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IRREVERSIBLE INHIBITION OF SERINE PROTEASES BY PEPTIDYL DERIVATIVES OF α-AMINOALKYLPHOSPHONATE DIPHENYL ESTERS

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SUMMARY: Peptidyl α-aminoalkylphosphonate diphenyl esters have been synthesized and shown to be effective inhibitors of serine proteases. Extending the peptide chain from a single α-aminoalkylphosphonate residue ($k_{obs}/[I]$=2.5-260 $M^{-1}s^{-1}$) to a tripeptide or tetrapeptide derivative ($k_{obs}/[I]$=7,000-17,000 $M^{-1}s^{-1}$) resulted in 65-2800 improvement in inhibitory potency and increased specificity. The rate of inactivation of chymotrypsin by MeO-Suc-Ala-Ala-Pro-HNCH(CH_2Ph)P(O)(OPh)$_2$ was decreased 5 fold in the presence of the substrate Suc-Val-Pro-Phe-NA (0.119 mM). Phosphonylated serine proteases are extremely stable since the half-life for reactivation was >48 hrs for the inhibited elastases and at least 10 hrs for chymotrypsin.

Phosphorylating agents such as diisopropylfluorophosphate (DFP) are classical inhibitors for serine proteases and have been used diagnostically in the identification and classification of new proteolytic enzymes (1). The mechanism of inactivation involves phosphorylation of the active site serine (2) and a single covalent bond between the active site serine and the inhibitor phosphorus atom has been found in the crystal structure of diisopropylphosphoryl trypsin (3). The phosphorylated enzymes are considered to be good transition state analogues due to the presence of the tetrahedral phosphorus atom (4) and have proved to be useful tools for X-ray and ^{31}P NMR investigations (5). Most reported inhibitors are organophosphorus fluoridates or *p*-nitrophenyl esters, since only compounds with good leaving groups at the phosphorus atom are able to react with the active site serine. In the case of *p*-nitrophenyl esters some selectivity was observed (6), but generally organic phosphate derivatives are nonspecific serine proteases inhibitors and extremely toxic due to reaction with acetylcholinesterase.

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Abbreviations: HLE, human leukocyte elastase; iPr, *iso*-propyl; NA, 4-nitroanilide; Ph, phenyl; PPE, porcine pancreatic elastase.

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To enhance the selectivity Lamden and Bartlett (7) reported the synthesis of some phosphonate derivatives of amino acids where the representative phenylalanine related structure Z-NH-CH(CH_2Ph)P(O)(O-i-Pr)F had an inactivation rate constant of 180,000 $M^{-1}s^{-1}$ with chymotrypsin, but only 160 $M^{-1}s^{-1}$ with porcine pancreatic elastase. The presence of a phosphorus-fluorine bond makes this α-aminoalkylphosphonic acid derivative extremely unstable and it undergoes rapid hydrolysis during the enzyme assays. In a continuation of this study, Bartlett et al.,(8) reported the synthesis of phosphonates with different leaving groups and peptide analogs incorporating a tetrahedral phosphorus moiety in the place of the scissile carbonyl group of an extended peptide substrate. The peptide at the leaving group side of the inhibitor significantly decreased inhibitory potency and the inhibition rate constants for the tetrapeptides were only 12-27 $M^{-1}s^{-1}$. Here we report that peptides with substrate related sequences containing C-terminal α-aminoalkylphosphonate diphenyl esters specifically and irreversibly inactivate serine proteases including bovine chymotrypsin, porcine pancreatic and human leukocyte elastases, to give extremely stable inhibitor-enzyme complexes.

MATERIALS AND METHODS

Human leukocyte elastase (HLE) was supplied to us by Dr. James Travis and his research group at the University of Georgia. Porcine pancreatic elastase (PPE) was purchased from Worthington, bovine chymotrypsin from the Sigma Chemical Company. N-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES), triphenyl phosphite, benzyl carbamate, aldehydes and all common chemicals and solvents were obtained from the Aldrich Co., Milwaukee WI. MeO-Suc-Ala-Ala-Pro-Val-NA (9), Suc-Ala-Ala-Ala-NA, and Suc-Val-Pro-Phe-NA (10) were prepared as previously described.

Synthesis of Inhibitors. Diphenyl α-(N-benzyloxycarbonylamino)alkyl-phosphonates were obtained by the method described earlier (11) and converted by the dicyclohexylcarbodiimide coupling method to the desired tri- and tetrapeptide inhibitors. The final products were purified by thin layer chromatography (TLC) on silica gel. The purity of each compound was checked by ^{1}H-NMR, ^{31}P-NMR, IR, mass spectroscopy, TLC and elemental analysis, and in each case the analytical results were consistent with the proposed structure. Racemic diphenyl α-aminoalkylphosphonates were used for the synthesis of the peptide derivatives and the products are mixtures of diastereromers. Full details of the syntheses will be presented elsewhere. The UV spectrum and ^{31}P NMR of several inhibitors (**1, 3, 4, & 5**) in HEPES buffer containing 10% Me_2SO or Me_2SO-d_6 showed no changes after three days incubation indicating no hydrolysis.

Enzyme Inactivation-Incubation method. Inactivation was initiated by adding a 25-50 μL aliquot of inhibitor in Me_2SO to 0.5 ml of a buffered enzyme solution (0.1-2.0 μM) such that the final Me_2SO concentration was 5-10 % v/v at 25°C. Aliquots were removed periodically and diluted into substrate solution (40-200 fold dilution). The residual enzyme activity was measured spectrophotometrically in 0.1 M HEPES, 0.5 M NaCl, pH 7.5 using the following substrates: chymotrypsin, Suc-Val-Pro-Phe-NA (0.476 mM) (10); HL elastase, MeO-Suc-Ala-Ala-Pro-NA (0.482 mM) (10); PP elastase, Suc-Ala-Ala-Ala-NA (0.714 mM) (10). Peptide *p*-nitroanilide hydrolyses were monitoring at 410 nm (12). First order inactivation rate constants (k_{obs}) were obtained from plots of ln v_t /v_o vs. time. Inactivation rate constants shown in Table 1 are typically

the average of the duplicate or triplicate experiments and inhibitor concentration are shown in Table 1.

Dephosphonylation Kinetics. The dephosphonylation rate constants of inactivated enzyme (<5% of activity in most cases) were measured after removal of excess inhibitor by centrifuging with Centricon-10 microconcentrators twice at 0 °C for 1 hr after the addition of fresh buffer. The solution was warmed to 25 °C, aliquots were removed at intervals, and the enzymatic activity was assayed as described above. The half time for dephosphonylation was obtained from plots of ln $(v_o - v_t)$ vs. time where v_o is the enzymatic rate under the same conditions for the uninhibited enzyme.

Determination of Inactivation Rates in the Presence of Substrate-Progress Curve Method. In some cases, $k_{obs}/[I]$ values were determined in the presence of substrate as described by Tian and Tsou (13). For example inactivation of chymotrypsin (0.109 μM) by MeO-Suc-Ala-Ala-Pro-NH-$CH(CH_2Ph)P(O)(OPh)_2$ (1.5-4.0 μM) in the presence of 0.476, 0.357, and 0.119 mM Suc-Val-Pro-Phe-NA was measured by addition of a 0.01 mL aliquot of enzyme to a substrate and inhibitor solution containing 10% of Me_2SO. The increase in absorbance was monitored (410 nM) with time until no further release of *p*-nitroaniline was observed. The $k_{obs}/[I]$ values were calculated from plots of $\log([P]_\infty - [P]_t)$ vs. time, where $[P]_\infty$ and $[P]_t$ are the concentration of *p*-nitroaniline after total inactivation and at time t, respectively.

RESULTS

Inactivation kinetics. The second order rate constants ($k_{obs}/[I]$) for inactivation of chymotrypsin, PPE, and HLE by diphenyl α-aminoalkylphosphonate derivatives are reported in **Table 1.** The psuedo first-order inactivation plots remained linear for greater than four half-lives. Inactivation of the three serine proteases by diphenyl α-(N-benzyloxycarbonylamino)alkylphosphonates **1** and **4** (analogues of Val and Phe respectively) was very slow. However, clear selectivity was observed since the phenylalanine analog **4** is most reactive with chymotrypsin with a rate constant of 260 $M^{-1}s^{-1}$, reacted slowly with HLE (6.0 $M^{-1}s^{-1}$), and did not

TABLE 1. RATE CONSTANTS ($k_{obs}/[I]$) FOR THE INACTIVATION OF SERINE PROTEASES BY DIPHENYL 1-(N-BENZYLOXY-CARBONYLAMINO)ALKYLPHOSPHONATES AND PEPTIDES WITH C-TERMINAL DIPHENYL 1-AMINOALKYLPHOSPHONATE RESIDUES[a]

	Inactivator	Chymotrypsin[b]		PPE[c]		HLE[d]	
		[I] (μM)	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)	[I] (μM)	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)	[I] (μM)	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)
1	Z-HN-CH(i-Pr)P(O)(OPh)$_2$	50	0.4	50	2.5	26	90
2	Z-Ala-Ala-HN-CH(i-Pr)P(O)(OPh)$_2$	240	2.4	24.0	340	14.0	1300
3	MeO-Suc-Ala-Ala-Pro-HN-CH(i-Pr)P(O)(OPh)$_2$	180	21	9	7100	4.9	7100
4	Z-NH-CH(CH_2Ph)P(O)(OPh)$_2$	58	260	58	NI	30	6.0
5	MeO-Suc-Ala-Ala-Pro-HN-CH(CH_2Ph)P(O)(OPh)$_2$	11.0	11,000	220	0.34	120	7.2
6	Z-Phe-Pro-HN-CH(CH_2Ph)P(O)(OPh)$_2$	5.0	17,000	93	NI	51	1.5

[a]Conditions were 0.1 Hepes, 0.5 M NaCl, pH 7.5 at 25 °C. Rate constants were measured as described under Materials and Methods. [b]Enzyme concentration 1.9 μM. [c]Enzyme concentration 1.7 μM. [d]Enzyme concentration 0.32 μM. NI, less than 5% inhibition after 4 hrs.

react with PPE. In contrast, the valine analog **1** is most reactive toward HL elastase (90 $M^{-1}s^{-1}$) and PP elastase (2.5 $M^{-1}s^{-1}$), but is a very poor inhibitor for chymotrypsin (0.4 $M^{-1}s^{-1}$). The tripeptide valine derivative Z-Ala-Ala-HN-CH(i-Pr)P(O)$(OPh)_2$ **2** was a much more effective elastase inhibitor and had rate constants of 340 $M^{-1}s^{-1}$ with PPE (136 fold improvement compared to **1**) and 1300 $M^{-1}s^{-1}$ with HLE (14 fold improvement). Addition of another aminoacid residue again significantly improved inhibitory potency and the tetrapeptide valine derivative **3** inhibited PPE and HLE (7100 $M^{-1}s^{-1}$ for both) respectively 2800 and 79 fold better than **1**. The tetrapeptide **3** also inhibited chymotrypsin at a 340 fold lower rate, but at the concentrations used to effectively inhibit the elastases (9 or 4.9 μM), **3** did not inhibit chymotrypsin. The peptide phosphonate analogs of phenylalanine were the most effective inhibitors of chymotrypsin with rate constants of 11,000 $M^{-1}s^{-1}$ for **5** and 17,000 $M^{-1}s^{-1}$for **6**, which are respectively 42 and 65 fold increases in rate when compared to the amino acid derivative **4**. These peptide chymotrypsin inhibitors are quite specific since **6** does not react with PPE and inhibits only HLE very slowly.

Substrate Protection. Addition of substrate to the enzyme incubation mixture resulted in significant decreases in inactivation rate constants. The rate constants ($k_{obs}/[I]$) for the inactivation of chymotrypsin (0.109 μM) by **5** in presence of 0.476, 0.357 and 0.119 mM Suc-Val-Pro-Phe-NA were 900, 1070 and 2200 $M^{-1}s^{-1}$ respectively compared to 11,000 $M^{-1}s^{-1}$ in the absence of the substrate.

Dephosphonylation Kinetics. Less than 5% of the enzymatic activity was regained after removal of excess inhibitor by diluting with buffer and centrifuging twice at 0 °C. This is consistent with the formation of a covalent enzyme-inhibitor adduct. Chymotrypsin inactivated by **4** and **5** regained activity slowly upon further standing 25 °C ($t_{1/2}$= 10 and 16 hrs respectively). With the elastases all inhibitors gives very stable inhibitor-enzyme complexes with reactivation half-lives greater than 48 hrs.

DISCUSSION

There are at least three factors which should be considered in designing new therapeutically useful organophosphorus irreversible serine proteases inhibitors: reactivity, chemical stability and specificity. Reactivity and chemical stability are determinated by the electrophilicity of the phosphorus atom. Organophosphorus inhibitor with very electronegative leaving groups will have increased reactivity toward serine proteases, but also decreased stability toward hydrolysis. For example, attachment of a fluorine atom to phosphorus has resulted in some excellent inhibitors, but these also undergo very fast nonenzymatic hydrolysis. Decreasing the electronegativity of the

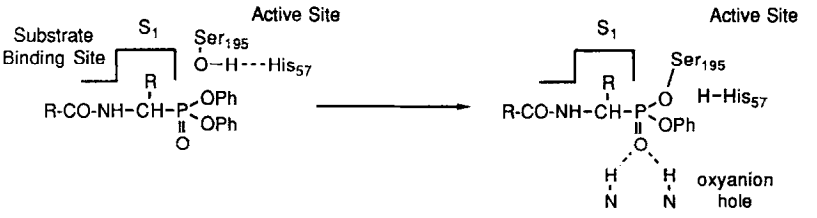

Figure 1. Proposed scheme for the reaction of a serine protease with a peptidyl derivative of an α-aminoalkylphosphonic acid diphenyl ester.

leaving group substantially increases the chemical stability of the inhibitor, but at the cost of lower inactivation rates. Specificity results when there are structural and stereochemical similarities between a natural serine protease substrate and the potential organophosphorus inhibitor, and could provide the ability to discriminate not only between different serine proteases but also between serine proteases and other serine hydrolases such as acetylcholine esterase. Thus, an ideal irreversible serine protease organophosphorus inhibitor represents a delicate balance between several competing factors which determines its specificity and reactivity.

The peptide derivatives of α-aminoalkylphosphonate diphenyl esters reported here are one solution to the problem of designing an ideal organophosphorus serine protease inhibitor. The presence of the two phenoxy groups on the phosphonate moiety makes the phosphorus atom sufficient electrophilic to undergo reaction with the active site serine of serine proteases. The available evidence is consistent with formation of a covalent bond due to phosphonylation of the active site serine by the diphenyl α-aminoalkylphosphonate derivatives (Figure 1). The rate of inactivation of chymotrypsin by **5** is decreased significantly in the presence of a substrate and this demonstrates active site involvement. In addition, the enzyme-inhibitor complex is extremely stable with the elastases ($t_{1/2}$ = >48 hrs). In the case of chymotrypsin inactivated by **4** and **5**, enzyme activity was very slowly regained with a half-lives of about 10 hrs.

Peptide derivatives of α-aminoalkylphosphonic diphenyl esters are hydrolytically stable and no changes were observed in either the UV spectrum or ^{31}P-NMR spectrum after incubation in buffer for 3 days. The fluoridates of α-aminoalkylphosphonic acids reported earlier (7) are potent inhibitors of serine proteinases but are too hydrolytically unstable to be considered as therapeutically useful compounds. Tetrapeptides with the α-amino alkylphosphonate moiety inside of the peptide chain are irreversible chymotrypsin inhibitors and have K_I values close to the K_M value for the corresponding peptide substrate (8). However, they inhibit chymotrypsin very poorly with the rate constants of 12-27 $M^{-1}s^{-1}$. This can be explained partially by the fact that the phosphoramides, unlike a carboxylic amide,

prefers the cis conformation of the PO and NH groups (14). This destroys the similarity between the inhibitor and a substrate at least in the region of the scissile bond. Additionally, the presence of the phosphonamidate or the phosphonic acid alkylester moiety in these derivatives significantly decreases the electrophilicity of the phosphorus atom, decreases reactivity toward nucleophiles, and results in the low inactivation rate constants.

The inhibition rate constants of serine proteases by α-aminoalkylphosphonic diphenyl esters may be moderate compared with the related hydrolytically unstable phosphonyl fluorides reported by Bartlett (7), but are quite respectably compared to other peptidyl irreversible serine protease inhibitors. For example, with elastase the inhibition rates are better than those observed with other peptidyl irreversible inhibitors such as peptide chloromethyl ketones ($k_{obs}/[I]$ values of 1-1560 $M^{-1}s^{-1}$, 15). Peptide chloromethyl ketones have little potential as therapeutic agents because of the probable toxicity which should result from their ability to alkylate various cellular nucleophiles (15). Diphenyl α-aminoalkylphosphonate derivatives do not suffer from this limitation and also show low reactivity with the serine hydrolase acetylcholine esterase (less than 5% inhibition after 3 hrs at the concentrations given in **Table 1** for **1**, **2**, & **5**).

The specificity of peptide inhibitors is determined mainly by interaction of the P_1 amino acid residue with the S_1 pocket (16) of the target enzyme. The peptide diphenyl esters with a P_1 phosphonate structurally related to phenylalanine, such as **4**, **5**, & **6**, are specific for chymotrypsin. The tripeptide derivative **6**, which contains a sequence which is preferred in substrates by a number of chymotrypsin-like enzymes (17), is indeed the best chymotrypsin inhibitor which we have discovered and has a inhibition rate constant of 17,000 $M^{-1}s^{-1}$. It is also quite specific since it reacts with the HLE at least 10,000 times slower than with chymotrypsin and does not react with PPE. Peptide phosphonate diphenyl esters related to valine (**2** & **3**) are at least 300-500 fold more reactive with elastases than with chymotrypsin. The tripeptide **3** is one of the better peptide irreversible inhibitors which has been reported for PPE (15).

Racemic mixtures of diphenyl α-aminoalkylphosphonates were used in the synthesis and the peptides shown in Table **1** are nonequivalent mixtures of diastereromers as shown by ^{31}P NMR measurements. It is likely that the enzymes are reacting with only one stereoisomer, while the second isomer reacts more slower or not at all. Preliminary experiments suggest that the correct isomer (same stereochemistry as an L-amino acid residue) reacts faster with serine proteases. It is possible that the other isomer is effectively decreasing the inhibition rate constants due to non-productive binding to the enzyme and thus the rate constants reported in table **1** should be improved once the pure diastereromers are isolated.

It is clear that peptide derivatives of α-aminoalkylphosphonic diphenyl esters are useful serine protease inhibitors and have potential utility as therapeutic agents. They are hydrolytically stable and form extremely stable irreversibly inhibited derivatives with several serine proteases. Resolution of the diastereromers and synthesis of peptides with more specific amino acid sequences should significantly improve the potency and specificity of the inhibitors. Additional specificity could also be introduced by replacing the phenoxy groups with other alcohols or phenols as long as the pK_a of the leaving group is close to that of phenol.

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