Novel Peptidyl α-Keto Amide Inhibitors of Calpains and Other Cysteine Proteases

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A series of new dipeptidyl α-keto amides of the general structure R_1-L-Leu-D,L-AA-CONH-R_2 were synthesized and evaluated as inhibitors for the cysteine proteases calpain I, calpain II, and cathepsin B. They combine 10 different N-protecting groups (R_1), 3 amino acids residues in P1 (AA), and 44 distinct substituents on the α-keto amide nitrogen (R_2). In general, calpain II was more sensitive to these inhibitors than calpain I, with a large number of inhibitors displaying dissociation constants (K_i) in the 10−100 nM range. Calpain I was also effectively inhibited, but very low K_i values were observed with a smaller number of inhibitors than with calpain II. Cathepsin B was weakly inhibited by most compounds in this study. The best inhibitors for calpain II were Z-Leu-Abu-CONH-CH_2-CHOH-C_6H_5 (K_i = 15 nM), Z-Leu-Abu-CONH-CH_2-2-pyridyl (K_i = 17 nM), and Z-Leu-Abu-CONH-CH_2-C_6H_3(3,5(OMe)$_2$) (K_i = 22 nM). The best calpain I inhibitor in this study was Z-Leu-Nva-CONH-CH_2-2-pyridyl (K_i = 19 nM). The peptide α-keto amide Z-Leu-Abu-CONH-$(CH_2)_2$-3-indolyl was the best inhibitor for cathepsin B (K_i = 31 nM). Some compounds acted as specific calpain inhibitors, with comparable activity on both calpains I and II and a lack of activity on cathepsin B (e.g., **40**, **42**, **48**, **70**). Others were specific inhibitors for calpain I (e.g., **73**) or calpain II (e.g., **18**, **19**, **33**, **35**, **56**). Such inhibitors may be useful in elucidating the physiological and pathological events involving these proteases and may become possible therapeutic agents.

Introduction

Calpains and calcium-activated neutral cysteine proteases widely distributed in animal cells and tissues. Two types of calpains have been identified, calpain I (or μ-calpain) and calpain II (or m-calpain), which require micro- and millimolar concentrations of calcium, respectively, for optimal enzyme activity *in vitro*. Proteolysis by calpains is a vital regulatory mechanism mediating biological responses elicited by the elevation of calcium. Calpains are able to degrade cytoskeletal proteins and hormone receptors as well as activate some kinases and phosphatases (for a review on calpain, see ref 1). Increasing evidence suggests that calpain participates in the initiation and/or expression of multiple degenerative conditions. Enhanced calpain activity has been described in conjunction with cell injury elicited by physical damage,[2] hypoxia,[3] and ischemia.[4−7] Therefore, specific calpain inhibitors may be useful therapeutic agents in some degenerative disease states.

Several types of low molecular weight inhibitors have been shown to effectively inhibit calpain. Acting by affinity labeling, peptidyl diazomethyl ketones[8] and peptidyl fluoromethyl ketones[9] irreversibly inhibit calpain. E-64, a naturally occurring epoxide, and its derivatives (E-64-c, E-64-d)[10] irreversibly inhibit cysteine proteases, including calpain, by covalent bond formation with the active site cysteine residue. Peptidyl aldehydes[11] and carbonyl derivatives, such as α-diketones,[12,13] α-keto acids,[13,14] α-keto esters,[12−14] and α-keto amides,[13−15] reversibly inhibit cysteine proteases as well as serine proteases, possibly due to hemithioacetal formation with the SH group of the active site cysteine, in the case of cysteine proteases, or due to hemiacetal formation with the OH group of the active site serine, in the case of serine proteases.

In a preceding article,[14] we explored the inhibitory potencies of dipeptidyl and tripeptidyl α-keto esters, α-keto amides, and α-keto acids. The influence of the type and number of amino acid residues, the nature of the N-protecting group, and differences between the ester or amide functional group were studied. Our results showed that, in general, peptidyl α-keto acids were more inhibitory toward both calpains than α-keto amides which, in turn, were more effective than α-keto esters *in vitro*. Lacking hydrophobicity, α-keto acids had quite poor membrane penetration as illustrated in a platelet membrane permeability assay. They were also highly unstable *in vivo* due to their sensitivity to esterases. After demonstrating that N-monosubstituted peptide α-keto amides were much more potent inhibitors than the corresponding N,N-disubstituted α-keto amides, we hypothesized that the N-H on the α-keto amide functional group formed a hydrogen bond with an amino acid residue of the active site of calpain. Noteworthy was the fact that α-keto amides with hydrophobic alkyl groups or alkyl groups with an attached phenyl group had the lowest K_i values, which suggest the existence of a hydrophobic pocket on the S′ [16] side of the active site.

The protective effect of two dipeptide α-keto amides against ischemic brain damage, using an animal model of ischemia, has been reported.[6,7] Focal ischemia was induced using a variation of the middle cerebral artery

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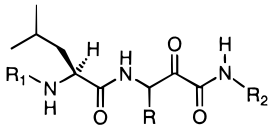


Figure 1. Structure of a dipeptidyl α-keto amide (abbreviated as R_1-Leu-AA-CONH-R_2).

occlusion method. One compound, Z-Leu-Abu-CONH-Et (**2**), was perfused directly onto the infarcted cortical surface.[7] Alternately, Z-Leu-Abu-CONH-$(CH_2)_3$-4-morpholinyl (**42**) was infused through the internal carotid artery.[6] After a delay, animals were sacrificed and the infarct volume was quantified. With **2**, a 75% reduction in infarct volume was achieved, while the maximum obtained with **42** was 32% using a different dosing protocol. A dose-dependent neuroprotective effect could be demonstrated in both cases. The morpholine compound **42** (AK295) is also able to attenuate motor dysfunction in rats following brain injury.[17] The significant improvement in behavioral outcome measurements suggest that **42** could be used in the treatment of brain injuries.

We have now investigated the inhibitory potencies of α-keto amides toward cysteine proteases using a series of new compounds. In addition, nine other α-keto amides, whose syntheses and inhibitory potencies toward cysteine proteases have been published elsewhere,[14] are presented here for comparison purposes. All α-keto amides share the general formula R_1-L-Leu-D,L-AA-CONH-R_2[18] (Figure 1). They combine 10 different R_1 groups, including heterocyclic (**75**−**80**) and nonheterocyclic aromatic (**1**−**74**, **81**−**89**) groups, 3 different amino acids (AA) in the P1 position (Abu, Phe, or Nva) and 44 different R_2 groups including alkyl, hydroxy, or alkoxy groups (**1**−**10**, **51**−**53**), arylalkyl groups (**11**−**32**, **54**−**65**, **72**), and heterocyclic derivatives (**33**−**50**, **66**−**71**, **73**, **74**).

Chemistry

We have previously reported synthetic methods for the preparation of peptidyl α-keto amides.[14] The procedure (Scheme 1) involves a modified Dakin−West reaction to prepare peptidyl α-enol esters **90**, which are then converted to α-keto esters **91** by reaction with alkoxides. The α-carbonyl group of the α-keto ester is then protected as a 1,3-dithiolane derivative (**92**) followed by reaction with an excess amount of the appropriate amine (R_2-NH_2) to yield the α-keto amide **94**. As previously reported, the 1,3-dithiolane protection group was unexpectedly lost during the reaction or workup procedure. This method (**91** → **92** → **94**) was used with compounds **1**−**48**, **51**−**70**, and **72**−**74** and as the final steps in the preparation of **81**−**89**. Many of the amines used in the coupling reaction were commercially available. Others, including 2-amino-1-(4-methoxyphenyl)-ethanol, 2-amino-1-(3,4,5-trimethoxyphenyl)ethanol, 2-amino-1-[4-(*N*,*N*-dimethylamino)phenyl]-ethanol, 2-amino-1-(pentafluorophenyl)ethanol, 2-amino-1-[3-(trifluoromethyl)phenyl]ethanol, 2-amino-1-(3-phenoxyphenyl)ethanol, 2-amino-1-(4-phenoxyphenyl)ethanol, 2-amino-1-[4-(benzyloxy)phenyl]ethanol, 2-amino-1-[3-[4-(trifluoromethyl)phenoxy]phenyl]ethanol, 2-amino-1-[3-(3,4-dichlorophenoxy)phenyl]ethanol, 2-amino-1-[3,4-bis(benzyloxy)phenyl]ethanol, 2-amino-1-(1-naphthyl)-ethanol, and 2-amino-1-(2-naphthyl)ethanol, were prepared using the trimethylsilyl cyanide method.[19]

The appropriate aldehyde was treated with trimethylsilyl cyanide and followed by reduction with $LiAlH_4$ to give the amine as a racemic mixture. 2-(Aminomethyl)quinoline[20] and 1-(aminomethyl)isoquinoline[21] were prepared by palladium-catalyzed hydrogenation of the corresponding nitrile derivatives. 1-(3-Aminopropyl)-1,2,3,4-tetrahydroquinoline[22] and 2-(3-aminopropyl)-1,2,3,4-tetrahydroisoquinoline[23] were prepared by refluxing the corresponding unsubstituted tetrahydro derivatives with acrylonitrile in acetic acid or toluene followed by catalytic hydrogenation or lithium aluminum hydride reduction of the resulting *N*-cyanoethyl derivatives.

8-(Aminomethyl)caffeine[24] was synthesized in seven steps according to the reported procedures. Three peptidyl α-keto amides **94** were prepared from the corresponding α-keto acids **93** by coupling using CDI (compound **49**) or DCC−HOBt (compounds **51** and **71**) and the appropriate amine in low yield. The starting peptidyl α-keto acids Z-Leu-Abu-CO_2H and Z-Leu-Phe-CO_2H (**93**) were prepared by alkaline hydrolysis of the corresponding α-keto esters **91** in high yield as previously reported.[14]

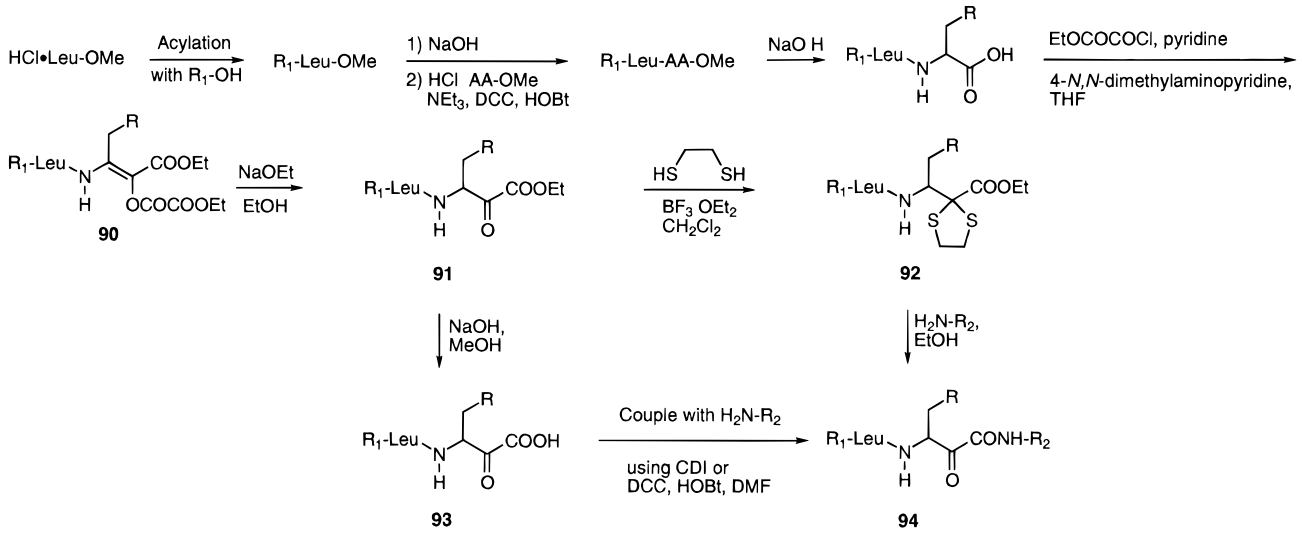
Several ketoamides (**75**−**80**) were prepared from **2** by deblocking the Z group and coupling HBr·Leu-Abu-CONHEt with the appropriate acid R_1-OH (Scheme 2). The synthesis of **81**−**89** involved coupling of R_1-Leu-OH with AA-OMe followed by hydrolysis to R_1-Leu-AA-OH which was then converted to the keto amide as described in Scheme 1.[14]

Results and Discussion

We have designed a series of new α-keto amides as specific calpain inhibitors. Because our previous results indicated that cysteine proteases possessed a hydrogen bond donor in the S1′ subsite,[14] we synthesized α-keto amides having one or several heteroatoms in an effort to further explore the H-bonding capacity of the S′ subsites. To take advantage of the hydrophobic pocket we previously identified in the S′ region,[14] we chose to incorporate the heteroatoms into aromatic structures (**14**−**33**, **35**−**40**, **44**−**49**, **56**−**69**, **72**, **73**, **84**, **85**, **87**, **88**). The hydrophobicity and H-bonding capacity of another region of the active site, the S3 and S4 subsites, were also explored using various heterocyclic (**75**−**80**) or nonheterocyclic aromatic (**1**−**74**, **82**−**89**) blocking groups on the N-terminus of the dipeptide α-keto amide inhibitors. The importance of the nature of the amino acid in the P1 position was investigated using 29 compounds which were analogs in all respects, except that they contained either an α-aminobutyric acid (Abu), a phenylalanine, or a norvaline (Nva) in P1.

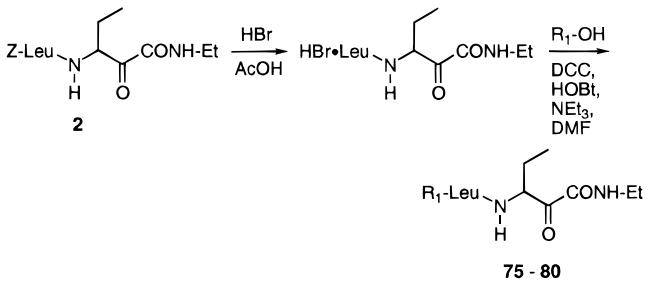
The inhibitory potencies of the peptidyl α-keto amides toward several cysteine proteases including calpain I, calpain II, and cathepsin B were investigated, and the results are presented in Tables 1 and 2. Data for the serine proteases pancreatic elastase and α-chymotrypsin and the cysteine protease papain are presented in the Supporting Information. Standard errors on K_i are not given individually but correspond to 5−15% of the given values. Some compounds were tested on bovine cathepsin B and others on human cathepsin B, depending on enzyme availability. After verification with several inhibitors that the measured K_i values did not differ significantly from one enzyme to the other, we chose to use both indiscriminately for comparison purposes.

Scheme 1[a]



[a] AA = Abu, Nva, Phe; R = Me, Et, Ph; R_1, R_2 = see Tables 1 and 2.

Scheme 2[a]



[a] R_1 = see Tables 1 and 2.

Calpain I Inhibition. Changing the substituent on the nitrogen atom of the α-keto amide function resulted in only a slight variation of inhibitory potency toward calpain I (less than 5-fold change with most compounds). The nitrogen substituents giving the best calpain I inhibitors were a pyridyl group, Z-Leu-Nva-CONH-CH_2-2-pyridyl (**73**, K_i = 19 nM), and phenyl-substituted derivatives of CH_2-CHOH-Ph (i.e., Z-Leu-Abu-CONH-CH_2-CHOH-C_6F_5, **23**, K_i = 50 nM; Z-Leu-Abu-CONH-CH_2-CHOH-C_6H_4-3-OC_6H_4(3-CF_3), **28**, K_i = 70 nM).

In contrast, modification of the N-terminal blocking group induced a large decrease in the inhibitory potency (up to 32-fold). The N-terminal blocking groups which gave the best calpain I inhibitors were the 1-naphthyl-CO (**83**), 1-isoquinolinyl-CO (**79**), and 2-quinolinyl-CO (**78**) derivatives which had inhibitory potencies in the same order of magnitude as the benzyloxycarbonyl analog **2** (K_i = 0.30, 0.35, 0.50, and 0.21 μM, respectively). Notably poor inhibition results were obtained with the morpholinyl derivative 4-morpholinyl-CO-Leu-Abu-CONHEt (**80**, K_i = 7.9 μM), and the bulky branched derivative Ph_2CHCO-Leu-Abu-CONH-Et (**82**, K_i = 5.0 μM).

The compounds possessing an Abu in the P1 position had a modest advantage over those with Phe, while compounds with a Nva residue in P1 were sometimes favored and sometimes disfavored. For example, Z-Leu-Nva-CONH-CH_2-2-pyridyl (**73**) is the best calpain I inhibitor presented in this paper with a K_i value of 19 nM, while the analogs with an Abu or a Phe residue in place of the Nva in P1 are weak calpain I inhibitors (0.64 and 0.65 μM, respectively). Another compound, Z-Leu-Nva-CONH-CH_2-CHOH-Ph (**72**), is a poor calpain I inhibitor (K_i = 7.8 μM), while Abu (**19**) and Phe (**56**) analogs have better inhibitory potencies, with K_i values of 1.1 and 1.3 μM, respectively.

Calpain II Inhibition. Among the various proteases tested, calpain II was most potently inhibited by this series of α-keto amides. A large number (27) of inhibitors have K_i values in the 10−100 nM range, whereas only five inhibitors have values this low with calpain I and only six with cathepsin B. Among the former are Z-Leu-Abu-CONH-CH_2-CHOH-Ph (**19**, K_i = 15 nM), Z-Leu-Abu-CONH-$(CH_2)_2$-Ph (**12**, K_i = 22 nM), and Z-Leu-Phe-CONH-CH_2-2-quinolinyl (**67**, K_i = 23 nM).

The introduction of heteroatoms into the amide nitrogen substituent of the α-keto amide or into the N-terminal blocking group of the inhibitors did not produce a large increase in affinity. This indicates that no additional significant H bonds are being formed between either the S or S′ sites of the enzyme and the inhibitor. Alternatively, the heteroatoms in the inhibitors may be in the wrong location to interact with H bond acceptors or donors in the enzyme. The existence of a hydrophobic pocket in S1′ was confirmed since α-keto amides R_1-L-Leu-D,L-AA-CONH-R_2 with R_2 = CH_2-CH_2-Ph or CHOH-CH_2-Ph (**12**, **19**, **55**, **56**) were excellent calpain II inhibitors, with K_i values ranging from 15 to 50 nM. But when the phenyl rings of these compounds were substituted (**14**−**17**, **20**−**32**, **57**−**65**), the affinity for calpain II decreased and ranged from 46 nM for Z-Leu-Abu-CONH-CH_2-CH_2-Ph(4-OMe) (**17**) to 670 nM for Z-Leu-Phe-CONH-CH_2-CHOH-Ph(3,4-$(OCH_2Ph)_2$) (**65**) indicating a very definite size for the hydrophobic pocket. Heterocyclic derivatives such as Z-Leu-Abu-CONH-CH_2-2-furyl (**33**, K_i = 33 nM), Z-Leu-Abu-CONH-CH_2-2-pyridyl (**25**, K_i = 17 nM), or Z-Leu-Phe-CONH-CH_2-2-quinolinyl (**67**, K_i = 23 nM) were also good calpain II inhibitors.

Variation of the N-terminal blocking group did not produce a large variation in the inhibitory potencies of the α-keto amides toward calpain II. None of the derivatives tested gave better inhibitory results than the parent benzyloxycarbonyl group. As observed with calpain I, the morpholinyl derivative 4-morpholinyl-CO-Leu-Abu-CONHEt (**80**, K_i = 9.9 μM) and the bulky branched derivative Ph_2CHCO-Leu-Abu-CONH-Et (**82**, K_i = 1.2 μM) were quite poor inhibitors.

Table 1. Inhibition of Cysteine Proteases and Platelet Membrane Permeability by Peptidyl α-Keto Amides R_1-Leu-AA-CONH-R_2[a]

compound				K_i (mM)			IC (μM) plat
no.	R_1	AA	R_2	Cal I[b]	Cal II[b]	Cat B[c,d]	memb permeability[e]
1	Z	Abu	Me	0.28	0.083	0.33[c]	
2	Z	Abu	Et[14]	0.25	0.21	2.4[d]	100
3	Z	Abu	*n*-Pr[14]		0.25	8.0[c]	70
4	Z	Abu	$(CH_2)_2OH$	0.80	0.078	4.5[d]	78
5	Z	Abu	$(CH_2)_5OH$	0.50	0.051	0.28[d]	54
6	Z	Abu	$(CH_2)_2O(CH_2)_2OH$	0.65	0.16	2.0[d]	58
7	Z	Abu	$CH_2CH(OCH_3)_2$	0.50	0.10	0.19[c]	
8	Z	Abu	$CH_2CH(OC_2H_5)_2$	0.20	0.16	0.11[c]	
9	Z	Abu	CH_2-C_6H_{11}[14]	0.68	0.044		
10	Z	Abu	CH_2-C_6H_7(1,3,3-$(CH_3)_3$-5-OH)	0.42	0.069	0.89[d]	103
11	Z	Abu	$CH_2C_6H_5$[14]	0.20	0.35	2.0[d]	30
12	Z	Abu	$(CH_2)_2C_6H_5$[14]	0.20	0.022	1.3[d]	50
13	Z	Abu	$(CH_2)_3C_6H_5$[14]	0.80	0.043	0.20[d]	100
14	Z	Abu	$(CH_2)_2C_6H_4$(4-OH)	0.38	0.060	nldp[d,f]	23
15	Z	Abu	$(CH_2)_2C_6H_4$(2-OCH_3)	0.13	0.16	0.63[d]	
16	Z	Abu	$(CH_2)_2C_6H_4$(3-OCH_3)	0.11	0.086	0.31[d]	
17	Z	Abu	$(CH_2)_2C_6H_4$(4-OCH_3)	0.12	0.046	0.44[d]	
18	Z	Abu	$CH_2C_6H_3$(3,5-$(OCH_3)_2$)	2.3	0.022	1.8[d]	30
19	Z	Abu	$CH_2CH(OH)Ph$	1.1	0.015	0.37[d]	69
20	Z	Abu	$CH_2CH(OH)C_6H_4$(4-OCH_3)	0.24	nldp[f]	0.12[c]	
21	Z	Abu	$CH_2CH(OH)C_6H_2$(3,4,5-$(OCH_3)_3$)	0.38	0.22	0.22[c]	
22	Z	Abu	$CH_2CH(OH)C_6H_4$(4-$N(CH_3)_2$)	0.33	0.41	0.072[c]	
23	Z	Abu	$CH_2CH(OH)C_6F_5$	0.050	0.20	0.15[c]	
24	Z	Abu	$CH_2CH(OH)C_6H_4$(3-CF_3)	0.35	0.18	0.11[c]	
25	Z	Abu	$CH_2CH(OH)C_6H_4$(3-OPh)	0.90	0.59	0.12[c]	
26	Z	Abu	$CH_2CH(OH)C_6H_4$(4-OPh)	0.10	0.29	0.21[c]	
27	Z	Abu	$CH_2CH(OH)C_6H_4$(4-OCH_2Ph)	0.080	0.12	0.11[c]	
28	Z	Abu	$CH_2CH(OH)C_6H_4$-3-OC_6H_4(3-CF_3)	0.070	0.28	0.30[c]	
29	Z	Abu	$CH_2CH(OH)C_6H_4$-3-OC_6H_3(3,4-Cl_2)	0.27	0.12	0.10[c]	
30	Z	Abu	$CH_2CH(OH)C_6H_3$(3,4-$(OCH_2Ph)_2$)	0.23	0.10	0.32[c]	
31	Z	Abu	$CH_2CH(OH)$-1-$C_{10}H_7$	0.12	0.17	0.063[c]	
32	Z	Abu	$CH_2CH(OH)$-2-$C_{10}H_7$	0.35	0.11	0.063[c]	
33	Z	Abu	CH_2-2-furyl	0.80	0.033	6.0[d]	58
34	Z	Abu	CH_2-2-tetrahydrofuryl	0.33	0.066	4.5[d]	22
35	Z	Abu	CH_2-2-pyridyl	0.64	0.017	3.0[d]	110
36	Z	Abu	CH_2-3-pyridyl		0.12	1.2[d]	>300
37	Z	Abu	CH_2-4-pyridyl	1.1	0.11	6.4[d]	54
38	Z	Abu	$(CH_2)_2$-2-pyridyl	0.41	0.47	0.20[d]	70
39	Z	Abu	$(CH_2)_2$-2-(*N*-methylpyrrole)	0.16	0.076	1.2[d]	
40	Z	Abu	$(CH_2$-3-1-imidazolyl	0.29	0.068	9.9[d]	
41	Z	Abu	$(CH_2)_2$-4-morpholinyl	1.0	0.16	2.5[d]	23
42	Z	Abu	$(CH_2)_3$-4-morpholinyl	0.14	0.041	6.9[d]	45
43	Z	Abu	$(CH_2)_3$-1-pyrrolidin-2-one	1.2	0.27	2.0[d]	
44	Z	Abu	$(CH_2)_2$-3-indolyl	0.30	0.050	0.031[c]	180
45	Z	Abu	CH_2-2-quinolinyl	0.13	0.60	0.45[c]	
46	Z	Abu	CH_2-1-isoquinolinyl	0.25	0.15	0.30[d]	
47	Z	Abu	$(CH_2)_3$-1-tetrahydroquinolinyl	0.37	0.20	0.094[c]	
48	Z	Abu	$(CH_2)_3$-2-tetrahydroisoquinolinyl	0.31	0.19	8.0[d]	
49	Z	Abu	CH_2-8-caffeinyl	32.0	4.6	1.7[c]	
50	Z	Abu	$(CH_2)_2NH$-biotinyl	0.65	0.28	0.66[c]	
51	Z	Phe	Me	0.35	0.15	0.57[c]	
52	Z	Phe	Et[14]	0.20	0.039	6.0[d]	22
53	Z	Phe	*n*-Pr[14]	0.35	0.050	3.0[d]	31
54	Z	Phe	CH_2Ph[14]		0.046	4.6	>300
55	Z	Phe	$(CH_2)_2Ph$[14]	0.052	0.024	9.3	100
56	Z	Phe	$CH_2CH(OH)Ph$	1.3	0.050	2.1[d]	44
57	Z	Phe	$CH_2CH(OH)C_6H_4$(4-$N(CH_3)_2$)	0.62	0.31	0.31[c]	
58	Z	Phe	$CH_2CH(OH)C_6F_5$	0.70	0.35	0.21[c]	
59	Z	Phe	$CH_2CH(OH)C_6H_4$(3-CF_3)	0.46	0.29	0.14[c]	
60	Z	Phe	$CH_2CH(OH)C_6H_4$(3-OPh)	0.60	0.34	0.64[c]	
61	Z	Phe	$CH_2CH(OH)C_6H_4$(4-OPh)	0.20	0.17	2.1[c]	
62	Z	Phe	$CH_2CH(OH)C_6H_4$(4-OCH_2Ph)	0.20	0.24	0.38[c]	
63	Z	Phe	$CH_2CH(OH)C_6H_4$-3-OC_6H_4(3-CF_3)	0.18	0.45	0.12[c]	
64	Z	Phe	$CH_2CH(OH)C_6H_4$-3-OC_6H_3(3,4-Cl_2)	0.59	0.12	0.64[c]	
65	Z	Phe	$CH_2CH(OH)C_6H_3$(3,4-$(OCH_2Ph)_2$)	0.48	0.67	20[c]	
66	Z	Phe	CH_2-2-pyridyl	0.65	0.27	0.80[d]	
67	Z	Phe	CH_2-2-quinolinyl	0.11	0.023	0.34[d]	
68	Z	Phe	$(CH_2)_3$-1-tetrahydroquinolinyl	0.38	0.26	0.14[c]	
69	Z	Phe	$(CH_2)_3$-2-tetrahydroisoquinolinyl	0.22	0.20	4.5[d]	
70	Z	Phe	$(CH_2)_3$-4-morpholinyl	0.12	0.33	13[d]	54
71	Z	Phe	$(CH_2)_2NH$-biotinyl	0.22	0.16	0.23[c]	
72	Z	Nva	$CH_2CH(OH)Ph$	7.8	11	3.4[c]	
73	Z	Nva	CH_2-2-pyridyl	0.019	0.12	0.75[d]	
74	Z	Nva	$(CH_2)_3$-4-morpholinyl	0.25	0.10	4.2[d]	18

[a] All of the peptide α-keto amide inhibitors are diastereomeric at the α-carbon of the P1 residue; peptide α-keto amides **19**−**32**, **56**−**65**, and **72** possess an additional chiral center (racemic mixture) on the P′ side. [b] 25 mM Tris, pH 8.0, 10 mM $CaCl_2$, 5% DMSO. [c] Bovine cathepsin B, 20 mM sodium acetate, pH 5.2, 0.5 mM dithiothreitol, 2% DMSO. [d] Human cathepsin B, 20 mM sodium acetate, pH 5.2, 0.5 mM dithiothreitol, 2% DMSO. [e] 14.7 mM HEPES, 0.4 mM sodium phosphate, 12 mM sodium bicarbonate, pH 7.35, 0.137 M NaCl, 3 mM KCl, 1 mM $MgCl_2$, 20 mM glucose, 2% DMSO. [f] nldp = gives nonlinear Dixon plots.

Table 2. Inhibition of Cysteine Proteases and Platelet Membrane Permeability by Peptidyl α-Keto Amides R_1-Leu-AA-CONH-R_2[a]

compound				K_i (μM)			IC_{50} (μM) plat memb permeability[e]
no.	R_1	AA	R_2	Cal I[b]	Cal II[b]	Cat B[c,d]	
75	2-furyl-CO	Abu	Et	0.85	0.58	1.2[c]	
76	3-pyridyl-CO	Abu	Et	1.30	0.83	0.88[c]	
77	2-pyrazinyl-CO	Abu	Et	1.4	0.48	0.35[c]	
78	2-quinolinyl-CO	Abu	Et	0.50	0.30	0.096[c]	
79	1-isoquinolinyl-CO	Abu	Et	0.35	0.11	0.22[c]	
80	4-morpholinyl-CO	Abu	Et	7.9	9.9	4.3[c]	
81	$Ph(CH_2)_2$-CO	Abu	Et	1.9	0.13	0.22[c]	
82	Ph_2CH-CO	Abu	Et	5.0	1.2	0.55[c]	
83	1-$C_{10}H_7CH_2$-CO	Abu	Et	0.30	0.25	0.35[d]	
84	Ph_2CH-CO	Abu	$CH_2CH(OH)Ph$	0.75	0.20	0.17[c]	180
85	Ph_2CH-CO	Abu	CH_2-2-pyridyl	0.5	0.09	2.8[d]	88
86	Ph_2CH-CO	Abu	$(CH_2)_3$-morpholinyl	0.8	0.11	2.3[d]	82
87	Ph_2CH-CO	Phe	$CH_2CH(OH)Ph$	10	0.73	0.24[c]	
88	Ph_2CH-CO	Phe	CH_2-2-pyridyl	1.1	0.36	2.2[d]	>300
89	Ph_2CH-CO	Phe	$(CH_2)_3$-morpholinyl	0.76	0.074	3.8[d]	

[a] All of the peptide α-keto amide inhibitors are diastereomeric at the α-carbon of the P1 residue; peptide α-keto amides **84** and **87** possess an additional chiral center (racemic mixture) on the P′ side. [b] 25 mM Tris, pH 8.0, 10 mM $CaCl_2$, 5% DMSO. [c] Bovine cathepsin B, 20 mM sodium acetate, pH 5.2, 0.5 mM dithiothreitol, 2% DMSO. [d] Human cathepsin B, 20 mM sodium acetate, pH 5.2, 0.5 mM dithiothreitol, 2% DMSO. [e] 14.7 mM HEPES, mM sodium phosphate, 12 mM sodium bicarbonate pH 7.35, 0.137 M NaCl, 3 mM KCl, 1 mM $MgCl_2$, 20 mM glucose, 2% DMSO.

We observed that Abu in P1 is slightly favored over the other two amino acids in our study. In the large majority of cases (13), replacement of the Abu residue by a Phe did not produce a significant change in inhibitory potency (<2-fold). However, with seven pairs of inhibitors, replacement of the Abu by Phe resulted in a decrease in affinity, while in two cases, it resulted in an increase in affinity.

Cathepsin B Inhibition. The peptidyl α-keto amides presented here displayed a lower affinity toward cathepsin B, since they were designed primarily to target calpains. However, the hypothetical H bond donor or acceptor that we have been probing for in the active site of the cysteine proteases is likely to be present in the S′ sites of cathepsin B. While alkyl substituents on the nitrogen (R_2) were poor inhibitors, hydroxy or alkoxy analogs (**5**, **7**, **8**) were more potent, with K_i values ranging from 51 to 160 nM. Similarly, unsubstituted phenyl derivatives (**11**, **12**, **19**, **55**, **56**) in R_2 showed poor inhibitory potencies (i.e., Z-Leu-Abu-CONH-CH_2-CH_2-Ph, **12**, $K_i = 1.3$ μM; Z-Leu-Phe-CONH-CH_2-CHOH-Ph, **56**, $K_i = 2.1$ μM) compared to analogs having the phenyl ring substituted with a group carrying halogens or heteroatoms (**15**−**18**) (i.e., Z-Leu-Abu-CONH-CH_2-CH_2-Ph(3-OMe), **16**, $K_i = 0.31$ μM; Z-Leu-Phe-CONH-CH_2-CHOH-Ph(3-CF_3), **59**, $K_i = 0.14$ μM). Almost all compounds having $R_2 = CH_2CHOHPh$ (**19**−**32**, **56**−**65**, **72**) were good cathepsin B inhibitors with K_i values as low as 63 nM (Z-Leu-Abu-CONH-CH_2-CHOH-1-naphthyl, **31**, or 2-naphthyl, **32** or 72 nM (Z-Leu-Abu-CONH-CH_2-CHOH-Ph(4-NMe_2), **22**). Conversely, heterocycles in P′ (**33**−**50**, **66**−**71**, **73**, **74**) resulted in a decreased inhibitory potency, although this was not observed with the pyridyl (**28**, $K_i = 200$ nM), indolyl (**44**, $K_i = 31$ nM), or tetrahydroquinolinyl (**47**, $K_i = 94$ nM) derivatives. Derivatives of imidazole, $(CH_2)_n$-4-morpholinyl, and tetrahydroquinoline should be positively charged at the pH of the inhibitory assay (pH 6). Extremely poor inhibitory results were obtained with all positively charged compounds in the P′ region (**40**−**42**, **48**, **69**, **70**, **74**), with K_i values as high as 13 μM.

Cathepsin B has dipeptidyl carboxypeptidase activity and interacts specifically with the C-terminal carboxyl group at the P2′ subsite of such substrates. This dipeptidyl carboxypeptidase activity is due to the presence of S2′ His-110 and His-111 residues which can form salt bridges with the substrate (Figure 3).[25] The poor inhibitory results obtained with our P′ positively charged inhibitors can be explained by an electrostatic repulsion effect between the inhibitors and these two histidine residues. Interestingly, this repulsive interaction is not observed with calpains I and II. Therefore, placement of positively charged residues in P2′ may be a useful technique for increasing the specificity of calpain inhibitors.

Modification of the N-terminal blocking group of the dipeptidyl α-keto amide chain led to rather small increases (up to 3.4-fold) or decreases (up to 3.6-fold) in the inhibitory potency of the α-keto amides. The best result was obtained with 2-quinolinyl-CO-Leu-Abu-CONH-Et (**78**, $K_i = 96$ nM). As observed with calpain II, no real preference for an Abu, Phe, or Nva residue in the P1 position was observed.

Selectivity. Considerable specificity for calpain was obtained in the dipeptide α-keto amides with Leu-Abu, Leu-Phe, or Leu-Nva sequences in the P2−P1 position. Serine proteases, such as α-chymotrypsin and pancreatic elastase, and cysteine proteases, papain and cathepsin B, are, in general, poorly inhibited. The specificity of serine proteases is determined primarily by the P1 residue, with chymotrypsin preferring aromatic amino acid residues and elastase, small hydrophobic residues. Peptidyl α-keto amides having an Abu residue in P1 position (**1**−**50**, **75**−**86**) are not inhibitors for α-chymotrypsin even at a high inhibitor concentration (150 μM). Pancreatic elastase is inhibited by such compounds but with poor K_i values, ranging from 10 μM for Z-Leu-Abu-CONH-CH_2-CHOH-Ph (**19**) up to 190 μM for Z-Leu-Abu-CONH-CH_2-C_6H_3(3,5-$(OMe)_2$) (**18**). Peptidyl α-keto amides with a Phe residue in the P1 position (**51**−**72**, **87**−**89**) are not pancreatic elastase inhibitors at concentrations as high as 100 μM. Only a few of these P1 Phe derivatives have been tested with α-chymotrypsin, and some are effective inhibitors with rather low K_i values. For example, Z-Leu-Phe-CONH-CH_2-CH_2-Ph (**55**) inhibits chymotrypsin with a K_i value of 0.8 μM. Despite its high affinity toward α-chymotrypsin, compound **55** remains principally, a calpain inhibitor, being 15- and 33-fold more active on calpains I and II, respectively. In general, the lack of inhibitory potency of dipeptidyl α-keto amides toward serine proteases can be explained by the small size of the inhibitors, with

pancreatic elastase in particular preferring longer peptide chains.

The cysteine proteases cathepsin B and papain preferentially cleave peptide substrates having a bulky residue such as Phe in the P2 position and a small hydrophobic residue in P1. Papain was, in general, very poorly inhibited by dipeptide α-keto amides having a Leu residue in P2 and a Phe or an Abu residue in P1, with affinity values greater than 38 μM. Cathepsin B was far more sensitive to their inhibitory potency, with one K_i value as low as 31 nM (**44**). However, the main target was calpains. Calpains preferentially cleave peptide substrates having a bulky residue such as Phe, Arg, Met, Abu, or Nva in the P1 position. Val or Leu residues are favored in the P2.

Specificity for calpain I, calpain II, or both calpains was obtained in various dipeptide α-keto amides having a Leu residue in P2 and a Phe, Abu, or Nva residue in P1. Among the α-keto amides specific for calpain I is Z-Leu-Nva-CONH-CH_2-2-pyridyl (**73**), having a K_i value of 19 nM toward calpain I and being 6.3- and 40-fold poorer with calpain II and cathepsin B, respectively. Other derivatives are specific for calpain II. For example, Z-Leu-Abu-CONH-CH_2-CHOH-Ph (**19**), Z-Leu-Abu-CONH-CH_2-2-pyridyl (**35**), and Z-Leu-Abu-CONH-CH_2-C_6H_3 (3,5-(OMe)$_2$) (**18**) have K_i values of 15, 17, and 22 nM toward calpain II and are 73-, and 38-, and 100-fold more potent on calpain II than on calpain I, respectively. They have respectively 25-, 180-, and 82-fold poorer affinity for cathepsin B. Some α-keto amides, such as Z-Leu-Abu-CONH-$(CH_2)_3$-2-tetrahydroisoquinolinyl (**48**) and Z-Leu-Phe-CONH-CH_2-CHOH-C_6H_3(3,4-(OCH_2Ph)$_2$) (**65**), are highly specific for both calpains without discriminating between calpains I and II.

Inhibitory Mechanism. The mechanism of inhibition of cysteine proteases by α-keto amides involves the formation of a reversible enzyme−inhibitor complex prior to the attack of the active site cysteine residue on the keto carbonyl group of the α-keto amides. This leads to the formation of a stable but reversible tetrahedral hemithioketal adduct (Figure 2). In addition to interactions with the S3−S1 subsites of the enzyme, we have proposed that the active site histidine is hydrogen-bonded to the carboxamide group.[14] In the vicinity of this hydrogen-bonding group, the S′ subsites contains a hydrophobic region or regions which are interacting with hydrophobic P′ nitrogen substituents in our inhibitors. It is also likely that there are additional hydrogen-bonding sites (donors or acceptors) in the S′ subsites, since many of the more potent calpain inhibitors have either hydrogen bond donor or acceptor groups (2-pyridyl, $CH_2CHOHPh$, etc.). However, this structural feature is not required since many derivatives lacking a hydrogen-bonding group are also potent calpain inhibitors.

Platelet Membrane Permeability Assay. The membrane penetrance of a number of dipeptide α-keto amide inhibitors was investigated. Treatment of rat platelets with a calcium ionophore results in the elevation of intracellular calcium concentration, activation of calpain, and calpain-mediated cleavage of the cytoskeletal proteins including spectrin. Using spectrin cleavage as an indicator or calpain inhibition, the membrane penetrance of the inhibitors can be inferred. Z-Leu-Phe-CONH-Et (**52**), Z-Leu-Nva-CONH-$(CH_2)_3$-4-morpholinyl (**74**), Z-Leu-Abu-CONH-$(CH_2)_2$-4-morpholinyl (**41**), Z-Leu-Abu-CONH-CH_2-2-tetrahydrofuryl (**34**), and Z-Leu-Abu-CONH-$(CH_2)_2$-C_6H_4(4-OH) (**14**) displayed the highest membrane penetrance in this assay, with IC_{50} values of 18−23 μM.

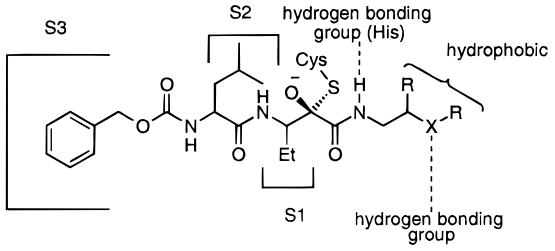


Figure 2. Proposed interaction of a dipeptidyl α-keto amide (Z-Leu-Abu-CONH-R_2) with the active site of calpain.

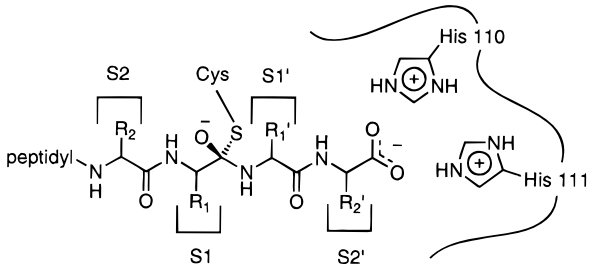


Figure 3. Proposed interaction of a substrate with the active site of cathepsin B.

Conclusion

A series of dipeptide α-keto amides with the general structure R_1-L-Leu-D,L-AA-CONH-R_2 are effective inhibitors of cysteine proteases. The majority of the inhibitors have inhibitory potency toward the cysteine proteases tested in the order: calpain II > calpain I > cathepsin B. In general, replacing the benzyloxycarbonyl blocking group at the N-terminus did not increase the potency of these inhibitors. None of the P1 amino acid residues (Abu, Nva, Phe) examined was clearly favored with calpain II or cathepsin B, although Nva appeared to be the best choice for calpain I. Introduction of a hydrophobic group (i.e., phenyl ring) on the P′ side improved inhibitory potency toward calpain II indicating a hydrophobic pocket at the S1′ site of the enzyme. Introduction of heteroatoms on the P′ side of the inhibitors increased potency toward cathepsin B but had less affect with the calpains.

Stroke ranks third in the number of deaths in the United States and is a major cause of disability. Postocclusion administration of the calpain keto amide inhibitor Z-Leu-Abu-CONH-$(CH_2)_3$-4-morpholinyl (**42**) gives a dose-dependent neuroprotective effect after focal brain ischemia in rats. This suggests that calpain proteolysis plays an important role in brain degeneration associated with cerebral ischemic events. Selective calpain inhibitors may provide a rational, novel, and viable means of treating such neurodegenerative diseases.

Experimental Section

Chemistry. Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. The purity of each compound was checked by TLC, mp, ^{1}H NMR, and MS. TLC was performed on Baker Si250F silica gel plates. Melting points were obtained on a Büchi capillary apparatus and are not corrected. ^{1}H NMR spectra were determined on a Varian Gemini 300 spectrom-

Table 3. Physical Properties of α-Keto Amides

no.	mp (°C)	% yield[a] (solvent)[c]	TLC R_f (solvent)[d]	formula	FAB MS[b]	anal.
1	125−127	37 (B)	0.39 (B)	$C_{20}H_{29}N_3O_5$	392	C,H,N
4	151−154	40 (C)	0.42 (C)	$C_{21}H_{31}N_3O_6$	422	C,H,N
5	122−123	42 (C)	0.54 (C)	$C_{24}H_{37}N_3O_6$	464	C,H,N
6	103−105	34 (C)	0.42 (C)	$C_{23}H_{35}N_3O_7$	466	C,H,N
7	99−102	25 (B)	0.47 (B)	$C_{23}H_{35}N_3O_7$	466	C,H,N
8	100−103	36 (B)	0.37 (B)	$C_{25}H_{39}N_3O_7$	494	C,H,N
10	59−61	51 (C)	0.55 (C)	$C_{29}H_{45}N_3O_6$	532	C,H,N
14	151−153	60 (C)	0.56 (C)	$C_{27}H_{35}N_3O_6$	498	C,H,N
15	101−103	71 (A)	0.47 (A)	$C_{28}H_{37}N_3O_6$	512	C,H,N
16	99−100	56 (A)	0.46 (A)	$C_{28}H_{37}N_3O_6$	512	C,H,N
17	152−155	50 (A)	0.46 (A)	$C_{28}H_{37}N_3O_6$	512	C,H,N
18	153−155	45 (A)	0.44 (A)	$C_{28}H_{37}N_3O_7$	528	C,H,N
19	152−154	50 (C)	0.48 (C)	$C_{27}H_{35}N_3O_6$	498	C,H,N
20	128−129	26 (F)	0.56 (H)	$C_{28}H_{37}N_3O_7$	528	C,H,N
21	170−172	29 (C)	0.54 (C)	$C_{30}H_{41}N_3O_9$	588	C,H,N
22	104 dec	23 (E)	0.41 (E)	$C_{29}H_{40}N_4O_6$	541	C,H,N
23	167−171	66 (C)	0.28 (C)	$C_{27}H_{30}N_3O_6F_5$	588	C,H,N
24	(ss)[e]	72 (C)	0.48 (C)	$C_{28}H_{34}N_3O_6F_3$	566	C,H,N
25	(oil)	67 (C)	0.54 (C)	$C_{33}H_{39}N_3O_7$	590.3378	C,H,N
26	55−60	48 (B)	0.22 (B)	$C_{33}H_{39}N_3O_7$	590	C,H,N
27	59−62	39 (B)	0.40 (B)	$C_{34}H_{41}N_3O_7$	604	C,H,N
28	97−101	57 (C)	0.40 (C)	$C_{34}H_{38}N_3O_7F_3$	658	C,H,N
29	63−67	55 (B)	0.28 (B)	$C_{33}H_{37}N_3O_7Cl_2$	658.2764	C,H,N
30	101−104	60 (C)	0.48 (C)	$C_{41}H_{47}N_3O_8$	710	C,H,N
31	63−71	15 (H)	0.48 (H)	$C_{31}H_{37}N_3O_6$	548	C,H,N
32	137−140	17 (F)	0.39 (H)	$C_{31}H_{37}N_3O_6$	548	C,H,N
33	138−139	43 (B)	0.68 (C)	$C_{24}H_{31}N_{3O6}$	458	C,H,N
34	126−128	35 (B)	0.59 (B)	$C_{24}H_{35}N_3O_6$	462	C,H,N
35	117−119	50 (C)	0.50 (C)	$C_{25}H_{32}N_4O_5$	469	C,H,N
36	122−123	35 (C)	0.54 (C)	$C_{25}H_{32}N_4O_5$	469	C,H,N
37	124−126	45 (C)	0.55 (C)	$C_{25}H_{32}N_4O_5$	469	C,H,N
38	128−130	53 (C)	0.60 (C)	$C_{26}H_{34}N_4O_5$	483	C,H,N
39	120−123	16 (A)	0.34 (A)	$C_{26}H_{36}N_4O_5$	485	C,H,N
40	52−55	27 (C)	0.33 (C)	$C_{25}H_{35}N_5O_5$	486	C,H,N
41	124−126	55 (C)	0.49 (C)	$C_{25}H_{38}N_4O_6$	491	C,H,N
42	125−126	42 (C)	0.50 (C)	$C_{26}H_{40}N_4O_6$	505	C,H,N
43	(ss)	33 (C)	0.51 (C)	$C_{26}H_{38}N_4O_6$	503	C,H,N
44	(ss)	18 (A)	0.47 (A)	$C_{29}H_{36}N_4O_5$	521.2745	nd[f]
45	135−138	16 (H)	0.27 (H)	$C_{29}H_{34}N_4O_5$	519	C,H,N
46	121−125	12 (H)	0.34 (H)	$C_{29}H_{34}N_4O_5$	519	C,H,N
47	(oil)	40 (A)	0.26 (B)	$C_{31}H_{42}N_4O_5$	551	C,H,N
48	(ss)	20 (B)	0.51 (B)	$C_{31}H_{42}N_4O_5$	551	C,H,N
49	171−177 dec	30 (B)	0.35 (C)	$C_{28}H_{37}N_7O_7$	584	C,H,N
50	188−192 dec	42 (D)	0.41 (D)	$C_{31}H_{46}N_6O_7S$	647	C,H,N
51	151−153	58 (B)	0.37 (B)	$C_{25}H_{31}N_3O_5$	454	C,H,N
56	164−166	46 (C)	0.72 (C)	$C_{32}H_{37}N_3O_6$	560	C,H,N
57	130 dec	22 (C)	0.68 (C)	$C_{34}H_{42}N_4O_6$	603	C,H,N
58	191−192	47 (B)	0.45 (B)	$C_{32}H_{32}N_3O_6F_5$	650	C,H,N
59	(ss)	42 (B)	0.48 (C)	$C_{33}H_{36}N_3O_6F_3$	628	C,H,N
60	(ss)	50 (B)	0.25 (B)	$C_{38}H_{41}N_3O_7$	652	C,H,N
61	146−149	30 (A)	0.20 (A)	$C_{38}H_{41}N_3O_7$	652	C,H,N
62	133−134	49 (B)	0.45 (B)	$C_{39}H_{43}N_3O_7$	666	C,H,N
63	142−143	52 (B)	0.23 (B)	$C_{39}H_{40}N_3O_7F_3$	720	C,H,N
64	136−137	41 (B)	0.40 (B)	$C_{38}H_{39}N_3O_7Cl_2$	721	C,H,N
65	149−152	45 (B)	0.42 (B)	$C_{46}H_{49}N_3O_8$	772	C,H,N
66	144−146	41 (B)	0.40 (B)	$C_{30}H_{34}N_4O_5$	531	C,H,N
67	131−135	33 (H)	0.30 (H)	$C_{34}H_{36}N_4O_5$	581	C,H,N
68	115−120	40 (A)	0.58 (B)	$C_{36}H_{44}N_4O_5$	613	C,H,N
69	107−111	51 (B)	0.62 (C)	$C_{36}H_{44}N_4O_5$	613	C,H,N
70	155−156	40 (C)	0.55 (C)	$C_{31}H_{42}N_4O_6$	567	C,H,N
71	204−206 dec	35 (D)	0.42 (D)	$C_{36}H_{48}N_6O_7S$	709	C,H,N
72	75−77	54 (C)	0.56 (C)	$C_{28}H_{37}N_3O_6$	512.3378	nd
73	65−70	50 (C)	0.55 (C)	$C_{26}H_{34}N_4O_5$	483.2656	nd
74	108−110	37 (C)	0.23 (C)	$C_{27}H_{42}N_4O_6$	519.3208	nd
75	58−59	39 (A)	0.51 (C)	$C_{18}H_{27}N_3O_5$	366	C,H,N
76	57−61	49 (D)	0.56 (C)	$C_{19}H_{28}N_4O_4$	377	C,H,N
77	51−56	18 (C)	0.33 (C)	$C_{18}H_{27}N_5O_4$	378	C,H,N
78	56−59	45 (H)	0.48 (H)	$C_{23}H_{30}N_4O_4$	427	C,H,N
79	104−106	46 (H)	0.47 (H)	$C_{23}H_{30}N_4O_4$	427	C,H,N
80	(oil)	33 (C)	0.45 (C)	$C_{18}H_{32}N_4O_5$	385.0209	nd
81	134−136	72 (A)	0.23 (A)	$C_{22}H_{33}N_3O_4$	404	C,H,N
82	78−83	24 (C)	0.40 (C)	$C_{27}H_{35}N_3O_4$	467	C,H,N
83	201−203	67 (A)	0.47 (A)	$C_{25}H_{33}N_3O_4$	440	C,H,N
84	178−180	30 (A)	0.40 (H)	$C_{33}H_{39}N_3O_5$	558	C,H,N
85	161−163	9 (J)	0.30 (C)	$C_{31}H_{36}N_4O_4$	529	C,H,N
86	170−174	25 (J)	0.25 (C)	$C_{32}H_{44}N_4O_5$	565	C,H,N
87	192−196	16 (M)	0.41 (L)	$C_{38}H_{41}N_3O_5$	620	C,H,N
88	160−162	9 (K)	0.33 (L)	$C_{36}H_{38}N_4O_4$	591	C,H,N
89	158−160	20 (M)	0.45 (L)	$C_{37}H_{46}N_4O_5$	627	C,H,N

[a] Yields are for the conversion of the peptide keto ester to keto amide. [b] Compounds which are oils or semisolids have high-resolution mass spectral data (FAB), while the solids have C, H, and N analyses. [c] Solvent in the purification of the product by column chromatography. [d] Solvent used in the TLC. [e] ss = semisolid. [f] nd = not determined.

Table 4. Proton NMR Data ($CDCl_3$) for Selected Compounds[a]

$$C_6H_5CH_2OCO\text{-}NH^b\text{-}CH^c(R_1)\text{-}CO\text{-}NH^d\text{-}CH^e(R_2)\text{-}CO\text{-}CO\text{-}NH^f\text{-}R_3$$

$$R_1 = \text{-}CH_2CH(CH_3)_2;\ R_2 = \text{-}C_2H_5 \text{ or } \text{-}CH_2C_6H_5$$

no.	H^b	H^c	H^d	H^e	H^f	other major signals
7	6.85, brd	5.26, m	5.36 brs	4.26, m	7.11, brs	5.12 s, 2H (OC**H₂**Ph); 4.52, t, 1H (C**H**(OEt)₂); 3.72, m, 2H; 3.54, m, 2H; 3.44, q, 2H; 1.22, t, 6H
16	6.77, brs	5.20, brs	5.56, brd	4.20, m	7.07, brs	5.09, s, 2H (OC**H₂**Ph); 3.75, s, 3H (OCH_3); 3.53, m, 2H (CH_2N); 2.81, m, 2H(C**H₂**CH_2N)
19	6.30, brs	5.17, m	5.25, m	4.22, m	7.19, brs	5.11, s, 2H (OC**H₂**Ph); 4.86, m, 1H (C**H**OH); 3.73 & 3.41, d of m, 2H (C**H₂**CHOH); 2.95, brs, 1H (OH)
22	6.70, d	5.18, m	5.18, m	4.20, m	6.70, d	5.10, s, 2H (OC**H₂**Ph); 4.75, brs, 1H (C**H**OH); 3.65 & 3.38, d of m, 2H (C**H₂**CHOH); 2.95, s, 6H ($N(CH_3)_2$)
23[f]	8.18, brs	5.05, m	5.96, m	4.11, m	8.69, brs	5.01, s, 2H (OC**H₂**Ph); 4.86, brs, 1H (C**H**OH); 3.44 & 3.25, d of m, 2H (C**H₂**CHOH)
26	6.77, brs	5.15, m	5.25, m	4.22, m	7.12, s	5.11, s, 2H (OC**H₂**Ph); 4.86, brs, 1H (C**H**OH); 3.67 & 3.41, d of m, 2H (C**H₂**CHOH)
27	6.94, brd	5.10, m	5.20, m	4.22, m	7.04, s	5.11, s, 2H (OC**H₂**Ph); 5.06, s, 4H (OC**H₂**Ph); 4.84, brs, 1H (C**H**OH); 3.70 & 3.39, d of m, 2H (C**H₂**CHOH)
28	6.72, brs	5.10, m	5.20, m	4.21, m	7.09, s	5.11, s, 2H (OC**H₂**Ph); 4.88, brs, 1H (C**H**OH); 3.70 & 3.40, d of m, 2H (C**H₂**CHOH)
31	6.99, brs	5.45, m	5.68, m	4.28, m	7.05, brs	5.25, brs, 1H (C**H**OH); 5.07, s, 2H (OC**H₂**Ph); 3.96 & 3.36, d of m, 2H (C**H₂**CHOH)
35	6.68, brd	5.15, m	5.29, m	4.22, m	7.21, brs	8.57, d, 1H; 8.02, s, 1H; 7.68, t, 1H; 7.34, m, 6H; 5.11, s, 2H (OC**H₂**Ph); 4.59, d, 2H (CH_2N)
42	6.62, brd	5.15, m	5.25, brs	4.21, m	8.71, brs	5.12, s, 2H (OC**H₂**Ph); 3.77, t, 4H (CH_2O); 3.42, m, 2H (CH_2N); 2.48, t, 6H (CH_2N)
45	6.77, brd	5.22, brs	5.36, brs	4.26, m	8.47, brs	8.14, m, 2H; 7.81, d, 1H; 7.74, t, 1H; 7.56, t, 1H; 7.34, m, 6H; 5.13, s, 2H (OC**H₂**Ph); 4.78, d, 2H (CH_2N)
50	7.96, m	4.91, m	7.66, d	4.12, m	8.68, d 8.26, m	6.43, d, 1H; 6.36, s, 1H; 4.29, t, 1H; 4.12, m, 1H; 3.07, m, 1H; 2.82, d of d, 1H; 2.56, s, 1H (H of the ring of biotin); 3.20−3.07, m, 4H (NH(C**H₂**)₂NH); 2.09, t, 2H (NHCOC**H₂**)
56	6.54, brs	5.04, brs	5.50, m	4.15, m	7.09, brs	5.09, s, 2H (OC**H₂**Ph); 4.91, brs, 1H (C**H**OH); 3.73 & 3.44, d of m, 2H (C**H₂**CHOH); 3.31 & 3.10, d of m, 2H (Phe)
61	6.88, brs	5.40, m	5.74, m	4.40, m	7.26, brs	5.35, s, 2H (OC**H₂**Ph); 5.11, brs, 1H (C**H**OH); 3.98 & 3.68, d of m, 2H (C**H₂**CHOH); 3.56 & 3.33, d of m, 2H (Phe)
63	6.58, brs	5.35, m	5.45, m	4.12, m	7.11, brs	7.68, s, 1H; 7.58, t, 2H; 7.50, m, 1H; 7.36, s, 5H; 5.08, s, 2H (OC**H₂**Ph); 3.75 & 3.35, d of m, 2H (C**H₂**CHOH); 3.28 & 3.08, d of m, 2H (Phe)
67	6.75, brs	5.22, m	5.64, m	4.20, m	8.47, brs	8.13, d of d, 2H; 7.81, d, 1H; 7.73, t, 1H; 7.56, t, 1H; 7.40−7.15, m, 10H; 7.10, d, 1H; 4.78, d, 2H (**CH₂**-C_9H_6N); 3.36 & 3.13, d of m, 2H (Phe)
70	6.51, brs	5.03, brs	5.56, m	4.16, m	8.78, brs	3.72, t, 4H (CH_2O); 3.44, q, 2H (CH_2N); 3.37 & 3.13, d of m, 2H (Phe); 2.46, m, 6H (CH_2N)
71	7.87, m	5.22, m	5.05, m	4.10, m	8.73, d of d	6.41, s, 1H; 6.35, s, 1H; 4.28, t, 1H; 8.32, d of d; 4.10, m, 1H; 3.07, m, 1H; 2.78, d of d, 1H; 2.55, s, 1H (H of the ring of biotin); 3.20, m, 4H (NH(C**H₂**)₂NH); 2.06, t, 2H (NHCOC**H₂**)
77	7.11, d	5.30, m	7.11, d	4.81, m	8.23, brs	9.40, d, 1H; 8.78, s, 1H; 8.57, s, 1H
79	7.37, m	5.33, m	7.12, brd	4.87, m	7.81, m	9.52, d, 1H; 8.66, t, 1H; 8.46, d, 1H; 7.81, m, 1H; 7.66, m, 2H
81	6.78, brs	5.22, m	5.92, brs	4.48, m	6.90, brs	3.32, q, 2H (NCH_2); 2.92, t, 2H (CH_2CO); 2.50, t, 2H (C**H₂**Ph); 1.20, t, 3H (NCH_2C**H₃**)

[a] Solvent was DMSO-d_6.

eter. Chemical shifts are expressed in ppm relative to internal tetramethylsilane. Mass spectra were obtained on a Varian MAT 112S spectrometer. Column chromatography was performed on silica gel (32−63 μm) using the following solvent systems: A, $CHCl_3$:CH_3OH = 30:1; B, $CHCl_3$:CH_3OH = 20:1; C, $CHCl_3$:CH_3OH = 10:1; D, $CHCl_3$:CH_3OH = 5:1; E, AcOEt:hexane = 6:1; F, AcOEt:hexane = 3:2; G, AcOEt:hexane = 2:1; H, AcOEt:hexane = 1:1; I, butanal:AcOH:H_2O = 4:1:1; J, $CHCl_3$:AcOEt = 7:3; K, $CHCl_3$:AcOEt = 9:1; L, AcOEt:CH_3-OH = 9:1; M, AcOEt.

The physical properties of the peptide α-keto amides inhibitors are given in Table 3. Proton NMR data for the inhibitors are given in Table 4.

Z-Leu-Abu-CONH-CH_2-2-pyridyl (35): General Procedure for the Synthesis of Keto Amides from Keto Esters. This procedure was used for the synthesis of compounds **1**−**48**, **51**−**70**, and **72**−**74** using the route shown in Scheme 1 (**91** → **92** → **94**). The procedure was also used as the final steps in the synthesis of **81**−**89**.

Z-Leu-Abu-CONH-CH_2-2-pyridyl was synthesized from the 1,3-dithiolane derivative of Z-Leu-Abu-COOEt (**92**, R = Me, R_1 = Z) and 2-(aminomethyl)pyridine as described earlier for the synthesis of other keto amide derivatives with the Z-Leu-Abu sequence.[14] The synthesis of **92** was described earlier.[14] Thus, after a mixture of **92** (1,3-dithiolane derivative of Z-Leu-Abu-CO_2Et; 1 g, 2.07 mmol) and the amine (6.7 g, 61.9 mmol) in ethanol (5 mL) was stirred overnight at room temperature, the solvent was evaporated and AcOEt (50 mL) was added. This mixture was filtered to remove a white precipitate. The filtrate was washed with water (4 × 20 mL) and saturated NaCl (2 × 20 mL) and dried over $MgSO_4$. Chromatography on a silica gel column using solvent C followed by precipitation from AcOEt/hexane afforded a yellow solid.

Z-Leu-Abu-CONH-CH_2-8-caffeinyl (49) *via* the Keto Acid Using CDI. Z-Leu-Abu-CONH-CH_2-8-caffeinyl was prepared from Z-Leu-Abu-COOH (**93**, R = Me, R_1 = Z)[14] and 8-(aminomethyl)caffeine (Scheme 1, **93** → **94**).[23] Thus, CDI (0.32 g, 1.98 mmol) was added to a solution of Z-Leu-Abu-COOH (0.5 g, 1.32 mmol) in DMF (5 mL), and the mixture was stirred for 2 h at room temperature. The reaction mixture was then added to a stirred suspension of 8-(aminomethyl)-caffeine (0.36 g, 1.58 mmol) in DMF (20 mL) and the reaction mixture stirred for 3 days at room temperature. After evaporating DMF, AcOEt (30 mL) and water (10 mL) were added, and the AcOEt layer was washed with H_2O (5 × 20 mL) and saturated NaCl (2 × 20 mL), dried over $MgSO_4$, and

concentrated. Chromatography on a silica gel column using solvent B followed by precipitation from AcOEt/hexane afforded a yellow solid.

Z-Leu-Abu-CONH-$(CH_2)_2$NH-biotinyl (50) *via* the Keto Acid Using DCC/HOBt: Procedure for Compounds 50 and 71. Biotin (1 g, 4.1 mmol) was dissolved in 20 mL of DMF at 70 °C and then cooled to 40 °C, CDI (0.97 g, 6 mmol) in 3 mL of DMF was added, and a white precipitate appeared. After the mixture was stirred at room temperature for 2 h, ethylenediamine (1.34 mL, 20 mmol) in 10 mL of DMF was added and stirring continued for another 3 h. After evaporating the DMF, the semisolid residue was dissolved in 50 mL of refluxing methanol and the unreacted biotin was removed by filtration. The solution was evaporated to dryness. The residue was washed with $CHCl_3$ to remove the imidazole and then dissolved in 6 mL of water, acidified to pH 3.0 with 1 N HCl, and evaporated to dryness. The crude biotinylethylenediamine hydrochloride was crystallized from methanol to give 1.04 g of biotinylethylenediamine hydrochloride (81% yield): long spot on TLC, $R_f = 0.21$ (I); mp 241−242 °C; ^{1}H NMR (DMSO-d_6) 8.05 (brs, 1H), 7.86 (brs, 3H), 6.42 (brd, 2H), 4.31 (t, 1H), 4.12 (t, 1H), 3.25 (m, 2H), 3.09 (m, 1H), 2.83 (m, 3H), 2.56 (d, 1H), 2.05 (t, 2H), 1.53−1.28 (m, 6H).

To a stirred solution of Z-Leu-Abu-CO_2H (**93**, R = Me, R_1 = Z; 0.6 g, 1.58 mmol)[14] in DMF (15 mL) were added HOBt (0.22 g, 1.58 mmol) and DCC (0.49 g, 2.38 mmol), and stirring was continued for 2 h at room temperature. TEA (0.28 mL, 2.03 mmol) was added to a stirred solution of biotinylethylenediamine hydrochloride (0.6 g, 1.85 mmol) in DMF (10 mL) at 0−5 °C and stirred for 2 h at room temperature. This solution was then added to the DCC/HOBt reaction mixture and stirred for 3 days. After filtration, the filtrate was evaporated to get a semisolid which was washed with H_2O (30 mL), 1 M HCl (30 mL), and H_2O (30 mL) and dried under vacuum. Chromatography on a silica gel column using solvent D afforded a yellow solid.

2-Furyl-CO-Leu-Abu-CONHEt (75): General Procedure for Compounds 75−80. A solution of hydrogen bromide in acetic acid (30 wt %, 1.52 mL, 7.40 mmol) was added to Z-Leu-Abu-CONHEt (**2**; 1 g, 2.47 mmol) at room temperature. The mixture was vigorously stirred for 1 h, during which all the keto amide dissolved in the acetic acid. The reaction was quenched with Et_2O (30 mL), and then the mixture was filtered. The semisolid product was triturated and washed successively with Et_2O (5 × 30 mL). After removal of solvent, the Leu-Abu-CONHEt·HBr was dried under vacuum, leaving a very hygroscopic solid (70% yield). ^{1}H NMR ($CDCl_3$) showed the loss of the Z group.

DCC (0.44 g, 2.13 mmol) and HOBt (0.29 g, 2.13 mmol) were added to a stirred solution of 2-furoic acid (0.24 g, 2.13 mmol) in DMF (10 mL), and the mixture was stirred for 2 h at room temperature. TEA (0.2 mL, 1.42 mmol) was added to a stirred solution of Leu-Abu-CONHEt·HBr (0.5 g, 1.42 mmol) in DMF (5 mL) at 0−5 °C and stirred for 3 min. This solution was then added to the DCC/HOBt reaction mixture at 0−5 °C and stirred overnight at room temperature. After evaporating DMF and adding AcOEt (40 mL), the precipitate was filtered, and the filtrate was washed with 0.25 M HCl (10 mL), H_2O (20 mL), 10% Na_2CO_3 (3 × 20 mL), H_2O (20 mL), and saturated NaCl (2 × 20 mL), dried over $MgSO_4$, and concentrated. Chromatography on a silica gel column with solvent C afforded a yellow solid.

1-$C_{10}H_7CH_2$-CO-Leu-Abu-CONHEt (83): General Procedure for Compounds 81−89. Leucine methyl ester hydrochloride (3.9 g, 26.8 mmol) was acylated with R_1-OH (R_1 = 1-$C_{10}H_7CH_2$-CO; 5 g, 26.8 mmol; R_1 = Ph_2CHCO or $PhCH_2$-CH_2CO for **81**, **82**, **84**−**89**) in the presence of TEA (3 g, 29.7 mmol) and CDI (4.8 g, 29.5 mmol) by stirring the mixture in DMF (20 mL) for 2 hours at room temperature to form R_1-Leu-OMe (7.5 g, 23.96 mmol, 89% yield): ^{1}H NMR ($CDCl_3$) 0.70 (d, 3H), 0.76 (d, 3H), 1.26 (m, 2H), 1.46 (m, 1H), 3.61 (s, 3H), 4.04 (m, 2H), 4.59 (m, 1H), 5.61 (d, 1H), 7.46−7.53 (m, 4H), 7.86−7.96 (m, 3H).

The methyl ester was then hydrolyzed with 1.2 equiv of NaOH in methanol (30 mL) for 3 h at room temperature to give a quantitative yield of R_1-Leu-OH (R_1 = 1-$C_{10}H_7CH_2$-CO; 7.16 g, 23.96 mmol) followed by coupling with AA-OMe·HCl (AA = Abu; 4.62 g, 30 mmol) in the presence of TEA (4.22 mL, 30 mmol), HOBt (3.4 g, 25 mmol), and DCC (6.7 g, 32.5 mmol) in DMF for 16.5 h at room temperature to give the dipeptidyl methyl ester R_1-Leu-AA-OMe (1-$C_{10}H_7CH_2$-CO-Leu-Abu-OMe): ^{1}H NMR ($CDCl_3$) 0.65−0.89 (m, 9H), 1.10−1.90 (m, 5H), 3.72 (s, 3H), 3.95−4.14 (m, 2H), 4.43 (dd, 2H), 5.70 (d, 1H), 6.59 (dd, 1H), 7.39−7.55 (m, 4H), 7.79−7.98 (m, 3H).

The dipeptidyl acid (R = Me, R_1 = 1-$C_{10}H_7CH_2$-CO) was quantitatively obtained by hydrolysis of the corresponding dipeptidyl methyl ester with NaOH in methanol followed by the Dakin−West procedure to give the dipeptidyl enol ester **90**.[14] Subsequent hydrolysis by NaOEt and protection of the α-keto group with 1,2-ethanedithiol gave the corresponding α-keto ester **91** and its 1,3-dithiolane derivative **92**.[14]

The final product **83** (1.13 g, 2.59 mmol, 67% yield) was prepared by reacting **92** (2 g, 3.87 mmol) with ethylamine (3.5 g, 77 mmol) under the reaction conditions described earlier for **35**.

Biochemistry. The materials, enzymes, enzyme kinetic assays, and platelet membrane permeability assay have been described previously.[14] With calpain, initial velocities (t = 30−60 s) of Suc-Leu-AMC hydrolysis were determined at room temperature after a delay of 30 s. Five or more concentrations of inhibitor (not exceeding 2 × K_{obs}) and two fixed concentrations of substrate were utilized. K_i values were determined by Dixon plots. The average of triplicate assays, plotted as $1/v$ versus I, gave intersecting lines with a correlation coefficient ≥0.95. No other attempt was made to correct for slow binding or autolysis. The intra-assay variation in the platelet membrane assay is approximately 5%. An internal standard was always run to control for inter-assay variation. The total variance in this assay is less than 15%.

Supporting Information Available: Inhibition studies of peptidyl α-keto amides on porcine pancreatic elastase, bovine α-chymotrypsin, and papaya latex papain (3 pages). Ordering information is given on any current masthead page.

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(18) Abbreviations: Abu, α-aminobutyric acid; AcOEt, ethyl acetate; *n*-Bu, *n*-butyl; Bzl, benzyl; -C_6H_7 (1,3,3-$(CH_3)_3$-5-OH), 1,3,3-trimethyl-5-hydroxylcyclohexyl; 8-caffeinyl, 8-substituted derivative of caffeine or 8-substituted 1,3,7-trimethylxanthine; Cal I, calpain I; Cal II, calpain II; Cat B, cathepsin B; CDI, 1,1-carbonyldiimidazole; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; Pap, papain; Ph, phenyl; TEA, triethylamine; TLC, thin layer chromatography; Z, benzyloxycarbonyl. The peptidyl α-keto amides RCO-L-Leu-D,L-NHCH(R′)-CO-CONHR″ are abbreviated as R_1-L-Leu-D,L-AA-$CONHR_2$ or simply as R_1-Leu-AA-$CONHR_2$.

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