

In Vitro Antifungal and Fungicidal Activities and Erythrocyte Toxicities of Cyclic Lipodepsinonapeptides Produced by *Pseudomonas syringae* pv. *syringae*†

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Recent increases in fungal infections, the few available antifungal drugs, and increasing fungal resistance to the available antifungal drugs have resulted in a broadening of the search for new antifungal agents. Strains of *Pseudomonas syringae* pv. *syringae* produce cyclic lipodepsinonapeptides with antifungal activity. The in vitro antifungal and fungicidal activities of three cyclic lipodepsinonapeptides (syringomycin E, syringotoxin B, and syringostatin A) against medically important isolates were evaluated by a standard broth microdilution susceptibility method. Erythrocyte toxicities were also evaluated. All three compounds showed broad antifungal activities and fungicidal actions against most of the fungi tested. Overall, the cyclic lipodepsinonapeptides were more effective against yeasts than against the filamentous fungi. Syringomycin E and syringostatin A had very similar antifungal activities (2.5 to >40 µg/ml) and erythrocyte toxicities. Syringotoxin B was generally less active (0.8 to 200 µg/ml) than syringomycin E and syringostatin A against most fungi and was less toxic to erythrocytes. With opportunities for modification, these compounds are potential lead compounds for improved antifungal agents.

Fungal infections, once dismissed as a nuisance, are now a major health concern. Opportunistic fungal infections are increasingly important causes of morbidity and mortality in hospitalized patients. Patients at risk of developing invasive fungal infections are those with AIDS and other immunocompromised conditions, those receiving broad-spectrum antibiotics or cytotoxic therapy, and patients with intravascular catheters. Efforts to combat these infections are hampered by a lack of drugs, increasing resistance, a growing list of pathogens, and lagging research (26). A limited number of agents are available to treat systemic mycoses: mainly, amphotericin B (AmB), the triazoles, and flucytosine (22). Increases in the incidence of fungal infections have prompted a search for new antifungal agents with broad antifungal activities and fungicidal actions, a low likelihood of resistance development, and minimal toxicity.

The syringomycins, syringotoxins, and syringostatins were the first recognized cyclic lipodepsinonapeptides (CLPs) produced by the plant bacterium *Pseudomonas syringae* pv. *syringae*. Individual *P. syringae* pv. *syringae* strains produce a single CLP group. For example, the syringomycins are produced by *P. syringae* pv. *syringae* B301D (24), SCI (12), and M1 (1); the syringotoxins are produced by certain citrus isolates (2, 11); and the syringostatins are produced by the lilac isolate, strain SY12 (16). Within each group, predominant forms are synthesized by the producing organism. These include syringomycin E (SR-E), syringotoxin B (ST-B), and syringostatin A (SS-A). All of the predominant forms inhibit the growth of yeasts such as *Rhodotorula pilimanae* and *Saccharomyces cerevisiae* (29). Another group of CLPs, the pseudomycins, produced by strain 16H, were characterized more recently (3), and its predominant form, pseudomycin A, has antifungal activities (13).

The CLPs are composed of a nonapeptide moiety with the

C-terminal sequence dehydroaminobutanoic acid–Asp(3-OH)–Thr(4-Cl) and an N-terminal Ser *N*-acylated by a long-chain unbranched 3-hydroxy fatty acid and *O*-acylated by the C-terminal carboxyl to form a macrolactone ring (Fig. 1). The five amino acids between the N-terminal Ser and the C-terminal tripeptide form the variable region of the peptide moiety.

The CLPs target the fungal plasma membrane. SR-E alters several membrane functions such as membrane potential, protein phosphorylation, H⁺-ATPase activity, and cation transport fluxes (4, 5, 27, 31, 32). These effects are likely related to channel formation in the plasma membrane (10, 14). Recent molecular genetic studies with *S. cerevisiae* indicate that lipids are involved in the action of SR-E (8, 28).

Many strains of *P. syringae* pv. *syringae* produce CLPs; as a result, a variety of these metabolites occur in nature. This variety as well as the unique mechanism of action and potential for chemical modifications make the CLPs attractive lead compounds for development as clinically useful antifungal agents. In the study described here we evaluated the in vitro antifungal and fungicidal activities of SR-E, ST-B, and SS-A against a variety of clinical fungal isolates and their erythrocyte toxicities.

MATERIALS AND METHODS

Antifungal drugs. SR-E, ST-B, and SS-A were produced from cultures of *P. syringae* pv. *syringae* B301D, PS268, and SY12, respectively. Strains B301D and PS268 were grown in potato dextrose broth (31). Strain SY12 was grown in syringomycin minimal medium supplemented with 100 µM arbutin (A 4256; Sigma Chemical Co., St. Louis, Mo.) and 0.1% fructose (SRM_{AF}) (19, 23). SR-E, ST-B, and SS-A were purified by high-performance liquid chromatography as described previously (5). Solubilized AmB containing 35% sodium deoxycholate (A 9528; Sigma Chemical Co.) and ketoconazole (K-1003; Sigma Chemical Co.) were used as test standards.

Cultures. Most of the fungal strains used in the tests were clinical isolates obtained from the Fungus Testing Laboratory, University of Texas Health Science Center at San Antonio, and the remaining isolates were American Type Culture Collection strains.

Medium. Liquid RPMI 1640 (RPMI) medium with L-glutamine and without sodium bicarbonate (R-6504; Sigma Chemical Co.) buffered with 0.165 M MOPS (morpholinepropanesulfonic acid; 34.54 g/liter) was used for in vitro antifungal

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TABLE 1. Activities of SR-E, ST-B, SS-A, AmB, and ketoconazole against fungal isolates

Organism (no. of isolates)	MIC ($\mu\text{g/ml}$) range ^a				
	SR-E	ST-B	SS-A	AmB	Ktz ^b
<i>Candida albicans</i> (20)	2.5–5	3.2–12.5	2.5–5	≤0.04–0.3	≤0.02–10
<i>Candida kefyr</i> (1)	2.5	3.2	2.5	0.3	≤0.02
<i>Candida krusei</i> (2)	10	12.5–25	10	0.3–0.6	0.15
<i>Candida lusitanae</i> (2)	2.5	6.25	5	0.3	≤0.02
<i>Candida parapsilosis</i> (2)	2.5	6.25–12.5	2.5–5	0.6	≤0.02
<i>Candida rugosa</i> (2)	5–20	3.2–25	10–20	0.3–1.25	≤0.02
<i>Candida tropicalis</i> (2)	2.5–5	3.2	2.5–5	0.3–1.25	0.08–0.6
<i>Cryptococcus neoformans</i> (14)	2.5–10	0.8–6.25	2.5–10	0.08–1.25	0.04–0.6
<i>Saccharomyces cerevisiae</i> (1) ^c	2.5	6.25	2.5	0.3	0.15
<i>Aspergillus fumigatus</i> (16)	10–20	6.25–25	5–40	0.15–1.25	0.3–>10
<i>Mucor</i> spp. (5)	10–>40	6.25–100	10–>40	≤0.02–0.15	0.6–>10
<i>Microsporium</i> spp. (2)	6.25–12.5	25–200	2.5–5	0.04–0.3	0.8–1.6
<i>Trichophyton</i> spp. (3)	3.1–6.25	25–200	2.5–5	0.3–0.6	≤0.4–3.1

^a Values were obtained from triplicate determinations.

^b Ktz, ketoconazole.

^c The strain was tested more than once on different days.

which is known for its fungicidal action, also showed fungicidal activity against most of the strains tested. Ketoconazole, which is not considered fungicidal, showed fungicidal activity only against *Candida krusei*.

Erythrocyte toxicity. All three CLPs caused lysis of sheep erythrocytes and were more toxic than AmB to the erythrocytes (Fig. 2). ST-B was the least toxic of the three CLPs. The kinetics of hemolysis differed between the CLPs and AmB.

DISCUSSION

SR-E, ST-B, and SS-A all displayed fungicidal activities. Previously, a fourth *P. syringae* CLP, pseudomycin A, was also shown to be fungicidal, although the numbers and kinds of fungal organisms tested were limited in comparison with those tested in the present study (13). These activities probably reflect the natural role of these metabolites in plant environments as agents that promote bacterial survival against fungal competitors (29). The *P. syringae* pv. *syringae* CLPs are significantly more toxic to fungi than to plant tissues and bacteria (15).

There was some variability in susceptibility between fungal species. All three CLPs were more active against the yeasts

than against the filamentous fungi. A similar difference was observed with pseudomycin A (13). This difference could be due to differences in the lipid compositions of the membranes of yeasts and filamentous fungi (30). Lipids are important for the action of SR-E (8, 28). Although it was inhibited by all four CLPs, *C. neoformans* was particularly susceptible to ST-B.

In addition to their antifungal properties, the CLPs caused erythrocyte lysis. As is well documented (6, 18), the widely used antifungal agent AmB also elicited erythrocyte lysis. The lytic activity profiles of the three CLPs paralleled their antifungal activities. SR-E and SS-A were more active than ST-B. Conceivably, the more positive net charge of SR-E and SS-A imparted by three basic amino acids (ST-B has two basic amino acids) could account for this difference as well as ST-B's higher fungicidal activity against *C. neoformans*.

A significant finding was that AmB-resistant *C. rugosa* (6) was susceptible to the CLPs. This is likely due to differences in the mechanisms of action between AmB and the CLPs, although both agents bind membrane sterols and perturb membrane function (6, 8, 28). Chemical differences between the two classes of compounds probably account for their distinctive actions on membranes. The CLPs are water-soluble lipodepsi-

TABLE 2. Fungicidal activities of SR-E, ST-B, SS-A, AmB, and ketoconazole against fungal isolates

Organism (no. of isolates)	MFC ($\mu\text{g/ml}$) range ^a				
	SR-E	ST-B	SS-A	AmB	Ktz ^b
<i>Candida albicans</i> (20)	2.5–10	3.2–12.5	2.5–10	0.15–0.3	0.3–>10
<i>Candida kefyr</i> (1)	2.5	3.2	2.5	0.3	1.25
<i>Candida krusei</i> (2)	10	12.5–50	10–20	0.6	0.15
<i>Candida lusitanae</i> (2)	2.5–5	6.25–12.5	5	0.6	≤0.02
<i>Candida parapsilosis</i> (2)	2.5	12.5–25	2.5–10	1.25	0.04
<i>Candida rugosa</i> (2)	10–20	6.25–50	10–>20	0.6–2.5	≤0.02–0.08
<i>Candida tropicalis</i> (2)	5	12.5	5	0.6–1.25	2.5–10
<i>Cryptococcus neoformans</i> (14)	2.5–10	0.8–12.5	2.5–10	0.15–1.25	0.08–>10
<i>Saccharomyces cerevisiae</i> (1) ^c	2.5	12.5–25	5	1.25	0.6–1.25
<i>Aspergillus fumigatus</i> (16)	10–>20	12.5–>50	5–40	0.6–2.5	2.5–>10
<i>Mucor</i> spp. (5)	20–>40	25–>100	40–>40	≤0.02–0.3	2.5–>10
<i>Microsporium</i> spp. (2)	6.25–12.5	25–200	5	0.08–0.3	25–>25
<i>Trichophyton</i> spp. (3)	6.25–25	25–200	5	0.3–0.6	12.5–>25

^a Values were obtained from triplicate determinations. MFC, minimum fungicidal concentration.

^b Ktz, = ketoconazole.

^c The strain was tested more than once on different days.

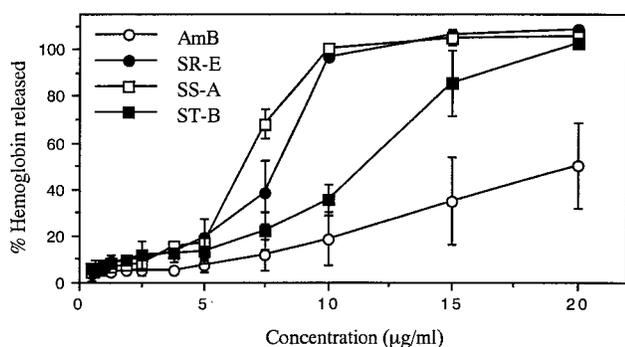


FIG. 2. Release of hemoglobin by erythrocytes induced by AmB, SR-E, SS-A, or ST-B. Sheep erythrocytes were from a single lot, and each point represents the mean \pm standard deviation of three experiments.

nonapeptides, whereas AmB is a cyclic polyene and is relatively more hydrophobic.

In conclusion, although they were not as active as AmB and ketoconazole in vitro, the *P. syringae* pv. *syringae* CLPs show potential as lead compounds for the development of effective antifungal agents. They are fungicidal against important human pathogenic yeasts, are water soluble, and have unique mechanisms of action. Several chemical sites could be modified in an attempt to enhance their antifungal activities and reduce their toxicities.

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