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

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Abstract

A series of peptidyl α -ketoacids and α -ketoesters were 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 wide spread 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 pseudo-dipeptides **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**. ¹H NMR analysis of the crude products showed **1a-e** to be diastereomerically pure. However, column chromatographic purification (silica gel) as well as

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basic hydrolysis of the ester functionality led to epimerization of the chiral center at P₁ to yield **2a-e** as pairs of diastereomers. The diastereomeric ratios of the compounds as determined by ¹H NMR spectrometry are shown in Table 1. Racemization of the compounds is consistent with previous 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 H₂₇₂.⁹ Compounds **1e** and **2e** also made hydrogen bonding interactions with Q₁₀₉, G₂₀₈, G₂₇₁, and H₂₇₂ at the active site of μ-calpain. Hydrogen bonding between the NH of the P₁-P₂ amide bond of calpain inhibitors and G₂₇₁ 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 8 hydrogen bonds that were facilitated by two water molecules (Figure 1). The carboxylate group of **2e** was juxtapositioned to the imidazole ring of H₂₇₂ 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 H₂₇₂. On the contrary, α-ketoester **1e** formed 9 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 H₂₇₂ 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 size of the *N*-terminal group on μ-calpain inhibition. Our data showed that large planar aromatic groups are preferred over non-planar 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 S₃ 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 non-planar 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 pseudo-dipeptides 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 Et₂O or EtOAc. The combined extracts were washed successively with saturated NaHCO₃ solution, 0.5N HCl, and H₂O followed by drying (MgSO₄). 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 CH₂Cl₂ and the mixture was stirred at RT for 5-6 h. Sodium thiosulfate pentahydrate (14-28 equiv.) in saturated NaHCO₃ solution was added and stirred for 10 min. The mixture was extracted with CH₂Cl₂ and the organic extract was washed with NaHCO₃ solution, water, and dried (MgSO₄). 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 (1-2hr). The MeOH was removed under vacuum, water was added, and the solution was washed with CH₂Cl₂. The aqueous layer was recovered and acidified with 2N HCl and extracted with EtOAc. The combined EtOAc extracts were washed with water and dried (MgSO₄) 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. ¹H NMR (CDCl₃, 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 + CH₃OH)⁺. Anal. (C₂₆H₃₈N₂O₅) 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. ¹H NMR (CDCl₃, 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. (C₂₉H₄₀N₂O₅) 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. ¹H NMR (CDCl₃, 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. (C₃₁H₃₄N₂O₅) 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. ¹H NMR (CDCl₃, 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. (C₃₂H₃₆N₂O₅) C, H, N.

***N*-(9-Fluoreneacetyl)-L-leucyl-2-oxo-3(*R,S*)-amino-4-phenylbutanoic acid methyl ester (1e)**

¹H NMR (CDCl₃, 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. (C₃₂H₃₄N₂O₅) C, H, N.

***N*-(3-Cyclohexylpropionyl)-L-leucyl-2-oxo-3(*R,S*)-amino-4-phenylbutanoic acid (2a)**

¹H NMR (CDCl₃, 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. (C₂₅H₃₆N₂O₅ · 0.25 H₂O) 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. ¹H NMR (CDCl₃, 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. (C₂₈H₃₈N₂O₅ · 0.25 H₂O) 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. ¹H NMR (CDCl₃, 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. (C₃₀H₃₂N₂O₅ · 0.5 H₂O) 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. ¹H NMR (CDCl₃, 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. (C₃₁H₃₄N₂O₅ · 0.5 H₂O) 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. ¹H NMR (CDCl₃, 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. (C₃₁H₃₂N₂O₅ · 0.5 H₂O) 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; ¹H NMR (CDCl₃, 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); ¹³C NMR (CD₃OD, 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 ¹H NMR (CD₃OD, 300 MHz) 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); ¹³C NMR (CD₃OD, 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 afford rod-like white solid: mp 109 °C ¹H NMR (CDCl₃, 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 ^1H 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 ^1H 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. ^1H 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. ^1H 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. ^1H 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. ^1H 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. ^1H 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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 DE, DeMartino GN. Calcium-activated neutral protease (calpain) system: structure, function, and regulation. *Physiol Rev* 1991;71:813–847. [PubMed: 2057527]
3. Bano D, Young KW, Guerin CJ, Lefevre R, Rothwell NJ, Naldini L, Rizzuto R, Carafoli E, Nicotera P. Cleavage of the plasma membrane $\text{Na}^+/\text{Ca}^{2+}$ exchanger in excitotoxicity. *Cell* 2005;120:275–285. [PubMed: 15680332]
4. Zatz A, Starling A. Calpains and disease. *N Engl J Med* 2005;352:2413–2423. [PubMed: 15944426]
5. Donkor IO. A survey of calpain inhibitors. *Curr Med Chem* 2000;12:1171–1188. [PubMed: 11032966]
6. Neffe AT, Abell AD. Developments in the design and synthesis of calpain inhibitors. *Curr Opin Drug Discov Devel* 2005;6:684–700.
7. Takahashi, K. Intracellular Calcium-Dependent Proteolysis. Mellgren, RL.; Murachi, T., editors. CRC Press; Boca Raton, FL: 1990. p. 55-74.
8. Moldoveanu T, Hosfield CM, Lim D, Elce JS, Jia Z, Davies PL. A Ca^{2+} switch aligns the active site of calpain. *Cell* 2002;108:649–660. [PubMed: 11893336]
9. Cuerrier D, Moldoveanu T, Inoue J, Davies PL, Campbell RL. Calpain inhibition by alpha-ketoamide and cyclic hemiacetal inhibitors revealed by X-ray crystallography. *Biochemistry* 2006;45:7446–7452. [PubMed: 16768440]
10. Harbeson SL, Abelleira SM, Akiyama A, Barrett R III, Carroll RM, Straub JA, Tkacz JN, Wu C, Musso GF. Stereospecific synthesis of peptidyl alpha-keto amides as inhibitors of calpain. *J Med Chem* 1994;37:2918–2929. [PubMed: 8071940]
11. Lescop C, Herzner H, Siendt H, Bolliger R, Hennebohle M, Weyermann P, Briguët A, Courdier-Fruh I, Erb M, Foster M, Meier T, Magyar JP, 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. [PubMed: 16185867]
12. Li Z, Patil GS, Golubski ZE, Hori H, Tehrani K, Foreman JE, Eveleth DD, Bartus RT, Powers JC. 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. [PubMed: 8230139]
13. Angelastro MR, Marquart AL, Mehdi S, Koehl JR, Vaz PB, Peet NP. The synthesis of ketomethylene pseudopeptide analogues of dipeptide aldehyde inhibitors of calpain. *Bioorg Med Chem Lett* 1999;9:139–140. [PubMed: 10021915]
14. Dixon M. The graphical determination of K_m and K_i . *Biochem J* 1972;129:197–202. [PubMed: 4630451]

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.

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.

Scheme 1.

Reagents: (a) EDC, HOBT, NMM, DMF; (b) 1N NaOH/CH₃OH; (c) **7**, EDC, HOBT, NMM, DMF; (d) Dess-Martin Reagent/CH₂Cl₂.

Table 1Structure, 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

Compd.	R ¹	R ²	K _i (nM) ^b	DR (S,S:R,S) ^c
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 versus I (inhibitor concentration) to give intersecting lines with correlation coefficient ≥ 0.95 .

^c DR = Diastereomeric Ratio of the compounds as determined by ¹H NMR spectrometry in CDCl₃.

^d MDL28170 (Calbiochem).