SIMAOMICIN (LL-D42067), A NOVEL ANTIBIOTIC FROM *ACTINOMADURA MADURAE*

I. TAXONOMY, FERMENTATION AND BIOLOGICAL ACTIVITY

W. M. MAIESE, J. KORSHALLA, J. GOODMAN, M. J. TORREY,
S. KANTOR[†], D. P. LABEDA[††] and M. GREENSTEIN

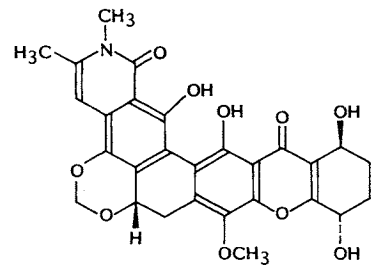
Medical Research Division, American Cyanamid Company, Lederle Laboratories,
Pearl River, New York 10965, U.S.A.
[†]Agricultural Research Division, American Cyanamid Company,
Princeton, New Jersey 08540, U.S.A.
[††]Culture Collection Laboratory, USDA-NRRL,
Peoria, Illinois 61604, U.S.A.

(Received for publication March 12, 1990)

A new antibacterial antibiotic, designated simaomicin α (LL-D42067α) was isolated from the fermentation broth of an actinomycete strain. Based on cultural, physiological, morphological and chemical characteristics, culture LL-D42067 was identified as a new subspecies of *Actinomadura madurae*. Simaomicin α demonstrated potent activity against Gram-positive bacteria and was active *in vivo* against a variety of *Eimeria* species causing coccidiosis in chickens.

In the course of our search for novel antibacterial antibiotics produced by microorganisms, a culture designated LL-D42067 was found to produce a novel antibiotic, simaomicin α (Fig. 1). This antibiotic exhibited potent activity against Gram-positive bacteria, weak active against fungi and was inactive against Gram-negative bacteria. Simaomicin α is the most potent naturally-occurring anticoccidial agent reported for the treatment of *Eimeria tenella* infections in chickens. This paper describes the taxonomy of the producing culture, fermentation and biological activity of simaomicin α.

Fig. 1. The structure of simaomicin α (LL-D42067α).



Materials and Methods

Microorganism

Culture LL-D42067 was isolated from a soil sample collected at San Simao, Brazil. This culture was deposited at the Northern Regional Research Center's Culture Collection Laboratory under the accession No. NRRL 15734.

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 and whole-cell composition were analyzed by the methods of LECHEVALIER and LECHEVALIER[3].

Media and Fermentation

Culture LL-D42067 was stored as a frozen seed suspension at −70°C in growth medium. To prepare seed inoculum for the production of the antibiotic, 1.0 ml of a thawed suspension was inoculated into a

500-ml Erlenmeyer flask containing 100 ml of seed medium consisting of yeast extract 0.5%, N-Z-Amine type A (Sheffield Chemical Company) 0.5%, dextrin 2.0%, glucose 1.0%, and $CaCO_3$ 0.1%. After 48 hours incubation at 32℃ on a rotary shaker at 210 rpm, 100 ml of this suspension was added into a 12-liter fermentor containing 10 liters of seed medium. Following 48 hours incubation at 32℃ (aeration: 1 vol/vol/minute, 450 rpm), the contents from this fermentor were inoculated into a 410-liter fermentor containing 300 liters of seed medium (aeration: 0.75 vol/vol/minute, 250 rpm). After 48 hours growth, these 300 liters were used to inoculate a production fermentor containing 3,000 liters of a medium consisting of dextrin 3.0%, glucose 0.5%, Nutrisoy 1.5%, corn steep liquor 0.5%, $CaCO_3$ 0.5%, and antifoam 0.3%. This fermentation was carried out at 28℃ for up to 144 hours (aeration: 0.66 vol/vol/minute, 450 rpm). The pH of the seed and production media used in these studies was adjusted to 6.8~7.0 prior to sterilization. Antibiotic production was monitored by a paper-disk agar diffusion assay using *Bacillus subtilis* and by analytical HPLC.

Isolation

Simaomicin α was isolated from the fermentation broth by solvent extraction. The fermentation broth was acidified to pH 3.0 with 6 N HCl, filtered, and the filtrate extracted with acetone. The acetone extract was concentrated to an aqueous residue, and the product was extracted into ethyl acetate. The combined ethyl acetate extracts were washed with 5% $NaHCO_3$, 0.1 N HCl, and water prior to concentration under reduced pressure to an oily residue. The residue was dissolved in 2.5 ml of THF-MeOH (9:1) for chromatography on a C_8 reversed-phase HPLC column (2.5×2.5 cm) packed with 10 μm C_8 bonded silica. The mobile phase consisted of 35% acetonitrile in 0.05 M NH_4OAc buffer, pH 4.5. The column was monitored by UV absorbance at 254 and 405 nm[4].

In Vitro Antimicrobial Activity

The *in vitro* antimicrobial activity of simaomicin α was determined by the agar dilution method employing Mueller-Hinton medium for bacteria and Sabouraud dextrose agar for fungi. The lowest concentration that inhibited growth after 18 hours incubation at 35℃ was recorded as the MIC.

In Vivo Activity in Chickens

Commercial type cockerels (Avian Services, Inc., Frenchtown, N.J.) were used. Birds were 3 days of age at inoculation and were maintained in rooms heated to 30~32℃. Medication was mixed into the feed using a laboratory blender. Water and feed were available to the birds *ad libitum* during the tests. A commercial coccidiosis vaccine consisting of live oocysts of 8 species of drug sensitive *Eimeria* affecting chickens was used at 80 times the recommended immunizing dose level. Tests were terminated at 14 days after inoculation, the birds sacrificed, then necropsied for lesion evaluation.

Results

Taxonomic Studies of the Producing Culture

Culture LL-D42067 was isolated from a soil sample collected at San Simao, Brazil. Microscopic examination of the culture revealed short chains of conidia on aerial hyphae which were slightly hooked to short spirals. Electron microscopic examination showed the spores were smooth, distinguishing this culture from *Actinomadura madurae*.

Table 1. Cultural characteristics of LL-D42067 on ISP morphological media.

Agar medium	Aerial mycelium	Vegetative mycelium[a]	Soluble pigment
Yeast extract-malt extract (ISP 2)	White, sparse	Medium orange-brown 153	None
Inorganic salts-starch (ISP 4)	Colorless	Colorless	None
Glucose-asparagine (ISP 5)	Colorless	Colorless	None
Oatmeal (ISP 3)	Sparse, pinkish-white	Light orange-brown 152	None

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

The cultural characteristics of culture LL-D42067 grown at 28℃ for 14~31 days on various media are described in Table 1. Whole cell analysis showed that the strain contained the *meso*-isomer of diaminopimeric acid and the sugar 3-*O*-methyl-D-galactose (madurose); thus, it falls into whole-cell pattern type B. The cell-wall composition was of type III (*meso*-DAP, glutamic acid, alanine, muramic acid and glucosamine) and the phospholipid pattern of type PIV (phosphatidyl ethanolamine and/or methylethanolamine plus unknown glucosamine-containing phospholipids). These data support the assignment of this culture to the genus *Actinomadura*. The PIV phospholipid type is not typical for *A. madurae*, which usually has PI.

The carbohydrate utilization patterns and physiological reactions of culture LL-D42067 are summarized in Tables 2 and 3, respectively. A comparison of the reactions in the GORDON test series of *A. madurae*[5] and LL-D42067 revealed differences only in amylase production and acid from glycerol and raffinose. Since amylase production and raffinose utilization have been found to be variable in *A. madurae*[6], glycerol remains the only physiological

Table 2. Comparison of carbohydrate utilization reactions of LL-D42067 with related *Actinomadura* sp.

Carbohydrate	LL-D42067	*A. madurae*[6]	*A. verrucosospora*[6,13]
Arabinose	+	+	+
Fructose	+	+	+
Inositol	−	Variable	Variable
Mannitol	+	+	+
Raffinose	−	−	−
Rhamnose	+	+	+
Sucrose	+	+	+
Xylose	+	+	+

+: Utilized, −: not utilized.

Table 3. Physiological reactions of culture LL-D42067 and *Actinomadura madurae*.

	LL-D42067	*A. madurae*[5]		LL-D42067	*A. madurae*[5]
Degradation/Transformation of			Growth at		
Casein	+	+	10℃	−	−
Xanthine	−	−	45℃	+	−
Hypoxanthine	+	+	53℃	−	−
Tyrosine	+	+	Acid from		
Adenine	−	−	Adonitol	+	+
Production of			Arabinose	+	+
Amylase	−	+	Cellobiose	+	+
Gelatinase	+	+	Dextrin	+	ND
Phosphatase	−	ND	Dulcitol	−	−
Nitrate reductase	+	+	Erythritol	−	−
Urease	−	−	Fructose	+	ND
Esculinase	+	+	Galactose	+	+
Growth on/in			Glucose	+	+
5% Sodium chloride	−	ND	Glycerol	−	+
Salicylate	−	ND	Inositol	−	+
Lysozyme broth	−	−	Lactose	−	+
Utilization			Maltose	−	+
Acetate	+	+	Mannitol	+	+
Benzoate	−	−	Mannose	+	+
Citrate	−	−	Melibiose	−	−
Lactate	+	ND	α-Methyl-D-glucoside	−	−
Malate	+	+	Raffinose	Variable	−
Mucate	−	−	Rhamnose	+	+
Oxalate	−	ND	Salicin	+	ND
Propionate	−	ND	Sorbitol	−	−
Pyruvate	+	ND	Sucrose	+	ND
Succinate	+	+	Trehalose	+	+
Tartrate	−	−	Xylose	+	+
			β-Methyl-D-xyloside	+	ND

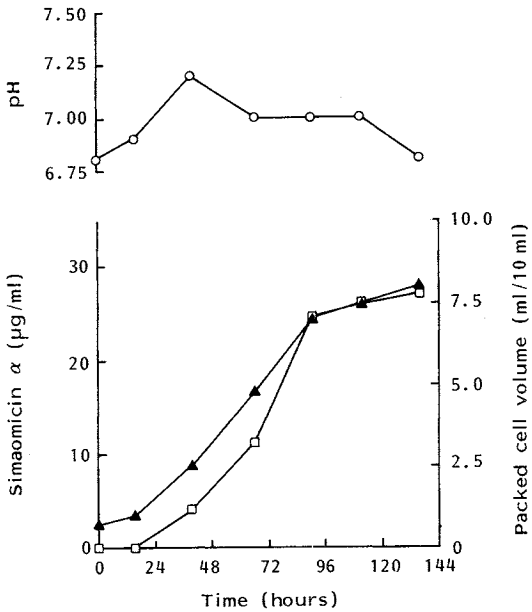
+: Utilized, −: not utilized, ND: not determined.

difference. Since strain LL-D42067 is the same as *A. madurae* in all properties tested except for its glycerol reaction and its PIV phospholipid pattern, it has been assigned to the taxon *A. madurae* as a subspecies designated *simaoensis*.

Fermentation and Isolation

Culture LL-D42067 was grown in a 3,000-liter fermentor at 28℃ for 144 hours. A typical time course for the production of simaomicin α is presented in Fig. 2. Antibiotic production started at approximately 20~24 hours post-inoculation and reached a maximum at 130~140 hours into the fermentation cycle. The antibiotic was distributed in both the mycelium and the growth medium and was recovered by ethyl acetate extraction of the acidified whole broth. Purification was achieved by chromatography on silica gel developed with methylene chloride.

Fig. 2. Fermentation profile of culture LL-D42067.
○ pH, □ simaomicin α, ▲ growth.



Biological Properties

The antimicrobial spectrum of simaomicin α is presented in Table 4. The antibiotic exhibited potent antibacterial activity against Gram-positive bacteria

Table 4. *In vitro* antimicrobial activity of simaomicin α.

Test organism (No. strains)	MIC (μg/ml)
Staphylococcus aureus (5)	≦0.06
S. epidermidis (2)	≦0.06
Streptococcus faecalis (1)	≦0.06
Streptococcus (*Enterococcus* sp.) (2)	≦0.06
S. mutans (2)	≦0.06
S. sanguis (1)	≦0.06
Micrococcus luteus (1)	≦0.06
Bacillus cereus (1)	≦0.06
Candida albicans (1)	256
Saccharomyces cerevisiae (1)	32
Escherichia coli (2)	512
Klebsiella pneumoniae (1)	512
Morganella morganii (1)	512
Acinetobacter calcoaceticus (1)	512

Table 5. *In vivo* activity of simaomicin α against a spectrum of chicken coccidia.

Dosage[a]	Reduction of lesion scores (%)[b]				
	Eimeria tenella	*E. acervulina*	*E. brunetti*	*E. necatrix*	*E. maxima*
Simaomicin α					
1.25	100	95	100	100	100
1.00	100	91	100	100	100
0.75	74	86	100	100	100
0.40	52	62	89	100	72
0.20	32	24	65	82	0
Maduramicin					
5.00	35	24	75	59	21

[a] g per metric ton of drug administered in feed.

[b] % reduction of lesion scores: $\frac{100-(\text{Score of treated group})}{\text{Score of infected control}} \times 100$.

Scoring is from 0=no intestinal involvement to 3=maximal intestinal involvement.

with MIC values equal to or less than 0.06 μg/ml and moderate activity against the fungi tested. However, no activity was observed against Gram-negative bacteria.

The anticoccidial activity of simaomicin α is presented in Table 5. This antibiotic demonstrated activity against all of the commercially important species of chicken coccidia and almost completely prevented lesions at 1 g/ton of medicated feed.

Discussion

Culture LL-D42067, identified as a new subspecies of *A. madurae*, was found to produce a new antibiotic, simaomicin α. This compound possessed potent activity against Gram-positive bacteria and moderate activity against fungi. With an optimal dosage of 1 g/ton in the diet of chickens, simaomicin α is the most potent naturally-occurring anticoccidial agent reported[7]. Simaomicin α contains a xanthone unit and is structurally related to a small family of antibiotics including LL-E19085[8], lysolipin[9], the actinoplanones[10], and the cervinomycins[11,12]. Simaomicin α is the only member of this structurally related class of antibiotics reported to have anticoccidial activity.

Acknowledgments

The authors are grateful to the personnel of the Microbial Physiology, Microbial Chemistry, and Antimicrobial Chemotherapy Departments for their contributions and helpful suggestions.

References

1) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
2) GORDON, R. E.; S. K. MISHRA & D. A. BARNETT: Some bits and pieces of the genus *Nocardia*: *N. carnea*, *N. vaccinii*, *N. transvalensis*, *N. orientalis* and *N. aerocolonigenes*. J. Gen. Microbiol. 109: 69~78, 1978
3) LECHEVALIER, M. P. & H. A. LECHEVALIER: The chemotaxonomy of actinomycetes. *In* Actinomycete Taxonomy. No. 6. *Eds*., A. DIETZ & D. W. THAYER, pp. 227~291, Soc. for Ind. Micro. Special Publication, 1980
4) LEE, T. M. & G. T. CARTER: Structure determination of simaomicins α and β, extremely potent, novel anticoccidial agents produced by *Actinomadura*. J. Chem. Soc. Chem. Commun. 1989: 1771~1772, 1989
5) GORDON, R. E.; D. A. BARNETT, J. E. HANDERHAN & C. HOR-NAY PANG: *Nocardia coeliaca*, *Nocardia autotrophica*, and the nocardin strain. Int. J. Syst. Bacteriol. 24: 54~63, 1974
6) GOODFELLOW, M.; G. ALDERSON & J. LACEY: Numerical taxonomy of *Actinomadura* and related species. J. Gen. Microbiol. 112: 95~112, 1979
7) KANTOR, S. & E. S. JOHNSON: D42067 alpha: A potent non-polyether ionophore anticoccidial. Program and Abstracts of the 26th Intersci. Conf. on Antimicrob. Agents Chemother., No. 223, p. 137, New Orleans, Sept. 28~Oct. 1, 1986
8) MAIESE, W. M.; M. P. LECHEVALIER, H. A. LECHEVALIER, J. KORSHALLA, J. GOODMAN, M. J. WILDEY, N. KUCK & M. GREENSTEIN: LL-E19085α, a novel antibiotic from *Micromonospora citrea*: Taxonomy, fermentation and biological activity. J. Antibiotics 42: 846~851, 1989
9) DOBLER, M. & W. KELLER-SCHIERLEIN: Metabolites of microorganisms. 162nd communication. The crystal and molecular structure of lysolipin I. Helv. Chim. Acta 60: 178~185, 1977
10) KOBAYASHI, K.; C. NISHINO, J. OHYA, S. SATO, T. MIKAWA, Y. SHIOBARA & M. KODAMA: Actinoplanones C, D, E, F and G, new cytotoxic polycyclic xanthones from *Actinoplanes* sp. J. Antibiotics 41: 741~750, 1988
11) ŌMURA, S.; Y. IWAI, K. HINOTOZAWA, Y. TAKAHASHI, J. KATO, A. NAKAGAWA, A. HIRANO, H. SHIMIZU & K. HANEDA: Cervinomycin A_1 and A_2, new antibiotics active against anaerobes, produced by *Streptomyces cervinus* sp. nov. J. Antibiotics 35: 645~652, 1982
12) ŌMURA, S.; A. NAKAGAWA, K. KUSHIDA & G. LUKACS: Structure of cervinomycin, a novel antianaerobic antibiotic. J. Am. Chem. Soc. 108: 6088~6089, 1986
13) NONOMURA, H. & Y. OHARA: Distribution of actinomycetes in soil, (XI) some new species of the genus *Actinomadura* LECHEVALIER *et al.* J. Ferment. Technol. 49: 904~912, 1971