A NEW ANTIBIOTIC, LEUCINOSTATIN, DERIVED FROM *PENICILLIUM LILACINUM*

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A new antibiotic, leucinostatin, was isolated from the culture filtrate of *Penicillium lilacinum*. The antibiotic was obtained in the form of white prisms, decomposing at 131~136°C, and showing optical rotation $[\alpha]_D^{22}+644°$ (*c* 0.5, methanol). The ultraviolet absorption spectrum showed only an inflection at 220 nm in methanol. The antibiotic gave a positive reaction to biuret and negative to ninhydrin. Upon hydrolysis, it gave a positive reaction to ninhydrin, yielded leucine predominantly, and the peptide nature of the antibiotic was assumed. Leucinostatin was found to be active against some gram-positive bacteria and a wide range of fungi. It was also cytotoxic to HeLa cell culture and showed some inhibitory effect on EHRLICH subcutaneous solid tumor. The LD_{50} by intraperitoneal injection in mice was calculated to be 1.6 mg/kg. The antibiotic manifested a significant hypotensive effect on rabbit blood pressure without affecting epinephrine and acetylcholine responses.

During the course of searching for biologically-active secondary metabolites of fungi, the culture filtrate of strain A-267 was found to exhibit cytotoxicity to HeLa cell culture, antitumor activity on EHRLICH solid carcinoma, and some other antibacterial and antifungal activities. From the results of detailed taxonomical studies, the strain was identified as *Penicillium lilacinum*. The biologically-active principle in the culture filtrate was isolated in colorless crystalline form. It was a peptide being predominantly composed of leucine and unidentified amino acids. The antibiotic was thus named leucinostatin. The present paper describes fermentation, isolation, physicochemical properties and biological activities of leucinostatin.

Fermentation and Isolation of Leucinostatin

Penicillium lilacinum A-267 was maintained on modified CZAPEK's agar containing 1% glucose instead of 3% sucrose. The strain was cultured in ADYE's medium[1] which was used for the production of aflatoxin with slight modification. The medium contained per 1,000 ml of distilled water: sucrose, 50 g; corn steep liquor, 10 g; KH_2PO_4, 10 g; $MgSO_4 \cdot 7H_2O$, 2 g; $Na_2B_4O_7 \cdot 10H_2O$, 0.7 mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.5 mg; $Fe_2(SO_4)_3 \cdot 6H_2O$, 10 mg; $CuSO_4 \cdot 5H_2O$, 0.3 mg; $MnSO_4 \cdot H_2O$, 0.11 mg; $ZnSO_4 \cdot 7H_2O$, 17.6 mg. The pH should be between 4.3 and 4.5. Adjust, if necessary, to pH 4.3~4.5 with 1 N NaOH or 1 N HCl.

Maximum production of leucinostatin was attained after 72-hour incubation at 27°C on a rotary shaker or in a 100-liter stainless steel fermentor containing 50 liters

of the same medium, which was agitated 250 r.p.m. and aerated with 0.5 v/v/min. After incubation, the culture filtrate was adjusted to pH 2.0 with 1 N HCl and twice extracted with one-half volume of ethylacetate. The solvent layers were combined and concentrated *in vacuo* below 50°C. The concentrated solvent layer was washed with 5% $NaHCO_3$ solution and concentrated to dryness. The crude substance was loaded on a silica gel column and successively developed with chloroform and solvent mixtures of chloroform and methanol. Active fractions developed by a mixture of chloroform and methanol (100 : 5) were combined, concentrated and further subjected to Sephadex LH-20 column chromatography. White powder was obtained from the active fractions developed with methanol. This white powder was recrystallized several times from a mixture of ether and petroleum ether.

Physicochemical Properties

Leucinostatin melts at 131~136°C with decomposition. The optical rotation is $[\alpha]_D^{22}$ +644° (*c* 0.5, methanol). The elementary analysis gave the following composition: C 57.40; H 8.89; N 11.87; O 21.74. The molecular weight as determined by vapor pressure osmometry is 1568. The ultraviolet absorption spectrum in methanol which is shown in Fig. 1 has only a shoulder at 220 nm ($E_{1cm}^{1\%}$ 128). The infrared spectrum in KBr tablet is shown in Fig. 2, and the bands at 3300, 1655 and 1540 cm^{-1} correspond to amide functions. It is soluble in lower alcohols, esters, acetone and chloroform, sparingly soluble in ether and benzene and insoluble in *n*-hexane, cyclohexane, petroleum ether and water. The antibiotic gives a positive reaction to biuret but negative to ninhydrin. The nitrogen content, the biuret reaction, and the absorption bands corresponding to amide linkages in the infrared absorption spectrum suggested the peptide nature of leucinostatin. Hydrolysis in 6 N HCl at 105°C for 48 hours in a sealed tube and subsequent amino acid analysis revealed the predominant presence of leucine and four

Fig. 1. Ultraviolet absorption spectrum of leucinostatin (in methanol)

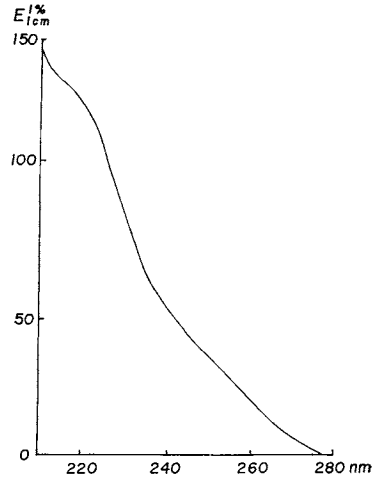
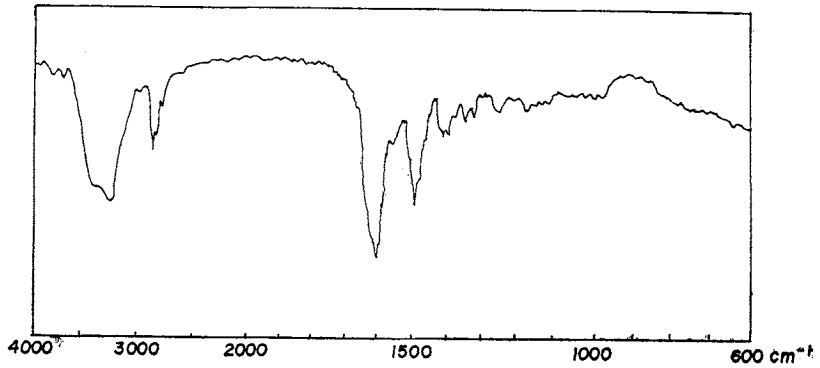


Fig. 2. Infrared absorption spectrum of leucinostatin (KBr tablet)



unidentified amino acids in leucinostatin.

Biological Properties

In vitro antibacterial and antifungal spectra of leucinostatin were determined by employing 14 strains of gram-positive and -negative bacteria and 23 strains of fungi. The results are shown in Tables 1, 2 and 3 with pertinent descriptions of media, incubation period and temperature. Leucinostatin is moderately active against some gram-positive bacteria. *Sarcina lutea* and *Bacillus subtilis* were found to be most sensitive to the antibiotic and mycobacteria were less susceptible. The antibiotic is inactive against gram-negative bacteria. Leucinostatin is fairly active on a wide range of fungi including both pathogenic and non-pathogenic strains. Among 5 strains of *Candida albicans*, strain YU 1200 was inhibited at a concentration as low as 1.0 mcg/ml, while minimal inhibitory concentration for *C. albicans* IFM strain was 10 mcg/ml. As is shown in Table 4, leucinostatin is rather heat stable and the decrease in its activity is only negligible or two-fold at most by heating at 60°C for 30 minutes

Table 1. Antimicrobial spectrum of leucinostatin

Test organism	Medium*	MIC (mcg/ml)
Staphylococcus aureus FDA 209 P	N	75
Staphylococcus albus	N	10
Bacillus subtilis	N	5
Sarcina lutea	N	2.5
Streptococcus pyogenes	B	10
Streptococcus faecalis	B	10
Mycobacterium sp. 607	N	50
Mycobacterium phlei	N	50
Serratia marcescens	N	100
Escherichia coli F_1	N	>100
Salmonella typhosa	N	>100
Proteus vulgaris	N	>100
Shigella dysenteriae	N	>100
Pseudomonas aeruginosa	N	>100

Medium*: N=nutrient agar, B=blood agar
Incubation: 48 hours for *Mycobacterium* and 24 hours for others at 37°C

Table 2. Antifungal spectrum of leucinostatin (1)

Test organism	MIC (mcg/ml)
Candida albicans IFM strain	10
Candida albicans Saito	2.5
Candida albicans Nakagawa	2.5
Candida albicans 7 N	2.5
Candida albicans YU 1200	1.0
Candida guilliermondii	5.0
Candida krusei	10
Candida parakrusei	10
Candida tropicalis	2.5
Candida stellatoidea	5.0
Cryptococcus neoformans	0.5
Sporotrichum schenckii	2.5
Trichophyton mentagrophytes	1.0
Trichophyton rubrum	1.0
Microsporum gypseum	5.0

Medium: 4% SABOURAUD agar
Incubation: 72 hours for *Sporotrichum schenckii*, *Trichophyton rubrum* and 48 hours for others at 37°C

Table 3. Antifungal spectrum of leucinostatin (2)

Test organism	Medium*	MIC (mcg/ml)
Saccharomyces cerevisiae	S	5
Zygosaccharomyces salsus	S	10
Torula rubra Saito	Cz	0.5
Penicillium glaucum	Cz	5.0
Aspergillus oryzae	Cz	25.0
Aspergillus niger	Cz	5.0
Mucor mucedo	Cz	1.0
Rhizopus nigricans	Cz	1.0

Medium*: S=SABOURAUD agar, Cz=CZAPEK DOX agar
Incubation: 24 hours for *Mucor mucedo*, *Rhizopus nigricans* and 48 hours for others at 27°C

Table 4. Heat stability of leucinostatin

Temp.	Time (min.)	MIC (mcg/ml)			
		C. albicans 7 N	*C. albicans* YU 1200	*T. mentagrophytes*	*T. rubrum*
60°C	10	10	5	1	1
	30	10	5	2.5	2.5
100°C	10	10	5	2.5	2.5
	30	25	10	2.5	2.5

Incubation: 48 hours for *C. albicans* and 72 hours for *Trichophyton* at 37°C

Table 5. Effect of serum on leucinostatin activity

Test organism	Concentration of serum and MIC (mcg/ml)				
	30%	10%	5%	1%	0%
C. albicans 7 N	5.0	5.0	2.5	2.5	2.5
C. albicans YU 1200	2.5	2.5	1.0	1.0	1.0
T. mentagrophytes	1.0	1.0	1.0	1.0	1.0
T rubrum	1.0	1.0	1.0	0.5	1.0

Incubation : 48 hours for *C. albicans* and 72 hours for *Trichophyton* at 37℃

Table 6. Ultraviolet sensitivity* of leucinostatin

Test organism	MIC (mcg/ml)		
	0 min.	10 min.	30 min.
C. albicans	2.5	10	25
C. albicans YU 1200	1.0	5	10
T. mentagrophytes	1.0	2.5	5
T. rubrum	1.0	2.5	5

* exposure time in minutes for a Mitsubishi sterilization lamp GL-15 (2537 Å) at 17 cm distance
Incubation : 48 hours for *C. albicans* and 72 hours for *Trichophyton* at 37℃

Table 7. Effect of leucinostatin on EHRLICH subcutaneous solid tumor

Dose (mg/kg/day)	No. of injections	Av. body wt. change (g) test/control	Tumor wt. (mg) test/control	T/C	No. of deaths
0.25	7	−3.6/5.8	384/778	0.49	0/10
0.5	2	−1.1/5.8	291/778	0.36	5/10

or at 100°C for 30 minutes. The incorporation of up to 30% bovine serum into medium also did not affect leucinostatin activity significantly as is shown in Table 5. The activity of leucinostatin in methanol dropped to approximately one-half of the original solution after 10-minute irradiation of ultraviolet light and to one-tenth after 30 minutes (Table 6). Leucinostatin is cytotoxic to HeLa cell culture at a concentration of 0.05 mcg/ml. LD_{50} to mice by intraperitoneal route was calculated to be 1.6 mg/kg. When mice was implanted intraperitoneally, or subcutaneously at left inguinal region, with 1×10^6 EHRLICH ascites tumor cells and treatment was initiated intraperitoneally after 24 hours, leucinostatin did not show any antitumor effect on ascite form of the tumor. Up to a dose of 0.25 mg per mouse, however, it showed some inhibitory effect on the subcutaneous solid form of the same tumor as is shown in Table 7. When the blood pressure of the carotid artery of rabbit was recorded by a pressure transducer, the intravenous administration of 0.5 mg/kg of the antibiotic showed prolonged hypotensive effect without affecting the responses of 5 mcg/kg of epinephrine and 2 mcg/kg of acetylcholine.

Discussion

The results of the present experiments clearly indicate the peptide nature of leucinostatin. Leucinostatin is readily differentiated from known peptide antibiotics derived from fungi by its molecular weight, peculiar amino acid composition and other physicochemical and biological characteristics. More recently, a peptide antibiotic, lilacinin, was isolated from the culture filtrate of *Penicillium lilacinum*[2]. Lilacinin is a water-soluble peptide antibiotic melting at 102~103°C. Its molecular weight is determined as 748, and it is much less active against gram-positive bacteria than fungi and is also differentiated from leucinostatin.

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