Evidence for a New Enzyme-Catalyzed Reaction Other Than β-Lactam Hydrolysis in Turnover of a Penem by the TEM-1 β-Lactamase†

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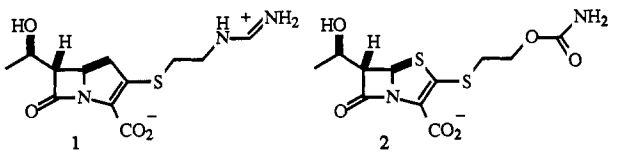
Abstract: The mechanism of turnover of the penem Sch 34343 by the TEM-1 β-lactamase, a class A β-lactamase, has been investigated. In contrast with carbapenems, Sch 34343 does not inhibit the β-lactamase in the course of turnover and the kinetics were not biphasic. Sch 34343 is converted to a compound A, which subsequently gives rise to a compound B; both conversions were mediated by the β-lactamase. The two compounds were purified, and their structures were determined. Compound A, the immediate product of turnover, was shown to share all structural elements of Sch 34343, except the β-lactam moiety was hydrolyzed. The structural assignment of this compound constitutes the first direct report for the existence of "penemoic acid" as a product of hydrolysis of penems by β-lactamases. Compound B was shown to be 2-hydroxy-3-mercapto-3-[[2-(carbamoyloxy)ethyl]thio]propenoic acid (**4**). The conversion of compound A to compound B by β-lactamase was active site-directed, the rates showed saturation, and a rate enhancement of 10^7-fold was measured for this reaction compared to the corresponding nonenzymic buffer-catalyzed reaction. Two plausible mechanisms for this unprecedented reaction for β-lactamases are offered.

Synthetic penem β-lactams were predicted correctly by Woodward to possess a broad spectrum of activity against bacteria;[1] as a consequence, they are compounds of considerable medicinal interest. However, the mechanism(s) for turnover of penems by β-lactamases has not been studied in any detail. The overriding assumption has been that β-lactamases should turn over penems similarly to their structural cognates, carbapenems. Knowles and colleagues had shown a biphasic profile for hydrolysis of carbapenems by the TEM β-lactamase.[2,3] It was demonstrated that upon acylation at the active site of the β-lactamase (at Ser-70)[4] by carbapenems, the Δ^2-pyrroline ring undergoes a tautomerization to the Δ^1-derivative, which subsequently deacylates more slowly than the Δ^2-tautomer from the active site. We have provided a more detailed analysis of these processes for imipenem (**1**) recently.[5] We report here on our investigation of turnover of the penem Sch 34343 (compound **2**) by the TEM-1 β-lactamase and highlight an unprecedented reaction for β-lactamases in this process.



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Experimental Section

Proton 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 parts per million. Infrared and mass spectra were recorded on Nicolet DX and Kratos MS 80RFA spectrometers, respectively. Benzylpenicillin was purchased from the Sigma Chemical Co. Sch 34343 was a generous gift from the Schering-Plough Corp. Kinetic measurements were carried out on a Hewlett-Packard 452 diode array instrument. The titration of the thiol in compound **4** by 5,5′-dithiobis(2-nitrobenzoic acid) was carried out as described by Ellman.[6] The method of Charnas *et al.* was used in the preparation of the TEM-1 β-lactamase modified in the active site by clavulanate.[7]

Quantification of the Spectral Changes of Sch 34343 (Compound 2) in the Course of Its Turnover by the TEM-1 β-Lactamase. A solution of compound **2** (100 μM) in 100 mM sodium phosphate buffer, pH 7.0, was incubated with the TEM-1 β-lactamase (2 μM) at room temperature. The enzymic hydrolysis of **2** was accompanied by prominent changes in its UV chromophores at 256 and 322 nm. The changes in the spectra, and the extinction coefficients, were evaluated after compounds **3** and **4** were purified by HPLC (*vide infra*).

The values for the kinetic parameters (k_{cat} and K_m) were determined from Lineweaver–Burk plots of the initial steady-state velocities at concentration ranges of 25–100 and 50–600 μM for compounds **2** and **3**, respectively. For these measurements the changes in chromophores at 256 nm were monitored. Five different concentrations, all in duplicate, were used in each experiment. All rate determinations were performed in 100 mM sodium phosphate, pH 7.0. For the measurement of the apparent second-order rate for the buffer-catalyzed degradation of **3** as a function of the phosphate ion concentration, phosphate concentrations of 10, 100, and 500 mM (all at pH 7.0) were used.

To verify whether compound **2** inhibited β-lactamase in the course of the turnover chemistry, the following experiment was carried out. A solution of compound **2** (50 μM) and TEM-1 β-lactamase (2 μM) was incubated in 100 mM phosphate buffer, pH 7.0, at room temperature. Aliquots (10 μL each) were removed at different time intervals and were diluted into cuvettes containing benzylpenicillin (990 μL, 2 mM) in 100 mM sodium phosphate, pH 7.0. The initial rate of hydrolysis of benzylpenicillin was then monitored at 240 nm immediately. The enzymic activity in the course of turnover of **2** did not change compared to the activity in the absence of **2**.

Isolation and Characteristics of Compound A. A solution of 100 mg of Sch 34343 (**2**) in 100 mM sodium phosphate, pH 7.0 (10 mL), was incubated with 6 mg of the TEM-1 β-lactamase at room temperature for 5–6 h. The solution was subsequently passed through an Amicon ultrafiltration device (YM10 membrane) to remove the protein. The excess substrate was separated from the mixture by initially purifying the filtrate by HPLC (VYDAC C_{18}, 4.6 mm × 25 cm) using a gradient of 2–98% methanol in 0.1% TFA. The effluent was monitored at 256 nm by a Perkin-Elmer variable wavelength LC-95 detector. The fractions

† This paper is dedicated to Professor Josef Fried for his friendship, inspiration, and leadership as a scholar and teacher.

(1) Woodward, R. B. *Philos. Trans. R. Soc. London, B* **1980**, *289*, 239.
(2) Easton, C. J.; Knowles, J. R. *Biochemistry* **1982**, *21*, 2857.
(3) Charnas, R. L.; Knowles, J. R. *Biochemistry* **1981**, *20*, 2732.
(4) The amino acid numbering is according to the convention proposed by Ambler *et al.*: 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.
(5) Zafaralla, G.; Mobashery, S. *J. Am. Chem. Soc.* **1992**, *114*, 1505.
(6) Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70.
(7) Charnas, R. L.; Fisher, J.; Knowles, J. R. *Biochemistry* **1978**, *17*, 2185.

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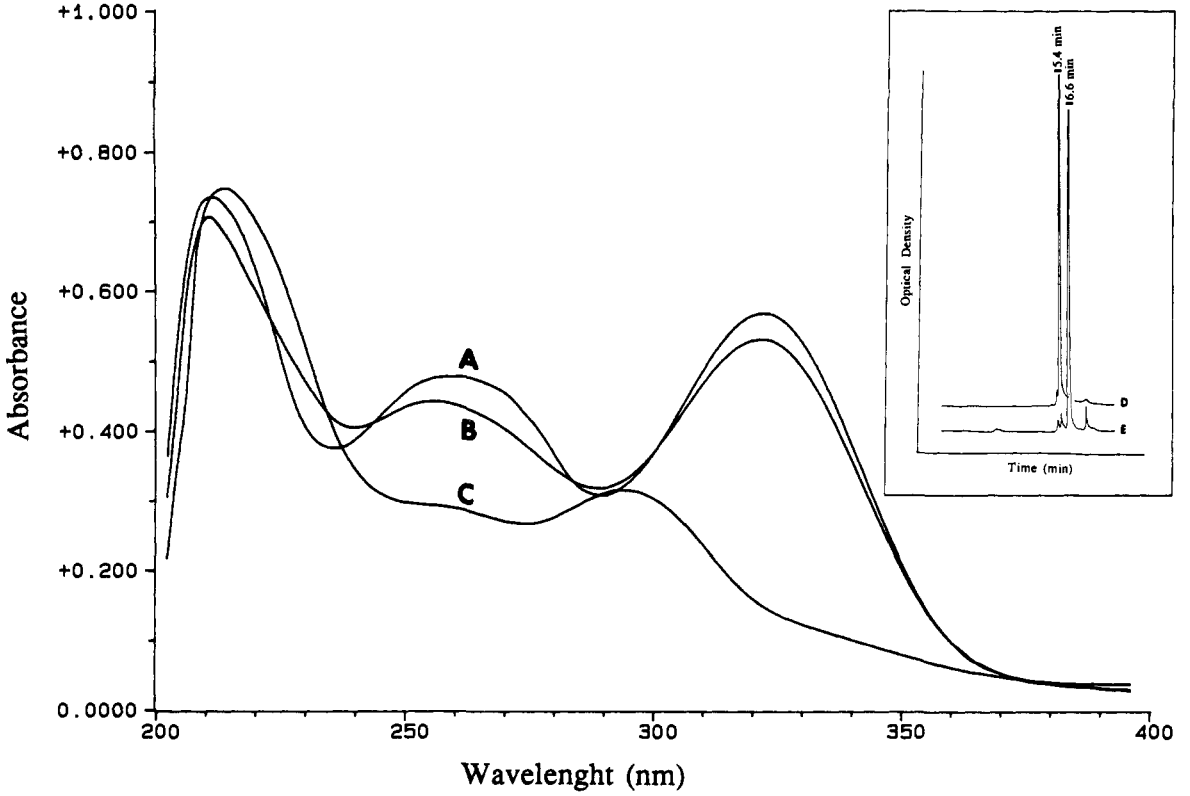
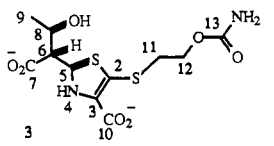


Figure 1. The ultraviolet spectra of 100 μM Sch 34343 (trace A), 100 μM compound A (trace B), and 100 μM compound B (trace C). The inset indicates the HPLC traces (C_4 column) of compounds A (trace D) and compound B (trace E) after the β-lactamase reactions.

that eluted at 2% methanol were pooled and concentrated *in vacuo* and then further purified on a VYDAC C_4 (1.0 × 25 cm) column using the same gradient of 2–98% methanol in 0.1% TFA. The fraction containing the major peak (t_R = 15.4 min) was evaporated *in vacuo*, and the residue was dissolved in 1 mL of water and then lyophilized to give compound A as an off-white powder: UV (100 mM sodium phosphate, pH 7.0) 210 (ϵ = 7050 M^{-1} cm^{-1}), 256 (4420 M^{-1} cm^{-1}), 322 nm (5320 M^{-1} cm^{-1}); IR (KBr pellet) 3436, 3394, 1752, 1703, 1668, 1600, 1500 cm^{-1}; ^1H NMR (DMSO-d_6)[8] δ 1.12 (d, 3H, C_9 methyl, J = 6.3 Hz) 3.05–3.22 (m, SCH_2, 2H), 3.75 (dd, 1H, C_6 methine, J = 1.5 and 6.3 Hz), 3.94 (quint$_{app}$, 1H, C_8 methine, J = 6.3 Hz), 4.1 (t, 2H, CH_2O, J = 6.3 Hz), 5.65 (d, 1H, C_5 methine, J = 1.5 Hz), 6.55 (br s, 2H, NH_2); ^{13}C NMR (DMSO-d_6) δ 176.2 (C_7), 163.7 (C_{10}), 159.1 (C_{13}), 155.5 (C_2), 120.7 (C_3), 73.9 (C_5), 66.9 (C_8 or C_{12}), 66.7 (C_8 or C_{12}), 64.9 (C_{11}), 37.4 (C_6), 24.3 (C_9); FAB^+ MS 353 (M + H, 3), 352 (M, 14), 335 (3), 249 (33), 232 ([249 − 17], 34), 129 ([double fragmentation at C_2–SR and C_5–C_6 bonds], 3), 108 (3), 103 (6).

Isolation and Characteristics of Compound B. Compound A (23 mg) was dissolved in 100 mM sodium phosphate, pH 7.0 (3.0 mL), and then incubated with 2 mg of the TEM-1 β-lactamase at room temperature for 4 h. Subsequently, the solution was passed through an Amicon ultrafiltration device (YM10 membrane). The filtrate was purified on a VYDAC C_4 column as described above for compound A, and the fraction containing the major peak (t_R = 16.6 min) was evaporated *in vacuo* and lyophilized to yield compound B: UV (100 mM sodium phosphate, pH 7.0) 214 (ϵ = 7480 M^{-1} cm^{-1}), 256 (2940 M^{-1} cm^{-1}), 296 nm (3170 M^{-1} cm^{-1}); IR (KBr pellet) 3360 (br s), 1717, 1675, 1600 (w), 1463 cm^{-1}; ^1H NMR (DMSO-d_6) δ 3.24 (t, 2H, SCH_2, J = 6.3 Hz), 4.17 (t, 2H, CH_2O, J = 6.3 Hz), 6.54 (br s, 3H, OH and NH_2, D_2O-exchangeable), 8.92 (s, <1H, SH, D_2O-exchangeable); FAB^+ MS 239 (M, 4), 221 (M − 18, "ortho effect," 40), 179 (20). A total of 1.2 equiv of free thiol was titrated by the Ellman reagent [5,5′-dithiobis(2-nitrobenzoic acid)] at pH 7.2. The compound gave a negative ninhydrin test.

(8) The numbering system for **3** is given below:



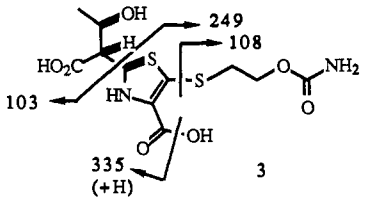
Results and Discussion

We noted that compound **2**, in contrast to carbapenems (including **1**), does not inhibit the enzyme in the course of its turnover. This observation suggests that, after acylation of Ser-70, the acyl–enzyme intermediate undergoes deacylation readily. We investigated the nature of the products of turnover of **2** by UV spectrophotometry (Figure 1). The TEM-1 β-lactamase converts penem **2** to a species with a UV spectrum similar to that of **2** itself, except that it shows smaller extinction coefficients at 256 nm (ϵ = 4420 M^{-1} cm^{-1}) and 322 nm (ϵ = 5320 M^{-1} cm^{-1}) (*i.e.*, compound A), with kinetic parameters k_{cat} = 0.04 s^{-1}, K_m = 73 μM (k_{cat}/K_m = 548 M^{-1} s^{-1}); compound A is subsequently converted to a different species with chromophores at 256 nm (ϵ = 2940 M^{-1} cm^{-1}) and 296 nm (ϵ = 3170 M^{-1} cm^{-1}) (*i.e.*, compound B). Both compounds A and B were purified by HPLC and their structural features analyzed by spectroscopic methods.

As stated above, with the exception of somewhat smaller extinction coefficients, essentially no change in the UV chromophore was noted for compound A, compared to **2**. Furthermore, both ^1H and ^{13}C NMR of compound A retained all features of the corresponding spectra for **2**. Some minor chemical shift changes in the ^{13}C NMR were noted for the conversion of **2** to compund **A** [δ 172.6 to 176.1 (C_7), δ 34.2 to 37.4 (C_6), and δ 73.9 to 70.5 (C_5)]. The significant changes in the ^1H NMR between **2** and compound A were for the C_6 methine (from δ 3.53 to 3.75) and the C_5 methine (from δ 5.65 to 5.50) signals. The IR spectrum for compound A showed the disappearance of the β-lactam carbonyl stretching band at 1766 cm^{-1} and the formation of a strong signal at 1752 cm^{-1}, which is typical for saturated carboxylic acids.[9] The FAB^+ MS gave the parent ion corresponding to structure **3** (plus a proton), in addition to a fragmentation pattern (shown below). These data are all consistent with structure **3** for compound A.

The ^1H-NMR spectrum of compound B is remarkably simplified, compared to that of **3**. The two methylene chemical

(9) Silverstein, R. M.; Bassler, G. C.; Morill, T. C. In *Spectrometric Identification of Organic Compounds*, 5th ed.; John Wiley & Sons, Inc.: New York, 1991; p 117.



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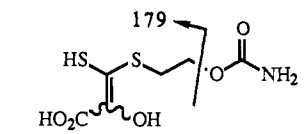
shifts of the side chain attached to C_2 at δ 3.24 and 4.17 are retained; also, approximately four D_2O-exchangeable hydrogens were noted. It is significant that the compound retains a strong UV chromophore and that the IR signal of an α,β-unsaturated carboxylic acid at 1717 cm^{-1} is seen, but not the saturated carboxylic acid signal of **3**. The FAB mass spectrum provided conclusive support for the assignment of structure **4** for compound B. The parent ion and one crucial fragmentation at 179 m/z were seen. The free thiol in **4** was readily titrated by the Ellman reagent. The geometric position of the hydroxyl and the carboxyl groups on the sp^2 carbon is not known with certainty. However, a strong M - 18 peak (40%) in the FAB^+ MS spectrum for **4** argues for an "ortho effect",[10] suggesting that the carboxyl and thiol functions may be *cis* to each other, as shown below for **5**. The possibility that compound B may actually have the structure shown for **9** (*vide infra*) was ruled out on the basis of the IR analysis. The tandem asymmetric and symmetric carboxylate stretch bands at 1600–1590 cm^{-1} (strong) and 1400 cm^{-1} (weak), respectively, which are typical of amino acids, were not seen. The observed weak band at 1600 cm^{-1} for compound B is typical for tetrasubstituted alkene functionalities, such as that present in structure **4**. Our assignment of structure **4**, rather than **9**, was further supported by a negative ninhydrin test; compound **9** would be expected to give a positive ninhydrin test. Whereas **9** may indeed be an intermediate *en route* to **4** (*vide infra*), its isolation and detection cannot be carried out easily; enammonium/enamine compounds such as **9** are known to undergo hydrolysis exceedingly fast as neutral pH.[11]



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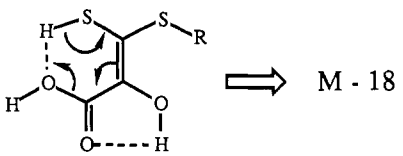

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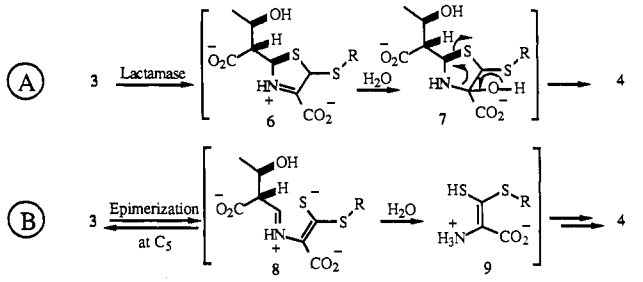
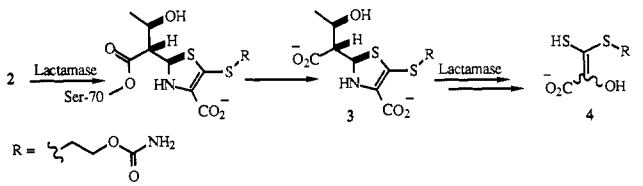
Compound **3** is not unlike the immediate products of turnover of penicillins or cephalosporins, in which only the lactam bond has undergone hydrolysis.[11-15] However, we have discovered that the subsequent formation of **4** from **3** is mediated by β-lactamase as well, a reaction that is unprecedented for β-lactamases. The following experiments, carried out with purified compound **3**, support this assertion: (i) The conversion showed saturation, as

Scheme I



is typical for any enzyme-mediated reaction, with kinetic parameters k_{act} = 0.035 s^{-1}, K_m = 625 μM (k_{cat}/K_m = 56 M^{-1} s^{-1}) at pH 7.0. (ii) In the absence of β-lactamase, **3** is converted to **4** in a phosphate buffer-mediated process (pH 7.0) with an apparent bimolecular rate constant $k_2 = 3.4 \times 10^{-6}$ M^{-1} s^{-1}, indicating a rate enhancement of 10^7-fold by the enzyme.[17] (iii) β-Lactamase modified in the active site by clavulanate, a mechanism-based inactivator for class A β-lactamases, did not enhance the rate of turnover of **4** beyond the background buffer-mediated reaction. Therefore, the immediate product from hydrolysis of penem **2** (*i.e.*, **3**) is formed and released into solution, but binds to the active site again to give rise to species **4** in a somewhat slower process. Compound **3** is unlikely to build up *in vivo* to a concentration that can successfully compete with penem **2** for binding to the active site; however, the results reported herein suggest that the catalytic machinery of β-lactamase can be recruited to enhance significantly a reaction other than what it has evolved for.



We wish to point out that the existence of a Δ^4-thiazoline intermediate such as **3**, the so-called penemoic acid, has been alluded to only by indirect evidence in chemical fragmentations of penems.[18,19] It has been suggested that imines such as Δ^3-thiazolines are in general the more stable tautomers in protic solvents.[20] However, both compounds **3** and **4** appear to be thermodynamically stable compounds because of the conjugation of the lone electron pairs of the three heteroatoms into the π-orbitals of the alkene function, and the ability to form intramolecular hydrogen bonds (see structure **5**). Furthermore, the stability of enediols, especially α,β-unsaturated ones such as **4**, is well documented.[21] Two plausible mechanisms for conversion of **3** to **4** are offered in Scheme I. One (route A) involves tautomerization of the double bond in **3** to the Δ^3-thiazoline product **6** by the β-lactamase. As alluded to earlier, such tautomerization has been reported for carbapenems while the substrate is tethered to Ser-70.[2,3,5] The nonenzymic addition of water to the iminium function of **6** should proceed exceedingly fast at neutral to acidic pH, as evidenced by model studies.[22]

(10) Silverstein, R. M.; Bassler, G. C.; Morill, T. C. In *Spectrometric Identification of Organic Compounds*, 5th ed.; John Wiley & Sons, Inc.: New York, 1991; p 27.
(11) Stamhuis, E. J. In *Enamines*; Cook, A. G., Ed.; Marcel Dekker Inc.: New York, 1969; pp 101–113.
(12) Grabowski, E. J. J.; Douglas, A. W.; Smith, G. B. *J. Am. Chem. Soc.* **1985**, *107*, 267.
(13) Page, M. I. *Acc. Chem. Res.* **1984**, *17*, 144.
(14) Page, M. I.; Procter, P. *J. Am. Chem. Soc.* **1984**, *106*, 3829.
(15) Faraci, W. S.; Pratt, R. F. *J. Am. Chem. Soc.* **1984**, *106*, 1489.
(16) Woodward, R. B.; Neuberger, A.; Trenner, N. R. In *The Chemistry of Penicillin*; Clarke, H. T., Johnson, R., Robinson, R., Eds.; Princeton University Press: Princeton, NJ, 1949; p 415.
(17) Degradation of **3** as a function of sodium phosphate buffer concentration (pH 7.0) was studied over several days. The nonenzymic reaction was first-order with respect to sodium phosphate concentrations.
(18) Visentin, G.; Peronne, E.; Borghi, D.; Rizzo, V.; Alpegiani, M.; Bedeschi, A.; Corigli, R.; Rivola, G.; Franceschi, G. *Heterocycles* **1992**, *33*, 859.
(19) Iwata, H.; Tanaka, R.; Ishiguro, M. *J. Antibiot.* **1990**, *43*, 901.
(20) De Jeso, B.; Pommier, J. C.; *J. Chem. Soc., Chem. Commun.* **1977**, 565.
(21) For a comprehensive review, see: Hesse, G. In *Methoden der Organischen Chemie* (Houben-Weyl); Georg Thieme Verlag: Stuttgart, 1978; vol. VI/1d, pp 217–298.
(22) Hauser, C. R.; Lednicer, D. *J. Org. Chem.* **1959**, *24*, 46. Böhme, H.; Haake, M. *Adv. Org. Chem.* **1976**, *9*, Part 1, 107.

Subsequently, the carbinolamine intermediate **7** yields the stable enol form of the resultant ketone (**7**→**4**).[23] The second possibility (route B) involves the displacement of the thiolate at position 1 (**3**→**8**), followed by the hydrolysis of the iminium moiety to give **9**. Compound **9** would have to undergo tautomerization to its corresponding iminium species, followed by hydrolysis to yield **4**. The equilibrium shown for the interconversion of **3** and **8** has been reported for penicillins,[24] anhydropenicillin,[25] and some penems,[18] which leads to epimerization at C_5. We hasten to add that we have not noticed any such epimerization at C_5 of **3** at neutral pH within 5 h.

(23) On stereoelectronic grounds, conversion of **7** to **4** should be stepwise since an antiperiplanar relationship is not observed for the two bonds that undergo scission in **7**: Grob, C. A. *Angew. Chem., Int. Ed. Engl.* **1969**, *8*, 535.

(24) Ghebre-Sellassie, I.; Hem, S. L.; Knevel, A. M. *J. Pharm. Sci.* **1984**, *73*, 125.

In conclusion, we have provided the first direct evidence for the existence of the "penemoic acid" (*i.e*, **3**) in turnover of a penem by the TEM-1 β-lactamase. Furthermore, we have shown that the degradation of the "penemoic acid", a type of process that previously would have been assumed to be nonenzymic, is facilitated by β-lactamase as well.

Acknowledgment. We are indebted to Drs. George Miller and Stuart McCombie of the Schering-Plough Corp. for providing us with a sample of Sch 34343. This work was supported by National Institutes of Health Grant AI 33170.

(25) Pratt, R. F.; Surh, Y. S.; Shaskus, J. J. *J. Am. Chem. Soc.* **1983**, *105*, 1006.