Peptidyl inverse esters of *p*-methoxybenzoic acid: a novel class of potent inactivator of the serine proteases

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A series of novel synthetic peptides, containing a C-terminal β -amino alcohol linked to *p*-methoxybenzoic acid via an ester linkage, have been prepared and tested as inhibitors against typical members of the serine protease family. For example, the sequences Ac-Val-Pro-NH-CH-(CH₂-C₆H₅)-CH₂O-CO-C₆H₄-OCH₃ (**I**) and Ac-Val-Pro-NH-CH-[CH-(CH₃)₂]-CH₂O-CO-C₆H₄-OCH₃ (**II**), which fulfil the known primary and secondary specificity requirements of chymotrypsin and elastase respectively, have been found to behave as exceptionally potent irreversible inactivators of their respective target protease. Thus **I** was found to inactivate chymotrypsin with an overall second-order rate constant (k_2/K_1) of approx. $6.6 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$, whereas **II** is an even more potent inactivator of human neutrophil

elastase, exhibiting a second-order rate constant of inactivation of approx. $1.3 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$. These values represent the largest rate constants ever reported for the inactivation of these proteases with synthetic peptide-based inactivators. On prolonged incubation in substrate-containing buffers, samples of the inactivated proteases were found to regain activity slowly. The first-order rate constants for the regeneration of enzymic activity from chymotrypsin and human neutrophil elastase inactivated by **I** and **II** respectively were determined to be approx. $5.8 \times 10^{-5} \text{ s}^{-1}$ and approx. $4.3 \times 10^{-4} \text{ s}^{-1}$. We believe that the most likely mechanism for the inactivation and regeneration of enzymic activity involves the formation and subsequent slow hydrolysis of long-lived acyl enzyme intermediates.

INTRODUCTION

The serine proteases are known to play a diverse role in many normal and pathological states. Thus in addition to their familiar homeostatic roles, for example in dietary protein turnover, coagulation, fibrinolysis and complement activation, their inopportune activation can result in the exacerbation, if not the triggering, of diseases such as emphysema, cystic fibrosis, cancer, systemic lupus erythematosus and myocardial infarction (reviewed in [1,2]). As a consequence of this, much effort has been directed towards the development of selective inactivators for this class of protease because such reagents would permit the unequivocal assessment of the contribution of this protease family to the processes listed above and might even provide a potential source of therapeutic agents.

One class of inactivator that has attracted considerable attention is the so-called inverse substrate inhibitors. The first reported application of these reagents was the efficient inactivation of trypsin by *p*-amidinophenol esters of substituted benzoic acids (Figure 1A) [3]. Since this original report, a variety of heterocyclic inverse or 'alternate substrate' inactivators have been described such as benzoxazinones [4–6], protio enol lactones [7,8] and substituted cephalosporins [9]. These reagents function as inactivators of the serine proteases by forming long-lived acyl enzyme derivatives with the active site serine residue. The enhanced stability of these acyl enzymes, compared with those arising from the hydrolysis of normal amide or ester derivatives, is derived from a decrease in the electrophilicity of the acyl carbonyl grouping due to inductive effects and/or steric shielding

of this grouping from attack by water, thus retarding deacylation and the regeneration of active enzyme.

It is also well established that specific and potent inhibitors of this class of protease can be obtained by using peptides in which the C-terminal amide (-CONH-) or acid (-CO₂H) functional groups have been replaced by electrophilic moieties such as aldehydic (-CHO) [10,11], chloromethyl ketone (-COCH₂Cl) [12,13], trifluoromethyl ketone (-COCF₃) [14,15] and glyoxal (-COCHO) [16]. By choosing appropriate amino acids to occupy the P₁ to P_n positions of the inhibitor (nomenclature of Schechter and Berger [17]), so as to fulfil the primary and subsite specificity of individual members of this protease family, it has been possible to produce reagents that exhibit exquisite selectivity of action (see, for example, [13]).

Consequently we reasoned that by synthesizing peptides of the general structure shown in Figure 1(B), in which the C-terminal amino acid has been replaced by a β -amino alcohol, these could be further chemically elaborated to give inverse esters, for example, of *p*-methoxybenzoic acid (pMOBA) (Figure 1C), thus combining the best features of the peptide-based inhibitors listed above with those of the inverse substrate inactivators. By the appropriate choice of the P₁ amino alcohol residue, it should be possible to selectively introduce pMOBA into the active site, for example, of chymotrypsin (P₁ = phenylalaninol), trypsin (P₁ = argininol) and elastase (P₁ = valinol), by fulfilling the primary specificity requirements of each protease.

This paper reports the synthesis and kinetic testing of a series of such peptides, designed as inactivators of chymotrypsin and human neutrophil elastase (HNE) and provides initial evidence

Abbreviations used: Boc, t-butyloxycarbonyl; Cbz, benzyloxycarbonyl; DIPEA, di-isopropylethylamine; DMF, dimethylformamide; HNE, human neutrophil elastase; Mec, 4-methylcoumarin-7-yl; NMM, N-methylmorpholine; -Phe-ol, -NH-CH-(CH₂-C₆H₅)-CH₂OH; -Phe- ψ -(CH₂-O)-, -NH-CH-(CH₂-C₆H₅)-CH₂O-; pMOBA, *p*-methoxybenzoic acid (*p*-CH₃O-C₆H₄-CO₂H); succ-, succinyl; THF, tetrahydrofuran; -Val-ol, -NH-CH-[CH-(CH₃)₂]-CH₂OH; -Val- ψ -(CH₂-O)-, -NH-CH-[CH-(CH₃)₂]-CH₂O-.

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Figure 1 Comparison of the general structure of the peptide alcohols prepared in the present study (B; R = isopropyl or benzyl; P = peptidyl portion such as -Val-Pro-) that were used for the synthesis of esters of pMOBA (C) with those of the first inverse inactivators of trypsin (A) and chymotrypsin (D), first described by Tanizawa et al. [3] and Jencks and Caplow [34] respectively

that such an approach might well yield a class of potent and discriminating inactivators for this class of protease.

MATERIALS AND METHODS

Materials

All t-butyloxycarbonyl (Boc) amino acids were purchased from NovaBiochem Ltd. (Nottingham, U.K.), as were *N*- α -Boc-Phe- and *N*- α -Boc-Val-derivatized Merrifield polystyrene resins. Isopropenyl chloroformate and dimethylaminopyridine were purchased from Fluka Ltd. (Buchs, Switzerland). *N*-Methylmorpholine (NMM), succ-Ala-Ala-Pro-Phe-NHMec (where succ- and Mec stand for 4-methylcoumarin-7-yl and succinyl respectively), CH₃O-succ-Ala-Ala-Pro-Val-NHMec and chymotrypsin were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). HNE was supplied by Mr. T. Johnston (Division of Biochemistry, Queen's University of Belfast), and was purified from purulent sputum obtained from children suffering from cystic fibrosis, essentially in accordance with the procedures of Martodam et al. [18]. Tetrahydrofuran (THF; Labscan, Stillorgan, Eire) was dried over sodium wire and then distilled from calcium hydride and finally stored over the latter. Dimethylformamide (DMF; Aldrich Chemical Company, Poole, Dorset, U.K.) was stored over 4A molecular sieves (to remove any traces of water and dimethylamine), for at least 4 days before use. All other materials were of A.R. grade and purchased from either Sigma or Aldrich.

Synthesis of putative inhibitors

The preparation of the inverse substrate inhibitors was achieved with a combination of solid-phase and solution peptide synthesis methodologies. In essence, the target peptide sequences were rapidly constructed on a solid-phase support, with standard Merrifield 'Boc chemistry' methodologies; they were then cleaved from the solid support as their C-terminal alcohol derivatives. The following series of peptide alcohols were prepared: Ac-Val-Pro-Phe-ol [where Phe-ol stands for -NH-CH-

(CH₂-C₆H₅)-CH₂OH], Boc-Val-Pro-Phe-ol, Boc-Ala-Pro-Phe-ol and benzyloxycarbonyl (Cbz)-Ala-Ala-Phe-ol (chymotrypsin-directed sequences); and Ac-Val-Pro-Val-ol {where Val-ol stands for -NH-CH-[CH-(CH₃)₂]-CH₂OH} and Boc-Val-Pro-Val-ol (HNE-directed sequences). These were then used for the preparation of ester derivatives of pMOBA. Although Scheme 1 illustrates the synthetic scheme employed for the synthesis of an HNE-targeted sequence containing a C-terminal valine-derived β -amino alcohol (valinol), this methodology was also followed for the preparation of the chymotrypsin-directed sequences containing a C-terminal phenylalanine-derived β -amino alcohol (phenylalaninol). The synthetic protocols employed are detailed below.

Preparation of peptides containing C-terminal β -amino alcohols

The target peptide sequences were synthesized, on a 0.25 mmol scale, with standard Merrifield-Boc solid phase synthesis protocols [19], on a manual bubbler system (see, for example, [20]). *N*- α -Boc-valine- and *N*- α -Boc-phenylalanine-derivatized Merrifield polystyrene resins were used for the synthesis of the elastase- and chymotrypsin-directed sequences respectively. In essence, the syntheses were begun by removing the *N*- α -Boc protecting groups from each of the derivatized resins with a solution of trifluoroacetic acid [20% (v/v) in dichloromethane], for 30 min at room temperature. This 'standard deprotection cycle' was employed before the coupling of subsequent *N*- α -Boc-protected amino acids. After standard washing steps, each of the resins was then neutralized with di-isopropylethylamine (DIPEA). The subsequent *N*- α -Boc-protected amino acids to be coupled were activated by reaction with 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate in DMF containing 1 equiv. of 1-hydroxybenzotriazole and 3 equiv. of DIPEA [21]. The completeness of the coupling reactions at each stage was assessed by the Kaiser ninhydrin test [22]. The desired peptide sequences were then constructed by repeating the deprotection and coupling steps for the appropriate number of cycles. N-acetylated sequences were generated by first performing the standard deprotection cycle on the appropriate resin-bound peptides, followed by reaction with acetic anhydride (3-fold

Scheme 1 Outlining of synthetic scheme for preparation of peptides

Conditions: **i**, TFA/DCM (20% v/v), 30 min at room temperature, followed by neutralization with DIPEA; **ii**, Boc-Pro-OH (3 equiv.)/2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (3 equiv.)/1-hydroxybenzotriazole (3 equiv.)/DIPEA (6 equiv.), 1 h at room temperature; **iii**, repeat step **i**; **iv**, Boc-Val-OH (3 equiv.)/1-hydroxybenzotriazole (3 equiv.)/DIPEA (6 equiv.), 1 h at room temperature; **v**, repeat step **i**; **vi**, acetic anhydride (3 equiv.)/DIPEA (3 equiv.), 1 h at room temperature; **vii**, LiBH₄ (6 equiv.) in THF/EtOH (6:1, v/v), 36 h at room temperature; **viii**, react with mixed anhydride formed by reaction of MOBA (1.5 equiv.)/isopropenyl chloroformate (1.0 equiv.)/NMM (1.5 equiv.), in dry dichloromethane, containing dimethylaminopyridine (0.1 equiv.), for 2–3 h at –10 to –15 °C and then at room temperature overnight.

molar excess based on initial resin loading) in DMF containing 3 molar equivalents of DIPEA. The progress of this ‘acetylation’ step was monitored by removing samples of resin (5 mg) and subjecting these to the Kaiser ninhydrin test. On completion of each synthesis, the peptide-resins were thoroughly washed five times with 50 ml of DMF followed by five washes with 50 ml of dichloromethane; they were then dried for at least 3 h under vacuum before use in the next stage.

The protected target peptide sequences were then reductively cleaved in almost quantitative yield from the solid support by using LiBH₄ (generated *in situ*), as their C-terminal amino alcohol derivatives, essentially in accordance with the procedure of Mergler and Nyfeler [23]. This was achieved as follows. Peptide resin (0.2 mmol, based on initial resin loading) was added to a round-bottomed flask (25 ml) containing a solution (10 ml) of THF/ethanol (6:1, v/v). Lithium bromide (86.5 mg, 1 mmol) and sodium borohydride (37.5 mg, 1 mmol) were ground to a fine powder in a mortar and were then added to the suspension of peptide-resin in the THF/ethanol mixture. The reaction mixture was then stirred for 36 h at room temperature. The resin was then filtered, washed twice with 5 ml of THF and the filtrate and washings were combined. After removal of solvents under reduced pressure, the peptide alcohols were extracted into 100 ml of ethyl acetate and washed three times with 100 ml of NaHCO₃, three times with 100 ml of NaCl and three times with 100 ml of NaHSO₄. The extract was dried over MgSO₄ and the ethyl acetate removed under reduced pressure. The isolated products were analysed by TLC [solvent systems: A, chloroform/methanol, 1:1 (v/v); B, ethyl acetate/cyclohexane, 1:1 (v/v)] and electrospray MS and were used without further purification.

Synthesis of the peptidyl pMOBA esters

pMOBA (152 mg, 1.1 mmol) was dissolved in dry THF (approx. 20 ml), and the resulting solution was cooled in a methanol/ice bath. To this chilled solution was added, with vigorous stirring, isopropenyl chloroformate (132 μ l, 1.0 mmol) and NMM (101 μ l, 1.0 mmol) [24]. The formation of the mixed anhydride between the aryl carboxylic acid and chloroformate proceeded for 20 min.

The required tripeptide alcohol was dissolved in dry DCM (approx. 50 ml), along with dimethylaminopyridine (1.2 mg, 0.1 mmol), and the resultant solution was added to the mixed anhydride with vigorous stirring. The reaction mixture was left to come to ambient temperature, over a period of 3–4 h, and the reaction was continued overnight at room temperature, with stirring. After removal of solvents under reduced pressure, the product was extracted into 100 ml of ethyl acetate and washed three times with 100 ml of NaHCO₃, three times with 100 ml of NaCl and three times with 100 ml of NaHSO₄. The extract was dried over MgSO₄ and the ethyl acetate removed under reduced pressure. The products were recrystallized from ethyl acetate/hexane, and were analysed with TLC (solvent systems as above) and electrospray MS.

Inactivation studies

Chymotrypsin

Chymotrypsin (10 μ l of approx. 0.1 μ M stock solution in 1 mM HCl) was added to a solution (1 ml) of succ-Ala-Ala-Pro-Phe-NHMec (50 μ M) and the inhibitor under study (concentration range 10 nM to 10 mM) in 50 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl and maintained at 37 °C. The rate of substrate hydrolysis was monitored continuously by measuring the rate of increase of fluorescence at 455 nm (λ_{exc} 383 nm) in a Perkin-Elmer MPF-44B spectrofluorimeter.

HNE

A solution (1 ml) of HNE (approx. 0.1–0.5 nM) in 50 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl, was placed in a cuvette maintained at 37 °C and housed in the sample compartment of a Perkin-Elmer MPF-44B spectrofluorimeter. To this were added, simultaneously, solutions (0.5 ml each) of 10–25 nM Ac-Val-Pro-Val- ψ -(CH₂-O)-CO-C₆H₄-OCH₃ {where Val- ψ -(CH₂-O)- stands for -NH-CH-[CH-(CH₃)₂]-CH₂O-} and CH₃O-succ-Ala-Ala-Pro-Val-NHMec (50 μ M) in the same buffer. The addition was achieved by mixing the solutions of substrate and inhibitor, with two syringes linked to a single barrel, that was introduced to the sample cuvette (containing the

enzyme solution) via the air inlet in the cell housing. Mixing was complete in less than 1 s.

Determination of first-order rate constants for regeneration of enzymic activity

To a solution (1 ml) of chymotrypsin (approx. 1 nM) or HNE (approx. 1 nM) in 50 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl (buffer A) and maintained at 37 °C, was added Ac-Val-Pro-Phe- ψ -(CH₂-O)-CO-C₆H₄-OCH₃ [where Phe- ψ -(CH₂-O)- stands for -NH-CH-(CH₂-C₆H₅)-CH₂O-] (10 μ l of a 2 mM stock solution in DMF) or Ac-Val-Pro-Val- ψ -(CH₂-O)-CO-C₆H₄-OCH₃ (10 μ l of a 2 mM stock solution in DMF) respectively. After 1 min, samples (50 μ l) of the inactivated proteases were added to an equal volume of 200 mM sodium phosphate buffer, pH 5.4, to stop the reaction with inhibitor. The inactivated proteases were then freed from excess inhibitor by gel-permeation chromatography on a G-25 spin column. Samples of each inactivated protease (typical volume 40–80 μ l) were then added to a fresh solution (1 ml) of buffer A, maintained at 37 °C, containing the respective fluorogenic substrate for either chymotrypsin or HNE (each used at a final concentration of 50 μ M); the formation of product was measured continuously as described above. By reference to control samples of each protease incubated in the absence of inhibitor, the fractional activity of each of the inactivated samples was evaluated as a function of time and the first-order rate constant for the regeneration of activity was determined from the resultant semi-logarithmic plots.

Determination of the operational molarity of the chymotrypsin solution

Chymotrypsin was titrated with 4-methylumbelliferyl- β -trimethylammonium cinnamate in accordance with the procedure of Jameson et al. [25].

Determination of K_m and V_{max} for the chymotrypsin-catalysed hydrolysis of succ-Ala-Ala-Pro-Phe-NHMec and the HNE-catalysed hydrolysis of CH₃O-succ-Ala-Ala-Pro-Phe-NHMec

To determine the K_m and V_{max} values for the chymotrypsin- and HNE-catalysed hydrolysis of their respective fluorogenic substrates, substrate concentrations spanning a range 0.2–5 times the K_m were used. It was ensured that the determinations of the kinetic constants were performed under the exact conditions used to monitor the inactivation processes in the preceding section. K_m and V_{max} values were determined with the least-squares method (see pp. 209–306 in [26]).

Evaluation of kinetic parameters

The kinetic constants for the irreversible inactivation of chymotrypsin by Ac-Val-Pro-Phe- ψ -(CH₂-O)-CO-C₆H₄-OCH₃, Boc-Val-Pro-Phe- ψ -(CH₂-O)-CO-C₆H₄-OCH₃, Boc-Ala-Pro-Phe- ψ -(CH₂-O)-CO-C₆H₄-OCH₃ and Cbz-Ala-Ala-Phe- ψ -(CH₂-O)-CO-C₆H₄-OCH₃, and HNE by Ac-Val-Pro-Val- ψ -(CH₂-O)-CO-C₆H₄-OCH₃ and Boc-Val-Pro-Val- ψ -(CH₂-O)-CO-C₆H₄-OCH₃, were determined as described previously [27].

RESULTS AND DISCUSSION

Synthesis of inverse esters

The preparation of the peptidyl inverse esters of pMOBA used in the present study was achieved with a combination of solid-phase and standard classical solution peptide synthesis methodologies

as outlined in Scheme 1. The peptide alcohols that were used to esterify pMOBA were prepared by the concomitant reduction and cleavage of their respective C-terminal acid that was derivative-linked via an ester bond to Merrifield-type resin, using LiBH₄ [23]. Each of the peptide alcohols was obtained in high yield (typically 80–90 %, based on initial resin loading), was found to be homogeneous by TLC and exhibited the correct molecular mass when subjected to electrospray mass spectrometry (see Table 1). The utility of the method was demonstrated by the successful synthesis of peptide alcohols containing various N-terminal protecting groups such as Boc-, Cbz- or Ac-.

Each of the N-protected peptide alcohols was then used to esterify pMOBA. This was achieved by reacting each of the alcohols in turn with the mixed anhydride formed by the reaction of pMOBA and isopropenyl chloroformate. This esterification reaction was catalysed by the addition of a small amount (0.1 mequiv.) of dimethylaminopyridine [24]. The use of this chloroformate for the preparation of ester derivatives of carboxylic acids is to be preferred to the more commonly used isobutyl- and ethyl chloroformates because it produces acetone, as a by-product of the formation of the mixed anhydride, which cannot undergo a competitive reaction with the desired alcohol to form inappropriate esters, unlike isobutyl alcohol and ethanol produced by the aforementioned chloroformates. After recrystallization from ethyl acetate/hexane, each of the target ester sequences was obtained in modest yield (16–34 %, based on the amount of starting alcohol) as a homogeneous product (as determined by TLC analysis employing two different solvent systems) and their identity was confirmed by electrospray MS (see Table 1).

Inactivation studies

Before commencement of the inactivation studies, the hydrolytic stability of the various peptidyl inverse esters was examined. It was found that incubation of each of the analogues (performed with a final concentration of 20 nM, the lowest concentration employed in the inactivation studies) in 50 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl, at 37 °C for up to 2 h, resulted in no diminution of inhibitory effectiveness (results not shown). These observations are entirely consistent with the known hydrolytic stability of aliphatic esters of arylcarboxylic acids.

Initial inactivation studies on the peptidyl esters of pMOBA were performed with the Kitz–Wilson method [28]. However, the rates of inactivation were too rapid to be measured by this method. For example, the incubation of α -chymotrypsin (approx. 0.1 nM) with Ac-Val-Pro-Phe- ψ -(CH₂-O)-CO-C₆H₄-OCH₃ (**I**) (20 nM) in 50 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl, at 37 °C, resulted in the virtually instantaneous inactivation of the protease. Similarly, the incubation of HNE (approx. 0.1 nM) with Ac-Val-Pro-Val- ψ -(CH₂-O)-CO-C₆H₄-OCH₃ (**II**) (10 nM) under identical conditions also resulted in the instantaneous inactivation of this protease. The remaining phenylalaninol-containing peptides that were examined in this study, although not as effective as **I**, also brought about the very rapid inactivation of α -chymotrypsin. Consequently, we performed the subsequent inactivation studies in the presence of competing substrate in an attempt to make the kinetic analysis more amenable to study.

Figure 2 shows the time course for the formation of product (7-amino-4-methylcoumarin, NH₂-Mec) released from the substrate succ-Ala-Ala-Pro-Phe-NH-Mec (50 μ M) by α -chymotrypsin-catalysed hydrolysis in the presence of various concentrations of **I** (12.5–62.5 nM). Similar progress curves were

Table 1 Yields, electrospray MS data and TLC data for synthesized peptide analogues

Yields of peptide alcohols were determined for the crude materials cleaved from the resin and are based on initial resin loading. Yields of the ester derivatives were determined for the purified materials, isolated after recrystallization from ethyl acetate/hexane. TLC solvent systems used: A, chloroform/methanol (1:1, v/v); B, ethyl acetate/cyclohexane (1:1, v/v).

Peptide species [yield; R_f (A), R_f (B)]	Molecular mass (Da)	
	Determined	Required
Alcohol derivatives		
Ac-Val-Pro-Phe-ol (90%; 0.80, 0.69)	411.4 ($M+Na$) ⁺	389.48
Ac-Val-Pro-Val-ol (80%; 0.40, 0.62)	364.3 ($M+Na$) ⁺	341.48
Boc-Val-Pro-Val-ol (86%; 0.43, 0.67)	421.6 ($M+Na$) ⁺	398.5
Boc-Val-Pro-Phe-ol (87%; 0.89, 0.77)	469.6 ($M+Na$) ⁺	446.55
Boc-Ala-Pro-Phe-ol (92%; 0.83, 0.71)	419.7 ($M+H$) ⁺	418.49
Cbz-Ala-Ala-Phe-ol (85%; 0.92, 0.78)	449.6 ($M+Na$) ⁺	426.47
Ester derivatives		
Ac-Val-Pro-Phe- ψ -(CH ₂ -O)-CO-C ₆ H ₄ -OCH ₃ (37%; 0.65, 0.55)	524.6 ($M+H$) ⁺	523.5
Ac-Val-Pro-Val- ψ -(CH ₂ -O)-CO-C ₆ H ₄ -OCH ₃ (24%; 0.61, 0.34)	497.6 ($M+Na$) ⁺	475.62
Boc-Val-Pro-Val- ψ -(CH ₂ -O)-CO-C ₆ H ₄ -OCH ₃ (16%; 0.88, 0.75)	533.5 ($M+H$) ⁺	532.2
Boc-Val-Pro-Phe- ψ -(CH ₂ -O)-CO-C ₆ H ₄ -OCH ₃ (26%; 0.72, 0.56)	581.7 ($M+H$) ⁺	580.69
Boc-Ala-Pro-Phe- ψ -(CH ₂ -O)-CO-C ₆ H ₄ -OCH ₃ (30%; 0.70, 0.49)	575.6 ($M+Na$) ⁺	552.63
Cbz-Ala-Ala-Phe- ψ -(CH ₂ -O)-CO-C ₆ H ₄ -OCH ₃ (27%; 0.56, 0.47)	561.6 ($M+H$) ⁺	560.4

Figure 2 Best fit of the data to the equation for irreversible inhibition [27] of chymotrypsin by Ac-Val-Pro-Phe- ψ -(CH₂-O)-CO-C₆H₄-OCH₃ in the presence of succ-Ala-Ala-Pro-Phe-NHMec

The inactivations were performed with a fixed concentration of substrate (50 μ M) and the following concentrations of inhibitor: ▲, 0; ○, 12.5; □, 31.5; ■, 41.5; ●, 62.5 nM.

obtained for the HNE-catalysed hydrolysis of CH₃O-succ-Ala-Ala-Pro-Val-NHMec in the presence of **II** (Figure 3). These progress curves are indicative of the action of an active-site-directed irreversible inhibitor operating through the kinetic scheme shown in Scheme 2 [27,29].

Data from these progress curves were fitted, by non-linear regression analysis [30], to the following integrated rate equation:

$$[P] = A[1 - \exp(-k_{app}t)] \quad (1)$$

This represents a first-order process, of apparent rate constant k_{app} and amplitude A , for the formation of product P as a function of time t [27]. The values of A and k_{app} for five different inhibitor concentrations were then determined; from these the inhibitor constant K_i and first-order rate constant k_2 for the reaction shown in Scheme 2 were evaluated [27]. These are listed in Table 2.

Figure 3 Best fit of the data to the equation for irreversible inhibition [27] of HNE by Ac-Val-Pro-Val- ψ -(CH₂-O)-CO-C₆H₄-OCH₃ in the presence of CH₃O-succ-Ala-Ala-Pro-Val-NHMec

The inactivations were performed with a fixed concentration of substrate (50 μ M) and the following concentrations of inhibitor: ○, 0; □, 10.0; ●, 12.5; ▼, 15.0; ■, 17.5 nM. Each set of data points shown is the mean of four separate determinations and, for the sake of clarity, the error bars representing the estimates of the S.E.M. have been omitted.

It is clear from Table 2 that these peptidyl esters of pMOBA are exceptionally efficient inactivators of their target protease. For example, the most efficient chymotrypsin inactivator, Ac-Val-Pro-Phe- ψ -(CH₂-O)-CO-C₆H₄-OCH₃ (**I**), inactivated the enzyme with an overall second-order rate constant (k_2/K_i) of approx. $6.6 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$. This peptidyl sequence binds chymotrypsin, before covalent complex formation, with a K_i of approx. 6.0 nM. This represents a very potent interaction between enzyme and inhibitor, and the basis for this high affinity has yet to be examined. The formation of the putative acyl enzyme intermediate E-I from the Michaelis-type complex EI (our evidence in support of this proposed mechanism is discussed below) then takes place with a determined first-order rate constant of approx. 0.04 s^{-1} .

Similarly, Ac-Val-Pro-Val- ψ -(CH₂-O)-CO-C₆H₄-OCH₃ (**II**) is

Scheme 2 Kinetic scheme for the irreversible inactivation of an enzyme in the presence of competing substrate

a superb inactivator of HNE because it brings about inactivation of the protease with an even greater rate constant of approx. $1.3 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$. Indeed, the rate of inactivation was so rapid, even in the presence of competing substrate, that we had to adopt the rapid-mixing method detailed in the Materials and methods section to follow the progress of the reaction. To the best of our knowledge this second-order rate constant is the greatest ever reported for the inactivation of HNE by a synthetic peptide-based inhibitor. For example, it is approx. 500-fold greater than that reported for the inactivation of HNE by the exactly analogous peptidyl diphenyl phosphonate Boc-Val-Pro-NH-CH[CH(CH₃)₂]-P(=O)(OPh)₂, which, again to the best of our knowledge, was the previous most efficient synthetic (peptide-based) inactivator of this protease [31].

A particularly satisfying finding was that the chymotrypsin-specified sequence **I** was found to be completely inactive against HNE and, conversely, the HNE-specified sequence **II** was without effect when tested against chymotrypsin (each peptide was incubated for 30 min at 37 °C with the 'inappropriate' protease in the absence of competing fluorogenic substrate, at a concentration of 50 μM). To put this into perspective, incubation of chymotrypsin with 50 μM **I** or HNE with the same concentration of **II** would result in the complete inactivation of each protease in less than 10 ms (this figure was arrived at on the basis that the half-life for the interaction of each protease with its respective inhibitor was of the order of 1 ms, and that 'complete' inhibition would require 10 half-lives). Such a result is entirely in keeping with the known primary specificity of both proteases. Whereas chymotrypsin can accommodate aromatic amino acids in its primary binding pocket, peptides containing aromatic residues at the P₁ position are excluded from gaining entry into the active site of HNE by steric clashes with residues Val-190, Phe-192,

Ala-213 and Val-216, and with the disulphide bridge between Cys-191 and Cys-220 in the latter [32].

For both the chymotrypsin-specified and elastase-specified sequences, there is only a modest decrease in inhibitor effectiveness on replacement of the smaller and more polar acetyl grouping with the bulky and hydrophobic Boc grouping at the P₄ position of the inhibitor; respective second-order rate constants were $6.6 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $1.8 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ for Ac-Val-Pro-Phe- γ -(CH₂-O)-CO-C₆H₄-OCH₃ and Boc-Val-Pro-Phe- γ -(CH₂-O)-CO-C₆H₄-OCH₃ (chymotrypsin-directed), and $1.3 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $3.1 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ for Ac-Val-Pro-Val- γ -(CH₂-O)-CO-C₆H₄-OCH₃ and Boc-Val-Pro-Val- γ -(CH₂-O)-CO-C₆H₄-OCH₃ (HNE-directed) respectively. In contrast, the presence of proline at P₂ and valine at P₃ are obviously important in determining inhibitor effectiveness in the chymotrypsin-directed inverse esters because the sequential replacement of each of these residues by alanine resulted in substantial decreases in inhibitor efficacy of approximately two orders of magnitude on each substitution; respective second-order rate constants were $1.1 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $5.5 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$ for Boc-Ala-Pro-Phe- γ -(CH₂-O)-CO-C₆H₄-OCH₃ and Cbz-Ala-Ala-Phe- γ -(CH₂-O)-CO-C₆H₄-OCH₃ respectively. These results are entirely in keeping with those from previous studies on the inactivation of this protease with peptidyl chloromethyl ketones [33], although it is conceivable that the presence of the Cbz group at the P₄ position of Cbz-Ala-Ala-Phe- γ -(CH₂-O)-CO-C₆H₄-OCH₃ could also contribute to the decrease in inhibitor efficacy.

We believe that these peptide derivatives function as irreversible inactivators of their target proteases by forming long-lived acyl enzyme intermediates, most probably with the active-site serine residue. This is based on the following observations.

First, because the inverse esters contain no electrophilic grouping capable of causing alkylation, the observed irreversible inactivation can be explained only on the basis of the transfer of the pMOBA (anisoyl) grouping to the target protease, resulting in the acylation of the enzyme. The possible alternative explanation, that the peptide alcohols generated during the course of 'normal enzymic turnover' of the inverse esters bind tightly to their target protease thus causing a progressive and apparently time-dependent inhibition, can be discarded because none of the peptide alcohols exhibited any competitive reversible inhibition, even when tested at concentrations as high as 200 μM against both proteases (results not shown). This result is in agreement with the work of Thompson, who studied the inhibition of pig pancreatic elastase with peptide aldehydes [10]. Thompson demonstrated that whereas the peptide aldehydes Ac-Ala-Pro-NHCH(CH₃)CHO and Ac-Pro-Ala-Pro-NHCH(CH₃)CHO

Table 2 Kinetic constants for the inactivation of chymotrypsin and HNE by peptidyl inverse substrates derived from pMOBA

Values are means $\pm$ S.E.M. for four determinations. Abbreviation: n.d., not determined.

Protease	Inhibitor sequence	Kinetic constants		
		k_2 (s ⁻¹)	K_i (nM)	k_2/K_i (M ⁻¹ · s ⁻¹)
Chymotrypsin	Ac-Val-Pro-Phe- γ -(CH ₂ -O)-CO-C ₆ H ₄ -OCH ₃	0.04	6.0	$(6.6 \pm 0.8) \times 10^6$
	Boc-Val-Pro-Phe- γ -(CH ₂ -O)-CO-C ₆ H ₄ -OCH ₃	0.04	20.0	$(1.8 \pm 0.3) \times 10^6$
	Boc-Ala-Pro-Phe- γ -(CH ₂ -O)-CO-C ₆ H ₄ -OCH ₃	0.02	166.6	$(1.1 \pm 0.1) \times 10^5$
	Cbz-Ala-Ala-Phe- γ -(CH ₂ -O)-CO-C ₆ H ₄ -OCH ₃	n.d.	n.d.	$(5.5 \pm 0.6) \times 10^2$
	Ac-Val-Pro-Val- γ -(CH ₂ -O)-CO-C ₆ H ₄ -OCH ₃	No inhibition		
HNE	Ac-Val-Pro-Phe- γ -(CH ₂ -O)-CO-C ₆ H ₄ -OCH ₃	No inhibition		
	Ac-Val-Pro-Val- γ -(CH ₂ -O)-CO-C ₆ H ₄ -OCH ₃	n.d.	n.d.	$(1.3 \pm 0.6) \times 10^7$
	Boc-Val-Pro-Val- γ -(CH ₂ -O)-CO-C ₆ H ₄ -OCH ₃	n.d.	n.d.	$(3.1 \pm 6.5) \times 10^6$

Figure 4 Best fit of the data for the exponential recovery of amidase activity from chymotrypsin inactivated by Ac-Val-Pro-Phe- ψ -(CH₂-O)-CO-C₆H₄-OCH₃ (●) and HNE inactivated by Ac-Val-Pro-Val- ψ -(CH₂-O)-CO-C₆H₄-OCH₃ (■)

Figure 5 Best fit of the data for the exponential recovery of amidase activity from chymotrypsin inactivated by Ac-Val-Pro-Phe- ψ -(CH₂-O)-CO-C₆H₄-OCH₃ (●) and Cbz-Ala-Ala-Phe- ψ -(CH₂-O)-CO-C₆H₄-OCH₃ (○) respectively

inhibited the protease with K_i values of 62 and 0.8 μ M respectively, the exactly analogous peptide alcohols Ac-Ala-Pro-NHCH(CH₃)CH₂OH and Ac-Pro-Ala-Pro-NHCH(CH₃)CH₂OH were exceedingly poor reversible inhibitors, exhibiting K_i values of 7000 and 600 μ M respectively.

Secondly, if this acyl transfer mechanism is correct, one would expect to see a regeneration of enzymic activity, due to deacylation of the anisoylated protease, on prolonged incubation in the presence of excess substrate. This is exactly what was observed. Thus, when samples of inactivated protease were freed from excess inhibitor, via gel filtration employing a G-25 spin column, and were then added to substrate-containing buffers (see the Materials and methods section for details), an exponential regeneration of enzymic activity was observed. Figure 4 illustrates typical curves for the regeneration of enzymic activity from HNE inactivated by **II** and chymotrypsin inactivated by **I** respectively. From Figure 4 it is clear that the amidase activity of HNE is regenerated much more rapidly than that of chymotrypsin. We determined the first-order rate constants for the regeneration of active HNE and chymotrypsin to be approx. 4.3×10^{-4} and $5.8 \times 10^{-5} \text{ s}^{-1}$ respectively. These correspond to half-lives for the presumed anisoylated chymotrypsin and anisoylated HNE of approx. 198 min and approx. 27 min respectively.

Thirdly, the regeneration curves for the recovery of chymotrypsin amidase activity from samples of the protease inactivated by each of the peptide inverse esters containing the phenylalanine-derived C-terminal alcohol were virtually superimposable on one another. For example, Figure 5 illustrates the almost identical regeneration curves for the recovery of chymotrypsin inactivated by Ac-Val-Pro-Phe- ψ -(CH₂-O)-CO-C₆H₄-OCH₃ and Cbz-Ala-Ala-Phe- ψ -(CH₂-O)-CO-C₆H₄-OCH₃, implying that active protease was regenerated from a common enzyme-inhibitor complex in each instance. Given that the peptidyl portion differs markedly between these two inhibitor sequences, it seems reasonable to suggest that this enzyme-inhibitor complex is most probably the acyl chymotrypsin derivative formed by transfer of the anisoyl grouping -CO-C₆H₄-OCH₃ of each inverse ester to the protease.

Finally, the first-order rate constants determined for the regeneration of active chymotrypsin inactivated by the peptide inverse esters of pMOBA are in excellent agreement with that for

the deacylation of anisoylated chymotrypsin (prepared by reaction with the imidazolidine derivative of pMOBA (Figure 1D), determined by Jencks and Caplow [34].

In conclusion, we have provided some preliminary experimental evidence in support of our contention that it is possible to develop selective and highly potent inactivators of the serine proteases by using peptidyl inverse esters. Given the great variety of synthetic transformations that can be performed on the aryl acid moiety, it should be possible to influence the rate at which the acyl enzyme intermediates de-acylate via fine-tuning of the inductive and steric properties of the aromatic nucleus. Consequently it should be possible to produce inactivated species that regenerate enzymic activity even more slowly than the (presumed) anisoylated derivatives discussed in the present study. Additionally, given that one can vary the peptidyl portion of the inhibitor as well, it should be possible to generate inactivators not only with enhanced potency but also with pronounced selectivity of action.

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