KUNDRYMYCIN, A NEW TUMOR-INHIBITORY ANTIBIOTIC. I
CULTURE TAXONOMY, FERMENTATION AND PRODUCTION

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An actinomycete, isolated from soil, was selected for investigation on the basis of its ability to produce an agent inhibiting WALKER 256 tumors in rats. This agent is a new antibiotic that has been designated "kundrymycin". Increased kundrymycin yield was obtained with media development and strain selection. Improvement was based on results obtained in *in vivo* tumor inhibition tests. Antibiotic yield in an 800-gallon (3,000-liter) tank fermentation was 4.2 mg/ml.

In June 1966, during the course of screening *Streptomyces* culture filtrates for *in vivo* antitumor properties, inhibition of WALKER 256 carcinosarcoma growth in rats[1] was noted with a filtrate from organism C-35,101. This organism was isolated from a soil sample collected in Michigan, U. S. A. Comparison of C-35,101 with published descriptions of other species of *Streptomyces* suggests that it is a new species. It has been designated *Streptomyces metachromogenes* sp. n. and has been deposited with the American Type Culture Collection, Rockville, Md., as ATCC 21,440. The name "kundrymycin" (NSC-114,573) has been proposed for the crystalline antibiotic isolated from fermentation broths of C-35,101. Chemical characterization suggests that it is a novel substance. A culture description of C-35,101, analytical procedures and fermentation conditions are presented. Isolation procedures, chemical properties and biological characterization of kundrymycin are described in a separate communication[2].

Taxonomic Description

An inoculum for both the parent strain C-35,101 and high-kundrymycin-producing variant 8 was prepared for diagnostic studies according to a procedure described by GOTTLIEB[3]. On solid media both organisms exhibit yellowish brown to yellowish gray aerial mycelium. Spores are arranged in chains of more than 10 spores, with open and closed spirals. Electron micrographs of spores formed on CARVAJAL's oatmeal agar[4] exhibit a hairy to spiny spore surface (Plate 1). The dimensions of spores are 0.71 μ by 1.4~2.1 μ.

Plate 1. Electron-micrograph of spores of *S. metachromogenes* sp. n. strain C-35,101, variant 8 (×13,000)

* Deceased January 1, 1971.

Table 1. Cultural characteristics of *Streptomyces metachromogenes* sp. n. strain C-35,101 (parent)

Medium	Description *	
Yeast extract agar[4]	Aerial mycelium :	Abundant, floccose, spores in spirals, Gravel plate 13 A-4 (light grayish yellowish brown #79) to Flesh plate 11 A-2 (yellowish gray #93)
	Vegetative mycelium :	Reddish tan
	Reverse :	Clay plate 13 J-8 (moderate yellowish brown #77)
	Soluble pigment :	None
Carvajal's oatmeal agar[4]	Aerial mycelium :	Abundant, floccose, spores in spirals, Atmosphere plate 12 A-3 (light grayish yellowish brown #79) to Piping Rock plate 13 A-2 (light olive gray #112)
	Vegetative mycelium :	Orange
	Reverse :	Feuille Morte plate 5 A-12 (brownish orange #54), no change with 0.05 N HCl, reddish purple with 0.05 N NaOH
	Soluble pigment :	Faint yellowish orange, no change with 0.05 N HCl, faint yellowish purple with 0.05 N NaOH
Glycerol asparagine agar[7]	Aerial mycelium :	Scant, spores in spirals, Traprock plate 15 A-2 (dark gray #266)
	Vegetative mycelium :	Rust brown
	Reverse :	Rose Beige 2 plate 5 A-10 (light brown #57), no change with 0.05 N HCl, grayish violet with 0.05 N NaOH
	Soluble pigment :	A trace of pink, no change with 0.05 N HCl or 0.05 N NaOH
Inorganic salts-starch agar[4]	Aerial mycelium :	Moderate, floccose, spores in spirals Bisque plate 11 A-3 (yellowish gray #93)
	Vegetative mycelium :	Red-brown
	Reverse :	Pheasant plate 4 B-11 (moderate orange #53), no change with 0.05 N HCl, violet with 0.05 N NaOH
	Soluble pigment :	Trace of pink

* Plate numbers refer to color blocks described by Maerz and Paul[5]. ISCC-NBS[6] designation is given in parentheses.

Growth characteristics of parent strain C-35,101 (Table 1) and variant 8 (Table 2) were obtained from cultures incubated at 28°C for 14 days on agar media in a cross-hatched pattern. Color names are listed according to color blocks of Maerz and Paul[5]. These names are converted to the ISCC-NBC color name and number[6].

Strain C-35,101 utilizes D-glucose, D-fructose, D-mannitol, D-xylose, rhamnose, sucrose, L-arabinose, raffinose and *i*-inositol when tested by the procedure of Pridham and Gottlieb[8]. Starch is hydrolyzed in inorganic salts-starch agar[4].

Hydrogen sulfide is produced in peptone iron agar (Difco Laboratories Detroit, Michigan) plus 0.1 % yeast extract (Baltimore Biological Laboratory Baltimore, Maryland). Melanin-like pigment is produced in that medium as well as in Tryptone yeast extract broth[3] and tyrosine agar[9]. Strain C-35,101 grows well on tomato paste oatmeal agar[4] at 28°C but does not grow at 50°C.

Members of the genus *Streptomyces* showing a hairy to spiny spore surface are cited by Shirling and Gottlieb[10,11,12]. These organisms do not produce a pH indicator pigment in diagnostic media. In such media strain C-35,101 and variant 8 produce a single pH indicator pigment (Tables 1 and 2) which is designated kundrymycin, a novel antitumor agent.

An organism that appears to be closely related to strain C-35,101 is *Streptomyces misawanensis* (Hamada *et* Okami), strain MA944-A5, producing aquayamycin[13]. Kun-

Table 2. Cultural characteristics of *Streptomyces metachromogenes* sp. n. strain C-35,101, high-kundrymycin-producing variant 8

Medium	Description *	
Yeast extract agar	Aerial mycelium :	Abundant velvety, spores in spirals Beaver plate 15 A-6 (brownish gray # 64)
	Vegetative mycelium :	Not exposed
	Reverse :	Piccadilly plate 7 H-10 (grayish reddish brown # 46) orange brown with 0.05 N HCl, purple brown with 0.05 N NaOH
	Soluble pigment :	Light brown
CARVAJAL'S oatmeal agar	Aerial mycelium :	Abundant velvety, spores in spirals Atmosphere plate 12 A-3 (light grayish yellowish brown # 79)
	Vegetative mycelium :	Dark orange
	Reverse :	Gypsy plate 6 B-12 (strong brown # 55) no change with 0.05 N HCl, dark purple with 0.05 N NaOH
	Soluble pigment :	Yellowish orange, no change with 0.05 N HCl, purple with 0.05 N NaOH
Glycerol asparagine agar	Aerial mycelium :	None
	Vegetative mycelium :	Dark red brown
	Reverse :	Vassar Tan plate 6 A-11 (strong brown # 55), no change with 0.05 N HCl, purple with 0.05 N NaOH
	Soluble pigment :	Light pink, no change with 0.05 HCl, faint violet pink with 0.05 N NaOH
Inorganic salts-starch agar	Aerial mycelium :	Moderate floccose, spores in spirals, Bisque plate 11 A-3 (yellowish gray # 93)
	Vegetative mycelium :	Dark reddish brown
	Reverse :	Mexico plate 5 D-11 (moderate reddish brown # 43), no change with 0.05 N HCl, purple with 0.05 N NaOH
	Soluble pigment :	Faint pink

* Plate numbers refer to color blocks described by MAERZ and PAUL[5]. ISCC-NBC[6] designation is given in parentheses.

drymycin and aquayamycin are related antitumor antibiotics, but they differ in certain properties such as molecular weight. In addition, these actinomycetes differ morphologically; namely, the projections on the spore surface of C-35,101 are longer than those of strain MA944-A5.

In view of these differences, strain C-35,101 is considered a new species and is given the name *Streptomyces metachromogenes* sp. n. Variant 8 is designated the type culture for this species.

Analytical Procedures

In early studies culture samples for analysis were centrifuged to remove mycelium. Subsequent findings indicated that the mycelium contained significant quantitites of kundrymycin and that the amount of kundrymycin in the supernatant increased as the content in the mycelium increased. To estimate kundrymycin in both mycelium and in solution, cultures were shaken for 30 minutes with methanol (1 part culture sample, 29 parts methanol). Methanol was removed by centrifugation, and the sediment was re-extracted twice with 15 ml portions of methanol. Antitumor activity was measured with the intramuscularly-transplanted WALKER 256 carcinosarcoma in rats[1].

Antibiotic activity was measured by means of a cylinder type agar diffusion assay. The assay organism was *Bacillus subtilis* ATCC 6633 seeded into Streptomycin Assay

Agar with Yeast Extract (Baltimore Biological Laboratory, Baltimore, Maryland). Assay plates were incubated at 30°C for 18 hours. When kundrymycin became available in crystalline form, levels from 0.2 to 10 μg/ml were used as reference concentrations for quantitative bioassays.

A colorimetric procedure for estimation of kundrymycin is based on a measurement of the blue color of kundrymycin in 0.1 N NaOH. Crystalline kundrymycin was dissolved in methylene chloride and diluted appropriately in methanol containing 20 % methylene chloride to give reference solutions. Immediately after addition of methanolic NaOH, reference and unknown solutions at appropriate concentrations were read with a Bausch and Lomb spectrophotometer set at a wave length of 548 mμ. Kundrymycin is unstable in alkaline solution, but concentrations from 10 to 50 μg/ml read within 1 minute after addition of alkali gave a linear curve with a correlation coefficient greater than 0.999.

Thin layer chromatography of culture extracts failed to give evidence of other pH indicator compounds that might interfere with a colorimetric assay. A preferred silica gel thin-layer solvent system for kundrymycin consists of methanol - toluene - formic acid (95 : 5 : 0.5). To obtain a bioautograph, thin-layer chromatograms were overlayed with Streptomycin Assay Agar with Yeast Extract seeded with *B. subtilis* ATCC 6633. After incubation for 18 hours at 30°C a 1.25 % aqueous solution of 2,3,5-triphenyl 2H-tetrazolium chloride containing 1.25 % glucose was sprayed on the surface of the agar. Areas containing *B. subtilis* growth became red; while zones of inhibition remained colorless. Five μg of kundrymycin on a thin-layer plate gave a yellow spot at Rf 0.78 that coincided with a zone of bacterial inhibition. Chromatography of culture samples showed evidence of other zones of inhibition; however, these zones were not investigated further.

Table 3. Antitumor activity with kundrymycin production media

Production media		Walker 256 carcinosarcoma Tumor test* Highest active dilution giving 58% or greater tumor weight inhibition
1. Corn starch	4.0 %	1/2
Peanut meal	2.0 〃	
$CaCO_3$	0.4 〃	
2. Glucose hydrate	4.0 〃	1/8
Pharmamedia	1.0 〃	
Peanut meal	1.0 〃	
$CaCO_3$	0.5 〃	
3. Glucose hydrate	5.0 〃	1/8
Pharmamedia	2.0 〃	
Safflower meal	1.0 〃	
$CaCO_3$	0.5 〃	
4. Glucose hydrate	4.0 〃	1/16
Menhaden fishmeal	1.0 〃	
Peanut meal	2.0 〃	
$CaCO_3$	0.5 〃	
5. Glucose hydrate	6.0 〃	1/25
Soybean flour	3.0 〃	
$CaCO_3$	0.5 〃	

* Tests conducted with culture samples clarified by centrifugation.

Fermentation Conditions

Strain C-35,101 was grown on an agar slant medium consisting of 2 g glucose, 20 g oatmeal, 2 g soy peptone, 20 g agar and 1 liter distilled water. After 7 days of incubation at 28°C, spores were transferred to a 500-ml Erlenmeyer flask with 100 ml of vegetative medium consisting of 30 g Cerelose (glucose monohydrate from Corn Products Sales Co., Englewood Cliffs, New Jersey, U. S. A.), 10 g Nutrisoy (soybean flour from Archer Daniels Midland Co., Minneapolis, Minnesota,

U. S. A), 10 g Pharmamedia (cottonseed embryo meal from Traders Oil Mill Co., Fort Worth, Texas, U. S. A.), 3 g $CaCO_3$ and 1 liter distilled water. The culture was incubated at 27°C on a Gyrotory tier shaker (Model G53, New Brunswick Scientific Co., Inc., New Brunswick, New Jersey, U. S. A.) set at 212 r.p.m. describing a 2-inch diameter circle. After 48 hours, 4 ml of vegetative culture was transferred to 100 ml of production medium.

A series of media was evaluated for production of antitumor activity with conditions as described for preparation of vegetative inoculum, except that the shaker was set at 230 r.p.m. and samples were taken from 72 to 168 hours. Productivity was evaluated when the highest dilution of clarified culture sample giving *in vivo* tumor inhibition was determined. Representative production media, showing progressive improvement in antitumor activity, are listed in Table 3.

Production of antitumor activity with C-35,101 was detected for the first time in Medium 1 (Table 3). Early trials indicated that glucose hydrate gave better activity than corn starch. Paired combinations of soybean flour, peanut meal, linseed meal, Pharmamedia, safflower meal and Menhaden fish meal were tested in media containing 4~6 % glucose hydrate and 0.5 % $CaCO_3$. The best results, however, were obtained with Medium 5 (Table 3), giving antitumor activity at a 1/25 dilution of clarified broth.

Further yield improvement resulted from evaluation of 20 isolated strains of C-35,101, obtained by selection of colonies from platings without prior mutagenic treatment. Two isolates gave antitumor activity in Medium 5 at a 1/50 dilution. One of these, designated Variant 8, appeared superior and was selected for further study.

Variant 8 was used to produce kundrymycin in tank fermentors with Medium 5. A tank with 10 gallon (*ca.* 38 liters) of production culture was agitated with an impeller speed of 375 r.p.m. and air was supplied at 0.7 ft^3/min. (*ca.* 20 liters/min.). A tank with 800 gallon (*ca.* 3,000 liters) of production culture was agitated with an impeller speed of 155 r. p. m. and air was supplied at 75 ft^3/min. (*ca.* 2,900 liters/min.). At 166 hours kundrymycin yield was 4.2 mg/ml.

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