SPENOLIMYCIN, A NEW SPECTINOMYCIN-TYPE ANTIBIOTIC

I. DISCOVERY, TAXONOMY AND FERMENTATION

JAMES P. KARWOWSKI*, MARIANNA JACKSON, THOMAS A. BOBIK,
JOSEPH F. PROKOP and ROBERT J. THERIAULT

Pharmaceutical Products Division, Abbott Laboratories,
North Chicago, Illinois 60064, U.S.A.

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Spenolimycin, a new spectinomycin-type antibiotic, was discovered in the fermentation broth of a new actinomycete named *Streptomyces gilvospiralis* sp. nov. strain AB634D-177. Although a small amount of spectinomycin is coproduced by strain AB634D-177, the culture is different from other known spectinomycin-producing actinomycetes. Spenolimycin was obtained by conventional submerged culture in 14-liter fermentors with a peak antibiotic titer of 140 μg/ml.

Spenolimycin is a new antibiotic isolated from the culture broth of *Streptomyces gilvospiralis* sp. nov. The antibiotic is coproduced with, and is structurally related to, spectinomycin. Spenolimycin displays broad spectrum activity similar to that of spectinomycin.

Many antibiotics are found in nature as mixtures of structurally related congeners. Even though spectinomycin was discovered in 1961[1,2], until now only one other related, naturally-occurring aminocyclitol antibiotic, dihydrospectinomycin[3], has been reported. Spenolimycin is the third member of this unique structural class to be obtained by fermentation.

This paper reports the discovery of spenolimycin, the taxonomy of the producing culture, and its production by fermentation. The isolation, structure determination, and biological properties of spenolimycin are described in accompanying papers[4,5].

Materials and Methods

Microorganisms

The producing culture was isolated from a soil sample collected in West Haven, Connecticut, U.S.A. The isolate was originally referred to as strain AB634D-177 and later designated *Streptomyces gilvospiralis* sp. nov. The bacterial strains used to obtain the activity spectra of spenolimycin were from the stock culture collection in our laboratory and from the ATCC.

Taxonomic Studies

Methods adopted by the International Streptomyces Project (ISP)[6] were used for taxonomic characterization and carbohydrate utilization studies. Cultural characteristics were determined on media recommended by the ISP and WAKSMAN[7]. Observations were made after incubation at 28°C for 14 days. Whole-cell hydrolysates were prepared and analyzed by the methods described by KUTZNER[8].

Fermentation Studies

Well sporulated slants of strain AB634D-177 grown on ATCC medium 172[9] were used to inoculate the seed medium which consisted of Cerelose (Corn Products) 0.1%, soluble starch 2.4%, yeast extract (Difco) 0.5%, Tryptone (Difco) 0.5%, beef extract (Inolex) 0.3% and $CaCO_3$ 0.4%, in distilled water. The pH was adjusted to 7.0 prior to sterilization. Inoculum for antibiotic production was prepared in two stages. The culture was incubated for 96 hours in a 25×150 mm culture tube containing 10 ml of

Table 1. Antimicrobial activity of 5-day spenolimycin fermentation broth.

Organisms	Agar diffusion zone (mm), 18 hours		
	pH 8[a]	pH 6.5[b]	pH 7.2[c]
Staphylococcus aureus ATCC 6538P	28	14	
Escherichia coli SS	30	20	
E. coli ATCC 26	25	16	
Proteus vulgaris ATCC 6897	23	12	
Klebsiella pneumoniae ATCC 8045	27	18	
Pseudomonas aeruginosa BMH 1	18	0	
P. aeruginosa BMH 10	31	21	
Bacteroides fragilis 784			14
Neisseria gonorrhoeae 708			16

[a] Streptomycin assay agar with yeast extract(BBL).
[b] Penassay agar (Difco).
[c] *B. fragilis* was grown anaerobically on Wilkins Chalgren agar. *N. gonorrhoeae* was grown in a 7% CO_2 atmosphere on GC agar base (Difco) supplemented with 1% (v/v) IsoVitalex (BBL) and 1% (w/v) hemoglobin.

Table 2. Paper chromatography of fermentation broth and basic antibiotics.

Antibiotic	Rf Value
Destomycins A and B	0.00
Dihydrostreptomycin	0.00
Fortimicin A	0.10
Gentamicin C complex	0.12
Kanamycin	0.00
Neomycin	0.00
Neamine	0.00
Nebramycin complex	0.00
Ribostamycin	0.00
Sisomicin	0.00
Spectinomycin	0.28
Streptolin complex	0.02
Streptothricin	0.00
Streptomycin	0.02, 0.16
5-Day fermentation broth	0.31, 0.50

seed medium, and then 5 ml was transferred to a 500-ml Erlenmeyer flask containing 100 ml of the same medium. After 72 hours, 5% (v/v) inoculum was transferred to production fermentors. Seed tubes and flasks were incubated at 26°C and agitated on a rotary shaker at 250 rpm (3.2 cm stroke).

Fermentations were performed in New Brunswick 14-liter fermentors charged with 10 liters of medium and sterilized for 1 hour at 121°C. The fermentors were incubated at 26°C, aerated at 1.0 v/v/minute, and agitated at 300 rpm. The production medium consisted of Cerelose (Corn Products) 1.0%, peptone (Difco) 0.5%, yeast extract (Difco) 0.5%, $CaCO_3$ 0.1% and P-2000 antifoam (Dow) 0.01% (v/v), in distilled water. The pH of the production medium was adjusted to 7.3 prior to sterilization.

Packed cell volumes were determined by centrifuging at $600 \times g$ for 20 minutes in 15 ml conical tubes. Glucose concentrations were determined by the method of HOFFMAN[10].

Paper Chromatography/Bioautography

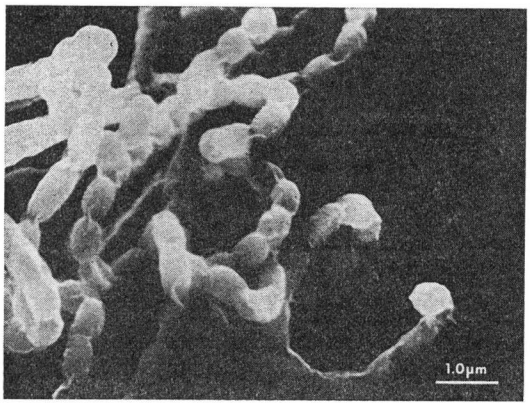
Fermentation broths and partially purified antibiotic preparations were applied to 1 cm wide What-

Table 3. Antimicrobial activity of partially purified spenolimycin against aminoglycoside-resistant bacteria.

Organisms	Resistance markers[a]	Agar diffusion zone (mm), 18 hours[b]
Escherichia coli ATCC 26	No known resistance	26
E. coli R_3	AMP, CP, GM, KM, NM, SM, TC	18
E. coli 76-2	AMP, CP, GM, KM, SM, TOB	39
Proteus vulgaris ATCC 6897	No known resistance	21
P. vulgaris U-953	AMK, FOT, GM, TOB	31
Klebsiella pneumoniae ATCC 8045	No known resistance	26
K. pneumoniae 4262	GM, TOB	30
Enterobacter cloacae 5053	No known resistance	21
E. cloacae ST-10	FOT, GM	0
Pseudomonas aeruginosa BMH 1	No known resistance	22
P. aeruginosa B-15352	GM	20

[a] AMK=Amikacin, AMP=ampicillin, CP=chloramphenicol, FOT=fortimicin, GM=gentamicin, NM=neomycin, KM=kanamycin, SM=streptomycin, TC=tetracycline, TOB=tobramycin.
[b] Streptomycin assay agar with yeast extract (BBL) at pH 8.0.

Fig. 1. Scanning electron micrograph of aerial mycelium of strain AB634D-177 (inorganic salts - starch agar, 14 days, 30°C).



man No. 1 paper strips. The strips were developed ascending for 18 hours in a solvent system consisting of 2% (w/v) *p*-toluenesulfonic acid and 2% (v/v) piperidine in BuOH saturated with water. Partially purified antibiotic was prepared by passing culture filtrate at pH 6.5 through a column of Amberlite XAD-2 resin (Rohm and Haas) and eluting with MeOH.

Antibiotics were detected by bioautography against *Staphylococcus aureus* ATCC 6538P and *Klebsiella pneumoniae* ATCC 8045.

Antimicrobial Activity

The spectrum of antimicrobial activity of fermentation broth and partially purified antibiotic preparations was determined against a panel of Gram-positive and Gram-negative bacteria using the agar diffusion method. The titer of antibiotic in fermentation broth was determined using a bioassay in which 25 μl of the broth was pipetted onto paper discs which were placed on agar plates seeded with *K. pneumoniae* ATCC 8045. Spenolimycin sulfate, factored for free base equivalents, was the reference standard.

Table 4. Cultural characteristics of strain AB634D-177.

Medium	Cultural characteristics	Medium	Cultural characteristics
Yeast extract - malt extract agar	G*: Excellent; flat AM: Yellowish white (92)** R: Strong yellowish brown (74) SP: None	Tyrosine agar	G : Excellent; raised AM: Pale yellow green (121) R: Dark yellowish brown (78) SP: Moderate, yellowish brown (77)
Oatmeal agar	G: Excellent; flat, small colonies AM: Pale yellow (89) R: Dark orange yellow (72) SP: None	Nutrient agar	G: Excellent AM: Yellowish white (92) R: Dark orange yellow (72) SP: None
Inorganic salts - starch agar	G: Moderate AM: Pale yellow green (121) R: Yellowish gray (93) SP: None	Calcium malate agar	G: Moderate AM: None R: Light yellow (86) SP: None
Gycerol - asparagine agar	G: Excellent AM: Yellowish white (92) R: Light yellowish brown (76) SP: None	CZAPEK's agar	G: Moderate AM: Yellowish white (92) R: Yellowish gray (93) SP: None
Peptone - yeast extract iron agar	G: Excellent; smooth AM: None R: Deep brown (56) SP: None		

* Abbreviations: G=growth; AM=aerial mycelium; R=reverse; SP=soluble pigment.

** Color and number in parenthesis follow the color standard in KELLY, K. L. & D. B. JUDD: ISCC-NBS Color-Name Charts Illustrated with Centroid Colors. US Dept. of Comm. Suppl. to Cir. 553, Washington, D.C.

Table 5. Physiological characteristics of strain AB-634D-177.

Test	Reaction
Gelatin liquefaction	Positive
Cellulose decomposition	Negative
Starch hydrolysis	Positive
Nitrate reduction	Negative
Melanin formation	Positive on synthetic tyrosine agar
	Negative on complex media
NaCl tolerance	0~10%
Temperature range	Growth at 15~32°C
	(No growth at 4 or 40°C)
pH range	5~11
	(No growth at pH 4.0)

Table 6. Utilization of carbohydrates by strain AB634D-177.

Carbon source	Growth
None	−
D-Glucose	++
L-Arabinose	++
Cellulose	−
D-Fructose	++
D-Galactose	++
Inositol	−
Mannitol	++
Raffinose	++
L-Rhamnose	++
Sucrose	−
D-Xylose	++

++ Good utilization, − does not utilize.

Table 7. Comparison of *Streptomyces* sp. AB634D-177 with spectinomycin producers.

Characteristic	*Streptomyces* sp. AB634D-177	*Streptoverticillium flavopersicum* NRRL 2820	*Streptomyces spectabilis* NRRL 2494	*Streptomyces hygroscopicus* subsp. *sagamiensis* ATCC 21703	*Streptomyces* sp. ATCC 21853
Spore chain morphology	Spiral	Verticil	Straight~flexuous	Spiral	Spiral
Spore surface	Smooth	Smooth	Smooth	Warty	Smooth
Chromogenicity	−	+	+	−	−
Color of aerial mycelium	Yellow	Pinkish-beige	Red	Gray	Blue
Carbon source utilization					
Inositol	−	+	+	−	+
Mannitol	+	−	+	+	−
Raffinose	+	−	+	−	−
Rhamnose	+	−	−	−	−
Xylose	+	−	+	+	+

Results and Discussion

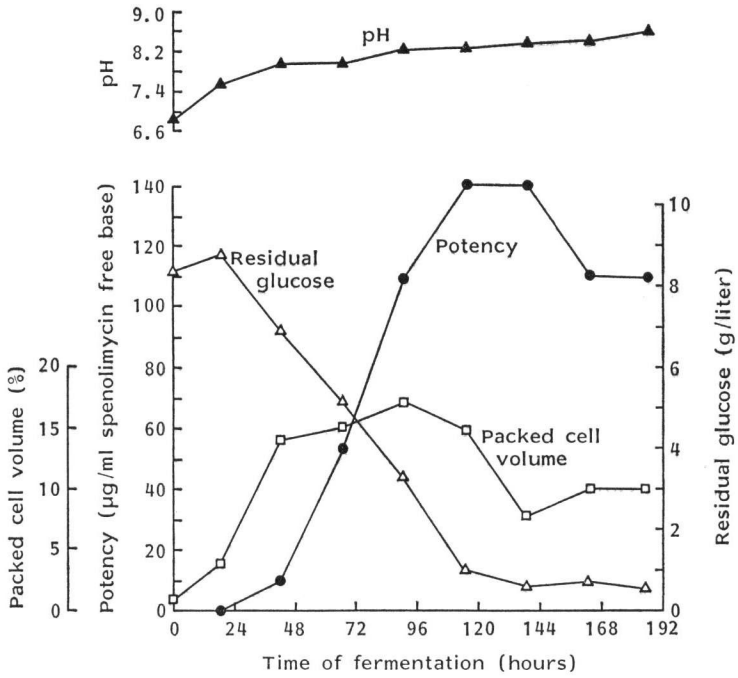
Discovery of Spenolimycin

Spenolimycin is a broad spectrum antibiotic inhibiting Gram-positive and Gram-negative bacteria as indicated in Table 1. Larger zones of inhibition at pH 8 than at pH 6.5 indicate that it is basic. A major and a minor antibiotic were observed in fermentation broths by paper chromatography followed by bioautography. Table 2 shows the Rf values of the two components compared with those of a number of known basic antibiotics. The Rf 0.31 component was eventually shown to be spectinomycin[4]. The major antibiotic moved to Rf 0.5 and appeared to be novel. Our interest was heightened when partially purified antibiotic preparations showed activity against aminoglycoside resistant bacteria (Table 3).

Taxonomy of Strain AB634D-177

The vegetative mycelium is branched and does not fragment. Spores occur on aerial hyphae in chains of more than 20 in a loose or open spiral configuration. Scanning electron microscopy revealed

Fig. 2. Time course of fermentation in a 14-liter fermentor.



that the spores have a smooth surface and are ellipsoidal with average dimensions of 0.5×0.8 μm (Fig. 1). Whole-cell analysis revealed the presence of LL-diaminopimelic acid and no diagnostic sugars. The microscopic observations and type I cell wall indicate that strain AB634D-177 belongs to the genus *Streptomyces*.

Cultural and physiological characteristics of strain AB634D-177 are shown in Tables 4 and 5. Its carbohydrate utilization pattern is shown in Table 6. The appearance of AB634D-177 on most media resembles *Streptomyces griseus*[11,12]. It shares with this species the unusual feature of producing melanin-like pigment on tyrosine agar but not on peptone-yeast extract iron agar[13]. It differs, however, most significantly from *S. griseus* in spore chain morphology and in its ability to utilize arabinose, raffinose and rhamnose. Although a culture with spiral spore chains was named *Streptomyces griseus* var. *spiralis* in 1962[14], classification keys published in 1974 and 1975[12,15~17] use spore chain morphology as one of the four to seven basic characteristics for differentiating *Streptomyces* species.

One *Streptoverticillium*[2] and three *Streptomyces*[1,18,19], have been reported to produce spectinomycin. Even though strain AB634D-177 produces spectinomycin in addition to spenolimycin, it is clearly different from any of these cultures (Table 7).

A resemblance to any other *Streptomyces* species was not found. Strain AB634D-177 is therefore designated a new species for which the name *Streptomyces gilvospiralis* sp. nov. is proposed in recognition of its pale yellow aerial mycelium and spiral spore chains.

Fermentation

The time course of the spenolimycin fermentation in 14-liter fermentors is shown in Fig. 2. Peak antibiotic yields of 140 μg/ml were obtained between 114 and 138 hours.

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