

O-Aryloxycarbonyl Hydroxamates: New β -Lactamase Inhibitors That Cross-Link the Active Site

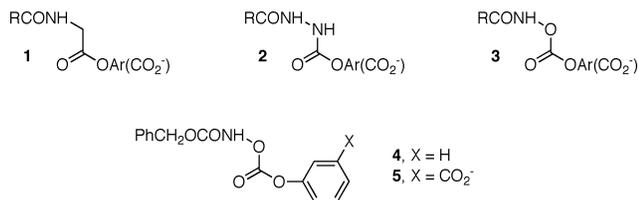
Pauline N. Wyrembak,[†] Kerim Babaoglu,[‡] Ryan B. Pelto,[†] Brian K. Shoichet,[‡] and R. F. Pratt^{*†}

Department of Chemistry, Wesleyan University, Middletown, Connecticut 06459, and Department of Pharmaceutical Chemistry, University of California, San Francisco, 1700 4th Street, San Francisco, California 94158-2330

Received April 4, 2007; E-mail: rpratt@wesleyan.edu

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

The depsipeptides of general structure **1** are β -lactamase substrates.^{3,4} Although the aza analogues **2** display little or no substrate activity,⁵ we were encouraged to try the oxa analogues **3** because of the inhibitory properties of vanadate/hydroxamic acid complexes.⁶ 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 Lössén rearrangement.⁷ An NMR spectrum of ¹⁵N-**4** in DMSO-*d*₆ showed a ¹⁵N 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*₀) of 2.5 × 10⁻⁴ and 2.78 × 10⁻⁴ s⁻¹, respectively, were obtained.



Compound **4** inhibited, essentially irreversibly, the class C β -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 enzyme⁴ which can also partition to a dead end complex EI'. These fits, shown as solid

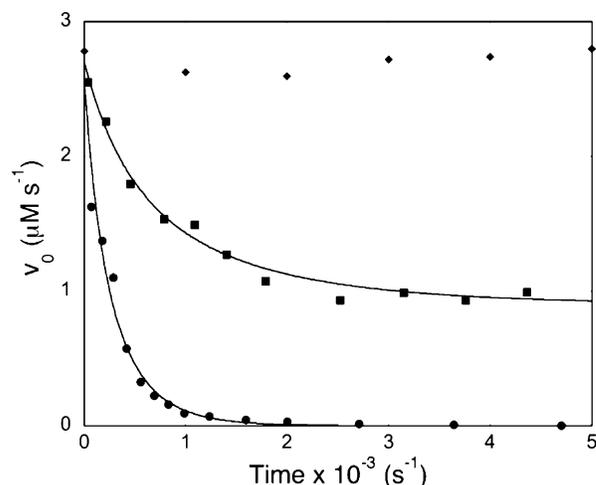


Figure 1. Activity of the P99 β -lactamase (0.25 μ M) as a function of time in the presence of **4** (0 μ M, \blacklozenge ; 0.5 μ M, \blacksquare ; 2.5 μ M, \bullet).

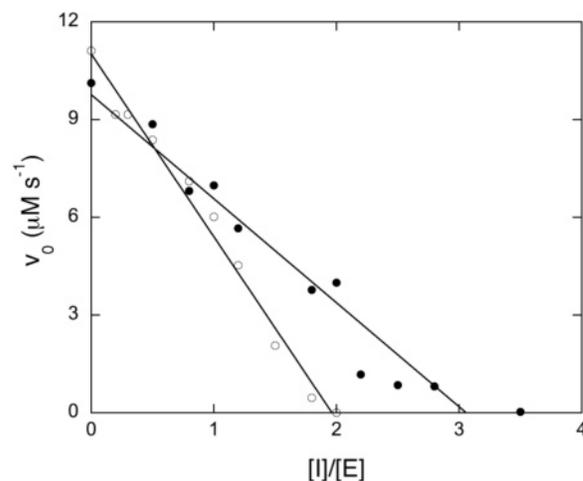
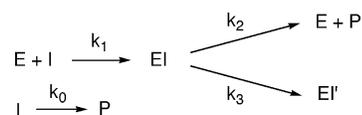


Figure 2. Activity of the P99 β -lactamase (0.25 μ M) after complete reaction with **4** (\bullet) and **5** (\circ) at various concentrations (0–1.0 μ M).

Scheme 1



lines in the figures, yielded *k*₁ and *k*₂/*k*₃ values of 6.1 ± 0.2 × 10³ s⁻¹ M⁻¹ 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*₁ and *k*₂/*k*₃ of 5.4 ± 0.3 × 10³ s⁻¹ M⁻¹ and 1.00 ± 0.05, respectively. It is interesting that **5**, bearing the *m*-carboxy sub-

[†] Wesleyan University.

[‡] University of California, San Francisco.

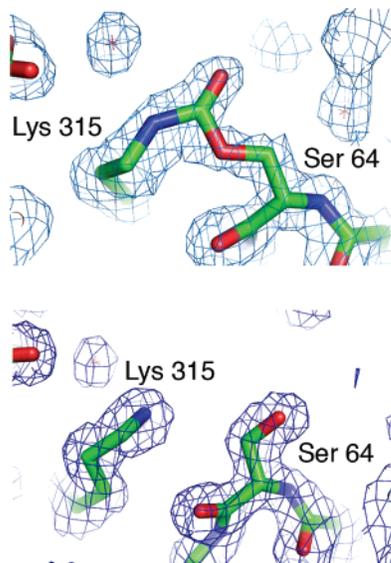


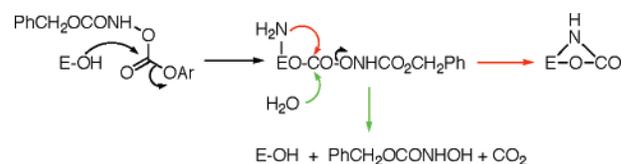
Figure 3. Top: crystal structure of the active site of the AmpC β -lactamase after inhibition by **4**, showing the carbamate cross-link between Ser64 and Lys315. The electron density is contoured at the 3σ level. Bottom: the same view of the wild-type enzyme,¹⁰ showing a clear gap between Ser64 and 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.⁸ 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.⁹

An electrospray mass spectrum of the inhibited enzyme was obtained. Enzyme (10 μ 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 β -lactamase was also obtained, as described in the Supporting Information. The only observable difference from the structure of the native enzyme¹⁰ was at the active site. In monomer A of the structure, the O_γ 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_ϵ of Lys315 (Figure 3). Tyr150 has moved aside slightly to accommodate insertion of a carbonyl, but O_ζ remains within hydrogen-bonding distance of the inserted carbonyl oxygen (Figure

Scheme 2



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

The mechanism of inhibition of the P99 β -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 β -lactamase active site. Inhibition of class A β -lactamases by clavulanic acid and penicillin sulfones has been shown to involve cross-linking of the active site serine to the conserved Ser130.^{11,12}

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

Acknowledgment. This research was supported by the National Institutes of Health through Grant AI 17986 to R.F.P. and GM 63815 to B.K.S. K.B. is supported by a Ruth L. Kirschstein National Research Service Award fellowship (GM 076883).

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>.

References

- (1) Georgopapadakou, N. *Exp. Opin. Invest. Drugs* **2004**, *13*, 1307.
- (2) Buynak, J. D. *Biochem. Pharmacol.* **2006**, *31*, 930.
- (3) Pratt, R. F.; Govardhan, C. P. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 1302.
- (4) Govardhan, C. P.; Pratt, R. F. *Biochemistry* **1987**, *26*, 3385.
- (5) Cabaret, D.; Garcia Gonzalez, M.; Wakselman, M.; Adediran, S. A.; Pratt, R. F. *Eur. J. Org. Chem.* **2001**, 141.
- (6) Bell, J. H.; Pratt, R. F. *Biochemistry* **2002**, *41*, 4329.
- (7) Renfrow, W. B., Jr.; Hauser, C. R. *J. Am. Chem. Soc.* **1937**, *59*, 2308.
- (8) Ahn, Y.-M.; Pratt, R. F. *Bioorg. Med. Chem.* **2004**, *12*, 1539.
- (9) Nagarajan, R.; Pratt, R. F. *Biochemistry* **2004**, *43*, 9664.
- (10) Usher, K. C.; Blaszcak, L. C.; Weston, G. S.; Shoichet, B. K.; Remington, S. J. *Biochemistry* **1998**, *37*, 16082.
- (11) Brown, R. P. A.; Aplin, R. T.; Schofield, C. J. *Biochemistry* **1996**, *35*, 12421.
- (12) Kuzin, A. P.; Nukaga, M.; Nukaga, Y.; Hujer, A.; Bonomo, R. A.; Knox, J. R. *Biochemistry* **2001**, *40*, 1861.

JA072370U