Stereospecific Synthesis of Peptidyl α-Keto Amides as Inhibitors of Calpain

Scott L. Harbeson,* Susan M. Abelleira, Alan Akiyama, Robert Barrett, III, Reneé M. Carroll, Julie Ann Straub, Jaroslaw N. Tkacz, Chichih Wu, and Gary F. Musso

Alkermes, Inc., 64 Sidney Street, Cambridge, Massachusetts 02139-4136

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Peptidyl α-keto amides have been synthesized and tested as inhibitors of the cysteine protease calpain. A stereospecific synthesis was devised in which Cbz-dipeptidyl-α-hydroxy amides were oxidized with TEMPO/hypochlorite to the corresponding α-keto amides. This oxidation was accomplished in good yields and without epimerization of the chiral center adjacent to the ketone. The potent inhibition of porcine calpain I by the L,L diastereomers, combined with the poor inhibition by the L,D diastereomers, established the requirement for the all-L stereochemistry of the active inhibitor. The early lead inhibitors were very hydrophobic and, therefore, poorly soluble in aqueous solutions. Using the stereospecific route, new compounds were prepared with polar groups at the C- and N-termini. These modifications resulted in more soluble inhibitors that were still potent inhibitors of calpain. Studies of the stability of these α-keto amides showed that absolute stereochemistry can be maintained in acidic and unbuffered environments but general base-catalyzed epimerization of the chiral center adjacent to the ketone occurred rapidly. The α-hydroxy precursors were inactive as inhibitors of calpain, which supports the hypothesis that the α-keto compounds reversibly form an enzyme-bound tetrahedral species that results from the nucleophilic addition of the catalytic thiol of calpain to the electrophilic ketone of the inhibitor.

Introduction

Following either global (e.g., cardiac arrest, drowning) or focal (e.g., stroke) cerebral ischemia, a cascade of biochemical actions is initiated that results in a delayed degeneration of neurons.[1-4] One therapeutic strategy that has been proposed for the treatment of the neurodegeneration resulting from cerebral ischemia is the inhibition of intracellular calpains (calcium activated neutral proteases). These proteases are activated by the increased intracellular calcium concentrations associated with ischemic events.[5-8] Several neuronal structural proteins have been shown to be good substrates for activated calpain; therefore, the activation of calpain that occurs during ischemia may result in the degradation of these structural proteins. Calpain inhibitors may be useful therapeutics since the inhibition of activated calpain could also limit the delayed neuronal damage associated with ischemia.[6-12] Although calpain displays a marked preference for large protein substrates,[7,13] several reports of small molecule inhibitors of calpain have recently appeared. These inhibitors can be classified as either irreversible (such as peptidyl halomethyl ketones,[14] epoxides,[15] peptidyl-*O*-acylhydroxylamines,[16] and peptidyl diazomethylketones[17]) or reversible (such as peptidyl aldehydes,[18a,b-20] peptidyl diketones,[22] peptidyl α-keto esters,[21-23] α-keto acids,[22,23] and α-keto amides[22,23]).

Our approach to the development of calpain inhibitors is based upon the initial work of Powers[22] and others[23] using peptidyl α-keto amides as reversible inhibitors of cysteine proteases. This previous work had shown that dipeptidyl α-keto esters, α-keto amides and α-keto acids, where P_2 was leucine [Schecter and Berger nomenclature[24]], were selective, potent inhibitors of calpain.[21,22] The Dakin–West chemistry used by Li et al.[22] to synthesize these dicarbonyl inhibitors limited not only the choice of residues at P_1 and P_2 but also the types of functionality at both the N- and C-termini. This synthetic route also yielded a mixture of diastereomers due to epimerization of the chiral center at P_1. The L,L diastereomer, which mimics a dipeptide substrate, should be the active inhibitor. To unequivocally establish this, we devised a synthetic route that would yield inhibitors of defined stereochemistry. This synthetic route also allowed the synthesis of inhibitors with greater structural diversity.

In order to effectively inhibit calpains *in vivo*, an inhibitor must penetrate cell membranes. An earlier study had shown that peptidyl α-keto acid inhibitors, although potent *in vitro* inhibitors of calpain, were ineffective against intracellular calpain in platelets due to poor membrane permeance.[22] To insure membrane permeability, our inhibitor design focused on the preparation of neutral, hydrophobic compounds. Others had observed that the ester bond of α-keto esters was rapidly metabolized *in vivo*;[22] therefore, we focused our efforts on peptidyl α-keto amides. The hydrophobic α-keto amides were expected to penetrate cell membranes; however, the hydrophobicity of these inhibitors would have to be balanced with aqueous solubility to obtain a potential therapeutic agent. Furthermore, the stability of these α-keto amide inhibitors was a concern since not only would the electrophilic ketone be expected to react with nucleophiles but the chiral center adjacent to the ketone could be prone to epimerization. The synthesis of these inhibitors as single diastereomers was an important first step to answering these concerns.

In this report we present the stereospecific synthesis of peptidyl α-keto amides and the inhibitory potency of these single diastereomers against calpain. Using this synthetic strategy, inhibitors with different C- and N-terminal functional groups were prepared and used to expand our understanding of calpain inhibitor structure–activity. Several of these structural modifications

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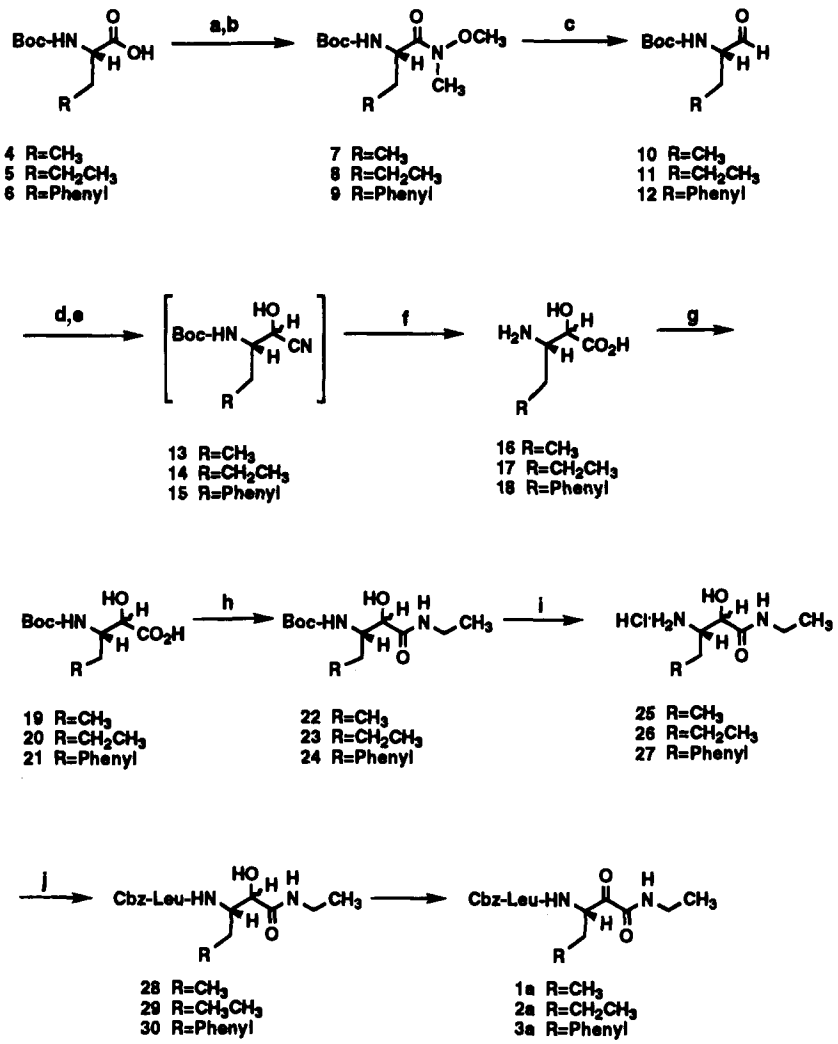
Scheme 1[a]



[a] Reagents: (a) CDI or PyBOP; (b) $HNOCH_3(CH_3)$–HCl, DIEA; (c) $LiAlH_4$; (d) $NaHSO_3$; (e) KCN; (f) concentrated HCl/dioxane (1:1); (g) Boc_2O, 1 N NaOH; (h) ethylamine (aqueous), EDC, HOBt; (i) 4 N HCl/dioxane; (j) Cbz-Leu, EDC, HOBt, DIEA; (k) TEMPO, NaOCl(aq), KBr.

yielded inhibitors with enhanced water solubility. Finally, we describe the physicochemical properties of single diastereomer inhibitors with respect to their overall stability, relative solubility, and sensitivity toward epimerization

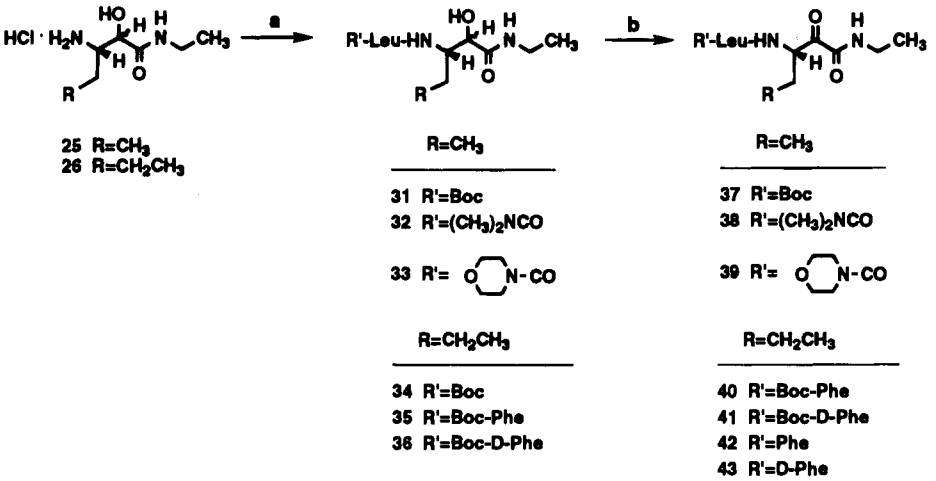
Results

Stereospecific Synthesis of α-Keto Amides 1a–3a, 3b. Compounds **1a–3a** were synthesized from the corresponding Boc-protected amino acids as shown in Scheme 1. The Boc-amino acids were converted to the amides **7–9** using *N,O*-dimethylhydroxylamine[25] and either carbonyldiimidazole (CDI) or PyBOP in anhydrous THF/DMF. Extractive workup provided the desired amides in yields of 82–99%. Amides **7-9** were reduced with LAH[26,27] to the Boc-amino aldehydes **10-12** in yields of 85–99%.

The α-hydroxy-β-amino acids **16–18** were prepared from the aldehydes according to established procedures.[28,29] The aldehydes were converted to the cyanohydrins **13–15,** which were isolated without purification and directly hydrolyzed in refluxing 1:1 concentrated HCl/dioxane (v/v). Isolation of the α-hydroxy-β-amino acids **16–18** in yields of 52–65% was accomplished either by crystallization or ion-exchange chromatography. The α-hydroxy-β-amino acids **16–18** were protected with the Boc group by treatment with di-*tert*-butyl dicarbonate in dioxane with 1 N NaOH as the base. The modest yields of 46–79% for this step were due to the aqueous solubility of some of the product acids, which resulted in losses during the workup. The Boc-protected α-hydroxy-β-amino acids **19–21** were then coupled to ethylamine using EDC to provide the ethyl amides **22–24** in yields ranging from 66 to 86%. Treatment of **22–24** with 4 N HCl/dioxane removed the Boc protecting group, and the resulting amine hydrochlorides **25–27** were coupled directly to Cbz-Leu to provide **28–30** in yields of 67–83%.

Oxidation of the α-hydroxy amides **28–30** to the desired α-keto amides was achieved using the nitroxyl radical TEMPO as a catalyst in a biphasic reaction mixture of dichloromethane and aqueous sodium hypochlorite.[30,31] This mild oxidation procedure provided the α-keto amides **1a-3a** in yields of 50–75% after recrystallization and without detectable epimerization of the chiral center at P_1. These compounds were purified by recrystallization since silica gel chromatography epimerized the chiral center at P_1 and yielded a mixture of two diastereomers. In all cases, final products were assessed for diastereomer content by both

Scheme 2[a]



[a] Reagents: (a) R'-Leu or Boc-Phe or Boc-D-Phe, EDC, HOBt, DIEA; (b) TEMPO, NaOCl(aq), KBr.

HPLC and ^{1}H-NMR. In the absence of silica gel chromatography, both techniques showed that the amount of the L,D diastereomer was below the level for accurate quantitation (less than 10% by NMR and less than 1% by HPLC). Compound **3b**, the L,D diastereomer of **3a**, was synthesized from the Boc-D-Phe by the same methodology shown in Scheme 1.

Several α-keto amides were synthesized in which the N-terminal Cbz group was replaced with other protecting groups or suitably protected amino acids. The synthesis of these inhibitors is shown in Scheme 2. Compounds **25** and **26** were coupled to Boc-Leu to yield **31** and **34**, respectively. Compound **31** was purified by silica gel chromatography and then oxidized to **37** using TEMPO/NaOCl(aq). Removal of the Boc-protecting group from **34** with 4 N HCl/dioxane followed by coupling of the amine to either Boc-Phe or Boc-L-Phe provided **35** and **36**, respectively, which were oxidized with TEMPO/NaOCl (aq) to **40** and **41**, respectively. Removal of the Boc groups from **40** and **41** yielded the free amines **42** and **43**. Alternatively, compound **25** was coupled to either [(*N*,*N*-dimethylamino)carbonyl]-Leu or morpholinocarbonyl-Leu to provide **32** and **33**, respectively, which were then oxidized as before to yield **38** and **39**. These derivatives of leucine were prepared according to standard literature procedures.[32]

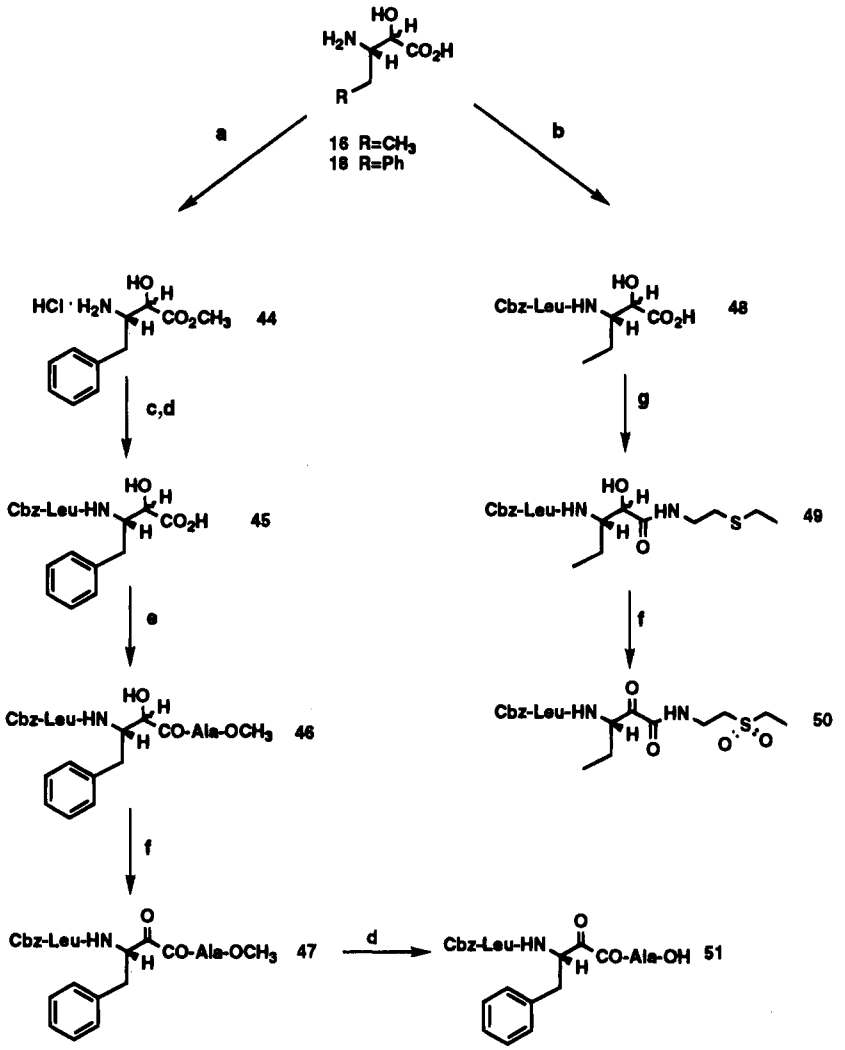
Two alternate synthetic routes for the preparation of compounds with different C-terminal groups are shown in Scheme 3. In route a, the α-hydroxy-β-amino acid **18** was converted to the methyl ester **44** via a Fischer esterification. Coupling of **44** to Cbz-Leu-OSu provided the Cbz-Leu-β-amino-α-hydroxy methyl ester, which was saponified without purification to the acid **45**. Compound **45** was then coupled to alanine methyl ester to yield **46**, which was then oxidized as previously described to yield **47**. This route was generally applicable to other amino acids (data not shown). The methyl ester of **47** was then saponified with 1 N NaOH in methanol to yield **51**. As predicted by the pH stability studies (*vide infra*), the basic conditions of the saponification resulted in partial epimerization of the chiral center at P_1 as evidenced by the appearance of multiple amide resonances in the ^{1}H-NMR spectrum of **51**. Alternatively, as shown in route b, the α-hydroxy-β-amino acid **16** was coupled directly to Cbz-Leu-OSu to yield **48**, which was converted to **49** by coupling to (ethylthio)-ethylamine. In this case, the TEMPO-catalyzed oxidation simultaneously converted the hydroxyl to the ketone and the sulfide to the sulfone to provide **50**. Route b involved fewer synthetic steps and would be expected to proceed in a higher overall yield; however, compound **48** was contaminated with Cbz-Leu resulting from hydrolysis of the *N*-hydroxysuccinimide ester during the coupling reaction. Chromatographic purification of either **48** or **49** prior to the final oxidation was required, whereas a majority of the peptidyl α-hydroxy amides synthesized by route a could be isolated without the need for chromatographic purification. For this reason, we found route a to be preferable to route b.

Synthesis of α-Keto Amides via the Dakin–West Reaction. The inhibitors shown in Table 1 (mixtures of epimers at P_1) were synthesized by a modification of the published procedure.[22] The α-keto ester precursors were synthesized using the Dakin–West reaction as previously described; however, the esters were isolated by extractive workup and trituration with hexane in place of column chromatography. The α-keto esters were then directly converted to the α-keto ethyl amides by treatment with ethylamine (aqueous) in EtOH. Crystallization provided the desired compounds **1a,b-3a,b** in yields of 60–70% and overall purity ≥98%. Both ^{1}H-NMR and HPLC showed the products to be mixtures of diastereomers in varying ratios: **1a:1b** (L,L:L,D) = 40:60; **2a:2b** (L,L:L,D) = 40:60; and **3a:3b** (L,L:L,D) = 60:40. The identity of the L,L diastereomers was established by independent synthesis as previously described.

Enzyme Inhibition. Compounds **1a,b-3a,b**, as mixtures of diastereomers, were assayed with calpain I from human and porcine erythrocytes and with calpain II from human placenta, bovine heart, and porcine kidney (Table 1). The compounds that were synthesized as single diastereomers were assayed with porcine calpain I and these K_i values are shown in Table 2. The K_i values for **1a,b-3a,b** are also shown for comparison. As noted in Table 2, several compounds were slow-binding inhibitors.

Physicochemical properties. As several of the calpain inhibitors synthesized were neutral and hydrophobic, the suitability of these compounds to be tested *in vitro* or *in vivo* required an evaluation of their aqueous solubility limits. Inhibitors were dissolved in a minimal amount of dimethylacetamide (DMA) at ~30

Scheme 3[a]



[a] Reagents: (a) $SOCl_2$/MeOH; (b) Cbz-Leu-OSu; (c) Cbz-Leu, EDC, HOBt, DIEA; (d) 1 N NaOH, MeOH; (e) Ala-OCH_3·HCl, EDC, HOBt, DIEA; (f) TEMPO, NaOCl(aq), KBr; (g) $H_2N(CH_2)_2SCH_2CH_3$, EDC, HOBt.

Table 1. Inhibition of Calpains by α-Keto Amides Synthesized as Diastereomeric Mixtures

compound	K_i (nM)				
	hCal I[a]	pCal I[b]	hCal II[c]	pCal II[d]	bCal II[e]
Cbz-L-Leu-(D,L)-Abu-CO-NHEt (1a,b)	210 ± 20	109 ± 15	138 ± 17	122 ± 14	138 ± 15
Cbz-L-Leu-(D,L)-Nva-CO-NHEt (2a,b)	66 ± 8	78 ± 9	73 ± 5	42 ± 5	50 ± 5
Cbz-L-Leu-(D,L)-Phe-CO-NHEt (3a,b)[f]	74 ± 6	89 ± 6	35 ± 2	51 ± 3	40 ± 3

[a] Human calpain I from erythrocytes. [b] Porcine calpain I from erythrocytes. [c] Human calpain II from placenta. [d] Porcine calpain II from kidney. [e] Bovine calpain II from heart. [f] Slow-binding kinetics observed with five calpains tested.

mg/mL and diluted with saline, and the pH was adjusted to 2.75 such that the final concentration of DMA was 3%. After incubation, the test mixture was centrifuged and a sample of the supernatant was analyzed by HPLC. Resultant inhibitor concentration was calculated from the area percent integrated from the HPLC chromatogram after calibrating the instrument response factor with standard concentrations of the corresponding inhibitor. These results are presented in Table 2. As seen from this table, many of the inhibitors proved sparingly soluble in this test vehicle; accordingly, these inhibitors were only tested in the enzyme inhibition assays that used DMSO as a cosolvent. Compounds **40** and **41** were found to be poorly soluble during the enzyme assays; therefore, we did not feel that the solubility of these compounds should be determined. Likewise, **42** and **43**, as hydrochloride salts, were very soluble and a determination of the upper limit of solubility was not warranted. Quantities of **1a** were available, and this compound was sufficiently soluble for further *in vivo* testing;[6] therefore, additional physiochemical studies were initiated using **1a** as a model for the inhibitor series.

We developed an HPLC-based assay to assess epimerization rates and evaluate chiral purity of the inhibitors. Using this HPLC technique, we could detect approximately a 1% contamination of the other diastereomer (i.e., the L,D diastereomer). We chose a representative single diastereomer inhibitor, **1a**, for these studies. The data, as presented in Table 3, show that epimerization is general base-catalyzed and that mildly acidic pH improves chiral stability.

The reactivity of **1a** with alcohols was studied by ^{1}H-NMR using CD_3OD. The formation of a methanol

Table 2. Inhibition Constants with Porcine Calpain I and Solubility Properties for Single Isomer Inhibitors

compound	K_i (nM)	solubility (mg/mL)
Cbz-L-Leu-L-Abu-CO-NHEt (**1a**)	77 ± 10	0.08
Cbz-L-Leu-(D,L)-Abu-CO-NHEt (**1a,b**)	109 ± 15	ND[a]
Cbz-L-Leu-L-Nva-CO-NHEt (**2a**)	32 ± 3	<0.02
Cbz-L-Leu-(D,L)-Nva-CO-NHEt (**2a,b**)	78 ± 9	ND
Cbz-L-Leu-L-Phe-CO-NHEt (**3a**)[b]	36 ± 2	<0.02
Cbz-L-Leu-(D,L)-Phe-CO-NHEt (**3a,b**)[b]	89 ± 6	ND
Cbz-L-Leu-D-Phe-CO-NHEt (**3b**)	>1500[c]	ND
Boc-L-Leu-L-Abu-CO-NHEt (**37**)	170 ± 15	0.72
$(CH_3)_2$N-CO-L-Leu-L-Abu-CO-NHEt (**38**)	333 ± 29	0.77
Mpl[d]-CO-L-Leu-L-Abu-CO-NHEt (**39**)	119 ± 16	0.63
Boc-L-Phe-L-Leu-L-Nva-CO-NHEt (**40**)	61 ± 2	ND
Boc-D-Phe-L-Leu-Nva-CO-NHEt (**41**)	93 ± 4	ND
L-Phe-L-Leu-L-Nva-CO-NHEt (**42**)	116 ± 5	ND
D-Phe-L-Leu-L-Nva-CO-NHEt (**43**)	244 ± 11	ND
Cbz-L-Leu-L-Phe-CO-L-Ala-OMe (**47**)[b]	92 ± 6	ND
Cbz-L-Leu-L-Abu-CO-NHEt-SO_2-Et (**50**)	129 ± 15	0.73
Cbz-L-Leu-L-Phe-CO-L-Ala-OH (**51**)[b]	718 ± 84	ND
Cbz-L-Leu-L-Pheψ[CH(OH)CO-NH]Et (**30**)	>350000	ND

[a] Not determined. [b] Slow-binding inhibitor. [c] Activity may be due to epimerization occurring during the course of the assay. [d] Mpl = morpholino.

Table 3. Epimerization Rates of **1a** As Measured by HPLC

pH	buffer concentration (mM)[a]	final ratio L,L:L,D (15 days)	k_f (min^{-1})
10	50	50:50	1.4×10^{-3}
10	10	51:49	8.6×10^{-4}
10	3	48:52	1.9×10^{-4}
7	50	51:49	2.9×10^{-3}
7	10	50:50	3.9×10^{-4}
7	3	49:51	6.9×10^{-5}
3	50	82:18	ND[b]
3	10	>95:5	ND[b]
3	3	>95:5	ND[b]

[a] Initial concentration of phosphate buffer prior to mixing with DMSO. [b] The epimerization rate was too slow to measure in the time frame of this study.

hemiketal was assessed by monitoring the change in shift of the proton α to the ketone (β to the amide). After 30 min at room temperature in CD_3OD, the formation of hemiketals was apparent. The reaction reached equilibrium (12% ketone remaining) in less than 47 h. It was also noted from ^{1}H-NMR studies in D_2O-DMSO-d_6 (60:40) that the keto amides rapidly form hydrates; an equilibrium value of 44% ketone was achieved within 1 h. While this phenomenon was not extensively studied, it was noted that an equilibrium between the ketone and the hydrate was rapidly reached (no change from the initial value was observed).

Discussion

Peptidyl α-keto amides have been prepared as single diastereomers using TEMPO/NaOCl (aq) as the oxidant for converting the α-hydroxy amides to the α-keto amides without epimerization of the chiral center adjacent to the ketone. A modified version of the published Dakin–West procedure[22,33] was used initially for inhibitor synthesis. However, this procedure provided a mixture of diastereomers that could not be resolved by silica gel chromatography due to the interconversion of diastereomers catalyzed by silica gel. With several of these compounds, repeated recrystallization yielded a product enriched in one diastereomer, the L,L diastereomer (data not shown). We believe this enrichment was due to the poorer solubility of the L,L diastereomer. The observation that the initial products **1a,b** and **2a,b** were enriched in the L,D diastereomer indicates that this diastereomer may be thermodynamically favored at some step of the synthesis. These issues were not pursued since recrystallization did not appear to be a viable route to quantities of inhibitor for *in vitro* and *in vivo* studies. An unambiguous stereospecific synthesis was the preferred route.

The α-hydroxy-β-amino acids **16–18** (Scheme 1) were the key intermediates in the stereospecific synthesis of the α-keto amide inhibitors. The stereochemistry of the starting amino acid determined the stereochemistry of the P_1 residue in the final product. This synthetic approach to the α-hydroxy-β-amino acids is well-established in the literature.[28,29] Several changes, however, have been made to the standard methodology for the LAH reduction of the Weinreb amide to the aldehyde,[27] i.e., the conversion of **7** to **10**. In agreement with others,[26] we found that an inverse addition of the Weinreb amide to excess LAH followed by quenching of the excess LAH with ethyl acetate and $KHSO_4$(aq) yielded the desired aldehyde with minimal epimerization (<1%). There have been several reports that these Boc-amino aldehydes are prone to racemization;[34a,b] however, the aldehydes could be stored dry for months at −20 °C without appreciable racemization (i.e., less than 1%).[35] Rigorous control of the stereochemistry of the chiral aldehyde is important since the chirality of this center in the final product is critical for inhibitory activity.

TEMPO/NaOCl(aq) has been reported to be a mild, economical oxidant[30,31] that maintains chiral integrity in oxidations of primary alcohols to aldehydes; therefore, we were pleased to find that the oxidation of the α-hydroxy amides using this reagent proceeded in good yields and maintained the optical purity at the C-α of the P_1 amino acid residue. Several other methods have been previously reported for the oxidation of peptidyl hydroxyls to ketones, notably the Swern oxidation[36] and Dess–Martin periodinane.[37] The Swern oxidation was not suitable due to its potential to cause epimerization and reported poor yields of similar compounds.[28,38] Although high yields and chiral integrity were generally reported with Dess–Martin periodinane,[38,39] its lack of commercial availability and potential handling hazards made this reagent less desirable.

As shown by the K_i values in Table 1, the three peptidyl α-keto amides tested did not show a pronounced selectivity for either calpain I or calpain II. Additionally, there did not appear to be any notable difference in K_i values for calpains isolated from various mammalian species. This same lack of selectivity was observed for several other α-keto amides that were synthesized and tested (data not shown). For this reason, we chose to assay all single isomer inhibitors using the commercially available porcine calpain I from erythrocytes. These results are shown in Table 2. Many of these compounds were potent inhibitors of porcine calpain I. Since others had reported that leucine at P_2 is an important recognition element in calpain binding,[22,40] all of the compounds prepared incorporated this residue. The other moiety that is critical for inhibitory activity is the ketone, since the α-hydroxy amide precursors (see compound **30**, Table 2) were inactive as calpain inhibitors. This requirement of the ketone for inhibitory activity supports the pro-

posed mechanism[22] that these inhibitors form an enzyme-bound, tetrahedral species that results from the nucleophilic addition of the active site cysteine sulfhydryl to the electrophilic ketone. This proposed mechanism mimics the catalytic mechanism of cysteine proteases in which the cysteine sulfhydryl attacks the amide carbonyl of the scissile bond.

Direct comparison of our data with the previously published work of Li et al.[22] was complicated by the fact that the relative proportions of L,L versus L,D diastereomers in the inhibitors prepared by Dakin–West chemistry could vary. As we clearly demonstrated by synthesizing both the L,L and L,D diastereomers **3a** and **3b**, respectively, the active inhibitor was the L,L diastereomer. For this reason, experimentally determined K_i values will be dependent upon the amount of the L,L diastereomer when one is working with a mixture of diastereomers.

In some cases, the K_i values that are reported in Tables 1 and 2 may not be true dissociation constants due to the conditions of the enzyme assays. Since the inhibitors were synthesized as the active L,L diastereomer, the actual concentration of inhibitor was known, and in several cases this was similar to the concentration of enzyme (40–60 nM) in the assays. Inhibitors assayed under these "tight-binding" conditions do not follow Michaelis–Menten kinetics since the concentration of free inhibitor is not constant.[41a,b] A K_i value obtained under these conditions is dependent upon the concentration of enzyme in the assay, and this K_i is not a true dissociation constant. In the cases of tight-binding inhibitors such as **1a**, **2a, 3a,** and **40**, we are currently modifying the assay to allow lower enzyme concentrations, which will then eliminate the tight-binding conditions.

As noted in Table 2, compounds **3a**, **47**, and **51** were slow-binding inhibitors.[42] Slow-binding inhibitors do not show a steady-state initial velocity; instead, these inhibitors exhibit a slow onset of inhibition with time until a steady-state velocity is attained. These inhibitors all have a phenylalanine side chain at P_1 indicating that this bulky substituent is required for slow binding to calpains. The slow-binding phenomenon could be due to a conformational change of the enzyme that is required for optimum binding of the bulky benzyl substituent; however, the synthesis of analogs of **3a** bearing other bulky groups and more detailed kinetic analyses will be required to address this issue. The kinetic behavior of these slow-binding inhibitors may cause the reported K_i values to be larger than the true dissociation constants. If the rate of enzyme-catalyzed substrate hydrolysis in the presence of the inhibitor did not attain a steady-state value during the time course of the assay, then the K_i value will be artificially increased. The assay time could not be lengthened to check this since autolysis of the enzyme becomes significant after 3–4 min. This autolysis results in a loss in enzyme activity, which further complicates the slow-binding phenomenon. For these reasons, we have recently started to evaluate slow, tight-binding inhibitors such as **3a** and **47** by analysis of the progress curves for the onset of inhibition using an equation derived for slow, tight-binding inhibition. Details of these studies will be reported elsewhere.

In the series of Z-Leu-Xxx-keto ethyl amides, our data confirm that compounds with L-Phe, L-Abu, and L-Nva at P_1 were all potent inhibitors of calpain I. In all cases, any structural modifications of **1a-3a** decreased the inhibitory activity; however, some of these changes resulted in increased solubility. Compounds **38**, **39**, and **50** were all slightly less active than **1a** (4×, 1.5×, and 1.7×, respectively) whereas the increased solubility (10×, 8×, and 9×, respectively) was much greater than the loss in inhibitory activity. Whether this increased solubility can compensate for the loss in potency will be determined in future *in vivo* studies.

It is of particular interest that modifications at the P_1' and P_3 positions had only modest effects upon K_i. Replacement of the Cbz of **2a** with either Boc-Phe (**40**) or Boc-D-Phe (**41**) decreased affinity 2-fold and 3-fold, respectively. The observation that a D residue at P_3 had such a small effect was very surprising since proteases are usually very strict in their requirements for L-amino acids in a peptide substrate. Another surprising feature was the activity of **42**. Calpains cleave large peptides and proteins, and yet this inhibitor with a free N-terminus was only 3.6× less active than **2a**. Compound **47** showed that replacing the C-terminal ethyl amide with an alanine methyl ester decreased affinity even though adding this amino acid residue, which should bind on the enzyme at P_1', was expected to enhance affinity. Taken together, these results indicate that a dipeptide is the optimum size for these α-keto amides. The inhibitory potency shown by these dipeptide α-keto amides makes identifying a non-peptide, small molecule inhibitor a reasonable goal for future studies.

Saponification of the methyl ester of **47** yielded **51**, which was at least 10-fold weaker as an inhibitor than **47**. Some of this loss in potency is due to base-catalyzed epimerization at the P_1 position that occurred during the saponification. However, ^{1}H-NMR showed that approximately 15% of the sample was the inactive diastereomer, which is not sufficient to explain the 10-fold loss in activity. Li et al.[22] had reported that the α-keto acid, Z-Leu-Phe-CO_2H, was a very potent inhibitor of calpain and suggested that the carboxylic acid may interact with a histidyl residue in the active site. If this postulate is true, then increasing the distance between the ketone and the acid, as in the case of compound **51,** may perturb the ability of the ketone to interact with the free sulfhydryl of the active-site cysteine, possibly due to the interaction of the carboxylate with an active-site histidine residue.

The α-keto amide moiety is essential for inhibitory activity; however, our studies showed that this structural feature is very susceptible to epimerization. As shown in Table 3, epimerization was more rapid at high buffer concentrations ($t_{1/2}$ = 2 h at pH = 7 in 50 mM phosphate buffer) as seen in the rate difference between 50 and 3 mM phosphate buffer. No significant difference was seen between pH 7 and 10 in the phosphate buffered solutions tested; however, epimerization was much slower at pH 3. Similar to previous reports of hemi-ketal formation of α-keto amides in methanol,[28] ^{1}H-NMR analysis indicated a comparable reaction of the α-keto amide with this solvent. These findings stress the importance of proper solvent or buffer selection for *in vitro* and *in vivo* studies with these compounds.

Summary

Stereospecific syntheses of peptidyl α-keto amides have been achieved using TEMPO/hypochlorite as the final oxidation reagent. Several potent inhibitors of calpain were synthesized, and three important structural features were identified: a leucyl residue at P_2, an electrophilic ketone at the scissile bond, and a hydrophobic residue at P_1. Various extensions at P_3 or P_1' did not drastically affect the potency of these compounds as inhibitors of calpain. However, these extensions did increase the aqueous solubility of these compounds. In assessing the chiral stability of the C-α of the residue at P_1, we found that under general base conditions, epimerization proceeds rapidly; however, chirality can be maintained in unbuffered or slightly acidic conditions. Our studies also clearly showed that the L,L diastereomer is the active inhibitor. As a class of compounds, peptidyl α-keto amides appear to be effective inhibitors of calpain. These potent inhibitors of calpain should provide useful probes for the assessment of calpain's role in the neurodegenerative cascade that accompanies head trauma and ischemic events such as stroke.

Experimental Section

General Methods. All chemicals were reagent grade or better. TLC analysis was performed using EM Science precoated silica gel 60 F_{254} plates (0.25-mm thickness), and compounds were visualized by one or more of the following: iodine, UV, phosphomolybdic acid (PMA), or ninhydrin. Flash chromatography was performed on EM Science's silica gel 60 (200–400 mesh). ^{1}H-NMR spectra were obtained on Bruker AM 500 or AM 600 instruments at the National Center for NMR Application, Colorado State University, Ft. Collins, CO., operating at 500 or 600 MHz, respectively; either a Bruker 300- or 500-MHz NMR at Harvard University, Cambridge, MA; or a 360-MHz instrument operated by Spectral Data Services, Champaign, IL. Chemical shifts are reported relative to TMS, and coupling constants (J) are in Hz. Mass spectral analysis (FAB) was performed at M-Scan Inc., West Chester, PA on a VG-ZAB-2SE instrument. Elemental analyses were performed at Oneida Research Services, Inc, Whitesboro, NY. Melting points were determined using a Mel-Temp II from Laboratory Devices, USA, and are uncorrected.

General Synthetic Procedure 1: Synthesis of α-Keto Amides via Dakin–West Chemistry. The dipeptidyl α-keto esters were prepared as described previously.[22] The α-keto ester (1 equiv) was dissolved in EtOH (10 mL/mmol), and to this solution was added ethylamine (70% aqueous solution, 10 equiv). After stirring for 13 h at room temperature, the reaction mixture was diluted into EtOAc and washed with saturated $NaHCO_3$, 1 N HCl, and saturated NaCl. The organic phase was dried with anhydrous $MgSO_4$, suction filtered, and concentrated *in vacuo* to yield a yellow residue that was crystallized from ethyl ether.

Cbz-Leu-(D,L)-Abu-CONH-CH_2CH_3 (1a,b). Yield: 60%. TLC: R_f = 0.37 (CH_2Cl_2:MeOH 20:1). MS: $(M + H)^+$ = 406. 500-MHz ^{1}H-NMR (DMSO-d_6) δ 0.87 (m, 9H), 1.05 (dt, 3H), 1.35–1.67 (m, 4H), 1.78 (m, 1H), 3.14 (m, 2H), 4.12 (m, 1H), 4.89 (m, 1H), 5.03 (s, 2H), 7.35 (m, 6H), 8.22 (d, 0.4H), 8.26 (d, 0.6H), 8.68 (q, 1H). Anal. ($C_{21}H_{31}N_3O_5$) C, H, N.

Cbz-Leu-(D,L)-Nva-CONH-CH_2CH_3 (2a,b). Yield: 67%. TLC: R_f = 0.34 (CH_2Cl_2:MeOH 20:1). MS: $(M + H)^+$ = 420. 500-MHz ^{1}H-NMR (DMSO-d_6) δ 0.85 (m, 9H), 1.04 (dt, 3H), 1.20–1.52 (m, 5H), 1.55–1.75 (m, 2H), 3.14 (m, 2H), 4.05–4.17 (m, 1H), 4.97 (m, 1H), 5.02 (s, 2H), 7.34 (m, 6H), 8.22 (d, 0.4H), 8.27 (d, 0.6H), 8.68 (q, 1H). Anal. ($C_{22}H_{33}N_3O_5$) C, H, N.

Cbz-Leu-(D,L)-Phe-CONH-CH_2CH_3 (3a,b). Yield: 71%. TLC: R_f = 0.58 (CH_2Cl_2:MeOH 20:1). MS: $(M + H)^+$ = 468. 500-MHz ^{1}H-NMR (DMSO-d_6) δ 0.73–0.87 (m, 6H), 1.05 (dt, 3H), 1.12–1.28 (m, 2H), 1.37–1.57 (m, 2H), 2.68–2.86 (m, 1H), 3.07–3.2 (m, 2H), 4.06 (m, 1H), 5.0 (m, 2H), 5.2 (m, 1H), 7.18–7.38 (m, 11H), 8.30 (d, 0.6 H), 8.37 (d, 0.4H), 8.70 (t, 0.6H), 8.76 (t, 0.4H). Anal. ($C_{26}H_{33}N_3O_5$) C, H, N.

General Synthetic Procedure 2: Synthesis of Boc-Aminoacyl Weinreb Amides 7–9 [*N*-*tert*-Boc-Nval-N(CH_3)-OCH_3 (8)]. Boc-L-Nval (5) (25.0 g, 115.0 mmol) was coupled to *N,O*-dimethyhydroxylamine-hydrochloride (12.2g, 126.5 mmol) by the following procedure. Boc-L-Nva was dissolved in anhydrous THF (200 mL) and placed under argon. Carbonyldiimidazole (22.4 g, 138 mmol) was then added portionwise over a 10-min period and the solution was stirred for 30 min under argon at room temperature. *N,O*-Dimethylhydroxylamine-hydrochloride was dissolved in DMF (75 mL) with DIEA (21 mL, 15.6 g, 120 mmol) and added to the Boc amino acid solution. The reaction was allowed to stir overnight at room temperature. The reaction solution was concentrated *in vacuo*, diluted into ethyl acetate (300 mL), and washed with 1 N HCl (3 × 100 mL), saturated $NaHCO_3$ (3 × 100 mL), and saturated NaCl (1 × 100 mL). The organic phase was dried with anhydrous $MgSO_4$ and suction filtered and the filtrate concentrated *in vacuo* to yield **8** as a clear viscous oil that was dried overnight *in vacuo*: 23.2 g, 82%. TLC: R_f = 0.78 (1:1 hexane:EtOAc). MS: $(M + H)^+$ = 247.

General Synthetic Procedure 3: Synthesis of *N*-*tert*-Boc Amino Aldehydes 10–12 [*N*-*tert*-Boc-norvalinal (11)]. Compound **8** (20.8 g, 84.5 mmol) was dissolved in 125 mL of anhydrous ethyl ether. Lithium aluminum hydride (3.5 g, 92 mmol) was added to 125 mL of anhydrous ethyl ether and chilled (ice bath) in a round-bottom flask equipped with a pressure-equalizing dropping funnel and thermometer. The amide solution was added to the dropping funnel, placed under argon, and added to the LAH suspension at a rate in which the temperature was not allowed to exceed 5 °C. After all the amide was added, the reaction mixture was allowed to stir for 30 min at which point EtOAc (50 mL) was added dropwise to destroy any excess LAH. $KHSO_4$ (5%) (50 mL) was then added dropwise to quench the complex, while the temperature was maintained at less than 5 °C. The mixture was washed with 1 N HCl (3 × 100 mL), saturated $NaHCO_3$ (3 × 100 mL), and saturated NaCl (1 × 100 mL). The organic phase was dried with anhydrous $MgSO_4$ and suction filtered and the filtrate concentrated *in vacuo* to yield 11 as a clear viscous oil: 15.3 g, 90%. TLC: R_f = 0.42 (4:1 EtOAc:hexane). MS: $(M + H)^+$ = 202.

General Synthetic Procedure 4: Synthesis of α-Hydroxy β-Amino Acids 16–18 [2(*R,S*)-Hydroxy-3(*S*)-amino-4-phenylbutanoic Acid (18)]. *N*-*tert*-Boc-L-phenylalaninal **12** (16.7 g, 67 mmol) was dissolved in MeOH (100 mL) and chilled to 5 °C. Sodium bisulfite (7.0 g, 67 mmol) was dissolved in deionized water (150 mL) and chilled to 5 °C before addition to the aldehyde solution. This mixture was stirred overnight at 5 °C. NaCN (4.0 g, 81 mmol) was dissolved in deionized water (100 mL) and added with ethyl acetate (300 mL) to the above mixture. The reaction was allowed to stir for 5 h at room temperature. The organic layer was collected, dried with anhydrous $MgSO_4$, and suction filtered, and the filtrate was concentrated *in vacuo* to yield the crude cyanohydrin as a clear, colorless, viscous syrup. This crude cyanohydrin was hydrolyzed without further purification. The crude cyanohydrin was dissolved in 1,4-dioxane (250 mL), concentrated HCl (250 mL), and 10 mL of anisole; the solution was gently refluxed, with stirring, overnight. The hydrolysis reaction was allowed to cool to room temperature and then concentrated *in vacuo* to a brown paste. The residue was dissolved in deionized water (100 mL) and washed with ethyl ether (3 × 50 mL). The aqueous phase was then placed on a Dowex 50X8–100 column (H^+ form; 25 × 1.8 cm). The column was washed with deionized water until the pH was 5.5 and then eluted with 2 M ammonium hydroxide (ca. 1.5 L). The eluant was dried *in vacuo* to yield **18** as an off-white, amorphous solid: 6.8 g, 52%. TLC: R_f = 0.34 (4:1:1 butanol:HOAc:water). MS: $(M + H)^+$ = 196.

General Synthetic Procedure 5: Synthesis of *N*-*tert*-Boc-β-amino-α-hydroxy Ethyl Amides 22–24 [2(*R,S*)-Hydroxy-3(*S*)-(*N*-*tert*-Boc-amino)-4-phenylbutanoic Acid

Table 4. Analytical Data for Synthetic Intermediates in the Synthesis of 1a-3a.

compound	yield (%)	TLC R_f	TLC solvent1	MS *m/e* $(M + H)^+$	^{1}H-NMR
7	77	0.77	2:1 EtOAc Hex	247	yes
9	99	0.43	1:1 EtOAc–Hex	309	yes
16	65	0.15	4:1:1 BAW	134	ND[a]
17	61	0.23	4:1:1 BAW	147	ND[a]
22	66	0.39, 0.43[b]	9:1 DCM–MeOH	261	yes
23	66	0.33, 0.40[b]	9:1 DCM–MeOH	275	yes
28	70	0.20, 0.26[b]	20:1 DCM–MeOH	408	yes
29	67	0.30	20:1 DCM–MeOH	422	yes

[a] Not determined. [b] Resolution of diastereomers observed.

Ethyl Amide (24)]. Compound **18** (1.4 g, 7.2 mmol) was dissolved in 1 N NaOH (9 mL). To this solution was added a solution of di-*tert*-butyl dicarbonate (1.6 g, 7.3 mmol) in dioxane (9 mL). The reaction was stirred at room temperature while the pH was maintained between 10 and 11 with 1 N NaOH. After 4 h, the reaction was diluted into deionized water (80 mL) and washed with ethyl ether (2 × 25 mL). The aqueous phase was chilled in an ice bath and acidified to pH 2 with 1 N HCl. This mixture was extracted with ethyl ether (3 × 30 mL). The organic phase was dried with anhydrous $MgSO_4$ and suction filtered and the filtrate concentrated *in vacuo* to yield **21** as a clear colorless oil: 1.7 g, 79%. MS: $(M + H)^+ = 296$. Compound **21** was converted to the ethyl amide without further purification.

Compound **21** (1.7 g, 5.7 mmol) was dissolved in 0.5 M HOBt in DMF (12 mL) and cooled in an ice bath. EDC (1.2 g, 6.25 mmol) was added followed by 70% aqueous ethylamine solution (800 μL, 9.9 mmol). The solution was stirred overnight. The solution was diluted into EtOAc (50 mL) and washed with 1 N HCl (3 × 50 mL), saturated $NaHCO_3$ (3 × 50 mL), and saturated NaCl (1 × 50 mL). The organic phase was dried with anhydrous $MgSO_4$ and suction filtered and the filtrate concentrated *in vacuo* to yield **24** as an off white solid: 1.6 g, 86%. TLC: $R_f = 0.38$ (9:1 EtOAc:hexane). MS: $(M + H)^+ = 323$. 500 MHz ^{1}H-NMR (DMSO-d_6) δ 0.99 (m, 3H), 1.28 (d, 9H), 2.63 (dd, 1H), 2.79 (dd, 1H), 3.10 (m, 2H), 3.77 (m, 0.5H), 3.92–4.05 (m, 1.5H), 5.67 (d, 0.5H), 5.76 (d, 0.5H), 6.12 (d, 0.5H), 6.57 (d, 0.5H), 7.12–7.29 (m, 5H), 7.82 (dt, 1H).

General Synthetic Procedure 6: Synthesis of Cbz-Leu-α-hydroxy-β-amino Acid Ethyl Amides 28–30 [*N*-Cbz-leucyl-3(*S*)-amino-2(*R,S*)-hydroxy-4-phenylbutyric Acid Ethyl Amide (30)]. The Boc group was cleaved by dissolving compound **24** (3.35 g, 10.4 mmol) in 4 N HCl/dioxane (40 mL) and stirring for 30 min at room temperature. The white semisolid reaction mixture was dried *in vacuo* to yield the hydrochloride salt of the amine. This amine was coupled to Cbz-Leu without further purification.

Cbz-Leu (3.0 g, 11.3 mmol) was dissolved in 1M HOBt in NMP (11.3 mL, 11.3 mmol HOBt) and added to a flask containing EDC (2.2 g, 11.5 mmol) and the amine hydrochloride **27** (3.2 g, 10.4 mmol). To this mixture was added DMF (10 mL) and diisopropylethylamine (2.0 mL, 11.5 mmol), and the reaction mixture was allowed to stir overnight at room temperature. The reaction solution was diluted into EtOAc (150 mL), and the resulting milky mixture was washed with 1 N HCl (3 × 50 mL), saturated $NaHCO_3$ (3 × 50 mL, and saturated NaCl (1 × 50 mL). The organic phase was dried with anhydrous $MgSO_4$ and suction filtered, and the filtrate was concentrated to a viscous syrup on a rotary evaporator. This syrup was dried *in vacuo* to a brittle foam which was redissolved in the minimum amount of ethyl ether and allowed to crystallize in an ice bath. The solid was isolated by suction filtration, washed with cold ethyl ether, and dried *in vacuo* to yield **30:** 4.05 g, 83%. TLC: $R_f = 0.18$ (25:1 CH_2Cl_2:MeOH). MS: $(M + H)^+ = 470$. 500-MHz ^{1}H-NMR (DMSO-d_6) δ 0.80 (m, 6H), 0.98 (t, 3H), 1.23–1.38 (m, 2H), 1.52 (m, 1H), 2.63 (dd, 1H), 2.82 (dd, 1H), 3.03 (m, 2H), 3.77 (m, 1H), 3.98 (td, 1H), 4.20 (q, 1H), 5.05 (s, 2H), 6.10 (d, 1H), 7.13–7.37 (m, 10H), 7.42 (d, 1H), 7.48 (d, 1H), 7.63 (t, 3H).

The other intermediates prepared in the course of α-keto amide synthesis were isolated and characterized. The characterization of these intermediates is summarized in Table 4.

General Synthetic Procedure 7: Oxidation to the α-Keto Amides 1a-3a, 3b [Cbz-Leu-Phe-CONH-CH_2CH_3 (3a)]. In a 25-mL round-bottom flask was placed TEMPO (5 mg), potassium bromide (25 mg), water (0.11 mL), and CH_2Cl_2 (5 mL). As this mixture was stirred, compound **30** (1.0 g, 2.13 mmol) was added, and the reaction mixture was stirred in an ice bath until the hydroxy amide was dissolved. To a 5.25% sodium hypochlorite solution (commercial bleach, 25 mL) was added $NaHCO_3$ (300 mg), and this was stirred until the solid was dissolved. Over a period of ca. 5 min, 3.4 mL of the bleach solution was added with vigorous stirring to the above reaction mixture in an ice bath. The reaction mixture became a solid mass requiring the addition of 5–10 mL of CH_2Cl_2 to obtain a slurry. After 20 min, another portion of TEMPO (5 mg) was added, and additional bleach solution was added as above. When the addition was complete, TLC showed no starting material remaining. The reaction was diluted into EtOAc (100 mL) and washed with 0.5 N HCl (3 × 30 mL), saturated $NaHCO_3$ (3 × 30 mL), and saturated NaCl (1 × 30 mL). The organic phase was dried with anhydrous $MgSO_4$, suction filtered, and concentrated *in vacuo* to a white solid. This solid was vigorously stirred with ethyl ether (ca. 10 mL) and then isolated by suction filtration to obtain **3a** as a white solid: 0.75 g, 75%. TLC: $R_f = 0.58$ (20:1 CH_2Cl_2:MeOH). Mp: 156–157 °C. MS: $(M + H)^+ = 468$. 600-MHz ^{1}H-NMR (DMSO-d_6) δ 0.85 (dd, 6H), 1.03 (t, 3H), 1.36 (m, 2H), 1.57 (m, 1H), 2.83 (dd, 1H), 3.12 (m, 3H), 4.07 (q, 1H), 5.02 (q, 2H), 5.20 (m, 1H), 7.20–7.40 (m, 11H), 8.28 (d, 1H), 8.68 (t, 1H). Anal. ($C_{26}H_{33}N_3O_5$) C, H, N.

Cbz-Leu-Abu-CONH-CH_2CH_3 (1a). Yield: 81%. TLC: $R_f = 0.38$ (20:1 CH_2Cl_2:MeOH). Mp: 159–160 °C. MS: $(M + H)^+ = 406$. 500-MHz ^{1}H-NMR (DMSO-d_6) δ 0.84–0.93 (m, 9H), 1.04 (t, 3H), 1.43 (t, 2H), 1.54 (m, 1H), 1.64 (m, 1H), 1.78 (m, 1H), 3.15 (m, 2H), 4.1 (q, 1H), 4.87 (m, 1H), 5.05 (s, 2H), 7.30–7.45 (m, 6H), 8.23 (d, 1H), 8.67 (t, 1H). Anal. ($C_{21}H_{31}N_3O_5$) C, H, N.

Cbz-Leu-Nva-CONH-CH_2CH_3 (2a). Yield: 67%. TLC: $R_f = 0.34$ (20:1 CH_2Cl_2:MeOH). Mp: 169–170 °C. MS: $(M + H)^+ = 420$. 500-MHz ^{1}H-NMR (DMSO-d_6) δ 0.87 (m, 9H), 1.04 (t, 3H), 1.25–1.52 (m, 5H), 1.60–1.75 (m, 2H), 3.15 (m, 2H), 4.10 (q, 1H), 4.95 (m, 1H), 5.02 (s, 2H), 7.25–7.40 (m, 6H), 8.23 (d, 1H), 8.69 (t, 1H). Anal. ($C_{22}H_{33}N_3O_5$) C, H, N.

Cbz-Leu-D-Phe-CONH-CH_2CH_3 (3b). Yield: 84%. TLC: $R_f = 0.34$ (20:1 CH_2Cl_2:MeOH). Mp: 166–167 °C. MS: $(M + H)^+ = 468$. 500-MHz ^{1}H-NMR (DMSO-d_6) δ 0.77 (dd, 6H), 1.06 (t, 3H), 1.15 (m, 1H), 1.23 (m, 1H), 1.39 (m, 1H), 2.72 (dd, 1H), 3.12–3.21 (m, 3H), 4.05 (m, 1H), 4.99 (q, 2H), 5.19 (m, 1H), 7.22-7.36 (m, 11H), 8.36 (d, 1H), 8.76 (t, 1H). Anal. ($C_{26}H_{33}N_3O_5$) C, H, N.

N-(Boc-Leu)-3(*S*)-amino-2(*R,S*)-hydroxypentanoic Acid Ethyl Amide (31). Compound **25** (0.176 g, 0.894 mmol) was dissolved in anhydrous DMF (25 mL) and cooled on an ice bath for 10 min. To this solution was added Boc-Leu·H_2O (0.267 g, 1.07 mmol), DIEA (0.46 mL, 2.68 mmol), and HOBt (0.133 g, 0.984 mmol), and the reaction mixture was allowed to equilibrate for 30 min. After this time EDC (0.188 g, 0.984 mmol) in anhydrous DMF (10 mL) was added and the reaction mixture was allowed to react overnight. The reaction mixture was concentrated *in vacuo* and the resulting residue redissolved in $CHCl_3$ (100 mL). The solution was washed twice with 50 mL of both saturated $NaHCO_3$ (2 × 50 mL) and brine (2 × 50 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The crude material was purified by silica gel column chromatography (9:1 $CHCl_3$:

MeOH as the eluent) to obtain **31** as a white solid: 0.184 g, 55.1%. TLC: R_f = 0.42 (9:1 $CHCl_3$:MeOH).

***N*-[[(*N*,*N*-Dimethylamino)carbonyl]-Leu]-3(*S*)-amino-2(*R*,*S*)-hydroxypentanoic Acid Ethyl Amide (32).** Compound **25** (0.176 g, 0.894 mmol) was coupled to *N*-[(*N*,*N*-dimethylamino)carbonyl]-L-Leu (0.217 g, 1.07 mmol) as described for compound **31**. The crude product was purified by silica gel column chromatography (9:1 $CHCl_3$:MeOH as the eluent) to yield **32** as a white solid: 0.204 g, 66.5%. TLC: R_f = 0.38 (9:1 $CHCl_3$:MeOH).

***N*-[*N*-(Morpholinocarbonyl)-Leu]-3(*S*)-amino-2(*R*,*S*)-hydroxypentanoic Acid Ethyl Amide (33).** Compound **25** (0.201 g, 1.02 mmol) was coupled to *N*-(morpholinocarbonyl)-Leu (0.30 g, 1.23 mmol) as described for compound **31**. The crude product was purified by silica gel column chromatography (90:10 $CHCl_3$:MeOH as the eluent) to yield **33** as a white solid: 0.241 g, 61.0%. TLC: R_f = 0.36 (9:1 $CHCl_3$:MeOH).

***N*-(Boc-Leu)-3(*S*)-amino-2(*R*,*S*)-hydroxyhexanoic Acid Ethyl Amide (34).** Boc-Leu·H_2O (1.30 g, 5.22 mmol) was coupled to compound **26** (1.00 g, 4.75 mmol) with EDC (1.0 g, 5.22 mmol) according to procedure 6 to yield **34**: 1.65 g, 4.26 mmol, 89.6%. MS: $(M + H)^+$ = 388. 600-MHz ^{1}H-NMR (DMSO-d_6) δ 0.78-0.88 (m, 9H), 1.00 (q, 3H), 1.20–1.48 (m, 15H), 1.56 (m, 1H), 3.04–3.15 (m, 2H), 3.80 (m, 1H), 3.85 (q, 0.5H), 3.95 (q, 0.5H), 4.01 (m, 0.5H), 4.09 (m, 0.5H), 5.67 (d, 0.5H), 5.85 (d, 0.5H), 6.94 (d, 0.5H), 7.34 (d, 0.5H), 7.62 (t, 0.5H), 7.78 (t, 0.5H).

***N*-(Boc-Phe-Leu)-3(*S*)-amino-2(*R*,*S*)-hydroxyhexanoic Acid Ethyl Amide (35).** The Boc protecting group was cleaved from **34** (1.65 g, 4.26 mmol) with 4 N HCl/dioxane (10 mL, 40 mmol HCl), and the resulting amine hydrochloride salt (0.30 g, 0.93 mmol) was coupled to Boc-Phe (0.27 g, 1.02 mmol) according to procedure 6 to yield **35**: 270 mg, 54%. TLC: R_f = 0.52 and 0.55, 2 diastereomers (9:1 CH_2Cl_2:MeOH). MS: $(M + H)^+$ = 535. 600-MHz ^{1}H-NMR (DMSO-d_6) δ 0.78-0.89 (m, 9H), 1.00 (q, 3H), 1.15–1.50 (m, 15H), 1.60 (m, 1H), 2.72 (m, 1H), 2.96 (m, 1H), 3.01–3.19 (m, 2H), 3.81 (m, 1H), 4.02 (m, 0.5H), 4.07–4.16 (m, 1.5H), 4.30 (q, 0.5H), 4.35 (q, 0.5H), 5.65 (d, 0.5H), 5.75 (d, 0.5H), 6.94 (m, 1H), 7.18-7.31 (m, 5H), 7.40 (d, 0.5H), 7.58 (d, 0.5H), 7.67 (t, 0.5H), 7.77 (t, 0.5H), 7.95 (m, 1H). Anal. ($C_{28}H_{46}N_4O_6$) C, H, N.

***N*-(Boc-D-Phe-Leu)-3(*S*)-amino-2(*R*,*S*)-hydroxyhexanoic Acid Ethyl Amide (36).** The same procedure as above was used to couple Boc-D-Phe to the amine derived from compound **34** to yield compound **36**: 320 mg, 64%. TLC: R_f = 0.52 (9:1 CH_2Cl_2:MeOH). MS: $(M + H)^+$ = 535. 600-MHz ^{1}H-NMR (DMSO-d_6) δ 0.78–0.86 (m, 9H), 1.00 (m, 3H), 1.15–1.50 (m, 16H), 2.74 (m, 1H), 2.90 (m, 1H), 3.04–3.14 (m, 2H), 3.82 (m, 1H), 4.00 (m, 0.5H), 4.05 (m, 0.5H), 4.16-4.30 (m, 2H), 5.60 (d, 0.5H), 5.70 (d, 0.5H), 6.85 (d, 1H), 6.95 (d, 0.5H), 7.18–7.32 (m, 5H), 7.34 (d, 0.5H), 7.51 (d, 0.5H), 7.64 (t, 0.5H), 7.72 (t, 0.5H), 8.00 (m, 1H). Anal. ($C_{28}H_{46}N_4O_6$) C, H, N.

Boc-Leu-Abu-CONH-CH_2CH_3 (37). Compound **31** (0.0823 g, 0.22 mmol) was oxidized according to procedure 7. Recrystallization from EtOAc:hexane yielded **37** as a white solid: 0.067 g, 82.3%. TLC: R_f = 0.52 (9:1 $CHCl_3$:MeOH). HPLC analysis was performed on a Vydac C_4 column (4.6 × 250 mm) at 60 °C, using a gradient of 25–35% B/30 min (A = 0.1% TFA in water, B = 0.1% TFA in acetonitrile. The product had a retention time of 21.05 min and a purity of 99.1%. Mp: 157–158 °C. 600-MHz ^{1}H-NMR (DMSO-d_6) δ 8.66 (t, 1H), 8.06 (d, 1H), 6.85 (d, 1H), 4.88 (m, 1H), 3.99 (m, 1H), 3.12 (m, 2H), 1.77 (m, 1H), 1.60 (m, 1H), 1.51 (m, 1H), 1.35 (br s, 11H), 1.02 (t, 3H), 0.86 (m, 9H). MS: $(M + H)^+$ = 372. Anal. ($C_{18}H_{33}N_3O_5$) C, H, N.

***N*-[(*N*,*N*-Dimethylaminocarbonyl)-Leu]-Abu-CONH-CH_2CH_3 (38).** Compound **32** (0.100 g, 0.291 mmol) was oxidized according to procedure 7. Recrystallization from EtOAc:hexane yielded **38** as a white solid: 0.048 g, 48%. TLC: R_f = 0.43 (9:1 $CHCl_3$:MeOH). Mp: 135–136 °C. HPLC analysis was performed on a Vydac C_4 column (4.6 × 250 mm) at 60 °C using a linear gradient of 15–25% B over 30 min (A = 0.1%TFA in water, B = 0.1% TFA in acetonitrile). The product had a retention time of 14.49 min and a purity of 97%. 600-MHz ^{1}H-NMR (DMSO-d_6) δ 8.65 (t, 1H), 8.10 (d, 1H), 6.07 (d, 1H), 4.85 (m, 1H), 4.20 (m, 1H), 3.12 (m, 2H), 2.77 (s, 6H), 1.77 (m, 1H), 1.63 (m, 1H), 1.48 (m, 2H), 1.40 (m, 1H), 1.02 (t, 3H), 0.85 (m, 9H). MS: $(M + H)^+$ = 343. Anal. ($C_{16}H_{30}N_4O_4$) C, H, N.

***N*-(Morpholinocarbonyl)-Leu-Abu-CONH-CH_2CH_3 (39).** Compound **33** (0.122 g, 0.317 mmol) was oxidized according to procedure 7. Recrystallization from EtOAc:hexane yielded **39** as a white solid: 0.048 g, 40%. TLC: R_f = 0.32 (9:1 $CHCl_3$:MeOH). Mp: 142–144 °C. HPLC analysis was performed on a Vydac C_4 column (4.6 × 250 mm) at 60 °C using a linear gradient of 15–25% B over 30 min (A = 0.1% TFA in water, B = 0.1% TFA in acetonitrile). The product had a retention time of 14 min and a purity of 97.8%. 600-MHz ^{1}H-NMR (DMSO-d_6) δ 8.65 (t, 1H), 8.10 (d, 1H), 6.41 (d, 1H), 4.85 (m, 1H), 4.20 (m, 1H), 3.51 (m, 4H), 3.26 (m, 4 H), 3.12 (m, 2H), 1.75 (m, 1H), 1.62 (m, 1H), 1.48 (m, 2H), 1.40 (m, 1H), 1.02 (t, 3H), 0.85 (m, 9H). MS: $(M + H)^+$ = 385. Anal. ($C_{18}H_{32}N_4O_5$) C, H, N.

Boc-Phe-Leu-Nva-CONH-CH_2CH_3 (40). Compound **35** (255 mg, 0.48 mmol) was oxidized according to procedure 7 to yield **40**: 141 mg, 0.26 mmol, 55%. TLC: R_f = 0.27 (20:1 CH_2Cl_2:MeOH). Mp: 182–183 °C. MS: $(M + H)^+$ = 533. 600-MHz ^{1}H-NMR (DMSO-d_6) δ 0.86–0.91 (m, 9H), 1.04 (t, 3H), 1.23–1.49 (m, 15H), 1.68 (m, 1H), 2.71 (m, 1H), 2.94 (m, 1H), 3.13 (m, 2H), 4.15 (m, 1H), 4.42 (m, 1H), 4.98 (m, 1H), 6.91 (d, 1H), 7.18–7.29 (m, 5H), 7.87 (d, 1H), 8.29 (d, 1H), 8.70 (t, 1H). Anal. ($C_{28}H_{44}N_4O_6$) C, H, N.

Boc-D-Phe-Leu-Nva-CONH-CH_2CH_3 (41). Compound **36** (255 mg, 0.48 mmol) was oxidized according to procedure 7 to yield **41**: 222 mg, 0.42 mmol, 55%. TLC: R_f = 0.38 (20:1 CH_2Cl_2:MeOH). Mp: 178–179 °C. MS: $(M + H)^+$ = 533. 600-MHz ^{1}H-NMR (DMSO-d_6) δ 0.76–0.91 (m, 9H), 1.04 (t, 3H), 1.21–1.44 (m, 15H), 1.70 (m, 1H), 2.75 (m, 1H), 2.87 (m, 1H), 3.14 (m, 2H), 4.18 (q, 1H), 4.34 (m, 1H), 4.95 (m, 1H), 6.98 (d, 1H), 7.05–7.38 (m, 5H), 8.04 (d, 1H), 8.18 (d, 1H), 8.68 (t, 1H). Anal. ($C_{28}H_{44}N_4O_6$) C, H, N.

Phe-Leu-Nva-CONH-CH_2CH_3·HCl (42). The Boc protecting group was cleaved from **40** (70 mg, 0.13 mmol) while stirring with 4 N HCl/dioxane (1 mL, 4 mmol HCl) for 30 min at room temperature. The solvent was removed *in vacuo*, and the resulting white solid was triturated with diethyl ether and dried *in vacuo* to yield **42**: 41 mg, 67%. TLC: R_f = 0.20 (85:10:5 $CHCl_3$:MeOH:HOAc). MS (FAB, high-resolution, calcd for M + H, $C_{23}H_{37}N_4O_4$, 433.2813): $(M + H)^+$ = 433.2833. 600-MHz ^{1}H-NMR (DMSO-d_6) δ 0.87–0.93 (m, 9H), 1.04 (t, 3H), 1.31–1.52 (m, 5H), 1.65–1.72 (m, 2H), 2.95 (dd, 1H), 3.14 (m, 3H), 4.07 (s, 1H), 4.43 (q, 1H), 5.01 (m, 1H), 7.27 (m, 5H), 8.20 (br s, 3H), 8.47 (d, 1H), 8.72 (t, 1H), 8.76 (d, 1H).

D-Phe-Leu-Nva-CONH-CH_2CH_3·HCl (43). The Boc protecting group was cleaved from **41** (100 mg, 0.19 mmol) with 4 N HCl/dioxane (1 mL, 4 mmol HCl) for 30 min at room temperature. The solvent was removed *in vacuo*, and the resulting white solid was triturated with diethyl ether and dried *in vacuo* to yield **43**: 54 mg, 60%. TLC: R_f = 0.22 (85:10:5 $CHCl_3$:MeOH:HOAc). MS (FAB, high-resolution, calcd for M + H, $C_{23}H_{37}N_4O_4$, 433.2813): $(M + H)^+$ = 433.2831. 600-MHz ^{1}H-NMR (DMSO-d_6) δ 0.74 (d, 3H), 0.79 (d, 3H), 0.88 (t, 3H), 1.17–1.26 (m, 2H), 1.31 (m, 2H), 1.40 (m, 1H), 1.49 (m, 1H), 1.68 (m, 1H), 7.25–7.33 (m, 5H), 8.33 (br s, 3H), 8.47 (d, 1H), 8.68 (t, 1H).

Methyl 3(*S*)-Amino-2(*R*,*S*)-hydroxy-4-phenylbutanoate·Hydrochloride (44). MeOH (40 mL) was chilled in an ice bath, to which thionyl chloride (5.0mL, 68.5mmol) was carefully added. When the addition was complete, **18** (4.0 g, 17.3 mmol) was added and the stoppered flask was allowed to come to room temperature and was stirred overnight. The resulting solution was diluted into MeOH and twice rotory evaporated to dryness. The glassy foam was redissolved in a minimum amount of MeOH and the compound crystallized upon addition of ethyl ether. The solid was isolated by suction filtration, washed with cold ether, and dried *in vacuo* to yield **44** as a white, crystalline solid: 2.72 g, 64%. TLC: R_f = 0.59 (butanol:HOAc:water, 4:1:1). MS: $(M + H)^+$ = 210.

***N*-(Cbz-Leu)-3(*S*)-amino-2(*R*,*S*)-hydroxy-4-phenylbutanoic Acid (45).** Compound **44** (1.75 g, 7.14 mmol) was dissolved in THF (20 mL) containing DIEA (1.4 mL, 14.3 mmol). Cbz-leucine *N*-hydroxysuccinimide ester (3.2 g, 113.2

mmol) was dissolved in 1,4-dioxane (20 mL) and added to the solution of the methyl ester. The reaction was allowed to stir at room temperature overnight. The reaction mixture was diluted into EtOAc (100 mL) and washed with 1 N HCl (3 × 80 mL), saturated aqueous $NaHCO_3$ (3 × 80 mL), and saturated NaCl (1 × 80 mL). The organic phase was dried with $MgSO_4$ and suction filtered, and the filtrate was concentrated on a rotary evaporator to an off-white solid that was saponified without purification.

The crude ester was dissolved in MeOH (16 mL) and to this solution was added 1 N NaOH (16 mL). The reaction was stirred for 1 h, after which a white solid mass formed. The reaction mixture was dissolved in 100 mL of water and washed with ethyl ether (3 × 50 mL). The aqueous phase was acidified with solid sodium bisulfate to approximately pH 2.0 and extracted with EtOAc (3 × 50 mL). The EtOAc layer was dried with anhydrous $MgSO_4$, suction filtered, and dried on a rotary evaporator to a clear colorless glass. The compound was recrystallized from a minimum amount of ethyl ether to yield **45**: 2.83 g, 89.6%. TLC: R_f = 0.42 and 0.32 ($CHCl_3$:MeOH:HOAc 85:10:5). MS: $(M + H)^+$ = 443. 500-MHz ^{1}H-NMR (DMSO-d_6) δ 0.78–0.85 (m, 6H), 1.33 (m, 2H), 1.53 (m, 1H), 2.66 (dd, 1H), 2.83 (dd, 1H), 3.88 (s, 1H), 3.99 (m, 1H), 4.28 (br q, 1H), 7.15–7.37 (m, 6H), 7.58 (d, 1H).

***N*-[*N*-(Cbz-Leu)-3(*S*)-amino-2(*R,S*)-hydroxy-4-phenylbutanoyl]-Ala-OCH_3 (46).** Compound **45** (175.0 mg, 0.4 mmol) was coupled to Ala-OCH_3·HCl salt (0.059 g, 0.42 mmol) with EDC (120 mg, 6.3 mmol) according to procedure 6. Recrystallization from ethyl ether provided **46:** 0.34 g, 81%. TLC: R_f = 0.24 (20:1 CH_2Cl_2:MeOH). MS: $(M + H)^+$ = 528. 500-MHz ^{1}H-NMR (DMSO-d_6) δ 0.80 (m, 6H), 1.28 (m, 5H), 1.50 (m, 1H), 2.60 (dd, 1H), 2.86 (dd, 1H), 3.61 (s, 3H), 3.86 (m, 1H), 3.96 (m, 1H), 4.24 (m, 2H), 5.02 (s, 2H), 6.05 (d, 1H), 7.13–7.43 (m, 11H), 7.53 (d, 1H), 8.10 (d, 1H).

Cbz-Leu-Phe-CO-Ala-OCH_3 (47). Compound **46** (0.26 g, 0.50 mmol) was oxidized according to procedure 7. Recrystallization from EtOAc yielded **47:** 0.168 g, 64%. TLC: R_f = 0.47 (20:1 CH_2Cl_2:MeOH). Mp: 149–150 °C. MS (FAB, high resolution, calcd for $C_{28}H_{36}N_3O_7$, 526.2551): $(M + H)^+$ = 526.2501. 500-MHz ^{1}H-NMR (DMSO-d_6) δ 0.85 (dd, 6H), 1.33 (d, 3H), 1.37 (t, 2H), 1.57 (m, 1H), 2.83 (dd, 1H), 3.10 (dd, 1H), 3.65 (s, 3H), 4.07 (q, 1H), 4.34 (qt, 1H), 5.02 (m, 2H), 5.22 (m, 1H), 7.15–7.40 (m, 11H), 8.30 (d, 1H), 9.10 (d, 1H).

***N*-(Cbz-Leu)-3(*S*)-amino-2(*R,S*)-hydroxypentanoic Acid (48).** Compound **16** (2.58 g, 19.4 mmol) was dissolved in saturated $NaHCO_3$ (40 mL). To this solution was added a solution of Cbz-Leu-ONSu (8.8 g, 24.3 mmol) in dioxane (20 mL). The reaction was stirred at room temperature overnight. The solution was concentrated *in vacuo* and the residue dissolved in EtOAc (100 mL) and washed with 1 N HCl (3 × 50 mL) followed by saturated NaCl (1 × 50 mL). The organic phase was dried with anhydrous $MgSO_4$ and suction filtered and the filtrate concentrated *in vacuo* to an off-white semisolid. This material was purified by silica gel column chromatography (91:8:1 $CHCl_3$:MeOH:HOAc eluant) to yield **48**: 5.60 g, 76%. TLC: R_f = 0.28, 0.39, two diastereomers (85:10:5 $CHCl_3$:MeOH:HOAc). MS: $(M + H)^+$ = 381.

***N*-(Cbz-Leu)-3(*S*)-amino-2(*R,S*)-hydroxypentanoic Acid (Ethylthio)ethyl Amide (49).** Compound **48** (50 mg, 0.131 mmol) was coupled to 2-(ethylthio)-ethylamine hydrochloride (38 mg, 0.263 mmol) with EDC (52 mg, 0.263 mmol) according to procedure 6 to yield **49** as an off-white solid: 0.0413 g, 33%. TLC: R_f = 0.24, 0.30 (20:1 $CHCl_3$:MeOH). MS: $(M + H)^+$ = 468. 600-MHz ^{1}H-NMR (DMSO-d_6) δ 0.75–0.90 (m, 9H), 1.15 (m, 3H), 1.30–1.50 (m, 4H), 1.50–1.70 (m, 2H), 2.53–2.60 (m, overlaps with DMSO), 3.20 (m, 2H), 3.80–4.10 (m, 3H), 5.00 (s, 2H), 5.80 (m, 0.5 H), 5.91 (m, 0.5H), 7.35 (m, 4H), 7.42 (d, 0.5H), 7.48 (m, 1.5H), 7.85 (t, 0.5H), 7.92 (t, 0.5H). Anal. ($C_{23}H_{37}N_3O_5S$) C, H, N.

Cbz-L-Leu-L-Abu-CONH-CH_2CH_2-SO_2-Et (50). Compound **49** (41 mg, 0.088 mmol) was oxidized according to procedure 7 to yield 18 mg of the crude product. This material was further purified by preparative reverse-phase HPLC on a Vydac C_4 column (250 × 4.6 mm) at 60 °C eluting with a gradient system (eluant A = 0.1% aqueous TFA, eluant B = 0.1% TFA in CH_3CN) which ran from 25% to 35% eluant B over 40 min at 1 mL/min to yield **50:** 7.7 mg, 17%. TLC: R_f = 0.32 (CH_2Cl_2:MeOH, 20:1). MS (FAB, high resolution, calcd for $C_{23}H_{35}N_3O_7S$, 498.2274): $(M + H)^+$ = 498.2325. 600-MHz ^{1}H-NMR (DMSO-d_6) δ 0.88 (m, 9H), 1.20 (t, J = 7.5 Hz, 3H), 1.41 (m, 3H), 1.58 (m, 2H), 1.80 (m, 1H), 3.12 (q, J = 7.5 Hz, 2H), 3.52 (m, 2H), 4.10 (dt, J = 8.4 Hz, 0.5H), 4.87 (m, 1H), 5.02 (s, 2H), 7.35 (m, 5H), 7.40 (d, J = 8.4 Hz, 1H), 8.28 (d, J = 6.6 Hz, 1H), 8.85 (t, J = 5.6 Hz, 1H).

Cbz-Leu-Phe-CO-Ala-OH (51). Compound **47** (75 mg, 0.14 mmol) was suspended in MeOH (1.2 mL) and stirred while 1 N NaOH (0.3 mL) was added dropwise. After being stirred for 10 min (clear yellow solution), the reaction was diluted into deionized water (10 mL) and washed with EtOAc (1 × 10 mL). The aqueous phase was acidified with 1 N HCl to pH 2 and then extracted with EtOAc (3 × 10 mL). The organic phase was dried over $MgSO_4$, suction filtered, and concentrated to a yellow solid. This solid was triturated with diethyl ether to yield **51** as a white solid: 17 mg, 24%. TLC: R_f = 0.65 (91:8:1 $CHCl_3$:MeOH:HOAc). MS (FAB, high resolution, calcd for $C_{27}H_{34}N_3O_7$, 512.2395): $(M + H)^+$ = 512.2378. 360-MHz ^{1}H-NMR (DMSO-d_6) δ 0.76–0.87 (m, 6H), 1.15–1.60 (m, 6H), 2.69–2.92 (m, 1H), 3.16 (m, 1H), 4.06 (m, 1H), 4.28 (m, 1H),5.00 (m, 2H), 5.21 (m, 1H), 7.10–7.40 (m, 11H), 8.28 (d, 0.25H), 8.34 (d, 0.75H), 8.86 (d, 0.25H), 8.93 (d, 0.75H), 12.55 (br, 1H).

Enzymology. Calpain activity was monitored using a continuous spectrofluorometic assay with succinyl-Leu-Tyr-4-methylcoumaryl-7-amide (SLY-AMC) at 25 °C.[40] Porcine calpain I from erythrocytes and porcine calpain II from kidney were purchased from Nacalai Tesque, Kyoto, Japan. Human calpain I and II were isolated from erythrocytes and placenta, respectively, as previously described.[43,44] Bovine calpain I was kindly provided by Dr. Dorothy Croall (U. Maine, Orono). Substrate (SLY-AMC) was purchased from Bachem Bioscience. Spectrofluorescent changes were measured using a Hitachi F4500 fluorescence spectrophotometer. Substrate (0.2-2 mM) was prepared in 50 mM MOPS, 5 mM $CaCl_2$, 5 mM β-mercaptoethanol, 1% DMSO, pH 7.5. Stock inhibitor solutions (approximately 1 mM) were prepared fresh each day in 1:1 (v/v) DMSO: 10 mM HCl and kept at 4 °C. The stock inhibitor solutions were diluted into substrate buffer immediately prior to the assay to provide concentrations in the range 100–500 nM. Calpain was diluted to 0.18 mg/mL in 20 mM HEPES, 2 mM EDTA, 2 mM EGTA, 5 mM DTT, pH 7.5. The kinetic analysis was initiated by first incubating 0.9 mL of substrate solution with 0.05 mL of inhibitor (or buffer as a control). The reaction was initiated by addition of calpain solution (0.05 mL), and the increase in fluorescence (λ_{ex} = 380 nm, λ_{em} = 460 nm) was monitored for 3–10 min. The initial velocities were then used to obtain the values of V_{max}, K_m, and K_i by an iterative least-squares fit[45] of the data to the equation for competitive inhibition:[46]

$$V = \{V_{max}[\text{SLY-MCA}]\}/\{(1 + [\text{Inh}]/K_i)K_m + [\text{SLY-MCA}]\}$$

Epimerization Studies. Epimerization rates were assessed chromatographically using compound **1a** prepared as a single L,L isomer. Compound **1a** (2 mM) was dissolved in DMSO. Kinetic analysis of each condition was initiated by the addition of an equal volume of aqueous phosphate buffer at various ionic strengths and pH values. Analytical samples for each time point were obtained by removing a 200-μL aliquot and extracting the inhibitor using 800 μL of methylene chloride. A 700-μL portion of the organic phase was removed and dried under a stream of nitrogen. The resultant pellet was dissolved in 60 μL of dioxane for subsequent analysis. Analysis and quantification of diastereomer levels were performed on a Hewlett-Packard 1050 series HPLC using a variable-wavelength UV detector monitoring absorbance at 210 nm. Analysis was conducted on a 25-μL portion of the sample over a Macherey-Nagel Nucleosil Chiral-2 column (4 × 250 mm) using a mobile phase of hexane:dioxane:acetonitrile (86:11:3, v/v) at a flow rate of 1 mL/min.

At pH 7 and 10, the experimentally measured ratio of the L,L diastereomer to L,D diastereomer at 15 days was assumed

to represent the thermodynamic equilibrium for the calcualtion of rate constants. At pH 3, it was evident that equilibrium was not reached, therefore exact rate constants could not be calculated. The forward rate constant, k_f, was calculated for a first-order reversible reaction according to the following equation:

$$t = -0.5k_f^{-1} \ln\{[2(\%\text{L,L isomer}_t)/\%\text{L,L isomer}_{t=0}] - 1\}$$

At pH 3, k_f values could not be calculated by this equation.

Interaction of α-Keto Amides with Alcohol. Hemiketal formation of **1a** was studied using a Bruker 300-MHz ^{1}H-NMR. The compound was dissolved at 5 mg/mL in CD_3OD, and the proton spectra were monitored over time at room temperature. At $t = 0$, initial spectral interpretation is as follows: δ (ppm) 7.45–7.2 (m, 5H, Cbz aromatic), 5.10 (s, 2H, CH_2 of Cbz), 5.05 (m, 1H, αCH of Abu), 4.2 (dd, 1H, αCH of Leu), 3.28 (2 × m, 2H, CH_2N of ethylamide), 1.95(m, 1H, γCH of Leu), 1.80–1.45 (m, 3H, βCH_2 of Leu and Abu), 1.15 (t, 3H, γCH of Abu), 1.05–0.8 (overlapping t, 9H, δCH_3 of Leu, CH_3 of ethylamide). Proton resonances assigned to the Cbz group remained constant over time; however, the remainder of the spectra rapidly became extremely complex with evidence of multiple chemical species.

Solubility Measurements. Solubility of various peptidyl α-keto amides was evaluated by first dissolving (with sonication, if necessary) 3 mg of inhibitor in dimethylacetamide (DMA) to a stock concentration of 30 mg/mL. A portion was diluted with 0.9% saline to a final concentration of 3% DMA (final inhibitor concentration ≤0.9 mg/mL). The pH of the mixture was adjusted to 2.75 with 0.1 N HCl, sonicated for 5 min in a sonic bath, and allowed to sit at room temperature for 15 min. The sample was then centrifuged for 5 min (16500*g*) in a microfuge and the supernatant sampled for HPLC analysis. Standard solutions of inhibitor were prepared at known concentrations in acetonitrile for quantitation. HPLC quantification of peak area integrated from the signal at 210 nm for each sample was measured on a Hewlett-Packard 1090M HPLC system using a Vydac C_4 reverse-phase column (4.6 × 250 mm, 5-μm particle size) maintained at 60 °C at a flow rate of 1 mL/min. Gradients of 15–50% acetonitrile using an aqueous buffer of 0.1% TFA were employed to elute the various inhibitors.

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