

# Using Steric Hindrance to Design New Inhibitors of Class C $\beta$ -Lactamases

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## Summary

$\beta$ -lactamases confer resistance to  $\beta$ -lactam antibiotics such as penicillins and cephalosporins. However,  $\beta$ -lactams that form an acyl-intermediate with the enzyme but subsequently are hindered from forming a catalytically competent conformation seem to be inhibitors of  $\beta$ -lactamases. This inhibition may be imparted by specific groups on the ubiquitous R<sub>1</sub> side chain of  $\beta$ -lactams, such as the 2-amino-4-thiazolyl methoxyimino (ATMO) group common among third-generation cephalosporins. Using steric hindrance of deacylation as a design guide, penicillin and carbacephem substrates were converted into effective  $\beta$ -lactamase inhibitors and antiresistance antibiotics. To investigate the structural bases of inhibition, the crystal structures of the acyl-adducts of the penicillin substrate amoxicillin and the new analogous inhibitor ATMO-penicillin were determined. ATMO-penicillin binds in a catalytically incompetent conformation resembling that adopted by third-generation cephalosporins, demonstrating the transferability of such sterically hindered groups in inhibitor design.

## Introduction

The impact of bacterial resistance on antimicrobial chemotherapy is a pressing public health problem [1–3]. Among the antibiotic classes most affected are the  $\beta$ -lactams, such as the penicillins and cephalosporins, which are also among the most prescribed. The most widespread resistance mechanism to these antibiotics is the expression of  $\beta$ -lactamases [4], which hydrolyze these drugs, thereby inactivating them. All  $\beta$ -lactams share the same core four-membered lactam ring from which they take their name. It is this core structure that is hydrolyzed by  $\beta$ -lactamases. Intriguingly, substitutions distant from the lactam ring can convert a  $\beta$ -lactamase substrate into a  $\beta$ -lactamase inhibitor. For instance, amoxicillin and cephalothin are excellent substrates for class C  $\beta$ -lactamases such as AmpC, whereas cloxacillin and ceftazidime (Figure 1) are either inhibitors or very poor substrates for these enzymes. Structural studies

[5–10] suggest that these latter  $\beta$ -lactams act as inhibitors because they otherwise would form unfavorable steric interactions in the acyl adduct with class C  $\beta$ -lactamases and so are distorted away from a catalytically competent conformation. Thus, although they rapidly form covalent adducts in the initial acylation phase of the hydrolytic reaction, bulky groups on the R<sub>1</sub> side chains of these  $\beta$ -lactams force them to adopt catalytically incompetent conformations within the acyl adduct, preventing the deacylation step of the reaction from taking place, effectively trapping the enzyme. The action of these inhibitors may thus be distinguished from mechanism-based “suicide” substrates, such as clavulanate, which rely on secondary chemical reactions within the enzyme, and also from classical noncovalent substrate-based inhibitors, such as the dihydrofolate reductase inhibitor methotrexate or the trypsin inhibitor benzamidine, which rely on steric and electrostatic *complementarity* to the active site.

An example of a chemical group that appears to force a catalytically incompetent conformation in the binding site of AmpC  $\beta$ -lactamase is the 2-amino-4-thiazolyl methoxyimino (ATMO) group common among third-generation cephalosporins, such as cefotaxime and ceftazidime (Figure 1). The structure of the acyl-adduct of ceftazidime with AmpC suggested that this ATMO group, which occurs at the distal end of the molecule, forces the dihydrothiazine ring of the cephalosporin into a configuration where it destabilizes the formation of the deacylation transition state relative to good substrates, thereby making it an inhibitor or very poor substrate of AmpC [10]. In counterpoint to these structural studies, a series of elegant enzymological studies of third-generation cephalosporins has suggested that part of their ability to inhibit class C  $\beta$ -lactamases is conferred by an internal electronic rearrangement that displaces the R<sub>2</sub> side chain peculiar to cephalosporins. This R<sub>2</sub> group sits at the C3' position of the dihydrothiazine ring (Figure 1) of cephalosporins and has itself become the subject of much synthetic effort in novel  $\beta$ -lactam design. When the lactam ring is opened as a result of attack by the serine nucleophile (Ser64 in AmpC), the lone pair electrons on the formerly lactam nitrogen are free to rearrange, leading to the departure of the displaceable R<sub>2</sub> side chain (Figure 2). The resulting ring structure is thought to be more stable to hydrolytic attack [11–14]. In cephalosporins that are rapidly hydrolyzed, this rearrangement happens slower than hydrolysis of the acyl-enzyme species and consequent product formation and thus does not affect how well the  $\beta$ -lactam might inhibit the enzyme. For molecules that are intrinsically slow to deacylate, for instance because of steric interactions in their R<sub>1</sub> side chains, this rearrangement will happen before deacylation, thereby further stabilizing the acyl adduct against deacylation. In such cases, the  $\beta$ -lactam will be a slow substrate, to the point where it may be considered an inhibitor of the  $\beta$ -lactamase.

If steric hindrance plays a dominant role in the inhibition of class C  $\beta$ -lactamases by third-generation cepha-

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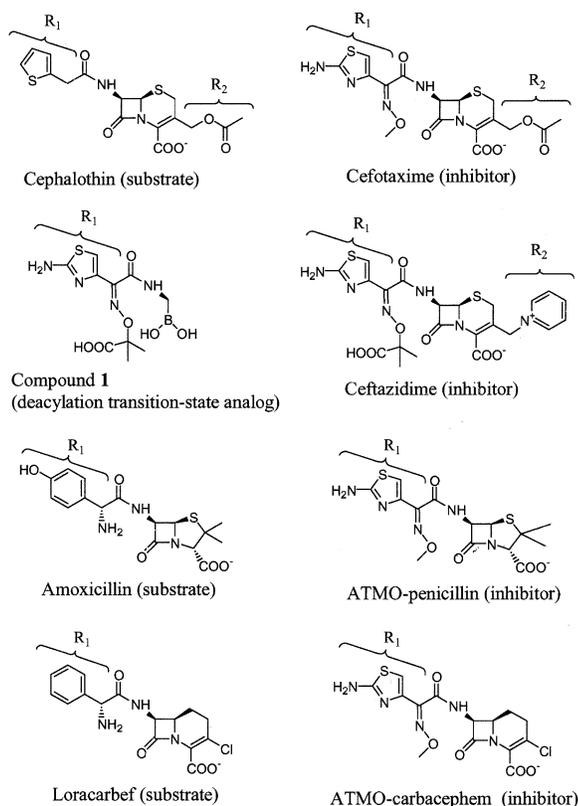


Figure 1. Class C  $\beta$ -Lactamase Substrates and Inhibitors Discussed in This Paper

losporins, then introduction of the ATMO group should convert other classes of  $\beta$ -lactams from substrates to inhibitors. Conversely, it may be that such side chains will only confer inhibition to  $\beta$ -lactams that also have a displaceable  $R_2$  side chain, which are generally restricted to the cephalosporins and oxacephem. Here, we investigate this question by introducing the ATMO group onto penicillin and carbacephem scaffolds, neither of which has a displaceable  $R_2$  side chain. The new

Table 1. Kinetic Data for Hydrolysis of Analogous  $\beta$ -Lactams by AmpC  $\beta$ -Lactamase

Compound	ATMO		
	$R_1$ group	$k_{cat}$ ( $s^{-1}$ ) <sup>a</sup>	$K_M$ ( $\mu M$ ) <sup>a</sup>
Cephalothin	–	263	31
Cefotaxime	+	0.0448	0.80 <sup>b</sup>
Penicillin G	–	133	5.0
ATMO-penicillin	+	<0.0045	0.90 <sup>b</sup>
Loracarbef	–	118	24
ATMO-carbacephem	+	0.0323	0.080 <sup>b</sup>

<sup>a</sup>Kinetic constants are accurate to within 20%.

<sup>b</sup> $IC_{50}$  reported instead of  $K_M$  [14].

$\beta$ -lactams, which we will refer to as ATMO-penicillin and ATMO-carbacephem (Figure 1), were indeed effective inhibitors, with  $IC_{50}$  values of 900 nM and 80 nM, respectively. To investigate the structural basis for this inhibition, the structure of the ATMO-penicillin/AmpC complex was determined by X-ray crystallography to 1.72 Å resolution and compared to the structure of AmpC in complex with a substrate, amoxicillin, which was determined to 1.87 Å resolution. To investigate the biological relevance of these inhibitors, the efficacy of ATMO-carbacephem was investigated in bacterial cell culture. These studies suggest a structural basis for the actions of these and potentially other sterically hindered inhibitors of class C  $\beta$ -lactamases, one that may have use in the design of new agents to overcome bacterial resistance.

## Results

### Enzymology

The AmpC-catalyzed hydrolysis of the  $\beta$ -lactams cefotaxime, ATMO-penicillin, and ATMO-carbacephem (Figure 1) were monitored by UV-Vis spectroscopy (Table 1). Consistent with the notion that steric hindrance can confer inhibition across  $\beta$ -lactam families, all of these ATMO-bearing  $\beta$ -lactams were good inhibitors for AmpC. Indeed, the  $k_{cat}$  of ATMO-penicillin was so low that we were only able to assign an upper bound of

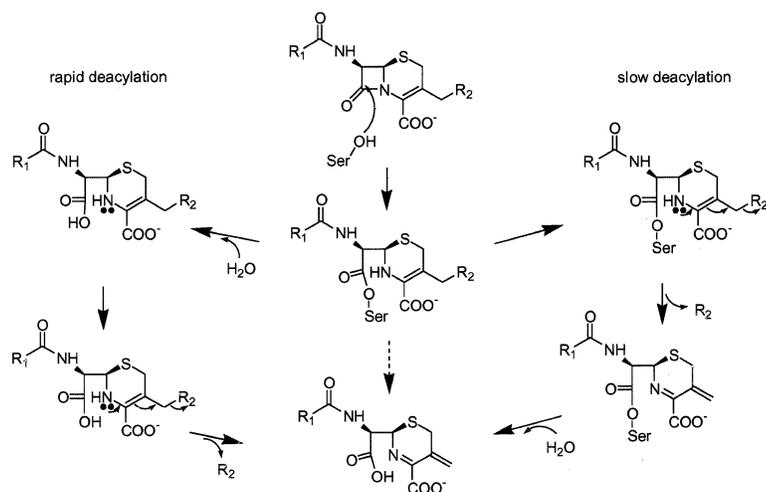


Figure 2. Two Possible Routes of Cephalosporin Hydrolysis by Class C  $\beta$ -Lactamases

The center scheme shows the overall reaction pathway, involving nucleophilic attack by a catalytic serine residue and eventual deacylation leading to hydrolyzed antibiotic, departed  $R_2$  leaving group, and free enzyme. The scheme on the left depicts the case where deacylation occurs rapidly, freeing the hydrolyzed antibiotic, which subsequently undergoes the electronic rearrangement that leads to the departure of the  $R_2$  group. The scheme on the right depicts the case where deacylation occurs more slowly, and so the electronic rearrangement and departure of the  $R_2$  group occurs while the  $\beta$ -lactam is still covalently bound to the enzyme.

Table 2. Data Collection and Refinement Statistics

	AmpC + Amoxicillin	AmpC + ATMO-Penicillin
Space group	C2	C2
Unit cell dimensions (Å, deg)	$a = 118.33, b = 76.85$ $c = 97.99, \beta = 116.51$	$a = 118.66, b = 76.73$ $c = 98.17, \beta = 116.19$
Number of complexes per asymmetric unit	2	2
Resolution (Å)	1.87	1.72
Number of observed reflections	225,850	270,758
Number of unique reflections	63,012	81,383
Completeness (%)	97.0 (97.0) <sup>a</sup>	97.0 (95.3) <sup>a</sup>
R <sub>merge</sub> (%)	5.6 (37.3) <sup>a</sup>	3.5 (24.3) <sup>a</sup>
$\langle I/\sigma_I \rangle$	30.70 (3.92) <sup>a</sup>	31.49 (4.36) <sup>a</sup>
Number of working reflections	56,774	74,290
Resolution range for refinement (Å)	20.0–1.87 (1.91–1.87) <sup>a</sup>	20.0–1.72 (1.76–1.72) <sup>a</sup>
Number of protein residues	715	715
Number of water molecules	353	598
Rmsd for bond lengths (Å)	0.0057	0.0126
Rmsd for bond angles (deg)	1.34	1.71
R <sub>cryst</sub> (%)	19.9	17.8
R <sub>free</sub> (%)	22.3 <sup>b</sup>	19.9 <sup>c</sup>
Average B-factor (Å <sup>2</sup> )		
Protein	29.16	23.62
Ligand <sup>d</sup>	35.70	29.46
Solvent	29.60	29.94

<sup>a</sup> Values in parentheses are for the highest-resolution shell used in refinement.

<sup>b</sup> R<sub>free</sub> was calculated with 2048 reflections set aside randomly.

<sup>c</sup> R<sub>free</sub> was calculated with 2324 reflections set aside randomly.

<sup>d</sup> Catalytically competent conformation only.

0.0045 s<sup>-1</sup> for this value. As is common with  $\beta$ -lactam inhibitors of  $\beta$ -lactamases, the  $K_M$  was too low to be determined for these ATMO-bearing inhibitors. Following Pratt and colleagues [14], we use the inhibition IC<sub>50</sub> values for these compounds as proxies for  $K_M$  rates when calculating turnover rates.

To isolate the role of bulky R<sub>1</sub> groups from possible influences due to R<sub>2</sub> side chain displacement on the inhibitory effect of these compounds, the turnover rates of  $\beta$ -lactam substrates from the same  $\beta$ -lactam families were determined. Cephalothin (for comparison to cefotaxime), penicillin G (for comparison to ATMO-penicillin), and loracarbef (for comparison to ATMO-carbacephem) were good substrates for AmpC, with  $k_{cat}$  values of 263 s<sup>-1</sup>, 133 s<sup>-1</sup>, and 118 s<sup>-1</sup>, respectively, about 10<sup>4</sup>- to 10<sup>5</sup>-fold faster than their ATMO-bearing analogs (Table 1).

### Crystal Structure of AmpC in Complex with Amoxicillin

The crystal structure of amoxicillin (Figure 1) covalently bound to AmpC was determined to a resolution of 1.87 Å (Table 2). 91.3% of the amino acid residues were in the “most favored” regions of the Ramachandran plot, and the remaining 8.7% were in “additionally allowed” regions. The final R<sub>cryst</sub> and R<sub>free</sub> values of the refined model were 19.9% and 22.3%, respectively.

Due to the high resolution of this structure, we were able to distinguish two distinct conformations of amoxicillin in its covalent complex. The predominant conformation (Figure 3A), modeled at 75% occupancy, shows the  $\beta$ -lactam carbonyl oxygen oriented in a catalytically competent conformation in the “oxyanion” [15] or “electrophilic” [16] hole formed by the backbone amide groups of Ser64 and Ala318. The other conformation shows this oxygen swung “out” of the hole in a catalyti-

cally incompetent conformation; the rest of the ligand position remains mostly unperturbed in the active site. The transient existence of multiple conformations of the acyl-enzyme species has been suggested previously by both Fourier transform infrared spectroscopic (FTIR) [17, 18] and crystallographic [19] studies. Further discussion will focus on the catalytically competent conformation of the ligand.

Key hydrogen-bonding interactions in the active site (Figure 3C) closely resemble those typically seen in covalent complexes of  $\beta$ -lactams with AmpC. These include the key interactions between the amide group of the R<sub>1</sub> side chain (Figure 1) and the conserved residues Gln120, Asn152, and Ala318 [5–7, 19, 20]. The putative deacylating water, Wat402, is also clearly observed and is stabilized by its interaction with Thr316. The position of Wat403, also important in the catalytic mechanism of AmpC [19], is also nearly identical to that seen in other substrate complexes [6, 19].

### Crystal Structure of AmpC in Complex with ATMO-Penicillin

The crystal structure of ATMO-penicillin (Figure 1) covalently bound to AmpC was determined to a resolution of 1.72 Å (Table 2). 92.8% of the amino acid residues were in the “most favored” regions of the Ramachandran plot, and 7.2% were in “additionally allowed” regions. The final R<sub>cryst</sub> and R<sub>free</sub> values of the refined model were 17.8% and 19.9%, respectively. As with the amoxicillin complex, two distinct conformations of the acyl-enzyme species were captured in the crystal structure: a more predominant conformation with the  $\beta$ -lactam carbonyl oxygen in the oxyanion/electrophilic hole (Figure 3B) and a lower occupancy (25%) conformation with the  $\beta$ -lactam carbonyl oxygen swung out of the oxyanion/

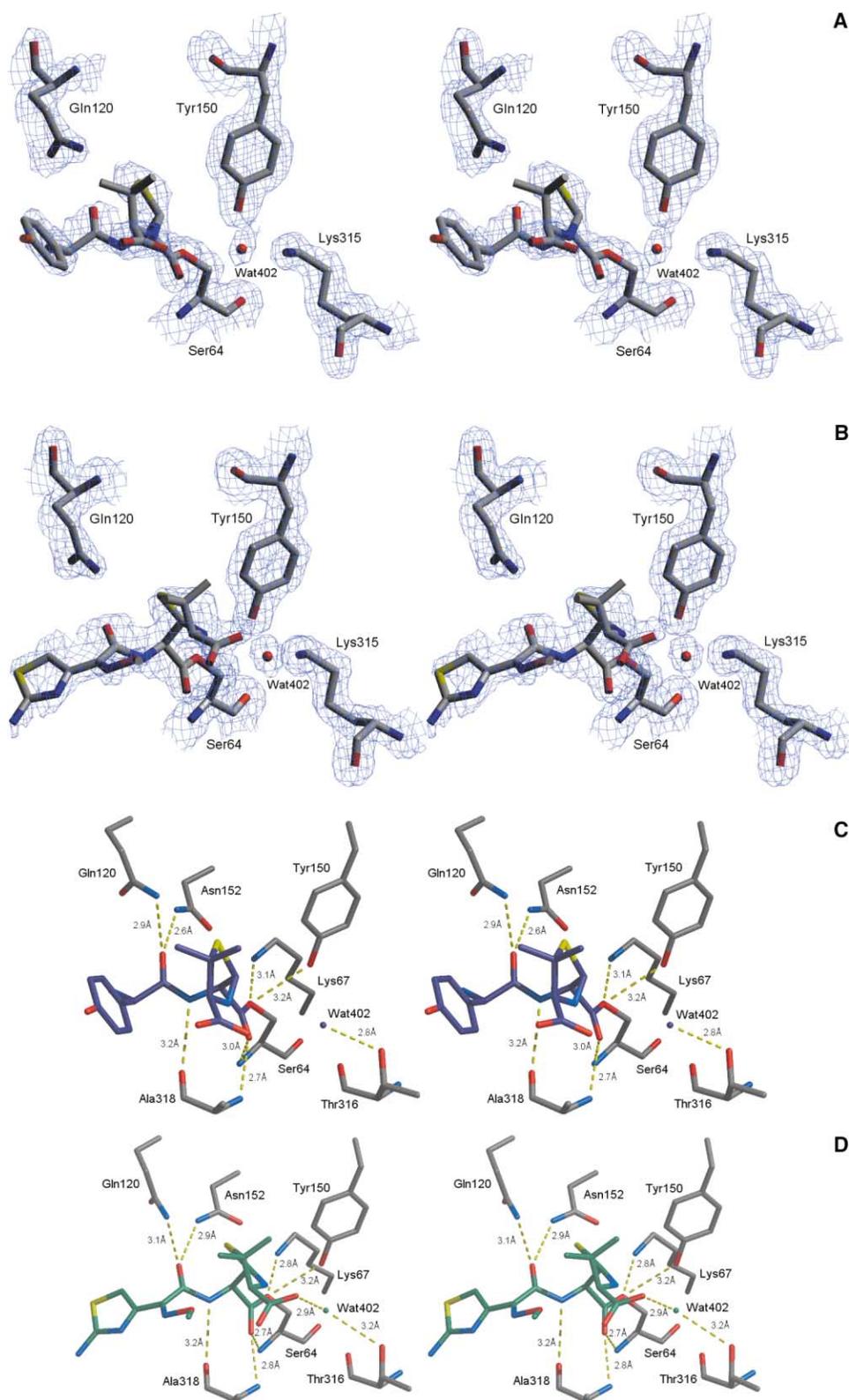


Figure 3. The Active Site of AmpC Covalently Bound to a Substrate and an Inhibitor  $\beta$ -Lactam

$2|F_o|-|F_c|$  electron density of the refined models of AmpC in complex with the (A) substrate amoxicillin and the (B) inhibitor ATMO-penicillin, contoured at  $1\sigma$ . Carbon atoms are colored gray, oxygen atoms red, nitrogens blue, and sulfurs yellow. The putative deacylating water, Wat402, is shown as a red sphere. Key hydrogen bond interactions in the active site of AmpC covalently bound to (C) amoxicillin and (D) ATMO-penicillin. Amoxicillin carbon atoms are colored indigo, and ATMO-penicillin carbons are sea green. Wat402 is the deacylating water and is colored the same as ligand carbons.

Table 3. Minimum Inhibitory Concentrations ( $\mu\text{g/ml}$ ) of Representative  $\beta$ -Lactams against Several Bacteria

Bacteria	Cephalothin	Cefotaxime	Loracarbef	ATMO-Carbacephem
<i>E. coli</i> not expressing AmpC $\beta$ -lactamase	8	0.00781	4	0.25
<i>E. coli</i> expressing AmpC $\beta$ -lactamase	64	0.0313	8	1
<i>E. cloacae</i> expressing $\beta$ -lactamase	>2048	64	256	64
<i>S. aureus</i> expressing $\beta$ -lactamase	0.25	1	2	2

electrophilic hole. The position of key active site residues has not changed significantly from the position adopted in the AmpC/amoxicillin complex, with an rmsd of 0.18 Å for those residues shown in Figures 3C and 3D.

Key hydrogen-bonding interactions in the active site (Figure 3D) in this complex resemble other covalent complexes of  $\beta$ -lactams with AmpC. A key difference between ATMO-penicillin and substrate complexes, such as those with loracarbef [6], cephalothin [19], and amoxicillin (above), is that the entire inhibitor is rotated such that the C3 carboxylate and the thiazolidine ring nitrogen occupy positions different from those adopted by substrate  $\beta$ -lactams. This conformation resembles that adopted by ceftazidime in its complex with AmpC and, like ceftazidime, seems to owe to interactions between the ATMO group and highly conserved residues at the distal end of the AmpC site, such as Val211 and Tyr221 [10]. The putative deacylating water is still observed in this complex, stabilized by both Thr316 and a  $\beta$ -lactam carboxylate oxygen.

### Microbiology

Serial dilution assays in ligand culture were performed to examine the efficacy of sterically hindered  $\beta$ -lactams against clinically relevant pathogens (Table 3). Against  $\beta$ -lactamase-expressing *Escherichia coli*, cefotaxime had an MIC of 0.031  $\mu\text{g/ml}$  and ATMO-carbacephem had an MIC of 1  $\mu\text{g/ml}$ , both much improved compared to the analogous  $\beta$ -lactams cephalothin and loracarbef, which had MIC values of 64 and 8  $\mu\text{g/ml}$ , respectively. When tested against a clinical isolate of *Enterobacter cloacae* that hyperproduces  $\beta$ -lactamase, ATMO-carbacephem and cefotaxime were again much more effective than their analogous parent compounds. Against a clinical isolate of *Staphylococcus aureus* that expresses a class A  $\beta$ -lactamase, ATMO-carbacephem was just as effective (2  $\mu\text{g/ml}$ ) as loracarbef, whereas cefotaxime was slightly worse (1  $\mu\text{g/ml}$  versus 0.25  $\mu\text{g/ml}$ ) than cephalothin, as might be expected of a third-generation cephalosporin. The ATMO-bearing  $\beta$ -lactamase inhibitors also showed good efficacy as antibiotics themselves against  $\beta$ -lactamase-negative *E. coli*, with MIC values of 0.008  $\mu\text{g/ml}$  and 0.25  $\mu\text{g/ml}$  for cefotaxime and ATMO-carbacephem, respectively.

### Discussion

Sterically hindered inhibitors are recognized well enough by an enzyme to form a covalent adduct, but once in that adduct are distorted away from a catalytically competent conformation because such a conformation would require steric clashes between the ligand and the enzyme. It is therefore difficult for the intermediate to move on to the next stage of the reaction—

hydrolytic attack in the case of  $\beta$ -lactams bound to  $\beta$ -lactamases. If conformational distortion is indeed a useful design principal, it should be possible to transfer groups that confer it to other families of  $\beta$ -lactams, converting substrates into inhibitors. The ability to convert two different classes of substrates for AmpC, a penicillin and a carbacephem, into submicromolar inhibitors by the addition of an appropriate  $R_1$  group from a third class, the cephalosporins, is thus consistent with this idea and suggests that the bulky ATMO group of the third-generation cephalosporins is the primary determinant of inhibition for all three classes of inhibitors. This may be understood in detail by considering the kinetic and structural attributes of these inhibitors.

### Enzymology

The hydrolytic rate constants,  $k_{\text{cat}}$ , for cefotaxime, ATMO-penicillin, and ATMO-carbacephem are low enough,  $10^4$ - to  $10^5$ -fold reduced compared to their analogous substrates, that they may be usefully considered to be inhibitors of AmpC (Table 1). When tested in the presence of a good substrate such as cephalothin or nitrocefin, these ATMO-containing  $\beta$ -lactams have  $\text{IC}_{50}$  values ranging from 900 nM for ATMO-penicillin to 80 nM for ATMO-carbacephem (Table 1). In a series of elegant enzymological studies [11–14, 21], Pratt and colleagues have shown that the inhibition conferred by the ATMO group in third-generation cephalosporins is coupled to the ability of the  $R_2$  side chain of these  $\beta$ -lactams to depart when in the acyl-adduct complex, rendering the resulting adduct less susceptible to hydrolytic attack (Figure 2). Others have posited that it is not the displaceability of this  $R_2$  side chain but rather the electron-withdrawing inductive effect that this group has on the  $\beta$ -lactam core [22, 23] that is more important. For ATMO-penicillin and ATMO-carbacephem, which do not have a displaceable  $R_2$  group, neither of these explanations is true; the entire inhibitory effect may be attributed to the addition of the ATMO side chain. This may be understood quantitatively by comparing the deacylation rate constants,  $k_{\text{cat}}$ , for the ATMO-bearing inhibitors to their analogous substrates that lack this group. The ratios of the turnover rates (Table 1) for each pair of  $\beta$ -lactams (ATMO-bearing inhibitor divided by non-ATMO-bearing substrate) can be compared:

$$(I) \frac{\text{cefotaxime } k_{\text{cat}}}{\text{cephalothin } k_{\text{cat}}} \quad (II) \frac{\text{ATMO-penicillin } k_{\text{cat}}}{\text{penicillin G } k_{\text{cat}}} \quad (III) \frac{\text{ATMO-carbacephem } k_{\text{cat}}}{\text{loracarbef } k_{\text{cat}}}$$

Were the ratio (I) in the cephalosporin pair (with a readily displaceable acetate  $R_2$  group) lower than that for the penicillin (II) or carbacephem (III) pairs (which have no displaceable  $R_2$  group), then the rapid departure of the cephalosporin  $R_2$  group would be key to trapping the complex in its acyl-enzyme state (since cefotaxime and

cephalothin have identical  $R_2$  groups), and the inhibitory effect of the ATMO group would not be fully transferable to  $\beta$ -lactams that lack an  $R_2$  group. However, this is not observed: the  $k_{\text{cat}}$  ratio is actually larger for the cephalosporin pair ( $1.71 \times 10^{-4}$ ) than for the penicillin pair ( $3.38 \times 10^{-5}$ ) and very similar to the carbacephem pair ( $2.73 \times 10^{-4}$ ), indicating that the ATMO-containing analogs for these  $\beta$ -lactams are just as or *more* stable to hydrolysis compared to their analogous substrate than is the cephalosporin (Table 1). Qualitatively, the same pattern may be seen if the  $\text{IC}_{50}$  values for cefotaxime, ATMO-penicillin, and ATMO-carbacephem are compared – the value for ATMO-carbacephem is 11-fold lower than that of cefotaxime and the value for ATMO-penicillin is comparable to cefotaxime (we note that  $\text{IC}_{50}$  values convolute acylation and deacylation rate constants, and so must be interpreted with care). These results demonstrate the transferability of bulky  $R_1$  groups that have thus far been used only in the context of cephalosporins and monobactams (in the case of aztreonam). The synthesis of new  $\beta$ -lactams that incorporate these groups thus need not be limited to cephalosporins, but in fact should be general to any compound with the  $\beta$ -lactam core, such as the penicillins and carbacephems demonstrated here.

#### Crystal Structure of AmpC in Complex with Amoxicillin

To understand the structural bases of how the ATMO group confers inhibition to the penicillins, it was first necessary to determine the structure of a penicillin substrate of AmpC. This complex between wild-type AmpC and amoxicillin is the first structure of a penicillin substrate in complex with a class C  $\beta$ -lactamase. Penicillin substrates bind in the active site of AmpC (Figure 4A) in a nearly identical manner as the carbacephem substrate loracarbef [6] and the cephalosporin substrate cephalothin [19]. In this, the class C  $\beta$ -lactamases appear different from the class A  $\beta$ -lactamases, where cephalosporins and penicillins are seen to adopt different conformations in the active site [24]. The key interactions between the  $R_1$  amide group and conserved active site residues Gln120, Asn152, and Ala318 are retained, as is the positions of the  $\beta$ -lactam carbonyl oxygen. The five-membered penicillin ring overlays closely with the six-membered loracarbef and cephalothin rings, as do the ubiquitous carboxylate substituents on each of these rings. The putative deacylating water, Wat402, occupies a nearly identical location as that seen in the loracarbef and cephalothin complexes, implying a catalytic mechanism identical to that seen with the cephalosporins [19]. Overlaying the structure of the amoxicillin/AmpC complex with that of a deacylation transition-state analog bearing the ceftazidime  $R_1$  side chain, compound 1 (Figure 1), in complex with AmpC [10] shows that the  $\beta$ -lactam ring nitrogen is positioned to stabilize the deacylation transition state complex, being 3.0 Å from the expected position of the deacylating water in the high-energy intermediate (Figure 4B). In short, we see that the catalytically competent conformation of amoxicillin closely resembles that of carbacephem and

cephalosporin substrates, and that the hydrolytic mechanism seems to be shared among these different classes of  $\beta$ -lactams.

#### Crystal Structure of AmpC in Complex with ATMO-Penicillin

The acyl-adduct structure of AmpC covalently bound to ATMO-penicillin closely resembles that when bound to the third-generation cephalosporin ceftazidime (Figure 1). Like ceftazidime, the bulky ATMO group on the penicillin derivative appears to force the thiazolidine ring (the analog of the dihydrothiazine ring on the cephalosporin) into a conformation where it would block the formation of the high-energy deacylation intermediate. Although the ATMO  $R_1$  group is somewhat smaller than that of ceftazidime, a comparison of the binding modes of ATMO-penicillin and ceftazidime indicates that the  $R_1$  groups bind very similarly, with nearly identical positions for the amide groups, aminothiazole rings, and methoxime substituents. The additional bulk of the 1,1-dimethyl-1-carboxylate group of ceftazidime appears to only further displace the six-membered cephalosporin ring relative to the five-membered penicillin ring of ATMO-penicillin.

The fundamental mechanism of inhibition observed is the same as was observed with cloxacillin [6], moxalactam [6, 9], and ceftazidime [10], namely the destabilization of the deacylation transition state. In contrast to the 3.0 Å distance between the  $\beta$ -lactam ring nitrogen of amoxicillin and the position of the deacylating water in the high-energy intermediate state, the analogous distance in the ATMO-penicillin complex would be a mere 1.7 Å (Figure 4C). These atoms would be so close in space as to be in van der Waals violation; thus the formation of the deacylation transition state would be destabilized by the position of the ring nitrogen relative to the degree of stabilization afforded to the transition state by the substrates.

Of interest to the specialist will be the two conformers that we observe in both the amoxicillin and ATMO-penicillin complexes with AmpC. FTIR spectroscopic studies [17, 18] suggest that, in their acyl-adducts with  $\beta$ -lactamases, the carbonyl group that forms the ester with the catalytic serine (Ser64 in AmpC) can adopt more than one conformation. Until recently, there had been no crystallographic evidence for such libration within the acyl adduct. Recent X-ray structures involving both class A [25] and class C [19]  $\beta$ -lactamases have suggested that such alternate conformations can indeed be observed crystallographically. The resolution of the structures reported here is sufficient to discern both the canonical conformation, with the carbonyl oxygen in the “oxyanion” [15] or “electrophilic” [16] hole (modeled at 75% occupancy in each structure), and the conformation with the carbonyl oxygen flipped out of the hole (modeled at 25% occupancy). Given the FTIR data [17, 18], these alternate conformations may be kinetically relevant. As a crystallographic matter, they may partly explain why it has been possible to capture the acyl-adduct of the substrate amoxicillin in the wild-type crystal, as the activity of the enzyme clearly is slowed in the crystal environment.

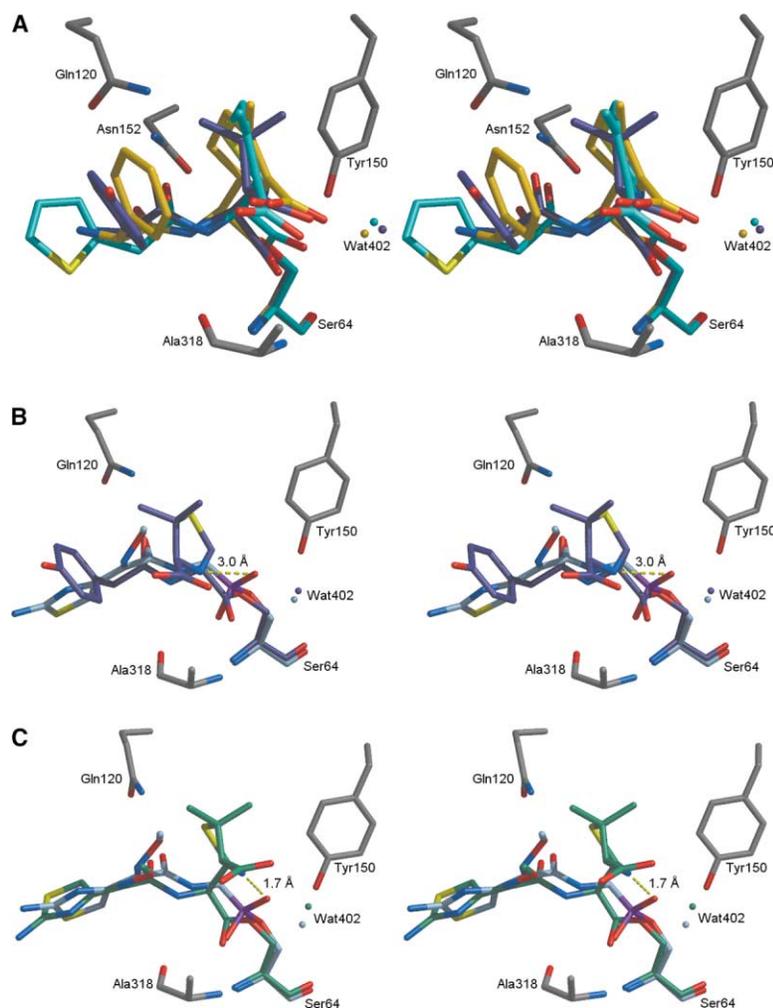


Figure 4. Steric Hindrance and Inhibition

AmpC in complex (A) with the substrates amoxicillin (in indigo), loracarbef [6] (in gold), and cephalothin [19] (in cyan). AmpC complexes with (B) amoxicillin (in indigo) and (C) ATMO-penicillin (in sea green) overlaid with the deacylation transition state analog 1 [10] (in light blue). (The dimethyl-carboxylate group of the transition state analog has been omitted for clarity.) The distance between the ring nitrogen and the presumed position of the hydrolytic water in the deacylation high-energy intermediate is indicated.

### Implications for Inhibitor Design

Perhaps the result with the most therapeutic implications to emerge from the high-resolution structure of ATMO-penicillin in complex with AmpC is insight into the transferability of bulky  $R_1$  groups responsible for inhibition. ATMO and other bulky groups have become mainstays in the design of synthetic third- and fourth-generation cephalosporins, with little obvious attempt to apply this chemical design to other families of  $\beta$ -lactams. Examination of the binding modes of several ligands with very similar bulky  $R_1$  substituents shows nearly identical modes of binding—and consequently inhibition—among various  $\beta$ -lactam families: monobactams [5], cephalosporins [10], and now penicillins. The size of the ligand's second ring, whether zero-membered in the case of monobactams, five-membered in the case of penicillins, or six-membered in the case of the cephalosporins (and, presumably, carbacephems as well), does not seem to affect the key mechanism of inhibition, namely destabilization of the deacylation transition state relative to substrate deacylation.

The use of destabilizing groups as components of  $\beta$ -lactamase inhibitors is a recurring synthetic theme, and similar inhibition has been demonstrated before in

compounds with bulky  $6(7)\alpha$  groups [6, 9, 25]. Of course, steric bulk alone is an overly simplistic description for these effects. A relatively small group in the right position, such as the  $7\alpha$ -methoxy in moxalactam, can convert a substrate into an inhibitor, whereas a bulky group in the wrong position can leave the  $\beta$ -lactam a substrate. For the steric hindrance approach to be effective, the groups must be introduced in such a way as to force an unfavorable interaction with the enzyme in the acyl adduct. Given the structure of a substrate-enzyme complex, the design of a distorting bulky group is typically straightforward, indeed much more so than the design of complementary interactions, which are the focus of classical structure-based inhibitor design. By adding bulky substituents to a conserved core essential for recognition by the enzyme (the  $\beta$ -lactam ring), good substrates can be turned into potent inhibitors.

Because the pharmacological and synthetic properties of the various  $\beta$ -lactam families are well understood, these insights may be useful in designing new leads for drug discovery against  $\beta$ -lactamases. Indeed, in cell culture studies ATMO-carbacephem was much more effective against gram-negative bacteria that express class C  $\beta$ -lactamases (Table 3) than was loracarbef or

cephalothin and was about as effective as ceftazidime. Although ATMO-carbacephem was no better than loracarbef against a *Staphylococcus aureus* clinical isolate that expresses a class A  $\beta$ -lactamase and worse than the traditional amoxicillin/clavulanate, its MIC values are low enough to be interesting as a lead compound.

## Significance

$\beta$ -lactamases are important therapeutic targets because of their prominent role in resistance to  $\beta$ -lactam antibiotics, such as penicillins and cephalosporins. The characteristics separating  $\beta$ -lactam substrates from  $\beta$ -lactam inhibitors of these enzymes have been the subject of much research. Here we find that the substitution of an appropriate bulky side chain in place of a small one at the 6(7) $\beta$  position is sufficient to convert  $\beta$ -lactam substrates into potent inhibitors of class C  $\beta$ -lactamases. X-ray crystal structures demonstrate that this bulky side chain forces the inhibitor to adopt a catalytically incompetent conformation in the acyl-enzyme complex, blocking further reaction. These molecules are thus able to proceed through the first half of the  $\beta$ -lactamase mechanism but are unable to continue through the second, hydrolytic half of the reaction, and so remain bound in the acyl-adduct, effectively trapping the enzyme. We distinguish these sterically hindered  $\beta$ -lactams from mechanism-based inhibitors, whose covalent adducts typically form through off-pathway reactions. Steric hindrance provides a simple criterion for the design of new  $\beta$ -lactamase inhibitors, and it may be that such a design strategy could be applied to other enzymes that proceed through a covalent adduct as part of their catalytic mechanism. These inhibitors also show some promise as antibiotics against resistant bacteria, with one of the new  $\beta$ -lactams active against  $\beta$ -lactamase-expressing strains of *Enterobacter cloacae*, *Escherichia coli*, and *Staphylococcus aureus* that are resistant to most other  $\beta$ -lactam antibiotics.

## Experimental Procedures

### Chemical Synthesis

Reactions were carried out with continuous stirring under a positive pressure of nitrogen except where noted. Reagents and solvents were purchased and used without further purification. Thin layer chromatography was performed with 0.25 mm silica gel 60 plates with a 254 nm fluorescent indicator from E. Merck (Mumbai, India). Plates were developed in a covered chamber and visualized by ultraviolet light or by treatment with 5% phosphomolybdic acid in ethanol followed by heating. Flash chromatography was carried out with silica gel 60, 230–400 mesh (0.040–0.063 mm particle size) purchased from EM Science (Cincinnati, OH). High performance liquid chromatography (HPLC) purifications were performed using Waters (Milford, MA) X-terra C18 columns with the specified solvent system and flow rate. NMR spectra are reported as chemical shifts in parts per million (ppm) downfield from a tetramethylsilane internal standard (0 ppm).  $^1\text{H}$  NMR spectra were recorded in the solvent indicated on a Varian Mercury spectrometer at 400.21 MHz. Electrospray mass spectra (ES/MS) were recorded on a Micromass (Beverly, MA) Platform LCZ spectrometer.

### 6-[Z-(2-Aminothiazol-4-Yl)-Methoximinoacetamido]-Penicillanic Acid, Ammonium Salt ("ATMO-Penicillin")

Z-(2-aminothiazol-4-yl)-methoximinoacetic acid (1.01 g, 5 mMol) was slurried in *N,N*-dimethylformamide (15 ml) at room temperature.

2-Chloro-4,6-dimethoxy-1,3,5-triazine (966 mg, 5.5 mMol) and *N*-methylmorpholine (0.583 ml, 5.3 mMol) were added. The mixture was stirred at room temperature for 30 min, at which time the system was a homogeneous solution of active ester. In a separate flask, the tosylate salt of allyl penicillanate [26] (2.31 g, 5.4 mMol) was slurried in acetonitrile (11 ml). *N*-methylmorpholine (1.21 ml, 11 mMol) was added and the mixture was stirred until homogeneous. The penicillin solution was then added to the ATMO-active ester solution via syringe over  $\sim$ 2 min, and the resulting acylation mixture was stirred at room temperature for 12 hr. The reaction mixture was diluted with ethyl acetate and partitioned with 0.2 M phosphate buffer at pH 4. The organic phase was washed with additional 0.2 M phosphate buffer at pH 4 ( $3\times$ ) and brine ( $1\times$ ), dried (anhydrous magnesium sulfate), and finally concentrated in vacuo to an oil.

The crude ATMO-penicillin allyl ester (2.23 g) was adsorbed on silica gel-60 (10 g) and chromatographed over silica gel-60 (10 g) using a gradient elution of chloroform to 10% methanol in chloroform. Appropriate fractions were combined and concentrated in vacuo to yield an oil (1.83 g, 83%) with spectral characteristics (ES/MS,  $^1\text{H}$  NMR) consistent with ATMO-penicillin allyl ester.

To a stirred solution of palladium(II)acetate (25 mg, 0.11 mMol) in acetonitrile (2 ml) was added a solution of triphenylphosphine (140 mg, 0.53 mMol) in acetonitrile (1.3 ml). A thick yellow-green precipitate formed in  $\sim$ 15 min. To the vigorously stirred precipitate was added tri(*n*-butyl)stannane (66  $\mu\text{l}$ , 0.25 mMol). Stirring was continued for 30 min, at which point a solution of ATMO-penicillin allyl ester (1.83 g, 4.16 mMol) in ethyl acetate (15 ml) was added in one portion. The reaction mixture immediately became homogeneous. A solution of sodium 2-ethylhexanoate in ethyl acetate (0.5 M, 10 ml) was added dropwise over  $\sim$ 5 min, during which time a precipitate formed. The crude precipitated ATMO-penicillin sodium salt was isolated by low-speed centrifugation of the reaction mixture. The solid was washed with ether ( $3\times$ ) and dried in vacuo to afford the crude salt (1.05 g).

Pure ATMO-penicillin was isolated as the ammonium salt by preparative HPLC and lyophilization of relevant fractions. Method: 19 mm  $\times$  300 mm Waters X-terra C18 column (5  $\mu\text{m}$ ), 20 ml/min, gradient elution 5%–40% methanol in aqueous ammonium carbonate over 30 min. ES/MS (positive ion) 400.1 [M + H], 240.9 [ $\beta$ -lactam "vertical cleavage"] (negative ion) 398.1 [M – H];  $^1\text{H}$  NMR in Dimethylsulfoxide- $d_6$  (shift, multiplicity/integration, J Hz) 1.39, s 3H; 1.49, s 3H; 3.74, s 3H; 5.39, d 1H; J = 3.9; 5.43, d/d 1H, J = 3.9/7.4; 6.68, s 1H; 9.36, d 1H; J = 7.4.

### 7-[Z-(2-Aminothiazol-4-Yl)-Methoximinoacetamido]-1-Carba(Dethia)-3-Chloro-Cephem-4-Carboxylic Acid, Ammonium Salt ("ATMO-Carbacephem")

This was prepared in an analogous manner, with the analytical sample isolated by preparative HPLC. ES/MS (positive ion) 385.2 [M + H], 406.9 [M + Na]; (negative ion) 383.1 [M – H];  $^1\text{H}$  NMR in Dimethylsulfoxide- $d_6$  (shift, multiplicity/integration, J Hz) 1.81, m 2H; 2.70, m 2H; 3.69, s 3H; 3.78, m 1H; 5.11, d/d 1H; J = 5.0/7.3; 6.65, s 1H; 9.27, d 1H; J = 7.3.

## Enzymology

*Escherichia coli* AmpC  $\beta$ -lactamase was expressed and purified to homogeneity as previously described [16]. AmpC-catalyzed  $\beta$ -lactam hydrolysis was monitored in an HP8453 UV/visible spectrophotometer. For cephalothin (Sigma, St. Louis, MO) and cefotaxime (Sigma), reactions were monitored at 265 nm, penicillin G (Sigma) and ATMO-penicillin at 235 nm, loracarbef at 262 nm, and ATMO-carbacephem at 260 nm. The reaction buffer used during the assays was 50 mM Tris-HCl (Fisher, Fair Lawn, NJ) at pH 7 in doubly deionized water.  $k_{\text{cat}}$  and  $K_M$  values were determined at an enzyme concentration of 1.75 nM, with enzyme concentration determined, from more concentrated stocks, based on an  $\epsilon_{280}$  of 0.098  $\mu\text{M}^{-1}\text{cm}^{-1}$ . Where  $K_M$  values could not be determined because they were so low (i.e., for molecules that behaved more like inhibitors),  $\text{IC}_{50}$  values were determined against 200  $\mu\text{M}$  cephalothin for ATMO-penicillin and cefotaxime and 200  $\mu\text{M}$  nitrocefin (Oxoid, Ogdensburg, NY) in the case of ATMO-carbacephem [14]. Except for compounds synthesized specifically for this study (ATMO-penicillin, ATMO-carbacephem), all compounds were used as supplied from the manufacturers without further purification.

### Crystal Growth and Structure Determination

Purified AmpC was crystallized in 1.7 M potassium phosphate (KP) buffer at pH 8.7 as described [9]. AmpC crystals were harvested and placed in a 6  $\mu$ l drop of 1.7 M KP, (pH 8.7) and soaked with excess ligand (50–70 mM) for 15 min and then transferred to a fresh drop for another 15 min to obtain the acyl-enzyme complexes presented here. The crystals were then transferred to a pH 8.7 cryoprotectant solution containing 20% sucrose, 1.7 M KP, and excess ligand (50 mM) for 10–15 s before being flash frozen in liquid nitrogen.

Diffraction data were collected at DND-CAT beamline 5-IDB at the Advanced Photon Source at 100 K using a Mar-CCD detector. Reflections were indexed, integrated, and scaled using HKL software [27] (Table 2). The complexes crystallized in the C2 space group, with two AmpC molecules per asymmetric unit, each containing 358 amino acid residues. Out of a possible 716 residues, 715 were included in each final model. The initial model was built by molecular substitution using an apo AmpC structure (Protein Data Bank ID code 1KE4) without solvent molecules. This model was refined with CNS [28] using rigid body, simulated annealing, positional minimization, and individual B factor refinement, with the maximum likelihood target, and included bulk solvent correction and a  $2\sigma$  cutoff for the data. Manual model building into  $\sigma$ -A weighted electron density maps using O [29] alternated with rounds of positional and B factor refinement in CNS.

Molecule two in the asymmetric unit exhibited stronger electron density for the ligands in both structures. Ligands were built into the  $2|F_o|-|F_c|$  and  $|F_o|-|F_c|$  difference density in this monomer for both structures. Simulated-annealing omit density was used to confirm the placement of the ligands in the active site. The relative occupancies of the two conformations for each ligand were determined based on optimization of the difference density for each and also by comparing the refined thermal factors of the ligand atoms with those of Ser64, to which they are covalently bound.

### Microbiology

The effectiveness of cephalothin, cefotaxime, loracarbef, and ATMO-carbacephem was tested against bacteria growing in liquid culture. The compounds were dissolved in 50 mM Tris buffer, and serial dilutions were performed into Luria Broth (Difco, Detroit, MI). Each broth solution was then inoculated with bacterial cells from an overnight culture that had been diluted to give an inoculum concentration of approximately  $5 \times 10^5$  cfu/ml. The MIC of the test compounds was determined against both AmpC-expressing and AmpC-negative laboratory strains of JM109 *Escherichia coli* and  $\beta$ -lactamase-expressing clinical isolates of *Enterobacter cloacae* and *Staphylococcus aureus*. In *E. coli*, AmpC was expressed off of the high-expression vector pOGO295, which is the same vector used for protein expression and purification [16].

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#### Accession Numbers

Structure factors and coordinates for amoxicillin and ATMO-penicillin in complex with AmpC have been deposited in the Protein Data Bank with ID codes 1LL9 and 1LLB, respectively.