GILVOCARCINS, NEW ANTITUMOR ANTIBIOTICS

1. TAXONOMY, FERMENTATION, ISOLATION AND BIOLOGICAL ACTIVITIES

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Gilvocarcin V and gilvocarcin M, a group of antitumor antibiotics with a novel skeleton, were discovered in culture broths of *Actinomycete* DO-38. The producing organism, subsequently determined to be a new species and named *Streptomyces gilvotanareus* (NRRL 11382). Gilvocarcin V and M were isolated by ethyl acetate extraction and chromatography on silica gel. The antibiotics are active against Gram-positive bacteria and experimental tumors such as mouse sarcoma 180 and mouse leukemia P388.

Gilvocarcin, a group of antibiotics with a novel carbon skeleton[1,2], was discovered in the course of a continuing search for antitumor antibiotics of microbial origin. Because these antibiotics are yellow in color and are produced by *Streptomyces gilvotanareus*, they have been named gilvocarcin. As described in the separate paper[2], the names gilvocarcin V and gilvocarcin M are based on their structure in which vinyl group and methyl group are borne respectively.

This paper describes taxonomic studies of the producing organism, the production of gilvocarcin by fermentation, the sequence of chromatographic procedures used to isolate them in an essentially pure form, and their biological activity.

Materials and Methods

Fermentation

Strain F3 of *Streptomyces gilvotanareus* was selected by single-spore isolation for its ability to produce high yield of gilvocarcin. Inocula for jar fermentations were prepared in a seed medium consisting 10 g glycerol, 10 g peptone, 1 g yeast extract and 1 g K_2HPO_4 per liter of tap water adjusted to a final pH of 6.8. The seed flasks were inoculated with stock cultures maintained in a deep freezer (−70°C) or spores on agar, and incubated at 28°C for 48 hours on a rotary shaker. A 5% vegetative seed was used to inoculate into a jar fermentor containing a production medium.

Medium A used for the production of gilvocarcin V and M contained per liter: 60 g soluble starch, 10 g glucose, 20 g soy bean meal, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$ and 3 g NaCl; after adjustment of pH to 7.0, 0.5% $CaCO_3$ was added.

Medium B used for the selective production of gilvocarcin V contained per liter: 60 g fructose, 10 g glucose, 20 g L-leucine, 5 g corn steep liquor, 10 g NaCl, 5 g NH_4Cl, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $ZnSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.01 g $MgCl_2 \cdot 4H_2O$ and 0.025 g $CuSO_4 \cdot 5H_2O$; after adjustment of pH to 7.0, 0.5% $CaCO_3$ was added.

Gilvocarcin Assay

Because gilvocarcin V and M sedimented with mycelium by centrifugation of culture broth, amounts of gilvocarcin in broth and of partially purified samples were determined by the following

procedure. To 2 ml of culture broth in a test tube, 1 ml of acetone and 1 ml of ethyl acetate were added, and mixed vigorously. After centrifugation at $1200 \times g$ for 10 minutes, a portion (usually 10 μl) of upper layer was applied on a silica gel plate and developed with a mixture of ethyl acetate and acetic acid (9: 1 v/v). The amount of each components of gilvocarcin were determined by measuring the intensity of absorbance at 395 nm with a Shimadzu CS-900 chromatoscanner.

Determination of Biological Activities

The minimal inhibitory concentration of gilvocarcin, dissolved in DMF - MeOH (1: 1), against Gram-positive and Gram-negative bacteria were determined in a nutrient agar (Eiken Chemical Co. Ltd., Japan) by two-fold serial dilution method.

The acute toxicity (LD_{50}) were calculated from the number of survivors at 14 days after a single intraperitoneal injection of gilvocarcin suspended in 0.85% NaCl solution containing 0.2% Tween 80 into *dd*Y mice.

Antitumor activity against mouse experimental tumors determined as described previously[3].

Results

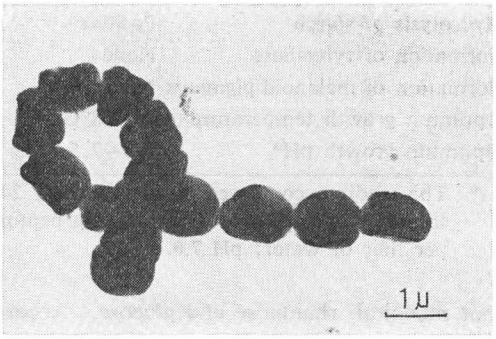
Taxonomy

Actinomycete strain DO-38 was isolated from a soil sample collected in Kochi-shi, Kochi, Japan. The strain has been deposited at Nothern Regional Research Laboratories, Peoria, Illinois, U.S.A. and has been assigned accession number NRRL 11382.

The taxonomic characterization was carried out according to the methods used in the International Streptomyces Project (ISP)[4]. The various kinds of media were inoculated with the washed mycelia suspended in 0.85% saline obtained from a culture shaken at 28°C for 72 hours in a liquid medium consisted of 10 g glucose, 24 g soluble starch, 3 g beef extract, 5 g yeast extract, 5 g peptone per liter of water, pH 7.0 prior to sterilization.

Strain DO-38 grew well on various media as shown in Table 1. The abundant aerial mycelium with simple branching and spiral spore chains were observed by microscopy. The mature spore chains were generally long with 10~30 or more spores per chain. The spores were oval in shape ($0.8 \sim 0.9 \times 1.1 \sim 1.2$ μ) and smooth surface as seen by electron microscope (Fig. 1).

Fig. 1. Spores of *Streptomyces gilvotanareus* grown on oatmeal agar; electron micrograph.



The aerial mycelium was white (colorless) to gray colored on agar media. The golden color occasionally occurred in aged cultures and melanine was not produced on peptone-yeast extract-iron agar and tyrosine agar. These morphological and cultural characteristics are listed in Table 1.

The physiological characteristics of strain DO-38 and utilization of carbon sources are shown in Tables 2 and 3. The temperature range for growth and the pH range for growth were observed after cultivation of 2 days and the action upon milk and decomposition of cellulose was observed after one month. All other observations were made after 20 days.

According to the method of KÜSTER's classification[6], analysis of above results placed strain DO-38 to *Streptomyces flavescens*, for it showed white aerial mycelium on oatmeal agar and no mel-

Table 1. Cultural characteristics of strain DO-38.

Medium	Growth of colony	Color of colony*		Mycelium	
		Surface	Reverse	Growth & color of mycelium	Pigment
CZAPECK's agar (WAKSMAN No. 1)	Good flat	Camel (3ie)	Camel (3ie)	Good Ash (5fe)	Coral rose (6ic)
Glucose-asparagine agar (WAKSMAN No. 2)	Good flat	Oatmeal (2ec)	Egg shell (2ca)	Good Covert gray (2fe)	None
Yeast extract-malt extract agar (ISP No. 2)	Good flat	Light mustard tan (2ie)	Oatmeal (2ec)	Good Silver gray (3fe)	None
Oatmeal agar (ISP No. 3)	Good raised	Mustard tan (2lg)	Mustard tan (2lg)	Good White (a)	Dull gold (2ng)
Inorganic salt-starch agar (ISP No. 4)	Good flat	White (a)	White (a)	Good Silver gray (3fe)	None
Glycerol-asparagine agar (ISP No. 5)	Good raised	Oatmeal (2ec)	Egg shell (2ca)	Good Ash (5fe)	None
Peptone-yeast extract-iron agar (ISP No.6)	Moderate flat	Bamboo (2gc)	Oatmeal (2ec)	None	None
Tyrosine agar (ISP No.7)	Good raised	Light mustard tan (2ie)	Light mustard tan (2ie)	Good Natural (3dc)	None
Nutrient agar	Moderate flat	Light mustard tan (2ie)	Light mustard tan (2ie)	Poor White (a)	None

* Color designation from Color Harmony Manual.[5]

Table 2. Physiological properties of strain DO-38.

Liquefaction of gelatin	Weakly positive
Liquefaction of milk	Negative
Peptonization of milk	Negative
Decomposition of cellulose	Weakly positive
Hydrolysis of starch	Positive
Formation of tyrosinase	None
Formation of melanoid pigments	None
Optimum growth temperature*	28~38°C
Optimum growth pH*	6.8~7.5

* The medium composed of 10 g glucose, 24 g soluble starch, 3 g beef extract, 5 g peptone per liter of water; pH 7.0.

Table 3. Utilization of carbohydrates by strain DO-38.

D-Arabinose	−	Sucrose	+
D-Xylose	−	Inositol	+
D-Glucose	±	Raffinose	−
D-Fructose	+	L-Rhamnose	±
D-Mannitol	±		

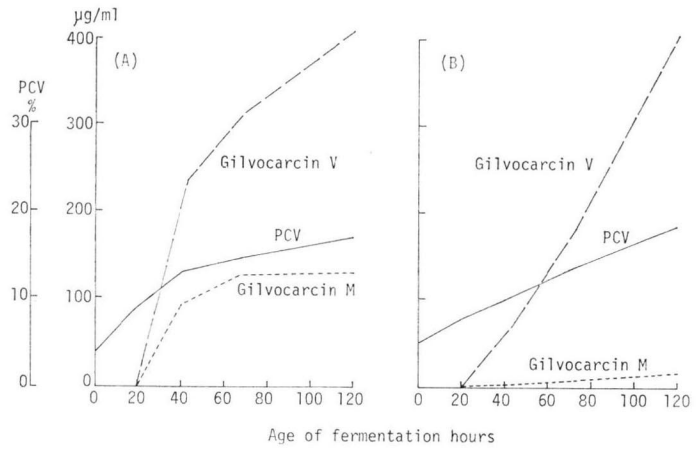
anine pigment on peptone-yeast extract-iron agar. However strain DO-38 has spiral spore chains and does not utilizes arabinose and utilizes mannitol, rhamnose as well as glucose. On the other hand, *Streptomyces flavescens* has flexious spore chains and utilizes arabinose but not mannitol, rhamnose and glucose. Accordingly strain DO-38 was assigned to a new *Streptomyces* species for which was chosen the name *gilvotanareus* to denote the color in aged cultures.

Fermentation

Fig. 2-A shows a typical time course of gilvocarcin production in a 5-liter jar fermentor containing 3 liters of medium A. Cell growth continued throughout the production of gilvocarcin but growth rate decreased after about 40 hours. During 120 hours of fermentation, 410 μg/ml gilvocarcin V and 130 μg/ml of gilvocarcin M were accumulated.

The contents of gilvocarcin V and gilvocarcin M varied with culture media, in particular, with the source of nitrogen. Fig. 2-B shows the selective production of gilvocarcin V (over 90% of all gilvocarcin) with the production medium B. As described below, separation of gilvocarcin V from

Fig. 2. Time course of gilvocarcins production.



gilvocarcin M by chromatographic procedure was a rather difficult task. Therefore the production medium B was advantageous for the isolation of gilvocarcin V.

Isolation of Gilvocarcins

Gilvocarcins were extracted with ethyl acetate from the broth filtrate or aqueous acetone extract of the mycelium. The greenish-yellow extract was washed with water and concentrated on a rotary evaporator until crude crystals of gilvocarcins were obtained. The crude crystals were dissolved in tetrahydrofuran and the solution was evaporated with silica gel (Kanto Chemical Co.) to dryness *in vacuo*. The crude gilvocarcins absorbed on silica gel powder was applied to silica gel column, and chromatographed with $CHCl_3$, then $CHCl_3$ - methylisobutylketone (1: 1). Each fraction of eluate was tested by silica gel TLC using the solvent system of ethyl acetate - acetic acid (9: 1) (Table 4). The fractions which contained gilvocarcin V was collected and concentrated to dryness on a rotary evaporator. Gilvocarcin V could be recrystallized by dissolving in hot acetone - methanol or by dissolving in DMSO and adding ethanol.

Table 4. Chromatographic mobility of gilvocarcins on silica gel TLC.

Solvent system	Rf value of gilvocarcin	
	V	M
Ethyl acetate - acetic acid (9 : 1)	0.45	0.32
Ethyl acetate	0.60	0.51
Methylisobutylketone	0.20	0.10
Acetone	0.92	0.92
Methanol	0.68	0.68
$CHCl_3$	0	0
$CHCl_3$ - methanol (9 : 1)	0.30	0.30

Isolation of gilvocarcin M by chromatography was difficult, because gilvocarcin M is practically insoluble in ethyl acetate or methylisobutylketone that can separate gilvocarcin V and gilvocarcin M on silica gel. Thus, the mixture of gilvocarcin V and M was converted to tetrahydropyranyl derivatives, and then vinyl group of the gilvocarcin V derivative was oxidized to aldehyde by osmium tetroxide and sodium periodate. The mixture of tetrahydropyranyl gilvocarcin M and oxidized tetrahydropyranyl gilvocarcin V was applied to silica gel column and chromatographed with $CHCl_3$. The fractions which contained the gilvocarcin M derivative were collected and concentrated on a rotary evaporator. Acid hydrolysis of the concentrate gave pure gilvocarcin M, which was recrystallized from acetone - methanol.

Biological Properties

Table 5 shows the minimal inhibitory concentration of gilvocarcin V and M against Gram-positive and Gram-negative bacteria. Gilvocarcin V is highly active against *Staphylococcus aureus*. The antibacterial activity of V was about one orders greater than that of M. In another test of paper-disk agar-diffusion method, gilvocarcin V showed clear inhibitory zones against *Bacillus subtilis* in spite of its insolubility in water. The inhibitory zone increased with increasing amount of gilvocarcin V; range of 0.02 μg/disk to 10 μg/disk.

The LD_{50} values of gilvocarcin V was about 300 mg/kg when administered intravenously. Gilvocarcin V was not toxic to mice at a single dose of 1,000 mg/kg by other administration routes; oral or intraperitoneal.

Table 5. Antimicrobial activity of gilvocarcins.

Test organism	MIC (μg/ml)	
	Gilvocarcin V	Gilvocarcin M
Staphylococcus aureus ATCC 6538P	0.05	1.6
Bacillus subtilis No. 10707	0.78	25
Klebsiella pneumoniae ATCC 10031	100	100
Escherichia coli ATCC 26	25	100
Shigella sonnei ATCC 9290	100	100

Medium: Nutrient agar (Eiken Chemical Co., Ltd.,).

Table 6. Antitumor activity of gilvocarcin V.

	Dose (mg/kg)	Sarcoma 180 (T/C)	P-388 (ILS %)
Gilvocarcin V	50	N.T.	32
	100	0.81	35
	200	0.76	47
	400	0.46	57
Mitomycin C	4.2	0.39	113

Table 6 shows the effect of gilvocarcin on lymphocytic leukemia P388. The life span of the gilvocarcin V-treated mice has been significantly increased to that of control animals.

Table 6 shows the ratio of the tumor volumes of gilvocarcin V-treated and control mice at 7 days after inoculation of 5×10^6 cells of sarcoma 180. Gilvocarcin V at a single dose of 400 mg/kg (ip) induced the significant tumor regression (T/C 0.46).

Further studies on the antitumor activity of gilvocarcin will be described in elsewhere.

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References

1) HIRAYAMA, N.; K. TAKAHASHI, K. SHIRAHATA, Y. OHASHI & Y. SASADA: Crystal and molecular structure of antibiotic gilvocarcin M. Bull. Soc. Chem. Japan, submitted.
2) TAKAHASHI, K.; M. YOSHIDA, F. TOMITA & K. SHIRAHATA: Gilvocarcins, new antitumor antibiotics. 2. Structural elucidation. J. Antibiotics 34: 271~275, 1981
3) TAMAOKI, T.; M. KASAI, K. SHIRAHATA, S. OHKUBO, M. MORIMOTO, K. MINEURA, S. ISHII & F. TOMITA: Tetrocarcins, novel antitumor antibiotics. II. Isolation, characterization and antitumor activity. J. Antibiotics 33: 946~950, 1980
4) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Intern. J. Syst. Bacteriol. 16: 313~340, 1966
5) Container Corporation of America: Color Harmony Mannual, 4th edition, Chicago, U.S.A., 1958
6) KÜSTER, E.: Simple working key for the classification and identification of named taxa included in the international *Streptomyces* project. Intern. J. Syst. Bacteriol. 22: 139~148, 1972