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L-LEUCINTHIOL - A POTENT INHIBITOR OF LEUCINE AMINOPEPTIDASE

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L-leucinthiol (2-amino-4-methyl-1-pentanethiol) was designed as an inhibitor of leucine aminopeptidase by analogy with sulfhydryl inhibitors of other zinc-containing peptidases. It was synthesized from L-leucinol and shown to be a potent competitive inhibitor of the microsomal aminopeptidase from porcine kidney (K_I = 2.2 x 10^{-8}M). The results suggest that the mechanism of aminopeptidase may be similar to that of other metalloproteases.

Zinc proteases are known to be involved in many physiologically important processes such as the inactivation of neuropeptides, the destruction of connective tissue and the control of blood pressure (1). The development of an effective treatment for hypertension based on specific inhibition of the angiotensin-converting enzyme (2,3) has caused a surge of interest in the design and synthesis of inhibitors for this class of enzymes (4-7). In general, the practice is to incorporate into the inhibitor, as far as possible, those structural features which determine normal enzyme-substrate recognition. In addition, a functional group is introduced at an appropriate location to form a strong ligand to the zinc atom. Functional groups which have been successfully used for this purpose include carboxylates (3,8), thiols (2), hydroxamates (4) and phosphates (6). Among the enzymes which have been studied most extensively (i.e. carboxypeptidase, thermolysin and angiotensin-converting enzyme) almost equally effective inhibitors can be designed with the same functional groups provided that the inhibitor contains the structural determinants appropriate for the substrate specificity of the particular enzyme (9,10).

Although leucine aminopeptidase is a zinc-containing enzyme, the pattern of its sensitivity to inhibitors does not correspond closely to those of other

Abbreviations: DMF, dimethylformamide; DTT; dithiothreitol; TFA, trifluoroacetyl, and Ts, toluenesulfonyl.

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metalloproteases. Until recently, the most effective inhibitors of this enzyme have been chloromethyl ketones (11), o(t-butyl)threonyl peptides (12), phenylsulfinylphenyl acetates (13), and microbial products such as bestatin and amastatin (14,15). These compounds are structurally far removed from typical inhibitors of zinc proteases. However, we found unexpectedly that amino acid hydroxamates were strong inhibitors of the microsomal leucine aminopeptidase from pig kidney (16). This observation, which has been confirmed for aminopeptidase from other sources (17,18), would indicate that aminopeptidase might be similar in its catalytic site to other zinc proteases. Further conflicting evidence is provided by the very recent finding that α-amino aldehydes are potent inhibitors suggesting a similarity to serine and sulfhydryl proteases (19). In an attempt to resolve the discrepancy, we decided to examine inhibitors with other functional groups.

METHODS

Various details concerning the source of leucine aminopeptidase and the study of its interaction with inhibitors have been given elsewhere (16). Owing to the high affinity of L-leucinthiol, the enzyme concentration used for K_i determination had to be reduced to 0.86 nM.

Trifluoroacetyl imidazole (24 mmoles) was added slowly to a cooled solution of L-leucinthiol (24 mmoles) in dry pyridine (24 ml). After 30 minutes, a solution of toluenesulfonyl chloride (48 mmoles) in 22 ml pyridine was added with cooling. After overnight incubation, the product was extracted from aqueous phosphoric acid into methylene chloride. Evaporation and recrystallization from methanol and water gave the intermediate (I) in 73% yield, m.p. 101°C. R_f = 0.46 in CH_2Cl_2. (TLC was performed on silica gel and all compounds were identified by NMR).

Thiolacetic acid (4.5 mmoles) was mixed with KOH (4 mmoles) in dry DMF (2.3 ml) and added to a cooled solution of compound I (2.3 mmoles) in 20 ml DMF. After overnight incubation, deionized water (58 ml) was added at 0°. The thiolester (II) was filtered off as a precipitate and recrystallized from aqueous methanol, m.p. 48°C. R_f = 0.56 in CH_2Cl_2. Yield 70%.

Hydrolysis of (II) (0.74 mmole) was conducted for 2 hr. at 50°C in 5 ml 2 N aqueous NaOH containing 50% ethanol. An air stream was used to oxidize the product and evaporate the alcohol. The disulfide form of L-leucinthiol (III) was extracted into methylene chloride, converted into the dihydrochloride and recrystallized from dioxane-ethanol, m.p. 275-279°C (decomp). Yield 82%. R_f = 0.60 in $CH_2Cl_2/CH_3OH/HCO_2H$ (70:30:1).

RESULTS AND DISCUSSION

In a preliminary survey, we compared a number of commercially available compounds structurally related to glycine in order to find the most suitable

Table I
Inhibition of leucine aminopeptidase

Compound	K_i (μM)
$NH_2 - CH_2 - SO_3H$	>10,000
$NH_2 - CH_2 - CH_2 - CO_2H$	6,400
$NH_2 - CH_2 - CO - NHOH$[a]	2,100
$NH_2 - CH_2 - PO_3H_2$	700
$NH_2 - CH_2 - CH_2 - SH$	3.0
$NH_2 - CH - CH_2 - SH$ (L-leucinthiol) $CH_2 - CH(CH_3)_2$	0.022

[a] Reference 16

functional group. The results (Table I) show clearly that the thiol group is by far the most effective for inhibition. In fact, considering its very small size, β-mercaptoethylamine interacts remarkably strongly with this enzyme. We therefore proceeded to synthesize L-leucinthiol and this compound proved to be the most potent synthetic inhibitor available for aminopeptidase. The inhibition was strictly competitive as shown in the Lineweaver-Burk plots (Fig. 1).

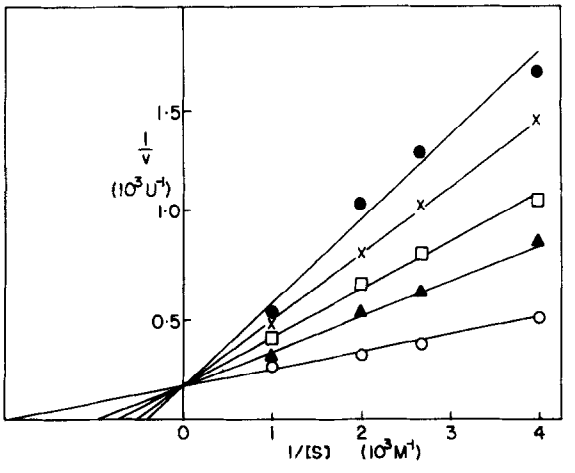
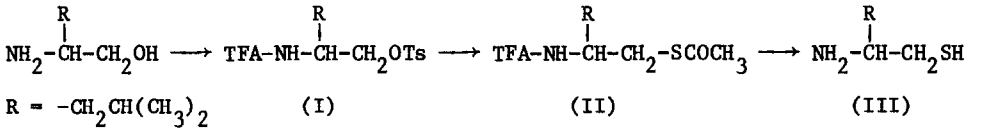


Fig. 1. Inhibition of aminopeptidase by L-leucinthiol. Activities (v) were measured in 0.2 M Tris HCl buffer pH 7.5 at 37°C with different concentrations [S] of leucine p-nitroanilide as substrate. The levels of L-leucinthiol used were ▲, 25 nM; □, 50 nM; ×, 75 nM; ●, 100 nM and ○, 0 nM. Dithiothreitol (0.5 mM) was used to maintain the inhibitor in the reduced state and has negligible effect on activity.

It is interesting to note that the leucine side chain increases the affinity by a factor of 100-150 which was also found for the hydroxamate series (16).

The synthesis of L-leucinthiol was accomplished essentially in three steps from its oxygen analog as follows:



First, the amino group was protected by trifluoroacetylation in pyridine and the hydroxyl group was activated by conversion to the tosyl derivative. Displacement of the tosyl group by thiolacetic acid followed by alkaline hydrolysis gave the desired product which was isolated in the more stable disulfide dihydrochloride form. The disulfide had no effect on the enzyme and showed inhibitory activity only after incubation with dithiothreitol. In fact, the inhibition disappeared upon prolonged exposure to air but could be restored by further addition of dithiothreitol (Fig. 2). Similar effects were observed with β-mercaptoethylamine. The results indicate that the reduced thiol is the active form of these inhibitors.

Leucinthiol, like leucine hydroxamate (16), protects aminopeptidase from inactivation by EDTA. This effect could be used to follow the interaction

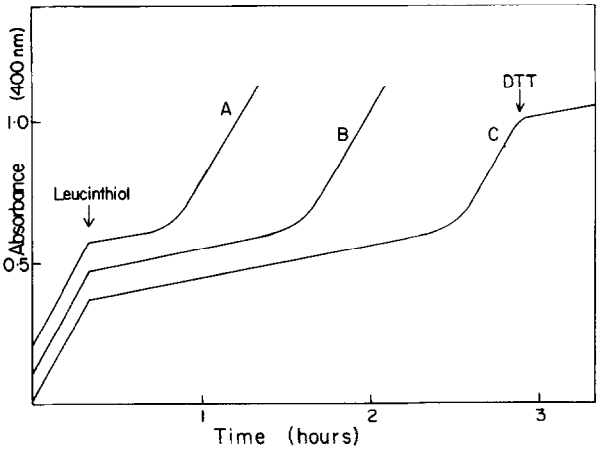


Fig. 2. Dependence of inhibition on dithiothreitol. Time course of the enzyme reaction was recorded at the following concentrations of dithiothreitol: A, 7.5 μM, B, 10 μM, and C, 15 μM. At the first arrow, L-leucinthiol was added to all samples to a final concentration of 0.5 μM. At the second arrow, fresh dithiothreitol was added (0.5 mM).

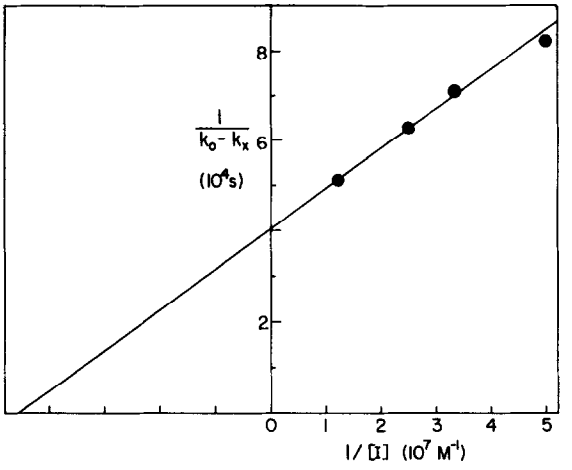


Fig. 3. Effect of leucinthiol on the EDTA-inactivation rate of the enzyme. k_x represents the first-order rate constants at different concentrations ([I]) of leucinthiol. k_o, the corresponding value in the absence of leucinthiol has a value of about $3 \times 10^{-5} s^{-1}$ at 37°C.

between the inhibitor and the enzyme. If k_o represents the first-order rate constant for the inactivation of the free enzyme and k_∞ that of the enzyme-inhibitor complex, then the observed rate constant k_x at any inhibitor concentration is given by $k_x([E] + [EI]) = k_o[E] + k_\infty [EI]$. Using the relationship $[E][I] = K_i[EI]$ it can be shown that $(k_o - k_\infty)/(k_o - k_x) = 1 + K_i/[I]$ therefore a plot of $1/(k_o - k_x)$ against $1/[I]$ gives a straight line with vertical intercept of $1/(k_o - k_\infty)$ and horizontal intercept of $-1/K_i$. The results provide an independent estimate for K_i of 2.2×10^{-8} M identical to that determined above (Fig. 3). The extrapolated value of k_∞ is close to zero indicating essentially complete protection.

Leucinthiol contains the hydrophobic side chain and the equivalent of the α-amino group of specific substrates. The thiol is located at the carbonyl position of the scissile peptide bond of normal substrates. The high affinity of this inhibitor suggests that the carbonyl is normally co-ordinated to the zinc atom in the active site. Thus the geometry of this site and the catalytic mechanism are likely to be similar to those of carboxypeptidase and thermolysin which have been characterized more extensively. There are indications that aminopeptidase, in common with most proteolytic enzymes, contains subsites which contribute to substrate binding (20,21). By introducing substituents

which can interact with these additional sites, it is expected that even more potent inhibitors can be obtained. Since aminopeptidase is believed to participate in the inactivation of enkephalin (22), these inhibitors may have significant physiological effects and potential pharmacological interest.

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