An Antibiotic Lethal to Fungi

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ABSTRACT

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An antibiotic produced by *Pseudomonas* sp. and identified as tropolone was lethal to established colonies of many fungi, including plant parasitic species of *Helminthosporium*, *Alternaria*, *Fusarium*, *Diplodia*, *Piricularia*, *Cladosporium*, *Rhizoctonia*, and *Pythium* and human parasitic *Trichophyton mentagrophytes* and *T. rubrum*. It was also lethal to an actinomycete, a yeastlike *Capnodium*, and a *Mycobacterium*. Polyene and other antifungal antibiotics, the antifungal activity of *Bacillus uniflagellatus*, and several chemical fungicides were strongly inhibitory but not lethal to the fungi tested.

Among isolations of fungi from Bermuda grass (*Cynodon dactylon* (L.) Pers.) I found a bacterium (*Pseudomonas* sp., ATCC 31099) that produced an antibiotic lethal to fungi. The bacterium was placed in the genus *Pseudomonas* and the antibiotic was identified as the seven-membered ring compound tropolone (5). The antibacterial activities of tropolone and of the isopropyltropolone, B-thujaplicin, have been reported (8,9). Extractions from woods of the family Cupressaceae that contain isopropyltropolone and other tropolones have been tested against wood-rotting fungi (3,6). However, the activity of tropolone (2-hydroxy-2,4,6-cycloheptatriene-1-one) against fungi had not been reported.

This study sought to determine the spectrum of activity of this antibiotic against fungi, to test it against microorganisms with a history of resistance to antibiotics, and to compare it with other antifungal antibiotics and several chemical fungicides.

MATERIALS AND METHODS

The *Pseudomonas* antibiotic was produced in 600 ml of potato-dextrose broth (PDB) in 2-L flasks at 22 C in either standing or shake culture. PDB was prepared with 300 g of peeled, diced potatoes, 23 g of dextrose, and 2 L of distilled water. A broth was defined as having standard activity when two assay disks saturated with a ×20 concentrate and placed on 15 ml of potato-dextrose agar (PDA) prevented growth of the test fungi, *Helminthosporium cynodontis* and *Fusarium roseum*. Assay disks were infested with the test fungi by having both surfaces touched to a sporulating culture or by immersion in a suspension containing about 1×10^5 conidia per milliliter. Standing and shake cultures of the *Pseudomonas* sp. reached standard activity about 14 and 10 days after seeding, respectively. Standing cultures aged longer than 14 days sometimes met the criterion of standard activity in ×7.5 and ×10 concentrates.

The ×20 concentrates of 14- to 30-day-old filtrates of the *Pseudomonas* culture, prepared by chloroform extraction or by evaporation at about 54 C, showed activity equal to that of 0.02 M of tropolone. One hundred milliliters of filtrate was extracted with 30 ml of ethyl ether. The water phase was separated and extracted with 25 ml of chloroform four times. The chloroform was evaporated and the residue dissolved in 5 ml of sterile water.

Synthetic tropolone was obtained from the Aldrich Chemical Company; the antibiotics cycloheximide, neomycin sulfate, endomycin, and filipin from The Upjohn Company; stendomycin salicylate from Eli Lilly & Company; the saprophytic *Mycobacterium* from Baton Rouge General Hospital; and *Trichophyton mentagrophytes* and *T. rubrum* from a Baton Rouge dermatology clinic. Schleicher and Schuell 1/4-in.-diameter assay discs were used.

RESULTS

Activity of *Pseudomonas* antibiotic. The *Pseudomonas* occurred in a plate of *H. cynodontis* colonies that were abnormal and appeared diseased. Transfers from the colonies failed to grow on fresh media, unlike all diseased helminthosporia I have studied previously, which grew readily from mycelial transfers (4). The *Pseudomonas* was streaked on PDA plates opposite transfers of *H. cynodontis*. Within 48 hr, fungal colonies stopped growing nearest to the bacterial streak. *H. cynodontis* cells lysed, as evidenced by frequent bleb formation, the mycelium flattened, and the colony color differed from that of the control fungus (Fig. 1). Transfers (3-mm cubes of mycelial mat plus agar) of the treated colonies were made after about 10 days and placed on fresh PDA; all transfers failed to grow (Fig. 2).

I tested the antibiotic against several genera of fungi by seeding a small bit of mycelium or conidia on one side of a PDA plate and streaking the *Pseudomonas* on the other (Fig. 2). Three replicate plates of each genus were treated in each of two experiments. The first signs of inhibition of the sensitive fungi developed 36–48 hr after seeding; growth stopped completely after about 72 hr. Two transfers, consisting of 3-mm cubes of mycelial mat plus agar, were taken from each treated fungal colony and placed on fresh PDA plates about 1 wk after the treatment began. The antibiotic produced by the *Pseudomonas* was lethal to species of *Helminthosporium*,

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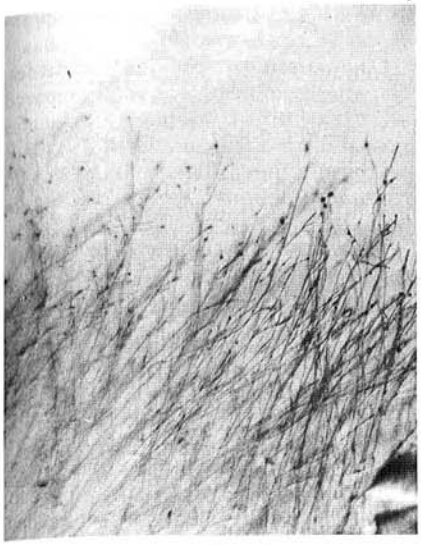

Fig. 1. Bleb formation produced by *Pseudomonas* filtrates or synthetic tropolone in *Helminthosporium cynodontis*.

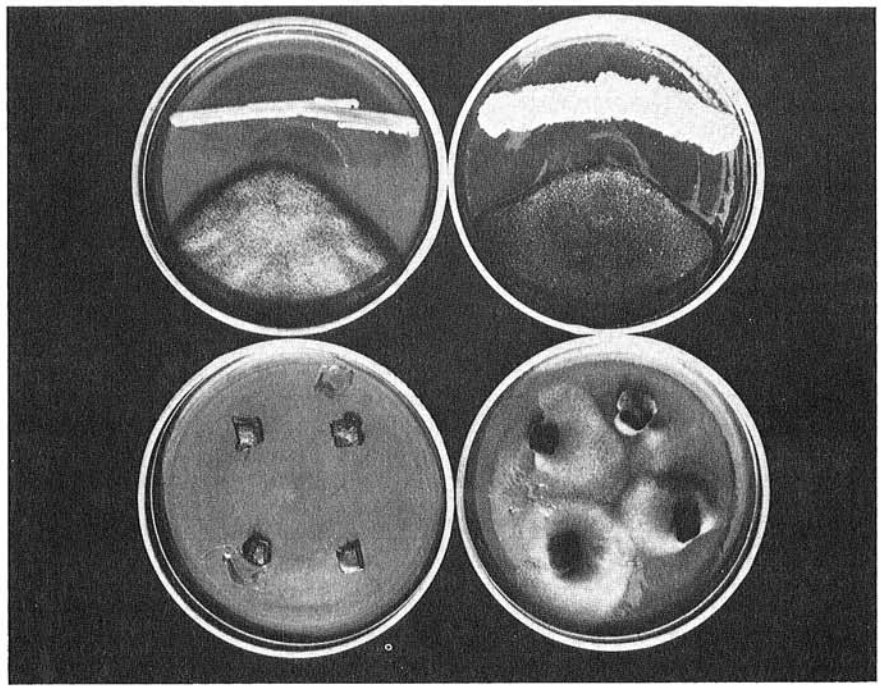

Fig. 2. The antibiotic from *Pseudomonas* (top left) and *Bacillus uniflagellatus* (top right) streaked in plates with *Helminthosporium cynodontis*. Subcultures of *H. cynodontis* treated with the *Pseudomonas* failed to grow (lower left); those treated with *B. uniflagellatus* grew readily (lower right).

Alternaria, *Fusarium*, *Diplodia*, *Piricularia*, *Cladosporium*, *Rhizoctonia*, and *Pythium*; all 12 subcultures of each of these treated fungi failed to grow. The antibiotic was strongly inhibitory but not lethal to a *Colletotrichum* sp., which was moderately inhibitory to growth of the *Pseudomonas*. Species of *Aspergillus*, *Penicillium*, *Trichoderma*, and *Rhizopus* were only slightly inhibited by the antibiotic, and all subcultures grew. All untreated control transfers grew readily.

The unusual activity of the *Pseudomonas* against fungi prompted tests with an actinomycete, *Streptomyces ipomoea*; a yeastlike sooty mold, *Capnodium* sp.; two fungal pathogens of humans, *Trichophyton mentagrophytes* and *T. rubrum*; and a saprophytic *Mycobacterium*. The *Pseudomonas* was streaked on the opposite sides of PDA plates that contained 7-day-old colonies of *S. ipomoea* or fresh transfers of *T. mentagrophytes*, *T. rubrum*, or *Capnodium* in three experiments with equal numbers of control plates. The *Mycobacterium* was placed on PDA plate alone and opposite streaks of the *Pseudomonas*. *S. ipomoea*, *T. mentagrophytes*, *T. rubrum*, and *Capnodium* stopped growing 2–3 days after the *Pseudomonas* was streaked; control colonies grew normally. There was little or no detectable growth of the treated or control *Mycobacterium* on PDA.

None of 60 subcultures of treated *S. ipomoea*, *T. mentagrophytes*, or *T. rubrum* grew; only 18 of 60 subcultures of treated *Capnodium* contained viable cells. All control subcultures grew normally. Treated *Mycobacterium* was placed on slants of Petragnani medium but failed to grow in any of three trials, while control *Mycobacterium* grew vigorously.

Comparison with other antibiotics. Polyene and other antifungal antibiotics produce impressive zones of inhibition against fungi. Fungicidal properties, however, are usually expressed as the failure of conidia to grow following treatment in liquid media with various concentrations of antibiotics (2). The tropolone was considered unusual because it was lethal to established fungal colonies, and I wanted to compare this characteristic with that of other antifungal antibiotics.

I determined the concentrations of the polyene and other antifungal antibiotics, the *Pseudomonas* filtrates, and the synthetic tropolone that would prevent or nearly prevent propagule growth in the test fungi, *F. roseum* and *H. cynodontis*. The concentrations were then increased 50% in a test against 3-day-old fungal colonies; final concentrations per milliliter of medium were 1.8 mg of cycloheximide, neomycin sulfate, endomycin, pimaricin, and synthetic tropolone; 2.7 mg of stendomycin salicylate and filipin; and ×0.75 equivalent concentration of 14- to 30-day-old *Pseudomonas* filtrates.

After 24 hr, fungal colonies treated with the polyene and other antifungal antibiotics stopped growing. Four days later, those treated with cycloheximide, neomycin sulfate, endomycin, and stendomycin salicylate had little or no further growth; those treated with pimaricin grew slightly; and those treated with filipin were the least affected (Table 1). About 4 days after the treatment began, transfers (3-mm cubes of mycelial mat plus agar) were removed from the leading edge of the fungal colonies nearest the assay disks. All transfers from colonies treated with antibiotics grew; those treated with cycloheximide, the most inhibitive of the antifungal antibiotics used, grew only slightly after 24 hr, but hundreds of new hyphae radiated from all areas of the transfers after 72 hr.

Mycelial cells at the leading edge of colonies treated with concentrates of the *Pseudomonas* filtrate and with synthetic tropolone were lysed 24 hr after treatment started. All transfers from the treated colonies failed to grow regardless of whether they were taken from the edge or from deep within the fungal colonies (Table 1). Striking differences between the colonies treated with tropolone and those treated with the polyene and other antifungal antibiotics were apparent when viewed at ×10 under a dissecting microscope. Colonies treated with tropolone were flattened, discolored, lifeless, and dotted with brownish droplets of exudate; colonies treated with the polyene and other antifungal antibiotics were not discolored, and the mycelial mats generally thickened at the inhibited edge to form a "mycelial cliff" above the agar surface.

Colonies of 7-day-old *S. ipomoea*, 3-day-old *T. mentagrophytes* and *T. rubrum*, and 1-day-old *Capnodium* were treated with the polyene and other antifungal antibiotics. The concentrations are listed in Table 1. *S. ipomoea* was significantly inhibited only by endomycin. It continued to grow in the presence of the other antibiotics to a size nearly that of the untreated control colonies. *T. rubrum* was more strongly inhibited by several of the antibiotics, especially neomycin

Table 1. Activity of antibiotics against 3-day-old colonies of *Helminthosporium cynodontis* and *Fusarium roseum*

Antibiotic	Concentration	Inhibition zone (mm)[a]	Growth of subcultures[b]	Lethal zone (mm)[a]
Cycloheximide	1.8 mg/ml	10	40/40	0
Neomycin sulfate	1.8 mg/ml	8	40/40	0
Endomycin	1.8 mg/ml	8	40/40	0
Pimaricin	1.8 mg/ml	4	40/40	0
Stendomycin salicylate	2.7 mg/ml	5	40/40	0
Filipin	2.7 mg/ml	2	40/40	0
Pseudomonas filtrate	×0.75 filtrate	10	0/40	30
Synthetic tropolone	1.8 mg/ml	10	0/40	30

[a] Average from 10 plates in two experiments.
[b] No. growing/no. transferred.

Table 2. Growth of five plant pathogenic fungi treated with chemical fungicides[a]

Fungicide[b]	*Helminthosporium cynodontis*	*Fusarium roseum*	*Colletotrichum graminicola*	*Diplodia zeae*	*Piricularia oryzae*
Benomyl	G	O	G	O_g	O
Elanco 291	O_g	O_g	O_g	O_g	O_g
CGA 64250	O	O	O_g	O_g	O
Fentin hydroxide	O	G	O_g	O_g	O
Chlorothalonil	O_g	O	G	G	O
Dichloran	O_g	G	G	O_g	O_g
Captan	O_g	O	G	O	O_g
Thiophanate methyl	G	G	G	O	O_g
Mancozeb	O	O	O	O	O
Chloroneb	O_g	G	O_g	O_g	O_g
Biloxazol	G	G	O_g	G	O_g

[a] G = growth; O = no growth; O_g = growth after transfer to fresh media.
[b] Concentration of 1 mg a.i./ml.

Table 3. Comparison of activity of tropolone and chemical fungicides against five plant pathogenic fungi

	Mean inhibition zone (mm)[b]				
Agent[a]	*Helminthosporium cynodontis*	*Fusarium roseum*	*Colletotrichum graminicola*	*Diplodia zeae*	*Piricularia oryzae*
Benomyl	17	14	19	18	20
Elanco 291	15	10	9	15	14
CGA 64250	20	21	14	22	17
Fentin hydroxide	18	11	18	15	19
Chlorothalonil	3	2	7	11	5
Dichloran	4	8	15	16	14
Captan	16	8	15	20	4
Thiophanate methyl	18	6	15	15	18
Mancozeb	19	10	16	16	20
Chloroneb	18	13	15	19	21
Biloxazol	16	13	13	6	16
Tropolone	22	19	23	22	22

[a] Final concentration of all fungicides was 3.3 mg a.i./ml; concentration of tropolone was 1.7 mg/ml.
[b] Average from four plates in two experiments.

sulfate, than was *T. mentagrophytes*. *Capnodium* colonies were inhibited about equally by all of the antifungal antibiotics except cycloheximide, which was particularly inhibitive.

Transfers were made from colonies of *S. ipomoea*, *T. mentagrophytes*, *T. rubrum*, and *Capnodium* treated with the antibiotics in three separate trials. All transfers grew; those from colonies of *Capnodium* treated with cycloheximide showed almost no growth after 24 hr, but were growing after 72 hr.

The bacterium *Bacillus uniflagellatus*, which produces marked antifungal activity, was compared with the *Pseudomonas*. Each bacterium was streaked opposite *H. cynodontis* or *F. roseum* on PDA. The test was repeated three times. The leading edge of colonies treated with *B. uniflagellatus* thickened, whereas those treated with the *Pseudomonas* lysed. Treated colonies were subcultured 10 days after treatment with transfers taken from the colony edge nearest to the bacterial streak. Each of the 60 transfers from colonies treated with *B. uniflagellatus* grew, but all subcultures from colonies treated with the *Pseudomonas* failed to grow (Fig. 2).

Comparison with chemical fungicides. The activity of tropolone was compared with that of 11 chemical fungicides against established colonies of five plant pathogenic fungi, *H. cynodontis*, *F. roseum*, *Colletotrichum graminicola*, *Diplodia zeae*, and *Piricularia oryzae*. It was first determined that all of the fungicides, at 1 mg a.i./ml, prevented growth of "seeds" of some of the five test fungi. A seed was obtained by touching a sterile assay disk on both surfaces to a sporulating culture or dipping it into a suspension that contained approximately 1×10^5 conidia per milliliter. Fungi that showed no growth after 1 wk were transferred, via the seeded disk, to fresh PDA to determine whether the fungicides were merely inhibitive or were lethal to the seeds.

CGA 64250 and mancozeb not only prevented growth of seeds of all of the test fungi, but the seeded assay disk failed to grow when transferred to fresh PDA (Table 2). Failure of the transferred seeds to grow on fresh media was interpreted as lethality to the young hyphae of germinating conidia. Benomyl, fentin hydroxide, chlorothalonil, and captan were lethal to two of the five fungal seeds and thiophanate methyl was lethal to one. Elanco 291, dichloran, chloroneb, and biloxazol were strongly inhibitive to many of the fungal seeds but were lethal to none.

I incorporated the fungicides (at 3.3 mg a.i./ml) and tropolone (at 1.7 mg a.i./ml) into media via saturated assay disks that were placed opposite 60- to 90-hr-old colonies of the five fungi. Seven days later, I measured the zones of inhibition produced by the fungicides and immediately subcultured 3-mm cubes of the treated colonies. The zones showed that some fungicides were strongly active against some of the fungi (Table 3), but all subcultures from fungicide-treated colonies grew (*data not shown*). However, none of the subcultures of colonies treated with tropolone grew.

I also compared the minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC) of tropolone, cycloheximide, and CGA 64250 for *H. cynodontis*, *F. roseum*, and *T. mentagrophytes*. Each assay tube contained PDB and 1×10^5 conidia. The MIC and MFC of CGA 64250 were as low or lower than those of tropolone for these fungi. Tropolone was more inhibitive and fungicidal to conidia of the three test fungi than was cycloheximide (Table 4).

DISCUSSION

Six polyene and other antifungal antibiotics and 11 chemical fungicides prevented or nearly prevented conidial growth of several test fungi. Some of the materials were lethal to conidia, but none was lethal to significant numbers of cells

Table 4. Minimum inhibitory and fungicidal concentrations (μg/ml) of tropolone, cycloheximide, and CGA 64250 for conidia of *Helminthosporium cynodontis, Fusarium roseum,* and *Trichophyton mentagrophytes*

Organism	Tropolone		Cycloheximide		CGA 64250	
	MIC[a]	MFC[b]	MIC	MFC	MIC	MFC
H. cynodontis	1	5	10	50	1	1
F. roseum	5	10	50	100	1	1
T. mentagrophytes	10	50	100	500	1	50

[a] Minimum inhibitory concentration.
[b] Minimum fungicidal concentration.

of established colonies when used at rates 1.5–3 times greater than the rates used against conidia. Mature fungal cells that were part of an established colony survived treatment with antifungal agents better than conidia.

An evaluation of the activity of tropolone based on the standard MIC and MFC would not have identified the magnitude of its fungicidal properties. Many materials, antibiotic and not antibiotic, have MIC and MFC similar to those of tropolone (1,2,7). CGA 64250 prevented growth of and was lethal to conidia of three test fungi at concentrations as low or lower than those of tropolone.

This study investigated activity only; any applications of tropolone are contingent upon studies of possible toxicity to animals and plants. Nevertheless, it is useful to discuss where tropolone might fit in with established methods of control of fungal infections.

Chemical fungicides are one of the most effective methods of plant disease control; their mechanism of action is mostly one of protection, ie, prevention of fungal infection. Activity as lethal as that of tropolone against fungi is not needed for effective protection. However, tropolone might be valuable in controlling plant diseases where fungi in seed or storage organs (such as tubers, bulbs, and corms) must be destroyed before healthy plant growth can occur.

Control of bacterial infections in man and animals by antibacterial antibiotics is one of the great landmarks in medicine. Antifungal antibiotics have not been nearly as effective in the control of fungal infections. Antibacterial antibiotics are aided in their activities, however, by certain defense mechanisms of the body—antibody system, phagocytes, elevated temperature—that have little influence on fungal infections.

It is thus likely that antibiotics must be lethal to be effective against most fungal infections in man. The polyene and other antifungal antibiotics studied were strongly inhibitory but not lethal to *T. mentagrophytes* and *T. rubrum* and to many other fungi in vitro. According to a Baton Rouge dermatology clinic, *T. mentagrophytes* is the most common pathogen isolated from infected nails in the Louisiana area. Theoretically, local applications of tropolone, an antibiotic lethal to *T. mentagrophytes*, should be effective in controlling such infections.

LITERATURE CITED

1. Gold, W., Stout, H. A., Pagano, J. F., and Donovick, R. 1955–1956. Amphotericins A and B, antifungal antibiotics produced by a streptomycete. Antibiot. Annu. 579-586.
2. Harrison, E. F. 1970. Haloprogin: A topical antifungal agent. Appl. Microbiol. 19:746.
3. Hubert, E. E. 1931. An Outline of Forest Pathology. John Wiley & Sons, New York. 543 pp.
4. Lindberg, G. D. 1959. A transmissible disease of *Helminthosporium victoriae*. Phytopathology 49:29-32.
5. Lindberg, G. D., Whaley, H. A., and Larkin, J. M. 1980. Production of tropolone by a *Pseudomonas*. J. Nat. Prod. 43:592-594.
6. Sowder, A. M. 1929. Toxicity of water-soluble extractives and relative durability of water-treated wood flour of western red cedar. Ind. Eng. Chem. 21:981-984.
7. Taber, W. A., Vining, L. C., and Waksman, S. A. 1954. Candidin, a new antifungal antibiotic produced by *Streptomyces viridoflavus*. Antibiot. Chemother. (Washington, DC) 4:455.
8. Trust, T. J. 1975. Antibacterial activity of tropolone. Antimicrob. Agents Chemother. 7:500-506.
9. Trust, T. J., and Coombs, R. W. 1973. Antibacterial activity of B-thujaplicin. Can. J. Microbiol. 19:1341-1346.