Pergamon

Bioorganic & Medicinal Chemistry Letters 11 (2001) 2615–2617

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Potent Peptide α-Ketohydroxamate Inhibitors of Recombinant Human Calpain I

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Received 16 April 2001; accepted 24 July 2001

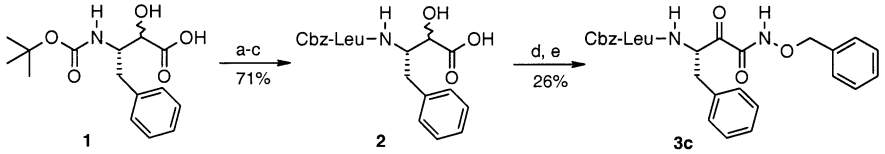
Abstract—A series of potent dipeptide and tripeptide α-ketohydroxamic esters was prepared as inhibitors of recombinant human calpain I. Compound **3c**, a Cbz-Leu-Phe hydroxamate, displayed the greatest potency against calpain I ($IC_{50} = 6$ nM), while two compounds, **3l** and **3m**, both possessing the Cbz-Leu-Leu-Phe sequence, were the most potent ($IC_{50} = 0.2$ μM) in a MOLT-4 cell assay.

Calpains, calcium-activated cysteine proteases widely distributed in mammalian cells, have been identified in two major forms: calpain I and calpain II. It is believed that calpain I is activated during a biochemical cascade that leads to a delayed degeneration of neurons following ischemia.[1] Calpain is implicated in many nervous system disorders such as stroke, severe head trauma, and other neurological diseases. This understanding has led to the search for potent, membrane permeable inhibitors as pharmacological targets. A variety of inhibitor structures, both reversible (aldehyde, hydroxyoxazolidine and α-ketocarbonyl)[2] and irreversible (diazomethyl ketone, fluoromethyl ketone, epoxysuccinate, and acyloxymethyl ketone),[3] have been reported. The most potent reversible inhibitors show IC_{50} values less than 10 nM against calpain I.[2] We report on a novel series[4] of peptide α-ketohydroxamates,[5] compounds **3a–n**, and their inhibitory activities against recombinant human calpain I and in a whole-cell MOLT-4 assay.

The title compounds were synthesized following one of two routes. In General Method A (Scheme 1), the P_1 *N*-BOC-β-amino-α-hydroxy acid **1**[6] was esterified and *N*-deprotected in a single step with thionyl chloride in methanol. This was then coupled to the desired *N*-protected P_2 amino acid (or P_3–P_2 dipeptide) using standard BOP/HOBt procedures. Hydrolysis of the resulting ester with LiOH gave the α-hydroxy acid **2** with the appropriate P_2 (or P_3–P_2) moiety of the peptide in place. Formation of the hydroxamate was achieved with BOP/HOBt and the appropriate hydroxylamine. Subsequent oxidation with the Dess–Martin periodinane[7] afforded α-ketohydroxamates, compounds **3a–f**,[8] **3h**, **3i**, **3l**, and **3m**.

For General Method B (Scheme 2), the P_1 *N*-BOC-β-amino-α-hydroxy acid **4** was first coupled to the hydroxylamine with BOP/HOBt and then *N*-deprotected with HCl to give the corresponding β-amino-α-hydroxy-hydroxamate **5**. Standard peptide coupling reagents

General Method A



Scheme 1. (a) $SOCl_2$, MeOH; (b) Cbz-Leu-OH, BOP, HOBt, DMF; (c) LiOH, MeOH; (d) H_2NOBn, BOP, HOBt, DMF; (e) Dess–Martin.

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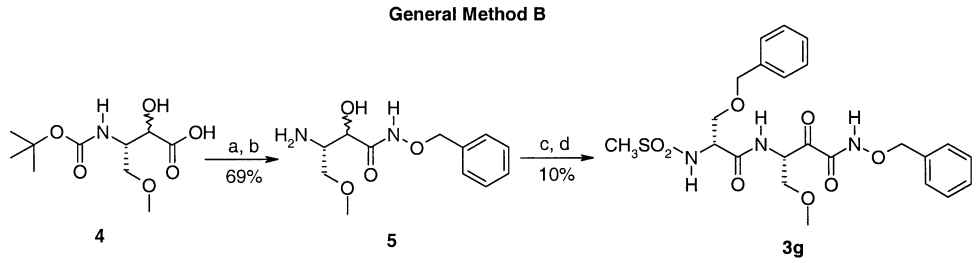
PII: S0960-894X(01)00526-1



Scheme 2. (a) H_2NOBn, BOP, HOBt, DMF; (b) HCl, dioxane; (c) CH_3SO_2-D-Ser(Bn)-OH, EDCI, HOBt, NMM, DMF; (d) Dess–Martin.

(EDCI/HOBt) were used to add the desired *N*-protected amino acid to the P_2 portion. Oxidation under Dess–Martin conditions yielded the α-ketohydroxamates, compounds **3g**, **3j**, **3k**, and **3n**.

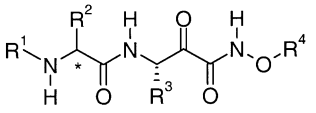
Table 1 summarizes the potency of the peptide α-ketohydroxamates **3** evaluated in this study. The activity as inhibitors of purified human recombinant calpain I at 20 °C was determined utilizing Suc-Leu-Tyr-MNA (0.2 mM; $K_m = 0.4$ mM) as substrate, as previously described.[9] The cell permeability was evaluated by measuring the spectrin breakdown product in intact MOLT-4 cells (human leukemia T-cell line) in which calpain was activated by ionomycin.[10]

Hydroxamates with the Cbz-Leu-Phe peptide sequence, compounds **3a**–**f**, are generally better inhibitors of the calpain I enzyme than the other hydroxamates investigated. The activity does not vary greatly as the relative size of the *O*-substituent of the hydroxamic ester is increased from methyl to pentafluorobenzyl. In contrast, in a series of Cbz-Leu-Phe α-ketoamides described by Li et al.,[2] the phenethyl amide ($K_i = 0.052\,\mu M$) is approximately 300-fold more potent than the *n*-propyl analogue ($K_i = 15.0\,\mu M$). Compound **3a**, the methyl hydroxamate, is approximately isosteric with the corresponding α-ketoamide Cbz-Leu-Phe-CONHEt. The activity of these two compounds are nearly identical in our calpain I enzyme assay ($IC_{50} = 10$ and 11 nM, respectively) and in the MOLT-4 cell assay ($IC_{50} = 0.9$ and 0.8 μM, respectively).

The three dipeptides with the unnatural D-Ser-benzyl ether at P_2, **3g**–**i**, were prepared to further investigate this template previously reported from our laboratories.[11] The hydroxamates **3h** and **3i** are about 2–3 times more potent than their corresponding α-ketoamides, CH_3SO_2-D-Ser(Bn)-Phe-CONHBn and CH_3SO_2-D-Ser(Bn)-Phe-CONHEt ($IC_{50} = 28$ and 56 nM vs. $IC_{50} = 56$ and 180 nM, respectively). Compound **3g**, bearing a Ser-methyl ether at P_1, is five times less potent than **3h**, containing Phe P_1, which is consistent with P_1 site affinity criteria for the calpain enzyme.[1,2]

Compounds **3j** and **3k**, both with Val at P_2, are potent in both the calpain I enzyme assay and the MOLT-4 cell assay. The Nle at P_1 for **3k** results in only a minor loss of activity compared to **3j** containing P_1 Phe. Compound **3n**, with P_2–P_1 Phe–Nle, is 10-fold less active than **3k**, which is consistent with previous observations that peptides with P_2 Phe are not preferred for calpain binding.[1,2] The N-capping group in **3n** is PhCO rather than Cbz, but this should not significantly affect the potency.[1,2]

Table 1. Calpain inhibitory activity



Compd	R^1	R^2	R^3	R^4	Calpain I IC_{50} (nM)	MOLT-4 IC_{50} (μM)
3a[a]	BnOCO	L-$CH_2CH(CH_3)_2$	Bn	CH_3	10	0.9
3b	BnOCO	L-$CH_2CH(CH_3)_2$	Bn	CH_2CH_3	19	1.1
3c	BnOCO	L-$CH_2CH(CH_3)_2$	Bn	Bn	6	0.6
3d	BnOCO	L-$CH_2CH(CH_3)_2$	Bn	$CH_2C_6F_5$	17	2.9
3e	BnOCO	L-$CH_2CH(CH_3)_2$	Bn	*t*-Bu	26	3.9
3f	BnOCO	L-$CH_2CH(CH_3)_2$	Bn	(4-Methyl-cyclohexyl)	21	26%[b]
3g	CH_3SO_2	D-CH_2OBn	CH_2OCH_3	Bn	152	ND[c]
3h	CH_3SO_2	D-CH_2OBn	Bn	Bn	28	0.9
3i	CH_3SO_2	D-CH_2OBn	Bn	CH_2CH_3	56	ND
3j	BnOCO	D-$CH(CH_3)_2$	Bn	Bn	12	0.6
3k	BnOCO	L-$CH(CH_3)_2$	$(CH_2)_3CH_3$	Bn	21	0.5
3l	Cbz-Leu	L-$CH_2CH(CH_3)_2$	Bn	CH_3	20	0.2
3m	Cbz-Leu	L-$CH_2CH(CH_3)_2$	Bn	Bn	17	0.2
3n	PhCO	L-Bn	$(CH_2)_3CH_3$	CH_2CH_3	193	ND

[a]For comparison: Cbz-Leu-Phe-CONHEt calpain I $IC_{50} = 11$ nM and MOLT-4 $IC_{50} = 0.8$ M.
[b]% Inhibition at 1 μM.
[c]Not determined.

The tripeptides in the series, **3l** and **3m**, are also potent inhibitors of the calpain I enzyme, but more importantly, are the most potent inhibitors identified in our MOLT-4 cell assay to date. Therefore, these compounds have greater cell permeability than the dipeptide α-ketohydroxamates in our assay.[12] This result is consistent with α-ketoamide and α-ketoester tripeptides described by Li et al.[2] in a platelet membrane permeability assay.

We have described a series of novel peptide α-ketohydroxamates that are potent inhibitors of recombinant human calpain I. The activity of this series compares favorably to that of previously described α-ketoamides.[2] However, substituent changes on the P′ hydroxamates do not affect the potency as significantly as changes to the P′ amide substituents. These α-ketohydroxamates are highly membrane permeable as demonstrated in a whole-cell MOLT-4 assay.

Acknowledgements

The authors would like to thank John Mallamo, Sankar Chatterjee, and Derek Dunn for their support, and helpful discussions. We would also like to thank Mark Ator, Donna Bozyczko-Coyne, Satish Mallya, Beth Ann McKenna, Teresa M. O'Kane, and Shobha E. Senadhi for performing the biological assays. We would also like to thank SmithKline Beecham for partial support in this project.

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12. One reviewer suggested there could be a correlation of the MOLT-4 cell assay IC_{50} values and some biophysical property such as lipophilicity. Calculation of the log P values gave a range of 4.5 to 7.3 ± 1 for compounds with $IC_{50} < 1$ μM. Although there appears to be no correlation with these data at this time, we gratefully thank the reviewer for bringing this to our attention.