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FIRST HYDROXAMATE INHIBITORS FOR CARBOXYPEPTIDASE A. *N*-ACYL-*N*-HYDROXY-β-PHENYLALANINES

Dong H. Kim,* and Yonghao Jin

Center for Biofunctional Molecules and Department of Chemistry
Pohang University of Science and Technology
San 31 Hyojadong, Pohang 790-784, Korea

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Abstract: A series of *N*-acyl-*N*-hydroxy-β-Phe were designed, synthesized, and shown to have potent inhibitory activity for carboxypeptidase A (CPA). They are the first examples of CPA inhibitors having a hydroxamate functionality.

Inhibitors of zinc-containing proteases can be prepared by incorporation of a zinc ligating functionality into the structural frame of a substrate-like small molecule that can be recognized and accommodated by the target enzyme.[1] Among these ligands, hydroxamate that is known to coordinate to zinc ion in a bidentate fashion[2] has been most widely utilized.[3] The hydroxamate-type inhibitors thus obtained are in general highly effective reversible competitive inhibitors for zinc proteases of a wide variety.[4] Despite such frequent uses of hydroxamate for designing inhibitors for zinc proteases, no hydroxamate inhibitor has yet been reported for carboxypeptidase A (CPA)[5], a prototypic zinc protease that has been used as a model enzyme for designing inhibitors of medicinal interest. In this communication we wish to describe first examples of CPA inhibitors having a hydroxamate moiety. They were prepared by incorporating the hydroxamate group into β-Phe to give *N*-acyl-*N*-hydroxy-β-Phe.

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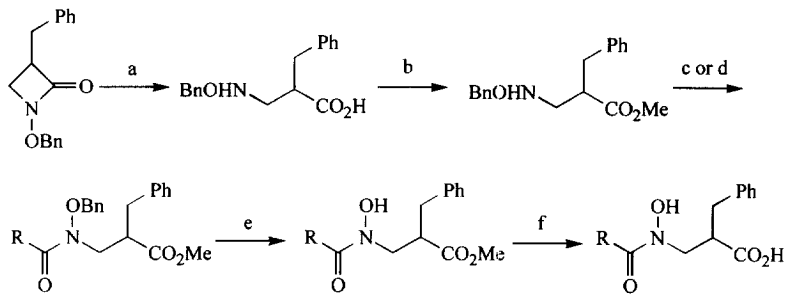
The inhibitors were readily prepared in good yields starting with *N*-benzyloxy-3-benzyl-2-azetidinone [6,7,8] as outlined in Scheme 1 and they were found to be competitive inhibitors for CPA when evaluated by the method of Lineweaver-Burk plot.[9] Inhibitory constants (K_i) were estimated from the respective Dixon plots.[10] Figure 1 exemplifies the Dixon plots. Table 1 summarizes structures and physical properties of the inhibitors evaluated in this study along with their kinetic constants obtained for the inhibition of CPA.[11]



Scheme 1. Reagents, conditions, and (yields): (a) LiOH (1.2 eq), THF-MeOH-H_2O (99%); (b) CH_2N_2, diethyl ether (100%); (c) Ac_2O (8.0 eq), HCO_2H, 0 °C (100%); (d) acyl chloride (1.0 eq), TEA (1.1 eq), 0 °C, CH_2Cl_2 (>95%); (e) H_2, Pd/C, MeOH (>97%); (f) LiOH (1.2 eq), THF-MeOH-H_2O (>95%).

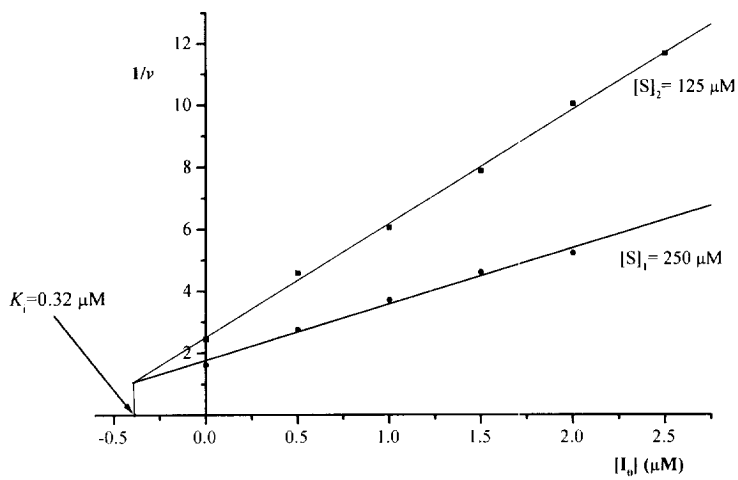


Figure 1. The Dixon plot of data for the inhibition of CPA-catalyzed hydrolysis of Hipp-L-Phe by 2-benzyl-3-(*N*-hydroxy-*N*-phenylacetyl)aminopropanoic acid (**5**).

Table 1. Structures of *N*-acyl-*N*-hydroxy-β-amino acids and their inhibitory constants determined for the inhibition of carboxypeptidase A

Compd No.	Structure	mp (°C)	K_i (μM)
1	$HC(=O)N(OH)CH_2CH(CH_2Ph)CO_2H$	oil	0.98
(*R*)-**1**	(*R*)-$HC(=O)N(OH)CH_2CH(CH_2Ph)CO_2H$	oil	0.56
(*S*)-**1**	(*S*)-$HC(=O)N(OH)CH_2CH(CH_2Ph)CO_2H$	oil	4.95
2	$CH_3C(=O)N(OH)CH_2CH(CH_2Ph)CO_2H$	oil	5.90
3	$CH_3CH_2C(=O)N(OH)CH_2CH(CH_2Ph)CO_2H$	85-86	5.82
4	$PhC(=O)N(OH)CH_2CH(CH_2Ph)CO_2H$	116-117	6.50
5	$PhCH_2C(=O)N(OH)CH_2CH(CH_2Ph)CO_2H$	108-109	0.32
6	$PhCH_2CH_2C(=O)N(OH)CH_2CH(CH_2Ph)CO_2H$	131-132	1.20
7	$PhCH_2CH_2CH_2C(=O)N(OH)CH_2CH(CH_2Ph)CO_2H$	113-114	1.16

N-Formyl-*N*-hydroxy-β-Phe is a potent reversible inhibitor having the K_i value of 0.98 μM. When the formyl group was replaced with a bulkier group such as an acetyl or propanoyl group, the potency was reduced progressively (Table 1). It has been known that there exists a hydrophobic pocket at the S_1 subsite region of CPA.[5] The X-ray crystallographic studies revealed that the aromatic side chain of Tyr-198 present in the region undergoes an aromatic edge-face interaction with the aromatic side chain of the P_1 residue of substrates or inhibitors.[5] It is therefore thought that incorporation of an aromatic ring in the acyl moiety would improve the inhibitory potency. Indeed, as can be seen from Table 1, hydroxamates having a phenylalkyl group show higher inhibitory potency compared with those without the phenyl ring. The most potent inhibitor in the series is *N*-phenylacetyl-*N*-hydroxy-β-Phe (**5**) with the K_i value of 0.32 μM. The reduced potency observed with **4** suggests that in the accommodation of the aromatic ring of the inhibitors by the S_1 subsite pocket a full interaction may only be attained when the ring is anchored deep in the S_1 subsite pocket in consistent with the explanation that the aromatic side chain of Tyr-198 undergoes π–π interactions with the aromatic side chain of the P_2 residue.[5] The high inhibitory potency shown by **6** and **7** may be explained by the presence of another hydrophobic pocket next to the S_1 subsite (Figure 2c). It was thought to be of interest to know the stereochemistry in the CPA inhibition. We have synthesized optically active

N-formyl-*N*-hydroxy-β-Phe and evaluated them to find that the (*R*)-form[12] is about 10 times more potent than its enantiomer (Table 1). This inhibitory stereochemistry is consistent with that reported for thermolysin.[13]

Figure 2 depicts the binding mode of substrate and the hydroxamate type inhibitors to the active site of CPA: the carboxylate in the ligands hydrogen bonds to the protonated guanidinium moiety of Arg-145 and the hydroxamate functionality chelates the active site zinc ion.[14] The aromatic ring of the benzyl group at the α-position to the carboxylate is accommodated in the primary substrate recognition pocket (S_1'). The aromatic ring of the *N*-acyl moiety is fitted in the S_1 hydrophobic pocket to result in an improvement of the binding affinity of the inhibitor. As the alkyl chain gets longer, the anchoring of the phenyl ring in the S_1 subsite pocket becomes difficult. Thus, in the case of **6** having a phenylpropanoyl group, the S_1 subsite pocket may not be able to accommodate the terminal phenyl ring due to its insufficient depth. It is conceivable that the phenyl ring would instead be fitted into the other hydrophobic pocket present perhaps in the S_2 subsite region (Figure 2c).

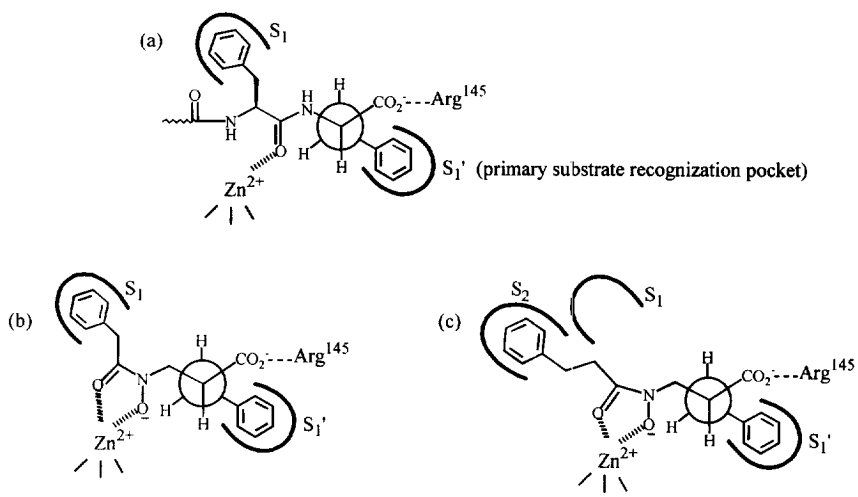


Figure 2. Schematic representations of the binding mode of substrate (a) and hydroxamate-type inhibitors **5** (b) and **6** (c).

It is of interest to compare the structure-activity relationships found with the present inhibitors for CPA with those reported for thermolysin,[13] a thermally stable zinc-containing endopeptidase isolated from *Bacillus thermoproteolyticus*. In contrast to the case of thermolysin in which the inhibitory potency reduced abruptly when the *N*-formyl group in *N*-formyl-*N*-hydroxy–β-Phe-NHMe is replaced with an acetyl group, in the case of CPA such a replacement brought about only modest reduction in potency. The difference in the

structure-activity relationships for the inhibitions of the two enzymes suggests that the structures of the active sites of these enzymes are markedly different each other although both share common catalytic features. Whereas CPA that bears a hydrophobic pocket at the S_1 subsite region possesses a relatively broad ligand specificity, the substrate as well as inhibitor specificity of thermolysin is much more restricted. As a thermostable enzyme, thermolysin is structurally rigid and thus resists induced conformational changes upon binding of inhibitors.[15] In fact, the thermolysin crystal structure is known to contain four Ca^{+2} which stabilize the enzyme against thermal denaturation.[16] On the other hand, CPA as a digestive enzyme is structurally less rigid than thermolysin and its active site is more open.[15a, 17] The interesting observations made with respect to the inhibitory specificity for both enzymes reveal that in the design of enzyme inhibitors it is of considerable importance to know the degree of structural rigidity of the target enzyme.

The present work demonstrates that hydroxamates derived from β-Phe are potent competitive inhibitors for CPA and that the structure-activity relationships found with these inhibitors for CPA are markedly different from those reported for thermolysin, although both enzymes are known to share common catalytic features.

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