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Sensitivity of *Aeromonas hydrophila* Carbapenemase to Δ^3-Cephems: Comparative Study with Other Metallo-β-Lactamases

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Ceftriaxone and ceftriaxone *S*-oxide behaved as inactivators against the metallo-β-lactamase of *Aeromonas hydrophila* AE036 and as substrates for the zinc β-lactamase produced by *Bacillus cereus* (569/H/9) and *Stenotrophomonas maltophilia* ULA 511. Moreover, RO 09-1428, a catechol-cephalosporin, was not recognized by the *A. hydrophila* enzyme. Panipenem, cephalosporin C, cephalosporin C-γ-lactone, and loracarbef were substrates for the three studied β-lactamases.

Class B β-lactamases are metalloproteins which require a divalent transition metal ion to exert their catalytic activity, most often a zinc ion (1). These enzymes rapidly hydrolyze carbapenem antibiotics. Zinc β-lactamases exhibit a broad activity spectrum against β-lactam compounds except for monobactams (6). The production of metallo-β-lactamases by pathogenic strains such as *Aeromonas hydrophila*, *Bacteroides fragilis*, *Stenotrophomonas maltophilia*, *Serratia marcescens*, and *Pseudomonas aeruginosa* is likely to result in clinical problems (9). A detailed understanding of the mechanism of action of these enzymes (3, 4) is important for the design of drugs which would remain effective against them.

In this paper, we investigated the interaction between metallo-β-lactamases and cephalosporins and carbapenems in order to find potentially poor substrates of metallo-β-lactamases. Special attention was devoted to the *A. hydrophila* AE036 carbapenemase, which is an enzyme poorly active against the other β-lactam compound families and which is inactivated by cephamycin and moxalactam (7). For comparison purposes, the metallo-β-lactamases produced by *S. maltophilia* ULA 511 and *Bacillus cereus* 569/H/9 were also included in this study.

Purified β-lactamases were prepared for kinetic assays as described in reference 6. Antibiotics used in the assays were as follows: ceftriaxone ($\delta\varepsilon_M^{260} = -9{,}400\ M^{-1}\ cm^{-1}$), ceftriaxone *S*-oxide ($\delta\varepsilon_M^{260} = -5{,}000\ M^{-1}\ cm^{-1}$), the cephalosporin RO 09-1428 ($\delta\varepsilon_M^{255} = -18{,}550\ M^{-1}\ cm^{-1}$; kindly given by R. L. Then, Hoffmann-La Roche, Basel, Switzerland), loracarbef ($\delta\varepsilon_M^{260} = -11{,}300\ M^{-1}\ cm^{-1}$; Eli Lilly, Indianapolis, Ind.), imipenem ($\delta\varepsilon_M^{300} = -9{,}000\ M^{-1}\ cm^{-1}$; Merck Sharp & Dohme Research Laboratories, Rahway, N.J.), panipenem ($\delta\varepsilon_M^{297} = -7{,}400\ M^{-1}\ cm^{-1}$; Sankyo Co. Ltd. Biological Research Laboratories, Tokyo, Japan), cephalosporin C ($\delta\varepsilon_M^{260} = -5{,}200\ M^{-1}\ cm^{-1}$; Sigma Chemical Co., St. Louis, Mo.), nitrocefin ($\delta\varepsilon_M^{482} = -15{,}000\ M^{-1}\ cm^{-1}$; Unipath, Milan, Italy). Cephalosporin C-γ-lactone ($\delta\varepsilon_M^{260} = -4{,}600\ M^{-1}\ cm^{-1}$) was synthesized as previously described (10). The structures of the β-lactam compounds are shown in Fig. 1.

The hydrolysis of the antibiotics was monitored by observing the absorbance variation resulting from the opening of the β-lactam ring with a Lambda 2 spectrophotometer (Perkin-Elmer, Rome, Italy) connected to a PC-compatible Epson PSE30 microcomputer via an RS232C serial interface. All the reactions were performed in 30 mM cacodylate buffer (pH 6.5)–50 μM $ZnCl_2$ at 30°C. The reaction volume was equal to 0.6 ml. K_m and catalytic constant (k_{cat}) values were determined by analyzing the complete hydrolysis time courses as described by De Meester et al. (5). For high values of K_m, initial hydrolysis rates were monitored. The computer program ENZFITTER (Elsevier Biosoft, Cambridge, United Kingdom) was used to calculate kinetic parameters by the Hanes-Woolf plot. In some cases, only the ratio k_{cat}/K_m was measured. When the K_m value was too small ($K_m < 10$ μM), it was measured as K_i in competition experiments by using 200 μM nitrocefin as the reporter substrate. In the case of *A. hydrophila* AE036 enzyme, 200 μM imipenem was used. The initial rate of hydrolysis of the reporter substrate was measured in the presence of different concentrations of the studied compounds. In that case, the k_{cat} value was obtained by monitoring the hydrolysis of the antibiotic at high concentration.

Values for the different kinetic parameters (k_{cat}, K_m, and k_{cat}/K_m) are reported in Table 1. We noted that the Zn-dependent β-lactamases produced by *B. cereus* 569/H/9 and *S. maltophilia* hydrolyzed all the tested compounds. Their catalytic activities against cephalosporin C-γ-lactone were decreased by a factor of approximately 40 compared to their activities against cephalosporin C. The presence of a free carboxylic group in position C-4 seems to have a impact on the k_m and k_{cat} values. The opposite phenomenon was observed for the *A. hydrophila* β-lactamase. Imipenem and panipenem were good substrates, while loracarbef behaved as a poor substrate for the *A. hydrophila* β-lactamase. The catechol-cephalosporin RO 09-1428 did not affect *A. hydrophila* metalloenzyme activity, as demonstrated by the hydrolysis rate for 100 μM imipenem, which was found to remain constant when measured in the presence of 1 mM cephalosporin. Ceftriaxone and ceftriaxone *S*-oxide behaved as inactivators against the *A. hydrophila* β-lactamase. The determination of the inactivation parameters was carried out by analyzing the hydrolysis of 200 μM imipenem, used as a reporter substrate, in the presence of increasing inactivator concentration (25 to 300 μM for both compounds).

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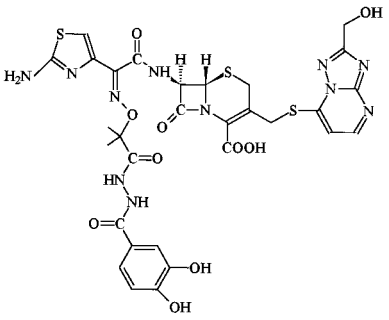
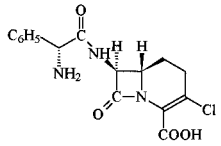
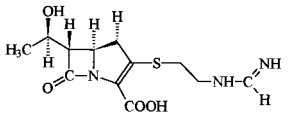


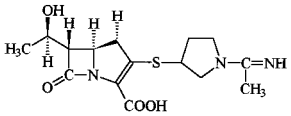
Ro 09-1428



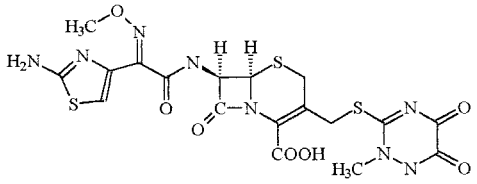
Loracarbef



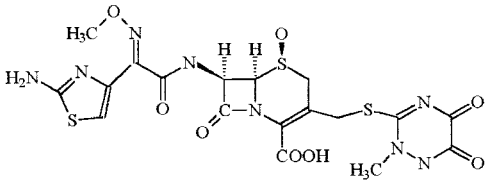
Imipenem



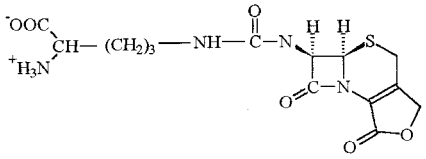
Panipenem



Ceftriaxone



Ceftriaxone (S) - oxide



Cephalosporin C$_\gamma$-lactone

FIG. 1. Structures of the β-lactam compounds used in the study.

Inactivation was complete and the inactivation constants were calculated on the basis of the following scheme:

$$E + C \underset{}{\overset{K}{\rightleftarrows}} EC \xrightarrow{k_2} EC^*$$

where E, C, EC, and EC* are the enzyme, the inactivator, the Henri-Michaelis-Menten complex, and the stable inactive complex, respectively; K is the dissociation constant of the noncovalent complex EC; and k_2 is the acylation rate constant.

The inactivation rate constant k_i was given by the following equation:

$$k_i = \frac{k_2[C]}{K\dfrac{(K_m + [S])}{K_m} + [C]} \quad (1)$$

TABLE 1. Kinetic parameter values for the three metallo-β-lactamases

Antibiotic	Kinetic parameter value[a] for the metallo-β-lactamase of:								
	A. hydrophila AE036			*B. cereus* 569/H			*S. maltophilia* ULA-511		
	K_m (μM)[b]	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	K_m (μM)[b]	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	K_m (μM)[b]	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
Cephalosporin C	125 ± 5	0.004	32	12 ± 0.7	185	1.54×10^7	25 ± 1	62	2.50×10^6
Ceftriaxone	NH[c]	NH	NH	12 ± 0.7	104	8.67×10^6	0.8 ± 0.01	8	1.00×10^7
Ceftriaxone *S*-oxide	NH	NH	NH	10 ± 0.7	30	3.00×10^6	0.7 ± 0.01[d]	1.8	2.57×10^6
Cephalosporin C-γ-lactone	90 ± 2	0.034	3.80×10^2	230 ± 3	19	8.26×10^4	>1,500	>90	6.15×10^4[e]
Loracarbef	45 ± 3	0.006	1.33×10^2	280 ± 8	32	1.14×10^5	1.4 ± 0.002[d]	4.3	3.07×10^6
RO 09-1428	NI[f]	NI	NI	65 ± 3	19	2.92×10^5	11 ± 0.02	7	6.36×10^5
Imipenem	90 ± 3	171	1.90×10^6	>1,000	>100	1.20×10^5	57 ± 2	1,100	1.93×10^7
Panipenem	290 ± 10	445	1.53×10^6	300 ± 3	255	8.50×10^5	35 ± 2	89	2.54×10^6

[a] For all data, the standard deviation values were less than 10% of the means of five independent measurements.
[b] Values are means ± standard deviations.
[c] NH, no hydrolysis.
[d] K_m was determined as K_i by the competition method. For more details, see the text.
[e] Only the k_{cat}/K_m ratio value was determined (v_0 remained proportional to S_0 up to the highest utilized substrate concentration).
[f] NI, no interaction.

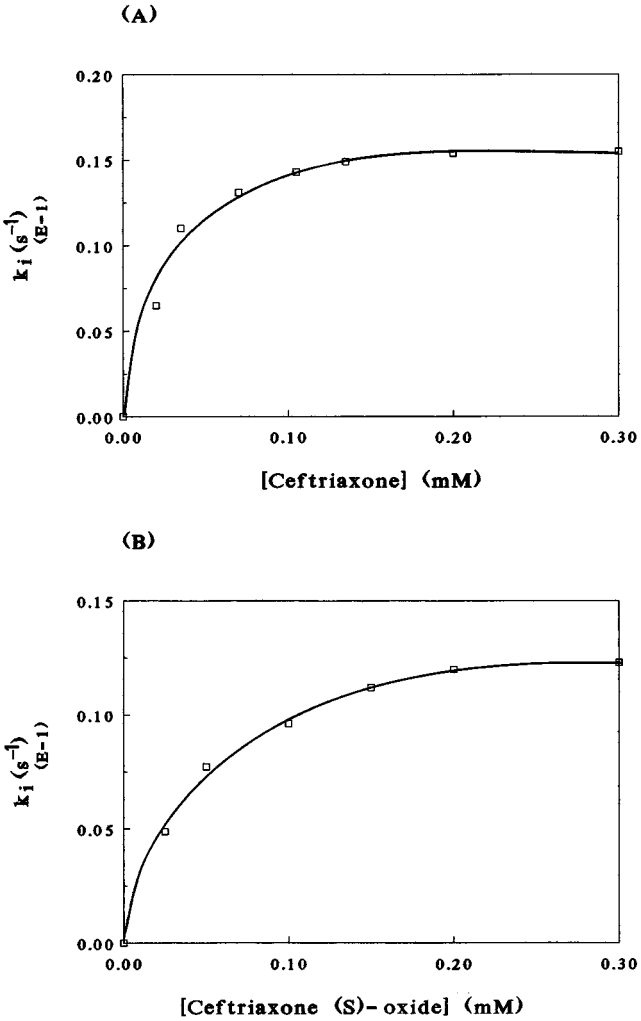


FIG. 2. Inactivation of the *A. hydrophila* enzyme. The pseudo-first-order inactivation rate constant (k_i) is plotted as a function of ceftriaxone (A) and ceftriaxone *S*-oxide (B) concentrations. Each point is the mean ± standard deviation for five measurements.

where [*S*] is the concentration of the reporter substrate and k_2, C, and *K* are as defined above.

In both cases (i.e., with ceftriaxone and ceftriaxone *S*-oxide as inhibitors), the dependence of the k_i values on the inactivator concentration was not linear (Fig. 2A and B). In these conditions, the linearization of equation 1 allowed us to calculate the *K* and k_2 parameters with the help of the ENZFITTER program. The values obtained for ceftriaxone were as follows: $K = 25 \pm 1$ μM, $k_2 = 0.0174\ s^{-1}$, and $k_2/K = 7 \times 10^2\ M^{-1}\ s^{-1}$. For the interaction between ceftriaxone *S*-oxide and the *Aeromonas* β-lactamase, the kinetic parameters were $K = 45 \pm 2$ μM, $k_2 = 0.0143\ s^{-1}$, and $k_2/K = 3.2 \times 10^2\ M^{-1}\ s^{-1}$. The acylation efficiencies of the metallo-β-lactam compounds are greater than those previously observed for β-lactam compounds cefoxitin and moxalactam (7). Tazobactam represents the only other β-lactam antibiotic which is able to inactivate a metallo-β-lactamase, e.g., the CcrA enzyme produced by *B. fragilis* (2). Ceftriaxone and ceftriaxone *S*-oxide also exhibited a higher level of activity than cilastatin, an inhibitor of renal dehydropeptidase I (8). Surprisingly, cefotaxime (an oximinocephem) is a rather good substrate for the *A. hydrophila* β-lactamase (6). The different natures of the leaving groups in C-3 between cefotaxime and the ceftriaxone derivatives might explain the differences in their behavior against the enzyme. From the data presented here, it appears that the use of efficient Δ^3-cephem inhibitors for the metallo-β-lactamase of *A. hydrophila* AE036 could represent an interesting strategy to fight this particular pathogen.

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