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Nuances of Mechanisms and Their Implications for Evolution of the Versatile β-Lactamase Activity: From Biosynthetic Enzymes to Drug Resistance Factors

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Abstract: β-Lactamases of classes A and C are believed to have evolved from bacterial enzymes involved in biosynthesis of the peptidoglycan, the so-called penicillin-binding proteins. All these enzymes undergo acylation at an active-site serine by β-lactam antibiotics as a common feature. However, the fate of the acyl-enzyme species is different for β-lactamases and penicillin-binding proteins; deacylation is rapid for the former, whereas it is slow for the latter. It is believed that the acquisition of the ability to deacylate the acyl-enzyme intermediate led to the evolution of β-lactamase activity, which is indispensable for the survival of bacteria in the face of challenge by β-lactam antibiotics. The mechanisms of deacylation of acyl-enzyme intermediates for β-lactamases are examined as a means to investigate structural factors in evolutionary descendency of classes A and C of β-lactamases from penicillin-binding proteins. It is known that in class A β-lactamases the hydrolytic water approaches the acyl-enzyme intermediate from the α-face, a process which is promoted by Glu-166 of these enzymes. An approach from the β-face for class C β-lactamase has been proposed. The process of activation of the hydrolytic water is not entirely understood at the present for these enzymes. Two compounds, *p*-nitrophenyl (2*R*,5*R*)-5-prolylacetate (**2**) and *p*-nitrophenyl (1*S*,3*S*)-3-carboxycyclopentylacetate (**3**), were synthesized as mechanistic probes to explore whether the hydrolytic water molecule actually approaches the acyl-enzyme species from the β-face and to investigate a notion that the ring amine at the acyl-enzyme intermediate may promote the hydrolytic reaction. Compound **2** acylates the active site serine of the Q908R β-lactamase (a class C enzyme), and the intermediate undergoes deacylation. On the other hand, compound **3** only acylates the active site, and not having the requisite amine in its structure, the intermediate resists deacylation. Both compounds serve as substrates for the class A TEM-1 β-lactamase, as they were expected, since the approach of the hydrolytic water molecule is from the α-face in this enzyme and is not promoted by the substrate itself. We conclude that substrate-assisted catalysis applies for the class C β-lactamases. On the basis of the evidence discussed, the knowledge of the crystal structures for the classes A and C of β-lactamases and the *Steptomyces* R61 DD-peptidase/transpeptidase (a PBP), it is proposed herein that evolution of classes A and C of β-lactamases from a primordial penicillin-binding protein should have been independent events; hence, the process does not represent a linear descendency of one β-lactamase from the other.

The β-lactamase activity is the primary means for bacterial resistance to β-lactam antibiotics. Of the four known classes of β-lactamases, class A enzymes are most common among pathogens, whereas class C enzymes are second most common. On the basis of similarity of the three-dimensional fold for β-lactamases of known structures with penicillin-binding proteins (PBPs), enzymes involved in cellwall biosynthesis in bacteria, these proteins are believed to be related by common ancestry. Both classes A and C of β-lactamases, as well as the known PBPs, undergo acylation at an active-site serine residue

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by β-lactam antibiotics. The rate of deacylation of this acyl-enzyme intermediate from the active site of penicillin-binding proteins is slow, thereby the bacterium is deprived of the biosynthetic function of these enzymes, an event that results in bacterial death. However, β-lactamases are capable of undergoing deacylation in a facile manner, completing the turnover necessary for hydrolysis of the β-lactam antibiotics.

Availability of crystal structures from the Brookhaven Protein Data Bank for several of these proteins (two PBPs, three class A β-lactamases, and one class C β-lactamase) has stimulated research on the mechanisms of these important bacterial enzymes. Despite the availability of the structural information, the details of the catalytic mechanisms for all these proteins remain elusive. Furthermore, evolutionary relationship among these proteins is not known. Lobkovsky *et al*.[1] have proposed on the basis of analysis of crystal structures that class C β-lactamases arose from PBPs and, subsequently, class A enzymes arose from class C enzymes (linear evolution). Insofar as all these proteins undergo acylation by β-lactam antibacterials, we decided to investigate the processes for deacylation, both to delineate the pertinent mechanisms, as well as to gain a mechanistic handle on the subject of the evolutionary diversification of β-lactamases from PBPs. We disclose herein that the classes A and C of β-lactamases have developed entirely distinct approaches for catalyzing the deacylation step of their respective acyl-enzyme intermediates. As it is inconceivable to see how such disparate diversification in the catalytic machinery can arise from a linear evolutionary process, we conclude that this mechanistic information supports an alternative model for the evolution of this family of proteins. This model states that the two classes of β-lactamases evolved independently from the ancestral PBP(s).

Experimental Section

Hydrogen and carbon NMR spectra were obtained at 300 and 75 MHz, respectively, using a Gemini-300 Varian spectrometer, or at 500 and 125 MHz, respectively, using U-500 Varian spectrometer; chemical shift values (δ) are given in parts per million. Infrared and mass spectra were recorded on Nicolet DX and Kratos MS 80 RFA spectrometers, respectively. Optical rotation was measured on JASCO DIP-370 polarimeter. Melting points were taken on an Electrothermal apparatus and are uncorrected. Thin-layer chromatograms were made on silica gel. The purification protocols for the TEM-1[2] and Q908R[3] β-lactamases have been described previously. Kinetic measurements were carried out on a Hewlett-Packard 452 diodearray instrument. All enzyme assays were performed in 100 mM sodium phosphate buffer, pH 7.0. (5*R*,2*R*)-1-*N*-Benzyl-5-[(methoxycarbonyl)methyl]pyrrolidine-2-carboxylic acid *tert*-butyl ester (**7**)[4] and (1*R*,3*S*)-cyclopentanedicarboxylic acid 1-methyl ester (**13**)[5] were synthesized according to the literature methods. Modeling was performed according to the methodology reported previously with AMBER force field.[6]

(5*R*,2*R*)-5-[(Methoxycarbonyl)methyl]pyrrolidine-2-carboxylic Acid *tert*-Butyl Ester (8). A suspension of 35 mg of 5% Pd/C in methanol (2 mL) was stirred under an atmosphere of hydrogen for 10 h. A solution of **7** (110 mg, 0.33 mmol) in methanol (5 mL) was added to the suspension, and the mixture was stirred for 15 h. The mixture was filtered, and the filtrate was evaporated *in vacuo* to give a solid residue. The product was purified by silica gel column chromatography (methanol/chloroform/aqueous ammonia, 1/30/0.1) to afford the title compound (76 mg; yield, 95%): mp 114 °C; R_f 0.28 (methanol/chloroform/aqueous ammonia, 1/30/0.1); ^{1}H NMR ($CDCl_3$) δ 1.29 (9H, s, CH_3 *t*-Bu), 1.27−1.36 (1H, unresolved m, C3 or C4 methylene), 1.61−1.74 (1H, m, C3 or C4 methylene), 1.76−1.88 (1H, m, C3 or C4 methylene), 1.98−2.11 (1H, m, C3 or C4 methylene), 2.34 (2H, d, J = 7 Hz, $-CH_2CO_2R$), 3.42−3.50 (1H, unresolved m, C5 methine), 3.51 (3H, s, CH_3 methoxy), 3.61 (1H, dd, J = 6 and 8 Hz, C2 methine), 6.54 (1H, broad s, amine); ^{13}C NMR ($CDCl_3$) δ 27.6, 29.1, 30.6, 40.1, 51.1, 54.3, 59.5, 80.8, 172.0, 173.7; $[\alpha]^{25}_D$ +112° (*c* 0.805, ethyl acetate); CI MS 244 (M + H, 45%).

(5*R*,2*R*)-1-*N*-(*tert*-Butoxycarbonyl)-5-(carboxymethyl)pyrrolidine-2-carboxylic Acid *tert*-Butyl Ester (10). An aqueous solution of NaOH (90 μL, 0.4 mmol) was added dropwise to the solution of **8** (100 mg, 0.4 mmol) in dioxane (7 mL) at ice−water temperature, and the mixture was stirred at this temperature for 6 h. The solvent was evaporated *in vacuo*, giving a yellowish residue, which was dissolved in water. The pH of the solution was adjusted to 2.0 by the addition of concentrated HCl, and the product was extracted into ethyl acetate (4×, 120 mL each). The combined organic fraction was dried over anhydrous $MgSO_4$, the mixture was filtered, and the filtrate was evaporated to dryness to give a pale yellow solid (82 mg); mp 124 °C.

The product of the above reaction (152 mg, 0.65 mmol) was dissolved in 10 mL of water/dioxane mixture (1/2), and the pH of the solution was adjusted to 9.0 by the addition of 6 N NaOH. Di-*tert*-butyl dicarbonate (214 mg, 0.98 mmol) was added to the mixture with stirring at ice−water temperature, and the reaction was allowed to progress at that temperature for 24 h. Dioxane was removed by evaporation *in vacuo*. The pH of the aqueous solution was adjusted to 2.0 with concentrated HCl, and the product was extracted into ethyl acetate (4×, 120 mL each). The combined organic fraction was dried over anhydrous $MgSO_4$, the solid was filtered, and the filtrate was evaporated to dryness. The product was purified by silica gel column chromatography (methanol/chloroform, 1/20) to afford the title compound as a white solid (180 mg; yield, 83%): mp 104 °C; R_f 0.72 (methanol/chloroform, 1/20); ^{1}H NMR ($CDCl_3$) δ 1.40 (9H, s, *t*-Bu), 1.43 (9H, s, *t*-Bu), 1.70−1.79 (1H, m, C3 or C4 methylene), 1.83−1.94 (1H, m, C3 or C4 methylene), 2.02−2.11 (1H, m, C3 or C4 methylene), 2.15−2.25 (1H, m, C3 or C4 methylene), 2.41 (1H, dd, J = 10 and 16 Hz, C6 methylene), 3.14 (1H, dd, J = 4 and 16 Hz, $-CH_2CO_2H$), 4.09 (1H, m, C5 methine), 4.22 (1H, m, C2 methine), 10.41 (1H, broad s, carboxylic acid); ^{13}C NMR ($CDCl_3$) δ 27.9, 28.2, 28.7, 30.0, 38.6, 54.9, 60.6, 80.5, 81.2, 153.6, 172.3, 176.3; $[\alpha]^{22}_D$ +98° (*c* 0.617, CH_2Cl_2); EI MS 330 (M^+, 1%).

(5*R*,2*R*)-1-*N*-(*tert*-Butoxycarbonyl)-5-[[(*p*-nitrophenoxy)carbonyl]-methyl]pyrrolidine-2-carboxylic Acid *tert*-Butyl Ester (11). *p*-Nitrophenol (90 mg, 0.65 mmol), 1,3-dicyclohexylcarbodiimide (128 mg, 0.62 mmol) and 1-hydroxybenzotriazole monohydrate (95 mg, 0.62 mmol), were added to the solution of **10** (209 mg, 0.63 mmol) in ethyl acetate (20 mL). The reaction mixture was stirred for 24 h at room temperature. The mixture was filtered, and the solvent was removed by evaporation. The residue was redissolved in ethyl acetate (20 mL) and filtered again. The solvent was removed, and the residue was purified by silica gel column chromatography (hexane/ethyl acetate, 5/2) to afford the title compound as a yellow oil (185 mg; yield, 65%): R_f 0.51 (hexane/ethyl acetate, 5/2); ^{1}H NMR ($CDCl_3$) δ 1.38 (9H, s, *t*-Bu), 1.41 (9H, s, *t*-Bu), 1.81−1.90 (1H, m, C3 or C4 methylene), 1.81−2.02 (1H, m, C3 or C4 methylene), 2.17−2.30 (2H, broad m, C3 or C4 methylene), 2.69 (1H, dd, J = 8.5 and 16 Hz, C6 methylene), 3.40 (1H, dd, J = 5 and 16 Hz, $-CH_2CO_2R$), 4.19 (1H, m, C5 methine), 4.40 (1H, m, C2 methine), 7.32 (2H, d, J = 9 Hz, aromatic), 8.25 (2H, d, J = 9 Hz, aromatic); ^{13}C NMR ($CDCl_3$) δ 28.0, 28.3, 28.8, 30.1, 39.0, 55.0, 60.7, 80.4, 81.2, 122.5, 125.1, 145.3, 153.5, 155.5, 169.3, 172.1; EI MS 329 (M − *p*-nitrophenol, 1%).

(5*R*,2*R*)-5-[[(*p*-Nitrophenoxy)carbonyl]methyl]pyrrolidine-2-carboxylic Acid (2). Compound **11** (100 mg, 0.22 mmol) was dissolved in a mixture of 5 mL of freshly distilled trifluoroacetic acid and 1 mL of anisole. The reaction mixture was stirred for 8 min at ice−water temperature, after which trifluoroacetic acid was removed *in vacuo*. Both water and ethyl acetate, 2 mL each, were added to the resulting brown oil. The aqueous fraction was lyophilized resulting in the title

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compound as a yellow solid (41 mg; yield, 60%): mp 94 °C dec; ^{1}H NMR (D_2O) δ 1.64−1.80 (1H, m, C3 or C4 methylene), 2.11−2.32 (3H, broad m, C3 or C4 methylene), 3.17 (2H, dd, J = 4 Hz, $-CH_2$-CO_2R), 4.02 (1H, m, C5 methine), 4.27 (1H, m, C2 methine), 7.26 (2H, d, J = 9 Hz, aromatic), 8.18 (2H, d, J = 9 Hz, aromatic); ^{13}C NMR (D_2O) δ 35.7, 40.3, 41.3, 48.5, 69.3, 85.3, 135.3, 145.6, 154.6, 168.3, 183.0; $[\alpha]^{25}_D$ +134° (*c* 0.743, H_2O); FAB MS 295 (M, 58%).

(1*R*,3*S*)-3-[[(Benzyloxy)carbonyl]methyl]cyclopentanecarboxylic Acid Methyl Ester (14). The general procedure for one-carbon homologation of the carboxylic acid, as well as the issue of configuration retention are described elsewhere.[7] Oxalyl chloride (3.6 mL, 20 mmol) was added under stirring dropwise to the solution of **13** (730 mg, 4.3 mmol) in dry CH_2Cl_2 (10 mL) under an atmosphere of nitrogen. A catalytic amount of DMF (one drop) was added, and the reaction mixture was stirred at room temperature for 2.5 h under nitrogen. The solvent and excess oxalyl chloride were removed *in vacuo* to give the crude acyl chloride as a brown oil. The acyl chloride was attached to high vacuum for 6 h and was taken to the next step without any purification: ^{1}H NMR ($CDCl_3$) δ 1.96−2.45 (6H, unresolved m, C2, C4 and C5 methylene), 2.88 (1H, m, C3 methine), 3.29 (1H, m, C1 methine), 3.70 (3H, s, methyl).

(Trimethylsilyl)diazomethane (4.5 mL of 2 M solution in hexanes) was added dropwise to a stirred solution of the acyl chloride in a 1:1 mixture of anhydrous THF and acetonitrile (20 mL total) under an atmosphere of dry nitrogen at ice−water temperature. The reaction mixture was stirred for 5 h, after which another portion of the (trimethylsilyl)diazomethane solution was added (4 mL), and the mixture was allowed to progress for another 5 h. The volatiles were removed *in vacuo* to give the crude diazo ketone as a brown oil. After the crude product was kept under high vacuum for 6 h, it was dissolved in a 1:1 mixture of benzyl alcohol and 2,4,6-trimethylpyridine (4 mL total). The solution was stirred under an atmosphere of dry nitrogen at 180−185 °C for 7 min. The crude product was purified by silica gel column chromatography (benzene/hexane/diethyl ether, 1/1/0.2) to afford the title compound as a colorless oil (801 mg; yield, 68%): R_f 0.46 (benzene/hexane/diethyl ether, 1/1/0.25); ^{1}H NMR ($CDCl_3$) δ 1.25−1.54 (2H, broad m, C2 methylene), 1.82−1.95 (3H, broad m, C4 or C5 methylene), 2.19−2.23 (1H, m, C4 or C5 methylene), 2.25−2.39 (1H, m, C3 methine), 2.42 (2H, d, J = 8 Hz, $-CH_2CO_2R$), 2.73−2.85 (1H, m, C1 methine), 3.64 (3H, s, methyl), 5.10 (2H, s, $-CH_2$Ph), 7.33 (5H, m, aromatic); ^{13}C NMR ($CDCl_3$) δ 28.8, 29.7, 31.9, 36.4, 36.7, 39.8, 43.3, 51.6, 66.1, 128.1, 128.5, 136.0, 172.7, 176.6; $[\alpha]^{24}_D$ +61° (*c* 1.012, ethyl acetate); EI MS 276 (M, 1%).

(1*S*,3*S*)-3-(Carboxymethyl)cyclopentanecarboxylic Acid (15). A solution of NaOH (16 mL of 1 M) was added to a solution of **14** (200 mg, 0.73 mmol) in dioxane (16 mL), subsequent to which, the reaction mixture was stirred for 24 h at room temperature. The solvent and low boiling components were removed *in vacuo* to give the crude intermediate in the form of a viscous oil. The oil was fractionated between water and ethyl acetate. The aqueous fractions were concentrated *in vacuo* to result in a white crystalline solid. The product was dissolved in 1 M HCl (1.5 mL) and was heated at 180 °C for 6 h in a sealed tube, as described by Berson and Reynolds-Warnhoff.[8] The product was extracted into ethyl acetate (2×, 40 mL each). The combined organic fraction was evaporated *in vacuo* to give the title compound as white crystals (110 mg; yield, 88%): mp 131 °C; ^{1}H NMR (D_2O) δ 1.34−1.43 (1H, m, C2 methylene), 1.49−1.55 (1H, m, C2 methylene), 1.89−1.99 (3H, broad m, C4 or C5 methylene), 2.19−2.26 (1H, m, C4 or C5 methylene), 2.28−2.35 (1H, m, C3 methine), 2.44 (2H, d, J = 4 Hz, $-CH_2CO_2H$), 2.82−2.89 (1H, m, C2 methine); ^{13}C NMR (D_2O) δ 28.8, 31.8, 36.2, 36.4, 39.4, 43.3; $[\alpha]^{23}_D$ +84° (*c* 1.006, H_2O); EI MS 172 (M^+, 1%).[9]

(1*S*,3*S*)-3-[[(*p*-Nitrophenoxy)carbonyl]methyl]cyclopentanecarboxylic Acid (3). *p*-Nitrophenol (115 mg, 0.83 mmol), 1,3-dicyclohexylcarbodiimide (164 mg, 0.8 mmol), and 1-hydroxybenzotriazole monohydrate (121 mg, 0.8 mmol) were added to a solution of **15** (139 mg, 0.81 mmol) in ethyl acetate (25 mL). The reaction mixture was stirred for 24 h at room temperature. The solution was filtered, and the filtrate was concentrated to dryness *in vacuo*. The residue was redissolved in ethyl acetate, and the solution was filtered again. The solvent was evaporated, and the residue was purified by silica gel column chromatography (hexane/ethyl acetate, 1/1) to afford the title compound as a yellowish solid (70 mg; yield, 30%): R_f 0.48 (hexane/ethyl acetate, 2/3); mp 87 °C dec; ^{1}H NMR ($CDCl_3$) δ 1.41−1.52 (1H, m, C2 methylene), 1.59−1.68 (1H, m, C2 methylene), 1.97−2.13 (4H, broad m, C4 and C5 methylene), 2.33−2.43 (1H, m, C3 methine), 2.49 (2H, d, J = 3 Hz, CH_2CO_2R), 3.08−3.13 (1H, m, C1 methine), 7.27 (2H, d, J = 6 Hz, aromatic proton), 8.27 (2H, d, J = 6 Hz, aromatic proton), 10.84 (1H, broad s, carboxylic acid); ^{13}C NMR ($CDCl_3$) δ 29.4, 32.6, 35.2, 37.5, 40.2, 45.9, 119.1, 125.5, 141.5, 127.2, 161.2, 173.3; $[\alpha]^{23}_D$ +96° (*c* 0.914, H_2O); CI MS 172 (M + H − *p*-nitrophenol, 1%).

Turnover Kinetics. Compounds **2** and **3** experienced some residual hydrolysis in neutral buffered solutions. Hence, all rates for enzymatic turnover of these compounds were obtained by subtracting the background hydrolysis rate from the corresponding rates of the enzyme-catalyzed reactions. The k_{cat} and K_m parameters for turnover of compounds **2** and **3** by the TEM-1 β-lactamase were determined from Lineweaver−Burk plots. The standard assay conditions were as follows. Portions of the stock solutions of compounds in dioxane were added to the 1-mL assay containing 100 mM phosphate buffer pH 7.0 and 33 nM TEM-1 enzyme to give final concentrations of 1.0−5.0 mM for **2**, and 0.5−2.0 mM for **3**. The initial rates for hydrolyses were monitored at 455 nm ($\Delta\epsilon_{455}$ 1114 M^{-1} cm^{-1} and 1121 M^{-1} cm^{-1} for compounds **2** and **3**, respectively). The enzymes are fully active in the presence of 10% dioxane as reported earlier.[6b] The k_{cat} and K_m values for turnover of compound **2** by the Q908R enzyme were determined similarly. Portions of the stock solution of compound **2** in dioxane were added to the 1-mL assay containing 100 mM sodium phosphate buffer, pH 7.0, and 80 nM of the Q908R enzyme to give final substrate concentrations of 1−10 mM, prior to monitoring of the rates of hydrolysis.

Inactivation Experiments. A solution of **3** in dioxane was added to a solution of the Q908R β-lactamase (0.2 μM) in 100 mM sodium phosphate buffer, pH 7.0 (final dioxane concentration of 10%), in a total volume of 50 μL, to give final concentrations of **3** in the range of 0.2−3.0 mM. Aliquots (10 μL) of the inactivation mixture were diluted into the 1-mL assay mixture containing 0.1 mM cephaloridine in the same buffer. The initial rate of hydrolysis of cephaloridine was monitored at 295 nm ($\Delta\epsilon_{295}$ cephaloridine 1000 M^{-1} cm^{-1}).

The enzyme inactivation process was accompanied by the rapid release ("burst") of *p*-nitrophenolate, a process which was dependent on the concentration of the enzyme that was being inactivated. A portion of the Q908R β-lactamase (4.5 or 12.7 μM final concentration) was added to 1-mL assay containing compound **2** (1 mM) in 100 mM sodium phosphate buffer, pH 7.0. The change of the absorbance due to release of *p*-nitrophenolate as a function of time was monitored at 455 nm. The rates of release of *p*-nitrophenolate were corrected for the nonenzymic hydrolysis of **2**.

Product Analysis. Compounds **2** and **3** were hydrolyzed by the TEM-1 β-lactamase. The protein was separated from the low molecular weight products by ultrafiltration in an Amicon device. The products were analyzed by HPLC, and were compared to the authentic standards for each case. The conditions were as follows: Waters C-4 1.0 × 25 cm, 5−95% linear acetonitrile gradient, 0.1% TFA, over 40 min, 1 mL/min, 205 and 405 nm; *p*-nitrophenolate, t_R = 38 min (205 nm or 405 nm); (5*R*,2*R*)-5-(carboxymethyl)pyrrolidine-2-carboxylic acid, t_R = 14 min (205 nm); (1*S*,3*S*)-3-(carboxymethyl)cyclopentanecarboxylic acid (**15**), t_R = 31 min (205 nm); (1*S*,3*S*)-3-[[(*p*-nitrophenoxy)carbonyl]-methyl]cyclopentanecarboxylic acid (**3**), t_R = 45 min (Waters C-4 1.0 × 25 cm, 5−95% linear acetonitrile gradient, 0.1% TFA, over 40 min; 95% acetonitrile, 0.1% TFA, over 10 min, 1 mL/min, 205 or 405 nm).

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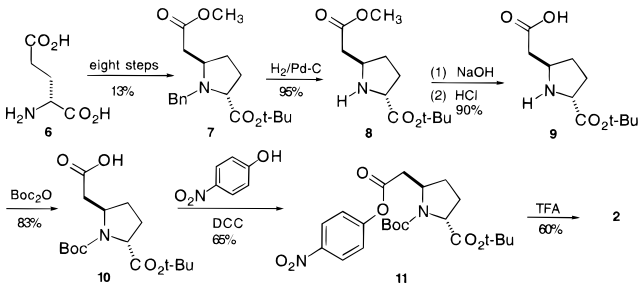
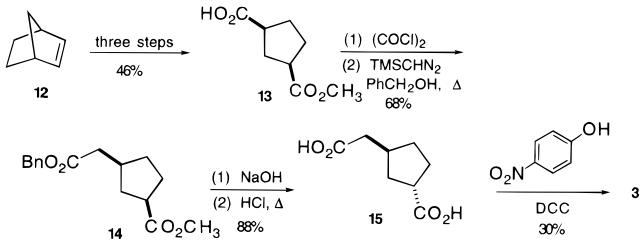
(9) The optical rotation for (1*S*,3*S*)-3-(carboxymethyl)cyclopentanecarboxylic acid (**15**) was $[\alpha]^{23}_D$ = +84° (*c* = 1.006, H_2O) and that for (1*R*,3*S*)-3-(carboxymethyl)cyclopentanecarboxylic acid was $[\alpha]^{23}_D$ = +38° under the same conditions. Furthermore, (1*R*,3*S*)-3-(carboxymethyl)cyclopentanecarboxylic acid showed NOE of 2% between C3 and C1 hydrogens, whereas (1*S*,3*S*)-3-(carboxymethyl)cyclopentanecarboxylic acid (**15**) did not give any NOE.

Scheme 1

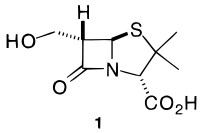


Scheme 2



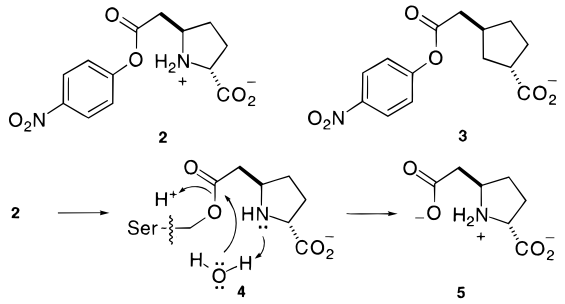
Results and Discussion

A molecular probe, 6α-(hydoxymethyl)penicillanic acid (**1**), was designed in our earlier work in a computer-aided process with the help of the crystal structure for the *Escherichia coli* TEM-1 β-lactamase, a prototypic class A enzyme. This molecule was designed to prevent the approach of the presumed hydrolytic water from the α-face of the acyl-enzyme intermediate.[10] The compound acylated the enzyme readily, but resisted deacylation, as was expected. The crystal structure for the acyl-enzyme intermediate for **1**, the only acyl-enzyme intermediate for turnover of a substrate by a native class A β-lactamase,[11] supported the design paradigms, indicating that the approach of the hydrolytic water is indeed from the α-face, and is promoted by Glu-166 as a general base.[12]



1

Interestingly, there is no counterpart to Glu-166 in class C β-lactamases.[1] Earlier work from our laboratory[13] and information from the crystal structures for the *Citrobacter freundii*,[14] and for the *Enterobacter cloacae* P99 β-lactamases,[1,15] both class C β-lactamases, had suggested that the approach of the hydrolytic water may be from the β-face of the acyl-enzyme species for these enzymes. It occurred to us that if this were the case, the formerly β-lactam nitrogen, now a secondary amine at the acyl-enzyme intermediate stage, is ideally positioned to serve as a general base in promoting the water molecule for approach to the acyl carbonyl from the β-face.[16] To test this possibility, compounds **2** and **3** were designed. Both compounds are surrogates of a minimal "β-lactam" molecule. Compound **2** was expected to acylate the active-site serine of the class C β-lactamase, followed by deacylation (**2** → **4** → **5**), should the



ring nitrogen promote the deacylation step. On the other hand, compound **3** was anticipated solely to acylate the active site, and not having the requisite amine in its structure, the intermediate should resist deacylation. Both compounds would be expected to serve as substrates for the class A TEM-1 β-lactamase, since the approach of water in this enzyme is from the α-face, but also the process is not substrate-assisted, as suggested here for the class C enzymes.

Compounds **2** and **3** were synthesized in multiple steps according to the Schemes 1 and 2. Compounds **7** and **13** were the key starting materials for the syntheses of the target compounds, which were prepared from D-Glu (**6**)[4] and norbornylene (**12**)[5] according to literature methods, respectively.

As expected, compounds **2** and **3** were both substrates for the class A TEM-1 β-lactamase (**2**, $K_m = 3.52 \pm 0.35$ mM, $k_{cat} = 1.1 \pm 0.2$ s^{-1}, $k_{cat}/K_m = 312 \pm 65$ M^{-1} s^{-1}; **3**, $K_m = 0.92 \pm 0.08$ mM, $k_{cat} = 0.17 \pm 0.03$ s^{-1}, $k_{cat}/K_m = 185 \pm 36$ M^{-1} s^{-1}). On the other hand, compound **2** was a substrate for the class C Q908R[17] β-lactamase ($K_m = 5.56 \pm 0.23$ mM, $k_{cat} = 0.050 \pm 0.006$ s^{-1}, $k_{cat}/K_m = 9.0 \pm 1.1$ M^{-1} s^{-1}), but compound **3** served as an inactivator for the enzyme ($K_I = 6.2 \pm 0.7$ mM, $k_{inact} = 0.21 \pm 0.06$ s^{-1}, $k_{inact}/K_I = 34 \pm 5$ M^{-1} s^{-1}).[18] Compound **3** acylated the enzyme efficiently, as evidenced by the rapid release of *p*-nitrophenolate, in a process which was directly dependent on concentration of the enzyme (Figure 1),

(10) Miyashita, K.; Massova, I.; Taibi, P.; Mobashery, S. *J. Am. Chem. Soc.* **1995**, *117*, 11055.

(11) Maveyraud, L; Massova, I.; Birck, C.; Miyashita, K.; Samama, J. P.; Mobashery, S. *J. Am. Chem. Soc.* **1996**, *118*, 7435.

(12) Our work with compound **1** complemented earlier mutagenesis results (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. *Prot. Eng.* **1991**, *4*, 805), which support these conclusions.

(13) Bulychev, A.; Massova, I.; Lerner, S. A.; Mobashery, S. *J. Am. Chem. Soc.* **1995**, *117*, 4797.

(14) Oefner, C.; Darcy, A.; Daly, J. J.; Gubernator, K.; Charnas, R. L.; Heinze, I.; Hubschwerlen, C.; Winkler, F. K. *Nature* **1990**, *343*, 284.

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

(16) Deprotonated Tyr-150 has been suggested as the general base for deacylation of the acyl-enzyme intermediate in class C enzymes by one group (Dubus, A.; Ledent, P.; Lamotte-Brasseur, J.; Frère, J. M. *Proteins: Struct., Funct., Genet.* **1996**, *25*, 473), whereas the results of another group indicated that this is not so (Dubus, A.; Normark, S.; Kania, M.; Page, M. G. P. *Biochemistry* **1994**, *33*, 8577). The ring nitrogen, as will be outlined in the text, plays an important role in the deacylation step.

(17) 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.

(18) The enzyme activity gradually recovered from inhibition over several hours ($k_{re} = 5.7 \times 10^{-5}$ s^{-1}). This rate of deacylation (i.e., recovery of activity) is comparable to those for PBPs acylated by the β-lactam antibiotics, which are devoid of the catalytic machinery for deacylation (typically in the range of 10^{-4} to 10^{-6} s^{-1}): Wilkin, J. M.; Jamin, M.; Joris, B.; Frère, J. M. *Biochem. J.* **1993**, *293*, 195. Wilkin, J. M.; Dubus, A.; Joris, B.; Frère, J. M. *Biochem. J.* **1994**, *301*, 477.

(19) The pH-dependence plots for k_{cat} and k_{cat}/K_m for catalysis by the class C enzymes are bell shaped and indicate the potential involvement of a group at the basic limb of catalysis which titrates with a pK_a value of ~10: Page M. I.; Vilanova, B.; Layland, N. J. *J. Am. Chem. Soc.* **1995**, *117*, 12092. Although the authors interpreted this finding as probably due to Tyr-150, we believe that if the decline in the catalytic ability at the basic limb of the pH profile is due to a titratable function, it is also consistent with the ring amine as such a residue. The alternative is that both the ring amine and the side chain of Tyr-150 are titrating simultaneously. Here are some typical pK_a values relevant to this discussion: 10.7 ± 0.2 for proline, 8.8 ± 0.2 for thiazolidine, and 11.3 ± 0.2 for pyrrolidine. The dependence of pH of reactions with **2** and **3** could not be studied because of the instability of the *p*-nitrophenyl esters at both acidic and basic conditions.

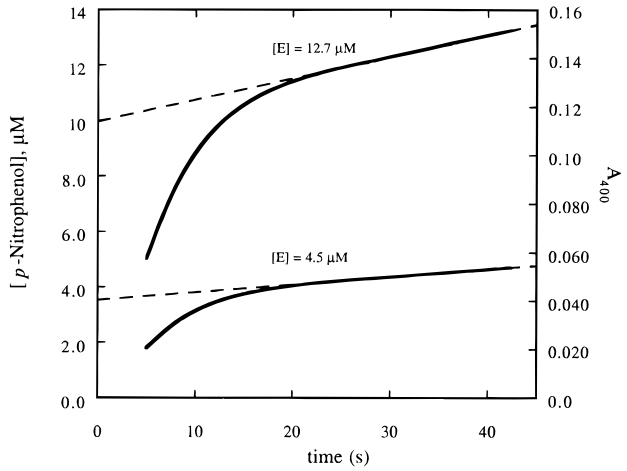
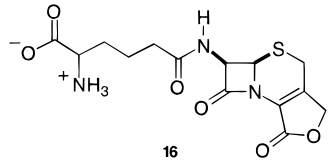


Figure 1. Release of *p*-nitrophenol due to modification of the active site of the Q908R β-lactamase (at specified concentrations) by compound **3**. The measurements were made in 100 mM sodium phosphate, pH 7.0, with concentration of compound **3** fixed at 6.7 mM in each case. The extrapolated points for the "burst size" are proportional to the respective enzyme concentrations.

and the acylated enzyme resisted deacylation. These observations are consistent with our proposal that the ring amine in β-lactam antibiotics would play an important role in the deacylation step.[19] Hence, in contrast to class A β-lactamases, substrate-assisted catalysis would appear to be operating for class C enzymes.[20]

We were prompted to prepare and study compounds **2** and **3** on the basis of our initial notion that the ring nitrogen might serve as the general base in promoting the deacylation step. However, we are aware that the electrostatic contribution of the ring nitrogen to the stabilization of the transition state for deacylation may also be a factor. That is to say, if the ring nitrogen is not the general base for promotion of water in the hydrolytic process, its electrostatic contribution to the reaction energy profile is still quite significant for class C β-lactamases, but not so for class A β-lactamases. In essence, the collective nature of the active-site functionalities may be considered perturbed in the absence of the ring nitrogen, hence affecting the catalytic ability of the enzyme to undergo deacylation after modification of the active site by compound **3**. Deprotonated Tyr-150 has been suggested as the general base for deacylation of the acyl-enzyme intermediate in class C enzymes by one group, whereas the results of another group showed some significant effects on the catalytic parameters for turnover of substrates, but were inconsistent with this conclusion.[16] There are no crystal structures for a typical acyl-enzyme intermediate available for a class C β-lactamases, so we have generated an energy-minimized structure for the acyl-enzyme intermediate of cephalothin, a first-generation cephalosporin, in the active site of the crystal structure for the class C enzyme from *E. cloacae* P99 (Figure 2). We believe that because of the close interactions of the side chains of Lys-315 and Lys-67 with that of Tyr-150, it is likely that the Tyr-150 side chain may exist in its ionized phenolate form. The phenolate contribution to the electrostatic nature of the surface of the active site should be significant, which is consistent with the results of mutagenesis experiments at this position, indicating this residue to be indeed important for the enzyme, even if it may not be the general base. It is worthy of a note that the distance between the side-chain oxygen of Tyr-150 and the ring nitrogen of the acyl-enzyme intermediate is in excess of 5 Å, precluding any direct interaction between the two, although they would both be influenced by one another in an electrostatic sense.

We note that Tyr-150, ring nitrogen, and the substrate carboxylate are all within a hydrogen-bonding distance to an active-site water molecule. Comparative work of Waley with the cephalosporin lactone **16** and typical β-lactam substrates had indicated some influence by the carboxylate of the β-lactam substrates on enzymic hydrolyses.[21] This information in conjunction with results of mutagenesis at position 150 and our findings reported herein indicate a collective influence by these functionalities on the deacylation step of the hydrolytic mechanism. When the class C enzyme is acylated by **3**, its catalytic machinery is impaired sufficiently such that enzyme inactivation results. The activity returns over several hours, suggesting that deacylation occurs, albeit sluggishly.[18] Similarly, the results of Waley indicated that the absence of the substrate carboxylate (as in lactone **16**) affected hydrolysis.[21] The work of Pratt and colleagues with non-β-lactam peptide and depsipeptide substrates for class C β-lactamases is interesting in that the portion of the substrate corresponding to the substrate ring is released in the course of turnover.[22] These are substrates which either are hydrolyzed by entirely different mechanism(s), or they rely solely on the functionalities of the enzyme (e.g., Tyr-150) to facilitate the slow deacylation. In summary, we believe that activation of the hydrolytic water molecule in the active site of class C enzymes would potentially rely on the functions of the substrate carboxylate, ring nitrogen and Tyr-150; the ring nitrogen would potentially serve as the active-site general base, which activates the water molecule for the deacylation step, or it affects the electrostatic properties of the active site so significantly that its absence from the active site impairs catalysis.



16

A recent parsimony analysis of β-lactamases indicated that divergence of classes A and C of these enzymes from a common ancestor was an early event in their diversification of function.[23] Bacteria have been in existence for over 3.5 billion years,[24] and the chances are that PBPs have been serving their biosynthetic activities from the early beginnings of bacterial life. The advent of antibiotics, such as β-lactams, by microorganisms took place in order to give the producing organisms an advantage over the nonproducing bacteria. The biosynthetic development of antibacterials is an old one. Indeed, it is accepted that the resistance mechanisms to antibacterials arose in response to the challenge by antibiotic-producing organisms, well predating the selection pressure due to clinical use of antibiotics in the modern era.[25] Reconstruction of the biochemical evolutionary paths for

(20) We have observed that under the conditions that the kinetic experiments were carried out (100 mM phosphate buffer pH 7.0, room temperature), compound **2** underwent nonenzymic hydrolysis approximately 5-fold faster than did **3**. It is plausible that the ring nitrogen may also be involved in enhancing this solvent-mediated hydrolysis.

(21) Waley, S. G. In *The Chemistry of β*-Lactams; Page, M. I., Ed.; Blackie Academic: London, 1992; pp 216−217.

(22) Pazhanisamy, S.; Govardhan, C. P.; Pratt, R. F. *Biochemistry* **1989**, *28*, 6863. Murphy, B. P.; Pratt, R. F. *Biochemistry* **1991**, *30*, 3640. Xu, Y.; Soto, G.; Hirsch, K. R.; Pratt, R. F. *Biochemistry* **1996**, *35*, 3595. Adediran, S. A.; Deraniyagala, S. A.; Pratt, R. F. *Biochemistry* **1996**, *35*, 3604.

(23) Bush, K.; Jacoby, G. A.; Medeiros, A. A. *Antimicrob. Agents Chemother*. **1995**, *39*, 1211.

(24) Holland, H. D. *Science* **1997**, *275*, 38.

(25) Abraham, E. P.; Chain, E. *Nature* **1940**, *146*, 837. Massova, I.; Mobashery, S. *Acc. Chem. Res.* **1997**, *30*, 162.

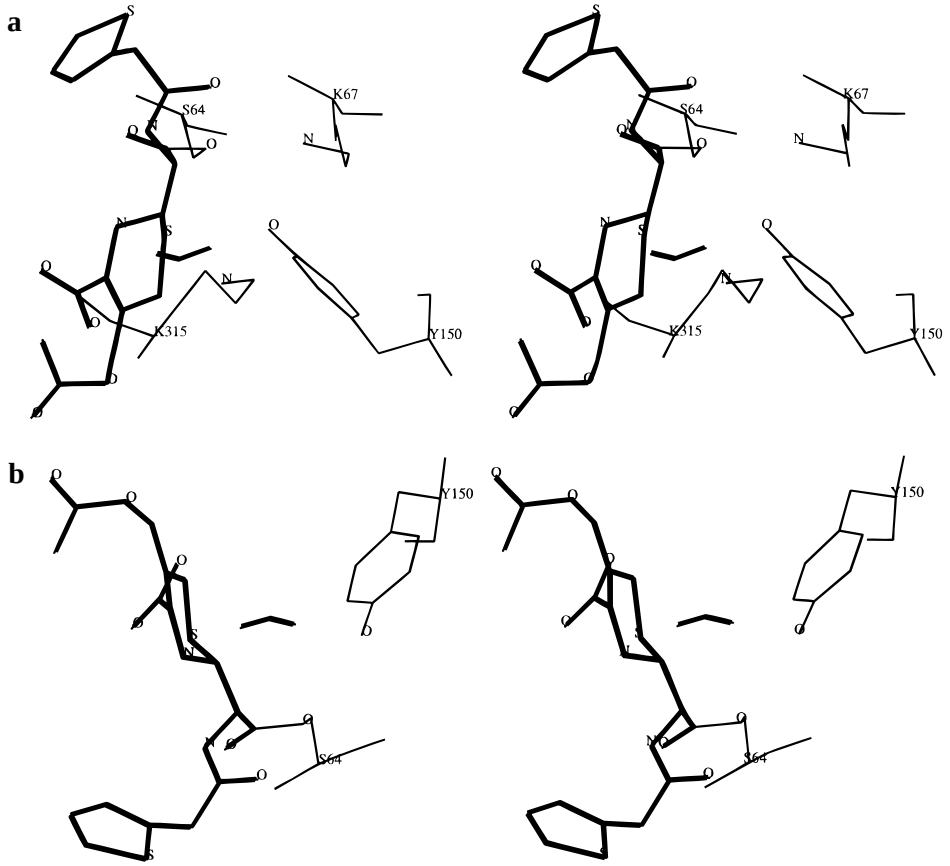


Figure 2. (A) Stereoview of the energy-minimized structure for the acyl-enzyme intermediate for cephalothin in the active site of the class C β-lactamase from *E. cloacae* P99. Cephalothin and the hydrolytic water molecule are shown in bold. Because of the fleeting existence of the acyl-enzyme species, the acetoxy group at the C3 position is retained; the expulsion of this group is known to take place once the product of hydrolysis is released into solution. (B) A different perspective, showing the proximity of the side chain of Tyr-150, the substrate ring nitrogen and carboxylate to the hydrolytic water, all of which are within a hydrogen-bonding distance.

such a highly evolved system presents difficulties since any such analysis has to be carried out on the basis of our knowledge of the properties of the extant modern descendants. However, β-lactamases present a special case for such analyses since by now over 200 members of this family of enzymes have been identified,[23] and that several crystal structures for representative enzymes are available. Analysis of the details of mechanisms of β-lactamases would be revealing in understanding the incremental evolutionary steps which would lead to the modern forms of these enzymes. Such an undertaking is attempted in this manuscript.

The suggestion that a certain ancestral bacterial biosynthetic enzyme (e.g., a PBP) may have given rise to the resistance enzymes was put on firm ground on the basis of the observation of the general conservation of the three-dimensional topology of the DD-peptidase/transpeptidase from *Streptomyces* R61, a PBP, with the crystal structures of β-lactamases.[26] We have used the mechanistic features of β-lactamases as they pertain to the deacylation step as a tool in gaining insight into the evolutionary pathways for these related enzymes. Nature has selected two distinct pathways for deacylation of the acyl-enzyme intermediates in catalysis by classes A and C of β-lactamases. The approach of water to the acyl carbonyl is from the α-face for the class A β-lactamases, whereas for the class C enzymes it is from the β-face. An interesting aspect of the structures for classes A and C of β-lactamases and the PBPs is that the general scaffolding for the active-site functionalities is remarkably preserved. In this vein, the information from the crystal structure for DD-peptidase/transpeptidase from *Streptomyces* R61 is informative. We note that the active site of this PBP contains two important crystallographic water molecules (Figure 3). When the structure of this enzyme is superimposed on those of the class A β-lactamases (including the TEM-1 enzyme), the position of the hydrolytic water of the class A enzymes is identical to that of Wat-1 of DD-peptidase/transpeptidase from *Streptomyces* R61 (Figure 3). Hence, in the course of evolution of the class A enzymes, the ancestral enzyme underwent restructuring near the active site to make available Glu-166 for activating Wat-1 for hydrolysis, a water molecule which already existed in the active site of the PBP. On the other hand, in evolution of the class C enzymes nature took a different ploy. Instead of relying solely on a basic function in the active site, as was the case for the class A enzymes, these enzymes exploited the ring amine of the acyl-enzyme species in facilitating the deacylation step. We hasten to add that Tyr-159 of DD-peptidase/transpeptidase (which is devoid of deacylation machinery) corresponds spatially to Tyr-150 of the class C β-lactamases, so Tyr-150 was not put in place to facilitate the deacylation step in class C enzymes. The water molecule on the β-face of the acyl-enzyme species (Wat-2) can serve as the potential hydrolytic water molecule (Figure 3). This water molecule is positioned less than 0.5 Å away from a suitable interaction with the ring amine of the acyl-enzyme species in the crystal structure of DD-peptidase/transpeptidase (Figure 3). Therefore, nature would require the restructuring of the environment around this water molecule to permit its approach to the ring amine and the carbonyl of the

(26) Kelly, J. A.; Dideberg, O.; Charlier, P.; Wery, J. P.; Libert, M.; Moews, P. C.; Knox, J. R.; Duez, C.; Fraipont, C.; Joris, B.; Dusart, J.; Frère, J. M.; Ghuysen, J. M. *Science* **1986**, *231*, 1429.

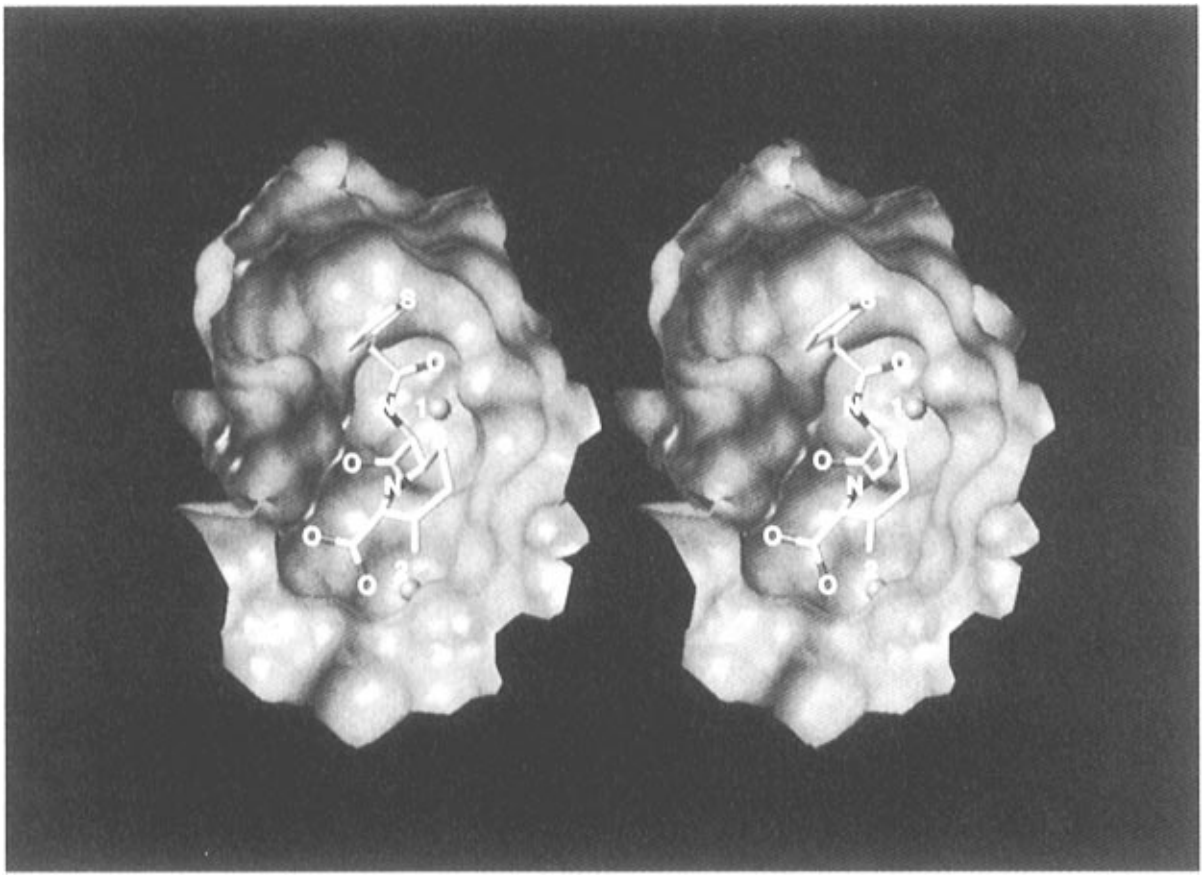
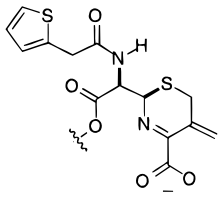


Figure 3. The active site of DD-peptidase/transpeptidase from *Streptomyces* R61 modified by cephalothin (see below for schematic of the structure), shown as the water-accessible surface from the crystal structure (Kuzin, A. P.; Liu, H.; Kelly, J. A.; Knox, J. R. *Biochemistry* **1995**, *34*, 9532). Two active-site water molecules are shown as spheres at 2 and 5 o'clock positions. Because of the longevity of the acyl-enzyme species, the acetoxy group appended to the C3 methyl is expelled.



acyl-enzyme species during its metamorphosis to a class C β-lactamase.

On the basis of conservation of structural topology, Lobkovsky *et al*. suggested that evolution of these enzymes was a sequential one;[1] that is to say that the PBP gave rise to the larger class C enzymes, which in turn underwent change to become class A enzymes. Our findings reported in this manuscript do not support this conclusion. A linear evolution of one β-lactamase from another intuitively becomes impossible, since the existing deacylation machinery in the first enzyme would have to be dismantled first, and then another should be put into place, if the linear evolution had taken place. We suggest here that in light of the mechanistic evidence presented, evolution of classes A and C of β-lactamases from an ancestral PBP was likely to have been two independent events.[27]

Note Added in Proof: Subsequent to submission of this manuscript, a structural survey of two β-lactamases and one penicillin-binding protein by Knox *et al.* has appeared in the literature (Knox, J. R.; Moews, P. C.; Frère, J. M. *Chemistry & Biology* **1996**, *3*, 937), which is relevant to the question of independent evolution for the classes A and C of β-lactamases.

Acknowledgment. This work was supported by the National Institutes of Health. I.M. is the recipient of the Rumble and Heller Predoctoral Fellowships. We are indebted to Professors Judith Kelly and James Knox for supplying us the coordinates for the of DD-peptidase/transpeptidase from *Streptomyces* R61 prior to its availability from the Protein Data Bank.

Supporting Information Available: The ^{1}H and ^{13}C NMR spectra for compounds **2** and **3** (4 pages). See any current masthead page for ordering and Internet access instructions.

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(27) We have carried out a multiple amino acid sequence alignment of 73 distinct β-lactamases of all four classes with 80 known PBPs. The alignment indicated that PBPs diversified into six distinct clusters. As branching points from the PBPs, β-lactamases were sequestered. For example, all class A β-lactamases were more closely related to one group of PBPs, and similarly all class C β-lactamases were more closely related to a different group of PBPs. This observation further supports our notion for a nonlinear process for the evolution of β-lactamases from PBPs. (Massova and Mobashery, unpublished results).