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Reaction of Clavams with Elastase Reveals a General Method for Inhibiting 'Serine' Enzymes

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Abstract—Ester derivatives of clavulanic acid acylate Ser-195 of the serine protease porcine pancreatic elastase to form stable malonyl semi-aldehyde derivatives, analogous to those formed in the inhibition of β-lactamases by clavulanic acid itself. Formation of such derivatives maybe a general way of inhibiting 'serine' enzymes.

Introduction

Soon after the introduction of β-lactam compounds as antibacterials, it became clear that bacteria had evolved resistance mechanisms mediated by β-lactamases[1–3] which catalyse the hydrolysis of β-lactams to give biologically inactive ring-opened products.[4,5] Presently the most clinically important β-lactamases employ a catalytic mechanism involving a nucleophilic serine residue and proceeding via a hydrolytically labile acyl–enzyme intermediate.[5] Clavulanic acid (**1**) is an important serine β-lactamase inhibitor which itself possesses insufficient antibacterial activity and is consequently administered in combination with an antibiotic.[6–8]

Clavulanic acid (**1**) inhibits Class A serine β-lactamases via a mechanism involving fragmentation of both its four and five-membered rings[9–13] (Fig. 1). Spectroscopic and mass spectrometric studies on the clavulanate mediated inhibition of the TEM β-lactamase from *Escherichia coli* were consistent with a process involving initial acylation and

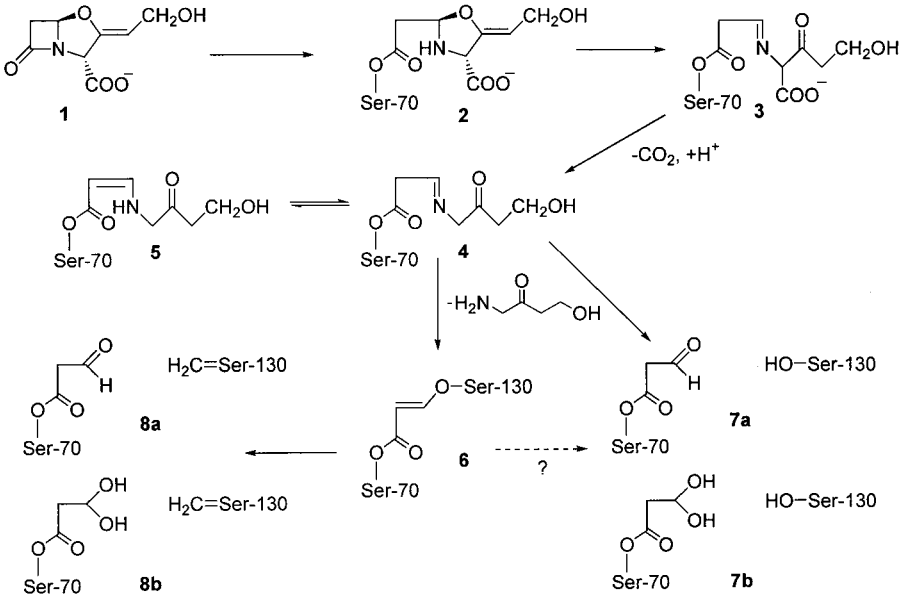

Figure 1. Proposed mechanism for the inhibition of TEM β-lactamase by clavulanic acid.[9–13]

Keywords: clavams; elastase; serine enzymes.

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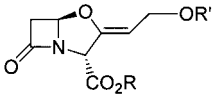
ring-opening of the β-lactam to give **2**. Subsequent ring-opening of the five-membered ring is followed by decarboxylation to give the imine/enamine acyl–enzyme species **4**. Loss of a four carbon fragment can either result in the formation of a relatively stable malonyl semi-aldehyde acyl–enzyme complex **7a** which can be reversibly hydrated to give the diol **7b**. Alternatively, cross-linking with Ser-130 can occur to form a vinyl ether species **6**, the existence of which was predicted by molecular modelling studies.[14] On prolonged standing hydrolysis of **6** results in elimination from Ser-130 to give a dehydroalanyl residue and the formation of an analogous aldehyde acyl–enzyme complex with Ser-70 (**8a**). A cryo-crystallographic study on a trapped enzyme-clavulante complex using the *Staphylococcus aureus* PC1 β-lactamase proved consistent with the formation of *cis*-enamine attached to Ser-70, as well as the *trans*-isomer of the decarboxylated enamine.[15] The stability of aldehydes **7a** and **8a** may be due to their preferential reaction to form hydrates (**7b**, **8b**) rather than undergo ester hydrolysis. Displacement of the hydrolytic water, rather than 'safe reaction', has also been investigated as a strategy for inhibiting serine β-lactamases. Thus, 6α-hydroxyl penicillins have been shown to be inhibitors of TEM β-lactamase.[16,17]

In recent years β-lactams compounds have also found utility as inhibitors of other 'serine' enzymes and, in particular, elastase which is a member of the chymotrypsin sub-family of serine proteases.[18] Elastases have been shown to be inhibited by a variety of mono- and bicyclic β-lactams,[19] some of which have been shown to act via 'double hit' mechanisms (i.e. covalent cross-linking) involving both Ser-195 and His-57.[18–20] Recently, a new class of monocyclic azetidinones with a hydroxyethyl substituent at C-3 were shown to inhibit elastase via acylation and fragmentation involving a retro-aldol process to give a stable ester derivative at Ser-195.[21] Although a water molecule was observed in the 'correct' position for hydrolysis of a normal peptide,[22,23] the complex was reasoned to be stable due to the presence of an α,β-unsaturated ester.

We speculated that the inhibition of serine β-lactamases involving formation of aldehydes such as **7a** might be general to enzymes employing hydrolytically labile acyl–enzyme complexes. Thus, the inhibition of porcine pancreatic elastase (PPE) by clavulanic acid (**1**) and three ester derivatives (**9**, **10**, **11**) was examined.



1 R=H; R'=H
9 R=CH_2Ph; R'=H
10 R=CH_2PhNO_2; R'=H
11 R=CH_2OMe; R'=Me

Results and Discussion

As anticipated from prior work on cephalosporins,[20] clavulanic acid (**1**) itself did not inhibit PPE, but both the benzyl ester (**9**) and *p*-nitrobenzyl ester (**10**) were found to be inhibitors with IC_{50} values of 184 and 187 μM, respectively, under standard assay conditions. No inhibition was observed with the methoxymethyl derivative (**11**). It was found that the inhibitory potency of **9** and **10** increased

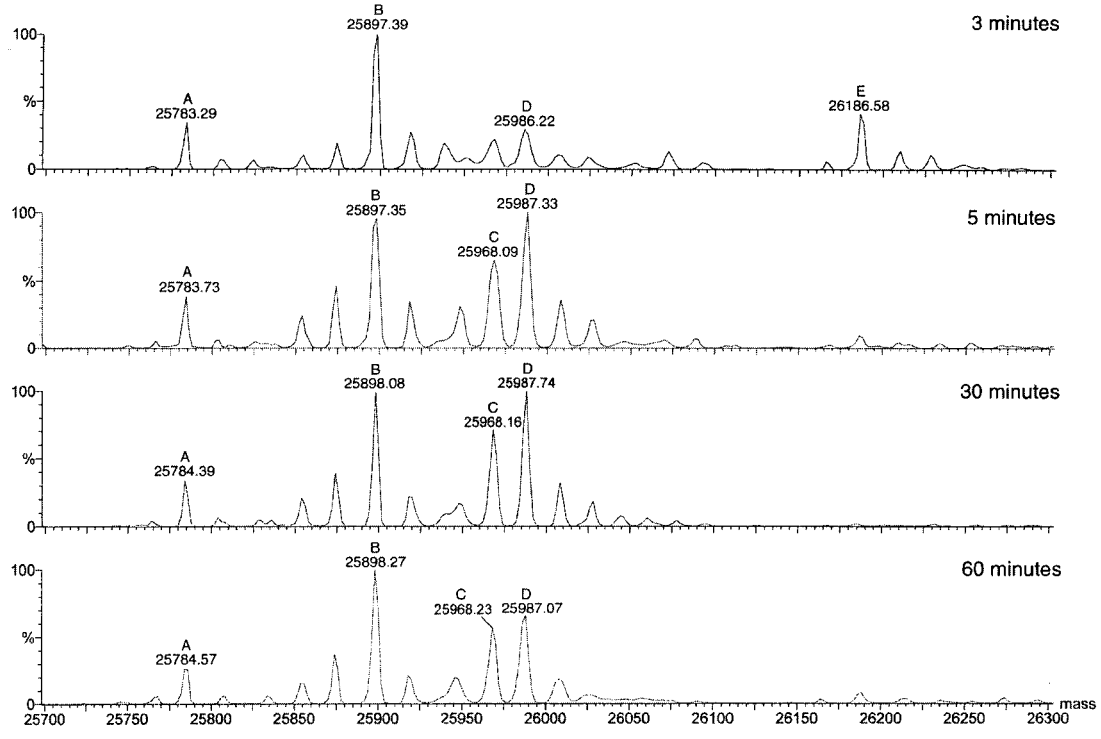


Figure 2. ESIMS time course of the incubation between PPE and the benzyl ester derivative (**9**). Species A corresponds to PPE with loss of its C-terminal asparagine residue[24] (the pattern of adduct peaks for this truncated form of PPE mirrors that of native PPE). Species B corresponds to native PPE and species C and D to the aldehyde and hydrated aldehyde adducts respectively. Species E corresponds to the initial acyl-enzyme complex formed between PPE and the benzyl ester derivative (**9**). Peak assignments are consistent with the errors (standard deviations) on individual peaks (≤1.5 Da).

significantly with pre-incubation of the clavam with PPE indicating that the inhibition was at least partially irreversible. In the case of the benzyl ester derivative (**9**), 43, 87 and 93% inhibition was observed after pre-incubation times of 15, 30 and 60 min, respectively. It is likely that the lack of inhibition observed for clavulanic acid (**1**) is due to the presence of a negative charge on the carboxylic acid at C-3 which prevents binding within the PPE active site.

^{1}H NMR (500 MHz) analysis in D_2O/CD_3CN of the incubation of PPE with the benzyl ester derivative (**9**) demonstrated the production of resonances assigned as arising from clavulanic acid (**1**) and benzyl alcohol. Their identity was subsequently confirmed by doping experiments. These products were just visible after a 1 h incubation and their concentration was observed to increase after 3 and 12 h. This indicated that the ester could be hydrolysed by PPE but was unlikely alone to explain the inhibitory activity of **9**. The lack of any other detected peaks in the NMR spectrum suggested that any inhibitory complex was not readily hydrolysable. Similar ^{1}H NMR analysis using the methoxymethyl ester derivative (**11**) showed that hydrolysis of the ester side chain was faster with complete hydrolysis observed after overnight incubation.

Electrospray ionisation mass spectrometry (ESIMS) analysis of the incubation between the (^{t}Bu)H_3N^+ salt of clavulanic acid (**1**) and PPE at time points of between 3 min and 5 h revealed no mass increments relative to native PPE. In contrast, repeats of the ESIMS time course using the benzyl ester derivative (**9**) (Fig. 2) demonstrated a peak at 26,187 Da after 3 min which corresponds to formation of an initial acyl–enzyme complex between **9** and PPE. After 5 min the intensity of this peak had significantly decreased and two clear additional peaks at 25,968 and 25,987 Da were apparent. These adducts correspond to mass increments of ca. 70 and 88 Da, respectively. After 60 min of incubation, the intensities of both peaks began to fall and after 5 h they had almost disappeared. The 20 h time point showed no further change had occurred.

Similar results were observed with the *p*-nitrobenzyl ester (**10**) except that the formation of the 70 and 88 Da adducts appeared to be slightly faster with significant signals present after 3 min. There was no clear evidence for formation of an acyl–enzyme complex formed by ester cleavage, consistent with the proposal that the inhibition of PPE by clavam derivatives occurs via β-lactam cleavage. Presumably, during ester hydrolysis an acyl–enzyme complex is formed only transiently. Somewhat surprisingly, formation of the 'aldehyde' adducts was also observed with ESIMS analysis of the methoxymethyl ester derivative (**11**), which was not observed to be an inhibitor under standard assay conditions.

The ca. 70 and 88 Da adducts correspond to similar adducts (**7a/b**) detected in the inhibition of TEM β-lactamase by clavulanic acid (**1**)[9] and are likely to represent analogous aldehyde and hydrated aldehyde species. After initial acylation of Ser-195 of PPE by the clavam derivatives and subsequent opening of the β-lactam ring, decarboxylation presumably cannot occur due to the presence of the ester functionality on C-3. This explains the absence of any adducts analogous to the acyl–enzyme species **4** seen with TEM β-lactamase inhibition.[9] The ^{1}H NMR experiments demonstrated that PPE-catalysed hydrolysis of the clavam esters occurs. The lack of inhibition observed for clavulanic acid (**1**) suggests that this catalytic process competes with inhibition via acylation by the β-lactam ring. Similar hydrolysis of a side-chain ester has also been observed with a series of monocyclic γ-lactam inhibitors of elastase.[22] The rapid PPE-catalysed hydrolysis of the methoxymethyl ester derivative (**11**) observed by ^{1}H NMR to give a free carboxylic acid may explain why it was not found to be an inhibitor in the kinetic studies. The rapid reaction (<3 min) to form the 70 and 88 Da adducts as observed by ESIMS may indicate that under the unbuffered conditions necessary for the ESIMS analysis, hydrolysis of the methyl ester on C-3 was sufficiently slow for acylation by the β-lactam ring to compete.

A possible mechanism for the inhibition of PPE by the esterified derivatives of clavulanic acid is shown in Fig. 3. It is assumed that the intermediate imine is hydrolysed by reaction with a water molecule within the active site to give rise to the 'final' inhibitory aldehyde species.

Crystallographic studies on the structure of acyl–enzyme complexes of serine proteases have been reported including

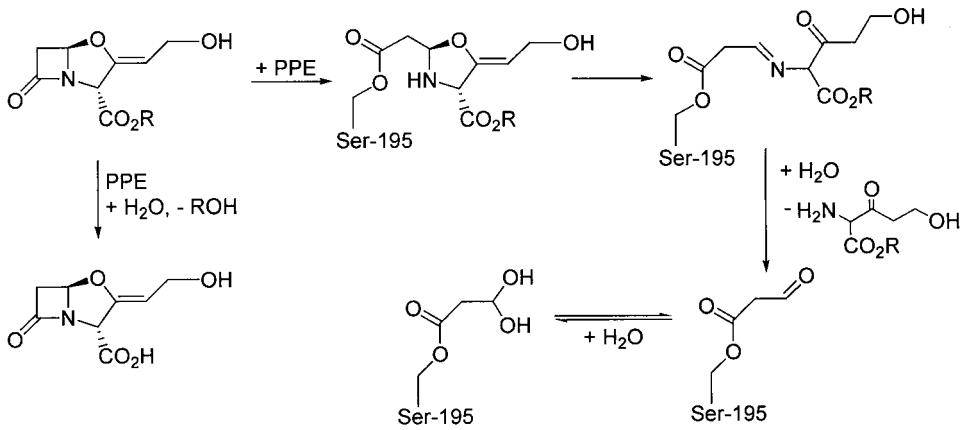


Figure 3. Proposed outline mechanism for the inhibition of PPE by ester derivatives of clavulanic acid. The imines may be in equilibrium with the isomeric enamines (*E*/*Z* ratio unknown). PPE also catalyses the hydrolysis of the esters to give clavulanic acid (**1**) itself.

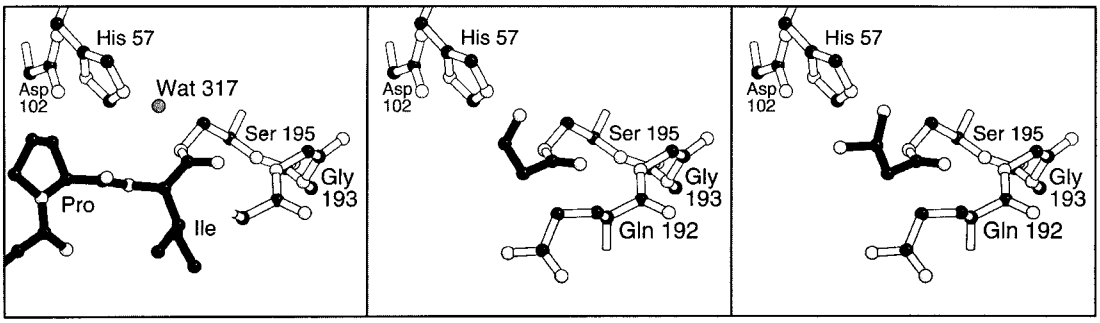


Figure 4. Model structure of the malonyl semi-aldehyde complex (middle) and its hydrated form (right) in PPE. The acyl-enzyme complex between PPE and β-casomorphin-7[23] is shown for comparison (left). The location of the hydrolytic water (Wat-317) can be seen to be almost coincident with the oxygen of the aldehyde carbonyl. In all cases the inhibitor molecule is shown in black and the enzyme in white. The figure was prepared using Bobscript.

one formed between a natural heptapeptide and PPE.[23] This structure reveals a water molecule (Wat-317) positioned above the ester carbonyl, apparently poised for nucleophilic attack, which does not occur due to the pH (ca. 5) of the crystal. Using this structure as a template it seems that a malonyl semi-aldehyde could readily displace or 'soak up' the hydrolytic water by reaction with its aldehyde to form a hydrate (Fig. 4), which would be in position to form a hydrogen bond with $N_{\epsilon 2}$ of His-57. Note that in addition to formation of the aldehyde derivatives, hydration of the intermediate imine may also serve to 'protect' the ester linkage.

The fact that analogous aldehydes are formed by reaction of different clavam derivatives with both PPE and TEM β-lactamase, suggests that their formation may be more widely applicable to enzyme inhibition. It is possible that the generation of an enzyme-X–$COCH_2CR{=}Y$ species (X=O, serine proteases; X=S, cysteine proteases; Y=O, S, NR etc.) may be a general way of inhibiting enzymes proceeding via hydrolytically unstable acyl–enzyme complexes. Clavam derivatives appear to be a way of delivering a malonyl semi-aldehyde derivative, which would otherwise be unstable and probably toxic. The generation of other templates capable of delivering the same functionality is a challenge for synthetic chemists.

There are several ways in which the potency of these clavulanic acid (**1**) derivatives as protease inhibitors may be enhanced. Changing the C-3 ester to a non-hydrolysable functionality would remove the second pathway of nucleophilic attack by Ser-195. Secondly, addition of an alkyl group α to the carbonyl of the β-lactam ring has been shown to improve acylation by PPE in a range of monocyclic β- and γ-lactam inhibitors.[18,22,25–27] When the alkyl group is correctly located in the S_1 subsite of elastase it ensures proper localisation of the β-lactam carbonyl within the oxyanion hole and thus optimises successful nucleophilic attack by Ser-195.

Experimental

Enzyme assays

Enzyme assays were performed using a Shimadzu 1601PC spectrophotometer equipped with a thermostatted multi-cell transport system. The hydrolysis of the *para*-nitroanilide substrate (Suc-AlaAlaProAla-pNa, 100 μM) was measured at 405 nm and at a constant temperature of 25°C in 0.1 M Tris–HCl buffer (pH 7.5). At least quadruplicate measurements of the initial rate were determined at five inhibitor concentrations and data analysed using standard kinetic equations programmed into EXCEL (Microsoft Corp.) and GRAFIT (Erithacus Inc.). To aid solubility the clavam inhibitors were dissolved in DMSO to give a final concentration in the assay of 10% (v/v).

NMR experimental procedures

^{1}H NMR analyses were performed at 500 MHz on a Bruker AMX500 instrument. Samples (450–500 μl) of PPE (ca. 3 mg) and clavulanic acid derivative (ca. 3 mg) were dissolved in 10% (v/v) CD_3CN in D_2O. The temperature was regulated at 303 K and the spectra were referenced to internal MeCN at 2.05 ppm.

Electrospray ionisation mass spectrometry

Electrospray ionisation mass spectra were recorded on a Micromass BioQ II-ZS triple quadrupole mass spectrometer equipped with an electrospray interface. PPE at 80 pmol μl^{-1} was incubated with one equivalent of inhibitor. Samples (5 μl) were removed at fixed time points, diluted with water:acetonitrile (1:1 v/v) containing 0.2% (v/v) formic acid to give a protein concentration of 5 pmol μl^{-1} and analysed immediately. The resulting electrospray mass spectra were calibrated relative to native PPE and processed using the MaxEnt algorithm and the spectra reported as centroided data.

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