IDENTIFICATION OF PRODUCER AND BIOLOGICAL ACTIVITIES OF NEW ANTIBIOTICS, MIMOSAMYCIN AND CHLOROCARCINS

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A strain of *Streptomyces*, No. 314 identified as a *Streptomyces lavendulae* produced under a novel condition of culture, four new antibiotics, mimosamycin and chlorocarcins A, B, and C. Among the components of chlorocarcin complex, chlorocarcin A was found to be most biologically active. This antibiotic inhibited the growth of *Staphylococcus aureus* FDA 209P and *Corynebacterium diphtheriae* at the concentrations of 0.1 and 0.003 mcg/ml, respectively. Chlorocarcin A also exhibited antitumor activity on EHRLICH carcinoma, ascitic and solid forms, and mouse leukemia L1210. Mimosamycin proved to be mainly active on *Mycobacterium tuberculosis* and inactive on the experimental murine tumors.

As described in the preceding paper[1], the *Streptomyces* strain No. 314 produced, in addition to streptothricin, four new antibiotics, mimosamycin and chlorocarcins A, B, and C, which were determined by a more sensitive and rational antitumor antibiotic screening procedure. In this paper, identification of the producing strain and biological activities of the antibiotics are described.

Producing Strain

Classification studies of *Streptomyces* sp. No. 314

1. Morphological Characteristics

The strain No. 314 was grown on glucose-asparagine agar and inorganic salts-starch agar plates at 27°C for 7~14 days. Sporophores of the strain No. 314 are abundantly branched and form open loops (Section Retinaculiaperti, Plate 1).

Plate 1. Sporophores of strain No. 314 (Inorganic-salts starch agar, 27°C, 14 days) ×800

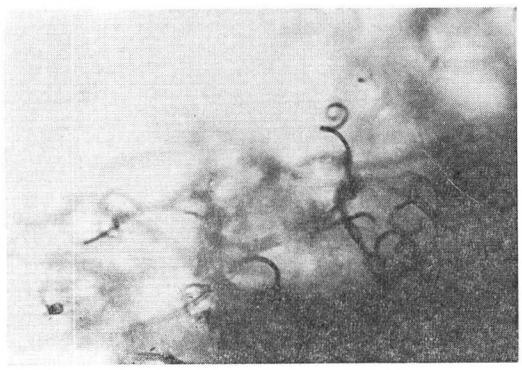


Plate 2. Spores of strain No. 314 (Oatmeal agar, 27°C, 14 days) The bar equals 1 μ.

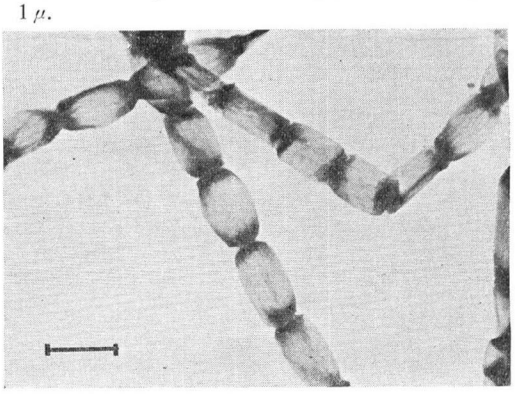


Table 1. Comparison of cultural characteristics of strain No. 314 and *S. lavendulae* ISP 5069 (IMRU-3440)

Medium	Strain No. 314	*S. lavendulae* ISP 5069 (IMRU-3440)
Sucrose-nitrate agar	VM abundant, spreading, light olive (1 1/2 ie)*	abundant, spreading, colorless to faint brown
	AM abundant, white to ivory (2 db) with lavender shade (5 ge to 4 ig)	abundant, white to ivory (2 db) with lavender shade (5 ge to 4 ig)
	SP none to faint brown	faint brown
Glucose-asparagine agar	VM abundant, spreading, colorless to light olive (1 1/2 ie)	abundant, spreading, grayish blue (10 pn)
	AM abundant, grayish pink (5 ec) with lavender shade (5 ge to 4 ig)	abundant, white later becoming to reddish gray with lavender shade (5 ge)
	SP none to faint brown	none to faint brown
Glycerol-asparagine agar	VM abundant, spreading, colorless	abundant, spreading, dark olive (1 1/2 pn)
	AM moderate, faint brownish gray (2 fe) to silver gray (3 fe)	moderate, faint brownish gray (2 fe) to silver gray (3 fe)
	SP none	light brown
Tyrosine agar	VM abundant, spreading, faint brown to mustard brown (2 ni)	abundant, spreading, faint brown to mustard brown (2 ni)
	AM abundant, reddish gray (7 ge) with lavender shade (5 ge to 4 ig)	abundant, reddish gray (7 ge) with lavender shade (5 ge to 4 ig)
	SP none to faint brown	none to faint brown
Calcium hydroxy-succinate agar	VM abundant, spreading, faint brown to dark olive (1 nl)	abundant, spreading, bluish brown (3 pn)
	AM moderate, thin, powderly, white later becoming to shell tint (3 ba)	none
	SP none to faint brown	faint olive brown
Inorganic salts-starch agar	VM moderate, colorless	moderate, colorless to black olive (2 pn)
	AM abundant, spreading, yellowish gray (3 dc)	abundant, spreading, yellowish gray (3 dc)
	SP none	none
Nutrient agar	VM abundant, spreading, glistening surface, camel (3 ie)	abundant, spreading, glistening surface, camel (3 ie)
	AM none	none
	SP brown	brown
Yeast extract-malt extract agar	VM abundant, spreading, much folded, colorless to faint brown	abundant, spreading, colorless to faint brown
	AM abundant, grayish pink (5 ec) with lavender shade (5 ge to 4 ig)	abundant, grayish pink (5 ec) with lavender shade (5 ge to 4 ig)
	SP oak brown (4 pi)	oak brown (4 pi)
Oatmeal agar	VM moderate, colorless	moderate, dusty blue (15 ni)
	AM moderate, grayish pink (5 ec) with lavender shade (5 ge to 4 ig)	moderate, rose wood with lavender shade (5 ge to 4 ig)
	SP none	none
Egg medium	VM spreading, much folded, chocolate brown (4 pn)	spreading, much folded, chocolate brown (5 pn)
	AM none	none
	SP faint brown to chocolate brown (4 pl)	faint brown to chocolate brown (4 pl)

VM: vegetative mycelium AM: aerial mycelium SP: soluble pigment (diffusible pigment)
* Color Harmony Manual code.

Spores on oatmeal agar were observed under an electron-microscope. Conidia are oval to cylindrical in shape, averaging 0.8~1.0 μ by 0.8~2.0 μ in size and the spore surface is smooth (Plate 2).

2. Cultural and Physiological Characteristics

The media used in this study were prepared according to the recommendations in "The Actinomycetes" by WAKSMAN[2] and those of the International Streptomyces Project (ISP)[3]. The growth on yeast extract-malt extract agar was used to inoculate each medium studied. Unless otherwise stated, all cultures were incubated at 27°C for 3 weeks. The cultural characteristics and physiological properties of the strain No. 314 are shown in Tables 1 and 2.

Table 2. Comparison of physiological properties of strain No. 314 and *S. lavendulae* ISP 5069 (IMRU-3440)

	Strain No. 314	*S. lavendulae* ISP 5069 (IMRU-3440)
Utilization of carbon source (27°C, 21 days)	+: D-glucose, L-arabinose, maltose, sucrose, Na-acetate, Na-citrate, Na-succinate	+: D-glucose, maltose, sucrose, Na-acetate, Na-citrate, Na-succinate
	±: D-fructose, D-xylose	±: D-fructose
	−: *i*-inositol, D-mannitol, L-rhamnose, L-lactose, raffinose	−: L-arabinose, *i*-inositol, D-mannitol, L-rhamnose, L-lactose, raffinose, D-xylose
Liquefaction of gelatin	positive (at 18°C, 21 days)	positive (at 18°C, 21 days) brown soluble pigment
Nitrate reduction	positive (at 27°C, 21 days)	positive (at 27°C, 21 days)
Milk peptonization and coagulation	positive (at 27°C, 21 days)	positive (at 27°C, 21 days)
Starch hydrolysis	positive (at 27°C, 21 days)	positive (at 27°C, 21 days)
Chromogenicity	brown pigment on peptone-yeast extract-iron agar and nutrient agar, slightly soluble brown pigment to tyrosine agar (ISP)	brown pigment on peptone-yeast extract-iron agar, nutrient agar, and tyrosine agar

+: utilization positive ±: utilization doubtful −: utilization negative

Some characteristics are: Color of mature spores is lavender (Red color series) on most synthetic agar media. Starch is hydrolyzed, nitrate is reduced to nitrite, and proteolytic activities are positive on gelatin and milk. Chromogenicity is doubtful on tyrosine agar, but positive on peptone-yeast extract-iron agar and on nutrient agar.

Strain No. 314 produced streptothricin complex in the culture filtrate. These characteristics were compared with those of known species described by WAKSMAN and ISP coworkers, and the strain No. 314 was found to resemble *S. lavendulae*. As a result of comparative studies of the strain No. 314 and *S. lavendulae* ISP 5069 (IMRU 3440) (Tables 1 and 2), it was found that the strain No. 314 differed from *S. lavendulae* in the utilization of L-arabinose, in the color of vegitative mycelium on glycerol asparagine agar and inorganic-salts starch agar, and in addition, in the production of the antibiotics, chlorocarcins and mimosamycin. These slight differences, however, are considered to be strain characteristics and insufficient to create a new species. As a result, strain No. 314 was identified as *Streptomyces lavendulae* WAKSMAN *et* HENRICI 1948.

Biological Activities

Antimicrobial Activity

Standard strains of bacteria and fungi from our laboratory were used for the present experiments. For the majority of gram-positive and negative bacteria, 0.5% glucose nutrient agar was used. Blood agar was used for *Streptococcus pyogenes*, *S. salivarius* and *Brucella abortus*. SABOURAUD's dextrose agar was employed for fungi and SAUTON's liquid medium for the strains of *Mycobacterium tuberculosis* and *M. bovis*.

Minimum inhibitory concentrations were determined after 24-hour incubation at 37°C for gram-positive and negative bacteria except for several species described below in which 2 or 3 days' incubation at 27°C for fungi was employed. The MICs for *Nocardia*, *Corynebacterium*, and *Lactobacillus* were determined after 2-day incubation and for *M. tuberculosis* and *M. bovis* were estimated after 3-week incubation at 37°C (Tables 3 and 4). Chlorocarcins A, B, and C were found mainly active on gram-positive bacteria. Among chlorocarcin components, chlorocarcin A was most active and inhibited *S. aureus* FDA 209P, *Staphylococcus albus*, *Sarcina lutea* and *Corynebacterium diphtheriae* at the concentrations of 0.1 mcg/ml, 0.1 mcg/ml, 0.05 mcg/ml, and 0.003 mcg/ml, respectively. Mimosamycin was also active on some gram-positive bacteria. However, mycobacteria were most sensitive to the compound, and the growth of both streptomycin-sensitive and resistant strains of *M. tuberculosis* was completely inhibited at the concentration of 3.125 mcg/ml. All the antibiotics are inactive on gram-negative bacteria and fungi.

Table. 3. Antimicrobial spectra of satellite antibiotics of *Streptomyces lavendulae* No. 314 (1)

Test organism	Mimosamycin (MIC, mcg/ml)
Staphylococcus aureus FDA 209 P	12.5
Staphylococcus albus	50.0
Staphylococcus citreus	25.0
Streptococcus faecalis	25.0
Sarcina lutea	12.5
Bacillus subtilis PCI 219	25.0
Corynebacterium diphtheriae	6.25
Corynebacterium xerosis	6.25
Lactobacillus arabinosus	100.0
Mycobacterium sp. 607	25.0
Mycobacterium avium	25.0
Mycobacterium tuberculosis H 37 Rv+	3.125
Mycobacterium tuberculosis Matsudo+	1.56
Mycobacterium tuberculosis SM-R+	1.56
Mycobacterium bovis 10+	6.25
Mycobacterium bovis BCG+	3.125
Nocardia asteroides	12.5
Escherichia coli F_1	>100.0
Salmonella typhimurium	>100.0
Pseudomonas aeruginosa	>100.0
Candida albicans 7 N	50.0
Saccharomyces cerevisiae	100.0
Aspergillus niger	>100.0
Aspergillus oryzae	>100.0
Penicillium glaucum	>100.0
Mucor mucedo	>100.0
Trichophyton mentagrophytes	>100.0

Agar dilution streak method
Medium: 0.5% glucose nutrient agar 37°C, 24 or 48 hours for bacteria.
1% glucose SABOURAUD agar 27°C, 48 or 72 hours for fungi.
+ SAUTON medium 37°C, 3 weeks.

Effect on Transplanted Animal Tumors

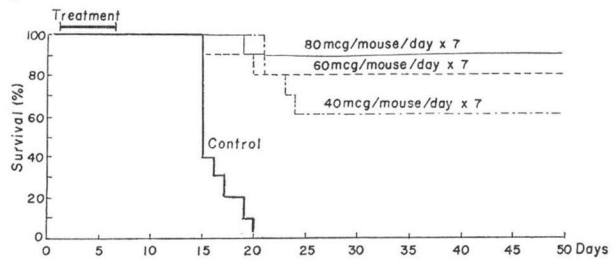
The effect of chlorocarcin A and mimosamycin on transplanted animal tumors was determined using EHRLICH carcinoma (ascitic and solid forms) and mouse leukemia L1210. With EHRLICH ascitic carcinoma, tumor cells (2×10^6) were inoculated intraperitoneally into each mouse of the ddy strain weighing 20~23 g. Daily intraperitoneal administration of the antibiotics was initiated 24 hours after the

Table 4. Antimicrobial spectra of satellite antibiotics of *Streptomyces lavendulae* No. 314 (2)

Test organism	MIC (mcg/ml)	
	Chlorocarcin A	Chlorocarcin B
Staphylococcus aureus FDA 209 P	0.1	12.5
Staphylococcus aureus Smith	0.02	12.5
Staphylococcus albus	0.1	50.0
Staphylococcus citreus	0.1	25.0
Streptococcus faecalis	25.0	>100.0
Streptococcus pyogenes Coock+	6.25	6.25
Streptococcus pyogenes 090 R+	50.0	100.0
Streptococcus salivarius+	6.25	50.0
Sarcina lutea	0.05	6.25
Bacillus subtilis PCI 219	0.1	25.0
Bacillus cereus	50.0	50.0
Corynebacterium diphtheriae	0.003	0.05
Corynebacterium xerosis	0.003	0.05
Mycobacterium sp. 607	100.0	>100.0
Mycobacterium phlei	100.0	>100.0
Mycobacterium avium	100.0	>100.0
Nocardia asteroides	25.0	50.0
Escherichia coli F_1	>100.0	>100.0
Salmonella typhimurium	>100.0	>100.0
Shigella dysenteriae Shiga	>100.0	>100.0
Klebsiella pneumoniae	100.0	50.0
Brucella abortus	>100.0	>100.0
Serratia marcescens S-32	>100.0	>100.0
Pseudomonas aeruginosa	>100.0	>100.0
Candida albicans 7 N	>100.0	>100.0
Saccharomyces cerevisiae	>100.0	>100.0
Rhodotorula glutinis	>100.0	>100.0
Aspergillus niger	>100.0	>100.0
Aspergillus oryzae	>100.0	>100.0
Penicillium glaucum	>100.0	>100.0
Mucor mucedo	>100.0	>100.0
Trichophyton mentagrophytes	>100.0	>100.0

Agar dilution streak method.
Medium: 0.5% glucose nutrient agar, 37°C, 24 or 48 hours for bacteria.
1% glucose SABOURAUD agar, 27°C, 48 or 72 hours for fungi.
+ Blood agar, 37°C, 24 hours.

Fig. 1. Effect of chlorocarcin A on EHRLICH ascites tumor



implantation and continued for 7 days. As shown in Fig. 1, chlorocarcin A showed a marked prolongation of life span at the daily dose of 2 mg/kg and higher. With 4 mg/kg, the ascites did not increase and 90 % of the treated mice were cured completely.

In the case of the solid form of EHRLICH carcinoma, 2×10^6 cells were implanted subcutaneously at right inguinal regions of mice. The mice were treated in the same way as ascitic form. All the mice were sacrificed 14 days after implantation, and the tumors were dissected out and weighed. The percent inhibition of tumor growth was assessed by measuring the tumor weight. The results are shown in Table 5. The inhibition was 62 % at the daily dose of 3 mg/kg. With 4 mg/kg, the tumor growth was further inhibited by 72 %. On the other hand, mimosamycin did not show any antitumor activities at a dose of 25 mg/kg.

Table 5. Effect of chlorocaricn A on EHRLICH solid tumor

Dose (mcg/mouse/day)	Number of injection	Survivors	Body weight change	Tumor weight test/control (%)
20	7	10/10	+ 9.8	65.1
40	7	10/10	+ 9.1	45.5
60	7	10/10	+ 9.1	38.0
80	7	10/10	+ 8.3	27.8
Control	7	10/10	+11.3	—

For preliminary test against L 1210 mouse leukemia, BDF_1 mice were inoculated with 1×10^5 tumor cells. Intraperitoneal injection of chlorocarcin A was started 24 hours after the implantation, and was continued for 12 days. With the daily dose of 3 mg/kg and 4 mg/kg, T/C values of 156 % and 162 % were obtained respectively.

Discussion

In addition to the production of streptothricin group antibiotics, the strain No. 314 produces chlorocarcins and mimosamycin. To date, a limited number of *Streptomycetes* which produce old and new types of streptothricin group antibiotics have been reported. They are *S. candidus* (LL-AB664[8]), *S. griseochromogenes* (SF-701[9]), *S. hygroscopicus* (E-749-C,[10] LL-AC541[11]), *S. microflavus* (streptothricin[12]), *S. lavendulae* (akimycin,[13] neothricin,[14] R-4H,[15] streptothricin,[16] yazumycin[17]), *S. olivoreticuli* (BD-12,[18] BY-81[18]), *S. racemochromogenes* (racemomycin,[19]) and *S. sclerogranulatus* (sclerothricin[20]).

Among these known species of producers, *S. lavendulae* and *S. racemochromogenes* resemble the strain No. 314 most closely. According to the description of *S. racemochromogenes*[19] and to our taxonomic studies (unpublished data), it seems that *S. racemochromogenes* is a synonym of or is very closely related to *S. lavendulae*. As a result of comparative studies of the strain No. 314 and *S. lavendulae* ISP 5069, we identified the strain No. 314 as *Streptomyces lavendulae*.

The results reported here also showed that, among these antibiotics, chlorocarcin A is the most bioactive. The antibiotic proved to be active on gram-positive bacteria. The only gram-negative bacteria, which is relatively sensitive to chlorocarcin A was found to be *Klebsiella pneumoniae*. Chlorocarcin A is also highly active on various types of murine tumors. Our preliminary experiment against L 1210 showed that chlorocarcin A has antitumor activity similar to or somewhat better than azaserine. As reported in the preceding paper, these antibiotics have a relatively low toxicity. Their high biological activity, therefore, seems to warrant further investigations for chemotherapeutic agents.

Acknowledgements

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