

A KINETIC STUDY ON THE INTERACTION BETWEEN TAZOBACTAM (A PENICILLANIC ACID SULPHONE DERIVATIVE) AND ACTIVE-SITE SERINE β -LACTAMASES

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The interaction between tazobactam and several chromosome- and plasmid-encoded (TEM, SHV, PSE types) class A and C β -lactamases was studied by spectrophotometry. Tazobactam behaved as a competitive inhibitor or inactivator able to restore in several cases the efficiency of piperacillin as a partner β -lactam. A detailed kinetic analysis permitted measurement of the acylation efficiency for some cephalosporinases and broad-spectrum β -lactamases; the presence of a turn-over of acyl-enzyme complex was also evaluated.

Keywords: β -lactamase; Inhibitor; Tazobactam; Kinetics

INTRODUCTION

The production of β -lactamase represents one of the major causes of resistance to β -lactam antibiotics. To date, two different strategies have been

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used to overcome β -lactamase-mediated resistance: (i) development of β -lactamase-stable compounds such as metoximino cephalosporins, cephamycins, monobactams and carbapenems; (ii) use of suicide inhibitors associated with a β -lactam-labile antibiotic. The success of the latter strategy depends on the ability of the suicide inhibitor to inactivate the enzyme. The former approach has led to new forms of β -lactamase-mediated resistance such as stable derepression of class C cephalosporinases or the emergence of extended spectrum variants of TEM, SHV and AmpC plasmid-derived families.¹ Since these enzymes are able to determine resistance to many newer antibiotics there is a new interest in drugs inhibiting β -lactamase activity.

Despite enormous research efforts, only three compounds are widely used at the clinical level. Clavulanic acid was the first β -lactamase inhibitor to be introduced into clinical practice; two other compounds, sulbactam and tazobactam, are also used in therapy. Tazobactam has been reported to inhibit many of the most common β -lactamases; it is used in association with piperacillin which has intrinsically greater activity than other penicillins previously used in combination with inhibitors.²⁻⁴

In the present study, we have evaluated the kinetic interactions between tazobactam and chromosome/plasmid-encoded β -lactamases belonging to class A and C active-site serine enzymes purified in our laboratory in order to determine the effectiveness of this inhibitor.

METHODS

Chemicals, Media and Antimicrobials

Chemicals were of the purest analytical grade and obtained from commercial sources. Culture media and nitrocefin were from Unipath, Milan, Italy. Tazobactam was kindly supplied by Lederle, Catania, Italy.

The strains used in this study were all clinical isolates coming from immunocompromised patients and belonged to the collection of the University of L'Aquila. The identification of the investigated strains was performed by conventional methods.

In Vitro Susceptibility Testing

Minimum inhibitory concentration (MIC) determinations for selected organisms were performed using macrodilution broth procedures and a final inoculum of 5×10^5 CFU/ml as recommended by the National Committee for Clinical Laboratory Standards reference manual.⁵

Enzyme Purification

The β -lactamases used in the present study are listed in Table I. The enzymes from *Citrobacter diversus* ULA-27, *Mycobacterium fortuitum* D316, *Serratia marcescens* TEM-AQ (*Escherichia coli* TEM-AQ), *Acinetobacter baumannii* ULA-187 and *Providencia stuartii* (*E. coli* TEM-60) were purified as previously reported.⁶⁻¹⁰ The enzyme from *Morganella morganii* MN89 was purified to homogeneity and the detailed procedures are reported elsewhere (Amicosante, unpublished data).

The β -lactamases produced by *E. coli* (PSE-1 and PSE-4), *Pseudomonas aeruginosa* PSE-2, *Klebsiella pneumoniae* ULA-4, (*E. coli* ULA-4, SHV-type by oligotyping), *P. aeruginosa* ULA-18 and *P. aeruginosa* ULA-21 were purified as follows. Bacteria were grown in BHI medium (2L) for 18 h at 37°C with orbital shaking (120 rpm). Cells were harvested by centrifugation at 10,000 \times g for 15 min at 4°C, washed with 50 mM sodium phosphate buffer pH 7.0 and disrupted by sonication (4 cycles of 40 s, 60 W, in ice bath). Crude extracts were centrifuged at 105,000 \times g for 30 min and the cleared supernatants of PSE-1, PSE-2 and PSE-4 were dialysed overnight against 30 mM Tris-HCl buffer pH 8.0. The material was then loaded onto a Sepharose Q FF column (XK 26/20, Pharmacia Biotech, Milan, Italy), equilibrated with the same buffer. The crude extracts of *E. coli* ULA-4, *P. aeruginosa* ULA-18 and *P. aeruginosa* ULA-21 were separately loaded onto a Sepharose S-FF column equilibrated with the sodium/potassium phosphate buffer 50 mM pH 6.8. The β -lactamases were eluted at 3 ml/min over a linear gradient 0–0.5 M NaCl in 60 min. The active fractions were pooled and loaded onto a Superdex 75 (XK 26/70 Pharmacia Biotech, Milan, Italy)

TABLE I Main characteristics of the β -lactamases used in this study

Enzyme source	Gene location	Molecular class*	Km [§] (μ M)	pI
<i>C. diversus</i> ULA-27	Chromosome	A ⁶	20 [¶]	6.8
<i>M. fortuitum</i> D316	Chromosome	A ¹⁸	115	4.9
<i>E. coli</i> TEM-AQ	Plasmid-ESBL [†]	A ⁸	120	5.5
<i>E. coli</i> TEM-60	Plasmid-ESBL [†]	A ¹⁰	20	6.4
<i>E. coli</i> ULA-4	Plasmid-ESBL [†]	A ¹	25	8.5
<i>E. coli</i> PSE-1	Plasmid	A ¹	48	5.7
<i>E. coli</i> PSE-4	Plasmid	A ¹	53	5.4
<i>A. baumannii</i> ULA-187	Chromosome	C ⁹	50	>9.0
<i>M. morganii</i> MN89	Chromosome	C	120	6.3
<i>P. aeruginosa</i> ULA-18	Chromosome	Undetermined	200	>9.0
<i>P. aeruginosa</i> ULA-21	Chromosome	Undetermined	45	>9.0

* According to the Ambler's classification.¹⁸ [†] SHV-type hybridization. [‡] ESBL = extended spectrum β -lactamase. [¶] For *C. diversus* ULA-27, cefazoline was used as a substrate reporter. [§] Km values of nitrocefin which were used as reporter substrate.

equilibrated with 50 mM sodium phosphate buffer pH 7.0. The fractions exhibiting β -lactamase activity were concentrated by ultrafiltration with YM10 membranes (cutoff 10 kDa) (Amicon Corp., Beverly, Mass.).

The purity of enzymes was determined by SDS-polyacrilamide gel electrophoresis analysis performed by the method of Laemmli¹¹ and each enzyme was estimated to be at least 90% pure (data not shown).

Determination of β -Lactamase Activity

The β -lactamase activity was monitored in a Perkin-Elmer Lambda 2 spectrophotometer by measuring the hydrolysis of 100 μ M nitrocefim ($\Delta\epsilon_M^{482} = 15,000 \text{ M}^{-1} \text{ cm}^{-1}$) at 482 nm. One enzyme unit represents the amount of protein that hydrolyses 1 μ mol of nitrocefim per minute at 30°C in 50 mM sodium phosphate buffer pH 7.0. The protein concentration was estimated by the method of Bradford¹² using bovine serum albumin as standard.

Enzyme Inhibition Studies

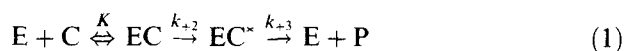
The interaction of tazobactam with each enzyme was studied using 100–200 μ M nitrocefim or cefazoline as reporter substrate.¹³ The inhibition constant K_i , when a competitive model was assessed, was determined at various tazobactam concentrations by measuring the initial rate of substrate hydrolysis with or without inhibitor. Under these conditions, a plot of v_0/v_i versus tazobactam concentration yielded a straight line whose slope was $K_m/(K_m + S) \times K_i$.

Tazobactam Behaving as Poor Substrate

When tazobactam behaved as a poor substrate, the k_{cat} value was determined from the initial rate at saturating substrate concentration (250 μ M) and the K_m value was calculated as K_i .

Tazobactam Behaving as Transient Inhibitor

The accumulation of the acyl-enzyme (EC*) allowed calculation of the individual kinetic constants on the basis of the following scheme:



where k_{+2} and k_{+3} are the first-order acylation and deacylation constants and K the dissociation constant of the Henri–Michaelis complex.

The individual parameters k_{+2} , k_{+3} and K of the reaction were derived from the dependence of k_i (first-order rate constant relative to the EC* accumulation) versus tazobactam concentration as reported by Frère *et al.*¹⁴ and Galleni *et al.*¹⁵ on the basis of the following equation:

$$k_i = k_{+3} + k_{+2}[C]/[C] + K(K_m + [S]/K_m) \quad (2)$$

where $[S]$ and K_m represent the concentration and the K_m of the reporter substrate respectively.

Linear variations of k_i versus tazobactam concentrations indicated that the range of inhibitor concentrations used was below K ; the k_{+2}/K and k_{+3} values were calculated as slope of the line and the extrapolation to $I=0$ respectively.

When possible, the k_{+3} value was also calculated by direct hydrolysis of tazobactam at 233 nm ($\Delta\varepsilon_M^{233} = +3600 \text{ M}^{-1} \text{ cm}^{-1}$) in the presence of large quantities of enzyme.

When tazobactam behaved as an inactivator the kinetic constants were derived as reported above. If a measurable k_{+3} was observed, the value was calculated by monitoring the complete enzyme reactivation after incubation with the inhibitor at 1:1000 ratio. Aliquots (5 μl) were withdrawn and diluted in 600 μl of buffered 200 μM nitrocefin (120 fold dilution).

RESULTS

In Table II, we report the kinetic values for the PSE, TEM and SHV derived enzymes with tazobactam. Regarding the PSE β -lactamases (Table II) (of clinical relevance in *P. aeruginosa*) we were able to measure the acylation efficiency value for PSE-4 enzyme. After prolonged incubation (30 min), the acyl-enzyme complex regained enzyme activity, the half-life of the complex E–I was estimated as around 2 h. The PSE-1 enzyme has a high affinity for tazobactam ($K_i = 10 \mu\text{M}$).

Concerning the TEM-derived enzymes, TEM-AQ hydrolysed tazobactam and a k_{cat} value was measured by direct hydrolysis; on the basis of the k_{cat}/K ratio the compound could be considered as a substrate equivalent to benzylpenicillin. The TEM-60 from *P. stuartii*, was acylated in a very fast manner and the rate was not measurable because the E–I complex formation occurred within the dead-time of the reaction. Moreover, the half-life of the E–I

TABLE II Tazobactam as poor substrate

Enzymes	K _m * (μM)	k _{cat} (s ⁻¹)	k _{cat} /K _m (M ⁻¹ s ⁻¹)
<i>E. coli</i> PSE-1	10	n.d.	< 10
<i>E. coli</i> TEM-AQ	0.34	8 · 10 ⁻²	2.3 · 10 ⁵
<i>E. coli</i> TEM-60	1.8 · 10 ⁻²	4.2 · 10 ⁻⁴	2.3 · 10 ⁴
<i>E. coli</i> (SHV)	1.2 · 10 ⁻²	< 10 ⁻⁴	< 8.4 · 10 ³

n.d. = not determined. SD values were below 10%. *K_m determined as K_i.

TABLE III Tazobactam as transient inactivator or inactivator of class A and C β-lactamases

Enzymes	K _i (μM)	k ₊₂ /K (M ⁻¹ s ⁻¹)	k ₊₃ (s ⁻¹)	K (μM)	k ₊₂ (s ⁻¹)
<i>C. diversus</i> ULA-27	n.d.	1.9 · 10 ³	No turn-over	1.2	2.2 · 10 ⁻³
<i>M. fortuitum</i> D316	20	1.1 · 10 ³	1.4 · 10 ⁻²	n.d.	n.d.
<i>E. coli</i> PSE-4	6.7 · 10 ⁻²	2.9 · 10 ⁴	1.3 · 10 ⁻³	6.9 · 10 ⁻¹	2.0 · 10 ⁻²
<i>M. morgani</i> MN-89	2.3	8.2 · 10 ²	No turn-over	n.d.	n.d.
<i>A. baumannii</i> ULA-187	3.3 · 10 ²	62	No turn-over	26	1.6 · 10 ⁻²
<i>P. aeruginosa</i> ULA-18	31*	1.3 · 10 ²	4.1 · 10 ⁻³	n.d.	n.d.
<i>P. aeruginosa</i> ULA-21	18*	1.1 · 10 ²	2.0 · 10 ⁻³	n.d.	n.d.

n.d. = not determined. SD values were below 10%. *K_i calculated equal to k₊₃K/k₊₂.

complex was calculated as 40 min, a time sufficient to restore the efficiency “*in vivo*” of a partner β-lactam such as piperacillin.

The SHV-derived ESβL from *K. pneumoniae* ULA-4 was competitively inhibited with a K_i value similar to that for the TEM-60 enzyme. As reported in Table III, the PSE-4 class A β-lactamase was efficiently acylated by tazobactam and we could compute all the individual kinetic constants (Figures 1–3). A turn-over was observed and the k₊₃ could be estimated to be equal to 1.3 · 10⁻³s⁻¹. Moreover, the calculated k_i value (k_i = k₊₃K/k₊₂ = 0.044 μM) was in good agreement with the K_i determined at the steady-state (K_i = 0.067 μM). The two chromosome-encoded class A enzymes produced by *C. diversus* ULA-27 and *M. fortuitum* D316 respectively showed similar low values of acylation efficiency (k₊₂/K = 10³ M⁻¹ s⁻¹). The *M. fortuitum* D316 was transiently inactivated. The acylation efficiency of the enzyme was determined by studying a plot of the k_i values versus different tazobactam concentrations. Figure 3 where a linear dependence of k_i was obtained; the k₊₂/K was computed as the slope of the curve. The persistence of the enzyme–inhibitor complex (E–I) at the steady-state before the break-down occurred was estimated to be 72 s (half-life) for *M. fortuitum* β-lactamase. In the case of *C. diversus* ULA-27 enzyme, the result indicated a process of inactivation without turn-over. Similarly for the class C enzyme,

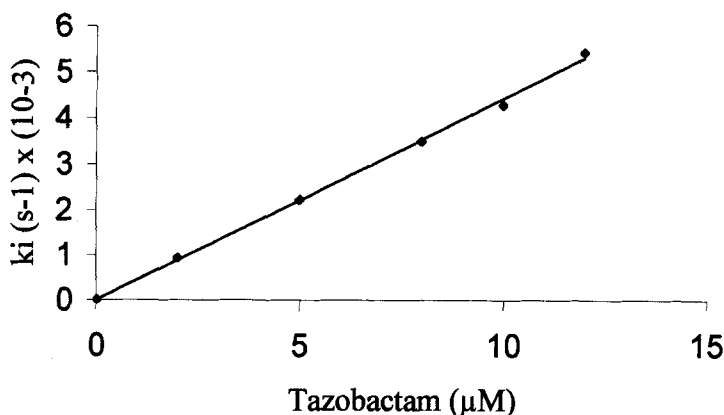


FIGURE 1 Inactivation rate constant (k_i) of *M. morgani* MN89 class C β -lactamase as a function of tazobactam concentration. The plot represent the fitting of data with $k_{+3} = 0$.

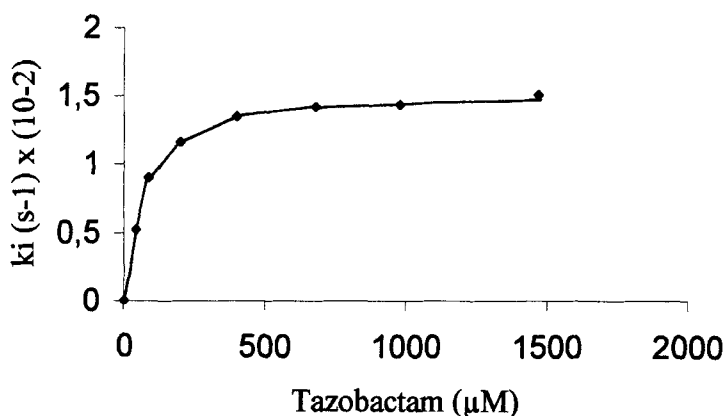


FIGURE 2 Variation of the inactivation rate constant (k_i) versus tazobactam concentration for *A. baumannii* ULA-187 class C β -lactamase.

M. morgani MN-89 and *A. baumannii* ULA-187 inactivation occurred but no turn-over was detected. *M. morgani* inactivation constants k_i were linearly dependant on the tazobactam concentration (Figure 1) and the k_{+2}/K value could be estimated. For *A. baumannii* the plot of k_i versus tazobactam concentration gave a hyperbolic curve (Figure 2). Under these conditions, we were able to calculate the individual kinetic constants k_{+2} and K (Table III). Tazobactam behaved as a poor inactivator ($k_{+2}/K = < 200 \text{ M}^{-1} \text{ s}^{-1}$) for *P. aeruginosa* ULA-18 and ULA-21 cephalosporinases. In both systems

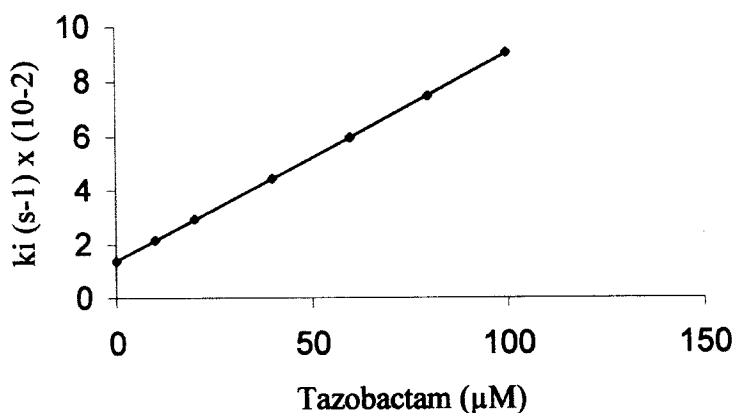


FIGURE 3 Inactivation rate constant k_i of *M. fortuitum* D316 class A β -lactamase as a function of tazobactam concentration. The plot represents the fitting of data with $k_{+3} \neq 0$.

TABLE IV MICs relative to piperacillin and piperacillin–tazobactam combinations towards selected strains used in this work

Strain	Piperacillin ($\mu\text{g/ml}$)	Piperacillin–tazobactam* ($\mu\text{g/ml}$)
<i>A. baumannii</i> ULA-187	64	1
<i>E. coli</i> Bla-div	> 256	16
<i>E. coli</i> TEM-AQ	> 256	16
<i>E. coli</i> TEM-60	256	1
<i>E. coli</i> PSE-1	> 256	8
<i>E. coli</i> PSE-4	> 256	4
<i>E. coli</i> ULA-4	64	< 0.5

* Tazobactam was used at 4 $\mu\text{g/ml}$.

a transient inactivation phenomenon was observed and a small turn-over could be measured. Moreover, a putative K_i value could be calculated as $K_i \cong k_{+3}K/k_{+2}$.

In Table IV are reported the “*in vitro*” activities of piperacillin and piperacillin–tazobactam combination against the selected strains. All the bacteria are resistant to piperacillin except *E. coli* TEM-AQ; tazobactam restores the susceptibility of the other studied strains.

DISCUSSION

Several studies have demonstrated that inhibitors actively working against cell-free enzymes could have their potential reduced in the presence of a permeability barrier and this point cannot be considered a trivial issue when

low-permeable species are encountered in clinical environments. As pointed out by Livermore,³ several factors should be assessed in order to elucidate the usefulness of β -lactam-inhibitor combinations against β -lactamase-producing strains. Akova *et al.*² reported a comparison between clavulanic acid and tazobactam as inhibitors and inducers of AmpC-type β -lactamases in *P. aeruginosa*, *E. cloacae*, *C. freundii*, *S. marcescens* and *M. morgani* strains. The authors concluded that tazobactam was able to inhibit cephalosporinases and restore the microbiological activity of piperacillin. However, the inhibitory activity was reduced against *P. aeruginosa* and *E. cloacae* strains.

Despite several hundreds of reports on susceptibility testing with β -lactam/ β -lactamase inhibitor combinations only a few researchers described in detail the kinetic interactions between tazobactam and β -lactamases.¹⁶ Bush *et al.*¹⁷ reported parameters relevant to various molecular classes of β -lactamases and noted that the I_{50} for the P99 cephalosporinase was 3-fold higher than the K_i for *M. morgani* enzyme and several-fold lower than the K_i value reported for the *A. baumannii* enzyme.

The use of β -lactams in combination with a β -lactamase inhibitor is reduced because of the catalytic heterogeneity of serine-active site β -lactamases mainly present in Gram-negative bacteria and with reduced permeability. The appearance of metallo- β -lactamases (Ambler Class B enzymes) able to hydrolyse inhibitors poses a great concern for the future of antibacterial chemotherapy.

This study analysed in detail the kinetic interaction between tazobactam and several class A and C β -lactamases purified in our laboratory. MIC results indicated that tazobactam was a useful inhibitor to restore piperacillin susceptibility towards all the tested strains. Kinetics parameters relative to the enzyme inactivation by tazobactam showed these to be quite heterogeneous. With chromosomal β -lactamases from *C. diversus* ULA-27, *M. morgani* MN89 and *A. baumannii* ULA-187 no turn-over was detected even if acylation efficiency (k_{+2}/K) was 52-fold higher in *C. diversus* ULA-27 with respect to *A. baumannii* ULA-187. The β -lactamases produced by *M. fortuitum* D316 and *P. aeruginosa* ULA-18 and ULA-21 seemed quite different regarding the acylation (measured as k_{+3}) which is not negligible and 10-fold larger with respect to the *C. diversus* ULA-27 enzyme.

The plasmid-encoded β -lactamases as reported in Table II appear to be really heterogeneous with respect to the interaction with the inhibitor. This finding leads to the conclusion that the appearance of new TEM-derived enzymes or the diffusion of chromosome-encoded enzymes able to hydrolyse inactivators or insensitive could represent a real problem in the therapy of bacterial-associated infections.

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References

- [1] K. Bush, G.A. Jacoby and A.A. Medeiros (1995) *Antimicrob. Agents Chemother.*, **39**(6), 1213–1233.
- [2] M. Akova, Y. Yang and D.M. Livermore (1990) *J. Antimicrob. Chemother.*, **25**, 199–208.
- [3] D.M. Livermore (1993) *J. Antimicrob. Chemother.*, **31**(Suppl. A.), 9–21.
- [4] D.M. Livermore, H.Y. Chen and G. Bonfiglio (1992) *Fortschr. Antimikrob. Antineoplast. Chemotherapie*, **11**, 379–388.
- [5] National Committee for Clinical Laboratory Standards (1990) Reference methods for the determination of minimum inhibitory concentrations (MICs) of aerobic bacteria by broth macrodilution, broth microdilution, and agar dilution. Approved Standard M7-A2. National Committee for Clinical Laboratory Standards, Villanova, Pa.
- [6] G. Amicosante, A. Oratore, N. Franceschini, M. Maccarrone, R. Strom, M. Galleni *et al.* (1988) *Biochem. J.*, **254**, 885–890.
- [7] G. Amicosante, N. Franceschini, B. Segatore, A. Oratore, L. Fattorini, G. Orefici *et al.* (1990) *Biochem. J.*, **271**, 729–734.
- [8] M. Perilli, A. Felici, N. Franceschini, A. De Santis, L. Pagani, F. Luzzaro, A. Oratore, G.M. Rossolini, J.R. Knox and G. Amicosante (1997) *Antimicrob. Agents Chemother.*, **41**(11), 2374–2382.
- [9] M. Perilli, A. Felici, A. Oratore, G. Cornaglia, G. Bonfiglio, G.M. Rossolini *et al.* (1996) *Antimicrob. Agents Chemother.*, **40**, 715–719.
- [10] N. Franceschini, M. Perilli, B. Segatore, D. Setacci, G. Amicosante, A. Mazzariol and G. Cornaglia (1998) *Antimicrob. Agents Chemother.*, **42**(6), 1459–1462.
- [11] U.K. Laemmli (1970) *Nature (Lond.)*, **227**, 680–685.
- [12] M.M. Bradford (1976) *Anal. Biochem.*, **72**, 248–254.
- [13] F. De Meester, B. Joris, G. Reckinger, C. Bellefroid-Bourguignon and J.M. Frère (1997) *Biochem. Pharmacol.*, **14**, 2393–2403.
- [14] J.M. Frère, C. Dormans, V.M. Lenzini and C. Duychaerts (1982) *Biochem. J.*, **207**, 429–436.
- [15] M. Galleni, N. Franceschini, B. Quinting, L. Fattorini, G. Orefici, A. Oratore *et al.* (1994) *Antimicrob. Agents Chemother.*, **38**, 1608–1614.
- [16] E.E. Stobberingh (1990) *Chemotherapy*, **36**, 209–214.
- [17] K. Bush, C. Macalintal, B.A. Rasmussen, V.J. Lee and Y. Yang (1993) *Antimicrob. Agents Chemother.*, **37**, 851–858.
- [18] R.P. Ambler (1980) *Phil. Trans. Roy. Soc. Ser. B*, **289**, 321–331.