The efficiency of C-4 substituents in activating the β-lactam scaffold towards serine proteases and hydroxide ion†

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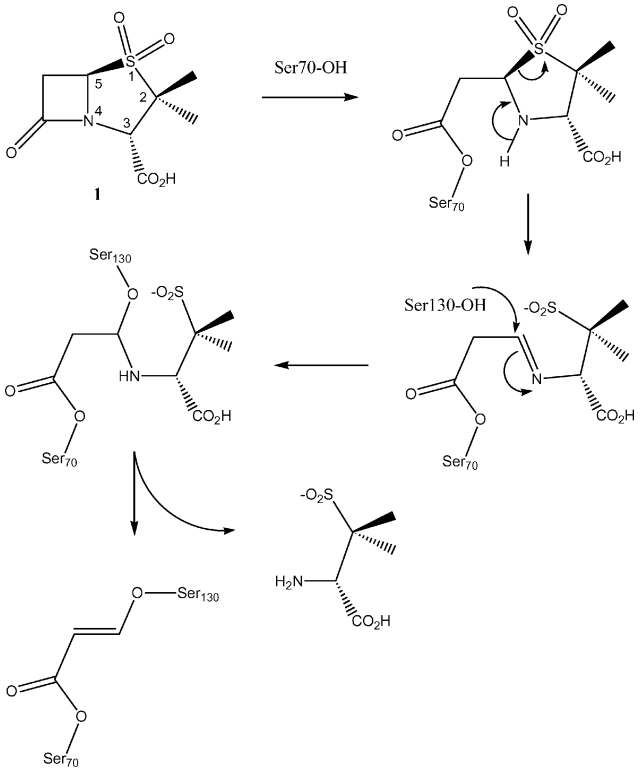
The presence of a leaving group at C-4 of monobactams is usually considered to be a requirement for mechanism-based inhibition of human leukocyte elastase by these acylating agents. We report that second-order rate constants for the alkaline hydrolysis and elastase inactivation by *N*-carbamoyl monobactams are independent of the pK_a of the leaving group at C-4. Indeed, the effect exerted by these substituents is purely inductive: electron-withdrawing substituents at C-4 of *N*-carbamoyl-3,3-diethylmonobactams increase the rate of alkaline hydrolysis and elastase inactivation, with Hammett ρ_I values of 3.4 and 2.5, respectively, which indicate the development of a negative charge in the transition-states. The difference in magnitude between these ρ_I values is consistent with an earlier transition-state for the enzymatic reaction when compared with that for the chemical process. These results suggest that the rate-limiting step in elastase inactivation is the formation of the tetrahedral intermediate, and that β-lactam ring-opening is not concerted with the departure of a leaving group from C-4. Monobactam sulfones emerged as potent elastase inhibitors even when the ethyl groups at C-3, required for interaction with the primary recognition site, are absent. For one such compound, a 1 : 1 enzyme–inhibitor complex involving porcine pancreatic elastase has been examined by X-ray crystallography and shown to result from serine acylation and sulfinate departure from the β-lactam C-4.

Introduction

β-Lactams are potent inhibitors of a wide range of enzymes that contain a catalytic serine residue, including the hepatitis C virus serine protease, penicillin-binding proteins (PBPs), β-lactamases and human leukocyte elastase (HLE).[1–7] The efficiency of β-lactams as enzyme inhibitors depends on the molecular recognition by the protein as well as on the intrinsic chemical reactivity of the β-lactam, both of which affect the rate at which these inhibitors acylate the serine residue.[8]

Among the most extensively studied enzymatic reactions of β-lactams is the inhibition of class A β-lactamases by penam sulfone inhibitors such as sulbactam, **1** (Scheme 1), tazobactam and their analogues.[9–15] Reaction of penam sulfones with the catalytic Ser70 involves β-lactam ring-opening and leads to the departure of a sulfinate leaving group from C-5 and formation of an imine (Scheme 1).[1,14] The imine then undergoes a cascade of reactions including nucleophilic attack by the Ser130 hydroxyl group to form a stable acrylate ester capable of preventing hydrolysis of the acyl-enzyme, and thus leading to irreversible inhibition.[11] The oxidation state of the sulfur atom in the penam plays a role in potency: while sulbactam is a potent β-lactamase inhibitor, its penicillanate counterpart is only a substrate of β-lactamase,



Scheme 1

while the corresponding sulfoxides are both substrate and weak inactivators.[10] It is worth noting that β-lactamase inhibition by clavulanic acid, **2**, requires intramolecular hydrogen bonding from

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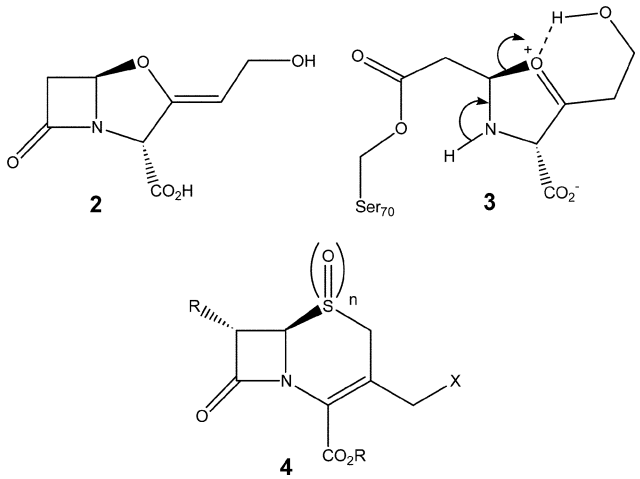
the C-9 hydroxyl group to assist the departure of an oxonium oxygen from C-5, *i.e.* **3** (species **3** results from proton transfer from a water molecule, Wat673, to the carbon–carbon double bond of the acyl-enzyme).[16] These results suggest that departure of a good leaving group from the C-5 carbon atom of penams and clavams is required to achieve irreversible inhibition of enzymes containing a catalytic serine residue.[10,17]

Cephalosporins are time-dependent inhibitors of HLE inhibitors, and, similarly to penams, the oxidation state of the sulfur atom also plays a role in potency, with sulfones (**4**, n = 2) showing considerably greater activity than the corresponding sulfides (**4**, n = 0) or β-sulfoxides (**4**, n = 1), while α-sulfoxides are inactive.[18–21] However, the identity of the species giving rise to inhibition remains to be defined. X-Ray crystallography indicates that inactivation of HLE by cephalosporin sulfones containing a good leaving group at C-3′ (*e.g.* **4**, X = AcO) preferably involves reaction with His57 at this position rather than sulfinic acid departure from C-6 and thiazine ring-opening.[22]



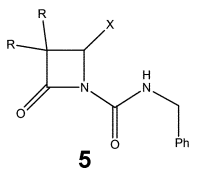
Monobactams containing potential leaving groups at C-4 have also been developed as mechanism-based inhibitors of HLE,[23–25] although the requirement of leaving-group departure to achieve irreversible inactivation is still controversial. Crystallography[26] and mass spectrometry[27] studies indicate that the reaction of monobactams containing a C-4 aryloxy substituent with HLE involves the departure of a phenol, possibly concerted with C–N bond fission and β-lactam ring-opening.[28] However, the observation that the second-order rate-constants for HLE inactivation by monobactams with no leaving group at C-4 do not differ significantly from those of monobactams containing a phenol[28] suggests that leaving group ability is not essential to inactivate the enzyme. Understanding how β-lactam substituents affect molecular recognition by the enzyme and "chemical reactivity" is most useful in designing more potent enzyme inhibitors. We report here a study on the chemistry of elastase inactivation by model monobactams, **5** (Scheme 2, Table 1) with the objective of elucidating how C-4 substituents contribute to the chemical reactivity towards the catalytic serine. The results herein presented show that the effect exerted by these substituents is largely inductive and that sulfones at C-4 are particularly efficient in activating the β-lactam scaffold towards elastase.

Results and discussion

1. Chemistry

The synthesis of compounds **5** used the appropriate 4-acetoxyazetidin-2-one **6**[24] as the starting material to give direct access to the differently C-4-substituted azetidin-2-one key intermediates **7–10** (Scheme 2). Reaction of 4-acetoxyazetidin-2-ones **6** with phenol[24] (or thiols[29]) and sodium hydroxide in acetone at room temperature gave the corresponding C-4-substituted β-lactams **7** and **8**, respectively. Alternatively, **8a** was synthesised by refluxing **6a** with thiophenol in benzene.[30] Treatment of thioethers **8** with 3-chloroperbenzoic acid (MCPBA) yielded the

Table 1 Second-order rate constants, k_{OH^-}, for the hydroxide-catalyzed hydrolysis of 4-substituted azetidin-2-ones, **5**, at 25 °C and the kinetic parameters for the time-dependent inactivation of porcine pancreatic elastase (**5a–d**) and human leukocyte elastase (**5e–k**) in pH 7.2 buffer at 25 °C



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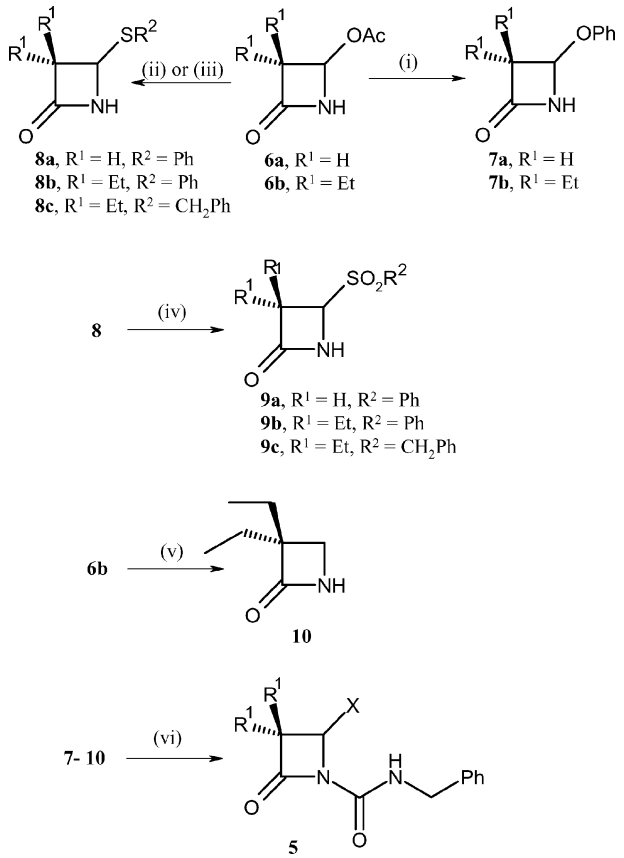
Compound	R	X	pK_a (XH)	(k_{inact}/K_i)/M^{-1} s^{-1}	$10^2\,k_{OH^-}$/M^{-1} s^{-1}	EREF[f]
5a	H	H	35[53]	NI[f]	13.0	—
5b	H	OPh	9.92[54]	11.5[a]	99.0	11.6
5c	H	SPh	6.52[54]	22.0[a]	44.1	50.0
5d	H	SO_2Ph	1.29[55]	290[a]	416	70.0
5e	Et	H	35[53]	310[b]	0.164	1.89×10^5
5f	Et	OPh	9.92[54]	2280[b]	0.722	3.15×10^5
5g	Et	SPh	6.52[54]	5760[b,c]	0.713	8.08×10^5
5h	Et	SO_2Ph	1.29[55]	15 200[b,d]	14.2	1.07×10^5
5i	Et	SCH_2Ph	9.27[54]	4464[b]	0.362	1.23×10^6
5j	Et	SO_2CH_2Ph	1.45[55]	11 741[b]	12.7	9.24×10^4
5k	Et	OC_6H_4–4-CO_2H	8.90[54]	8320[b,e]	ND[f]	ND[f]

[a] Against PPE. [b] Against HLE. [c] **5g**: $K_i = 0.633$ μM, $k_{inact} = 3.64 \times 10^{-3}$ s^{-1}. [d] **5h**: $K_i = 0.267$ μM, $k_{inact} = 4.06 \times 10^{-3}$ s^{-1}. [e] **5k**: $K_i = 0.454$ μM, $k_{inact} = 3.78 \times 10^{-3}$ s^{-1}. [f] NI: no inhibition, ND: not determined, EREF: enzyme rate enhancement factor.



Scheme 2 *Reagents and conditions*: (i) PhOH, NaOH, acetone; (ii) R^2SH, NaOH, acetone; (iii) PhSH (2 mol equiv.), C_6H_6, reflux; (iv) MCPBA, DCM; (v) $NaBH_4$, EtOH; (vi) $PhCH_2NCO$, TEA, DCM.

corresponding sulfones **9** (Scheme 2). The C-4 unsubstituted intermediate **10** was synthesised by reduction of **6b** with $NaBH_4$ in ethanol at 0 °C.[31] Finally, reaction of intermediates **7–10** with benzyl isocyanate gave the desired compounds **5** in good yield.

2. Enzyme inhibition studies

The series of β-lactams, **5a–d**, lacking substituents at the C-3 position was prepared to assess the impact of a leaving group at C-4 on porcine pancreatic elastase (PPE) inhibition when interaction with the S_1 primary recognition site[32] of elastase is reduced. PPE is a readily available elastase that shares a conserved catalytic triad consisting of Ser195, His57 and Asp102 with HLE.[33] The primary specificity pocket of PPE is slightly less hydrophobic and smaller than that of HLE, showing preference for small aliphatic side chains such as alanine.[33] The kinetic studies carried out at pH 7.2 and 25 °C, using the incubation method,[34] showed that β-lactams **5b–d** containing leaving groups are time-dependent inhibitors of PPE, while the C-4-unsubstituted counterpart **5a** is inactive up to a concentration of 1 mM. The second-order rate constants for PPE inactivation, k_{inact}/K_i (Table 1) were determined from the plots of pseudo-first-order inhibition rate constant, k_{obs}, *versus* [I] (Fig. 1). The sulfone derivative **5d** proved to be a potent inhibitor of PPE, with a k_{inact}/K_i, value of 290 M^{-1} s^{-1}, thus suggesting that the sulfone group is a very powerful activator of the β-lactam carbonyl carbon atom towards the Ser195 hydroxyl group, even in

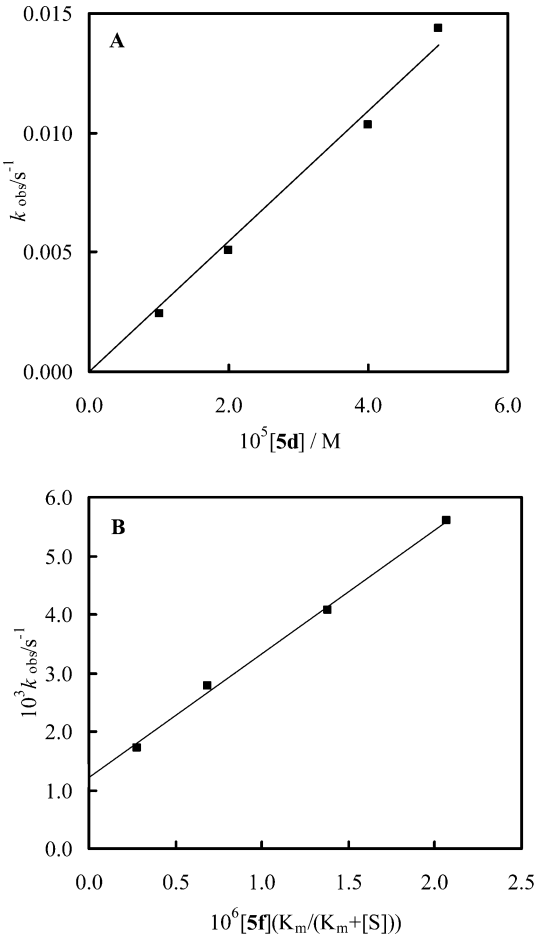


Fig. 1 Dependence of k_{obs} for enzyme inhibition on inhibitor concentration: (A), **5d**, porcine pancreatic elastase; (B), **5f**, human leucocyte elastase.

the absence of an adequate molecular recognition moiety at C-3 in the β-lactam scaffold.

Further evidence of the efficiency of **5d** as an irreversible inhibitor of PPE comes from the titration of enzyme activity. The number of equivalents required to inactivate PPE was calculated by plotting the fraction of remaining enzyme activity, v/v_0, after a 30 min incubation period *versus* the initial ratio of inhibitor to enzyme, *i.e.* $[I]/[E]_0$. For levels of inhibition of up to 90% of the original enzyme activity, the extent of inactivation was found to depend linearly on the inhibitor-to-enzyme molar ratio (Fig. 2), and extrapolation of the line to $v/v_0 = 0$ shows that it required approximately 1.2 equiv. of **5d** to completely inactivate PPE. Interestingly, such high inhibitory efficiency is similar to that reported for HLE inactivation by 3,3-diethylmonobactams and cephalosporins.[33,35]

In contrast to the C-3-unsubstituted series, their 3,3-diethyl counterparts **5e–j** were completely inactive against PPE in concentrations up to 0.5 mM. However, β-lactams **5e–j** inhibited HLE very efficiently in a time-dependent fashion. The pseudo-first-order rate-constants, k_{obs}, for HLE inactivation were determined using the progress-curve method.[36] For compound **5f**, a linear dependence of k_{obs} on inhibitor concentration was observed (Fig. 1B); and correction for the concentration and Michaelis constant of the substrate yielded the second-order rate constant for

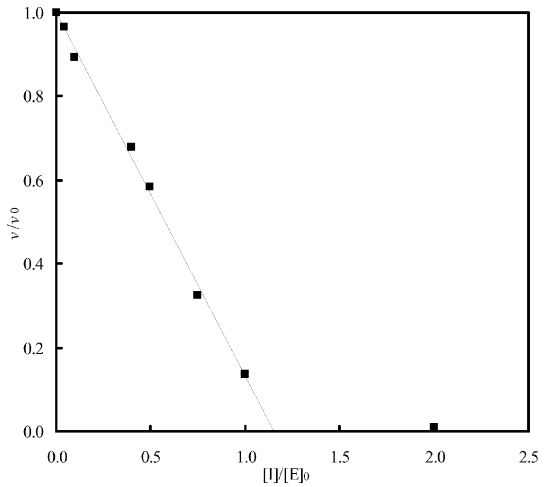
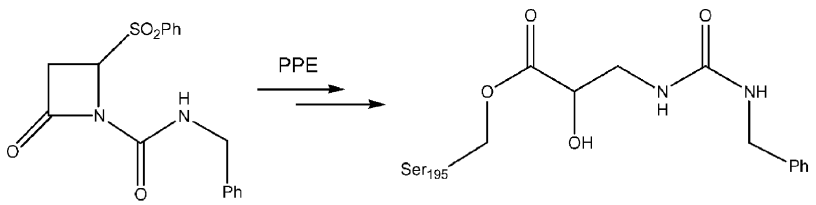


Fig. 2 Inactivation of porcine pancreatic elastase as a function of the molar ratio of inhibitor **5d** to enzyme. PPE (10 μM) and various amounts of inhibitor **5d** (20–0.4 μM) in 0.1 M HEPES buffer, pH 7.25, were incubated at 25 °C for 30 min, and aliquots were withdrawn for assay.

inhibition, k_{inact}/K_i, as the slope (Table 1). For compounds **5g** and **5h**, the individual kinetic parameters K_i and k_{inact} were obtained by determining k_{obs} as a function of the inhibitor concentration,[28] while for the remaining β-lactams (**5e**, **5i** and **5j**) the k_{inact}/K_i values were determined by calculating k_{obs}/[I] (Table 1). The second-order rate-constants for HLE inactivation, k_{inact}/K_i, are within the range of 10^3 to 10^4 M^{-1} s^{-1}, which reflects the stringent S_1 subsite specificity of this enzyme toward hydrophobic substituents with three or four carbon atoms.[25,31,33,37]

3. X-Ray crystallography studies

PPE was incubated with **5d** for 30 min. Good quality crystals were grown and subjected to X-ray analysis.[38] The electron density maps at the catalytic site indicate that PPE inactivation by **5d** involves β-lactam ring-opening, as revealed by the ester formed with Ser195 Oγ (Fig. 3). Moreover, inspection of the maps calculated at 1.66 Å resolution shows that acylation of PPE by **5d** also involves the departure of sulfinate from C-4. Interestingly, the crystal structure reveals the presence of a hydroxyl group at C-3 (β-lactam numbering) (Scheme 3). This hydroxyl group establishes H-bonds to water molecules while the ester carbonyl group is H-bonded to the NH backbone of Gln192. Furthermore, the adjacent urea NH groups are within H-bonding distance to water molecules, as shown in Fig. 3. This was a somewhat unexpected result when compared with those from previous X-ray crystallographic studies with other β-lactams containing leaving groups at C-4. For example, inactivation of PPE by 4-aryloxy-3,3-diethyl-β-lactams leads to a carbinolamine acyl-enzyme, resulting



Scheme 3 Covalent enzyme–inhibitor complex (see Fig. 3).

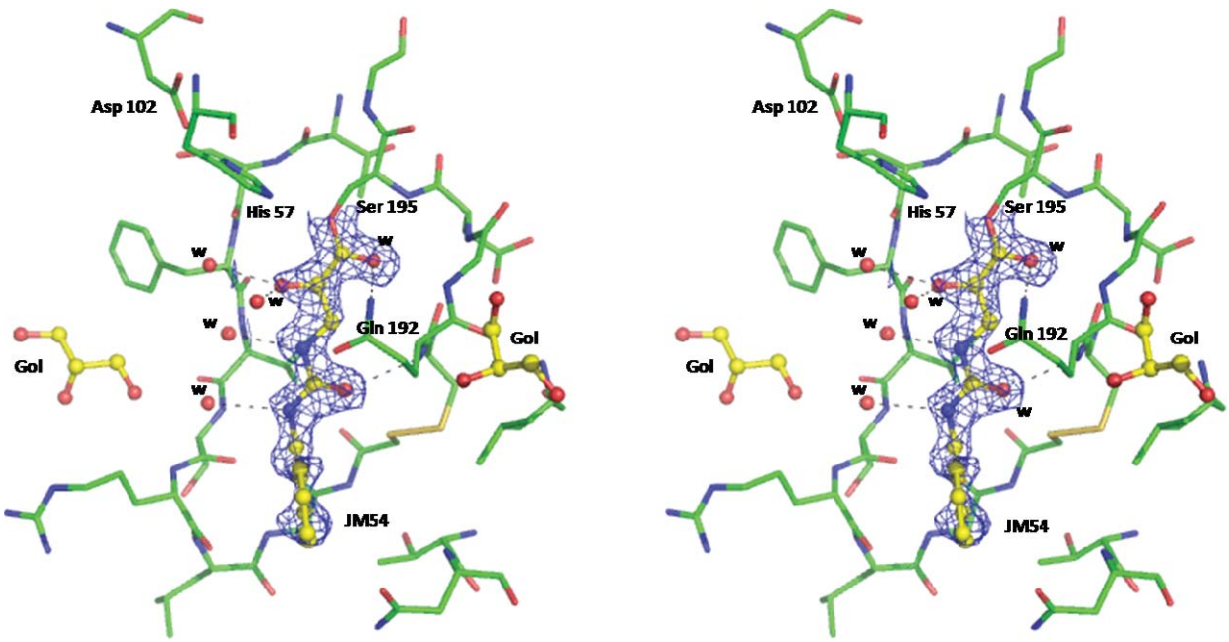
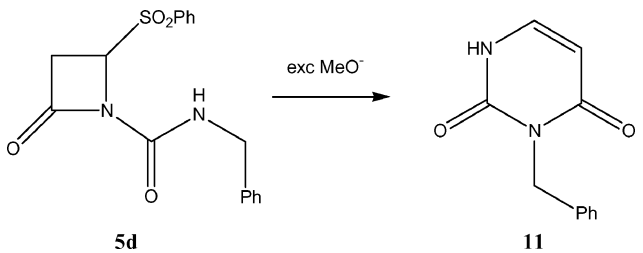


Fig. 3 A stereoview of the $|F_0| - |F_c|$ electron density map calculated, with Ser195 and inhibitor **5d** (code JM54) omitted from the model. The omit map is contoured at 3σ and shows the acyl-enzyme covalently linked to Ser195 with the corresponding interactions with PPE active site.

from reaction of water at C-4 of the imine intermediate.[26] When a single 1-hydroxyethyl substituent is present at C-3, an enamine acyl-enzyme is formed, presumably from a retro-aldol reaction on the imine precursor.[26]

We wondered if this pattern of reactivity was inherent to **5d** or a function of the enzyme active site. Therefore we undertook reaction of inhibitor **5d** with sodium methoxide in methanol, which has been reported as a good model reaction for the acylation of serine enzymes.[39,40] The product of this reaction was 3-benzylpyrimidine-2,4(1*H*,3*H*)-dione, **11**, in 80% yield (Scheme 4). This result is consistent with MeO^--catalyzed β-lactam ring opening followed by pyrimidine ring formation and phenylsulfinate elimination (or first the elimination then the ring closure). Clearly, the enzyme active site must preclude the ring closure process; moreover, the imine resulting from Ser195 acylation and phenylsulfinate elimination would be expected to add water to C-4 (β-lactam numbering) rather than to the C-3 atom that is evident in Fig. 3, thus suggesting that a different pathway may be available in the active site of PPE subsequent to β-lactam ring-opening. Although the nature of such a pathway is currently unclear, it is unlikely to be the rate-limiting step in the enzyme inactivation process (see below). In this regard, it is of interest to note that the distance between C-4 (β-lactam numbering) of the PPE–**5d** complex and the C-α of Gln192 is only 3.4 Å; this close approach might suggest a reason as to why the presence of a hydroxyl group in the C-4 position is precluded.

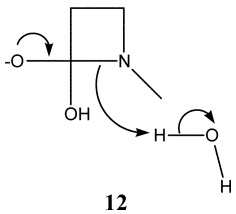


Scheme 4

4. Alkaline hydrolysis

It has been suggested that the magnitude of the second-order rate-constant, k_{OH^-}, for the alkaline hydrolysis of potential inhibitors of enzymes containing a catalytic serine is a crude indicator for their ability to be effective and therapeutically useful acylating agents.[39,41,42] Comparison of the k_{OH^-} values presented in Table 1 reveals that the reactivity of β-lactams **5** correlates poorly to the pK_a of the leaving group at C-4 of the β-lactam moiety. A poor correlation between log k_{OH^-} values for the 3,3-diethyl series **5e–j** and the pK_a of the leaving group at C-4 was observed, corresponding to a β_{lg} value of −0.05, indicating that there is essentially no change in the effective charge on the leaving group on going from the ground state to the transition state. These results are comparable to those reported for the alkaline hydrolysis of the vinylogous cephalosporins containing a potential leaving group at C-3′ (**4**, $n = 0$), for which a β_{lg} value close to zero was also determined.[43]

In contrast, log k_{OH^-} values correlate with σ_I values for the substituents at C-4, including those that are not expelled, yielding Hammett ρ_I values of 2.8 and 3.4 for the series **5a–d** and **5e–j**, respectively (Fig. 4). The Hammett ρ_I values are large and positive, which indicates a substantial build-up of negative charge in the transition-state and that the effect exerted by C-4 substituents on the alkaline hydrolysis of **5** is purely inductive. The solvolysis of penicillins and cephalosporins involves decomposition of the tetrahedral intermediate *via* general-acid-catalysed N-protonation, *e.g.* **12**, or complexation with a metal ion, to effect C–N bond cleavage.[44] For compounds **5**, N-protonation is thermodynamically unfavourable. However, ureido anion expulsion from the tetrahedral intermediate may occur at a rate that is comparable or even faster than that for hydroxide ion, and thus the reaction may involve the rate-limiting attack of hydroxide on the β-lactam carbonyl atom.



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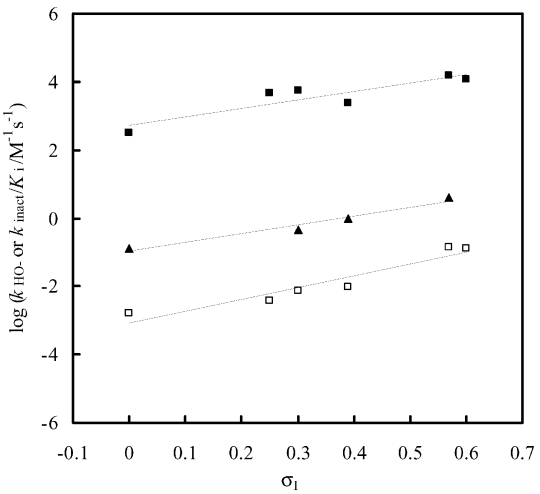

Fig. 4 Plots of second-order rate-constants for the alkaline hydrolysis of C-3-unsubstituted β-lactams **5a–d** (▲) and 3,3-diethyl-β-lactams **5e–j** (□), and of second-order rate-constants for HLE inactivation by 3,3-diethyl-β-lactams **5e–j** (■), against σ_I values for the X substituents in C-4.

The higher ρ_I value determined for the 3,3-diethyl series when compared to that of the C-3 unsubstituted series is consistent with the formation of a high energy tetrahedral intermediate in which the two C–O bonds are eclipsed with the ethyl groups at C-3. According to the Hammond postulate, this implies that formation of such an intermediate involves a later transition state in which there is significant negative charge build-up, thereby being more susceptible to the electronic effects of the C-4 substituents. The ρ_I value determined here for the monobactams **5** is higher than the corresponding ρ_I value of 1.35 reported for the alkaline hydrolysis of cephalosporins,[44] a difference that almost certainly reflects the shorter distance between the electron-withdrawing substituents and the nitrogen atom in the β-lactam scaffold of **5**.

5. Structure–activity relationships

Insight into how a particular substituent promotes the ability of the enzyme to use its catalytic apparatus to increase the rate of acylation through β-lactam ring-opening can be obtained by the enzyme rate enhancement factor (EREF); this is the ratio of second-order rate-constant for enzyme inactivation to the second-order rate-constant for the alkaline hydrolysis, *i.e.* $(k_{inact}/K_i)/k_{OH^-}$.[39,45] Remarkably, the 3,3-diethyl β-lactams **5e–j** exhibit EREF values *ca.* 10^4 times higher than those of their C-3 unsubstituted analogues **5b–d** (Table 1). The differences in EREF between the two series of β-lactams largely reflect the importance of the alkyl substituents at C-3 in promoting efficient molecular recognition by the S_1 site of the enzyme. This interaction probably positions the β-lactam carbonyl within the oxyanion hole, facilitating successful nucleophilic attack by Ser195, and thus enhancing the rate of β-lactam ring-opening.[31] Clearly, the favourable binding energy between the enzyme and the C-3 substituents overcomes the strain energy in the tetrahedral intermediate *en route* to the acyl-enzyme.

As with alkaline hydrolysis, enzyme inactivation efficiency is largely independent on the pK_a of the leaving group at C-4. For example, the second-order rate constant for PPE inactivation by the 4-phenylsulfonyl derivative **5d** is only 25 times higher than that of its 4-phenoxy analogue **5b**, despite the 9-fold difference between the leaving group abilities of phenylsulfinate and phenolate. Similarly, the 3,3-diethyl-4-phenylsulfonyl counterpart **5h** is only 6 times more potent as HLE inhibitor than its 4-phenoxy analogue **5f**. However, a good correlation was found between the logarithm of the second-order rate-constants of HLE inactivation and the σ_I values for the substituents at C-4, including X = H (**5e**), which corresponds to a ρ_I value of 2.5 for compounds **5e–j** (Fig. 4). This ρ_I value is higher than that reported for HLE inactivation by cephalosporin sulfones (**4**, R = MeO, $n = 2$; $\rho_I = 1.83$[20]), again reflecting the shorter distance between the substituents and the nitrogen atom in the β-lactam scaffold **5**. Compared with the alkaline hydrolysis of **5** (ρ_I of 3.4), the HLE reaction seems to involve an earlier transition-state with less negative charge buildup. This effect can be ascribed, at least in part, to the favourable non-covalent interactions with the primary specificity pocket, which allows the enzyme to stabilize the transition-state and to make full use of its catalytic machinery to increase the rate of acylation through β-lactam ring-opening. This is consistent with the EREF values for **5e–j** ranging from 10^5 to 10^6, which indicate that HLE is facilitating the expulsion of the urea anion from the tetrahedral intermediate when compared to the hydroxide-induced hydrolysis.

In conclusion, the effectiveness of monobactams as elastase inhibitors is strongly dependent on the effect of C-3 substituents on the molecular recognition by the enzyme as well as on the effect of C-4 substituents on chemical reactivity that leads to serine acylation. The rate-limiting step in elastase inactivation is the formation of the tetrahedral intermediate, and β-lactam ring-opening is not concerted with the departure of a leaving group from C-4. Monobactam sulfones emerged as very potent inhibitors of elastase due to the strong electron-withdrawing properties of the sulfone, although the departure of sulfinate from C-4 might be relevant to the chemistry of enzyme inactivation.

Experimental

General

Melting points were determined using a Kofler camera Bock Monoscope M and are uncorrected. The infrared spectra were collected on a Nicolet Impact 400 FTIR infrared spectrophotometer and the NMR spectra on a Bruker 400 Ultra-Shield (400 MHz) in $CDCl_3$; chemical shifts, δ, are expressed in ppm, coupling constants, J, are expressed in Hz. Low-resolution mass spectra were recorded using VG Mass Lab 20–250, VG Quattro or HP5988A mass spectrometers. Elemental analyses were performed by Medac Ltd, Brunel Science Centre, Englefield Green, Egham, TW20 0JZ, UK, or by ITN, Chemistry Unit, Sacavém, Portugal. UV-vis assays were recorded either on Shimadzu UV-1603 or UV-2100 PC spectrophotometers. TLC was performed on Merck aluminium plates, silica gel 60 F_{254}, and visualized by UV light and/or iodine. Preparative column chromatography was performed on silica gel 60 from Merck (70–230 mesh ASTM). DCM, TEA and benzene were purified and dried before use. Solvents and buffer materials for enzyme assays were of analytical reagent grade and were purchased from Merck or Sigma. PPE, MeO-Suc-Ala-Ala-Pro-Val-*p*NA and *N*-Suc-Ala-Ala-Ala-*p*NA were purchased from Sigma, and HLE was purchased from Calbiochem.

General procedure for the synthesis of *N*-carbamoylazetidin-2-one derivatives

Benzyl isocyanate (4.2 mmol) and subsequently triethylamine (4.2 mmol) were slowly added to a solution of the appropriate azetidin-2-one **7–10** (3.5 mmol) in dichloromethane (3 mL), The reaction was stirred at room temperature and monitored by TLC. After completion of the reaction, the solution was evaporated under reduced pressure. The product was purified by column chromatography.

***N*-Benzylcarbamoylazetidin-2-one, 5a.** Purified by column chromatography on silica gel (dichloromethane–ethyl acetate 9.5 : 0.5); 66%; m.p. 62–64 °C (lit.,[46] oil); ν_{max} (film) 3335, 1764, 1685 cm^{-1}; δ_H 3.08 (2H, t, $J = 4.8$); 3.68 (2H, t, $J = 4.8$); 4.50 (2H, d, $J = 6.0$); 6.89 (1H, brs); 7.28–7.38 (5H, m); δ_C 36.06, 37.21, 43.70, 127.59, 127.64, 128.72, 137.93, 150.59, 167.00; ESI-MS m/z 205.37 (MH^+); Anal. calcd. for $C_{11}H_{12}N_2O_2$: C, 64.69; H, 5.92; N, 13.72; found: C, 64.55; H, 6.10; N, 13.66.

***N*-Benzylcarbamoyl-4-phenoxyazetidin-2-one, 5b.** Purified by column chromatography on silica gel (elution with dichloromethane–ethyl acetate 9.5 : 0.5); 85%; m.p. 129–130 °C; ν_{max} (film) 3368, 1780, 1708 cm^{-1}; δ_H 3.17 (1H, dd, $J =$ 16.0, 1.2); 3.46 (1H, dd, $J = 16.0$, 4.4); 4.50 (1H, dd, $J = 14.4$, 5.6); 4.55 (1H, dd, $J = 14.4$, 5.6); 6.07 (1H, dd, $J = 4.4$, 1.2); 6.88 (1H, brs); 7.10 (1H, t, $J = 7.2$); 7.15 (2H, d, $J = 8.0$); 7.31–7.39 (7H, m); δ_C 43.76, 45.51, 78.76, 117.14, 123.21, 127.00, 128.78, 129.72, 137.56, 156.14, 164.42; ESI-MS m/z 297.52 (MH^+); Anal. calcd. for $C_{17}H_{16}N_2O_3$: C, 68.91, H, 5.44, N, 9.45; found, C, 69.12, H, 5.61, N, 9.19.

***N*-Benzylcarbamoyl-4-phenylthioazetidin-2-one, 5c.** Purified by column chromatography on silica gel (elution with dichloromethane–ethyl acetate 9.5 : 0.5); 80%; m.p. 78–79 °C, ν_{max}

(film) 3366, 3036, 2908, 1773, 1698 cm^{-1}; δ_H 2.88 (1H, dd, J = 16.4, 2.8); 3.40 (1H, dd, J = 16.4, 5.6); 4.47 (1H, dd, J = 15.0, 6.0); 4.54 (1H, dd, J = 15.0, 6.0); 5.29 (1H, dd, J = 5.6, 2.8), 6.81 (1H, br*s*); 7.30–7.39 (9H, m); 7.55 (1H, dd, J = 8.4, 1.6); δ_C 43.68, 43.99; 56.69; 127.69, 127.72, 128.77, 129.29, 129.31, 129.36, 135.25, 137.83, 149.63, 165.43; ESI-MS m/z 334.84 (MNa^+); Anal. calcd. for $C_{17}H_{16}N_2O_2S$: C, 65.36; H, 5.16; N, 8.97; found C 65.12; H 5.11; N 8.98.

***N*-Benzylcarbamoyl-4-phenylsulfonylazetidin-2-one, 5d.** Purified by column chromatography on silica gel (elution with dichloromethane–ethyl acetate 9.5 : 0.5); 81%; m.p. 159–161 °C, ν_{max} (film) 3374, 3064, 3031, 2978, 1789, 1711, 1316, 1150 cm^{-1}; δ_H 3.48 (1H, dd, J = 16.8, 6.0); 3.67 (1H, dd, J = 16.8, 2.8); 4.33 (1H, dd, J = 14.8, 6.0); 4.39 (1H, dd, J = 14.8, 6.0); 5.20 (1H, dd, J = 6.0, 2.8), 6.65 (1H, brs); 7.23 (2H, dd, J = 8.0, 1.2); 7.31–7.38 (3H, m); 7.58 (2H, t, J = 8.0); 7.74 (1H, dt, J = 8.0, 1.2); 7.96 (2H, dd, J = 8.0, 0.8); δ_C 39.43, 43.86, 65.84, 127.58, 127.79, 128.77, 129.28, 129.39, 134.88, 136.75, 137.28, 148.39, 164.27; EI-MS m/z: 344.00 (M^+); Anal. calcd. for $C_{17}H_{16}N_2O_4S$: C, 59.29; H, 4.68; N, 8.13; found C, 59.41; H, 4.55; N, 8.01.

***N*-Benzylcarbamoyl-3,3-diethylazetidin-2-one, 5e.** Purified by column chromatography on silica gel (elution with dichloromethane–ethyl acetate 9.5 : 0.5); 83% as a colourless oil, ν_{max} (film) 3364, 3071, 3031, 2969, 2924, 2875, 1757, 1702 cm^{-1}; δ_H 1.00 (6H, t, J = 7.6); (4H, q, J = 7.6); 3.42 (2H, s); 4.50 (2H, d, J = 6.0); 6.93 (1H, brs); 7.28–7.38 (5H, m); δ_C 8.70, 25.71, 43.75, 47.04, 59.31, 127.44, 127.54, 127.65, 128.65,128.71, 137.98, 150.95, 172.83; ESI-MS m/z 261.10 (MH^+); Anal. calcd. for $C_{15}H_{20}N_2O_2$: C, 69.20, H, 7.74, N, 10.76; found C, 68.95; H, 7.80; N, 10.59.

***N*-Benzylcarbamoyl-3,3-diethyl-4-phenoxyazetidin-2-one, 5f[25].** Purified by column chromatography on silica gel (elution with dichloromethane–ethyl acetate 9.5 : 0.5); 81%; m.p. 58–59 °C; ν_{max} (film) 3366, 3064, 3031, 2970, 2939, 2880, 1770, 1710 cm^{-1}; δ_H 1.00 (3H, t, J = 7.6 Hz); 1.06 (3H, t, J = 7.6); 1.77–1.84 (3H, m); 1.99 (1 H, dq, J = 14.4, 7.2); 4.47 (1H, dd, J = 15.6, 6.0); 4.51 (1H, dd, J = 15.6, 6.0); 5.68 (1H, s); 6.93 (1H, brs); 7.07 (1H, t, J = 7.2); 7.22–7.35 (9H, m); δ_C 8.58, 8.84, 21.23, 23.86, 43.75, 64.35, 86.76, 117.76, 123.17, 127.63, 127.67, 128.76, 129.67, 137.66, 150.14, 157.47, 172.23; ESI-MS m/z 353.29 (MH^+); Anal. calcd. for $C_{21}H_{24}N_2O_3$: C, 71.57, H, 6.86, N, 7.95; found 71.84, 6.78, 8.18.

***N*-Benzylcarbamoyl-3,3-diethyl-4-phenylthioazetidin-2-one, 5g.** Purified by column chromatography on silica gel (elution with dichloromethane–ethyl acetate 9.5 : 0.5); 70%; m.p. 80–81 °C; ν_{max} (film) 3361, 3061, 2968, 1758, 1701 cm^{-1}; δ_H 0.94 (3H, t, J = 8.0 Hz); 1.06 (3H, t, J = 8.0); 1.75–1.98 (3H, m); 1.99 (1 H, dq, J = 14.4, 7.2); 4.51 (1H, dd, J = 15.6, 6.0); 4.55 (1H, dd, J = 15.6, 6.0); 5.06 (1H, s); 6.95 (1H, brs); 7.28–7.39 (8H, m); 7.77 (2H, d, J = 8.0); δ_C 8.43, 9.01, 23.11, 24.74, 43.74, 63.95, 71.42, 127.60, 127.65, 128.17, 128.74, 129.19, 133.24, 137.71, 150.13, 172.04; EI-MS m/z 368.15 (M^+); Anal. calcd. for $C_{21}H_{24}N_2O_2S$: C, 68.45, H, 6.56, N, 7.60; found, C, 68.22, H, 6.65, N, 7.88.

***N*-Benzylcarbamoyl-3,3-diethyl-4-phenylsulfonylazetidin-2-one, 5h.** Purified by column chromatography on silica gel (elution with dichloromethane–ethyl acetate 9.5 : 0.5); 63%; m.p. 142–144 °C; ν_{max} (film) 3366, 3060, 3041, 2971, 1778, 1711, 1312, 1151 cm^{-1}; δ_H 1.01 (3H, t, J = 7.2 Hz); 1.06 (3H, t, J = 7.2); 1.68 (1 H, dq, J = 14.4, 7.2); 1.95 (1 H, dq, J = 14.4, 7.2); 2.20 (1 H, dq, J = 14.4, 7.2); 2.47 (1 H, dq, J = 14.4, 7.2); 4.21 (1H, dd, J = 14.8, 6.0); 4.33 (1H, dd, J = 14.8, 6.0); 4.75 (1H, s); 6.66 (1H, brs); 7.14 (2H, d, J = 7.6); 7.19–7.28 (3H, m); 7.47 (2H, t, J = 7.6); 7.61 (1H, t, J = 7.6); 7.85 (2H, t, J = 7.6); δ_C 8.56, 8.90, 20.86, 25.12, 43.85, 67.05, 74.72, 127.43, 127.69, 128.74, 128.88, 129.18, 134.39, 137.36, 139.84, 148.99, 171.49; EI-MS m/z 400.05 (M^+); Anal. calcd. for $C_{21}H_{24}N_2O_4S$: C, 62.98, H, 6.04, N, 6.99; found, C, 63.11, H, 6.25, N, 7.12.

***N*-Benzylcarbamoyl-3,3-diethyl-4-benzylthioazetidin-2-one, 5i.** Purified by column chromatography on silica gel (elution with dichloromethane–ethyl acetate 9.5 : 0.5); 53% as a yellow oil, ν_{max} (film) 3365, 1759, 1701 cm^{-1}; δ_H 0.56 (3H, t, J = 7.4); 0.85 (3H, t, J = 7.4); 1.44–1.69 (4H, m); 3.99 (1H, d, J = 12.8); 4.06 (1H, d, J = 12.8); 4.31 (1H, dd, J = 15.3, 6.1); 4.36 (1H, dd, J = 15.3, 6.3); 4.70 (1H, s); 6.91 (1H, s); 7.10–7.27 (10H, m); δ_C 7.67; 8.90; 22.41; 24.44; 37.37; 43.61; 62.99; 65.84; 127.63; 127.72; 127.81; 129.01; 129.07; 129.80; 138.79; 139.62;150.96; 172.35; HPLC-ESIMS m/z (MH^+) 383.52; Anal. calcd. for $C_{22}H_{26}N_2O_2S$; C, 69.08; H, 6.85; N, 7.32; found C, 69.10, H, 6.90; N, 7.30.

***N*-Benzylcarbamoyl-3,3-diethyl-4-benzylsulfonylazetidin-2-one, 5j.** Purified by column chromatography on silica gel (elution with diethyl ether–light petroleum 1 : 1; 52%; m.p. 93–94 °C; ν_{max} (film) 3365, 1778, 1313, 1165 cm^{-1}; δ_H 0.79 (3H, t, J = 7.4); 1.01 (3H, t, J = 7.5); 1.62 (1 H, dq, J = 14.5, 7.3); 1.84 (1H, dd, J = 14.5, 7.3); 2.08 (1H, dq, J = 14.5, 7.4); 2.52 (1H, dq, J = 14.6, 7.4); 4.48 (1H, d, J = 13.9); 4.53 (2H, d, J = 5.9); 4.62 (1H, s); 5.01 (1H, d, J = 13.9); 6.95 (1H, t, J = 5.9); 7.19–7.59 (10H, m); δ_C 8.27, 8.87, 20.90, 24.48, 44.12, 62.27, 66.46, 69.06, 127.59, 127.89, 128.91, 129.07, 131.37, 137.08, 150.20, 171.22; EI-MS m/z 413.8 (MH^+); Anal. calcd. for $C_{22}H_{26}N_2O_4S$; C, 63.75; H, 6.32; N, 6.76; found C, 63.70, H, 6.40; N, 6.60.

Large scale reaction of *N*-benzylcarbamoyl-4-phenylsulfonylazetidin-2-one with excess sodium methoxide

N-Benzylcarbamoyl-4-phenylsulfonylazetidin-2-one (0.29 mmol), **5d**, was added to a solution of sodium methoxide (1.5 mmol) in methanol (15 mL). The reaction mixture was stirred at room temperature and monitored by TLC. The solvent was removed under reduced pressure and the residue was taken up in water (20 mL), acidified with 10% HCl until pH 2 and extracted with ethyl acetate (3 × 30 mL). After drying and evaporating off the solvent, the residue was purified by column chromatography on silica gel (elution with dichloromethane–ethyl acetate 8 : 2), to yield the product, 3-benzylpyrimidine-2,4(1*H*,3*H*)-dione, **11**, as a white solid (79%); m.p. 177–179 °C (lit.,[47] 181–182 °C); ν_{max} (film) 3084, 2965, 1625, 1601 cm^{-1}; δ_H 5.12 (2H, s); 5.81 (1H, dd, J = 7.2, 1.2); 7.14 (1H, dd, J = 7.2, 6.0); 7.26–7.34 (3H, m); 7.46 (2H, d, J = 7.2); 9.35 (1H, brs); δ_C 43.71, 102.26, 127.70, 128.46,128.80, 136.49, 138.17, 163.12; EI-MS m/z 202.05 (M^+); Anal. calcd. for $C_{11}H_{10}N_2O_2$, C, 65.34; H, 4.98; N, 13.85; found, C, 65.55, H, 5.25, N, 13.81.

Chemical kinetics

All kinetic measurements were carried out at 25.0 ± 0.1 °C and with an ionic strength adjusted to 0.5 M by addition of $NaClO_4$. Due to substrate solubility problems all buffers contained 20% (v/v) acetonitrile. Rate constants were determined using UV spectrophotometry by recording the decrease of substrate absorbance at fixed wavelength (**5a**, 220; **5b**, 250; **5c**, 260; **5d**, 255; **5e**, 224; **5f**, 238; **5g**, 260; **5h**, 225; **5i**, 240; **5j**, 240 nm), using a spectrophotometer equipped with a temperature controller. In a typical run, the reaction was initiated by adding a 15 μL aliquot of a 10^{-2} M stock solution of substrate in acetonitrile to a cuvette containing 3 mL of the buffer solution. The pseudo-first-order rate constants, k_{obs}, were obtained by least-squares treatment of $\log(A_t - A_\infty)$ data, where A_t and A_∞ represent the absorbance at time t and at time infinity, respectively. Rate constants derived using this method were reproducible to ±5%.

Enzyme inactivation by the progress curve method

Inactivation of HLE was assayed at 25 °C by mixing 10 μL of HLE stock solution (2 μM in 0.05 M acetate buffer, pH 5.5) to a solution containing 10 μL of inhibitor in DMSO (200 μM), 20 μL of substrate MeO-Suc-Ala-Ala-Pro-Val-*p*NA (50 mM in DMSO) and 960 μL of 0.1 M HEPES buffer, pH 7.2, and the absorbance was continuously monitored at 410 nm for 20 minutes. Control assays, in which the inhibitor was omitted, ran linearly. The pseudo-first order rate constants, k_{obs}, for the inhibition of HLE were determined according to the slow-tight binding inhibition model[36] and involved the fitting of product concentration as a function of time to eqn (1) by non-linear regression analysis using the routine ENZFIT (developed at the Faculty of Pharmacy, Lisbon):

$$A = v_s t + \frac{(v_i - v_s)(1 - e^{-k_{obs}t})}{k_{obs}} + A_0 \quad (1)$$

where A is the absorbance at 410 nm, A_0 is the absorbance at $t = 0$, v_i is the initial rate of change of absorbance, v_s is the steady-state rate and k_{obs} is the first-order rate constant for the approach to the steady-state. The individual kinetic parameters K_i and k_{inact} were obtained by determining k_{obs} (in duplicate or triplicate) as a function of the inhibitor concentration and by fitting the experimental data to eqn (2).[25] When a linear dependence of k_{obs} on inhibitor concentration was observed (*e.g.* for **5f**), correction for substrate concentration and Michaelis constant yielded the second-order rate constant for inhibition (k_{inact}/K_i) as the slope, and the first-order rate for the dissociation of the E·I complex (k_{off}) as the intercept (eqn (3)) (see Fig. 1B). For the remaining β-lactams the k_{inact}/K_i values were determined in duplicate or triplicate by calculating $k_{obs}/[I]$ and then correcting for the substrate concentration and Michaelis constant using eqn (4).

$$k_{obs} = \frac{k_{inact}[I]}{K_i(1 + [S]/K_m) + [I]} \quad (2)$$

$$k_{obs} = k_{off} + \frac{(k_{inact}/K_i)[I]}{1 + [S]/K_m} \quad (3)$$

$$\frac{k_{obs}}{[I]} = \frac{k_{inact}/K_i}{1 + [S]/K_m} \quad (4)$$

Enzyme inactivation by the incubation method

Inhibition of PPE was assayed by Kitz and Wilson's incubation method.[34] In a typical experiment, 50 μL of inhibitor solution in DMSO was incubated at 25 °C with 750 μL of 0.1 M HEPES buffer, pH 7.2, and 200 μL of PPE solution (50 μM in 0.1 M HEPES buffer, pH 7.2). Aliquots (100 μL) were withdrawn at different time intervals and transferred to a cuvette thermostatted at 25 °C, containing 895 μL of 0.1 M HEPES buffer, pH 7.2, and 5 μL of *N*-Suc-Ala-Ala-Ala-*p*NA (12.5 mM in DMSO). The absorbance was monitored at 390 nm for 60 seconds and the gradients of the slopes obtained of initial rate used as a measure of enzyme activity. The values of k_{obs} for compounds **5b–d** were determined in duplicates or triplicates from plots of $\ln(v/v_0)$ *versus* incubation time, where v is the initial rate at time t and v_0 is the initial rate of the control incubation without inhibitor. The plots of k_{obs} *versus* [I] were linear and the potency of the inhibitors was determined in terms of the bimolecular rate constant $k_{inact}/K_i = k_{obs}/[I]$.

Partition ratio

PPE solutions were incubated at 25 °C with different concentrations of **5d** solutions in 0.1 M HEPES buffer, pH 7.2, in a final volume of 1 mL. After 30 minutes incubation, a 100 μL aliquot of the reaction mixture was withdrawn and assayed for remaining enzyme activity as described previously.

X-Ray crystallographic studies

Porcine pancreatic elastase (PPE) was incubated with inhibitor **5d** for 30 min. Good quality crystals were grown in 200 mM sodium sulfate and 100 mM sodium acetate at pH 5.1 (293 K) using the sitting drop vapour-diffusion method.[38] X-Ray diffraction data were measured at EMBL X11 beamline at the DORIS storage ring, DESY, Hamburg (Germany), to 1.66 Å resolution. The data were integrated with MOSFLM[48] and scaled using SCALA.[49] Relevant statistics on data collection and processing are given in Table S1 (Supplementary material†). The crystals soaked with the inhibitor solution belong to the same space group and show similar cell parameters as the native ones, so the structure was solved by Fourier synthesis. The initial rigid body refinement step yielded an *R*-work and *R*-free of 27.7 and 30.5%, respectively. Further crystallographic refinement was performed with Refmac[50] and the electron density maps were inspected with COOT.[51] The refined model of PPE in complex with inhibitor **5d** shows an *R*-factor of 15.6% and *R*-free of 18.6%.

The structure is generally well defined within the electron density maps, showing an average *B* factor of 10.5 $Å^2$ for all protein atoms. The final model comprises 240 amino acid residues, 316 water molecules, the inhibitor **5d** without the C-4 $PhSO_2^-$ leaving group, two glycerol molecules, one sulfate ion and one sodium ion, which is hexa-coordinated to side-chain atoms of Glu80, Asp77, Gln75, Asn72, Glu70 and a water molecule. Alternate conformations were modelled for the side chains of Gln23, Gln75, Val83 and Ser189, with 50% occupancy each. All protein residues lie within allowed regions of the Ramachandran plot, and the relevant refinement statistics are presented in Table 2. The overall protein structure of the **5d**–PPE complex is very similar to that of the native enzyme (PDB code 1QNJ) and superposition of the

Table 2 Data collection and crystallographic refinement statistics for the porcine pancreatic structure bound to **5d**

Resolution range/Å	37.95–1.66
Refined model	
R-factor (%)	15.6
R-free (%)	18.6
No. of non-H protein atoms	1833
No. of solvent molecules	320
Average *B*-factor/Å^2	
Protein only	10.5
Inhibitor only	24.4
Solvent molecules	25.9
Ramachandran plot	
Residues in most favoured regions (%)	87.4
Residues in additional allowed regions (%)	12.6
Root-mean-square deviations	
Bond angles/°	0.01
Angle lengths/Å	1.22

C-α atoms shows a r.m.s. deviation of 0.089 Å. The numbering of **5d**–PPE complex follows the common practice of using the bovine chymotrypsinogen A numbering.[52]

The coordinates of **5d**–PPE complex have been deposited in the Protein Data Bank (PDB) with identification code 2V35 and the corresponding structure factors with id code 2V35SF.

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