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D-Stereospecificity in the inhibition of thermolysin by *N*-acyl-*N*-hydroxy-α-amino acid esters

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Abstract: Non-peptidic substrate analog inhibitors for thermolysin, *N*-acyl-*N*-hydroxyleucine methyl esters which were designed by incorporating the bidentate metal coordinating moiety of hydroxamate into leucine methyl ester showed 'D-stereospecificity' in inhibition of the enzyme, and the inhibitory potency was sharply curtailed as the alkyl group in the hydroxamate moiety became bulkier. © 1997 Published by Elsevier Science Ltd

The high binding affinity of hydroxamic acids for metal ions such as zinc[1] has been extensively utilized as a metal coordinating functionality in designing inhibitors for metalloenzymes.[2] These inhibitors are substrate analogs in which the scissile peptide bond of the substrate is replaced with a hydroxamate moiety. Thermolysin is a prototypical zinc-containing endopeptidase, representing a large number of medicinally important enzymes such as endopeptidase 24.11, angiotensin converting enzyme, and matrix metalloproteases.[3] The stereospecificity in the inhibition of thermolysin is thus thought to be of considerable importance especially for inhibitor design of zinc-containing enzymes of medicinal interest because the stereospecificity shown with thermolysin may be translated to those enzymes. In this communication we wish to report that contrary to the L-stereochemistry shown for substrates, compounds having the D-stereochemistry are active in the inhibition of thermolysin by hydroxamate-containing substrate analogs.

These inhibitors were readily prepared starting with chiral *N*-hydroxyleucine methyl ester.[4] The latter was treated with TMSCl in the presence of 2,6-lutidine followed by addition of acyl chloride, and then water to give the desired products in over 85% yield.[5] The present one-pot procedure for the preparation of chiral hydroxamic acids constitutes a significant improvement over the existing one[6] in terms of convenience and yield as well as purity of product.

Structures of hydroxamates prepared for the study are shown in Table 1 together with their thermolysin inhibitory constants determined according to the procedure of Feder[7] using *N*-(3-[2-furyl]acryloyl)-Gly-Leu-NH_2 as substrate. Two distinctive features are apparent from examination of the Table. Firstly, it can be seen that the thermolysin inhibitory activity rests exclusively on acylhydroxyamino acid esters derived from D-Leu. It is worth noting the report of Holmes and Matthews[8] that in the X-ray crystal structure of thermolysin inhibited by L-Leu-NHOH, the hydroxamate moiety binds the zinc ion as a bidentate ligand and the isobutyl side chain occupies the S_1' hydrophobic pocket. In this mode of binding, leucine in the inhibitor is shown to be as if it were the D-form, that is, the isobutyl side chain is projected in the spatial direction where that of the unnatural D-Leu would be. The reason why the stereochemistry has to be reversed in the inhibition of thermolysin by the hydroxamate inhibitors is not immediately apparent, but one may possibly find its origin in the bidentate complexing mode of the hydroxamate to the zinc ion. This is supported by the observation that in the case of *N*-hydroxyleucine methyl ester, which lacks the acyl group, the L-isomer binds preferably (Table 1).

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Table 1. Structures of *N*-acyl-*N*-hydroxyleucine methyl esters and their inhibitory constants determined for the inhibition of thermolysin

Compd No.	Inhibitor	K_i (μM)[a]
1	L-HN-Leu-OMe OH	3200
2	D-HN-Leu-OMe OH	20700
3	HCO-L-N-Leu-OMe OH	3070[b]
4	HCO-D-N-Leu-OMe OH	44
5	CH_3CO-L-N-Leu-OMe OH	c
6	CH_3CO-D-N-Leu-OMe OH	6570
7	CH_3CH_2CO-L-N-Leu-OMe OH	c
8	CH_3CH_2CO-D-N-Leu-OMe OH	13400
9	CH_2=CHCO-L-N-Leu-OMe OH	c
10	CH_2=CHCO-D-N-Leu-OMe OH	1500

[a] The inhibitory constants were determined according to the literature method.[7] [b] The inhibitory activity shown by 3 may actually be due to 4 which is present as an impurity (commercially available L-leucine contains about 1.5% of D-leucine as impurity.[c] No inhibitory activity was obsvered in the presence of inhibitor at the concentration of up to 30 mM.

The other noted structural feature of the hydroxamate inhibitors of thermolysin is the dependence of the inhibitory potency on the size of the alkyl group in the *N*-acyl portion of the molelcules: the highest binding affinity (K_i=44 μM) was exhibited when the *N*-acyl group is formyl i.e., **4**, and the K_i value increases rapidly as the alkyl group becomes larger (Table 1), suggesting that at the active site there is no locus that can accommodate the bulky alkyl groups. This explanation is supported by the X-ray crystal structure of the inactivated thermolysin by *N*-chloroacetyl-*N*-hyroxyleucine methyl ester, in which it is shown that the methylene unit next to the carbonyl group of the covalently linked inhibitor experiences severe steric hindrance by the extended segments of Ala-113–Asn-116 which constitute the wall of the active site.[9]

It is clear from the inspection of molecular models of substrate and hydroxamate inhibitor that if the hydroxamate inhibitor which bears the L-configuration binds to the active site in the same fashion as the substrate does with the isobutyl side chain being accommodated in the S_1' hydrophobic pocket, the bidendate chelating moiety of hydroxamate would be positioned too far from the zinc for complexing. In constrast, the zinc chelating hydroxamate of D-*N*-acyl-*N*-hydroxy-Leu-OMe would lie within the coordination sphere of the zinc ion upon binding to the active site. Furthermore, the stereochemically reversed binding of the present hydroxamate inhibitors would place the alkyl group of the hydroxamate moiety at a site other than the S_1 subsite pocket, where the alkyl may experience steric interactions with the wall of the active site.[9]

We now propose a schematic representation for the active site of thermolysin, which assists one to visualize not only the substrate stereospecificity, but also the inhibitory stereochemistry manifested by hydroxamate inhibitors synthesized for the present study. The proposed representations of the thermolysin active site which is occupied by a substrate and *N*-acyl-*N*-hydroxy-Leu-OMe are shown in Figs 1 and 2, respectively. In the representations, the enzyme-bound substrate and inhibitor are shown by the Newman's projection as they better represent the spatial orientation of the functionalities attached to the P_1' carbon atom, and thus enable one to envision the topology of the active site of the enzyme.

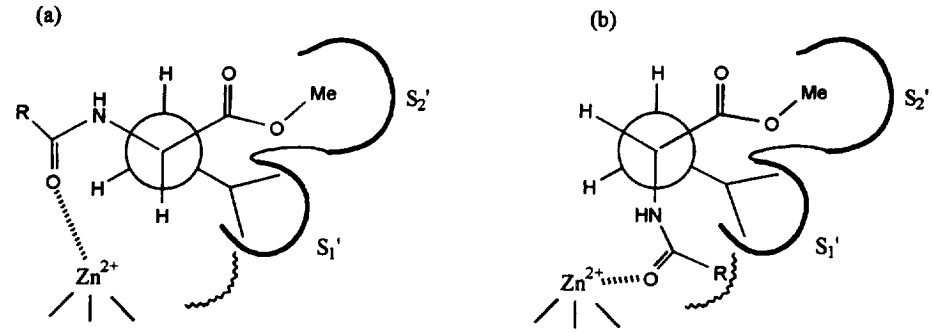

Figure 1. Schematic representation of the active site of thermolysin, which is occupied by substrate (RCO-Leu-OMe). While L-RCO-Leu-OMe binds to the active site of thermolysin in a productive mode (a), the binding of D-RCO-Leu-OMe to the active site is prohibitive due to severe steric interactions of the R group with the wall of the active site cavity of the enzyme (b).

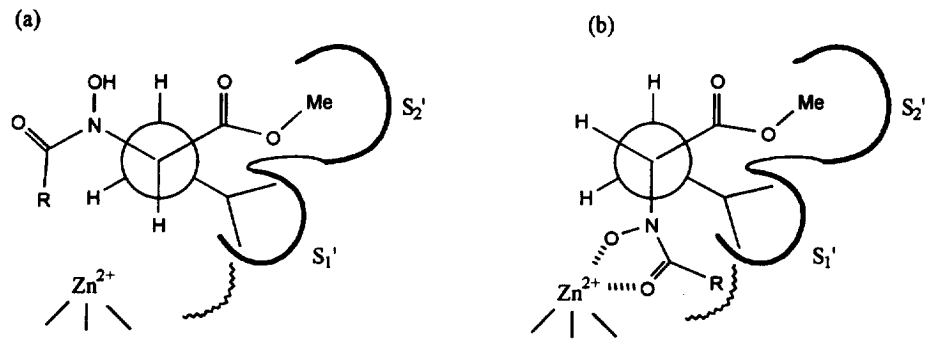

Figure 2. Schematic representation of the active site of thermolysin, which is occupied by hydroxamate inhibitor. While the hydroxamate bidentate chelating moiety of L-RCO-N(OH)-Leu-OMe would be positioned too far from the zinc ion for complexing when the inhibitor binds the enzyme (a), the zinc chelating hydroxamate in D-RCO-N(OH)-Leu-OMe would lie within the coordination sphere of the zinc ion to form a tightly bound complex (b).

In conclusion, structurally simple non-peptide thermolysin substrate analog inhibitors which were designed by incorporating a bidentate metal-coordinating moiety of hydroxamate into leucine methyl ester show the D-stereospecificity in the thermolysin inhibition, and the inhibitory potency is sharply curtailed as the alkyl group in the hydroxamate moiety becomes bulkier. A schematic representation for the active site of thermolysin has been proposed with which one can now visualize the L-stereospecificity of substrate and the 'D' inhibitory stereospecificity manifested by hydroxamate inhibitors.

Acknowledgements

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5. To a solution of D-*N*-hydroxyleucine methyl ester (0.89 g, 5.5 mmol) in THF (30 ml) was added 2,6-leutidine (1.3 ml, 11.0 mmol) and TMSCl (1.4 ml, 11.0 mmol) at 0°C and the resulting mixture

was stirred for 6 h at room temperature. The mixture was cooled to 0°C and acetyl chloride (0.43 ml, 6.05 mmol) was added dropwise with stirring. The stirring was continued for 0.5 h at 0°C and 0.5 h at room temperature. A small amount of water (1 ml) was added, and the resulting solution was stirred for 1 h at room temperature, after which THF was evaporated *in vacuo*. Ethyl acetate (50 ml) was added to the residue, and the organic layer was washed successively with 10% citric acid, 5% $NaHCO_3$ solution, water then dried ($MgSO_4$), and evaporated *in vacuo* to afford a crude product which was purified by column chromatography to give 0.99 g (88%) of **6** as an oil. IR (neat) 3180, 1743, 1629 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 5.30–5.24 (dd, 1H), 3.73 (s, 3H), 2.20 (s, 3H), 2.10–1.90 (m, 1H), 1.70–1.55 (m, 2H), 1.00–0.90 (m, 6H); $[\alpha]_D^{23}$=+49.0 (c=1.0, MeOH).

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