MARTINOMYCIN, A NEW POLYETHER ANTIBIOTIC PRODUCED BY *Streptomyces salvialis*

I. TAXONOMY, FERMENTATION AND BIOLOGICAL ACTIVITY

VALERIE S. BERNAN*, DEBORAH A. MONTENEGRO, JOSEPH J. GOODMAN, MAHENDER R. ALLURI, GUY T. CARTER, DARREN R. ABBANAT, CEDRIC J. PEARCE, WILLIAM M. MAIESE and MICHAEL GREENSTEIN

Natural Products Research Section, Medical Research Division, American Cyanamid Company, Pearl River, NY 10965, U.S.A.

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Actinomycete culture LL-D37187 has been found to produce the new polyether antibiotic martinomycin[1]. Taxonomic studies, including morphological, physiological, and cell wall chemistry analyses, revealed that culture LL-D37187 is a novel streptomycete species, and the proposed name is *Streptomyces salvialis*. Martinomycin exhibits activity against the Southern Army Worm (*Spodoptera eridania*) and Gram-positive bacteria.

An actinomycete designated LL-D37187 was isolated from a soil sample collected in Pomeroy, Washington. Fermentation broths of the culture showed activity against Gram-positive bacteria and brine shrimp. These activities have been attributed to the new polyether antibiotic martinomycin[1]. In this paper, we report the taxonomy and fermentation of the producing organism and the biological activities of martinomycin.

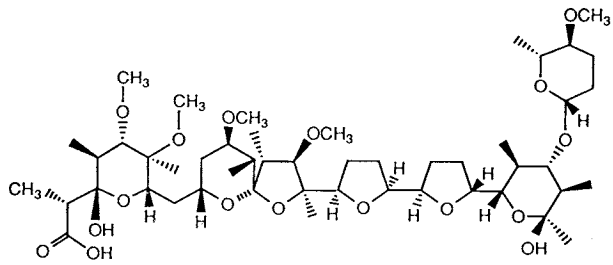
Materials and Methods

Microorganisms

Culture LL-D37187 was compared to *Streptomyces armeniacus* ATCC 15676, and *Streptomyces gibsonii* ATCC 6852. These cultures were received from the American Type Culture Collection (ATCC).

Isolation

Culture LL-D37187 was isolated from a soil sample collected under a sage brush in Pomeroy, Washington. The soil was suspended in water, and the suspension was sonicated for one minute at 30 watts of acoustic power. The suspension was then heated in a water bath at 60°C for five minutes. The



Martinomycin

culture was isolated by inoculating the pretreated sample onto a novobiocin-containing (25 μg/ml) agar medium consisting of 2% soluble starch, 0.1% potassium nitrate, 0.05% magnesium sulfate 7-hydrate, 0.05% potassium phosphate dibasic, 0.05% sodium chloride, 0.001% ferrous sulfate 7-hydrate, 1.5% agar, cycloheximide (50 μg/ml), and nystatin (50 μg/ml). The selection plate was incubated at 28°C for 7~10 days. The culture has been deposited with the Northern Regional Research Center's Culture Collection Laboratory under the accession No. NRRL 21087.

Physiological Characteristics

Physiological studies were carried out as described by the International Streptomyces Project (ISP)[2] and GORDON *et al.*[3]. For the evaluation of cultural characteristics, the strains were incubated from 14 to 28 days at 28°C.

Morphological Observations

Cultural characteristics were recorded after 7, 14, and 28 days at 28°C based on the International Streptomyces Project (ISP)[2]. The color and hue numbers indicated are those of the National Bureau of Standard Centroid Color Charts, Publication 440, Washington, D.C. Scanning electron micrographs were prepared using a modified method of Locci[4] and photographed using a JEOL 6300 V scanning electron microscope.

Chemotaxonomic Analysis

The culture was cultivated in a 250-ml Erlenmeyer flask containing 50 ml of a medium containing 1% yeast extract and 1% glucose. The flask was incubated at 28°C, 200 rpm for 72 hours. The mycelium was harvested by centrifugation, thoroughly washed with sterile distilled water, and lyophilized to dryness. Whole-cell sugars were determined by the GC/MS method of SADDLER *et al.*[5]. Phospholipids were extracted and were determined by two-dimensional thin-layer chromatography by the method of MINNIKIN *et al.*[6].

2,6-Diaminopimelic acid (DAP) analysis. The content of DAP stereoisomers in the biomass was determined by GC/MS analysis of *N*-heptafluorobutyryl *n*-butyl ester derivatives obtained from 10 mg of biomass by a modification of the method of SONESSON[7]. GC/MS analyses were carried out with a Hewlett-Packard 5890 Series II gas chromatograph interfaced to a 5971A mass spectrometric detector. A chiral capillary column (Chirasil-Val 25 m, 0.25 mm i.d., Alltech) was used to separate the isomers. For the analysis, the injector temperature was maintained at 250°C, and the column flow rate was approximately 1 ml/minute. The initial column temperature (70°C) was held for 1 minute and was then raised to 200°C at 7°C/minute. The final temperature was held for 15 minutes. Detection was either by total ion current or single ion monitoring of diagnostic ions (*e.g. m/z* 278). Under these conditions the retention times for the DAP isomers were: DD 30.3 minute, DL (*meso*) 30.9 minute, LL 31.0 minute. *Meso* and LL forms were not completely resolved by this procedure; however, relative abundances could be determined by integration of the area under the respective peaks. Monosaccharide standards and 2,6-diaminopimelic acid (racemic) were obtained from Sigma Chemicals, St. Louis, MO, U.S.A. Heptafluorobutyric anhydride (HFBA) was obtained from Pierce Chemical Co., Rockford, IL, U.S.A.

Fatty acid analysis. The preparation and analysis of whole cell fatty acid methyl esters were carried out according to the Microbial Identification System[8].

Production of Martinomycin

The culture of strain LL-D37187 was maintained as frozen vegetative material in a liquid medium containing 10% glycerol. The frozen vegetative material was then used as the source of inoculum for a seed medium consisting of: 1.0% glucose, 2.0% dextrin, 0.5% yeast extract, 0.5% N-Z Amine type A, and 0.1% $CaCO_3$. After cultivation for 3 days at 28°C at 200 rpm, this primary seed stage was used as a 10% inoculum for a 50 ml fermentation flask. The production medium consisted of 2.0% molasses, 1.5% dextrin, 1.5% nutrisoy, and 0.1% Mississippi Lime. The fermentation was carried out at 28°C, 200 rpm and the antibiotic was recovered by extraction of the whole broth with an equal volume (50 ml) of ethyl acetate. The production of martinomycin was followed by HPLC analysis. Antimicrobial activity was determined by measuring the diameter of the zone of growth inhibition of *Bacillus subtilis* in agar diffusion assays.

HPLC Analysis of Martinomycin in Fermentation Broth

The whole broth (25 ml) was extracted with ethyl acetate (25 ml) followed by centrifugation on a IECCentra-8R (International Equipment Co., U.S.A.) at 4000 rpm for 15 minutes. The phases were separated, and the organic layer was concentrated to dryness under reduced pressure and the residue was redissolved in 250 μl of the mobile phase. HPLC analysis was performed on a Hewlett-Packard 1090 M system fitted with a Hewlett-Packard 1047A refractive index detector and a 35900 interface. The detector temperature was set to 30°C and the detector range set to 8×10^{-5} RIU/FS. Injections of 25 μl were made onto a Whatman C18 reverse phase column (Partisil 5 ODS-3, 4.6 × 100 mm), which was eluted isocratically with a premixed solution of acetonitrile - 0.1 M NH_4OAc (90 : 10) at a flow rate of 1 ml/minute martinomycin eluted with a retention time of 4.4 minute.

Antibacterial Activity

The *in vitro* antibacterial activity of martinomycin was determined by agar dilution method following the recommendations of the National Committee for Clinical Laboratory Standards[13]. Eight Gram-positive isolates and four *Escherichia coli* strains were tested on antibiotic containing Mueller-Hinton II agar supplemented with 5% sheep blood. The lowest concentration that inhibited growth of a bacterial strain after 18 hours of incubation at 35°C was recorded as the minimum inhibitory concentration (MIC).

Brine Shrimp Assay

Brine shrimp (*Artemia salina*) eggs (25 mg) were placed in a 125-ml Erlenmeyer flask containing 25 ml of a salt solution (0.95% Instant Ocean, 50 mM MOPS, pH 7.2). The eggs were then incubated at room temperature for 48 hours for maximum hatching. One hundred microliters of hatched shrimp were pipetted into a 96 well plate containing the test compounds and incubated in the dark. Plates were observed microscopically using an Olympus zoom stereoscope for any changes in mobility and/or viability. These observations occurred at 1 hour, 5 hours, and 24 hours after addition of the test compound.

Results and Discussion

Taxonomic Studies of the Producing Culture

A summary of cultural characteristics of strain LL-D37187 on various media is presented in Table 2. White aerial mycelium was formed on yeast extract - malt extract and oatmeal agars, while yellow-white aerial mycelium was formed on inorganic salts - starch medium. No growth was noted on glycerol - asparagine agar. Substrate mycelium ranged from pink-yellow to yellow-white depending upon the ISP medium employed. No soluble pigments or melanin-like pigments were produced under any conditions. The organism could grow on yeast extract - malt extract agar containing $\geq 5\%$ salt. Optimum temperature for growth was 22 ~ 45°C.

Table 1. Comparison of morphological, phisiological, and chemotaxonomic characteristics of strain LL-D37187 with *Streptomyces armeniacus* (ATCC 15676) and *Streptomyces gibsonii* (ATCC 6852).

Characteristic	LL-D37187	*S. armeniacus*	*S. gibsonii*
Spore chain morphology	Spirals	Spirals	Spirals
Fragmentation ofsubstrate mycelium	None	None	None
Zoospores and Sporangia	None	None	None
Spore number per chain	15 ~ 20	8 ~ 10	8 ~ 10
Spore shape	Cylindrical	Oval	Oval
Spore surface	Smooth	Smooth	Smooth
Temperature growth	<45°C	<45°C	<50°C
Salt tolerance	≥5%	≥5%	≥5%
DAP isomer	LL-DAP	LL-DAP	LL-DAP

Table 2. Comparison of cultural characteristics of strain LL-D37187 with *Streptomyces armeniacus* (ATCC 15676) and *Streptomyces gibsonii* (ATCC 6852).

Agar medium		LL-D37187[a]	*S. armeniacus*	*S. gibsonii*
Yeast-malt (ISP 2)	G:	Abundant	Abundant	Abundant
	AM:	White (263)	None	None
	SM:	Pink-yellow to medium yellow (89, 87)	Light brown (57)	Yellow-white dark yellow (92, 88)
	SP:	None	None	None
Oatmeal (ISP 3)	G:	Abundant	Abundant	Abundant
	AM:	White (263)	White (263)	White (263)
	SM:	Pink-yellow (89)	Light yellow-brown (76)	Yellow-white to gray-yellow (92, 90)
	SP:	None	None	None
Inorganic salts-starch (ISP 4)	G:	Sparse	Abundant	Abundant
	AM:	Yellow-white (92)	White (263)	White (263)
	SM:	Yellow-white (92)	Light yellow-brown (76)	Yellow-white (92)
	SP:	None	None	None
Glycerol-Asparagine (ISP 5)	G:	No growth	Sparse	Sparse
	AM:	None	None	White (263)
	SM:	Colorless	Yellow-white (92)	Colorless
	SP:	None	None	None

G, growth; AM, aerial mycelium; SM, subtrate mycelium; SP, soluble pigment.
[a] ISCC, National Bureau of Standard Centroid Color Charts, Publication 440, Washington, D.C., 1976.

Table 3. Comparison of biochemical reactions of strain LL-D37187 with *Streptomyces armeniacus* (ATCC 15676) and *Streptomyces gibsonii* (ATCC 6852).

	LL-D37187	*S. armeniacus*	*S. gibsonii*		LL-D37187	*S. armeniacus*	*S. gibsonii*
Utilization of Carbon Sources:				Citrate	+	+	+
D-Glucose	+	+	+	Lactate	+	+	±
L-Arabinose	+	+	+	Malate	+	+	+
Sucrose	−	+	−	Mucate	−	−	−
D-Xylose	±	+	+	Oxalate	−	+	−
i-Inositol	±	+	±	Proprionate	−	+	+
D-Mannitol	−	+	+	Pyruvate	+	+	+
β-D-Fructose	+	+	+	Succinate	+	+	+
α-L-Rhamnose	+	+	−	Tartrate	±	−	−
Raffinose	−	−	−	Acid Production from:			
Cellulose	−	−	−	Arabinose	+	+	±
Hydrolysis of:				Dulcitol	−	−	−
Casein	+	+	+	Erythritol	+	+	+
Xanthine	−	+	+	Glucose	+	+	+
Hypoxanthine	+	+	+	Inositl	−	±	−
Tyrosine	±	±	+	Lactose	−	+	+
Adenine	−	−	−	Mannitol	−	+	+
Esculin	+	−	+	Mannose	+	+	+
Production of:				Methyl-α-D-glucoside	−	−	+
Urease	+	−	+	Melibiose	−	+	+
Melanin-like pigments	−	−	−	Raffinose	−	+	−
Decarboxylation of:				α-L-Rhamnose	+	+	−
Acetate	−	+	+	Sorbitol	−	−	−
Benzoate	−	−	−	Trehalose	+	+	+

+: positive, -: negative, ±: weak.

Fig. 1. Scanning electron micrograph of culture LL-D37187 cultured on yeast extract-malt extract agar. Total magnification: 14,000.

Bar represents 1 μm.

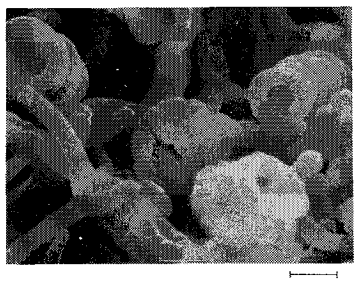


Table 4. Fatty acid composition of strain LL-D37187 and *Streptomyces armeniacus* (ATCC 15676) and *Streptomyces gibsonii* (ATCC 6852).

Fatty acid	LL-D37187 amount (%)	*S. armeniacus* amount (%)	*S. gibsonii* amount (%)
14:0 Iso	4.0	4.5	20.0
15:0 Iso	6.8	3.0	5.9
15:0 Anteiso	40.0	15.4	25.0
16:0 Iso	24.0	40.0	37.0
16:1 Iso G[a]	—	10.0	—
16:1 Iso H	5.5	—	3.10
17:0 Anteiso	15.0	11.5	4.2
17:1 Anteiso C	—	4.7	—

[a] Letters designate an undetermined double bond position.

Biochem characteristics of strain LL-D37187 and its utilization of various carbon sources are shown in Table 3. The culture utilized all carbon sources tested except sucrose, mannitol, raffinose, and cellulose. The culture degraded casein, hypoxanthine, tyrosine, and esculin, but not xanthine or adenine.

Microscopic examination of the organism revealed an extensively branching substrate mycelium as well as abundant aerial mycelium, which is then transformed into spirals of cylindrical, smooth arthrospores. No fragmentation of the substrate mycelium was noted. The aerial mycelium, which had between 15~20 spores per chain, is classified as *Spirales*. The spores, as examined by scanning electron microscopy, were cylindrical in shape with smooth surfaces. Sporangia, zoospores, and sclerotia were not observed (Fig. 1).

Whole-cell analysis showed that the cell wall of strain LL-D37187 contained >90% of the LL isomer of diaminopimelic acid (DAP). Additionally, the strain contained only phosphatidylethanolamine, indicating a type PII phospholipid pattern. The fatty acid pattern was a type 2c pattern, consisting mainly of saturated *iso* and *anteiso* fatty acids (Table 4).

Based on these characteristics, it was determined that strain LL-D37187 belonged to the genus *Streptomyces*, and it was therefore compared to two closely related *Streptomyces* species: *Streptomyces armeniacus*, ATCC 15676, and *Streptomyces gibsonii*, ATCC 6852.[9,10] Many differences were seen in the macromorphological, chemotaxonomic, and physiological studies. Morphologically strain LL-D37187 was distinguishable from the other two *Streptomyces* by possessing long chains of cylindrical spores, producing white aerial mycelium on ISP 2 and producing pink-yellow substrate mycelium on both ISP 2 and 3. Some of the distinctions in physiological tests included the utilization of D-mannitol, hydrolysis of xanthine, decarboxylation of acetate, and acid production from lactose, mannitol, and melibiose. Additionally, a dendrogram was constructed using the fatty acid profiles of the three strains to compare their relatedness. This analysis revealed that although strain LL-D37187 is indeed a *Streptomyces*, it is distinct from the two closely related strains (Fig. 2). Therefore, from the many differences observed, it was concluded that strain LL-D37187 is a novel species of *Streptomyces*, and the proposed name is *Streptomyces salvialis*. sp. nov. (*salva*. L. deriv. of the herb sage, *alis*. L. deriv. pertaining to or having a connection with).

Fermentation

Culture LL-D37187 was fermented over a period of 10 days and the production of martinomycin was

Fig. 2 Cluster analysis of strain LL-D37187, *S. armeniacus*, ATCC 15676, and *S. gibsonii*, ATCC 6852 by fatty acid profiles.

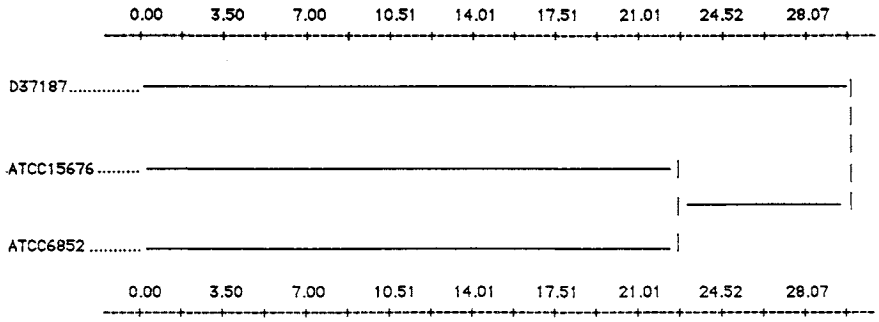


Fig. 3. Time course of martinomycin production by culture LL-D37187.

○ PCV; ■ pH; ● antibacterial activity, *B. subtilis*; △ martinomycin concentration.

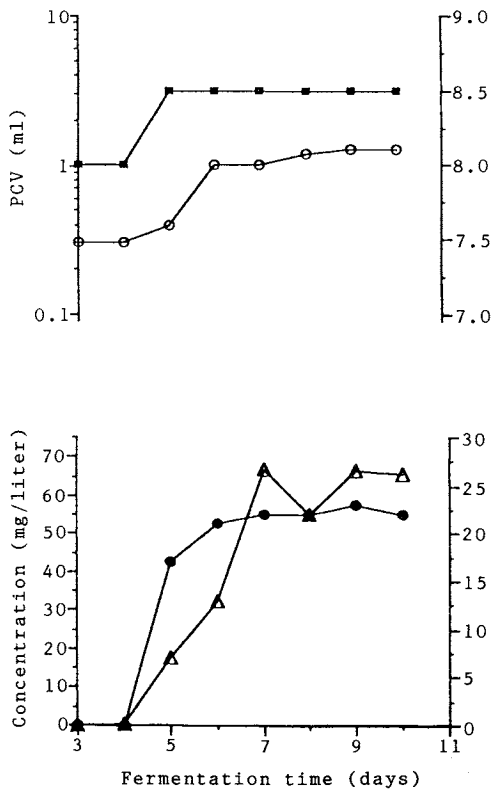


Table 5. Antimicrobial activity of martinomycin.

Test Organism	MIC range (μg/ml)
Staphylococcus aureus (11 strains)	0.12～0.50
Staphylococcus haemolyticus (2 strains)	0.12～0.50
Staphylococcus Coagulase Negative (2 strains)	1.5
Bacillus cereus	0.5
Enterococcus faeceum (6 strains)	0.12～0.50
Enterococcus faecalis	0.5
Streptococcus Group A	0.12
Streptococcus pneumoniae (3 strains)	0.06
Escherichia coli (4 strains)	>128

followed by HPLC analysis, as described in Materials and Methods. A time course for growth and bioactivity is shown in Fig. 3. The maxium yield of martinomycin (67 μg/ml) peaked at day 7 and remained constant throughout the fermentation. Antimicrobial activity, as gauged by zones of inhibition versus *B. subtilis* on agar plates, paralleled the production of martinomycin.

Biological Activity

The antimicrobial activity of martinomycin as determined by agar dilution is presented in Table 5. The compound demonstrated a pattern typical of polyether antibiotics, with potent activity against Gram-positive bacteria and none against Gram-negative species. Evaluation of the compound in the brine shrimp assay revealed that martinomycin exhibited similar potency when compared to the known polyether septamycin[11] (Table 6). Martinomycin was also evaluated for insecticidal activity against the southern army worm (*Spodoptera eridania*). A 10 ppm solution of martinomycin applied to the surface of bean leaves gave an 80% kill of third instar larvae.

Table 6. Comparison of bioactivity in the brine shrimp assay of martinomycin and septamycin[11].

Sample	Concentration (μg/ml)	Brine shrimp activity		
		1 hour	5 hours	24 hours
Martinomycin	1,000	+/−	+	+
	500	+/−	+/−	+
	250	+/−	+/−	+
	125	+/−	+/−	+
	62	−	−	+
	32	−	−	+/−
	16	−	−	−
Septamycin[a]	1,000	+	+	+
	500	+/−	+/−	+
	250	−	+/−	+
	125	−	+/−	+
	62	−	−	+
	32	−	−	+/−
	16	−	−	+/−
	8	−	−	−

Nemadectin α was used as the control compound and was active in one and five hours at a concentration of 1.0 μg/ml and active in 24 hours at 0.001 μg/ml[12].

Legend

+ = active; dead/paralyzed.

+/− = Slightly active; decreased motility.

− = Inactive; no effect.

[a] See Ref 11.

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