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PEPTIDE ARGININOL "INVERSE SUBSTRATES" OF ANISIC ACID: NOVEL INHIBITORS OF THE TRYPSIN-LIKE SERINE PROTEINASES

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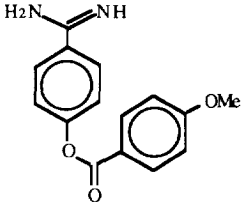
Abstract: Peptides containing a C-terminal argininol residue linked, via an ester bond, to anisic acid have been synthesized as putative inhibitors of trypsin-like serine proteinases. The most potent analogue, Boc-Ile-Glu-Gly-Arg-ψ-(CH_2-O)-CO-C_6H_4-OMe, that was modelled on a known recognition sequence for the clotting enzyme factor Xa, was found to inactivate the protease with a second-order rate constant of ~ 4.5 x 10^5 $M^{-1}.min^{-1}$.

Introduction: The first reported application of inverse esters for the efficient inactivation of trypsin by *p*-amidinophenol esters of substituted benzoic acids, including those of *p*-methoxy benzoic acid (anisic acid) (**1**), was made by Tanizawa et al.,[1]. These authors had postulated that the *p*-amidinophenol grouping would function as an arginine mimic thus fulfilling the primary specificity of trypsin and, as a consequence, would deliver the aryl acyl moiety into the active site of the proteinase. Since this original report, a number of other workers have demonstrated that derivatives of this type also inactivate a variety of trypsin-like proteinases such as plasmin, urokinase and tissue plasminogen activator[2]. These reagents function as inactivators of the serine proteinases by forming long-lived acyl enzyme derivatives with the active-site serine residue. The enhanced stability of these acyl enzymes, over those arising out of 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 regeneration of active enzyme.



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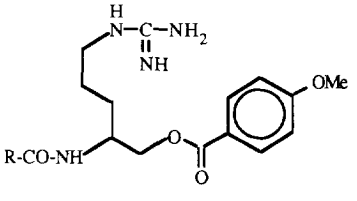

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We have reasoned that peptides of general structure (**2**), which contain a C-terminal argininol residue linked, via an ester bond, to anisic acid, should also be capable of introducing the anisoyl acid function into the active-site of the trypsin-like serine proteinases. Furthermore, by altering the nature of the peptidyl portion R in

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these structures so as to fulfil the secondary specificity requirements of individual members of this proteinase family, it should be possible to obtain reagents that exhibit pronounced selectivity of action for individual members within this subclass. This principle of 'affinity labelling' has been elegantly exploited by Shaw in his studies on the selective inactivation of the plasma trypsin-like enzymes by peptides containing a C-terminal arginine chloromethane[3] and, more recently, by Semple et al., in their studies on the inhibition of thrombin by peptides containing a C-terminal argininal residue[4].

This paper reports on the combination of solid phase and classical solution methodologies for the rapid synthesis of these peptide argininol inverse esters and details their inhibitory activities against a variety of trypsin-like serine proteinases .

Synthetic considerations: The following peptide argininol inverse esters were synthesized; Boc-Ile-Glu-Gly-Arg-ψ–(CH_2-O)-CO-C_6H_4-OMe, Boc-(D)-Phe-Pro-Arg-ψ–(CH_2-O)-CO-C_6H_4-OMe and Ac-Arg-Gln-Arg-ψ(CH_2-O)-CO-C_6H_4-OMe. The first two analogues were designed as putative inhibitors of the blood coagulation cascade trypsin-like serine proteases factor Xa and thrombin, respectively, and were based on known peptidyl arginine chloromethane inactivators of these proteinases[3]. The third was synthesized as a putative inactivator of the mast cell proteinase tryptase; this sequence was also based on a peptidyl arginine chloromethane, previously identified by our laboratory as a potent inactivator of this trypsin-like proteinase[5].

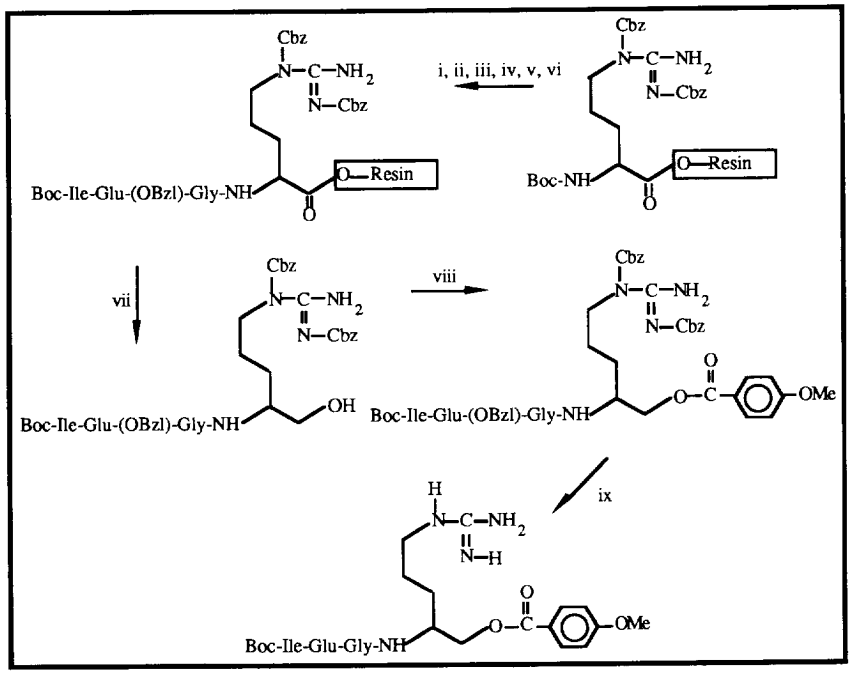
Their syntheses were achieved using a combination of solid phase and standard classical solution peptide synthesis methodologies, as outlined in **Scheme 1**. Although this particular illustrated example is for the synthesis of Boc-Ile-Glu-Gly-Arg-ψ–(CH_2-O)-CO-C_6H_4-OMe, the same general method was employed for each of the peptides synthesized.

In essence, the target peptide sequences were synthesised, on a 0.25 mmol scale, using standard Merrifield-Boc solid phase synthesis protocols, on a manual bubbler system[6] and employing HBTU-mediated couplings[7]. The tri-functional amino acids Arg and Glu were incorporated into the peptides as their *N*-α-Boc-(bis-Cbz)-Arg-OH and *N*-α-Boc-Glu-(OBzl)-OH derivatives, respectively. All other amino acids were incorporated as their simple *N*-α-Boc-derivatives. No difficulties were encountered in coupling any of the residues in the peptides synthesised, as indicated by negative Kaiser ninhydrin tests[8], performed after each of the single couplings.

The protected target peptide sequences were then reductively cleaved from the solid support, using $LiBH_4$ (generated *in situ*), as their *C*-terminal amino alcohol derivatives, essentially according to the procedure of Mergler and Nyfeler[9]. Each of the peptide alcohols were obtained in high yield (typically 80-90%, based on initial resin loading), were found to be homogeneous by TLC and exhibited the correct molecular mass when subjected to electrospray mass spectrometry (EMS).

The peptide alcohols were then reacted in turn with *p*-anisoyl chloride (1.5 molar equivalents), in dry pyridine at 0°C, to yield, after standard workup, the inverse substrate esters in moderate yield (35-55%)[10]. These were judged to be pure by TLC and exhibited the correct $(M + H)^+$ molecular ions when subjected to EMS.

Scheme 1



Conditions: **i**, TFA/DCM (20%, v/v), 30 min at R.T, followed by neutralisation with DIPEA; **ii**, Boc-Gly-OH (3 equiv.) + HBTU (3 equiv.) + HOBt (3 equiv.)+ DIPEA (6 equiv.), 1 hr at R.T; **iii**, repeat step i; **iv**, Boc-Glu-(O-Bzl)-OH (3 equiv.) + HBTU (3 equiv.) + HOBt (3 equiv.) + DIPEA (6 equiv.), 1 hr at R.T; **v**, repeat step i; **vi**, Boc-Ile-OH (3 equiv.) + HBTU (3 equiv.) + HOBt (3 equiv.) + DIPEA (6 equiv.), 1 hr at R.T; **vii**, $LiBH_4$ (6 equiv.) in THF/EtOH (6:1, v/v), 36 hr at R.T; **viii**, p-MeO-C_6H_4-COCl (1.5 equiv.) in dry pyridine for 3 hr at R.T; **ix**, Pd/C (10% w/w) + H_2, 2-3 hr at R.T.

Inactivation studies: The peptide argininol inverse esters were examined for their ability to block the formation of 7-amino 4-methyl coumarin (NH_2Mec) released from the appropriate fluorogenic substrate of their respective target proteinase. For example, **Figure 1** shows typical progress curves for the factor Xa-catalysed hydrolysis of Boc-Ile-Glu-Gly-Arg-NHMec (50 μM), in the presence of varying concentrations (0.1-5.0 μM) of Boc-Ile-Glu-Gly-Arg-ψ–(CH_2-O)-CO-C_6H_4-OMe[11]. These progress curves are typical of the action of an active-site-directed irreversible inhibitor, operating via the mechanism listed below[12,13]. Similar progress curves were obtained for the inactivation of trypsin by the sequences Boc-(D)-Phe-Pro-Arg-ψ–(CH_2-O)-CO-C_6H_4-OMe.

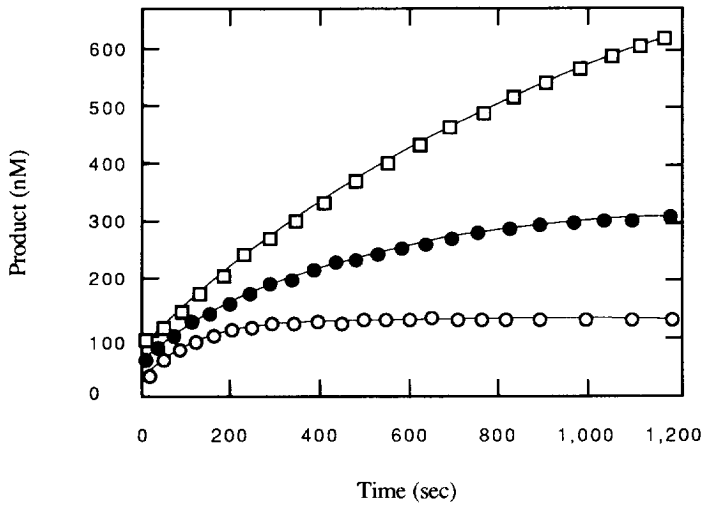
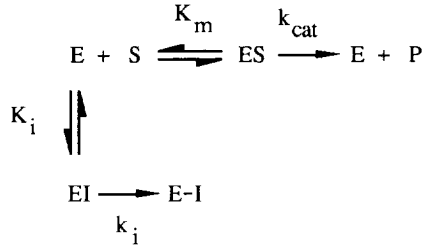


Figure 1. Progress curves for the formation of 7-amino 4-methylcoumarin generated by the factor Xa-catalysed hydrolysis of Boc-Ile-Glu-Gly-NHMec in the presence of 0.5 μM (□), 1.0 μM (●) and 5.0 μM (○) Boc-Ile-Glu-Gly-Arg-ψ-$(CH_2\text{-}O)$-CO-C_6H_4-OMe.



In this scheme, the proteinase **E** catalyses the hydrolysis of substrate **S**, to generate the fluorescent product **P**. This reaction is characterised by the standard kinetic constants K_m and k_{cat}. In the presence of competing inhibitor **I**, the proteinase binds the inhibitor to form the Michaelis complex **EI** (the affinity of this interaction is characterised by the inhibitor constant K_i), which is then covalently modified with first-order rate constant k_i, to form the inactivated complex **E-I**.

Data from progress curves such as those shown in **Figure 1**, were fitted, using non-linear regression analysis [14], to the integrated rate equation **1**.

$$[P] = A.\,(1\text{-}\exp(\text{-}k_{app}.t)) \qquad (1)$$

This rate equation 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 [12]. The values of A and k_{app} for 5 different inhibitor concentrations were then determined and, from these, the inhibitor specificity constant k_i / K_i was evaluated for each of the peptide argininol inverse esters against their particular target proteinase[12]. These are are recorded in **Table 1**.

Table 1

Proteinase Species	Inverse ester	k_i / K_i ($M^{-1}.min^{-1}$)
Factor Xa	Boc-Ile-Glu-Gly-Arg-ψ–(CH_2-O)-CO-C_6H_4-OMe	$4.5 \pm 0.5 \times 10^5$
Thrombin	Boc-(D)-Phe-Pro-Arg-ψ–(CH_2-O)-CO-C_6H_4-OMe	Reversible[a]
Trypsin	Boc-(D)-Phe-Pro-Arg-ψ–(CH_2-O)-CO-C_6H_4-OMe	$2.5 \pm 0.4 \times 10^5$
Tryptase	Ac-Arg-Gln-Arg-ψ–(CH_2-O)-CO-C_6H_4-OMe	N.I.

[a] A K_i value of 14 ± 0.2 μM was obtained for the interaction of this inhibitor with thrombin
N.I. Not inhibited

From **Table 1** it can be seen that the peptidyl inverse esters synthesised in the present study exhibit a complex pattern of inhibitory activity. For example, whereas Boc-Ile-Glu-Gly-Arg-ψ–(CH_2-O)-CO-C_6H_4-OMe and Boc-(D)-Phe-Pro-Arg-ψ–(CH_2-O)-CO-C_6H_4-OMe function as potent time-dependent irreversible inactivators of Factor Xa and trypsin, respectively, the latter peptide which was designed specifically to inactivate thrombin, behaves only as a competitive reversible inhibitor. Additionally, Ac-Arg-Gln-Arg-ψ–(CH_2-O)-CO-C_6H_4-OMe which is modelled on an exceptionally potent chloromethyl ketone (Ac-Arg-Gln-Arg-CH_2Cl) inactivator of tryptase, developed in our laboratory[5], exhibited no inhibitory activity whatsoever, when tested against this protease. This suggests that there might be subtle differences in active-site geometry between the different members of the trypsin-like serine proteinase family and that these could be exploited so as to achieve the selective inactivation of some members of this subclass, using peptidyl inverse esters.

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10. We have also examined an alternative synthetic route that involves the activation of anisic acid with *iso*propenyl chloroformate to yield the mixed carbonic anhydride (see, for example, Jouin, P.; Castro, B.; Zeggaf, C.; Pantaloni, A.; Senet, J.P.; Lecoller, S.; Sennyey, G. *Tet. Lett.*s. **1987,** 28, 1661.) which is then reacted with the peptidyl alcohol in the presence of a catalytic amount (~ 0.1 meq.) of dimethyl amino pyridine (DMAP), to yield the inverse ester. However, this method generally resulted in lower yields of the desired material.
11. Inactivation studies were carried out in 200 mM-Tris/HCl buffer, pH 7.8, containing 100 mM-NaCl and 1 mM-$CaCl_2$, maintained at 37°C. The rate of substrate hydrolysis was monitored continuously by measuring the rate of increase of fluorescence at 455 nm (λexcitation 383 nm).
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