Kinetics and Mechanism of the Serine β-Lactamase Catalyzed Hydrolysis of Depsipeptides†

Chandrika P. Govardhan and R. F. Pratt*
Department of Chemistry, Wesleyan University, Middletown, Connecticut 06457
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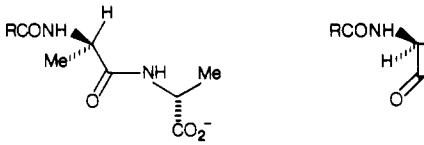
ABSTRACT: Steady-state kinetic parameters have been determined for the hydrolysis of a series of acyclic depsipeptides (ester analogues of acyl-D-alanyl-D-alanine peptides) catalyzed by representative class C (*Enterobacter cloacae* P99) and class A (*Bacillus cereus* I, TEM-2, and *Staphylococcus aureus* PC1) β-lactamases. The best of these substrates, and the one most used in this work, was *m*-[[(phenylacetyl)-glycyl]oxy]benzoic acid, whose rates of cleavage could be followed spectrophotometrically. The P99 enzyme also catalyzed the methanolysis of these substrates in aqueous methanol solutions. Quantitative evaluation of the effects of methanol on the kinetics of the competing hydrolysis and methanolysis reactions, and on the product distribution, supports a reaction mechanism involving an acyl-enzyme intermediate whose formation is rate-determining under conditions of substrate saturation. Consideration of the variation of these kinetic parameters with the structure of the depsipeptides and comparison with the analogous parameters for bicyclic β-lactam substrates suggest that a variety of substrate binding modes exist on this enzyme. The class A enzymes, *B. cereus* β-lactamase I and the TEM-2 β-lactamase, catalyze depsipeptide and benzylpenicillin hydrolyses but not methanolysis. The acyl-enzyme derived from both types of substrate is thus shielded from external nucleophiles; the shielding is therefore not an effect, direct or indirect, of the thiazolidinyl group in the penicilloyl-enzyme. The class A β-lactamase of the PC1 plasmid of *S. aureus* is distinctly different from the above two representatives of that class, in that it does catalyze methanolysis of depsipeptides (but not of benzylpenicillin). The methanolysis kinetics suggest that deacylation is rate-determining at saturation, a conclusion supported by the demonstration of an intermediate during the hydrolysis of *m*-[[(phenylacetyl)glycyl]oxy]benzoate, subsequent to leaving-group departure. The β-lactamases have thus been shown to catalyze the hydrolysis of specific depsipeptides with comparable facility to that demonstrated by D-alanyl-D-alanine carboxypeptidase/transpeptidases. The former enzymes, however, differ in being unable to cleave the analogous peptides.

There are two types of enzyme, both of bacterial origin, that specifically catalyze the nucleophilic cleavage of the β-lactam ring of β-lactam antibiotics, the D-alanyl-D-alanine transpeptidase/carboxypeptidases (abbreviated DD-peptidases below), which are involved in bacterial cell wall biosynthesis and are covalently inhibited by β-lactam antibiotics, and the β-lactamases, which catalyze the hydrolysis of β-lactams and are responsible for a major part of bacterial resistance to these antibiotics. On the basis of the structural resemblance between their substrates, D-alanyl-D-alanine terminating peptides **1** and their inhibitors, the penicillins **2**, Tipper and Strominger (1965) suggested that the two types of enzyme might be evolutionarily related and thus should show similarities of structure and catalytic mechanism. Since then much evidence has accumulated [see, for example, reviews by Waxman and Strominger (1983) and Coulson (1985)] in support of this hypothesis. For example, both types of enzyme (excluding the small number of zinc metallo-β-lactamases and DD-peptidases) contain an active site serine hydroxyl group which is acylated by their respective substrates in the formation of an acyl-enzyme intermediate during turnover. The same serine hydroxyl group of the DD-peptidases is also acylated by β-lactam antibiotics in the reaction leading to inhibition.



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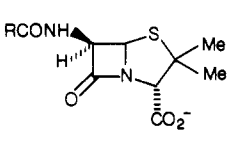

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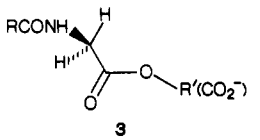

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3a, R = $PhCH_2$-, OR′= D-lactate
3b, R = $PhCH_2$-, OR′= D-mandelate
3c, R = $PhCH_2$-, R′= *o*-carboxyphenyl
3d, R = $PhCH_2$-, R′= *m*-carboxyphenyl
3e, R = $PhCH_2$-, R′= *p*-carboxyphenyl
4, R = *N*,*N*′-diacetyl-L-lysyl, OR′= D-lactate
5, R = Ph-, OR′= D-phenyllactate

Although there is significant amino acid sequence homology between the different types of enzyme in the region closely adjacent to the active site serine, this is not apparently so throughout the rest of the protein. The ambiguity of this result with respect to the evolution of these enzymes has been recently dispelled to a considerable extent by the discovery through X-ray crystallography that the number and arrangement of secondary structural elements is very similar in *Bacillus licheniformis* β-lactamase (Kelly et al., 1986), *Bacillus cereus* β-lactamase I (Samraoui et al., 1986), and *Streptomyces* R61 DD-peptidase (Kelly et al., 1982, 1985).

The remaining gap in the picture of substrate complementarity between the two types of enzyme, viz., the inability of β-lactamases to catalyze reactions of acyclic peptides (Saz et al., 1961; Pratt et al., 1980), has been, if not filled, at least illuminated by our demonstration (Pratt & Govardhan, 1984) that β-lactamases do catalyze [as do DD-peptidases of course (Rasmussen & Strominger, 1978)] the hydrolysis and aminolysis by specific amino acids of depsipeptide analogues **3** of

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D-alanyl-D-alanine peptides, where the alcohol leaving group was a D-α-hydroxy acid and the side chain, R, benzyl. It was also noted in that work that (phenylacetyl)glycyl esters (lacking, like penicillins, the methyl group on the penultimate amino acid residue) were, in general, better β-lactamase substrates than the corresponding phenylacetyl-D-alanyl esters. This paper now explores in more detail the kinetics and mechanism of the class A and class C β-lactamase-catalyzed hydrolysis of several depsipeptides (**3a–e**, **4**, **5**). The comparisons with β-lactam turnover and with the DD-peptidases are discussed.

Experimental Procedures

Materials. The β-lactamase I of *B. cereus* (strain 569/H/9) and the β-lactamases of the TEM-2 plasmid (from *Escherichia coli*, strain W 3310), of *Staphylococcus aureus* PC1, and of *Enterobacter cloacae* P99 were obtained from the Centre for Applied Microbiology and Research (Porton Down, England) and used as supplied. Benzylpenicillin was purchased from Sigma Chemical Co. Deacetylcephalothin was a generous gift of Eli Lilly and Co. and nitrocefin of Glaxo Research Ltd. Methanol was Baker analyzed reagent grade.

The depsipeptide substrates were prepared by the general method of Losse and Klengel (1971). Details of these syntheses and the properties of the purified substrates are reported below. Melting points are uncorrected and were taken in open capillary tubes in a Mel-temp melting point apparatus. Mass spectra and NMR spectra were obtained by means of a Perkin-Elmer RMU-6E and a Varian XL-200 spectrometer, respectively. Elemental analyses were performed by the MicAnal Laboratory, Tucson, AZ.

(Phenylacetyl)glycine. To a stirred solution of glycine (9.7 g, 0.13 mol) and sodium hydroxide (10.4 g, 0.26 mol) in 75 mL of water at 0 °C was added redistilled phenylacetyl chloride (20.0 g, 0.13 mol; Fluka) dropwise over 45 min. The mixture was stirred for a further 2 h at 0 °C and then activated charcoal added. After the charcoal had been removed by filtration, the filtrate was acidified to pH 1.5 with concentrated HCl. The precipitated product was isolated by filtration and recrystallized from water: yield 48%; mp 143–144 °C [lit. mp 142–143 °C (James et al., 1972)].

D-Lactic Acid. Cold concentrated sodium nitrite solution (12.0 g dissolved in the minimum volume of water) was added dropwise over a period of 1 h to a vigorously stirred solution at 0 °C of D-alanine (10.0 g, 0.11 mol) in 11.0 mL of glacial acetic acid and 2.5 mL of water. Vigorous evolution of nitrogen was observed. The solution was then heated on a steam bath for 30 min to complete the reaction. Spectrophotometric assays for D-lactate [using *Lactobacillus leichmannii* D-lactate dehydrogenase (Sigma)] and for L-lactate [using rabbit muscle L-lactate dehydrogenase (Boehringer Mannheim)], as described by Bergmeyer et al. (1983), showed that the crude reaction mixture contained a 68% yield of D-lactate and no L-lactate. This solution was concentrated to one-third of the original volume and used directly in the synthesis below.

Benzyl D-Lactate. One equivalent of benzyl chloride was added to the solution of D-lactate described above, and the mixture was heated under reflux for 5 h at 115 °C. After the reaction mixture had cooled, it was extracted with two 35-mL portions of ethyl acetate. The ethyl acetate solution was then dried over $MgSO_4$, the solvent removed by rotary evaporation, and the residue fractionally distilled under reduced pressure, yielding, after foreruns of benzyl alcohol and benzyl acetate, a 40% yield of benzyl D-lactate, bp 148–152 °C (1 Torr).

Benzyl [(Phenylacetyl)glycyl]-D-lactate. (Phenylacetyl)-glycine (4.19 g, 21.8 mmol) was suspended in 30 mL of freshly dried tetrahydrofuran (THF) (distilled from $LiAlH_4$) and stirred at 0 °C. 1,1′-Carbonyldiimidazole (3.31 g, 20.4 mmol) was added as a solid and the mixture stirred at 0 °C for 1 h. During that time, the acid dissolved and carbon dioxide was evolved. Benzyl D-lactate (2.4 g, 13.3 mmol) in 13 mL of THF was added to the stirred mixture. The mixture was stirred at 0 °C for 3 days and then evaporated to dryness. The residue was dissolved in ethyl acetate and washed successively with water, 10% aqueous citric acid, water, saturated aqueous $NaHCO_3$, and water. The ethyl acetate was dried over $MgSO_4$ and evaporated to dryness, yielding the required product as a colorless solid (3.6 g, 10.1 mmol, 76%). The NMR spectrum confirmed the structure: ^1H NMR (C^2HCl_3) δ 1.50 (d, J = 7 Hz, 3, CH_3), 3.66 (s, 2, PhCH_2), 4.00, 4.17 (AB of ABX system, J = 20, 7, 6 Hz, 2, NHCH_2), 5.19 (q, J = 7 Hz, 1, CH), 5.20 (s, 2, OCH_2), 5.90 (br, 1, NH), 7.4 (m, 5, Ar H).

(Phenylacetyl)glycyl-D-lactic Acid (**3a**). The benzyl ester of (phenylacetyl)glycyl-D-lactic acid (3.6 g) was dissolved in 135 mL of 95% ethanol and added to 266 mg of 10% Pd/C catalyst and hydrogenated at 30 lb/in.^{2} pressure at room temperature in a Parr hydrogenator for 2 h. The reaction mixture was then filtered and evaporated to dryness. The crude sample was recrystallized from methylene chloride to give white crystals of (phenylacetyl)glycyl-D-lactic acid (0.95 g): mp 120–121 °C; ^1H NMR (C^2HCl_3) δ 1.50 (d, J = 7 Hz, 3, CH_3), 3.60 (s, 2, PhCH_2), 4.17, 4.03 (AB of ABX system, J = 20, 7, 6 Hz, 2, NHCH_2), 5.03 (q, J = 7 Hz, 1, CH), 6.61 (br, 1, NH), 7.35 (m, 5, ArH); mass spectrum, m/e 265 (M^+). Anal. Calcd for $C_{13}H_{15}NO_5$: C, 58.85; H, 5.70; N, 5.28. Found: C, 58.44; H, 5.43; N, 5.26.

Trichloroethyl D-Mandelate. A solution of D-mandelic acid (15.0 g, 0.098 mol), trichloroethanol (24.5 g, 0.164 mol), and *p*-toluenesulfonic acid monohydrate (1.87 g, 0.01 mol) in 175 mL of toluene was heated under reflux in a Dean and Stark apparatus for 24 h. After the mixture had cooled, it was concentrated on a rotary evaporator and the residue taken up into ethyl acetate. The ethyl acetate solution was washed with sodium bicarbonate solution and water, dried over $MgSO_4$, and evaporated to dryness. The residue was fractionally distilled under reduced pressure, yielding 7.1 g (26%) of trichloroethyl D-mandelate: bp 100 °C (5 Torr); ^1H NMR (C^2HCl_3) δ 3.28 (d, J = 7 Hz, 1, OH), 4.80 (s, 2, CH_2), 5.38 (d, J = 7 Hz, 1, CH), 7.35 (m, 5, Ar H).

(Phenylacetyl)glycyl-D-mandelic Acid (**3b**). The trichloroethyl ester of this compound was prepared as a colorless solid in 68% yield by the procedure described above for the D-lactate benzyl ester. The product had the following ^1H NMR spectrum: (C^2HCl_3) δ 3.65 (s, 2 H, PhCH_2), 4.17, 4.25 (AB of ABX system, J = 16, 7, 6 Hz, 2, NHCH_2), 4.92, 5.07 (AB q, J = 12 Hz, 2, CH_2CCl_3), 5.95 (br t, J = 7 Hz, 1, NH), 6.08 (s, 1, CH), 7.4 (br m, 10, Ar H).

Subsequent removal of the trichloroethyl protecting group was carried out by using zinc and 90% acetic acid, as described by Marinier et al. (1973). The crude acid product was recrystallized from methylene chloride to give fine white crystals of (phenylacetyl)glycyl-D-mandelic acid, mp 145–148 °C, in 32% overall yield: ^1H NMR (C^2HCl_3) δ 3.62 (s, 2, PhCH_2), 4.15 (d, J = 7 Hz, 2, NHCH_2), 5.94 (s, 1, CH), 6.80 (br t, 1, NH), 7.4 (m, 10, Ar H); mass spectrum, m/e 327 (M^+). Anal. Calcd for $C_{18}H_{17}NO_5$: C, 66.04; H, 5.24; N, 4.28. Found: C, 65.71; H, 5.15; N, 4.44.

o-[[(Phenylacetyl)glycyl]oxy]benzoic Acid (**3c**). This material was prepared from (phenylacetyl)glycine and benzyl salicylate (Matheson Coleman and Bell) as described for the D-lactic acid derivative. The final product, mp 131–132 °C,

was recrystallized from acetonitrile: ^{1}H NMR (C^2HCl_3/[$^2H_6$]Me_2SO) δ 3.66 (s, 2, PhCH_2), 4.36 (d, J = 7 Hz, 2, NHCH_2), 6.58 (br t, 1, NH), 7.1–8.1 (m, 9, Ar H). Anal. Calcd for $C_{17}H_{14}NO_5$: C, 65.38; H, 4.51; N, 4.49. Found: C, 65.20; H, 4.74; N, 4.43.

m-[[(*Phenylacetyl*)*glycyl*]*oxy*]*benzoic Acid* (**3d**). This was prepared as for the ortho compound. Benzyl *m*-hydroxybenzoate was obtained as described by Cavallito and Buck (1943). The final depsipeptide, mp 172–174 °C, was recrystallized from aqueous ethanol and had the following ^{1}H NMR spectrum: (C^2HCl_3) δ 3.67 (s, 2, PhCH_2), 4.29 (d, J = 7 Hz, 2, NHCH_2), 6.82 (br t, J = 7 Hz, 1, NH), 7.2–8.0 (m, 9, Ar H). Anal. Calcd as above for the ortho compound. Found: C, 65.08; H, 4.67; N, 4.15.

p-[[(*Phenylacetyl*)*glycyl*]*oxy*]*benzoic Acid* (**3e**). This was prepared as for the meta compound and after recrystallization from aqueous ethanol had a mp of 194–196 °C and ^{1}H NMR spectrum (C^2HCl_3/[$^2H_6$]Me_2SO) δ 3.64 (s, 2, PhCH_2), 4.24 (d, J = 7 Hz, 2, NHCH_2), 7.2–8.0 (m, 9, Ar H). Anal. Calcd as above for the ortho compound. Found: C, 64.60; H, 4.54; N, 4.33.

*N,N′-Diacetyl-*L*-lysylglycyl-*D*-lactic Acid* (**4**). *N,N′*-Bis(benzyloxycarbonyl)-L-lysine was converted to its hydroxysuccinimide ester and this then reacted with glycine, both steps following the general method of Anderson et al. (1964), to give, after recrystallization from 95% ethanol, *N,N′*-bis(benzyloxycarbonyl)-L-lysylglycine, mp 152–155 °C. The latter compound was coupled with benzyl D-lactate via the procedure described above for (phenylacetyl)glycyl-D-lactate, yielding benzyl *N,N′*-bis(benzyloxycarbonyl)-L-lysylglycyl-D-lactate as a colorless oil. The identity of this material was confirmed by ^{1}H NMR: (C^2HCl_3) δ 1.48 (br m, 4, lysyl γ,δ-CH_2), 1.50 (d, J = 7 Hz, 3, CH_3), 1.85 (br m, 2, lysyl β-CH_2), 3.17 (br m, 2, lysyl ε-CH_2), 4.12 (d, J = 7 Hz, glycyl CH_2), 4.16 (m, 1, lysyl α-CH), 4.92 (br, 1, NH), 5.06 (s, 2, CH_2O), 5.11 (s, 2, CH_2O), 5.17 (s, 2, CH_2OCOCH), 5.20 (q, J = 7 Hz, 1, $CH$$CH_3$), 5.24 (br, 1, NH), 6.64 (br, 1, NH), 7.4 (m, 15, Ar H).

Benzyl *N,N′*-bis(benzyloxycarbonyl)-L-lysylglycyl-D-lactate (5.0 g) was dissolved in 30 mL of methanol, added to 325 mg of 10% Pd/C, and hydrogenated at 35 lb/in.^{2} pressure at room temperature for 18 h. An equal amount of fresh catalyst was then added and the hydrogenation repeated. The catalyst was then removed by filtration and the solution evaporated to dryness to give L-lysylglycyl-D-lactate as a colorless glass: ^{1}H NMR (^2H_2O) δ 1.43 (d, J = 7 Hz, 3, CH_3), 1.51 (m, 2, lysyl γ-CH_2), 1.73 (quint, J = 7 Hz, 2, lysyl δ-CH_2), 1.92 (q, J = 4 Hz, lysyl β-CH_2), 3.01 (t, J = 7 Hz, 2, lysyl ε-CH_2), 3.97 (t, J = 7 Hz, 1, lysyl α-CH), 4.11, 4.26 (AB q, J = 17 Hz, 2, glycyl CH_2), 4.91 (q, J = 7 Hz, 1, lactate CH).

L-Lysylglycyl-D-lactate was acetylated following the general procedure of Nieto and Perkins (1971). The crude triethylammonium salt of the product was applied to a Dowex 50W-X4 (H^+ form) cation-exchange column and eluted with water. Fractions containing absorption at 220 nm were pooled and freeze-dried to yield, in 46% yield from the benzyl ester, the free acid form of the required product. This was converted into a solution of the sodium salt by titration with sodium hydroxide, and the colorless salt isolated by freeze-drying. The ^{1}H NMR of the product was as follows: (^2H_2O) δ 1.47 (m, 4, lysyl γ,δ-CH_2), 1.53 (d, J = 7 Hz, 3, CHCH_3), 1.80 (t, J = 7 Hz, 2, lysyl β-CH_2), 2.00 (s, 3, $COCH_3$), 2.07 (s, 3, $COCH_3$), 3.20 (t, J = 7 Hz, 2, lysyl ε-CH_2), 4.13 (s, 2, glycyl CH_2), 4.25 (t, J = 7 Hz, 1, lysyl α-CH), 5.14 (q, J = 7 Hz, 1, $CH$$CH_3$).

*Hippuryl-*D*-phenyllactic Acid* (**5**). The sodium salt of hippuryl-DL-phenyllactic acid (Sigma) (0.5 g) and sodium bicarbonate (1.0 g) were dissolved in the minimum volume of water, and 50 μL of bovine carboxypeptidase A (Sigma, Type II-DFP) added. After 30 min at room temperature (when the ^{1}H NMR spectrum of a control sample showed that the reaction was complete), the reaction mixture was loaded onto a QAE-Sephadex anion-exchange column (35 × 2.3 cm, sodium form) and eluted with a sodium chloride gradient (0–1.0 M). One hundred 5-mL fractions were collected and assayed by absorbance at 255 nm and by P99 β-lactamase. Fractions containing the required ester were pooled, acidified, and extracted with ethyl acetate. Evaporation of the dried extracts yielded hippuryl-D-phenyllactic acid, pure by ^{1}H NMR: (C^2HCl_3) δ 3.13, 3.24 (AB of ABX, J = 16, 8, 4 Hz, 2, PhCH_2), 4.26 (d, J = 7 Hz, 2, NHCH_2), 5.37 (X of ABX, J = 8, 4 Hz, 1, CH), 7.14 (t, J = 7 Hz, 1, NH), 7.46, 7.60 (m, 10, Ar H). An aqueous solution of the free acid was carefully titrated with sodium hydroxide to pH 6.5 and then freeze-dried, yielding the sodium salt as a stable, colorless, fluffy solid, which was used in the kinetic studies.

m-[(*Phenylacetyl*)*glycinamido*]*benzoic Acid* (**6**). The hydroxysuccinimide ester of (phenylacetyl)glycine was prepared and coupled to *m*-aminobenzoic acid by the methods of Anderson et al. (1964). The colorless crystalline product was recrystallized from aqueous ethanol: mp 206–207 °C; ^{1}H NMR (^2H_2O, HCO_3^-) δ 3.74 (s, 2, PhCH_2), 4.08 (s, 2, glycyl CH_2), 7.2–7.9 (m, 9, Ar H).

(*Phenylacetyl*)*glycyl-*D*-phenylalanine* (**7**). This compound was prepared in the same way as the above amide and also recrystallized from aqueous ethanol: mp 143–145 °C; ^{1}H NMR (^2H_2O, HCO_3^-) δ 2.90, 3.18 (AB of ABX, J = 16, 8, 4 Hz, 2, CHCH_2), 3.64 (s, 2, PhCH_2), 3.80, 3.86 (AB q, J = 15 Hz, glycyl CH_2), 4.46 (X of ABX, J = 8, 4 Hz, 1, CH), 7.2–7.5 (m, 10, Ar H).

Analytical Methods. Absorption spectra and spectrophotometric steady-state reaction rates were measured by means of a Cary 219 spectrophotometer. β-Lactamase activity was routinely estimated against benzylpenicillin by the spectrophotometric method of Waley (1974). All kinetics experiments were performed at 25 °C in 0.1 M potassium phosphate buffer at pH 7.5, unless otherwise stated. Specific activities of the various enzymes were, under these conditions, 140 units/mg (*S. aureus* PC1), 65 units/mg (*E. cloacae* P99), 1700 units/mg (TEM-2), and 2400 units/mg (*B. cereus* I). Pre-steady-state kinetics measurements were obtained from a Durrum D-110 spectrophotometer (Anderson & Pratt, 1981, 1983). The β-lactamase concentrations were determined spectrophotometrically, assuming their extinction coefficients to be 1.95×10^4 M^{-1} cm^{-1} at 276.5 nm for the PC1 enzyme (Carrey & Pain, 1978), 5.42×10^4 M^{-1} cm^{-1} at 280 nm for P99 (Cartwright & Waley, 1984), 2.90×10^4 M^{-1} cm^{-1} at 280 nm for TEM-2 (Fisher et al., 1980), and 2.85×10^4 M^{-1} cm^{-1} at 280 nm for the *B. cereus* enzyme (Imsande et al., 1970). Aqueous methanol solutions were prepared by making up measured volumes of methanol to the required volumes with aqueous buffer.

Steady-State Kinetics. Michaelis–Menten parameters were obtained by the method of Wilkinson (1961) from initial velocity measurements of the hydrolysis of the various depsipeptides. The reactions were followed spectrophotometrically at 330 nm (Δε = 120 M^{-1} cm^{-1}) for **3c**, 314 nm (Δε = 50 M^{-1} cm^{-1}) for **3d**, 300 nm (Δε = 40 M^{-1} cm^{-1}) for **3e**, 268 nm (Δε = 130 M^{-1} cm^{-1}) for **5**, and 275 nm (Δε = 2600 M^{-1} cm^{-1}) for deacetylcephalothin. The spectrophotometric method was

also used to obtain V_T values for **3d**. The hydrolyses of **3a**, **3b**, and **4** were followed spectrophotometrically with an indicator method (Pratt et al., 1985).

Pre-Steady-State Kinetics. The reaction of **3d** at (close to) saturating concentrations (1.88, 3.75, and 7.5 mM) with the PC1 β-lactamase (14 μM) was followed spectrophotometrically at 290 nm ($\Delta\epsilon$ = 2000 M^{-1} cm^{-1}) in the stopped-flow instrument. The resulting absorbance changes were fitted to a first-order equation by using a nonlinear least-squares program (Johnson et al., 1976).

Detection of an Acyl-Enzyme Formed on Interaction of **3d** *with the PC1 β-Lactamase.* Aliquots of a reaction mixture, consisting of 2.5 mM **3d** and 7.7 μM β-lactamase incubated together for 30 s, were diluted into an assay mixture (1.0 mL) containing 0.61 mM benzylpenicillin, and the hydrolysis of the latter was followed spectrophotometrically as described above. The initial rate of benzylpenicillin hydrolysis was low, but it increased exponentially with time to that representing full activity. The first-order rate constant for this process was obtained as described by Faraci and Pratt (1985).

Acyl Transfer to Methanol. ^1H NMR spectroscopy was used to detect and determine the extent of acyl transfer to methanol when this cosolvent ([$^2H_4$]methanol (Aldrich) in the NMR experiments) was present in the β-lactamase-catalyzed-hydrolysis reaction mixtures. The concentrations of enzymes and substrates used were ca. 5 μM and 10 mM, respectively, and 0.1 M $NaHCO_3$ was present as a buffer. With depsipeptides **3a–e** and **4**, the appearance of a resonance at 4.00 ppm (**3a–e**) and 4.02 ppm (**4**), corresponding to the glycyl methylene resonance of the methyl ester product (see below), was taken as diagnostic of the presence of acyl transfer to methanol. The extent of methanolysis in any particular case was determined after the reaction was complete from a peak height (integration gave the same result) comparison of the above-mentioned resonance with that of the corresponding glycyl resonance of the hydrolysis product [δ 3.74 (**3a–e**) and 3.76 (**4**)].

In one case the methanolysis product was further characterized. Thus, the P99 β-lactamase was added, to a final concentration of 1.8 μM, to an aqueous solution containing **3d** (13 mM), methanol (12.5% v/v), and sodium bicarbonate (0.1 M). The mixture was stirred at room temperature for 20 min. Ethyl acetate extraction then yielded methyl (phenylacetyl)glycinate: ^1H NMR (C^2HCl_3) δ 3.64 (s, 2, PhCH_2), 3.74 (s, 3, OCH_3), 4.30 (d, J = 7 Hz, NHCH_2), 6.06 (br, 1, NH), 7.35 (m, 5, Ar H); mass spectrum, m/e 207 (M^+).

Methyl hippurate, formed on methanolysis of **5**, was also detected and determined through its ^1H NMR spectrum [(C^2HCl_3) δ 3.82 (s, 3, OCH_3), 4.26 (d, J = 7 Hz, 2, CH_2), 6.82 (br t, 1, NH), 7.5 (m, 5, ArH)] and mass spectrum [m/e 193 (M^+)].

Methanolysis Kinetics (V_H Determinations). Initial rates of P99 β-lactamase-catalyzed methanolysis and hydrolysis of **3d**, at **3d** concentrations of 1.79 mM (≈K_m) and 13.6 mM (≈7.5 K_m), were determined by an NMR method. For the data at the lower concentration, reaction mixtures were prepared by the addition of 5-μL aliquots of an enzyme solution (21.9 μM) to 4.6-mL samples of solutions of **3d** containing methanol concentrations between 0 and 3.22 M. A 2.0-mL aliquot was withdrawn after 50 s (at which time about 10% of the substrate had reacted) and added to 1.0 mL of acetonitrile, a procedure that was shown to immediately and irreversibly inactivate the enzyme. Each reaction mixture was then freeze-dried. The dry residues were taken up in 0.45 mL of ^2H_2O immediately prior to NMR spectroscopy. Small amounts (ca. 5%) of background hydrolysis prior to addition of the enzyme were accounted for with zero-time samples. The relative amounts of hydrolysis and methanolysis were determined by the relative areas of the glycyl methylene resonances, as described above. The same procedure was employed at the higher substrate concentration except that smaller reaction volumes (0.23 mL) could be used. The product ratios after complete reaction were also determined in this way except that three times as much enzyme was used and the samples were freeze-dried directly, with no acetonitrile addition, after 30 min.

Hydroxide-Catalyzed Hydrolysis. The pseudo-first-order hydroxide ion catalyzed hydrolyses of **3c–e** were followed spectrophotometrically in 5 mM potassium hydroxide solution (μ = 1.0 with KCl) at 25 °C. Second-order rate constants were obtained by division of the pseudo-first-order rate constants by the hydroxide ion concentration.

Peptide Inhibition Constants. These were obtained from spectrophotometric (486 nm) total progress curves of nitrocefin (32.6 μM) hydrolysis in the presence and absence of the peptides (Waley, 1982) in 20 mM 3-(N-morpholino)-propanesulfonate buffer at 25 °C. The peptide concentrations were 12.3 and 24.5 mM for **6** and 29.5 and 39.3 mM for **7**. Steady-state parameters for the hydrolyses of nitrocefin and **3d** were determined under the same conditions.

RESULTS

Steady-State Parameters for Depsipeptide Hydrolysis. A survey of the steady-state parameters for the *E. cloacae* P99 β-lactamase (a class C enzyme) catalyzed hydrolysis of the depsipeptides is given in Table I. Also shown here are comparable parameters for hydrolysis of one of these depsipeptides, **3d**, catalyzed by the class A β-lactamases of *B. cereus*, the PC1 plasmid of *S. aureus*, and the TEM-2 plasmid. For comparison, steady-state parameters, determined under the same conditions, for two good β-lactam substrates, benzylpenicillin and deacetylcephalothin, are also presented.

Acyl Transfer to Methanol. The P99 β-lactamase also catalyzed acyl transfer from depsipeptides to solvent methanol. The extent of this methanolysis reaction, determined by the ^1H NMR method under specific conditions, is indicated in the data of Table II, where a comparable figure for benzylpenicillin is also presented. The class A β-lactamases were tested with respect to methanolysis of **3d**, with the results also shown in Table II.

Kinetics of the P99 β-Lactamase-Catalyzed Solvolysis in Aqueous Methanol. The effect of methanol on the rates of the P99 β-lactamase-catalyzed solvolysis of **3d** was examined. The spectrophotometric method in this case (see Experimental Procedures) measures the rate of release of *m*-hydroxybenzoate and thus the rate of the total reaction, hydrolysis plus methanolysis, V_T. The observed initial rates of reaction of **3d** at concentrations of 1.9 and 13.1 mM were invariant with methanol concentration to 3 M (Figure 1).

Similarly, the solvolysis of benzylpenicillin was studied. Under saturating conditions (0.45 mM benzylpenicillin) the spectrophotometric rates of total β-lactam ring cleavage, V_T, increased with methanol concentration, as shown in Figure 1.

The initial rates of hydrolysis of **3d**, V_H, in the presence of competing methanolysis, were determined by the NMR method and shown (in reciprocal form for reasons made clear below) in Figure 2. Also shown in this diagram is the product ratio, determined after completion of the reaction, as a function of methanol concentration.

It is now well established that the mechanism of the β-lactamase reaction in both class A and class C β-lactamases

Table I: Kinetics of Hydroxide Ion and β-Lactamase-Catalyzed Depsipeptide Hydrolysis

enzyme	substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})	K_s (mM)	k_2 (s^{-1})	k_3 (s^{-1})	k_{OH} (s^{-1} M^{-1})
P99	**3a**	≥100	≥9.2	178 ± 13	≥100	≥9.2	≥9.2	
	3b	24.0 ± 6.0	53.0 ± 5.0	2200 ± 144	24.0 ± 6.0	53.0 ± 5.0	>53.0	0.36[d]
	3c	14.2 ± 2.3	43.0 ± 2.5	3000 ± 520	14.2 ± 2.3	43.0 ± 2.5	>43.0	1.2
	3d	1.9 ± 0.5	101.0 ± 7.0	(5.4 ± 1.4) × 10^4	1.9 ± 0.5	101.0 ± 7.0	>101.0	15.2
	3e	13.8 ± 2.5	107.0 ± 15.0	7780 ± 1770	13.8 ± 2.5	107.0 ± 15.0	>107.0	21.3
	4	94.0 ± 13.0	18.6 ± 1.9	198.0 ± 6.0	94.0 ± 13.0	18.6 ± 1.9	>18.6	
	5	27.0 ± 5.0	81.0 ± 8.0	3000 ± 630	27.0 ± 5.0	81.0 ± 8.0	>81.0	
	benzylpenicillin	0.015 ± 0.003	25.0 ± 0.8	(1.66 ± 0.34) × 10^6	>0.015	>25.0	25.0 ± 0.8	0.10[d]
	deacetylcephalothin	0.54 ± 0.14	610 ± 50	(1.13 ± 0.29) × 10^6	*a*	*a*		
TEM-2	**3d**	1.8 ± 0.3	24.0 ± 0.0	(1.33 ± 0.04) × 10^4	*a*	*a*		
	benzylpenicillin[b]	0.02	2000	10^8				
BC1	**3d**	6.0 ± 0.5	1.9 ± 0.0	313 ± 7	*a*	*a*		
	benzylpenicillin[c]	46	2000	4.3 × 10^7	*a*	*a*		
PC1	**3d**	0.072 ± 0.005	0.11	1500 ± 100	>5	>10	0.11	
	benzylpenicillin	≤0.010	30.0	≥3 × 10^6	*a*	*a*		

[a] Not defined, see text. [b] 30 °C; Fisher et al. (1980). [c] Hardy & Kirsch (1984). [d] Pratt & Govardhan (1984).

Table II: Extent of Acyl Transfer from Depsipeptides to Methanol[a]

enzyme	substrate	% methanolysis	k_4/k_3
P99	**3a**	57	21.0
P99	**3b**	61	24.8
P99	**3c**	59	22.8
P99	**3d**	66	30.8
P99	**3e**	65	29.4
P99[b]	**4**	80	53.0
P99	**5**	63	27.0
P99[b]	benzylpenicillin	53	16.0
TEM	**3d**	0	0
BC1	**3d**	0	0
PC1	**3d**	42	11.5
PC1	benzylpenicillin	0	0

[a] [$^2H_4$]Methanol concentrations were 3.5 M unless otherwise noted. [b] [$^2H_4$]Methanol concentrations 3.9 M.

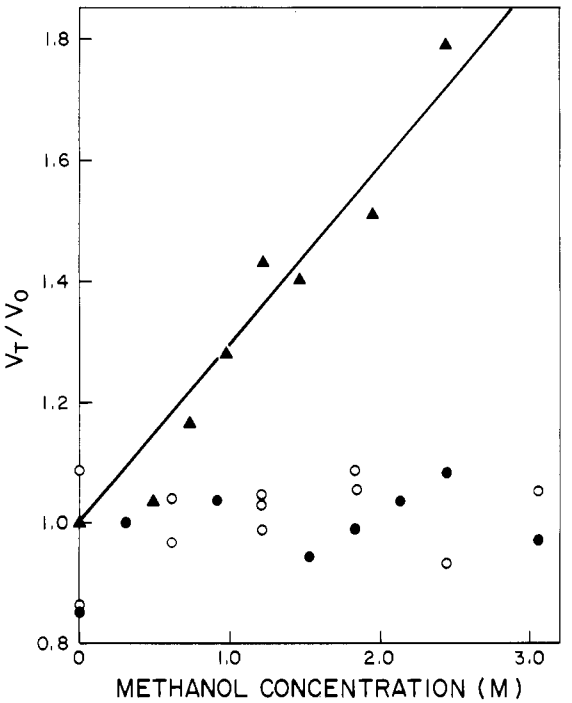


FIGURE 1: Initial rates of total solvolysis (V_T) of 0.45 mM benzylpenicillin (▲) and **3d**, the latter at concentrations of 1.9 mM (●) and 13.1 mM (○), catalyzed by the P99 β-lactamase, as a function of methanol concentration. The rates are shown as their ratios to V_0, the solvolysis (hydrolysis) rate in the absence of methanol.

involves a covalent acyl-enzyme intermediate, where the active-site nucleophile is a specific serine hydroxyl group (Knott-Hunziker et al., 1979, 1982; Cohen & Pratt, 1980; Fisher et al., 1980; Anderson & Pratt, 1981; Joris et al., 1984). It thus seemed reasonable to consider the depsipeptide reactions in terms of such a mechanism, Scheme I (where E·$R''CO_2R'$ represents the Michaelis complex of enzyme and substrate, E–COR'' the acyl-enzyme intermediate, $R''CO_2R$ the alcoholysis product, and $R''CO_2^-$ the hydrolysis product). We will first consider our results with the P99 enzyme. If deacylation were rate-determining under conditions of sub-

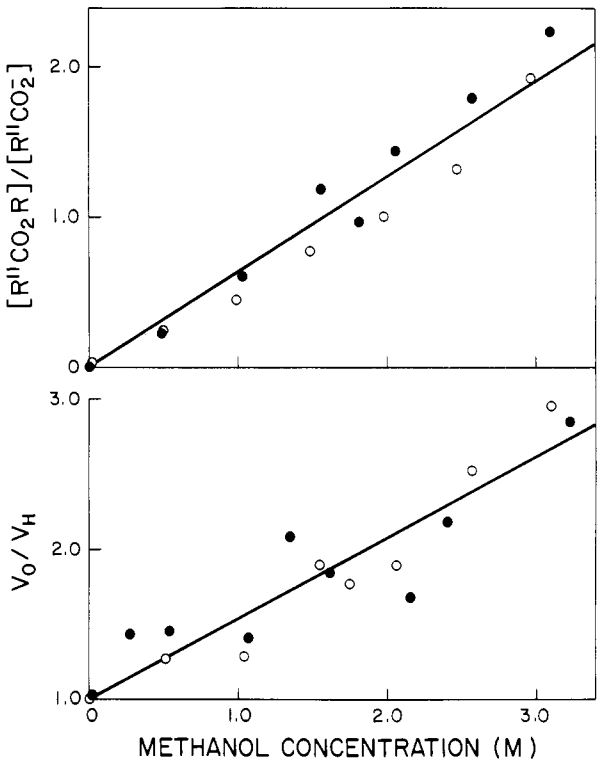
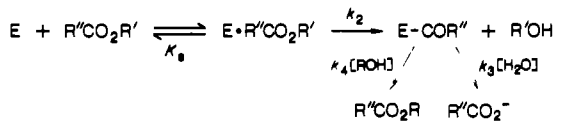
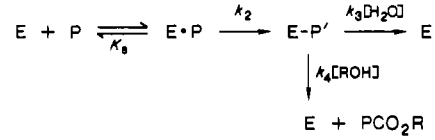


FIGURE 2: Plot of V_0/V_H as a function of methanol concentration for the P99 β-lactamase catalyzed solvolysis of **3d** at concentrations of 1.79 mM (●) and 13.6 mM (○); V_H is the initial rate of hydrolysis at a given methanol concentration and V_0 the initial rate of solvolysis (hydrolysis) in the absence of methanol. Also shown, in the upper panel, is a plot of the methanolysis to hydrolysis product ratio as a function of methanol concentration from these same experiments.

Scheme I



Scheme II



strate saturation, then substrates with the same acyl group and chemically very different alcohol leaving groups should generate the same acyl-enzyme and thus yield identical k_{cat} values. This seems close to being true, given the k_{cat} values for compounds **3a** and **3c–e** (Table I), but not quite. One might conclude perhaps that deacylation was completely rate determining for the better leaving groups (in **3d** and **3e**) and not quite rate determining for the poorer pair (**3a** and **3c**). Such considerations, taken alone, can, however, be misleading, as further experiments proved.

Knott-Hunziker et al. (1982) have shown that during catalysis of benzylpenicillin hydrolysis by other class C enzymes, hydrolysis of the acyl- (penicilloyl-) enzyme is rate-determining at saturation, i.e., in Scheme II, in the absence of ROH and where E–P′ represents the acyl-enzyme and PCO_2^- the penicilloate hydrolysis product, $k_{cat} = k_3[H_2O]$. A major source of evidence for this conclusion was the fact that addition of an alcohol cosolvent (methanol or ethanol) led to an increase in k_{cat} and, at the same time, to the quantitatively corresponding appearance of α-methyl benzylpenicilloate as a product. It was proposed that, in the presence of the mixed solvent, the acyl-enzyme partitioned between hydrolysis and methanolysis. This is also shown in Scheme II, where PCO_2R is the methanolysis product.

We therefore explored the application of this acyl-enzyme-trapping technique with depsipeptides and the P99 enzyme. First, the presence of methanolysis products was sought by NMR, with the results shown in Table II. The P99 β-lactamase-catalyzed reaction of benzylpenicillin in methanol/water mixtures does in fact yield the methanolysis product, suggesting that Scheme II does apply with this enzyme also. Furthermore, as Figure 1 shows, the rate does increase linearly with methanol concentration. The steady-state rate equation relevant to Scheme II is given in eq 1, where V_T is the initial rate of disappearance of S

$$V_T = \frac{k_{cat}E_0S_0}{K_m + S_0} \quad (1)$$

and where

$$K_m = K_s\left(\frac{k_3[H_2O] + k_4[MeOH]}{k_2 + k_3[H_2O] + k_4[MeOH]}\right)$$

$$k_{cat} = \frac{k_2(k_3[H_2O] + k_4[MeOH])}{k_2 + k_3[H_2O] + k_4[MeOH]}$$

Under saturating conditions and if deacylation is rate-determining, i.e., $k_2 \gg k_3[H_2O] + k_4[MeOH]$, V_T will be given by

$$V_T = (k_3[H_2O] + k_4[MeOH])E_0$$

which may also be expressed as eq 2, where V_0 is the initial rate in the absence of methanol. (In contrast, if acylation

$$V_T/V_0 = 1 + k_4[MeOH]/k_3[H_2O] \quad (2)$$

were rate-determining, the equation $V_T/V_0 = 1$ would apply at all methanol concentrations.) The data of Figure 1 yield a k_4/k_3 ratio of 16.2, and thus, since $k_3 = 0.413\ s^{-1}\ M^{-1}$ (from V_0), $k_4 = 6.7\ s^{-1}\ M^{-1}$. Also, Scheme II predicts that, at any time, eq 3 will hold. The data of Table II yield for benzyl-

$$[PCO_2R]/[PCO_2^-] = k_4[MeOH]/k_3[H_2O] \quad (3)$$

penicillin a k_4/k_3 value of 16.0, which is in excellent agreement with the value from the kinetic data and thus in support of Scheme II. These results with benzylpenicillin nicely agree with the findings of Knott-Hunziker et al. (1982), where k_4/k_3 values of 31 and 6.3 were found for the *Pseudomonas aeruginosa* 18S and ampC β-lactamases, respectively. Thus, solvolysis of a penicilloyl-enzyme intermediate is rate-determining at saturation for all three enzymes.

The data of Table II for the depsipeptides **3a–e** indicate first that methanolysis of the depsipeptides is catalyzed by the β-lactamase and, second, that the extent of methanolysis is leaving group independent. The latter result indicates a common intermediate on the paths of hydrolysis and methanolysis which thus must occur subsequent to departure of the hydroxy acid leaving group. This is of course suggestive of an acyl-enzyme and Scheme I. Interpreted in terms of Scheme I and an equation analogous to eq 3, an averaged partition ratio (k_4/k_3) of 26 ± 5 can be derived from the data of Table II.

The effect of methanol on the kinetics of the depsipeptide reaction was also determined. Equation 1 predicts that V_T will increase with methanol concentration unless $K_m \gg S_0$ [in which case $V_T = (k_2/K_s)E_0S_0$] or $k_3[H_2O] + k_4[MeOH] \gg k_2$ (in which case $V_T = k_2E_0S_0$). At $S_0 \approx K_m$ and $S_0 \approx 6K_m$, V_T for **3d** was invariant with methanol (Figure 1), supporting the latter explanation.

According to eq 1, V_H, the initial rate of hydrolysis in the presence of the cosolvent methanol, is given by eq 4, where

$$V_H = \frac{k_2k_3[H_2O]E_0S_0/(k_2 + k_3[H_2O] + k_4[MeOH])}{K_m + S_0} \quad (4)$$

K_m is as in eq 1. In reciprocal form this equation yields eq 5. If acylation were rate-determining, this equation would

$$\frac{1}{V_H} = \left[K_s\left(\frac{k_3[H_2O] + k_4[MeOH]}{k_2 + k_3[H_2O] + k_4[MeOH]}\right) + S_0\right]\left(\frac{k_2 + k_3[H_2O] + k_4[MeOH]}{k_2k_3[H_2O]E_0S_0}\right) \quad (5)$$

reduce to eq 6, where V_0 [$=k_2E_0S_0/(K_s + S_0)$] is the initial

$$\frac{V_0}{V_H} = (1 + k_4[MeOH]/k_3[H_2O]) \quad (6)$$

rate in the absence of methanol. Conversely, if deacylation were rate-determining, eq 5 would reduce to eq 7, where V_0

$$\frac{V_0}{V_H} = \left[1 + \left(\frac{K_m^\circ}{K_m^\circ + S_0}\right)\left(\frac{k_4[MeOH]}{k_3[H_2O]}\right)\right] \quad (7)$$

is the initial rate in the absence of methanol and K_m° is the Michaelis constant under these conditions.

Equations 6 and 7 both predict a linear dependence of V_0/V_H on methanol concentration, but only in the case of eq 7 is the dependence substrate concentration dependent. The data of Figure 2 show that the slope of the V_0/V_H vs. [MeOH] plots does not change as S_0 varies between K_m° and $6K_m^\circ$. Thus eq 6 better fits the data. If it is assumed that Scheme I and eq 6 apply, a value of 29.4 ± 3.9 for the ratio k_4/k_3 can be derived from the kinetic data of Figure 2. The product ratio $[R''CO_2R]/[R''CO_2^-]$, determined under the conditions of the kinetics experiment and also plotted in Figure 2, yields, by an equation analogous to eq 3, a k_4/k_3 ratio of 34.8 ± 2.8, which

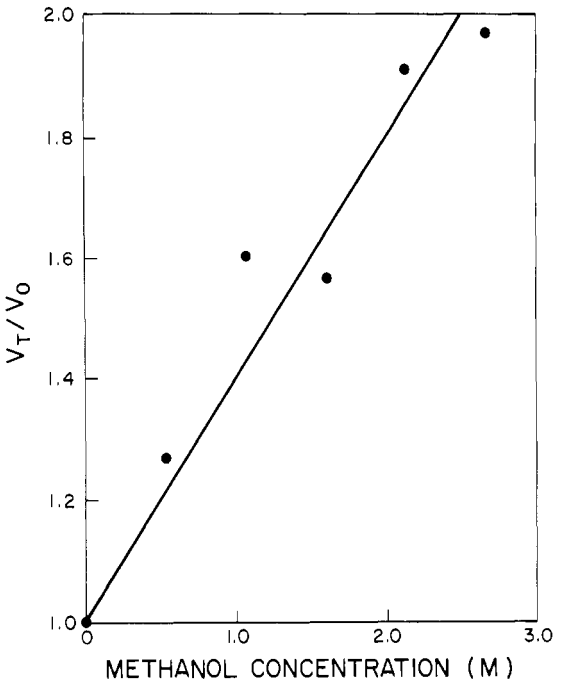


FIGURE 3: Initial rates of total solvolysis (V_T) of 1.29 mM **3d**, catalyzed by the PC1 β-lactamase, shown as their ratios to V_0, the solvolysis (hydrolysis) rate in the absence of methanol.

clearly is in good agreement with both that derived from the rate measurements and also that from the NMR experiments of Table II. Note that the reaction conditions were slightly different in the latter experiment. This result shows that the ratio of methanolysis to hydrolysis does not change with substrate concentration and is an excellent test of the mechanism of Scheme I. The additional result is that enzyme acylation is rate-determining under maximal velocity conditions. If k_{cat} is in fact k_2 for **3d** (and thus $K_m = K_s$), this must be true for **3a**, **3c**, and **3e** also, since the k_{cat} values for these compounds are less than or equal to that of **3d** (Table I) and the same acyl-enzyme would be involved.

Kinetics of Class A β-Lactamase-Catalyzed Solvolysis in Aqueous Methanol. The rate of disappearance of 5–10 mM **3d** (V_T) in the presence of either the *B. cereus* β-lactamase (3.2 μM) or the TEM-2 β-lactamase (1.5 μM) was not affected by methanol, in concentrations up to 2.1 M. In contrast, the initial rates of the PC1 β-lactamase (1.29 μM) catalyzed reaction of 1.29 mM **3d** increased with methanol concentration as shown in Figure 3.

Evidence for an Accumulating Acyl-Enzyme on Interaction of **3d** *with the PC1 β-Lactamase.* Under the conditions described above, a stoichiometric (equivalent to the enzyme concentration) burst of *m*-hydroxybenzoate was produced in an exponential fashion on mixing the PC1 β-lactamase and **3d** in the stopped-flow spectrophotometer. A plot of the observed pseudo-first-order rate constants vs. substrate concentration was linear within experimental uncertainty, yielding a slope (k_2/K_s) of 1900 s^{-1} M^{-1}.

Dilution of mixtures of **3d** and the PC1 β-lactamase into a benzylpenicillin assay mixture demonstrated the existence and accumulation of an intermediate species that reverted to free enzyme with a first-order rate constant of 0.13 s^{-1}.

Inhibition by Peptides. The peptides **6** and **7**, the former of which is the peptide analogue of **3d**, appeared to be competitive inhibitors of nitrocefin hydrolysis by the P99 β-lactamase. Under comparable conditions the K_m value of **3d** was 0.56 mM, while the competitive inhibition constants of **6** and **7** were 5.4 and 4.85 mM, respectively. Enzyme concentrations that, under the conditions of the steady-state experiments described above, led to rapid hydrolysis of the depsipeptides had no effect on the peptides; the latter are thus much poorer than the analogous depsipeptides as β-lactamase substrates.

Discussion

Our previously reported experiments showed that specific acyclic depsipeptides of structure **3**, i.e., acyl-D-alanyl-D-alanine analogues, were substrates of β-lactamases from all three classes, A, B, and C (Pratt & Govardhan, 1984). Quantitative data to this effect for reaction of several depsipeptide substrates **3a–e**, **4**, and **5**, with the P99 (class C) β-lactamase and three class A enzymes are shown in Table I. The immediate conclusion from these data, as it was from a smaller amount of similar data in the earlier paper referred to above, is that depsipeptides are in general poor (with respect to the best bicyclic β-lactam substrates) substrates of β-lactamases. All values of k_{cat}/K_m determined are at least an order of magnitude lower than that for benzylpenicillin and in most cases much lower than that. Inspection suggests that in the case of the class C enzyme the difference lies mainly in K_m, while with the class A enzymes both k_{cat} and K_m are significantly less favorable for the depsipeptides. The remaining experiments in this paper were designed to determine the meaning of the steady-state parameters and thus to achieve a clearer definition of the differences between the interactions of depsipeptides and of penicillins with these β-lactamases.

The results obtained with the class C P99 β-lactamase will first be discussed. The methanolysis experiments described above showed that this enzyme catalyzes acyl transfer from depsipeptide substrates to methanol. Detailed kinetic analysis of this reaction suggested that Scheme I obtained and that enzyme acylation is rate-determining to both methanolysis and hydrolysis under conditions of substrate saturation.

We can now look a little more closely at the data of Table I, making comparisons between the individual depsipeptides and between the depsipeptides as a group and the β-lactams. With respect to benzylpenicillin, it should be noted that, as discussed in Results, deacylation is rate-determining at saturation, i.e., k_{cat} must be k_3 (25 s^{-1}), and thus the acylation rate must be significantly greater, i.e., $k_2 > 25$ s^{-1}, and K_s must thus be significantly greater than K_m, i.e., >0.015 mM [since, for Scheme I, $K_m = K_s(k_3/k_2)$]. Unfortunately, since the acyl-enzyme presumably derived on interaction of deacetylcephalothin with the P99 enzyme did not appear to be accessible to methanol, the steady-state kinetics of this compound cannot be further dissected at present. Nonetheless, from the k_{cat} value it is clear that the acyl-enzyme from the cephalosporin substrate must hydrolyze at least an order of magnitude faster than that from benzylpenicillin. The P99 enzyme is in this sense at least (Knott-Hunziker et al., 1982) a cephalosporinase.

The noncovalent binding ($1/K_s$) of the depsipeptides to the P99 enzyme may be weaker than that of the β-lactams, but not by more than an order of magnitude and possibly not at all. One factor which might be expected to contribute, in general, to weaker binding by depsipeptides (and peptides) arises from the greater flexibility of the depsipeptides than that of bicyclic β-lactams. The acyclic compounds have two significant degrees of freedom not available to penicillins, rotations about the terminal C_α–O and about the penultimate C_α–CO bonds. Rotation about both of these is probably facile and relatively unrestricted (DeCoen et al., 1981; Lamotte-Brasseur et al., 1984; Labischinski et al., 1985). Empirical methods (Andrews et al., 1984) suggest that in the extreme case of selection of a particular conformer from a system with

two degrees of unrestricted internal rotational freedom the binding strength with respect to a rigid analogue would be reduced by about 1.4 kcal/mol, i.e., by a factor of about 30 through the loss of rotational entropy on binding.

The major difference in shape between the β-lactams and the depsipeptides **3a** and **3b**, not eliminated by facile single-bond rotations, is the geometry of the scissile acyl function, the pyramidal nitrogen of the β-lactam vs. the planar oxygen of the depsipeptide. Although the barrier to rotation about ester linkages is less than that about amides, it is still around 10 kcal/mol (Allinger & Chang, 1977), and thus the rotation of about 45° required to optimally overlap the penicillin and depsipeptide structures **3a** and **3b** would cost some 7 kcal/mol, i.e., lead to some (1.4×10^5)-fold weaker binding. Since this difference is not apparent, it is unlikely that **3a** and **3b** are significantly strained in the dominant noncovalent binding mode. That this is true is also indicated by the comparably strong binding of the much more torsionally resistant peptides **6** and **7**. The slightly weaker binding of **6** with respect to its ester analogue **3d** may reflect the absence of a suitable hydrogen-bond acceptor for the amide N–H at the binding site. Of course, since these peptides are not substrates of the P99 β-lactamase, it might be argued that, whereas peptides bind only in a nonproductive mode, depsipeptides also bind, even more weakly, in a distorted but productive fashion.

It is noteworthy that the aromatic depsipeptides **3c–e** bind to the enzyme more strongly than **3a** and **3b**. Although the aromatic ring itself may be important for favorable hydrophobic interactions (for example, compare the K_s value of **3a** with that of **3b**), consideration of the K_s values of the group **3c–e** suggests that more must be involved. Cohen (1983) has suggested that much data on the relative antibiotic effectiveness of β-lactams can be rationalized by the proposition that the distance between the β-lactam carbonyl oxygen atom and the carbon atom of the 3 (in penicillins)-carboxylate group is crucial to receptor recognition. Since the relevant receptors are presumably DD-peptidases [Lamotte-Brasseur et al. (1984) have also shown, it might be noted, that distances similar to the critical one described above occur in the most stable conformers of D-alanyl-D-alanine peptides] and because of the probable structural and functional homologies between the DD-peptidase and β-lactamase active sites (Tipper & Strominger, 1965; Waxman & Strominger, 1983; Pratt & Govardhan, 1984; Kelly et al., 1986; Samraoui et al., 1986), this parameter should be considered with respect to the latter enzymes also. The present results suggest that, at least with respect to initial noncovalent binding, this distance parameter is not critical to productive interaction with the P99 β-lactamase. Although depsipeptides **3a–c** and **4** can be arranged in reasonable conformations where this distance is penicillin-like (although the relative orientations of the two functional groups vary), this is not true of **3d** and **3e**; consequently, it is quite striking that **3d** is bound significantly more tightly than the other depsipeptides. It seems likely, therefore, that the conformation of the enzyme and/or substrate is quite different in the noncovalent complexes of at least some of these substrates. It should be mentioned in this regard that although **3a** and **3b** are also substrates of the *Streptomyces* R61 DD-peptidase (Pratt et al., 1985) **3c–e** are not, perhaps indicating a less flexible and more specific active site in the DD-peptidases.

The relative chemical reactivity of the depsipeptides is not well reflected in the enzyme acylation rate constants, k_2. On the basis of chemical reactivity toward a standard nucleophile, hydroxide ion (Table I), one might have expected k_2 to increase in the order **3a**, **3b** < **3c** < **3d**, **3e**, with more than 1 order of magnitude between the extremes. In reality (Table I), a leveling effect is seen, with no more than a factor of 2 separating **3a** and **3e**. This must reflect the factors discussed above with respect to the binding of these substrates, i.e., that it is not identical and uniformly productive. One might speculate, for example, that the enzyme distortion needed to accommodate the carboxylate groups of **3d** and **3e** affects the position of the scissile ester bond with respect to the serine nucleophile in such a way as to apparently decrease the reactivity of these esters. The parameter k_2/K_s ($=k_{cat}/K_m$) does not accurately reflect the order of chemical reactivity either. Again, this suggests that the productive binding modes are different (rather than one identical productive mode among a variable number of nonproductive ones). The significantly larger k_{cat}/K_m values for the penicillin and cephalosporin are also not understandable on a purely chemical basis without the proposal of a more effective binding mode for bicyclic β-lactams than for the depsipeptides. Conformational and electrostatic potential analysis of β-lactams and acyclic analogues by Ghuysen and co-workers (Lamotte-Brasseur et al., 1984) suggested to them that different modes of binding of these two classes of molecules to DD-peptidases must occur; this may well be true for β-lactamases also.

In contrast to the conclusions above with respect to chemical reactivity, the inability of acyclic peptides to acylate β-lactamases, as observed here and previously (Pratt et al., 1980; Joris et al., 1985), and thus to turn over, since deacylation (see below) would of course be facile, probably does reflect their chemically inert nature, either directly, i.e., through their poor susceptibility to nucleophilic attack, or indirectly, in the higher barrier to amide bond torsion and thus the much smaller likelihood of the peptide achieving the productive binding mode.

The rates of deacylation of the (phenylacetyl)glycyl-β-lactamase must be significantly greater than the measured acylation rates (k_{cat}), i.e., $k_3 > 100\ s^{-1}$. This result means that the deacylation of the penicilloyl-enzyme ($k_3 = 25\ s^{-1}$) must be significantly slower than that of the (phenylacetyl)glycyl enzyme. This order of reactivity does not follow on the basis of the electronic effects of the acyl group substituents, but must result from steric effects, either directly or indirectly through an induced protein conformation. It is interesting to note that the same difference in deacylation rates, although probably to a greater degree, is seen in the hydrolysis rates of the acyl-enzymes generated from the DD-peptidases with acyclic substrates on one hand and penicillins on the other; here, of course, the effect probably leads to the antibiotic action of penicillin. Despite the rate differences, however, the data of Table II show that the relative accessibility to water and methanol of the acyl group of the acyl-enzymes arising from depsipeptides and from penicillins is very similar. This perhaps indicates that the effect of the thiazolidinyl substituent in the latter complex stems from differences in the active-site conformation rather than from direct steric interference with nucleophile approach. It should also be noted that the acyl-enzyme generated from deacetylcephalothin (a) hydrolyzes much more rapidly than the penicilloyl-enzymes and (b) is much less susceptible to methanolysis. Both of these features suggest a somewhat different acyl-enzyme, perhaps characteristic of the class C cephalosporinases. It should be noted, however, that Knott-Hunziker et al. (1982) report some methanolysis of cephalosporin C in the presence of the *P. aeruginosa* 18S β-lactamase.

Depsipeptide **4** illustrates the effect on the hydrolysis kinetics of changing the acyl group from one optimal for a β-lactamase to one (*N*,*N*'-diacetyl-L-lysyl) optimal for substrate models for the *Streptomyces* DD-peptidases. The effect of this change on β-lactamase turnover is not great, however, either on the steady-state parameters (Table I) or the methanol/water partitioning of the acyl-enzyme (Table II). Although a wider panel of structures should be surveyed, it is clear that the acyl-group binding site of the P99 β-lactamase does not specifically favor the *N*,*N*'-diacetyl-L-lysyl group.

The depsipeptide **5** is significant since it is a D-lactate substrate, of reactivity comparable to **3**, where the hydrolysis can be followed spectrophotometrically; it has the further advantage of being commercially available, as a DL mixture, which can be used directly in assays. Methanolysis experiments indicate that for **5** also acylation of the P99 β-lactamase is rate-determining under saturating conditions. This depsipeptide is also a substrate of the *Streptomyces* R61 DD-peptidase and can be used in a spectrophotometric assay for this enzyme [see, for example, Faraci and Pratt, (1986)], although it is significantly less sensitive than assays using *N*,*N*'-diacetyl-L-lysyl-D-alanyl-D-lactate (Pratt et al., 1985).

The results of experiments with the depsipeptides and class A β-lactamases are interestingly different from those with the P99 enzyme. As noticed earlier with **3a** and **3b** (Pratt & Govardhan, 1984), the TEM-2 and *B. cereus* I β-lactamases appear somewhat poorer than the P99 enzyme as catalysts of **3d** hydrolysis (Table I). On the other hand, TEM-2 and the *B. cereus* enzyme are very effective penicillinases, much more so than the P99 enzyme. Consequently, the depsipeptides are, relative to penicillins, much poorer substrates of these enzymes than they are of the P99 enzyme.

Direct comparison of other kinetic parameters of Table I between **3d** and benzylpenicillin is not possible for the TEM-2 and *B. cereus* I β-lactamases since it is not known for the penicillins just what step, acylation or deacylation, is rate-determining under conditions of substrate saturation. The latter cannot be determined from application of the methanol partitioning method, as it can be for class C enzymes, since, as is well-known (Brenner et al., 1981; Anderson & Pratt, 1981; Knott-Hunziker et al., 1982), the penicilloyl-enzymes of the TEM-2 and *B. cereus* β-lactamases have not yet been trapped with nucleophiles other than water and, specifically, do not undergo methanolysis. This might suggest that in these cases the acyl-group acceptor must specifically bind prior to reaction. It is possible, for example, that a specifically occluded water molecule might be the acceptor. It might be noted here also that, although methanol and ethanol can replace water in nucleophilic attack on the penicilloyl-enzyme intermediate of class C enzymes, other small nucleophiles, like ammonia or hydroxylamine cannot (Knott-Hunziker et al., 1982). Hence here too there is a clear indication of acceptor specificity. Our earlier work with depsipeptides and the P99 enzyme (Pratt & Govardhan, 1984) also pointed in this direction: certain amino acids could function as acyl-group acceptors but peptide amines could not.

The data of Table II show that methanol is also not an acceptor of the (phenylacetyl)glycyl-enzyme intermediates generated on interaction of **3d** with either the TEM-2 or *B. cereus* I β-lactamase. This result appears to strongly further demonstrate the sheltered nature of the acyl group in these acyl-enzymes. In addition, it shows that the thiazolidinyl substituent of the penicilloyl-enzyme is not, directly or indirectly, alone responsible for the isolation of the acyl group. There is, of course, thus no evidence for the occurrence of acyl-enzymes in these reactions, any more than there is with benzylpenicillin, but the results of studies with poorer β-lactam substrates (Fisher et al., 1980; Anderson & Pratt, 1981, 1983) imply their existence with β-lactams at least.

Despite the uncertain meaning of the steady-state parameters for TEM-2 and *B. cereus* enzymes [although most indications suggest $k_{cat} = k_2$ (Fisher, 1984; Bicknell & Waley, 1985)], the data of Table I show, at least, that deacylation of their penicilloyl-enzymes is much more facile than that of the P99 enzyme. If this is so for the depsipeptides also, then k_{cat} must be k_2 for the hydrolysis of **3d** catalyzed by the former two enzymes. Acylation of these enzymes by **3d** must therefore be much slower than by benzylpenicillin, pointing to poorly productive binding by **3d** in these cases also.

The results given in Tables I and II show that the *S. aureus* PC1 β-lactamase is clearly distinct from the other class A enzymes discussed above. Not only are the K_m and k_{cat} values for **3d** much smaller than those of the other enzymes but, more importantly, solvolysis of **3d** yields substantial quantities of methyl (phenylacetyl)glycinate. Further, the rate of solvolysis of **3d** is accelerated by methanol in a linear fashion (Figure 3). These data strongly suggest the existence of an acyl-enzyme intermediate, whose breakdown is rate-determining at saturation and which can be trapped by methanol as well as water, i.e., Scheme I applies. The product distribution data of Table II yields a value of 11.5 for k_4/k_3, while the kinetic data of Figure 3 give, via eq 2, a value of 22.0 ± 2.0. The discrepancy between these numbers probably reflects some contribution to the latter from a nonspecific activation of this enzyme, unlike the P99 enzyme, by small organic molecules; methanol (and acetonitrile) does (do) accelerate benzylpenicillin hydrolysis by the PC1 enzyme, although no methyl α-penicilloate is produced.

The existence of an accumulating covalent intermediate in this case was confirmed in two ways. First, in stopped-flow experiments, a stoichiometric burst of *m*-hydroxybenzoate was observed on mixing enzyme and depsipeptide. The measured rate constants yielded $k_2/K_s = 1.9 \times 10^3\ s^{-1}\ M^{-1}$, in good agreement with the k_{cat}/K_m value from steady-state experiments (Table I); separation of k_2 and K_s was not possible because of the high substrate concentrations required, but the lower limits established ($k_2 > 10\ s^{-1}$, $K_s > 5$ mM) suggest that these parameters may well be comparable to those of the other β-lactamases. In a second type of experiment an inactive (with respect to benzylpenicillin hydrolysis) intermediate was found to form on incubation of **3d** and the PC1 β-lactamase. The rate constant for conversion of this species to active enzyme agrees well with k_{cat}, indicating that this process is rate-determining under conditions of saturating substrate.

Although methanolysis of **3d** could thus be catalyzed by the PC1 β-lactamase, methanol had no effect on the products of benzylpenicillin hydrolysis in the presence of this enzyme. The penicilloyl-enzyme here also must be shielded from nucleophiles other than water, although in this case the thiazolidinyl substituent may be directly or indirectly responsible. In any event, no evidence is available from these experiments as to whether a penicilloyl-enzyme accumulates or not, although the results with **3d** suggest that it might. The PC1 β-lactamase is known to form longer lived acyl-enzymes than do the other class A β-lactamases with cephalosporin substrates (Anderson & Pratt, 1981, 1983; Faraci & Pratt, 1985).

It has been suggested (Fisher, 1984) as a generalization that with class C β-lactamases deacylation is rate-determining at saturation, while with class A β-lactamases acylation is. It seems likely, however, that this difference is very much en-

zyme- and substrate-dependent. Thus, although the P99 class C β-lactamase forms an accumulating acyl-enzyme with benzylpenicillin, it does not with the depsipeptides. Conversely, although the PC1 (class A) enzyme may or may not yield a stable penicilloyl-enzyme, it certainly does form an accumulating acyl-enzyme during **3d** turnover.

As a final comparison it would be appropriate to compare the above results with those obtained for the same range of substrates, viz., penicillin, acyclic depsipeptide, and acyclic peptide, with representative DD-peptidases. Currently such data are available for the membrane-bound DD-peptidases from *B. subtilis* (PBP5), *S. aureus* (PBP4), *E. coli* (PBP5) (Waxman & Strominger, 1982), and *Streptomyces* K15 (Leyh-Bouille et al., 1986; Nguyen-Distèche et al., 1986). These can be compared with the present results with β-lactamases and with data for the soluble *Streptomyces* R61 DD-peptidase (Kelly et al., 1986) already referred to above.

The novel conclusion from such a comparison is that D-lactate depsipeptides are hydrolyzed at not greatly dissimilar rates by all of these enzymes. There is much evidence for an acyl-enzyme intermediate in all cases, whose hydrolysis is commonly rate determining at saturation (the main exception to the latter are the β-lactamases). Consequently, the serine nucleophile of these active sites is comparably reactive in each case. In contrast, the analogous peptides hydrolyze only in the presence of the DD-peptidases. Since the serine hydroxyl group appears to have comparable nucleophilicity in both the DD-peptidases and β-lactamases, the inability of amides to acylate β-lactamase active sites may lie in the absence of an electrophilic catalyst from the latter enzymes that is present in the former.

All indications are that, in both classes of enzymes, access of the acyl acceptor to the acyl-enzyme is carefully controlled, i.e., there are specific binding sites for acceptors, including water in cases where it is an acceptor. It is apparently not possible for the DD-peptidases to permit access of water to the penicilloyl-enzyme in the way β-lactamases do, perhaps because such a modification would in some way disrupt the ability of the enzyme to cleave peptides.

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Medium Viscosity Regulates the Activity of Membrane-Bound and Soluble Phospholipase A_2[†]

Saul Yedgar,* Nurith Reisfeld, David Halle, and Itzhak Yuli

Department of Biochemistry, Hebrew University, Hadassah Medical School, Jerusalem, Israel, and Department of Membrane Research, The Weizmann Institute, Rehovot, Israel

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ABSTRACT: Medium viscosity is a regulator of very low density lipoprotein production by cultured hepatocytes; their secretion and synthesis are inversely proportional to the extracellular fluid viscosity. The possibility that the mechanism of this extracellular effect on cell function involves modulation of cell membrane component(s) was considered. Along with this assumption, we studied the effect of medium viscosity on the activity of phospholipase A_2 (PLA_2), an enzyme present in the cell surface membrane, and the activity has been correlated with cellular secretion. We have found that culture medium viscosity inhibits the activity of PLA_2 in the plasma membrane of cultured liver cells, concomitantly with the inhibition of lysosomal enzyme and lipoprotein secretion. It was also found that the degradation of liposomal phosphatidylcholine by soluble snake venom PLA_2 is inversely proportional to the solvent viscosity. The possibility that the effect of medium viscosity on the enzymatic reaction involves the modulation of dynamic properties of membrane phospholipids was then considered. This hypothesis was examined by monitoring the fluorescence depolarization of fluorophores incorporated into phospholipid vesicles. No significant effect of the solvent viscosity on the phospholipid bilayer was observed. It is proposed that the regulation of cellular secretion by extracellular fluid viscosity involves modulation of the cell membrane PLA_2 activity.

The viscosity of blood and plasma is elevated in numerous pathological conditions and is considered a risk factor for coronary heart disease (Lowe et al., 1981). It has been studied mainly in relation to circulation and hemodynamics. In recent years, more attention has been paid to the role of fluid viscosity in biochemical and cellular processes, as it has been shown that solvent viscosity is an important determinant in protein dynamics and enzyme–substrate interaction (Gavish & Werber, 1979; Breece, et al., 1980; Mckinnie & Olson, 1981; Sawicki & Khaleque, 1983). The relevance of extracellular fluid viscosity in cell function has been demonstrated by Yedgar et al., who showed that viscosity is a regulator of lipoprotein metabolism, both in vivo and in cultured hepatocytes (Yedgar et al., 1982, 1985). Increasing the plasma viscosity of hyperlipidemic rats markedly reduced plasma triglyceride and cholesterol levels (Yedgar et al., 1985). In hepatocyte cultures, the viscosity of the extracellular fluid has been shown to be a regulator of very low density lipoprotein (VLDL)[1] production (Yedgar et al., 1982). Increasing medium viscosity linearly inhibited secretion and synthesis of protein and lipid VLDL components, while their cellular levels remained unaltered (Yedgar et al., 1982). Concordant with these findings, we observed that medium viscosity had an immediate inhibitory effect on the secretion of lysosomal enzymes from cultured liver cells (Yedgar et al., 1986b), indicating that the viscosity affects primarily exocytosis prior to its effect on synthesis. In these studies, medium viscosity was modulated by the addition

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* Address correspondence to this author at the Department of Biochemistry, Hebrew University, Hadassah Medical School.

[1] Abbreviations: PLA_2, phospholipase A_2; PC, phosphatidylcholine; C_6-NBD-PC, 1-acyl-2-[[*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-caproyl]phosphatidylcholine; C_6-NBD-FA, C_6-NBD fatty acid; DOPC, dioleoylphosphatidylcholine; HMEM, HEPES-buffered minimum essential medium; DME, Dulbecco-modified Eagle's medium; Dex, Dextran T-500; Xa, xanthan gum; MeC, methylcellulose; DPH, 1,6-diphenyl-1,3,5-hexatriene; ANS, 1-anilinonaphthalene-8-sulfonic acid; *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; VLDL, very low density lipoprotein; 4MU, 4-methylumbelliferone; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TLC, thin-layer chromatography.

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