ISOLATION AND CHARACTERIZATION OF SATELLITE ANTIBIOTICS, MIMOSAMYCIN AND CHLOROCARCINS FROM *STREPTOMYCES LAVENDULAE*, STREPTOTHRICIN SOURCE

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Under novel conditions of culture, *Streptomyces lavendulae* No. 314 was found to produce new antibiotics, chlorocarcins and mimosamycin in addition to streptothricin. The antibiotics are extracted from culture filtrate with organic solvents and further purified by silica gel chromatography. Chlorocarcins A, B, and C are closely related basic antibiotics which are active against gram-positive bacteria, EHRLICH carcinoma and L 1210 leukemia of mice. Mimosamycin is a neutral antibiotic which is mainly active against mycobacteria. Production, isolation and physicochemical properties of the antibiotics are described.

As the new sources of streptomycetes and streptoverticillia starting to dwindle, the chances to find new antibiotics seem to have become increasingly poor, and a current screening techniques among streptomycetes often lead to known substances. On the other hand, the application of more sensitive and selective detection techniques has continued to provide new and interesting antibiotic substances. A sensitive and rational antitumor cell screening system was developed recently in our laboratory employing suspension culture of L 1210 murine leukemia cell line.[1] During the course of the antitumor cell screening using this system, the culture filtrate of a strain of streptomyces, designated as No. 314, was found to be cytocidal and antibacterial with activity against *Bacillus subtilis* and *Escherichia coli*. The major antibiotic produced by the strain in a submerged state under regular culture conditions proved to be a streptothricin complex[2] which apparently is devoid of antitumor activity. During the study of the detailed culture conditions for the production of satellite antibiotics of streptothricin, our group have isolated mimosamycin, an antibiotic which is mainly active on mycobacteria including streptomycin-resistant strains of human tubercle bacilli and three antitumor antibiotics, chlorocarcins A, B, and C. This paper describes the production of these antibiotics and their physicochemical characterization.

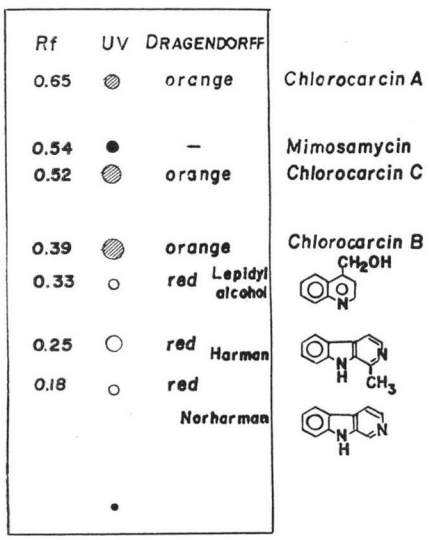
Antibiotic Production

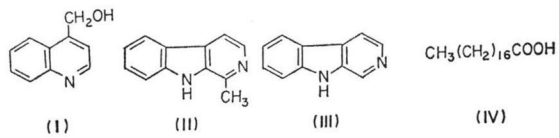
The organism used in this investigation was *Streptomyces* sp. No. 314 which was later identified as a strain of *Streptomyces lavendulae*.[3] The culture was maintained in the lyophilized state. A freshly grown slant on glucose-asparagine agar (KRAINSKY) was used for the inoculum of laboratory-scale antibiotic production. Laboratory fermentation was run in 500-ml shake

flasks (SAKAGUCHI flask) at 28°C on a 125-stroke reciprocal shaker with an 8-cm throw. One hundred ml of the medium of the following composition (g/liter): glucose, 1; soluble starch, 10; Polypeptone (Wako), 10; meat extract (Wako), 5; NaCl, 3; and Silicone KM72, 10 ml (pH 7.0) was placed in each shake flask. The fermentation was carried out at 27°C for 72 hours. The production of antibiotics in a pilot plant scale was carried out by growing the organism at 27°C in aerated stainless steel 20-liter jar fermenters. An inoculum of the culture No. 314 was prepared by growing the organisms in shake flasks for 24 hours in the medium described above. One per cent inoculum was used to seed 15-liters of the production medium which contained the following ingredients (g/liter): glucose, 5; soluble starch, 5; Polypeptone, 10; meat extract, 5; NaCl, 3 (pH 7.0~7.2).

The time course of the antibiotic production was followed during the fermentation by assaying samples of fermentation beer by an agar-diffusion or an agar-dilution method. *E. coli* F1 grown on nutrient agar was used for the assay of the streptothricin complex. For the assay of chlorocarcins or mimosamycin, 10 ml of the fermentation beer was extracted with an equal volume of ethyl acetate at pH 8.0. The solvent layer was washed with a small amount of phosphate buffer solution (pH 8.0) and concentrated *in vacuo* to dryness. The residue dissolved in 10 ml methanol was used for the analytical procedure. Test organisms were *B. subtilis* PCI 219 grown on nutrient agar for chlorocarcins and *Mycobacterium smegmatis* ATCC 607 grown on 3% glycerol agar for mimosamycin. Thin-layer chromatography studies were also run to separate the satellite antibiotics with the solvent mixture, acetone and chloroform (1:1). This was carried out using precoated glass plates (5×20 cm or 20×20 cm) with silica gel (Kieselgel $60F_{254}$, Merck) and spots were detected by UV light or spraying with DRAGENDORFF reagent. The antibiotics were readily detected by ultraviolet quenching or with DRAGENDORFF reagent. A thin-layer chromatogram of the basic components in the chloroform extract of a *S. lavendulae* culture filtrate is shown in Fig. 1. Chlorocarcins A, B, C and mimosamycin had the Rf values of 0.65, 0.39, 0.52 and 0.54, respectively. Other basic components produced by the organisms which had the Rf values of 0.33, 0.25 and 0.18, were also isolated and were identified as 4-hydroxymethylquinoline [I], 1-methyl-β-carboline (harman, **II**)[4,5] and β-carboline (norharman, **III**), respectively by direct comparison with authentic samples.

Fig. 1. TLC of basic components of $CHCl_3$ extract. Solvent system: Acetone-$CHCl_3$ 1:1





Time courses of the fermentation under two different conditions of agitation by jar

Fig. 2. Fermentation time course of *Streptomyces lavendulae* No. 314. Agitation: 250 rpm. Aeration: equal volume. Temperature: 27°C. Pressure: 0.5 kg/cm².

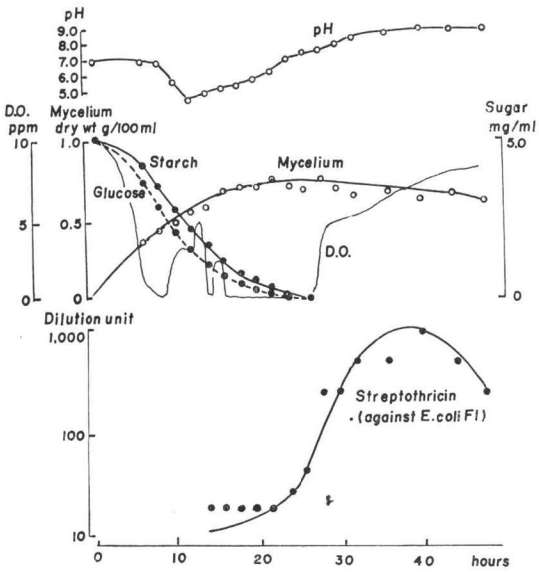
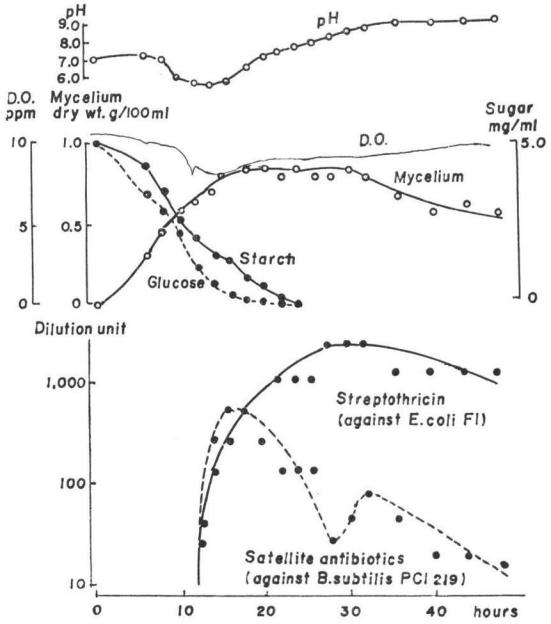


Fig. 3. Fermentation time course of *Streptomyces lavendulae* No. 314. Agitation: 550 rpm. Aeration: equal volume. Temperature: 27°C Pressure: 0.5 kg/cm².



fermenters are presented in Figs. 2 and 3. No significant difference in the drop of the pH and mycelial growth was observed between 250 rev/min and 550 rev/min of agitation. With 250 rev/min agitation, however, were observed a steep decrease in the concentration of dissolved oxygen and only streptothricin production which reached its maximum at 38 hours of fermentation and lasted as long as 48 hours. On the other hand, with 550 rev/min the concentration of dissolved oxygen did not decrease significantly and there was a rapid rise in the production of satellite antibiotics within 14 hours. Antibiotic production reached its maximum between 16~18 hour-fermentation, followed by a rapid decay. There was a second rise in the content of the solvent-extractable satellite antibiotics during the period of 30 to 38 hours.

Extraction and Purification of the Satellite Antibiotics

Eight hundred liters of a 20-hour culture of strain No. 314 produced under the aforementioned viogorously stirred culture conditions was filtered, adjusted to pH 8.0 with dilute sodium hydroxide and concentrated by a thin-film evaporator Hitachi-Contro Processer to 1/2 or 1/3 of the original volume. The concentrate was extracted with an equal amount of chloroform and the resulting solution was vacuum concentrated to dryness.

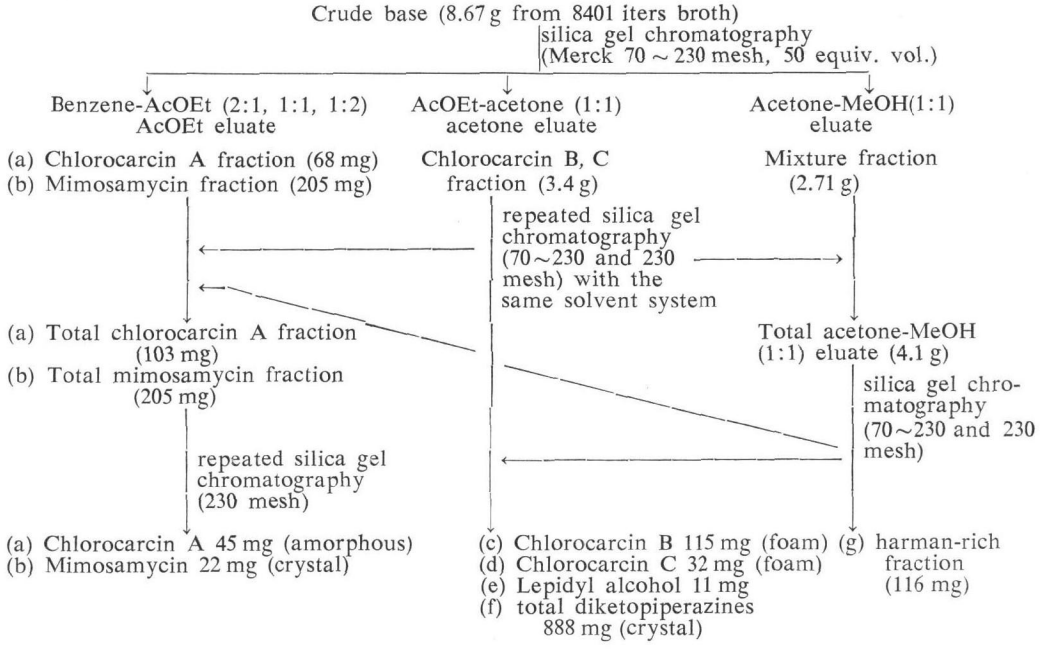
The crude extract was taken up into 100 ml of ethyl acetate and the ethyl acetate solution was successively extracted with 5% $NaHCO_3$, 1 N Na_2CO_3 and 1 N NaOH. From the aqueous layer, was isolated stearic acid (**IV**), which was identical with an authentic sample in all respects. The ethyl acetate layer was extensively extracted with 1 N HCl. This acidic solution was again made strong alkaline (pH 10) with concentrated NH_4OH and extracted with chloro-

form. The chloroform extract containing basic components was vacuum dried.

Removal of solvent from the ethyl acetate layer produced a brown syrup which mainly contained neutral components. The syrup was subjected to counter extraction between 10 % aqueous methanol and *n*-hexane.

The combined extracts of 10 % aqueous methanol was concentrated to dryness *in vacuo* and the residue was dissolved in a small amount of ethyl acetate. The ethyl acetate solution was washed with 1 N HCl and 1 N NaOH successively. The fraction containing mostly neutral components was thus obtained and concentrated into a syrup under reduced pressure.

Fig. 4. Fraction of satellite antibiotics of *Streptomyces lavendulae* No. 314.



Further purification of basic and neutral components and the procedure for the isolation of chlorocarcins and mimosamycin are shown in the scheme presented in Fig. 4. Column chromatography was effected using silica gel (Merck, 70~230 or 230 mesh) or Florisil (Floridin Co. 100~200 mesh).

Physicochemical Properties of Mimosamycin and Chlorocarcins

Physicochemical properties of mimosamycin and chlorocarcins are described below. In these experiment, melting points were determined on a Yanagimoto Micro-melting point apparatus (hot stage type) and are uncorrected. Optical rotations were measured on a Jasco DIP-4 polarimeter. Ultraviolet absorption spectra were determined on a Hitachi 323 spectrometer and infrared absorption spectra were taken on a Jasco IRA-2 spectrometer. NMR spectra were determined on a Hitachi R-24A (60 MHz) or Jeol PS-100 (100 MHz) with tetramethylsilane (TMS) as a internal standard (*s*, singlet: *d*, doublet; *t*, triplet: *m*, multiplet). Low-resolution- and GC-mass spectra were obtained on a Shimadzu-LKB 9000 GC-MS spectrometer and high-resolution mass spectra were determined on a Joel JMS-01SG-2 double focus mass spectrometer. ORD and CD spectra were recorded on a Jasco J-20 spectropolarimeter.

Gas-liquid chromatography (GLC) was carried out on a Shimadzu GC-4BM gas chromatography with FID using a glass column (2 m×3 mm) packed with 10 % OV-1 on Chromosorb W 80~100 mesh.

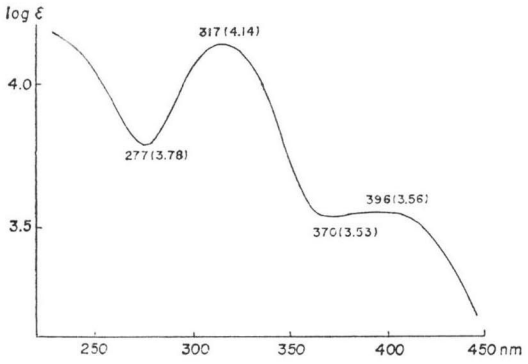
Mimosamycin

Mimosamycin is a neutral substance obtained as mimosa-yellow prisms melting at 227~231°C and has the specific rotation of $[\alpha]_D^{24}$ −1.8° (*c* 1.0, chloroform). It is readily soluble in lower alcohols, chloroform, ethyl acetate and acetone, slightly soluble in ethyl ether and *n*-hexane and insoluble in water.

The antibiotic gives a positive reaction to EHRLICH reagent (orange) and negative response to ninhydrin and DRAGENDORFF reagents.

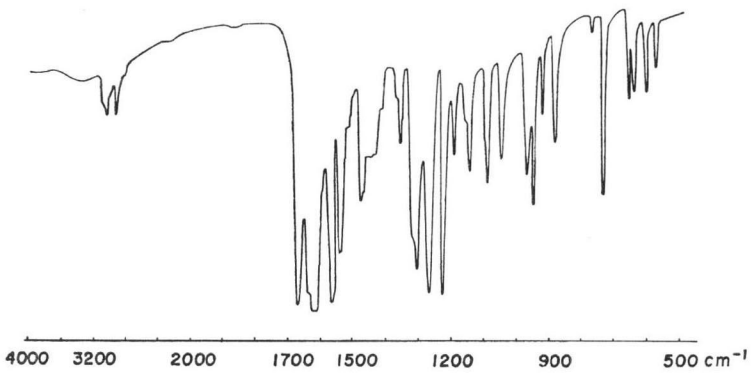
The elementary analysis and the consideration of the low-resolution mass spectrum with a parent ion at 233 established the molecular formula as $C_{12}H_{11}NO_4$ (M.W. 233.22): C, 61.80; H, 4.75; N, 6.01. Found: C, 61.51; H, 4.79; N, 5.87. Moreover, this molecular formula was confirmed by the exact mass determination (233.0691, calcd. 233.0688).

Fig. 5. Ultraviolet absorption spectrum of mimosamycin in methanol.



The ultraviolet absorption spectrum of mimosamycin in methanol had a shoulder and two maxima as shown in Fig. 5. UV λ_{max}^{MeOH} nm (log ε): 230 sh. (4.16), 317 (4.14), 396 (3.56). UV λ_{min}^{MeOH} nm (log ε): 277 (3.78), 370 (3.53). The infrared absorption spectrum in KBr is shown in Fig. 6. IR ν_{max}^{KBr} cm^{-1}: 1685, 1655, 1635, 1585. NMR (60 MHz, $CDCl_3$) δ: 2.10 (3H, s), 3.69 (3H, s), 4.20 (3H, s), 7.12 (1H, s), 8.28 (1H, s).

Fig. 6. Infrared absorption spectrum of mimosamycin in KBr.



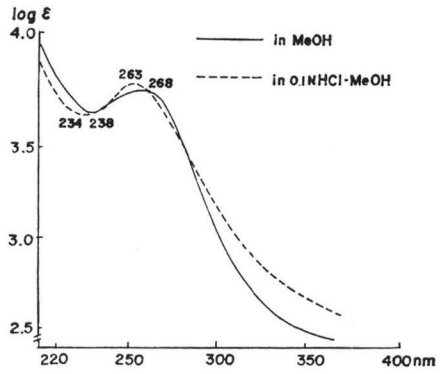
Chlorocarcin A

Chlorocarcin A is a basic substance obtained as a yellowish brown oil and has the specific

rotation of $[\alpha]_D^{28}$ −4.0° (*c* 1.0, methanol).

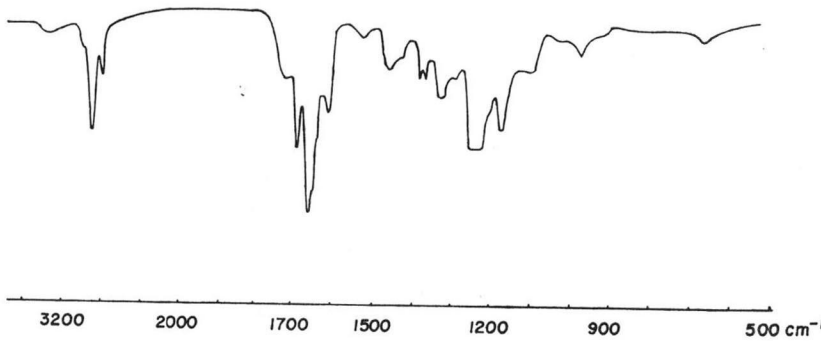
It is readily soluble in lower alcohols, chloroform, ethyl acetate, acetone and ethyl ether, slightly soluble in *n*-hexane and insoluble in water. The antibiotic gave positive reaction to DRAGENDORFF reagent and negative one to ninhydrin, $FeCl_3$ and anthrone reagents.

Fig. 7. Ultraviolet absorption spectra of chlorocarcin A.



Chlorocarcin A hydrochloride was obtained as a yellow powder melting at 140~144°C (dec.). The elementary analysis of the HCl salt and the consideration of the mass spectrum of the free base with a parent ion at 535 established the molecular formula as $C_{24}H_{26}N_3O_9Cl \cdot 2HCl \cdot H_2O$: C, 45.97; H, 4.79; N, 6.73; Cl, 16,98. Found: C, 45.74; H, 4.73; N, 6.74; Cl, 16.14. The ultraviolet absorption spectrum of chlorocarcin A free base in methanol had one strong maximum as shown in Fig. 7. UV λ_{max}^{MeOH} nm(log ε): 268 (3.83);

Fig. 8. Infrared absorption spectrum of chlorocarcin A in $CHCl_3$.

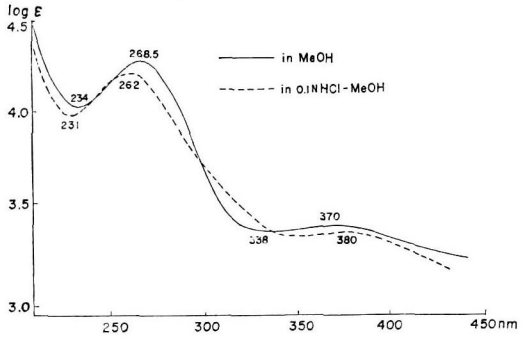


λ_{min}^{MeOH} nm (log ε): 238 (3.68); $\lambda_{max}^{0.1N\ HCl-MeOH}$ nm (log ε): 263 (3.85); $\lambda_{min}^{0.1N\ HCl-MeOH}$ nm (log ε): 234 (3.68). The infrared absorption spectrum in chloroform is shown in Fig. 8. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1685, 1665, 1610. NMR (100 MHz, $CDCl_3$) δ: 1.23 (3H, s), 1.95 (3H, d J=7Hz), 2.26 (3H, d J=7Hz), 4.05(3H, s), 6.70 (1H, s). Circular dichroism spectrum (*c* 1.89×10^{-4}, MeOH) showed the following maxima: $\Delta\varepsilon$(nm): −7.70 (358) (negative maximum): −0.45 (315) (positive maximum): −24.36 (279) (negative maximum).

Chlorocarcin B

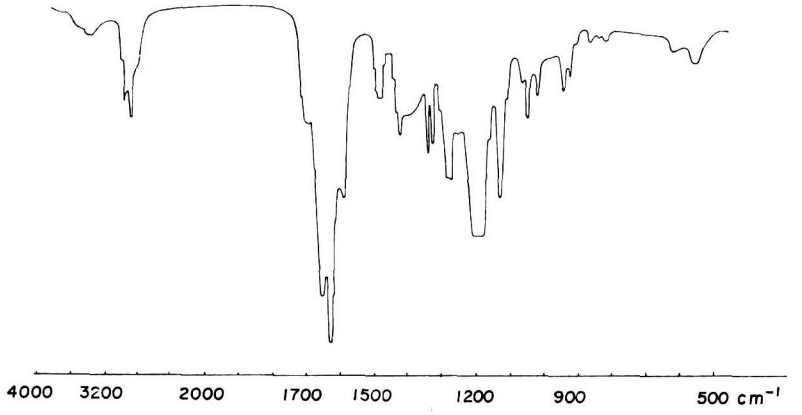
Chlorocarcin B is also a basic substance obtained as a reddish brown glass melting at 78 ~81°C and has the specific rotation of $[\alpha]_D^{22}$ −106.9° (*c* 0.57, chloroform). It is readily soluble in lower alcohols, chloroform, ethyl acetate, acetone, benzene and ethyl ether, slightly soluble in *n*-hexane and insoluble in water. The antibiotic gave positive reaction to DRAGENDORFF and MEYER reagents and negative response to ninhydrin and EHRLICH reagents. The elementary analysis of the base and the consideration of the low resolution mass spectrum with a parent

Fig. 9. Ultraviolet absorption spectra of chlorocarcin B.



ion at 553 established the molecular formula as $C_{29}H_{32}N_3O_6Cl \cdot 5/4H_2O$: C, 60.46; H, 6.04; N, 7.29; Cl, 6.07. Found: C, 60.89; H, 6.20; N, 6.71; Cl, 5.20. This molecular formula was confirmed by the exact mass determination ($C_{29}H_{32}N_3O_6Cl^{35}$ 553.2003, calcd. 553.1979). The ultraviolet absorption spectrum of chlorocarcin B in methanol (Fig. 9) had one strong and another broad maxima which shifted to shorter and longer wave lengths in acidic methanol respectively. UV λ_{max}^{MeOH} nm (log ε):

Fig. 10. Infrared absorption spectrum of chlorocarcin B in $CHCl_3$.



268.5 (4.25), 370 (3.38); λ_{min}^{MeOH} nm (log ε): 234 (3.99), 338 (3.34); $\lambda_{max}^{0.1N\ HCl-MeOH}$ nm (log ε): 262 (4.18), 380 (3.34); $\lambda_{min}^{0.1N\ HCl-MeOH}$ nm (log ε): 231 (3.95), 354 (3.32). The infrared absorption spectrum of chlorocarcin B is shown in Fig. 10; IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1715, 1683, 1655, 1612. NMR (100 MHz, $CHCl_3$) δ: 1.28 (3H, m), 1.92 (3H, s), 2.04 (3H, s), 2.27 (3H, s), 2.50 (3H, s), 4.04 (3H, s), 4.08 (3H, s), 4.42 (1H, s), 6.84 (1H, d J=8Hz).

Circular dichroism spectrum (c 8.63×10^{-5}, MeOH) showed the following maxima: $\Delta\varepsilon$(nm): −2.45(360) (negative maximum), −0.352 (320) (positive maximum), −13.0 (278) (negative maximum).

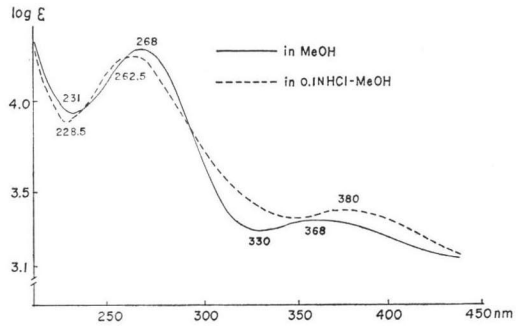
Chlorocarcin C

Chlorocarcin C is a closely related substance to chlorocarcin B. It is also obtained as a reddish brown glass melting at 79~84°C and has the specific rotation of $[\alpha]_D^{24}$ −16.0 (c 0.52, chloroform).

The solubility and color reactions of chlorocarcin C are the same as those of chlorocarcin B. The elementary analysis and the consideration of the low resolution mass spectrum with a parent ion at 567 established the molecular formula as $C_{30}H_{34}N_3O_6Cl \cdot 3/2H_2O$: C, 60.59; H, 6.27; N, 7.06. Found: C, 60.88, 60.88; H, 6.11, 6.13; N, 6.54, 6.63.

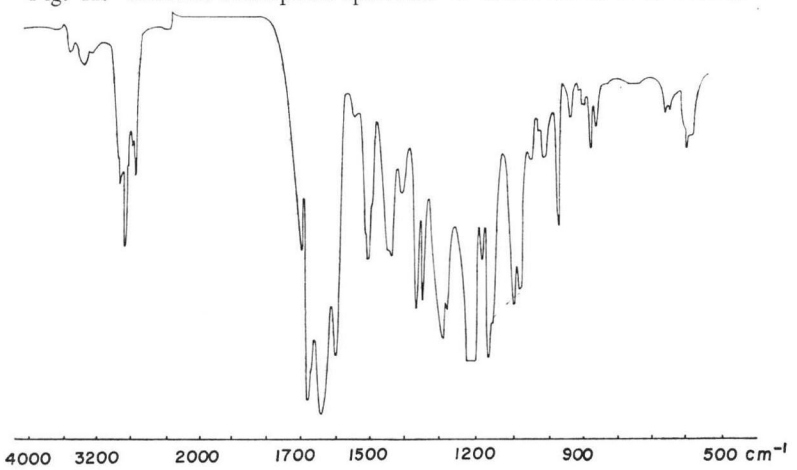
This molecular formula was also confirmed by the exact mass determination ($C_{30}H_{34}N_3O_6Cl^{35}$

Fig. 11. Ultraviolet absorption spectra of chlorocarcin C.



567.2106, calcd. 567.2135). The ultraviolet absorption spectrum of chlorocarcin C in methanol (Fig. 11) also had two absorption maxima. UV λ_{max}^{MeOH} nm (log ε): 268 (4.26), 368 (3.34); λ_{min}^{MeOH} nm (log ε): 231 (3.91), 330 (3.30); $\lambda_{max}^{0.1N\ HCl-MeOH}$ nm (log ε): 262.5 (4.22), 380 (3.40); $\lambda_{min}^{0.1N\ HCl-MeOH}$ nm (log ε): 228.5 (3.87), 351 (3.36). The infrared absorption spectrum of chlorocarcin C is shown in Fig. 12; IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1718, 1683, 1655, 1618. NMR (100 MHz, $CDCl_3$) δ: 1.42 (3H, s),

Fig. 12. Infrared absorption spectrum of chlorocarcin C in $CHCl_3$.



2.01 (3H, s), 2.15 (3H, s), 2.35 (3H, s), 2.59 (3H, s), 3.59 (3H, s), 4.05 (3H, s), 4.07 (3H, s), 6.71 (1H, d J=8Hz).

Circular dichroism spectrum (c 9.38×10^{-5}, MeOH) showed the following maxima: $\Delta\varepsilon$(nm): −3.88 (360) (negative maximum), −2.26 (310) (positive maximum), −24.5 (273) (negative maximum).

Biological Properties of the Satellite Antibiotics

Mimosamycin was found to be mainly active against mycobacteria including streptomycin-sensitive and resistant strains of human tubercle bacilli. It shows relatively low toxicity and mice tolerated intravenous administration of 25 mg per kg of the antibiotic. The antibiotic, however, showed no antitumor activity.

Among chlorocarcins, chlorocarcin A proved to be the most bioactive. It inhibited a number of gram-positive bacteria at a concentration range of 0.003~50 mcg per ml. The antibiotic was found to be highly active on murine tumors such as EHRLICH carcinoma, ascites and solid forms, and L 1210 leukemia at the doses below 80 mcg per mouse per day. Mice well tolerated intravenous injection of 25 mg/kg of chlorocarcin A hydrochloride and 250 mg/kg of chlorocarcin B base. Some of their biological activities are compared in Table 1.

Table. 1. Biological characteristics of mimosamycin and chlorocarcins.

	Mimosamycin	Chlorocarcin A	Chlorocarcin B	Chlorocarcin C
Antimicrobial activity[1]	*Mycobacterium* ⫲	gram-positive ⫲	gram-positive +~⫲	gram-positive +~⫲
Cytostatic activity[2]	−	0.05	10	10

1): + slight, ⫲ moderate, ⫲ strong
2): L1210 suspension culture, 100% cell killing, mcg/ml.

The detailed biological activity of these antibiotics are reported in a separate communication.

Discussion

Chlorocarcins are chlorine-containing antibiotics, and compared with known chlorine-containing compounds. These antibiotics are: avilamycin,[6] chloramphenicol,[7] chlortetracycline,[6] cineromycin A,[8] curamycin,[9] exfoliatin[10] and vancomycin.[11] However, the present experimental results of elementary analysis, melting point, optical rotation, infrared spectrum and biological activity, differentiate chlorocarcins from these antibiotics.

Mimosamycin is also differentiated from the known antibiotics by its physico-chemical and biological properties.

The above experimental results as well as the discoveries of interesting antibiotics as minor components by others eloquently tell of possible presence of satellite antibiotics or other accessary bioactive compounds of new class in the culture of even already known actinomycetes which might be masked by other major antibiotic or only revealed under a novel culture condition. Although such compounds are often produced in trace amounts at first, it is a common history of antibiotic research that the extraordinary increase of the production of microbial secondary metabolites can be attained by means of the improvement of the producing strain and culture conditions. Furthermore, once their chemical structures are elucidated, they can be also chemically synthesized. The isolation of these components, however, requires a screening system that is sensitive and specific enough for the detection of their biological activity.

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References

1) ISHIWATA, K.: Studies on the experimental cancer chemotherapy employing established cell line of mouse leukemia L 1210. Chiba Med. J. 51: 81~89, 1975
2) JOHNSON, A.W. & J.W. WESTLEY: The streptothricin group of antibiotics. 1. The general structural pattern. J. Chem. Soc. 1962: 1642~1655, 1962
3) MIKAMI, Y.; K. YOKOYAMA, A. ÔMI & T. ARAI: Identification of producer and biological activities of new antibiotics, mimosamycin and chlorocarcins. J. Antibiotics 29: 408~414, 1976
4) PERKIN, W.H. & R. ROBINSON: Harmin and harmalin. J. Chem. Soc. 115: 967~971, 1919
5) TAKEUCHI, T.; K. OGAWA, H. IINUMA, H. SUDA, K. UKITA, T. NAGATSU, M. KATO, H. UMEZAWA

& O. TANABE: Monoamine oxidase inhibitors isolated from fermented broths. J. Antibiotics 26: 162~167, 1973
6) UMEZAWA, H.: Index of antibiotics from actinomycetes. University of Tokyo Press, 1967
7) GOTTLIEB, D.; P.K. BHATTACHARYYA, H.W. ANDERSON & H.E. CHARTER: Some properties of an antibiotic obtained from a species of *Streptomyces*. J. Bact. 55: 409~417, 1948
8) MIYAIRI, N.; M. TAKAHASHI, K. SHIMIZU & H. SAKAI: Studies on new antibiotics, cineromycins A and B. J. Antibiotics, Ser. A 19: 56~62, 1966
9) GALMARINI, O. & V. DEULOFEU: Curamycin I. Isolation and characterization of some hydrolysis products. Tetrahedron 15: 76~86, 1961
10) UMEZAWA, H.; S. TAKAHASHI, T. TAKEUCHI, K. MAEDA & Y. OKAMI: On a new antibiotic, exfoliatin, produced by a strain of streptomyces. J. Antibiotics 5: 466, 1952
11) MCCORMICK, M.H.; W.M. STARK, G.E. PITTENGER, R.C. PITTENGER & J.M. MCGUIRE: Vancomycin, a new antibiotic. 1. Chemical and biological properties. Antibiotics Ann. 1955/56: 606~611, 1956