

STUDIES ON PEPTIDE ANTIBIOTICS, LEUCINOSTATINS

II. THE STRUCTURES OF LEUCINOSTATINS A AND B

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Structures **I** and **II** have been assigned to leucinostatins A and B based on fast atom bombardment, secondary ion, field desorption and chemical ionization mass spectrometry, NMR studies and chemical degradation methods of the intact antibiotics and their acid hydrolysis products.

The essential difference between leucinostatins A and B is concluded to be the replacement of (2*S*)-*N*¹,*N*¹-dimethylpropane-1,2-diamine in leucinostatin A by (2*S*)-*N*¹-methylpropane-1,2-diamine in leucinostatin B. This was further confirmed from the evidence that methylation with methyl iodide led each antibiotic to the identical compound which was named leucinostatin A-M (**III**).

The antibiotic leucinostatin having antimicrobial activity against bacteria and fungi, antitumor activity and uncoupling effect on rat liver mitochondrial function, was isolated from the culture filtrate of *Paecilomyces lilacinus* A-267.

In the first report on leucinostatin¹⁾, the antibiotic was described as a single entity. The subsequent studies clarified that leucinostatin was not a single substance, but a mixture of two closely related components, leucinostatins A and B.

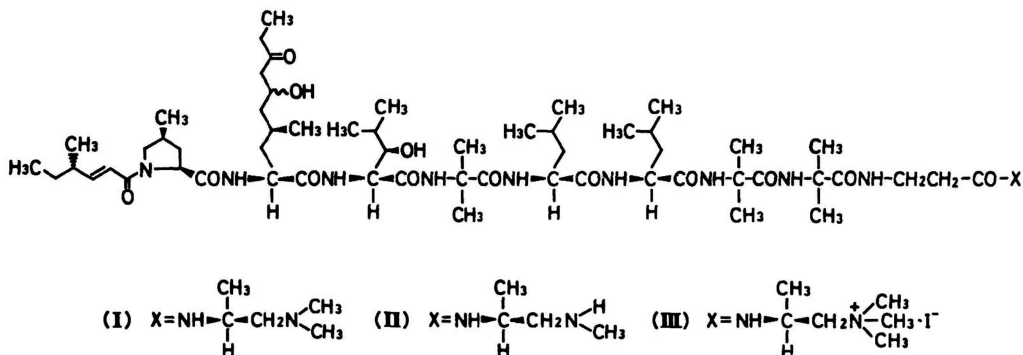
In the preceding paper²⁾ we have described the separation, physicochemical and biological properties of both components.

After separation into each component, preliminarily structural studies of leucinostatin A have been performed and we have reported its structure³⁾.

The structure of leucinostatin A (**I**) is shown in Fig. 1 along with the structures of leucinostatins B (**II**) and A-M (**III**) which were recently elucidated. The antibiotics, **I** and **II**, are basic peptides which contain an unsaturated fatty acid, nine amino acid residues and a basic component joined together by amide linkages.

In independent studies, ISOGAI *et al.*^{4,5)} reported structure of an antibiotic P168 produced by *Paecilomyces lilacinus* (Thom) Samson. SATO *et al.*⁶⁾ reported structure of an antibiotic 1907-II which was produced together with 1907-VIII by *Paecilomyces lilacinus* strain No. 1907. The five antibiotics including leucinostatins A and B, can be divided into two groups, one having a molecular weight of 1,217 (leucinostatin A, P168 and 1907-VIII) and the other 1,203 (leucinostatin B and 1907-II). Of these antibiotics, except for 1907-VIII whose structure has been reported to be an *N*-methylated compound of 1907-II, the primary structures of leucinostatins A and B are the same as those of P168 and 1907-II, respectively. However, further identification between both antibiotics could not be made owing to the lack of stereochemical studies on the constituents of P168 and 1907-II as follows. The stereochemical

Fig. 1. Structures of leucinostatins A (I), B (II) and A-M (III).



studies on the constituent fatty acid and amine of antibiotic P168 have been remained open, and there were no descriptions about the absolute stereochemistry of all the chiral constituents of antibiotic 1907-II. Furthermore, the amino acid sequence of antibiotic 1907-II has been elucidated only by in-beam EI mass spectrometry.

In our structural studies on leucinostatins, stereochemistry of all their chiral constituents has been established. In this paper we wish to present our experimental results leading to structural elucidations of I and II, as well as their constituents in details.

Results and Discussion

Constituents of Leucinostatins A and B

Each antibiotic was found to be composed of a fatty acid, amino acids and a basic component by acid hydrolysis of them.

Fatty Acid Moiety

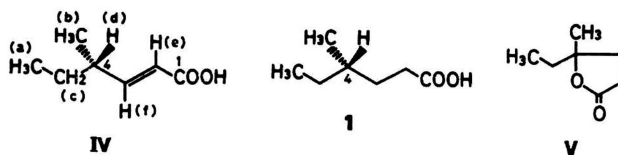
Hydrolysis of leucinostatin A (I) with 6 N HCl at 110°C for 20 hours gave an oily material which was extracted with ether and examined by gas-liquid chromatography (GLC). Fig. 2 shows the gas-liquid chromatograms of the extract prepared after hydrolysis for 20 hours (a) and 1 hour (b). The extract was found to be a mixture of two major materials, IV and V, which was subjected to further purification by preparative thin-layer chromatography (TLC). After distillation *in vacuo*, the two materials were obtained as colorless oil.

Structure of IV, which was slightly fluorescent under UV, was determined to be (4*S*)-(2*E*)-4-methylhex-2-enoic acid on the basis of the following spectral data. The electron impact (EI) and field ionization (FI) mass spectra gave the molecular ion peak at m/z 128. The bands at 3600~2400, 1685 and 1640 cm^{-1} in IR spectrum suggested the presence of an α,β -unsaturated carboxylic acid system in the molecule. The methylated product of IV with diazomethane gave the molecular ion peak at m/z 142. The 100 MHz ^1H NMR spectrum and decoupled spectra of IV were recorded. A triplet (3H, $J=7$ Hz, $-\text{CH}_2\text{CH}_3$) at 0.89 ppm and a doublet (3H, $J=7$ Hz, $-\text{CHCH}_3$) at 1.05 ppm were assigned to Ha and Hb, respectively, because both signals collapsed into singlets on irradiating each proton signal at 1.45 (Hc) and 2.26 (Hd) ppm. From the decoupled spectra obtained by irradiating the respective proton signal at 2.26, 5.77 and 6.98, the presence of $-\text{CHCH}=\text{CHCO}$ system was indicated. It was further confirmed that the signals of olefinic protons, a doublet (1H, $J=16$ Hz) at 5.77 ppm and a double doublet (1H,

$J=16, 8$ Hz) at 6.98 ppm, were assigned to He and Hf, respectively, and they had *E*-geometry. On the basis of the above data, the primary structure of IV was deduced to be (*2E*)-4-methylhex-2-enoic acid.

In order to determine the absolute configuration of IV, it was hydrogenated over palladium charcoal to afford a saturated acid, 4-methylhexanoic acid, $[\alpha]_D^{20} +7.6^\circ$ (c 0.15, CHCl_3); IR $\nu_{\text{max}}^{\text{CHCl}_3}$ cm^{-1} 3500~2400 and 1710; $^1\text{H NMR}$ $\delta_{\text{ppm}}^{\text{CDCl}_3}$ 0.86~0.95 (6H, $-\text{CH}_2\text{CH}_3$ and $-\overset{|}{\text{C}}\text{HCH}_3$), 1.06~1.88 (5H, methylene and methine), 2.38 (2H, t, $J=8$ Hz, $-\text{CH}_2\text{CH}_2\text{COOH}$); MS m/z 109 ($\text{M}^+ - 29$), 74, 71 (base peak) and 60. These data were identical with those of (*4S*)-4-methylhexanoic acid (**1**), including specific rotation $[\alpha]_D^{20} +7.4^\circ$, described previously⁷⁾. This good agreement led us to conclude that C-4 had *S*-configuration.

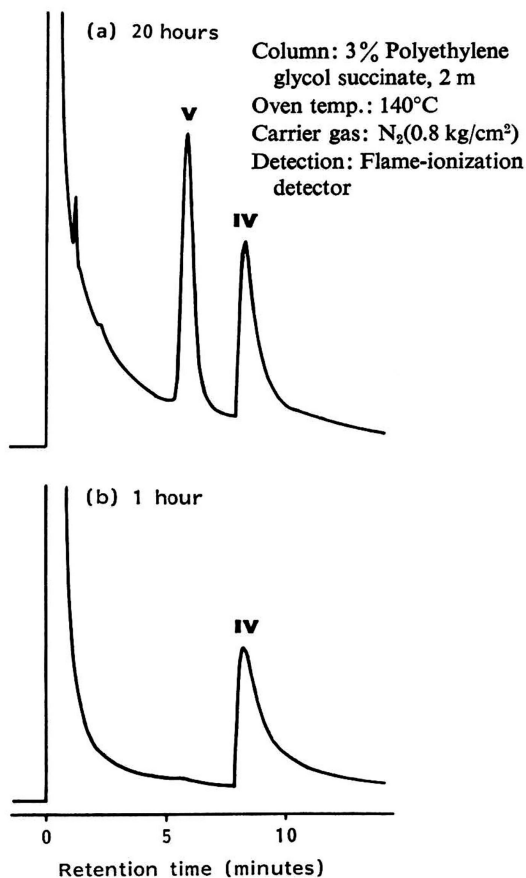
The other oily substance (V) was deduced to be 4-hydroxy-4-methylhexanoic-1,4-lactone by spectral analyses: IR $\nu_{\text{max}}^{\text{CHCl}_3}$ 1770 cm^{-1} ; EI-MS m/z 128 (M^+); CI-MS (NH_3) m/z 146 ($\text{M} + \text{NH}_3$)⁺; $^1\text{H NMR}$ $\delta_{\text{ppm}}^{\text{CDCl}_3}$ 0.98 (3H, t, $J=7$ Hz), 1.39 (3H, s), 1.72 (2H, q, $J=7$ Hz), 2.02 (2H, m) and 2.60 (2H, m).



However, the IR spectrum of I did not show the absorption of lactone while its $^1\text{H NMR}$ spectrum showed the two olefinic proton signals assignable to an α, β -unsaturated bond of the fatty acid IV. This suggests that V is formed from IV during acid hydrolysis. The results obtained by the following experiments supported the above consideration as well as the presence of the fatty acid of IV in I. The antibiotic I was hydrogenated to give dihydroleucinostatin A, FD-MS m/z 1,220 (MH^+), which subsequently gave (*4S*)-4-methylhexanoic acid (**1**) by acid hydrolysis.

It has been reported that longer hydrolysis of antibiotics containing β -hydroxy fatty acid constituents, e.g. EM49⁸⁾ and 333-25⁹⁾, resulted in extensive destruction of the fatty acids and formation of a mixture consisting largely of α, β -unsaturated fatty acids and butyrolactones. Similar observations were made in our work. As shown in Fig. 2, two peaks were found in the ethereal extract from the

Fig. 2. Gas-liquid chromatograms of the ethereal extract obtained by the hydrolysis of leucinostatin A for 20 hours (a) and 1 hour (b).



hydrolysis for 20 hours, but the sample prepared by the hydrolysis for 1 hour gave primarily one peak corresponding to **IV**. On the other hand, in structural studies on fatty acid moiety of leucinostatin **B** (**II**) carried as in the case of **I**, the fatty acid **IV** and the butyrolactone **V** were identified in the ethereal extract from the hydrolysate of **II**. Thus, it was concluded that the fatty acid **IV** was the same in **II** as well as in **I**.

Amino Acid Moieties

When the acid hydrolysate of **I** was analyzed by an amino acid analyzer, leucine, α -aminoisobutyric acid, β -alanine and two unusual amino acids, **VI** and **VII**, and an unknown basic component which appeared at the position immediately behind ammonia, were found (Fig. 3). Similarly, the same five amino acids as above, and an unknown basic component which differed from that of **I** and being observed immediately before ammonia, appeared in the hydrolysate of **II**. Thin-layer chromatograms of the hydrolysates of both **I** and **II** gave seven ninhydrin-positive constituents. Fig. 4 shows the example of **I**. On the basis of the result obtained from the thin-layer chromatogram, the constituent amino acids of **I** were isolated from the acid hydrolysate by a combination of cellulose column chromatography and preparative cellulose TLC. In addition to the five amino acids mentioned above, also an unknown amino acid (**VIII**) was isolated.

The isolated amino acids, α -aminoisobutyric acid (Aib), and β -alanine (β -Ala) were identified by

Fig. 3. Amino acid analysis of the hydrolysate of leucinostatin A.

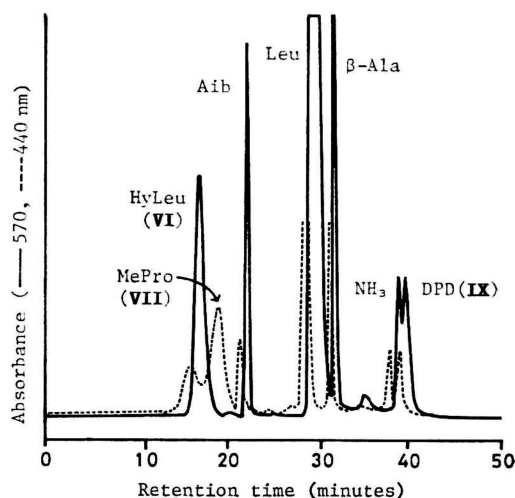
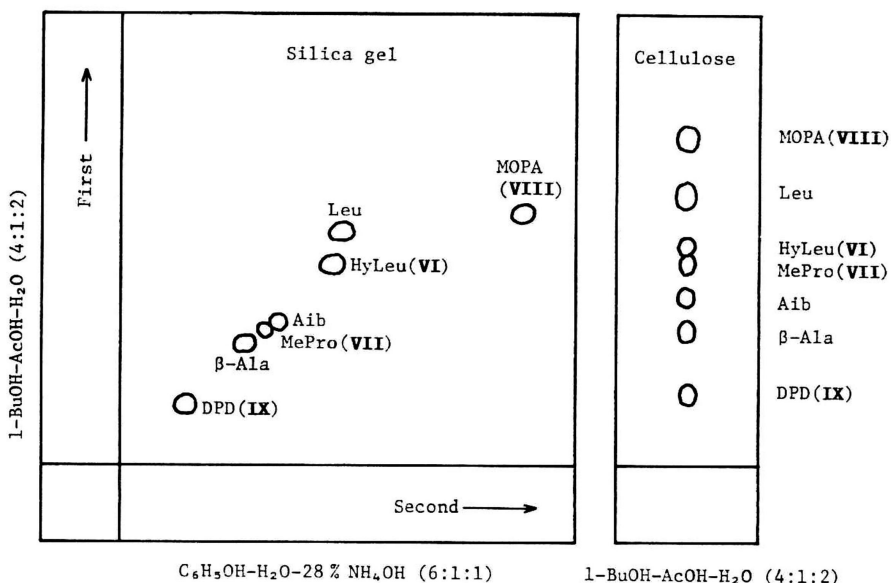


Fig. 4. Thin-layer chromatograms of the hydrolysate of leucinostatin A.

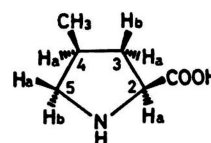


direct comparisons with authentic samples. Leucine (Leu) was deduced to be L-form from its specific rotation and its oxidation by L-amino acid oxidase.

The amino acid (VI) showed the following specific rotation and spectral data: $[\alpha]_D^{25} -4.4^\circ$ (c 0.09, H_2O); mp $210\sim 215^\circ C$; FD-MS m/z 148 (MH^+); 1H NMR $\delta_{ppm}^{D_2O}$ 1.10 (3H, d, $J=7$ Hz, $-\overset{|}{C}HCH_3$), 1.07 (3H, d, $J=7$ Hz, $-\overset{|}{C}HCH_3$), 1.76 (1H, heptet, $J=7$ Hz, $-\overset{|}{C}H(CH_3)_2$), 3.80 (1H, dd, $J=3.5, 7$ Hz, $-\overset{|}{C}HOH$) and 3.92 (1H, d, $J=3.5$ Hz, $N-\overset{|}{C}HCO$). Moreover, the IR spectrum of VI was in good agreement with that of L-threo- β -hydroxyleucine reported in the literature¹⁰. Thus, the structure of the amino acid VI was identified to be L-threo- β -hydroxyleucine (HyLeu).

Physico-chemical properties and spectral data of the amino acid VII were as follows: mp $242\sim 248^\circ C$; a yellow coloration with ninhydrin; $[\alpha]_D^{25} -77^\circ$ (c 0.66, H_2O); FD-MS m/z 130 (MH^+); 1H NMR $\delta_{ppm}^{D_2O}$ 1.08 (3H, d, $J=6$ Hz, $-\overset{|}{C}HCH_3$), 1.60 (1H, q, $J=4$ Hz, $J_{2a-3b}=9$ Hz, H_{3b}), 2.42 (2H, complex multiplet, H_{3a}, H_{4a}), 2.94 (1H, q, $J_{5a-4a}=9.5$ Hz, $J_{5a-5b}=11.6$ Hz, H_{5a}), 3.48 (1H, q, $J=11.6$ Hz, $J_{5b-4a}=8.1$ Hz, H_{5b}) and 4.12 (1H, t, $J=9$ Hz, H_{2a}); IR ν_{max}^{KBr} cm^{-1} 3600 \sim 2400, 1639 and 1570. These data were in good agreement with those reported^{11,12} for cis-4-methyl-L-proline. This amino acid has been also isolated from an antibiotic I.C.I. 13,959¹³.

The structure of an unidentified amino acid (VIII) has been reported as (2*S*,4*S*,6*S*)-4-methyl-6-(2-oxobutyl)-2-piperidinecarboxylic acid (MOPA) in this journal¹⁴. That compound VIII could not be detected by the amino acid analyzer might be due to the low coloration efficiency of this amino acid in the ninhydrin reaction and to its partial degradation during the hydrolysis.



VII

After removal of the fatty acid moiety, the acid hydrolysate of I was subjected to measurement of FD mass spectra. Fig. 5 shows the FD mass spectrum (emitter current at 19 mA) of the hydrolysate. The mass spectrum showed protonated molecular ions at m/z 90 (Ala), 104 (Aib), 130 (MePro), 132 (Leu) and 148 (HyLeu). However, the protonated molecular ion peaks of MOPA and of the basic component could not be observed at any emitter current. The composition of these ions were confirmed by high resolution FD mass spectrometry (see Table 1). The isolation followed by structural studies of the constituent amino acids of leucinostatin B (II) gave the conclusion that it was comprised of identical amino acids. Amino acid analyses of I and II substantiated the above conclusion. Table 2 shows quantitation of the constituent amino acids of I and II. An exact molar ratio of the amino acids in each antibiotic could not be obtained because of the low coloration efficiency of Aib (*ca.* 8% of alanine) and the failure to observe the peak of MOPA.

Fig. 5. FD mass spectrum of the total water soluble hydrolysate of leucinostatin A.

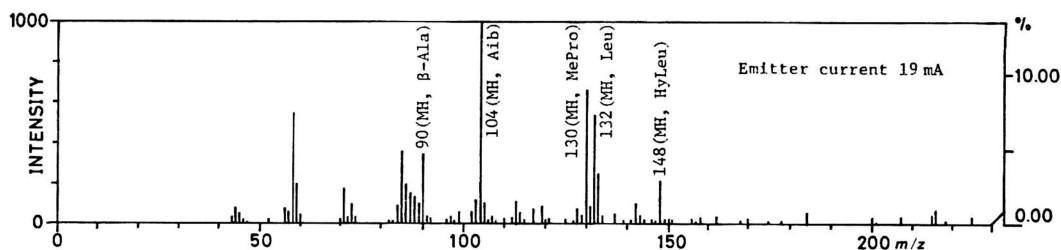


Table 1. High resolution FD-MS data of the aqueous constituents obtained from acidic hydrolysis of leucinostatin A.

Measured mass	Δ , mmu	Composition	Assignment
90.0564	0.9	C ₃ H ₈ NO ₂	β -Ala+H
104.0727	1.7	C ₄ H ₁₀ NO ₂	Aib+H
130.0879	1.2	C ₆ H ₁₂ NO ₂	MePro+H
132.1035	1.1	C ₆ H ₁₄ NO ₂	Leu+H
148.0969	-0.4	C ₆ H ₁₄ NO ₃	HyLeu+H

Table 2. Amino acid analyses of leucinostatin A and B.

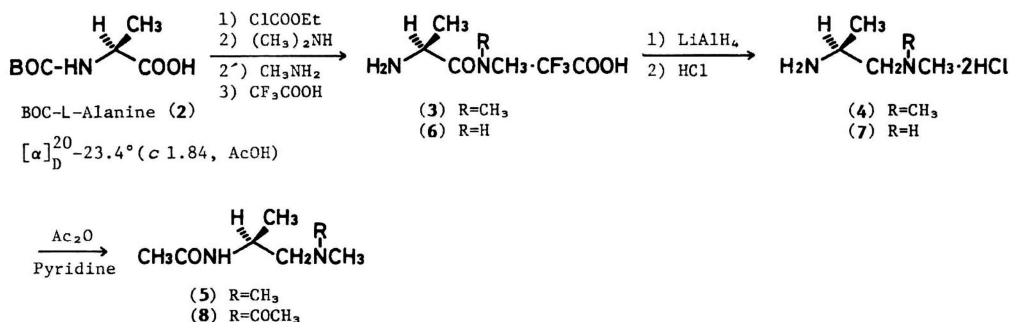
Amino acid	Leucinostatin A		Leucinostatin B	
	nmol	Molar ratio	nmol	Molar ratio
HyLeu	18.65	1	28.15	1
Aib	28.19	1~2	34.75	1~2
Leu	54.19	2~3	83.40	2~3
β -Ala	17.45	1	22.00	1
MePro	31.60	1~2	42.93	1~2

The Basic Component Moiety

Leucinostatin A: The basic component (**IX**) of **I** was located at the lowest R_f value of 0.26 on TLC (Fig. 4); it showed a yellowish brown color on the reaction with ninhydrin reagent. It was isolated by preparative cellulose TLC developed with a solvent system of 1-BuOH - AcOH - H₂O (4:1:2) and further purified on a Sephadex LH-20 column followed by vacuum distillation. Finally, **IX** was crystallized as dihydrochloride: mp 174~176°C; $[\alpha]_D^{20}$ -2.76° (*c* 1.085, MeOH); CI-MS (*i*-C₄H₁₀) *m/z* 103 (MH⁺), 205 (2M+H)⁺; ¹H NMR $\delta_{ppm}^{CDCl_3}$ 1.50 (3H, d, *J*=6 Hz, -CHCH₃), 3.00 (6H, s, -N(CH₃)₂), 3.40 (2H, m, NCH₂) and 3.95 (1H, m, NCH-). Component **IX** was characterized as a basic compound containing a tertiary amino group because of a positive DRAGENDORFF reaction. The structural study of **IX** was accomplished through the spectral data of its acetyl derivative and of **IX** itself. Acetylation of **IX** with acetic anhydride - methanol or acetic anhydride - pyridine gave the corresponding *N*-monoacetylated compound (**X**), bp 107°C (oven temperature) (5 mmHg); $[\alpha]_D^{20}$ -3.38° (*c* 0.609, MeOH); CI-MS (*i*-C₄H₁₀ or NH₃) *m/z* 145 (MH⁺); EI-MS *m/z* 58 (CH=N(CH₃)₂) base peak; IR $\nu_{max}^{CHCl_3}$ cm⁻¹ 3430, 1660 and 1510; ¹H NMR $\delta_{ppm}^{CDCl_3}$ 1.19 (3H, d, *J*=8 Hz, -CHCH₃), 1.98 (3H, s, COCH₃), 2.30 (1H, dd, *J*=5, 12 Hz, -CH₂N), 2.34 (6H, s, N(CH₃)₂), 2.64 (1H, dd, *J*=11, 12 Hz, -CH₂N), 4.08 (1H, m, NCH-(CH₃)₂) and 6.38 (1H, br, NH). On irradiating the proton signal at 4.08 ppm, a doublet at 1.19 ppm collapsed to a singlet, and the double doublet signals at 2.30 and 2.64 ppm were transformed into one doublet, respectively. From these data, the primary structures of **IX** and **X** were deduced to be *N*¹,*N*¹-dimethylpropane-1,2-diamine and *N*²-acetyl-*N*¹,*N*¹-dimethylpropane-1,2-diamine, respectively.

Next a synthetic study (Fig. 6) was undertaken to determine the configuration of C-2 of **IX** and to confirm the structure presented here. BOC-*L*-alanine (**2**) was converted to alanine dimethylamide trifluoroacetic acid salt (**3**) *via* the subsequent steps: 1) synthesis of the mixed anhydride by the reaction

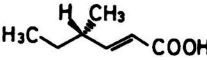
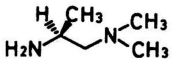
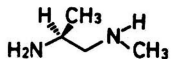
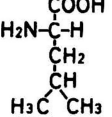
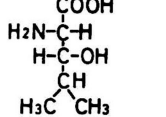
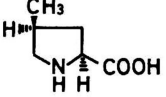
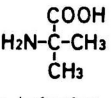

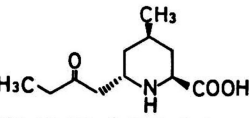
Fig. 6. Syntheses of the basic components of leucinostatins A and B.



with $\text{ClCOOC}_2\text{H}_5$, 2) reaction of the mixed anhydride with $(\text{CH}_3)_2\text{NH}$, 3) removal of BOC group with CF_3COOH . (2*S*)-*N*¹,*N*¹-Dimethylpropane-1,2-diamine·2HCl (4) was obtained by reduction of 3 with LiAlH_4 , followed by the addition of methanol containing hydrogen chloride. The amine (4) was acetylated with acetic anhydride - pyridine to give (2*S*)-*N*²-acetyl-*N*¹,*N*¹-dimethylpropane-1,2-diamine (5), $[\alpha]_D^{20} -3.4^\circ$ (*c* 0.56, MeOH). All data, including IR, NMR, MS, R_f value on TLC, and specific rotation were completely identical for the synthetic compound (5) and the compound X. The evidence shows that C-2 of the component IX has *S*-configuration. Consequently, the basic component IX of I is (2*S*)-*N*¹,*N*¹-dimethylpropane-1,2-diamine (DPD) (4).

Leucinostatin B: Isolation and structural study of the basic component of II were carried as in the case of I. Through purification by preparative cellulose TLC, followed by Sephadex LH-20 column chromatography and vacuum distillation, the basic component was obtained as dihydrochloride (XI), a pale yellow oil: $[\alpha]_D^{15} -0.74^\circ$ (*c* 0.8, MeOH); IR $\nu_{\text{max}}^{\text{film}}$ cm^{-1} 3600~2400, 1620 and 1470; CI-MS (*i*-C₄H₁₀) *m/z* 89 (MH⁺), 72 (MH⁺-NH₃); ¹H NMR $\delta_{\text{ppm}}^{\text{CDCl}_3, \text{OD}}$ 1.45 (3H, d, *J*=7 Hz, -CHCH₃), 2.77 (3H, s, NCH₃), 3.38 (2H, d, *J*=7 Hz, -CHCH₂N) and 3.70 (1H, m). From these data, the primary structure of XI was elucidated to be *N*¹-methylpropane-1,2-diamine. In order to confirm the structure for XI and to determine the configuration of C-2 of the molecule, the amine was acetylated to yield compound (XII), a colorless oil: bp 144°C (oven temperature) (4 mmHg); $[\alpha]_D^{20} -20.2^\circ$ (*c* 0.61, MeOH); CI-MS (*i*-C₄H₁₀) *m/z* 173 (MH⁺); IR $\nu_{\text{max}}^{\text{CHCl}_3}$ cm^{-1} 3400, 1660, 1625 and 1525; ¹H NMR $\delta_{\text{ppm}}^{\text{CDCl}_3}$ 1.14 (3H, d, *J*=7 Hz, -CHCH₃), 1.93 (3H, s, COCH₃), 2.12 (3H, s, COCH₃), 2.87 (1H, dd, *J*=13, 3 Hz), 3.08 (3H, s, NCH₃), 4.05 (1H, t, *J*=13 Hz), 4.20 (1H, m) and 6.48 (1H, br, CONH). From these data, the primary structure

Table 3. Constituents of leucinostatins A and B.

	Leucinostatin A	Leucinostatin B
Fatty acid	 (4 <i>S</i>)-(2 <i>E</i>)-4-Methylhex-2-enoic acid (IV)	
Basic components	 (2 <i>S</i>)- <i>N</i> ¹ , <i>N</i> ¹ -Dimethylpropane-1,2-diamine (DPD)	 (2 <i>S</i>)- <i>N</i> ¹ -Methylpropane-1,2-diamine (MPD)
Amino acids	 L-Leucine  L-threo-β-Hydroxy-leucine (VI)	 <i>cis</i> -4-Methyl-L-proline (VII)
	 α-Aminoisobutyric acid  β-Alanine	 (2 <i>S</i> ,4 <i>S</i> ,6 <i>S</i>)-4-Methyl-6-(2-oxobutyl)-2-piperidine-carboxylic acid (MOPA) (VIII)

of **XII** was established to be N^1, N^2 -diacetyl- N^1 -methylpropane-1,2-diamine. Subsequently, syntheses of **XI** and **XII** were accomplished by means of the procedure shown in Fig. 6. BOC-L-alanine (**2**) was transformed to alanine methylamide trifluoroacetic acid salt (**6**) in three steps, but methylamine was used instead of dimethyl amine in the second step. Reduction gave (2*S*)- N^1 -methylpropane-1,2-diamine·2HCl (**7**), and acetylation of **7** led to the expected (2*S*)- N^1, N^2 -diacetyl- N^1 -methylpropane-1,2-diamine (**8**), $[\alpha]_D^{20} -18.5^\circ$ (c 2.59, MeOH). The fact that all data including IR, NMR, MS and specific rotation of the synthetic product (**8**) and the natural compound **XII** were completely identical with each other, demonstrated that C-2 of the compound **XI** had *S*-configuration. Consequently, the structure of the constituent basic component of **II** was concluded to be (2*S*)- N^1 -methylpropane-1,2-diamine (MPD, **7**). The structures of the constituents of leucinostatins A and B are summarized in Table 3.

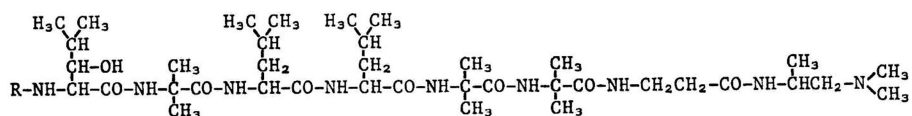
Structure of Leucinostatin A

The molecular formula of leucinostatin A (**I**) was established to be $C_{62}H_{111}N_{11}O_{13} \cdot HCl$ by FD and FAB mass spectrometric analyses and elemental analysis. The ^{13}C NMR spectrum of **I** exhibited nine carbon signals between 177.2 and 169.9 ppm assignable to amide. From the intensity of the signal at 174.1 ppm it was estimated that the antibiotic contains ten amide carbonyls. The following evidence suggested that **I** had to be a straight chain: **I** was negative for ninhydrin, neither *N*- and *C*-terminal residue was detected by the methods used with 2,4-dinitrofluorobenzene, dansyl chloride and diazomethane, and as its constituents **I** had the fatty acid **IV** and the amine (DPD) (**IX**).

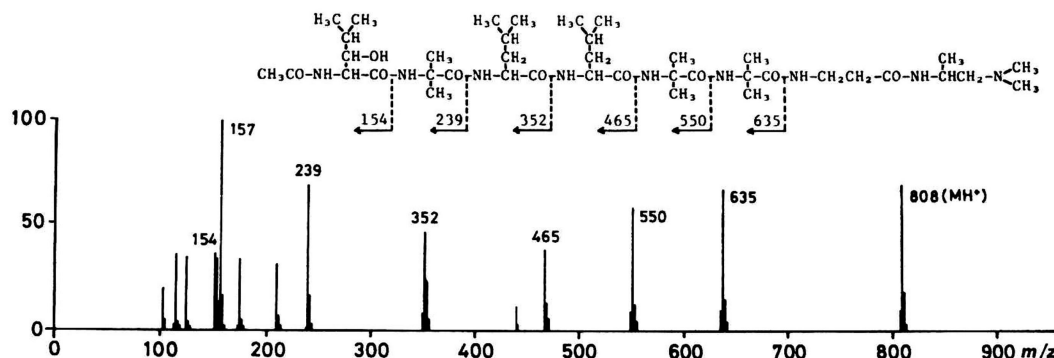
To clarify the amino acid sequence, **I** was partially hydrolyzed with 6*N* HCl, at room temperature for 40 hours, yielding mainly two fragments, **XIII** and **XIV**. Each fragment was isolated and purified by Sephadex LH-20 column chromatography. Structures of the fragments were elucidated by a combination method of chemical degradation, dansyl-EDMAN degradation, and mass spectrometric analysis.

Fragment **XIII**

Acid hydrolysis of **XIII** gave HyLeu, Aib, Leu, β -Ala and DPD, whose presence was suggested from the signal at δ 3.02 ppm (s, $N(CH_3)_2$) observed in the 1H NMR spectrum. In the carbonyl region of the ^{13}C NMR spectrum of **XIII**, seven carbon signals appeared between 175.3 and 177.9 ppm, which account for this fragment to be a heptapeptide. *N*-Terminal residue was determined to be HyLeu by dansylation. Dansyl-EDMAN degradation of **XIII** clarified the partial sequence to be HyLeu-Aib-Leu-Leu, but it did not offer further corroborating evidence for the amino acid sequence. In order to analyze the sequence by mass spectrometry, **XIII** was derivatized acetyl derivative (**XV**). Fig. 7 shows the CI ($i-C_4H_{10}$) mass spectrum defining the amino acids sequence of the acetate. The spectrum gave the protonated molecular ion at m/z 808 accompanied by the fragment ion peaks at m/z 635, 550, 465, 352, 239 and 154 derived from the cleavage at the peptide bond. The first six residues (acetyl-HyLeu-Aib-Leu-Leu-Aib-Aib) were defined by the fragment ions described above. Furthermore, the peak at m/z 635 was considered to be the ion arisen by elimination of β -Ala-DPD moiety ($C_8H_{15}N_3O$, 172) from the MH^+ , m/z



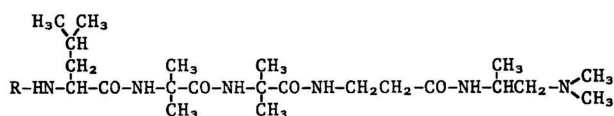
9 R=COCH₃ 10 R=H

Fig. 7. The CI($i\text{-C}_4\text{H}_{10}$) mass spectrum of the acetyl derivative (XV) of fragment XIII.

808, on the basis of the fact that β -Ala and DPD were the constituents of fragment XIII. From the above consideration, the entire sequence of the acetate XV and subsequently the structure of fragment XIII were established to be **9** and **10**, respectively. The result means that DPD was the C-terminal residue of this antibiotic.

Fragment XIV

Acid hydrolysis of XIV gave Aib, β -Ala, DPD and Leu, which was detected as *N*-terminal residue by dansylation. By dansyl-EDMAN degradation, the partial sequence being Leu-Aib-Aib was clarified, but it did not allow further step. Then, after acetylation of XIV, the obtained acetate (XVI) was subjected to CI mass spectrometric analysis. The mass spectrum gave the intense protonated molecular ion peak at m/z 499 and the sequence-determining fragment ions. The sequence of Ac-Leu-Aib-Aib was indicated by a series of major peak at m/z 156, 241 and 326. By taking into account the result of the hydrolytic experiment mentioned above, the peaks at m/z 326, 259 and 344 were assigned to the following ions, $\text{MH}^+ - (\beta\text{-Ala-DPD})$, $(\text{Aib-}\beta\text{-Ala-DPD})^+$ and $(\text{Aib-Aib-}\beta\text{-Ala-DPD})^+$, respectively. These ions indicate that the sequence of β -Ala-DPD must be situated in a position adjacent to Aib. Thus, the structures of the acetate XVI and the original fragment XIV were established to **11** and **12**, respectively.



11 R=COCH₃ **12** R=H

Since no informations corresponding to *N*-terminal side of the antibiotic were obtained from this experiment, another partial hydrolysis was tried. The hydrolysis was carried out with 2 N HCl at 140°C for 2 hours, and the hydrolysate was separated into an ethyl acetate soluble fraction and an unextracted aqueous fraction.

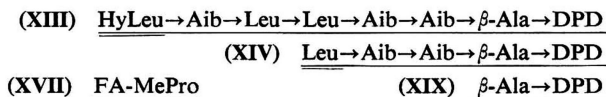
The organic solvent fraction, fragment XVII, was esterified with HCl - MeOH to give an oily compound XVIII having the following properties: negative for ninhydrin reaction; UV $\lambda_{\text{max}}^{\text{EtOH}}$ nm 215 ($\log \epsilon$ 4.0); IR $\nu_{\text{max}}^{\text{CHCl}_3}$ cm⁻¹ 1740 (ester), 1655, 1600, 1455 and 1430; CI-MS ($i\text{-C}_4\text{H}_{10}$) 507 ($2\text{M}+\text{H}$)⁺, 254 (MH^+), 222, 194, 111 and 84. In the ¹H NMR spectrum ($\beta_{\text{ppm}}^{\text{CDCl}_3}$), the signals suggesting the presence of

the fatty acid IV: 6.84 (1H, dd, $J=8, 15$ Hz), 6.02 (1H, d, $J=15$ Hz), 0.88 (3H, t, $J=7$ Hz), 1.02 (3H, t, $J=7$ Hz) and 2.04 (1H, m), and MePro moiety: 1.10 (1H, d, $J=7$ Hz), 4.42 (1H, m), 3.81 (1H, m), 3.23 (1H, t, $J=10$ Hz) and 2.30 (2H, m), were observed. This consideration was confirmed by hydrolyzing XVII to yield IV and MePro. From the data mentioned above, the structures of the compound XVIII and the original fragment XVII were deduced to be 13 and 14, respectively. They mean that *N*-terminal residue of this antibiotic is constituted with the fatty acid IV. On the other hand, the fragment XIX whose structure was determined to be β -Ala-DPD (15) by spectral and acid degradation studies, was isolated from the aqueous fraction.



Fig. 8. Sequence of the fragments (XIII), (XIV), (XVII) and (XIX) obtained by partial hydrolyses of I.

The methods of determination are indicated as follows; singly underlined: mass spectrometry, doubly underlined: dansylation, arrow: dansyl-EDMAN analysis. FA = (4*S*)-(2*E*)-4-methylhex-2-enoic acid. DPD = (2*S*)-*N*¹,*N*¹-dimethylpropane-1,2-diamine.



The sequences of the four fragments obtained by partial hydrolyses of leucinostatin A are summarized in Fig. 8.

As the *N*- and *C*-terminal of leucinostatin A were blocked with the fatty acid (FA) IV and DPD, respectively, the amino acid MOPA should be placed between the fragments XVII and XIII. The above-mentioned components constitute a peptide, C₆₂H₁₀₉N₁₁O₁₂, which corresponds to the dehydration product of leucinostatin A.

Alumina treatment of diacetyl compound obtained by acetylation of I gave the *O*-monoacetyl derivative (XX); FD-MS m/z 1,264 ($M \cdot Na^+$) and 1,242 (MH^+); IR $\nu_{\text{max}}^{\text{CHCl}_3}$ cm⁻¹ 1745 (ester), 1680 and 1660 (conjugated carbonyl). In the 400 MHz ¹H NMR spectrum (Fig. 9), the signals at δ (ppm) 2.06 (3H, s, COCH₃), 5.10 (1H, t, $J=6$ Hz, -CHOCOCH₃), 6.15 (1H, d, $J=17.4$ Hz) and 6.78 (1H, m), which had not been observed in I, were observed. They suggested that acetylation occurred at the position of the hydroxyl group of HyLeu and α,β -unsaturated ketone system was newly formed. The chemical shifts at 131.6 (d), 144.2 (d) and 200.8 (s) ppm observed in the ¹³C NMR spectrum can be ascribed to newly formed α,β -unsaturated ketone system. These data suggested that the amino acid MOPA is present in I as 2-amino-6-hydroxy-4-methyl-8-oxodecanoic acid (AHMOD, 16), which, upon hydrolysis, is converted into an α,β -conjugated ketone by elimination of water and then cyclized to MOPA by MICHAEL addition¹⁵⁾. On the basis of these results, the structure of XX could be presented as 17. Consequently, the structure of

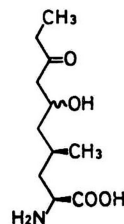
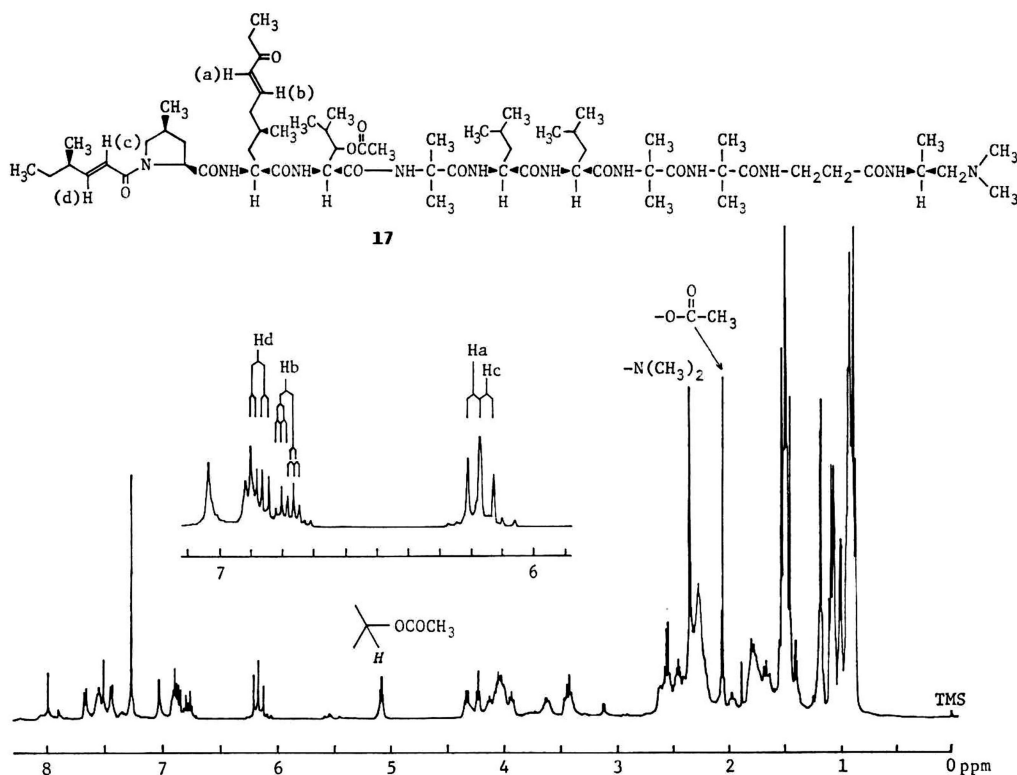


Fig. 9. 400 MHz ^1H NMR spectrum of the *O*-monoacetyl derivative (XX) of I.

leucinostatin A was determined to be I.

Recently, the direct comparisons between leucinostatin A and antibiotic P168 (a gift of Prof. A. SUZUKI) have been carried out and it was concluded that the two antibiotics were identical.

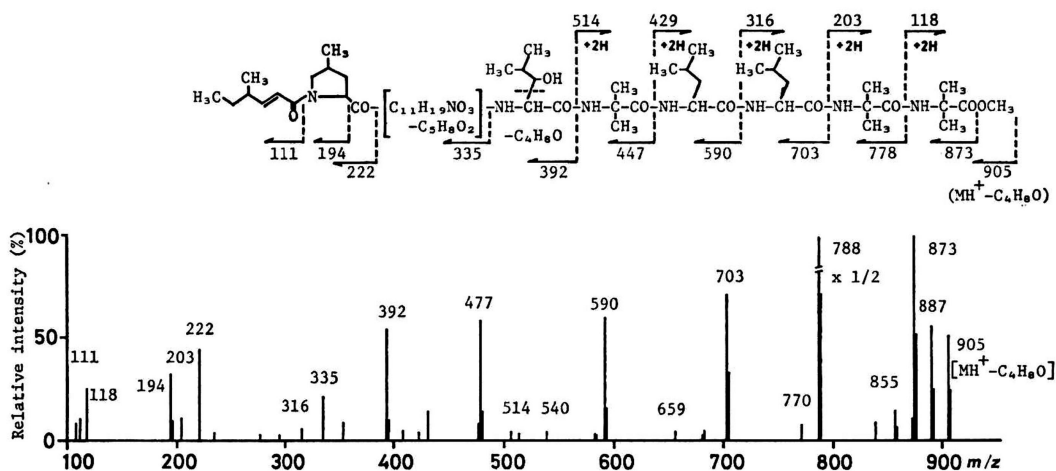
Structure of Leucinostatin B

The composition of $\text{C}_{61}\text{H}_{109}\text{N}_{11}\text{O}_{13} \cdot \text{HCl}$ for leucinostatin B (II) was established by elemental analysis, FAB and FD mass spectrometric analyses, m/z 1,204 (MH^+). The ^1H NMR and ^{13}C NMR spectra of II were closely related to those of I. Signals suggesting the presence of an *N*-methyl group (δ_{H} 2.46, s), a γ -monosubstituted α,β -unsaturated amide (δ_{H} 6.18, d, $J=16$ Hz, and 6.86, dd, $J=16, 8$ Hz; δ_{C} 120.1, d, 151.9, d, and 166.9, s) were found in the spectra. Furthermore, the ^{13}C NMR spectrum exhibited signals assignable to a ketone (δ_{C} 211.7) as well as eight amides between δ_{C} 175.2 and 173.9 ppm. Then, it was estimated that ten amide carbonyls were contained in II as well as I. To elucidate the sequence of amino acids, II was partially hydrolyzed with a mixture of HCl and HCOOH (1:1), at 37°C for 30 hours, giving mainly two fragments, XXI and XXII from the ethyl acetate-soluble fraction and three fragments, XXIII, XXIV and XXV from the water-soluble fraction.

By direct comparisons between the methyl esters of XXI and XVIII (13), it was found that the fragments XXI obtained here and XVII (14) obtained from I were identical to each other. As can be seen in I, this fragment is anticipated to be the *N*-terminal moiety of II.

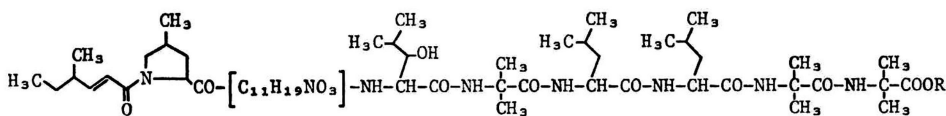
Fragment XXII

On hydrolysis of XXII, HyLeu, Aib, Leu, MePro and the fatty acid IV were identified in the hydroly-

Fig. 10. The CI(*i*-C₄H₁₀) mass spectrum of the methyl ester (XXVI) of fragment XXII.

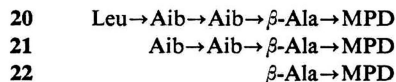
sate. The ¹³C NMR spectrum showed the presence of ten amides and an α,β-unsaturated carbonyl system in the molecule. Since the FD mass spectrum gave the cluster ion at *m/z* 999 ($M \cdot Na^+$), the molecular weight of XXII was considered to be 976.

Application of dansyl-EDMAN degradation method to this fragment has failed. Then, structural elucidation of XXII was carried out for its methyl ester (XXVI) by CI mass spectrometry (Fig. 10). There are no peaks in the molecular ion region. However, a series of fragment ions containing *N*- and *C*-termini were found and they enabled us to establish the amino acid sequence of XXVI as **18** except for that of [AHMOD+HyLeu] part. This result led to the structure of XXII to **19** which showed that the release of C₃H₅O₂ moiety from AHMOD had occurred already at the stage of partial hydrolysis. The peak at *m/z* 905 in the spectrum are presumed to be arisen by elimination of C₄H₅O[(CH₃)₂CHCHO] from the side chain of HyLeu.



18 R=CH₃ **19** R=H

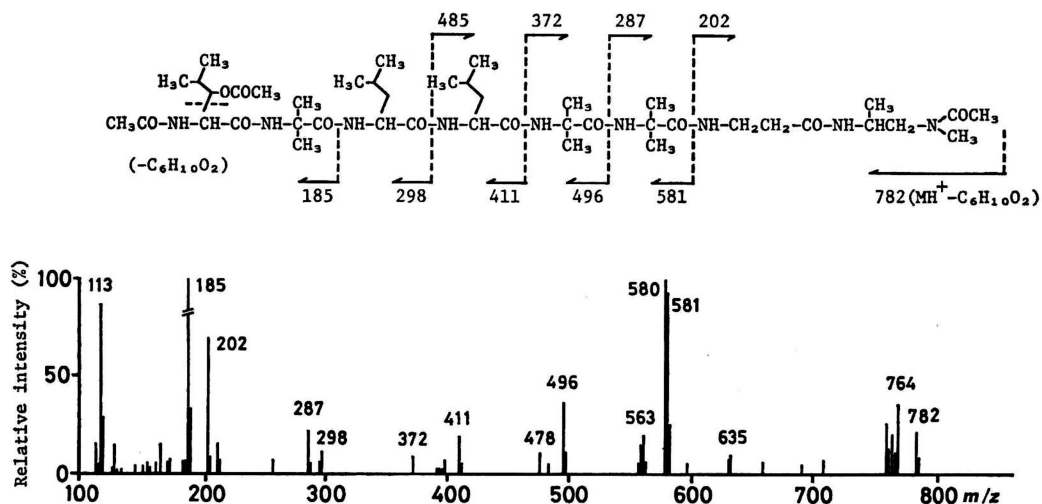
The structures of the fragments, XXIII, XXIV and XXV were deduced to be **20**, **21** and **22** respectively from acidic hydrolysis and dansyl-EDMAN degradation method.



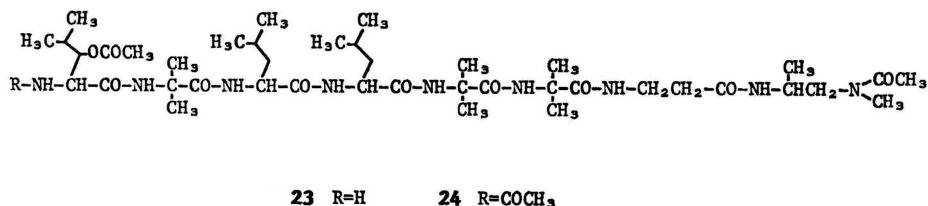
Partial hydrolysis (6 N HCl, 25°C, 20 hours) of an acetylated compound of **II** gave two fragments, XXVII and XXVIII.

Fragment XXVII

FD-MS, *m/z* 812 (MH⁺). The ¹³C NMR spectrum showed that eight amide carbonyls were contained in the molecule. By acid hydrolysis, it was found that the fragment was composed of HyLeu,

Fig. 11. The CI(*i*-C₄H₁₀) mass spectrum of the acetate (XXIX) of fragment XXVII.

Leu, Aib, β -Ala and MPD. Dansyl-EDMAN degradation method clarified the partial sequence to be HyLeu-Aib-Leu, but it did not proceed further. Further structural study of XXVII was made for its acetyl derivative (XXIX) by mass spectrometry. The FD spectrum of XXIX gave an intense peak at m/z 896 (MH⁺). On the other hand, the CI spectrum did not give the peak of MH⁺, but it gave a series of the sequence-determining fragment ions as illustrated in Fig. 11. Consequently, the structures of XXVII and its acetate XXIX were established to be **23** and **24**, respectively.

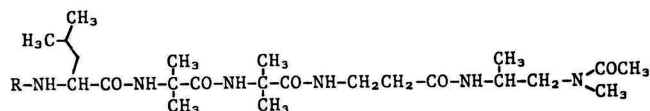
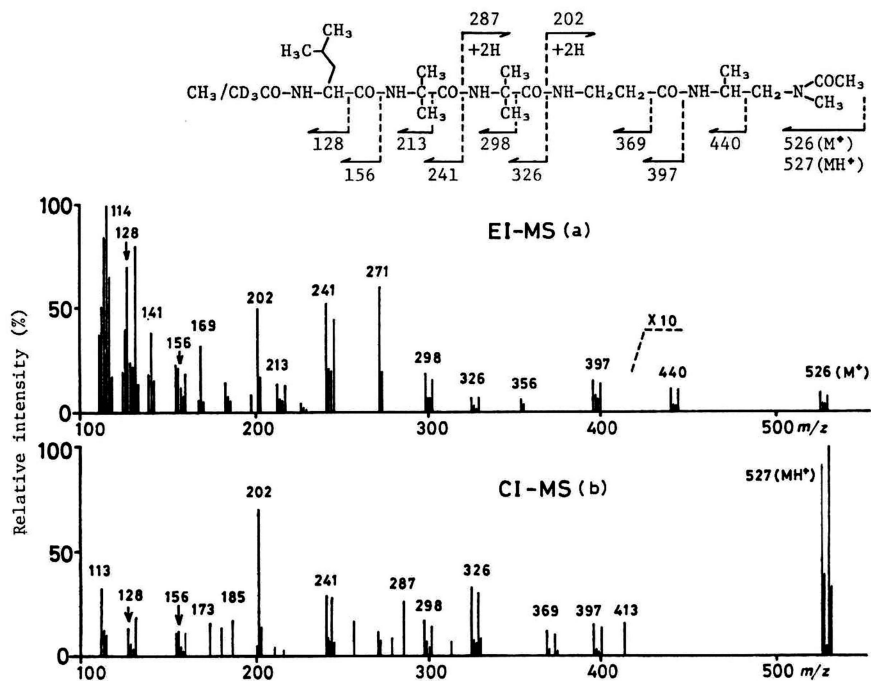


The peak at m/z 782 in the CI spectrum is considered to be formed by cleavage of C₆H₁₀O₂ moiety from HyLeu.

Fragment XXVIII

FD-MS, m/z 485 (MH⁺). The constituents of XXVIII were clarified to be Leu, Aib, β -Ala and MPD by acid hydrolysis. Dansyl-EDMAN degradation method gave the partial sequence, Leu-Aib-Aib, but it did not proceed furthermore.

To determine the total structure of this fragment, we have tried the mixed acylation method which have been used in mass spectrometry for peptides^{16,17}. The fragment XXVIII was acetylated with an equimolar mixture of acetic anhydride and perdeuterioacetic anhydride to give the CH₃CO/CD₃CO-mixed acetylated compound (XXX), which was subjected to EI and CI mass spectrometric analyses. Fig. 12 shows the EI (a) and CI (b) spectra for XXX; doublets separated by 3 amu indicate fragments containing the *N*-terminal acetyl group with its isotopic marker. The total sequence of XXX was defined to be **25** by a series of the principal CH₃/CD₃ doublets arisen from the cleavage on either side of

Fig. 12. The EI and CI(*i*-C₄H₁₀) mass spectra of the CH₃CO/CD₃CO-mixed acetylated compound (XXX).

25 R=CH₃CO/CD₃CO 26 R=H

Fig. 13. Sequences of the fragments (XXI)~(XXV), (XXVII) and (XXVIII) obtained by partial hydrolyses of II and the acetyl-II.

Singly underlined: mass spectrometric analysis.

Doubly underlined: dansylation. Arrow: dansyl-EDMAN analysis.

* Detected after hydrolysis.

FA=(4S)-(2E)-4-methylhex-2-enoic acid.

MPD=(2S)-N¹-methylpropane-1,2-diamine.

(XXI) FA-MePro

(XXII) FA-MePro—[AHMOD + HyLeu]—Aib—Leu—Leu—Aib—Aib

(XXIII) Leu—Aib—Aib—[β-Ala—MPD]*

(XXIV) Aib—Aib—[β-Ala—MPD]*

(XXV) β-Ala—MPD—Ac

(XXVII) HyLeu—Aib—Leu—Leu—Aib—Aib—β-Ala—MPD—Ac

(XXVIII) Leu—Aib—Aib—β-Ala—MPD—Ac

(27) FA-MePro — [Y] — HyLeu — Aib — Leu — Leu — Aib — Aib — β-Ala — MPD

carbonyl groups. The result led the structure of XXVIII to 26.

The sequences of the five fragments obtained by the partial hydrolyses of II and acetyl-II are summarized in Fig. 13.

Placement of the amino acid VIII instead of [Y] in the sequence (27) constitutes a peptide, $C_{61}H_{107}N_{11}O_{12}$, which corresponds to the dehydration product of II and suggests that VIII must be present as the amino acid, AHMOD, as in the case of I. However, the C-terminal amine XI has the possibility of being linked to β -Ala in two ways. In order to establish the structure unambiguously, II was selectively N-methylated.

Treatment of II with methyl iodide in ethanol gave the trimethyl ammonium compound: $[\alpha]_D^{20} -20.8^\circ$ (*c* 1.0, MeOH); IR ν^{CHCl_3} cm^{-1} 3275, 1650 and 1540; 1H NMR δ^{CDCl_3} 3.27 (9H, s, $N(CH_3)_3$), 6.10 (1H, d, $J=15$ Hz, $-CH=CHCO$), 6.70 (1H, dd, $J=15, 8$ Hz, $-CHCH=CHCO$); ^{13}C NMR δ^{CDCl_3} 53.8 ($N(CH_3)_3$). From the above spectral data, the structure of the compound was established to be leucinostatin A-M (III), being the methyl iodide adduct of leucinostatin A. The secondary ion mass spectrum (SIMS) of III showed the ion peak at m/z 1,232, corresponding to the mass of the cationic part of III.

The ammonium compound III was identified as the N-methylated compound of I, obtained by treatment with methyl iodide in ethanol, by the spectral (IR, 1H NMR, ^{13}C NMR and SIMS) comparisons including the specific rotation. These results revealed the structure of leucinostatin B as II²¹.

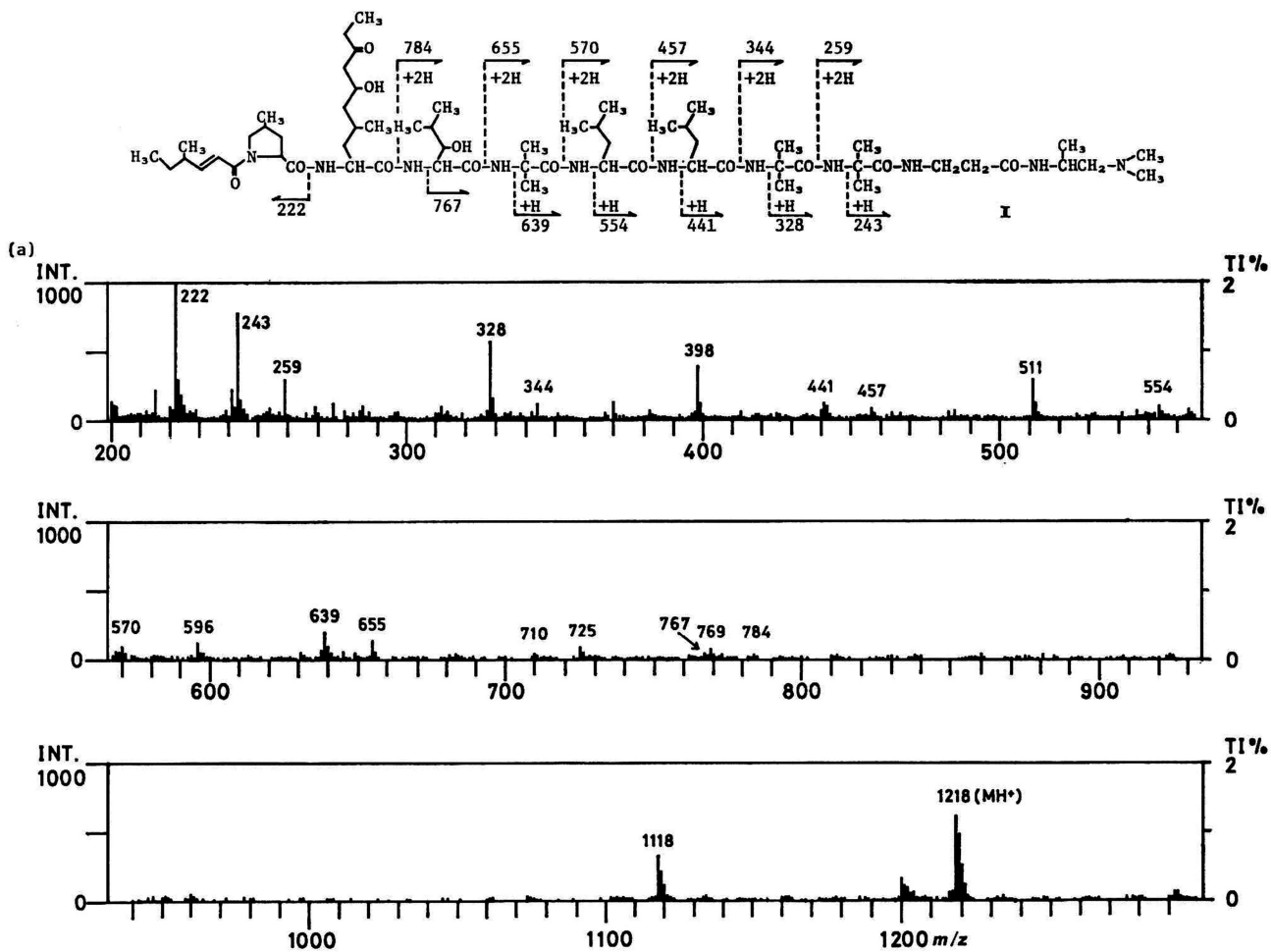
The structures of leucinostatins A and B, in which stereochemistry for all chiral constituents has been defined, were thus established. The essential difference between leucinostatins A and B was concluded to be the replacement of the basic component DPD in leucinostatin A by MPD in leucinostatin B.

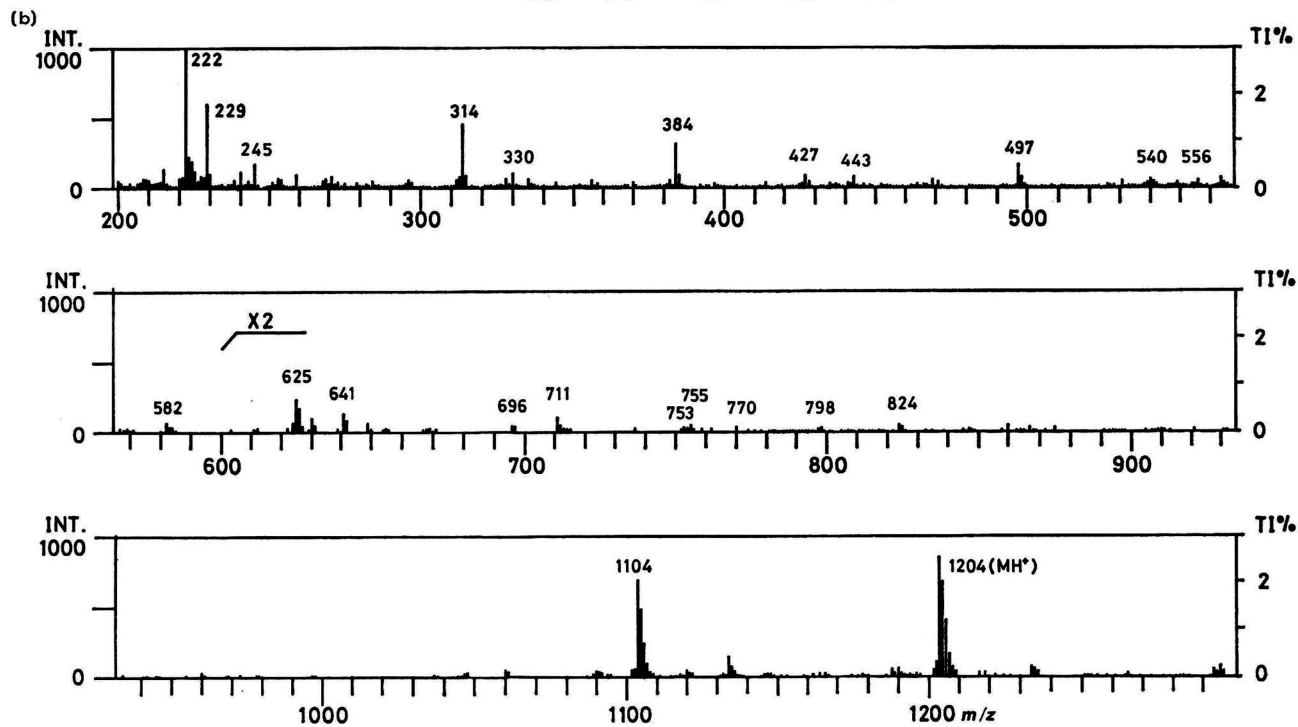
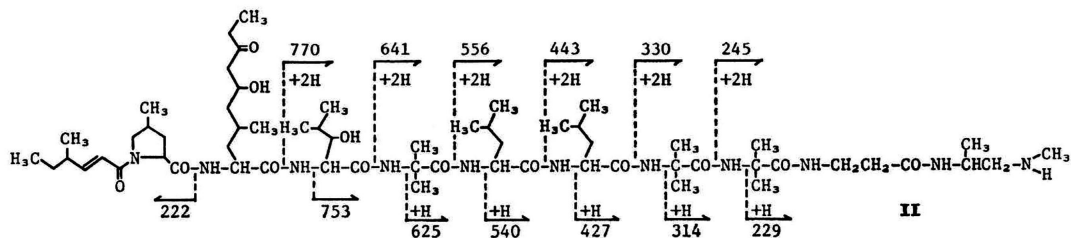
We also employed FAB mass spectrometry¹⁸⁻²⁰⁾ in the present study of leucinostatins. The positive ion FAB mass spectra of leucinostatins A and B are shown in Fig. 14. The spectra are simple to interpret, defining the entire sequence of amino acids, except for the moiety of AHMOD. The intense protonated molecular ion peaks at m/z 1,218 for I and 1,204 for II were observed in each spectrum. Fragmentations predominantly occur at the positions between α -carbon and NH in amino acids rather than peptide bonds. When the fragment ions in both spectra are compared, most of the fragment ion peaks observed in the spectrum of leucinostatin B, except for the ion at m/z 222, shift to positions 14 mass lower. The peak at m/z 222 commonly encountered in the both spectra comes from N-terminal side (FA-MePro) of each individual antibiotic. These data also support that structures of the antibiotics differ in only the constituent amine as concluded above. A series of intense fragment ions at m/z 398, 511, 596 and 725 in Fig. 14 (a) are estimated to be formed by elimination of isopropyl group from the ions at m/z 441, 554, 639 and 769, respectively. The same considerations can be made to the ions at m/z 384, 497, 582 and 711 in Fig. 14 (b). The intense peaks at m/z 1,118 and 1,104, observed in each spectrum are considered to be arisen by elimination of $C_6H_8O_2$ moiety from AHMOD residue of the antibiotics. The great potential of FAB-MS for sequence analysis of peptidal compounds is amply demonstrated by these experiments.

Acknowledgment

We are indebted to Prof. A. SUZUKI, Tokyo University, for the generous gift of antibiotic P168.

Fig. 14. The FAB mass spectra of leucinostatins A (a) and B (b).





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