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Interactions between non-classical β-lactam compounds and the β-lactamases of *Actinomadura* R39 and *Streptomyces albus* G

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6-Aminopenicillanic acid, 7-aminocephalosporanic acid, mecillinam and quinacillin have varying substrate activities for both the R39 β-lactamase (excreted by *Actinomadura* R39) and the G β-lactamase (excreted by *Streptomyces albus* G). Cefoxitin and quinacillin sulphone are not recognized by the G β-lactamase and are weak inactivators of the R39 β-lactamase. *N*-Formimidoylthienamycin is a poor substrate for the G β-lactamase and a potent inactivator of the R39 β-lactamase. The high value of the bimolecular rate constant for enzyme inactivation is mainly due to a very low dissociation constant (1 μM). Clavulanate is an inactivator of both G and R39 β-lactamases. The reaction with this latter enzyme is a branched pathway where normal turnover and permanent enzyme inactivation occur concomitantly. Between 28 and 43 molecules of clavulanate are hydrolysed before one of them has the opportunity to inactivate one molecule of enzyme.

The β-lactamases and D-alanyl-D-alanine peptidases (carboxypeptidase/transpeptidases) have the common property of catalysing the opening of the β-lactam ring of the penicillins and cephalosporins. At this time, one Zn^{2+}-containing and several serine β-lactamases (Knott-Hunziker *et al.*, 1979; Fisher *et al.*, 1980; Cartwright & Coulson, 1980; Hill *et al.*, 1980) and similarly one Zn^{2+}-containing and several serine D-alanyl-D-alanine peptidases (Frère *et al.*, 1976; Dideberg *et al.*, 1980; Duez *et al.*, 1981*b*; Waxman *et al.*, 1980) have been characterized. Whatever their mechanistic properties, the β-lactamases usually efficiently hydrolyse β-lactam antibiotics into inactive compounds. Recently, however, β-lactam compounds have been discovered that inactivate the serine β-lactamases. In general, β-lactam compounds are also effective inactivators of the serine D-alanyl-D-alanine peptidases (Ghuysen *et al.*, 1979). The reaction between the Zn^{2+}-containing D-alanyl-D-alanine peptidase and penicillins (Δ^3-cephalosporins), however, is essentially reversible and enzyme inactivation occurs only under extreme conditions (Frère *et al.*, 1978).

The preceding paper (Kelly *et al.*, 1981) describes the effects of several non-classical β-lactam compounds (6-aminopenicillanic acid, 7-aminocephalosporanic acid, mecillinam, quinacillin sulphone, cefoxitin, clavulanic acid and *N*-formimidoylthienamycin) on the Zn^{2+}-containing G D-alanyl-D-alanine peptidase secreted by *Streptomyces albus* G and the serine R61 and R39 D-alanyl-D-alanine peptidases secreted by *Streptomyces* R61 and *Actinomadura* R39 respectively. The present paper describes the effects of the same non-classical β-lactam compounds on the β-lactamases secreted by *Streptomyces albus* G (for convenience, termed the G β-lactamase; Duez *et al.* 1981*a*) and *Actinomadura* R39 ('the R39 β-lactamase'; Johnson *et al.*, 1975). *Streptomyces* R61 secretes exceedingly low amounts of β-lactamase, preventing isolation of this enzyme (J.-M. Frère & K. J. Johnson, unpublished work).

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Materials and methods

β-Lactamases

The G β-lactamase (mol.wt. 30000) and the R39 β-lactamase (mol.wt. 15000) were isolated as previously described (Duez *et al.*, 1981*a*; C. Duez & J. M. Frère, unpublished work).

β-Lactam compounds

Nitrocefin (chromogenic cephalosporin 87/312) was a gift from Glaxo Group Research, Greenford, Middlesex, U.K., and cephalothin from Eli Lilly and Co., Indianapolis, IN, U.S.A. The other β-lactam compounds used are those studied by Kelly *et al.* (1981).

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Buffer

All the experiments, including the measurements of β-lactamase activity, were carried out in 25mM-sodium phosphate buffer, pH 7.0, supplemented, in the case of the G β-lactamase, with 10% glycerol and 10% (v/v) ethylene glycol.

Measurements of β-lactamase activity

Nitrocefin is an excellent substrate (see below) for both G and R39 β-lactamases; it was used routinely for the determinations of enzyme activity. Enzyme samples (up to 50μl) were added to 350μl of 0.1mM-nitrocefin in phosphate buffer and the absorbance of the solutions was monitored at 482nm. Cephalothin was also used as substrate (see the Results section) and the hydrolysis was monitored by absorbance measurements made at 260nm.

Hydrolytic activities of the β-lactamases

The catalysed hydrolysis of the β-lactam compounds (except *N*-formimidoylthienamycin) was assayed using the starch/I_2 method as described by Johnson *et al.* (1975). The absorption coefficient to be applied in each case was determined by using, as standard, preparations of the particular compound under study that had been completely hydrolysed by a large excess of β-lactamase or by incubation in 0.1M-potassium phosphate buffer, pH 12.0. Degradation of *N*-formimidoylthienamycin was estimated on the basis of the decreased absorbance at 297nm ($\Delta\varepsilon = 7900\,M^{-1}\cdot cm^{-1}$). Steady-state kinetic experiments were performed by incubating together fixed concentrations of the enzyme and various concentrations of the β-lactam compound under investigation, at 30°C and in a total volume of 30–60μl. Whenever possible, substrate concentrations around the K_m value were used. The reactions were stopped by addition of 0.2ml of 1M-sodium acetate, pH 4.0.

Inactivation of the β-lactamases

The enzymes were incubated at 30°C in the presence of various concentrations of the β-lactam inactivators. Samples (up to 10μl) were removed after increasing times of incubation and the residual enzyme activity was determined using nitrocefin as substrate.

Spectra

The spectra were recorded at 25°C using a Cary 17 double-beam recording spectrophotometer.

Reaction pathway and kinetic parameters

The simple model

$$E + I \underset{}{\overset{K}{\rightleftharpoons}} EI \xrightarrow{k_2} EI^* \xrightarrow{k_3} E + Ps$$

where E = enzyme, I = β-lactam compound, K = dissociation constant, k_2 and k_3 = first-order rate constants, EI = Henri Michaelis complex, EI* = inactivated intermediate and Ps = degradation product(s), best explains the interaction between the β-lactam compounds and the D-alanyl-D-alanine peptidases (Frère *et al.*, 1975; Fuad *et al.*, 1976). It was assumed that the same underlying mechanism governs the reactions catalysed by the β-lactamases. With good β-lactam substrates, the kinetic parameters that are available experimentally are K_m (M), $k_{cat.}$ (s^{-1}) and catalytic efficiency [$k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)]. If the formation of the Henri Michaelis complex is a rapid equilibrium process, then the catalytic efficiency becomes a valid measure of the k_2/K ratio, i.e. the bimolecular rate constant for the formation of the intermediate EI*. With β-lactam inactivators (whether the enzymes are β-lactamases or D-alanyl-D-alanine peptidases), the reaction stops at the level of the intermediate EI* and the k_2/K ratio can be determined directly.

Results

Table 1 gives the values of the kinetic parameters

Table 1. *Kinetic parameters for the interactions between β-lactam compounds and the G and R39 β-lactamases*

	R39 β-lactamase			G β-lactamase		
	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)
Benzylpenicillin*	64	0.065	980000	460	0.74	620000
Nitrocefin	210	0.085	2800000	120	0.13	930000
6-Aminopenicillanic acid*	50	0.072	700000	350	0.6	600000
7-Aminocephalosporanic acid	1.6	2	800	2.5	1.6	1500
Mecillinam	150	2.5	60000	140	3.3	43000
Quinacillin	37	0.7	53000	5	1.0	5000
Quinacillin sulphone	Inactivator ($k_2/K = 0.8\,M^{-1}\cdot s^{-1}$)			No interaction up to 5mM		
Cefoxitin	Inactivator ($k_2/K = 0.4\,M^{-1}\cdot s^{-1}$)			No interaction up to 5mM		
N-Formimidoylthienamycin	Inactivator ($K = 1\,\mu M$; $k_2 = 2\times 10^{-3}\,s^{-1}$; $k_2/K = 2000\,M^{-1}\cdot s^{-1}$)			0.2	0.077	3000
Clavulanate	Complex interaction; see the text			Inactivator ($k_2/K = 300\,M^{-1}\cdot s^{-1}$)		

* From Duez *et al.* (1981*b*) and C. Duez & J.-M. Frère (unpublished work).

for the interactions between the G and R39 β-lactamases and the selected, non-classical, β-lactam compounds. Table 1 also includes, for comparative purposes, the kinetic parameters obtained with benzylpenicillin and nitrocefin.

6-Aminopenicillanic acid, 7-aminocephalosporanic acid, mecillinam and quinacillin

These four β-lactam compounds had varying substrate activities for the two β-lactamases under consideration.

Cefoxitin and quinacillin sulphone

These two β-lactam compounds had no effect on the G β-lactamase (at least at a 5mM concentration) but inactivated the R39 β-lactamase. The second-order rate constants for the enzyme inactivation were $0.4\,M^{-1}\cdot s^{-1}$ with cefoxitin and $0.8\,M^{-1}\cdot s^{-1}$ with quinacillin sulphone. The pseudo-first order rate constants (k_a) were proportional to the concentrations of cefoxitin up to 4mM and to the concentrations of quinacillin sulphone up to 1mM.

N-formimidoylthienamycin

N-Formimidoylthienamycin was a poor substrate for G β-lactamase. Assuming that this compound and nitrocefin bind to the same enzyme site, competition experiments (Fig. 1*a*) yielded a K_m for *N*-formimidoylthienamycin of 160μM. This value was in fair agreement with the 77μM value obtained by direct measurements of the kinetic parameters.

N-Formimidoylthienamycin was not a substrate of the R39 β-lactamase; no change in the absorbance at 297nm was observed after 30min of incubation at 30°C of a 0.15mM-*N*-formimidoylthienamycin solution containing 12μg of enzyme (indicating a v value smaller than 0.003μmol/min per mg of protein). However, when the R39 β-lactamase, *N*-formimidoylthienamycin and nitrocefin were incubated together (Fig. 1*b*), the initial rates of hydrolysis of nitrocefin were decreased and a progressive inactivation of the R39 β-lactamase was observed. Assuming that the interaction was competitive, an approximate value of 0.5μM was found for the dissociation constant K between the enzyme and *N*-formimidoylthienamycin. The inactivation of the R39 enzyme by this β-lactam compound was then investigated by the direct procedure described in the Materials and methods section. The experiments yielded a K value of 1.4μM and a k_2 value of $0.2 \times 10^{-3}\,s^{-1}$.

Clavulanate

Clavulanate inactivated the G β-lactamase with a second-order rate constant of $300\,M^{-1}\cdot s^{-1}$. Effective inactivation occurred at clavulanate concentrations as low as 20μM. After complete inactivation of the enzyme and elimination of the excess of clavulanate by dialysis at 4°C, further incubation of the reaction mixture for 3h at 30°C did not result in any recovery of the enzyme activity. Attempts to regenerate the free enzyme with neutral hydroxyl-

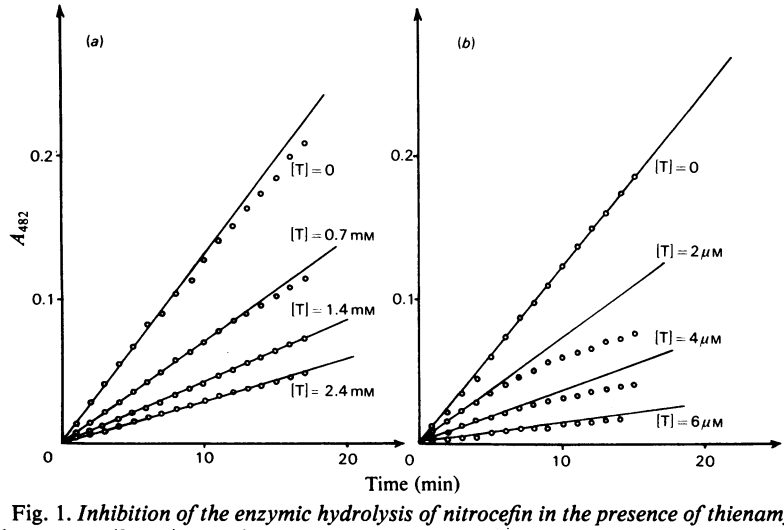

Fig. 1. *Inhibition of the enzymic hydrolysis of nitrocefin in the presence of thienamycin*

(*a*) The G β-lactamase (2.1ng) was incubated at 30°C in 350μl of 25mM-sodium phosphate buffer, pH7.0 (containing 10% glycerol and 10% ethylene glycol) in the presence of 0.1mM-nitrocefin either alone or supplemented with thienamycin (T) at concentrations ranging from 0.7 to 2.4mM. The hydrolysis of nitrocefin was monitored by the change in A_{482}. (*b*) The R39 β-lactamase (0.6ng) was incubated at 30°C in 350μl of 25mM-sodium phosphate buffer, pH7.0, in the presence of 0.1mM-nitrocefin either alone or supplemented with thienamycin (T) at concentrations ranging from 2 to 6μM.

amine failed, but neutral hydroxylamine was found to inactivate the free enzyme itself.

The interaction between clavulanate and the R39 β-lactamase resembled, in several respects, that between clavulanate and the *Escherichia coli* RTEM β-lactamase (Charnas *et al.*, 1978; Fisher *et al.*, 1978). Both the fate of the enzyme and that of clavulanate depended on the ratio of clavulanate concentration to enzyme concentration (Fig. 2). Below a certain ratio, enzyme inactivation was partially reversible and clavulanate was completely destroyed essentially by normal turnover. Above this ratio, enzyme inactivation was complete and irreversible, and clavulanate was only partially hydrolysed by the enzyme. The rate constant for the enzyme recovery (at low [clavulanate]/[enzyme] ratios) was determined by incubating the R39 β-lactamase (0.14 μg) in the absence and in the presence of 10 μM- and 20 μM-clavulanate ([clavulanate]/[enzyme] ratios of 0, 16 and 32), in a total volume of 15 μl, for 10 min at 30°C, after which time each sample was supplemented with cephalothin as substrate (Fig. 3). Absorbance measurements at 260 nm permitted estimation of a pseudo-first-order rate constant of $1 \times 10^{-3} s^{-1}$ for the reactivation of the enzyme. As shown by the data of Fig. 3, the recovery of enzyme activity was not complete; the higher the [clavulanate]/[enzyme] ratio, the lower the extent of enzyme regeneration.

The irreversible inactivation of the R39 β-lactamase by clavulanate gave rise to a complex exhibiting a characteristic absorbance at 280 nm. A 300 μl sample of the enzyme (0.35 mg/ml) was incubated with 9 mM-clavulanate for 3 h at 30°C and the excess of clavulanate was eliminated by dialysis at 4°C. The dialysed solution contained 0.25 mg of protein/ml (as determined by the method of Lowry *et al.*, 1951), had no enzyme activity (even after further incubation at 30°C) and exhibited the spectrum shown in Fig. 4. The reaction mixture was then incubated with 30 μl of neutral 1 M-hydroxylamine; as a result of this treatment, 7% of the original enzyme activity was maximally recovered (within 5 min) and, concomitantly, the peak at 280 nm disappeared. This low extent of enzyme recovery could be attributed, at least in part, to some denaturation process, as the following experiment suggests. The R39 β-lactamase (1.4 μg) and 0.5 mM-clavulanate ([clavulanate]/[enzyme] = 50) were incubated together in 20 μl of buffer, at 30°C for 20 min, after which time the reaction mixture was diluted 20-fold in the same buffer containing neutral 26 mM-hydroxylamine. Under these conditions, 66% of the original enzyme activity was recovered after 25 min of incubation. No reactivation was observed in another enzyme sample similarly treated, except that the buffer used for the dilution was free of hydroxylamine. At this time, either scheme (*a*) or (*b*) of Fig. 5 [these schemes are similar to those presented by Fisher *et al.* (1978) for the interaction between clavulanate and the RTEM β-lactamase] best explains the above kinetic and spectroscopic observations.

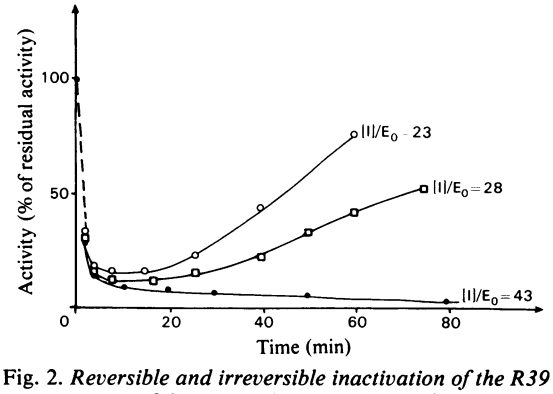

Fig. 2. *Reversible and irreversible inactivation of the R39 β-lactamase by clavulanate*

A fixed amount of enzyme (2.8 μg) was incubated for increasing times at 30°C, in the presence of 200 (○), 260 (□) and 400 (●) μl (final volumes) of a 20 μM solution of clavulanate made in 25 mM-sodium phosphate, pH 7.0. Samples (5 μl) were removed after increasing times of incubation and assayed for enzymic activity using nitrocefin as substrate. $[I]/E_0$ represents the molar ratio of total inhibitor to total enzyme.

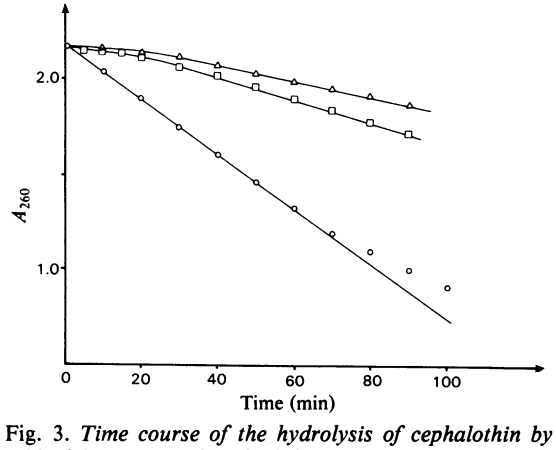

Fig. 3. *Time course of the hydrolysis of cephalothin by R39 β-lactamase that had been pre-incubated in the presence of 0* (○), *10* (□) *and 20* (△) *μM-clavulanate*

The enzyme (0.14 μg in 15 μl of buffer) was incubated with 0, 10 and 20 μM-clavulanate. After 10 min at 30°C, 400 μl of a 0.25 mM-cephalothin solution in buffer was added to each sample. The hydrolysis of cephalothin was monitored by absorbance measurements at 260 nm.

Discussion

Some of the non-classical β-lactam antibiotics investigated in the present study are substrates of the G and R39 β-lactamases. The G β-lactamase has, in the indicated order, decreasing $k_{cat.}$ values for 6-aminopenicillanic acid and mecillinam, 7-aminocephalosporanic acid and quinacillin, and finally *N*-formimidoylthienamycin. With the R39 β-lactamase, the $k_{cat.}$ values are high for mecillinam, moderate for 6-aminopenicillanic acid and quinacillin and low for 7-aminocephalosporanic acid. The occurrence of a given acyl side chain has varying effects on the catalytic constants of the reactions, depending on the nature of the bicyclic fused-ring system to which it is attached. Attachment of a C_6H_5–$CH(NH_2)$–CO side chain to 6-aminopenicillanic acid has no effect on the $k_{cat.}$ and K_m parameters of the reactions catalysed by the G β-lactamase and causes a 7–8-fold increase of both parameters of the reactions catalysed by the R39 β-lactamase, so that the enzyme catalytic efficiency ($k_{cat.}/K_m$) is virtually unaltered (Table 2). When the same side chain is attached to 7-aminocephalosporanic acid, the K_m is little affected, whereas the $k_{cat.}$ value is considerably increased, especially for the R39 enzyme (Table 2).

The G β-lactamase seems to be unable to recognize cefoxitin and quinacillin sulphone, but is rather efficiently inactivated by clavulanate ($k_2/K = 300\,M^{-1}\cdot s^{-1}$). The enzyme inactivation appears to follow a simple, non-branched pathway:

$$E + I \overset{K}{\rightleftharpoons} EI \xrightarrow{k_3} EI^*$$

The R39 β-lactamase is efficiently inactivated by *N*-formimidoylthienamycin. The high value ($k_2/K = 2000\,M^{-1}\cdot s^{-1}$) of the bimolecular rate constant for enzyme inactivation is mainly due to a very low

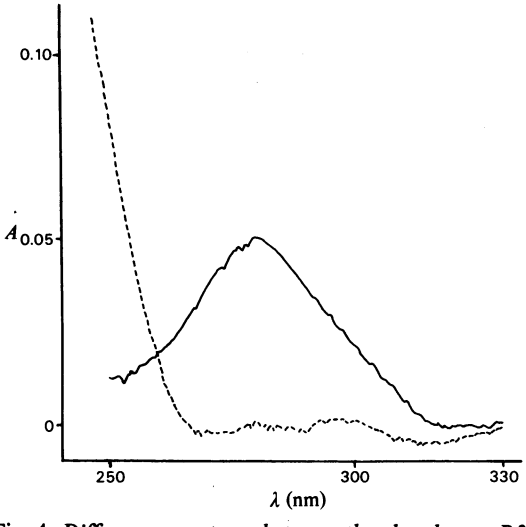

Fig. 4. *Difference spectrum between the clavulanate-R39 β-lactamase complex and the free R39 β-lactamase, before and after hydroxylamine treatment*

The spectrum of the dialysed clavulante-R39 β-lactamase complex was recorded between 330 and 250nm against a 0.25mg/ml solution of the enzyme (——). The complex was enzymically inactive. Both solutions were then supplemented with 30μl of neutral 1M-hydroxylamine and the difference spectrum recorded after maximal recovery of the enzyme activity (– – – –).

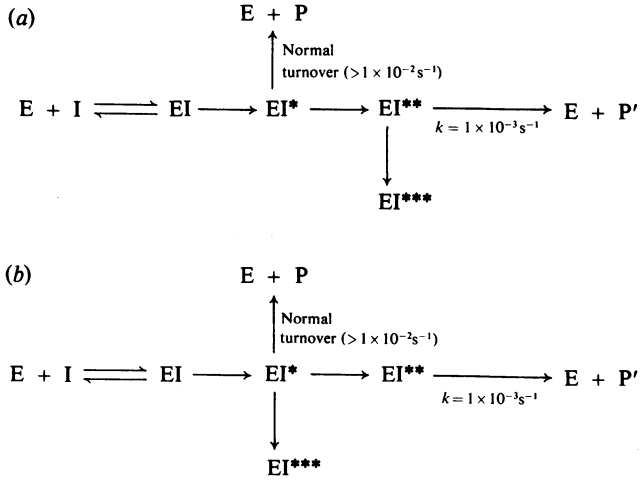

Fig. 5. *Possible schemes for the interaction between clavulanate and the R39 β-lactamase*

EI* is the intermediate formed during normal turnover to hydrolysis [P = hydrolysed product(s)]. Several different EI*** species may be formed since reactivation by hydroxylamine is not complete.

K value (1 μM) and not to a high k_2 vlaue ($2 \times 10^{-3} s^{-1}$). The R39 β-lactamase is also inactivated by quinacillin sulphone and cefoxitin. The k_2/K values are rather low but since K must be higher than 1 mM, it follows that k_2 cannot be much lower than that found with *N*-formimidoylthienamycin. Finally, the interaction between the R39 β-lactamase and clavulanate is a complex phenomenon; it involves (i) a normal catalysed hydrolysis pathway through the intermediate EI*, (ii) the rearrangement of this intermediate EI* into a second inactivated complex EI** that, in turn, spontaneously breaks down with a half-life of about 10 min ($k = 1 \times 10^{-3} s^{-1}$), and (iii) the formation of another or several other inactivated and stable complex(es) EI*** from which the enzyme can be partially regenerated by treatment with neutral hydroxylamine. One irreversible enzyme-inactivating event occurs about every 28–43 turnovers. With the RTEM β-lactamase, about 150 molecules of clavulanate are hydrolysed before one of them has the opportunity to inactivate 1 molecule of enzyme (Fisher & Knowles, 1980).

A β-lactam compound (for example cefoxitin; see Table 1) can be a substrate for a given β-lactamase, an inactivator of another β-lactamase and an inactivator of a D-alanyl-D-alanine peptidase. Assuming that the same underlying mechanism governs these interactions (see the Materials and methods section), $k_{cat.}/K_m$ for a substrate is equivalent to k_2/K for an inactivator. It has been shown previously that the R39 β-lactamase and the R39 D-alanyl-D-alanine peptidase (which are secreted by the same *Actinomadura* strain R39) have roughly comparable $(k_{cat.}K)/(K_m k_2)$ values for several classical penicillins and Δ^3-cephalosporins, suggesting that these two enzymes might be somehow functionally related (C. Duez & J.-M. Frère, unpublished work). A similar observation has been made during the present study with 7-aminocephalosporanic acid, quinacillin sulphone and *N*-formimidoylthienamycin (Table 3). However, other non-classical β-lactam compounds do not behave in this way. 6-Aminopenicillanic acid, mecillinam and quinacillin are much better substrates of the R39 β-lactamase than inactivators of the R39 D-alanyl-D-alanine peptidase, and cefoxitin is a much better inactivator of the R39 D-alanyl-D-alanine peptidase than of the R39 β-lactamase (Table 3). If one excepts cephalosporin C (C. Duez & J.-M. Frère, unpublished work), the $(k_{cat.}K)/(K_m k_2)$ ratios largely deviate from unity for β-lactam compounds that are good substrates of the β-lactamase and

Table 2. *Effects of the attachment of the side chain C_6H_5-CH(NH_2)-CO to 6-aminopenicillanic acid (6-APA), giving rise to ampicillin, or to 7-aminocephalosporanic acid (7-ACA), giving rise to cephaloglycine, on the catalytic constants of the reactions catalysed by the G and R39 β-lactamases*

For the $k_{cat.}$ and K_m values of ampicillin and cephaloglycine, see Duez *et al.* (1981*a*).

Enzyme	Pair of β-lactam compounds	Ratios between the catalytic constants for each pair of β-lactam compounds: $k_{cat.}$	K_m	$k_{cat.}/K_m$
R39 β-lactamase	Ampicillin/6-APA	6.9	8.0	0.85
	Cephaloglycine/7-ACA	86	1.4	60
G β-lactamase	Ampicillin/6-APA	1.6	1.5	1.1
	Cephaloglycine/7-ACA	14	2.4	5.9

Table 3. *Comparison of the kinetic parameters for the interactions between various β-lactam compounds and the R39 D-alanyl-D-alanine peptidase and R39 β-lactamase*

(S) indicates that the corresponding β-lactam compound is a substrate. (I) indicates that the corresponding β-lactam is an inactivator. All the β-lactam compounds are inactivators of the R39 D-alanyl-D-alanine peptidase.

	R39 D-alanyl-D-alanine peptidase* K_2/K (A)	R39 β-lactamase k_2/K or $k_{cat.}/K_m$ (B)	A/B
6-Aminopenicillanic acid	1200	700000 (S)	0.0017
7-Aminocephalosporanic acid	200	800 (S)	0.25
Mecillinam	32	60000 (S)	0.0005
Quinacillin	400	53000 (S)	0.0075
Quinacillin sulphone	10	0.8 (I)	12.5
Cefoxitin	7000	0.4 (I)	17500
N-Formimidoylthienamycin	10000	2000 (I)	5
Clavulanate	30	>300 (I)	<0.1
Benzylpenicillin	300000	980000 (S)	0.3

* From Kelly *et al.* (1981).

rather poor inactivators of the D-alanyl-D-alanine peptidase.

At this time, the mechanistic properties of the R39 and G β-lactamases are unknown. They are insensitive to EDTA and thiol-specific reagents; they are inactivated by clavulanate and, for the R39 enzyme, by cefoxitin and quinacillin sulphone. They might be serine enzymes similar to the β-lactamases of *Escherichia coli* (Fisher *et al.*, 1980) and *Staphyloccus aureus* (Cartwright & Coulson, 1980), and the β-lactamase I of *Bacillus cereus* (Knott-Hunziker *et al.*, 1979).

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