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Isolation and Characterization of Leupeptins produced by *Actinomycetes*

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Leupeptins which have anti-plasmin activity and inhibit trypsin, papain and blood coagulation have been obtained from cultured broths of various species of *Actinomycetes*. Two leupeptins which were leupeptins Pr ($C_{21}H_{40}O_4N_6$) and Ac ($C_{20}H_{38}O_4N_6$) were isolated and characterized. They gave their di-*n*-butyl acetals, dihydroleupeptins and leupeptin acids. Physical and chemical properties of these leupeptins and their derivatives were described.

Mixtures of new compounds which showed anti-plasmin activity were obtained from cultured broths of various species of *Streptomyces* and named leupeptins. Besides the anti-plasmin activity, they showed anti-inflammatory effect and inhibition of trypsin, papain and blood coagulation as reported in a previous paper.[2]

Ten strains of *Streptomyces* which were isolated from soil samples and confirmed to produce leupeptins were classified as *Streptomyces roseus* (strains No. MA839-A1, MB262-M1), *Streptomyces roseochromogenes* (MA943-M1, MB260-A2, MB456-AE1), *Streptomyces chartreusis* (MB58-MG1), *Streptomyces albireticuli* (MB26-A1), *Streptomyces thioluteus* (MB321-A1), *Streptomyces lavendulae* (MB172-A2) and *Streptomyces noboritoensis* (MB46-AG1). Production of leupeptins was also confirmed in other strains which should be classified into other more than 11 species. Therefore, productivity of leupeptins is not species-specific and leupeptins are produced widely by various species of *Streptomyces*.

In the present paper, production, isolation and characterization of leupeptins produced by *Streptomyces roseus* MA839-A1 are described.

The cultural conditions of *S. roseus* MA839-A1 were studied determining the anti-plasmin activity as described in another paper.[2] Glucose and starch were suitable carbon sources for the production of leupeptins, and peptone and NZ-amine Type A (Sheffield Chemical) were suitable nitrogen sources. Therefore, the leupeptins-producing organism was shake-cultured at 27—29° for 24—72 hr in a medium containing 1—2% glucose, 1% starch, 2% peptone and 0.5% sodium chloride (adjusted to pH 7.0). The seed culture was prepared by the shaking culture in the same medium for 2 days. The same medium was employed also for the production in stainless steel fermentors. The 50% inhibition concentrations (ID_{50}) of anti-plasmin activities of these cultured broths were at 0.03—0.01 ml per milliliter in the assay system. The yield of about 300—1000 μg of leupeptins per milliliter of the broths was estimated by calculation from the anti-plasmin activities.

Leupeptins in the culture filtrate were adsorbed on carbon and eluted with 80% aqueous methanol at pH 2. The active eluate was concentrated to dryness yielding a brownish powder (ID_{50}: 95 μg/ml, yield: 30—40% from the filtrate). Leupeptins in the brownish powder were extracted by processes of adsorption on a carbon column followed by elution with 0.02N hydrochloric acid in 80% methanol yielding a yellowish powder shown the higher activity

1) Location: *14-23, Kamiosaki 3-chome, Shinagawa-ku, Tokyo.*
2) T. Aoyagi, T. Takeuchi, A. Matsuzaki, K. Kawamura, S. Kondo, M. Hamada, K. Maeda and H. Umezawa, *J. Antibiotics* (Tokyo), **22**, 283 (1969).

(ID_{50}: 53 μg/ml, yield: 70—80% from the brownish powder). Further purification was achieved by alumina column chromatography developed with methanol, and a white powder of mixture of leupeptins hydrochlorides was obtained (ID_{50}: 12 μg/ml, yield: 80—90% from the yellowish powder).

Ion-exchange resin processes were found to be more efficient for extraction of leupeptins than the carbon adsorption processes. Leupeptins in the culture filtrate were also adsorbed on a column of Lewatit CNP resin (Farbenfabriken Bayer A. G., pH 6.0 with sodium hydroxide) and eluted with 1N hydrochloric acid in 80% methanol. Leupeptins in the eluate was extracted into butanol and the evaporation of the butanol layer under reduced pressure yielded a brownish powder (ID_{50}: 13 μg/ml, yield: 75% from the culture filtrate). Further purification was achieved by Dowex 1×2 (Cl form) resin chromatography developed with water and a white powder of mixture of leupeptins hydrochlorides was obtained (ID_{50}: 11 μg/ml, yield: 91% from the brownish powder).

Separation of the mixture into two leupeptin groups which were designated leupeptins Pr and Ac was accomplished by silicic acid (Mallinckrodt) column chromatography, using butanol–butyl acetate–acetic acid–water (4:8:1:1 in volume) as the developing solvent, of di-*n*-butyl acetal derivatives which were prepared by refluxing the mixture of leupeptins hydrochlorides in butanol for 2—3 hr. Di-*n*-butyl acetal of leupeptin Pr was faster moving than di-*n*-butyl acetal of leupeptin Ac in the chromatography. Di-*n*-butyl acetals of leupeptins Pr and Ac were converted to hydrochlorides of leupeptin Pr (ID_{50}: 8 μg/ml, yield: 25—50%) and hydrochlorides of leupeptin Ac (ID_{50}: 12 μg/ml, yield: 25—50%) by heating at 60° for 3 hr in 0.01N hydrochloric acid. The amount of leupeptin Ac obtained was 1.5—3 times more than that of leupeptin Pr.

As reported in the another paper,[3] the structures of one of leupeptin Pr and one of leupeptin Ac were determined by its chemical syntheses to be propionyl-L-leucyl-L-leucyl-DL-argininal and acetyl-L-leucyl-L-leucyl-DL-argininal. Leupeptins Pr and Ac were suggested by the amino acid analysis to be mixtures of leupeptins in which one or two of leucine was substituted with isoleucine or valine. It was not successful to separate each of these leupeptins. However, main components of leupeptins produced by the strain MA839-Al were propionyl- and acetyl-L-leucyl-L-leucyl-DL-argininals (leupeptins Pr-LL and Ac-LL) and therefore the properties of these leupeptins examined on samples which were shown by amino acid analysis to contain only slight amount of other leupeptins.

The most purified leupeptin Pr hydrochloride which was shown to contain mainly leupeptin Pr-LL by the result of the amino acid analysis (leucine 1.00, isoleucine 0.10, valine 0) showed the following properties: A white powder melting at the wide range of 110—140°, $[\alpha]_D^{22}$ −56° (c=1, methanol), p$K_{a'}$ is more than 12. The elemental analysis indicates that the formula of the hydrochloride is $C_{21}H_{40}O_4N_6 \cdot HCl \cdot H_2O$. It has no characteristic ultraviolet absorption. The IR spectrum shows absorption corresponding to the aldehyde (1720—1730 cm^{-1}) and strong amide bands (1650, 1540 cm^{-1}) as shown in Fig. 1. The NMR spectrum (100 MHz) in $(CD_3)_2$ SO exhibits the existence of N-propionyl group in the molecule depending on peaks at δ 1.00 (triplet, 3H) and at δ 2.12 (quartet, 2H).

The most purified leupeptin Ac hydrochloride which was shown to contain mainly leupeptin Ac-LL by the result of amino acid analysis (leucine 1.00, isoleucine 0.04, valine 0.01) showed the following properties: A white powder melting at the wide range of 75—110°, $[\alpha]_D^{22}$ −52° (c=1, methanol), p$K_{a'}$ is more than 12. The elemental analysis indicates that the formula of the hydrochloride is $C_{20}H_{38}O_4N_6 \cdot HCl \cdot H_2O$. It has no characteristic ultraviolet absorption. The IR spectrum is similar to that of leupeptin Pr hydrochloride as shown in Fig. 2. On the NMR spectrum (100 MHz) in $(CD_3)_2$ SO, a peak at δ 1.85 (singlet, 3H) shows the existence of N-acetyl group.

3) K. Kawamura, S. Kondo, K. Maeda and H. Umezawa, *Chem. Pharm. Bull.* (Tokyo), **17**, 1902 (1969).

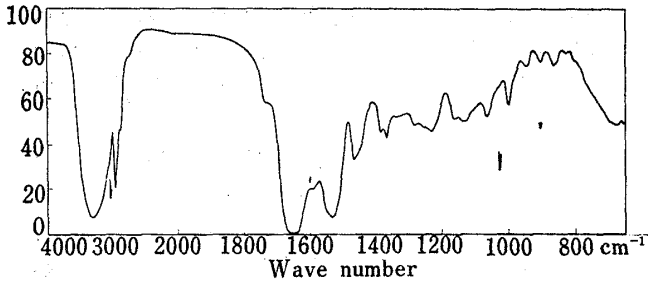


Fig. 1. IR Spectrum of Leupeptin Pr Hydrochloride (KBr)

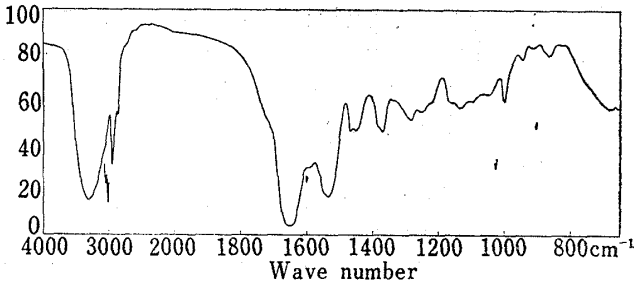


Fig. 2. IR Spectrum of Leupeptin Ac Hydrochloride (KBr)

The hydrochlorides of leupeptins Pr and Ac are soluble in water, methanol, ethanol, butanol, acetic acid, dimethylformamide and dimethylsulfoxide, and scarcely soluble in ethyl acetate, acetone, chloroform, carbon tetrachloride, ethyl ether and *n*-hexane.

Leupeptins Pr and Ac give positive Rydon-Smith,[4] red tetrazolium, 2,4-dinitrophenylhydrazine, Sakaguchi, diacetyl,[5] and pentacyanoaquoferriate[5] reactions, and negative ninhydrin, Jaffe,[5] ferric chloride, anthrone, Molisch, and benzidine reactions.

The paper chromatography of leupeptins Pr and Ac by ascending method revealed a single spot detected by Rydon-Smith, red tetrazolium or Sakaguchi reaction at *Rf* 0.9—0.95 developing with the upper layer of butanol–acetic acid–water (4:1:5 in volume), 0.9—0.95 with *n*-propanol–water (7:3) and 0.4—0.6 with ethyl acetate–methanol–water (4:1:1). On high-voltage paper electrophoresis (3500 V, 15 min) using formic acid–acetic acid–water (25:75:900), leupeptins Pr and Ac moved 5—6 cm toward the cathode from the starting point. On thin layer chromatography using silica gel G (E. Merck), leupeptins Ac and Pr gave a single spot at *Rf* 0.6—0.7 with *n*-propanol–water (7:3), 0.2—0.4 with chloroform–methanol (7:3), 0.2—0.3 with acetone–water (1:1) and 0.3—0.4 with ethyl acetate–acetic acid–water (60:17:17). Leupeptins Pr and Ac could not be distinguished by paper and thin-layer chromatographies and by high-voltage paper electrophoresis at the conditions described above. On thin layer chromatography of silica gel G using butanol–butyl acetate–acetic acid–water (4:2:1:1 in volume) as a developing solvent, leupeptins Pr and Ac were separated as each two spots at *Rf* 0.45—0.50 and 0.35—0.45 for leupeptin Pr, and *Rf* 0.35—0.45 and 0.30—0.35 for leupeptin Ac, respectively. The extract from each spots was detected as respective two spots on the same thin-layer chromatography. The possible mechanism for the results will be discussed in the following paper.[3]

Leupeptin Pr di-*n*-butyl acetal hydrochloride is a white powder melting at 70—90°, $[\alpha]_D^{23}$ −46° (*c*=2, methanol), p$K_{a'}$ is more than 12. The molecular formula is established as $C_{29}H_{58}O_5N_6$ by the parent peak *m/e*=570 in the mass spectrum. Leupeptin Pr di-*n*-butyl acetal picrate is a yellow crystalline powder melting at 60—90°.

Leupeptin Ac di-*n*-butyl acetal hydrochloride is a white powder melting at 70—90°, $[\alpha]_D^{23}$ −43° (*c*=2, methanol), p$K_{a'}$ is more than 12. The molecular formula is derived from the high-resolution mass spectrum of its hydrochloride: calcd. mol. wt. for $C_{28}H_{56}O_5N_6$, 556.431; found, *m/e* 556.429±0.003. Leupeptin Ac di-*n*-butyl acetal picrate is a yellow crystalline powder melting at 60—100°.

Leupeptins Pr and Ac di-*n*-butyl acetals hydrochlorides have no characteristic ultraviolet absorption. The IR spectra show the strong absorption at 1070 cm^{-1} attributed to the acetal. On thin layer chromatography using Silica gel G and butanol–butyl acetate–acetic acid–water (4:2:1:1), leupeptins Pr di-*n*-butyl acetal and Ac di-*n*-butyl acetal gave each single spot at *Rf* 0.65 and 0.60, respectively.

4) H.N. Rydon and P.W.G. Smith, *Nature*, **169**, 922 (1952).
5) J. Smith, "Chromatographic Techniques. Clinical and Biochemical Applications," William Heinemann Medical Books, Ltd., London, 1958, p. 149.

Leupeptins Pr and Ac were converted to dihydroleupeptins by reduction with sodium borohydride, and to leupeptin acids as crystals by oxidation with potassium permanganate.

Dihydroleupeptin Pr hydrochloride is a white powder melting at 85—120°, $[\alpha]_D^{20}$ −43° (c=1, methanol). Dihydroleupeptin Ac hydrochloride is a white powder melting at 85—120°, $[\alpha]_D^{20}$ −41° (c=1, methanol).

Dihydroleupeptins Pr and Ac hydrochlorides have no characteristic ultraviolet absorption. The IR spectra show the absorption at 1050 cm^{-1} for the primary alcohol. On thin layer chromatography using silica gel G and butanol–butyl acetate–acetic acid–water (4:2:1:1), dihydroleupeptins Pr and Ac gave each single spot at *Rf* 0.35 and 0.27, respectively.

Leupeptin Pr acid is white crystals melting at 256—259° under decomposition. $[\alpha]_D^{30}$ −53° (c=2.3, methanol). The amino acid analysis (molar ratio) gives leucine 1.68, isoleucine 0.26 and arginine 1.00.

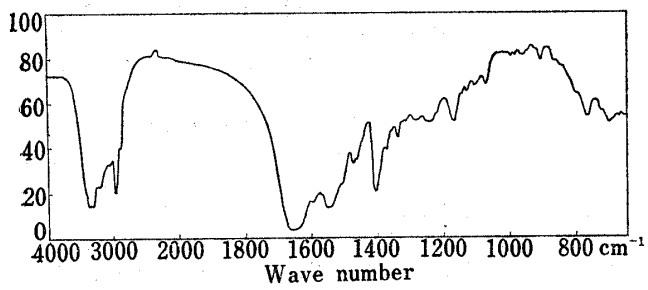


Fig. 3. IR Spectrum of Leupeptin Pr Acid (KBr)

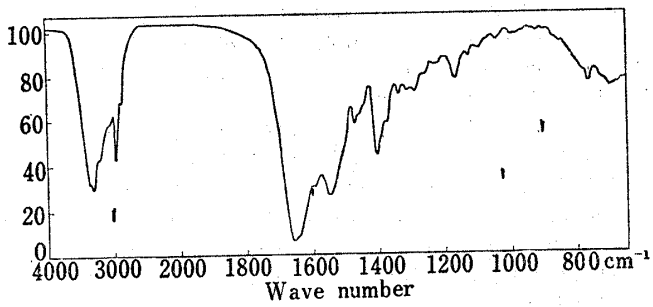


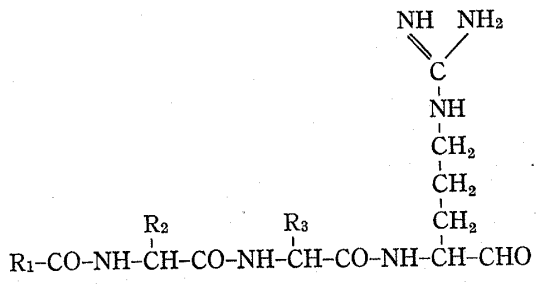
Fig. 4. IR Spectrum of Leupeptin Ac Acid (KBr)

Leupeptin Ac acid is white crystals melting at 260—265° under decomposition. $[\alpha]_D^{30}$ −51° (c=2.2, methanol). The amino acid analysis (molar ratio) gives leucine 1.77, isoleucine 0.20, valine 0.02 and arginine 1.00.

Leupeptins Pr and Ac acids have no characteristic ultraviolet absorption. The IR spectra are shown in Fig. 3 and 4. On thin-layer chromatography using Silica gel G and butanol–butyl acetate–acetic acid–water (4:2:1:1), leupeptins Pr and Ac acids gave each single spot at *Rf* 0.38 and 0.30, respectively.

Leupeptins produced in a synthetic medium containing 1% glucose, 1% starch, 0.2% NH_4NO_3, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.001% $FeSO_4 \cdot 7H_2O$, 0.26% leucine, 0.4% arginine monohydrochloride and 0.3% glycine, gave only leucine by the amino acid analysis, but leupeptins produced in an organic medium, contained isoleucine and valine in addition to leucine.

Since the foregoing results suggested that leupeptins produced by *Actinomycetes* contained various analogues having such as isoleucine and valine instead of 2 moles of leucine, the general formula of leupeptins can be written as the following.



R_1,R_2,R_3: alkyl radicals

Experimental

Isolation of Leupeptins——1) Carbon Process: *Streptomyces roseus* MA839-Al was cultured at 27° for 71 hr in 85 flasks (dispense volume in a flask:125 ml) by a reciprocal shaker (130 strokes/min). The medium contained 1% glucose, 1% starch, 2% Polypepton (Daigo Eiyo Co.) and 0.5% sodium chloride and adjusted to pH 7.0 with 2N sodium hydroxide. Vegetative inoculum, 1% by volume, grown for 2 days in the same medium was used. The culture broth was collected (10 liters, ID_{50}: 0.015 ml/ml) and centrifuged at 3000 rpm for 10 min. To the supernatant (9 liters, pH 7.3), 135 g of carbon (Wako Pure Chem.) was added. After stirring for 30 min, the carbon was separated by filtration and washed with 6 liters of water and eluted with 1500 ml and 1200 ml of acidic 80% methanol (pH 2 with 2N hydrochloric acid). The active eluate was neutralized with Amberlite IR 45 (OH form) and concentrated to dryness yielding 22 g of a brownish powder (ID_{50}: 95 μg/ml, yield of activity: 38%). The brownish powder was dissolved in 500 ml of water (pH 7.0) and poured over a column of carbon (Wako Pure Chem., 110 g). The column was washed with 2 liters of water and 2 liters of 0.02N hydrochloric acid, and eluted with 0.02N hydrochloric acid in 80% methanol. The eluate was neutralized with Amberlite IR 45 (OH form) and concentrated to dryness yielding 8.9 g of a yellowish powder (ID_{50}: 53 μg/ml, yield of activity: 72%). The yellowish powder (7.5 g) was dissolved in 50 ml of methanol and poured over a column of alumina (Woelm, acidic, 750 g), and the column was developed with methanol. The eluate which gave positive Rydon–Smith, Sakaguchi and red tetrazolium reactions, was concentrated to dryness yielding 2.3 g of leupeptins hydrochlorides (ID_{50}: 12 μg/ml, total yield of activity: 24%).

2) Resin Process: *Streptomyces roseus* MA839-Al was cultured in a 2000 liters fermentor (dispense volume: 1500 liters). The medium contained 2% glucose, 1% starch, 2% peptone, 0.5% NaCl and 0.2% KH_2PO_4, and sterilized at 110° for 30 min. Vegetative inoculum, 0.13% by volume, grown for 48 hr in the same medium was used. The temperature was maintained at 27°, the air-flow rate was held constant at 1500 liters per minutes and the agitation was operated at 200 rpm. The culture broth (1500 liters, pH 7.15, ID_{50}: 0.015 ml/ml) was harvested after 53.5 hr and filtered using 45 kg of Dicalite as a filter acid. The filtrate was passed through a column of Lewatit CNP resin (Farbenfabriken Bayer A.G., pH 6.0 with sodium hydroxide, 180 liters). The column was washed with water and eluted with 300 liters of 1N hydrochloric acid in 80% methanol and 200 liters of 0.2N hydrochloric acid in 80% methanol. The active eluate (565 liters) was adjusted to pH 5.2 with 6N sodium hydroxide and concentrated to 90 liters. The concentrate was extracted with butanol (80 liters) and the extract was concentrated to dryness yielding 979.1 g of a brownish powder (ID_{50}: 13 μg/ml, yield of activity: 75%).

The brownish powder (164 mg) in 2 ml of water was decolorized and purified by a resin chromatography of Dowex 1×2 (100—200 mesh, Cl form, 10 ml) developing with water. The eluate, which gave positive Rydon-Smith and Sakaguchi reactions, was concentrated to dryness yielding 126 mg of a white powder of leupeptins hydrochlorides (ID_{50}: 11 μg/ml, yield of activity: 91%).

Separation of Leupeptins Pr and Ac Di-*n*-butyl Acetals——The leupeptins hydrochlorides (19.2 g, ID_{50}: 15 μg/ml) was refluxed in 270 ml of butanol for 2 hr. The butanol solution was washed with 270 ml of water and concentrated to dryness yielding 23.4 g of a crude powder. The crude powder (14.0 g) was dissolved in 20 ml of butanol–butyl acetate–acetic acid–water (4:8:1:1) and subjected to a column chromatography of silicic acid (Mallinckrodt, 900 g) developing with the same solvent. The first eluate (150 ml), which gave Rydon–Smith and Sakaguchi reactions, was concentrated to dryness yielding 1.5 g of leupeptin Pr di-*n*-butyl acetal hydrochloride as a white powder, mp 70—90°. *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, 55.73; H, 9.68; N, 13.56; Cl, 5.86. The second eluate (500 ml) was concentrated to dryness yielding 5.3 g of a mixture containing leupeptins Pr and Ac di-*n*-butyl acetals (hydrochloride). The third eluate (1400 ml) was concentrated to dryness yielding 3.4 g of leupeptin Ac di-*n*-butyl acetal hydrochloride as a white powder, mp 70—90°. *Anal.* Calcd. for $C_{28}H_{56}O_5N_6 \cdot HCl \cdot \frac{1}{2}H_2O$: C, 55.84; H, 9.71; N, 13.96; Cl, 5.89. Found: C, 56.03; H, 9.67; N, 13.68; Cl, 6.16.

Leupeptin Pr Hydrochloride——Leupeptin Pr di-*n*-butyl acetal hydrochloride (93 mg) was dissolved in 5 ml of 0.01N hydrochloric acid and heated at 60° for 3 hr. The solution was neutralized to pH 6.0 with Amberlite IR 45 (OH form) and concentrated to dryness yielding 72 mg of leupeptin Pr hydrochloride as a white powder, mp 110—140°. *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, 51.13; H, 8.77; N, 17.10; Cl, 6.80.

Leupeptin Ac Hydrochloride——Leupeptin Ac di-*n*-butyl acetal hydrochloride (1.0 g) was dissolved in 50 ml of 0.01N hydrochloric acid and treated by the same procedure described above. Then, a white powder of leupeptin Ac hydrochloride (800 mg) was obtained. mp 75—110°. *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, 50.34; H, 8.57; N, 16.76; Cl, 6.99.

Leupeptin Pr Di-*n*-butyl Acetal Picrate——To a solution of leupeptin Pr di-*n*-butyl acetal hydrochloride (48 mg) in 2 ml of water, 31 mg of sodium picrate in 1 ml of water was added at 40—50°. After the mixture had been kept overnight at room temperature, the oily syrup was separated by decantation and dried in reduced pressure. The residue was washed with 2 ml of cold water and a yellow crystalline picrate of leupeptin Pr di-*n*-butyl acetal (50 mg) was obtained. The picrate was recrystallized with hot water. mp

60—90°. *Anal.* Calcd. for $C_{29}H_{58}O_5N_6 \cdot C_6H_3O_7N_3$: C, 52.55; H, 7.69; N, 15.76. Found: C, 52.32; H, 7.80; N, 15.43.

Leupeptin Ac Di-*n*-butyl Acetal Picrate——To a solution of leupeptin Ac di-*n*-butyl acetal hydrochloride (100 mg) in 2 ml of water, 88 mg of sodium picrate in 1 ml of water was added at 40—50°. A yellow crystalline picrate of leupeptin Ac di-*n*-butyl acetal (109 mg) was obtained by the same procedure as described in the previous section. mp 60—100°. *Anal.* Calcd. for $C_{28}H_{56}O_5N_6 \cdot C_6H_3O_7N_3$: C, 51.96; H, 7.57; N, 16.04. Found: C, 51.78; H, 7.51; N, 15.67.

Dihydroleupeptins Pr Hydrochloride and Ac Hydrochloride——To the solution of leupeptins hydrochlorides (10.1 g) in 150 ml of water on an ice water bath under stirring, 287 mg of $NaBH_4$ in 5 ml of cold water was added dropwise for 10 min. After stirring for 1 hr, the reaction mixture was adjusted to pH 6.0 with 0.5N hydrochloric acid and concentrated to dryness yielding 10.6 g of a white powder. The powder (7.9 g) was dissolved in 20 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, 500 g) and the column was developed with the same solvent. The first eluate (240 ml), which gave positive Rydon–Smith and Sakaguchi reactions, was concentrated to dryness yielding 1.1 g of dihydroleupeptin Pr hydrochloride as a white powder, mp 85—120°. *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, 51.21; H, 8.90; N, 16.05; Cl, 7.26. The second eluate (240 ml) was concentrated to dryness yielding 1.1 g of a mixture containing dihydroleupeptins Pr and Ac hydrochlorides. The third eluate (1100 ml) was concentrated to dryness yielding 2.0 g of dihydroleupeptin Ac hydrochloride as a white powder, mp 85—120°. *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, 50.27; H, 8.89; N, 16.58; Cl, 6.74.

Leupeptins Pr Acid and Ac Acid——To a solution of leupeptins hydrochlorides (2.5 g) in 50 ml of water, 650 mg of potassium permanganate in 40 ml of water was added dropwise under stirring at room temperature for 1 hr. After the mixture had been stirred for 1 hr, the precipitate appeared was removed by filtration. The filtrate was concentrated to dryness (3.0 g) and the residue was extracted with 20 ml of methanol. The methanol extract was concentrated to dryness yielding 2.4 g of a white powder. The powder was dissolved in 10 ml of water and subjected to a column chromatography of Dowex 1×2 (100—200 mesh, OH form, 200 ml) developing with water. The eluate (220 ml), which gave positive Rydon–Smith and Sakaguchi reactions, was concentrated to dryness yielding 1.2 g of leupeptin acids as a mixture. The leupeptin acids in 10 ml of butanol–butyl acetate–acetic acid–water (6:6:1:1) was subjected to a column chromatography of silicic acid (Mallinckrodt, 150 g) and the column was developed with the same solvent. The first eluate (300 ml) was concentrated to dryness yielding 247 mg of leupeptin Pr acid as a white powder, which was crystallized from a mixture of methanol and ether. mp 256—259° decomp. *Anal.* Calcd. for $C_{21}H_{40}O_5N_6$: C, 55.24; H, 8.83; N, 18.41. Found: C, 54.65; H, 8.81; N, 17.68. The second eluate (400 ml) from the column was concentrated to dryness yielding 214 mg of leupeptin acids as a mixture. The third eluate (600 ml) was concentrated to dryness yielding 535 mg of leupeptin Ac acid as a white powder, which was crystallized from a mixture of methanol and ether. mp 260—265° (decomp.). *Anal.* Calcd. for $C_{20}H_{38}O_5N_6$: C, 54.28; H, 8.66; N, 18.99. Found: C, 54.76; H, 9.14; N, 18.24.

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