U-43,120; A NEW ANTITUMOR ANTIBIOTIC

I. PRODUCTION, BIOLOGICAL ACTIVITY, MICROBIOLOGICAL ASSAY AND TAXONOMY OF THE PRODUCING MICROORGANISM

L. J. HAŇKA and A. DIETZ

Research Laboratories, The Upjohn Company
Kalamazoo, Michigan, U. S. A.

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A new antitumor antibiotic, U-43,120 was discovered. It is produced by fermentation of a new species of *Streptomyces*, designated *Streptomyces paulus* DIETZ sp. n. Its antimicrobial activity is limited to bacteria. A microbiological assay with *Bacillus subtilis* was developed that can detect concentrations of 1~2 μg/ml of the drug in fermentation liquors. U-43,120 was active *in vivo* against P-388 leukemia in mice.

During the screening program for new antitumor drugs, a new antibiotic U-43,120 was discovered. This communication describes the producing microorganism, the fermentation conditions, a suitable microbiological assay and the biological activity of the new drug. The isolation and chemical characterization of U-43,120 was described by P. F. WILLEY.[1]

Materials and Methods

Taxonomy

A new species of *Streptomyces* isolated from soil has been characterized as *Streptomyces paulus* DIETZ sp. n. The methods used were those cited by DIETZ,[2]* DIETZ and MATHEWS[3] and SHIRLING and GOTTLIEB.[4]

Production

Stock cultures of the producing microorganism were frozen plugs made from a Petri plate with heavy surface growth and kept in a liquid nitrogen storage tank. The seed medium contained 25 g of cerelose (Corn Product Sales Co., Detroit, MI) and 25 g of Pharmamedia (Traders Oil Mill Co., Fort Worth, TX) per liter of tap water. It was inoculated with the stock culture and incubated on a rotary shaker for 48 hours at 28°C and used at a rate of 5% (v/v).

The production medium for U-43,120 contained 10 g/liter of cerelose, 30 g/liter of malt extract, 20 g/liter of liquid peptone (Wilson Protein Technology, Calumet City, Ill.) and 5 g/liter of corn steep liquor. The fermentation was carried out at 28°C in 500-ml non-stippled flasks, containing 100 ml of medium on a rotary shaker at 250 rpm (2.5″ stroke).

In Vitro Evaluation

A broad antimicrobial evaluation of U-43,120 was done by a disc-plate assay method using the microorganisms and cultivation media previously described.[7]

Microbiological Assay

The fermentation titers were estimated by a disc-plate assay with *Bacillus subtilis* UC-564 cultivated in a completely synthetic medium.[7] The molten agar was inoculated with a suspension containing 1.5×10^{10} spores/ml at a rate of 0.5 ml/liter. The fermentation liquors were applied at appropriate dilutions to the 12.7 mm paper discs (Carl Schleicher & Schuell Co., Keene, NH). The assay plates were incubated for 16~20 hours at 37°C and the zones of inhibition were recorded.

* Modified. Reference color from ISCC-NBS Color Names Chart for NBS Circular 553[5] only. The Color Harmony Manual[6] is no longer available.

In Vivo Studies

The *in vivo* evaluation of U-43,120 was done in mice inoculated with P-388 leukemia according to the protocol of the National Cancer Institute. The testing was done at the Illinois Institute of Technology in Chicago.

Results and Discussion

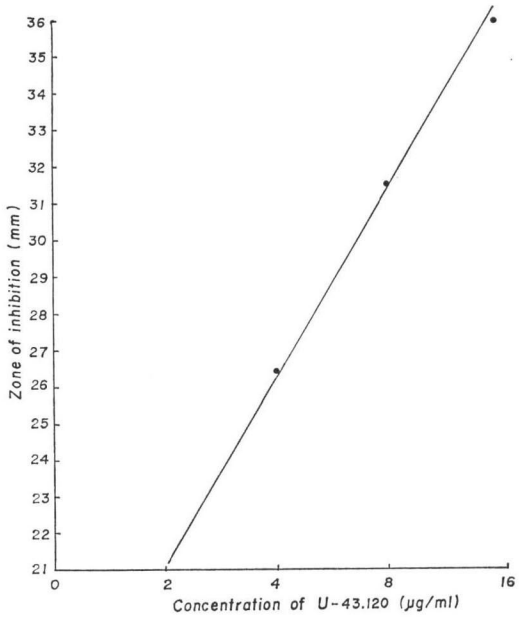
Taxonomy

Color Characteristics. Aerial growth cream to olive. Melanin negative. Appearance on Ektachrome is given in Table 1. Reference color characteristics are given in Table 2. The culture may be placed in the yellow color group of TRESNER and BACKUS.[8]

Table 1. Appearance of *Streptomyces paulus* on Ektachrome[18]

Agar medium	Surface	Reverse
BENNETT'S	Cream-white	Tan
CZAPEK's sucrose	Cream-white	Colorless
Maltose-tryptone	Cream-white	Tan
Peptone-iron	Cream-white	Tan
0.1% Tyrosine	Trace cream-white	Tan
Casein starch	Trace cream-white	Very pale tan

Fig. 1. Disc-plate assay with *B. subtilis* for U-43,120



Microscopic Characteristics. Spore chains long, flexuous (RF) in the sense of PRIDHAM *et al.*[9] Spore chains may be in tufts. Spores, examined with the scanning electron microscope, appear spherical with a smooth surface. The procedure was that cited by DIETZ and MATHEWS.[3]

Cultural and Biochemical Characteristics. Cultural and biochemical characteristics are cited in Table 3.

Carbon Utilization. In the synthetic medium of PRIDHAM and GOTTLIEB,[10] *S. paulus* growth on the control (basal medium without added compound) was moderate. Growth was good on D-xylose, L-arabinose, D-fructose, D-galactose, D-glucose, D-mannose, maltose, cellobiose, dextrin, soluble starch, glycerol, D-mannitol, salicin, sodium citrate, and sodium succinate; moderate on rhamnose, sucrose, lactose, raffinose, inulin, dulcitol, D-sorbitol, inositol sodium tartrate, and sodium acetate; poor on sodium formate and sodium oxalate. The culture did not grow on phenol, cresol, or sodium salicylate. In the synthetic medium of SHIRLING and GOTTLIEB[4] growth was poor on the basal medium without added compounds, good on the D-glucose control and on D-xylose, D-mannitol, and D-fructose; fair on rhamnose; poor on L-arabinose, sucrose, and inositol. There was no growth on raffinose and cellulose.

Temperature. *S. paulus* had good aerial growth on BENNETT's, CZAPEK's sucrose, maltose-tryptone, and HICKEY-TRESNER agars at 18°C, 24°C, 28°C and fair growth on these media at 32°C and 37°C. The culture did not grow at 45°C or 55°C.

Table 2. Reference color characteristics of *Streptomyces paulus*

Agar medium	Determination	ISCC-NBS color-name charts illustrated with centroid colors[5]		
		Chip No.	Color	
BENNETT'S	S	90	gy. y.	Grayish yellow
		92	y. white	Yellowish white
	R	95	m.Ol.Br.	Deep olive
	P	72	d.O.Y.	Dark orange yellow
CZAPEK's sucrose	S	92	y.white	Yellowish white
	R	92	y.white	Yellowish white
	P	—	—	—
Maltose-tryptone	S	92	y.white	Yellowish white
		93	y.gray	Yellowish gray
	R	95	m.Ol.Br.	Deep olive
	P	94	I.Ol.Br.	Olive
HICKEY-TRESNER	S	92	y.white	Yellowish white
		90	gy.y.	Grayish yellow
	R	77	m.y.Br.	Yellowish brown
	P	76	I.y.Br.	Dull yellowish brown
Yeast extract-malt extract (ISP-2)	S	89	p.y.	Pale yellow
		90	gy.y.	Pale yellow
	R	96	d.Ol.Br.	Dark brown
	P	95	m.Ol.Br.	Dark brown
Oatmeal (ISP-3)	S	92	y.white	Yellowish white
	R	87	m.Y.	Yellow Brazil Wood (yellow)
		88	d.Y.	Yellow
	P	87	m.Y.	Yellow Brazil Wood (yellow)
Inorganic-salts starch (ISP-4)	S	90	gy.y.	Pale yellow
		92 to 105 (edge)	y.white gy.g.Y	Yellowish white Grayish greenish yellow
	R	95	m.Ol.Br.	Dark brown
	P	91	d.gy.Y.	Dark grayish yellow
Glycerol-asparagine (ISP-5)	S	92	y.white	Yellowish white
	R	72	d.O.Y.	Dark orange yellow
	P	72	d.O.Y.	Dark orange yellow

Antibiotic-Producing Properties. The culture produces U-43,120.

Source. Soil.

Type Culture. *Streptomyces paulus* sp. n. UC® 5231.

Based on these results, this culture may be placed in the Helvolus series of GAUZE,[11] the Streptomyceten mit griseus-Luftmycel of HÜTTER,[12] the variants of *Actinomyces albus* of KRASIL'NIKOV,[13] or the "Yellow series (17.43b)" of PRIDHAM and TRESNER in BERGEY's Manual, 8th Edition.[14] The soil isolate is differentiated from species in the references cited by the characteristics noted in the tables. In Table 4 the culture is differentiated from *Streptomyces albidoflavus*[14,15] and *Streptomyces globisporus*[15,16,17] the cultures to which it appeared similar.

S. albidoflavus is reported to have spiral spore chains by GAUZE[11] and is placed in his "Albus

Table 3. Cultural and biochemical characteristics of *Streptomyces paulus*.

	Medium	Surface	Reverse	Other characteristics
Agar	Peptone-iron	Pale pink	Yellow-tan with red edge	No pigment Melanin negative
	Calcium malate	Trace pale yellow	Pale yellow	No pigment Malate slightly solubilized under growth
	Glucose-asparagine	Cream	Yellow	Pale yellow pigment
	Skim milk	—	Tan	Tan pigment Casein solubilized
	Tyrosine	Cream	Tan	Tan pigment Tyrosine solubilized
	Xanthine	Cream	Cream olive	Pale yellow-tan pigment Xanthine solubilized
	Nutrient starch	Cream	Cream olive	Pale yellow-tan pigment Starch solubilized
	Yeast extract-malt extract	Pale olive-cream	Tan-brown	Pale tan pigment
	Peptone-yeast extract-iron (ISP-6)	Very slight trace white	Pale yellow tan	No pigment
	Tyrosine (ISP-7)	Cream	Yellow tan	No pigment
Gelatin	Plain	Cream white	—	Yellow pigment 1/4 Liquefaction complete
	Nutrient	White	—	Yellow pigment Liquefaction complete
Broth	Synthetic nitrate	Trace white aerial growth on thin surface pellicle	—	Poor compact bottom growth Nitrate not reduced to nitrite
	Nutrient nitrate	Cream aerial growth on surface pellicle	—	Yellow pigment No bottom growth Nitrate reduced to nitrite
	Litmus milk	Blue-gray aerial growth on surface pellicle Blue-gray-green surface ring	—	Peptonization-partial to complete Litmus reduced pH 7.4～7.6

Series." *Streptomyces globisporus* of KRASIL'NIKOV[18] belongs to the variants of *Actinomyces globisporus*.

The culture characterized is considered to be a new species of *Streptomyces*. The consideration is justified by the differences noted in Table 4 and in the references cited for the cultures with which it was compared. The new soil isolate is designated *Streptomyces paulus* sp. n. It is understood that this culture is the type species and that it will become the type variety should cultures with similar properties be isolated.

Production

The results of a typical fermentation carried out in 500 ml fermentation flasks are presented in Table 5. Samples of fermentation liquors were collected on days 2～5 and the potency was estimated by microbiological assay against *B. subtilis*.

In Vitro Antimicrobial Activity. The inhibition by U-43,120 (1 mg/ml) of different microorganisms is presented in Table 6. The numbers in the body of the table are diameters of the zones of inhibition (mm) around a 13.6-mm disc.

Table 4. Comparison of *Streptomyces paulus*, *Streptomyces albidoflavus*, and *Streptomyces globisporus*

Test condition	*Streptomyces paulus* UC 5231	*Streptomyces albidoflavus*		*Streptomyces globisporus*	
		UC 2190 (CBS)*	CBS 416.34 (ISP 5455)(15)	UC 5398 (NRRL B-2872)(17)	INMI 2302 (ISP-5199)(16)
Spore chain morphology	Section Rectiflexibilis (RF long)	Section Rectiflexibilis (RF long)	Section Rectiflexibilis (RF short)	Section Rectiflexibilis (RF long)	Section Rectiflexibilis (RF long)
Spore surface	Smooth	Smooth	Smooth	Smooth	Smooth
Spore chains	Abundant	Abundant	Sparse	Abundant	Good
Aerial mass color	Yellow	Yellow	White or gray	Yellow	Yellow
Carbon utilization					
D-Glucose	Good	Good	Good	Good	Good
L-Arabinose	Poor (doubtful)	Poor	Good	Good	Good
Sucrose	Poor (doubtful)	Negative	Poor	Good	Good
D-Xylose	Very good	Very good	Doubtful	Very good	Negative
Inositol	Poor	Negative	Doubtful	Very good	Negative
D-Fructose	Very good	Good	Doubtful	Very good	Good
D-Mannitol	Very good	Very good	Negative	Negative	Good
Rhamnose	Good	Negative	Negative	Very good	Good
Raffinose	Negative	Negative	Negative	Negative	Negative
Calcium malate agar	Malate slightly solubilized	Malate solubilized	—	Malate not solubilized	—
Peptone-iron agar	Pale pink aerial growth	No aerial growth	—	Cream white aerial growth	—
Plain gelatin	Complete liquefaction	No liquefaction	—	Trace liquefaction	—
Nutrient gelatin	Complete liquefaction	Trace liquefaction	—	Complete liquefaction	—
Litmus milk	Litmus reduced pH 7.4~7.6	Litmus reduced pH 6.9	—	Litmus reduced pH 7.7	—
CZAPEK's sucrose agar	Good aerial growth	No aerial growth	—	Fair aerial growth	Excellent
Antibiotic produced	U-43,120	None cited	None cited. The culture is reported to exhibit antibacterial and antifungal activity.	None cited	None cited. The culture is reported to exhibit antibacterial and antifungal activity.

* Received in 1954 before CBS cultures were numbered. (probably CBS 416.34=type strain)

It appears that the *in vitro* activity of U-43,120 is mostly limited to Gram-positive bacteria. There was no activity against fungi.

Microbiological Assay. The microbiological disc-plate assay for U-43,120 with *B. subtilis* cultivated in a synthetic medium is presented in Fig. 1.

It appeared that the lowest detectable concentration was about 1~2 μg/ml. The linear part of the curve was between 2 and 16 μg/ml.

In Vivo Antitumor Evaluation. The drug was administered (i.p.) on 9 consecutive days (1~9) at 5 levels to groups of mice inoculated with P-388 leukemia. The results of the study are presented in Table 7.

The drug was toxic to the mice at the two highest levels used. It has demonstrated *in vivo* activity at 50, 25 and 12.5 mg/kg since any increase in life span over 25% is considered significant.

Table 5. Fermentation of U-43,120 in 500-ml flasks

Time (hours)	Diameter of the zone of inhibition (mm)	pH
48	trace	7.2
72	27	6.4
96	31	5.8
120	34	6.0

As a rule the highest titre was reached following 5 days of incubation at the specified conditions. The methods of isolation were described by P. F. WILEY.[1]

Table 6. Microbiological spectrum of U-43,120

Microorganism	Taxonomic designation	Zones of inhibition
Bacillus cereus	Gram-positive bacterium	24
Bacillus subtilis	Gram-positive bacterium	17
Lactobacillus casei	Gram-positive bacterium	19
Micrococcus luteus	Gram-positive bacterium	31
Staphylococcus aureus	Gram-positive bacterium	29
Streptococcus faecalis	Gram-positive bacterium	16
Mycobacterium phlei	Gram-positive bacterium	15
Propionibacterium thoenii	Gram-positive bacterium	28
Klebsiella pneumoniae	Gram-negative bacterium	0
Salmonella gallinarum	Gram-negative bacterium	trace
Azotobacter vinelandii	Gram-negative bacterium	15
Rhodopseudomonas spheroides	Gram-negative bacterium	19
Chromobacterium violaceum	Gram-negative bacterium	18
Saccharomyces cerevisiae	Yeast	0
Trigonopsis variabilis	Yeast	0
Torulopsis albida	Yeast	0
Glomerella cingulata	Filamentous fungus	0
Penicillium oxalicum	Filamentous fungus	0
Ochromonas danica	Protozoan	17
Crithidia fasciculata	Protozoan	0
Chlorella vulgaris	Alga	0
Prototheca zopfii	Alga	0

Table 7. Evaluation of U-43,120 against P-388 leukemia in mice

Dose (mg/kg)	Increase in life span (%)
150	toxic
100	toxic
50	50
25	50
12.5	39

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References

1) WILEY, P. F.: A new antibiotic, U-43,120. J. Antibiotics 29: 587~589, 1976
2) DIETZ, A.: *Streptomyces steffisburgensis* sp. n. J. Bact. 94: 2022~2026, 1967
3) DIETZ, A. & J. MATHEWS: Classification of *Streptomyces* spore surfaces into five groups. Appl. Microbiol. 21: 527~533, 1960
4) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
5) KELLY, K. L. & D. B. JUDD: The ISCC-NBS method of designating colors and a dictionary of color names. U. S. Dept. of Comm. Circ. 553, Washington, D. C., 1955
6) JACOBSON, E.; W. C. GRANVILLE & C. E. FOSS: Color harmony manual. 3rd Edition, Container Corporation of America, Chicago, 1948
7) HAŇKA, L. J.: Correlative microbiological assays. Advances in Appl. Microbiol. 15: 147~156, 1972
8) TRESNER, H. D. & E. J. BACKUS: System of color wheels for streptomycete taxonomy. Appl. Microbiol. 11: 335~338, 1963
9) PRIDHAM, T. G.; C. W. HESSELTINE & R. G. BENEDICT: A guide for the classification of streptomycetes according to selected groups. Placement of strains in morphological sections. Appl. Microbiol. 6: 52~79, 1958
10) PRIDHAM, T. G. & D. GOTTLIEB: The utilization of carbon compounds by some Actinomycetales as an aid for species determination. J. Bact. 56: 107~114, 1948
11). GAUZE, G. F.; T. P. PREOBRAZHENSKAYA, E. S. KUDRINA, N. O. BLINOV, I. D. RYABOVA & M. A. IVESHNIKOVA: Problems in the classification of antagonistic actinomycetes. State Publishing House for Medical Literature, Moscow. English edition translated by F. DANGA; D. GOTTLIEB (ed.). The American Institute of Biological Sciences, Washington, D. C., 1957
12) HÜTTER, R.: Systematik der Streptomyceten. S. Karger, Basel, p. 382, 1967
13) KRASIL'NIKOV, N. A.: Keys to Actinomycetales. Academy of Sciences of the U. S. S. R., Institute of Microbiology, 1941. English edition published for the U. S. Department of Agriculture and the National Science Foundation, Washington, D. C., by the Israel Program for Scientific Translations. Jerusalem, 1966
14) BUCHANAN, R. E. & N. E. GIBBONS: BERGEY's Manual of Determinative Bacteriology. 8th Edition, The Williams and Wilkins Co., Baltimore, 1974
15) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type culture of *Streptomyces*. IV. Species descriptions from the second, third and fourth studies. Int. J. Syst. Bacteriol. 19: 391~512, 1969
16) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. III. Additional species descriptions from first and second studies. Int. J. Syst. Bacteriol. 18: 280~399, 1964
17) SUGIMOTO, H.: Lysis of yeast cell wall by enzymes from streptomycetes. Agr. Biol. Chem. 31: 1111~1123, 1967
18) DIETZ, A.: Ektachrome transparencies as aids in actinomycete classification. Ann. N. Y. Acad. Sci. 60: 152~154, 1954