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A Simple Method for Semi-Synthesis of Peptidyl Argininals as Potent Inhibitors of Trypsin-like Proteases[1)]

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A simple method was developed for the conversion of leupeptin (acetyl–Leu–Leu–argininal) to other peptidyl argininals. Argininal dibutylacetal, a key intermediate, was produced by enzymatic digestion of leupeptin dibutylacetal with Pronase E and was isolated by column chromatography on CM-52 cellulose. N_α-Blocked peptides (or amino acids) were connected to the α-amino group of this intermediate by conventional methods, and finally the acetal protecting group was removed by mild acid treatment to recover the essential aldehyde function. This novel method is applicable to large-scale preparation of many kinds of peptidyl argininals, which are promising candidates as specific inhibitors of trypsin-family proteases.

Keywords——argininal dibutylacetal; leupeptin (Ac–Leu–Leu–ArgH); Pronase E; protease inhibitor; Z–Val–Pro–argininal

Introduction

Since the discovery of leupeptin (Ac–Leu–Leu–L-ArgH),[2)] peptidyl argininals have been of increasing interest due to their great potential as strong and specific inhibitors of individual trypsin-family proteases.[3,4)] However, their usage has been limited by the difficulty generally encountered in the synthesis of the L-argininal moiety that is essential for their inhibitory action. The difficulty mainly arises from the functional and stereochemical instability of the moiety.[5)] The chromatographic purification of leupeptin and other peptide aldehydes using silica gel is usually detrimental because of the easy racemization of the amino aldehyde residues.[6)]

The finding that leupeptin can be regenerated without racemization from its dibutylacetal derivative (Ac–Leu–Leu–L-ArgH$(OBu)_2$) by mild acid treatment[7)] led us to achieve successful semi-syntheses of many leupeptin analogs including radioactive leupeptin (Ac–[U-^{14}C]–Leu–Leu–L-ArgH),[7)] pyroglutamyl–Leu–L-ArgH,[8)] and pyroglutamyl–Lys–Leu–L-ArgH.[9)] The last compound was found to afford a useful biospecific affinity adsorbent for purification of urokinase, when immobilized on gel matrix through the ε-amino group of its lysine residue.[9)] In the synthesis of these analogs, the starting material employed was H–Leu–ArgH-$(OBu)_2$ obtained as a thermolysin-digestion product[10)] of Ac–Leu–Leu–L-ArgH$(OBu)_2$. Therefore, all the compounds synthesized were the derivatives of leupeptin in which P_3 and P_4 subsite residues[11)] had been substituted. The residue at the P_2 position of peptidyl argininals is thought to be more important than those at the P_3 and P_4 residues in the expression of discriminative inhibitory activity against various trypsin-like proteases. In order to obtain leupeptin analogs with substitution at the P_2 position by a similar method to that adopted in the above-mentioned studies, H–ArgH$(OBu)_2$ should be a key starting material.

In this study, we established a simple method to prepare H–ArgH$(OBu)_2$ by cleaving

leupeptin dibutylacetal with Pronase E. Semi-synthesis of Z–Val–Pro–L-ArgH is also described as a typical example of syntheses of compounds from this starting material.

Results and Discussion

Enzymatic Cleavage of Leupeptin Dibutylacetal

The enzymatic digestion of leupeptin dibutylacetal (Leup(OBu)$_2$) to yield H–ArgH-(OBu)$_2$ was examined by thin-layer chromatography (TLC) on silica-gel plates. The substrate was prepared by treating leupeptin with 1-BuOH in the presence of *p*-toluenesulfonic acid and was a diastereomeric mixture, Ac–Leu–Leu–DL-ArgH(OBu)$_2$, due to the inevitable racemization at the ArgH moiety during acetal formation.[7] Among several protease specimens tested, Pronase E, a commercial preparation consisting of several proteases produced by *Streptomyces griseus*, was the best one to form H–ArgH(OBu)$_2$ (see lane 1 in Fig. 1). It was initially expected that *Streptomyces griseus* proteases A (SGPA) and B (SGPB), which are known to be components of Pronase E, would both be suitable agents to split the Leu–ArgH(OBu)$_2$ bond of the substrate, producing H–ArgH(OBu)$_2$ directly because the activity of these enzymes has been reported to depend mainly on the subsite contacts with the P_2–P_4 residues of substrates.[12] Indeed, either of the enzymes was found to cleave the substrate only at the Leu–ArgH(OBu)$_2$ bond, but the reaction was very slow. Even after a 96-h incubation with SGPA, more than 80% of the substrate remained in the reaction mixture (lane 2). Similarly, incubation with SGPB gave only a trace amount of H–ArgH(OBu)$_2$ (lane 3). On the other hand, H–ArgH(OBu)$_2$ was efficiently produced when H–Leu–ArgH (OBu)$_2$, a sole

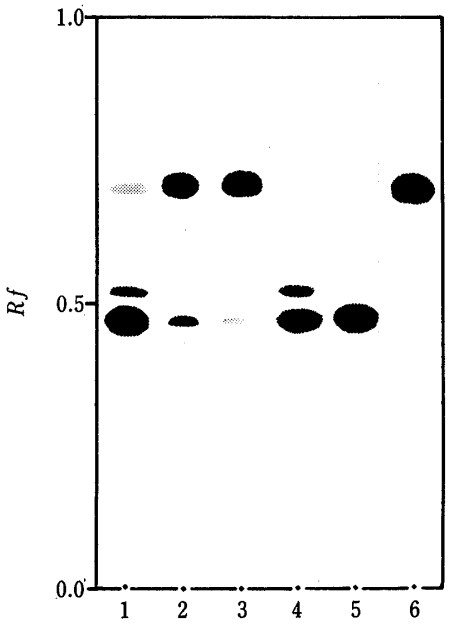

Fig. 1. Silica-Gel TLC Analysis of Protease Reactions on Leup(OBu)$_2$

Fifty mg of Leup(OBu)$_2$ and 2.5 mg of one of the enzyme preparations described below were incubated in 0.1 M *N*-ethylmorpholine–HCl, pH 8.0, containing 0.02 M $CaCl_2$ and 0.02 M $MnCl_2$ at 40 °C. After 72 h, an aliquot of 1 μl of the supernatant was spotted on a gel plate and developed with 1-BuOH : AcOBu : AcOH : H_2O (2 : 1 : 1 : 1). Compounds were visualized with the Sakaguchi reagent. Lane 1, Leup(OBu)$_2$ + Pronase E; lane 2, Leup(OBu)$_2$ + SGPA; lane 3; Leup(OBu)$_2$ + SGPB; lane 4, H–Leu–L-ArgH-(OBu)$_2$ + Pronase E; lane 5, H–ArgH(OBu)$_2$; lane 6, Leup(OBu)$_2$.

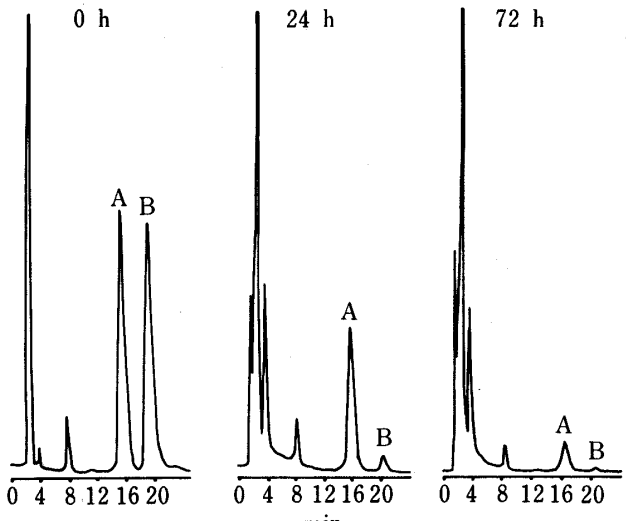

Fig. 2. Time Course of the Hydrolysis of Ac–Leu–Leu–L-ArgH(OBu)$_2$ (Peak B) and Ac–Leu–Leu–D-ArgH(OBu)$_2$ (Peak A) with Pronase E

HPLC conditions: column, 150 × 4 mm i.d. packed with Nucleosil 7C-18; mobile phase, 0.05 M NaH_2PO_4 : CH_3CN : MeOH = 60 : 40 : 9 (v/v); flow rate, 1.0 ml/min; monitoring wavelength, 205 nm at 0.32 full scale.

$Arg(OBu)_2$-containing fragment found in the thermolysin digest of $Leup(OBu)_2$, was treated with Pronase E (lane 4). Because it has been reported that Pronase E also contains large amounts of neutral endopeptidase and aminopeptidase,[13] the former of which has a substrate specificity similar to that of thermolysin,[14] the production of $H–ArgH(OBu)_2$ from $Leup(OBu)_2$ with Pronase E (lane 1) is considered to be mainly attributable to the cooperative action of these two enzymes; the neutral endopeptidase specifically splits the Leu–Leu bond first, and the aminopeptidase removes leucine from the resulting $H–Leu–ArgH(OBu)_2$.

Susceptibility to Pronase E was compared between the diastereomeric isomers of $Leup(OBu)_2$ by analytical high-performance liquid chromatography (HPLC). As shown in Fig. 2, Ac–Leu–Leu–L-$ArgH(OBu)_2$ was digested more rapidly than Ac–Leu–Leu–D-$ArgH(OBu)_2$, but the latter was also cleaved steadily. Since the Pronase aminopeptidase has been reported to have also the ability to cleave peptide bonds involving D-amino acid residues, though at greatly reduced rates,[15] $H–ArgH(OBu)_2$ as the final product may be a mixture of the optical antipodes.

Preparation of $H–ArgH(OBu)_2$

Pronase E (1.25 g) was added in several portions to 25 g of $Leup(OBu)_2$ suspended in 2000 ml of 0.1 M *N*-ethylmorpholine–HCl buffer, pH 8.0, containing 0.02 M $CaCl_2$ and 0.02 M $MnCl_2$, and the mixture was incubated for 72 h at 40 °C. The resulting solution was directly applied to a column of HP-20 (500 ml). The column was washed with water (2000 ml) to remove salts, and the adsorbed material was eluted with MeOH (220 ml). The eluate was concentrated to afford a yellow powder (15.2 g), which (in portions of 3 g each) was subjected to column chromatography on CM-52 cellulose (500 ml) with 0.1 M sodium phosphate buffer, pH 7.8. The elution profile is shown in Fig. 3. The fractions (Nos. 51—80), which contained material showing a Sakaguchi-positive spot with $Rf=0.47$ on TLC (see Fig. 3 inset), were pooled and applied to a column of HP-20 (250 ml) to desalt them. By repeating this procedure, 4.8 g of $H–ArgH(OBu)_2$ was obtained as a white powder. The isolation process is shown in Chart 1, and the physico-chemical data for $H–ArgH(OBu)_2$ prepared in this way are summarized in Table I. As described below, this product was a mixture of L- and D-isomers, as expected.

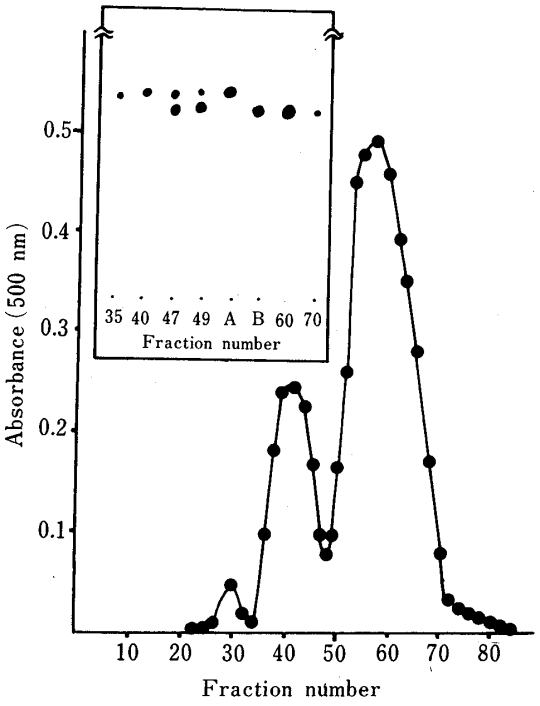

Fig. 3. Elution Profile of a Pronase E Digest of $Leup(OBu)_2$ from a CM-52 Cellulose Column (3.75 × 45 cm)

Effluent fractions of 15 ml were collected. An aliquot (20 μl) from each of the fractions was mixed with 0.007% 8-hydroxyquinoline : 1 M NaOH (1.5 ml) and 0.05% *N*-bromosuccinimide : H_2O (0.5 ml), and the absorbance at 500 nm was measured.

Fig. 3 Inset. Silica-Gel TLC of the Reaction Products

Aliquots (1 μl each) of effluent fractions from the chromatographic column of CM-52 were spotted and developed with 1-BuOH : AcOBu : AcOH : H_2O (2 : 1 : 1 : 1). Compounds were visualized with the Sakaguchi reagent. (A, $H–Leu–ArgH(OBu)_2$; B, $H–ArgH(OBu)_2$)

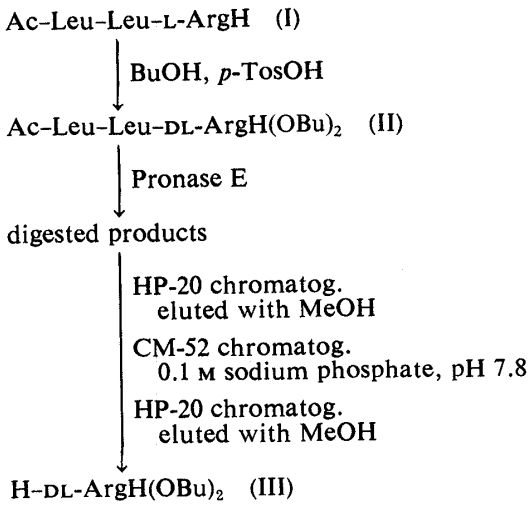

Chart 1

TABLE I. Physicochemical Properties of H–DL-ArgH(OBu)$_2$

Melting point	164—166 °C
Optical rotation	$[\alpha]_{578}^{25}$ +0.5° (c=0.8, AcOH)
Molecular formula	$C_{14}H_{32}N_4O_2$ (M_r 288)
Mass spectrometry	FD-MS m/z 289 (MH^+, 100.0%), 290 (17.3%), 291 (1.9%), 159 (1.0%)
HR-MS Calcd	289.2604 (MH^+)
Found	289.2606 (FAB-MS)
IR (KBr) (cm^{-1})	3250, 2930, 2860, 1670, 1640, 1630, 1470, 1400
Rf (Silica gel)	0.17 (1-BuOH : AcOBu : AcOH : H_2O=4 : 2 : 1 : 1), 0.47 (1-BuOH : AcOBu : AcOH : H_2O=2 : 1 : 1 : 1)
Positive reaction	Sakaguchi, ninhydrin, iodine vapor

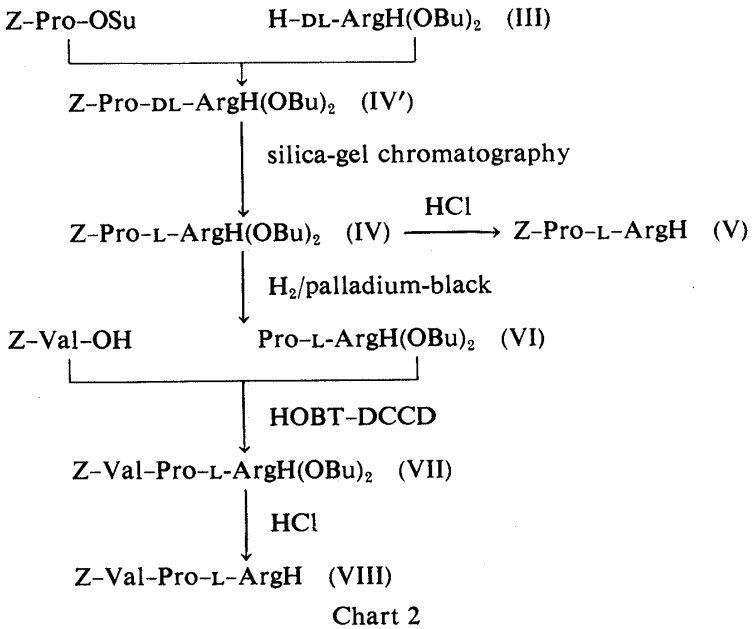

Chart 2

Synthesis of Z–Val–Pro–L-ArgH

Using H–ArgH(OBu)$_2$ as a starting material, we synthesized several peptidyl argininals. The process will be explained in the case of Z–Val–Pro–L-ArgH as a typical example (Chart

2). The details are presented in Experimental. First, H–ArgH(OBu)$_2$ was coupled with Z–Pro–OSu. The reaction product was found to be a mixture of Z–Pro–L-ArgH(OBu)$_2$ and Z–Pro–D-ArgH(OBu)$_2$, which were separable from each other by silica-gel chromatography. A small portion of each of the isolated isomers was treated with acid (1 M HCl : CH_3CN) to regenerate the aldehyde group and examined for inhibitory activity against urokinase to determine which was the isomer with the L-ArgH moiety. The Z protecting group was then removed from Z–Pro–L-ArgH(OBu)$_2$ by hydrogenation over palladium black and the product was coupled with Z–Val–OH by using of HOBT–DCCD. The product was purified by silica-gel chromatography with the same solvent system as described above. Z–Val–Pro–L-ArgH(OBu)$_2$ thus obtained was confirmed to be homogeneous by TLC on silica gel. It exhibited a base peak at $(MH)^+$, either on field desorption mass spectrometry (FD-MS) or on high-resolution mass spectrometry (HR-MS) with fast atom bombardment, and gave Z–Val–Pro–L-ArgH after acid treatment.

Utility of ArgH-Containing Compounds as Protease Inhibitors

Leupeptin and various analogs, prepared either from H–ArgH(OBu)$_2$ as described above or from H–Leu–ArgH(OBu)$_2$ as reported previously,[7,8] have been used in the investigation of the roles of proteases in fertilization. For example, the fertilization of the solitary ascidian (*Halocynthia roretzi*) was found to suffer the strongest inhibition with Z–Val–Pro–L-ArgH and Z–Phe–Leu–L-ArgH among ten kinds of ArgH-containing compounds examined. The activity of spermosin isolated from spermatozoa of this sea animal was specifically inhibited with Z–Val–Pro–L-ArgH, while the activity of acrosin, another trypsin-like protease isolated from the same source, was susceptible to most of the ten compounds, among which Z–Phe–Leu–L-ArgH was the best inhibitor. It is suggested therefore that both spermosin and acrosin are involved in fertilization of the ascidian.[16] A similar approach allowed us to conclude that a trypsin-like protease found in eggs of the sea urchin (*Strongylocentrotus intermedius*), the activity of which was highly sensitive to Z–Phe–ArgH and Z–Phe–Leu–L-ArgH, participates at least in the formation of the fertilization membrane.[17]

The present study has thus provided a simple route to many kinds of ArgH-containing inhibitors which should be extremely useful in the study of the biological functions of trypsin-like proteases.

Experimental

Materials and General Methods——Leupeptin was manufactured by fermentation of *Streptomyces roseus* by Nippon Kayaku Co., Ltd., Tokyo, Japan. Leup(OBu)$_2$, H–Leu–L-ArgH(OBu)$_2$, and H–Leu–D-ArgH(OBu)$_2$ were prepared by the method described in a previous paper.[7] Pronase E was purchased from Kaken Seiyaku Co., Ltd., Tokyo, Japan. SGPA and SGPB were isolated from Pronase E essentially as described by Narahashi.[13] Urokinase used in this paper was prepared by the method described in a previous paper.[9] Melting points were determined with a Shibata melting point apparatus and are uncorrected. Infrared (IR) spectra were measured with a Hitachi 260-10 spectrometer. Optical rotations were measured with a Perkin-Elmer 241 polarimeter. FD-MS was carried out for the identification of reaction products by using a JEOL JMS-DX 300 mass spectrometer equipped with a carbon-graphite needle emitter (emitter current, 15—20 mA, acceleration voltage, 3 kV). HR-MS with fast atom bombardment (FAB) were measured with an MM-ZAB-HF mass spectrometer (VG-Analytical, England). Xenon was used for the primary beam (8 kV).

Determination of L-Configuration of the Argininal Moiety by Checking Inhibition of Urokinase Activity——The ArgH-C_α configuration of Z–Pro–ArgH was determined by examining the inhibitory effect on urokinase, which is known to be susceptible only to peptidyl argininals with an L-ArgH moiety. The two isomers of Z–Pro–ArgH were dissolved in 50% aqueous methanol at various concentrations, and an aliquot (25 μl) from each of the solutions (and a blank solution) was mixed with 450 μl of 0.05 M Tris–HCl buffer, pH 8.0, containing 0.1 M NaCl. Then 50 μl of urokinase solution (500 IU/ml) and 50 μl of 1 mM glutaryl–Gly–Arg–4-methylcoumaryl-7-amide were added to the mixture. The enzyme activity was determined by measuring the fluorescence due to 7-amino-4-methyl-coumarin, liberated during a 10-min reaction at 37 °C, with excitation at 380 nm and emission at 460 nm. The results were

expressed in terms of IC_{50}, that is, the concentrations (μg/ml) of test compounds required to inhibit 50% of the enzyme activity.

Z–Pro–L-ArgH(OBu)$_2$ (IV)——H–DL-ArgH(OBu)$_2$ (III) (324 mg), which had been prepared as already described, was dissolved in CH_2Cl_2 (10 ml) containing triethylamine (140 μl). After addition of Z–Pro–OSu (415 mg), the solution was stirred at room temperature for 20 h. The resulting solution was found to contain two Sakaguchi-positive components, A and B, with Rf=0.65 and 0.56, respectively, by TLC on a silica-gel plate using 1-BuOH : AcOBu : AcOH : H_2O (4 : 2 : 1 : 1) (called solvent A hereafter). The solution was then concentrated under reduced pressure and applied to a column of silica gel (150 ml). From the column, components A and B were separately eluted with 1-BuOH : AcOBu : AcOH : H_2O (8 : 8 : 1 : 1). Since only A of the two components showed strong inhibitory activity toward urokinase after regeneration of its aldehyde function (see the next section), it was assigned as Z–Pro–L-ArgH(OBu)$_2$. The column eluate containing component A was then evaporated to dryness to give 257 mg (45%) of hygroscopic material. $[\alpha]_{578}^{24}$ −60.3° (c=0.3, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3400, 2970, 2890, 1670, 1560, 1420, 1360. Rf 0.65 (solvent A), a single positive spot either with the Sakaguchi reagent or with iodine vapor. FD-MS m/z (relative intensity): 520 (MH^+, 100.0), 521 (32.4), 522 (6.0), 523 (0.8). HR-MS m/z: Calcd for $C_{27}H_{46}N_5O_5$: 520.3499. Found: 520.3493. Component B showed the same pattern with a base peak at 520 (MH^+) on FD-MS.

Z–Pro–L-ArgH (V)——A part of IV (23.5 mg) was treated with 4 ml of 1 M HCl : CH_3CN (1 : 2, v/v) at 37 °C for 2 h. The reaction mixture was adjusted to pH 4.8 with Dowex WGR (OH^-). The filtrate was concentrated to dryness to give 16 mg (89%) of white powder, mp 120—123 °C. $[\alpha]_{578}^{26}$ −77.7° (c=0.5, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3350, 2960, 2890, 1660, 1550, 1420, 1360. Rf 0.53 and 0.41 (solvent A), major two spots reactive either to the Sakaguchi reagent or to iodine vapor. As described previously,[7] peptidyl argininals show more than two spots in TLC on silica-gel plates. FD-MS m/z (relative intensity): 390 (MH^+, 100.0), 391 (26.7), 392 (6.3). HR-MS m/z: Calcd for $C_{19}H_{28}N_5O_4$: 390.2142. Found: 390.2114. IC_{50} for urokinase: 24 μg/ml.

Pro–L-ArgH(OBu)$_2$ (VI)——IV (150 mg) was hydrogenated in 10 ml of MeOH over palladium black for 5 h. The filtrate was concentrated to dryness to give 106 mg (93%) of hygroscopic material. $[\alpha]_{578}^{26}$ −58.1° (c=0.7, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3300, 2970, 2890, 1660, 1560, 1440, 1410. Rf 0.11 (solvent A), a single spot either with the Sakaguchi reagent or with iodine vapor. HR-MS m/z: Calcd for $C_{19}H_{40}N_5O_3$: 386.3131. Found: 386.3124.

Z–Val–Pro–L-ArgH(OBu)$_2$ (VII)——DCCD (22.7 mg) was added to a solution containing VI (42.2 mg), N-ethylmorpholine (11.1 μl), Z–Val–OH (25.1 mg), and HOBT (14.9 mg) in CH_2Cl_2 (3 ml) under ice cooling. The mixture was stirred for 1 h under ice cooling and for an additional 16 h at room temperature. The reaction mixture was concentrated to dryness. The residue was dissolved in ethylacetate as thoroughly as possible, and dicyclohexylurea was removed by filtration. The filtrate was successively washed with saturated aqueous $NaHCO_3$, water, 10% citric acid, and water. The organic phase was dried with $MgSO_4$ and evaporated to dryness. The residue was dissolved in 25% MeOH and applied to a column of HP-20 (10 ml) that had been equilibrated with 25% MeOH. The column was washed with 100 ml of 25% MeOH, then the adsorbed material was eluted with MeOH. The Sakaguchi positive fractions were pooled and concentrated to give 41.0 mg (63%) of hygroscopic material. $[\alpha]_{578}^{25}$ −54.8° (c=0.3, MeOH). Rf 0.65 (solvent A), a single spot either with the Sakaguchi reagent or with iodine vapor. FD-MS m/z (relative intensity): 619 (MH^+, 100.0), 620 (45.7), 621 (9.0), 622 (1.6), 511 (2.1).

Z–Val–Pro–L-ArgH (VIII)——VII (20 mg) was treated with 6 ml of 1 M HCl : CH_3CN (1 : 2, v/v) at 37 °C for 2 h. The reaction mixture was adjusted to pH 4.8 with Dowex WGR (OH^-). The filtrate was concentrated and lyophilized to give 14 mg (88%) of white powder. mp 135—137 °C. $[\alpha]_{578}^{26}$ −68.9° (c=0.2, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3350, 2970, 1710, 1660, 1530, 1450. Rf 0.43, 0.30 (solvent A), positive spots either with the Sakaguchi reagent or with iodine vapor. FD-MS m/z (relative intensity): 489 (MH^+, 100.0), 490 (42.1), 491 (16.1), 492 (3.3). HR-MS m/z: Calcd for $C_{24}H_{37}N_6O_5$: 489.2826. Found: 489.2864. IC_{50} for urokinase: 10 μg/ml.

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References and Notes

1) Abbreviations used are those recommended by the IUPAC-ICB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 2458 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1976). Other abbreviations used in this paper are: ArgH = argininal, ArgH(OBu)$_2$ = argininal dibutylacetal, Z = benzyloxycarbonyl, DCCD = dicyclohexylcarbodiimide, HOBT = 1-hydroxybenzotriazole, SGPA = *Streptomyces griseus* protease A, SGPB = *Streptomyces griseus* protease B. All the amino acid residues described in their abbreviated forms without any other specification are of L-configuration except in the case of ArgH.
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