

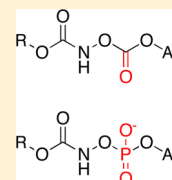
Covalent Inhibition of Serine β -Lactamases by Novel Hydroxamic Acid Derivatives

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Supporting Information

ABSTRACT: The effectiveness of β -lactam antibiotics is greatly limited by the ability of bacteria to produce β -lactamases. These enzymes catalyze the hydrolysis of β -lactams and thus loss of their antibiotic activity. The search for inhibitors of β -lactamases began soon after β -lactams were introduced into medical practice and continues today. Some time ago, we introduced a new class of covalent serine β -lactamase inhibitors, the *O*-aryloxycarbonyl hydroxamates, that inactivated these enzymes by a unique mechanism in which the active site became cross-linked. We describe in this paper some new variants of this class of inhibitor. First, we investigated compounds in which more polar hydroxamates were incorporated. These were generally not more active than the original compounds against representative class A and class C β -lactamases, but one of them, 1-(benzoyl)-*O*-(phenoxycarbonyl)-3-hydroxyurea, was significantly more stable in solution, thus revealing a useful platform for further design. Second, we describe a series of *O*-(arylphosphoryl) hydroxamates that are also irreversible inactivators of class A and class C β -lactamases, by phosphorylation of the enzyme, as revealed by mass spectra. These compounds did not, however, cross-link the enzyme active site. A striking feature of their structure–activity profile was that hydroxamate remained the leaving group on enzyme phosphorylation rather than aryloxide, even though the aryloxide was intrinsically the better leaving group, as indicated by pK_a values and demonstrated by the products of hydrolysis in free solution. Model building suggested that this phenomenon arises from the relative affinity of the enzyme active site components for the two leaving groups. The results obtained for both groups of inhibitors are important for further optimization of these inhibitors.



Bacterial resistance to β -lactam antibiotics continues to increase through evolution of β -lactamases.¹ These enzymes have become able to catalyze hydrolytic destruction of essentially all β -lactam antibiotics.² Class C β -lactamases are of concern in Gram-negative bacteria, particularly enterobacteria.^{3–5} The efficacy of β -lactams can be restored, however, by administration of a specific β -lactamase inhibitor together with a threatened β -lactam. There has been, and continues to be, much research directed toward new β -lactamase inhibitors.⁶ Most of the effective β -lactamase inhibitors known to date, including those that are currently used in clinical practice, are themselves β -lactams and act as mechanism-based inhibitors.⁷ These, too, eventually succumb to β -lactamase evolution.⁸

The broad specificity and genetic plasticity of β -lactamases, necessary to their biological function, dictate the situation in which potent broad spectrum noncovalent inhibitors are probably not possible; tellingly, none have been discovered from exhaustive natural product screens.⁹ Thus, the only useful β -lactamase inhibitors are likely to be of the covalent type. Although covalent inhibitors have had a spotty reputation in pharmaceutical circles,¹⁰ many clinically employed drugs are enzyme inhibitors of this kind, and there are recent calls for more attention to be paid to them.¹¹

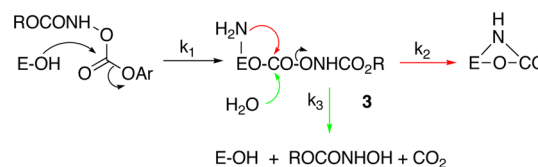
A number of non- β -lactam, covalent inhibitors of β -lactamases that, in principle, should not be susceptible to β -lactamases have been described over the years, including various acylating agents,^{12–15} phosphorylating agents, sulfonating agents, and boronic acids, for example.¹² Recently, we described a new class of covalent β -lactamase inhibitors of general structure 1.^{15,16} These compounds are acylating agents and direct analogues of the

acyclic substrates 2 but have the new feature of two possible leaving groups, as indicated in structure 1.



A study of 1 suggested that inhibition of the class C *Enterobacter cloacae* P99 β -lactamase was initiated by nucleophilic attack on the reactive carbonyl group by the active site serine hydroxyl, followed by loss of the aryloxide leaving group to yield acyl-enzyme 3 (Scheme 1). This acyl-

Scheme 1



enzyme was then attacked by the amine of Lys 315, displacing the second leaving group, the hydroxamate, to yield an inert cross-linked active site.¹⁵

A similar rationale has now led to phosphates 4 as direct analogues of known phosphonate inhibitors 5.

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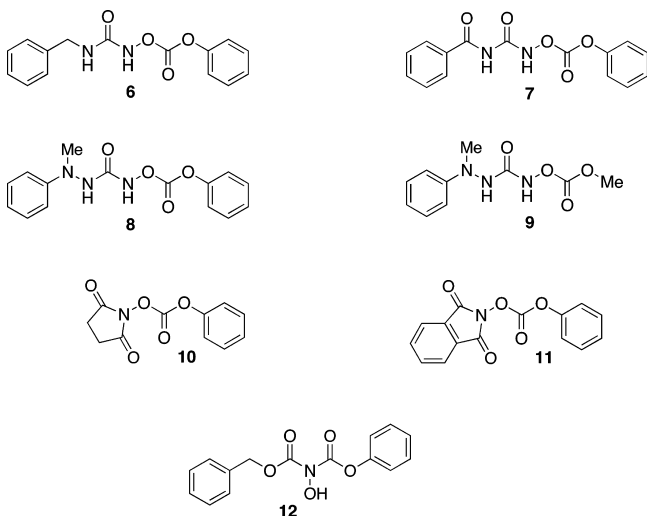
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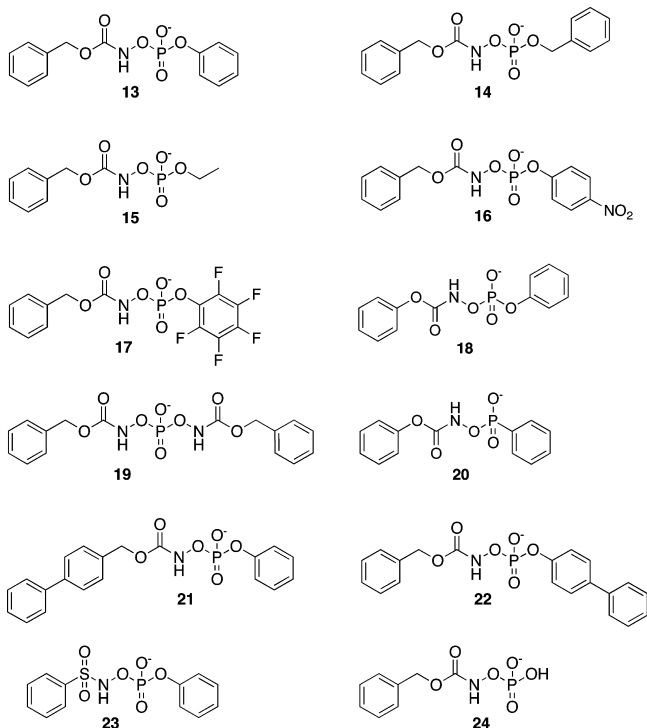




We have previously shown that electron-withdrawing groups on the aryloxy ring of **1** lead to more effective inhibitors.¹⁶ Structure–activity studies of **1** suggested that these compounds may react with the β -lactamase active site with the amido side chain in an orientation different from that of normal substrates.¹⁶ To probe for unusual polar interactions of the side chain, compounds **6–12** were prepared and evaluated as described below.



The dual leaving group potential of phosphyl inhibitors **4** was explored by means of compounds **13–24**.



EXPERIMENTAL PROCEDURES

The class C P99 β -lactamase from *E. cloacae* P99 and the TEM-2 β -lactamase from *Escherichia coli* were purchased from the Centre for Applied Microbiology and Research (Porton Down,

Wiltshire, U.K.). Compounds **25** and **26** were synthesized by R. Peltó as part of a previous study in this laboratory.¹⁶ Elemental analyses were conducted by Atlantic Microlab, Inc. (Norcross, GA). Electrospray mass spectra of enzyme complexes were obtained by the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois (Urbana, IL).

Synthesis. (*O*-Phenoxycarbonyl)-1-benzyl-3-hydroxyurea (**6**). A solution of imidazole (0.20 g, 3.01 mmol) in DCM (3 mL) was added dropwise to 1-benzyl-3-hydroxyurea (0.5 g, 3.01 mmol) (preparation described in the Supporting Information) dissolved in dimethoxyethane (10 mL). The resulting solution was stirred at room temperature for 15 min and the temperature then reduced to -20°C under a dry nitrogen atmosphere. Phenyl chloroformate (0.47 g, 3.01 mmol) in DCM (2 mL) was added dropwise over 1 min and the mixture stirred for approximately 90 min at 0°C . The imidazole hydrochloride precipitate was removed by filtration and the filtrate reduced to dryness by rotary evaporation. The residue was dissolved in ethyl acetate, precipitating more imidazole hydrochloride, which was removed by filtration. The solvent was then removed by evaporation and the residue further dried overnight under an oil pump vacuum. The crude solid product was recrystallized from a mixture of benzene and cyclohexane (1/1) in 6.6% yield: mp $108\text{--}110^{\circ}\text{C}$; ^1H NMR (d_6 -DMSO) δ 4.25 (d, $J = 7.20$ Hz, 2H), 7.22 (m, 8H), 7.45 (t, $J = 7.1$ Hz, 2H), 7.59 (t, $J = 6.9$ Hz, 1H), 10.5 (s, 1H); FTIR (KBr) 1813 cm^{-1} ; HRMS (ES⁺) 287.1031 ($M + \text{H}^+$), calcd for $\text{C}_{15}\text{H}_{15}\text{N}_2\text{O}_4$ 287.1032.

N-(Benzylloxycarbonyl)-*O*-(phenylphosphoryl)-hydroxylamine (**13**). Triethylamine (0.48 g, 4.76 mmol) in dry DCM (3 mL) was added very slowly dropwise to a precooled solution of phenyl dichlorophosphate (1.0 g, 4.76 mmol) and benzyl alcohol (0.51 g, 4.76 mmol) in dry DCM (12 mL) over a period of 1 h at -20°C under nitrogen. The reaction mixture was then stirred for an additional 1 h at that temperature. Benzyl *N*-hydroxycarbamate (0.79 g, 4.76 mmol) in dry DCM (12 mL) was added, followed by dropwise addition of triethylamine (0.48 g, 4.76 mmol) in dry DCM (3 mL). This reaction mixture was stirred for an additional 2 h until it reached room temperature. The solvent was evaporated to dryness, the residual oil dissolved in ethyl acetate, and the solution washed several times with 5% aqueous HCl. The organic layer was separated, dried over Na_2SO_4 , and evaporated to dryness to yield the benzyl ester of the required product as a colorless oil. This material was dried under an oil pump vacuum overnight. Then, without purification, the ester was subjected to debenzylation as described by Saady et al.¹⁷ Thus, DABCO (0.05 g, 0.48 mmol) was added to a stirred solution of the ester (0.20 g, 0.48 mmol) in anhydrous toluene (2 mL), under an argon atmosphere. The reaction mixture was heated under reflux for 2 h before the solvent was evaporated and the residue dissolved in aqueous HCl (5%, 10 mL). This solution was extracted with ethyl acetate and the organic layer dried over Na_2SO_4 . The solvent was then evaporated under reduced pressure to yield **13**. The crude product was purified by recrystallization from an ethyl acetate/hexane mixture (1/1) in 60% yield: mp $108\text{--}110^{\circ}\text{C}$; ^1H NMR (d_6 -DMSO) δ 5.12 (s, 2H), 7.17 (m, 3H), 7.36 (m, 7H), 10.93 (s, 1H); ^{31}P NMR (d_6 -DMSO) δ -4.14 ; HRMS (ES⁺) 324.0645 ($M + \text{H}^+$), calcd for $\text{C}_{14}\text{H}_{15}\text{NO}_6\text{P}$ 324.0637.

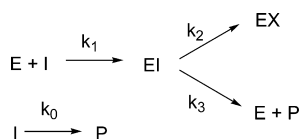
Kinetics Methods. Absorption spectra and spectrophotometric reaction rates were measured with Hewlett-Packard 8452A and 8452E spectrophotometers. Enzyme concentrations were determined spectrophotometrically. Kinetics experiments were conducted at 25°C in a 20 mM 3-morpholinopropanesulfonic acid

(MOPS) buffer solution at pH 7.5. Stock solutions of the inhibitors were prepared in DMF and diluted to 1–5% DMF in reaction mixtures.

Spontaneous Hydrolysis. Spontaneous hydrolysis rates of 6–12 were measured spectrophotometrically in replicate at appropriate concentrations and wavelengths (Table S1 of the Supporting Information). The full progress curves were then fit to a first-order rate equation and the rate constants thus obtained averaged.

P99 β -Lactamase Inactivation. The P99 β -lactamase (0.5 μ M) was incubated with inhibitor (10–500 μ M) in 100 μ L of buffer containing 0.1% BSA. At appropriate times, aliquots (5 μ L) of the reaction mixture were diluted to 1 mL in MOPS buffer containing cephalothin (300 μ M), and hydrolysis of the latter was monitored spectrophotometrically at 280 nm over a period of time. The measured initial rates, proportional to the remaining free enzyme concentration, were plotted versus incubation time. These data were fit to Scheme 2 with Dynafit.¹⁸

Scheme 2



With compounds such as 8 in which the spontaneous reaction was too rapid for the procedure described above to be employed, various concentrations of the inhibitor were incubated with the enzyme until the reaction was complete. The reaction mixture was then assayed for β -lactamase activity against cephalothin (300 μ M). These data, initial rates versus concentration, were also fit to Scheme 2 with Dynafit.¹⁸

Active Site Titration with 13. The P99 β -lactamase (1.0 μ M) was incubated with a series of concentrations (0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 μ M) of 13 in buffer containing 0.1% BSA. After 24 h, the reaction mixtures were assayed for β -lactamase activity against 300 μ M cephalothin and the initial rates thus determined plotted against inhibitor concentration to obtain the partition ratio (k_3/k_2) from the abscissa intercept. The data were fit in Kaleidograph.

Mass Spectra of EI Complexes. The P99 β -lactamase (20 μ M, 100 μ L) was incubated with 6 (1.0 mM; 5 μ L of a 20 mM stock solution in DMF was added) for 15 min. A small aliquot was taken and assayed for enzyme activity to confirm inactivation. The reaction was then quenched with 50% aqueous trichloroacetic acid (final concentration of 5%). The reaction mixture was allowed to stand on ice for 30 min and centrifuged at 2000 rpm for 8 min and the supernatant decanted. The precipitate was washed with 5% trichloroacetic acid (2 \times 50 μ L) followed by acetone (2 \times 50 μ L). The solid residue was dried under an oil pump vacuum and subjected to electrospray mass spectrometry. A control sample without inhibitor was prepared in the same way.

The P99 β -lactamase (20 μ M) was incubated with phosphates 13 (500 μ M) and 19 (1.2 mM) (total volumes of 100 μ L) for 15 and 30 min, respectively. Small aliquots were taken and assayed for enzyme activity to confirm inactivation. The reaction mixtures were then prepared for mass spectroscopy as described above.

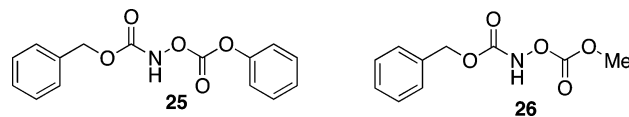
NMR Study of the Hydrolysis of 13. A solution of 13 in $^2\text{H}_2\text{O}$ containing 20 mM NaHCO_3 at p²H 7.2 was heated at 70 $^\circ\text{C}$ for 24 h. ^1H NMR spectra (300 MHz) were recorded at suitable intervals as the hydrolysis reaction proceeded.

Molecular Modeling. The crystal structure of the P99 β -lactamase (Protein Data Bank entry 1XX2), including crystallographic water molecules, was used as the starting point for computations using Discovery studio version 2.5 (Accelrys). The CHARMM force field was employed for all computations. The partial charges of the enzyme were assigned by Discovery studio. Structures and partial charges of the PO_5 nucleus of the pentacoordinated phosphorus species were derived from the results of high-level calculations.¹⁹ The positions of the phosphorus atom and the oxygen atoms bound to it were fixed because molecular mechanics parameters for this type of structure were not available. The phosphorane dianion structure (with neutral Tyr 150) was chosen because it represents a close analogue of the intermediate immediately formed on general base (Tyr 150 anion)-catalyzed attack of the serine hydroxyl group on a phosphate monoanion. The pH was set to 7.5, and the total charge on the complex was -2.0 . The side chains of Lys 67 and Lys 315 were cationic. The enzyme–substrate complex was hydrated with a 20 \AA sphere of water molecules centered at the active site serine. The hydrated structure was energy-minimized using the steepest descent algorithm (250 steps) followed by the conjugate gradient method (750 steps) for 1000 steps. The energy-minimized structures were heated for 10000 steps (10 ps) to 300 K. The output of the heating run was then subjected to a 1 ns production run. Several of the 100 saved structures were selected from the production output and energy-minimized using the protocol mentioned above.

RESULTS AND DISCUSSION

Acyl hydroxamates 6–12 were prepared by acylation of the corresponding hydroxamic acids, as previously described.¹⁶ Phosph(on)ate diesters 13–23 were prepared by reaction of appropriate phosphyl chlorides with the required alcohols and hydroxamic acids in the presence of base. Where appropriate, the benzyl group was used to protect one phosphyl oxygen and subsequently removed by DABCO aminolysis.¹⁷ Phosphate monoester 24 was prepared by condensation of phosphoric acid with benzyl *N*-hydroxycarbamate.²⁰ Details of the synthesis of 6 and 13 are presented in Experimental Procedures and those of the other compounds in the Supporting Information.

Acyl derivatives 6–12 hydrolyzed spontaneously in aqueous buffer. Observed first-order rate constants for this reaction of 6–12, under the conditions employed at pH 7.5, are listed in Table 1. For comparison, data for parent compound 25 and methoxycarbonyl analogue 26 are included. Immediately noticeable from Table 1 is the high spontaneous reactivity of many of the new compounds, most of them more reactive than 25 itself. The differences in reactivity among 25, 6, 7, and 8, and between 9 and 26, probably reflect the relative effectiveness of the hydroxamate carbonyl as an intramolecular nucleophile in catalyzing the breakdown of these compounds (Scheme 3).



Nucleophilic amido-carbonyl participation in systems analogous to these is well-documented.^{21,22} The carbonyl of 1, as a carbamate, is a poor nucleophile²³ but would be enhanced by the replacement of the carbamate oxygen with nitrogen as in 6 and 8 (and in 9 vs 26). Acylation of this nitrogen, as in 7, would weaken the nucleophilicity of the hydroxamate carbonyl, leading to the slow, spontaneous reaction of this compound.

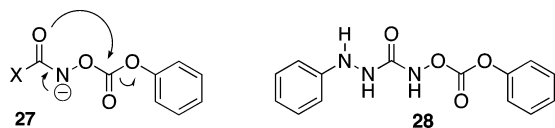
Table 1. Rate Constants for Inhibition of the P99 β -Lactamase by Acyl Hydroxamates

| inhibitor | k_0 (s^{-1}) ^a | k_1 ($M^{-1} s^{-1}$) | k_3/k_2 ^b |
|-------------------|--------------------------------------|---------------------------|------------------------|
| 25 ^{c,d} | 2.5×10^{-4} | 6100 ± 200 | 2.0 ± 0.1 |
| 6 | 0.0191 ± 0.0001 | 1640 ± 50 | ND ^f |
| | $[(2.41 \pm 0.01) \times 10^{-4}]^e$ | $[400 \pm 9]^e$ | 7.7 |
| 7 ^c | $(8.35 \pm 0.04) \times 10^{-5}$ | 320 ± 160 | 580 ± 15 |
| | $[(7.27 \pm 0.13) \times 10^{-5}]^e$ | $[800 \pm 69]^e$ | 21 |
| 8 | 0.0643 ± 0.0005 | 7480 ± 250 | ND ^f |
| | $[(2.54 \pm 0.02) \times 10^{-4}]^e$ | $[1700 \pm 350]^e$ | 2.05 |
| 9 | $(2.28 \pm 0.01) \times 10^{-3}$ | 0.87 ± 0.01 | ND ^f |
| 26 ^d | very slow | 0.19 ± 0.01 | ND ^f |
| 10 | $(3.66 \pm 0.01) \times 10^{-3}$ | 201 ± 1 | ND ^f |
| 11 | $(7.2 \pm 0.1) \times 10^{-3}$ | 4320 ± 420 | ND ^f |
| 12 | $(2.93 \pm 0.03) \times 10^{-5}$ | 64 ± 7 | ND ^f |

^aSpontaneous hydrolysis rate constant. ^b k_3/k_2 is the turnover/inactivation ratio (Scheme 1). ^cInhibition data fit to Scheme 1. ^dFrom ref 16. ^epH 5.0. ^fNot determined. Small values of k_3/k_2 (≤ 20) for these compounds could not be precisely determined because of the high spontaneous hydrolysis rates and/or the low rates of inhibition.

The combination of a poor intramolecular nucleophile and a poor leaving group in **26** greatly impedes the spontaneous breakdown of **26**.²⁴

The lability of both **6** and **8** is significantly lower at pH 5 (Table 1). This suggests that the reactive entity in Scheme 3 may actually be anion **27**. Compound **25** has an acidic NH with a pK_a of 6.8,¹⁶ and the current compounds would be expected to be comparably acidic, with some variation dictated by the details of the structure. Compounds **10** and **11** hydrolyze rapidly in solution, probably from direct water and hydroxide attack; the hydroxamate leaving group would be much better in these cases (pK_a values of 6.0 and 7.1, respectively²⁵) because of the two activating acyl groups on the hydroxamate nitrogen.



Compounds **6–12**, in general, were time-dependent, irreversible inactivators of the class C *E. cloacae* P99 β -lactamase (see Figure 1, for example) and of the class A TEM-2 enzyme. Second-order rate constants of inactivation of the P99 enzyme were obtained as described in Experimental Procedures, and the results are listed in Table 1. It is unlikely that the (transiently stable) dioxazolinone product from the reaction of **27** is a significant inhibitor.²⁶ The results show that, at pH 7.5, only compound **8** is (slightly) more effective than parent compound **25**; **6** and **7** are markedly less effective inactivators. The same positive effect of hydrazine incorporation is seen upon comparison of methyl esters **9** and **26**. This may be due to the more effective hydrogen bond acceptor (hydrazide carbonyl) present in **8** and **9**. The demethylated analogue of **8**, compound **28**, may also be an effective inhibitor, but attempts to synthesize it failed, probably because of its instability.

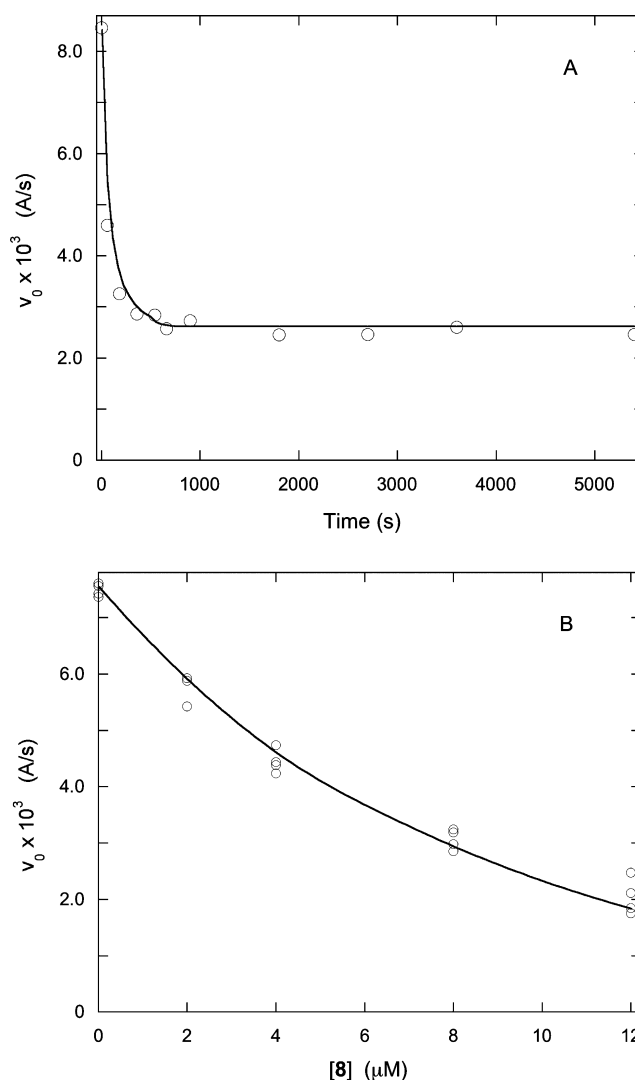
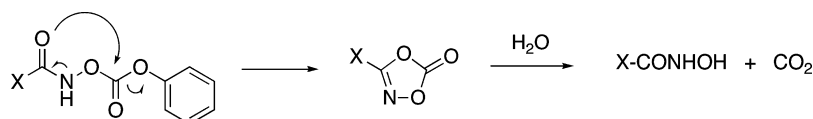
Scheme 3


Figure 1. (A) Activity of the P99 β -lactamase (0.15 μ M) as a function of time in the presence of **6** (10.0 μ M). (B) Activity of the P99 β -lactamase (0.15 μ M) after complete reaction with **8** at various concentrations.

The value of an aromatic ring on the hydroxamic acid moiety is apparent in the comparison of **10** and **11**. The imide moiety of **10** and **11** does not, however, provide them any advantage as inhibitors over the original amide of **25**.

It is also interesting to note at this stage the activity of *N,N*-diacyl-hydroxylamine **12**, in comparison with that of the *N,O*-diacyl isomer **25**. As recorded in Table 1, **12** inhibited the P99 enzyme less effectively than did **25**. This order of activity is in accord with the order of spontaneous hydrolytic lability (Table 1) and the carbonyl stretch frequencies in the infrared spectra of these molecules (the highest-frequency carbonyl is 1764 cm^{-1} for **12** and 1808 cm^{-1} for **25**¹⁶).

As described in the introductory section, inactivation of the P99 β -lactamase by **25** is accompanied by irreversible cross-linking

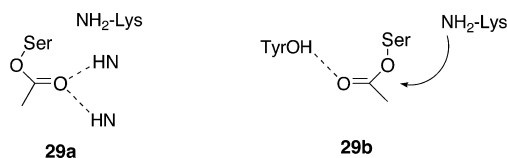
of the active site by insertion of an inhibitor-derived carbonyl moiety between the active site Ser 67 and Lys 315. It is likely that the inhibitors described in this paper also follow this mechanism. A mass spectrum of the inactive complex of **6** with the enzyme showed, as with **25**, addition of a small fragment with a mass of 25 ± 5 , consistent with Scheme 1.

The effectiveness of **25** as an inhibitor at pH 7.5 is limited by its existence as an unreactive anion at that pH.¹⁶ It is more effective in the pH range of 5–7 where it gains a proton to form the reactive neutral species (Scheme 1). Values of k_1 for **6**–**8** at pH 5 are also listed in Table 1. It can be seen that **7** also becomes more effective at pH 5, but **6** and **8** become less effective. These changes probably reflect the resultant of the effects of the NH pK_a 's of **6** and **8**, which are likely to be higher than that of **25**, and the pK_a of the enzyme active site whose protonation reduces its reactivity.¹⁶

The practical effectiveness of the new inhibitors is greatly restricted by their instability in solution [k_0 (Table 1)], where the exception is **7**, which is much more stable, as discussed above. Although *N,N*-diacyl compound **12** is, like **7**, more stable, it is also a poorer inhibitor than its *N,O*-diacyl analogue **25** (Table 1). As also described above, pH is important, because the stability of **6** and **8** does increase markedly at lower pH values.

The other parameter that is important in defining the inhibitory potency of these compounds is the partition ratio k_3/k_2 (Scheme 1), favoring hydrolysis of the intermediate acyl-enzyme versus inactivation. This was small, ~ 2 , for **25**. It is also small (≤ 20) for **6** and **8**–**12**, although it is difficult to accurately determine because of the large background hydrolysis rates at pH 7.5. The interesting exception is **7**, for which the turnover rate greatly exceeds the inactivation rate. As discussed previously,¹⁶ partitioning of the acyl-enzyme is probably determined by its relative occupancy of two conformations of the inhibitor in the active site (Scheme 4), one (**29a**) in which the acyl-enzyme carbonyl is held in the oxyanion hole in the manner of a normal substrate, leading to hydrolysis. In the other (**29b**), the carbonyl is hydrogen-bonded to the Tyr 150 hydroxyl; this conformation leads to attack by Lys 315 and inactivation by cross-linking. It seems that the acyl-enzyme from compound **7**, unlike the others, and possibly due to an interaction of the benzoyl carbonyl oxygen with a component of the active site, e.g., Asn 152 or Gln 120, prefers conformation **29a** and hydrolysis. More structural information is needed to design inhibitors with a preference for **29b**, but **7** would be a useful place to begin.

Scheme 4



Compound **6** was also, like **25**, an inactivator of the class A TEM-2 β -lactamase, with a k_1 value of $240 \pm 15 \text{ M}^{-1} \text{ s}^{-1}$, cf. $13.4 \text{ M}^{-1} \text{ s}^{-1}$ for **25**.¹⁶ The significantly higher value for **6** may signal the formation of an additional hydrogen bond between **6**, containing an additional NH hydrogen bond donor in the side chain, and the polar active site of the TEM-2 enzyme, which contains, for example, Glu 166 and Asn 171.

In contrast to the acyl derivatives, the phosphates are stable to spontaneous hydrolysis at neutral pH and room temperature.

The hydrolysis of **13** at 70 °C in 20 mM NaHCO_3 was monitored for 4 days by ^1H NMR, which showed approximately two-thirds of it hydrolyzed to phenol and **24** at that time. It is interesting that the first leaving group appeared to be phenoxide (phenol, pK_a of 10.0²⁷) rather than the hydroxamate (benzyl *N*-hydroxycarbamate, pK_a of 9.2²⁸) (see further discussion below). The better 4-nitrophenoxide leaving group of **16** also left first in the spontaneous hydrolysis of this compound.

Phosphylates **13**–**22** are stoichiometric 1/1 irreversible inactivators of the P99 β -lactamase (see Figure 2, for example).

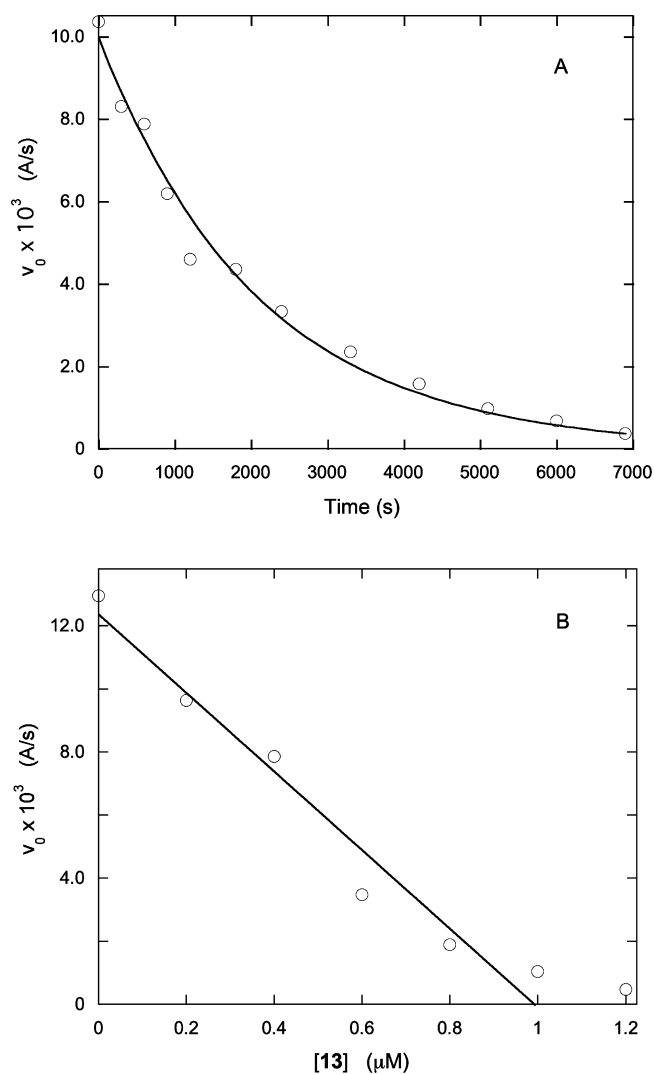


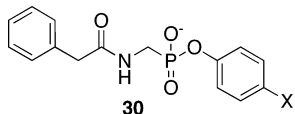
Figure 2. (A) Activity of the P99 β -lactamase ($0.5 \mu\text{M}$) as a function of time in the presence of **13** ($10.0 \mu\text{M}$). (B) Activity of the P99 β -lactamase ($1.0 \mu\text{M}$) after complete reaction with **13** at various concentrations.

Second-order inactivation constants are listed in Table 2. The k_1 value of $49 \text{ M}^{-1} \text{ s}^{-1}$ for **13** is smaller than the value of $200 \text{ M}^{-1} \text{ s}^{-1}$ for the close phosphonate analogue **30** ($X = \text{H}$), a difference that is typical for nucleophilic attack at the phosphorus of a phosphate versus a phosphonate.^{29,30} Although the values of k_1 listed in Table 2 generally appear to be low, it has been shown that structural optimization of **30** can lead to k_1 values of at least $10^5 \text{ M}^{-1} \text{ s}^{-1}$ for both class A and class C β -lactamases.³¹ One important improvement in **30** lay in adding a better aryloxy leaving group. For example, the k_1 value for **30** ($X = \text{NO}_2$) is $5.6 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$.³²

Table 2. Rate Constants for Inhibition of the P99 β -Lactamase by Phosphylates

| phosphate | k_i ($M^{-1} s^{-1}$) | phosphate | k_i ($M^{-1} s^{-1}$) |
|-----------|---------------------------|-----------|---------------------------|
| 13 | 49 ± 3 | 19 | 6.7 ± 0.2 |
| 14 | 31 ± 1 | 20 | 1100 ± 6 |
| 15 | 3.1 ± 0.1 | 21 | 12.0 ± 1.4 |
| 16 | 0.89 ± 0.17 | 22 | 93 ± 1 |
| 17 | 3.5 ± 0.4 | 23 | NI ^a |
| 18 | 130 ± 1 | 24 | NI ^a |

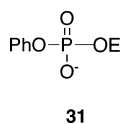
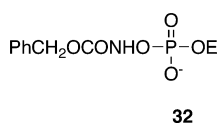
^aNo inhibition observed under the conditions employed.



Consequently, we investigated the effect of changing the aryloxy leaving group of **13** (although the hydroxamate is also a potential leaving group). In **14** and **15**, the phenoxide of **13** is replaced with poorer alkoxide leaving groups, and in **16** and **17**, it is replaced with much better leaving groups. Counter-intuitively, the alkoxy leaving groups produced only a small decrease in rate, and even more surprisingly, the *p*-nitro and pentafluoro substitutions of the phenoxide led to decreases in rate (Table 2). These observations are incompatible with rate-determining nucleophilic displacement of phenoxide in **13** by the active site serine hydroxyl. The observations are, however, in accord with nucleophilic attack on phosphorus with displacement of the hydroxamate to yield phosphoryl-enzyme **31**. A concerted associative mechanism would be expected for nucleophilic displacement at a phosphate diester monoanion,³³ which, in this case, as indicated by the substituent effects, would involve a transition state with more bond breaking (to hydroxamate) than formation (to Ser OH). Alternatively, general acid catalysis for departure of the hydroxamate could be indicated, but this seems unlikely for such a good leaving group and is thought to be unavailable for phosphyl transfer reactions at the P99 β -lactamase active site.³² Rate-significant bond breaking to hydroxamate is also suggested by the activity of **18** being higher than that of **13** (the anion of PhOCONHOH, pK_a of 8.4,²⁸ should be a better leaving group than that of PhCH₂OCONHOH, pK_a of 9.2²⁸).

Compounds **13**–**24** had low activity against the class A TEM-2 or the class D OXA-1 β -lactamases, which resembles the situation with **30** and these enzymes.³⁴ Nonetheless, as with **30**,³¹ structural optimization might well lead to useful inhibitors.

A mass spectrum (Figure S1A of the Supporting Information) of the inert complex derived from the reaction of the P99 β -lactamase with **13** showed a mass enhancement of 155 amu compared with that of the original enzyme. This corresponds well to addition of (PhPO₂ minus H) (155 amu), as in **31**, and thus loss of the hydroxamate from phosphorus. Notably, the phenoxide group was also not lost, and thus, cross-linking did not occur.


31

32

Thus, contrary to observations in the carbonyl system and to observations in free solution (see above), the hydroxamate has

been the first to leave from the phosphoryl reaction center. This must reflect the differences in the transition state geometry and electronic structure between the acylation and phosphorylation transition states. It is possible, however, to achieve a hydroxamoyl phosphoryl enzyme **32** by means of symmetrical phosphate **19**. This compound was, however, a weaker inhibitor than **13**, implying that neither hydroxamate moiety interacts strongly with the enzyme. The enzyme inactivated by **19** yielded a mass spectrum (Figure S1B of the Supporting Information) with an increase of 234 amu versus the free enzyme mass. This correlates well with addition of (PhCH₂OCONHOPO₂ minus H) (230 amu) to the enzyme.

The reactivity of phosphyl hydroxamates with β -lactamases does, however, seem to be a predictable function of the phosphyl group. As would be expected,^{28,29,35} phosphonate **20** is a more effective inhibitor than phosphate **18**. The ability of hydroxamate as a leaving group is also seen in the results for **20**, which is a considerably better inhibitor of the P99 enzyme than 4-nitrophenyl phenylphosphonate ($k_i = 2.9 M^{-1} s^{-1}$).³⁶

Additional hydrophobicity in the hydroxamate moiety, as in **21**, produces a decrease in inhibitory activity as compared with that of **13**, while such an addition to the aryloxy moiety, as in **22**, leads to a small increase. This difference in response presumably reflects the nature of the respective binding sites (see below). The inactivity of **23**, like that of the analogous acyl derivative, is probably due to the dissociation of the NH to form an unreactive dianion.¹⁶ Finally, the inactivity of **24** is likely due to the poor electrophilicity of phosphorus in this molecule, even as a monoanion, and the absence of general acid catalysis^{32,37} to facilitate a dissociative mechanism involving quasi-free metaphosphate.

We employed molecular modeling to understand the results described above with the phosphates in terms of active site structure. We began with the premise that the mechanism of the reaction between the β -lactamase and a phosphate diester involved bimolecular displacement at phosphorus with a trigonal bipyramidal pentacoordinated phosphorus intermediate.³³ In the reactive complex, one would expect the incoming serine oxygen nucleophile to take one apical position and a leaving group, either hydroxamate or phenoxide, to take the other. We also considered the possibility of pseudorotation of the first-formed pentacoordinated intermediate, but this did not seem to lead to more strongly interactive structures. Although pseudorotation at an enzyme active site is unlikely and, to the best of our knowledge, not unambiguously proven to occur for any enzyme,^{38,39} it may be more likely to arise when unnatural active site ligands are investigated or promiscuous or moonlighting enzymes.⁴⁰ The participation of a sixth phosphorus ligand is also a possibility but not one yet demonstrated in any particular case.⁴⁰

The experimental evidence (see above) suggests that the hydroxamate must prefer the second apical position even when the phenoxide is apparently a better leaving group, such as the 4-nitrophenoxide in **16** (4-nitrophenol, pK_a of 7.1)²⁷ and the pentafluorophenoxide in **17** (pentafluorophenol, pK_a of 5.5).⁴¹ A combination of model building, molecular dynamics, and molecular mechanics energy minimizations led to the structures of Figure 3 as best representing the likely pentacoordinated intermediates.

In Figure 3A, phenoxide is in the (apical) leaving group position and the hydroxamate in the equatorial. Neither group seems to enjoy any interaction with the enzyme that would confer specificity. In Figure 3B, the hydroxamate is certainly in a good position to leave. It has no direct interaction with the enzyme, and

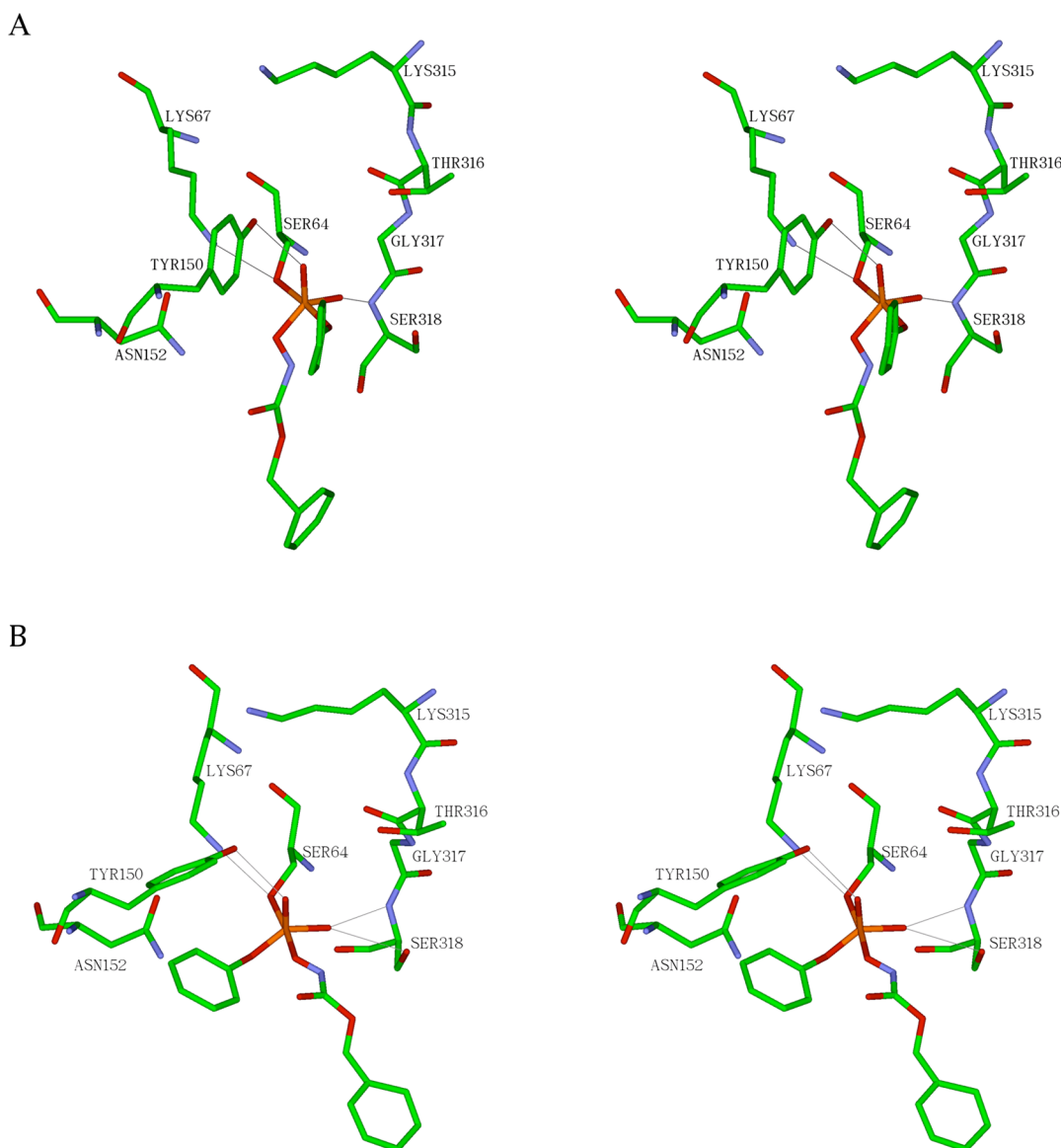


Figure 3. Stereoviews of energy-minimized pentacoordinated phosphorus intermediate structures formed on reaction of **13** with the P99 β -lactamase. Only heavy atoms are shown. (A) Phenoxide in the (apical) leaving group position. (B) Hydroxamate in the (apical) leaving group position.

thus, its rate of departure is most directly influenced by its intrinsic leaving group ability, as expressed to a first approximation by its conjugate acid pK_a . The presence of additional hydrophobic character may therefore not greatly affect its departure as seen in the results with **21**. It is interesting to note here that the phenylacetamido moiety of **30** does appear in models⁴² to interact more strongly, by hydrogen bonding, with the enzyme (occupying the classical side chain amide binding site comprising the side chain NH_2 group of Asn 152 and the backbone carbonyl oxygen of Ser 318), than the carbamate group of **13**. In the case of **30**, the aryloxy leaving group, in an apical position, appears to interact with the aromatic ring of Tyr 150, in a manner similar to that of the aryloxy of **13** in Figure 3B (see below).

The equatorial disposition of the other potential leaving group, aryloxy or alkoxide, however, is also favorable to the structure of Figure 3B (cf. Figure 3A). The phenoxide ring of **13** is able to interact favorably with both Tyr 150 (hydrophobic/quadrupole) and Asn 152 (dipole/quadrupole). The quadrupolar interactions would be enhanced if the electron

density on the phenoxide ring were decreased, as in **16** and **17**. It is therefore possible that the aryloxy group would prefer to remain in the equatorial (nonleaving) position in the complexes derived from **16** and **17**, as well as from **13**, rather than take up the apical (leaving group) position. The ability of enzyme active sites to select the leaving group of a phosphoryl transfer reaction to yield a kinetic rather than a thermodynamic product has been previously observed in a substrate analogue^{43,44} but not, to the best of our knowledge, in an inhibitor. It should be noted that an extended hydrophobic aryloxy might reorient to allow hydrophobic interaction with Tyr 221 rather than Tyr 150, as seen in a variety of boronic acid and noncovalent inhibitors.^{45,46} The aryl group of arylphosphonyl inhibitors,³⁶ also in an equatorial position, appears to interact with Tyr 150 in a manner similar to that of the aryloxy leaving group of **13** shown in Figure 3B. The poorer alkoxide phosphorus ligands of **14** and **15** would naturally take up equatorial positions as the hydroxamate prepared to depart from the apical position. The structure of Figure 3B, therefore, seems to better accommodate the experimental results.

CONCLUSIONS

O-Aryloxycarbonyl hydroxamates, **1**, inactivate serine β -lactamases by cross-linking the active site (Scheme 1).¹⁶ The new derivatives, **6–12**, noting particularly those containing side chains elaborated with polar hydrogen bonding functionality (**6–9**), appear to inhibit by the same mechanism used by **1** and generally have similar activity as inhibitors. The new compounds, however, are generally less stable in aqueous solution than **25**, with the interesting exception of hydroxyurea derivative **7**. Further structure–activity studies based on **7** may lead to a useful new class of inhibitors.

Phosphyl analogues **13–24** also represent a new class of class C β -lactamase inhibitors, with the advantage of being quite stable in solution. These compounds phosphorylate the active site but do not cross-link it. The competition between hydroxamate and aryloxide as the leaving group from phosphorus was found to favor hydroxamate, which does not appear to significantly interact with active site residues. Aryloxide does appear to interact with the active site through hydrophobic and quadrupolar interactions but only when placed in an equatorial (nonleaving group) position of trigonal bipyramidal intermediates. Counterintuitively then, and in contrast to the situation with acylation inhibitors **1**, addition of electron-withdrawing groups to the aryloxide produced poorer rather than better inhibitors (**16** and **17** vs **13**). Better inhibitors should therefore be sought by optimizing the hydroxamate leaving group and by enhancing the interactions of the aryloxide with the active site in pentacoordinated phosphorus intermediates (Figure 3B).

ASSOCIATED CONTENT

Supporting Information

Mass spectra of complexes of the P99 β -lactamase with **13** and **19**, a table of analytical wavelengths, and synthetic details for the preparation of compounds **6–12** and **14–24**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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ABBREVIATIONS

BSA, bovine serum albumin; DABCO, 1,4-diazabicyclo[2.2.2]-octane; DCM, dichloromethane; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; ESMS, electrospray mass spectrometry; FTIR, Fourier transform infrared; MOPS, 3-(N-morpholino)-propanesulfonic acid; NMR, nuclear magnetic resonance.

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