FOR THE RECORD

Specificity and reversibility of the transpeptidation reaction catalyzed by the *Streptomyces* R61 D-Ala-D-Ala peptidase

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Abstract

The specificity of the *Streptomyces* R61 penicillin-sensitive D-Ala-D-Ala peptidase has been re-examined with the help of synthetic substrates. The products of the transpeptidation reactions obtained with Gly-L-Xaa dipeptides as acceptor substrates are themselves poor substrates of the enzyme. This is in apparent contradiction with the classically accepted specificity rules for D-Ala-D-Ala peptidases. The Gly-L-Xaa dipeptide is regenerated by both the hydrolysis and transpeptidation reactions. The latter reaction is observed when another Gly-L-Xaa peptide or D-Alanine are supplied as acceptors. Utilization of substrates in which the terminal -COO^- group has been esterified or amidated shows that a free carboxylate is not an absolute prerequisite for activity. The results are discussed in the context of the expected reversibilty of the transpeptidation reaction.

Keywords: D-Ala-D-Ala peptidase; transpeptidation; reversibility; specificity; β-lactam antibiotic; penicillin binding protein; enzymes; active sites; thermodynamics; hydrodynamics; kinetics; mechanism

At the present time, the *Streptomyces* R61 DD-peptidase is certainly the best studied penicillin-sensitive enzyme (McDonough et al. 2002; Anderson et al. 2003; Silvaggi et al. 2003, 2004, 2005; Josephine and Pratt 2004; Nagarajan and Pratt 2004; for review, see Frère 2004). Numerous kinetic and structural data have been accumulated over the years. In the carboxypeptidase reaction, the enzyme cleaves the C-terminal D-Ala residue of peptides of general structure R-D-Ala-D-Ala (the "donor") according to a three-step pathway

$$E + C \overset{K}{\Leftrightarrow} EC \overset{k_2}{\rightarrow} \underset{(+\,P_2)}{EC^*} \overset{k_3(H2O)}{\longrightarrow} E + P_1$$

where E is the enzyme; C, the substrate; K, the dissociation constant of EC; and k_2 and $k_3(H_2O)$, first order rate constants. EC* is an acylenzyme intermediate (E-O-D-Ala-R), P_2 is the C-terminal amino acid of the substrate (D-Alanine), and P_1 is the R-D-Ala peptide. With β-lactams, the same reaction pathway prevails, but there is no P_2 and the decay of the acylenzyme is very slow (low $k_3(H_2O)$ values, of the order of 10^{-4} sec^{-1} or lower). With peptide substrates, the kinetic parameter k_{cat}/K_m is equal to k_2/K and acylation has been shown to be rate-limiting,

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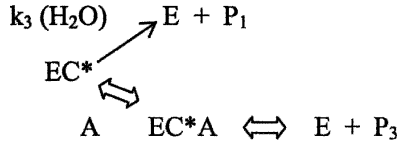
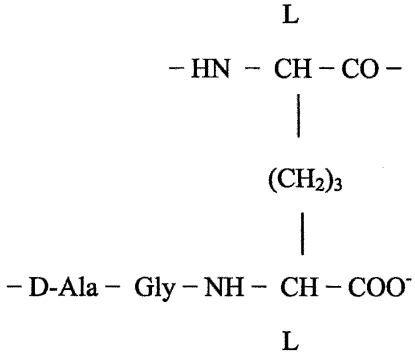
even with the most specific (and best) substrate described so far (Anderson and Pratt 2000). With β-lactams, the rate of acylation rests on the k_2/K ratio, a second-order rate constant, which can thus be directly compared with the k_{cat}/K_m ratio of substrates.

When a suitable acceptor is present, the EC* acylenzyme is partitioned between hydrolysis and transfer reactions (Frère et al. 1973). Good acceptors are D-amino acids or dipeptides of general structure Gly-L-Xaa (Perkins et al. 1973). The most simple partitioning scheme would be

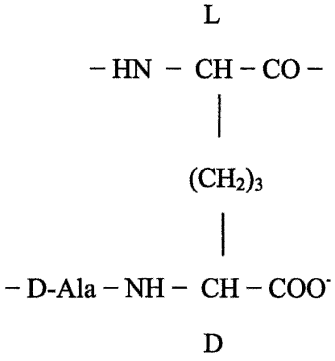


where A is the acceptor and P_3 is the transpeptidation product. The data of Jamin et al. (1993), however, indicate that the situation might be more complicated.

The Gly-L-Xaa acceptors reflect the physiological transpeptidation reaction in *Streptomyces* whose peptidoglycan peptide cross-link is -D-Ala-Gly-LL-A_2pm where A_2pm is diaminopimelic acid, the distal -NH-CH-CO- group of which is part of a second stem peptide.



In contrast, the *Actinomadura* R39 peptidoglycan contains a direct -D-Ala-D(meso)-A_2pm



cross-link, and consequently, the only good acceptors are D-amino acids (Ghuysen et al. 1973).

Other compounds of general structure R-CO-NH-CHR_1-CO-X-CHR_2-COO^- (where R_1 and R_2 can be H or CH_3 and X = O or S) also serve as good donor substrates and, when X = S, deacylation becomes rate-limiting at saturation by the donor so that the presence of a good acceptor increases the k_{cat} value in a concentration-dependent mode (Jamin et al. 1991).

Early experiments indicated that the transpeptidation products obtained with dipeptides might be substrates of the R61 enzyme, in agreement with the fact that the transpeptidation reaction is expected to be reversible. However, the results were far from clear (in particular, the transpeptidation reaction did not appear to be reversible since no Ac_2 L-Lys-D-Ala-D-Ala could be detected upon incubation of Ac_2 L-Lys-D-Ala-Gly-L-Ala with D-Alanine) and, in the present contribution, this problem was re-examined with the help of newly synthesized R-D-Ala-Gly-Xaa peptides.

This investigation was of special interest since penicillin-sensitive DD-peptidases are supposed to be highly specific for -DAla-DAla C termini. For enzymes produced by bacteria such as *Streptomyces*, this specificity thus seems to be in contradiction with a possible reversibility of the transpeptidation reaction.

Results

Dipeptides as acceptors

As observed before with Gly-L-Ala (Jamin et al. 1991), the k_{cat} value for the donor substrate S2a (see below for the structure) increased in the presence of the dipeptides Gly-L-Leu and Gly-L-Gln, but the k_{cat}/K_m value decreased with increasing concentrations of the dipeptide (Fig. 1). Nevertheless, acceleration factors could be computed for the three dipeptides. The acceleration factor was defined as $\Delta k_{cat}/\Delta[A]$ in the linear part of the k_{cat} vs. [A] curve, where [A] is the acceptor concentration. Table 1 compares the acceleration factors of the dipeptides with those observed before with D-amino acids and shows that Gly-L-Leu can be considered as one of the most efficient acceptors.

Transpeptidation products as substrates

In early experiments, Frère et al. (1973) isolated the tetrapeptide Ac_2-L-Lys-D-Ala-Gly-L-Ala after reaction of Ac_2-L-Lys-D-Ala-D-Ala with Gly-L-Ala in the presence of the R61 enzyme and showed that it was ~5% as efficient as Ac_2-L-Lys-D-Ala-D-Ala as a substrate for the DD-carboxypeptidase. However, the possibility of a contamination of the enzyme preparation by a nonspecific peptidase could not be rejected and the tetrapeptide did not seem to undergo transpeptidation reactions since regeneration of the tripeptide could not be observed in the presence of D-Alanine. Moreover, the products of the hydrolysis reaction were not

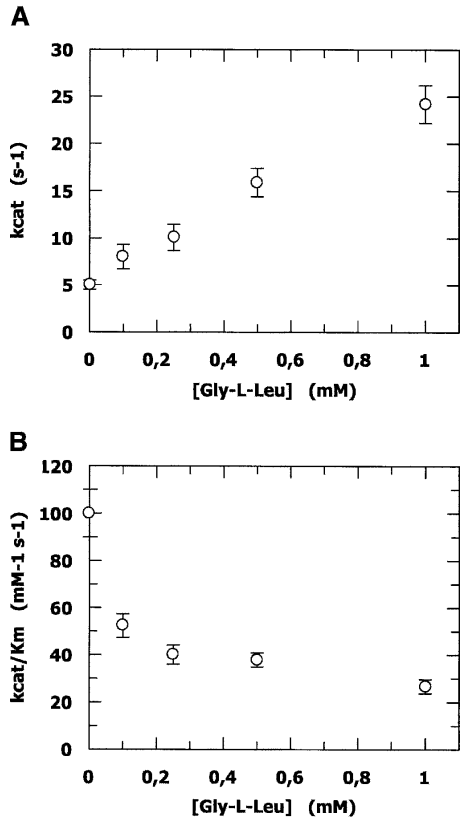


Figure 1. Effects of acceptor concentration on the k_{cat} (*A*) and k_{cat}/Km (*B*) values. The donor substrate was S2a and the acceptor Gly-L-Leu. For experimental conditions, see Materials and Methods.

identified and it remained possible that a sequential release of L-Alanine and Glycine had occurred.

The problem was re-examined with the help of three newly synthetized tetrapeptides, Ac_2-L-Lys-D-Ala-Gly-L-Xaa, where Xaa was Ala, Leu, or Gln.

Firstly, the three compounds behaved as poor substrates of the R61 enzyme, but hydrolysis was significant (Table 2). Initial rates remained proportional to the substrate concentrations up to 15 mM so that only k_{cat}/K_m values could be derived. HPLC analysis revealed that the only reaction products were Ac_2-L-Lys-D-Ala and the dipeptide.

Secondly, when the enzyme was first incubated with a 10-fold molar excess of benzylpenicillin, no reaction was observed, showing that the dipeptide-releasing activity was penicillin sensitive. Moreover, no hydrolysis occurred when the R61 enzyme was replaced by the *Actinomadura* R39 DD-peptidase, an enzyme that only utilizes D-amino acids as acceptors (Ghuysen et al. 1973).

Thirdly, the R61 enzyme could also utilize the tetrapeptides in transpeptidation reactions: When Ac_2-L-Lys-D-Ala-Gly-L-Gln was incubated in the presence of the enzyme and Gly-L-Ala, the formation of Ac_2-L-Lys-D-Ala-Gly-L-Ala was observed in addition to Ac_2-L-Lys-D-Ala and Gly-L-Gln. The transpeptidation/hydrolysis ratio increased with the concentration of the acceptor substrate, Gly-L-Ala, and decreased with that of the donor (Table 3) as observed before with other substrates (Jamin et al. 1993). When Ac_2-L-Lys-D-Ala-Gly-L-Leu was used as a donor and 40 mM D-Alanine as an acceptor, the formation of Ac_2-L-Lys-D-Ala-D-Ala was detected. The T/H ratios were 0.7 and 0.53, with 2.5 and 5 mM donor, respectively. This experiment showed that the transpeptidation was clearly reversible.

Substrates devoid of a free carboxylate

The experiments described above show that a free carboxylic group in α of the hydrolyzed or transferred peptide bond is not absolutely necessary for the R61 DD-peptidase. To shed more light on this point, compounds were synthetized, in which the carboxylic goups of S2a or S2d were amidated or esterified, yielding compounds 1–4.

$$C_6H_5-CO-NH-CHR_1-CO-S-CH_2-CO-R_2$$

S2a: $R_1=H, R_2=OH$

1: $R_1=H, R_2=OCH_3$

2: $R_1=H, R_2=NH_2$

S2d: $R_1=CH_3, R_2=OH$

3: $R_1=CH_3, R_2=OCH_3$

4: $R_1=CH_3, R_2=NH_2$

The thiolesters were chosen as substrates because they exhibit much higher k_{cat}/K_m values than the peptides, thus making the detection of low activities easier. The compounds were also tested as substrates of the *Enterobacter cloacae* P99 class C β-lactamase, an enzyme that retains a non-negligible activity on derivatives of benzylpenicillin where the free carboxylic group has been esterified or

Table 1. *Acceleration factors*

Acceptor	Acceleration factors ($M^{-1}s^{-1}$)
D-Alanine	3340
D-Phenylalanine	15,500
D-Leucine	9700
Gly-Gly	490
Gly-L-Ala	1160
Gly-L-Leu	23,000
Gly-L-Gln	1200
Gly-Gly-Gly	230

The acceleration factor is defined as $\Delta k_{cat}/\Delta[A]$ (where [A] is the acceptor concentration) in the linear part of the k_{cat} vs. [A] graph. The values for the D-amino acids, Gly-Gly and Gly-Gly-Gly are those of Jamin et al. (1991). SD values are ± 15%.

Table 2. *Hydrolysis of the tetrapeptides by the R61 enzyme at 37°C*

Substrate	k_{cat}/K_m ($M^{-1}s^{-1}$)
Ac_2-L-Lys-D-Ala-Gly-L-Ala	147 ± 15
Ac_2-L-Lys-D-Ala-Gly-L-Gln	67 ± 10
Ac_2-L-Lys-D-Ala-Gly-L-Leu	12 ± 2

For comparison, the k_{cat}/K_m value for Ac_2-L-Lys-D-Ala-D-Ala is 4000 $M^{-1}s^{-1}$.

amidated (Varetto et al. 1991), and which also hydrolyzes the thiolesters S2a and S2d. The results (Table 4) show that the free carboxylate increases the activity of the R61 enzyme at least 60-fold when R_1 = H (S2a group) and 300-fold when $R_1 = CH_3$ (S2d group), but that the behavior of the P99 enzyme is quite different.

Discussion

The results obtained with both the tetrapeptides and the modified thiolesters show that a free carboxylate in α of the scissile substrate bond is not an absolute prerequisite for the R61 enzyme. However, the effects of the various modifications can be quite different according to the structure of the initial compound. Somewhat surprisingly, amidation of compound S2a and of benzylpenicillin is much more detrimental than esterification, although the amide is more isosteric to the free acid (or salt) than the ester. Interestingly, this is not true for the derivatives of thiolester S2d, which is the best acylating agent among those studied here. The structure of the complex formed between an inactive form of R61 and a highly specific substrate (McDonough et al. 2002) shows that the terminal -COO^- group of the substrate establishes a strong ionic interaction with Arg285 and a good H-bond (0.28 nm) with Thr299. These interactions are expected to significantly contribute to the binding and productive positioning of the substrate in the enzyme active site. Clearly, if -COO^- is replaced by CO-X, where X is OCH_3, NH_2, or NH-CHR-COO^-, the strong ion pair can at best become a much weaker ion–dipole interaction. Manual positioning of a -D-Ala-Gly-L-Ala C terminus in place of D-Ala-D-Ala shows that it is not impossible that the -COO^- of L-Xaa might lay at a reasonable distance of the Arg285 guanidinium, but this remains highly speculative.

For the P99 β-lactamase, the results confirm those obtained with penicillin derivatives (Varetto et al. 1991); the disappearance of the free carboxylate is much less detrimental; it is even favorable in the case of S2d. However, and just as above, the poorer activities versus the amides of S2a and benzylpenicillin remain surprising.

Among the tested dipeptides, Gly-L-Leu was clearly the best acceptor, exhibiting one of the highest acceleration factors (Table 1). However, the tetrapeptide containing this dipeptide at its C terminus was also the poorest substrate among the studied tetrapeptides. This might be taken as an indication of the fact that the C-terminal -Gly-L-Xaa of the tetrapeptide substrate does not bind in the same site as (or in a similar way to) the Gly-L-Xaa dipeptide when it binds to the acylenzyme before transpeptidation occurs. In the R61-products complex (EP_1P_2) solved by McDonough et al. (2002), the D-Alanine-COO^- remains in interaction with Arg285 and Thr299, but its amino group does not seem to be well-positioned to perform a nucleophilic attack on the acylenzyme scissile ester bond. It is quite possible that this complex does not supply an adequate insight into the structure of the acylenzyme-acceptor complex (EC*A) productive for transpeptidation. Indeed, at neutral pH, the D-Alanine amino group is protonated and one expects the removal of a proton to be a perequisite for transpeptidation to occur. In the enzyme-products complex, the D-Alanine amino group does not seem to interact with any protein group that might fulfill this function. It could thus be suggested that there is a significant movement of some side chains at the levels of the EC* and/or EC*A complexes when compared with the free enzyme or to the EP_1P_2 complex. The pK_a's of the amino groups of the free amino acids are about two pH units higher than those of the dipeptides (Frère et al. 1973) and, if one takes account of the percentage of base form, all of the amino acids listed in Table1 are considerably better acceptors than even the best peptide. Again, this might indicate different positionings of the amino acids and the dipeptides in the EC*A complexes prior to transpeptidation.

The decrease of k_{cat}/K_m for the global reaction with increasing concentrations of the dipeptide acceptors was tentatively explained by Anderson and Pratt (2000) by a competition between these compounds for the site where

Table 3. *Transpeptidation/hydrolysis (T/H) ratios at 37°C with Ac_2-L-Lys-D-Ala-Gly-L-Gln as donor and Gly-L-Ala as acceptor*

[Donor] (mM)	[Acceptor] (mM)	T/H
5	1	0.94
	2	1.26
	5	1.35
	10	1.8
	20	1.9
	40	2.2
1	20	2.7
2		2.6
4		2.2
6		1.8
8		0.8
10		0.66

SD values are ±5%.

Table 4. *Effects of the disappearance of the free carboxylic group on the activity of the R61 DD-peptidase and the P99 β-lactamase*

	k_{cat}/K_m (or k_2/K) ($M^{-1}s^{-1}$)	
Compound	R61 DD-peptidase	P99 β-lactamase
S2a	100,000	2400
1	1640 ± 50	460 ± 40
2	< 10	16 ± 2
S2d	700,000	240
3	1600 ± 100	350 ± 50
4	2400 ± 200	320 ± 30
benzylpenicillin	10,000	32×10^6
benzylpenicillin methyl ester	20	5×10^6
benzylpenicillin amide	< 0.1	70,000

Experiments were performed at 37° and 30°C for the R61 and P99 enzymes, respectively. The values for benzylpenicillin and its derivatives are from Varetto et al. (1991) and, for R61, were obtained as second-order acylation rate constants.

the acylated (in our case) or the glycinated (in their case) ε-NH- group of the donor substrate bind. Although these authors supplied interesting indirect data in favor of this hypothesis, all attempts to bind Gly-L-Xaa peptides to the R61 crystals have unfortunately failed (J.A. Kelly, pers. comm.). The decrease of the T/H ratio in the presence of increasing *donor* concentrations (Table 3) is also impossible to explain on the basis of a simple model in which the acylenzyme is just partitioned between hydrolysis and transpeptidation. The results obtained here with a tetrapeptide are in perfect agreement with those obtained earlier with a thiolester (Jamin at al. 1993).

A detailed model accounting for the behavior of the R61 enzyme in the transpeptidation reaction thus remains to be built despite the structural details obtained recently for complexes with very specific substrates and products. From a kinetic point of view, building such a model is made even more difficult by the fact that all of the transpeptidation products have now been shown to be substrates of the enzyme and that at each turnover, part of the acylenzyme irreversibly decays into hydrolysis products.

The present work, however, brings important additional and new insights into the functioning of the enzyme. Firstly, the transpeptidation products obtained with dipeptides are substrates of both the carboxypeptidase and transpeptidase activities. In both cases, the C-terminal dipeptide is released and the possible contamination by a nonspecific peptidase is made very unlikely by the fact that the reaction is penicillin sensitive. Finally, in the presence of both the tetrapeptide product and D-Alanine, the classical Ac_2-L-Lys-D-Ala-D-Ala tripeptide is regenerated, a result which demonstrates the reversibility of the transpeptidation reaction, in agreement with the fact that the equilibrium constant of this reaction is expected to be close to unity (if one takes account of the different pK_a's of the acceptors) and in contrast to earlier results obtained by one of us. This discrepancy is probably due to the utilization, in the present study, of a well-characterized, pure substrate and of a vastly improved analytical method.

Materials and methods

Enzymes

The *Streptomyces* R61 and the *Actinomadura* R39 DD-peptidases were purified as described previously (Granier et al. 1994). The class C β-lactamase from *Enterobacter cloacae* P99 was also purified as described previously (Joris et al. 1985).

Substrates

Tetrapeptides

The peptides were synthesized on a advanced ChemTech 9O apparatus (solid phase peptide synthesis) using 9-fluorenylmethoxycarbonyl (Fmoc)-strategy on an amino acid Wang resin (Advanced ChemTech). The L-amino acids were introduced into the sequences as preactivated pentafluorophenyl esters (OPfp) (threefold excess) in the presence of 1-hydroxybenzotriazole (HOBt) (threefold excess also, i.e., 1 eq). The D-amino acid was introduced as a free form using a threefold excess of Fmoc-amino acid with one equivalent of HBTU (2-(1H-Benzotriazole-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate) and two equivalents of DIPEA (N,N-Diisopropylethylamine). Acetylation of the N-terminal peptide was realized with acetic anhydride introduced as an amino acid. The side-chain protections were as follows: Lys(-t-butyloxy-carbonyl or tBoc), Gln(-trityl or Trt). The synthesis was performed in dimethylformamide (DMF) by using the conventional coupling procedure (Atherton and Sheppard 1989). The efficiency of coupling was checked by the Kaiser test. Deprotection of the Fmoc-group was carried out with 20% piperidine in DMF for 15 min. Finally, the protected peptide was cleaved from the resin by a 90-min treatment with TFA/water (95/5, v/v). After filtration, the solution was evaporated and TFA removed in vacuo. The peptides were redissolved in water and freeze-dried. They were purified by HPLC (Pharmacia SMART system) on a 2.1 mm, 15-cm PTC-C18 column (Brownlee) using a linear gradient from 5% to 100% solvent B over 50 min. Solvent A was 0.1% TFA in water and Solvent B, 0.08% TFA in 70% acetonitrile. Fractions were collected using the automated peak fractionation tool embedded in the software of the instrument, and it was verified that the experimental masses corresponded to the calculated ones by nano-ESMS and MS/Ms on a Q-TOF apparatus (Micromass) after dilution to about 1 pmol/μL in a 1/1 mixture (v/v) of acetonitrile and 0.1% formic acid.

Modified thiolesters

The modified thiolesters (compounds 1–4) similar to those described by Payne et al. (1997) were prepared by a classical procedure involving the condensation of the carboxylic acid

group with the corresponding thiol in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) and a catalytic amount of p-dimethylaminopyridine.

Hydrolysis and transfer reactions with the thiolester substrates

All measurements were performed in 10 mM sodium phosphate buffer (pH 7.0). The absorbance measurements were made with an UVIKON 860 spectrophotometer linked to a microcomputer via an RS232 interface. In hydrolysis and aminolysis reactions, the disappearance of substrate S2a was monitored at 250 nm using a $\Delta\varepsilon$ value of $-2000\ M^{-1}\ cm^{-1}$. K_m and k_{cat} values were determined either by analyzing complete time courses with the help of the integrated Henri-Michaelis equation (De Meester et al. 1987) or by recording initial rates at different substrate concentrations and analyzing these results according to the Hanes' linearization of the Henri-Michaelis equation. Donor substrate S2a concentrations ranged from 25 μM to 1 mM, the concentrations of Gly-L-Ala and Gly-L-Gln acceptors from 1 to 40 mM and those of Gly-L-Leu from 0.1 to 1 mM. The final enzyme concentrations ranged from 5.4 to 27 nM.

Hydrolysis of amidated and esterified thiolester substrates was monitored at 412 nm in the presence of 1.2 mM 5,5′-dithiobis(2-nitrobenzoic acid) using a $\Delta\varepsilon$ value of $13{,}600\ M^{-1}\ cm^{-1}$. The k_{cat}/K_m values were obtained from initial rates of hydrolysis at substrate concentrations ([S] < Km) ranging from 0.1 to 10 mM. The final enzyme concentrations ranged from 100 to 400 nM for the R61 DD-peptidase and from 0.33 to 1.3 μM for the *Enterobacter cloacae* P99 class C β-lactamase. Incubations were performed at 37°C with the DD-peptidase and 30°C with the β-lactamase. For the R61 DD-peptidase, the buffer was 10 mM sodium phosphate (pH 7.0) and for the β-lactamase, 50 mM sodium phosphate (pH 7.0).

Hydrolysis and transfer reactions with the tetrapeptide substrates

The reactions between the tetrapeptide substrates and the R61 and R39 DD-peptidases were monitored by separating the products and substrate on an ET 250/8/4 Nucleosil-5 C18 column (Machery-Nagel) coupled to a Waters Alliance HPLC system (Waters). Quantification was achieved by integrating the areas of the various peaks at 214 nm with the use of Ac_2-L-Lys-D-Ala-D-Ala and Ac_2-L-Lys-D-Ala as standards. Eluent A was 0.1% (v/v) trifluoroacetic acid in water and eluent B 0.1% (v/v) trifluoroacetic acid in acetonitrile. The flow rate was 1 mL/min. Elution was performed under isocratic conditions (A/B = 97/3, v/v). The k_{cat}/K_m parameters were determined by recording initial rates at different substrate concentrations and analyzing the data by direct fitting to equation $v = k_{cat}\ E_0\ S_0/K_m$. The retention times were as follows: Gly-L-Gln, 3.2 min; Gly-L-Leu, 12.5 min; Ac_2-L-Lys-D-Ala, 21.0 min; Ac_2-L-Lys-D-Ala-D-Ala, 27.8 min; Ac_2-L-Lys-D-Ala-Gly-L-Ala, 40.7 min; and Ac_2-L-Lys-D-Ala-Gly-L-Gln, 25.8 min.

With substrate Ac_2-L-Lys-D-Ala-Gly-L-Leu, elution was performed under isocratic conditions (97:3, v/v) for 40 min, followed by a 20-min linear acetonitrile gradient (3%–100%). The retention times for the hydrolysis product (Ac_2-L-Lys-D-Ala), for the transfer product Ac_2-L-Lys-D-Ala-D-Ala, and for Ac_2- L-Lys-D-Ala-Gly-L-Leu were 21.0, 27.8, and 48.6 min, respectively.

In the hydrolysis experiments, the substrates (4–15 mM) were incubated at 37° with 2–4 μM R61 for 15–30 min in 10 mM sodium phosphate (pH 7.0). In the transpeptidation experiments, the enzyme was 1–2 μM and the incubation performed for 30 min at 37°. With R39, the enzyme concentration was 6 μM and the incubation time 30 min in 10 mM phosphate (pH 7.5), containing 5 mM $MgCl_2$ and 50 mM NaCl. The total volume was 100 μL in all cases, of which 80 μL were injected into the column.

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