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Serendipitous Discovery of α-Hydroxyalkyl Esters as β-Lactamase Substrates†

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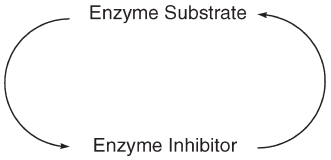
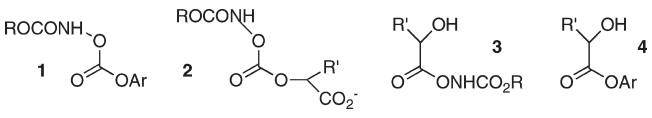
ABSTRACT: *O*-(1-Carboxy-1-alkyloxycarbonyl) hydroxamates were found to spontaneously decarboxylate in aqueous neutral buffer to form *O*-(2-hydroxyalkylcarbonyl) hydroxamates. While the former molecules do not react rapidly with serine β-lactamases, the latter are quite good substrates of representative class A and C, but not D, enzymes, and particularly of a class C enzyme. The enzymes catalyze hydrolysis of these compounds to a mixture of the α-hydroxy acid and hydroxamate. Analogous compounds containing aryloxy leaving groups rather that hydroxamates are also substrates. Structure−activity experiments showed that the α-hydroxyl group was required for any substantial substrate activity. Although both D- and L-α-hydroxy acid derivatives were substrates, the former were preferred. The response of the class C activity to pH and to alternative nucleophiles (methanol and D-phenylalanine) suggested that the same active site functional groups participated in catalysis as for classical substrates. Molecular modeling was employed to explore how the α-hydroxy group might interact with the class C β-lactamase active site. Incorporation of the α-hydroxyalkyl moiety into novel inhibitors will be of considerable interest.

The unexpected discovery of a new class of substrates for an enzyme opens up a period of recollection and reflection. How does the newly discovered structural motif facilitate catalysis; i.e., how does it interact with the enzyme active site? Does the enzyme catalyze the reaction of the new substrate in the same way as that of classical substrates, and how might it be incorporated into new inhibitors? These questions arise with particular immediacy for enzymes with medical implications such as the β-lactamases, which continue to represent a serious barrier to future clinical application of the β-lactam antibiotics (*1*). The discovery of acyclic depsipeptide substrates of the β-lactamases (*2*), for example, led directly to the development of phosphonate inhibitors (*3*).



Recently, we described a new class of β-lactamase inhibitors, the *O*-aryloxycarbonyl hydroxamates, **1**. These molecules were found to be effective against all serine β-lactamases, although particularly so against representative class C enzymes (*4*, *5*).



As an extension of this structural class, we prepared the analogues **2**, which also incorporate the carboxylate moiety that is found in good β-lactamase substrates and which interacts with specific active site residues (*6*−*8*). As we found and describe in this paper, compounds of structure **2** rearrange spontaneously in solution more rapidly than they inhibit β-lactamases but on doing so form α-hydroxyalkyl esters **3** that are substrates of β-lactamases. Extension of **3** to **4** also yielded β-lactamase substrates. The ability of α-hydroxyalkyl esters to react with serine β-lactamases has not been reported previously, to the best of our knowledge. In this paper, we describe an initial survey of the reactivity of **3** and **4** with serine β-lactamases.

MATERIALS AND METHODS

Synthetic reagents were, in general, purchased from Sigma-Aldrich. *tert*-Butyl D-lactate was purchased from Fluka, anhydrous D-lactic acid from Bachem, Boc-D-phenylalanine 4-nitrophenyl ester from Chem-Impex, (*R*)-(−)-2-hydroxy-4-phenylbutyric acid from AnaSpec Inc., D-4-biphenylalanine from PepTech Corp., *N*-methylhydroxylamine hydrochloride from Acros, and [^{15}N]hydroxylamine hydrochloride from Isotec. Phosgene was purchased as a 20% solution in toluene from Sigma-Aldrich.

The class C P99 β-lactamase from *Enterobacter cloacae* and the class A TEM-2 β-lactamase from *Escherichia coli* W3310 were purchased from the Centre for Applied Microbiology and Research (Porton Down, Wiltshire, U.K.). The class D OXA-1 β-lactamase was generously provided by M. Nukaga (Jyosai International University, Chiba, Japan) and the class C ampC enzyme by B. Shoichet (University of California, San Francisco, CA). The *Streptomyces* R61 and *Actinomadura* R39 DD-peptidases were generous gifts from J.-M. Frère and P. Charlier (University of Liège, Liège, Belgium).

A Varian Gemini-300 MHz NMR[1] spectrometer was used to collect ^{1}H NMR spectra, and a Perkin-Elmer 1600 FTIR instrument was used to obtain IR spectra. Elemental analyses were

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[1]Abbreviations: AMPSO, 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid; BSA, bovine serum albumin; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; Centa, 7β-[(thien-2-yl)acetamido]-3-[(4-nitro-3-carboxyphenylthio)methyl]-3-cephem-4-carboxylic acid; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DMSO, dimethyl sulfoxide; ESMS, electrospray mass spectrometry; IR, infrared; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NMR, nuclear magnetic resonance; PBP, penicillin-binding protein; TAPS, *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid; TBDMS, *tert*-butyldimethylsilyl; TFA, trifluoroacetic acid.

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conducted by the Desert Analytics Laboratory. Routine ESI mass spectra were recorded using a Thermo LCQ Advantage instrument.

Syntheses. (*i*) *N*-(*Benzyloxycarbonyl*)-*O*-(*1-D-carboxyethoxycarbonyl*)*hydroxylamine* (**2**, $R = CH_2Ph$, $R' = Me$). (*a*) *1-D-(tert-Butoxycarbonyl)ethyl Chloroformate*. Phosgene as a 20% solution in toluene (7 mL, 14 mmol) was stirred under nitrogen at 0 °C, and *tert*-butyl D-lactate (0.98 g, 6.6 mmol) in methylene chloride (5 mL) was added dropwise, followed by DMAP (0.81 g, 6.6 mmol), dissolved in methylene chloride (5 mL). The mixture was stirred for 20 min and the precipitate of DMAP hydrochloride removed by filtration. The resulting chloroformate solution was used directly in the following acylation step.

(b) *N*-(Benzyloxycarbonyl)-*O*-(1-D-carboxyethoxycarbonyl)hydroxylamine. Benzyl *N*-hydroxycarbamate (0.43 g, 2.5 mmol) in methylene chloride (4 mL) was stirred at 0 °C under an atmosphere of dry nitrogen. Anhydrous pyridine (0.21 mL, 2.5 mmol) was added and the mixture stirred for 10 min. This was followed by dropwise addition of the chloroformate (0.43 mg, 2.8 mmol). The ensuing mixture was stirred for a further 25 min and filtered and the solvent evaporated from the filtrate. The crude product was collected as a colorless oil (0.48 g) that was purified on silica gel with a hexane/ethyl acetate mixture (2:1) as the eluent. The colorless solid product was recrystallized from a benzene/cyclohexane mixture to yield 0.28 g (33%) of colorless needlelike crystals (mp 79 °C): ^{1}H NMR ($CDCl_3$) δ 8.38 (s, 1H), 7.34 (s, 5H), 5.19 (s, 2H), 4.90 (q, 1H), 1.50 (d, 3H), 1.43 (s, 9H); IR (KBr) 1792, 1759, 1702 cm^{-1}. Anal. Calcd for $C_{16}H_{21}NO_7$: C, 56.63; H, 6.24; N, 4.13. Found: C, 56.42; H, 6.23; N, 4.04.

(c) Deprotection. The *tert*-butyl-protected derivative described above (265 mg, 0.75 mmol) was dissolved in trifluoroacetic acid (10 mL) and the mixture stirred for 30 min. The solvent was evaporated and the free acid obtained quantitatively as a colorless oil, which proved to be recalcitrant to crystallization attempts. The oil was used directly in kinetic and analytical assays. ^{1}H NMR (CD_3CN) δ 9.15 (s, 1H), 7.41 (s, 5H), 5.21 (s, 2H), 5.06 (q, 1H, J = 5.7 Hz), 1.54 (d, 3H, J = 6.3 Hz); IR (neat) 1790, 1736, 1721 cm^{-1}; ES(−)MS m/z 282.3 ($M - H^+$).

The ^{15}N isotopomer of this compound was prepared as described above but using benzyl [^{15}N]-*N*-hydroxycarbamate (*4*) as the starting material.

(*ii*) *N*-(*Benzyloxycarbonyl*)-*O*-(*1-D-carboxy-2-phenylethoxycarbonyl*)*hydroxylamine* (**2**, $R = R' = CH_2Ph$). (*a*) *tert-Butyl 2-D-Hydroxy-3-phenylpropanoate*. *tert*-Butyl 2-D-hydroxy-3-phenylpropanoate was prepared from D-3-phenyllactic acid following the procedure of Yang et al. (*9*). Thus, acetyl chloride (2.47 mL) was added dropwise to solid D-3-phenyllactic acid (500 mg, 3 mmol) at 0 °C. The mixture was brought to reflux at 60 °C and stirred for 4 h. The reaction mixture was then dried by rotary evaporation, leaving a pale yellow oil that was taken into diethyl ether (15 mL) and washed with water (2 × 15 mL). The ether layer was dried over Na_2SO_4 and the solvent evaporated. The resulting oil was dissolved in methylene chloride (7.5 mL) and stirred at 0 °C. *tert*-Butyl alcohol (0.45 g) and DMAP (0.122 g, 1 mmol) were added, followed by DCC (0.83 g, 4 mmol) dissolved in methylene chloride (2.5 mL). The reaction mixture was allowed to warm to room temperature as it was stirred for 12 h. The mixture was filtered and the filter cake washed with methylene chloride. The combined solutions were washed with water (2 × 10 mL), and the organic layer was dried over $MgSO_4$. The solvent was evaporated under reduced pressure, yielding 636 mg of a pale yellow oil. The oil was dissolved in a mixture of methanol (3.5 mL) and water (5 mL), followed by addition of potassium carbonate (1.24 g, 9 mmol), and the mixture stirred vigorously for 12 h at room temperature. The methanol was removed by rotary evaporation and the remaining aqueous layer extracted with methylene chloride (2 × 10 mL). The combined washings were dried over $MgSO_4$, and the solvent was evaporated to yield 398 mg (58% overall) of a pale yellow oil.

1-D-(*tert*-Butoxycarbonyl)-2-phenylethyl chloroformate was then prepared by the method described above for 1-D-(*tert*-butoxycarbonyl) ethyl chloroformate, yielding a colorless oil (65% yield): IR (neat) 1781, 1744 cm^{-1}.

(b) *N*-(Benzyloxycarbonyl)-*O*-[1-D-(*tert*-butoxycarbonyl)-2-phenylethoxycarbonyl]hydroxylamine. The procedure for **2** ($R = CH_2Ph$, $R' = Me$), described above, was also followed for this synthesis. The crude oil obtained from the reaction of benzyl *N*-hydroxycarbamate with 1-D-(*tert*-butoxycarbonyl)-2-phenylethyl chloroformate was purified by chromatography on silica gel preparative plates with a hexane/ethyl acetate mixture (2:1) as the eluent, yielding 152 mg (33%) of a colorless oil: ^{1}H NMR ($CDCl_3$) δ 7.86 (s, 1H), 7.35 (m, 5H), 7.26 (m, 5H), 5.21 (s, 2H), 5.07 (dd, 1H, J = 5.1 Hz), 3.18 (t, 2H, J = 5.1 Hz), 1.40 (s, 9H); IR (neat) 1794, 1732 cm^{-1} (broad, two peaks); ES(+)MS m/z 438.0 ($M + Na^+$).

(c) Deprotection. The *tert*-butyl-protected compound (20 mg, 0.05 mmol) was dissolved in trifluoroacetic acid (4 mL) and the mixture stirred for 30 min under a flow of nitrogen. The trifluoroacetic acid was then evaporated, quantitatively yielding the free acid as an oil: ^{1}H NMR (CD_3CN) δ 9.10 (s, 1H), 7.35 (m, 5H), 7.26 (m, 5H), 5.20 (m, 1H), 5.17 (s, 2H), 3.22 (m, 2H); IR (neat) 1792, 1732, 1665 cm^{-1}; ES(−)MS m/z 358.07 ($M - H^+$).

(*iii*) α-*Hydroxy Esters*. Enantiomerically pure α-hydroxy esters (**6**−**20**) were synthesized from their corresponding lactate derivatives by a common method, as reported below for **7**, except where otherwise noted. The hydroxyl and carboxyl groups of lactic acid derivatives were first protected as *tert*-butyldimethylsilyl (TBDMS) ethers and esters, respectively. The protected acids were converted to the corresponding acid chlorides as reported by Weinger (*10*). Coupling of the acid chlorides to either a *N*-hydroxycarbamate or alcohol and phenol was accomplished with pyridine as the base. The silyl ethers were then deprotected with $KHSO_4$ in aqueous methanol (*11*). The products were generally initially purified on silica gel with a hexane/ethyl acetate mixture (3:1) as the eluent and finally recrystallized from a benzene/cyclohexane mixture (1:1).

(*iv*) *N*-(*Benzyloxycarbonyl*)-*O*-(*2-hydroxy-3-phenylpropanoyl*)*hydroxylamine* (*7*) (*and* ***8***). (*a*) *2-D-TBDMSO-3-phenylpropionyl Chloride*. A solution of D-3-phenyllactic acid (500 mg, 3 mmol) in DMF (7.5 mL) was stirred at room temperature. TBDMS chloride (0.9 g, 6 mmol) and imidazole (0.61 g, 8.9 mmol) were added, and the reaction mixture was stirred overnight. The reaction mixture was then diluted with hexanes and washed sequentially with water (25 mL), saturated $NaHCO_3$ (25 mL), and brine (25 mL). The organic layer was then dried over $MgSO_4$ and the solvent removed under reduced pressure. The protected product was taken into dry methylene chloride (4 mL) containing one drop of catalytic DMF and stirred at 0 °C. Oxalyl chloride (1.2 mL, 14 mmol) was added dropwise and the mixture stirred for 1 h at 0 °C, followed by 1 h at room temperature. The solvent was removed by rotary evaporation and the acid chloride used directly for acylation.

(b) Acylation Reaction. The protected acid chloride (181 mg, 0.61 mmol) in methylene chloride (1.5 mL) was added dropwise to a stirred solution of benzyl *N*-hydroxycarbamate (95 mg, 0.61 mmol) and pyridine (49 μL, 0.61 mmol), dissolved in a mixture of ethyl acetate (6 mL) and methylene chloride (3 mL) at 0 °C, under dry nitrogen. The reaction mixture was then stirred for a further 1 h at 0 °C, the mixture filtered, and the solvent removed under vacuum. The resulting oil was fractionated on silica gel with a hexane/ethyl acetate mixture (3:1) as the eluent, yielding 160 mg (62%) of a clear oil. This silyl ether (94 mg) was then dissolved in a mixture of methanol (10 mL) and water (3 mL) containing 15 mg of potassium bisulfate. The mixture was stirred for 5 days and filtered, and the methanol was removed by rotary evaporation. The aqueous mixture was extracted with ethyl acetate and the organic portion dried over $MgSO_4$. The resulting product was then purified further on silica gel with a hexane/ethyl acetate mixture (3:1) as the eluent, yielding a colorless solid that was recrystallized from a benzene/cyclohexane mixture (1:1). Thus, 33 mg (48%) of **7** as colorless crystals (mp 110−112 °C) was obtained: ^{1}H NMR (CD_3CN) δ 8.97 (s, 1H), 7.39 (m, 5H), 7.30 (m, 5H), 5.19 (s, 1H), 4.52 (dd, 1H), 3.01 (m, 2H); ^{13}C NMR δ 173.97, 157.80, 138.26, 137.26, 129 (m), 71.88, 71.45, 69.02; IR (KBr) 1804, 1721 cm^{-1}; ES(−)MS *m*/*z* 314.33 ($M - H^+$). Anal. Calcd for $C_{17}H_{17}NO_5$: C, 64.75; H, 5.44; N, 4.44. Found: C, 64.33; H, 5.44; N, 4.43.

Analytical and Kinetic Methods. Absorption spectra and spectrophotometric reaction rates were measured with a Hewlett-Packard 8453 UV−vis spectrophotometer. Steady state kinetics were assessed at 25 °C, buffered in 20 mM 3-morpholinopropanesulfonic acid (MOPS) at pH 7.5, unless otherwise noted. The substrates were prepared in concentrated acetonitrile stock solutions and diluted to ≤5% acetonitrile in assays. Acetonitrile alone at these concentrations had no effect on initial rate measurements.

(*i*) *^{15}N gHSQC Spectroscopy*. Two-dimensional (2D) ^{15}N gHSQC spectra of [^{15}N]**2** (R = $PhCH_2$, R′ = Me) (10 mM) and its reaction products in d_6-DMSO were recorded at 25 °C on a Varian Unityplus 500 spectrometer. The data acquisition parameters were as follows: 10 kHz spectral width, 2 kHz 2D spectral width, 1 s acquisition time, 1 s delay time, 2 × 64 increments, and 8 repetitions. Chemical shifts were referenced to formamide (108.5 ppm) as an internal standard (*12*).

(*ii*) *Spontaneous Hydrolysis*. The spontaneous hydrolysis rates of the compounds were measured spectrophotometrically in triplicate at concentrations of 50−500 μM at appropriate wavelengths as reported in Table S1 of the Supporting Information. The full progress curves were fit to a pseudo-first-order rate equation by means of a nonlinear least-squares program and the triplicate rate constants averaged.

(*iii*) *pK_a Determination*. The pK_a of ester **7** (100 μM) was determined spectrophotometrically by monitoring the change in absorbance of the molecule at 233 nm as a function of pH. The pH was varied from 4.0 to 10.0 in increments of 0.5 at 25 °C. A mixed buffer system containing sodium acetate, MES, MOPS, AMPSO, TAPS, and CAPS (20 mM each) was employed. A constant ionic strength of 1.0 was maintained with sodium chloride.

(*iv*) *Isolation of the Reaction Intermediate from Spontaneous Hydrolysis of N-(Benzyloxycarbonyl)-O-(1-D-carboxyethoxycarbonyl)hydroxylamine*. *N*-(Benzyloxycarbonyl)-*O*-(1-D-*tert*-butylcarboxyethoxycarbonyl)hydroxylamine (30 mg, 88 μmol) was freshly deprotected when a solution of it in trifluoroacetic acid (4 mL) was stirred for 30 min. The trifluoroacetic acid was removed by rotary evaporation, yielding a clear colorless oil that was further dried by means of an oil pump. The resulting free acid was taken into CD_3CN (1 mL) and then diluted into D_2O (10 mL) containing 20 mM MOPS buffer adjusted to an apparent pH of 7.1 (pD 7.5). The subsequent spontaneous reaction was monitored by proton NMR, to determine the time of maximum accumulation of the intermediate. The methylene resonance was conveniently monitored for this purpose. At this time, the sample was extracted with methylene chloride (2 × 4 mL), the combined extracts were washed with cold distilled water (3 × 3 mL), and the organic fraction was dried over Na_2SO_4. The solvent was then removed by rotary evaporation, yielding 5 mg of a colorless semisolid: ^{1}H NMR ($CDCl_3$) δ 8.08 (s, 1H, NH), 7.35 (s, 5H), 5.22 (s, 2H), 4.49 (q, 1H, J = 7.1 Hz), 1.50 (d, 3H, J = 7.1 Hz); IR 1735, 1798 cm^{-1}; ES(−)MS *m*/*z* 238.1 ($M - H^+$).

(*v*) *P99 β-Lactamase Steady State Kinetics*. The enzyme (final concentration of 0.1−2.0 μM) was added to a buffered solution of the substrate (0.01−2.5 mM) and the subsequent hydrolysis monitored spectrophotometrically at an appropriate wavelength (Table S1 of the Supporting Information). Initial rates were measured and corrected for spontaneous hydrolysis by subtraction, where necessary, and the data were fit by means of a nonlinear least-squares program to the Michaelis−Menten equation to determine k_{cat} and K_m. Alternatively, where saturation was not achieved, the data were fit linearly to determine k_{cat}/K_m.

(*vi*) *K_m Values by Competition*. The P99 β-lactamase (with the stock solution containing 1 mg/mL BSA) was diluted into a buffered solution (final concentration of 1 nM) containing the substrate cephalothin (50 μM; K_m = 15 μM) and an α-hydroxy ester (0−1 mM). The hydrolysis of cephalothin was monitored at 278 nm ($\Delta\varepsilon$ = 4000 M^{-1} cm^{-1}), and the initial rates were determined. The initial rates were plotted as a function of the α-hydroxy ester concentration and the data fit to the steady state equation for competitive inhibition (eq 1) by means of a nonlinear least-squares program. The K_i value obtained should correspond to the K_m value of the α-hydroxy ester as a substrate.

$$v_0 = V_{max}S_0/[S_0 + K_m(1 + I/K_i)] \quad (1)$$

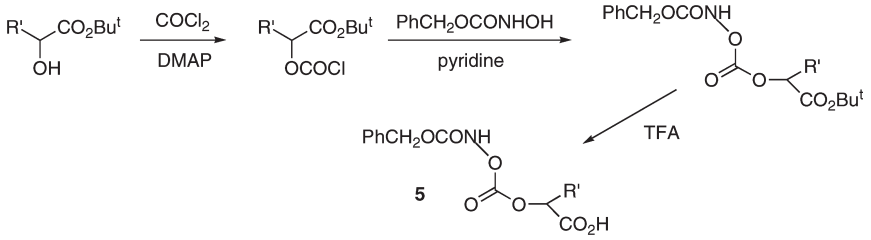
(*vii*) *Methanolysis of the P99 β-Lactamase Acyl Enzyme*. The effect of methanol on the initial rates of solvolysis of substrate **7** (700 μM; K_m = 71 μM) by the P99 β-lactamase (0.1 μM) was determined spectrophotometrically (233 nm), under close to saturating substrate conditions. Methanol concentrations of up to 2.5 M in aqueous MOPS buffer (20 mM) were employed; this concentration of methanol has been shown to have a negligible effect on enzyme activity (*13*). The effect of methanol (0−2.5 M) on the initial rates of solvolysis of **14** (800 μM; $K_m \sim 700$ μM) in the presence of the P99 β-lactamase (0.5 μM) was similarly determined spectrophotometrically at 230 nm. The initial rates of the enzyme-catalyzed solvolysis of **7** were plotted as a function of methanol concentration and fit to eq 2 [derived from Scheme 4, Results and Discussion (*14*)] by means of a nonlinear least-squares program.

$$v/v_0 = \frac{\alpha\beta(1 + S_0/K_m)}{\alpha\beta + [H_2O]_0(k_2/k_3 + \beta)(S_0/K_m)} \quad (2)$$

where $\alpha = k_2/k_3 + [H_2O]_0$ and $\beta = [H_2O] + (k_4/k_3)[MeOH]$.

(*viii*) *Aminolysis of the P99 β-Lactamase Acyl Enzyme*. To investigate the aminolysis of the acyl enzyme derived from

Scheme 1



compound **20**, two complementary methods were employed. First, the turnover of **20** (1 mM, ~5K_m) by the P99 β-lactamase (0.1 μM), in the presence of D-phenylalanine (0−40 mM), was monitored by following the release of *m*-hydroxybenzoate at 290 nm. Second, at a constant concentration of D-phenylalanine (20 mM), the aminolysis of the substrate (0−3 mM) by the β-lactamase (0.1 μM) was monitored (290 nm). Initial rates were measured in each case, and the two data sets were fit simultaneously to Scheme 4 by means of Dynafit (*15*).

(*ix*) *pH−Rate Profiles*. pH−rate profiles were obtained in a mixed buffer system of sodium acetate, MES, MOPS, AMPSO, TAPS, and CAPS (20 mM each) with a constant ionic strength of 1.0 maintained with NaCl. Experiments were conducted at 25 °C. Compound **7** was hydrolyzed over a pH range of 6.0−9.5, and k_{cat}/K_m was determined by initial rates as described above, with the spontaneous hydrolysis taken into account. For pH 6.0−8.0 and 8.5−9.5, hydrolysis was monitored at 233 and 250 nm, respectively, because of pH-dependent changes in the extinction coefficients. Because the solubility of **7** diminished at pH values below its pK_a (7.6), and the change in the extinction coefficient also decreased, rate measurements were not possible below pH 6. Values of k_{cat}/K_m in the obtained range, however, were plotted against the pH to obtain a curve, which was fit to eq 3 by means of a nonlinear least-squares program. The more soluble compound **20** was employed to obtain a full pH profile (pH range of 4.0−9.5) in the same manner.

$$k_{obs} = k_1^{max} K_{a1} h/(K_{a1} h + K_{a1} K_{a2} + h^2) \quad (3)$$

(*x*) *Kinetics with Other Enzymes*. Experiments for determining steady state parameters for turnover of the α-hydroxy esters (50−1000 μM) by the TEM-2 (0.01- 2.0 μM) and OXA-1 β-lactamases were performed as described above for the P99 enzyme. In the case of the OXA-1 enzyme, the buffer consisted of 20 mM MOPS, 50 mM $NaHCO_3$ and 0.1% gelatin, maintained at pH 7.5. Hydrolyses of compounds **7** (≤1 mM) and **20** (≤1 mM) in the presence of this enzyme (0.5 and 1.0 μM, respectively) were monitored as described above. Competitive inhibition experiments were performed as described above with compound **20** (3 mM) and the OXA-1 enzyme (0.1 μM) using the substrates benzylpenicillin (100 μM, monitored at 230 nm) and Centa (10 μM, monitored at 410 nm) by the techniques described above. Compound **15** (5 μM) was also tested for competitive inhibition against the substrate cephalothin (50 μM) with the OXA-1 enzyme (0.2 μM).

Hydrolysis of compounds **7** (500 μM), **8** (500 μM), and **20** (3.0 mM), in the presence of the *Streptomyces* R61 DD-peptidase (0.5 μM), was monitored spectrophotometrically as described above. Hydrolysis of **7** (500 μM), **8** (500 μM), and **20** (1.0 mM) was also studied in the presence of the *Actinomadura* R39 DD-peptidase (0.4, 0.4, and 1.0 μM, respectively). Competitive inhibition experiments were performed with **20** (1.0 mM), monitoring the turnover of *m*-carboxyphenyl *N*-phenylacetyl-D, L-alaninate (3 mM) by the R39 enzyme (0.1 μM) at 305 nm.

(*xi*) *Molecular Modeling*. Simulations were performed on a SGI workstation running InsightII (Accelrys). The crystal structure of a phosphonate-bound P99 β-lactamase (Protein Data Bank entry 1BLS) (*16*), after removal of the phosphonate ligand, was the starting point for building the tetrahedral intermediate of acylation (**21**/**22**). In this construct, Tyr 150 was neutral and both Lys 67 and Lys 315 were cationic. Partial charges on the atoms of the substrate, as the anionic intermediate, were calculated using a model of the adduct, which included the nucleophilic Ser 64 but not the rest of the protein. The model was subjected to a 1000-step steepest gradient energy minimization prior to the use of MNDO level calculations from the MOPAC module of InsightII, to calculate the partial charges.

Both *R* and *S* isomers of the chiral reaction center were constructed. Hydrogens were added to the Protein Data Bank structure, and the pH of the enzyme complex was set to 7.5. The partial charges of the protein residues were assigned with InsightII. The total charge on each complex was −1.0. The active site was hydrated with a 15 Å sphere of water centered on O_γ of the nucleophilic Ser 64. The models were energy-minimized with 1000 steepest gradient steps followed by molecular dynamics of 10000 equilibration steps and 90000 production steps. Conformations that were found to contain specific hydrogen bonding interactions with the α-hydroxyl group of the substrate were then energy minimized by 1000 steepest descent steps, followed by 2000 conjugate gradient steps. Relative quantitative evaluation of the models was achieved by means of calculations of ligand interaction energies, E_{int} (*17*).

RESULTS AND DISCUSSION

The synthesis of compounds of general structure **2**, as their *tert*-butyl esters, was achieved largely in the manner successfully employed for **1**, from the reaction of a hydroxamic acid with a chloroformate in the presence of a tertiary amine base (Scheme 1) (*5*). The *tert*-butyl ester carboxyl protecting group was removed by trifluoroacetic acid treatment immediately before use because it was found that the free acids were unstable, to decarboxylation as subsequently demonstrated (see below). That compound **5** (R′ = Me) was indeed an *O*-acyl hydroxamate, rather than *N*-acyl, was demonstrated by ^{15}N gHSQC spectroscopy, in which direct coupling between a ^{15}N nucleus resonating at 158.8 ppm and a proton at 11.8 ppm was observed.

Incubation of **2** (R = CH_2Ph, R′ = Me) with the P99 β-lactamase led to the data presented in Figure 1 which shows an initial loss of enzyme activity, measured against the good substrate, cephalothin, followed by a slower restoration of activity. This result is very different from that obtained with the aryl derivatives, **1**, in which the enzyme became irreversibly inactivated (*4*, *5*); the mechanism of the reaction of **2** with the enzyme,

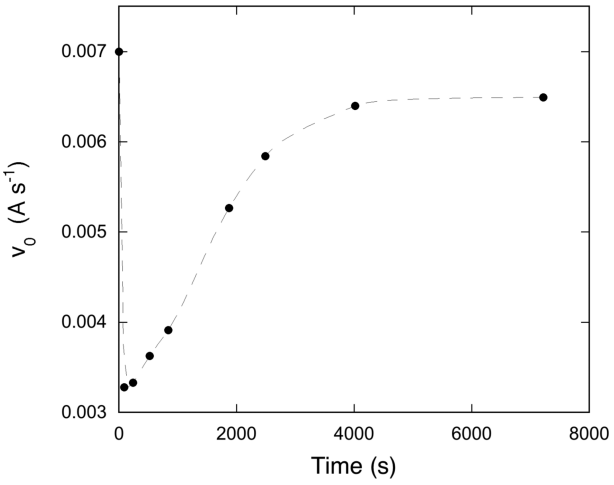


FIGURE 1: Activity of the P99 β-lactamase (0.25 μM) as a function of time after being mixed with **2** (R = $PhCH_2$, R′ = Me) (100 μM).

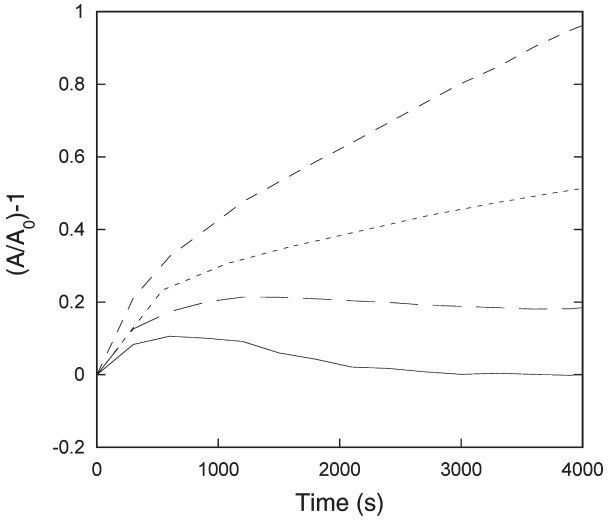


FIGURE 2: Absorbance changes at 250 nm upon reaction of **2** (R = $PhCH_2$, R′ = Me) (0.4 mM) in the absence (– – –) or presence of the P99 β-lactamase [(---) 0.25, (———) 0.5, and (—) 1.25 μM].

which exhibits a transiently stable intermediate, must therefore be different from that of **1**, despite the common *O*-acyl hydroxamate structure. Thus, we obtained an immediate indication of new active site chemistry and therefore a new class of substrates and/or inhibitors.

Direct spectrophotometric observation of the reaction between the P99 enzyme and **2** (R = CH_2Ph, R′ = Me) gave data such as those depicted in Figure 2. In the absence of enzyme, a burst phase is observed followed by a slower reaction. In the presence of enzyme, a second, enzyme-catalyzed, phase of the reaction is inserted between the two phases described in the previous sentence. The first phase, observed in both the absence and presence of enzyme, is not enzyme-catalyzed. The nature of the slower spontaneous reaction is unknown at present. It appeared to require the hydroxamate leaving group and represents a process with a large extinction coefficient change at 250 nm, but one that does not produce an amount of product that can be detected by ^{1}H NMR. One interpretation of these data, and one that we pursued, was that **2** in aqueous buffer was not an effective enzyme substrate or inhibitor, at least over several minutes, but spontaneously rearranged to a molecule that is a β-lactamase substrate.

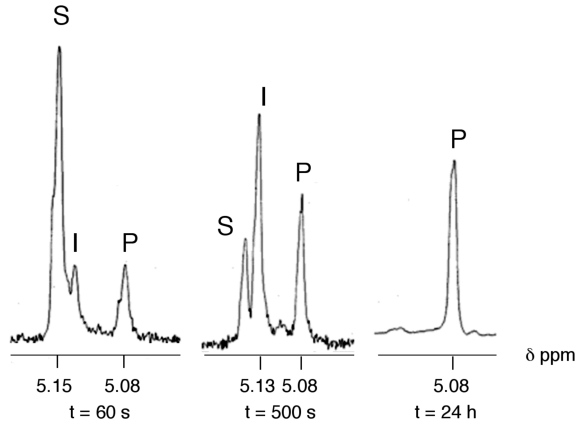


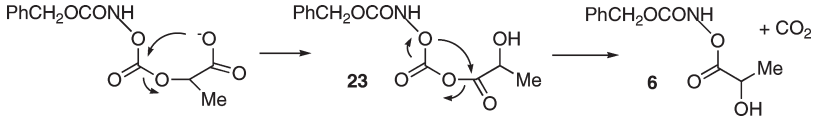
FIGURE 3: ^{1}H NMR spectral changes in the methylene region on spontaneous reaction of **2** (R = $PhCH_2$, R′ = Me) (S) at pH 7.5. Resonance peaks from intermediate I and final product P (benzyl *N*-hydroxycarbamate) appeared as the reaction proceeded. The signal corresponding to the former subsequently disappeared as that of the latter continued to rise.

A ^{1}H NMR experiment (Figure 3) showed that during the reaction of **2** (R = CH_2Ph, R′ = Me) in neutral aqueous buffer in the absence of an enzyme, there was an intermediate, I, between the starting material, S, and the final hydrolysis product(s), P. The final product spectrum showed the expected mixture of benzyl *N*-hydroxycarbamate and lactate. The intermediate was isolated by extraction from a reaction mixture, as described in Materials and Methods, and identified on the basis of its spectra (see Materials and Methods) as **6**, an α-hydroxycarboxylic acid anhydride with benzyl *N*-hydroxycarbamate. This identification was confirmed by independent synthesis (Materials and Methods). The rates of spontaneous and enzyme-catalyzed reactions of the isolated intermediate and the independently synthesized material (**6**) were identical. These kinetics were also in accord with the observations of Figure 1, which indicated that reaction of **2** (R = $PhCH_2$, R′ = Me) in the presence of the P99 β-lactamase involves a transiently stable acyl enzyme species, probably derived from reaction of the enzyme with **6**. Reaction of **2** (R = R′ = $PhCH_2$) was similarly shown to yield **7**.

Thus, in aqueous solution, **2** rearrange to α-hydroxyalkyl esters **3**. A likely rearrangement path from **2** (R = $PhCH_2$, R′ = Me) to **6** is shown in Scheme 2. We are unaware of a strong precedent for the first step, although the probably more thermodynamically favorable reverse reaction has been described previously (*18*). It is likely that **23** would be a very transient intermediate, and we did not observe it in the experiments described in this paper. Precedent for the second step is found in the rearrangement of mixed anhydrides of carboxylic and carbonic acids to esters with loss of carbon dioxide (*19*, *20*).

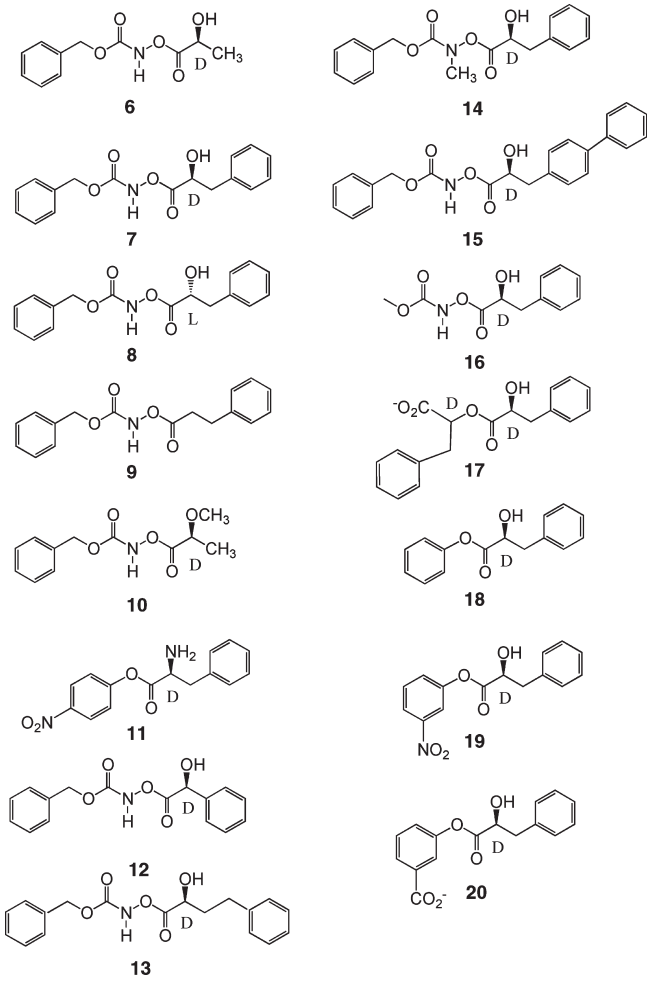
If interpreted correctly, the results described above suggested that α-hydroxycarboxylic acid esters are β-lactamase substrates. Because the α-hydroxyalkyl moiety has not previously been noticed to have β-lactamase affinity, we proceeded to investigate further. Synthesis of **3** and **4** (Scheme 3) was achieved by reaction of α-hydroxy acid chlorides, where the α-hydroxy group was protected by *tert*-butyldimethylsilylation, with either hydroxamic acids or alcohols and phenols in the presence of pyridine. Desilylation of the product was affected by its treatment with $KHSO_4$ in aqueous methanol. Thus, compounds **6**–**20** were prepared. They were purified by recrystallization if they were solids or by flash chromatography on a silica gel if not. As noted

Scheme 2



above, compounds **6** and **7** were identical to the intermediates isolated from the reaction of **2** (R = CH_2Ph, R′ = Me) and **2** (R = R′ = CH_2Ph), respectively. Compounds **6**−**20** were then examined for their reactivity with serine β-lactamases.

All of the compounds **6**−**20** are susceptible to spontaneous hydrolysis in a neutral aqueous solution. ^1H NMR studies showed that the hydrolysis products were the α-hydroxy acid and the leaving group hydroxyl compound. Rate constants for this process in 20 mM MOPS (pH 7.5) are listed in Table 1 and can be seen to range between 10^{-5} and 10^{-4} s^{-1} (excluding the very reactive amino acid ester **11**). For perspective on these rate constants, it might be noted that the spontaneous hydrolysis rate constants of benzylpenicillin and clavulanic acid under similar conditions are 1.5×10^{-5} (*21*) and 8.0×10^{-5} s^{-1} [the hydrolysis of clavulanic acid (85 μM) in 25 mM MOPS buffer (pH 7.5, 25 °C) was monitored spectrophotometrically at 260 nm; the resulting trace was fitted to a first-order reaction scheme], respectively.



As *O*-acyl hydroxamates, **6**−**10**, **12**, **13**, **15**, and **16** would be expected to have an acidic NH proton (*5*). Spectrophotometric titration of **7** indeed yielded a pK_a value of 7.58 ± 0.03, slightly higher than those of **1** [6.8−7.2 (*5*)]. Thus, at pH 7.5, roughly equal amounts of the neutral and anionic forms of **7**, and presumably of its analogues, **8**−**10**, **12**, **13**, **15**, and **16**, would be present in solution.

Most of the compounds in the group of **6**−**20** were found to be quite effective substrates of the P99 β-lactamase, not as effective as the best β-lactam substrates ($k_{cat}/K_m \geq 10^6$ s^{-1} M^{-1}), but comparable to previously described depsipeptides (*2*, *13*, *14*). Steady state rate parameters for the new compounds are listed in Table 1. It can be seen that the best of these substrates is **7** with a k_{cat}/K_m value of 1.2×10^5 s^{-1} M^{-1}. This compound is a considerably better substrate than **6**, presumably because of addition of the hydrophobic phenyl group. Further hydrophobic extension of the acyl moiety, as seen in **13**, did not lead to a rate constant larger than that of **7**. Although **15** was prepared, the details of its interaction with the P99 β-lactamase could not be obtained directly because of a combination of its low water solubility (≤50 μM) and the small absorption change accompanying hydrolysis. An experiment in which its inhibition (at 20 μM) of cephalothin turnover by the enzyme was assessed showed that its K_m value must be higher than that of **7**. Aggregation in solution might, of course, also be a problem with **15**. Comparison of the parameters for **16** and **7** suggests that some degree of hydrophobic bulk in the leaving group is also favorable for reaction with the enzyme.

The importance of the α-hydroxy group to the recognition and turnover of these compounds by the β-lactamase is illustrated by the results with compounds **9** and **10**. Compound **9**, a direct analogue of **7**, but lacking the hydroxyl group, was not detectably a substrate at enzyme concentrations of ≤2 μM. No time-dependent inactivation of the enzyme (0.25 μM) by **9** (200 μM) was detected, eliminating the possibility that a refractory acyl enzyme formed. An experiment for detecting inhibition of cephalothin turnover by **9** showed that its K_i value, and thus the K_m value of **9** as a substrate, must be ≥0.5 mM. Along the same lines, it was found that **10**, where the hydroxyl group of **6** is replaced with a methoxy group, is a much poorer substrate than **6**. Finally, compound **11**, in which the hydroxyl group of **7** is replaced with an amine (**11** would probably be mainly in the neutral form at pH 7.5) and a better leaving group is present [pK_a values of benzyl *N*-hydroxycarbamate and *p*-nitrophenol of 14.3 and 9.4, respectively, in a dioxane/water mixture (*22*)], is clearly a much poorer substrate than **7**. Only an upper limit to k_{cat}/K_m for **11** could be obtained because no enzyme-catalyzed reaction was observed over the rapid spontaneous hydrolysis. Taken together, these results attest to the benefits of the α-hydroxy group in determining the ability of these esters to be P99 β-lactamase substrates. The results described above also suggest that the hydroxyl group may act as a hydrogen bond donor in its productive complex with the enzyme.

Comparison of the data for **7** and **8** shows that the D-α-hydroxy enantiomer is preferred to the L, although the latter retains significant activity, suffering mainly in the K_m parameter. Previous investigations of class C β-lactamases with acyclic substrates have generally shown preferences for the L enantiomer in the acyl fragment adjacent to the scissile bond (*23*, *24*), for structural reasons that have been discussed (*24*). The preference for the D enantiomer observed in this work suggests that the acyl substituents of **3** may interact with the active site rather differently than those of, say, **24** (see below).

The electronic quality of the leaving group is also important. The aliphatic ester **17**, even when bearing a carboxylate and a

Scheme 3

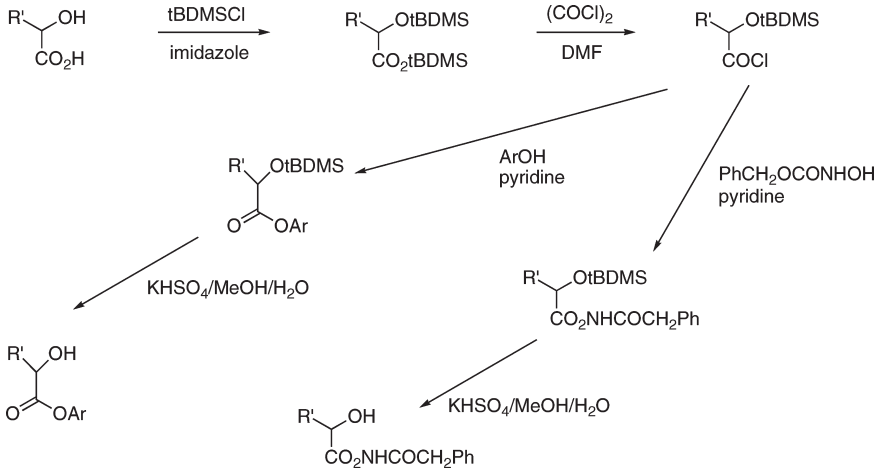


Table 1: Rate Parameters for Spontaneous Hydrolysis of α-Hydroxy Esters and Their Steady State Turnover by the P99 β-Lactamase

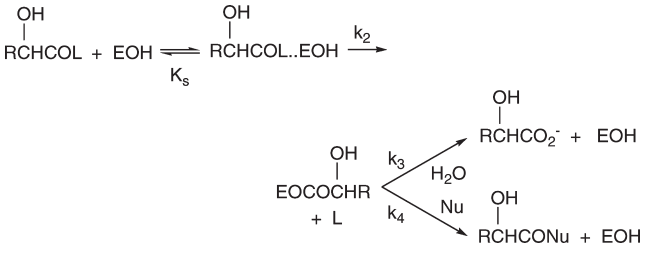
	k_{sp} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
6	$(8.6 \pm 1.9) \times 10^{-5}$	520 ± 140	1.5 ± 0.2	2.8×10^3
7	$(8.2 \pm 0.8) \times 10^{-5}$	71 ± 10	8.7 ± 0.3	1.2×10^5
8	ND[a]	340 ± 60	5.9 ± 0.3	1.74×10^4
9	$(6.5 \pm 0.2) \times 10^{-5}$	NR[b]		
10	$(1.64 \pm 0.02) \times 10^{-5}$	$\geq 10^3$	≥ 0.05	46 ± 1
11	$(6.8 \pm 1.7) \times 10^{-3}$			$\leq 3 \times 10^3$
12	$(6.0 \pm 0.7) \times 10^{-5}$	460 ± 240	3.5 ± 0.7	7.5×10^3
13	$(6.4 \pm 2.1) \times 10^{-5}$	20 ± 2	1.5 ± 0.1	5.5×10^4
14	$(9.0 \pm 0.6) \times 10^{-5}$	660 ± 340	21.0 ± 5.7	3.2×10^4
15	ND[a]	insoluble/NR[b]		
16	$(5.6 \pm 2.2) \times 10^{-5}$	480 ± 100	7.4 ± 0.7	1.5×10^4
17	$(2.6 \pm 0.1) \times 10^{-5}$	NR[b]		
18	$(2.0 \pm 0.2) \times 10^{-5}$	1010 ± 120	10.4 ± 0.7	1.0×10^4
19	$(9.0 \pm 0.5) \times 10^{-5}$	320 ± 60	10.9 ± 0.8	3.4×10^4
20	$(2.0 \pm 0.5) \times 10^{-5}$	172 ± 16	6.9 ± 0.2	3.9×10^4

[a]Not determined. [b]No reaction observed.

phenyl ring in the leaving group, which might have been expected to enhance specificity, was not observably a substrate. On the other hand, the aryl esters **18**−**20** were comparable in reactivity to the hydroxamates. The limited effect of nitro group substitution (**19** vs **18**) may reflect either a steric problem with the nitro group or the presence of significant electrophilic catalysis. The latter has been noted previously in acylation of the P99 β-lactamase active site (*14*, *25*) and, indeed, may be present with **1** (*5*). The result with compound **20** is distinctive. The addition of the *m*-carboxylate to **18** has an effect on k_{cat}/K_m much weaker than that found in acyclic substrates such as **24** (see below); in the latter, the *m*-carboxylate is placed to interact with the same active site functional groups as the carboxylate of β-lactams (*6*−*8*). It seems likely that the leaving group in acylation of the enzyme by **4** is placed differently compared to that in acylation by **24**.

If, as is likely, the hydrolysis of α-hydroxy esters is catalyzed by the P99 β-lactamase by a double-displacement mechanism involving an acyl enzyme intermediate (Scheme 4), the observation of quite similar k_{cat} values for **7**, **16**, and **18**−**20**, which have the same acyl group, suggests that deacylation may be rate-determining in turnover of these molecules under substrate saturation conditions. This would not be surprising because rate-determining deacylation is a common feature of catalysis by the P99 β-lactamase (*13*, *14*).

Scheme 4



Support for rate-determining deacylation in these cases was obtained from measurement of the kinetic effect of alternative nucleophiles. At substrate concentrations where a significant amount of the acyl enzyme intermediate would be expected to accumulate, i.e., $\geq K_m$, alternative nucleophiles, Nu, would directly accelerate turnover if enzyme deacylation were rate-determining. This effect has been observed with many substrates of the P99 β-lactamase, including β-lactams (*13*), in the presence of methanol, and acylic substrates in the presence of methanol and D-amino acids (*26*). In this case, turnover of **7** was linearly accelerated by addition of methanol (Figure S1 of the Supporting Information), from which data the partition ratio k_4/k_3 was calculated to be 22.6 ± 0.8. This value is interestingly similar to those for more classical substrates, 25 ± 10 (*8*, *14*, *24*, *27*). Similarly, D-phenylalanine (but not L-phenylalanine) accelerated turnover of **20** quite dramatically (Figure 4). From these data and with the assumption of Scheme 4, the following kinetic constants were obtained by curve fitting: $K_s = 2.85$ mM, $k_2 = 113$ s^{-1}, $k_3 = 7.3$ s^{-1}, and $k_4 = 1600$ s^{-1} M^{-1}. These data support the proposition that Scheme 4 describes the turnover of α-hydroxy esters by the P99 β-lactamase and suggests that the deacylation step may often be the slow one.

A pH−rate profile for hydrolysis of **20** by the P99 β-lactamase yielded the bell-shaped curve in Figure 5 and the associated pK_a values of 5.75 ± 0.20 and 9.37 ± 0.19. These values are comparable to those from classical substrates (*27*−*30*) and indicate that the same active site functional group assembly is probably responsible for catalysis. The profile for hydrolysis of **7**, which could be studied spectrophotometrically only at pH values above the pK_a because of the absence of a measurable spectral change at lower pH, yielded a pK_a for decreasing activity of 7.6 ± 0.2 (Figure 5). This correlates well with the pK_a obtained with **1** (*5*) and strongly suggests that the reactive form of **7** is the

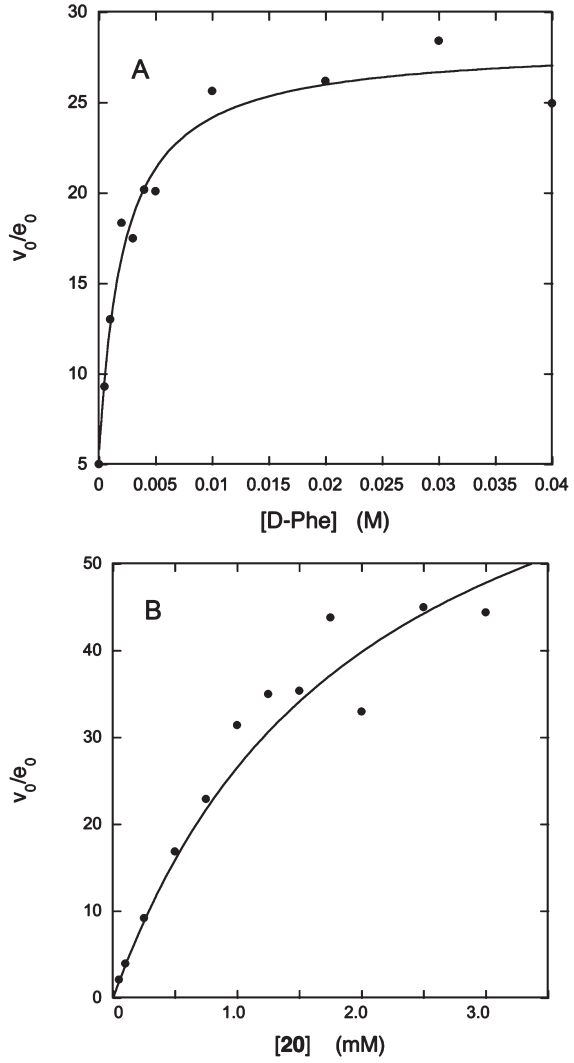


FIGURE 4: Initial rates of reaction of **20** in the presence of the P99 β-lactamase (0.1 μM) and D-phenylalanine. (A) Variation of the rate at a fixed concentration of **20** (1.0 mM) and varying D-phenylalanine concentrations. (B) Effect of varying the concentration of **20** at a fixed D-phenylalanine concentration (20 mM). The fitted lines were calculated as described in the text.

neutral ester rather than the nitrogen-based anion. In further support of this conclusion, the data in Table 1 indicate that **14**, the N-methylated derivative of **7**, is also an effective substrate. The apparently rather higher k_{cat} value of **14** versus what would be expected on the basis of the discussion given above (**14** has the same acyl group as **7** and **20**) may be an artifact; because of the low solubility of **14** ($\leq$0.8 mM), rate measurements at concentrations greater than K_m were not possible, and hence, the k_{cat} and K_m values should be seen as rather uncertain. Deacylation of the acyl enzyme derived from **14**, like that from **7**, is likely to be rate-determining at saturation because methanol acceleration of the reaction of the former was observed at a concentration close to K_m (data not shown).

Structural Considerations. Molecular modeling was used to explore possible interactions of the α-hydroxyl group with active site residues and thus, perhaps, rationalize the activity of these compounds as substrates. Tetrahedral intermediate **21** was constructed at the P99 β-lactamase active site. This represents the *R* configuration at the tetrahedral carbon generated by nucleophilic attack by the active site serine hydroxyl group at the substrate carbonyl. The *R* configuration has the α-hydroxyacyl moiety in

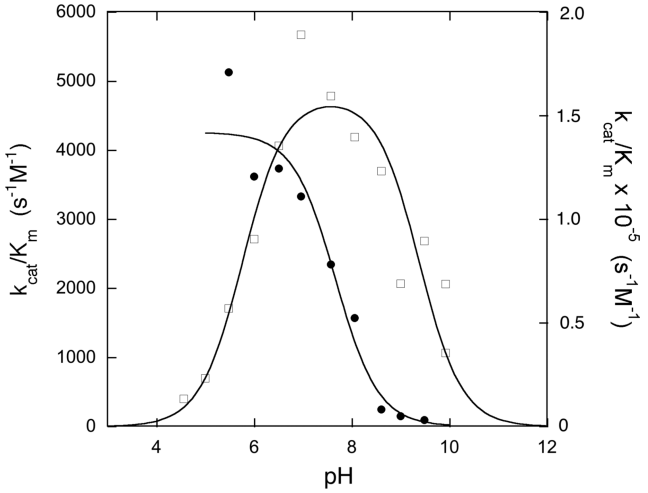
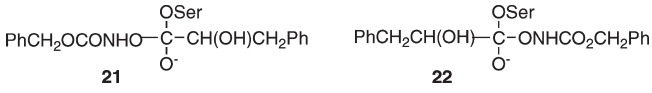


FIGURE 5: pH−rate profiles for hydrolysis of **7** (●) and **20** (□) in the presence of the P99 β-lactamase. The fitted lines were calculated as described in the text.

the normal acyl (side chain) site (*16*, *31*, *32*) and the hydroxamate in the leaving group site (*31*, *33*). A variety of starting conformations of the α-hydroxyacyl group were chosen and stable conformers sought by a combination of molecular dynamics and energy minimization computations. In all of the thereby derived structures, the usual positioning of active site residues was observed; viz., the Lys 67 and Tyr 150 side chain functional groups closely associated with either Ser 64 O_γ or the leaving group (hydroxamate or phenolic) oxygen, and placement of the oxyanion in its usual hole composed of the backbone NH groups of Ser 64 and Ser 318 (*31*, *34*). The reactivity of **3** and **4** would still, therefore, be controlled by the pK_a values of these residues.



The most favorable (by the criterion of ligand interaction energy values, E_{int}) conformations obtained showed the α-hydroxyl group hydrogen bonded as an acceptor to the Lys 67 terminal ammonium group (Figure 6A) or, more favorably, as a donor to the Ser 318 carbonyl oxygen (Figure 6B). In the former case, the phenyl group of the substrate was situated in such a way that it formed an amide NH−π complex (*35*, *36*) with the side chain of Asn 152 and, in the latter, hydrophobic contact with Tyr 221. Another apparently stable structure, similar to that depicted in Figure 6B but in which the α-hydroxyl group is hydrogen-bonded to Ser 318 O_γ, was also noted. This structure, however, seems unlikely to be generally important because **7** was also found to be a good substrate of the ampC β-lactamase, another class C enzyme, but in which Ser 318 is replaced with Ala. In none of these structures does there appear to be any particular very specific interaction with the hydroxamate leaving group except that of Tyr 150 with the oxygen more proximal to the reaction center (see Figure 6B, for example).

The available structure−activity data can then be assessed in terms of these structures. First, if the α-hydroxyl group is acting as a hydrogen bond donor, as suggested by the experimental data given above, the model in Figure 6B would be more likely. The limited effect of the *m*-carboxylate in **20**, when compared with **18** and **19**, for example, is, however, rather striking. The *m*-carboxylate in phenaceturate substrates such as **24** has been shown to

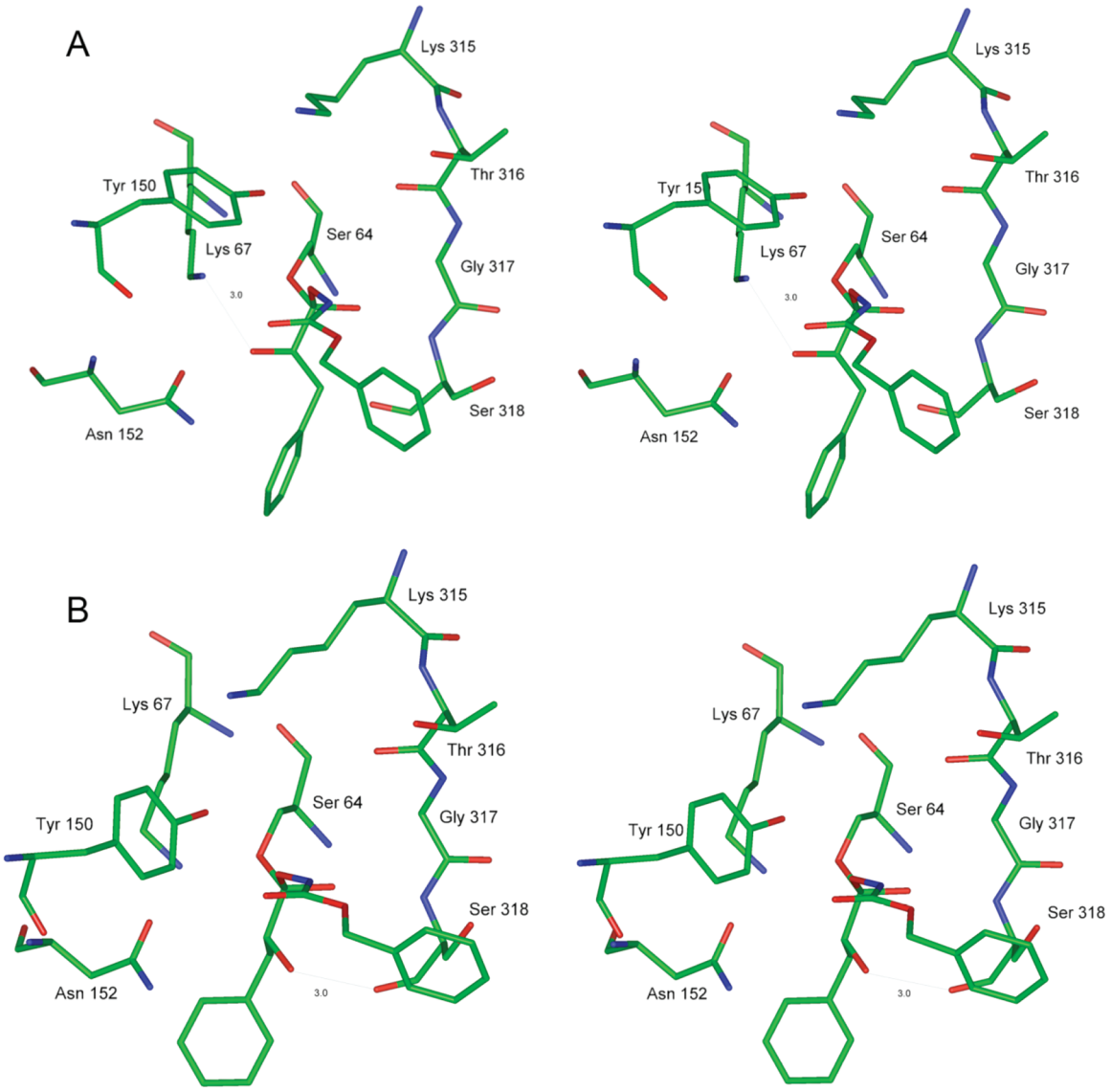
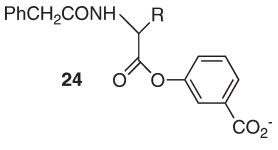
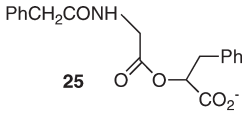


FIGURE 6: Stereoviews of energy-minimized tetrahedral intermediate structures formed upon reaction of **7** with the P99 β-lactamase. Only heavy atoms are shown. Panels A and B show alternative conformations of the *R* adduct (**21**).

enhance catalysis rather more markedly (*13*), probably by its interaction with hydrogen bond donors adjacent to the active site (*8*). Also, although the phenyllactate **25** is a substrate of the P99 β-lactamase (*13*), the α-hydroxyacyl analogue **17** is not. These observations rather suggest that the orientation of the leaving groups of the two series of substrates when bound to the enzyme may be different. One further possibility with respect to this issue is, of course, that the *S* tetrahedral intermediate (**22**) rather than the *R* intermediate (**21**) is formed, where the relative positions of the acyl group and leaving group are reversed. Models of the *S* configuration were constructed (e.g., Figure S1 of the Supporting Information), but none led to structures in which the α-hydroxyl group interacted directly with the enzyme.





A final point relating to structure was also noted above, the clear preference of the P99 β-lactamase for the D enantiomer **7** versus the L enantiomer **8**, which is rather unusual if the reaction is thought to take place through the positioning of **3** as for classical substrates (Figure 6A,B). Another abnormal result, relating to this last point, is the fact that **7**, **8**, and **20** (the other compounds were not tested) were not substrates of the *Streptomyces* R61 and *Actinomadura* R39 DD-peptidases. The R61 peptidase, in particular, has a protein fold and active site structure very similar to those of the P99 β-lactamase (*37*, *38*). Phenaceturates such as **24** (R = H) are substrates of this enzyme, as are α-substituted analogues such as **24** (R = Me or OMe), where there is an absolute stereochemical preference for one enantiomer at the α-position, thought to be D on the basis of the structure of natural substrates of this enzyme (*24*, *33*). This enzyme, unlike the β-lactamase, has a specific binding pocket for small D-α-substituents (*33*, *39*, *40*). The pocket is hydrophobic, however, reflecting a preference for the methyl group of its natural substrate, an acyl-D-alanyl-D-alanine peptide. The placement of a hydroxyl group in this site may well be unfavorable. The β-lactamase, which does not have such a pocket (*33*, *39*, *41*), is forced to react with D-α-substituted substrates, which it does, bound in a different conformation (*24*). In the case presented here, the polar D-substituent (OH) is most likely hydrogen

bonded to either Lys 67 (Figure 6A) or Ser 318 (Figure 6B) of the β-lactamase. The structures of Figure 6 can each accommodate an L-α-hydroxy substrate by redirection of the alkyl group.

Although the discussion given above seems to favor occupation of the active site by α-hydroxy esters as shown in Figure 6B, direct evidence of the orientation of these substrates during reaction with the enzyme will most likely come from the crystal structure of an acyl enzyme derived from **7** or from a poorer substrate or inhibitor containing the α-hydroxylalkyl moiety.

Other β-Lactamases. The class A TEM-2 β-lactamase did catalyze hydrolysis of the α-hydroxy esters **3** and **4**, although less efficiently than the class C enzymes. For example, k_{cat}/K_m values for **7**, also the best substrate of the group of **6**−**20**, and **20** were 2.4×10^3 and 460 s^{-1} M^{-1}, respectively; in both cases, K_m values were >1 mM. This result may just reflect the generally lower reactivity of this class A enzyme with acyclic substrates (*13*, *24*). The TEM-2 β-lactamase, as did the P99 enzyme, preferred the D enantiomer **7** to the L enantiomer **8**; the k_{cat}/K_m value for **8** was 540 s^{-1} M^{-1}.

A class D β-lactamase, OXA-1, did not catalyze hydrolysis of **7** and **20** at all. This enzyme is, however, an even poorer catalyst of the hydrolysis of acyclic substrates in general than class A (TEM) and C (P99) enzymes (*27*). It is possible that interaction of the α-hydroxy group with the carboxylated active site lysine, which is believed to be an essential general acid/base in substrate turnover (*42*, *43*), precludes reaction; no significant fast reversible or slow inhibition of the OXA-1 enzyme by **7** or by the more hydrophobic **15** was, however, detected.

Conclusions. In distinction from their aryl analogues **1**, the *O*-acyloxycarbonyl hydroxamates **2** were not good irreversible inhibitors of β-lactamases. An investigation of the reactions of **2** (R = CH_2Ph, R′ = Me or CH_2Ph) in aqueous solution, however, led to the serendipitous discovery that α-hydroxy esters **3** and **4** are new substrates of class C β-lactamases. The class A TEM-2 β-lactamase also catalyzes their turnover, although less efficiently. The hydroxamate leaving group appears to be superior to simple aryloxy leaving groups in these substrates. It seems likely that the α-hydroxyl group enforces a specific orientation of these compounds at the β-lactamase active site that may differ from that of classical substrates. Incorporation of the α-hydroxyalkyl moiety into other platforms, for example, β-lactams and phosphonates, will be interesting.

SUPPORTING INFORMATION AVAILABLE

Synthetic details for the preparation of **6** and **9**−**20**, analytical absorption data for hydrolysis of **7**−**20**, kinetic data for the P99 β-lactamase-catalyzed methanolysis of **7**, and a stereoview of an energy-minimized *S* tetrahedral intermediate structure formed upon reaction of **7** with the P99 β-lactamase (**22**). This material is available free of charge via the Internet at http://pubs.acs.org.

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