Inhibition Studies of Some Serine and Thiol Proteinases by New Leupeptin Analogues

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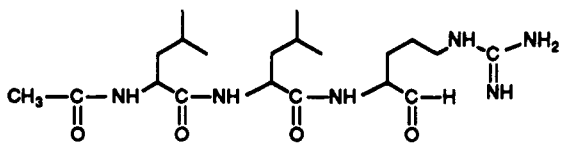
Fifteen tripeptide analogues of leupeptin containing either a C-terminal argininal or lysinal were synthesized. The synthetic analogues were tested, using spectrophotometric assay techniques, as inhibitors of trypsin, kallikrein, thrombin, plasmin, and cathepsin B. The lysinal analogues were fairly selective as inhibitors of cathepsin B activity. Acetyl-L-leucyl-L-valyl-L-lysinal (2l) showed a stronger inhibition of cathepsin B (IC_{50} = 4 nanomolar) than leupeptin. Acetyl-L-phenylalanyl-L-valyl-L-argininal (2i) was found to be a good inhibitor of cathepsin B (IC_{50} = 0.039 μM), thrombin (IC_{50} = 1.8 μM), and plasmin (IC_{50} = 2.2 μM).

Leupeptin (1) is known to be an efficient reversible inhibitor of trypsin-like serine and thiol proteinases.[1-9] Leupeptin has recently been used to study tumor metastasis,[10-12] neuromuscular conditions,[13-17] and immunological dysfunctions.[18,19] However, leupeptin lacks the selectivity among proteases of similar substrate specificities needed to be an efficient biochemical tool. Also leupeptin can cause a variety of responses in similar systems depending on the proteinases involved. For example, in several studies leupeptin was found to promote synapse formation and improve neuromuscular recovery during conditions of nerve trauma.[13,14,16] In other studies leupeptin has been shown to cause degeneration of neuronal processes and formation of dense bodies with fine morphologies similar to lipofuscin of aged tissue.[20,21] This variation of neurological responses caused by leupeptin is primarily due to its lack of selectivity among the proteinases involved.



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Attempts have been made to improve selectivity of leupeptin's activity through the utilization of analogues.[22-26] Saito et al. found that a tripeptide aldehyde with norleucine at the C-terminus had a neurite initiating effect on PC 12h cells, but leupeptin and a tripeptide aldehyde with methionine at the C-terminus had no such action.[25] Recently, attempts have been made to find inhibitors of cysteine proteinases, especially those which can penetrate cell membranes.[12,18,24] Lysosomal cysteine proteinases, such as cathepsin B, have been linked to tumor generation, atherosclerosis, and several neurological disorders.[11,12,18,27] Carbobenzyloxyvalylphenylalaninal (MDL) and carbobenzyloxyleucylnorleucinal (Calpeptin) were found to inhibit cathepsin B but not trypsin-like enzymes.[24]

Leupeptin analogues with the traditional argininal C-terminus have also been isolated.[23] Our earlier work on the synthesis and assay of leupeptin analogues demonstrated that slight variations in the basic side chain of the C-terminal amino aldehyde can improve the selectivity among trypsin-like enzymes.[22] We have since prepared leupeptin analogues containing a greater variety of alterations in the P_2 and P_3 positions[28] with C-terminal argininal or lysinal groups. Previously, we discovered that the nature of the N-terminal protecting group is important to the activity of the inhibitor.[22] Therefore, our newest leupeptin analogues (2a-o) contain an N-terminal acetyl protecting group, like that in natural leupeptin. The inhibitory activities of these analogues have been assayed with five proteolytic enzymes. These are trypsin, kallikrein, plasmin, thrombin, and cathepsin B. The synthesis and biological activities of these compounds are reported below.

Synthesis

The preparation of amino aldehydes from amino acids with basic side chains, such as arginine or lysine, are seldom reported in the literature. The reports of leupeptin analogues describe inhibitors which, for the most part, contain either a neutral, rather than basic, amino acid at the C-terminus[24,25] or involve isolated, rather than synthesized, analogues. Someno and Aoyagi have reported a semisynthetic method involving the enzymatic cleavage of argininal dibutyl acetal from protected leupeptin.[26,29] However, we have found this to be a very expensive and low-yield method.

In our earlier work, we reported a synthetic method which involved a diisobutylaluminum hydride reduction of protected arginine, lysine, or ornithine methyl esters.[22] We utilized this method for the preparation of our newest analogues with a C-terminal lysinal. However, we abandoned this method for the preparation of the argininal analogues. Preparation of a protected argininal resulted from the direct cyclization of (*tert*-butoxycarbonyl)nitro-L-arginine to the δ-lactam using 1,1'-carbonyldiimidazole. The lactam was then immediately reduced to the cyclic carbinol amine (Scheme I). The cyclized carbinol-amine exists in equilibrium with the open-chain and hydrated aldehydes.[22,30] Without isolation the protected amino aldehyde was converted to the semicarbazone derivative (3). The N-terminal protecting group was removed, and the amino aldehyde semicarbazone was condensed with the mixed anhydrides of a series of carbobenzyloxy-protected dipeptide acids. The N-terminal carboben-

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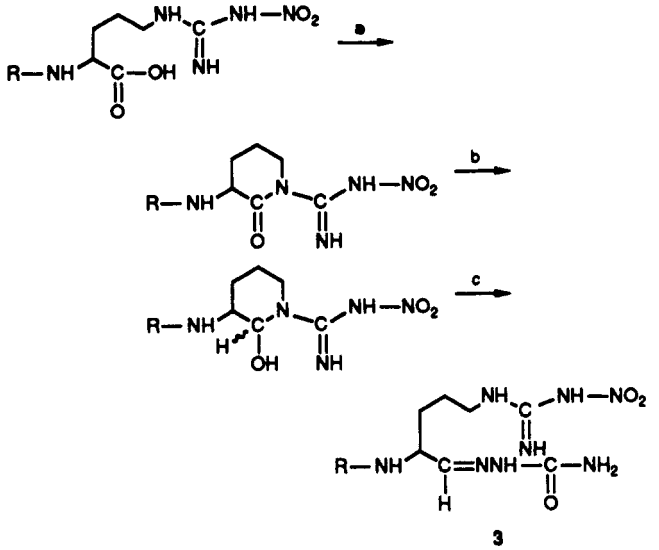
Scheme I[a]



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[a] (a) 1,1'-Carbonyldiimidazole, -20 °C, dry THF; (b) 1 M lithium aluminum hydride in THF; (c) semicarbazide hydrochloride and sodium acetate.

Table I. Physical Constants of Leupeptin Analogues

no.	compound	mp, °C	$[\alpha]^{25}_{Na}$ (c 1.0, methanol)
2a	acetyl-Val-Leu-argininal	151–153	-31.7
2b	acetyl-Leu-Val-argininal	152–154	-35.2
2c	acetyl-Leu-Ile-argininal	163–165	-29.8
2d	acetyl-Ile-Leu-argininal	158–160	-23.5
2e	acetyl-Leu-Phe-argininal	170–172	-31.6
2f	acetyl-Phe-Leu-argininal	175–177	-41.6
2g	acetyl-Leu-Tyr-argininal	186–190	-16.3
2h	acetyl-Tyr-Leu-argininal	192–195	-19.7
2i	acetyl-Phe-Val-argininal	166–168	-38.5
2j	acetyl-Leu-Leu-lysinal	150–153	-43.1
2k	acetyl-Val-Leu-lysinal	159–161	-27.6
2l	acetyl-Leu-Val-lysinal	160–163	-22.5
2m	acetyl-Leu-Phe-lysinal	167–170	-37.5
2n	acetyl-Phe-Leu-lysinal	173–175	-33.6
2o	acetyl-Ile-Leu-lysinal	148–151	-38.9

zyloxy groups were removed from the peptides and replaced by *N*-acetyl protecting groups. The side chain and semicarbazone protecting groups were removed lastly to form the leupeptin analogues (**2a–o**) as hydrochloride salts. Physical constants were documented (Table I). All spectroscopic data were consistent with the structures although ^{1}H NMR indicate that considerable cyclization occurs in both the argininal and lysinal type leupeptin analogues in dimethyl sulfoxide. This does not preclude the existence of the free aldehyde in aqueous solutions. The ^{1}H NMR spectra indicates that the argininal analogues exist primarily as the carbinol–amine in dimethyl sulfoxide, while the spectra of the lysinal analogues show both the cyclic iminium and the carbinol–amine (Figure 1).

Biological Activities and Discussion

The leupeptin analogues were assayed for their inhibitory activities of four serine proteinases and one thiol proteinase with similar substrate specificities (Table II). The assays were performed by traditional spectrometric assay techniques using commercially available chromogenic substrates.

The changes made in the basic side chain of the C-terminal aldehyde caused great variation in activity. The lysinal analogues (**2j–o**) tended to be fairly selective for cathepsin B inhibition with IC_{50} values as high as 4 nM

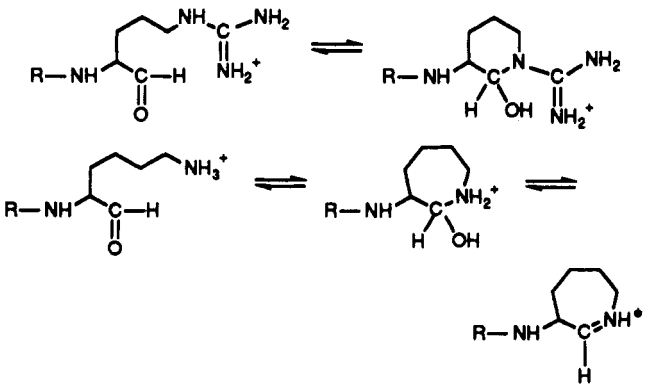

Figure 1. Cyclization of argininal (top) and lysinal (bottom) leupeptin analogues.

Table II. Inhibition Data

	IC_{50} in micromolar concentrations				
compound	trypsin[a]	kallikrein[b]	thrombin[c]	plasmin[d]	cathepsin B[e]
leupeptin	8.1	40	12	3.7	0.31
2a	130	19	170	65	100
2b	NA[f]	16	NA	NA	130
2c	9.1	35	18	11	0.15
2d	NA	54	94	67	NA
2e	NA	41	8.2	3.6	1.1
2f	37	16	71	7.7	1.5
2g	NA	25	66	5.6	1.6
2h	NA	26	35	78	22
2i	12	28	1.8	2.2	0.039
2j	NA	NA	NA	18	0.13
2k	NA	NA	NA	NA	28
2l	45	NA	140	13	0.004
2m	NA	NA	NA	NA	0.10
2n	NA	NA	NA	NA	18
2o	NA	NA	NA	17	2.0

[a] Substrate = benzoyl-L-arginine ethyl ester. [b] Substrate = benzoyl-L-arginine ethyl ester. [c] Substrate = benzoyl-Phe-Val-arginine *p*-nitroanilide. [d] Substrate = D-Val-L-Leu-L-lysine *p*-nitroanilide. [e] Substrate = Cbz-L-lysine *p*-nitrophenyl ester. [f] NA = not significantly active (concentrations of 100 μM or greater cause a decrease by 25% or less of enzymatic activity).

for acetyl-Leu-Val-lysinal (compared to leupeptin at 0.31 μM). The lysinal analogues did not inhibit trypsin or kallikrein and showed very little inhibition of thrombin and plasmin activities. The general preference by trypsin for the argininal analogues is in agreement with crystallographic studies by Bode,[31] which show a direct binding at the S_1 site of arginine side chains and an indirect binding of lysine side chains.

The changes made in the P_2 and P_3 side chains also caused a variation in activity, especially in the case of trypsin inhibition. Of the nine argininal analogues (**2a–i**), five were not active as trypsin inhibitors. The alterations made in the P_2 and P_3 side chains of the arginine analogues caused very little change in activity in the case of kallikrein inhibition. This is in agreement with crystallographic studies of kallikrein that show kallikrein as a dimer with two somewhat flexible active sites.[31] Selectivity was achieved for cathepsin B inhibition through the changes made in the P_2 and P_3 side chains in acetyl-Phe-Val-argininal (**2i**) which shows an inhibition of cathepsin B activity 100–1000 times stronger than for the other enzymes tested. It has not yet been determined whether the increased activity if **2i** for cathepsin B inhibition is due to a tighter binding of the P_2 and P_3 side chains by the enzyme, or rather to an increase in the equilibrium concentration of the uncyclized free aldehyde (Figure 1). NMR studies of this equilibrium, and the rates

of interconversion, while varying the steric and electronic effects of the P_2 and P_3 side chains under a broad range of pH conditions is currently underway.

Summary

Several analogues of leupeptin were synthesized and assayed for their inhibition of trypsin-like serine and thiol proteinases. The argininal analogues were prepared by the cyclization of (*tert*-butoxycarbonyl)nitroarginine to the δ-lactam, followed by reduction to the cyclic aldehyde with lithium aluminum hydride. The lysinal analogues were prepared by diisobutylaluminum hydride reduction of the protected lysine methyl ester. Both the lysine and arginine aldehydes were protected as semicarbazones and incorporated into peptides. Deblocking then resulted in a series of tripeptides with argininal or lysinal at the C-terminus.

The leupeptin analogues were analyzed by spectrophotometric assay techniques for their inhibitory activities of trypsin, kallikrein, thrombin, plasmin, and cathepsin B. the lysinal analogues (**2j–o**) were very selective inhibitors of cathepsin B activity with acetyl-Leu-Val-lysinal (**2l**) showing significantly greater inhibition than leupeptin. Selectivity for cathepsin B activity was also achieved with acetyl-Phe-Val-argininal (**2i**).

The changes made in the P_2 and P_3 side chains showed great variation in activity for most of the enzymes tested, particularly trypsin. However, kallikrein did not show significant variation in activity with the changes made in the argininal analogues. The inhibitory activities of the analogues described compare well with other low molecular weight inhibitors with a minimum of functionality.

Experimental Section

All amino acids, protected amino acids, carbobenzyloxy-protected dipeptide acids, enzymes, and substrates were obtained from Sigma Chemical Co. unless otherwise specified. Capillary melting points were determined on a Hoover melting point apparatus and are uncorrected. NMR spectra are consistent with the structures reported and were recorded on a Hitachi Perkin-Elmer R-24B 60MHz, a Varian EM-390, or a Varian XL-200 spectrometer. All peptides and peptide derivatives were homogeneous by thin-layer chromatography unless otherwise indicated. Spectrophotometric assays were conducted on a Coleman/Hitachi Model 124 spectrometer and data recorded on a Kipp & Zonen BD41 Recorder.

N^{α}-(*tert*-Butoxycarbonyl)-N^{G}-nitro-L-argininal Semicarbazone (3). A solution of 5.0 g (16.2 mmol) of N^{α}-(*tert*-butoxycarbonyl)-N^{G}-nitro-L-arginine in 100 mL of freshly distilled (over Na) tetrahydrofuran was chilled to 5 °C in an ice/water bath under nitrogen atmosphere and treated with 2.63 g (16.2 mmol) of 1,1′-carbonyldiimidazole. The mixture was stirred at 5 °C under nitrogen for 1 h. The clear solution was then chilled to −40 °C (alcohol/water, 1/1, dry ice bath). A 32-mL portion of 1 M lithium aluminum hydride in tetrahydrofuran was slowly added over 20 min. The mixture was stirred at −40 °C under nitrogen for an additional 25 min. The reaction was then quenched by the addition of 100 mL of 0.1 M HCl. The mixture was partitioned between the phases of 200 mL of dichloromethane and 200 mL of 10% sodium potassium tartrate. The organic layer was washed with 100 mL of distilled water and dried over anhydrous magnesium sulfate. The organic layer was then concentrated under reduced pressure to form a viscous colorless oil (weight 4.2 g). The crude aldehyde was dissolved in 100 mL of ethanol. A solution of sodium acetate (16 mmol) and semicarbazide hydrochloride (16 mmol) in 50 mL of distilled water was added to the alcoholic solution. The mixture was heated to reflux and then stirred at room temperature for 18 h. The mixture was then diluted with 200 mL of distilled water and extracted three times with 200-mL portions of ethyl acetate. The organic layers were pooled, dried over anhydrous magnesium sulfate, and concentrated under reduced pressure. The crude semicarbazone was purified by silica gel chromatography using ethyl acetate/methanol (95/5, v/v) as the mobile phase to give 3.2 g (57%) of **3**: $[\alpha]^{25}_{Na} = -33.1°$ (*c* 1.0 methanol); mp 117–119 °C; $R_f = 0.22$ (ethyl acetate/methanol, 9/1, v/v); ^{1}H NMR (CD_3CN, 60 MHz) δ 1.1 (s, 9 H), 1.7 (m, 4 H), 3.2 (t, *J* = 7 Hz, 2 H), 4.1 (m, 1 H), 6.0 (m, 3 H), 7.1 (d, *J* = 7 Hz, 1 H), 7.7 (m, 2 H).

N^{G}-Nitro-L-argininal Semicarbazone Trifluoroacetate (4). A solution containing 1.1 g (2.8 mmol) of N^{α}-(*tert*-butoxycarbonyl)-N^{G}-nitro-L-argininal semicarbazone (**3**), in 30% trifluoroacetic acid in chloroform (by volume), was stirred at 0 °C for 4 h. The solvent was evaporated under reduced pressure, and the crude product was crystallized from methanol/ether to yield 0.95 g (92%) of **4**: mp 135–136 °C; $R_f = 0.23$ (1-butanol/water/acetic acid, 7/3/1, v/v/v); ^{1}H NMR (CD_3OD, 60 MHz) δ 1.8 (m, 5 H), 3.4 (t, *J* = 7 Hz, 2 H), 4.2 (m, 1 H), 5.8 (m, 2 H), 7.1 (d, *J* = 7 Hz, 1 H), 7.8 (m, 3 H), 9.4 (m, 1 H).

Coupling Procedure. A solution of 1 mmol of the appropriate carbobenzoxy-protected dipeptide acid in 10 mL of anhydrous *N*,*N*-dimethylformamide was chilled to −15 °C with vigorous stirring and treated with 0.140 mL (1 mmol) of triethylamine. After 30 min, 0.097 mL (1 mmol) of ethyl chloroformate was added and the mixture was stirred at −15 °C for an additional 45 min. A precooled solution containing 0.386 g (1 mmol) of N^{G}-nitroargininal semicarbazone trifluoroacetate (**4**) and 0.140 mL (1 mmol) of triethylamine in 10 mL of anhydrous *N*,*N*-dimethylformamide was added. The resulting mixture was stirred at 0 °C for 4 h and overnight at room temperature. The mixture was then partitioned between the phases of ethyl acetate and 0.1 M NaOH. The organic layer was extracted with 10% aqueous $NaHCO_3$, distilled water, 0.1 M HCl, and distilled water again. The organic layer was then dried over anhydrous magnesium sulfate and concentrated under reduced pressure. The tripeptide was then solidified by addition of ether and recrystallized from methanol/ether.

Carbobenzyloxy-L-valyl-L-leucyl-N^{G}-nitroargininal semicarbazone (5a): mp 118–120 °C; $R_f = 0.40$ (ethyl acetate/methanol, 9/1, v/v); ^{1}H NMR (DMSO-d_6, 60 MHz) δ 1.1 (d, *J* = 7 Hz, 12 H), 1.3 (m, 8 H), 3.4 (m, 2 H), 3.6 (m, 1 H), 4.6 (m, 3 H), 4.9 (m, 2 H), 5.1 (s, 2 H), 6.1 (m, 2 H), 6.9 (d, *J* = 7 Hz, 1 H), 7.4 (s, 5 H), 8.2 (m, 3 H), 9.4 (m, 1 H). Anal. ($C_{26}H_{42}N_{10}O_7$) C, H, N.

Carbobenzyloxy-L-leucyl-L-valyl-N^{G}-nitroargininal semicarbazone (5b): mp 126–127 °C; $R_f = 0.36$ (ethyl acetate/methanol, 9/1, v/v); ^{1}H NMR (DMSO-d_6, 60 MHz) δ 1.0 (d, *J* = 7 Hz, 12 H), 1.4 (m, 8 H), 3.2 (m, 2 H), 3.5 (m, 1 H), 4.4 (m, 3 H), 4.8 (m, 2 H), 5.2 (s, 2 H), 6.0 (m, 2 H), 7.0 (d, *J* = 7 Hz, 1 H), 7.3 (s, 5 H), 8.0 (m, 3 H), 9.5 (m, 1 H). Anal. ($C_{26}H_{42}N_{10}O_7$) C, H, N.

Carbobenzyloxy-L-leucyl-L-isoleucyl-N^{G}-nitroargininal semicarbazone (5c): mp 128–130 °C; $R_f = 0.72$ (ethyl acetate/methanol, 9/1, v/v); ^{1}H NMR (DMSO-d_6, 90 MHz) δ 0.9 (m, 9 H), 1.3 (m, 14 H), 3.3 (m, 2 H), 3.5 (m, 1 H), 4.4 (m, 3 H), 4.9 (m, 2 H), 5.2 (s, 2 H), 6.1 (m, 2 H), 6.9 (d, *J* = 7 Hz, 1 H), 7.2 (s, 5 H), 7.9 (m, 3 H), 9.3 (m, 1 H). Anal. ($C_{27}H_{44}N_{10}O_7$) C, H, N.

Carbobenzyloxy-L-isoleucyl-L-leucyl-N^{G}-nitroargininal semicarbazone (5d): mp 131–133 °C; $R_f = 0.69$ (ethyl acetate/methanol, 9/1, v/v); ^{1}H NMR (DMSO-d_6, 90 MHz) δ 1.0 (m, 9 H), 1.4 (m, 14 H), 3.1 (m, 2 H), 3.3 (m, 1 H), 4.3 (m, 3 H), 5.0 (m, 2 H), 5.3 (s, 2 H), 6.1 (m, 2 H), 7.1 (d, *J* = 7 Hz, 1 H), 7.3 (s, 5 H), 8.1 (m, 3 H), 9.5 (m, 1 H). Anal. ($C_{27}H_{44}N_{10}O_7$) C, H, N.

Carbobenzyloxy-L-leucyl-L-phenylalanyl-N^{G}-nitroargininal semicarbazone (5e): mp 117–119 °C; $R_f = 0.10$ (ethyl acetate/methanol, 9/1, v/v); ^{1}H NMR (DMSO-d_6, 60 MHz) δ 0.9 (m, 6 H), 1.6 (m, 7 H), 3.0 (m, 4 H), 3.5 (m, 1 H), 4.5 (m, 3 H), 4.8 (m, 2 H), 5.1 (s, 2 H), 6.0 (m, 2 H), 6.9 (d, *J* = 7 Hz, 1 H), 7.1 (s, 5 H), 7.3 (s, 5 H), 7.8 (m, 3 H), 9.6 (m, 1 H). Anal. ($C_{30}H_{42}N_{10}O_7$) C, H, N.

Carbobenzyloxy-L-phenylalanyl-L-leucyl-N^{G}-nitroargininal semicarbazone (5f): mp 105–107 °C; $R_f = 0.33$ (ethyl acetate/methanol, 9/1, v/v); ^{1}H NMR (DMSO-d_6, 60 MHz) δ 0.8 (m, 6 H), 1.6 (m, 7 H), 3.1 (m, 4 H), 3.3 (m, 1 H), 4.5 (m, 3 H),

5.0 (m, 2 H), 5.3 (s, 2 H), 6.0 (m, 2 H), 7.1 (d, J = 7 Hz, 1 H), 7.3 (s, 5 H), 7.5 (s, 5 H), 8.0 (m, 2 H), 9.5 (m, 1 H). Anal. ($C_{30}H_{42}N_{10}O_7$) C, H, N.

Carbobenzyloxy-L-leucyl-L-tyrosinyl-N^G-nitroargininal semicarbazone (5g): mp 137–140 °C; R_f = 0.15 (ethyl acetate/methanol, 9/1, v/v); ^{1}H NMR (DMSO-d_6, 60 MHz) δ 0.8 (m, 6 H), 1.5 (m, 7 H), 3.1 (m, 4 H), 3.5 (m, 1 H), 4.5 (m, 3 H), 5.1 (m, 2 H), 5.3 (s, 2 H), 5.9 (m, 2 H), 6.3 (s, 1 H), 7.0 (d, J = 7 Hz, 1 H), 7.3 (s, 5 H), 7.7 (m, 4 H), 8.0 (m, 2 H), 9.3 (m, 1 H). Anal. ($C_{30}H_{42}N_{10}O_8$) C, H, N.

Carbobenzyloxy-L-tyrosinyl-L-leucyl-N^G-nitroargininal semicarbazone (5h): mp 143–146 °C; R_f = 0.19 (ethyl acetate/methanol, 9/1, v/v); ^{1}H NMR (DMSO-d_6, 60 MHz) δ 0.8 (m, 6 H), 1.4 (m, 7 H), 3.2 (m, 4 H), 3.6 (m, 1 H), 4.4 (m, 3 H), 5.0 (m, 2 H), 5.2 (s, 2 H), 6.0 (m, 2 H), 6.8 (s, 1 H), 7.1 (d, J = 7 Hz, 1 H), 7.3 (s, 5 H), 7.9 (m, 4 H), 8.1 (m, 2 H), 9.5 (m, 1 H). Anal. ($C_{30}H_{42}N_{10}O_8$) C, H, N.

Carbobenzyloxy-L-phenylalanyl-L-valyl-N^G-nitroargininal semicarbazone (5i): mp 100–102 °C; R_f = 0.38 (ethyl acetate/methanol, 9/1, v/v); ^{1}H NMR (DMSO-d_6, 60 MHz) δ 0.9 (m, 6 H), 1.4 (m, 5 H), 3.0 (m, 4 H), 3.4 (m, 1 H), 4.4 (m, 3 H), 5.1 (m, 2 H), 5.3 (s, 2 H), 5.9 (m, 2 H), 7.1 (d, J = 7 Hz, 1 H), 7.3 (s, 5 H), 7.6 (s, 5 H), 8.1 (m, 2 H), 9.3 (m, 1 H). Anal. ($C_{29}H_{40}N_{10}O_7$) C, H, N.

General Deblocking Procedure. A dilute solution containing 0.50 mmol of the fully protected tripeptide (**5a–i**) in 200 mL of methanol was treated with a slurry of 10 mg of 10% palladium on carbon in 2.0 mL of 0.10 M HCl. The mixture was stirred vigorously under a hydrogen atmosphere (1 atm) at room temperature for 24 h. The catalyst was removed by filtration through a fine mesh funnel, and the solution was concentrated under reduced pressure. Excess water was removed by the addition and subsequent evaporation of 100 mL of acetonitrile. The crude material was recrystallized from methanol/ether. The resulting dihydrochloride salt of the tripeptide semicarbazone was dissolved in 10 mL of anhydrous N,N'-dimethylformamide and chilled to 0 °C. The solution was treated with 40 μL (0.50 mmol) of anhydrous pyridine. The solution was stirred for 30 min at 0 °C. A 47-μL (0.50-mmol) portion of acetic anhydride was then added. The mixture was stirred at 0 °C for 3 h and overnight at room temperature. Distilled water (100 μL) was added to quench the reaction. Cold tetrahydrofuran (100 mL at 0 °C) was added to precipitate the product. The crude solid was purified via Sephasorb H-P chromatography (100- × 5-cm column) using methanol as the mobile phase. The fractions showing both a negative Ninhydrin test and a positive Sakaguchi test were pooled and concentrated under reduced pressure. The acetyl tripeptide semicarbazone was redissolved in 5 mL of methanol and 1 mL of 0.5 M HCl, chilled to 0 °C, and treated with 75 μL (1.0 mmol) of 37% formaldehyde. The mixture was stirred at 0 °C for 4 h. The crude product was solidified by the addition of 100 mL of cold anhydrous tetrahydrofuran. The crude solid was purified via Sephasorb H-P chromatography (100- × 5-cm column) using methanol as the mobile phase. The fractions demonstrating both positive Sakaguchi and 2,4-DNP tests were pooled and concentrated under reduced pressure. The resulting acetyl tripeptide aldehydes (**2a–i**) were solidified by the addition of anhydrous ether and recrystallized from methanol/ether.

Acetyl-L-valyl-L-leucyl-L-argininal hydrochloride (2a): mp 151–153 °C; $[\alpha]^{25}_{Na}$ = −31.7° (c 1.0, methanol); R_f = 0.35 (ethanol/water, 6/4, v/v, on Whatman MKC$_{18}$F reversed phase TLC); ^{1}H NMR (DMSO-d_6, 200 MHz) δ 0.9 (d, J = 7 Hz, 12 H), 1.3 (m, 8 H), 2.5 (s, 3 H), 2.9 (t, J = 7 Hz, 2 H), 4.1 (m, 3 H), 4.6 (m, 1 H), 5.4 (m, 2 H), 6.0 (m, 0.6 H, carbinolamine), 7.6 (m, 3 H), 9.9 (s, 0.4 H, CHO). Anal. ($C_{19}H_{37}N_6O_4Cl$) C, H, N.

Acetyl-L-leucyl-L-valyl-L-argininal hydrochloride (2b): mp 152–154 °C; $[\alpha]^{25}_{Na}$ = −35.2° (c 1.0, methanol); R_f = 0.31 (ethanol/water, 6/4, v/v, on Whatman MKC$_{18}$F reversed-phase TLC); ^{1}H NMR (DMSO-d_6, 200 MHz) δ 0.8 (d, J = 7 Hz, 12 H), 1.2 (m, 8 H), 2.5 (s, 3 H), 3.0 (t, J = 7 Hz, 2 H), 4.2 (m, 3 H), 4.8 (m, 1 H), 5.3 (m, 2 H), 6.1 (m, 0.7 H, carbinolamine), 7.4 (m, 3 H), 10.0 (s, 0.3 H, CHO). Anal. ($C_{19}H_{37}N_6O_4Cl$) C, H, N.

Acetyl-L-leucyl-L-isoleucyl-L-argininal hydrochloride (2c): mp 163–165 °C; $[\alpha]^{25}_{Na}$ = −29.8° (c 1.0, methanol); R_f = 0.27 (ethanol/water, 6/4, v/v, on Whatman MKC$_{18}$F reversed phase TLC); ^{1}H NMR (DMSO-d_6, 200 MHz) δ 0.8 (m, 9 H), 1.2 (m, 13 H), 2.6 (s, 3 H), 3.0 (t, J = 7 Hz, 2 H), 4.3 (m, 3 H), 4.7 (m, 1 H), 5.6 (m, 2 H), 6.1 (m, 0.6 H, carbinolamine), 7.4 (m, 3 H), 10.1 (s, 0.4 H, CHO). Anal. ($C_{20}H_{39}N_6O_4Cl$) C, H, N.

Acetyl-L-isoleucyl-L-leucyl-L-argininal hydrochloride (2d): mp 158–160 °C; $[\alpha]^{25}_{Na}$ = −23.5° (c 1.0, methanol); R_f = 0.25 (ethanol/water, 6/4, v/v, on Whatman MKC$_{18}$F reversed-phase TLC); ^{1}H NMR (DMSO-d_6, 200 MHz) δ 0.9 (m, 9 H), 1.2 (m, 13 H), 2.5 (s, 3 H), 2.9 (t, J = 7 Hz, 2 H), 4.0 (m, 3 H), 4.5 (m, 1 H), 5.3 (m, 2 H), 6.0 (m, 0.8 H, carbinolamine), 7.5 (m, 3 H), 10.0 (s, 0.2 H, CHO). Anal. ($C_{20}H_{39}N_6O_4Cl$) C, H, N.

Acetyl-L-leucyl-L-phenylalanyl-L-argininal hydrochloride (2e): mp 170–172 °C; $[\alpha]^{25}_{Na}$ = −31.6° (c 1.0, methanol); R_f = 0.56 (ethanol/water, 6/4, v/v, on Whatman MKC$_{18}$F reversed-phase TLC); ^{1}H NMR (DMSO-d_6, 200 MHz) δ 0.9 (d, J = 7 Hz, 6 H), 1.3 (m, 7 H), 2.4 (s, 3 H), 3.0 (m, 4 H), 4.2 (m, 3 H), 4.8 (m, 1 H), 5.7 (m, 2 H), 6.1 (m, 1 H, carbinolamine), 7.0 (s, 5 H), 7.5 (m, 3 H). Anal. ($C_{23}H_{37}N_6O_4Cl$) C, H, N.

Acetyl-L-phenylalanyl-L-leucyl-L-argininal hydrochloride (2f): mp 175–177 °C; $[\alpha]^{25}_{Na}$ = −41.6° (c 1.0, methanol); R_f = 0.63 (ethanol/water, 6/4, v/v, on Whatman MKC$_{18}$F reversed-phase TLC); ^{1}H NMR (DMSO-d_6, 200 MHz) δ 1.0 (d, J = 7 Hz, 6 H), 1.4 (m, 7 H), 2.3 (s, 3 H), 3.1 (m, 4 H), 4.3 (m, 3 H), 5.0 (m, 1 H), 5.6 (m, 2 H), 6.0 (m, 0.7 H, carbinolamine), 7.1 (s, 5 H), 7.7 (m, 3 H), 9.9 (s, 0.3 H, CHO). Anal. ($C_{23}H_{37}N_6O_4Cl$) C, H, N.

Acetyl-L-leucyl-L-tyrosinyl-L-argininal hydrochloride (2g): mp 186–190 °C; $[\alpha]^{25}_{Na}$ = −16.3° (c 1.0, methanol); R_f = 0.87 (ethanol/water, 6/4, v/v, on Whatman MKC$_{18}$F reversed-phase TLC); ^{1}H NMR (DMSO-d_6, 200 MHz) δ 1.0 (d, J = 7 Hz, 6 H), 1.3 (m, 7 H), 2.4 (s, 3 H), 3.2 (m, 4 H), 4.3 (m, 3 H), 4.9 (m, 1 H), 5.5 (m, 2 H), 6.0 (m, 1 H, carbinolamine), 6.7 (s, 1 H), 7.2 (m, 3 H), 7.8 (m, 4 H). Anal. ($C_{23}H_{37}N_6O_5Cl$) C, H, N.

Acetyl-L-tyrosinyl-L-leucyl-L-argininal hydrochloride (2h): mp 192–195 °C; $[\alpha]^{25}_{Na}$ = −19.7° (c 1.0, methanol); R_f = 0.92 (ethanol/water, 6/4, v/v, on Whatman MKC$_{18}$F reversed-phase TLC); ^{1}H NMR (DMSO-d_6, 200 MHz) δ 0.9 (d, J = 7 Hz, 6 H), 1.2 (m, 7 H), 2.5 (s, 3 H), 3.0 (m, 4 H), 4.3 (m, 3 H), 5.0 (m, 1 H), 5.8 (m, 2 H), 6.1 (m, 0.5 H, carbinolamine), 6.5 (s, 1 H), 7.1 (m, 3 H), 7.9 (m, 4 H), 10.1 (s, 0.5 H, CHO). Anal. ($C_{23}H_{37}N_6O_5Cl$) C, H, N.

Acetyl-L-phenylalanyl-L-valyl-L-argininal hydrochloride (2i): mp 166–168 °C; $[\alpha]^{25}_{Na}$ = −38.5° (c 1.0, methanol); R_f = 0.70 (ethanol/water, 6/4, v/v, on Whatman MKC$_{18}$F reversed-phase TLC); ^{1}H NMR (DMSO-d_6, 200 MHz) δ 1.0 (d, J = 7 Hz, 6 H), 1.4 (m, 5 H), 2.4 (s, 3 H), 3.0 (m, 4 H), 4.3 (m, 3 H), 4.7 (m, 1 H), 5.8 (m, 2 H), 6.1 (m, 0.6 H, carbinolamine), 7.0 (s, 5 H), 7.7 (m, 3 H), 10.0 (s, 0.4 H, CHO). Anal. ($C_{22}H_{35}N_6O_4Cl$) C, H, N.

Acetyl-L-leucyl-L-leucyl-L-lysinal hydrochloride (2j): mp 150–153 °C; $[\alpha]^{25}_{Na}$ = −43.1° (c 1.0, methanol); R_f = 0.70 (methanol/water, 7/3, v/v, on Whatman MKC$_{18}$F reversed-phase TLC); ^{1}H NMR (DMSO-d_6, 200 MHz) δ 0.8 (d, J = 7 Hz, 12 H), 1.1 (m, 12 H), 2.3 (s, 3 H), 2.9 (t, J = 7 Hz, 2 H), 4.0 (m, 3 H), 4.9 (m, 1 H), 5.5 (m, 1 H), 6.3 (m, 0.9 H, CHO cyclized), 9.9 (s, 0.1 H, CHO). Anal. ($C_{20}H_{39}N_4O_4Cl$) C, H, N.

Acetyl-L-valyl-L-leucyl-L-lysinal hydrochloride (2k): mp 159–161 °C; $[\alpha]^{25}_{Na}$ = −27.6° (c 1.0, methanol); R_f = 0.67 (methanol/water, 7/3, v/v, on Whatman MKC$_{18}$F reversed-phase TLC); ^{1}H NMR (DMSO-d_6, 200 MHz) δ 0.9 (d, J = 7 Hz, 12 H), 1.2 (m, 10 H), 2.2 (s, 3 H), 3.0 (t, J = 7 Hz, 2 H), 4.2 (m, 3 H), 5.3 (m, 2 H), 6.4 (m, 0.8 H, CHO cyclized), 10.0 (s, 0.2 H, CHO). Anal. ($C_{19}H_{37}N_4O_4Cl$) C, H, N.

Acetyl-L-leucyl-L-valyl-L-lysinal hydrochloride (2l): mp 160–163 °C; $[\alpha]^{25}_{Na}$ = −22.5° (c 1.0, methanol); R_f = 0.66 (methanol/water, 7/3, v/v, on Whatman MKC$_{18}$K reversed-phase TLC); ^{1}H NMR (DMSO-d_6, 200 MHz) δ 1.0 (d, J = 7 Hz, 12 H), 1.3 (m, 10 H), 2.4 (s, 3 H), 3.1 (t, J = 7 Hz, 2 H), 4.1 (m, 3 H), 4.9 (m, 1 H), 5.2 (m, 1 H), 6.5 (m, 0.8 H, CHO cyclized), 9.9 (s, 0.2 H, CHO). Anal. ($C_{19}H_{37}N_4O_4Cl$) C, H, N.

Acetyl-L-leucyl-L-phenylalanyl-L-lysinal hydrochloride (2m): mp 167–170 °C; $[\alpha]^{25}_{Na}$ = −37.5° (c 1.0, methanol); R_f = 0.51 (methanol/water, 7/3, v/v, on Whatman MKC$_{18}$F reversed-phase TLC); ^{1}H NMR (DMSO-d_6, 200 MHz) δ 0.9 (d, J = 7 Hz, 6 H), 1.3 (m, 9 H), 2.4 (s, 3 H), 3.0 (m, 4 H), 4.2 (m, 3 H), 4.9 (m, 1 H), 5.7 (m, 1 H), 6.5 (m, 0.9 H, CHO cyclized), 10.0 (s, 0.1 H, CHO). Anal. ($C_{23}H_{37}N_4O_4Cl$) C, H, N.

Acetyl-L-phenylalanyl-L-leucyl-L-lysinal hydrochloride (2n): mp 173–175 °C; $[\alpha]^{25}_{Na}$ = −33.6° (c 1.0, methanol); R_f = 0.43 (methanol/water, 7/3, v/v, on Whatman $MKC_{18}F$ reversed-phase TLC); ^1H NMR (DMSO-d_6, 200 MHz) δ 0.8 (d, J = 7 Hz, 6 H), 1.4 (m, 9 H), 2.5 (s, 3 H), 3.1 (m, 4 H), 4.3 (m, 3 H), 5.2 (m, 1 H), 5.6 (m, 1 H), 6.5 (m, 0.8 H, CHO cyclized), 10.0 (s, 0.2 H, CHO). Anal. ($C_{23}H_{37}N_4O_4Cl$) C, H, N.

Acetyl-L-isoleucyl-L-leucyl-L-lysinal hydrochloride (2o): mp 148–151 °C; $[\alpha]^{25}_{Na}$ = −38.9° (c 1.0, methanol); R_f = 0.86 (methanol/water, 7/3, v/v, on Whatman $MKC_{18}F$ reversed-phase TLC); ^1H NMR (DMSO-d_6, 200 MHz) δ 0.9 (m, 9 H), 1.1 (m, 14 H), 2.4 (s, 3 H), 3.0 (t, J = 7 Hz, 2 H), 4.3 (m, 3 H), 4.8 (m, 1 H), 5.3 (m, 1 H), 6.6 (m, 0.9 H, CHO cyclized), 10.0 (s, 0.1 H, CHO). Anal. ($C_{20}H_{39}N_4O_4Cl$) C, H, N.

Inhibitor Activity Measurements. Rate measurements were determined with the aid of a Coleman/Hitachi Model 124 spectrophotometer equipped with temperature-controlled cuvette holders. The assays were performed using commercially available (Sigma) enzymes and chromogenic substrates in 1-cm quartz cuvettes. In each assay the buffer was kept at reaction temperature. The buffer and enzyme, with or without inhibitor, were preincubated for 2.5 min prior to the addition of the substrate. The inhibition of the enzyme activity was measured at least three times at four or more inhibitor concentrations. The average change in absorbance at each concentration was utilized in the calculations of percent inhibition. All values were within 0.05 standard deviations from the mean. The percent inhibition of the enzymatic reactions were calculated as follows:

$$\% I = (A - B)/A \times 100$$

where A = the change in absorbance without inhibitor and B = the change in absorbance with inhibitor. The concentration of the inhibitor inducing a 50% inhibition (IC_{50}) was obtained in each case by plotting the percent inhibition against the log of the inhibitor concentration. Standard errors for the linear regression plots were calculated and are in each case less than 7%.

Inhibition of Trypsin Activity. A mixture of 0.20 mL of 0.1 mg/mL trypsin (type I from bovine pancreas) in 0.001 M aqueous HCl, 2.55 mL of 0.1 M sodium phosphate buffer (pH 7.6), and 0.05 mL of dimethyl sulfoxide with or without inhibitor was incubated at 37 °C for 2.5 min. The addition of 0.20 mL of 0.008 M benzoyl-L-arginine ethyl ester in distilled water initiated the reaction. The cuvette was shaken, and the change in absorbance was measured at 253 nm over 1 min.

Inhibition of Kallikrein Activity. A mixture of 0.10 mL of a 0.32 BAEE units/mL human plasma kallikrein solution, 0.65 mL of 0.50 M tris(hydroxymethyl)aminomethane buffer (pH 8.0) containing 0.011 M sodium chloride, and 0.05 mL of dimethyl sulfoxide with or without inhibitor was incubated at 37 °C for 2.5 min. The substrate (0.20 mL of a 0.04 M benzoyl-L-arginine ethyl ester in distilled water) was added and the cuvette shaken. The change in absorbance was measured at 253 nm over 1 min.

Inhibition of Thrombin Activity. A mixture of 0.25 mL of a 19.7 NIH units/mL thrombin solution (from bovine plasma) solution and 0.58 mL of a 0.01 M tris(hydroxymethyl)aminomethane buffer (pH 8.0), with or without inhibitor, was incubated at 37 °C for 2.5 min. The reaction was initiated by the addition of 0.100 mL of a 0.0005 M benzoyl-L-phenylalanyl-L-valyl-L-arginine-p-nitroanilide solution in 6.8% dimethyl sulfoxide/buffer. The mixture was shaken, and the change in absorbance was measured at 405 nm for 1 min.

Inhibition of Plasmin Activity. A mixture of 6.24 mg/mL plasmin solution (from bovine plasma) and 0.55 mL of a 0.45 M tris(hydroxymethyl)aminomethane buffer (pH 8.0) with 0.011 M NaCl, with or without inhibitor, was incubated at 37 °C for 2.5 min. The reaction was then initiated by the addition of the substrate (0.05 mL of a 0.0031 M D-valyl-L-leucyl-L-lysine p-nitroanilide). The change in absorbance was measured at 405 nm over 1 min.

Inhibition of Cathepsin B Activity. A solution of 0.015 mL of 0.163 mg/mL cathepsin B from bovine spleen in 0.10 M sodium phosphate buffer (pH 6.0), containing 0.10 mg/mL DL-dithiothreitol, and 0.935 mL of 0.10 M sodium phosphate buffer (pH 6.0), with or without inhibitor, was incubated at 30 °C for 2.5 min. The substrate (0.050 mL of 0.0024 M N^{α}-carbobenzyloxy-L-lysine p-nitrophenyl ester in distilled water) was added to the mixture to initiate the enzymatic reaction. The mixture was shaken, and the change in absorbance at 405 nm was measured for 1 min.

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