Cell Wall Active Antifungal Compounds Produced by the Marine Fungus *Hypoxylon oceanicum* LL-15G256

III. Biological Properties of 15G256γ

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15G256γ is a cyclic lipopeptide antifungal agent discovered in a mechanism of action screen for cell wall acting antifungal agents. The compound shows moderate activity in both greenhouse tests against plant disease caused by pathogenic fungi and in *in vitro* tests against human fungal pathogens. Microscopic examination of treated fungi suggests that the compound acts by the inhibition of cell wall biosynthesis. However, *in vitro* inhibition of *Neurospora crassa* glucan and chitin synthase were only observed at high drug concentrations suggesting that 15G256γ may act on a novel cell wall target.

Attempts to discover novel and commercially useful antifungal antibiotics have been ongoing for over fifty years. Since fungi are eucaryotes and thus in many ways similar to their animal and plant hosts, a major challenge has been the identification of compounds that show selective action against fungal cells and lack activity against plant and animal cells. The cell walls of most fungi contain chitin and β-glucans, polymers that are not synthesized by animal cells[6,12]. Although plant cells have cell walls, plant cell walls contain different polysaccharides and callose, a β(1-3) glucose polymer that appears to be synthesized by an enzyme unrelated to fungal β(1-3) glucan synthase[7]. Therefore, inhibitors of the synthesis of fungal cell wall polysaccharides should show fungal selectivity as well as have desirable toxicological and environmental properties.

Although many of the most useful antibacterial agents act *via* the inhibition of bacterial cell wall biosynthesis, researchers have been less successful in developing commercially useful antifungal cell wall inhibitors. The polyoxins, a family of nucleoside-peptide antibiotics, inhibit chitin biosynthesis in a number of phytopathogenic fungi and have been used in Japan for many years as agricultural fungicides[13]. Dimethomorph is an agricultural chemical fungicide active against fungi in the class oomycetes with a reported anti-cell wall mechanism of action[2,15]. Although none of the current antifungals used in clinical medicine act by the inhibition of fungal cell wall synthesis, at least two research groups have recently placed compounds of this type into clinical trial studies. These compounds are semisynthetic derivatives of the echinocandins and pneumocandins, cyclic lipopeptide antibiotics that produce antifungal activity by the inhibition of cell wall β-glucan synthesis[4,5,8].

Our research program to identify novel antifungal agents is based in part on research to identify new cell wall targets and the development of novel screening protocols for the discovery of cell wall active antibiotics[11,16,17]. Antibiotic 15G256γ was identified during the course of screening over 80,000 fermentations[1,21]. Vijayakumar and coworkers[23] have recently described the identification of arthrichitin, a compound which is

identical to 15G256γ. In this paper, we describe biological properties of 15G256γ and provide preliminary information on the mechanism of action of this antibiotic.

Materials and Methods

Tests for Activity Against Phytopathogenic Fungi

Compound 15G256γ was dissolved in methanol/acetone (v/v) and was diluted to the desired concentration with water and amended with a surfactant. For *in vivo* evaluations, test solutions were sprayed on 8 ~ 13 cm tall plants in pots to near runoff. Zero to three days after drying, plants were inoculated with plant pathogenic fungi, in most cases placed in a high humidity chamber for 1 ~ 2 days, and returned to the greenhouse for disease development. Disease control assessments were made 2 ~ 14 days later. For *in vitro* evaluations, test solutions were added to potato dextrose broth (Difco, Detroit, MI) in microtiter plates, which were then inoculated with spore or mycelial suspensions of assay fungi. Plates were scored for fungal growth following incubation.

Tests for Activity Against Human Fungal Pathogens

Eight strains of dermatophytic fungi were grown on modified Sabouraud Dextrose agar (BBL, Cockeysville, MD) at 30°C for 4 to 7 days. The inoculum for MIC tests was prepared by scraping fungal growth from plates and making a suspension of this material in distilled water that gave a 75% transmittance at 530 nm[10]. Three strains of yeast were used as controls for comparative agents and were prepared by methods recommended by NCCLS[20].

Reference antifungal agents were obtained from the following sources: fluconazole, Pfizer Inc., New York, NY; amphotericin B, miconazole, nystatin, and griseofulvin, Sigma Chemical Company, St. Louis, MO. Each drug was dissolved in DMSO, and serial two-fold dilutions were made in sterile distilled water. The drug dilutions were then added to melted RPMI 1640 medium (Gibco BRL, Grand Island, NY) containing 1% purified agar (BBL) and 0.165 M MOPS buffer, pH 7.0 (Sigma) prior to pouring into petri dishes. The plates were inoculated with fungal suspensions using a Steer's replicator. The plates were incubated at 30°C and were examined daily for growth up to 7 days. The MIC was defined as the lowest concentration of drug needed to inhibit the growth of the organism when compared to a control plate containing no drug.

Enzyme Analyses

Neurospora crassa glucan synthase membrane preparations (holoenzyme) and assays were carried out according to the procedure of KANG and CABIB[14]. Chitin synthase membranes and assays were prepared and performed according to YARDEN and YANOFSKY[24]. 15G256γ was dissolved in DMSO at the appropriate concentration, which was then diluted 1/40 for the glucan synthase assay and 1/25 for the chitin synthase assay.

Results

Identification of 15G256γ in Screening for Cell Wall Active Agents

A *Neurospora crassa* based assay was used to screen over 80,000 fermentations to identify cell wall biosynthesis inhibiting antifungal antibiotics[16]. This assay is based upon the use of a *N. crassa* strain carrying the *os-1* mutation. The *os-1* mutation is pleiotropic, producing sensitivity to growth in the presence of high salt concentration, resistance to dicarboximide fungicides and permitting the growth of fungal cells that lack a cell wall. This latter property is exploited in the screen design: compounds that inhibit cell wall biosynthesis are detected by their ability to induce protoplast formation. During the validation of this assay, a panel of antifungal agents with different mechanisms of action was tested. Compounds that inhibited non-cell wall targets were inactive. The chitin synthesis inhibitors polyoxin ABG complex and nikkomycin XZ complex were active, while aculeacin, an inhibitor of glucan biosynthesis was inactive. It is not known whether aculeacin is inactive because it lacks adequate intrinsic activity against *N. crassa*, or because of the incorporation of L-sorbose into the assay medium. L-Sorbose is a glucan biosynthesis inhibitor in *N. crassa*, and the detection of glucan synthesis inhibitors might be suppressed by the presence of this compound.

In addition to 15G256γ, five known antibiotics were detected as actives during the course of screening 80,000 fermentation samples: cystargin, bacillomycin, iturin, cyclothiazomycin and surfactin[3,9,18,19,22]. Polyoxins and related nucleoside-peptide compounds were not detected because of the addition of a peptide mixture to the assay medium. Polyoxins enter fungal cells *via* the action of peptide permeases and the peptides present in the media act as permease competitors. All of the detected known active compounds are cyclic peptides or cyclic lipopeptides. While antifungal activity has been reported for all of these compounds, only cystargin has been

Table 1.

(a) *In vivo* activity of antibiotic 15G256γ against selected phytopathogenic fungal species.

Target organism and host	Rate in μg/ml			
	500	250	125	62.5
Podosphaera leucotricha on apple	—	73	23	0
Venturia inaequalis on apple	85	—	40	—
Cochliobolus sativus on barley	89	80	41	29
Botrytis cinerea on broad bean	86	73	60	55
B. cinerea on bell pepper	95	—	60	—
Uromyces appendiculatus on bean	100	98	96	94
Cercospora beticola on sugar beet	90	—	85	—
Plasmopara viticola on grape vine	100	—	85	—
Puccinia recondita f. sp. *tritici* on wheat	100	—	93	83
Erysiphe graminis f. sp. *tritici* on wheat	100	94	80	0

Table values are percent disease control. —: compound not tested.

(b) *In vitro* activity of antibiotic 15G256γ against selected phytopathogenic fungal species

Test organism	ED_{90} (μg/ml)
Botrytis cinerea	50
Cochliobolus sativus	0.78

reported to have activity against a cell wall target. A number of biological characterization studies were performed to determine whether 15G256γ had useful fungicidal activity and acted by inhibiting cell wall biosynthesis.

Greenhouse Activity of 15G256γ

Preliminary testing of crude preparations of antibiotic 15G256γ suggested that the compound had broad spectrum antifungal activity with moderate potency inhibiting the growth of phytopathogenic fungi belonging to a variety of taxonomic groups: Ascomycetes, Basidiomycetes, Deuteromycetes and Oomycetes. Purified 15G256γ was therefore tested in the greenhouse on a broad spectrum of plant diseases. As shown in Table 1a, 15G256γ produced good disease control against a wide variety of fungal pathogens although control commonly required the use of the compound at high application rates, in the range of 500 μg/ml in a foliar spray. In some diseases such as bean rust caused by *Uromyces appendiculatus*, however, 90% disease control could be achieved with a ten fold lower application rate of 15G256γ.

To determine whether the lack of potency was due to low intrinsic fungicidal activity, 15G256γ was tested *in vitro* to determine growth inhibition potency against *Botrytis cinerea* and *Cochliobolus sativus*. Although an application of 500 μg/ml was required for both fungi to achieve 90% disease control *in vivo*, *in vitro* growth inhibition ED90 values were 50 μg/ml for *B. cinerea* and 0.78 μg/ml for *C. sativus* (Table 1b). This result suggests that factors other than low intrinsic fungicidal activity may be responsible for the high rates of compound application required for plant disease control.

Activity of 15G256γ Against Human Fungal Pathogens

To expand the characterization of 15G256γ, it was also tested for antifungal activity against human fungal pathogens. The MICs obtained in these tests with 15G256γ and a variety of antifungal agents are listed in Table 2. The MICs of 15G256γ ranged from 2~16 μg/ml for the dermatophytic fungi. 15G256γ was more active against some organisms than was fluconazole but in general was less active than the other agents tested. 15G256γ showed the best activity against the griseofulvin-resistant strain of *Trichophyton rubrum* ATCC 44697 (MIC of 2 μg/ml). Therefore, similar to results obtained with phytopathogenic fungi, 15G256γ showed broad spectrum activity with moderate potency against medically important fungal pathogens.

Table 2. Activity of 15G256γ and comparative agents against pathogenic dermatophytic fungi and yeast species.

Fungal pathogen	Strain	Reading	MIC (μg/ml)					
			FLU[a]	AMP. B	MIC	NYS	GRS	15G256γ
Trichophyton rubrum	ATCC 30484	4 days	16	0.5	0.12	2	0.5	8
T. rubrum	ATCC 28190	4 days	0.25	0.5	0.06	2	1	16
T. rubrum	ATCC 44697	7 days	2	0.5	0.06	1	16	2
T. mentagrophytes	ATCC 24198	4 days	64	0.12	1	0.25	2	16
T. mentagrophytes	E 0153 (clinical isolate)	4 days	64	1	1	4	2	16
T. mentagrophytes	ATCC 52015	4 days	32	0.25	0.25	2	0.5	16
Epidermophyton floccosum	ATCC 52063	4 days	2	0.06	0.06	0.12	0.25	8
Microsporum audoinii	ATCC 26340	6 days	16	0.12	0.25	1	0.06	8
Candida albicans	ATCC 90028	24 hours	0.12	0.12	0.06	0.5	64	16
C. parapsiliosis	ATCC 90018	24 hours	0.12	0.5	0.06	0.25	64	16
C. glabrata	ATCC 90030	24 hours	0.12	0.5	0.06	0.25	64	16

[a] FLU: fluconazole, AMP. B: amphotericin B, MIC: miconazole, NYS: nystatin, GRS: griseofulvin.

Structural Abnormalities Observed in Fungi Treated with 15G256γ

Since antibiotic 15G256γ induced protoplast formation in the *Neurospora crassa os-1* screening strain, phytopathogenic fungi were treated with 15G256γ and examined microscopically for the production of morphological effects. The Ascomycete *Cochliobolus sativus* was selected for this experiment because 15G256γ shows high *in vitro* potency against this species. Fungal spores were incubated in the presence of either polyoxin B complex or 15G256γ, allowed to grow for 48 hours, examined under light microscopy and compared with an untreated control culture. The spores of the untreated control sample germinated normally to produce long, straight and highly branched hyphal extensions (Figure 1A). Spores germinated in the presence of polyoxin produced hyphae with a "beaded" appearance and produced a large number of highly swollen protoplast-like structures (Figure 1B). These effects have been reported previously and interpreted to be the result of a weakening of the cell wall leading to varying amounts of hyphal bulging, presumably caused by the relatively unopposed osmotic pressure of the cytoplasm. Interestingly, the hyphal morphology in the 15G256γ treatment was almost indistinguishable from the polyoxin treated material (Figure 1C), consistent with the two compounds having similar mechanisms of action. Also in agreement with a fungal cell wall site of action, 15G256γ lacked activity against *Escherichia coli* and *Bacillus subtilis* when tested at 200 μg/disk in disk diffusion assays. Since the polyoxins are known to act *via* the inhibition of chitin synthase, 15G256γ was tested for *in vitro* effects against cell wall biosynthetic enzymes.

In Vitro Activity of 15G256γ Against Cell Wall Biosynthetic Enzymes

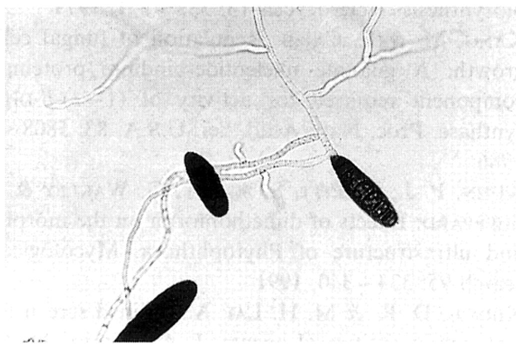
Since the above experiments suggested that 15G256γ acted by inhibiting fungal cell wall biosynthesis, we assayed the activity of the two enzymes that are the most likely targets of such compounds. *In vitro* chitin and β-(1-3)-glucan synthase assays were performed using membrane preparations. Figure 2 shows that although inhibition of both enzymes occurs at high concentrations of 15G256γ (circa 500 μg/ml), the IC_{50} for chitin synthase is approximately half that for glucan synthase. The compound has a slight stimulatory effect on chitin synthase activity at low concentrations.

To correlate these enzyme inhibiton data with growth effects, the *Neurospora crassa* strain used as the source for the enzyme preparations was tested for sensitivity to 15G256γ. No effects on growth or morphology were observed at drug concentrations of 3.125 μg/ml or lower. At a concentration of 6.25 μg/ml, microscopic examination of fungal hyphae revealed the production of abnormal morphology (similar to the effects shown in Figure 1). In addition, cellular debris was observed at a drug concentration of 12.5 μg/ml. The morphological effects and production of cellular debris became more pronounced as drug concentration was increased.

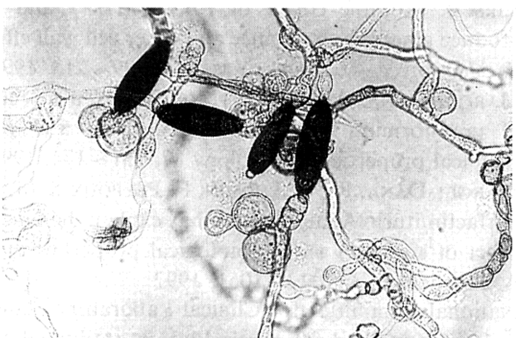
Fig. 1. Microscopic examination of 15G256γ treated fungi.

A) untreated, B) treated with 250 μg/ml polyoxin B, C) treated with 250 μg/ml 15G256γ

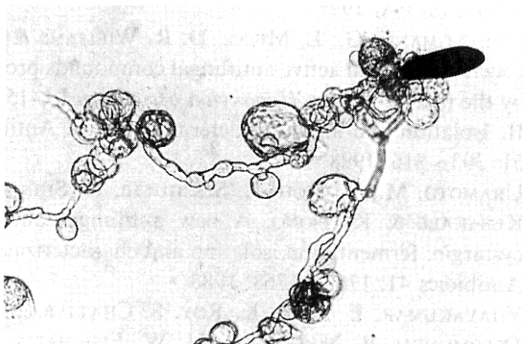
(A)



(B)



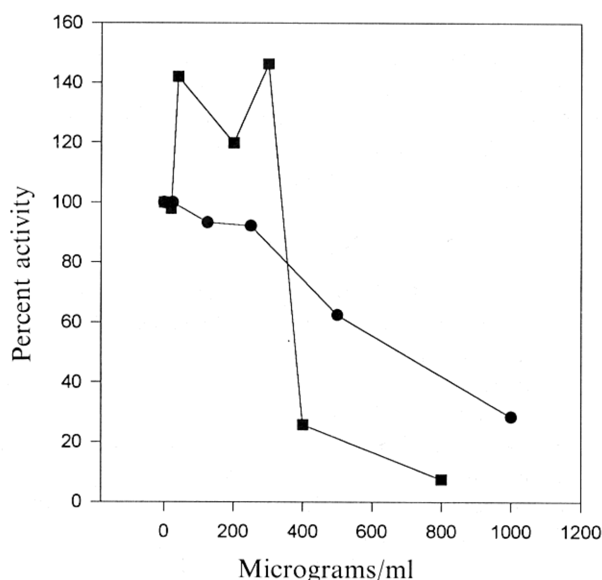
(C)



Cultures of the phytopathogenic fungus *Cochliobolus sativus* were grown for 48 hours and then examined by phase contrast microscopy.

Therefore, significant growth and morphological effects were produced by concentrations of 15G256γ that had little or no inhibitory activity against either enzyme. Unless this antibiotic is actively concentrated by fungal cells, these data suggest that 15G256γ may be acting on one or more novel cell wall targets.

Fig. 2. Activity of *in vitro Neurospora crassa* chitin (■) and glucan (◆) synthase in the presence of 15G256γ (percent activity *versus* untreated).



Discussion

The antibiotic 15G256γ is a structurally novel cyclic lipopeptide antifungal with moderate activity against a range of phytopathogenic fungi and human fungal pathogens. The morphological effects produced by 15G256γ suggest that this compound acts *via* the inhibition of cell wall biosynthesis. This agrees with studies of the structurally related cyclic lipopeptide echinocandins, which have been shown to inhibit fungal cell wall biosynthesis by acting on glucan synthase[4,5,8]. However, our studies indicated that the inhibition of chitin synthase and glucan synthase only occurred at drug concentrations over ten fold greater than the concentration needed to produce significant alterations in hyphal morphology. It is possible that 15G256γ acts on a chitin synthase isozyme whose activity was not detected in the *in vitro* assay employed in this study. Differential activity by antibiotics on chitin synthase isozymes has been reported previously[11]. It is also possible that 15G256γ affects regulatory processes or the cell cycle in a fashion that results in cell wall abnormalities. Distinguishing among these possibilities would require additional experimentation.

Although this compound lacks commercial utility because of its low potency, it might be possible to synthesize an analog with improved potency. Analog

synthesis efforts with the structurally more complex echinocandin and pneumocandin cyclic lipopeptide antibiotics have resulted in compounds that are currently being tested in clinical trials[4,5,8].

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