New Substrates for β-Lactam-Recognizing Enzymes: Aryl Malonamates†

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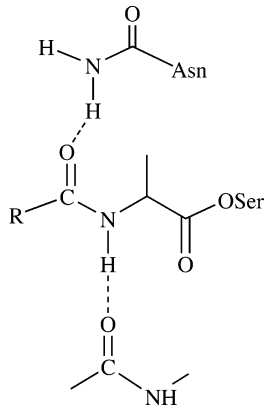
ABSTRACT: Aryl malonamates are demonstrated to be novel substrates of a broad range of β-lactam-recognizing enzymes. These compounds are isomers of the aryl phenaceturates, which are well-known substrates of these enzymes, but the new compounds contain a retro-amide side chain. Several lines of evidence, including comparisons of steady-state kinetic parameters between enzymes and a detailed investigation of the methanolysis kinetics, solvent deuterium isotope effects, and pH−rate profile for turnover of a retro substrate by the *Enterobacter cloacae* P99 β-lactamase, suggested that the new substrates are likely to be hydrolyzed by the same chemical mechanisms as "normal" substrates. Molecular modeling indicated that the retro-amide group fits snugly into the active site of the P99 β-lactamase by hydrogen bonding to the conserved lysine-67 residue. The retro-amide side chain may represent a lead to novel mechanism-based and transition state analogue inhibitors.

The search for β-lactamase inhibitors to potentiate β-lactam antibiotics continues unabated (*1*, *2*). Since many of the best known β-lactamase inhibitors are of the mechanism-based variety (*3*, *4*), new motifs for inhibitors can, in principle, be derived from new substrates. Crystal structures of β-lactamases and the related DD-peptidases that are now available suggest possible directions to be followed. For example, crystal structures of covalent adducts of these enzymes with substrates (*5*−*8*) and transition state analogue inhibitors (*9*, *10*) show that the classical amido side chain is specifically hydrogen bonded to a backbone carbonyl oxygen of the β-2 strand and the side chain NH of a generally conserved asparagine residue, as in **1** (R represents an alkyl



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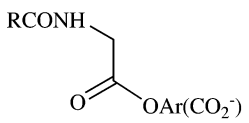
or aryl group). β-Lactams lacking this amido side chain are generally poor β-lactamase substrates and poor antibiotics (i.e., poor DD-peptidase inhibitors). In certain instances, however, these poor substrates can be mechanism-based inhibitors (*3*).

It occurred to us that a retro-amide (*11*) side chain might be able to enforce a different hydrogen-bonding pattern at the active site and thus new chemistry. Few examples of β-lactams containing the retro side chain are to be found in the literature (*12*−*16*), and these have only modest antibiotic activity. No studies of such molecules with specific enzymes have been reported. Retro-amide moieties have been incorporated into a variety of bioactive peptides. It is unusual, however, to find that the product is reactive as an enzyme substrate; indeed, one major reason for such incorporation has been to reduce protease susceptibility (*17*).

Previous studies have shown that acyclic aryl phenaceturates, **2**, are good substrates of both β-lactamases and DD-peptidases (*18*−*20*). Since it seemed possible that the flexibility afforded by acyclic species might allow the retro side chain to more productively adapt to the active site, we undertook a study of compounds of general structure **3**, the



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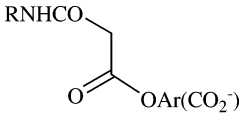

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retro-amide analogues of **2**. In this paper, we describe the synthesis of two such compounds and their activity as substrates of a variety of typical β-lactam-recognizing enzymes and present some discussion of their possible interactions with the active sites of these enzymes.

EXPERIMENTAL PROCEDURES

Materials. The class C β-lactamase of *Enterobacter cloacae* P99, the class A β-lactamases, TEM-2 from *Escherichia coli* W3310 and PCl from *Staphylococcus*

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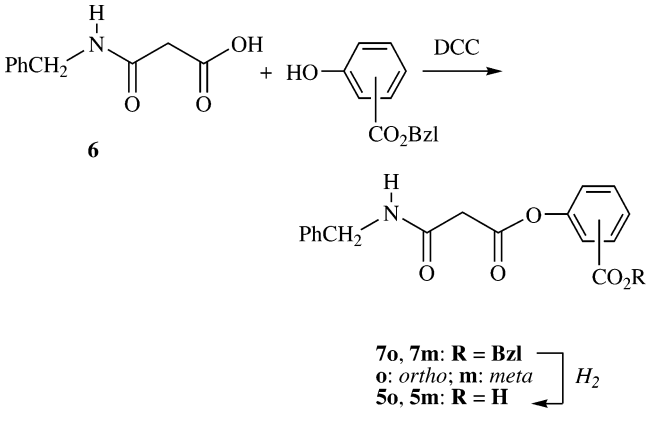
Scheme 1



aureus, and the class B β-lactamase II of *Bacillus cereus* [as a mixture with the class A β-lactamase I (*21*)] were purchased from the Centre for Applied Microbiology and Research (Porton Down, Wiltshire, U.K.). The class A β-lactamase I activity was removed from the *B. cereus* mixture by titration with 6β-bromopenicillanic acid (*22*). The class D OXA-1 β-lactamase was generously provided by Dr. Michiyoshi Nukaga of the University of Connecticut. The DD-peptidase of *Actinomadura* R61 was a gift of Dr. J.-M. Frère of the University of Liège, Liège, Belgium. The DD-peptidase of *Streptomyces* R39 was provided by Dr. P. Charlier, also of the University of Liège. PBP3 of *Neisseria gonorrhoeae* was kindly provided by Dr. R. A. Nicholas of the University of North Carolina at Chapel Hill. PBP5 of *E. coli* and the depsipeptides **4o** and **4m** were available from previous studies in this laboratory (*18*, *19*, *23*).

*Synthesis of the Retro-Depsipeptides **5o** and **5m***. The syntheses of **5o** and **5m** were achieved in two steps from *N*-benzylmalonamic acid (**6**) (Scheme 1). Condensation (dicyclohexylcarbodiimide) of **6** with benzyl 2- or 3-hydroxybenzoate gave **7o** and **7m**, respectively, which were converted to **5o** and **5m** by catalytic hydrogenation.

N-Benzylmalonamic Acid (6) (25). Ethyl *N*-benzylmalonamate (*24*) (773 mg, 3.5 mmol) was dissolved in ethanol (15 mL), a solution of 1 N NaOH (15 mL) was added, and the mixture was stirred at room temperature overnight. The ethanol was evaporated, and then the aqueous solution was washed with ethyl acetate, acidified with 50% HCl, and extracted with ethyl acetate. The organic layer was dried ($MgSO_4$), and the solvent was evaporated. The title acid, 594 mg (88% yield), was obtained: R_f[1] 0.35 (acetic acid/ethyl acetate, 2.5/97.5); mp 85.5 °C; ^{1}H NMR (acetone-d_6) δ 3.40 (s, 2H, $NHCOCH_2$), 4.46 (d, 2H, $C_6H_5CH_2NH$), 7.23−7.33 (m, 5H, ArH), 8.10 (br s, 1H, NH).

*Benzyl 3-[[2-(Benzylaminocarbonyl)ethanoyl]oxy]benzoate (**7m**).* To a solution of 96.5 mg (0.5 mmol) of *N*-benzylmalonamic acid in dichloromethane (5 mL) under an argon atmosphere were added 103 mg (0.5 mmol) of DCC and 114 mg (0.5 mmol) of benzyl 3-hydroxybenzoate. The reaction mixture was stirred at room temperature overnight. The white precipitate (dicyclohexylurea) was removed by filtration. The solvent was evaporated, and the crude product was purified by chromatography (silica gel; pentane/ethyl acetate, 7/3) to give 155 mg (77% yield) of the title product **7m**: R_f 0.20 (pentane/ethyl acetate, 7/3); mp 89 °C; ^{1}H NMR ($CDCl_3$) δ 3.63 (s, 2H, $NHCOCH_2$), 4.51 (d, 2H, $C_6H_5CH_2$-NH), 5.37 (s, 2H, $COOCH_2C_6H_5$), 7.26−7.50 (m, 12H, ArH), 7.79 (t, 1H, ArH), 7.99 (dt, 1H, ArH); ^{13}C NMR ($CDCl_3$) δ 41.54 ($COCH_2CO$), 43.89 ($NHCH_2$), 67.22 ($COOCH_2C_6H_5$), 122.80−137.77 (CH Ar), 150.22 (C Ar), 164.47−165.44 and 167.78 (CO). Anal. Calcd for $C_{24}H_{21}NO_5$: C, 71.45; H, 5.25; N, 3.47. Found: C, 71.44; H, 5.23; N, 3.53.

*Benzyl 2-[[2-(Benzylaminocarbonyl)ethanoyl]oxy]benzoate (**7o**).* *N*-Benzylmalonamic acid (189 mg, 1 mmol) was dissolved in DMF (3 mL) at −15 °C. DCC (206 mg, 1 mmol) and benzyl 2-hydroxybenzoate (228 mg, 1 mmol) were then added. The reaction was allowed to proceed at room temperature for 2 days. The mixture was then poured into a 10% HCl solution and extracted with ethyl acetate. The organic layer was washed twice with water and dried ($MgSO_4$). The crude product was purified by silica gel chromatography (pentane/ethyl acetate, 1/1) and recrystallized from diethyl ether to give 114 mg of the required product **7o** (30% yield): R_f 0.66 (pentane/ethyl acetate, 1/1); mp 82.6 °C; ^{1}H NMR ($CDCl_3$) δ 3.53 (s, 2H, $NHCOCH_2$), 4.50 (d, 2H, $C_6H_5CH_2NH$), 5.19 (s, 2H, $COOCH_2C_6H_5$), 7.09 (dd, 1H, ArH), 7.22−7.33 (m, 11H, ArH), 7.55 (dt, 1H, ArH), 8.04 (dd, 1H, ArH); ^{13}C NMR ($CDCl_3$) δ 41.63 ($COCH_2CO$), 43.94 ($NHCH_2$), 67.32 ($COOCH_2C_6H_5$), 122.79−138.05 (CH Ar), 150.40 (C Ar), 164.37−164.68 and 168.15 (CO). Anal. Calcd for $C_{24}H_{21}NO_5$: C, 71.45; H, 5.25; N, 3.47. Found: C, 71.51; H, 5.24; N, 3.48.

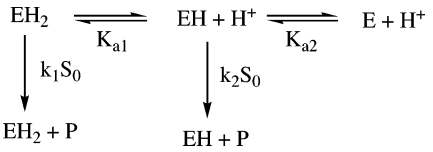
*3-[[2-(Benzylaminocarbonyl)ethanoyl]oxy]benzoic Acid (**5m**).* The benzyl ester **7m** (187 mg) was dissolved in ethyl acetate, 10% Pd/C (55 mg) was added, and the solution was hydrogenated at room temperature for 16 h under a 3.45×10^5 Pa H_2 pressure. The reaction mixture was filtered and the solvent evaporated. The crude product was washed with pentane and ether. Recrystallization from acetone gave 133 mg of product **5m** (91% yield): R_f 0.75 (acetone); mp 178 °C; ^{1}H NMR (acetone-d_6) δ 3.67 (s, 2H, $NHCOCH_2$), 4.48 (d, 2H, $C_6H_5CH_2NH$), 7.23−7.42 (m, 6H, ArH), 7.57 (t, 1H, ArH), 7.80 (t, 1H, ArH), 7.95 (dt, 1H, ArH); ^{13}C NMR (acetone-d_6) δ 43.80 ($COCH_2CO$), 43.92 ($NHCH_2$), 123.82−140.09 (CH Ar), 151.93 (C Ar), 165.84−166.89 and 167.57 (CO). Anal. Calcd for $C_{17}H_{15}NO_5$: C, 65.17; H, 4.83; N, 4.47. Found: C, 65.04; H, 4.77; N, 4.55.

*2-[[2-(Benzylaminocarbonyl)ethanoyl]oxy]benzoic Acid (**5o**).* **5o** was prepared in the same manner as **5m**; 37 mg of benzyl ester **7o** gave 27.6 mg (95% yield) of recrystallized (acetone) product **5o**: R_f 0.36 (pentane/ethyl acetate, 1/1); mp 153 °C; ^{1}H NMR (acetone-d_6) δ 3.64 (s, 2H, $NHCOCH_2$), 4.46 (d, 2H, $C_6H_5CH_2NH$), 6.95 (d, 1H, ArH), 7.19−7.41 (m, 6H, ArH), 7.53 (t, 1H, ArH), 7.89 (d, 1H, ArH); ^{13}C NMR (acetone-d_6) δ 43.27 ($NHCH_2$), 43.68 ($COCH_2CO$), 118.05−139.67 (CH Ar), 151.80 (C Ar), 166.86−167.47 and 168.05 (CO). Anal. Calcd for $C_{17}H_{15}NO_5$: C, 65.17; H, 4.83; N, 4.47. Found: C, 65.18; H, 4.81; N, 4.65.

Analytical and Kinetic Methods. Absorption spectra and spectrophotometric reaction rates were measured with a Hewlett-Packard 8452A spectrophotometer. Solution condi-

[1] Abbreviations: AMPSO, 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NMR, nuclear magnetic resonance; R_f, retardation factor; TAPS, *N*-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane. Standard three-letter abbreviations for amino acids are used.

Scheme 2



tions for the kinetic runs were as follows: 0.1 M MOPS buffer, pH 7.5 at 25 °C (P99, TEM, and PCl β-lactamases and the R61 DD-peptidase); 0.1 M pyrophosphate buffer, pH 8.5 at 37 °C (PBP3 and PBP5); 0.1 M Tris buffer, pH 7.7 at 37 °C (R39 DD-peptidase). Stock solutions of all depsipeptides were prepared in DMSO, and aliquots of this were added to cuvettes. The total DMSO concentration in all reaction mixtures was 5% v/v. Initial rates of enzyme-catalyzed reactions were monitored spectrophotometrically at 300 nm ($\Delta\epsilon = 950$ cm^{-1} M^{-1}) or 290 nm ($\Delta\epsilon = 1760$ cm^{-1} M^{-1}) after addition of small aliquots of stock enzyme solutions to substrate solutions. Steady-state kinetic parameters were obtained from initial rates by the method of Wilkinson (*26*). In cases where no signs of enzyme saturation were obtained, k_{cat}/K_m values were obtained from progress curves by means of the Dynafit program (*27*).

The pH profile of k_{cat}/K_m for the P99-catalyzed hydrolysis of **5m** was obtained as a function of pH under V/K ($S_o \ll K_m$) substrate concentration conditions. Control of pH was achieved by means of mixed buffers containing 20 mM each of acetate, MES, MOPS, AMPSO, and TAPS and where an ionic strength of 1.0 was maintained with NaCl. The classical bell-shaped pH−rate profile was fitted to Scheme 2 and the derived rate equation (eq 1) by means of a nonlinear least-squares program to obtain the pK_a values of the catalytic groups.

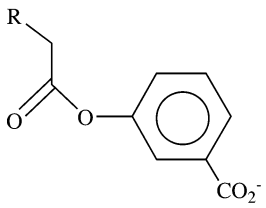
$$k_{cat}/K_m = k_1K_{a1}[H^+]/\{[H^+]^2 + K_{a1}[H^+] + K_{a1}K_{a2}\} \quad (1)$$

Solvent deuterium kinetic isotope effects were obtained as described previously (*20*, *28*). The effect of methanol on the initial rates of solvolysis of **5o** and **5m** in the presence of the P99 β-lactamase was determined spectrophotometrically in aqueous methanol/MOPS buffer solutions as described previously (*18*, *19*). Methanol concentrations between 0 and 2.5 M were employed. The concentrations of **5o** and **5m** were 2.5 and 1.0 mM, respectively, and initial rates were obtained at 330 and 290 nm, respectively. The partition ratio k_4/k_3 (Scheme 3) was then determined from least-squares fits to the data, as previously described (*19*). The effect of D-phenylalanine on the rates of disappearance of **5o** and **5m** in the presence of the P99 enzyme was also determined; concentrations of the substrates were as for the methanolysis experiments, and those of D-phenylalanine ranged to 30 mM.

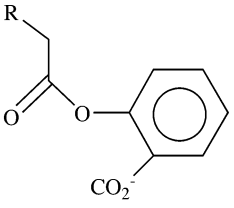
Molecular Modeling. The computations were set up essentially as previously described (*29*) and run on an SGI Octane 2 computer with Insight II 2000 (MSI, San Diego, CA). The starting point for the simulations of the P99 β-lactamase was the crystal structure with a phosphonate inhibitor covalently attached to the active site serine residue [PDB file 1bls (*9*)]. The retro-amide acyl group of **5m** was constructed using the Builder module, and calculated partial atomic charges were applied to it (*29*). A variety of initial conformations of the ligand were explored by means of molecular dynamics, and a typical snapshot of the predominant conformation was selected for energy minimization. The protonation state of active site residues was as described previously (*29*). A similar protocol was adopted for the TEM β-lactamase where the deacylation transition state model was built from the crystal structure of a boronate complex [PDB file 1ERO (*30*)]. Dynamics runs were performed with Glu 166 both protonated and unprotonated.

RESULTS AND DISCUSSION

The retro-depsipeptides **5m** and **5o**, analogues of the previously studied β-lactamase and DD-peptidase substrates **4m** and **4o**, were prepared as described in Experimental



4m R = $PhCH_2CONH$
5m R = $PhCH_2NHCO$



4o R = $PhCH_2CONH$
5o R = $PhCH_2NHCO$

Procedures (Scheme 1). The new compounds were soluble in water at neutral pH to concentrations around 20 mM and slowly hydrolyzed. In 100 mM MOPS buffer at pH 7.5 and 25 °C, the pseudo-first-order rate constants of hydrolysis of **5m** and **5o** were 5.4×10^{-6} s^{-1} and 3.8×10^{-5} s^{-1}, respectively. The corresponding values for **4m** and **4o** were 1.0×10^{-5} s^{-1} (*31*) and 5.6×10^{-5} s^{-1}, respectively. These values suggest little intramolecular catalysis by the amido side chain but some by the *o*-carboxylate; the latter is likely to be due to general base catalysis (*32*).

The important result then obtained was that **5m** and **5o** are, like **4m** and **4o** (*18*, *19*), substrates of a variety of β-lactamases and DD-peptidases. This was observed first in NMR experiments where the hydrolysis of **5m** and **5o** was found to be much more rapid in the presence than in the absence of β-lactamases. The products of hydrolysis in all cases were the hydroxybenzoic acid and *N*-benzylmalonamic acid. Subsequently, the quantitative spectrophotometric determination of the initial rates led to the steady-state kinetic parameters of Tables 1 and 2 for these hydrolysis reactions; comparable kinetic parameters for the classical depsipeptides **4m** and **4o** are also provided in Tables 1 and 2 for comparison.

The remarkable feature of the data of Tables 1 and 2 is the overall general similarity between the kinetic parameters of the retro compounds **5m** and **5o** and their "normal" amide analogues **4m** and **4o**, respectively. Although there are certainly differences in particular parameters in specific cases, there are no quantitative differences greater than an order of magnitude in favor of the classical substrates. For a given enzyme, the retro compound is, in general, not significantly poorer as a substrate than the original depsipeptide. Further, the change in k_{cat}/K_m, i.e., in the free energy of the acylation transition state, from enzyme to enzyme for a given substrate, is very similar for the retro compounds as for the originals. This suggests that the mechanism of hydrolysis employed by the various enzymes, i.e., the nature of the active site groups and their role in catalysis, is the same for the retro

Table 1: Steady-State Kinetic Parameters for the Hydrolysis of Compounds **4m** and **5m** by β-Lactamases

	4m			**5m**		
enzyme[a]	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}\,M^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}\,M^{-1}$)
P99	125[b]	0.23	5.4×10^5	41 ± 2	0.17 ± 0.02	2.45×10^5
TEM	25.4[b]	2.2	1.2×10^4	28 ± 1	1.42 ± 0.06	1.95×10^4
PCl	0.030[c]	0.19	1.6×10^2	0.112 ± 0.003	0.029 ± 0.003	3.9×10^3
OXA-1	3.6 ± 0.3	5.1 ± 0.5	7.1×10^2	0.27 ± 0.01	1.0 ± 0.1	2.7×10^2
BCII	1[d]	19 ± 2		3.1[d]	9.3 ± 0.6	

[a] Abbreviations: P99, class C β-lactamase of *E. cloacae* P99; TEM, class A TEM-2 β-lactamase; PCl, class A β-lactamase from *S. aureus* PCl; OXA-1, class D β-lactamase; BCII, class B β-lactamase II from *B. cereus*. [b] Data from ref *28*. [c] Data from ref *31*. [d] Value relative to that of **4m**.

Table 2: Steady-State Kinetic Parameters for the Hydrolysis of Compounds **4o** and **5o** by β-Lactamases

	4o			**5o**		
enzyme[a]	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}\,M^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}\,M^{-1}$)
P99	139 ± 6	11.8 ± 0.8	1.2×10^4	41 ± 4	0.8 ± 0.1	5.4×10^4
TEM		n[c]		≥ 0.0072	≥ 0.2	36
PCl		nd[d]			n	
OXA-1	4.8 ± 0.3	5.3 ± 0.4	9.1×10^2	3 ± 1	11 ± 5	2.55×10^2
BCII		>10	1[b]		>0.5	2.7[b]

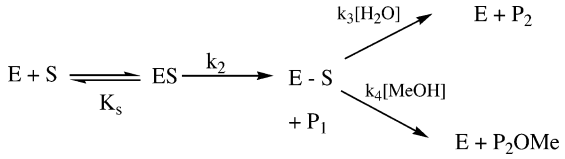
[a] The enzyme abbreviations are as for Table 1. [b] Value relative to that of **4o**. [c] n = not a substrate. [d] nd = not determined.

compounds as for the originals. The class B enzyme, a metallo-β-lactamase rather than a serine β-lactamase, is actually a little more efficient in catalyzing the hydrolysis of **5m** and **5o** than of **4m** and **4o**, respectively. The K_m values are high, however, indicating little affinity between these depsipeptide substrates and the class B active site.

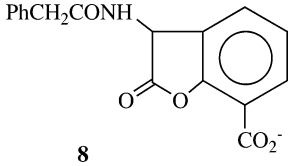
There are a few significant differences evident from the data of Tables 1 and 2. First, both *m*-carboxyphenyl esters, **4m** and **5m**, are better class A and class C β-lactamase substrates than are their *o*-carboxyphenyl analogues, **4o** and **5o**; on the other hand, however, there is little specific preference (k_{cat}/K_m) for the meta isomers by the class D β-lactamase. The general difference in steady-state parameters between the class A TEM and the PCl β-lactamase has been noted previously (*18*, *33*); the PCl β-lactamase generally has difficulty with deacylation.

The data for the P99 β-lactamase indicate that, within experimental uncertainty, k_{cat} for **5m** is the same as that for **5o**. This is suggestive of a common rate-determining step which most likely would be deacylation. This is certainly to be expected since k_{cat} for the P99 β-lactamase generally correlates with the deacylation step and, in particular, has been shown to be so for **4m** (*19*); the similarity of k_{cat} values between **4m** and **4o** suggests that deacylation is also rate determining for the latter compound under saturating conditions. Direct evidence for rate-determining deacylation in the case of the retro compounds **5m** and **5o** was obtained from methanolysis experiments. The acyl-enzyme derived from **4m** is well-known to partition between hydrolysis and methanolysis in aqueous methanol solutions [Scheme 3 (*19*)]. In Scheme 3, E−S represents the acyl-enzyme intermediate and P_2 and P_2OMe are the hydrolysis and methanolysis products, respectively, of this intermediate. In the presence of methanol, the apparent k_{cat} for **4m** increases with methanol concentration as deacylation is enhanced. This same result has now been observed with **5m** and **5o** (data not shown). Treatment of these data by the previously employed methods (*19*) yielded values of k_4/k_3, the partition ratio in favor of

Scheme 3



methanolysis, of 15 and 68 for **5m** and **5o**, respectively. These values are comparable to those previously obtained for **4m** (viz., 28) (*19*) and for the benzofuranone **8** (viz., 55)



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(*34*). ^{1}H NMR studies showed the presence of a methanolysis product in reaction mixtures from **5m** just as they did with **4m** (*19*). Access of methanol to the acyl-enzyme derived from the retro compounds **5m** and **5o** is therefore not very different from that to comparable acyl-enzymes with normal side chains. This result also suggests a similar catalytic process.

A pH−rate profile for the hydrolysis of **5m** in the presence of the P99 β-lactamase also indicated mechanistic similarity. Figure 1 shows the k_{cat}/K_m profile obtained for **5m** as described in Experimental Procedures. This profile yielded pK_a values, probably those of free enzyme, of 5.86 ± 0.10 and 9.63 ± 0.12. Note that the former value is too high to correspond to that of free substrate. A pH−rate profile for the hydrolysis of **4m**, obtained under identical conditions, yielded pK_a values of 6.27 ± 0.11 and 9.26 ± 0.12 (*20*); a profile for dansylcephalothin yielded values of 5.92 ± 0.16 and 9.26 ± 0.35 (*35*). These results suggest that a combination of the same basic and acidic functional groups is required for the hydrolysis of **5m** as for other substrates. Unambiguous

Table 3: Steady-State Kinetic Parameters for the Hydrolysis of Compounds **4m**, **5m**, and **5o** by DD-Peptidases

enzyme[a]	parameter	**4m**	**5m**	**5o**
R61	k_{cat} (s^{-1})	1.51	0.7 ± 0.3	n[c]
	K_m (mM)	0.76	10 ± 3	
	k_{cat}/K_m ($s^{-1} M^{-1}$)	2.0×10^3 [b]	70	
R39	k_{cat} (s^{-1})	≥ 4.2	0.363 ± 0.001	≥ 0.37
	K_m (mM)	≥ 1.0	0.227 ± 0.002	≥ 1.0
	k_{cat}/K_m ($s^{-1} M^{-1}$)	$(4.2 \pm 0.1) \times 10^3$	1.6×10^3	$(3.7 \pm 0.7) \times 10^2$
PBP3	k_{cat} (s^{-1})	≥ 5.6	≥ 3.3	≥ 0.8
	K_m (mM)	≥ 1.0	≥ 1.0	≥ 1.0
	k_{cat}/K_m ($s^{-1} M^{-1}$)	$(5.6 \pm 0.1) \times 10^3$	$(3.3 \pm 0.1) \times 10^3$	$(8.0 \pm 0.2) \times 10^2$
PBP5		n	n	nd[d]

[a] Abbreviations: R61, DD-peptidase from *Streptomyces* R61; R39, DD-peptidase from *Actinomadura* R39; PBP3, penicillin-binding protein 3 of *N. gonorrhoeae*; PBP5, penicillin-binding protein 5 of *E. coli*. [b] Data from ref *20*. [c] n = not a substrate. [d] nd = not determined.

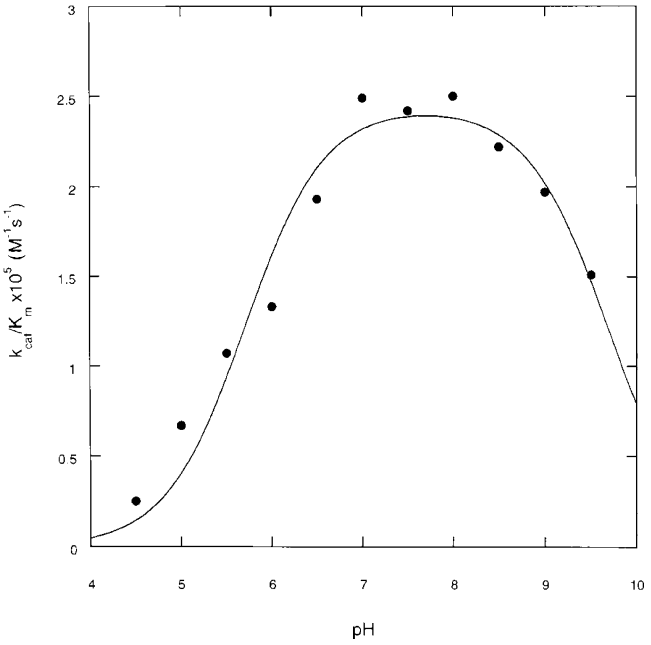

FIGURE 1: pH−rate profile for hydrolysis of compound **5m** by the P99 β-lactamase.

identification of these has not been achieved although tyrosine-150 and lysine-315 are possible candidates (*9*).

As another probe of mechanism, solvent deuterium kinetic isotope effects were determined for turnover of **5m** by the P99 β-lactamase. Values of these parameters are available for **4m** (*28*): $^{D}V/K = 0.83$ and $^{D}V = 1.44$. The former of these values has been interpreted to indicate a very specific combination of fractionation factors in the free enzyme and the latter to indicate some degree of proton transfer in deacylation (*20*, *28*). The values of these parameters for **5m** and the same enzyme were 0.75 ± 0.05 and 1.61 ± 0.14, respectively. A comparison of these values with those of **4m** provides further evidence that the P99 β-lactamase catalyzes the hydrolysis of **4m** and **5m** by the same mechanism and with very similar V/K and V transition states, at least with respect to solvent proton disposition.

More distinctive is the situation with aminolysis. The acyl-enzyme derived from **4m** is subject to facile aminolysis by D-amino acids (*36*, *37*). For example, the partition ratio in favor of aminolysis (versus hydrolysis) of this acyl-enzyme by D-phenylalanine is 7.9×10^4 (*37*). Aminolysis of **5m** by D-phenylalanine was much less facile, however, with a partition ratio of 350, some 220-fold less than that of **4m**. Aminolysis of **5m** by the smaller amine D-alanine was also inefficient, with a partition ratio of 15. These results indicate that the approach of D-amino acid to the acyl-enzyme derived from **5m** is much less favorable than to that from **4m**. It is likely therefore that a D-phenylalanine or D-alanine peptide derived from **5m** would be a much poorer substrate of the P99 β-lactamase than one derived from **4m** (*37*). The more stringent stereoelectronic requirements for peptide hydrolysis by these enzymes are well-known.

Insight into how the retro side chain might fit productively into the active site of these β-lactamases was achieved by model building accompanied by molecular dynamics exploration of accessible conformations and energy minimization by means of a molecular mechanics force field. A resulting energetically favorable structure is shown in Figure 2. This

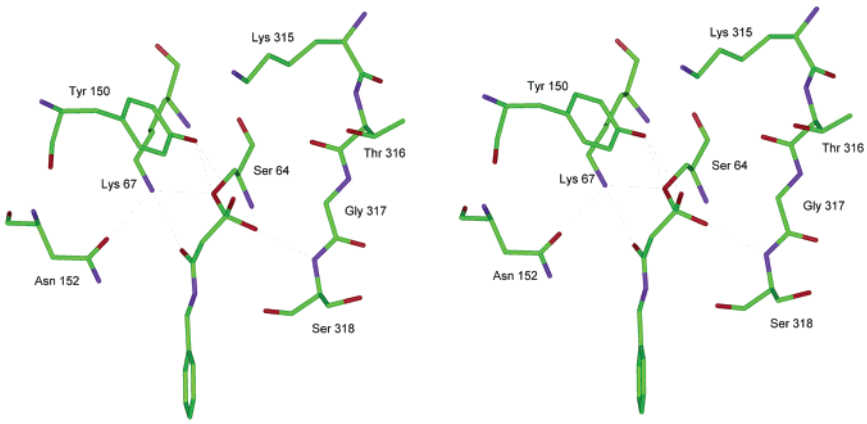

FIGURE 2: Stereoview of an energy-optimized model of the acylation tetrahedral intermediate formed on reaction of compound **5m** with the P99 β-lactamase.

represents the tetrahedral intermediate for deacylation of the acyl-enzyme formed from **5m** or **5o** and the P99 β-lactamase. Comparison with a similar structure derived from **4m** (*29*) shows much similarity. Both have much in common with the structure of a phosphonate transition state analogue (*9*). Features related to catalysis are thought to include positioning of the tetrahedral C−O$^-$ in the oxyanion hole formed by backbone NH groups of Ser 64 and Ser 318, the presence of the hydroxyl of Tyr 150 within hydrogen-bonding distance of the tetrahedral C−OH and Ser 64 O_γ, and that of the Lys 67 ammonium ion within hydrogen-bonding distance of the side chain carbonyl of Asn 152 and Ser 64 O_γ. The novel feature of the structure shown in Figure 2 is the position of the retro-amide side chain. The normal side chain of **4m** is found hydrogen-bonded, on one side, to the side chain NH_2 of Asn 152 and, on the other, to the backbone carbonyl of Ser 318 (**1**). These interactions are not as easily achieved with a retro-amide side chain, and as seen in Figure 2, an apparently stable tetrahedral intermediate structure can be achieved with hydrogen bonding between the retro- amide carbonyl oxygen and the ammonium terminus of Lys 67. This interaction might assist catalysis by stabilizing the position of the substrate with respect to the catalytic apparatus and in a more direct fashion than indirectly via Asn 152 as in substrates with normal side chains. On the other hand, the NH of the side chain amide does not appear to interact with the enzyme but is directed out into solution.

Simulations of the interaction of tetrahedral intermediates derived from reaction of **4m** and **5m** with the class A TEM β-lactamase revealed much greater ligand mobility. No specific interactions between the enzyme and the side chains were observed that were stable over time. This may explain why **4m** and **5m** are poorer substrates of the TEM enzyme than of the P99 enzyme.

The reactivity of **5m** and **5o** with typical DD-peptidases was also explored. These enzymes, the evolutionary parents of β-lactamases, catalyze the hydrolysis and aminolysis of acyclic peptide substrates (*38*). They also catalyze hydrolysis and aminolysis of (thio)depsipeptide analogues of these substrates, including **4m** (*20*, *37*, *39*). Table 3 shows the results of experiments designed to quantitatively determine the extent of turnover of **5m** and **5o** with representative low molecular weight [classes A, B, and C (*38*)] DD-peptidases. These results show that, in general, **5m**, and to a lesser extent **5o**, is a hydrolysis substrate of DD-peptidases in cases where **4m** is also a substrate, viz., the R61 DD-peptidase, the R39 DD-peptidase, and *N. gonorrhoeae* PBP3. Productive interactions of the retro-amide side chain with DD-peptidase active sites are therefore also possible, although in the particular combinations of enzyme and substrate examined to date, these interactions are less favorable than with substrates with normally oriented amides. The reaction of the R61 DD-peptidase with **5m** in the presence of D-phenylalanine yielded a partition ratio (aminolysis versus hydrolysis) some 20-fold smaller than this ratio for **4m** (*37*). This suggests that peptide substrates with retro-amide side chains may also be poorer substrates of DD-peptidases.

General Conclusions. Rather unexpectedly, the depsipeptides **5m** and **5o** containing a retro-amide (with respect to normal substrates) side chain interact productively as substrates with a wide range of β-lactam-recognizing enzymes. We are unaware of examples of malonamate substrates of traditional proteases; the retroamide moiety adjacent to the reaction center may not be accommodated at the active sites of these enzymes as well as at those of the β-lactam-recognizing enzymes. The two compounds tested in the present work are generally as effective as substrates of the latter enzymes, within an order of magnitude, as their analogues **4m** and **4o** with the normal side chain orientation. The similar change of the steady-state parameters of **4m** and **5m** with enzyme, the methanolysis result with the P99 β-lactamase, and the solvent deuterium kinetic isotope effect and pH rate profile with this enzyme suggest that enzyme-catalyzed hydrolysis of the retro and normal compounds occurs by the same chemical mechanism. Molecular modeling indicates how the retro-amide side chain may fit into the class C β-lactamase active site, where the novel interaction that may account for the productivity of the interaction is between the amide carbonyl group and the conserved lysine of the active site of this enzyme. The P99 β-lactamase and the R61 DD-peptidase catalyze aminolysis of the retro compound by D-phenylalanine much more weakly than of the normal compounds; DD-peptidase activity against retro peptide substrates would therefore be much smaller than against the normal peptides, reflecting probably the much greater sensitivity of the peptidase reaction to structural variation.

Optimization of side chain structure on these retro substrates may lead to new mechanism-based and transition state analogue inhibitors with novel scope for structural elaboration. It is possible that the issue of retro-β-lactams should also be revisited.

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