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Novel Mechanism of Inhibiting β-Lactamases by Sulfonylation Using β-Sultams

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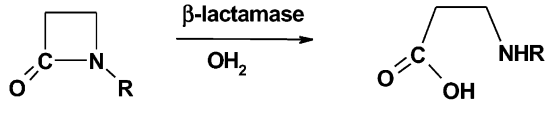
Abstract—β-Sultams are the sulfonyl analogues of β-lactams and *N*-acyl β-sultams are novel inactivators of the class C β-lactamase of *Enterobacter cloacae* P99. The rates of inactivation show a similar pH-rate dependence as that exhibited by the β-lactam antibiotics and with ESIMS data it is suggested that β-sultams sulfonylate the active site serine residue to form a sulfonate ester.

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 catalyse the hydrolysis of the β-lactam to give the ring opened and bacterially inert β-amino acid (Scheme 1). Bacteria are evolving which produce new β-lactamases which can catalyse the hydrolysis of β-lactams previously resistant to enzyme degradation. For example, when the carbapenems, such as imipenem, were first introduced in the 1970's they were seen as versatile broad-spectrum antibacterials resistant to hydrolysis by most β-lactamases. However, 'carbapenemases' are now increasingly produced by a variety of bacteria[2] and there are over 200 different β-lactamases which have now been identified.[3]

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 non-covalently bound intermediates.[4] On the basis of their amino acid sequences, the serine β-lactamases are sub-divided into three classes—A, C and D—whereas the class B β-lactamases consist of the zinc enzymes.[3–5] The class C β-lactamases of Gram-negative bacteria are widely expressed and are not significantly inhibited by clinically used β-lactamase inhibitors such as clavulanic acid. 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 four class A[6] and two class C[7] 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.

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.[4] In class C β-lactamases 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[7–9] (Scheme 2, B = TyrO$^-$). The pK_a of the ionising group acting as a general base obtained from pH-rate profiles is 6.3 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 undergoing dissociation.[9] In common with serine proteases, the serine β-lactamases possess an oxyanion pocket which donates two hydrogen bonds to the β-lactam



Scheme 1.

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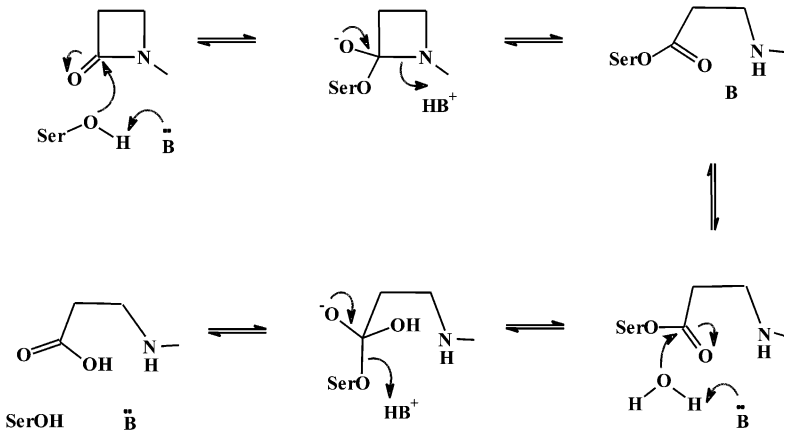


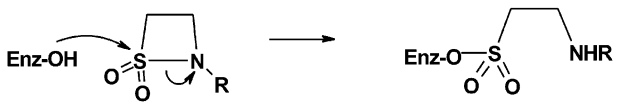
Scheme 2.

carbonyl oxygen, inducing polarisation to facilitate nucleophilic attack and stabilising the oxyanion of the tetrahedral intermediate.

There is an obvious interest in finding new inhibitors of the β-lactamases and the sulfonation of serine enzymes offers an interesting but largely unexplored strategy for inhibition as an alternative to the traditional mechanism-based acylation process. In addition to their normal acyl substrates, many serine enzymes are known to react with other electrophilic centres such as phosphonyl derivatives.[10] The main reason why sulfonation is not a well studied process is because sulfonyl derivatives are much less reactive than their acyl counterparts. For example, sulfonamides are extremely resistant to alkaline and acidic hydrolysis and, in general, sulfonyl transfer reactions are 10^2–10^4 fold slower than the corresponding acyl transfer process.[11] However, we have recently shown that β-sultams (**1**), which are the sulfonyl analogues of β-lactams are 10^2–10^3 fold more reactive than β-lactams in their rates of alkaline and acid hydrolysis[12] and therefore we wished to explore the possibility of using β-sultams as a novel mechanism of inhibiting serine β-lactamases (Scheme 3).

Results and Discussion

P99 β-Lactamase is a serine class C β-lactamase enzyme derived from the Gram negative bacteria *Enterobacter cloacae* and is capable of hydrolysing a wide variety of β-lactam based substrates at a rapid rate.[9] *N*-Benzoyl-β-sultam (**2**) 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 substrate cephaloridine at various times. Enzyme activity, manifested as the relative rate of enzyme catalysed substrate hydrolysis, decreased with time in an exponential manner to give apparent pseudo



Scheme 3.

first-order rates of inactivation. The 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 *N*-benzoyl-β-sultam (**2**) is $163\,M^{-1}\,s^{-1}$ showing that *N*-acyl-β-sultams are a novel class of β-lactamase inhibitors.

The rate of inactivation of P99 β-lactamase by *N*-benzoyl-β-sultam (**2**) shows a sigmoidal dependence on the pH of the incubation solution (Fig. 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. From the pH-rate profile it appears that the inactivation of P99 β-lactamase by *N*-benzoyl-β-sultam (**2**) involves the same catalytic groups as used for the hydrolysis of the substrate. 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 which ionises with a pK_a of 6.3 ± 0.2, which is good evidence for active-site directed inhibition. Elements of selectivity in the inhibition of β-lactamase are demonstrated by the introduction of a 4-isopropyl substituent (**3**) (k_i $0.07\,M^{-1}\,s^{-1}$) which decreases the rate of inactivation by over 2×10^3. There is 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.

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 *N*-benzoyl-β-sultam (**2**) retards the rate of inhibition, indicative of the β-sultam reacting at the active site. Electrospray ionisation-mass spectrometry (ESIMS) of solutions of P99 β-lactamase incubated with *N*-benzoyl-β-sultam (**2**) reveal both the native enzyme (M_r $39{,}184 \pm 2$ Da) and enzyme bound to one equivalent of β-sultam (MW $39{,}395 \pm 2$ Da). The mass difference of $+211 \pm 4$ is consistent with the sulfonylation of the active site serine residue Ser-64 to form an inactive sulfonyl enzyme (Scheme 3). The enzyme bound hydrolysis product would give a mass of 39,412. There is X-ray crystallographic

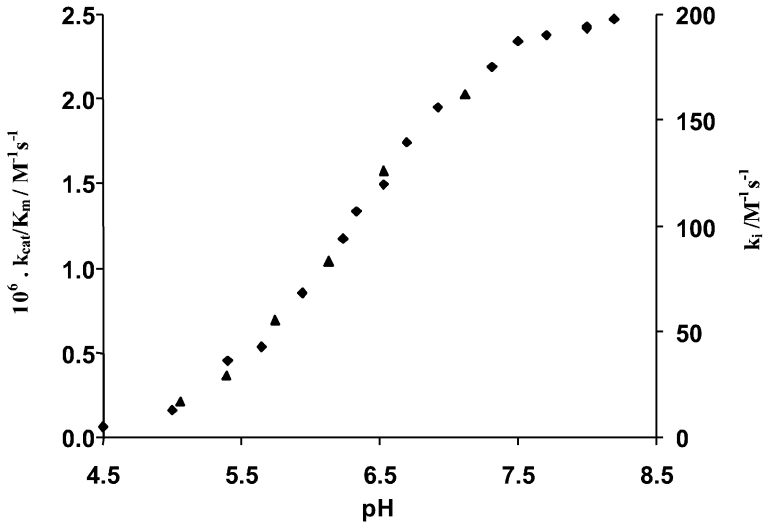


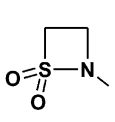
Figure 1. Plot of k_{cat}/k_m values (◆) for the hydrolysis of cephaloridine by P99 β-lactamase and k_i values (▲) for the inactivation of P99 β-lactamase by *N*-benzoyl-β-sultam (**2**) against pH.

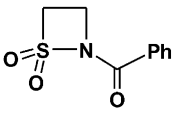
evidence for the sulfonylation of the active site serine of elastase inactivated by β-sultams.[13] Non-covalent binding of one equivalent of the ring opened hydrolysis product of **2** would lead to a mass difference of +228. Enzyme inactivation is irreversible and there is no return of enzyme activity over 4 days.

N-Acylsulfonamides have been used previously to inactivate serine enzymes.[14] 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 are formally *N*-acylsulfonamides inactivation occurs by sulfonylation as a result of serine nucleophilic attack on the sulfonyl centre and displacement of the amide as a leaving group. Thus *N*-acyl β-sultams appear to be unusual in undergoing preferential S–N over C–N bond fission with a serine enzyme.

As active-site directed inhibition is occurring in the inactivation of P99 β-lactamase by *N*-benzoyl β-sultam (**2**) it appears that the catalytic machinery of P99 β-lactamase is catalysing the sulfonyl transfer process, particularly general base catalysis presumably by Tyr-150. 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.[11,15] Although C–N bond fission in the hydrolysis of amides requires general acid catalysis to facilitate amine expulsion, the reactivity of *N*-acyl-β-sultams and their amide leaving groups may allow ring opening to occur without *N*-protonation. 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 Tyr-150. 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 catalyse phosphoryl transfer reactions, via trigonal bipyramidal intermediates.[15]

In summary, β-sultams are a novel class of inactivators of class C β-lactamases which appear to sulfonylate the active site serine residue to form a sulfonate ester using the enzyme's normal catalytic apparatus.


(1)



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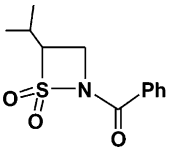

(3)

Acknowledgements

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