TWO ANTIBIOTICS (LAVENDULIN AND ACTINORUBIN) PRODUCED BY ACTINOMYCES

I. Isolation and Characteristics of the Organisms[1]

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Two strains of *Actinomyces* showing antibacterial properties of special interest were isolated from greenhouse soil during a search for antibiotics effective against gram-negative and acid-fast microorganisms. One strain, designated as A-10, appears to be a variant of *Actinomyces lavendulae*. Although a similarity to streptothricin (Waksman, 1945) was noted in the spectrum obtained from limited antibacterial tests in which the unpurified product of A-10 was used, the culture differed so much in appearance, nutritional requirements, and other respects from *A. lavendulae*, a known producer of streptothricin, as to merit further study. More extensive antibacterial tests, performed later with the purified antibiotic, showed it to be distinct from streptothricin. We have named it *lavendulin*.

The second strain of *Actinomyces* was found to produce an antibacterial substance resembling streptothricin and streptomycin but distinct from either of these. This strain of *Actinomyces*, designated as A-105, resembles in some respects *Actinomyces erythreus*, *A. fradii*, *A. albosporeus*, and *A. californicus* without being unmistakably one, or a variant of any, of these species. We have named the antibiotic produced by strain A-105 *actinorubin* because of the characteristic red mycelium which the organism forms on many media.

This paper describes the methods by which the strains of *Actinomyces* were isolated, their cultural characteristics, the cultural conditions which were observed to yield maximum production of the antibiotic substances, and their antibacterial properties *in vitro*. The methods for the chemical purification of lavendulin and actinorubin will be described elsewhere by Junowicz-Kocholaty and Kocholaty. The chemotherapeutic studies on lavendulin and actinorubin will be published by one of us (Morton, 1947).

Methods used in isolating from nature organisms which inhibit bacteria. The methods used for isolating and testing A-10, A-105, and A-82, the latter an organism producing an antismegmatis factor (Kelner and Morton, 1946), were similar. Our procedures were adapted from those described by Waksman, Bugie, and Schatz (1944) and in general resembled those described by Emerson *et al.* (1946). The actinomycetes were isolated from greenhouse soil (a rich and convenient source) by plating the soil in nutrient agar and selecting colonies

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of actinomycetes for isolation. The selection of colonies of actinomycetes was made entirely at random, no attempt being made to pick colonies differing from one another in appearance, nor to favor colonies which were inhibiting other soil organisms growing in the same plate. We assumed that different strains of the same species of *Actinomyces* differed in antibiotic potentialities and that colonies apparently inactive on the primary isolation plate might be active producers of antibiotics after prolonged incubation. Preliminary enrichment of the soil with dead or living bacteria was not found necessary, nor was the soil plated out on washed agar containing living bacteria as the sole nutrient. This confirmed the findings of Waksman and Schatz (1946), who showed such techniques to be of little value. The preliminary screening of the isolates for antibiotic activity was made by growing them on plain nutrient agar for 4 to 8 days at room temperature, after which their action against *Escherichia coli*, and sometimes *Mycobacterium smegmatis*, was determined by streaking the bacteria on the same plate and observing zones of inhibition.

Actinomycetes markedly inhibiting one or both bacteria were regrown, and tested this time against eight species, *Staphylococcus aureus*, *E. coli*, *Eberthella typhosa*, *M. smegmatis*, *Neisseria catarrhalis*, *Bacillus mycoides*, and *Bacillus subtilis*. This limited spectrum was selected as likely to indicate the possibility of previously unknown antibiotics. Actinomycetes that it seemed desirable to study further were grown in fluid media at 28 C. This was a criterion used in the screening process, for the many organisms which showed great activity on agar but produced little or no antibiotic in fluid media were considered to be of no practical value. The cultures were first grown in three representative fluid media—nutrient broth, nutrient broth plus glucose, and starch tryptone broth. For stationary cultures the media were made semisolid with 0.25 per cent agar. Isolates failing to form antagonistic substances in one of the media of at least 20 dilution units per ml against *Escherichia coli* or *Mycobacterium smegmatis* were discarded, i.e., a 1:20 dilution of the *Actinomyces* culture in agar failed to inhibit the growth of these two microorganisms. The spectrum of the more promising antagonists was redetermined, using the agar dilution assay method. The relative sensitivities of the eight bacteria were usually similar to those obtained in the first spectrum, but often marked differences appeared. By systematic variation of the best of the three media it was often, but not always, possible to increase the yield 2 to 20 times. No chemical purification of the antagonistic substance was attempted unless it was possible to obtain crude filtrates assaying at least 100 dilution units per ml of agar against *E. coli*. The majority of antagonists isolated yielded filtrates assaying no more than 20 or 40 dilution units, and unless the titer was easily raised, these were abandoned.

The cultural characteristics of the *Actinomyces* cultures, and such properties of the antibiotic as the detailed spectrum, stability to heat, acid, and alkali, relation of activity to pH, salts, etc., yielded additional evidence bearing on the novelty of a crude antibiotic. It was recognized, however, that definitive chemical analyses of the purified antibiotics was also necessary in determining their newness.

One of the tests used in evaluating a crude antibiotic was its toxicity when injected intraperitoneally into mice in 1- or 2-ml portions. It was realized that the amount of active substance present in the injected material might be too small to produce evidence of an inherent toxicity. Similarly, with an impure preparation an observed toxicity could not be attributed with certainty to the active principle. Nevertheless the experience of Fleming (1929), who demonstrated the low toxicity of penicillin before its chemical purification, indicated the usefulness of the test.

Mice tolerated sterile culture media such as the three afore-mentioned basic media used for actinomycetes, as well as glucose yeast-extract peptone broth, Czapek, and Raulin's medium (pH 4). Media containing corn steep were toxic. Filtrates of cultures of *Actinomyces antibioticus* (crude actinomycin) and *Penicillium flexuosum* (probably crude clavacin) killed mice. The great majority of the filtrates of strains of *Actinomyces*, which inhibited *E. coli* or *M. smegmatis* at a dilution of 1:20 or 1:40, did not kill mice. Toxic filtrates were not common. When a choice had to be made between antibiotics, both equally promising in other respects, the toxicity was the determining factor. Several cultures were discarded on this basis.

All assays at this stage were done by the agar dilution method, in Difco nutrient agar of final pH 7.3. The growth in the assay agar of some organisms, such as A-105 which developed at 37 C, was suppressed by adding crystal violet in the final concentration of 1:1,000,000 to the nutrient agar, which did not alter the end point but did facilitate reading of the plates. Crystal violet agar was also useful for the assaying of nonsterile filtrates from bacilli and aspergilli.

Cultural requirements of strain A-10. Strain A-10 cannot be grown for more than a few generations on glucose asparagine agar, a medium on which a streptothricin-producing *A. lavendulae*, which we have examined, grows well. On a richer medium, such as nutrient agar, which supported its growth, sporulation of A-10 was poor. A search was made, therefore, for a medium rich enough to permit growth and to stimulate sporulation of *Actinomyces* A-10. Tryptone starch agar fulfilled these requirements; it was further enriched without sacrifice of its sporulation-stimulating properties. The final medium (M-130) contained 5 g tryptone, 10 g starch, 3 g Difco beef extract, 2 g glucose, 1.2 g Na_2HPO_4, 0.8 g KH_2PO_4, 0.1 g $FeSO_4 \cdot 7H_2O$, and 15 g agar. The ingredients were dissolved in 1,000 ml potato extract made by simmering 400 g white potato slices for 40 minutes in 1,000 ml water (distilled water, or preferably, spring water). The decanted liquid is used.[3] Not only A-10 but most of the 200 strains of *Actinomyces* isolated during the investigation grew and sporulated well on this medium.

Media for the production of lavendulin. Titers of 200 to 400 dilution units against *E. coli* were formed by A-10 when grown in stationary culture at 28 C

[3] In at least two cases potato extract was also useful as a substitute for corn steep in media for the production of antibiotics. Either fresh potato extract or corn steep stimulated antibiotic production by a *Bacillus* (H-40), and either fresh or dried potato extract (kindly prepared for us by the Difco Laboratories) or corn steep stimulated antibiotic production by an *Actinomyces* (R-13).

on a medium containing 5 g Difco peptone, 3 g Difco beef extract, 10 g glucose, 20 ml molasses ("Brer Rabbit," green label), 2.5 g agar, and 1,000 ml distilled water; pH 6.0. The peptone could not be replaced by tryptone without reduction in the yield of antibiotic. A detailed study of nutritional requirements for stationary cultures was not made. The maximum titer is reached about the sixth day, at which time the pH is about 5.6. The medium at this time is covered by a thick, gray, partially submerged pellicle.

In shake cultures a study was made of the effect of beef extract on the yield of antibiotic. Table 1 shows that in the presence of beef extract the yield is reduced; the medium also becomes much more acid, suggesting that growth factors or salts contained in the beef extract affect the type of carbohydrate metabolism.

TABLE 1

Effect of varying the composition of the culture medium on the production of lavendulin

MEDIUM	DAYS	2	3	4
M-120*	pH†	4.5	4.5	4.2
	Activity‡	40	20	
M-10.6§	pH	8.2	8.2	8.2
	Activity	200	100	40
M-10.0‖	pH	5.8	7.4	7.8
	Activity	200	200	200 (400)
M-10.5¶	pH	7.4	7.4	7.4
	Activity	400 (1,000)	400 (1,000)	1,000

* M-120—5 g Difco peptone, 3 g Difco beef extract, and 10 g glucose.

† The pH of the culture at the time of assay.

‡ Activity expressed in dilution units per ml of agar against *E. coli*. The figures in parentheses indicate almost complete inhibition of *E. coli* at the dilution indicated.

§ M-10.6—M-120 without beef extract.

‖ M-10.0—5 g Difco peptone, 3 g Difco beef extract, 10 g glucose, and 20 ml molasses.

¶ M-10.5—M-10.0 without beef extract.

Our best medium for the production of antibiotic is one composed of 5 g Difco peptone, 10 g glucose, and 20 ml molasses in 1,000 ml distilled water. Titers of 1,000 dilution units against *E. coli* have been obtained consistently.

Cultural characteristics of Actinomyces, strain A-105. Strain A-105 is characterized by ability to grow well on Czapek agar, by lack of soluble pigment, by failure to blacken peptone media; by a yellowish to intense red vegetative mycelium and by white to pink aerial mycelium. Oval conidia are borne in chains, and spirals are abundant in many media. Optimum temperature for growth is 37 C; very poor growth was obtained at 15 C. Action in milk is variable; gelatin is liquefied. On nutrient agar containing 3 to 5 per cent glycerol, the mycelium was yellow to an intense fuchsinlike red color, especially on first isolation, and with no spores. The red mycelium is characteristic on calcium malate agar, on Czapek agar, and also on most media in the presence of a *Bacillus* contaminant.

Media for the production of actinorubin. Crude actinorubin for chemical study was produced by surface cultivation at 28 to 30 C on a medium composed of 5 g Difco tryptone, 1.2 g Na_2HPO_4, 0.8 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4 \cdot 7H_2O$, 10 g white sugar (commercial or purified sucrose), 2.5 g agar, and 1,000 ml distilled water; final pH was 6.8 to 7.2.

In surface culture, tryptone could be replaced, with somewhat reduced yields, by asparagine, "phytone," or dehydrated skim milk, but not by Difco peptone, neopeptone, nitrates, or gelatin. In the presence of Difco peptone, the medium became alkaline very much more quickly than with tryptone. This increase in alkalinity may be indirectly the cause of reduced antibiotic production. White sugar (sucrose) could be replaced by brown sugar, molasses, or lactose with an equal or a somewhat reduced yield of antibiotic. Starch and glycerol were unsatisfactory.

Unless iron was added to the medium, antibiotic production was very low, although growth was relatively unaffected. As little as 5 mg $FeSO_4 \cdot 7H_2O$ per liter of medium gave good yields of the antibiotic. Use of 20 mg $FeSO_4 \cdot 7H_2O$ decreased the yield slightly and caused the pH of the medium to fall to 5.6 to 6.0 within 7 days, as compared to pH 7.0 to 7.4 for media containing 10 mg $FeSO_4 \cdot 7H_2O$. Slight changes in the concentration of $MgSO_4$ and KCl had little effect.

Little or no antibiotic was produced in the white sugar tryptone medium (agar omitted) in shake culture, apparently because of very poor growth. Good yields were obtained, however, if 10 ml molasses ("Brer Rabbit," green label) per liter were substituted for the white sugar. Equal or somewhat reduced yields were also obtained if brown sugar, lactose, or starch was substituted. There was no antibiotic produced in plain nutrient broth. In a molasses tryptone medium containing only 25 per cent as much phosphate as usual, the yield was reduced by about one-half. The importance of the carbohydrate-nitrogen balance is indicated by rapid alkalinization and reduced yield when 10 instead of 5 g tryptone per liter of medium were used.

In surface cultivation on the white sugar tryptone medium, 200 to 400 dilution units per ml against *E. coli* were obtained in 8 to 10 days. The pH tended to rise to 7.2 to 7.5 in the first 3 to 5 days, slowly drop to 6.6 to 6.8 at about the tenth day, then slowly rise during the next week to about 7.8. In brown sugar and molasses media, the maximum production of antibiotic was reached in 5 to 6 days. The white sugar medium was preferred because of its greater simplicity and because the maximum titer, once reached, persisted longer.

Table 2 shows two experiments in shake culture in molasses tryptone medium at a temperature of 28 C, and illustrates the variation encountered. Yellow to salmon-colored, moderately coarse pellets were produced in shake culture in the molasses tryptone medium.

Bacterial spectrum of lavendulin and actinorubin. Partially purified preparations of the two antibiotics (Junowicz-Kocholaty and Kocholaty, to be published) were used for a comprehensive study of the action of lavendulin and actinorubin on a variety of bacterial cultures. Serial twofold dilutions of the active substances were made in Difco nutrient broth adjusted so as to have a reaction of pH 7.3 after sterilization. One loopful of the culture under test was

inoculated into each dilution tube and a control tube of the culture medium. Incubation of the tests was at 37 C for 18 to 20 hours. The antibiotics were standardized against *E. coli*, strain P216. The smallest amount of the antibiotic per ml of Bacto-nutrient broth, pH 7.3, which prevented growth of *E. coli* under the conditions of the test was designated as one dilution unit. The results of testing the two antibiotics against many strains of microorganisms are summarized in table 3.

From the data listed in table 3 it appears that, in general, actinorubin and lavendulin are similar in their antibacterial action against most strains of microorganisms. However, the differences in the susceptibilities of *Corynebacterium diphtheriae*, P1, *Micrococcus aurantiacus*, P103, and *Sarcina lutea*, P6, to the two antibiotics were greater than the anticipated experimental error.

At the time our tests were being conducted, Price, Nielsen, and Welch (1946) reported on the sensitivity of *Bacillus circulans* to streptomycin. We obtained a subculture of this strain of *B. circulans* from these authors and tested its sen-

TABLE 2

Production of actinorubin in shake culture with tryptone molasses medium

DAYS	1	2	3	4	5	6	7	8	9	16
pH*		7.0	7.0	7.2	7.8	8.0			8.0	
Activity†		400	400(1,000)	400(1,000)		200(400)			200(400)	
pH	6.8	7.0	7.4	7.8	7.8		7.8	8.0	7.8	
Activity	10	200(400)	100(200)	100	40(100)		40(100)	40	40	10(20)

* The pH of the culture at the time of assay.

† The dilution units per ml of agar determined against *E. coli* equal the inhibiting dilution. The figures in parentheses indicate almost complete inhibition of *E. coli* at the dilution indicated.

sitivity to streptomycin sulfate, lavendulin, and actinorubin in Difco nutrient broth, pH 7.3, by our technique of assaying. The sample of streptomycin sulfate was assayed against the strain of *E. coli* employed for standardization of lavendulin and actinorubin and under the same conditions employed for assaying these antibiotics. *B. circulans* was 32 times more resistant to actinorubin and 2.5 times more resistant to lavendulin than to streptomycin. Another member of the genus *Bacillus*, *B. megatherium*, P97, was tested against the three antibiotics under identical conditions. It was 16 times more resistant to actinorubin than to streptomycin. Its resistance to lavendulin and streptomycin was of the same order. From the results obtained by testing *B. circulans* and *B. megatherium* against streptomycin and actinorubin it appears that the two antibiotics are different.

In a personal communication from an investigator (Dr. E. J. Pulaski) working in another laboratory with many of our cultures, it was stated that *Chromobacterium violaceum*, P104, and *Serratia marcescens*, P4, were equally susceptible to streptomycin. The differences in susceptibility of these two cultures to actinorubin and lavendulin (table 3) suggest that both actinorubin and lavendulin are different from streptomycin.

TABLE 3

Bacterial spectrum of actinorubin and lavendulin

ORGANISM	STRAIN	NUMBER OF DILUTION UNITS/ML INHIBITING TEST ORGANISM*	
		Actinorubin	Lavendulin
Aerobacter aerogenes	P41	0.25	0.5
Alcaligenes faecalis	P61	2	4
Bacillus anthracis	P60	4	4
Bacillus anthracis	P119	2	8
Bacillus cereus	P109	32	64
Bacillus mesentericus	P112	0.5	0.5
Bacillus mycoides	P156	0.25	0.06
Bacillus mycoides	P33A	8	8
Bacillus mycoides, Waksman	P34A	16	32
Bacillus subtilis	P7	0.01	0.03
Bacillus subtilis, Merck	P23A	0.5	0.125
Bacillus subtilis, Koch	P219	8	16
Brucella abortus†	P18A, P62	4	16
Brucella melitensis†	P80	8	8
Brucella suis†	P64	16	32
Chromobacterium violaceum	P104	0.015	0.025
Corynebacterium diphtheriae	P1	0.015	4
Corynebacterium xerose	P83	0.004	0.007
Diplococcus pneumoniae‡	P27, P29	>128	128
Eberthella typhosa	P115	0.25	0.015
Eberthella typhosa, nonmotile	P11	0.5	1
Escherichia coli	P216	1	1
Escherichia communior	P218	0.5	1
Gaffkya tetragenae	P43	0.03	0.015
Klebsiella pneumoniae	P163A	0.125	0.03
Micrococcus aurantiacus	P103	0.007	0.000007
Mycobacterium smegmatis	P49	1	1
Mycobacterium tuberculosis, bovis§	Ravenel	8	6
Neisseria catarrhalis	P66	0.03	0.06
Proteus vulgaris	P98	1	2
Pseudomonas aeruginosa	P186A	32	128
Salmonella enteritidis	P51	0.25	0.25
Salmonella paratyphi	P198A	0.25	0.06
Salmonella schottmuelleri	P35	0.125	0.125
Sarcina lutea	P6	0.007	0.5
Serratia marcescens	P4	4	4
Shigella dysenteriae	P52A	1	2
Shigella paradysenteriae, Flexner	P21	1	2
Staphylococcus aureus	P210	0.06	0.015
Streptococcus pyogenes‡	P24	128	128
Streptococcus, alpha‡	P169	>128	>128
Streptococcus, gamma‡	P26	128	128
Trichophyton interdigitale		8	16
Vibrio comma	P215	0.5	0.25

* One dilution unit is the smallest amount of the antibiotic per ml of Difco nutrient broth, pH 7.3, which prevents growth of *E. coli* under the conditions of the test as described in the text.

† Difco tryptose broth. Incubation period, 48 hours.

‡ Difco nutrient broth, pH 7.3, to which were added 0.5 per cent NaCl and 5 per cent sterile, defibrinated, normal horse blood.

§ Test made using the medium described by Crumb (1946) and the technique described by Wells (1946).

In some earlier work we tested streptothricin and a streptothricinlike compound for their antibacterial spectra. It was found that *Corynebacterium xerose* was about as resistant as *E. coli* to streptothricin. The great susceptibility of this strain to actinorubin and lavendulin suggests that these two antibiotics are different from streptothricin.

Development of tolerance of E. coli to actinorubin, lavendulin, streptothricin, and streptomycin. *E. coli*, P216, was inhibited by 0.06 units of streptomycin per ml of Difco nutrient broth, pH 7.3, for 48 hours at 37 C. At 48-hour intervals a new series of tubes of nutrient broth containing serially decreasing amounts of streptomycin was inoculated with 0.1-ml amounts of the culture growing in the maximum amount of streptomycin in the previous series of tubes. In the second and fourth series there was a gradual increase in the resistance of *E. coli* to streptomycin, each series of cultures being about twice as resistant as the previous series. The fifth serial transfer of the culture in the presence of streptomycin showed an abrupt change in tolerance for the drug, there being about a 16-fold increase. The organisms in the eighth series were over 100 times more resistant than those in the fifth series. In the twelfth serial transfer *E. coli* grew nearly as well in the presence of 1,600 units of streptomycin per ml of nutrient broth as in the nutrient broth without streptomycin. This represented a more than 26,600-fold increase in resistance of the culture to streptomycin. When this streptomycin-fast strain of *E. coli* was tested against lavendulin, actinorubin, and streptothricin, it was found to be nearly as susceptible to the three drugs as was the normal culture of *E. coli*. In the case of streptothricin, growth of the streptomycin-fast strain took place one tube earlier in the series than did the normal strain, whereas in the case of lavendulin and actinorubin growth took place two tubes earlier in the series than did the normal culture. Although these results represent a 2-fold or 4-fold increase in resistance, they are within the range of experimental error. When growth of the streptomycin-fast strain occurred in any of the tubes, it was similar to that produced by the normal strain of *E. coli*. In these results, also, streptomycin differed from streptothricin, actinorubin, and lavendulin.

During 10 serial transfers of *E. coli*, P216, in Difco nutrient broth (pH 7.3) containing actinorubin by a technique described above for streptomycin, the culture developed a 64-fold increase in resistance to actinorubin. When this actinorubin-fast strain was tested against streptomycin, streptothricin, and lavendulin, it appeared to be slightly more resistant to all three compounds. The actinorubin-fast strain was 16 times more resistant to streptomycin and 32 times more resistant to streptothricin and lavendulin. Growth of the actinorubin-fast strain was about one-half as vigorous as that of the normal strain.

During 10 serial transfers of *E. coli*, P216, in Difco nutrient broth (pH 7.3) containing lavendulin by a technique described above for streptomycin, the culture developed a 32-fold increase in resistance to lavendulin. When this lavendulin-fast strain was tested against streptomycin, streptothricin, lavendulin, and actinorubin, it was found that the increase in resistance to each of the four antibiotics was of the same order. Growth of the lavendulin-fast strain was about one-half as vigorous as that of the normal strain.

During 10 serial transfers of *E. coli*, P216, in streptothricin (medium and technique as above), the culture developed only an 8-fold increase in resistance to the antibiotic. When the streptothricin-fast strain was tested against streptomycin, actinorubin, lavendulin, and streptothricin, it was found that the increase in resistance to each of the four antibiotics was of the same order. Growth of the streptothricin-fast strain was about one-half as vigorous as that of the normal strain.

From these studies on drug fastness of *E. coli* to streptomycin, streptothricin, actinorubin, and lavendulin, it is readily apparent that actinorubin and lavendulin are distinct from streptomycin. Differences between actinorubin, lavendulin, and streptothricin were not readily detected by this method of testing. The usefulness of this method of testing antibiotics was pointed out by Eisman, Marsh, and Mayer (1946).

Effect of NaCl and blood upon the activity of actinorubin and lavendulin. Antibacterial tests were made in Difco nutrient broth, pH 7.3, employing twofold dilutions of the antibiotics and *E. coli* as the test organism, as described previously. When NaCl was added to the medium to the concentration of 0.8 per cent, 128 times more actinorubin and 256 times more lavendulin were required to inhibit the test organism as compared to the amounts of the antibiotics required to inhibit the test organism in the absence of added NaCl. The addition of 10 per cent sterile, defibrinated horse blood to the Difco nutrient broth containing 0.8 per cent NaCl did not alter the activity of the antibiotics.

Preliminary tests of the action of actinorubin and lavendulin in mice were promising (Kelner, Kocholaty, Junowicz-Kocholaty, and Morton, 1946) and are being reported elsewhere (Morton, 1947).

SUMMARY

Two strains of *Actinomyces* have been isolated which produce substances that appear to be different from previously described antibiotics. One of these new substances has been named *actinorubin*, the other *lavendulin*.

A description of the two strains of *Actinomyces* is given as well as conditions of growth for maximum production of the antibiotic substances.

The bacterial spectrum of actinorubin and lavendulin is given. The two substances appear to be different from each other and from streptomycin and streptothricin.

Much greater resistance of *Escherichia coli* to streptomycin could be developed than resistance to actinorubin or lavendulin.

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