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Synthesis and kinetic evaluation of peptide α-keto-β-aldehyde-based inhibitors of trypsin-like serine proteases

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Abstract

New, synthetic peptide analogues bearing a C-terminal basic α-keto-β-aldehyde moiety were prepared as novel inhibitors of the trypsin-like serine proteases. The compounds, Ac-Leu-Leu-Arg-COCHO, Ac-Arg-Gln-Arg-COCHO and Boc-Val-Leu-Lys-COCHO were evaluated kinetically against trypsin and three other trypsin-like serine proteases, tryptase, plasmin and thrombin, all of which are implicated as mediators of important disease processes. Results illustrate that α-keto-β-aldehydes are potent inhibitors, with similar potency to comparable peptide aldehydes, and intriguingly, appear to act, in some instances, by a novel mechanism of action. Ac-Leu-Leu-Arg-COCHO, an analogue of the natural product leupeptin, is a potent, tight-binding inhibitor of trypsin ($K_{i(final)} = 1.9\ \mu M$), plasmin ($K_{i(final)} = 4.9\ \mu M$) and tryptase ($K_{i(final)} = 1.2\ \mu M$) and an irreversible inactivator of thrombin (k_{2nd} 4500 $M^{-1}.min^{-1}$). Boc-Val-Leu-Lys-COCHO was found to be a tight-binding inhibitor of its target protease plasmin ($K_{i(final)} = 3.1\ \mu M$) and was inactive against thrombin. Ac-Arg-Gln-Arg-COCHO was a slow-binding inhibitor of tryptase ($K_{i(final)} = 1.6\ \mu M$) and also irreversibly inactivated trypsin ($k_{2nd} = 8920\ M^{-1}\ min^{-1}$). Peptides or peptidomimetics with a C-terminal basic α-keto-β-aldehyde function thus provide a useful new molecular template for the development of new therapeutic agents against a wide range of disorders, such as coagulopathies and asthma, which may be mediated by the aberrant activity of trypsin-like serine proteases.

Introduction

Serine proteases have been shown to play a multifarious role in the aetiology of a number of important pathophysiological conditions, including coagulopathies (Vacca 1998), tumour invasion and metastasis (Andreasen et al 1997), COPD (chronic obstructive pulmonary disease) (Anderson & Shinagawa 1999) and asthma (Walls 1995). As a consequence, there has been considerable effort expended in the search for small molecule inhibitors of these enzymes as potential therapeutic agents.

One class of compounds which have received considerable attention are peptide aldehydes which act by mimicking the tetrahedral transition state of peptide bond hydrolysis, through the formation of a tetrahedral hemiacetal with the active site serine residue (Ortiz et al 1991).

The first reported peptide aldehydes, including leupeptin (Ac-Leu-Leu-Arg-CHO; Figure 1), were originally isolated from *Actinomycetes* spp (Umezawa 1971). These have subsequently been used as a template for the development of the more selective agents described below. Leupeptin has gained particular significance as a

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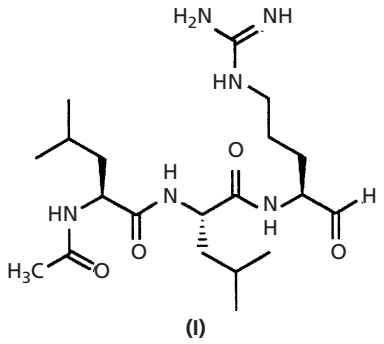


Figure 1 Structure of leupeptin (Ac-Leu-Leu-Arg-CHO).

broad spectrum inhibitor, and is still widely used as a chemical probe for the study of the role of serine and cysteine proteases in cellular and other biological systems.

A further development in the field of protease inhibitors bearing electrophilic carbonyl systems was the introduction of compounds containing C-terminal α, β-dicarbonyl systems, such as α-keto amides, esters and acids (Angelastro et al 1990). Bearing such observations in mind, we reported the first synthesis and kinetic evaluation of compounds bearing a C-terminal α-keto-β-aldehyde (glyoxal) group as inhibitors of both cysteine and serine proteases (Walker et al 1993). Whereas compounds such as peptide chloromethyl ketones contain an electrophilic carbonyl and an alkylating group, the peptide α-keto-β-aldehydes, uniquely, possess two highly electrophilic carbonyl groups at both the α and β positions. This work demonstrated that such compounds are substantially more active against the cysteine proteases (compared with their serine counterparts) and additionally, some 10-fold more potent against cysteine proteases, than peptide aldehydes, and provided the impetus for our development of dipeptide α-keto-β-aldehydes as exceptionally potent and selective slow, tight-binding inhibitors of the cysteine proteases, cathepsins B, L and S (Lynas et al 2000; Walker et al 2000). We now wish to report their synthesis and kinetic characterization against a number of pathophysiologically important trypsin-like serine proteases. This report illustrates that α-keto-β-aldehydes bearing a C-terminal basic residue are potent inhibitors of trypsin-like serine proteases and in some cases display some remarkable kinetic characteristics. These new compounds, incorporating suitable peptide or peptidomimetic targeting motifs, may provide a molecular template for the development of new, selective serine protease inhibitors, with therapeutic potential.

Materials and Methods

Materials

Bovine trypsin (sequencing grade), human plasmin, bovine thrombin, *N*-α-benzyloxycarbonyl-Gly-Gly-Arg-7-amino-4-methylcoumarin (Z-GGR-NHMec), *N*-α-*tert*-butyloxycarbonyl-Val-Leu-Lys-7-amino-4-methylcoumarin (Boc-VLK-NHMec) and *N*-α-*tert*-butyloxycarbonyl-Val-Pro-Arg-7-amino-4-methylcoumarin (Z-VPR-NHMec) were purchased from Sigma-Aldrich (Poole, UK). Human lung tryptase was purchased from BioAss (Munich, Germany). All other biological materials were purchased from either Sigma-Aldrich or ICN Biomedicals (High Wycombe, UK).

N-α-(9-fluorenylmethoxycarbonyl)-(L)-leucine (Fmoc-Leu-OH), *N*-α-(9-fluorenylmethoxycarbonyl)-(*N*-ω-2, 2, 5, 7, 8-pentamethylchroman-6-sulphonyl)-(L)-arginine (Fmoc-Arg(Pmc)-OH), *N*-α-(9-fluorenylmethoxycarbonyl)-(L)-valine (Fmoc-Val-OH), *N*-α-(9-fluorenylmethoxycarbonyl)-(*N*-β-trityl)-(L)-glutamine Fmoc-Gln(Trt)-OH, 2-(1*H*-benzotriazol-1-yl)-1, 1, 3, 3-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt) and *N*, *N*-diisopropylethylamine (DIPEA) were obtained from Novabiochem Ltd (Nottingham, UK). *N*-α-(9-fluorenylmethoxycarbonyl)-(*N*-ϵ-*tert*-butyloxycarbonyl)-(L)-lysine and Fmoc-Arg(Pmc)-OH linked to 2-methoxy-4-alkoxybenzyl alcohol derivatized polstyrene resin (200–400 mesh) (Fmoc-Lys(Boc)-SASRIN and Fmoc-Arg(Pmc)-SASRIN) were purchased from Bachem Feinchemikalien Ltd (Bubendorf, Switzerland). All other materials were analytical grade and purchased from either Sigma-Aldrich or Fluka.

Curve-fitting analysis was performed using the program GRAFIT (DataSoft, Cambridge, UK) on an IBM PC.

Solid/solution-phase synthesis of inhibitors

Synthesis of peptide α-keto-β-aldehydes was carried out as previously reported, with some minor modifications (Lynas et al 2000). A representative scheme for the synthesis of *N*-α-acetyl-Arg-Gln-Arg-COCHO (**II**) is outlined in Figure 2.

In brief, the desired target peptide sequences (Ac-Leu-Leu-Arg-, Boc-Val-Leu-Lys- and Ac-Arg-Gln-Arg-) were prepared (typically on a 0.25 mmol scale) on a SASRIN polystyrene-based resin containing a super acid-sensitive linker (2-methoxy-4-alkoxybenzyl alcohol) employing standard *N*-α-Fmoc and acid labile orthologous group protection solid-phase synthetic pro-

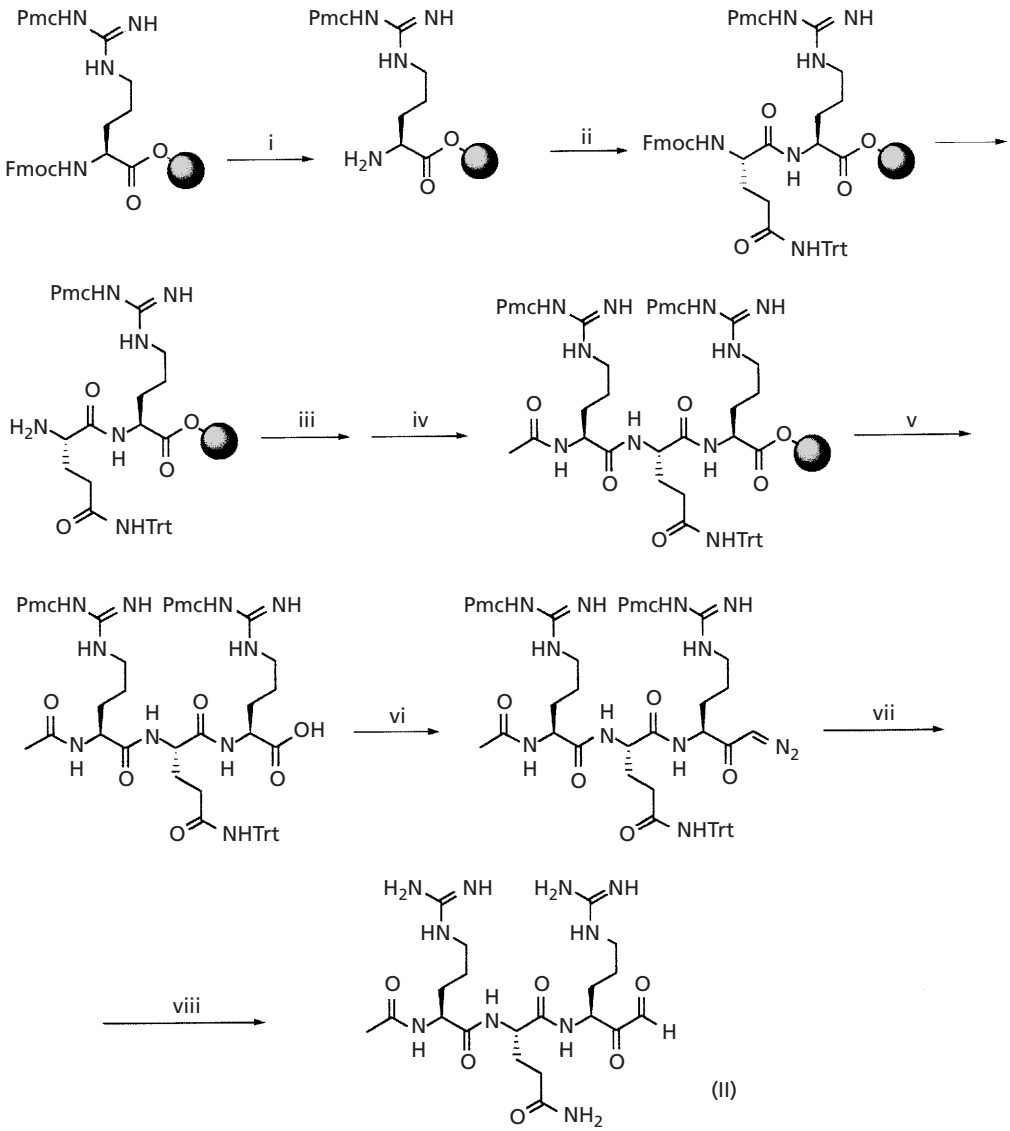


Figure 2 Solid/solution-phase synthesis of Ac-Arg-Gln-Arg-COCHO. i, 20% (v/v) piperidine–DMF, 45 min. ii, Fmoc-Gln(Trt)-OH–HBTU–HOBt–DIPEA (1:1:1:2), 1.5 h, then repeat i. iii, Fmoc-Arg(Pmc)-OH–HBTU–HOBt–DIPEA (1:1:1:2), 1.5 h, then repeat i. iv, 20% (v/v) $Ac(O)_2$ in 20% (v/v) DIPEA–DMF, 15 min. v, 1% (v/v) TFA–DCM, 2×30 min, neutralize. vi, isobutyl chloroformate (1.1 equiv.), *N*-methylmorpholine (1.2 equiv.), 0°C, 15 min, then add ethereal diazomethane, stir at room temperature overnight. vii, Dimethyldioxirane in moist acetone. viii, TFA–triisopropylsilane–thioanisole (95:2.5:2.5, v/v/v), 3 h at room temperature.

tocols (Walker 1994). Upon completion of the synthesis, peptides were cleaved, as their fully protected derivatives, from the solid support by treatment with trifluoroacetic acid (TFA) (1% v/v) in dichloromethane. After neutralization and standard workup, the protected peptide free acids were then converted into their corresponding diazomethyl ketone derivatives by reaction of their unsymmetrical anhydrides with ethereal diazomethane (Ye & McKervey 1992). Finally, the peptidyl diazoketones were treated with a solution of dimethyldioxirane in moist acetone, which we have previously demonstrated leads to formation of C-terminal α-keto-β-aldehydes, in almost quantitative yield, by oxidative cleavage of the diazo group (Lynas et al 1998), followed by removal of protecting groups by treatment (1.5–3.0 h) with 95% (v/v) trifluoroacetic acid in the presence of an

appropriate scavenger mixture (2.5% (v/v)) triisopropylsilane, 2.5% (v/v) thioanisole, 3 h; Ac-Arg-Gln-Arg-COCHO (**II**)); (2.5% (v/v)) anisole, 2.5% (v/v) thioanisole, 1.5 h; Ac-Leu-Leu-Arg-COCHO (**III**)) and (2.5% (v/v)) anisole, 2.5% (v/v) H_2O, 1.5 h; Boc-Val-Leu-Lys-COCHO (**IV**)). Invariably, the peptidyl α-keto-β-aldehydes were obtained as their hydrates.

Inhibition assays: progress curve methods

Trypsin
Enzyme (5 μL of a 0.1 μM stock solution in 1 mM HCl) was added to a cuvette containing substrate (Z-GGR-NHMec) (50 μM) and inhibitor under study (0.1–100 μM) in 50 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl and 0.05% (w/v) Brij 35, maintained at 37°C (final volume 1 mL). Each determination was carried out in triplicate. The rate of hydrolysis of substrate was monitored continuously by measuring the rate of increase of fluorescence, due to production of 7-amino-4-methylcoumarin (NH_2Mec) at 455 nm (excitation 383 nm).

Plasmin
Enzyme (10 μL of a 0.1 μM stock solution in 1 mM HCl) was assayed in the presence of Boc-VLK-NHMec (50 μM) and inhibitor under study (25–200 μM) exactly as described for trypsin.

Tryptase
Enzyme (5 μL of a 0.75 μM stock solution in 1 mM HCl) was assayed in the presence of Boc-VPR-NHMec (50 μM) and inhibitor under study (1–100 μM) exactly as described for trypsin, with the exception that the buffer contained 25 mM NaCl.

Thrombin
Enzyme (5 μL of a 0.05 μM stock solution in 1 mM HCl) was assayed in the presence of Boc-VPR-NHMec (50 μM) and inhibitor under study (25–200 μM) exactly as described for trypsin with the exception that the buffer also contained 1 mM $CaCl_2$.

Enzyme inhibition: incubation method

Trypsin
Enzyme (10 μL of a 0.1 μM stock solution in 1 mM HCl) was incubated with (**II**) (0.1–10 μM) for 30 min in 1 mL of buffer. A sample (10 μL) was removed after this time and added to buffer containing substrate (Z-GGR-NHMec (50 μM)) (990 μL) as described previously.

Thrombin
Enzyme (10 μL of a 0.05 μM stock solution in 1 mM HCl) was incubated with (**III**) (10–100 μM) in buffer (1 mL) for 30 min at 25°C. A sample (10 μL) was removed after this time and added to buffer containing substrate (Boc-VPR-NHMec (50 μM)) (990 μL) as described previously.

Kinetic data analysis

The K_m and k_{cat} values for protease-catalysed hydrolysis of the respective fluorogenic substrates for each of the trypsin-like serine proteases under investigation, in the appropriate buffer system, were determined by monitoring the rate of hydrolysis of a series of concentrations of substrate spanning the range 0.2–5 times K_m and applying the least-squares method directly to the Michaelis-Menten equation (Roberts 1977).

Inhibitors exhibiting slow, tight-binding kinetics

The progress curves of inhibitors exhibiting slow, tight-binding kinetics were analysed according to the models described by Morrison (1982). One model describes rapid formation of one enzyme–inhibitor complex prior to slow formation of another enzyme–inhibitor complex while the other describes a single slowly formed complex.

For both models, the amount of product, P, formed by the remaining active enzyme at time, t, is described by Equation 1.

$$P = v_s t + (v_0 - v_s)[1 - e(-kt)]/k + d \quad (1)$$

v_s is the steady-state rate of product formation at equilibrium (assuming no change in substrate concentration), v_0 is the initial velocity, k is the apparent first-order rate constant and d is the offset of P from zero at time = 0.

For each trial of a given set of enzyme, substrate [S] and inhibitor [I] concentrations, two assays were performed. In the first, enzyme was added to a mixture of substrate and inhibitor. In the second, enzyme and inhibitor were pre-incubated before the addition of substrate. The progress curves for two such assays are characterized by the same steady-state rate, v_s, and the same rate constant, k, but the tangents to the curves at time zero differ, since one reflects a rate before formation of complex, whereas the other reflects a rate prior to the relief of inhibition by substrate. Parameters were determined by simultaneous analysis of the paired assay data. The overall inhibition constant, designated K_i,

was determined using the final steady-state rates and a rearrangement of the equation for competitive inhibition:

$$v_s = v_{max}[S]/[S] + K_m(1 + [I]/K_i) \quad (2)$$

Irreversible inhibitors

Evaluation of the inhibitor constant, K_i, and the apparent first-order rate constant, k_i, for irreversible inactivation was carried out using the methods of Tian & Tsou (1982). This method is based on the assumption that formation of product in the presence of an irreversible modifier should approach an asymptote. If $[P\infty]$ represents the concentration of the product (in this case NH_2Mec) formed at a time approaching infinity, then:

$$[P\infty] = V[S]\underline{K}/(1 + [S]\underline{K})A[Y] \quad (3)$$

in which $\underline{K}$ is the inverted Michaelis Constant, A is the apparent second-order rate constant and [Y] is inhibitor concentration. Since [S] and [Y] are known and V and $\underline{K}$ can be determined in separate experiments, A can be calculated from $[P\infty]$ immediately. Tian & Tsou also determined that:

$$\log([P\infty] - [P]) = \log[P\infty] - 0.43A[Y]t \quad (4)$$

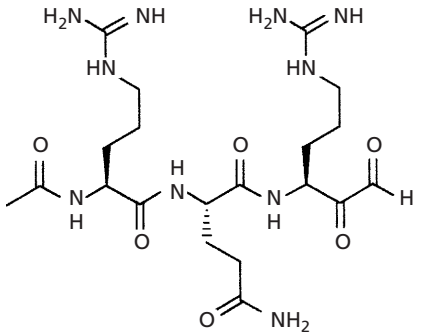
where [P] is the product concentration at time, t. Data can be plotted as $\log([P\infty] - [P])$ against t. As [Y] is known, the apparent inhibition rate constant can be directly read from the slope of the straight line thus obtained.

Results

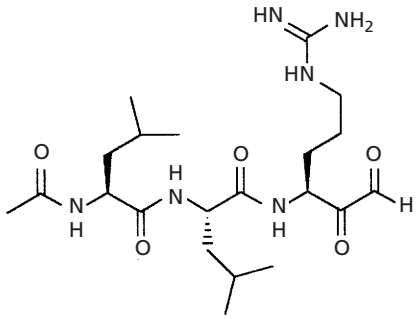
The compounds prepared are illustrated in Figure 3. Kinetic constants, including $K_{i(final)}$ and second-order inactivation constants (k_{2nd}) obtained for the inhibition of trypsin, plasmin, thrombin and tryptase are summarized in Table 1.

Ac-Arg-Gln-Arg-COCHO (II)

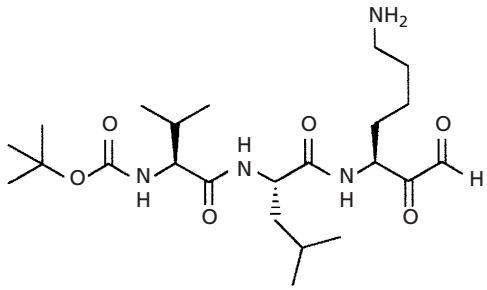
Against tryptase, Ac-Arg-Gln-Arg-CHO behaved as a slow, tight-binding inhibitor. K_i values were calculated to be 13.4 $\mu M_{(initial)}$ and 1.6 $\mu M_{(final)}$. Ac-Arg-Gln-Arg-CHO had no activity against either plasmin or thrombin. The compound was also tested for comparison against bovine trypsin. Intriguingly, when data from progress curves for the trypsin-Ac-Arg-Gln-Arg-COCHO reaction were analysed, a very poor fit was obtained with the slow, tight-binding kinetics equation (Morrison



(II)



(III)



(IV)

Figure 3 Peptide α-keto-β-aldehydes prepared for this study: Ac-Arg-Gln-Arg-COCHO (**II**); Ac-Leu-Leu-Arg-COCHO (**III**); and Boc-Val-Leu-Lys-COCHO (**IV**).

Table 1 Inhibition constants for the inactivation of trypsin-like serine proteases by peptide α-keto-β-aldehydes.

Inhibitor	$K_{i(final)}$ (μM)			
	Trypsin	Thrombin	Plasmin	Tryptase
Ac-Leu-Leu-Arg-COCHO	1.90 ± 0.15	4500[a]	4.90 ± 0.36	1.20 ± 0.11
Ac-Arg-Gln-Arg-COCHO	8920[a]	N.I.	nd	1.60 ± 0.11
Boc-Val-Leu-Lys-COCHO	nd	N.I.	3.10 ± 0.25	nd

[a]k_{2nd} (M^{-1} min^{-1}). Values are ± s.e.m. for 3 determinations. nd = not determined; N.I. = no inhibition at 200 μM.

1982). On closer examination, the final rate was close to zero. As a result, data points from these progress curves were re-examined by fitting to the equation for first-order inactivation in the presence of substrate (Tian & Tsou 1982). This resulted in an excellent fit. From the resultant plots it was possible to calculate the apparent second-order rate constant to be 4500 M^{-1} min^{-1}. The irreversible nature of this interaction was further established when samples of a solution of trypsin inhibited with the compound were diluted 200-fold with buffer containing substrate, and no turnover was observed.

Ac-Leu-Leu-Arg-COCHO (III)

With the exception of the reaction with thrombin, Ac-Leu-Leu-Arg-COCHO typically behaved as a classical, reversible, slow, tight-binding inhibitor against target proteases. Figure 4 shows a typical series of progress curves, in this case against the serine protease plasmin. After the data obtained from these curves were fitted to the equation for slow, tight-binding kinetics, K_i values were calculated using the protocols outlined in the Materials and Methods section.

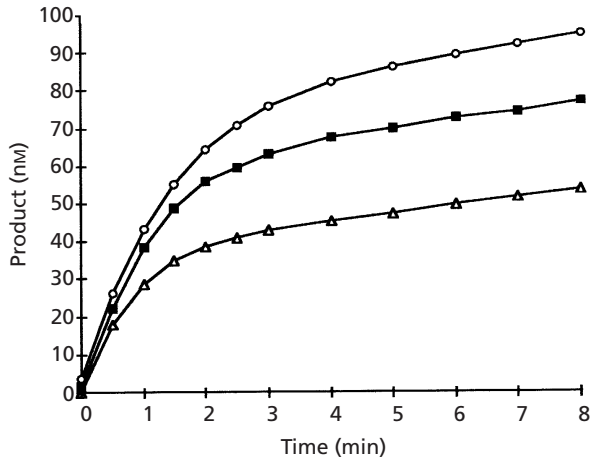


Figure 4 Progress curves for the plasmin-catalysed hydrolysis of Boc-Val-Leu-Lys-NHMec in the presence of varying concentrations of Ac-Leu-Leu-Arg-COCHO: 20 μM (○); 25 μM (■) and 50 μM (△).

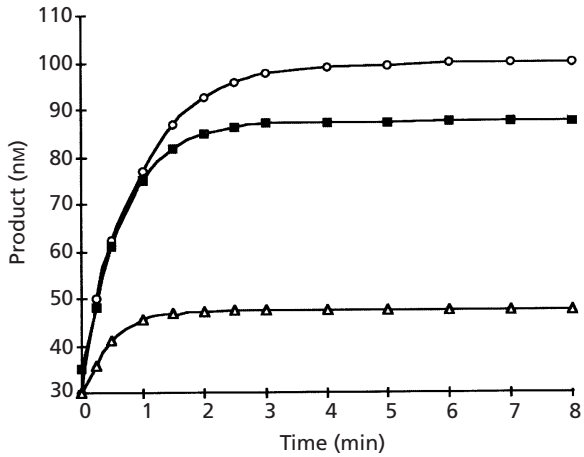


Figure 5 Progress curves for the thrombin-catalysed hydrolysis of Boc-Val-Pro-Arg-NHMec in the presence of varying concentrations of Ac-Leu-Leu-Arg-COCHO: 5 μM (○); 75 μM (■); and 125 μM (△).

As with the trypsin-Ac-Arg-Gln-Arg-COCHO interaction, the final rate of inactivation was close to zero (Figure 5). Curve-fitting analysis demonstrated the thrombin-Ac-Leu-Leu-Arg-COCHO interaction to be irreversible. The apparent second-order rate constant, k_i/K_i, in this instance, was found to be 8920 M^{-1} min^{-1}. The irreversible nature of this interaction was also further established when samples of solution of thrombin-Ac-Leu-Leu-Arg-COCHO were diluted 200-fold with buffer containing substrate, and no turnover was observed.

Ac-Val-Leu-Lys-COCHO (IV)

This was prepared as a plasmin inhibitor. Progress curves, obtained from the inactivation of human plasmin with the inhibitor, were indicative of slow, tight-binding inhibition. After fitting the data to the appropriate equation, K_i values were calculated to be 8.5 $\mu M_{(initial)}$ and 3.1 $\mu M_{(final)}$, respectively. The inhibitor was found to be inactive against thrombin when tested up to 200 μM.

Discussion

While peptide aldehydes have been shown to be superb inactivators of both serine and cysteine proteases, a judicious choice in peptide targeting motifs during compound design has enabled researchers to develop many series of such compounds as potent inhibitors with exquisite selectivity for individual trypsin-like serine proteases, including thrombin (Tapparelli et al 1993), plasmin (McConnell et al 1993), kallikrein (Garrett et al 1998) and urokinase plasminogen activator (Tamura et al 2000).

Successive studies, in this laboratory, have illustrated that compounds bearing the closely analogous C-terminal α-keto-β-aldehyde grouping have greatly improved biological activity compared with peptide aldehydes. Indeed α-keto-β-aldehydes bearing the Cbz-Phe-Xaa- motif have been demonstrated to be among the most potent, reversible synthetic inhibitors of the cysteine proteases cathepsin L (Xaa = Tyr(OBut)) (Lynas et al 2000) and cathepsin S (Xaa = Leu) (Walker et al 2000) reported to date.

A previous study from this laboratory reported the limited activity of a series of α-keto-β-aldehydes against chymotrypsin and pancreatic elastase. Although dipeptide α-keto-β-aldehydes effectively inhibit chymotrypsin, which preferentially cleaves after hydrophobic aromatic amino acids, the sequence Cbz-Pro-Val-COCHO, surprisingly, had no activity against pancreatic elastase (Walker et al 1993). We reasoned that these differences may be due to subtle differences in active site interactions and therefore wished to investigate the possibility that this phenomenon may occur with other serine proteases, as such differences may be a potential means to introduce selectivity for individual enzymes. In light of these observations, we wished to investigate the potential of α-keto-β-aldehydes containing a basic C-terminal residue (Lys or Arg) at the P_1 position to inhibit enzymes of the important trypsin-like serine protease family. We have synthesized and characterized kinetically the α-keto-β-aldehyde analogue of the natural product leupeptin (Ac-Leu-Leu-Arg-CHO), for comparative purposes, and two other sequences, Ac-Arg-Gln-Arg-, which imparts selectivity for tryptase over thrombin in a chloromethyl ketone series (J. McIlroy, unpublished results) and Boc-Val-Leu-Lys-, which has been shown to impart selectivity for plasmin.

A number of findings were immediately apparent from this work. Firstly, peptide α-keto-β-aldehydes, as expected, are much poorer inhibitors of the serine proteases compared with cysteine proteases, with activity ranging in the low micromolar range (Table 1). Generally, α-keto-β-aldehydes bearing dipeptide motifs selective for cysteine proteases display very low nanomolar activity (Lynas et al 2000; Walker et al 2000). Secondly, although previously reported inhibitory data for leupeptin is quoted as IC50 values and therefore not directly comparable, it can be seen that our more rigorous K_i determinations illustrate that α-keto-β-aldehydes have activity in a similar range to their aldehyde counterparts (e.g. McConnell et al (1993) have reported IC50 values in the low micromolar range for leupeptin against trypsin (8.1 μM), thrombin (12.0 μM) and plasmin (3.7 μM). Additionally, the inactivity of the compounds Ac-Arg-Gln-Arg-COCHO and Ac-Val-Leu-Lys-COCHO against thrombin illustrates that high selectivity can be introduced between enzymes.

The most interesting results obtained in this work concerned the apparent irreversible inhibition of trypsin (by Ac-Arg-Gln-Arg-COCHO) and thrombin (by Ac-Leu-Leu-Arg-COCHO). The mechanism behind this phenomenon is unclear, although it may be a consequence of the fact that peptide α-keto-β-aldehydes, unlike α-keto esters, amides and acids, have the distinctive property of possessing two (as opposed to one) highly electrophilic carbonyl groups. α-Keto-β-aldehydes most probably act through the formation of a tetrahedral intermediate between the active site serine residue and the α-carbonyl group. Thus the β-aldehyde group may be accessible to additional interactions within the active site region, perhaps interacting with free amino or imidazole groups. Basjuz et al (1998) have recently reported the observation that trypsin and thrombin can be inhibited by a compound containing the C-terminal β-homoarginine aldehyde moiety, Boc-(D)Phe-Pro-Arg-CH_2CHO (IC50 = 0.19 μM (thrombin); 1.76 μM (trypsin)). While such compounds were shown to be substantially less potent than the comparable peptide α-aldehydes, this work clearly indicates that both enzymes can interact with the β-aldehyde group. The nature of the inhibition in this instance was not reported. Thus, a combination of electrophilic car-

bonyls, selective peptide targeting motifs and possible synergistic intermolecular interactions between protease and inhibitor may confer distinctive properties on the α-keto-β-aldehydes against some serine proteases.

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