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Kinetics of Action of a Two-Stage Pro-Inhibitor of Serine β-Lactamases

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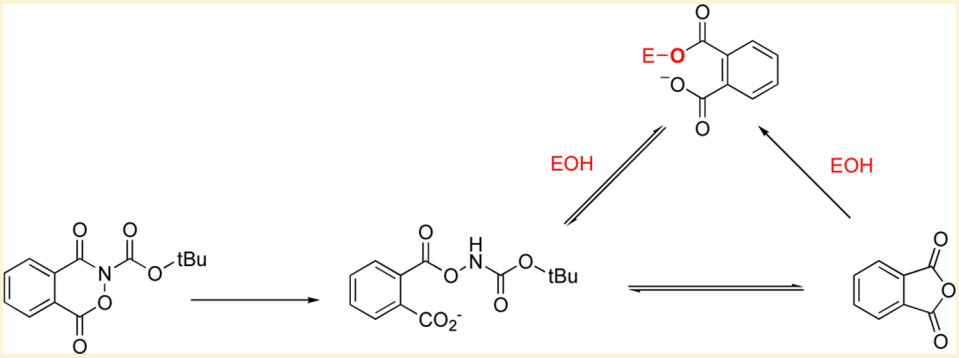
Supporting Information



ABSTRACT: β-Lactamase inhibitors are important in medicine in the protection of β-lactam antibiotics from β-lactamase-catalyzed destruction. The most effective inhibitors of serine β-lactamases covalently modify the enzyme active site. We have recently studied *O*-acyl and *O*-phosphyl hydroxamates as a new class of such inhibitors. In this paper, we describe our studies of the *N*-acyl derivatives of a cyclic *O*-acyl hydroxamic acid, 3*H*-benzo[*d*][1,2]oxazine-1,4-dione, and, in particular, the *N*-*tert*-butoxycarbonyl derivative. This compound is not a β-lactamase inhibitor itself but undergoes spontaneous hydrolysis in aqueous solution, yielding an *O*-phthaloyl hydroxamic acid, which is a β-lactamase inhibitor. This compound spontaneously, but reversibly, cyclizes in solution to form phthalic anhydride, which is also a β-lactamase inhibitor. Both inhibitors react to form the same transiently stable phthaloyl−enzyme complex. Thus, we have a two-step cascade, beginning with a pro-inhibitor, in which each step leads to a different inhibitor, presumably with different enzyme specificities. The kinetics of these transformations have been elucidated in detail. The phthaloyl derivatives, where the free carboxylate is important for facile reaction with the enzyme, represent a new lead for serine β-lactamase inhibitors. Analogues can be conveniently constructed *in situ* by reaction of nucleophiles with phthalic anhydrides and then screened for activity. Active hits may then become new leads.

β-Lactamases catalyze the hydrolysis of β-lactams and are thereby the main source of bacterial resistance to these antibiotics. Inhibitors of these enzymes have demonstrated potential in medical practice where they are used in combination with β-lactams.[1] The β-lactamase inhibitor of such a pair eliminates the resistance produced by these enzymes, allowing the β-lactam to reach its DD-transpeptidase target. The continuing evolution of β-lactamases, enforced by the selective pressure of both β-lactams and β-lactamase inhibitors, has provided the impetus for the ongoing search for new β-lactamase inhibitors.[2−5]

The search for broad-spectrum β-lactamase inhibitors is complicated by the fact that these are two very different groups of β-lactamases, the serine β-lactamases (classes A, C, and D) that belong to the β-lactam-recognizing enzyme superfamily[6] and the metallo-β-lactamases (class B), members of a distinct superfamily of metalloenzymes that exhibit a variety of functions.[7] Catalysis by the serine enzymes involves a covalent acyl−enzyme intermediate, whereas that by the metalloenzymes comprises attack on the substrate by a metal ion-coordinated water molecule. Broad-spectrum inhibitors that cover both of these major groups of enzymes are difficult to achieve. Some progress in this direction has been made,[8] but no candidates have yet been brought forward for development. It has been easier to achieve potent inhibitors of each group separately, with design focusing on acyl transfer chemistry in the case of the serine enzymes and on the metal ion in that of the metallo-β-lactamases.

Most effective inhibitors of the serine β-lactamases interact covalently with the active site, either as transition state analogues or as mechanism-based inhibitors.[9] Many are β-lactams themselves, such as clavulanic acid, sulbactam, and tazobactam,[1,2] which are currently used in medical practice. Such inhibitors remain, however, susceptible to evasion by mutant β-lactamases.[10] Thus, inhibitors that are not β-lactams are of considerable interest. Examples of these investigated to date include boronic acids, and various phosphonyl, sulfonyl, and acyl derivatives.[5] Recently, we have described aryloxy-

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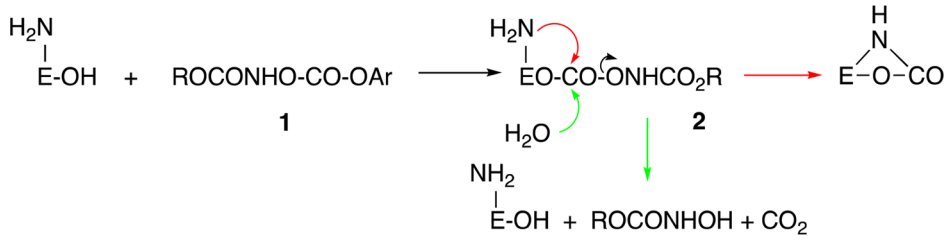
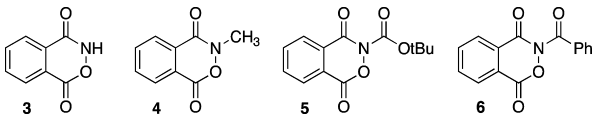
Scheme 1



carbonyl hydroxamates **1**, which are general inhibitors of serine β-lactamases.[11,12] These compounds first acylate the active site serine residue with release of the aryloxide. Irreversible interaction then follows by intramolecular aminolysis by a conserved active site lysine residue (**2**) with displacement of the hydroxamate leaving group and formation of a cross-linked active site (Scheme 1).

In view of these results, we have explored other types of *O*-acyl hydroxamates in more detail. Recently, we described some new acylic variants, including a series of phosphyl hydroxamates.[13] In the work presented here, we explore the potential of some cyclic analogues. We have thus prepared compounds **3**–**6** and assessed their activities as β-lactamase inhibitors.



Although none of these molecules, in themselves, turned out to be significant inhibitors of β-lactamases, *N*-acyl derivatives **5** and **6** generated inhibitors *in situ* by interesting chemical rearrangements that we describe in this paper. Thus, **5** and **6** were found to be pro-inhibitors. Pro-inhibitors, as leads to pro-drugs, are of considerable significance to many classes of drugs,[14] including β-lactams.[15,16] In many cases, the active drug is more efficiently delivered to the site of action in a pro-drug form, to be released in its active form after the important pharmaceutical, pharmacokinetic, and pharmacodynamic barriers have been finessed.[17] In many cases, the pro-drug is a chemically simple derivative from which the active principle can be released by spontaneous or enzyme-catalyzed hydrolysis. In some cases, however, the conversion to the active form involves a more complicated rearrangement. Both modes are illustrated by the molecules described in this paper.

EXPERIMENTAL PROCEDURES

The class C P99 β-lactamase from *Enterobacter 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.). The *Streptomyces* R61 and *Actinomadura* R39 DD-peptidases were generously supplied by J.-M. Frère of the University of Liège (Liège, Belgium) and P. Charlier (University of Liège).

The following hydroxamic acids and anhydrides were purchased commercially and used as received: **16**, **18**, **20**, **21**, **23**, **24**, **26**, and **45** (Sigma-Aldrich), **17**, **27**, **42**–**44**, **46**, **48**, and **49** (Acros), **19**, **34**, and **40** (Alfa-Aesar), **22**, **39**, and **50** (TCI America), **32** and **35** (Fluka), **25** (Mallinckrodt), **36** (Calbiochem), and **47** (Matheson Coleman & Bell).

Hydroxamic acids **28**–**31**, **33**, **37**, and **38** were synthesized following the procedure described previously.[13] The synthetic procedure for phthalic anhydride **41** is given in the Supporting Information.

Synthesis. Compounds **3**–**6** were prepared as described by Zinner et al.[18]

*tert-Butyl 1,4-Dioxo-1H-benzo[d][1,2]oxazine-3(4H)-carboxylate (**5**).* The crude solid product was recrystallized from a dioxane/ether mixture (1:1) to afford compound **5** in 60% yield as a colorless solid: mp 226–228 °C (lit.[18] 226–228 °C); ^{1}H NMR (d_6-DMSO) δ 1.56 (s, 9H), 8.02 (m, 2H), 8.16 (m, 2H); FTIR (KBr) 1780, 1762, 1713 cm^{-1}; ES(+)MS 301.00 (M + H$^+$).

*3H-Benzo[d][1,2]oxazine-1,4-dione (**3**).* This compound was obtained in 75% yield as an off-white solid after recrystallization from a cyclohexane/benzene mixture (1:1): mp 222–224 °C (lit.[18] 225 °C); ^{1}H NMR (d_6-DMSO) δ 8.04 (m, 2H), 8.20 (d, J = 6.0 Hz, 2H); FTIR (KBr) 1768, 1677 cm^{-1}; ES(−)MS 162.00 (M − H$^+$).

*3-Methyl-3H-benzo[d][1,2]oxazine-1,4-dione (**4**).* The crude solid product was recrystallized from ethanol to afford **4** in 40% yield as a colorless solid: mp 99–100 °C (lit.[18] 120 °C); ^{1}H NMR (d_6-DMSO) δ 3.56 (s, 3H), 7.95–8.16 (m, 4H); FTIR (KBr) 1762, 1657 cm^{-1}; ES(+)MS 178.07 (M + H$^+$).

*3-Benzoyl-3H-benzo[d][1,2]oxazine-1,4-dione (**6**).* The crude product was recrystallized from a benzene/cyclohexane mixture (1:1) to afford **6** in 60% yield as a colorless solid: mp 136–138 °C; ^{1}H NMR (d_6-DMSO) δ 7.49–8.23 (m, 9H); FTIR (KBr) 1764, 1754, 1701 cm^{-1}; ES(+)MS 268.07 (M + H$^+$).

*N-(tert-Butoxycarbonyl)-O-(2-carboxybenzoyl)-hydroxylamine (**7**) and Its ^{15}N Isotopomer.* A solution of triethylamine (0.57 mL, 4.13 mmol) in DCM (2 mL) was added dropwise to a solution of *tert*-butyl *N*-hydroxycarbamate (0.5 g, 3.75 mmol) in DCM (20 mL) at 0 °C. A solution of phthalic anhydride (0.61 g, 4.13 mmol) and DMAP (45 mg, 0.10 mmol) in DCM (5 mL) was then added dropwise over a period of 10 min. The reaction mixture was stirred for a further 4 h to room temperature. The organic layer was washed twice with water, after which the organic phase was dried over Na_2SO_4 and evaporated under reduced pressure. The crude solid was purified by preparative silica gel thin layer chromatography with an ethyl acetate/hexane/ether/acetic acid mixture (4:45:50:1) as the solvent. The resulting material was recrystallized from a dioxane/benzene/cyclohexane mixture (1:10:3) to afford **7** in 35% yield as a colorless crystalline solid: mp 96–98 °C; ^{1}H NMR (d_6-DMSO) δ 1.42 (s, 9H), 7.69 (s, 3H), 7.8 (m, 1H), 10.98 (s, 1H), 13.38 (s, 1H); FTIR (KBr) 1790, 1698, 1667 cm^{-1}; HRMS (ES+) 304.0799 (M + Na$^+$), calcd for $C_{13}H_{15}NO_6Na$ 304.0797.

The ^{15}N isotopomer of this compound was prepared as described above but using *tert*-butyl [^{15}N]-*N*-hydroxycarbamate[12] as the starting material: ^{1}H NMR (d_6-DMSO) δ 1.42 (s,

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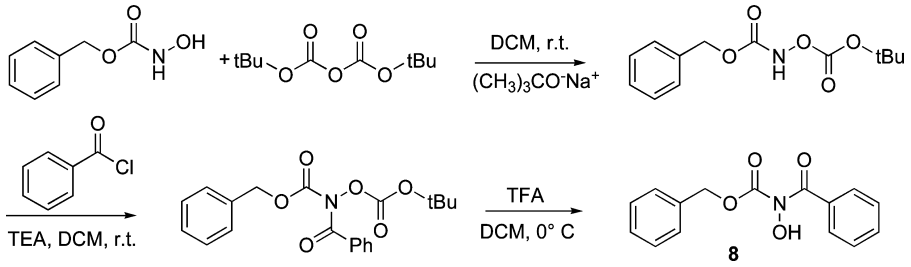
Scheme 2



9H), 7.69 (s, 3H), 7.8 (m, 1H), 11.00 (d, J = 92.1 Hz, 1H), 13.4 (s, 1H).

N-(Benzyloxycarbonyl)-N-(benzoyl)hydroxylamine (**8**) *and N-(tert-butoxycarbonyl)-O-(benzoyl)hydroxylamine* (**9**) were prepared by standard procedures,[19] the former as shown in Scheme 2. Experimental details are provided in the Supporting Information.

Kinetic Methods. Absorption spectra and spectrophotometric reaction rates were obtained from Hewlett-Packard 8452A and 8452E spectrophotometers. Enzyme concentrations were determined spectrophotometrically. Kinetic experiments were conducted at 25 °C in a 20 mM MOPS buffer solution at pH 7.5. The inhibitors were prepared in concentrated DMF stock solutions and diluted to 1−5% DMF in reaction mixtures.

Spontaneous Hydrolysis Reactions. The spontaneous hydrolysis rates of the potential inhibitors were measured spectrophotometrically in replicate at appropriate wavelengths and concentrations (Table 1). The full progress curves were then fit to a first-order rate equation and the rate constants thus obtained from averaged replicates (Table 1).

Table 1. Rate Constants for Spontaneous Hydrolysis of Acyl Hydroxamates and Phthalic Anhydrides

compd	k_0 (s^{-1})	λ (nm)	$\Delta\varepsilon$ (cm^{-1} M^{-1})
3	$<1 \times 10^{-6}$ [a]	250−300	–
4	$(7.6 \pm 0.1) \times 10^{-4}$	300	2100
5	0.034 ± 0.001	260	3125
6	0.029 ± 0.001	300	5000
7	$(4.0 \pm 0.1) \times 10^{-3}$ [b]	260	1475
8	$(2.2 \pm 0.1) \times 10^{-3}$	300	635
9	$(2.0 \pm 0.2) \times 10^{-6}$	250	1775
10	0.015 ± 0.001	300	2250
39	$(8.1 \pm 0.1) \times 10^{-3}$	260	40000
41	$(8.6 \pm 0.1) \times 10^{-3}$	300	2275

[a]No observed reaction. [b]Cyclization to **10** (Scheme 3).

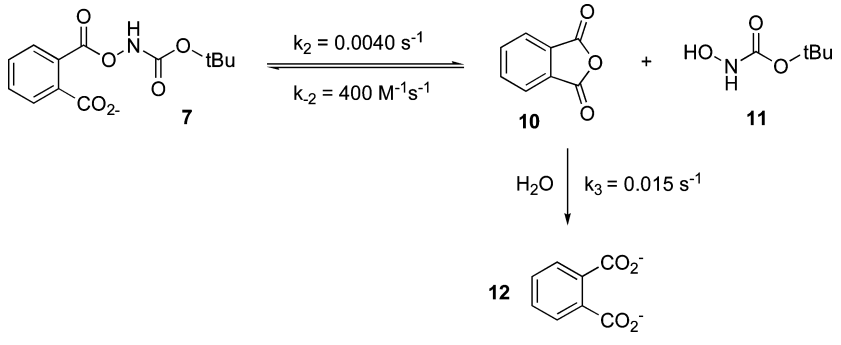
Identification of the Intermediates of Spontaneous Reactions by ^1H NMR. MOPS buffer (20 mM, pH 7.5, 20 mL) was added to the stirred solution of the reactant (5 mg) dissolved in acetonitrile (2 mL). The reaction mixture was then stirred for an appropriate time period (7 min for **7** and 3 or 90 min for **5**). The reaction was quenched with 0.1 M HCl (~3 mL) to pH <2. The resulting solution was extracted twice with ethyl acetate. The organic layer was separated, dried over Na_2SO_4, and evaporated under reduced pressure. This residue was dried under an oil pump vacuum overnight. ^1H NMR spectra were obtained with d_6-DMSO as the solvent. The same sample was used for mass spectrometric analysis.

Equilibrium Experiments with Compound **7**. To determine the spontaneous reaction rate of **7**, three experiments were conducted separately: (i) reaction of **7** alone (200 and 400 μM), (ii) same as (i) in the presence of 500 μM **11**, and (iii) reaction of **10** (200 and 400 μM) in the presence of 500 μM **11**. All reactions were monitored spectrophotometrically at 260 nm. All six curves were simultaneously fit to Scheme 3 using Dynafit.[20]

Direct Turnover by the P99 β-Lactamase. The enzyme (final concentrations of 1−20 μM) was added to the buffered solution of the inhibitory substrate (final concentrations of 100 μM for **10** and 200 μM for **7**) and the subsequent hydrolysis monitored spectrophotometrically at an appropriate wavelength (Table 1). The measured initial rates were plotted against enzyme concentration and fit to a linear equation.

Time-Dependent Enzyme Inhibition. The P99 β-lactamase (0.5 μM) was incubated with inhibitor (500 μM) in buffer (100 μL) containing 0.1% BSA. At appropriate times, aliquots (5 μL) of the reaction mixture were diluted to 0.50 mL in MOPS buffer containing cephalothin (300 μM), and hydrolysis of the latter was monitored spectrophotometrically at 280 nm over a suitable period of time. The measured initial rates, proportional to the remaining free enzyme concentration, were plotted against the time of incubation. These data were fit to

Scheme 3



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appropriate reaction schemes (see Results and Discussion) using Dynafit.[20]

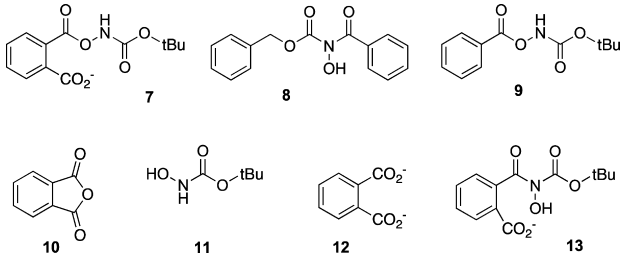
Competitive Enzyme Inhibition Experiments. CENTA (50 μM; K_m = 19.5 μM) was employed as the substrate, and its hydrolysis was monitored at 410 nm.[21] The P99 β-lactamase concentration was 1.0 nM, and concentrations of the inhibitor were 10−300 μM. Total progress curves were fit to an appropriate scheme (see Results and Discussion) using Dynafit.[20] This experiment was also conducted to screen combinations of various anhydrides (100 μM) and nucleophiles (100 μM).

Mass Spectrum of the EI Complex. The P99 β-lactamase (20 μM, 100 μL) was incubated with **7** (200 μM) for 50 s. 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%). A sample for mass spectrometry was prepared as previously described.[13]

Kinetics with Other Enzymes. The competitive inhibition experiment described above was also performed with the TEM-2 β-lactamase (5.0 nM) and **10** (500 μM) alone as well as with **10** (500 μM) in the presence of **11** (500 μM). The hydrolysis of the substrate nitrocefin (200 μM; K_m = 86 μM) was monitored at 600 nm.[22] This experiment was also performed with the *Streptomyces* R61 DD-peptidase (0.2 μM) and *Actinomadura* R39 DD-peptidase (0.3 μM), employing *m*-carboxyphenyl *N*-phenylacetylglycinate (K_m = 0.76 mM at 2.0 mM and $K_m \gg$ 1 mM at 2.0 mM, respectively, monitored at 310 nm) as the substrate.[23]

RESULTS AND DISCUSSION

Spontaneous Reactions in Solution. The pseudo-first-order rate constants of spontaneous reactions of **3**−**10** in MOPS buffer at pH 7.5 were obtained from spectrophotometric measurements (see Experimental Procedures) and are listed in Table 1. Compounds **4** and **9** hydrolyzed in a simple one-step exponential fashion. The product from hydrolysis of **4** arose from nucleophilic attack on the acyl hydroxamate carbonyl to yield the hydroxamic acid product.[24,25] Presumably, this reaction would also occur for **3**, but in this case, the hydrolysis was extremely slow at pH 7.5 because , as indicated by absorption spectra taken at pH 1−13, the compound probably exists as an inert anion at that pH (cf. **1**[12]). The hydrolysis of **9** also yielded the hydroxamic acid (**11**) and carboxylate (benzoate). The *N*,*N*-diacylhydroxylamine **8** hydrolyzed in a simple one-step process to yield benzoate and the hydroxamic acid (benzyl *N*-hydroxycarbamate), as indicated by ^{1}H NMR spectra taken during the reaction. It appeared to be surprisingly reactive (Table 1) compared with the *N*,*O*-diacyl compound **9**, possibly because of intramolecular general base catalysis by the anion of **8**.



The rapid spontaneous reaction of **7** in aqueous buffer was first monitored by ^{1}H NMR spectroscopy. This identified phthalic anhydride **10** as a transient intermediate and *tert*-butyl *N*-hydroxycarbamate **11** and phthalate **12** as final products (Figure S1 of the Supporting Information). Identical spectra (Figure S1 of the Supporting Information) were obtained from a mixture of **10** and **11**, indicating the presence of a reversible equilibrium between these species and **7**. These results were suggestive of Scheme 3. The rate constants of Scheme 3 were determined from spectrophotometric rate measurements. Reaction mixtures were set up in three different ways: (i) beginning with **7** alone, (ii) beginning with **7** together with **11**, and (iii) beginning with **10** in the presence of **11**. Data from these were fit simultaneously to Scheme 3 (Figure S2 of the Supporting Information) to obtain values of k_2 and k_{-2}, given the independently measured rate constant for hydrolysis of **10** to **12** (viz. 0.015 s^{-1}). Direct hydrolysis of **7** was assumed to be slow in view of the results for **9**. The rate constants thus obtained are shown in Scheme 3. At millimolar concentrations of **11**, the equilibrium lies in favor of **7** (vs **10**), and thus, under these conditions, the hydrolysis of **10** will be impeded by the presence of **11**. The situation displayed in Scheme 3 has previously been observed with monoaryl phthalates.[26]

Spontaneous reaction of **5** in solution is complicated. Quenched reaction samples gave ^{1}H NMR spectra (Figure S3 of the Supporting Information) showing the presence of **7**, **3**, **12** and **13**. This result was supported by ES(−) mass spectra of quenched reaction mixtures that showed peaks at 162 amu (**3**) and 165 amu (**12**). An ES(+) spectrum showed a peak at 282 amu that corresponds to **7** and/or **13**. The likely presence of **13** as well as **7** is indicated by a ^{1}H NMR peak at 10.2 ppm [attempts to independently synthesize **13** were unsuccessful, but the close analogue **8** was prepared; the ^{1}H NMR spectrum of **8** contained a peak at 10.6 ppm (OH)]. On further reaction, the peak at 10.2 ppm did disappear with time at a rate comparable to that of **7** (Figure S3 of the Supporting Information). These spectra suggested that **5** reacts in solution to form ∼10% each of **3** and **13**, with the remainder converted to **7**. This information was used in fitting the spectrophotometric data, as described below.

The absorption at 260 nm of a solution of **5** at pH 7.5 decreases with time in two phases (Figure S4 of the Supporting Information). These data were fit to Scheme 4, by fixing k_2, k_{-2}, and k_3 to the values of Scheme 3 and the rate constants for conversion of **5** to **3** and **5** to **13** to the value of 0.1k_1, the latter assignment based on the NMR data described above. The hydrolyses of **3** and **13**, thought to represent minor contributions to the absorption changes at 260 nm (that of **3** certainly does, from the independent study of **3**, described above), were omitted from the fit. Compound **13** does react further, as noted above, perhaps by cyclization to form **10** (Scheme 4), but probably also directly to **12** because **8** hydrolyzes quite readily to benzoate (see above). Compound **13** is kinetically less accessible from **10** ($\alpha \ll 1$) than is **7** because addition of **11** to **10** appears to immediately generate essentially only **7**, as indicated by NMR spectra (Figure S1C of the Supporting Information).

The value of k_1 (0.034 ± 0.001 s^{-1}), obtained from the fit of the data of Figure S4 of the Supporting Information, shows that **5** is quite unstable in solution, rapidly ($t_{1/2}$ = 20 s) converting to (mainly) **7**, yielding the first kinetic phase noted above and seen in Figure S4 of the Supporting Information. Compound **7** then proceeds via **10** to **12** (Scheme 3) in the second, slower phase.

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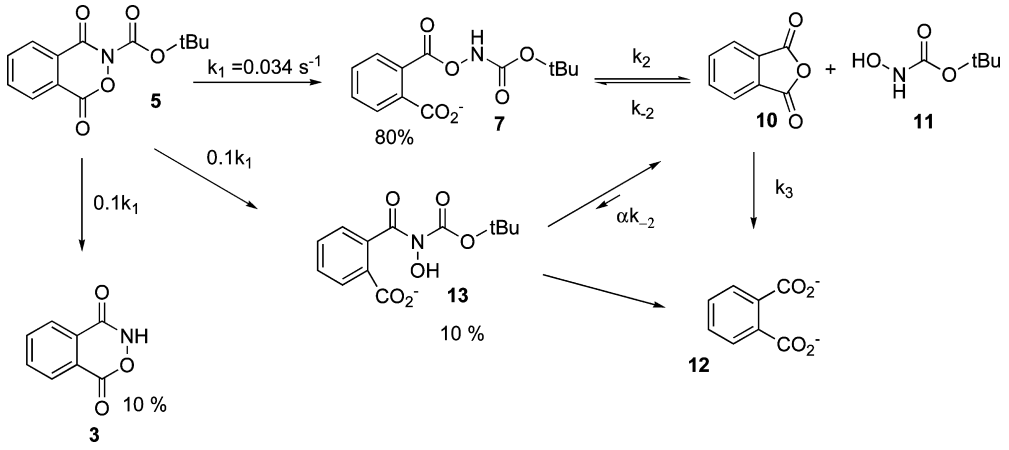
Scheme 4



Inhibition of the P99 β-Lactamase. Preliminary experiments indicated that incubation of the P99 β-lactamase with either **5** or **6** led to transient inhibition of the enzyme. Because of the time dependence of this phenomenon and the instability of **5** and **6** in solution (see above), it was necessary to carefully assess the various species of Scheme 4 for inhibitory activity. Because the enzyme activity loss was transient and full activity was eventually recovered, inhibitory substrates seemed to be involved.

Phthalic anhydride, **10**, is certainly a transient inhibitor of the β-lactamase. Figure 1A shows the activity of the enzyme as a function of time after addition of phthalic anhydride. The enzyme activity is lost in a rapid initial phase and then slowly recovered. This data could be fit to Scheme 5 to give a k_5 value of $(9.0 \pm 2.3) \times 10^{-3}$ s^{-1}. The value of k_4 was obtained by monitoring direct turnover of **10** spectrophotometrically (Figure 1B), which yielded a k_4 value of 800 ± 70 M^{-1} s^{-1}. In Scheme 5, we assume, on the basis of all precedent,[27−29] that the transient inactivation of the P99 enzyme occurs by covalent modification of the active site serine residue. To support this assertion, we quenched a reaction mixture at an appropriate time (see Experimental Procedures). A mass spectrum of the precipitated protein yielded a mass of 39338 amu, which is as expected for phthaloylation of the enzyme (mass of 39190 amu).

Reactivation of the transiently inactivated enzyme could occur either by recylization to re-form **10** (dashed arrow, k_{-4}) or by direct hydrolysis (solid arrow, k_5) (Scheme 5). The latter is thought to dominate for reasons given below. The effective thermodynamic dissociation constant, K_i, of phthalic anhydride is given by k_5/k_4 and is thus 11 μM. We are unaware of any previous study of the inactivation of a serine hydrolase by phthalic anhydride. Isatoic anhydride, however, has been shown to be a class C β-lactamase inhibitor (see below).[30]

The transient inhibition caused by **10** is also readily demonstrated by competition experiments with a chromogenic substrate. Figure 2 shows the results of such an experiment in which the lag in turnover and the recovery of activity are clearly visible. Fitting of the data to a reaction scheme involving a combination of Scheme 5 with steady state turnover of the substrate CENTA yielded a k_5/k_4 or K_i value (only the ratio was available from numerical fitting of Scheme 5 to the data of Figure 2) of 0.26 ± 0.03 μM. This value is clearly much smaller than that obtained from the direct experiment described above. The difference is readily seen qualitatively in the lags prior to reactivation in Figure 2 that are much more extended than that observed in Figure 1A. The difference arises because turnover of a cephalosporin substrate by the P99 enzyme generates a different free enzyme form that reacts with acylating agents

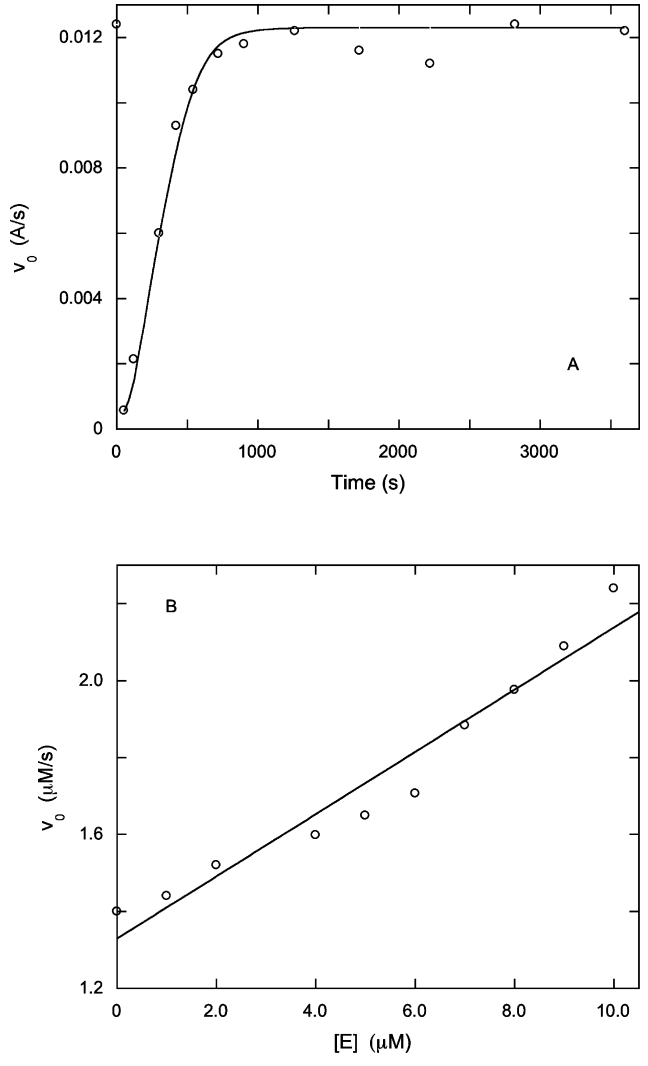


Figure 1. (A) Activity of the P99 β-lactamase (0.5 μM) as a function of time in the presence of **10** (0.50 mM). The data were fit (—) to Scheme 5. (B) Initial velocity of hydrolysis of **10** (0.10 mM) as a function of β-lactamase concentration.

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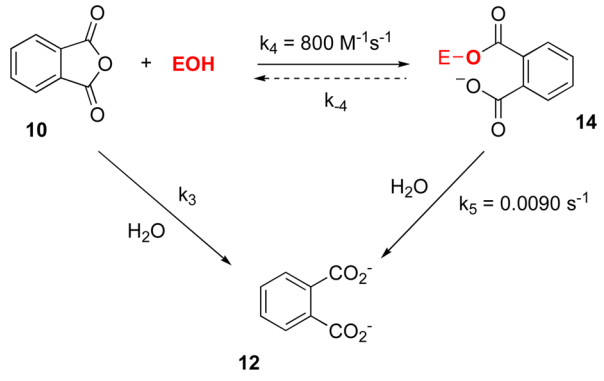
Scheme 5



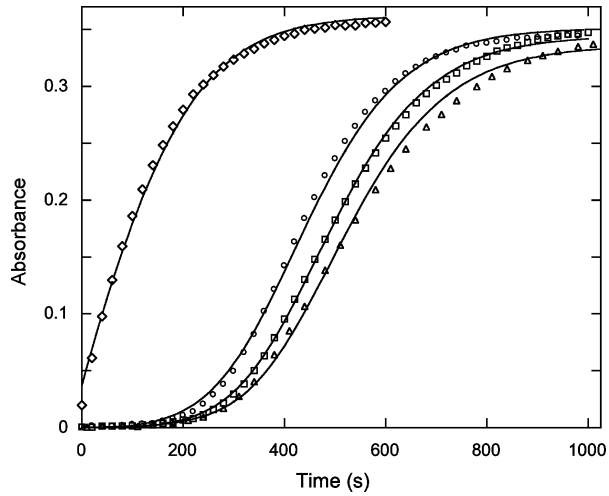


Figure 2. Turnover of CENTA (50 μM) by the P99 β-lactamase (1.0 nM), alone (◇) and in the presence of **10** [0.10 (○), 0.20 (□), and 0.30 mM (△)]. The solid lines represent the fit of the data to Scheme 5.

and/or substrates in a manner different from that of the original free enzyme.[30−32] This experimental protocol is introduced here because, as described below, it is convenient to qualitatively demonstrate the relative effectiveness of a variety of anhydride−nucleophile combinations by this method.

Because **7**, an intermediate in Scheme 4, could be independently synthesized (Experimental Procedures), its inhibitory activity could be assessed directly. It is worth noting at this point that the compound synthesized, by reaction of **10** with the hydroxamic acid **11**, was shown to be the *O*-acyl product **7** rather than the *N*-acyl analogue **13**. This was proven by synthesis of the ^{15}N isotopomer of **7** and the observation of direct $^{15}N-H$ coupling (92.1 Hz) in the ^{1}H NMR spectrum (see Experimental Procedures). The high-frequency carbonyl at 1790 cm^{-1} is also indicative of O-acylation. Acylation of hydroxamic acids, under conditions of mild base catalysis, generally yields the O-acylated material, at least as a kinetic product.[12,33]

Compound **7** also appeared to be a transient inhibitor of the P99 enzyme, although, in view of the facile interconversion of **7** and **10** (Scheme 3), its activity could not be taken for granted. Experiments to monitor enzyme activity versus time and turnover were conducted in a manner similar to that described for **10**, with the results shown in panels A and B of Figure 3, respectively. In addition, taking advantage of the interconversion of **7** and **10**, we conducted an experiment beginning with **10** and a concentration of **11** sufficient to rapidly drive most of

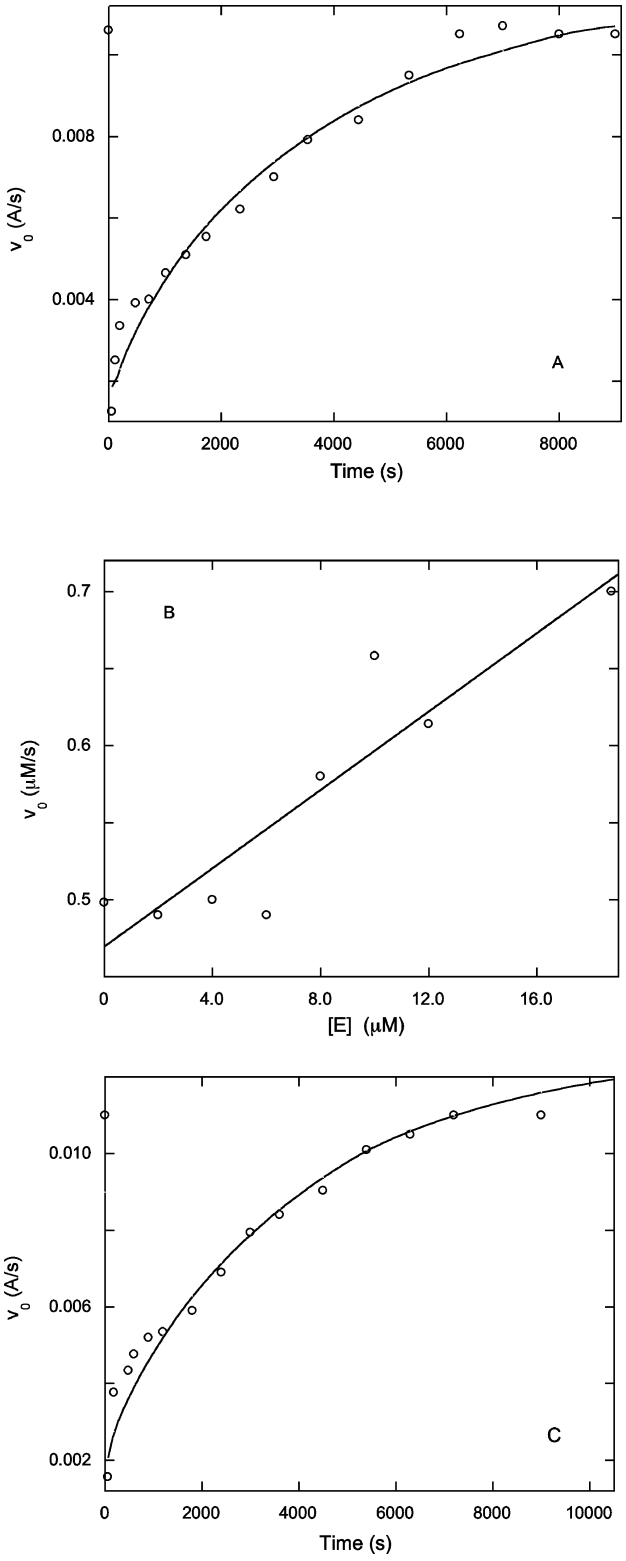


Figure 3. (A) Activity of the P99 β-lactamase (0.50 μM) as a function of time in the presence of **7** (0.50 mM). The data were fit (—) to Scheme 6. (B) Initial velocity of hydrolysis of **7** (0.20 mM) as a function of β-lactamase concentration. (C) Activity of the P99 β-lactamase (0.50 μM) as a function of time in the presence of a mixture of **10** (0.50 mM) and **11** (0.50 mM). The data were fit (—) to Scheme 6.

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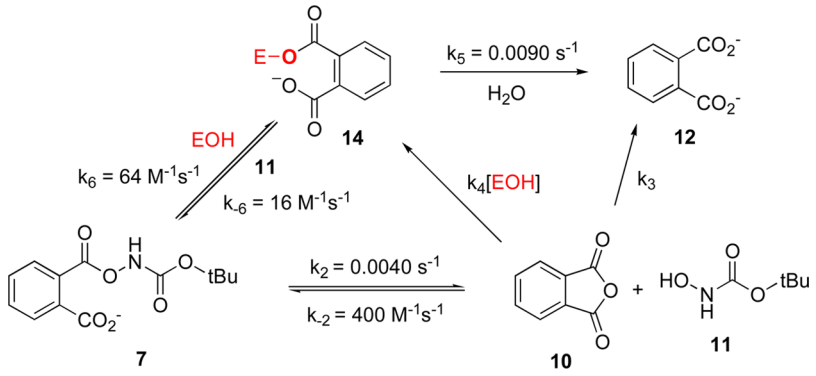
Scheme 6



10 (>90%) to **7**. The β-lactamase inhibitory activity of this mixture was also monitored as a function of time (Figure 3C). The similarity of panels A and C of Figure 3, given the position of the equilibrium between **10** and **7** [$K_2 = k_{-2}/k_2 = 10$ μM (discussed above)], strongly indicates that **7** is itself an inhibitor. These data were evaluated in terms of Scheme 6.

Linear least-squares analysis of the data in Figure 3B yielded a k_6 value of 64 ± 11 M^{-1} s^{-1}. Incorporation of this value for k_6 and the other previously determined rate constants shown in Schemes 4 and 5 into the fits of panels A and C of Figure 3 yielded k_{-6} values of 19 ± 17 and 14 ± 6 M^{-1} s^{-1}, respectively. A value for the equilibrium constant K_6 (= k_{-6}/k_6) of ~0.25 for the nucleophile-assisted dissociation of **14** was thus established.

As quite clearly indicated by Figure 3C and the analysis presented above, therefore, **7** is directly an inhibitor of the β-lactamase as well as indirectly via **10**. Although more stable in solution than the anhydride (k_2 = 0.0040 s^{-1} vs k_5 = 0.015 s^{-1}), **7** is less effective as a β-lactamase inhibitor (k_6 = 64 M^{-1} s^{-1} vs k_4 = 800 M^{-1} s^{-1}). The combination of **10** and **11** can be more effective than **10** alone (compare Figures 1A and 3C, for example), however, because of the equilibrium in favor of the more stable **7**. It is noteworthy that **9**, an analogue of **7** lacking the *o*-carboxylate, is not detectably an inhibitor of the P99 enzyme. Apparently, as is often true with β-lactamase substrates, the presence of a carboxylate substantially enhances reactivity;[34,35] the best β-lactam substrates all possess such a carboxylate, which is known to interact with active site functional groups.[36,37] The relative chemical inertness of **9** (Table 1) is undoubtably also a factor. In contrast, the chemically more reactive *N*,*N*-diacyl analogue **8** is measurably an inhibitor, although it lacks the carboxylate [k_i = 20 ± 2 M^{-1} s^{-1} (data not shown)]. It is possible, however, that the OH or O^- group of **8** also favorably interacts with the enzyme active site during acylation. Although the carboxylate group appears to be advantageous for enzyme acylation, it seems less necessary for deacylation. The deacylation rate constant [k_5 = 0.0090 s^{-1} (Scheme 5)] is very similar to that oberved for deacylation of the benzoyl-enzyme, generated from benzoyl phosphates.[38] Presumably, the enzyme is able to directly catalyze, albeit slowly, the hydrolysis of both of these species.

The thermodynamic cycle of Scheme 6 (**7** ⇔ **10** ⇔ **14** ⇔ **7**) also permits calculation of k_{-4}, the rate constant for breakdown of **14** by recyclization to **10** (see Scheme 5). Thus, k_{-4} = $(K_6k_2k_4)/k_{-2}$ = 0.0020 s^{-1}. This indicates that some breakdown of **14** does occur by recyclization to **10**, although the major path is by direct hydrolysis (k_5).

Finally, as noted above, the original compound **5** also appears to cause transient inhibition of the P99 β-lactamase. Compound **5** itself, however, is not a significant inhibitor. This can be seen, for example, in Figure 4A, where reaction of **5** in the presence of the substrate CENTA and the P99 β-lactamase shows burst kinetics. Fitting of this data to Scheme 7, employing only the rate constants determined above, indicates that **5** has little or no inhibitory activity, and the latter arises only after generation of **7** and **10**. In support of this interpretation, the data of Figure 4B, which shows the recovery of enzyme activity in the presence of **5**, can also be fit by Scheme 7, using the previously determined rate constants, i.e., also not requiring active inhibition by **5**. Compound **5** is therefore a pro-inhibitor, giving rise spontaneously in solution to the inhibitors **7** and, subsequently, **10**.

The experiments described above show that new inhibitors of the P99 β-lactamase can be generated by addition of nucleophiles to phthalic anhydride (Scheme 8). The effectiveness of the generalized inhibitor **15** can be semiquantitatively assessed by an experiment similar to that shown in Figure 2. Thus, enzyme was added to a mixture of phthalic anhydride, nucleophile, and the substrate CENTA. The enzyme-catalyzed hydrolysis of CENTA was followed yielding data like those shown in Figure 5.

Nucleophiles yielding traces identical to that of phthalic anhydride alone probably did not react with the anhydride and thus did not perturb its level of inhibition. Nucleophiles such as **17** that add to form an adduct that was not inhibitory, or less so than **10** itself, yielded traces to the left of that for **10** alone (Figure 5), while those that produced more inhibitory adducts, such as the original **11** and **34**, yielded traces to the right of **10** (Figure 5). A graph showing the results of such experiments with the nucleophiles **16**–**38** is shown in Figure 6. The relative effectiveness of the various compounds derives from a combination of K_2 (and the nucleophile concentration, of course) and k_6/k_4. Figure 6 suggests that, in general, amines and hydroxylamines, phenols, phosphates, and carboxylates generate no or inactive adducts, whereas hydroxamates, as originally discovered, generate adducts that are active inhibitors. Those more effective than **11** are mainly aromatic hydroxamic acids, although **36** is an interesting outlier. A good inhibitor **15** would therefore be generated by a good nucleophile (K_2), yielding an effective acylator (k_6) of the active site, where the intrinsic effectiveness of **15** would be a function of the interactions between all of the components of **15**, including the carboxylate group, with the enzyme active site.

It is possible that nucleophiles **16**–**19**, interpreted above as yielding inactive adducts, may alternatively act by nucleophilic deacylation of **14**. This seems less likely than the original interpretation because nonspecific nucleophiles do not

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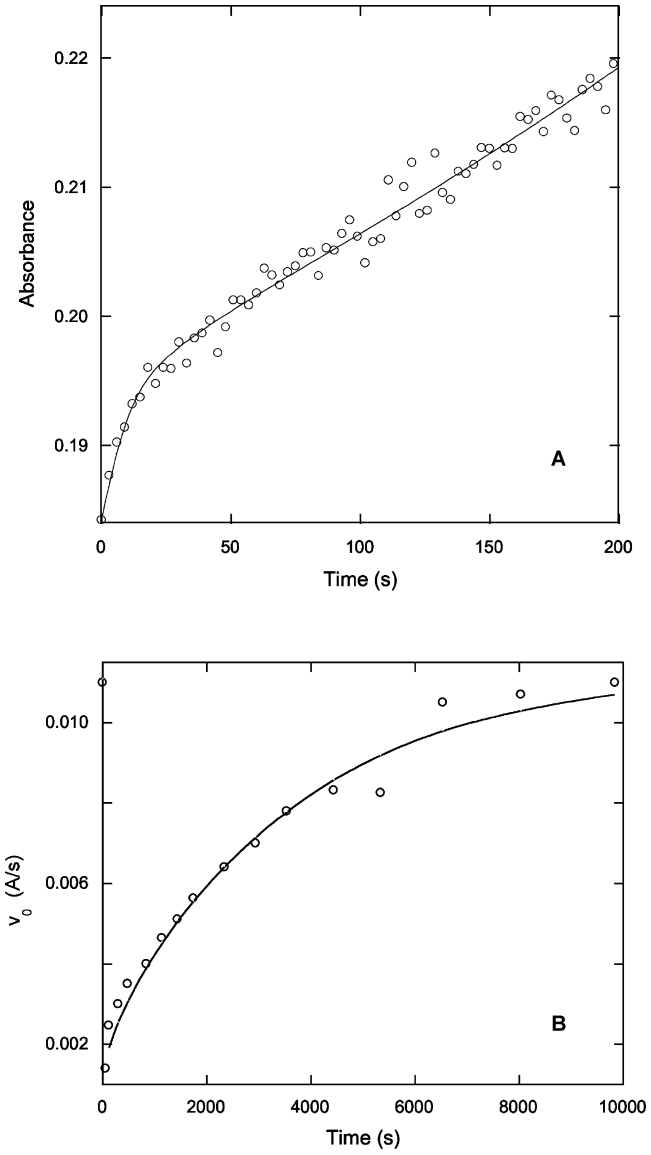
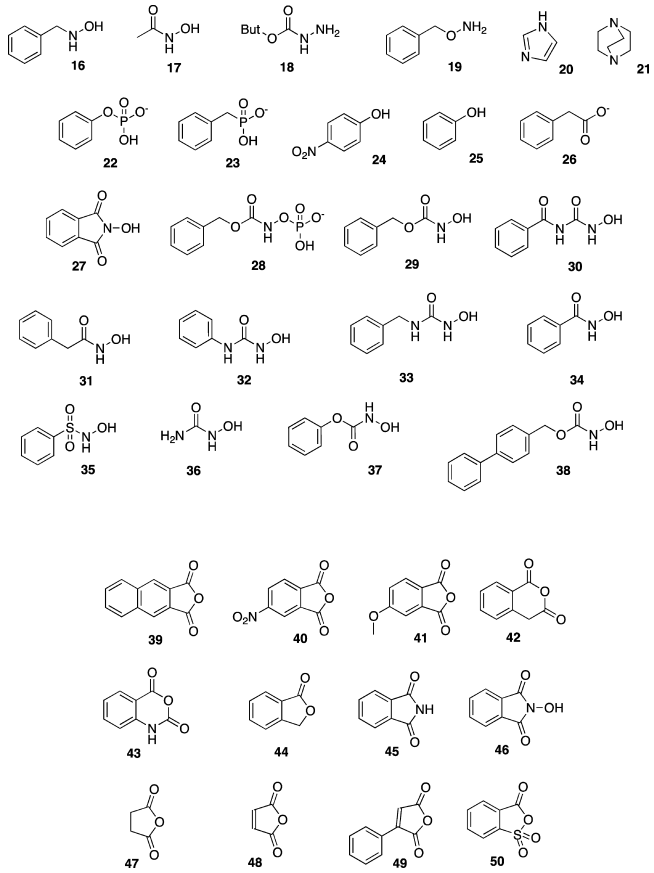


Figure 4. (A) Initial part of the progress curve for hydrolysis of CENTA (50 μM) by the P99 β-lactamase (1.0 nM) in the presence of **5** (25 μM). The data were fit (—) to Scheme 7. (B) Activity of the P99 β-lactamase (0.50 μM) as a function of time in the presence of **5** (0.50 mM). The data were fit (—) to Scheme 7.

generally cleave acyl derivatives of the P99 enzyme.[39] At any event, the product **15** thus obtained must still be less active than **10**.

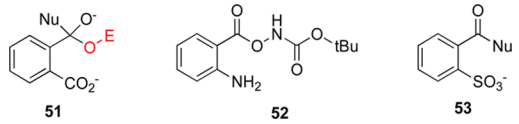
Variation of the phthalic anhydride moiety is also possible, and thus, compounds **39**–**50** were screened in a manner similar to that used for the combinations described above (Figure S5 of the Supporting Information). Of the compounds tested, only the substituted phthalic anhydrides **39** and **41** had significant activity, alone and in the presence of **11**. The nitro compound **40** was too unstable in aqueous solution to produce measurable inhibition. The hydrophobic **39** was more effective in combination with **11** than **10** itself. It seems likely that even more effective inhibition (faster acylation and slower deacylation) could be achieved from structure–activity studies of a further variety of anhydride–nucleophile combinations.

Compound **6** behaved very much like **5**, showing transient enzyme inhibition, for example, when added to solutions



containing a mixture of CENTA and the P99 β-lactamase (see above). It is likely that hydrolytic activation of **6** occurs, as with **5**. A quantitative analysis of the activity of **6** has not yet been performed.

One particularly important result obtained from the studies described above is that compound **7**, but not **9**, is a significant inhibitor of the P99 β-lactamase, giving rise to the acyl-enzyme **14**. The carboxylate is thus important, relating, presumably, to the general presence of carboxylate groups on β-lactamase substrates and inhibitors.[32−34] The transition state analogue motif **51** may, presumably, also be approached in a number of classical ways.[40]



Isatoic anhydride (**43**) is reported to be a class C β-lactamase inhibitor.[28] In our hands, it appeared to be less effective than **10**, and its activity was not enhanced by **11**. Presumably, **52**, the adduct likely derived from **43**, is also not a strong inactivator, emphasizing again the importance of the *o*-carboxylate group in **7**. Finally, the extremely reactive **50** was an irreversible inhibitor. An *o*-sulfonate rather than a carboxylate must impede hydrolysis of **14** by the enzyme. The sulfonates **53** may, therefore, have interesting inhibitory activities.

The class A TEM-2 β-lactamase and the *Streptomyces* R61 and *Actinomadura* R39 DD-peptidases were also transiently inhibited by **10**, but more slowly than the P99 β-lactamase, and the inhibition was weakened by the presence of **11**. Presumably, **7** is not an effective inhibitor of these enzymes, although other variants of **15** might be.

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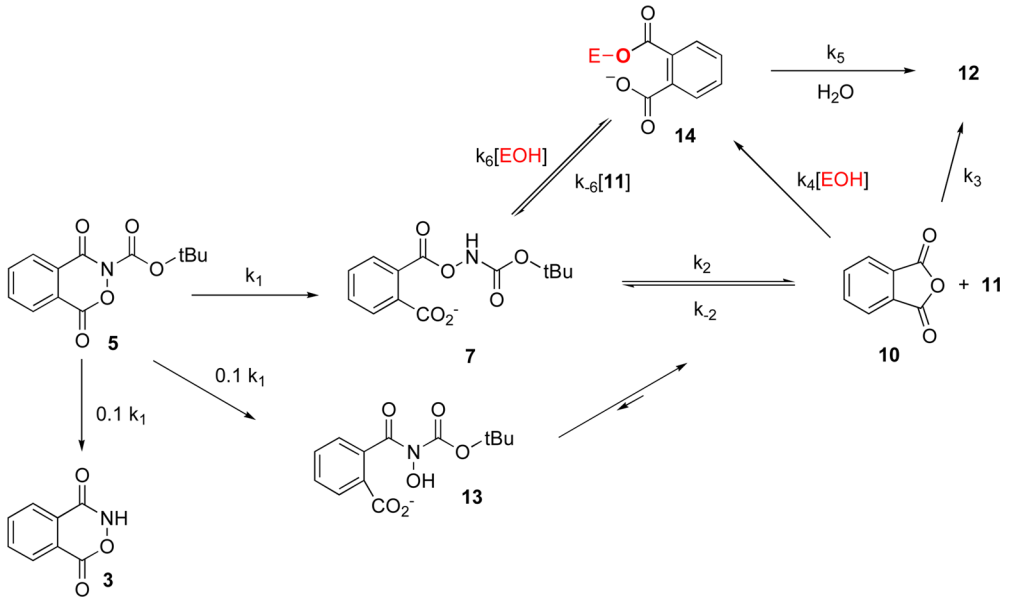
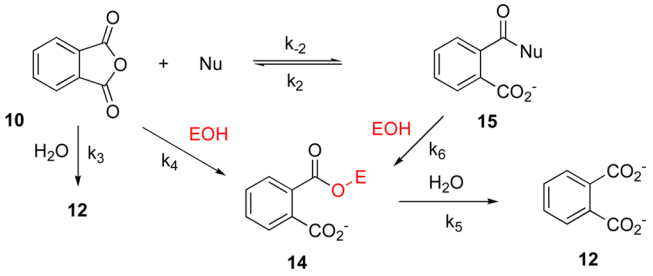
Scheme 7



Scheme 8



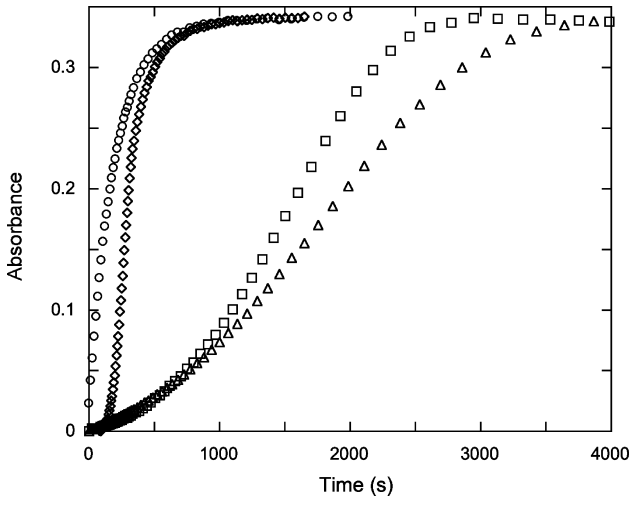


Figure 5. Turnover of CENTA (50 μM) by the P99 β-lactamase (1.0 nM) in the presence of **10** (0.10 mM) alone (◇) and upon addition of nucleophiles **17** (○), **11** (□), or **34** (△), each at 0.10 mM.

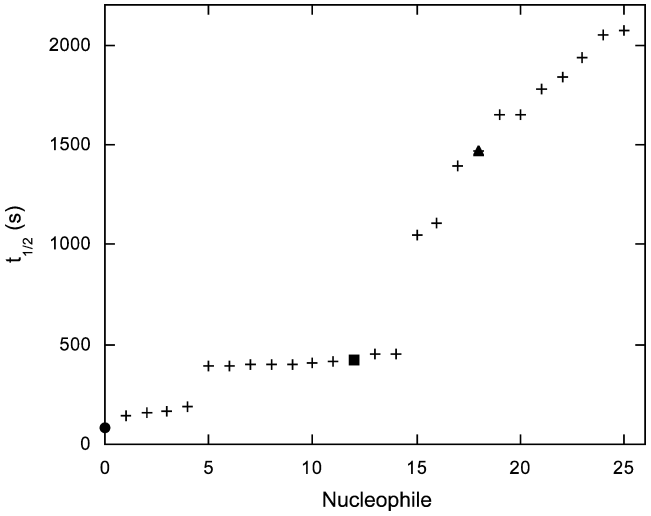


Figure 6. Relative activity (estimated as $t_{1/2}$, the time to half-consumption of the substrate) of a series of nucleophiles (0.10 mM each) added to **10** (0.1 mM) in the presence of the substrate CENTA (50 μM) and the P99 β-lactamase (1.0 nM). The filled circle represents the absence of both **10** and nucleophile, the filled square **10** alone, and the filled triangle **10** with the nucleophile **11**. The register of nucleophiles is as follows: **1**–**11**, **16**–**26**; **13**–**25**, **27**–**38**.

Conclusions. Although the cyclic hydroxamates **3** and **4** were not β-lactamase inhibitors, their *N*-acyl derivatives, **5** and **6**, respectively, had interesting inhibitory properties. Compound **5** was not itself inhibitory, but upon spontaneous reaction in aqueous solution, it yielded, first, the phthaloyl monoderivative **7**, which was inhibitory. Compound **7** reacted with the P99 β-lactamase yielding a transiently inhibited enzyme ($t_{1/2} \sim 1.25$ min), which is almost certainly a covalent phthaloyl-enzyme. Phthalic acid monoderivatives **15** may represent new leads to β-lactamase inhibitors; the carboxylate group of **7** was important for its activity, presumably providing the negative charge found in most β-lactamase substrates and inhibitors. In a second stage of reaction, the initially formed **7** cyclized to form phthalic anhydride **10**, which is also a β-lactamase inhibitor, forming the same transiently stable (phthaloyl) intermediate that **7** did. Compound **6** was also a transient inhibitor, presumably through similar chemistry. Although **5** and **6** appear to be too spontaneously reactive themselves to become practical inhibitors, it is possible that suitable chemically deactivated analogues may have interesting pro-drug properties. The two-stage activation of the new pro-

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inhibitors leads to two different inhibitors which, presumably, would have different enzyme specificity. Incorporation of a β-lactam into Nu of **15** would be a nice final touch.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic details for the preparation of compounds **8** and **9** and Figures S1−S5 illustrating the products and kinetics of spontaneous reactions of **5** and **7**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

BSA, bovine serum albumin; CENTA, 7β-[(thien-2-yl)-acetamido]-3-[(4-nitro-3-carboxyphenylthio)methyl]-3-cephem-4-carboxylic acid; DCM, dichloromethane; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; ESMS, electrospray mass spectrometry; FTIR, Fourier transform infrared; MOPS, 3-(*N*-morpholino)propanesulfonic acid; mp, melting point; NMR, nuclear magnetic resonance; TEA, triethylamine; TFA, trifluoroacetic acid.

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