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INHIBITORY STEREOCHEMISTRY OF *N*-CHLOROACETYL-*N*-HYDROXYLEUCINE METHYL ESTER FOR THERMOLYSIN[1]

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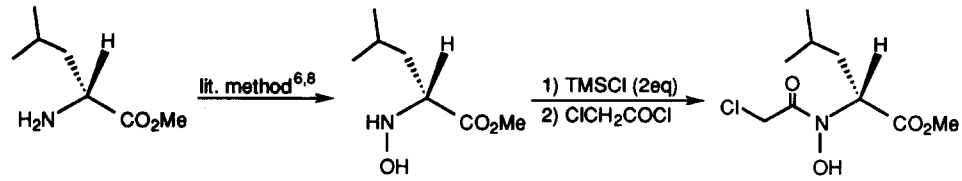
Abstract : The previously reported irreversible inhibition of thermolysin by (DL)-*N*-chloroacetyl-*N*-hydroxyleucine methyl ester, an active site directed inactivator, is found to be due to the D-enantiomer. Thus, while the D-enantiomer inactivated the enzyme, no inhibitory activity was found with the L-enantiomer. A possible explanation is presented for the reversed stereochemistry in the inactivation reaction compared with that of substrate.

In general, each stereoisomer of chiral drugs elicits dissimilar pharmacological effect many times of opposite property.[2] This chiral pharmacology is of great concern to many, ranging from drug designers and pharmaceutical manufacturers to drug regulatory authorities. In view of the fact that a majority of currently used therapeutic agents are displaying their pharmacological effects through interactions with enzymes,[3] such chiral pharmacology may possibly find its origin in the stereochemistry associated with the inhibition of enzyme. Accordingly, understanding of the inhibitory stereochemistry is of extreme importance to medicinal chemists. Although stereospecificity of enzymic catalytic reactions has long been recognized, stereochemistry involved in the inhibition of enzyme is poorly understood and cannot be predicted. In fact, the inhibitory stereochemistry is often shown to be the opposite of the enzymic stereospecificity.[4] We wish to report that the inhibition of thermolysin by *N*-chloroacetyl-*N*-hydroxyleucine methyl ester (**1**) is one of such examples.

As part of our ongoing project for the development of novel methodology for enzyme inhibitor design, we evaluated derivatives of L-leucine including *N*-chloroacetyl-*N*-hydroxyleucine methyl ester (**1**) as potential inhibitors for thermolysin, a prototypic zinc-containing endopeptidase.[5] The latter compound in a racemic form was previously reported by Rasnick and Powers[6] to be an active site directed inactivator for the enzyme. To our surprise, however, none of the L-leucine derivatives exhibited any noticeable inhibitory activity against the enzyme. We, then, decided to evaluate derivatives of D-leucine for thermolysin inhibitory activity, The D-enantiomer of the racemic inactivator (**1**) of Rasnick and Powers was thus synthesized for the evaluation, starting with methyl ester of D-leucine following the literature method[6,8] with some modifications (Scheme 1).

Scheme 1



We found that the D-form is indeed a potent irreversible inhibitor for thermolysin, showing a time-dependent loss of the enzyme activity at various concentrations as seen in Figure 1. Rasnick and Powers[6] had previously demonstrated using racemic **1** that the inactivation takes place at the active site of the

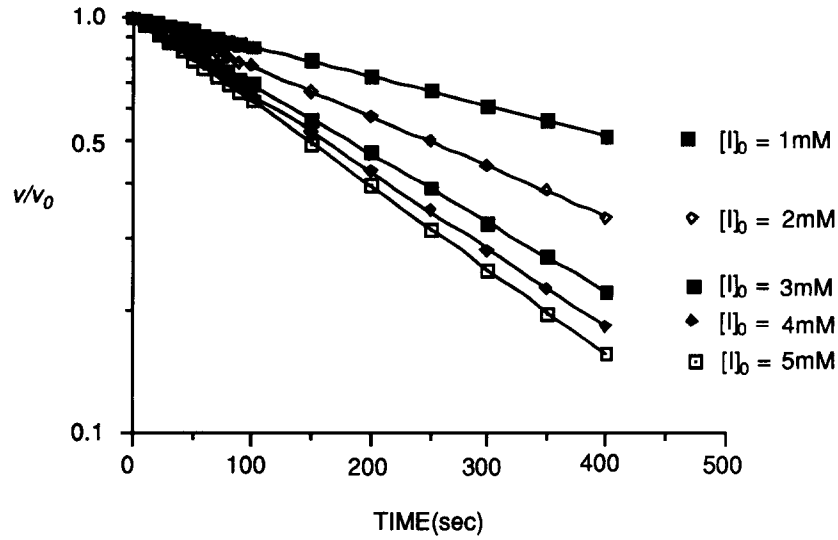


Figure 1. Semilogarithmic plots of the velocity ratio of the substrate hydrolysis after incubating thermolysin with D-**1** of various concentrations *vs* time of incubation give straight lines with a slope of $-k_{obs}$ for each inhibitor concentration.

enzyme. Kinetic parameters for the inhibition with D-**1** were determined by the method of Kitz and Wilson:[9] The inactivation reaction of the enzyme and its kinetic expression can be described by equations (1) and (2), respectively:

$$E + I \underset{}{\overset{K_i}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_{inact}} E\text{–}I \qquad (1)$$

$$\frac{1}{k_{obs}} = \frac{1}{k_{inact}} + \frac{K_i}{k_{inact}}\left(\frac{1}{[I]}\right) \qquad (2)$$

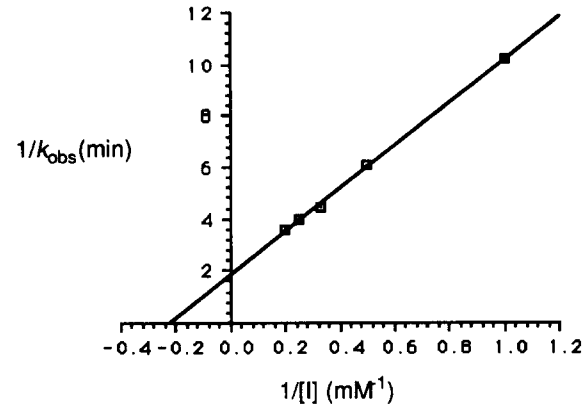


Figure 2. Double reciprocal plot of k_{obs} *vs* $[I]_O$ gives a straight line whose y-intercept corresponds to $1/k_{inact}$ and x-intercept shows $-1/K_i$.

where k_{obs} represents the observed rate constant for the hydrolysis of assay substrate (furylacryloyl-Gly-L-Leu-NH_2) by thermolysin in the presence of the inhibitor, and was obtained directly from the progress curve of the enzymic reaction using a microcomputer which is interfaced to the monitoring UV spectrophotometer. From the double reciprocal plot of k_{obs} *vs* $[I]_O$ (Figure 2) and equation (2), the values of K_i and k_{inact} for D-**1** were calculated to be 4.5×10^{-3} M and 9×10^{-3} sec^{-1}, respectively. These kinetic parameters for D-**1** compare favorably with those reported[6] for the racemic **1**, *i.e.*, $K_i = 7.5 \times 10^{-3}$ M and $k_{inact} = 7.5 \times 10^{-3}$ sec^{-1}. The result strongly suggests that the inhibitory activity observed with (DL)-**1** by the previous investigators was mainly due to the D-enantiomer in the racemic mixture.

Rasnick and Powers[6] had suggested that inhibitor **1** inactivates thermolysin *via* alkylation of the carboxylate of Glu-143 to form an ester linkage, which was subsequently ascertained by Holmes et al,[10] who elucidated by the X-ray crystallographic method the structure of the inactivated thermolysin that was obtained by soaking the enzyme in the solution of racemic **1**. However, as to the stereochemistry of the covalently bound inhibitor, these investigators seemed uncertain, making an only statement that the D-enantiomer fits better to the electron density map.[10] The present kinetic study supports the stereochemical assignment of Holme et al[10] for the covalently bound **1**.

There are two principal binding sites and a catalytically important carboxylate group at the active site of the enzyme:[5] The hydrophobic pocket which accommodates a hydrophobic side chain such as the isobutyl group of L-Leu, and the zinc ion that is coordinated to His-142, His-146, Glu-166 and a water molecule, and ligates to the scissile carboxamide oxygen of substrate when the enzyme forms the Michaelis complex with the substrate.[5] The carboxylate of Glu-143 is known to function as the general base which activates the zinc bound water molecule for the nucleophilic attack at the carbonyl carbon of the scissile carboxamide of the substrate.[5] Hydroxamic acid and its derivatives, which have a planar conformation with their C—N bond having a partial double bond character[11] as in an amide bond, are known to ligate to a zinc ion in a bidentate

fashion.[10,12] On the basis of the active site model of the enzyme and the established binding mode of hydroxamates to zinc ion, one can visualize the observed inhibitory stereochemistry of thermolysin by **1**. In examining molecular models of each enantiomer of **1** in comparison with that of substrate having L-leucine at the P_1' site, we found that when the isobutyl side chain and the carbomethoxy group of **1** were superimposed onto the corresponding moieties of the substrate, the hydroxamic moiety of the D-**1** only was rested in the general region where the zinc-binding carbonyl carbon of the substrate would lie, suggesting that only the hydroxamate of the D-enantiomer can ligate to the active site zinc. Therefore, the alkylation of the catalytic carboxylate can occur only with **1** having D-configuration. Our ongoing work would shed some additional light on this reserved stereochemistry in the inactivation of thermolysin.

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