Emergence of methicillin-resistant \textit{Staphylococcus aureus} (MRSA) has created challenges in treatment of nosocomial infections. The recent clinical emergence of vancomycin-resistant MRSA is a new disconcerting chapter in the evolution of these strains. \textit{S. aureus} normally produces four PBPs, which are susceptible to modification by \beta-lactam antibiotics, an event that leads to bacterial death. The gene product of \textit{mecA} from MRSA is a penicillin-binding protein (PBP) designated PBP 2a. PBP 2a is refractory to the action of all commercially available \beta-lactam antibiotics. Furthermore, PBP 2a is capable of taking over the functions of the other PBPs of \textit{S. aureus} in the face of the challenge by \beta-lactam antibiotics. Three cephalosporins (compounds 1–3) have been studied herein, which show antibacterial activities against MRSA, including the clinically important vancomycin-resistant strains. These cephalosporins exhibit substantially smaller dissociation constants for the preacylation complex compared with the case of typical cephalosporins, but their pseudo-second-order rate constants for encounter with PBP 2a (\(k_2/K\)) are not very large (\(\leq 200 \text{ m}^{-1} \text{s}^{-1}\)). It is documented herein that these cephalosporins facilitate a conformational change in PBP 2a, a process that is enhanced in the presence of a synthetic surrogate for cell wall, resulting in increases in the \(k_2/K\) parameter and in more facile enzyme inhibition. These findings argue that the novel cephalosporins are able to co-opt interactions between PBP 2a and the cell wall in gaining access to the active site in the inhibition process, a set of events that leads to effective inhibition of PBP 2a and the attendant killing of the MRSA strains.

\textit{Staphylococcus aureus} was exquisitely sensitive to penicillins in the early years of the use of \beta-lactams in the clinic. In the 1940s, resistance to first generation penicillins emerged from a profusion of strains containing a class A \beta-lactamase. In response to the \beta-lactamase challenge, a second generation of penicillins that included methicillin was introduced in 1959. By 1961, a strain of \textit{S. aureus} emerged first in the United Kingdom and subsequently in other parts of the world, which was resistant to methicillin and other \beta-lactams. This strain became known as methicillin-resistant \textit{S. aureus} (MRSA). The recent emergence of variants of MRSA that are resistant to glycopeptide antibiotics, such as vancomycin, due to the acquisition of the \textit{vsaA} gene from Enterococcus, has created a new and disconcerting chapter in the evolution of this organism. MRSA is one of the most serious infectious disease agents globally. The need for novel antibiotics to treat this organism is genuine.

The basis for MRSA resistance to \beta-lactam antibiotics is complex (1–5). Nonetheless, a primary factor is the acquisition of the gene \textit{mecA}, which encodes a penicillin-binding protein (PBP) designated PBP 2a. Whereas \beta-lactam antibiotics can inhibit their target PBPs in penicillin-sensitive \textit{S. aureus} strains, a process that impairs the final steps of cell wall biosynthesis and leads to bacterial death, PBP 2a is refractory to inhibition by all commercially available \beta-lactam antibiotics (5–8). Hence, whereas \beta-lactam antibiotics inhibit the regular \textit{S. aureus} PBPs, the \beta-lactam-insensitive PBP 2a takes over their biosynthetic functions. Hence, MRSA acquires a survival advantage to the challenge by \beta-lactam antibiotics. The basis for PBP 2a resistance to inhibition is complex. The rate constant for acylation of the active site serine by the \beta-lactam is attenuated, and the dissociation constant for the preacylation \beta-lactam-enzyme complex is less favorable (6, 9). An additional reason for PBP 2a \beta-lactam resistance would appear to be the lack of access of the \beta-lactam to its active site. The active site of PBP 2a is normally closed and must open to bind to the bacterial cell wall, its substrate (10, 11). We recently documented that in concert with interactions with cell wall fragments outside the active site, the active site becomes more accessible by a conformational change (10).

The clinical difficulties in treatment of MRSA have prompted the pharmaceutical industry to search for new \beta-lactams active against the PBP 2a of MRSA (12–14). We report herein our analyses with three novel cephalosporins, 1–3 (Fig. 1), discovered at the Sandoz Antibiotic Research Institute of Vienna. Cephalosporin 1 is currently undergoing clinical trials for treatment of MRSA infections. These cephalosporins are found to have significantly smaller dissociation constants for the preacylation complex with PBP 2a compared with other \beta-lactam antibiotics, as will be discussed. However, the rate constants for the PBP 2a acylation by these three are similar to those for other \beta-lactams. Moreover, the rate constants for PBP 2a acylation by these cephalosporins are enhanced considerably in the presence of an analogue of a fragment of bacterial cell wall (compound 4). This suggests that these cephalosporins might be co-opting interactions of the cell wall with PBP 2a \textit{in vivo} to gain access to the active site of this enzyme in the course of covalent inhibition that leads to bacterial death.

**EXPERIMENTAL PROCEDURES**

The cloning and purification of soluble PBP 2a has been described (6, 10).
Inhibition of PBP 2a of MRSA

Determination of the Kinetic Parameters for Interactions of Cephalosporins with PBP 2a—The first-order rate constants for the interactions between the cephalosporins 1–3 and PBP 2a of MRSA were evaluated using a Cary 50 UV spectrophotometer (Varian Inc.) at room temperature. The reaction between PBP 2a and nitrocefin was monitored by the formation of the acyl-enzyme species at 500 nm (Δε₅₀₀ = +15,900 cm⁻¹ M⁻¹). The experiments were carried out in 25 mM Hepes, 1 M NaCl (pH 7.0) buffer. Nitrocefin (120 μM) was used as the reporter molecule to determine the apparent first-order rate constants for acylation by the nonchormogenic (or poorly chromogenic) cephalosporins 1–3 at varying concentrations (10–120 μM) in the presence and absence of compound 4 (0.5–2 mM) by the methods of Graves-Woodward et al. (15). Compounds 1–3 were dissolved in 25 mM Hepes, 1 M NaCl (pH 7.0) buffer. UV-spectroscopic analysis of the compounds in the range of 200–350 nm at room temperature for 4 days indicated no change, consistent with the stability of the antibiotics within this duration.

The deacylation rate constants (kᵣ) were determined using BOCILLIN FL as a reporter molecule. A typical reaction mixture (60 μl) contained 15 μM PBP 2a and cephalosporins 1, 2, or 3 at a concentration 2-fold higher than its Kᵣ value. The mixture was incubated at room temperature for 45 min in 25 mM Hepes, 1 M NaCl (pH 7.0) buffer. The excess cephalosporin was removed by passing the mixture through a Micro Bio-Spin®6 column (Bio-Rad). Deacylation rate constants for cephalosporins 1–3 were determined both in the presence and absence of the cell wall surrogate compound 4. In the experiments in the presence of compound 4, it (1.5 mM) was added to the resultant mixture immediately following the removal of excess antibiotic by passing the mixture through a Micro Bio-Spin®6 column (Bio-Rad). An aliquot (3 μl) of the mixture was diluted 5-fold with the same buffer and incubated at room temperature for different time intervals. The amount of free protein, liberated from the acyl-enzyme species, was assayed by the addition of BOCILLIN FL to afford a final concentration of 40 μM and incubated for an additional 20 min at room temperature. SDS sample buffer (15 μl) was added to the reaction mixture, which was then boiled for 3 min. The samples (30 μl in total) were loaded onto a 10% SDS-polyacrylamide gel, which was developed and then scanned using a Storm840® Fluorimager.

Circular Dichroism—The CD spectra of PBP 2a (3 μM in 25 mM Hepes, 1 M NaCl, pH 7.0) were recorded on a stopped-flow circular dichroism spectrometer (AVIV INSTRUMENTS INC-202 SF) with a 2-mm path length in the absence and presence of a 6 μM concentration of each of the cephalosporins 1–3 at 25 °C. The nearly negligible contribution of each cephalosporin by itself was subtracted in each case. The ensuing conformational changes were then monitored for 96 h.

Bacteria—The strain panel was comprised of S. aureus COL, S. aureus COLVA, S. aureus VR5 isolated in Michigan (16), and S. aureus VR5 isolated in Pennsylvania (17). All four strains were obtained from the Network on Antimicrobial Resistance in Staphylococcus aureus. Also screened was a clinical isolate of vancomycin-resistant Enterococcus faecalis (E. faecalis 99) obtained from Wayne State University Medical Center.

Susceptibility Testing—MICs were determined four times in each case by a broth microdilution method using cation-adjusted Mueller-Hinton II broth and procedures from the National Committee for Clinical Laboratory Standards (NCCLS) (18).

Solubility of the Acyl-enzyme Species of Cephalosporins 1–3 and PBP 2a—A typical reaction mixture (100 μl) contained 9 μM PBP 2a and 20 μM cephalosporins 1, 2, or 3. The mixture was incubated at room temperature for 30 min in 25 mM Hepes, 1 M NaCl (pH 7.0) buffer. Aliquots (8 μl) of the mixture were diluted 3-fold with the same buffer and incubated for different time intervals (0–96 h). The samples were centrifuged (14,000 rpm) to pellet any protein that might have precipitated (none was visually detected). SDS sample buffer (15 μl) was added to the supernatant, and the mixture was boiled for 3 min and loaded onto a 10% SDS-polyacrylamide gel. PBP 2a (3 μl), in the absence of the cephalosporins, was run alongside as a control. The gel was treated with Coomassie Blue, destained, and scanned (Epson Perfection, 3200 Photo). The density of each PBP 2a band corresponding to a different time point was determined using ImageJ software. The results for each cephalosporin indicated that the acyl-enzyme complex remained fully soluble over the course of 4 days.

Nondenaturing PAGe of the Acyl-enzyme Species of Cephalosporins 1–3 and PBP 2a—A typical reaction mixture (60 μl) contained 9 μM PBP 2a and 20 μM cephalosporins 1–3 in 25 mM Hepes, 1 M NaCl (pH 7.0) buffer. Aliquots (8 μl) of the mixture were diluted 3-fold with the same buffer and incubated for different time intervals (0–96 h). Nondenaturing sample buffer (15 μl) was added to aliquots of the sample, loaded onto a 5% nondenaturing polyacrylamide gel, and run alongside two controls, PBP 2a (3 μl) by itself and PBP 2a (3 μl) in the presence of ceftazidime (15 μl). Gels were run at 4 °C after 3, 24, and 96 h to monitor the occurrence of any oligomerization within the three samples. Each gel was treated with Coomassie Blue, destained, and scanned (Epson Perfection, 3200 Photo). The density of each PBP 2a band corresponding to the acyl-PBP 2a species (in the presence of cephalosporins 1–3) was compared with the two controls. There was no difference in the appearance of the bands for PBP 2a bound to antibiotics 1–3, for PBP 2a bound to ceftazidime, or for PBP 2a by itself. The results indi-
Inhibition of PBP 2a of MRSA

**TABLE 1**

Kinetic parameters for interactions of cephalosporins (nitrocefin, cefepime, ceftazidime, and antibiotics 1–3) with PBP 2a in the absence and presence of compound 4

| Cephalosporin  | $k_1$ | $K_s$ | $k_d/K_s$ | $k_d$ |
|----------------|-------|-------|-----------|-------|
|                | h$^{-1}$ | M$^{-1}$ h$^{-1}$ |                           | h$^{-1}$ |
| Cefepime       | 0.15 ± 0.2 | 135 ± 12 | 105 ± 15 | 35 ± 10 |
| Cefazidime     | 0.5 ± 0.2 | 190 ± 25 | 19 ± 3   | 7 ± 0.1 |
| Nitrocefin     | 0 ± 0.1  | 180 ± 15 | 0 ± 1    | 1 ± 0.1 |

*The kinetic values for cefepime and ceftazidime (6) have been reported (6); these parameters are being given here for comparison with those of cephalosporins 1–3.

In this minimal scheme, $E$ represents PBP 2a, $EI$ is the noncovalent preacylation complex, $E-I$ is the covalent acyl-enzyme species, and $P$ denotes the product of $E-I$ hydrolysis and restoration of enzyme activity. In the case of a typical PBP, the acylation rate constant ($k_2$) is rapid, but deacylation ($k_3$) is slow (19–22). As the results with nitrocefin (a chromogenic cephalosporin), cefepime, and ceftazidime (two clinically used cephalosporins) with PBP 2a indicate (Table 1), the acylation rate constants are slower, by over 4 orders of magnitude, than the corresponding values for $\beta$-lactam-sensitive PBPs (19–23). The deacylation rate constants ($k_3$) are slower yet, with $t_{1/2}$ of 26–77 h. Furthermore, the dissociation constants ($K_d$) for the preacylation complexes are elevated to values that preclude in vivo saturation of PBP 2a. Hence, these $\beta$-lactams (as with other examples) are ineffective PBP 2a inhibitors both by their modest dissociation constants and attenuated rate constants for acylation. A similar trend was noted for PBP 5 of Enterococcus faecium with some $\beta$-lactam antibiotics (24).

In the discovery program for the three ABRI cephalosporins, compounds were screened for inhibition of PBP 2a in 96-well assays. This assay identifies molecules with enhanced ability to form the inhibited complex. Accordingly, we observe that the $K_s$ values for these compounds are significantly lower than those of the other cephalosporins nitrocefin, cefepime, and ceftazidime. The corresponding values for 1, 2, and 3 are $32 ± 2$, $26 ± 1$, and $30 ± 2$ M$^{-1}$ (Table 1). Despite the substantially improved $K_s$ values for cephalosporins 1–3, their respective $k_d/K_s$ parameters, the pseudo-second-order rate constants for the bimolecular encounter between PBP 2a and the cephalosporins, are merely $205 ± 12$, $180 ± 20$, and $145 ± 8$ M$^{-1}$ s$^{-1}$, respectively. These values are smaller than we had expected for antibacterials that are effective in vivo (discussed further below).

Two additional data are noteworthy. First, the $k_2$ values for these compounds are comparable with those for the other cephalosporins (Table 1). Hence, their improved biological activity does not result from enhanced acylation of PBP 2a for the antibiotic by itself. Second, in contrast to the three other cephalosporins, we could not detect any deacylation of the ABRI cephalosporin acyl-enzyme species over 3 days of monitoring. Therefore, these cephalosporins acylate PBP 2a and give a complex that does not allow for the recovery of PBP 2a activity. This lack of deacylation may also contribute to the effectiveness of these antibiotics.

Returning to the issue of the small values for $k_d/K_s$, it is conceivable that the copy number of PBP 2a (800 molecules/S. aureus bacterium (25)) is so low that even these rather modest $k_d/K_s$ parameters are sufficient for inhibition of PBP 2a (and anti-MRSA activity). However, our earlier findings with a bacterial cell wall mimic indicated that the active site opening of PBP 2a, from a closed to an open conformation, was enabled by the binding of the cell wall mimic to an allosteric site outside of the transpeptidase active site (10). This possibility was considered for the ABRI cephalosporins.

Compound 4 is an analogue of a fragment of the S. aureus cell wall, whose synthesis was reported recently by our laboratory (10). Compound 4 possesses the N-acetyglycosamine-N-acetylmuramic acid disaccharide backbone of the cell wall. To the N-acetylmuramic acid residue is appended the pentapeptide (N-acetylmuramic acid-L-Ala-y-D-Gln-I-Lys-d-Ala-d-Ala) linker unit that is typical of S. aureus. The peptidoglycan of S. aureus also has a pentaglycyl moiety appended to the L-Lys side chain that participates in the PBP 2a-catalyzed cross-linking reaction. The omission of the pentaglycl segment from the structure of 4 was deliberate, since we did not want 4 to be turned over by PBP 2a.

The results for the ABRI cephalosporins with PBP 2a in the presence of compound 4 are intriguing (Table 1). As the concentration of the cell-wall analogue increased, the values for $k_d/K_s$ increased for each of antibiotics 1–3. The $K_d$ (dissociation constant) for interaction of compound 4 outside the transpeptidase active site of PBP 2a is $1.2 ± 0.2$ mM (10). We argued previously that the effective concentrations of the cell wall components that the PBP's experience on the bacterial cytoplasmic membrane are high (an entropic effect) (26). However, it is likely that the $K_d$ value for a larger fragment of peptidoglycan than compound 4 (i.e. a true polymeric cell wall) or for an analogue with the pentaglycl moiety appended to the L-Lys residue would be lower (more favorable) than those measured for compound 4. Nonetheless, compound 4 is a useful surrogate for the bacterial cell wall, since it does bind to the requisite allosteric binding site outside of the active site with demonstrated saturation (10). The results with compound 4 indicate that PBP 2a is predisposed to enhanced inhibition by the ABRI cephalosporins in the presence of the cell wall. It is important to note that these effects are consistent and set trends for all three cephalosporins (Table 1). As the concentration of the cell wall fragment increases, the values of $k_d$ decrease (enhance acylation), and those of $K_d$ decrease (more favorable binding). The absolute magnitude of these parameters is not as important (since compound 4 is a mere cell wall surrogate) as the consistent trends that they establish. Despite the fact that compound 4 is not a perfect substitute for the cell wall of S. aureus, the values measured for $k_d/K_d$ at concentrations of 4 that approach saturation (>$K_d$) of PBP 2a are within the range that should be significant in vivo (i.e. $2000–4000$ M$^{-1}$ s$^{-1}$) (27). We believe that these values should even be larger in the presence of a true polymeric cell wall, and they would contribute to the action of the novel cephalosporins in vivo.

Our previous study described time-dependent changes in the CD spectrum of PBP 2a in the presence of oxacillin and ceftazidime, during the first few $t_{1/2}$ values of the acylation process, documenting the exist-
Imbalance of conformational changes during the acylation event. This was followed by the ultimate restoration to the CD spectrum of the mixture to that of the active enzyme, indicative of the completion of the deacylation event in due time (6). A similar set of changes in the CD spectrum is observed for PBP 2a in the presence of cephalosporins 1–3 (Fig. 2), except the process for the onset is more rapid, and there is no recovery to the native state. The issue of lack of recovery of activity (and of the coincident altered CD spectrum) is addressed below. However, we were able to fit the data from the early parts of the CD spectra to a first-order process for the conformational change. In essence, the formation of the noncovalent complex of the antibiotic and PBP 2a sets off the conformational change that leads to acylation of the active site. The limitation of this method is the fact that the CD spectral acquisition is a relatively slow process, and we had as few as four data points for the extraction of the rate constants. As such, these rate constants should be considered approximations. However, it is interesting that the observed first-order rate constants from the CD measurements ($k_{\text{obs}}$) of 0.004, 0.002, and 0.001 s$^{-1}$ for antibiotics 1, 2, and 3, respectively) are close to the corresponding values for the first-order acylation rate constants ($k_A$) in the absence of compound 4; Table 1) of the respective antibiotics.

During protein folding, many of the events leading to a large fraction of secondary and tertiary structural rearrangements occur in milliseconds, whereas additional changes in the environment around aromatic residues, detectable only by near-UV CD, tend to remain flexible and unpacked for a somewhat longer duration (28). Furthermore, flexibility within the folded state of the protein can contribute to additional conformations, depending on the type of ligand interaction (29). CD studies by Gruebele have shown how even a slight alteration to a protein’s secondary structure, one perhaps attributable to a protein-ligand interaction, can shift residues away from the binding site in the folded complex in a matter of seconds, which structurally may not appear to be dramatically different from the native state but makes a significant difference in the CD spectrum (30, 31). It is likely that such a conformational change is being observed when antibiotics 1–3 encounter PBP 2a.

Parenthetically we state here that since the change in the PBP 2a CD spectrum is also seen with penicillins (6), the effect that we observe with cephalosporins 1–3 cannot be attributed to the well-characterized tautomerization of the dihydrothiazine double bond of the cephalosporins upon acylation of the enzyme (32, 33). Furthermore, the chromophore for the cephalosporins is weak, compared with the signals from the protein, such that it does not influence any of the events seen in Fig. 2 (see the flat lines at the top of A, C, and E).

One other conceivable explanation for the change in the CD of PBP 2a upon exposure to antibiotics 1–3 could be precipitation of the acyl-enzyme species from solution. The lack of observation of enhanced noise anticipated for protein precipitation in the course of the CD data acquisition argued against this possibility. Nonetheless, as described under “Experimental Procedures,” we performed a specific experiment to show that concentration of the acylated PBP 2a in solution did not diminish over 4 days of incubation (data not shown). There was no

**FIGURE 2.** A, the far-UV circular dichroism measurement of PBP 2a in the presence of antibiotics. PBP 2a (3 μM; ●), antibiotic 1 (6 μM, solid line), and PBP 2a (3 μM) with antibiotic 1 (6 μM) at 10 min (△), 1 h (■), 24 h (●), 72 h (○). B, change in molar ellipticity of PBP 2a (3 μM) at 208 (●) and 222 (○) as a function of time (200 min) for antibiotic 1 (6 μM). The inset shows the change in molar ellipticity of the PBP 2a (3 μM) at 208 (●) and 222 (○) over the longer duration (4320 min) for antibiotic 1 (6 μM). C, the far-UV circular dichroism spectrum of PBP 2a (3 μM; ●), antibiotic 2 (6 μM; solid line), and PBP 2a (3 μM) with antibiotic 2 (6 μM) at 10 min (△), 1 h (■), 24 h (●), 72 h (○). D, change in molar ellipticity of PBP 2a (3 μM) at 208 (●) and 222 (○) as a function of time for antibiotic 2. E, the far-UV circular dichroism spectrum of the PBP 2a (3 μM; ●), antibiotic 3 (6 μM; solid line), and PBP 2a (3 μM) with antibiotic 3 (6 μM) at 10 min (△), 1 h (■), 24 h (●), or 72 h (○). F, change in molar ellipticity of PBP 2a (3 μM) at 208 (●) and 222 (○) as a function of time for antibiotic 3. The inset shows the change in molar ellipticity of the PBP 2a (3 μM) at 208 (●) and 222 (○) over the longer duration (4320 min) for antibiotic 3 (6 μM).
evidence for any protein precipitation. Another conceivable contributor
to the CD changes could be aggregation or oligomerization of the pro-
tein on acylation by the antibiotics over the course of the 4 days of
monitoring. Admittedly, this was a remote possibility, but we investi-
gated it by monitoring the acylated proteins over the duration of the 4
days by nondenaturing gels. PBP 2a is acylated by these antibiotics and
remains stably in solution as a monomeric protein (data not shown).

The essence of the observations of the data of Fig. 2 is the fact that these
novel cephalosporins rapidly (in a matter of minutes, as opposed to over
the course of hours seen with other cephalosporins) cause a conforma-
tional change necessary for access to the active site. This feature differ-
etiates these antibacterials from other cephalosporins (including the
clinically used cefepime and ceftazidime) studied by us previously (6).
Furthermore, this process follows first-order kinetics, also inconsistent
with the possibility of aggregation or oligomerization.

The minimum inhibitory concentrations (MICs) of antibiotics 1–3
were determined with four MRSA strains (Table 2). *S. aureus* COL (34)
is a well characterized MRSA strain whose entire genome has been
sequenced (35). *S. aureus* COLVA was engineered by transferring the
vancomycin resistance determinants from the clinical staphylococcal
isolate VRS1 into *S. aureus* COL (36), and it is resistant to both oxacillin
(methicillin) and vancomycin, as reflected by the MIC data. The strains
*S. aureus* VRS1 and *S. aureus* VRS2 are vancomycin-resistant MRSA
strains isolated in Michigan (16) and in Pennsylvania (17). These are the
first ever vancomycin-resistant MRSA strains isolated from the clinic.

An MIC value of 4 μg/ml or less for vancomycin is highly desirable. We
also include in this table the MIC for a vancomycin-resistant clinical
isolate of *E. faecalis* (*E. faecalis* 99). Oxacillin, not methicillin, is used for
treatment of infections from methicillin/oxacillin-susceptible staphylo-
cocci (oxacillinMICs ≤2 μg/ml for *S. aureus* and ≤0.25 μg/ml for
coagulase-negative staphylococci) infections in the United States. Both
antibiotics produce similar MICs against various staphylococcal strains.
As expected, the MRSA strains are oxacillin-resistant (MIC > 128
μg/ml).

All three cephalosporins demonstrated good antibacterial activities
against these strains (Table 2). It is noteworthy that the MIC values for
cephalosporins 1 and 2 for resistant strains are below the standard suscep-
tibility breakpoints for various clinically important β-lactam antibiotics
against oxacillin-susceptible strains of *S. aureus* (≤8 μg/ml for cefepime,
ceftazidime, and aztreonam and ≤4 μg/ml for imipenem) (37).

The data presented here indicate that cephalosporins 1–3 have good
activities against strains of MRSA (and one strain of vancomycin-resis-
tant *E. faecalis* that was also examined). We present evidence that the
dissociation constants for the preacylation complexes of PBP 2a with
these antibiotics are lower, but the first-order acylation rate constants
for PBP 2a by these antibiotics are not any different than those of other
cephalosporins (Table 1). Whereas the pseudo-second-order rate con-
stants (k₂/Kₙ) for the encounter of these cephalosporins and PBP 2a are
small, we have documented that in the presence of an analogue of the
cell wall (compound 4), this meaningful parameter, a quantitative meas-
ure of enzyme inactivation efficacy, is enhanced significantly. This enhancement takes the $k_2/K_s$ values for cephalosporins 1–3 to a level that is clinically relevant (27). In addition, it is likely that the effect of the actual *S. aureus* polymeric cell wall on enhancement of the $k_2/K_s$ values would be even more than what we have measured with the surrogate compound 4.

Furthermore, we have documented that interactions of cephalosporins 1–3 with PBP 2a cause a rapid adjustment in the conformation of the protein, such that access to the active site of the enzyme becomes more facile. We surmise that, based on the measured $k_2/K_s$ values, the interactions of the cell wall with PBP 2a might be required for the antibiotics to be effective. Hence, cephalosporins 1–3 might conceivably co-opt the existing interactions of the cell wall with PBP 2a to achieve access to the active site. We add that Lu et al. (9) analyzed one $\beta$-lactam antibiotic that has shown activity against MRSA previously. The kinetics of interactions of this antibiotic with PBP 2a were evaluated by mass spectrometry ($k_2 = 0.39 \text{ s}^{-1}, K_s = 0.22 \text{ mM}, k_2/K_s = 1750 \text{ M}^{-1} \text{ s}^{-1}$) (9). Whereas the $K_s$ value for this antibiotic was 1 order of magnitude higher than those studied by us in this respect, the measured $k_2$ was more rapid in that case. The effect of the cell wall fragments on PBP 2a was not evaluated in that study; nor were the issues of conformational changes investigated.

The interaction between the cell wall and PBP 2a is believed to facilitate the requisite conformational change. We have documented such an allosteric binding site for the cell wall, which binds to PBP 2a in a saturable manner. Although the knowledge of the location of this binding site and how this binding event alters the protein structure is presently lacking, we speculate that the collective three-dimensional structures of cephalosporins 1–3, especially the abundance of heteroatoms at the C$_3$ and C$_7$ functionalities, are the contributing factors.

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**TABLE 2**

| Microorganism          | MIC 1 | MIC 2 | MIC 3 | VAN  | OXA  |
|------------------------|-------|-------|-------|------|------|
| *S. aureus* COL (NARSA)| 2     | 1     | 8     | 2    | 256  |
| *S. aureus* COLVA (NARSA)| 2    | 1    | 8     | 1000 | 256  |
| *S. aureus* VRS1 (NARSA)| 4    | 2    | 16    | 1000 | 512  |
| *S. aureus* VRS2 (NARSA)| 4    | 2    | 16    | 64   | 128  |
| *E. faecalis* 99       | <0.5  | <0.5  | <0.5  | 256  | 32   |
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