Breakdown of the stereospecificity of DD-peptidases and β-lactamases with thiolester substrates

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With peptide analogues of their natural substrates (the glycopeptide units of nascent peptidoglycan), the DD-peptidases exhibit a strict preference for D-Ala-D-Xaa C-termini. Gly is tolerated as the C-terminal residue, but with a significantly decreased activity. These enzymes were also known to hydrolyse various ester and thiolester analogues of their natural substrates. Some thiolesters with a C-terminal leaving group that exhibited L stereochemistry were significantly hydrolysed by some of the enzymes, particularly the *Actinomadura* R39 DD-peptidase, but the strict specificity for a D residue in the penultimate position was fully retained. These esters and thiolesters also behave as substrates for β-lactamases. In this case, thiolesters exhibiting L stereochemistry in the ultimate position could also be hydrolysed, mainly by the class-C and class-D enzymes. However, more surprisingly, the class-C *Enterobacter cloacae* P99 β-lactamase also hydrolysed thiolesters containing an L residue in the penultimate position, sometimes with a higher efficiency than the D isomer.

INTRODUCTION

Recently, the availability of direct spectrophotometric assays for monitoring the activity of active-site serine pencillin-sensitive DD-peptidases [1–3] has greatly facilitated analysis of the enzymic properties of these proteins involved in the synthesis of the bacterial cell-wall peptidoglycan. The assays depend on the utilization of ester and thiolester analogues of the natural D-alanyl-D-alanine-terminated peptide substrates of these enzymes. Of the thiolesters examined, a very simple compound, carboxymethylbenzoylamidothioacetate (Table 1, S2a), was found to be particularly useful and widely recognized by the enzymes [2]. For some high-molecular-mass penicillin-binding proteins, the corresponding derivative of benzoyl-D-alanine (S2d), however, behaved as a significantly better substrate [3]. The present study was undertaken to analyse the structural requirements of a set of DD-peptidases and penicillin-binding proteins for this type of thiolester substrate. As a first approach, the R^1 and R^2 side chains (see Table 1) were modified. The enzymes studied were the DD-peptidases of *Streptomyces* R61 and *Actinomadura* R39 and PBP2x of *Streptococcus pneumoniae*.

The same chromogenic thiolesters are also hydrolysed by another group of active-site serine penicillin-recognizing enzymes, the β-lactamases [2]. These enzymes are divided into three molecular classes, A, C and D, on the basis of their primary structures [5]. Representative members of each class have been included in our analysis: class A, the TEM-1 β-lactamase and those of *Bacillus licheniformis* 749/C and *Streptomyces albus* G; class C, the AmpC β-lactamases of *Escherichia coli* K12 and *Enterobacter cloacae* P99; class D, the OXA-2 β-lactamase.

Surprisingly, the utilization of racemic mixtures showed that, in some cases, the strict stereospecificity exhibited by the DD-peptidases for their peptide substrates was far from absolute for their activity with thiolesters. In this respect, some unexpected results were also obtained with a class-C β-lactamase.

To simplify the nomenclature, the following abbreviations have been used: Bz, benozyl; Thg, thioglycollate; Thl, thiolactate; Phl, phenyl-lactate (see Table 1).

MATERIALS AND METHODS

Enzymes

The *Streptomyces* R61 and *Actinomadura* R39 DD-peptidases were purified as described previously [6,7] and *Strep. pneumoniae* PBP2x as described by Jamin *et al.* [8]. Purification of the β-lactamases was performed by the methods of Matagne *et al.* [9] (*B. licheniformis* and *S. albus* G), Raquet *et al.* [10] (TEM-1), Dubus *et al.* [11] (*E. coli* K12), Joris *et al.* [12] (*Ent. cloacae* P99) and Ledent *et al.* [13] (OXA-2). Pronase was purchased from Boehringer.

Substrates

The synthesis of thiolesters Bz-Gly-Thg (S2a), Bz-Gly-Thl (S2c), Bz-Ala-Thg (S2d) and Bz-Ala-Thl (S2e) has been described previously [2,3]. Substrates Bz-Val-Thg (S2Val), Bz-Leu-Thg (S2Leu) and Bz-Phe-Thg (S2Phe) were obtained by benzoylation of DL-valine, DL-leucine or DL-phenylalanine, followed by coupling to mercaptoacetic acid as described by Adam *et al.* [2]. The final products were racemic mixtures. Similarly, racemic Bz-Ala-Thg (S2d) was obtained using DL-alanine as a starting compound. Substrate S1e (hippuryl DL-phenyl-lactate or Bz-Gly-Phl) was purchased from Sigma. The structures are detailed in Table 1. In the Tables, the stereochemistry of the various substrates is shown as follows: (residue 2) (residue 1). For instance, the L isomer of S1e is (NA)(L) where NA means non-asymmetric and the D isomer of Bz-Ala-Thg (S2d) is (D) (NA). Substrate Bz-Ala-Thl (S2e) as a (D)(D/L) mixture was a gift from UCB Bioproducts (Braine-l'Alleud, Belgium). The isomers were separated on a C_{18} reverse-phase column (Nucleosil 7 μm; 4 mm × 250 mm; M.N. Düren) in 0.1% trifluoroacetic acid using a linear gradient (0–100% in 20 min) of acetonitrile and a flow rate of 1 ml/min. Unfortunately, the (D)(L) isomer was lost during the process. The (D)(D) isomer solution was dry-evaporated under vacuum, the solid residue dissolved in 10 mM sodium phosphate buffer, pH 7.0, and pH adjusted to 7.0 with NaOH.

Abbreviations used: Bz, benzoyl; Thg, thioglycollate; Thl, thiolactate; Phl, phenyl-lactate; NA, non-asymmetric.
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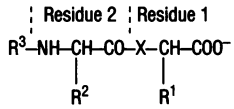
Table 1 Structures of the natural substrates of DD-peptidases and of their ester and thiolester analogues



NA, non-asymmetric; Bz, benzoyl; Phl, phenyl-lactate; Thg, thioglycollate; Thl, thiolactate.

R^3	R^2	X	R^1	Stereochemistry Residue 2	Stereochemistry Residue 1	Short name of substrate
Complex peptide*	CH_3	NH	CH_3	D	D	Natural substrate R^3-D-Ala-D-Ala
C_6H_5–CO–	H	O	$-CH_2-C_6H_5$	NA	D/L	S1e Bz-Gly-Phl
C_6H_5–CO–	H	S	H	NA	NA	S2a Bz-Gly-Thg
C_6H_5–CO–	CH_3	S	H	D/L	NA	S2d Bz-Ala-Thg
C_6H_5–CO–	H	S	CH_3	NA	D/L	S2c Bz-Gly-Thl
C_6H_5–CO–	CH_3	S	CH_3	D	D	S2e Bz-D-Ala-D-Thl
C_6H_5–CO–	$-CH(CH_3)(CH_3)$	S	H	D/L	NA	S2Val Bz-Val-Thg
C_6H_5–CO–	$-CH_2-CH(CH_3)(CH_3)$	S	H	D/L	NA	S2Leu Bz-Leu-Thg
C_6H_5–CO–	$-CH_2-C_6H_5$	S	H	D/L	NA	S2Phe Bz-Phe-Thg

* Note that, in some cases, a natural ester substrate, R-D-Ala-O–CHX–COO⁻ (where X = CH_3 or C_2H_5), has also been tentatively identified [22]. *In vivo*, R_3 represents the nascent peptidoglycan side chain, i.e. glycan-L-Ala-D-GluL-Xaa where L-Xaa is a diamino acid. *In vitro*, shorter peptides where $R^3 = N^{\alpha}N^{\epsilon}$-diacetyl-L-Lys ($Ac_2$-L-Lys) or N^{α}-acetyl-L-Lys (α-Ac-L-Lys) have also been utilized.

Kinetic methods

Hydrolysis of the various substrates was monitored spectrophotometrically as described previously [2,3]. The kinetic parameters were derived by the analysis of complete time courses with the help of the integrated Henri–Michaelis equation [14] or by measuring initial rates and fitting the data to the Henri–Michaelis equation with the help of the Enzfitter program (Elsevier Biosoft, Cambridge, U.K.). For the β-lactamases, the K_m values were generally quite high and only the $k_{cat.}/K_m$ ratios could be determined. Fitting the curves to sums of two exponentials and to unsolved differential equations was also performed with the help of the Enzfitter or SIMFIT [15] program respectively. Hydrolysis of the substrates by the various enzymes follows the three-step model.

$$E+S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_2} ES^* \xrightarrow{k_3} E+P$$

where ES* is the acyl-enzyme.

When complete time courses were utilized, it was verified, as before [2], that no product inhibition occurred at least at concentrations corresponding to complete hydrolysis of the substrates and that the various enzymes remained stable during the time necessary to complete the experiments. Moreover, when one of the isomers was first hydrolysed with the help of a second enzyme, the kinetic parameters were not significantly different from those determined directly with the enzyme being investigated. This further indicated that both the isomer hydrolysed by the second enzyme and its products had no effect on the hydrolysis of the remaining substrate by the enzyme under investigation. Differences were only observed when the enzyme being examined hydrolysed both isomers. In these cases, however, the data could be fitted to a sum of two exponentials (the R39 enzyme and S2c, Bz-Gly-Thl); the parameters found for the D isomer, corresponding to the faster phenomenon, were in good agreement with those found after hydrolysis of the L isomer by Pronase (see also the Results section). In the case of the interaction between the P99 β-lactamase and S2Phe (Bz-D/L-Phe-Thg), for which the most unexpected results were recorded, it was carefully verified that Bz-D/L-Phe did not behave as an inhibitor of the reactions. When the K_m values were high, the $k_{cat.}/K_m$ values were directly deduced from first-order analyses of the curves at $[S] \ll K_m$.

RESULTS

R61 and R39 DD-peptidases

Stereospecificity

Although these enzymes only hydrolyse D-Ala-D-Xaa and to a lesser degree D-Ala-Gly C-termini [16,17], when the substrates were esters or thiolesters, hydrolysis of both L and D isomers on the C-terminal group was observed in some cases. In fact, it was found that the 'complex kinetics' reported earlier for racemic mixtures [2] were sometimes due to the slow hydrolysis of the L isomer after completion of that of the D-isomer. The parameters for the D isomer were obtained after hydrolysis of the L isomer

Table 2 Kinetic parameters for the hydrolysis of esters and thiolesters by R61 and R39 DD-peptidases

–, Not determined. Unless otherwise stated (IR = initial rate), values were deduced from complete time courses. Results are means ± S.E.M. (at least 3 determinations).

Substrate and stereo chemistry	R61			R39		
	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)
S1e (NA) (D)	4.6 ± 0.3	900 ± 50	5200 ± 50 (IR)‖	0.33 ± 0.05	50 ± 15	6600 ± 900
(NA) (L)	–	–	< 10	–	–	180 ± 20*
S2a (NA) (NA)	5 ± 0.1	50 ± 1	100000 ± 1000	0.3 ± 0.03	30 ± 2	10000 ± 500
S2c (NA) (D)	5.4 ± 0.6	52 ± 7	104000 ± 1000	0.35 ± 0.08	28 ± 7	13000 ± 1000
(NA) (L)	–	–	1800 ± 50*	[0.35 ± 0.08]	235 ± 12	1500 ± 400†
				0.17 ± 0.02	90 ± 10	1900 ± 200‡
						1500 ± 200*
S2d (D) (NA)	50 ± 3.5	116 ± 17	430000 ± 2000	5.6 ± 0.1	15 ± 0.4	336000 ± 11000
(L) (NA)		No detectable hydrolysis	< 100		No detectable hydrolysis	< 100
S2e (D) (D)	75 ± 5	540 ± 80	140000 ± 10000	7.5 ± 1	220 ± 20	35000 ± 2000
S2Val (D) (NA)	3.3 ± 1	390 ± 100	8800 ± 1000	0.9 ± 2	500 ± 20	1800 ± 200
S2Leu (D) (NA)	25 ± 5	1300 ± 200	20000 ± 2000§	0.05 ± 0.02	700 ± 300	75 ± 5 (IR)
S2Phe (D) (NA)	1.2 ± 0.2	5400 ± 500	200 ± 20 (IR)	–	–	< 4 (IR)

* Obtained by fitting the later part of the curve to a first-order equation (at [S] < K_m for S2c).
†‡ Obtained by fitting complete time courses to the differential equations and setting $V_L = V_D$ (†) or without such an assumption (‡).
§ Complete time courses and initial rates were used, yielding similar results.
‖ The leaving group, phenyl-lactate, is a good acceptor in the transacylation reaction and, when complete time courses are monitored, k_{cat} and K_m increase with the initial substrate concentration.

with Pronase. Those for the L isomer were deduced from the later part of the time courses after hydrolysis of the D isomer was completed (Table 2). With the R39 enzyme and Bz-Gly-Thl (S2c), the difference between the L and D forms was less important and the $k_{cat.}/K_m$ value for the L form was computed both from the later points of the curve and by fitting the experimental data to the following differential equations where V_D and K_{m_D} were those measured above.

$$\frac{dS_D}{dt} = -\frac{V_D[S_D]}{[S_D] + K_{m_D}\left(1 + \frac{[S_L]}{K_{mL}}\right)}$$

$$\frac{dS_L}{dt} = -\frac{V_L[S_L]}{[S_L] + K_{m_L}\left(1 + \frac{[S_D]}{K_{m_D}}\right)}$$

and

$$\frac{dP}{dt} = -\left(\frac{dS_D}{dt} + \frac{dS_L}{dt}\right)$$

The two methods yielded similar $k_{cat.}/K_m$ values for the L isomer. Figure 1 depicts the results obtained when substrate S2c was hydrolysed by the R39 enzyme alone or by successive additions of Pronase and the R39 enzyme. Interestingly, on reaction with 125–500 μM substrate S2c, the fluorescence quenching of the R61 enzyme could be fitted to a single exponential over a short (1 s) period whereas that of the R39 enzyme was biexponential, with the first phase being completed over about 10 s. The latter result, which indicates some accumulation of acyl-enzyme with the L isomer [18], shows that k_3 is smaller than or similar to k_2 in the interaction between R39 and the L isomer of substrate S2c. Moreover, the $k_{cat.}$ value obtained for the L isomer was very similar to that for the D isomer, probably reflecting the hydrolysis rate of the acyl-enzyme, which is identical for both isomers. With the ester Bz-Gly-Phl (S1e), only the R39 enzyme significantly hydrolysed the L form.

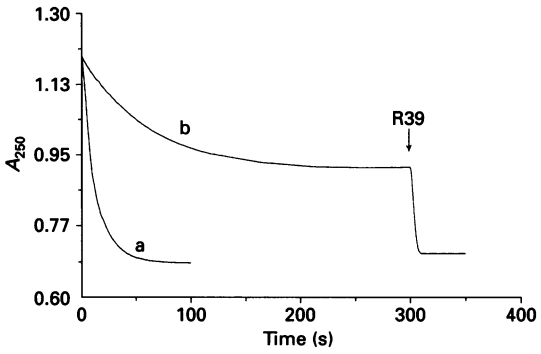


Figure 1 Hydrolysis of 250 μM racemic S2c by the *Actinomadura* R39 enzyme

Curve a was obtained with 35 μg of R39 enzyme in a total volume of 500 μl. In a second experiment (curve b) Pronase (400 μg) was first added, followed by the R39 enzyme (35 μg).

Side chains on the second residue

Table 2 shows that, for both enzymes, the best substrate was Bz-D-Ala-Thg (S2d), with the natural D-alanine residue in the central position. Surprisingly, Bz-D-Ala-D-Thl (S2e) was not better than S2d, in contrast with the behaviour of these enzymes with the peptides, where replacement of a C-terminal glycine by a D-alanine increased $k_{cat.}/K_m$ values by a factor of about 10 [16,17].

When the racemic S2d was submitted to the action of Pronase, hydrolysis of about 50% of the substrate was observed. Subsequent addition of the R39 DD-peptidase resulted in the hydrolysis of the residual 50%. Conversely, when the R39 enzyme was added first, 50% of the compound was hydrolysed and the addition of Pronase completed the hydrolysis (not shown). These

Table 3 Hydrolysis of thiolester substrates by *Strep. pneumoniae* PBP2x

Data for substrates S2a–S2e are taken from ref. [8]. –, Not determined. The values were determined on the basis of complete time courses (S2a–S2d) or, for the other four compounds, from initial rates determined at at least two different initial substrate concentrations, and are means ± S.E.M.

Substrate	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)
S2a	0.47 ± 0.04	0.8 ± 0.1	610 ± 150
S2c	0.4 ± 0.04	0.13 ± 0.03	3200 ± 1000
S2d	30 ± 5	5.6 ± 0.9	5000 ± 1400
S2e	–	–	4900 ± 200
S2Val	–	–	< 5
S2Leu	–	–	< 100
S2Phe	> 0.07	> 0.1	700 ± 100

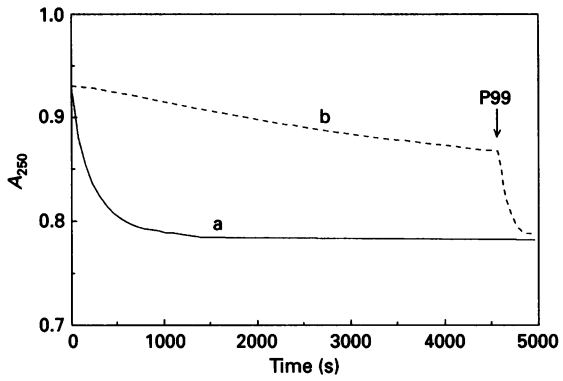


Figure 2 Hydrolysis of 100 μM racemic S2Phe by the P99 β-lactamase

Curve a was obtained within 200 μg of P99 enzyme. In a second experiment (curve b, dashed line), the R61 enzyme (20 μg) was first added, followed by the P99 enzyme. In a third experiment (not shown), Pronase (40 μg) was first added, followed by the P99 enzyme and a similar result was obtained, i.e. the P99 enzyme hydrolysed the D isomer left intact after Pronase treatment.

results indicate that, for each enzyme, the specificity for the L (Pronase) or D isomer (DD-peptidase) was conserved and the parameters obtained with optically pure S2d and the R39 enzyme were identical.

Although hydrolysis of substrates with more bulky side chains was significantly less efficient, fluorescence quenching was observed with Bz-Val-Thg (S2Val) and Bz-Leu-Thg (S2Leu), indicating an accumulation of acyl-enzyme, and, in consequence, it could be concluded that k_2 was larger than or similar to k_3. As with S2d, no hydrolysis of the L isomer was detected with these compounds.

PBP2x

The values for substrates Bz-Gly-Phl (S1e), Bz-Gly-Thg (S2a), Bz-Gly-Thl (S2c), Bz-Ala-Thg (S2d) and Bz-Ala-Thl (S2e) have been published previously [8]. As shown in Table 3, of the compounds with a larger side chain in position 2, only Bz-Phe-Thg (S2Phe) was significantly hydrolysed. With substrate Bz-Gly-Thl (S2c), no hydrolysis of the L isomer was observed ($k_{cat.}/K_m < 100\ M^{-1} \cdot s^{-1}$).

β-Lactamases

The results obtained with the various enzymes representing the three classes of active-site serine β-lactamases are summarized in Table 4 and compared with those already published. The K_m values were generally very high, so that the individual $k_{cat.}$ and K_m values could only be determined in one case. The stereospecificity of some enzymes for substrates Bz-Gly-Thl (S2c), Bz-Ala-Thg (S2d) and Bz-Phe-Thg (S2Phe) was, however, more

Table 4 $k_{cat.}/K_m$ values ($M^{-1} \cdot s^{-1}$) of the β-lactamases with ester and thioester substrates (at 30 °C)

The data for the OXA-2 enzyme were usually obtained under initial-rate conditions except for substrate S2c for which the complete time courses were fitted to a sum of two exponentials. For the other enzymes, complete time courses were utilized unless otherwise stated (IR, initial rate), Results are means ± S.E.M. (at least 3 determinations).

Substrate and stereo chemistry	Class-A enzymes			Class-C enzymes		Class-D enzyme
	B. licheniformis	TEM-1	*S. albus* G	*E. coli* K12	*Ent. cloacae* P99	OXA-2
S1e (NA) (D)	80 ± 10*	50 ± 10	310 ± 20*	23000 ± 1000‡	20000 ± 1000*	< 5
(NA) (L)	–	–	–	–	10 ± 0.5‖	–
S2a (NA) (NA)	4500 ± 200*	5200 ± 200†	15600 ± 1000*	2900 ± 300	2400 ± 200	200 ± 20
S2c (NA) (D)	6200 ± 200	5700 ± 700	11400 ± 600*	4300 ± 200	3600 ± 200‖	265 ± 30
(NA) (L)	9 ± 2	–	–	–	360 ± 30‖	50 ± 5
S2d (D) (NA)	–	100 ± 10 (IR)	< 10 (IR)	310 ± 40§	240 ± 30‖	16 ± 2
(L) (NA)	–	–	< 10 (IR)	310 ± 40§	225 ± 20‖	< 1
S2Val (D/L) (NA)	< 10	< 10 (IR)	< 5 (IR)	< 10 (IR)	–	< 5
S2Leu (D/L) (NA)	< 10	< 10 (IR)	< 5 (IR)	< 10 (IR)	–	< 5
S2Phe (D) (NA)	< 10	< 10 (IR)	< 5 (IR)	–	330 ± 60‖	< 5
(L) (NA)	< 10	< 10 (IR)	< 5 (IR)	–	1200 ± 250‖	< 5

* Data from ref. [2].
† Data from ref. [11].
‡ $k_{cat.} = 30\ s^{-1}$; $K_m = 1.3$ mM (± 10%).
§ Data for the racemic mixture; the reaction time course was first-order within the limits of experimental error.
‖ Data for the D isomer obtained after hydrolysis of the L isomer by Pronase; data for the L isomer after hydrolysis of the D isomer by the R61 DD-peptidase.

closely examined. As with the R61 and R39 DD-peptidases, some cases were found where the L isomer of substrates S2c and S2d were hydrolysed. With substrate Bz-Phe-Thg (S2Phe), the *Ent. cloacae* P99 enzyme exhibited a completely unexpected preference for the L isomer.

When both D and L isomers were hydrolysed, the individual $k_{cat.}/K_m$ values were determined as follows.

(1) Bz-Gly-Thl (S2c) + *B. licheniformis*: the difference was so large that the values could be determined individually from the early and late parts of the curve.

(2) Bz-Gly-Thl (S2c) + *Ent. cloacae* P99 and OXA-2: the curves were fitted to a sum of two exponentials. A similar value for the D isomer was also obtained after complete hydrolysis of the L isomer by Pronase.

(3) Bz-Ala-Thg (S2d) + *Ent. cloacae* P99: the value for the D isomer was obtained after hydrolysis of the L isomer by Pronase and that for the L isomer after hydrolysis of the D isomer by the R61 DD-peptidase.

(4) Bz-Phe-Thg (S2Phe) + *Ent. cloacae* P99: the curves were fitted to a sum of two exponentials. The value for the D isomer was determined after hydrolysis of the L isomer by Pronase and that for the L isomer after hydrolysis of the D isomer by the R61 DD-peptidase (Figure 2).

DISCUSSION

The specificity of the R61 and R39 enzymes for their peptide substrates has been examined in detail [16,17]. These studies have underlined a strong preference for D-Ala-D-Xaa-terminated peptides. As shown in Table 5, the only substitution that appears to be compatible with good activity is that of a glycine residue for the C-terminal D-residue, but this nevertheless results in a 10-fold decrease in enzyme efficiency. The penultimate position is even more specific, with a nearly complete loss of activity, even with the D-Ala→Gly substitution. By contrast, with the thiolesters, both positions can be successfully occupied by a glycine residue or the equivalent thioglycollate. Substrate Bz-D-Ala-Thg (S2d), which is strictly analogous to R-D-Ala-Gly, is an even better substrate than Bz-D-Ala-D-Thl (S2e; analogous to R-D-Ala-D-Ala) for both enzymes. Thus, if residue 2 is D-alanine, the thioglycollate is a better substrate than the D-thiolactate derivative, but the difference disappears if residue 2 is glycine. The L-thiolactate derivative behaves as a significantly poorer substrate but, with the R39 enzyme, the $k_{cat.}/K_m$ value is nonetheless more than 10% of that observed with the D isomer. In the penultimate position, a D-alanine yields the very best substrates for both enzymes if residue 1 is a thioglycollate. The requirement for a D-alanine in this position is clearer for the R39 than the R61 enzyme, with which a glycine and, to a lesser degree, a D-leucine residue still yield rather high values. In both cases, L-alanine is, as expected, strongly detrimental to recognition by the enzymes. The active sites thus exhibit a strong preference for D-alanine, but as above, the structure of residue 1 seems to influence the specificity for residue 2, indicating that the contributions of the two side chains are not simply additive.

The spectacular decrease in the stereospecificity of the enzymes with the thiolesters can probably be explained by a larger conformational freedom for these compounds where the rotation around the CO–S bond is energetically much less 'expensive' than around the CO–NH bond of peptides.

An additional interesting observation concerns the residue 3 group. With a D-Ala-D-Ala-terminated peptide, replacement of the benzoyl side chain by $N^{\alpha}N^{\epsilon}$-diacetyl-L-lysyl greatly increases the $k_{cat.}/K_m$ values (N. Rhazi, M. Jamin and J.-M. Frère, unpublished work). As shown by Wilkin et al. [19], the same substitution does not result in a similar increase with the thiolesters. In fact, the benzoyl side chain consistently yields better thiolester substrates, confirming that the actual substrate quality depends on a subtle interplay between at least three binding sites which contribute to the positioning of the scissile –CO–NH– or –CO–S– bond with respect to the active-site serine hydroxy group and possibly to slight modifications in the enzyme structure responsible for a more or less adequate alignment of the other catalytic side chains. To explain similar results, Xu et al. [1] have proposed the presence of distinct binding sites for the phenylacetyl and Ac_2-L-Lys side chains of thiolesters. However, in spite of recent progress in acquiring knowledge of the three-dimensional structure of the R61 enzyme (J. A. Kelly, personal communication), the details of its catalytic mechanism remain elusive.

PBP2x does not hydrolyse simple peptides and it is thus impossible to perform the same comparisons as above. The data

Table 5 Ratios of the $k_{cat.}/K_m$ values observed after modification of residues 2 and 1 in the hydrolysis of peptides and thiolesters by the R61 and R39 DD-peptidases

For comparison purposes, the k_{cat}/K_m values are respectively 4600 $M^{-1} \cdot s^{-1}$ and 50000 $M^{-1} \cdot s^{-1}$ for the R61 and R39 enzymes with $N^{\alpha}N^{\epsilon}$-diacetyl-L-Lys-D-Ala-D-Ala.

	Peptides			Thiolesters		
	R61	R39		R61	R39	
Res 1 = D-Ala / Res 1 = Gly	13	10	Res 1 = D-Thl / Res 1 = Thg	0.3 1	0.12 1.3	(Res 2 = D-Ala) (Res 2 = Gly)
Res 1 = D-Ala / Res 1 = L-Ala	> 1000	> 500	Res 1 = D-Thl / Res 1 = L-Thl	60	8	(Res 2 = Gly)
Res 2 = D-Ala / Res 2 = Gly	650	> 500	Res 2 = D-Ala / Res 2 = Gly	4.3 1.4	34 3	(Res 1 = Thg) (Res 1 = D-Thl)
Res 2 = D-Ala / Res 2 = L-Ala	> 1000	> 500	Res 2 = D-Ala / Res 2 = L-Ala	≫ 1000	≫ 1000	(Res 1 = Thg)

presented here, however, indicate a significant increase in the $k_{cat.}/K_m$ values if at least one of the two C-terminal residues is the D isomer. Although the enzyme appears as generally more stereospecific than the R39 enzyme, as the ratio (Res 1 = D-Thl)/(Res 1 = L-Thl) was certainly larger than 30, it does not exhibit a very strong preference for D-alanine over glycine as residue 2 as long as residue 1 is D-Thl. A surprising observation was the relatively high $k_{cat.}/K_m$ value for S2Phe which indicated that the active site could accommodate an aromatic side chain at that position but not a branched aliphatic one, rather unusual behaviour.

The situation was somewhat different for the β-lactamases. Here, the highest $k_{cat.}/K_m$ values were orders of magnitude lower than with their good β-lactam substrates. All the enzymes showed a strong (class C, class D) to nearly exclusive (class A) preference for glycine as residue 2. For the class-A enzyme, replacement of this residue by D-alanine results in a nearly complete loss of activity (Gly/D-Ala > 1000). All the enzymes indifferently hydrolyse thiolesters with Thg or D-Thl as residue 1 but the class-C and class-D enzymes also significantly recognize L-Thl in this position. Among the compounds with larger R^2 side chains, the only detectable hydrolysis was found for S2Phe by the class-C enzyme from *Ent. cloacae*. The two class-C enzymes which, as expected, exhibited very similar behaviour [20], generally recognized a wider range of substrates than their class-A and class-D counterparts, in agreement with their usually broader substrate specificity, at least if only the acylation step, characterized by $k_{cat.}/K_m$, is considered. The most unexpected results, however, were the similar hydrolysis rates of the L and D isomers of Bz-Ala-Thg (S2d) and the significant preference for the L isomer of Bz-Phe-Thg (S2Phe), although the $k_{cat.}/K_m$ values remained lower than with S2a and S2c, which contain a glycine residue. This observation might, at first sight, appear to corroborate the hypothesis of Bishop and Weiner [21], who suggested that class-C enzymes might be more closely related to the LD- than to the DD-peptidases. However, the natural substrates of LD-peptidases contain aliphatic rather than aromatic side chains and, although these enzymes have not been as closely studied as DD-peptidases, the absence of activity of the *E. coli* K12 AmpC β-lactamase on Bz-Val-Thg (S2Val) and Bz-Leu-Thg (S2Leu) seems to contradict such a relationship. Moreover, the three-dimensional structures of the P99 β-lactamase and the R61 DD-peptidase are clearly similar [4], with the conserved structural and functional elements situated in equivalent positions. Little is known, however, about the primary and tertiary structures of the LD-peptidases, and detailed studies of these enzymes should supply interesting data in the general context of relationships between the three groups of protein. Nevertheless, the inverted D/L specificity of the P99 β-lactamase suggests unexpected differences between the binding sites of the DD-peptidases and class-C β-lactamases. For the latter enzymes, the ester Bz-Gly-Phl (S1e) remains the best non-β-lactam substrate, and it would be interesting to analyse the behaviour of its thiolester equivalent, containing thiomandelate as a leaving group.

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