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Use of the Chromosomal Class A β-Lactamase of *Mycobacterium fortuitum* D316 To Study Potentially Poor Substrates and Inhibitory β-Lactam Compounds

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Sixteen different compounds usually considered β-lactamase stable or representing potential β-lactam inhibitors and inactivators were tested against the β-lactamase produced by *Mycobacterium fortuitum*. The compounds exhibiting the most interesting properties were BRL42715, which was by far the best inactivator, and CGP31608 and ceftazidime, which were not recognized by the enzyme. These compounds thus exhibited adequate properties for fighting mycobacterial infections. Although cloxacillin, dicloxacillin, cefoxitin, and CP65207-2 exhibited poor inhibitory efficiency against the enzyme, they were also rather poor substrates and might be considered potential antimycobacterial agents. By contrast, CGP31523A and ceftamet were good substrates.

Mycobacterium fortuitum D316 produces a chromosomal class A β-lactamase whose peculiar substrate profile includes many cephalosporins and penicillins (1). Most of the enzyme is bound to the bacterial envelope. A small amount is released in the medium (9, 18).

In mycobacteria, a decrease in the diffusion rate of the antibiotic toward its membrane-bound targets can be attributed to the cell wall mycolic acids (8, 13). This phenomenon, in combination with β-lactamase production, drastically increases resistance to penicillins and cephalosporins. A better understanding of these two factors represents the basis for the design of improved drugs which should ideally escape the inactivating action of β-lactamases and exhibit increased diffusion rates and affinities for their natural targets, the penicillin binding proteins.

The aim of this work was to study the interactions between the *M. fortuitum* β-lactamase and some potential inactivators or compounds generally considered β-lactamase stable. Clavulanic acid, BRL42715, CGP31608, CP65207-2, and HRE664 belong in the first category; the other compounds belong in the second.

MATERIALS AND METHODS

Chemicals and antibiotics. Nitrocefin was purchased from Unipath. Penem CP65207-2 (12) was a gift from A. C. Bostook and J. Kemp, Pfizer Central Research, Sandwich, Kent, United Kingdom. Penem CGP31608 (20) and cephalosporins CGP7174 (cefsulodin), CGP17520, and CGP31523A were gifts from A. Matter and D. R. K. Scheibli, CIBA GEIGY Ltd., Basel, Switzerland. Penem HRE664 (5) and cefpirome were gifts from F. Basani, Hoechst Italia, Milan, Italy. Ceftamet and cefteram were gifts from R. L. Then, Hoffmann-La Roche Ltd., Basel, Switzerland. BRL42715 and clavulanic acid lithium salt were gifts from T. Farmer, Smith-Kline Beecham. Cloxacillin and dicloxacillin were purchased from Sigma, St. Louis, Mo. Ceftazidime was a gift from Glaxo Italia, Verona, Italy, and cefoxitin was purchased from Merck Sharp & Dohme Italia, Rome, Italy. The structures of the β-lactam antibiotics used in this work are shown in Fig. 1 (7).

Strain and enzymes. *M. fortuitum* D316, a strain obtained by nitrosoguanosidine mutagenesis of *M. fortuitum*, overproduces a class A β-lactamase (9). The enzyme was purified to homogeneity as described by Amicosante et al. (1).

Determination of the kinetic parameters. All kinetic experiments were performed at 30°C in 50 mM sodium phosphate (pH 7.4) containing 20 μg of bovine serum albumin per ml and 0.2 M KCl. The other experimental conditions are summarized in Table 1. Values represent averages of five different experiments performed at various concentrations. The data were collected with a microcomputer-linked Perkin-Elmer Lambda 2 spectrophotometer.

Compounds not recognized by the enzyme (ceftazidime and CGP31608). The direct hydrolysis of the antibiotics by the β-lactamase was studied by incubating 0.05 to 2 μg of enzyme with 100 μM ceftazidime or CGP31608. Since no hydrolysis was detected, the effect of the β-lactams on the enzyme activity was tested as follows. (i) A total of 0.05 μg of enzyme was added to 450 μl of 100 μM nitrocefin containing concentrations of ceftazidime and CGP31608 up to 5 mM. The hydrolysis rate of nitrocefin (which is a good substrate) was monitored at 482 nm. (ii) The enzyme (0.3 μM) was incubated with 5 mM ceftazidime or CGP31608 for 30 min at 30°C. Aliquots (5 μl each) were withdrawn, and the residual activity was estimated by measuring the rate of nitrocefin (100 μM) hydrolysis.

Compounds behaving as substrates. For the compounds behaving as substrates (listed in Table 2), the usual steady-state

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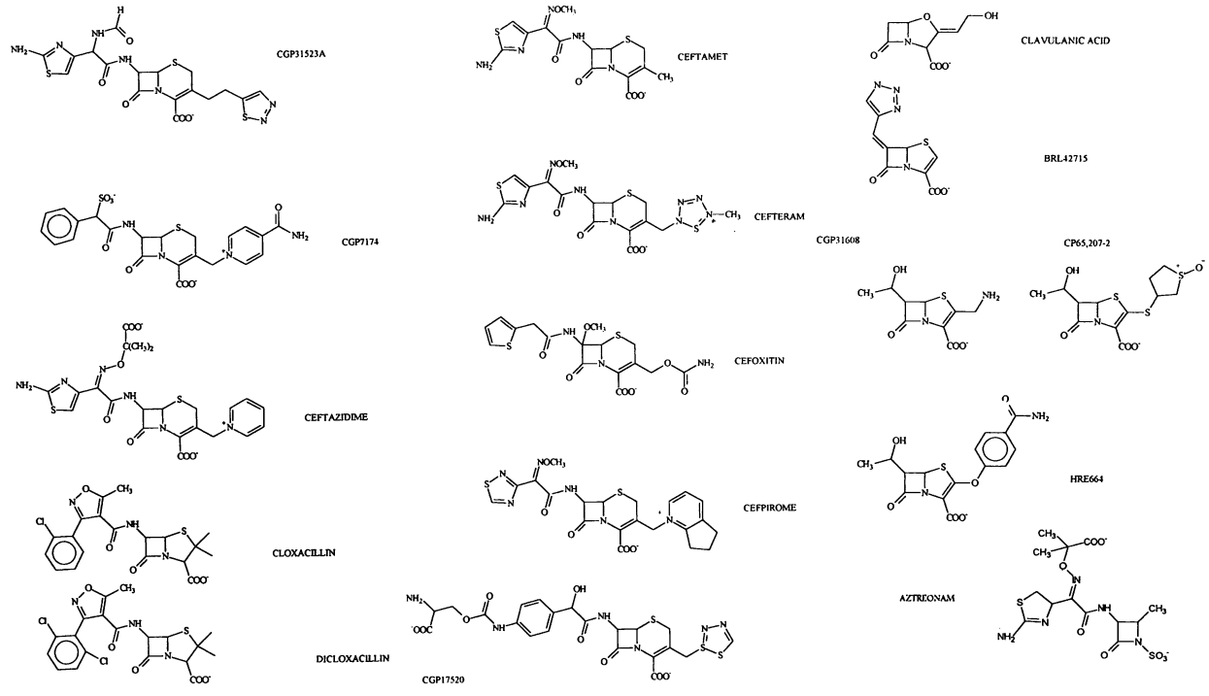

FIG. 1. Structures of the β-lactam antibiotics studied in this work.

parameters k_{cat} and K_m were determined as follows. For CGP31523A, complete hydrolysis time courses were analyzed (method A) (6).

The enzyme was added to 450 μl of substrate solutions (1 to 2 mM). The conditions were such that the reaction was completed in 2 to 3 min. The absence of substrate-induced inactivation was demonstrated by modifying both substrate and enzyme initial concentrations. That phenomenon results in decreased K_m and k_{cat} values when the enzyme concentration is increased.

The analysis of the curves was done according to the integrated Henri-Michaelis equation:

$$\frac{1}{t}\ln\frac{S_0}{S_t} = \frac{1}{K_m}\cdot\left(V - \frac{(S_0 - S_t)}{t}\right) \quad (1)$$

TABLE 1. Experimental conditions for kinetic studies[a]

Antibiotic	Antibiotic concn (mM)	Enzyme (μg)	Wavelength (nm)	δε ($M^{-1}cm^{-1}$)	Method(s)
CGP31523A	1–2	0.01–0.05	265	−4,300	A
CGP7174	0.1–3.8	0.055–2	262	−6,800	B
Ceftamet	0.05–0.3	0.055–2	260	−6,300	B
Cefteram	0.1–0.7	0.055–2	260	−4,400	B
Cefpirome	0.1–0.9	0.055–2	260	−4,500	B
Cefoxitin	0.1–10	0.055–5.5	260	−500	C, D
CGP17520	0.16–4 (*0.5–1*)	0.055	265	−4,000	C, D
Aztreonam	0.1–2 (*0.2*)	0.012–0.12	318	−750	C, D
Cloxacillin	0.1–0.6 (*0.2–0.4*)	0.055–10	230	+500	D, E
Dicloxacillin	0.1–0.6 (*0.2–0.4*)	0.055–10	230	+500	D, E
CP65207-2	0.07–1 (*1*)	0.055–10	327	−3,900	D, E
HRE664	0.14–0.7 (*0.5*)	0.055–2	265	−1,600	D, E
BRL42715	10^{-6}–5×10^{-4} (*0.1*)	0.055–0.55	350	+2,000	D, E, F
Clavulanic acid	0.001–10	0.055–10	265	+2,000	C
Ceftazidime	0.1–5	0.055–2	260	−9,000	D
CGP31608	0.1–5	0.055–2	320	−3,000	D

[a] The total sample volume was 450 μl. The numbers in italics represent the concentration range utilized for the k_{cat} value determination.

TABLE 2. Kinetic parameters for interaction between *M. fortuitum* β-lactamase and compounds behaving as substrates[a]

Antibiotic	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}s^{-1}$)
Cefpirome[b]	1	2	0.5
Ceftamet[b]	0.48	0.044	11
Cefteram[b]	5	0.26	20
CGP31523A[c]	410	0.8	500
CGP7174[b]	2.6	1.3	2
Cefoxitin[d]	0.015	0.9	0.016
Aztreonam[d]	1.2	0.230	5.2
CGP17520[d]	0.01	0.1	0.1
Cefazolin[e]	165	0.141	1,170

[a] Standard deviations did not exceed 5 to 10%.
[b] k_{cat} and K_m were calculated from Hanes plots (method B).
[c] K_m and k_{cat} were determined by using complete hydrolysis time curves (method A).
[d] K_m was determined as K_i in competition experiments (method F).
[e] The kinetic parameters for a typical good substrate (cefazolin) are also shown.

where S_0 and S_t represent the substrate concentrations at times 0 and t, respectively, V represents the maximum rate of the reaction, and K_m represents the Henri-Michaelis constant. The K_m value was obtained from the slope of the line $1/t \ln (S_0/S_t)$ versus $(S_0-S_t)/t$ and that of V/K_m by extrapolation at $(S_0-S_t)/t = 0$. The k_{cat} value was deduced from the value of the maximal rate of hydrolysis (V).

For cefpirome, ceftamet, cefteram, and CGP7174, initial rates were determined and the kinetic parameters were obtained by using the Hanes-Woolf linearization of the Henri-Michaelis equation (method B) (16)

$$\frac{[S]}{v} = \frac{K_m}{V} + \frac{[S]}{V} \quad (2)$$

where v is the initial rate measured at a substrate concentration, [S].

The enzyme (0.055 μg) was added to 450 μl of substrate solution (0.05 to 0.3 mM), and the initial rates were determined. The enzyme and substrate concentrations are listed in Table 1.

The other three compounds (cefoxitin, aztreonam, and CGP17520) were rather stable in response to β-lactamase action. The k_{cat} values were determined from the initial rates calculated at saturating substrate concentrations (i.e., the highest concentrations listed in Table 1), and the K_m values were determined from experiments involving competition between the poor substrate and nitrocefin. Under these conditions,

$$\frac{v_0}{v_i} = 1 + \frac{K_m \cdot I}{(K_m + S) \cdot K_i} \quad (3)$$

where v_0 and v_i represent the initial rates of hydrolysis of nitrocefin in the absence and presence of the poor substrate, respectively, I is the poor substrate concentration, K_i is the inhibition constant, and K_m and S are the Henri-Michaelis constant and the concentration of nitrocefin, respectively.

The plot v_0/v_i versus I yielded a line of slope $K_m/(K_m + S) \cdot K_i$ (method C).

Compounds behaving as transient inactivators. In compounds behaving as transient inactivators, a rather stable acylenzyme (EC*) was found to accumulate and the interactions were studied on the basis of scheme 1. The slow breakdown of EC* regenerated the free enzyme (low value of k_{+3}):

$$E + C \underset{}{\overset{K}{\rightleftharpoons}} E \cdot C \xrightarrow{k_{+2}} EC^* \xrightarrow{k_{+3}} E + P \quad \text{(scheme 1)}$$

where E, C, E · C, and P are the enzyme, substrate, Henri-Michaelis complex, and hydrolysis product, respectively; k_{+2} and k_{+3} are the first-order acylation and deacylation rate constants, respectively; and K is the dissociation constant of the Henri-Michaelis complex.

The values of the first-order rate constant (k_i) characterizing the rate of EC* accumulation were obtained by monitoring the hydrolysis of nitrocefin utilized as a reporter substrate (method D [6]) whose hydrolysis time course was monitored at 482 nm.

It can be shown that

$$(v_t - v_{ss}) = (v_0 - v_{ss})\, e^{-k_i t} \quad (4)$$

where v_0, v_t, and v_{ss} are the rates of utilization of the reporter substrate at times 0 and t and after the steady state has been established, respectively. For more details, see reference 6.

The individual parameters, k_{+2}, k_{+3}, and K, can be derived from the dependence of k_i upon [C]

$$k_i = k_{+3} + \frac{k_{+2}[C]}{C + K\left(\frac{K_m + [S]}{K_m}\right)} \quad (4)$$

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

If the k_i values vary linearly with [C], indicating that the range of studied concentrations is well below K, equation 4 is simplified:

$$k_i = k_{+3} + \frac{k_{+2}[C] \cdot K_m}{K \cdot (K_m + [S])} \quad (5)$$

The k_{+2}/K value is then obtained from the slope of the line, and k_{+3} is obtained from the extrapolation at [C] = 0.

Conversely, if [C] is much larger than $K \cdot (K_m + [S])/K_m$, $k_i = k_{+2} + k_{+3}$ and is independent of [C].

Typically, 0.055 μg of enzyme was added to 450 μl of 100 μM nitrocefin containing increasing concentrations of cloxacillin, dicloxacillin, CP65207-2, or HRE664 (0.2 to 1.4 mM). For each concentration, the k_i value was derived from the hydrolysis curves of the reporter substrate. Less than 20% of the nitrocefin was hydrolyzed. The concentration ranges of the different antibiotics are reported in Table 1. Alternatively, the k_{+3} value was obtained by directly measuring the hydrolysis of the poor substrate C with large enzyme concentrations (method E). Ten micrograms of the enzyme was incubated with the poor substrate (100 to 300 μM). The hydrolysis rates were measured at the steady state. In that case, the k_{cat} value was equal to k_{+3}.

Inactivators (BRL42715 and clavulanic acid). (i) BRL42175. The direct hydrolysis of BRL42715 was monitored at 350 nm, a wavelength at which the hydrolysis product exhibited an absorbance maximum. One hundred microliters of enzyme (1 mg/ml) was incubated at 30°C with 900 μl of 100 μM BRL42715 for 60 min. The k_{+3} value was obtained by monitoring the reactivation of the enzyme in the presence of the reporter substrate (method F). The enzyme was first completely inactivated, and the reaction mixture was diluted to reduce the inactivator concentration to a negligible value. The activity was then monitored until complete reactivation.

In the case of complete reactivation,

$$\frac{(v_f - v_t)}{(v_f - v_0)} = e^{-k_{+3}t} \quad (6)$$

where v_0, v_t, and v_f are the rates of the reporter substrate hydrolysis at times 0 and t and after complete reactivation, respectively.

The k_{+3} value for the interaction between BRL42715 and the β-lactamase was also determined as follows. One hundred microliters of enzyme (1 mg/ml) was incubated with 100 μl of 30 μM BRL42715 at 30°C for 10 min. Aliquots (5 μl each) were withdrawn after increasing periods and added to 1 ml of 100 μM nitrocefin.

The possibility of a branched pathway was also investigated. The reaction is represented by scheme 2 (19):

$$\mathrm{E+C} \underset{}{\overset{K}{\rightleftharpoons}} \mathrm{E\cdot C} \xrightarrow{k_{+2}} \mathrm{EC^*} \xrightarrow{k_{+3}} \mathrm{E+P} \qquad \text{(scheme 2)}$$
$$\mathrm{EC^*} \xrightarrow{k_{+4}} \mathrm{EC^{**}}$$

where the initial noncovalent enzyme inactivator complex (E · C) is converted to a covalent adduct (EC*) which can either rearrange to produce an inactive enzyme (EC**) or break down to yield active enzyme (E) and products (P).

The reaction was followed by monitoring the hydrolysis of a reporter substrate in the presence of various concentrations of the inactivator. Ten microliters of β-lactamase (0.055 μg/ml) was added to 450 μl of 100 μM nitrocefin containing increasing concentrations of BRL42715 (5 to 90 nM). The k_i values were calculated from the hydrolysis curves, and the individual constants k_{+2} and K were derived.

The ratio $(k_{+3} + k_{+4})/k_{+3}$ represents the ratio between the number of productive turnovers and those leading to enzyme inactivation (11, 15); it was calculated from experiments in which the ratio between the initial inactivator concentration (C_0) and enzyme concentration (E_0) was such that incomplete inactivation occurred. This was done by monitoring the residual activity after incubating the enzyme (1.66 μM) with increasing concentrations of BRL42715 (0.16 μM to 1.66 mM).

Clavulanic acid. As shown by Fisher et al. (10) for the TEM β-lactamase, the interaction between clavulanic acid and some class A β-lactamases appears to be more complex, involving at least three different covalent adducts (scheme 3):

$$\mathrm{EC^*_i} \quad (k_{+6})$$
$$\mathrm{E + C} \underset{}{\overset{K}{\rightleftharpoons}} \mathrm{E\cdot C} \xrightarrow{k_{+2}} \mathrm{EC^*} \xrightarrow{k_{+3}} \mathrm{E + P} \qquad \text{(scheme 3)}$$
$$\mathrm{EC^*} \xrightarrow{k_{+4}} \mathrm{EC^{**}} \xrightarrow{k_{+5}} \mathrm{E + P'}$$

where EC* represents the transient covalent enzyme, EC** and $\mathrm{EC^*_i}$ represent modified covalent adducts, and P′ represents the hydrolysis product of the modified complex EC**.

The β-lactamase (0.2 μM) was incubated with various clavulanic acid concentrations (2 μM to 1.1 mM). Aliquots (10 μl each) were withdrawn after increasing periods, and their residual activity was determined.

The transformation of EC* to EC** results in the appearance of a new absorption band at 265 nm. In order to detect the formation of EC**, the β-lactamase (30 μM) was incubated with a high concentration of clavulanic acid (500 μM). The A_{265} was determined at 10-min intervals for 2 h.

The inhibition constant characterizing the interaction between the β-lactamase and clavulanic acid was obtained by incubating 5.5 ng of enzyme with 450 μl of 100 μM nitrocefin containing increasing concentrations of clavulanic acid (1 to 10 μM) and by recording product accumulation for 2 min (i.e., before inactivation became detectable). In that case, the inhibition constant was calculated by method C.

RESULTS

Compounds not recognized by the enzyme. At concentrations up to 100 μM, no hydrolysis of ceftazidime and CGP31608 could be detected after 60 min in the presence of 3 μM enzyme, indicating k_{cat}/K_m values lower than 50 $M^{-1}s^{-1}$. In competition experiments, high concentrations (5 mM) of the two compounds did not affect the rate of hydrolysis of 100 μM nitrocefin. Moreover, after a 30-min preincubation of 0.3 μM enzyme in the presence of 5 mM antibiotic, no inactivation could be detected.

Compounds behaving as substrates. The results for compounds behaving as substrates are summarized in Table 2.

Good substrates. CGP31523A was a very good substrate of the enzyme. It was hydrolyzed with high efficiency (k_{cat} = 410 s^{-1}) and the K_m value was rather high (K_m = 0.8 mM). The parameters were derived by method A (Table 1). A low K_m value was obtained for the interaction between the β-lactamase and ceftamet, which was also rather stable in the presence of the enzyme. Cefpirome, cefteram, and CGP7174 exhibited low k_{cat}/K_m values. The kinetic parameters were derived with the help of method B.

Poor substrates. Cefoxitin, aztreonam, and CGP17520 were poor substrates of the enzyme. The low k_{cat} values did not allow a complete determination of the kinetic parameters by direct hydrolysis of these antibiotics. Only k_{cat} was so obtained (method D). The presence of a burst was only detected during the hydrolysis of cefoxitin. The k_{cat} values given in Table 2 were determined after the steady state was established (≈1 min). In the presence of the reporter substrate, no transient acylenzyme accumulation was detected. Method F was used to calculate the K_m value. Cefoxitin was poorly recognized by the enzyme (k_{cat}/K_m = 16 $M^{-1}s^{-1}$). CGP17520 exhibited a somewhat higher k_{cat}/K_m value, and aztreonam was relatively well hydrolyzed.

Poor substrates behaving as transient inactivators. The results for poor substrates behaving as transient inactivators are presented in Table 3. The experimental conditions and the methods utilized for the determination of the kinetic parameters are listed in Table 1. The kinetic parameters obtained for cloxacillin and dicloxacillin were similar. For these compounds, accumulation and slow hydrolysis of a transient acylenzyme were observed. The kinetic parameters were obtained by method C. The variation in k_i versus the antibiotic concentration was linear. The second-order acylation rate constant (k_{+2}/K) and k_{+3} were obtained. The acylation efficiency was

TABLE 3. Kinetic parameters for interaction between *M. fortuitum* β-lactamase and poor substrates behaving as transient inactivators[a]

Antibiotic	k_{cat} (s^{-1})	k_{+3} (s^{-1})	k_{+2} (s^{-1})	k_{+2}/K ($M^{-1}s^{-1}$)	K (μM)	K_m (mM)[b]
Cloxacillin[c]	0.05	0.02	ND[d]	150	ND	0.13
Dicloxacillin[c]	0.02	0.02	ND	40	ND	0.5
CP65207-2	0.03	0.022	ND	80	ND	0.25
HRE664	0.005	ND	0.022	1,400	16	0.003

[a] Standard deviations did not exceed 10%.
[b] K_m was calculated as $(k_{+3} \cdot K)/k_{+2}$.
[c] Presence of a burst during the direct hydrolysis of the antibiotic. In that case, the values of k_{cat} were determined after the establishment of the steady state. k_{+3} values were obtained by extrapolation as shown in Fig. 2 for dicloxacillin.
[d] ND, not determined. The plots of k_i versus [C] were linear (k_{+2}/K values) and were extrapolated to [C] = 0 (k_{+3}).

very poor (k_{+2}/K = 40 to 150 $M^{-1}s^{-1}$). The k_{+3} values were also determined as k_{cat}s by direct hydrolysis of the antibiotic (method D). The presence of a burst was detected during the hydrolysis of both isoxazolyl penicillins. The k_{+3} values given in Table 3 were determined after the steady state was established (≈1 min). When both methods were utilized, the directly determined k_{cat} values (method D) were in good agreement with those derived by method C. Figure 2 shows the determination of the deacylation constant (k_{+3}) and of the second-order acylation rate constant (k_{+2}/K) for dicloxacillin.

The kinetic parameters for CP65207-2 and HRE664 were similarly determined. With the latter, the k_i value exhibited a hyperbolic dependency upon the concentration and it was possible, on the basis of method C, to derive the individual constants k_{+2} and K from a plot of $1/(k_i - k_{+3})$ versus 1/[HRE664]. The k_{+3} value was the k_{cat} value determined from the direct hydrolysis of the antibiotic.

Inactivators. (i) Interaction with BRL42715. A transient acylenzyme was found to accumulate, and slow hydrolysis was observed by monitoring the variation of A_{350} (method D) (2). The k_{cat} value was 4×10^{-4} s^{-1}. By monitoring the reactivation of the enzyme, a similar value of 10^{-4} s^{-1} was obtained (method E).

An approximate value of 2 was found for the $(k_{+3} + k_{+4})/k_{+4}$ ratio. When a reporter substrate was utilized, accumulation of the acylenzyme could be monitored by determining the enzyme inactivation (method C). Figure 3 shows the variation of the pseudo-first-order inactivation rate constant with the antibiotic concentration. From these data, the acylation constant (k_{+2} = 0.04 s^{-1}) and the acylation efficiency ($k_{+2}/K = 3.8\ 10^6\ M^{-1}\ s^{-1}$) were calculated. These values were computed with the help of the Enzfitter program (14). Since the rearrangement of the acylenzyme appeared to be rapid (14a), it is likely that the k_{cat} value represented the rate of hydrolysis of the rearranged acylenzyme.

(ii) Interaction with clavulanic acid. By using initial rate measurements of nitrocefin hydrolysis, a K_i value of 3.2 μM was found (method C). Longer incubations resulted in a slow inactivation of the enzyme. Figure 4 presents the progressive decrease in enzyme activity for different clavulanic acid/enzyme molar ratios. Even at high antibiotic concentrations and after a 24-h incubation, the enzyme was never totally inactivated (3 to 5% of residual activity). The modification of the transient acylenzyme into a chromophoric complex was monitored at 265 nm (3, 4, 8, 9). At a high (500 μM) concentration of clavulanic acid, a slow increase in A_{265} was observed, which might correspond to the accumulation of the modified acylenzyme.

In conclusion, scheme 4 with $k_{+6} = 0$ seems to hold for both inactivators, with the following implications. For BRL42715, $k_{cat} = k_{+5}$ and the values of k_{+2}/K and k_{+4} were very high. For clavulanate, k_{+5} is also nonnegligible and k_{+4} probably is very

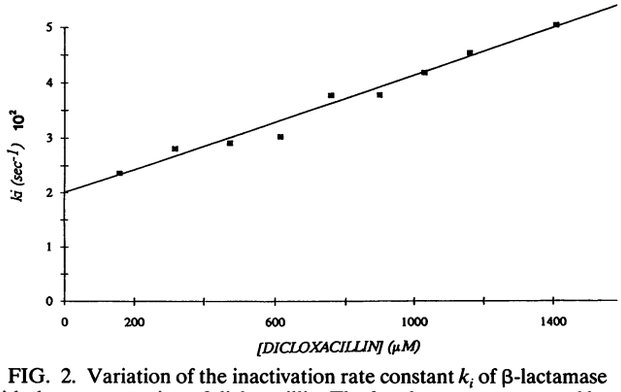

FIG. 2. Variation of the inactivation rate constant k_i of β-lactamase with the concentration of dicloxacillin. The k_i values were measured by the reporter substrate method (method D). The enzyme was added to a mixture (500 μl) of dicloxacillin and 100 μM nitrocefin. The concentration range of dicloxacillin is given in Table 1. The absorbance variations were recorded at 482 nm. The k_i values were derived by analyzing the curves as described by De Meester et al. (6).

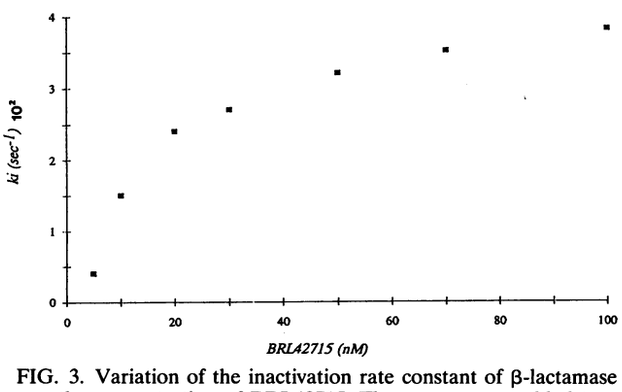

FIG. 3. Variation of the inactivation rate constant of β-lactamase versus the concentration of BRL42715. The enzyme was added to a mixture of BRL42715 and 100 μM nitrocefin. The total reaction volume was 500 μl. The absorbance variations were recorded at 482 nm. k_i values were derived by analyzing the curves as described by De Meester et al. (6). The curve of k_i as a function of the antibiotic concentration represents the fitting of the data to equation 2, with $k_{+3} = 0$. The BRL42715 concentration range used in these experiments is given in Table 1.

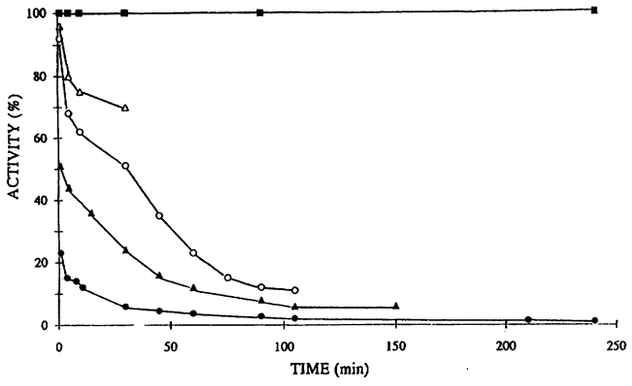

FIG. 4. Variation of β-lactamase activity with incubation time for different clavulanic acid/β-lactamase molar ratios. *M. fortuitum* β-lactamase (0.2 μM) was incubated with various clavulanic acid concentrations (0.2 μM to 1.1 mM), and the residual activity was determined for different clavulanic acid/β-lactamase molar ratios. ■, C/E = 1 and 10; △, C/E = 110; ○, C/E = 550; ▲, C/E = 1,100; ●, C/E = 5,500.

small. The measured K_i value corresponds to the K_m of the horizontal branch, i.e., $k_{+3} \cdot K/(k_{+2} + k_{+3})$.

DISCUSSION

The incredible success of β-lactam therapy has led to extensive use of these compounds in hospitals for the last 40 years. Such a dramatic selection pressure resulted in the emergence of β-lactamase producers or of bacteria exhibiting decreased permeability to the drugs. Previous studies indicated the presence of a chromosomal class A β-lactamase in *M. fortuitum* D316 associated with a low level of permeability to β-lactams because of the presence of the mycolic acid layer (1, 13, 21). Mutants producing penicillin binding proteins with reduced penicillin affinity were also isolated (9).

The *M. fortuitum* β-lactamase is still poorly studied. The enzymes synthesized by the mutant, overproducing D316 strain and the wild-type FC1 strain were purified to homogeneity. Comparison of the sequences of the two genes (17) indicated a few differences which did not seem to reflect the catalytic properties of the two enzymes (15a), and, in consequence, the D316 enzyme can be considered to be a typical *M. fortuitum* β-lactamase.

Two strategies have been developed to counter the production of β-lactamases by pathogenic strains: the synthesis of β-lactamase-stable compounds and the utilization of β-lactamase inactivators in synergy with classical, β-lactamase-sensitive antibiotics. In this contribution, compounds belonging to both categories were studied in order to identify molecules which might be used for efficiently inhibiting the growth of pathogenic mycobacteria.

On the basis of their kinetic parameters, β-lactams which interact with the enzyme according to scheme 1 can be divided into the following categories (note that $k_{cat}/K_m = k_{+2}/K$ and thus supplies an estimation of the acylation efficiency): (i) compounds exhibiting high k_{cat}/K_m and high k_{cat} values, which are good substrates; (ii) compounds exhibiting high k_{cat}/K_m and low k_{cat} values, which are poor substrates; and (iii) compounds exhibiting low k_{cat}/K_m and low k_{cat} values, which are poor to very poor substrates.

For the *M. fortuitum* enzyme, cefteram and CGP31523A belonged to the first category and were thus likely to be inefficient antimycobacterial compounds. None of the molecules studied could really be considered members of the second group. Ceftamet (k_{cat}/K_m = 11,000 $M^{-1}s^{-1}$) and, to a lesser degree, aztreonam (k_{cat}/K_m = 6,000 $M^{-1}s^{-1}$), CGP7174 (k_{cat}/K_m = 2,000 M^{-1} s^{-1}), and HRE664 (k_{cat}/K_m = 1,400 M^{-1} s^{-1}) were nearest to fulfilling the criteria, but the k_{cat} values for the first three compounds were relatively high and with HRE664, the k_{+2}/k_{+3} ratio was such that even at saturating concentrations, about 20% of the enzyme would remain free. The other compounds belonged to the third category, and among them, dicloxacillin, cefoxitin, ceftazidime, and CGP31608 were the poorest substrates. None of these could be considered even fair transient inactivators, because the k_{cat}/K_m values were so low that acylenzyme accumulation only occurred significantly at high substrate concentrations.

Although the behavior of the various compounds was certainly dependent upon the structures of the side chains, it was not possible to establish clear structure-activity relationships.

BRL42715 was the only inactivator exhibiting a high level of acylation efficiency. The accumulation of a transient acylenzyme was observed. The k_{cat} value obtained by directly monitoring the hydrolysis of the substrate was similar to that obtained by monitoring the enzyme reactivation.

Although clavulanic acid exhibited a good affinity (K_i = 3.2 μM) for the enzyme, the subsequent inactivation was very slow. Moreover, a small fraction of the enzyme always escaped inactivation (3 to 5% residual activity), indicating that all of the covalent adducts on the reaction pathway were unstable. The interaction between clavulanic acid and class A β-lactamases has been extensively studied, and the presence of distinct acylenzymes has been demonstrated (3, 4, 10). The appearance of an intermediate absorbing at 265 nm could be visualized with the *M. fortuitum* enzyme. As previously described, a branched-pathway mechanism could be proposed to characterize the phenomenon.

In conclusion, BRL42715 was the most efficient inactivator of the enzyme, while ceftazidime and CGP31608 escaped its hydrolytic activity. To a lesser degree, cefpirome, cefoxitin, CGP17520, dicloxacillin, and CP65207-2 could also be considered potentially interesting compounds for fighting mycobacterial infections. However, their diffusion rate through the mycolic acid layer and their efficiencies in penicillin binding protein inactivation remain to be evaluated.

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