Brief Articles

Structural Basis for the Potent Calpain Inhibitory Activity of Peptidyl α-Ketoacids

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A series of peptidyl α-ketoacids and α-ketoesters was synthesized and studied as μ-calpain inhibitors. Docking studies revealed that the μ-calpain inhibitory activity of the compounds is influenced by hydrogen bonding interactions and the potential for ionic interaction with active site residues as well as placement of a planar *N*-terminal capping group into the S_3 pocket of the enzyme.

Introduction

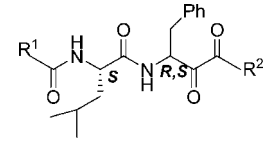
Calpains are a family of intracellular calcium-dependent cysteine proteases with widespread expression in a variety of cells and tissues.[1] μ-Calpain (calpain I) and m-calpain (calpain II) are the major calpain isoforms, which are ubiquitously distributed in mammalian cells. These calpain isoforms catalyze the partial hydrolysis of a wide variety of target proteins including cytoskeletal proteins, receptors, and integral membrane proteins.[1–3] The enzymes have been implicated in several physiological processes and a variety of degenerative diseases.[1–4] The implication of calpain in human disease has aroused interest in the search for calpain inhibitors as tools for studying the enzyme and as potential therapeutic agents.[5,6]

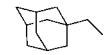
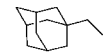
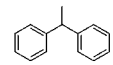
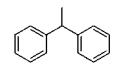
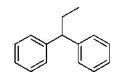
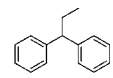
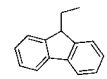
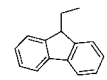
The broad substrate specificity of the major calpains suggests that the enzymes tolerate a wide variety of side chain residues within their active site pockets.[7] Recent crystallographic studies have revealed the structure of calpain with and without bound inhibitors and have made it possible to rationalize the structural basis for ligand induced inhibition of the enzyme.[8,9] We report here the synthesis and structural basis for the potent calpain inhibitory activity of a series of peptidyl α-ketoacids that incorporate lipophilic residues as the P_2 *N*-capping substituent (Table 1).

Results and Discussion

Chemistry. Compounds **1a**−**e** and **2a**−**e** were synthesized (Scheme 1) by employing general coupling procedures for the solution phase synthesis of peptides. Thus, coupling the appropriate carboxylic acid **4a**−**e** with L-leucine methyl ester hydrochloride (**5**) using EDC/HOBT as the coupling agent and DMF/NMM mixture as solvent afforded pseudodipeptides **6a**−**e**, which were hydrolyzed with 1N NaOH in MeOH and coupled with β-amino-α-hydroxy ester **7** to give **8a**−**e**. Compound **7** was synthesized as previously reported.[10] Dess−Martin oxidation of **8a**−**e** gave α-ketoesters **1a**−**e**. ^{1}H NMR analysis of the crude products showed **1a**−**e** to be diastereomerically pure. However, column chromatographic purification (silica gel) as well as basic hydrolysis of the ester functionality led to epimerization of the chiral center at P_1 to yield **2a**−**e** as pairs of diastereomers. The diastereomeric ratios of the compounds as determined by ^{1}H NMR spectrometry are shown in Table 1. Racemization of the compounds is consistent with previous

Table 1. Structure, Diastereomeric Ratio, and μ-Calpain[a] Inhibitory Activity of Compounds **1a**−**e** and **2a**−**e**



Compd.	R^1	R^2	K_i (nM)[b]	DR (*S,S*:*R,S*)[c]
1a		OCH_3	1048 ± 19	60:40
2a		OH	50 ± 6	58:42
1b		OCH_3	450 ± 10	65:35
2b		OH	41 ± 7	67:33
1c		OCH_3	944 ± 3	75:25
2c		OH	108 ± 9	75:25
1d		OCH_3	2150 ± 20	100:0
2d		OH	63 ± 4	69:31
1e		OCH_3	1200 ± 15	57:43
2e		OH	8 ± 0.5	53:47
3	MDL28170[d]		10 ± 0.3	

[a] Porcine erythrocyte μ-calpain (Calbiochem). [b] K_i values were determined by Dixon plots using the average of triplicate assays and plotting $1/v$ vs I (inhibitor concentration) to give intersecting lines with correlation coefficient 0.95. [c] DR = Diastereomeric ratio of the compounds as determined by ^{1}H NMR spectrometry in $CDCl_3$. [d] MDL28170 (Calbiochem).

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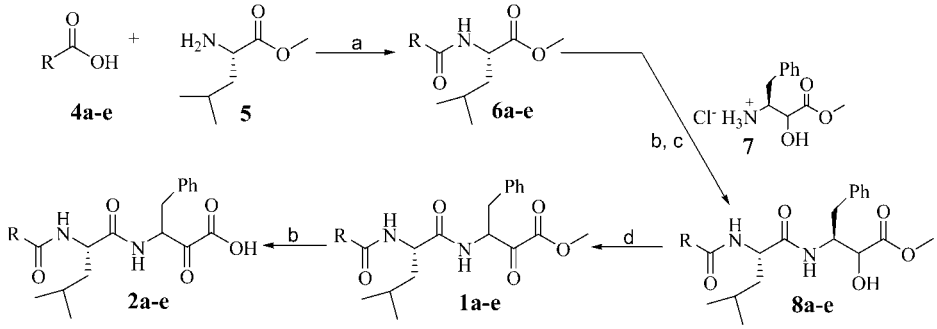
Scheme 1[a]



[a] Reagents: (a) EDC, HOBT, NMM, DMF; (b) 1N NaOH/CH_3OH; (c) 7, EDC, HOBT, NMM, DMF; (d) Dess−Martin Reagent/CH_2Cl_2.

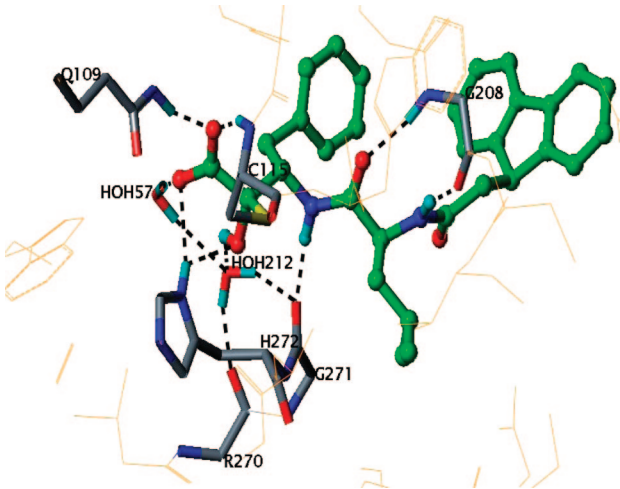


Figure 1. α-Ketoacid **2e** (the *S*,*S*-diastereomer) docked into the active site of μ-calpain. Hydrogen atoms are removed for clarity except those involved in hydrogen bonding. The inhibitor is shown as ball and stick. Hydrogen bonds are shown as black dashed lines; all protein residues are colored orange except those involved in the hydrogen bonding.

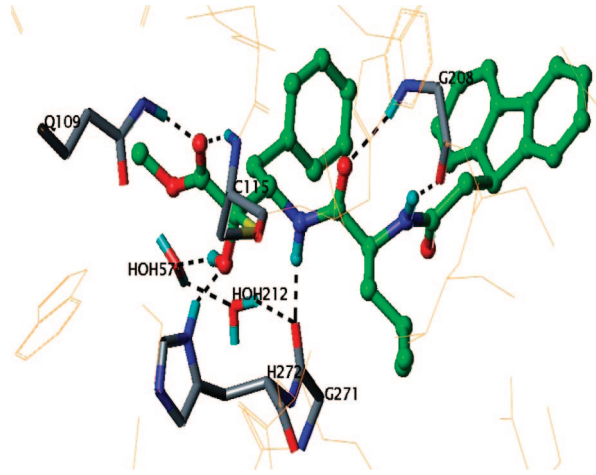


Figure 2. α-Ketoester **1e** (the *S*,*S*-diastereomer) docked into the active site of μ-calpain. Hydrogen atoms are removed for clarity except those involved in hydrogen bonding. The inhibitor is shown as ball and stick. Hydrogen bonds are shown as black dashed lines; all protein residues are colored orange except those involved in the hydrogen bonding.

reports, which indicate that α-keto carbonyl compounds are prone to racemization in the presence of base.[11]

Calpain Inhibition and Docking Studies. The μ-calpain inhibitory potency (K_i values) of **1a**−**e** and **2a**−**e** (as diastereomeric mixtures) were determined employing a fluorometric assay with Suc-Leu-Tyr-AMC as substrate and the peptidyl aldehyde calpain inhibitor, MDL28170, as positive control. The inhibition data (Table 1) showed that the α-ketoacids are better inhibitors of μ-calpain compared to the corresponding α-ketoesters, suggesting that the carboxylic acid moiety is very important for potent inhibition of the enzyme. For example, α-ketoacid **2e** (K_i = 8 nM), which was the most potent member of the series, was 150-fold more potent than its methyl ester derivative **1e**. This is consistent with the results of Li et al.,[12] who reported that the calpain inhibitory activity of peptidyl calpain inhibitors with α-keto carbonyl electrophilic centers follows the order α-ketoacids > α-ketoamides > α-ketoesters. To determine the structural basis for the potent calpain inhibitory activity of peptidyl α-ketoacids compared to peptidyl α-ketoesters, we docked the *S*,*S*-diastereomers of **1e** and **2e** into the active site of μ-calpain. This study revealed that water molecules play an important role in the binding of the inhibitors to the enzyme by serving as bridges to facilitate formation of a network of hydrogen bonds between the inhibitors and active site residues (Figures 1 and 2). This is consistent with the reported cocrystal structure of mini-calpain in complex with an α-ketoamide inhibitor, which showed a water bridge between the ketoamide hydrogen and H_{272}.[9] Compounds **1e** and **2e** also made hydrogen bonding interactions with Q_{109}, G_{208}, G_{271}, and H_{272} at the active site of μ-calpain. Hydrogen bonding between the NH of the P_1−P_2 amide bond of calpain inhibitors, and G_{271} has been shown to be critical for inhibition of the enzyme. The absence of such an interaction was shown to decrease calpain inhibition by 250-fold.[13] Compound **2e** formed 12 hydrogen bonds with active site residues, which included a network of eight hydrogen bonds that were facilitated by two water molecules (Figure 1). The carboxylate group of **2e** was juxtapositioned to the imidazole ring of H_{272} and formed a hydrogen bond with the NH group of the imidazole ring, suggesting the possibility for ionic interaction between the carboxylate of **2e** and H_{272}. On the contrary, α-ketoester **1e** formed nine hydrogen bonds with active site residues (compared to 12 for α-ketoacid **2e**) (Figure 2). Esterification of the carboxylate group eliminated the possibility for ionic interaction between **1e** and the enzyme. This is supported by the absence of the hydrogen bond that was observed between **2e** and H_{272} and may account in part for the 150-fold difference in the μ-calpain inhibitory activity between **1e** and **2e**, the 30-fold difference in activity between **1d** and **2d**, the 10-fold difference in activity between **1c** and **2c** or **1b** and **2b**, and the 20-fold difference between **1a** and **2a**.

It has been reported that peptidyl aldehydes with hydrophobic *N*-terminal capping groups are good inhibitors of calpain.[4,5] To further explore the structural requirements of the *N*-terminal capping group that is important for potent inhibition of μ-calpain, we investigated the effect of the molecular shape and molecular

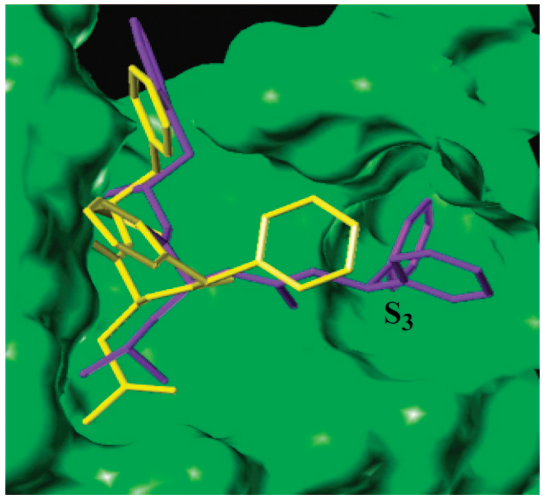


Figure 3. Compounds **2c** (yellow) and **2d** (purple) docked into the active site of μ-calpain. Notice that the diphenyl group of **2c** unlike that of **2d** does not effectively access the S_3 pocket. The *S*,*S*-diastereomers were studied.

size of the *N*-terminal group on μ-calpain inhibition. Our data showed that large planar aromatic groups are preferred over nonplanar saturated substituents (**2b** vs **2e**). Compound **2e** with the planar fluorene moiety was over 5-fold more potent than **2a** and **2b** with cyclohexyl and adamantyl groups, respectively. The docking studies also revealed that the *N*-terminal capping group occupied a large pocket at the S_3 subsite of the enzyme (Figure 3). It was also noticed that the extent to which the *N*-terminal capping substituent occupied this pocket (as determined by the linker length) influenced μ-calpain inhibition. Thus, **2d** with a diphenylpropionyl group was about twice as potent as **2c** with a diphenylacetyl group as the *N*-terminal capping substituent (Figure 2).

In summary, our data suggest that the structural basis for the potent calpain inhibitory activity of peptidyl α-ketoacids is derived from a network of hydrogen bonding interactions and the possibility for ionic interaction between the inhibitor and active site residues of the enzyme. Esterification of the carboxylic acid group significantly reduced calpain inhibition due in part to diminished potential for hydrogen bonding and ionic interaction interactions with active site residues. Additionally, large planar aromatic groups are preferred over nonplanar saturated groups as the *N*-terminal capping substituent. Furthermore, efficient positioning of the *N*-terminal capping group into a large pocket at the S_3 subsite of the enzyme potentiates μ-calpain inhibition.

Experimental Section

General Procedure for the Synthesis of Pseudodipeptides 6a−e and α-Hydroxy Esters 8a−e. A solution of the appropriate carboxylic acid (1 equiv) in DMF was cooled with an ice-bath and EDC (1.15 equiv), HOBt (1.15 equiv), NMM (2.3 equiv), and the appropriate amine hydrochloride (1 equiv) were added consecutively. The reaction mixture was stirred overnight at RT. DMF was removed in vacuo, water was added to the residue, and the mixture was extracted with Et_2O or EtOAc. The combined extracts were washed successively with saturated $NaHCO_3$ solution, 0.5N HCl, and H_2O, followed by drying ($MgSO_4$). The solvent was removed, and the crude product was purified by column chromatography.

General Procedure for the Synthesis of α-Ketoesters 1a−e. Dess−Martin reagent (2−4 equiv) was added to a cooled (ice-bath) solution of the appropriate α-hydroxy ester **8a−e** in CH_2Cl_2 and the mixture was stirred at RT for 5−6 h. Sodium thiosulfate pentahydrate (14−28 equiv) in saturated $NaHCO_3$ solution was added and stirred for 10 min. The mixture was extracted with CH_2Cl_2, and the organic extract was washed with $NaHCO_3$ solution, water, and dried ($MgSO_4$). The solvent was removed, and the crude product was purified by column chromatography.

General Procedure for the Synthesis of α-Ketoacids 2a−e. A solution of the appropriate ester **1a−e** in MeOH was stirred with 1N NaOH solution until TLC showed completion of reaction (1−2 h). The MeOH was removed under vacuum, water was added, and the solution was washed with CH_2Cl_2. The aqueous layer was recovered and acidified with 2N HCl and extracted with EtOAc. The combined EtOAc extracts were washed with water and dried ($MgSO_4$), followed by evaporation of the solvent to give the desired acid.

***N*-(3-Cyclohexylpropionyl)-L-leucyl-2-oxo-3(*R*,*S*)-amino-4-phenylbutanoic Acid Methyl Ester (1a).** Column chromatographic purification of the crude product with EtOAc/hexanes (4:6) as solvent gave a diastereomeric mixture of **1a** as white solid in 80.3% yield. ^{1}H NMR ($CDCl_3$, 300 MHz) Diastereomer 1: δ 7.22 (m, 5H), 6.73 (d, J = 6.6 Hz, 0.60H), 5.75 (m, 1H), 5.33 (m, 1H), 4.42 (m, 1H), 3.84 (s, 1.8H), 3.23 (m, 1H), 3.00 (m, 1H), 2.16 (m, 2H), 1.49 (m, 10H), 1.19 (m, 4H), 0.89 (m, 8H). Diastereomer 2: δ 7.22 (m, 5H), 6.80 (d, J = 6.9 Hz, 0.40H), 5.75 (m, 1H), 5.33 (m, 1H), 4.42 (m, 1H), 3.85 (s, 1.20H), 3.23 (m, 1H), 3.00 (m, 1H), 2.16 (m, 2H), 1.49 (m, 10H), 1.19 (m, 4H), 0.89 (m, 8H). ESI MS: *m*/*z* 513.6 $(M + Na + CH_3OH)^+$. Anal. ($C_{26}H_{38}N_2O_5$) C, H, N.

***N*-(1-Adamantylacetyl)-L-leucyl-2-oxo-3(*R*,*S*)-amino-4-phenylbutanoic Acid Methyl Ester (1b).** The crude product was purified by column chromatography with EtOAc/hexanes (2:3) as solvent to give 0.71 g (94.6%) of **1b** as a mixture of diastereomers. ^{1}H NMR ($CDCl_3$, 300 MHz) Diastereomer 1: δ 7.23 (m, 5H), 6.77 (d, J = 6.9 Hz, 0.54H), 5.68 (d, J = 8.1 Hz, 1H), 5.35 (m, 1H), 4.43 (m, 1H), 3.84 (s, 1.62H), 3.23 (m, 1H), 3.02 (m, 1H), 1.92 (m, 5H), 1.59 (m, 15H), 0.90 (m, 6H). Diastereomer 2: δ 7.23 (m, 5H), 6.88 (d, J = 6.9 Hz, 0.46H), 5.68 (d, J = 8.1 Hz, 1H), 5.35 (m, 1H), 4.43 (m, 1H), 3.85 (s, 1.38H), 3.23 (m, 1H), 3.02 (m, 1H), 1.92 (m, 5H), 1.59 (m, 15H), 0.90 (m, 6H). ESI MS: *m*/*z* 551.3 $(M + Na + CH_3OH)^+$. Anal. ($C_{29}H_{40}N_2O_5$) C, H, N.

***N*-(2,2-Diphenylacetyl)-L-leucyl-2-oxo-3(*R*,*S*)-amino-4-phenylbutanoic Acid Methyl Ester (1c).** Column chromatographic purification of the crude product with EtOAc/hexanes (2:3) as solvent gave **1c** as white solid in 88% yield. ^{1}H NMR ($CDCl_3$, 300 MHz) Diastereomer 1: δ 7.25 (m, 13H), 7.08 (m, 2H), 6.78 (d, J = 6.6 Hz, 0.60H), 5.90 (m, 1H), 5.29 (m, 1H), 4.88 (s, 0.60H), 4.47 (m, 1H), 3.79 (s, 1.80H), 3.14 (m, 1H), 2.90 (m, 1H), 1.36 (m, 3H), 0.80 (m, 6H). Diastereomer 2: δ 7.25 (m, 13H), 7.08 (m, 2H), 6.92 (d, J = 6.9 Hz, 0.40H), 5.90 (m, 1H), 5.29 (m, 1H), 4.94 (s, 0.40H), 4.47 (m, 1H), 3.87 (s, 1.20H), 3.14 (m, 1H), 2.90 (m, 1H), 1.36 (m, 3H), 0.80 (m, 6H). ESI MS: *m*/*z* 569.2 $(M + Na + CH_3OH)^+$. Anal. ($C_{31}H_{34}N_2O_5$) C, H, N.

***N*-(3,3-Diphenylpropionyl)-L-leucyl-2-oxo-3(*S*)-amino-4-phenylbutanoic Acid Methyl Ester (1d).** Compound **1d** was obtained as white solid in 89% yield after column purification with EtOAc/hexanes (2:3) as solvent. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.51 (m, 15H), 6.57 (d, J = 6.9 Hz, 1H), 5.60 (d, J = 8.1 Hz, 1H), 5.25 (m, 1H), 4.50 (m, 1H), 4.27 (m, 1H), 3.82 (s, 3H), 3.14 (m, 1H), 2.89 (m, 3H), 1.38 (m, 1H), 1.15 (m, 2H), 0.72 (m, 6H). ESI MS: *m*/*z* 551.3 $(M + Na + CH_3OH)^+$. Anal. ($C_{32}H_{36}N_2O_5$) C, H, N.

***N*-(9-Fluoreneacetyl)-L-leucyl-2-oxo-3(*R*,*S*)-amino-4-phenylbutanoic Acid Methyl Ester (1e).** ^{1}H NMR ($CDCl_3$, 300 MHz) Diastereomer 1: δ 7.73 (m, 2H), 7.31 (m, 11H), 6.74 (d, J = 7.2 Hz, 0.55H), 5.62 (d, J = 8.1 Hz, 0.55H), 5.29 (m, 1H), 4.41 (m, 2H), 3.84 (s, 1.65H), 3.22 (m, 1H), 2.99 (m, 1H), 2.77 (m, 1H), 2.59 (m, 1H), 1.34 (m, 3H), 0.81 (m, 6H). Diastereomer 2: δ 7.73 (m, 2H), 7.31 (m, 11H), 6.66 (d, J = 6.9 Hz, 0.45H), 5.55 (d, J = 8.1 Hz, 0.45H), 5.29 (m, 1H), 4.41 (m, 2H), 3.82 (s, 1.35H), 3.22 (m, 1H), 2.99 (m, 1H), 2.77 (m, 1H), 2.59 (m, 1H), 1.34 (m, 3H), 0.81 (m, 6H). ESI MS: *m*/*z* 551.3 $(M + Na + CH_3OH)^+$. Anal. ($C_{32}H_{34}N_2O_5$) C, H, N.

***N*-(3-Cyclohexylpropionyl)-L-leucyl-2-oxo-3(*R*,*S*)-amino-4-phenylbutanoic Acid (2a).** ^{1}H NMR ($CDCl_3$, 300 MHz) Diastereomer 1: δ 7.22 (m, 6H), 6.47 (d, J = 8.3 Hz, 0.60H), 5.36 (m, 0.6H),

4.53 (m, 1H), 3.33 (m, 1H), 3.00 (m, 1H), 2.21 (m, 2H), 1.51 (m, 10H), 1.15 (m, 4H), 0.85 (m, 8H). Diastereomer 2: δ 7.22 (m, 6H), 6.24 (d, J = 8.4 Hz, 0.40H), 5.25 (m, 0.4H), 4.53 (m, 1H), 3.33 (m, 1H), 3.00 (m, 1H), 2.21 (m, 2H), 1.51 (m, 10H), 1.15 (m, 4H), 0.85 (m, 8H). ESI MS: m/z 442.9 $(M - 1)^+$. Anal. ($C_{25}H_{36}N_2O_5 \cdot 0.25H_2O$) C, H, N.

***N*-(1-Adamantylacetyl)-L-leucyl-2-oxo-3(*R*,*S*)-amino-4-phenylbutanoic acid (2b).** Column chromatographic purification with EtOAc/hexanes (1:1) as solvent gave a diastereomeric mixture of **2b** as foamy white solid in 92% yield. ^{1}H NMR ($CDCl_3$, 300 MHz) Diastereomer 1: δ 7.23 (m, 6H), 6.23 (d, J = 8.4 Hz, 0.67H), 5.33 (m, 0.67H), 4.51 (m, 1H), 3.31 (m, 1H), 3.01 (m, 1H), 1.94 (m, 5H), 1.57 (m, 15H), 0.84 (m, 6H). Diastereomer 2: δ 7.23 (m, 6H), 6.02 (d, J = 8.4 Hz, 0.33H), 5.22 (m, 0.33H), 4.51 (m, 1H), 3.31 (m, 1H), 3.01 (m, 1H), 1.94 (m, 5H), 1.57 (m, 15H), 0.84 (m, 6H). ESI MS: m/z 480.9 $(M - 1)^+$. Anal. ($C_{28}H_{38}N_2O_5 \cdot 0.25H_2O$) C, H, N.

***N*-(2,2-Diphenylacetyl)-L-leucyl-2-oxo-3(*R*,*S*)-amino-4-phenylbutanoic Acid (2c).** Compound **2c** was obtained in 97% yield as diastereomeric mixture of a white foamy solid. ^{1}H NMR ($CDCl_3$, 300 MHz) Diastereomer 1: δ 7.15 (m, 16H), 6.16 (d, J = 8.1 Hz, 0.75H), 5.08 (m, 2H), 4.46 (m, 1H), 3.21 (m, 1H), 2.74 (m, 1H), 1.25 (m, 3H), 0.83 (m, 6H). Diastereomer 2: δ 7.15 (m, 16H), 6.06 (d, J = 8.1 Hz, 0.25H), 5.08 (m, 2H), 4.46 (m, 1H), 3.21 (m, 1H), 2.74 (m, 1H), 1.25 (m, 3H), 0.83 (m, 6H). ESI MS: m/z 499.1 $(M - 1)^+$. Anal. ($C_{30}H_{32}N_2O_5 \cdot 0.5H_2O$) C, H, N.

***N*-(3,3-Diphenylpropionyl)-L-leucyl-2-oxo-3(*R*,*S*)-amino-4-phenylbutanoic Acid (2d).** Compound **2d** was obtained in 99.7% yield as diastereomeric mixture of a white foamy solid. ^{1}H NMR ($CDCl_3$, 300 MHz) Diastereomer 1: δ 7.15 (m, 16H), 6.36 (d, J = 8.4 Hz, 0.69H), 5.27 (m, 0.69H), 4.41 (m, 2H), 3.23 (m, 1H), 2.92 (m, 3H), 1.04 (m, 3H), 0.62 (m, 6H). Diastereomer 2: δ 7.15 (m, 16H), 6.11 (d, J = 8.4 Hz, 0.31H), 5.12 (m, 0.31H), 4.41 (m, 2H), 3.23 (m, 1H), 2.92 (m, 3H), 1.04 (m, 3H), 0.62 (m, 6H). ESI MS: m/z 512.9 $(M - 1)^+$. Anal. ($C_{31}H_{34}N_2O_5 \cdot 0.5\ H_2O$) C, H, N.

***N*-(9-Fluoreneacetyl)-L-leucyl-2-oxo-3(*R*,*S*)-amino-4-phenylbutanoic Acid (2e).** Compound **2e** was obtained in 78% yield as diastereomeric mixture of a white foamy solid. ^{1}H NMR ($CDCl_3$, 300 MHz) Diastereomer 1: δ 7.68 (m, 2H), 7.26 (m, 12H), 6.09 (d, J = 8.1 Hz, 0.53H), 5.25 (m, 0.53H), 4.37 (m, 2H), 3.27 (m, 1H), 2.84 (m, 3H), 1.34 (m, 3H), 0.75 (m, 6H). Diastereomer 2: δ 7.68 (m, 2H), 7.26 (m, 12H), 5.75 (d, J = 7.8 Hz, 0.47H), 5.09 (m, 0.47H), 4.37 (m, 2H), 3.27 (m, 1H), 2.84 (m, 3H), 1.34 (m, 3H), 0.75 (m, 6H). ESI MS: m/z 510.9 $(M - 1)^+$. Anal. ($C_{31}H_{32}N_2O_5 \cdot 0.5\ H_2O$) C, H, N.

***N*-(3-Cyclohexylpropionyl)-L-leucine (6a).** 3-Cyclohexylpropionic acid (0.688 g, 2.8 mmol) was coupled with L-leucine methyl ester hydrochloride (0.755 g, 2.8 mmol) to give yellow viscous oil, which upon recrystallization from hexanes at freezer temperature afforded *N*-(3-cyclohexylpropionyl)-L-leucine methyl ester as short rod-like crystals in 98% yield. This was hydrolyzed to give a white solid, which upon recrystallization from EtOAc/hexanes gave **6a** as rods in 97% yield: mp 175−176 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 5.86 (d, J = 8.1 Hz, 1H), 4.62 (m, 1H), 2.28 (m, 2H), 1.81−1.52 (m, 10H), 1.27−1.04 (m, 5H), 0.99 (d, J = 3.2 Hz, 3H), 0.97 (d, J = 3.2 Hz, 3H), 0.90 (m, 1H). ^{13}C NMR (CD_3OD, 75 MHz): δ 176.6, 176.2, 51.9, 41.6, 38.5, 34.4, 34.4, 34.3, 34.2, 26.5, 27.7, 27.4, 26.1, 23.4, 21.7.

***N*-(1-Adamantylacetyl)-L-leucine (6b).** 1-Adamantaneacetic acid (1.94 g, 10 mmol) was coupled with L-leucine methyl ester hydrochloride (1.817 g, 10 mmol) to give *N*-(1-adamantylacetyl)-L-leucine methyl ester as light-yellow solid in 98% yield. This was hydrolyzed to give **6b** as white solid in 94% yield: mp 204−205 °C. ^{1}H NMR (CD_3OD, 300 MHz): δ 4.41 (m, 1H), 2.00 (s, 2H), 1.95 (m, 3H), 1.76−1.59 (m, 15H), 0.97 (d, J = 6.5 Hz, 3H), 0.93 (d, J = 6.5 Hz, 3H). ^{13}C NMR (CD_3OD, 75 MHz): δ 174.7, 172.5, 50.5, 50.1, 42.3, 40.1, 36.5, 32.5, 28.8, 24.6, 21.9, 20.2.

***N*-(2,2-Diphenylacetyl)-L-leucine (6c).** Diphenylacetic acid (2.12 g, 10 mmol) was coupled with L-leucine methyl ester hydrochloride (1.82 g, 10 mmol) to give *N*-(2,2-diphenylacetyl)-L-leucine methyl ester in 93% yield. This was hydrolyzed to give **6c** (98% yield), which was recrystallized from hexanes/EtOAc/MeOH to afforded rod-like white solid: mp 109 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.35−7.23 (m, 10H), 6.01 (d, J = 7.9 Hz, 1H), 5.00 (s, 1H), 4.62 (m, 1H), 1.67−1.43 (m, 3H), 0.87 (d, J = 6.3 Hz, 3H) 0.86 (d, J = 6.2 Hz, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 176.6, 172.7, 139.0, 138.9, 129.0, 128.9, 128.8, 127.5, 127.4, 58.9, 51.2, 40.8, 25.0, 22.8, 21.8.

***N*-(3,3-Diphenylpropionyl)-L-leucine (6d).** Diphenylpropionic acid (2.26 g, 10 mmol) was coupled with L-leucine methyl ester hydrochloride (1.82 g, 10 mmol) to give *N*-(3,3-diphenyl-propionyl)-L-leucine methyl ester. This was hydrolyzed to give 99% of **6d**, which was recrystallization from EtOAc/hexanes to give a white solid: mp 115−116 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.70 (brs, 1H), 7.36−7.15 (m, 10H), 6.00 (d, J = 8.2 Hz, 1H), 4.59−4.47 (m, 2H), 3.02 (dd, J = 14.1, 7.0 Hz, 1H), 2.96 (dd, J = 14.1, 9.0 Hz, 1H), 1.52 (m, 1H), 1.33(m, 1H), 1.15 (m, 1H), 0.79 (d, J = 6.6 Hz, 3H) 0.76 (d, J = 6.5 Hz, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 176.4, 171.8, 143.3, 143.2, 128.6, 127.9, 127.6, 126.7, 126.6, 50.6, 47.4, 43.0, 41.0, 24.3, 22.8, 21.6.

***N*-(9-Fluoreneacetyl)-L-leucine (6e).** 9-Fluoreneacetic acid (1.45 g, 6.47 mmol) was coupled with L-leucine methyl ester hydrochloride (1.18 g, 6.47 mmol) to give *N*-(9-fluoreneacetyl)-L-leucine methyl ester in 94% yield. This was hydrolyzed to give **6e** (97%), which was recrystallized from EtOAc/hexanes/MeOH as white solid: mp 139−140 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.71 (d, J = 7.3 Hz, 2H), 7.48 (t, J = 6.8 Hz, 2H), 7.35 (m, 2H), 7.26 (m, 2H), 5.80 (d, J = 8.0 Hz, 1H), 4.60 (m, 1H), 4.43 (t, J = 6.9 Hz, 1H), 2.76 (dd, J = 15.0, 6.9 Hz, 1H), 2.68 (dd, J = 15.0, 7.0 Hz, 1H), 1.61 (m, 1H), 1.53−1.36 (m, 2H), 0.89 (d, J = 3.6 Hz, 3H), 0.87 (d, J = 3.6 Hz, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 176.4, 171.8, 145.9, 140.7, 127.5, 127.5, 127.3, 127.1, 124.4, 119.9, 50.8, 43.7, 40.8, 40.1, 24.6, 22.8, 21.5.

***N*-(3-Cyclohexylpropionyl)-L-leucyl-2-hydroxy-3(*R*,*S*)-amino-4-phenylbutanoic Acid Methyl Ester (8a).** *N*-(3-Cyclohexylpropionyl)-L-leucine (**6a**) was coupled with (*S*)-methyl 3-amino-2-hydroxy-4-phenylbutanoate hydrochloride (**7**)[10] to give **8a** as a mixture of diastereomers in 80% yield after column chromatographic purification with EtOAc/hexanes (3:7) as eluent. ^{1}H NMR ($CDCl_3$, 300 MHz) Diastereomer 1: δ 7.21 (m, 5H), 6.53 (d, J = 8.7 Hz, 0.50H), 5.73 (d, J = 8.2 Hz, 0.52H), 4.60 (m, 1H), 4.37 (m, 1H), 4.29 (m, 0.51H), 3.71 (s, 1.56H), 3.48 (d, J = 5.6 Hz, 0.51H), 2.92 (d, J = 7.9 Hz, 1H), 2.81 (d, J = 7.4 Hz, 1H), 2.14 (m, 2H), 1.54 (m, 10H), 1.23 (m, 4H), 0.87 (m, 8H). Diastereomer 2: δ 7.21 (m, 5H), 6.46 (d, J = 9.2 Hz, 0.50H), 5.64 (d, J = 8.1 Hz, 0.48H), 4.60 (m, 1H), 4.37 (m, 1H), 4.10 (m, 0.49H), 3.61 (s, 1.44H), 3.38 (d, J = 4.6 Hz, 0.49H), 2.92 (d, J = 7.9 Hz, 1H), 2.81 (d, J = 7.4 Hz, 1H), 2.14 (m, 2H), 1.54 (m, 10H), 1.23 (m, 4H), 0.87 (m, 8H).

***N*-(1-Adamantylacetyl)-L-leucyl-2-hydroxy-3(*R*,*S*)-amino-4-phenylbutanoic Acid Methyl Ester (8b).** *N*-(1-Adamantylacetyl)-L-leucine (**6b**) was coupled with **7** to give 0.95 g (63.6%) of **8b** as a mixture of diastereomers. ^{1}H NMR ($CDCl_3$, 300 MHz) Diastereomer 1: δ 7.24 (m, 5H), 6.64 (br, 1H), 5.73 (d, J = 8.4 Hz, 0.57H), 4.56 (m, 1H), 4.38 (m, 1H), 4.10 (m, 0.59H), 3.70 (s, 1.89H), 3.52 (d, J = 4.5 Hz, 0.56H), 2.88 (m, 2H), 1.91 (m, 5H), 1.54 (m, 15H), 0.92 (m, 6H). Diastereomer 2: δ 7.24 (m, 5H), 6.64 (br, 1H), 5.65 (d, J = 7.8 Hz, 0.43H), 4.56 (m, 1H), 4.38 (m, 1H), 4.28 (m, 0.41H), 3.59 (d, J = 5.7 Hz, 0.44H), 3.57 (s, 1.11H), 2.88 (m, 2H), 1.91 (m, 5H), 1.54 (m, 15H), 0.92 (m, 6H).

***N*-(2,2-Diphenylacetyl)-L-leucyl-2-hydroxy-3(*R*,*S*)-amino-4-phenylbutanoic Acid Methyl Ester (8c).** *N*-(2,2-Diphenylacetyl)-L-leucine (**6c**) (1.0 g, 3.07 mmol) was coupled with **7** (0.75 g, 3.07 mmol) to give 1.23 g (77.6%) yield of **8c** as a diastereomeric mixture after column chromatographic purification of the crude product. ^{1}H NMR ($CDCl_3$, 300 MHz) Diastereomer 1: δ 7.23 (m, 15H), 6.62 (br, 1H), 6.03 (d, J = 8.1 Hz, 0.56H), 4.89 (d, J = 4.28 Hz, 1H), 4.49 (m, 2H), 4.07 (m, 0.57H), 3.66 (s, 1.71H), 3.48 (d, J = 4.8 Hz, 0.54H), 2.81 (m, 2H), 1.41 (m, 3H), 0.84 (d, J = 6.3 Hz, 6H). Diastereomer 2: δ 7.23 (m, 15H), 6.62 (br, 1H), 5.93 (d, J = 7.8 Hz, 0.44H), 4.89 (d, J = 4.28 Hz, 1H), 4.49 (m, 2H),

4.26 (m, 0.43H), 3.59 (d, J = 5.4 Hz, 0.46H), 3.55 (s, 1.29H), 2.81 (m, 2H), 1.41 (m, 3H), 0.84 (d, J = 6.3 Hz, 6H).

***N*-(3,3-Diphenylpropionyl)-L-leucyl-2-hydroxy-3(*R*,*S*)-amino-4-phenylbutanoic Acid Methyl Ester (8d).** *N*-(3,3-Diphenylpropionyl)-L-leucine (**6d**) (1.02 g, 3 mmol) was coupled with **7** (0.737 g, 3 mmol), followed by column chromatographic purification using EtOAc/hexanes (3:7) as solvent to give 1.2 g (75.4%) of a diastereomeric mixture of **8d** as white solid. ^{1}H NMR ($CDCl_3$, 300 MHz) Diastereomer 1: δ 7.23 (m, 15H), 6.43 (d, J = 9.3 Hz, 0.64H), 5.70 (d, J = 8.4 Hz, 0.64H), 4.51 (m, 2H), 4.17 (m, 2H), 3.69 (s, 1.92H), 3.43 (d, J = 4.8 Hz, 0.59H), 2.86 (m, 4H), 1.22 (m, 3H), 0.71 (m, 6H). Diastereomer 2: δ 7.23 (m, 15H), 6.49 (d, J = 8.7 Hz, 0.36H), 5.59 (d, J = 7.8 Hz, 0.36H), 4.51 (m, 2H), 4.17 (m, 2H), 3.59 (s, 1.08H), 3.55 (d, J = 5.7 Hz, 0.41H), 2.86 (m, 4H), 1.22 (m, 3H), 0.71 (m, 6H).

***N*-(9-Fluoreneacetyl)-L-leucyl-2-hydroxy-3(*R*,*S*)-amino-4-phenylbutanoic Acid Methyl Ester (8e).** *N*-(9-Fluoreneacetyl)-L-leucine (**6e**) (1.012 g, 3 mmol) was coupled with **7** (0.736 g, 3 mmol) and the crude product was purified by column chromatography using EtOAc/hexanes (4:6) as solvent to give 1.12 g (70.7%) of **8e** as a mixture of diastereoisomers. ^{1}H NMR ($CDCl_3$, 300 MHz) Diastereomer 1: δ 7.74 (d, J = 7.2 Hz, 2H), 7.47 (d, J = 7.5 Hz, 2H), 7.27 (m, 9H), 6.53 (d, J = 9.3 Hz, 1H), 5.67 (d, J = 7.8 Hz, 0.61H), 4.48 (m, 3H), 4.12 (m, 0.61H), 3.69 (s, 1.80H), 3.56 (d, J = 4.8 Hz, 0.61H), 2.84 (m, 3H), 2.56 (m, 1H), 1.38 (m, 3H), 0.86 (d, J = 3.2 Hz, 6H). Diastereomer 2: δ 7.74 (d, J = 7.2 Hz, 2H), 7.47 (d, J = 7.5 Hz, 2H), 7.27 (m, 9H), 6.53 (d, J = 9.3 Hz, 1H), 5.52 (d, J = 8.1 Hz, 0.39H), 4.48 (m, 3H), 4.29 (m, 0.39H), 3.61 (d, J = 5.4 Hz, 0.39H) 3.59 (s, 1.20H), 2.84 (m, 3H), 2.56 (m, 1H), 1.38 (m, 3H), 0.86 (d, J = 3.2 Hz, 6H).

***μ*-Calpain Inhibition Assay.** The assay mixture consisted of 50 mM Tris HCl (pH 7.4), 50 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 0.2 mM or 1.0 mM Suc-Leu-Tyr-AMC (Calbiochem), 1 mg porcine erythrocyte μ-calpain (Calbiochem), varying concentrations of inhibitor dissolved in DMSO (2%), and 5 mM $CaCl_2$ in a final volume of 250 μL in microtiter plates. Assays were initiated by addition of $CaCl_2$, and the increase in fluorescence (λ_{ex} = 370 nm, λ_{em} = 440 nm) was monitored at ambient temperature. MDL28170 and buffer with 2% DMSO were used as controls. K_i values were determined according to Dixon's method.[14]

Docking Studies. μ-Calpain (PDB accession number 2G8J) was retrieved from the Brookhaven Protein Data Bank. The structures of the inhibitors (as the *S*,*S*-diastereomers) were constructed using Sybyl 8.0 and energetically minimized using Tripos force field with Gasteiger−Huckel charges and docked into the active site of the enzyme. The complexes of the inhibitors with μ-calpain were further minimized using the Tripos force field in Sybyl 8.0 and the Powell method for 5000 steps or until the gradient was lower than 0.01 kcal mol^{-1} Å^{-1}. All simulations were performed in a vacuum with a dielectric constant ϵ = 1 using the Tripos force field. The inhibitors were allowed to be fully flexible, while in the enzyme, only residues within 10 Å radius of the inhibitor were made fully flexible, the other residues being kept fixed.

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Supporting Information Available: Elemental analysis data for target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Suzuki, K.; Sorimachi, H.; Yoshizawa, T.; Kinbara, K.; Ishiura, S. Calpain: novel family members, activation, and physiologic function. *Biol Chem.* **1995**, *376*, 523–529.
(2) Croall, D. E.; DeMartino, G. N. Calcium-activated neutral protease (calpain) system: structure, function, and regulation. *Physiol. Rev.* **1991**, *71*, 813–847.
(3) Bano, D.; Young, K. W.; Guerin, C. J.; Lefeuvre, R.; Rothwell, N. J.; Naldini, L.; Rizzuto, R.; Carafoli, E.; Nicotera, P. Cleavage of the plasma membrane Na+/Ca^{2+} exchanger in excitotoxicity. *Cell* **2005**, *120*, 275–285.
(4) Zatz, A.; Starling, A. Calpains and disease. *N. Engl. J. Med.* **2005**, *352*, 2413–2423.
(5) Donkor, I. O. A survey of calpain inhibitors. *Curr. Med. Chem.* **2000**, *12*, 1171–1188.
(6) Neffe, A. T.; Abell, A. D. Developments in the design and synthesis of calpain inhibitors. *Curr. Opin. Drug Discovery Dev.* **2005**, *6*, 684–700.
(7) Takahashi, K. *In Intracellular Calcium-Dependent Proteolysis*; Mellgren, R. L. , Murachi, T. , Ed.; CRC Press: Boca Raton, FL, 1990; pp 55−74.
(8) Moldoveanu, T.; Hosfield, C. M.; Lim, D.; Elce, J. S.; Jia, Z.; Davies, P. L. A Ca^{2+} switch aligns the active site of calpain. *Cell* **2002**, *108*, 649–660.
(9) Cuerrier, D.; Moldoveanu, T.; Inoue, J.; Davies, P. L.; Campbell, R. L. Calpain inhibition by alpha-ketoamide and cyclic hemiacetal inhibitors revealed by X-ray crystallography. *Biochemistry* **2006**, *45*, 7446–7452.
(10) Harbeson, S. L.; Abelleira, S. M.; Akiyama, A.; Barrett, R., III.; Carroll, R. M.; Straub, J. A.; Tkacz, J. N.; Wu, C.; Musso, G. F. Stereospecific synthesis of peptidyl alpha-keto amides as inhibitors of calpain. *J. Med. Chem.* **1994**, *37*, 2918–2929.
(11) Lescop, C.; Herzner, H.; Siendt, H.; Bolliger, R.; Hennebohle, M.; Weyermann, P.; Briguet, A.; Courdier-Fruh, I.; Erb, M.; Foster, M.; Meier, T.; Magyar, J. P.; von Sprecher, A. Novel cell-penetrating alpha-keto-amide calpain inhibitors as potential treatment for muscular dystrophy. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 5176–5181.
(12) Li, Z.; Patil, G. S.; Golubski, Z. E.; Hori, H.; Tehrani, K.; Foreman, J. E.; Eveleth, D. D.; Bartus, R. T.; Powers, J. C. Peptide alpha-keto ester, alpha-keto amide, and alpha-keto acid inhibitors of calpains and other cysteine proteases. *J. Med. Chem.* **1993**, *36*, 3472–3480.
(13) Angelastro, M. R.; Marquart, A. L.; Mehdi, S.; Koehl, J. R.; Vaz, P. B.; Peet, N. P. The synthesis of ketomethylene pseudopeptide analogues of dipeptide aldehyde inhibitors of calpain. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 139–140.
(14) Dixon, M. The graphical determination of K_m and K_i. *Biochem. J.* **1972**, *129*, 197–202.

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