Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 5, No. 10, pp. 1043-1048, 1995

0960-894X/95 $9.50+0.00

0960-894X(95)00162-X

MECHANISTIC SUPPORT FOR THE STEPWISE PROCESS FOR INACTIVATION OF CLASS A β-LACTAMASES BY CLAVULANATE

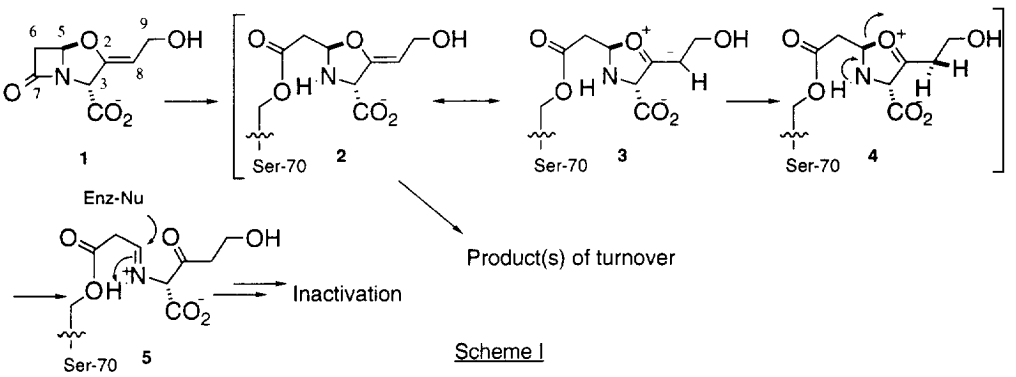
Kazuyuki Miyashita and Shahriar Mobashery*

Department of Chemistry, Wayne State University, Detroit, MI 48202-3489

Abstract: We proposed recently a stepwise process for the inactivation chemistry of class A β-lactamases by clavulanate (*J. Am. Chem. Soc.* **1993**, *115*, 4435). A requisite protonation by an active-site water molecule was suggested to be a critical chemical step in enzyme inactivation. The details of this proposed stepwise mechanism have been explored further by three synthetic analogues of clavulanate.

Because of their effectiveness and safety β-lactam antibiotics play a major role in the treatment of bacterial infections. The success of β-lactam antibiotics has resulted in selection of resistance to these antibacterial agents.[1] Most high-level resistance to β-lactams involves the production of β-lactamases, which hydrolyze, and thereby inactivate, various β-lactam antibiotics. A strategy which has been used successfully in the clinic is the use of a mechanism-based β-lactamase inactivator, such as clavulanate (**1**), sulbactam, or tazobactam, administered together with a penicillin.[2,3] The inactivator inactivates β-lactamase, thereby preserving the antibacterial activity of the penicillin.

Because of the clinical importance of clavulanate, its mechanism of action has been the subject of much investigation.[4-9] We proposed recently a stepwise mechanism for the inactivation chemistry of class A β-lactamases by the clinically used clavulanate (Scheme I).[8] We suggested that a structurally conserved water molecule in class A β-lactamases, Wat-673 (Fig. 1),[10] provides the necessary proton for ring opening (**2** → **5**), and that the protonation of the enol ether would most likely take place with the resonance structure **3**. Furthermore, we proposed that the proton transfer from Wat-673 may either take place directly, or via the C_3 carboxylate of clavulanate. This second proposal was offered in light of the distance of 3.9 Å between the water and the site of protonation and the fact that the carboxylate is hydrogen bonded to Wat-673. We had suggested that in a six-membered transition, the transfer of water could be mediated by the carboxyl function.



Scheme I

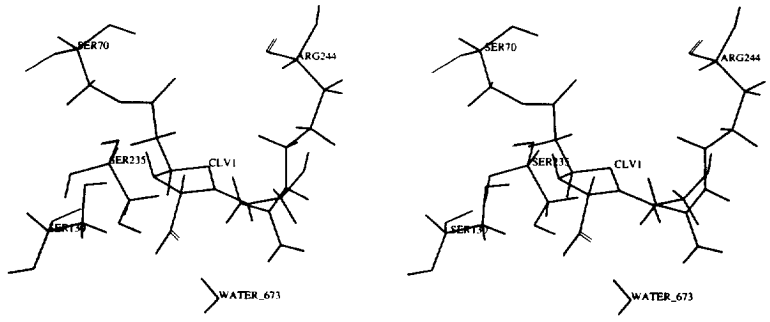
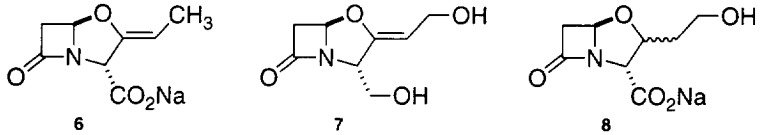


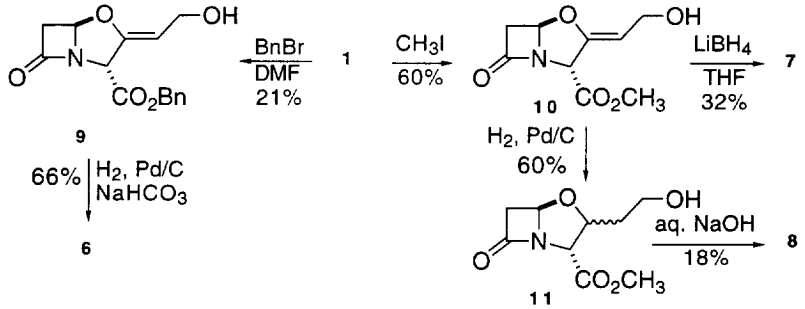
Figure 1. Stereoview of the energy-minimized structure for the acyl-enzyme intermediate of clavulanate in the active-site of β-lactamase. Hydrogen atoms are shown.

In an attempt to address a number of issues raised by this proposed mechanism, we have prepared compounds **6**, **7**, and **8**. Compound **6** lacks the C_9 hydroxyl and was intended to test the role of this function in the inactivation chemistry. The C_3 carboxylate of clavulanate was reduced to the hydroxymethyl function to give compound **7** to test whether the carboxylate of clavulanate would be necessary for enzyme inactivation. Finally, the mixture of the two dihydroclavulanates (**8**) was synthesized to examine whether the enol ether is a critical functionality for the inactivation chemistry, as is suggested by Scheme I.



6 7 8

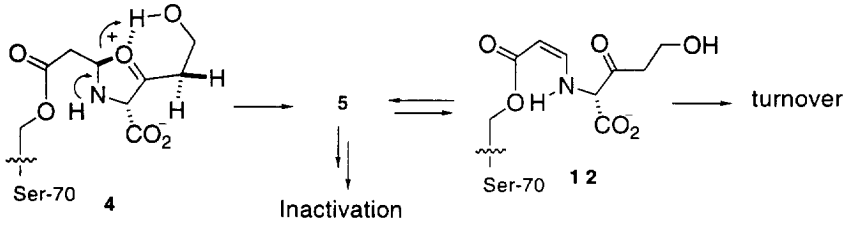
Clavulanate (1)was stirred with benzyl bromide in DMF to give the corresponding benzyl ester (**9**). Compound **9** was hydrogenolyzed in the presence of Pd/C to give **6**. Similarly to the preparation of **9**, compound **10** was synthesized by allowing the reaction of iodomethane with clavulanate. This key intermediate was reduced with $LiBH_4$ to give **7**, or by hydrogenation in the presence of Pd/C to yield 11.[11] Saponification of 11 afforded compound **8**.



As shown in Fig. 2A, inactivation of the TEM-1 β-lactamase—a prototypic Gram-negative class A β-lactamase—by clavulanate is biphasic.[6,8] The biphasic nature of irreversible inactivation kinetics is a

consequence of competing processes for the formation of the inactivated species and the so-called transiently inhibited species (**12**);[6] the rapid initial phase of irreversible inactivation (Scheme I) is slowed down by branching at species **5** (**5** → **12**). Species **12** was suggested to turn over more slowly because of the unsaturation of the ester function.[4,5] We suggested recently that this tautomerization reaction (**5** → **12**) may be mediated by Glu-166 via the structurally conserved Wat-712.[8]

The C_9 hydroxyl of clavulanate is introduced in a rather circuitous manner during biosynthesis of clavulanate.[12] The C_9 methylene comes from the $C\delta$ of ornithine. The $C\delta$ amine from the ornithine side chain, which is introduced into a clavulanate precursor, is removed by oxidative deamination, and the resultant aldehyde is reduced to the hydroxymethyl function in a process that also achieves epimerization at C_3 and C_5. It would appear that considerable effort is expended to acquire the C_9 hydroxyl. A likely function for the C_9 hydroxyl group is the stabilization by an intramolecular hydrogen bond of the ground state for the enol ether function. However, we note that 9-deoxyclavulanate (**6**) shows a measurable rate attenuation for both the first and the second phases of irreversible inactivation (Fig. 2B).[13] One implication of this observation is that the C_9 hydroxyl group of clavulanate forms a hydrogen bond with the active site of the enzyme in the course of inactivation. This possibility was refuted in the models for both the precatalytic ("Michaelis") complex as well as the acyl-enzyme intermediate (Fig. 1), as reported by us previously.[8] The C_9 hydroxyl was shown to point in the direction of the active-site opening without any interactions with the protein. Nonetheless, we evaluated the dissociation constant as a measure of affinity of the TEM-1 β-lactamase for **6**. The dissociation constant for **6** was 0.80 ± 0.04 μM, which compares well with the value of 0.40 ± 0.02 μM measured for clavulanate by Imtiaz *et al.*[8] with the TEM-1 enzyme. Therefore, affinity of the enzyme for **6** has not been reduced within the limits of such determinations and a different factor has to account for the inactivation rate attenuation. We suggest here that the C_9 hydroxyl of clavulanate may facilitate the departure of the proposed oxonium oxygen by forming an intramolecular hydrogen bond as depicted below.[14] This hydrogen bonding is not essential for the inactivation chemistry, but would speed it up. Because turnover of clavulanate, irreversible inactivation and transient inhibition of the enzyme all proceed concurrently, we have found it difficult to measure the rates for these events with high accuracy. However, the partition ratio (*i.e.*, k_{cat}/k_{inact}) may be evaluated readily by the titration method.[15] The partition ratio for **6** was evaluated at 390 ± 13, and that for clavulanate was measured at 160 ± 3. It is reasonable to assume that k_{cat} for clavulanate (**1**) and **6** should be essentially the same, hence the difference in the partition ratios is likely to be due to the differences in k_{inact} values. Therefore, we suggest that such intramolecular hydrogen bond would lower the energy barrier for irreversible inactivation by as much as 0.5 kcal/mol.[16]



Compound **7** was synthesized to test whether a direct proton transfer to C_8 or a proton transfer via the inactivator carboxylate is involved in the inactivation chemistry. As indicated by Fig. 2C, compound **7** inactivated the enzyme in the characteristic biphasic manner, however, the rate of irreversible inactivation in the second phase was substantially decreased. Portions of the enzyme were inactivated by **7** and the preparations were dialyzed overnight, during which time full recovery from transient inhibition had taken place. The enzyme preparations showed progressive irreversible inactivation as a function of time. These findings indicate that the C_3 carboxylate is not required for the inactivation chemistry and that protonation of C_8 take place directly. However, as reported previously,[8] the carboxylate of clavulanate makes hydrogen bonds to the structurally conserved Wat-673, and the side chains of Ser-130, Ser-235, and Arg-244. These interactions, which would be largely absent with the hydroxymethyl group of **7**, are obviously necessary for proper sequestration of clavulanate in the active site in the course of the inactivation chemistry. Since irreversible inactivation of the enzyme is slowed down—but not entirely eliminated—the enzyme relies more heavily on transient inhibition with compound **7**. The rate of recovery from transient inhibition was calculated at $(2.0 \pm 0.7) \times 10^{-3}$ s^{-1} according to the method of Glick *et al.*[17] This value agrees well with the rate of $(3.8 \pm 0.3) \times 10^{-3}$ s^{-1} reported for the same process for clavulanate, as measured for the TEM-2 β-lactamase.[6] Therefore, it would appear that compound **7** both irreversibly inactivates, as well as transiently inhibits the TEM-1 β-lactamase, as would clavulanate. We add here that the TEM-1 β-lactamase when inactivated by clavulanate, compounds **6** or **7** showed three protein species on inspection by isoelectric focusing (data not shown); there were two major and one minor bands in the protein gel. These results are analogous to the findings reported by Charnas *et al.* for clavulanate inactivation of the TEM-2 β-lactamase.[4]

Compounds **8** lack the olefin moiety in the enol ether function of clavulanate. As suggested earlier for the inactivation chemistry of clavulanate, protonation of the enol ether function is likely to take place on the C_8 carbon for the resonance structure **3**.[8] Therefore, compounds **8** would be expected not to serve as inactivators for β-lactamase, and indeed they do not. Incubation of concentrations of **8** as high as 20 mM (Fig. 2D) with the TEM-1 β-lactamase resulted in no irreversible inactivation of the enzyme. The mixture of compounds **8** served as a fairly good substrate for the TEM-1 β-lactamase ($k_{cat} = 8.0 \pm 0.1$ s^{-1}, $K_m = 2.2 \pm 0.5$ μM, $K_s = 11.6 \pm 1.9$ μM, $k_{cat}/K_m = 3.7 \times 10^6$ $M^{-1}s^{-1}$, $k_2 = 42$ s^{-1} and $k_3 = 10$ s^{-1}). The small decrease in enzyme activity noted in the earlier parts of incubation shown in Fig. 2D is fully reversible and is a consequence of relatively slow deacylation from the active site in the course of turnover of **8**. It would appear that deacylation is the rate-limiting step in turnover of **8**. Compounds **8** provided the opportunity to study turnover in the absence of inactivation, since both turnover and inactivation chemistries proceed rapidly and concurrently with clavulanate and cannot be investigated independently.

An interesting observation has been made by Lobkovsky *et al.* on the lack of effective inactivation of class C β-lactamases by clavulanate.[18] Consistent with our mechanistic explanation,[8] they observe that a counterpart to Wat-673 of class A enzymes is found in the crystal structure for the class C β-lactamase from *Enterobacter cloacae* P99; however, the position of this water molecule in relation to the acyl-enzyme complex of clavulanate is such that it cannot serve as the source of proton in the inactivation chemistry. Lobkovsky *et al.* suggest that this may be at the roots of the ineffective inactivation of class C enzymes by clavulanate.

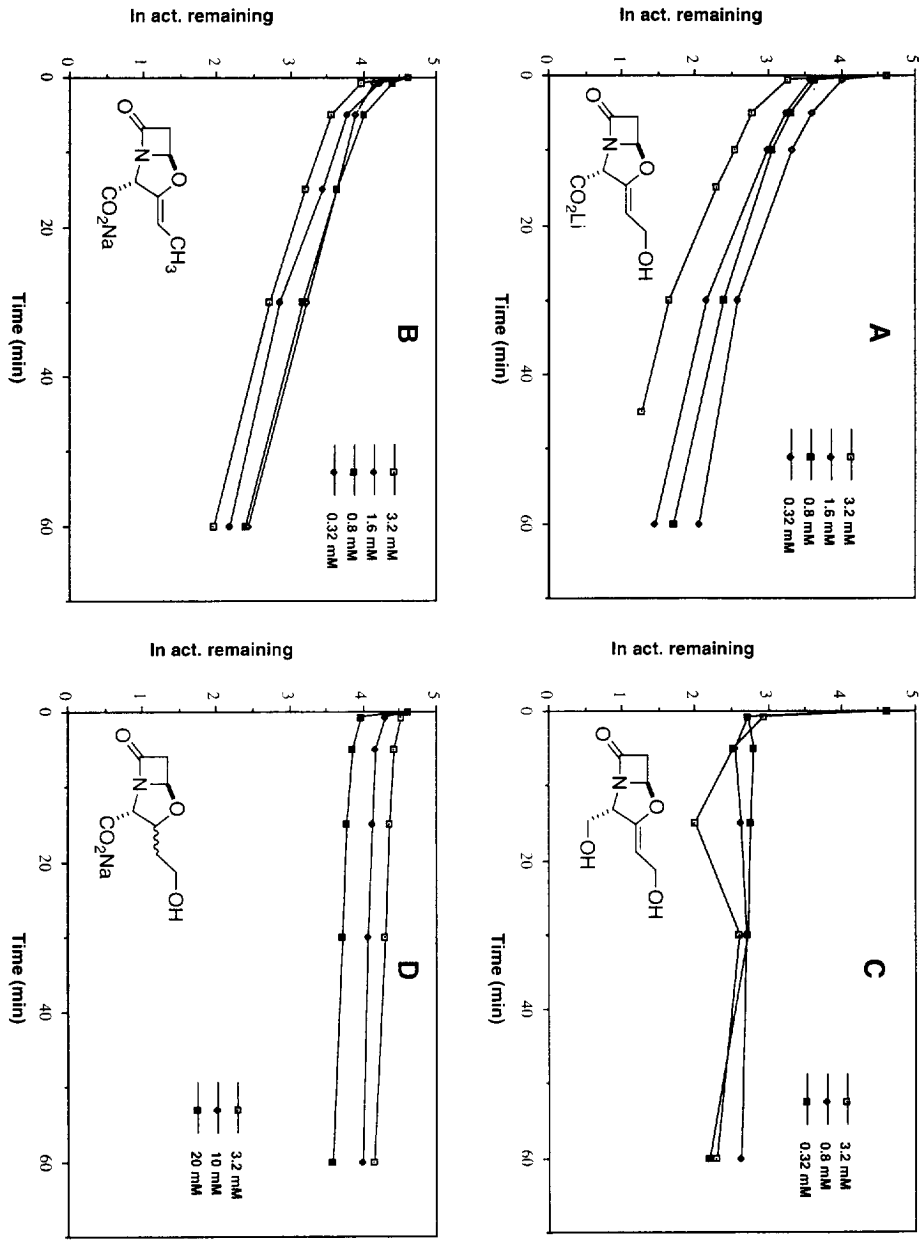


Figure 2. Plots of enzyme activity as a function of time for (A) clavulanate, (B) compound **6**, (C) compound **7**, and (D) compounds **8**.

We have shown that the Arg-244-Ser mutant derivative of the TEM-1 β-lactamase is resistant, both *in vivo* and *in vitro*, to inactivation by clavulanate.[8] We suggested that this mutant β-lactamase, which was prepared in the laboratory, does not have the ability to retain Wat-673 in the active site with high affinity, hence the chemistry of inactivation has been impaired. This mutant β-lactamase has now been detected in clinical isolates.[19] β-Lactamases are often plasmid-encoded, and since plasmid exchange occurs promiscuously among bacterial populations, this would facilitate rapid acquisition of the drug-resistance phenotype among bacterial flora. Such dissemination of the resistance phenotype would herald an anticipated selection for organisms that are resistant to the combination therapy of clavulanate and penicillins.

Acknowledgment: This work was supported by the National Institutes of Health. We thank Drs. D. Knowles, J.B. Harbridge, and J. Gleason of SmithKline Beecham for providing us with a generous sample of clavulanate.

References and Notes

(1) Neu, H.C. *Science* **1992**, *257*, 1064.
(2) Leigh, D. A., Bradnock, K. & Marriner, J. M. (1981) *J. Antimicrob. Chemother.* **7**, 229-236.
(3) Moellering, R. C. (1991) *Rev. Infect. Dis. 13*, S723-S726.
(4) Charnas, R.L.; Fisher, J.; Knowles, J.R. *Biochemistry* **1978**, *17*, 2185.
(5) Charnas, R.L.; Knowles, J.R. *Biochemistry* **1981**, *20*, 3214.
(6) Fisher, J.; Charnas, R.L.; Knowles, J.R. *Biochemistry*, **1978**, *17*, 2180.
(7) Rizwi, I.; Tan, A.K.; Fink, A.L.; Virden, R. *Biochem. J.* **1989**, *258*, 205.
(8) Imtiaz, U.; Billings, E.; Knox, J. R.; Manavathu, E. K.; Lerner, S. A.; Mobashery, S. *J. Am. Chem. Soc.* **1993**, *115*, 4435-4442.
(9) Sawai, T.; Yamaguchi, A. *Diag. Microbiol. Infect. Dis.* **1989**, *12*, 121S. Knowles, J.R. *Acc. Chem. Res.* **1985**, *18*, 97; and the references cited therein.
(10) The consensus amino-acid numbering system of class A β-lactamases has been used throughout the manuscript (Ambler, R. P.; Coulson, A. F. W.; Frère, J. M.; Ghuysen, J. M.; Joris, B.; Forsman, M.; Levesque, R. C.; Tiraby, G.; Waley, S. G. *Biochem. J.* **1991**, *276*, 269). The numbering system for the water molecules is that of the related *Bacillus licheniformis* enzyme, as proposed by Knox and Moews (Knox, J. R.; Moews, P. C. *J. Mol. Biol.* **1991**, 220, 435).
(11) Reduction of **10** to **11** and **9** to **6** have been reported: Brown, A.G.; Corbett, D.F.; Goodacre, J.; Harbridge, J.B.; Howarth, T.T.; Ponsford, R.J.; Stirling, I.; King, T.J. *J. Chem. Soc. Perkin Trans.* I **1984**, 635.
(12) Iwata-Reuyl, D.; Townsend, C.A. *J. Am. Chem. Soc.* **1992**, *114*, 2763. Janc, J.W.; Egan, L.A.; Townsend, C.A. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 2313. Baldwin, J.E.; Goh, K.C.; Schofield, C.J. *Tetrahedron Lett.* **1994**, *35*, 2779. Baldwin, J.E.; Fujishima, Y.; Goh, K.C.; Schofield, C.J. *Tetrahedron Lett.* **1994**, *35*, 2783.
(13) We add here that Charnas and Knowles reported that 9-deoxyclavulanate behaved virtually the same as clavulanate in inactivation of the TEM-2 β-lactamase (ref. 5). Our results with this compound stand in contrast to the earlier report.
(14) It should be noted that despite the formal charge of +1 on the oxygen of the oxonium moiety electronegativity arguments clearly indicate that the oxygen atom bears a net negative charge density (Bach, R.D.; Owensby, A.L.; Gonzales, C.; Schlegel, H.B.; McDouall, J.J.W. *J. Am. Chem. Soc.* **1991**, *113*, 6001).
(15) Silverman, R. In *Mechanism-Based Enzyme Inactivation: Chemistry and Enzymology*; CRC Press, Boca Raton, 1988; p 22.
(16) Calculated from $\Delta\Delta G = -RT\ln(k_{inact,6}/k_{inact,1})$.
(17) Glick, B.R.; Brubacher, L.J.; Leggett, D.J. *Can. J. Biochem.* **1978**, *56*, 1055.
(18) Lobkovsky, E; Moews, P.C.; Liu, H.; Zhao, H.; Frère, J.-M.; Knox J.R. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 11257.
(19) Vedel, G.; Belaoaouaj, A.; Gilly, L.; Labia, R.; Phillippon, A.; Nevot, P.; Paul, G. *J. Antimicrob. Chemother.* **1992**, *30*, 449.Zhou, X.Y.; Bordon, F.; Sirot, D.; Kitzis, M.D.; Gutmann, L. *Antimicrob. Agents Chemother.* **1994**, *38*, 1085.

(*Received in USA* 9 *March* 1995; *accepted* 7 *April* 1995)