⑲ Europäisches Patentamt
European Patent Office
Office européen des brevets

⑪ Publication number: 0 209 855
A2

⑫ EUROPEAN PATENT APPLICATION

㉑ Application number: 86109849.9

㉒ Date of filing: 17.07.86

㊿ Int. Cl.⁴: A61K 31/195 , A61K 31/415

㉚ Priority: 18.07.85 US 756252

㊸ Date of publication of application:
28.01.87 Bulletin 87/05

㊹ Designated Contracting States:
AT BE CH DE FR GB IT LI LU NL SE

㉛ Applicant: WICHITA STATE UNIVERSITY
1845 Fairmount
Wichita Kansas 67208(US)

㉜ Inventor: Groutas, William C., Dr.
1703 Red Oaks
Wichita, Kansas 67207(US)

㉞ Representative: Henkel, Feiler, Hänzel & Partner
Möhlstrasse 37
D-8000 München 80(DE)

㊴ Amino acid derivatives useful for deactivating enzymes.

㊼ Certain amino-acid derivatives having three or four carbon straight alkyl chains are disclosed as effective inhibitors of human leukocyte elastase and therefore useful in preventing the imbalance of this proteolytic enzyme in vivo. The compounds specifically are L-valine, L-norvaline, L-norleucine, and L-methionine methyl ester azolides and sulfonate salts, and these compounds plus L-leucine and L-phenylalanine methyl ester sulfonate salts. The sulfonate salts were found to be excellent inhibitors in that they embody both inhibitory and anti-oxidant activity.

EP 0 209 855 A2

Xerox Copy Centre

AMINO ACID DERIVATIVES USEFUL FOR DEACTIVATING ENZYMES

This invention relates to the discovery that certain amino acid derivatives are capable of irreversibly deactivating the proteolytic enzyme human leukocyte elastase (HLE). The inhibitors of this invention have been found to bond to primary specificity sites to thereby irreversibly eliminate enzyme activity.

Pulmonary emphysema is a disease characterized by alterations in the physiological lung function related to the loss of elastic recoil. There is an uncontrolled degradation of lung connective tissue coincident with the development of emphysema.

It has been hypothesized that the development of emphysema stems from a proteinase proteinase-inhibitor imbalance. Human alpha-1-proteinase inhibitor (alpha-1-PI) is a defense protein that controls that activity of proteolytic enzymes, and that of HLE in particular. The inhibitory activity of alpha-1-PI is drastically reduced when the reactive site methionine residue is oxidized to the sulfoxide form by exogenous oxidants such as ozone, oxidants in cigarette smoke, or oxygen derived endogenous oxidants. The resulting proteinase proteinase-inhibitor imbalance often results in uncontrolled degradation of lung connective tissue. Accordingly, an ideal inhibitor of HLE would embody both inhibitory and anti-oxidant activity.

It is known that aliphatic isocyanates can be used to selectively inhibit serine proteinases. However the high toxicity of isocyanates renders them undesirable from a pharmacological standpoint. There are, however, certain compounds known to function as latent isocyanates which do not possess high toxicity.

Selective inhibition is necessary so that an inhibitory agent will not affect closely related proteinases. Although HLE, cathepsin G, porcine pancreatic elastase (PPE) and alphachymotrypsin have many common features, such as similar catalytic apparatus, and extended binding site and a preference for hydrophobic substances (or inhibitors), they differ from each other in their substrate specificities. These diferences in substrate preference arise from variations in the size of the binding cleft at the catalytic sites. Studies have shown that PPE can accommodate small hydrophic side chains at its primary specificity site S_1. The S_2-S_4 subsites of PPE are similar to those of HLE while S_1 subsite of HLE is larger, enabling the accommodation of larger side chains at S_1. The N-terminal of HLE is homologous to that of PPE.

It was reported in Biochemical and Biophysical Research Communications, Vol. 45, No. 4, Pages 1890-94 (1980) that certain imidazole-N-carboxamides actively inhibited PPE. Specifically, the n-butyl derivative was found to be particularly capable of binding to the active site of the enzyme. These compounds were known to dissociate into isocyanates and imidazole under very mild conditions. Such compounds however were not found to be effective with HLE.

Subsequently it was reported at the American Chemical Society Division of Medicinal Chemistry, 183rd ACS National Meeting, March 28-April 2, 1982, that irreversible inhibition of PPE by amino acid derivative imidazole-N-carboxamides had been discovered. Particularly active were those derived from DL-norvaline and L-valine methyl esters. The inhibitor derived from glycine ethel ester however was found to be inactive, while that derived from D-valine methyl ester exhibited marginal activity. It was speculated that the compounds functioned via an enzyme induced generation of an isocyanate moiety at the active sight of the enzyme.

The interaction of a serine proteinase with alpha-1-PI results in the formation of a hightly stable 1:1 complex involving the active site serine of the proteinase and a methionine-serine peptide bond in the alpha-1-PI. When the critical methionine residue at the active site of the alpha-1-PI is oxidized to the corresponding sulfoxide, the proteinase inhibitor is inactivated, creating an imbalance.

It has been discovered, however, that certain amino-acid-derived azolides are effective inhibitors of HLE, but not of PPE. These compounds may be effective based upon the fact that the active site of HLE is hydrophobic and prefers a valine, norvaline or norleucine residue at its S_1 subsite.

Because HLE prefers hydrophobic substrates, potential inhibitors had been chosen based upon this important fact. Consequently, inhibitors reported were highly hydrophobic with limited solubility in aqueous media. It has now been discovered, however, that sulfonate salts of certain amino acids demonstrate for the first time that ionic compounds can function as inhibitors of HLE. While it is not known with certainty, it is believed that the group bearing the charge must be disposed on the leaving group side. It is likely that the sulfonate group is not occupying the S_1 site, but rather is pointed away from the surface of the enzyme and toward the aqueous milieu.

Accordingly, these ionic derivatives of the methyl esters of L-valine, L-norvaline, and L-norleucine were found to be very potent inhibitors. In addition, the sulfonate salts of methyl esters of L-leucine, L-methionine and L-phenylalanine also inactivate HLE, but not as efficiently.

Accordingly, it is an object of this invention to provide pharmaceutically effective inhibitory compounds for HLE.

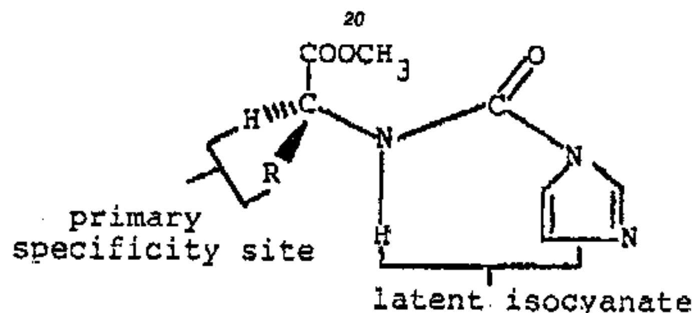
It is another object of this invention to provide pharmaceutically effective inhibitory compounds which exhibit both inhibitory and anti-oxidant activity.

It is another object of this invention to provide amino acid derivatives which either function as latent isocyanates or as ionic compounds derived from isocyanates which will irreversibly inhibit the enzymatic activity of HLE.

These and other objects will become readily apparent with reference to the following description.

As noted above, in recent years attempts to map the active sites of certain proteolytic enzymes has proven successful. This work paved the way for the development of irreversible and reversible inhibitors that can discriminate between individual members of this class of enzymes. These studies have demonstrated that properly designed small organic molecules can be used to inhibit selectively individual serine proteases, even those these molecules cannot participate in secondary subsite interactions.

It has previously been shown that aliphatic isocyanates can be used to inhibit selectively the serine proteases. However, the high toxicity of isocyanates has prompted investigation into the inhibitory activity of compounds that function as latent isocyanates. Certain azolides of the formula identified below derived from appropriate amino acid esters have been found to irreversibly inhibit both PPE and HLE and demonstrate that masked isocyanates can be used as inhibitors.



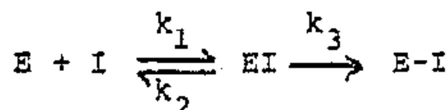
The compounds of this invention and related compounds also evaluated, as will be subsequently described, were synthesized using known techniques. The following is an example:

L-N-(Imidazol-1-ylcarbonyl)norvaline Methyl Ester. A 1.36g(0.02 mol) sample of imidazole was mixed with 3.14g (0.02mol) of L-norvaline methyl ester isocyanate in 20 mL of anhydrous ethyl ether under a nitrogen atmosphere. The reaction mixture was refluxed with stirring for two hours. Removal of the solvent in vacuo left a colorless oil which solidified upon refrigeration. The isolated solid was homogeneous by TLC(silica gel:ethyl acetate/chloroform, 1:1), melted at 86-88 °C, and was obtained in 96% yeild (4.3g). Anal. - ($C_{10}H_{15}N_3O_3$)C, H, N.

The enzyme assays used to evaluate inhibitors were performed as follows: PPE was assayed spectrophotometrically at 347.5nm with (tert-butoxycarbonyl)-alanyl-p-nitrophenol in 0.05M phosphate buffer, pH 6.5, 25 °C, or by using succinyl-L-trialanyl-p-nitroanilid in 0.1M Tris buffer, pH 8.0, at 410nm. HLE was assayed spectrophotometrically at 410nm with succinyl-L-trialanyl-p-nitroanilid in 0.1M Tris buffer, pH 8.0.

The appropriate amount of inhibitor in acetonitrile was mixed with PPE in phosphate buffer solution and placed in a constant temperature bath. Aliquots were withdrawn at different time intervals and transferred to a cuvette containing 100µL of substrate, 140µL of acetonitrile, and 2mL of phosphate buffer, pH 6.5. After incubating for 30 seconds, the absorbance change was monitored for two minutes at 347.5nm. The inhibitor-to-enzyme ratios varied between 50 and 200, and the incubation intervals were shortened or lengthened depending upon the potency of the inhibitor. Controlled reactions for 100% PPE activity were run in the presence of acetonitrile. A similar procedure was followed for HLE.

The reaction of an irreversible inhibitor with PPE and HLE may be illustrated by the scheme shown below:



where EI is the noncovalently bound enzyme-inactivator complex and E-I is the final product with the inhibitor covalently bound to the enzyme. The kinetics data were analyzed according to known procedures. The apparent pseudo-first-order inactivation rate constants were determined from the slopes of semilogarithmic plots of enzymatic activity remaining versus time using the following equation:

$$\ln(E_t/E_o) = k_{obsd}t$$

where E_t/E_o is the amount of active enzyme remaining after time "t". Inhibition is generally expressed in terms of k_3/K_i, $M^{-1}s^{-1}$, wherein K_i is the disassociation constant for the enzyme inhibitor complex and k_3 is a limiting rate constant for the irreversible loss of enzymatic activity.

Table I below illustrates the inhibition found with assays of nine amino-acid-derived azolides against PPE and HLE.

TABLE I

Compd	R	Porcine pancreatic elastase k_3/K_i, $M^{-1}s^{-1}$	Human leukocyte elastase k_3/K_i, $M^{-1}s^{-1}$
1	H		
2	L-CH_3		
3	L-$(CH_3)_2CH$	2.8	193
4	D-$(CH_3)_2CH$		
5	L-$CH_3CH_2CH_2$	6.3	500
6	D-$CH_3CH_2CH_2$		
7	L-$(CH_3)_2CHCH_2$	0.5	
8	L-CH_3CH_2-$CH(CH_3)$	1.0	
9	DL-CH_3CH_2-CH_2CH_2	2.2	167

With attention to Table I above, the glycine derivative, Compound 1, failed to deactivate either PPE or HLE. Compound 2 showed marginal inhibitory activity, but the valine derivative, Compound 3, was found to be an excellent inhibitor against HLE and a poor inhibitor of PPE. As anticipated, the corresponding D-isomer, Compound 4, had no effect on either PPE or HLE, reflecting unproductive

binding and/or wrong stereochemical alignment of the inhibitor reactive site and the catalytic residues. HLE apparently shows a preference for a 3 or 4 straight alkyl chain at the P_1 site and, accordingly, the norvaline derivative, Compound 5, and the norleucine derivative, Compound 9, were found to be effective inhibitors of HLE. Thus, Compounds 3, 5, and 9 show an avidity for HLE, but not PPE.

In addition to the compounds evaluated above, a methionine derivative, N-(1H-imidazol-1-ylcarbonyl)-methionine methyl ester, was evaluated. This compound exhibited a remarkable specificity and high potency toward HLE, but PPE was unaffected by the inhibitor under the same conditions. While it is not intuitively apparent why such a high specificity is shown by the methionine derivative, it is likely that the side chain of methionine is highly complementary to the S_1 subsite of HLE. Steric effects probably play a large role as far as the lack of inhibitory activity toward PPE is concerned. This finding is of pharmaceutical significance since the isosteric replacement of a CH_2 by sulfur gives rise to a highly biospecific inhibitor.

As noted above, the foregoing derivatives are hydrophobic and, therefore, only weakly soluble in aqueous solution. It has been discovered, however, that excellent inhibitory activity against HLE can be exhibited with ionic compounds.

Monopotassium salts were produced by admixing the appropriate amino acid ester isocyanate and an aqueous solution of potassium metabisulfite in dioxane. The monopotassium sulfonate salts had the following formula:

$R\text{-}CH(COOCH_3)NH\text{-}CO\text{-}HSO_3^{\,-}K^+$.

Table II below summarizes the results of enzyme assays against HLE for each of the enumerated compounds. The assay procedure used was the procedure identified relative to the tests above in Table I.

TABLE II

Compound	Precursor Amino Acid	R	k_2/K_i $M^{-1}sec^{-1}$
1	Gly	H	inactive
2	L-Ala	CH_3	inactive
3	L-Val	$(CH_3)_2CH$	800
4	L-Norval	$CH_3CH_2CH_2$	860
5	D-Norval	$CH_3CH_2CH_2$	inactive
6	L-Norleu	$CH_3CH_2CH_2CH_2$	920
7	L-Leu	$(CH_3)_2CHCH_2$	260
8	L-Met	$CH_3SCH_2CH_2$	260
9	L-Phe	$PhCH_2$	530

To summarize, the sulfonate salts of L-valine, L-norvaline, and L-norleucine methyl esters proved to be very potent inhibitors of HLE. In addition, the derivatives of L-leucine, L-methionine, and L-phenylalamine also deactivate HLE but were not as effective. This is in agreement with what is presently known about the primary specificity site (S_1 subsite) of HLE. The active site of HLE is hydrophobic and prefers a valine, norvaline, or norleucine residue at its S_1 subsite. In further tests, for example, L-norvaline had no effect on chymotrypsin and PPE under comparable conditions, attesting to the high specificity of the inhibitor. Thus, selective inhibition can be achieved by manipulating the side chain that binds to the S_1 subsite of each serine proteinase, since the topographical features of the active sites of the various serine proteases are known to be different. As

expected, the inhibitor derived from D-norvaline methyl ester showed no inhibitory activity toward HLE. The compounds derived from glycine and L-alanine were also devoid of any inhibitory activity. The lack of inhibitory activity of these compounds can be ascribed to poor binding. Accordingly, the amino acid sulfonate salts of this invention demonstrate that ionic compounds can function as inhibitors of HLE provided the group bearing the charge is on the leaving-group side. As noted above, it is unlikely that the sulfonate group occupies the S_1 subsite. It is likely that this group is pointing away from the surface of the enzyme and toward the aqueous milieu. The high potency and specificity exhibited by these inhibitors, as well as their ease of preparation, render them highly useful as enzyme probes and pharmacological agents.

The L-norvaline azolide, Compound 5, Table I, was also evaluated in vivo.

In Experiment 1, the tolerance of 30-40 gram male ICR mice to intraperitoneal (IP) or intratracheal (IT) injection of inhibitor was evaluated. Table III below summarizes the results thereof:

TABLE III

Treatment	Volume	Survival	Total Inhibitor Injected
IP 20% DMSO	2.0 ml	+	0
IP 20% DMSO + 0.02 M Inhibitor	2.0 ml	+	40 μmoles
IP 100% DMSO	0.02 ml	+	0
IP 100% DMSO + 2.0 M Inhibitor	0.02 ml	+	40 μmoles
IT Saline + 5% DMSO	0.02 ml	+	0
IT Saline + 5% DMSO + 0.05 M Inhibitor	0.02 ml	+	1 μmole

As can be seen above, all animals survived and no animals showed abnormal pulmonary pathology.

Animals were also used to evaluate the effect of simultaneous intratracheal installation of PPE with the above inhibitor compound. The ICR mice in this experiment were used to evaluate the effect of the inhibitor in preventing emphysema induction. Table IV below illustrates the results of these tests:

TABLE IV

Treatment	Inhibitor	Emphysema
IT 34 µg PPE	-0-	Yes
IT -0-	1 µmole	No
IT 34 µg PPE	1 µmole	No

All animals were sacrificed ten days after installation. As can be seen, the inhibitor prevented emphysema when given simultaneously with PPE. Therefore, the free functional enzyme did not dissassociate in vivo.

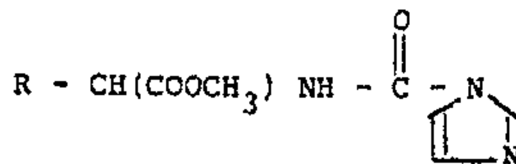
To summarize, it has been discovered that azolide derivatives of certain amino acids and the sulfonate salts thereof are highly specific inhibitors for HLE and, therefore, are useful pharmacological agents in the prevention of the HLE imbalance which results in emphysema. The L-valine, L-norvaline, and L-leucine, and methionine azolides prove to be highly effective. In addition, the L-valine, L-norvaline, L-norleucine, and L-leucine, L-methionine and L-phenylalamine sulfonate salts also prove to be effective inhibitors of HLE. Related alkyl amino acids prove to be ineffective.

The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The present embodiment is therefore to be considered in all respects as illustrative and not restrictive, the scope of the invention being indicated by the appended claims rather than by the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.

Claims

1. Method for deactivating the proteolytic enzyme human leukocyte elastase in vivo comprising:

administering an inhibitory effective amount of an amino acid methyl ester derivative having the formula:



wherein R is a member selected from the group consisting of L $(CH_3)_2CH$-; L CH_3-CH_2-CH_2-; DL CH_3-CH_2-CH_2-CH_2-; or L CH_3-S-CH_2-CH_2-.

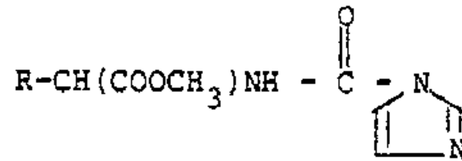
2. Method for deactivating the proteolytic enzyme human leukocyte elastase in vivo comprising:

administering an inhibitory effective amount of an amino acid methyl ester sulfonate derivative having the formula:

R -CH($COOCH_3$) NH -CO -$HSO_3^-K^+$

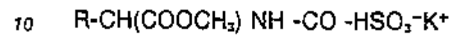
wherein R is a member selected from the group consisting of L$(CH_3)_2CH$-; L-CH_3-CH_2-CH_2-; DL-CH_3-CH_2-CH_2-CH_2-; L CH_3-S-CH_2-CH_2-; L$(CH_3)_2CH$-CH_2-; or L C_6H_5-CH_2.

3. An inhibitor composition effective to selectively deactivate the proteolytic enzyme human leukocyte elastase comprising: an amino acid methyl ester derivative having the formula:



wherein R is a member selected from the group consisting of L $(CH_3)_2CH$-; L CH_3-CH_2-CH_2-; DL CH_3-CH_2-CH_2-CH_2-; or L CH_3-S-CH_2-CH_2-, in a pharmaceutically acceptable dosage form.

4. An inhibitor composition effective to selectively deactivate the proteolytic enzyme human leukocyte elastase comprising: an amino acid methyl ester sulfonate derivative having the formula:



R-CH($COOCH_3$) NH -CO -$HSO_3^-K^+$

wherein R is a member selected from the group consisting of: L$(CH_3)_2$; L CH_3-CH_2-CH_2-; DL CH_3-CH_2-CH_2-CH_2-; L CH_3-S-CH_2-CH_2-; L$(CH_3)_2CH$-CH_2-; or L C_6H_5-CH_2-, in a pharmaceutically acceptable dosage form.