Reactivity and selectivity in the inhibition of elastase by 3-oxo-β-sultams and in their hydrolysis†

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3-Oxo-β-sultams are both β-sultams and β-lactams and are a novel class of time-dependent inhibitors of elastase. The inhibition involves formation of a covalent enzyme–inhibitor adduct with transient stability by acylation of the active-site serine resulting from substitution at the carbonyl centre of the 3-oxo-β-sultam, C–N fission, and expulsion of the sulfonamide. The lead compound, *N*-benzyl-4,4-dimethyl-3-oxo-β-sultam **1** is a reasonably potent inhibitor against porcine pancreatic elastase with a second-order rate constant of 768 M^{-1} s^{-1} at pH 6, but also possesses high chemical reactivity with a half-life for hydrolysis of only 6 mins at the same pH in water. Interestingly, the hydrolysis of 3-oxo-β-sultams occurs at the *sulfonyl* centre with S–N fission and expulsion of the amide leaving group, whereas the enzyme reaction occurs at the *acyl* centre. Increasing selectivity between these two reactive centres was explored by examining the effect of substituents on the reactivity of 3-oxo-β-sultam towards hydrolysis and enzyme inhibition. The inhibition activity against porcine pancreatic elastase has a much higher sensitivity to substituent variation than does the rate of alkaline hydrolysis. A difference of 2000-fold is observed in the second-order rate constants, k_i, for inhibition whereas there is only a 100-fold difference in the second-order rate constants, k_{OH}, for alkaline hydrolysis within the series. The higher sensitivity of enzyme inhibition to substituents than that of simple chemical reactivity indicates a significant degree of molecular recognition of the 3-oxo-β-sultams by the enzyme.

Introduction

Elastase is a proteolytic enzyme that catalyzes the hydrolysis of the fibrous protein elastin, which comprises an appreciable percentage of all protein content in tissues such as arteries, ligaments and the lung. Three distinct types of human elastase have been identified – human neutrophil elastase (HNE), pancreatic elastase II (PE-II) and macrophage metalloelastase (MME).[1] The imbalance between HNE and its endogenous inhibitors leads to excessive elastin proteolysis and destruction of connective tissues in a number of inflammatory diseases such as pulmonary emphysema, adult respiratory distress syndrome, chronic bronchitis, chronic obstructive pulmonary disease and rheumatoid arthritis.[1,2] Inhibition of human neutrophil elastase is therefore an important approach to the treatment of the disease, and the search for suitable inhibitors has been intense.[3] Human neutrophil elastase is a member of the chymotrypsin family of serine proteases and, as such, has a rich family of classical inhibitors.[4] However, there is a need for the design of new inhibitors as currently available drugs have shown only limited effect on the diseases.[3] β-Lactams are well-known as potent inhibitors of some serine enzymes, including the bacterial penicillin binding proteins (PBPs) and Class A and Class C β-lactamases.[5] β-Lactams have also been structurally modified to develop active site-directed and mechanism-based inhibitors of HNE.[4,6,7] We have been interested in developing mechanism-based inhibitors of HNE and the related, but more readily available, porcine pancreatic elastase (PPE). PPE is a model enzyme that shares *ca.* 40% homology and the catalytic triad consisting of Ser-195, His-57 and Asp-102 with HNE.[8] Among different classes of inhibitor of elastase, acylating agents such as β-lactams and *trans*-fused γ-lactones[9] that inactivate elastase by formation of an acyl–enzyme adduct appear to be one of the most promising areas for the development of successful therapeutic agents.[10] We have shown that β-sultams, the sulfonyl analogues of β-lactams, are a novel class of effective inhibitors of serine enzymes by sulfonylation of the active-site serine residue.[11,12] We have also shown that 3-oxo-β-sultams, which are both β-sultams and β-lactams, are a new class of acylating agents that inactivate the elastase from porcine pancreas.[13] The inhibition involves the formation of an enzyme–inhibitor adduct with transient stability by acylation of the active-site serine resulting from substitution at the carbonyl center of the 3-oxo-β-sultam. The lead compound, *N*-benzyl-4,4-dimethyl-3-oxo-β-sultam **1**, is a reasonably potent inhibitor against porcine pancreatic elastase with a second-order rate constant of 768 M^{-1} s^{-1} at pH 6. However, the 3-oxo-β-sultam is chemically too reactive for studies towards therapeutic application – the half-life for the degradation of the 3-oxo-β-sultam by hydrolysis is only 6 mins at pH 6 in water. By contrast, increasing the rate of serine acylation by enhancing the intrinsic chemical reactivity of the β-lactams has been used as a strategy to design more potent inhibitors.[14]

Nucleophilic attack on *N*-benzyl-4,4-dimethyl-3-oxo-β-sultam **1** could involve either acylation or sulfonylation resulting from

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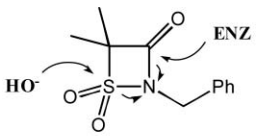
substitution at the carbonyl centre and expulsion of the sulfonamide or from substitution at the sulfonyl centre and expulsion of the amide, respectively. Interestingly, the hydrolysis of **1** and its enzyme reaction with elastase occur at different reaction centres; the enzyme reaction occurs at the *acyl* centre whereas the hydrolysis of 3-oxo-β-sultams occurs at the *sulfonyl* centre **1**.[13,15] Inhibition of elastase by **1** occurs by acylation of the active-site serine and so involves C–N fission and expulsion of sulfonamide.[13] By contrast, the alkaline hydrolysis of **1** occurs by hydroxide-ion attack on the sulfonyl centre with S–N fission.[15] This is a very rapid process and occurs with a second-order rate constant, k_{OH}, value of. *ca.* 2×10^5 M^{-1} s^{-1},[15] which is about 10^6 times greater than those of most clinically useful β-lactams.[16] Highly reactive inhibitors may decrease the selectivity towards the target enzyme and reduce bioavailability.

Nucleophilic substitution at acyl centres generally proceeds through the formation of an unstable tetrahedral intermediate **2** and it is usually assumed that the incoming nucleophile approaches at approximately the tetrahedral angle to the carbonyl group.[17] By contrast, sulfonyl-transfer reaction involves a transition state or intermediate with pentacoordinate trigonal bipyramidal geometry **3** and the incoming nucleophile is assumed to take the apical position.[18,19] The two processes, acyl and sulfonyl transfer, are expected to show different sensitivity to stereochemical and geometrical properties of the α-substituents and it is of interest to investigate the balance between the chemical reactivity and enzymatic activity of 3-oxo-β-sultams by changing the α-substituents.



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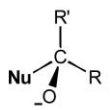

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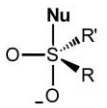

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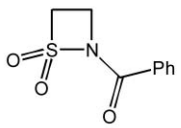

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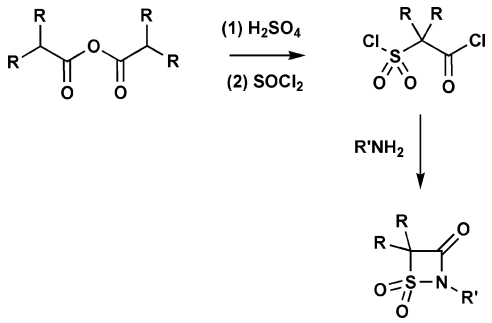
In addition to varying the α-substituents, 3-oxo-β-sultams with different nitrogen leaving groups have been synthesized in order to study the effect of the leaving group on the reactivity of these compounds as inhibitors of elastase. We now report that such modifications can retain inhibitory activity while decreasing dramatically the reactivity towards hydrolysis by hydroxide-ion.

Results and discussion

Chemistry

3-Oxo-β-sultams were prepared by a common strategy involving ring closure of the corresponding (chlorosulfonyl)acetyl chloride with a substituted amine (Scheme 1). The ring-closure step was monitored conveniently by using IR spectroscopy as the characteristic C=O IR stretching frequencies of the acyclic amide and 3-oxo-β-sultam were well-resolved and are 1690 and 1790 cm^{-1}, respectively. That the IR stretching frequency of the C=O double bond of 3-oxo-β-sultam is much higher than that of the acyclic amide and higher than the 1730–1760 cm^{-1} seen with monocyclic β-lactams indicates more C=O double bond character and possibly weaker amide resonance due to the competing electron withdrawal by the adjacent sulfonyl group.[20] Although reduced resonance in β-lactam amides in β-lactam antibiotics such as penicillins, has often been stated as the reason for their chemical reactivity and biological susceptibility, it is a fact that, contrary to expectation, the rate of alkaline hydrolysis of β-lactams is less than a hundred-fold greater than that of an analogous acyclic amide.[16,21] The second-order rate constant for the hydroxide-ion catalyzed hydrolysis of *N*-methyl β-lactam is only 3-fold greater than that for *N*,*N*-dimethyl acetamide.[21]



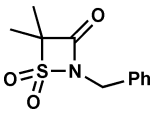
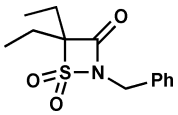
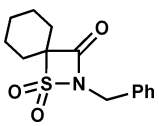
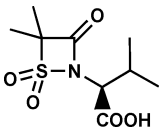
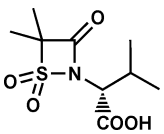
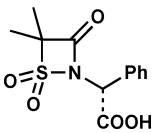
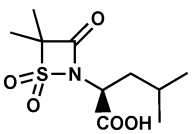
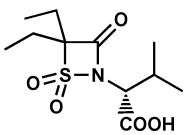
Scheme 1

The 3-oxo-β-sultam **1** is both a β-sultam and a β-lactam with a second-order rate constant for alkaline hydrolysis k_{OH} of 1.83×10^5 dm^3 mol^{-1} s^{-1} (Table 1) and hydroxide-ion attack occurs preferentially at the sulfonyl centre with expulsion of a carboxamide leaving group, as shown by product analysis.[15] That hydroxide-ion attacks the sulfonyl centre in **1** rather than the β-lactam carbonyl is consistent with the observation that β-sultams are 10^2 to 10^3-fold more reactive than β-lactams towards alkaline hydrolysis.[18] Although attack at the acyl centre may be expected to expel a better leaving group – the sulfonamide anion – the nature of the leaving group does not have a large effect on the relative rates of hydrolysis of imides and similar *N*-acyl sulfonamides.[15]

Increasing the steric bulk of the 4-substituent, α to the electrophilic sulfonyl centre, decreases the rate of alkaline hydrolysis (Table 1). For example, replacing the dimethyl groups by diethyl decreases the rate 10-fold and the spiro cyclohexyl group is 30-fold less reactive. 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.[22] In the elastase enzymes this binding pocket is relatively small and has a preference for small hydrophobic substituents.[22,23] The inhibition of elastase 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.[24,25] We therefore wished to investigate any differential effects of these substituents on chemical reactivity and enzyme binding.

A series of 3-oxo-β-sultams with *N*-α-carboxylate substituents were prepared and their rates of hydrolysis measured (Table 1). All of these compounds undergo hydrolysis by hydroxide-ion attack at the sulfonyl centre to give ring-opened products by S–N fission reaction. There is no evidence of intramolecular attack by the carboxylate oxyanion as the rate of the reaction

Table 1 The second-order rate constants for alkaline hydrolysis (k_{OH}) and the enzyme inhibition (k_i) of porcine pancreatic elastase (PPE) at pH 6.0 (0.1 M MES buffer) by 3-oxo-β-sultams in 1% acetonitrile, 1.0 M ionic strength at 30 °C

		$k_{OH}/M^{-1}\ s^{-1}$	$k_i/M^{-1}\ s^{-1}$	$k_i : k_{OH}$ ratio
1		1.83×10^5	768	64
5		1.86×10^4	275	227
6		5.99×10^3	5.85	15
7		3.89×10^4	2.54	1
8		Not determined	5.00	2
9		2.42×10^4	30.1	19
10		3.27×10^4	8.30	4
11		1.74×10^3	0.37	3

occurs at similar rates to those for 3-oxo-β-sultams lacking the α-carboxylate (Table 1). Introducing a carboxylate on the exocyclic carbon α to the nitrogen of the amine leaving group of **9** or **11** decreases the chemical reactivity of the 3-oxo-β-sultam by about 10-fold compared with **1** or **5**, respectively (Table 1). This decrease in chemical reactivity is of the magnitude expected due to the electrostatic repulsion between the incoming negatively charged nucleophile and the negative charge on the oxygens of the dissociated carboxylate.

Inhibition of elastase by 3-oxo-β-sultams

N-Benzyl-4,4-dimethyl-3-oxo-β-sultam **1** is a time-dependent inhibitor of porcine pancreatic elastase (PPE). The activity of PPE was monitored by measuring the rate of the hydrolysis of *N*-succinyl-(L-ala)$_3$-*p*-nitroanilide as substrate at 390 nm using a molar extinction coefficient change, $\Delta\varepsilon_{390}$, of $1.24 \times 10^4\ M^{-1}\ cm^{-1}$. The kinetic analysis was undertaken with the anilide substrate (0.08 mM) in 0.1 M pH 8.5 TAPS buffer at 30 °C in 1.5% methanol v/v and $I = 1.0$ M (KCl) in the assay cells. Inhibition was studied by incubating the *N*-benzyl-4,4-dimethyl-3-oxo-β-sultam **1** with PPE together in a buffered solution. Aliquots of the incubation solution containing enzyme and potential inhibitor were then removed after an incubation time and assayed for PPE activity against the anilide substrate in separate assay cells. 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 time. Incubation of the enzyme alone under exactly the same conditions but in the absence of β-sultam gave constant assay slopes throughout the experimental time frame. The enzyme activity at a given time was measured by the initial rate of hydrolysis of the substrate and expressed as a percentage of the initial slope relative to that for full enzyme activity measured in a control experiment in which there is no 3-oxo-β-sultam present. The decrease of enzyme activity against time in the inhibition of PPE with 2×10^{-5} and 4×10^{-5} M of *N*-benzyl-4,4-dimethyl-3-oxo-β-sultam **1** at pH 6.0 follows an exponential decay (Fig. 1). The concentration of the enzyme is much lower than that of the inhibitor at time zero and therefore the observed rate of inactivation is effectively pseudo-first order and gives the apparent first-order rate constant, k_{obs}. The values of k_{obs} show a first order dependence on the concentration of the 3-oxo-β-sultam indicative of the simple rate laws (eqn (1) and (2)) in which k_{obs} is the observed pseudo-first-order rate constant for inhibition in the presence of excess β-sultam, and k_i is the corresponding second-order rate constant for the inhibition. The second-order rate constant, k_i, was given by the slope of the linear plot of k_{obs} against the concentration of the 3-oxo-β-sultam (Fig. 2).

$$\text{Rate} = k_i[\text{enzyme}][\beta\text{-sultam}] \quad (1)$$

$$\frac{\text{Rate}}{[\text{enzyme}]} = k_{obs} = k_i[\beta\text{-sultam}] \quad (2)$$

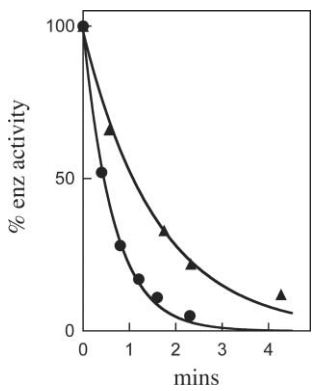


Fig. 1 The decrease of enzyme activity against time in the inhibition of PPE with 2×10^{-5} (▲) and 4×10^{-5} (●) M of *N*-benzyl-4,4-dimethyl-3-oxo-β-sultam **1** at pH 6.0, $I = 1$ M (KCl), 25 °C. The solid curves were the theoretical curve generated by fitting the data to a first-order decay equation.

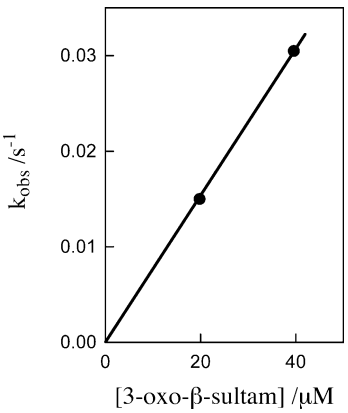


Fig. 2 Plot of observed first-order rate constant, k_{obs}, for the inhibition of PPE by *N*-benzyl-4,4-dimethyl-3-oxo-β-sultam **1** against the concentrations of the 3-oxo-β-sultam at pH 6.0, $I = 1$ M (KCl), 25 °C.

With some 3-oxo-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 normalized with respect to the decreasing β-sultam concentration using eqn (3) in which $[3\text{-oxo-β-sultam}]_t$ and $[3\text{-oxo-β-sultam}]_o$ are the concentration of the 3-oxo-β-sultam at time $= t$ and that applied in the reaction, respectively. The concentration of the 3-oxo-β-sultam at time $= t$ was determined spectrophotometrically in a separate experiment in which the hydrolysis of the 3-oxo-β-sultam in the same buffer as that in the inhibition experiment was followed.

$$= \text{observed enzyme activity} \times \frac{[3-\text{oxo}-\beta-\text{sultam}]_t}{[3-\text{oxo}-\beta-\text{sultam}]_o} \qquad (3)$$

(i) Acylation or sulfonylation? Inactivation of elastase by 3-oxo-β-sultams occurs with formation of a 1 : 1 complex. Analysis of solutions of PPE incubated with *N*-benzyl-4,4-dimethyl-3-oxo-β-sultam **1** in a 1 : 1 ratio by electrospray ionization-mass spectrometry (ESI-MS) shows the enzyme bound to one equivalent of β-sultam (M_{EI} 26 133 ± 1 Da) (98%). The native enzyme has a mass of 25 892 and therefore a mass difference of +241 is consistent with the formation of a covalent bond between the enzyme and β-sultam **1**. If inhibition was caused by the hydrolysis product binding to the enzyme then a mass of 26 148 would be expected. However, ESI-MS does not distinguish between acylation and sulfonylation of the enzyme. Previously we have shown by X-ray crystallography that *N*-acyl β-sultams inhibit elastase by sulfonylation of the active-site serine residue[25] whereas β-lactams inhibit the enzyme by acylation of the same residue.[26] The second-order rate constant, k_i, for the inactivation of PPE by *N*-benzyl-4,4-dimethyl-3-oxo-β-sultam **1** is 768 M^{-1} s^{-1} (Table 1), which is nearly 10^3 greater than that by *N*-benzoyl β-sultam **4** which inhibits elastase by a sulfonylation process. This large rate difference is indicative of a different mechanism of inhibition, possibly acylation by attack of the active-site serine on the β-lactam of **1** giving C–N fission and acylation to form an acyl enzyme (Scheme 2). This is supported by the recovery of full enzyme activity when elastase is inactivated by *N*-benzyl-4,4-diethyl-3-oxo-β-sultam **5** over a period of days, with a rate constant of 1.39×10^{-4} s^{-1} at pH 8.5, whereas the enzyme remains inactive for at least 4 weeks when inactivated by **4**. Interestingly, the rate of recovery of enzyme activity shows the same sigmoidal dependence on pH as that for the rate of hydrolysis (k_{cat}/K_m) of the anilide substrate (Fig. 3). Both reactions are critically dependent on the ionization of a group with a pK_a of 6.9. This observation suggests a common residue for the two processes of acylation and deacylation because the dependence of k_{cat}/K_m on pH reflects the acylation process, whereas that for recovery of activity is deacylation (Scheme 2). The much faster rate of enzyme inactivation by the 3-oxo-β-sultam **1** compared with *N*-benzoyl β-sultam **4** together with the recovery of enzyme activity suggests different mechanisms for the two processes. It thus appears that inactivation of elastase by the 3-oxo-β-sultam **1** is occurring by serine attack on the β-lactam centre to form an acyl enzyme of intermediate stability which is then slowly hydrolyzed in the conventional manner (Scheme 2).

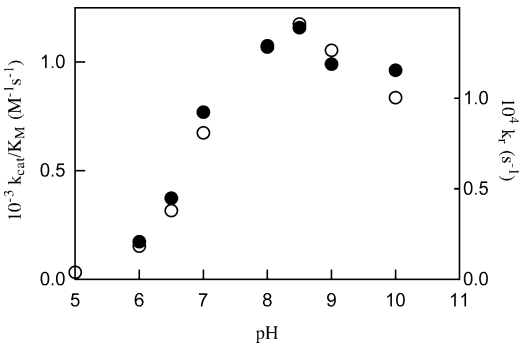
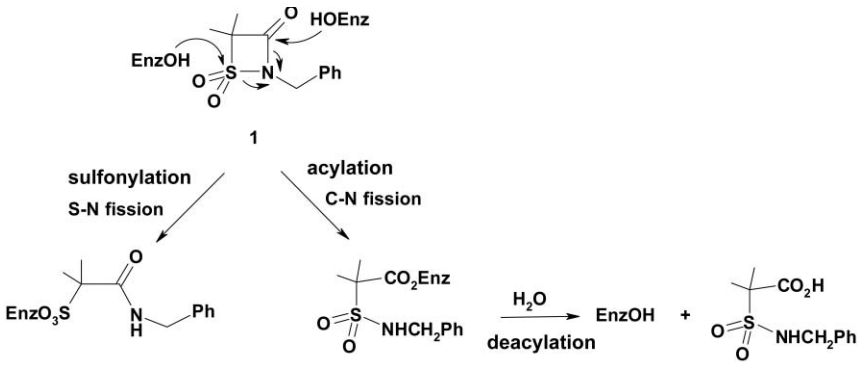


Fig. 3 The pH dependence of the rate constants for the PPE-catalyzed hydrolysis of *N*-succinyl-(L-ala)$_3$-*p*-nitroanilide (k_{cat}/K_m, open circles) and that for the enzyme recovery after inhibited with *N*-benzyl-4,4-diethyl-3-oxo-β-sultam **5** (k_r, filled circles) at different pHs. Rate constants were determined in 0.1 M aqueous buffer solution at 30 °C with 1.0 M ionic strength.

(ii) Variation of C-4 substituents. The rate of inhibition of PPE by *N*-benzyl-3-oxo-β-sultams shows a large dependence on the size of the C-4 substituents. The gem-dimethyl group at the C-4 position of *N*-benzyl-4,4-dimethyl-3-oxo-β-sultam **1** shows the largest rate constant, k_i, for inhibition and the rate constant decreases moderately by 3-fold in changing to gem-diethyl **5** and decreases more significantly by 100-fold on changing to a spirocyclohexyl substituent **6** (Table 1).

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 should ideally be relatively stable under *in vivo* conditions. The rate of inactivation can be considered to be controlled by two important factors: (i) the intrinsic "chemical" reactivity of the inhibitor and (ii) the molecular recognition reflected by the binding energy between the inhibitor and the target enzyme. The acylation process involves displacement of a leaving group by serine attacking the carbonyl group of the inhibitor, so changes in chemical reactivity brought about by substituents is usually considered in terms of the electrophilic nature of the inhibitor and the leaving group



Scheme 2

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. Although increasing the intrinsic chemical reactivity may lead to a faster rate of reaction with the target enzyme, it may also lead to greater hydrolytic and metabolic instability with consequent little change in selectivity. One method of indicating this selectivity is to use an 'enzyme rate-enhancement factor' (EREF)[27] which is the second-order rate constant for the inactivation of the enzyme, k_i, divided by that for hydrolysis of the inhibitor catalyzed by hydroxide ion, k_{OH}.

Although the 4,4-dimethyl derivative **1** has the largest rate constant, k_i, for inhibition, changing to 4,4-diethyl groups only decreases the inhibition activity against PPE by 3-fold but by 10-fold for chemical reactivity as measured by the rate of alkaline hydrolysis (Table 1). Consequently the 4,4-diethyl derivative has the largest EREF ratio of the rate constant for inhibition to that for alkaline hydrolysis. This suggests that the 4,4-diethyl derivative has the most favourable binding in the series. The comparison is complicated by the fact that the enzyme reaction and the chemical reaction involve different reaction sites on the 3-oxo-β-sultam and the substituent effect for the hydrolysis at the two reaction centres, acyl and sulfonyl, may not be the same. Nevertheless, the large decrease in the rate of inhibition of PPE by *N*-benzyl-3-oxo-β-sultams on increasing size of the C-4 substituents agrees with other results that have suggested the alkyl substituents at C-3 position of monocyclic β-lactam (which is the equivalent of the C-4 of 3-oxo-β-sultams) binds to the S_1 pocket of the enzyme,[24] and for which a small hydrophobic residue is preferred.[22,23] The preference shown by the C-4 substituents for the enzyme also confirms that the inhibition by 3-oxo-β-sultams is active-site directed.

(iii) Variation of the amine leaving group. The inhibition activity is severely affected by the carboxylate group; a 25-fold decrease of the second-order rate constant, k_i, for the inhibition by **9** compared with **1** is observed (Table 1). The decrease in the inhibition activity may be the result of a poorer binding of the dissociated carboxylate in the hydrophobic environment of the active site of PPE. Esterification of the *N*-α-carboxylate of the 3-oxo-β-sultams (**7** and **9**) with benzyl alcohol gave esters which could not be studied for inhibition because of poor solubility, even in 15% acetonitrile–water (v/v).

The amine leaving group was further modified by varying the substituent at the α-carbon. Increasing the length of the alkyl chain by one methylene unit has little or no effect on the chemical reactivity of the 3-oxo-β-sultam as **7** and **10** have similar second-order rate constants, k_{OH}, for their alkaline hydrolysis. Similarly, a phenyl group does not have any significant effect on chemical reactivity as shown by the similar second-order rate constants, k_{OH}, for the alkaline hydrolysis of **7** and **9**. However, the second-order rate constant for inactivation of elastase, k_i, varies 12-fold in the series; changing iso-propyl to iso-butyl increases k_i by 4-fold and changing it to phenyl gives an 12-fold increase in the second-order rate constant, k_i (Table 1). The highest EREF (k_i/k_{OH} ratio) in this series is shown by the phenyl substituted derivative **9**. Finally, incorporating a gem-diethyl group at C-4 and a valine nitrogen leaving group yield the most chemically stable 3-oxo-β-sultam **11** in the series with a second order rate constant of 1.74×10^3 $M^{-1}s^{-1}$ for alkaline hydrolysis, which is still highly reactive compared with a k_{OH} of 10^{-1} M^{-1} s^{-1} for acylating agents of enzymes that are therapeutically useful.[20] However, the inhibition activity of this compound is also the lowest in the series. The second-order rate constants, k_{OH} and k_i, for **11** are 100- and 2000-fold less reactive, respectively, than the most reactive 3-oxo-β-sultam **1**. The higher sensitivity of inhibition activity to substituents compared with that of chemical activity does indicate significant recognition of the 3-oxo-β-sultams by the enzyme.

In summary, 3-oxo-β-sultams are novel acylating agents that inactivate elastase with reasonable specificity. These compounds are unusual in that the enzyme reacts with the acyl centre whereas the hydrolysis reaction occurs at the sulfonyl centre despite the fact that β-sultams are about 10^3-fold more reactive than β-lactams. Variation of the structure by simple substituents leads to differences in rates of inactivation of up to 2000-fold. There is a large differential effect of substituents on the relative reactivities of hydrolysis and enzyme reaction.

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 F254 TLC plates used for initial investigations. Solvents were purified according to Perrin and Armarego.[28] Starting materials were purchased from Aldrich, Avocado and Lancaster, and were used without further purification.

Disodium 2-methyl-2-sulfonatopropanoate (S1). Concentrated H_2SO_4 (5.22 ml) was added dropwise to isobutyric anhydride (20 g, 127.0 mmol) and the mixture stirred for 30 minutes at 20–35 °C. The reaction mixture was then gently heated to 90 °C and stirred for approximately 7 hours. To check for the completion of the reaction: a solution of $BaCl_2$ (1 ml) was added to a sample of the reaction mixture, if no precipitation appeared then the reaction was considered complete. The hot reaction mixture was poured into ice-cold water (50 ml) and extracted with ether (3 × 40 ml). Ether extracts contain the isobutyric acid from the sulfonation. To the aqueous phase, a solution of NaOH (6.16 g) in water (20 ml) was added in small portions to adjust the pH to around pH 8. The solution was evaporated to dryness using reduced pressure rotary evaporation at 40 °C. The residue was dissolved in hot water (10 ml) and precipitated by addition of ethanol (50 ml) and the resulting precipitate was isolated by vacuum filtration. If the colour of the solution was dark brown–orange, then charcoal was added to the solution before the addition of the ethanol. A second crop was isolated by adding ethanol to the mother liquor. The product isolated (20.1 g, 75%) was carried through to the next step without any further purification.

2-(Chlorosulfonyl)-2-methylpropanoyl chloride (S2). Disodium 2-methylsulfonatopropionate (**S1**) (4.88 g, 23 mmol) was added to thionyl chloride (18.3 ml, 153 mmol) in small portions over 10 minutes at 0 °C with stirring, DMF (0.37 ml) was added dropwise over 2 minutes and the mixture was heated to 70 °C. After gas production was complete the mixture was heated for a further 5 hours at 70 °C. Excess thionyl chloride was evaporated under reduced pressure on a rotary evaporator at 40 °C, yielding a pale yellow residue which was dissolved in ether. The resultant NaCl was filtered off and the solvent was removed from the filtrate by reduced pressure rotary evaporation at 30 °C to yield a yellow oil (3.48 g, 74%). IR: ν_{max} (cm^{-1}) (neat) 3003, 1811, 1464, 1368, 1179, 1127. ^{1}H NMR: δ ($CDCl_3$): 2.0 (6H, s, 2 x CH_3). ^{13}C NMR: δ ($CDCl_3$): 169.29 (C=O), 85.44 (quaternary carbon), 22.07 (CH_3).

2-Benzyl-4,4-dimethyl-1,2-thiazetidin-3-one-1,1-dioxide (1). 2-(Chlorosulfonyl)-2-methylpropionyl chloride (**S2**) (1 g, 4.9 mmol) was dissolved in dry ether (200 ml) and the mixture was cooled to −78 °C. A solution of benzylamine (0.53 ml, 4.9 mmol) in ether (10 ml) was added dropwise over 20 minutes at −78 °C with stirring. The mixture was stirred for 10 minutes before triethylamine (1.35 ml, 14.7 mmol) in ether (10 ml) was added dropwise over 10 minutes to the mixture at −78 °C. The reaction mixture was stirred at −78 °C for 1 hour and then for 3 hours at room temperature and the resultant $Et_3N{\cdot}HCl$ salt filtered off. The solvent was removed by reduced pressure rotary evaporation at 30 °C to yield a creamy white residue which was purified using column chromatography (30 g silica) (3 : 2 hexane–EtOAc, R_f = 0.56) (0.76 g, 43%), m.p. 63–64 °C. IR: ν_{max} (cm^{-1}) ($CHCl_3$) 3020, 1772, 1339, 1216, 1182, 1122. ^{1}H NMR: δ ($CDCl_3$): 7.40–7.38 (5H, m, Ph), 4.6 (2H, s, CH_2), 1.8 (6H, s, 2 x CH_3). ^{13}C NMR: δ ($CDCl_3$): 164.36 (C=O), 133.17 (quaternary carbon), 128.96 (CH (Ph)), 128.52 (CH (Ph)), 128.1 (CH (Ph)), 83.03 (quaternary carbon), 44.47 (CH_2), 18.4 (CH_3 × 2).

(DL)-2′-(4,4-Dimethyl-1,1-dioxido-3-oxo-1,2-thiazetidin-2-yl) phenylacetic acid (9). Palladium (0.1 g, 10% Pd/C) was added to dry ethyl acetate (2 ml) and then to a solution of benzyl (DL)-2′-(4,4-dimethyl-1,1-dioxido-3-oxo-1,2-thiazetidin-2-yl) phenyl acetate (**S3**) (0.1 g, 0.268 mmol) in dry ethyl acetate (11 ml). The reaction vessel was flushed with nitrogen for 2 minutes followed by hydrogen for 1 minute. The solution was hydrogenated at atmospheric pressure for 2 hours before filtering through celite. The solvent was removed by reduced pressure rotary evaporation at 30 °C to yield a white solid (74 mg, 98%) m.p. 195–197 °C (decomposes to a dark oil). IR: ν_{max} (cm^{-1}) ($CHCl_3$) 3020, 2956, 1776, 1732, 1354, 1337, 1216, 1181, 1124. ^{1}H NMR: δ ($CDCl_3$): 7.47–7.37 (5H, m, Ph), 5.76 (1H, s, CH), 1.62 (3H, s, CH_3), 1.58 (3H, s, CH_3). ^{13}C NMR: δ ($CDCl_3$): 167.98 (C=O), 164.58 (C=O), 134.3 (quaternary carbon), 129.06 (CH (Ph)), 128.84 (CH (Ph)), 128.45 (CH (Ph)), 82.71 (quaternary carbon), 60.15 (CH), 17.8 (CH_3), 17.68 (CH_3). HREI-MS $[M+NH_4]^+$ for $C_{12}H_{13}NO_5S$ calc. 301.0853 measured 301.0855.

4-*Spiro*-cyclohexyl-3-oxo-β-sultam (S17). 1-(Chlorosulfonyl)-cyclohexane carbonyl chloride (**S16**) (1 g, 4.33 mmol) was dissolved in ether (10 ml) and added dropwise over 20 minutes (VERY SLOWLY) to liquid NH_3 (5.3 ml) in ether (10 ml) at −78 °C. The mixture was warmed to room temperature and stirred until all the solvent had evaporated. The residue was dissolved using $CHCl_3$ (5 ml) and water (5 ml) at 0–4 °C, and the pH of the solution was adjusted to pH 1 using dilute HCl. The organic layer was separated and the aqueous layer extracted with $CHCl_3$ (3 × 5 ml). The organic layers were combined and dried over sodium sulfate, and then the solvent was removed by reduced pressure rotary evaporation 30 °C to give a white solid (0.50 g, 65%) m.p. 61–62 °C. IR: ν_{max} (cm^{-1}) 3241, 2946, 2864, 1778, 1452, 1356, 1338, 1215, 1161, 1132, 754. ^{1}H NMR: δ ($CDCl_3$): 8.27 (1H, br s, NH), 2.41 (2H, m, cyclohexyl H), 2.01 (2H, m, cyclohexyl H), 1.91 (2H, m, cyclohexyl H) 1.71 (1H, m, cyclohexyl H), 1.6 (2H, m, cyclohexyl H), 1.44 (1H, m, cyclohexyl H). ^{13}C NMR: δ ($CDCl_3$): 163.29 (C=O), 86.75 (quaternary carbon), 28.11 (C_1/C_5 CH_2), 24.05 (C_3 CH_2), 22.62 (C_2/C_4 CH_2). HREI-MS [M–H] for $C_7H_{11}NO_3S$ calculated 188.0376 measured 188.0379.

***N*-Benzyl-4-*spiro*-cyclohexyl-3-oxo-β-sultam (6).** At 0 °C under nitrogen, 4-*spiro*-cyclohexyl-3-oxo-β-sultam (**S17**) (0.1 g, 0.53 mmol) in anhydrous THF (5 ml) was added to a suspension of NaH (0.015 g, 0.6 mmol) (pre-washed 2–3 times with petroleum ether) in DMF (2 ml). The mixture was stirred for 15 minutes and benzyl bromide (0.09 g, 0.526 mmol) was injected through a septum dropwise over 5 minutes. The mixture was allowed to warm-up to room temperature and stirred for 24 hours. The mixture was then cooled to 0 °C, ether (10 ml) was added, the solution was hydrolyzed with brine, and the pH was adjusted to 4–5 using dilute HCl. The organic layer was separated and the aqueous layer was extracted with ether (2 × 10 ml). The organic layers were combined, washed with saturated brine (2 ×

10 ml) and dried over Na_2SO_4. The solvent was removed by rotary evaporation at 30 °C and the resulting pale yellow oil was purified using column chromatography (silica 3 g) (1 : 8 : 1 ether–hexane–EtOAc) $R_f = 0.58$ (0.082 g 53%) m.p. 73–74 °C. IR: ν_{max} (cm^{-1}) ($CHCl_3$) 3020.6, 2944.8, 1768.8, 1335.8, 1215.9, 1168.4, 668. ^1H NMR: δ ($CDCl_3$): 7.40–7.36 (5H, m, Ph), 4.6 (2H, s, CH_2Ph), 2.37 (2H, m, cyclohexyl H), 1.99 (2H, m, cyclohexyl H), 1.89 (2H, m, cyclohexyl H) 1.7 (1H, m, cyclohexyl H), 1.59 (2H, m, cyclohexyl H), 1.42 (1H, m, cyclohexyl H). ^{13}C NMR: δ ($CDCl_3$): 163.71 (C=O), 133.4 (quaternary carbon), 128.84 (CH (Ph)), 128.34 (CH (Ph)), 127.97 (CH (Ph)), 87.53 (quaternary carbon), 44.09 (CH_2Ph), 27.91 (C_1/C_5 CH_2), 23.99 (C_3 CH_2), 22.75 (C_2/C_4 CH_2).

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 40 (Beckman, Fullerton, USA) or 3020 (Jenway, Dunmow, England) pH meter. Electrodes were semi-micro Ag/AgCl and calomel (Russell, 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 deionized 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 neutralization 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 2×10^{-5} M to 2×10^{-4} M to ensure pseudo-first-order conditions.

Hydroxide ion concentrations were calculated using pK_w (H_2O) = 13.883 at 30 °C.[29] Reactions studied by UV spectrophotometry were usually commenced by injections of acetonitrile or dioxane 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 dioxane 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 deionized 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 standardized 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 standardized 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 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 PPE and up to 5×10^{-3} M β-sultam. These conditions were created in a separate UV cell containing stock PPE suspension (200 μl), 0.1 M buffer (200 μl) and inhibitor stock solution in MeCN or MeCN alone (100 μl). Aliquots of this solution (50 μl) were assayed for PPE 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 PPE, 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.

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References

1 J. W. Skiles and A. Y. Jeng, *Expert Opin. Ther. Pat.*, 1999, **9**, 869–895.
2 H. Ohbayashi, *Expert Opin. Ther. Pat.*, 2005, **15**, 759–771.
3 K. M. Beeh and J. Beier, *Clin. Exp. Allergy*, 2006, **36**, 142–157.
4 J. C. Powers, J. L. Asgian, O. D. Ekici and K. E. James, *Chem. Rev.*, 2002, **102**, 4639–4750.
5 M. I. Konaklieva, *Curr. Med. Chem.: Anti-Infect. Agents*, 2002, **1**, 215–238.
6 M. I. Konaklieva and B. J. Plotkin, *Mini-Rev. Med. Chem.*, 2004, **4**, 721–739.
7 D. Leung, G. Abbenante and D. P. Fairlie, *J. Med. Chem.*, 2000, **43**, 305–341.
8 W. Bode, E. Meyer, Jr. and J. C. Powers, *Biochemistry*, 1989, **28**, 1951–1963.
9 N. O. Sykes, S. J. F. Macdonald and M. I. Page, *J. Med. Chem.*, 2002, **45**, 2850–2856.
10 H. Ohbayashi, *Expert Opin. Ther. Pat.*, 2002, **12**, 65–84.
11 M. I. Page, *Acc. Chem. Res.*, 2004, **37**, 297–303.
12 M. I. Page, P. S. Hinchliffe, J. M. Wood, L. P. Harding and A. P. Laws, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 4489–4492.
13 W. Y. Tsang, N. Ahmed, L. P. Harding, K. Hemming, A. P. Laws and M. I. Page, *J. Am. Chem. Soc.*, 2005, **127**, 8946–8947.
14 R. Moreira, A. B. Santana, J. Iley, J. Neres, K. T. Douglas, P. N. Horton and M. B. Hursthouse, *J. Med. Chem.*, 2005, **48**, 4861–4870.
15 N. Ahmed, W. Y. Tsang and M. I. Page, *Org. Lett.*, 2004, **6**, 201–203.
16 M. I. Page, *Adv. Phys. Org. Chem.*, 1987, **23**, 165–270.
17 M. I. Page, A. Williams, *Organic and Bio-organic Reaction Mechanisms*, Longman, Harlow, 1997.
18 N. J. Baxter, L. J. M. Rigoreau, A. P. Laws and M. I. Page, *J. Am. Chem. Soc.*, 2000, **122**, 3375–3385.
19 P. S. Hinchliffe, J. M. Wood, A. M. Davis, R. P. Austin, R. P. Beckett and M. I. Page, *J. Chem. Soc., Perkin Trans. 2*, 2001, 1503–1505.
20 J. M. Wood and M. I. Page, *Trends Heterocycl. Chem.*, 2002, **8**, 19–34.
21 W. Y. Tsang, N. Ahmed, K. Hemming and M. I. Page, *Can. J. Chem.*, 2005, **83**, 1432–1439.
22 L. Hedstrom, *Chem. Rev.*, 2002, **102**, 4501–4523.
23 P. D. Edwards and P. R. Bernstein, *Med. Res. Rev.*, 1994, **14**, 127–194.
24 R. A. Firestone, P. L. Barker, J. M. Pisano, B. M. Ashe and M. E. Dahlgren, *Tetrahedron*, 1990, **46**, 2255–2262.
25 P. S. Hinchliffe, J. M. Wood, A. M. Davis, R. P. Austin, R. P. Beckett and M. I. Page, *Org. Biomol. Chem.*, 2003, **1**, 67–80.
26 P. Taylor, V. Anderson, J. Dowden, S. L. Flitsch, N. J. Turner, K. Loughran and M. D. Walkinshaw, *J. Biol. Chem.*, 1999, **274**, 24901–24905.
27 M. I. Page and A. P. Laws, *Chem. Commun.*, 1998, 1609–1617.
28 D. D. Perrin, W. L. F. Armarego, *Purification of Laboratory Chemicals*, Pergamon Press, Oxford, 3rd edn, 1988, p. 391.
29 *Lange's Handbook of Chemistry*, ed. J. A. Dean, McGraw-Hill, New York, 11th edn, 1973, p. 1576.

