ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 2001, p. 2215–2223 Vol. 45, No. 8
0066-4804/01/$04.00+0 DOI: 10.1128/AAC.45.8.2215–2223.2001
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Kinetic Study of Two Novel Enantiomeric Tricyclic β-Lactams Which Efficiently Inactivate Class C β-Lactamases

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Received 19 July 2000/Returned for modification 26 January 2001/Accepted 3 May 2000

A detailed kinetic study of the interaction between two ethylidene derivatives of tricyclic carbapenems, Lek 156 and Lek 157, and representative β-lactamases and D-alanyl–D-alanine peptidases (DD-peptidases) is presented. Both compounds are very efficient inactivators of the *Enterobacter cloacae* 908R β-lactamase, which is usually resistant to inhibition. Preliminary experiments indicate that various extended-spectrum class C β-lactamases (ACT-1, CMY-1, and MIR-1) are also inactivated. With the *E. cloacae* 908R enzyme, complete inactivation occurs with a second-order rate constant, k_2/K', of 2×10^4 to 4×10^4 M^{-1} s^{-1}, and reactivation is very slow, with a half-life of >1 h. Accordingly, Lek 157 significantly decreases the MIC of ampicillin for *E. cloacae* P99, a constitutive class C β-lactamase overproducer. With the other serine β-lactamases tested, the covalent adducts exhibit a wide range of stabilities, with half-lives ranging from long (>4 h with the TEM-1 class A enzyme), to medium (10 to 20 min with the OXA-10 class D enzyme), to short (0.2 to 0.4 s with the NmcA class A β-lactamase). By contrast, both carbapenems behave as good substrates of the *Bacillus cereus* metallo-β-lactamase (class B). The *Streptomyces* sp. strain R61 and K15 extracellular DD-peptidases exhibit low levels of sensitivity to both compounds.

Since the introduction of benzylpenicillin in clinical trials about 60 years ago, the effectiveness of penicillins and related compounds (β-lactam antibiotics) has been continuously challenged by the emergence of resistant pathogenic strains. Although bacteria have developed several strategies for escaping the lethal actions of β-lactam antibiotics (13, 31, 42), the most common and often the most efficient mechanism is the synthesis of β-lactamases (14, 20, 29, 48). These enzymes, which are usually secreted into the external medium by gram-positive species and into the periplasm by their gram-negative counterparts, very efficiently catalyze the irreversible hydrolysis of the amide bond of the β-lactam ring, yielding biologically inactive products. Despite the large number (~300 [7]) of β-lactamases described to date, these enzymes are divided into only four classes, classes A, B, C, and D, on the basis of their amino acid sequences (35). Enzymes of classes A, C, and D are active-site serine β-lactamases, whereas class B enzymes are Zn^{2+} dependent.

Therefore, much effort has been devoted to the synthesis of molecules which would not be cleaved by β-lactamases of pathogenic strains and which have suitable physicochemical and pharmacodynamic profiles. Absolute stability has, however, not been achieved with any one drug. An alternative strategy to the use of these so-called β-lactamase-stable compounds rests on the use of two β-lactams in synergy: one is an efficient β-lactamase inactivator but a poor antibiotic, while the second is a good, but β-lactamase-sensitive, antibiotic. The former is able to potentiate the action of the latter by protecting it from enzymatic hydrolysis (31, 43). Thus, β-lactamase inactivators such as clavulanic acid, sulbactam, and tazobactam have been successfully used against bacteria that produce the ubiquitous and prevalent TEM-1 or TEM-2 and SHV-1 class A β-lactamases (38). These "wonder" drugs display, however, little or no activity against class B and C enzymes. In addition, bacterial susceptibility to such combinations has recently been challenged by the spontaneous appearance of new β-lactamases of the TEM family, which are resistant to the mechanism-based inactivators (9, 28, 46, 50).

Recently, by following a rational drug design approach, novel tricyclic carbapenem compounds (12) with potential inhibitory activity against serine β-lactamases were synthesized. In this paper we describe the kinetics of the interaction between two of these compounds (Lek 156 and Lek 157; Fig. 1) and representative β-lactamases of the four classes. *Streptomyces* sp. strains R61 and K15 D-alanyl–D-alanine peptidases (DD-peptidases), which are prototypes for penicillin-binding proteins (23), were also studied. The specificities of the interactions of Lek 156 and Lek 157 with active-site serine β-lactamases and DD-peptidases, which constitute two closely related families of penicillin-recognizing enzymes (26), have been determined. The influence of the compounds on the MIC of ampicillin for *Enterobacter cloacae* P99 was also evaluated.

MATERIALS AND METHODS

Compounds. Lek 156 and Lek 157 (Fig. 1) were prepared by Lek (Ljubljana, Slovenia), as described by Copar et al. (12). Tazobactam was a gift from Wyeth-

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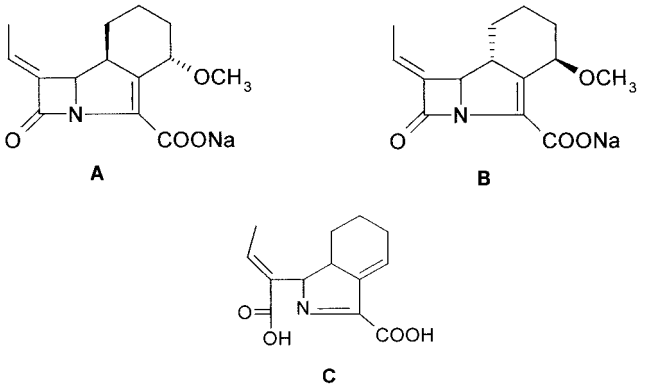

FIG. 1. Structures of Lek 157 {sodium (4S,8S,9R)-4-methoxy-10-[(E)-ethylidene]-11-oxo-1-azatricyclo[7.2.0.0^{3,8}]undec-2-en-2-carboxylate} (A), Lek 156 {sodium (4R,8R,9R)-4-methoxy-10-[(E)-ethylidene]-11-oxo-1-azatricyclo[7.2.0.0^{3,8}]undec-2-en-2-carboxylate} (B), and Lek 1A [3-(1-carboxy-1-propenyl)-3′,4,5,6-tetrahydro-3*H*-isoindole-1-carboxylate] (C), which is obtained from base- or enzyme-catalyzed hydrolysis of the first two compounds.

Ayerst Laboratories (West Chester, Pa.). Nitrocefin was purchased from Unipath (Basingstoke, United Kingdom). Ampicillin was purchased from Sigma Chemical Co. (St. Louis, Mo.). Phenylacetyl-D-alanyl-thio-lactate (S_2x) was a gift of Hoechst-Marion-Roussel (Romainville, France), and benzoyl-D-alanyl-thioglycolate (S_2d) was synthesized as described by Adam et al. (1).

Enzymes. The various enzymes studied in the present work are listed in Table 1. All enzyme preparations were at least 95% pure.

In vitro susceptibility tests. The MICs of ampicillin were determined in the absence or in the presence of Lek 156 or Lek 157 at final concentrations of 10 or 30 μg/ml by a dilution technique with Mueller-Hinton broth and a bacterial inoculum of approximately 5×10^5 CFU per tube, according to the guidelines of the National Committee for Clinical Laboratory Standards. The strain tested was *E. cloacae* P99, a strain which constitutively overproduces a class C β-lactamase (19). The P99 and the Q908 enzymes are virtually identical (24, 25). A control experiment was also performed in the presence of 10 μg of tazobactam per ml.

Experimental conditions for kinetic studies. All kinetic measurements were performed at 30°C. The buffer for β-lactamases of classes A and D was 50 mM sodium phosphate (pH 7.0), that for the class C β-lactamase was 10 mM HEPES (pH 7.0), that for the class B β-lactamase was 25 mM HEPES (pH 7.5) with 100 μM $ZnSO_4$; that for the DD-peptidase of *Streptomyces* sp. strain R61 was 10 mM sodium phosphate (pH 7.0) with 0.4 M NaCl, and that for the enzyme of *Streptomyces* sp. strain K15 was 25 mM sodium phosphate (pH 7.2) with 4 μM dithiothreitol. Dilutions of the β-lactamases below a concentration of 0.1 mg/ml were made with buffer solutions containing 0.1 mg of bovine serum albumin per ml.

Hydrolysis of Lek 156 and Lek 157 was directly monitored at 310 nm by using changes in ε_M ($\Delta\varepsilon_M$) values of −1,850 and −1,350 M^{-1} cm^{-1}, respectively.

Nitrocefin was used as the reporter substrate in all experiments performed with β-lactamases. The absorbance was monitored at 482 nm, and the conditions were chosen such that the level of use of nitrocefin was below 10%. The concentration of nitrocefin (100 μM) was such that the correction factor α (see equation 5 in the Evaluation of the Kinetic Results section) was ~5.3 for the β-lactamase of *E. cloacae* 908R (K_m of the reporter substrate [$K_{m,S}$] = 23 ± 2 μM), ~2.8 for the TEM-1 β-lactamase ($K_{m,S}$ = 55 ± 5 μM), and ~7.7 for the OXA-10 β-lactamase ($K_{m,S}$ = 15 ± 1 μM), so that no large errors were introduced [15]. These $K_{m,S}$ values were derived from the analysis of complete hydrolysis time courses obtained with nitrocefin and the respective enzymes (15).

TABLE 1. Enzymes used in the present work

Enzyme	Class	Reference[a]
TEM-1	Class A β-lactamase	44
NmcA	Class A β-lactamase	45
Bacillus cereus 569H (BcII)	Class B β-lactamase	8
E. cloacae 908R	Class C β-lactamase	41
OXA-10 (PSE-2)	Class D β-lactamase	2
Streptomyces sp. strain R61	DD-Peptidase	27
Streptomyces sp. strain K15	DD-Peptidase	27

[a] Those articles in which the purification procedures are described.

The reporter substrates for the *Streptomyces* sp. strain K15 and R61 DD-peptidases were 400 μM S_2x and S_2d, respectively, and hydrolysis was monitored in the presence of a chemical agent that reacts with thiol groups (49). 5,5′-Dithionitrobenzoic acid was used at 1.4 mM, and the absorbance was monitored at 412 nm. Protection by the reporter substrates did not have to be considered, since with both enzymes [S] was $\ll K_m$ (i.e., α was equal to 1), where [S] is the concentration of the reporter substrate.

With the *Streptomyces* sp. strain R61 DD-peptidase, inactivation was also monitored by measuring the quenching of fluorescence intensity (λ for excitation, 280 nm, λ for emission, 320 nm), as described by Frère et al. (22).

UV and visible spectroscopic measurements were performed on a Beckman DU-8 spectrophotometer. Intrinsic fluorescence emission was recorded with a Perkin-Elmer LS50 spectrometer.

Data analysis. Kinetic data were fitted by linear or nonlinear regression with the program GraFit (32).

Evaluation of the kinetic results. (i) Kinetic models. Both active-site serine β-lactamases and DD-peptidases generally hydrolyze their substrates according to a simple three-step acylation-deacylation pathway (23, 35, 48), as follows:

$$E + C \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EC \xrightarrow{k_2} EC^* \xrightarrow{k_3} E + P(s) \qquad \text{(model 1a)}$$

where E is the enzyme, C is the antibiotic, and P(s) is the inactive degradation product(s) of the antibiotic and where k_2 and k_3 are the first-order rate constants for acylation and deacylation, respectively. If k_3 is very low or equal to zero, the antibiotic becomes a transient or irreversible inactivator (model 1b) (37, 39). EC is the noncovalent Henri-Michaelis complex, and EC* is the acyl-enzyme. In most cases, the reaction can be quantitatively described by the Henri-Michaelis-Menten equation ($[E]_0 \ll [C]_0$), and the steady-state parameters are as follows:

$$k_{cat} = k_2 \cdot [k_3/(k_2 + k_3)] \qquad (1)$$

and

$$K_m = k_3 \cdot [K'/(k_2 + k_3)] \qquad (2)$$

where

$$K' = (k_{-1} + k_2)/k_{+1} \qquad (3)$$

and

$$k_{cat}/K_m = k_2/K' \qquad (4)$$

Equation 4 shows that the apparent second-order rate constant for substrate hydrolysis, k_{cat}/K_m (also called the specificity constant), corresponds to the apparent second-order rate constant for acyl-enzyme formation (k_2/K').

More complex interactions involving rearrangement of the initially formed acyl-enzyme (EC*) have been encountered (model 2a):

$$\begin{array}{l} E + C \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EC \xrightarrow{k_2} EC^* \xrightarrow{k_3} E + P \\ \qquad\qquad\qquad\quad \Big\downarrow k_4 \\ \qquad\qquad\qquad\quad FC^{**} \xrightarrow[k_5]{} E + P' \end{array} \qquad \text{(model 2a)}$$

where EC** is a second acyl-enzyme, in which the antibiotic moiety has rearranged. Note that this inactivated species might also rearrange back to EC* ($k_{-4} \neq 0$). It is, however, usually impossible to distinguish between the k_{-4} and the k_5 steps (because P→P′), and it is arbitrarily assumed that k_{-4} is equal to 0 (36, 40).

When k_3 is equal to 0 or k_3 is $\ll k_4$, all the reaction flux is channeled through the second acyl-enzyme, EC** (model 2a′). Irreversible inactivation of the enzyme occurs if k_3 and k_5 are equal to 0 (model 2b) or if k_5 is equal to 0 (model 2c).

Model 2a was proposed by Faraci and Pratt (16) to account for the interaction

between active-site serine β-lactamases and some cephalosporin-like compounds exhibiting a leaving group on C-3′. Models 2a, 2a′, and 2b were used to characterize the interactions of various enzymes with β-lactamase-stable compounds (37, 39, 40). With the class A β-lactamases of *Actinomadura* sp. strain R39 and *Streptomyces albus* G, both β-iodopenicillanate (21) and sulbactam (36) seemed to react according to model 2c. An even more complex mechanism was suggested to describe the interaction between both clavulanate (10, 11) and sulbactam (3, 4) and the TEM-2 β-lactamase. This model involves a third branch, which accounts for additional intramolecular events that lead to irreversible inactivation of the enzyme (5, 31).

(ii) Determination of kinetic parameters. Progressive inactivation of the *Streptomyces* sp. strain K15 and R61 DD-peptidases was monitored discontinuously by measuring the residual activity either after increasing periods of time at fixed inhibitor concentrations or after a fixed period of time at different inhibitor concentrations (30), to give pseudo-first-order rate constants for inactivation (k_i). With β-lactamases, inactivation can be monitored continuously with a reporter substrate (15). When inactivation is complete, a steady state ($v_{ss} = 0$) is eventually reached and the pseudo-first-order inactivation rate constant, k_i, can be computed (15). For all the models described above, with the exception of models 2a and 2c, the value of k_i is given by

$$k_i = k_r + (k_2 \cdot [C])/(K' \cdot \alpha + [C]) \quad (5)$$

where k_r takes the values of zero for models 1b and 2b, the value of k_3 for model 1a, and the value of k_5 for model 2a′. The correction factor α equal to $1 + [S]/K_{m,S}$ accounts for the protection of the enzyme by the reporter substrate (S).

Values of k_i were measured at various carbapenem concentrations [C], and when k_i exhibited a hyperbolic variation with [C] the individual values of k_2 and K' could be computed by nonlinear regression. When K' was too high, only the ratio k_2/K' could be obtained.

When k_3 (model 1a) or k_5 (model 2a′) is not negligible, inactivation is incomplete and the reporter substrate utilization reaches a steady state ($v_{ss} \neq 0$). Here, the K_m value for the carbapenem ($K_{m,C}$) can be computed from

$$v_0/v_{ss} = 1 + K_{m,S}/(K_{m,S} + [S]) \cdot [C]/K_{m,C} \quad (6)$$

which takes into account the competition between the two substrates at the steady state. v_0 is the initial rate of hydrolysis of the reporter substrate in the absence of carbapenem.

Much more complex kinetics prevail for models 2a and 2c, which describe branched pathway mechanisms. In both cases, the ratio of the k_3 and k_4 rate constants (k_3/k_4), Waley's partition ratio (47), represents the ratio of the number of productive turnovers to those reactions that lead to irreversible enzyme inactivation. As described previously (36), k_3/k_4 can be determined by measuring the residual activity after partial (and possibly transient) inactivation at low values of $[C]_0/[E]_0$, where $[C]_0$ and $[E]_0$ are the initial concentrations of antibiotic and enzyme, respectively.

Provided that k_3 is $\gg k_4$, conditions can be chosen in which one branch of the pathway has negligible effects on the other (21). Thus, under conditions in which hydrolysis can be neglected ($[C]_0/[E]_0 \gg k_3/k_4$), enzyme inactivation is also characterized by a pseudo-first-order inactivation rate constant (k_i), which can be measured as described above for the linear pathways. In the case of model 2c, the following equation applies:

$$k_i = (k_i)_{lim} \cdot [C]/([C] + K_m) \quad (7)$$

where

$$K_m = k_3 \cdot [K'/(k_2 + k_3 + k_4)] \quad (8)$$

and

$$(k_i)_{lim} = k_2 \cdot [k_4/(k_2 + k_3 + k_4)] \quad (9)$$

and thus

$$(k_i)_{lim}/K_m = (k_2/K') \cdot (k_4/k_3) \quad (10)$$

and

$$k_{cat}/(k_i)_{lim} = k_3/k_4 \quad (11)$$

where

$$k_{cat} = k_2 \cdot [k_3/(k_2 + k_3 + k_4)] \quad (12)$$

In this mechanism, $(k_i)_{lim}$ is a first-order rate constant characterizing the rate of the inactivation process at saturating inactivator concentrations. An even more complicated value of k_i is given for model 2a by equation 13:

$$k_i = k_5 + A \cdot \{[C]/(B + [C])\} \quad (13)$$

where

$$A = k_2 \cdot [k_4/(k_2 + k_3 + k_4)] \quad (14)$$

and

$$B = (k_3 + k_4) \cdot [K'/(k_2 + k_3 + k_4)] \quad (15)$$

In both models 2a and 2c, the steady-state parameters k_{cat} and K_m can be determined by initial rate measurements (k_{cat}) and competitive inhibition experiments (K_m), performed rapidly before the inactivation process becomes detectable (21).

RESULTS

Class A β-lactamases: NmcA and TEM-1. Both Lek 156 and Lek 157 are readily hydrolyzed by the NmcA enzyme, and the UV spectra of the products (P) are the same whether they are obtained with sodium hydroxide, the NmcA and TEM-1 enzymes, or the Zn^{2+}-containing *Bacillus cereus* II β-lactamase as hydrolytic agents (Fig. 2). Hydrolysis of the two compounds was monitored at 310 nm to avoid protein absorption at lower wavelengths. A major absorbance decrease ($\Delta\varepsilon_M$, −1,850 and −1,350 M^{-1} cm^{-1} for Lek 156 and Lek 157, respectively) is followed by a very slow and partial recovery in intensity ($\Delta\varepsilon_M \approx 1{,}000$ M^{-1} cm^{-1} for both compounds; Fig. 2A). This probably arises from the formation of the rearrangement product shown in Fig. 1C, in which the methoxy group of the cyclohexane ring has been eliminated. Indeed, mass spectrometry experiments (M. Vilar, B. Turk, and T. Solmajer, unpublished data) suggest that the major product in these hydrolysis experiments is the 3-(1-carboxy-1-propenyl)-3′, 4, 5, 6-tetrahydro-3*H*-isoindole-1-carboxylate (Lek 1A) rearrangement product (Fig. 1C), which lacks the methoxy group. In addition, it is reasonable to assume that the normal δ-2 pyrroline intermediate covalently rearranges into the tautomeric and thermodynamically more stable δ-1 pyrroline (31).

Incubation of NmcA with initial Lek 156 and Lek 157 concentrations up to 20,000 times that of the enzyme did not lead to inactivation, and the kinetic parameters k_{cat} and K_m are given in Tables 2 and 3. The very low K_m values were measured as K_i with 100 μM nitrocefin as the substrate (41) (Fig. 3), whereas the k_{cat} values were derived from initial rate measurements at saturating concentrations ($[C]_0 \gg K_m$).

The spectral changes shown in Fig. 2A suggest that the rearrangement process which follows the opening of the β-lactam ring is slow. As a consequence, it probably occurs in solution, after hydrolysis of the acyl-enzyme complex, in which case model 1a accounts for the interaction between NmcA and both Lek compounds.

With the TEM-1 enzyme ($[C]_0/[E]_0$, <100), a biphasic phenomenon is detected at the onset of hydrolysis of Lek 156 and Lek 157. Following a burst phase, a much slower turnover of the β-lactam is observed, suggesting a branched pathway as described by models 2a and 2c. With both Lek 156 and Lek 157, the inactivated complexes (EC**) obtained at high $[C]_0/[E]_0$ ratios are very stable, in agreement with model 2c. Experiments in which the residual activity of the enzyme was measured at various time intervals after partial (80 to 95%) inactivation indicates very large half-lives ($t_{1/2}$) for EC** ($t_{1/2}$

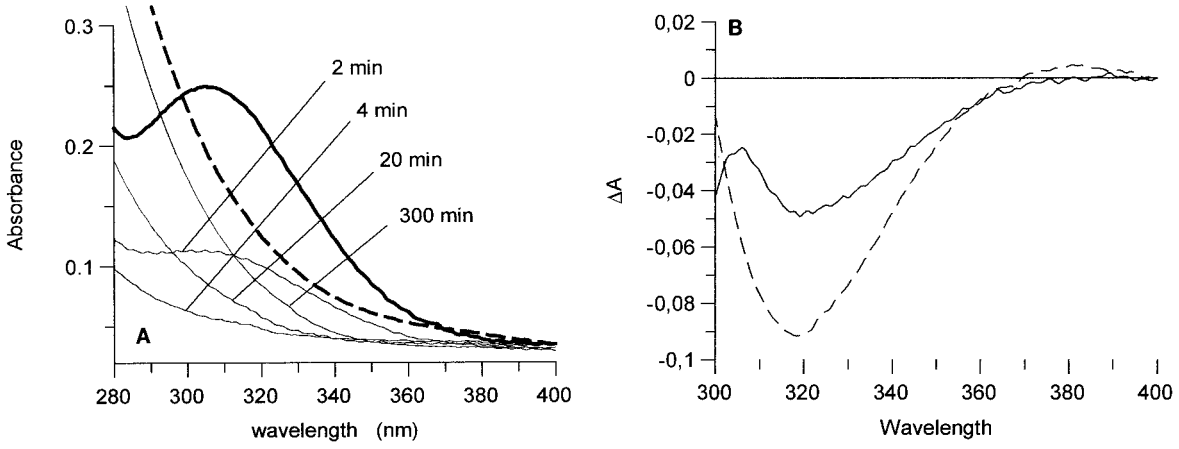

FIG. 2. Spectral changes observed during hydrolysis of 100 μM Lek 156. (A) Hydrolysis by 0.08 μM NmcA β-lactamase. The heavy solid line corresponds to the starting compound; the dashed line refers to the hydrolysis product obtained after long-term incubation (~24 h). The thin lines are the spectra obtained after intermediate periods of incubation. (B) Difference spectra of the final hydrolysis product against the intact Lek 156 (dashed line) and between the adduct obtained with 60 μM TEM β-lactamase and the starting compound (solid lines). ΔA, difference in absorbance.

> 4 h; i.e., k_5 [or k_{-4}] $< 5 \times 10^{-5}$ s^{-1}). This allows k_3/k_4 to be determined (see Materials and Methods section) with good accuracy (Tables 2 and 3).

Under conditions in which the horizontal branch of model 2c is observed ($[C]_0/[E]_0 < k_3/k_4$), the hydrolysis of both compounds by TEM-1 is also characterized by a significant absorbance decrease at 310 nm, followed by a very slow and partial recovery of intensity, as observed with NmcA. In addition, the difference in the spectra of the inactivated acyl-enzyme species and the starting compound (Lek 156 or Lek 157) indicates clear similarities between the spectrum of the complex (EC**) and that of the rearranged hydrolyzed compound (Fig. 2B). These results suggest that the inactivated species (EC**) contains a rearranged carbapenem molecule with properties similar to those of the final hydrolysis product (Fig. 1C). This leads to the conclusion that rearrangement of the opened β-lactam molecule occurs much faster in the enzyme active-site cavity than in solution (Fig. 2A).

The reporter substrate method was used to monitor enzyme inactivation at high values of $[C]_0/[E]_0$ (~10^3 to 10^5). With Lek 157, the rate of inactivation does not vary between 1 and 20 μM, indicating that K_m is $\ll$1 μM and yields a $(k_i)_{lim}$ value of $(8 \pm 0.5) \times 10^{-4}$ s^{-1}. With Lek 156, $(k_i)_{lim}$ was equal to $(4 \pm 0.4) \times 10^{-4}$ s^{-1} and K_m was equal to 0.02 ± 0.007 μM.

In short experiments (<60 s), K_m values (Tables 2 and 3) were measured as K_i with 100 μM nitrocefin as the substrate. With Lek 156, the K_m value obtained by inactivation measurements (0.02 μM) is in good agreement with that derived from competitive inhibition experiments (0.04 μM). The k_{cat} values (Tables 2 and 3) are derived from initial rate measurements at saturating concentrations ($[C]_0 \gg K_m$) and at low $[C]_0/[E]_0$ values (<40).

Values of k_2/K' and k_3/k_4 were calculated from equations 10 and 11 (Tables 2 and 3). The calculated and experimental values of k_3/k_4 are in good agreement for both compounds. The k_2/K' values are identical to the k_{cat}/K_m values calculated from the individual k_{cat} and K_m values. These data support the conclusion that the interaction between Lek 156 or Lek 157 and the TEM-1 β-lactamase is adequately described by model 2c.

Class C β-lactamase: *E. cloacae* 908R. The reaction observed with the *E. cloacae* 908R enzyme appears to be more simple. The two carbapenems form long-lived ($t_{1/2} > 1$ h) inactivated complexes with the β-lactamase, and titration measurements indicate that, in both cases, complete inactivation occurs at an equimolar (1:1) ratio. Thus, the interaction between Lek 156 or Lek 157 and the β-lactamase from *E. cloacae* 908R can be interpreted on the basis of a linear pathway. The presence of a putative leaving group at the C-3′ positions of these compounds suggests that the accumulated acyl-enzymes are the rearranged adduct (EC**) (model 2a′). The difference spectra (data not shown) between the inactivated species and the starting compounds are closely similar to those shown in Fig. 2B. Thus, the inactivated species obtained with the *E. cloacae* 908R β-lactamase probably involves the rearranged carbapenem molecules.

The rate of inactivation was measured as a function of [C] with nitrocefin as the reporter substrate. With Lek 157, a linear increase in k_i is found up to [C] equal to 20 μM, in which k_i is equal to ~0.13 s^{-1}, from which we obtain k_2/K' equal to 36,500 ± 500 M^{-1} s^{-1}, with k_2 being >0.2 s^{-1}, $\alpha K'$ being >20 μM (and, hence, K' being >4 μM), and k_r being $\leq 10^{-3}$ s^{-1}. In contrast, with Lek 156 a hyperbolic dependence of k_i on [C] is observed, from which k_2 equal to 0.37 ± 0.05 s^{-1} and K' equal to 16 ± 4 μM are derived (Fig. 4A), yielding a k_2/K' value of about 2.3×10^4 M^{-1} s^{-1}. At low Lek 156 concentrations, a similar value of k_2/K' (17,500 ± 700 M^{-1} s^{-1}) is obtained from the slope of the linear dependence of k_i on [C] (Fig. 4B). The K_m value for the hydrolysis of Lek 156 can also be derived from the same kinetic experiments. Only partial inactivation of the enzyme occurs, and thus, after establishment of the final steady state, v_{ss} can be measured at various values of [C]. Hence, by using equation 6, a K_m of 0.019 ± 0.003 μM, where K_m is equal to $(k_5 \cdot k_4 \cdot K')/[(k_2 \cdot k_5) + (k_2 \cdot k_4) + (k_4 \cdot k_5)]$, is determined (Fig. 4C).

In another series of experiments, we tried to measure the value of k_2 by directly monitoring the hydrolysis of Lek 156 at 310 nm. The size of the burst phase corresponds to the enzyme concentration, confirming that an equimolar concentration of antibiotic is sufficient to inactivate the enzyme. This further supports the hypothesis of a linear pathway. At substrate concentrations ranging from 200 to 1,500 μM (with $3 < [C]_0/[E]_0 < 30$), the progressive inactivation of the enzyme was found to be independent of [C] and yielded k_i equal to $(0.23 \pm 0.03) \times 10^{-2}$ s^{-1}. This value is about 2 orders of magnitude lower than that obtained for k_2 by the reporter substrate method. This apparent discrepancy can be explained on the basis of model 2a′. In this model, the inactivation of the enzyme observed by the reporter substrate method is due to the accumulation of the first acyl-enzyme species (EC*), whereas the enzyme inactivation followed by direct hydrolysis of the compound results from the accumulation of the second acyl-enzyme species (EC**). Thus, the values obtained by the two methods correspond to different rate constants, i.e., k_2 (~0.4 s^{-1}) and k_4 (~0.002 s^{-1}). In addition, the difference spectra between the inactivated species and Lek 156 and Lek 157, which are very similar to that obtained with Tem-1 (Fig. 2B), indicate that the rearranged hydrolyzed compounds are bound at the active-site cavity (EC**). These results show that model 2a′ adequately describes the interaction between the *E. cloacae* 908R β-lactamase and the two carbapenem molecules.

Finally, the recovery of activity after partial (80 to 95%) inactivation of the enzyme yielded k_r (k_5) values of $(1.5 \pm 0.1) \times 10^{-4}$ and $(0.4 \pm 0.07) \times 10^{-4}$ s^{-1} for Lek 156 (Fig. 5) and Lek 157, respectively. The values of the kinetic parameters determined with the *E. cloacae* 908R β-lactamase are given in Tables 2 and 3.

Although the inactivation pathways were not studied in detail, it could easily be shown that the extended-spectrum, plasmid-encoded ACT-1 and CMY-1 class C β-lactamases were inactivated after 5 min of contact with 1 μM Lek 157, while 20 μM Lek 157 was necessary for inactivation of MIR-1.

Class D β-lactamase: OXA-10. As with the TEM-1 β-lactamase, OXA-10 reacts according to a branched pathway with both compounds. Although complete enzyme inactivation is achieved at much lower $[C]_0/[E]_0$ ratios (k_3/k_4 equal to 4 and 2 for Lek 156 and Lek 157, respectively), the inactivated complexes (EC**) are not completely stable ($t_{1/2}$, ≈12 and 20 min for Lek 156 and Lek 157, respectively). Hence, we have analyzed the data according to model 2a.

The rate of inactivation by Lek 157 was measured as a function of [C], using the reporter substrate method, with 100 μM nitrocefin at a $[C]_0/[E]_0$ of $>> k_3/k_4$, and is shown in Fig. 6. Individual values of A and B can be calculated from equation 13, giving A/B equal to $(2.3 \pm 0.3) \times 10^5$ M^{-1} s^{-1}. From the value of k_3/k_4 and by use of equations 14 and 15, k_2/K' is equal to $(7 \pm 1) \times 10^5$ M^{-1} s^{-1}. Similar experiments performed with Lek 156 yielded A equal to $(68 \pm 5) \times 10^{-3}$ s^{-1} and B equal to 0.06 ± 0.01 μM, giving A/B equal to $(11 \pm 2) \times 10^5$ M^{-1} s^{-1} and, hence, a value of $(57 \pm 10) \times 10^5$ M^{-1} s^{-1} for k_2/K'.

Values of 0.04 (Lek 156) and 0.05 μM (Lek 157) corresponding to K_m equal to $k_5 \cdot (k_3 + k_4) \cdot (K'/[(k_2 \cdot k_5) + (k_2 \cdot k_4) + k_5 \cdot (k_3 + k_4)])$ in model 2a were measured in competitive inhibition experiments with 100 μM nitrocefin as the substrate.

TABLE 2. Kinetic parameters of β-lactamases for Lek 156[a]

Enzyme (model)	k_2 (s^{-1})	K' (μM)	k_2/K' (M^{-1} s^{-1})	k_{+4} (s^{-1})	k_r (s^{-1})	k_{cat} (s^{-1})	$(k_i)_{lim}$ (s^{-1})	$k_{cat}/(k_i)_{lim}$ (k_3/k_4)	k_3/k_4	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	$(k_i)_{lim}/K_m$ (M^{-1} s^{-1})
Nmc-A (1a; substrate)	ND[b]	ND	ND	—[c]	—	3 ± 0.3 (A)	—	—	—	0.53 ± 0.05 (B)	(5.6 ± 0.15) × 10^6 (C)	—
TEM-1 (2c; inhibitor)	ND	ND	(2.6 ± 0.3) × 10^6 (C)	ND	<5 × 10^{-5} (D)	0.094 ± 0.007 (A)	(4 ± 0.4) × 10^{-4} (E)	240 ± 30 (C)	260 ± 15 (F)	0.039 ± 0.002 (B)	(2.4 ± 0.2) × 10^6 (C)	(1 ± 0.1) × 10^4 (C)
BcII (Henri-Michaelis; substrate)	ND	ND	ND	—	—	1,500 ± 100 (A)	—	—	—	280 ± 40 (A)	(5.5 ± 1) × 10^6 (C)	—
908R (2a′; inhibitor)	0.37 ± 0.05 (E)	16 ± 4 (E)	(17.5 ± 0.7) × 10^3 (E)	(0.23 ± 0.03) × 10^{-2} (G)	(1.5 ± 0.1) × 10^{-4} (D)	$k_r = k_5$	—	—	—	0.019 ± 0.003 (H)	(8 ± 1.5) × 10^3 (C)	—
OXA-10 (2a; inhibitor)	ND	ND	(57 ± 10) × 10^5 (E)	ND	(10 ± 2) × 10^{-4} (D)	$k_r = k_5$	—	—	4 ± 0.1 (F)	0.04 ± 0.008 (B)	(2.5 ± 0.7) × 10^4 (C)	—

[a] The capital letters in parentheses indicate the method used to obtain the value: A, measured directly under initial rate conditions; B, competitive inhibition; C, calculated (see text); D, residual activity measurements (reactivation); E, reporter substrate; F, measured directly in partial inactivation experiments; G, measured by monitoring the hydrolysis of the carbapenem; H, reporter substrate, equation 6.

[b] ND, not determined.

[c] —, not applicable.

TABLE 3. Kinetic parameters of β-lactamases for Lek 157[a]

Enzyme (model)	k_2 (s^{-1})	K' (μM)	k_2/K' (M^{-1} s^{-1})	k_r (s^{-1})	k_{cat} (s^{-1})	$(k_2)_{lim}$ (s^{-1})	$k_{cat}/(k_2)_{lim}$ (k_3/k_4)	k_3/k_4	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	$(k_2)_{lim}/K_m$ (M^{-1}/s^{-1})
Nmc-A (1a; substrate)	ND[b]	ND	ND	—[c]	1.7 ± 0.1 (A)	—	—	—	3.6 ± 0.2 (B)	(4.7 ± 0.1) × 10^5 (C)	—
TEM-1 (2c; inhibitor)	ND	ND	(5 ± 3) × 10^6 (C)	<5 × 10^{-5} (D)	0.5 ± 0.05 (A)	(8 ± 0.5) × 10^{-4} (E)	620 ± 70 (C)	530 ± 10 (F)	0.08 ± 0.04 (B)	(6 ± 3) × 10^6 (C)	(1 ± 0.5) × 10^4 (C)
BcII (Henri-Michaelis; substrate)	ND	ND	ND	—	750 ± 150 (A)	—	—	—	45 ± 10 (A)	(1.7 ± 0.5) × 10^7 (C)	—
908R (2a′; inhibitor)	>0.2 (E)	>4 (E)	(36.5 ± 0.5) × 10^3 (E)	(4 ± 0.7) × 10^{-5} (D)	$k_r = k_5$	—	—	—	ND	ND	—
OXA-10 (2a; inhibitor)	ND	ND	(7 ± 1) × 10^5 (E)	(5.6 ± 0.5) × 10^{-4} (D)	$k_r = k_5$	—	—	2.2 ± 0.1 (F)	0.05 ± 0.01 (B)	(1.1 ± 0.25) × 10^4 (C)	—

[a] The capital letters in parentheses indicate the method used to obtain the value: A, measured directly under initial rate conditions; B, competitive inhibition; C, calculated (see text); D, residual activity measurements (reactivation); E, reporter substrate; F, measured directly in partial inactivation experiments.
[b] ND, not determined.
[c] —, not applicable.

FIG. 3. Dixon plot of inhibition of nitrocefin turn over by the NmcA β-lactamase in the presence of Lek 157. [C] is the concentration of Lek 157, and v is the initial rate of nitrocefin hydrolysis. A total of 5 to 10 μg of enzyme was added to 450 μl of 100 μM nitrocefin in 50 mM sodium phosphate (pH 7.0) in the presence of various concentrations of Lek 157 (0.4 to 21 μM). The slope of the line obtained by linear regression analysis of the data and under the assumption of a competitive phenomenon yielded K_i (K_m) equal to 3.6 ± 0.2 μM.

Finally, the values of k_5 (or perhaps k_{-4}; see model 2a) were determined in reactivation experiments (k_r) as described above, and values of (10 ± 2) × 10^{-4} and (5.6 ± 0.5) × 10^{-4} s^{-1} were obtained for Lek 156 and Lek 157, respectively.

Class B β-lactamase: the Zn^{2+}-containing enzyme from *B. cereus* (BcII). The kinetic parameter values (Tables 2 and 3) show that the two carbapenems are hydrolyzed very efficiently by the BcII enzyme. In the absence of any indication of a more complex situation, the simple Henri-Michaelis model was used, and initial rate measurements at various substrate concentrations allowed the individual k_{cat} and K_m values to be determined. Kinetics similar to those of the NmcA enzyme are observed by monitoring the change in absorbance at 310 nm.

DD-Peptidases of *Streptomyces* sp. strains R61 and K15. The interactions between the two DD-peptidases and Lek 156 and Lek 157 can be analyzed on the basis of a simple linear pathway (model 1a). Due to the very slow ($t_{1/2} > 4$ h) deacylation process, however, the rearrangement of the opened β-lactam compounds probably occurs at the level of the acyl-enzyme (EC* → EC**), in which case model 2a′ provides a better description of the phenomenon.

The kinetic parameters are listed in Table 4. In inactivation experiments with the *Streptomyces* sp. strain K15 and R61 DD-peptidases by using S_2x as the reporter substrate or fluorescence quenching, respectively, only the k_2/K' values could be determined. Reactivation experiments (using S_2x and S_2d for the K15 and R61 enzymes, respectively) allowed the deacylation rate constants (k_3) to be calculated.

In vitro susceptibility tests. Lek 156 and Lek 157 had no effect at concentrations up to 284 μg/ml. The MICs (Table 5) of ampicillin were significantly reduced by the presence of 30 μg of Lek 157 per ml or 10 μg of tazobactam per ml. The presence of Lek 156 also decreased the MIC of ampicillin, but with a lesser efficiency. However, it should be noted that Lek 156 was unstable even in pure phosphate buffer (pH 7). Under these conditions, a UV and visible spectrum indicated that

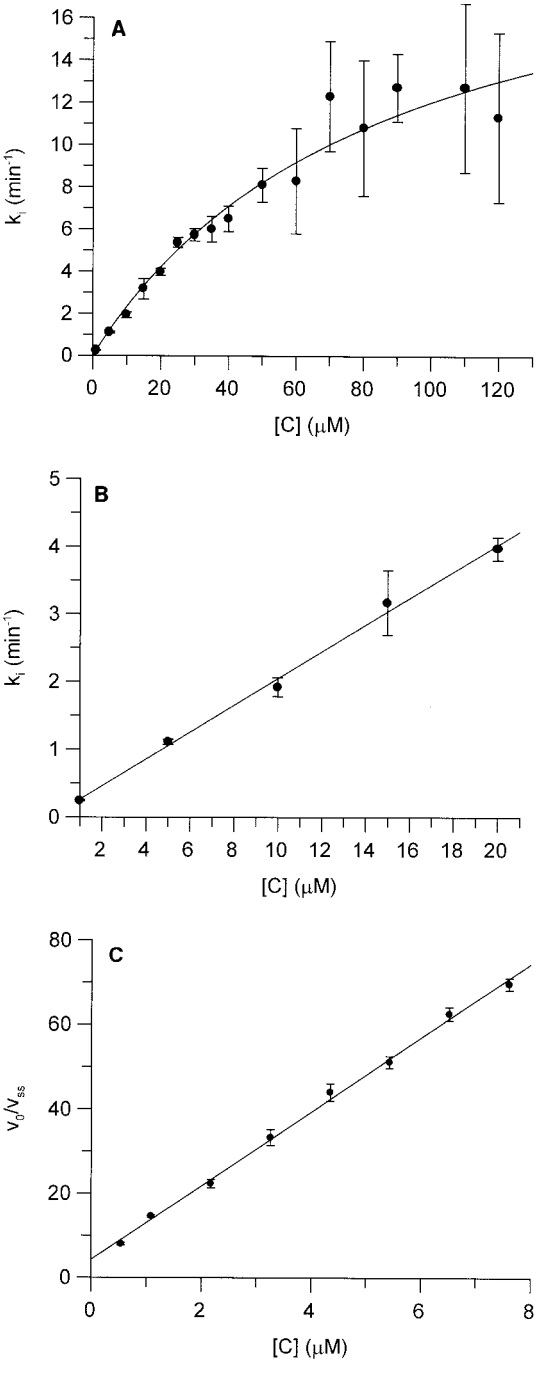

FIG. 4. Interaction between the *E. Cloacae* 908R β-lactamase and Lek 156. (A) Saturation phenomenon at high concentrations of the compound. The line is drawn from the fit of the data to equation 5 by using the following parameters: k_2 equal to 0.37 s^{-1} and K' equal to 16 μM (αK′ is equal to 86 μM). Note that above 50 μM, the k_i values (>0.135 s^{-1}) become very high so that the mixing dead time constitutes more that 50% of the complete time course, which explains the large errors. (B) The same data described for panel A but with low Lek 156 concentrations ([C]≪αK'), from which a k_2/K' value of 17,500 M^{-1} s^{-1} can be calculated. (C) Competitive inhibition between Lek 156 and the reporter substrate (100 μM nitrocefin) after establishment of the steady state, from which a K_m value of 0.02 μM is derived by linear regression of the data according to equation 6.

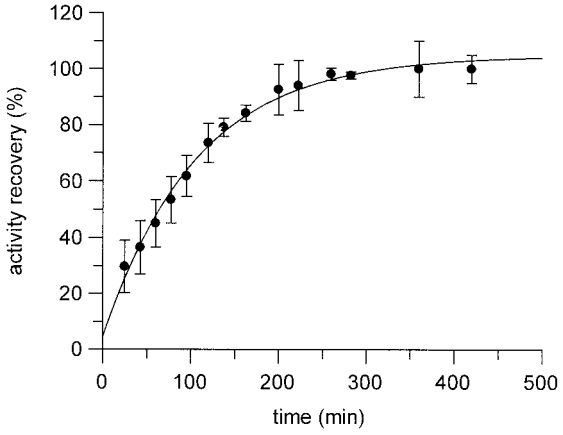

FIG. 5. Recovery of activity of the *E. cloacae* 908R β-lactamase after transient inactivation by Lek 156. The enzyme was incubated with an equimolar concentration of inactivator that resulted in ~95% inactivation at time zero, and the samples were assayed against 100 μM nitrocefin after various periods of time. Fitting of the data to a single exponential function gives a k_r value of 1.5×10^{-4} s^{-1}.

more than 50% of the compounds was degraded after 24 h at 20°C.

DISCUSSION

Both tricyclic carbapenem molecules tested in the present study, Lek 156 and Lek 157, are very efficient inactivators of the *E. cloacae* 908R class C β-lactamase. With this enzyme, the reaction pathway appears to be linear (model 2a′) due to negligible hydrolysis of the first covalent adduct ($k_3 \ll k_4$) and a very low k_5 value (1.5×10^{-4} s^{-1} or less). At saturating concentrations, the enzyme is rapidly inactivated ($k_2/K' \approx 2 \times 10^4$ to 4×10^4 M^{-1} s^{-1}), and the resulting species, EC**, although not completely stable, displays very low turnover values ($t_{1/2} \approx 1$ to 5 h). Preliminary experiments also indicate that both compounds efficiently inactivate various extended-spectrum class C β-lactamases. It is interesting that BRL 42715 (6, 17, 40), which is also a good inactivator of class C enzymes,

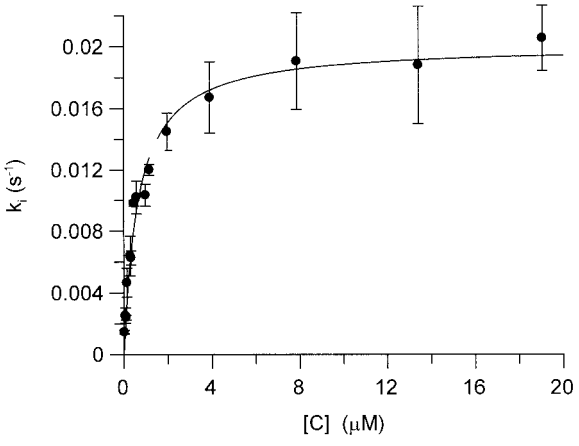

FIG. 6. Interaction between the OXA-10 β-lactamase and Lek 157. Variation of k_i with [C]. Fitting of the data to equation 13 leads to A equal to $(20 \pm 6) \times 10^{-3}$ s^{-1} and B equal to 0.085 ± 0.01 μM (αB equal to 0.66 μM).

TABLE 4. Kinetic parameters of the interaction of *Streptomyces* sp. strain K15 and R61 DD-peptidases with Lek 156 and Lek 157

Compound	*Streptomyces* sp. strain K15 DD-peptidase				*Streptomyces* sp. strain R61 DD-peptidase			
	k_2/K' (M^{-1} s^{-1})	K' (M)	k_2 (s^{-1})	k_3 (s^{-1})	k_2/K' (M^{-1} s^{-1})	K' (M)	k_2 (s^{-1})	k_3 (s^{-1})
Lek 156	0.57 ± 0.01	$>5 \times 10^{-3}$	$>3 \times 10^{-3}$	$\leq 5 \times 10^{-5}$	9 ± 0.6	$>3.5 \times 10^{-4}$	$>4 \times 10^{-3}$	$(3 \pm 0.5) \times 10^{-5}$
Lek 157	6.8 ± 0.2	$>5 \times 10^{-4}$	$>4 \times 10^{-3}$	$(4 \pm 2) \times 10^{-5}$	45 ± 1	$>1.5 \times 10^{-4}$	$>7 \times 10^{-3}$	$(2.2 \pm 0.2) \times 10^{-5}$

shares structural characteristics with the compounds studied in the present work.

In agreement with these observations, Lek 157 significantly reduced the MIC of ampicillin for *E. cloacae* P99, which overproduces a class C β-lactamase. The lesser decrease in the MIC observed with Lek 156 probably results from the intrinsic instability of this compound.

The NmcA β-lactamase (class A) has been chosen for its unusual catalytic properties (34). It is a very broad spectrum enzyme, hydrolyzing efficiently not only classical penams and cephems but also a wide range of β-lactam molecules usually considered resistant to class A β-lactamases. In particular, NmcA hydrolyzes imipenem (and related carbapenem antibiotics) with k_{cat} and K_m values of ~200 s^{-1} and ~0.6 mM ($k_{cat}/K_m = 2 \times 10^5$ M^{-1} s^{-1}), respectively (34, 45; Florence Mahy, Jean-Marie Frère, and Moreno Galleni, unpublished data). In the present case, both carbapenems are substrates of the NmcA enzyme, but the k_{cat} (~2 to 3 s^{-1}) and K_m (~0.5 to 4 μM) values are about 3 orders of magnitude lower than those obtained with imipenem.

In contrast to the NmcA enzyme, both compounds inactivate the TEM-1 β-lactamase ($t_{1/2} \approx$ 15 to 30 min for complete inactivation at high $[C]_0/[E]_0$ values) according to a branched pathway mechanism ($k_3 > k_{+4}$). Most interestingly, the rearranged adducts (EC**) are found to be very stable ($t_{1/2} > 4$ h). A similar mechanism is observed with the class D enzyme (OXA-10). In this case, however, the ratio between the number of productive turnovers and reactions that lead to enzyme inactivation (i.e., the partition ratio k_3/k_4) is quite low, and the enzyme is completely inactivated on a shorter time scale ($t_{1/2} \approx$ 2 to 4 min). Furthermore, the inactivated species (EC**), is not fully stable, and hydrolysis of the rearranged adduct (EC**) is observed ($k_5 \neq 0$; $t_{1/2} \approx$ 10 to 20 min).

Like all other known carbapenems, which are usually only very poorly hydrolyzed by the active-site serine β-lactamases (37), Lek 156 and Lek 157 are readily hydrolyzed by the Zn^{2+}-containing class B enzyme (18). With the two compounds, both the k_{cat}/K_m ($\geq 5 \times 10^6$ M^{-1} s^{-1}) and the k_{cat} (≥ 700 s^{-1}) values are remarkably high, which confirms the high degree of catalytic efficiency of this enzyme (18).

TABLE 5. MIC of ampicillin for *E. cloacae* P99

Compound	MIC (μg/ml)
Lek 156	>284
Lek 157	>284
Tazobactam	>256
Ampicillin	512
Ampicillin + Lek 156 (10 μg/ml)	256
Ampicillin + Lek 156 (30 μg/ml)	128
Ampicillin + Lek 157 (10 μg/ml)	64
Ampicillin + Lek 157 (30 μg/ml)	16
Ampicillin + tazobactam (10 μg/ml)	16

With the representative enzymes considered in the present work, the spectral properties of the final hydrolysis products are identical and correspond to Lek 1A (Fig. 1C), which is also obtained after sodium hydroxide hydrolysis. It is probable that a very slow rearrangement of the primary hydrolysis product, i.e., elimination of the methoxy group of the cyclohexane ring and tautomerization to the δ-2 pyrroline, occurs after its release from the enzyme. These two events are characterized by significant changes in the molecular extinction coefficient value of the molecules at 310 nm, i.e., *ca.* −1,500 M^{-1} cm^{-1} and *ca.* + 1,000 M^{-1} cm^{-1} for the β-lactamase-catalyzed opening of the β-lactam ring and the spontaneous rearrangement of the cleaved β-lactam, respectively. It appears, however, that the rearrangement takes place much faster when the opened β-lactam is trapped as a stable acyl-enzyme species, as is the case with the TEM-1, *E. cloacae* 908R, and OXA-10 enzymes.

The results indicate that both compounds are very poor acylating agents ($k_2/K' = 0.5$ to 50 M^{-1} s^{-1}) for the two model DD-peptidases. With imipenem, a semisynthetic carbapenem antibiotic, the k_2/K' values have been estimated to be 1,000 M^{-1} s^{-1} (for the *Streptomyces* sp. strain R61 enzyme [30]) and 100 to 200 M^{-1} s^{-1} (for the *Streptomyces* sp. strain K15 enzyme [33]). Imipenem is, however, a broad-spectrum antibiotic which very efficiently inactivates some of the essential penicillin-binding proteins of the pathogenic strains and is therefore often used as a last resort for patients in intensive care units. Thus, the intrinsic antibacterial activities of the new Lek carbapenem antibiotics, which have not yet been tested in vivo, cannot be predicted on the basis of the behaviors of the two DD-peptidases tested in the present study.

The present survey of the interaction between different β-lactamases and two novel enantiomeric carbapenem molecules confirms the very different behaviors of the enzymes. Although both compounds are readily hydrolyzed by the so-called carbapenem-hydrolyzing β-lactamases of class A (NmcA) and class B (BcII), they are efficient inactivators of the "classical" active-site serine β-lactamases. This is a quite unusual, specific, and interesting property, since most other mechanism-based inactivators of active-site serine enzymes (e.g., clavulanic acid and sulbactam) generally exhibit rather poor activity against class C enzymes. These enzymes may be associated with resistance profiles that include virtually all β-lactam antibiotics (7, 35), and the recent discovery of many new plasmid-mediated forms of genes encoding class C enzymes has now been recognized as a serious threat. Our kinetic data therefore suggest that further development of the tricyclic carbapenem compounds could be of clinical interest.

ACKNOWLEDGMENTS

The research of M.V., T.S., and B.T. was funded by the Ministry of Science and Technology of Slovenia (grant J1-7374) and Lek, d.d., Pharmaceutical Works. The research of J.-M.F., M.G., and A.M. was supported by the Belgian Government in the frame of the Pôles

d'Attraction Interuniversitaires (grant PAI P4/03). A.M. is a research associate of the National Fund for Scientific Research of Belgium.

We express our gratitude to R. Pain for critical reading of the manuscript and helpful discussions.

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