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β-Ketophosphonates as β-lactamase inhibitors: Intramolecular cooperativity between the hydrophobic subsites of a class D β-lactamase

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ABSTRACT

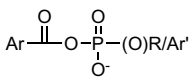
A series of aryl and arylmethyl β-aryl-β-ketophosphonates have been prepared as potential β-lactamase inhibitors. These compounds, as fast, reversible, competitive inhibitors, were most effective (micromolar K_i values) against the class D OXA-1 β-lactamase but had less activity against the OXA-10 enzyme. They were also quite effective against the class C β-lactamase of *Enterobacter cloacae* P99 but less so against the class A TEM-2 enzyme. Reduction of the keto group to form the corresponding β-hydroxyphosphonates led to reduced inhibitory activity. Molecular modeling, based on the OXA-1 crystal structure, suggested interaction of the aryl groups with the hydrophobic elements of the enzyme's active site and polar interaction of the keto and phosphonate groups with the active site residues Ser 115, Lys 212 and Thr 213 and with the non-conserved Ser 258. Analysis of binding free energies showed that the β-aryl and phosphonate ester aryl groups interacted cooperatively within the OXA-1 active site. Overall, the results suggest that quite effective inhibitors of class C and some class D β-lactamases could be designed, based on the β-ketophosphonate platform.

1. Introduction

The effectiveness of β-lactam antibiotics was compromised even at the beginning of their clinical application by the existence of β-lactamases.[1] These enzymes catalyze hydrolysis of β-lactams and thence the loss of their antibiotic activity. Over the last 60 years, the importance of β-lactamases to the resistance of bacteria to β-lactams has only increased through worldwide populations of pathogenic bacteria.[2,3] β-Lactamase inhibitors have been introduced into clinical practice to oppose the effect of β-lactamases, but those employed to date are of limited spectrum of activity and need to be supplemented by broader spectrum alternatives. The search for, design, synthesis, and testing of β-lactamase inhibitors therefore continues.[4,5]

There are four well-defined classes of β-lactamase.[6] One of these, class B, contains metallo-enzymes that have an essential metal ion at the active site as the central catalytic entity. The other three classes (A, C, and D) are serine enzymes where the mechanism of catalysis involves an active site serine residue whose side chain hydroxyl group initiates catalysis by acting as a nucleophile against the substrate. A covalent acyl-enzyme intermediate is thus generated which is subsequently hydrolyzed to regenerate the free enzyme. Of the four classes of β-lactamase, the most significant medically, for some time now, have been the classes A and C enzymes.[2,3] Many mutants of these enzymes, and particularly of class A, have arisen as threats to the effectiveness of β-lactam antibiotics. New variants of classes B and D have also appeared,[7,8] further threatening the future of β-lactam chemotherapy. The β-lactamase inhibitors employed commercially to date are, to a large degree, specific to the class A enzymes and their effectiveness is continually eroded by the appearance of β-lactamase variants capable of resisting or hydrolyzing them.[9,10] The research described below represents part of a project to explore new classes of non-β-lactam β-lactamase inhibitors and, primarily in this report, inhibitors of serine β-lactamases.

A series of aroyl phosphates (**1**) and diaroyl phosphates (**2**) represent a new lead to non-β-lactam inhibitors of β-lactamases.[11–14] These compounds inhibit serine β-lactamases (classes A, C, and D) by acylation of the active site, presumably of the nucleophilic serine; deacylation is then generally slow (Scheme 1).



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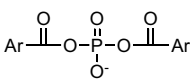

2

Class D β-lactamases, the oxacillinases, appear to be particularly susceptible to inhibition by the diaroyl phosphates, **2**.[13,14] The active site of these enzymes is known to be hydrophobic[15,16] and, consequently, inhibition was observed to be greatly enhanced by hydrophobic substituents. Specifically, enzyme acylation was strongly accelerated by hydrophobic substituents while deacyla-

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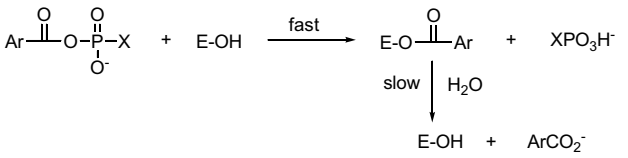
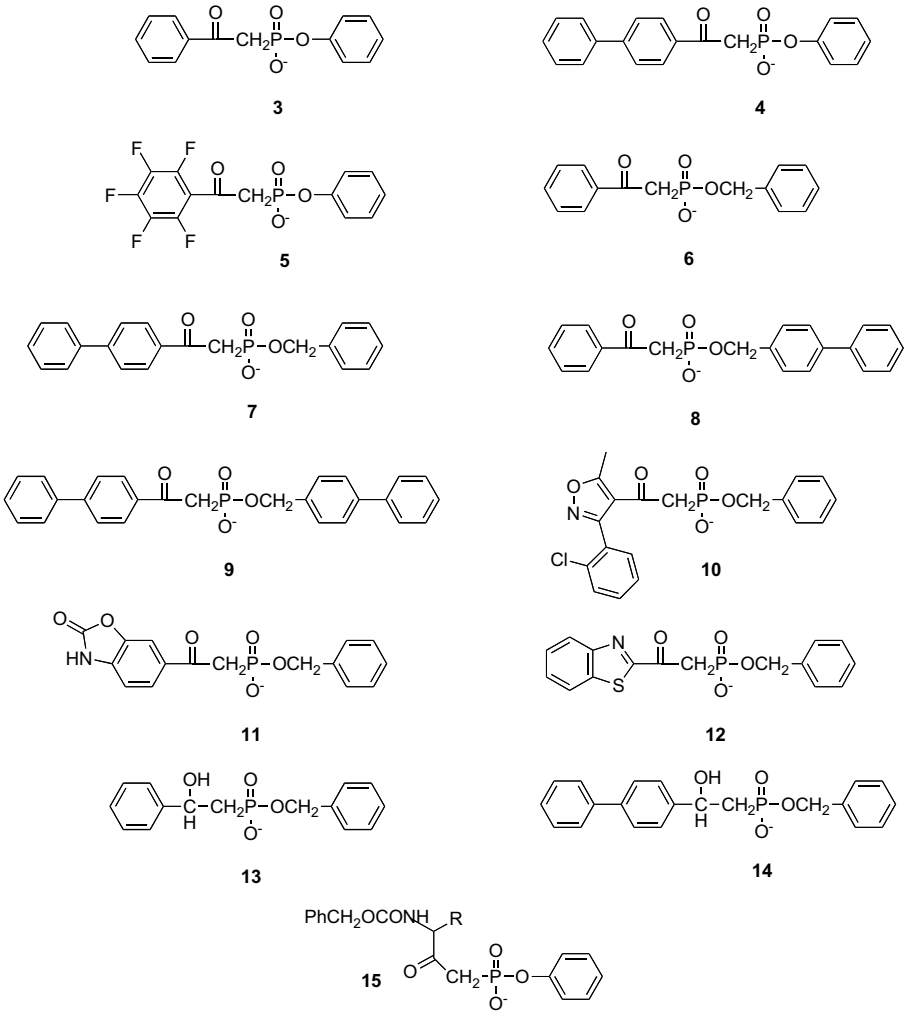


Scheme 1.

tion was more strongly influenced by electronic effects; the lack of significant hydrophobic influence on the latter presumably reflects the similarity of hydrophobic interactions in the acyl-enzyme intermediate and the deacylation transition state.

(en route to **10**), the reaction between an aryl carboxylic acid methyl ester and dibenzyl methylphosphonate (Scheme 2, route b) was employed.[18] Phenyl monoesters (**3**–**5**) were then accessible by alkaline hydrolysis (Scheme 2). Phenyl diesters were converted to benzyl and biphenylmethyl diesters by a literature procedure[19] and the latter compounds mono-dealkylated by iodide in a Finkelstein reaction to obtain the benzyl and biphenylmethyl monoesters **6**–**12**. The β-hydroxyphosphates **13** and **14** were obtained by addition of the dibenzyl methylphosphonate anion to aryl aldehydes to form the diester intermediates **18**, followed by mono-dealkylation as above (Scheme 2).

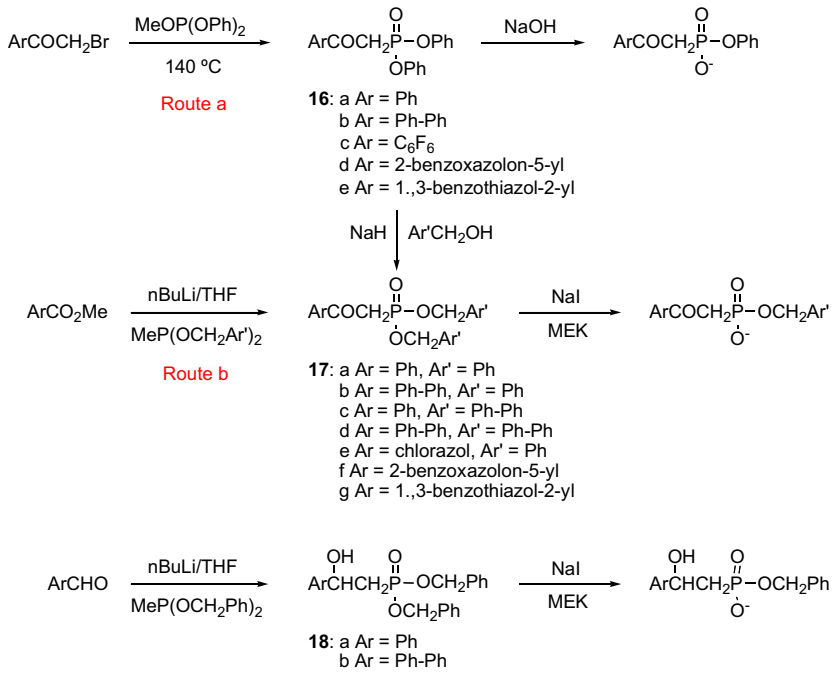
The phosphonates **3**–**14** inhibited class A, C, and D β-lactamases in a fast reversible fashion. ^{1}H NMR spectra of the β-ketophospho-



In order to better assess the influence of the hydrophobic effect on both the acyl donor (ArCO–) site and the leaving group (–OPO_2^-X) site, a series of aryl and arylmethyl β-ketophosphonates **3**–**12** have been prepared and tested as β-lactamase inhibitors. Of these, **3**–**5** represent non-hydrolyzable analogues of **1**, and **6**–**12**, of **2**. To assess the contribution of the planar carbonyl group, the reduced species **13** and **14** were also prepared. Few specific non-hydrolyzable inhibitors of β-lactamases are known. β-Ketophosphonates such as **15**, bearing amido side chains, have previously been established as β-lactamase inhibitors.[17]

2. Results and discussion

Diphenyl arylacylphosphonates were, in general, synthesized by the Michaelis–Arbuzov reaction (Scheme 2, route a). In one case, nates in ^2H_2O showed no evidence of carbonyl hydration. These carbonyl groups are not, therefore, strongly electrophilic, even in **5**. It is unlikely, therefore, that covalent tetrahedral carbonyl adducts were formed between these ketophosphonates and the β-lactamase active sites. Thus, fluorination of the aryl ketone in **5** did not lead to strong inhibition—the small improvement of **5** over **3** as an inhibitor is probably due to the greater hydrophobicity of the pentafluorophenyl group[20] (see below). It might be noted here, in passing, that no such adducts were observed with even more electrophilic ketophosphonates.[17] In one case, that of **4** and the OXA-1 β-lactamase, more detailed steady state experiments revealed competitive inhibition with a weaker non-competitive component ($K_{si} \geqslant 0.5$ mM). The results reported below assume simple competitive inhibition at concentrations comparable to the K_i values.



Scheme 2.

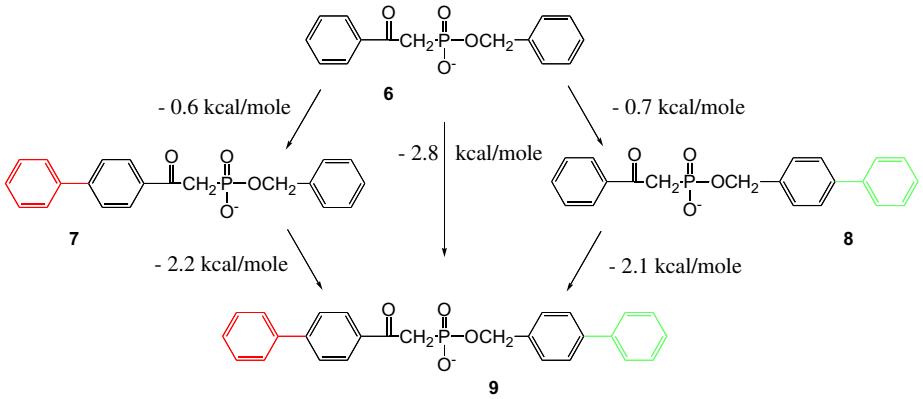
Table 1
Inhibition of β-lactamases by β-ketophosphonates

β-Lactamase, K_i (μM)[a]				
Phosphonate	OXA-1[b]	OXA-10	P99[b]	TEM[b]
3	1050	>1000	200	n
4	140	n	100	920
5	380	>1000	350	475
6	640	>1000	670	150
7	230	>1000	190	>1000
8	200	>1000	n	n
9	6	200	74	n
10	430	n	>1000	>1000
11	270	n	70	1500
12	170	n	24	3600
13	440	n	1080	>1000
14	300	n	260	>1000

n, not determined.
[a] Uncertainties ⩽10%.
[b] P99, class C β-lactamase of *Enterobacter cloacae* P99; TEM, class A β-lactamase from the TEM-2 plasmid; OXA-1 and OXA-10, class D β-lactamases from *E. coli*.

The β-lactamase inhibition data collected on **3**–**14** are reported in Table 1. The compounds appear to be quite effective inhibitors of the class D OXA-1 enzyme and the class C P99 enzyme. A survey of the OXA-1 data for the ketophosphonates **3**–**9** suggests an increase in effectiveness with increase in hydrophobicity of the substituents on either side of the phosphonyl moiety. This result is in accord with observations made with the diacyl phosphates **2** where it was shown that two separate hydrophobic groups could advantageously bind to the hydrophobic active site of the OXA-1 β-lactamase.[14]

The present data for compounds **6**–**9** can be analyzed to assess the degree of intramolecular cooperativity between the two hydrophobic sites. Scheme 3 shows free energy of binding differences (calculated from the data of Table 1) on changing phenyl to biphenyl substituents on each side of the phosphonate. It appears that there is approximately the same contribution to the free energy of binding on addition of one phenyl group on either side of the phosphonyl moiety of **6**. Addition of the second phenyl, however, and again to either side, increases the binding free energy considerably more than addition of the first (ca. 2.1 kcal/mol cf. ca. 0.6 kcal/mol). There must, therefore, be cooperativity between the second pair of phenyl groups on binding to the protein. The source of this cooperativity cannot be definitively identified at this



Scheme 3.

stage but a possible structural basis for it involving the enzyme active site structure is described below. It is also possible, however, that the phenomenon arises from differences between the structures of the compounds in free solution, whether they exist in an open or closed (hydrophobically stabilized) conformation, for example.

Molecular models of **9** bound to the active site of the OXA-1 β-lactamase were constructed as described in Section 3, and various possible conformations were explored by molecular dynamics simulations. In Figure 1, the two stablest structures found (by the criterion of interaction energy[14,21]) are shown. In the first of these (A), the carbonyl group of **9** appears hydrogen-bonded to the side chain functional groups of Lys 212 and Ser 115, both conserved active site residues, and the phosphonate moiety appears to interact with the hydroxyl groups of Thr 213 and Ser 258. The latter of these residues is not conserved among class D β-lactamases[22] and is replaced in OXA-10, for example, with arginine. In structure B, more favorable than A on the basis of overall interaction energy, the carbonyl group does not appear to directly interact with any protein residues, but the phosphonate appears to be hydrogen-bonded to Lys 212, Thr 213, and Ser 115. In neither A nor B does the ligand interact directly with the catalytic Lys70 carbamate/Ser 67 pair, which remains internally hydrogen-bonded.

In both A and B, the biphenyl groups are positioned close to the hydrophobic residues of the active site, Met 99, Trp 102, Val 117, Leu 255, and Ile 259, as also observed in the models of **2** (Ar = biphenyl).[14] With respect to the latter comparison also, it is interesting that the carbonyl group of **9** in structure B is well placed to be attacked by the hydroxyl group of Ser 67, aided by the Lys 70 carbamate acting as a general base. The carbonyl oxygen is also well placed to slip into the oxyanion hole (backbone NH groups of Ser 67 and Ala 215) after such attack. It is true that this structure is close to that obtained from the original structure building based on the model of **2** (Ar = Ph–Ph) bound to the enzyme (see Section 3), but the ligand did move considerably during the dynamics runs (e.g., structure A) and structure B was the lowest energy conformation found. It is not unlikely, therefore, that structure B represents a good model of the non-covalent Michaelis complex formed on binding of **2** to the OXA-1 β-lactamase.

Finally, it is noticeable that in complex A the biphenyl groups of **9** appear to interact with each other ('stack') to a greater degree than in B where the biphenyl 'arms' splay apart. The thermodynamic cooperativity described above on addition of the second phenyl groups to **7** and **8** might, therefore, more obviously arise from A. If B were the correct structure, however, the cooperativity would then likely involve changes in protein structure and/or dynamics, leading, for example, to a tightening of the complex. A crystal structure would be helpful in resolving this issue.

Addition of the 'OXA-specific' chlorazole substituent in **10** did not lead to strong inhibition. Apparently, the combination of this substituent and the phosphonate in **10** is not able to take advantage of the position occupied by the side chain of cloxacillin, a good

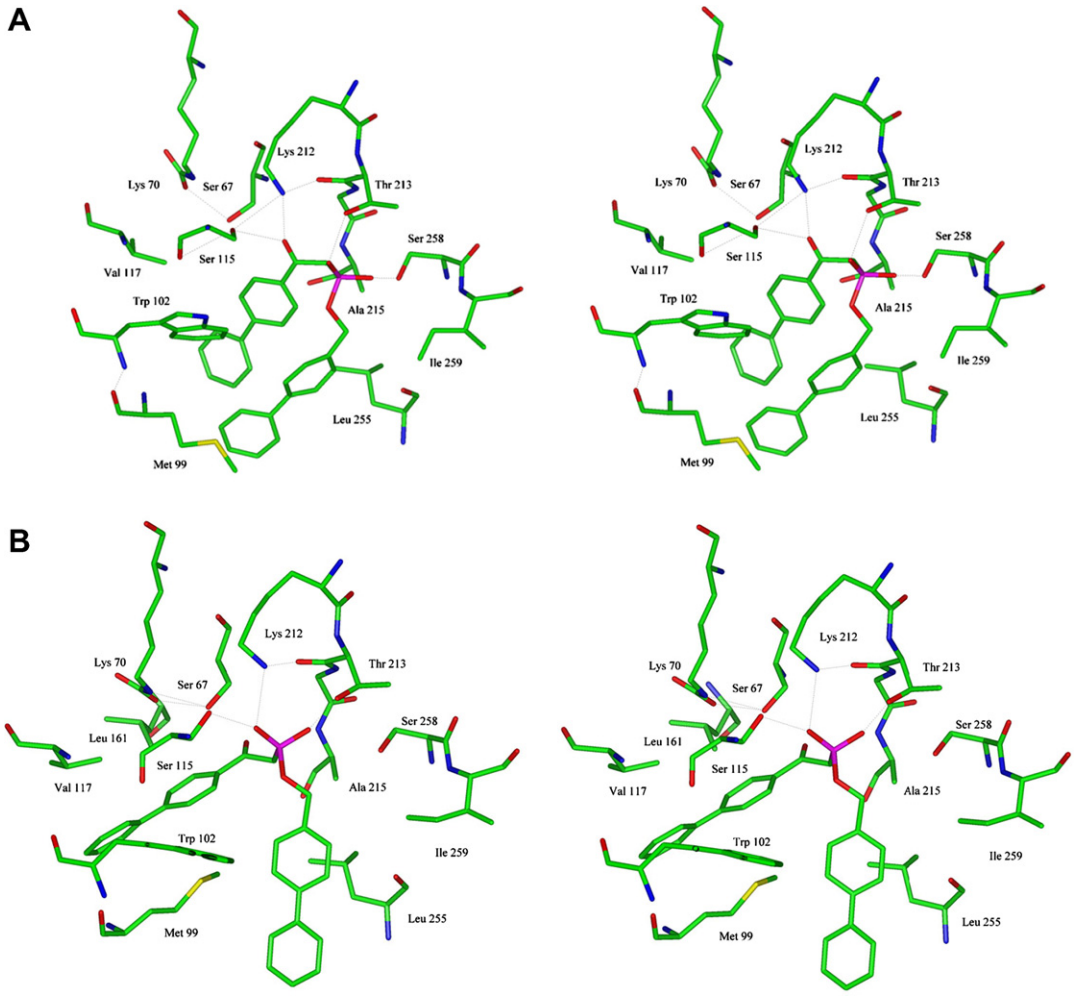


Figure 1. Stereoviews of energy-minimized complexes formed between the ketophosphonate **9** and the OXA-1 β-lactamase. The ligand is shown in the A (upper) and B (lower) conformations (see text); only heavy atoms are displayed.

OXA-1 substrate.[23] Replacement of the phenacyl group of **6** with the more polar heterocycles of **11** and **12** also did not lead to significantly stronger inhibition. Modeling had suggested, based on the above structure with **9**, that the heterocycle of **11** may be in a position to hydrogen bond to backbone amide groups surrounding the active site. Any such dual interaction, however, may have been counterbalanced by the diminished hydrophobic interaction.

Although the diacyl phosphates **2** were comparably effective inhibitors of the OXA-10 β-lactamase, a representative of another subclass of class D enzymes, as they were of OXA-1,[14] this was not true of **3**–**12**, which were much poorer inhibitors of the former enzyme. It may be that generally effective non-covalent inhibitors will be difficult to find for class D β-lactamases.

Compounds **3**–**12** were, however, moderately effective inhibitors of the class C P99 β-lactamase (Table 1). Additional hydrophobic (phenyl) groups slightly improved the activity over the baseline of **3** and this correlates with previous observations.[12,14,24] The addition of heterocycles, as seen in **12**, particularly, has more dramatic effects, again a known property of the class C active site. It is likely that optimization of heterocyclic substituents could achieve very potent phosphonate inhibitors of class C β-lactamases, as demonstrated by Shoichet et al. with boronates.[25]

The class A TEM β-lactamase is not effectively inhibited by these ketophosphonates, reflecting, most likely, the much more polar environment of its active site where Glu 166, Asn 170, and the hydrolytic water molecule are incorporated. It is possible that polar replacements for the aryl groups may help, although neither the heterocycles of **11** and **12** nor the phenylacetylamido side chain of previously examined ketophosph(on)ates[17] support this idea.

Finally, the results (Table 1) of reduction of the (planar) keto group of **6** and **7** to the (tetrahedral) alcohols of **13** and **14** does not suggest a route to more effective β-lactamase inhibition. It may be, as was suggested by our exploration of models of complexes of **13** (both enantiomers) with the OXA-1 β-lactamase (not shown), that alcohols simply make the inhibitors more flexible without supplying additional interactions to counteract this negative feature.

Thus, our survey of the β-ketophosphonates **3–12** suggests that quite effective inhibitors of the class D OXA-1 and class C P99 β-lactamases could be developed, based on this platform. It seems unlikely, however, on the basis of results now in hand, that a completely general non-covalent serine β-lactamase inhibitor of this class could be developed. On the other hand, since no other such molecules are currently known, further investigation of the β-ketophosphonates may be warranted.

3. Experimental

3.1. Synthesis

All chemical reagents were purchased from Aldrich, unless otherwise noted. Methyl diphenyl phosphite was purchased from Organometallics Inc. and 1-(1,3-benzothiazol-2-yl)-2-bromo-1-ethanone from Maybridge Chem. Co.

3.1.1. General procedure for synthesis of diphenyl β-ketophosphonates

3.1.1.1. Diphenyl phenacylphosphonate (16a). Bromoacetophenone (1.0 g, 5.02 mmol) and methyl diphenyl phosphite (1.25 g, 5.02 mmol) were mixed and stirred at 140 °C for 2.5 h under a dry N_2 atmosphere. The crude product thus obtained was subjected to flash chromatography with ethyl acetate/hexane (1:4) as eluant (R_f: 0.3). The product was obtained as a colorless solid in 81% yield. ^{1}H NMR ($CDCl_3$) δ 8.05 (d, J = 7.2 Hz, 2H), 7.62 (t, J = 7.2 Hz, 1H), 7.50 (t, J = 7.8 Hz, 2H), 7.30 (t, J = 7.8 Hz, 4H), 7.14–7.21 (m, 6H), 3.93 (d, J = 23.1 Hz, 2H). ^{31}P NMR ($CDCl_3$) δ 10.21.

Compounds **16b** and **16c** were prepared as described above for **16a** and purified as described below.

3.1.1.2. Diphenyl 4-biphenacylphosphonate (16b). The product was purified by flash chromatography using ethyl acetate/hexane (3:7) as eluant (R_f: 0.38). The product was obtained as a colorless solid in 80% yield. Mp 92–94 °C; ^{1}H NMR ($CDCl_3$) δ 8.12 (d, J = 8.7 Hz, 2H), 7.71 (d, J = 8.1 Hz, 2H), 7.63 (d, J = 6.6 Hz, 2H), 7.41–7.51 (m, 3H), 7.31 (t, J = 6.9 Hz, 4H), 7.15–7.20 (m, 6H), 3.96 (d, J = 23 Hz, 2H), ^{31}P NMR ($CDCl_3$) δ 10.26.

3.1.1.3. Diphenyl pentafluorophenacylphosphonate (16c). The crude product was subjected to flash chromatography using 30% ethyl acetate in hexane (3:7) as eluant (R_f: 0.30). The product was obtained as a colorless viscous oil (25%). ^{1}H NMR (DMSO-d_6) δ 7.32 (t, J = 7.8 Hz, 4H), 7.19 (t, J = 7.2 Hz, 2H), 7.13 (d, J = 8.1 Hz, 4H), 3.87 (d, J = 22.5 Hz, 2H). ^{19}F NMR (DMSO-d_6) δ −140.46 (d, J = 8.5 Hz, 2F), −147.47 (t, J = 9.3 Hz, 1F), −159.84 (t, J = 15 Hz, 2F). ^{31}P NMR (D_2O) δ 11.08.

3.1.2. General procedure for preparation of phenyl β-ketophosphonates

3.1.2.1. Sodium phenyl phenacylphosphonate (3). The title compound was obtained by alkaline hydrolysis of the diphenyl ester **16a**. Thus, to a solution of **16a** (0.2 g, 0.59 mmol) in dioxane (2 ml) was added an aqueous solution of NaOH (94 mg, 2.3 mmol) in 1 ml of water. The above mixture was stirred at room temperature for 1 h. The solvent was then reduced to one-third of its original volume by rotary evaporation and the pH of the solution adjusted to 8 with HCl. This solution was extracted with ethyl acetate. The aqueous layer was then acidified to pH 1 by addition of HCl and extracted with ether (in this case) or methylene chloride. The combined organic extracts were washed with water and dried over anhydrous $MgSO_4$. The solvent was then removed under reduced pressure and the acid thus obtained was converted into its sodium salt by the addition of an aqueous solution of $NaHCO_3$ (50 mg, 0.59 mmol). The solution was then freeze–dried and the crude product purified by aqueous Sephadex G10 chromatography. The appropriate fractions (characterized by UV absorption) were freeze–dried to yield the title compound in 58% yield. ^{1}H NMR (D_2O) δ 7.89 (d, J = 7.8 Hz, 2H), 7.55 (t, J = 7.5 Hz, 1H), 7.41 (t, J = 7.8 Hz, 2H), 7.20 (t, J = 7.8 Hz, 2H), 7.03 (t, J = 7.5 Hz, 1H), 6.91 (d, J = 8.7 Hz, 2H), 3.61 (d, J = 22.2 Hz, 2H). ^{31}P NMR (D_2O) δ 13.12; HRMS (ES^+) calculated for $C_{14}H_{13}O_4PNa$: 299.0449; found: 299.0443.

3.1.2.2. Sodium phenyl 4-biphenacylphosphonate (4). The title compound was obtained from **16b** in exactly the same way as **3** from **16a** in 50% yield. ^{1}H NMR (D_2O) δ 7.92 (d, J = 8.1 Hz, 2H), 7.63 (t, J = 8.4 Hz, 4H), 7.40 (t, J = 7.2 Hz, 2H), 7.33 (d, J = 7.5 Hz, 1H), 7.16 (t, J = 7.8 Hz, 2H), 6.99 (t, J = 7.2 Hz, 1H), 6.88 (d, J = 8.4 Hz, 2H), 3.60 (d, J = 22.2 Hz, 2H). ^{31}P NMR (D_2O) δ 9.37; HRMS (ES^+) calculated for $C_{20}H_{17}O_4PNa$: 375.0762; found: 375.0766.

3.1.2.3. Sodium phenyl pentafluorophenacylphosphonate (5). The title compound was obtained in 36% yield by employing the same procedure as for **3**. ^{1}H NMR (DMSO-d_6) δ 7.35 (t, J = 7.2 Hz, 2H), 7.09–7.19 (m, 3H), 3.72 (d, J = 21.6 Hz, 2H). ^{31}P NMR (D_2O) δ 7.90. ^{19}F NMR (D_2O) δ −141.73 (d, J = 20 Hz, 2F), −149.20 (t, J = 16 Hz, 1F), −161.63 (t, J = 16 Hz, 2F); HRMS (ES^+) calculated for $C_{14}H_7F_5O_4PNa_2$: 410.9798; found: 410.9796.

3.1.3. General procedure for the synthesis of bis[(bi)phenylmethyl] aryl ketophosphonates

3.1.3.1. Dibenzyl phenacylphosphonate (17a). Dry benzyl alcohol (0.37 g, 3.42 mmol) was added to a solution of NaH (60% suspension in toluene) (0.23 g, 5.68 mmol) in dry DMSO. This

mixture was stirred at room temperature for 15 min. To the above solution was added a dry DMSO solution of diphenyl phenacylphosphonate **16a** (0.4 g, 1.14 mmol). The reaction mixture was then stirred at room temperature for 4 h and quenched with saturated NH_4Cl solution (20 ml). The mixture was extracted with methylene chloride (2× 60 ml) and the combined organic solution was washed with brine (60 ml), dried over anhydrous $MgSO_4$, and concentrated to dryness. The crude product was subjected to flash chromatography using ethyl acetate/hexane (2:3) as the eluant (R_f: 0.38). The product was obtained as a colorless solid in 50% yield. ^{1}H NMR ($CDCl_3$) δ 7.97 (d, J = 7.5 Hz, 2H), 7.59 (t, J = 7.5 Hz, 1H), 7.44 (t, J = 7.8 Hz, 2H), 7.30–7.34 (m, 10H), 4.99–5.12 (m, 4H), 3.67 (d, J = 22.8 Hz, 2H); ^{31}P NMR ($CDCl_3$) δ 18.32.

3.1.3.2. Dibenzyl 4′-biphenacylphosphonate (17b). This compound was obtained from **16b** by essentially the same procedure as was used for **17a**. The crude product was recrystallized from 50% benzene/cyclohexane in 50% yield. Mp 79–80 °C; ^{1}H NMR ($CDCl_3$) δ 8.01 (d, J = 8.0 Hz, 2H), 7.64 (d, J = 8.0 Hz, 2H), 7.61 (d, J = 8.0 Hz, 2H), 7.48 (t, J = 8.0 Hz, 2H), 7.43 (t, J = 8.0 Hz, 1H), 7.29 (br, 5H), 5.07 (dAB q, J = 9.6, 13.6 Hz, 4H), 3.69 (d, J = 24.5 Hz, 2H). ^{31}P NMR ($CDCl_3$) δ 18.40.

3.1.3.3. Bis[(4-biphenyl)methyl] phenacylphosphonate (17c). The title compound was obtained from **16a** by essentially the same procedure as was used for the synthesis of **17a**. The biphenylmethanol that was found to be the only contaminant of the crude product was removed by flash chromatography using 40% ethyl acetate in hexane as the eluant (R_f: 0.4), followed by a methanol wash to obtain the pure product. The product was thus obtained as a colorless solid in 57% yield. Mp 84–86°C; ^{1}H NMR ($CDCl_3$) δ 7.98 (d, J = 7.2 Hz, 2H), 7.53–7.57 (m, 9H), 7.44 (t, J = 7.9 Hz, 6H), 7.35–7.37 (m, 6H), 5.06–5.18 (m, 4H), 3.71 (d, J = 22.5 Hz, 2H). ^{31}P NMR ($CDCl_3$) δ 18.57.

3.1.3.4. Bis(4-biphenylmethyl) 4′-biphenacylphosphonate (17d). The title compound was obtained from **16b** by essentially the same procedure as used for the synthesis of **17b**. The crude product, however, was purified by recrystallization from ethyl acetate. The product was obtained as a colorless solid in 38% yield. Mp 200–202 °C; ^{1}H NMR ($CDCl_3$) δ 8.03 (d, J = 8.7 Hz, 2H), 7.52–7.65 (m, 12H), 7.33–7.47 (m, 13H), 5.07–5.20 (m, 4H), 3.74 (d, J = 22.5 Hz, 2H). ^{31}P NMR ($CDCl_3$) δ 18.45.

3.1.3.5. Methyl 3-[2′-chlorophenyl]-5-methylisoxazole-4-carboxylate. Triethylamine (0.8 g, 7.8 mmol) was added to a solution of chlorazol chloride (2 g, 7.8 mmol) in methanol (150 ml). This mixture was stirred at room temperature for 5 h. The precipitated amine hydrochloride was removed by filtration, the filtrate concentrated under reduced pressure, and the residue taken up into ethyl acetate. The ethyl acetate solution was washed with aqueous sodium bicarbonate and brine, and dried over anhydrous $MgSO_4$. Removal of the solvent under reduced pressure afforded the product in 77% yield. Mp 52–54 °C; ^{1}H NMR ($CDCl_3$) δ 7.31–7.48 (m, 4H), 3.68 (s, 3H), 2.75 (s, 3H).

3.1.3.6. Dibenzyl 3-[2′-chlorophenyl]-5-methylisoxazole-4-acylphosphonate (17e). To a solution of dibenzyl methylphosphonate (2.6 g, 9.4 mmol) in dry THF, stirred at −78 °C under a dry N_2 atmosphere, was added 3.8 ml of 2.5 M BuLi in hexane (0.603 g, 9.4 mmol). After an hour of stirring at −78 °C, a solution of methyl 3-[2′-chlorophenyl]-5-methylisoxazole-4-carboxylate (0.28 g, 1.23 mmol) in 3 ml of dry THF was added dropwise to the above solution. The reaction mixture was stirred for 1 h at −78 °C after which the coolant bath was removed. The reaction mixture was then hydrolyzed with 10% aqueous acetic acid and extracted with ethyl acetate (3× 40 ml). The organic layer was washed successively with $NaHCO_3$, water, and brine and dried over anhydrous $MgSO_4$. The oil obtained after evaporation of the solvent was subjected to flash chromatography with 40% ethyl acetate in hexane (R_f: 0.4) as the eluant, to afford the product in 48% yield. ^{1}H NMR ($CDCl_3$) δ 7.18–7.45 (m, 14H), 4.84–5.09 (m, 4H), 3.02 (d, J = 22.2 Hz, 2H), 2.61 (s, 3H). ^{31}P NMR ($CDCl_3$) δ 21.52.

3.1.4. General procedure for the synthesis of benzyl and (4-phenyl)benzyl aryl ketophosphonates (6–10)

The phosphonate monoesters **6**–**10** were synthesized as shown in Scheme 2. Thus, for example, to a solution of dibenzyl phenacylphosphonate (0.214 g, 0.56 mmol) in MEK (10 ml), NaI (84 mg, 0.56 mmol) was added. The above solution was refluxed for 2.5 h. The solvent was removed under reduced pressure and the resulting solid was collected and washed with ice-cold acetone to remove starting materials. Trace solvents were removed from the filtered solid under reduced pressure.

3.1.4.1. Sodium benzyl phenacylphosphonate (6). The crude product from above was recrystallized from ethanol (95%) to yield a colorless solid in 57% yield. ^{1}H NMR (D_2O) δ 7.78 (d, J = 7.2 Hz, 2H), 7.47 (t, J = 7.2 Hz, 1H), 7.31 (t, J = 7.8 Hz, 2H), 7.12–7.21 (m, 3H), 7.03–7.10 (m, 2H), 4.63 (d, J = 10.2 Hz, 2H), 3.43 (d, J = 21.6 Hz, 2H). ^{31}P NMR (D_2O) δ 12.01; HRMS (ES^+) calculated for $C_{15}H_{15}O_4PNa$: 313.0606; found: 313.0594.

3.1.4.2. Sodium benzyl 4-biphenacylphosphonate (7). The crude product was recrystallized from 50% aqueous ethanol as a colorless solid in 65% yield. ^{1}H NMR (DMSO-d_6) 8.13 (d, J = 7.5 Hz, 2H), 7.71 (2d, J = 7.5 Hz, 4H), 7.48 (t, J = 7.5 Hz, 2H), 7.39 (t, J = 7.5 Hz, 1H), 7.28 (m, 5H), 4.69 (d, J = 6.9 Hz, 2H), 3.26 (d, J = 20.9 Hz, 2H). ^{31}P NMR (DMSO-d_6) δ 5.40; HRMS (ES^+) calculated for $C_{21}H_{19}O_4PNa$: 389.0919; found: 389.0919.

3.1.4.3. Sodium 4-biphenylmethyl phenacylphosphonate (8). The crude product was recrystallized from water/methanol (4:1) to yield a colorless solid in 77% yield. ^{1}H NMR (D_2O) δ 7.78 (d, J = 8.4 Hz, 2H), 7.54 (d, J = 8.1 Hz, 2H), 7.45 (d, J = 7.8 Hz, 3H), 7.38 (t, J = 7.8 Hz, 2H), 7.31 (t, J = 8.4 Hz, 3H), 7.17 (d, J = 7.5 Hz, 2H), 4.73 (d, J = 7.2 Hz, 2H), 3.47 (d, J = 21 Hz, 2H). ^{31}P NMR (D_2O) δ 12.12; HRMS (ES^+) calculated for $C_{21}H_{19}O_4PNa$: 389.0919; found: 389.0921.

3.1.4.4. Sodium 4-biphenylmethyl 4′-biphenacylphosphonate (9). The crude product was recrystallized from 95% ethanol to yield a colorless solid in 66% yield. ^{1}H NMR (DMSO-d_6) δ 7.95 (d, J = 8.0 Hz, 2H), 7.63 (d, J = 8.4 Hz, 2H), 7.56 (d, J = 8.0 Hz, 2H), 7.45 (d, J = 7.6 Hz, 5H), 7.17–7.31 (m, 7H), 4.95–4.98 (m, 2H), 3.92 (d, J = 22.4 Hz, 2H). ^{31}P NMR (DMSO-d_6) δ 14.21; HRMS (ES^+) calculated for $C_{27}H_{23}O_4PNa$: 465.1232; found 465.1233.

3.1.4.5. Sodium benzyl 3-[2′-chlorophenyl]-5-methylisoxazole-4-acylphosphonate (10). The crude product was purified by Sephadex G-10 chromatography. The appropriate fractions (characterized by UV absorption) were then freeze–dried to yield the title compound in 30% yield. ^{1}H NMR (D_2O) *δ 7.37–7.45 (m, 2H), 7.20–7.33 (m, 5H), 7.05–7.08 (m, 2H), 4.55 (d, J* = 6.9 Hz, 2H), 2.86 (d, J = 21.6 Hz, 2H), 2.51 (s, 3H). ^{31}P NMR (D_2O) δ 10.95; HRMS (ES^+) calculated for $C_{19}H_{18}ClNO_5P$: 406.0611; found: 406.0602.

3.1.5. Heterocyclic ketophosphonates

3.1.5.1. 2-Benzoxazolone-5-acyl bromide. The title compound was synthesized by a reported literature procedure.[26] Thus, anhydrous dimethyl formamide (2.1 ml, 33.5 mmol) was added dropwise to finely ground $AlCl_3$ (13.3 g, 0.1 mol), with stirring, under argon. The mixture was heated to 45 °C and benzoxazolone (1.35 g, 10.0 mmol) and bromoacetyl bromide (1.33 ml,

15.0 mmol) were added slowly. After 30 min, the mixture was heated to 95 °C for 4.5 h, poured into ice (0.5 kg), and stirred for 1 h. The precipitate was collected by filtration and washed with 0.5 l of water, dried, and recrystallized from methanol to give 2.1 g of a light brown solid in 82% yield, mp 204–205 °C (lit[26] 205 °C). ^{1}H NMR (DMSO-d_6) 12.16 (br s, 1H), 7. 91 (s, 1H), 7.89 (d, J = 8.1 Hz, 1H), 7.23 (1H, d, J = 8.1 Hz), 4.90 (s, 2H).

3.1.5.2. Diphenyl 2-Benzoxazolone-5-acylphosphonate (16d). The above bromoketone (0.88 g, 3.43 mmol) and methyl diphenyl phosphite (2.13 g, 8.58 mmol) were mixed together in DMF (0.5 ml) and stirred at 140 °C for 2.5 h under a dry N_2 atmosphere. The residue obtained was diluted with methylene chloride/ethyl acetate (1:1) and stored at room temperature for 1 h. The solid thus obtained was filtered, dried and recrystallized from ethyl acetate to give the product as a colorless solid in 80% yield. Mp 198–200 °C; ^{1}H NMR (DMSO-d_6) δ 12.15 (br s, 1H), 8.00 (s, 1H), 7.97 (d, J = 8.1 Hz, 1H), 7.39 (t, J = 7.8 Hz, 4H), 7.23 (d, J = 8.0 Hz, 2H), 7.13–7.19 (m, 5H), 4.35 (d, J = 23.0 Hz, 2H); ^{31}P NMR (DMSO-d_6) δ 13.45.

3.1.5.3. Diphenyl 2-(1′,3′-benzothiazol-2′-yl)-2-oxo-ethylphosphonate (16e). 1-(1′,3′-Benzothiazol-2′-yl)-2-bromo-1-ethanone (1 g, 3.9 mmol) and methyl diphenyl phosphite (0.97 g, 3.9 mmol) were mixed together and stirred at 140 °C for 2.5 h under dry N_2 atmosphere. The residue obtained was then subjected to flash chromatography using ethyl acetate/hexane (2:3) as eluant (R_f: 0.25). The product was thus obtained as a colorless solid in 35% yield. ^{1}H NMR ($CDCl_3$) δ 8.09 (d, J = 7.8 Hz, 1H), 7.91 (d, J = 7.8 Hz, 1H), 7.45–7.54 (m, 2H), 7.06–7.27 (m, 10H), 4.24 (d, J = 23.0 Hz, 2H); ^{31}P NMR ($CDCl_3$) δ 9.34.

Dibenzyl phosphonate esters **17f** and **17g** were prepared as described above for **17a**.

3.1.5.4. Dibenzyl 2-benzoxazolone-5-acylphosphonate (17f). The product was obtained as a pale yellow solid in 59% yield. Mp 120–122°C; ^{1}H NMR ($CDCl_3$) δ 9.55 (br s, 1H), 7.74 (d, J = 8.7 Hz, 1H), 7.71 (s, 1H), 7.34–7.38 (m, 10H), 6.75 (d, J = 7.8 Hz, 1H), 5.03–5.17 (m, 4H), 3.62 (d, J = 23.0 Hz, 2H); ^{31}P NMR ($CDCl_3$) δ 17.93.

3.1.5.5. Dibenzyl 2-(1′,3′-benzothiazol-2′-yl)-2-oxo-ethylphosphonate (17g). The product was obtained as a pale brown oil in 82% yield. ^{1}H NMR ($CDCl_3$) δ 8.13 (d, J = 7.8 Hz, 1H), 7.95 (d, J = 7.8 Hz, 1H), 7.51–7.60 (m, 2H), 7.20–7.30 (m, 10H), 5.02–5.16 (m, 4H), 4.05 (d, J = 22 .0 Hz, 2H); ^{31}P NMR ($CDCl_3$) δ 17.25.

Benzyl phosphonate monoesters **11** and **12** were prepared as described above for **6**.

3.1.5.6. Sodium benzyl 2-benzoxazolone-5-acyl phosphonate (11). The crude material obtained was recrystallized from ethanol/methanol (2:1) to yield the product in 55% yield. ^{1}H NMR (DMSO-d_6) δ 12.40 (br s, 1H), 7.90 (s, 1H), 7.82 (d, J = 8.4 Hz, 1H), 7.22–7.34 (m, 5H), 6.97 (d, J = 8.2 Hz, 1H), 4.74 (d, J = 7.2 Hz, 2H), 3.27 (d, J = 21.0 Hz, 2H); ^{31}P NMR ($CDCl_3$) δ 5.59; HRMS (ES^+) calculated for $C_{16}H_{15}NO_6P$: 348.0637; found: 348.0646.

3.1.5.7. Sodium benzyl 2-(1′,3′-benzothiazol-2′-yl)-2-oxo-ethylphosphonate (12). The crude material was recrystallized from ethanol/methanol (4:1) to yield the required product in 67% yield. ^{1}H NMR (DMSO-d_6) δ 8.16–8.22 (m, 2H), 7.56–7.65 (m, 2H), 7.19–7.26 (m, 5H), 4.75 (d, J = 6.9 Hz, 2H), 3.55 (d, J = 20.0 Hz, 2H); ^{31}P NMR ($CDCl_3$) δ 4.24; HRMS (ES^+) calculated for $C_{16}H_{15}NO_4PS$: 348.0459; found: 348.0467.

3.1.6. Synthesis of β-hydroxyphosphonates

The dibenzyl esters **18a** and **18b** were synthesized by modification of a procedure reported for a similar synthesis.[27] Thus, to a solution of dibenzyl methylphosphonate (1.56 g, 5.66 mmol) in anhydrous THF at −78 °C under dry N_2 atmosphere was added a 2.5 M hexane solution of nBuLi (2.25 ml, 5.6 mmol). The above solution was stirred at −78 °C for 30 min, after which the aldehyde (4.72 mmol) in anhydrous THF was added slowly to the stirred solution. The reaction was allowed to continue for another hour at −78 °C, and then another 30 min at room temperature. An aqueous saturated solution of NH_4Cl (10 ml) was added to the reaction mixture and the mixture was extracted with ethyl acetate. The organic layer was washed with brine and dried over $MgSO_4$. The ^{1}H NMR spectrum (below) showed it to contain the required product as the only organic component. This material was used for the subsequent synthesis without any further purification.

3.1.6.1. Dibenzyl (±) 2-hydroxy-2-phenylethylphosphonate (18a). The product was obtained as a colorless solid in 95% yield. Mp 62–65 °C; ^{1}H NMR ($CDCl_3$) δ 7.24–7.38 (m, 15H), 4.93–5.11 (m, 5H), 3.78 (br s, 1H), 2.13–2.32 (m, 2H); ^{31}P NMR ($CDCl_3$) δ 27.37.

3.1.6.2. Dibenzyl (±) 2-hydroxy-2-(4′-biphenyl)ethylphosphonate (18b). The product was obtained as a colorless solid in 94% yield. Mp 74–76 °C; ^{1}H NMR ($CDCl_3$) δ 7.24–7.38 (m, 15H), 7.47–7.50 (m, 4H), 4.90–5.07 (m, 5H), 3.69 (br s, 1H), 2.11–2.29 (m, 2H); ^{31}P NMR ($CDCl_3$) δ 27.32.

The phosphonate monoesters **13** and **14** were prepared as described above for **6**.

3.1.6.3. Sodium benzyl (±) 2-hydroxy-2-phenylethylphosphonate (13). The crude material was recrystallized from ethanol/diisopropyl ether (9:1) to afford the required product as a colorless solid in 60% yield. ^{1}H NMR (DMSO-d_6) δ 7.14–7.38 (m, 10H), 7.00 (br s, 1H), 4.77 (d, J = 8.0 Hz, 2H), 4.63–4.71 (m, 1H), 1.61–1.71 (m, 1H), 1.34–1.47 (q, J = 13.0 Hz, 1H); ^{31}P NMR ($CDCl_3$) δ 16.18; HRMS (ES^+) calculated for $C_{15}H_{17}O_4PNa$: 315.0762; found: 315.0763.

3.1.6.4. Sodium benzyl (±) 2-hydroxy-2-(4′-biphenyl)ethylphosphonate (14). The crude product was recrystallized from ethanol to afford the required material as a colorless solid in 72% yield. ^{1}H NMR (DMSO-d_6) δ 7.63 (d, J = 7.8 Hz, 2H), 7.55 (d, J = 8.1 Hz, 2H), 7.44 (t, J = 7.8 Hz, 2H), 7.25–7.39 (m, 8H), 7.09 (br s, 1H), 4.78 (d, J = 7.8 Hz, 2H), 4.71 (t, J = 9.1 Hz, 1H), 1.71 (t, J = 14.3 Hz, 1H), 1.44 (q, J = 12.8 Hz, 1H); ^{31}P NMR ($CDCl_3$) δ 16.18; HRMS (ES^+) calculated for $C_{21}H_{21}O_4PNa$: 391.1075; found: 391.1079.

3.2. Enzyme kinetics

The class C β-lactamase of *Enterobacter cloacae* P99 and the TEM-2 class A β-lactamase from *Escherichia coli* were purchased from the Centre for Applied Microbiology and Research (Porton Down, Wiltshire, UK) and used as received. The class D *E. coli* OXA-1 β-lactamase was generously provided by Dr. Michiyoshi Nukaga, Jyosai International University, Japan. The class D *E. coli* OXA-10 β-lactamase was a generous gift from Dr. J.-M. Frère of the University of Liège, Liège, Belgium. Cephalothin and 7α-aminocephalosporanic acid (7α-ACA) were purchased from Aldrich. Nitrocefin was purchased from Oxoid.

All kinetics experiments were carried out at 25 °C in a buffer containing 20 mM 3-morpholinopropanesulfonic acid (MOPS) at pH 7.50, and, for the experiments with the OXA-1 and OXA-10 β-lactamases, the buffer also contained 50 mM bicarbonate.

The activities of the β-keto and β-hydroxyphosphonates 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. The phosphonates **3**–**7** and **10**–**14** were sufficiently soluble in water that 10 mM stock solutions were readily

Table 2
Experimental conditions employed for spectrophotometric inhibition kinetics

Enzyme (concn)[a]	Substrate (concn, K_M)	Wavelength (nm)
P99 (2 μM)	ACA[b] (0.5 mM, 0.29 mM)	340
TEM (2 nM)	Cephalothin (0.1 mM, 0.12 mM)	278
	Nitrocefin (0.1 mM, 86 μM)	482[c]
OXA-1 (20 nM)	Nitrocefin (25 μM, 7.6 μM)	482
OXA-10 (5 nM)	Nitrocefin (80 μM, 22.6 μM)	482

[a] P99, class C β-lactamase of *Enterobacter cloacae* P99; TEM, class A β-lactamase from the TEM-2 plasmid; OXA-1 and OXA-10, class D β-lactamases from *E. coli*.
[b] ACA, 7-aminocephalosporanic acid.
[c] Employed for **11** and **12**.

obtained. The ketophosphonates **8** and **9**, however, were not soluble enough in water to provide stock solutions at this concentration. Hence a 5 mM stock solution of **8** was prepared in DMSO/water (1:1). The solubility of **9** was even lower than that of **8,** both in water and in DMSO, and thus only a 1 mM solution in DMSO could be obtained. Inhibitor concentrations were varied in the range of 0–1.0 mM. The data were fitted to a simple competitive inhibition equation to obtain the K_i values of Table 1.

3.3. Molecular modeling

The computations were performed by means of an sgi octane2 workstation with the InsightII 2000 suite of molecular modeling programs (Accelrys, San Diego, CA). The starting point for the structures was a previously published[14] model of **2** (Ar = Ph–Ph) covalently bound as a tetrahedral intermediate to the active site of the OXA-1 β-lactamase; this, in turn, was derived from a published[16] crystal structure of the enzyme [PDB entry 1M6K]. As a first step toward modeling a complex with the β-ketophosphonate **9**, the covalent bond between the active site serine and the carbonyl carbon was broken, thereby converting the serine side chain oxygen to a neutral hydroxyl, and hybridizing the tetrahedral carbon atom of the tetrahedral intermediate to an sp^2 carbonyl carbon and the attached oxygen to an sp^2 carbonyl oxygen. The remaining structural modification of the ligand was also achieved by means of the Builder module of InsightII. MNDO charges for the ligand were calculated from MOPAC and the charges on the protein were assigned by InsightII. A 15 Å sphere of water molecules, centered at Ser 67 Oγ, was added to each structure, supplementing the crystallographic water. Molecular dynamics simulations (200 ps) followed by energy minimization on representative snapshots led to the structures of Figure 1 as the most stable [lowest ligand–protein interaction energies (E_{int}[21])] structures.

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