Pergamon

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Bioorganic & Medicinal Chemistry Letters 13 (2003) 783–784

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Peptidyl Aldehyde Inhibitors of Calpain Incorporating P_2-Proline Mimetics

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Received 11 November 2002; revised 7 January 2003; accepted 10 January 2003

Abstract—Four new peptidyl aldehydes bearing proline mimetics at the P_2-position were synthesized and studied as inhibitors of calpain I, cathepsin B, and selected serine proteases. The ring size of the P_2-constraining residue influenced the inhibitory potency and selectivity of the compounds for calpain I compared to the other proteases.

The calcium-activated intracellular neutral cysteine protease, calpain I, has been implicated in a number of pathological conditions including cardiac ischemia and cerebral ischemia.[1–5] Inhibitors of calpain I are therefore of interest as therapeutic agents and as biomedical tools. A variety of peptide-based reversible inhibitors (e.g., aldehyde and α-ketocarbonyl) and irreversible inhibitors (e.g., expoxysuccinate, diazomethyl ketone, acyloxymethyl ketone, and halomethyl ketone) of calpain have been reported.[5,6] Structure–activity relationship studies of the active site of calpain suggest that the S_1-subsite of calpain can accommodate a wide variety of amino acids but the S_2-subsite of the enzyme appears to have a strict requirement for either L-Val or L-Leu.[5] However, recent studies[7,8] have demonstrated that calpain can accommodate D-amino acids at the P_2-position of inhibitors. In one such study, Tripathy et al.[8] incorporated D-proline at the P_2 position (**4b**) to obtain potent inhibitors of the enzyme. Peet et al.[9] have also reported peptidyl aldehydes with constrained P_1–P_2 units. As a part of our interest in probing the subsite specificities of calpain I,[10,11] we synthesized **4a–d** (Scheme 1) incorporating proline mimetics at the P_2 position of the inhibitors with the objective of investigating the effect of the ring size of the P_2 constraining residue on calpain I inhibition.

The procedure used to synthesize **4a–d** is summarized in Scheme 1.[12] Tosylation of the proline mimetics gave **2a–d** (27–78%), which were coupled with L-phenylalaninol in the presence of HOBt and BOP to give dipeptides **3a–d** (53–82%). Dess–Martin oxidation of the dipeptides afforded target compounds **4a–d** (62–95%). Compound **5** was synthesized in 63% yield using L-leucine methylester in place of the proline mimetics. The products were purified by column chromatography on silica gel and/or recystallization (**4a**, **4d**, and **5**) from hexanes/CH_2Cl_2. The diastereomeric ratios (see Table 1) of the compounds were determined by HPLC using a C_{18} reversed phase column with 6.2% aqueous TFA and acetonitrile as the mobile phase.

Tripathy et al.[8] have shown that **4b** and its isomer with L-proline at the P_2 position are equipotent inhibitors of calpain I. Compounds **4b** and **4c**, which could not be resolved into pure isomers were tested as diastereomeric mixtures. Peptidyl aldehydes are known to inhibit serine and cysteine proteases.[5] The compounds were therefore evaluated[13] as inhibitors of porcine erythrocyte calpain I and selected serine proteases (trypsin, thrombin and factor Xa) to determine selectivity for calpain I inhibition compared to serine protease inhibition. The compounds were poor inhibitors of serine proteases. They displayed IC_{50} values[14] greater than 100 μM (data not shown) versus the serine proteases. On the contrary, the compounds were potent inhibitors of calpain I with K_i values in the submicromolar range (Table 1). Thus, **4a–d** are selective inhibitors of cysteine proteases compared to serine proteases. The basis for the selectivity may be attributed to differences in the address regions of the enzymes as well as the high nucleophilicity of the catalytic site cysteine residue of cysteine proteases.

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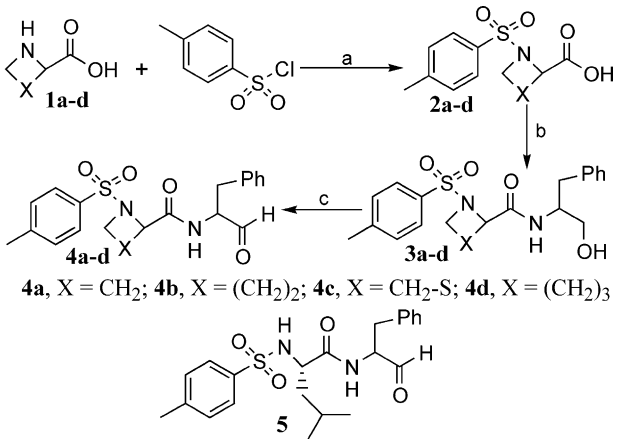
doi:10.1016/S0960-894X(03)00021-0



Scheme 1. Reagents and conditions: (a) $NaOH/H_2O/THF$; (b) phenylalaninol hydrochloride, NMM, HOBt, BOP, DMF; (c) Dess–Martin reagent.

Table 1. Diastereomeric ratios, K_i (μM) values[a] and selectivity ratios for inhibition of porcine calpain I and human liver cathepsin B by **4a–d** and **5**

Compd	Calpain I	Cathepsin B	SR[b]	DR[c] LL:DL
4a	0.25	2.75	11	100:0
4b	0.08	12.1	151	81:19[d]
4c	0.07	–	–	86:14
4d	0.54	5.26	10	100:0
5	0.02	0.07	4	–

[a]Values are means of triplicate experiments.
[b]SR is selectivity ratio to the nearest whole number. It was determined by dividing the K_i for cathepsin B inhibition by the K_i for calpain I inhibition.
[c]DR is diastereomeric ratio (P_1:P_2).
[d]DR for **4b** is that for the LD:DD isomers not the LL:DL isomers.

Cathepsin B, like calpain belongs to the papain superfamily of cysteine proteases and is known to be inhibited by peptidyl aldehydes and several calpain inhibitors.[5] We therefore assayed the compounds against cathepsin B to determined selectivity for calpain I. Generally, the compounds were better inhibitors of caplain I compared to cathepsin B. Compound **4b**, which showed 150-fold selectivity for calpain I over cathepsin B was the most selective member of the series (Table 1). The size of the P_2 residue influenced the calpain I inhibitory potency of the compounds. The k_i values for inhibition of calpain I ranged from 0.07 μM to 0.54 μM. Compounds with a five-membered ring at the P_2-position were the most potent members of the series. Thus, **4b** with a D-proline residue at the P_2-position and **4c** with a L-thiazolidine-4-carboxylic acid residue at this position were better inhibitors of calpain I compared to **4a** and **4d**, which had D-azetidine-2-carboxylic acid and L-pipecolic acid as the P_2 residue, respectively. The equipotency of **4b** and **4c** is in agreement with the observation that calpain can accommodate D- and L-proline at the P_2 position.[8] The conformational behavior of the proline mimetics may account for the observed difference in the potency of the compounds.[15] Compounds **4b** and **4c**, which were the most potent calpain I inhibitors of the series were less potent than **5**. This suggests that constraining the P_2 residue is detrimental to calpain I inhibition despite enhanced selectivity for calpain I compared to cathepsin B. A similar effect was observed when the L-leucine residue at the P_2-position of calpain I inhibitors was replaced with 2,3-methanoleucine stereoiomers.[10]

Acknowledgements

The study was supported by grant 5 K14HL03536 from the U.S. Public Health Service.

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12. L-Azetidine-2-carbozylic acid (**1a**), L-thiazolidine-4-carboxylic acid (**1c**), and DL-pipecolic acid (**1d**) were purchased from Aldrich. D-Proline (**1b**) was obtained from Advanced ChemTech.
13. The calpain assay 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, 2 μg porcine erythrocyte calpain I, varying inhibitor concentrations in DMSO (2%) and 5 mM $CaCl_2$ in a final volume of 250 μL. Assays were initiated by addition of $CaCl_2$ and the increase in fluorescence ($\lambda ex = 370$ nm, $\lambda em = 440$ nm) was monitored at amibient temperature. K_i values were by Dixon plots[16] of triplicate assays with correlation coefficient >0.95.
14. Serine protease inhibition assays: Each 200 μL reaction mixture contained buffer (20 mM Tris pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$, 0.1% BSA), inhibitor (0.1–100 μM), enzyme (40 nM trypsin, or 40 nM α-thrombin, or 10 nm FXa), and substrate (100 μM Bzl-Phe-Val-Arg-pNA for trypsin and α-thrombin assays, or 100 μM Bzl-Ile-Glu-Gly-Arg-pNA for FXa assay). The reaction mixture was incubated at 25 °C for 5 min prior to the addition of the substrate. After substrate addition, the hydrolytic rates were read for 2 min at 412 nm.
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