Mass Spectrometric Studies on the Inhibition of TEM-2β-Lactamase by Clavulanic Acid Derivatives

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Potassium clavulanate is the most important clinically used inhibitor of β-lactamases.[1] Despite a number of studies[2~5], including X-ray analysis[6], the complex mechanism of clavulanate **1** inhibition of β-lactamases is not fully understood. Using electrospray ionisation mass spectrometry (ESI-MS) studies we have recently showed that a minimum of four different modified proteins are formed upon incubation of clavulanate **1** with the TEM-2β-lactamase.[7] These exhibit mass increments relative to the unmodified TEM-2β-lactamase of 70, 88, 52 and 155 Da. The 70 and 88 Da mass increments are consistent with the production of aldehyde **2** and hydrated aldehyde **3**. HPLC-ESI-MS and chemical sequencing experiments demonstrated that the 52 Da mass increment resulted from cross-linking of the enzyme between Ser-70† and Ser-130 in the form of a vinyl ether **4**†† as proposed by IMTIAZ *et al.*[8] The 155 Da mass increment is clearly too small to result from modification by intact clavulanate **1** and instead corresponds to a decarboxylated species such as imine **5a** or enamine **5b**.

The presence of the C-9 alcohol has been shown to be unimportant for TEM-2 inhibition by clavams.[4] Analogues of clavulanate **1** differing only in the C-9 substituent thus offer the potential to test mechanistic proposals by differentiating between those inhibited forms that retain the oxazolidine ring of the clavulanate skeleton and those that do not. Forms of C-9 modified clavulanate-analogue-inhibited TEM-2 that retained the oxazolidine ring would be expected to exhibit a mass increment relative to unmodified TEM-2 enzyme diffferent from those detected in normal clavulanate-inhibited enzyme. Forms which did not retain the oxazolidine ring might be expected to be indistinguishable from comparable forms of clavulanate-inhibited enzyme.

ESI-MS spectra were acquired after incubation of TEM-2β-lactamase with *t*-butylammonium clavulanate **1**, potassium 9-*O*-methylclavulanate **6** and potassium 9-*N*-isobutylaminodeoxyclavulanate **7**. As previously reported in the case of clavulanate **1**[7], three mass increments were observed in each case: A, B and a broad series X. A corresponds to unmodified TEM-2 and was not visible in the case of the analogues **6** or **7**. The broad series X was clearly present in each case and corresponds to an approximate mass of 28985 Da. High resolution studies in the case of **1** have demonstrated the broad series X to be a superposition of three species with mass increments of 52, 70 and 88 Da, corresponding to **4**, **2** and **3**, respectively.

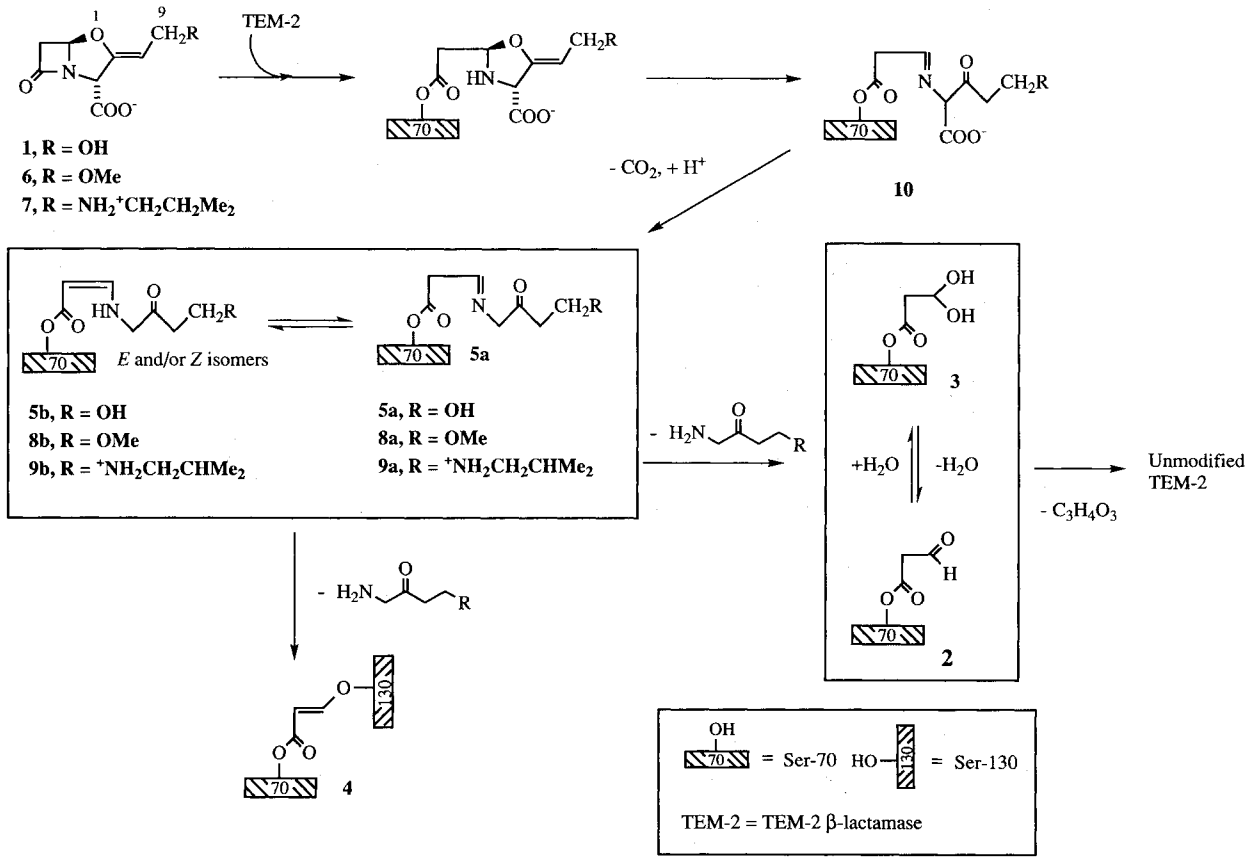
In contrast to the presence of series X in all three cases, differences existed between the largest apparent mass increments (B) resulting from the incubations of **1**, **6** and **7** with the TEM-2β-lactamase. Incubation of TEM-2β-lactamase with **1** results in an observed mass of 29067±6 Da, incubation with **6** in an observed mass of 29078±7 Da, and incubation with **7** in an observed mass of 29117±7 Da. These correspond to mass increments relative to unmodified enzyme of 155 for **1**, 173 for **6**, and 212 Da for **7**. The observed differences in mass increments between **1** and the analogues **6** and **7** may be accounted for by the difference in their C-9 substituents, resulting in the formation of **5**, **8** and **9**, respectively. Furthermore, as observed for **1**, in the cases of **6** and **7** there was no evidence for the presence of any mass increment corresponding to the mass of the intact clavam inhibitor, implying rapid decarboxylation of the each of the three clavam inhibitors occurs after binding to the β-lactamase.

These studies support a common mechanistic scheme for the inhibition of β-lactamases by clavulanates **1**, **6** and **7** in which acylation of Ser-70 by the β-lactam is followed by ring opening of the oxazolidine ring. Rapid decarboxylation of the resultant β-keto acid **10** gives the imines **5a** (R=OH), **8a** (R=OMe) or **9a** (R=$N^+H_2CHMe_2$) which may be in equilibrium with *E* and/or *Z* enamines **5b**, **8b** or **9b**, respectively. The imines **5a**, **8a** or **9a** are then hydrolysed to give the aldehyde **2** and hydrated aldehyde **3** with concomitant loss of the elements of the oxazolidine ring and its C-9 substituent. Aldehyde **2**/hydrated aldehyde **3** may undergo further slow hydrolysis to reform unmodified enzyme. Alternatively, nucleophilic attack of the alcohol of Ser-130 onto either the imine **5a**, **8a** or **9a** or enamines **5b**, **8b** or **9b** results in cross-linking to form a vinyl ether **4** *via* an addition-elimination mechanism.

† The ABL numbering scheme for β-lactamases is used (AMBLER *et al.*, 1991).

†† Under acidic conditions **4** reacts to form TEM-2β-lactamase modified by the conversion of Ser-130 to a dehydroalanine residue and with an aldehyde/hydrated aldehyde (analogous to **2**/**3**) linked to Ser-70. The latter undergo further hydrolysis to yield TEM-2 enzyme covalently modified only by the conversion of Ser-130 to a dehydroalanyl residue. The physiological relevance of this pathway is presently unclear.[7]

Scheme 1. Inhibition of the TEM-2β-lactamase by clavulanate **1**, and analogues **6**, and **7**.



Experimental

ESI-MS was performed using a VG Bio-Q triple quadrupole atmospheric pressure mass spectrometer equipped with an electrospray ionization interface. Solvent (acetonitrile : water 1 : 1 v/v) was supplied at a flow rate of 8 μl min^{-1} using an Applied Biosystems 140 A solvent delivery system. After incubation for 30 seconds in 20 mM Tris-HCl buffer, pH 7.8, reactions were quenched by addition of an equal volume of acetonitrile containing 2% (v/v) formic acid such that the final mixture was composed of water : acetonitrile : formic acid in proportions of 1 : 1 : 1%. The final protein concentration was typically 20 pmoles μl^{-1}. A 16 fold excess of *t*-butylammonium **1** and 18 fold excesses of potassium **6** and potassium **7** relative to enzyme were used. Mass spectra typically comprised of 15 10-second scans over the range 750 ~ 1500 Da. and were acquired using a cone voltage of 50 V and a source temperature of 50°C.

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