

## O-Aryloxycarbonyl Hydroxamates: New $\beta$ -Lactamase Inhibitors That Cross-Link the Active Site

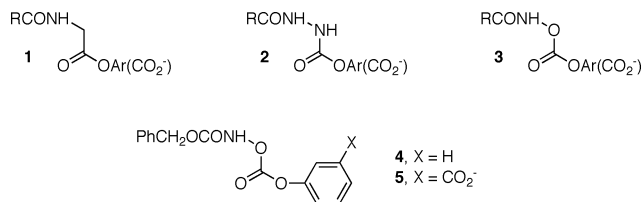
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The effective lifetime of  $\beta$ -lactams as antibiotics can be extended by concurrent treatment of patients with  $\beta$ -lactamase inhibitors.<sup>1,2</sup> The  $\beta$ -lactamase inhibitors in commercial production at present, however, are of limited spectrum and are largely specific to the class A enzymes.<sup>1</sup> New classes of inhibitor with broader specificity covering classes B, C, and D  $\beta$ -lactamases would be welcome since the prevalence of these enzymes, and thus  $\beta$ -lactam resistance among bacteria, continues to grow. We describe in this communication a new type of  $\beta$ -lactamase inhibitor with an unusual mechanism of action involving the covalent cross-linking of active site residues.

The depsipeptides of general structure **1** are  $\beta$ -lactamase substrates.<sup>3,4</sup> Although the aza analogues **2** display little or no substrate activity,<sup>5</sup> we were encouraged to try the oxa analogues **3** because of the inhibitory properties of vanadate/hydroxamic acid complexes.<sup>6</sup> Compounds **4** and **5** were therefore obtained from careful reaction of the appropriate hydroxamic acid and chloroformate (the latter carboxyl-protected in the case of **5**) in the presence of imidazole (Supporting Information). An alkoxy side chain was chosen since the alkyl or aryl analogues were unstable to the Lössen rearrangement.<sup>7</sup> An NMR spectrum of <sup>15</sup>N-**4** in DMSO-*d*<sub>6</sub> showed a <sup>15</sup>N resonance at 161.1 ppm coupled (*J* = 93 Hz) to a proton at 11.75 ppm. This clearly identifies the product as the *O*-acyl rather than *N*-acyl hydroxamic acid. Compounds **4** and **5** hydrolyzed in aqueous buffer (20 mM MOPS, pH 7.5), yielding benzyl *N*-hydroxycarbamate, the phenol, and, presumably, bicarbonate; pseudo-first-order rate constants (*k*<sub>0</sub>) of 2.5 × 10<sup>-4</sup> and 2.78 × 10<sup>-4</sup> s<sup>-1</sup>, respectively, were obtained.



Compound **4** inhibited, essentially irreversibly, the class C  $\beta$ -lactamase of *Enterobacter cloacae* P99 in a time-dependent fashion, as evident from Figure 1. At low inhibitor/enzyme concentration ratios, the final activity of the enzyme was not zero, which suggested that some turnover accompanied the inhibition reaction (background hydrolysis of **4** was not sufficient to explain the final activity). A greater excess of inhibitor did completely inactivate the enzyme (Figure 1). A plot of residual activity versus concentration of **4** (Figure 2) suggested that about two turnovers accompanied inhibition. These data were fitted to Scheme 1, where EI is likely to be a hydrolyzable acyl enzyme<sup>4</sup> which can also partition to a dead end complex EI'. These fits, shown as solid

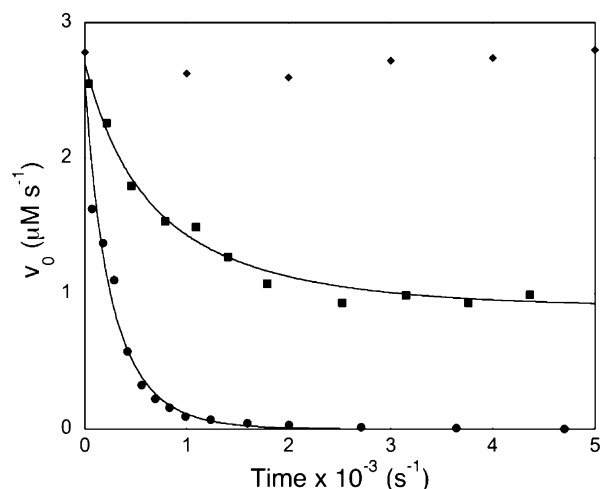


Figure 1. Activity of the P99  $\beta$ -lactamase (0.25  $\mu$ M) as a function of time in the presence of **4** (0  $\mu$ M,  $\blacklozenge$ ; 0.5  $\mu$ M,  $\blacksquare$ ; 2.5  $\mu$ M,  $\bullet$ ).

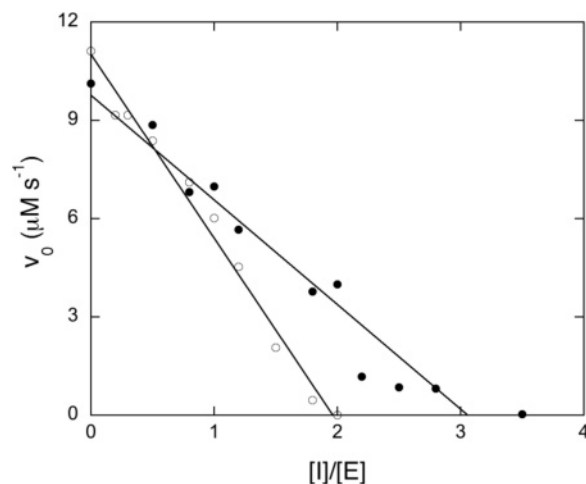
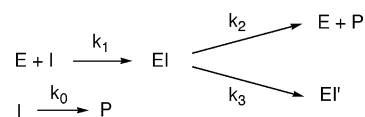


Figure 2. Activity of the P99  $\beta$ -lactamase (0.25  $\mu$ M) after complete reaction with **4** ( $\bullet$ ) and **5** ( $\circ$ ) at various concentrations (0–1.0  $\mu$ M).

### Scheme 1

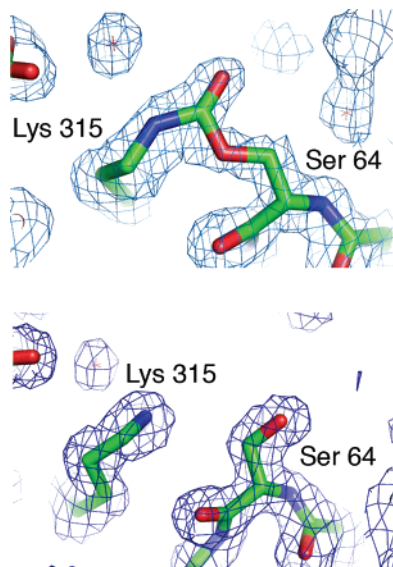


lines in the figures, yielded *k*<sub>1</sub> and *k*<sub>2</sub>/*k*<sub>3</sub> values of 6.1 ± 0.2 × 10<sup>3</sup> s<sup>-1</sup> M<sup>-1</sup> and 2.0 ± 0.1, respectively.

Compound **5** was also an inhibitor of the P99 enzyme (Figure 2). Experiments analogous to those described above yielded values of *k*<sub>1</sub> and *k*<sub>2</sub>/*k*<sub>3</sub> of 5.4 ± 0.3 × 10<sup>3</sup> s<sup>-1</sup> M<sup>-1</sup> and 1.00 ± 0.05, respectively. It is interesting that **5**, bearing the *m*-carboxy sub-

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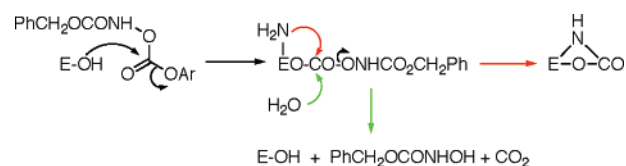
**Figure 3.** Top: crystal structure of the active site of the AmpC  $\beta$ -lactamase after inhibition by **4**, showing the carbamate cross-link between Ser64 and Lys315. The electron density is contoured at the  $3\sigma$  level. Bottom: the same view of the wild-type enzyme,<sup>10</sup> showing a clear gap between Ser64 and Lys315.

stituent, is not a better inhibitor than **4**. This result is contrary to what would be expected from comparable substitution in the depsipeptides **1**. A *m*-carboxy group in **1** is thought to interact specifically with the P99 active site.<sup>8</sup> The results suggest that **1** and **5** may not bind to the active site in the same way. It should be noted, however, that inactivation of the enzyme by **5** was competitively inhibited by *p*-nitrobenzene boronic acid, which is itself a competitive inhibitor of the P99 enzyme.<sup>9</sup>

An electrospray mass spectrum of the inhibited enzyme was obtained. Enzyme (10  $\mu$ M) and **4** (5 mM) were incubated together in MOPS buffer (above) for 5 min, after which time the enzyme was inactive. The protein was then precipitated with trichloroacetic acid, washed, and dried, and an ES+ mass spectrum obtained. The spectrum showed an increase in protein mass of 29, in good agreement with the mechanism of inactivation described below.

A 1.8 Å resolution crystal structure of the inhibited AmpC class C  $\beta$ -lactamase was also obtained, as described in the Supporting Information. The only observable difference from the structure of the native enzyme<sup>10</sup> was at the active site. In monomer A of the structure, the  $O_\gamma$  oxygen of Ser64 is flipped some 180° (Ser64 $C_\alpha C_\beta O_\gamma C$ ) and forms part of an unprecedented carbamate bridge to  $N_\epsilon$  of Lys315 (Figure 3). Tyr150 has moved aside slightly to accommodate insertion of a carbonyl, but  $O_\zeta$  remains within hydrogen-bonding distance of the inserted carbonyl oxygen (Figure

#### Scheme 2



S1, Supporting Information). Lys73 remains hydrogen bonded to Tyr150  $O_\zeta$ . Coordinates of the structure have been deposited in the RCSB protein data bank as entry 2P9V.

The mechanism of inhibition of the P99  $\beta$ -lactamase by **4** and **5** can thus, from the data available at present, be represented by the sequence shown in Scheme 2. This represents a novel cross-linking of the active site and a previously unobserved specific modification of one of the two conserved lysine residues of the  $\beta$ -lactamase active site. Inhibition of class A  $\beta$ -lactamases by clavulanic acid and penicillin sulfones has been shown to involve cross-linking of the active site serine to the conserved Ser130.<sup>11,12</sup>

We have also observed that **4** and **5**, and other derivatives of these compounds, inhibit the class A TEM  $\beta$ -lactamase. We plan further experiments to determine the scope of these compounds against  $\beta$ -lactam-recognizing enzymes.

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**Supporting Information Available:** Synthetic procedures for compounds **4** and **5** and the kinetics methods. Details of the crystallographic procedures and statistics are also provided. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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