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Reactivity of β-lactams and phosphonamidates and reactions with β-lactamase

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Abstract: Thermodynamically strained four membered cyclic ß-lactams do not show a corresponding kinetic effect in the rates of their ring opening reactions. Contrary to expectations, breaking the C-N bond in these strained ring structures appears to be a relatively difficult process. A four membered cyclic phosphonamidate, on the other hand, undergoes rapid hydrolysis in water with exclusive endocyclic P-N fission with a rate difference, compared with an acyclic analogue, of greater than 5×10^8. Two diastereoisomers of a phosphonamidate completely and irreversibly inactivate the class C ß-lactamase (P99) from *Enterobacter cloacae* in a time dependent manner. There is diastereoselectivity shown by the two inactivators and the more active one has proline displaced by the enzyme. Protonation of the phosphonamidate nitrogen is almost certainly required to expel neutral proline. Effective enzyme catalysed phosphonylation is surprising in view of the presumed different geometries for the reaction (trigonal bipyramidal) compared with that for acylation (tetrahedral).

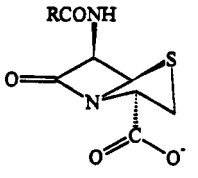
Chemical Reactivity of ß-Lactams

Four membered ß-lactams occur relatively rarely in nature, therefore it is not surprising that the biological activity of these compounds should be attributed to the chemical reactivity of the ß-lactam ring. Shortly after the introduction of penicillin to the medical world it was suggested that the antibiotic's activity was due to the inherent strain of the four-membered ring or to reduced amide resonance.[1] The non-planar butterfly shape of the penicillin molecule (1) could reduce amide resonance and thus increase the susceptibility of the carbonyl group to nucleophilic attack, compared with planar amides. However, the evidence to support an unusually strained or an amide-resonance inhibited ß-lactam in penicillin is ambiguous.[2]

It is estimated that resonance stabilises amides by about 19 kcal mol^{-1}. If delocalisation is completely inhibited in an amide then the rate of nucleophilic attack could occur up to 10^{13}-fold faster than in the analogous resonance-stabilised system. The strain energy of a four-membered ring is 26-29 kcal mol^{-1} and a reaction involving opening of the ß-lactam ring could therefore take place faster than the analogous bond fission process in a strain-free amide by a factor of up to 10^{20}, although the total strain energy of four-membered rings is probably not released until there is significant bond extension. If strain or resonance inhibition is even slightly significant in penicillins and cephalosporins their effects should therefore be easily observable.

The rates of alkaline hydrolysis of ß-lactams exhibit a first-order dependence on hydroxide ion concentration and show a Bronsted β_{lg} value of -0.44 (Figure 1) which is indicative of rate-limiting formation of the tetrahedral intermediate. It is only ß-lactams of weakly basic amines which show enhanced reactivity. The rate enhancement of ß-lactams compared with acyclic amides thus depends upon the basicity of the leaving group amine. ß-Lactams of weakly basic amines (pK_a of conjugate acid less than 4) are ca. 500-fold more reactive than an acyclic amide of the same amine. ß-Lactams of basic amines show very similar reactivity to that of analogous non-cyclic amides.

Fusing the ß-lactam ring to a five-membered ring to make 1-aza-bicyclo-[3.2.0]heptan-2-ones increases the reactivity by ca. 100-fold but does not significantly change the Bronsted β_{lg} value, which is -0.55 for the bicyclic system. Although the rate enhancement is substantial, it is hardly of the magnitude expected from the release of strain energy in opening a four-membered ring, or from a system in which amide resonance is significantly inhibited. It is worth noting that monocyclic ß-lactams of weakly basic amines can be as chemically reactive as penicillins and cephalosporins. It is not necessary to make the ß-lactam part of a bicyclic system to have a reactive amide.



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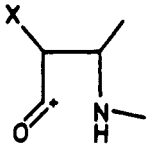

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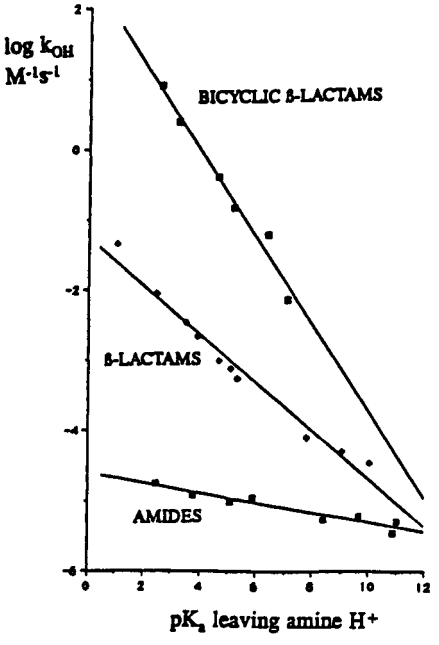
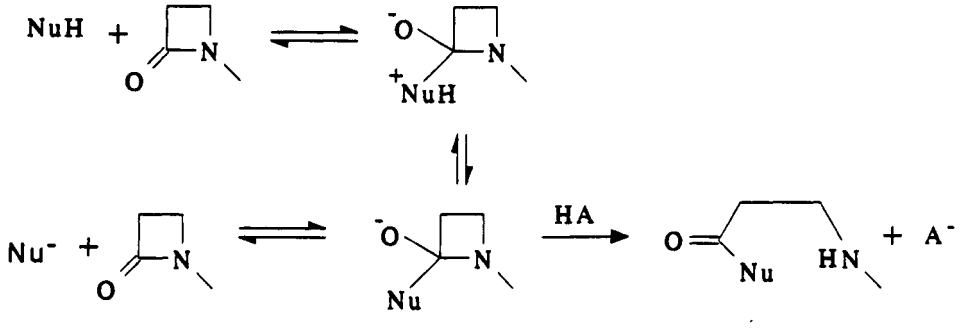


Figure 1 A plot of the second-order rate constants k_{OH} for alkaline hydrolysis against the pK_a of the conjugate acid of the leaving group amine

Remarkably, the logarithms of the pseudo first-order rate constants for the acid catalysed hydrolysis of some ß-lactam antibiotics and derivatives increase linearly with decreasing H_o values up to -5.[3] This is quite unlike the behaviour of other amides, for which the rate of hydrolysis passes through a maximum, attributed both to complete conversion of the amide into its O-conjugate acid and to decreasing water activity. This indicates that the ß-lactams are far less basic than normal amides for O-protonation and that a different mechanism of hydrolysis is operating. Neither the nitrogen nor the oxygen of the bicyclic ß-lactams is sufficiently basic for substantial conversion to the conjugate acid; the pK_a for O- or N-protonation must be < -5. This behaviour is not peculiar to bicyclic ß-lactams, since monocyclic ß-lactams show similar reactivities and behaviour. The mechanism of the acid catalysed hydrolysis of ß-lactams appears to be a unimolecular A-1 type process, with N-protonation of the ß-lactam followed by ring opening and formation of an acylium-ion (2).

Nucleophilic substitution at the carbonyl of ß-lactams is an acyl transfer process involving covalent bond formation between the carbonyl carbon and the nucleophile and C-N bond fission of the ß-lactam (Scheme 1). In these types of reactions the mechanism involves a two-step process.[1,2] Covalent bond formation to the incoming nucleophile occurs before C-N bond fission resulting in the reversible formation of a tetrahedral intermediate. Contrary to expectations, opening the four-membered ring is not a facile process.[4] In many of these nucleophilic substitution reactions the rate limiting step is not often the first addition step but a subsequent one which may sometimes even be ring opening itself.[2]

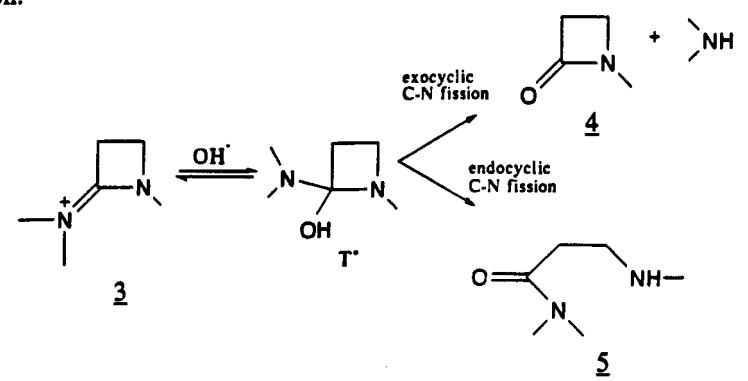


Scheme 1

Those reactions which involve the attack of a neutral nucleophile with an ionisable hydrogen (Scheme 1) invariably require general base catalysis to remove the proton.[1,2] The requirement for proton removal is paramount - and in extreme cases only the reaction of the anionic nucleophile is observed (Scheme 1). For example, there is no pH independent reaction of water with penicillin and alcohols react only through their anions.[5] The importance of general base catalysis is a reflection of the fact that contrary to expectations penicillins are not powerful acylating agents.[5]

Similarly, C-N bond fission requires protonation of the amine nitrogen.[1,2] Amine anion expulsion from the tetrahedral intermediate is an unlikely process even considering the release of strain energy facilitating ring opening and C-N bond fission. Consequently, general acid catalysed breakdown of the tetrahedral intermediate is often observed. It has been suggested that ring opening does not occur by stretching of the C-N bond but rather by a rotational motion.[4,6] This minimises strain effects and maximises favourable orbital interactions. The unusual mode of C-N bond fission could have interesting consequences in the enzyme catalysed hydrolysis of ß-lactams for the geometrical relationship of the proton donor in the protein and the amine leaving group.

The hydrolysis of azetidin-2-ylidenammonium salts (3) can give two products depending on which C-N bond is broken[4]. The alkaline hydrolysis proceeds by formation of the tetrahedral intermediate, T°, which can undergo exocyclic C-N bond fission to give the ß-lactam (4) and an amine, or endocyclic C-N bond fission to give the ß-amino amide (5) (Scheme 2). The breakdown of the tetrahedral intermediate T° is general acid catalysed and both exocyclic and endocyclic C-N bond fission require proton transfer to the departing amine nitrogen. Despite the release of strain on opening the four-membered ring, the endocyclic nitrogen still needs protonation to aid C-N bond fission.[4]

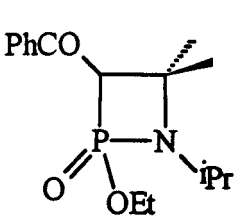


Scheme 2

The major product of the hydrolysis of azetidinyl amidinium salts is the ß-lactam. Partitioning of the tetrahedral intermediate T° favours exocyclic C-N bond fission rather than four-membered ring opening by endocyclic C-N bond fission, which would be expected to be facilitated by the release of strain energy. The apparent reluctance of the four-membered ring to open is not the result of differential basicities of the two nitrogens, entropic factors, or stereoelectronic effects.[4]

Reactivity of Four-Membered Cyclic Phosphonamidates

Four co-ordinate phosphorus derivatives usually have a tetrahedral geometry around phosphorus. However, there appears to be significant delocalisation of the nitrogen lone pair to the phosphoryl oxygen in phosphonamidates.[7] Nevertheless, the nitrogen attached to phosphoryl centres is more basic than the corresponding amide and protonation of phosphonamidates occurs on nitrogen rather than oxygen.[7] Phosphonamidates are, usually, relatively hydrolytically stable and only undergo ready hydrolysis at the extremes of pH, for P-N fission protonation of N is necessary.[7]



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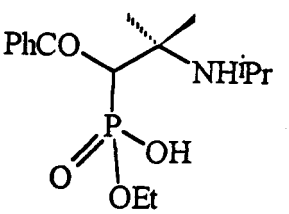

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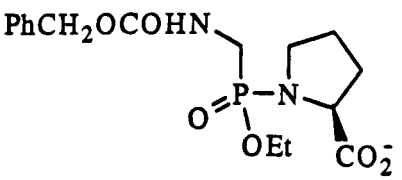
The four-membered cyclic phosphonamidate (6) contains a four-coordinate phosphorus with a CPN bond angle of 81°, the small bond angle about phosphorus allowing for the longer bonds between it and adjacent atoms.[8]. Hydrolysis occurs rapidly in water with exclusive endocyclic P-N fission to generate the corresponding ring opened amino phosphonate (7), easily identified by nmr in D_2O - in particular the ^1H-C-N-^{31}P coupling disappears upon P-N fission. The rate of hydrolysis above pH8 is first order in hydroxide and shows a second-order rate constant k_{OH} of 2.41 x 10^4 dm^3 mol^{-1} s^{-1} at 30° in water.[9] A corresponding acyclic phosphonamidate (8) undergoes hydroxide ion catalysed hydrolysis but with P-O fission to liberate ethanol and with a second order rate constant of 1.8 x 10^{-5} dm^3 mol^{-1} s^{-1}. Given P-N fission in the cyclic derivative but P-O fission in the acyclic system, the rate enhancement for P-N fission in the four-membered ring is at least 10^9 - a surprising contrast to the hydrolysis of ß-lactams and amides.

It has long been known that cyclic phosphate esters, eg ethylene phosphate, hydrolyse much faster than their acyclic analogues, although whether this is due to release of strain or difference in solvation energy remains controversial.[10] In the four-membered phosphonamidate (6) there is presumably ground-state strain due to the unfavourable endocyclic bond angle of 81° about phosphorus compared with an ideal tetrahedral geometry of 109°. Nucleophilic substitution at phosphoryl centres usually occurs through the formation of a trigonal bipyramidal (TBP) intermediate.[7] When phosphorus is contained in a four- or five-membered ring, the ring prefers to be attached apical/equatorial ie an approximately 90° endocyclic bond angle around phosphorus. Attack by hydroxide ion on phosphorus in (6) is therefore accompanied by a large relief in ground-state bond angle strain upon formation of the TBP intermediate, compared with the analogous acyclic derivative. This is in contrast to the ß-lactam/amide systems where there is relatively little relief of ground-state bond angle strain around the ß-lactam carbonyl carbon upon formation of the tetrahedral intermediate ie approximately 90° compared with 120° in the ground-state and 90° compared with 109° in the tetrahedral intermediate.

The phosphonamidate (6) also undergoes an acid catalysed hydrolysis and shows an entropy of activation of -9 cal K^{-1} mol^{-1}. This may be indicative of a unimolecular process similar to that observed for ß-lactams. The mechanism would then involve the intermediate formation of the three coordinate metaphosphate-type intermediate (9).



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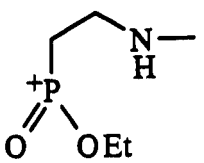

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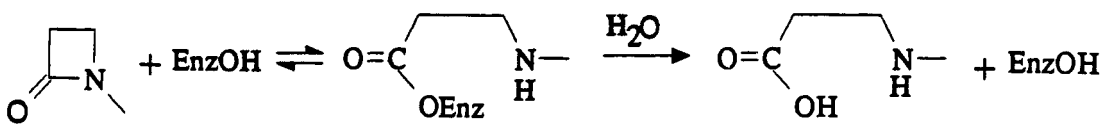
ß-Lactamase Enzymes

Many bacteria are resistant to the normally lethal action of ß-lactam antibiotics because of their ability to produce a ß-lactamase enzyme which catalyses the hydrolysis of the ß-lactam to its corresponding amino acid, devoid of antibacterial activity (Scheme 3). There are two main classes of ß-lactamases - the serine and the zinc enzymes.[2] Based on their primary structures there have been three common classes recognised - A, B and C. Class B is distinguished by the requirement of zinc ions for activity whereas classes A and C, although structurally distinguishable, act by using an active site serine and are characterised by the intermediate formation of an acyl-enzyme (Scheme 3).



Scheme 3

Investigating the efficiency and mechanism of enzyme catalysis often involves determining the effect on reactivity of changing substituents in the substrate or the enzyme and the reaction conditions such as the nature of the solvent. Changes in the substrate structure may modify its intrinsic chemical reactivity in terms of inductive, resonance and steric effects on the ease of bond-making and breaking, in addition to modifying its binding interactions with the enzyme, and hence may have a complex

effect on catalytic activity. In order to deduce the importance and role of interactions between the enzyme and substrate it is necessary to separate the intrinsic effects to give an enzyme rate enhancement factor (EREF) so that those effects resulting from specific interactions can be identified.[12]

The class A ß-lactamases are produced by both Gram-positive and Gram-negative bacteria. Crystal structures of the Gram-positive ß-lactamase from *Staphylococcus aureus*[13] and from *Bacillus licheniformis*[14] have been reported at 2.0 Å resolution. The crystal structure of a Gram-negative ß-lactamase, RTEM-1, from *Escherichia coli* at 2.5 Å resolution has been published.[15] The crystal structure of the class C ß-lactamase from *Citrobacter freundii* has been refined at 2.0 Å resolution.[16] There have been no structures reported of good inhibitors bound to ß-lactamase, although there has been a recent publication of the structure of an acyl-enzyme of a mutant RTEM-1 ß-lactamase (glu 166 asn) which catalyses the acylation but not the deacylation reaction.[17] Although useful, mechanistic conclusions based on crystal structures remain speculative.

Although the different ß-lactamase have topographically similar crystal structures there are significant differences in the relative orientations of secondary structural units and conformation of some of the loop regions. There are two closely packed domains and the polypeptide chain crosses twice from one domain to the other.[13] The active site serine 70 is located at the interface between the domains and at the amino terminus of a largely desolvated helix. Within the active site region surrounding the catalytically active serine 70 there is an invariant lysine residue (73 in class A) one helical turn after the serine. The serine oxygen to lysine nitrogen distance varies between 2.5 and 2.8 Å. There is also a conserved lysine residue (234 in class A and 315 in class C) located on the innermost strand of the ß-pleated sheet that borders the active site. This residue has been assumed to interact with the substrate carboxylate.[13] Another potential catalytic group within the active site is glu 166. The glu-166 carboxylate oxygen-lysine 73 nitrogen distance is 2.8 to 3.4 Å whereas the glu-166 oxygen and ser-70 oxygen are 3.5 to 4.0 Å apart. Glu-166 is apparently held pointing into the active site by the adjacent peptide being in the cis conformation.[13]

By analogy with the mechanism of acylation reactions of ß-lactams involving ring opening, the ß-lactamase catalysed reaction probably also involves the formation of a tetrahedral intermediate (Scheme 1). Although the acyl transfer process requires a number of steps involving proton transfer, little is known about the mechanism of these in the enzyme reaction and any consequent requirements for general acid-base catalysis.[11] The three common residues, besides serine 70, thought to be important are glu-166, lys-73 and lys-234. Their relative roles in catalysis remain speculative. It is assumed that any reaction catalysed by the enzyme, involving breaking a bond to nitrogen and expulsion of an amine will require protonation of the nitrogen.

Inhibition of ß-Lactamase with Phosphonamidates

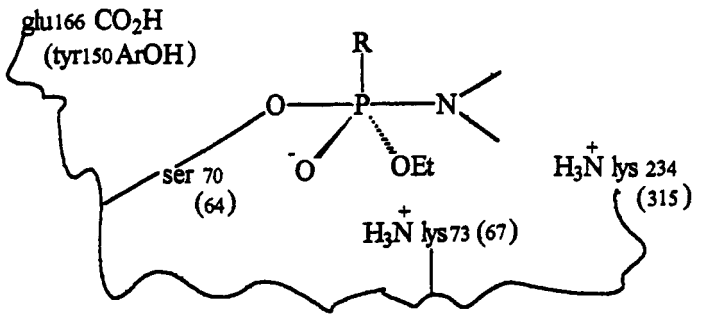
Four co-ordinate tetrahedral phosphorus derivatives are known to be inhibitors of serine enzymes either by acting as phosphorylating agents or as transition state analogues of the tetrahedral intermediate.[18] The phosphonamidates (8) exist as a pair of diastereoisomers which may be separated by HPLC. Both diastereoisomers completely and irreversibly inactivate the class C ß-lactamase (P99) from *Enterobacter cloacae*, but not class A ß-lactamases, in a time dependent manner.[19] The pseudo first-order rate constants for inactivation (k_{inact}), measured by the loss of activity of the ß-lactamase catalysed hydrolysis of benzylpenicillin, have a first-order dependence on the phosphonamidate concentration. Saturation kinetics are not observed, but the second-order rate constants for inactivation show a selectivity of 36 fold between the two diastereoisomers. However, the second-order rate constants are dependent on the enzyme concentration. For example, the rate constant for the most reactive diastereoisomer goes from about 300 M^{-1} s^{-1} to 10 M^{-1} s^{-1} upon changing enzyme concentration from 10^{-7} M to 10^{-6} M. This may be indicative of enzyme dimerisation with the dimer being less reactive towards inactivation than the monomer.

The time dependent inactivation of ß-lactamase suggests covalent bond formation between the enzyme and the phosphonamidates. This could be due to displacement of either ethanol or proline from the phosphonamidate (8) by the active site serine. In the absence of enzymes, the phosphonamidate (8) undergoes predominantly P-N cleavage in acid solution but P-O fission at high pH.[20] There is no evidence for ethanol formation during the inactivation of ß-lactamase by the more active diastereoisomer. Electrospray mass spectrometry (ESMS) indicates that the more active diastereoisomer becomes covalently linked to the enzyme during the inactivation. The positive ion ESMS mass transformed spectrum of the (P99) ß-lactamase shows a molecular mass Mr = 39,202 ± 5.3. After incubation with the more reactive phosphonamidate (8) the mass spectrum gives Mr = 39,442 ± 3.1 suggesting that the phosphonylated enzyme loses the proline residue (calculated Mr =

39,456). Inactivation thus appears to be accompanied by the formation of a covalently bound 1:1 enzyme:inactivator complex in which a proline residue has been displaced by a nucleophilic group on the enzyme - presumably the active site serine.

The second-order rate constant for deactivation of ß-lactamase by the phosphonamidate shows a sigmoidal dependence upon pH with an apparent pK_a of 6.3. The pH-rate profile for the ß-lactamase C catalysed hydrolysis of penicillins and cephalosporins is a typical bell-shape with two important ionisable groups of pK_a 6.1 and 10.3, indicating that the catalytically active form of the enzyme is EH. The pH dependence of inactivation is therefore compatible with, but does not prove, a reaction between the enzyme EH and the conjugate acid of the phosphonamidate or its kinetic equivalent. Direct displacement of the extremely poor leaving group the proline anion, via an $S_N2(P)$ mechanism is highly unlikely. Protonation of the phosphonamidate nitrogen is almost certainly required to expel neutral proline.[7] However, the pK_a of the protonated phosphonamidate nitrogen is less than zero so that the concentration of the protonated species present at pH 7 is very small. The observed loss of proline can be accounted for if a general acid is present in the enzyme's active site and which is in a position to be able to protonate the nitrogen once the trigonal bipyramidal intermediate has been formed (10). It is also possible that intramolecular proton transfer could occur from the undissociated carboxylic acid of the phosphonamidate (8) or, indeed, intermolecularly and non-enzymatically from the hydronium ion H_3^+O.

In the absence of the enzyme, the phosphonamidates (8) are stable under the conditions and timescale required for inactivation, the first order rate constant for the hydrolysis of (8) in water at pH 7 is 2×10^{-7} s^{-1}. The second-order rate constant for the hydroxide-ion catalysed hydrolysis is 2×10^{-5} M^{-1} s^{-1} and involves P-O bond fission so the enzyme rate enhancement factor for P-N fission is at least 10^6. Effective enzyme catalysed phosphonylation is, perhaps, surprising in view of the presumed different geometries for the reaction (trigonal bipyramidal (10)) compared with that for acylation (tetrahedral).[21] It is often assumed [21,22] that phosphonylating agents of serine enzymes usually require good leaving groups because these different geometries preclude general acid catalysed expulsion from the phosphorus apical position by the enzyme's general acid used for acylation reactions. If expulsion of proline occurred from the equatorial position then the geometries for acylation and phosphonylation would be more similar.



The amino-acid numbering is for class A ß-lactamase with those for class C in brackets.

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During the inactivation of the (P99) ß-lactamase by the less reactive diastereoisomer ethanol is displaced, incubation of this diastereoisomer with the enzyme gives a time dependent release of ethanol detectable by gas chromatography.

The four-membered phosphonamidate (6) is neither a substrate nor inhibitor of any of the classes of ß-lactamase.

The Role of the Carboxyl Residue in Penicillins and Cephalosporins

The roles of the lysine residues in the active site are of interest - are they there for recognition or do they participate in bond making and breaking? Their role as proton donors or acceptors has been suggested, although their anticipated pK_a's probably mitigate against this.[23] It has also been proposed that lys 315 (class C)/lys 234 (class A) may electrostatically stabilise the basic form of an ionisable group acting as a general base catalyst or lower the pK_a of serine 70 so that general base catalysis is not required.[24]

Most ß-lactam antibiotics contain a negatively charged substituent which is thought to be essential for molecular recognition. There is a carboxylate group at C3 in penicillins (1) and at C4 in cephalosporins. In class A ß-lactamases lysine 234 is a conserved residue and its amine side chain points into the presumed active site and, in its positively charged form, is thought to form an electrostatic interaction with the carboxylate group in penicillins (1) and cephalosporins. However, the replacement of the C3 carboxylate in penicillin by hydroxymethyl and aldehyde groups and of the C4 carboxylate in cephalosporins by both a lactone and an aldehyde gives derivatives which are still good substrates for *Bacillus cereus* ß-lactamase A. The enzyme rate-enhancement factors for the hydrolysis of the modified ß-lactams vary from 10^4 to 10^6. All the modified substrates show bell-shaped (k_{cat}/K_m)-pH profiles indicative of two catalytically important ionising residues on the protein of pK_a about 5 and 9. Although lys 234 is a highly conserved residue in class A ß-lactamases and has been traditionally thought to interact with the carboxylate of the ß-lactam antibiotic, it is not responsible for the decrease in enzyme activity at high pH corresponding to the pK_a of about 9.[25] Furthermore, there is no evidence of imine formation between the penicillin 3-aldehyde and ß-lactamase.

Summary

Four membered ß-lactams do not undergo ring opening reactions as readily as may be anticipated from their intrinsic strain energies. By contrast, the replacement of the three co-ordinate carbonyl carbon by a four co-ordinate phosphoryl phosphorous in four membered cyclic phosphonamidates causes an enormous increase in reactivity compared with an acyclic analogue. The role of proton transfer in the ß-lactamase catalysed hydrolysis of ß-lactams remains ambiguous. Acyclic phosphonamidates covalently inactivate ß-lactamase C by the displacement of proline at phosphorous. The reaction is enzyme catalysed and P-N fission is acid catalysed. The carboxylate residue at C3 in penicillins does not interact with a lysine residue in ß-lactamase to cause the decrease in enzyme activity at high pH.

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