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Structures and Syntheses of Leupeptins Pr-LL and Ac-LL

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The structures of two major components, leupeptins Pr-LL and Ac-LL, isolated from cultures of various species of *Actinomycetes*, have been investigated. Mild acid hydrolysis of leupeptins Pr-LL and Ac-LL gave propionyl- and acetyl-L-leucyl-L-leucine, whose structures were established by syntheses. Complete acid hydrolysis of leupeptin acids derived by oxidation of leupeptins gave L-leucine and DL-arginine. The structures of leupeptins Pr-LL and Ac-LL were determined to be propionyl- and acetyl-L-leucyl-L-leucyl-DL-argininal, respectively, and confirmed by the syntheses. Propionyl- and acetyl-L-leucyl-L-leucyl-L-arginine methyl esters were reduced to their alcohols with lithium borohydride and then oxidized to respective leupeptins Pr-LL and Ac-LL by sulfoxide–carbodiimide reaction.

As reported in the previous paper,[2] two major components, leupeptins Pr-LL and Ac-LL having physiological activities[3] were isolated from cultures of various species of *Streptomyces*.

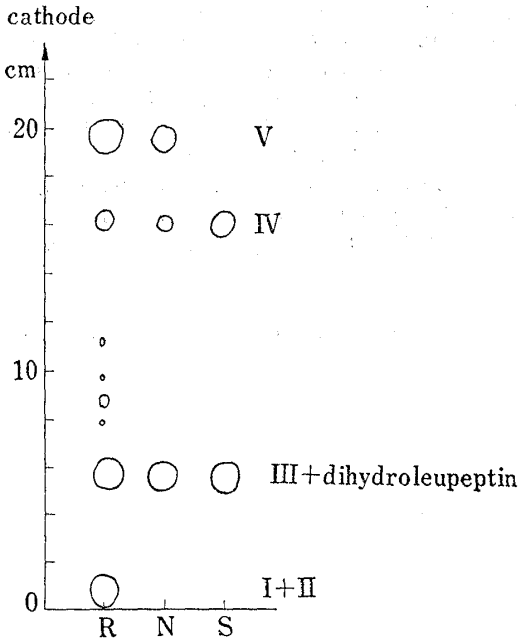


Fig. 1. High-voltage Paper Electrophoresis of Hydrolysate of Dihydroleupeptin Ac-LL

HCOOH–CH_3COOH–H_2O (25:75:900), 3500 V, 15 min
R: Rydon–Smith, N: ninhydrin, S: Sakaguchi

Their di-*n*-butyl acetals were prepared by refluxing in butanol, dihydroleupeptins by reduction with sodium borohydride, and leupeptin acids by oxidation with potassium permanganate.

Dihydroleupeptins Pr-LL and Ac-LL were oxidized to leupeptins Pr-LL and Ac-LL, respectively by sulfoxide–carbodiimide reaction reported by Pfitzner and Moffatt.[4]

Mild acid hydrolysis of leupeptin Pr-LL or its di-*n*-butyl acetal by refluxing for 30 min in 1N hydrochloric acid gave a white crystalline compound, mp 196—198°, $[\alpha]_D^{25}$ −56° (c=2, methanol), p$K_{a'}$ 5.9 in 67% dimethylformamide. The empirical formula $C_{15}H_{28}O_4N_2$ was shown by the elemental analysis and the titration. By NMR spectrum (100 MHz) in CD_3OD, the compound was suggested to be a propionylleucylleucine. Propionyl-L-leucyl-L-leucine (mp 197—199°, $[\alpha]_D^{21}$ −60°) was synthesized and the identity was determined.

Mild acid hydrolysis of leupeptin Ac-LL or its di-*n*-butyl acetal by the same condition

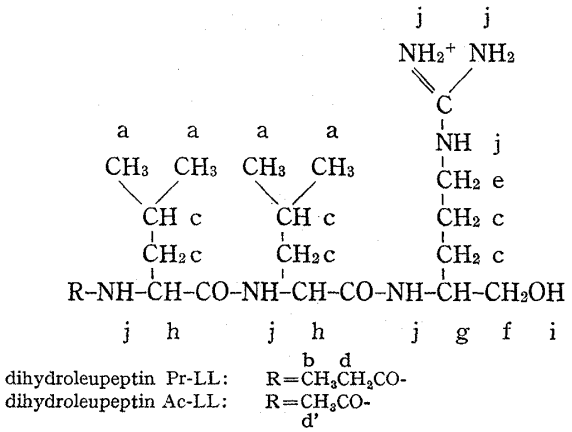
1) Location: *14-23, Kamiosaki 3-chome, Shinagawa-ku, Tokyo.*
2) S. Kondo, K. Kawamura, J. Iwanaga, M. Hamada, T. Aoyagi, K. Maeda, T. Takeuchi and H. Umezawa, *Chem. Pharm. Bull.* (Tokyo), **17**, 1896 (1969).
3) T. Aoyagi, T. Takeuchi, A. Matsuzaki, K. Kawamura, S. Kondo, M. Hamada, K. Maeda and H. Umezawa, *J. Antibiotics* (Tokyo), **22**, 283 (1969).
4) K.E. Pfitzner and J.G. Moffatt, *J. Am. Chem. Soc.*, **87**, 5661 (1965).

described above gave acetyl-L-leucyl-L-leucine (mp 190—191°, $[\alpha]_D^{25}$ −52°, $pK_{a'}$ 5.9, $C_{14}H_{26}O_4N_2$) as white crystals. This compound was compared with a synthetic sample (mp 191—192°, $[\alpha]_D^{22}$ −54°) for physical and chemical properties including mixed melting point. In 1956, Hara and others[5] reported that acetyl-L-leucyl-L-leucine was isolated from an ethyl acetate extract of a culture of *Streptomyces erythrochromogenes* producing sarkomycin.

Alkaline hydrolysis of dihydroleupeptin Ac-LL by refluxing for 1 hr in barium hydroxide–saturated water followed by neutralization with sulfuric acid gave more than five products shown by high-voltage paper electrophoresis, as shown in Fig. 1. These five hydrolysis products were separated by silicic acid column chromatography of the hydrolysate, and identified as acetyl-L-leucyl-L-leucine, urea, acetylleucylleucylornithinol, DL-argininol and DL-ornithinol. The signals of the NMR spectra (100 MHz) of dihydroleupeptins Pr-LL and Ac-LL hydrochlorides in $(CD_3)_2SO$ were assigned as shown in Table I.

Table I. NMR of Dihydroleupeptins (hydrochloride)
(100 MHz, $(CD_3)_2SO$)

Dihydroleupeptin Pr-LL		Dihydroleupeptin Ac-LL	
δ 0.85 d.d.	12H a	δ 0.85 d.d.	12H a
0.98 t.	3H b		
1.5 broad	10H c	1.5 broad	10H c
2.14 q.	2H d	1.85 s.	3H d′
3.1 broad	2H e	3.1 broad	2H e
3.3 broad	2H f	3.3 broad	2H f
3.6 broad	1H g	3.6 broad	1H g
4.2 broad	2H h	4.2 broad	2H h
4.70 t.	1H i	4.70 t.	1H i
7.2—8.2 broad	8H j	7.2—8.2 broad	8H j

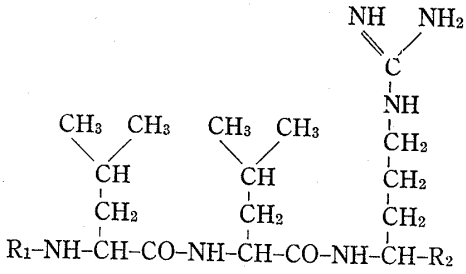


Complete acid hydrolysis of leupeptins Pr-LL and Ac-LL acids by refluxing with 6N hydrochloric acid for 10 hr yielded two main ninhydrin positive products which were separated by a column chromatography of Amberlite IRC 50 resin. One of which was obtained as crystals from the effluent of the column and identical with L-leucine. And the other was isolated from the eluate with 0.2N hydrochloric acid as a crystalline picrate, and identical with DL-arginine monopicrate.

From these results, the structure of leupeptin Pr-LL is determined to be propionyl-L-leucyl-L-leucyl-DL-argininal and leupeptin Ac-LL to be acetyl-L-leucyl-L-leucyl-DL-argininal. The structures of leupeptins Pr-LL and Ac-LL, and their derivatives are shown as in Fig. 2.

Leupeptins Pr-LL and Ac-LL were synthesized as shown in Chart 1. Crystalline propionyl- and acetyl-L-leucyl-L-leucyl-L-arginines (decomposed at 256—258° and 262—264°)

5) T. Hara, H. Yamada, K. Ida and Y. Yamada, *J. Antibiotics* (Tokyo), Ser. B, **9**, 184 (1956).



Leupeptins

Pr-LL: propionyl-L-leucyl-L-leucyl-DL-argininal

$R_1=CH_3CH_2CO-$ $R_2=-CHO$

Ac-LL: acetyl-L-leucyl-L-leucyl-DL-argininal

$R_1=CH_3CO-$ $R_2=-CHO$

Leupeptin acetals

Pr-LL: propionyl-L-leucyl-L-leucyl-DL-argininal di-*n*-butyl acetal

$R_1=CH_3CH_2CO-$ $R_2=-CH\langle^{OCH_2CH_2CH_2CH_3}_{OCH_2CH_2CH_2CH_3}$

Ac-LL: acetyl-L-leucyl-L-leucyl-DL-argininal di-*n*-butyl acetal

$R_1=CH_3CO-$ $R_2=-CH\langle^{OCH_2CH_2CH_2CH_3}_{OCH_2CH_2CH_2CH_3}$

Dihydroleupeptins

Pr-LL: propionyl-L-leucyl-L-leucyl-DL-argininol

$R_1=CH_3CH_2CO-$ $R_2=-CH_2OH$

Ac-LL: acetyl-L-leucyl-L-leucyl-DL-argininol

$R_1=CH_3CO-$ $R_2=-CH_2OH$

Leupeptin acids

Pr-LL: propionyl-L-leucyl-L-leucyl-DL-arginine

$R_1=CH_3CH_2CO-$ $R_2=-COOH$

Ac-LL: acetyl-L-leucyl-L-leucyl-DL-arginine

$R_1=CH_3CO-$ $R_2=-COOH$

Fig. 2. Structures of Leupeptins and Their Derivatives

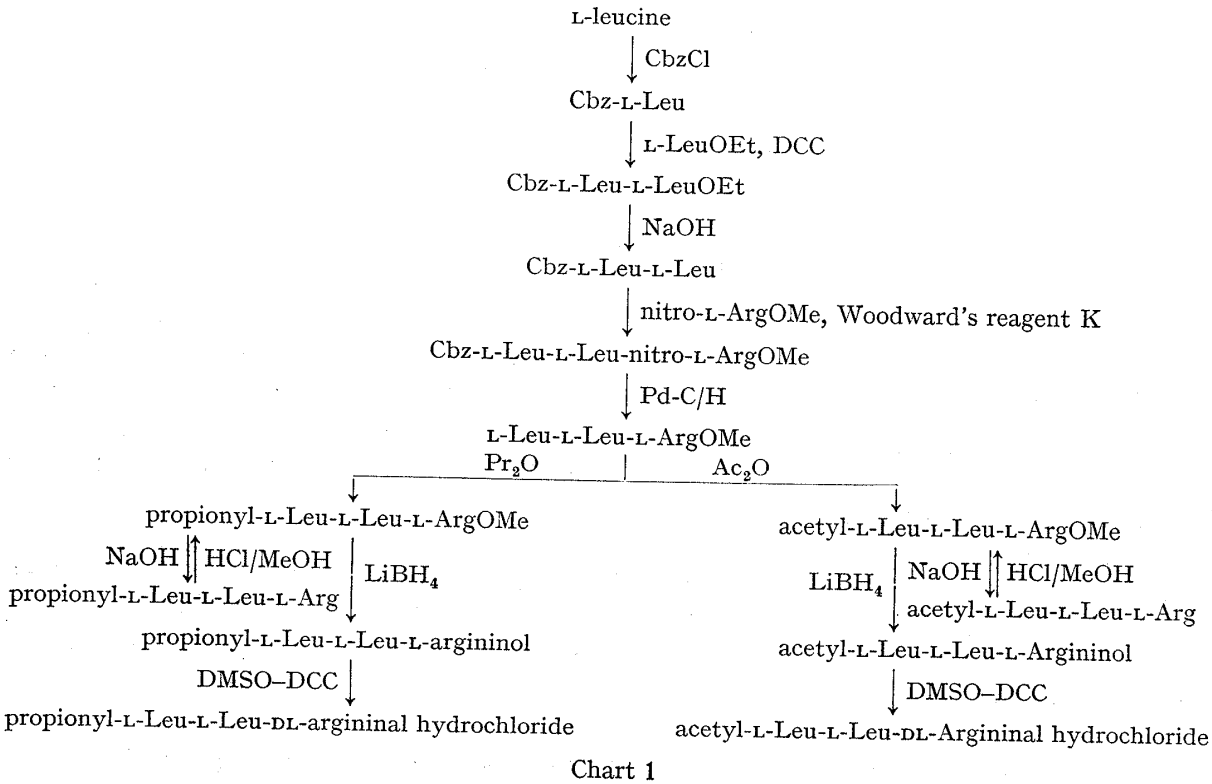


Chart 1

were converted to their methyl esters, reduced to their alcohols with lithium borohydride in dry tetrahydrofuran, and then oxidized to propionyl- and acetyl-L-leucyl-L-leucyl-DL-argininals by sulfoxide–carbodiimide reaction by the same procedure as described above for the partial syntheses of leupeptins from dihydroleupeptins. Synthetic propionyl- and acetyl-L-leucyl-L-leucyl-DL-argininals were identical with natural leupeptins Pr-LL and Ac-LL, respectively, in physical and chemical properties and also in the biological activities.

Leupeptins are derivatives of simple tripeptide, but the structures having an aldehyde group instead of a carboxyl group in the arginine moiety are unique and the first found in natural compounds.

On thin-layer chromatography of silicagel G using butanol–butyl acetate–acetic acid–water (4:2:1:1 in volume), synthetic or natural leupeptins Pr-LL and Ac-LL gave two spots,[2] respectively. This phenomena are compatible with the following NMR spectra of leupeptins Pr-LL and Ac-LL hydrochlorides in D_2O. A peak at δ 9.5 correspond to less than 1/5 proton, could reasonably be assigned to the aldehyde, and a doublet type peak at δ 5.4 corresponding to about 1/2 proton, is explained by the presence of an equilibrium such as between the aldehyde and oxazoline ring or *gem*-diol form. N-Acetyl argininal which was synthesized was also confirmed to show two spots on the thin-layer chromatography.

Experimental

Oxidation of Dihydroleupeptin Pr-LL to Leupeptin Pr-LL——According to sulfoxide–carbodiimide reaction reported by Pfitzner and Moffatt,[4] dihydroleupeptin Pr-LL (883 mg, 1.8 mmoles) was dissolved in anhydrous dimethylsulfoxide (10 ml) containing N,N′-dicyclohexylcarbodiimide (1.52 g, 7.4 mmoles) and anhydrous phosphoric acid (128 mg, 1.3 mmoles). Allowing to stand for 6.5 hr at room temperature, the reaction mixture was diluted with 150 ml of water and adjusted to pH 7.0 with 1N sodium hydroxide, and N,N′-dicyclohexylurea appeared was removed by filtration. The filtrate was subjected to a column chromatography using 20 g of carbon (Wako Pure Chem., Co.). The column was washed with 400 ml of water and eluted with 0.02N hydrochloric acid in 80% methanol. The eluate (220 ml), which gave positive Sakaguchi and red tetrazolium reactions, was neutralized with Amberlite IR 45 (OH from) and concentrated to dryness yielding 741 mg of a white powder. The white powder (726mg) was refluxed in 15ml of butanol for 3 hr. To the solution, 15 ml of butyl acetate and 30 ml of water were added and mixed by shaking. The upper layer of the mixture was separated and concentrated to dryness yielding 339 mg of the acetal hydrochloride as a crude powder. The crude powder was dissolved in butanol–butyl acetate–acetic acid–water (8:8:1:1 in volume) and subjected to a column chromatography using 30 g of silicic acid (Mallinckrodt) developing with the same solvent mixture. Fractions gave positive Sakaguchi reaction were combined (42 ml) and concentrated to dryness yielding 189 mg of leupeptin Pr-LL di-*n*-butyl acetal hydrochloride as a white powder, which melted at 80—105°, $[\alpha]_D^{22}$ −35° (c=1, methanol). *Anal.* Calcd. for $C_{29}H_{58}O_5N_6 \cdot HCl \cdot H_2O$: C, 55.70; H, 9.83; N, 13.44; Cl, 5.67. Found: C, 56.03; H, 9.69; N, 13.45; Cl, 5.91.

Leupeptin Pr-LL di-*n*-butyl acetal hydrochloride (77 mg) was dissolved in 7.5 ml of 0.01N hydrochloric acid. After heating at 60° for 3 hr, the solution was neutralized with Amberlite IR 45 (OH form) and concentrated to dryness yielding 63 mg of leupeptin Pr-LL hydrochloride as a white powder, which melted at 75—90°, $[\alpha]_D^{21}$ −46° (c=3, methanol). *Anal.* Calcd. for $C_{21}H_{40}O_4N_6 \cdot HCl \cdot H_2O$: C, 50.95; H, 8.76; N, 16.98; Cl, 7.16. Found: C, 50.85; H, 9.01; N, 16.71; Cl, 7.09.

Oxidation of Dihydroleupeptin Ac-LL to Leupeptin Ac-LL——Dihydroleupeptin Ac-LL (469 mg, 1.0 mmoles) was dissolved in anhydrous dimethylsulfoxide (5 ml) containing N,N′-dicyclohexylcarbodiimide (925 mg, 4.5 mmoles) and anhydrous phosphoric acid (52 mg, 0.5 mmoles). Allowing to stand for 20 hr at room temperature, the reaction mixture was diluted with 10 ml of water and adjusted to pH 6.0 with 1N sodium hydroxide, and N,N′-dicyclohexylurea was removed by filtration. The filtrate was passed through a column of Amberlite CG 50 (60 ml of 50% Na form). The column was washed with 300 ml of water and then eluted with 1N hydrochloric acid. The eluate (310 ml), which gave positive Sakaguchi and red tetrazolium reactions, was neutralized with Amberlite IR 45 (OH form). The neutralized eluate was concentrated to dryness and the residue was extracted with 10 ml of butanol yielding 195 mg of a white powder. The white powder (174 mg) was refluxed in 5 ml of butanol for 3 hr. The butanol solution was washed with 5 ml of water and concentrated to dryness yielding 113 mg of the acetal hydrochloride as a crude powder. The crude powder was subjected to the silicic acid column chromatography as similar manner as described in the previous section. A white powder of leupeptin Ac-LL di-*n*-butyl acetal hydrochloride (22 mg) was obtained, mp 60—90°, $[\alpha]_D^{25}$ −33° (c=1.1, methanol).

Leupeptin Ac-LL di-*n*-butyl acetal hydrochloride (20 mg) was dissolved in 2 ml of 0.01N hydrochloric acid. After heating at 60°, for 3 hr, the solution was neutralized with Amberlite IR 45 (OH form) and concentrated to dryness yielding 14 mg of leupeptin Ac hydrochloride as a white powder, mp 65—100°, $[\alpha]_D^{29}$ −42° (c=1, methanol). *Anal.* Calcd. for $C_{20}H_{38}O_4N_6 \cdot HCl \cdot H_2O$: C, 49.93; H, 8.59; N, 17.47; Cl, 7.37. Found: C, 50.17; H, 8.60; N, 17.48; Cl, 7.35.

Propionyl-L-leucyl-L-leucine from Leupeptin Pr-LL Di-*n*-butyl Acetal——A solution of 550 mg of leupeptin Pr-LL di-*n*-butyl acetal hydrochloride in 5.5 ml of 1N hydrochloric acid was refluxed for 30 min. On standing at room temperature, white crystals formed were collected by filtration and recrystallized from a mixture of ethanol and water to yield 137 mg of white crystals, mp 196—198°, $[\alpha]_D^{25}$ −56° (c=2, methanol), pK_a' 5.9 in 67% dimethylformamide. *Anal.* Calcd. for $C_{15}H_{28}O_4N_2$: C, 59.97; H, 9.40; N, 9.33; mol. wt. 300. 39. Found: C, 59.70; H, 9.43; N, 9.63; titration equivalent 309.

The NMR spectrum (100 MHz) in CD_3OD showed C-methyl at δ 0.94 (2 sets of doublet, 12H), propionyl at δ 1.12 (triplet, 3H) and δ 2.23 (quartet, 2H), α-methine of amino acid at δ 4.42 (triplet, 2H), and methylene and methine signals at δ 1.6 (broad, 6H). This compound was identical with synthetic propionyl-L-leucyl-L-leucine (mp 197—199°, $[\alpha]_D^{21}$ −60) in all respects such as mixed melting point, IR spectrum and optical rotation.

Acetyl-L-leucyl-L-leucine from Leupeptin Ac-LL Di-*n*-butyl Acetal——A solution of 240 mg of leupeptin Ac-LL di-*n*-butyl acetal hydrochloride in 2.5 ml of 1N hydrochloric acid was refluxed for 30 min. On standing at room temperature, white crystals formed were collected by filtration and recrystallized from a mixture of ethanol and water yielding 17 mg of white crystals, mp 190—191°, $[\alpha]_D^{22}$ −52° (c=1, methanol) (lit.[5]: mp 187—188°, $[\alpha]_D^{30}$ −56° (c=2.5, ethanol)), pK_a' 5.9 in 67% dimethylformamide. *Anal.* Calcd. for $C_{14}H_{26}O_4N_2$: C, 58.72; H, 9.15; N, 9.78; mol. wt., 286.36. Found: C, 58.39; H, 9.10; N, 9.73; titration equivalent, 305.

The NMR spectrum (100 MHz) in CD_3OD showed C-methyl at δ 0.93 (2 sets of doublet, 12H), acetyl at δ 1.95 (singlet, 3H), α-methine of amino acid at δ 4.42 (triplet, 2H), and methylene and methine signals at σ 1.6 (broad, 6H). This compound was identical with synthetic acetyl-L-leucyl-L-leucine (mp 191—192°, $[\alpha]_D^{22}$ −54°) in all respects such as mixed melting point, IR spectrum and optical rotation.

Alkaline Hydrolysis of Dihydroleupeptin Ac-LL——A solution of 1.094 g of dihydroleupeptin Ac-LL hydrochloride in 10 ml of barium hydroxide-saturated water was refluxed for 1 hr. The reaction mixture was neutralized with 1N sulfuric acid, and barium sulfate precipitated was removed by filtration. The filtrate was evaporated to dryness yielding 1.109 g of a white powder. The behavior of the filtrate on high-voltage paper electrophoresis is shown in Fig. 1. The electrophoresis was performed on Toyo filter paper No. 51, using acetic acid–formic acid–water (75:25:900 in volume) under 3500 V for 15 min (Savant Instruments, Inc., Model HV 5000-3). The white powder was dissolved in 5 ml of butanol–butyl acetate–acetic acid–water (6:6:1:1 in volume) and was subjected to a column chromatography of silicic acid (Mallinckrodt, 100 g). To the column, 1800 ml of butanol–butyl acetate–acetic acid–water (6:6:1:1) (fractions 1—155), 600 ml of butanol–butyl acetate–acetic acid–water (4:2:1:1) (fractions 156—209) and 730 ml of *n*-propanol–ethanol–0.1N HCl (5:4:1) (fractions 210—270) were passed successively. The eluate was collected in 10 ml fractions and each fraction was tested with Rydon–Smith, ninhydrin and Sakaguchi reactions. Fractions 6—21 were combined and concentrated to dryness yielding 200 mg of a white crystalline powder designated compound I. Compound I was recrystallized from a mixture of ethanol and water, and was identical with acetyl-L-leucyl-L-leucine in melting point, elemental analysis, and IR spectrum. Fractions 31—41 were combined and concentrated to dryness yielding 100 mg of a white powder designated compound II. To a solution of compound II (100 mg) in 0.8 ml of water, 0.14 ml of nitric acid was added. Allowing to stand in refrigerator, the solution deposited white crystals, which were identical with authentic urea nitrate in decomposition point, elemental analysis and IR spectrum. Fractions 155—190, which gave positive Rydon–Smith and ninhydrin reactions, were combined and concentrated to dryness yielding 175 mg of a white powder designated compound III. Compound III was acetylated with 8 ml of pyridine and 1.5 ml of acetic anhydride and crystallized from a mixture of ethanol and ethyl acetate, mp 216—220°, $[\alpha]_D^{26}$ −58° (c=1.4, methanol). *Anal.* Calcd. for $C_{23}H_{42}O_6N_4$: C, 58.70; H, 9.00; N, 11.91; mol. wt. 470.60. Found: C, 58.91; H, 8.97; N, 11.85; *m/e* 470 (mass spectrometry). The NMR spectrum (100 MHz) in $(CD_3)_2SO$ showed C-methyl at δ 0.85 (2 sets of doublet, 12H), methylene and methine at δ 1.4 (broad, 10H), N-acetyl at δ 1.77 (singlet, 3H) and at δ 1.84 (singlet, 3H), O-acetyl at δ 1.96 (singlet, 3H), methylene of $-CH_2-NH-CO-$ at δ 3.0 (broad, 2H), methylene of $-CH_2-O-CO-$ and methine of $-NH-CH<$ at δ 3.9 (broad, 3H), methine of $-NH-CH-CO$ at δ 4.2 (broad, 2H) and $-NH-$ signals at δ 7.5—8.0 (broad, 4H). The acid hydrolysate of compound III with 6N hydrochloric acid at 105° for 20 hr gave leucine and ornithinol on high-voltage paper electrophoresis. From these properties, the structures of the acetylated compound III and the compound III were determined to be triacetylleucylleucylornithinol and N-monoacetylleucylleucylornithinol, respectively. Fractions 226—227, which gave positive Rydon–Smith, ninhydrin and Sakaguchi reactions, were combined and concentrated to dryness yielding 40 mg of white powder designated compound IV. To a solution of compound IV in 3 ml of water, a solution of 102 mg of sodium picrate in 2 ml of water was added. Allowing to stand in a refrigerator, the mixture gave yellow crystals, which were identical with authentic DL-argininol dipicrate in decomposition point, elemental analysis, optical rotation, IR spectrum and high-voltage paper electrophoresis. Fractions 232—245, which gave positive Rydon–Smith and ninhydrin reactions, were combined

and concentrated to dryness yielding 46 mg of a white powder designated compound V. To a solution of the compound V in 3 ml of 83% ethanol at 50—60°, a solution of 150 mg of picric acid in 2 ml of ethanol was added. Allowing to stand overnight at room temperature, the mixture gave yellow needles, which were identical with authentic DL-ornithinol dipicrate in decomposition point, elemental analysis, optical rotation, IR spectrum and high-voltage paper electrophoresis.

Acid Hydrolysis of Leupeptin Acids and Isolation of L-Leucine and DL-Arginine——A mixture of leupeptins Pr-LL acid and Ac-LL acid (2.35 g) was dissolved in 50 ml of 6N hydrochloric acid and refluxed for 10 hr. The reaction mixture was concentrated to dryness, and the residue was dissolved in 50 ml of water and adjusted to pH 7.0 with aqueous ammonia. The solution was passed through a column of Amberlite IRC 50 (20 ml of 70% NH_4 form) and the effluent was concentrated to dryness. The residue was washed with 20 ml of methanol, and the methanol-insoluble part was recrystallized from a mixture of water and ethanol yielding 263 mg of L-leucine. $[M]_D^{26}$ −11.8° (c=2.2, water), $[M]_D^{26}$ +28.8° (c=1, acetic acid) (lit.[6]): $[M]_D$ −14.4° in water, $[M]_D$ +29.5° in acetic acid). *Anal.* Calcd. for $C_6H_{13}O_2N$: C, 54.94; H, 9.99; N, 10.67. Found: C, 54.97; H, 9.99; N, 10.58.

The column was washed with water and then eluted with 0.2N hydrochloric acid. The eluate, which gave positive Sakaguchi reaction, was adjusted to pH 6 with Amberlite IR 45 (OH form) and concentrated to dryness yielding 480 mg of a white powder. To a solution of the white powder in 1.5 ml of water, a solution of 640 mg of sodium picrate in 4 ml of water was added at 50—60°. Allowing to stand in a refrigerator, the mixture gave yellow crystals which were recrystallized from hot water, mp 206—212° decomp., $[\alpha]_D^{26}$ 0° (c=1.8, dimethylsulfoxide). *Anal.* Calcd. for $C_6H_{14}O_2N_4 \cdot C_6H_3O_7N_3$: C, 35.73; H, 4.25; N, 24.31. Found: C, 35.89; H, 4.35; N, 23.19. It was identical with authentic DL-arginine monopicrate in decomposition point, elemental analysis, optical rotation and IR spectrum.

Synthesis of Acetyl-L-leucyl-L-leucine——To a solution of carbobenzoxy-L-leucine (9.0 g, 34 mmoles), L-leucine ethyl ester hydrochloride (6.7 g, 34 mmoles) and triethylamine (4.8 ml, 34 mmoles) in 140 ml of chloroform, 7.0 g of N,N′-dicyclohexylcarbodiimide was added under cooling and stirring. Allowing to stand overnight in a refrigerator, the mixture was filtered to remove crystalline N,N′-dicyclohexylurea appeared. The filtrate was concentrated to dryness and the residue was dissolved in 150 ml of ethyl acetate. After removing insoluble N,N′-dicyclohexylurea by filtration, and the ethyl acetate solution was washed successively with 50 ml of 0.5N hydrochloric acid, 50 ml of water, 100 ml of 0.5M sodium bicarbonate and 50 ml of water. The solution was dried over anhydrous sodium sulfate and concentrated to dryness yielding 12.0 g of the crude powder. And, carbobenzoxy-L-leucyl-L-leucine ethyl ester crystallized from the mixture of 30 ml of methanol and 10 ml of water to obtain 8.4 g of crystals at 65% yield, mp 85.5—86.5°, $[\alpha]_D^{26}$ −50° (c=2, methanol). *Anal.* Calcd. for $C_{22}H_{34}O_5N_2$: C, 65.00; H, 8.43; N, 6.89. Found: C, 65.02; H, 8.67; N, 7.15.

Carbobenzoxy-L-leucyl-L-leucine ethyl ester (4.06 g, 10 mmoles) in a mixture of methanol (50 ml), water (15 ml) and acetic acid (4 ml) was hydrogenated in the presence of palladium carbon (4 g) containing 5% palladium for 2 hr. The syrupy L-leucyl-L-leucine ethyl ester acetate (2.95 g) obtained was dissolved in a mixture of chloroform (4 ml) and benzene (16 ml), and acetic anhydride (1.4 ml) was added.[7] After 9 hr at room temperature, ethanol (2 ml) was added and then ethyl acetate (60 ml). The solution was washed successively with 0.5N hydrochloric acid (20 ml), 0.5M sodium bicarbonate (40 ml) and water (20 ml), and dried over anhydrous sodium sulfate. Evaporation gave a residue (1.96 g) which was extracted with 20 ml of *n*-hexane. The extract was concentrated to dryness yielding 1.88 g of acetyl-L-leucyl-L-leucine ethyl ester as a white powder. The ester was hydrolyzed at room temperature by 1N sodium hydroxide (6.6 ml) in ethanol (10 ml) for 2 hr. The solution was made acid with 1N hydrochloric acid (7 ml) and acetyl-L-leucyl-L-leucine (1.36 g, 47% from carbobenzoxy-L-leucyl-L-leucine ethyl ester) crystallized. Recrystallization from ethanol-water gave white crystals, mp 191—192°, $[\alpha]_D^{22}$ −54° (c=2, methanol). *Anal.* Calcd. for $C_{14}H_{26}O_4N_2$: C, 58.72; H, 9.15; N, 9.78. Found: C, 58.71; H, 9.39; N, 9.67.

Synthesis of Propionyl-L-leucyl-L-leucine——To a solution of L-leucyl-L-leucine ethyl ester acetate (1.66 g) in a mixture of chloroform (2 ml) and benzene (8 ml), propionic anhydride (0.7 ml) was added. Propionyl-L-leucyl-L-leucine ethyl ester (1.32 g) was obtained and saponified and propionyl-L-leucyl-L-leucine (754 mg, 45% from carbobenzoxy-L-leucyl-L-leucine ethyl ester) crystallized, mp 197—199°, $[\alpha]_D^{21}$ −60° (c=2, methanol). *Anal.* Calcd. for $C_{15}H_{28}O_4N_2$: C, 59.97; H, 9.40; N, 9.33. Found: C, 60.13; H, 9.60; N, 9.26.

Synthesis of Acetyl-L-leucyl-L-leucyl-L-arginine——Carbobenzoxy-L-leucyl-L-leucine ethyl ester (8.13 g) was hydrolyzed at room temperature by 1N sodium hydroxide (22 ml) in ethanol (100 ml) for 3 hr. The solution was made acid with 1N hydrochloric acid (25 ml) and concentrated to about 50 ml. The concentrate was extracted twice with 80 ml of ethyl acetate. The extract was washed with water (40 ml) and dried over anhydrous sodium sulfate and concentrated to about 40 ml and added petroleum ether (200 ml). After the mixture had been cooled, the crude crystals (7.31 g) were obtained, and recrystallized from a mixture of ethyl

6) J.P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," Vol. 3, John Wiley & Sons, Inc., New York, N.Y., 1961, p. 1841.
7) N.A. Smart, G.T. Young and M.W. Williams, *J. Chem. Soc.*, **1960**, 3902.

acetate (20 ml) and petroleum ether (60 ml) to yield 6.45 g (85%) of carbobenzoxy-L-leucyl-L-leucine as white crystals, mp 93—95°, $[\alpha]_D^{26}$ −29° (c=2.9, methanol) (lit.[8]: mp 98—101°, $[\alpha]_D^{22}$ −24.7° (c=1, ethanol)). *Anal.* Calcd. for $C_{20}H_{30}O_5N_2$: C, 63.47; H, 7.99; N, 7.40. Found: C, 63.64; H, 7.66; N, 7.67.

Carbobenzoxy-L-leucyl-L-leucine (6.43 g, 17 mmoles) was dissolved in 250 ml of nitromethane and then cooled in an ice water bath. Triethylamine (2.38 ml, 17 mmoles) and Woodward's reagent K[9] (4.42 g, 17 mmoles) were added and the mixture was stirred for 1 hr. Then 4.6 g of N^G-nitro-L-arginine methyl ester hydrochloride[10] (17 mmoles) and triethylamine (2.38 ml, 17 mmoles) were added, and the mixture was stirred for 5 hr and kept overnight at room temperature. The reaction mixture was concentrated to dryness, and the residue was dissolved in ethyl acetate (400 ml). The ethyl acetate solution was then washed successively with water (200 ml), 0.5N HCl (100 ml), water (100 ml), 0.5M $NaHCO_3$ (200 ml) and water (100 ml), and dried over anhydrous sodium sulfate and concentrated to dryness yielding 6.6 g of carbobenzoxy-L-leucyl-L-leucyl-N^G-nitro-L-arginine methyl ester as a white powder, mp 75—85°.

The ester in a mixture of methanol (260 ml), water (40 ml) and acetic acid (20 ml) was hydrogenated in the presence of palladium carbon (10 g) containing 5% palladium for 14 hr. The syrupy L-leucyl-L-leucyl-L-arginine methyl ester diacetate (6.1 g) thus obtained was dissolved in a mixture of chloroform (80 ml) and benzene (40 ml), and acetic anhydride (3 ml) was added. After the mixture had been kept at room temperature for 8 hr, ethanol (20 ml) was added and then concentrated yielding 5.9 g of syrup. The syrup was hydrolyzed at room temperature by 1N sodium hydroxide (50 ml) in ethanol (50 ml) for 4 hr. The solution was neutralized with 1N hydrochloric acid (3 ml), and concentrated to dryness. The residue was extracted with water (60 ml) and the extract was subjected to a column chromatography of Dowex 1 X 2 (100—200 mesh, OH form, 210 ml) developing with water. The alkaline eluate giving positive Sakaguchi reaction was adjusted to pH 5.2 with Amberlite IRC 50 (H form) and concentrated to dryness yielding 250 mg of a white powder. The next neutral eluate was concentrated to dryness, yielding 2.54 g of a white powder. The powders were combined and crystallized from methanol–ether. Then, crystalline acetyl-L-leucyl-L-leucyl-L-arginine (1.67 g) was obtained. Total yield 22%, mp 262—264° decomp., $[\alpha]_D^{19}$ −47° (c=1, methanol). *Anal.* Calcd. for $C_{20}H_{38}O_5N_6$: C, 54.28; H, 8.66; N, 18.99. Found: C, 54.56; H, 8.78; N, 18.75.

Synthesis of Propionyl-L-leucyl-L-leucyl-L-arginine——Carbobenzoxy-L-leucyl-L-leucyl-N^G-nitro-L-arginine methyl ester (1.8 g, 3 mmoles) in a mixture of methanol (50 ml), water (10 ml) and propionic acid (5 ml) was hydrogenated in the presence of palladium carbon (2 g) containing 5% palladium for 18 hr to obtain 1.7 g of syrup. The syrup was dissolved in a mixture of chloroform (20 ml) and benzene (10 ml), and propionic anhydride (1 ml) was added. After the mixture had been kept at room temperature for 18 hr, ethanol (10 ml) was added and then concentrated yielding 1.6 g of syrup. The syrup was hydrolyzed and subjected to a column chromatography of Dowex 1 X 2 as described above. Crystalline propionyl-L-leucyl-L-leucyl-L-arginine (370 mg, 18% yield from carbobenzoxy-L-leucyl-L-leucine) was obtained, mp 256—258° decomp., $[\alpha]_D^{23}$ −55° (c=1.5, methanol). *Anal.* Calcd. for $C_{21}H_{40}O_5N_6$: C, 55.24; H, 8.83; N, 17.52. Found: C, 54.87; H, 8.72; N, 17.77.

Synthesis of Acetyl-L-leucyl-L-leucyl-L-argininol Hydrochloride——Acetyl-L-leucyl-L-leucyl-L-arginine (442 mg, 1 mmole) in 40 ml of 0.48% hydrogen chloride in dry methanol was kept at room temperature for 24 hr. The solution was concentrated to dryness yielding 518 mg of acetyl-L-leucyl-L-leucyl-L-arginine methyl ester hydrochloride. To the ester hydrochloride dried over P_2O_5, excess of $LiBH_4$ (400 mg, 18 mmoles) in tetrahydrofuran (40 ml) was added and the mixture was refluxed gently at 90° in an oil bath.[11] After 6 hr, the mixture was cooled and 9.5% hydrogen chloride in methanol (8 ml) was added to make a clear solution and concentrated to dryness. The residue was dissolved in 20 ml of water and extracted twice into 20 ml of butanol. The butanol extracts were combined and washed with 20 ml of water and concentrated to dryness yielding 320 mg of a white powder. The powder was dissolved in 10 ml of butanol–butyl acetate–acetic acid–water (6:6:1:1 in volume) and subjected to column chromatography of silicic acid (Mallinckrodt, 10 g) developing with the same solvent. The eluate, which gave Sakaguchi reaction, was concentrated to dryness yielding 150 mg (31%) of acetyl-L-leucyl-L-leucyl-L-argininol hydrochloride as a white powder, mp 60—75°, $[\alpha]_D^{20}$ −46° (c=1, methanol). *Anal.* Calcd. for $C_{20}H_{40}O_4N_6 \cdot HCl \cdot H_2O$: C, 49.73; H, 8.97; N, 17.40; Cl, 7.34. Found: C, 49.92; H, 8.79; N, 17.44; Cl, 6.98.

Synthesis of Propionyl-L-leucul-L-leucyl-L-argininol Hydrochloride——Propionyl-L-leucyl-L-leucyl-L-arginine (254 mg, 0.56 mmoles) was esterified and reduced with $LiBH_4$ and purified by silicic acid column chromatography as described above. A white powder of propionyl-L-leucyl-L-leucyl-L-argininol hydrochloride (110 mg, 40%) was obtained, mp 70—80°, $[\alpha]_D^{20}$ −38° (c=1, methanol). *Anal.* Calcd. for $C_{21}H_{42}O_4N_6 \cdot HCl \cdot H_2O$: C, 50.74; H, 9.13; N, 16.91; Cl, 7.13. Found: C, 50.97; H, 9.40; N, 17.02; Cl, 6.91.

Synthesis of Acetyl-L-leucyl-L-leucyl-DL-argininal (Leupeptin Ac-LL) Hydrocholride——Acetyl-L-leucyl-L-leucyl-L-argininol hydrochloride (42.2 mg, 0.09 mmoles) was dissolved in anhydrous dimethylsulfoxide

8) E.L. Smith, D.H. Spackman and W.J. Polglase, *J. Biol. Chem.*, **199**, 801 (1949).
9) W.B. Woodward, R.A. Olofson and H. Mayer, *J. Am. Chem. Soc.*, **83**, 1010 (1961).
10) K. Hofmann, W.D. Peckham and A. Rheiner, *J. Am. Chem. Soc.*, **78**, 238 (1956).
11) D.L. Swallow and E.P. Abraham, *Biochem. J.*, **72**, 326 (1959).

(0.5 ml) containing N,N′-dicyclohexylcarbodiimide (77.9 mg, 0.39 mmoles) and anhydrous phosphoric acid (6.9 mg, 0.07 mmoles). Allowing to stand at room temperature for 7.5 hr, the reaction mixture was diluted with 10 ml of water and adjusted to pH 7.0 with 1N sodium hydroxide and N,N′-dicyclohexylurea appeared was removed by filtration. The filtrate was subjected to column chromatography using 1 g of carbon (Woko Pure Chem., Co.). The column was washed with water and then eluted with 0.02N hydrochloric acid in 80% methanol. The eluate, which gave positive Sakaguchi and red tetrazolium reactions, was concentrated to dryness and 38.1 mg of a crude powder was obtained. Anti-plasmin activity[3]: ID_{50} 24 μg/ml. The crude powder (32.6 mg) in 2 ml of butanol was refluxed for 3 hr. To the solution, butyl acetate (2 ml) and water (4 ml) were added and mixed by shaking. The upper layer was concentrated to dryness yielding 21.1 mg of the di-*n*-butyl acetal hydrochloride, mp 60—90°. The acetal hydrochloride (20.0 mg) in 2 ml of 0.01N hydrochloric acid was heated at 60° for 3 hr. The solution was adjusted to pH 6.0 with Amberlite IR 45 (OH form) and concentrated to dryness yielding 14.3 mg of acetyl-L-leucyl-L-leucyl-DL-argininal hydrochloride as a white powder. Yield 40%. Anti-plasmin activity: ID_{50} 13 μg/ml, mp 65—100°, $[\alpha]_D^{28}$ −42° (c=1, methanol). *Anal.* Calcd. for $C_{20}H_{38}O_4N_6 \cdot HCl \cdot H_2O$: C, 49.93; H, 8.59; N, 17.47; Cl, 7.37. Found: C, 50.26; H, 8.46; N, 16.92; Cl, 7.61. The product was identical with leupeptin Ac-LL hydrochloride in anti-plasmin activity, and physical and chemical properties.

Synthesis of Propionyl-L-leucyl-L-leucyl-DL-argininal (Leupeptin Pr-LL) Hydrochloride——Propionyl-L-leucyl-L-leucyl-L-argininol hydrochloride (60.7 mg) was oxidized by sulfoxide-carbodiimide reaction and purified by carbon chromatography as described above. A crude powder of propionyl-L-leucyl-L-leucyl-DL-argininal hydrochloride (55.5 mg, ID_{50} 23 μg/ml) was obtained. The crude powder (52.7 mg) was derived to the di-*n*-butyl acetal hydrochloride (27.0 mg, mp 80—105°). The acetal hydrochloride (11.1 mg) was converted to propionyl-L-leucyl-L-leucyl-DL-argininal hydrochloride (8.8 mg) by hydrolysis. Total yield 40%. Anti-plasmin activity: ID_{50} 9 μg/ml, mp 75—90°, $[\alpha]_D^{21}$ −46° (c=3, methanol). *Anal.* Calcd. for $C_{21}H_{40}O_4N_6 \cdot HCl \cdot H_2O$: C, 50.95; H, 8.76; N, 16.98; Cl, 7.16. Found: C, 50.59; H, 8.91; N, 16.58; Cl, 7.09. The product was identical with leupeptin Pr-LL hydrochloride in anti-plasmin activity, and physical and chemical properties.