Reactivity of Penicillin-Binding Proteins with Peptidoglycan-Mimetic β-Lactams: What's Wrong with These Enzymes?†

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ABSTRACT: β-Lactams exert their antibiotic action through their inhibition of bacterial DD-peptidases (penicillin-binding proteins). Bacteria, in general, carry several such enzymes localized on the outside of their cell membrane to catalyze the final step in cell wall (peptidoglycan) synthesis. They have been classified into two major groups, one of high molecular weight, the other of low. Members of the former group act as transpeptidases *in vivo*, and their inhibition by β-lactams leads to cessation of bacterial growth. The latter group consists of DD-carboxypeptidases, and their inhibition by β-lactams is generally not fatal to bacteria. We have previously shown that representatives of the former group are ineffective at catalyzing the hydrolysis/aminolysis of peptidoglycan-mimetic peptides *in vitro* [Anderson et al. (2003) *Biochem. J. 373*, 949−955]. The theme of these experiments is expanded in the present paper where we describe the synthesis of a series of β-lactams (penicillins and cephalosporins) containing peptidoglycan-mimetic side chains and the kinetics of their inhibition of a panel of penicillin-binding proteins spanning the major classes (*Escherichia coli* PBP 2 and PBP 5, *Streptococcus pneumoniae* PBP 1b, PBP 2x and PBP 3, the *Actinomadura* R39 DD-peptidase, and the *Streptomyces* R61 DD-peptidase). The results of these experiments mirror and expand the previous results with peptides. Neither peptides nor β-lactams with appropriate peptidoglycan-mimetic side chains react with the solubilized constructs of membrane-bound penicillin binding proteins (the first five enzymes above) at rates exceeding those of generic analogues. Such peptides and β-lactams do react at greatly enhanced rates with certain soluble low molecular weight enzymes (R61 and R39 DD-peptidases). The former result is unexpected and interesting. Why do the majority of penicillin-binding proteins not recognize elements of local peptidoglycan structure? Possible answers are discussed. That this question needs to be asked casts fascinating shadows on current studies of penicillin-binding proteins for new drug design.

The biosynthesis of the bacterial cell wall has been intensively studied as a source of antibiotic targets. Although a number of inhibitors of the constitutive enzymes have been described (*1*−*3*), the most successful as antibiotics are the β-lactams. These compounds inhibit enzymes that catalyze the final transpeptidation step where the disaccharide pentapeptide monomer is incorporated into the cell wall (*4*). These enzymes are variously referred to as D-alanyl-D-alanine transpeptidases, DD-peptidases, and penicillin-binding proteins (PBPs[1]).

Bacteria generally maintain several PBPs in their cell membranes, each of which normally plays a different role in cell wall peptidoglycan synthesis (*5*, *6*). Structural studies of these enzymes, particularly by Ghuysen and co-workers, led to a classification scheme based on amino acid sequence homologies (*7*). First, two groups were distinguished, one of high molecular weight (HMW), the other of low (LMW). Each of these groups was divided into three classes: A, B, and C. High molecular weight classes A and B act as transpeptidases *in vivo* and are the killing targets of β-lactam antibiotics. Low molecular weight classes A−C contain D-alanyl-D-alanine carboxypeptidases. The latter enzymes are also inhibited by β-lactams, but their inhibition is not usually fatal to bacteria. Resistance to β-lactams can arise through PBP mutations and homologous recombinations (*8*, *9*).

Most PBPs, particularly the essential HMW class A and B enzymes, are attached to the outer leaflet of the cell

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[1] Abbreviations: CENTA, 7β-[(thien-2-yl)acetamido]-3-[(4-nitro-3-carboxyphenylthio)methyl]-3-cephem-4-carboxylic acid; DAP, 2,6-diaminopimelic acid; DME, dimethoxyethane; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; EDC, *N*-(dimethylaminopropyl)-*N*′-ethylcarbodiimide; ES MS, electrospray mass spectrometry; FTIR, Fourier transform infrared; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HMW, high molecular weight; HOBT, 1-hydroxybenzotriazole; LMW, low molecular weight; NMR, nuclear magnetic resonance; PNBn, *p*-nitrobenzyl; PBP, penicillin-binding protein; TFA, trifluoroacetic acid; THF, tetrahydrofuran.

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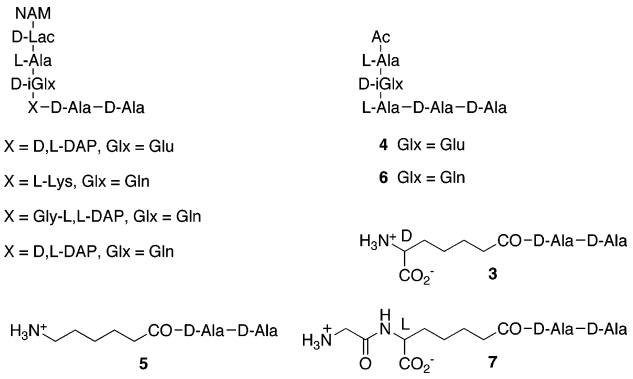
membrane by means of a short peptide extension. Deletion of this extension leads to soluble globular proteins that retain their penicillin-binding ability (*7*). X-ray crystal structures of several of these solubilized constructs have been obtained in recent years including examples of all the major classes referred to above (*10*−*16*), as well as of β-lactam-resistant mutants (*17*−*19*). These structures have revealed that PBP active sites have much in common, sharing similar functional groups and, presumably, similar mechanisms of catalysis (*20*). Less obvious from the structures, to date, are the sources of substrate structural specificity. Although the natural substrates of these enzymes must be, at least, oligopeptides, and although there is evidence in the structures for extended binding surfaces/clefts, there is little molecular detail available on how such substrates occupy the active sites in a productive manner.

The assertion contained in the last sentence of the previous paragraph is likely to be related to the observation that many PBPs, and particularly the essential HMW class A and B enzymes, do not, as solubilized constructs *in vitro*, catalyze the hydrolysis of peptidoglycan-mimetic peptides at any appreciable rate (*21*−*23*). Stated more directly, there is currently no known example of a HMW PBP efficiently catalyzing the hydrolysis and/or aminolysis of a peptidoglycan-mimetic peptide *in vitro*. Particularly relevant and well-studied examples here would be PBP 1b of *Escherichia coli* (*23*−*25*) and PBP 2x of *Streptococcus pneumoniae* (*26*, *27*). Class A LMW enzymes, such as *E. coli* PBP 5, also show very modest reactivity with peptides (*28*), even those including peptidoglycan-mimetic structure (*29*, *30*). Significantly higher peptidase activities have been observed with one particular LMW class C enzyme, *Neisseria gonorrhoeae* PBP 3 (*31*), but not, interestingly, in reaction with peptidoglycan-mimetic peptides (*29*).

Strikingly, however, despite the statements above, solubilized PBPs appear to be covalently inhibited by generic β-lactams at rates comparable to the analogous rates *in vivo*, after allowance is made, in the latter case, for permeability barriers (*32*, *33*).

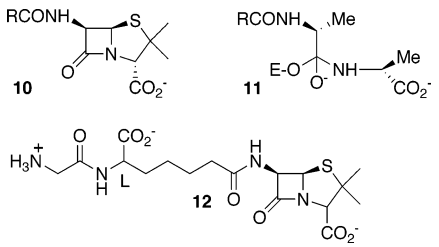
The stem peptides of *E. coli* and *S. pneumoniae* have structures **1** and **2**, respectively. We have previously shown that peptides **3** and **4** are not appreciably hydrolyzed by *E. coli* PBP 2 (HMW, class B) and PBP 5 (LMW, class A); nor are **5** and **6** hydrolyzed by *S. pneumoniae* PBP 2x (HMW, class B) (*29*). On the other hand, we have discovered



two cases where efficient turnover of peptidoglycan-mimetic peptides is evident (*29*, *34*). The peptide **7**, a direct mimic of the N-terminus of the *Streptomyces* stem peptide **8**, is an excellent substrate of the LMW class B DD-peptidase of *Streptomyces* R61 (k_{cat} = 69 s^{-1}, K_m = 7.9 μM, k_{cat}/K_m = 8.7 × 10^6 s^{-1} M^{-1}). Similarly, the LMW class C DD-peptidase of *Actinomadura* R39, where the stem peptide has structure **9**, is a very effective catalyst of hydrolysis of the peptide **3** (k_{cat} = 7.4 s^{-1}, K_m = 1.3 μM, k_{cat}/K_m = 5.7 × 10^6 s^{-1} M^{-1}). Neither of these enzymes, however, is a particularly effective catalyst of hydrolysis of the peptides **6** and **4**, respectively (k_{cat}/K_m values of 270 s^{-1} M^{-1} and 2900 s^{-1} M^{-1}, respectively (*29*)), suggesting that, in these cases at least, substrate specificity derives from the stem peptide N-terminus; this terminus, of course, represents the major source of variation in bacterial stem peptides (*35*).

X-ray crystal structures of the peptide **7** bound at the active site of the R61 DD-peptidase clearly showed the structural basis of the striking substrate specificity described above: the hydrophobic aminopimelate methylene chain is accommodated in a hydrophobic pocket while specific polar interactions with the enzyme are present at the N-terminus (*36*).

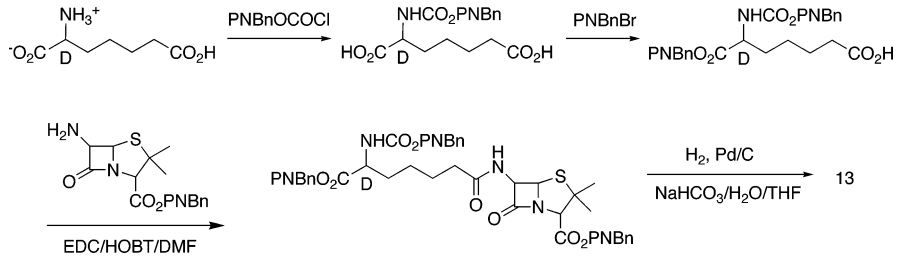
It is now generally appreciated that β-lactam antibiotics, such as the penicillins (**10**), rapidly and specifically acylate DD-peptidases because of their chemical reactivity and resemblance to the tetrahedral intermediates/transition states traversed in the hydrolysis or aminolysis of a D-alanyl-D-alanine peptide (**11**) (*4*, *37*). One might expect that if a



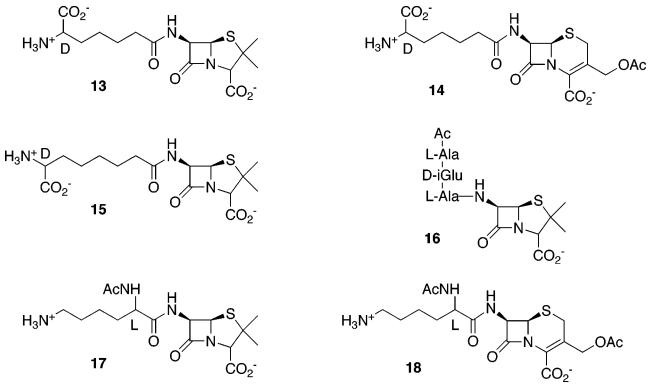
peptidoglycan-specific side chain R in **11** were important to peptide binding and reactivity, then the same side chain on the β-lactam **10** would demonstrate comparable specificity. This has been demonstrated to be true for the penicillin **12**, which is a powerful inhibitor (k_i = 1.0 × 10^7 s^{-1} M^{-1}) of the R61 DD-peptidase (*38*). A crystal structure of the β-lactam/enzyme complex shows the side chain of **12** bound to the active site in exactly the same way as that of the peptide **7** (*39*).

The reaction of β-lactams with PBPs involves acylation of the active site serine hydroxyl group (*40*). Largely because of steric hindrance (*20*), the acyl-enzyme thus formed is inert to hydrolysis; hence the antibiotic activity of β-lactams. Although, as described above, PBPs *in vitro* are not, in general, significantly reactive with peptides, they are, in general, acylated by β-lactams at appreciable rates, probably because of the intrinsically higher chemical reactivity of β-lactams than peptides (although the closer structural resemblance of **10** to a high energy intermediate than to a peptide may be more important). In order to further probe the specificity of PBPs for peptidoglycan-mimetic structural elements, we have expanded the theme of **12** to representatives of each of the PBP classes described above. Specifically, we describe the synthesis of the β-lactams **13**−**18**. The rates of inhibition of *E. coli* PBP 2 and PBP 5 by **13**−**16**, *S. pneumoniae* PBP 1b, 2x, and 3 by **17** and **18**, and the

Scheme 1



Actinomadura R39 DD-peptidase by **13**−**15** are then described and the results discussed with reference to the introduction above.



MATERIALS AND METHODS

Syntheses

6-β(D-α-Aminopimelyl)-aminopenicillanic acid (**13**) *(Scheme 1).*

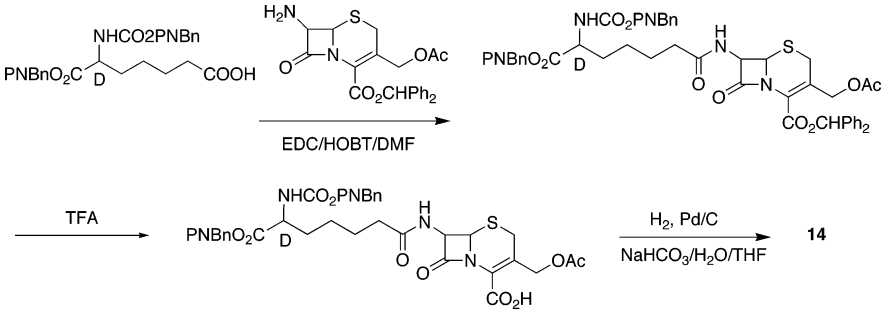
N-(p-Nitrobenzyloxycarbonyl)-D-α-aminopimelic Acid. D-α-Aminopimelic acid (*41*) (3.68 g, 0.021 mol) was dissolved in 2 M NaOH (21 mL, 0.042 mol), and dioxane (9 mL) was added. This solution was cooled to 0 °C with vigorous stirring while a solution of *p*-nitrobenzyl chloroformate (13.5 g, 0.06 mol) in dioxane (69 mL) and a solution of 2 M NaOH (42 mL, 0.084 mol) was added in five equal portions alternately, beginning with *p*-nitrobenzyl chloroformate, and with a 15 min delay after each set of additions. After the final addition, the resulting mixture was stirred at room temperature for 30 min. The crystalline byproduct, bis(*p*-nitrophenyl) carbonate, was removed by filtration and washed with water (70 mL). The filtrate and washings were combined and extracted with ethyl acetate. The aqueous layer was acidified to pH 2.5 and extracted with ethyl acetate (90 mL). This ethyl acetate extract was washed with saturated NaCl, dried over $MgSO_4$, and evaporated to dryness, leaving a yellow oil (5.84 g, yield 79%). ^{1}H NMR (d_6-DMSO) δ: 1.2−1.4 (2H, m), 1.40−1.56 (2H, m), 1.6−1.8 (2H, m), 2.2 (2H, t, J = 6.9 Hz), 3.9 (1H, m), 5.20 (2H, s), 7.62 (2H, d, J = 9.4 Hz), 7.76 (1H, d, J = 7.7 Hz), 8.25 (2H, d, J = 9.4 Hz).

p-Nitrobenzyl N-(p-Nitrobenzyloxycarbonyl)-D-α-aminopimelate. To a solution of *N*-(*p*-nitrobenzyloxycarbonyl)-D-α-aminopimelic acid (5.81 g, 16.4 mmol) in DMF (41 mL) at 75−85 °C (internal temperature) was added dicyclohexylamine (3.3 mL, 16.4 mmol), and the resulting solution was stirred for 5 min. A solution of *p*-nitrobenzyl bromide (3.19 g, 14.8 mmol) in DMF (9 mL) was then added in a single portion. Within seconds, a precipitate separated. The mixture was immediately cooled to room temperature, and ethyl acetate was added. The precipitate was removed by filtration, and the filtrate was washed with water and saturated NaCl, dried (MgSO4), and evaporated *in vacuo* to leave a yellow oil (7.94 g). This was further purified by two silica gel columns using 40:60 and 50:50 acetone/hexane respectively; yield 2.47 g (39%). ^{1}H NMR (d_6-DMSO) δ: 1.2−1.4 (2H, m), 1.40−1.56 (2H, m), 1.6−1.8 (2H, m), 2.2 (2H, t, J = 6.9 Hz), 4.12 (1H, m), 5.2 (2H, ABq, J = 3.0 Hz), 5.28 (2H, s), 7.59 (2H, d, J = 8.9 Hz), 7.62 (2H, d, J = 8.9 Hz), 8.0 (1H, d, J = 8.2 Hz), 8.2 (4H, d, J = 8.9 Hz).

Bis(p-nitrobenzyl) 6-β[N-(p-Nitrobenzyloxycarbonyl)-D-α-aminopimelyl]-aminopenicillanate. *p*-Nitrobenzyl *N*-(*p*-nitrobenzyloxycarbonyl)-D-α-aminopimelate (100 mg, 0.20 mmol) and *p*-nitrobenzyl 6-aminopenicillanate (*42*) (0.054 g, 0.148 mmol) were dissolved in 0.73 mL of anhydrous DMF and cooled to 0 °C in an ice bath. To this solution were added 1-hydroxybenzotriazole (0.0273 g, 0.208 mmol) and dicyclohexylcarbodimide (0.0341 g, 0.178 mmol), and the reaction mixture was stirred for 3 h at room temperature; it was then evaporated to dryness with a vacuum pump. The residue was dissolved in ethyl acetate and washed successively with equal portions of 10% aqueous citric acid, water, saturated aqueous $NaHCO_3$, water, and brine. The organic layer was dried over sodium sulfate and evaporated to dryness. The product was purified by preparative TLC on silica gel with 50/50 acetone/hexane as solvent; yield 53 mg (40%). ^{1}H NMR (d_6-DMSO) δ: 1.20−1.26 (2H, m), 1.36−1.46 (2H, m), 1.45 (3H, s), 1.54−1.66 (2H, m), 1.64 (3H, s), 2.23 (2H, t, overlapping solvent peak), 4.27 (1H, m), 4.54 (1H, s), 5.22 (2H, s), 5.30 (2H, s), 5.35 (2H, s), 5.48 (1H, d, J = 3.8 Hz), 5.60 (1H, dd, J = 3.8, 7.5 Hz), 7.54−7.62 (5H, m), 7.66 (1H, d, J = 8.6 Hz), 8.26 (1H, d, J = 8.6 Hz), 8.16−8.25 (5H, m).

6-β(D-α-Aminopimelyl)-aminopenicillanic acid (**13**). The triprotected penicillin from above (27.1 mg, 30.3 μmol) was dissolved in a solution of $NaHCO_3$ (5.09 mg, 60.6 μmol) in THF (3.1 mL) and water (3.1 mL); 10% Pd/C (54.2 mg) was then added and the mixture hydrogenated under 1 atm pressure at room temperature for 2.5 h. The suspension was filtered through a Celite pad into a cooled flask, and the filtered solid was washed with ethanol (3.1 mL) and water (3.1 mL). The combined filtrate was extracted with diethyl ether (2 × 3.1 mL) and the aqueous layer freeze-dried, yielding a colorless solid. The product, as a sodium salt, was initially purified by Sephadex G-10 column chromatography (eluent water) in 26 mg yield. A final purification step, carried out as the product was required, involved hplc (elution by 2% methanol in 0.03% aqueous NH_4HCO_3 from a Macherey-Nagel SS 250/0.5 in.″/10-nucleosil 300-7 C18

Scheme 2



reverse phase column; retention time 5.2 min at a flow rate of 3 mL/min). FTIR (KBr, cm^{-1}**)** 1761, 1658, 1590. ^{1}H NMR (D_2O) δ: 1.14−1.28 (2H, m), 1.36 (3H, s), 1.42−1.56 (2H, m), 1.50 (3H, s), 1.6−1.76 (2H, m), 2.20 (2H, ABX_2, J = 6.3, 7.3 Hz), 3.54 (1H, t, J = 6 Hz), 4.08 (1H, s), 5.31 (1H, d, J = 3.9 Hz), 5.40 (1H, d, J = 3.9 Hz). ES(+) MS *m*/*e*: 374.1, [M + 1].

7-β(D-α-Aminopimelyl)-aminocephalosporanic Acid (**14**) *(Scheme 2).*

*Diphenylmethyl p-Nitrobenzyl 7-β[N-(p-Nitrobenzyloxycarbonyl)-D-α-aminopimelyl]-aminocephalosporanate. N-(p-*Nitrobenzyloxycarbonyl)-D-α-aminopimelic acid (50 mg, 91.6 μmol) and diphenylmethyl 7-aminocephalosporanate (*43*) (35.6 mg, 76.3 μmol) were dissolved in 2 mL of anhydrous DMF and cooled to 0 °C in an ice bath. To this solution were added 1-hydroxybenzotriazole (14.0 mg, 107 μmol) and EDC (17.6 mg, 91.6 μmol), and the reaction was stirred for 6 h at room temperature. It was then evaporated to dryness by means of a vacuum pump. The residue was dissolved in ethyl acetate and washed successively with equal portions of 10% aqueous citric acid, water, saturated aqueous $NaHCO_3$, water, and brine. After evaporation of the organic solvent, the product was purified by preparative TLC on silica gel, with 50/50 acetone/hexane as solvent. The product was extracted from the plate with ethyl acetate in 47 mg (61%) yield. ^{1}H NMR (CD_3CN) δ: 1.22−1.36 (2H, m), 1.39−1.56 (2H, m), 1.60−1.78 (2H, m), 1.94 (3H, s), 2.24 (2H, t, 6.8 Hz), 3.55 (2H, ABq, J = 19.2 Hz), 4.26 (1H, m), 4.78 (2H, ABq, J = 12.9 Hz), 5.07 (1H, brs), 5.22 (2H, s), 5.26 (2H, s), 5.80 (1H, br s), 6.25 (1H, br d), 6.86 (1H, br d), 6.92 (1H, s), 7.3−7.48 (10H, m), 7.58 (4H, d, J = 7.3 Hz), 8.22 (4H, d, J = 7.3 Hz).

p-Nitrobenzyl 7-β[N-(p-Nitrobenzyloxycarbonyl)-D-α-aminopimelyl]-aminocephalosporanic Acid. The triprotected cephalosporin from above (47.6 mg) was stirred in trifluoroacetic acid (2 mL) at room temperature for 30 min. The acid was then removed by evaporation under vacuum, and the residue was partitioned between an aqueous layer containing $NaHCO_3$ (3 equiv) and ethyl acetate. The aqueous extraction was repeated twice, and the ethyl acetate layer was then dried ($MgSO_4$) and evaporated to dryness. The solid residue was further dried on a vacuum pump, extracted with benzene (the benzene extract discarded), and dried again; yield 25 mg (64%). ^{1}H NMR ($CDCl_3$) δ: 1.22−1.36 (2H, m), 1.39−1.56 (2H, m), 1.60−1.78 (2H, m), 1.94 (3H, s), 2.24 (2H, t, J = 6.8 Hz), 3.55 (2H, ABq, J = 19.6 Hz), 4.26 (1H, m), 4.78 (2H, ABq, J = 14.8 Hz), 5.03 (1H, d, J = 4.3 Hz), 5.20 (2H, s), 5.27 (1H, s), 5.80 (1H, (ABq), J = 4.3 Hz), 7.14 (1H, *d*, J = 7.3 Hz), 7.60 (4H, d, J = 7.3 Hz), 8.24 (4H, d, J = 7.3 Hz).

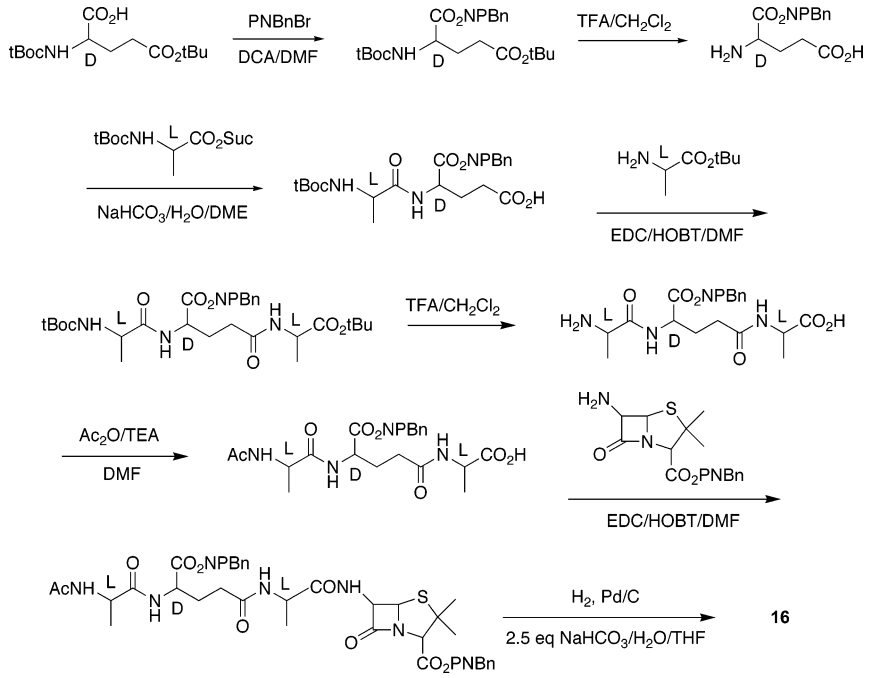
7-β(D-α-Aminopimelyl)-aminocephalosporanic Acid (**14**). *p*-Nitrobenzyl 7-*β*[*N*-(*p*-nitrobenzyloxycarbonyl)-D-α-aminopimelyl]-aminocephalosporanic acid (27.1 mg, 30.2 μmol) was dissolved in a solution of sodium bicarbonate (16.1 mg, 192 μmol, 2 equiv) in THF (16.4 mL) and water (8.2 mL). After addition of 10% Pd−C (159 mg) to the mixture, it was hydrogenated under 1 atm pressure at room temperature for 0.5 h. The resulting suspension was filtered through a Celite pad into a cooled flask, and the filtered solid was washed with ethanol (8.2 mL) and water (8.2 mL). The combined filtrate was extracted with diethyl ether (2 × 3.1 mL) and the aqueous layer freeze-dried, yielding a pale yellow solid. This salt was purified by hplc (elution by 4% methanol in 0.03% aqueous NH_4HCO_3 from a Macherey-Nagel SS 250/0.5 in./10-nucleosil 300-7 C18 reverse phase column; retention time 12 min at a flow rate of 3 mL/min); yield 7.6 mg (55%). FTIR (KBr, cm^{-1}): 1750, 1666, 1625. ^{1}H NMR (D_2O) δ: 1.16−1.32 (2H, m), 1.44−1.54 (2H, m), 1.62−1.74 (2H, m), 1.92 (3H, s), 2.18 (2H, ABX_2, J = 4.6, 7.4 Hz), 3.34 (2H, ABq, J = 18.0 Hz), 3.55 (1H, t, J = 5.8 Hz), 4.60 (2H, ABq, J = 13.0 Hz), 4.93 (1H, d, J = 4.5 Hz), 5.46 (1H, d, J = 4.5 Hz). ES(+) MS *m*/*e*: 452.5, [M + Na]$^+$.

6-β-(N-Acetyl-L-alanyl-γ-D-glutamyl-L-alanyl)-aminopenicillanic Acid (**16**) *(Scheme 3).*

γ-tert-Butyl α-p-Nitrobenzyl-N-Boc-D-glutamate. The starting material, Boc-D-α-Glu-OtBu, was dried overnight under vacuum in the presence of P_2O_5. Dicyclohexylamine (1.2 mL, 9.9 mmol) was added to a solution of this dried sample (3 g, 9.9 mmol) in DMF (21 mL) at 75−85 °C (internal temperature). A solution of 2.14 g of *p*-nitrobenzyl bromide (9.9 mmol) in DMF (5 mL) was added in a single portion to the hot solution, which was stirred for 5 min. A precipitate immediately separated, the mixture was cooled to room temperature, and ethyl acetate (10 mL) was added. The precipitate was removed by filtration and the ethyl acetate solution washed with water, saturated aqueous NaCl, and saturated aqueous $NaHCO_3$, dried over $MgSO_4$, and evaporated *in vacuo* to leave a yellow solid, which contained the required product along with some *p*-nitrobenzyl alcohol. The quantity of the required product was estimated to be 2.6 g (59%). ^{1}H NMR (d_6-DMSO) δ: 1.34 (9H, s), 1.66−1.84 (1H, m), 1.84−2.0 (1H, m), 2.24 (2H, t, J = 7.3 Hz), 4.06 (1H, q, J = 7.2 Hz), 5.26 (2H, s), 7.36 (1H, d, J = 8.2 Hz), 7.71 (2H, d, J = 8.2 Hz), 8.21 (2H, d, J = 8.2 Hz).

α-*p-Nitrobenzyl D-Glutamate.* The crude triprotected glutamate from above (3.54 g) was dissolved in CH_2Cl_2 (10.6 mL) and cooled to 0 °C, and TFA (53 mL) was slowly added. After complete addition, the solution was stirred for 2 h at room temperature. The TFA was removed by rotary evapo-

Scheme 3



ration and the residue further dried overnight under an oil pump vacuum.

α-*p-Nitrobenzyl N-Boc-L-Alanyl-D-glutamic acid.* A solution of α-*p*-nitrobenzyl D-glutamate (2.28 g, 8.09 mmol) and Boc-Ala-OSu (1.5 g, 5.09 mmol) in DME (11 mL) was mixed with $NaHCO_3$ (4 equiv, 32.3 mmol) in water (34 mL) at room temperature. This suspension was stirred overnight. To remove *p*-nitrobenzyl alcohol, the reaction mixture was extracted with ethyl acetate. It was then acidified at 4 °C with phosphoric acid to pH 2. Acidification was followed by several extractions with ethyl acetate. The combined ethyl acetate extracts were washed twice with water and dried over anhydrous Na_2SO_4. After evaporation of the solvent, the yield of the required product was 2.4 g, 100%. ^{1}H NMR (d_6-DMSO) δ: 1.16 (3H, d, J = 7.2 Hz), 1.34 (9H, s), 1.76−1.92 (1H, m), 1.92−2.08 (1H, m), 2.26 (2H, t, J = 6.9 Hz), 3.98 (1H, m), 4.34 (1H, AXY, J = 5.1, 8.9 Hz), 5.25 (2H, s), 6.91 (1H, d, J = 7.6 Hz), 7.62 (2H, d, J = 8.6 Hz), 8.22 (2H, d, J = 8.6 Hz), 8.25 (1H, d, J = 6.9 Hz).

α-*p-Nitrobenzyl N-Boc-L-Alanyl-D-γ-glutamyl-L-alanine tert-Butyl Ester.* α-*p*-Nitrobenzyl *N*-Boc-L-alanyl-D-γ-glutamic acid (2.45 g, 5.40 mmol) and AlaOtBu·HCl (981 mg, 5.40 mmol) were dissolved in DMF and cooled to 0 °C in an ice bath. To this solution was added triethylamine (756 μL, 5.40 mmol), and the mixture was stirred well, while a precipitate formed. At this stage, 1-hydroxybenzotriazole (875 mg, 6.48 mmol) was added. The mixture was stirred for 5 min and EDC (1.04 g, 5.4 mmol) added. The final reaction mixture was stirred at room temperature for 18 h. It was then evaporated to dryness with a vacuum pump. The residue was dissolved in ethyl acetate. This solution was washed successively with equal portions of 10% citric acid, water, saturated $NaHCO_3$, water, and brine and dried over anhydrous Na_2SO_4; yield 2.5 g, 95%. ^{1}H NMR (d_6-DMSO, 300 MHz) δ: 1.18 (3H, d, J = 7.0 Hz), 1.21 (3H, d, J = 7.0 Hz), 1.43 (18H, s), 1.78−1.92 (1H, m), 1.92−2.07 (1H, m), 2.15 (2H, t, J = 6.0 Hz), 3.96−4.14 (2H, 2 sets of overlapping quartets), 4.26−4.36 (1H, q, J = 6.0 Hz), 5.25 (2H, s), 6.89 (1H, d, J = 7.2 Hz), 7.62 (2H, d, J = 8.4 Hz), 8.13 (1H, d, J = 7.2 Hz), 8.21 (2H, d, J = 8.4 Hz), 8.28 (1H, d, J = 7.2 Hz).

α-*p-Nitrobenzyl L-alanyl-D-γ-glutamyl-L-alanine.* The protected tripeptide (481 mg, 0.83 mmol) was dissolved in the minimum volume (≤2 mL) of CH_2Cl_2. The solution was chilled to 0 °C in an ice bath with constant stirring. TFA (10.5 mL) was added slowly to the above solution. After the addition was complete, the reaction mixture was stirred for 2 h at room temperature. TFA was then removed by rotary evaporation. The required compound α-*p*-nitrobenzyl L-alanyl-D-γ-glutamyl-L-alanine was dried overnight on the vacuum pump.

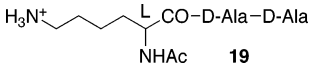
α-*p-Nitrobenzyl N-Acetyl-L-alanyl-D-γ-glutamyl-L-alanine.* Here we followed the acetylation procedure of Meroueh et al. (*44*). The crude residue from the previous step was dissolved in DMF (18.6 mL). To this solution were added triethylamine (448 μL, 3.32 mmol) and acetic anhydride 258 μL, 0.91 mmol), and the resultant mixture was stirred at room temperature overnight. The solvent was removed under vacuum. The residue was dissolved in ethyl acetate, washed with 10% citric acid followed by water, dried over anhydrous Na_2SO_4, and evaporated to dryness. The N-acetylated tripeptide was purified by silica gel column chromatography using 60/35/5 acetone/hexane/methanol as eluent; yield, 64.3 mg, 74%. ^{1}H NMR (d_6-DMSO) δ 1.22 (3H, d, J = 7.2 Hz), 1.17 (3H, d, J = 7.2 Hz), 1.82 (3H, s), 1.78−1.92 (1H, m), 1.92−2.07 (1H, m), 2.16 (2H, t, J = 7.5 Hz), 4.14 (1H, quint, J = 6.9 Hz), 4.22−4.33 (2H, m), 5.24 (s, 2H), 7.62 (2H, d, J = 8.4 Hz), 8.06 (1H, d, J = 8.0 Hz), 8.13 (1H, t, J = 7.2 Hz), 8.22 (2H, d, J = 8.7 Hz), 8.44 (1H, d, J = 4.8 Hz).

6-β[α-p-Nitrobenzyl N-Acetyl-L-alanyl-γ-D-glutamyl-L-alanyl]-aminopenicillanic Acid p-Nitrobenzyl Ester. α-*p*-Nitrobenzyl-*N*-acetyl-L-alanyl-D-γ-glutamyl-L-alanine (500 mg, 1.11 mmol) and *p*-nitrobenzyl 6-aminopenicillanate (*42*) (338 mg, 0.922 mmol) were dissolved in 5 mL of anhydrous DMF and cooled to 0 °C in an ice bath. To this solution were added 1-hydroxybenzotriazole (174 mg, 1.29 mmol) and

EDC (565 mg, 2.95 mmol), and the reaction mixture was stirred for 5 h at room temperature. The solution was then evaporated to dryness under vacuum. The residue was dissolved in ethyl acetate and washed successively with equal portions of citric acid, water, saturated aqueous $NaHCO_3$, and water, followed by brine. The ethyl acetate was dried and evaporated. Product yield: 158 mg (100%). ^{1}H NMR (d_6-DMSO) δ: 1.14 (6H, 2 overlapping d, $J = 6.9$ Hz), 1.35 (3H, s), 1.55 (3H, s), 1.95 (3H, s), 2.0 (2H, br s), 2.16 (2H, br s), 3.98 (1H, q, $J = 7.2$ Hz), 4.2−4.4 (overlapping q and m, 2H), 5.22 (2H, s), 5.32 (2H, s), 4.55 (1H, s), 5.49 (1H, d, $J = 4.2$ Hz), 5.55 (1H, d, $J = 4.2$ Hz), 7.59 (2H, d, $J = 8.7$ Hz), 7.68 (2H, d, $J = 6.6$ Hz), 8.02 (1H, d, $J = 7.8$ Hz), 8.18−8.24 (2 overlapping d, 4H), 8.39 (1H, d, $J = 7.5$ Hz), 8.56 (1H, d, $J = 7.5$ Hz).

6-β(N-Acetyl-L-alanyl-γ-D-glutamyl-L-alanyl)-aminopenicillanic Acid (**16**). The protected penicillin from above (158 mg, 0.2 mmol) was dissolved in freshly distilled THF (34 mL) and water (30 mL) containing $NaHCO_3$ (43.6 mg, 0.5 mmol). When the mixture became homogeneous, 10% Pd−C (315 mg) was added, and it was hydrogenated under 1 atm pressure at room temperature for 2.5 h. The suspension was filtered through Celite into a chilled receiving flask and the residue washed with ethanol (20 mL) and water (30 mL). The pH of the filtrate was checked to ensure that it was neutral or weakly basic. The filtrate was extracted with diethyl ether. After the extractions, the aqueous phase pH was checked again and found to be neutral. The aqueous phase was then freeze-dried to leave a pale yellow solid. This salt was purified by hplc (elution by 1% methanol in 0.03% aqueous NH_4HCO_3 from a Macherey-Nagel SS250/0.5 in./10-nucleosil 300-7 C_{18} reverse phase column; retention time 7.74 min at a flow rate of 3 mL/min); yield 15.3 mg. ^{1}H NMR (D_2O) δ: 1.23 (3H, d, $J = 7.1$ Hz), 1.25 (3H, d, $J = 7.1$ Hz), 1.37 (3H, s), 1.48 (3H, s), 1.90 (3H, s), 1.74−1.88 (1H, m), 1.96−2.10 (1H, m), 2.19 (2H, t, $J = 7.5$ Hz), 4.12 (1H, s), 4.15−4.20 (3H, m), 5.32 (1H, d, $J = 3.9$ Hz), 5.42 (1H, d, $J = 4.2$ Hz). ES(−) MS *m/e*: 528.3, [M − 1].

Penicillins **15** and **17**, the cephalosporin **18**, and the peptide **3** were prepared by routes analogous to those above, as described in the Supporting Information. The peptide **19** was purchased from Sigma-Aldrich Corp.



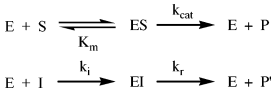
19

Kinetics Studies

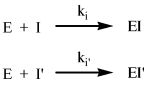
Materials. A purified sample of *E. coli* PBP 2 was obtained from Dr. H. Adachi (University of Tokyo, Tokyo, Japan). *S. pneumoniae* PBP 2x, PBP 1b, and PBP 3 (*10*−*12*) and *E. coli* PBP 5 (*13*) were prepared as described, as was the *Actinomadura* R39 DD-peptidase (*15*). The *S. pneumoniae* enzymes were generous gifts of Dr. Otto Dideberg and Dr. Andrea Dessen of CNRS/CEA/UJF, Grenoble, France.

E. coli PBP 2 and PBP 5. Rate constants for inhibition of PBP5 by **13**−**16** were obtained from competition experiments with the chromophoric substrate *N*-phenylacetylglycyl-D-thiolactate (**20**). Turnover of **20** (500 μM) was monitored spectrophotometrically at 257 nm ($\Delta\epsilon = 1.04 \times 10^3$ cm^{-1} M^{-1}) in a 0.1 M sodium pyrophosphate buffer containing 10% glycerol, pH 8.5, at 37 °C. The enzyme concentration was 0.74 μM, and the β-lactam concentrations varied between 0 μM and 50 μM. The total progress curves obtained were analyzed by means of the Dynafit program (*45*) and Scheme 4 (S and I represent **20** and the β-lactam, respectively; $k_{cat}/K_m = 550$ s^{-1} M^{-1}).

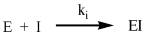
Scheme 4



Scheme 5



Scheme 6



For PBP 2, competition between **13** and the chromogenic β-lactam 6β-bromopenicillanic acid was monitored spectrophotometrically at 325 nm ($\Delta\epsilon = 6.1 \times 10^3$ cm^{-1} M^{-1}) (*46*). The buffer employed was 20 mM Hepes/0.2 M NaCl, pH 8.0. Concentrations of bromopenicillin and enzyme were 100 μM and 4.1 μM, respectively, and concentrations of **13** were 0−1.0 mM. Experiments were performed at 37 °C, and the total progress curves were fitted to Scheme 5 (I and I′ represent **13** and bromopenicillin, respectively.) The value of $k_{i'}$ for bromopenicillin was 33 s^{-1} M^{-1}.

S. pneumoniae PBP 1b, PBP 2x, and PBP 3. Acylation of the active sites of PBP 2x (*9*, *26*) and PBP 1b (*47*) is accompanied by a decrease in protein fluorescence. Therefore, in these cases, determinations of k_i could be made by direct measurement. For PBP 2x, excitation and emission wavelengths were 290 and 340 nm, respectively, while, for PBP 1b, the corresponding wavelengths were 290 and 350 nm, respectively. Thus, changes in the fluorescence emission of PBP 2x (0.26 μM) with time on addition of **17** (0.3−0.6 μM) were monitored directly. The reaction conditions were 10 mM phosphate/500 mM KCl buffer, pH 7.0, 37 °C. Total progress curves were fitted to Scheme 6. Experiments with PBP 1b were carried out in 20 mM Hepes/0.1 M NaCl/1 mM EDTA buffer, pH 7.0 and at 25 °C. Concentrations of enzyme and the β-lactams **17** and **18** were 0.56 μM and 2−100 μM, respectively.

The fluorescence spectrum of PBP 3 did not appear to change on addition of β-lactams. Therefore, competition experiments with the substrate **20** (0.5 mM) were performed as described above. The reaction conditions were 20 mM Hepes, 1 mM EDTA buffer, pH 7.0, 37 °C. Reaction mixtures contained enzyme (53 nM) and the β-lactams **17** and **18** (200−400 nM). The resulting total progress curves were analyzed in terms of Scheme 4.

The reactivity of PBP 3 and PBP 1b with the peptides **5** and **6** was assessed spectrophotometrically at 220−235 nm (*29*) in the Hepes buffer described immediately above, at 37 °C. Concentrations of PBP 3 were 53 nM and 10.5 μM and of PBP 1b 0.56 μM; peptide concentrations were 1−10 mM.

Actinomadura R39 DD-Peptidase. Rate constants of inhibition of the R39 DD-peptidase by the β-lactams **13**−**15** were obtained from competition experiments employing the peptide **3** (0.25 mM) as a chromophoric (220 nm) substrate. The

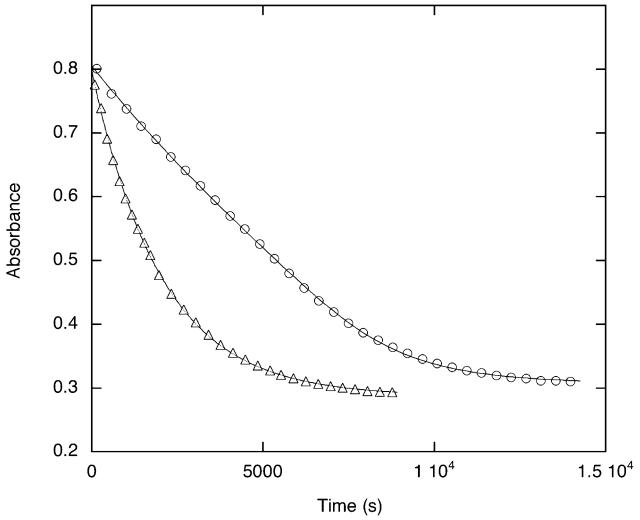


FIGURE 1: Reaction of *E. coli* PBP5 (0.74 μM) with the depsipeptide substrate **20** (0.5 mM), monitored spectrophotometrically at 257 nm, in the absence (△) and presence (○) of the penicillin **13** (20 μM). The points shown are experimental and the lines calculated as described in the text.

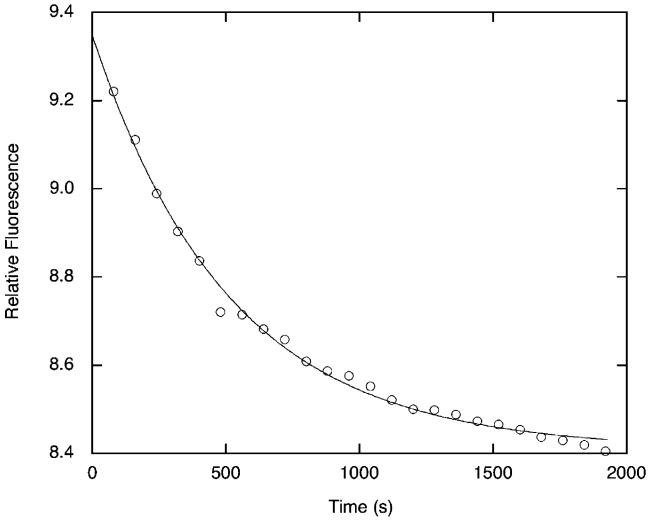


FIGURE 2: Reaction of *S. pneumoniae* PBP1b (0.56 μM) with the penicillin **17** (20 μM), monitored fluorimetrically at 350 nm (excitation at 290 nm). The points shown are experimental and the line is calculated as described in the text.

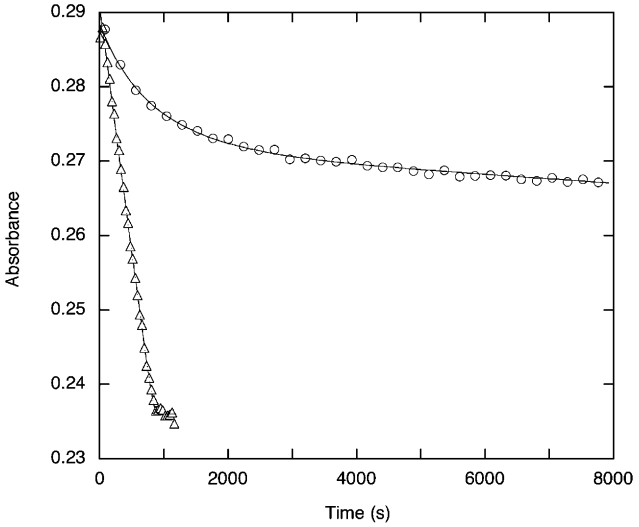


FIGURE 3: Reaction of R39 DD-peptidase (35 nM) with the peptide substrate **3** (0.25 mM), monitored spectrophotometrically at 220 nm, in the absence (△) and presence (○) of the penicillin **13** (50 nM). The points shown are experimental and the lines calculated as described in the text.

reaction conditions were 25 mM Tris/1.25 mM $MgCl_2$ buffer, pH 7.7, 37 °C. Under these conditions, the K_m value of **3** was 4.0 μM. For studies with **13** (50−60 nM), the enzyme concentration was 35 nM, and with **14** (10−20 nM) and **15** (50 nM), the enzyme concentration was 18.3 nM. Total progress curves were fitted to Scheme 4.

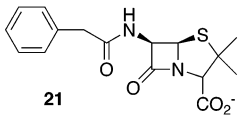
Where necessary for comparison, the inhibition rate constants for benzylpenicillin and cephalothin were obtained from experiments analogous to those described above.

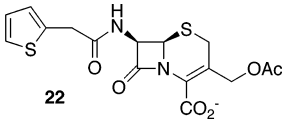
RESULTS AND DISCUSSION

The synthesis of the β-lactams **13**−**18** has been achieved by classical peptide chemistry, as described above. The side chain present in **13** and **14** is a direct mimic of the nucleophilic fork of the stem peptides of *E. coli* (**1**) and *Actinomadura* (**9**), respectively. The β-lactam in each case is, of course, an analogue of the D-alanyl-D-alanine C-terminus (*4*, *37*). The penicillin **15** is analogous to **13** and designed for the enzymes of the same species, but with the aliphatic methylene chain one carbon longer to allow greater flexibility. Penicillin **16**, where the side chain imitates the general stem peptide fork leading to NAM, was also designed, by virtue of the D-isoglutamic acid residue, as a probe of *E. coli* enzymes. Finally, **17** and **18** were designed to challenge the active sites of *S. pneumoniae* enzymes. β-Lactams with peptide side chains have been prepared previously by others, but they were not assessed as inhibitors of specific enzymes (*48*, *49*).

An example of the data used to obtain the inhibition rate constant k_i for inactivation of *E. coli* PBP 5 by the penicillin **13** is shown in Figure 1. This shows the results of a competition experiment where **13** and the reporter substrate **20** compete for the enzyme. The enzyme is obviously inhibited to some degree, but complete turnover of the substrate occurred because of the well-known significant ability of this particular PBP to catalyze hydrolysis of the penicilloyl-enzyme and thus hydrolyze the penicillin (*50*, *51*). The rate constants describing the interaction between **13** and the enzyme were obtained by fitting the data to Scheme 4, as described in Materials and Methods. The reaction between *S. pneumoniae* PBP 1b and the penicillin **14** was monitored directly by fluorescence changes (Figure 2). Figure 3 shows the results of a competition experiment between the peptide substrate **3** and the penicillin **13** for the R39 DD-peptidase. In this case, as is more typical, acylation by **13** was fast and turnover very slow. At the concentrations employed in all of these experiments, only second order inactivation reactions were observed.

The results of experiments, such as described above, designed to measure second order rate constants of inactivation (k_i) of the panel of enzymes by **13**−**18**, are shown in Table 1. For each enzyme, for comparison, the inactivation rate constants for the generic β-lactams, benzylpenicillin (**21**) and cephalothin (**22**), are also reported. The general result for the HMW PBPs (classes A and B) was that the "specific" peptidoglycan-mimetic β-lactams reacted more slowly with their respective enzymes than did their generic analogues.

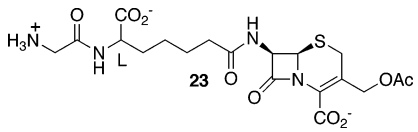




This result also applied, generally, to the class A LMW enzymes. An exception to this generalization is the greater reactivity of the cephalosporin **14** than cephalothin with *E. coli* PBP 5. This result is interesting, although **14** is only slightly more reactive than the penicillin **13** and the difference between the rates with **14** and cephalothin is not as great as observed in other cases (see below). The greater flexibility of **15**, designed to allow it a greater chance of reaching specific polar functional groups at the active site of *E. coli* PBPs, provided little enhanced reactivity. The penicillin **16** reacted more slowly with *E. coli* PBP 5 than **13**, showing that the segment of stem peptide reproduced in the side chain of **16** had even less affinity for the enzyme than did that of the N-terminus present in **13**.

The low reactivity of the PBPs described in the preceding paragraph with specific β-lactams is mirrored by their low reactivity with the corresponding specific peptides. For example, neither PBP 2 nor PBP 5 of *E. coli* catalyzes hydrolysis of **3** at any appreciable rate; nor does *S. pneumoniae* PBP 2x catalyze hydrolysis of **5** (*29*). *E. coli* PBP 5 shows modest activity against **4**, but neither *E. coli* PBP 2 nor *S. pneumoniae* PBP2x hydrolyzes **4** or **6**, respectively (*29*). As part of the present work, we have found that *S. pneumoniae* PBP 1b and PBP 3 do not catalyze hydrolysis of the peptides **5**, **6**, and **19**.

A very different result is obtained when the LMW class B and C enzymes are considered. As previously described (*38*), both **12** and the cephalosporin **23** react at least 2 orders of magnitude more rapidly than the respective generic β-lactams with the R61 DD-peptidase. A similar result is now



seen in the reactivity of **13** and **14** with the R39 DD-peptidase, suggesting, as with the R61 enzyme, the presence of specific interactions of the side chain with the R39 active site. The extended and more flexible analogue of **13**, the penicillin **15**, reacts somewhat more slowly than **13** with the R39 DD-peptidase. This result suggests that the optimal side chain length is indeed found in **13**. A similar effect of extended chain length was also observed with the R61 enzyme (*52*). As noted previously, these enzymes also catalyze hydrolysis of the peptides **3** (R39) and **7** (R61) very efficiently (*29*, *34*).

There appears to be, taking into account all of the enzymes considered above, a direct correlation between the effect of a peptidoglycan-mimetic side chain on the reactivity of a β-lactam and that of a D-alanyl-D-alanine peptide. Specifically, there is little side chain effect, on either β-lactam or peptide reactivity, with HMW PBPs or class A LMW PBPs. In contrast, such a side chain has a dramatic positive effect on the reactivity of both a β-lactam and peptide with a class B or C LMW PBP. As described in the introduction, crystal structures show that the peptidoglycan-mimetic side chain

Table 1: Rate Constants for Inactivation of Penicillin-Binding Proteins by β-Lactams

PBP (class) (source)	β-lactam	k_i (s^{-1} M^{-1})
PBP1b (HMW, A)	benzylpenicillin	$(1.30 \pm 0.07) \times 10^4$
(*S. pneumoniae*)	**17**	95 ± 16
	cephalothin	$(91 \pm 3) \times 10^3$
	18	34 ± 9
PBP 2x (HMW, B)	benzylpenicillin	$(1.3 \pm 0.3) \times 10^4$
(*S. pneumoniae*)	**17**	$(3.9 \pm 1.3) \times 10^3$
PBP 2 (HMW, B)	benzylpenicillin	190 ± 60
(*E. coli*)	**13**	≤ 25
PBP 5 (LMW, A)	benzylpenicillin	$(8.01 \pm 0.23) \times 10^3$
(*E. coli*)	**13**	$(2.16 \pm 0.04) \times 10^3$
	15	$(3.06 \pm 0.14) \times 10^3$
	16	$(5.80 \pm 0.22) \times 10^2$
	cephalothin	$(1.75 \pm 0.05) \times 10^2$
	14	$(4.7 \pm 0.9) \times 10^3$
PBP 3 (LMW, A)	benzylpenicillin	$(3.0 \pm 0.9) \times 10^4$
(*S. pneumoniae*)	**17**	$(1.20 \pm 0.16) \times 10^4$
R61 (LMW, B)	benzylpenicillin	1.37×10^4 [a]
(*Streptomyces* R61)	**12**	1.5×10^7 [a]
	cephalothin	1.4×10^3 [a]
	23	5.6×10^5 [a]
R39 (LMW, C)	benzylpenicillin	3×10^5 [b]
(*Actinomadura* R39)	**13**	$(6.8 \pm 0.3) \times 10^6$
	15	$(2.85 \pm 0.09) \times 10^6$
	cephalothin	6.5×10^4 [b]
	14	$(1.1 \pm 0.4) \times 10^7$

[a] Taken from ref *38*. [b] Taken from ref *68*.

of peptide **7** and the β-lactam **12** interact with the R61 DD-peptidase active site in what appears to be a tight and specific fashion. We conclude from the results described above that a specific side chain binding site must also be directly available in the R39 DD-peptidase active site, *but not in the other enzymes employed in this study*. X-ray crystallographic studies of appropriate complexes to examine these predictions are in progress.

There appear to be two possible explanations for the unexpected lack of recognition of stem peptide elements by the majority of the PBPs noted above. In these cases, either the region of the enzyme's active site specific to these elements is distorted or incomplete or the substrate recognition elements are elsewhere on the protein. These alternatives and their implications for future research are discussed below.

First, with respect to the enzyme active site, it may be noted that although there is no direct structural evidence at present for the presence or absence of peptidoglycan-mimetic side chain binding sites in these enzymes, apart from the R61 DD-peptidase (*36*, *39*), the general lack of reactivity of the solubilized constructs of these enzymes is striking. On one hand, they do, in general, react with β-lactams but, on the other, appear, at best, sluggishly reactive with peptides.

Studies of these enzymes under conditions designed to mimic those *in vivo*, e.g. as membrane fragments solubilized in detergents, or in mixed organic/aqueous solvents, have also yielded equivocal results. Although, under these conditions, a number of PBPs have been able to catalyze cross-linked peptidoglycan synthesis using undecaprenyl pyrophosphoryl disaccharide pentapeptide or disaccharide pentapeptide as substrate, the rates observed were much lower than required for bacterial growth rates (*21*−*25*). In explanation of these results, it has been suggested that the correct *in vivo* medium for these enzymes has not yet been reproduced, and, more particularly, that the likelihood that

these enzymes *in vivo* act as components of multiprotein complexes has not been sufficiently taken into account (*53*−*55*). There is evidence for association in solution of certain *E. coli* PBPs (*56*, *57* and references therein), and the transpeptidase activity of *E. coli* PBP1b appears to be greater when the enzyme is in dimeric form (*25*). It also appears well-established that *E. coli* PBP 2 is activated by the presence of the integral membrane protein rodA (*58*, *59*). It should also be noted that, *in vivo*, most PBPs, and certainly the lethal targets for β-lactams (HMW class A and B enzymes), are membrane-associated by an N-terminal peptide, and, in the case of HMW class A PBPs, by a secondary membrane association site located in the glycosyl transferase domain (*60*, *61*). Thus, there is certainly reason to believe that the structure of the extended active site of a PBP, both as a static and a dynamic entity, and therefore the enzyme activity, could be significantly different when present in solubilized transpeptidase domains in aqueous solution or in the crystal form from that *in vivo*. It does seem, however, that generic non-peptidoglycan-mimetic β-lactams react with PBPs *in vivo* as rapidly as with purified enzymes (*32*, *33*); indications of lower reactivity may derive from the presence of β-lactamases (*55*). This shows that although the specific recognition elements for natural substrates may not be present in the solubilized enzymes, the chemically reactive active site center is always present and, in solution, functional.

It is possible that, just as the active sites of these enzymes may be organized *in vivo* to react with peptide substrates rapidly, so they might also react rapidly *in vivo* with peptidoglycan-mimetic β-lactams such as **13**−**18**. Experiments to test this proposition are in progress.

It should be recalled at this point that the soluble *Actinomadura* R39 DD-peptidase, found to be an active DD-peptidase and rapidly inactivated by **13** and **14**, has an amino acid sequence and tertiary structure homologous to *E. coli* PBP 4 (*15*, *16*), which is, *in vivo*, a soluble protein only loosely associated with the cell membrane (*62*, *63*); the *Streptomyces* R61 DD-peptidase, as isolated, is also a water soluble protein. These enzymes, therefore, may be isolated in the complete active form and remain in that form.

A second explanation for the curious insensitivity of most PBPs to peptidoglycan mimetics is that an essential element of substrate recognition *in vivo* is centered at a region of the enzyme other than the transpeptidase active site. This implies that effective substrates may be larger than the molecules described here, and this is certainly conceivable since a likely substrate of many of these enzymes is the peptidoglycan polymer. Activation of enzymes by exosite recognition is well-known (*64*, *65*). The disaccharide pentapeptide itself (*23*) and its synthetic analogues (*30*), however, are apparently not large enough to achieve significant recognition. Perhaps a contribution from an adjacent peptidoglycan strand is required. There is some evidence for additional binding sites on certain PBPs, although only weak binding has been found to date (*66*, *67*). It may also be that different PBPs are activated by different elements of extended structure.

A combination of the results from this paper and from an earlier one (*29*) demonstrates that peptidoglycan-mimetic side chains have similar effects on the kinetics of reaction of both peptides and β-lactams with DD-peptidases. In certain cases (LMW class B and C enzymes), these side chains have a dramatic positive effect on reactivity, while in others (LMW class A and HMW enzymes) they have little or no effect. With respect to the latter group of enzymes, which include essentially all of the important β-lactam killing targets, the result is certainly a curious and unexpected one: the enzymes appear to show no specificity toward local peptidoglycan structure. The possible explanations for this are discussed above: the enzymes and/or the substrates are incomplete in some way that prevents them under *in vitro* conditions from behaving as they must *in vivo*. At this stage, therefore, because of the apparent absence of specific side chain binding sites in these latter enzymes, it is unclear whether chemical and structural studies *in vitro* can directly lead to new antibiotics. Much remains to be done for us to understand the action of these enzymes *in vivo*.

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SUPPORTING INFORMATION AVAILABLE

Synthetic details for the preparation of **15**, **17**, **18**, and the peptide **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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