Structure???reactivity relationships in the inactivation of elastase by ??-sultams

ARTICLE *in* ORGANIC & BIOMOLECULAR CHEMISTRY · JANUARY 2003

Impact Factor: 3.56 · DOI: 10.1039/b208079f

6 AUTHORS, INCLUDING:

Michael I Page
University of Huddersfield
205 PUBLICATIONS **4,387** CITATIONS

ARTICLE

OBC
www.rsc.org/obc

Structure–reactivity relationships in the inactivation of elastase by β-sultams

Paul S. Hinchliffe,[a] J. Matthew Wood,[a] Andrew M. Davis,[b] Rupert P. Austin,[b] R. Paul Beckett[c] and Michael I. Page*[a]

[a] *Department of Chemical and Biological Sciences, University of Huddersfield, Queensgate, Huddersfield, UK HD1 3DH*

[b] *AstraZeneca R&D, Charnwood, Bakewell Road, Loughborough, UK LE11 5RH*

[c] *British Biotech Pharmaceuticals Limited, Wellington Road, Oxford, UK OX4 5LY*

Received 20th August 2002, Accepted 30th September 2002
First published as an Advance Article on the web 27th November 2002

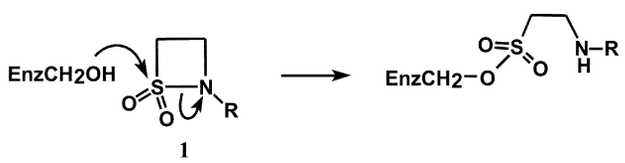
N-Acyl-β-sultams are time dependent irreversible active site directed inhibitors of elastase. The rate of inactivation is first order with respect to β-sultam concentration and the second order rate constants show a similar dependence on pH to that for the hydrolysis of a peptide substrate. Inactivation is due to the formation of a stable 1 : 1 enzyme inhibitor complex as a result of the active site serine being sulfonylated by the β-sultam. Ring opening of the β-sultam occurs by S–N fission in contrast to the C–N fission observed in the acylation of elastase by *N*-acylsulfonamides. Structure–activity effects are compared between sulfonylation of the enzyme and alkaline hydrolysis. Variation in 4-alkyl and *N*-substituted β-sultams causes differences in the rates of inactivation by 4 orders of magnitude.

Proteolytic enzymes are common targets for potential therapeutic application and their inhibition by mechanism based and active site directed agents is a fruitful area of study.[1] In general, this involves the covalent modification of an active site residue which is not then readily regenerated. For example, many inhibitors are acylating agents of the active site serine residue of serine proteases.[2] The mechanism of inhibition involves the displacement of a leaving group from the acylating agent to generate a relatively stable acyl enzyme which only reacts slowly with nucleophiles, such as water, to regenerate the enzyme and so this process leads to effective inhibition.

Human neutrophil elastase (HNE) is a serine enzyme which is one of the most destructive proteolytic enzymes, being able to catalyse the hydrolysis of the components of connective tissue. HNE is released in response to inflammatory stimuli and has a major role in protein digestion following phagacytosis. It has been implicated in the development of diseases such as emphysema, cystic fibrosis and rheumatoid arthritis and there have been numerous studies attempting to find small molecule inhibitors of HNE.[3] The enzyme belongs to the trypsin class and the structure of HNE has been determined by X-ray crystallography,[4] but many structural and inhibition studies have been conducted with the related, but more readily available, porcine pancreatic elastase (PPE).[5]

The majority of elastase inhibitors are based an other serine protease inhibitors. They are, for example, peptidyl fluoroketones[6] or ketones attached to a strongly electron withdrawing group, such as 2-benzoxazole, with suitable elements of molecular recognition.[7] An alternative strategy has been to use acylating agents that generate an acyl enzyme which does not turnover, or rearranges to a more stable adduct or even generates a second electrophilic 'trap' which subsequently reacts with a nucleophilic group on the enzyme. For example, bicyclic [3.3.0] systems containing a γ-lactone or γ-lactam which is *trans*-fused to the other 5-membered ring inhibit HNE by acylating the nucleophilic hydroxy group of serine-195 in the active site of the enzyme.[8] Interestingly, the classical β-lactams, traditionally used as anti-bacterial agents by inhibiting serine transpeptidases,[9] have also been shown to be mechanism based inhibitors of elastase when used as neutral derivatives.[10] ESI-MS and NMR studies have shown that the first step is an acylation process in which the four membered β-lactam ring is opened.[11] The requirements for inhibition of elastase by monocyclic lactams have been recently described.[12] They involve the initially formed acyl enzyme intermediate undergoing a conformational change to displace the hydrolytic water molecule, effectively generating a more stable complex.

In principle, sulfonation of serine proteases offers an interesting but largely unexplored strategy for inhibition as an alternative to the traditional mechanism-based acylation process. In addition to their normal acyl substrates, serine proteases are known to react with other electrophilic centres such as phosphonyl derivatives.[1] The main reason why sulfonation of serine enzymes is not a well studied process is because sulfonyl derivatives are much less reactive than their acyl counterparts.[13] For example, sulfonamides are extremely resistant to alkaline and acidic hydrolysis and, in general, sulfonyl transfer reactions are 10^2 to 10^4 fold slower than the corresponding acyl transfer process. However, we have recently shown that the rates of alkaline and acid hydrolysis of *N*-alkyl and *N*-aryl β-sultams are 10^2 to 10^3 fold greater than those for the corresponding β-lactams.[14] β-Sultams (**1**) show extraordinary rate enhancements of 10^9 and 10^7, respectively, compared with the acid and base catalysed hydrolysis of the corresponding acyclic sulfonamides.[15] In principle, therefore, β-sultams are excellent candidates to explore the mechanism of sulfonation and possible inhibition of serine protease enzymes. We recently demonstrated that ring opening of the β-sultam by elastase gives the sulfonate ester,[16] by analogy with the acyl enzyme intermediate formed during the hydrolysis of normal substrates (Scheme 1).



1

Scheme 1

Herein, we report further studies on this process and the effect of structural changes in the β-sultam on the rate of inactivation of elastase.

DOI: 10.1039/b208079f

Results and discussion

One of the major requirements for successful inhibition of an enzyme by covalent modification is that the rate of the inactivation must be faster than, or at least competitive with, the turnover of the natural substrate and the inhibitor itself must be relatively stable under *in vivo* conditions. The rate of inactivation is controlled by two important factors:

(i) the "chemical" reactivity of the inactivator

(ii) molecular recognition by the target enzyme.

Given that most modifications involve displacement of a leaving group by a nucleophile on the enzyme, chemical reactivity is usually considered in terms of the electrophilic nature of the inhibitor and the leaving group ability (nucleofugacity) of the group displaced. In addition to these electronic effects, the intrinsic chemical reactivity of a compound may be modified by steric and strain effects. Increasing intrinsic chemical reactivity may lead to a faster rate of reaction with the target enzyme but may also lead to greater hydrolytic and metabolic instability and lower specificity.

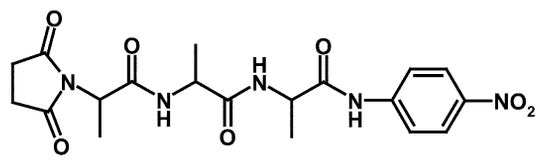
Obtaining maximum activity by molecular recognition involves using the full catalytic apparatus of the enzyme, as used for the natural substrate, and utilising the favourable non-covalent interactions at the various sub-sites of the enzyme and inhibitor.[17] The most desirable inhibitor may be one where molecular recognition is maximised using these favourable non-covalent interactions and where covalent modification of the enzyme, due to the inherent reactivity of the inhibitor, is minimised.

Improving the inhibition properties of a compound may therefore be achieved by modifying the structure to enhance either chemical reactivity or molecular recognition, or a combination of both. However, analysing these separate effects on the rate of inactivation when the inhibitor is modified is not straightforward. Modification of the inhibitor structure can affect the free energies of both the initial reactant state and the transition state of the reaction with the enzyme, whereas the observed variation in rate constants for various inhibitors reacting with the enzyme only reflects the *difference* in energies between these two states. Different substituents can affect the ease of bond-making and -breaking by classical electronic factors such as inductive, resonance and steric effects. However, the free energy of activation of an enzyme-catalysed reaction is also affected by the favourable binding energies between the protein and substrate substituents not directly involved with the reaction site. It is, therefore, important to separate these two effects before conclusions about the efficiency of inhibition can be made. For substrates we have suggested[18] that an 'enzyme rate-enhancement factor' (EREF) can be evaluated by dividing the second-order rate constant for the enzyme catalysed reaction, k_{cat}/K_m, by that for hydrolysis of the same substrate catalysed by hydroxide ion, k_{OH}. For inhibitors acting by covalent modification of the enzyme, a similar ratio of k_i/k_{OH} can be used where k_i is the rate constant for inactivation.

Alkaline hydrolysis of the PPelastase substrate, *N*-suc-(L-Ala)$_3$-*p*-nitroanilide (2)

An analysis of a particular enzyme's selectivity for various substrates or covalent inhibitors requires a knowledge of their reactivity in the absence of the enzyme. Although *N*-suc-(L-Ala)-*p*-nitroanilide (**2**) is an established and convenient substrate for porcine pancreatic elastase (PPelastase),[19] the magnitude and nature of its hydroxide ion catalysed hydrolysis has not been reported.

Below pH 13, *N*-suc-(L-Ala)$_3$-*p*-nitroanilide (**2**) undergoes hydroxide ion catalysed hydrolysis to give *p*-nitroaniline, for which the second-order rate constant, k_{OH}, is 7.19×10^{-3} M^{-1} s^{-1} at 30 °C. At pHs above *ca*. 13 there is a pH-independent reaction presumably due to a reversible ionisation of the



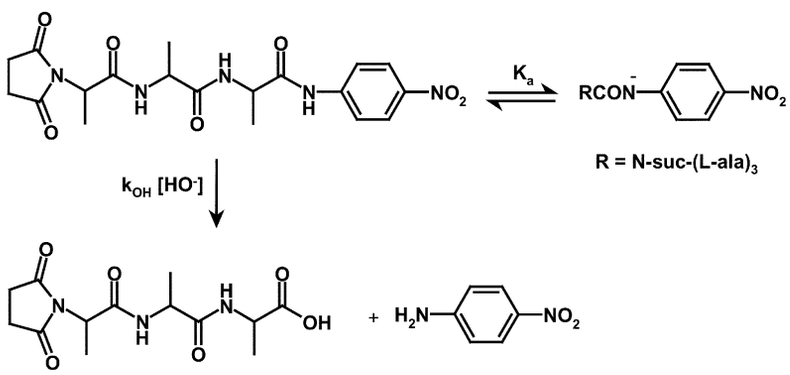
2

aryl-NH group of (**2**) consistent with the pK_a of simple *p*-nitroanilides.[20] The overall pathway for the hydroxide ion hydrolysis of *N*-suc-(L-Ala)$_3$ *p*-nitroanilide (**2**) is shown in Scheme 2.

Only the neutral compound undergoes hydroxide ion catalysed hydrolysis whilst the anion is relatively stable. The rate law for this reaction was determined based upon the fraction of the undissociated species present in solution [eqn. (1)], where [S] is the reactant concentration, k_{obs} is the observed first order rate constant for hydrolysis, k_{OH} is the second-order rate constant of hydroxide ion hydrolysis and $[H^+] / (K_a + [H^+])$ is the fraction of the anilide present in its undissociated form.

$$\text{Rate} / [S] = k_{obs} = k_{OH}[HO^-]([H^+] / (K_a + [H^+])) \quad (1)$$

When the solution pH is less than 13, $[H^+] \gg K_a$, eqn. (1) simplifies to $k_{obs} = k_{OH}[HO^-]$ and a first order dependence on hydroxide ion concentration is observed. At solution pHs greater than 13, $K_a \gg [H^+]$, eqn. (1) becomes $k_{obs} = k_{OH}(K_w / K_a)$ resulting in a pH independent rate. Modelling the experimental data to this equation using the Scientist software package gave the following parameters: $k_{OH} = 7.19 \times 10^{-3}$ M^{-1} s^{-1} and $K_a = 9.82 \times 10^{-14}$ M (pK_a 13.01). Direct titration using the absorbance change at 390 nm as a function of pH, immediately after injection of (**2**) into sodium hydroxide solutions, gave a reasonable sigmoidal plot from which was obtained a calculated pK_a, of 12.63 ± 0.07, in fairly good agreement with that determined kinetically.



Scheme 2

PPelastase catalysed hydrolysis of substrate, *N*-suc-(L-Ala)$_3$-*p*-nitroanilide (2)

(i) Initial rate data. Initial rate data for the hydrolysis of the anilide substrate (**2**) catalysed by PPelastase were collected at 390 nm using a molar extinction coefficient change, $\Delta\varepsilon_{390}$, of 1.24×10^4 M^{-1} cm^{-1}. The kinetic analysis was undertaken at 30 °C in 6% acetonitrile v/v, 1.5% methanol v/v and $I = 1.0$ M (KCl). The anilide substrate (**2**) was dissolved in 0.1 M pH 8.5 TAPS buffer at concentrations varying from 0.08 mM to 2 mM. Initial rates were collected up to a maximum time of 4 min. Rates of the enzyme reaction in units M s^{-1} were obtained by dividing the slope of the initial absorbance increase by the total change in molar extinction coefficient for the complete reaction. No effective saturation of the enzyme catalysed reaction was observed at substrate concentrations up to 2 mM. Hence it was not possible to obtain a reliable measurement for k_{cat} and K_m. However, using the initial rate data, the apparent values of k_{cat} and k_{cat} /$K_m$were obtained *via* a Lineweaver–Burke plot which gave an intercept realistically indistinguishable from zero. Nonetheless, this plot generated $k_{cat} = 7.82$ s^{-1}, $K_m = 4.92 \times 10^{-3}$ M and $k_{cat}/K_m = 1.59 \times 10^3$ M^{-1} s^{-1}. It is evident from these figures that although the anilide (**2**) is convenient for UV assays of PPelastase, it is only a moderately good substrate. The second-order rate constant k_{cat}/K_m is relatively low for an 'activated' substrate and the EREF is 2×10^5. The relatively high $K_m$value indicates a poor degree of enzyme-substrate binding.

(ii) Variation of pH. The activity of PPelastase was also determined in buffer solutions of pH 5 to pH 11. Enzyme activity was monitored using initial rates and the *N*-suc-(L-Ala)$_3$-*p*-nitroanilide (**2**) assay. The pH rate profile (Fig. 1) shows a typical bell shaped profile but, interestingly, there appears to

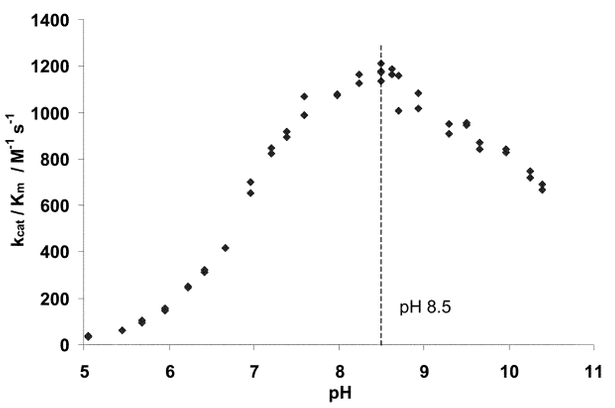


Fig. 1 Plot of PPelastase activity (k_{cat}/K_m) towards the substrate *N*-suc-(L-Ala)$_3$-*p*-nitroanilide (**2**) against pH. Initial rates measured at 1.52×10^{-6} M PPelastase, 6.0×10^{-5} M substrate, 390 nm and 30 °C in 0.1 M buffer, 6% MeCN v/v.

be more than a single ionisation occurring beyond the pH optimum. The profile is complex above pH 8.5 and either multiple ionisations or partial denaturation of the enzyme may be occurring. Modelling of the sigmoidal increase in enzyme activity up to pH 8.5 from pH 5 leads to a pK_a of the active-site's ionisable group of 6.88 ± 0.02. The enzyme is only active when this group is deprotonated, which is likely to be histidine-57 in the enzyme's catalytic triad and is reported to be responsible for similar increases in activity in many serine proteases.[2a,21]

Inhibition of PPelastase by *N*-acyl-β-sultams

Kinetics of inhibition. In order to test the inhibitory properties of *N*-acyl-β-sultams with respect to porcine pancreatic elastase (PPelastase), enzyme and β-sultam were incubated together in a buffered solution that was referred to as the incubation cell. Aliquots of the incubation solution containing enzyme and potential inhibitor were then removed from the incubation cell after an incubation time, t_i, and assayed for PPelastase activity against the anilide substrate (**2**) in separate assay cells at pH 8.5. These assays showed a time-dependent decrease in enzyme activity, given by the slope of absorbance at 390 nm (A_{390}) against time plots, and tended towards zero with increasing t_i. If the enzyme was incubated alone under exactly the same conditions but in the absence of β-sultam, the observed assay slope remained constant throughout the experimental time frame. By taking the slope of these control experiments as being equal to 100 % activity, the percentage activity of PPelastase remaining in the inhibitor experiments could be calculated and plotted against t_i. This gave apparent first-order rate constants for inactivation (Fig. 2) which were first order with respect to inhibitor concentration (Fig. 3). The

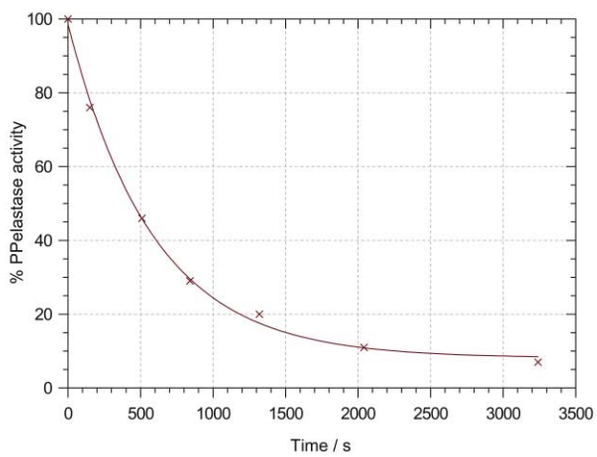


Fig. 2 Plot of % PPelastase activity against incubation time for the inactivation of PPelastase by *N*-benzoyl-β-sultam (**3**) at pH 6.75 and 30 °C in 0.04 M buffer, 20 % ACN v/v, 8×10^{-5} M PPelastase and 1.2×10^{-3} β-sultam.

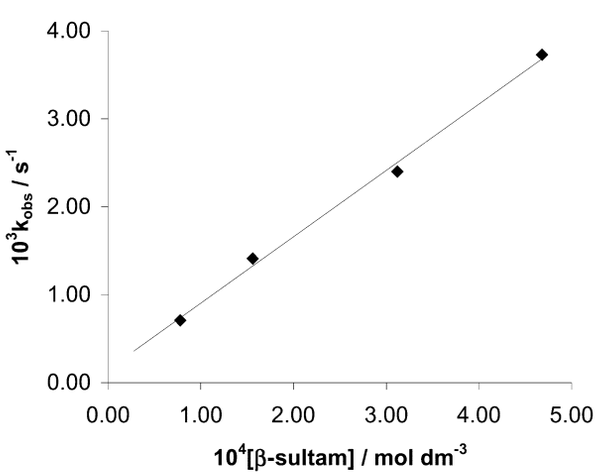


Fig. 3 Plot of the first order rate constant for the inactivation of PPelastase by *N*-benzoyl-β-sultam (**3**) corrected for hydrolysis at pH 6.1 against β-sultam concentration.

concentration of the enzyme is much lower than that of the inhibitor at time zero, and so the rate of inactivation is effectively pseudo-first order and the rate constant does not depend on the enzyme concentration. Second-order rate constants for inactivation, k_i, were obtained either by dividing the observed rate of inactivation by inhibitor concentration, k_{obs}/[I], when less than two inactivations were carried out or graphically from slopes of the plots of the observed rate of inactivation against several inhibitor concentrations (Fig. 3).

Table 1 Second-order rate constants for the inactivation of PPelastase by β-sultams (k_i) at pH 6, 7 and 8.5 and those for the alkaline hydrolysis, k_{OH}, of the β-sultam at 30 °C

β-Sultam	k_i at pH 6.1/M^{-1} s^{-1}	k_i at pH 7.0/M^{-1} s^{-1}	k_i at pH 8.5/M^{-1} s^{-1}	k_{OH}/M^{-1} s^{-1}	EREF at pH 8.5[a]
N-Bz (**3**)	0.85	2.34	4.06	1.46×10^4	6.1×10
4-iPr-*N*-Bz (**14**)	0.23	0.39	1.63	300	1.2×10^3
(*E*)-4-Ethylidene-*N*-Bz (**17**)	—	3.64	6.33	2.55×10^3	5.4×10^2
N-Carbamoyl (**4**)	—	2.13×10^{-2}	3.71×10^{-2}	265	3.1×10
4-iPr-*N*-carbamoyl (**15**)	—	9.14×10^{-3}	1.59×10^{-2}	3.80	9.1×10^2
4-Et-*N*-carbamoyl (**18**)	—	0.26	0.46	26.8	3.8×10^3
N-Cbz (**5**)	8.65	38	67	4.48×10^4	3.2×10^2
4-iPr-*N*-Cbz (**16**)	2.70	23.6	106	1.0×10^3	2.3×10^4

[a] EREF is k_i (inactivation)/k_{OH} at pH 8.5

With some *N*-acyl β-sultams and at high pH there were problems with the accurate determination of rate constants for the inactivation process because of the competing hydrolysis of these compounds. To correct for this, the results of the inactivations were normalised with respect to the decreasing β-sultam concentration using eqn. (2).

Normalised rate at time t =
(observed rate/ $[\text{Sultam}]_o$) × $[\text{Sultam}]_t$ (2)

The second-order rate constants k_i for the inactivation of PPelastase by various substituted β-sultams are given in Table 1.

Evidence for active-site sulfonation

(i) pH Dependence of inactivation. The three *N*-acyl-β-sultams, *N*-benzoyl (**3**), *N*-(*N*,*N*-methylphenyl)carbamoyl (**4**) and *N*-benzyloxycarbonyl-β-sultam (**5**) are all time dependent inhibitors of PPelastase and their second-order rate constants for inactivation are given in Table 1. Above pH 8, these rate constants are unreliable because of competing hydrolysis. However, below this pH the variation of the second-order rate constants for inactivation (k_i) with pH is very similar to that of the second-order rate constants for the hydrolysis of the anilide substrate (k_{cat}/K_m) catalysed by PPelastase (Fig. 4). This

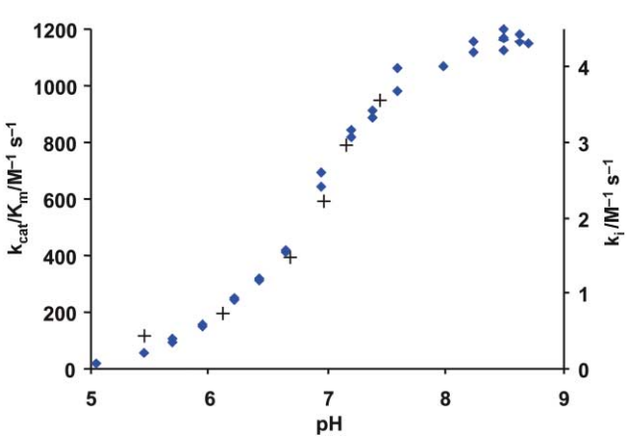
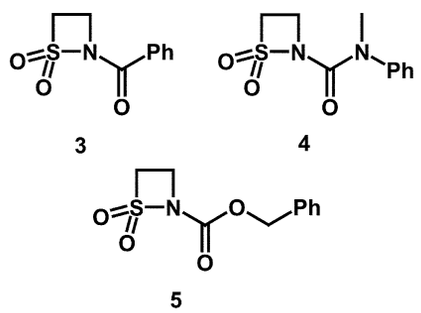


Fig. 4 Plot of k_{cat}/K_m for the hydrolysis of *N*-suc-(L-Ala)$_3$-*p*-nitroanilide by PPelastase (◆) and k_i for *N*-benzoyl-β-sultam (**3**) with PPelastase (+) against pH. 30 °C, I = 1.0 M (KCl), 6 % MeCN v/v and 1.5% MeOH v/v.

indicates that the rate of inactivation of PPelastase by these compounds is controlled by the same catalytic groups in the active-site that are used for substrate hydrolysis, *i.e.* active-site directed inhibition is occurring. The increase in enzyme activity towards inactivation with increasing pH shows an apparent pK_a of about 7 and is probably due to the dissociation of the protonated His-57 residue, crucial for effective catalysis of the substrate residue. The hydrolysis reaction shows a pK_a of 6.88, as described earlier.



(ii) ESIMS and X-ray crystallography data. Sulfonylation of the enzyme by β-sultams as shown in Scheme 1 was confirmed by ESIMS. Samples of PPelastase inactivated by *N*-benzoyl-β-sultam (**3**) were subjected to ESIMS analysis. Native PPelastase gave MW 25,904 Da (and 25943 (M + K)) which compares well to the literature ESIMS value of 25,898.[22] Incubation of the enzyme with *N*-benzoyl-β-sultam (**3**) (MW 211 Da) with PPelastase showed formation of the sulfonylated adduct at 26115 Da (and 26153 (M + K)).

Crystals of native PPelastase were soaked for 24 h at pH 5 in a saturated solution of *N*-benzoyl-β-sultam (**3**). After soaking, X-ray crystallography revealed that the crystals were isomorphous to native PPelastase and confirmed that ring opening of the β-sultam was part of the inactivation process (Fig. 5).[16] A sulfonate ester formed between Ser-195 and the inhibitor is observed along with the ethyl amino group projecting into the active site's P_1/P_2 region. Whilst one sulfonate oxygen atom was observed in the upper part of the S_1 pocket the other was located in the oxyanion hole within hydrogen bonding distance of the amido-nitrogen atoms of Ser-195 (3.02 Å) and Gly-193 (3.23 Å). Crucially, the side chain of His-57 has been displaced by approximately 90° from its native position, which is now occupied by two water molecules (Wat-342 and Wat-355). This effect is almost identical to that observed in the inhibition of PPelastase by γ-lactams.[22]

N-Acylsulfonamides have been used previously to inactivate serine enzymes.[8] However, the mechanism invariably involves acylation and C–N bond fission with the serine hydroxy group attacking the amide to displace the sulfonamide as the leaving group. Although the β-sultams reported here are also formally *N*-acylsulfonamides inactivation occurs by sulfonylation as a result of serine nucleophilic attack on the sulfonyl centre and displacement of the amide as a leaving group. This appears to be the first case of preferential S–N over C–N fission in the reaction of a N-acylsulfonamide with a serine protease.

(iii) Catalysis of sulfonyl transfer. As active-site directed inhibition is occurring in the inactivation of PPelastase by *N*-benzoyl β-sultam (**3**) it appears that the catalytic machinery of PPelastase is catalysing the sulfonyl transfer process. This indicates a significant degree of flexibility within the enzyme as the stereochemical requirements for catalysis of a reaction involving sulfonyl transfer with a trigonal bipyramidal

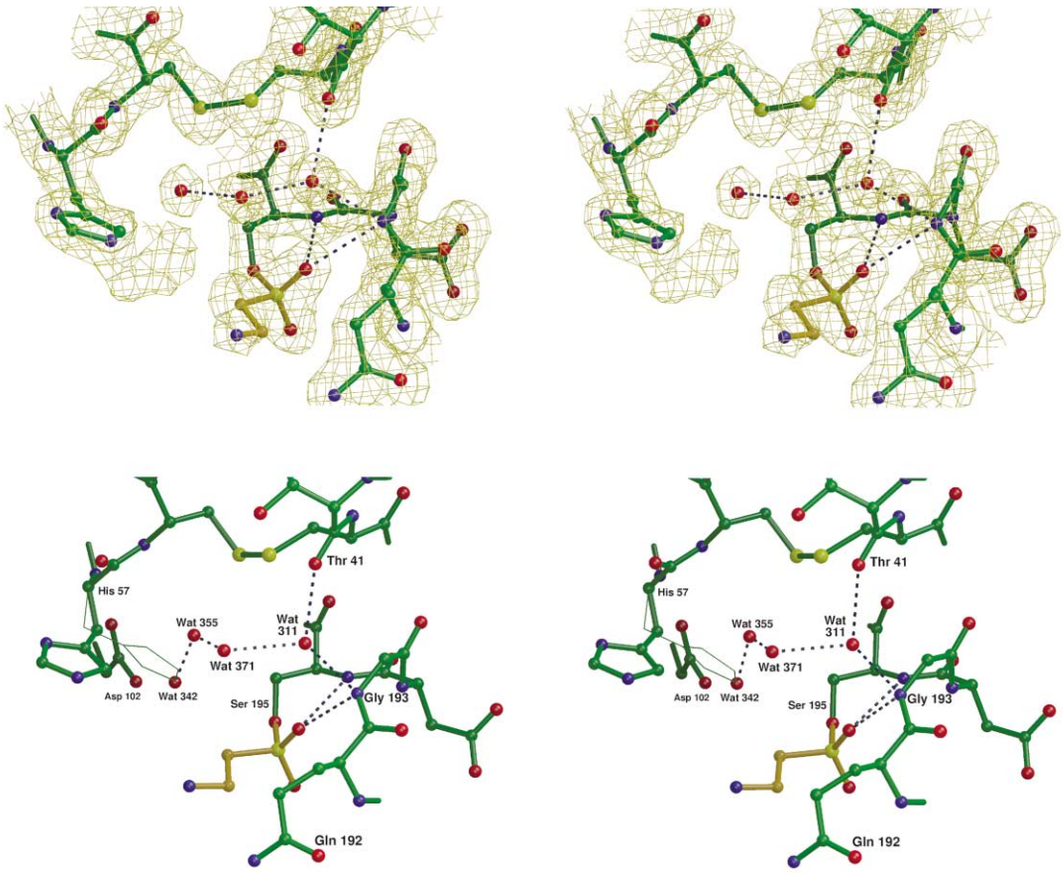
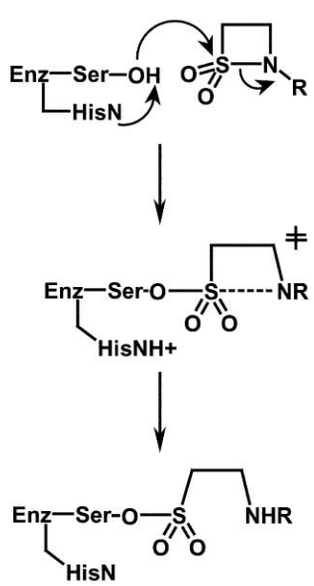


Fig. 5 Stereo views of the active site of PPelastase (in green) showing *N*-benzoyl- β-sultam (**3**) (in beige) covalently linked *via* a sulfonate ester to Ser-195 and the 'native' position of His-57 in thin lines. The top picture shows the $2mF_o - DF_c$ electron density map contoured at 1 σ. Wat-342 has not been included so that the 'extra' density extending from $N_{\varepsilon 2}$ of His-57 can be seen more clearly. The bottom picture shows the 'native' position of His-57 in thin lines.

arrangement in the transition state are significantly different to acyl transfer reactions involving a tetrahedral intermediate. For example, elastases possess an oxyanion hole used to stabilise the oxyanion in the tetrahedral intermediate (TI) formed after attack of the serine oxygen at a carbonyl centre. This oxyanion will lie at *ca.* 109° to the newly formed serine O–C bond. In contrast, the oxyanion formed by attack at the β-sultam sulfonyl centre is likely to occupy an equatorial position in a trigonal bipyramidal intermediate (TBPI) or transition state and will therefore lie at *ca.* 90° to the serine O–S bond. Although C–N bond fission in the hydrolysis of amides requires general acid catalysis to facilitate amine expulsion, the reactivity of *N*-acyl-β-sultams and their amide leaving groups may allow ring opening to occur without *N*-protonation. An outline possible mechanism for sulfonyl transfer from the β-sultam to the active site serine is shown in Scheme 3.

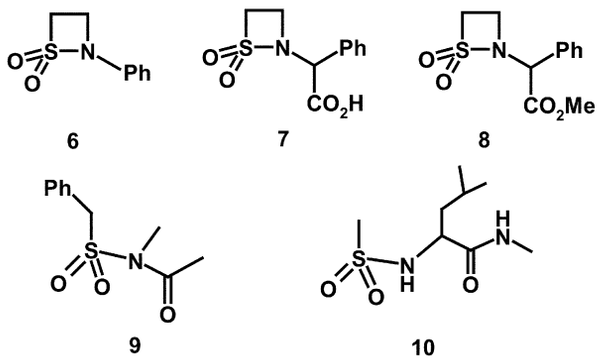
The preferred direction of nucleophilic attack at acyl centres is presumed to be at an approximately tetrahedral angle at either face. As this differs to that at sulfonyl centres, which is along the apical position bisecting the sulfonyl oxygens, attack of the serine group at the sulfonyl centre will alter the position of the other catalytic group, the histidine residue, relative to the rest of the molecule. In acyl transfer, the histidine residue has to be correctly positioned to protonate the leaving group nitrogen in the TI in its function as a general acid catalyst. The catalysis of sulfonyl transfer in *N*-acyl-β-sultams by elastase may therefore only be possible because ring opening occurs in a single step without general acid catalysis by the histidine residue. The relative position of this group in the active-site would therefore be less important. This apparent flexibility of enzymes has also been observed in the β-lactamase P99 which has the ability to catalyse phosphoryl transfer reactions, *via* trigonal bipyramidal intermediates.[23]



Scheme 3

(iv) Reactivation. The inactivation of the enzyme is irreversible; the resulting sulfonyl enzymes are stable for up to four weeks in the incubation solution at 30 °C. For some serine proteases such as α-chymotrypsin, inactivated by sulfonyl fluorides, it is possible to reactivate the enzyme above pH 8.5 or by initially unfolding the enzyme and then removing from 8 M urea.[24] The sulfonyl enzyme formed from *N*-benzoyl-β-sultam (**3**) and PPelastase could not be reactivated using a 500-fold excess of hydroxylamine or hydrogen peroxide. Human leucocyte elastase inhibited by monocyclic β-lactams *via* acylation

of the serine residue can be reactivated by hydroxylamine.[25] However, this reactivation can only occur if the hydroxylamine is added one minute after inhibitor. If added after ten minutes no reactivation occurs due to a postulated second reaction between the inhibitor molecule and His-57, generally involving expulsion of a leaving group, inactivating the enzyme permanently. A similar reaction in *N*-acyl-β-sultams is ruled out by the ESIMS results giving no evidence for mass loss and the fact that roughly equimolar mixtures of enzyme and inhibitor result in complete inactivation. This indicates that a single enzyme catalysed reaction results in inactivation. Inhibitors whose mechanism involves a second reaction exhibit partitioning between hydrolysis products and inactivated enzyme and characteristically require a specific molar excess of inhibitor for complete inactivation.[26]



Structure–activity relationships

The leaving group. The fact that the β-sultam has ring opened upon inactivation of the enzyme confirms the necessity of S–N fission for activity. However, it is important to separate the effects of substituents on chemical reactivity and molecular recognition. For example, N-substituents not only influence the rate of S–N bond fission and reaction through inductive effects, altering the electrophilicity of the sulfonyl centre, N-basicity and amine nucleofugacity, but also change binding energies through molecular recognition effects such as interactions between the substituent and a binding pocket in the enzyme. In our experiments, less reactive β-sultams such as *N*-phenyl-β-sultam (**6**) or *N*-alkyl-β-sultams such as *N*-carboxybenzyl-β-sultam (**7**) and *N*-methoxycarbonylbenzyl-β-sultam (**8**) do not inactivate PPelastase, nor do acyclic sulfonamides such as *N*-benzylsulfonyl-*N*-methylethanamide (**9**) and *N*-methylsulfonylisoleucinemethylamide (**10**). These acyclic compounds show no inhibitory activity against PPelastase at concentrations of 4 mM and 2 mM, respectively. The *N*-acylsulfonamide (**9**) could, in principle, act as either a sulfonylating or acylating agent. The absence of the four-membered ring enormously reduces the reactivity of the sulfonyl centres of both of these compounds, their second order rate constants for alkaline hydrolysis, k_{OH}, of *ca.* 1 M^{-1} s^{-1} (**9**) (for C–N fission) and *ca.* 1×10^{-8} M^{-1} s^{-1}(**10**), are smaller than those for analogous β-sultams by a factor of greater than 10^6. The four-membered ring is required for inactivation to ensure the sulfonyl centre is reactive enough. Acyclic sulfonyl fluorides are effective inhibitors of serine enzymes[27] as are sulfonate esters in their more reactive cyclic form.[28] There appears to be a threshold of chemical reactivity that must be reached for sulfonyl compounds to display effective enzyme inhibition. The least effective PPelastase inhibitor in the simple *N*-acyl-β-sultams is also the least reactive, the *N*-carbamoyl compound (**4**) with a k_{OH} of 265 M^{-1} s^{-1}. The *N*-acyl sulfonamide (**9**) probably does not inactivate PPelastase due to a combination of its low reactivity and its sterically bulky benzyl substituent. Although phenylmethanesulfonyl fluoride (PMSF) is an inhibitor of α-chymotrypsin, the S_1 pocket in α-chymotrypsin is much larger than in PPelastase. PMSF is also likely to be more reactive than the *N*-acyl sulfonamide (**9**) although direct k_{OH} comparisons are complicated by PMSF undergoing E1cB-type elimination reactions. However, the k_{OH} value of benzenesulfonyl fluoride is similar to that of (**9**) and *N*-phenyl-β-sultam (**6**) at 3.4 M^{-1} s^{-1}.[29]

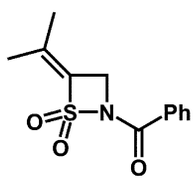
A comparison of the k_i values at pH 7 with the second-order rate constants for hydroxide ion hydrolysis, k_{OH} values (Table 1), for the *N*-benzoyl compound (**3**) and the *N*-carbamoyl compound (**4**) indicates that their relative effectiveness as PPelastase inhibitors is largely due to variations in their chemical reactivity. At pH 7, the *N*-benzoyl derivative (**3**) is a better inactivator by two orders of magnitude than the *N*-carbamoyl β-sultam (**4**) which is similar to the ratio of their k_{OH} values, 55. This indicates that there is no significant difference in binding between these two leaving groups and the enzyme in the inactivation of PPelastase. However, replacement of the *N*-carbamoyl residue by the *N*-benzyloxycarbonyl substituent increases the rate of inactivation by 10^3–10^4. The enzyme rate enhancement factors (EREFs) for *N*-benzoyl (**3**), *N*-carbamoyl (**4**) and *N*-benzyloxycarbonyl-β-sultam (**5**) are 61, 31 and 320 respectively. This suggests that of all the *N*-substituents the benzyloxycarbonyl moiety binds most favourably in the active-site of PPelastase, possibly *via* π–π interactions with the His-57 residue in the active-site or another aromatic residue in its vicinity. Such interactions have been observed between the *p*-toluene ring of *N*-arylsulfonyl-β-lactams and His-57.[30] Apparently the *N*-carbamoyl group of (**4**) does not reach sufficiently deeply into the S' sites of PPelastase's active-site and cannot interact as effectively with His-57. It both lacks one carbon atom on the side chain compared to that of (**5**) and free rotation is restricted by resonance of the urea moiety. There is also *cis–trans* isomerism of this urea moiety resulting in the *N*-carbamoyl compound (**4**) having two stereoisomers.

The ring substituents. The rates of enzyme inactivation observed could be increased if recognition elements are built into the structure of the β-sultams. β-Sultams (**3**), (**4**) and (**5**) are far from ideal as PPelastase inhibitors as they are relatively hydrolytically labile. The *N*-benzyloxycarbonyl compound (**5**) is the most potent of the simple *N*-acyl-β-sultams but the compound is too reactive to be an effective inhibitor above neutral pH. Effective substituent changes in the lead β-sultam structures which are similar to those that have been previously shown to increase recognition by PPelastase would serve two purposes. Firstly, they could improve binding to PPelastase and hence increase the rate of inactivation of the serine residue. Secondly, they may reduce the hydrolytic lability of the inhibitor, thus making it more stable in aqueous solution and in a biological environment.

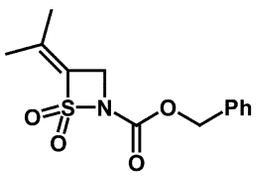
It is known that the α-chymotrypsin family of enzymes has a well-established binding pocket adjacent to the active serine residue, in the S_1 position. In the elastase enzymes this binding pocket is relatively small and has a preference for small hydrophobic substituents. The inhibition of PPelastase by monocyclic β-lactams has also been improved by the introduction of alkyl substituents at the 3-position which are thought to bind strongly in the S_1 subsite of elastase.[30,31]

Alkyl chains have therefore been added to the simple β-sultam ring at the 4-position with the intention of improving binding at the active-site of PPelastase. 4-Isopropylidene β-sultam (**11**) is unusual because the substituent causes nucleophiles to react at the acyl rather than the sulfonyl centre.[32] This switch of reactive centre is accompanied by a 10^4-fold reduction in reactivity towards hydroxide ion. Although N-acylsulfonamides may act as inhibitors of elastase by *acylation* and expulsion of the sulfonamide, 4-isopropylidene-*N*-benzoyl-β-sultam (**11**) and 4-isopropylidene-*N*-benzyloxycarbonyl-β-sultam (**12**) are both inactive towards PPelastase at concentrations up to 2 mM. Interestingly, 4-isopropylidene-*N*-(*N*,*N*-methyl phenyl)-

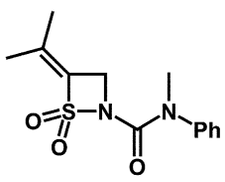
carbamoyl-β-sultam (**13**) displayed a small amount of inactivation of the enzyme with an estimated k_i value of 2×10^{-3} M^{-1} s^{-1} at pH 8.5. This is a reduction of approximately 10-fold compared to the parent compound (**4**) lacking the 4-isopropylidene substituent and is unexpected given the extremely low reactivity of this compound and the fact that it reacts with hydroxide ion at the acyl centre. It is not known whether (**13**) acts as a sulfonylating or acylating agent of PPelastase.



11

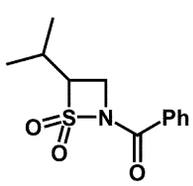


12

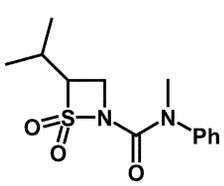


13

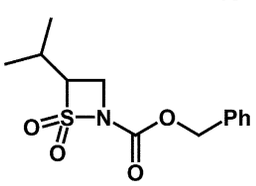
The introduction of the isopropyl substituent at the 4-position of the thiazetidine ring lowers the reactivity of β-sultams towards hydroxide ion by 50-fold—compare (**14**) with (**3**) (Table 1) and maintains the mechanism of S–N fission. In addition, unlike the 4-isopropylidene derivatives, the 4-isopropyl compounds are effective inhibitors. The second-order rate constants for inhibition obtained for 4-isopropyl-*N*-benzoyl-β-sultam (**14**), 4-isopropyl-*N*-(*N*,*N*-methylphenyl)carbamoyl-β-sultam (**15**) and 4-isopropyl-*N*-benzyloxycarbonyl-β-sultam (**16**) are summarised in Table 1.



14



15



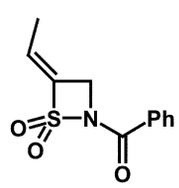
16

Whilst the 4-isopropyl substituent has decreased the rate of alkaline hydrolysis of *N*-benzoyl β-sultam (**3**) by 50-fold in (**14**), enzyme inhibitory activity was only decreased by 3-fold. If the EREF values are compared between the 4-isopropyl compound (**14**) and the simple *N*-benzoyl compound (**3**) then an increase of 20-fold is observed. This matches the increase in EREF of 30-fold observed between the 4-isopropyl-*N*-carbamoyl compound (**15**) and the simple *N*-carbamoyl compound (**4**). Therefore the 4-isopropyl substituent has increased the level of active site binding and made the β-sultams more reactive towards the enzyme in the presence of hydroxide ion. This is possibly a result of favourable interactions between the alkyl substituent and the hydrophobic S_1 binding pocket.

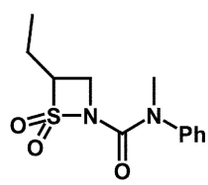
The best inactivator in this series is 4-isopropyl-*N*-benzyloxycarbonyl-β-sultam (**16**) with an EREF of 2.3×10^4, 70-fold greater than that of the *N*-benzyloxycarbonyl compound lacking the 4-isopropyl substituent (**5**). The combination of the 4-isopropyl and *N*-benzyloxycarbonyl groups creates favourable interactions for active-site binding. If the two isopropyl substituted compounds, 4-isopropyl-*N*-carbamoyl-β-sultam (**15**) and the chemically more reactive 4 isopropyl-*N*-benzyloxycarbonyl-β-sultam (**16**) are compared, there is an increase in EREF of 25-fold on going from the carbamoyl to the benzyloxycarbonyl *N*-substituent. As discussed for the simple *N*-benzyloxycarbonyl compound (**5**), this again suggests that of all the *N*-substituents the benzyloxycarbonyl moiety binds most favourably with PPelastase. This is possibly due to its longer length permitting more favourable π–π interactions with His-57 or another aromatic residue.

The introduction of the 4-isopropyl substituent has increased the effectiveness of the lead compounds as inhibitors of PPelastase. ESIMS analysis of 4-isopropyl-*N*-benzoyl-β-sultam (**14**) incubated with PPelastase in non-buffered aqueous solution confirmed that only 1 : 1 enzyme : inhibitor (EI) complexes were being formed, indicative of a good degree of selectivity. Native PPelastase was observed at MW 25 890 ± 2 Da and incubation with (**14**) gave a time dependent emergence of a second peak consistent with the formation of a mono-sulfonylated adduct (MW 26 148 ± 7 Da) up to a relative intensity of 80%. The observed mass difference of 258 is in good agreement with a calculated one of 254, consistent with sulfonylation of the enzyme. Only one equivalent of the inhibitor is observed to bind, compatible with the formation of a 1 : 1 EI complex. The conditions of the ionisation, 1 % formic acid, should result in protein unfolding as a result of the multiple side chain positive charges. As a result, bound complexes can probably be regarded as covalent because EI complexes formed from reversible inhibitors should dissociate under these conditions. The 4-isopropyl-*N*-benzoyl compound (**14**) has therefore irreversibly sulfonylated the enzyme. However, the precise location of the sulfonylation cannot be determined from these results. An X-ray crystal structure or further MS analysis of tryptic digests of the inactivated enzyme are required to determine whether or not Ser-195 is indeed sulfonylated as was observed with the simple *N*-benzoyl compound (**3**).[16]

In an attempt to take advantage of the smaller S_1 pocket present in PPelastase the following compounds were tested as inhibitors: (*E*)-4-ethylidene-*N*-benzoyl-β-sultam (**17**) and 4-ethyl *N*-(*N*,*N*-methylphenyl)carbamoyl-β-sultam (**18**). The kinetic results obtained are given in Table 1.



17



18

The k_i value of (*E*)-4-ethylidene-*N*-benzoyl-β-sultam (**17**) is similar to that for the unsubstituted *N*-benzoyl-β-sultam (**3**) despite a 5-fold decrease in reactivity towards hydroxide ion catalysed hydrolysis. The EREF values for the two compounds are 540 and 61 (Table 1) respectively. These values can also be compared to the EREF value for the 4-isopropyl-*N*-benzoyl compound (**14**) of 1.2×10^3 which is the least reactive of the three N-benzoyl β-sultams.

This suggests that the 4-isopropyl substituent binds slightly more favourably into the S_1 pocket of PPelastase than the 4-(*E*)-ethylidene substituent in the *N*-benzoyl series. This is surprising given the preference of the enzyme for alanine residues and may be due to the restricted rotation resulting from unsaturation.

The effectiveness of the ethyl substituent in increasing recognition was observed in 4-ethyl-*N*-(*N*,*N*-methylphenylcarbamoyl)-β-sultam (**18**). This compound shows relatively low k_i

values but is by far the least reactive β-sultam to display enzyme inhibition, being 10-fold less reactive than the simple *N*-carbamoyl compound (**4**). It therefore has the second highest selectivity towards PPelastase in the presence of hydroxide ion with an EREF of 3.8×10^3. This suggests that the additional methyl group in the 4-isopropyl compounds is unfavourable, possibly due to steric interactions in the active site.

In the *N*-carbamoyl series the presence of the alkyl substituent in the 4-position of the β-sultam ring increases the EREF value by an overall factor of 100-fold. The 4-ethyl substituted compound (**18**) is 4-fold more potent as an elastase inhibitor than the 4-isopropyl compound (**15**). This improvement is probably due to both the increased rotational mobility of the 4-ethyl substituent as a result of its lower bulk and improved binding in the S_1 active-site pocket. Based on a structural comparison to an alanine-containing peptide an *N*-methyl-β-sultam may show even greater binding.

It is notable, despite differing *N*-substituents, that the EREF value for the conformationally restricted 4-(*E*)-ethylidene-*N*-benzoyl-β-sultam (**17**) is nearly 10-fold lower than for the 4-ethyl compound (**18**). This suggests that the binding of β-sultams in the S_1 pocket requires the compound to have a degree of flexibility. This direct comparison has some justification despite differing leaving groups because the relative k_i values of the simple *N*-benzoyl (**3**) and *N*-carbamoyl (**4**) compounds was proportional to their reactivity, which may be indicative of similar binding of the *N*-substituent.

The EREF value for the 4-ethyl compound (**18**) is still 6-fold lower than that of 4-isopropyl-*N*-benzyloxycarbonyl-β-sultam (**16**)which has the highest EREF value of the series presented here. The inhibitor EREFs have been increased by *ca.* 400-fold from that of the original lead compound, *N*-benzoyl-β-sultam (**3**). This increase represents a combination of changes in chemical reactivity and molecular recognition.

The structure of an enzyme : inhibitor complex formed between a 3-ethyl-*N*-(*N*-benzyl)carbamoyl-β-lactam (**19**)compound and HLE has been modelled.[33] As expected the 4-ethyl group lies in the S_1 pocket but the β-lactam ring remains intact and the resulting oxyanion formed by attack of Ser-195 at the carbonyl is stabilised by the oxyanion hole. It is notable that the binding of the benzyl urea group in the so-called hydrophobic prime site (P_1') is improved by alkylation of the benzylic carbon. This is perhaps a further possible modification to the β-sultam structure although the hydrophobic pocket exploited may not be present in PPelastase.

In conclusion, *N*-acyl-β-sultams are a novel class of inhibitor compounds that inactivate serine enzymes by irreversible sulfonylation of the active site serine residue. They are inactive towards enzymes lacking active serine residues, indicating that specific sulfonylation of active site residues takes place. Variation in 4-alkyl and *N*-substituted β-sultams causes differences in the rates of inactivation by 4 orders of magnitude. Such structure–activity relationships highlight the possibilities for further development of this compound series. Improved enzyme binding will allow the application of more stable, and hence more selective, enzyme inhibitors.

Experimental

Synthesis

Melting points were determined on a Gallenkamp melting point apparatus and are uncorrected. Infrared measurements were recorded on a Perkin Elmer 1600 series FT-IR spectrometer. 400 MHz ^{1}H and 67 MHz ^{13}C NMR spectra were determined on a Bruker Advance 400 MHz spectrometer. 500 MHz ^{1}H and 100 MHz ^{13}C NMR spectra were determined on a Bruker AMX 500 spectrometer. GC-MS were determined on a Varian GC-MS with a Finnigan MAT ion trap detector. Mass spectrometry was performed on a Fisons Quattro VG quadrupole mass spectrometer. High resolution mass spectrometry was carried out by the University of Swansea, courtesy of the EPSRC. Elemental analyses were carried out at Medac Ltd. Fluka or Merck silica gel 60 was used for all chromatographic separations, with silica gel 60 F_{254} TLC plates used for initial investigations. Solvents were purified according to Perrin and Armarego.[34a] Starting materials were purchased from Aldrich, Avocado and Lancaster and were used without further purification.

1,2-Thiazetidine 1,1-dioxide (ethane β-sultam)

Taurinesulfonyl chloride (30.4 g, 169 mmol) was added to finely ground sodium carbonate (35.9 g, 339 mmol) in ethyl acetate (950 ml) and stirred at ambient temperature for 48 hours. The reaction mixture was filtered through celite and the solvent removed by reduced pressure rotary evaporation at 30 °C, giving a fine white powder (15.9 g, 89%). Mp 50–51 °C (Lit. 51–52 °C)[35] ^{1}H NMR: δ ($CDCl_3$ 3.39 (2H, dt, *J* 4 and 7, CH_2N), 4.32 (2H, dt, *J* 2 and 7, CH_2SO_2), 5.53 (1H, br s, *NH*). ^{13}C NMR: δ ($CDCl_3$ 60.6, 26.8. IR: ν_{max}(cm^{-1}): 3307, 3048, 3022, 2991, 2918, 1416, 1336, 1299, 1249, 1212, 1171, 1156, 1107, 966, 803, 760, 668, 615. *m*/*z* (GC-MS) (M^+H): 108, 77, 54, 42.

(1,1-Dioxo-1λ^6,2-thiazetidin-2-yl)(phenyl)methanone (3)

To a −5 °C stirred solution of 1,2-thiazetidine 1,1-dioxide (0.6 g, 5.6 mmol) in dry DCM (20 ml) was added DMAP (0.11 g, catalytic), and benzoyl chloride (1.18 ml, 10.2 mmol) dropwise. Triethylamine (1.88 ml, 13.5 mmol) was added dropwise over 10 minutes forming a pale yellow solution. The reaction mixture was stirred overnight before the addition of DCM (10 ml) and was then washed with water (15 ml) and brine (2 × 15 ml) before drying over Na_2SO_4. Reduced pressure rotary evaporation at 30 °C gave a pale orange solid (1.6 g) which was purified by column chromatography (75 g silica) (6 : 4 diethyl ether : DCM, R_f = .48) yielding a white powder (0.59 g, 49.9%). Mp 103–105 °C. ^{1}H NMR: δ ($CDCl_3$ 8.13 (2H br d, *Ph*), 7.62 (1H, br tt, *Ph*), 7.53 (2H, br t, *Ph*), 4.3 (2H, t, *J* 7.74, CH_2SO_2), 3.92 (2H, t, *J* 7.7, CH_2N). ^{13}C NMR: δ ($CDCl_3$) 167.4, 133.6, 132.1, 128.9, 128.4, 56.9, 30.9 IR; ν_{max}(cm^{-1}) 2997, 2924, 2854, 1670, 1448, 1337, 1197, 1165, 778, 715.9. HREI-MS [M+H] for $C_9H_9SO_3N$ calc. 212.0381 found 212.0385.

1,1-Dioxo-1$\lambda^6$2-thiazetidine-2-carboxylic acid benzyl ester (5)

To a −5 °C stirred solution of 1,2-thiazetidine 1,1-dioxide (0.6 g, 5.6 mmol) in dry DCM (20 ml) was added DMAP (0.11 g, catalytic), and benzyl chloroformate (1.21 ml, 8.5 mmol) dropwise. Triethylamine (1.9 ml, 13.8 mmol) was added dropwise over 10 minutes forming a pale yellow solution. The reaction mixture was stirred overnight before the addition of DCM (10 ml) and was then washed with water (15 ml) and brine (2 × 15 ml) before drying over Na_2SO_4. Reduced pressure rotary evaporation at 30 °C gave a pale yellow oil/solid (1.1 g) which was purified by column chromatography (50 g silica) (1 : 1 DCM : diethyl ether, R_f = 0.6) yielding a white powder (0.23 g, 17%). Mp 99–101 °C. ^{1}H NMR: δ ($CDCl_3$) 7.4 (5H, br, *Ph*), 5.3 (2H, s, CH_2Ph), 4.2 (2H, t, *J* 7.21, CH_2SO_2), 3.75 (2H, t, *J* 7.33, CH_2N) ^{13}C NMR: δ ($CDCl_3$) 151, 134.7, 128.6, 128.5, 128, 68.8, 57.9, 32. IR: ν_{max} (cm^{-1}) 3046, 2984, 1732, 1456, 1391, 1326, 1204, 1173, 1135, 1056, 952, 911, 780, 767, 699. HREI-MS [M+H] for $C_{10}H_{11}SO_4N$ calc. 259.0753 found 259.0749.

1,1-Dioxo-1λ^6,2-thiazetidine-2-carboxylic acid *N*-methyl-*N*-phenylamide (4)

To a −5 °C stirred solution of 1,2-thiazetidine 1,1-dioxide (0.6 g, 5.6 mmol) in dry DCM (20 ml) was added DMAP (0.11 g, catalytic), and methyl(phenyl)carbamoyl chloride (1.43 g, 8.4 mmol) dropwise. Triethylamine (1.88 ml, 13.5 mmol) was added dropwise over 10 minutes forming a pale yellow solution. The

reaction mixture was stirred overnight before the addition of DCM (60 ml) and was then washed with water (3 × 20 ml) and brine (2 × 20 ml) before drying over Na_2SO_4. Reduced pressure rotary evaporation at 30 °C gave a pale yellow solid/oil (1.5 g) which was purified by column chromatography (75 g silica) (1 : DCM : diethyl ether, R_f = 0.55) yielding a white powder (0.35 g, 26%). Mp 111–112 °C. ^{1}H NMR: δ ($CDCl_3$) 7.4 (5H, m, *Ph*), 4.02 (2H, t, *J* 7.75, CH_2SO_2), 3.58 (2H, dd, *J* 6.63 and 7.72, CH_2N), 3.38 (3H, s CH_3N). ^{13}C NMR: δ ($CDCl_3$) 152.6, 141.2, 129.6, 128.3, 128.1, 56.5, 39.3, 32.2. IR: ν_{max} (cm^{-1}) 2954.3, 2923.5, 2853.8, 1670.3, 1458.1, 1373.3, 1346.2, 1299.8, 1279, 1212.4, 1158, 1136. HREI-MS [M+H] for $C_{10}H_{12}SO_3N_2$ calc. 241.0647 found 241.0650.

2,4-Bis(*tert*-butyldimethylsilyl)-1,2-thiazetidine 1,1-dioxide

To a −78 °C stirred solution of 1,2-thiazetidine 1,1-dioxide (2.5 g, 23.3 mmol) in dry THF (190 ml) was added n-BuLi (1.6 M, 33 ml, 52.8 mmol) dropwise over 20 minutes. The mixture was stirred for 10 minutes before the addition of TBDMSCl (17.2 g, 114 mmol) in THF (10 ml). After a period of 30 minutes, the reaction mixture was allowed to warm to ambient over a further 30 minutes and was then quenched with glacial acetic acid (3.9 g, 65 mmol) in THF (4 ml). The organics were diluted with ethyl acetate (200 ml) and saturated NH_4Cl (200 ml). The aqueous layer was extracted a further 2 times with ethyl acetate (100 ml) before the combined organic extracts were washed with water (2 × 100 ml), brine (2 × 100 ml) and dried over anhydrous Na_2SO_4. Reduced pressure rotary evaporation at 30 °C gave a pale yellow oil (11.1 g) which was purified by column chromatography (400 g silica) (3:2 isohexane : ethyl acetate, R_f = 0.6) yielding a pale cream solid (6.14 g, 78%). Mp 89–91 °C. ^{1}H NMR: δ ($CDCl_3$) 4.20 (1H dd, *J* 9.0 and 5.5, $CHSO_2$), 3.45 (1H dd, *J* 9 and 4.5, *N*–CH_2), 3.17 (1H dd, *J* 4.5 and 5.3, NCH_2), 1.02 (9H, s, SiC(CH_3)$_3$), 0.96 (9H, s, SiC(CH_3)$_3$), 0.33 (3H, s, SiCH_3), 0.32 (3H, s, SiCH_3), 0.31 (3H, s, SiCH_3), 0.25 (3H, s, SiCH_3).

2-(*tert*-Butyldimethylsilyl)-4-isopropylidene-1,2-thiazetidine 1,1-dioxide

To a −78 °C stirred solution of diisopropylamine (2.79 ml, 19.9 mmol) in dry THF (130 ml) was added n-BuLi (1.6 M, 12.25 ml, 19.6 mmol) dropwise. The reaction mixture was stirred for 10 minutes before the addition of 2,4-bis(*tert*-butyldimethylsilyl)-12,-thiazetidine 1,1-dioxide (3.28 g, 9.79 mmol) in THF (15 ml) over 20 minutes. After stirring for a further 10 minutes, acetone (7.2 ml, 98.2 mmol) was added over 5 minutes and the mixture left to stir for 50 minutes. The reaction was quenched with saturated NH_4Cl (220 ml) and then extracted with ethyl acetate (200 ml + (2 × 100 ml)) before the combined organics were washed with water (2 × 100 ml), brine (2 × 100 ml) and dried over Na_2SO_4. Reduced pressure rotary evaporation at 30 °C yielded a pale yellow oil (5.3 g) which was purified by cloumn chromatography (200 g silica) (7 : 3 isohexane : ethyl acetate, R_f = 0.27) yielding a pale cream powder (0.5 g, 20%). Mp 100–101 °C. ^{1}H NMR: δ ($CDCl_3$) 3.78 (2H, s, CH_2N), 2.05 (3H, s, CH_3), 1.78 (3H, s, CH_3), 0.98 (9H, s, SiC(CH_3)$_3$), 0.28 (6H, s, SiCH_3). ^{13}C NMR : δ ($CDCl_3$) 142.5, 137.8, 40.7, 26.3, 21.2, 20.2, −0.55. IR: ν_{max} (cm^{-1}) 2965.2, 1672.7, 1672.7, 1449.6, 1314, 1282.1, 1162.8, 1037.4, 904.2, 841, 723.7, 647.8. *m*/*z* (EI-MS): (M+H) 261.8.

4-Isopropylidene-1,2-thiazetidine 1,1-dioxide

To a stirred solution of 2-(*tert*-buty1dimethylsilyl)-4-isopropylidene-1,2-thiazetidine 1,1-dioxide (0.204 g, 0.78 mmol) in THF (20 ml) was added TBAF (1 M, 0.78 ml, 78 mmol) in one portion. The reaction mixture was stirred for 3 minutes before quenching with saturated NaCl (30 ml), extracting with ethyl acetate (15 ml) and then the combined organics were dried over Na_2SO_4. Reduced pressure rotary evaporation at 30 °C gave a pale yellow oil (0.4 g) which was purified by column chromotography (25 g silica) (2 : 3 isohexane : ethyl acetate, R_f = 0.32) yielding a white powder (77mg, 67%). Mp 66–68 °C. ^{1}H NMR: δ ($CDCl_3$)5.40 (1H, br s, *NH*), 3.72 (2H, s, CH_2N), 2.08 (3H, s, CH_3), 1.70 (3H, s, CH_3). ^{13}C NMR: δ ($CDCl_3$)142.8, 140.8, 37.6, 21.4, 20.5. IR: ν_{max}(cm^{-1}) 3274.6, 1701.5, 1444.6, 1373.9, 1326.1, 1283.0, 1245.6, 1149.1, 1130.0, 1018.1, 940.7, 920.5, 869.1, 680.9.

(4-Isopropylidene-1,1-dioxo-1λ^6,2-thiazetidin-2-yl)phenylmethanone (11)

To a −5 °C stirred solution of 4-isopropylidene-1,2-thiazetidine 1,1-dioxide (77 mg, 0.523 mmol) in dry DCM (6 ml) was added DMAP (0.1 g, catalytic), and benzoyl chloride (0.091 ml, 0.78 mmol) dropwise. Triethylamine (0.18 ml, 1.26 mmol) was added dropwise over 10 minutes forming a pale yellow solution. The reaction mixture was stirred overnight before the addition of DCM (10 ml) and was then washed with water (15 ml) and brine (2 × 15 ml)before drying over Na_2SO_4. Reduced pressure rotary evaporation at 30 °C gave a pale yellow oil (0.4 g) which was purified by column chromotography (25 g silica) (3 : 2 isohexane : ethyl acetate, R_f = 0.36) yielding a white powder (93mg, 71%). Mp 82–84 °C. ^{1}H NMR: δ ($CDCl_3$) 8.01 (2H, d, J7.5, *Ph*), 7.61 (1H, t, *J* 7.4, *Ph*),7.51 (2H, d, *J* 7.6, *Ph*), 4.32 (2H, s, CH_2), 2.10 (3H, s, CH_3), 1.86 (3H, s, CH_3). ^{13}C NMR: δ ($CDCl_3$) 168, 143.3, 140.8, 133.9, 133.2, 129.2, 128.9, 40.9, 22.1, 20.8. IR: ν_{max} (cm^{-1}) 2923.8, 2854, 1670.1, 1600.1, 1376.8, 1337.2, 1283.5, 1199.7, 1165.4, 1125.5, 1032.1, 778, 715.9. HREI-MS [M+H] for $C_{12}H_{13}SO_3N$ calc. 252.0694 found 252.0690.

4-Isopropylidene-1,1-dioxo-1λ^6,2-thiazetidine-2-carboxylic acid benzyl ester (12)

To a −10 °C stirred solution of 4-isopropylidene-1,2-thiazetidine 1,1-dioxide (78.5 mg, 0.533 mmol) in dry DCM (6 ml) was added DMAP (0.1 g, catalytic), and benzyl chloroformate (0.12 ml, 0.795 mmol) dropwise. Triethylamine (0.19 ml, 1.33 mmol) was added dropwise over 10 minutes forming a pale yellow solution. The reaction mixture was stirred overnight before the addition of DCM (10 ml) and was then washed with water (15 ml) and brine (2 × 15 ml) before drying over Na_2SO_4. Reduced pressure rotary evaporation at 30 °C gave a pale yellow oil (0.2 g) which was purified by column chromatography (25 g silica) (3 : 2 isohexane : ethyl acetate, R_f = 0.32) yielding a white powder (108 mg, 73%. Mp 86–88 °C. ^{1}H NMR:δ ($CDCl_3$) 7.35 (5H, m *Ph*), 5.28 (2H, s, CH_2Ph), 4.10 (2H, s, CH_2N), 2.09(3H, s, CH_3), 1.80 (3H, s, CH_3). ^{13}C NMR: δ ($CDCl_3$), 142.8, 138.1, 135.3, 129, 128.92, 128.9, 128.4, 69, 41.3, 22.1, 20.8. IR: ν_{max} (cm^{-1}) 1731.2, 1445.2, 1386.2, 1315.1, 1248.0, 1179.3, 1153.0, 699.8. HREI-MS [M+NH_4] for $C_{13}H_{15}SO_3N$ calc. 299.1066 found 299.1069.

4-Isopropylidene-1,1-dioxo-1λ^6,2-thiazetidine-2-carboxylic acid *N*-methyl-*N*-phenylamide (13)

To a −5 °C stirred solution of 4-isopropylidene-1,2-thiazetidine 1,1-dioxide (120 mg 0.816 mmol) in dry DCM (10 ml) was added DMAP (0.1 g, catalytic) and *N*-methyl-*N*-phenylcarbamoyl chloride (0.21 g, 1.24 mmol) in THF (2 ml) dropwise. Triethylamine (0.27 ml, 1.89 mmol) was added dropwise over 10 minutes forming a pale yellow solution. The reaction mixture was stirred overnight before the addition of DCM (10 ml) and was then washed with water (15 ml) and brine (2 × 15 ml) before drying over Na_2SO_4. Reduced pressure rotary evaporation at 20 °C gave a pale yellow oil (0.45 g) which was purified by column chromatography (25 g silica) (DCM⟶7 : 3 DCM : diethyl ether, gradient elution) and preparative HPLC (water : ACN : MeOH, gradient elution) yielding a white powder (96 mg, 42%). Mp

102–104 °C. ^{1}H NMR: δ ($CDCl_3$): 7.35 (5H, m, *Ph*), 4.08 (2H, s, NCH_2), 3.39 (3H, s, NCH_3), 1.95 (3H, s, CH_3), 1.75 (3H, s, CH_3). ^{13}C NMR: δ ($CDCl_3$) 153.4, 142, 141.6, 137.4, 129.4, 128.6, 128.5, 41.9, 39.8, 21.7, 20.6. IR: ν_{max}(cm^{-1}) 2945.8, 1670.0, 1490.4, 1442.2, 1390.9, 1315.1, 1250.0, 1201.0, 1161.6, 1128.1, 1066.4, 954.8, 883.7, 762.1, 672.1. HREI-MS [M+H] for $C_{13}H_{16}SO_3N_2$ calc. 281.0960 found 281.0965.

2-(*tert*-Butyldimethylsilyl)-4-isopropyl-1,2-thiazetidine 1,1-dioxide

Palladium (0.35 g, 10% on C,) in ethyl acetate (1 ml) was added to a solution of 2-(*tert*-butyldimethylsilyl)-4-isopropylidene-1,2-thiazetidine 1,1-dioxide (0.45 g, 1.71 mmol) in dry ethanol (12 ml) under argon. The mixture was hydrogenated at 100 psi $H_2$for 24 hours before being filtered through a celite plug and rotary evaporated at 30 °C, yielding a white powder (278 mg, 62%). Mp 101–104 °C. ^{1}H NMR: δ ($CDCl_3$) 4.05 (1H, ddd, *J* 6.67, 8.59 and 11.04, $CHSO_2$), 3.29 (1H, dd, *J* 7.33 and 8.36, CH_2N), 2.87 (1H, dd, *J* 6.86 and 7.17, CH_2N), 2.20 (1H, d septet, *J* 6.55 and 10.97, CH (CH_3)$_2$), 1.07 (3H, d, *J* 6.43, CH_3CH), 0.86 (3H, d, *J* 6.8, CH_3CH), 0.85 (9H, s, C(CH_3)$_3$), 0.16 (6H, s, Si(CH_3)$_2$). *m*/*z* (EI-MS) (M^+H): 262.

4-Isopropyl-1,2-thiazetidine 1,1-dioxide

To a stirred solution of 2-(*tert*-butyldimethylsilyl)-4-isopropyl-1,2-thiazetidine 1,1-dioxide (0.278 g, 0.107 mmol) in THF (20 ml) was added TBAF (1 M, 1.7 ml, 1.7 mmol) in one portion, The reaction mixture was stirred for 3.5 minutes before quenching with a saturated aqueous NaCl solution (30 ml), extracting with ethyl acetate (15 ml) and then the combined organics were dried over Na_2SO_4. Reduced pressure rotary evaporation at 30 °C gave a pale yellow oil (0.8 g) which was purified by column chromatography (35 g silica) (2 : 3 isohexane : ethyl acetate, R_f = 0.35) yielding a white powder (123 mg, 77%). Mp 64–66 °C. ^{1}H NMR: δ ($CDCl_3$) 5.60 (1H, br s, *NH*), 4.22 (1H, ddd, *J* 6.79, 8.03 and 11.14, $CHSO_2$), 3.44 (1H, m, CH_2N), 3.03 (1H, dd, *J* 6.38 and 11.75, CH_2N), 2.32 (1H, ds, *J* 6.6 and 11.01, CH(CH_3)$_3$), 1.16 (3H, d, *J* 6.63, CH_3), 0.98 (3H, d, *J* 6.87, CH_3). ^{13}C NMR: δ ($CDCl_3$) 81.8, 35.2, 29.4, 20.5, 19.4. IR: ν_{max} (cm^{-1}) 3268.7, 2969.1, 2898.6, 2872.2, 1467.2, 1366.0, 1332.9, 1292.3, 1271.1, 1239.2, 1211.8, 1153.9, 1147.6, 1126.7, 986.0, 910.4, 842.2, 733.0, 645.7.

(4-Isopropyl-1,1-dioxo-1λ^6,2-thiazetidin-2-yl)phenylmethanone (14)

To a −10 °C stirred solution of 4-isopropyl-1,2-thiazetidine 1,1-dioxide (117 mg 0.785 mmol) in dry DCM (15 ml) was added DMAP (0.05 g, catalytic) and benzoyl chloride (0.14 ml, 1.18 mmol) dropwise. Triethylamine (0.27 ml, 1.89 mmol) was added dropwise over 10 minutes forming a pale yellow solution. The reaction mixture was stirred overnight before the addition of DCM (5 ml) and was then washed with water (12 ml) and brine (2 × 15 ml) before drying over Na_2SO_4. Reduced pressure rotary evaporation at 30 °C gave a pale yellow oil (0.4 g) which was purified by column chromatography (25 g silica) (chloroform column, eluting with 3 : 2 hexane : ethyl acetate, R_f = 0.3) yielding a white powder (144 mg, 72%). Mp 84–86 °C. ^{1}H NMR: δ ($CDCl_3$) 7.9 (2H, d, *J* 7.5, *Ph*), 7.50 (1H, t, *J* 7.5, *Ph*), 7.40 (2H, d, *J* 7.85, *Ph*), 4.06 (1H, ddd, *J* 6.34, 8.7 and 11.0 $CHSO_2$), 3.84 (1H, dd, *J* 8.35 and 8.5, CH_2N), 3.45 (1H, dd, *J* 6.4 and 8.0, CH_2N), 2.23 (1H, ds, *J* 6.6 and 11.2, CH(CH_3)$_2$), 1.09 (3H, d, *J* 6.6, CH_3), 0.93 (3H, d, *J* 6.7, CH_3). ^{13}C NMR: δ ($CDCl_3$) 167.9, 133.9, 132.9, 129.2, 128.9. 37.9, 29.7, 21.6, 19.2. IR: ν_{max}(cm^{-1}) 2961.0, 1665.4, 1581.7, 1468.1, 1449.6, 1391.8, 1327.6, 1289.4, 1209.6, 1181.7, 1164.4, 1028.6, 844.8, 795.7, 705.7, 657.7. HREI-MS [M+NH_4] for $C_{12}H_{15}SO_3N$ calc. 271.1116 found 271.1113.

4-Isopropyl-1,1-dioxo-1λ^6,2-thiazetidine-2-carboxylic acid benzyl ester (16)

To a −10 °C stirred solution of 4-isopropyl-1,2-thiazetidine 1,1-dioxide (22.2mg, 0.149 mmol) in dry DCM (2.5 ml) was added DMAP (0.02 g, catalytic) and benzyl chloroformate (0.04 ml, 0.265 mmol) dropwise. Triethylamine (0.05 ml, 0.35 mmol) was added dropwise over 5 minutes forming a pale yellow solution, The reaction mixture was stirred overnight before the addition of DCM (3 ml) and was then washed with water (2.5 ml) and brine (2 × 2.5 ml) before drying over Na_2SO_4. Reduced pressure rotary evaporation at 30 °C gave a pale yellow oil (70 mg) which was purified by column chromatography (8 g silica) (3 : 2 hexane : ethy1 acetate, R_f = 0.33) yielding a white powder (28.4 mg, 67%). Mp 93–94 °C. ^{1}H NMR: δ ($CDCl_3$) 7.35 (5H, m, *Ph*), 5.27 (2H, s, CH_2Ph), 4.09 (1H, ddd, *J* 6.0, 8.35 and 11.02, $CHSO_2$), 3.75 (1H, dd, *J* 6.94 and 8.49, CH_2N), 3.34 (1H, dd, *J* 6.4, CH_2N), 2.31 (1H, ds, *J* 6.68 and 11.05, CH(CH_3)$_2$), 1.19 (3H, d, *J* 6.42, CH_3), 1.0 (3H, d, *J* 6.72, CH_3). ^{13} C NMR: δ ($CDCl_3$) 153.2, 128.9, 128.4, 128.7, 128.4, 79.1, 69.1, 38.7, 29.4, 21.6, 19.4. IR: ν_{max} (cm^{-1}) 1732.0, 1455.8, 1386.3, 1341.4, 1319.7, 1207.9, 1185.5, 1170.5, 1127.9, 787.7, 769.5. HREI-MS [M+NH_4] for $C_{13}H_{18}SO_4N$ calc. 301.1222 found 301.1221.

4-Isopropyl-1,1-dioxo-1λ^6,2-thiazetidine-2-carboxylic acid *N*-methyl-*N*-phenylamide (15)

To a −10° stirred solution of 4-isopropyl-1,2-thiazetidine 1,1-dioxide (62.3mg 0.418 mmol) in dry DCM (5 ml) was added DMAP (0.05 g, catalytic) and *N*-methyl-*N*-phenylcarbamoyl chloride (0.11 g, 0.65 mmol) dropwise. Triethylamine (0.14 ml, 0.98 mmol) was added dropwise over 10 minutes forming a pale purple solution. The reaction mixture was stirred overnight at ambient temperature before the addition of DCM (10 ml) and was then washed with water (12 ml) and brine (2 × 12 ml) before drying over Na_2SO_4. Reduced pressure rotary evaporation at 30 °C gave a pale purple oil (0.12 g) which was purified by column chromatography (10 g silica) (3 : 2 hexane : ethy1 acetate, R_f = 0.31) yielding a white powder (87 mg, 74%). Mp 101–103 °C. ^{1}H NMR: δ ($CDCl_3$) 7.4 (2H, d, *J* 7.9, *Ph*), 7.36 (1H, t, *J* 7.37, *Ph*), 7.29 (2H, d, *J* 7.24, *Ph*), 3.85 (1H, ddd, *J* 6.67, 8.75 and 11.15, $CHSO_2$), 3.59 (1H, dd, *J* 7.31 and 8.35, CH_2N), 3.36 (3H, s, NCH_3), 3.31 (1H, dd, *J* 6.86, CH_2N), 2.23 (1H, ds, *J* 6.55 and 11.03 CH (CH_3)$_2$), 1.06 (3H, d, *J* 6.43, CH_3), 0.93(3H, d, *J* 6.67, CH_3). ^{13}C NMR: δ ($CDCl_3$) 153.2, 141.9, 129.9, 128.6, 77.1, 39.7, 38.9, 29.5, 21.5, 19.2. IR: ν_{max} (cm^{-1}) 2965.0, 1668.7, 1594.1, 1494.0, 1419.3, 1367.7, 1333.4, 1299.8, 1183.1, 1152.9, 1009.2, 829.0, 772.5, 697.0. HREI-MS [M+H] for $C_{13}H_{18}SO_3N_2$ calc. 283.1116 found 283.1113.

2-(*tert*-Butyldimethylsilyl)-4-(*E*/*Z*)-ethylidene-1,2-thiazetidine 1,1-dioxide

To a −78 °C stirred solution of diisopropylamine (4.5 ml, 32 mmol) in dry THF (170 ml) was added n-BuLi (1.6 M, 19.4 ml, 12.1 mmol) dropwise. The reaction mixture was stirred for 10 minutes before the addition of 2,4-bis(*tert*-butyldimethylsilyl)-1,2-thiazetidine 1,1-dioxide (5.2 g, 15.5 mmol) in THF (20 ml) over 20 minutes. After stirring for a further 10 minutes, acetaldehyde (8.7 ml, 142 mmol) was added over 10 minutes and the mixture left to stir for 1 hour, before allowing to warm to ambient over 30 minutes. The reaction was quenched with saturated NaCl (90 ml) before the addition of sodium bisulfite (400 ml, 40%) and was then stirred for a further 1 hour. The aqueous layer was extracted with ethyl acetate (2 × 200 ml) before the combined organics were washed with water (100 ml), brine (2 × 100 ml) and dried over Na_2SO_4. Reduced pressure rotary evaporation at 30 °C yielded a pale yellow oil (5.2 g) which was purified by column chromatography (200 g silica) (7 : 3 isohexane : ethyl acetate, R_f = 0.38 and 0.36) yielding a

pale yellow oil as a mixture of the two isomers *E* and *Z* (1.9 g, 50%). ^{1}H NMR: δ ($CDCl_3$) 6.38 (1H, tq, *J* 2.34 and 7.21, *CH*), 3.86 (2H, dq, 1.36 and 2.7, CH_2N), 1.8 (3H, dt, 1.36 and 7.2, CH_3), 0.98 (9H, s, $SiC(CH_3)_3$), 0.3 (6H, s, $Si(CH_3)_2$). ^{13}C NMR: δ ($CDCl_3$) 149.2, 125.6, 65.3, 39.9, 25.9, 18.3, 13.6. IR: ν_{max} (cm^{-1}) 3518.3, 2955.5, 2931, 2859.5, 1706.6, 1471.4, 1297.4, 1255.7, 1170.8, 1126.1, 1073.3, 1005.7, 905.6, 839.9, 825.4, 785.2, 686.2, 657.9. *m*/*z* (EI-MS) (M + H) 248.

4-(*E*/*Z*)-Ethylidene-1,2-thiazetidine 1,1-dioxide

To a stirred solution of 2-(*tert*-butyldimethylsilyl)-4-(*E*/*Z*)ethylidene-1,2-thiazetidine 1,1-dioxide (1.9 g, 7.7 mmol) in THF (80 ml) was added TBAF (1 M, 8.7 ml, 8.7 mmol) in one portion. The reaction mixture was stirred for 3 minutes, before quenching with saturated NaCl (100 ml), extracting with ethyl acetate (2 × 50 ml), washing with brine (50 ml) and then dried over Na_2SO_4. Reduced pressure rotary evaporation at 30 °C gave a pale yellow oil (2.5 g) which was purified by column chromatography (100 g silica) (2 : 3 isohexane : ethyl acetate, R_f = 0.3) yielding a clear colourless oil (0.92 g, 90%). ^{1}H NMR: δ ($CDCl_3$) 6.45 (0.5 H, qt, *J* 2.51 and 7.23, *CH*, *E*), 6.0 (0.5 H, qt, *J* 2.01 and 7.26, *CH*, *Z*), 5.66 (1H, br s *NH*), 3.86 (1H, m, CH_2N, *E*),3.83 (1H, m, CH_2N, *Z*), 2.03 (1.5 H, dt, 2.01 and 7.24, CH_3, *Z*), 1.8 (1.5 H, dt, 1.58 and 7.23, CH_3, *E*). ^{13}C NMR: δ ($CDCl_3$) 149.**148.9, 129.1**, 128.4, **37.6**, 36.8, **15.1**, 13.9 (*Z* isomer in **bold**). IR: ν_{max} (cm^{-1}) 3287.6, 2957.3, 2931.6, 2862.1, 2797.1, 1703.5, 1467.1, 1377.2, 1304.4, 1167.5, 1084.8, 944.5, 872.3, 728.

4-Ethyl-1,2-thiazetidine 1,1-dioxide

Palladium (0.3 g, 10% on C) in ethyl acetate (1 ml) was added to a solution of 4-(*E*/*Z*)-ethylidene-1,2-thiazetidine 1,1-dioxide (0.32 g, 2.4 mmol) in dry ethanol (25 ml) under argon. The mixture was hydrogenated for 72 hours before being filtered through a celite plug and rotary evaporated at 30 °C, yielding a pale yellow oil (98 mg, 30%).

^{1}H NMR: δ ($CDCl_3$) 5.8 (1H, br s, *NH*), 4.4 (1H, m, $CHSO_2$), 3.42 (1H, dd, *J* 6.27 and 8.05, CH_2N), 2.94 (1H, dd, *J* 6.12 and 6.12, CH_2N), 2.09 (1H, m, CH_2CH_3), 1.88 (1H, m, CH_2CH_3), 1.04 (3H, t, *J* 7.39, CH_3). ^{13} C NMR: δ ($CDCl_3$) 75.7, 35.2, 22.3, 11.1. IR: ν_{max} (cm^{-1}) 3583.8, 3291.7, 2971.7, 2880.4, 1637, 1461.4, 1383.6, 1307, 1241.2, 1196.6, 1161.4, 1040, 978.6, 850.6, 701.3.

2-(*tert*-Butyldimethylsilyl)-4-(*E*)-ethylidene-1,2-thiazetidine 1,1-dioxide

To a −78 °C stirred solution of diisopropylamine (4.28 ml, 30.5 mmol) in dry THF (180 ml) was added n-BuLi (1.6 M,19 ml, 30.4 mmol) dropwise. The reaction mixture was stirred for 10 minutes before the addition of 2,4-bis(*tert*-butyldimethylsilyl)-1,2-thiazetidine 1,1-dioxide (5 g, 14.9 mmol) in THF (20 ml) over 20 minutes. After stirring for a further 10 minutes, acetaldehyde (8.3 ml, mmol) was added over 10 minutes and the mixture left to stir for 1 hour before quenching with glacial acetic acid (15 ml) and stiring for a further 30 minutes. The aqueous layer was extracted with ethyl acetate (2 × 100 ml) before the combined organics were washed with brine (100 ml) and dried over Na_2SO_4. Reduced pressure rotary evaporation at 30 °C yielded a pale yellow oil (8.5 g) which was purified by column chromatography (200 g silica) (7 : 3⟶6 : 4 isohexane : ethyl acetate, R_f = 0.38) yielding a pale yellow oil as the product (0.4 g, 10.9%) and a second fraction determined to be starting material (R_f = 0.65, 3.8 g). Yield based upon total conversion, 45%. ^{1}H NMR: δ ($CDCl_3$) 6.38 (1H, tq, *J* 2.34 and 7.21, *CH*), 3.86 (2H, dq, 1.36 and 2.7, CH_2N), 1.8 (3H, dt, 1.36 and 7.2, CH_3), 0.98 (9H, s, $SiC(CH_3)_3$), 0.3 (6H, s, $Si(CH_3)_2$). ^{13}C NMR: δ ($CDCl_3$) 149.2, 125.6, 65.3, 39.9, 25.9, 18.3, 13.6. IR: ν_{max} (cm^{-1}) 3518.3, 2955.5, 2931, 2859.5, 1706.6, 1471.4, 1297.4, 1255.7, 1170.8, 1126.1, 1073.3, 1005.7, 905.6, 839.9, 825.4, 785.2, 686.2, 657.9.

4-(*E*)-Ethylidene-1,2-thiazetidine 1,1-dioxide

To a stirred solution of 2-(*tert*-butyldimethylsilyl)-4-(*E*)-ethylidene-1,2-thiazetidine 1,1-dioxide (0.4 g, 1.6 mmol) in THF (20 ml) was added TBAF (1 M, 1.7 ml, 1.7 mmol) in one portion. The reaction mixture was stirred for 3 minutes, before quenching with saturated NaCl (40 ml), extracting with ethyl acetate (2 × 25 ml), washing with brine (25 ml) and then dried over Na_2SO_4. Reduced pressure rotary evaporation at 30 °C gave a pale yellow oil (0.55 g) which was purified by column chromatography (20 g silica) (2 : 3 isohexane : ethyl acetate, R_f = 0.3) yielding a clear colourless oil (0.15 g, 71%). ^{1}H NMR: δ ($CDCl_3$) 6.44 (0.5 H, qt, *J* 2.52 and 7.23, *CH*), 5.65 (1H, br s, *NH*), 3.86 (1H, m, CH_2N,), 1.8 (1.5 H, dt, 1.59 and 7.22, CH_3). ^{13}C NMR: δ ($CDCl_3$) 149.1, 128.5, 36.8, 13.9. IR: ν_{max} (cm^{-1}) 328.4, 2957.2, 2931.1, 2860.7, 2797.0, 1703.4, 1467, 1377.1, 1304.4, 1166.9, 1084, 872.3, 728.2.

[4-(*E*/*Z*)-Ethylidene-1,1-dioxo-1λ^6,2-thiazetidin-2-yl](phenyl)-methanone (17)

To a −10 °C stirred solution of 4-(*E*/*Z*)-ethylidene-1,2-thiazetidine 1,1-dioxide (0.32 g 2.4 mmol) in dry DCM (35 ml) was added DMAP (0.1 g, catalytic) and benzoyl chloride (0.63 g, 3.7 mmol) dropwise. Triethylamine (0.85 ml, 6.1 mmol) was added dropwise over 10 minutes forming a pale yellow solution. The reaction mixture was stirred overnight at ambient temperature before the addition of DCM (25 ml) and was then washed with water (25 ml) and brine (2 × 25 ml) before drying over Na_2SO_4. Reduced pressure rotary evaporation at 30 °C gave a pale yellow oil (1 g) which was purified by column chromatography (35 g silica) (3 : 2 hexane : ethyl acetate, R_f = 0.28 and 0.22) yielding two fractions of white powders, *E* and *Z* isomers respectively [120 mg (*E*). Mp 92–94 °C] and [160 mg (*Z*). Mp 63–65 °C]. Overall 49% yield. *E* Isomer: ^{1}H NMR: δ ($CDCl_3$) 8.03 (2H, m, *Ph*), 7.62 (1H, m, *Ph*), 7.52 (2H, m, *Ph*), 6.65 (1H, qt, *J* 2.54 and 7.22 *CH*), 4.43 (2H, dq, 1.62 and 2.53, CH_2N), 1.93 (3H, dt, *J* 1.65 and 7.25, CH_3). ^{13}C NMR: δ ($CDCl_3$) 167.5, 143.8, 133.6, 132.6, 130.7, 128.9, 128.4, 40.1, 14.4. IR: ν_{max} (cm^{-1}) 2954.7, 2924.2, 2854, 1670.4, 1464.5, 1449.3, 1376.9, 1334.4, 1311.8, 1267.1, 1187, 1158.1, 946.6. HREI-MS [M+NH_4] for $C_{11}H_{11}SO_3N$ calc. 255.0803 found 255.0799.

Z Isomer: ^{1}H NMR: δ ($CDCl_3$) 8.03 (2H, m, *Ph*), 7.65 (1 H, m, *Ph*), 7.55 (2H, m, *Ph*), 6.27 (1H, qt, *J* 2.02 and 7.24, *CH*), 4.39 (2H, dq, 2.1 and 2.03, CH_2N), 2.15 (3H, dt, *J* 2.14 and 7.26, CH_3). ^{13}C NMR: 167.5, 143.2, 133.6, 132.6, 131.5, 128.9, 128.4, 40.9, 159. IR: ν_{max} (cm^{-1}) 3060.2, 2962.6, 1679.9, 1599.8, 1581.5, 1451.8, 1425.6, 1378.5, 1324.9 1256.2, 1216.6, 1162.3, 1136.2, 1126.7, 1028, 1017.6, 992.1, 840.8, 755.8, 710.9, 691.6, 635.4. HREI-MS [M+NH_4] for $C_{11}H_{11}SO_3N$ calc. 255.0803 found 255.0801.

Kinetics

Standard UV spectroscopy was carried out on a Cary 1E UV-visible spectrophotometer (Varian, Australia) equipped with a twelve compartment cell block. The instrument was used in double beam mode, allowing six reaction cells to be followed in a single run. The cell block was thermostatted using a peltier system. Stopped flow experiments used an SX 18 MV Spectrakinetic monochromator (Applied Photophysics, Leatherhead, England) equipped with an absorbance photomultiplier. The reagent syringes were thermostatted with a Grant thermostatted water circulator. pH-stat experiments were performed on a ABU 91 Autoburette (Radiometer, Copenhagen, Denmark), controlled by a VIT 90 video titrator. The SAM 90 sample station incorporated a machined aluminium E2000 sample block rotor thermostatted by a MGW Lauda M3 water

circulator. pH was measured by a pHG200–8 Glass pH electrode and a REF200 'Red Rod' reference electrode (Radiometer). Temperature was monitored by a T101 temperature sensor.

pH measurements; were made with either a ϕ40 (Beckman, Fullerton, USA) or 3020 (Jenway, Dunmow, England) pH meter. Electrodes were semi-micro Ag/AgCl and calomel (Russel, Fife, Scotland and Beckman respectively). A calibration of the pH meter was carried out at 30 °C using pH 7.00 ± 0.01, pH 4.01 ± 0.02 or pH 10.00 ± 0.02 calibration buffers.

ESIMS experiments were carried out on a VG Quattro SQ II (Micromass, Altrincham, England) and NMR experiments on a 400 MHz instrument (Bruker, Germany).

AnalaR grade reagents and deionised water were used throughout. Sodium hydroxide solutions were titrated prior to use against a 1.00 M ± 0.1% hydrochloric acid volumetric reagent (D.H. Scientific, Huddersfield, England) using phenolphthalein as an indicator.

Organic solvents were glass distilled prior to use and stored under nitrogen. For solution pHs ≥3 and ≤11 the pH was controlled by the use of ≤0.2 M buffer solutions of formate (pK_a 3.75), ethanoate (pK_a 4.72), 2-(*N*-morpholino)ethanesulfonic acid (MES) (pK_a 6.1), 3-(*N*-morpholino)propanesulfonic acid (MOPS) (pK_a 7.2), *N*-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid (TAPS) (pK_a 8.4), 3-(cyclohexylamino)-2-hydroxypropane-1-sulfonic acid (CAPSO) (pK_a 9.6), and 3-(cyclohexylamino)propane-1-sulfonic acid (CAPS) (pK_a 10.4). For general pH work, buffers were prepared by partial neutralisation of solutions of their sodium salts to the required pH. For the alcoholysis reactions, buffers were prepared by the addition of 0.25, 0.50 or 0.75 aliquots of 1 M NaOH to solutions of the alcohol. In all experiments temperatures were maintained at 30 °C and ionic strength at 1.0 M with AnalaR grade KCl unless otherwise stated. Reaction concentrations were generally within the range $\geq 2\times10^{-5}$ M to $\leq 2 \times 10^{-4}$M to ensure pseudo-first order conditions.

Hydroxide ion concentrations were calculated using pK_w (H_2O) = 13.883 at 30 °C.[34b] Reactions studied by UV spectrophotometry were usually commenced by injections of acetonitrile or dioxan stock solutions of the substrate (5–50 μl) into the cells containing pre-incubated buffer (2.5 ml). Final reaction cells contained ≤5% acetonitrile or dioxan v/v. The pH of the reaction cells was measured before and after each kinetic run at 30 °C, kinetic runs experiencing a change >0.05 units were rejected. Reactant disappearance or product appearance were followed at absorbance change maxima for individual compounds. The solubility of compounds was ensured by working within the linear range of absorbance in corresponding Beer–Lambert plots. If required, greater than 1% MeCN v/v was used to aid solubility. Pseudo-first order rate constants from exponential plots of absorbance against time or gradients of initial slopes were obtained using the Enzfitter package (Elsevier Biosoft, Cambridge, England) or the CaryBio software (Varian). pH-rate profiles were modelled to theoretical equations using the Scientist program (V2.02, Micromath Software Ltd, USA).

Reactions studied by stopped flow UV spectrophotometry used stock solutions prepared at twice the standard UV concentration in 1 M KCl. Hydroxide solutions, buffer solutions or solutions of nucleophilic reagents were prepared at twice the required concentration. The substrate solution and the reaction mixture were placed in separate syringes and thermostatted at 30 °C before pneumatic injection into the reaction into the reaction cell. Where applicable, the pH of solutions was measured prior to use. If greater than 1% acetonitrile v/v was required for solubility, then organic solvent concentration of all solutions used was fixed at the required reaction cell amount. The photomultiplier voltage was set to a maximum on deionised water and absorbance wavelengths common to the standard UV experiments were used. Pseudo-first order rate constants from exponential plots of absorbance against time were obtained using the supplied fitting software (Applied Photophysics).

For reactions studied by pH-stat standardised NaOH was delivered to a stirred sample solution (10 ml) held within the thermostatted sample station. All reactions were performed under nitrogen to prevent CO_2 absorption. Data were exported to a Windows PC *via* an RS232 interface and the terminal program (Microsoft Corp, USA). Conversion into an appropriate format was by means of an Excel (Microsoft Corp, USA) Macro and results were fitted to first order equations *via* the Enzfitter program (Elsevier Software). The titrant used was 0.01–0.1 M NaOH standardised prior to use by means of phenolphthalein titration against 1.00 M HCl (Volumetric reagent, D.H. Scientific). Reactions were performed in 1 M KCl, 5% MeCN v/v, with a pH set point of 6–7. Concentrations of sample were in the range of 1–2 mM with expected titrant added volumes of up to 1.0 ml.

Enzyme inhibition studies

Assays were performed at 30 °C in 0.1 M pH 8.5 TAPS buffer with I = 1.0 M (KCl). The substrate used was *N*-suc-(L-Ala)$_3$-*p*-nitroanilide (**2**) and stock solutions were made up in distilled methanol. Enzyme activity was monitored by following the appearance of the *p*-nitroaniline product at 390 nm. During inhibition studies a fixed assay substrate concentration of 6.0×10^{-5} M was used resulting in a constant methanol concentration of 1.5% v/v. Incubations of enzyme and inhibitor (500 μl) were carried out at 30 °C in 0.04 M buffer, 20% MeCN v/v, 8×10^{-5} M PPelastase and up to 5×10^{-3} M β-sultam. These conditions were created in a separate UV cell containing stock PPelastase suspension (200 μl), 0.1 M buffer (200 μ) and inhibitor stock solution in MeCN or MeCN alone (100 μ). Aliquots of this solution (50 μl) were assayed for PPelastase activity at various time intervals by injection into 0.1 M pH 8.5 TAPS (2.36 ml) containing MeCN (150 μl) and substrate solution (40 μl). This gave final assay conditions of 6.0×10^{-5} M substrate, 1.5×10^{-6} M PPelastase, 6 % MeCN v/v and 1.5 % MeOH v/v. In all cases assays of control incubations were performed at the same time as inhibitor incubations. These incubations substituted pure MeCN for the β-sultam solution but then matched the remaining methodology in all respects. Initial rates were monitored over up to eight minutes and the gradients of the slopes obtained used as a measure of enzyme activity. Gradients from inhibitor runs were normalised with respect to the first order exponential decrease in β-sultam concentration due to hydroxide ion catalysed hydrolysis at the pH of the incubation. This was done by dividing the observed gradient by the β-sultam concentration at the start of the experiments, time = 0, and multiplying by the β-sultam concentration at the time of the assay, time = *t*:

Normalised gradient at time *t* = (observed gradient/$[\text{Sultam}]_o$) × $[\text{Sultam}]_t$

The average control gradient was taken as 100% activity. This value and the normalised gradients were used to calculate % activity:

% Activity = (Normalised gradient/Average control gradient) × 100

Pseudo-first order rate constants of inactivation, K_{obs} values, were obtained from plots of % activity against time using the Enzfitter program (Elsevier Biosoft). The second order rate constants of inactivation, K_i values, were obtained by dividing the first order K_{obs} values by the concentration of β-sultam in the inactivation.

pK_a determinations

A solution of the compound was placed in a UV cell at 30 °C and typically titrated by means of additions of NaOH or HCl

(≤50 μl). Absorbance values at the wavelength of maximum change and the cell solution pH were measured after each injection of the 0.01, 0.05, 0.1 or 1.0 M standardised NaOH or HCl. Near the end point injections of the 0.01 M solution (5 μl) were made to ensure that sufficient pH values were covered in the proximity of the pK_a.

The results were analysed using the Enzfitter software package (Elsevier Biosoft), pK_{a1} determination equation, $A = [(A_{min} + A_{max}) \times (10^{(pH - pK_{a1})})]/[(10^{(pH - pK_{a1})}) + 1]$ where A = absorbance of solution at a particular pH and wavelength, A_{min} = minimum absorbance where 100% of dissociating species is in form with lowest absorption, A_{max} = maximum absorbance where 100% of dissociating species is in form with highest absorption, pH = solution pH and pK_a = pK_a of dissociating species.

^{1}H-NMR experiments

The β-sultam (15 mg) was dissolved in 50 : 50 v/v CD_3CN : D_2O (*ca.* 3 ml) and the ^{1}H-NMR spectra acquired at 400 MHz and 300 K. One drop additions of 4% v/v NaOD in D_2O were made to the NMR tube until complete conversion of reactants had occurred with spectra being acquired after each addition. In buffer solution, the β-sultam (15 mg) was dissolved in 50 : 50 v/v CD_3CN : 0.4 M buffer solution in D_2O (*ca.* 3 ml), the pD of which was measured before and after the experiment. The solutions were also routinely submitted for both positive and negative mode ESIMS-MS.

X-Ray crystal preparation, data collection, processing and structure refinement

PPE crystals were prepared as previously described.[36] A saturated solution of the *N*-benzoyl β-sultam (**3**) in sodium acetate buffer (pH 5.0, 50 mM), sodium sulfate (25 mM) and DMSO (10% v/v) was prepared. The mother liquor containing the crystals was exchanged with this solution over a 1 h period. After 24 h soaking in the inhibitor solution, the crystals were placed in a cryoprotectant solution containing 20% (v/v) glycerol and flash-cooled in liquid nitrogen.

Data were collected at 100 K using a Rigaku rotating anode generator (CuK_α radiation) and 345 mm Mar Research image plate detector to a resolution limit of 1.67 Å. The crystal belonged to the space group $P2_12_12_1$ with a = 50.77 Å, b = 57.61 Å, c = 74.48 Å. Raw data were processed with the programs MOSFLM and SCALA (CCP4 suite.[37] R_{merge} was 4.3% for all data (19.3% in the highest resolution shell, 1.76–1.67 Å). Data were 99.3% complete (96.6% in the highest resolution shell) with 92 216 reflections of which 25 847 were unique after reduction. The overall I/σ_1 was 20.1 and 4.8 in the highest resolution shell (1.68–1.67 Å).

The PPE:BCM7 structure (excluding the inhibitor) (PDB entry 1QIX) was used the starting model. The structure was refined using REFMAC (CCP4 suite) with a total of 4% of the reflections in the entire dataset randomly selected in order provide a test set for the R_{free} calculations. Models and electron density maps were displayed using the program O.[38] At the stage in refinement with the $F_o - F_c$ map showed clear and unambiguous density for the β-sultam derived atoms, they were included in the model and an appropriate PROTIN dictionary file created. The sulfonate ester bond was simulated solely with bond length restraints. Only the six atoms derived from the β-sultam ring were visible in the structure with the benzoyl group apparently disordered or missing. The side chains of three residues of PPE (Arg-61, Arg-125, Arg-223) were also disordered and were included as alanines in the model. During the latter stages of refinement, 141 water molecules were added along with the bound calcium and sulfate ions,[36] giving a final *R*-factor of 19.0% and free *R*-factor of 21.3%. The average *B*-factor for the atoms in the β-sultam inhibitor molecule was 20.3 Å^2.

Acknowledgements

For CASE awards we thank the EPSRC and AstraZeneca (JMW) and British Biotech (PSH). We also thank C J Schofield and R C Wilmouth for the X-ray crystal structure.

References

1 M. I. Page, Enzyme Inhibition in *Comprehensive Medicinal Chemistry*, ed. P. G. Sammes, Pergamon Oxford, 1990, Vol. 2, pp. 61–87.

2 (*a*) C. W. Wharton, The Serine Proteinases in *Comprehensive Biological Catalysis*, ed. M. Sinnott, Academic Press, London, 1997, Vol. 1, Ch. 11, pp. 345–379; (*b*) D. J. Underwood, B. G. Green, R. Chabin, S. Mills, J. B. Doherty, P. E. Finke, M. MacCoss, S. K. Shah, C. S. Burgey, T. A. Dickinson, P. R. Griffin, T. E. Lee, K. M. Swiderek, T. Covey, W. M. Westler and W, B. Knight, *Biochemistry*, 1995, **34**, 14344–14355; (*c*) J. A. Katzenellenbogen, R. Rai and W. Dai W, *Bioorg. Med. Chem. Lett. 2*, 1992, **11**, 1399–1404; (*d*) W. B. Knight, A. L. Maycock, B. G. Green, B. M, Ashe, P. Gale, H. Weston, P. E. Finke, W. K. Hagmann, S. K. Shah and J. B. Doherty, *Biochemistry*, 1992, **31**, 4980–4986; (*e*) C-M Kam, K. Fujikawa and J. C. Powers, *Biochemistry*, 1988, **27**, 2547–2557; (*f*) M. A. Navia, J. P. Springer, T. Y. Lin, H. R. Williams, R. A. Firestone, J. M. Pisano, J. B. Doherty, P. E. Finke and K. Hoogsteen, *Nature*, 1982, **327**, 79–82; (*g*) M. H. Gelb and R. H. Abeles, *J. Med. Chem.*, 1986, **29**, 585–589; (*h*) M. H. Gelb and R. H. Abeles, *Biochemistry*, 1984, **23**, 6569–6604.

3 W. A. Metz and N. P. Peet, *Prog. Inflammation Res.*, 1999, **9**, 853–868.

4 (*a*) W, Bode, A. Z. Wei, R. Huber, E. Meyer, J. Travis and S. Neuman, *EMBO J.*, 1986, **5**, 2453–2458; (*b*) M. A. Navia, B. M. McKeever, J. P. Springer, T. Y. Lin, H. R. Williams, E. M. Fluder, C. P. Dorn and K. Hoogsteen, *Proc. Natl. Acad. Sci. USA*, 1989, **86**, 7–11.

5 W. Bode, E. Meyer Jr. and J. C. Powers, *Biochemistry*, 1989, **28**, 1951–1962.

6 (*a*) L. H. Takahashi, R. Radhakrishan, R. E. Rosenfeld Jr., E. F. Meyer Jr. and D. A. Trainer, *J. Am. Chem. Soc.*, 1989, **111**, 3368–3374; (*b*) A. Renaud, P. Lestienne, D. L. Hughes, J. G. Bieth and J-L. Dimicoli, *J. Biol. Chem.*, 1983, **258**, 8312–8316.

7 (*a*) P. D. Edwards, E. F. Meyer Jr., J. Vijayalakshmi, P. A. Tuthill, D. A. Andisk, B. Gomes and A. Strimpler, *J Am. Chem. Soc.*, 1992, **114**, 1854–1863; (*b*) M. J. Costanzo, B. E. Maryanoff, L. R. Hecker, M. R. Schott, S. C. Yabut, H-C Zhang, P. Andrade-Gordon, J. A. Kauffman, J. M. Lewis, R. Krishnan and A. Tulinsky, *J. Med. Chem.*, 1996, **39**, 3039–3043; (*c*) P. R. Bernstein, B. C. Gomes, B. J. Kosmider, E. P. Vacek and J. C. Williams, *J. Med. Chem.*, 1995, **38**, 212–215; (*d*) P. D. Edwards, D. W. Andisik, A. M. Strimpler, B. C. Gomes and P. A. Tuthill, *J. Med. Chem.*, 1996, **39**, 1112–1124; (*e*) M. R. Angelastro, P. Bey, J. P. Burhart, T-M Chen, R. J. Cregge, S. L. Durham, R. A. Farr, S. L. Gallion, C. M. Hare, R. V. Hoffmann, E. W. Huber, M. L. Janusz, H-O. Kim, J. R. Koehl, A. L. Marquart, S. Mehdi, W. A. Metz, N. P. Peet, H. A. Schreuder, S. Sunder and C. Tardif, *J. Med. Chem.*, 1998, **41**, 2461–2480.

8 (*a*) S. J. F. Macdonald, D. B. Belton, D. M. Buckley, J. E. Spooner, M. S. Anson, L. A. Harrison, K. Mills, R. J. Upton, M. D. Dowle, R. A. Smith, C. Molloy and C. Risley, *J. Med. Chem.*, 1998, **41**, 3919–3922; (*b*) S. J. F. Macdonald, M. D. Dowle, L. A. Harrison, J. E. Spooner, P. Shah, M. R. Johnson, G. G. A. Inglis, G. D. E. Clarke, D. J. Belton, R. A. Smith, C. R. Molloy, M. Dixon, G. Murkitt, R. E. Godward, T. Skarzynski, O. M. P. Singh, K. A. Kumar, S. T. Hodgson, E. McDonald, G. W. Hardy, H. Finch, G. Fleetwood and D. C. Humphreys, *Bioorg. Med. Chem. Lett.*, 2001, **11**, 243–6; (*c*) S. J. F. Macdonald, M. D. Dowle, L. A. Harrison, P. Shah, M. R. Johnson, G. G. A. Inglis, G. D. E. Clarke, R. A. Smith, D. Humphreys, C. R. Molloy, A. Amour, M. Dixon, G. Murkitt, R. E. Godward, T. Padfield, T. Skarzynski, O. M. P. Singh, K. A. Kumar, G. Fleetwood, S. T. Hodgson, G. W. Hardy and H. Finch, *Bioorg. Med. Chem. Lett.*, 2001, **11**, 895–8.

9 *The Chemistry of β-Lactam Antibiotics*, ed. M. I. Page, Blackie, Glasgow, 1992.

10 (*a*) R. Chabin, B. G. Green, P. Gale, A. L. Maycock, H. Weston, C. P. Dorn, P. E. Finke, W. K. Hagmann, J. J. Hale, M. MacCross, S. K. Shah, D. J. Underwood, J. B. Doherty and W. B. Knight, *Biochemistry*, 1993, **32**, 8970; (*b*) C. Beauve, M. Bouchet, R. Touillaux, J. Fastrez and J- Marchand-Brynaert, *Tetrahedron*, 1999, **55**, 13301.

11 D. J. Underwood, B. G. Green, R. Chabin, S. Mills, J. B. Doherty, P. E. Finke, M. MacCoss, S. K. Shah, C. S. Burgey, T. A. Dickinson, P. R. Griffin, T. E. Lee, K. M. Swiderek, T. Covey, W. M. Westler and W. B. Knight, *Biochemistry*, 1995, **34**, 143344.
12 R. C. Wilmouth, S. Kassamally, N. J. Westwood, R. J. Sheppard, T. D. W. Claridge, R. T. Aplin, P. A. Wright, G. J. Pritchard and C. J. Schofield, *Biochemistry*, 1999, **38**, 7989.
13 (*a*) J. F. King, R. Rathore, J. Y. L. Lam, L. E. R. Gao and D. F. Klassen, *J. Am. Chem. Soc.*, 1992, **114**, 3028; (*b*) J. M. Wood and M. I. Page, *Trends Heterocycl. Chem.*, 2002, **8**, 19.
14 N. J. Baxter, A. P. Laws, L. J. M. Rigoreau and M. I. Page, *J. Am. Chem. Soc.*, 2000, **112**, 3375.
15 N. J. Baxter, A. P. Laws, L. J. M. Rigoreau and M. I. Page, *J. Chem. Soc., Perkin Trans. 2*, 1996, 2245.
16 M. Beardsell, P. S. Hinchliffe, J. M. Wood, R. C. Wilmouth, C. J. Schofield and M. I. Page, β-Sultams – a novel class of serine protease inhibitors, *Chem. Commun.*, 2001, 497–498.
17 M. I. Page, *Enzyme Catalysis in Comprehensive Medicinal Chemistry*, 1990, Vol. 2, ed. P. G. Sammes, Pergamon, Oxford, pp. 45–60.
18 (*a*) A. P. Laws, and M. I. Page, *J. Chem. Soc., Perkin Trans. 2*, 1989, 1577–1581; (*b*) A. P. Laws, N. J. Layland, D. G. Proctor and M. I. Page, *J. Chem. Soc., Perkin Trans 2.*, 1993, 17–21; (*c*) N. O. Sykes, S. J. F. McDonald and M. I. Page, *J. Med. Chem.*, 2002, **45**, 2850.
19 J. Bieth, B. Spiess and C. G. Wermuth, *Biochem. Med.*, 1974, **11**, 350.
20 I. B. Blagoeva, A. J. Kirby, I. I. Kochiyashki, A. H. Koedjikov, I. G. Pojarlieff and M. M. Toteva, *J. Chem. Soc., Perkin Trans. 2*, 2000, 1953.
21 A. R. Fersht, *Enzyme Structure, Mechanism*, W. H. Freeman, USA, 2nd edn., 1985, p. 405.
22 R. C. Wilmouth, S. Kassamally, N. J. Westwood, R. J. Sheppard, T. D. W. Claridge, R. T. Aplin, P. A. Wright, G. J. Pritchard and C. J. Schofield, *Biochemistry*, 1999, **38**, 7989.
23 M. J. Slater, A. P. Laws and M. I. Page, *Bioorg. Chem.*, 2001, **29**, 7795.
24 A. M. Gold and D. E. Fahrney, *Biochemistry*, 1964, **3**, 783.
25 R. A. Firestone, P. L. Barker, J. M. Pisano, B. M. Ashe and M. E. Dahlgren, *Tetrahedron*, 1990, **46**, 2255.
26 W. B. Knight, A. L. Maycock, B. G. Green, B. M. Ashe, P. Gale, H. Weston, P. E. Finke, W. K. Hagmann, S. K. Shah, D. Underwood and J. B. Doherty, *Biochemistry*, 1992, **31**, 4980.
27 (*a*) M. O. Lively and J. C. Powers, *Biochim. Biophys. Acta*, 1978, **525**, 171; (*b*) T. Yoshimura, L. N. Barker and J. C. Powers, *J. Biol. Chem.*, 1982, **257**, 5077; (*c*) D. E. Fahrney and A. M. Gold, *J. Am. Chem. Soc.*, 1963, **85**, 997.
28 (*a*) O. R. Zaborsky and E. T. Kaiser, *J. Am. Chem. Soc.*, 1970, **92**, 860; (*b*) E. T. Kaiser, *Intra-Sci. Chem. Rep.*, 1972, **6**, 5.
29 J. L. Kice, *Adv. Phys. Org. Chem.*, 1980, **17**, 65.
30 R. C. Wilmouth, N. J. Westwood, K. Anderson, W. Brownlee, T. D. W. Claridge, I. J. Clifton, G. J. Pritchard, R. T. Aplin and C. J. Schofield, *Biochemistry*, 1998, **37**, 17506.
31 R. A. Firestone, P. L. Barker, J. M. Pisano, B. M. Ashe and M. Dahlgren, *Tetrahedron*, 1990, **46**, 2255.
32 P. S. Hinchcliffe, J. M. Wood, A. M. Davis, R. P. Austin, R. P. Beckett and M. I. Page, *J. Chem. Soc., Perkin Trans. 2*, 2001, 1503.
33 P. E. Finke, S. K. Shah, D. S. Fletcher, B. M. Ashe, K. A. Brause, G. O. Chandler, P. S. Dellea, K. M. Hand, A. L. Maycock, D. G. Osinga, D. J. Underwood, H. Weston, P. Davies and J. B. Doherty, *J. Med. Chem.*, 1995, **38**, 2449.
34 (*a*) D. D. Perrin and W. L. F. Armarego, *Purification of Laboratory Chemicals*, 3rd edn., Pergamon, Oxford, 1988; (*b*) *Lange's Handbook of Chemistry*, ed. J. A. Dean, McGraw-Hill, 11th edn., 1973, pp. 5–7.
35 A. Champseix, J. Chanet-Ray, A. Etienne, A. Le Berre, J. Masson, C. Napierale and R. Vessiere, *Bull. Soc. Chim. Fr.*, 1985, 483.
36 E. Meyer, G. Cole, R. Radhakrishnan and O. Epp, *Acta. Crystallogr., Sect. B*, 1985, **B44**, 26–38.
37 S. Bailey, *Acta Crystallogr., Sect D*, 1994, **D50**, 760–3.
38 A. T. Jones, J. Y. Zou, S. W. Cowan and M. Kjeldgaard, *Acta Crystallogr., Sect A.*, 1991, **A47**, 110–9.