Penem BRL 42715: An Effective Inactivator for β-Lactamases

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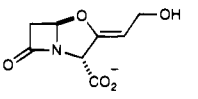
Abstract: The mechanism of inactivation of prototypic class A (TEM-1) and class C (Q908R) β-lactamases by penem BRL 42715 has been investigated by kinetic experiments and modeling computations. Both β-lactamases are inactivated extremely efficiently with $k_{inact}/K_i = 10^6-10^7\ M^{-1}\ s^{-1}$ and $k_{cat}/k_{inact} = 1-12$. The inhibitor constants were in the nanomolar range. The inactivation chemistry is equally efficient for the Arg-244-Ser mutant of the TEM-1 enzyme, which is resistant to inactivation by the clinical agent clavulanate. The inactivation chemistry is believed to involve acylation of the active-site serine, followed by a rearrangement to a dihydrothiazepine species. Inactivation is reversible in the case of the wild-type and mutant TEM-1 β-lactamases, but appears irreversible for the Q908R enzyme. Molecular modeling of the initial acylated species and the rearranged dihydrothiazepine species into the active sites of the crystal structures of the two enzymes provided insight into the chemistry of inactivation. Acylation is accompanied by a rotation about the C_5-C_6 bond which is critical for the proper positioning of an incipient thiolate upon its formation from the acyl-enzyme intermediate, en route to the dihydrothiazepine species. Structural information is provided for the reversibility of the inactivated TEM β-lactamase and the lack of such reversibility for the Q908 enzyme.

Because of their effectiveness and safety, β-lactam antibiotics play a central role in the treatment of bacterial infections.[1] However, clinical use of these antibiotics has been compromised by bacterial β-lactamases which hydrolyze the β-lactam moiety of these drugs as the primary mechanism of resistance to them.[2] Two types of β-lactamases, members of classes A and C, are of special concern for the resistance problem. These enzymes are believed to be related to one another from an evolutionary point of view; however, their size and details of their mechanisms are somewhat different.[3] One strategy that has been used with considerable success to overcome the catalytic function of these enzymes *in vivo* is the use of a mechanism-based inactivator, such as Clavulanate (**1**), sulbactam (**2**), or tazobactam (**3**), together with a penicillin; the inactivator inactivates the β-lactamase, thereby preserving the antibacterial activity of the penicillin. Clavulanate, sulbactam, and tazobactam are effective in inhibition of class A enzymes. Whereas tazobactam also inhibits class C enzymes *in vitro*, it is not sufficiently active to protect a penicillin in organisms harboring these enzymes.[4] The most active inhibitor of class C β-lactamases appears to be the investigational penem, BRL 42715 (**4**), a SmithKline Beecham product. As judged by microbiological analyses, it markedly enhances the activity of substrate β-lactams against standardized strains bearing class C β-lactamases under circumstances in which clavulanate, sulbactam, and tazobactam have no effect.[5] Parenthetically, it should be stated that although clavulanate, sulbactam, and tazobactam are active against class A β-lactamases of gram-negative bacteria, BRL 42715 is also more active than these compounds in enhancing activity of substrate β-lactams against strains bearing class A enzymes.[5] In this report, we have undertaken to investigate the kinetic features of inactivation of prototypical class A (TEM-1) and class C (Q908R) β-lactamases by BRL 42715. For both the TEM-1[6] and P99—a close analogue of Q908R[7]—β-lactamases crystal structures are available; these have allowed us to investigate the structural aspects of the inactivation chemistry as well.



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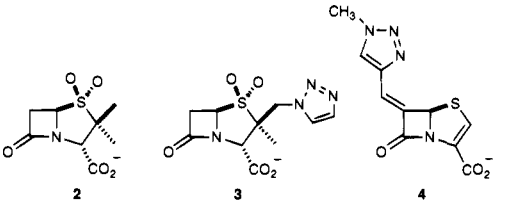

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Experimental Section

BRL 42715 (**4**) was obtained from the SmithKline Beecham Pharmaceutical Co. Penicillin G and cephaloridine were purchased from Sigma. Both the wild-type TEM-1 and Arg-244-Ser mutant β-lactamases were purified according to the method of Zafaralla *et al.*,[8] and the Q908R β-lactamase was purified by the procedure of Ross.[9]

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(3) 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.
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(6) (a) Strynadka, N. C. J.; Adachi, H.; Jensen, S. E.; Johns, K.; Sielecki, A.; Betzel, C.; Sutoh, K.; James, M. N. G. *Nature* **1992**, *359*, 700. (b) Jelsch, C.; Mourey, L.; Masson, J. M.; Samama, J. P. *Proteins: Struct. Funct. Genet.* **1993**, *16*, 364.
(7) The crystal structure report for the β-lactamase from *Enterobacter cloacae* P99 is given in ref 3. The P99 and Q908R β-lactamases are different only in four amino acids at sites remote from the active site. The two enzymes are believed to be virtually identical in both structure and mechanism.
(8) Zafaralla, G.; Manavathu, E. K.; Lerner, S. A.; Mobashery, S. *Biochemistry* **1992**, *31*, 3847.
(9) Ross, G. W. *Methods Enzymol.* **1975**, *43*, 678.

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All kinetic and spectral measurements were made on a Perkin-Elmer Lambda 3B or Hewlett-Packard 452 diode-array spectrometer. For a typical assay with the TEM β-lactamases, we utilized 1.0 mM penicillin G ($\Delta\epsilon_{240}$ = 570 M^{-1} cm^{-1}), and for the Q908R enzyme, 500 μM cephaloridine ($\Delta\epsilon_{295}$ = 1000 M^{-1} cm^{-1}) was used in 100 mM sodium phosphate buffer, pH 7.0.

Kinetic Experiments and Protein Modification. The experiments for the determination of k_{inact} and K_i were carried out by the method of Daniels *et al.*[10] For these experiments the concentrations of β-lactamase (20 nM of any of the three) and substrate (1.0 mM of penicillin G for the TEM enzymes, and 500 μM of cephaloridine for the Q908R enzyme) were kept constant, and the concentration of BRL 42715 was varied from 2 to 12 μM. Analysis of the data was performed according to the method of Daniels *et al.*[10]

The rate constant for recovery of activity for the fast phase was determined by the method of Koerber and Fink[11] and the computation of the data was performed according to Glick *et al.*[12] The following is a typical experiment. β-Lactamase (TEM-1 or Arg-244-Ser, 8.2 μM) was mixed with a solution of **4** (28 μM) and the mixture was incubated in an ice bath for 5 min, during which complete inactivation of the enzyme resulted. A 10-μL portion of this mixture was mixed into a solution of 2.0 mM penicillin G in 100 mM sodium phosphate and the recovery of activity was monitored at 240 nm ($\Delta\epsilon$ = 570 M^{-1} cm^{-1}) over 10−15 min.

In order to characterize the spectroscopic properties of the modified Q908 β-lactamase, the enzyme (10 μM, 300 μL) was inactivated by **4** in 100 mM sodium phosphate buffer, pH 7.0. The volume of the mixture was increased to 500 μL with the same buffer. A portion of the enzyme (150 μL) was dialyzed against 100 mM sodium phosphate buffer, pH 7.0, for 2 days with buffer changes every 4−5 h. The concentration of the modified enzyme was determined by the Bradford assay. Another portion of the inactivation mixture (250 μL) was loaded on a G-15 column (Pharmacia, 1 × 15 cm), previously equilibrated with 100 mM sodium phosphate buffer, pH 7.0. The eluent fractions with maximal absorbance at 280 nm were collected and combined. The TEM enzymes modified by **4** recovered activity over several hours, so dialysis could not be carried out as described. Hence, with the TEM β-lactamases only the G-15 column was used, as described above.

In the case of the inactivated Q908R β-lactamase, the protein was dialyzed against 100 mM phosphate buffer, pH 7.0. Subsequently, a solution of hydroxylamine was added to give a final concentration of 1.0 mM, and the protein was incubated at ice−water temperature. The activity was tested periodically; more than 90% of the activity was recovered within 2 h of incubation in the presence of hydroxylamine.

Thiol Titration. Titration of the thiol group was attempted with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) by the method of Ellman.[13] The inactivated Q908R β-lactamase was dialyzed against 100 mM sodium phosphate buffer, pH 7.0. Two portions (8 μM protein, 300 μL each) were titrated with DTNB (300 μM), one under denaturing conditions (supplemented to a final concentration of 0.1% SDS) and the other in 100 mM sodium phosphate buffer, pH 7.0 (non-denaturing condition). The absorbance change as a function of time at 412 nm was recorded and compared in each case. A control experiment in the absence of protein was also carried out.

Computational Models. Crystal coordinates for the TEM-1[6] and P99[3] β-lactamases were used in the three-dimensional modeling and energy minimization. The crystallographic water molecules were retained, and the active-site-bound inactivator(s) was then covered by the addition of Monte Carlo water molecules. The hydrogen atoms were added in the calculated positions and atomic charges were computed by the method of Gasteiger.[14] The energy minimization was performed by the geometry optimization algorithm MAXMIN2, using the Tripos force field by the Sybyl molecular modeling software in a Silicon Graphics R4000 Indigo computer. The Powell method[15] was used to determine the descending direction in the minimization. Minimization was performed in three stages: (1) the inhibitor and water molecules were allowed to move, (2) the inhibitor, water molecules, and the protein backbone were allowed to move, and (3) finally, the entire enzyme-inhibitor complex was allowed to minimize without any constraints. The minimization in a radius of 15 Å from the active site in each stage was continued until the change in energy was less than 0.001 (kcal mol^{-1})/Å between iterations. A dielectric constant of 1.0 was used for the calculations.

Results and Discussion

An important feature of the inactivation chemistry of **4** is that the process of inactivation is so fast that measurement of initial inactivation rates under standard steady-state conditions cannot be made. We have employed a technique by which the rate of inactivation is effectively slowed down by the presence of a competing substrate.[10] For these experiments we used the TEM and Q908R enzymes purified to homogeneity.

Compound **4** inactivates the TEM-1 β-lactamase in a process which is active-site directed, saturable, and involves covalent modification of the active site. The inactivation proceeds with the first-order rate constant of k_{inact} = 0.08 ± 0.02 s^{-1} and the inhibitor constant of K_i = 6 ± 1 nM (k_{inact}/K_i = 1.3 × 10^7 M^{-1} s^{-1}). The process of inactivation was extremely efficient as the partition ratio (k_{cat}/k_{inact})[16] was 1.1 ± 0.1, from which k_{cat} was calculated at 0.09 s^{-1}. After 1 day of dialysis of the inactivated protein against 100 mM sodium phosphate, pH 7.0, the entire activity was recovered. Subsequent investigation revealed that the recovery of activity was biphasic, and we were able to determine the rate constant for the fast phase (k_{rec}) at (8.7 ± 1.1) × 10^{-3} s^{-1}. At the end of the fast phase, approximately 20% of the activity had recovered. The rate of recovery of activity for the slow phase was substantially slower.

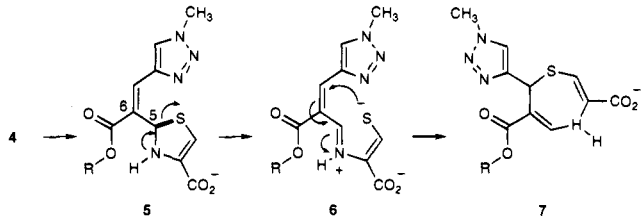
We reported recently that the Arg-244-Ser mutant TEM-1 β-lactamase was resistant to inactivation by clavulanate both *in vivo* and *in vitro*.[17] The same mutant β-lactamase was identified in a clinical bacterial isolate which was resistant to combinations of clavulanate and penicillins.[18] Distribution of the gene encoding this mutant enzyme among pathogens would herald an anticipated compromise for clinical utility of clavulanate in the future. However, the Arg-244-Ser mutant TEM-1 β-lactamase is inactivated effectively by penem **4** (k_{inact} = 0.024 ± 0.003 s^{-1}, k_{cat} = 0.029 s^{-1}, k_{cat}/k_{inact} = 1.2 ± 0.1, K_i = 230 ± 47 nM, and k_{inact}/K_i = 1.0 × 10^5 M^{-1} s^{-1}). The recovery of activity was again biphasic; the fast phase proceeded with a rate constant of k_{rec} = (4.2 ± 0.6) × 10^{-3} s^{-1}, which ended when approximately 30% of the activity was recovered.

Penem **4** likewise inactivated the class C Q908R β-lactamase with great efficiency. As with the TEM enzymes, the inactivation was active-site directed and saturable, and it involved a covalent modification of the enzyme. The inactivation chemistry proceeded with the following kinetic parameters: k_{inact} = 0.06 ± 0.01 s^{-1}, K_i = 40 ± 7 nM, and k_{inact}/K_i = 1.5 × 10^6 M^{-1} s^{-1}. A partition ratio (k_{cat}/k_{inact}) of 12 ± 1, which was measured, allowed for the calculation of k_{cat} at 0.73 s^{-1}. The activity of the enzyme did not recover after 2 days of dialysis; however, in the presence of 1 mM hydroxylamine >90% of the activity was recovered within 2 h.

Since the inactivated species for the Q908R β-lactamase was stable, we measured the UV spectrum of this protein. The

(10) Daniels, S. B.; Cooney, E.; Sofia, M. J.; Chakravarty, P. K.; Katzenellenbogen, J. A. *J. Biol. Chem.* **1983**, *258*, 15046.
(11) Koerber, S. C.; Fink, A. L. *Anal. Biochem.* **1987**, *165*, 75.
(12) Glick, B. R.; Brubacher, L. J.; Leggett, D. J. *Can. J. Biochem.* **1978**, *56*, 1055.
(13) Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70.
(14) Gasteiger, J.; Marsili, M. *Tetrahedron* **1980**, *36*, 3219. Gasteiger, J.; Marsili, M. *Org. Magn. Reson.* **1981**, *15*, 353.
(15) Powell, M. J. D. *Math. Prog.* **1977**, *12*, 241.
(16) Silverman, R. In *Mechanism-Based Enzyme Inactivation: Chemistry and Enzymology*; CRC Press: Boca Raton, FL, 1988; p 22.
(17) Imtiaz, U.; Billings, E.; Knox, J. R.; Manavathu, E. K.; Lerner, S. A.; Mobashery, S. *J. Am. Chem. Soc.* **1993**, *115*, 4435.
(18) Vedel, G.; Belaaouaj, A.; Gilly, L.; Labia, R.; Phillippon, A.; Nevot, P.; Paul, G. *J. Antimicrob. Chemother.* **1992**, *30*, 449.

nature of the inactivated species for the two β-lactamases should be the same, and the sole exception in the two cases should be the differential propensity of the two inactivated species to undergo hydrolysis. Inactivation of the Q908R β-lactamase was accompanied by the formation of a chromophore at 366 nm (ε 4000−7500, 100 mM sodium phosphate, pH 7.0) (Figure 1).[19] Reaction of **4** with the methoxide ion has been studied as a model reaction.[20] Broom *et al.* identified a dihydrothiazepine (**7**, R = methyl) as the product of this non-enzymic reaction.[20] This compound showed a UV chromophore at 360 nm (ε 4500, H_2O), consistent with our observations of the species formed in the active site of the β-lactamase.[21] We envision that **4** would acylate the active-site serine (Ser-70 in TEM-1 and Ser-64 in Q908R). The acylation may not take place concomitant with the departure of the thiolate from C_5, since the lactam and the C_5-S bonds are not antiperiplanar to one another.[22] Therefore, an intermediate such as **5** must exist. We have observed that such an acrylation event is accompanied by a rotation about the C_5-C_6 bond in our previous work on the chemistries of clavulanate[17] and sulbactam.[23] Our modeling of this process reveals that to be true also for **4**. This rotation is quite significant for proper positioning of the incipient thiolate upon its formation (**6**) for attack at the carbon backbone to yield **7**. The details of these processes have been investigated by generation of energy-minimized structures for **5** and **7** (R = active-site serine) in the crystal structure for the TEM-1 and Q908R β-lactamases.



Models for **5** and **7** for the TEM-1 β-lactamase are shown in Figure 2. We would like to add here that unlike the cases of clavulanate[17] and sulbactam,[23] fragmentation of compound **4** is not indicated during the course of enzyme inactivation, as shown by an increase in mass of the inactivated enzyme by 264 Da (*i.e.*, the mass of **4**).[24] Therefore, a rearrangement of structure takes place after active-site acylation, which, according to our UV analysis of the inactivated β-lactamase, should follow the chemistry described for the model system. The energy-minimized structure for **5** (Figure 2A) reveals that the C_3 carboxylate in **5** forms strong hydrogen bonds with the side-chain functions of Arg-244 and Ser-235. This picture is reminiscent of the contributions of Arg-244 and Ser-235 to interactions with other substrates[25] and inactivators[17,23] studied by our group and by others.[26] An intriguing observation is that

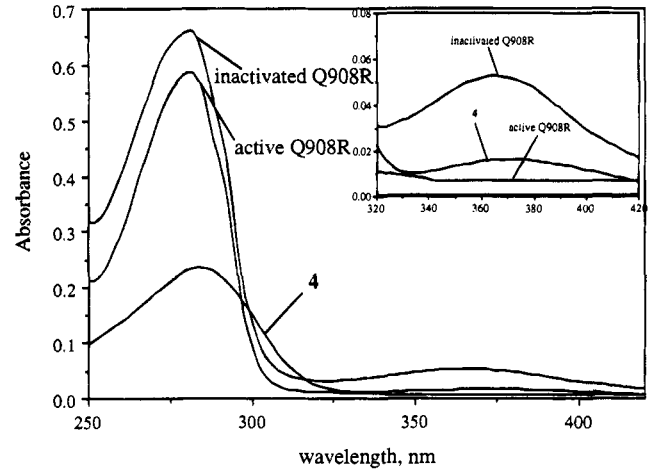


Figure 1. The UV profile of 9.3 μM of each of penem **4**, the Q908R β-lactamase, and the Q908R β-lactamase inactivated by penem **4**. The inset shows an expansion of the region between 320 and 420 nm.

the π orbitals in the functionality attached to the C_6 are forced to be nearly orthogonal to the π orbitals of the ester carbonyl in **5** for steric constraints of the active site; hence, no conjugation is possible between the two systems. This leads us to conclude that **5** would turn over readily because of the absence of the stabilization toward hydrolysis that would be provided by conjugation of the ester group; this is the most likely step that contributes to the partition ratio of 1 for this enzyme (*i.e.*, one turnover before one inactivation event).[27] In principle, there is not much of a difference between the structure of **5** and that of the acyl-enzyme intermediate for penicillin G, which turns over with a rate near the diffusion limit.[6a] The energy-minimized structure for **7** showed that the carboxylate relies more heavily on hydrogen bonding with Arg-244 and has moved away from the side chain of Ser-235 somewhat. Also, the absolute configuration at the stereogenic carbon to which the methyltriazole ring is attached can only be *S*. The reason for the relative longevity of **7** in the enzyme-active site rests entirely with conjugation of the ester carbonyl with the ring olefin moiety, which stabilizes it toward the hydrolytic reaction. We considered the possibility of species **6** as the inactivating species. The potential for conjugation of the extended unsaturation in **6** with the ester carbonyl is attractive for ester stabilization. Modeling indicated that because of the steric constraints of the active site such extended conjugation (flatness) was not permissible. Furthermore, analysis of the inactivated enzyme by the Ellman reagent[13] for the enethiolate moiety in **6** clearly indicated its absence.

The mechanism for deacylation of the acyl-enzyme intermediate for the class C β-lactamases is not well understood. There seems not to be a general base for activation of a water molecule to attack the acyl species.[3] Furthermore, the crystal structure for the P99 enzyme inactivated by phosphorylation—a proposed transition-state analogue for deacylation—suggests that the hydrolytic water would approach the acyl carbonyl from the opposite direction as for class A enzymes.[28] Hence, Wat-548 should be the hydrolytic water which is displaced by one of the phosphonyl oxygens. The conformations of species **5** and

(19) Because of a change in the UV profile of the enzyme after inactivation, the protein concentration could not be measured by the extinction coefficient for the enzyme. Hence, we used the Bradford assay for protein concentration determination, which permits the determination of protein concentration with as much as 200% error.

(20) Broom, N. J. P.; Farmer, T. H.; Osbourne, N. F.; Tyler, J. W. *J. Chem. Soc., Chem. Commun.* **1992**, 1663.

(21) Broom *et al.* (ref 20) also demonstrated that in the presence of a large excess of the β-lactamase from *Klebsiella pneumoniae* 1082E the dibasic form of **7** (R = Na) could be isolated.

(22) Crackett, P. H.; Stoodley, R. J. *Tetrahedron Lett.* **1984**, *25*, 1295.

(23) Imtiaz, U.; Billings, E. M.; Knox, J. R.; Mobashery, S. *Biochemistry* **1994**, *33*, 5728.

(24) Farmer, T. H.; Page, J. W. J.; Payne, D. J.; Knowles, D. J. C. *Biochem. J.* **1994**, *303*, 825.

(25) Zafaralla, G.; Manavathu, E. K.; Lerner, S. A.; Mobashery, S. *Biochemistry* **1992**, *31*, 3847. Imtiaz, U.; Manavathu, E. K.; Lerner, S. A.; Mobashery; S. *Antimicrob. Agents Chemother.* **1993**, *37*, 2438.

(26) Dubus, A.; Wilkin, J. M.; Raquet, X.; Normark, S.; Frère, J. M. *Biochem. J.* **1994**, *301*, 485.

(27) The same should be true for the *Klebsiella pneumoniae* 1082E β-lactamase (a class A enzyme), which Farmer *et al.* (ref 24) showed to turn compound **4** over several times before inactivation. Turnover would release the hydrolysis product of **5** in this case as well, which subsequently would rearrange in solution to give species **7** (R = Na).

(28) Lobkovsky, E.; Billings, E. M.; Moews, P. C.; Rahil, J.; Pratt, R. F.; Knox, J. R. *Biochemistry* **1994**, *33*, 6762.

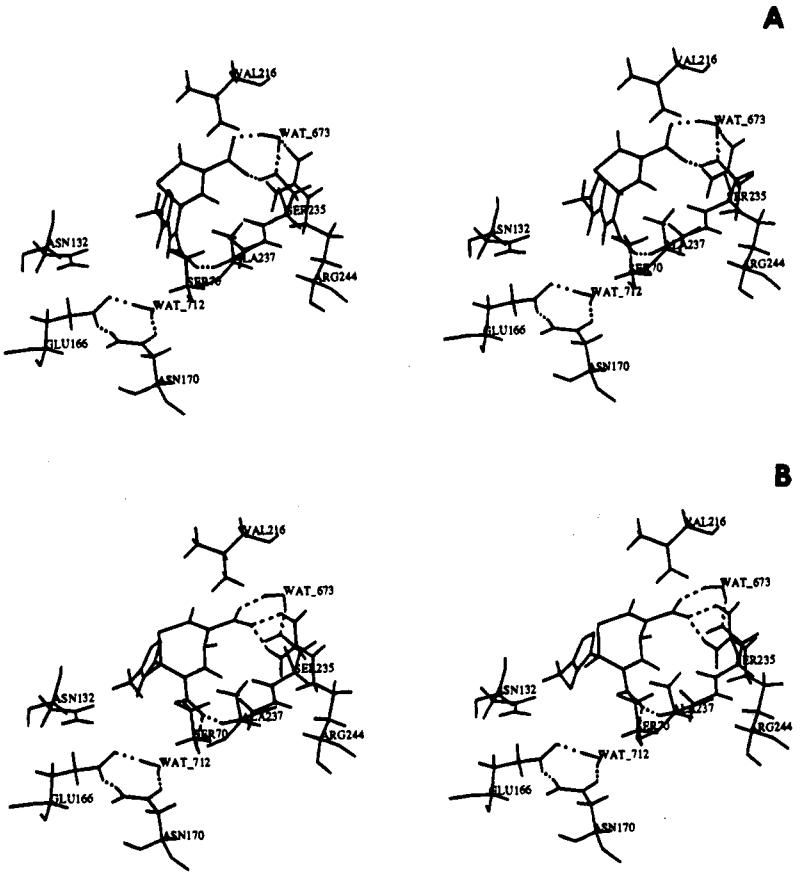


Figure 2. Stereoviews of the energy-minimized structures of species **5** (A) and of species **7** (B) in the active site of the TEM-1 β-lactamase; selected active-site residues are shown.

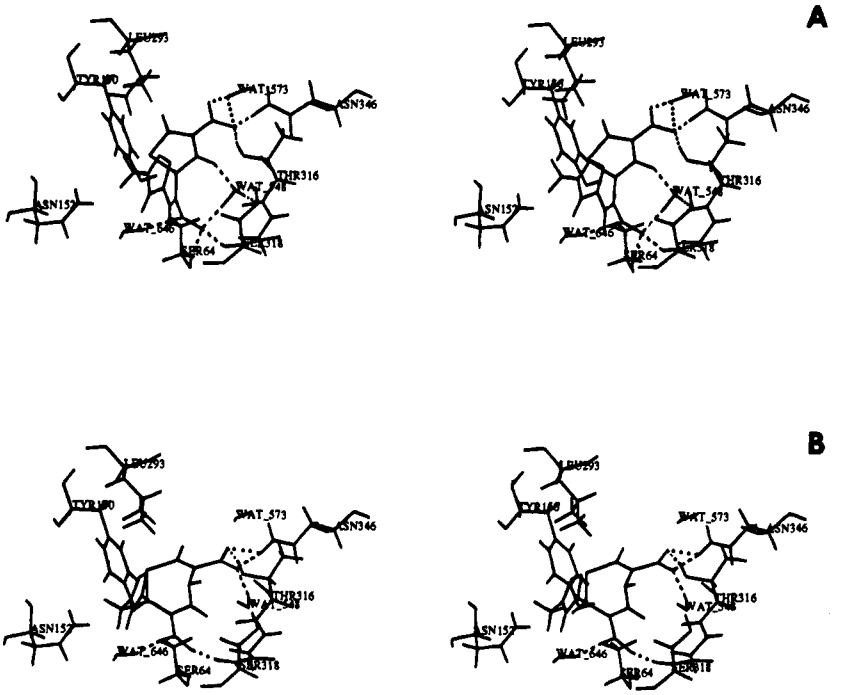


Figure 3. Stereoviews of the energy-minimized structures of species **5** (A) and of species **7** (B) in the active site of the P99 β-lactamase; selected active-site residues are shown.

7 in the active site of the P99 β-lactamase (Figure 3) are very similar to the corresponding structures for the TEM-1 enzyme (Figure 2). For **5**, Wat-548 is at a distance of 3.2 Å, allowing for deacylation to take place (*i.e.*, turnover); on the other hand, for structure **7** this water molecule has been displaced to a distance of 4.4 Å, and its angle of approach (60°) is not favorable for an attack for deacylation. This factor may be the reason that after the rearrangement of **5** to **7**, species **7** does not readily allow for the recovery of activity.

In conclusion, penem **4** is a molecule that inactivates both class A and class C β-lactamases very efficiently. The efficiency of inactivation stems from both low partition ratios

(1−12) and very rapid rates for inactivation. In terms of k_{inact}/K_i, the values for the wild-type and mutant TEM β-lactamases were 10^5-10^7 M^{-1} s^{-1}, which are faster than the corresponding values for inactivation of the TEM-1 enzyme by clavulanate (5.7×10^4 M^{-1} s^{-1})[29] and sulbactam (125 M^{-1} s^{-1})[23] and that for inactivation of the P99 enzyme by tazobactam (790 M^{-1} s^{-1}).[4] Furthermore, the partition ratios for inactivation of β-lactamases reported herein (*i.e.*, 1−12) are the lowest when compared to the clinically used clavulanate, sulbactam, and tazobactam, which are 160,[17] 10000,[23] and 125,[4] respectively.[30]

(29) Chamas, R. L.; Knowles, J. R. *Biochemistry* **1981**, *20*, 3214.

(30) Since the submission of this report, an article by Matagne *et al.* (Matagne, A.; Ledent, P.; Monnaie, D.; Felici, A.; Jamin, M.; Raquet, X.; Galleni, M.; Klein, D.; François, I.; Frère, J. M. *Antimicrob. Agents Chemother.* **1995**, *39*, 227) has been published which addresses the kinetics of inhibition of β-lactamase by BRL 42715. The kinetic analysis was carried out somewhat differently than our method, but our reported values are in good agreement with those reported by Matagne et al.

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