J|A|C|S
ARTICLES
Published on Web 11/18/2005

Different Transition-State Structures for the Reactions of β-Lactams and Analogous β-Sultams with Serine β-Lactamases

Wing Y. Tsang, Naveed Ahmed, Paul S. Hinchliffe, J. Matthew Wood, Lindsay P. Harding, Andrew P. Laws, and Michael I. Page*

Contribution from the Department of Chemical and Biological Sciences, The University of Huddersfield, Queensgate, Huddersfield, HD1 3DH, UK

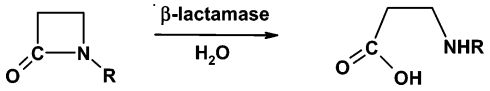
Received September 13, 2005; E-mail: m.i.page@hud.ac.uk

Abstract: β-Sultams are the sulfonyl analogues of β-lactams, and *N*-acyl β-sultams are novel inactivators of the class C β-lactamase of *Enterobacter cloacae* P99. They sulfonylate the active site serine residue to form a sulfonate ester which subsequently undergoes C−O bond fission and formation of a dehydroalanine residue by elimination of the sulfonate anion as shown by electrospray ionization mass spectroscopy. The analogous *N*-acyl β-lactams are substrates for β-lactamase and undergo enzyme-catalyzed hydrolysis presumably by the normal acylation−deacylation process. The rates of acylation of the enzyme by the β-lactams, measured by the second-order rate constant for hydrolysis, k_{cat}/K_m, and those of sulfonylation by the β-sultams, measured by the second-order rate constant for inactivation, k_i, both show a similar pH dependence to that exhibited by the β-lactamase-catalyzed hydrolysis of β-lactam antibiotics. Electron-withdrawing groups in the aryl residue of the leaving group of *N*-aroyl β-lactams increase the rate of alkaline hydrolysis and give a Bronsted β_{lg} of −0.55, indicative of a late transition state for rate-limiting formation of the tetrahedral intermediate. Interestingly, the corresponding Bronsted β_{lg} for the β-lactamase-catalyzed hydrolysis of the same substrates is −0.06, indicative of an earlier transition state for the enzyme-catalyzed reaction. By contrast, although the Bronsted β_{lg} for the alkaline hydrolysis of *N*-aroyl β-sultams is −0.73, similar to that for the β-lactams, that for the sulfonylation of β-lactamase by these compounds is −1.46, compatible with significant amide anion expulsion/S−N fission in the transition state. In this case, the enzyme reaction displays a later transition state compared with hydroxide-ion-catalyzed hydrolysis of the β-sultam.

Introduction

The susceptibility of β-lactam antibiotics to the hydrolytic activity of β-lactamase enzymes is the most common and growing form of bacterial resistance to the normally lethal action of these antibacterial agents.[1] β-Lactamases catalyze the hydrolysis of the β-lactam to give the ring-opened and bacterially inert β-amino acid (Scheme 1). Bacteria appear to be producing new β-lactamases that can catalyze the hydrolysis of β-lactams previously resistant to enzyme degradation. For example, when the carbapenems, such as imipenem, were first introduced in the 1970s they were seen as versatile broad-spectrum antibacterials resistant to hydrolysis by most β-lactamases. However, "carbapenamases" are now increasingly produced by a variety of bacteria,[2] and there are now over 500 different β-lactamases which have so far been identified.[3] Inducible enzymes may be chromosomal or plasmid encoded, but the more common constitutive β-lactamase production is predominantly plasmid mediated. Constitutive mutants of Gram-negative strains produce enormous amounts of enzyme so that periplasmic β-lactamase concentration may be up to 1 mM.[4]

Scheme 1

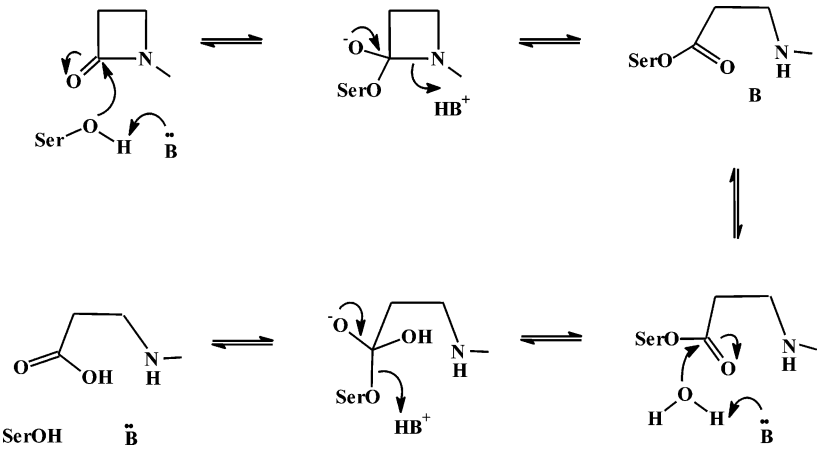


The main mechanistic division of β-lactamases is into serine and zinc enzymes.[1] The former have an active site serine residue, and the catalytic mechanism involves the formation of an acyl-enzyme intermediate, whereas the metallo enzymes appear to involve only noncovalently bound intermediates.[5] On the basis of their amino acid sequences, the serine β-lactamases are subdivided into three classes, A, C, and D, whereas the class B β-lactamases consist of the zinc enzymes.[3,5,6] The class C β-lactamases of Gram-negative bacteria are widely expressed but are not significantly inhibited by clinically used β-lactamase inhibitors such as clavulanic acid.

(1) Waley, S. G. In *The Chemistry of β-Lactams*; Page, M. I., Ed., Blackie: Glasgow, 1992; pp 198−226; Frère, J.-M. *Mol. Microbiol.* **1995**, *16*, 385−395.
(2) Payne, D. J. *J. Med. Microbiol.* **1993**, *39*, 93−99; Nordmann, P.; Mariotte, S.; Naas, T.; Labia, R.; Nicolas, M. H. *Antimicrob. Agents Chemother.* **1993**, *37*, 939−946; Naas. T.; Vandel, L.; Sougakoff, W.; Livermore, D. M.; Nordmann, P. *Antimicrob. Agents Chemother.* **1994**, *38*, 1262−1270.
(3) Bush, K.; Jacoby, G. A.; Medeiros, A. A. *Antimicrob. Agents Chemother.* **1995**, *39*, 1211−1233; Rasmussen, B. A.; Bush, K. *Antimicrob. Agents Chemother.* **1997**, *41*, 223−232; http://www.lahey.org/studies/ webt.asp.
(4) Hechler, U.; Van den Weghe, M.; Martin, H. H.; Frère, J.-M. *J. Gen. Microbiol.* **1989**, *135*, 1275−1290.
(5) Page, M. I.; Laws, A. P. *Chem. Commun.* **1998**, 1609−1617.
(6) Galleni, M.; Lamotte-Brasseur, J.; Rossolini, G. M.; Spencer, J.; Dideberg, O.; Frère, J.-M. *Antimicrob. Agents Chemother.* **2001**, *45*, 660−663.

10.1021/ja056124z CCC: $30.25

Scheme 2



The class A and class C enzymes are monomeric medium-sized proteins with M_r values of about 29 000 and 39 000, respectively.[1] The X-ray crystal structures of class A,[7] C,[8] and D[9] β-lactamases have been reported. The active site serine is situated at the N-terminus of the long, relatively hydrophobic, first α-helix of the all α-domain.

There are several highly conserved residues surrounding the active site that may be involved in substrate recognition and the catalytic processes of bond making and breaking. In class A enzymes, Glu 166 is, in the static crystal structure, hydrogen bonded to a conserved water molecule which in turn is hydrogen bonded to the active site serine. The mechanism of action of serine β-lactamases involves the formation of an acyl enzyme intermediate (Scheme 2) for which there is very strong evidence such as electrospray ionization mass spectroscopy (ESIMS),[10] infrared measurements,[11] trapping experiments,[12] the determination of the rate constants for their formation and breakdown,[13] and even an X-ray crystal structure.[14] Formation of the acyl enzyme intermediate requires at least two proton transfers: proton removal from the attacking serine and proton donation to the departing β-lactam amine. Despite the availability of a number of X-ray crystal structures of several class A and class C β-lactamases[7,8] and many site directed mutagenesis studies,[15] the identity of the catalytic groups involved in these proton-transfer steps remains controversial although the consensus in class A β-lactamases is that the general base is Glu166 and that it is used for both acylation and deacylation.[5,16,17] In principle, the general base that accepts a proton from the nucleophilic serine is not necessarily the same residue that subsequently acts as a general acid to donate a proton to the departing β-lactam nitrogen.[5] Conceptually this is neater, and our prejudices are reinforced by the juxtaposition of the nitrogen lone pair, the serine, and the general base−acid being on the same α (exo) face of the substrate in the tetrahedral intermediate.[17] Similarly, the hydrolysis of the acyl enzyme requires two proton-transfer steps—from water and to the departing serine—and only our preference for simplicity requires these to be one and the same residue *and* the same as that involved in the acylation step.

A major difference for class C β-lactamases compared with class A is that deacylation is often rate limiting so that the acyl enzyme intermediate may accumulate giving rise to low values of K_m.[5] Class C enzymes are also weak catalysts for the hydrolysis of non-β-lactam substrates such as esters and thioesters.[18] In class C β-lactamase, it has been suggested that the phenol of tyrosine 150 has a severely reduced pK_a and acts as a general base catalyst for proton removal from serine 64[8,18] although the results from site-directed mutagenesis of Tyr-150 are contradictory.[19] The pK_a of the ionising group acting as a general base obtained from pH-rate profiles is 6.1,[12] but the unusual shift in this pK_a in D_2O and the *inverse* kinetic solvent isotope effect on k_{cat}/K_m are indicative of a system with an abnormally high fractionation factor for the protonic state

(7) Kelly, J. A.; Dideberg, O.; Charlier, P.; Wery, J. P.; Libert, M.; Moews, P. C.; Knox, J. R.; Duez, C.; Fraipont, CL.; Joris, B.; Dusart, J.; Frère, J.-M.; Ghuysen, J. M. *Science* **1986**, *231*, 1429−1431; Samraoui, B.; Sutton, B. J.; Todd, R. J.; Artymiuk, P. J.; Waley, S. G.; Phillips, D. C. *Nature* **1986**, *320*, 378−380; Knox, J. R.; Moews, P. C. *J. Mol. Biol.* **1991**, *220*, 435−455; Moews, P. C.; Knox, J. R.; Dideberg, O.; Charlier, P.; Frère, J.-M. *Proteins* **1990**, *7*, 156−171; Herzberg, O.; Moult, J. *Science* **1987**, *236*, 694−701; Herzberg, O. *J. Mol. Biol.* **1991**, *217*, 701−719; Dideberg, O.; Charlier, P.; Wéry, J.-P.; Dehottay, P.; Dusart, J.; Erpicum, T.; Frère, J.-M.; Ghuysen, J.-M. *Biochem. J.* **1987**, *245*, 911−913; Lamotte-Brasseur, J.; Dive, G.; Dideberg, O.; Charlier, P.; Frère, J.-M.; Ghuysen, J.-M.; *Biochem. J.* **1991**, *279*, 213−221; Jelsch, C.; Mourey, L.; Masson, J.-M.; Samama, J.-P. *Proteins* **1993**, *16*, 364−383.

(8) Oefner, C.; D'Arcy, A.; Daly, J. J.; Gubernator, K.; Charnas, R. L.; Heinze, I.; Hubschwerlen, C.; Winkler, F. K. *Nature* **1990**, *343*, 284−288; Lobkovsky, E.; Moews, P. C.; Liu, H.; Zhao, H.; Frère, J.-M.; Knox, J. R. *Proc. Natl. Acad. Sci.* **1993**, *90*, 11257−11261; Lobkovsky, E.; Billings, E. M.; Moews, P. C.; Rahil, J.; Pratt, R. F.; Knox, J. R. *Biochemistry* **1994**, *33*, 6762−6772; Usher, K. C.; Blaszczak, L. C.; Weston, G. S.; Shoichet, B. K.; Remington, S. J. *Biochemistry* **1998**, *37*, 16082−16092.

(9) Maveyraud, L.; Golemi, D.; Kotra, L. P.; Tranier, S.; Vakulenko, S.; Mobashery, S.; Samama, J. P. *Struct. Fold. Des.* **2000**, *8*, 1289; Maveyraud, L.; Golemi-Kotra, D.; Ishiwata, A.; Meroueh, O.; Mobashery, S.; Samama, J. P. *J. Am. Chem. Soc.* **2002**, *124*, 2461−2465.

(10) Leung, Y.-C.; Robinson, C. V.; Aplin, R. T.; Waley, S. G. *Biochem. J.* **1994**, *299*, 671−678; Brown, R. P.; Aplin, R. T.; Schofield, C. J. *Biochemistry* **1996**, *35*, 12421−12432.

(11) Fisher, J.; Belasco, J. G.; Khosla, S.; Knowles, J. R. *Biochemistry* **1980**, *19*, 2895−2901; Wilkinson, A.-S.; Bryant, P. K.; Meroueh, S. O.; Page, M. G.; Mobashery, S.; Wharton, C. W. *Biochemistry* **2003**, *42*, 1950−1957.

(12) Page. M. I.; Vilanova, B.; Layland, N. J. *J. Am. Chem. Soc.* **1995**, *117*, 12092−12095.

(13) Christensen, H.; Martin, M. T.; Waley, S. G. *Biochem. J.* **1990**, *266*, 853−861.

(14) Strynadka, N. C.; Adachi, H.; Jensen, S. E.; Johns, K.; Sielecki, A.; Betzel, C.; Sutoh, K.; James, M. N. *Nature* **1992**, *359*, 700−705; Beadle, B. M.; Shoichet, B. K. *Antimicrob. Agents Chemother.* **2002**, *46*, 3978−3980.

(15) Matagne, A.; Frère, J.-M. *Biochim. Biophys. Acta* **1995**, *1246*, 109−127.

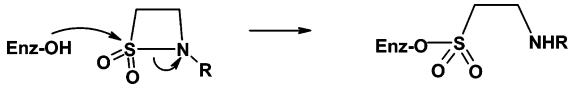
(16) Minasov, G.; Wang, X.; Shoichet, B. K. *J. Am. Chem. Soc.* **2002**, *124*, 5333−5340.

(17) Page, M. I. *Curr. Pharm. Des.* **1999**, *5*, 895−913.

(18) Damblon, C.; Zhao, G. H.; Jamin, M.; Ledent, P.; Dubus, A.; Vanhove, M.; Raquet, X.; Christiaens, L.; Frère, J.-M. *Biochem. J.* **1995**, *309*, 431−436; Xu, Y.; Soto, G.; Hirsch, K. R.; Pratt, R. F. *Biochemistry* **1996**, *35*, 3595−3603; Murphy, B. P.; Pratt, R. F. *Biochemistry* **1991**, *30*, 3640−3649.

(19) Dubus, A., Normark, S.; Kania, M.; Page, M. G. *Biochemistry* **1994**, *33*, 8577−8586; Dubus. A., Ledent, P., Lamotte-Brasseur, J., Frère, J.-M. *Proteins* **1996**, *25*, 473−485.

Scheme 3



undergoing dissociation.[12] The most likely basic group responsible for the acid limb of the pH-rate profile for both acylation and deacylation is Tyr-150, which is hydrogen-bonded to Lys-315 and Lys-67[8,12] (Scheme 2, B = TyrO$^-$). In common with serine proteases, the serine β-lactamases possess an oxyanion hole that donates two hydrogen bonds to the β-lactam carbonyl oxygen, inducing polarization to facilitate nucleophilic attack and stabilizing the oxyanion of the tetrahedral intermediate.

There is an obvious interest in finding new inhibitors of the β-lactamases, and the sulfonylation of serine enzymes (Scheme 3) offers an interesting but largely unexplored strategy for inhibition as an alternative to the traditional mechanism-based acylation process.[20] The main reason sulfonylation is not a well studied process is because sulfonyl derivatives are much less reactive than their acyl counterparts.[21] For example, sulfonamides are extremely resistant to alkaline and acid hydrolysis and, in general, sulfonyl transfer reactions are 10^2-10^4 fold slower than the corresponding acyl transfer process.[22] However, we have recently shown that β-sultams (1), which are the sulfonyl analogues of β-lactams (2), are 10^2-10^3 fold more reactive than β-lactams in their rates of alkaline and acid hydrolysis.[23,24] It is of interest to compare the efficiency and mechanisms of sulfonyl and acyl transfer from β-sultams and β-lactams, respectively, to serine β-lactamases. However, the maximum transition-state stabilization available for the "natural" substrate, brought about by the noncovalent interactions between the substrate and the enzyme, may not be available to a substrate with a less favorable geometry. This may be reflected in the transition-state structure as indicated by the Hammond Postulate:[25] the most favorable interactions producing an earlier transition state along the reaction coordinate, whereas the less favorable system induces a later transition state. We have investigated this possibility by comparing acyl and sulfonyl transfer reactions catalyzed by class C β-lactamases.

Results and Discussion

(i) Inhibition of β-Lactamase by β-Sultams. P99 β-Lactamase is a serine class C β-lactamase enzyme derived from the Gram-negative bacteria *Enterobacter clocae* with a molecular weight of 39 184 Da. It is capable of hydrolyzing a wide variety of β-lactam-based substrates at a rapid rate.[17] The high efficiency of P99 β-lactamase as a catalyst is exemplified by its large values of the second-order rate constant, $k_{cat}/K_m = 2.48 \times 10^6$ M^{-1} s^{-1}, for the "weak-binding" substrate cephaloridine (k_{cat} $= 1.16 \times 10^3$ s^{-1}, $K_m = 4.68 \times 10^{-4}$ M) and 3.97×10^6 M^{-1} s^{-1} for the tight-binding substrate benzylpenicillin ($k_{cat} = 29$ s^{-1}, $K_m = 7.30 \times 10^{-6}$ M).[12]

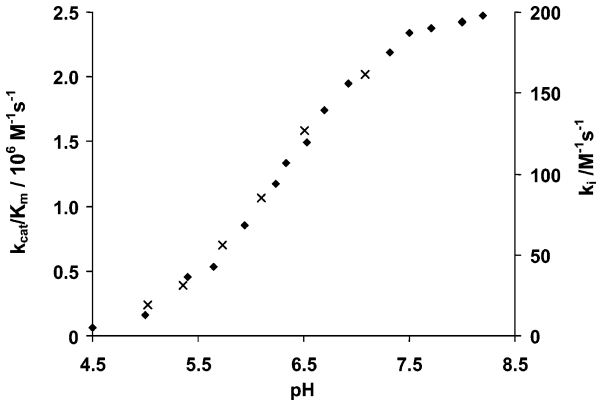
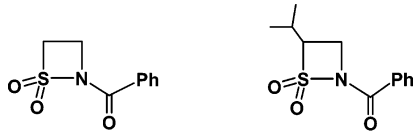


Figure 1. Plot of k_{cat}/K_m values (◆) for the hydrolysis of cephaloridine by P99 β-lactamase and k_i values (×) for the inactivation of P99 β-lactamase by **3** against pH.

N-Benzoyl β-sultam (3) was found to be a time-dependent inhibitor of P99 β-lactamase by incubating them together in a buffered solution and assaying aliquots for enzyme activity at pH 7 against the substrates benzylpenicillin or cephaloridine at various times. The rates of inactivation of P99 β-lactamase were the same whether enzyme activity was measured by the hydrolysis of benzylpenicillin or cephaloridine as substrate. Enzyme activity, manifested as the relative rate of enzyme-catalyzed substrate hydrolysis, decreased with time in an exponential manner to give apparent pseudo-first-order rates of inactivation. These first-order rate constants for inactivation exhibit a first-order dependence on β-sultam concentration and the corresponding second-order rate constants were obtained by dividing the observed pseudo first-order rates by the inhibitor concentration or graphically. At pH 7, the k_i value for the inactivation of P99 β-lactamase by **3** is 163 M^{-1} s^{-1}.

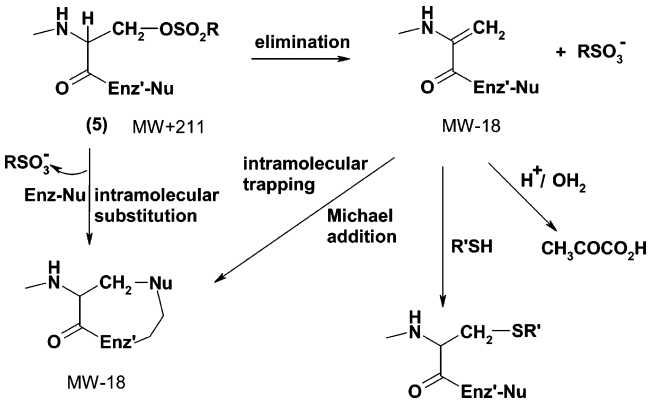


(3) (4)

The rate of inactivation of P99 β-lactamase by **3** shows a sigmoidal dependence on the pH of the incubation solution (Figure 1). For comparison, also shown in the same figure is the variation of k_{cat}/K_m for the hydrolysis of cephaloridine by P99 β-lactamase with pH. Both rate constants, k_i for inactivation and k_{cat}/K_m for hydrolysis, show a similar dependence on a catalytic group in the enzyme that ionizes with a pK_a of 6.5. From the pH rate profile it appears that the inactivation of P99 β-lactamase by **3** involves the same general base catalytic group as that used for the hydrolysis of the cephaloridine substrate. This is good evidence for active-site-directed inhibition and further evidence comes from competitive binding studies and ESIMS. In addition, elements of selectivity in the inhibition of β-lactamase are demonstrated by the introduction of a 4-isopropyl substituent (4) which decreases the rate of inactivation by over 2×10^3. There is also no inactivation by β-sultams of the metallo-enzyme class B β-lactamase *Bacillus cereus* 569/H (BcII) which contains a zinc (II) ion in its active site.

(20) Page, M. I. *Acc. Chem. Res*. **2004**, *37*, 297−303.
(21) Wood, J. M.; Page, M. I. *Trends Heterocycl. Chem.* **2002**, *8*, 19−34.
(22) King, J. F.; Rathore, R.; Lam, J. Y. L.; Guo, Z. R.; Klassen, D. F. *J. Am. Chem. Soc.* **1992**, *114*, 3028−3033.
(23) Baxter, N. J.; Rigoreau, L. J. M.; Laws, A. P.; Page, M. I. *J. Am. Chem. Soc.* **2000**, *122*, 3375−3385.
(24) Baxter, N. J.; Laws, A. P.; Rigoreau, L.; Page, M. I. *J. Chem. Soc., Perkin Trans 2* **1996**, 2245−2246.
(25) Page, M. I.; Williams, A. In *Organic and BioOrganic Mechanisms*; Longman, 1997.

Scheme 4



The presence of an excess of the tight binding substrate benzyl penicillin ($K_m = 7.30 \times 10^{-6}$ M) in the incubation solutions containing P99 β-lactamase and **3** retards the rate of inhibition. The inhibitor (3) exhibits no evidence of saturation kinetics and even as benzyl penicillin is turned over by the enzyme, the presence of the natural substrate in large excess and tight binding ensures that the β-sultam is prevented from reaching the enzyme's active site. The rate of inactivation of free P99 β-lactamase by the β-sultam is less than the rate of binding more substrate to the enzyme.

ESIMS of solutions of P99 β-lactamase incubated with **3** reveal both the native enzyme (MW 39 184 ± 2 Da) and enzyme bound to one equivalent of β-sultam (MW 39 395 ± 2 Da). The mass difference of +211 ± 4 is consistent with the sulfonylation of the active site serine residue Ser-64 to form an inactive sulfonyl enzyme (Scheme 3). Noncovalent binding of 1 equivalent of the ring-opened hydrolysis product of (3) would lead to a mass difference of +228. Enzyme inactivation is irreversible and there is no return of enzyme activity over 4 days. However, over a period of about an hour the mass reduces to that of the native enzyme less 18 (MW 39 166 ± 2 Da). This also occurs with other acyl substituted *N*-aroyl β-sultams and may be explained as follows.

It is likely that sulfonylation of the enzyme occurs at the active site hydroxyl group of Ser-64[26] to give an O-sulfonylated serine as has been shown by X-ray crystallography for the analogous reactions with the R61 D-ala D-ala transpeptidase[27] and elastase.[28] Scheme 4 shows a cartoon segment of the enzyme illustrating the sulfonylated serine residue and a nucleophile, Nu, at the active site. The inactive sulfonylated β-lactamase (5) may undergo a subsequent elimination reaction yielding dehydroalanine[29] or intramolecular nucleophilic displacement leading to cyclization and alkylation of a nucleophile in the active site (Scheme 4) both of which would yield MW(native − 18). These types of reactions have been demonstrated in proteins where a serine is phosphorylated[30] and in cases where an active site serine has been modified.[31] Displacement of the sulfonate anion could occur either directly by intramolecular nucleophilic substitution or indirectly by intramolecular Michael addition to the dehydroalanine residue (Scheme 4). If the observed MW(native − 18) is due to the formation of dehydroalanine, it should be detectable by Michael addition type trapping with thiols. After the P99 β-lactamase had been inhibited by the β-sultam (3), it was treated with ethanethiol and the ESIMS of the solution showed the presence of an ion with a mass increase of 65. The expected thiol adduct should show an increase in mass of 62 so the observed increase is compatible with the elimination of the sulfonate group from the sulfonylated enzyme to form an enzyme with a dehydroalanine residue at what is Ser 64 in the native enzyme. It is of interest to note that other serine enzymes sulfonylated with *N*-acyl β-sultams, R61 transpeptidase[27] and elastase,[28] do not undergo this elimination reaction. If the sulfonate is displaced by a side-chain lysine residue, so that Nu in Scheme 4 is then the amino group of lysine, or if the latter added to the dehydroalanine residue then protein degradation should yield some lysinoalanine. Amino acid analysis of the P99 β-lactamase after inactivation by (3) and alkali treatment gave no detectable lysinoalanine.[32−34]

N-Acylsulfonamides have been used previously to inactivate serine enzymes.[35] However, the mechanism invariably involves acylation and C−N bond fission with the serine hydroxyl group attacking the amide to displace the sulfonamide as the leaving group. Although the *N*-acyl β-sultams reported here are also formally *N*-acylsulfonamides, inactivation occurs by sulfonylation as a result of serine nucleophilic attack on the sulfonyl center and displacement of the amide as a leaving group. Thus *N*-acyl β-sultams appear to be unusual in undergoing preferential S−N over C−N fission with a serine enzyme.

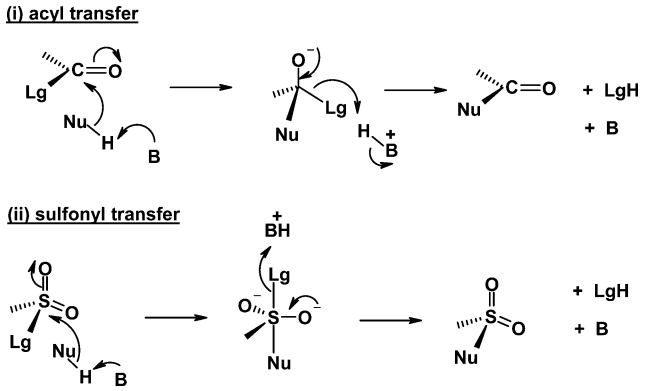
Nucleophilic substitution at acyl centers generally proceeds through the formation of an unstable tetrahedral intermediate (TI).[25] The reaction pathway involves a change in geometry as the carbonyl carbon is converted from three to four coordination. Furthermore, it is usually assumed that there is some preferential direction of nucleophilic attack such that the incoming nucleophile approaches at approximately the tetrahedral angle to the carbonyl group. If the leaving group is an amine and very basic, then bond fission and expulsion of the leaving group is often assisted by general acid catalysis.[20] By contrast, the associative mechanism for sulfonyl group transfer involves a transition state or intermediate with pentacoordinate trigonal bipyramidal geometry.[21] Here it is assumed that the preferential pathway involves the nucleophile taking up the apical position and the leaving group departing from an apical position of the trigonal bipyramidal intermediate (TBPI).[23] Given the preferential geo-

(26) Pratt, R. F. *Science* 1989, *246*, 917−919; Rahil, J.; Pratt, R. F. *Biochem. J.* **1991**, *275*, 793−795.
(27) Llinás, A.; Ahmed, N.; Cordaro, M.; Laws, A. P.; Frère, J.-M.; Delmarcelle, M.; Silvaggi, N. R.; Kelly, J. A.; Page, M. I. *Biochemistry* **2005**, *44*, 7738−7746.
(28) Hinchliffe, P. S.; Wood, J. M.; Davis, A. M.; Austin, R. P.; Page, M. I. *Org. Biomol. Chem.* **2003**, *1*, 67−80.
(29) Photaki, I. *J. Am. Chem. Soc.* **1963**, *85*, 1123−1126; Samuel, D.; Silver, B. L. *J. Chem. Soc.* **1963**, *85*, 1197−1198.
(30) Anderson, L.; Kelly, J. J. *J. Am. Chem. Soc.* **1959**, *81*, 2275−2276.
(31) Ako, H.; Foster, R. J.; Ryan, C. A. *Biochemistry* **1974**, *13*, 132−139; Weiner, H.; White, W. N.; Hoare, D. G.; Koshland Jr., D. E. *J. Am. Chem. Soc.* **1966**, *88*, 3851−3859.
(32) Galleni, M.; Lindberg, F.; Normark, S.; Cole, S.; Honore, N.; Joris, B.; Frère, J.-M. *Biochem. J.* **1988**, *250*, 753−760; Rahil, J.; Pratt, R. F. *Biochemistry* **1992**, *31*, 5869−5878.
(33) Madonna, M. J., Zhu, Y. F.; Lampen, J. O. *Nucleic Acids Res.* **1987**, *15*, 1877; Patchornik, A.; Sokolovsky, M. *J. Am. Chem. Soc.* **1964**, *86*, 1860−1861; Ziegler, K. *J. Biol. Chem.* **1964**, *239*, 2713−2714; Creamer, L. K.; Matheson, A. R. *N. Z. J. Dairy Sci. Technol.* **1977**, *12*, 253−259
(34) Cohen, S. A.; Pratt, R. F. *Biochemistry* **1980**, *19*, 3996−4003.
(35) Macdonald, S. J. F.; Belton, D. J.; Buckley, D. M.; Spooner, J. E.; Anson, M. S.; Harrison, L. A.; Mills, K.; Upton, R. J.; Dowle, M. D.; Smith, R. A.; Molloy, C. R.; Risley, C. *J. Med. Chem.* **1998**, *41*, 3919−3922; Macdonald, S. J. F., et al. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 243−246; Macdonald, S. J. F., et al. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 895−898.

Table 1. Second-Order Rate Constants, k_{OH}, for Hydroxide-Ion-Catalyzed Hydrolysis and k_i for the Inhibition of P99 β-lactamase by *N*-Aroyl β-Sultams at pH 7, 30 °C, and $I = 1.0$ mol dm^{-3}

N-aroyl β-sultams	*N*-*p*-nitro	*N*-*p*-chloro	*N*-benzoyl	*N*-*p*-methoxy
k_{OH}/M^{-1} s^{-1}	$(5.18 \pm 0.21) \times 10^4$	$(2.08 \pm 0.11) \times 10^4$	$(1.46 \pm 0.05) \times 10^4$	$(8.99 \pm 0.35) \times 10^3$
k_i /M^{-1} s^{-1}	$(1.50 \pm 0.17) \times 10^3$	$(3.69 \pm 0.25) \times 10^2$	$(1.63 \pm 0.02) \times 10^2$	48.7 ± 5.2

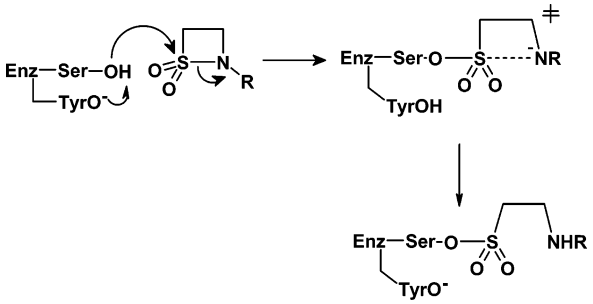
Scheme 5



metrical requirements for incoming nucleophiles and departing leaving groups for the two types of substitution, there must be a favored, relative position for the general base and general acid catalysts which is different for acyl and sulfonyl transfer (Scheme 5). It is often assumed, but with little actual supporting evidence, that enzymes catalyze reactions by an exquisite positioning of the catalytic groups involved in bond making and breaking.[36] However, for example, some β-lactamases efficiently catalyze the hydrolysis of both penicillins and cephalosporins even though there are significant geometrical structural differences, such as the distances and angles between the β-lactam carbonyl and nitrogen and the 3-carboxylic acid in penicillins and the 4-carboxylic acid in cephalosporins.[17] In addition, class C β-lactamases also catalyze reactions of carboxylic acid and phosphonic acid esters and amides.[18,23,37] If rigid positioning of catalytic groups within the active site was crucial then it is doubtful if an enzyme with a primary function as a catalyst for acyl transfer could be an effective catalyst for phosphonyl and sulfonyl transfer because of the geometrical differences in the displacement mechanisms.

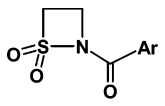
As active-site-directed sulfonylation is occurring in the inactivation of P99 β-lactamase by **3**, it appears that the catalytic machinery of P99 β-lactamase is catalyzing the sulfonyl transfer process. This indicates a significant degree of flexibility within the enzyme as the stereochemical requirements for catalysis of a reaction involving a trigonal bipyramidal arrangement for sulfonyl transfer are significantly different to those in reactions involving a tetrahedral intermediate required for acyl transfer. For example, P99 β-lactamase possesses an oxyanion hole used to stabilize the oxyanion in the tetrahedral intermediate (TI) formed after attack of the serine oxygen at a carbonyl center. 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 center is likely to occupy an equatorial

Scheme 6



position in a TBPI or transition state and will therefore lie at ca. 90° to the serine O−S bond. In addition there are significant differences in bond lengths and charge density distributions between the TI and TBPI of the two systems. Normally, C−N bond fission in the hydrolysis of amides requires general acid catalysis to facilitate amine expulsion, and in enzyme-catalyzed acyl transfer reactions, the proton donor has to be correctly positioned to protonate the leaving group nitrogen in the TI. The reactivity of *N*-acyl β-sultams and their amide leaving groups may allow ring opening to occur without *N*-protonation. An outline of a possible mechanism for sulfonyl transfer from the β-sultam to the active site serine is shown in Scheme 6. The catalysis of sulfonyl transfer in *N*-acyl β-sultams by P99 β-lactamase may therefore only be possible because ring opening occurs in a single step without general acid catalysis by the tyrosine or other 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 P99 β-lactamase which has the ability to catalyze phosphoryl transfer reactions, via trigonal bipyramidal intermediates.[37]

(ii) Structure−Activity Relationships in the Inactivation of β-Lactamase by *N*-Aroyl β-Sultams. The effect of changing the basicity of the leaving group in β-sultams on the rate of inactivation of β-lactamase was investigated by different aryl substituents in *N*-aroyl β-sultams (6). Electron-withdrawing



(6)

substituents in the *N*-acyl residue greatly increase the rate of inactivation and the second-order rate constants for inactivation, k_i, for a series of substituted derivatives are given in Table 1. These constants give a fairly unique opportunity to use linear free-energy relationships to help elucidate the reaction mechanism and the transition-state structure. The effective charge[38] on the nitrogen of *N*-aroyl β-sultams due to the inductive effect of the sulfonyl group is expected to be very similar to that in acyclic sulfonamides of 0.7+.[23,28] However, there is no data on the effect of an adjacent acyl group on this charge on the

(36) Menger, F. M. *Acc. Chem. Res.* **1985**, *18*, 128−134; Menger, F. M. *Acc. Chem. Res.* **1993**, *26*, 206−212; Dafforn, A.; Koshland, D. E., Jr. *Proc. Natl. Acad. Sci.* **1971**, *68*, 2463−2467; Kirby, A. J. *Acc. Chem. Res.* **1997**, *30*, 290−296; Kirby, A. J. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 706−724.

(37) Page, M. I.; Laws, A. P.; Slater, M. J.; Stone, J. R. *Pure Appl. Chem.* **1995**, *67*, 711−717; Laws, A. P.; Page, M. I.; Slater, M. J. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 2317−2322.

(38) Williams, A. *Adv. Phys. Org. Chem.* **1992**, *27*, 1−55.

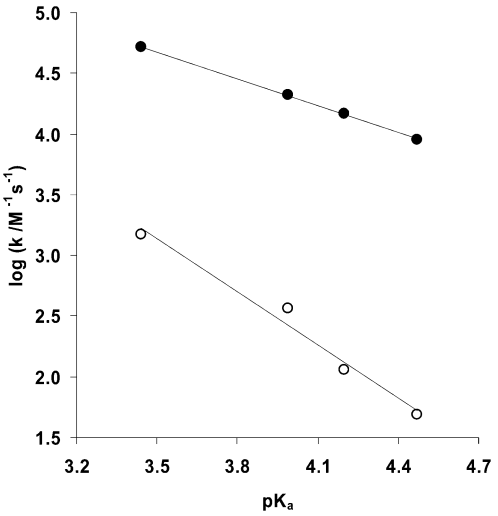
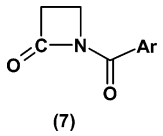


Figure 2. Bronsted-type plot of the second-order rate constants, k_{OH}, for the hydroxide-ion-catalyzed hydrolysis of *N*-aroyl β-sultams (●) and those for the inactivation of P99 β-lactamase by the same substrates (○) against the pK_a of the corresponding benzoic acid in 5 and 1% acetonitrile−water (v/v), respectively, at 30 °C, I = 1.0 M (KCl).

nitrogen in *N*-acylsulfonamides. If there is no leveling of resonance with the exocyclic amide, then presumably the charge could be as high as 1.4, as the effective charge on nitrogen in amides is also about 0.7+.[38] If say, amide resonance is reduced, then effectively *N*-acylsulfonamides could be treated as a sulfonamide to which is attached an electron withdrawing acyl group not involved in resonance stabilization. This would make the effective charge on the *N*-acylsulfonamide nitrogen greater than 0.7 but less than 1.4. A crude estimation may be obtained from the difference in σ and σ^- values for a para acyl substituent, i.e., 0.50 and 0.84, respectively.[25] The additional positive charge on the *N*-acylsulfonamide nitrogen due to an electron-withdrawing but nonresonating acyl substitutent would then be (0.5/0.84) × 0.7 = 0.42. This would make the effective charge on the *N*-acylsulfonamide nitrogen 1.1+. This will be used in subsequent discussions but a slightly smaller or larger value makes little difference to the conclusions.

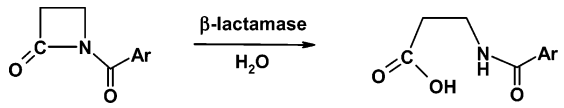
Although the pK_as of the amide leaving groups are not known, those of the corresponding carboxylic acids are readily available. Although it is probable that a Bronsted β_{lg} value for the leaving group based on the ionization of carboxylic acids would be larger than that based on the ionization of amides, this makes little difference to the conclusions of this study. A Bronsted-type plot of the logarithms of k_i against the pK_a of the analogous carboxylic acid is shown in Figure 2, the slope of which gives a Bronsted β_{lg} of −1.46, indicative of a large change in effective charge as the reactant is converted to its transition state. This value is indicative of a large development of negative charge on the nitrogen-leaving group compared with the relative positive effective charge of at least 1.1+ on nitrogen in the reactant. It suggests the leaving group is expelled as the amide anion and that there is significant S−N bond fission in the transition state (Scheme 6).

For comparison, the second-order rate constants, k_{OH}, for the alkaline hydrolysis of the same series of *N*-aroyl β-sultams are also given in Table 1. The corresponding Bronsted-type plot for these values generates a β_{lg} of −0.73 (Figure 2), indicative of a much smaller reduction in positive charge on the leaving nitrogen in the transition state for alkaline hydrolysis compared



(7)

Scheme 7



with the enzyme-catalyzed reaction. If a TBPI is formed along the reaction coordinate, then the effective charge on nitrogen is 0.7+ due to amide resonance with the exocyclic acyl group. The observed β_{lg} of −0.73 is thus compatible with a late transition state for rate-limiting formation of a TBPI or with its rate-limiting breakdown with little or no S−N fission. The alkaline hydrolysis of *N*-aryl β-sultams is thought to involve rate-limiting formation of a TBPI, whereas that for *N*-alkyl β-sultams occurs with rate-limiting breakdown with proton transfer to the departing amine nitrogen.[23] For *N*-acyl β-sultams, direct N protonation of the leaving group amide is thermodynamically unfavorable and amide anion expulsion is possible rather than protonation of the acyl oxygen and expulsion of the enol form of the amide. If an intermediate is formed in the reactions of *N*-acyl β-sultams and the rate of S−N bond fission and ring opening to expel the amide anion is greater than the rate of expulsion of hydroxide-ion, then formation of the intermediate will be rate limiting. Conversely, breakdown of the intermediate could become rate limiting if amide anion expulsion occurs at a slower rate than that for hydroxide-ion

Compared with the hydrolysis of *N*-acyl β-sultams by hydroxide-ion, β-lactamase appears to cause a move to a transition state much *later* along the reaction coordinate. The significantly different values of Bronsted β_{lg} indicate considerably more S−N bond fission in the transition state for the enzyme-catalyzed reaction. The enzyme appears to be using some of its catalytic machinery to facilitate the sulfonylation reaction, but the geometry of the displacement reaction (Scheme 6) is not ideal. The host of favorable noncovalent interactions, evolved by the enzyme to stabilize the transition state for the "natural" substrate, are not fully available to lower the activation energy for the β-sultams by the maximum amount. In terms of the Hammond Postulate,[25] this would be expected to lead to a later transition state as observed. It thus became of interest to see if compounds more structurally related to the "natural" one would show the opposite effect and exhibit an earlier transition state.

(iii) Structure−Activity Relationships in the Hydrolysis of *N*-Acyl β-Lactams. β-Sultams are the sulfonyl analogues of β-lactams, and it is of interest to compare their enzyme activity with similarly substituted *N*-acyl β-lactams (7), which are imides. Class C P99 β-lactamase does indeed catalyze the hydrolysis of *N*-acyl monocyclic β-lactams (7) to give the ring opened β-amidocarboxylic acid (Scheme 7). The second-order rate constants, k_{cat}/K_m, for a series of aryl-substituted derivatives are given in Table 2, but unlike the reaction with β-sultams, electron-withdrawing substituents in the *N*-acyl residue have little effect on the rate of the enzyme-catalyzed hydrolysis reaction. Despite the low level of molecular recognition, the pH-rate profile for the β-lactamase-catalyzed hydrolysis of **7** is sigmoidal and shows a dependence of enzyme activity on the

Table 2. Second-Order Rate Constants, k_{OH}, for Hydroxide-Ion-Catalyzed Hydrolysis and k_{Cat}/K_m for P99 β-lactamase Catalyzed Hydrolysis of *N*-Aroyl β-Lactams at pH 7, 30 °C, and I = 1.0 mol dm^{-3}

N-aroyl β-lactams	*N*-*p*-nitro	*N*-*p*-chloro	*N*-benzoyl	*N*-*p*-methoxy	*N*-*o*-carboxy
k_{OH} /M^{-1} s^{-1}	26.5 ± 1.2	13.8 ± 0.9	9.07 ± 0.58	7.27 ± 0.18	2.60 ± 0.08
k_{cat}/K_m/M^{-1} s^{-1}	1.77 ± 0.26	1.83 ± 0.09	1.96 ± 0.20	1.42 ± 0.07	4.74 ± 0.15

ionization of a group of pK_a 6.0, similar to that for the "normal" substrates such as benzylpenicillin and cephaloridine. The arguments used earlier to identify the effective charge on nitrogen in *N*-acylsulfonamides can be equally applied to imides and suggest that it is between 1.1+ and 1.7+. A Bronsted-type plot of the logarithms of k_{cat}/K_m against the pK_a of the analogous carboxylic acid is shown in Figure 3, the slope of which gives a Bronsted β_{lg} of −0.06. This value is much smaller than −1.46 seen with *N*-aroyl β-sultams (6) and is indicative of little development of negative charge on the nitrogen leaving group in the transition state compared with the relative positive effective charge on nitrogen in the reactant. It suggests C−N bond fission is not occurring in the transition state and that the rate-limiting step involves attack of the serine on the β-lactam carbonyl to form the tetrahedral intermediate (Scheme 2).

For comparison, the second-order rate constants, k_{OH}, for the alkaline hydrolysis of the *N*-acyl β-lactams (7) are also given in Table 2. Exocyclic C−N bond fission, as a result of hydroxide-ion attack on the exocyclic acyl center, is competitive with attack on the β-lactam carbonyl and the rate constants given in Table 2 are corrected for this and refer to those for β-lactam hydrolysis. The corresponding Bronsted-type plot for these values generates a β_{lg} of −0.55 that, relative to the enzyme-catalyzed hydrolysis, is indicative of more removal of the positive charge on the nitrogen on going from the reactant to the transition state. This is compatible with a late transition state for formation of the TI. For simple β-lactams of alkylamines or anilines alkaline hydrolysis is thought to involve rate-limiting formation of the tetrahedral intermediate. The rate of breakdown of the tetrahedral intermediate by C−N bond fission and ring opening probably involves proton transfer from water to nitrogen

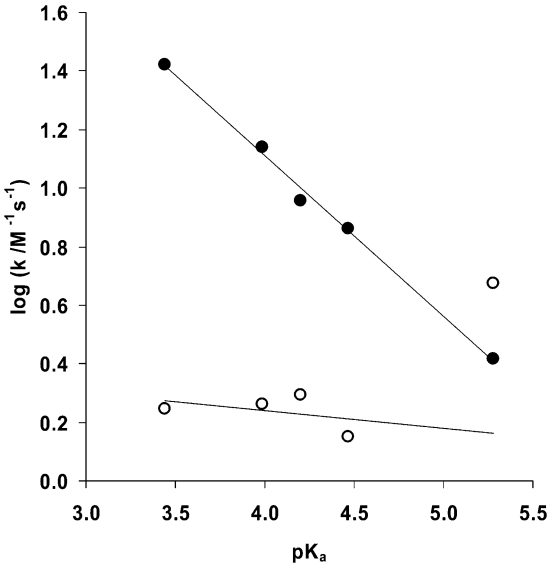
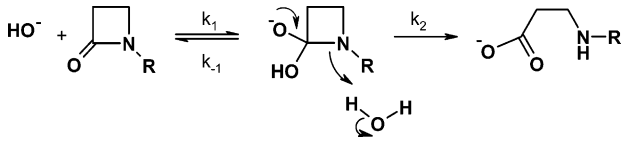


Figure 3. Bronsted-type plot of the second-order rate constants, k_{OH}, for the hydroxide-ion-catalyzed hydrolysis at the endocyclic center of *N*-aroyl β-lactams (●) and those for the hydrolysis catalyzed by P99 β-lactamase of the same substrate (○) against the pK_a of the corresponding benzoic acid in 1% acetonitrile−water (v/v) at 30 °C, I = 1.0 M (KCl).

Scheme 8



to expel the neutral amine,[39] the k_2 step, which is greater than the rate of expulsion of the poor leaving group, hydroxide-ion, the k_{-1} step, so making k_1, formation of the intermediate rate limiting (Scheme 8). For *N*-acyl β-lactams, N protonation is thermodynamically unfavorable, but amide anion expulsion may occur from the tetrahedral intermediate at a rate which is comparable or even faster than that for hydroxide ion, so that formation of the intermediate may also become rate limiting.

The second-order rate constants, k_{cat}/K_m, for the P99 β-lactamase catalyzed hydrolysis of *o*-carboxy **7** is 4.74 M^{-1} s^{-1}, which shows a 3-fold positive deviation from the Bronsted plot (Figure 3), whereas that for the hydroxide-ion-catalyzed hydrolysis, k_{OH}, fits its plot. This is indicative of a small recognition for the negatively charged carboxylate as occurs with normal β-lactam antibiotic substrates.[5]

Compared with the hydrolysis of *N*-acyl β-lactams by hydroxide-ion, β-lactamase appears to cause a move to a transition state earlier along the reaction coordinate. This suggests a reduced degree of bond making for the enzyme-catalyzed reaction between the incoming nucleophile and the β-lactam carbonyl carbon during formation of the tetrahedral intermediate. The enzyme must thus be facilitating expulsion of the amide anion from the *N*-acyl β-lactam compared with the latter's reaction with hydroxide-ion in solvent water and/or, despite the nominal weaker nucleophilicity of the serine hydroxyl, albeit enhanced by general base catalysis by Tyr-150 (Scheme 6), be causing an earlier transition state. This may be due to the stabilization brought about by the active site such as that of the oxyanion hole and compensation for the entropy loss in the bimolecular reaction.

Experimental Section

Synthesis. General Method for *N*-Aroyl β-Sultams. The aroyl chloride (2.6 g, 18.5 mmol) was added dropwise to a solution of 1,2-thiazetidine-1,1-dioxide (2 g, 18.6 mmol) and DMAP (0.15 g, 1.23 mmol) in anhydrous dichloromethane (50 mL) at −78 °C. The reaction mixture was stirred for 30 min before triethylamine (1.9 mL, 18.78 mmol) was added dropwise over 10 min at −78 °C forming a white precipitate. The mixture was then allowed to stir at room temperature for 24 h before the reaction mixture was filtered and the solvent removed by reduced pressure rotary evaporation at 30 °C. The pale-yellow oil was purified by column chromatography.

2-Benzoyl-1,2-thiazetidine-1,1-dioxide. Yield 1.61 g (41%); mp 95−96 °C; IR υ_{max} (cm^{-1}) ($CHCl_3$) 3021, 2995, 2918, 1674, 1450, 1355, 1326, 1216, 1162, 754.9; ^{1}H NMR δ ($CDCl_3$) 8.01 (2H, m, Ph), 7.56 (1H, m, Ph), 7.54 (2H, m, Ph), 4.31 (2H, t, J 7.45, CH_2-SO_2),

(39) Proctor, P.; Gensmantel, N. P.; Page, M. I. *J. Chem. Soc., Perkin Trans. 2.* **1982**, 1185−1192; Page, M. I. *Adv. Phys. Org. Chem.* **1987**, *23*, 165−270.

3.94 (2H, t, J 7.4, CH_2—NH); ^{13}C NMR δ ($CDCl_3$) 167.45 (C═O), 133.64 (quaternary carbon), 128.95 (CH (Ph)), 128.44 (CH (Ph)), 56.88 (CH_2—SO_2), 30.91 (CH_2—NH). High-resolution electrospray ionization mass spectrometry (HREI-MS) $[M + H]^+$ for $C_9H_9NO_3S$ calcd 212.0376, measured 212.0376.

2-(4′-Chlorobenzoyl)-1,2-thiazetidine-1,1-dioxide. Yield 0.89 g (39%); mp 84−86 °C; IR υ_{max} (cm^{-1}, neat) 1676, 1593, 1404, 1357, 1323, 1159, 1092, 1016; ^{1}H NMR δ ($CDCl_3$) 7.96 (2H, d, J 8.64,), 7.51 (2H, d, J 8.69,), 4.33 (2H, t, J 7.10, CH_2SO_2), 3.93 (2H, t, J 7.07, CH_2N); ^{13}C NMR 166.2 (C═O), 140.2 (quaternary carbon), 134.5 (quaternary carbon), 129.85 (ArCH), 129.34 (ArCH), 57.0 (CH_2SO_2), 30.9 (CH_2N); HREI-MS (EI) $C_9H_8ClNO_3S$ calcd 244.9908, measured 244.9905.

2-(4′-Nitrobenzoyl)-1,2-thiazetidine-1,1-dioxide. Yield 0.82 g (34%); mp 90−92 °C; IR υ_{max} (cm^{-1}) 3114, 3057, 1664, 1604, 1521, 1347, 1324, 1284, 1215, 1164; ^{1}H NMR δ ($CDCl_3$) 8.27 (2H, d, J 8.86), 8.06 (2H, d, J 8.83), 4.37 (2H, t, J 7.15, CH_2SO_2), 3.88 (2H, t, J 7.22, CH_2N); ^{13}C NMR 165.88 (C═O), 149.98 (quaternary carbon), 137.14 (quaternary carbon), 129.02 (ArCH), 123.45 (ArCH), 57.34 (CH_2SO_2), 30.86 CH_2N); HREI-MS (EI) $C_9H_8N_2O_5S$ calcd 256.0148, measured 256.0151

2-(4-Methoxybenzoyl)-1,2-thiazetidine-1,1-dioxide. Yield 1.2 g (53%); mp 98−99 °C; IR υ_{max} (cm^{-1}, Neat) 3035, 2977, 2841, 1662, 1606, 1513, 1327, 1262. 1201, 1156, 1031; ^{1}H NMR δ ($CDCl_3$) 8.01 (2H, d, J 8.95, Ha/Hc), 7.01 (2H, d, J 8.87, Hb/Hd), 4.27 (2H, t, J 7.35, CH_2SO_2), 3.98 (2H, t, J 7.22, CH_2N), 3.88 (3H, s, CH_3); ^{13}C NMR 163.95 (C═O), 132.25 (quaternary carbon), 130.67 (ArCH), 124.26 (quaternary carbon), 114.22 (ArCH), 55.56 (CH_2SO_2), 55.45 (CH_3), 30.72 (CH_2N).

General Method for *N*-Aroyl β-Lactams. To a −78 °C stirred solution of 2-azetidinone (0.5 g, 7.03 mmol) in dry dichloromethane (DCM) (20 mL) was added 4,4-(dimethylamino)pyridine (0.1 g, 0.82 mmol) and a solution of aroyl chloride (1.57 g, 8.46 mmol) in dichloromethane (10 mL) dropwise over 5 min. Triethylamine (0.98 mL, 7.02 mmol) was added dropwise over 10 min forming a white precipitate. The reaction mixture was stirred at −78 °C for 1 h and for a further 24 h at ambient temperature. DCM (10 mL) was added to the reaction mixture, and the mixture was washed with water (15 mL) and saturated brine (2 × 15 mL). The organic extract was dried over Na_2SO_4, and the solvent was removed by reduced pressure rotary evaporation at 30 °C to yield a pale-yellow oil, which was purified by column chromatography.

1-(4′-Methoxybenzoyl)-1-azetidin-2-one. Yield 0.6 g (42%); mp 125−127 °C; IR υ_{max} (cm^{-1}) ($CHCl_3$): 3020, 3009, 2975, 2912, 2842, 1784, 1668, 1606, 1325, 1259, 1195, 1108, 1028; ^{1}H NMR δ ($CDCl_3$) 7.99 (2H, d, J 8.88,), 6.92 (2H, d, J 8.97), 3.85 (3H, s, CH_3), 3.77 (2H, t, J 5.44, CH_2N), 3.02 (2H, t, J 5.45, CH_2CO); ^{13}C NMR δ ($CDCl_3$) 165.23 (C═O), 163.92 (C═O), 132.57 (quaternary carbon), 131.92 (ArCH), 123.76 (quaternary carbon), 113.948 (ArCH), 55.21 (CH_3) 36.45 (CH_2N), 34.44 (CH_2CO); HREI-MS $[M + H]^+$ for $C_{11}H_{11}NO_3$ calcd 206.0812, measured 206.0812.

1-(4′-Chlorobenzoyl)-1-azetidin-2-one. Yield 0.85 g (58%); IR υ_{max} (cm^{-1}) ($CHCl_3$) 3020, 1788, 1673, 1593, 1404, 1324, 1284, 1217, 1093; ^{1}H NMR δ ($CDCl_3$) 7.95 (2H, d, J 6.68,), 7.44 (2H, d, J 8.66), 3.77 (2H, t, J 5.5, CH_2N), 3.12 (2H, t, J 5.61, CH_2CO); ^{13}C NMR δ ($CDCl_3$) 165.01 (C═O), 163.84 (C═O), 139.51 (quaternary carbon), 131.14 (CH a/c), 130.1 (quaternary carbon), 128.44 (ArCH), 36.73 CH_2N), 34.99 (CH_2CO); HREI-MS $[M + H]^+$ for $C_{10}H_8NO_2Cl$ calcd 210.0316, measured 210.0316.

1-(4′-Nitrobenzoyl)-1-azetidin-2-one. Yield 1.23 g (80%); mp 130−131 °C; IR υ_{max} (cm^{-1}) ($CHCl_3$) 3020, 1787, 1686, 1599, 1523, 1397, 1309, 1252, 1204, 992; ^{1}H NMR δ ($CDCl_3$) 8.33 (2H, d, J 8.75, Hb/Hd), 8.14 (2H, d, J 8.74, Ha/Hc), 3.85 (2H, t, J 5.58, CH_2N), 3.2 (2H, t, J 5.57, CH_2CO); ^{13}C NMR δ ($CDCl_3$) 164.16 (C═O), 163.79 (C═O), 150.29 (quaternary carbon), 137.32 (quaternary carbon), 130.76 (CH b/d), 123.28 (CH a/c), 37.0 CH_2N), 35.5 (CH_2CO).

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 thermostated 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 thermostated with a Grant thermostated water circulator. pH stat experiments were performed on a ABU 91 Autoburet (Radiometer, Copenhagen, Denmark), controlled by a VIT 90 video titrator. The SAM 90 sample station incorporated an aluminum E2000 sample block rotor thermostated 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). The 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 meters. 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 6.99 ± 0.01, pH 4.01 ± 0.02, or pH 10.00 ± 0.02 calibration buffers.

AnalaR-grade reagents and deionized water were used throughout. Organic solvents were glass distilled prior to use and stored under nitrogen. 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. 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), MES (pK_a 6.1), MOPS (pK_a 7.2), TAPS (pK_a 8.4), CAPSO (pK_a 9.6), and CAPS (pK_a 10.4). Buffers were prepared by partial neutralization of solutions of their sodium salts to the required pH. 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 $\geq 2 \times 10^{-5}$ M ≤ 2×10^{-4} M to ensure pseudo-first-order conditions.

Hydroxide ion concentrations were calculated using pK_w (H_2O) = 13.83 at 30 °C.

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 preincubated 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. 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 modeled 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 thermostated at 30 °C before pneumatic injection into the reaction cell. Where applicable, the pH of solutions was measured prior to use. The photomultplier 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).

Enzyme Inhibition Studies. *Enterobacter cloacae* P99 β-lactamase was obtained from the Centre of Applied Microbiology and Research (Porton Down, UK).

Assays were performed at 30 °C in 0.1 M buffer with $I = 1.0$ M (KCl). The substrate was either benzylpenicillin (1.0×10^{-3} M) or cephaloridine (6.0×10^{-5} M), and enzyme activity was monitored by following the change in chromophore at 235 or 260 nm, respectively. Incubations of P99 β-lactamase and inhibitor were carried out at 30 °C in 0.10 M buffer and 8×10^{-5} M to 5×10^{-3} M β-sultam. Aliquots of this solution (50 μL) were assayed for P99 β-lactamase activity at various time intervals by injection into 0.1 M pH 7.0 MOPS and substrate. This gave final assay conditions of 6.0×10^{-5} M substrate and 1.5×10^{-8} M P99 β-lactamase. The assays of control incubations contained no β-sultam but matched the remaining methodology in all other 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 normalized with respect to the first-order exponential decrease in β-sultam concentration due to hydroxide-ion-catalyzed 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 experiment, time = 0, and multiplying by the β-sultam concentration at the time of the assay, time = t: normalized gradient at time t = (observed gradient/$[\text{sultam}]_0$) $\times$ $[\text{sultam}]_t$.

The average control gradient was taken as 100% enzyme activity. This value and the normalized gradients were used to calculate the percentage activity: % activity = (normalized gradient/average control gradient) $\times$ 100.

Pseudo-first-order rate constants of inactivation, k_{obs}, 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 incubation cell of the inactivation.

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

Lysinoalanine Analysis.

To a solution of P99 β-lactamase (1.27×10^{-8} M in deionized water) was added 1 equiv of **3**, and the reaction mixture was left at room temperature. The inhibition reaction was monitored by withdrawal of small aliquots of the reaction mixture, at fixed time intervals, and monitoring production of the inhibited enzyme using ESIMS. Maximum production of the species corresponding enzyme minus 18 mass units (E − 18) was observed after 90 min and corresponded to approximately 79% conversion of the stock enzyme. The pH of the solution was adjusted to 10.5 with the addition of the appropriate volume of 1 N NaOH and allowed to stand at this pH for a further 90 min. The resulting solution was neutralized with 1 N HCl, and the E − 18 complex was recovered from the excess salts by ultrafiltration through an ultrafiltration cartridge (cut off 10 000Da) and lyophilisation. The freeze-dried E − 18 complex was transferred to a pressure tube and combined with 10 mL of 6 N HCl. The contents were heated at 120 °C for 22 h. After heating the sample was cooled to room temperature and the amino acid solution neutralized using 1 N NaOH before being freeze dried. The lysinoalanine content of the protein hydrosylate was then determined by the method reported by Faist et al.[40]

Acknowledgment. We thank the University of Huddersfield for financial support.

Supporting Information Available: Complete refs 35b and 35c. This material is available free of charge via the Internet at http://pubs.acs.org.

JA056124Z

(40) Faist, V.; Drusch, S.; Kiesner, C.; Elmadfa, I.; Erbersdobler, H. F. *Int. Dairy J.* **2000**, *10*, 339−346.