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Synthesis and calpain inhibitory activity of peptidomimetic compounds with constrained amino acids at the P_2 position

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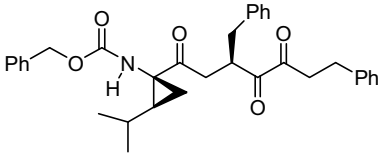
ABSTRACT

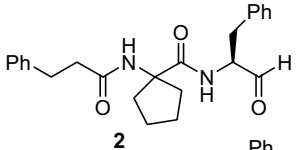
The effect of incorporating α,α′-diethylglycine and α-aminocyclopentane carboxylic acid at the P_2 position of inhibitors on μ-calpain inhibition was studied. Compound **3** with α,α′-diethylglycine was over 20-fold more potent than **2** with α-aminocyclopentane carboxylic acid. Additionally, **3** was over 35-fold selective for μ-calpain compared to cathepsin B, while **2** was 3-fold selective for cathepsin B compared to μ-calpain. Thus, the conformation induced by the P_2 residue influenced the activities of the compounds versus the closely related cysteine proteases, and suggests an approach to the discovery of selective μ-calpain inhibitors.

Calpain is a papain-like cytosolic cysteine protease that requires calcium ions for activation.[1] Several calpain isoforms have been reported of which μ-calpain and m-calpain are the most abundantly distributed in mammalian cells, and have been termed ubiquitous calpains.[1–3] Calpain is a promiscuous enzyme that catalyzes the limited proteolysis of a broad range of substrates in vivo.[2,3] The physiological role of the enzyme is evolving, and it has been shown to participate in signal transduction pathways.[4–7] Calpain has attracted considerable interest due in part to implication of the enzyme in a variety of pathological conditions including neurological disorders (e.g., stroke and Alzheimer's disease), cataract, and cancer.[8–13] This has led to the search for selective calpain inhibitors as potential therapeutic agents and as biochemical probes. Several compounds are known to inhibit the ubiquitous calpains. However, most of the inhibitors are not specific for the calpains because they also inhibit the closely related cysteine cathepsins (e.g., cathepsin B).[14,15] Hence there is a continuing need for new calpain inhibitors with improved selectivity for the enzyme.

Incorporation of constrained amino acids such as α,α′-dialkylglycines and α-aminocycloalkane carboxylic acids into a peptide restricts the conformational freedom of the peptide in the vicinity of the constrained amino acid.[16] This allows one to study the effect of local conformational constraints on bioactivity because peptides containing α,α′-dialkylglycine residues generally adopt fully extended conformation while those with α-aminocycloalkane carboxylic acids prefer a folded conformation.[16] We demonstrated in a previous study that incorporation of 2,3-methanoleucine stereoisomers at the P_2 position of peptidyl inhibitors influences μ-calpain inhibition. Compound **1** with a 2,3-methanoleucine stereoisomer of *E*-(2*S*,3*S*) configuration at the P_2 position (Fig. 1) was the most potent and selective member of the series albeit



1, *E*-(2S,3S)-Methanoleucine isomer



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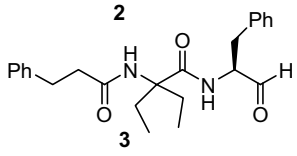

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Figure 1. Structures of calpain inhibitors with constrained amino acids.

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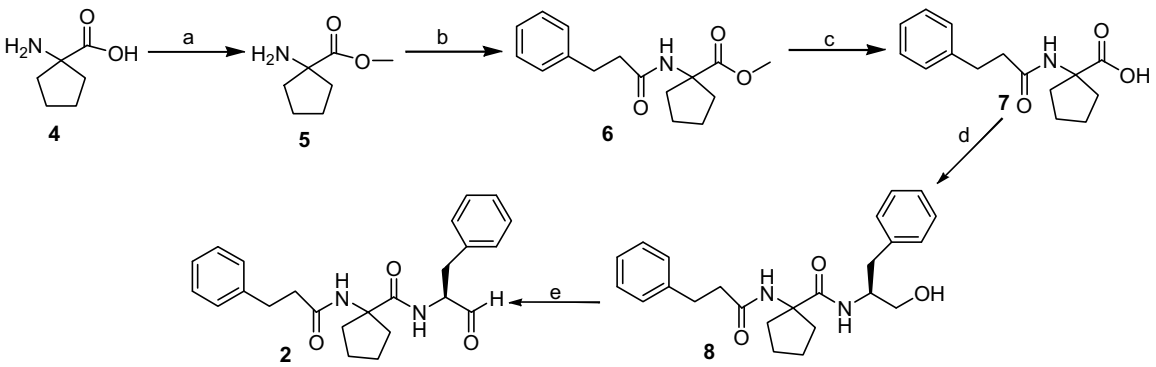
modest selectivity for μ-calpain versus cathepsin B.[17] This study showed that the S_2-subsite of μ-calpain is more stereosensitive than that of cathepsin B, which suggested that compounds that adopt different conformations would exhibit differential affinities for μ-calpain and cathepsin B. To further probe the active sites of these closely related cysteine proteases with the goal of generating selective μ-calpain inhibitors we studied the effect of incorporating α-aminocyclopentane carboxylic acid (as in **2**, Fig. 1) and α,α′-diethylglycine (as in **3**) at the P_2 position on inhibition of μ-calpain and cathepsin B.

Compounds **2** and **3** were synthesized using general coupling procedures as outlined in Schemes 1 and 2. 1-Amino-1-cyclopentane carboxylic acid **4** (Scheme 1) or diethylglycine **9** (Scheme 2) was refluxed in $SOCl_2$ and MeOH for 24 h to give esters **5** and**10**, respectively. The esters were coupled with 3-phenyl propionic acid, using EDC, NMM, and HOBT, to give the dipeptide esters **6** and **11**, which were hydrolyzed using 2 N NaOH to give the corresponding dipeptide acids **7** and **12**. Compound **7** was coupled with L-phenylalaninol in the presence of EDC, NMM, and HOBT to give tripeptide alcohol **8**. Compound **12** (Scheme 2) formed the cyclic intermediate **13** in the presence of EDC. This was coupled with L-phenylalaninol in the presence of EDC, NMM, and HOBT to give the tripeptide alcohol **14**. The tripeptide alcohols **8** and **14** were oxidized using Dess–Martin periodinane reagent to afford the target compounds **2** (Scheme 1) and **3** (Scheme 2), respectively. The final products were purified by flash column chromatography and/or recrystallization from hexanes/CH_2Cl_2 (1:1).[18] The compounds were evaluated[19] as inhibitors of porcine erythrocyte μ-calpain (Calbiochem) and human liver cathepsin B (Calbiochem) using ALLN as positive control. Table 1 shows the results of the study. The nature of the P_2 residue influenced the inhibitory activity of the compounds. Compound **3** with α,α′-diethylglycine at the P_2 position inhibited μ-calpain with K_i of 0.08 μM. It was over 20-fold better inhibitor of μ-calpain compared to **2** with α-aminocyclopentane carboxylic acid as the P_2 residue. Furthermore, **3** was over 35-fold selective for μ-calpain versus cathepsin B. Compound **2** on the contrary inhibited cathepsin B 3-fold better than **3**. Clearly, our data suggest that compounds that adopt extended conformation are better inhibitors of μ-calpain compared to those with folded conformation. This is consistent with recent X-ray crystallographic studies of the complexes of μ-calpain with bound inhibitors, which showed that the inhibitors occupy the active site pocket of the enzyme in extended conformation.[20–22] Thus, unlike μ-calpain, cathepsin B did not display significant preference for any of the two compounds suggesting that the enzyme is not very sensitive to the conformational differences induced by the constrained amino acids at the P_2 position of the inhibitors. This is consistent with our previous finding using compounds with 2,3-methanoleucine stereoisomers as the P_2 residue, which suggested

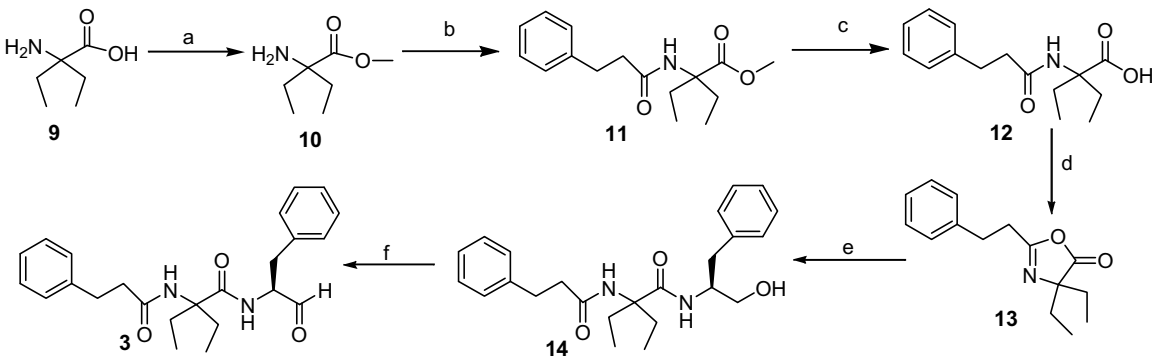
Table 1
Inhibition of porcine erythrocyte μ-calpain and human liver cathepsin B by compounds **2** and **3**

Compound	μ-Calpain K_i[a] (μM)	Cathepsin B K_i (μM)	SR[b]
2	1.94 ± 0.81	0.88 ± 0.01	0.45
3	0.08 ± 0.01	2.91 ± 0.62	36.37
ALLN[c]	0.19 ± 0.02	0.15 ± 0.01	0.79

[a] K_i values are means of triplicate determinations obtained by Dixon plots with correlation coefficient of ⩾0.95.
[b] SR is selectivity ratio, which was determined by dividing the K_i value for cathepsin B inhibition by that for calpain inhibition.
[c] ALLN was purchased from Calbiochem.



Scheme 1. Reagents: (a) $SOCl_2$, MeOH; (b) 3-phenylpropionic acid, EDC, NMM, HOBT; (c) 2 N NaOH, MeOH; (d) L-phenylalaninol, EDC, NMM, HOBT; (e) Dess–Martin reagent.



Scheme 2. Reagents: (a) $SOCl_2$, MeOH; (b) 3-phenylpropionic acid, EDC, NMM, HOBT; (c) 2 N NaOH, MeOH; (d) EDC, DMF; (e) L-phenylalaninol, EDC, NMM, HOBT; (f) Dess–Martin reagent.

that the S_2-subsite of cathepsin B is not as stereosensitive as that of μ-calpain.[17]

In summary, we have demonstrated using constrained amino acids as the P_2 residue that peptidomimetic compounds that adopt extended conformation are potentially potent and selective inhibitors of μ-calpain versus cathepsin B compared to related analogues that adopt folded conformation.

Acknowledgment

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18. *General coupling method*. A solution of the carboxylic acid (1 equiv) in CH_2Cl_2 was cooled in an ice-bath and EDC (1.05 equiv), HOBT (1.05 equiv), NMM (3 equiv), and the appropriate amine (1 equiv) were added consecutively. The reaction mixture was stirred overnight at room temperature. Water was added to the reaction mixture and extracted with CH_2Cl_2. The combined organic extracts were washed successively with saturated $NaHCO_3$solution (30 mL), 0.5 N HCl (30 mL), and then water (30 mL), and dried over Na_2SO_4. Evaporation of the solvent followed by column chromatographic purification afforded the desired amide.
General oxidation method. Dess–Martin periodinane reagent (1.05 equiv) was added to a solution of the appropriate alcohol (1 equiv) in CH_2Cl_2 (20 mL), and the reaction mixture was stirred for 2 h at room temperature. $Na_2S_2O_3{\cdot}5\ H_2O$ (14–28 equiv) in saturated $NaHCO_3$ solution was added and stirred for additional 10 min. The mixture was extracted with CH_2Cl_2 (3 × 30 mL). The combined CH_2Cl_2 extracts were washed with 0.5 N HCl (30 mL), water (30 mL), and dried over Na_2SO_4. The solvent was evaporated and crude product was purified by column chromatography.
1-(3-Phenyl-propionylamino)-cyclopentane carboxylic acid (1-benzyl-2-oxo-ethyl)-amide (**2**). Compound **8** was transformed to **2** as described under the general method for oxidation. The product was obtained as a white crystalline solid (0.5 g, 77%). Mp 148–150 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.57 (s, 1H), 7.29 (m, 11H), 5.46 (s, 1H), 4.55 (q, J = 9 Hz, 1H), 3.10 (m, 2H), 2.93 (t, J = 9 Hz, 2H), 2.63 (t, J = 7.5 Hz, 2H), 2.16 (m, 2H), 1.79 (m, 2H), 1.66 (m, 2H), 1.53 (m, 2H). Anal. ($C_{24}H_{28}N_2O_3$), C, H, N. Calcd: C 73.44, H 7.19, N 7.14. Found: C 73.21, H 7.24, N 7.04. ESI MS: m/z 447 $(M + Na + CH_3OH)^+$.
N-(1-Benzyl-2-oxo-ethyl)-2-ethyl-2-(3-phenyl-propionylamino)-butyramide (**3**). Compound **14** was transformed to **3** as described under the general method for oxidation. The product was obtained as a white crystalline solid (0.3 mg, 98.3%). Mp 98–100 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.62 (s, 1 H), 7.26 (m, 10H), 6.48 (s, 1H), 6.25 (d, J = 6.6 Hz, 1H), 4.74 (q, J = 6.9 Hz, 1H), 3.12 (d, J = 7.2 Hz, 2H), 2.94 (t, J = 7.5 Hz, 2H), 2.48 (m, 4H), 1.48 (m, 1H), 1.38 (m, 1H), 0.644 (t, J = 7.2 Hz, 3H), 0.54 (t,J = 7.2 Hz, 3H). Anal. ($C_{24}H_{30}N_2O_3$), C, H, N. Calcd: C 73.07, H 7.66, N 7.10. Found: C 72.99, H 7.75, N 7.10. ESI MS: m/z 449 $(M + Na + CH_3OH)^+$.
1-Amino-cyclopentane carboxylic acid methyl ester (**5**). $SOCl_2$ (1 mL) was added dropwise to a solution of 1-amino-1-cyclopentane carboxylic acid (0.4 g, 3.1 mmol) in methanol (10 mL) at 0 °C, and the mixture was refluxed for 3 h. It was cooled and evaporated in vacuo to give the product as a white solid (0.52 g, 93.5%). Mp 203–205 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.98 (s, 2H), 3.84 (s, 3H), 2.29 (m, 4H), 2.13 (m, 2H), 1.89 (m, 2H).
1-(3-Phenyl-propionylamino)-cyclopentane carboxylic acid methyl ester (**6**). Compound **6** was obtained from **5** and commercially available 3-phenyl propionic acid using the general coupling procedure. The product was obtained as a white solid (0.76 g, 99.4%). Mp 65–67 °C ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.25 (m, 5H), 5.86 (s, 1H), 3.70 (s, 3H), 2.93 (t, J = 9 Hz, 2H), 2.49 (t, J = 9 Hz, 2H), 2.20 (m, 2H), 1.87 (m, 2H), 1.72 (m, 4H).
1-(3-Phenyl-propionylamino)-cyclopentane carboxylic acid (**7**). NaOH (2 N, 15 mL) was added to a solution of **6** (0.66 g, 3.67 mmol) in MeOH (15 mL), and the mixture was stirred for 6 h at room temperature. The reaction mixture was cooled to 0 °C, ethyl acetate (50 mL) was added and it was acidified with 5% HCl (pH 4.5). The mixture was extracted with EtOAc (2 × 30 mL). The combined organic extracts were washed with brine (25 mL), dried with Na_2SO_4, and evaporated to give **7** as a white solid (0.53 g, 55.3%). Mp 170–172 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.18 (s, 1H), 7.21 (m, 5H), 2.89 (t, J = 8 Hz, 2H), 2.47 (t, J = 7.2 Hz, 2H), 2.13 (m, 2H), 1.88 (m, 2H), 1.67 (m, 4H).
1-(3-Phenyl-propionylamino)-cyclopentane carboxylic acid (1-benzyl-2-hydroxy-ethyl)-amide (**8**). Compound **8** was obtained from **7** and commercially available L-phenylalaninol using the general coupling procedure. The product was obtained as a white solid (0.69 g, 91.3%). Mp 50–52 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.24 (m, 10H), 6.64 (s, 1H), 5.84 (s, 1H), 3.63 (m, 1H), 3.45 (m, 1H), 3.05 (m, 1H), 2.96 (m, 3H), 2.83 (m, 2H), 2.5 (m, 2H), 2.25 (m, 1H), 1.94 (m, 1H), 1.71 (m, 4H), 1.55 (m, 2H).
2-Amino-2-ethylbutyric acid methyl ester (**10**). $SOCl_2$ (10 mL) was added dropwise to a solution of diethylglycine (0.7 g, 5.3 mmol) in methanol (30 mL) at 0 °C, and the mixture was refluxed for 24 h followed by solvent removal in vacuo. Further rinsing with $MeOH/CH_2Cl_2$ (2 × 30 mL) and evaporation afforded 10 as a semi-viscous yellowish green oil (0.92 g, 95.6%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.5 (s, 2H), 3.77 (s, 3H), 1.83 (q, J = 6 Hz, 4H), 0.877 (t, J = 7.2 Hz, 6H).
2-Ethyl-2-(3-phenyl-propionylamino)-butyric acid methyl ester (**11**). Compound **11** was obtained from **10** and commercially available 3-phenyl propionic acid using the general coupling procedure. The product was obtained as viscous oil (0.17 mg, 94.4%). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.25 (m, 5H), 6.32 (s, 1H), 3.74 (s, 3H), 2.96 (t, J = 6.2 Hz, 2H), 2.54 (t, J = 6.2 Hz, 2H), 2.45 (m, 2H), 1.75 (m, 2H), 0.64 (t, J = 7 Hz, 6H).
2-Ethyl-2-(3-phenyl-propionylamino)-butyric acid (**12**). NaOH (2 N, 50 mL) was added to a solution of **11** (0.8 g, 2.88 mmol) in MeOH (35 mL), and the mixture was stirred for 36 h at room temperature. EtOAc (50 mL) was added to the reaction mixture at 0 °C and then acidified with 5% HCl (pH 4.5). The mixture was extracted with EtOAc (2 × 50 mL), and the combined organic extracts were washed with brine, dried with Na_2SO_4 and evaporated to give **12** as a white powdery solid (0.66 g, 87%). Mp 200–202 °C. ^{1}H NMR (DMSO, 300 MHz): δ 7.49 (s, 1H), 7.21 (m, 5H), 2.78 (t, J = 9 Hz, 2H), 2.43 (t, J = 9 Hz, 2H), 1.80 (s, 1H), 1.78 (m, 4H), 0.62 (t, J = 6 Hz, 6H).
4,4-Diethyl-2-phenethyl-4H-oxazol-5-one (**13**). EDC (0.087 g, 0.46 mmol) was added to a solution of **12** (0.8 g, 2.88 mmol) in CH_2Cl_2/DMF (10:2) at 0 °C, and the mixture was stirred for 24 h at room temperature. The reaction mixture was diluted with CH_2Cl_2 (60 mL), washed with saturated $NaHCO_3$, 0.5 N HCl, and water successively, and dried with Na_2SO_4. Evaporation of the solvent gave **13** as colorless oil (0.92 g, 98.69%). Mp 180–182 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.27 (m, 5H), 3.05 (m, 2H), 2.87 (m, 2H), 1.76 (q, J = 6 Hz, 4H), 0.71 (t, J = 6 Hz, 6H).
N-(1-Benzyl-2-hydroxy-ethyl)-2-ethyl-2-(3-phenyl-propionylamino)-butyramide (**14**). Compound **14** was obtained from **13** and commercially available L-phenylalaninol using the general coupling procedure. The product was obtained as a white crystalline solid (0.6 mg, 94.6%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.28 (m, 10H), 6.06 (s, 1H), 6.09 (d, J = 7.2 Hz, 1H), 4.25 (m, 1H), 3.65 (m, 2H), 3.15 (s, 1H), 2.95 (m,3H), 2.81 (m, 1H), 2.53 (t, J = 6 Hz, 2H), 2.33 (m, 2H), 1.53 (m, 1H), 1.38 (m, 1H), 0.63 (t, J = 6 Hz, 3H), 0.45 (t, J = 6 Hz, 3H).
19. *Calpain inhibition assay*. The K_i values for inhibition of porcine erythrocyte μ-calpain (Calbiochem) activity was monitored as previously reported[23] in a buffer consisting 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), 2 μg of calpain from porcine erythrocyte (Calbiochem), varying concentrations of the inhibitor in DMSO (2% total concentration), and 5 mM $CaCl_2$ in a final volume of 250 μL in a microtiter plate. *Cathepsin B inhibition assay*. The K_i values for inhibition of human liver cathepsin B (Calbiochem) activity was determined as described for calpain using a reaction mixture containing 1 nM human liver cathepsin B (Calbiochem), 50 mM NaOAc (pH 6.0), 1 mM EDTA, 0.5 mM DTT, 50 μM or 250 μM of substrate (Z-Arg-Arg-AMC), and varying concentrations of inhibitor in DMSO (2% total concentration) in a final volume of 250 μL.
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