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CERTAIN MONOCYCLIC β-LACTAMS ARE β-LACTAMASE SUBSTRATES: NOCARDICIN A AND DESTHIOBENZYLPENICILLIN

R.F.Pratt, E.G.Anderson and I.Odeh
Department of Chemistry, Wesleyan University
Middletown, Connecticut 06457

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SUMMARY

The monocyclic β-lactams nocardicin A and desthiobenzylpenicillin are substrates of the β-lactamases of B.cereus 569/H/9, E.coli W3310 and S.aureus PC1. Thus the specificity of the β-lactamase active site does extend beyond bicyclic β-lactams.

INTRODUCTION

The production of β-lactamases (EC 3.5.2.6) represents an important defense mechanisms of bacteria against penicillins (1). An interesting feature of the β-lactamase active site is its apparent lack of affinity for anything other than a bicyclic β-lactam. Indeed no substrates are known for these enzymes other than bicyclic β-lactams and no small molecule is known to bind tightly to the active site, with a dissociation constant significantly below 1mM say, other than a bicyclic β-lactam.

A possible exception to the above statements is the monocyclic β-lactam antibiotic nocardicin A (1). This compound was first reported (2), without data, to have "sensitivity to some β-lactamases" although it was later shown (3) to be affected by only one of several β-lactamases. In the latter work neither the enzyme responsible nor the product of the reaction was characterised. On the other hand also it has been reported (4) that desthiobenzylpenicillin (2) is not a substrate of the B.cereus 569/H β-lactamase.

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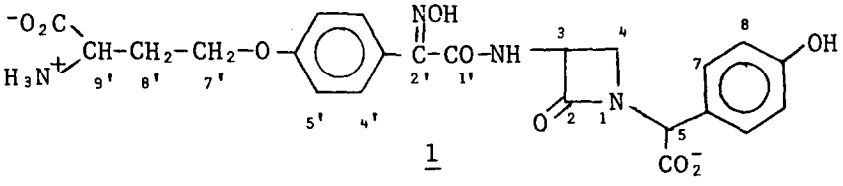
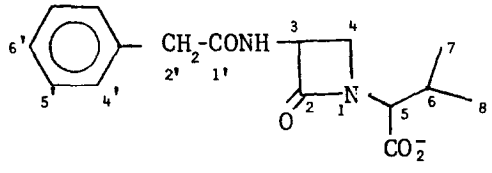
Since we have been interested in assessing the specificity of the β-lactamase active site beyond bicyclic β-lactams we thought it useful to examine the above results more fully with well-characterised enzymes. We find that both 1 and 2 are in fact substrates of several β-lactamases.



1



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MATERIALS AND METHODS

Materials. The β-lactamases of B.cereus (I and II), strain 569/H/9, E.coli, strain W3310 and S.aureus, strain PC1 were obtained and purified as previously described (5). Nocardicin A was the generous gift of the Fujisawa Pharmaceutical Co. Benzylpenicillin was purchased from Sigma Chemical Co. Desthiobenzylpenicillin was prepared as the benzylamine salt by desulfurization of benzylpencillin with Raney nickel (6); the nmr spectrum of the product, described below, is in accord with the given structure 2. Phenylacetyl-DL-alanyl-DL-valine, mp 178-9°, and benzyloxycarbonyl-D-alanyl-D-alanine, mp 151-2°, were prepared by standard methods.

Proton nmr spectra. These were obtained from either the 270 MHz Brüker spectrometer at the Southern New England High Field nmr facility at Yale University, New Haven, Connecticut or from the 200 MHz Varian XL200 instrument in this department. β-Lactamase activity against the various potential substrates was initially tested for by addition of ca 0.5 mg of the E.coli enzyme to a 10 mM solution of the substrate in 0.5 ml ^2H_2O also containing 30 mM $NaHCO_3$. A control sample without enzyme was also prepared. NMR spectra were then recorded after ca 12 hours at room temperature. The alkaline hydrolysis product was obtained by the addition of 10 µl of 35% NaO^2H in ^2H_2O to the control sample followed by incubation of the mixture at 57° for 24 hours. The hydrolysis mixture was then neutralised to pH 7 with ^2HCl before a final nmr spectrum was taken.

Spectrophotometric assays. Routine β-lactamase assays in 0.1 M phosphate buffer at pH 7.5 and at 30° were performed by the

spectrophotometric method of Waley (7). Spectrophotometric rate measurements with the other substrates were carried out under the same conditions and followed at 232 nm.

RESULTS

The proton nmr spectrum of 1 is given with structural assignments in Table I before and after treatment with the E.coli β-lactamase as described above. The spectrum of the control sample did not change. The spectrum of the neutralized alkaline hydrolysis product was identical to that of the enzyme incubated sample. The spectra of Table II are very close to those reported by Hashimoto et al. for 1 and its alkaline hydrolysis product (8). The spectral changes observed here, notably the upfield shifts

Table I

NMR Spectral Changes on Interaction of 1 with E.coli β-lactamase

Chemical Shift[a]		Assignment[b]
1	1/β-lactamase	
2.35(m)	2.37 (m)	8'
3.24(dd,J=2,6)	3.00 (dd,J=8,12)	4
3.85(t,J=6)	3.30 (dd,J=5,12)	4
3.91(dd,J=6,8)	3.94 (dd,J=5,8)	9'
4.24(t,br,J≈6)	4.24 (t,br,J=6)	7'
5.00(dd,J=2,6)	4.57 (dd,J=5,8)	3
5.33(s)	4.52 (s)	5
6.91,7.04,7.25,7.50(d,J=9)	6.91,7.04,7.30,7.56(d,J=9)	4',5',7,8

a ppm downfield (δ) from DSS; s=singlet, d=doublet, dd=double doublet m=multiplet, t=triplet, br=broad; coupling constants (J) are in hertz.
b in terms of the drawn structural formula of 1.

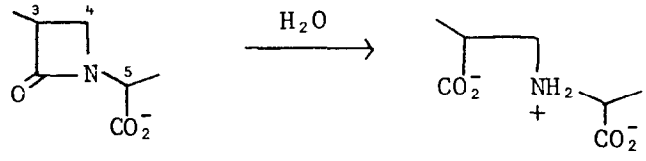
Table II

NMR Spectral Changes on Interaction of 2 with E.coli β-lactamase

Chemical Shift[a]		Assignment[b]
2	2/β-lactamase	
0.94, 0.95 (d,J=6)	0.93, 0.95 (d,J=6)	7,8
2.10 (m)	2.10 (m)	6
3.55 (dd,J=2,6)	3.05 (dd,J=6,14)	4
3.85 (t,J=6)	3.22 (dd,J=6,14)	4
3.96 (d,J=6)	3.29 (d,J=6)	5
3.62 (s)	3.67 (s)	2'
4.6-4.9[c]	4.30 (t,J=6)	3
7.35 (m)	7.35 (m)	4',5',6'

a as for Table I
b in terms of the drawn structural formula of 2
c not observed; presumably under ^{2}HOH peak.

of the 3, 4 and 5 protons, are in accord with the observed reaction being hydrolysis of the β-lactam ring (equation 1). These results,



(1)

taken together with the careful structural determinations of Hashimoto et al (8) clearly indicate that the E.coli β-lactamase catalyzes hydrolysis of the β-lactam of 1.

In Table II the nmr spectral results from the same type of experiment with 2 are presented. Again a spectral change is observed, only in the presence of the enzyme, and readily interpretable from the chemical shift changes as arising from hydrolysis of the β-lactam(equation 1). The final spectrum is again identical to that obtained from alkaline hydrolysis and neutralisation of the control sample. Thus desthiobenzylpenicillin is also a substrate of the E.coli β-lactamase.

In contrast to the above observations, the nmr spectra of the acyclic peptides phenylacetyl-DL-alanyl-DL-alanine and benzyloxycarbonyl-D-alanyl-D-alanine were unchanged after the incubation of these compounds with the E.coli enzyme; these compounds do not appear to be substrates.

The monocyclic β-lactam hydrolyses could also be followed by changes in absorption spectra at low wavelength. A decrease in absorbance is observed with both 1 ($\Delta\varepsilon/\varepsilon$=0.11) and 2 ($\Delta\varepsilon/\varepsilon$=0.5) at 232 nm; a similar decrease is of course observed with benzylpenicillin (7). Table III contains some initial rate data taken in this way for these hydrolyses catalyzed by various β-lactamases. In the case of the B.cereus β-lactamase I and 1, the Michaelis

Table III

Rates of β-lactam cleavage by various β-lactamases

Substrate	Conc.^{n}(mM)	β-lactamase	v^{a}
1	0.48	B.cereus I	0.47
1	0.48	B.cereus II	0.05
1	0.48	E.coli	1.5
1	0.48	S.aureus	$\leq 2 \times 10^{-3}$
2	1.0	B.cereus I	1.5×10^{-3}
2	1.0	E.coli	0.019
2	1.0	S.aureus	0.018

a initial rates expressed as moles substrate cleaved per mole of enzyme per second

parameters for the catalysis were determined to be K_m= 4.3 mM and k_{cat} = 6.7 sec^{-1}.

After the irreversible inactivation of B.cereus β-lactamase I towards benzylpenicillin hydrolysis brought about by the active site-directed covalent inhibitor 6-β-bromopenicillanic acid (9) the activity of the enzyme towards 1 was also lost. This is good evidence that the hydrolysis of 1 does occur at the β-lactamase active site.

The plots of absorbance *vs* time during the hydrolysis of 1 catalyzed by B.cereus β-lactamase I were not suggestive of more than a single phase to the reaction. Nor, on incubation of this enzyme with 1 (20 mM), was there any indication of a time-dependent inhibition, as judged by the enzyme activity immediately after aliquots of the incubation mixture were diluted into a benzylpenicillin solution under normal assay conditions.

DISCUSSION

The results described above clearly demonstrate that the monocyclic β-lactams 1 and 2 are β-lactamase substrates. This appears to be the first well-documented example of β-lactamase catalysis of any reaction but the hydrolysis of a bicyclic β-lactam. The data indicate that these substrates are quite poor both with respect to binding and to catalysis by comparison with the best

bicyclic substrates (in the case of B.cereus β-lactamase I and benzylpenicillin for example, K_m=0.08 mM and k_{cat}=2.3 x $10^3 sec^{-1}$ (10)) although 1 is comparable as a substrate of B.cereus β-lactamase I to a variety of cephalosporins and several of the A type penicillins (11). Interaction of these latter substrates with B.cereus β-lactamase I leads to slowly reversible (11, 12) inactivation processes but, as with cephalosporins, there seemed here no suggestion of A type substrate behavior by 1.

From the data of Table III it seems that 2 is in most cases but perhaps not all a much poorer substrate than 1. It is not known yet whether these differences largely reflect binding or catalysis.

The previous report by Baer and Mertes (4) that 2 is not a β-lactamase substrate presumably arose from a combination of (i) the small amount of enzyme they employed for what is in retrospect such a poor substrate and (ii) the titrimetric method they employed not being appropriate to detect hydrolysis of this substrate even if it occurred since, unlike the situation with penicillins, protons would not be released into solution at pH 7 on β-lactam hydrolysis. Our results are however in agreement with the conclusions of Baer and Mertes (4) that linear peptides are not β-lactamase substrates (although, as above, their method of detection was not appropriate).

It is noticeable from the above that the general order of β-lactamase catalytic activity, to the extent that it can be judged from the currently available data, viz. bicyclic β-lactam > monocyclic β-lactam > unstrained peptide, does follow the susceptibility of these compounds to nucleophilic cleavage (13). The inability of the β-lactamase active site to cleave peptides distinguishes it from that of the bacterial cell wall DD-carbox-

ypeptidases/transpeptidases which are able to cleave both penicillins and (specific) peptides (14). (Note however in passing that despite the ability of 1 to inhibit cell wall peptidoglycan synthesis it is apparently a poor, at best, inhibitor of DD-carboxypeptidase/transpeptidases (15, 16).) In spite then of the growing evidence for active site homology between these two classes of enzymes (17) there may yet be a chemically significant difference, e.g., the presence of an effective general acid catalyst in the carboxypeptidase/transpeptidases.

In summary then we have shown in this work a significant expansion of the β-lactamase active site specificity. On further development this could lead to more diverse substrates for β-lactamases and perhaps cell wall carboxypeptidase/transpeptidases and perhaps also to a further range of useful inhibitors.

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