Design, Synthesis, and Evaluation of a Potent Mechanism-Based Inhibitor for the TEM β-Lactamase with Implications for the Enzyme Mechanism

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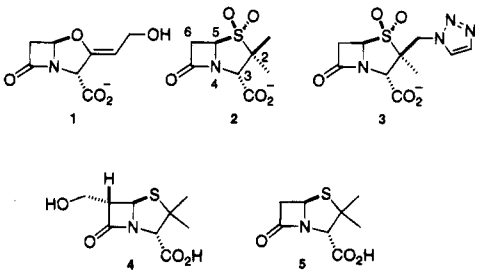
Abstract: The design, synthesis, and evaluation of 6α-(hydroxymethyl)penicillanic acid (**4**) as a new mechanism-based inhibitor for the class A TEM-1 β-lactamase is described. The design of this compound was aided by computer modeling, using the high-resolution crystal structure for the TEM-1 β-lactamase. The molecule is believed to displace the hydrolytic water molecule (Wat-712) with its hydroxymethyl function. This interaction would impart longevity to the acyl-enzyme intermediate, accounting for the onset of inhibition, a process that results in an 11-h period for recovery of 90% of activity for the inhibited enzymes. This molecule inhibited the TEM-1 β-lactamase rapidly, for which the kinetic parameters were evaluate. The molecule showed a partition ratio (*i.e.*, k_{cat}/k_{inact}) of 28 ± 2, a value which is lower than the corresponding parameter for all of the clinically used class A β-lactamase inactivators. The design concepts outlined for 6α-(hydroxymethyl)penicillanic acid as an inhibitor support the mechanistic roles proposed for Glu-166 and Wat-712 in the deacylation step for turnover chemistry by class A β-lactamases. Furthermore, the principles that we disclose herein for the design of compound **4** as an inhibitor for β-lactamases should be of general interest for designing specific inhibitors for any hydrolytic enzyme for which high-resolution crystal structure is available.

The activity of β-lactamases is the primary means for bacterial resistance to β-lactam antibiotics.[1] In the past several years, a series of non-classical β-lactam molecules have been developed which retained antibiotic activity despite the presence of β-lactamases in bacteria. However, the discovery of extended-spectrum β-lactamases presents a serious obstacle to the clinical viability of a number of these antibiotics.[2] Within the past few years mechanism-based inactivators for β-lactamases, such as clavulanate (**1**), sulbactam (**2**), and tazobactam (**3**), have been used in conjunction with penicillins. β-Lactamases are inhibited by these molecules, preserving the structural integrity of the penicillin, which kills the bacteria. Regrettably, within the past 2−3 years variants of β-lactamases have been identified in clinical isolates which are resistant to inhibition by these inactivators.[3] This phenomenon heralds the future compromise of therapy by the existing inactivator/antibiotic combinations. Therefore, development of new types of inactivators with distinct mechanisms of action is urgently needed to provide effective inhibition of these new enzymes in the immediate future. Toward that goal, we describe herein our design of 6α-(hydroxymethyl)penicillanic acid (**4**), as a potent inhibitor for the TEM-1 β-lactamase, a prototypic class A enzyme.



Experimental Section

Hydrogen- and carbon-NMR spectra were obtained at 300 and 75 MHz, respectively, using a Nicolet QE-300 spectrometer. Chemical shift values (δ) are given in ppm. Infrared and mass spectra were recorded on Nicolet DX and Kratos MS 80RFA spectrometers, respectively. Melting points were taken on a Hoover UniMelt apparatus and are uncorrected. Thin-layer chromatograms were made on silica gel. All other reagents were purchased from the Aldrich Chemical Co. The enzyme purification protocol for the wild-type TEM-1 has been described.[4] Kinetic measurements were carried out on a Hewlett-Packard 452 diodearray instruments. All enzyme assays were carried out in 100 mM sodium phosphate, pH 7.0. Modeling and energy-minimization protocols were according to the procedures reported earlier.[5,6] 6,6-Dibromopenicillanic acid (**7**) was synthesized according to a literature method.[7]

***p*-Nitrobenzyl 6,6-Dibromopenicillanate (8).** A suspension of 6,6-dibromopenicillanic acid (**7**, 7.0 g, 20 mmol), K_2CO_3 (2.7 g, 20 mmol), and *p*-nitrobenzyl bromide (4.7 g, 22 mmol) in 35 mL of DMF was stirred overnight. Subsequently, the reaction mixture was diluted with ethyl acetate and the solution was washed with saturated $NaHCO_3$ (2×), water, and saturated NaCl, dried over $MgSO_4$, and concentrated *in vacuo*

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to afford a yellow solid. The crude product was recrystalized from ethyl acetate at room temperature to give the title compound (6.1 g, 63%), mp 122−124 °C: IR (KBr) 1797, 1744, 1608, 1519, 1343 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.41 (3H, s, CH_3), 1.63 (3H, s, CH_3), 4.61 (1H, s, C_3 methine), 5.23−5.38 (2H, unresolved AB type, benzylic methylene), 5.79 (1H, s, C_5 methine), 7.57 (2H, d, J = 8.4 Hz, aromatic), 8.26 (2H, d, J = 8.4 Hz, aromatic); ^{13}C-NMR ($CDCl_3$) δ 25.84, 33.49, 58.52, 64.63, 66.04, 69.60, 80.80, 124.03, 129.02, 141.55, 148.05, 164.52, 166.28; EI HRMS 491.9000 (M^+, calcd for $C_{15}H_{14}Br_2N_2O_5S$ 491.8991).

***p*-Nitrobenzyl 6α-Bromo-6β-(hydroxymethyl)penicillanate (9).** A solution of methylmagnesium bromide in ether (3 M, 4.8 mL, 14.2 mmol) was added dropwise to a solution of *p*-nitrobenzyl 6,6-dibromopenicillanate (**8**, 7.0 g, 14.2 mmol) in dry THF (230 mL) at −78 °C, and the reaction mixture was stirred an additional 30 min after the completion of the addition at the same temperature. Formaldehyde gas, generated by heating paraformaldehyde (5.0 g), was passed with a stream of nitrogen into the stirred THF solution of metalated **8**, and the mixture was stirred for 2 h at −78 °C. Subsequent to the addition of a saturated NH_4Cl solution (ca. 3 mL), the reaction mixture was warmed to room temperature and the solvent was evaporated to near dryness *in vacuo*. Both water and ethyl acetate were added to the residue and the aqueous layer was further washed with additional portions of ethyl acetate (50 mL, 3×). The combined organic layer was washed with water and saturated NaCl, dried over $MgSO_4$, and concentrated *in vacuo* to afford a brown oil. The product was purified by silica-gel column chromatography ($CHCl_3$−ethyl acetate, 5/2) to afford the title compound as a yellow oil (720 mg, 11%): IR (neat) 3845, 1783, 1746, 1604, 1520, 1344 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.41 (3H, s, C_2 methyl), 1.66 (3H, s, C_2 methyl), 2.78 (1H, br s, OH), 4.05−4.23 (2H, unresolved AB type, CH_2OH), 4.59 (1H, s, C_3 methine), 5.23−5.35 (2H, unresolved AB type, benzylic methylene), 5.55 (1H, s, C_5 methine), 7.56 (2H, d, J = 8.7 Hz, aromatic), 8.24 (2H, d, J = 8.7 Hz, aromatic); ^{13}C-NMR ($CDCl_3$) δ 26.20, 32.71, 64.48, 65.00, 65.86, 70.04, 71.28, 71.85, 104.93, 123.99, 128.85, 141.78, 167.04, 168.83; EI MS 444, 446 (M^+, 0.5%, 0.5%).

***p*-Nitrobenzyl 6α-(hydoxymethyl)penicillanate (10).** Tributylphosphine (0.43 mL, 1.64 mmol) was added to a solution of **9** (500 mg, 1.09 mmol) in methanol (20 mL) and the reaction mixture was stirred for 1 h. After concentration under reduced pressure and purification by silica-gel column chromatography ($CHCl_3$−ethyl acetate, 3/1), the title compound was separated as the major component, which included some of the β-product (93% α:7% β) (93 mg; yield, 51%): IR (film) 3467, 1769, 1745, 1605, 1521, 1345 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.39 (3H, s, C_2 methyl), 1.61 (3H, s, C_2 methyl), 2.76 (1H, br s, OH), 3.50 (1H, m, C_6 methine), 3.91−4.05 (2H, m, CH_2OH), 4.53 (1H, s, C_3 methine), 5.20−5.31 (2H, unresolved AB type, benzylic methylene), 5.30 (1H, s, C_5 methine), 7.54 (2H, d, J = 8.7 Hz, aromatic), 8.21 (2H, d, J = 8.7 Hz, aromatic); ^{13}C-NMR ($CDCl_3$) δ 29.22, 32.61, 58.63, 63.81, 64.21, 65.06, 65.69, 69.66, 122.93, 128.79, 142.02, 147.86, 167.57, 172.76; EI HRMS 366.0886 (M^+, calcd for $C_{16}H_{18}N_2O_6S$ 366.0886).

6α-(Hydroxymethyl)penicillanic Acid (4). A solution of **10** (280 mg, 0.737 mmol) in methanol (8 mL), 0.1 M sodium phosphate buffer, pH 7.0 (1 mL), and water (2 mL) was added to a suspension of 10% Pd-C (200 mg) in methanol (5 mL) which had been stirred under an atmosphere of hydrogen for 3 h; the resultant suspension was stirred for 30 min under hydrogen. After removal of the catalyst by filtration through Celite, methanol was removed *in vacuo*. Saturated $NaHCO_3$ was added to the residue and the aqueous phase was washed with ethyl acetate and then acidified (to ca. pH 2) with 2.5 M HCl. After washes with ethyl acetate (3×), the combined organic layer was washed with water and saturated NaCl, dried over $MgSO_4$, and concentrated *in vacuo* to afford the product as a mixture of α- and β-isomers (47 mg, 81%; 93% α:7% β). Fractional crystallization from ethyl acetate afforded the title compound as colorless crystals (27 mg, 45%), mp 128 °C (sintered), 180 °C dec: IR (KBr) 3408, 3165, 1768, 1753 cm^{-1}; ^{1}H-NMR (acetone-d_6) 1.50 (3H, s, C_2 methyl), 1.61 (3H, s, C_2 methyl), 3.41 (1H, dt, J = 1.2, 4.5 Hz, C_6 methine), 3.89 (2H, d, J = 4.5 Hz, CH_2OH), 4.37 (1H, s, C_3 methine), 5.26 (1H, d, J = 1.2 Hz, C_6 methine); ^{13}C-NMR (acetone-d_6) δ 25.77, 28.68, 31.51, 58.06, 64.01, 64.44, 69.65, 168.44, 172.39; EI HRMS 231.0557 (M^+, calcd for $C_9H_{13}NO_4S_1$ 231.0565).

Kinetic Determinations. Inactivation experiments were commenced by the addition of a portion of a stock solution of **4** (5−100 μM final concentrations) in 100 mM sodium phosphate buffer, pH 7.0, at ice-water temperature. A 10−μL portion of the mixture was removed at various time intervals and was mixed with 990 μL of the assay mixture containing 2 mM benzylpenicillin in 100 mM sodium phosphate buffer to measure the residual enzyme activity. The activity was monitored at 240 nm until the substrate was exhausted. The activity of the enzyme increased during assay as the portion of the enzyme in the process of fast recovery of activity underwent hydrolysis. The highest steady-state rates for hydrolysis of the substrate were used in the calculations of the remaining enzyme activity. These experiments were carried out under conditions of excess substrates as described by Koerber and Fink.[8]

The dissociation constant for compound **4** was calculated by the method of Dixon.[9] Two concentrations of substrates (ampicillin, $\Delta\epsilon_{240}$ = 538 M^{-1} cm^{-1} at pH 7.0), 400 and 500 μM, were used. A series of assay mixtures containing both substrate and various concentrations of **4** (20−140 μM final concentrations) were prepared in 100 mM sodium phosphate buffer, pH 7.0. An aliquot of the enzyme was added to afford a final enzyme concentration of 5 nM in a total volume of 1.0 mL, followed by the immediate measurement of enzyme activity. The rates were calculated for the first 5% of substrate turnover.

The ratio k_{cat}/K_m for turnover of **4** by β-lactamase was determined as follows. To a solution of **4** (final concentration 30 μM) in 50 mM sodium phosphate buffer, 100 mM NaCl, pH 7.0, the enzyme was added to afford a final enzyme concentration of 0.2 μM, and the reaction was monitored at 230 nm for 1 h. The reaction rate was calculated from the slope ($\Delta\epsilon_{230}$ = 597 M^{-1} cm^{-1}) and the k_{cat}/K_m value was estimated according to the formula $k_{cat}/K_m = v/[E]_o[S]_o$ from the portion of the hydrolytic profile corresponding to less than 5% hydrolysis.

The partition ratio for **4** was determined by the titration method.[10] Various molar ratios of $[I]_o/[E]_o$ ranging from 1 to 100 in 100 mM sodium phosphate, pH 7.0, were incubated at 4 °C overnight. Subsequently, the remaining enzyme activity was assayed as described for the inactivation experiments. A plot of residual activity versus $[I]_o/[E]_o$ was linear, giving k_{cat}/k_{inact} as an intercept.[11]

The rate of recovery of enzyme activity from the fast hydrolyzing species on incubation of β-lactamase with **4** was measured as follows. β-Lactamase (2 μM) was incubated with **4** (50 μM) in 100 mM sodium phosphate buffer, pH 7.0, in an ice bath overnight. A 10−μL portion of the inhibited enzyme was added to the assay mixture and the recovery of activity was monitored immediately at 240 nm for several minutes at room temperature. The first-order rate (k_{rec}) for the fast step was calculated from the progress curve by the method of Glick *et al.*[12] The first-order rate constant (k_{rec}) for recovery of activity by the slower step was estimated from the $t_{1/2}$ of 5.5 h for essentially full recovery of activity (90%) at room temperature.

Circular-Dichroic Measurements. The CD spectra of the TEM-1 β-lactamase inhibited by **4** were determined at the beginning and at the end (after 11 h with 90% of the activity recovered) of the inhibition. The enzyme concentration was 7 μM and that for **4** was 100 μM. Similarly, the CD spectrum of **4** (100 μM) in the absence of protein was determined.

Spectrophotometric Monitoring of the Inhibition Time Course. The UV−vis spectra of the β-lactamase (5 μM) were determined after incubation with **4** (100 μM) for several hours. In one set of experiments, the excess inhibitor was removed by a series of sequential dilution and concentration in an Amicon apparatus, followed by the determination of the spectrum of the protein. We carried out an identical set of experiments without removing the inhibitor from the

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(10) Silverman, R. In *Mechanism-Based Enzyme Inactivation: Chemistry and Enzymology*; CRC Press, Boca Raton, 1988; p 22.
(11) The equation that describes the plot for determination of the partition ratio assumes an incubation to time infinity in its derivation. We take time infinity to be an overnight incubation. The rate of recovery of activity was intentionally attenuated for this experiment by incubation of the mixture at 4 °C, but obviously it cannot be eliminated entirely. Hence, the value for the partition ratio evaluated by this technique gives an overestimation of the true value (*i.e.*, the partition ratio should be lower than 28 for **4**).
(12) Glick, B. R.; Brubacher, L. J.; Leggett, D. J. *Can. J. Biochem.* **1978**, *56*, 1055.

mixture, and then recording the spectrum of the protein. The results of the two separate experiments were the same; no noticeable change in the spectrum of the enzyme during enzyme inhibition was registered.

Enzyme Inhibition Profile as a Function of Time. A 300−μL portion of a solution of β-lactamase (0.75 μM) and compound **4** (100 μM) was incubated in 100 mM sodium phosphate, pH 7.0, at room temperature. Aliquots (20 μM) were removed and diluted into 2.0 mM penicillin G (280 μL) in the same buffer periodically over 11 h. The activity of the enzyme was monitored at 240 nm immediately.

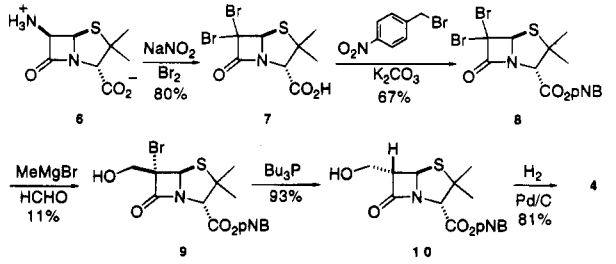
Results and Discussion

We recently investigated the interactions of sulbactam (**2**) in the active site of the TEM-1 β-lactamases.[13] This compound acylates the active-site serine, subsequent to which the sulfonate departs to give rise to an iminium moiety, which in turn traps an active-site nucleophilic residue and inactivates the enzyme. One serious limitation of sulbactam is the fact that the chemistry of inactivation is substantially slower than the process of turnover. Sulbactam is hydrolyzed 10 000 times (*i.e.*, k_{cat}/k_{inact} or partition ratio) before each inactivation;[13] therefore, high *in vivo* concentrations of the inactivator are needed. We also investigated the nature of the leaving group at C_5 of sulbactam.[13] The sulfone group of sulbactam was indispensable for inactivation of the enzyme. In fact, we showed that penicillanic acid (**5**) was merely a substrate for the TEM-1 β-lactamase (k_{cat}/K_m = 6.6 × 10^4 M^{-1} s^{-1}).[13]

It occurred to us that a molecule could be designed based on the minimal structure of penicillanic acid, which acylates the active-site serine, but would resist deacylation by being incorporated with a functionality that displaces the active-site hydrolytic water molecule. This would give increased longevity for the acyl-enzyme intermediate, which would be expected to provide effective inhibition of the enzyme. We hasten to add that deacylation of most typical acyl-enzyme intermediates for molecules with the penicillanic acid nucleus takes place rapidly. Indeed, enzymic hydrolysis takes place at the diffusion limit for a substrate such as penicillin G.[14]

The mechanism of deacylation for class A β-lactamases has been investigated.[15] The amino-acid residue Glu-166 has been suggested to be the general base which activates Wat-712,[16] a structurally conserved water molecule in class A β-lactamases, for attack at the carbonyl of the acyl-enzyme intermediate. We decided to explore the minimal structural parameter that may be used in a variant of penicillanic acid to displace the hydrolytic water molecule from the active site upon acylation, in order to retard deacylation. We generated the acyl-enzyme structure for penicillanate in the active site of the TEM-1 β-lactamase. The energy of the complex was minimized, after which the position of Wat-712 remained unchanged and favorably poised for the deacylation reaction (Figure 1A). Further exploration by molecular modeling of this structure suggested to us that the hydroxyl function of a hydroxymethyl group introduced at $C_{6\alpha}$ would overlap with the space where Wat-712 exists; the structure for the acyl-enzyme intermediate of this variant of penicillanic acid was energy minimized and is shown in Figure 1B. We also prepared an energy-minimized structure for the preacylation complex of this molecule, as shown in Figure 1C. This structure shows that the hydroxymethyl group would displace the water molecule prior to acylation and that the position of the hydroxymethyl moiety did not change much upon acylation (Figure 1B).[17] The hydroxyl group of the hydroxymethyl moiety made hydrogen bonds with Lys-73 and Glu-166 in both preacylated and acylated complexes. In each case the C_3 carboxyl was anchored by hydrogen bonds with the side chains of Ser-130, Lys-234, Ser-235, and Arg-244.

We set out to synthesize compound **4** for evaluation of its properties with the TEM-1 β-lactamase. The synthesis started with 6β-aminopenicillanic acid (**6**) which was converted to 6,6-dibromopenicillanic acid (**7**) according to a literature procedure.[7] The carboxyl group of **7** was protected as the *p*-nitrobenzyl ester to give **8**. The key intermediate for our synthesis, *p*-nitrobenzyl 6β-hydroxymethyl-6α-bromopenicillanate (**9**), was prepared by a slight variation of the method of DiNinno *et al.*[18] The bromo group in compound **9** was reduced by a literature method for reduction of halides using tributylphosphine.[19] Such reduction gave mostly inversion of stereochemistry, which after chromatography afforded compound **10**, along with 7% of the undesired β stereoisomer at C_6. Subsequently, hydrogenolysis of **10** over 10% Pd/C, and fractional crystallization, produced the desired compound **4**.[20]



Compound **4** inactivated the TEM-1 β-lactamase rapidly in a process which was saturable. Furthermore, the rate of the inactivation process was attenuated in the presence of penicillin G by 40% (50 μM inactivator and 2 mM substrate), further indicating that inactivation was active-site directed. The inactivation process appeared to be biphasic (Figure 2). For the fast phase, a standard double-reciprocal plot of the rates of enzyme inactivation as a function of the inactivator concentration furnished the parameters for inactivation (k_{inact} = 0.061 ± 0.001 min^{-1}, K_I = 36 ± 5 μM, k_{inact}/K_I = 1690 M^{-1} min^{-1}). The dissociation constant (K_i) for the molecule with the TEM-1 enzyme was evaluated at 48 ± 4 μM. The value for K_i is actually somewhat lower than K_s values for typical penicillin

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(15) Adachi, H.; Ohta, T.; Matsuzawa, H. *J. Biol. Chem.* **1991**, *266*, 3186. Escobar, W. A.; Tan, A. K.; Fink, A. L. *Biochemistry* **1991**, *30*, 10783. Delaire, M.; Lenfant, F.; Labia, R.; Masson, J. M. *Protein Eng.* **1991**, *4*, 805.

(16) The water molecules in the crystal coordinates for the TEM-1 β-lactamase which were made available to us were not numbered. Hence, 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).

(17) We tried to force Wat-712 to remain in the active site for the acyl-enzyme intermediate of **4**. For this, we added a water molecule near Glu-166 and Asn-170 of the acyl-enzyme species and we attempted energy minimization. The energy-minimization process failed to converge, but from the structures of the intermediary stages of the procedure it was evident that the forced presence of the water molecule caused unreasonable constraints on the conformation of the acyl-enzyme intermediate and the active-site residues.

(18) DiNinno, F.; Beattie, T. R.; Christensen, B. G. *J. Org. Chem.* **1977**, *42*, 2960.

(19) Chern, J. W.; Huang, M.; Tien, J. H.; Pai, S. H. *Heterocycles* **1988**, *27*, 1349.

(20) During the review of this manuscript, a referee astutely pointed out that compound **4** has been described in the patent literature. Our search of the patent literature revealed that compound **4** was reported as an intermediate in the syntheses of C_6-halomethyl penicillanic acid derivatives (Kellogg, M. S.; Hamanaka, E. S. German Patent 3,008,316. *CA* 94:84113).

(21) The partition ratios for the clinically used clavulanate, sulbactam, and tazobactam are 160 (ref 22), 10000 (ref 13), and 125 (Bush, K.; Macalintal, C.; Rasmussen, B. A.; Lee, V. J.; Yang, Y. *Antimicrob. Agents Chemother.* **1993**, *37*, 851), and the corresponding second-order rate constants for enzyme inactivation (*i.e.*, k_{inact}/K_i) are 5.7 × 10^4, 125, and 2.8 × 10^3 M^{-1} s^{-1}, respectively, given here for comparison.

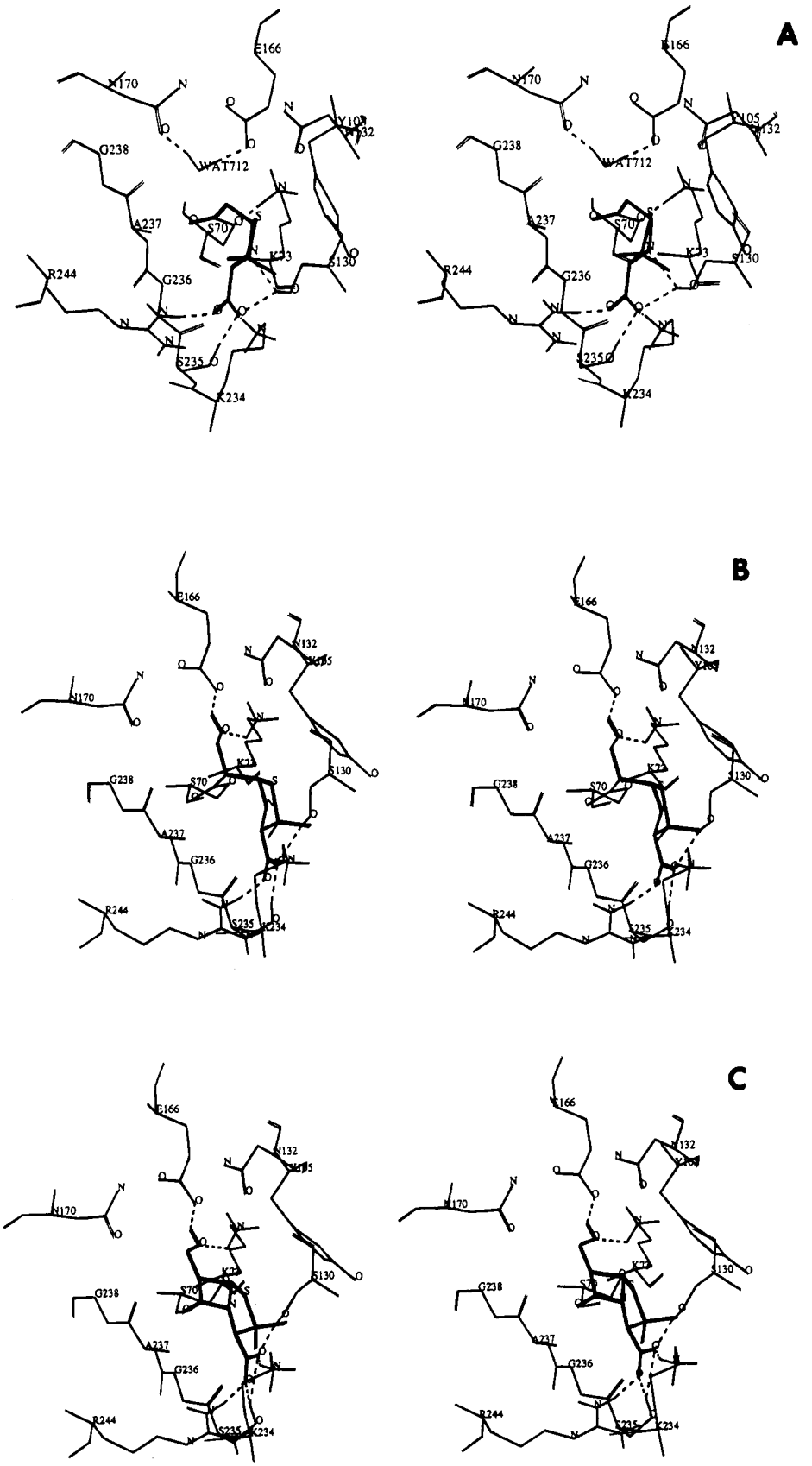

Figure 1. (A) Stereo view of the energy-minimized structure for the acyl-enzyme intermediate of penicillanate **5**; Wat-712 is positioned well for attack at the ester carbonyl. (B) Stereo views of the energy-minimized structure for the acyl-enzyme intermediate of compound **4** and (C) the preacylation complex of compound **4** in the active site of the TEM-1 β-lactamase. Compounds **4** and **5** are depicted in bold, and hydrogen atoms are shown only for moieties that make hydrogen bonds.

substrates for the TEM-1 β-lactamase.[4] The partition ratio (k_{cat}/k_{inact}) was measured by the titration[10,11] method to be 28 ± 2.[21] A number of mechanistic features for the inhibition chemistry of **4** are of interest. As judged by circular-dichroic analysis of the enzyme in the course of inhibition, we did not detect any conformational change for the protein. Therefore, the biphasic

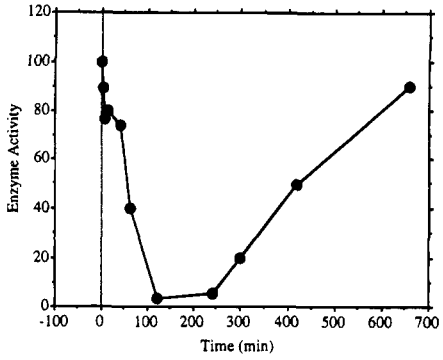

Figure 2. Time dependence of the inhibition profile of the β-lactamase by **4**.

inhibition is not due to a protein conformational change. The inhibited protein regains most of its activity in 11 h. Interestingly, the underlying reason for this biphasic profile for inactivation is distinct from that for inactivation by clavulanate[22] and sulbactam.[13] In contrast to the chemistries of clavulanate and sulbactam, no new chromophores formed in several hours of enzyme incubation in the presence of **4**, during which time the enzyme activity was inhibited, and subsequently restored. Therefore, formation of the so-called transiently inhibited species (**15**, in footnote 23)—a characteristic of the chemistry of clavulanate and sulbactam—is not seen with this inhibitor.[23] This indicates that the thiolate departure from the acyl-enzyme intermediate for **4**, a prerequisite for the formation of the transiently inhibited species, does not take place readily,[23] consistent with our findings with sulbactam and its analogues, indicating that a good leaving group such as a sulfonate is required for the inactivation chemistry of sulbactam-like compounds.[13] In addition, we could not detect the formation of either penicillamine (**14**, in footnote 23) or penicillamine disulfide (the potential product of air oxidation of penicillamine) in the course of the inhibition process;[23] these compounds may form on fragmentation of the penicillanate nucleus, subsequent to lactam hydrolysis. The R_f values for authentic DL-penicillamine and DL-penicillamine disulfide on silica-gel plates, eluted in $CHCl_3$/MeOH (1:1), were 0.35 and 0.11, respectively. None of those compounds were found in the incubation mixture at the beginning, in the middle, and at the end of incubation of **4** with the enzyme (up to 11 h). Thin-layer chromatography of the incubation mixture in the same elution solvent indicated the presence of a single ninhydrin-positive compound at R_f 0.48, which we attribute to 6α-(hydroxymethyl)penicilloate, the hydrolysis product of the lactam of **4** (compound **4** itself elutes at R_f 0.67).

The enzyme recovered activity in the presence of high substrate concentration in a process that was initially relatively fast, followed by a slower step. For the relatively brief faster phase of the recovery of activity a first-order rate constant of $k_{rec} = (3.3 \pm 0.3) \times 10^{-3}$ s^{-1} was evaluated. For the slower recovery of activity, we estimate $k_{rec} = 0.1$ h^{-1}, which accounts for a difference of approximately 120-fold for the rates of the two phases of recovery. We were able to estimate from initial rate measurements for hydrolysis the value for k_{cat}/K_m at 1200 M^{-1} s^{-1} for **4**. This value makes compound **4** a very poor substrate for the TEM-1 β-lactamase. From the parameters reported above, we calculated $k_{cat} = 0.029$ s^{-1} and $K_m = 24$ μM for turnover of compound **4**.[24]

In conclusion, we have shown that compound **4**, a simple structural variant of penicillanic acid, is an excellent inhibitor for the class A TEM-1 β-lactamase with a novel mechanism for inhibition. The basis for inhibition is the retarded rate of hydrolysis of the acyl-enzyme intermediate, which is in agreement with the proposal for Glu-166 serving as a general base in activation of Wat-712 for the deacylation step of the hydrolytic reaction. We would like to state that the principles that we disclosed herein for the design of compound **4** as an inhibitor for β-lactamases should be of general interest for *designing specific inhibitors for any hydrolytic enzyme for which high resolution crystal structure is available.*

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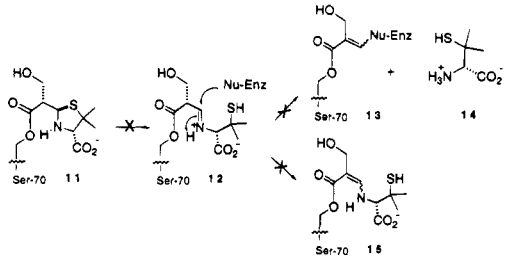
(22) Imtiaz, U.; Billings, E.; Knox, J. R.; Manavathu, E. K.; Lerner, S. A.; Mobashery, S. *J. Am. Chem. Soc.* **1993**, *115*, 4435 and the references cited therein.

(23) The mechanistic possibilities that were considered and ruled out for compound **4** were based on precedented reactions for clavulanate and sulbactam:



(24) These parameters are similar for turnover of imipenem, a clinically used carbapenem, by the TEM-1 β-lactamase ($K_m = 27$ μM, $k_{cat} = 0.04$ s^{-1}; Zafaralla, G.; Mobashery, S. *J. Am. Chem. Soc.* **1992**, *114*, 1505), and the class A β-lactamase from *Streptomyces albus* G (Matagne, A.; Lamotte-Brasseur, J.; Frère, J. M. *Eur. J. Biochem.* **1993**, *217*, 61). Imipenem is considered a poor substrate for class A β-lactamases.