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An Investigation into the Mechanism of Elastase Inhibition by Cephalosporins using Electrospray Ionisation Mass Spectrometry

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Abstract: Electrospray ionisation mass spectrometry was used to investigate the mechanism of inhibition of porcine pancreatic elastase (PPE) by two cephalosporins, L-658758 [1-[[3-(acetoxymethyl)-7α-methoxy-8-oxo-5-thia-1-azabicyclo[4.2.0.]oct-2-en-2-yl]carbonyl]proline S,S dioxide], and L-647957 [1-[[3-(acetoxymethyl)-7α-chloro-8-oxo-5-thia-1-azabicyclo[4.2.0.]oct-2-en-2-yl]carboxylic ^{t}butyl ester]S,S dioxide]. The mass shifts, observed as compared with native PPE, upon incubation with the inhibitors were consistent with the formation of an acyl enzyme complex followed by expulsion of the acetoxy group from the 3'-methylene position of the cephalosporin inhibitors. In the case of L-647957 the mass shifts also indicated loss of HCl. In contrast for L-658758, no evidence was accrued for loss of methanol, consistent with previous kinetic studies on human leukocyte elastase.

INTRODUCTION

Elastases are members of the serine protease enzyme family and possess the ability to cleave the connective tissue protein, elastin. Increased production or inadequate neutralisation of these enzymes is thought to result in uncontrolled proteolytic degradation of connective tissue components, a process that has been implicated in diseases such as pulmonary emphysema[1] and cystic fibrosis.[2] Human leukocyte elastase (HLE, EC 3.4.21.37) and porcine pancreatic elastase (PPE, EC 3.4.21.36) are examples of this class of enzyme. Extensive research has been carried out into their mode of action in an attempt to develop potent and specific inhibitors of HLE.

Following the initial discovery that benzyl clavulanate inhibited HLE,[3] a range of inhibitors based on the β-lactam nucleus has been developed by Merck, Sharp and Dohme. These include both monocyclic β-lactams[4-7] and bicyclic structures such as the cephalosporins.[3,4,8-10] This work is of major importance since it has pioneered the use of β-lactams for therapeutic uses other than as antibacterials.

The mechanism of elastase inhibition by the cephalosporins has been the subject of several studies.[11,12] Thus, X-ray crystallographic analysis of PPE inhibited by the 7α-chloro derivative, L-647957 (figure 1) has demonstrated both acylation of the active site serine and alkylation of the histidine in a 'double hit' mechanism. In addition, elimination of HCl was observed to occur to give complex (1) (figure 1). At present, no direct evidence exists concerning the relative timing of the elimination of HCl and the 3'-acetate group. In contrast, kinetic and [^{14}C]-labelling studies on the mechanism of inhibition of HLE by the 7α-methoxyl derivatives, L-658758 and L-659286 (figure 2), whilst supporting the 'double hit' mechanism, indicated that

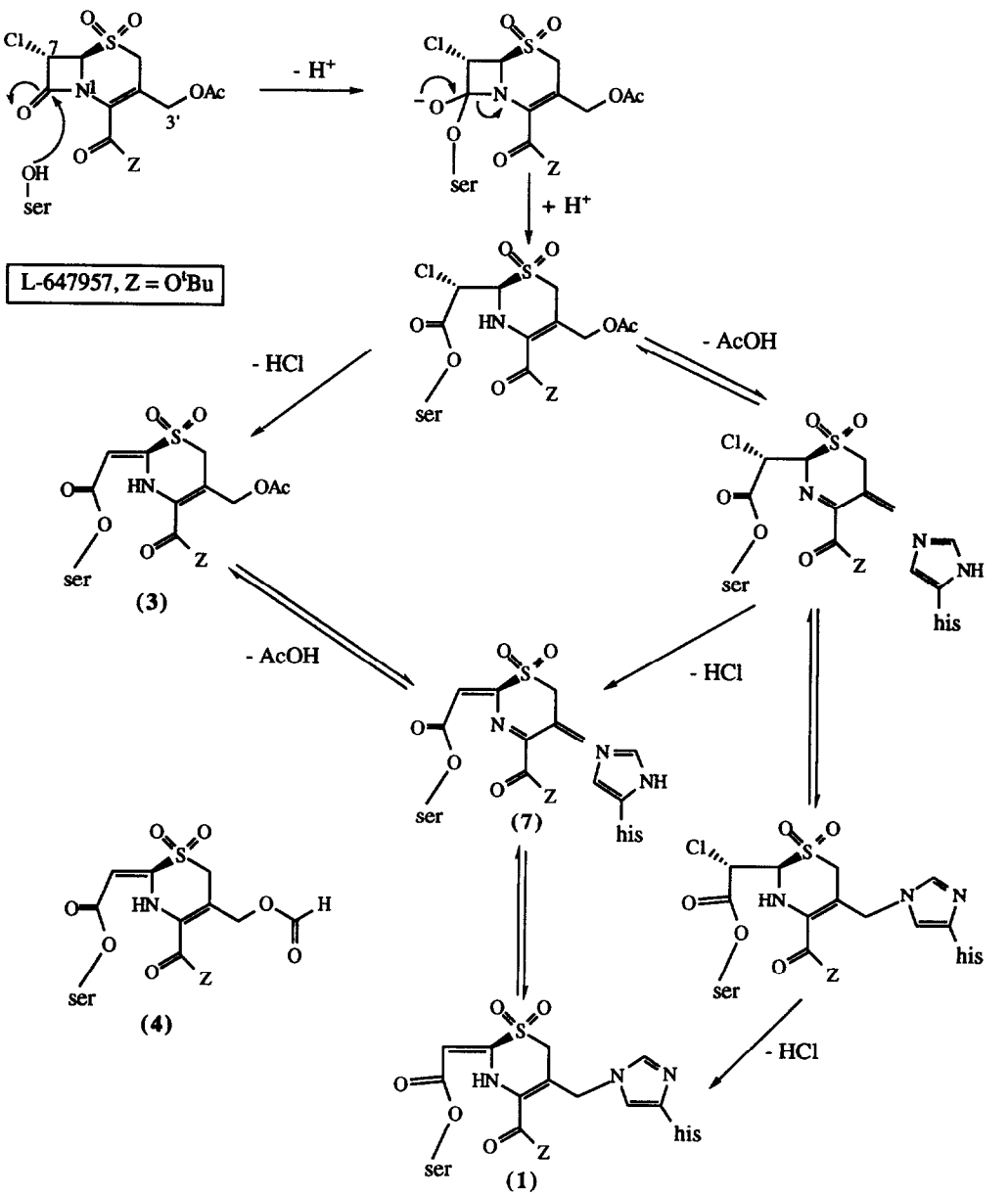
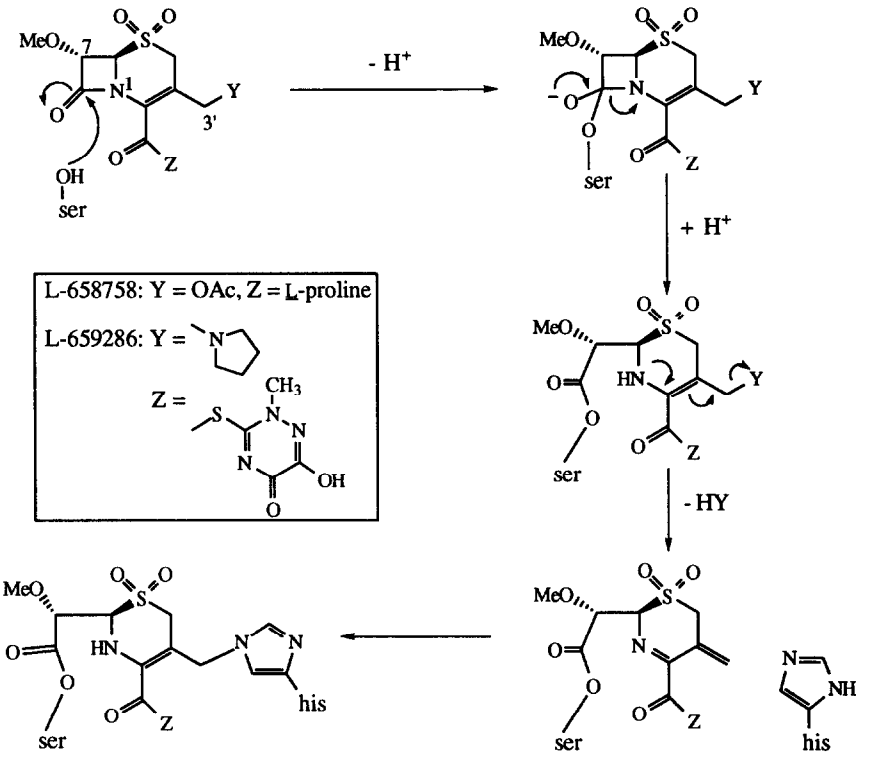


Fig.1 Inhibition of porcine pancreatic elastase by 7α-chloro cephalosporins.[11]

the methoxyl group was retained in the inhibited enzyme.[12] The proposed mechanism of inhibition by L-658758 and L-659286 is summarised in figure 2. It is not clear, however, whether the difference in behaviour observed for the reaction of the 7α-chloro cephalosporins with PPE compared with that of the 7α-methoxyl cephalosporins with HLE is due to differences in the intrinsic reactivity of the inhibitors or differences between the enzymes.[13]

Recent studies[14-16] have demonstrated the potential of electrospray ionisation mass spectrometry (ESI MS) for the analysis of intermediates in enzymic reactions. In particular we have reported the use of ESI MS to study the inhibition of PPE by a series of inhibitors[17] and Knight *et al*[18] have reported a study on the inhibition of HLE by a monocyclic β-lactam inhibitor. Herein, we reveal the results of our investigations, using ESI MS, into the inhibition of PPE by examples of both 7α-chloro and 7α-methoxyl cephalosporins.



(2)

Fig.2 Inhibition of human leukocyte elastase by 7α-methoxyl cephalosporin.[12]

RESULTS AND DISCUSSION

As we have reported previously,[17] the positive ion mass transformed spectrum of the commercial PPE [figures 3.1.1 and 3.2.1] used in these studies indicated the presence of two components with relative molecular masses corresponding to native PPE, component B (observed M_r = 25 899.4±3; calculated M_r = 25 898.1) and approximately 30% of a species, component A (observed M_r = 25 784.7±3; calculated M_r = 25 784.1) corresponding to PPE without the C-terminal asparagine (Asn) residue. In the inhibition studies described subsequently, analogous mass shifts were observed corresponding to binding to both of these species (the partial loss of a C-terminal Asn from the PPE has been confirmed by C-terminal sequencing[17]).

For the PPE/L-658758 system, the averaged observed mass shifts (species C and D, figure 3.1.2) were consistent with the loss of acetic acid from L-658758 (M_r = 416.4) and were independent of the quenching

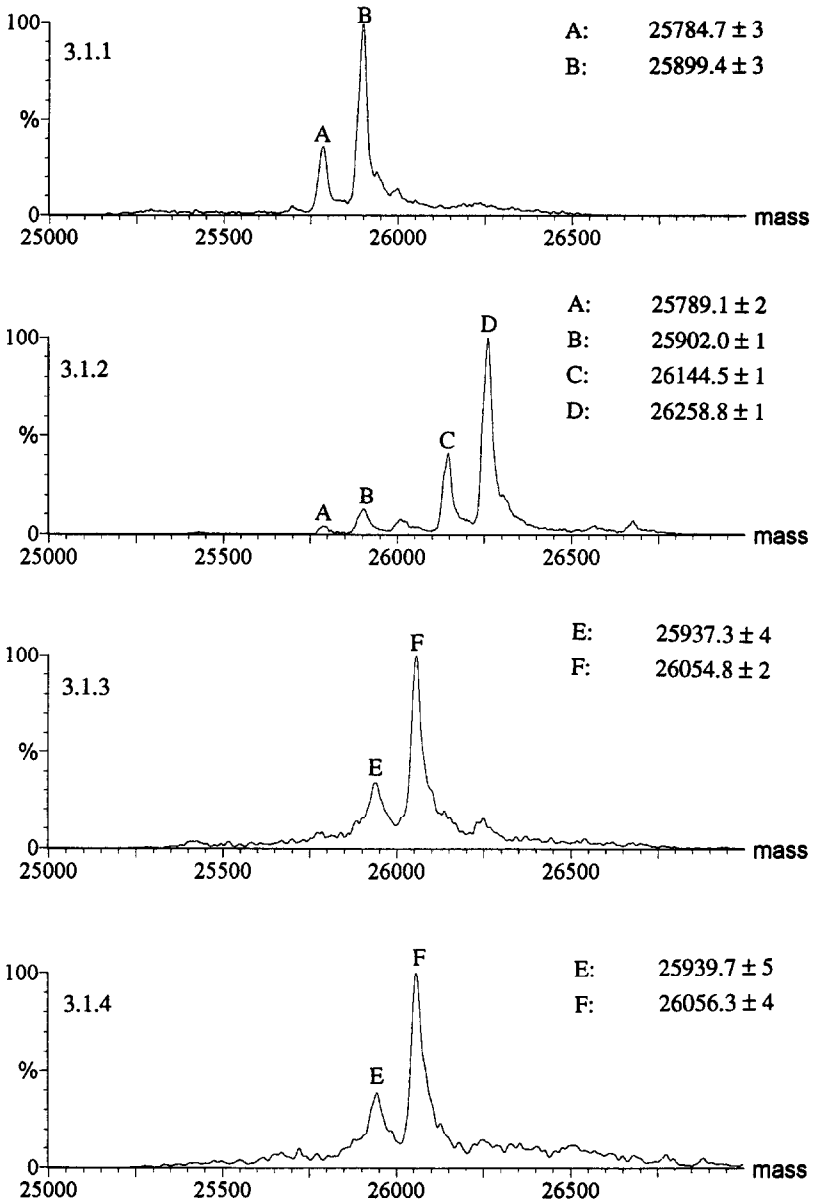


Fig. 3.1. Mass transformed ESI MS spectra for:

3.1.1) Native PPE; **3.1.2)** The inhibition of PPE by the cephalosporin inhibitor L-658758 [PPE:L-658758, 1:1, incubation time t = 5mins, quenching solution b) - CH_3CN + 2% HCO_2H]. Mass shifts: C-A = 355.4 ± 2Da; D-B = 356.8 ± 2Da. Calculated mass shift for formation of **(2)** (figure 2) = 356.4Da; **3.1.3)** The inhibition of PPE by phenylmethylsulfonyl flouride (PMSF) (PPE:PMSF, 1:3, t = 30mins); Mass shifts§: E-A = 152.6 ± 7Da; F-B = 155.4 ± 5Da. Calculated mass shift for PMSF-blocked PPE = 154.2Da; **3.1.4)** The inhibition of PPE by PMSF (PPE: PMSF, 1:3, t = 30mins) followed by incubation with L-658758 (PPE:L-658758, 1:1, t = 5mins, quenching solution b) - CH_3CN + 2% HCO_2H] used to quench incubation. Mass shifts§: E-A = 155.0 ± 8Da, F-B = 157.0 ± 7Da.

§ In experiments 3.1.3 and 3.1.4 where no native PPE was observed in the ESI MS spectra, the mass shifts were calculated using the observed values for native PPE and native PPE-asn given in figure 3.1.1.

solution used. This result provides evidence in support of the proposed 'double hit' mechanism[12] shown in figure 2, with the calculated mass shift (356.4Da) expected for the formation of the enzyme-inhibitor complex (**2**) being within 1% of the averaged observed mass shifts (table 1). In this case no evidence was accrued for the elimination of methanol from the inhibitor, consistent with the results of Knight *et al*[12] on the inhibition of HLE by 7α-methoxyl cephalosporins.

Table 1. Averaged Observed Mass Shifts as compared with Native PPE following incubation with the 7α-Methoxyl Cephalosporin L-658758 (figure 2) and quenching with two different solutions.

	Averaged Observed Mass Shift (Da)	
Quenching Solution	Based on PPE-Asn mass shifts	Based on PPE mass shifts
CH_3CN + 2% HCO_2H	355.9 ± 2 (5)	356.3 ± 2 (7)
CH_3CN	355.9 ± 3 (3)	356.9 ± 2 (4)

The number of ESI MS experiments over which the data was averaged is shown in parentheses. Compare averaged observed mass shifts shown above with the calculated mass shift for formation of (**3**) (Fig.2) = 356.4Da.

Incubation of the enzyme with PMSF resulted in the observation of mass shifts corresponding to PMSF-blocked enzyme (species E and F, figure 3.1.3). Subsequent incubation of the blocked enzyme with L-658758 and analysis by ESI MS led to the production of a spectrum (figure 3.1.4) in which no additional mass shifts were observed, implying that the active site of the enzyme is involved in the inhibition of PPE by L-658758.

Slow reactivation of HLE inhibited by L-658758 has been reported[12] and thus a time course study was conducted for the inhibition of PPE by L-658758. Analysis after a 3 minute incubation (PPE:L-658758, 1:3) indicated >95% of the enzyme was inhibited. Further analysis after 3.5 hours indicated the appearance of native PPE and native-asn PPE. After 18 hours peaks corresponding to the native PPE and native-asn PPE were dominant, although there was also evidence for partial degradation of the enzyme.

Quenching of the PPE/L-647957 system with CH_3CN resulted in the observation of a major series of mass shifts (species G and H, figure 3.2.2) with an averaged observed mass shift of 284.4 ± 2Da (averaged over 2 experiments). These mass shifts are consistent with loss of both acetic acid and HCl from L-647957 to give species (**1**) (figure 1, calculated mass shift = 283.3Da). This assignment of the mass shifts is in agreement with the enzyme-inhibitor complex characterised by X-ray crystallography for the PPE/L-647957 system.[11] It was generally found that ESI MS spectra obtained from experiments in which no acid was present in the quenching solution were of lower quality with less well resolved peaks and spectra weaker than those in which acid was present. The observation of other sets of minor mass shifts in non-acid quenched

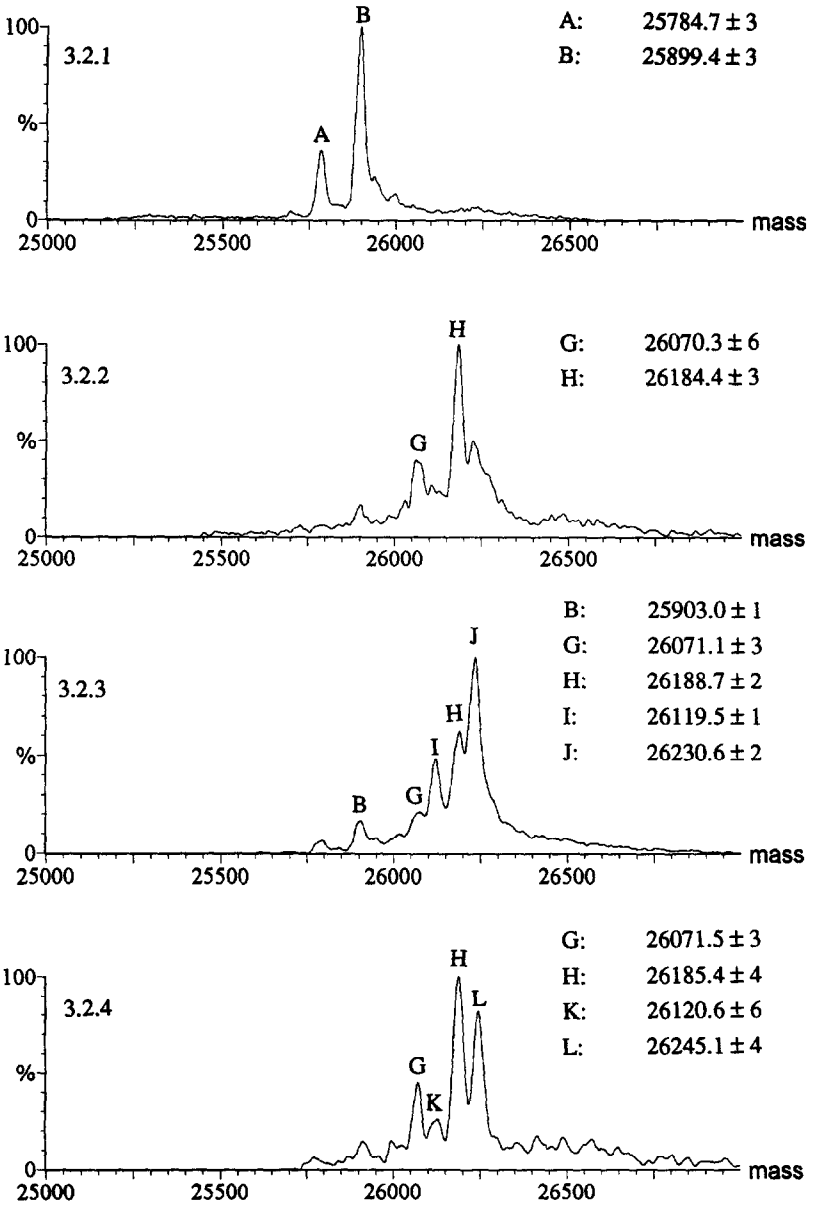


Figure 3.2. Mass transformed ESI MS spectra for:

3.2.1) Native PPE; **3.2.2)** The inhibition of PPE by L-647957 [PPE:L-647957, 1:3, incubation time t = 3mins,. quenching solution d) - CH_3CN]. Mass shifts: G-A = 285.6 ± 9Da, H-B = 285.1 ± 6Da; **3.2.3)** The inhibition of PPE by L-647957 [PPE:L-647957, 1:3, t = 3mins, quenching solution a) - CH_3CN + 8% formic acid]. Mass shifts: G-A = 282.7 ± 7Da; H-B = 285.7 ± 3Da; I-A = 331.1 ± 4Da; J-B = 327.6 ± 3Da. Based on the observation that native PPE has M_r = 25903.01 ± 0.7 in this experiment, a value for native PPE-asn (A) = 25 788.36 ± 3Da was used in calculating G-A and I-A; **3.2.4)** The inhibition of PPE by L-647957 [PPE:L-647957, 1:1, t = 3mins, quenching solutions c) - CH_3CN + 20% acetic acid]. Mass shifts: G-A = 286.8 ± 6Da; H-B = 286.1 ± 7Da; K-A = 335.9 ± 9Da; L-B = 345.7 ± 7Da. No spectra in which native PPE was present were obtained for these experiments. Mass shifts were calculated using the observed values for native PPE and PPE-asn given in figure 3.2.1.

experiments have been tentatively assigned to the addition of a residual amount of formic acid present in the electrospray interface to the enzyme:inhibitor complex (**1**) (figure 1). Pre-incubation of PPE with PMSF followed by incubation with L-647957 resulted in the observation of mass shifts corresponding to the PMSF-blocked enzyme only.

In the case of the PPE/L-647957 system, when formic acid was included in the quenching solution [quenching solutions a) and b) - see experimental section], mass shifts (species G and H, figure 3.2.3, table 2) corresponding to loss of HCl and acetic acid were observed as before. However, a second major series of mass shifts (species I and J, figure 3.2.3) corresponding to the loss of HCl and acetic acid and gain of formic acid was also observed (averaged observed mass shift are shown in table 2; calculated mass shift = 329.3 Da). An increase in the concentration of formic acid in the quenching solution led to an increase in the intensity of these species. Analogous mass shifts were not observed in the case of the PPE/L-658758 system when formic acid was included in the quenching solution or for native PPE when the concentration of formic acid was increased.

When acetic acid (up to 10%) was included in the quenching solution [solution c) - see experimental section] for the PPE/L-647957 system, a minor set of mass shifts (species K and L, figure 3.2.4, table 2) corresponding to the loss of HCl alone (calculated mass shift = 343.4Da) was present in addition to the major set (G and H, figure 3.2.4, table 2) corresponding to loss of HCl and acetic acid from L-647957. No evidence was accrued for the presence of mass shifts corresponding to the loss of HCl alone [(**3**), figure 1] for the PPE/L-647957 system when formic acid was included in the quenching solution.

Table 2. Averaged Observed Mass Shifts as compared with Native PPE following incubation with the 7α-Chloro Cephalosporin L-647957 (figure 1) and quenching with three different solutions.

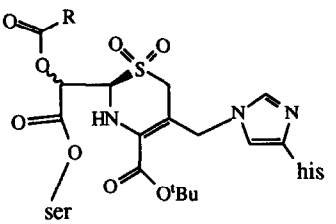
	Averaged Observed Mass Shifts (Da)			
Quenching Solution	Based on PPE - Asn mass shifts	Based on PPE mass shifts	Based on PPE - Asn mass shifts	Based on PPE mass shifts
CH_3CN + 2 or 8% HCO_2H Figure 3.2.3	species G 285.1 ± 3 (3)	species H 286.4 ± 2 (3)	species I 329.6 ± 1 (2)	species J 332.3 ± 3 (6)
CH_3CN + 2-20% CH_3CO_2H Figure 3.2.4	species G 287.1 ± 2 (4)*	species H 283.9 ± 4 (5)*	species K 338.8 ± 1 (2)*	species L 344.0 (2)*

* No spectra in which native PPE was present after incubation with L-647957 and quenching were obtained for these experiments. Mass shifts were calculated using the observed values for native PPE and PPE-asn given in Fig.3.2.1. Compare averaged observed mass shifts for species G and H with the calculated mass shift for formation of (**1**) (Fig 1) = 283.3Da; compare averaged observed mass shifts for species I and J with the calculated mass shift for formation of (**4**) (Fig.1) or (**5**) = 329.3Da; compare averaged observed mass shifts for species K and L with the calculated mass shift for formation of (**3**) (Fig.1) or (**6**) = 343.4Da.

This ESI MS study reinforces previous mechanistic proposals on the mode of inhibition of elastases by cephalosporins. In particular, the observation that both acetic acid and HCl are eliminated from L-647957 is consistent with the X-ray crystallographic studies on PPE.[11] The observed loss of acetic acid but not

methanol from L-658758, in our study on PPE, also agrees with studies on HLE using this inhibitor,[12] indicating a similar mode of inhibition of both PPE and HLE by L-658758. Loss of the 7α-group from the 7α-chloro cephalosporin (L-647957), but not from the 7α-methoxyl cephalosporin (L-658758) therefore probably reflects the better leaving group ability of the chloride versus the methoxyl group.

One possible explanation for the observation of a second major set of mass shifts (I and J, figure 3.2.3, table 2) when formic acid is included in the quenching solution is that the addition of the histidine to the C-3' methylene of the enzyme-inhibitor complex (**7**) (figure 1) is a reversible process, allowing addition of a formate group at the C-3' position to give (**4**) (figure 1). Similarly, inclusion of acetic acid in the quenching solution may lead, as a result of reversible histidine C-3' addition, to a significant concentration of complex (**3**) (figure 1) explaining the observation of species K and L (figure 3.2.4, table 2). However, addition of formate or acetate to the C-7 position of (**1**) (figure 1) to give complexes (**5**) and (**6**) respectively could also explain the observation of species I and J (figure 3.2.3, table 2) and species K and L (figure 3.2.4, table 2) when formic and acetic acid were included in the quenching solutions respectively.



(**5**) R = H

(**6**) R = CH_3

In contrast, the mass shifts observed for inhibition of PPE by the 7α-methoxyl cephalosporin (L-658758) were apparently independent of the quenching solution used, consistent with effectively irreversible histidine addition at the C-3' position of the inhibitor. This observed difference, with respect to the inclusion of formic or acetic acid in the quenching solution, for the two systems studied may result from the fact that in the case of L-647957 the 7α-chloro group is lost, a process which either renders histidine addition at C-3' reversible or enables nucleophilic attack at C-7.

The observation that cephalosporins functionalised with 7α-groups have increased elastase inhibition potency compared with their 7β-analogues has been rationalised[3] by proposing that the 7α-group fits into the S_1 pocket of elastases. Therefore the proposed reversibility of histidine addition in solution in the case of the 7α-chloro cephalosporin (L-647957) may stem from a loss of rigidity in the enzyme:inhibitor interactions resulting from the lack of a group in the S_1 pocket of PPE in enzyme:inhibitor complexes (**1**) and (**7**) (figure 1) after elimination of HCl. This contrasts with the 7α-methoxyl cephalosporin in which the 7α-group is retained and the enzyme:inhibitor complex is apparently formed effectively irreversibly.

EXPERIMENTAL

Materials. PPE was purchased from Universal Biological Ltd. (Serva Feinbiochemica), 30, Merton Road, London, SW18 1QY. The cephalosporin inhibitors L-658758 and L-647957 were kindly donated by Merck, Sharp and Dohme, Rahway, New Jersey, USA.

Methods. Electrospray mass spectra were measured on a VG BIO Q triple quadrupole atmospheric pressure mass spectrometer equipped with an electrospray interface. Samples (10μl) were injected into the electrospray source via a loop injector as a solution, typically 20pmol/μl, in one of four solvent systems at a flow rate of 2μl per minute [solvent systems used were a) water:CH_3CN + 8% HCO_2H (1:1); b) water:CH_3CN + 2% HCO_2H (1:1); c) water:CH_3CN + 2-20% CH_3CO_2H (1:1); d) water: CH_3CN (1:1)]. Sample solutions were prepared immediately prior to analysis by mixing in water (pH 7). The ratio of enzyme:inhibitor and the incubation time that resulted in optimal spectra were found to vary with the inhibitor used. Typical values for this ratio were between 1:1 and 1:3 with incubation times between 1 and 10 minutes. Aliquots were removed from the stock solution, mixed with an equal volume of one of the four quenching solutions and immediately analysed by ESI MS using a cone voltage of 50V [quenching solutions used were a) CH_3CN + 8% HCO_2H; b) CH_3CN + 2% HCO_2H; c) CH_3CN + 2-20% CH_3CO_2H; d) CH_3CN]. The mass spectrometer was scanned over a mass range 1100±450Da. The instrument was calibrated with horse heart myoglobin (20pmol/μl, M_r = 16 951.5).

Control experiments were carried out in which the enzyme was incubated with phenylmethylsulfonyl fluoride (PMSF),[19] a known serine protease active site acylating agent, prior to incubation with the cephalosporin inhibitors. Incubation with PMSF (ratio of PPE:PMSF = 1:1.5) for 15 minutes followed by further incubation with a second quantity of PMSF (1.5 equivalents) for 15 minutes (overall ratio of PPE:PMSF = 1:3) was found to result in >95% inhibition of PPE.

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