(6*R-trans*)-3-(iodomethyl)-8-oxo-7-[(phenoxyacetyl)amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid *tert*-butyl ester, 115622-66-7; (6*R-trans*)-3-(iodomethyl)-8-oxo-7-[(phenoxyacetyl)amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid allyl ester, 115622-74-7; [6*R*-[6α,7β(*Z*)]]-7-[[(2-tritylamino-4-thiazolyl)(methoxyimino)acetyl]amino]-3-(iodomethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid *tert*-butyl ester, 105514-47-4; [6*R*-[6α,7β(*Z*)]]-7-[[(2-tritylamino-4-thiazolyl)[[2-(1-*tert*-butylcarboxylate-1-methyl)ethoxy]imino]acetyl]amino]-3-(iodomethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid *tert*-butyl ester, 117333-74-1; [6*R*-(6α,7β)]-3-(iodomethyl)-7-[(2-thienylacetyl)amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid *tert*-butyl ester, 37553-14-3; (6*R-trans*)-7-(formylamino)-3-(iodomethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid *tert*-butyl ester, 122949-51-3; [6*R*-[6α,7β(*R**)]]-7-[[2-[[(4-ethyl-2,3-dioxopiperazinyl)carbonyl]amino]-2-phenylacetyl]amino]-3-(iodomethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (diphenylmethyl) ester, 122949-52-4; [6*R*-[6α,7β(*Z*)]]-7-[[(2-tritylamino-4-thiazolyl)[[(*tert*-butylcarboxylate)methoxy]imino]acetyl]amino]-3-(iodomethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid *tert*-butyl ester, 122949-57-9.

New Leupeptin Analogues: Synthesis and Inhibition Data

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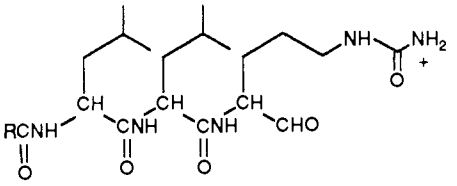
Syntheses of several tripeptide analogues of leupeptin containing C-terminal argininal, lysinal, or ornithinal units are presented. The synthetic analogues were tested as inhibitors of trypsin, plasmin, and kallikrein. (Benzyloxycarbonyl)-L-leucyl-L-leucyl-L-argininal (**2a**) was significantly less effective as an inhibitor of trypsin and plasmin activity than leupeptin. (Benzyloxycarbonyl)-L-leucyl-L-leucyl-L-lysinal (**2e**) and (benzyloxycarbonyl)-L-leucyl-L-leucyl-L-ornithinal (**2i**) display different inhibition characteristics than (benzyloxycarbonyl)-L-leucyl-L-leucyl-L-argininal (**2a**). While (benzyloxycarbonyl)-L-leucyl-L-leucyl-L-argininal (**2a**) showed moderate inhibition of all three enzymes tested, (benzyloxycarbonyl)-L-leucyl-L-leucyl-L-lysinal (**2e**) was less effective as an inhibitor of trypsin and plasmin activity. Of the three enzymes tested, (benzyloxycarbonyl)-L-leucyl-L-leucyl-L-ornithinal (**2i**) showed significant inhibition of kallikrein activity only. Modifications made in the composition and sequence of the P_2 and P_3 amino acids also resulted in variations in the inhibitory activity of the analogues. In general, plasmin showed a strong preference for inhibitors which contain an L-phenylalanyl-L-leucyl or an L-leucyl-L-valyl unit in the P_2 and P_3 positions.

Leupeptin (**1**), *N*-acetyl- or *N*-propionyl-L-leucyl-L-leucyl-DL-argininal, is a naturally occurring proteinase inhibitor isolated from the culture filtrates of various species of actinomyces.[1-3] Leupeptin has been shown to be a very

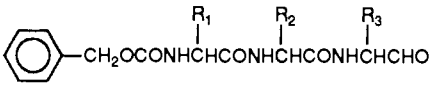


1: R = CH_3, CH_2CH_3

potent inhibitor of a number of proteolytic enzymes.[4-12] Leupeptin has also been shown to alter or suppress the symptoms of such disease conditions as rheumatoid arthritis,[1-13] muscular dystrophy,[14-18] allergic encephalomyelitis,[19] malaria,[20] and immunological dysregulations.[21,22] However, leupeptin is not selective among enzymes of similar substrate specificities, thus limiting its usefulness in the investigations of disease processes and as a therapeutic agent.

Several derivatives of leupeptin have been prepared that provide interesting insights into variation of biological activity with inhibitor structure.[23-25] The derivatives in which the C-terminal aldehyde of leupeptin was reduced to the alcohol, oxidized to the carboxylic acid, or protected as the dibutyl acetal[26] showed no inhibition of most enzymes that are strongly inhibited by leupeptin. Umezawa and co-workers studied the effect of minor variations in the amino acid composition and sequence of leupeptin.[23,25] Using a synthetic route which he later abandoned, Ume-

Table I. Inhibitors



no.	R_1	R_2	R_3
2a	$CH_2CH(CH_3)_2$	$CH_2CH(CH_3)_2$	$(CH_2)_3NHC(NH)NH_2$
2b	$CH_2CH(CH_3)_2$	CH_2Ph	$(CH_2)_3NHC(NH)NH_2$
2c	CH_2Ph	$CH_2CH(CH_3)_2$	$(CH_2)_3NHC(NH)NH_2$
2d	$CH_2CH(CH_3)_2$	$CH(CH_3)_2$	$(CH_2)_3NHC(NH)NH_2$
2e	$CH_2CH(CH_3)_2$	$CH_2CH(CH_3)_2$	$(CH_2)_4NH_2$
2f	$CH_2CH(CH_3)_2$	CH_2Ph	$(CH_2)_4NH_2$
2g	CH_2Ph	$CH_2CH(CH_3)_2$	$(CH_2)_4NH_2$
2h	$CH_2CH(CH_3)_2$	$CH(CH_3)_2$	$(CH_2)_4NH_2$
2i	$CH_2CH(CH_3)_2$	$CH_2CH(CH_3)_2$	$(CH_2)_3NH_2$
2j	$CH_2CH(CH_3)_2$	CH_2Ph	$(CH_2)_3NH_2$
2k	CH_2Ph	$CH_2CH(CH_3)_2$	$(CH_2)_3NH_2$
2l	$CH_2CH(CH_3)_2$	$CH(CH_3)_2$	$(CH_2)_3NH_2$

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[§] Genentech Corp.
[∥] Dept. of Biochemistry & Biophysics, Texas A&M University.

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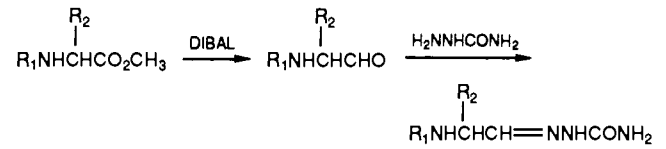
zawa prepared several leupeptin analogues with C-terminal argininal units.[23,24] These compounds were tested for their inhibition of plasmin fibrinogenolysis and papain caseinolysis and showed large variations in activity.[23] Later, after the development of a new semisynthetic route to leupeptin analogues involving an enzymatic cleavage of argininal dibutyl acetal from the protected leupeptin,[27] Umezawa prepared other analogues containing C-terminal argininal units.[25] Many of these new inhibitors show dramatic increases in their inhibitory activities of trypsin-like proteinases relative to leupeptin. For example, (benzyloxycarbonyl)-L-pyroglutamyl-L-leucyl-L-argininal was reported to be 10-fold more active than leupeptin as an inhibitor of trypsin and plasmin.[25] Also, DL-2-pipecolyl-L-leucyl-L-argininal was reported to be 25-fold more active than leupeptin as an inhibitor of kallikrein.[25] Ito and co-workers also found that by altering the side chain of the C-terminal aldehyde the specificity of the inhibitor could be changed considerably.[28] When the C-terminal argininal unit of leupeptin was replaced by phenylalaninal, tyrosinal, or tryptophanal, chymotrypsin was inhibited, but the inhibition of trypsin-like proteinases was lost. So it appears that the specificity within this mechanistic class of proteinases arises from the structure of the side chain of the C-terminal aldehyde and the composition and sequence of the peptide as a whole.

With these considerations in mind, we have undertaken the preparation of tripeptide analogues of leupeptin. Our goal was to study the effects of three distinct types of structural alterations (Table I) on the inhibitory activities and specificities among trypsin-like serine proteinases on the analogues. The first type of structural alteration studied was the N-terminal protecting group. Although there are no significant differences reported in the activities of *N*-acetyl- and *N*-propionylleupeptins,[29] there have been instances reported in the literature where the nature of the N-terminal protecting group altered the activity of trypsin-like enzyme inhibitors.[25,30,31] The second type of structural alteration examined in our leupeptin analogues was the effect of minor variations in the length and basicity of the side chain on the C-terminal aldehyde. The third type of structural change involved variations in the composition and sequence of the amino acids in the P_2 and P_3 positions[32] on the inhibitors. The synthesis and biological activities of these compounds are reported below.

Scheme I



4a: R_1 = Cbz, R_2 = $(CH_2)_4NH$—BOC
b: R_1 = Cbz, R = $(CH_2)_3NH$—BOC
c: R_1 = BOC, R_2 = $(CH_2)_3NHC(NH)NHNO_2$

Table II. Chemical Data

no.	compound[a]	mp, °C	$[\alpha]^{25}_{Na}$, deg
2a	Cbz-Leu-Leu-argininal	164–166	−24.7
2b	Cbz-Leu-Phe-argininal	176–178	−12.3
2c	Cbz-Phe-Leu-argininal	170–172	−15.5
2d	Cbz-Leu-Val-argininal	160–162	−16.4
2e	Cbz-Leu-Leu-lysinal	179–181	−39.9
2f	Cbz-Leu-Phe-lysinal	142–144	−33.3
2g	Cbz-Phe-Leu-lysinal	148–150	−18.8
2h	Cbz-Leu-Val-lysinal	134–136	−26.8
2i	Cbz-Leu-Leu-ornithinal	155–157	−37.6
2j	Cbz-Leu-Phe-ornithinal	133–135	−28.6
2k	Cbz-Phe-Leu-ornithinal	149–152	−18.7
2l	Cba-Leu-Val-ornithinal	139–141	−18.2

[a] Cbz = benzyloxycarbonyl.

Synthesis

The preparation of peptide aldehydes containing basic amino acids, especially arginine, in the C-terminal position is seldom seen in the literature. In his early synthesis of leupeptin,[4] Umezawa reduced the peptide containing a C-terminal arginine methyl ester to the alcohol with lithium borohydride and then reoxidized it to the aldehyde via DMSO/DCC.[29] This low-yield procedure was later abandoned by Umezawa in favor of a semisynthetic route involving an enzymatic cleavage of argininal dibutyl acetal from the protected leupeptin.[27] Ito and co-workers prepared *N*-(benzyloxycarbonyl)-N^G-nitroargininal semicarbazone using a diisobutylaluminum hydride reduction of the corresponding methyl ester.[33] Leupeptin analogues containing phenylalaninal, tyrosinal, and tryptophanal were prepared in reasonable yields by using this reduction technique;[34] however, no leupeptin analogues containing

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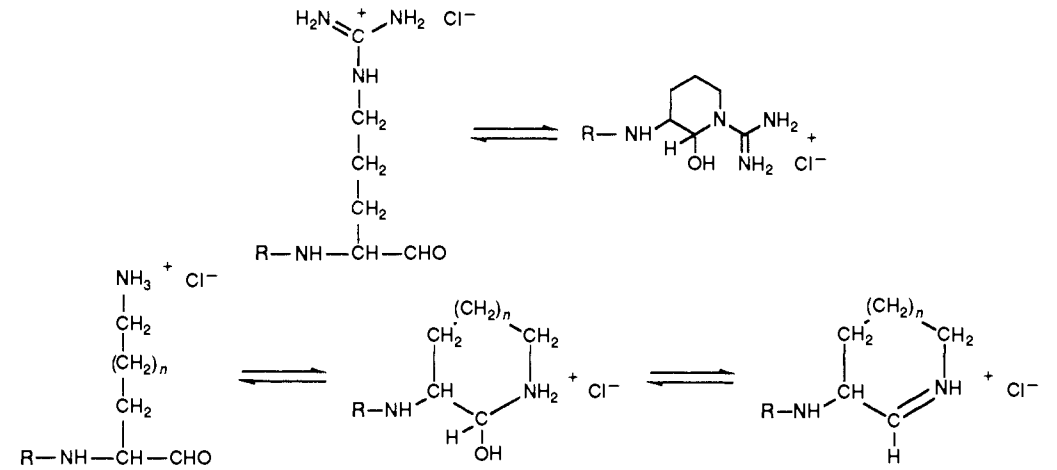

Figure 1. Cyclization of argininal (top), lysinal, and ornithinal (bottom) leupeptin analogues.

C-terminal argininal units prepared in this manner were reported. Galpin and co-workers in their development of chymostatin analogues have reported the synthesis of several peptide aldehydes which contain arginine as one of the amino acid units.[35-37] However, in these peptide aldehydes arginine occupies a P_2 or P_3 position rather than the C-terminal P_1 position.

Synthesis of the leupeptin analogues was accomplished through the use of lysine, ornithine, and arginine amino aldehyde derivatives prepared by diisobutylaluminum hydride reduction of the corresponding amino acid methyl esters (**3**). The three fully protected amino acid esters were each reduced directly to the aldehyde by careful treatment with diisobutylaluminum hydride using harsher conditions than is typically required for other amino acid esters. Without isolation the protected aldehydes were immediately converted to the semicarbazone derivatives (Scheme I) (**4a-c**).

In the preparation of lsyine and ornithine analogues, the N^{α}-benzyloxycarbonyl groups were removed by catalytic hydrogenolysis.[38] The resultant α-amino aldehyde semicarbazones (**5a,b**) were coupled to a series of benzyloxycarbonyl dipeptide acids via a mixed carbonic anhydride procedure[38] to yield the fully protected tripeptide aldehyde derivatives (**6a-h**). The *tert*-butoxycarbonyl groups were removed and the aldehyde was deprotected by treatment with formalin/HCl to obtain the lysinal and ornithinal leupeptin analogues (**2e-l**) in 25-65% yield (Table II).

The synthesis of the argininal analogues was performed by a deprotection/reprotection approach. Treatment of N^{α}-(*tert*-butoxycarbonyl)-N^{G}-nitroargininal semicarbazone (**4c**) with trifluoroacetic acid provided N^{G}-nitroargininal semicarbazone trifluoroacetate (**7**), which was condensed with a series of benzyloxycarbonyl-protected dipeptides to yield the fully protected tripeptide aldehyde derivatives (**8a-d**). The N-terminal benzyloxycarbonyl and the N^{G}-nitro groups were removed by catalytic hydrogenolysis to yield the tripeptide aldehyde semicarbazone dihydrochloride salts. Reacylation of the N-terminus with benzylchloroformate and pyridine provided the benzyloxycarbonyl-protected argininal semicarbazone hydrochlorides. Removal of the semicarbazone was effected by the treatment with 37% formalin and hydrochloric acid to provide the argininal analogues (**2a-d**) (Table II).

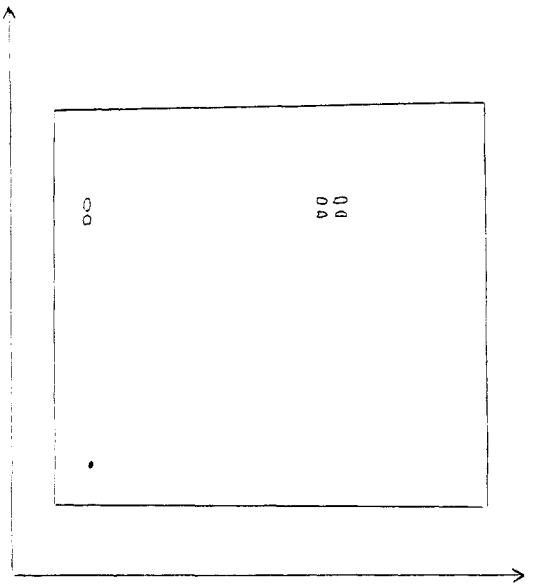

Figure 2. Representative two-dimensional thin-layer chromatogram of leupeptin analogues (1-butanol/water/acetic acid, 7/3/1, v/v/v).

All spectroscopic data are cosistent with the structures although ^{1}H and ^{13}C NMR spectral data show that in methanolic solutions considerable cyclization occurs. The cyclized carbinolamine (Figure 1) appears to be the major form of the lysinal analog (**2e-h**), although trace amounts of the free aldehyde were also observed in the NMR. Although no evidence of the iminium structure was seen in any of the lysinal analogues, it was identified by ^{13}C NMR in the ornithinal analogues (**2i-l**) by resonances at 162-163 ppm. Resonances for the carbinolamine were also observed at 137-138 ppm in the ornithinal analogs (**2i-l**); however, no evidence of the free aldehyde was observed by NMR in any of the methanolic solutions of the ornithinal analogues. Assignments of NMR resonances were based on spectral data of similar systems.[39] This, however, does not preclude the existence of the free aldehyde or hydrated aldehyde form of the analogues in aqueous solutions. Since the free aldehyde is believed to be the form which is bound by the enzyme,[34,40] an equilibrium would

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Table III. Inhibition Data

no.	inhibitor	I_{50}, μM trypsin	plasmin	kallikrein
1	leupeptin[a]	5.2	19.4	32.4
2a	Cbz-Leu-Leu-argininal	163.8	127.8	36.0
2b	Cbz-Leu-Phe-argininal	107.1	79.9	33.3
2c	Cbz-Phe-Leu-argininal	45.9	10.2	25.7
2d	Cbz-Leu-Val-argininal	81.4	10.2	11.2
2e	Cbz-Leu-Leu-lysinal	195.7	183.7	19.0
2f	Cbz-Leu-Phe-lysinal	400[b]	47.0	20.3
2g	Cba-Phe-Leu-lysinal	400[b]	8.9	25.6
2h	Cbz-Leu-Val-lysinal	400[b]	18.5	14.8
2i	Cbz-Leu-Leu-ornithinal	400[b]	>400[b]	29.3
2j	Cbz-Leu-Phe-ornithinal	400[b]	>400[b]	82.4
2k	Cbz-Phe-Leu-ornithinal	400[b]	241.6	111.8
2l	Cbz-Leu-Val-ornithinal	400[b]	432.0	53.8

[a] Acetyl-L-leucyl-L-leucyl-argininal. [b] No inhibition at 400 μM.

shift in that direction under the testing conditions. Evidence for the existence of an equilibrium state between two major forms of each inhibitor was seen by two-dimensional thin-layer chromatography (Figure 2). Using butanol, water, and acetic acid as the mobile phase, two structures were partitioned on silica gel; however, a second elution of the same solvent partitioned each of the first two spots into two new spots. This indicates that a fairly slow equilibrium, in which there are two major components, exists in this solvent mixture. Similar observations of both the cyclization and the thin layer chromatography effect were made by Umezawa in his initial studies of leupeptin.[4]

Biological Activities and Discussion

Although Umezawa saw no real differences in the inhibitory activities of *N*-acetyl and *N*-propionyl leupeptins,[29] we have now discovered that the use of a N-terminal benzyloxycarbonyl protection in (benzyloxycarbonyl)-L-leucyl-L-leucyl-L-argininal (**2a**) did result in a decrease in the inhibition of trypsin and plasmin from that of leupeptin (Table III). This might be due to steric restrictions at the S_4 binding sites in these enzymes.

The alterations made in the basic side chain of the C-terminal aldehyde also caused significant variation in selectivity. The argininal analogues (**2a–d**) tended to be better inhibitors of trypsin and plasmin than were the lysinal and ornithinal analogues. The general preference by trypsin for arginine peptides is in agreement with crystallographic studies by Bode[41] which show direct binding at the S_1 site of arginine side chains and indirect binding of lysine side chains. The lack of major variation in the inhibition of kallikrein hydrolysis with the alterations made in the side chain of the C-terminal aldehyde of the leupeptin analogues is also in agreement with crystallographic studies[42] which show a more flexible S_1 binding site present in kallikrein. Also, the use of the N-terminal benzyloxycarbonyl group in **2a** did not seem to cause as dramatic a decrease in activity from that of leupeptin in the case of kallikrein inhibition as it did with trypsin and plasmin inhibition. These properties can be useful in the design of selective kallikrein inhibitors. For example, (benzyloxycarbonyl)-L-leucyl-L-leucyl-L-ornithinal (**2i**) shows an inhibition of kallikrein comparable to that of leupeptin (Table III); however, it shows no significant inhibition of trypsin or plasmin.

The variations made in the composition and sequence of the amino acids in the P_2 and P_3 positions of the leupeptin analogues involved the usage of phenylalanine and valine, which are similar in hydrophobic nature to leucine. Our results show that the use of L-phenylalanine in the P_2 position, as in **2b,f,j**, caused a slight increase in activity for plasmin inhibition from those containing L-leucine in the P_2 position (**2a,e,i**). It was also discovered that those analogues of leupeptin containing an L-phenylalanyl-L-leucyl or an L-leucyl-L-valyl unit in the P_3 and P_2 positions proved to be much better inhibitors of plasmin than did the analogues of other peptide sequences (Table III). Some of these analogues are better inhibitors of these enzymes than leupeptin itself, despite having N-terminal benzyloxycarbonyl protecting groups.

Summary

Several analogues of leupeptin were prepared through the use of three key amino aldehyde derivatives. These amino aldehyde derivatives were synthesized and then coupled to a series of benzyloxycarbonyl-protected dipeptide acids. Deprotection of the resulting tripeptide aldehyde derivatives provided leupeptin analogues containing either a C-terminal argininal, lysinal, or ornithinal. The leupeptin analogues were analyzed by traditional spectrophotometric assay techniques for their inhibitory activities of trypsin, plasmin, and kallikrein.

The use of a N-terminal benzyloxycarbonyl protecting group caused a decrease from that of leupeptin in the inhibition of trypsin and plasmin activity. Of the three enzymes tested, the ornithinal analogues showed significant inhibition of kallikrein activity only. The analogues containing either an L-phenylalanyl-L-leucyl or an L-leucyl-L-valyl unit in the P_3 and P_2 positions were found to be much better inhibitors of plasmin than were analogues containing other peptide sequences. The inhibitors described here compare well to other low molecular weight inhibitors that possess minimal functionality. Further testing of these inhibitors and others containing different N-terminal protecting groups and peptide sequences with other trypsin-like proteinases is currently under way and will be reported in due course.

Experimental Section

All amino acids and protected amino acids were obtained from Sigma Chemical Co. unless otherwise noted. The human urinary kallikrein used was obtained from The Peptide Institute, Osaka, Japan. All other enzymes, enzyme substrates, and the benzyloxycarbonyl-protected dipeptides were purchased from Vega Biochemicals. 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 Varian T-60, EM-390, or XL-200 spectrometer. All peptides and peptide derivatives were homogeneous by HPLC and thin layer chromatographic analysis unless otherwise indicated. HPLC was performed on an Altex Ultrasphere-ODS C_{18} reverse-phase column (200 mm × 4.5 mm).

N^{α}-(Benzyloxycarbonyl)-N^{ϵ}-(*tert*-Butoxycarbonyl)-L-lysinal Semicarbazone (4a). A stirred solution of N^{α}-(benzyloxycarbonyl)-N^{ϵ}-(*tert*-butoxycarbonyl)-L-lysine methyl ester (13 mmol) in 150 mL of anhydrous toluene was chilled to −50 °C under a nitrogen atmosphere and slowly treated with 33 mL of a 1 M diisobutylaluminum hydride suspension in hexanes. The mixture was stirred at −50 °C for 3 h and then quenched with 100 mL of 0.1 M aqueous hydrochloric acid. The reaction mixture was extracted three times with 150-mL portions of ethyl acetate. The organic phase was washed with water, dried over anhydrous magnesium sulfate, and evaporated under reduced pressure to yield a clear oil (3.9 g). The crude aldehyde was dissolved in 50 mL of 80% ethyl alcohol and treated with 13 mmol of semicarbazide hydrochloride and 13 mmol of sodium acetate. The mixture was refluxed for 45 min and then stirred overnight at room temperature. The solution was then diluted with 100 mL of water and extracted with ethyl acetate. The organic phase was

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washed with water, dried over anhydrous magnesium sulfate, and evaporated under reduced pressure. The crude semicarbazone product was purified by silica gel chromatography using ethyl acetate/ethanol, (99/1, v/v) as the mobile phase. Compound **4a** had the following: 73% yield; $[\alpha]^{25}_{Na} = -27.9°$ (*c* 1.0, methanol); $R_f = 0.32$ (ethyl acetate/methanol, 9/1, v/v); ^{1}H NMR ($CDCl_3$, 90 MHz) δ 1.3 (s, 9 H), 1.4 (m, 6 H), 3.1 (t, J = 7 Hz, 2 H), 4.4 (m, 1 H), 4.9 (m, 1 H), 5.2 (s, 2 H), 5.7 (m, 1 H), 6.3 (m, 2 H), 7.1 (d, J = 7 Hz, 1 H), 7.4 (s, 5 H), 10.1 (m, 1 H). Anal. Calcd for $C_{20}H_{31}N_5O_5$: C, 56.94; H, 7.41; N, 16.62. Found: C, 57.01; H, 7.67; N, 16.73.

N^{α}-(Benzyloxycarbonyl)-N^{δ}-(*tert*-butoxycarbonyl)-L-ornithinal Semicarbazone (4b). The title compound was synthesized in analogy to **4a** except that N^{α}-(benzyloxycarbonyl)-N^{δ}-(*tert*-butoxycarbonyl)-L-ornithine methyl ester was used instead of N^{α}-(benzyloxycarbonyl)-N^{ϵ}-(*tert*-butoxycarbonyl)-L-lysine methyl ester. Compound **4b** had the following: 71% yield; $[\alpha]^{25}_{Na} = -22.7°$ (*c*, 1.0, methanol); $R_f = 0.21$ (ethyl acetate/methanol, 9/1, v/v); ^{1}H NMR ($CDCl_3$, 90 MHz) δ 1.3 (s, 9 H), 1.6 (m, 4 H), 3.0 (t, J = 7 Hz, 2 H), 4.2 (m, 1 H), 4.8 (m, 1 H), 5.0 (s, 2 H), 5.3 (m, 1 H), 6.0 (m, 2 H), 7.3 (d, J = 7 Hz, 1 H), 7.4 (s, 5 H), 10.1 (m, 1 H). Anal. Calcd for $C_{19}H_{29}N_5O_5$: C, 56.10; H, 7.17; N, 17.19. Found: C, 56.30; H, 7.23; N, 17.25.

N^{α}-(*tert*-Butoxycarbonyl)-N^{G}-nitro-L-argininal Semicarbazone (4c). A solution of 5.0 g (15 mmol) of N^{α}-(*tert*-butoxycarbonyl)-N^{G}-nitro-L-arginine methyl ester in 150 mL of anhydrous tetrahydrofuran was chilled to −25 °C under a nitrogen atmosphere, vigorously stirred, and slowly treated with 60 mL of 1 N diisobutylaluminum hydride in hexanes. The mixtures was stirred at −25 °C for 2 h, quenched with 100 mL of 0.1 M aqueous hydrochloric acid, and extracted twice with ethyl acetate. The organic phase was then washed with water and dried over anhydrous magnesium sulfate. The solvent was evaporated under reduced pressure to give 3.7 g of an oily solid. The crude material was dissolved in 50 mL of 70% ethanol and treated with 1.4 g of semicarbazide hydrochloride and 1.1 g of sodium acetate. The mixture was heated to reflux for 30 min and then allowed to stir at room temperature overnight. The solution was then diluted with 100 mL of water and extracted with ethyl acetate. The organic phase was washed with water, dried over anhydrous magnesium sulfate, and evaporated under reduced pressure to give a white solid. The crude product was purified by silica gel chromatography using ethyl acetate/ethanol (95/5, v/v) as the mobile phase to give 2.8 g (52%) of **4c**: $[\alpha]^{25}_{Na} = -33.2°$ (*c* 1.0, methanol); mp = 117–119 °C; $R_f = 0.22$ (ethyl acetate/methanol, 9/1 v/v); ^{1}H NMR (CD_3CN, 90 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), 9.6 (7, 3 H). Anal. Calcd for $C_{12}H_{24}N_8O_5$: C, 39.97; H, 6.72; N, 31.10. Found: C, 39.85; H, 6.76; N, 30.96.

N^{G}-Nitro-L-argininal Semicarbazone Trifluoroacetate (7). A solution containing 0.4 g (1.1 mmol) of N^{α}-(*tert*-butoxycarbonyl)-N^{G}-nitro-L-argininal semicarbazone (**4c**), in 25% trifluoroacetic acid in methanol (v/v), was stirred at 0 °C for 3 h. The solvent was evaporated under reduced pressure and the crude product was crystallized from methanol/ether to yield 0.28 g (86%) of **7**: mp = 134–136 °C; $R_f = 0.22$ (1-butanol/water/acetic acid, 7/3/1, v/v/v); ^{1}H NMR (D_2O, 90 MHz) δ 1.8 (m, 5 H), 3.3 (t, J = 7 Hz, 2 H), 4.2 (m, 1 H), 7.1 (d, J = 7 Hz, 1 H).

(Benzyloxycarbonyl)-L-leucyl-L-leucyl-N^{G}-nitroarginal Semicarbazone (8a). A solution of 0.21 g (0.58 mmol) of (benzyloxycarbonyl)-L-leucyl-L-leucine in 5 mL of anhydrous *N*,*N*-dimethylformamide was chilled to −15 °C with vigorous stirring and treated with 0.08 mL (0.57 mmol) of triethylamine. After a period of 10 min, 0.06 mL (0.62 mmol) of ethyl chloroformate was added and the mixture was stirred for an additional 30 min at −15 °C. A precooled solution containing 0.22 g (0.57 mmol) of N^{G}-nitroarginal semicarbazone trifluoroacetate (**7**) and 0.08 mL (0.057 mmol) of triethylamine in 6 mL anhydrous *N*,*N*-dimethylformamide was added. The resulting mixture was stirred at 0 °C for 3 h and overnight at room temperature. The solution was then partitioned between the two phases of ethyl acetate and 1 M aqueous hydrochloric acid. The organic layer was washed twice with equal volumes of 1 M aqueous hydrochloric acid, 5% aqueous sodium bicarbonate, and distilled water. The organic phase was then dried over anhydrous magnesium sulfate and evaporated under reduced pressure to obtain the solid tripeptide **8a**. The crude material was recrystallized in methanol/ether to yield 0.22 g (35%) of **8a**. The title compound had the following: mp = 125–126 °C; $R_f = 0.76$ (ethyl acetate/methanol, 9/1, v/v); ^{1}H NMR (CD_3CN, 90 MHz) δ 0.9 (d, J = 7 Hz, 12 H), 1.7 (m, 10 H), 3.0 (m, 1 H) 3.3 (t, J = 7 Hz, 2 H), 4.4 (m, 3 H), 4.7 (m, 2 H), 5.2 (s, 2 H), 6.0 (m, 2 H), 7.1 (d, J = 7 Hz, 1 H), 7.4 (s, 5 H), 7.7 (m, 2 H), 8.4 (m, 1 H), 9.6 (m, 1 H). Anal. Calcd for $C_{27}H_{44}N_{10}O_7$: C, 52.25; H, 7.14; N, 22.57. Found: C, 52.63; H, 7.08; N, 22.78.

(Benzyloxycarbonyl)-L-leucyl-L-phenylalanyl-N^{G}-nitroarginal Semicarbazone (8b). This peptide was synthesized in analogy to **8a** except (benzyloxycarbonyl)-L-leucyl-L-phenylalanine instead of (benzyloxycarbonyl)-L-leucyl-L-leucine was used in the coupling reaction. The title compound had the following: 51% yield; mp = 117–119 °C; $R_f = 0.11$ (ethyl acetate/methanol, 9/1, v/v); ^{1}H NMR (CD_3CN, 60 MHz) δ 0.9 (m, 6 H), 1.6 (m, 7 H), 3.1 (m, 4 H), 3.5 (m, 1 H), 4.5 (m, 3 H), 4.9 (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), 9.7 (m, 1 H). Anal. Calcd for $C_{30}H_{42}N_{10}O_7$: C, 55.03; H, 6.47; N, 21.39. Found C, 54.88; H, 6.87; N, 21.67.

(Benzyloxycarbonyl)-L-phenylalanyl-L-leucyl-N^{G}-nitroarginal Semicarbazone (8c). This peptide was prepared in the same manner as **8a** except (benzyloxycarbonyl)-L-phenylalanyl-L-leucine was used instead of (benzyloxycarbonyl)-L-leucyl-L-leucine in the coupling reaction. The title compound displayed the following: 57% yield; mp = 105–107 °C; $R_f = 0.33$ (ethyl acetate/methanol, 9/1, v/v); ^{1}H NMR (CD_3CN, 60 MHz) δ 0.7 (m, 6 H), 1.5 (m, 7 H), 3.0 (m, 4 H), 3.3 (m, 1 H), 4.5 (m, 3 H), 4.9 (m, 2 H), 5.2 (s, 2 H), 5.9 (m, 2 H), 7.0 (d, J = 7 Hz, 1 H), 7.2 (s, 5 H), 7.3 (s, 5 H), 7.9 (m, 2 H), 9.5 (m, 1 H). Anal. Calcd for $C_{30}H_{42}N_{10}O_7$: C, 55.03; H, 6.47; N, 21.39. Found: C, 54.85; H, 6.23; N, 21.10.

(Benzyloxycarbonyl)-L-leucyl-L-valyl-N^{G}-nitroarginal Semicarbazone (8d). This peptide was prepared by the same method as 8a except (benzyloxycarbonyl)-L-leucyl-L-valine was used instead of (benzyloxycarbonyl)-L-leucyl-L-leucine in the coupling reaction. The title compound had the following: 30% yield; mp = 125–127 °C; $R_f = 0.38$ (ethyl acetate/methanol, 9/1, v/v) ^{1}H NMR (CD_3OD, 60 MHz) δ 1.0 (d, J = 7 Hz, 12 H), 1.4 (m, 8 H), 3.1 (m, 2 H), 3.4 (m, 1 H), 4.4 (m, 3 H), 4.9 (m, 2 H), 5.2 (s, 2 H), 5.8 (m, 2 H), 7.0 (d, J = 7 Hz, 1 H), 7.3 (s, 5 H), 7.9 (m, 3 H), 9.6 (m, 1 H). Anal. Calcd for $C_{26}H_{42}N_{10}O_7$: C, 51.47; H, 6.98; N, 23.09. Found: C, 51.62; H, 7.25; N, 23.18.

(Benzyloxycarbonyl)-L-leucyl-L-leucyl-L-argininal Hydrochloride (2a). A solution of 0.09 g (0.14 mmol) of **8a** in 50 mL of methanol and 0.3 mL of 1 M aqueous hydrochloric acid containing 0.05 g of 10% palladium on carbon was stirred under a hydrogen atmosphere for 18 h. The catalyst was removed by filtration and the solvent was evaporated in vacuo to yield a clear oil. The oil was then triturated with ether to give a white powder (0.07 g), which was both ninhydrin and Sakaguchi positive. The powder was then dissolved in 5 mL of methanol and treated with 0.01 mL (0.13 mmol) of pyridine. After cooling to 0 °C, the solution was treated with 0.025 mL (0.13 mmol) of benzyl chloroformate. The resulting mixture was stirred at 0 °C for 2 h and overnight at room temperature. The solvent was evaporated under reduced pressure and the crude material was triturated with ether and then purified via Sephasorb H-P chromatography using methanol as the mobile phase. The fraction showing a negative ninhydrin reaction and a positive Sakaguchi reaction was collected and the solvent evaporated. (Benzyloxycarbonyl)-L-leucyl-L-leucyl-L-argininal semicarbazone hydrochloride (0.05 g) was then dissolved in 5 mL of methanol and 1 mL of 0.3 M aqueous hydrochloric acid, chilled to 0 °C, and treated with 0.2 mL of 37% formalin. The mixture was stirred at 0 °c for 2.5 h, diluted with saturated aqueous sodium chloride (50 mL) and extracted with ethyl acetate. The organic phase was dried over anhydrous sodium sulfate, and evaporated under reduced pressure. The crude product was purified via Sephadex LH-20 chromatography using methanol as the mobile phase. The fractions showing a positive 2,4-dinitrophenylhydrazine reaction and a positive Sakaguchi reaction were combined and evaporated to give 0.03 g (62% yield) of **2a**. The homogeneity of the product was confirmed by reverse-phase HPLC (5 μm, C_{18} column, methanol/water, 6/4, v/v). The product exhibited the following: HPLC R_v = 2.69 mL (methanol/water, 6/4, v/v, C_{18}, 5 μm); mp = 164–166 °C; $[\alpha]^{25}_{Na}$

= −24.7° (c 1.0, methanol); R_f = 0.63 (cyclized), 0.62 (uncyclized) (1-butanol/water/acetic acid, 7/2/1, v/v/v); ^{1}H NMR (CD_3OD, 200 MHz) δ 0.9 (d, J = 7 Hz, 12 H), 1.5 (m, 10 H), 3.2 (m, 3 H), 4.2 (m, 3 H), 5.2 (s, 2 H), 5.4 (m, 2 H), 6.0 (m, 0.5 H, carbinolamine), 7.3 (s, 5 H), 7.8 (m, 4 H), 9.4 (s, 0.5 H, CHO); ^{13}C NMR (CD_3OD, 200 MHz) δ 21.75, 21.94, 22.40, 22.53, 23.40, 23.48, 25.88, 30.55, 35.39, 41.67, 41.80, 58.00, 60.35, 66.78, 128.64, 129.41, 129.95, 130.02, 138.67 (carbinolamine), 158.25 (C=N), 158.89 (C=O, Cbz), 174.40, 175.77 (amide bonds) 193.83 (CHO). Anal. Calcd for $C_{26}H_{45}N_6O_6Cl$: C, 54.49; H, 7.91; N, 14.66. Found: C, 54.72; H, 7.83; N, 14.92.

(Benzyloxycarbonyl)-L-leucyl-L-phenylalanyl-L-argininal Hydrochloride (2b). The title compound was prepared in analogy to **2a** except that **8b** was used instead of **8a** in procedure. The title compound displayed the following: HPLC R_v = 2.95 mL (methanol/water, 6/4, v/v, C_{18}, 5 μm); mp = 176–178 °C; $[\alpha]^{25}_{Na}$ = −12.3° (c 1.0, methanol); R_f = 0.58 (cyclized), 0.56 (uncyclized) (1-butanol/water/acetic acid, 7/2/1, v/v/v); ^{1}H NMR (CD_3OD, 200 MHz) δ 0.9 (m, 6 H), 1.4 (m, 7 H), 3.2 (m, 5 H), 4.2 (m, 3 H), 5.1 (s, 2 H), 5.3 (m, 2 H), 5.9 (m, 0.4 H, carbinolamine), 7.1 (s, 5 H), 7.3 (s, 5 H), 7.7 (m, 4 H), 9.4 (s, 0.6 H, CHO); ^{13}C NMR (CD_3OD, 200 MHz) δ 20.63, 21.18, 23.20, 25.64, 30.51, 35.81, 40.67, 41.67, 58.20, 60.41, 66.73, 67.45, 127.60, 128.78, 128.96, 129.29, 129.40, 129.57, 130.25, 130.53, 138.33 (carbinolamine), 157.50 (C=N), 161.10 (C=O, Cbz), 174.70, 175.41 (amide bonds), 192.50 (CHO). Anal. Calcd for $C_{29}H_{43}N_6O_6Cl$: C, 57.37; H, 7.14; N, 13.84. Found: C, 57.46; H, 7.32; N, 13.98.

(Benzyloxycarbonyl)-L-phenylalanyl-L-leucyl-L-argininal Hydrochloride (2c). The title compound was synthesized in the same manner as **2a** except that **8c** was used instead of **8a** in the procedure. The title compound had the following: HPLC R_v = 2.87 mL (methanol/water, 6/4, v/v, C_{18}, 5 μm); mp = 170–172 °C; $[\alpha]^{25}_{Na}$ = −15.5 (c 1.0, methanol); R_f = 0.79 (cyclized), 0.76 (uncyclized) (1-butanol/water/acetic acid, 7/2/1, v/v/v); ^{1}H NMR (CD_3OD, 200 MHz) δ 0.9 (m, 6 H), 1.5 (m, 7 H), 3.1 (m, 5 H), 4.4 (m, 3 H), 5.0 (s, 2 H), 5.3 (m, 2 H), 6.1 (m, 0.5 H, carbinol amine), 7.0 (s, 5 H), 7.3 (s, 5 H), 7.9 (m, 2 H), 9.5 (s, 0.5 H, CHO); ^{13}C NMR (CD_3OD, 200 MHz) δ 21.94, 22.05, 23.45, 26.02, 29.54, 34.59, 38.58, 41.69, 42.27, 57.85, 58.10, 66.82, 66.83, 127.86, 128.67, 129.28, 129.49, 129.98, 130.14, 130.59, 130.69, 138.40 (carbinolamine), 157.70 (C=N), 162.75 (C=O, Cbz), 174.30, 174.60 (amide bonds), 190.10 (CHO). Anal. Calcd for $C_{29}H_{43}N_6O_6Cl$: C, 57.37; H, 7.14; N, 13.84. Found: C, 57.42; H, 7.05; N, 13.56.

(Benzyloxycarbonyl)-L-leucyl-L-valyl-L-argininal Hydrochloride (2d). The title compound was prepared by the same method as **2a** except that **8d** was used instead of **8a** in the procedure. The title compound exhibited the following: HPLC R_v = 2.56 mL (methanol/water, 6/4, v/v, C_{18}, 5 μm); mp = 160–162 °C; $[\alpha]^{25}_{Na}$ = −16.4° (c 1.0, methanol); R_f = 0.58 (cyclized), 0.56 (uncyclized) (1-butanol/water/acetic acid, 7/2/1, v/v/v); ^{1}H NMR (CD_3OD, 200 MHz) δ 0.9 (d, J = 7 Hz, 12 H), 1.4 (m, 8 H), 2.9 (t, J = 7 Hz, 2 H), 4.1 (m, 4 H), 5.1 (s, 2 H), 5.4 (m, 2 H), 6.0 (m, 0.6 H, carbinolamine), 7.3 (s, 5 H), 7.7 (m, 3 H), 9.6 (s, 0.4 H, CHO); ^{13}C NMR (CD_3OD, 200 MHz) δ 19.67, 19.79, 21.65, 21.87, 24.80, 29.90, 31.20, 33.45, 34.85, 41.55, 41.68, 60.58, 60.72, 66.80, 67.55, 128.45, 129.35, 129.67, 130.73, 138.45 (carbinolamine), 157.50 (C=N), 159.50 (C=O, Cbz), 173.55, 175.59 (amide bonds), 189.95 (CHO). Anal. Calcd for $C_{25}H_{43}N_6O_6Cl$: C, 53.71, H, 7.75; N, 15.03. Found: C, 53.99; H, 7.53; N, 14.87.

***N*$^\epsilon$-(*tert*-Butoxycarbonyl)-L-lysinal Semicarbazone Hydrochloride (5a).** A solution of 0.84 g (2.1 mmol) of **4a** in 50 mL of methanol and 0.5 mL of 1 M aqueous hydrochloric acid containing 0.7 g of 10% palladium on carbon was stirred under a hydrogen atmosphere for 3.5 h. The catalyst was removed by filtration and the solvent was evaporated under reduced pressure to yield a clear oil. The crude product was triturated with ether, providing 0.61 g (73% yield) of **5a**. The title compound had the following: mp = 165–167 °C; R_f = 0.42 (1-butanol/water/acetic acid, 7/2/1, v/v/v); ^{1}H NMR (CD_3CN/D_2O, 1/1, v/v, 90 MHz) δ 1.4 (s, 9 H), 1.5 (m, 6 H), 3.2 (t, J = 7 Hz, 2 H), 4.1 (m, 1 H), 7.3 (d, J = 7 Hz, 1 H).

***N*$^\delta$-(*tert*-Butoxycarbonyl)-L-ornithinal Semicarbazone Hydrochloride (5b).** The title compound was prepared in analogy to **5a** except that **4b** was used instead of **4a** in the deblocking reaction. Compound **5b** exhibited the following: 76% yield; R_f = 0.11 (ethyl acetate/methanol, 9/1, v/v); ^{1}H NMR (CD_3CN/D_2O, 1/1, v/v, 60 MHz) δ 1.2 (s, 9 H), 1.4 (m, 4 H), 2.9 (t, J = 7 Hz, 2 H), 3.9 (m, 1 H), 7.0 (d, J = 7 Hz, 1 H).

(Benzyloxycarbonyl)-L-leucyl-L-leucyl-*N*$^\epsilon$-(*tert*-butoxycarbonyl)-L-lysinal Semicarbazone (6a). A solution of 0.7 g (1.9 mmol) (benzyloxycarbonyl)-L-leucyl-L-leucine in 30 mL of anhydrous chloroform was chilled to −15 °C with vigorous stirring and treated with 0.3 mL (2.1 mmol) of triethylamine. After 10 min, 0.2 mL (2.1 mmol) of ethyl chloroformate was added. The reaction mixture was stirred under a nitrogen atmosphere at −15 °C for 30 min. A precooled solution of 0.50 g (1.8 mmol) of **5a** and 0.3 mL (2.1 mmol) of triethylamine in 30 mL of anhydrous chloroform was added. The resulting mixture was stirred at 0 °C for 3 h and overnight at room temperature. The solution was washed with equal volumes of 1 M aqueous hydrochloric acid, 5% aqueous sodium bicarbonate, and water, dried over anhydrous magnesium sulfate, and evaporated in vacuo. Purification of the crude product via Sephadex LH-20 chromatography using methanol as the mobile phase gave 0.35 g (31% yield) of **6a**. The title compound had the following: R_f = 0.1 (ethyl acetate); ^{1}H NMR ($CDCl_3$, 90 MHz) δ 0.9 (d, J = 7 Hz, 12 H), 1.4 (s, 9 H), 1.6 (m, 12 H), 3.2 (m, 2 H), 3.6 (m, 1 H), 4.4 (m, 3 H), 5.0 (m, 2 H), 5.2 (s, 2 H), 6.3 (m, 2 H), 7.2 (d, J = 7 Hz, 1 H), 7.4 (s, 5 H), 10.1 (m, 1 H). Anal. Calcd for $C_{32}H_{53}N_7O_7$: C, 59.33; H, 8.24; N, 15.13. Found: C, 59.39; H, 8.32; N, 15.24.

(Benzyloxycarbonyl)-L-leucyl-L-phenylalanyl-*N*$^\epsilon$-(*tert*-butoxycarbonyl)-L-lysinal Semicarbazone (6b). This peptide was prepared in analogy to **6a** except (benzyloxycarbonyl)-L-leucyl-L-phenylalanine was used instead of (benzyloxycarbonyl)-L-leucyl-L-leucine in the coupling procedure. The title compound exhibited the following: 35% yield; R_f = 0.2 (ethyl acetate); ^{1}H NMR ($CDCl_3$, 90 MHz) δ 0.9 (d, J = 7 Hz, 6 H), 1.3 (s, 9 H), 1.6 (m, 9 H), 3.3 (m, 4 H), 3.8 (m, 1 H), 4.3 (m, 3 H), 5.0 (m, 3 H), 5.3 (s, 2 H), 6.4 (m, 2 H), 7.2 (d, J = 7 Hz, 1 H), 7.3 (s, 10 H), 10.2 (m, 1 H). Anal. Calcd for $C_{35}H_{51}N_7O_7$; C, 61.65; H, 7.54; N, 14.38. Found: C, 61.39; H, 7.67; N, 14.43.

(Benzyloxycarbonyl)-L-phenyl-L-leucyl-*N*$^\epsilon$-(*tert*-butoxycarbonyl)-L-lysinal Semicarbazone (6c). This peptide was prepared by the same method as **6a** except that (benzyloxycarbonyl)-L-phenylalanyl-L-leucine was used instead of (benzyloxycarbonyl)-L-leucyl-L-leucine in the coupling procedure. The title compound displayed the following: 35% yield; R_f = 0.1 (ethyl acetate); ^{1}H NMR ($CDCl_3$, 90 MHz) δ 0.7 (m, 6 H), 1.4 (s, 9 H), 1.6 (m, 9 H), 3.0 (m, 4 H), 3.5 (m, 1 H), 4.6 (m, 3 H), 5.0 (m, 3 H), 5.2 (s, 2 H), 6.1 (m, 2 H), 7.0 (d, J = 7 Hz, 1 H), 7.2 (s, 5 H), 7.4 (s, 5 H), 10.0 (m, 1 H). Anal. Calcd for $C_{35}H_{51}N_7O_7$: C, 61.65; H, 7.54; N, 14.38. Found C, 61.72; H, 7.71; N, 14.34.

(Benzyloxycarbonyl)-L-leucyl-L-valyl-*N*$^\epsilon$-(*tert*-butoxycarbonyl)-L-lysinal Semicarbazone (6d). This peptide was prepared in the same manner as **6a** except that (benzyloxycarbonyl)-L-leucyl-L-valine was used instead of (benzyloxycarbonyl)-L-leucyl-L-leucine in the coupling procedure. The title compound had the following: 31% yield; R_f = 0.3 (ethyl acetate/methanol, 9/1, v/v); ^{1}H NMR ($CDCl_3$, 90 MHz); δ 0.9 (d, J = 7 Hz, 12 H), 1.2 (s, 9 H), 1.4 (m, 10 H), 3.2 (m, 2 H), 3.6 (m, 1 H), 4.4 (m, 3 H), 4.8 (m, 3 H), 5.0 (s, 2 H), 6.2 (m, 2 H), 7.1 (d, J = 7 Hz, 1 H), 7.3 (s, 5 H), 10.0 (m, 1 H). Anal. Calcd for $C_{31}H_{51}N_7O_7$: C, 58.75; H, 8.11; N, 15.47. Found: C, 59.11; H, 8.24; N, 15.33.

(Benzyloxycarbonyl)-L-leucyl-L-leucyl-*N*$^\delta$-(*tert*-butoxycarbonyl)-L-ornithinal Semicarbazone (6e). This peptide was prepared in analogy to **6a** except **5b** was used instead of **5a** in the coupling procedure. The title compound exhibited the following: 43% yield: R_f = 0.1 (chloroform/methanol, 9/1, v/v); ^{1}H NMR (CD_3CN, 90 MHz) δ 0.9 (d, J = 7 Hz, 12 H), 1.4 (s, 9 H), 1.6 (m, 10 H), 3.2 (t, J = 7 Hz, 2 H), 3.8 (m, 2 H), 4.4 (m, 3 H), 4.8 (m, 2 H), 5.2 (s, 2 H), 6.2 (m, 2 H), 7.0 (d, J = 7 Hz, 1 H), 7.3 (s, 5 H), 10.0 (m, 1 H). Anal. Calcd for $C_{31}H_{51}N_7O_7$: C, 58.75; H, 8.11; N, 15.47. Found: C, 58.63; H, 8.07; N, 15.53.

(Benzyloxycarbonyl)-L-leucyl-L-phenylalanyl-*N*$^\delta$-(*tert*-butoxycarbonyl)-L-ornithinal Semicarbazone (6f). This peptide was synthesized in the same manner as **6e** except that (benzyloxycarbonyl)-L-leucyl-L-phenylalanine was used instead of (benzyloxycarbonyl)-L-leucyl-L-leucine in the coupling procedure. The title compound displayed the following: 41% yield; R_f = 0.28 (ethyl acetate); ^{1}H NMR ($CDCl_3$, 60 MHz) δ 0.9 (d, J = 7 Hz, 6 H), 1.3 (s, 9 H), 1.6 (m, 7 H), 3.0 (m, 4 H), 3.8 (m, 1

H), 4.4 (m, 3 H), 5.2 (s, 2 H), 5.7 (m, 2 H), 7.0 (d, J = 7 Hz, 1 H), 7.1 (s, 5 H), 7.3 (s, 5 H), 10.1 (m, 1 H). Anal. Calcd for $C_{34}H_{49}N_7O_7$: C, 61.16; H, 7.40; N, 14.67. Found: C, 60.98; H, 7.52; N, 14.75.

(Benzyloxycarbonyl)-L-phenylalanyl-L-leucyl-N^{δ}-(*tert*-butoxycarbonyl)-L-ornithinal Semicarbazone (6g). This peptide was produced by the same method as **6e** except that (benzyloxycarbonyl)L-phenylalanyl-L-leucine was used instead of (benzyloxycarbonyl)-L-leucyl-L-leucine in the coupling reaction. The title compound had the following: 32% yield; R_f = 0.43 (ethyl acetate); ^{1}H NMR ($CDCl_3$, 60 MHz) δ 0.9 (m, 6 H), 1.3 (s, 9 H), 1.4 (m, 7 H), 1.8 (m, 1 H), 3.0 (m, 4 H), 3.6 (m, 2 H), 4.2 (m, 3 H), 5.0 (s, 2 H), 5.2 (m, 1 H), 5.9 (m, 2 H), 7.0 (d, J = 7 Hz, 1 H), 7.1 (s, 5 H), 7.3 (s, 5 H), 10.0 (m, 1 H). Anal. Calcd for $C_{34}H_{49}N_7O_7$: C, 61.15; H, 7.40; N, 14.67. Found: C, 61.11; H, 7.54; N, 14.70.

(Benzyloxycarbonyl)-L-leucyl-L-valyl-N^{δ}-(*tert*-butoxycarbonyl)-L-ornithinal Semicarbazone (6h). This peptide was prepared in analogy to **6e** except (benzyloxycarbonyl)-L-leucyl-L-valine was used instead of (benzyloxycarbonyl)-L-leucyl-L-leucine in the coupling procedure. The title compound exhibited the following: 67% yield; R_f = 0.38 (chloroform/methanol, 9/1, v/v); ^{1}H NMR (CD_3CN, 60 MHz) δ 0.9 (m, 12 H), 1.2 (s, 9 H), 1.4 (m, 8 H), 3.0 (m, 2 H), 3.6 (m, 1 H), 4.3 (m, 3 H), 4.8 (m, 3 H), 5.1 (s, 2 H), 5.8 (m, 2 H), 7.0 (d, J = 7 Hz, 1 H), 7.3 (s, 5 H), 10.0 (m, 1 H). Anal. Calcd for $C_{30}H_{49}N_7O_7$: C, 58.14; H, 7.97; N, 15.82. Found: C, 57.89; H, 8.06; N, 15.73.

(Benzyloxycarbonyl)-L-leucyl-L-leucyl-L-lysinal Hydrochloride (2e). A solution of 0.16 g (0.25 mmol) of **6a** in 10 mL of methanol and 0.5 mL of 0.1 M aqueous hydrochloric acid was cooled to 0 °C and treated with 0.3 mL of 37% formalin. The mixture was stirred at 0 °C for 2 h, diluted with 25 mL of water, and extracted three times with ethyl acetate. The organic layers were combined, washed with distilled water, and dried over anhydrous magnesium sulfate. The solvent was evaporated under reduced pressure, and the crude product was purified via Sephadex chromatography (LH-20) using methanol as the mobile phase. The fraction showing a positive (2,4-dinitrophenyl)hydrazine reaction was concentrated and treated with 4 M hydrochloric acid in anhydrous dioxane. The solution was stirred at 0 °C for 3 h. The solvent was then evaporated under reduced pressure to give a white solid. The homogeneity of the product was confirmed by reverse-phase HPLC (5 μm, C_{18} column, methanol/water, 7/3, v/v). The title compound displayed the following: HPLC R_v = 4.15 mL (methanol/water, 7/3 v/v C_{18}, 5 μm); $[\alpha]^{25}_{Na}$ = −39.9° (c 1.0, methanol); mp = 179–181 °C; R_f = 0.51 (cyclized), 0.49 (uncyclized) (1-butanol/water/acetic acid, 7/2/1, v/v/v); ^{1}H NMR (CD_3OD, 200 MHz) δ 0.9 (d, J = 7 Hz, 12 H), 1.5 (m, 12 H), 3.1 (t, J = 7 Hz, 2 H), 3.6 (m, 3 H), 4.3 (m, 3 H), 4.9 (m, 2 H), 5.1 (s, 2 H), 6.0 (m, 0.7 H, CHO cyclized), 7.3 (s, 5 H); ^{13}C NMR (CD_3OD, 80 MHz) δ 21.74, 21.88; 22.35, 22.42, 23.42, 23.49, 27.86, 28.01, 31.22, 35.42, 35.64, 46.31, 46.85, 58.05, 60.45, 66.67, 67.75, 128.74, 129.22, 129.50, 138.03 (carbinolamine), 158.53 (C=O, Cbz), 174.33, 175.61 (amide bonds). Anal. Calcd for $C_{26}H_{45}N_4O_6Cl$: C, 57.29; H, 8.32; N, 10.27. Found C, 57.63; H, 8.21; N, 10.68.

(Benzyloxycarbonyl)-L-leucyl-L-phenylalanyl-L-lysinal Hydrochloride (2f). This compound was prepared in analogy to **2e** except that **6b** was used instead of **6a** in the deblocking procedure. The title compound exhibited the following: HPLC R_v = 3.26 mL (methanol/water, 7/3, v/v, C_{18}, 5 μm); $[\alpha]^{25}_{Na}$ = −33.3° (c 1.0, methanol); mp = 142–144 °C; R_f = 0.55 (cyclized) 0.53 (uncyclized) (1-butanol/water/acetic acid, 7/2/1, v/v/v); ^{1}H NMR (CD_3OD, 200 MHz) 0.7 (d, J = 7 Hz, 6 H), 1.4 (m, 9 H) 3.2 (m, 4 H), 3.5 (m, 3 H) 4.3 (m, 3 H), 4.9 (m, 2 H), 5.2 (s, 5 H), 6.0 (m, 0.6 H, CHO-cyclized), 7.1 (s, 5 H), 7.4 (s, 5 H), 9.3 (s, 0.4 H, CHO); ^{13}C NMR (CD_3OD, 80 MHz) δ 20.95, 21.35, 23.47, 27.98, 28.06, 31.10, 35.69, 40.41, 46.31, 46.85, 55.15, 60.50, 66.79, 66.85, 127.68, 127.82, 128.75, 129.04, 129.23, 129.51, 130.10, 130.35, 138.07 (carbinolamine), 159.20 (C=O, Cbz), 174.65, 175.50 (amide bonds), 190.50 (CHO). Anal. Calcd for $C_{29}H_{43}N_4O_6Cl$: C, 60.14; H, 7.48; N, 9.67. Found: C, 60.38; H, 7.15; N, 9.87.

(Benzyloxycarbonyl)-L-phenylalanyl-L-leucyl-L-lysinal Hydrochloride (2g). This compound was synthesized by the same method as **2e** except that **6c** was used instead of **6a** in the deblocking procedure. The title compound had the following: HPLC R_v = 3.24 mL (methanol/water, 7/3, v/v, C_{18}, 5 μm); $[\alpha]^{25}_{Na}$ = −18.75° (c, 1.0, methanol); mp = 148–150 °C; R_f = 0.6 (cyclized), 0.58 (uncyclized) (1-butanol/water/acetic acid, 7/2/1, v/v/v); ^{1}H NMR (CD_3OD, 200 MHz) δ 0.9 (c, J = 7 Hz, 6 H), 1.5 (m, 9 H), 3.0 (m, 4 H), 3.6 (m, 1 H), 3.9 (m, 1 H), 4.5 (m, 3 H), 4.8 (m, 2 H), 5.0 (s, 2 H), 5.8 (m, 0.9 H, CHO-cyclized), 7.0 (s, 5 H), 7.3 (s, 5 H), 9.2 (s, 0.1 H, CHO); ^{13}C NMR (CD_3OD, 80 MHz) δ 21.85, 22.01, 23.45, 28.00, 28.82, 30.37, 35.77, 38.59, 45.89, 46.84, 57.84, 58.00, 65.80, 66.82, 127.79, 128.66, 129.00, 129.49, 129.71, 129.85, 130.00, 130.36, 138.30 (carbinolamine), 162.00 (C=O, Cbz), 174.29, 174.59 (amide bonds). Anal. Calcd for $C_{29}H_{43}N_4O_6Cl$: C, 60.14; H, 7.48; N, 9.67. Found: C, 60.27; H, 7.35; N, 9.74.

(Benzyloxycarbonyl)-L-leucyl-L-valyl-L-lysinal Hydrochloride (2h). This compound was produced by the same method as **2e** except that **6d** was used instead of **6a** in the deblocking procedure. The title compound displayed the following: HPLC R_v = 3.39 mL (methanol/water, 7/3, v/v, C_{18}, 5 μm); mp = 134–136 °C; $[\alpha]^{25}_{Na}$ = −26.8° (c 1.0 methanol); R_f = 0.21 (cyclized), 0.17 (uncyclized) (1-butanol/water/acetic acid, 7/2/1, v/v/v); ^{1}H NMR (CD_3OD, 200 MHz) δ 0.9 (d, J = 7 Hz, 12 H), 1.3 (m, 10 H), 3.0 (m, 2 H), 3.6 (m, 2 H), 4.4 (m, 3 H), 4.9 (m, 2 H), 5.2 (s, 2 H), 5.9 (m, 1 H, CHO-cyclized), 7.3 (s, 5 H). ^{13}C NMR (CD_3OD, 80 MHz) δ 19.75, 19.99, 21.84, 21.91, 27.75, 28.06, 31.23, 31.74, 33.41, 34.95, 45.78, 46.05, 60.60, 60.70, 66.78, 66.87, 128.37, 128.79, 129.04, 129.51, 138.19 (carbinolamine), 158.44 (C=O, Cbz), 173.51, 175.60 (amide bonds), 188.23 (CHO). Anal. Calcd for $C_{25}H_{43}N_4O_6Cl$: C, 56.54; H, 8.16; N, 10.55. Found: C, 56.91; H, 8.05; N, 10.89.

(Benzyloxycarbonyl)-L-leucyl-L-leucyl-L-ornithinal Hydrochloride (2i). This compound was produced in the same manner as **2e** except that **6e** was used instead of **6a** in the deblocking procedure. The title compound exhibited the following: HPLC R_v = 6.59 mL (methanol/water, 7/3, v/v, C_{18}, 5 μm); mp = 155–157 °C dec; $[\alpha]^{25}_{Na}$ = −37.6° (c 1.0, methanol); R_f = 0.71 (cyclized), 0.69 (uncyclized) (1-butanol/water/acetic acid, 7/3/1, v/v/v); ^{1}H NMR (CD_3OD, 200 MHz) δ 0.9 (d, J = 7 Hz, 12 H), 1.4 (m, 10 H), 3.0 (m, 2 H), 3.6 (m, 2 H), 4.3 (m, 3 H), 4.9 (m, 2 H), 5.2 (s, 2 H), 6.1 (m, 1 H, CHO-cyclized), 7.3 (s, 5 H); ^{13}C NMR (CD_3OD, 80 MHz) δ 20.95, 21.80, 21.95, 22.32, 23.39, 23.45, 27.00, 31.15, 36.15, 36.35, 44.85, 45.15, 57.93, 60.35, 67.70, 67.95, 128.65, 128.98, 129.37, 129.65, 137.80 (carbinolamine), 158.25 (C=O, Cbz), 162.90 (C=N, iminium), 174.40, 174.75 (amide bonds). Anal. Calcd for $C_{25}H_{41}N_4O_5Cl$: C, 58.52; H, 8.05; N, 10.92. Found: C, 58.15; H, 8.16; N, 10.65.

(Benzyloxycarbonyl)-L-leucyl-L-phenylalanyl-L-ornithinal Hydrochloride (2j). This compound was produced in analogy to **2e** except that **6f** was used instead of **6a** in the deblocking procedure. The title compound had the following: HPLC R_v = 3.43 mL (methanol/water, 7/3, v/v, C_{18}, 5 μm); mp = 133–135 °C; $[\alpha]^{25}_{Na}$ = −28.6° (c 1.0, methanol); R_f = 0.18 (cyclized), 0.13 (uncyclized) (1-butanol/water/acetic acid, 7/2/1, v/v/v); ^{1}H NMR (CD_3OD, 200 MHz) δ 0.9 (d, J = 7 Hz, 6 H), 1.4 (m, 7 H), 3.0 (m, 4 H), 3.4 (m, 1 H), 3.8 (m, 1 H), 4.2 (m, 3 H), 4.8 (m, 2 H), 5.1 (s, 2 H), 6.1 (m, 1 H, CHO-cyclized), 7.0 (s, 5 H), 7.2 (s, 5 H); ^{13}C NMR (CD_3OD, 200 MHz) δ 21.38, 21.72, 23.90, 26.73, 31.06, 35.96, 40.15, 44.71, 45.63, 57.93, 60.55, 67.70, 67.79, 127.65, 127.71, 128.57, 128.65, 128.74, 128.91, 129.37, 130.22, 137.30 (carbinolamine), 160.32 (C=O, Cbz), 162.95 (C=N, iminium), 174.70, 175.45 (amide bonds). Anal. Calcd for $C_{28}H_{39}N_4O_5Cl$: C, 61.47; H, 7.18; N, 10.24. Found: C, 61.09; H, 7.30; N, 9.95.

(Benzyloxycarbonyl)-L-phenylalanyl-L-leucyl-L-ornithinal Hydrochloride (2k). This compound was prepared by the same method as **2e** except that **6g** was used instead of **6a** in the deblocking procedure. The title compound displayed the following: HPLC R_v = 3.68 mL (methanol/water, 7/3, v/v, C_{18}, 5 μm); mp = 149–152 °C; $[\alpha]^{25}_{Na}$ = −18.7° (c 1.0, methanol); R_f = 0.49 (cyclized), 0.44 (uncyclized) (1-butanol/water/acetic acid, 7/2/1, v/v/v); ^{1}H NMR (CD_3OD, 200 MHz) δ 0.9 (d, J = 7 Hz, 6 H), 1.5 (m, 7 H), 3.0 (m, 4 H), 3.4 (m, 1 H), 3.6 (m, 1 H), 4.3 (m, 3 H), 4.8 (m, 2 H), 5.0 (s, 2 H), 6.1 (m, 1 H, CHO-cyclized), 7.1 (s, 5 H), 7.3 (s, 5 H). ^{13}C NMR (CD_3OD, 200 MHz) δ 21.05, 21.92, 23.37, 26.70, 31.05, 36.35, 38.51, 44.71, 45.01, 57.84, 57.93, 67.69, 67.96, 127.72, 128.58, 128.90, 129.04, 129.18, 129.37, 130.02, 130.22, 137.90 (carbinolamine), 159.59 (C=O, Cbz), 163.80 (C=N, iminium), 174.30, 174.61 (amide bonds). Anal. Calcd for $C_{28}H_{39}N_4O_5Cl$: C, 61.47; H, 7.18; N, 10.24. Found: C, 61.12; H, 7.32; N, 9.89.

(Benzyloxycarbonyl)-L-leucyl-L-valyl-L-ornithinal Hydrochloride (2l). This compound was prepared by using the same procedure as that described for **2e** except that **6h** was used instead

of **6a** in the deblocking reactions. The title compound exhibited the following: HPLC R_v = 3.66 mL (methanol/water, 7/3 v/v, C_{18}, 5 μm); mp = 139–141 °C; $[\alpha]^{25}_{Na}$ = −18.2° (*c* 1.0, methanol); R_f = 0.53 (cyclized), 0.46 (uncyclized) (1-butanol/water/acetic acid, 7/2/1, v/v/v); ^{1}H NMR (CD_3OD, 200 MHz) δ 0.9 (m, 12 H), 1.4 (m, 8 H), 3.0 (t, *J* = 7 Hz, 2 H), 3.6 (m, 2 H), 4.3 (m, 3 H), 4.7 (m, 2 H), 5.1 (s, 2 H), 6.0 (m, 1 H, CHO-cyclized), 7.3 (s, 5 H). ^{13}C NMR (CD_3OD, 200 MHz) δ 19.65, 19.89, 21.65, 21.86, 27.20, 31.28, 31.82, 33.52, 35.05, 44.51, 45.37, 60.60, 60.90, 67.65, 67.73, 128.61, 128.84, 129.01, 129.50, 138.30 (carbinolamine), 157.60 (C=O, Cbz), 162.80 (C=N, iminium), 173.55, 175.59 (amide bonds). Anal. Calcd for $C_{24}H_{39}N_4O_5Cl$: C, 57.76; H, 7.88; N, 11.23. Found: C, 57.38; H, 7.98; N, 10.89.

Inhibitor Activity Measurements. Rate measurements were performed with the aid of a Beckman Model DU UV-visible spectrophotometer in 1-cm quartz cuvettes thermostated at 37 °C. The inhibition of the enzyme activity was measured four times at five or more inhibitor concentrations. The average change in absorbance at each inhibitor concentration was utilized in the calculation of percent inhibition. All values were within 0.002 standard deviations from the mean. The percent inhibition of the reactions were calculated as follows:

$$\% \text{ inhibition} = (A - B)/A \times 100$$

where A = the change in absorbance without inhibitor and B = the change in absorbance with inhibitor.

The concentration inducing a 50% inhibition was obtained by plotting the percent inhibition versus the log of the inhibitor concentration. The standard error for the linear-regression plots was calculated and in each case is less than 5%.

Inhibition of Kallikrein Activity. To a 37 °C mixture of 0.02 mL of a 0.3 units/mL human urinary kallikrein solution was added 0.45 mL of 0.46 M tris(hydroxymethyl)aminomethane buffer (pH 8.0) containing 0.0115 M sodium chloride and 0.05 mL of 1% dimethyl sulfoxide/water with or without an inhibitor, 0.1 mL of the substrate, benzoylarginine ethyl ester (0.5 mM). The mixture was shaken and incubated at 37 °C for 2 min. The change in absorbance was then measured at 253 nm over 10 min.

Inhibition of Plasmin Activity. A solution of 0.02 mL of a 0.25 casein units/mL plasmin solution in 50% glycerol in 2 mM aqueous hydrochloric acid containing 5 g/L carbowax (mol wt 6000) and 0.45 mL of a 0.5 M tris(hydroxymethyl)aminomethane buffer (pH 8.0) containing 0.12 M sodium chloride and 0.05 mL of 1% dimethyl sulfoxide/water, with or without an inhibitor, was maintained at 37 °C. To this solution D-valyl-L-leucyl-L-lysine *p*-nitroanilide (0.1 mL of 3.35 mM in water) was added. The mixture was shaken and incubated at 37 °C for 1 min. The change in absorbance at 405 nm was then measured over 10 min.

Inhibition of Trypsin Activity. To a 37 °C mixture of 0.1 mL of 0.01 g/L trypsin in 0.001 N aqueous hydrochloric acid, 2.6 mL of 0.046 M tris(hydroxymethyl)aminomethane buffer containing 0.0115 M $CaCl_2$ (pH 8.1) and 0.1 mL 1% dimethyl sulfoxide/water with or without an inhibitor was added 0.2 mL of tosyl-L-arginine methyl ester (0.01 M). The mixture was shaken and incubated at 37 °C for 2 min. The change in absorbance was measured at 247 nm over 10 min.

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Registry No. **2a**·HCl, 83039-48-9; **2a** (free base), 114376-85-1; **2a** (semicarbazone), 122314-25-4; **2b**·HCl, 83134-12-7; **2b** (free base), 122405-43-0; **2b** (semicarbazone), 122314-26-5; **2c**·HCl, 122406-12-6; **2c** (free base), 83039-66-1; **2c** (semicarbazone, 122314-27-6; **2d**·HCl, 122314-38-9; **2d** (free base), 122405-44-1; **2d** (semicarbazone), 122314-28-7; **2e**·HCl, 122314-35-6; **2e** (free base), 122405-45-2; **2f**·HCl, 122314-36-7; **2f** (free base), 122405-46-3; **2g**·HCl, 122314-37-8; **2g** (free base), 122405-47-4; **2h**·HCl, 122406-13-7; **2h** (free base), 122314-43-6; **2i**·HCl, 122314-39-0; **2i** (free base), 122405-48-5; **2j**·HCl, 122314-40-3; **2j** (free base), 122405-49-6; **2k**·HCl, 122314-41-4; **2k** (free base), 122405-50-9; **2l**·HCl, 122314-42-5; **2l** (free base), 122405-51-0; **3a**, 2389-49-3; **3b**, 32393-52-5; **3c**, 112208-06-7; **4a**, 122314-18-5; **4b**, 122314-19-6; **4c**, 122314-20-9; **5a**, 122314-16-3; **5b**, 122314-17-4; **6a**, 122332-80-3; **6b**, 122314-29-8; **6c**, 122314-30-1; **6d**, 122314-31-2; **6e**, 122332-79-0; **6f**, 122314-32-3; **6g**, 122314-33-4; **6h**, 122314-34-5; **7**, 122314-15-2; **8a**, 122314-21-0; **8b**, 122314-22-1; **8c**, 122314-23-2; **8d**, 122314-24-3; Z-Lys(BOC)-H, 122314-13-0; Z-Orn(BOC)-H, 122314-14-1; BOC-Arg(NO_2)-H, 71413-14-4; Z-Leu-Leu-OH, 7801-71-0; Z-Leu-Phe-OH, 6401-63-4; Z-Phe-Leu-OH, 4313-73-9; Z-Leu-Val-OH, 7801-70-9; trypsin, 9002-07-7; plasmin, 9001-90-5; kallikrein, 9001-01-8.

DNA Intercalating Properties of Tetrahydro-9-aminoacridines. Synthesis and ^{23}Na NMR Spin–Lattice Relaxation Time Measurements

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A series of 9-(arylamino)-1,2,3,4-tetrahydroacridines, including the tetrahydro *m*-AMSA [*N*-[4-(acridin-9-ylamino)-3-methoxyphenyl]methanesulfonamide] derivative, has been synthesized. ^{23}Na NMR spin–lattice relaxation rate ($1/T_1$) measurements have been used to study whether these hydrogenated acridines were capable of intercalative binding to calf thymus DNA. The results have been compared to corresponding measurements for 9-aminoacridine, *m*-AMSA, and $MgCl_2$. All compounds studied were capable of intercalative binding to DNA. However, it was found that the interaction was strongly influenced by substituents on the 9-arylamino group. Thus, tetrahydro *m*-AMSA was found to intercalate much more weakly with DNA than *m*-AMSA. Removal of the 3′-methoxy substituent of the 9-arylamino group resulted in intercalation in DNA that was almost as strong as that for *m*-AMSA.

A large variety of drugs are known to interact strongly with nucleic acids. Many of them bind to double-stranded DNA through intercalation as first described by Lerman.[1] A great deal of effort has been devoted to the synthesis of several classes of compounds with specific intercalating properties to obtain agents of clinical importance.

9-Aminoacridines constitute a class of compounds that are now recognized for their ability to interact with DNA by intercalation. These compounds show great biological activity and several of them are of clinical importance. More than 500 fully aromatized 9-(arylamino)acridine derivatives have been found to be antitumor active compounds. In particular, the *N*-[4-(9-acridinylamino)-phenyl]methanesulfonamide (AMSA) has shown a broad spectrum of activity against animal tumors.[2] *N*-[4-(9-

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