The Production of Polyenic Antibiotics by Soil Streptomycetes

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SUMMARY: Sixty *Streptomyces* isolates which produce polyene antibiotics were isolated from 25 soil samples collected in different parts of the world. Of these isolates 25 produced tetraenes, 23 produced pentaenes, 1 produced a hexaene, and 15 produced heptaenes. One isolate produced simultaneously a tetraene and a pentaene, another produced a tetraene and a heptaene, and a third produced a tetraene, a pentaene and a heptaene. The antibiotics showed activity against a wide range of fungi; the minimum inhibitory concentrations against *Saccharomyces cerevisiae*, *Aspergillus niger*, *Mucor racemosus*, *Candida albicans*, *Fusarium culmorum* and *Trichophyton mentagrophytes* are given for 10 of them. The distribution of activity between culture filtrate and mycelium is given for 14 of the antibiotics.

Hazen & Brown (1951) described the isolation of an antifungal antibiotic (fungicidin) from a *Streptomyces* sp. It was active against a wide range of yeasts and other fungi, but not against bacteria. The ultraviolet (u.v.) absorption spectrum of the antibiotic in ethanol showed sharp maxima at 290, 305 and 320 m μ .; the frequency differences between these absorption peaks is characteristic of a series of conjugated double bonds, and the chromophoric grouping of the compound is of polyene type. Many other antibiotics of similar properties have been described in recent years in publications from the U.S.A. or Japan, and they may be grouped together on the basis of certain general properties:

- (1) They inhibit the growth of a wide range of fungi, including yeasts, but are inactive against bacteria.
- (2) They show a relatively high toxicity when injected into animals, but are much less toxic when given by mouth.
- (3) They are of low solubility in water but dissolve more readily in aqueous solutions of the lower alcohols, and are easily soluble in aqueous pyridine.
- (4) They exhibit characteristic ultraviolet absorption spectra typical of polyenic chromophores.

These properties are fairly general for the whole series of compounds, although there are some wide differences, particularly in their biological properties. Several workers have classified the polyenic antifungal antibiotics into a number of different groups according to the nature of their ultraviolet absorption spectra (Vining, Taber & Gregory, 1955; Utahara, Okami, Nakamura & Umezawa, 1954; Oroshnik, Vining, Mebane & Taber, 1955; Pledger & Lechevalier, 1956), and we have used a similar method of classification in a screening programme for antifungal antibiotics produced by various *Streptomyces* spp. The scheme we have used is shown in Table 1, together with the

Table 1. *The classification of polyene antibiotics according to their ultraviolet absorptions*

Amphotericins A and B: Vandeputte, Wachtel & Stiller (1956); antibiotic 1968: Oswald, Reedy & Randall (1956); antimycin: Raubitschek, Acker & Waksman (1952); ascosin: Hickey, Corum, Hidy, Cohen, Nager & Kropp (1952); candicidin: Lechevalier, Acker, Corke, Haenseler & Waksman (1953); candidin: Taber, Vining & Waksman (1954); candimycin: Shibata, Honjo, Tokui & Nakazawa (1954); chromin: Wakaki, Hamada, Akanabe & Asahina (1953); eurocidin: Okami, Wahara, Nakamura & Umezawa (1954); filipin: Whitfield, Brock, Amman, Gottlieb & Carter (1955); flavacid: Takahashi (1953); fradycin: Swart, Romano & Waksman (1950); fungichromatin: Tytell, McCarthy, Fisher, Bolhofer & Charney (1955); fungichromin: Tytell *et al.* (1955); fungicidin: Hazen & Brown (1951); mediocidin: Okami *et al.* (1954); rimocidin: Davisson, Tanner, Finlay & Solomons (1951); trichomycin: Hosoya, Komatsu, Soeda & Sonoda (1952).

Group no.	Polyene type	Absorption maxima (m μ)	Published antibiotics
1	Tetraene	290, 305, 318	Fungicidin, antimycin, rimocidin, chromin, amphotericin A
2A	Pentaene	318, 333, 351	Fungichromatin, eurocidin
2B	Pentaene	325, 340, 358	Fungichromin, filipin
3	Hexaene	340, 356, 378	Mediocidin, flavacid, fradycin
4	Heptaene	360, 378, 405	Ascosin, candicidin, candidin, trichomycin, candimycin, amphotericin, B, antibiotic 1968

recorded antibiotics placed in their respective classes. During 1952–3 we carried out a screening programme for antifungal antibiotics that might be used clinically and found a wide range of polyene antibiotics from micro-organisms derived from soil samples collected in different parts of the world. The details of our methods and a selection of our results are given below.

METHODS

Pure cultures of the *Streptomyces* spp. were isolated from agar soil dilution plates. Four media were generally used for each soil—Bennett's, potato-peptone, yeast extract and paraffin agar. The compositions of these media were:

(1) Bennett's agar medium: NZ amine (enzymic casein digest from Sheffield Chemical Co. Inc., U.S.A.) 0.2 g., Difco beef extract 0.1 g., Difco yeast extract 0.1 g., glucose 1 g., agar 1.5 g., distilled water to 100 ml.; pH adjusted to 7.8 ± 0.1 .

(2) Potato-peptone agar medium: aqueous extract from 20 g. of over-cooked potatoes in 100 ml. of water 50 ml., peptone 0.5 g., dipotassium monohydrogen phosphate 0.2 g., sodium chloride 0.2 g., ferrous sulphate heptahydrate 0.001 g., agar 1.5 g., distilled water to 100 ml.; pH adjusted to 6.4 ± 0.1 .

(3) Yeast extract agar medium: Difco yeast extract 1 g., Evans peptone 1 g., agar 1.5 g., distilled water to 100 ml.; pH adjusted to 7.7 ± 0.1 .

(4) Paraffin agar medium: ammonium nitrate 0.4 g., liquid paraffin B.P. 0.1 g., dipotassium monohydrogen phosphate 0.6 g., potassium dihydrogen phosphate 0.2 g., zinc sulphate heptahydrate 0.0049 g., ferrous sulphate

heptahydrate 0.00054 g., manganous chloride tetrahydrate 0.0046 g., copper sulphate pentahydrate 0.00025 g., sodium borate 0.000094 g., ammonium molybdate 0.00002 g., agar 1.5 g., distilled water to 100 ml.; pH adjusted to 7.0 ± 0.1 . The medium was kept hot to melt the agar and was vigorously stirred throughout the pouring of the plates.

The medium which was successful in producing the largest number of *Streptomyces* colonies varied with the soil population, but paraffin agar proved to be selective for *Streptomyces* spp. and sometimes permitted their isolation from soils containing a high content of spreading bacteria and fungi. The number of colonies isolated from any one soil varied from about 30 to 200/g. soil.

A primary screening for antifungal activity was carried out by an agar disc method. The *Streptomyces* isolates were grown, in small aluminium cups with loose fitting caps, on medium 1, 2 or 3 above. After 5 days of incubation at 28°, cork borer plugs of the grown cultures were transferred to test plates seeded with *Saccharomyces cerevisiae* and *Aspergillus niger*. The plates were incubated overnight at 37° and the *Streptomyces* which showed good inhibition zones were selected for investigation of their ability to produce antibiotics in submerged aerated culture. In this secondary screening, 2 ml. portions of a 48 hr. shake-flask culture in glycerol meat broth were used to inoculate duplicate flasks of 6 different media distributed in 40 ml. quantities in 250 ml. conical flasks. These flasks were incubated on a reciprocating shaker for 5 days at 28° and were sampled aseptically at daily intervals for assessment of their antibiotic content by a cup plate method using as test organism *Saccharomyces cerevisiae* NCTC 4614 in 2% (w/v) glucose nutrient agar with 8 mm. diameter punched holes. When conditions had been found whereby a culture fluid gave an inhibition zone of more than 20 mm. overall diameter, six 250 ml. flasks containing 40 ml. medium were grown for extraction purposes. The six cultures were bulked and filtered without addition of a filter aid. The separated mycelium was extracted with 2×100 ml. 80% (v/v) acetone in water, a stirring time of 15 min. being allowed for each extraction. The acetone was removed from the extract by concentration to small bulk (5 ml.) *in vacuo*; 20 ml. *n*-butanol was added to the aqueous extract and the water was removed by further concentration *in vacuo* to 5 ml. The antibiotic present in this extract was precipitated by the addition of 6 vol. diethyl ether and the resulting solid isolated by centrifugation, washed with ether to remove traces of butanol and dried *in vacuo* at 40° for 1 hr. The bulked culture filtrate was extracted with 2×100 ml. *n*-butanol, the combined butanol solutions were concentrated to 100 ml. *in vacuo*, and the solid was isolated as above. The solid preparations were examined in aqueous solution or in 40% (v/v) pyridine in water for antibiotic activity, but ethanolic solutions were used for measurement of the ultraviolet absorption spectra in a Unicam model SP. 500 spectrophotometer. We found it satisfactory to take the absorption spectra at the butanolic concentrate stage, for the impure nature of the samples at this stage did not preclude recognition of the polyene entities, since sharp maxima were observed. It was necessary to dilute the solutions before

Table 2. *Distribution in soils of streptomycete isolates which produce polyene antibiotics*

Sample no.	Soil location	No. of polyene producing isolates	Type of polyene produced				
			Group 1	Group 2A	Group 2B	Group 3	Group 4
1	Argentina	3	2	3	.	.	1
2		1	.	.	.	1	
3		1	1	.	.	.	
4		2	2	.	.	1	
5		2	.	.	.	2	
6		1	.	.	1	.	
7		4	1	3	.	.	
8		2	.	2	.	.	
9	Brazil	1	.	.	.	1	
10	Burma	9	.	9	.	.	
11	Ceylon	1	.	.	.	1	
12		1	1	.	.	.	
13	England	1	1	.	.	.	
14	India	4	1	.	.	3	
15	Iraq	3	1	.	.	2	
16	Kenya	1	.	.	.	1	
17	Malaya	1	.	1	.	.	
18	Nigeria	1	.	.	1	.	
19		1	.	.	1	.	
20		2	2	.	.	.	
21		13	13	.	.	.	
22	Pakistan	2	.	.	.	2	
23	Sierra Leone	1	.	1	.	.	
24		1	.	.	1	.	
25		1	.	1	.	.	
Totals		60	25	20	3	1	15

Table 3. *Minimum inhibitory concentrations of different polyene-antibiotic preparations against 6 fungi*

Test organisms: *Saccharomyces* (S.) *cerevisiae*, *Aspergillus* (A.) *niger*, *Mucor* (M.) *racemosus*, *Candida* (C.) *albicans*, *Fusarium* (F.) *culmorum*, *Trichophyton* (T.) *mentagrophytes*.

Anti-biotic no.	Polyene group	Estimated purity by ultraviolet absorption (%)	Test organism					
			<i>S. cerevisiae</i>	<i>A. niger</i>	<i>M. racemosus</i>	<i>C. albicans</i>	<i>F. culmorum</i>	<i>T. mentagrophytes</i>
			Minimum inhibitory concentration at 48 hr. (μg/ml.)					
A. 432	1	20-30	1.2	4.4	4.4	3.2	2.2	11.6
A. 228	2A	20-30	3.4	4.4	5.0	4.2	6.75	4.5
A. 688	2A	20-30	1.4	8.9	5.8	> 3.25	8.9	9.75
A. 695	2A	20-30	2.2	20.0	2.2	2.2	6.6	20.0
A. 789	2A	20-30	1.5	4.4	2.9	6.6	5.3	12.0
A. 246	2B	80-90	0.66	0.66	4.4	2.2	2.2	4.4
A. 772	2B	20-30	2.96	4.44	10.0	4.44	2.96	4.44
A. 583	4	40-50	0.10	18.4	0.54	0.16	18.4	14.8
A. 570	4	20-30	0.48	16.0	5.3	1.43	16.0	16.0
A. 571	4	20-30	1.5	18.4	5.4	3.6	14.2	15.8

measurement; ethanol was used, as the absorption spectra did not vary greatly with solvent and the addition of ethanol gave clear solutions.

The antibiotic contents of the culture filtrates and mycelial extract samples were estimated by an agar cup-plate assay with *Saccharomyces cerevisiae* as the test organism. For the first extract of each antibiotic a sample of culture filtrate was used as an arbitrary assay standard. Usually enough solid was isolated from this extraction to enable all subsequent work on the antibiotic to be assayed in terms of the solid preparation in 40 % (v/v) pyridine in water. The minimum inhibitory concentrations against six selected fungi were determined by duplicate serial two-thirds or one-third dilutions in 2 % glucose broth at pH 6.0: 48 hr. broth cultures diluted 1/100 were used as inocula and dilution tubes were incubated at 28° for 48 hr. The test organisms used were *Saccharomyces cerevisiae*, *Aspergillus niger*, *Mucor racemosus*, *Candida albicans*, *Fusarium culmorum* and *Trichophyton mentagrophytes*.

RESULTS

In 25 soil samples from various localities 60 polyene antibiotic-producing isolates were found. One isolate produced simultaneously antibiotics in groups 1 and 2A, another produced antibiotics in groups 1 and 4, and a third isolate produced antibiotics in groups 1, 2A and 4. Three isolates produced antibiotics which were not of a polyene nature. In two of these the antibiotic was identified as cycloheximide (Whiffen, Bohonos & Emerson, 1946) but the third one was not characterized. The locations of the soil samples and the polyene-producing isolates obtained therefrom are given in Table 2, together with the types of polyene they produce. A selection of some results for the minimal inhibitory concentrations of the antibiotics against 6 fungi and yeasts is given in Table 3.

The relative amounts of the antibiotics contained in the mycelia and culture filtrates of the organisms grown in different media were determined by plate assay against *Saccharomyces cerevisiae*. Whole cultures and culture filtrates were assayed directly, and the mycelia were extracted exhaustively with 80 % (v/v) acetone in water, the acetone being then removed *in vacuo* before dilution and addition to the plates. Some of the results are shown in Table 4.

Antibiotics of group 1 assayed as whole culture always gave an accurate assessment of the total antibiotic present, that is, all the antibiotic was available for assay purposes. This was not true of antibiotics in the other groups, where the figures obtained by whole culture assay were always lower than the amount of antibiotic activity recovered by extraction. In these cases part of the antibiotic is held in a bound form by the mycelium and is not available for assay purposes.

DISCUSSION

It seems from our results that there is a wide distribution throughout the world of streptomycetes which produce polyene antibiotics, though we can draw no conclusions about their abundance. The distribution of polyenes found by us differs markedly from that described by Pledger & Lechevalier (1956),

but this is not surprising in view of the limited range of soils they examined. Further, they stated that their method of screening favoured the selection of *Streptomyces griseus* strains, a limitation that does not apply to our methods of isolation. We think it noteworthy that most antifungal antibiotics revealed by our screening programme are of a polyene nature. The ratio of polyene antibiotics to non-polyene types found was 20 to 1.

Table 4. *Distribution of activity of polyene antibiotics between culture filtrates and mycelia*

Antibiotic no.	Polyene type	Activity (%)	
		Culture filtrate	Mycelium
A. 284	1	50	50
A. 288	1	31	69
A. 387	1	0	100
A. 432	1	7	93
A. 862	1	67	33
A. 228	2A	55	45
A. 341	2A	78	22
A. 786	2A	57	43
A. 789	2A	24	76
A. 246	2B	40	60
A. 772	2B	47	53
A. 4	4	40	60
A. 571	4	7	93
A. 583	4	40	60

There does not appear to be any correlation of polyene type with geographical location, although the number of soil samples investigated is too small for drawing any firm conclusions. We consider any such relationship rather improbable in view of the general kind of distribution we have found. Further, the fact that one isolate can produce more than one type of polyene would appear inconsistent with any such relationship, even though all the isolates which produced more than one polyene originated in the Argentine.

The results obtained with the limited number of test organisms used show that there are small differences between antibiotics in the same group and that members of groups 1, 2A and 2B cannot be distinguished by the test organisms used. Group 4 antibiotics differ markedly from those of groups 1, 2A and 2B by their relative inactivity against three of the four filamentous fungi and their high activity against yeasts. In the *Saccharomyces cerevisiae* plate assay it was noted that antibiotics of groups 1, 2A and 2B gave satisfactory dose-response curves (2.5 mm. diam. for doubling dilutions) and sharp inhibition zones. Antibiotics of group 4 and the one group 3 antibiotic gave poor dose-response curves (about 1.5 mm. for doubling dilutions) and zones with diffuse edges. A point of distinction between antibiotics of group 1 and those of the other groups was in the reliability of the whole culture assay values. Whereas the group 1 antibiotics always gave reliable results when the cultures were assayed on plates, the members of the other groups generally gave by this assay figures well below the true antibiotic contents of the cultures, as determined by extractions. However, there was no relationship between the type

of polyene and the distribution of activity between culture filtrate and mycelium. The widely differing values for the proportions of antibiotic in culture filtrates and mycelia, and in particular the high filtrate contents, are somewhat surprising, since the pure antibiotics have low solubilities in water. It would have appeared reasonable to expect accumulations of the antibiotics in the mycelia, but this did not occur, and there must be active solubilizing agents present in culture media used.

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