Transpeptidation Reactions of a Specific Substrate Catalyzed by the *Streptomyces* R61 DD-Peptidase: Characterization of a Chromogenic Substrate and Acyl Acceptor Design†

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ABSTRACT: The *Streptomyces* R61 DD-peptidase, a functional model for penicillin-binding proteins, catalyzes the hydrolysis and aminolysis of D-alanyl-D-alanine-terminating peptides by specific amines. In vivo, this reaction completes bacterial cell wall biosynthesis. For in vitro studies of this enzyme to date, various nonspecific acyl-donor substrates have been employed. Recently, however, a peptidoglycan-mimetic peptide substrate, glycyl-L-α-amino-ϵ-pimelyl-D-alanyl-D-alanine, has been described that is much more specific for this enzyme. In this paper, we describe the synthesis and kinetic characterization of an analogous thiolester substrate, 3-(*N*-glycyl-L-cysteinyl)-propanoyl-D-alanyl-D-thiolactate, that the enzyme hydrolyzes and aminolyzes very efficiently ($k_{cat}/K_m = 1.0 \times 10^7$ s^{-1} M^{-1}). Direct or indirect, by means of a thiol trap, spectrophotometric monitoring of the reactions of this substrate is readily achieved. Deacylation of the enzyme is rate-determining under substrate saturation conditions, and therefore the aminolysis reaction can be directly studied. The results show that D-amino acids and certain Gly-L-Xaa dipeptides and tripeptides may act as acyl acceptors at the active site of the enzyme. D-Phenylalanine and Gly-L-Phe were the most effective D-amino acid and dipeptide acceptors, respectively. On the basis of the dual specificity of the active site for acceptors (D-amino acids and Gly-L-Xaa peptides), "dual function" acceptors were designed and synthesized. Two of these, aminomalon-(*N*-ethyl)amide and aminomalon-(*N*-phenethyl)amide, were particularly effective. It did seem, however, that the observed rates of reaction of these very effective acceptors may be limited by some common, possibly physical, step. More extended, peptidoglycan-like, acceptors were found to be essentially unreactive. The reasons for this counterintuitive behavior are discussed.

The target of β-lactam antibiotics has been established to be the class of enzymes that catalyze the final stage of bacterial cell wall biosynthesis (*1*). In this step, a terminal D-alanyl-D-alanine moiety of one peptidoglycan strand reacts with the amine terminus of another to yield free D-alanine and a peptide cross-linked cell wall (Scheme 1). The enzymes that catalyze this step, variously known as DD-peptidases, transpeptidases, or penicillin-binding proteins, continue to be excellent targets for antibacterial agents. Further development of their potential in this regard will stem from rational application of the structural and functional information that has been accumulated about them in recent years (*2*−*6*).

As described in the accompanying paper (*7*), functional studies of these enzymes have been limited to a considerable degree by the absence of specific, tight-binding ligands, i.e., substrates and non-β-lactam inhibitors. It would seem logical to suppose that such ligands should be achievable by the coupling of peptidoglycan-mimetic elements with D-alanyl-D-alanine derivatives and analogues. There is little evidence to date, however, that such incorporation does lead to enhanced interaction with the DD-peptidases, in vitro at least. We have identified one particular example, however, where inclusion of specific elements of peptidoglycan structure led to enhanced interaction with a DD-peptidase.

Scheme 1

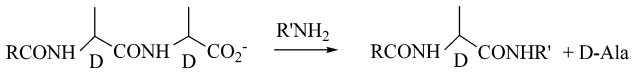
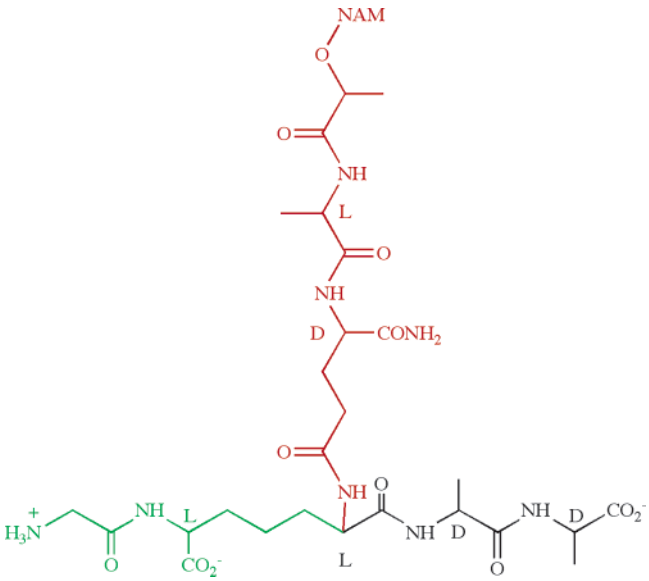


Chart 1



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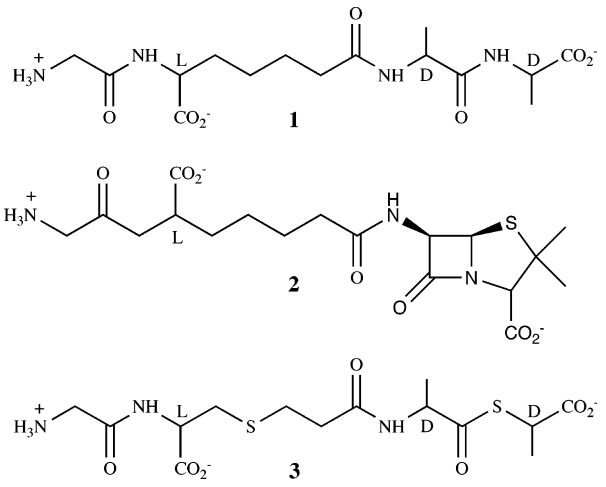
The stem peptide of *Streptomyces* sp. peptidoglycan has the structure depicted in Chart 1. Peptides containing the "main chain" (red) as a side chain to D-alanyl-D-alanine are poor substrates of the *Streptomyces* R61 DD-peptidase, and of DD-peptidases in general (*8*). On the other hand, a peptide containing the "amino terminus" (green), such as **1**, is an excellent substrate of the R61 peptidase (*9*), and a β-lactam including such a side chain, such as **2**, is an excellent inhibitor of this enzyme (*10*). Both **1** and **2** react with the

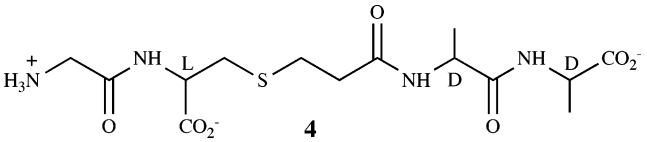


enzyme at rates approaching diffusion-controlled and some 10^2-10^3 times more rapidly than the best previously described nonspecific analogues. Enhanced activity in an "amino terminus" substrate has also been observed with the *Actinomadura* R39 DD-peptidase (*8*).

One might also anticipate that peptidoglycan-mimetic elements in the acyl group acceptor, $R'NH_2$ in Scheme 1, would also enhance its reactivity, and thus the proportion of transpeptidation products. The extended substrate binding site that includes such an acyl acceptor site should, in principle, also be available for inhibitor design. To date, exploration of the potential of this idea has been very limited. In the accompanying paper (*7*), initial experiments designed to explore this terrain with **1** as the acyl donor substrate and the R61 DD-peptidase as the enzyme have been described. In particular, we demonstrated how both D-amino acid and Gly-Xaa dipeptide acceptors might separately bind at the active site. Direct application of **1** to kinetic studies is limited, however, by its weak, low wavelength absorption spectrum that precludes its direct observation in the presence of peptide or aromatic acyl acceptors. In this paper, we describe the synthesis and application of the specific thiolester **3**, which has been designed to expedite studies of the transpeptidase reaction with specific acyl donor and acceptor substrates. Reaction of this compound can be followed either directly at wavelengths between 240 and 260 nm or indirectly at longer wavelengths by means of a chromogenic thiol trap. With the aid of **3**, we have been able to more closely define the acyl acceptor specificity of the *Streptomyces* R61 DD-peptidase and thereby design effective "dual function" acceptors. These results should be useful in the design of inhibitors of this enzyme and may indicate a direction to take in pursuit of new inhibitors of DD-peptidases in general, i.e., of new antibiotics.

MATERIALS AND METHODS

Materials. The DD-peptidase of *Streptomyces* R61 was generously supplied by Dr. J. -M. Frère of the University of Liège (Liège, Belgium). Sources of amino acids and peptides were as described in the accompanying paper (*7*). The peptide Gly-L-Lys(Ac-L-Ala-D-iGln)[1] was purchased from Multiple Peptide Systems, and **4** was prepared by Dr. Rajesh Nagarajan in this laboratory (*11*). Syntheses of aminomalonyl-L-alanine (**5**) and its methyl ester (**6**), aminomalon-(*N*-ethyl)-amide (**7**), aminomalon-(*N*-phenethyl)amide (**8**), and *N*-ethyl-D-asparagine (**9**) are described in the Supporting Information.

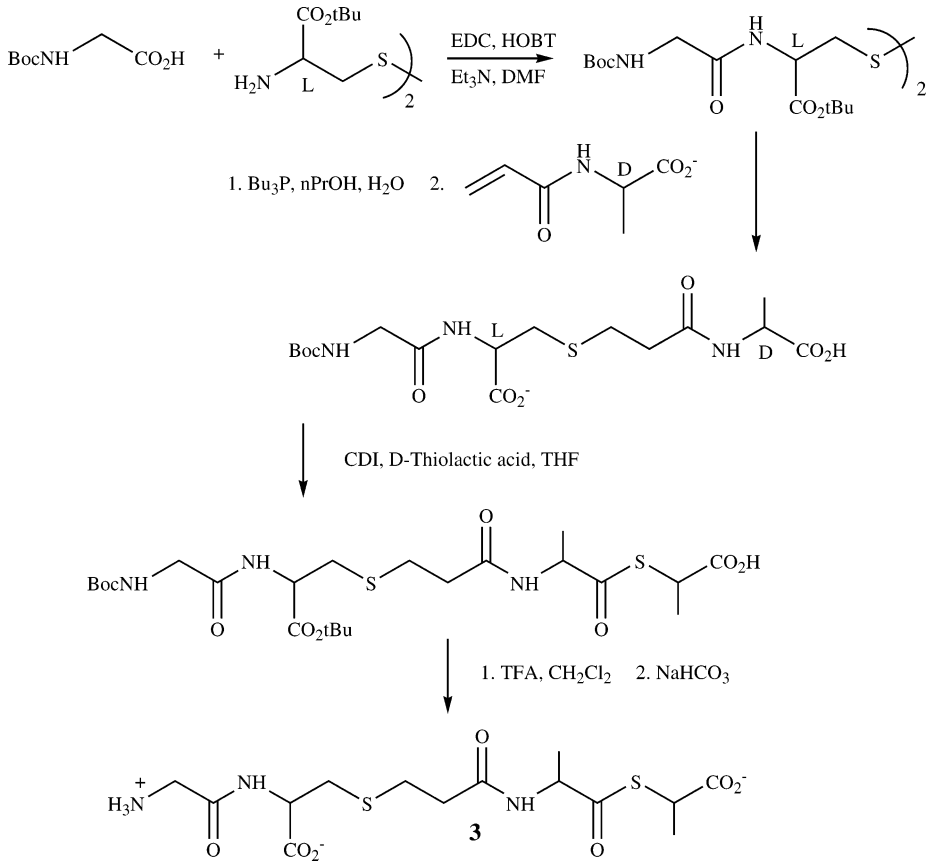


3-(N-Glycyl-L-cysteinyl)-propanoyl-D-alanyl-D-thiolactate (3). This compound was prepared as outlined in Scheme 2. First, di-(*N*-t-Boc-glycyl)-L,L-cystine di-*tert*-butyl ester was prepared by coupling t-Boc-glycine with L,L-cystine di-*tert*-butyl ester. Thus, t-Boc-glycine (806 mg, 4.61 mmol) and cystine di-*tert*-butyl ester (1.0 g, 2.35 mmol) were dissolved in dry DMF (25 mL) and the solution cooled with stirring to 0 °C. *N*-Hydroxybenzotriazole (699 mg, 5.17 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (991 mg, 5.17 mmol), and triethylamine (0.65 mL, 8.9 mmol) were then added successively. Reaction was allowed to proceed for 20 h at 4 °C. Subsequently, the DMF was removed by evaporation under reduced pressure and the residue partitioned between ethyl acetate (20 mL) and water (10 mL). The aqueous layer was extracted twice more with 20 mL portions of ethyl acetate. The combined ethyl acetate extracts were washed successively with 0.2 M $KHSO_4$ solution, water, 0.4 M $NaHCO_3$ solution, and water (2×25 mL each). Evaporation of ethyl acetate from the dried (Na_2SO_4) solution gave the required product as a colorless solid (42%). ^{1}H NMR (d_6-DMSO): δ 1.38 (9H, s, t-Bu), 1.41 (9H, s, t-Bu), 2.92 (1H, dd, $J = 8.2$, 13.5 Hz, CH_2S), 3.11 (1H, dd, $J = 5.3$, 13.7 Hz, CH_2S), 3.57 (2H, d, $J = 6.2$ Hz, CH_2N), 4.45 (1H, m, CH), 6.94 (1H, t, $J = 6.2$ Hz, CH_2*NH*), 8.22 (1H, d, $J = 8.2$ Hz, CH*NH*).

Di-(*N*-t-Boc-glycyl)-L,L-cystine di-*tert*-butyl ester was then reduced to the protected cysteine and this, in situ, added to *N*-acryloyl-D-alanine, prepared as described by Iwakura et al. (*12*). The cystine diester (466 mg, 0.7 mmol) was dissolved in *n*-propanol (4 mL) under a nitrogen atmosphere. Water (13 μL), followed by tributylphosphine (256 μL, 0.91 mmol), was then added. After 1 h of reaction at room temperature, *N*-acryloyl-D-alanine (200 mg, 1.4 mmol), dissolved in water (4 mL) at pH 9.0 (adjusted with ammonium hydroxide) was added, and the subsequent reaction was allowed to proceed at room temperature for 22 h. The propanol was then removed by rotary evaporation and the remaining aqueous layer extracted with ethyl acetate (3×20 mL). The aqueous solution was chilled in an ice bath and its pH reduced to 2.0 by addition of phosphoric acid.

[1] Abbreviations: standard three-letter abbreviations for α-amino acids; Xaa, any α-amino acid; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; NMR, nuclear magnetic resonance.

Scheme 2



The acidified solution was extracted with ethyl acetate (3 × 20 mL), and the combined ethyl acetate extracts were washed with water (2 × 20 mL) and dried over sodium sulfate. On evaporation of the solvent, the required product, 3-(*N*-t-Boc-glycyl-L-cysteinyl)-propanoyl-D-alanine mono-*tert*-butyl ester, was obtained as a colorless glass (56%). ^{1}H NMR (d_6-DMSO): δ 1.24 (3H, d, J = 7.2 Hz, CH_3), 1.38 (9H, s, tBu), 1.41 (9H, s, tBu), 2.38 (2H, t, J = 7.2 Hz, CH_2), 2.69 (2H, t, J = 7.2 Hz, CH_2), 2.74 (1H, dd, J = 6.3, 14.1 Hz, *CH*$_2$ CH), 2.85 (1H, dd, J = 5.4, 13.5 Hz, *CH*$_2$ CH), 3.57 (2H, d, J = 6.2 Hz, CH_2N), 4.19 (1H, quint, J = 7.2 Hz, *CH* CH_3), 4.38 (1H, m, *CH* NH), 6.98 (1H, t, J = 6.3 Hz, CH_2*NH*), 8.12 (1H, d, J = 8.1 Hz, CH*NH*), 8.21 (1H, d, J = 7.2 Hz, CH*NH*).

The above peptide was then condensed with D-thiolactic acid, the latter prepared by the method of Strijtveen and Kellog (*13*). Thus, the peptide (1.15 g, 2.47 mmol) was dissolved in dry THF (12 mL) and chilled, with stirring in an ice bath, under a nitrogen atmosphere. 1,1′-Carbonyldiimidazole (469 mg, 2.89 mmol) was then added and the mixture stirred for 30 min. At this time, D-thiolactic acid (238 μL, 2.7 mmol) was added. The reaction mixture was then stirred for 4 days at 4 °C. To prepare the mixture for workup, the solvent was removed by rotary evaporation. The residue was partitioned between ethyl acetate (40 mL) and 10% citric acid solution (25 mL). The organic layer was washed further with citric acid solution (2 × 25 mL) and then with water (3 × 25 mL). After the organic solution was dried over sodium sulfate, the solvent was removed by rotary evaporation. The residue was purified by flash chromatography on silica gel with 1/5 ethyl acetate/hexane containing 0.1% trifluoroacetic acid as solvent. The final yield was 20%. ^{1}H NMR (d_6-DMSO): δ 1.25 (3H, d, J = 7.2 Hz, CH_3), 1.35 (3H, d, 7.2 Hz, CH_3), 1.35 (s, 9H, tBu), 1.50 (s, 9H, tBu), 2.44 (2H, t, J = 7.2 Hz, CH_2), 2.73 (2H, t, J = 7.2 Hz, CH_2), 2.78 (1H, dd, J = 6.9, 13.5 Hz, *CH*$_2$ CH), 2.87 (1H, dd, J = 5.5, 13.5 Hz, *CH*$_2$CH), 3.57 (2H, d, J = 6.2 Hz, CH_2N), 3.96 (1H, q, J = 7.2 Hz, CHS), 4.35 (1H, m, *CH*CH_2), 4.38 (1H, quint, J = 7.2 Hz, *CH*CH_3), 6.97 (1H, t, J = 6.1 Hz, NH), 8.14 (1H, d, J = 7.8 Hz, NH), 8.61 (1H, d, J = 6.2 Hz, NH).

Finally, **3** was obtained by deprotection of the above material. The thiolactate (170 mg, 0.30 mmol) was dissolved in methylene choride (0.4 mL) and chilled to ice temperature with stirring. Trifluoroacetic acid (2.3 mL) was added slowly, and stirring continued for 2 h to room temperature. Volatiles were removed by evaporation under vacuum. The product was dissolved in water (0.5 mL) and converted to the disodium salt by addition of 2 equiv of $NaHCO_3$. The solid salt was then obtained by freeze-drying the solution. ^{1}H NMR (D_2O): δ 1.39 (3H, d, J = 6.9 Hz, CH_3), 1.44 (3H, d, J = 7.2 Hz, CH_3), 2.63 (2H, t, J = 6.9 Hz, CH_2), 2.86 (2H, t, J = 6.9 Hz, CH_2), 2.91 (1H, dd, J = 8.4, 13.8 Hz, *CH*$_2$ CH), 3.10 (1H, dd, J = 4.1, 13.8 Hz, *CH*$_2$CH), 3.88 (2H, s, CH_2N), 3.95 (1H, q, J = 7.2 Hz, CH), 4.41 (1H, dd, 4.5, 8.4 Hz, *CH* CH_2), 4.51 (1H, q, J = 6.9 Hz, CH). Mass spectrum (ES+): *m*/*e* 410.1.

Kinetics. All kinetics studies were performed at 25 °C. The buffers employed were 10 mM phosphate (pH 7.6) and 10 mM Tris (pH 8.9). Steady state studies of the hydrolysis and aminolysis of **3** were obtained spectrophotometrically, either directly at 240 nm ($\Delta\epsilon$ = 2230 cm^{-1} M^{-1}), following disappearance of **3**, or indirectly at 324 nm ($\Delta\epsilon$ = 1.98 × 10^4 cm^{-1} M^{-1}), following the appearance of 4-pyridinethione

generated by the rapid reaction of thiolactate with 4,4′-dipyridyl disulfide (1mM) (*14*). The latter, more sensitive, method was used for the determination of the K_m value for hydrolysis of **3**. The latter method was also employed when the reactions of **3** with nucleophiles that absorbed strongly at 240 nm, e.g., phenylalanine and its peptides, were studied. Typical reaction mixtures (100 μL) contained enzyme (1.0−2.5 nM), **3** (0−100 μM), and, where required, amino acid or peptide acyl acceptors (0−100 mM). Ratios of hydrolysis to aminolysis products were obtained from hplc experiments as described in the accompanying paper (*7*).

Detection of acyl-enzymes by quenching of the enzyme fluorescence was achieved by means of a Spex Fluoromax 2 spectrofluorimeter. Reaction mixtures (100 μL) typically contained enzyme (0.2 μM) and either **3** (2.0 mM) or **4** (2.0 mM). Fluorescence intensity at 325 nm (excitation at 280 nm) was monitored as a function of time after initiation of the reaction by addition of enzyme. The data from all of the above kinetics experiments was fitted to the appropriate reaction scheme by means of the Dynafit program (*15*).

Molecular Modeling. This was carried out using the Insight II 2000 suite of programs (Accelrys, San Diego, CA), as described in the accompanying paper (*7*).

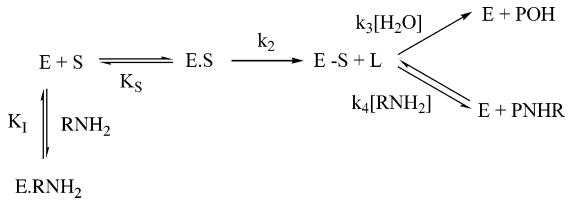
RESULTS AND DISCUSSION

The chromogenic substrate **3** was prepared as shown in Scheme 2. It contains a close analogue of the glycyl-L-α-aminopimelyl side chain of the specific peptide substrate **1** but with a D-thiolactate leaving group. The pimelyl side chain was replaced by thiapimelyl for ease of synthesis (*11*). The peptide analogous to **3**, viz., **4**, has also been prepared in this laboratory (*11*). It is a good substrate of the R61 DD-peptidase, but somewhat poorer than **1**, probably because of a smaller hydrophobic interaction between its side chain and the enzyme (*11*).

The R61 DD-peptidase catalyzes hydrolysis of **3** with release of D-thiolactate. This was demonstrated by a ^{1}H NMR experiment where the enzyme was added (final concentration 27 nM) to a solution of **3** (2.3 mM) and $NaHCO_3$ (20 mM) in D_2O (600 μL). NMR spectra as a function of time showed the rapid disappearance of **3** from solution and the appearance of D-thiolactate; the spectrum of the other product was consistent with it being 3-(*N*-glycyl-L-cysteinyl)-propanoyl-D-alanine. This hydrolysis reaction can be conveniently monitored spectrophotometrically at wavelengths between 240 and 260 nm, or indirectly at 324 nm with the use of 4,4′-dipyridyl disulfide to trap the thiolactate (*13*). At 25 °C in 10 mM phosphate buffer at pH 7.6, the steady state kinetics parameters for hydrolysis of **3** catalyzed by the R61 DD-peptidase were $k_{cat} = (42 \pm 3)$ s^{-1}, $K_m = (4.0 \pm 0.6)$ μM, $k_{cat}/K_m = 1.05 \times 10^7$ s^{-1} M^{-1}. These parameters indicate that **3**, like **1** and **2**, reacts with the enzyme at close to or at a diffusion controlled rate and, like **1**, is an excellent substrate. Comparison of the rate parameters obtained for **3** with those of **4**, the peptide bearing the same acyl group, is interesting and is discussed below. The latter values are $k_{cat} = (48 \pm 8)$ s^{-1}, $K_m = (260 \pm 100)$ μM; $k_{cat}/K_m = 1.85 \times 10^5$ s^{-1} M^{-1} (*11*).

In terms of the well-established reaction scheme for turnover of substrates by the R61 DD-peptidase (*16*) (Scheme 3), the comparison referred to above suggests, first, that

Scheme 3



deacylation may be the common rate-determining step for turnover of **3** and **4** under conditions of substrate saturation. In Scheme 3, E.S represents the noncovalent enzyme/substrate complex, E-S the acyl-enzyme, and L the leaving group, either D-alanine (from **4**) or D-thiolactate (from **3**).

The acyl-enzyme may be either hydrolyzed to POH, or, in the presence of a suitable acyl acceptor, aminolyzed to PNHR. The latter step is shown as reversible since the product PNHR will often also be a (poor) substrate and thus can react to slowly regenerate E-S and thus, finally, POH (*7*). In certain cases, RNH_2 may also be an inhibitor (K_I) (see below). The similarity of the k_{cat} values of **3** and **4** suggests that a common rate-determining step obtains, which presumably must be hydrolysis of the common acyl-enzyme, i.e., $k_{cat} = k_3[H_2O]$ in each case. The lower k_{cat}/K_m value for **4** would then reflect the slower acylation of the enzyme (k_2/K_s) by the chemically less reactive amide (k_2 for **4** would be less than that for **3**, but each would be greater than k_3).

The conclusion that deacylation is rate-determining for turnover of the amide **4** is rather unexpected. Usually, with this enzyme, turnover of amides is rate-limited by enzyme acylation while that of (thiol)esters is rate-limited by deacylation (*9*, *17*, *18*). Certainly, at saturation, turnover of **1** and the nonspecific amide *N*,*N*′-diacetyl-L-lysyl-D-alanyl-D-alanine appear limited by acylation (*17*).

In order to clarify this issue, two further experiments were carried out. First, the effects of **3** and **4** on the fluorescence of the enzyme were examined. It is well-known that acylation of the R61 DD-peptidase by either β-lactams or depsipeptide substrates leads to partial quenching of the enzyme fluorescence. Thus, an accumulating acyl-enzyme can be detected (*18*, *19*). On the other hand, with peptide substrates such as **1**, or *N*,*N*′-diacetyl-L-lysyl-D-alanyl-D-alanine, where acylation is rate-determining and no acyl-enzyme accumulates, no such quenching of fluorescence is observed (*9*, *17*). The results of such experiments for **3** and **4** at pH 7.6 are shown in Figure 1. An instantaneous quenching of enzyme fluorescence was observed, followed by a slower return of fluorescence intensity, presumably as the substrates hydrolyzed and free enzyme was restored. The data could be quantitatively fitted to Scheme 4 as shown in the figure. Product inhibition was included in the scheme since it becomes significant as the reaction proceeds at the initial substrate concentrations employed (*9*, *11*). This experiment strongly supports the proposition that $k_2 > k_3[H_2O]$ for both **3** and **4**.

The effect of alternative nucleophiles was also examined. As shown in the accompanying paper (*7*), the reactions of **1** in the presence of D-amino acids (RNH_2) as acyl acceptors could be interpreted in terms of Scheme 3. If, as deduced above, $k_2 > k_3[H_2O]$ for **3**, then the observed steady state rate of reaction of **3** (release of thiolactate) should increase with $[RNH_2]$. This was found to be true for glycine,

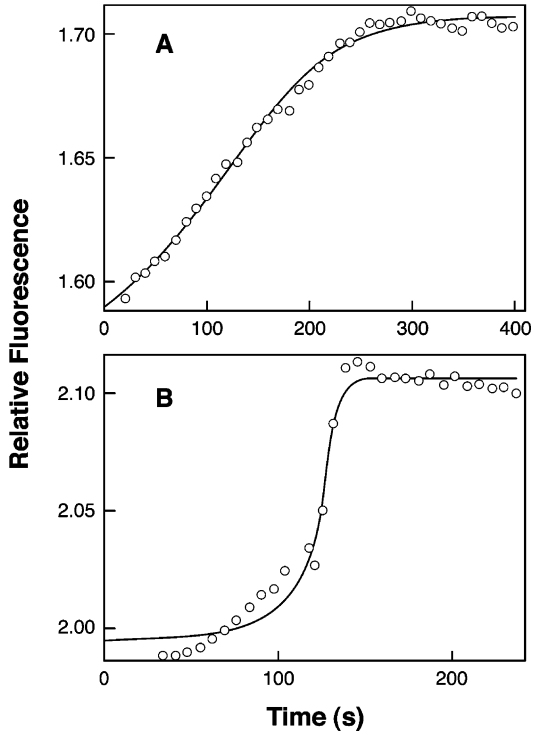


FIGURE 1: Fluorescence intensity as a function of time after mixing the R61 DD-peptidase (0.2 μM) with **4** (2.0 mM), panel A, or **3** (2.0 mM), panel B. The lines represent fits of Scheme 4 to the data (see text).

Scheme 4

$$E + S \underset{K_m}{\rightleftharpoons} ES \xrightarrow{k_{cat}} EP \overset{K_p}{\rightleftharpoons} E + P$$

D-alanine, D-norleucine, and D-phenylalanine as nucleophiles. Figure 2 shows the data for D-alanine and glycine at pH 7.6. The data in each case show an approach to saturation in $[RNH_2]$. This can be interpreted in terms of Scheme 3 as reflecting a change in rate-determining step from deacylation to acylation. If this were happening, the rate at saturating $[RNH_2]$ would be $k_2[E]_0[S]_0/(K_s + [S]_0)$ and, if, as is likely (cf. the K_m value for **4**), $[S]_0 \ll K_s$, the saturation rate would be given by $(k_2/K_s)[E]_0[S]_0$, which is equal to $(k_{cat}/K_m)[E]_0[S]_0$; the latter was found to be true. The data of Figure 2 give k_4/k_3 values of $(1.6 \pm 0.3) \times 10^4$ and $(1.0 \pm 0.4) \times 10^3$ for D-alanine and glycine, respectively.

The applicability of Scheme 3 to both **3** and **4** was demonstrated by an experiment with an amine acceptor. One would expect the same acyl-enzyme to be generated from **3** and **4** and hence the same k_4/k_3 ratio for a given acceptor. Product analysis was conducted as described in Materials and Methods for reaction between **3** and **4** (1 mM each) and glycine (100 mM and 200 mM) in the presence of the R61 DD-peptidase (15 nM and 30 nM for **3** and **4**, respectively) at pH 7.6. After 20 min, hplc data showed that aminolysis/hydrolysis ratios were 1.9 and 2.1 for **3** and **4**, respectively, in 100 mM glycine, and 3.2 and 3.4, respectively, in 200 mM glycine. The agreement between the ratios for **3** and **4** is good evidence for a common acyl-enzyme intermediate. The ratios correspond to a k_4/k_3 value of $(1.0 \pm 0.1) \times 10^3$ for glycine, in good agreement with the value from kinetics data (see above).

In a similar vein, the value of k_4/k_3 for D-alanine from its inhibition of hydrolysis of **4** [in an experiment similar to that shown in Figure 1 of the accompanying paper (*7*)] was $(1.1 \pm 0.2) \times 10^4$. Again a common acyl-enzyme is strongly implied.

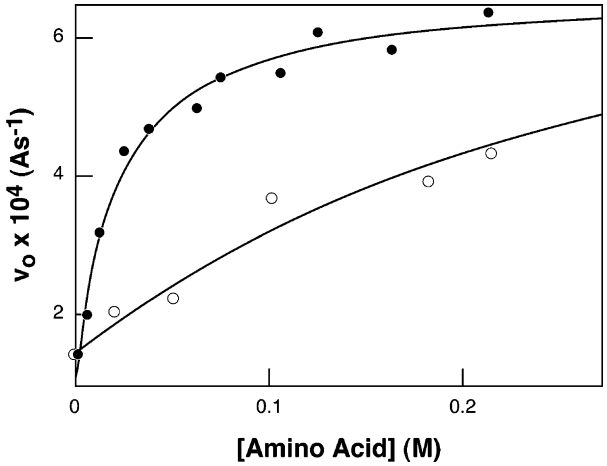


FIGURE 2: Steady state initial velocities (absorbance change/s) of reaction of **3** (34 μM) with the R61 DD-peptidase (1.25 nM) in the presence of D-alanine (●) or glycine (○).

Table 1: Transpeptidation Rate Constants and Inhibition Constants for the R61 DD-Peptidase-Catalyzed Reaction between the Thiodepsipeptide **3** and Amino Acids or Peptides at pH 8.9

acyl acceptor	highest concn (mM)	$(k_4/k_3) \times 10^{-4}$	K_I (mM)
D-Ala	70	3.0 ± 0.8	*a*
L-Ala	50	≤ 0.14	*a*
Gly	100	1.2 ± 0.3	*a*
D-Nle	70	3.4 ± 0.9	*a*
D-Phe	60	4.7 ± 1.1	*a*
Gly-L-Ala	120	1.30 ± 0.65	43 ± 10
Gly-L-AlaNH$_2$	50	0.038 ± 0.005	*a*
Gly-L-Phe	50	13.0 ± 6.5	11 ± 3
Gly-D-Phe	50	0.46 ± 0.19	22 ± 5
Gly-L-Nle	10	$\leq 2^b$	0.8 ± 0.1
Gly-L-Lys(Ac)	40	$\leq 0.3^b$	14 ± 4
Gly-L-Lys(Ac-L-Ala-D-iGln)	20	$\leq 0.2^b$	4.3 ± 0.4
Gly-L-Ala-Gly	60	0.49 ± 0.09	*a*
Gly-L-Ala-L-Phe	15	1.9 ± 0.9	*a*
Gly-L-Phe-L-Ala	20	2.5 ± 1.5	*a*
D-Ala-L-Ala	50	≤ 0.02	*a*

[a] No inhibition observed. [b] No rate acceleration observed.

The kinetics data obtained from application of a variety of alternative amine nucleophiles is presented in Table 1. This data was obtained at pH 8.9 where the alternative acyl acceptors were more effective (*7*). The data show, first, that D(but not L)-amino acids are good acyl acceptors in the reaction of the DD-peptidase with **3**, as they are with **1** [accompanying paper (*7*)]. With the assumption that the R61 DD-peptidase is indeed a transpeptidase in vivo, the expected nucleophile in deacylation would be the amine terminus of the stem peptide (Chart 1). Gly-L-Xaa peptides therefore could be small molecule analogues of the stem peptide. Such dipeptides were in fact acyl-acceptors with **3**, as they have been demonstrated to be of **1** (*7*). The dipeptides Gly-L-Ala and, especially, Gly-L-Phe were also good acceptors. Loss of the terminal carboxylate, as in Gly-L-AlaNH$_2$ and Gly-L-PheNH$_2$, and inversion in the C-terminal amino acid, as in Gly-D-Phe, had large negative effects on the ability of these acceptors. It is noticeable, however, that tripeptides, where a carboxylate is restored, albeit at a greater formal

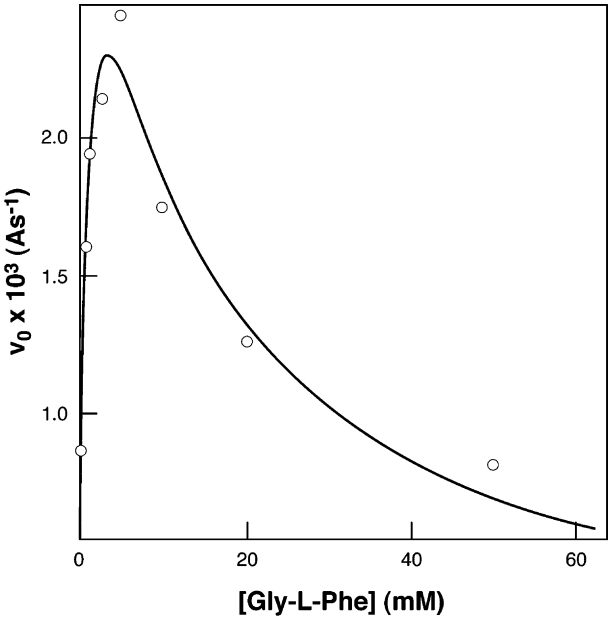


FIGURE 3: Steady state initial velocities (absorbance change/s) of reaction of **3** (34 μM) with the R61 DD-peptidase (0.68 nM) in the presence of Gly-L-Phe.

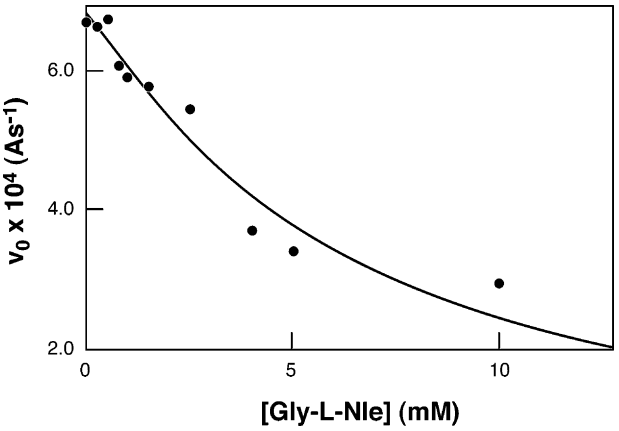
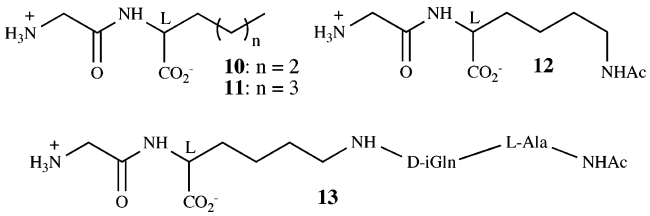


FIGURE 4: Steady state initial velocities (absorbance change/s) of reaction of **3** (34 μM) with the R61 DD-peptidase (5 nM) in the presence of Gly-L-Nle.

distance from the nucleophilic center, are quite good acceptors, particularly again when the aromatic ring of Phe is included. The results, overall, are qualitatively similar to those obtained with **1** [accompanying paper (*7*)], although the significantly larger k_4/k_3 values for **3** are noteworthy (see below).

The Gly-L-Xaa peptides were also, in general, inhibitors of turnover of **3**; see, for example, the data for Gly-L-Phe shown in Figure 3. At higher Gly-L-Phe concentrations, above 5 mM, after the initial rate acceleration, inhibition of the reaction was observed. These data were fitted to Scheme 3, where competitive inhibition by the dipeptide is included. It is likely that Gly-L-Xaa peptides also bind in the acyl donor site, preventing the binding of **3**. Indeed, such binding was proposed to explain the inhibition of reaction of β-lactams with this enzyme (*9*). The interaction in that case was noncompetitive since shorter Gly-L-Xaa species could also bind to the enzyme/β-lactam complex. Frère et al. have observed noncompetitive inhibition of hydrolysis of the nonspecific peptide *N*,*N*′-diacetyl-L-lysyl-D-alanyl-D-alanine by Gly-L-Ala (*20*). In some cases of Gly-L-Xaa, for example Gly-L-Nle, inhibition dominated the effect of the dipeptide on rates (Figure 4) and no rate acceleration was observed. This inhibition likely arises from competition between Gly-L-Xaa and **3** for the acyl donor site because of the structural similarity between these peptides and the N-terminus of α-glycyl-L-aminopimelyl peptides (*9*).

If the R61 DD-peptidase were a transpeptidase in vivo, one would expect that more extended glycyl peptides, mimicking the stem peptide itself, would also be acyl acceptors. Thus, the peptides glycyl-L-norleucine (**10**), *N*-(glycyl)-D,L-α-aminocaprylic acid (**11**), α-*N*-glycyl-ϵ-*N*-acetyl-L-lysine (**12**), ϵ-*N*-glycyl-α-(*N*-acetyl-L-alanyl-D-isoglutaminyl)-L-lysine (**13**), and **1** itself were examined. Curiously, these compounds



show little or no sign of accelerating the enzyme-catalyzed hydrolysis of **3** (see Table 1 for upper limits to k_4/k_3 values); all, however, do inhibit the hydrolysis (see K_I values in Table 1) as do the smaller dipeptides described above and, as there, presumably by competition with the substrate at the acyl donor side chain site. Similarly, only inhibition was observed when the reaction of **3** was monitored in the presence of **1**. Even in a less aqueous medium [17:58:25, water:ethylene glycol:glycerol (*20*−*22*)], hplc/mass spectral analysis revealed no transpeptidation products at 50% complete reaction of 8 mM **1** with 0.16 μM DD-peptidase. The ineffectiveness of Gly-L-Nle and Gly-D,L-α-aminocaprylic acid as acceptors, in view of the reactivity of Gly-L-Phe, the best of the dipeptides examined, is very curious. It has been suggested (*7*) that Gly-L-Phe may bind in an orientation different from that of Gly-L-Ala, because of the hydrophobicity of the phenyl ring in the former peptide, but the reason why such binding should not be available to Gly-L-Nle and **10**−**13** is not apparent.

The lack of acyl acceptor activity in **1** and **10**−**13** is striking and contrasts with the observations of Ghuysen and co-workers with similar peptidoglycan-mimetic acceptors (*20*−*22*). A significant difference between these earlier observations and the current results lies in the nature of the acyl donor. All previous studies of this question, in vitro, have been made with less specific donor substrates, lacking, particularly, the glycyl-L-amino acid N-terminus. That this difference is significant is suggested in the present experiments by the higher k_4/k_3 values obtained for **3** than for the more specific substrate **1**. Another example of this phenomenon may be seen in experiments with the potential acceptors D-lactate and D-phenyllactate (close analogues of the acceptors D-alanine and D-phenylalanine, respectively). Jamin et al. have shown that D-lactate accelerated the hydrolysis of hippuryl thioglycolate with formation of hippuryl D-lactate (*18*). Neither D-lactate nor D-phenyllactate, however, accelerated hydrolysis of **3**, and no product of nucleophilic displacement was evident by hplc analysis. It may be, therefore, that with specific acyl donors, which presumably include *Streptomyces* stem peptides, the R61 DD-peptidase

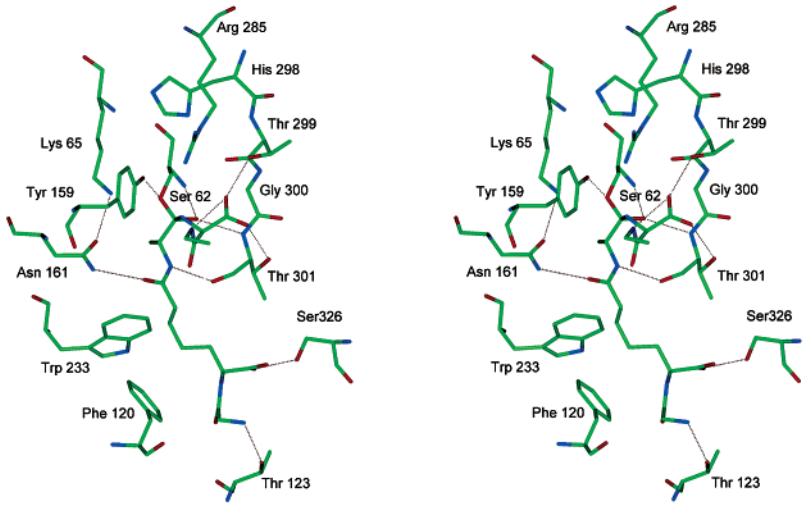
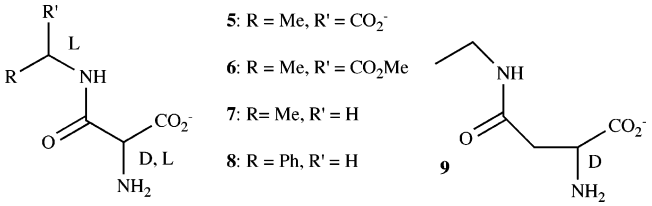


FIGURE 5: Stereoview of an energy-minimized tetrahedral intermediate complex structure formed on reaction of the acyl acceptor **7** with the acyl enzyme formed on reaction of the R61 DD-peptidase with **1**. Only heavy atoms are shown.

does not catalyze transpeptidation to extended amine acceptors and therefore may not be a transpeptidase in vivo.

As discussed in the accompanying paper (*7*), the bacterial DD-peptidases must have a dual function acyl acceptor binding site, one that is able to specifically accommodate the D-alanine leaving group, and also the glycyl-L-amino acid acceptor. Since the enzyme employs a double displacement mechanism with an acyl-enzyme intermediate, the two binding modes must overlap to some degree. Since D-Ala-L-Ala is not an acceptor (Table 1), it seemed likely that the carboxyl of a dipeptide acceptor does not occupy the carboxylate site of the acceptor D-alanine [see Figure 8 of the accompanying paper (*7*)].We hypothesized, therefore, that a combination or dual function acceptor may be more effective than either of the two separately. The molecules **5** and **6** were, therefore, prepared, and tested as acyl acceptors against **3**. Of these, **5** did not accelerate the hydrolysis of **3**,



but **6** was a good acceptor [$k_4/k_3 = (8 \pm 3) \times 10^4$]. This result shows that the concept of a dual function acceptor may have merit, but two carboxylate groups cannot be present and optimally occupy the same sites that they do in individual acceptors. On the basis of these results, **7** and **8** were prepared, the former containing the methyl group of Gly-L-Ala and the latter the benzyl group of Gly-L-Phe, both good dipeptide acceptors. The homo analogue of **7**, *N*-ethyl-D-asparagine, **9**, was also prepared.

The malonyl C−H bond of **5**−**8** was found to be labile under the reaction conditions used for kinetics measurements (NMR spectra of **7** in 10 mM Tris buffer at pH 8.9, for example, showed that the malonyl hydrogen exchanged with solvent deuterium with a half-time of about 40 min), so separation of the epimers at this center was not attempted. It is assumed that the reactive form of these molecules has the configuration of a D-amino acid at this position.

Both **7** and **8** were very good acceptors, with k_4/k_3 values of $(1.3 \pm 0.5) \times 10^5$ each, but **9** was less effective [$k_4/k_3 = (1.3 \pm 0.2) \times 10^4$]. The latter result attests to the specificity of **7** and **8**. It is striking that **7** and **8** are essentially equally effective as acceptors despite the presence of the additional phenyl group on **8** [compare the relative reactivity of Gly-L-Ala and Gly-L-Phe (Table 1)] and that, within experimental uncertainty, the most effective Gly-L-Xaa species, Gly-L-Phe, also has the same value for this parameter. This value of k_4/k_3 corresponds to a k_4 value of 1.2×10^5 s^{-1} M^{-1}. It is possible that this rate constant represents an upper limit, where, with very efficient acceptors, the transpeptidation reaction is limited by some common physical process such as a conformational change. Crystal structures of analogues of reaction intermediate complexes along the reaction coordinate of transpeptidation have been interpreted (*23*, *24*) in terms of an induced fit mechanism where the initial binding of a specific side chain induces a reactive conformation of the α-2 helix at the reaction center. This induced tension appears to relax as the reaction progresses. It is possible that the rate of aminolysis of the acyl-enzyme by specific acceptors may become limited by this conformational relaxation. Irrespective of this point, however, it is clear that the "dual function" acceptors **7** and **8** are very reactive and thus the rationale for their design, viz., to take advantage of the close, but separate alignment of D-amino acid and Gly-L-Xaa acceptors at the active site, may be correct.

A molecular model was constructed of **7** at the active site of the enzyme. This was accomplished by removing the terminal carboxylate from the Gly-L-Ala peptide in a previously constructed model of a complex of this peptide with the enzyme [Figure 6A of the accompanying paper (*7*)], and adding a D-amino acid carboxylate to the glycyl α-carbon. The model was then subjected to energy minimization with the result shown in Figure 5. Essentially all of the strong interactions previously noted (*7*) between protein and ligand were retained. The malonyl carboxylate remained in a position similar to that seen in the D-alanine adduct described in the accompanying paper (*7*, Figure 5A), with strong interactions with the side chain hydroxyl groups of Thr 299 and Thr 301; the interaction with Arg 285 seemed weaker, however, with the closest nitrogen of the arginine

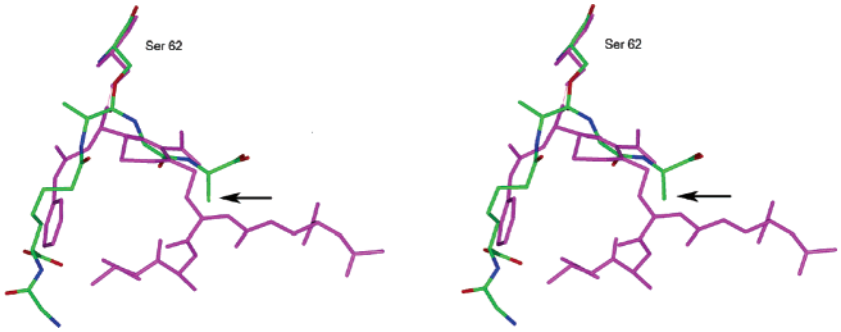
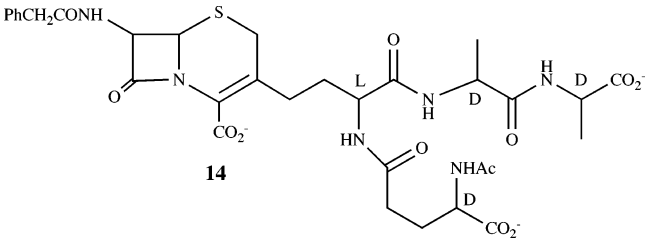


FIGURE 6: Stereoview of an overlap between the crystal structure of a covalent complex of the R61 DD-peptidase with the cephalosporin **14** and a model tetrahedral intermediate derived from nucleophilic attack of Gly-L-Ala on the acyl enzyme derived from **1**. Only heavy atoms of the ligand and Ser 62 are shown. The L-Ala methyl group of Gly-L-Ala is indicated by an arrow. A complete *Streptomyces* peptidoglycan acyl acceptor would extend this methyl group by four more carbon atoms followed by D-Ala-D-Ala.

side chain 3.9 Å from a carboxylate oxygen. The *N*-ethylamide terminus moved to some extent during energy minimization from a position with the methyl group adjacent to Leu 332 to one closer to Tyr 159, as seen in the Gly-D-Phe model (*7*, Figure 7). These positions remained essentially unchanged during molecular dynamics simulations. The ligand **7**, therefore, can occupy the active site in a way reminiscent of both D-alanine and Gly-L-Phe.

Lee et al. have described the crystal structure of a covalent complex between the R61 DD-peptidase and a cephalosporin (**14**) containing a peptidomimetic substituent at the 3′-position (*25*). The aim in the preparation of this β-lactam



14

was to produce a model of a DD-peptidase with the acyl acceptor substrate bound. Although elegant in concept, **14** has several flaws with respect to the aim. Some of these have been also noted by Frère (*26*). First, an accurate model of a *Streptomyces* acceptor would have the nucleophilic terminus extended by an additional glycyl residue (in **14**, the nucleophilic terminus, represented by the β-lactam nitrogen, mimics an α,ϵ-diaminopimelic acid terminus), and D-isoglutamyl should be D-isoglutaminyl (*8*). Further, and probably more seriously, the twist created about the C_6-C_7 bond on acylation of the enzyme and concomitant β-lactam ring-opening move the β-lactam carboxylate away from Arg 285. In crystal structures of a complex between an accurate peptidoglycan-mimetic peptide substrate and the R61 DD-peptidase (*23*), the terminal (D-Ala) carboxylate strongly interacts with Arg 285. This is seen also in a product complex (*23*) and in models (*27*). Finally, the geometry of the dihydrothiazine ring in the covalent complex of **14** with the enzyme also serves to direct the peptide substituent in a direction that it would not necessarily go if unfettered. A result of this is to place the terminal D-Ala carboxylate of **14** in hydrogen bonding distance of the backbone NH of Asn 327, where it would seriously and negatively impact upon the pimelyl carboxylate of the donor strand; in all available crystal structures (*23*, *24*, *28*), the latter residue also hydrogen bonds to Asn 327. Thus, there is reason to doubt whether the enzyme complex with **14** really does represent a good model of a *Streptomyces* acceptor strand bound to the R61 DD-peptidase. An overlap diagram, illustrating the issues discussed above, is shown in Figure 6.

CONCLUDING DISCUSSION

Preparation of the peptidoglycan-mimetic thiolester **3** produced an excellent substrate of the R61 DD-peptidase whose reaction with the enzyme, like that of the peptide **1**, may well be diffusion-controlled. Experiments to prove the latter have not been yet possible because of the enzyme's adverse reaction to the common viscosogens (*10*). The chromogenic properties of **3** permitted technically simple studies of the transpeptidation reaction: enzyme deacylation is rate-determining at saturation so that the aminolyis reaction could be studied directly. As with the peptide **1**, D-amino acids and Gly-L-Xaa dipeptides were good acyl acceptors, where the best of the former employed was D-Phe and the best of the latter Gly-L-Phe. Extension of the active dipeptides to tripeptides produced molecules with some acceptor ability (but see below). Consideration of the structure of these acceptors permitted design of the "dual function" acceptors **7** and **8**. These were excellent acceptors, of similar reactivity to Gly-L-Phe. The apparent upper limit to the activity of these molecules suggests that, with very good acceptors, the deacylation rate may be limited by a common physical step. This issue will be further investigated. Although there was no evidence for measurable noncovalent binding of these acceptors prior to the aminolysis reaction, specificity therefore arising seemingly in a negative, exclusionary fashion, almost by default, it is still possible that incorporation of the structural elements of the dual function acceptors into an extended molecule may be advantageous to inhibitor development. Curiously, more extended peptidogycan-mimetic analogues were extremely poor acyl acceptors in the transpeptidation reaction. This finding may be an extension of the observation made above, i.e., that the thermodynamics of acceptor binding are neutral, at best, perhaps from the canceling out of environmental interactions on binding. More extended (and flexible) and peptidoglycan-like acceptors may be even less likely to bind and react under these circumstances for entropic reasons, unless, as perhaps occurs in vivo, the acceptor is immobilized in its specific locale. Alternatively, the R61 DD-peptidase may not be a

transpeptidase in vivo, but rather a pure carboxypeptidase or a β-lactam trap (*29*).

SUPPORTING INFORMATION AVAILABLE

Details of the synthesis of the acyl acceptors **5**−**9**. This material is available free of charge via the Internet at http://pubs.acs.org.

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