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An Alternate Synthesis of Leupeptins and Their Analogs

HIROMICHI SAEKI, YOSHIKAZU SHIMADA, NORIO KAWAKITA, BUNJI SHIMIZU, EIJI OHKI,[1a] KENJI MAEDA, and HAMAO UMEZAWA[1b]

Central Research Laboratories, Sankyo Co., Ltd.[1a] *and Institute of Microbial Chemistry*[1b]

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It was found that lithium aluminum hydride reduction of N^{α},N^{G}-disubstituted arginine lactam (**3a**—**c**) at a low temperature gave a corresponding argininal derivative (**4a**—**c**). Analogous reduction of acetyl-L-leucyl-L-leucyl-N-benzyloxycarbonyl-L-arginine lactam (**8b**) or its dipeptide analog (**13**) yielded leupeptin Ac-LL (**1a**) or acetylleucylargininal (**11**) respectively. Antiplasmin activities of these synthetic leupeptins were also reported.

Leupeptins produced by various strains of *Actinomycetes* show strong inhibition of proteases such as plasmin, trypsin and papain and, in addition, show anti-inflammatory effects.[2] The structure of the two major components, leupeptin Ac-LL (acetyl-L-leucyl-L-leucyl-argininal, **1a**) and leupeptin Pr-LL (propionyl-L-leucyl-L-leucyl-argininal, **1b**) has been elucidated[3] and their chemical syntheses have already been announced.[3,4] Further, because of their interesting biological activities, an improved synthesis was desirable and we report herein an additional study on the synthesis of leupeptin Ac-LL (**1a**) and its dipeptide analog.

It has been found that activation of the carboxyl group of N^{α},N^{G}-disubstituted arginine (**2**) sometimes results in formation of a δ-lactam, 1-guanyl-3-aminopiperidone-2 (**3**).[5,6] The resulting lactam (**3**) can be regarded as a tertiary amide amenable to conversion into an aldehyde by treatment with lithium aluminum hydride.[7] Consequently, it was presumed that the δ-lactam (**3**) would be converted into an argininal derivative by metal hydride reduction and this conversion would find an application in a synthesis of leupeptins and their analogs. First, reduction of N^{α}-benzyloxycarbonyl (Cbz)-N^{G}-nitro-L-arginine lactam[5] (**3a**), which was prepared from N^{α}-Cbz-N^{G}-nitro-L-arginine (**2a**) by the mixed anhydride method, was attempted; treatment of **3a** with lithium aluminum hydride in tetrahydrofuran at a low temperature (−15——20°) yielded an aldehyde (**4a**) as crystals, which formed a crystalline semicarbazone. The resulting aldehyde (**4a**) and its semicarbazone were identical with the samples reported earlier.[4] Reduction of N^{α},N^{G}-di-Cbz-L-arginine lactam[6] which was analogously prepared from the corresponding arginine derivative (**2b**), with lithium aluminum hydride similarly proceeded to give a crystalline aldehyde (**4b**) whose structure was also confirmed by its elementary analysis and 2,4-dinitrophenylhydrazine test. The infrared (IR) and nuclear

1) Location: *a) Hiromachi 1-2-58, Shinagawa-ku, Tokyo; b) Kamiosaki 3-14-23, 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); T. Aoyagi, S. Miyata, M. Nanbu, E. Kojima, M. Matsuzaki, M. Ishizuka, T. Takeuchi, and H. Umezawa, *ibid.*, **22**, 558 (1969).
3) K. Kawamura, S. Kondo, K. Maeda, and H. Umezawa, *Chem. Pharm. Bull.* (Tokyo), **17**, 1902 (1969).
4) B. Shimizu, A. Saito, A. Ito, K. Tokawa, K. Maeda, and H. Umezawa, *J. Antibiotics* (Tokyo), **25**, 515 (1972).
5) M. Bodanszky and J.T. Sheehan, *Chem. Ind.* (London), **1960**, 1268.
6) L. Zervas, T.T. Otani, M. Winitz, and J.P. Greenstein, *J. Am. Chem. Soc.*, **81**, 2878 (1959); L. Zervas, M. Winitz, and J.P. Greestein, *ibid.*, **83**, 3300 (1961).
7) G. Wittig and P. Hornberger, *Ann. Chem.*, **577**, 11 (1952); F. Weygand and R. Mitgau, *Chem. Ber.*, **88**, 301 (1955); W. Reid and F.J. Königstein, *Ann. Chem.*, **622**, 37 (1959); H.C. Brown and A. Tsukamoto, *J. Am. Chem. Soc.*, **83**, 2016, 4549 (1961); H.A. Staab and H. Bräunling, *Ann. Chem.*, **654**, 118 (1962); H. Kuzuhara and H.G. Fletcher Jr., *J. Org. Chem.*, **32**, 2531 (1967).

magnetic resonance (NMR) spectra of these aldehydes (**4a** and **4b**) did not show the presence of a free aldehyde function, suggesting cyclization into a piperidine ring as shown in Chart 1.[8]

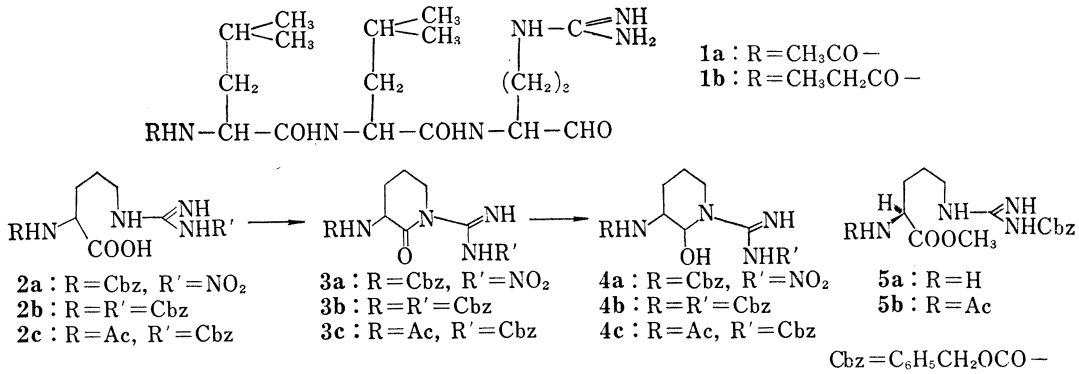

Chart 1

Next, N^{α}-acylated argininals including leupeptin Ac-LL (**1a**) were prepared by means of a method similar to that described above. As a starting material, N^{G}-Cbz-L-arginine methyl ester (**5a**) prepared by a modification of Zervas' method[10] was selected for the following reason. As described in a preceding paper,[3] purification of leupeptins at a final stage presents formidable difficulties because they hardly crystallize and their chromatographic purification is wasteful and limited. Therefore, the synthesis of some N^{G}-protected argininal derivative, which is easily purified and readily generates a leupeptin under mild conditions, was desirable. Thus, it was presumed that a protection of guanidino group with benzyloxycarbonyl group would be most useful for this purpose, because a benzyloxycarbonyl group is removable by hydrogenation on palladium charcoal in dilute hydrochloric acid without any damage to the formed aldehyde function.

In order to examine whether acylamino groups of the δ-lactams could survive during treatment with lithium aluminum hydride, we carried out the reduction of N^{α}-acetyl-N^{G}-Cbz-arginine δ-lactam (**3c**) as a model experiment. Acetylation of the arginine ester (**5a**) with excess acetic anhydride gave a mixture of N^{α}-acetate (**5b**) and N^{α},N^{G}-diacetate, while a controlled acetylation of **5a** with acetic anhydride in methanol gave the N^{α}-acetate (**5b**) exclusively. The acetate (**5b**) was saponified into an acid (**2c**) which was successively cyclized with ethyl chloroformate and triethylamine, giving a crystalline lactam (**3c**). Treatment of **3c** with two molar equivalents of lithium aluminum hydride in tetrahydrofuran at a low temperature afforded a crystalline aldehyde (**4c**). The lactam (**3c**) and aldehyde (**4c**) did not show optical activity and this fact suggested that a racemization had occured at the stage of formation of **3c**.

N^{G}-Cbz-L-arginine methyl ester (**5a**) and *t*-butoxycarbonyl-L-leucine[11] (BOC-L-leucine) were coupled by the mixed anhydride method in the usual manner to give a dipeptide (**6a**) whose hydrochloride was obtained as crystals. The BOC-dipeptide (**6a**) thus obtained was saponified with sodium hydroxide in methanol and the resulting acid (**6b**) was cyclized into a lactam (**7a**) by the mixed anhydride method as described above. Determination of optical purity of the arginine moiety in the lactam (**7a**) thereby obtained was carried out in the follow-

8) These structures were already discussed in previous papers.[4,9]
9) K. Maeda, K. Kawamura, S. Kondo, T. Aoyagi, T. Takeuchi, and H. Umezawa, *J. Antibiotics* (Tokyo), **24**, 402 (1971).
10) L. Zervas, M. Winitz, and J.P. Greenstein, *J. Org. Chem.*, **22**, 1515 (1957).
11) R. Schwyzer, P. Sieber, and H. Kappeler, *Helv. Chim. Acta*, **42**, 2622 (1959).

ing way: The lactam (**7a**) was hydrolyzed with aqueous sodium hydroxide and the resulting acid was hydrogenated over palladium charcoal in diluted hydrochloric acid, giving BOC-leucylarginine hydrochloride which was successively hydrolyzed in boiling 6N hydrochloric acid into a mixture of leucine and arginine. The total quantity of arginine was determined by Sakaguchi's method[12] and that of L-arginine was determined by a bioassay using *Streptococcus faecalis* R, ATCC 8043,[13] according to the method of Henderson and Snell,[14] or by an enzymatic method[15] using L-arginine-decarboxylase.[16] The result thus obtained indicated that the arginine was 100% optically pure, in other words, "L", and consequently it was established that the lactam (**7a**) is BOC-leucyl-N^G-Cbz-L-arginine δ-lactam.

On removal of the BOC group from the lactam (**7a**) in trifluoroacetic acid, an amine (**7b**) was formed as crystals. Coupling of **7b** with BOC-L-leucine by the mixed anhydride method afforded a tripeptide (**8a**). Removal of the BOC-group from **8a** and successive acetylation gave a lactam (**8b**) which would be a precursor of leupeptin Ac-LL (**1a**). Further, the following represents another preparation of the lactam (**8b**) which was carried out for the purpose of determining the optical purity of leucine in **8b**. Acetyl-L-leucyl-L-leucine ethyl ester[3] was converted into an azide in an usual manner and coupled with N^G-Cbz-L-arginine methyl ester (**5a**), giving a tripeptide (**9**). Saponification of **9** with potassium hydroxide and the analogous conversion of the resulting acid into a lactam gave **8b** identical with the sample obtained as above. On the basis of these facts, the formed lactam (**8b**) can be designated as acetyl-L-leucyl-L-leucyl-N^G-Cbz-L-arginine δ-lactam.

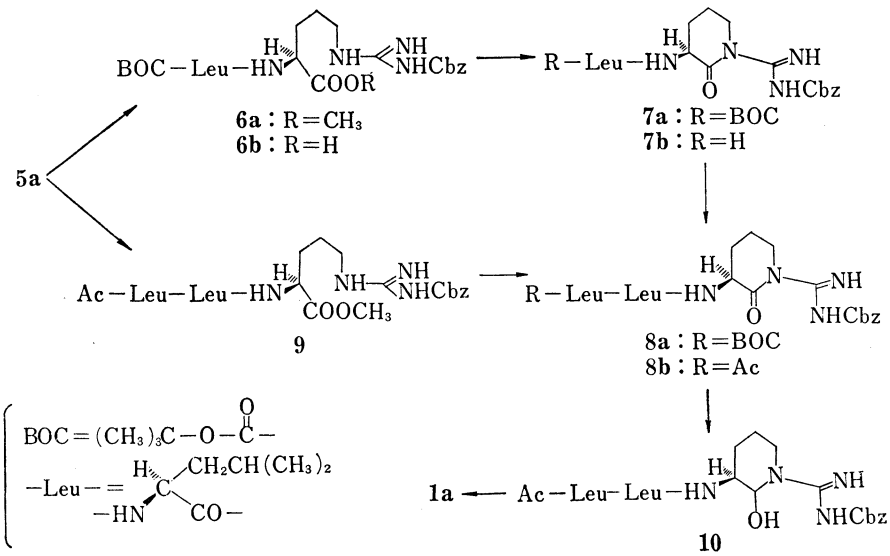

Chart 2

Reduction of the lactam (**8b**) with about 5 molar equivalents of lithium aluminum hydride in tetrahydrofuran afforded a crystalline aldehyde (**10**). The aldehyde (**10**) was suspended in dilute hydrochloric acid and hydrogenated on palladium charcoal. The reaction mixture was adjusted to pH 6 with Amberlite IR-45 (OH^-) and freeze-dried, giving a colorless powder

12) S. Sakaguchi, *J. Biochem.* (Tokyo), **5**, 25 (1925); C.J. Weber, *J. Biol. Chem.*, **86**, 217 (1930).
13) Arginine Assay Medium-Bacto, Difco Labs., Detroit, Michigan, U.S.A. was used as a medium.
14) L.M. Henderson and E.E. Snell, *J. Biol. Chem.*, **172**, 15 (1948).
15) E.F. Gale, in "Methods of Enzymatic Analysis," ed. by H-U. Bergmeyer, Academic Press, 1963, p. 373.
16) Procurable from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

of leupeptin Ac-LL (**1a**) hydrochloride. The sample thus obtained was almost pure on thin-layer chromatograms except for contamination by a trace amount of a more polar peptide.

It was presumed that the argininal moiety of leupeptin Ac-LL (**1a**) thus obtained might be liable to racemization because of a *keto-enol* equilibrium of the aldehyde function; therefore, the optical purity of the argininal part was studied as follows. Oxidation of **1a** thereby synthesized with potassium permanganate in water and successive hydrolysis with hydrochloric acid gave a mixture of leucine and arginine. The total quantity of arginine and that of L-arginine were determined by the methods described above and the results showed that the L-arginine content in the acid derived from **1a** was 82—86%. Therefore, the synthetic leupeptin Ac-LL was considered to contain for the most part acetyl-L-leucyl-L-leucyl-L-argininal.

The synthetic leupeptin Ac-LL (**1a**) thus obtained had $[\alpha]_D^{22}$ $-68\pm1°$ (MeOH)[17] and also showed a strong antiplasmin activity whose 50% inhibition concentration (ID_{50}) was 6 μg/ml.[18]

Synthesis of several leupeptin analogs and their biological activities has been reported in preceding paper.[4,9] We also attempted a preparation of a dipeptide analog, acetyl-L-leucyl-L-argininal (**11**) in the following way. Exchange of the BOC group in BOC-L-leucyl-N^G-Cbz-L-arginine methyl ester (**6a**) with acetyl group in an usual manner or coupling of N^G-Cbz-L-arginine methyl ester (**5a**) with acetyl-L-leucylazide[19] yielded a crystalline acetyl derivative (**12**). However, **12** unusually resisted hydrolysis under ordinary conditions and drastic treatment of **12** with alkali resulted in a formation of complex products. Accordingly, acetylation of the leucylarginine δ-lactam (**7b**) was carried out and gave an acetate (**13**). Analogous treatment of **13** with lithium aluminum hydride afforded an aldehyde (**14**) ,which gave acetyl-L-leucyl-L-argininal (**11**) as a colorless hydrochloride on removal of the protecting group. The ID_{50} of antiplasmin activity in **11** was 18 μg/ml.[18]

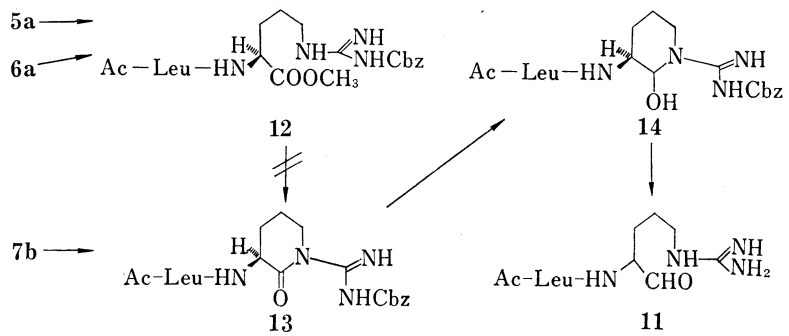

Chart 3

Experimental

Melting points are not corrected. Infrared spectra were recorded on a Hitachi EPI-S2 spectrometer, nuclear magnetic resonance spectra on a Varian A-60 spectrometer, and optical rotations on a Perkin-Elmer 141 automatic polarimeter in 1 dm tubes, respectively. Thin-layer chromatography (TLC) was performed on TLC-plates Silica Gel F_{254} pre-coated (E. Merck AG, layer thickness, 0.25 mm) and detection of spots was carried out by UV-irradiation or spraying Rydon-Smith reagent.[20] Solvents were removed by a rotating flash evaporator at diminished pressure and usually at 35—50°.

N^α-Benzyloxycarbonyl-N^G-nitro-L-arginine Lactam (3a)——To a solution of N^α-benzyloxycarbonyl-N^G-nitro-L-arginine (**2a**) (15 g) and triethylamine (7.05 ml) in 150 ml of CH_2Cl_2 was added ethyl chlorofor-

17) Natural leupeptin Ac-LL has $[\alpha]_D^{22}$ $-52°$[3] or $-42°$[9] as reported.
18) These determinations were carried out by Dr. T. Aoyagi, Institute of Microbial Chemistry.
19) N.A. Smart, G.T. Young, and M.W. Williams, *J. Chem. Soc.*, **1960**, 3902.
20) H.N. Rydon and P.W.G. Smith, *Nature*, **169**, 922 (1952).

mate (4.26 ml) with cooling at 0° and stirring. After 10 min stirring, triethylamine (7.05 ml) was further added to the mixture and stirring was continued for 30 min at 0° and another 30 min at room temperature. The mixture was washed with aqueous $NaHCO_3$ and H_2O, dried over anhyd. $MgSO_4$ and evaporated, leaving 11.5 g of a crystalline mass, mp 145—147°, which was recrystallized from EtOH to give 9.1 g of **3a**, mp 146—147.5°, $[\alpha]_D^{22}$ *ca.* 0° (*c*=5, DMF) (reported,[5] mp 145—147.5°, $[\alpha]_D^{20}$ −4° (*c*=2, DMF)).

N^{α}-Benzyloxycarbonyl-N^{G}-nitro-L-argininal (4a)——A solution of **3a** (3.25 g) in 50 ml of anhyd. tetrahydrofuran (THF) was cooled at −15——20° and $LiAlH_4$ (380 mg) was added to the solution with stirring. After 15 min, a few drops of H_2O were added to decompose the excess reagent. After filtration, the mixture was concentrated *in vacuo* to dryness. The residue was dissolved in 100 ml of $CHCl_3$ and the solution was washed with H_2O and dried over anhyd. $MgSO_4$, then evaporated to leave crude **4a** (2.31 g) as a sirup. A solution of this sirup (2 g) in $CHCl_3$ was charged on 40 g of alumina (E. Merck, Grade II—III) and the column was eluted with MeOH–$CHCl_3$ (1: 50, v/v). The glassy sirup obtained after evaporation of the solvent was triturated with AcOEt–hexane, giving a powder. The powder thus obtained was covered with AcOEt and by rubbing the vessel wall with a spatula and standing formed crystals of **4a**, mp 119—123°, $[\alpha]_D^{25}$ −1° (*c*=3.1, MeOH) (reported,[4] mp 122—124°, $[\alpha]_D^{22}$ −1.6°)). *Anal.* Calcd. for $C_{14}H_{19}O_5N_5$: C, 49.84; H, 5.68; N, 20.76. Found: C, 49.83; H, 6.00; N, 20.68.

To a solution of the crude sirup of **4a** (307 mg) in 1 ml of EtOH, H_2O was added to a slight turbidity. After addition of semicarbazide hydrochloride (111 mg) and sodium acetate (160 mg), the mixture was allowed to stand for 10 min at room temperature and diluted with H_2O to separate an oil. The solvent was removed by decantation and the residual oil was dissolved in EtOH and filtered. To the filtrate was added H_2O gradually to turbidity. The mixture was allowed to stand overnight and gave a crystalline semicarbazone (103 mg), mp 105—108°, which was identified with the authentic sample[4] by mixed melting point, thin-layer chromatography and infrared spectrometry.

N^{α},N^{G}-Dibenzyloxycarbonyl-L-argininal (4b)——To a stirred solution of N^{α},N^{G}-dibenzyloxycarbonyl-L-arginine lactam[6] (**3b**) (720 mg) in 10 ml of THF was added $LiAlH_4$ (70 mg) with cooling at −10——15°. After being stirred for 1 hr with cooling, the mixture was diluted with 20 ml of $CHCl_3$ and the combind filtrate and washings was washed with saturated aqueous NaCl, dried over $MgSO_4$, and evaporated, leaving 640 mg of a crystalline mass, whose recrystallization from EtOH gave **4b** as a crystalline powder, mp 128—131°, $[\alpha]_D^{21}$ +5.3° (*c*=2, $CHCl_3$). IR ν_{max}^{Nujol} cm^{-1}: 3450, 3300 (NH, OH), 1690, 1660, 1600, 1540. *Anal.* Calcd. for $C_{22}H_{26}O_5N_4$: C, 61.96; H, 6.15; N, 13.14. Found: C, 61.71; H, 6.33; N, 13.19.

N^{G}-Benzyloxycarbonyl-L-arginine Methyl Ester (5a)——The dihydrochloride of **5a** obtained according to the method of Zervas, *et al.*[10] was dissolved in H_2O and the solution was basified with K_2CO_3 (solid) and extracted with $CHCl_3$. The extract was dried over anhyd. $MgSO_4$ and evaporated *in vacuo* to leave a crystalline mass whose recrystallization from AcOEt gave **5a** as needles, mp 116—117°. *Anal.* Calcd. for $C_{15}H_{22}O_2N_4$: C, 55.88; H, 6.88; N, 17.38. Found: C, 56.24; H, 6.46; N, 17.78.

N^{α}-Acetyl-N^{G}-benzyloxycarbonyl-L-arginine Methyl Ester (5b)——A mixture of **5a** (644 mg), MeOH (4 ml), and Ac_2O (0.4 ml) was allowed to stand for 8 min at room temperature, diluted with 50 ml of $CHCl_3$, and neutralized with cold aqueous $NaHCO_3$. The $CHCl_3$ layer was collected, dried and evaporated *in vacuo* to a thick oil (*ca.* 900 mg), which revealed two spots on thin-layer chromatogram. The oil was crystallized by trituration with AcOEt and gave 624 mg of **5b** as a crystalline powder, mp 150—153°, $[\alpha]_D^{23}$ −1° (*c*= 1.8, $CHCl_3$). *Anal.* Calcd. for $C_{17}H_{24}O_5N_4$: C, 56.03; H, 6.64; N, 15.38. Found: C, 56.37; H, 6.61; N, 15.51.

The mother liquor left by collection of **5b** was evaporated and the residue was chromatographed on 5 g of silica gel (Wakogel Q-22). Elution with MeOH–AcOEt (1: 35, v/v) and removal of the solvent gave 125 mg of crystals which was recrystallized from AcOEt–petroleum ether, giving N^{α},N^{G}-diacetyl-N^{G}-benzyloxycarbonyl-L-arginine methyl ester as crystals of mp 109—110°. NMR ($CDCl_3$) δ ppm: 1.97 (3H, singlet, acetyl), 2.19 (3H, singlet, acetyl). *Anal.* Calcd. for $C_{19}H_{26}O_6N_4$: C, 56.14; H, 6.45; N, 13.79. Found: C, 56.31; H, 6.31; N, 13.78.

The column was then eluted with MeOH–AcOEt (7: 93, v/v) and removal of the solvent gave 107 mg of a second crop of **5b**, mp 150—153°.

N^{α}-Acetyl-N^{G}-benzyloxycarbonyl-L-arginine (2c)——To an ice-cold solution of **5a** (0.61 g) in 5 ml of MeOH, a cooled solution of NaOH (0.15 g) in 4 ml of H_2O was added and the resulting mixture was allowed to stand for 2 hr at room temperature. After neutralization, the mixture was evaporated to dryness below 40° (bath temp.). The residue was triturated with EtOH and H_2O and was cooled in a refrigerator overnight, giving 303 mg of crude **2c**. The mother liquor was concentrated at a low temperature to yield 152 mg of a further crop of **2c**. The combined crystals were recrystallized from a large amount of MeOH gave pure **2c** as a crystalline powder, mp 185—189° (with bubbling). IR ν_{max}^{Nujol} cm^{-1}: 2520, 1735. *Anal.* Calcd. for $C_{16}H_{22}O_5N_4$: C, 54.84; H, 6.33; N, 15.99. Found: C, 54.51; H, 6.38; N, 15.80.

N^{α}-Acetyl-N^{G}-benzyloxycarbonyl-DL-arginine Lactam (3c)——To an ice-cold suspension of **2c** (625 mg) in 24 ml of THF containing triethylamine (0.27 ml) was added ethyl chloroformate (0.30 ml) with stirring and the mixture was stirred for 30 min. After addition of triethylamine (0.27 ml), stirring was continued for another 20 min at 0° and the reaction mixture was diluted with a mixture of 50 ml of $CHCl_3$ and 5 ml of H_2O, shaken and filtered. The solid was washed with sat. aqueous NaCl and the organic layer was collected from the combined filtrate and washings. The aqueous layer was extracted with $CHCl_3$ and the

combined organic layer and extracts were dried over $MgSO_4$ and evaporated *in vacuo*, leaving a crystalline mass which was recrystallized from EtOH to give 343 mg of **3c** as needles or platelets, mp 159—160°, $[\alpha]_D^{20}$ 0° (c=2.6, $CHCl_3$). *Anal.* Calcd. for $C_{16}H_{20}O_4N_4$: C, 57.82; H, 6.07; N, 16.86. Found: C, 57.77; H, 6.01; N, 16.76.

N$^{\alpha}$-Acetyl-N^{G}-benzyloxycarbonyl-DL-argininal (4c)——To a solution of **3c** (332 mg) in 20 ml of THF, $LiAlH_4$ (76 mg) was added in one portion with cooling at −15——20° and with vigorous stirring. The mixture was kept at this temperature with stirring for 40 min, then diluted with 50 ml of $CHCl_3$ and 15 ml of H_2O successively and filtered. The filtrate was washed with sat. aqueous NaCl and washings were extracted with $CHCl_3$. The combined $CHCl_3$ solution was dried and evaporated to a powder (266 mg) which crystallized on trituration with AcOEt. The resulting crystals (206 mg) which contained AcOEt as a component of the crystals were dissolved in $CHCl_3$ and the solution was diluted with hexane to a slight turbidity. On rubbing the vessel wall with a spatula, crystals of **4c** appeared. They melted at 107—110°. NMR (DMSO-d_6): 1.83 ppm (3H, singlet, acetyl). *Anal.* Calcd. for $C_{16}H_{22}O_4N_4 \cdot 1/2CHCl_3$: C, 50.29; H, 5.73; N, 14.22. Found: C, 50.25; H, 5.92; N, 14.02.

***t*-Butoxycarbonyl-L-leucyl-N^{G}-benzyloxycarbonyl-L-arginine Methyl Ester (6a)**——To a cooled solution of BOC-L-leucine monohydrate[11] (5 g) and triethylamine (3.0 ml) in 90 ml of THF, ethyl chloroformate (4.5 ml) was added at −15——20° with stirring. After 10 min stirring, a solution of N^{G}-benzyloxycarbonyl-L-arginine methyl ester[10] (**5a**) (6 g) in 50 ml of CH_2Cl_2 was addded dropwise to the mixture and the mixture was stirred for 30 min at −15°, diluted with sat. aqueous NaCl and then extracted with $CHCl_3$. The extract was washed with aqueous $NaHCO_3$ and H_2O, dried and evaporated to give a crude hydrochloride of **6a** (11.7 g) as a powder.

The crude hydrochloride of **6a** was dissolved in a small amount of AcOEt, and ether was added gradually to the solution to turbidity. Precipitates obtained by standing several days were collected and triturated with AcOEt to give a colorless powder of hydrochloride of **6a**, mp 130—138°, $[\alpha]_D^{20}$ −26.7° (c=1.2, MeOH). *Anal.* Calcd. for $C_{26}H_{41}O_7N_5 \cdot HCl$: C, 54.59; H, 7.40; N, 12.24. Found: C, 54.60; H, 7.49; N, 11.90.

***t*-Butoxycarbonyl-L-leucyl-N^{G}-benzyloxycarbonyl-L-arginine Lactam (7a)**——To a solution of the crude hydrochloride of **6a** (11.7 g) in 80 ml of MeOH was added a solution of NaOH (1 g) in 15 ml of H_2O with cooling (ice bath). The mixture was set aside for 1 hr at room temperature, diluted with ice water, acidified with 2N HCl to pH 4—5, and extracted with $CHCl_3$. The extract was washed with H_2O, dried and evaporated to give 11.7 g of **6b** as a glassy powder. The powder of **6b** thus obtained and triethylamine (4.0 ml) was dissolved in 80 ml of CH_2Cl_2 and cooled at −5——10°. To the solution, ethyl chloroformate (2.2 ml) was added with stirring, and the resulting mixture was kept at −5——10° for 15 min, diluted with cold sat. aqueous NaCl and 100 ml of $CHCl_3$. After vigorous shaking, the organic layer was separated, dried and filtered with activated carbon powder. The filtrate was evaporated to an oil which crystallized on trituration with hexane and following standing overnight at room temperature, giving 7.2 g of curde of **7a**. Recrystallization from PrOH–hexane afforded needles of mp 170—171°, $[\alpha]_D^{20}$ −46.3° (c=1.0, $CHCl_3$). *Anal.* Calcd. for $C_{25}H_{37}O_6N_5$: C, 59.62; H, 7.41; N, 13.19. Found: C, 59.48; H, 7.11; N, 14.17.

Determination of L-Arginine Content in 7a——To an ice-cold solution of **7a** (504 mg) in acetone, NaOH (40 mg) dissolved in H_2O (5 ml) was added. After standing at room temperature for 1 hr, the mixture was concentrated to remove acetone and acidified with AcOH and then extracted with $CHCl_3$. The extract was washed with H_2O and dried and evaporated to dryness, leaving a powder (520 mg). The powder was dissolved in a mixture of dil. HCl (0.18N, 22 ml) and MeOH (15 ml) containing 10% Pd-C (0.25 g) and hydrogenated for 1 hr at room temperature. After filtration, the mixture was evaporated and the residue was refluxed in 20 ml of 6N HCl for 8 hr which were found by means of TLC to be enough for the peptide to be completely hydrolyzed. The hydrolyzate was decolorized and evaporated to leave 267 mg of powder. A solution of the powder (135 mg) in 50 ml of H_2O was analyzed as follows: Total arginine (by Sakaguchi's method), 1080 μg/ml; L-arginin (by bioassay), 1050 μg/ml; (by enzymatic analysis), 1010 μg/ml.

L-Leucyl-N^{G}-benzyloxycarbonyl-L-arginine Lactam (7b)——A solution of **7a** (5.0 g) in 25 ml of trifluoroacetic acid was kept at room temperature for 10 min and evaporated at low temperature (below 45°, bath temp.). The residual oil was disssolved in H_2O and $CHCl_3$. The two-layer solution was basified by adding Na_2CO_3 (solid) with shaking and the organic layer was separated. The aqueous layer was extracted with 50 ml of $CHCl_3$. The combined organic layer and extracts were washed with H_2O, dried and evaporated to an oil, which gave crystals of **7b** on trituration with hexane. For purification, the crystals were dissolved in $CHCl_3$ and hexane was added to a slight turbidity. Then the mixture was set aside to afford pure crystals of **7b** which began to decompose with browning at 196° and did not melt at 300°. *Anal.* Calcd. for $C_{20}H_{29}O_4N_5$: C, 59.53; H, 7.25; N, 17.36. Found: C, 59.22; H, 7.23; N, 17.05.

***t*-Butoxycarbonyl-L-leucyl-L-leucyl-N^{G}-benzyloxycarbonyl-L-arginine Lactam (8a)**——To a cooled and stirred solution of BOC-L-leucine hydrate[11] (2.5 g) and triethylamine (1.50 ml) in 45 ml of THF, ethyl chloroformate (2.25 ml) was added at −20° and the resulting mixture was stirred for 5 min with cooling. To the mixture, a solutuion of **7b** (4.0 g) in 100 ml of $CHCl_3$ was dropped in the course of 10 min with keeping the temperature at −20°. After having been stirred for 10 min, the reaction mixture was diluted with 80 ml of H_2O and extracted with 80 ml of $CHCl_3$. The extract was washed with aqueous $NaHCO_3$ and H_2O, dried and evaporated to give an oil (6.9 g). The oil was triturated with hexane containing a small amount

of PrOH to crystallize, and allowed to stand in a refrigerator. Resultant crystals (4.7 g) were collected and recrystallized from PrOH–hexane, to give **8a** as fine needles of mp 200—201°, $[\alpha]_D^{20}$ −57.0° (c=1.1, $CHCl_3$). *Anal.* Calcd. for $C_{31}H_{48}O_7N_6$: C, 60.37; H, 7.89; N, 13.63. Found: C, 60.00; H, 7.69; N, 13.77.

Acetyl-L-leucyl-L-leucyl-N^G-benzyloxycarbonyl-L-arginine Methyl Ester (9)——A solution of Ac-L-leucyl-L-leucine ethyl ester[3] (5 g) and $NH_2NH_2 \cdot H_2O$ (1.2 g) in 30 ml of MeOH was allowed to stand for 24 hr and then evaporated, leaving crystals which was recrystallized to give Ac-L-leucyl-L-leucylhydrazide (4.8 g) as needles of mp 210—213°, $[\alpha]_D^{24}$ −71.9° (c=1.2, MeOH). *Anal.* Calcd. for $C_{14}H_{29}O_3N_4$: C, 55.97; H, 9.40; N, 18.65. Found: C, 55.76; H, 9.17; N, 18.37.

To a solution of the hydrazide (3.4 g) in 50 ml of H_2O and 5 ml of conc. HCl, $NaNO_2$ (0.85 g) was added with cooling and stirring. Precipitated azide was extracted with 80 ml of ether. The extract was rapidly dried and concentrated to a volume of 20 ml. The concentrated solution was added to a solution of **5a** (3.25 g) in $CHCl_3$. The resultant solution was set aside for 3 days, diluted with $CHCl_3$, washed with dil. HCl, dil. $NaHCO_3$ and H_2O successively, dried and evaporated to give an oily substance (6 g). The oil was chromatographed on 120 g of silica gel (Wakogel Q-22) using MeOH–$CHCl_3$ (1: 25, v/v) as eluant to give **9** as a powder (4.2 g), $[\alpha]_D^{24}$ −37° (c=1.3, MeOH). *Anal.* Calcd. for $C_{29}H_{46}O_7N_6 \cdot H_2O$: C, 57.22; H, 7.95; N, 13.81. Found: C, 56.73; H, 7.90; N, 13.70.

Acetyl-L-leucyl-L-leucyl-N^G-benzyloxycarbonyl-L-arginine Lactam (8b)——i) A solution of 4.7 g of **8a** in 35 ml of trifluoroacetic acid was allowed to stand at room temperature for 15 min and then evaporated *in vacuo* below 40°. The residue was dissolved in 100 ml of $CHCl_3$ and cooled at 0°. The solution was shaken with sat. aqueous $NaHCO_3$ until its pH was adjusted to 8. Then, to the mixture was added 2.7 ml of Ac_2O with stirring and the mixture was stirred for 50 min, diluted with $CHCl_3$ and sat. aqueous $NaHCO_3$. The $CHCl_3$ layer was washed with dil. $NaHCO_3$ and H_2O, dried and evaporated to give crude crystals of **8b** (4.3 g). Recrystallization from AcOEt afforded needles of mp 200—203°, $[\alpha]_D^{20}$ −69.6° (c=1.0, $CHCl_3$). *Anal.* Calcd. for $C_{28}H_{42}O_6N_6$: C, 60.19; H, 7.58; N, 15.04. Found: C, 59.98; H, 7.62; N, 15.47.

ii) A solution of **9** (2.8 g) in 0.5N KOH in MeOH (30 ml) was kept for 24 hr at room temperature, then diluted with H_2O to dissolve the precipitates formed and washed with $CHCl_3$. The aqueous layer was acidified with dil. HCl and extracted with $CHCl_3$. The extract was washed with H_2O, dried and evaporated to afford 2 g of acetyl-L-leucyl-L-leucyl-N^G-benzyloxycarbonyl-L-arginine. Reprecipitation in AcOEt gave a solid of mp 131—151°.

To a cooled solution of the arginine derivative (576 mg) thus obtained and triethylamine (0.14 ml) in a mixture of 10 ml of CH_2Cl_2 and 4 ml of THF, ethyl chloroformate (0.22 ml) was added with stirring at −10—−20°. After 5 min, triethylamine (0.14 ml) was added and the mixture was stirred at −15° for another 25 min. Then, the mixture was diluted with sat. aqueous NaCl and extracted with 20 ml of $CHCl_3$. The extract was dried and evaporated to give a solid (527 mg). Recrystallization from AcOEt gave crude **8b**, mp 185—192°, and further recrystallization afforded crystals of mp 200—201° which were identified with the sample obtained as above by mixed melting point and infrared spectrometry.

Acetyl-L-leucyl-L-leucyl-N^G-benzyloxycarbonyl-L-argininal (10)——To a cooled solution of **8b** (1.12 g) in 80 ml of THF, $LiAlH_4$ (390 mg) was added at one portion with stirring at 0—10°. After having been stirred for 1 hr with cooling, the mixture was diluted with $CHCl_3$ and H_2O to decompose the excess reagent, and filtered. The filtrate was shaken with sat. aqueous NaCl and the organic layer was dried and evaporated to a crystalline mass (1.05 g). The product was dissolved in THF and the solution was diluted with hexane to precipitate a solid which was recrystallized from AcOEt, giving **10** as a powder, mp 141—145° with bubbling, $[\alpha]_D^{20}$ −44° (c=1.0, MeOH). IR ν_{max}^{Nujol} cm^{-1}: 3300 (NH, OH), 1640, 1610 (shoulder), 1550 (shoulder), 1535 (broad). *Anal.* Calcd. for $C_{23}H_{44}O_6N_6 \cdot 1/4H_2O$: C, 59.50; H, 7.94; N, 14.87. Found: C, 59.32; H, 8.06; N, 15.15.

Acetyl-L-leucyl-L-leucyl-L-argininal (Leupeptin Ac-LL, 1a)——Hydrogen was slowly bubbled through a stirred mixture of **10** (1.00 g), 10% Pd-C (0.4 g) and 2N HCl (2.5 ml) in 40 ml of H_2O for 20 min at room temperature. The mixture was filtered, the catalyst was washed with H_2O and the filtrate and washings were adjusted to pH 6 with Amberlite IR-45 (OH^-) and filtered. The filtrate was freeze-dried to give a hydrochloride of **1a** (695 mg) as a colorless powder which was almost pure on TLC (BuOH:AcOBu:AcOH:H_2O= 4: 2:1:1), mp 136—145° (bubbling) with preliminary softening at 92—100°, $[\alpha]_D^{22}$ −69.0° (c=3.1, MeOH) (another lot, $[\alpha]_D^{24}$ −67.8° (c=1.1, MeOH)). *Anal.* Calcd. for $C_{20}H_{38}O_4N_6 \cdot HCl$: C, 49.93; H, 8.59; N, 17.47; Cl, 7.37. Found: C, 49.85; H, 8.88; N, 17.52; Cl, 7.69.

Determination of L-Argininal Content in 1a——To a solution of **1a** (510 mg) in 10 ml of H_2O was added dropwise a solution of $KMnO_4$ (133 mg) in 12 ml of H_2O with stirring at room trmperature. After being stirred for 1 hr, the mixture was filtered through activated carbon bed, and the filtrate was evaporated to dryness. A solution of the residual powder in H_2O (100 mg/25 ml) was analyzed as follows: Total arginine (by Sakaguchi's method), 760 μg/ml; L-Arginine (by bioassay), 650 μg/ml; (by enzymatic analysis), 610 μg/ml. These data indicated that L-argininal content in **1a** were *ca.* 86%. Another lot was also analyzed as 82%.

Acetyl-L-leucyl-N^G-benzyloxycarbonyl-L-arginine Methyl Ester (12)——i) Conversion of the BOC group of **6a** into acetyl group was accomplished as described in the case of the conversion of **8a** into **8b**. Thus, 1.6 g of the crude hydrochloride of **6a** gave 1.4 g of a crude hydrochloride of **12** as a powder. To a solution

of this powder in 5 ml of MeOH, was added a solution of NaOH (150 mg) in 5 ml of H_2O. After a few minutes standing crystallization occurred. After further standing for 30 min, 5 ml of H_2O was added to the mixture which was set aside in a refrigerator overnight. The resulting crystals (0.9 g) were collected and recrystallized from EtOH, giving **12**, mp 174—178°, $[\alpha]_D^{23}$ −25.3° (c=0.86, $CHCl_3$). *Anal.* Calcd. for $C_{23}H_{35}O_6N_5$: C, 57.84; H, 7.39; N, 14.67. Found: C, 57.83; H, 7.74; N, 14.80.

ii) A solution of **5a** (7.3 g) and acetyl-L-leucylazide[19] prepared from acetyl-L-leucylhydrazide (5.3 g) in 30 ml of $CHCl_3$ was allowed to stand at room temperature for 20 hr and then washed with dil. HCl, H_2O and dil. $NaHCO_3$ successively. After being dried, the mixture was evaporated to a crystalline mass. Two recrystallizations from EtOH gave 2.95 g of **12** as granular crystals, mp 174—179°, which were identified with the sample prepared before by mixed melting point test and infrared spectrometry.

Acetyl-L-leucyl-N^G-benzyloxycarbonyl-L-arginine Lactam (13)——To a solution of **7b** (1.6 g) in 70 ml of $CHCl_3$ was added a mixture of MeOH (1 ml) and Ac_2O (1 ml) and, after standing for a few min, the mixture was washed with sat. aqueous $NaHCO_3$ to be neutralized. The $CHCl_3$ layer was collected, washed with H_2O, dried and evaporated, giving a crystalline mass which was recrystallized from AcOEt to afford **13** as silky crystals of mp 177—180°, $[\alpha]_D^{20}$ −32.5° (c=1.0, $CHCl_3$). *Anal.* Calcd. for $C_{22}H_{31}O_5N_5$: C, 59.31; H, 7.01; N, 15.72. Found: C, 59.04; H, 7.02; N, 15.51.

Acetyl-L-leucyl-N^G-benzyloxycarbonyl-L-argininal (14)——To a cooled solution of **13** (890 mg) in 20 ml of THF, $LiAlH_4$ (156 mg) was added at one portion with stirring at −15—−20°. After being stirred at −15° for 20 min, the mixture was diluted with 100 ml of $CHCl_3$ and 10 ml of H_2O to decompose the excess reagent, and filtered. The organic layer separated was dried and evaporated to give an oil which was dissolved in a minimum amount of AcOEt with boiling. After cooling to room temperature, the mixture was diluted with hexane to deposit a powder. Decantation of the solvent gave 795 mg of **14** as a powder which was recrystallized from AcOEt to give pure **14**, mp 110—115° (with bubbling), $[\alpha]_D^{20}$ −28.5° (c=1.1, $CHCl_3$). IR ν_{max}^{Nujol} cm^{-1}: 3300 (NH, OH), 1640, 1620 (broad), 1530. *Anal.* Calcd. for $C_{22}H_{33}O_5N_5 \cdot 1/4H_2O$: C, 58.46; H, 7.47; N, 15.49. Found: C, 58.49; H, 7.52; N, 15.89.

Acetyl-L-leucyl-L-argininal (11)——Hydrogen was slowly bubbled through a stirred mixture of **14** (500 mg), 10% Pd-C (0.25 g) and 0.1N HCl (21 ml) for 15 min at room temperature. After filtration, the catalyst was washed with H_2O. The filtrate and washings were adjusted to pH 5—6 with Amberlite IR-45 (OH^-) and filtered. The filtrate was freeze-dried to give hydrochloride of **11** as a colorless powder (356 mg) which was almost pure on TLC, mp 130—140° (bubbling), with prelimiary softening at *ca.* 96°, $[\alpha]_D^{24}$ −59.3° (c=2.9, MeOH). *Anal.* Calcd. for $C_{14}H_{27}O_3N_5 \cdot 1/2H_2O$: C, 46.86; H, 8.15; N, 19.51; Cl, 9.88. Found: C, 46.64; H, 8.14; N, 18.96; Cl, 10.45.

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