AZIRINOMYCIN. I
MICROBIAL PRODUCTION AND BIOLOGICAL CHARACTERISTICS

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A new antibiotic, azirinomycin, has been discovered in the culture broth of newly isolated strains of actinomycetes. It was produced by submerged culture in shaken Erlenmeyer flasks in complex organic media. Azirinomycin and its methyl ester were found to exhibit broad spectrum antibiotic activity, *in vitro*, against both Gram-positive and Gram-negative bacteria. Both azirinomycin and its methyl ester were toxic to mice, and failed to protect them against lethal bacterial infections.

Azirinomycin, a new antibiotic, has been found in the culture broth of newly isolated strains of actinomycetes. The original isolate from soil found to produce it has been identified as a strain of *Streptomyces aureus*. It was produced by submerged culture in shaken Erlenmeyer flasks in complex organic media. It exhibits activity against both Gram-positive and Gram-negative bacteria in agar diffusion assays.

This report will present biological data relevant to a description of the antibiotic, its preparation by fermentation, and the nature of producing microorganisms. Purification and chemical identification of azirinomycin as 3-methyl-2-(2H)azirinecarboxylic acid are reported by MILLER, *et al.*[1]. Although the antibiotic is unstable in solution, especially when purified, it was possible to determine biological activities of freshly-prepared solutions. Biological charcteristics were also determined for the more stable, chemically-prepared, methyl ester.

Materials and Methods

Taxonomic Studies

The cultural characteristics of the organism which produces azirinomycin were determined by the use of the media and methods described by WAKSMAN[7], PRIDHAM, HESSELTINE and BENEDICT[3], PRIDHAM and GOTTLIEB[2], and the International Committee on Bacterial Nomenclature[6].

Fermentation Studies

The antibiotic was produced in submerged culture in 2-liter baffled Erlenmeyer flasks which contained 350 ml of medium and were incubated for 3~4 days on a rotary shaker

(145 rpm) at 28°C. The fermentation flasks were inoculated with 2.5% of vegetative growth. The inoculum was developed for 24~48 hours at 28°C in shaken 250 ml baffled Erlenmeyer flasks containing 50 ml of a yeast autolysate dextrose broth inoculated with a suspension of cells scraped from the surface of an agar slant culture.

In Vitro Studies

The antibiotic potency of broth or purified samples of azirinomycin was determined by disc-plate, agar-diffusion, assays in nutrient agar supplemented with 0.2% yeast extract and seeded with a suspension of cells of *Proteus vulgaris* MB-838 as described previously[5]. A slope of approximately 4.5 to 5.0 was observed in these assays with the samples diluted with distilled water.

The biological characteristics of azirinomycin were investigated by the use of standardized antibacterial spectrum and cross-resistance assays performed as previously described[4]. These tests were sufficient to distinguish it from known antibiotic compounds. Final identification of the antibiotic was achieved by extraction and chemical characterization as described by MILLER, *et al.*[1].

The presence of antibiotic on paper-electrophoresis strips was determined by bioautography with the use of thin-agar plates prepared in the same manner as the assay plates. Paperstrip electrophoresis was performed in a refrigerated unit operated for 2.5 hours at 600 volts with 0.165 M phosphate buffer at pH 7.0 on strips of Schleicher and Schuell SS-598 filter paper, 52 cm in length.

In Vivo Studies

For the *in vivo* tests female white swiss mice weighing from 19 to 21 g, were infected intraperitoneally with 5~50 LD_{50} of the test organism and were treated by the same route at the time of infection and again 6 hours later. Five mice were treated with each of the three or four concentrations of antibiotic used, and controls for the virulence of the test organisms and the toxicity of the antibiotic were included in each test. All untreated control mice died within 36 hours of infection. Surviving mice were observed daily over a period of 7 days, at which time the test was considered complete.

Results and Discussion

Taxonomy

Taxonomic studies of actinomycete culture MA-2941 revealed that it was a *Streptomyces* which formed sporophores with compact spirals as side branches of the mycelium. Spores were spherical to oval averaging 0.9 μ in diameter to 0.9 μ in width by 1.2 μ in length. The culture grew well at 28°C, moderately at 37°C, but did not grow at 50°C. These characteristics, plus those reported in Tables 1 and 2 were compared with those of known species, as described by WAKSMAN[7] and presented in BERGEY'S Manual of Determinative Bacteriology (7th Edition). Good agreement with these descriptions of *Streptomyces aureus* was found with the exception that a brown, soluble pigment was observed in our culture on some synthetic, as well as organic, media. Under our test conditions there was no coagulation but complete peptonization of milk and no reduction of nitrate to nitrite. The significance of these differences is questionable on the basis of the differences in descriptions in the cited references. On the basis that the differences observed are not sufficient to justify a new species, we have named our culture *Streptomyces aureus* strain MA-2941.

Fermentation

The presence of azirinomycin in the fermentation broth samples was determined by disc-plate assay against *P. vulgaris* MB-838. The response in this assay was linear

Table 1. Cultural characteristics of *Streptomyces aureus* MA-2941—Producer of azirinomycin

Medium	Vegetative growth	Aerial mycelium	Soluble pigment	Observations
Tomato paste-oatmeal agar	Flat, brown	Moderate-beige center with cream edging	Brown	
Glycerol-asparagine agar	Tan to brown	Very sparse-whitish	Dark brown	
CZAPEK-DOX agar	Tan to brown	Very sparse-whitish	Dark brown	
Egg albumen agar	Tan Reverse-orange	Velvety, cream-colored, moderate	Tan	
Synthetic starch agar	Flat, dark brown	Moderate-mostly along edge of growth, velvety, pinkish beige	Brown	
Nutrient starch agar plates	Brown	None	Brown	Hydrolysis of starch-good
Skim milk agar	Good, flat, dark brown	Sparse, whitish	Brown	Hydrolysis of casein
Nutrient tyrosine agar	Good, flat, dark brown	None	Brown	Tyrosine decomposed
Calcium malate agar	Cream, flat, spreading	None	None	
Peptone-iron-yeast extract agar	Dark grey	None	Dark brown to black	Melanin positive, H_2S produced
Potato plug	Good, wrinkled brown	None	Dark brown	
LOEFFLER's blood serum slant	Moderate, grey	None	Dark grey	No liquefaction
Skim milk	Dark brown ring	Sparse, whitish	Dark brown	No coagulation; complete peptonization; pH 6.45 (Control pH 6.20)
Litmus milk	Heavy dark brown ring	Sparse, whitish	Dark brown	No coagulation; complete peptonization; pH 6.2 (Control pH 6.5)
Microaerophilic growth (Yeast extract-dextrose stabs-40 mm depths)	Dark brown	Moderate; greyish white	Dark brown	Growth on surface-no growth along stab line; aerobic
Gelatin stab	Flaky brown vegetative growth settling to bottom of tube	None	Dark greenish-brown	Complete liquefaction
Nutrient gelatin agar plates	Dark brown	None	Brown	Liquefaction-good

Table 2. Carbohydrate utilization by *Streptomyces aureus* MA-2941*

Compound	Growth	Compound	Growth	Compound	Growth
Cellulose	−	Lactose	+	Raffinose	+
Glucose	+	Malate	−	Rhamnose	+
Arabinose	+	Maltose	+	Sucrose	+
Fructose	+	Mannose	+	Xylose	+
Inositol	+	Mannitol	+		

* Growth tests performed in PRIDHAM-GOTTLIEB Basal Synthetic Medium plus 1% carbohydrate, incubated for 3 weeks at 28℃.
Symbols: +=utilization; −=no utilization.

when plotted as a function of the log of the drug concentration over a range from 100~1,000 micrograms per ml. The most active preparation of azirinomycin, estimated to be 50 % pure on the basis of chemical analysis, prepared by extraction and purification, produced a zone of 25 mm diameter at a concentration of 600~700 mcg/ml. The inhibition zone diameter for four fermentations averaged 27 mm which corresponds to 400 μg/ml of antibiotic.

Table 3. Media used in fermentation for azirinomycin

Medium	Composition	
Agar slant medium	Distiller's solubles	40.0 g
	Dextrose	20.0 g
	Agar	25.0 g
	Distilled water	1,000 ml
	pH 7.0	
Inoculum medium	Ardamine (Yeast Products Co.)	10.0 g
	Dextrose	10.0 g
	Phosphate buffer*	2 ml
	$MgSO_4 \cdot 7H_2O$	0.05 g
	Distilled water	1,000 ml
	pH : adjust to 6.5	
Fermentation medium	Tomato paste	20.0 g
	Oatmeal (Gerber's)	20.0 g
	Distilled water	1,000 ml

* Phosphate buffer solution : KH_2PO_4 91.0 g, Na_2HPO_4 95.0 g, distilled water 1,000 ml.

Media utilized for various stages of culture handling and production of azirinomycin are detailed in Table 3. In the usual experiments, initial inoculum was a slant culture which was used to inoculate a primary seed medium flask; this was incubated on the shaker for 1 day at 28°C. Second stage seed flasks were inoculated from the first stage, shaken for 1 day at 28°C, and used to inoculate production flasks. Production fermentations were performed in the tomato paste oatmeal medium under conditions outlined in methods. Activity was monitored by the disc-plate assay during fermentation and the fermentation broth was normally harvested after 3 or 4 days of incubation.

Antimicrobial Activity

The antibacterial spectrum of azirinomycin was determined by the agar diffusion method. Results obtained with the acid and chemically-prepared methyl ester of azirinomycin are presented in Table 4. Tests performed against 15 cultures, representing 13 genera, revealed 10 of these organisms representing 8 genera, to be sensitive to azirinomycin. It is of interest that the spectrum of activity includes both Gram-positive and Gram-negative microorganisms and even extends to a strain of *Pseudomonas aeruginosa* which is resistant to a wide variety of antibiotics. The methyl ester of azirinomycin demonstrated an even wider spectrum of antimicrobial activity since it inhibited all of the 15 strains tested. On the basis of these data it is clear that both azirinomycin acid, the natural product, and the chemically-prepared azirinomycin methyl ester are broad spectrum antibacterial agents. However, the antibacterial spectra of these two agents are quite different. The acid is most active versus *Staphylococcus aureus* followed by *Proteus vulgaris*, *Bacillus subtilis* and *Streptococcus faecalis*. In contrast, the methyl ester shows its lowest activities against one of the *Staphylococcus aureus* cultures and *Streptococcus faecalis*.

The pattern of cross-resistance of azirinomycin and its methyl ester was investigated with a series of *E. coli* strains resistant *in vitro* to a variety of antibiotics (Table 5). These data are useful for the evaluation of the characteristics of the antibiotic and for use in differentiation of one antibiotic from another. Since these

Table 4. Antibacterial spectrum of azirinomycin

Test organism	Inhibition zone (diameter, mm)*	
	Acid 0.3 mg/ml	Methyl ester 10 mg/ml
Escherichia coli MB-60	11	27
Bacillus sp. MB-633	15	32
Proteus vulgaris MB-1012	19	22
Pseudomonas aeruginosa MB-979	13	27
Serratia marcescens MB-252	7	28
Staphylococcus aureus MB-108	24	23
Bacillus subtilis MB-964	19	29
Sarcina lutea MB-1108	14	24
Staphylococcus aureus MB-698	24	16
Streptococcus faecalis MB-753	19	14
Alcaligenes faecalis MB-10	7	24
Brucella bronchiseptica MB-965	7	29
Salmonella gallinarum MB-1287	13	29
Vibrio percolans MB-1272	7	29
Xanthomonas vesicatoria MB-815	7	25

* Assays were performed with 7 mm filter paper discs on nutrient agar +0.2% yeast extract medium.

Table 5. Cross-resistance spectrum of azirinomycin

Escherichia coli strain*	Inhibition zone (diameter, mm)**	
	Acid 0.3 mg/ml	Methyl ester 10 mg/ml
Susceptible parent culture (MB-60)	11	27
Streptomycin-resistant	13	31
Streptothricin-resistant	16	28
D-Cycloserine-resistant	13	28
Pleocidin-resistant	16	31
Chloramphenicol-reistant	14	30
Chlortetracycline-resistant	13	37
Oxytetracycline-resistant	18	34
Neomycin-resistant	17	20
Tetracycline-resistant	12	17
Viomycin-resistant	7	28
Polymyxin-resistant	15	25
Grisein-resistant	14	27

* Tests were performed against a series of *E. coli* strains isolated from the same parent culture after exposure to the individual antibiotics.
** Assays were performed with 7 mm paper discs on nutrient agar +0.2% yeast extract medium.

Table 6. Special effects spectrum of azirinomycin

Escherichia coli MB-60 with addition noted	Inhibition zone (diameter, mm)*	
	Acid 0.3 mg/ml	Methyl ester 10 mg/ml
Control (Nutrient agar +0.2% yeast extract)	11	27
0.1 M Phosphate buffer, pH 5.0	24	62
0.1 M Phosphate buffer, pH 7.0	7	23
0.1 M Phosphate buffer, pH 9.0	7	14
Blood plasma, 20%	7	11
Cation-exchange resin, 1% (Dow ET91)	14	49

* Assays were performed with 7 mm filter paper discs.

resistant cultures are prepared *in vitro*, the interrelationships do not necessarily have any significance for *in vivo* tests of antibiotics. In general, both the acid and methyl ester are broadly active against all the *E. coli* strains tested. The acid appears to be cross-resistant with viomycin, while the methyl ester is cross-resistant with neomycin and tetracycline. Differences in antibacterial spectrum and cross-resistance response between the acid and the methyl ester might be expected on the basis of differences in stability and solubility characteristics of the two substances.

The data on antibacterial activity and cross-resistance spectrum for both azirinomycin acid and azirinomycin methyl ester have been compared for profile of response in a computer comparison program. The results of these tests demonstrated that azirinomycin was distinct from all antibiotics previously tested in our laboratories. This procedure for comparison of azirinomycin with previously observed antibiotics was facilitated by the inclusion of information presented in Table 6. These data indicate that both the acid and the methyl ester are considerably more active at acidic pH and are somewhat less active in the presence of 20% blood plasma. The addition of a soluble cation-exchange resin to assay media results in some increase in activity. This pattern of results has been quite frequently observed with acidic antibiotics. In addition to the foregoing data, one further, very interesting, characteristic of the acid was its rapid movement in paper strip electrophoresis. This result might be expected, in

retrospect, since the compound is a small acidic molecule. Broth samples and partially purified preparations of azirinomycin were normally found to move approximately 12 cm under the conditions of electrophoresis employed. This rate of movement by electrophoresis is very unusual and has been observed, in our laboratory, with only one other antibiotic compound (phosphonomycin).

In Vivo Activity

Sodium salt was tested in mice by scheduling the fermentation, extraction, and testing to proceed without delay to avoid the problems caused by the chemical instability of azirinomycin. A 50 % pure sample of azirinomycin was toxic when 2 intraperitoneal injections, of 6 mg each, were given 6 hours apart. Two doses of 3 mg each were tolerated, but this level failed to protect mice against lethal infections of *Staphylococcus aureus* or *Proteus vulgaris*.

The methyl ester was found to be toxic at 5 mg per dose and failed to protect mice against a lethal *Proteus vulgaris* infection at non-toxic levels. The urine of mice dosed with 3 mg of ester was examined by thin-layer chromatography and bioassay. Neither the ester nor the free acid was detected. This result is taken to indicate that either the substance is degraded *in vivo* or rapid excretion does not occur.

Failure of azirinomycin to protect against infection at non-toxic levels may be a reflection of its reduced activity at neutral pH and in the presence of blood plasma.

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