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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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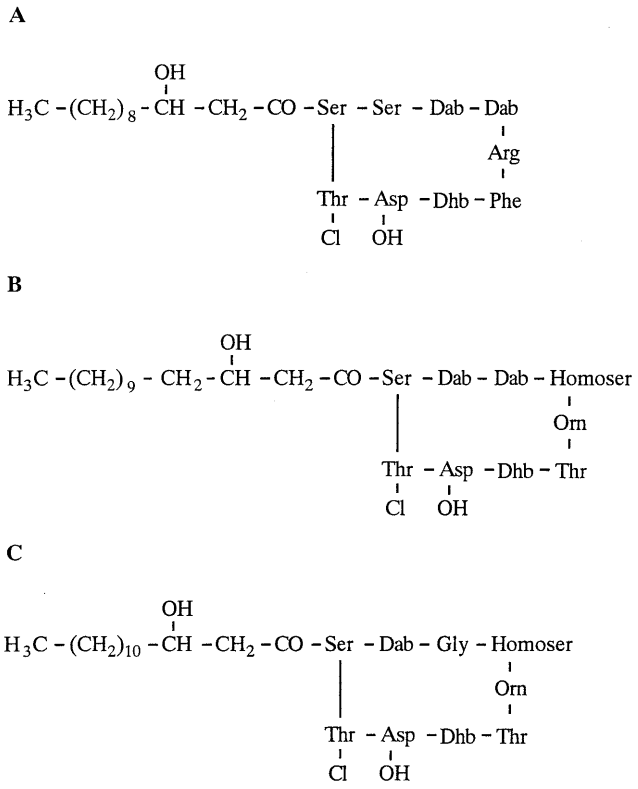

FIG. 1. Structures of SR-E (A), SS-A (B), and ST-B (C). Dab, diaminobutanoic acid; Dhb, dehydroaminobutanoic acid.

tests. The medium was adjusted to pH 7.0 with 10 M NaOH and was filter sterilized.

Drug dilutions. All stock drug solution concentrations were at least 10-fold higher than the highest concentration tested. AmB, ketoconazole, and the test compounds (SR-E, ST-B, and SS-A) were dissolved in sterile distilled water, 0.2 N HCl, and 0.001 N HCl, respectively. Each stock solution was diluted to two times the highest concentration of drug tested with RPMI medium. Twofold dilutions were made with RPMI medium.

Preparation of inoculum suspensions. All isolates were subcultured at least twice before use on Sabouraud dextrose agar (SDA; BBL, Becton and Dickinson and Co., Cockeysville, Md.) to ensure purity and viability. *Candida* spp. and *S. cerevisiae* were grown for 24 h at 35°C. Isolates of *Cryptococcus neoformans* were grown for 48 h at 35°C. Yeast cells from at least five 1-mm-diameter colonies were suspended in 5 ml of sterile 0.85% saline. The resulting suspension was vortexed for 15 s. The turbidity of each mixed suspension was measured at 530 nm and was adjusted to 1×10^6 to 5×10^6 CFU/ml by the spectrophotometric method of the National Committee for Clinical Laboratory Standards (20). The final transmission of each yeast suspension ranged from 85 to 87%.

Aspergillus fumigatus and *Mucor* spp. were grown for 1 week at 35°C. The dermatophytes, *Microsporum* spp. and *Trichophyton* spp., were grown for 2 weeks at 30°C. Fungal spores were washed from the plates of all good spore formers (*A. fumigatus*, *Mucor* spp., *Microsporum canis*, and *Trichophyton* spp.) by placing 5 ml of 0.85% saline with 0.2% Tween 80 on the plates and mixing with an inoculating loop. The spore-mycelium suspension was drawn off with a pipette and was allowed to settle for at least 5 min. For the poor spore-forming species *Microsporum audouinii*, the procedure was the same, except that the spore-mycelium suspension was homogenized with a ground glass tissue homogenizer and was then allowed to settle for at least 5 min. A small amount of the supernatant was transferred to 5 ml of sterile saline and vortexed. The stock suspensions of all filamentous fungi were measured at 530 nm and were adjusted to 85 to 87% transmission. The concentration (1×10^6 to 5×10^6 CFU/ml) was determined by counting with a hemacytometer or by plate counts on SDA. All stock suspensions were diluted 1,000-fold with RPMI medium or to two times the final desired test inoculum (the 2× inoculum). The 2× inoculum was diluted 1:1 when the wells were inoculated, and the desired inoculum size was achieved.

Broth microdilution test. Broth microdilution tests (20) were performed in triplicate with sterile, disposable, multiwell microdilution plates (96 round U-bottom wells; Falcon 3077; Becton and Dickinson Labware, Lincoln Park, N.J.). The drugs (2× concentrations) were dispensed into the wells of rows number 1 to 10 of the microdilution plates in 100-μl volumes, from the highest to the lowest drug concentration. Each well was inoculated with 100 μl of the corresponding 2×-concentrated fungal suspension. The wells of row 11 contained the inoculum, with drug-free medium used as a positive growth control, and the wells of row 12 contained uninoculated drug-free medium, which was used as a sterility control.

Incubation and scoring of MICs. All cultures were incubated without shaking at the temperature used during subculture. Incubation times were 48 h for *Candida* spp., *S. cerevisiae*, *A. fumigatus*, and *Mucor* spp; 72 h for *C. neoformans*; and 7 days for *Microsporum* and *Trichophyton* spp. The growth in the microdilution wells was scored as follows: 0, no growth; 1, slightly hazy; 2, prominent decrease in turbidity; 3, slight reduction in turbidity; and 4, no reduction in turbidity compared with the growth control (drug-free) well. The MICs of SR-E, ST-B, SS-A, and AmB were defined as the lowest concentrations at which scores of 0 were observed. Score ranges from triplicate determinations were recorded. The MIC of ketoconazole was described as the lowest concentrations at which a score of 2 was observed (9).

Minimum fungicidal concentrations. Minimum fungicidal concentrations were determined by subculturing 10 μl from each well with a drug concentration higher than the MIC, equal to the MIC, and the next drug concentration lower than the MIC on drug-free SDA when the MICs were read. Incubation temperatures were the same as those used for the MIC determinations. Incubation times were 24 h for *Candida* spp., *S. cerevisiae*, and *Mucor* spp.; 48 h for *C. neoformans* and *A. fumigatus*; and 7 days for *Microsporum* and *Trichophyton* spp. The minimum fungicidal concentration was the lowest concentration with three or fewer colonies per plate for the yeasts and no growth for the filamentous fungi (17). Score ranges from triplicate determinations were recorded.

Erythrocyte toxicity. Sheep erythrocyte hemolysis was used to assess the erythrocyte toxicities of the CLPs and AmB. Erythrocytes (MicroBio Products, Inc., Salt Lake City, Utah) were washed four times with phosphate-buffered-saline (PBS) by centrifugation at $800 \times g$ for 10 min and adjusted to 10^8 cells per ml (7). Erythrocytes and a 2× concentration of SR-E, ST-B, or SS-A in PBS were mixed in a 1:1 ratio, and the mixture was incubated at 37°C for 1 h. After incubation, the cells were pelleted by centrifugation at $800 \times g$ for 10 min, and the supernatant was collected and the A_{550} was determined. To verify that the compound did not affect the absorbance reading, the pellet was washed with PBS and lysed with distilled water, and the absorbance of the supernatant was determined after centrifugation. Distilled water and PBS were used as lysis and hemoglobin retention controls, respectfully (21).

RESULTS

Antifungal activity. The CLPs showed a broad range of antifungal activity against the fungal isolates (Table 1). SR-E and SS-A had similar activity profiles (the exception was the activity profile against *Microsporum* spp.) and, overall, were more active than ST-B. One strain of *C. neoformans* did not follow this pattern and was more susceptible to ST-B than either SR-E or SS-A. This strain was very susceptible to ST-B (0.8 μg/ml) and was somewhat resistant to AmB (1.25 μg/ml). This differential susceptibility to ST-B also occurred with *Candida tropicalis* and *Candida rugosa*. One strain of *C. tropicalis* and one strain of *C. rugosa* showed resistance to AmB, but they were still susceptible to the CLPs.

SR-E, SS-A, and ST-B were more active against yeasts (MIC range, 0.8 to 25 μg/ml) and were least active against the filamentous fungi (*A. fumigatus*, 5 to 40 μg/ml; *Mucor* spp., 6.25 to 100 μg/ml). In addition, ST-B was not as active against the dermatophytes *Microsporum* and *Trichophyton* spp. (MIC range, 25 to 200 μg/ml).

For the control organism, *S. cerevisiae* ATCC 36375, the MICs of AmB and ketoconazole were as expected (25). The MICs of AmB and ketoconazole, which were used as test standards, were within or close to the range of the expected MICs for the clinical isolates (20, 25). These clinical isolates showed a wide range of susceptibilities to AmB (MICs, ≤0.02 to 1.25 μg/ml) and ketoconazole (MICs, ≤0.02 to >10 μg/ml). The MICs of AmB and ketoconazole were generally lower than those of the CLPs. One strain of *C. albicans* showed resistance (MIC, 10 μg/ml) to ketoconazole, but it was susceptible to the other compounds tested.

Fungicidal activity. All three CLPs showed fungicidal activity against most of the organisms tested (Table 2). The minimum fungicidal concentrations were within twofold dilutions of the respective MICs except for those for *Mucor* spp. AmB,

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

Organism (no. of isolates)	MIC (μg/ml) range[a]				
	SR-E	ST-B	SS-A	AmB	Ktz[b]
Candida albicans (20)	2.5–5	3.2–12.5	2.5–5	≤0.04–0.3	≤0.02–10
Candida kefyr (1)	2.5	3.2	2.5	0.3	≤0.02
Candida krusei (2)	10	12.5–25	10	0.3–0.6	0.15
Candida lusitaniae (2)	2.5	6.25	5	0.3	≤0.02
Candida parapsilosis (2)	2.5	6.25–12.5	2.5–5	0.6	≤0.02
Candida rugosa (2)	5–20	3.2–25	10–20	0.3–1.25	≤0.02
Candida tropicalis (2)	2.5–5	3.2	2.5–5	0.3–1.25	0.08–0.6
Cryptococcus neoformans (14)	2.5–10	0.8–6.25	2.5–10	0.08–1.25	0.04–0.6
Saccharomyces cerevisiae (1)[c]	2.5	6.25	2.5	0.3	0.15
Aspergillus fumigatus (16)	10–20	6.25–25	5–40	0.15–1.25	0.3–>10
Mucor spp. (5)	10–>40	6.25–100	10–>40	≤0.02–0.15	0.6–>10
Microsporum spp. (2)	6.25–12.5	25–200	2.5–5	0.04–0.3	0.8–1.6
Trichophyton 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 (μg/ml) range[a]				
	SR-E	ST-B	SS-A	AmB	Ktz[b]
Candida albicans (20)	2.5–10	3.2–12.5	2.5–10	0.15–0.3	0.3–>10
Candida kefyr (1)	2.5	3.2	2.5	0.3	1.25
Candida krusei (2)	10	12.5–50	10–20	0.6	0.15
Candida lusitaniae (2)	2.5–5	6.25–12.5	5	0.6	≤0.02
Candida parapsilosis (2)	2.5	12.5–25	2.5–10	1.25	0.04
Candida rugosa (2)	10–20	6.25–50	10–>20	0.6–2.5	≤0.02–0.08
Candida tropicalis (2)	5	12.5	5	0.6–1.25	2.5–10
Cryptococcus neoformans (14)	2.5–10	0.8–12.5	2.5–10	0.15–1.25	0.08–>10
Saccharomyces cerevisiae (1)[c]	2.5	12.5–25	5	1.25	0.6–1.25
Aspergillus fumigatus (16)	10–>20	12.5–>50	5–40	0.6–2.5	2.5–>10
Mucor spp. (5)	20–>40	25–>100	40–>40	≤0.02–0.3	2.5–>10
Microsporum spp. (2)	6.25–12.5	25–200	5	0.08–0.3	25–>25
Trichophyton 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.

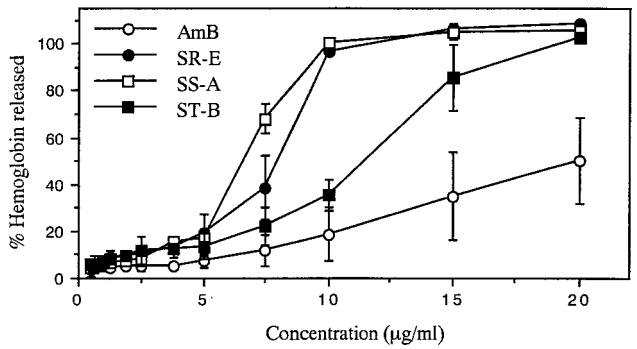

FIG. 2. Release of hemoglobin by erythrocytes induced by AmB, SR-E, SS-A, or ST-B. Sheep erythrocytes were from a single lot, and each point represents the mean ± standard deviation of three experiments.

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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