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Synthesis and Evaluation of Ketophosph(on)ates as β-Lactamase Inhibitors

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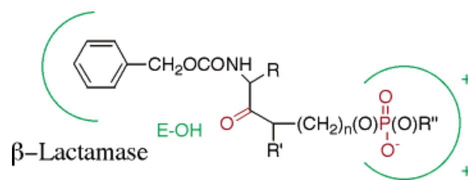
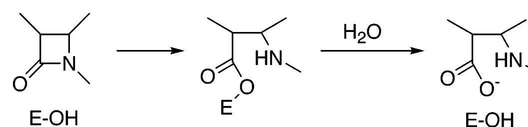


A series of amidoketophosph(on)ates of general structure $PhCH_2OCONHCH(R)COCHR'(CH_2)_n(O)P(O_2^-)(O)R''$ (R = H, CH_3; R′ = H, CH_3; n = 0, 1; R″ = H, CH_3, Et, Ph) have been prepared as a potential source of β-lactamase inhibitors. The phosphonates (n = 0) were obtained by means of the Arbuzov reaction while most of the phosphates were achieved from reaction of phosph(or/on)ic acids with the appropriate diazoketone $PhCH_2OCONHCH(R)COCR'N_2$. The electrophilicity of the carbonyl group in the resulting phosph(on)ates was assessed by the degree of hydration in aqueous solution, determined from NMR spectra. These compounds inhibited typical class C and class D β-lactamases, particularly the latter group, but showed no activity against class A enzymes. To enhance the carbonyl electrophilicity, an α-difluorinated analogue (R = H, CHR′ = CF_2, n = 0, R″ = Et) was also prepared, but no enhanced inhibitory activity was observed. All evidence suggested that these compounds inhibited in the carbonyl form rather than by formation of tetrahedral adducts at the β-lactamase active site. They show promise as leads to specific class D β-lactamase inhibitors.

Introduction

The β-lactam antibiotics are an important defense against bacterial infections. β-Lactams kill bacteria by inhibiting the final cross-linking step in cell wall synthesis and thus fatally weakening the walls of growing bacterial cells. β-Lactamases arose through bacterial evolution to combat the chemical peril to bacteria of β-lactam antibiotics.[1] These enzymes specifically catalyze the hydrolysis of β-lactams, thereby rendering them ineffective as antibiotics. On the basis of amino acid homology and active site structure, β-lactamases have been grouped into four classes.[2] Of these, three classes A, C, and D, are serine enzymes that employ a double displacement mechanism of catalysis (Scheme 1) and are thought to have evolved from bacterial transpeptidases/DD-peptidases,[3] and Class B β-lactamases are zinc-containing metalloenzymes that have a broad spectrum of activity.[4] Classes A and C contain the best-studied β-lactamases and, at present, are also the most important with respect to human disease. Class D β-lactamases, however, capable of hydrolyzing second generation penicillins, such as oxacillin and cloxacillin, and penems, have gained attention in recent years owing to their extended spectrum of activity in clinical isolates.[5]

SCHEME 1



Since β-lactamase-induced antibiotic resistance has emerged as a real threat to the use of the β-lactam antibiotics, there has been considerable interest in the development of β-lactamase inhibitors. Inhibition of β-lactamases remains a viable approach

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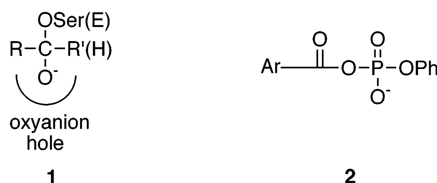
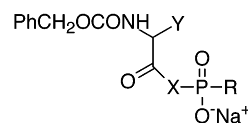
to retrieving the effectiveness of a variety of β-lactam antibiotics in medicine.[6] The β-lactamase inhibitors used clinically at present are, however, ineffective against several well-known enzymes.[7]

A wide variety of inhibitors of serine β-lactamases have been obtained from natural sources or designed and synthesized. The most effective of these are mechanism-based inhibitors or transition state analogues, where the latter class includes, particularly, a variety of boronates and phosphonates.[3,8] Another group of transition state analogues that are known to be effective against serine hydrolases are electrophilic carbonyl compounds.[9] The mechanism of action of these compounds as inhibitors is generally thought to involve addition of the active site nucleophilic serine hydroxyl group to the electrophilic carbonyl group to form a tetrahedral anionic adduct, **1**, that mimics a high-energy tetrahedral species of normal catalysis.[10] Examples of such inhibitors that have been tested against β-lactamases include suitable aldehydes,[11] trifluoromethyl ketones,[11,12] α-ketoacid derivatives,[11] and α-ketoheterocycle analogues.[13] The results, to date, have, however, been disappointing and no potent β-lactamase inhibitors of this type have yet emerged.



A study in this laboratory of the reaction of acyl phosph(on)ates, **2**, with serine β-lactamases demonstrated that these compounds could rapidly acylate the enzyme active site.[14] In some cases, depending on the acyl group involved, subsequent deacylation was rapid while in others it was slow such that the acyl phosph(on)ates behaved as inhibitory substrates. Molecular modeling suggested that the leaving group phosphate moiety interacted strongly with the active site of these enzymes, particularly with the conserved lysine residue in the leaving group site.[14] On the basis of the above observations, we envisioned that the favorable active site interactions of the phosphyl group could be taken advantage of by its incorporation into an electrophilic ketone with an amido side chain to obtain new inhibitors of β-lactamases. We therefore decided to investigate the potential of ketophosph(on)ates as inhibitors of β-lactamases. Compounds **3**−**11** were therefore synthesized and



	X	R	Y
3	CH_2	OPh	H
4	CH_2	ONa	H
5	CH_2O	OPh	H
6	CH_2O	OMe	H
7	CH_2O	Ph	H
8	CH_2O	ONa	H
9	CH_2O	ONa	D-Me
10	$CH(CH_3)O$	ONa	H
11	CH_2CH_2	ONa	H
12	CF_2	OEt	H
13	$NHCH_2$	ONa	H

evaluated. Further, it is well-known that introduction of fluorine atoms α to a carbonyl group increases the electrophilicity of the carbonyl carbon, thereby increasing its ability to react with nucleophiles in a facile manner.[9a] Further, α-difluorophosphonates and phosphates are isoelectronic and isosteric with very similar pK_a values.[15] The molecule **12**, with fluorine α to both the carbonyl and phosphonyl moieties, was therefore also prepared. The peptide **13**, not susceptible to stable carbonyl addition, was prepared as a mechanistic control.

Results and Discussion

The new series of compounds **3**−**12** were prepared by reactions that, in view of our results, should now be routinely available for the synthesis of a wide range of analogous compounds. All syntheses commenced with *N*-acylglycines to which the phosphyl moiety was attached in various ways. The syntheses of **3**−**11** and the ^{13}C-isotopomer of **8** involved conversion of the *N*-acylglycine (via a mixed anhydride) to the corresponding diazoketone by means of the Arndt−Eistert reaction. The phosphonate **3** could then be obtained by way of the bromide **16** (Scheme 2) and the Arbuzov reaction. Hydrolysis of **3**, catalyzed by phosphodiesterase I then yielded the dianion **4** (Scheme 3). The phosphates **5**−**10** were obtained by reaction of the relevant diazoketone with phosphonic or phosphoric acids (Scheme 4), as described by Bischofberger et al.[16] and employed by Dai et al. to prepare analogous potential dioxygenase inhibitors.[17] Compound **20**, the ^{13}C-carbonyl-labeled isotopomer of **8**, was prepared in the same way as **8**, as described in the Supporting Information. Compound **11**, the homologue of **3**, was prepared according to Scheme 5. The additional carbon was incorporated by formaldehyde addition to the Wittig reagent **26**. Michael addition of dimethyl phosphite to the alkene **27** then yielded the basic framework of the final product. The α-difluorinated compound **12** was obtained from the copper bromide catalyzed reaction of a zinc complex of the diethyl difluoromethylphosphonate anion with the appropriate glycyl chloride (Scheme 6). The amide analogue **13** was

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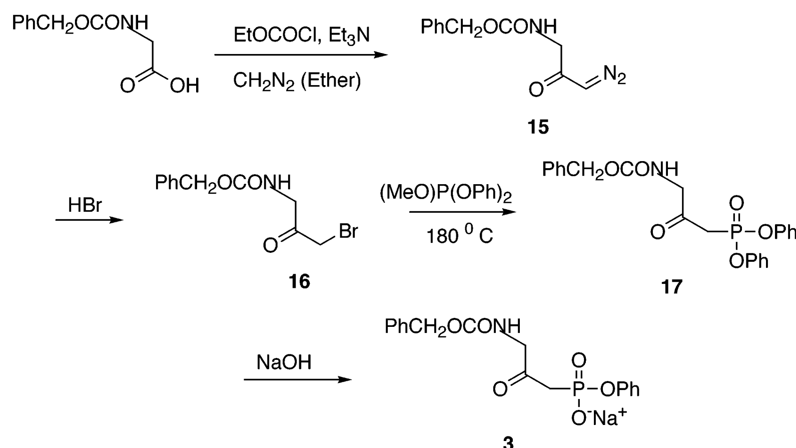
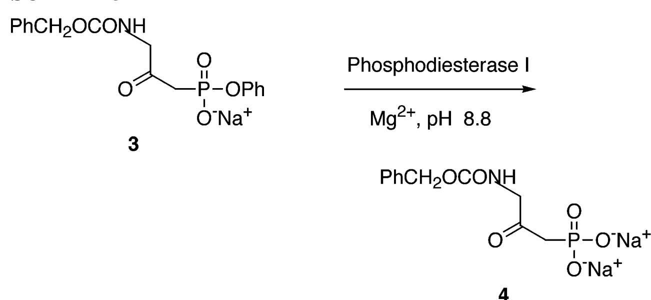
SCHEME 2



SCHEME 3

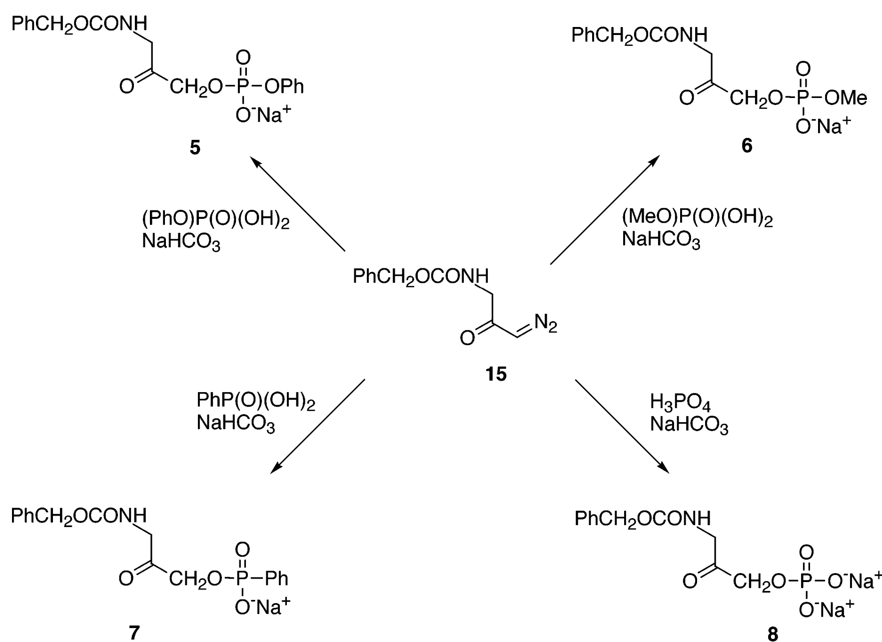


obtained from standard peptide chemistry. All compounds were purified by aqueous chromatography as the sodium salts; yields were not, in general, optimized—since we were interested in purity rather than yield, only the purest chromatography fractions were selected.

In a number of cases, ^{1}H NMR spectra of the ketones **3**−**12** in deuterium oxide solution showed the presence of two species. The nature of the spectral difference between these species—characterized by differences between the chemical shifts of methylene groups α to the carbonyl of ca. 0.8 ppm—indicated that, as anticipated, most of these ketones were hydrated in aqueous solution. This conclusion was supported by the ^{13}C spectrum of the ^{13}C (ketone carbonyl) isotopomer of **8** in D_2O that displayed resonances at 208.9 and 94.9 ppm, typical of ketone and hydrate species, respectively. The extent of hydration of **3**−**12** was estimated quantitatively from the ^{1}H NMR spectra and the results are presented in Table 1. The phosphonates **3**, **4**, and **11** are, like acetone and the amide **13**, not discernibly hydrated (≤2%) in solution. The phosphates **5**−**10**, by virtue, presumably, of the oxygen atom β to the carbonyl, are significantly hydrated (27−44%); in comparison, hydroxyacetone is ca. 3% hydrated in aqueous solution[18] (cf. 0.2% for acetone[19]). Finally, **12**, the difluoroketone, was 74% hydrated in solution, considerably more so than the hydrogenated analogue **3**. The significantly hydrated compounds, at least, should have a sufficiently electrophilic carbonyl for reaction with the active site serine of the β-lactamases.

The ketophosph(on)ates **3**−**13** were found to inhibit typical class C and class D β-lactamases. Quantitative results from these experiments are presented in Table 1. Although these compounds were not very effective against the class C enzyme, they

SCHEME 4



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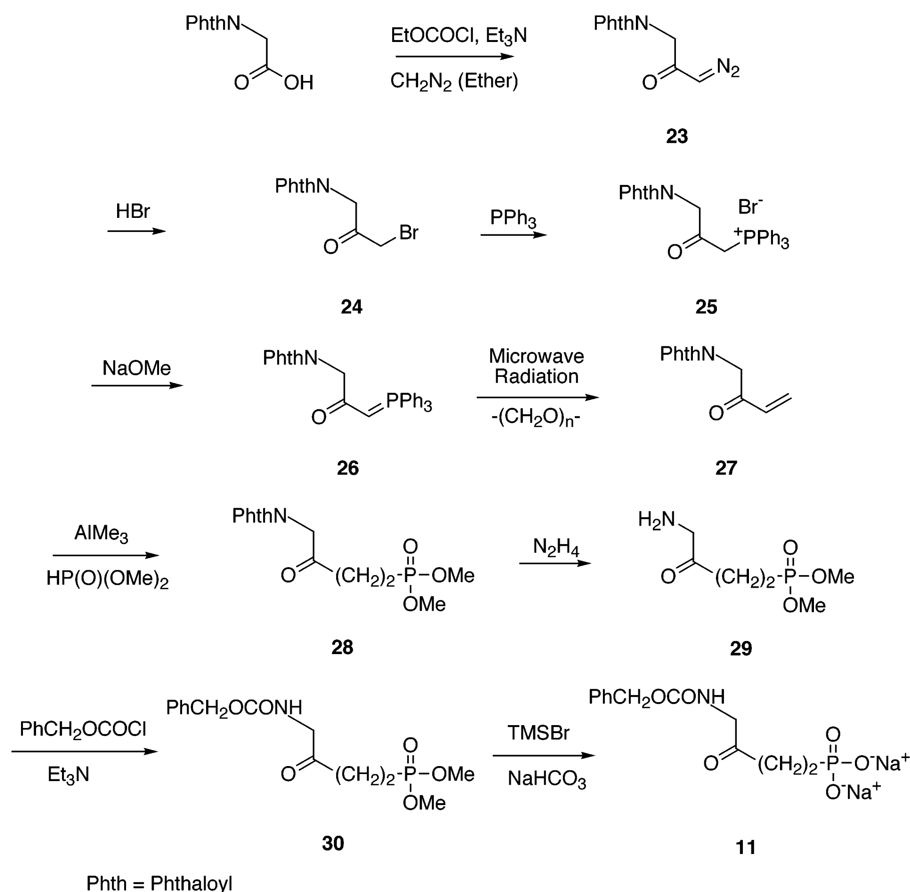

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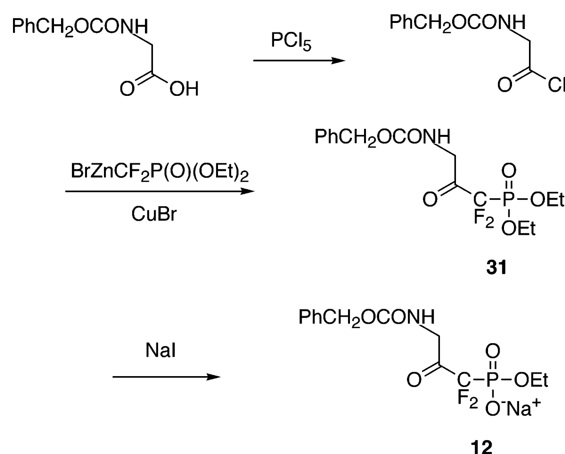

TABLE 1. Hydration and Inhibition Data for the Ketophosph(on)ates 3−13

inhibitor	% hydrated	K_i (mM)[a] P99[b]	OXA-1[b]	OXA-10[b]
3	0	1.59	0.13	0.077
4	0	0.73	0.15	
5	36	0.70	0.13	0.11
6	36	1.13	0.24	
7	43	5.0	0.19	0.15
8	44	2.0	0.10	0.60
9	42	2.1	0.30	0.36
10	27	0.97	0.088	0.20
11	0	1.23	0.60	0.47
12	74	0.95	0.93	
13	0	0.93	0.43	

[a] Uncertainties in K_i values are ±20%. [b] P99: class C β-lactamase of *Enterobacter cloacae* P99. OXA-1, OXA-10: class D β-lactamases.

were more so against the class D enzymes—the K_i value of **3** against OXA-10 was 77 μM while that of **10** against OXA-1 was 88 μM. It is likely that the addition of further hydrophobic substituents to these compounds would increase their effectiveness against the class D β-lactamases and probably also against class C.[20] Neither the class A TEM enzyme nor the class B β-lactamase II of *B. cereus* was significantly inhibited by **3**−**13**. With respect to the latter observation, it might also be noted that no transition state analogue inhibitor of class B β-lactamases has yet been devised—a puzzling situation. The classical class A β-lactamase, TEM, in general, interacts only weakly with acyclic substrates[21] and thus, perhaps, its poor response to **3**−**12**. It is interesting to note, however, that class D β-lactamases, for which **3**−**12** appear to be the more effective inhibitors, have active site structures similar to those of class A.[22] There are

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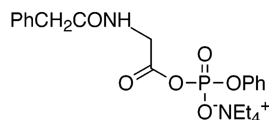
important differences, however, which may be related to the different level of interactions with **3**−**12**. Activity against bacterial DD-peptidases/transpeptidases is also problematic since **3**−**13** had little effect on the DD-peptidase of *Streptomyces* R61.

The relative affinity of **3**−**13** for the various β-lactamases does, however, correlate closely with the activity of these enzymes against the structurally very similar substrate **14**; values



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of k_{cat}/K_m for this substrate were 1.7×10^6, 1.0×10^6, 1880, and 400 s^{-1} M^{-1}, for the OXA-1, P99,[14c] TEM,[14c] and PCl[14c] β-lactamases, respectively. These values correspond to the free energies of enzyme acylation, a process that is usually thought of as involving a tetrahedral intermediate, but which may be a more concerted reaction—involving little loss of carbonyl double bond character—in the presence of a good leaving group,[23] especially if assisted by hydrogen bonding.[14]

Although it was hoped that **3**−**12** would form tetrahedral adducts with serine β-lactamases, the evidence suggests that they do not. The peptide **13**, which cannot form a stable tetrahedral adduct, was essentially as effective as **3**−**12** against the P99 and OXA-1 enzymes. A ^{13}C NMR spectrum (not shown) of a mixture of ^{13}C-labeled **8** (0.8 mM) with the P99 β-lactamase (1.0 mM) showed a free carbonyl resonance at ca. 208 ppm but no tetrahedral carbon at ca. 100 ppm, strongly suggesting that any bound **8** was in the carbonyl form. Finally, the fluoroketone **12**, although containing a far more electrophilic carbonyl than **3**, as shown by the hydration studies described above, was no better as an inhibitor than **3**−**11**. This situation contrasts, for example, with that for acetylcholinesterase, a serine acyl hydrolase, where the inhibitory power of ketones increased dramatically on successive α-fluorination.[24] Similar observations were made with the human cytomegalovirus protease.[25] In the latter case, only a carbonyl resonance was observed in the ^{13}C NMR spectrum of the complex of the enzyme with a ketone inhibitor whereas a hemiketal resonance was observed with a fluorinated analogue.

The reason for the apparently different relative abilities of serine acyl hydrolases to form tetrahedral adducts with carbonyl compounds is not well understood. Presumably, it is, to some degree, a function of the position of the tetrahedral adduct with respect to the other specific binding sites—of the amido and phosph(on)ate sites in the present case. It may be that the binding of the amide and phosph(on)ate together favor trigonal carbon rather than tetrahedral, in which case modification of the former may lead to better inhibitors.

It should also be noted that calculations of the interaction energies between tetrahedral adducts and the class C β-lactamase active site—principally with the conserved lysines, Lys 64 and Lys 315, both assumed to be cationic—suggested that tetrahedral adducts generated from esters (i.e. substrates), boronic acids, and phosphonate esters yielded more strongly interacting species than did carbonyl compounds; the presence of an oxygen atom rather than carbon bonded to the tetrahedral atom in the leaving group position was important in this regard.[11] Inspection of models also suggests that the presence of hydrogen or fluorine atoms directly adjacent to the tetrahedral center may disrupt the mobility of the Lys 64-Tyr 150 catalytic apparatus.

A novel series of amido ketophosph(on)ates has thus been prepared. Although these compounds are not, in general, powerful β-lactamase inhibitors, the results obtained do suggest how better inhibitors might be obtained, particularly of class D where few effective inhibitors are currently available.[26] We are pursuing these leads.

Experimental Section

Materials and Methods. See the Supporting Information.

Syntheses. Sodium Phenyl 3-(*N*-Benzyloxycarbonyl)amino-2-oxopropylphosphonate (3). See Scheme 2.

Diphenyl 3-(*N*-Benzyloxycarbonyl)amino-2-oxopropylphosphonate (17). Compounds **15** and **16** were obtained as described in the Supporting Information. The title compound **17** was obtained by Arbuzov reaction of **16** with methyl diphenyl phosphite. Thus, a mixture of **16** (0.4 g, 1.4 mmol) and methyl diphenyl phosphite (0.35 g, 1.4 mmol) was heated to 180 °C for 2 h. The crude mixture was purified by silica gel chromatography with ethyl acetate:hexane (2:3) as the eluant. The pure title compound was obtained as a colorless solid. Yield 26%; mp 220 °C dec; ν_{max} (KBr) cm^{-1} 3275, 1717, 1255; ^1H NMR (DMSO-d_6) δ 7.60 (t, J = 5.4 Hz, 1H), 7.18−7.44 (m, 15 H), 5.00 (s, 2H), 4.20 (d, J = 6 Hz, 2H), 3.75 (d, J = 22.2 Hz, 2H); ^{31}P NMR (DMSO-d_6) δ 12.76.

Sodium Phenyl 3-(*N*-Benzyloxycarbonyl)amino-2-oxopropylphosphonate (3). The required compound was obtained from alkaline hydrolysis of **17**.[27] To a solution of **17** (0.26 g, 0.6 mmol) in dioxane (4 mL) was added an aqueous solution of NaOH (0.104 g, 2.6 mmol, in 1.0 mL). The mixture was stirred for 1 h at room temperature. The pH of the reaction mixture was then brought to 8 by addition of aqueous $NaHCO_3$ solution. The mixture was then extracted with ethyl acetate and the aqueous layer was lyophilized. The product was purified by Sephadex G-10 chromatography. The appropriate fractions (characterized by UV absorption) after lyophilization yielded **3** in 12% yield from **17**. ^1H NMR (D_2O) δ 7.34−7.5 (m, 7H), 7.15−7.25 (m, 3H), 5.17 (s, 2H), 4.24 (s, 2H), 3.21 (d, J = 22 Hz, 2H); ^{31}P NMR (D_2O) δ 7.11; exact mass (ES^+) MH^+ calculated for $C_{17}H_{18}NO_6PNa$ 386.0769, found 386.0754.

Disodium 3-(*N*-Benzyloxycarbonyl)amino-2-oxopropylphosphonate (4) (see Scheme 3). A solution of sodium phenyl 3-(*N*-benzyloxycarbonyl)amino-2-oxo-1-propylphosphonate (**3**) (0.5 g, 1.3 mmol) in 5 mL of carbonate−bicarbonate buffer (pH 9), containing 10 mM Mg^{2+}, was stirred at 37 °C. Snake venom phosphodiesterase I (500 mg, Sigma Chemical Co., Type IV) from *Crotalus atrox* was added,[28] and the solution was maintained at 37 °C. The incubation was continued until the reaction was complete (3 days) as monitored by ^1H NMR spectra. The solution was then diluted 2-fold with water and transferred to a Centriprep-10 concentrator. This was then centrifuged at 3000 g for 40 min. The filtrate thus obtained was lyophilized. The crude product was then purified by anion-exchange chromatography, using Sephadex-QAE A25 ion-exchange resin with an ammonium bicarbonate concentration gradient (0−2.5 M). This material was further purified by Sephadex G-10 chromatography. The appropriate fractions (characterized by UV absorption) after lyophilization yielded **4** (9%). ^1H NMR (D_2O) δ 7.21−7.34 (m, 5H), 4.97 (s, 2H), 4.06 (s, 2H),

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2.79 (d, $J = 20.7$ Hz, 2H); ^{31}P NMR (D_2O) δ 12.22; exact mass (ES$^+$) MH$^+$ calculated for $C_{11}H_{13}NO_6PNa_2$ 332.0276, found 332.0280.

Phosph(on)ates 5−8. See Scheme 4.

Sodium Phenyl 3-(*N*-Benzyloxycarbonyl)amino-2-oxopropylphosphate (5). To a solution of the diazoketone **15** (0.2 g, 0.85 mmol) in benzene/THF mixture (2:1) (1.0 mL) was added phenyl phosphate (0.15 g, 0.85 mmol), obtained from phenyl dichlorophosphate by a general procedure reported by Kluger et al.[29] The reaction mixture was stirred for 10 h at room temperature. An aqueous solution of $NaHCO_3$ (72 mg, 0.85 mmol) was added to the above mixture and the pH was brought to 8. After an extraction with ethyl acetate, the aqueous solution was lyophilized. The product was purified by Sephadex G-10 chromatography. The appropriate fractions (characterized by UV absorption) after lyophilization yielded **7** (9%). Ketone: ^{1}H NMR (D_2O) δ 7.1−7.3 (m, 8H), 6.95−7.05 (m, 2H), 4.95 (s, 2H), 4.53 (d, $J = 7.5$ Hz, 2H), 3.97 (s, 2H). Hydrate: ^{1}H NMR (D_2O) δ 7.1−7.3 (m, 8H), 6.95−7.05 (m, 2H), 4.91 (s, 2H), 3.72 (d, $J = 5.1$ Hz, 2H), 3.16 (s, 2H); ^{31}P NMR (D_2O) δ −3.21, −3.65; exact mass (ES$^+$) MH$^+$ calculated for $C_{17}H_{18}NO_7PNa$ 402.0719, found 402.0701.

Sodium Methyl 3-(*N*-Benzyloxycarbonyl)amino-2-oxo-1-propylphosphate (6). To a solution of the diazoketone **15** (0.2 g, 0.85 mmol) in a benzene/THF mixture (2:1) (1 mL) was added methyl phosphate (0.095 g, 0.85 mmol), obtained from methyl dichlorophosphate by a general procedure reported by Kluger et al.[29] The reaction mixture was stirred for 10 h at room temperature. An aqueous solution of $NaHCO_3$ (72 mg, 0.85 mmol) was added to the above mixture and the pH was brought to 8. After an extraction with ethyl acetate, the aqueous solution was lyophilized. The product was purified by Sephadex G-10 chromatography. The appropriate fractions (characterized by UV absorption) after lyophilization yielded **6** in 12% yield. Ketone: ^{1}H NMR (D_2O) δ 7.4−7.5 (m, 5H), 5.17 (s, 2H), 4.58 (d, $J = 8.1$ Hz, 2H), 4.22 (s, 2H), 3.62 (d, $J = 10.8$ Hz, 3H). Hydrate: ^{1}H NMR (D_2O) δ 7.4−7.5 (m, 5H), 5.19 (s, 2H), 3.80 (d, $J = 5.1$ Hz, 2H), 3.59 (d, $J = 11$ Hz, 3H), 3.41 (s, 2H); ^{31}P NMR (D_2O) δ −1.00, −1.41; exact mass (ES$^+$) MH$^+$ calculated for $C_{12}H_{16}NO_7PNa$ 340.0562, found 340.0546.

Sodium 3-(*N*-Benzyloxycarbonyl)amino-2-oxopropylphenylphosphonate (7). A mixture of the diazoketone **15** (50 mg, 0.215 mmol) and phenylphosphonic acid (34 mg, 0.215 mmol) in benzene/THF (1:1) mixture (1 mL) was heated at 40 °C for 12 h. The solvent was removed, and the residue was redissolved in ethyl acetate. To this mixture was added an aqueous solution of $NaHCO_3$ (18 mg, 0.215 mmol) and the pH was taken to 8. After an extraction with ethyl acetate, the aqueous layer was lyophilized. The product was purified by Sephadex G-10 chromatography. The appropriate fractions (characterized by UV absorption) after lyophilization afforded **7** in 29% yield. Ketone: ^{1}H NMR (D_2O) δ 7.4−7.8 (m, 10H), 5.13 (s, 2H), 4.52 (d, $J = 7.2$ Hz, 2H), 4.12 (s, 2H); ^{31}P NMR (D_2O) δ 7.00. Hydrate: ^{1}H NMR (D_2O) δ 7.4−7.8 (m, 10H), 5.09 (s, 2H), 3.68 (d, $J = 5.4$ Hz, 2H), 3.30 (s, 2H); ^{31}P NMR (D_2O) δ 4.66; exact mass (ES$^+$) MH$^+$ calculated for $C_{17}H_{18}NO_6PNa$ 386.0769, found 386.0764.

Disodium 3-(*N*-Benzyloxycarbonyl)amino-2-oxopropylphosphate (8) To a solution of the diazoketone **15** (0.2 g, 0.85 mmol) in ether (1 mL) was added an 85% aqueous solution of orthophosphoric acid (58 μL). The reaction mixture was stirred for 12 h at room temperature. An aqueous solution of $NaHCO_3$ (0.16 g, 1.9 mmol) was added to the above mixture. After an extraction with ethyl acetate, the aqueous solution was lyophilized. The product was purified by Sephadex G-10 chromatography. The appropriate fractions (characterized by UV absorption) after lyophilization afforded **8** in 18% yield. Ketone: ^{1}H NMR (D_2O) δ 7.4−7.5 (m, 5H), 5.14 (s, 2H), 4.52 (d, $J = 6.9$ Hz, 2H), 4.24 (s, 2H). Hydrate: ^{1}H NMR (D_2O) δ 7.4−7.5 (m, 5H), 5.13 (s, 2H), 3.77 (d, $J = 6.9$ Hz, 2H), 3.34 (s, 2H); ^{31}P NMR (D_2O) δ 1.16, −0.63; exact mass (ES$^+$) MH$^+$ calculated for $C_{11}H_{13}NO_7PNa$ 348.0225, found 348.0222.

Disodium *R*-3-(*N*-Benzyloxycarbonylamino)-2-oxo-butylphosphate (9). To a solution of the diazoketone **21** (0.21 g, 0.85 mmol), whose synthesis is described in the Supporting Information, in ether (1 mL) was added an 85% aqueous solution of orthophosphoric acid (98 mg, 58 μL). On stirring the reaction mixture, N_2 evolution took place. The reaction mixture was stirred for 12 h at room temperature. An aqueous solution of $NaHCO_3$ (0.16 g, 1.9 mmol) was then added to the above mixture to take the pH of the solution to 8. After an extraction with ethyl acetate, the aqueous solution was lyophilized. The product was purified by Sephadex G-10 chromatography. The appropriate fractions (characterized by UV absorption) after lyophilization yielded **9** (12%). Ketone: ^{1}H NMR (D_2O) δ 7.34−7.44 (m, 5H), 5.08 (s, 2H), 4.59 (d, $J = 7.5$ Hz, 2H), 4.37 (q, $J = 7.5$ Hz, 1H), 1.29 (d, $J = 7.2$ Hz, 3H). Hydrate: ^{1}H NMR (D_2O) δ 7.34−7.44 (m, 5H), 5.08 (s, 2H), 3.71−3.82 (m, 3H), 1.12 (d, $J = 7$ Hz, 3H). ^{31}P NMR (D_2O) δ −0.69, −0.96; exact mass (ES$^+$) MH$^+$ calculated for $C_{12}H_{15}NO_7PNa_2$ 362.0382, found 362.0376.

Disodium *RS*-4-(*N*-Benzyloxycarbonyl)amino-3-oxo-2-butylphosphate (10).

2-Diazo-4-(*N*-benzyloxycarbonyl)aminobutan-3-one (22). The synthesis of the title compound was achieved by employing a general procedure reported in the literature.[16,28] *N*-(Benzyloxycarbonyl)glycine (3.77 g, 18 mmol) in anhydrous THF (90 mL) with triethylamine (1.82 g, 18 mmol) was stirred at −20 °C under a dry N_2 atmosphere. To the above solution was added ethyl chloroformate (1.95 g, 18 mmol) in anhydrous THF (15 mL). The solution was stirred for 30 min and then allowed to warm to −10 °C. The precipitated triethylamine hydrochloride was removed by filtration. To the solution of the mixed anhydride thus obtained was added an ethereal solution of diazoethane (27 mmol), which was obtained from *N*-ethyl-*N*-nitroso-N′-nitroguanidine, as reported.[30] The solution was stirred at −5 °C for a further 3 h and then allowed to warm to room temperature. The reaction mixture was evaporated to one-third of its original volume and diluted with ether. The solution was then washed with saturated bicarbonate solution and brine and dried over $MgSO_4$. The solvent was removed and the crude product thus obtained was purified by silica gel chromatography, using ethyl acetate:hexane (1:2) as eluant, in 39% yield. ν_{max} (KBr) (cm^{-1}) 2083, 1736; ^{1}H NMR (DMSO-d_6) δ 7.72 (t, $J = 7.0$ Hz, 1H), 7.31−7.37 (m, 5H), 5.07 (s, 2H), 4.05 (d, $J = 7.3$ Hz, 1H), 1.97 (br s, 3H).

Disodium *RS*-4-(*N*-Benzyloxycarbonyl)amino-3-oxo-2-butylphosphate (10). This compound was prepared from the diazoketone **22** in the same way that **9** was obtained from **21**. The product was purified by Sephadex G-10 chromatography. The appropriate fractions (characterized by UV absorption) after lyophilization yielded **10** in 16% yield. Ketone: ^{1}H NMR (D_2O) δ 7.14−7.30 (m, 5H), 4.97 (s, 2H), 4.5−4.6 (m, 1H), 4.19 (s, 2H), 1.23 (d, $J = 6.9$ Hz, 3H). Hydrate: ^{1}H NMR (D_2O) δ 7.14−7.30 (m, 5H), 4.97 (s, 2H), 3.51 (s, 2H), 3.23−3.45 (m, 1H), 1.11 (d, $J = 5.4$ Hz, 3H); ^{31}P NMR (D_2O) δ −0.47, −1.83; exact mass (ES$^+$) MH$^+$ calculated for $C_{12}H_{15}NO_7PNa$ 362.0382, found 362.0373.

Disodium 4-(*N*-Benzyloxycarbonyl)amino-3-oxobutylphosphonate (11). See Scheme 5.

1-Phthalimido-2-oxo-3-butene (27). A mixture of the ylide **26** (see the Supporting Information) (100 mg, 0.216 mmol) and paraformaldehyde (40 mg, 1.33 mmol) in DMF (20 mL) was irradiated by means of a domestic microwave oven (Sunbeam, SBM 7500W) for 1 min and then cooled to room temperature. This operation was repeated 4 times. The solvent was then removed at reduced pressure. The crude product was purified by silica gel chromatography with acetone:hexane (2:3) as the eluant. Yield 8%; mp 80−84°C; ^{1}H NMR ($CDCl_3$) δ 7.88 (dd, $J = 3.3$ Hz, 6 Hz,

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2H), 7.74 (dd, $J = 3$ Hz, 5.4 Hz, 2H), 6.44 (ABX, $J = 18.0$, 9.6, 2.0 Hz, 2H), 6.02 (ABX, $J = 9.6$ Hz, 2.0 Hz, 1H), 4.8 (s, 2H).

Dimethyl 4-Phthalimido-3-oxo-butylphosphonate (28). The title compound was synthesized by following a procedure reported by Green.[31] Thus, to a solution of dimethyl phosphite (66.5 mg, 0.46 mmol) in methylene chloride (1 mL) at 0 °C was added a 2 M toluene solution of trimethylaluminum (0.23 mL, 0.465 mmol). After the above solution had been stirred for 20 min, the vinyl ketone **27** (100 mg, 0.46 mmol) was added. The resulting solution was allowed to warm to room temperature for over an hour and stirred overnight. The solution was then poured into a 5% aqueous solution of HCl and extracted with methylene chloride. The combined organic extracts were dried over $MgSO_4$ and then concentrated to yield the title compound. The product was purified by preparative thin-layer chromatography in 63% yield. Mp 100−106 °C; ^{1}H NMR ($CDCl_3$) δ 7.88 (dd, $J = 3.3$ Hz, 5.4 Hz, 2H), 7.75 (dd, $J = 3$ Hz, 6 Hz, 2H), 4.52 (s, 2H), 3.75 (d, $J = 10.5$ Hz, 6H), 2.84 (dt, $J = 12.0$ Hz, 7.5 Hz, 2H), 2.09 (dt, $J = 18.0$ Hz, 7.4 Hz, 2H); ^{31}P NMR ($CDCl_3$) δ 30.23.

Dimethyl 4-amino-3-oxobutylphosphonate (29). This compound was obtained by hydrazinolysis[32] of **28**. Thus, to a stirred suspension of **28** (0.2 g, 0.6 mmol) in methanol (2 mL) was added 98% anhydrous hydrazine (20 μL, 0.6 mmol). When the addition was completed, a homogeneous solution was obtained. The reaction mixture was then stirred for 24 h at room temperature and the resulting solid was removed by filtration and washed with methanol. The filtrate was concentrated at 0°C to yield the title compound which was used immediately as described below.

Dimethyl 4-(*N*-Benzyloxycarbonyl)amino-3-oxobutylphosphonate (30). To a stirred solution of **29** (59 mg, 0.5 mmol) in $CHCl_3$ (2 mL) at 0 °C was added benzyl chloroformate (0.13 g, 0.73 mmol), followed by the addition of triethylamine (74 mg, 0.73 mmol). The above mixture was stirred at room temperature for 12 h before it was diluted with $CHCl_3$ (5 mL) and washed with 20 mL portions of 2 N H_2SO_4. The combined organic layers were dried over $MgSO_4$ and concentrated to yield (67%) the title compound. ^{1}H NMR ($CDCl_3$) δ 7.27−7.30 (m, 5H), 5.38 (br t, 1H), 5.05 (s, 2H), 4.04 (d, $J = 5.1$ Hz, 2H), 3.66 (d, $J = 11$ Hz, 6H), 2.67 (dt, $J = 7.5$ Hz, 12.9 Hz, 2H), 2.01 (dt, $J = 7.8$ Hz, 18 Hz, 2H); ^{31}P NMR ($CDCl_3$) δ 29.84.

Disodium 4-(*N*-Benzyloxycarbonyl)amino-3-oxobutylphosphonate (11). The title compound was obtained by the demethylation of the dimethyl phosphonate **30** with TMSBr.[33] Thus, to a solution of **30** (96 mg, 0.29 mmol) in dry methylene chloride (3 mL) was added TMSBr (76 μL, 0.59 mmol) dropwise. The mixture was then stirred for 3 h, after which the volatiles were evaporated under reduced pressure to give bis(trimethylsilyl)-4-(*N*-benzyloxycarbonyl)amino-3-oxobutylphosphonate. This silylated product was then stirred with acetone (3 mL) and water (0.2 mL) for an hour. The solvent was then evaporated and the product obtained was treated with an aqueous solution of $NaHCO_3$ (49 mg, 0.58 mmol) and thereby the pH brought to 8. This solution was extracted with ethyl acetate. The aqueous layer was lyophilized and the product purified by Sephadex G-10 chromatography. The appropriate fractions (characterized by UV absorption) after lyophilization afforded **11** in 18% yield. ^{1}H NMR (D_2O) δ 7.27 (m, 5H), 4.97 (s, 2H), 3.97 (s, 2H), 2.56 (dt, $J = 8.4$ Hz, 12 Hz, 2H), 1.58 (dt, $J = 8.4$ Hz, 17.1 Hz, 2H); ^{31}P NMR (D_2O) δ 23.72; exact mass (ES^+) MH^+ calculated for $C_{12}H_{15}NO_6PNa_2$ 346.0432, found 346.0441.

Sodium Ethyl 3-(Benzyloxycarbonyl)amino-2-oxo-1,1-difluoropropylphosphonate (12). See Scheme 6.

Diethyl 3-(*N*-Benzyloxycarbonyl)amino-1,1-difluoro-2-oxopropylphosphonate (31). *N*-(Benzyloxycarbonyl)glycyl chloride was prepared by modification of the procedure reported by Bose et al.[34] Thus, a mixture of *N*-(benzyloxycarbonyl)glycine (2.56 g, 12.2 mmol) and PCl_5 (2.56 g, 12.2 mmol) was dissolved in dry DME (25 mL) and stirred at 0 °C under N_2 atmosphere for 30 min. Triethylamine (1.69 mL, 12.2 mmol) was then added slowly to the above solution and the precipitated triethylamine hydrochloride was removed by filtration under N_2; a solution of *N*-(benzyloxycarbonyl)glycyl chloride was thus obtained. To this solution was added [(diethoxyphosphinyl)difluoromethyl]zinc bromide (24.4 mmol), obtained by employing the procedure reported by Burton et al.,[35] followed by the addition of copper(I) bromide (0.35 g, 1.2 mmol). The above suspension was stirred at room temperature under dry N_2 atm for 4 h. The solvent was then evaporated under reduced pressure. The residue obtained was then taken into CH_2Cl_2, and the solution was successively washed twice with water, with $NaHCO_3$ solution, and finally with brine, and dried over $MgSO_4$. The solvent was removed to obtain an oil that was purified by silica gel chromatography, with ethyl acetate:hexane (35:65) as eluant, to obtain the title compound **31** in 6% yield. Ketone: ^{1}H NMR ($CDCl_3$) δ 7.3−7.4 (m, 5H), 5.28 (br t, 1H), 5.15 (s, 2H), 4.51 (d, $J = 4.8$ Hz, 2H), 4.31 (quint, $J = 6.9$ Hz, 4H), 1.38 (t, $J = 7.2$ Hz, 6H); ^{19}F NMR ($CDCl_3$) δ −119.69 (d, $^2J_{PF} = 95$ Hz); ^{31}P NMR ($CDCl_3$) δ 3.28 (t, $^2J_{PF} = 96$ Hz). Hydrate: ^{1}H NMR ($CDCl_3$) δ 7.3−7.4 (m, 5H), 5.28 (br t, 1H), 5.15 (s, 2H), 4.10 (quint, $J = 6.9$ Hz, 4H), 3.57 (d, $J = 4.8$ Hz, 2H), 1.36 (t, $J = 7.2$ Hz, 6H); ^{19}F NMR ($CDCl_3$) δ −122.26 (d, $^2J_{PF} = 97$ Hz); ^{31}P NMR ($CDCl_3$) δ 7.79 (t, $^2J_{PF} = 96$ Hz).

Sodium Ethyl 3-(Benzyloxycarbonyl)amino-2-oxo-1,1-difluoropropylphosphonate (12). The synthesis of this compound was achieved by a procedure employed by Marecek and Griffith.[36] To a solution of **31** (44 mg, 0.117 mmol) in acetone was added sodium iodide (17.6 mg, 0.117 mmol). The mixture was heated gently at 50 °C for 4 h. The solvent was then removed under reduced pressure. The residue obtained was redissolved in ether and the solution extracted with water. The aqueous extract was then lyophilized to obtain the crude product, which was purified by Sephadex G-10 chromatography. The appropriate fractions (characterized by UV absorption) after lyophilization yielded **12** in 52% yield. Ketone: ^{1}H NMR (D_2O) δ 7.24−7.32 (m, 5H), 5.02 (s, 2H), 4.33 (s, 2H), 3.93 (quint, $J = 7.50$ Hz, 2H), 1.13 (t, $J = 7.2$ Hz, 3H); ^{19}F NMR (D_2O) δ − 119.69 (d, $^2J_{PF} = 87$ Hz); ^{31}P NMR (D_2O) δ 4.65 (t, $^2J_{PF} = 88$ Hz). Hydrate: ^{1}H NMR (D_2O) δ 7.24−7.32 (m, 5H), 5.01 (s, 2H), 4.33 (quint, $J = 6.9$ Hz, 2H), 3.43 (s, 2H), 1.13 (t, $J = 7.2$ Hz, 3H); ^{19}F NMR (D_2O) δ −122.25 (d, $^2J_{PF} = 88$ Hz); ^{31}P NMR (D_2O) δ 4.47 (t, $^2J_{PF} = 88.7$ Hz); exact mass (ES^+) MH^+ calculated for $C_{13}H_{16}NO_6PF_2Na$ 374.0581, found 374.0570.

Disodium *N*-[*N'*-(Benzyloxycarbonyl)aminoacetyl]aminomethylphosphonate (13). To a solution of aminomethylphosphonic acid (1.0 g, 9 mmol) in water (15 mL) was added sodium bicarbonate (2.5 g) and the mixture was stirred at room temperature until all the components were dissolved. To the above solution was added Z-glycine *N*-hydroxysuccinimide ester (2.76 g, 9 mmol) and the reaction mixture was stirred at room temperature for 26 h.[37] The mixture was then diluted with water (15 mL) and the pH adjusted to 8. This solution was extracted with ethyl acetate and the aqueous layer was lyophilized. The excess salts were removed by desalting chromatography, using Dowex Ion-Retardation 11A8 resin, and further purified by ion-exchange chromatography, using Sephadex QAE ion-exchange resin with an ammonium bicarbonate

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TABLE 2. Experimental Conditions Employed for Spectrophotometric Inhibition Kinetics

enzyme (concn)[a]	substrate (concn, K_m)[b]	wavelength (nm)
P99 (0.1 μM)	ACA (0.5 mM, 0.29 mM)	310
TEM (2 nM)	Cephalothin (0.1 mM, 0.12 mM)	278
PC1 (6nM)	Nitrocefin (20 μM, 16 μM)	482
OXA-1 (0.24 μM)	CENTA (50 μM, 5.0 μM)	410
OXA-10 (50 nM)	CENTA (100 μM, 25 μM)	410
BCII (37 nM)	m-CPP (0.5 mM, 20 mM)	292
R61 (60 nM)	m-CPP (1.0 mM, 0.8 mM)	292

[a] P99: class C β-lactamase of *Enterobacter cloacae* P99. TEM: class A β-lactamase from the TEM-2 plasmid. PC1: class A β-lactamase of *Staphylococcus aureus* PC1. OXA-1, OXA-10: class D β-lactamases from *E. coli*. BCII: class B β-lactamase from *Bacillus cereus*. R61: DD-peptidase from *Streptomyces* R61. [b] ACA: 7β-aminocephalosporanic acid. CENTA: 7β-[(thien-2-yl)acetamido]-3-[(4-nitro-3-carboxyphenylthio)methyl]-3-cephem-4-carboxylic acid. m-CPP: *m*-carboxyphenyl phenaceturate.

concentration gradient (0−2 M). The product was further purified by Sephadex G-10 column chromatography. The appropriate fractions (characterized by UV absorption) after lyophilization yielded **13** (18%). ^{1}H NMR (D_2O) δ 7.22−7.32 (m, 5H), 4.96 (s, 2H), 3.69 (s, 2H), 3.07 (d, $J = 12.6$ Hz, 2H); ^{31}P NMR (D_2O) δ 10.65; exact mass (ES$^+$) MH$^+$ calculated for $C_{11}H_{14}O_6N_2PNa_2$ 347.0385, found 347.0376.

Kinetics. All kinetics experiments were carried out at 25 °C in a buffer containing 20 mM 3-morpholinopropanesulfonic acid (MOPS) at pH 7.50. β-Lactamase concentrations were determined spectrophotometrically, using the following values for their extinction coefficients: *S. aureus* PC1, 1.95×10^4 M^{-1} cm^{-1} at 276.5 nm;[38] *E. coli* TEM-2, 2.90×10^4 M^{-1} cm^{-1} at 280 nm;[39] *Enterobacter cloacae* P99, 7.10×10^4 M^{-1} cm^{-1} at 280 nm.[40]

The activities of the ketophosph(on)ates as β-lactamase inhibitors were obtained from spectrophotometric measurements of initial rates of reaction of appropriate substrates when catalyzed by the various enzymes. Experimental details are provided in Table 2. Inhibitor concentrations were varied in the range of 0−1.2 mM. The data were fitted to a simple competitive inhibition equation to obtain the K_i values of Table 1.

The steady-state parameters for the reaction of the OXA-1 β-lactamase (0.1 μM) with the acyl phosphate **14** (5.0 μM, 50.0 μM) were obtained directly from spectrophotometric total progress curves (270 nm). These were fitted to a Michaelis−Menten scheme by means of the Dynafit program.[41]

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Supporting Information Available: Materials and methods: synthetic details for compounds **15**, **16**, **18**−**21**, **23**−**26**; NMR spectra of all new compounds and important intermediates. This material is available free of charge via the Internet at http://pubs.acs.org.

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