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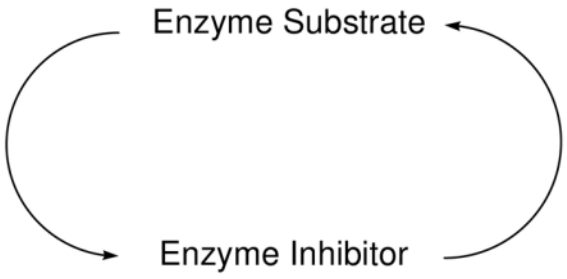
Serendipitous Discovery of α-Hydroxyalkyl Esters as β-Lactamase Substrates†

Ryan B. Pelto and **R. F. Pratt***
Department of Chemistry, Wesleyan University, Middletown, CT 06459

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 classes 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-hydroxyacid 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 reaction of the new substrate in the same way as that of classical substrates, and how (Figure 1) 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).



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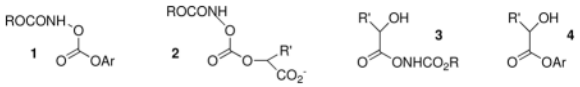
*Corresponding Author: Dr. R.F. Pratt, address above, telephone 860-685-2629; rpratt@wesleyan.edu; Fax: 860-685-2211.

SUPPORTING INFORMATION AVAILABLE

Synthetic details for the preparation of **6** and **9**–**20**. Analytical absorption data for hydrolysis of **7**–**20**. Kinetics data for the P99 β-lactamase-catalyzed methanolysis of **7**. Stereoview of an energy-minimized tetrahedral intermediate structure formed on reaction of **7** with the P99 β-lactamase: the S-adduct (22). This material is available free of charge via the Internet at http://pubs.acs.org

Recently, we described a new class of β-lactamase inhibitors, the O-aryloxy-carbonyl 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 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 corporation, 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 Dr. Michiyoshi Nukaga, Jyosai International University, Japan, and the class C ampC enzyme by Dr. Brian Shoichet of the University of California, San Francisco. The *Streptomyces* R61 DD-peptidase and *Actinomadura* R39 DD-peptidases were generous gifts from Dr. J-M. Frère and Dr. P. Charlier of the University of Liège, Liège, Belgium.

A Varian Gemini-300 MHz NMR 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 carried out by Desert Analytics Laboratory. Routine ESI mass spectra were collected using a Thermo LCQ Advantage instrument.

Syntheses

N-(Benzyloxycarbonyl)-O-(1-D-carboxy-ethoxycarbonyl)hydroxylamine (2, R = CH2Ph, R′ = Me)

1-D-(t-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 minutes and the precipitate of DMAP hydrochloride removed by filtration. The resulting chloroformate solution was used directly in the following acylation step.

N-(Benzyloxycarbonyl)-O-(1-D-carboxy-ethoxycarbonyl)hydroxylamine: Benzyl N-hydroxycarbamate (0.43 g, 2.5mmol) in methylene chloride (4 ml) was stirred at 0 °C and under an atmosphere of dry nitrogen. Anhydrous pyridine (0.21 ml, 2.5 mmol) was added

and the mixture stirred for 10 minutes. This was followed by dropwise addition of the chloroformate (0.43 mg, 2.8 mmol). The ensuing mixture was stirred for a further 25 minutes, filtered and the solvent evaporated from the filtrate. The crude product was collected as a colorless oil (0.48 g) which was purified on silica gel with hexanes:ethyl acetate (2:1) as eluent. The colorless solid product was recrystallized from benzene/cyclohexane to yield 0.28 g (33%) of colorless needle-like crystals (mp 79 °C). ^{1}H NMR ($CDCl_3$): δ8.38 (s, 1H), 7.34 (s, 5H), 5.19 (s, 2H), 4.90 (q, 1H), 1.50 (d, 3H), 1.43 (s, 9H). IR (KBr, cm^{-1}): 1792, 1759, 1702. Elemental Anal. Calc 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.

Deprotection: The *tert*-butyl protected derivative from above (265 mg, 0.75 mmol) was dissolved in trifluoroacetic acid (10 ml) and stirred for 30 minutes. The solvent was evaporated and the free acid was obtained quantitatively as a colorless oil, which proved 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, cm^{-1}): 1790, 1736, 1721. 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 starting material.

N-(Benzyloxycarbonyl)-O-[1-D-carboxy-2-phenyl-ethoxycarbonyl]hydroxylamine (2, R = R′ = CH2Ph)

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 hours. The reaction mixture was then dried by rotary evaporation, leaving a pale yellow oil which 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 was allowed to warm to room temperature as it stirred for 12 hours. 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 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 hours 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 evaporated to yield 398 mg (58% overall) of a pale yellow oil.

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

N-(Benzyloxycarbonyl)-O-[1-D-(t-butoxycarbonyl)-2-phenyle-thoxycarbonyl] 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-(*t*-butoxycarbonyl)-2-phenylethyl chloroformate, was purified by chromatography on silica gel preparative plates with hexanes/ethyl acetate (2:1) as eluent, yielding 152 mg (33%) of colorless oil. ^{1}H NMR ($CDCl_3$): δ 7.86 (s, 1H), 7.35 (m,

5H), 7.26 (m, 5H), 5.21 (s, 2), 5.07 (dd, 1H, J = 5.1 Hz), 3.18 (t, 2H, J = 5.1 Hz), 1.40 (s, 9H). IR (neat, cm^{-1}): 1794, 1732 (broad, two peaks). ES(+)MS m/z: 438.0 ($M+ Na^+$).

Deprotection: The *tert*-butyl protected compound (20 mg, 0.05 mmol) was dissolved in trifluoroacetic acid (4 ml) and stirred for 30 minutes under a flow of nitrogen. The trifluoroacetic acid was then evaporated, quantitatively yielding the free acid as an oil. ^1H 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, cm^{-1}): 1792, 1732, 1665. ES(−)MS m/z: 358.07 ($M - H^+$).

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/phenol was accomplished with pyridine as base. The silyl ethers were then deprotected with $KHSO_4$ in aqueous methanol (11). The products were generally initially purified on silica gel with hexanes/ethyl acetate (3:1) as eluent and finally recrystallized from benzene/cyclohexane (1:1).

N-(Benzyloxycarbonyl)-O-(2-hydroxy-3-phenylpropanoyl)hydroxylamine (7) [and (8)]

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 was stirred overnight. The reaction mixture was then diluted with hexanes and washed sequentially with water (25 ml), saturated $NaHCO_3$ (25ml) 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 one hour at 0 °C, followed by one hour at room temperature. The solvent was removed by rotary evaporation and the acid chloride used directly for acylation.

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 hr at 0 °C, the mixture filtered, and the solvent removed under vacuum. The resulting oil was fractionated on silica gel with hexanes/ethyl acetate (3:1) as 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 five days, filtered, and the methanol 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 hexanes/ethyl acetate (3:1) elution, yielding a colorless solid which was recrystallized from benzene/cyclohexane (1:1). Thus, 33 mg (48%) of **7** as colorless crystals (mp 110–112 °C) was obtained. ^1H 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, cm^{-1}) 1804, 1721. 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 carried out at 25 °C, buffered in 20 mM 3-morpholinopropanesulfonic acid (MOPS) at a pH of 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.

15N 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 taken at 25 °C on a Varian Unityplus 500 spectrometer. The data acquisition parameters were 10 kHz spectral width, 2 kHz 2D spectral width, a one second acquisition time, one second delay time, 2 × 64 increments, and 8 repetitions. Chemical shifts were referenced to formamide (108.5 ppm) as an internal standard (12).

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 (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.

pKa determination—The pKa of the 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 20 mM each of sodium acetate, MES, MOPS, AMPSO, TAPS and CAPS was employed. A constant ionic strength of 1.0 was maintained with sodium chloride.

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 by stirring a solution of it in trifluoroacetic acid (4 ml) for 30 minutes. The trifluoroacetic acid was removed by rotary evaporation yielding a clear colorless oil which 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 dried over Na_2SO_4. The solvent was then removed by rotary evaporation yielding 5 mg of a colorless semi-solid. ^{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 (cm^{-1}): 1735, 1798. ES(−)MS m/z: 238.1 ($M\text{-}H^+$).

P99 β-Lactamase Steady-State Kinetics—The enzyme (final concentration 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). Initial rates were measured, corrected for spontaneous hydrolysis by subtraction, where necessary, and the data 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 was fit linearly to determine k_{cat}/K_m.

K_m values by Competition—The P99 β-lactamase (with the stock solution containing 1 mg/ml BSA) was diluted into a buffered solution (1 nM final concentration) 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 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 of the α-hydroxy ester as a substrate.

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

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 up to 2.5 M in aqueous MOPS buffer (20 mM) were employed; this concentration of methanol has been shown to have negligible effect upon enzyme activity (13). The effect of methanol (0–2.5 M) on the initial rates of solvolysis of **14** (800 μM, K_m = ca. 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 equation 2 [derived from Scheme 4, Results and Discussion (14)], by means of a nonlinear least-squares program.

$$v/v_0 = \frac{\alpha\beta(1 + S_0/K_m)}{\alpha\beta + [H_2O]_0(k_2/k_3 + \beta)(S_0/K_m)}$$
$$\text{where } \alpha = k_2/k_3 + [H_2O]_0$$
$$\text{and } \beta = [H_2O] + (k_4/k_3)[MeOH] \quad (2)$$

Aminolysis of the P99 β-Lactamase Acyl-Enzyme—To investigate the aminolysis of the acyl-enzyme derived from compound **20**, two complementary methods were employed. First, the turnover of **20** (1 mM, ca. 5x K_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 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 the Dynafit program (15).

pH-Rate profiles—pH-Rate profiles were obtained in a mixed buffer system of 20 mM each sodium acetate, MES, MOPS, AMPSO, TAPS and CAPS with a constant ionic strength of 1.0 maintained with NaCl. Experiments were carried out 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 pH 8.5–9.5, hydrolysis was monitored at 233 nm and 250 nm, respectively, due to pH dependent changes in the extinction coefficients. Because the solubility of **7** diminished at pH values below its pKa (7.6), and the change in 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 equation 3 by means of a nonlinear least-squares program. The more soluble compound **20** was employed to obtain a full pH profile (pH range 4.0–9.5) in the same manner.

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

Kinetics with other enzymes—Experiments to determine 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** (≤ 1mM) and **20** (≤ 1mM) in the presence of this enzyme (0.5 μM 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 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 μM, 0.4 μM 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.

Molecular Modeling—Simulations were performed on a SGI workstation running the program Insight II (Accelrys). The crystal structure of a phosphonate bound P99 β-lactamase (PDB 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 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 without the rest of the protein. The model was given a 1000 step steepest gradient energy minimization prior to employing 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 PDB structure and the pH of the enzyme complex was set to 7.5. The partial charges of the protein residues were assigned by Insight II. 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 serine 64. The models were energy-minimized with 1000 steepest gradient steps followed by molecular dynamics of 10,000 equilibration steps and 90,000 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, followed by 2000 conjugate gradient steps. Relative quantitative evaluation of the models was made by means of calculations of ligand interaction energies, E_{int} (17).

RESULTS AND DISCUSSION

The synthesis of compounds of general structure **2**, as their t-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 teriary amine base (Scheme 1) (5). The t-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, where 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 of 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**, where the enzyme became irreversibly inactivated (4,5); the mechanism of reaction of **2** with the enzyme, 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 thence a new class of substrates/inhibitors.

Direct spectrophotometric observation of the reaction between the P99 enzyme and **2** (R = CH_2Ph, R′=Me) gave data such as shown 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 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 detectable by ^1H 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.

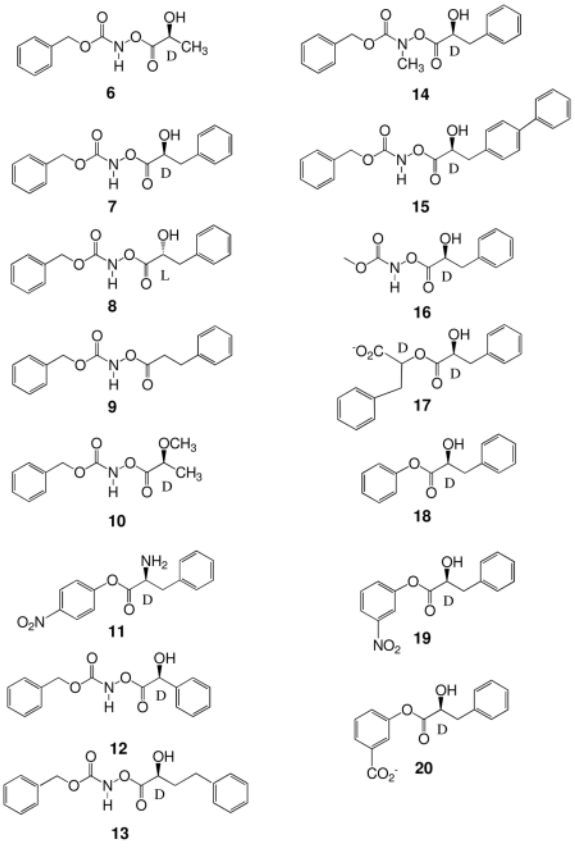
A ^1H NMR experiment (Figure 3) showed that during the reaction of **2** (R = CH_2Ph, R′ = Me) in neutral aqueous buffer in the absence of an enzyme, there was an intermediate, I, between the starting material, S, and the final hydrolysis product(s), P. The final product spectrum showed the expected 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 strong precedent for the first step, although the probably more thermodynamically favorable reverse reaction has been described (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. Since 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 t-butyldimethylsilylation, with either hydroxamic acids or alcohols/

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 silica gel if not. As noted above, compounds **6** and **7** were identical to the intermediates isolated from 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 neutral aqueous solution. ^{1}H 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} s^{-1} 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} s^{-1} (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 pKa 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 **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 presented 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 larger rate constant 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 where 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 up to 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 to detect inhibition of cephalothin turnover by **9** showed that its K_i value, and thus the Km 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 by methoxy, is a much poorer substrate than **6**. Finally, compound **11**, where the hydroxyl group of **7** is replaced by an amine (**11** would probably be mainly in the neutral form at pH 7.5), and where a better leaving group is present [pKa values of benzyl N-hydroxycarbamate and p-nitrophenol are 14.3 and 9.4, respectively, in dioxane/water (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 L-preferences in the acyl fragment adjacent to the scissile bond (23,24), for structural reasons that have been discussed (24). The preference for D observed in the present 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 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 of comparable 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 much less effect on k_{cat}/K_m than is found in acyclic substrates such as **24** (see below); in the latter, the *m*-carboxylate is placed so as 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 to that in acylation by **24**.

If, as is likely, the hydrolysis of α-hydroxyesters is catalyzed by the P99 β-lactamase by a double displacement mechanism involving an acyl-enzyme intermediate (Scheme 4), the observation of quite similar kcat 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 since rate-determining deacylation is a common feature of catalysis by the P99 β-lactamase (13,14).

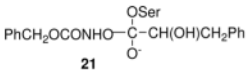
Support for rate-determining deacylation in these cases was obtained from measurement of the kinetic effect of alternative nucleophiles. At substrate concentrations where significant acyl-enzyme would be expected to accumulate, i.e. ≥ K_m, alternative nucleophiles, Nu, should directly accelerate turnover 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 the present case, turnover of **7** was linearly accelerated by addition of methanol (Figure S1, 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-hydroxyesters 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 of Figure 5 and the associated pKa 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 only be studied spectrophotometrically at pH values above the pKa because of the absence of a measurable spectral change at lower pH, yielded a pKa for decreasing activity of 7.6 ± 0.2 (Figure 5). This correlates well with the pKa obtained with **1** (5) and strongly suggests that the reactive form of **7** is the neutral ester rather than the nitrogen-based anion. In further support of this conclusion, the data of Table 1 indicates that **14**, the N-methylated derivative of **7**, is also an effective substrate. The apparently rather higher k_{cat} value of **14** than would be expected on the basis of the discussion above – **14** has the same acyl group as **7** and **20** – may be an artifact; because of the low solubility of **14**, ≤ 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 since methanol acceleration of 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 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 was 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 pKa values of these residues.



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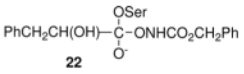

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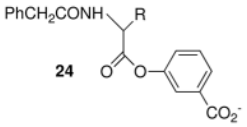
The most favorable (by the criterion of ligand interaction energy values, E_{int}) conformations obtained showed the α-hydroxyl group hydrogen bonded as an acceptor to the Lys 67 terminal ammonium group (Figure 6A), or, more favorably, as a donor to the Ser 318 carbonyl oxygen (Figure 6B). In the former case, the phenyl group of the substrate was situated in such a way as to form 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 of Figure 6B, but where the α-hydroxyl group is hydrogen-bonded to Ser 318 O_γ, was also noted. This structure, however, seems unlikely to be generally important since **7** was also found to be a good substrate of the ampC β-lactamase, another class C enzyme, but where Ser 318 is replaced by Ala 318. 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 above, the model of 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 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 (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, Supplementary Information) but none led to structures where the α-hydroxyl group interacted directly with the enzyme.



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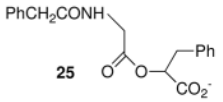

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A final point relating to structure was also noted above, the clear preference of the P99 β-lactamase for the D enantiomer **7** *vs* the L enantiomer **8**, which is rather unusual if reaction is thought to take place through the positioning of **3** as for classical substrates (Figures 6A and 6B). 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 that 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, 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 present case, 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 above seems to favor occupation of the active site by α-hydroxy esters as shown in Figure 6B, direct evidence for 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 **6** – **20**, and **20** were 2.4×10^3 $s^{-1}M^{-1}$ and 460 $s^{-1}M^{-1}$, respectively; in both cases, Km 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, **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, 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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	infra-red
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-butyl-dimethylsilyl
TFA	trifluoroacetic acid

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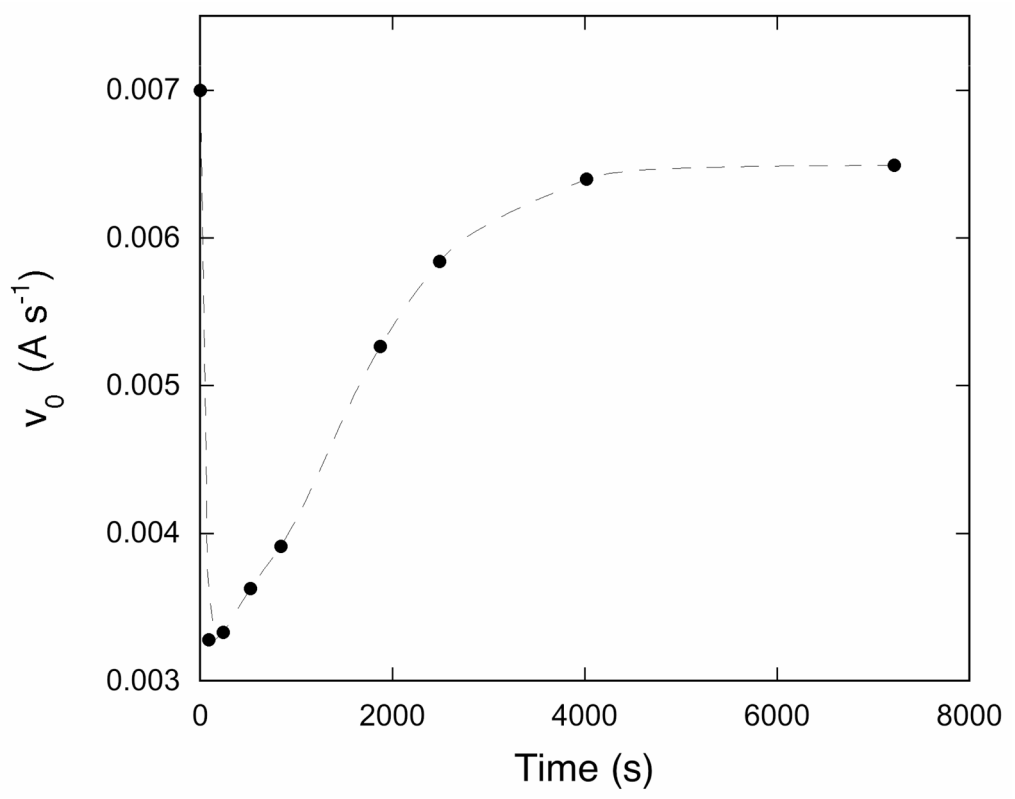


Figure 1.
Activity of the P99 β-lactamase (0.25 μM) as a function of time after mixing with **2** (R = $PhCH_2$, R′ = Me) (100 μM).

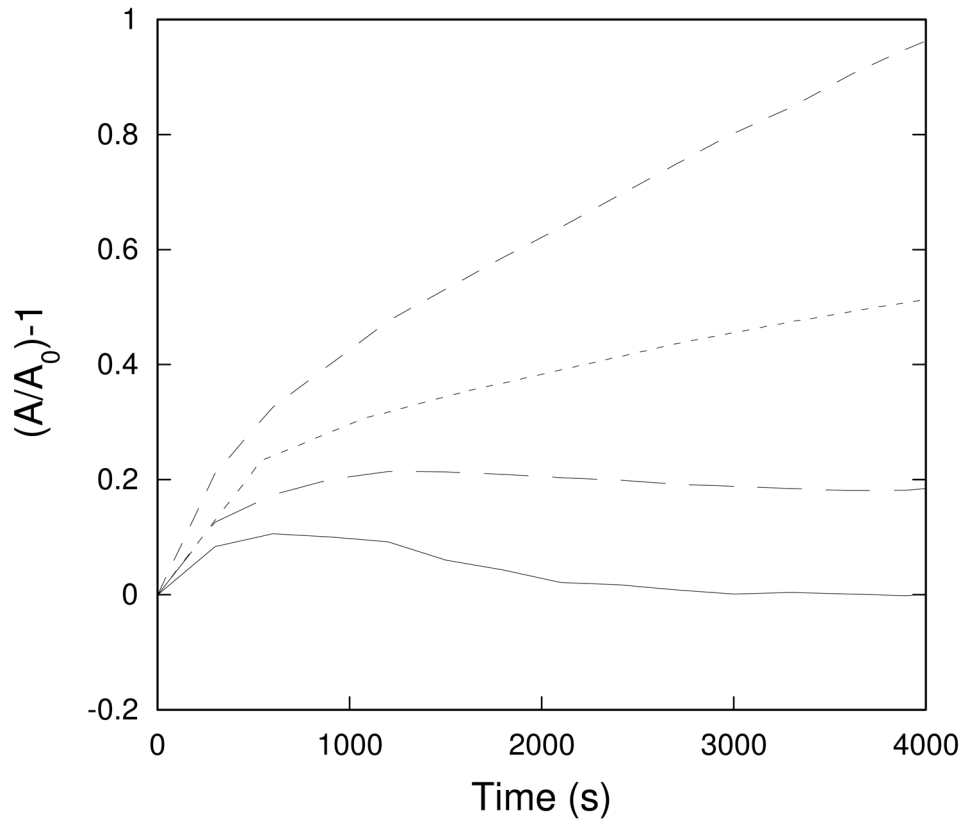


Figure 2.
Absorbance changes at 250 nm on reaction of **2** (R = $PhCH_2$, R′ = Me) (0.4 mM) in the absence of (– – –) and in the presence of the P99 β-lactamase (----, 0.25 μM; — —, 0.5 μM; ——, 1.25 μM).

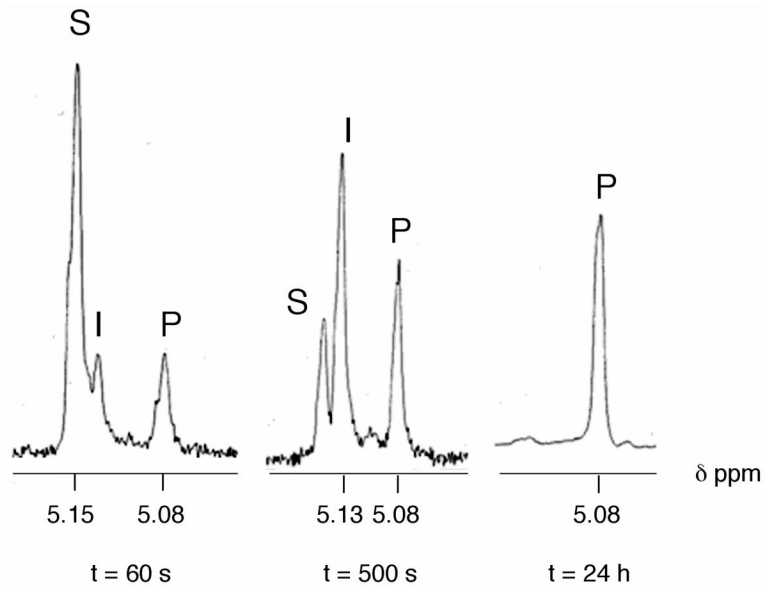


Figure 3.

^{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 the 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.

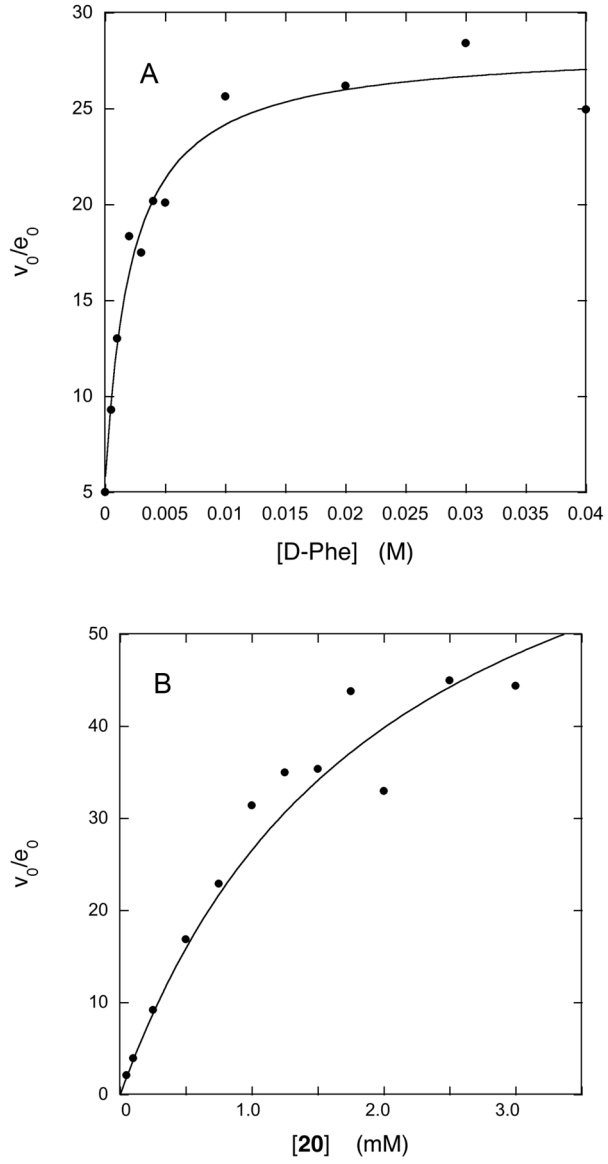


Figure 4.

Initial rates of reaction of **20** in the presence of the P99 β-lactamase (0.1 μM) and D-phenylalanine. The upper plot shows variation of the rate at fixed concentration of **20** (1.0 mM) and varying D-phenylalanine concentration, while the lower shows the 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.

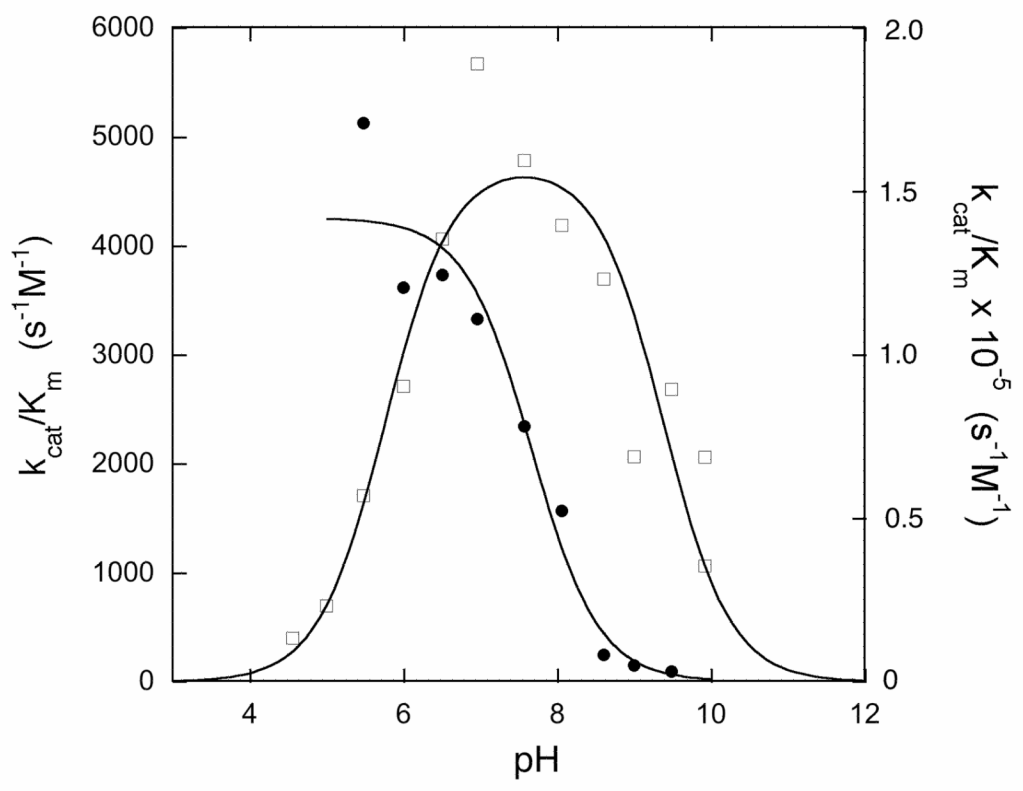


Figure 5.
pH-profiles for hydrolysis of **7** (●) and **20** (□) in the presence of the P99 β-lactamase. The fitted lines were calculated as described in the text.

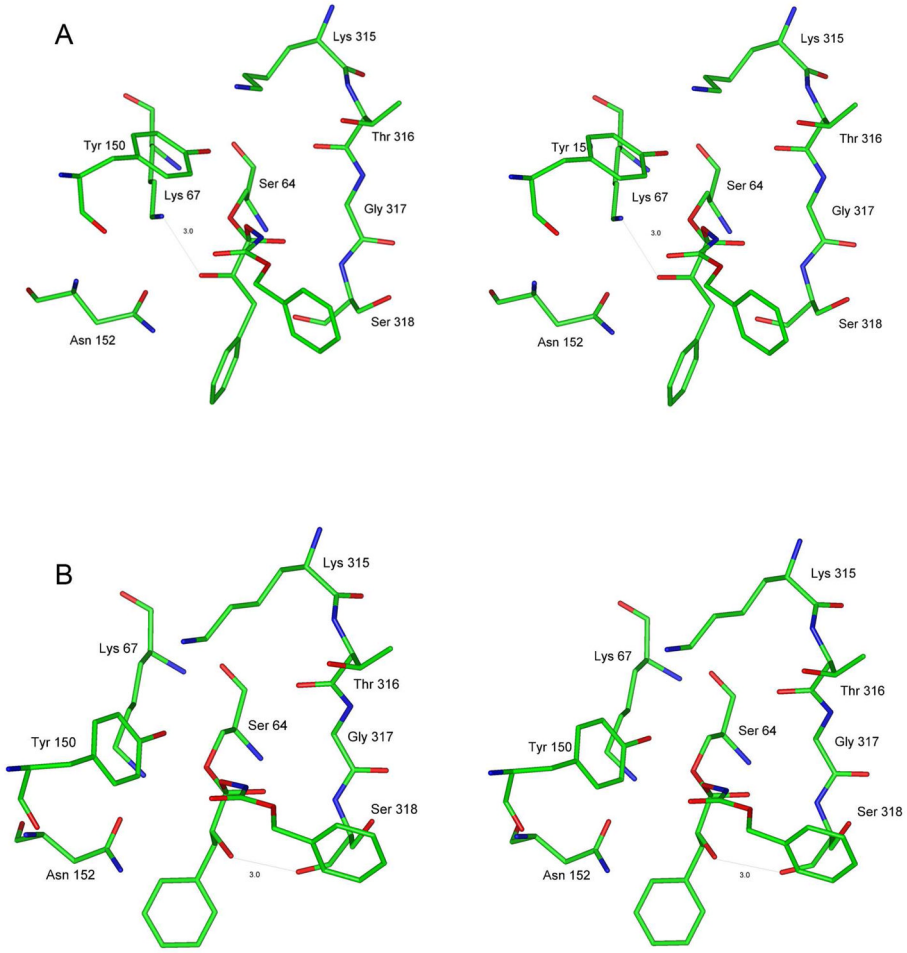
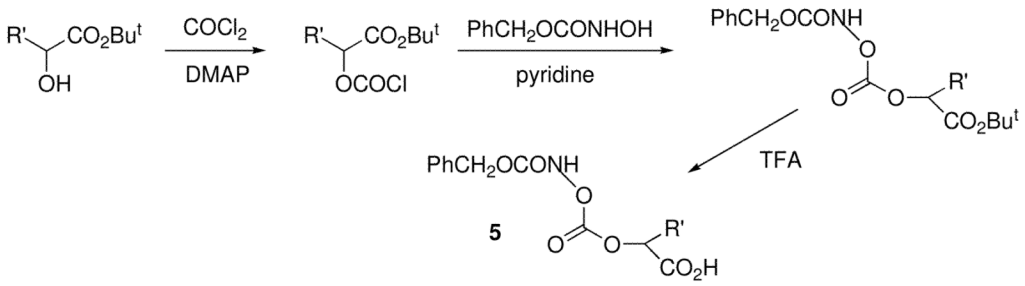
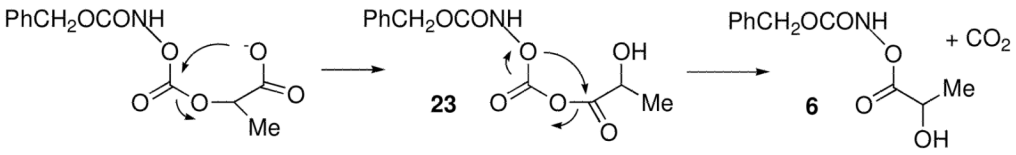


Figure 6.

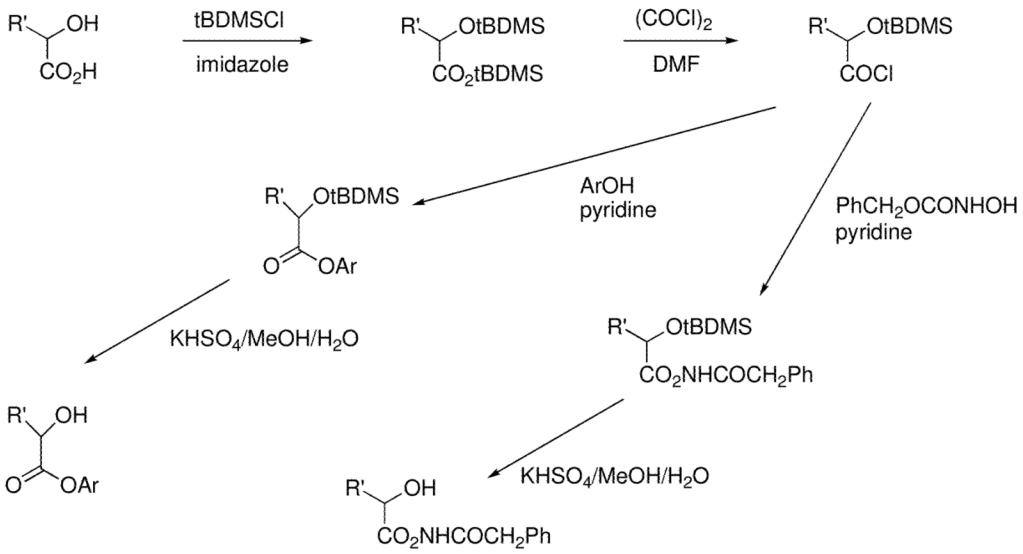
Stereoviews of energy-minimized tetrahedral intermediate structures formed on reaction of **7** with the P99 β-lactamase. Only heavy atoms are shown. A, B: Alternative conformations of the R-adduct (21).



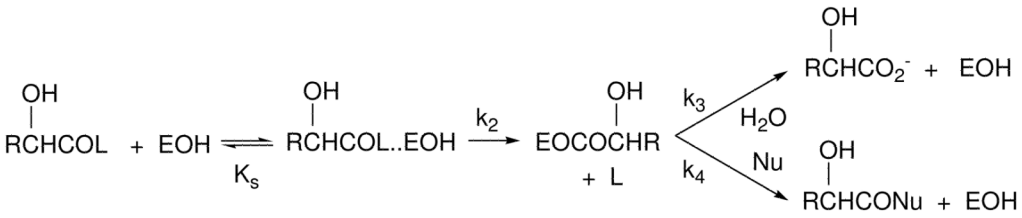
Scheme 1.



Scheme 2.



Scheme 3.



Scheme 4.

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	340 ± 60	5.9 ± 0.3	1.74×10^4
9	$(6.5 \pm 0.2) \times 10^{-5}$		NR	
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		Insoluble/NR	
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	
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

NR = no reaction observed, ND = not determined