# Effectiveness of Tetrahedral Adducts as Transition-State Analogs and Inhibitors of the Class C $\beta$-Lactamase of *Enterobacter cloacae* P99

**Kieran Curley and R. F. Pratt***

*Contribution from the Chemistry Department, Wesleyan University, Middletown, Connecticut 06459*

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**Abstract:** Previous studies have shown that $\beta$-lactamases are inhibited by boronates and phosphonates, both of which form covalent tetrahedral adducts with the active-site serine residue. These have been interpreted, as have similar complexes formed with serine proteinases, as transition-state analog structures. Not all molecules capable of forming such tetrahedral adducts are good inhibitors of serine $\beta$-lactamases, however. In this paper, a series of molecules potentially capable of forming anionic tetrahedral adducts at the active site [$PhCH_2CONHCH_2M(XY)^-OSer$] have been assessed as sources of transition-state analogs and as inhibitors of the class C $\beta$-lactamase of *Enterobacter cloacae* P99. It was found by experiment that the aldehyde, the silanetriol, and the α-keto acid (and its methyl ester) of the series were significantly poorer inhibitors than the structurally analogous boronate. This result was explored computationally. From the starting point of the crystal structure of a phosphonate adduct (Lobkovsky et al. *Biochemistry* **1994**, *33*, 6762), the various inhibitors were introduced into the active site and the complete structure relaxed by energy minimization in a force field. Tetrahedral structures derived from analogous substrates were similarly treated, and the final structures were analyzed both structurally and energetically. From the point of view of energy, it was found that the boronate (M = B, X = Y = OH), phosphonate (M = P, X = Y = O), and carbon substrate-derived analog (M = C, X = O, Y = OH) interacted comparably strongly in a noncovalent sense with the active-site residues, while the aldehyde (M = C, X = O, Y = H), silicate (M = Si, X = O, Y = OH), and α-keto acid derivatives (M = C, X = O, Y = COR) interacted more weakly. The order of energies of interaction between the tetrahedral ligands and the active site was shown to best correlate with the electrostatic interactions of the $MXY^-$ moiety with the two conserved lysine residues of the $\beta$-lactamase active site, here Lys 67 and Lys 315. There appeared to be no positive correlation between the interaction energy of $X^-$ with the oxyanion hole and the total interaction energy; the oxyanion hole therefore appears to contribute uniformly to the ligand binding but not to discrimination between ligands. There was, however, a correlation between the active site interaction energies and the interaction energy between $MXY^-$ and the H2(α2) helix dipole. The H2 helix may therefore contribute selectively toward catalysis and inhibition. The structures were interpreted in terms of the mechanism of Oefner et al. (Oefner et al. *Nature (London)* **1990**, *343*, 284). The relationship of the calculations to the measured inhibitory properties of the parent molecules is discussed as well as projections to further inhibitor design.

## Introduction

Specific phosphonate monoanions of structure **1** (Scheme 1) have been shown to inhibit a variety of serine $\beta$-lactamases.[1,2] They act by covalent modification of the active-site serine hydroxyl group (Scheme 1), yielding an inert phosphonyl derivative **2** after displacement of a good leaving group L.[3]

**Scheme 1**

$$EOH + \underset{\mathbf{1}}{R_1CONHCH_2PO_2{}^-L} \rightarrow \underset{\mathbf{2}}{EOPO_2{}^-CH_2NHCOR_1} + H^+ + L^-$$

Following prior thought on the inhibition of other serine hydrolases by phosphonyl and phosphoryl derivatives,[4] it was believed likely that the tetrahedral phosphonyl moiety in **2** would closely resemble the analogously positioned tetrahedral carbon atom and its ligands in the high energy tetrahedral intermediates and transition states of normal catalysis by these enzymes. If this were true then one would predict strong noncovalent interactions between the inhibitor moiety of **2** and the enzyme leading to, *inter alia*, significant stabilization of the protein. The latter prediction was demonstrated to be true in one particular combination of $\beta$-lactamase and phosphonate.[5] Crystal structures of phosphonates bound to a class A[6] and to a class C $\beta$-lactamase[7] showed the expected covalent attachment of the phosphorus atom to the active site serine hydroxyl oxygen atom and were interpreted in terms of tetrahedral intermediate/transition-state structures.

It is well-known that a variety of other inhibitors of serine hydrolases also produce tetrahedral species covalently bound to the active-site serine residue. These include boronic acids, sulfonic and arsonic acid derivatives, aldehydes, and keto acid derivatives and are generally collectively referred to as examples of transition-state analog inhibitors.[4,8,9] Many of these classes

* To whom correspondence should be addressed. Fax: (860) 685-2211. E-mail: rpratt@wesleyan.edu.

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of molecule might be expected, in principle, to also inhibit serine $\beta$-lactamases. Indeed, it is already known that boronic acids are quite effective inhibitors of $\beta$-lactamases, in general,[10] and in particular when designed to target a specific $\beta$-lactamase.[11] There has, however, been little investigation of the potential of other tetrahedral analogs as $\beta$-lactamase transition state analogs and inhibitors.

The present paper describes a survey of the interactions of a range of potential inhibitors of the type indicated above with a class C $\beta$-lactamase, that of *Enterobacter cloacae* P99. This enzyme is quite strongly inhibited by phosphonate monoester monoanions,[3] and the crystal structure of an enzyme−inhibitor complex is available.[7] There were two related but distinct issues addressed. First, which of the potential inhibitors is likely to generate a structure having close resemblance to a tetrahedral intermediate or transition state of the analogous acyl transfer reaction? The criteria used to judge this were both structural and energetic. Does the tetrahedral analog position itself in the active site in a similar fashion to the carbon analog of an analogous substrate and are the nature and strength of its interactions with active site functional groups also similar? The second issue is whether any or all of the original chemical species giving rise to the tetrahedral active site adducts are likely to be inhibitors of the enzyme in question. This is a more complicated question since, in general, it contains implications concerning the kinetics and thermodynamics of formation and breakdown of the adduct.

With these issues in mind, an assessment of compounds **1** and **3**−**9** ($R_1$ = $PhCH_2$ in all cases) as sources of transition-state analog structures and inhibitors of the P99 $\beta$-lactamase was undertaken. These compounds would be expected to

$R_1CONHCH_2PO_2^-L$ **1**  $R_1CONHCH_2B(OH)_2$ **3**

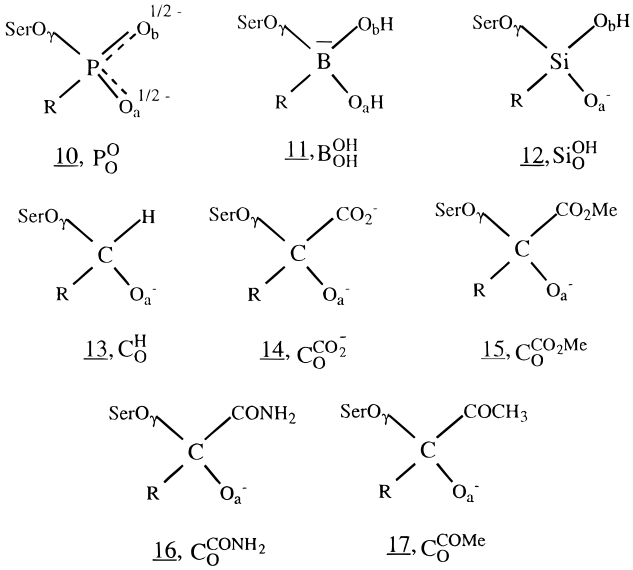
$R_1CONHCH_2Si(OH)_3$ **4**  $R_1CONHCH_2CHO$ **5**

$R_1CONHCH_2COCO_2^-$ **6**  $R_1CONHCH_2COCO_2Me$ **7**

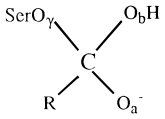
$R_1CONHCH_2COCONH_2$ **8**  $R_1CONHCH_2COCOCH_3$ **9**

generate the tetrahedral anionic species **10**−**17**, respectively, (R = $PhCH_2CONHCH_2$ in all cases), at the $\beta$-lactamase active site, where the oxyanion would presumably be stabilized by the oxyanion hole.[12,13] For ease of reference, these species will also be represented in the text henceforward as $M^Y_X$, where M represents the central tetrahedral atom and X and Y are the variable ligands and where X represents the occupant of the oxyanion hole. These types of molecule have all previously been examined with reference to other serine hydrolases, as indicated above, with the exception of the silanetriol **4**, which represents a novel potential inhibitor capable of generating a tetrahedral anion at the active site.

We were unable on the basis of qualitative chemical intuition to predict the relative effectiveness of **3**−**9** as $\beta$-lactamase inhibitors and are unaware of a successful comparable prediction for the classical serine hydrolases. Consequently, the aim of the present endeavor was to use computational methods to




compare **10**−**17** structurally and energetically. As a control or reference structure **18** was employed. This represents the high




18, $C^{OH}_O$

$R_1CONHCH_2COL'$ **19**

energy tetrahedral structure presumably generated during hydrolysis of the $\beta$-lactamase substrate **19**[14,15] (the tetrahedral intermediate traversed during hydrolysis of the acyl enzyme) and would certainly be expected to interact strongly with the active site in a manner relevant to normal catalysis by the enzyme. The structures **10**−**18** were generated from the crystal structure of **2**[7] where the enzyme was the P99 $\beta$-lactamase. The interactions of the energy-minimized structures of **10**−**18** with the enzyme were compared qualitatively and quantitatively (to the level allowed by the computational method; another aim of the study, incidentally, was to assess whether the low-level theory available in a typical commercial package could yield at least qualitative insight into the problem) and an attempt then made to predict the performance of **3**−**9** as inhibitors. These predictions were compared with experimental measurements. We believe that the goal of the exercise was achieved—the results do give a qualitative and at least semiquantitative understanding of the elements of the class C $\beta$-lactamase active site that are involved in the stabilization of the tetrahedral intermediates and presumably transition states of the acyl-transfer reaction catalyzed by this enzyme and, thus, information related to the mechanism of catalysis and to the design of transition-state analog inhibitors.

## Experimental Section

**Computational Methods.** The starting point for the computations was the crystal structure of the class C $\beta$-lactamase from *E. cloacae* P99 with the phosphonate covalently bound to the active site serine residue as in **20**[7] set up on an IBM 530H computer with INSIGHT II, version 2.2.0 (Biosym Technologies, Inc., San Diego, CA). Two conformations of the arylacetamido side chain were observed in the crystal structure, suggesting that a substrate or inhibitor side chain may experience significant flexibility in the large, nonspecific site available for it. The conformation with the aryl group on the right, closer to the

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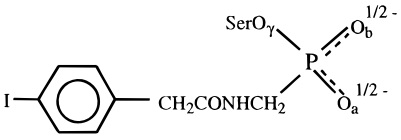


20

B-3 strand, as seen in Figure 4 of Lobkovsky et al.,[7] was arbitrarily chosen as the starting point for the present calculations. Significant side chain movement was in fact observed during the energy-minimization procedure. A pH of 7.0 was assumed (all Asp and Glu residues anionic and lysines cationic) giving a net charge of +2 to the protein and thus of +1 to the complexes with monoanionic inhibitors bound. The side chain of Tyr 150 was assumed to be neutral (protonated). The iodine atom on the phosphonate was replaced by hydrogen.

Atomic charges on the protein were assigned by INSIGHT II. The charges on the inhibitor moiety were generated by MNDO calculations (MOPAC 6.0) on a *N*-acetylglycylserylisoleucinamide tripeptide (thus including amino acids adjacent in the amino acid sequence to the active-site serine) with the inhibitor bound to the serine residue. These appeared to be in generally good agreement with those for similar compounds from higher level theory.[16−20] Bond lengths, except those to the central tetrahedral atom (M henceforward), were initially those of the crystal structure. Bond lengths to C and P were derived from INSIGHT II, while those for B and Si were from the most relevant small molecule crystal structures.[21] The bond lengths to B and Si were constrained to these values since no force field parameters were available for these elements. A list of the important charges and bond lengths employed are given in Table S1.[22]

In each case, the enzyme−inhibitor complex was then hydrated with a 15 Å sphere of water molecules centered at the active site Ser O$_\gamma$. The water molecules were then equilibrated to the complex, whose atoms were constrained to their initial positions, by means of a molecular dynamics simulation within the DISCOVER module (version 2.9) of INSIGHT II. The consistent valence force field (CVFF), a temperature of 300 K, and a dielectric constant of 1.0 were employed. The molecular dynamics simulation was run for 8000 steps (8 ps) with coordinates saved every 200 steps. Typically, the energy decreased to a steady value after about 1000 steps. A typical snapshot from the latter half of the simulation was selected for further computation.

The protein−inhibitor complex with its 15 Å sphere of water molecules, both now unconstrained, was then subjected to energy minimization by means of 1000 steps by the method of steepest descents and then 2000 steps by the method of conjugate gradients, both within the CVFF force field. No Morse and cross terms were employed, and thus, a harmonic potential was used for bond-stretching terms. After this procedure was completed, the final derivative of energy with respect to structural perturbation was in the range of 0.05−0.08 kcal/Å. Interaction energies, including all nonbonded interactions, could be obtained directly from the minimized structure by means of the DISCOVER program.

**Experimental Determination of Inhibition Constants.** The P99 $\beta$-lactamase was obtained from the Centre for Applied Microbiology and Research (Porton Down, Wilts., U.K.). The chromophoric substrate PADAC (7-(thienyl-2-acetamido)-3-[[2-[[4-(*N*,*N*-dimethylamino)phenyl]azo]pyridiniumyl]methyl]-3-cephem-4-carboxylic acid) was purchased from Calbiochem. All kinetic runs were performed at 25 °C in 20 mM MOPS buffer at pH 7.5. Absorption spectra and spectrophotometric rates were obtained from either a Perkin-Elmer Lambda 4B or a Hewlett-Packard HP8452 spectrophotometer. Inhibition constants $K_I$ were obtained from measurements of the effect of the inhibitor on the rate of enzyme-catalyzed PADAC hydrolysis under $S_o \ll K_m$ conditions (enzyme and PADAC concentrations were 16 nM and 16 $\mu$M, respectively).

The free keto acid **6** was prepared as described by Cornforth and Cornforth.[23] It was converted into its methyl ester **7** by diazomethane treatment. The silane **4** was prepared as its tris(2-methoxyethyl) ester by phenylacetylation and transesterification of triethoxy(aminomethyl)-silane, the latter obtained by the method of Noll et al.[24] Details of the synthesis and hydrolysis of this ester will be published elsewhere. Only a lower limit to a $K_i$ value was achievable for **4** because of its polymerization at concentrations above 0.1 mM. The aldehyde **5** was prepared in this laboratory by Mr. Andre Pearson according to the procedure described by Brutschy et al.[25] $^1$H NMR spectra of **5** and **6** equilibrated in neutral aqueous $^2H_2O$ showed them to exist as mixtures of the carbonyl and carbonyl hydrate forms where the latter made up *ca*. 90% and 32%, respectively, of the total.

## Results and Discussion

The experimentally determined thermodynamic inhibition constants, $K_I$, for **4**−**7** are presented in Table 1. Also shown there is an estimate of $K_I$ for **3** from values for boronates[10,26] with a kinetically similar[27,28] class C $\beta$-lactamase. Boronates have certainly been shown to strongly inhibit the P99 enzyme,[29] so there is no reason to suspect that the value for $K_I$ for **3** reported in Table 1 is not a good estimate for this enzyme.

The molecules **3**−**9** were assumed, by analogy with what is known to occur with other serine hydrolases, to act as inhibitors by rapid and reversible addition to the active site serine hydroxyl group of the P99 $\beta$-lactamase. In addition to the phosphonylation of the active site serine, as described in the Introduction, the crystal structure of this enzyme inhibited by **1** shows the amido side chain hydrogen-bonded into the active site in the manner postulated for substrates and observed in the crystal structures of related $\beta$-lactam-recognizing enzymes with poor substrates or of weakly active mutants with good substrates.[30−32] It seems likely therefore that **3**−**9** would occupy the active site in an analogous fashion as represented in the structures **10**−**17**. The general layout of the active site is shown schematically in Figure 1.

An arylboronate has been shown by $^9$B NMR to form a tetrahedral adduct with the active-site serine hydroxyl group of the P99 enzyme.[33] The electrophilicity of the carbonyl groups of **5**−**9** is demonstrated by the observation by $^1$H NMR of the substantial hydration of **5** and **6** in aqueous solution, to an extent expected of an amidoaldehyde[34] and a pyruvic acid derivative bearing an electron-withdrawing substituent.[35] It seems likely

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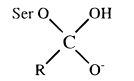
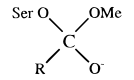
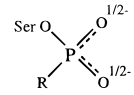
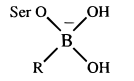
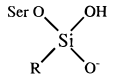
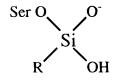
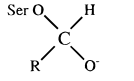
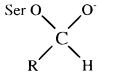
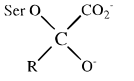
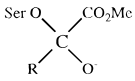
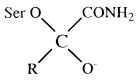
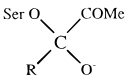
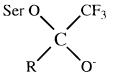
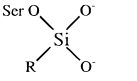
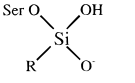
**Table 1.** Interaction Energies and Inhibition Constants of $\beta$-Lactamase Ligands[a]

| | | $\delta_I E_{int}$(kcal/mole) | $E_{H2}$(kcal/mole)[b] | $K_I$(mM)[c] |
|---|---|---|---|---|
|  | 18 | 0[d] | -24.6 | |
|  | 23 | +17.7 | -21.4 | |
|  | 10 | +2.2 | -8.9 | |
|  | 11 | -0.4 | -21.4 | $\leq 10^{-3}$ |
|  | 12 | +25.3 | -6.5 | $\geq 1$ |
|  | 24 | +36.5 | -1.4 | $\geq 1$ |
|  | 13 | +32.4 | -16.0 | 17 |
|  | 25 | +13.5 | -8.8 | 17 |
|  | 14 | -79.3 | -10.8 | 7 |
|  | 15 | +48.2 | -5.6 | 3 |
|  | 16 | +43.4 | -7.0 | |
|  | 17 | +28.1 | -13.7 | |
|  | 27 | +62.3 | +0.90 | |
|  | 28 | -130.0 | | |
|  | 29[e] | -42.1 | | |

[a] R is $PhCH_2CONHCH_2$ in every case. [b] Interaction energy between MXY and the H2 helix dipole. [c] These values represent the inhibition constants of the original compounds. [d] Absolute calculated value −120.4 kcal/mol. [e] Tyr 150 $O^-$.

therefore that formation of **13**−**17** by hydroxyl additions to **5**−**9** would be facile. Similarly, the generation of **12** from **4** should also, in principle, be readily achieved since oxygen ligand exchange at silicon in aqueous solution is facile.[36,37] It will therefore be assumed henceforward in this paper that any inhibition of the P99 $\beta$-lactamase by **3**−**9** would derive from the formation of the structures **11**−**17**. These structures should have much in common with that of the phosphonate analog **10**.

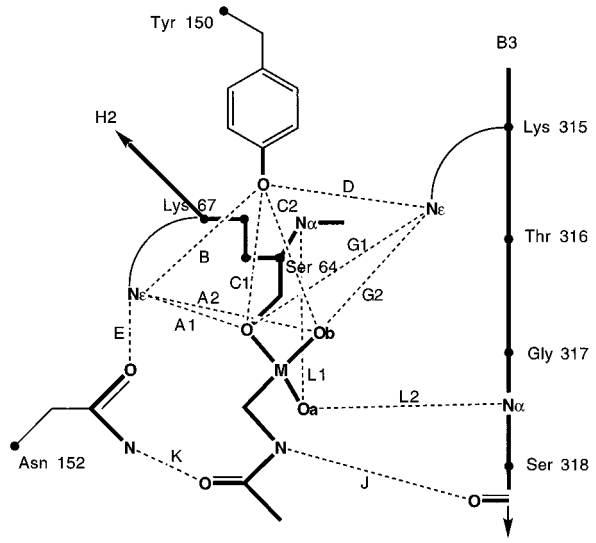
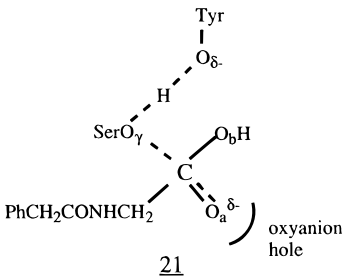



**Figure 1.** General layout of the inhibitors at the active site of the P99 $\beta$-lactamase. The inhibitor (bold lines) is attached to Ser 64 at the C-terminus of helix 2 (bold line, H2), adjacent to $\beta$-strand 3 (bold line, B3). Side chains of Lys 67, Asn 152, Tyr 150, and Lys 315, which interact with the inhibitor, are also depicted. Bold points represent the α-carbons of amino acid residues and Nα the backbone nitrogen atoms comprising the oxyanion hole. The distances listed in Table S2[22] are indicated by letter-labeled dotted lines.

The striking result made evident in the experimental data of Table 1 is that the boronate appears to be by far the best inhibitor in the thermodynamic sense of those examined by experiment. Direct comparison with the phosphonate, an inhibitor for kinetic rather than thermodynamic reasons,[3] cannot be made from these data. Even the silicate, which might have been expected to closely resemble a *gem*-diol moiety when bound to Ser $O_\gamma$, was apparently only a weak inhibitor at best. The remainder of this paper will look into the structural basis for these results.

**Enzyme−Inhibitor Interactions: Structure.** The crystal structure of the covalent complex formed on reaction between the *E. cloacae* P99 class C $\beta$-lactamase and a phosphonate inhibitor (**1**) has been interpreted as a transition-state analog.[7] The structure appears to most resemble **21**, the transition state for breakdown of the tetrahedral intermediate involved in hydrolysis of an acyl enzyme. This would occur as part of a mechanism first proposed by Oefner et al.[30] where the hydroxyl group of Tyr 150 plays a key role as a general acid/base catalyst—in **21** as a general acid assisting departure of the active



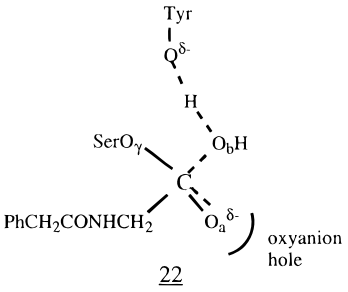

21

(36) Knight, C. T. G.; Thompson, A. R.; Kunwar, A. C.; Gutowsky, H. S.; Oldfield, E.; Kirkpatrick, R. J. *J. Chem. Soc., Dalton Trans.* **1989**, 275−281.

(37) McNeil, K. J.; DiCaprio, J. A.; Walsh, D. A.; Pratt, R. F. *J. Am. Chem. Soc.* **1980**, *102*, 1859−1865.

site nucleophile, the Ser 64 oxygen. Also present, close by but not shown in **21**, are the conserved ammonium ions of Lys 67 and Lys 315, which are believed to largely act as electrostatic catalysts, decreasing the p$K_a$ of the hydroxyl group of Tyr 150, such that its anion can be a credible base at neutral pH,[30] and stabilizing the anionic tetrahedral intermediates.[7,38,39] In the phosphonate analog $P_O^O$, the proton corresponding to that in flight in the transition state is likely to be localized on Tyr 150 and, because of the p$K_a$ of phosphonate monoesters, $O_b$ is also an anion.

In order to employ $P_O^O$ as a starting point to computationally explore the interactions of active-site functional groups with potential transition-state analogs, it was first necessary to allow a theoretical model of $P_O^O$ to relax in the presence of the force field to be employed in subsequent calculations. This process, described in the Experimental Section, led to no extensive changes to the protein structure (root mean square (rms) difference between all heavy atoms in the crystal structure and relaxed model was 1.72 Å and all backbone heavy atoms 1.42 Å), but the small changes [rms differences for the eight major active site residues (see below) were 0.58 and 0.51 Å for all heavy atoms and backbone heavy atoms, respectively] in the region of the active site were interesting (Figure 2, Table S2[22]—the distances reported are those of Figure 1). The Tyr 150 hydroxyl group has moved from Ser 64 $O_\gamma$ to very firmly hydrogen bond with the phosphonyl oxygen $O_b$. The Lys 67 and Lys 315 ammonium ions have moved in more closely to $O_b$ and Ser 64 $O_\gamma$. This structure can be interpreted as an analog of transition state **22**, that for formation of the tetrahedral intermediate in deacylation, where the Tyr 150 anion is acting as a general base catalyst. The lysines would then be interpreted




22

as involved in increasing the acidity of the water molecule H−$O_b$−H in the transition state and the electrophilicity of the carbonyl carbon, the latter particularly through the close approach of the Lys 73 ammonium ion to Ser 64 $O_\gamma$. It might be noted that the analogous transition state of serine proteinases—that of formation of the tetrahedral intermediate in deacylation—is thought to be closely imitated by the crystal structures of phosphomonoester derivatives of $\gamma$-chymotrypsin[40] and $\beta$-trypsin[41] where the general base catalyst of the active site, the histidine imidazole group of the "catalytic triad," in protonated form, is hydrogen bonded to the phosphoryl oxygen corresponding to $O_b$.

Although it cannot be claimed that the computational result described above proves that the $\beta$-lactamase−phosphonate complex in solution has the minimized structure described above and in Table S2,[22] the result does suggest that the reaction path between the crystal structure and the minimized structure, and

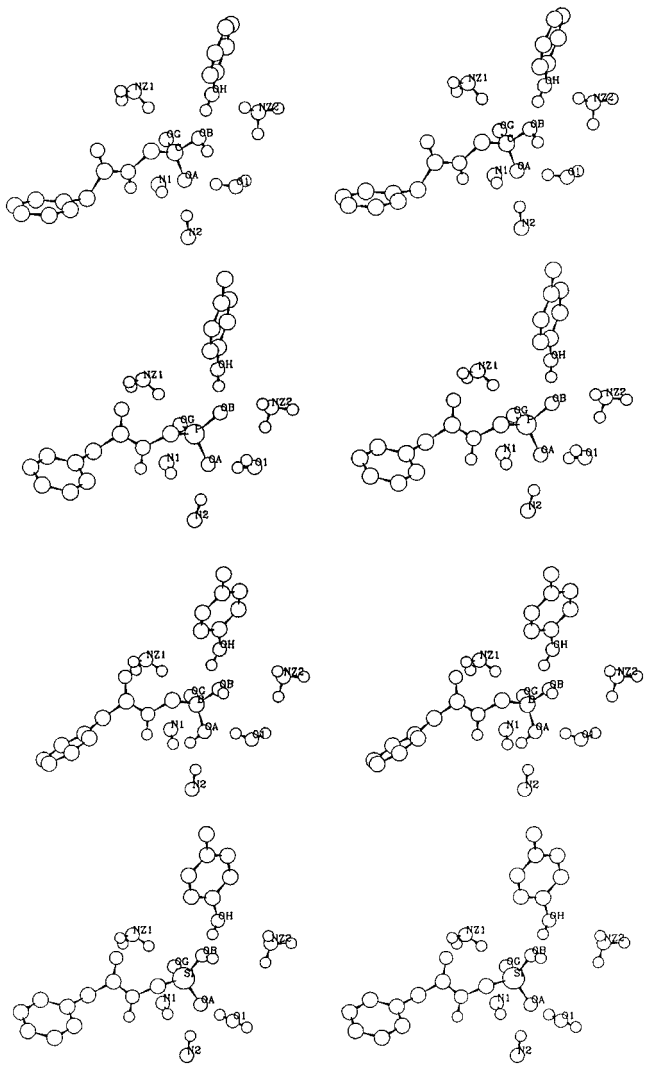
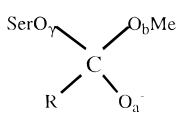


**Figure 2.** Energy-minimized structures corresponding to $C_O^{OH}$, $P_O^O$, $B_{OH}^{OH}$, and $Si_O^{OH}$ (top to bottom). The inhibitor moiety is shown with the termini of the side chains of the important amino acid residues Lys 67, Tyr 150, and Lys 315, the NH components of the oxyanion hole, and the water molecule generally found close to $O_a$ (Figure 1).

by implication between the transition states **21** and **22**, is a facile one as would be anticipated in terms of the mechanism of Oefner et al.[30]

The relationship between the minimized phosphonate structure and a reaction intermediate or transition state is further demonstrated by inspection of the minimized tetrahedral carbon species $C_O^{OH}$ and **23**, $C_O^{OMe}$. As Figure 2 and the data in Table




23, $C_O^{OMe}$

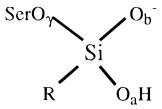
S2[22] indicate, the energy-minimized structure (again beginning from the crystal structure) containing the covalently-bound ligand moiety $C_O^{OH}$ is very similar to that containing the phosphonate, particularly with respect to the positions of Tyr 150, hydrogen-bonded to $O_b$, and Lys 67, and thus also seems closely related to transition state **22**. The smaller negative charge on $O_b$ does result in the Lys 315 ammonium ion staying significantly further away than in the phosphonate structure. In this regard, it is possible that the phosphonate gives a better representation of the transition state— depending on how the negative charge is in fact distributed in **22**.

(38) Knap, A. K.; Pratt, R. F. *Biochem. J.* **1991**, *273*, 85−91.
(39) Ellerby, L. M.; Escobar, W. A.; Fink, A. L.; Mitchinson, C.; Wells, J. A. *Biochemistry* **1990**, *29*, 5797−5806.
(40) Harel, M.; Su, C.-T.; Frolow, F.; Ashani, Y.; Silman, I.; Sussman, J. L. *J. Mol. Biol.* **1991**, *221*, 909−918.
(41) Kossiakoff, A. A.; Spencer, S. A. *Biochemistry* **1981**, *20*, 654−664.

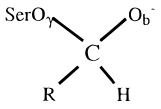
Strikingly different (Table S2[22]) is the distribution of functional groups in the structure $C_O^{OMe}$, which represents an acylation tetrahedral intermediate. As above, Lys 315 is distant but, in contrast to what is observed in $P_O^O$ and $C_O^{OH}$, Tyr 150 is hydrogen bonded to Ser $O_\gamma$ rather than $O_b$, and Lys 67 $N_\epsilon$ is more distant. The overall effect here then is of **21**, which in acylation would represent a transition state for formation of the tetrahedral intermediate. Again, one gets the impression of the facile interconvertibility of **21** and **22** presumably by concerted motion of Tyr 150 and Lys 67.

Three tetrahedral structures $B_{OH}^{OH}$, $Si_O^{OH}$, and $C_O^H$ that would likely be generated from the three inhibitors **3**−**5**, respectively, will now be considered. The energy-minimized structures (Figure 2, Table S2[22]) have much in common with those of $P_O^O$, $C_O^{OH}$, and $C_O^{OMe}$. In particular, $Si_O^{OH}$ and $C_O^H$ are quite similar to the latter three. They differ from $C_O^{OH}$ mainly in the looser clustering of the lysines and Tyr 150 about the ligand oxygen atoms. The structure derived from the much better inhibitor, the boronate **3**, differs from $C_O^{OH}$, $Si_O^{OH}$, and $C_O^H$ by having, like $C_O^{OMe}$, Tyr 150 hydrogen-bonded to Ser $O_\gamma$ rather than to $O_b$. A rather striking difference between $B_{OH}^{OH}$ and the other tetrahedral structures described above lies in its minimal occupancy of the oxyanion hole, defined by the distances $L_1$ and $L_2$ to the backbone nitrogen atoms of Ser 64 and Ser 318, respectively. All except the boronate show close interaction with Ser 318 and much looser interaction (if any) with Ser 64. The boronate does not closely interact with either. This presumably relates to the smaller size of the boronate moiety, the smaller negative charge on $O_a$ (Table S1[22]), and the presence of a proton on $O_a$.

In view of the close proximity of the lysine residues to the anionic tetrahedron of the bound inhibitor and the energy calculations described below, it was realized that alternative structures to some of the adducts should also be considered. These involve placement of the oxyanion outside rather than inside the oxyanion hole. For the silanetriol **4**, for example, the bound structure **24**, $Si_{OH}^O$ rather than $Si_O^{OH}$ might be considered and for the aldehyde **5**, **25** $C_H^O$ rather than $C_O^H$.




24, $Si_{OH}^O$




25, $C_H^O$

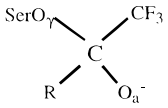
There is considerable precedent for this variation in the complexes of aldehydes with other hydrolases. An X-ray crystal structure of the complex between protease A and the aldehyde inhibitor chymostatin, for example, showed two enantiomeric structures where the tetrahedral carbinol oxygen was either in or out of the oxyanion hole.[42] NMR studies of complexes of trypsin and leupeptin showed the presence of two forms in solution that, on the basis of NOE measurements, were proposed to represent analogs to the alternative structures $C_O^H$ and $C_H^O$.[43] Energy-minimized structures corresponding to $Si_{OH}^O$ and $C_H^O$ were therefore also obtained (Table S2[22]) for adducts of the P99 $\beta$-lactamase with **4** and **5**. After minimization, both $Si_{OH}^O$ and $C_H^O$, perhaps counter-intuitively, resemble $B_{OH}^{OH}$ and $C_O^{OMe}$ rather than $C_O^{OH}$, in having the Tyr 150 hydroxyl (as well as the Lys 67 ammonium ion) hydrogen-bonded to Ser $O_\gamma$ rather than to $O_b$. The Lys 73 ammonium ion has moved closer to $O_b$ in $Si_{OH}^O$ than in $Si_O^{OH}$, however, and to a distance in $C_H^O$ similar to that in $C_O^{OH}$. Although all of these structures seem reasonable, it is not possible, immediately at least, to predict by inspection alone the order of effectiveness of the interaction of the ligands with the enzyme or an order of potency of the parent compounds as inhibitors.

Another point of some uncertainty with respect to the aldehyde adduct, and perhaps to some of the other structures, is whether the stablest form at neutral pH would have the oxygen at $O_a$ (or $O_b$) protonated (neutral) or not. There seems some controversy in the literature on this point in relation to both serine and cysteine proteinases. The most direct evidence seems to be that from NMR studies of aldehyde adducts. Although earlier studies of such adducts were interpreted in terms of a protonated hemiacetal oxygen,[44,45] the most detailed study that we are aware of, that of the leupeptin/trypsin complex,[43] suggests a hemiacetal p$K_a$ of 4.69 in the complex. The presence of an anion in trifluoro ketone complexes also seems well established,[46] but here of course the intrinsic p$K_a$ of the carbinol would be lower than in the case of the aldehyde. It seems likely, particularly in view of the higher positive electrostatic potential of the class C $\beta$-lactamase active site than that of chymotrypsin at least,[7] that the aldehyde adduct $C_O^H$ would indeed contain an anionic ligand at neutral pH. Calculations in fact did indicate only weak interaction between the active site and a neutral hemiacetal ligand.

The dicarbonyl compounds **6**−**9** differ from the inhibitors described above in having a carbonyl group in place of $O_b$. They are therefore not structurally as similar to $C_O^{OH}$ as are **10**−**13** but do have, in their putative adducts with the active site serine, a tetrahedral carbon atom directly bound to Ser $O_\gamma$. Details of the minimized structures $C_O^{CO_2^-}$, $C_O^{CO_2Me}$, $C_O^{CONH_2}$, and $C_O^{COMe}$ are also presented in Table S2.[22] It can be seen that the major difference between structures $C_O^{CO_2^-}$, $C_O^{CO_2Me}$, and $C_O^{CONH_2}$ and those described above lies with the position of Tyr 150, whose hydroxyl group, while still remaining close, but not hydrogen bonded to Ser $O_\gamma$, now interacts with substituent heteroatoms on the carbonyl carbon atom. Thus, in $C_O^{CO_2^-}$, the Tyr 150 hydroxyl group (and the Lys 315 ammonium ion) are strongly hydrogen-bonded to a carboxylate oxygen atom; the other carboxylate oxygen is directed into the solvent. In $C_O^{CO_2Me}$ the Tyr 150 hydroxyl is hydrogen-bonded to the ester alkyl oxygen (the ester carbonyl oxygen is directed into solution) while in $C_O^{CONH_2}$, Tyr 150 interacts with the amide carbonyl and the amide nitrogen is oriented toward the solution. The structure of $C_O^{COMe}$, the diketone adduct, differs from those of $C_O^{CO_2^-}$, $C_O^{CO_2Me}$, and $C_O^{CONH_2}$ in that the carbonyl oxygen is directed toward the external solution and, no doubt because of this, the Tyr 150 hydroxyl group appears to have little interaction with the inhibitor moiety.

Finally, the interaction of the trifluoro ketone **26** in its tetrahedral adduct with Ser $O_\gamma$, **27**, $C_O^{CF_3}$ was assessed. This


26




27, $C_O^{CF_3}$

compound has not been synthesized but was of interest because of other serine hydrolase precedent.[47] The energy-minimized

(42) Delbaere, L. T. J.; Brayer, G. D. *J. Mol. Biol.* **1985**, *183*, 89−103.
(43) Ortiz, C.; Tellier, C.; Williams, H.; Stolowich, N. J.; Scott, A. I. *Biochemistry* **1991**, *30*, 10026−10034.
(44) Shah, D. O.; Gorenstein, D. G. *Biochemistry* **1983**, *22*, 6096−6101.
(45) Bachovchin, W. W.; Yong, W. Y. L.; Farr-Jones, S.; Shenvi, A. B.; Kettner, C. A. *Biochemistry* **1988**, *27*, 7689−7697.
(46) Liang, T.-C.; Abeles, R. H. *Biochemistry* **1987**, *26*, 7603−7608.
(47) Imperiali, B.; Abeles, R. H. *Biochemistry* **1986**, *25*, 3760−3767.

**Table 2.** Interaction Energies between Representative Ligands and the Amino Acids of the Active Site

| | ligand $E_{EOI(noncov)}$ (kcal/mol) | | | |
|---|---|---|---|---|
| residue | $C_O^{OH}$ | $P_O^O$ | $B_{OH}^{OH}$ | $Si_O^{OH}$ |
| Ser64 | −7.8 | 11.6 | −1.4 | 8.7 |
| Lys67 | −36.5 | −39.7 | −53.3 | −22.1 |
| Tyr150 | −8.0 | −14.2 | −7.9 | −6.6 |
| Asn152 | −4.3 | −3.7 | −4.1 | −4.5 |
| Lys315 | −37.5 | −55.0 | −38.1 | −25.0 |
| Thr316 | −5.4 | −1.7 | 2.2 | 0.0 |
| Gly317 | −7.0 | −2.3 | −1.4 | −4.5 |
| Ser318 | −9.2 | −8.8 | −5.6 | −10.2 |
| total | −115.7 | −113.8 | −109.6 | −64.2 |

structure (Table S2[22]) was similar to those above, but Tyr 150 did not significantly interact with the bound ligand.

The arrangement of water molecules in the vicinity of the ligand should also be mentioned. Essentially all minimized structures had one external water molecule hydrogen-bonded to $O_a$. Anionic $O_b$, in $Si_{OH}^O$ and $C_H^O$, each accommodated two strongly hydrogen-bonded water molecules. The dicarbonyl species in general had additional water molecules associated with the terminal carbonyl group—the carboxylate of $C_O^{CO_2^-}$, in particular, had four associated water molecules and the carbonyl oxygens of $C_O^{CO_2Me}$ and $C_O^{COMe}$ one each. Although this arrangement of directly interacting water molecules was generally reproducible by the molecular dynamics procedure, variants were observed, particularly those where the ligand appeared drawn out of the active site by a second water molecule interacting with $O_a$, apparently supplanting the oxyanion hole, and by another inserted between Lys 315 and $O_b$. Although the total interaction energy between ligand and enzyme, as defined by eq 1 (see below), was similar to that generated in the structures described by the data of Table S2,[22] direct interactions between the ligand and functional groups of the active site were weaker. They were, however, compensated for by direct interaction between the enzyme functional groups themselves. Since our interest principally lay with the closest complexes between ligand and enzyme, such as would presumably occur in transition states and their analogs, these more open complexes are not considered further in this paper.

**Inhibitor−Enzyme Interactions: Energy.** Interaction energies between the covalently-bound inhibitor moiety and the enzyme were obtained as described in the Experimental Section. It was evident from the calculations that essentially all of the quantitatively significant interactions, particularly when differences between inhibitors were considered, involved eight amino acid residues directly adjacent to the bound inhibitor, *viz*. Ser 64, Lys 67, Tyr 150, Asn 152, Lys 315, Thr 316, Gly 317, and Ser 318. Thus, the calculated energies presented include interactions only with these entities. An exception to this generalization, however, may be provided by the H2 helix. This point is taken up below.

The data of Table 2 give as a representative sample the calculated direct energies of noncovalent interaction of the ligands $C_O^{OH}$, $P_O^O$, $B_{OH}^{OH}$, and $Si_O^{OH}$ with the active site components. It is evident at once that the strongest interactions are electrostatic in nature, between the negatively charged ligands and the positively charged Lys 67 and Lys 315 residues. The absolute magnitude of the numbers of course strongly reflects the value of the dielectric constant assumed in the electrostatics calculations. An increase of the assumed dielectric constant to 20, however, gave essentially the same qualitative picture.

In order to more rigorously compare the energies $E_{int}$ of noncovalent interaction of different ligands with the enzyme, it is necessary to take into account not only the direct interaction energies between the enzyme and ligand, $E_{EOI(noncov)}$, but also internal energies $E_E$ of the enzyme (reduced to the eight amino acids noted above) and the ligand, $E_I$. The internal energies of E and I include contributions from deviations from ideal geometry and from nonbonded interactions. The sum of these contributions to $E_{int}$ is presented in Table 1 in the form of a difference $\delta_I$ (eq 1) between its value for a ligand I and that of the carbon ligand shown in $C_O^{OH}$, the tetrahedral intermediate derived from a substrate, as the reference ligand.

$$\delta_I E_{int} = \delta_I E_E + \delta_I E_I + \delta_I E_{EOI(noncov)} \quad (1)$$

These results show that in terms of interaction energy between the enzyme and the ligand, the boronate and the phosphonate have the largest values, both very similar to that of the tetrahedral carbon intermediate analog. One might therefore conclude that these are closer to tetrahedral intermediate analogs than are the other ligands, and therefore, the structure of the enzyme in these complexes should closely resemble that in an actual tetrahedral intermediate. It is interesting to note that the acylation tetrahedral intermediate $C_O^{OMe}$ interacts much less strongly with the enzyme than does the deacylation intermediate $C_O^{OH}$. One can perhaps correlate this result with the fact that the methyl ester **19** (L′ = OMe) is a very poor $\beta$-lactamase substrate, which must reflect a very slow acylation rate since deacylation, through $C_O^{OH}$, as seen in derivatives of **19** where L′ contains a carboxylate group, is facile.[14,15] The best $\beta$-lactam substrates also contain a carboxylate on the leaving group, although it is certainly not essential to good substrates.[48,49]

By the measure of the interaction energies of Table 1, none of the other tetrahedral derivatives are close analogs of the tetrahedral intermediate. The tetrahedral adduct of the aldehyde does interact more strongly with the enzyme when the oxyanion is out of rather than in the oxyanion hole. The reverse is true, however, for the silicate, which interacts more strongly when the oxyanion is in the hole. The dicarbonyl derivatives $C_O^{CO_2Me}$, $C_O^{CONH_2}$, and $C_O^{COMe}$ and the trifluoro ketone $C_O^{CF_3}$ interact much less strongly than $C_O^{OH}$, presumably because of the positively charged carbon rather than negative oxygen ($O_b$) directly adjacent to the tetrahedral carbon. The ketoacid anion $C_O^{CO_2^-}$, on the other hand, interacts much more strongly with the enzyme than do any of the others, by virtue of its dianionic nature: the coulombic interaction energy of the carboxylate group with the lysines amounts to −151 kcal/mol in these calculations. This result further emphasizes the positive electrostatic potential of the $\beta$-lactamase active site[7] and the value of a second negative charge in the leaving group of a substrate. The position of the additional carboxylate in $C_O^{CO_2^-}$, however, is not typical of good substrates, and thus, $C_O^{CO_2^-}$, despite its strong interaction with the enzyme, is probably not a good tetrahedral intermediate/transition state analog. This compound is discussed further below.

In order to localize as much as possible the source of the enzyme's ability to discriminate between the various tetrahedral species, the interaction energies of Table 1 were dissected further. Figure 3 shows plots of the correlation between the coulombic interaction between the Lys 67 and Lys 315 side chains, separately and as a sum, with the MXY moiety of each of the ligands and the total interaction energy between ligand and enzyme, $E_{int}$. This procedure yields a clear positive correlation to which both Lys 67 and Lys 315 contribute to a comparable degree. Individual deviations from these conclu-

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(49) Varetto, L.; DeMeester, F.; Monnaie, D.; Marchand-Brynaert, J.; Dive, G.; Jacob, F.; Frére, J.-M. *Biochem. J.* **1991**, *278*, 801−807.

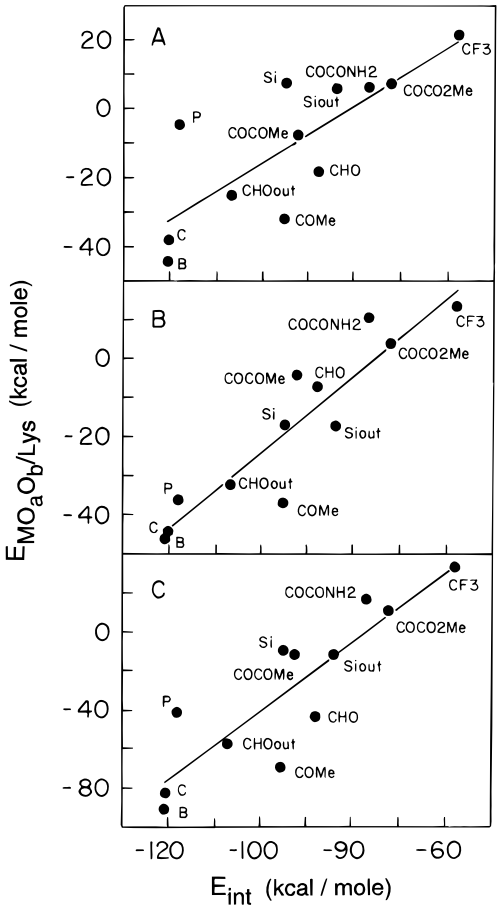


**Figure 3.** Plots of interaction energies of MXY with Lys 67 (A), Lys 315 (B), and the sum of the two (C) against the total interaction energy of the inhibitor with the eight important amino acids of the active site (see text). The species bearing the descriptor "out" have the oxyanion in the $O_b$ position (Figure 1), out of the oxyanion hole.

sions correlate to specific structural features, e.g., the weak interaction of $P_O^O$ and $Si_O^{OH}$ with Lys 67 derives from compensation between the negative charge on $O_a$ and $O_b$ and the large positive charge on phosphorus and silicon, respectively, where the latter is closer than the oxygens to Lys 67. Notably, the tetrahedral intermediates $C_O^{OH}$ and $C_O^{OMe}$ as well as the boronate $B_{OH}^{OH}$ interact strongly with both lysines.

In Figure 4 a plot of the interaction energy between $O_a$ and the oxyanion hole (Ser 64 plus Ser 318) *vs* $E_{int}$ is shown. It is clear from comparison of Figures 3 and 4 that the coulombic interaction energies of MXY with the lysines is the dominant determinant of the order of total interaction energies and that the coulombic interaction energies of $O_a$ with the oxyanion hole alone is not. In particular, the relative strengths of interaction of $O_a$ of the boronate and silicate complexes, $B_{OH}^{OH}$ and $Si_O^{OH}$, respectively, with the oxyanion hole are inverted with respect to the relative total interaction energies. The favorable interactions of the oxygens around silicon with the oxyanion hole and lysine residues is offset by the unfavorable interactions of the latter with the highly positive charge on silicon. Thus, it appears that the *distribution* of charge in these tetrahedral species, its position in space relative to the charges, and particularly the positive charges, of the active site, dictates the order of interaction energies between these species and the enzyme.

It has been noted by $\beta$-lactamase crystallographers[13,50] that the nucleophilic serine of the active site lies at the N-terminus of an extended α-helix, denoted α2 or H2, and the suggestion

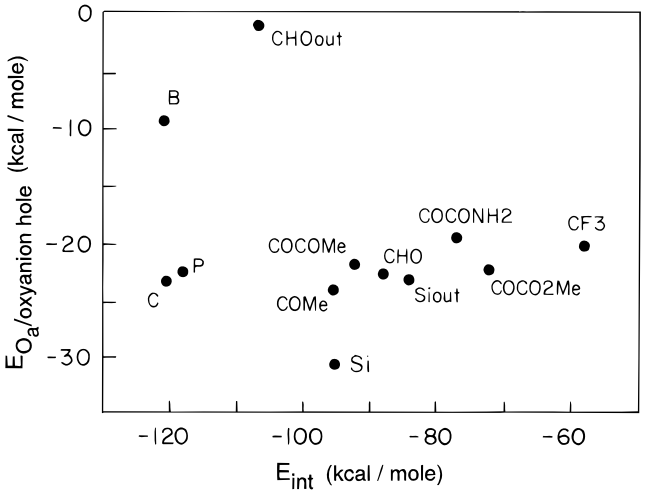


**Figure 4.** Plot of interaction energies of $O_a$ (Figure 1) with the oxyanion hole (backbone NH groups of Ser 64 and Ser 318) against the total interaction energy of the inhibitor with the eight important amino acids of the active site (see text). The species bearing the descriptor "out" have the oxyanion in the $O_b$ position, out of the oxyanion hole.

was made that catalysis might be facilitated by the electrostatic influence of the helix dipole whose positive end would be at the N-terminus.[51−53] Similar suggestions were made earlier with respect to the proteinases subtilisin and papain.[52,53] In these enzymes also the nucleophilic serine/cysteine residue is found at the N-terminus of an α-helix, and the backbone NH of the nucleophilic residue provides one half of the oxyanion hole. One role proposed for the helix dipole in such circumstances is stabilization of the anionic tetrahedral intermediate.[52,53]

In view of this background, we have made estimates of the interaction energy between the H2 helix dipole of the P99 $\beta$-lactamase and the MXY moiety of the tetrahedral anions **10**−**17**, **18**, **23**−**25**, and **26**. In order to do this, the axis of the helix was defined by means of an algorithm developed by Sklenar et al.[54] and incorporated into a local MD toolchest.[55] Then, following Sheridan et al.,[56] a positive point charge of +0.5 electronic units was placed on the helix axis 0.3 Å from the N-terminal Cα of the helix in the N → C direction, and the required electrostatic interaction energies, $E_{H2}$, were calculated. The helix was assumed to include the residues from Gly 63 and Ala 79.[57] The calculated interaction energies are presented in Table 1. The representation of the helix dipole in terms of point charges is of course an approximation, particularly when interactions with nearby charges are in question, but the order of interaction energies was of greatest interest for the purposes of this paper. More sophisticated treatments of helix dipoles have of course been described.[58,59]

From the helix interaction energies of Table 1, two points stand out. First, the interaction energies, compared with the individual interaction energies of Table 2, for example, are, in some cases, quite substantial and suggest that the H2 helix dipole

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may indeed significantly stabilize tetrahedral anions at the active site and, in particular, the tetrahedral transition states of catalysis. As with the lysine interactions described above, the charges on the atoms of MXY and their relative distances to the point charge representing the helix dipole are crucial to the interaction energies calculated; in all cases, distances to the point charge increased in the order X (in the oxyanion hole) < M < Y.

Second, the order of the calculated energies broadly correlates with $\delta_I E_{int}$ and the inhibition data—the boronate interacts comparably strongly to the carbon diol species whereas the silanol, the aldehyde, and keto ester interact more weakly. Thus, electrostatic interactions of ligands with the H2 helix dipole may be an additional and discriminating contributor to their effectiveness as substrates or inhibitors.

**Enzyme Inhibition.** Although one might suspect that $\delta_I E_{int}$ would give a good indication of the abilities of **1**, **3**−**9**, and **26** as inhibitors of the P99 $\beta$-lactamase, the real assessment of inhibitory power, at least in a thermodynamic sense, must come from evaluation of their performance in the reaction of eq 2. It

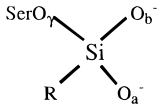
$$EOI_{aq} + H_2O \overset{K_I}{\rightleftharpoons} EOH_{aq} + HOI_{aq} \qquad (2)$$

should be noted that the writing of the inhibition equilibrium as a reaction between hydrated species as in eq 2 is not meant to carry any implication as to the *mechanism* of the reaction; e.g., formation of $C_O^H$ no doubt occurs through addition of the active site serine hydroxyl group to the free aldehyde **5** rather than to the hydrate. It is clear from eq 2 that the affinity of I for E in $EOI_{aq}$ must be balanced against the affinity of each separately for the aqueous solvent. Although an accurate calculation of absolute $K_I$ values, incorporating solvent interaction energies would require theory far beyond that used in this work,[60−65] it is interesting to consider the assumptions that would allow $\delta_I E_{int}$ to usefully indicate inhibitory power.[22] Reference to the available data in Table 1 suggests that at least a semiquantitative relationship between $\delta_I E_{int}$ and $\delta_I \Delta G_I^O$ (eq 2) does hold. The calculated interaction energies $\delta_I E_{int}$ predict that the boronate should be a much better inhibitor than the silane, the aldehyde, and the neutral dicarbonyl compounds. This result is correct and could not have been predicted, we feel, on simple inspection of the phosphonate crystal structure and the structures of the potential inhibitors. The slightly better performance of **7** as an inhibitor than the aldehyde **5**, contrary to the calculations, might reflect the poorer performance of the approximations of equations S6−S8[22] in $C_O^{CO_2Me}$ where a significantly different substituent replaces $O_b$.

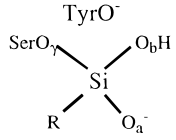
The calculations also predict that the phosphonate is a good inhibitor thermodynamically, but this refers to the neutral phosphonic acid as the free ligand (eq 2) and would likely not be true (as observed) at neutral pH where the compound is completely dissociated to the anion. Another interesting prediction is that the more effective interaction of the aldehyde **5** with the enzyme would occur with the oxyanion out of rather than in the oxyanion hole, $C_H^O$ rather than $C_O^H$; this is not true of the silicate, however. The trifluoromethyl ketone **26** is predicted to be a very poor inhibitor (compounded further by its likely complete hydration in water[66]).

The keto acid **6** is also a relatively weak inhibitor despite the calculated strong interaction (Table 1) between its tetrahedral adduct $C_O^{CO_2^-}$ and the enzyme. This discrepancy presumably reflects the compensating strong interaction between its carboxylate group with water in the free ligand. Indeed, calculation of the energy of interaction between energy-minimized HOI species and a 15 Å sphere of water for those inhibitors yielding monoanionic adducts yielded values around −50 kcal/mol, while that for **6** was −160 kcal/mol. Although, again, it is certainly not argued that these numbers represent good estimates of hydration free energies, they do indicate that the carboxylate of **6** might well be more strongly stabilized in free solution than in $C_O^{CO_2^-}$.

This same factor probably contributes significantly to the poor activity of the silanetriol **4** as an inhibitor, not only on forming the monoanionic adduct $Si_O^{OH}$, which interacts weakly with the enzyme (Table 1), but also as the dianion **28**, $Si_O^O$. This species contributes a tight (Table S2[22]), strongly interacting (Table 1) ligand but apparently to no inhibitory avail, and here also the calculated interaction energy with solvent in free solution is high (−181 kcal/mol). The basic strength of such a dianion may also be too high for it to exist stably as $Si_O^O$ (the p$K_a$ of a silanetriol monoanion in free solution would probably be 12−13[67]), and **29**, where proton transfer from Tyr 150 to $O_b$ has occurred, may be the more stable dianionic structure. The ligand in **29**, however, interacts more weakly with the enzyme than does $Si_O^O$ (Table 1).




**28**, $Si_O^O$




**29**

## Overview

The calculations reported above achieve an intriguing degree of rapport with experiment and chemical intuition. The energy-minimized structures $P_O^O$, $B_{OH}^{OH}$, $Si_O^{OH}$, $C_O^H$, and $C_O^{OH}$ can be readily interpreted in terms of the tetrahedral intermediates/transition states of class C $\beta$-lactamase catalysis in the mechanism proposed by Oefner and co-workers on the basis of crystal structures.[7,30] The parameter chosen for quantitative comparison of transition state analogs, $\delta_I E_{int}$, which may be termed a comparative interaction energy, has maximal (negative) value with $C_O^{OH}$, a tetrahedral intermediate traversed during hydrolysis of a substrate, and with $P_O^O$ and $B_{OH}^{OH}$, derived from known phosphonate and boronate inhibitors, respectively. The adducts $P_O^O$ and $B_{OH}^{OH}$ can be considered transition-state analogs both structurally and energetically. They may achieve this status, however, for different reasons, the boronate perhaps because of the more even charge distribution, reminiscent of the substantial charge dispersal in a transition state, and the phosphonate perhaps because of its larger size (P−O bond length 1.6 Å), resembling the expanded nature of a transition state. The $\delta_I E_{int}$ values indicate lesser transition state analogy for the aldehyde $C_O^H$ and silicate $Si_O^{OH}$ adducts. The importance of charge distribution in this result is demonstrated by Figure 3 where it is shown that $\delta_I E_{int}$ is largely dictated by electrostatic interactions between the central MXY moiety and the conserved

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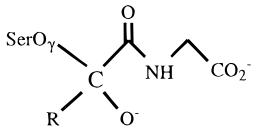
(in all $\beta$-lactam-recognizing enzymes) active site lysine residues, here Lys 67 and Lys 315. The oxyanion hole does not seem to contribute differentially at the tetrahedral stage of catalysis (Figure 4) but may do so at other points along the reaction coordinate. Discrimination between the inhibitors may also occur, however, through their interaction with the H2 helix of the protein.

It is interesting to compare these results with those from serine proteinases. In the latter enzymes, boronates are also generally considerably more effective as inhibitors than aldehydes, particularly when subsite specificity is included into the molecules.[45,68,69] Bachovchin and co-workers[44] discussed this difference in terms of two factors, the additional hydrogen bonding to the active site histidine available in boronates by virtue of the additional hydroxyl group and the different distribution of negative charge [although they, and others,[70,71] appear to consider the negative charge in boronates to be localized on boron (cf. Table S1[22])]. These clearly and directly correspond to the findings reported here, where the charge distribution and position of MXY was found to be crucial. It is likely that the placement of MXY in the serine proteinase active site with respect to the histidinium cation would be an important contributor to the relative effectiveness of tetrahedral adducts as inhibitors of this class of enzyme.[41,46,72]

By means of substantial but not unreasonable approximations (eqs S6−S8[22]), $\delta_I E_{int}$ can also be used to estimate the order of effectiveness of the parent species **3**−**5** as inhibitors, in the thermodynamic sense, of the P99 $\beta$-lactamase in aqueous solution. These approximations would be reasonable for a closely similar series of inhibitors. In the present case, **3** was correctly predicted to be a considerably better inhibitor than **4** and **5** (neither of which had been previously tested as $\beta$-lactamase inhibitors). With a larger number of inhibitors, $\delta_I E_{int}$ could be scaled to measured $K_I$ values as Jedrzejus et al.,[73] for example, have done with neuraminidase inhibitors.

The values of $\delta_I E_{int}$ also suggest that the dicarbonyl species **7**−**9** and the trifluoromethyl ketone **26** would not be effective inhibitors of the P99 $\beta$-lactamase, and this was shown to be true for the keto ester **7**. Although the dianionic ligand moieties in the keto acid and silicate complexes $C_O^{CO_2^-}$ and $Si_O^O$, respectively, interact much more strongly with the active site than the monoanions, they are not inhibitors under normal circumstances most likely because of their competing strong interaction with water. Other examples of ineffective carboxylates have been rationalized similarly.[65]

Two avenues of inhibitor design are suggested by these results. First, and most obvious, is the procedure of, beginning with any of the minimized structures, building further functionality at the position $O_b$ in order to achieve further effective interaction with the enzyme, particularly with the Lys 315 and Thr 316 side chains. This, of course, would represent the position of a substrate leaving group (and the best substrates do contain a carboxylate group here). For example, the carboxylate oxygens of the energy-minimized structure **30** do




**30**

in fact interact strongly with the side chains of Tyr 150, Lys 315, and Thr 316. Whether the carboxylate of **30** would have a net negative free energy of interaction with the active site with respect to aqueous solution or, as for $C_O^{CO_2^-}$, not, cannot easily be judged from these calculations. It is possible that neutral polar substituents would be more effective. A similar approach was adopted by Martin and Jones[11] in their elaboration of the boronate theme where the inclusion of a carboxylate did enhance binding.

A second approach questions whether in all cases MXY is optimally placed at the active site. The correlations of Figure 3, where the slope of the best fitting straight line is greater than unity, suggests that there may be a tension between MXY and the remainder of the ligand, presumably the side chain, which could be relaxed by side-chain manipulation. In exploratory experiments, we took the minimized phosphonate structure $P_O^O$, replaced the side chain R with a methyl group, and energy-minimized again to see if a position of MXY could be found whose interaction with the active site was stronger than in $P_O^O$. Methylphosphonates are poor $\beta$-lactamase inhibitors,[3,74] but this is by kinetic measurement (reflecting the free energy of the pentacoordinated intermediate) and may not directly reflect the degree of interaction MXY with the active site in the tetrahedral product. An energy-minimized methylphosphonate structure ($P_O^O$, R = Me) where the $PO_aO_b^-$ moiety interacted more strongly with Lys 67 and Lys 315 by 6 kcal/mol (cf. Table 2) was achieved. Apparently, further optimization of this aspect of $P_O^O$ as a transition state analog is possible. No improvement in the position of MXY for $Si_O^{OH}$ was found by this procedure, however.

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**Supporting Information Available:** Table S1 containing the charges on the ligands employed in the calculations and Table S2 containing the distances of Figure 1 for the energy-minimized structures. An analysis of the relationship between $\delta_I E_{int}$ and $\delta_I \Delta G_I^O$ (4 pages). See any current masthead page for ordering and Internet access instructions.

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