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Antibiotics Against Plant Disease

VIII. Screening for Nonpolyenic Antifungal Antibiotics Produced by Streptomycetes[1]

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ABSTRACT

LINDENFELSER, L. A. (Northern Regional Research Laboratory, Peoria, Ill.), ODETTE L. SHOTWELL, MARILYN J. BACHLER, GAIL M. SHANNON, AND T. G. PRIDHAM. Antibiotics against plant disease. VIII. Screening for nonpolyenic antifungal antibiotics produced by streptomycetes. Appl. Microbiol. **12**:508–512. 1964.—In a survey of *Streptomyces* species, methods were designed and followed that would specifically select strains capable of producing heat-stable, nonpolyenic, antifungal antibiotics. Of 500 strains grown in shaken flasks, 240 of the culture liquors contained active factors as demonstrated by paper-disc assay against *Mucor ramannianus*. Culture filtrates and mycelial extracts of the active strains were examined by ultraviolet spectrophotometry; 166 were nonpolyenic as determined by absorption spectra. Heat-stability tests of the nonpolyenic antibiotics over a broad pH range revealed that 15 were stable under all test conditions, 70 moderately stable, and 81 unstable. Culture liquors containing stable, nonpolyenic antifungal agents were chromatographed with eight solvent systems in an attempt to identify the antibiotics. The producing cultures were studied by cross-antagonism tests to discover similarities with producers of known antibacterial antibiotics. Two of the antibiotics produced by promising strains were identified as cycloheximide and musarin. Six antibiotics, presumably new, were detected.

Although much emphasis has been placed on screening for antibacterial antibiotics, less effort has been placed on antifungal antibiotics. Many antifungal agents obtained from streptomycetes are polyenes (Ball, Bessell, and Mortimer, 1957; Oroshnik et al., 1955; Pledger and Lechevalier, 1956; Utahara et al., 1954; Vining, Taber, and Gregory, 1955). In spite of the large number of polyenes normally encountered in antibiotic screening programs, only a few prove useful in the treatment of mycoses of animals or plants. Among those effective are trichomycin, nystatin, pimaricin, pentamycin, and amphotericin B.

Somewhat more than 50 nonpolyenic antifungal antibiotics were reported from streptomycetes. Řeháček (1964) attempted to classify many of these compounds on the basis of their ultraviolet absorption spectra and solubilities in organic solvents, and brought together some of their chemical and physical data. Cycloheximide, probably the best known of the nonpolyenic antifungal antibiotics, is on the market for control of plant diseases.

Polyene-type antibiotics exhibit the instability typical of conjugated unsaturated compounds, especially to light and oxygen. This instability adds to the difficulty of isolating, purifying, and characterizing these antibiotics. Because of these difficulties, and because of greater effectiveness possibly against plant pathogens under actual field conditions, we initiated a screening program directed specifically toward selection of nonpolyenic antifungal agents. At the same time, we are aware of the possibility that nonpolyenic antifungal antibiotics also might be of value in control of animal mycoses.

MATERIALS AND METHODS

The cultures used in the survey were obtained mainly as isolates from soil samples procured both in the United States and abroad. All cultures were handled similarly, according to the procedures given below.

Shaken-flask production of active materials. A series of fermentations in shaken flasks was made with each culture. Composition of the media was varied systematically with each successive run (Table 1). The first run in the series consisted of a single-flask fermentation for each culture, and the medium employed was a modification of A-4h of Warren, Prokop, and Grundy (1955). The pH of the medium, which was not adjusted, was 6.7 to 6.85 after sterilization. Special nutrient 4-S (A. E. Staley Mfg. Co., Decatur, Ill.) was weighed directly into individual 300-ml Erlenmeyer flasks, and 100-ml quantities of the remaining ingredients in water were dispensed into the flasks. All media were sterilized for 30 min at 121.5 C, inoculated, and incubated at 28 C on a rotary shaker having a displacement of 2.5 in. (6.35 cm) and operating at 200 rev/min. Samples were taken at 72, 96, and 120 hr; the filtrates were assayed by the method described below.

In the next step of the procedure, streptomycete cultures producing active factors in the single-flask screen were put through a "five-flask" run. The composition of the media used is given in Table 1. Each ingredient, other than nutrient 4-S and Cerelose, was omitted individually from four of the flasks. One flask contained the

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complete primary medium as a control. Whenever $CaCO_3$ was omitted, the pH of the medium was adjusted to 7 with 1 N NaOH before sterilization. Samples of the culture liquors taken during the fermentation were assayed to determine which of the five media allowed production of most antibiotic activity for each strain.

In the next step, a "nine-flask" run, the carbon-nitrogen ratio (proportion of Cerelose and nutrient 4-S) was determined for maximal activity. An example cited in Table 1 is no. 5 of the five-flask run. Use of the one-, five-, and nine-flask procedure usually disclosed a medium that promoted increased antibiotic activity against the test fungus. Later, it was found that culture liquors of less potency ordinarily were adequate for the various tests. To expedite the work, all streptomycetes were run initially through the single-flask screen only. The five- and nine-flask series were reserved for streptomycetes showing the greatest promise.

Inocula for shaken flasks consisted of 2- to 5-day-old broth cultures grown in yeast extract medium (Pridham et al., 1957). Each broth culture was prepared by transferring a loopful of spores from a stock slant to 10 ml of sterile yeast extract broth in a test tube (25 by 150 mm). In cases where few or no aerial hyphae or spores were apparent, a small quantity of agar containing some mycelia was removed from the stock slant and was triturated in the broth. The broth tubes were incubated for 2 to 5 days at 28 C on a rotary shaker. Coarse pellets of mycelial growth which occurred were macerated with a sterile pipette; this action usually resulted in a satisfactory inoculum, although in some cases after triturating it was desirable to return the tubes to the shaker for an additional day. It was soon learned that inocula could be prepared a few weeks in advance and held in cold storage until needed. Shaken flasks were seeded with 1% of the appropriate broth cultures.

TABLE 1. *Media employed in shaken-flask screening*

Shaken-flask run	Flask no.	Staley's 4-S[a]	Cerelose[b]	Curbay BG[c] (0.5%)	Glycerol (0.25%)	NaCl (0.5%)	$CaCO_3$ (0.1%)	pH adjustment
		%	%					
One-flask	1	1.5	1.5	+	+	+	+	−
Five-flask	1	1.5	1.5	+	+	+	+	−
	2	1.5	1.5	−	+	+	+	−
	3	1.5	1.5	+	−	+	+	−
	4	1.5	1.5	+	+	−	+	−
	5[d]	1.5	1.5	+	+	+	−	+
Nine-flask	1	1.0	1.0	+	+	+	−	+
	2	1.0	1.5	+	+	+	−	+
	3	1.0	2.0	+	+	+	−	+
	4	1.5	1.0	+	+	+	−	+
	5	1.5	1.5	+	+	+	−	+
	6	1.5	2.0	+	+	+	−	+
	7	2.0	1.0	+	+	+	−	+
	8	2.0	1.5	+	+	+	−	+
	9	2.0	2.0	+	+	+	−	+

[a] Staley's special nutrient 4-S (soya meal).
[b] Commercial glucose.
[c] Dried molasses fermentation solubles.
[d] Flask of highest activity in a hypothetical run; note that $CaCO_3$ was omitted also from the media in the nine-flask screen.

Preparation of culture liquors and mycelia for testing. At the end of a given shaken-flask run, the culture liquor in which highest antibiotic activity for each streptomycete was demonstrated was filtered, and both the filtrate and mycelia were retained for further processing and testing. The mycelia were removed from the filter papers with a spatula, washed once with distilled water, and then extracted by suspending the damp mycelia in absolute methanol (volume of methanol was one-fifth the original volume of liquor) and agitating for 2 hr on a rotary shaker. The mycelia then were removed by filtration through Whatman no. 12 filter paper, and the methanol extract was retained. Culture filtrates were lyophilized and stored at −15 C, along with the methanol extracts.

Assay methods. The screening organism was *Mucor ramannianus* Moeller, NRRL 1839. This strain has a number of advantages over other fungal cultures as an assay organism. It is apparently more sensitive to most antifungal antibiotics than are many other test fungi; inhibition zones are usually more sharply defined; it grows rapidly and requires only overnight incubation; it produces a more homogeneous growth in assay plates than do most fungi; and spore suspensions for inocula are easily prepared and are stable for long periods.

Spore suspensions of *M. ramannianus* for seeding assay plates were prepared by first cultivating the organism on malt extract-agar (Haynes, Wickerham, and Hesseltine, 1955) slants at 25 C for 10 days. Slant surfaces then were flooded with physiological NaCl solution, and the spores were dislodged by scraping the agar surfaces with a sterilized loop. After suspensions were filtered through sterile hospital gauze to remove any large fragments, they were diluted further with sterile physiological saline to give a reading of 50 in a Lumetron colorimeter (red filter, 650 mμ). Standardized spore suspensions were dispensed in sterile screw-capped bottles, each containing about 36 glass beads. Such suspensions remained viable for over 2 years when stored in a refrigerator.

All assays were conducted by a paper-disc technique, with the use of a layer of seeded agar as described by Lindenfelser and Pridham (1962). The medium employed was Mucor synthetic agar (MSA) (Pridham et al., 1956) containing 1% agar. Assay discs [0.5 in. (1.27 cm); Carl Schleicher & Schuell Co., Keene, N.H.] were saturated with culture filtrate and applied to the seeded agar. Assay discs saturated with methanol extracts of mycelia were allowed to dry for at least 2 hr at room temperature before being placed on the agar. All assay dishes were incubated overnight at 30 C, and inhibition zones were measured on

an antibiotic zone reader (Fisher-Lilly; Fisher Scientific Co., St. Louis, Mo.).

Ultraviolet absorption studies. Ultraviolet absorption spectra for classification of polyene antibiotics were employed by several workers (Ball et al., 1957; Oroshnik et al., 1955; Pledger and Lechevalier, 1956; Utahara et al., 1954; Vining et al., 1955). To determine the practicality of this test for our work, a number of culture liquors from known polyene and nonpolyene antibiotic-producing cultures were analyzed with a Cary model 14 recording spectrophotometer. In these trial tests, both culture filtrates and methanol extracts of mycelia produced ultraviolet absorption maxima characteristic of the particular antibiotics present in the samples.

In screening culture liquors from unidentified streptomycetes, however, in a number of instances polyene peaks occurred with methanol extracts of mycelia, but no comparable peaks were obtained with the corresponding filtrates. In such cases, the filtrates were extracted with 1-butanol to concentrate the antibiotics, and the extracts were examined spectrophotometrically. Each time, polyene peaks were obtained.

Ultraviolet absorption maxima were obtained even when minute amounts of polyenic antibiotics were present. Much less antibiotic was required to exhibit ultraviolet absorption than to cause inhibition of fungi. For example, a methanol extract containing an antifungal polyene gave no inhibition zones at dilutions greater than 1:40, but clearly demonstrated ultraviolet absorption peaks in dilutions as high as 1:1,280. Impurities in the crude samples apparently did not interfere; absorption maxima were sharp and polyene entities were easily recognized.

All samples were diluted before analysis with a spectrophotometer. Culture filtrates were diluted with distilled water, and the extracts were diluted with the solvent employed in the extractions. Dilutions of 1:40 usually were satisfactory, but some of the polyene-containing samples required further dilution, in some instances, as high as 1:160. Absorption spectra of the samples were obtained over the wavelength range of 220 to 440 mμ. Culture liquors containing polyenes were classified as to the polyenic nature of the antibiotic (e.g., pentaene and hexaene). Absorption maxima used as criteria for classification of polyenes were: tetraenes, 290 to 291, 303 to 306, and 318 to 320; pentaenes, 318 to 324, 333 to 338, and 346 to 358; hexaenes, 339 to 341, 356 to 358, and 377 to 380; and heptaenes, 358 to 365, 376 to 380, and 399 to 405. Polyene-producing strains were eliminated at this point; the nonpolyene-producing strains were retained for stability studies.

Stability studies. One objective of this screening project was to discover antibiotics that were heat-stable. Therefore, each active nonpolyenic culture filtrate was divided into four portions; portions were adjusted to pH 5, 7, and 9, and one was left unadjusted as a control. The pH of unadjusted filtrates ranged from 5 to 8.5; the majority were between 6.5 and 7.5. Each sample then was further divided into three additional parts. One of these parts was autoclaved for 20 min at 121.5 C, the second was subjected to flowing steam in an Arnold sterilizer for 10 min, and the third was untreated. The heated specimens were cooled immediately after removal from the autoclave or steamer and were assayed.

Culture filtrates were classified on the basis of antibiotic stability after assaying by the paper-disc technique. Those retaining their activities were classed as "stable"; filtrates that retained partial activity were considered as "moderately stable"; and those that lost most, or all, activity were designated "unstable."

Paper-strip chromatography and cross-antagonism tests. All antibiotic-containing culture liquors that, as tested, were nonpolyenic and stable were chromatographed on paper to determine whether any of the antibiotics were new. Lyophilized culture filtrates were reconstituted with distilled water to 5× concentrations based on the total solids content of the original culture filtrates. Such concentration minimized the volume of material applied to paper strips. Each reconstituted filtrate was run in eight solvent systems, and the paper chromatographic results were compared with those of known antibiotics. Where similarities occurred, admixtures were run in selected solvent systems.

Paper chromatography was carried out by the conventional descending technique, with strips [0.5 by 18.5 in. (1.27 by 46.9 cm)] of Whatman no. 1 filter paper. Liquid preparations were applied to the strips with micro pipettes in quantities of 2 to 80 μliters. Not more than 10 μliters were applied at any one time; spots were dried between applications with warm air from an electric hair dryer. Strips were equilibrated for 1 hr in sealed jars before addition of solvents. Solvent systems employed were: (i) water-saturated 1-butanol; (ii) water-saturated 1-butanol containing 2% *p*-toluene sulfonic acid; (iii) water-saturated 1-butanol containing 2% *p*-toluene sulfonic acid and 2% piperidine; (iv) 1-butanol-saturated water; (v) water-saturated methylisobutyl ketone containing 2% *p*-toluene sulfonic acid; (vi) water-saturated methylisobutyl ketone; (vii) water-saturated methylisobutyl ketone containing 2% piperidine; and (viii) aqueous 10% NH_4Cl. Developed strips dried at room temperature were placed on trays of MSA seeded with *M. ramannianus*. After 1 hr, the strips were removed from the agar surfaces. Inhibition zones usually were visible after overnight incubation, but a 24- to 36-hr period was better if trays were to be photographed for permanent records. Reproductions of bioautographs were made with a Polaroid Land camera. Identification of antifungal-antibiotic factors was based principally on paper chromatographic data.

As an aid in presumptive identification of an antifungal agent, cross-antagonism and antibacterial spectra were determined for each promising strain. These tests were conducted by the method of Lyons and Pridham (1962).

The data obtained were compared with those available for producers of known antibiotics, and were interpreted in view of known relationships of certain antibacterial antibiotics with antifungal agents.

RESULTS

Results of the three-step screening procedure as applied to 500 streptomycetes are summarized in Tables 2 to 5. The numbers of polyenes and nonpolyenes found in the culture filtrates and mycelial extracts as determined from ultraviolet absorption spectra were classified by activity groups (Table 2). Also classified by activity groups were the types of polyenes represented (Table 3). Of the 74 polyenes, 2 tetraenes, 26 pentaenes, 15 hexaenes, and 24 heptaenes were detected as apparent single entities. Also, one tetraene-hexaene, one tetraene-heptaene, and five pentaene-heptaene combinations produced by single strains were detected. Results of stability tests are illustrated in Table 4. Table 5 summarizes the results obtained by the examination of 500 cultures through the various steps in the screening procedure. Paper chromatographic and cross-antagonism data revealed that, of the 15 stable nonpolyenic antibiotics obtained from the original 500 cultures, 7 were possibly new, and 2 of these were identical. Of the remaining eight, two were identified as cycloheximide and musarin, and six are still under study. The highest number of presumed new antibiotics was five in group I; groups II and III contained only one each.

TABLE 2. *Summary of screening and ultraviolet absorption studies*

Activity group*	No. of polyene-producing strains detected	No. of nonpolyene-producing strains detected	Total no. of strains
I	46	58	104
II	20	40	60
III	8	68	76
Nonactive	—	—	260
Total	74	166	500

* Activity groups were classified by diameters of inhibition zones when assayed against *Mucor ramannianus* NRRL 1839: I, >25 mm; II, 20 to 25 mm; and III, trace to 20 mm.

TABLE 3. *Classification of polyenes in activity groups*

Polyene type	Activity group*		
	I	II	III
Tetraene	2	0	0
Pentaene	8	12	6
Hexaene	12	3	0
Heptaene	17	5	2
Tetraene-hexaene	1	0	0
Tetraene-heptaene	1	0	0
Pentaene-heptaene	5	0	0
Total	46	20	8

* Same classification as in Table 2.

TABLE 4. *Stability of nonpolyenic antibiotics*

Activity group*	Total tested	No. stable	No. moderately stable	No. unstable
I	62	11	28	23
II	40	3	19	18
III	64	1	23	40
Total	166	15	70	81

* Same classification as in Table 2.

DISCUSSION

Apparently, in an antifungal screening program, the greatest immediate potential lies in the group of highest activity, that is, culture liquors producing inhibition zones of 25 mm or more. Five of the presumed new antibiotics were in this category.

In the chromatography of culture liquors, difficulty was encountered because of what is believed to be a contaminating substance in the filter paper. The contaminant may have been chemically similar to that described by Dubois et al. (1956). During irrigation of blank strips, the substance moved with the solvent fronts, and produced distinct zones of inhibition on the bioautographs at that point. The "artifact" zones were most frequently encountered with methylisobutyl ketone solvent systems and, to a lesser degree, with the water-saturated 1-butanol combinations and the 10% aqueous NH_4Cl. The contaminant was difficultly soluble, because it was not entirely removed by irrigation of the strips with water, numerous organic solvents, and aqueous solutions of inorganic compounds. The eluate collected during prolonged irrigations contained this inhibitory substance. Although extensive tests were not made, the substance was primarily antifungal because the inhibition phenomenon was not observed with the bacteria or yeasts we normally employ as test organisms. Inasmuch as the artifact zones appeared only sporadically, unspotted strips were run through all steps, as controls, along with those containing the antibiotic preparations.

Cultures found to produce polyenic antibiotics were not discarded. Because some strains could yield mixtures of polyenic and nonpolyenic agents, such mixtures would

TABLE 5. *Summary of results from screening of 500 streptomycete cultures*

Classification of culture liquors	Activity group*			Total, all groups
	I	II	III	
Active	104	60	76	240
Nonpolyenic	58	40	68	166
Stable, nonpolyenic	11	3	1	15
Containing possibly new antibiotics	5	1	1	7

* Same classification as in Table 2.

not be apparent from ultraviolet absorption spectra. Chromatographic study of these materials with appropriate solvent systems might indicate the presence of polyene-nonpolyene combinations.

Also, detection spectrophotometrically of a single type of polyene in a given filtrate or extract does not necessarily indicate the presence of a single entity only. For example, ultraviolet absorption spectra of the candicidins, ascosin, and other known polyenes yield only heptaene peaks, whereas it was demonstrated that samples of some of these materials contain more than one component (Craveri et al., 1960). In a number of the samples examined, inflections in the ultraviolet spectral curves suggest that there may be polyenic types present in addition to the major components. This point would require verification through further chromatographic and chemical studies. So far, the criteria employed in this screening project (e.g., ultraviolet absorption spectra of crude filtrates and extracts) allowed a quicker selection of promising strains and eliminated much labor in early phases of the work.

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LITERATURE CITED

BALL, S., C. J. BESSELL, AND A. MORTIMER. 1957. The production of polyenic antibiotics by soil streptomycetes. J. Gen. Microbiol. **17**:96–103.

CRAVERI, R., O. L. SHOTWELL, R. G. DWORSCHACK, T. G. PRIDHAM, AND R. W. JACKSON. 1960. Antibiotics against plant disease. VII. The antifungal heptaene component (F-17-C) produced by *Streptomyces cinnamomeus* f. *azacoluta*. Antibiot. Chemotherapy **10**:430–439.

DUBOIS, M., K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.

HAYNES, W. C., L. J. WICKERHAM, AND C. W. HESSELTINE. 1955. Maintenance of cultures of industrially important microorganisms. Appl. Microbiol. **3**:361–368.

LINDENFELSER, L. A., AND T. G. PRIDHAM. 1962 Natural variation of *Streptomyces viridochromogenes* (Krainsky) Waksman et Henrici. Develop. Ind. Microbiol. **3**:245–256.

LYONS, A. J., JR., AND T. G. PRIDHAM. 1962. Proposal to designate strain ATCC 3004 (IMRU 3004) as the neotype strain of *Streptomyces albus* (Rossi-Doria) Waksman and Henrici. J. Bacteriol. **83**:370–380.

OROSHNIK, W., L. C. VINING, A. D. MEBANE, AND W. A. TABER. 1955. Polyene antibiotics. Science **121**:147–149.

PLEDGER, R. A., AND H. LECHEVALIER. 1956. Survey of the production of polyenic substances by soil streptomycetes. Antibiot. Ann. 1955–1956, p. 249–254.

PRIDHAM, T. G., L. A. LINDENFELSER, O. L. SHOTWELL, F. H. STODOLA, R. G. BENEDICT, C. FOLEY, R. W. JACKSON, W. J. ZAUMEYER, W. H. PRESTON, JR., AND J. W. MITCHELL. 1956. Antibiotics against plant disease. I. Laboratory and greenhouse survey. Phytopathology **46**:568–575.

PRIDHAM, T. G., P. ANDERSON, C. FOLEY, L. A. LINDENFELSER, C. W. HESSELTINE, AND R. G. BENEDICT. 1957. A selection of media for maintenance and taxonomic study of *Streptomyces*. Antibiot. Ann. 1956–1957, p. 947–953.

ŘEHÁČEK, Z. 1964. Classification of nonpolyenic antifungal antibiotics produced by actinomycetes. Antimicrobial Agents and Chemotherapy—1963, p. 530–540.

UTAHARA, R., Y. OKAMI, S. NAKAMURA, AND H. UMEZAWA. 1954. On a new antifungal substance, Mediocidin, and other antifungal substances of *Streptomyces* with three characteristic absorption maxima. J. Antibiotics (Tokyo) Ser. A **7**:120–124.

VINING, L. C., W. A. TABER, AND F. J. GREGORY. 1955. The candidin-candicidin group of antifungal antibiotics. Antibiot. Ann. 1954–1955, p. 980–987.

WARREN, H. B., JR., J. F. PROKOP, AND W. E. GRUNDY. 1955. Nonsynthetic media for antibiotic producing actinomycetes. Antibiot. Chemotherapy **5**:6–12.