Potent Mechanism-Based Inhibition of the TEM-1 β-Lactamase by Novel N-Sulfonyloxy β-Lactams

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Abstract: A novel class of *N*-sulfonyloxy β-lactam molecules are described as potent mechanism-based inactivators for the bacterial TEM-1 β-lactamase, a prototypic class A enzyme. These molecules inactivate the enzyme with k_{inact}/K_i values in the range of $1-7 \times 10^4$ M^{-1} s^{-1} and partition ratios (i.e., k_{cat}/k_{inact}) of 2−7. The mechanism of action of these inactivators was investigated. These molecules acylate the active-site serine of the TEM-1 β-lactamase, a process that results in the release of the sulfonate attached to the lactam nitrogen, giving rise to a proposed β-amino cinnamoyl derivative as the inhibitory species. This species undergoes gradual hydrolysis with concomitant recovery of activity, the rate constants for which were evaluated.

Wide-spread—and often indiscriminate—clinical use of antimicrobials over the past five decades has led to emergence and spread throughout the world of bacterial strains that are often resistant to several families of antibiotics concurrently.[1] There exist currently bacterial strains that cannot be treated with any of the existing drugs, a fact that has created a state of crisis in treatment of such organisms in the clinic.[1] The primary means of bacterial resistance to β-lactam antibiotics is the ability of bacteria to produce β-lactamases, which hydrolyze the β-lactam moiety of these antibacterial agents, hence rendering them ineffective.[2] A strategy that has been used effectively to combat the harmful effect of this family of enzymes is the coadministration of a β-lactam antibiotic along with a mechanism-based inactivator, such as clavulanate, sulbactam, or tazobactam, for β-lactamases; the inactivator inactivates the β-lactamase, thereby preserving the antibacterial activity of the β-lactam antibiotic. Unfortunately, mutations in the genes for class A β-lactamases, the most prevalent group of these bacterial enzymes, have been identified that preserve the catalytic activity of the enzymes, yet render the enzymes resistant to the inactivation chemistry.[3] This recent resistance phenomenon to inactivators necessitates a vigilant pursuit of new types of enzyme inactivators as a vanguard against such new variants of β-lactamases. New enzyme inactivators would be indispensable for future developments in this area to provide an acceptable level of chemotherapeutic care in the immediate future. We disclose herein our findings with a novel class of potent monobactam mechanism-based inactivators for class A β-lactamases, which exhibit a distinct mechanism for their inhibition chemistry.

Experimental Section

Penicillin G was purchased from Sigma. The wild-type TEM-1 β-lactamase was purified according to the method of Zafaralla *et al.*[4] All kinetic and spectral measurements were made on a Perkin-Elmer Lambda 3B or Hewlett-Packard 452 diode-array spectrometer. Melting points were taken on a Thomas-Hoover capillary melting apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR were obtained on a General Electric GN-300 spectrometer and a Varian VXR 500S spectrometer. Chemical shifts are reported in parts per million relative to tetramethylsilane. Infrared spectra were recorded on a Perkin-Elmer 1420 spectrophotometer. Mass spectra were recorded on an AEI Scientific Apparatus MS 902 and Finnigan MAT Model 8430 spectrometers. Analytical thin-layer chromatography was carried out using commercially available aluminum-backed 0.2-mm silica gel 60 F-254 plates. Flash-silica-gel column chromatography was performed using Merck silica gel 60 (230−400 mesh). Solvents for flash chromatography were all distilled. Elemental analyses were performed by the M-H-W Laboratories, Phoenix, AZ. Compounds **4**[5] and **1**[6] were synthesized as described previously. The synthesis and characterization of the 4(*R*) enantiomer of **1** were essentially identical to those reported for the racemate,[6] except that the synthesis started from the commercially available methyl (*R*)-3-hydroxy-3-phenylpropionate (Eastman). The optical rotation of the 4(*S*) enantiomer of **1** was determined: $[\alpha]^{24}_D = -18.90°$ (*c* 2.3, ethyl acetate).

Ethyl 3-Hydroxy-3-phenylpropionate-3-*d* (13). Ethyl benzoylacetate (compound **12**, 20.0 g, 0.1 mol) was dissolved in methanol (575 mL) and was stirred in an ice-water bath. Sodium borodeuteride (2.25 g, 0.05 mol) was added, and the reaction was stirred for 20 min. The reaction mixture was poured into 200 mL of ethyl acetate and was stirred for 10 additional min. The mixture was diluted with ether (450 mL), followed by the addition of brine (200 mL). The mixture was washed with 1.1 M citric acid (2 × 150 mL), 5% sodium bicarbonate (2 × 150 mL), and brine (1 × 150 mL) and dried over anhydrous magnesium sulfate. The organic layer was concentrated *in vacuo* to dryness to yield 12.8 g (66%) of the title compound as a yellow oil: ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 7.38−7.26 (m, 5 H, aromatic), 4.16 (q, 2 H, OCH_2CH_3), 3.42 (br s, 1 H, OH), 2.74 (d, J = 16.3, 1 H, CH_2), 2.66 (d, J = 16.2 Hz, 1 H, CH_2), 1.24 (t, 3 H, OCH_2CH_3); ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 171.92, 142.52, 128.17, 127.40, 125.45, 69.62 (t), 60.50, 43.20, 13.82; IR (neat) 3480, 3075, 3040, 2980, 2940, 2910, 1760, 1495, 1450, 1375, 1335, 1190, 1095, 1035, 760, 700 cm^{-1}; HRMS calcd for $C_{11}H_{12}O_3D$ = 196.1084, found 196.1085.

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(1) Neu, H. C. *Science* **1992**, *257*, 1065. Davies, J. *Science* **1994**, *264*, 375.
(2) Neu, H. C. In *The Chemistry of β-Lactams*; Page, M. I., Ed.; Blackie Academic: London 1992; pp 101−128.
(3) Vedel, G.; Belaaouaj, A.; Gilly, L.; Labia, R.; Phillippon, A.; Nevot, P.; Paul, G. *J. Antimicrob. Chemother.* **1992**, *30*, 449. Sirot, D.; Chanal, C.; Henquell, C.; Labia, R.; Sirot, J.; Cluzel, R. *J. Antimicrob. Chemother.* **1994**, *33*, 1117. Belaaouaj, A.; Lapoumeroulie, C.; Caniça, M. M.; Vedel, G.; Névot, P.; Krishnamoorthy, R.; Paul, G. *FEMS Microbiol. Lett.* **1994**, *120*, 75. Brun, T.; Péduzzi, J.; Caniça, M. M.; Paul, G.; Névot, P.; Barthélémy, M.; Labia, R. *FEMS Microbiol. Lett.* **1994**, *120*, 111.
(4) Zafaralla, G.; Manavathu, E. K.; Lerner, S. A.; Mobashery, S. *Biochemistry* **1992**, *31*, 3847.
(5) Teng, M.; Miller, M. J. *J. Am. Chem. Soc.* **1993**, *115*, 548.
(6) Teng, M.; Miller, M. J.; Nicas, T. I.; Grissom-Arnold, J.; Cooper, R. D. G. *Bioorg. Med. Chem.* **1993**, *1*, 151.

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***O*-Cbz-3-hydroxy-3-phenylpropiohydroxamate-3-*d* (14).** Hydroxylamine hydrochloride (5.34 g, 0.08 mol) was dissolved in methanol (120 mL) and was cooled in an ice bath. Potassium hydroxide (4.32 g, 0.08 mol) was added, and the mixture was stirred for 30 min. The reaction mixture was filtered, and the filtrate was added to a solution of ethyl 3-hydroxy-3-phenyl propionate-3-*d* (12.2 g, 0.06 mol) in methanol (180 mL). The solution was stirred overnight and was subsequently concentrated *in vacuo*. The resultant white solid was suspended in dry acetonitrile (300 mL), and benzyl chloroformate (11.8 g, 0.07 mol) was added dropwise. The mixture was stirred overnight, and then the solvent was removed to give a pink solid, which was recrystallized from ethyl acetate in hexanes to yield 6.82 g of the product (34%) as a white solid: mp 121−122 °C; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 9.20 (br s, 1 H, NH), 7.41−7.28 (m, 10 H, aromatic), 5.28 (s, 2 H, CH_2Ph), 2.71 (d, J = 15.2, 1 H, CH_2), 2.61 (d, J = 15.1 Hz, 1 H, CH_2), 1.60 (br s, 1 H, OH); ^{13}C-NMR (CD_3CN, 125 MHz) δ 170.03, 155.15, 144.62, 135.78, 129.66, 129.63, 129.40, 129.29, 128.46, 126.66, 72.00, 42.97, CDPhOH was not observed; IR (film) 3360, 3140, 2970, 1780, 1675, 1385, 1245, 1230, 1105, 980, 955, 775, 740, 695 cm^{-1}; HRMS calcd for $C_{11}H_{16}DNO_5$ (MH^+) 317.1248, found (FAB) 317.1263.

***O*-Cbz-2-azetidinone-4-*d* (15).** Compound **14** (6.0 g, 18.9 mmol) and triphenyl phosphine (7.24 g, 27.6 mmol) were dissolved in tetrahydrofuran (100 mL). A solution of diethyl azodicarboxylate (4.8 g, 27.6 mmol) in tetrahydrofuran (60 mL) was added dropwise, and the reaction was stirred overnight. The solution was concentrated, and the residue was chromatographed using 1:3 ethyl acetate/hexanes. The title compound was isolated as a yellow oil, which solidified on standing (4.08 g, 72%): mp 40−42 °C; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 7.41−7.32 (m, 10 H, aromatic), 5.20 (s, 2 H, CH_2Ph), 3.32 (d, J = 13.9, 1 H, CH_2), 2.75 (d, J = 13.9 Hz, 1 H, CH_2); ^{13}C-NMR (C_6D_6, 125 MHz) δ 164.46, 153.98, 136.27, 128.92, 128.84, 128.72, 128.67, 128.55, 126.74, 71.54, 60.40 (t), 42.28; IR (neat) 3100, 3070, 3040, 2970, 1805, 1780, 1450, 1280, 1230, 1220, 1190, 1050, 960, 940, 740, 700 cm^{-1}; HRMS calcd for $C_{17}H_{14}NO_4D$ = 299.1142, found 299.1131.

4-Phenyl *N*-(Tosyloxy)-2-azetidinone-4-*d* (6). A solution of compound **15** (260 mg, 0.9 mmol) in methanol (6 mL) was hydrogenolized over 10% Pd/C (10 mg) under an atmosphere of hydrogen. The suspension was stirred for 45 min and was then filtered over a thin layer of Celite. The filtrate was concentrated *in vacuo* to yield 130 mg of the product of hydrogenolysis, which was stirred into a solution of *p*-toluenesulfonyl chloride (0.17 g, 0.9 mmol) in dry acetonitrile (4 mL). Triethylamine (0.05 mL, 0.06 mmol) was added dropwise to the mixture, and the reaction was stirred for 20 min. The mixture was filtered over a thin layer of silica gel, which was subsequently washed with a 1:1 mixture of ethyl acetate in hexanes. The filtrate was concentrated, and the materials were applied to flash chromatography in 3:1 ethyl acetate in hexanes to obtain 144 mg (52%) of the desired product. This material was recrystallized from ethyl acetate in hexanes to yield 64 mg (23%) of the titled compound as a white solid. Mass spectral analysis indicated >96% deuterium incorporation: mp 110−110.5 °C dec; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 7.79 (d, J = 8.4 Hz, 2 H, aromatic), 7.36−7.24 (m, 7 H, aromatic), 3.24 (d, J = 14.4 Hz, 1 H, CH_2), 2.75 (d, J = 14.4 Hz, 1 H, CH_2), 2.44 (s, 3 H, CH_3); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 165.2, 146.3, 135.2, 130.8, 129.9, 129.01, 128.99, 128.86, 126.82, 42.19, 21.81, the CDPh carbon was not observed; IR (KBr) 3090, 3070, 3030, 1810, 1595, 1450, 1385, 1285, 1195, 1180, 1090, 1035, 945, 815, 780, 750, 700, 690, 655 cm^{-1}; HRMS calcd for $C_{16}H_{15}NO_4SD$ = 318.0784, found 318.0772.

Ethyl Benzoylacetate-d_2 (16). Ethyl benzoylacetate (16.5 g, 0.08 mmol) was dissolved in ethanol-*d* (60 mL), and triethylamine (1.3 mL, 0.009 mol) was added. The reaction was concentrated, and the same procedure was repeated two more times. Subsequent to the last removal of the solvent, the resultant yellow oil was placed under vacuum overnight to yield 17.0 g (~100%) of **16**. ^{1}H-NMR analysis indicated that approximately 30% ethanol-*d* was still in the oily sample and that incorporation of deuterium in the product was approximately 96%: ^{1}H-NMR (C_6D_6, 300 MHz) δ 7.75−7.72 (d, J = 7.4 Hz, 2 H, aromatic), 7.65 (dd, J = 1.6, 6.4 Hz, 1H aromatic), 7.09−6.98 (m, 2 H, aromatic), 3.94 (q, J = 7.5 Hz, 2 H, OCH_2CH_3), 0.88 (t, J = 7.2 Hz, 3 H, OCH_2CH_3); IR (neat) 3060, 2980, 2935, 1735, 1680, 1630, 1595, 1575, 1560, 1445, 1305, 1275, 1250, 1020, 770, 745, 695 cm^{-1}; HRMS calcd for $C_{11}H_{10}O_3D_2$ = 194.0912, found 194.0903.

Ethyl 3-Hydroxy-3-phenylpropionate-d_2. Compound **16** (16.0 g, 0.08 mol) was dissolved in methanol-*d* (70 mL) and was cooled in an ice bath. Sodium borohydride (1.6 g, 0.04 mol) was added, and the mixture was stirred for 30 min. The mixture was diluted with ether (200 mL) and ethyl acetate (50 mL). The organic layer was washed with 1 M citric acid (100 mL), then with water until the aqueous layer was pH 7, and brine. The organic layer was dried over anhydrous magnesium sulfate, filtered, and concentrated to yield 11.5 g (71%) of the title compound as a yellow oil: ^{1}H-NMR (C_6D_6, 300 MHz) δ 7.27−7.05 (m, 5 H, aromatic), 5.01 (br s, 1 H, C*H*(OH), 3.85 (q, J = 7.14 Hz, 2 H, OCH_2CH_3), 3.22−3.06 (br m, 1 H, OH), 0.86 (t, J = 7.14 Hz, 3 H, OCH_2CH_3); ^{13}C-NMR (C_6D_6, 75 MHz) δ 158.06, 129.72, 114.52, 113.61, 112.01, 56.37, 46.49, 29.29 (quintet), 16.13; IR (neat) 3460, 3025, 2480, 1730, 1450, 1365, 1260, 1115, 1055, 1020, 770, 750, 695 cm^{-1}; HRMS calcd for $C_{11}H_{12}O_3D_2$ = 196.1068, found 196.1062.

***O*-Benzyl 3-Hydroxy-3-phenylpropiohydroxamate-d_2.** A suspension of *O*-benzylhydroxylamine hydrochloride (0.7 g, 4.4 mmol) in anhydrous (5 mL) methylene chloride was cooled in an ice bath, followed by the dropwise addition of trimethylaluminum (2.2 mL, 4.4 mmol, 2 M solution in hexanes) over 10 min. The mixture was stirred at room temperature for 1 h and was then cooled again in an ice bath. A solution containing 3-hydroxy-3-phenylpropionate-d_2 (0.5 g, 2.5 mmol) in anhydrous methylene chloride (25 mL) was added dropwise over 15 min. The solution was warmed to room temperature and was stirred overnight. A 5% aqueous solution of sodium potassium tartrate was added, and the mixture was stirred for 20 min, followed by filtration. The aqueous portion of the filtrate was washed with methylene chloride (3 × 50 mL), and the combined organic layer was washed with the 5% aqueous sodium potassium tartrate solution (2 × 20 mL), water (2 × 20 mL), and brine (1 × 20 mL). The organic layer was dried over anhydrous magnesium sulfate, filtered, and concentrated to give 0.68 g (100%) of the crude product, which was recrystallized from ethyl acetate in hexanes to yield 0.37 g (54%) of the title compound as a white solid: mp 120-121 °C; ^{1}H-NMR ($CDCl_3$ 300 MHz) δ 8.59 (br s, 1 H, NH), 7.43−7.22 (m, 10 H, aromatic), 5.05 (br s, 1 H, C*H*(OH), 4.86 (br s, 2 H, CH_2Ph), 3.56 (br s, 1 H, OH), some of the isomeric hydroximate was also seen in the spectrum; ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 169.28, 142.64, 135.11, 129.26, 128.81, 128.61, 127.90, 127.70, 125.54, 78.30, 70.57, CD_2 was not observed; IR (film) 3445, 2890, 1635, 1485, 1450, 1335, 1320, 1050, 960, 760, 740, 695 cm^{-1}; HRMS calcd for $C_{16}H_{15}NO_3D_2$ = 273.1365, found 273.1366.

4-Phenyl *O*-Benzyl-2-azetidinone-d_2. A solution of diethyl azodicarboxylate (8.0 g, 46 mmol) in tetrahydrofuran (80 mL) was added dropwise to a solution of 4-phenyl *O*-benzyl-2-azetidinone-d_2 (9.0 g, 32 mmol) and triphenylphosphine (12.1 g, 46 mmol) in tetrahydrofuran (120 mL). The solution was stirred overnight, following which it was concentrated, and the product mixture was subjected to flash chromatography using 1:3 ethyl acetate in hexanes to give 7.67 g (94%) of the desired product as a yellow-brown oil, which solidified upon standing: mp 38−39 °C; ^{1}H-NMR (C_6D_6, 300 MHz) δ 7.17−6.98 (m, 10 H, aromatic), 4.75 (d, J = 11.4 Hz, 1 H, OCH_2Ph), 4.63 (d, J = 11.4 Hz, 1 H, OCH_2Ph), 4.08 (s, 1 H, C*H*Ph); ^{13}C-NMR (C_6D_6, 125 MHz): δ 164.31, 138.13, 135.98, 129.03, 128.86, 128.64, 128.54, 128.50, 126.85, 77.83, 59.88, 41.49 (quintet); IR (film) 3050, 3020, 3030, 1775, 1630, 1445, 1310, 1075, 995, 730, 715, 695 cm^{-1}; HRMS calcd for $C_{16}H_{13}NO_2D_2$ = 255.1228 found 255.1227.

4-Phenyl *N*-(Tosyloxy)-2-azetidinone-4-d_2. A suspension of 4-phenyl *O*-benzyl-2-azetidinone-d_2 (120 mg, 0.78 mmol) and 10% Pd/C (19 mg) in methanol-*d* (5 mL) was placed under an atmosphere of hydrogen. The solution was stirred for 45 min, following which it was filtered over a thin layer of Celite. The filtrate was concentrated *in vacuo* to yield 134 mg of a residue, which was stirred into a solution of *p*-toluenesulfonyl chloride (150 mg, 0.78 mmol) in dry acetonitrile-d_3 (4 mL). Triethylamine (0.04 mL, 0.05 mmol) was added dropwise, and the reaction was stirred 30 min. The solution was concentrated, and the residue was subjected to flash chromatography using 3:1 ethyl acetate in hexanes. The product was obtained as a white residue (297 mg), which was recrystallized from ethyl acetate and hexanes to yield 57 mg (23%) of the title compound as a white solid: mp 109−110 °C dec; ^{1}H-NMR (C_6D_6, 300 MHz) δ 7.79 (d, J = 8.4 Hz, 2 H, aromatic), 7.02−6.96 (m, 6 H, aromatic), 6.63 (d, J = 8.0 Hz, 2 H, aromatic)

4.41 (br s, 1H, CHPh), 1.75 (s, 3 H, CH_3); ^{13}C-NMR (C_6D_6, 75 MHz): δ 165.17, 145.75, 136.13, 131.97, 129.91, 129.33, 128.89, 128.84, 127.15, 61.88, 21.24, the CD_2 carbon was not observed; IR (KBr) 3060, 3030, 1810, 1595, 1450, 1385, 1190, 1180, 1080, 975, 815, 770, 735, 695 cm^{-1}; HRMS calcd for $C_{16}H_{13}NO_4SD_2$ = 319.0848, found 319.0840; mass spectral analysis indicated >98% d_2 incorporation.

4-Phenyl *N*-(4-Methoxyphenylsulfonyloxy)-2-azetidinone (2). A suspension of 4-phenyl *O*-Cbz-2-azetidinone (200 mg, 6.7 mmol) and 10% Pd/C (10 mg) in methanol (6 mL) was placed under an atmosphere of hydrogen. The mixture was stirred for 1 h and was subsequently filtered over a thin layer of Celite. The filtrate was concentrated *in vacuo* to yield 90 mg of a residue. A portion of the solid (10.9 mg) was added to a solution of 4-methoxybenzenesulfonyl chloride (115 mg, 0.6 mmol) in dry acetonitrile (5 mL) at ice-water temperature. Triethylamine (0.05 mL, 0.2 mmol) was added dropwise, and the reaction mixture was stirred for 30 min. The solution was concentrated, and the residue was subjected to flash chromatography using 1:3 ethyl acetate in hexanes. The purified residue was recrystallized from ethyl acetate in hexanes to yield 15 mg (23%) of the title compound as white solid: mp 109−111 °C dec; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 7.85 (d, *J* = 8.9 Hz, 1 H, aromatic), 7.37−7.35 (m, 3 H, aromatic), 7.29−7.25 (m, 2 H, aromatic), 6.96 (d, *J* = 8.9 Hz, 1 H, aromatic), 7.18 (s, 5 H, aromatic), 4.96 (dd, *J* = 3.3, 6.2 Hz, 1 H, CHPh), 3.89 (s, 3 H, OCH_3), 3.24 (dd, *J* = 6.1, 14.4 Hz, 1 H, CH_2), 2.76 (dd, *J* = 3.1, 14.4 Hz, 1 H, CH_2); ^{13}C-NMR ($CDCl_3$, 300 MHz) δ 165.1, 135.4, 131.4, 129.1, 128.9, 126.9, 114.6, 62.0, 55.7, 42.3; IR (neat) 1800, 1695, 1375, 1265, 1195, 1170, 740, 690 cm^{-1}; HRMS calcd for $C_{16}H_{15}NO_5S$ (MH^+) = 334.0749, found (MH^+) 334.0765.

4-Phenyl *N*-(2-Naphthalenylsulfonyloxy)-2-azetidinone (3). A suspension of 4-phenyl *O*-Cbz-2-azetidinone (115 mg, 0.4 mmol) and 10% Pd/C (11 mg) in methanol (6 mL) was placed under an atmosphere of hydrogen. The mixture was stirred for 30 min and was then filtered over a thin layer of Celite. The filtrate was concentrated to yield 90 mg of a residue which was mixed into a solution of 2-naphthalenesulfonyl chloride (74.4 mg, 0.3 mmol) in dry acetonitrile (5 mL) at ice-water temperature. Triethylamine (0.05 mL, 0.2 mmol) was added dropwise, and the reaction was stirred for 25 min. Subsequently, the solution was concentrated, and the residue was subjected to flash chromatography using 1:3 ethyl acetate in hexanes. The resultant white solid from column chromatography was recrystallized from ethyl acetate in hexanes to yield 36 mg of the title compound as a white solid (25%): mp 110−111 °C dec; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 8.47 (br s, 1 H, aromatic), 7.96−7.89 (m, 3 H, aromatic), 7.84−7.80 (m, 1 H, aromatic), 7.71−7.65 (m, 1 H, aromatic), 7.18 (s, 5 H, aromatic), 4.90 (dd, *J* = 3.3, 6.2 Hz, 1 H, CHPh), 3.24 (dd, *J* = 6.2, 14.5 Hz, 1 H, CH_2), 2.76 (dd, *J* = 3.3, 14.5 Hz, 1 H, CH_2); HRMS calcd for $C_{19}H_{15}NO_4S$ (MH^+) = 354.0800, found (MH^+) 354.0802. Anal. Calcd for $C_{19}H_{15}NO_4S$: C, 64.58; H, 4.28; N, 3.96. Found: C, 64.58; H, 4.36; N, 3.78.

Assay of Enzyme Activity. A 1.0 mL assay mixture typically consisted of 2 mM penicillin G in 100 mM sodium phosphate, pH 7.0. Hydrolysis of the β-lactam of penicillin G ($\Delta\epsilon_{240}$ = 570 M^{-1} cm^{-1}) was monitored at 240 nm upon the addition of enzyme (typically 10^{-8} M).

Kinetic Experiments. Inactivation experiments were performed according to the method of Bush *et al.*[7] An aliquot of the stock solution of any of the inactivators (75−μM final concentration) was added to the TEM-1 β-lactamase (6 μM) in 100 mM sodium phosphate, pH 7.0 at 4 °C. Portions (10 μL) were removed from the mixture at time intervals and were diluted 100-fold into the assay mixture containing 2.0 mM penicillin G. The enzyme activity was monitored until penicillin G had been entirely depleted. The remaining enzyme activity was calculated from the initial linear portion of the hydrolysis curve.

The partition ratios for inactivators were determined by the titration method.[8] Several buffered mixtures containing various molar ratios of $[I]_o/[E]_o$ ranging from 0.2 to 100 in 100 mM sodium phosphate, pH 7.0 were incubated for 15 min at 4 °C. Subsequently, the remaining enzyme activity was determined. The extent of nonspecific inactivation of each enzyme was taken into account by measuring the activity of enzyme incubated under identical set of conditions in the absence of the inactivator.

Rates of hydrolysis of inactivated enzyme species were measured under conditions of excess substrate (2.0-mM penicillin G), as described by Koerber and Fink.[9] A solution of a given inactivator in *p*-dioxane was mixed with the TEM-1 β-lactamase to result in a final concentration of 20 μM and an $[I]_o/[E]_o$ value above the partition ratio for the given inactivator. The mixture was incubated at room temperature for 5 min to give complete inactivation. A 10−μL portion of this mixture was mixed with the solution of penicillin G (2.0 mM) in 100 mM sodium phosphate, pH 7.0. Hydrolysis of penicillin G was monitored at 240 nm ($\Delta\epsilon_{240}$ = 570 M^{-1} cm^{-1}). The computation of the rate constants were performed according to the method of Glick *et al.*[10]

Michaelis−Menten parameters for turnover (K_m and k_{cat}) for compound **4** were evaluated by a Lineweaver−Burk plot. The concentrations of **4** were varied from 10 to 150 μM. A portion of the enzyme was added to a solution of **4** to achieve a final concentration of 60 nM for the enzyme in a total volume of 1.0 mL, and hydrolysis was monitored at 256 nm ($\Delta\epsilon_{256}$ = 8900 M^{-1} cm^{-1}).

The dissociation constant (K_i) for compound **4** for the TEM-1 β-lactamase was calculated by method of Dixon.[11] A series of assay mixtures containing both penicillin G as the substrate (either 50 or 100 μM) and various concentrations of compound **4** as the inhibitor (0.1−10.0 μM) were prepared in 100 mM sodium phosphate, pH 7.0. A portion of the enzyme was added to give a final concentration of 60 nM in a total volume of 1.0 mL. The enzyme activity was determined immediately.

Detection of Tosylate. A solution of β-lactamase (final concentration 11.2 μM) in 100 mM sodium phosphate, pH 7.0 was mixed with a solution of **1** in *p*-dioxane (final concentration 55 μM). The mixture was incubated for 4 h at room temperature, after which the low molecular weight species were separated from the protein by ultrafiltration through an Amicon device, and they were analyzed for the presence of tosylate by HPLC: t_R = 7.7 min (Beckman ODS 4.6 mm × 25 cm, linear gradient of 5−95% acetonitrile in 0.1% TFA over 30 min, 1.0 mL/min). The effluent was monitored at 220 nm on a Rainin Dynamax UV detector, and the area under the peak was integrated by Dynamax MacIntegrator. Known concentrations of tosylate were similarly injected into the HPLC, and the peak areas were integrated for comparison with that of the reaction mixture.

Computational Models. Crystal coordinates for the TEM-1[12] β-lactamase was used in the three-dimentional modeling and energy minimization. The crystallographic water molecules were retained, and the active-site-bound inactivator(s) was then covered by the addition of more water molecules. The hydrogen atoms were added in the calculated position. MOPAC MNDO ESP charges were used for inactivators[13] and Kollman charges[14] for the protein. The energy minimization was performed by the geometry optimization algorithm MAXMIN2, using the Tripos force field by the Sybyl molecular modeling software in a Silicon Graphics R4000 Indigo computer. The Powell method[15] was used to determine the descending direction in the minimization. Minimization was performed in three stages: (1) the inhibitor and water molecules were allowed to move, (2) the inhibitor, water molecules, and the protein backbone were allowed to move, and (3) finally, the entire enzyme-inhibitor complex was allowed to minimize without any constraints. The minimization in a radius of 15 Å from the active site in each stage was continued until the change in energy gradient was less than 0.01 kcal/mol/Å between iterations. A dielectric constant of 1.0 was used for the calculations.

Results and Discussion

The currently known mechanism-based inactivators for β-lactamases all have a leaving group at C_4 (numbering according to the convention for azetidinones) of the lactam ring. We have

(7) Bush, K.; Macalintal, C.; Rasmussen, B. A.; Lee, V. J.; Yang, Y. *Antimicrob. Agents Chemother.* **1993**,*37*, 851.

(8) Silverman, R. In *Mechanism-Based Enzyme Inactivation: Chemistry and Enzymology*; CRC Press: Boca Raton, FL, 1988; p 22.

(9) Koerber, S. C.; Fink, A. L. *Anal. Biochem.* **1987**, *165*, 75.

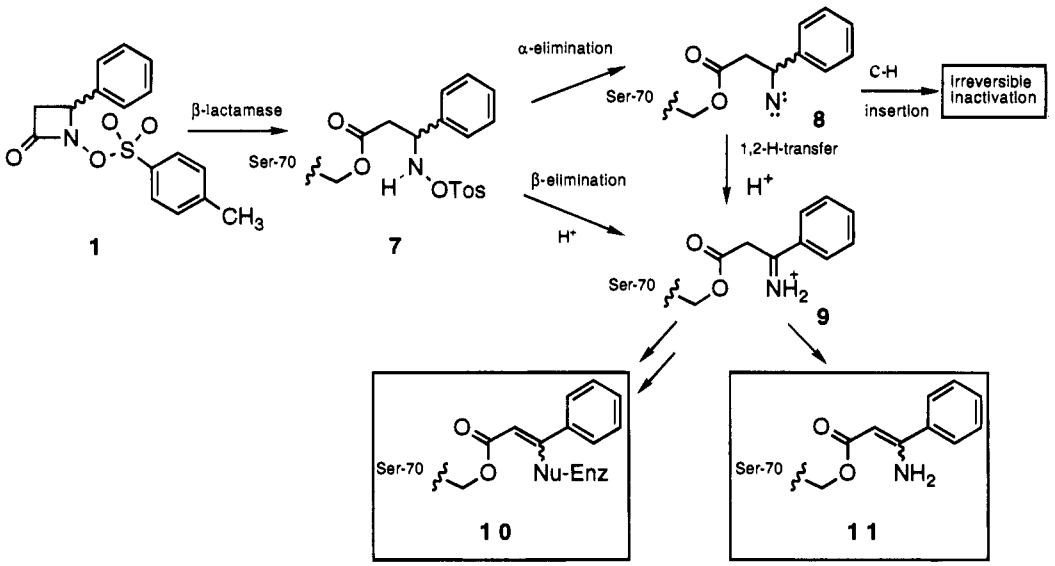
(10) Glick, B. R.; Brubacher, L. J.; Leggett, D. J. *Can. J. Biochem.* **1978**, *56*, 1055.

(11) Dixon, M. *Biochem. J.* **1953**, *55*, 170.

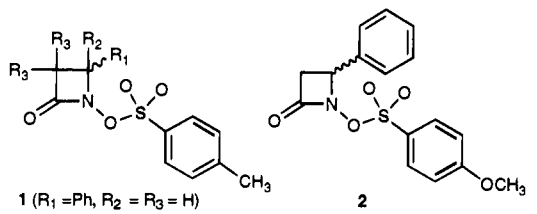
(12) Strynadka, N. C. J.; Adachi, H.; Jensen, E. E.; Johns, K.; Selecki, A.; Betzel, C; Sutoh, K.; James, M. N. G. *Nature* **1992**, *359*, 700. Subsequently, the crystal structure for the TEM-1 enzyme was reported also by the Samama group as well: Jelsch, C.; Mourey, L.; Masson, J. M.; Samama, J. P. *Proteins: Structure, Function and Genetics* **1993**, *16*, 364.

(13) Stewart, J. J. P. QCPE program No. 455.

Scheme 1

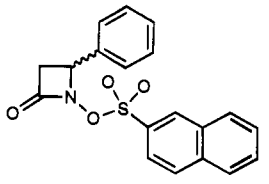


conceived of a new approach for inactivator design, where the leaving group is positioned on the lactam nitrogen. Compounds **1**–**6** are six such molecules. We envisioned that these compounds would acylate the active-site serine of β-lactamases (Ser-70 of class A enzymes), generating the acyl-enzyme intermediate (**7**, Scheme 1). Acylated hydroxamates, similar to our tosylated hydroxamate (**7**), are known to undergo α-elimination with ease to give rise to nitrenes,[16,17] which might insert themselves into C–H bonds in the protein active site.[18] Such a reaction would result in irreversible inactivation of the enzyme. Alternatively, a β-elimination of the tosylate of **7** may give rise to the iminium species **9**. Trapping of a nucleophilic side chain of an active-site amino acid by the iminium moiety of **9**, followed by deprotonation α to the ester and departure of the ammonium ion—as reported for the chemistries of clavulanate[19] and sulbactam[20]—may result in irreversible inactivation (**10**). Alternatively, if a tautomerization takes place before there is a chance for nucleophile capture, a reversible inhibition by a species such as **11** may ensue. Such a tautomerization is precedented for class A β-lactamase inhibition.[19,20] We hasten to add that a 1,2-hydrogen shift may readily convert the nitrene **8** to species **9** as well, a reaction which finds precedent in the literature.[17]



1 (R_1 =Ph, R_2 = R_3 = H)
4 (R_1 = Me, R_2 = R_3 = H)
5 (R_1 =Ph, R_2 = D, R_3 = H)
6 (R_1 =Ph, R_2 = H, R_3 = D)

2



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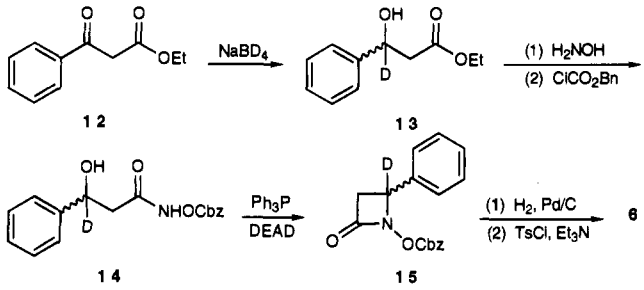
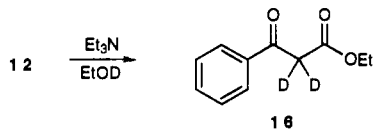
The following features of compounds **1**–**6** are of interest. Molecular modeling of the acyl-enzyme intermediate (*e.g.*, **7**) into the active site of the crystal structure for the TEM-1 β-lactamase revealed that the two non-hydroxamate sulfonate oxygens virtually exist in the same space that the C_3 carboxylate oxygens of a penicillin would occupy after enzyme acylation—hence the sulfonate oxygens serve as surrogates for the carboxylate oxygens. We have previously documented the importance of the carboxylate oxygens in electrostatic interactions with the active site of the TEM-1 β-lactamase, as a prototypic class A enzyme, with both substrates[4,21] and inactivators.[19,20] Figure 1 shows the energy-minimized structure for the acyl-enzyme intermediate of the 4(*S*) enantiomer of **1**. As will be discussed later, the rate-limiting step appears to be the loss of the leaving group (*i.e.*, the sulfonate) from the acyl-enzyme intermediate, so the structure presented in Figure 1 is relevant for the discussion of the interactions in the active site. The sulfonate makes hydrogen bonds to the side chains of amino acid residues 130, 235, and 244 just as do the carboxylates of typical β-lactam molecules (Figure 1A).[4,19,20] Furthermore, this structure reveals that the C_4 phenyl and the aromatic function of the sulfonate make specific interactions with hydrophobic regions of the enzyme (Figure 1B). The 4(*R*) phenyl group of **1** would interact with the hydrophobic side chains of Gly-238 and Ala-237. Alternatively, the 4(*S*) phenyl group in the other enantiomer, as depicted in Figure 1A, would interact with the side chain of Tyr-105 and the aromatic function of the sulfonate in the inhibitor itself. An interesting finding from these analyses is that the aromatic function of the sulfonate fits in a loosely defined hydrophobic pocket made by the side chains of residues Tyr-105 and Val-216 (Figure 1B). The existence of this hydrophobic binding pocket for binding of inhibitors has not been described previously.

(14) Weiner, S. J.; Kollman, P. A.; Case, D. A.; Singh, U. C.; Ghio, C.; Alagona, G.; Profeta, S.; Weiner, P. *J. Am. Chem. Soc.* **1984**, *106*, 765.
(15) Powell, M. J. D. *Mathematical Programming* **1977**, *12*, 241
(16) Lwowski, W. In *Nitrene*; Lwowski, W., Ed.; Interscience, New York, 1970; pp 1–11.
(17) Lewis, F. D.; Saunders, W. H. In *Nitrene*; Lwowski, W., Ed., Interscience: New York, 1970; pp 47–97.
(18) Inhibition of serine and cysteine proteases by *N*-peptidyl-*O*-acyl hydroxylamines has been reported: Demuth, H. U.; Neumann, U.; Barth, A. *J. Enzyme Inhib.* **1989**, *2*, 239. Demuth, H. U.; Stockel, A.; Schierhorn, A.; Fittkau, S.; Kirschke, H.; Bromme, D. *Biochim. Biophys. Acta* **1993**, *202*, 265. Steinmetz, A. C. U.; Demuth, H. U.; Ringe, D. *Biochemistry* **1994**, *33*, 10535; and the references cited therein.
(19) Imtiaz, U.; Billings, E.; Knox, J. R.; Manavathu, E. K., Lerner, S. A.; Mobashery, S. *J. Am. Chem. Soc.* **1993**, *115*, 4435.
(20) Imtiaz, U.; Billings, E. M.; Knox, J. R.; Mobashery, S. *Biochemistry* **1994**, *33*, 5728.
(21) Imtiaz, U.; Manavathu, E. K.; Lerner, S. A.; Mobashery, S. A *Antimicrob. Agents Chemother.* **1993**, *37*, 2438.

Compounds **1**−**4**, and the deuterated analogues **5** and **6**, were each prepared in a few steps. We described here syntheses of the deuterated molecules, but the syntheses of non-deuterated compounds follow the same general scheme. The synthesis of compound **6** started with the incorporation of deuterium at the C_3 position of ethyl benzoylacetate. Reduction of the ketone function in **12** by sodium borodeuteride afforded compound **13**. Reaction of hydroxylamine with **13**, followed by that of the resultant hydroxamate with benzyl chloroformate gave intermediates **14**. Hydroxamate **14** was then subjected to Mitsunobu conditions (PPh_3 and DEAD) to generate the *O*-Cbz β-lactam **15**. Sequential hydrogenolysis and tosylation of **15** furnished the desired compound **6**. Compound **6** had >96% deuterium incorporated.



To prepare compound **5**, first, deuteration of **12** was carried out by equilibration of the compound in the presence of ethanol-*d* and triethylamine to give **16**. According to the ^{1}H-NMR spectrum of **16**, deuterium incorporation was in excess of 96%. Subsequently, **16** was reduced with sodium borohydride, and the remainder of the synthesis was essentially as described for **6**. Compounds **1**−**3** were each prepared by the reaction of the non-deuterated analogue of **15** under the same conditions that produced **6**, except a different sulfonyl chloride was employed to give the corresponding final products.



These compounds inactivated the TEM-1 β-lactamase in a process which was active-site directed, saturable, and involved covalent modification of the active site. Inactivation was extremely rapid, such that dissociation constants for none of the compounds, except for **4** (the relatively poor inactivator), could be measured. Furthermore, the nature of the kinetics was such that we could not use the standard competition experiments with a substrate to slow down the rate of inactivation.[22] Therefore, we have evaluated the rates of inactivation by measuring the second-order rate constant for inactivation by the method of Bush *et al.*[7] To be able to carry out these measurements, we incubated the enzyme in the presence of concentrations of an inactivator which did not generally exceed the given partition ratio. As shown in Table 1, compounds **1**−**6** inactivate the TEM-1 β-lactamase very efficiently, as judged by the low partition ratios (k_{cat}/k_{inact}) as well as fast second-order inactivation rate constants.

Compound **4** is the worst inactivator in the series, indicating that the C_4 phenyl group is important for function, when compared to the results for **1**. Since the enzyme turns **4** over better (k_{cat}/k_{inact} = 70) than the other molecules in the series, we were able to evaluate some kinetic parameters for **4** that could not be measured for the other compounds: $K_m = 25 \pm 1$ μM, $k_{cat} = 0.046 \pm 0.001$ s^{-1}, $k_{inact} = 6.6 \times 10^{-4}$ s^{-1}, $K_i = 1.0 \pm 0.3$ μM. On the other hand, compound **3** is the best inactivator, both in terms of the fastest rate for inactivation and the lowest partition ratio, indicative of the importance of the hydrophobicity at the sulfonate function. This finding is consistent with the proposed fitting of the aromatic group of the sulfonate in the hydrophobic depression created by the side chains of Val-216 and Tyr-105 (Figure 1B). This suggests that the hydrophobic interaction of the larger naphthyl group of **3** is more favorable at this site than that of the smaller phenyl group of **1**. We have also synthesized and studied the 4(*S*) enantiomer of **1**. This compound inactivates the TEM-1 β-lactamase virtually as efficiently as the racemate [$k_{inact}/K_i = (1.47 \pm 0.18) \times 10^4$ M^{-1}s^{-1}, $k_{cat}/k_{inact} = 4 \pm 1$, $k_{H_2O} = (1.7 \pm 0.4) \times 10^{-3}$ s^{-1}], indicating that both enantiomers are active molecules. Our modeling indicated that the C_4 phenyl group in both the 4(*S*) and 4(*R*) enantiomers of **1** would fit in the enzyme active site and each would make favorable interactions with the protein (see Figure 1 for the picture for the 4(*S*) enantiomer).

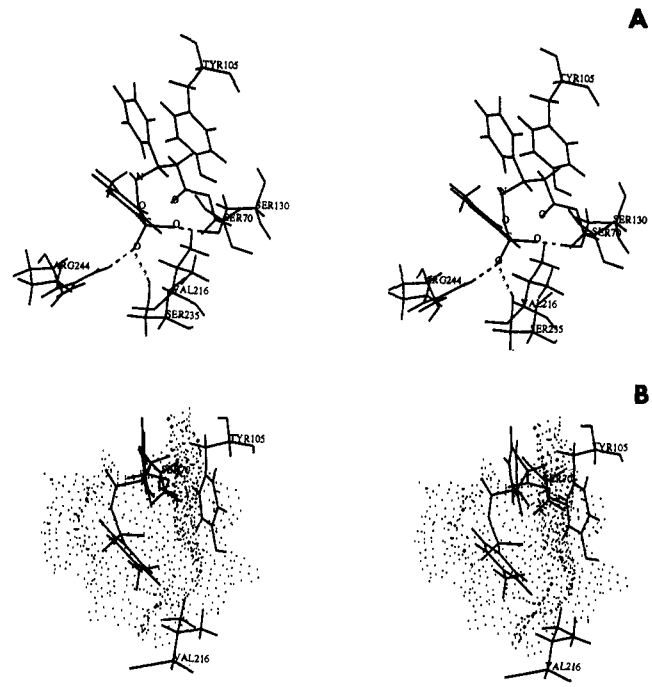


Figure 1. (A) Energy-minimized structure for the acyl-enzyme intermediate of the 4(*S*) enantiomer of **1** in the active site of the TEM-1 β-lactamase showing the interactions with several active-site residues; hydrogen atoms are shown and hydrogen bonds to the side chains of amino-acid residues 130, 235, and 244 are indicated as broken lines. (B) Approximately the same perspective as in stereoview A, showing the Connolly solvent-accessible surface of the active site and the proposed hydrophobic binding pocket created by the side chain functions of amino-acid residues Tyr-105 and Val-216.

Subsequent to inactivation, the entire activity of the enzyme recovered after 3 h. This indicates that nitrene insertion into a C−H bond in the protein is not taking place but does not rule out that species **8** may exist. Also, it refutes the possibility for the formation of **10** (Scheme 1). Species **11** is the most plausible possibility for the inactivated species. The enzyme upon inactivation exhibits a chromophore at 276 nm (ϵ 4085 M^{-1} cm^{-1}, 100 mM sodium phosphate, pH 7.0), which is consistent with species **11** (Figure 2). It is noteworthy that *trans*-cinnamic acid possesses a chromophore at 274 nm (ϵ 1920 M^{-1} cm^{-1}, methanol). Furthermore, α,β-unsaturated species similar to **11** have been reported as the socalled transiently inhibited species for β-lactamases inhibited by both sulbac-

(22) Daniels, S. B.; Cooney, E.; Sofia, M. J.; Chakravarty, P. K.; Katzenellenbogen, J. A. *J. Biol. Chem.* **1983**, *258*, 15046.

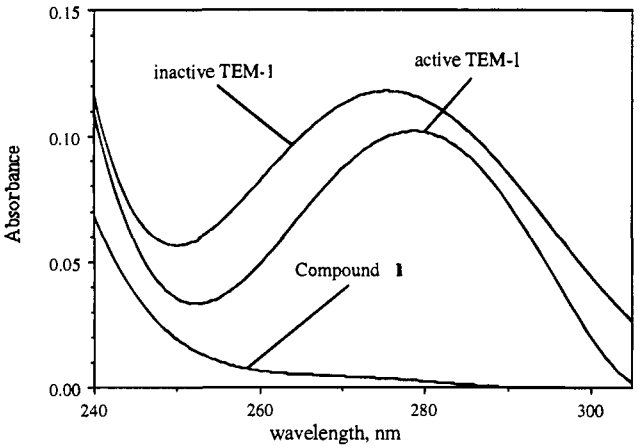


Figure 2. The UV profile of 4.4 μM of each of compound **1**, the TEM-1 β-lactamase, and the TEM-1 β-lactamase inactivated by compound **1** in the region between 240−310 nm.

Table 1. Kinetic Parameters for Interactions of Compounds **1-6** with the TEM-1 β-Lactamase[a]

	k_{inact}/K_i (M^{-1} s^{-1}) × 10^{-3}	k_{cat}/k_{inact}	k_{H_2O} (s^{-1}) × 10^3
1	11.2 ± 0.8	5 ± 0.5	2.1 ± 0.3
2	7.4 ± 0.2	7 ± 1	2.0 ± 0.3
3	72.5 ± 1.4	2 ± 0.3	1.8 ± 0.2
4	0.6[b]	70 ± 10	>10[b]
5	15.2 ± 1.5		2.1 ± 0.2
6	11.2 ± 1.1		2.3 ± 0.4

[a] The experiments were carried out in 100 mM sodium phosphate, pH 7.0 at 3 °C. [b] These values are estimates.

tam[20,23] and clavulanate.[19,23] We measured the rate of hydrolysis of species **11**. The rates of hydrolysis (k_{H_2O}, Table 1) of all compounds, except **4**, are essentially identical. This is to be expected as species **11** is shared by all. The inhibited species for compound **4** underwent hydrolysis substantially faster (Table 1).

Compounds **5** and **6** were prepared to investigate whether reactions **7** → **9**, **8** → **9**, or **9** → **11** were rate-limiting. No deuterium-isotope effect was seen in interactions of these two compounds with the TEM-1 β-lactamase. This is consistent with the model systems for the α-elimination route, which show that the rate-limiting step is the departure of the leaving group (e.g., **7** → **8**) in known cases.[17] Once the β-lactam moiety is cleaved, by either enzyme acylation or hydrolysis, the loss of the sulfonate leaving group is expected to be a rapid process. The presence of free tosylate was detected in the medium after incubation of **1** with the TEM-1 β-lactamase by HPLC, consistent with the expectation from the mechanism offered in Scheme 1. This experiment was carried out under conditions at which the concentration of inactivator **1** was kept at five-fold (*i.e.*, the partition ratio) above that of the enzyme. The concentration of the free tosylate detected in solution after incubation in the presence of the enzyme came to within 86% of the predicted value from the concentration of inactivator. This experiments shows the validity of our expectation for the loss of the leaving group in the course of both turnover and inactivation chemistries for these compounds.

In conclusion, we have demonstrated that compounds **1**−**6** are very efficient inactivators of the TEM-1 β-lactamase, a representative class A enzyme. The efficiency of inactivation stems from both low partition ratios as well as very rapid rates for inactivation. To put this in perspective, k_{inact}/K_i values for inhibition of the TEM-1 β-lactamases by these compounds were in the range of 1−7 × 10^4 M^{-1} s^{-1}, which are as fast as the corresponding values for inactivation of the TEM-1 enzyme by clavulanate (5.7 × 10^4 M^{-1} s^{-1})[24] and faster than the case of sulbactam (125 M^{-1} s^{-1}),[20] and tazobactam (2.8 × 10^3 M^{-1} s^{-1}).[7] The partition ratios for inactivation of the TEM-1 β-lactamase are the lowest for the best of the compounds reported herein (*i.e.*, 2−7), compared to the clinically used clavulanate, sulbactam, and tazobactam, which are 160,[19] 10 000,[20] and 125,[7] respectively.

Acknowledgment. This work was supported by the National Institutes of Health Grant AI 33170 (to S.M.) and Eli Lilly and Co. (to M.J.M.). The use of the Lizzadro Magnetic Resonance Center for the NMR studies at Notre Dame is gratefully acknowledged. TAG was an NSF-REU program participant (summer 1994). We are indebted to Professor Michael James for providing us with the crystal coordinates for the TEM-1 β-lactamases.

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(23) Knowles, J. R. *Acc. Chem. Res.* **1985**, *18*, 97 and the references cited therein.
(24) Charnas, R. L.; Knowles, J. R. *Biochemistry* **1981**, *20*, 3214.