N-(Phenylacetyl)glycyl-D-aziridine-2-carboxylate, an Acyclic Amide Substrate of β-Lactamases: Importance of the Shape of the Substrate in β-Lactamase Evolution†

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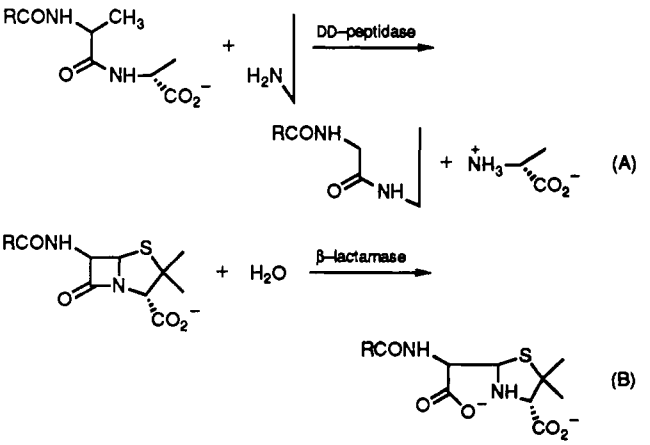
ABSTRACT: Certain acyclic depsipeptides, but not peptides, are substrates of typical β-lactamases [Pratt, R. F., & Govardhan, C. P. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1302]. This may reflect either the greater chemical reactivity of depsipeptides (and of β-lactams, the natural substrates) than peptides or the greater ease of distortion of the depsipeptide (ester) than the peptide (amide) group into a penicillin-like conformation. The latter explanation has been shown to be more likely by employment of a novel β-lactamase substrate, *N*-(phenylacetyl)glycyl-D-aziridine-2-carboxylate, which combines a high chemical reactivity with a close to tetrahedral amide nitrogen atom. Although this substrate was better (higher k_{cat}/K_M) than a comparable depsipeptide for β-lactamases, it was poorer than the depsipeptide for the *Streptomyces* R61 D-alanyl-D-alanine peptidase (which catalyzes specific peptide hydrolysis). It therefore seems likely that one vital feature of the putative evolution of a DD-peptidase into a β-lactamase would have been modification of the active site to, on one hand, accommodate bicyclic β-lactams and, on the other, exclude productive binding of planar acyclic amides. Certain serine β-lactamases and the R61 DD-peptidase also catalyze methanolysis and aminolysis by D-phenylalanine of the *N*-acylaziridine. The latter reaction, the first amide aminolysis shown to be catalyzed by a β-lactamase, is a very close analogue of the transpeptidase reaction of DD-peptidases. The methanolysis reaction appeared to proceed by way of the same acyl-enzyme intermediate as formed from depsipeptides possessing the same acyl moiety as the aziridine. The kinetics of methanolysis were employed to determine whether acylation or deacylation was rate limiting to the hydrolysis reaction under saturating substrate concentrations. The kinetics of the aminolysis reaction, catalyzed by the *Enterobacter cloacae* P99 β-lactamase, showed the characteristics of, and were interpreted in terms of, a sequential mechanism previously deduced for depsipeptides and this enzyme [Pazhanisamy, S., & Pratt, R. F. (1989) *Biochemistry 28*, 6875–6882]. This mechanism features two separate binding sites, only one of which is productive. Strikingly, the binding of the *N*-acylaziridine to the nonproductive site was very tight, such that essentially all hydrolysis at substrate concentrations above $0.1K_m$ proceeded via the ternary complex; this could also be true of penicillins.

The interaction of β-lactam antibiotics with bacteria is mediated by two groups of enzymes, the DD-peptidases on one hand, which catalyze biosynthesis of cell-wall peptidoglycan (Scheme IA) and which are the primary targets of these antibiotics, and the β-lactamases on the other, which catalyze hydrolysis of β-lactams (Scheme IB), leading to loss of their antibiotic activity, and provide much of the resistance of bacteria to these antibiotics.

The inhibition of DD-peptidases by β-lactams has long been known (Cooper, 1956; Blumberg & Strominger, 1974) to involve covalent interaction between the two. Since both DD-peptidases and β-lactamases react specifically and covalently with β-lactams, and because of the structural resemblance between their natural substrates, D-alanyl-D-alanine-terminating peptides and β-lactams, respectively, Tipper and Strominger (1965) suggested that β-lactamases were evolutionary descendents of DD-peptidases.

There is now much evidence, both structural and functional, for this evolutionary relationship. Although there is only limited evidence for homology on a primary level (Waxman & Strominger, 1983; Joris et al., 1988), it seems likely that there is much secondary and tertiary similarity (Kelly et al., 1986; Samraoui et al., 1986). It is also now clear that a number of elements of primary structure, adjacent to the active sites, have been conserved (Joris et al., 1988). These include,

Scheme I



first, a serine residue [Ser-70, according to the numbering system of Ambler (1980), originally devised for class A β-lactamases] whose hydroxyl group is the primary active-site nucleophile during turnover of β-lactams by β-lactamases and inhibition of DD-peptidases by β-lactams (Scheme II). Also apparently important functional groups are the ammonium ions of Lys-73 and -237 (the latter is replaced by a His in at least one case) and also, perhaps, the carboxyl(ate) of Glu-166. The catalytic role of these functional groups has been recently discussed (Herzberg & Moult, 1987; Moews et al., 1990;

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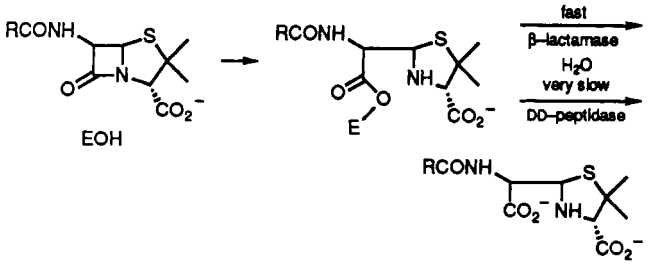
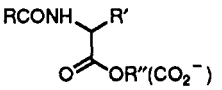
Scheme II



Ellerby et al., 1990; Knap & Pratt, 1991).

Functionally, there is further overlap between the two types of active sites. Both DD-peptidases (Nguyen-Disteche et al., 1986) and β-lactamases (Pratt & Govardhan, 1984) catalyze the hydrolysis and aminolysis, by specific amino acid and peptide amines, of depsipeptides of general structure **1**. The



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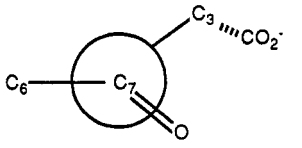
latter reaction is obviously directly analogous to the in vivo transpeptidase reaction (Scheme IA). The two enzymes also share the same unusual steady-state kinetics of the aminolysis reaction (Frere et al., 1973; Pazhanisamy et al., 1989) for which a new interpretation has recently been provided (Pazhanisamy & Pratt, 1989a).

The essential differences between the two groups of enzymes, which must have been crucial factors in the evolutionary process producing a β-lactamase from a DD-peptidase, given that it did in fact occur, are as follows: (1) β-Lactamases have the ability to catalyze hydrolysis of acyl-enzymes formed on reaction with β-lactam antibiotics (Scheme II). It has been suggested (Govardhan & Pratt, 1987; Herzberg & Moult, 1987) that this development involved the appearance in β-lactamases of a specific and possibly substrate-occluded binding site for water. (2) β-Lactamases do not have the ability to catalyze the hydrolysis and aminolysis of acyclic amides (peptides). This would likely be an important feature of β-lactamase evolution since the interference in cell-wall biosynthesis created by peptide-hydrolyzing β-lactamases could be very serious, particularly since β-lactamases, as protective enzymes, are generally produced by cells in much larger quantities than DD-peptidases.

On consideration of the differences between bicyclic β-lactams and acyclic peptides that could be selected for in the evolution of a β-lactamase, the difference in chemical reactivity is striking (Pratt et al., 1980). Bicyclic β-lactams of course are much more susceptible to nucleophilic amide cleavage than are acyclic peptides. It seemed possible therefore that chemically more reactive acyclic compounds might be β-lactamase substrates. This turned out to be correct, as shown by the depsipeptide β-lactamase substrates **1** (Pratt & Govardhan, 1984). Further investigation showed however that such depsipeptides had very similar reactivity with both DD-peptidases and β-lactamases, which suggests comparable nucleophilic reactivity of the active-site serine hydroxyl groups (Govardhan & Pratt, 1987). It also seems likely that β-lactams, like acyclic peptides but perhaps unlike depsipeptides, do require acid catalysis of leaving group departure (Page, 1987). Thus the loss of a general acid catalytic group in β-lactamases (Pratt et al., 1980) seems unlikely.

These considerations appear to leave substrate shape as the most likely characteristic of substrate structure to be selected for. In bicyclic β-lactams, the dihedral angle across the amide bond is about 140° (**2**). This tetrahedral-like geometry of the amide nitrogen is related to chemical reactivity and antibiotic effectiveness (Sweet & Dahl, 1970; Woodward, 1980; Boyd, 1982; Frère et al., 1982; Page, 1987). In acyclic peptides, of course, the corresponding angle is 180° (**3**). It is not difficult to imagine evolution of an active site from one that is specific to planar peptide substrates to one specific to those that were nonplanar (**4**) and that would no longer productively accommodate the former substrates (**5**). The presence of direct interaction with the oxyanion hole (as shown in diagrams **4** and **5**; Murphy & Pratt, 1988; Herzberg & Moult, 1987; Moews et al., 1990) and/or a general acid catalyst (not shown) could control this specificity.



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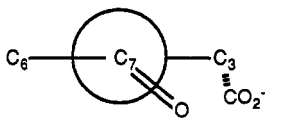

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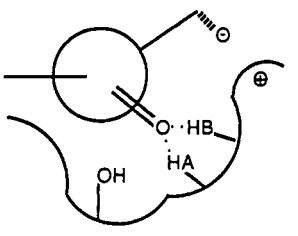

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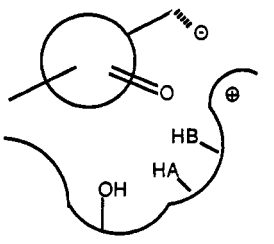

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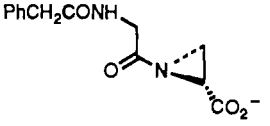
In order to test this hypothesis of the importance of substrate shape, we designed a new β-lactamase (and DD-peptidase) substrate, N-(phenylacetyl)glycyl-D-aziridine-2-carboxylate (**6**). Its structure, as an N-acylaziridine, allows the combination of high chemical reactivity with respect to normal peptides and, because of the nature of the bonding in the aziridine ring, a close to tetrahedral amide nitrogen atom, where the dihedral angle referred to above is about 150° (Shibaeva et al., 1968; Zacharis & Trefonas, 1968; Vilkov et al., 1969).

This paper reports on the synthesis of **6** and its reactivity with typical β-lactamases and one DD-peptidase, in comparison with that of the most closely analogous compound, the depsipeptide **7**, where the dihedral angle across the scissile bond is, in the ground state at least, 180°. Comparisons with β-lactams and peptides are also made.



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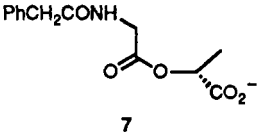

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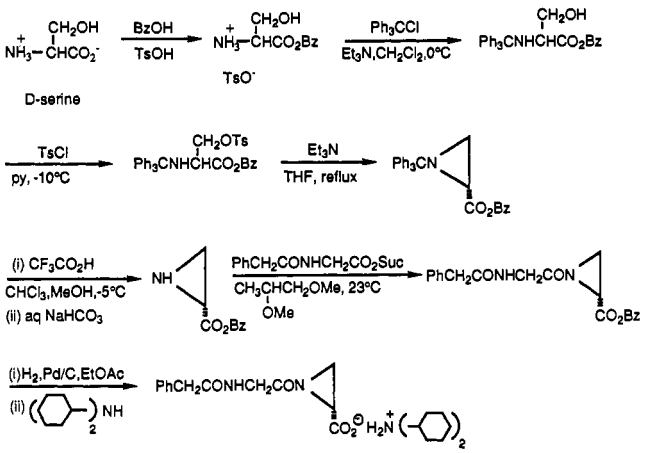
Experimental Procedures

Materials. The β-lactamase I of *Bacillus cereus* (strain 569/H/9) and the β-lactamases of the TEM-2 plasmid (from *E. coli*, strain W3310), of *Staphylococcus aureus* PCI, and of *E. cloacae* P99 were obtained from the Centre for Applied Microbiology and Research (Porton Down, England) and used as supplied. The β-lactamase II of *B. cereus* was separated from β-lactamase I (the mixture was also purchased from the above source) by the method of Davies et al. (1974). The water-soluble D-alanyl-D-alanine transpeptidase/carboxypeptidase (DD-peptidase) of *Streptomyces* R61 was a generous gift from Drs. J.-M. Ghuysen and J.-M. Frère (University of Liege, Liege, Belgium). Benzylpenicillin was purchased from Sigma Chemical Co. *N*-(Phenylacetyl)glycyl-D-phenylalanine

Scheme III: Synthesis of *N*-(Phenylacetyl)glycyl-D-aziridine-2-carboxylate



and *N*-(phenylacetyl)glycyl-D-lactic acid were previously prepared in this laboratory by Drs. S. Pazhanisamy and Chandrika Govardhan, respectively. Authentic sodium DL-aziridine-L-carboxylate was a gift from Dr. Segei Hasegawa of Mitsui Toatsu Chemicals, Inc., Tokyo, Japan.

Chemical reagents for the synthesis described below were obtained from Aldrich and Baker Chemical Co. ^{1}H NMR spectra were obtained on a Nicolet QE-300 spectrometer. Low-and high-resolution mass spectra were obtained from Drs. Michael Lee and Steve Khlor, respectively, both of Bristol-Myers Squibb Pharmaceutical Research and Development Division, Wallingford, CT.

N-(Phenylacetyl)glycyl-D-aziridine-2-carboxylate Dicyclohexylammonium Salt. This compound was prepared (Scheme III) by acylation of benzyl D-aziridine-2-carboxylate. The latter was obtained essentially by the procedure described by Nakajima et al. (1978) for the L-isomer. First, D-serine benzyl ester benzenesulfonate, mp 94–96 °C [lit. (Koehn & Kind, 1965) mp 97–98 °C], was prepared from D-serine (Sigma) by the method described for the L-serine analogue by Koehn and Kind (1965). This compound was converted into the *N*-trityl derivative in quantitative yield by reaction with trityl chloride and triethylamine in dry chloroform, as described by Nakajima et al. (1978). The ^{1}H NMR spectrum of the oily product was appropriate for *N*-trityl-D-serine benzyl ester: (C^2HCl_3) δ 2.18 (1 H, br s, OH), 3.57 (2 H, m, CH_2O), 3.72 (1 H, br s, CH), 4.64 (1 H, d, J = 12.3 Hz, CHPh), 4.78 (1 H, d, J = 12.3 Hz, CH'Ph), 7.4 (21 H, m, ArH, NH). The *N*-trityl derivative was cyclized to benzyl *N*-trityl-D-aziridine-2-carboxylate by treatment with *p*-toluenesulfonyl chloride in dry pyridine, followed by triethylamine (Nakajima et al., 1978). The product was purified by chromatography on silica gel in chloroform and recrystallized from ethyl acetate, mp 113–116 °C [lit. (Nakajima et al., 1978) mp 114–116 °C], in 60% yield from *N*-trityl-D-serine benzyl ester and gave the following ^{1}H NMR spectrum: (C^2HCl_3) δ 1.41 (1 H, dd, J = 6.4, 1.2 Hz, C3-H), 1.92 (1 H, dd, J = 6.1, 2.6 Hz, C3-H), 2.28 (1 H, dd, J = 2.4, 1.5 Hz, C2-H), 5.18 (1 H, d, J = 12.2 Hz, CHPh), 5.24 (1 H, d, J = 12.2 Hz, CH'Ph), 7.37 (20 H, m, ArH). The *N*-trityl group was then removed by treatment with trifluoroacetic acid/methanol (Nakajima et al., 1978) to give benzyl D-aziridine-2-carboxylate, an oil, in 75% yield, with the following ^{1}H NMR spectrum: (C^2HCl_3) δ 1.86 (1 H, d, J = 4.9 Hz, C3'-H), 2.02 (1 H, br s, C3-H), 2.56 (1 H, q, J = 2.6 Hz, C-2), 5.18 (2 H, AB q, J = 12.5 Hz, CH_2Ph), 7.56 (5 H, br s, ArH).

Phenaceturic acid was prepared as previously described (Govardhan & Pratt, 1987) and converted to the *N*-hydroxysuccinimide ester, mp 173.5–174 °C, by the general method of Anderson et al. (1964). A solution of 0.93 g (3.2 mmol) of the *N*-hydroxysuccinimide ester and 0.57 g (3.2 mmol) of benzyl D-aziridine-2-carboxylate in 12 mL of dried (CaH_2) 1,2-dimethoxyethane was stirred at room temperature for 1 h. Water (20 mL) was then added and the mixture extracted with diethyl ether. After being dried over sodium sulfate, the ether solution was evaporated to dryness, giving a quantitative yield of a colorless solid (recrystallized from 2-propanol), mp 84–85 °C, with the following ^{1}H NMR spectrum: (C^2HCl_3) δ 2.56 (2 H, m, C3-H_2), 3.25 (1 H, dd, J = 3.2, 2.0 Hz, C2-H), 3.58 (2 H, s, CH_2Ph), 4.05 (2 H, d, J = 5.1 Hz, CH_2CO), 5.07 (2 H, s, OCH_2Ph), 6.1 (1 H, br s, NH), 7.2 (10 H, m, ArH). The infrared spectrum (film) showed two carbonyl absorptions at 1738 and 1652 cm^{-1}. The properties of this compound are essentially the same as those reported by Henery-Logan and Limberg (1966), although the stereochemistry of their product was not defined.

Finally, and as described by Henery-Logan and Limberg (1966), **6** was obtained in 63% yield by hydrogenation of its benzyl ester over 10% Pd/C in ethyl acetate. The dicyclohexylammonium salt of the product, mp 133–136 °C [lit. (Henery-Logan & Limberg, 1966) mp 133.5–136.5 °C], was precipitated by diethyl ether. Its structure was supported by its ^{1}H NMR spectrum [(^2H_2O, HCO_3^-) δ 1.1–2.2 (22 H, m, dicyclohexyl), 2.36 (1 H, br s, C3-H), 2.50 (1 H, d, J = 4.6 Hz, C3-H), 3.05 (1 H, br q, C2-H), 3.69 (2 H, s, CH_2Ph), 4.07 (2 H, s, CH_2CO), 7.36 (5 H, m, ArH)] and high-resolution mass spectrum (M + 1, 444.2856; theoretical for $C_{26}H_{37}N_3O_4$, 444.2862). This dicyclohexylammonium salt was used for the kinetic studies. For product determinations by ^{1}H NMR spectroscopy, it was convenient to remove the dicyclohexylammonium ion. This was done by treatment of the dicyclohexylammonium salt in aqueous solution with Dowex 50X4-400 ion-exchange resin in the Na^+ form. The ^{1}H NMR spectrum of the sodium salt (^2H_2O, HCO_3^-) was identical with that of the dicyclohexylammonium salt less the dicyclohexylammonium ion resonances.

Analytical Methods. Absorption spectra and spectrophotometric steady-state reaction rates were measured by means of Cary 219 and Perkin-Elmer Lambda 4B spectrophotometers. β-Lactamase activity was routinely estimated against benzylpenicillin by the spectrophotometric method of Waley (1974).

The hydrolysis of *N*-(phenylacetyl)glycyl-D-aziridine-2-carboxylate was followed spectrophotometrically at 230 nm, where the extinction coefficient change was 695 M^{-1} cm^{-1}. The initial rates of hydrolysis of *N*-(phenylacetyl)glycyl-D-lactate to phenaceturate and D-lactate were determined spectrophotometrically under a nitrogen atmosphere by an indicator method that followed proton release (Pratt et al., 1985). The indicators used were phenol red (37 μM, pH 7.5, followed at 550 nm), bromocresol purple (50 μM, pH 6.0, followed at 585 nm), and thymol blue (173 μM, pH 9.0, followed at 593 nm). The indicator responses were calibrated by the addition of aliquots of 0.100 M hydrochloric acid to solutions of the indicators in the appropriate buffers. Separate calibrations were performed in methanol solutions. The hydrolysis of *N*-(phenylacetyl)glycyl-D-alanine was followed spectrophotometrically at 230 nm, where the extinction coefficient change, determined from alkaline hydrolysis, was 178 M^{-1} cm^{-1}. All experiments were performed at 25 °C unless otherwise noted.

Enzyme concentrations were determined spectrophotometrically by application of published extinction coefficients: 1.95×10^{-4} M^{-1} cm^{-1} at 276.5 nm for the PCI β-lactamase

(Carrey & Pain, 1978), 5.42×10^4 M^{-1} cm^{-1} at 280 nm for the P99 β-lactamase (Joris et al., 1985), 2.90×10^4 M^{-1} cm^{-1} at 280 nm for the TEM-2 β-lactamase (Fisher et al., 1980), 2.85×10^4 M^{-1} cm^{-1} at 280 nm for *B. cereus* β-lactamase I (Imsande et al., 1970), 2.2×10^4 M^{-1} cm^{-1} at 280 nm for *B. cereus* β-lactamase II (Bicknell et al., 1986), and 3.9×10^4 M^{-1} at 280 nm for the R61 DD-peptidase (Nieto et al., 1973). Aqueous methanol solutions were prepared by making up measured volumes of methanol to the required volumes with aqueous buffer.

Product Determinations. As described previously for depsipeptides (Pratt & Govardhan, 1984; Govardhan & Pratt, 1987), ^1H NMR spectroscopy could be used to detect and determine the products of hydroxide ion, acid, and β-lactamase catalyzed hydrolysis of **6**. Thus, ca. 1-mg samples of **6** were dissolved in ^2H_2O and either KO^2H (7.2 μL of 40% KO^2H solution, final concentration 0.1 M) or ^2HCl (7.3 μL of 37% ^2HCl solution, final concentration 0.1 M) was added. In typical experiments with β-lactamases, 0.1–0.2-mg samples of the enzyme were added to NMR tubes containing **6** (ca. 1 mg) in 0.5 mL of $NaHCO_3$ (0.1 M) in ^2H_2O. Sodium (trimethylsilyl)propanesulfonate was employed as an internal standard. Spectra were obtained at suitable time intervals until the reactions were complete. The reaction of **6** in the presence of the P99 β-lactamase at pH 4.9 (50 mM acetate buffer) was also examined.

^1H NMR spectroscopy was also used to 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, as described previously (Govardhan & Pratt, 1987). Similarly, the extent of aminolysis of **6** by D-phenylalanine could be determined (Pratt & Govardhan, 1984; Pazhanisamy et al., 1989).

Kinetics of Hydroxide Ion and Acid-Catalyzed Hydrolysis. Pseudo-first-order hydrolyses of **6** (ca. 1 mM) in acidic (10–100 mM HCl) or alkaline (5–20 mM NaOH) solutions (μ = 1.0 with KCl) were followed spectrophotometrically.

Steady-State Enzyme Kinetics. Michaelis–Menten parameters were obtained by the method of Wilkinson (1961) from spectrophotometric initial velocity measurements. Hydrolyses of **6**, catalyzed by the TEM-2, PCI, and *B. cereus* I β-lactamases, were carried out in 0.1 M phosphate buffer at pH 7.5. Enzyme concentrations were 1.0, 5.3, and 9.1 μM, respectively, and concentrations of **6** ranged to 20 mM. Succinate buffer (214 mM, pH 6.0, μ = 1.0 with NaCl) was used with *B. cereus* β-lactamase II (2.9 μM), and MOPS (20 mM, pH 7.5) was used for the P99 β-lactamase (0.10 μM). Hydrolysis kinetics of the TEM-2 and P99 β-lactamases were also obtained at pH 9.0 in TAPS buffer (20 mM), with enzyme concentrations of 1.2 and 0.22 μM, respectively. Reactions of the R61 DD-peptidase (1.5 μM) were studied in 0.1 M phosphate buffer (containing 0.02% gelatin), pH 7.0, at 37 °C. The same buffers were employed for the kinetic studies of the hydrolysis of depsipeptide **7**, with the indicator method described above. Enzyme concentrations were 21.7 μM (TEM-2), 60.5 μM (PCI), 17.7 μM (*B. cereus* I), 25.5 μM (*B. cereus* II), 0.38 μM (P99), and 0.86 μM (R61 DD-peptidase), and depsipeptide concentrations ranged to 20 mM.

The effects of methanol on the initial rates of reaction of **6** in the presence of the P99 β-lactamase and the R61 DD-peptidase were determined in aqueous methanol solutions, essentially as described above. The extinction coefficient change at 230 nm appeared unaffected by the presence of methanol, although methanolysis did occur (see Results). The concentrations of **6** employed were 12.9 and 6.5 mM with the P99 β-lactamase and R61 DD-peptidase, respectively. Methanol concentrations up to 3 M could be used with the P99 β-lactamase but only up to 1.5 M with the DD-peptidase before time-dependent loss of activity, presumably through denaturation, became a problem. Initial rates of reaction of the depsipeptide **7**, catalyzed by the above two enzymes in aqueous methanol, were measured by using the indicator method described above. Concentrations of **7** employed were 6.0 and 18.1 mM with the P99 β-lactamase and 2.9 and 17.6 mM with the DD-peptidase.

Steady-state kinetics of the aminolysis of **6** by D-phenylalanine, catalyzed by the P99 β-lactamase, were also determined spectrophotometrically as described above. At fixed concentrations of **6** (3.6, 8.6, 10.9, and 13.3 mM) initial rates were determined with D-phenylalanine concentrations ranging to 25 mM. At fixed D-phenylalanine concentration (16 mM), initial rates were determined with **6** concentrations up to 10 mM. In both types of experiments the enzyme concentration was 0.22 μM.

Activity of β-Lactamases against **6** *after Reaction with 6β-Bromopenicillanic Acid.* The P99, PCI, TEM-2, and *B. cereus* I β-lactamases were inactivated toward benzylpenicillin by 6β-bromopenicillanic acid (Pratt & Loosemore, 1978). Their activities (at concentrations of 0.13, 3.8, 1.7, and 6.4 μM, respectively) against 5.6 mM **6** were then determined spectrophotometrically, as described above.

Susceptibility of β-Lactamases to Irreversible Inhibition by **6**. Samples of the TEM-2, PCI, *B. cereus* I, and P99 β-lactamases were incubated at pH 7.5 in the buffers described above or in 200 mM acetate buffer at pH 5.0 with 4.4 mM **6**. The activity of each enzyme against benzylpenicillin was monitored over a 75-min period.

Determination of the Ratio (V_H/V_A) *of the Initial Rate of Hydrolysis to Aminolysis of* **6** *by D-Phenylalanine, Catalyzed by the P99 β-Lactamase.* Incubation mixtures contained D-phenylalanine (3.5 mM), **6** (2–10 mM), and P99 β-lactamase (0.25 μM) in 20 mM MOPS buffer at pH 7.5. At various times, up to that corresponding to 10% of the total reaction, 90-μL samples were withdrawn from the reaction mixtures and the reactions quenched in 40 μL of acetonitrile; the quenched mixtures were then held at dry ice temperature until HPLC analysis was performed. For analysis, samples (50 μL) were injected into a Waters analytical HPLC system fitted with a Du Pont Zorbax CN column, with a mobile phase of 10% acetonitrile in an aqueous mixture of 0.05 M KCl and 0.008 M HCl and a flow rate of 1.0 mL min^{-1}. The components of the reaction mixture were thus separated [retention times were 3.94, 5.14, 5.75, and 15.38 min for D-phenylalanine, phenaceturic acid, **6**, and *N*-(phenylacetyl)glycyl-D-phenylalanine (**8**), respectively] and could be detected through their absorbance at 225 nm. Calibration of the absorbances with standard samples of the components allowed their concentrations in the quenched solutions to be determined. Linear plots of concentrations of phenaceturate and **8** with time yielded initial rates of hydrolysis and aminolysis, respectively.

Results

The spectral evidence and the hydrolysis product analyses (below) appear sufficient to confirm the identity of the new potential substrate as *N*-(phenylacetyl)glycyl-D-aziridine-2-carboxylic acid (**6**), where the stereochemistry of the carboxylic acid substituent is defined by that in the starting material (Nakajima et al., 1978).

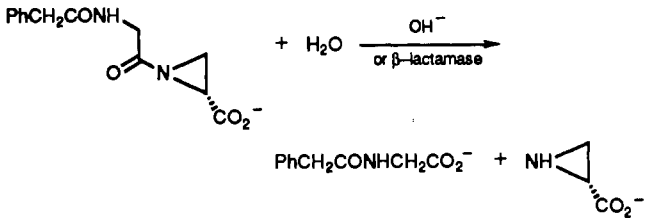
^1H NMR and UV absorption experiments showed that **6** is a substrate of all β-lactamases examined and of the R61

Table I: Steady-State Parameters for β-Lactamase-Catalyzed Hydrolyses

		enzyme					
		β-lactamase[b]					
		class A			class B	class C	DD-peptidase[b]
substrate	steady-state parameter[a]	TEM	BCI	PCI	BCII	P99	R61
acylaziridine **6** (k_{OH} = 8.0 s^{-1} M^{-1})	k_{cat} (s^{-1})	20.7	5.4	0.14	0.4	130	4.5
	K_M (mM)	10.7	45	0.5	0.1	3.4	7.6
	k_{cat}/K_M (s^{-1} M^{-1})	1.9×10^3	120	280	4×10^3	3.9×10^4	600
depsipeptide **7** (k_{OH^-} = 0.3 s^{-1} M^{-1})	k_{cat} (s^{-1})	>0.006	>0.001	>0.01	>0.08	39.6	3.2
	K_M (mM)	>11	>11	>11	>15	16.1	2.9
	k_{cat}/K_M (s^{-1} M^{-1})	0.54	0.1	0.9	5.3	2.5×10^3	1.1×10^3
	$(k_{cat}/K_M)(6)/(k_{cat}/K_M)(7)$	4000	1200	300	750	15	0.55
depsipeptide **11**[c] (k_{OH} = 15.2 s^{-1} M^{-1})	k_{cat} (s^{-1})	24.0	1.9	0.11		101	
	K_M (mM)	1.8	6.0	0.07		1.9	
	k_{cat}/K_M (s^{-1} M^{-1})	1.3×10^4	3.1×10^2	1.5×10^3		5.4×10^4	
benzylpenicillin[c] (k_{OH} = 0.1 s^{-1} M^{-1})	k_{cat} (s^{-1})	2000	2000	30	880[d]	14[e]	1.4×10^{-4}[f]
	K_M (mM)	0.02	0.05	≤0.01	0.8[d]	6×10^{-4}[e]	1.0×10^{-5}[f]
	k_{cat}/K_M (s^{-1} M^{-1})	10^8	4×10^7	$\geq 3 \times 10^6$	1.1×10^6[d]	2.3×10^7[e]	1.3×10^4[f]

[a] From initial rate measurements at 25 °C in 0.1 M phosphate buffer, pH 7.5 (TEM, BCI, PCI), 200 mM succinate, pH 6.0 (BCII), 20 mM MOPS, pH 7.5 (P99), or 20 mM phosphate, pH 7.0 and 37 °C (R61). The standard deviations on these parameters were less than 20% of the value given. [b] TEM, the β-lactamase of the TEM plasmid of *E. coli*; BCI, *B. cereus* β-lactamase I; PCI, the β-lactamase of the PCI plasmid of *S. aureus*; BCII, *B. cereus* β-lactamase II; P99, the β-lactamase of *E. cloacae* P99; R61, the DD-peptidase of *Streptomyces* R61. [c] Govardhan and Pratt (1987) except where otherwise noted. [d] 30 °C; Bicknell et al. (1983). [e] Galleni and Frère (1988). [f] 37 °C; Frère and Joris (1985).

Scheme IV



DD-peptidase. β-Lactamases inhibited with respect to benzylpenicillin hydrolysis by 6β-bromopenicillanic acid did not catalyze any reaction of **6**, suggesting that the normal β-lactamase active site is employed in the turnover of **6**. No sign of irreversible or slowly reversible inhibition of the β-lactamases by **6** was observed.

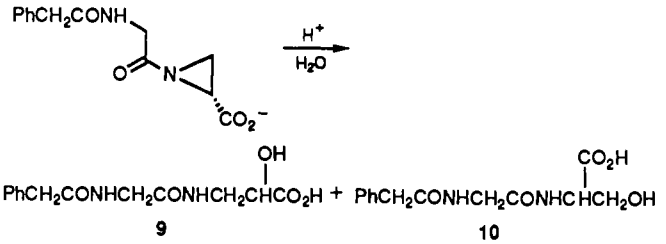
^{1}H NMR studies showed that the sole products of alkaline and β-lactamase-catalyzed hydrolysis of **6** were phenaceturate and aziridine-2-carboxylate (by comparison with the spectra of authentic samples). Hence only amide hydrolysis occurred under these conditions (Scheme IV). The latter was also true of the β-lactamase-catalyzed reaction at pH 4.9 (P99 β-lactamase), as well as at the pH 9.0. The depsipeptide **7** has been shown to yield phenaceturate and D-lactate under these conditions (Pratt & Govardhan, 1984).

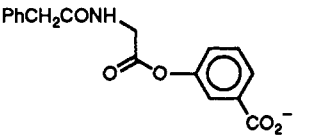
Acid-catalyzed hydrolysis of **6** did not yield the amide hydrolysis products. The complex ^{1}H NMR spectrum of the product solution suggested the presence, as expected (Clifford-King et al., 1982; Slebocka-Tilk & Brown, 1987), of one or perhaps both of the amides **9** and **10**, resulting from aziridine ring opening (Scheme V).

Reaction of both **6** and **7** in aqueous methanol solutions, catalyzed by the P99 and PCI β-lactamases and by the R61 DD-peptidase, generated methyl phenaceturate as well as the hydrolysis products. This methanolysis product could be detected and determined by ^{1}H NMR (Govardhan & Pratt, 1987). Similarly, the P99 β-lactamase-catalyzed aminolysis of **6** by D-phenylalanine yielded *N*-(phenylacetyl)glycyl-D-phenylalanine, also identified by ^{1}H NMR (Pratt & Govardhan, 1984; Pazhanisamy et al., 1989).

The acid- and base-catalyzed hydrolyses of **6** were first order in H^+ and OH^- concentrations, respectively, with second-order rate constants of 0.24±0.01 $s^{-1}M^{-1}$ and 7.8±0.8 $s^{-1}M^{-1}$, respectively. The latter number gives a measure of the susceptibility of the N-acylaziridine carbonyl group to nucleophilic attack. It is thus significantly more reactive than the D-lactate **7** and benzylpenicillin (see Table I) but less so than the depsipeptide **11**, which is also a β-lactamase substrate (Govardhan & Pratt, 1987).

Scheme V



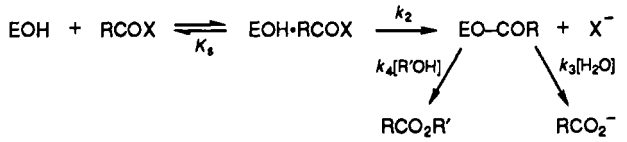


11

Steady-State Parameters for Depsipeptide **7** *and Acylaziridine* **6** *Hydrolysis.* A survey of the steady-state parameters for the β-lactamase and R61 DD-peptidase-catalyzed hydrolyses of **6** and **7** is given in Table I. For comparison, steady-state parameters for the hydrolysis of benzylpenicillin and the depsipeptide **11** (Govardhan & Pratt, 1987) are also presented. The R61 DD-peptidase (3.65 μM) did not appear to catalyze the hydrolysis of the peptide *N*-(phenylacetyl)-glycyl-D-alanine (10 mM).

Enzyme-Catalyzed Methanolysis and Rate-Determining Steps. The serine β-lactamase and DD-peptidase-catalyzed hydrolysis of specific substrates proceeds by a double-displacement mechanism with an acyl-enzyme intermediate (Scheme VI) (Waxman & Strominger, 1983; Frère & Joris, 1985; Pratt, 1989; Govardhan & Pratt, 1987). In certain cases, e.g., class C β-lactamases with β-lactams (Knott-Hunziker et al., 1982) and depsipeptides (Govardhan & Pratt, 1987), the acyl-enzyme can undergo alcoholysis as well as hydrolysis. In such a case, and where formation of the acyl-enzyme is rate determining, the total rate will not be accelerated by alcohol,

Scheme VI



but where deacylation of the enzyme is rate determining, it will be. Thus a means of determining the rate-determining step is available. Another indication of this point comes from the variation of k_{cat} with leaving group. If k_{cat} is independent of the nature of the leaving group, deacylation is likely to be rate determining and vice versa (although there could be a change in rate-determining step in the latter case). This simple correlation assumes that all kinetically significant steps are shown in Scheme VI.

The k_{cat} values of Table I (comparing the values for **6**, **7**, and **11**) suggest that acylation of the P99 β-lactamase by all three acyclic substrates is probably rate determing [since it is known to be so for **11** (Govardhan & Pratt, 1987)] and that deacylation is rate determining in the case of **6** and the PCI β-lactamase [as it is known to be for **11** (Govardhan & Pratt, 1987)]. Deacylation of the TEM-2 β-lactamase after acylation by **6** and **11** may well be rate determining. The situation is less clear with the B. cereus I β-lactamase, although rate-determining acylation, with **6** at least, seems more likely. Similarly unclear is the situation for the R61 DD-peptidase, but this is illuminated by the methanolysis data described below.

Initial rates of disappearance of **6**, catalyzed by the P99 β-lactamase, were not accelerated by added methanol (data not shown). This strongly suggests rate-determining acylation, as deduced above, and as occurs with **11**. Product analysis by ^{1}H NMR gave a k_4/k_3 value (Scheme VI) of 17.6 that is sufficiently close to the value of 22.5±4.3 obtained previously (Govardhan & Pratt, 1987) as to support a common acyl-enzyme intermediate. (Note that the latter figure has been recalculated from that given previously, on the basis of 47.5 M ^2H_2O rather than 55.5 M.)

Initial rates of disappearance of **7**, in the presence of the P99 β-lactamase, as determined by the indicator method, apparently decreased on addition of methanol. This is due to the fact that the indicator method measures only the rate of hydrolysis rather than the total rate of disappearance of **7**; methanolysis does not lead to proton release. Since the decrease in the measured rate did not change with substrate concentration (data not shown), rate-determining acylation is indicated (Govardhan & Pratt, 1987). Qualitatively, these same results were obtained for the effect of methanol on the rates of disappearance of **6** and **7** catalyzed by the R61 DD-peptidase. Here too, rate-determining acylation presumably obtains.

Aminolysis of Acylaziridine **6** *by* D-*Phenylalanine, Catalyzed by the P99 β-Lactamase.* ^{1}H NMR studies indicated that the P99 β-lactamase efficiently catalyzed aminolysis of **6** by D-phenylalanine. Kinetic measurements showed that the total rate of reaction of **6**, V_T, increased both with **6** concentration at a fixed concentration of D-phenylalanine and with D-phenylalanine concentration at a fixed concentration of **6**. At high concentrations of both varied reagents, indications of saturation were observed (Figure 1). The curves of Figure 1B could be fitted with the empirical equation (eq 1), where

$$V_T = (v_0 K_M^{app} + V_{MAX}^{app}[A])/(K_M^{app} + [A]) \quad (1)$$

v_0 is the (hydrolysis) rate at the particular aziridine concentration in the absence of D-phenylalanine (A). V_{MAX}^{app} is the rate at saturating D-phenylalanine concentration, and K_M^{app} is the D-phenylalanine concentration at half the maximal rate, i.e., the apparent dissociation constant of D-phenylalanine. Since analysis of the data of Figure 1B showed that both V_{MAX}^{app} and K_M^{app} increased with the concentration of **6**, it was clear that a situation similar to that observed for the P99 β-lactamase-catalyzed aminolysis of **11** by D-phenylalanine obtained (Pazhanisamy et al., 1989). Consequently, measurements were made of V_H/V_A, the ratio of initial rates of hydrolysis to aminolysis, as a function of *N*-acylaziridine concentration. This parameter has been shown to be a powerful indicator of the kinetic mechanism (Frère, 1973; Pazhanisamy et al., 1989). In the present instance, V_H/V_A increased linearly with *N*-acylaziridine concentration (data not shown). All of the data were then fitted to the complete empirical equation (eq 2),

$$V_T = \frac{b_1 V_{MAX}^H[Az] + b_2 V_{MAX}^H[Az]^2 + a_1[A][Az]}{b_1 K_M^H + (b_1 + b_2 K_M^H)[Az] + b_2[Az]^2 + [A]} \quad (2)$$

which was shown (Pazhanisamy et al., 1989) to apply in the case of **11**. In this equation, V_{MAX}^H and K_M^H are the steady-state parameters for P99 β-lactamase-catalyzed hydrolysis of **6** (Az) in the absence of D-phenylalanine (as given in Table I), and a_1, b_1, and b_2 are empirical parameters. Equation 3 follows

$$V_H/V_A = \frac{b_1 V_{MAX}^H}{a_1[A]} + \frac{b_2 V_{MAX}^H[Az]}{a_1[A]} \quad (3)$$

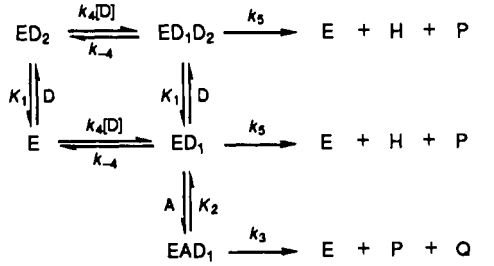
from eq 2. Application of eq 3 to the V_H/V_A measurements yielded good estimates of b_1/a_1 and b_2/a_1 that were then used to obtain, by nonlinear least-squares fitting (Johnson et al., 1976), the best possible estimates of the three empirical parameters of eq 2 from the data obtained from rate measurements at varying D-phenylalanine concentrations and at fixed **6** concentrations, and vice versa. The solid lines of Figure 1 show the fit to these data, where the optimized parameters yielding these lines were $a_1 = 0.33$ min^{-1}, $b_1 = 6.4 \times 10^{-3}$, and $b_2 = 112$ M^{-1}. In Table II, values are given for the rate and equilibrium constants of Scheme VII, derived from these empirical parameters. This scheme, which applied for **11** (Pazhanisamy et al., 1989), is assumed to apply in the present case on the basis of the fit of the data to eqs 2 and 3. The con-

Table II: Kinetic Parameters of Scheme VII for P99 β-Lactamase-Catalyzed Aminolysis of *N*-Acylaziridine **6** and Depsipeptide **11**

parameter	substrate **6**	substrate **11**[a]
$k_4 \times 10^{-4}$ (s^{-1} M^{-1})	2.17	30.0
K_1 (μM)	5.8	1000
$(k_3/K_2) \times 10^{-5}$ (s^{-1} M^{-1})	34	1.1

[a] These are data from Pazhanisamy and Pratt (1989a).

Scheme VII[a]



[a] Abbreviations: D, acyl donor (depsipeptide or **6**); A, amino acid (D-phenylalanine); H, hydrolysis product (phenaceturate); P, common hydrolysis and aminolysis product; Q, aminolysis product **8**.

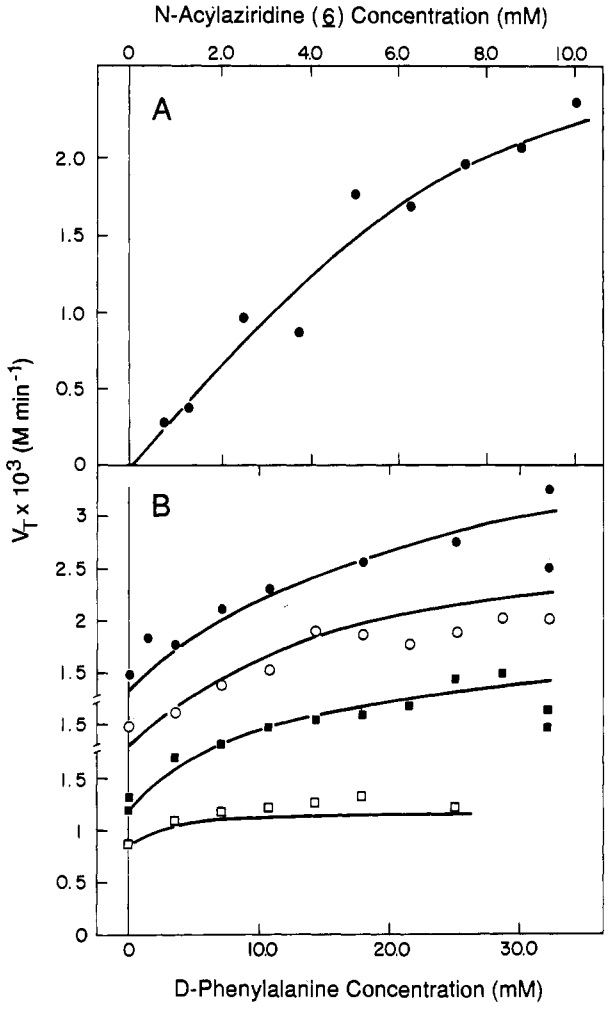


FIGURE 1: Initial total velocity, V_T, of the reactions of **6** in D-phenylalanine solutions, catalyzed by the P99 β-lactamase (0.22 μM). (A) Reaction of varying concentrations of **6** at constant D-phenylalanine concentration (16 mM). (B) Reaction of varying D-phenylalanine concentrations at fixed concentrations of **6**: (●) 13.3 mM, (○) 10.9 mM, (■) 8.6 mM, and (□) 3.6 mM. The points are experimental, and the lines are calculated from eq 2, as described in the text.

version relationships are $a_1 = k_4E_0$, $b_1 = k_4(K_2/k_3)$, and $b_2 = (k_4/K_1)(K_2/k_3)$ (Pazhanisamy & Pratt, 1989a). The analogous rate and equilibrium constants for **11** (Pazhanisamy & Pratt, 1989a) are also given in Table II for comparison.

Discussion

We show in this paper that *N*-(phenylacetyl)glycyl-D-aziridine-2-carboxylate (**6**) is a substrate both of β-lactamases, where it is the first acyclic amide substrate described, and of a DD-peptidase. The β-lactamases include three class A enzymes, one class B, and one class C. The DD-peptidase was the soluble enzyme from *Streptomyces* R61, which has long been used, notably by Ghuysen et al. (1979), as a model DD-peptidase. It should be mentioned that **6**, in an uncertain enantiomeric state, was also prepared some time ago by Henery-Logan and Limburg (1966), who reported that it had slight, if any, antibiotic activity; in retrospect, from several viewpoints, the latter finding is not surprising.

The reaction of **6**, catalyzed by the above enzymes, is the anticipated hydrolysis of the amide bond (Scheme IV), as is also observed in alkaline hydrolysis. There is no indication in the interactions of **6** with these enzymes of acid-catalyzed nucleophilic cleavage of the aziridine ring (Clifford-King et al., 1982; Slebocka-Tilk & Brown, 1987) such as was observed in acid-catalyzed hydrolysis, either in turnover or in leading to enzyme inhibition. Any *N*-protonated aziridine formed during catalysis must have only fleeting existence and/or poor access to nucleophiles, enzymic and otherwise.

The data of Table I indicate that **6** is a better (k_{cat}/K_M) substrate of the β-lactamases than is the depsipeptide **7**, although it is generally poorer than the depsipeptide **11**. This suggests that protonation of the leaving group, which is presumably required for **6** but perhaps not for the depsipeptides, particularly **11**, is available to **6** and may well be enzyme catalyzed. In cases of the serine β-lactamases (classes A and C) where identification of the rate-determining step was possible, particularly those of the class A PCI β-lactamase and the class C P99 enzyme, the rate-determining step was the same for **6** as for **11** (deacylation and acylation, respectively). The latter result further indicates the absence of any difficulties in ejecting the amine leaving group. Although **6**, as an *N*-acylaziridine, is more reactive toward nucleophilic cleavage than is **7** and benzylpenicillin, as indicated by the k_{OH^-} values of Table I, this does not seem to be a direct indicator of substrate ability, as discussed previously with respect to depsipeptides (Govardhan & Pratt, 1987) and β-lactams (Frère et al., 1988).

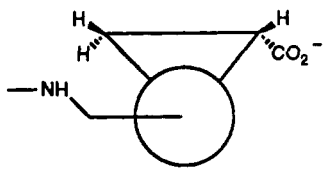
The *N*-acylaziridine **6** is also a substrate of the R61 DD-peptidase of comparable ability to the depsipeptide **7** and with acylation probably rate determining in each case. Here too departure of the leaving group from **6** apparently posed no problem.

The most interesting parameter of Table I is probably the ratio of k_{cat}/K_M for **6** to that of **7**, as a measure of the specificity of the enzyme toward **6** with respect to **7**. This ratio reveals that the β-lactamases strongly prefer **6** to **7**. The degree of this preference, by the TEM and P99 β-lactamases at least, was essentially the same at pH 9.0 as at pH 7.5. Conversely, the DD-peptidase prefers the lactate **7**, although weakly. The former preference can be interpreted as the selectivity of the β-lactamase active site for "bent" substrates with tetrahedral geometry at the leaving group heteroatom (**4**), i.e., *against* planar substrates (**5**). This suggests, as discussed in the introduction, that the β-lactamase active site has evolved to exclude planar peptides from a productive binding mode and therefore that the substrate shape was in fact a major determinant of β-lactamase evolution.

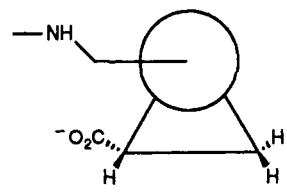
A similar trend in specificity is seen on comparison of k_{cat}/K_M values for benzylpenicillin with those of **7**, although in this case the DD-peptidase slightly prefers benzylpenicillin to **7**. It is worth reflecting at this stage on just why the distorted substrates, **6** and penicillins, are accepted by DD-peptidases at all. The best answer to this question still seems to be that introduced by Lee (1971). A DD-peptidase binds these molecules and cannot easily evolve to prevent such binding because of the similarity in structure between them and the tetrahedral transition states of the acyl-transfer reactions catalyzed by the DD-peptidase. Although β-lactams thus bind sufficiently well in a productive mode to DD-peptidases, they do not bind as tightly as classical transition-state analogues because they do not have tetrahedral geometry at the carbonyl carbon atom. The transition state formed on nucleophilic attack on the β-lactam does, however, strongly resemble that on attack on a peptide, and hence β-lactams acylate DD-peptidases. Conversely, however, the β-lactamase *can* strongly select against acyclic peptides since no planar species arises during β-lactamase catalysis.

Another point of interest that arises on consideration of Table I is why **6**, despite its favorable β-lactam-like geometry

and greater chemical reactivity, is still a much poorer substrate of β-lactamases than benzylpenicillin. One factor involved here must be its greater flexibility both about the C_6–C_7 bond (penicillin numbering) and about the amide C–N bond [barriers to rotation (and inversion) in *N*-acylaziridines are probably less than 6 kcal/mol (Anet & Osyany, 1967; Boggs & Gerig, 1968)], which puts it at an entropic disadvantage with respect to the rigid penicillin. This point has been previously noted with respect to depsipeptides (Govardhan & Pratt, 1987). Perhaps more important in the present case is the strong likelihood that the penicillin-like conformation of **6** is not the most stable one. The cis relationship between the phenylacetamido substituent and the aziridine ring in the most



12



13

penicillin-like conformation **12** brings the 3-endo-hydrogen of the aziridine ring to an uncomfortable 2.5–3.0 Å from the phenylacetamido nitrogen atom; in solution a more extended trans relationship between these substituents would likely obtain, such as in the rotamer **13**. The conformers arising from facile inversion at nitrogen would probably be intermediate in stability between **12** and **13**. The energy difference between **12** and **13** would also contribute to the advantage of the similarly strained but rigid penicillin.

A related question that should be mentioned in passing is that, given the essentially complete inability of β-lactamases to catalyze peptide hydrolysis, how is it that the presumably planar depsipeptides **1** are substrates, albeit rather poor ones? Their reactivity with β-lactamases can probably now be seen to arise, not so much from the intrinsic chemical reactivity of esters per se, but rather from a related property, the ease, relative to peptides, of rotation about the scissile bond into a more penicillin-like conformation. This interpretation has also been previously raised (Govardhan & Pratt, 1987).

Finally, with respect to Table I, comment should be made on the competence of the class C P99 β-lactamase in depsipeptide turnover, where it is much more effective than the class A β-lactamases and comparable to the DD-peptidase. This result may correlate with the greater relative effectiveness of class C enzymes in general against cephalosporins. Cephalosporins, of course, have a less tetrahedral β-lactam nitrogen atom than the penicillins (Sweet & Dahl, 1970; Boyd, 1982), and distortion of the depsipeptide to this conformation is presumably more readily achieved. The class C β-lactamases also seem closer than class A β-lactamases to the R61 DD-peptidase in primary structure (Joris et al., 1988) and thus both structurally and functionally may be more DD-peptidase-like than the class A enzymes.

Another significant result that should be considered is the inability of the R61 DD-peptidase to catalyze hydrolysis of *N*-(phenylacetyl)glycyl-D-alanine, the peptide analogue of **7**. That this compound is not a substrate is, however, in accord with much data of Ghuysen et al. (1979) from this enzyme, wherein it is clear that peptidase activity is a strong function of peptide structure. First, it seems likely that the substitution of glycine for the D-alanine of the best substrates would lead to some activity loss. More important however would seem to be the N-terminal acyl group where anything significantly different from an *N*,*N*-diacetyl-L-lysyl group, a mimic of the presumed natural substrate, induces little peptidase activity. Thus, the specificity of the enzyme with respect to this acyl substituent on peptide substrates is very different from that with respect to the apparently corresponding side chains of inhibitory penicillins. This type of observation led to suggestions that the side chains, at least of good substrates on one hand and inhibitory β-lactams on the other, are bound in different sites on the enzyme (Ghuysen et al., 1979, 1981).

An alternative explanation, but partly complementary, would be that the DD-peptidase active site is able, as discussed above, to selectively bind "distorted" substrates, i.e., those tetrahedral at the scissile bond nitrogen atom (and at the carbonyl carbon, of course). When such transition-state-like substrates, for example penicillins, **6**, and distorted depsipeptides, are bound, the active-site functional groups are well placed for catalysis. In this way DD-peptidases would appear to be more β-lactamase-like than previously considered. With planar substrates such as peptides however, only very specific structures, those with the N-terminal acyl group resembling those of natural substrates, are able to bind in a way that induces the active-site functionality to be productively positioned. This view would extend the picture of β-lactamases as enzymes where induced fit or conformational adaptation (Citri et al., 1976; Citri, 1981) was important to the parent DD-peptidases. In such cases, crystal structures of the free enzymes must be interpreted cautiously.

Evolution of a β-lactamase would then involve loss of the ability of planar substrates (peptides) to induce a reactive active-site conformation, while maintaning the productive binding of distorted species. The important difference between the two groups of enzymes would then be located, not in the area where bond breaking and making occurs, but in the side-chain binding sites. The accommodation of water for acyl-enzyme hydrolysis would presumably be a separate problem, as described in the introduction.

The P99 β-lactamase also catalyzes aminolysis of **6** by D-phenylalanine, ust as it does the depsipeptides **7** and **11**. This result suggests that the binding of **6** has much in common with that of the depsipeptides. The reaction, an amide aminolysis, is the closest analogue to the transpeptidase reaction yet achieved by a β-lactamase (and the closest possible in the light of the above discussion). The steady-state kinetics of this reaction were analyzed in terms of Scheme VII, which has been shown to apply to the analogous reaction of **11**. It is of interest to compare the derived rate constants of Scheme VII for **6** with those of **11** (Table II). There are several points of interest here. First, the binding of **6**, like that of **11**, is slow, much too slow to represent diffusion-controlled association. This is suggestive of the binding of a transition-state analogue where such slow binding is commonly seen (Morrison & Walsh, 1988; Schloss, 1988). As noted previously however (Pazhanisamy & Pratt, 1989a), β-lactam binding must be faster, suggesting that the slow binding of **6** and **11**, partially at least, represents the binding of unfavorable conformations of these flexible molecules, the latter either taken from solution or achieved by induction on binding. The rate of aminolysis (k_3/K_2) of **6** is somewhat larger than that of **11**, although this observation cannot be interpreted at present, partly because of lack of knowledge of the mechanism of the aminolysis reaction.

Probably the most interesting result, however, is the value of K_1, the dissociation constant of the substrate from the second [and probably nonproductive (Pazhanisamy & Pratt, 1989a)] binding site. The binding of **6** to this site is much stronger than that of **11**. This observation also cannot be interpreted

in any structural sense at present, although if this second binding site does represent the acceptor (amino acid) binding site, as previously suggested (Pazhanisamy & Pratt, 1989a), the more peptidelike **6** may well fit it better than **11**. It should also be noted, however, that **6** would thereby also bind much more strongly than the good acceptor D-phenylalanine (Pazhanisamy & Pratt, 1989b). Irrespective of all this, the important point is that the hydrolysis of **6**, except at low micromolar concentrations ($0.1K_m$), must largely proceed via the ternary complex. This may well be true too of penicillins, which resemble **6** much more than they do **11**. The reality and significance of this second binding site is an important focus of further research.

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Mechanism-Based Inactivation of Bovine Adrenal Cytochromes P450 C-21 and P450 17α by 17β-Substituted Steroids†

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ABSTRACT: A series of progesterone derivatives has been studied as potential inactivators of the bovine adrenocortical cytochromes P450, P450 17α, and P450 C-21. Replacement of the 21-methyl group of progesterone with a difluoromethyl group resulted in a selective inactivator of P450 C-21 in a reconstituted system. The loss of 21-hydroxylase activity caused by this compound exhibits a number of characteristics of mechanism-based inactivation including NADPH dependence, pseudo-first-order kinetics, saturability, irreversibility, and protection by substrate. In addition to the difluoro compound, 21,21-dichloroprogesterone, the acetylenic compound pregn-4-en-20-yn-3-one, and the olefinic compound pregna-4,20-dien-3-one all inactivate P450 C-21. In contrast, the only compound to inactivate the rabbit adrenal progesterone 21-hydroxylase is 21,21-dichloroprogesterone. In binding studies, the 21,21-dihalo steroids produce a greater maximal type I spectral shift of P450 C-21 than the two 17β-unsaturated steroids. The dihalo compounds inactivate P450 C-21 by both heme destruction and protein modification as shown by significant decreases in residual 21-hydroxylase activity and spectrally detectable P450 after incubation with P450 C-21 in a reconstituted system. Liquid chromatographic and mass spectral analyses of the organic extracts from these incubations showed that 21-pregnenoic acid is a major metabolite of the dihalo compounds with a partition ratio of 5 nmol of acid produced/nmol of P450 C-21 inactivated. This supports the hypothesis that inactivation proceeds in part through an acyl halide intermediate. In contrast, the acetylenic compound pregn-4-en-20-yn-3-one inactivates P450 C-21 mainly by protein modification, producing an NADPH-dependent irreversible type I spectral shift. The stoichiometry of inactivation is approximately 1.5 nmol of compound bound/nmol of enzyme inactivated, indicating selective modification of the enzyme at or near the substrate binding site.

Cytochromes P450 are a gene superfamily of hemoproteins that catalyze the oxidation of lipophilic substrates to more water-soluble products (Nebert et al., 1989). The numerous P450 forms in the liver are generally inducible by and carry out the metabolism of xenobiotics (Conney, 1986). In contrast, the P450 forms present in the adrenal gland are more limited in number and substrate specificity and utilize steroids as their endogenous substrates. Two key microsomal enzymes of the adrenal cortex are P450 C-21 (P450XXIA1)[1] and P450 17α (P450XVIIA1), which catalyze the hydroxylation of progesterone at the 21- and 17α-positions, respectively. The metabolic products of these reactions are important precursors in the production of mineralocorticoids, glucocorticoids, and sex hormones (Miller, 1988).

Although these and other P450s have been extensively studied at the biochemical and molecular level, the question of why certain P450 forms display superior catalytic activity and/or strict regioselectivity with a particular substrate has remained largely unanswered. Several approaches have been utilized by other laboratories to investigate the basis for differences in progesterone 21-hydroxylation by related P450s

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[1] The major cytochromes P450 referred to in this report, their steroid hydroxylase activities, and gene designations are as follows: P450 C-21 (P450XXIA1), an adrenal steroid 21-hydroxylase encoded by the bovine *CYP21A1* gene; P450 17α (P450XVIIA1), an adrenal steroid 17α-hydroxylase encoded by the bovine *CYP17* gene; PB-C (P450IIC6), a hepatic progesterone 21-hydroxylase encoded by the rat *CYP2C6* gene. Rabbit adrenal 21-hydroxylase will be referred to as such since the gene designation is presently unknown.

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