Pergamon

Bioorganic & Medicinal Chemistry Letters 10 (2000) 2497–2500

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Synthesis and Calpain Inhibitory Activity of α-Ketoamides with 2,3-Methanoleucine Stereoisomers at the P_2 Position

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Received 3 August 2000; accepted 28 August 2000

Abstract—A series of novel ketoamides incorporating all four 2,3-methanoleucine stereoisomers at the P_2 position was synthesized. The compounds displayed a wide variation in K_i values for inhibition of calpain I depending on the configuration of the P_2 methanoleucine residue. However, similar variation in cathepsin B inhibition was not observed suggesting that the S_2 pocket of calpain I is more stereosensitive than that of cathepsin B. © 2000 Published by Elsevier Science Ltd.

Introduction

Calpain is a cytosolic calcium-activated neutral protease that belongs to the papain superfamily of cysteine proteases.[1] Two major isoforms of calpain (calpains I and II), which differ in their calcium sensitivity under in vitro conditions, are known.[2] Excessive activation of intracellular calpain occurs as a result of increased intracellular calcium concentrations associated with ischemic events.[3] Calpain activation under ischemic conditions may result in degradation of the structural proteins. The enzyme has therefore been implicated in a number of pathological conditions including neurological disorders (e.g., stroke), cataract, cardiac ischemia, and thrombotic platelet aggregation.[1,3–5] The potential involvement of calpain in a variety of disease states has fueled the search for selective cell permeable calpain inhibitors as biomedical tools for studying the cellular role of calpain and as potential therapeutic agents for treating conditions such stroke where considerable evidence links overactivation of calpain to cellular damage.[6,7] Calpain inhibitors may be broadly grouped into active site directed inhibitors (or domain II binding inhibitors) and allosteric inhibitors (or domain IV binding inhibitors). Several calpain inhibitors that inactivate the enzyme by binding to the active site have been reported.[8] However, several of these inhibitors are not selective for calpain since they also inhibit other cysteine proteases such as cathepsin B and cathepsin L. Our interest in developing novel active site directed selective inhibitors of calpain led us to study the effect of incorporating 2,3-methanoleucine units at the P_2 position of calpain inhibitors on the potency and selectivity of the inhibitors. 2,3-Methanoamino acids are structurally constrained and have only two possible side-chain rotatory angles, $\chi = 0°$ and $\chi = 120°$ corresponding to the *Z*- and *E*-configurations, respectively. Incorporation of such amino acids into a peptide effectively constrains the proximal conformation of the peptide and also makes the peptide resistant to proteolytic cleavage. In this report we describe the synthesis and calpain I inhibitory activity of α-ketoamides (**1**–**4**) incorporating 2,3-methanoleucine stereoisomers (**6**–**9**) at the P_2 position of the inhibitors.

Synthesis of the P_2-modified analogues **1**–**4** required all four 2,3-methanoleucine stereoisomers **6**–**9** (Fig. 1). Others and us have previously reported the asymmetric synthesis and stereochemical purity of the stereoisomers.[9,10] Compounds **1**–**5** were synthesized as outlined in Scheme 1. Boc-protected phenylalanine **10** was coupled with *O*,*N*-dimethylhydroxylamine hydrochloride using ethylcarbodiimide (EDC) as the coupling agent in the presence of *N*-methyl morpholine (NMM) to give Weinreb amide **11**, which was reduced with LAH to give Boc-protected amino aldehyde **12** as previously reported.[11] Aldehyde **12** was transformed to cyanohydrin **13**, the nitrile group of which was hydrolyzed to give α-hydroxy-β-amino acid **14**. Boc protection of the amino group of **14** followed by coupling with phenethylamine and subsequent deprotection gave α-hydroxy-β-amino amide **17**. Coupling of **17** with either

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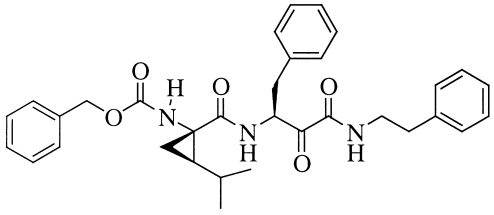
0960-894X/00/$ - see front matter © 2000 Published by Elsevier Science Ltd.
PII: S0960-894X(00)00518-7

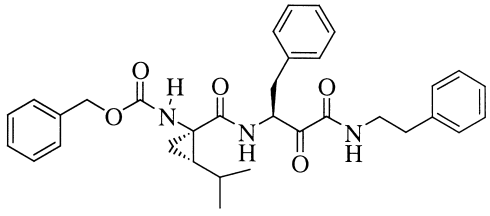
Cbz-protected L-leucine or Cbz-protected 2,3-methanoleucine derivatives **6**–**9** followed by Dess–Martin oxidation of the secondary alcohol gave the corresponding α-ketoamides **1**–**5**.[12]

Results and Discussion

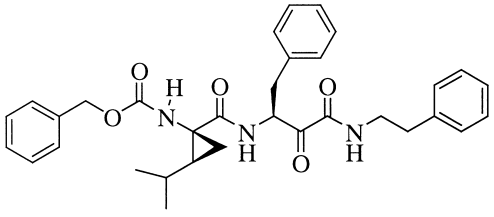
The compounds were evaluated as inhibitors of calpain I from porcine erythrocytes and cathepsin B from human liver.[13] Table 1 shows the results of the study. Compound **5** was previously reported by Li et al.[14] as a potent α-ketoamide inhibitor of calpain I ($K_i = 0.052\,\mu M$). In our assay system **5** inhibited calpain I with a K_i value of 0.07 μM. The configuration of the 2,3-methanoleucine stereoisomers had pronounced effects on inhibition of calpain I compared to cathepsin B. By analyses of Dixon plots,[15] the inhibition was found to be competitive with K_i values for inhibition of calpain I ranging from 0.75 to 40 μM. The K_i values for inhibition of cathepsin B on the other hand did not show a similar wide variation ($K_i = 1.26$–$6.78\,\mu M$) suggesting that the S_2 pocket of cathepsin B did not discriminate between the stereoisomers. Additionally, the *E*-isomers (**3** and **4**) were better inhibitors of calpain I than the *Z*-isomers (**1** and **2**). Indeed, **3** with an *E*-(2*S*,3*S*) configuration at the P_2-position was over 50-fold a better inhibitor of calpain I than **2** with a P_2 *Z*-(2*R*,3*S*) configuration. On the contrary, with respect to cathepsin B inhibition, no such dramatic configurational



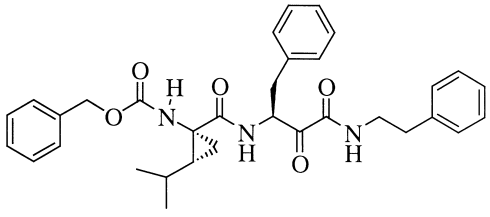
Z-(2*S*,3*R*)-**1**



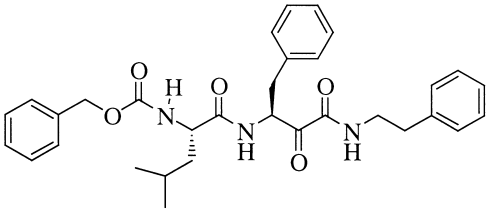
Z-(2*R*,3*S*)-**2**



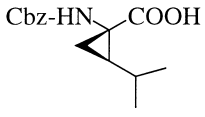
E-(2*S*,3*S*)-**3**



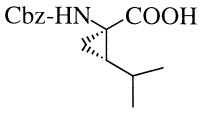
E-(2*R*,3*R*)-**4**



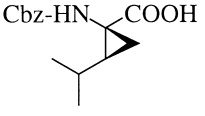
5



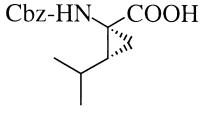
(-)-(2*S*,3*R*)-Cbz-*E*-cyclo-Leu (**6**)



(+)-(2*R*,3*S*)-Cbz-*E*-cyclo-Leu (**7**)

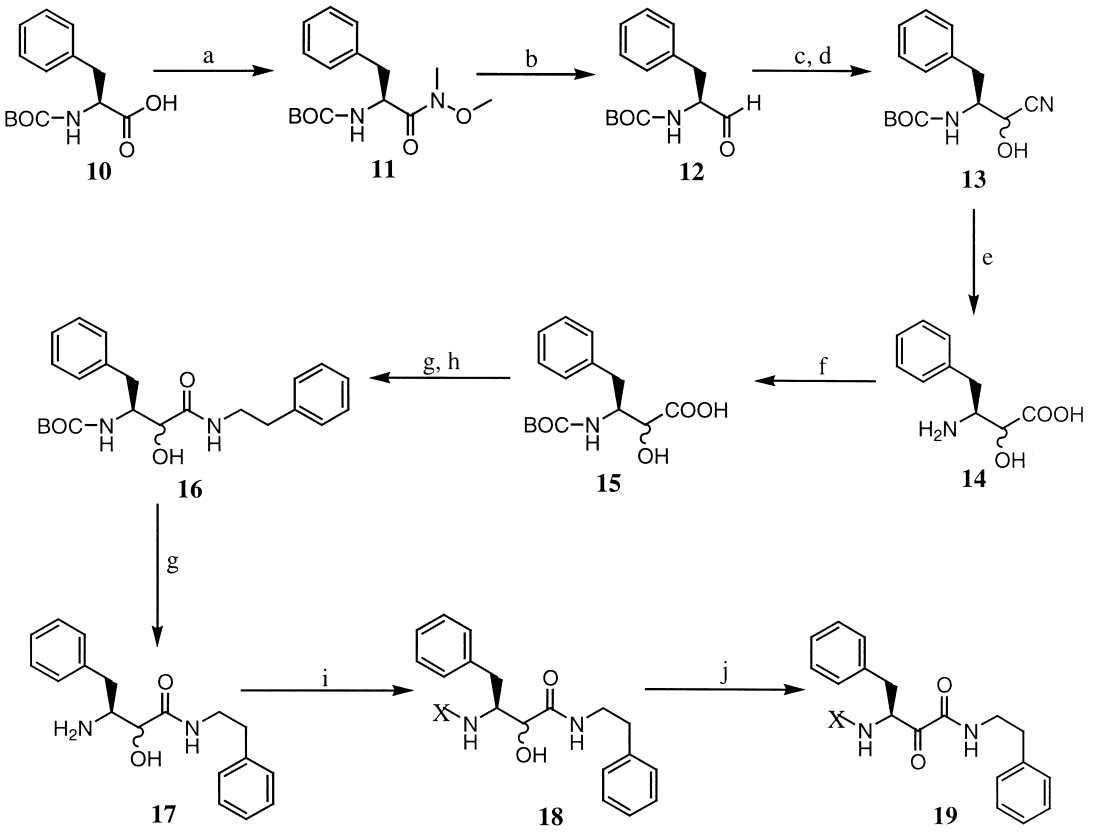


(+)-(2*S*,3*S*)-Cbz-*Z*-cyclo-Leu (**8**)



(-)-(2*R*,3*R*)-Cbz-*Z*-cyclo-Leu (**9**)

Figure 1. Structures of target compounds **1**–**5** and 2,3-methanoleucine stereoisomers **6**–**9** from which they were derived.



Scheme 1. Reagents: (a) CH_3NHOCH_3/EDC/NMM. (b) $LiAlH_4$. (c) $NaHSO_3$. (d) NaCN. (e) HCl/Dioxane. (f) $(t\text{-BuOCO})_2O$/NaOH. (g) $PhCH_2CH_2NH_2$/EDC/HOBt. (h) HCl/Dioxane. (i) X/EDC/HOBt (X = Cbz-L-leucine, **6**, **7**, **8**, or **9**). (j) Dess–Martin reagent.

Table 1. Inhibition of porcine erythrocyte calpain I and human liver cathepsin B by compounds **1**–**5**[a]

Compound	K_i (μM)		Selectivity ratio[d]
	Calpain I[b]	Cathepsin B[c]	
1 [*Z*-(2*S*,3*R*)]	11.13	5.05	0.5
2 [*Z*-(2*R*,3*S*)]	40.00	4.56	0.1
3 [*E*-(2*S*,3*S*)]	0.75	6.78	9.0
4 [*E*-(2*R*,3*R*)]	6.15	1.26	0.2
5	0.07	0.30	4.0

[a]K_i values were determined by Dixon plots using the average of triplicate assays and plotting $1/v$ versus I to give intersecting lines with correlation coefficient ≥ 0.95.
[b]50 mM Tris HCl pH 7.4, 50 mM NaCl, 10 mM DTT, 1 mM EDTA, 1 mM EGTA, 2% DMSO.
[c]20 mM NaOAc pH 5.2, 0.5 mM DTT, 2% DMSO.
[d]Selectivity ratios were determined by dividing the K_i values for cathepsin B inhibition by those for calpain I inhibition.

discrimination was observed. Replacement of the P_2-leucine residue of **5** with the 2,3-methanoleucine stereoisomers resulted in at least a 10-fold decrease in calpain I inhibitory potency. However, **3**, which was the most potent calpain I inhibitor of the series, was 9-fold selective for calpain I over cathepsin B. Compound **5** displayed only 4-fold selectivity between the two enzymes. Thus, despite the 10-fold decrease in potency, incorporation of the *E*-(2*S*,3*S*) 2,3-methanoleucine stereoisomer at the P_2-position of **5** enhanced selectivity for calpain I over cathepsin B.

Acknowledgements

The study was supported in part by grant 5 K14 HL03536 from the U. S. Public Health Service (IOD) and a University of Tennessee College of Pharmacy Faculty Development Grant (IOD).

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12. All of the novel ketoamides had satisfactory analytical data, ^{1}H, ^{13}C NMR, and elemental analyses. Compound **1**: Mp 194.0–194.8 °C. ^{1}H NMR ($CDCl_3$) δ 0.79 (s, 1H, $(CH_3)_2$-CHCHC(CH_2)), 0.99 (d, 3H, J = 6.5 Hz, $(CH_3)_2$CHCH-C(CH_2)), 1.06 (d, 3H, J = 6.5 Hz, $(CH_3)_2$CHCHC(CH_2)), 1.15–1.30 (m, 1H, $(CH_3)_2$CHCHC(CH_2)), 1.43–1.52 (m, 1H, $(CH_3)_2$-CHCHC(CH_2)), 1.70–C(CH_2)), 1,70–1.78 (m, 1H, $(CH_3)_2$-CHCHC(CH_2)), 2.84–2.96 (m, 2H, $PhCH_2CH_2NH$), 3.06–3.16 (m, 1H, $PhCH_2CHCO$), 3.30–3.41 (m, 1H, $PhCH_2CHCO$), 3.51–3.72 (m, 2H, $PhCH_2CH_2NH$), 5.03–5.19 (m, 3H, $PhCH_2OCONH$, $PhCH_2CHCO$), 5.44–5.57 (m, 1H, CONH), 6.91 (s, 1H, CONH), 6.99 (s, 1H, CONH), 7.04–7.37 (m, 15H, Ph-H); ^{13}C NMR ($CDCl_3$) δ 22.14, 22.68, 28.02, 35.35, 35.87, 37.10, 39.83, 40.43, 55.89, 126.80, 127.03, 128.33, 128.49, 128.59, 128.68, 128.79, 129.43, 135.67, 135.82, 138.02, 159.07, 171.51, 194.98. Anal. ($C_{33}H_{37}N_3O_5$) C, H, N. Compound **2**: Mp 155.2–156.0 °C. ^{1}H NMR ($CDCl_3$) δ 0.85–1.04 (m, 6H, $(CH_3)_2$CHCHC(CH_2)), 1.11–1.21 (m, 1H, $(CH_3)_2$CHCHC-(CH_2)), 1.38–1.70 (m, 3H, $(CH_3)_2$CHCHC(CH_2)), 2.84–2.93 (m, 2H, $PhCH_2CH_2NH$), 3.01–3.10 (m, 1H, $PhCH_2CHCO$), 3.22–3.37 (m, 1H, $PhCH_2CHCO$), 3.51–3.73 (m, 2H, $PhCH_2CH_2NH$), 5.09 (s, 2H, $PhCH_2OCONH$), 5.20 (s, 1H, $PhCH_2CHCO$), 5.47–5.61 (m, 1H, CONH), 6.93 (s, 1H, CONH), 7.05 (s, 1H, CONH), 7.21–7.39 (m, 15H, Ph-H); ^{13}C NMR ($CDCl_3$) δ 20.10, 22.09, 22.14, 26.66, 35.38, 36.99, 39.56, 40.46, 55.61, 67.28, 126.76, 127.04, 128.20, 128.39, 128.61, 128.70, 128.75, 129.28, 135.81, 138.11, 156.42, 159.19, 170.01, 195.25. Anal. ($C_{33}H_{37}N_3O_5$) C, H, N. Compound **3**: ^{1}H NMR ($CDCl_3$) δ 0.75–0.96 (m, 6H, $(CH_3)_2$CHCHC(CH_2)), 1.01–1.07 (m, 1H, $(CH_3)_2$CHCHC(CH_2)), 1.12–1.30 (m, 1H, $(CH_3)_2$CHCHC(CH_2)), 1.35–1.52 (m, 2H, $(CH_3)_2$CHCHC-(CH_2)), 2.84–2.93 (m, 2H, $PhCH_2CH_2NH$), 3.00–3.08 (m, 1H, $PhCH_2CHCO$), 3.23–3.33 (m, 1H, $PhCH_2CHCO$), 3.51–3.64 (m, 2H, $PhCH_2CH_2NH$), 5.09–5.16 (m, 2H, $PhCH_2OCONH$), 5.21 (s, 1H, $PhCH_2CHCO$), 5.47–5.56 (m, 1H, CONH), 6.88 (s, 1H, CONH), 7.03 (s, 1H, CONH), 7.21–7.36 (m, 15H, Ph-H); ^{13}C NMR ($CDCl_3$) δ 20.72, 22.06, 22.12, 26.54, 28.08, 35.37, 37.24, 39.64, 40.35, 40.42, 55.95, 67.23, 126.77, 127.05, 128.09, 128.28, 128.58, 128.63, 128.69, 128.77, 129.26, 135.82, 135.95, 138.09, 156.31, 159.30, 170.26, 195.26. Anal. ($C_{33}H_{37}N_3O_5$) C, H, N. Compound **4**: Mp 154.0–154.9 °C. ^{1}H NMR ($CDCl_3$) δ 0.73–0.79 (m, 1H, $(CH_3)_2$CHCHC(CH_2)), 0.96 (d, 3H, J = 6.4 Hz, $(CH_3)_2$CHCHC(CH_2)), 1.04 (d, 3H, J = 6.4 Hz, $(CH_3)_2$CHCHC(CH_2)), 1.13–1.33 (m, 1H, $(CH_3)_2$-CHCHC(CH_2)), 1.43–1.51 (m, 1H, $(CH_3)_2$CHCHC(CH_2)), 1.68–1.78 (m, 1H, $(CH_3)_2$CHCHC(CH_2)), 2.84–2.92 (m, 2H, $PhCH_2CH_2NH$), 3.16–3.36 (m, 2H, $PhCH_2CHCO$), 3.50–3.70 (m, 2H, $PhCH_2CH_2NH$), 4.95–5.25 (m, 3H, $PhCH_2OCONH$, $PhCH_2CHCO$), 5.46–6.80–6.91 (m, 2H, CONH), 7.04–7.37 (m, 15H, Ph-H, 1H, CONH); ^{13}C NMR ($CDCl_3$) δ 22.05, 22.13, 28.08, 35.38, 35.94, 37.39, 39.82, 40.42, 55.75, 67.39, 126.80, 127.08, 128.09, 128.16, 128.27, 128.38, 128.58, 128.63, 128.68, 128.78, 129.27, 129.47, 135.53, 135.85, 138.06, 156.26, 159.19, 171.69, 195.41. Anal. ($C_{33}H_{37}N_3O_5$) C, H, N.

13. 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 calpain I (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 amibient temperature using a SPECTRAmax Gemini fluorescence plate reader (Molecular Devices). K_i values were determined by Dixon plots.[15] The average of triplicate assays, plotted as $1/v$ versus I, gave intersecting lines with correlation coefficient ≥0.95. No other attempt was made to correct for slow binding or autolysis.

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