Use of Electrospray Mass Spectrometry to Investigate the Inhibition of β-Lactamases by 6-Halogenopenicillanic Acids

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Electrospray mass spectrometry has been used to investigate the mechanism of inhibition of class A and C β-lactamases by 6-halogenopenicillanic acids; in the case of the 6β-halogen substituted penicillanic acids the molecular mass differences observed between the unreacted and the inhibited β-lactamases are consistent with the formation of an enzyme bound dihydrothiazine derivative, as previously proposed.

The most important mode of bacterial resistance to the β-lactam antibiotics is hydrolysis of the lactam ring as mediated by β-lactamases. Mechanically they may be subdivided into either metallo-enzymes or those that contain a nucleophilic active site serine residue. The serine type can be further subdivided, on the basis of sequence, into either class A or C.[1,2]

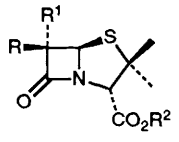
Recently we reported[3] mass spectrometry studies, using the electrospray technique,[4,5] on the hydrolysis of several β-lactams by the class C β-lactamase, from *Entereobacter cloacae* P99. In several cases we were able to observe molecular mass increases upon treatment of the P99 β-lactamase with 'poor' substrates (*e.g.* carbenicillin, aztreonam) corresponding to the formation of an acyl enzyme intermediate in the catalytic hydrolysis of the lactam ring. Analogous results were observed for the class A β-lactamase I from *Bacillus cereus* with the 'poor' substrate, cloxacillin.[3] Herein, we report the extension of this work to the study of the inhibition of class A and C β-lactamases by the potent irreversible inhibitors of these enzymes, the 6β-halogenopenicillanic acids, for example 6β-bromo-**1** and 6β-iodo-**2** penicillanic acid.[6] Previous mechanistic studies have provided strong evidence for the production of an active site serine bound dihydrothiazine derivative.[7–9]

Previously we reported[3] that analysis of the electrospray mass spectrometry (ESMS) data for the P99 β-lactamase at pH 3.5, indicated a single molecular mass of 39203.4 ± 2 (calculated from sequence data = 39206.4). In contrast analysis of the β-lactamase I enzyme (class A) from *B. cereus* showed two mass series.† The major series had a mass of 28941.8 ± 3, which is the same as the calculated mass from sequence data (28941.8).[3] The minor series (*ca.* 10%) had a mass of 28813.7 ± 4, a difference of *ca.* 128. Similar results were obtained when the assay was carried out under neutral (pH 7, MeCN–H_2O, 1:1) conditions (observed M_rs = 28941.9 ± 1 and 28810.3 ± 6). It was reasoned that the most likely explanation for the minor series is the post translational cleavage of an *N*-terminal lysine residue from the β-lactamase I (calculated M_r = 28813.7; difference = 128). β-Lactamase I has been previously reported to have a ragged *N*-terminus.[11] This hypothesis was confirmed by *N*-terminal sequencing, which showed the presence of two β-lactamase I sequences, differing only in the *N*-terminal amino acid (lysine or histidine).

† β-Lactamase I from *B. cereus* was purified according to previously published protocols.[10] The electrospray mass spectra were acquired on a VG BIO Q triple quadrupole atmospheric pressure mass spectrometer equipped with an electrospray interface (VG Biotech, Tudor Rd., Altrincham, Cheshire, UK). Unless otherwise stated the following standard conditions were used: samples (10 μl) were injected into the electrospray source as a solution (15–25 pmol μl^{-1}), in water–acetonitrile (1:1) containing 1% formic acid. Sample solutions were prepared immediately prior to analysis by mixing 1:1 molar proportions of the β-lactamase and the inhibitor in water at pH 7. Aliquots were taken at 3 and 5 min, mixed with an equal volume of acetonitrile containing 2% (v/v) formic acid (resultant pH 3.5) and analysed immediately.

Incubation of both the P99 β-lactamase and the β-lactamase I, with four different 6-halogeno substituted pencillanic acids was then carried out and the resultant products examined by ESMS. In the case of the 6α-bromopenicillanic acid salt **4** and the 6,6-dichloropenicillanic acid salt **5** no shift was observed in the mass spectra for either enzyme. This was anticipated since neither of these compounds are efficient substrates or inhibitors of β-lactamases, under the conditions of these experiments. Treatment of the β-lactamases with 6β-bromo- **1** and 6β-iodo- **2** penicillanic acid salts, however, led to the production of a new species with a significant mass shift, relative to the untreated enzyme. For example when a 3:1 ratio of **1**: P99 β-lactamase was used approximately 65% of the native enzyme was converted to a new series of peaks, which corresponded to a mass shift of +200 (Table 1, entry 2). With a 1:1 ratio of **2**: P99 β-lactamase approximately 60% of the native enzyme also underwent a similar mass shift (Table 1, entry 2).

In the case of the β-lactamase I from *B. cereus*, using the higher ratio of the inhibitors (**1** or **2**) to the enzyme (100:1) reported in the original kinetic investigations,[9] the native enzyme was completely converted to a complex new series of peaks (Table 1, entries 3 and 4). It is probable the complexity of the spectra observed was due to the replacement of protons by metal ions. For example when these conditions were used for the inhibition of the β-lactamase I by **2** (Table 1, entry 4, Fig. 1), the observed ESMS for the major component consisted of a series of peaks, which corresponded to mass shifts of 199 + 38*n* (*n* = 0 to 10), consistent with replacement of protons by potassium ions from the excess inhibitor **2**. Addition of 4% formic acid prior to analysis (Table 1, entry 5)



1; R = Br, R^1 = H, R^2 = Na
2; R = I, R^1 = H, R^2 = K
4; R = H, R^1 = Br, R^2 = K
5; R = R^1 = Cl, R^2 = K

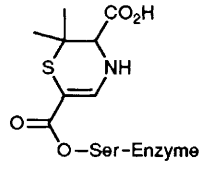


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Table 1 Mass shifts observed for the inhibition of β-lactamases by 6-halogenopenicillanic acids

Entry	Inhibitor	Enzyme[a]	Ratio of inhibitor: enzyme	Mass shift[b]	Approx. % inhibition
1	**2**	P99	1:1	+199	60
2	**1**	P99	1:3	+200	65
3	**1**	BCI	1:100	+199	>95
4	**2**	BCI	1:100	+199	>95
5	**2**	BCI[c]	1:100	+199	>95
6	**2**	BCI	1:10	+199	>95

[a] P99 = β-Lactamase form *E. cloacae* P99; BCI = β-lactamase I from *B. cereus*. [b] Errors ± <1. [c] Assayed in the presence of 4% formic acid.

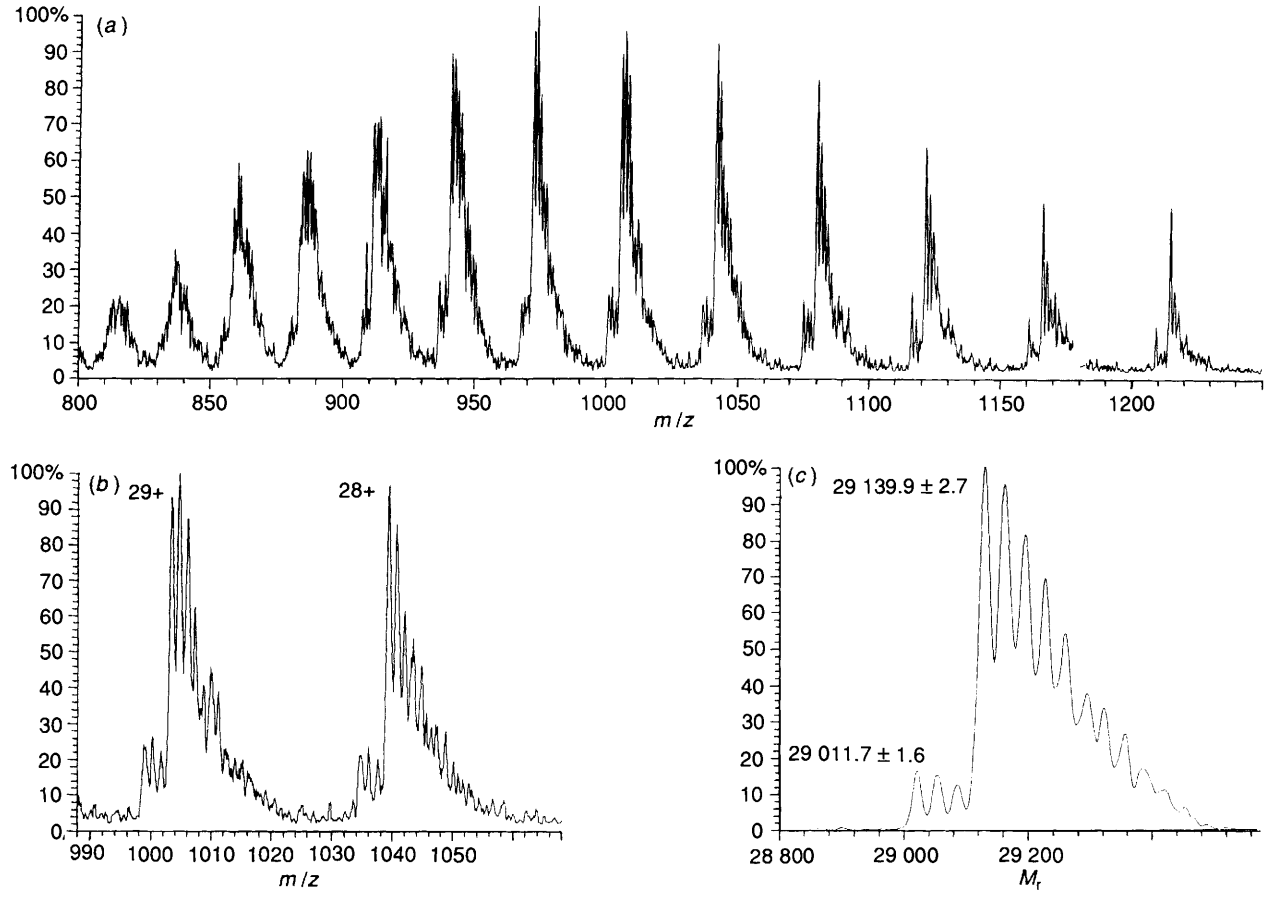

Fig. 1 ESMS of β-lactamase I from *B. cereus* with **2** (1:100); (*a*) From 800 to 1250; (*b*) over two charge states from 990 to 1070; (*c*) the mass transformed spectrum

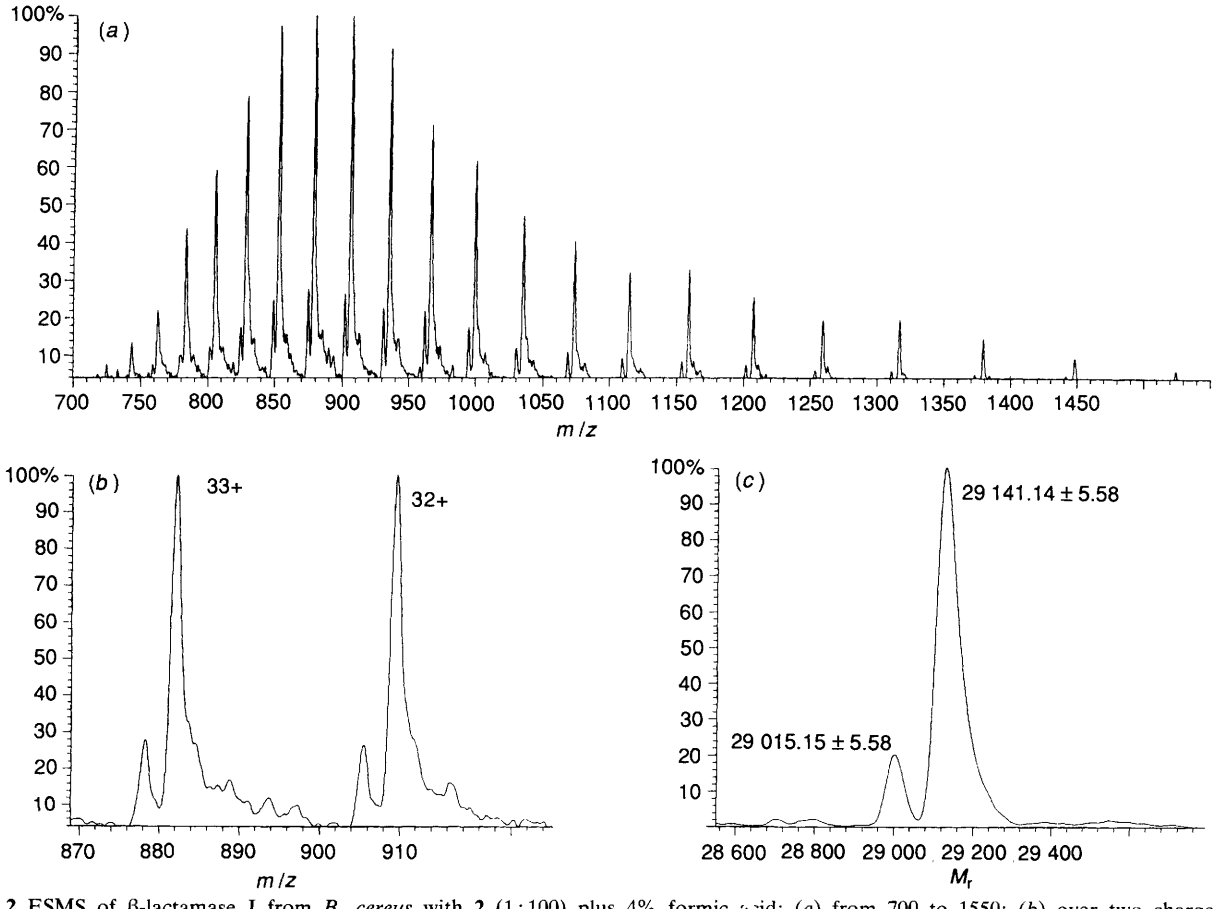

Fig. 2 ESMS of β-lactamase I from *B. cereus* with **2** (1:100) plus 4% formic acid; (*a*) from 700 to 1550; (*b*) over two charge states from 870 to 925; (*c*) the mass transformed spectrum

or the use of a lower ratio of inhibitor to enzyme (10 : 1) (Table 1, entry 6) removed the salt adduct species (Fig. 2) and gave mass shifts of 199. Similar mass shifts were observed when incubations were carried out for 3, 5 and 10 min and when the ESMS assays were carried out at either pH 3.5 or 7.

In each case where a mass shift of the β-lactamase was observed upon incubation with a 6-halogenopenicillanic acid salt, the value of the shift observed was close to that calculated for the production of an enzyme bound dihydrothiazine derivative such as **3** (Table 1). Thus, these experiments provide further powerful evidence for the previously proposed mode of inhibition[7–9] of the class A and C β-lactamases by the 6β-halogenopenicillanic acids and further exemplify the power of electrospray mass spectrometry as a rapid and useful probe for the mode of action of enzyme inhibitors.

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