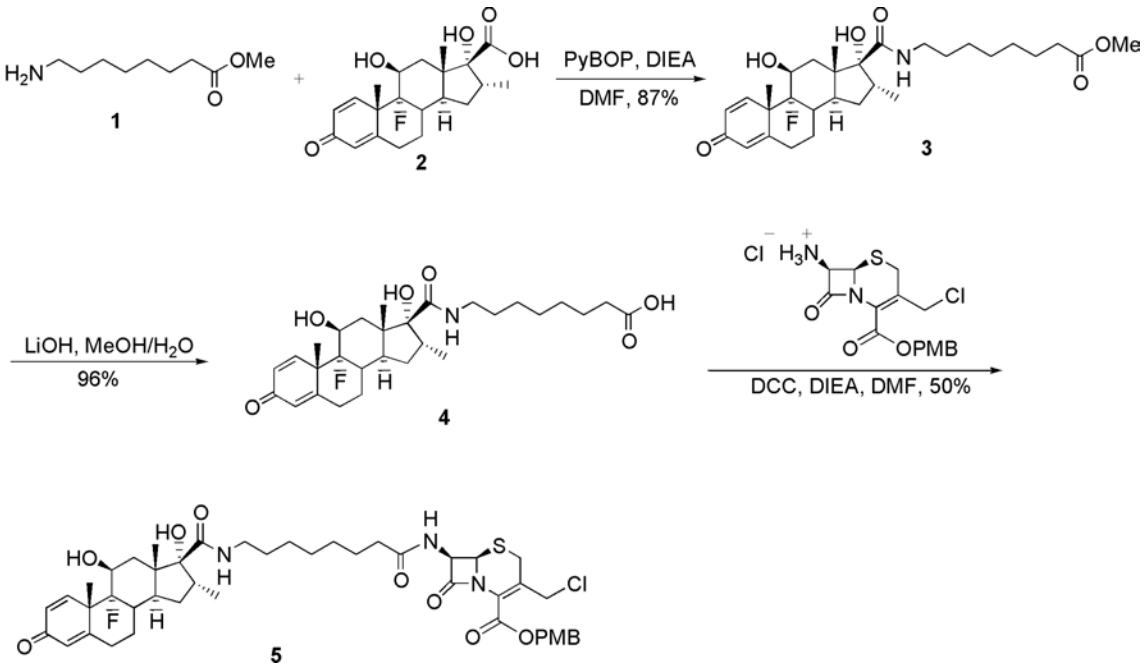
Supporting information for Baker *et al.* (2002) *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.262420099

Supporting Text

General Methods for Chemical Synthesis. Unless otherwise noted, reagents were purchased from Aldrich and used without further purification: *N*,*N*-dimethylacetamide (DMA), *N*,*N*-dimethylformamide (DMF), diisopropylethylamine (DIEA), and trifluoroacetic acid (TFA) were from Sure Seal bottles. 7-Amino-3-chloromethyl-3-cephem-4-carboxylic acid *p*-methoxybenzyl ester hydrochloride (ACLE) was graciously donated by the Otsuka Chemical Co. (Osaka). Benzotriazole-1-yl-oxytrispyrrolidinophosphonium hexafluorophosphate (PyBOP) was purchased from Advanced ChemTech. All moisture- or oxygen-sensitive reactions were performed under a positive pressure of nitrogen as noted. All column purifications were done by using flash chromatography on silica gel from Sorbent Technologies (standard grade) or Whatman. Analytical TLC was conducted by using silica gel precoated plates from EM Science (Silica Gel 60 F_{254}) and visualization by UV light (254 nm) or ninhydrin stain (as noted). Organic solvents were removed *in vacuo* by using a rotary evaporator equipped with a vacuum pump [approximately 1 torr (133 Pa)]. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker 300-MHz or 400-MHz Fourier-transform NMR spectrometer in the Chemistry Department of Columbia University. ^{1}H NMR spectra are tabulated in the following order: chemical shift calculated with reference to solvent standards based on tetramethylsilane, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet, m, multiplet; br, broad), coupling constant(s) in Hz, and number of protons. Infrared (IR) spectra were recorded on a Perkin Elmer 1600 series Fourier-transform IR spectrometer in a potassium bromide pellet. Fast atom bombardment (FAB) high-resolution mass spectra (HRMS) were recorded on a JMS-HX110A mass spectrometer; low-resolution electron spray ionization (ESI) mass spectra were recorded on a JMS-LC mate mass spectrometer.



Scheme 2.

Synthesis of 1. 7-Aminoheptanoic acid (0.20 g, 1.26 mmol) was dissolved in anhydrous methanol (4 ml) and stirred in a dry ice/CCl_4 bath. Thionyl chloride (0.184 ml, 2.52 mmol, 2.0 eq) was slowly added, and the resulting solution was stirred approximately 5 min more in the ice bath, and then at room temperature for the remaining reaction time. The reaction was complete in approximately 30 min, with completion monitored by TLC (5:1 CH_2Cl_2/MeOH) and ninhydrin stain. The solvent was removed *in vacuo*, leaving a white solid. MeOH (ACS grade, 5 ml) was used to wash the solid (three times), evaporated off after each wash, leaving the desired compound as a white solid (quantitative yield). R_f = 0.4 in 5:1 CH_2Cl_2/MeOH; ^{1}H NMR (400-MHz, CD_3OD) δ 3.67 (s, 3), 2.94 (t, J = 7.5 Hz, 2), 2.36 (t, J = 7.4 Hz, 2), 1.66 (m, 4), 1.40 (m, 6). MS m/z 173.8 (M^+).

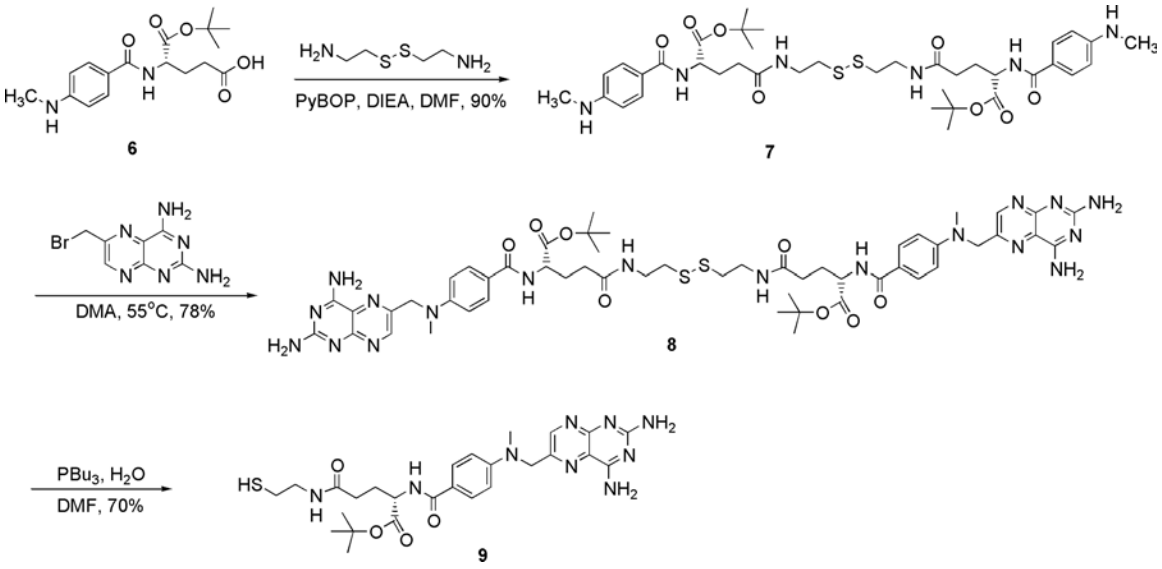
Synthesis of 2. Compound **2** was synthesized as reported (1) to give **2** as a white solid in 93% yield.

Synthesis of 3. Compounds **1** (188 mg, 0.5 mmol) and **2** (86 mg, 0.5 mmol) were combined with PyBOP (330 mg, 63 mmol, 1.1 eq). Under a N_2 atmosphere, DMF (2.0 ml) and DIEA (1.5 ml, 8.6 mmol, 5.0 eq) were added, and the reaction was stirred at room temperature overnight. After reaction completion the DMF was removed *in vacuo* and the residue was dissolved in CH_2Cl_2 (50 ml), washed with 1:1 brine/0.1 M $NaHSO_4$ (50 ml, three times), dried with anhydrous Na_2SO_4, and purified by silica gel column chromatography (50:1 CH_2Cl_2/MeOH). The product was obtained in 87% yield, R_f = 0.44 in 10:1 CH_2Cl_2/MeOH; ^{1}H NMR (400-MHz, CD_3OD), δ 7.56 (t, J = 5.8 Hz, 1), 7.44 (d, J = 10.0 Hz, 1), 6.30 (dd, J = 1.9, 10.0 Hz, 1), 6.10 (s,1), 4.26 (dd, J = 1.9, 11 Hz, 1), 3.67 (s, 3), 3.28 (m, 1), 3.15 (m, 2), 2.74 (m, 1), 2.49 (m, 1), 2.41 (m, 1), 2.35 (t, J = 7.4 Hz, 2), 2.22 (m, 2), 1.91 (m, 1), 1.78 (q, J = 12.2 Hz, 1), 1.7-1.3 (m, 15), 1.22 (m, 1), 1.11 (s, 3), 0.91 (d, J = 7.2, 3); ^{13}C NMR (100-MHz, CD_3OD), δ 187.77, 174.80, 174.38, 169.94, 154.88, 128.67, 124.02, 102.52, 100.78, 87.22, 72.34, 71.97, 51.15, 49.63, 49.41, 44.04, 39.69, 36.14, 35.53, 35.10, 34.90, 34.00, 32.64, 31.50, 30.01, 29.32, 29.26, 28.07, 27.12, 25.19, 22.86, 17.04, 14.44; MS m/z 534.0 (MH^+); HRMS, m/z 534.3211 (MH^+), calculated 534.3231; IR 3404 (br), 2930, 2860, 2521, 1732, 1656, 1622, 1523, 1442, 1372, 1296, 1244, 1200, 1174, 1128, 1104, 1070, 1035, 1011, 982, 924, 889, 820.

Synthesis of 4. Compound **3** (1,529 mg, 2.869 mmol) was dissolved in MeOH (10 ml). LiOH (241 mg, 5.737 mmol) aqueous solution (2 ml) was added to the solution and the reaction was stirred at room temperature for approximately 1 h. Then H_2O (10 ml) was added to the reaction, and stirring was continued for another hour. MeOH was then removed *in vacuo*, the residue was take up in 1 M NaOH (50 ml) and brine (30 ml) and washed with CH_2Cl_2 (50 ml, three times). The aqueous solution was acidified to pH = 1 with 6 M HCl and extracted with 3:1 CH_2Cl_2/EtOAc (50 ml, five times). The organic phases were combined and dried with anhydrous Na_2SO_4. The solvent was removed under reduced pressure to yield **4** (1,432 mg) in 96% yield. R_f = 0.35 in 10:1 CH_2Cl_2/MeOH; ^{1}H NMR (400-MHz, CD_3OD); δ 7.55 (t, J = 5.8 Hz, 1), 7.44 (d, J = 10.2 Hz, 1), 6.30 (dd, J = 1.8, 10.1 Hz, 1), 6.10 (s, 1), 4.26 (d, J = 9.2 Hz, 1), 3.29 (m, 1), 3.15 (m, 2), 2.74 (dt, J = 6.0, 13.6 Hz, 1), 2.60-2.37 (m, 2), 2.31 (t, J = 7.4 Hz, 2), 2.22 (m, 2), 1.90 (m, 1), 1.78 (q, J = 12.2 Hz, 1), 1.7-1.3 (m, 15), 1.22 (m, 1), 1.12 (s, 3), 0.91 (d, J =

7.2 Hz, 3); ^{13}C NMR (100-MHz, CD_3OD), δ 187.77, 176.47, 174.32, 169.96, 154.93, 128.71, 124.07, 102.55, 100.81, 87.24, 72.35, 71.97, 49.65, 49.42, 44.07, 39.75, 36.15, 35.54, 35.10, 34.91, 34.17, 32.68, 31.54, 30.04, 29.39, 28.09, 27.17, 25.27, 22.98, 17.10, 14.51; MS *m*/*z*, 520.4 (MH^+); HRMS, *m*/*z* 520.3093 (MH^+), calculated 520.3074; IR 3417 (br), 2932, 2862, 2345, 1710, 1662, 1622, 1529, 1454, 1396, 1373, 1354, 1298, 1240, 1194, 1142, 1125, 1101, 1072, 1032, 1015, 986, 950, 928, 890, 819.

Synthesis of 5. Compound **4** (22 mg, 0.0424 mmol), ACLE (17.1 mg, 0.0424 mmol), and dicyclohexylcarbodiimide (10 mg, 0.047 mmol) were combined and dissolved in DMF (0.1 ml). DIEA (5.5 mg, 0.0424 mmol) were then added to the solution, and the reaction was stirred at room temperature for 4 h. The reaction was diluted with CH_2Cl_2 (20 ml), washed with 1:1 brine/1 M $NaHSO_4$ (20 ml, two times). The organic phase was dried with anhydrous Na_2SO_4 and purified by silica gel column chromatography with 60:1 to 30:1 CH_2Cl_2/MeOH. A slightly yellow solid was obtained in 39% yield. R_f = 0.55 in 10:1 CH_2Cl_2/MeOH; ^{1}H NMR (400-MHz, CD_3OD); δ 7.44 (d, *J* = 10.1 Hz, 1), 7.35 (d, *J* = 8.8 Hz, 2), 6.93 (d, *J* = 8.8 Hz, 2), 6.30 (dd, *J* = 1.8, 10.1 Hz, 1), 6.10 (s, 1), 5.76 (d, *J* = 4.9 Hz, 1), 5.27 (d, *J* = 11.8 Hz, 1), 5.20 (d, *J* = 11.8 Hz, 1), 5.11 (d, *J* = 4.9 Hz, 1), 4.54 (d, *J* = 11.5, Hz 1), 4.49 (d, *J* = 11.5 Hz, 1), 4.26 (d, *J* = 11.0 Hz, 1), 3.81 (s, 3), 3.75 (d, *J* = 18.1 Hz, 1), 3.60 (d, *J* = 18.1 Hz, 1), , 3.29 (m, 1), 3.14 (m, 2), 2.75 (dt, *J* = 6.0, 13.6 Hz, 1), 2.6-2.1 (m, 6), 2.0-1.2 (m, 18), 1.1 (s, 3), 0.91 (d, *J* = 7.2 Hz, 3). ^{13}C NMR (100-MHz, CD_3OD), δ 187.77, 175.58, 174.23, 169.95, 165.13, 161.60, 160.27, 154.91, 130.54, 128.70, 127.35, 126.99, 125.75, 124.04, 113.90, 102.57, 100.83, 87.22, 72.33, 71.96, 68.11, 59.81, 58.42, 54.91, 49.63, 49.41, 44.06, 43.31, 30.58, 36.16, 35.60, 35.53, 35.09, 34.90, 32.65, 31.51, 29.98, 29.27, 28.08, 27.10, 25.98, 22.95, 17.10, 14.49; MS *m*/*z* 534.4 (MH^+); HRMS, *m*/*z* 534.3211 (MH^+), calculated 534.3231; IR 3416 (br), 3044, 2930, 2860, 1785, 1726, 1360, 1627, 1517, 1450, 1389, 1361, 1300, 1244, 1170, 1099, 1065, 1030, 1020, 982, 950, 928, 889, 820.



Scheme 3.

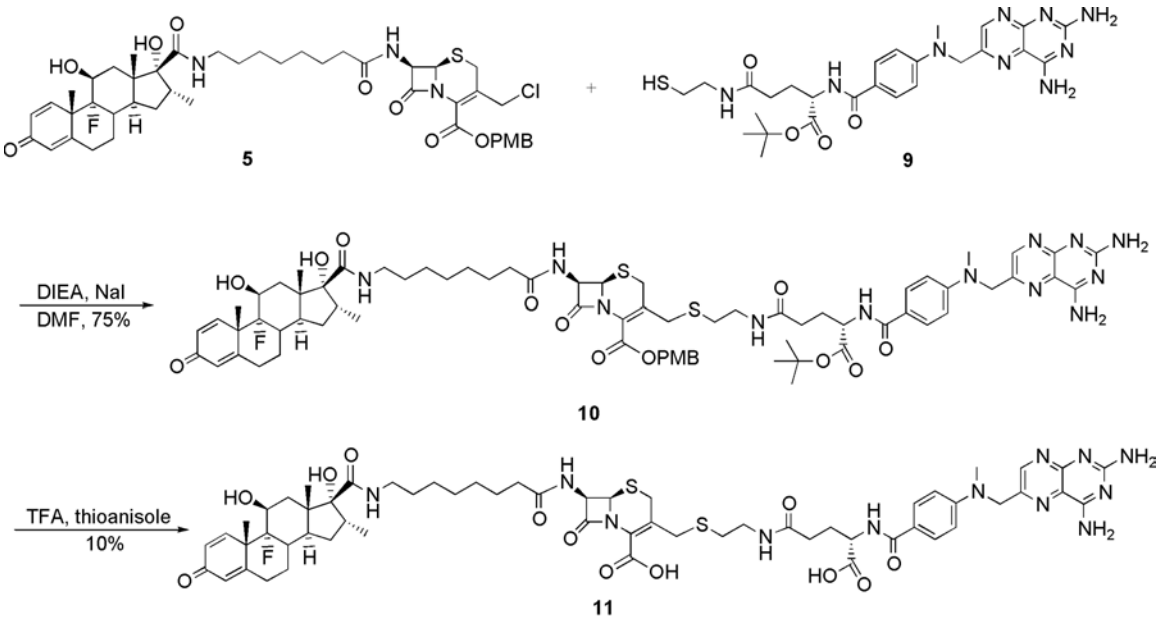
Synthesis of 6. Compound **6** was synthesized as reported (2) to give a white solid in 57% yield.

Synthesis of 7. Compound **6** (340 mg, 1.0 mmol), cystamine dihydrochloride (113 mg, 0.50 mmol), and PyBOP (720 mg, 1.38 mmol) were dissolved in DMF (3.0 ml). DIEA (520 mg, 4.0 mmol) was added and the reaction was stirred at room temperature overnight. DMF was removed *in vacuo* and the crude product was purified by silica gel column chromatography with 30:1 CH_2Cl_2/MeOH in 93% yield. R_f = 0.40 in 30:1 CH_2Cl_2/MeOH; ^{1}H NMR (400-MHz, CD_3OD) δ 7.72 (d, J = 8.8 Hz, 4), 6.60 (d, J = 8.8 Hz, 4), 4.47 (dd, J = 4.5 Hz, 2), 3.46 (t, J = 6.2 Hz, 4), 2.81 (s, 6), 2.77 (t, J = 6.7 Hz, 4), 2.38 (t, J = 7.2 Hz, 4), 2.22 (m, 2), 2.07 (m, 2), 1.49 (s, 18); ^{13}C NMR (100-MHz, CD_3OD), δ 174.00, 171.79, 169.10, 153.55, 129.15, 120.48, 110.99, 81.89, 53.99, 39.09, 37.73, 32.79, 29.38, 27.65, 27.50; MS, *m*/*z* 789.5 (MH^+); HRMS, *m*/*z* 789.3649 (MH^+), calculated 789.3679; IR 3353, 2972, 2933, 2869, 2467, 2360, 1734, 1640, 1606, 1575, 1535, 1423, 1367, 1350, 1289, 1246, 1211, 1156, 1117, 1084, 1012, 916, 839, 767, 697, 583.

Synthesis of 8. Compound **7** (394 mg, 0.50 mmol) and the hydrobromide salt of 2,4-diamino-6-bromomethylpteridine (435 g, 1.1 mmol) were dissolved in DMA (1.25 ml)

and stirred in a 55°C oil bath overnight. The reaction was diluted with CH_2Cl_2 and the product was purified by silica gel column chromatography with 10:1 and 5:1 CH_2Cl_2/MeOH in 78% yield. ^{1}H NMR (400-MHz, CD_3OD) δ 8.65 (s, 2), 7.76 (d, J = 9.0 Hz, 4), 6.85 (d, J = 9.0 Hz, 4), 4.93 (s, 4), 4.45 (dd, J = 4.5 Hz, 2), 3.41 (t, J = 6.6 Hz, 4), 3.29 (s, 6), 2.72 (t, J = 6.7 Hz, 4), 2.38 (t, J = 6.7 Hz, 4), 2.21 (m, 2), 2.10 (m, 2), 1.47 (s, 18); ^{13}C NMR (100-MHz, CD_3OD) δ 176.1, 173.8, 171.0, 165.5, 165.1, 156.6, 154.0, 151.1, 150.0, 131.2, 124.3, 123.1, 113.5, 83.8, 57.5, 55.6, 40.7, 40.6, 39.3, 34.2, 29.2, 28.9.

Synthesis of 9. The disulfide compound **8** (440 mg, 0.387 mmol) was dissolved in DMF (1.9 ml) and water (0.2 ml). After being purged with N_2, tributylphosphine (0.107 ml, 0.426 mmol) was added, and the reaction was stirred at room temperature overnight. The product was purified by silica gel column chromatography with 20:1 and 10:1 CH_2Cl_2/MeOH in 75% yield. ^{1}H NMR (400-MHz, CD_3OD) δ 8.61 (s, 1), 7.78 (d, J = 9.8 Hz, 2), 6.89 (d, J = 9.7 Hz, 2), 4.89 (s, 2), 4.47 (m, 1), 3.31 (m, 2), 3.28 (s, 3), 2.55 (t, J = 6.9 Hz, 2), 2.38 (m, 2), 2.23 (m, 1), 2.09 (m, 1), 1.49 (s, 9); ^{13}C NMR (100-MHz, CD_3OD) δ 174.19, 171.55, 168.76, 163.19, 161.07, 151.91, 149.21, 148.79, 129.38, 122.51, 121.01, 111.73, 82.45, 55.91, 53.80, 43.23, 39.45, 32.67, 27.93, 23.93; MS, m/z 570.3 (MH^+); HRMS, m/z 570.2590 (MH^+) calculated 570.2611; IR 3335 (br), 2974, 2928, 2350, 1726, 1635, 1606, 1554, 1511, 1450, 1365, 1135, 1344, 1205, 1151, 1104, 919, 831, 762.



Scheme 4.

Synthesis of 10. Compound **5** (78.5 mg, 0.10 mmol), compound **9** (57 mg, 0.10 mmol), and sodium iodide (75 mg, 0.5 mmol) were added to a 2-ml round-bottom flask. The flask was then purged with N_2. DMF (0.40 ml) and DIEA (0.019 ml, 0.11 mmol) were then added, and the reaction was stirred at room temperature for 3 h. The reaction mixture was diluted with CH_2Cl_2 (10 ml) and loaded onto a silica gel column. The column was washed with CH_2Cl_2, then 30:1 CH_2Cl_2/MeOH, and the product was eluted with 14:1 CH_2Cl_2/MeOH in 75% yield. ^{1}H NMR (400-MHz, 4:1 $CD_3OD/CDCl_3$) δ 8.56 (s, 1), 7.76 (d, J = 8.9 Hz, 2), 7.41 (d, J = 10.1 Hz, 1), 7.31 (d, J = 8.6 Hz, 2), 6.84 (m, 4), 6.29 (dd, J = 1.8, 10.1 Hz, 1), 6.08 (s, 1), 5.66 (d, J = 4.6 Hz, 1), 5.16 (m, 2), 5.05 (d, J = 5 Hz, 1), 4.48 (m, 1), 4.25 (d, J = 9.3 Hz, 1), 3.79 (m, 4), 3.60 (q, J = 17.9 Hz, 2), 3.35 (d, J = 15.3 Hz, 2), 3.27 (m, 3), 3.22 (s, 3), 3.12 (m, 2), 2.69 (m, 1), 2.54 (t, J = 6.8 Hz, 2), 2.5-1.0 (m, 10), 1.86 (m, 1), 1.75 (q, J = 11.8 Hz, 1), 1.69-1.17 (m, 25), 1.10 (s, 3), 0.90 (d, J = 7.2 Hz, 3); ^{13}C NMR (75 MHz, 4:1 $CD_3OD/CDCl_3$) δ 188.14, 175.79, 174.38, 174.05, 171.95, 170.00, 168.94, 165.46, 163.70, 163.07, 162.33, 160.32, 155.10, 154.86, 152.10, 149.36, 147.91, 131.51, 130.76, 129.30, 128.96, 127.41, 124.42, 124.31, 122.47, 121.45, 114.06, 111.77, 102.86, 100.55, 87.24, 82.16, 72.28, 71.79, 67.95, 59.58, 58.67, 55.79, 55.04, 53.85, 49.50, 43.90, 39.64, 39.03, 35.98, 35.59, 35.35, 34.91, 34.66, 33.10,

32.70, 32.60, 31.42, 30.59, 29.74, 29.03, 27.84, 27.64, 27.33, 26.88, 25.73, 22.89, 22.81, 17.01, 14.41; MS, *m/z* 1403.9 (MH^+), 1425.9 (MNa^+); IR 3383 (br), 3040, 2932, 2862, 2515, 2365, 1780, 1727, 1663, 1627, 1610, 1560, 1540, 1515, 1445, 1368, 1300, 1246, 1156, 1102, 1031, 925, 890, 825, 766, 731.

Synthesis of 11. To compound **10** (35 g, 0.025 mmol) was added thioanisole (31 µl, 0.25 mmol) followed by TFA (0.15 ml). The reaction was stirred at room temperature for 15 min. Then the reaction mixture was concentrated *in vacuo* and the residue was dissolved in 2:1 DMF/H_2O and purified by reversed-phase HPLC on a Whatman Partisil 10 ODS-2 column with an initial 100% aqueous mobile phase for 5 min, followed by a CH_3CN gradient (0% to 20% over 10 min, 20% to 60% over 86 min, 60% to 100% over 20 min) to produce **11** and its Δ^2 isomer in 10.0% yield. MS *m*/*z* 1227.50 (MH^+).

Small Molecule Concentration Calibration. The small molecules were dissolved in DMF to final concentrations of 1-10 mM. The exact concentrations were then determined by Beer's law, using an extinction coefficient at 383 nm of $\varepsilon = 6700\ cm^{-1} \bullet M^{-1}$ at 20°C in DMF [determined from a solution of methotrexate (Mtx) of known concentration in DMF]. Small molecule solutions were stored at –80°C and allowed to come to room temperature before use.

General Methods for Molecular Biology. Standard protocols for molecular biology and yeast genetics were used (3, 4). Restriction enzymes, Vent DNA polymerase, T4 DNA polymerase, calf intestinal alkaline phosphatase, and T4 DNA ligase were purchased from New England Biolabs; *Pfu* Turbo polymerase, from Stratagene. The dNTPs used in PCR were purchased from Pharmacia Biotech. XL1-Blue cells were purchased from Stratagene. BL21(DE3) cells and the plasmid pET26b were purchased from Novagen. Nitrocefin was purchased from Becton Dickinson (Cockeysville, MD). The transformation of *Escherichia coli* was carried out by electroporation using a Bio-Rad *E. coli* pulser. Oligonucleotides were purchased from the Great American Gene Company (Ramona, CA). The phrog used to transfer yeast cells was purchased from Dan-Kar (Wilmington, MA). 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) was

purchased from Diagnostic Chemicals (Oxford, CT). Methotrexate was from the National Cancer Institute (NCI; Bethesda, MD). All other chemicals were purchased from Aldrich or Sigma. All solutions were made with distilled water prepared from a Milli Q water purification system (Millipore). For PCR, an MJ Research PTC-200 Peltier thermal cycler was employed. UV/Vis measurements were taken with a Perkin Elmer UV/Vis Lambda 19 spectrophotometer. Sequencing was performed by GeneWiz (New York). J. Frere (Université de Liège, Liège, Belgium) kindly provided the gene encoding the P99 cephalosporinase; H. Madhani (University of California, San Francisco), the vector p425MET25; and M. Carlson (Columbia University, New York), the strain FY251.

Plasmid Construction. The constitutive alcohol dehydrogenase (ADH) promoter in pMW3eDHFR was replaced with the inducible GAL1 promoter from the plasmid pMW102. The ADH promoter was cut out of pMW3eDHFR by restriction digestion with *Aat*II and partial restriction digestion with *Hin*dIII to obtain a 6,931-bp fragment cut at the *Aat*II site upstream of the ADH promoter and at the *Hin*dIII site just downstream of the ADH promoter. The GAL1 promoter was obtained from pMW102 by restriction digestion with *Aat*II and *Hin*dIII to obtain a 3,597-bp piece from the *Aat*II site upstream of the GAL1 promoter and the *Hin*dIII site just downstream of the GAL1 promoter. The two DNA fragments were ligated together to give pKB521 (2μ, *HIS3*, kan^R). The amp^R marker in p425MET25 was replaced with a $spec^R$ marker from pJL74 by a blunt-end ligation. Plasmid p425MET25 was digested with *Sca*I and *Bsa*I, and the 1.1-kb $spec^R$ fragment from pJL74 was obtained by digestion of the plasmid with *Bam*HI and *Pst*I. Both the vector and insert were then treated with T4 DNA polymerase (NEB, according to the supplier's protocol), and the vector subsequently was treated with calf intestinal alkaline phosphatase (NEB, supplier's protocol). The vector and insert were ligated to give the shuttle vector pVC167 (2μ, *LEU2*, $spec^R$) upon selection for growth on spectinomycin (50 μg/ml) and not ampicillin. The P99 cephalosporinase gene contained three point mutations $Ile^{16} \rightarrow$ Val, $Ala^{88} \rightarrow$ Pro, and $Ala^{299} \rightarrow$ Val, and two silent mutations Thr^{42} ACA → ACG and Ala^{292} GCA → GCG and was amplified by PCR from the plasmid pNU602. For subcloning into the bacterial expression vector pET26b (kan^R), a 1,107-bp fragment encoding the P99 cephalosporinase was generated by PCR by using

the primers 5'-GCA TAC GTC CAT ATG ACG CCA GTG TCA GAA AAA (*Nde*I site underlined, coding strand, VWC167) and 5'-GCA TTG CTG AAG CTT AGT GGT GGT GGT GGT GGT GCT GTA GCG CCT CGA GG (*Hin*dIII, noncoding strand, His_6-tag, VWC69). This PCR fragment was inserted between the *Nde*I and *Hin*dIII sites in pEt26b to generate the plasmid pSG430 (kan^R). For subcloning into the shuttle vector pVC167, a 1,107-bp fragment encoding the P99 cephalosporinase was generated by PCR using the primers 5'-GCA TAC GTC CTG CAG ATG ACG CCA GTG TCA GAA AAA C (*Pst*I, coding strand, VWC28ampC) and 5'-GCA TTG CTG AAG CTT TTA ATG ATG ATG ATG ATG ATG CTG TAG CGC CTC GAG (*Hin*dIII, noncoding strand, His_6-tag, VWC31ampC). This PCR fragment was inserted between the *Pst*I and *Hin*dIII sites in pVC167 to generate the plasmid pVC172 ($spec^R$). The $Ser^{64} \rightarrow$ Ala variant of P99 cephalosporinase was generated by QuikChange site-directed mutagenesis according to the manufacturer's protocol (Stratagene). For mutation of plasmid pVC172, the primers 5'-CTG TTC GAG CTG GGT GCT ATA AGT AAA ACC TTC (coding strand, VWC554) and 5'-GAA GGT TTT ACT TAT AGC ACC CAG CTC GAA CAG (noncoding strand, VWC555) were used to generate plasmid pCB827. For mutation of pSG430, the primers 5'-CAG ACC CTG TTC GAG CTG GGT GCT ATA AGT AAA ACC TTC ACC (coding strand, VWC204) and 5'-GGT GAA GGT TTT ACT TAT AGC ACC CAG CTC GAA CAG GGT CTG (noncoding strand, VWC205) were used to generate plasmid pDS984. All genes amplified by PCR were confirmed to be correct by sequencing.

Enzyme Purification. P99 cephalosporinase engineered to have a C-terminal His_6 tag was purified from strain BL21(DE3) carrying plasmid pSG430. A cell pellet from a 5-ml culture known to express protein on the basis of analysis by SDS/PAGE was resuspended in 1 ml of LB medium. A 1:1000 dilution of this solution was then used to inoculate 50 ml of LB medium containing 30 μg/ml kanamycin. This culture was grown at 37°C with vigorous shaking to an OD_{600} of 0.6, at which time expression of the enzyme was induced by adding isopropyl thiogalactoside (IPTG) to a final concentration of 0.5 mM. After growth for an additional 3 h the cells were harvested by centrifugation and stored at –80°C. The pellet was thawed at room temperature, and the cells were lysed by adding 2

ml of BugBuster Protein Extraction Reagent (Novagen) and following the protocol supplied by the manufacturer. The protein was then purified under standard conditions using a Ni-NTA Spin Kit (Qiagen). Fractions containing protein were then dialyzed against PBS, pH 7.3. The enzyme concentration was determined based on the A_{280} assuming an $\varepsilon = 71{,}000\ cm^{-1} \bullet M^{-1}$ as reported. The purified protein was stored at 4ºC. An identical procedure was followed for purification of the P99 $Ser^{64} \rightarrow$ Ala cephalosporinase mutant. About 60 μg of protein was obtained from 50 ml of culture. The proteins were judged to be >95% pure on the basis of Coomassie blue staining of an SDS/PAGE gel.

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