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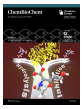

How Clavulanic Acid Inhibits Serine β-Lactamases

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Clavulanic acid is a medicinally important inhibitor of serine β-lactamases (SBLs). We report studies on the mechanisms by which clavulanic acid inhibits representative Ambler class A (TEM-116), C (*Escherichia coli* AmpC), and D (OXA-10) SBLs using denaturing and non-denaturing mass spectrometry (MS). Similarly to observations with penam sulfones, most of the results support a mechanism involving acyl enzyme complex formation, followed by oxazolidine ring opening without efficient subsequent scaffold fragmentation (at pH 7.5). This observation contrasts with previous MS studies, which identified clavulanic acid scaffold fragmented species as the predominant SBL bound products. In all the SBLs studied here, fragmentation was promoted by acidic conditions, which are commonly used in LC–MS analyses. Slow fragmentation was, however, observed under neutral conditions with TEM-116 on prolonged reaction with clavulanic acid. Although our results imply clavulanic acid scaffold fragmentation is likely not crucial for SBL inhibition in vivo, development of inhibitors that fragment to give stable covalent complexes is of interest.

Introduction

β-Lactams are amongst the most important antibacterials in use, but their efficacy is increasingly compromised by resistance, commonly by production of β-lactamases.[1] The >4300 β-lactamases identified to date,[2] can be grouped into Ambler classes A, C, and D, which employ a nucleophilic serine in catalysis (serine β-lactamases, SBLs) and class B, which use one or two zinc ions in catalysis (metallo β-lactamases, MBLs).[3] MBL mediated resistance is growing, but SBLs are presently more commonly encountered in the clinic.[3b]

One successful strategy to combat β-lactamase resistance is the co-administration of a β-lactam antibiotic with a β-lactamase inhibitor.[4] Although in recent times synthetic β-lactamase inhibitors without a β-lactam have been developed (e.g. diazabicyclooctanes[5] and cyclic boronates[6]), SBL inhibitors containing a β-lactam, such as clavulanic acid[7] and the penam sulfones sulbactam[8] and tazobactam,[9] are most commonly used. The combination of the natural product clavulanic acid and amoxicillin (Augmentin) has been a particularly successful treatment and is on the World Health Organization's list of essential medicines.[10]

Clavulanic acid ($C_8H_9NO_5$, Figure 1) is produced by fermentation and has an apparently simple structure, with two chiral centers; however, its ring strained and oxidised nature means it is both a chemically interesting and challenging molecule. Relatively few structure activity relationship studies have been reported on clavulanic acid compared to other important β-lactam classes, i.e. penams, cephems, carbapenems and monobactams.[11] Like the penam sulfones, clavulanic acid is a potent inhibitor of many class A and D SBLs, but is less active

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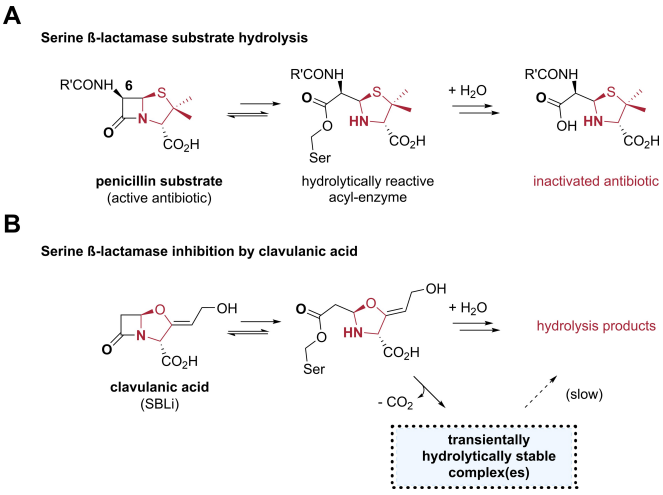

Figure 1. Clavulanic acid is a mechanism-based inhibitor of serine β-lactamases. (A) Outline mechanism for penicillin hydrolysis as catalyzed by serine β-lactamases (SBLs); reaction proceeds via a hydrolytically labile acyl-enzyme complex. (B) Clavulanic acid is an SBL inhibitor that reacts to give one or more hydrolytically stable complex(es), the nature of which was the focus of our work.

versus class C SBLs and is inactive against most MBLs, though inhibition of MBLs by it has been rarely observed.[4,12]

SBL inhibition by clavulanic acid and penam sulfones proceeds via a bifurcating mechanism: at the acyl-enzyme stage, reaction proceeds either to: (i) give hydrolysed turnover products, or (ii) give relatively hydrolytically stable species (Figure 1). We have shown that with the penam sulfones sulbactam, tazobactam, and enmetazobactam, which had previously been proposed to inhibit SBLs through extensive scaffold fragmentation,[13] that fragmentation can be induced by the acidic conditions employed in commonly used denaturing protein LC–MS analysis, and is likely not relevant for SBL inhibition under physiological conditions.[14] Since our early electrospray ionisation MS studies revealed the potential for similar fragmentation of the clavulanic acid scaffold on reaction with TEM-2[15] we reinvestigated reaction of clavulanic acid with representative class A, C and D SBLs (TEM-116, the *Escherichia coli* AmpC ($AmpC_{EC}$), and OXA-10) using denaturing and non-denaturing intact protein observed MS methods.

Results

Inhibition Studies

Dose-dependence studies on SBL inhibition by clavulanic acid employing the reporter substrate FC-5[16] (Figure S1 and Table S1) showed potent inhibition of TEM-116 (pIC_{50} 7.02 ± 0.03) and OXA-10 (pIC_{50} 6.21 ± 0.02) with 10 min pre-incubation of the inhibitor and enzyme. By contrast, $AmpC_{EC}$ inhibition was very weak (pIC_{50} 2.78 ± 0.02) under the same conditions. While TEM-116 inhibition was apparently independent of the tested pre-incubation times (0–360 min), with $AmpC_{EC}$ and OXA-10 clavulanic acid, the potency increased substantially with increased pre-incubation time, that is $AmpC_{EC}$: pIC_{50} 2.37 ± 0.05 at 0 min; 3.52 ± 0.03 at 60 min; and pIC_{50} 4.22 ± 0.03 at 360 min; OXA-10: pIC_{50} 5.75 ± 0.02 at 0 min; 6.66 ± 0.04 at 60 min; and pIC_{50} 6.88 ± 0.04 at 360 min (Table S1). As with the penam sulfones,[14] addition of excess bicarbonate (to ensure catalytically important Lys70 carbamylation[17]) did not substantially influence OXA-10 inhibition by clavulanic acid, under the tested conditions (pIC_{50} 6.21 ± 0.02 and 6.28 ± 0.05 with or without added bicarbonate, respectively, Table S1). No clear pH dependence of clavulanic acid inhibition of $AmpC_{EC}$ was observed within the tested range (pH 7.0–8.0, Table S1).

More detailed kinetic analyses (Figure S2 and Table S2) revealed substantially slower pseudo first-order rate constants (k_{inact}/K *or* k_2/K) for clavulanic acid inhibition of $AmpC_{EC}$ and OXA-10 (3.0 ± 0.4 $M^{-1}s^{-1}$ and 108.7 ± 3.7 $M^{-1}s^{-1}$, respectively) compared to TEM-116 (16.5 ± 0.3×10^3 $M^{-1}s^{-1}$). The dissociation rates (k_{off}) were also substantially slower for $AmpC_{EC}$ ((13.67 ± 0.04)×10^{-6} s^{-1} and (61.57 ± 0.28)×10^{-6} s^{-1}, for $AmpC_{EC}$ and OXA-10, respectively) compared to TEM-116 ((0.34 ± 0.01)×10^{-3} s^{-1}), corresponding to increased half-lives of the inhibited species (845 ± 2 min and 188 ± 1 min for $AmpC_{EC}$ and OXA-10, respectively, compared to 34 ± 1 min for TEM-116, Table S2). The partition ratio (k_{cat}/k_{inact}) for clavulanic acid inhibition of TEM-116 (Table S2) was comparable to those previously observed for penam sulfones (11 for clavulanic acid compared to 9 for tazobactam, Table S2 and[14]), while for OXA-10 the partition ratio was significantly improved (68 for clavulanic acid compared to 160 for tazobactam, Table S2 and[14]). With $AmpC_{EC}$, inactivation by clavulanic acid was too slow to determine a partition ratio.

Intact Protein MS Studies

Due to the potency and clinical efficacy of clavulanic acid, the nature of the transient hydrolytically stable species resulting from the bifurcating reaction of SBLs with it has been of interest. Spectroscopic[13c,18] and some crystallographic[13h,18b,19] studies with clavulanic acid (mass: 199 Da) indicated that SBL inhibition involves opening of the oxazolidine ring to give imine/enamine species without fragmentation of the clavulanic acid 'scaffold', followed by decarboxylation (i.e. **3a**–**5a** in Figure 2), to give one or more hydrolytically stable imine/enamine species (**3b**–**5b** in Figure 2), with a mass shift of + 155 Da relative to the unmodified protein. Early direct injection or LC–MS based intact protein ESI-MS studies under denaturing conditions[13e,h,15,20] as well as other crystallographic studies[19a,20b,21] have suggested inhibition may involve substantial scaffold fragmentation of the initially formed acyl-enzyme complex, leading to species corresponding to + 88, + 70, or + 52 Da mass shifts, potentially correlating to **6**, **7**, and **8** in Figure 2, or equivalent mass species.

To further investigate the mechanism by which clavulanic acid inhibits SBLs, clavulanic acid reacted samples of TEM-116, $AmpC_{EC}$, and OXA-10 were analysed using liquid chromatography-MS (LC–MS), solid phase extraction-MS (SPE-MS), and non-denaturing (native) MS (Figure 3). As for the penam sulfones,[14] and in accord with prior direct injection and LC–MS studies with clavulanic acid[13c,e,h,15a,20,22], LC–MS analysis on reaction of SBLs with 500 equivalents of clavulanic acid for 20 min at room temperature manifested species with clear mass shifts of + 88, + 70, and + 52 Da (Figure 3), corresponding to **6**, **7**, or **8** in Figure 2, or equivalent mass shift species. By contrast, SPE-MS and native MS analysis showed clear evidence for + 155 Da adducts (Figure 3), corresponding to decarboxylated species **3b**--**5b** (Figure 2), or equivalent mass species. As with the penam sulfones,[14] addition of 0.1% (*v*/*v*, final concentration) formic acid to TEM-116 and OXA-10 samples reacted with clavulanic acid induced apparently rapid fragmentation of the clavulanic acid derived + 155 Da species to give + 88, + 70, and + 52 Da species (likely corresponding to **6**–**8** in Figure 2) within 30 min of formic acid addition, as evident in SPE-MS analyses (Figure S3). In the case of $AmpC_{EC}$, addition of formic acid accelerated the reformation of the apparently unmodified enzyme (Figure S3).

SPE-MS time-course studies were conducted on the reactions of clavulanic acid (Figure 4) to investigate potential clavulanic acid derived acyl-enzyme complex fragmentation on prolonged reaction, as observed on reaction of TEM-116 and OXA-10 with the penam sulfones.[14] In agreement with the

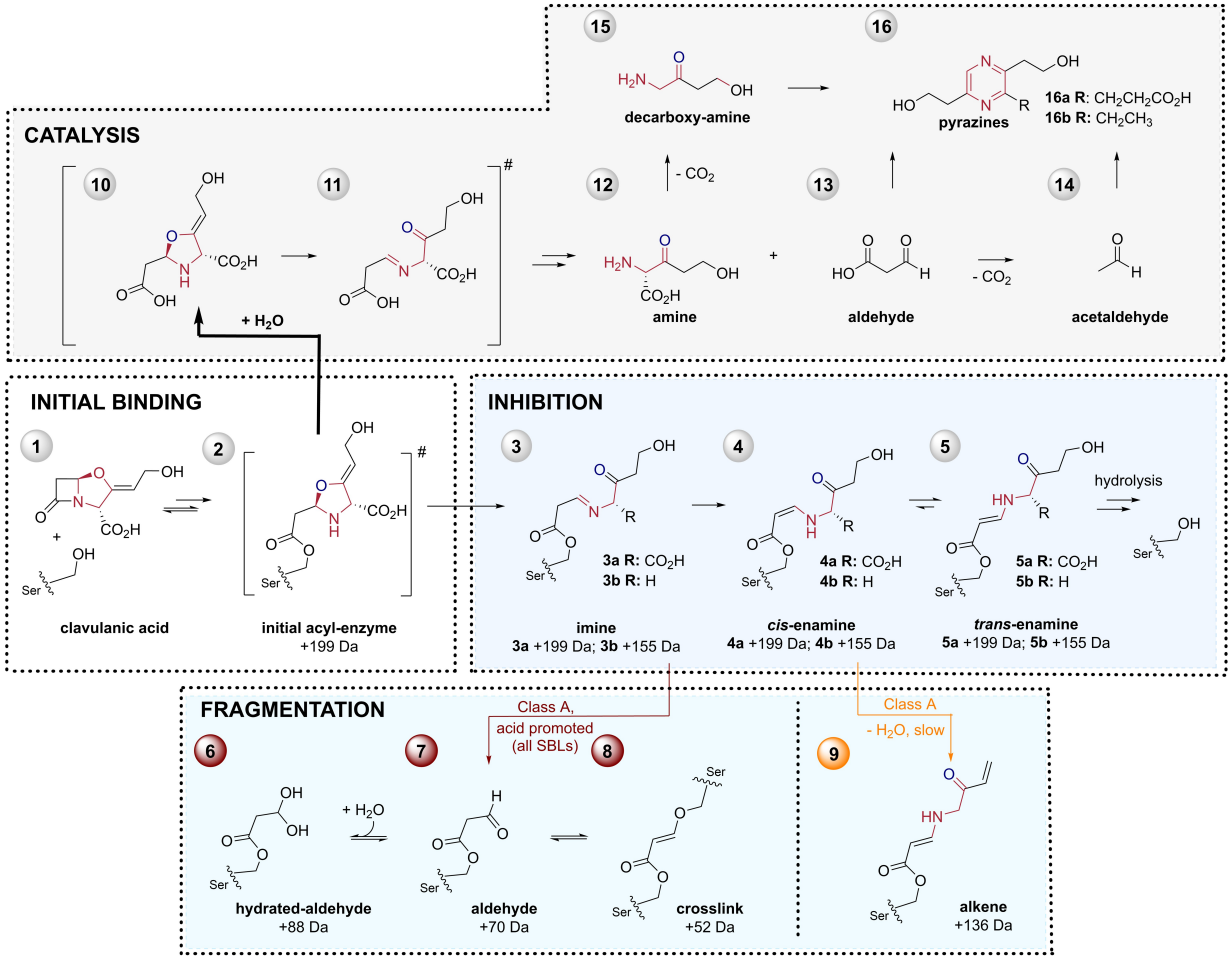

Figure 2. Pathways for reaction of clavulanic acid with serine β-lactamases. An acyl-enzyme **2** species is formed which can undergo hydrolysis to give **10**, which reacts in solution to give **12**–**16**. Alternatively, oxazolidine opening can occur to give **3a**–**5a**, decarboxylation (likely preferentially of **3a**) can occur to give **3b**–**5b**, which are relatively stable to hydrolysis; these species likely reflect those most important in inhibition in solution. In some cases, fragmentation of **3b**–**5b** can occur in an acid promoted manner to give **6**–**8**. Dehydration of **3b**–**5b** may also occur to give **9**.

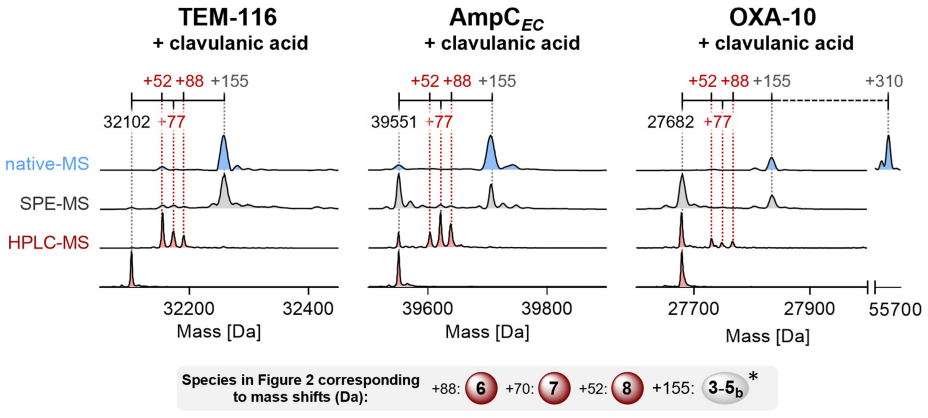

Figure 3. The method of mass spectrometric analysis influences the products observed on reaction of clavulanic acid with serine β-lactamases. For liquid chromatography (LC) MS analyses,TEM-116, $AmpC_{EC}$ and OXA-10 (1 μM) were incubated with potassium clavulanate (500 μM) in 50 mM Tris pH 7.5 at room temperature for 20 min. For solid phase-extraction (SPE) MS, TEM-116, $AmpC_{EC}$ and OXA-10 (3 μM) were incubated with potassium clavulanate (300 μM) in 50 mM Tris pH 7.5 at room temperature for 20 min. For LC-analyses, modified proteins were eluted using a mobile phase of 0.1% (v/v) aqueous formic acid over approx. 4–5 min; elution using the SPE-method was done using the same mobile phase and was complete within 4 sec. For non-denaturing (native) MS analyses, $AmpC_{EC}$, OXA-10, or TEM-116 (1 μM, 2 μM, or 0.5 μM, respectively) were incubated with the tert-butylamine clavulanate salt ($AmpC_{EC}$: 500 μM; TEM-116: 100 μM, OXA-10: 50 μM) in 500 mM ammonium acetate, pH 7.5, at room temperature for 20 min, then sprayed into the spectrometer. As reported[24] OXA-10 was predominantly dimeric.

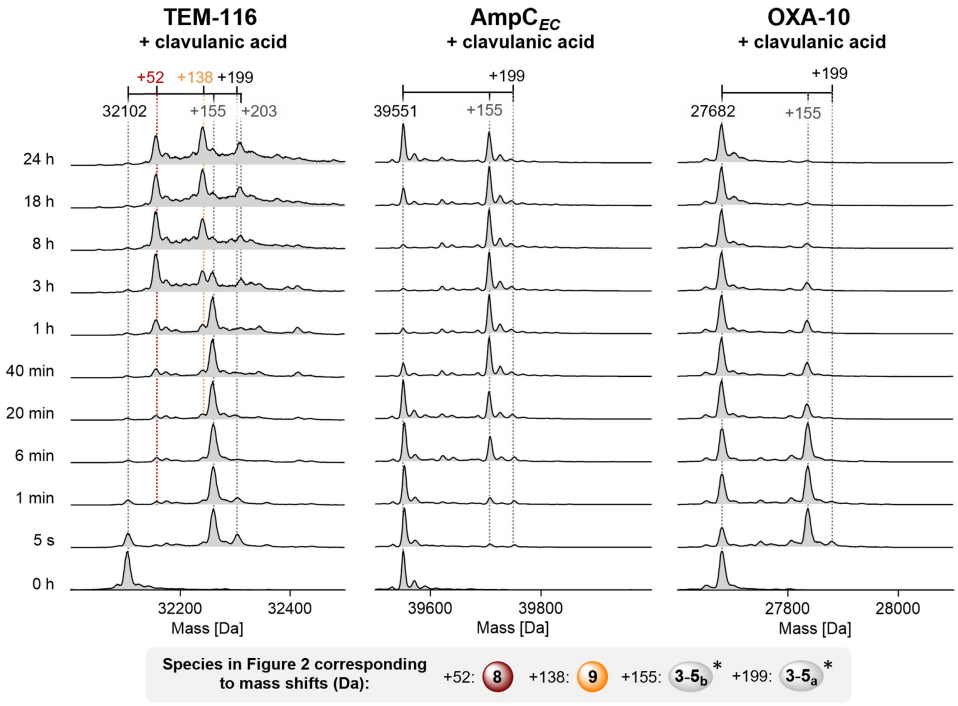

Figure 4. Solid phase extraction coupled to MS analysis (SPE-MS) time courses for the reactions of TEM-116, AmpC$_{EC}$, and OXA-10 with clavulanic acid. TEM-116, AmpC$_{EC}$, and OXA-10 (3 μM) were incubated with potassium clavulanate (1.5 mM) at room temperature. Buffer: 50 mM Tris buffer, pH 7.5. Mass-spectra were acquired without the inhibitor (0 h) and after the indicated incubation times. Deconvoluted spectra are shown, obtained using the maximum entropy algorithm in the MassHunter Workstation Qualitative Analysis B.07.00 programme (Agilent). Proposed structures corresponding to the labelled mass shifts are given in Figure 2.

kinetic studies (Figure S2 and Table S2), modification of TEM-116 by clavulanic acid was significantly faster than with AmpC$_{EC}$ and OXA-10 (Figure 4). Notably, at early timepoints (5 s–6 min) a small amount of a +199 Da species was observed, corresponding to a species with the intact mass of clavulanic acid (e.g. **2**–**5a** in Figure 2, or equivalent mass species), as reported in MS studies on reaction of clavulanic acid with the Bla$_{MAB}$ β-lactamase from mycobacteria.[23]

Our SPE-MS observations are in agreement with a bifurcating pathway, with turn-over of the acyl-enzyme **2** and/or carboxylated-imine **3a** (or equivalent mass species), and with inhibition resulting from decarboxylation to give **3b**–**5b**. Notably, while only traces of the +199 Da species were observed on reaction with AmpC$_{EC}$ and OXA-10, a higher abundance of the +199 Da species was apparent with TEM-116 (Figure 4). After ~6 min the +199 Da species was not observed with TEM-116, with the +155 Da adduct dominating with all three of the tested SBLs. With AmpC$_{EC}$ and OXA-10, the +155 Da adduct slowly reacted to regenerate the unmodified enzymes (within 8–18 h). By contrast, a low abundant +52 Da species was observed with TEM-116 with time course studies over 8 h, potentially corresponding to **8** (or equivalent mass species), as well as a +136 Da species, potentially indicating dehydration of **3b**–**5b** (or equivalent mass species) to give alkene **9** (or equivalent mass species, Figure 2). While the +155 Da species appeared stable over the remaining time (24 h), the +52 Da species appeared to react to give a +203 Da species, which is equivalent to the addition of a second clavulanic acid molecule accompanied by decarboxylation (+155 Da relative to the +52 Da species, Figure 4). By contrast with our results with the penam sulfones,[14] and reported work on TEM-2 and SHV-1 (both class A SBLs) inhibition by clavulanic acid[15a,22], under our conditions no dehydration to give a −18 Da species was observed by SPE-MS on reaction of TEM-116, AmpC$_{EC}$, or OXA-10 with clavulanic acid.

To investigate the possibility of acid-catalysed interference in the SPE-MS studies, native MS studies were conducted, analysing equivalent samples over a period of 4 h, by direct injection into the mass spectrometer without addition of acid. Due to limitations of the experimental set-up, the shortest timepoint was acquired approximately 1 min after mixing. At this, and all following, timepoints no evidence was accrued for species with a +199 Da mass shift on reaction of TEM-116, AmpC$_{EC}$, or OXA-10 with clavulanic acid. In agreement with the results of the SPE-MS studies (Figure S4 and S5), a predominant +155 Da species was observed. A +52 Da mass shift species was observed to accumulate over time in the case of TEM-116, though +70, +88, and −18 Da species were not observed.

NMR Studies

We investigated the products of the reactions of TEM-116, AmpC$_{EC}$, and OXA-10 with clavulanic acid in solution by ^{1}H NMR spectroscopy (700 MHz, Figure S6–S8). No evidence for the non-fragmented oxazolidine ring/imine/enamine species **10** and **11**

(Figure 2) was accrued. The fragmentation products amino acid **12** and the natural product malonic acid semialdehyde **13** were observed (Figure S6–S8). These observations suggest that hydrolysis of the nascent acyl-enzyme **2** or imine/enamine intermediates (**3**–**5**, Figure 2) is followed by efficient fragmentation in solution, consistent with reports on non-enzymatic hydrolysis of clavulanic acid.[25] Whilst with OXA-10 evidence for amino acid **12** (Figure 2) was observed at early time points (5–10 min), with TEM-116 and $AmpC_{EC}$ a mixture of both amino acid **12** and amine **15** (Figure 2) was observed. In all three cases only the decarboxylated species was observed on prolonged reaction (1.5–48 h, Figure S6–S8). Identification of the amino acid **12** was further supported by HMBC studies showing correlation of the α-proton to both the carboxylic acid (C1 carbon, 168 ppm) and the ketone carbon (C3 carbon, 202 ppm, Figure S10). Over 24 h, aldehyde **13** was observed to undergo decarboxylation to give acetaldehyde **14**. Both aldehydes **13** and **14** were apparently in equilibrium with their hydrated diols (**13b** and **14b**, respectively, Figure S6 and S7). Amine **15** was observed to undergo condensation with aldehydes **13** or **14** to give pyrazines (**16a** and **16b**, Figure 2), consistent with studies on non-enzymatic clavulanic acid hydrolysis.[25]

Discussion

The first clinically applied focused SBL inhibitor to be discovered, clavulanic acid, remains the most used β-lactam antibiotic protecting agent. Intriguingly, clavulanic acid, like the penam sulfones, is closely structurally related to the penicillins and related bicyclic β-lactam antibiotics, but lacks the C6 amido sidechain and features an oxazolidine ring with C-2 exocyclic alkene fused to the β-lactam instead of the thiazolidine ring of the penicillins (Figure 1).[7] The clavulanic acid scaffold, however, confers potent inhibition to many class A and D SBLs, as previously reported[7,9] and shown here (Figure S1 and S2 and Table S1 and S2). Derivatives of clavulanic acid have also been shown to inhibit other nucleophilic enzymes such as proteases,[7] and in some cases clavulanic acid can inhibit MBLs.[12] The mechanism of SBL inhibition by clavulanic acid has thus been the focus of extensive studies, some of which identified substantial scaffold fragmentation of initially formed acyl-enzyme species. Recent studies, however, have provided evidence that SBL inhibition by the penam sulfones sulbactam, tazobactam, and enmetazobactam does not involve previously proposed[13] analogous scaffold fragmentation, which instead is induced by acidic conditions during the employed LC–MS analysis[14]; as reported here we thus re-investigated the role of fragmentation in clavulanic acid inhibition.

Similarly to our studies with the penam sulfones, the combined LC-, SPE-, and native MS results suggest that with clavulanic acid, the previously reported[13e,h,15,20] rapid fragmentation of the nascent acyl-enzyme species to give +52, +70 and +88 Da species is promoted by commonly used acidic LC–MS conditions. However, while with $AmpC_{EC}$ and OXA-10, predominantly species with a +155 Da mass shift were observed (likely corresponding to the decarboxylated species **3b**–**5b**, or equivalent mass species, Figure 2) over 24 h, the SPE-MS results with TEM-116 give evidence for slow fragmentation of the +155 Da species, leading to +52 Da and +136 Da species, potentially corresponding to a crosslinked species **8** and alkene **9** (or equivalent mass species, Figure 2), respectively, the latter of which has been overserved crystallographically on prolonged reaction of the *Mycobacterium tuberculosis* class A β-lactamase BlaC with clavulanic acid[19a]. While the reactions leading to +52 Da and +136 Da species are considerably slower than previously reported fragmentation[13e,h,15,20], at least in the case of the +52 Da species, they occur on a faster time scale than observed with the penam sulfones,[14] and may thus be more biologically relevant for clavulanic acid conferred inhibition of TEM-116 and, by implication, potentially other class A SBLs. By contrast with the small amounts of +52 Da fragments observed on TEM-116 reaction with penam sulfones,[14] the +52 Da species observed on reaction of TEM-116 with clavulanic acid appeared stable towards hydrolysis, consistent with previous suggestions of further reaction of a nascent hydrolytically unstable +52 Da species to give a hydrolytically stable species with equivalent mass[26] and/or addition of a second clavulanate molecule accompanied by decarboxylation to give +203 Da species which also appear stable towards hydrolysis (Figure 4).

The NMR based solution studies (Figure S6–S8) support efficient SBL catalysis to proceed via hydrolysis of acyl-enzyme species resulting from non-fragmented clavulanic acid (e.g. **2**, **3a**--**5a** in Figure 2), while transient inhibition is achieved through decarboxylation to give **3**–**5b** (or equivalent mass species, Figure 2), as evident in the SPE- and non-denaturing-MS studies (Figure 3 and 4 and Figure S4).

Conclusions

The overall results presented here and in some previous reports[13c,h,18,19] support the proposal that clavulanic acid conferred SBL inhibition proceeds via a bifurcating mechanism, involving initial formation of an acyl-enzyme species, which is labile to hydrolysis, but which can also undergo oxazolidine ring opening followed by decarboxylation to give substantially more hydrolytically stable species (Figure 2). The results imply that, at least in general, more extensive scaffold fragmentation reactions do not occur under biologically relevant conditions or only occur slowly, meaning they are unlikely to be important for inhibition. However, in some cases such scaffold fragmented species appear to be very stable, meaning the development of inhibitors that undergo such fragmentations is of interest.

Experimental Section

Materials

Unless stated otherwise potassium clavulanate from Sigma Aldrich was used. tert-Butylamine clavulanate salt, used for native MS studies, was from Guangpu Biotech Ltd. FC-5 was prepared as reported.[16]

Enzyme Production

Recombinant $AmpC_{EC}$, TEM-116 with a *N*-terminal His tag and OXA-10 with a cleavable *N*-terminal His tag were produced and purified as described[27]; purity (>95%) was demonstrated by SDS-PAGE and mass spectrometry analyses. Concentrations of purified proteins were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific).

Inhibition Studies

Kinetic assays were performed as described[14] using either nitrocefin (measuring absorbance at $\lambda_{Abs}=486$ nm, using path-length correction) or FC-5[16] (measuring fluorescence intensity at $\lambda_{ex}=380$ nm and $\lambda_{em}=460$ nm) as a reporter substrate and either BMG LABTECH PHERAstar or CLARIOstar plate readers. Unless stated otherwise, reactions were in 50 mM phosphate buffer pH 7.5, containing 0.01% (*v/v*) aqueous Triton X-100 (and 10 mM $NaHCO_3$ for experiments with OXA-10) and performed at least in triplicate.

IC_{50}s were determined as reported.[16] TEM-116 (1 nM), $AmpC_{EC}$ (500 pM), or OXA-10 (250 pM) were incubated with varying amounts of potassium clavulanate for the indicated times, then assayed using 5 μM FC-5. The apparent inhibitory constant K_{iapp} and the second order rate constant k_{inact}/K (or k_2/K) were determined using modifications of reported methods.[28] SBLs were reacted with the reporter substrate (nitrocefin or FC-5) in the presence of potassium clavulanate in varied concentrations. Reactions were initiated by SBL addition and immediately monitored for 60–120 s (until the reaction profiles plateaued). For TEM-116 (1 nM) assays were carried out in competition with nitrocefin (50 μM); for $AmpC_{EC}$ (100 nM) and OXA-10 (50 nM), FC-5 (5 μM) was used. Dissociation constants (k_{off}) were determined by the jump dilution method.[29] SBLs (3 μM) were incubated with potassium clavulanate at room temperature, then serially diluted in assay buffer (to final concentrations of TEM-116: 30 pM; $AmpC_{EC}$ 10 pM, OXA-10: 10 pM) and assayed using 25 μM FC-5. Data were analyzed as reported.[14] Non-linear regression analysis was performed with Prism 9 (GraphPad Software).

SPE Electrospray Ionisation MS Assays

$AmpC_{EC}$, OXA-10, or TEM-116 (3 μM) in 50 mM Tris pH 7.5 were incubated with potassium clavulanate (1.5 mM) at room temperature. Mass spectra were acquired in the positive ion mode using an integrated autosampler/solid phase extraction (SPE) RapidFire365 system (Agilent Technologies) coupled to an Agilent 6550 Accurate Mass QTOF mass spectrometer. After the indicated time, 50 μl of the solution was loaded onto a C4 SPE cartridge (Agilent Technologies), which was then washed with buffer A (100% (*v/v*) water 0.1% (*v/v*) formic acid), then eluted into the mass spectrometer in buffer B (15% (*v/v*) water 85% (*v/v*) acetonitrile 0.1% (*v/v*) formic acid). The cartridge was re-equilibrated in buffer A in between samples. Data were analyzed using MassHunter Qualitative Analysis V.7 (Agilent Technologies) using the maximum entropy deconvolution algorithm.

HPLC Electrospray Ionisation MS Assays

$AmpC_{EC}$, OXA-10, or TEM-116 (1 μM) in 50 mM Tris pH 7.5 were incubated with potassium clavulanate (500 μM) at room temperature for 20 min. Samples were analyzed using a Xevo G2-S mass spectrometer (Waters Corporation) coupled to a Aquity-UPLC system (Waters Corporation), equipped using a ProSwift™ RP-4H 1 mm 50 mm column (Thermo Fisher Scientific). Samples were loaded onto the column in 95% (*v/v*) water 5% (*v/v*) acetonitrile 0.1% (*v/v*) formic acid and eluted using a gradient to 5% (*v/v*) water 95% (*v/v*) acetonitrile 0.1% (*v/v*) formic acid, then introduced directly into the ESI source. The retention times of all proteins were ~4–5 min. Data were analyzed using MassLynx 4.1 (Waters Corporation), with deconvolution using the MaxEnt1 algorithm.

Non-denaturing MS Assays

$AmpC_{EC}$, OXA-10, or TEM-116 (1 μM, 2 μM, or 0.5 μM, respectively) were incubated with clavulanic acid tert-butylamine salt ($AmpC_{EC}$: 500 μM; TEM-116: 100 μM, OXA-10: 50 μM) in 50 mM ammonium acetate pH 7.5. Samples (3 μl) were loaded into in-house prepared gold-coated capillary needles (Harvard Apparatus) and were injected into a Q-Exactive UHMR Hybrid Quadrupole-Orbitrap spectrometer (Thermo Fisher Scientific).[30] Instrument parameters: capillary voltage 1.2 kV, S-lens RF 200%, mass range from 1,000–12,000 m/z, capillary temperature 60 °C, resolution of the instrument 17,500 at m/z = 200 (transient time: 64 ms). The noise level was 3, rather than the default of 4.64. Instrument calibration was performed using a 10 mg ml$^{-[1]}$ solution of CsI in water. Data were analyzed using Xcalibur 4.1 (Thermo Fisher Scientific), and deconvoluted using UniDec.[31]

Nuclear Magnetic Resonance Spectroscopy

^{1}H NMR spectra were recorded using a Bruker Avance III 700 MHz spectrometer, equipped with a Bruker 5 mm TCI H/C/N He cryoprobe. Samples were prepared using 50 mM sodium phosphate, pH 7.5, 10% (*v/v*) D_2O. The water signal was suppressed using presaturation or excitation sculpting[32] with the Perfect Echo pulse sequence. Turnover assays were performed using 1 mM potassium clavulanate and 10 μM $AmpC_{EC}$, TEM-116, or OXA-10. Chemical shift assignments were made on the basis of ^{1}H, COSY, HSQC, and HMBC spectra as well as previous reports.[25,33]

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Conflict of Interests

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: Antimicrobial resistance · Clavulanic acid · Mechanism-based inhibition · Penam sulfone · Serine β-lactamase inhibitor

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