytotoxicity of SRE (■), SYRA (▲), and RL<sub>Zonix</sub> (◆).



CHAPTER 3  
PHYSICAL-CHEMICAL PROPERTIES OF SRE AND SRE-RHAMNOLIPID  
MIXTURES

**Introduction**

In recent years, the need for new and effective agrofungicides has emerged due to decreases in the effectiveness and safety of currently used products (10).

The small cyclic lipodepsipeptide syringomycin E (SRE) contains a long, unbranched-3-hydroxy fatty acid with a positively charged and hydrophilic cyclic ring of nine amino acids at the C terminus (5, 13). Previous studies showed that SRE has inhibitory activity against fungi and yeast such as *Botrytis cinerea*, *Geotrichum candidum* and *Rhodotorula pilimanae* (9, 17)

The antifungal activity of SRE against a wide range of yeast and fungi, elected it to be a possible agrofungicide. However, its chances to be developed as a commercial fungicide agent will strongly depend on its stability under different conditions.

A comprehensive examination of the literature revealed no publications that describe the chemical stability of SRE over a range of pHs and temperatures. However, Hu et al. (7) examined the stability of crude extracts of *Pseudomonas* species under high temperature, and acidic and alkaline pHs. The results revealed good stabilities of the extract to heat and acid but not to alkaline pHs (7). Furthermore, based on earlier studies on SRE activity, it has been observed that SRE solutions had long shelf-lives and remained stable for years under dry freezing conditions (unpublished observations, J. Takemoto, Utah

State University). In addition, examination of SRE's structural features suggests that it would be relatively stable to different temperatures and acidic pHs.

Rhamnolipids are glycolipids formed by rhamnose linkage to fatty acids of saturated or unsaturated alkyl chain between C8 and C12 (6). Recent studies have shown that rhamnolipids are active against zoosporic plant pathogens such as *Pythium* and *Phytophthora* species (16) and against fungal strains such as *Fusarium solani* and *Penicillium funiculosum* (6). In addition, the biosurfactants are known to be effective at extreme temperature and pH values (2). However, very little information has been reported on stability of rhamnolipids under different temperatures and pHs. Thaniyavarn et al. (18) have tested the surface tension activity of rhamnolipids over a wide range of pHs, temperatures, and NaCl concentrations. The results showed a significant stability of rhamnolipids over all conditions tested.

SRE and SRE + rhamnolipid (SYRA) formulations are potential candidates for postharvest disease control and possibly other fungicidal applications in agriculture such as seed treatment and fruit storage. In Chapter 2, studies on the bioactive properties of both SRE and SYRA were described. The results showed strong antifungal activities against yeast and plant pathogenic filamentous fungi. It is important to mention that most of the experiments were administered in vitro under controlled or optimal conditions. However, if used in the field these compounds may be exposed to proteolysis and variety of environmental and climate conditions such as high temperatures, humidity and different pHs that may abolish or alter their antifungal activities. Their usefulness as agrofungicides requires sufficient stability when subjected to such conditions.



The aim of this study is to examine the physical and chemical stability of SRE and SYRA. To further explore their stabilities and candidacies as agrofungicides, SRE and SYRA formulations were exposed to different temperatures and pHs, and subjected to autoclaving, ultraviolet light, sonication, and proteolysis. The treated solutions were tested for antifungal activities and analyzed by RP-HPLC in the cases of temperature and proteolysis treatments. In addition, SRE and SYRA were mixed with mineral oil and their activities were tested. The overall results showed significantly high stability of SRE and SYRA under the tested conditions.

## Materials and Methods

### SRE and SYRA

SRE was purified from *P.syringae* pv.*syringae* strain B301D by the method of Bidwai et al. (3). *P.syringae* pv.*syringae* strain B301D was grown in non-commercial potato dextrose casamino acids medium in 4- or 8-liter cultures as described by Zhang and Takemoto (19). The SRE was extracted from cultures using acidified acetone and purified using the chromatographic methods described by Bidwai et al. (3). HPLC column fractions were tested for bioactivity by applying 10  $\mu$ l aliquots on a fresh lawn of *R. pilimanae* on potato-dextrose agar plates, incubation at 28°C, and observing cleared growth inhibition areas (19). Commercial rhamnolipid (Zonix) (RL<sub>Zonix</sub>) was obtained from Jeneil Biotech, Inc. RL<sub>Zonix</sub> is an 8.5% (wt/vol) solution of rhamnolipid analogs in water. Two forms of rhamnolipids are in RL<sub>Zonix</sub>, R1 (C<sub>26</sub>H<sub>48</sub>O<sub>9</sub>) has the molecular formula of  $\alpha$ -L-rhamnopyranosyl- $\beta$ -hydroxydecanoyl- $\beta$ -hydroxydecanoate, and R2 (C<sub>32</sub>H<sub>58</sub>O<sub>13</sub>)

is 2-O- $\alpha$ -L-rhamnopyranosyl- $\alpha$ -L-rhamnopyranosyl-  $\beta$  hydroxydecanoate. SYRA was prepared by combining SRE and RL<sub>Zonix</sub> at 1:3 (wt/wt) ratios, respectively. Two formulations were used: SYRA<sub>0.6, 1.95</sub> (0.6 mg SRE and 1.95 mg RL<sub>Zonix</sub> per ml) and SYRA<sub>0.25,0.75</sub> (0.25 mg SRE and 0.75 mg RL<sub>Zonix</sub> per ml).

### **Antifungal Activities**

**Agar disk diffusion method.** The disk diffusion assay was done accordingly to the method described by the National Committee for Clinical Laboratory Standards (1) protocols for antifungal testing with modification (11, 15). *R. pilimanae* was grown in potato dextrose broth (PDB) and adjusted to  $5 \times 10^4$  CFU/ml (8, 14), and transferred onto solid agar medium. The culture was spread over the surface as a thin film (14). Four mm diameter sterilized paper disks were deposited on the surface and SRE and SYRA<sub>0.6,1.95</sub> or SYRA<sub>0.25,0.75</sub> were applied on disk1 and disk 2, respectively (7 to 10  $\mu$ l aliquots).

**HPLC analysis.** The high performance reverse-phase liquid chromatography (RP-HPLC) analysis was performed by the method determined by Bidwai et al. with modification (3). The treated SRE and SYRA were loaded onto C18 reverse-phase column for HPLC analysis. The samples were eluted by a 25 min (0.5ml per min) linear gradient of the 70% of 0.1% trifluoroacetic acid in water, and 30% of 0.1% trifluoroacetic acid in isopropanol. Eluted SRE and SYRA were detected by UV detection at 240 nm.

**Effect of temperature.** SRE (1mg/ml) and SYRA<sub>0.25,0.75</sub> solutions were prepared and incubated at 4°, 28°, 50°, and 65°C. At 50°C, and 65°C the activities were tested as described previously every day for 17 days. At 4°C and 28°C the activities were tested each day for 60 days. The solutions were also autoclaved for 15, 30 and 45 min and their

activities were tested against *R. pilimanae* and 50 µl of the treated SRE solutions or SYRA<sub>0.25,0.75</sub> were analyzed by RP-HPLC as described previously (3). In the case of SYRA<sub>0.25,0.75</sub>, two sample variations were prepared and used in these experiments. Sample A was prepared by mixing SRE (0.25mg/ml) and RL<sub>Zonix</sub> (0.75 mg/ml) after autoclaving the individual solutions separately. Sample B was prepared by mixing SRE (0.25 mg/ml) and RL<sub>Zonix</sub> (0.75 mg/ml) before autoclaving.

**Effect of pH on SRE and SYRA activity.** The effect of pH on the activities of SRE (2.4 mg/ml) and SYRA<sub>0.6,1.95</sub> was examined using the disk diffusion assay against *R. pilimanae* as described before (12). The solutions were adjusted to pH values of 4, 5, 6, and 7 by the addition of 1N HCl or 1N NaOH and incubated at room temperature for 24 h. Ten µl of SRE and SYRA<sub>0.6,1.95</sub> were applied on the disks. The plates were incubated at 28°C for 48 h and the zones of inhibition were measured.

**Effects of ultraviolet light and sonication on SRE and SYRA activities.** The effects of UV light and sonication on SRE and SYRA activities were studied using the disk diffusion assay against *R. pilimanae* (12). One hundred µl SRE (2.4 mg/ml) and SYRA<sub>0.6,1.95</sub> were added to 96 well-plates and irradiated using a Fisher Biotech ultraviolet hand-held lamp (Fisher Scientific) for 60 min at a wave length of 320 nm. Ten µl of SRE (2.4 mg/ml) and SYRA<sub>0.6,1.95</sub> were taken every 10 min for 60 min and applied on disks. The plates were incubated at 28°C for 48 h and the zones of inhibition were measured.

Two hundred µl of SRE (2.4 mg/ml) and SYRA<sub>0.6,1.95</sub> in 2 ml glass tubes were sonicated for different time intervals in a water bath sonicator ( Bransonic ultrasonic Model 1210, 50-60Hz). The 10 µl samples were taken every 10 min for 60 min and

tested for activity as described previously. The plates were incubated at 28°C for 48 h and the zones of inhibition were measured.

**Trypsin treatment.** To study the effect of protease on SRE and SYRA activities, the solutions were dissolved in PBS buffer, pH 7.4 in 1.5 mL capacity microcentrifuge tubes (20). Trypsin (bovine pancreas, Sigma, St. Louis) was added at ratio of 1:25 (enzyme/peptide, w/w). SRE (1mg/ml) and SYRA<sub>0.25,0.75</sub> were mixed with 40 µg/ml and 10 µg/ml of trypsin, respectively. The tubes were incubated at 37°C for 2 h and stopped by adding trypsin inhibitor aprotinin (Sigma) (10µg/ml). The treated samples were examined for their activities against *R. pilimanae* and analyzed by RP-HPLC as described earlier.

**Effect of mineral oil on SRE and SYRA activities.** SRE and SYRA<sub>0.25,0.75</sub> were mixed with mineral oil at a ratio of 1:1 (vol/vol) and incubated at room temperature for 1 h. Ten µl of SRE and SYRA<sub>0.25,0.75</sub> were tested by disk diffusion assay as described earlier. The plate was incubated at 28°C for 48 h and the zones of inhibition were measured.

## Results and Discussion

### Physico-chemical Stability of SRE and SYRA

The use of SRE and SYRA as fungicides agents requires that they be sufficiently stable. Their useful applications will necessitate long-term stability, as well as resistance to environmental conditions such as temperature, ultraviolet light, and pH variations.

The results show that SRE and SYRA<sub>0.25,0.75</sub> are stable at 4°C and 28°C (Fig. 3.1). However, at 55°C and 65°C, SYRA<sub>0.25,0.75</sub> activity began to decrease after 4 days and was completely lost after 6 and 11 days at 65°C and 55°C, respectively (Fig. 3.1). In

contrast, SRE remained completely active at these temperatures. It is important to note that at 4°C and 28°C SRE and SYRA<sub>0.25,0.75</sub> activities were very stable after 60 days of incubation (data not shown).

To further explore the effect of temperature on SRE and SYRA<sub>0.25,0.75</sub> activities. SRE and SYRA<sub>0.25,0.75</sub> were autoclaved for 15, 30, and 45 min at 121°C (Table. 3.1, Fig. 3.2). SRE showed full inhibitory activity against *R. pilimanae* at each time. In contrast the activity of SYRA<sub>0.25,0.75</sub> (sample B) declined when autoclaved. However, when SYRA<sub>0.25,0.75</sub> prepared with separately autoclaved solutions of SRE and RL<sub>Zonix</sub> (sample A), it was active. To further examine the effect of autoclaving on SRE and SYRA<sub>0.25,0.75</sub> HPLC analyses were performed on treated samples (Fig. 3.3.). HPLC profiles of RL<sub>Zonix</sub> showed that its structure was not affected by autoclaving. However, 60 % of SRE was degraded. as a consequence of autoclaving. In the case of sample B, the SRE structure was completely degraded (Fig. 3.3). It appears that RL<sub>Zonix</sub> interaction with SRE forms a complex that is heat sensitive. It could be speculated that the two molecules physically interacted via their hydrophobic and hydrophilic moieties, respectively, and that high heat (e.g. autoclaving) disrupts the interactions that are mainly hydrophobic interactions and hydrogen bonding. SRE and RL<sub>Zonix</sub> each showed significant long term stability. In the case of SYRA<sub>0.25,0.75</sub>, the long term stability was very good at 4°C and 28°C, but, the stability began to decline at 55°C and 65°C. These observations are important for considerations of storage and transportation of SYRA formulations.

To further explore the physical properties of SRE and SYRA<sub>0.6,1.95</sub>, these two were exposed to UV light, sonication, and varied pHs levels. The results showed UV light and sonication had no effects on antifungal activities against *R. pilimanae* were not

affected by UV light (Fig. 3.5). SRE and SYRA<sub>0.6,1.95</sub> antifungal activities were also stable at pHs between 4 and 7 (Fig. 3.4). However with SYRA<sub>0.6,1.95</sub>, I observed a moderate increase in activity at pH 5 and 6 (Fig. 3.4). This difference in activity is perhaps due to the net charge of RLs. RL is an anionic biosurfactant with a pKa value of 5.6 (2). It was reported by Aranda et al. (2) that at pH 7.4, 98.4 % of the rhamnolipid forms are negatively charged, whereas, at pH 4, 97 % are neutral. Possibly, the ratio between the anionic and neutral forms have contributed to the activity of SYRA<sub>0.6,1.95</sub>. This information is important for considering pH constraints when combining SRE and RL<sub>Zonix</sub> to make SYRA formulations for applications. The overall results indicate a good degree of stability for both SRE and SYRA<sub>0.6,1.95</sub> at pH range of 4 to 7.

The successful use of SRE and SYRA formulations as fungicidal agents may depend on environmental and climate conditions such as humidity, radiation, and temperature. Wet and humid conditions may require repetitive treatments to reach the desired control. In addition, this may enhance the effectiveness of fungicides by facilitating its spread and penetration into the pathogen. However, repetitive applications might be costly. Perhaps using mineral oil to deliver these agents may reduce the cost. SRE and SYRA were mixed with mineral oil and tested for activity against *R. pilimanae*. The results showed that SRE and SYRA<sub>0.25,0.75</sub>, were active after mixing them with mineral oil (Table. 3.2.).

### **Effects of Trypsin**

To examine whether SRE and SYRA formulations are stable to protease treatment, SRE and SYRA<sub>0.25,0.75</sub> were pretreated with trypsin and their antimicrobial activities were tested. The results showed that trypsin treatment did not alter SRE and

SYRA<sub>0.25,0.75</sub> activities against *R. pilimanae*. Moreover, no degradation was observed in the trypsin-treated samples. HPLC analyses showed that the peaks were almost identical to the control (Fig. 3.6). The trypsin is known to cleave at the carboxyl side of both lysine and arginine that are connected to other amino acids. Interestingly, trypsin did not cleave the Arg-Phe bond in SRE. Perhaps the nine amino acids cyclic ring and the structural conformation of SRE prevented and protected the molecule from trypsin active site. This result is consistent with observations of Fernandez-Lopez et al. (4) on chemically synthesized cyclic D,L-alpha-peptides.

In conclusion, the results of this study provide information on the activities and chemical stabilities of SRE and SYRA formulations under a variety of physical conditions and chemical treatments. Overall, SRE and the SYRA show long term stabilities. Their broad spectrum antifungal properties and physical stabilities reinforce the prediction that they are promising candidates for use as agrofungicides.

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Table. 3.1. Effect of autoclaving on SRE and SYRA<sub>0.25,0.75</sub> activities

Zone of inhibition (mm)			
Autoclave time	15 minutes	30 minutes	45 minutes
2.4mg/mlSRE- Autoclaved	22	20	20
SYRA <sub>0.25,0.75</sub> Mixture B	NI	NI	NI
2.6mg/ml RL Autoclaved	NI	NI	NI
SYRA <sub>0.25,0.75</sub> Mixture A	34	32	29

NI= no inhibition

Table.3.2. Effect of mineral oil on SRE and SYRA<sub>0.25,0.75</sub> activities.

	Zone of inhibition (mm)	
	SRE	SYRA <sub>0.25,0.75</sub>
With mineral oil	22	35
Without mineral oil	22	35

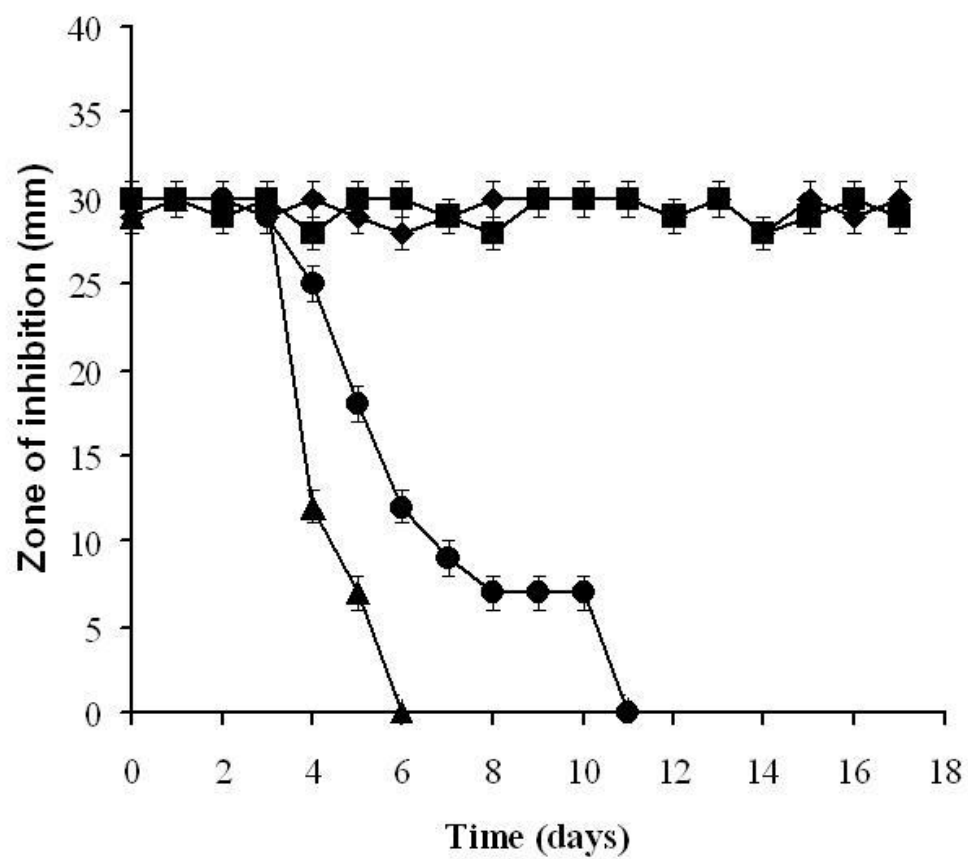


Fig. 3.1. Effect of temperature on SYRA<sub>0.25,0.75</sub> activity 4°C(■), 28°C(◆), 55°C(●), 65°C(▲).SRE activity (○) 4°C, 28°C, 55°C, 65°C (SRE activity was the same for all temperatures)

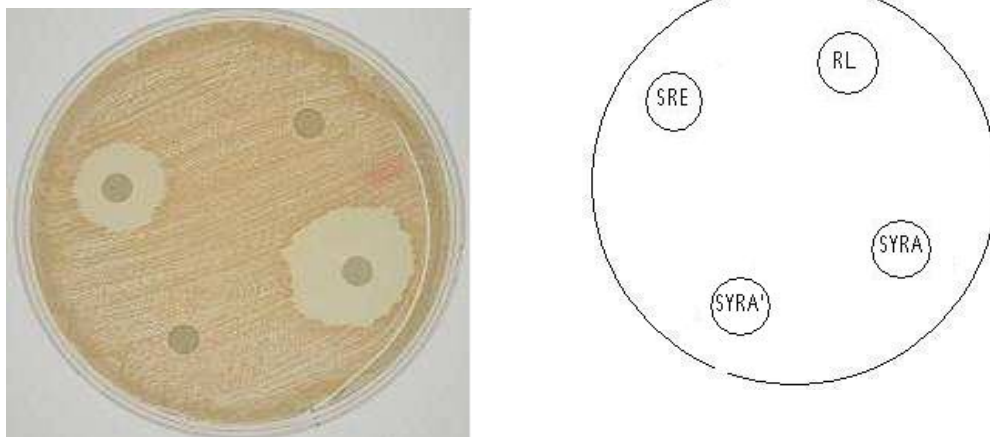
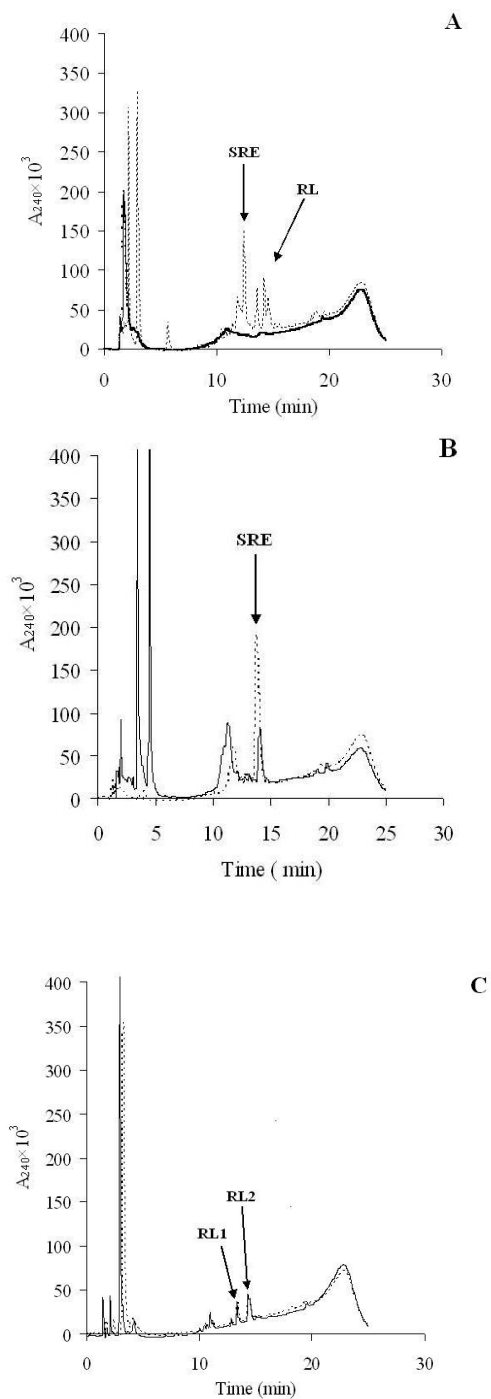


Fig. 3.2. The antifungal activity of SRE, RL, and SYRA against *R. pilimanae*.after autoclaving. Ten  $\mu$ l of SRE, RL, SYRA<sub>0.25,0.75</sub> mixture A (SYRA) prepared after autoclaving the components SRE and RL<sub>Zonix</sub> and SYRA<sub>0.25,0.75</sub> mixture B (SYRA') mixed before autoclaving) were applied on disks placed on a lawn of *R. pilimanae*.



**Fig. 3.3. HPLC analyses of : (A) SRE 1mg/ml, (B) RLZonix 0.75mg/ml (the arrows represent rhamnolipid isomers RL1 and RL2), and (C) SYRA0.25,0.75 . (---) before autoclaving and (—) after autoclaving. Shown are the HPLC profiles obtained using a C18 column with a 25 minute of 0-70% gradient 2-propanol in 0.1% TFA.**

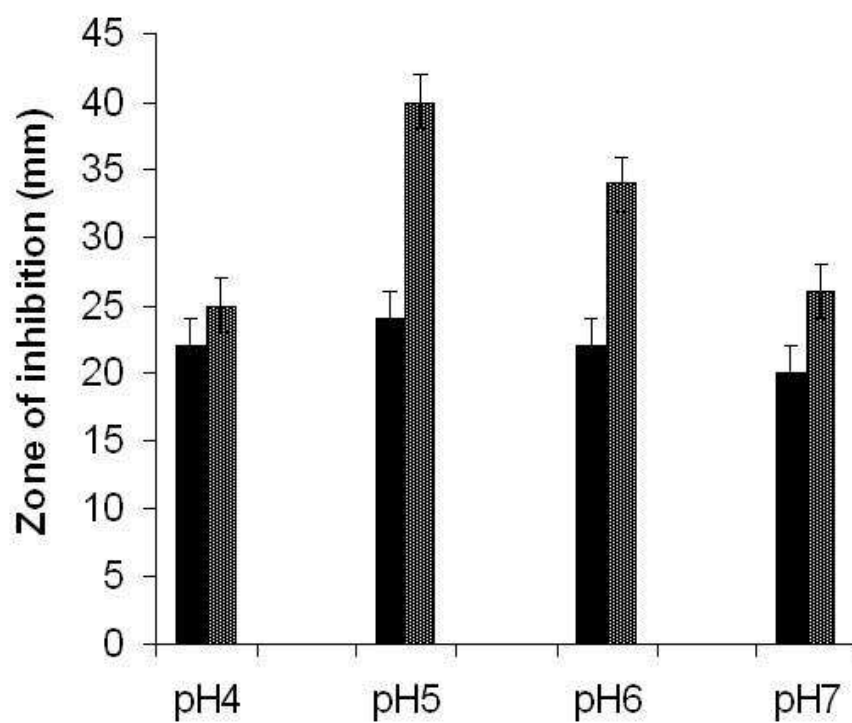


Fig. 3.4. The effect of pH on SRE (■), and SYRA<sub>0.6,1.95</sub> (▨) antifungal activity against *R. pilimanae*

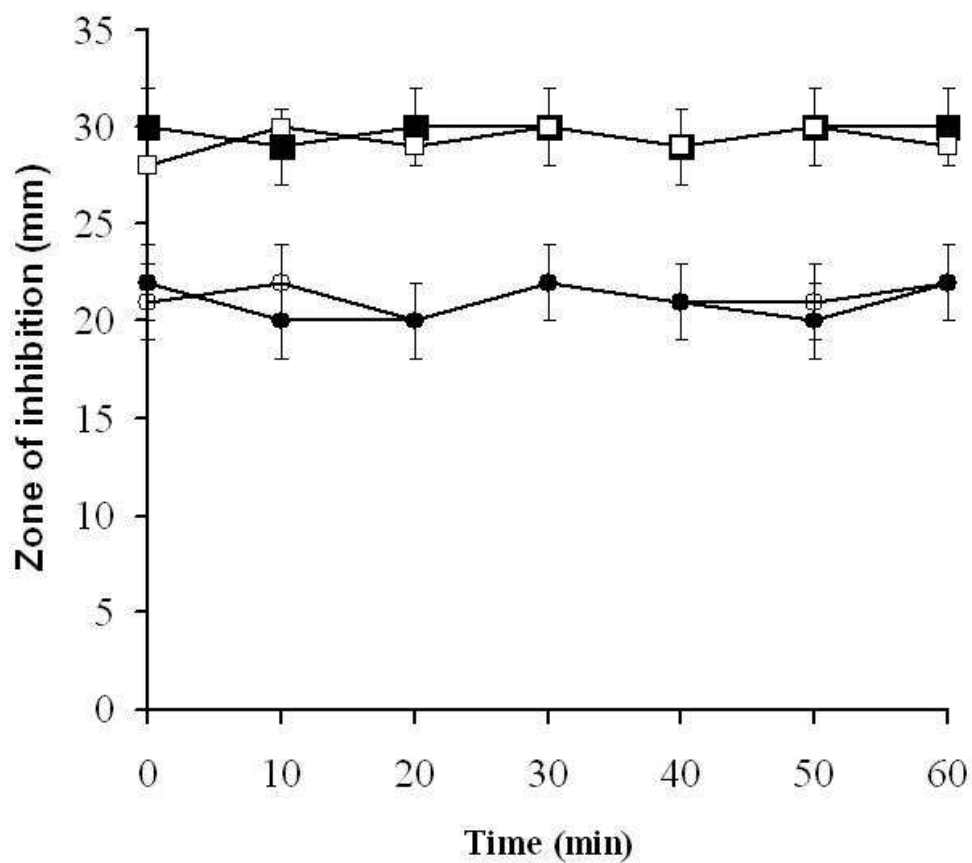
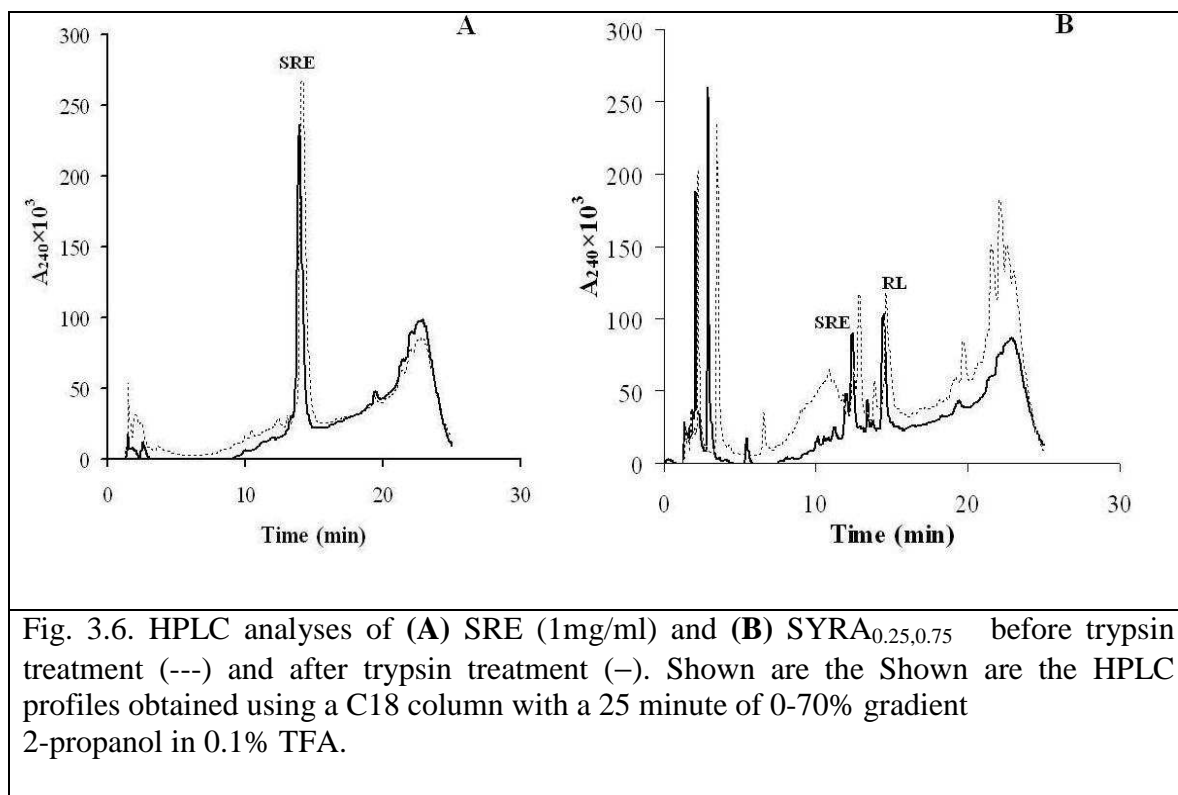


Fig. 3.5. Effect of UV exposure and sonication on SRE and SYRA<sub>0.6,1.95</sub> activities. SRE: UV exposure (●), sonication (○). SYRA<sub>0.6,1.95</sub>: UV exposure (■), sonication (□)





## CHAPTER 4

MECHANISM OF ACTION OF SRE- RL<sub>zonix</sub> COMBINATION**Introduction**

Syringomycin E is a cyclic lipodepsinonapeptide produced by the phytopathogenic strains of *Pseudomonas syringae* pv. *syringae* (Takemoto, 1992). SRE has inhibitory activity against fungi and yeast such as *Botrytis cinerea*, *Geotrichum candidum* and *Rhodoturula pilimanae* (Takemoto *et al.*, 1992; Lavermicocca, *et al.*, 1997). The mechanism of action of SRE is well studied. SRE acts on yeast and plant plasma membranes to cause numerous cellular effects with increases in cellular K<sup>+</sup> efflux and transient Ca<sup>2+</sup> fluxes among the most prominent. The effects are consistent with SRE's ability to form ion-conducting voltage sensitive channels in membrane bilayers. However, ion channel formation in the native plasma membrane as the primary cause of growth inhibition has not yet been determined. In addition, studies with lipid biosynthesis yeast mutants have revealed that sphingolipids and sterols modulate the fungicidal activity of SRE (Cliften *et al.*, 1996; Grilley *et al.*, 1998; Stock *et al.*, 2000). For instance, among the genes that promote SRE action, the *SYR2* is gene required for sphingolipid C-4 hydroxylation, *ELO2* and *ELO3* are responsible for very long fatty acid elongation, *FAH1*, responsible for  $\alpha$ -hydroxylation of the amide linked very long chain fatty acids, and *IPT1*, which encodes the enzyme that catalyzes the terminal yeast sphingolipid biosynthetic step (Cliften *et al.*, 1996, Grilley *et al.*, 1998; Stock *et al.*, 2000). In addition, *SMR1* (identical to *ERG3*), which encodes the C-5,6 desaturase required for ergosterol biosynthesis (Taguchi *et al.*, 1994) is also needed for SRE action.

SRE lipidic pore formation has been proposed as the mechanistic basis for SRE action on membranes (Malev *et al.*, 2002).

Rhamnolipids are produced by *Pseudomonas aeruginosa* and are formed by rhamnose linkage to fatty acids of saturated or unsaturated alkyl chain between C8 and C12 (Haba *et al.*, 2002). Rhamnolipids possess antimicrobial activities (Haba *et al.*, 2002). They are active against zoosporic plant pathogens (Stanghellini *et al.*, 1997) and other fungal species such as *Fusarium solani* and *Penicillium funiculosum* (Haba *et al.*, 2002). The mechanism of action of rhamnolipids against fungi is still unknown. However, Stanghellini and Miller (Stanghellini & Miller, 1997) suggested that the rhamnolipids inhibit the zoosporic fungi by disrupting the zoospore plasma membrane.

Combinations of existing fungicides show some promise for use as next generation and more effective antifungal strategies (Rex *et al.*, 1995). The current work is part of a general study on the antifungal properties and efficacy of combined SRE and RL<sub>zonix</sub> formulations. I hypothesize that SYRA the combination of SRE and RL<sub>zonix</sub> also inhibit fungi by mechanisms that are similar to those for SRE action. Therefore, I studied the effects of SYRA on *Saccharomyces cerevisiae* strains deficient in various steps of sphingolipid and sterol biosynthesis to investigate whether the antifungal action of SYRA, like SRE, depends on sphingolipids and sterols.

To further examine the basis for the fungicidal activity of SYRA, I studied the channel-forming properties of SRE, RL<sub>zonix</sub>, and SYRA in lipid bilayers in comparison with those of SRE. The results suggest that sphingolipids and sterols are important for the killing action of SYRA in a manner similar to the mechanism of action of SRE, and I found that SYRA and SRE have similar single-channel pore forming properties.

## Materials and Methods

### SRE and SYRA

SRE was purified from *P.syringae* pv.*syringae* strain B301D by the method of (Bidwai *et al.*, 1987). *P.syringae* pv.*syringae* strain B301D was grown in non-commercial potato dextrose casamino acids medium in 4- or 8-liter cultures as described by Zhang and Takemoto (1987). The SRE was extracted from cultures using acidified acetone and purified using the chromatographic methods described by Bidwai *et al.* (Bidwai *et al.*, 1987). HPLC column fractions were tested for bioactivity by applying 10  $\mu$ l aliquots on a fresh lawn of *R. pilimanae* on potato-dextrose agar plates, incubation at 28°C, and observing cleared growth inhibition areas (Zhang & Takemoto, 1987). Commercial rhamnolipid Zonix (RL<sub>Zonix</sub>) was obtained from Jeneil Biotech, Inc. RL<sub>Zonix</sub> is an 8.5% (wt/vol) solution of rhamnolipid analogs in water. Two forms of rhamnolipids are in RL<sub>Zonix</sub>, R1 (C<sub>26</sub>H<sub>48</sub>O<sub>9</sub>) has the molecular formula of  $\alpha$ -L-rhamnopyranosyl- $\beta$ -hydroxydecanoyl- $\beta$ -hydroxydecanoate, and R2(C<sub>32</sub>H<sub>58</sub>O<sub>13</sub>) is 2-O- $\alpha$ -L-rhamnopyranosyl- $\alpha$ -L-rhamnopyranosyl- $\beta$  hydroxydecanoate. A formulation was prepared by combining SRE and RL<sub>Zonix</sub> at 1:3 (wt/wt) ratios (0.25 mg SRE and 0.75 mg RL<sub>Zonix</sub> per ml), respectively, and designated SYRA<sub>0,25,0.75</sub>.

### Yeast strains and maintenance

SRE-sensitive *S. cerevisiae* strains 8A-1B, W303C, and BY4741 and isogenic SRE-resistant and lipid biosynthesis mutant strains  $\Delta$ *erg3*,  $\Delta$ *syr2*,  $\Delta$ *ipt1*,  $\Delta$ *elo2*,  $\Delta$ *elo3*,  $\Delta$ *skn1* and  $\Delta$ *fah1* were described previously. SRE-resistant strains  $\Delta$ *syr2* and  $\Delta$ *elo3* are single-gene disruptants isogenic to parental strains W303C and 8A-1B, respectively. All

other SRE-resistant mutants are single gene disruptants isogenic to parental strain BY4741. The strains were grown at 28°C and maintained at 5°C in yeast extract-peptone dextrose (YPD) broth or on agar medium (Hama *et al.*, 2000).

### **Effects on yeast growth**

**Replica plating.** The effects of SRE, RL<sub>Zonix</sub>, and SYRA<sub>0,25,0.75</sub> on the growth of yeast strains were determined using the replica plate method as previously described (Hama, *et al.*, 2000). Freshly grown cells of yeast parental and lipid mutant strains were replica-plated onto YPD agar containing different concentrations of SRE, RL<sub>Zonix</sub>, and SYRA<sub>0,25,0.75</sub> and incubated for 2 to 3 days at 28°C. Growth was determined by visual inspection of the agar plates.

**Disk diffusion assay.** The disk diffusion assay was done accordingly to the method described by the National Committee for Clinical Laboratory Standards (NCCLS) protocols for antifungal testing with modification (NCCLS, 1997,2002). Different yeast parental and lipid mutant strains were grown on YPD for 48 h at 28°C and 50 µl of cell suspension was transferred and spread uniformly using a cotton swab onto YPD agar (Steinbach *et al.*, 2004). Four mm diameter sterilized paper disks were deposited on the surface and 10 µl of SRE (0.25 mg/ml), RL<sub>Zonix</sub> (0.75 mg/ml), and SYRA<sub>0,25,0.75</sub> were applied onto each disk. The plates were incubated for 24 to 48 h and the zones of inhibition were measured.

**Microbroth dilution assay.** Minimal inhibitory concentrations (MICs) were determined by the microbroth dilution assay according to methods outlined by the NCCLS with modification (NCCLS, 1997, 2002). Yeast parental and lipid mutant strains were grown to a final concentration of 10<sup>8</sup> CFU/ml and suspended at a final concentration

of  $5 \times 10^5$  CFU/ml. Cell suspensions (25  $\mu$ l) were added to 25 $\mu$ l aliquots of twofold serial dilutions of SRE, RL<sub>Zonix</sub>, and SYRA<sub>0,25,0.75</sub> and YPD broth media were dispensed (100  $\mu$ l total volume) in wells of 96-well polystyrene microtiter plates (Fisher Scientific, catalog no. 21-377-203). The plates were incubated for 24 h at 28°C. The MICs were determined by visual inspection of the 96-well plate.

### **Lipid bilayer channel formation**

Lipid bilayer electrophysiological experiments were performed using 1,2-dioleoyl-sn-glycero-3-phosphorylcholine (DOPC), (Avanti Polar Lipids) as previously described (Blasko *et al.*, 1998, Malev *et al.*, 2002). Solutions of 0.1 M of NaCl were buffered with 5 mM MOPS (Sigma) to pH 6. Bilayer lipid membranes were prepared by the monolayer-opposition technique (Montal & Muelle; 1972) on a 50–100  $\mu$ m diameter aperture in the 10  $\mu$ m thick Teflon film separating two (*cis* and *trans*) compartments of a Teflon chamber (Montal & Mueller *et al.*, 1972; Bezrukov & Vodyanoy, 1993). The membrane-forming solution was DOPC. A pair of Ag/AgCl electrodes with agarose/2 M KCl bridges was used to apply transmembrane voltages and to measure single channel currents. Current measurements were carried out using an Axopatch 200B amplifier (Axon Instruments) in the voltage clamp mode. The data were filtered with a low-pass 8-pole Model 9002 Bessel filter (Frequency Devices) at 1 kHz and directly recorded into computer memory with a sampling frequency of 5 kHz. Data were analyzed using pClamp 9.2 (Axon Instruments) and Origin 7.0 (Origin Lab). Single channel conductance was calculated as the mean single-channel current divided by the applied transmembrane voltage. All experiments were performed at room temperature.

SRE (5  $\mu$ g/ml) and RL<sub>Zonix</sub> (4  $\mu$ g/ml) were added to the aqueous phase as follows :

- 1- At t=0, SRE was added to *cis* side compartment
- 2- At t=0, RL<sub>Zonix</sub> was added to *cis* side compartment, then at t=10 min, SRE was added to the same side.
- 3- At t=0, SRE was added to the *cis* side compartment, then at t=10 min, RL<sub>Zonix</sub> was added to the opposite side *trans* side.
- 4- At t=0, RL<sub>Zonix</sub> was added to the *trans* side, then at t=10 min, SRE was added to the *trans* side.

## Results

### Susceptibility of SRE-resistant lipid biosynthesis mutants

The growth of *S. cerevisiae* sphingolipid and sterol biosynthetic mutants in the presence of different concentrations of SRE and SYRA<sub>0,25,0.75</sub> was examined. On replica-plated YPD agar medium SRE (0.75 µg/ml) and SYRA<sub>0,25,0.75</sub> (0.5µg/ml of SRE and 1.5 µg/ml of RL<sub>Zonix</sub>) inhibited the growth of parental yeast strains (W303C, BY4741, and 8A-1B) (Fig. 4.1) but not the lipid mutant strains ( Fig. 4.2). SYRA<sub>0,25,0.75</sub> was the most effective inhibitor against the *S. cerevisiae* strains (Table. 4.1, Fig. 4.2). The MICs (of equivalent SRE levels in SYRA<sub>0,25,0.75</sub>) were 2 to 4 fold lower than the SRE MICs (of SRE alone) (Table. 4.1) against all *S. cerevisiae* strains tested. Although all of the mutants were less susceptible to the SRE and SYRA<sub>0,25,0.75</sub> compared to their parental strains, there was some variation in the extent of resistance among the tested mutants. For example, the corresponding SRE-resistant isogenic mutant strains with deletions in genes for sphingolipid ( $\Delta$ *syr2*,  $\Delta$ *ipt1*, and  $\Delta$ *skn1*) or ergosterol ( $\Delta$ *erg3*) biosynthesis were less susceptible to inhibition by SYRA<sub>0,25,0.75</sub> compare to the other mutants (  $\Delta$ *fah1*,  $\Delta$ *elo3*,

and  $\Delta elo2$ ). However, in the case of SRE,  $\Delta syr2$  and  $\Delta erg3$  were the most resistant compared to the other strains. Overall, the patterns of relative susceptibility and resistance of the lipid mutants to SYRA<sub>0,25,0.75</sub> and SRE were similar. At relatively high concentrations of SRE (3.9  $\mu\text{g/ml}$ ) and SYRA<sub>0,25,0.75</sub> (1,95 $\mu\text{g/ml}$  SRE and 5,85  $\mu\text{g/ml}$  RL<sub>Zonix</sub>) neither the wild type nor the mutants grew.

### **Ion channel formation**

To study the influence of RL<sub>Zonix</sub> on SRE interaction with the plasma membrane, I examined the membrane channel forming properties of SRE in the presence and absence of RL<sub>Zonix</sub>. The effects on ion conductance across planar lipid bilayers were measured. Figure. 4.3 shows time courses of the integral macroscopic conductance of the bilayers doped with SRE alone and SRE with RL<sub>Zonix</sub>.

The results showed that the negative potential application drove the increase of the membrane conductance. Remarkably, the addition of RL<sub>Zonix</sub> to both sides of the bilayer (cis and trans) caused a significant increase in membrane conductance indicating an increase in the number of pores formed by the combined mixture of SRE and RL<sub>Zonix</sub>. However, after 40 min of the imposing voltage, the conductance decreased indicating the closing or disappearance of the pores. As reported previously (Szabo *et al.*, 2004) (Malev *et al.*, 2002), SRE produced a smaller steady-state conductance and no subsequent decrease was observed after 40 min. No change in conductance was observed when RL<sub>Zonix</sub> was applied alone. In contrast to the differences in macroscopic conductance, the properties of the transmembrane single channels formed by SRE with or without RL<sub>Zonix</sub> were the same (Fig. 4.3).



## Discussion

It is known that SRE interacts with membranes resulting in lipidic pore formation that in yeast leads to ion leakage, membrane dysfunction, and possibly other cell damaging effects. It is also known that growth inhibition by SRE is modulated by sphingolipids and sterols (Grilley *et al.*, 1998; Cliften *et al.*, 1996; Stock *et al.*, 2000; Hama *et al.*, 2000) Sphingolipid and sterol biosynthetic mutants of yeast were less susceptible to SRE as compared to the isogenic wild type strains. The same effect was observed with SYRA. However, the presence of RL<sub>Zonix</sub> rendered the wild type and the mutants cell more sensitive to SRE. the effect was greatest in yeast with mutations in either *SYR2*, the gene responsible for C4 hydroxylation of sphingolipids, or *SMRI*, which encodes the C-5, 6 desaturase required for ergosterol biosynthesis. It is unclear whether the RL<sub>Zonix</sub> interaction with the plasma membrane was affected by the sphingolipid and sterol biosynthetic mutation such as the lack of C-4 hydroxylation or C5, 6 desaturase as it was shown for SRE action. However, the presence of RL<sub>Zonix</sub> facilitated SRE binding to the plasma membrane. Thus, SRE was more effective and it increased its activity.

To further explore and understand the effect of RL<sub>Zonix</sub> on SRE action, the transmembrane channel properties of SRE+ RL<sub>Zonix</sub> were examined and compared to those of SRE channels. SRE caused voltage-dependent macroscopic conductance increases due to the formation of ion permeable channels in the membrane bilayers. However, when RL<sub>Zonix</sub> was added, a significant increase in the conductance level was observed. This increase is mainly due to the total numbers of channels formed. I suggest that the observed increase in the number of channels is due most likely to the

enhancement and favorable binding of SRE in the presence of RL<sub>Zonix</sub>. However, after 40 min a decrease in conductance was observed as a consequence of pore inactivation in the planar membrane. This perhaps is triggered by the dissociation of SRE+ RL<sub>Zonix</sub> complex from the plasma membrane. The nature of interaction between SRE, RL<sub>Zonix</sub>, and the plasma membrane, however, is not clear. One possibility is that the penetration of RL<sub>Zonix</sub> into the plasma membrane (in which the hydrophobic portion interacts with lipids and the negatively charged polar head points outward in a carpet-like manner (Oren & Shai, 1998) attracts more SRE binding to the membrane to give an increase in number of channels (Fig. 4.4). It is important to mention that the nature of the membrane plays an important role in how RL<sub>Zonix</sub> binds and inserts into the membrane (Aranda, *et al.*, 2007). Aranda, *et al.*, (2007) suggested that a high presence of cholesterol in the membrane rendered the binding of diRL more difficult; however, the presence of phosphatidylethanolamine enhanced its binding and partitioning into the membrane. Another possibility is that SRE interacts with the plasma membrane with a barrel-stave mechanism as described by Oren et al. (1998) for linear amphipathic peptides. The positive charge of the SRE polar head and pore formation results in a decrease in the motional order of the lipids and as a consequence, RL<sub>Zonix</sub> binding and insertion into the membrane is enhanced (Aranda *et al.*, 2007) with an increase in the number of channels.

In summary, the present study suggests that SYRA inhibits yeast by the same mechanism as does SRE by itself. Its antifungal action is promoted by sphingolipids and sterols of the plasma membrane and involves pore formation. The results also provide an explanation for the antifungal synergy between SRE and RL<sub>Zonix</sub>. The addition of RL<sub>Zonix</sub> results in an increase in the number of membrane channels formed by SRE.

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Table. 4.1 Antimicrobial activities of SRE and SYRA against yeast lipid biosynthetic mutants.

Organisms	Zone of inhibition (mm)		MIC ( $\mu\text{g/ml}$ )	
	SRE	SYRA	SRE	SYRA (SRE/ $RL_{\text{Zonix}}$ )
<i>Saccharomyces cerevisiae</i>				
W303C	14	25	0.97	0.48/1.44
$\Delta\text{syr2}$	10	7	3.9	1.95/5.85.
$\Delta\text{elo3}$	14	19	1.95	0.97/2.91
BY4741	13	24	0.97	0.48/1.44
$\Delta\text{ipt1}$	12	17	1.95	1.95/5.85
$\Delta\text{fah1}$	12	22	1.95	0.97/2.91
$\Delta\text{elo2}$	12	22	1.95	0.97/2.91
$\Delta\text{skn1}$	12	22	1.95	0.97/2.91
$\Delta\text{8A-1B}$	15	24	0.97	0.48/1.44
$\Delta\text{smr1}$	10	8	3.9	1.95/5.85

MICs values were obtained from triplicate determinations

The zone of inhibition: The error was  $\pm 2$  mm, as determined from triplicate determinations

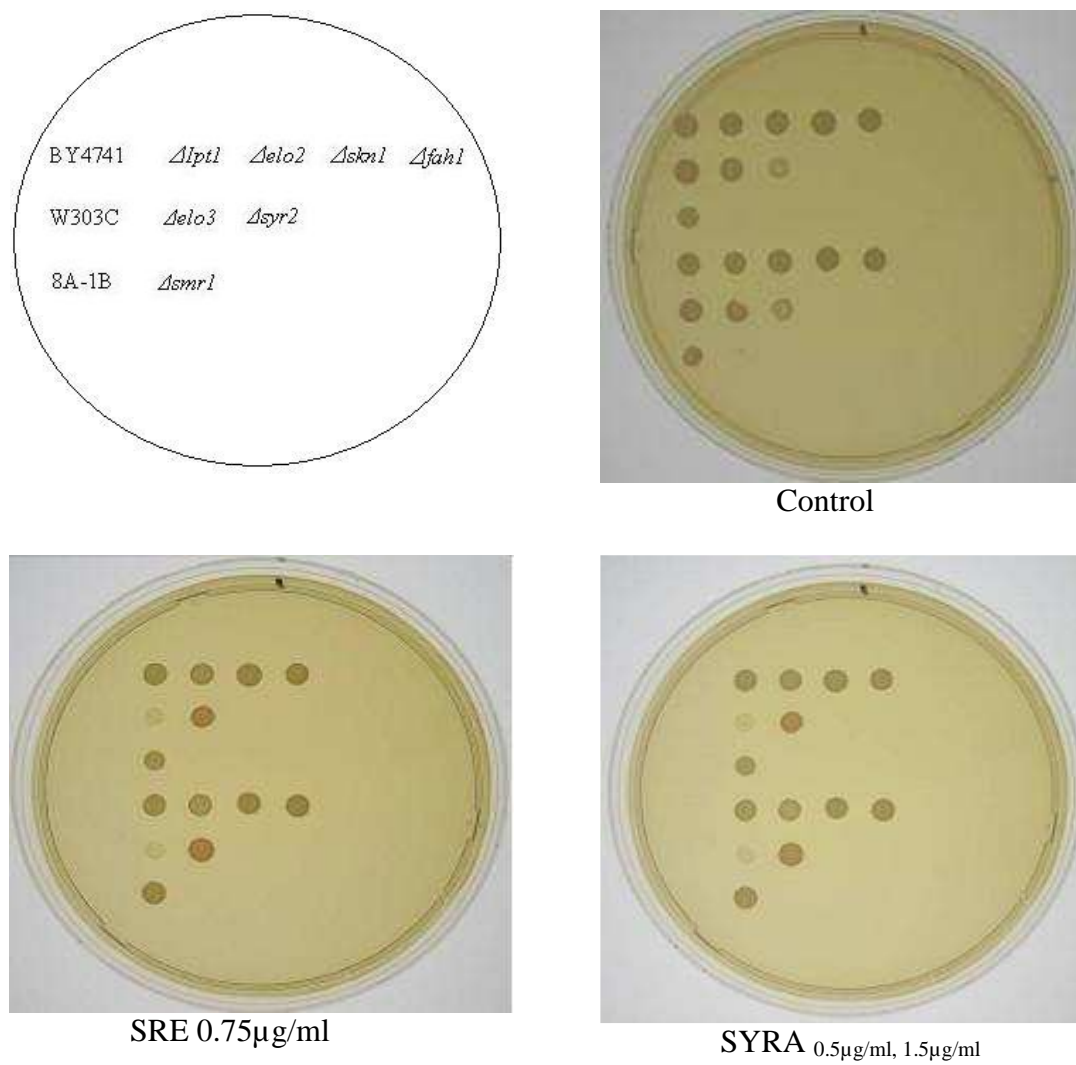


Fig. 4.1 .Effect of SRE and SYRA on growth of yeast lipid biosynthetic mutants. Sphingolipid and ergosterol biosynthesis mutant and parental strains were replica plated onto YPD agar with or without: SRE (0.75  $\mu\text{g/ml}$ ) or SYRA (0.5  $\mu\text{g/ml}$  SRE, 1.5  $\mu\text{g/ml}$  RL) the plates were incubated at 28°C for 48 h.

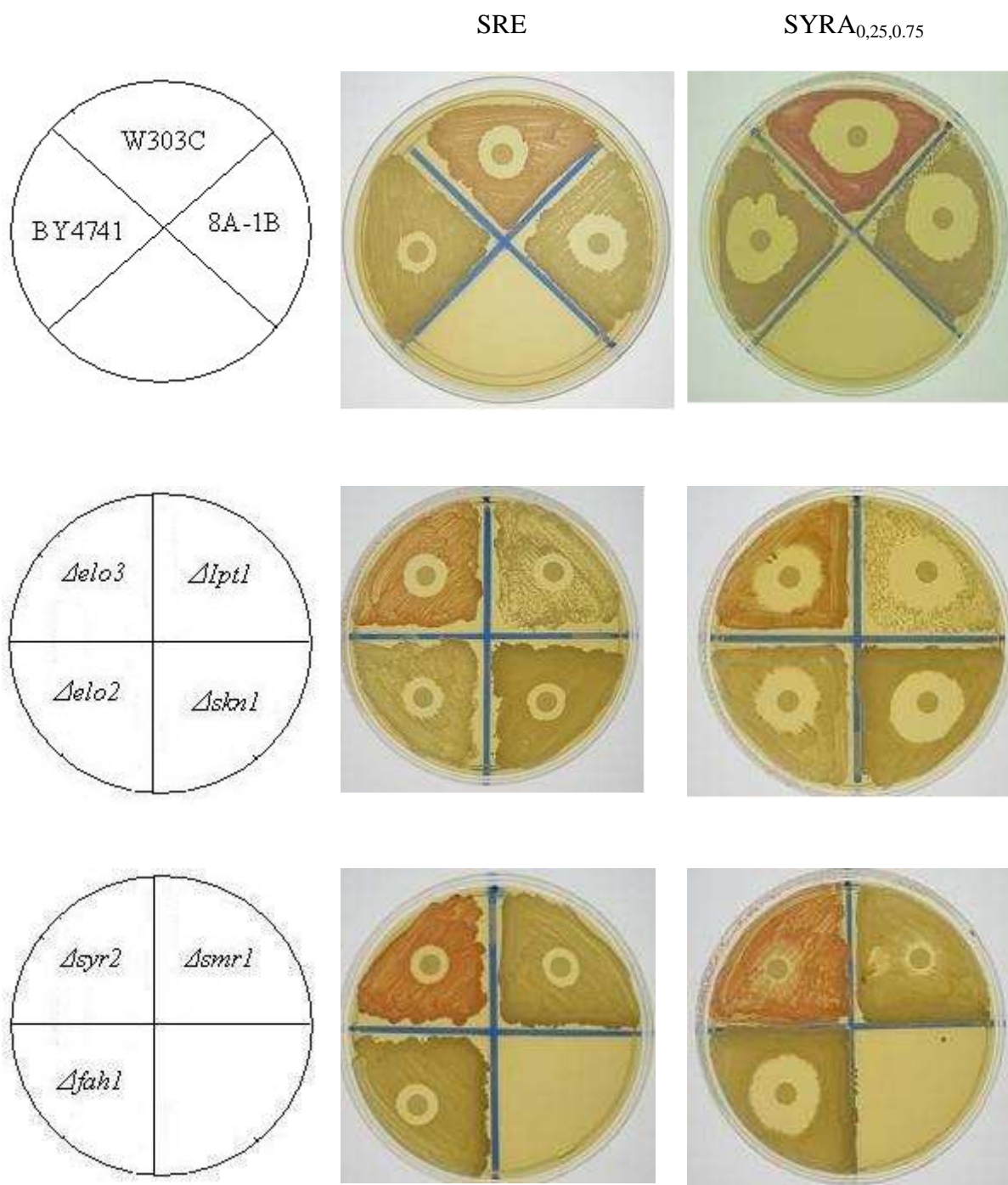


Fig. 4.2 . SRE and SYRA<sub>0,25,0.75</sub> inhibition against yeast lipid biosynthetic mutants. Ten  $\mu$ l of SRE (0.25mg/ml) and SYRA<sub>0,25,0.75</sub> were applied on the disks. The plates were incubated for 48 h. RL<sub>Zonix</sub> showed no inhibition.



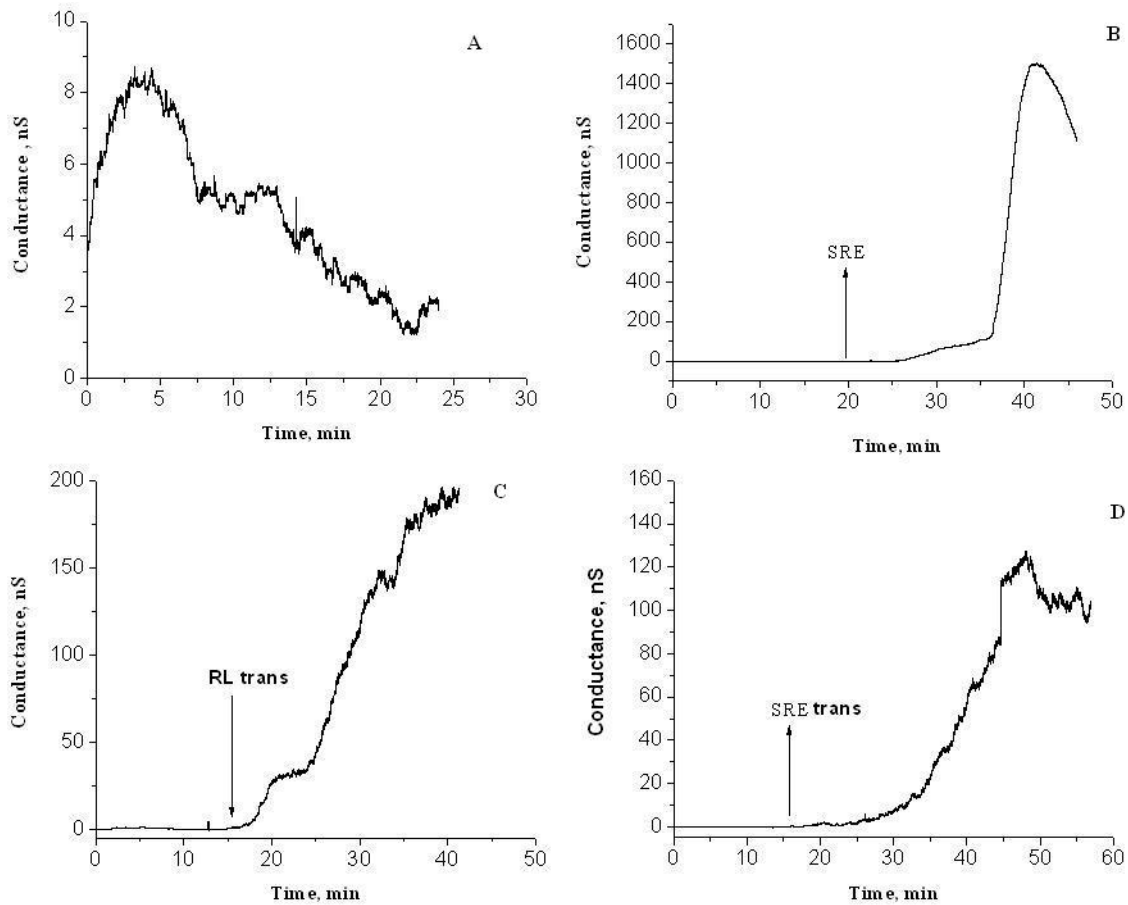


FIG.4.3. Time course of ion conductance of bilayers doped with A)- SRE, B)- SRE and RL applied to Cis side. C)- SRE applied to cis side and RL to trans side , recorded at the applied voltage of  $-50$  mV

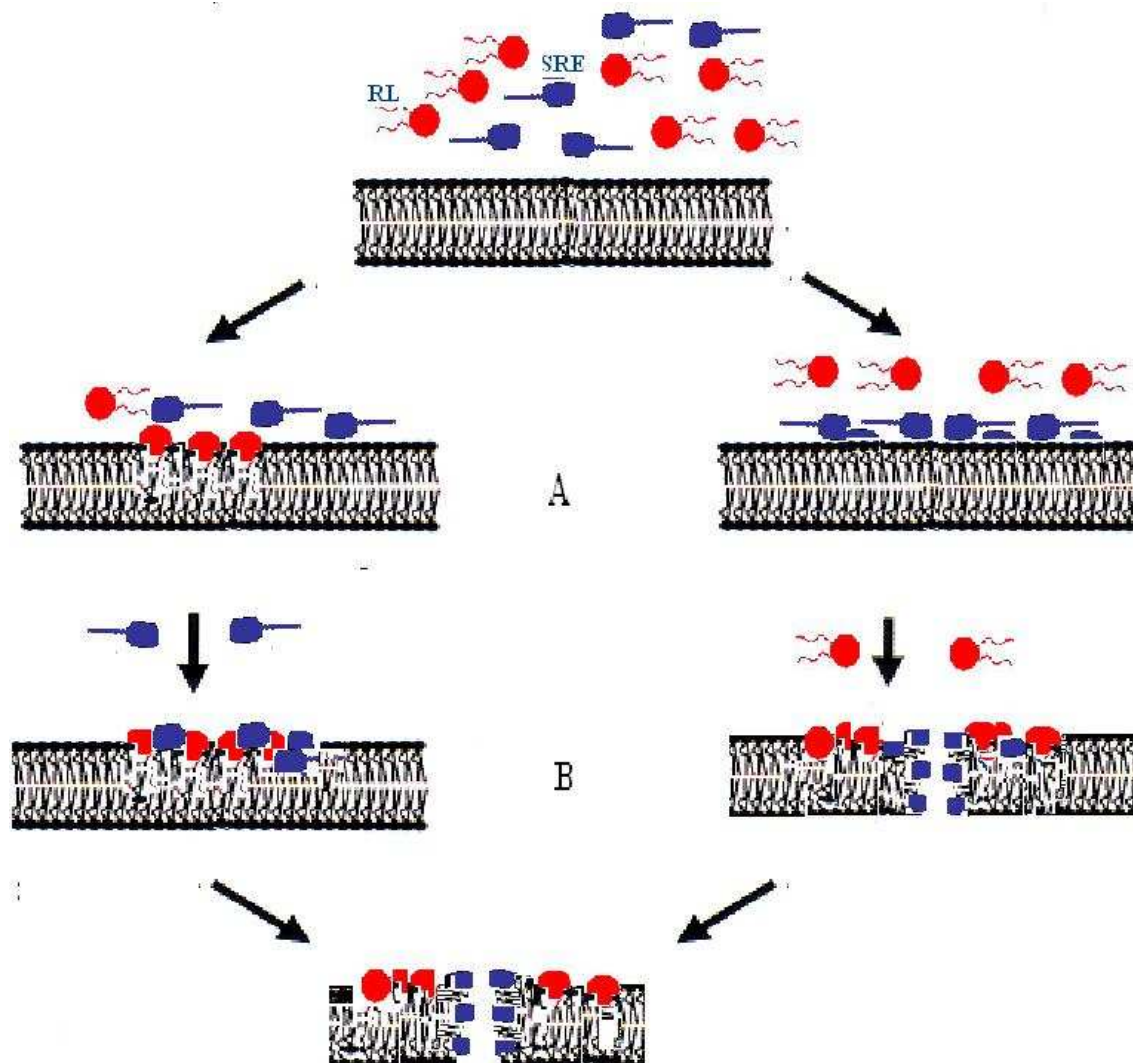


Fig. 4.4. A cartoon illustrating on the mechanism of SRE and RL combination and membrane pore formation. Red symbol represents RL and blue symbol represents SRE. Left: A- the RL interacted with the plasma membrane with their hydrophobic embedded in the membrane and hydrophilic portion on the surface. B- the binding of RL attracted more SRE to the surface of the plasma membrane. Right: A- the syringomycin interacts with the plasma membrane. B- The binding of SRE resulted in channel formation and a decrease in the motional order of the lipids as a consequence,  $RL_{Zonix}$  binding and insertion into the membrane is enhanced with an increase in the number of channels.

CHAPTER 5  
MECHANISMS OF YEAST GROWTH INHIBITION  
BY SYRINGOPEPTINS

**Introduction**

In addition to well-characterized cyclic lipodepsinonapeptides, *Pseudomonas syringae* pv. *syringae* produces larger cyclic lipodepsipeptides known as the syringopeptins (SPs). These compounds are composed of 22 or 25 amino acids (SP22 and SP25, respectively) with an octadepsipeptide ring structure and a 3-hydroxy fatty acyl chain. Isoforms of SP22 and SP25 possessing fatty acyl chains composed of ten and twelve carbons are designated A and B homologs, respectively. The SPs are phytotoxic, and they have antimicrobial activities against Gram-positive bacteria (Lavermicocca *et al.*, 1992; Grgurina *et al.*, 2005, Chapter 6). They are also antifungal although typically less potent in this regard than the cyclic lipodepsinonapeptides (Iacobellis *et al.* 1992; Lavermicocca *et al.*, 1997; Grgurina *et al.*, 2005). Nevertheless, SP22 analogs show strong inhibitory activities against certain fungal yeasts including the pathogen *Candida albicans* (Grgurina *et al.*, 2005).

The mechanisms of action of the cyclic lipodepsinonapeptides, particularly syringomycin E (SRE), are well studied. SRE acts on yeast and plant plasma membranes to cause numerous cellular effects with increases in cellular K<sup>+</sup> efflux and transient Ca<sup>2+</sup> fluxes among the most prominent. The effects are consistent with SRE's ability to form ion-conducting voltage sensitive channels in membrane bilayers. However, ion channel formation in the native plasma membrane as the primary cause of growth inhibition has

not yet been determined. In addition, studies with yeast have revealed that sphingolipids and sterols (lipids that occur predominantly in the plasma membrane) modulate the fungicidal activity of SRE (Cliften *et al.*, 1996; Grilley *et al.*, 1998; Stock *et al.*, 2000). SRE lipidic pore formation has been proposed as the mechanistic basis for SRE action on membranes (Malev *et al.*, 2002). Less is known of the mechanisms of action of the SPs. A few studies have shown that the SPs are capable of forming ion conducting membrane channels (Hutchison & Gross, 1997; Dalla Serra *et al.*, 1999; Agner *et al.*, 2000). In lipid bilayers and erythrocytes, SP22A channels do not thermally inactivate as do SRE channels – a phenomenon related to SP22A's greater effectiveness in ordering membrane lipids (Szabo *et al.*, 2004). In plant systems, SPs have been shown to cause electrolyte leakage in leaf tissues (Iacobellis 1992; Lavermicocca *et al.*, 1997), to increase the permeability of tonoplasts (Carpaneto *et al.*, 2002), close stomata (Di Giorgio *et al.*, 1996) and modify H<sup>+</sup> fluxes across mitochondria and plasma membranes (Di Giorgio *et al.*, 1994;1996). Finally, SPs interact with bacterial cell surface teichoic acids (Chapter 6Bensaci), but the mechanism of interaction with bacterial membranes is still unknown. Despite recognition that the SPs have antifungal activities, there have been no published studies on the mechanisms of action of SPs against yeasts or other fungi.

In this study, we address the mechanisms of action of SPs against yeast. We show that the physiological responses of yeast to the SPs and the membrane lipid requirements for fungicidal action resemble those of SRE. This is so despite the large structural differences between the SPs and SRE molecules. To examine the basis for the fungicidal activity of SPs, we studied the channel-forming properties of the SPs in lipid bilayers in comparison with those of SRE. We found that SP22A and SP25A produce higher

macroscopic conductance than SRE but, surprisingly, have similar single-channel properties.

## **Materials and methods**

### **Yeast strains and maintenance**

*Saccharomyces cerevisiae* SRE-sensitive strains KZ1-1C, 8A-1B, W303C, and BY4741 and SRE-resistant and lipid biosynthesis mutant strains  $\Delta erg3$ ,  $\Delta syr2$ ,  $\Delta ipt1$ ,  $\Delta elo2$ ,  $\Delta elo3$  and  $\Delta fah1$  were described previously (Taguchi *et al.*, 1994, Hama *et al.*, 2000, Stock *et al.*, 2000). SRE-resistant strains  $\Delta erg3$  (formerly  $\Delta syr1$ , (Taguchi *et al.*, 1994)) and  $\Delta fah1$  are single-gene disruptants isogenic to parental strains 8A-1B and BY4741, respectively. All other SRE-resistant mutants are single gene disruptants isogenic to parental strain W303C. The strains were grown (28°C) and maintained (5°C) in yeast extract-peptone dextrose (YPD) broth or on agar medium (Hama *et al.*, 2000).

### **Purification of SRE, SP22A and SP25A**

SRE was purified from *Pseudomonas syringae* pv. *syringae* strains B301D or M1 by previously described methods of (Bidwai *et al.*, 1987; Adetuyi *et al.*, 1995). SP22A and SP25A were purified from extracts of strains *P. syringae* pv. *syringae* B301D and M1 respectively, using methods described earlier (Chapter 6).

### **Effects on yeast growth**

The effects of SP22A, SP25A and SRE on the growth of yeast strains were determined using two methods. A replica plate method was used as previously described (Hama *et al.*, 2000). Freshly grown cells of yeast parental and lipid mutant strains were

replica-plated onto YPD agar medium containing different concentrations (between 0.61 and 2.06  $\mu\text{M}$ ) of SRE, SP22A, and SP25A and incubated for 2 to 3 days at 28°C. Growth was determined by visual inspection of the agar plates. For measuring effects on growth in liquid batch cultures, parental yeast strain KZ1-1C was first grown in YPD broth medium at 28°C in 125 mL capacity Erlenmeyer flasks with rotary shaking for 48h to a density of  $\sim 8 \times 10^8$  cells  $\text{ml}^{-1}$ . The cells were centrifuged and resuspended in YPD broth medium to give  $2.4 \times 10^7$  cells  $\text{ml}^{-1}$  and SRE, SP22A or SP25A were added at designated concentrations (between 0.81 and 9.33  $\mu\text{M}$ ). The suspensions were incubated with rotary shaking at 28°C and samples removed hourly for direct cell counts. The number of cells was counted using a hemocytometer and light microscope and generation times determined from calculated specific growth rate constants.

### **K<sup>+</sup> efflux**

Whole cell K<sup>+</sup> efflux rates were determined as changes in extracellular K<sup>+</sup> concentrations. Yeast strain KZ1-1C was grown in YPD broth medium with rotary shaking at 28°C to a density of  $10^8$  cells  $\text{ml}^{-1}$ . Cells were suspended in 2 mM Tris/MES buffer, pH6.5, and 0.1M-glucose with or without SRE, SP22A or SP25A (at concentrations of 1, 10 or 100  $\mu\text{M}$ ) to  $A_{600}$  nm of 1 in 125 ml capacity Erlenmeyer flasks with rotary shaking (200 rpm) at 28°C (Takemoto *et al.*, 1991). At 5 min after suspension, 1 ml samples were withdrawn, centrifuged in an Eppendorf microcentrifuge for 30 sec, and the supernatant fractions were recovered. K<sup>+</sup> concentrations were determined by atomic absorption spectroscopy (AA/AE spectrophotometer 457, Instrumentation Laboratories).

### **Ca<sup>2+</sup> uptake**

The net cellular uptake of  $^{45}\text{Ca}^{2+}$  was measured using strain KZ1-1C as described previously (Takemoto *et al.*, 1991). Cells were grown in YPD broth medium to a density of  $1 \times 10^7$  cells  $\text{ml}^{-1}$ , harvested by centrifugation, and then suspended in YPD broth medium to a density of  $4 \times 10^7$  cells  $\text{ml}^{-1}$ . Ten mL aliquots were dispensed in 125 ml capacity Erlenmeyer flasks with radioactive  $^{45}\text{CaCl}_2$  (Amersham Radiochemicals) and incubated with rotary shaking (200 rpm) at  $28^\circ\text{C}$ . The specific radioactivity of  $^{45}\text{Ca}^{2+}$  was adjusted to 4mCi (148MBq) per mmole of  $\text{CaCl}_2$  (Takemoto *et al.*, 1991). SRE, SP22A or SP25A were added 5 min following addition of  $^{45}\text{CaCl}_2$ . At designated times, cell samples (200  $\mu\text{l}$ ) were collected on glass fiber filters (0.45  $\mu\text{M}$  pore size), the filters were quickly washed twice with ice-cold water, and the radioactivity on the filters determined using a liquid scintillation counter.

### **Channel formation in lipid bilayers**

Lipid bilayer electrophysiological experiments were performed using 1,2-dioleoyl-sn-glycero-3-phosphorylcholine (DOPC) (Avanti Polar Lipids) as previously described (Blasko *et al.*, 1998; Malev *et al.*, 2002). Solutions of 0.1 M NaCl were buffered with 5 mM MOPS (Sigma) to pH 6. Bilayer lipid membranes were prepared by the monolayer-opposition technique (Montal & Mueller, 1972) on a 50–100  $\mu\text{m}$  diameter aperture in the 10  $\mu\text{m}$  thick Teflon film separating two (*cis* and *trans*) compartments of a Teflon chamber. SP22A, SP25A, or SRE were added to the aqueous phase of the *cis*-side compartment. A pair of Ag/AgCl electrodes with agarose/2 M KCl bridges was used to apply transmembrane voltages and to measure single channel currents. All experiments were performed at room temperature. Current measurements were carried out using an Axopatch 200B amplifier (Axon Instruments) in the voltage clamp mode. The data were

filtered with a low-pass 8-pole Model 9002 Bessel filter (Frequency Devices) at 1 kHz and directly recorded into computer memory with a sampling frequency of 5 kHz. Data were analyzed using pClamp 9.2 (Axon Instruments) and Origin 7.0 (Origin Lab). Current transition histograms were generated for each tested voltage. Histogram peaks were fitted with the normal distribution function. Single channel conductance was calculated as the mean single-channel current divided by the applied transmembrane voltage.

## Results

### Yeast growth inhibition by SP22A and SP25A

The growth of *S. cerevisiae* strain KZ1-1C in batch cultures with YPD broth medium was completely inhibited by SP22A and SP25A at concentrations of 4.66  $\mu\text{M}$  and 8.24  $\mu\text{M}$ , respectively (Table 5. 1). In contrast, SRE gave complete inhibition at 0.81  $\mu\text{M}$ . In all cases, growth did not resume after 48h indicating that inhibition was fungicidal.

### Susceptibility of SRE-resistant lipid mutants

On replica-plated YPD agar medium SP22A and SP25A inhibited the growth of parental yeast strains (W303C, BY4741, and 8A-1B) at 1.16  $\mu\text{M}$  and 2.06  $\mu\text{M}$ , respectively (Fig. 5.1). Corresponding SRE-resistant isogenic mutant strains with deletions in genes for sphingolipid ( $\Delta\text{syr2}$ ,  $\Delta\text{ipt1}$ ,  $\Delta\text{elo2}$  and  $\Delta\text{elo3}$ ) or ergosterol ( $\Delta\text{erg3}$ ) biosynthesis were less susceptible to inhibition by SP22A and SP25A at these respective concentrations (Fig. 5. 1). In contrast, sphingolipid  $\alpha$ -hydroxylase mutant  $\Delta\text{fah1}$  was more susceptible to SP22A than to SRE or SP25A. This mutant strain was nevertheless



resistant to SP22A concentrations below 1  $\mu\text{M}$  that were still inhibitory to isogenic strain BY4741 (data not shown). Overall, the patterns of relative susceptibility and resistance of the lipid mutants to SP22A and SP25A were similar to those shown previously for SRE (Clifften *et al.*, 1996; Hama *et al.*, 2000; Stock *et al.*, 2000).

### **Effects on $\text{K}^+$ efflux**

As previously observed for SRE (Takemoto *et al.*, 1991), SP22A and SP25A stimulated  $\text{K}^+$  efflux from cells of strain KZ1-1C (Fig. 5.2). Higher concentrations of SP22A and SP25A were required to give the effect as compared to SRE. SRE stimulated  $\text{K}^+$  efflux as low as 1  $\mu\text{M}$ . But, 10 and 100  $\mu\text{M}$  of SP22A and SP25A, respectively, were required to achieve similar degrees of  $\text{K}^+$  efflux.

### **Effects on $\text{Ca}^{2+}$ uptake**

As previously observed for SRE (Takemoto *et al.*, 1991), SP22A and SP25A stimulated  $\text{Ca}^{2+}$  uptake by strain KZ1-1C cells (Fig. 5.3). As with  $\text{K}^+$  efflux (Fig. 5.2), higher concentrations of SP22A and SP25A, as compared to SRE, were required to produce the effect.  $\text{Ca}^{2+}$  uptake was observed within 15 min after 5  $\mu\text{M}$  SRE addition. But, 40  $\mu\text{M}$  and 120  $\mu\text{M}$  were the minimal concentrations of SP22A and SP25A, respectively, required for observing  $\text{Ca}^{2+}$  uptake. At these concentrations,  $\text{Ca}^{2+}$  uptake was not evident until about 20 min and 30 min after addition of SP22A and SP25A, respectively.

### **Channel formation in lipid bilayers**

To examine the membrane channels properties of SRE and SPs, their effects on ion conductance across planar lipid bilayers were measured. Figure 5.4-A shows time courses of the integral conductance of the bilayers doped with SP22A, SP25A, and SRE. In all three cases, negative potential application drove the increase of the membrane conductance after 5 min delay followed by stable conductance levels for 10 minutes. Positive voltages produced membrane conductance decreases (not shown), indicating closing of the pores. SP22A produced slightly higher conductance than SP25A (10 individual observations). SRE produced smaller steady-state conductance levels, even at concentrations of SRE that were higher than those of the SPs (17  $\mu\text{M}$  of SRE vs. 0.1  $\mu\text{M}$  of SP22A and 0.11  $\mu\text{M}$  of SP25A).

The single-channel recordings with all three lipodepsipeptides were similar (Fig. 5.4). In all cases, two types of single-channel conductance fluctuations were observed, small and large, differing in the levels of conductance 5 to 6 fold. The dwell times of the large channels were longer than the small ones. The values of the single-channel conductance for the small channels over the range of  $\pm 200$  mV were similar for all three lipodepsipeptides (Fig. 5.4-C). Thus, the properties of the transmembrane channels, formed by SRE, SP22A, and SP25A were essentially the same.

### **Discussion**

Despite the structural differences between SRE, SP22A, and SP25A, all three lipodepsipeptides displayed similar physiological and model membrane-permeabilizing activities. They stimulated  $\text{Ca}^{2+}$  uptake and  $\text{K}^{+}$  efflux by yeast cells. SRE, however, was

active at lower concentrations compared to the SPs, which parallels its relatively higher fungicidal activities. As in the case of SRE, it is not yet known how the SPs cause changes in fluxes. However, it is likely that they both form pores in the plasma membrane as a key step in their action that leads to ion leakage, membrane dysfunction, and possibly other cell-damaging effects. It is also shown that growth inhibition by the SPs is modulated by sphingolipids and sterols, which also is the case with inhibition by SRE (Cliften *et al.*, 1996; Hama *et al.*, 2000). Sphingolipids and sterol biosynthetic mutants of yeast were less susceptible to SP22A and SP25A as compared to the isogenic wild type strains. By analogy to SRE action, it is suggested that the SPs also impart fungicidal activities by forming lipidic pores that are influenced by specific sphingolipids and sterols (Malev *et al.*, 2002; Takemoto, 2003; Kaulin *et al.*, 2005).

To further explore their resemblance, the transmembrane channel properties of SP22A and SP25A were examined and compared to those of SRE channels. SP22A and SP25A caused voltage-dependent macroscopic conductance increase due to the formation of ion-permeable channels in the membrane bilayers. Both SPs were ~150 times more effective in channel formation than SRE. At the same time, despite large structural differences, the properties of single channel formed by all three cyclic lipodepsipeptides were very close (Fig. 4.5). Thus, the differences in macroscopic transmembrane conductance levels were mostly due to differences in the total numbers of channels formed by SRE vs. SP22A and SP25A. We suggest that the observed differences in the membrane activity of SPs and SRE most likely stem from the structural differences in their molecules, and, subsequently, from differences in interaction with the DOPC bilayers. As opposed to the SPs, SRE lacks a hydrophobic peptide domain. SP22 and

SP25 have hydrophobic peptide domains of fourteen and seventeen amino acids, respectively (Ballio et al., 1991). As a consequence, the SPs will interact more favorably with the lipid bilayer than will SRE. It is not known precisely how the SPs and SRE interact with membrane lipids to form channels (Malev *et al.*, 2002). However, the electrostatic and hydrophobic interactions between SP22A and the lipid membrane are known to increase lipid order as a prelude to stable pore formation (Szabo *et al.*, 2004). In addition, it was shown that unlike SRE, the SP22A pore was not thermally inactivated suggesting a higher degree of affinity between SP22A and membrane lipids.

In summary, the present study suggests that although different in fungicidal potencies, SRE and the two SPs kill yeast cells by the same mechanism, involving lipid-dependent membrane pore formation as a crucial step. Like SRE, the action of the SPs against yeast is promoted by sphingolipids and sterols of the plasma membrane. Our observations also show that the structures of pores formed by SRE and the SPs in membranes are very similar despite large structural differences between these molecules.

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TABLE. 5.1. Effects of SRE, SP22A, and SP25A on batch culture growth of *S. cerevisiae* strain KZ-IC\*

Additions to medium ( $\mu\text{M}$ )	<sup>+</sup> Generation time (hr)
None	<b>5.33</b>
SRE	
0.81	$\infty$
SP22A	
2.33	<b>8.66</b>
4.66	$\infty$
9.33	$\infty$
<b>SP25A</b>	
<b>2.06</b>	<b>6.3</b>
<b>4.12</b>	<b>8.66</b>
<b>8.24</b>	$\infty$

\* Growth was determined by microscopic direct cell counts during exponential growth in YPD broth medium.

<sup>+</sup> Calculated as 0.693 divided by the specific growth rate (cell doubling  $\text{hr}^{-1}$ ) during exponential growth.

$\infty$  = infinite

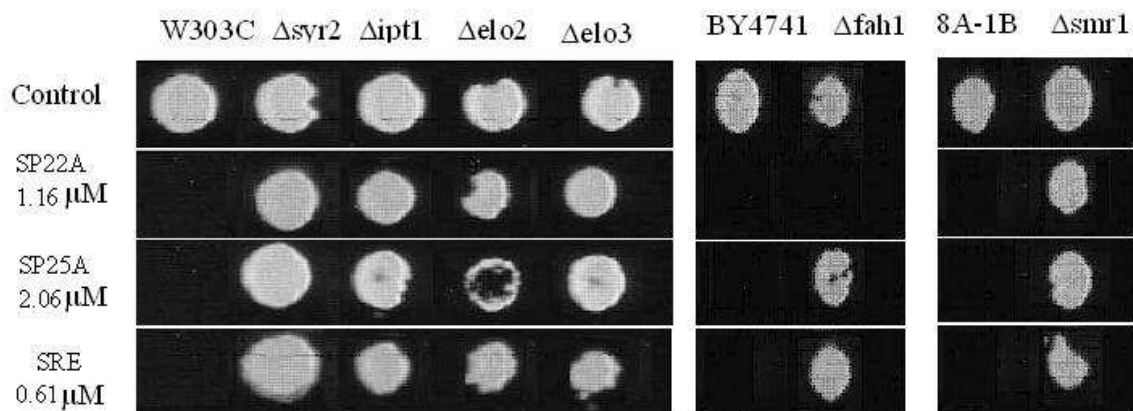


FIG. 5.1. Effect of SRE and SPs on growth of yeast lipid biosynthetic mutants. Sphingolipid and ergosterol biosynthesis mutant and parental strains were replica plated onto YPD agar with or without: SP25A (2.06 $\mu M$ ), SP22A (1.16 $\mu M$ ), or SRE (0.61 $\mu M$ ). The plates were incubated at 28°C for 48 h.

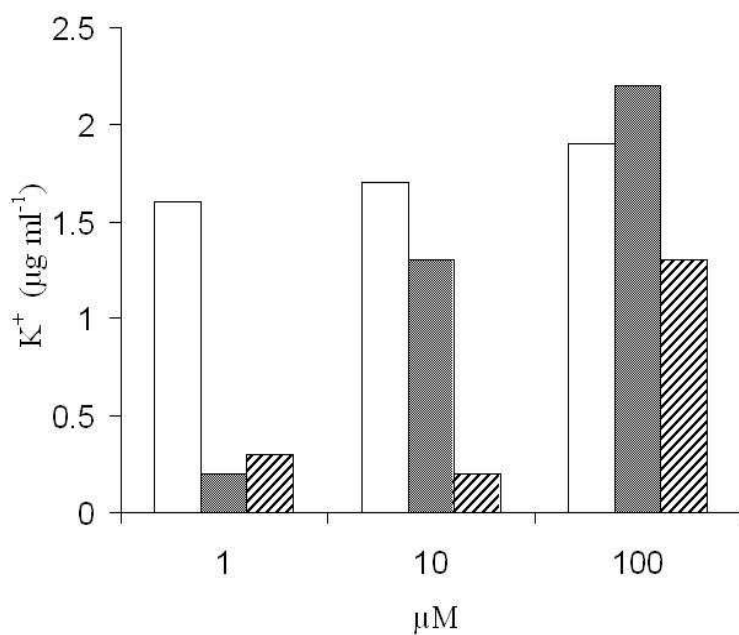


FIG. 5.2. Effects of SRE (blank bars), SP22A (grey bars) and SP25A (striped bars) on K<sup>+</sup> efflux by yeast strain KZ1-1C cells growing in YPD medium. The cells were exposed to designated concentrations of SRE, SP22A or SP25A and extracellular K<sup>+</sup> concentrations measured. The same results were obtained from three separate experiments. Results from a single experiment are presented. Control efflux was similar to SP25A at 1µM.

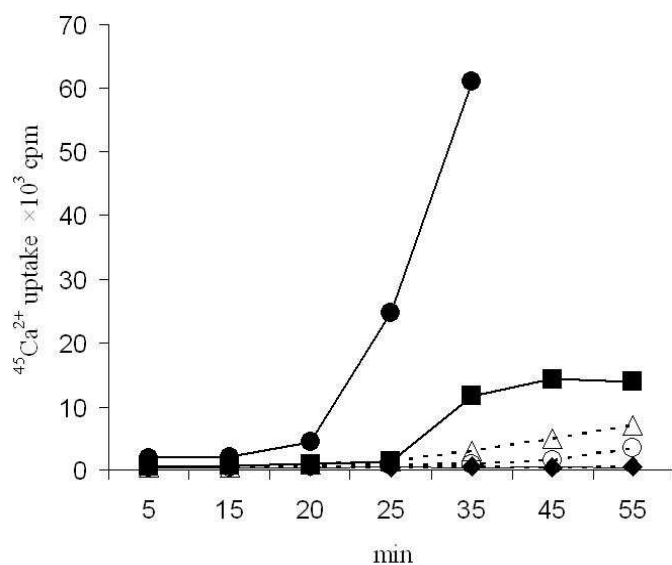


FIG. 5.3. Effects of SRE, SP22A and SP25A on  $^{45}\text{Ca}^{2+}$  uptake by strain KZ1-1C cell suspensions. SRE and SPs were added 5 min following addition of  $^{45}\text{CaCl}_2$ . SP22A concentrations were 10 (◆), 40 (■); SP25A (dashed lines) concentrations were 10 (◆), 40 (○), and 120 (Δ)  $\mu\text{M}$ ; SRE concentration was 5  $\mu\text{M}$  (●). Total  $\text{CaCl}_2$  concentration was 50  $\mu\text{M}$ . The same results were obtained from two separate experiments. Results from a single experiment are presented.

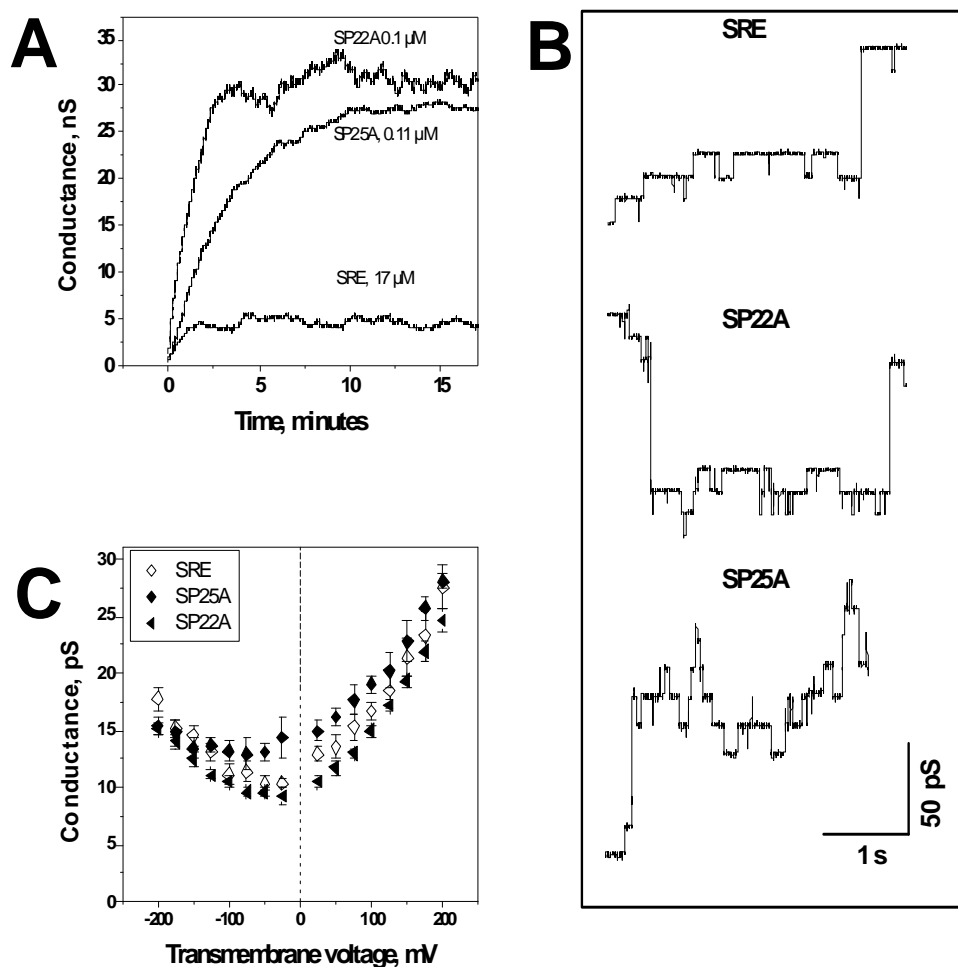


FIG. 5.4. (A) Time courses of the ion conductance of the bilayers doped with SRE, SP22A, and SP25A, recorded at the applied voltage of  $-100$  mV. (B) Records of conductance fluctuations at  $-150$  mV transmembrane potential of SRE, SP22A, and SP25A-modified bilayers. (C) conductance-voltage curves for SRE, SP22A and SP25A small channels

CHAPTER 6  
SYRINGOPEPTIN SP25A INHIBITION OF GRAM-POSITIVE  
BACTERIA AND ROLE OF TEICHOIC ACID  
D-ALANYLATION

**Introduction**

The syringopeptins are secondary metabolites produced by many strains of the plant-associated bacterium *Pseudomonas syringae* (Ballio et al., 1991; Bender et al., 1999). They are cyclic lipodepsipeptides, with their peptide portions containing either 22 [syringopeptins (SP)22] or 25 (SP25) amino acids that are predominantly hydrophobic and largely of the d configuration (Ballio et al., 1991; Isogai et al., 1995) (Fig. 6.1). A common N-terminus dehydroaminobutyric acid (Dhb) is amide linked to a 3-hydroxylated acyl chain containing 10 or 12 carbons to give A and B homologs, respectively. The C-terminus carboxyl group is ester linked to a Thr hydroxyl to form an eight-membered amino acid lactone ring with a net cationic charge. SP25 homologs, SP25A and SP25B, are produced by strains of *P. syringae* pv. *syringae* isolated from millet (strain B359; Ballio et al., 1991), citrus (strain B427; Ballio et al., 1991), and wheat (strain M1; Adetuyi et al., 1995), and by the wheat pathogen *P. syringae* pv. *atrofaciens* (Vassilev et al., 1996). Variants differing in the C-terminal residue (Phe vs. Tyr) are produced by a laurel-infecting *P. syringae* strain (Scaloni et al., 1997). SP22 A and B homologs are produced by the pear isolate *P. syringae* pv. *syringae* strain B301 (Ballio et al., 1991), and variants are produced by *P. syringae* pv. *syringae* isolates from sugar cane [SP(SC; Isogai et al., 1995)], bean (SPPhv; Grgurina et al., 2002), and apple

(SP508; Grgurina et al., 2005). All syringopeptin-producing *P. syringae* strains produce one type of syringopeptin together with a smaller, nine amino acid-containing cyclic lipodepsipeptide – either syringomycin, syringotoxin, syringostatin, or pseudomycin (Bender et al., 1999; Takemoto et al., 2003). The syringopeptins are phytotoxic and also antimicrobial. In plant systems, they perturb mitochondria and protoplasts of tobacco leaves (Iacobellis et al., 1992; Hutchison & Gross, 1997) and form ion channels in sugar beet vacuoles (Carpaneto et al., 2002). They are also antifungal (Iacobellis et al., 1992; Lavermicocca et al., 1997), particularly when combined with cell wall-degrading enzymes (Fogliano et al., 2002). SP22A and SP508A have antibacterial activities against several Gram-positive bacteria such as *Bacillus megaterium*, *Staphylococcus aureus*, and *Mycobacterium smegmatis* but not against Gram-negative bacteria (Lavermicocca et al., 1997; Grgurina et al., 2005). In contrast, the antibacterial spectrum for SP25A is less well known, although it is reported to inhibit *B. megaterium* and to inhibit *Micrococcus luteus* marginally (Lavermicocca et al., 1997). The molecular basis for the inhibitory specificity of syringopeptins against Gram-positive bacteria is not known. The most probable physiological targets of the syringopeptins are membranes, and lipid bilayer studies suggest that the inhibitory mechanism of action involves formation of membrane pores (Hutchison & Gross, 1997; Dalla Serra et al., 1999).

Teichoic acids are major cell surface components of Gram-positive bacteria that confer high densities of negative charges (phosphate groups) on the cell envelope surface. Such anionic sites might serve as initial ionic interaction sites for the cationic 'head groups' of the syringopeptins. The charge densities of peptidoglycan-linked wall teichoic acids and membrane-linked lipoteichoic acids are modulated by esterification with d-

alanine. The enzymes that catalyze and regulate teichoic acid d-alanylation are encoded by five genes of the *dlt* operon (Perego et al., 1995). Genes *dltA* and *dltC* encode a d-alanine-d-alanyl carrier protein ligase and d-alanyl carrier protein, respectively. Both *dltB* and *dltD* encoded proteins facilitate the transport of activated d-alanine across the membrane for incorporation into lipoteichoic acid. The function of the fifth gene of the *B. subtilis* *dlt* operon, *dltE*, remains unknown and defects in *dltE* do not lead to impairment of d-alanylation. d-alanine moieties of lipoteichoic acid serve as precursors for d-alanylation of wall-linked teichoic acids (Haas et al., 1984). Studies with *dlt* mutants reveal that teichoic acid d-alanylation is not essential for viability (Perego et al., 1995), but the process influences susceptibility to lysis (Peschel et al., 1999), acid (Boyd et al., 2000), and antimicrobial peptides (Peschel et al., 2000; Kristian et al., 2005).

In the present study, the antimicrobial spectrum of SP25A and it is shown that it specifically inhibits Gram-positive bacteria is explored further. This includes *B. megaterium*, which was previously shown to be sensitive to this syringopeptin (Iacobellis et al., 1992; Lavermicocca et al., 1997). Data are reported that reveal a role for d-alanylation of teichoic acids in modulating the susceptibility of *B. subtilis* to SP25A and other syringopeptins. This is consistent with the cationic character of the cyclic peptide portion of SP25A, and it provides an explanation for its higher degree of specificity for Gram-positive bacteria.



## Materials and Methods

### Syringopeptin purification

SP25A, SP22A, and SP508A were purified from cultures of *P. syringae* pv. *syringae* strains M1 (Adetuyi et al., 1995), B301D (DeVay et al., 1968) and 508 (Grgurina et al., 2005), respectively, following previously described methods (Ballio et al., 1991). Cultures were extracted with acidified acetone (0.4% HCl), and extracts were chromatographed on Amberlite XAD-2 (Bidwai et al., 1987). Final purification was achieved by preparative HPLC using a Varian 5000 system and an Alltech reverse-phase C18 silica column (Bidwai et al., 1987). For growth inhibition studies, dry and purified syringopeptins were each gravimetrically weighed, mixed in 0.001N HCl at a concentration of 10 mg mL<sup>-1</sup> (4.6, 4.5, and 4.1 mM for SP22A, SP508A, and SP25A, respectively), and diluted accordingly with distilled water before use.

### Organisms and culture conditions

The following organisms were obtained from the American Type Culture Collection (Manassas, VA): *M. smegmatis* ATCC 14468, *Staphylococcus aureus* ATCC 6538, *B. megaterium* ATCC 14381, *B. subtilis* ATCC 1965, *Alcaligenes faecalis* ATCC 8750, *Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 13315, *Pseudomonas aeruginosa* ATCC 15442, *Salmonella enterica* (serovar typhimurium) ATCC 14028, *Serratia marcescens* ATCC 8100, and *Citrobacter freundii* ATCC 8090. *Listeria monocytogenes* ATCC 82302 and a laboratory strain of *Listeria innocua* were obtained from B. Weimer (Department of Nutrition and Food Sciences, Utah State University). *Streptococcus agalactiae* and *Streptococcus pyogenes* strains were obtained from the

Department of Biology Culture Collection, Utah State University. *Mycobacterium smegmatis* was grown at 37°C in Middlebrook 7H9 broth supplemented with 0.2% glycerol and 10% Middlebrook oleic acid-albumin-dextrose-catalase enrichment. *Staphylococcus aureus* was grown at 37°C in Mueller–Hinton medium (Difco), and *B. megaterium* and *B. subtilis* were grown in Luria–Bertani (LB) medium (Sambrook et al., 1989) at 37°C. *Listeria monocytogenes*, and *L. innocua* were grown on brain heart infusion medium (Difco) at 37°C. *Alcaligenes faecalis*, *E. coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella enterica serovar typhimurium*, *Serratia marcescens*, and *C. freundii* were grown on LB medium at 37°C. *Streptococcus agalactiae* and *Streptococcus pyogenes* were cultured on blood agar medium as described by Jasir et al. (2003). *Bacillus subtilis* teichoic acid d-alanylation mutant strains and wild-type JH642 (*trpC2*, *phe-1*) (Perego et al., 1995) were obtained from Z. Pragai (University of Newcastle Upon Tyne). The mutant strains were deleted in *dltB* and *dltD* by insertional mutagenesis with integrative vectors pDLT72 (derived from pJM103) and pDLT74A (derived from JM105A), respectively. Both mutants are deficient in d-alanine esterification of lipoteichoic acids and wall teichoic acids (Perego et al., 1995). A third *dlt* operon mutant strain disrupted by pDLT76 (derived from pDLT74A) and with a truncated *dltD* gene and completely defective *dltE* still incorporates d-alanine into lipoteichoic acid and wall teichoic acids at wild-type levels (Perego et al., 1995). Wild-type strain JH642 was grown in LB medium, and the *dlt* mutant strains were grown in LB medium in the presence of 5 µg mL<sup>-1</sup> chloramphenicol, 2 µg mL<sup>-1</sup> kanamycin, and 1 µg mL<sup>-1</sup> erythromycin (Perego et al., 1995).

### **Effects of SP25A on batch culture growth of *B. subtilis***

*Bacillus subtilis* strains ATCC 1965, JH642 (*trpC2*, *phe-1*), pDLT72, and pDLT74A were each initially grown in 25 mL nutrient broth in 125 mL capacity Erlenmeyer flasks with gyratory shaking (200 r.p.m.) in a New Brunswick G26 incubator shaker for 24 h at 37°C. Ten microlitres of each of the cultures were transferred into 100 mL of fresh nutrient broth medium in 250 mL capacity Erlenmeyer flasks containing 0 (control), 5, or 10 µg mL<sup>-1</sup> of SP25A. The flasks were incubated at 37°C with gyratory shaking in a New Brunswick G76 water bath incubator (speed setting at 6). Cell turbidities at designated times were measured at an absorbance wavelength of 660 nm with a Shimadzu UV1201 spectrophotometer using 1 cm path length cuvettes.

### **Determination of minimal inhibitory concentration (MIC) values**

The Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS)-approved standard reference methods for microbroth dilution susceptibility assays were performed to obtain MIC values (NCCLS, 1993, 2003). Bacteria, except for *M. smegmatis* and *Streptococcus agalactiae* and *Streptococcus pyogenes*, were grown to a final concentration of 10<sup>8</sup> CFU mL<sup>-1</sup> and suspended to a final concentration of 5 × 10<sup>5</sup> CFU mL<sup>-1</sup>. Cell suspensions (25–50 µL) and 10 µL aliquots of twofold dilutions of SP25A were deposited in wells of 96-well polystyrene plates. The plates were incubated at 37°C for 16–20 h. *Mycobacterium smegmatis* cells were grown to a density of 1.0 McFarland unit (c. 3 × 10<sup>8</sup> CFU mL<sup>-1</sup>; Liu & Nikaido, 1999), and *Streptococcus agalactiae* and *Streptococcus pyogenes* colonies grown on blood agar plates were suspended to 0.5 McFarland units (c. 1.5 × 10<sup>8</sup> CFU mL<sup>-1</sup>) in saline (Jasir et al., 2003).

Inocula were diluted to  $5 \times 10^4$  CFU mL<sup>-1</sup>, the cells were dispensed, and twofold dilution series of SP25A (initial concentration 1.0 mg mL<sup>-1</sup>) were applied to 96-well polystyrene plates. MICs were determined after 48-h incubation at 37°C. Minimum bacteriocidal concentrations were determined as described by Perry et al. (1999). After MIC determinations, 100 µL aliquots were removed from each microtiter plate well and spread-plated on appropriate agar medium. All plates were incubated at the optimal temperature for each organism and growing colonies were counted after 24–48 h. The minimal bacteriocidal concentration values were determined as the lowest concentrations of SP25A that yielded a >99.9% killing of the organism. Half-maximal inhibitory concentration (IC<sub>50</sub>) values were determined as the SP25A concentration yielding 50% killing.

#### **Determination of disk diffusion zone of inhibition (ZI) diameters**

Disk diffusion assays were performed according to CLSI-approved standard reference methods (NCCLS, 1993) with modification. Bacteria were grown in appropriate media for 24–48 h. The culture densities were adjusted to 0.5 McFarland units, and cultures were spread on agar plate medium surfaces. Sterilized paper disks (0.5 cm diameter) were placed on the inoculated agar medium surfaces. Ten microlitres aliquots of SP25A solutions were applied to the disks, and the plates were incubated for 16–24 h at optimal temperature (37 or 28°C) before examination and measurement of the diameters of the cleared ZIs. To examine pH effects on syringopeptin activities using disk diffusion assays, *B. subtilis* strains JH642 (*trpC2*, *phe-1*), *dltB*, *dltD*, and *dltE* mutant strains were spread-plated onto surfaces of LB agar medium adjusted to pHs 7.0, 9.0, or

5.8, syringopeptins SP25A, SP22A, or SP508A applied to disks as described above, and the LB agar medium plates incubated at 37°C for 16–24 h.

## **Results and Discussion**

### **Inhibition of bacteria by SP25A**

Previous studies by Lavermicocca et al. (1997) showed that SP25A inhibited the growth of *B. megaterium* but not of several Gram-negative bacteria. In the present study, the inhibitory effects of SP25A against additional Gram-positive bacteria were examined, and its effects against Gram-negative bacteria were re-evaluated (Table. 6.1). MICs ranged between 1.95 and 7.8  $\mu\text{g mL}^{-1}$  for all Gram-positive bacteria tested. The inhibition by SP25A was bacteriocidal as the minimal bacteriocidal concentrations were identical to the MICs.  $\text{IC}_{50}$  values were twofold lower than the corresponding MIC values. In contrast and consistent with the previous observations (Lavermicocca et al., 1997), none of the Gram-negative bacterial species tested were inhibited by SP25A (Table. 6.1).

### **Effects of SP25A on *B. subtilis* teichoic acid d-alanylation mutants**

The basis for SP25A's specificity against Gram-positive bacteria was studied using *B. subtilis* teichoic acid d-alanylation *dlt* mutants. SP25A at 5  $\mu\text{g mL}^{-1}$  inhibited *dlt* operon wild-type *B. subtilis* strains JH642 (*trpC2*, *phe-1*) to varying degrees when grown with aeration at 37°C in nutrient broth (Fig.6.2). Strain JH642 grew slowly with 5  $\mu\text{g mL}^{-1}$  SP25A with a generation time of  $6.97 \pm 0.07$  h compared with a generation time of  $1.58 \pm 0.41$  h when grown with no added SP25A. In contrast, d-alanylation mutant

strains pDLT74A (*dltB* deletion) and pDLT72 (*dltD* deletion) (both constructed in strain JH642) were completely inhibited by  $5 \mu\text{g mL}^{-1}$  SP25A. Therefore, the lack of d-alanylation of teichoic acids led to a higher degree of sensitivity to SP25A. SP25A at  $10 \mu\text{g mL}^{-1}$  completely inhibited the growth of these strains as well as *B. subtilis* strain ATCC 1965. The higher sensitivities of the *dltB* and *dltD* mutants were also evident from microbroth dilution susceptibility and disk diffusion assays. Both mutants showed approximately fourfold lesser MIC values ( $0.98 \mu\text{g mL}^{-1}$ ) and twofold larger ZI diameters (19–20 mm) compared with the parent wild-type strain JH642 ( $3.95 \mu\text{g mL}^{-1}$  and 10 mm, respectively) (Table.6.1). With both assays, the mutant strain inactivated in *dltE* served as an integrative vector control, and it showed susceptibilities comparable to the parental strain JH642 (Table. 6.1). Effect of pH on SP25A inhibition of *B. subtilis* strains and comparisons with SP22A and SP508A. Alkaline pH promotes the hydrolytic removal of d-alanine from teichoic acids (MacArthur & Archibald, 1984; Hyrylainen et al., 2000). As d-alanylation has the effect of masking the phosphate-negative charges of teichoic acids, it was speculated that alkaline pHs will increase the potential for interaction and therefore susceptibility of *B. subtilis* to cationic SP25A. Such an effect was observed. In disk diffusion assays (Table.6.2), SP25A gave larger ZI diameters (30–50%) with *B. subtilis* wild-type strain JH642 at pH 9.0 as compared with pH 7.0 and 5.8. In contrast, the ZI diameters with the *dltB* and *dltD* mutants were comparable or slightly larger (10–15%) at pH 9.0 as compared with pH 7.0 and 5.8 (Table. 6.2). The integrative vector control *dltE* mutant displayed the same degrees of inhibition as parental strain JH642 at all three pHs. The alkaline pH effect was also analyzed with syringopeptins, SP22A and SP508A. With these syringopeptins, the ZI diameters were essentially the

same at pHs 7.0 and 9.0 with all strains tested (parental wild type and *dltB* and *dltD* mutants; Table 2). However, SP22A displayed higher degrees of growth inhibition of the *dltB* and *dltD* mutants at acidic pH. At pH 5.8, the ZI diameters were 30–40% larger for both mutants as compared with the cases at pH 7.0 and 9.0. Although uncertain, it is conceivable that the chemical stabilities of SP22A and SP508A were compromised at pH 9.0. These findings expand upon previous reports concerning SP25A's inhibitory activity against *B. megaterium* (Iacobellis et al., 1992; Lavermicocca et al., 1997), and they solidify the notion that the antibacterial spectrum of the syringopeptins is confined to Gram-positive bacteria (Grgurina et al., 2002, 2005). The variability in MICs (ranging from 1.95 to 7.8  $\mu\text{g mL}^{-1}$ ) among the Gram-positive species is likely due to the evolving variations in cell surface structure and charge distribution that occur with these organisms (Peschel & Sahl, 2006). Like SP22A and SP508A (Grgurina et al., 2005), SP25A is inhibitory to *M. smegmatis*, raising the prospect that these lipopeptides may be developed as antimycobacterial therapeutics.

This study shows that the anionic charges of teichoic acids are important for SP25A's inhibitory action against *B. subtilis*. In contrast, SP22A and SP508A are less reliant on teichoic acid charge as the *dlt* mutant strains were as susceptible as the parental wild-type strain to these syringopeptins. Two principal structural differences between SP25A vs. SP22A and SP508A may account for this disparity: (1) SP25A possesses valine instead of dehydroaminobutyrate at the fourth position of the cyclic portion of the peptide (from the N-terminus), and (2) SP25A has an additional three amino acids in the hydrophobic portion of the peptide. It is speculated that one or both of these features promotes interaction between the cationic charges of the SP25A cyclic peptide moiety

and the anionic teichoic acid phosphate groups and that such interactions are weaker or lacking with SP22A and SP508A. Differences in the modes of interactions of these syringopeptins with the cell surface were also evident by the increased level of inhibition by SP22A and SP508A at acidic pH, but not by SP25A. The acidic effect seen with the former two syringopeptins remains unexplained as well as a mechanism to account for their specific growth inhibition of Gram-positive bacteria. Of relevance is the observation that growth inhibition by positively charged polylysine is not altered in *dlt* mutants of *Staphylococcus* species (Peschel et al., 1999). Apparently, cationic properties alone are not sufficient for peptides to inhibit selectively the growth of Gram-positive bacteria even when lacking teichoic acid d-alanines.

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Table 6.1. SP25A inhibitory activities against bacterial species and strains.

Organism	<sup>1</sup> MIC (µg/ml)	<sup>2</sup> ZI diameter(mm)
<i>M. smegmatis</i> ATCC14468	1.95	25
<i>S. aureus</i> ATCC6538	1.95	17
<i>B. megaterium</i> ATCC14381	1.95	13
<i>B. subtilis</i> ATCC1965	3.9	11
<i>L. monocytogenes</i> ATCC82302	3.9	ND
<i>L. innocua</i>	3.9	ND
<i>B. subtilis</i> JH642	3.9	10
<i>B. subtilis dltB</i> mutant strain (Perego, et al., 1995)	0.98	19
<i>B. subtilis dltD</i> mutant strain (Perego, et al., 1995)	0.98	20
<i>B. subtilis dltE</i> mutant strain (Perego, et al., 1995)	3.9	11
<i>A. faecalis</i> ATCC8750	>250	<1
<i>E. coli</i> ATCC25922	>250	<1
<i>P. vulgaris</i> ATCC13315	>250	<1
<i>P. aeruginosa</i> ATCC15442	>250	<1
<i>S. typhimurium</i> ATCC14028	>250	<1
<i>S. marcescens</i> ATCC8100	>250	<1
<i>C. freundii</i> ATCC8090	>250	<1

<sup>1</sup> MIC values represent two or three matching values from three experiments. The initial undiluted SP25A concentration was 5 mg per ml.

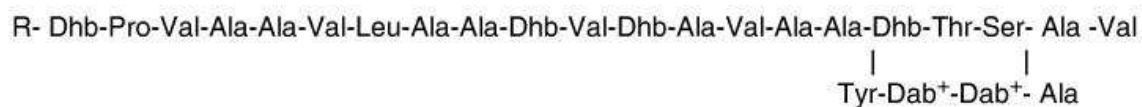
<sup>2</sup> Error was +/- 2 mm from duplicate determinations.

ND= not determined

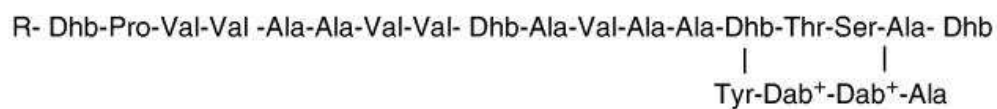
Table 6. 2. Inhibition of *Bacillus subtilis* strains by SP25A, SP22A and SP508A at pH 7.0, 5.8, and 9.0

Strain	* ZI diameter (mm)								
	SP25A			SP22A			SP508A		
	7.0	5.8	9.0	7.0	5.8	9.0	7.0	5.8	9.0
JH642 <sup>+</sup>	10	13	15	9	9	10	9	8	10
<i>dltB</i> mutant <sup>+</sup>	19	19	22	10	13	9	8	11	7
<i>dltD</i> mutant <sup>+</sup>	20	18	22	9	13	10	8	11	10
<i>dltE</i> mutant <sup>+</sup>	11	13	14	8	9	8	9	9	8

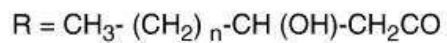
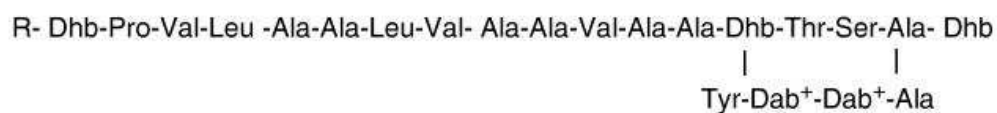
SP25A



SP22A



SP508A




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Fig. 6.1. Structures of SP25A, SP22A, and SP508A. R is 3-hydroxydodecanoyl in SP508A and 3-hydroxydecanoyl in SP22A and SP25A

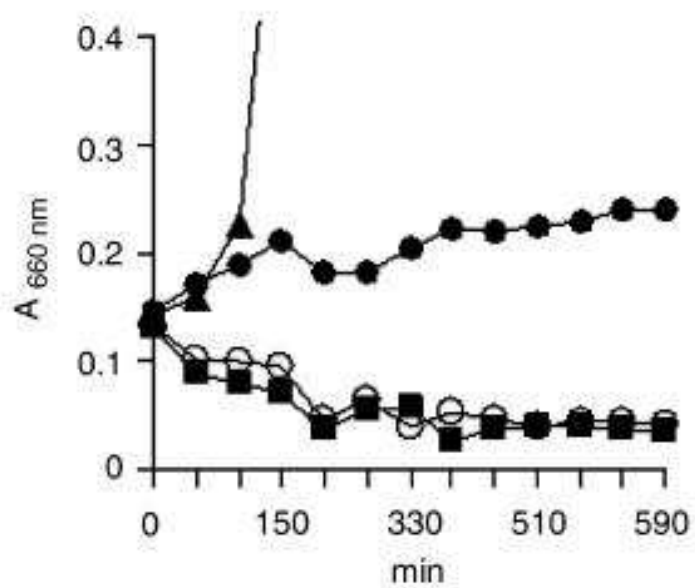


Fig. 6.2. Inhibitory effects of SP25A ( $5 \mu\text{g mL}^{-1}$ ) on the growth of *Bacillus subtilis* strains JH642 (●), *dltB* mutant (○), and *dltD* mutant (■) strains and the growth of *B. subtilis* JH642 in the absence of SP25A (▲).  $A_{660 \text{ nm}}$  is optical absorbance at 660 nm.



## CHAPTER 7

## SUMMARY AND FUTURE DIRECTIONS

The need for new antifungals and fungicidal biocontrol agents is growing due to the narrowing spectrum of fungal targets and increasing resistance to existing antifungals such as amphotericin B and the azoles (11, 12). One approach to develop novel antifungals is to combine known antifungal compounds.

The focus of this research was to examine the bioactive properties of SRE and RL<sub>Zonix</sub> against bacteria, yeast, and filamentous fungi. Previous studies have shown that SRE has inhibitory activity against yeast and fungi ( MICs range, 6.25-40 µg/ml) (13, 7, 15) . However, no activity against bacteria was reported. Moreover, Haba et al. (1, 6) have shown that RLs have antibacterial activities against certain fungi, Gram-negative and Gram-positive bacteria. Although no activity against yeast was observed. In this study I report strong synergistic antifungal activities between SRE and RL<sub>Zonix</sub> in the mixtures (SYRAs). The SRE minimal inhibitory concentrations (MICs) were lowered when combined with RL<sub>Zonix</sub>. However, no activity was observed against bacteria.

In vitro cytotoxicity and erythrocyte lysis were also investigated. The hemolytic activity and cytotoxicity of SYRA was dose dependent. However, the concentrations of SRE in SYRA that caused hemolysis and cytotoxicity were 3 to 5 times higher than the MICs. In addition, the honeybee toxicity effect of SYRA was also studied. The results showed no toxicity at concentrations higher than the MICs. The synergistic antifungal interaction between SRE and RL occurred at low concentrations of SRE. As a result, the

effective antifungal concentration of SYRA was not toxic. These results present new findings and potential uses for alternative therapeutics against pathogenic yeasts and fungi in various applications such as agriculture and medicine.

To further explore their candidacies as agrofungicides, SRE and SYRA formulations were exposed to different temperatures and pHs, and subjected to autoclaving, ultraviolet light, sonication, and proteolysis. The treated solutions were tested for antifungal activities and analyzed by RP-HPLC in the cases of temperature and proteolysis treatments. In addition, SRE and SYRA were mixed with mineral oil and their activities were tested. The overall results showed significantly high stabilities of SRE and SYRA under the tested conditions. The data indicate a possible exploitation of SYRA as agrofungicide.

Finally, I studied the mechanism of action of SYRA using *Saccharomyces cerevisiae* strains deficient in various steps of sphingolipid and sterol biosynthesis as a model. I also studied the channel-forming properties of SYRA in lipid bilayers in comparison with those of SRE. Previously, it was shown that SRE acts on yeast and plant plasma membranes to cause numerous cellular effects with increases in cellular  $K^+$  efflux and transient  $Ca^{2+}$  fluxes among the most prominent. The effects are consistent with SRE's ability to form ion-conducting voltage sensitive channels in membrane bilayers. In addition, studies with yeast have revealed that sphingolipids and sterols (lipids that occur predominantly in the plasma membrane) modulate the fungicidal activity of SRE (4, 5, 14). SRE lipidic pore formation has been proposed as the mechanistic basis for SRE action on membranes (10). In this study the results suggest that SYRA inhibits yeast by the same mechanism as does SRE by itself. Its antifungal

action is promoted by sphingolipids and sterols of the plasma membrane and involves pore formation. In addition, the present data provide an explanation for the antifungal synergy between SRE and RL<sub>Zonix</sub>. The addition of RL<sub>Zonix</sub> resulted in an increase in the number of membrane channels formed by SRE. I suggest that the observed increase in the number of channels is due most likely to the enhancement and favorable binding of SRE in the presence of RL<sub>Zonix</sub>. However, the nature of interaction between SRE, RL<sub>Zonix</sub>, and the plasma membrane is not clear. One possibility is that the penetration of RL<sub>Zonix</sub> into the plasma membrane attracts more SRE binding to the membrane to give an increase in number of channels. It would be of interest to induce RL into the lipid membrane and study the attraction, insertion and orientation of SRE in the lipid membrane using NMR spectroscopy or circular dichroism (CD) spectroscopy (8, 3). Another approach will be to look directly at the SRE's pore structure in the presence and absence of RL using mercury porosimetry analyses and circular dichroism (CD) spectroscopy (8). It's noteworthy to mention that the nature of the membrane plays an important role in how RL<sub>Zonix</sub> binds and inserts into the membrane (2). Perhaps studying the characterization of a single channel in membrane bilayers using different ratios of lipids and solid-state NMR spectroscopy will help to define the interaction of SRE and RL with the plasma membrane and the effects of membrane composition on their antimicrobial activity.

In conclusion, these studies show strong synergism between SRE and RL<sub>Zonix</sub> against fungi and yeast including phytopathogenic species. The antifungal spectrum, low toxicity, chemical and physical stabilities suggests that SYRA is a potential candidate for fungicidal agricultural applications and antifungal therapy in medicine. However,

additional studies such as in vivo examination of SRE and RL mixtures are needed to evaluate the significance of the combination as a potential antifungal therapy or agricultural fungicide. To illustrate SYRA candidacy as antifungal agent, SYRA can be tested against aspergillosis and candidiasis in animal models. Additionally, further studies in pharmacokinetics and pharmacodynamics can be performed to develop strategies dosing for an optimize treatment (9). Furthermore, to explore the possibility of SYRA as a biocontrol agent against phytopathogenic fungi, infected plants or infected seeds can be used as a model. In addition, SYRA can also be tested as a postharvest control agent by studying the effect of SYRA on infected and wounded fruit.

The studies reported here offer a new approach for developing new antifungal agents. For example, combining rhamnolipids with other lipodepsipeptides such as syringotoxins, syringostatins, and syringopeptins and with other amphipathic compounds may lead to discoveries of novel and effective fungicidal formulations for future applications in agriculture and medicine.

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APPENDICES

## APPENDIX A

ANTIMICROBIAL ACTIVITY OF SP25A- RL<sub>Zonix</sub> AND GRAMICIDIN- RL<sub>Zonix</sub>  
COMBINATIONS

Disk diffusion tests were done according to NCCLS methods with modification {Washington, 1995 #55}. Tested organisms were grown in appropriate media (Bacteria strains were grown on Luria-Bertani medium except *S. aureus* was grown on Mueller-Hinton medium and *R. pilimanae* on potato dextrose broth ( PDB) for 24 to 48 h, and the cell densities were adjusted to 0.5 McFarland standard. A 100 µl aliquot of each culture was spread over the surface of the appropriate agar growth medium as a thin film. A 6 mm diameter sterilized paper disk was placed and pressed down onto the agar {Perego, 1995 #31}. The SP25A- RL<sub>Zonix</sub>, and gramicidin-RL<sub>Zonix</sub> combinations were prepared by adding SP25A or gramicidin (both at 1 mg/ml) together with RLZonix (1 mg/ml) in ratios of 1:3 [wt/wt] ,1:2 [wt/wt] and 3:1 [wt/wt], respectively. Ten µl of the mixtures were applied onto the disks. The plates were incubated for 24 to 48 h and the zones of inhibition were measured.



Table. Antimicrobial activities of SP25A (1mg/ml)- RL<sub>Zonix</sub> (1mg/ml) combinations.

	Zone of inhibition (mm)				
	SP25A	RL <sub>Zonix</sub>	SP25A-RL <sub>Zonix</sub> 1:2	SP25A-RL <sub>Zonix</sub> 1:3	SP25A-RL <sub>Zonix</sub> 3:1
<i>R. pilimanae</i>	10	>1	>1	>1	9
<i>S. aureus</i>	20	>1	9	>1	15
<i>B. subtilis</i>	12	>1	8	>1	9
<i>E. coli</i>	>1	>1	>1	>1	>1

Table. Antimicrobial activities of gramicidin (1mg/ml)-RL<sub>Zonix</sub> (1mg/ml) combinations.

	Zone of inhibition (mm)				
	Gramicidin	RL <sub>Zonix</sub>	Gramicidin- RL <sub>Zonix</sub> 1:2	Gramicidin- RL <sub>Zonix</sub> 1:3	Gramicidin- RL <sub>Zonix</sub> 3:1
<i>R. pilimanae</i>	>1	>1	>1	>1	>1
<i>S. aureus</i>	25	>1	15	10	18
<i>B. subtilis</i>	23	>1	10	>1	15
<i>E. coli</i>	>1	>1	>1	>1	>1

## APPENDIX B

MECHANISM OF ACTION OF SP25A- RL<sub>Zonix</sub> COMBINATION

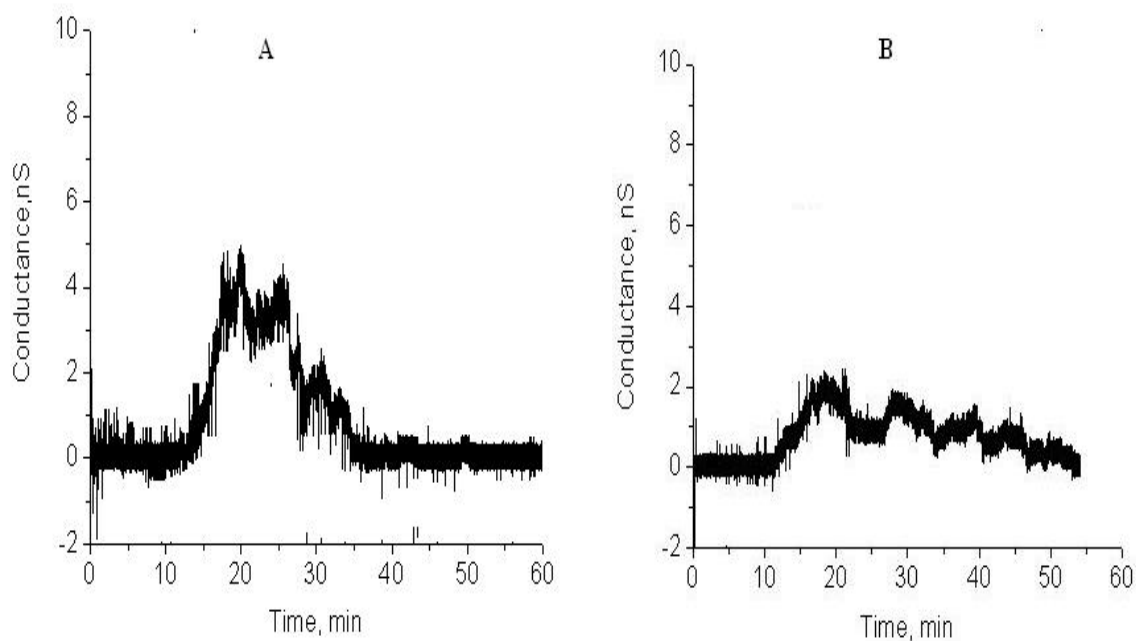
To study the influence of RL<sub>Zonix</sub> on SP25A interaction with the plasma membrane, I examined the membrane channel forming properties of SP25A in the presence and absence of RL<sub>Zonix</sub>. The effects on ion conductance across planar lipid bilayers were measured. The figure shows time courses of the integral macroscopic conductance of the bilayers doped with SP25A alone and SP25A with RL<sub>Zonix</sub>. The results showed that the negative potential application drove the increase of the membrane conductance. Remarkably, the addition of RL<sub>Zonix</sub> to cis side of the bilayer caused no changes in the membrane conductance.

Lipid bilayer electrophysiological experiments were performed using 1,2-dioleoyl-sn-glycero-3-phosphorylcholine (DOPC), (Avanti Polar Lipids) as previously described (Malev, *et al.*, 2002) (Blasko, *et al.*, 1998). Solutions of 0.1 M of NaCl were buffered with 5 mM MOPS (Sigma) to pH 6. Bilayer lipid membranes were prepared by the monolayer-opposition technique (Montal & Mueller, 1972) on a 50–100  $\mu\text{m}$  diameter aperture in the 10  $\mu\text{m}$  thick Teflon film separating two (*cis* and *trans*) compartments of a Teflon chamber (Bezrukov & Vodyanoy, 1993) (Montal & Mueller, 1972). The membrane-forming solution was DOPC. A pair of Ag/AgCl electrodes with agarose/2 M KCl bridges was used to apply transmembrane voltages and to measure single channel currents. Current measurements were carried out using an Axopatch 200B amplifier (Axon Instruments) in the voltage clamp mode. The data were filtered with a low-pass 8-pole Model 9002 Bessel filter (Frequency Devices) at 1 kHz and directly recorded into

computer memory with a sampling frequency of 5 kHz. Data were analyzed using pClamp 9.2 (Axon Instruments) and Origin 7.0 (Origin Lab). Single channel conductance was calculated as the mean single-channel current divided by the applied transmembrane voltage. All experiments were performed at room temperature.

SP25A (1  $\mu\text{g/ml}$ ) and RL<sub>Zonix</sub> (4  $\mu\text{g/ml}$ ) were added to the aqueous phase as follows :

- 5- At  $t=0$ , SP25A was added to cis side compartment
- 6- At  $t=0$ , RL<sub>Zonix</sub> was added to cis side compartment, then at  $t=10$  min, SP25A was added to the same side.



Time courses of the ion conductance of the bilayers doped with A)- SP25A applied to cis side. B)- SP25A and RL<sub>Zonix</sub> applied to Cis side. recorded at the applied voltage of  $-50$  mV

## APPENDIX C

ANTIFUNGAL ACTIVITY OF *PSEUDOMONAS SYRINGAE* PV SYRINGAE  
SYRINGOMYCIN EXTRACT AND RHAMNOLIPID  
COMBINATIONS

Syringomycin extracts were purified from *P.syringae* pv.*syringae* strain M1 by the method of Bidwai et.al {Bidwai, 1987 #242}. Five-hundred ml-capacity Erlenmeyer flasks containing 250ml of PDB medium were inoculated with 1 ml of an actively growing culture. The flasks were incubated at room temperature without shaking for 7 to 14 days. The flasks were chilled to 4°C and 250 ml of cold acidified acetone was added (4 ml HCl per liter acetone). The debris and cells were removed by centrifugation. The supernatant was then concentrated by rotary evaporation to 400 ml. Then, 600 ml of acetone were added and the suspension was stirred overnight at 4°C. Finally, the suspension was centrifuged and the supernatant was removed and concentrated, then diluted to 1 l with 0.1% trifluoroacetic acid (TFA). The TFA solution was applied to an Amberlite XAD-2 (20 to 50 mesh) column (3×25 cm). The crude extract was eluted with 0.1% TFA using a nonlinear gradient of 2-propanol. The 2-propanol was removed using a rotary evaporator and the solution was concentrated and lyophilized. The sample was dissolved in 15 ml of water:acetone (40:60 v/v). The final extract was diluted  $2^{-1}$  to  $2^{-4}$  with water before mixing (1:1 v/v) with 1 mg per ml rhamnolipid (Zonix or RL<sub>Zonix</sub>). Commercial RL<sub>Zonix</sub> was obtained from Jeneil Biotech, Inc. RL<sub>Zonix</sub> is an 8.5% (wt/vol) solution of rhamnolipid analogs in water. Two forms of rhamnolipids are in RL<sub>Zonix</sub>, R1 (C<sub>26</sub>H<sub>48</sub>O<sub>9</sub>) has the molecular formula of  $\alpha$ -L-rhamnopyranosyl- $\beta$ -hydroxydecanoyl- $\beta$ -

hydroxydecanoate, and  $R_2(C_{32}H_{58}O_{13})$  is 2-O- $\alpha$ -L-rhamnopyranosyl- $\alpha$ -L-rhamnopyranosyl- $\beta$  hydroxydecanoate.

The combinations were prepared by mixing  $RL_{Zonix}$  with diluted syringomycin extract.

Method similar to those described by the NCCLS protocols for antifungal testing {Washington, 1995 #55} was used. *R. pilimanae* was grown on PDB medium and adjusted to  $5 \times 10^4$  CFU per ml {Shin, 2003 #63} {Johnson, 1999 #65}. A 100 $\mu$ l of the culture was transferred onto solid agar medium. The culture was spread over the surface as a thin film {Shin, 2003 #63}. Four mm diameter sterilized paper disk was deposited on the surface. Different mixtures of diluted crude extract and  $RL_{Zonix}$  were prepared and applied on a disk (7 to 10  $\mu$ l aliquots). The plate was incubated at 28°C for 24 h and the zone of inhibition was measured {Cuenca-Estrella, 2003 #61}.

The antifungal activity of crude extract is shown in Table (Appendix C). The crude extract and  $RL_{Zonix}$  showed strong antifungal synergism against *R. pilimanae*. The zones of inhibition of the combinations were greater than the crude extract applied alone. The strongest activity was observed when 3 mg/ml of  $RL_{Zonix}$  was combined with 1/16 of the crude extract.

Table (Appendix C). Antifungal activity of syringomycin extract and RL<sub>Zonix</sub> combinations against *R. pilimanae*.

RL <sub>Zonix</sub> (1 mg/ml)		Zone of inhibition (mm)				
		--	18.75	3	0.850	0.250
Dilution of syringomycin extract (1 mg per ml) in mixed with RL <sub>Zonix</sub> (1 mg/ml)	--	--	--	--	--	--
1	15	ND	ND	ND	ND	
½	14	ND	ND	ND	ND	
¼	13	38	30	20	15	
1/8	12	44	42	21	15	
1/16	12	36	45	20	15	

ND: not determined

## APPENDIX D

### POSTHARVEST CONTROL OF FUNGI USING SRE AND SYRA

Postharvest fruit biocontrol assays were done by methods described by Bull et al. {Bull, 1998 #151} and Zhou et al. {Ting Zhou, 2001 #261} with modification. Peaches (variety E lberta) free of wounds and pesticide-free were obtained from a local home garden (Jon Takemoto, North Logan, Utah). The fruits were washed using sterile water. The fruits were wounded using a sterile steel rod and 20 $\mu$ l of spore ( $1 \times 10^8$  spore/ml) suspension was placed in the wound and allowed to dry. SRE (40  $\mu$ g/ml) and SYRA (SRE 40  $\mu$ g/ml, RL<sub>Zonix</sub> 120  $\mu$ g/ml) were then applied. The fruits were kept at room temperature and photographs recorded after 3 days. Each tray held three fruits and each experiment was done three times.

The treatment with SRE and SYRA provided significant reduction in the growth of tested fungi and it was effective in controlling *Botrytis cinerea* and *Rhizopus stonifer* on peaches.

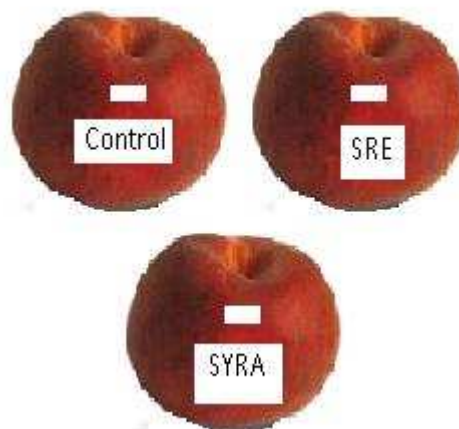
**A****B****C**

Fig. Biocontrol assay. *Botrytis cinerea* (A), *Rhizopus stonifer* (B), *Penicillium oxalicum* (C). 20 $\mu$ l of spore were dropped on the wound. The 20 $\mu$ l of SRE (40  $\mu$ g/ml) and SYRA ( SRE 40  $\mu$ g/ml, RL<sub>Zonix</sub> 120  $\mu$ g/ml ) were applied after the spore suspension was dried.