LL-D49194 ANTIBIOTICS, A NOVEL FAMILY OF ANTITUMOR AGENTS: TAXONOMY, FERMENTATION AND BIOLOGICAL PROPERTIES

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A novel family of antitumor antibiotics, designated LL-D49194, was isolated from the fermentation broth of an actinomycete strain identified as *Streptomyces vinaceus-drappus*. LL-D49194 α_1 and β_2 were active against Gram-positive and inactive against Gram-negative bacteria *in vitro*. The β_1 component was not active against either Gram-positive or Gram-negative bacteria. These antibiotics exhibited significant *in vivo* activities against several murine tumors, albeit with differing potencies.

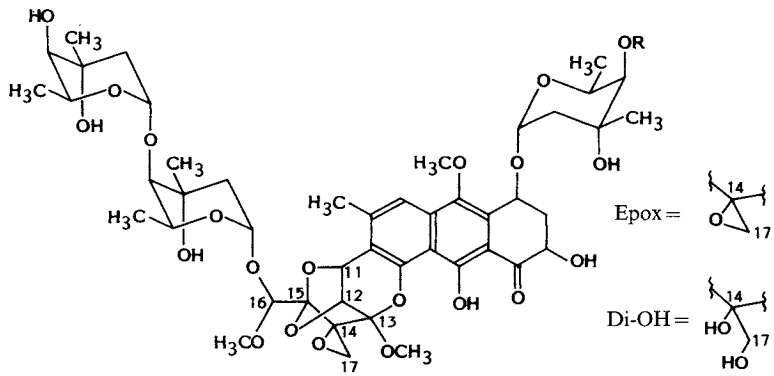
In the course of our search for novel antitumor agents produced by microorganisms, a culture designated LL-D49194 was isolated from a soil sample and was found to produce a new family of antitumor compounds (Fig. 1). This paper describes the taxonomy of the producing culture, fermentation and biological activities of these agents.

Materials and Methods

Microorganism

Culture LL-D49194 was isolated from a soil sample collected in La Encanada, Peru. This culture was

Fig. 1. Chemical structures of LL-D49194 α_1, β_1 and β_2.



LL-D49194 components	R	C-14—C-17
α_1	CH_3CO	Epox
β_1	CH_3CO	Di-OH
β_2	H	Epox

deposited with the Northern Regional Research Center's Culture Collection Laboratory under the accession No. NRRL 15735.

Taxonomic Studies

The taxonomic studies were carried out as described by the International Streptomyces Project (ISP)[1] and GORDON *et al.*[2]. For the evaluation of cultural characteristics, the strains were incubated for 14~31 days at 28°C. Cell wall composition was analyzed by the methods of LECHEVALIER and LECHEVALIER[3].

Media and Fermentation

Culture LL-D49194 was stored as a frozen suspension at −70°C in growth medium. A 1.5-ml aliquot of thawed suspension was used to inoculate 100 ml of seed medium (yeast extract 0.5%, beef extract 0.3%, Tryptose 0.5%, glucose 1.0% and $CaCO_3$ 0.1%) in a 500-ml Erlenmeyer flask. This seed inoculum was incubated on a rotary shaker (5 cm orbit) at 200 rpm, 28°C, for 48 hours and was then added to a 3 liter fermenter containing 1 liter of seed medium. Following 48 hours incubation (aeration: 1 v/v/m, 450 rpm), the contents of this fermenter were inoculated into 30 liters of seed medium (molasses 2.0%, peptone 0.5%, glucose 1.0% and $CaCO_3$ 0.1%) in a 41-liter fermenter. This culture was grown at 32°C for up to 48 hours (aeration: 1 v/v/m, 350 rpm). The contents from this fermenter were inoculated into a 410-liter fermenter containing 300 liters of seed medium (aeration: 0.75 v/v/m, 250 rpm). After 48 hours growth at 32°C, these 300 liters were used to inoculate a production fermenter containing 3,000 liters of medium (glucose 3.0%, molasses 1.0%, peptone 0.5% and $CaCO_3$ 0.1%). This fermentation was carried out at 28°C for up to 150 hours (aeration: 0.66 v/v/m, 450 rpm). The pH of the seed and production media used in these studies was adjusted to 6.8~7.0 prior to sterilization. Microbial growth was determined by packed cell volume. Antibiotic production was monitored by the biochemical prophage induction assay (BIA)[4,5] and analytical HPLC[6].

Isolation and TLC/Bioautography

The BIA active compounds produced by culture LL-D49194 were associated with the mycelium and were recovered by extracting the whole fermentation broth with ethyl acetate[6]. Antimicrobial and BIA activities on the TLC plates were detected by bioautography.

Antibacterial Activity

The *in vitro* antibacterial activities of the LL-D49194 antibiotics against a spectrum of Gram-positive and Gram-negative bacteria were determined by an agar dilution method employing Mueller-Hinton medium. The lowest concentration of antibiotic that inhibited growth of a bacterial strain after 18 hours of incubation at 35°C was recorded as the MIC.

Antitumor Activity

The antitumor activity of each component was determined in male BDF_1 mice against P388 leukemia and B16 melanoma. The P388 leukemia test was initiated by ip injection of 10^6 cells per mouse in 0.5 ml of dilute ascitic fluid. For B16 melanoma, a 1 g portion of tumor was homogenized in 10 ml of balanced salt solution, and a 0.5-ml aliquot was implanted intraperitoneally into each mouse. The antibiotics were administered intraperitoneally days 1, 5 and 9 after initiation of the P388 test and by the same route on days 1 through 9 for B16. The antitumor activity was expressed as T/C values (median survival time of the treated group/median survival time of the untreated group × 100), with a value of ≥ 125 being considered significant.

Results

Taxonomic Studies of the Producing Culture

Culture LL-D49194 was isolated from a soil sample collected in La Encanada, Peru. Examination of the culture grown at 28°C for 14~31 days on various media revealed that spores were formed in coiled chains on aerial sporophores. The spores were ovoid (0.4~0.5 by 0.9~1.0 μm), and the surface of the

mature spores was smooth when observed by scanning electron microscopy. A summary of the culture's growth characteristics on various media is presented in Tables 1~3. Whole cell analysis showed that the strain contained the L,L-isomer of diaminopimelic acid, placing it in the type I cell wall group of LECHEVALIER *et al.*[7]. Utilization of carbon sources is summarized in Table 4. From the macromorphological, chemotaxonomic and physiological studies, it was concluded that culture LL-D49194 taxonomically resembles the reference strain of *Streptomyces vinaceus-drappus* and is designated as a strain of this species.

Fermentation

Culture LL-D49194 was grown in a 3,000-liter fermenter at 28°C for 150 hours. A typical time course for the production of the antitumor antibiotic is shown in Fig. 2. Antibiotic production started at

Table 1. Comparison of LL-D49194 with *Streptomyces* reference cultures.

Culture	Spore mass color	Soluble pigments	Reverse color
S. bottropensis NRRL 12051	Light grayish yellow-brown	Reddish-brown	Brownish black
S. vinaceus-drappus NRRL 2363	Light gray to pinkish gray	None	Moderate orange-yellow
LL-D49194	Light gray to pinkish gray	None	Moderate orange-yellow

Table 2. Cultural characteristics of LL-D49194.

Medium	Amount of growth	Aerial mycelium and/or spores[a]	Soluble pigment	Reverse color
Glycerol-asparagine agar	Good to moderate	Slightly raised colonies with white aerial mycelia becoming 264. Light gray to 10. Pinkish gray in sporulated areas	None	Light yellow
Hickey-Tresner agar	Good	Slightly raised colonies with white aerial mycelia becoming 264. Light gray to 10. Pinkish gray in sporulated areas; sporulation heavy	None	Yellowish brown
Inorganic salts-starch agar	Good	Relatively flat growth with very heavy sporulation, 264. Light gray	Brownish	Deep yellow
Oatmeal agar	Moderate	Flat powdery growth with 264. Light gray to 10. Pinkish gray with 265. Medium gray patches	None	Yellowish white
Tomato paste-oatmeal agar	Good	Raised colonies with white aerial mycelia becoming 264. Light gray to 10. Pinkish gray where sporulated	Brownish	
Yeast extract-malt extract agar	Good	Raised, ridged colonies with white aerial mycelia becoming 10. Pinkish gray in sporulated areas	None	Moderate orange-yellow

[a] ISCC, National Bureau of Standard Centroid Color Charts, Publication 440, Washington, D.C., 1976.

approximately 24~30 hours after inoculation and reached maximum titers at approximately 120 hours.

Antibacterial Activity

The antibacterial activities of the α_1, β_1 and β_2 components of LL-D49194 are shown in Table 5. The α_1 and β_2 components exhibited activity against Gram-positive bacteria with MIC values in the μg/ml range. None of the three LL-D49194 components tested had activity against the Gram-negative bacteria. The β_1 antibiotic was essentially inactive against the test bacteria.

Antitumor Activity

The antitumor activities of the α_1, β_1 and β_2 components were determined in male BDF_1 mice against P388 leukemia and B16 melanoma. The results are summarized in Table 6. These agents exhibited activity against both tumors; however α_1 and β_2 were significantly more potent than β_1.

Table 3. Physiological reactions of LL-D49194.

Medium	Incubation period (days)	Amount of growth	Physiological reaction
Peptone-iron agar	7	Good	No blackening
	14	Good	No blackening
Tyrosine agar	7	Good	No blackening
	14	Good	No blackening
Litmus milk	7	Good	Slight proteolysis
	14	Good	Moderate proteolysis
Nutrient gelatin	7	Good	No proteolysis
	14	Good	No proteolysis
Nitrate broth	7	Good	Reduction

Table 4. Carbon source utilization of LL-D49194.

Carbon source	Utilization
Arabinose	++
Fructose	+++
Galactose	+++
Glucose	++
Inositol	+++
Mannitol	++
Raffinose	+
Rhamnose	+++
Salicin	++
Sucrose	++
Xylose	+++

+: Poor utilization, ++: fair utilization, +++: good utilization.

Fig. 2. Fermentation profile of culture LL-D49194.

● α_1, ○ β_2, □ growth, △ pH.

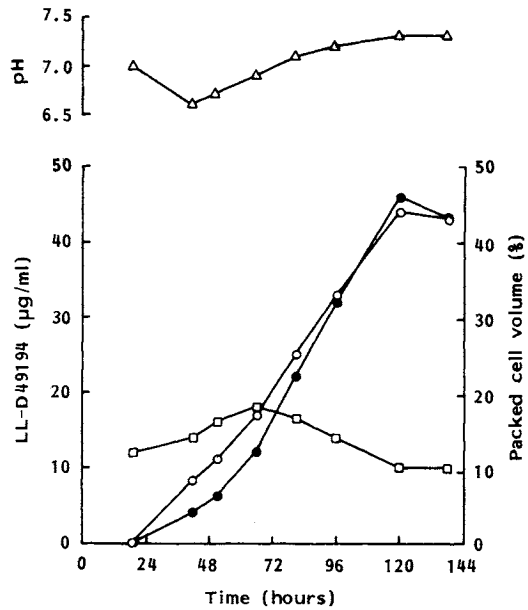


Table 5. *In vitro* antibacterial activity of LL-D49194 α_1, β_1 and β_2.

Organism	MIC (μg/ml)		
	α_1	β_1	β_2
Gram-negative bacteria:			
Escherichia coli Stfd-79-20	>128	>128	>128
E. coli No. 311	>128	>128	>128
Klebsiella pneumoniae AD	>128	>128	>128
Acinetobacter calcoaceticus K-77-1	>128	>128	>128
Pseudomonas aeruginosa SSC-78-13	>128	>128	>128
P. aeruginosa 12-4-4	>128	>128	>128
P. aeruginosa ATCC 27853	>128	>128	>128
Gram-positive bacteria:			
Staphylococcus aureus SSC-79-18	1	512	2
S. aureus Smith	0.5	512	1
S. aureus ATCC 25923	2	512	8
Micrococcus luteus PCI 1001	0.5	>512	1
Enterococcus sp. OSU-75-1	2	>512	8
Enterococcus sp. SSC-81-1	2	>512	4
Bacillus subtilis ATCC 6633	0.5	>512	0.5

Table 6. Antitumor activity of LL-D49194 against P388 leukemia and B16 melanoma in mice.

Compound	Dose (mg/kg)	Median survival (days)[a]	T/C × 100 (%)	Compound	Dose (mg/kg)	Median survival (days)[a]	T/C × 100 (%)
P388 Leukemia[b]:				B16 Melanoma[c]:			
LL-D49194 α_1	0.5	18.5	156	LL-D49194 α_1	0.25	31.5	153
	0.25	19.0	165		0.125	35.0	171
	0.125	16.5	144		0.06	34.0	165
	0.06	16	144		0.03	27.0	132
LL-D49194 β_1	400	20.5	178	LL-D49194 β_1	200	45.0	219
	200	19.0	165		100	34.5	168
	100	19.5	169		50	36.5	178
	50	18.0	156	LL-D49194 β_2	0.5	36.0	176
	25	16.0	139		0.25	35.5	173
LL-D49194 β_2	2	16.5	143		0.125	35.0	170
	1	15.5	134		0.06	27.0	131
	0.5	13.0	113	Cisplatin	0.5	27.0	131
Cisplatin	1.5	21.0	182		0.25	27.5	134
	0.8	19.5	170		0.125	23.5	114
	0.4	15.0	130	Control	0	20.5	100
Control	0	11.5	100				

[a] Median survival time.
[b] 30 days for P388.
[c] 60 days for B16.

Discussion

A novel complex of antitumor antibiotics, designated LL-D49194, was isolated from the fermentation broth of an actinomycete, *S. vinaceus-drappus*. The α_1 and β_2 components were active against Gram-positive bacteria and exerted antitumor effects against both P388 leukemia and B16 melanoma in mice. The β_1 component was inactive against the test bacteria; however, it did possess antitumor activity. Preliminary studies indicate that the antitumor effect of α_1 is related to its ability to bind to and damage DNA[8]. The LL-D49194 family of antitumor antibiotics are related structurally to but are distinct from the trioxacarcins produced by *Streptomyces bottropensis*[9,10].

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