PRODUCTION AND BIOLOGICAL ACTIVITY OF MARCELLOMYCIN, AN ANTITUMOR ANTHRACYCLINE ANTIBIOTIC, AND TAXONOMY OF THE PRODUCING ORGANISM

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An actinomycete, isolated from a soil sample from Ontario, Canada, was studied taxonomically and named *Actinosporangium bohemicum* sp. nov. strain C-36,145. This strain was found to produce a complex mixture of ε-pyrromycinone glycosides having antitumor properties. Marcellomycin, a member of this complex, was selected for further study. Conditions for production of this antibiotic were developed in flask studies and scaled-up to the 3,000-liter fermentor stage.

A microorganism was isolated from a soil sample collected in Ontario, Canada during a screening program for the isolation of novel antitumor agents. This organism, designated strain C-36,145, was cultured in shake-flasks and samples were tested for *in vivo* antitumor activity. Tumor inhibitory effects were observed first with Walker 256 carcinosarcoma[1] carried as a solid intramuscular implant.

With further investigation activity against transplanted mouse leukemias P388[2] and L1210[3] was obtained with crude culture extracts. The antitumor active products of strain C-36,145 were found to be glycosides of ε-pyrromycinone and this mixture was designated as the bohemic acid complex[4]. Strain C-36,145 was studied taxonomically and was named *Actinosporangium bohemicum* sp. nov. This

Table 1. Other actinomycetes producing pyrromycinone glycosides.

Name of organism	Strain No.	Product name
Streptomyces cinereoruber var. *fructofermentans*	ETH 6143	Cinerubins[5,6]
Streptomyces niveoruber	ETH 17860, ETH 17403	Cinerubins[5,6]
Streptomyces bobiliae	ATCC 3310	Cinerubin[5]
Streptomyces sp.	DOA 1205	Pyrromycin[7]
Actinomycete	No. A220	Rutilantin[8]
Streptomyces ryensis	No. 44, ATCC 29805	Ryemycins[9]
Streptomyces tauricus	R1A 1417, ATCC 27470	Tauromycetins[10,11]
Streptomyces galilaeus	JA 3043	Galirubins[12,13]
Streptomyces diastatochromogenes	IMET JA 10081/9	Trypanomycin A_2[14]
Streptomyces galilaeus	MA 144–M1, ATCC 31133	Cinerubins, pyrromycin,[15] MA-144 group
Streptomyces sp.	ME 505-HEI, ATCC 31273	Rhodirubins[16]
Streptomyces griseorubiginosus	No. 4915	Cinerubins[9]

organism was deposited in the American Type Culture Collection, Rockville, Md. with the accession number ATCC 31127. Other organisms producing ε-pyrromycinone glycosides are listed in Table 1.

The following anthracyclines were isolated from cultures of strain C-36,145; musettamycin, marcellomycin, rudolphomycin, mimimycin, collinemycin and alcindoromycin.[17,18] Their structures were given by DOYLE[19] along with other anthracycline oligosaccharides. Among the members of the bohemic acid complex marcellomycin was selected for further study since antitumor activity and preliminary results of toxicological evaluation were encouraging.[20] To aid in production of larger amounts of material studies were undertaking to increase the yield of marcellomycin with strain C-36,145.

Materials and Methods

Strain C-36,145 was isolated from a soil sample from Ontario, Canada in the course of a screening program for discovery of new antitumor agents. *Actinosporangium violaceum* ATCC 15813, *Streptomyces massasporeus* ATCC 19785, and *Actinopycnidium caerulium* ATCC 15812 were obtained from the American Type Culture Collection, Rockville, Maryland. The procedures of SHIRLING and GOTTLIEB[21] were used to characterize strain C-36,145 for taxonomic studies. The nitrate reduction test was described by GOTTLIEB[22] and cell wall analysis was performed according to the procedures of YAMAGUCHI[23] and LECHEVALIER and LECHEVALIER[24].

Cinerubin A and cinerubin B were obtained from the National Cancer Institute Bethesda, MD. Procedures for isolation of the bohemic acid complex from cultures of strain C-36,145 and the preparation of marcellomycin, musettamycin, pyrromycin, ε-pyrromycinone and η-pyrromycinone are described by NETTLETON, *et al.*[4] Sterigmatocystin was prepared from cultures of *Aspergillus versicolor* M-1073[25].

For maintenance or fermentation studies strain C-36,145 was cultured on agar slant media consisting of either 2 g glucose, 20 g oatmeal, 2 g soy peptone and 20 g agar made up to one liter with distilled water, or 4 g glucose, 4 g yeast extract, 10 g malt extract and 20 g agar made up to one liter with distilled water. Slant cultures were incubated at 27°C for 144 hours. Spores and mycelium from a slant culture were transferred to 500-ml Erlenmeyer flasks containing 100 ml of sterile vegetative medium. A typical vegetative medium consists of 30 g glucose, 10 g soybean flour, 10 g Pharmamedia (Traders Oil Mill Co., Fort Worth, Texas) and 3 g $CaCO_3$ made up to one liter with distilled water. The vegetative culture was incubated at 27°C for 48 hours on a rotary shaking machine (Gyrotory tier shaker, model G53 New Brunswick Scientific Co., Inc., New Brunswick, New Jersey) describing a 5.1-cm diameter circle. For the fermentation phase four ml of culture was transferred to a 500-ml flask containing 100 ml of sterile production medium and the culture was incubated on a similar shaker with prescribed experimental conditions.

Strain C-36,145 was cultivated in 14-liter stir-jar fermentors containing ten liters of sterile production medium. Four hundred ml of vegetative culture was prepared as described previously and was transferred aseptically to the stir-jar fermentor installed in a Fermentor Drive Assembly (Model FS-614, New Brunswick Scientific Co., New Brunswick, N.J.). The temperature was maintained at 27°C, the air flow rate was 6 liters/minute and the agitator was set at 360 rev/minute. Hodag F1 antifoam (Hodag Chemical Corp., Skokie, Illinois) was fed automatically as required to control foaming.

A 48-liter tank fermentor with 37.8 liters of production medium was inoculated with 1.89 liters of vegetative culture agitated with an impeller speed of 300 rev/minute, aerated at 85 liters/minute and incubated at 27°C. Hodag F1 antifoam was used to control foaming. A 3,800-liter tank fermentor with 3,000 liters of production medium was inoculated with 152 liters of vegetative culture, agitated with an impeller speed of 155 rev/minute, aerated at a rate of 1,400 liters/minute and incubated at 27°C. Polypropylene glycol was used to control foaming.

Clarified culture samples of strain C-36,145 were prepared for biological testing by centrifugation to remove mycelium. To prepare a mycelial extract, ten ml sample of culture was centrifuged and the supernatant was replaced with an equal volume of acetone. The mycelium was resuspended, shaken

for thirty minutes on a reciprocal shaker and centrifuged to remove mycelium. A third procedure, designated a whole culture extract, consisted of extracting a culture sample for 30 minutes with an equal amount of acetone.

Antibiotic activity of culture samples, extracts and isolated bohemic acid components were measured with cylinder type agar diffusion assays. The assay organism, *Bacillus subtilis* ATCC 6633, was added as a spore suspension to Streptomycin Assay with Yeast Extract (Baltimore Biological Laboratory, Baltimore, Maryland). Assay plates were incubated at 30°C for 18 hours. A marcellomycin preparation, found by high pressure liquid chromatography[4] to be 98+% pure, was used as a standard and was given an assigned potency of 980 units/mg for the purpose of microbiological assays. To estimate marcellomycin content C-36,145 culture samples were extracted and the extract was analyzed by high pressure liquid chromatograph.[4]

The procedure to test for induction of lysogenic *Escherichia coli* W1709 (λ) was described by BRADNER, *et al.*[26] Phenobarbitol induced liver microsomes were obtained from Litton Bionetics Laboratory Products, Kensington, Maryland.

Thin-layer chromatography was carried out with 20×20 cm Uniplates precoated with a 250 μm layer of silica gel G (Analtech, Inc., Newark, Delaware). Bioautographs of thin-layer chromatograms with *B. subtilis* ATCC 6633 were prepared as described in an earlier publication[27].

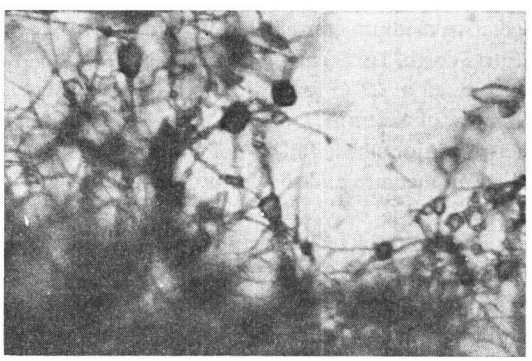
Procedures for measurement of antitumor activity against lymphocytic leukemia P388 and L1210 leukemia and Walker 256 carcinosarcoma with test compounds have been described[3]. To test activity with clarified culture samples 0.5 ml of appropriate dilutions was administered daily for nine days.

Results

Characterization of Strain C-36,145

When grown on glucose-asparagine agar, tyrosine agar, yeast extract-malt extract agar and oatmeal agar, strain C-36,145 produces a branched, unfragmented mycelium typical of members of the genus *Streptomyces* (Fig. 1). On these media there are sporangium-like structures, 8 to 11 μm in diameter that are difficult to distinguish from the sporangium of members of the genus *Streptosporangium*. Electron micrographs of these spore masses failed to reveal a sporangial wall. Spores enclosed in a viscid covering are smooth or warty ellipsoidal and non-motile. These spores masses, therefore, are designated pseudosporangia, which are the predominant spore structure of strain C-36,145. In addition there are sporophores in the form of hooks, loops and irregular open spirals.

Fig. 1. Photomicrograph of strain C-36,145 (×500).



Crude and purified cell wall preparations were found to contain LL-diaminopimelic acid and glycine with two dimensional thin-layer chromatography and liquid chromatography. No diagnostic carbohydrate was found with gas chromatographic analysis trimethylsilylation.

Cultural characteristics are given in Table 2. Aerial mycelium, when fully developed, is grayish pink. In several media the substrate mycelium has pH indicator pigment that is reddish orange with acid and purple with base. Melanin pigment in tyrosine agar and in peptone-yeast extract-iron agar. Optimal growth temperature on yeast extract-malt extract agar is 28°C. There is no growth at 10°C or 43°C. Growth is slow at 50°C and growth is moderate at 20~30°C.

Table 2. Cultural characteristics of strain C-36,145.

Medium	Aerial mycelium	Substrate mycelium	Diffusible pigment
Glycerol - asparagine agar	Moderate, light gray to pale pink	Rose, orange with 0.05 N HCl, purple with 0.05 N NaOH	Reddish pink
Inorganic salts - starch agar	Scant, grayish pink	Reddish orange to deep red, orange with 0.05 N HCl, purple with 0.05 N NaOH	Light orange, partially light yellow
Tyrosine agar	Poor, grayish	Brown to deep purplish brown	Dark brown
Yeast extract - malt extract agar	Moderate, grayish pink	Deep red, orange tan with 0.05 N HCl slightly purple with 0.05 N NaOH	Light brown
Oatmeal agar	Moderate, grayish pink	Vivid reddish orange, no change with 0.05 N HCl, purple with 0.05 N NaOH	Vivid orange
Peptone - yeast extract - iron agar	None	Black	Black

Table 3. Comparison of strain C-36,145 with possibly related actinomycetes on yeast extract - malt extract agar.

Organism	Aerial mycelium	Substrate mycelium	Diffusible pigment
Actinosporangium violaceum ATCC 15813	White to pale lavender	Amber	Amber
Actinopycnidium caerulium ATCC 15812	White to blue	Reddish blue	Dark blue
Streptomyces massasporeus ATCC 19785	White to purple	Purple	Amber purple
Strain C-36,145	Grayish pink	Deep red	Light brown

Carbon sources utilized by strain C-36,145 include L-arabinose, D-xylose, D-ribose, L-rhamnose, D-glucose, D-galactose, D-fructose, D-mannose, sucrose, maltose, lactose, D-melibiose, raffinose, soluble starch, glycerol, inositol and D-mannitol. Strain C-36,145 does not utilize D-arabinose, D-melezitose, cellulose, sorbitol and dulcitol.

Gelatin is liquefied but casein is not hydrolyzed. Nitrate is reduced in CZAPEK's medium. At 0.5~4.0% NaCl growth is moderate while at 8% NaCl growth is negligible.

In Table 3 strain C-36,145 is compared with three actinomycete strains showing fused spore masses.

Production of Antitumor and Antibiotic Activity with C-36,145

After isolation from soil, strain C-36,145 was cultured initially in medium 1 (Table 4) for four days in shake-flask culture. Undiluted culture fluid inhibited Walker 256 carcinosarcoma giving a 66% reduction in tumor growth compared to the control tumor group. With the same culture medium after incubation for five days activity was observed against the P388 tumor system with 1/8 and 1/16 dilutions

Table 4. Media for production of bohemic acid complex.

Medium No.		Medium No.	
1. Glucose	30.0 g	6. Glucose	50.0 g
Soybean flour	10.0 g	Soybean flour	10.0 g
Safflower meal	10.0 g	Safflower meal	10.0 g
$CaCO_3$	3.0 g	$CaCO_3$	3.0 g
Distilled water to	1.0 liter	Distilled water to	1.0 liter
2. Glucose	30.0 g	7. Glycerol	60.0 ml
Soybean flour	20.0 g	Safflower meal	20.0 g
Peanut meal	10.0 g	$CaCO_3$	10.0 g
$CaCO_3$	3.0 g	Distilled water to	1.0 liter
Distilled water to	1.0 liter		
3. Glycerol	50.0 ml	8. Glycerol	50.0 ml
Soybean flour	20.0 g	Menhaden fishmeal**	20.0 g
Peanut meal	10.0 g	$CaCO_3$	10.0 g
$CaCO_3$	10.0 g	Distilled water to	1.0 liter
Distilled water to	1.0 liter		
4. Glycerol	50.0 ml	9. Glycerol	60.0 ml
Soybean flour	20.0 g	Menhaden fishmeal	10.0 g
Biorex collagen*	10.0 g	Linseed meal	10.0 g
$CaCO_3$	10.0 g	$CaCO_3$	10.0 g
Distilled water to	1.0 liter	Distilled water to	1.0 liter
5. Glycerol	50.0 ml	10. Glycerol	50.0 ml
Safflower meal	20.0 g	Cottonseed embryo meal	20.0 g
$CaCO_3$	10.0 g	$CaCO_3$	10.0 g
Distilled water to	1.0 liter	Tap water to	1.0 liter

* Dubuque Packing Co., Dubuque, Iowa
** Zapata Haynie Corp., Baltimore, MD.

Table 5. Activity against P388 tumor system in the extracellular fluid of C-36,145 cultures.

Fermentation vessel	Medium*	Hours of incubation	% Increase in median survival time in days** (Test group/control group × 100)					
			1/8 dil	1/16 dil	1/25 dil	1/50 dil	1/100 dil	1/200 dil
Flask	1	120	140	135, 120	120	110	—	—
Flask	2	96	130	130, 120	125	130		
Stir-jar 14 liter capacity	3	162	—	—	123	135	112	—
Fermentor 48 liter capacity	4	110	—	—	—	148, 137	156	96

* See Table 4 for medium composition.
** A value ≥125 is considered significant antitumor activity.

of culture fluid (Table 5). When other media were tested with strain C-36,145 in flasks and tanks, improved production of antitumor activity was obtained (Table 5).

Concomitant with increased antitumor activity, there was an increase in levels of antibiotic activity against *Bacillus subtilis* ATCC 6633. Both the extracellular fluid and acetone extracts of mycelium inhibited *B. subtilis* with agar diffusion assays (Table 6).

Table 6. Distribution of antibiotic activity in extracellular fluid and mycelium of C-36,145 cultures*.

Days of incubation	Antibiotic yield** (Units/ml)					
	210 Rev/minute***		230 Rev/minute		250 Rev/minute	
	Extracellular fluid	Mycelial extract	Extracellular fluid	Mycelial extract	Extracellular fluid	Mycelial extract
6	22	26	26	18	18	11
7	35	35	52	42	30	48
8	33	35	43	35	33	48
9	50	86	55	105	33	61

* Production medium 5 (Table 2).
** Agar diffusion assays with *Bacillus subtilis* ATCC 6633 with marcellomycin as an assay standard (1,000 units/mg).
*** Shaker speed.

A mixture of anthracycline antibiotics was isolated from flasks, stir-jars and tanks with strain C-36,145 cultured in media 1~4 listed in Table 2[4].

Among the isolated antibiotics marcellomycin, a pyrromycinone triglycoside, was selected for further study. Therefore, conditions were developed to enhance production of marcellomycin specifically.

Culture extracts were chromatographed with a thin-layer system that separates marcellomycin from some but not all pyrromycinone glycosides (Table 7). High pressure liquid chromatography is suitable to separate marcellomycin from other bohemic acid components and to quantitate marcellomycin content. In a flask culture with me-

Table 7. Chromatography of marcellomycin and related compounds.

Compound	Rf value	Detection method
Cinerubin B	0.93	Color and bioactive zone*
η-Pyrromycinone	0.90	Color
ε-Pyrromycinone	0.77	Color
Cinerubin A	0.56	Color and bioactive zone
Marcellomycin	0.35	Color and bioactive zone
Pyrromycin	0.17	Color and bioactive zone

Silica gel thin-layer plate (Analtech, Inc., Newark, Delaware).
Solvent system: Toluene - ethyl acetate - methanol (3:1:1).
* Bioautograph with *Bacillus subtilis* ATCC 6633.

Table 8. Induction of lysogenic *E. coli* W1709 (λ) with anthracyclines.

Anthracycline	Phage count/control*												
	Concentration in the test (μg/ml)												
	200	100	50	25	12.5	6.2	3.1	1.6	0.8	0.4	0.2	0.05	0.025
Bohemic acid complex	0.2	0.3	0.2	0.8	1.1	0.9	0.8						
Pyrromycin			0.4	1.0	0.9	0.8	1.0						
Aklavin (1-deoxypyrromycin)			0.5	1.2	1.1	1.2	1.1						
Aclacinomycin A			0.05		0.1		0.2		0.9	1.3			
Daunorubicin						18.8	11.3	6.3	3.4	2.0	1.6		
Adriamycin						19.3	14.1	10.0	3.0	2.1	1.4		
Carminomycin						5.5	11.0	6.3	3.0	2.2	1.4		
Figaroic acid complex									13.9		15.8	6.3	3.5

* Phage count/control value of 3.0 or greater is significant induction activity.

dium 7 (Table 4) marcellomycin yield was 127 μg/ml after 240 hours of incubation. On the other hand the addition of 0.005 M potassium phosphate resulted in the production of less than 7 μg/ml.

Glycerol gave superior production of marcellomycin compared to glucose, corn starch or sucrose. Safflower meal, cottonseed embryo meal, linseed meal, Menhaden fishmeal, soybean flour peanut meal and collagen were tested individually as nitrogen sources. Safflower meal, cottonseed embryo meal, linseed meal and Menhaden fishmeal were suitable for marcellomycin production. A combination of fishmeal and linseed meal gave the best results. With medium 9 (Table 4) marcellomycin was 280 μg/ml in 264 hours of incubation. A yield of 160 μg/ml was obtained at 118 hours in a 37-liter tank fermentation with medium 10. With the same medium the yield was 126 μg/ml at 111 hours in a 3,000-liter tank fermentation.

Table 9. Induction of lysogenic *E. coli* W-1709 (λ) with and without microsomes.

		Phage count/control*	
		Without microsomes	With microsomes
Marcellomycin	370 μg/ml	0.4	1.2
	37	0.9	1.0
	3.7	1.0	1.0
Sterigmatocystin	1,000	3.7	8.5
	100	1.1	3.0
Mitomycin C	1.0	20.9	11.0
	0.1	5.0	2.4

* A phage count/control value of 3.0 or greater is significant induction activity.

Testing of Marcellomycin with Lysogenic *Escherichia coli* Strain W1709 (λ)

The antitumor activity of marcellomycin has been reported in detail[25]. Certain other antitumor anthracyclines such as daunomycin, adriamycin and carminomycin I induce lysogenic phage production with *Escherichia coli* (Table 8). Therefore, marcellomycin was tested with and without treatment with microsomes for the property to induce bacteriophage. In Table 9 the data indicate that marcellomycin does not induce *E. coli* strain W1709 (λ) with or without treatment with liver microsomes at levels as high as 370 μg/ml. The activity of sterigmatocystin is enhanced with liver microsomes at 1 mg/ml confirming the observation of MOREAU *et al*[29]. Mitomycin C induces *E. coli* strain W1709 (λ) without microsomes and activity is reduced with microsomes.

Discussion

In 1961 the genus *Actinosporangium* was proposed by KRASIL'NIKOV and TSI-SHEN[30] to include those actinomycetes having sporangia consisting of mucous masses formed by the fusion of several sporophores. These investigators designated strain 3810 which exhibits such sporangia as *Actinosporangium violaceus* and placed this species in the family *Actinoplanaceae*. Extensive examination of these spore masses failed to indicate a sporangial wall. The cell wall analysis of this strain was found to be typical of the genus *Streptomyces* having LL-diaminopimelic acid and no DD-diaminopimelic acid.[28] Additional members of the genus *Actinosporangium* were described[31,32] but cell wall analyses have not been reported. *Actinosporangium* has been listed as a valid genus name[33]. In view of the cell wall analysis of strain 3810 it would be more appropriate to place the genus *Actinosporangium* in the family *Streptomycetaceae* rather than *Actinoplanaceae*.

DNA-DNA homology studies indicate that *Actinosporangium violaceum* DSM 43159 is closely related to members of the genus *Streptomyces*.[34] The usefulness of maintaining a separate genus *Actinosporangium* will be determined with the examination of more strains having morphology similar to strains 3810 and C-36,145.

Strain 3810 does not produce diffusible pigments in diagnostic media while strain C-36,145 produces

reddish orange diffusible pigments of the anthracycline type.

HUSSEIN and KRASIL'NIKOV in 1969[32] described 33 actinomycete strains having smooth spores enclosed in mucous sporangia without a sporangial membrane. Each of these strains was distributed among four species, *Actinosporangium calcenogenum*, *A. granulosum*, *A. violatum* and *A. aurantiacus*, on the basis of the color of the substrate mycelium. All these strains were reported to have beige or dark beige aerial mycelium. On the other hand strain C-36,145 has grayish pink aerial mycelium.

Streptomyces massasporeus strain 602 ATCC 15813[35,36] and *Actinopycnidium caerulium* ATCC 15812[37,38] reported to show fused spore masses were different from C-36,145 on yeast extract - malt extract agar in regard to color of aerial and substrate mycelium (Table 3).

Since strain C-36,145 shows significant differences from those organisms previously described it is placed in a new species designated *Actinosporangium bohemicum*.

Phosphate stimulates production of cinerubins with *Streptomyces griseorubiginosus*[9] and daunomycinone glycosides with *Streptomyces* sp.[39] but inhibits marcellomycin production. With strain C-36,145 phosphate appears to enhance other bohemic acid components possibly cinerubin A and B at the expense of marcellomycin and rudolphomycin. Since glucose and phosphate are commonly used in production media, this practice might explain why marcellomycin was not observed by other investigators examining cinerubin cultures with the exception of *Streptomyces galilaeus* MA 144-M1 ATCC 31133[15].

Marcellomycin and other components in the bohemic acid complex do not induce *E. coli* strain W1709 (λ). Microsomal treatment, which can enhance inducing activity of sterigmatocystin, failed to have such an effect on marcellomycin. Conversely, daunomycin, adriamycin, carminomycin and the mixture of carminomycinone glycosides called figaroic acid complex[40] were effective inducers without microsomal treatment (Table 8). Other anthracyclines such as aklavinone glycosides, nogalomycin type, rhodomycin type and pyrromycinone glycosides also fail to induce *E. coli* strain W1709 (λ). Aclacinomycin[41] and marcellomycin[42] are not mutagenic with *Salmonella typhimurium* tests while adriamycin is mutagenic[41]. All these findings are consistent with differing actions on nucleic acid synthesis. For example, adriamycin is more active than aclacinomycin in causing DNA cleavage[43] whereas aclacinomycin and several pyrromycinone oligosaccharides cause greater inhibition of RNA synthesis, particularly nucleolar RNA synthesis, than adriamycin[44].

The fact that marcellomycin had antitumor effects in several experimental systems, had a possibly more favorable toxicity profile as suggested by preliminary studies[20,45] and a differing mechanism of action compared to adriamycin and daunomycin has lead to clinical trials of marcellomycin which are now underway.

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