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A novel series of urea-based peptidomimetic calpain inhibitors

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Abstract—A series of peptide aldehyde derivatives in which the P_2 chiral carbon has been replaced with nitrogen were synthesized as urea-based peptidomimetic inhibitors of μ-calpain. The compounds mirrored the general SAR of peptidyl aldehyde calpain inhibitors but displayed greater selectivity for μ-calpain over cathepsin B.

Calpain is of considerable interest because of its implication in numerous physiological[1,2] and pathological events.[1,3–6] This has led to the search for calpain inhibitors as pharmacological agents for modulating calpain action.[7,8] For example, calpain inhibitors are of interest as potential therapy for cardiac ischemia,[1,9,10] cerebral ischemia,[1,8,9,11] cancer,[12,13] and cataracts.[14,15] Most calpain inhibitors are peptide substrate analogues in which the scissile amide bond of the substrate has been replaced with an electron-deficient center (e.g., an aldehyde) for covalent interaction with the catalytic site cysteine of calpain.[7,8,16] The inhibitors generally display poor selectivity for calpain.[7] To overcome this problem, several groups have embarked on the search for peptidomimetic inhibitors of calpain.[7,8] As part of this effort, we herein report the synthesis, μ-calpain inhibitory activity, and selectivity of a series of urea-based peptidomimetic compounds **1**–**12** (Table 1). Our objective was to study the effect on μ-calpain inhibition and selectivity when the geometry of the α-carbon of the P_2 amino acid residue of a peptidyl calpain inhibitor is changed from chiral and tetrahedral to achiral and trigonal planar via replacement of the P_2 chiral carbon with nitrogen (Fig. 1). The R^1–R^3 substituents were also varied to study the general SAR of the urea-based compounds.

Compound **1** was synthesized by transformation of Boc-L-phenylalanine **15** to Weinreb amide **16** (89%) (Scheme 1). Deprotection of **16** followed by reacting with isopropyl isocyanate[17] afforded urea **17** (94%), which was reduced with LAH to give urea-based peptidomimetic aldehyde **1** (63%). Compounds **2**–**12** were synthesized as outlined in Scheme 2. Reductive amination of the appropriate aldehyde and the corresponding amine with $NaBH_3CN$ at rt gave secondary amine **20** (41–63%). The secondary amine that was used for the synthesis of **5** was obtained by refluxing a mixture of excess isopropyl amine and 1-chloro-4-phenylbutane in anhydrous EtOH for 48 h.[18] The secondary amines were purified by column chromatography and reacted with the appropriate methyl ester isocyanate **21** to afford urea methyl ester **22** (83–96%).[17] Following purification via column chromatography, the methyl ester functional group was hydrolyzed (2 N NaOH) to give acid **23** (82–99%), which was transformed to Weinreb amide **24** (43–68%) followed by LAH reduction to give urea-based peptidomimetic aldehydes **2**–**12** (74–96%) in over 95% *ee*.

Earlier SAR studies of calpain inhibitors suggested that for potent inhibition of calpain the P_2 residue of the inhibitor must be either L-valine or L-leucine. However, recent studies from our laboratory[19] and those of others[20,21] have demonstrated that peptidyl aldehydes with D-amino acids at the P_2-position are effective inhibitors of calpain. To further study the significance of the geometry of the P_2 residue on the potency and selectivity of peptidyl calpain inhibitors, we replaced the P_2 α-carbon with a nitrogen atom thus changing the geometry of the P_2-position from chiral and tetrahedral to achiral and trigonal planar to afford compounds **1**–**12**. The compounds were studied as inhibitors of porcine erythrocyte μ-calpain (Calbiochem) and human liver cathepsin B

Keywords: Calpain inhibitors; Peptidomimetic; Urea-based calpain inhibitors; Peptidyl aldehyde inhibitors.
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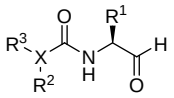
Table 1. μ-Calpain inhibitory activity of urea-based compounds **1**–**12** and peptidyl aldehyde calpain inhibitors **13** and **14**



Compound	X	R^1	R^2	R^3	μ-Calpain[a], K_i[b] (μM)	Cathepsin B[c], K_i[b] (μM)	SR[d]
1	N	Bn	*i*-Pr	H	13% inh.[e]		
2	N	Bn	*i*-Pr	$PhCH_2$	6.82		
3	N	Bn	*i*-Pr	$Ph(CH_2)_2$	2.35		
4	N	Bn	*i*-Pr	$Ph(CH_2)_3$	1.16		
5	N	Bn	*i*-Pr	$Ph(CH_2)_4$	0.40		
6	N	Bn	*i*-Pr	$PhCH_2O(CH_2)_2$	0.23	22.1	96.1
7	N	Bn	Me	$PhCH_2O(CH_2)_2$	6.69		
8	N	Bn	Et	$PhCH_2O(CH_2)_2$	1.15		
9	N	Bn	*i*-Bu	$PhCH_2O(CH_2)_2$	0.15	33.3	222
10	N	Bn	Bn	$PhCH_2O(CH_2)_2$	6.65		
11	N	H	*i*-Bu	$PhCH_2O(CH_2)_2$	88.7		
12	N	*i*-Pr	*i*-Bu	$PhCH_2O(CH_2)_2$	9.27		
13[f]	CH	Bn	*i*-Bu	$PhCH_2OCO$	0.015	0.12	8
14[f,g]	CH	Bn	*i*-Pr	$PhCH_2OCO$	0.01	0.02	2

[a] μ-Calpain is porcine erythrocyte calpain (Calbiochem).
[b] The K_i values are the average of duplicate determinations.
[c] Cathepsin B is from human liver.
[d] SR = selectivity ratio, which was determined by dividing the K_i value for cathepsin B inhibition by the K_i value for μ-calpain inhibition.
[e] 100 μM of **1** inhibited μ-calpain activity by 13%.
[f] Peptidyl calpain inhibitors with L-amino acid residues at the P_2-position.
[g] MDL28170 (calpain inhibitor III) was purchased from Calbiochem.

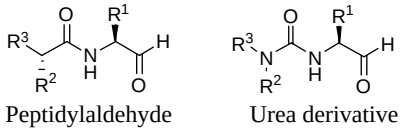
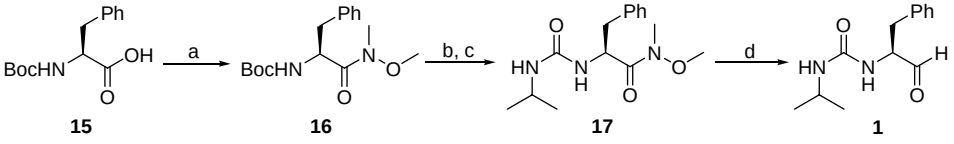
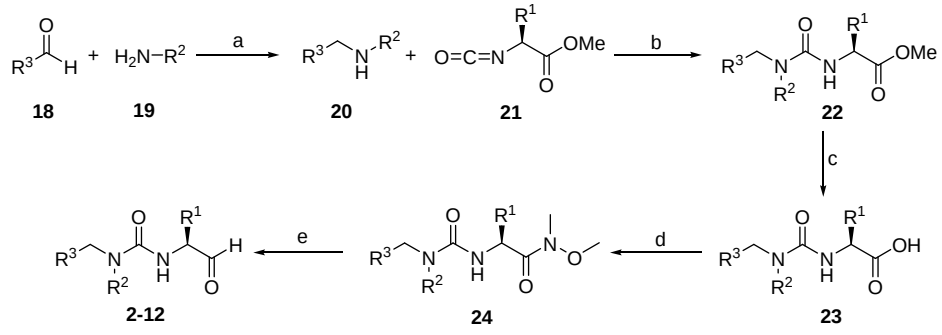


Figure 1. General structures of peptidyl aldehyde calpain inhibitors and the proposed urea derivatives.

(Calbiochem). Assays using synthetic fluorogenic substrates were employed to determine the K_i values for inhibition of the enzymes.[22,23] The results of the study are shown in Table 1. Compound **1**, which lacks an R^3 substituent for interaction with the S_3 subsite of μ-calpain, was a poor inhibitor of the enzyme. Introduction of an R^3 benzyl group (as in **2**) enhanced calpain



Scheme 1. Synthesis of compound **1**. Reagents: (a) EDC, $HCl{\cdot}NH(OCH_3)CH_3$, TEA, CH_2Cl_2; (b) TFA, CH_2Cl_2; (c) TEA, isopropyl isocyanate, toluene; (d) LAH, THF.



Scheme 2. Synthesis of compounds **2**–**12**. Reagents and condition: (a) MeOH, $NaBH_3CN$; (b) TEA, toluene, 30 °C; (c) 2 N NaOH, MeOH; (d) CDI, $HCl{\cdot}NH(OCH_3)CH_3$, DIEA, THF; (e) LAH, THF.

inhibition. Increasing the alkyl chain to four methylene groups (as in **5**) further increased μ-calpain inhibition. We attribute the increase in potency with increased chain length to favorable interaction with the S_3 subsite of μ-calpain. Introduction of a heteroatom into the alkyl chain appeared to enhance potency (**5** vs. **6**) presumably due to hydrogen bonding in the S_3 pocket of the enzyme and/or a change in the conformation of the molecule. A benzyl group was preferred as the R^1 substituent (e.g., **9** > **12** > **11**), while the *iso*-butyl group was the preferred R^2 substituent (e.g., **9** > **6** > **8** > **7**). Thus, the SAR of the urea-based calpain inhibitors mirrored that of peptidyl aldehyde calpain inhibitors.[7,8,16] However, the urea-based inhibitors were generally less potent than the corresponding peptide-based analogues. For example, **9** was 10-fold less potent than the closely related peptidyl aldehyde inhibitor **13**. The decrease in potency is consistent with the observation that peptide substrates of calpain that bind to the enzyme with L-Leu residue occupying the S_2 subsite are efficiently cleaved by the enzyme[24–26] and that peptide aldehydes with P_2 L-Leu residues are potent inhibitors of calpain.[25–28]

Most peptide calpain inhibitors lack selectivity for the enzyme because they equally inhibit other cysteine proteases such as the cathepsins. Only a few active site-directed calpain inhibitors with good selectivity for the enzyme compared to the closely related cathepsins are known.[7] We therefore tested compounds **6** and **9**, which were the most potent members of the series against human liver cathepsin B to determine if changing the geometry at the P_2-position from chiral/tetrahedral to achiral/trigonal planar will favor binding to μ-calpain over cathepsin B. As shown in Table 1, compounds **6** and **9** were markedly selective for μ-calpain over cathepsin B. Compound **9**, which was the most potent member of the series, was also the most selective inhibitor. It was over 220-fold selective for μ-calpain compared to cathepsin B, while the equivalent peptidyl aldehyde inhibitor **13** was only 8-fold selective for μ-calpain over cathepsin B. Thus, despite the apparent 10-fold decrease in μ-calpain inhibitory potency of the urea-based compounds compared to their peptide-based analogues, the urea backbone appears to be a good scaffold for the discovery of active site-directed μ-calpain inhibitors with enhanced selectivity for the enzyme.

In summary, our results suggest that transformation of the geometry of the α-carbon of the P_2 amino acid residue of peptide aldehyde calpain inhibitors from chiral/tetrahedral to achiral/trigonal planar by replacement of the P_2 chiral carbon with nitrogen maintains the general SAR of peptide aldehyde calpain inhibitors. The change in geometry at the P_2-position also improves the selectivity of the inhibitors for μ-calpain over cathepsin B albeit a 10-fold decrease in μ-calpain inhibitory potency. We are currently in the process of synthesizing active site-directed μ-calpain inhibitors that incorporate the urea scaffold with the objective of improving μ-calpain inhibitory potency, while retaining the high selectivity of the inhibitors for the enzyme.

Acknowledgments

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22. *Calpain assay:* μ-Calpain activity was monitored in a reaction mixture containing 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 porcine erythrocyte μ-calpain (Calbiochem), varying concentrations of inhibitor dissolved in DMSO (2% total concentration), and 5 mM $CaCl_2$ in a final volume of 250 μL in a polystyrene microtiter plate. 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 using a SPECTRAmax Gemini fluorescence plate reader (Molecular Devices). The K_i values were estimated from the semi-reciprocal

plot of the initial velocity versus the concentration of the inhibitor according to the method of Dixon.[29] The correlation coefficients for the Dixon plots were above 0.95. No other attempt was made to correct for slow binding or autolysis. The reported K_i values are the average of duplicate determinations.

23. *Cathepsin B assay:* K_i values for inhibition of human liver cathepsin B were determined as described for calpain using a reaction mixture containing 14 ng human liver cathepsin B (Calbiochem), 20 mM NaOAc (pH 6.0), 1 mM EDTA, 0.5 mM DTT, 50 μM or 250 μM Z-Arg-Arg-AMC, and varying concentrations of inhibitor dissolved in DMSO (2% total concentration) in a final volume of 200 μL.
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