STUDIES ON PEPTIDE ANTIBIOTICS, LEUCINOSTATINS

I. SEPARATION, PHYSICO-CHEMICAL PROPERTIES AND BIOLOGICAL ACTIVITIES OF LEUCINOSTATINS A AND B

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Leucinostatin, a peptide antibiotic, was separated by silica gel and alumina column chromatography into two related components designated as leucinostatin A hydrochloride ($C_{61}H_{111}N_{11}O_{13}\cdot HCl$) and leucinostatin B hydrochloride ($C_{61}H_{109}N_{11}O_{13}\cdot HCl$). Physico-chemical as well as biological properties of the two separated components were analyzed. These properties pointed to closely resembling chemical structures.

In a previous paper[1], leucinostatin, isolated from the culture filtrate of *Paecilomyces lilacinus* A-267 as white prisms, has been characterized to be a single entity. However, in the course of determination of the chemical structure of leucinostatin, it was found that the antibiotic was a complex of two components which could be separated on thin-layer and by high performance liquid chromatography. The thin-layer and high performance liquid chromatograms of leucinostatin are shown in Figs. 1 and 2,

Fig. 1. Thin-layer chromatograms of leucinostatins A and B hydrochlorides.
Solvent system;
A: MeOH - CH_3COCH_3 (1: 4)
B: BuOH - AcOH - H_2O (4: 1: 2)
Detection by UV (254 nm)

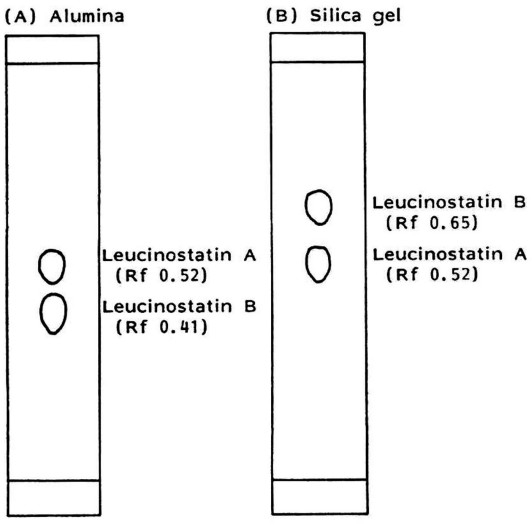
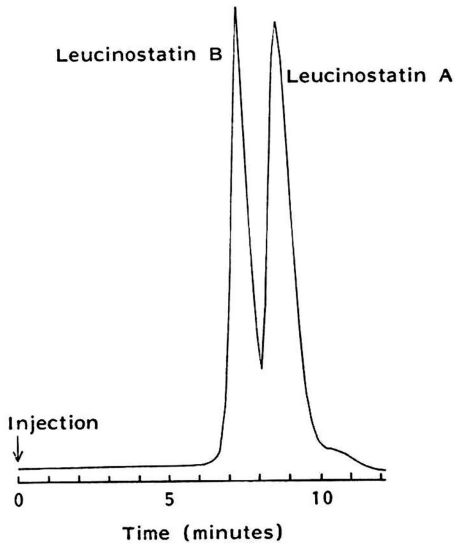


Fig. 2. HPLC of leucinostatins A and B hydrochlorides.
Packing: μBondapak C_{18}, flow rate: 1 ml/minute, solvent: 1% triethylamine-phosphoric acid - MeOH (2: 8), UV range: 254 nm (sens. 0.5), chart speed: 1 cm/minute.



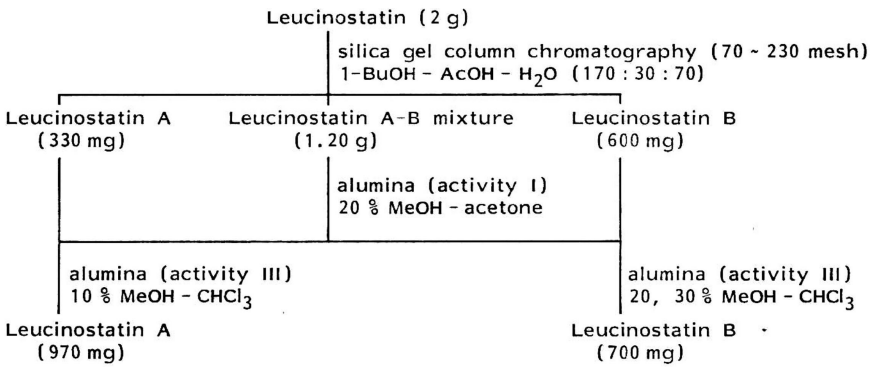
respectively. The two components were named leucinostatin A and leucinostatin B in accordance with their Rf values on alumina thin-layer chromatogram (Fig. 1-A).

Separation, physico-chemical and biological properties of leucinostatins-A and B are described in this paper.

Separation of Leucinostatins A and B

In a preceding paper[1], the isolation and purification procedures of leucinostatin were reported. Separation of two components, leucinostatins A and B, from leucinostatin complex was achieved by silica gel and alumina column chromatography as shown in Fig. 3. Silica gel column chromatography was carried out as follows: Two grams of leucinostatin complex dissolved in a small amount of a solvent mixture of 1-butanol - acetic acid - water (4: 1: 2) was charged on a Silica gel (Merck, 70~230 mesh, 300 g) column (3.0×80 cm). The column was developed with a solvent mixture of 1-butanol - acetic acid - water (170: 30: 70). The eluent was made to flow through the column at a flow rate of 5 ml/minute. The eluates were collected in 75 ml fractions, each checked by silica gel thin-layer chromatography (TLC). Fractions containing only leucinostatin B component were eluted first, collected and evaporated *in vacuo* to give a syrup (600 mg). Fractions of a mixture of leucinostatin B and A were subsequently eluted, and evaporation of the solvent gave 1.2 g of the mixture as a syrup. Fractions containing only leucinostatin A component were finally eluted and collected. The solvent was evaporated to dryness to give leucinostatin A as a syrup (330 mg). A portion of the mixture obtained as described above was subsequently treated as follows. The mixture dissolved in a small amount of 20% methanol in acetone, used as the eluent, was subjected to alumina column chromatography (Woelm, activity I, 500 g, 3.0× 90 cm). Eluates were collected in fractions of each 50 ml. The elution of each component was monitored by TLC on silica gel plates. The mixture was completely separated into the two components by this method. Fractions containing leucinostatin A were eluted first, followed by those of leucinostatin B. Each component was obtained as a syrup after evaporation of the solvent. The samples of leucinostatins A and B obtained from silica gel and alumina chromatography were combined prior to further purification by chromatography. The combined fractions of leucinostatin A were dissolved in a small amount of 10% methanol in chloroform, poured onto a top of a column (3.0×20 cm), packed with alumina (activity III), and eluted with the solvent mixture described above. Fractions containing leucinostatin A were combined and evaporated *in vacuo* to dryness, yielding 970 mg of a white powder. Purification of the combined fractions of leucinostatin B was carried out in a similar manner except that

Fig. 3. Separation procedure of leucinostatins A and B hydrochlorides.



20 and 30% methanol in chloroform were used as an eluent; 700 mg of leucinostatin B was obtained as a white powder.

Physico-chemical Properties

The separated samples of leucinostatins A and B were found to be hydrochlorides. The salts were

Fig. 4. IR spectra of leucinostatins A and B hydrochlorides.

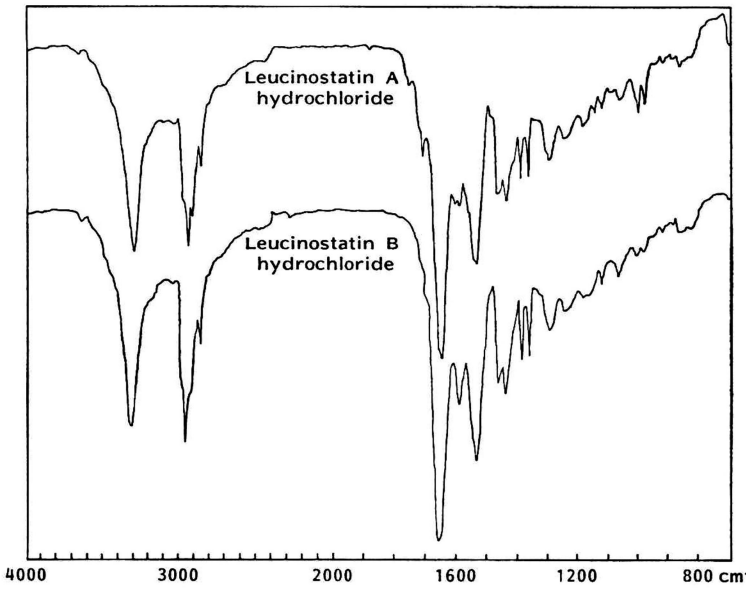
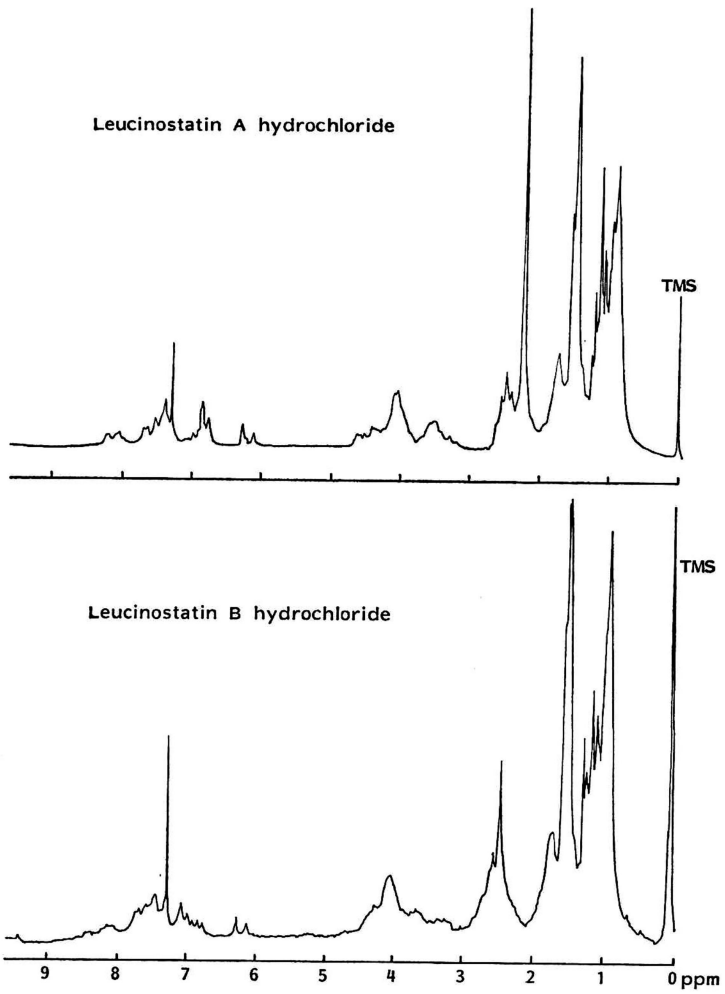


Table 1. Physico-chemical properties of leucinostatins.

	Leucinostatin A hydrochloride		Leucinostatin B hydrochloride	
Melting point (°C)	98~101		132~140	
Optical rotation $[\alpha]_D^{20}$	−11° (*c* 0.1, MeOH)		−31° (*c* 0.091, MeOH)	
UV λ_{max}^{EtOH} nm (log ε)	202 (4.05)		204 (4.38)	
	220 (sh. 3.83)		213 (4.27)	
Molecular weight	1,217		1,203	
FD-MS, *m/z*	1,218 $(M+H)^+$		1,204 $(M+H)^+$	
FAB-MS, *m/z*	1,218 $(M+H)^+$		1,204 $(M+H)^+$	
Elemental analysis	Found	Calcd. for $C_{62}H_{111}N_{11}O_{13}\cdot HCl$	Found	Calcd. for $C_{61}H_{109}N_{11}O_{13}\cdot HCl$
C	59.55	59.33	59.24	59.04
H	9.45	9.00	9.53	8.93
N	12.45	12.28	12.38	12.41
Color reaction				
DRAGENDORFF	Positive		Positive	
Ninhydrin	Negative		Positive	
^{13}C NMR $CDCl_3$, ppm	211.0, 177.2, 176.4, 176.1, 175.7, 175.5, 174.1, 173.9, 172.6, 166.9, 150.6, 120.9, 77.2, 74.6, 63.6, 62.4, 60.8, 56.4, 56.0, 55.8, 54.7, 50.6, 45.4, 44.3, 40.1, 39.6, 39.3, 38.5, 37.9, 37.6, 36.6, 34.5, 33.7, 30.3, 29.6, 29.0, 27.5, 27.0, 25.7, 24.7, 22.8, 22.6, 22.3, 21.4, 19.8, 18.7, 18.5, 17.4, 16.5, 11.7, 7.6		211.7, 176.8, 176.4, 175.7, 175.2, 174.3, 173.9, 172.6, 166.9, 151.9, 120.1, 77.1, 64.0, 63.7, 60.4, 56.5, 56.2, 55.5, 54.9, 50.0, 42.2, 39.7, 39.3, 38.0, 37.5, 36.8, 34.7, 34.0, 33.7, 30.5, 29.6, 29.0, 27.6, 27.1, 27.0, 26.4, 25.0, 24.8, 22.9, 22.7. 22.3, 22.0, 21.6, 21.3, 20.2, 19.7, 18.7, 18.0, 17.8, 16.6, 11.7, 7.6	

recrystallized from a mixture of ether (or benzene) and *n*-hexane to give colorless prisms. The physicochemical properties of leucinostatin hydrochlorides are summarized in Table 1. Leucinostatins A and B hydrochlorides are soluble in methanol, ethanol, ethyl acetate, acetone, chloroform and dimethyl sulfoxide, sparingly soluble in ethyl ether and benzene, and insoluble in *n*-hexane, petroleum ether and water. The IR absorption spectra indicated that these antibiotics are peptides (Fig. 4). The ultraviolet spectra of leucinostatins A and B hydrochlorides in ethanol resemble each other: for instance, leucinostatin A hydrochloride shows maxima at the following wave lengths (log ε): 202 (4.05) and 220 nm (sh 3.83) suggesting the presence of conjugated amide moieties in its molecule. Field desorption (FD) and fast atom bombardment (FAB) mass spectrometric analyses of leucinostatins A and B hydrochlorides gave protonated molecular ion peaks as base peaks, at *m/z* 1,218 and 1,204, respectively. The ^{1}H NMR (100 MHz) and ^{13}C NMR (25 MHz) spectra were recorded with a Jeol JNM FX-100 spectrometer in deuterochloroform using tetramethylsilane (TMS) as an internal reference. The ^{1}H NMR spectra of the antibiotics are shown in Fig. 5. The spectrum of leucinostatin A hydrochloride shows absorption at δ 6.18 and 6.82 indicating the presence of olefinic protons. Corresponding signals were also recognized

Fig. 5. ^{1}H NMR spectra (100 MHz) of leucinostatins A and B hydrochlorides.



in the spectrum of leucinostatin B hydrochloride. The ^{13}C NMR data of both antibiotics are given in Table 1.

Acid hydrolyses of the antibiotics showed that each antibiotic was comprised of an unsaturated fatty acid, six kinds of amino acids and a basic substance. Of the amino acids three were identified as leucine, α-aminoisobutylic acid and β-alanine, the others were unusual amino acids. The constituent fatty acid and amino acids were the same in both of the antibiotics and only the basic substances were differed. Structural studies on the unidentified constituents of leucinostatins will be discussed in the following publication[2].

Biological Properties

In vitro antimicrobial spectra of leucinostatins A and B hydrochlorides were obtained by agar dilution method under identical conditions. The results are given in Tables 2 and 3. As can be seen from the tables both of the antibiotics were fairly active against yeasts including pathogenic and non-pathogenic strains and filamentous fungi, and were moderately active against Gram-positive bacteria. Furthermore, the data showed that the minimum inhibitory concentrations (MIC) of the two antibiotics against the sensitive microorganism are similar. The Gram-negative bacteria examined were insensitive to the antibiotics.

Both antibiotics are quite toxic. The acute toxicities determined by intraperitoneal LD_{50} values for leucinostatins A and B hydrochlorides were found to be 1.8 mg/kg body weight. The oral LD_{50} of leucinostatins A and B hydrochlorides amounted to 5.4 mg and 6.3 mg/kg body weight, respectively. These data show that leucinostatins can be classified into a class of very toxic mycotoxins such as citreoviridin,

Table 2. Antifungal spectrum of leucinostatins A and B.

Test organism	Medium*	MIC (μg/ml)	
		Leucinostatin A	Leucinostatin B
Candida albicans IFM strain	S	10.0	7.5
C. albicans Saito	S	2.5	2.5
C. albicans Nakagawa	S	2.5	1.25
C. albicans 7N	S	1.25	1.25
C. albicans YU 1200	S	1.25	1.0
Candida quilliermondii	S	2.5	2.5
Candida krusei	S	10.0	10.0
Candida parakrusei	S	10.0	7.5
Candida tropicalis	S	2.5	1.25
Candida stellatoidea	S	2.5	2.5
Cryptococcus neoformans	S	1.0	0.5
Sporothrix schenckii	S	2.5	2.5
Trichophyton mentagrophytes	S	1.0	0.5
Trichophyton rubrum	S	0.5	0.5
Microsporum gypseum	S	7.5	5.0
Saccharomyces cerevisiae	S	7.5	7.5
Zygosaccharomyces salsus	S	10.0	7.5
Torula rubra Saito	S	1.0	1.0
Penicillium glaucum	Cz	7.5	5.0
Aspergillus oryzae	Cz	25.5	25.5
Aspergillus niger	Cz	5.0	2.5
Mucor mucedo	Cz	1.0	1.0
Rhizopus nigricans	Cz	1.0	1.0

* Medium: S; Sabouraud agar, Cz; Czapek-Dox agar.

luteoskyrin, aflatoxin B_1, cytochalasins and trichothecenes[3].

The effect of leucinostatins on mitochondria was examined. Rat liver mitochondria were isolated by the method of HOGEBOOM[4]. The respiratory rate of mitochondria was measured with a Clark oxygen electrode (Yellow Spring Instruments) at 25°C in a medium consisting of 250 mM mannitol, 10 mM KH_2PO_4, 10 mM Tris·HCl, 10 mM KCl, 2 mM $MgCl_2$ and 0.2 mM EDTA, pH 7.4. Fig. 6 shows the effect of leucinostatins A and B hydrochlorides on state 4 of mitochondria using succinate as substrate. Both

Table 3. Antibacterial spectrum of leucinostatins A and B hydrochlorides.

Test organism	Medium*	MIC (μg/ml)	
		Leucinostatin A	Leucinostatin B
Staphylococcus aureus FDA 209P	N	62.5	50.0
Staphylococcus albus	N	12.5	10.0
Bacillus subtilis	N	6.25	6.25
Micrococcus luteus	N	2.5	2.5
Streptococcus pyogenes	B	10.0	10.0
Streptococcus faecalis	B	10.0	7.5
Mycobacterium sp. 607	N	50.0	31.25
Mycobacterium phlei	N	62.5	50.0
Serratia marcescens	N	100.0	100.0
Escherichia coli F_1	N	>100.0	>100.0
Salmonella typhosa	N	>100.0	>100.0
Proteus vulgaris	N	>100.0	>100.0
Shigella dysenteriae	N	>100.0	>100.0
Pseudomonas aeruginosa	N	>100.0	>100.0

* Medium: N; nutrient agar, B; blood agar.

Fig. 6. Effect of leucinostatins A and B hydrochlorides on the respiration of rat liver mitochondria.
Mitochondria: 0.9 mg protein/ml. Total volume of the reaction mixture: 3 ml. Dotted lines indicate the respiration rate without any drug. Leucinostatins A and B were added at indicated point at three different concentrations separately.

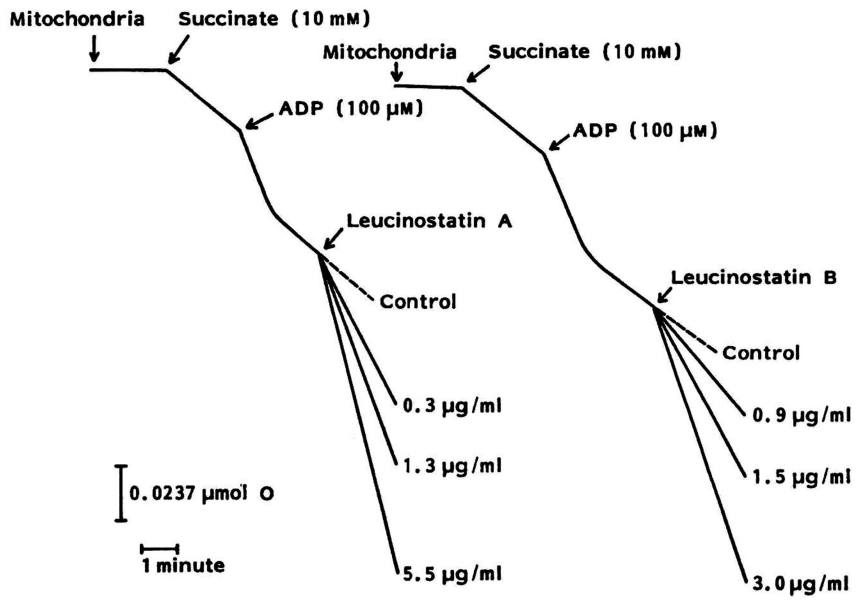
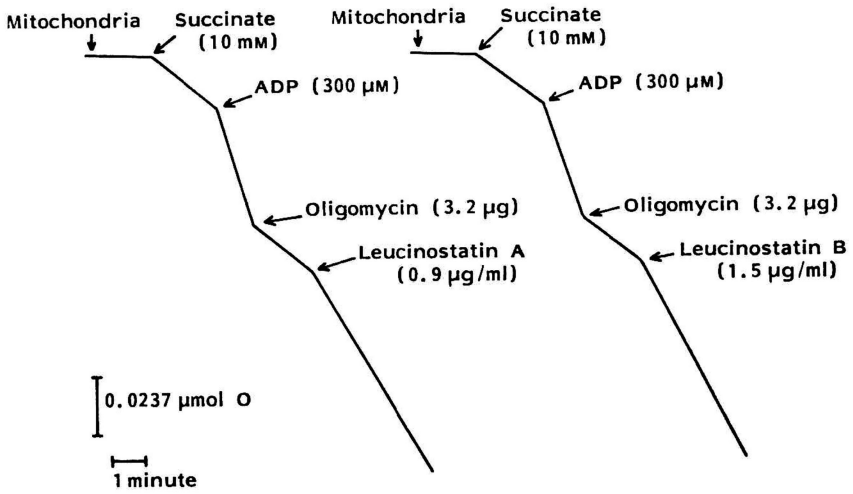


Fig. 7. Effect of leucinostatins A and B hydrochlorides on oligomycin-inhibited respiration of rat liver mitochondria.

Substrate: 10 mM succinate. Mitochondria: 0.9 mg protein/ml. ADP: Adenosine 5'-diphosphate, disodium salt. Total volume of the reaction mixture: 3 ml.



of the antibiotics stimulated the respiration of state 4 of mitochondria. The minimum concentration to obtain this effect was 0.3 μg/ml with leucinostatin A hydrochloride, and 0.9 μg/ml with leucinostatin B hydrochloride. The effect was dose-dependent. Leucinostatin A hydrochloride released almost completely the oligomycin-inhibited respiration of mitochondria at a concentration of 0.9 μg/ml, as shown in Fig. 7. The same result was obtained at 1.5 μg/ml with leucinostatin B hydrochloride. The effect of the antibiotics were characteristic of that of the uncouplers of oxidative phosphorylation in mitochondria[5,6]. Thus, it was concluded that leucinostatins act as uncouplers on mitochondria.

References

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