Inhibition of β -Lactamase II of *Bacillus cereus* by Penamaldic Derivatives of Penicillins

Pilar Gutiérrez Navarro,* Bartolomé Quintero Osso, Raquel García Ortiz,
Pedro J. Martínez de las Parras, María I. Martínez Puente, and
M. Carmen Cabeza González

Department of Physical Chemistry, School of Pharmacy, University of Granada, E-18071 Granada, Spain

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The penamaldic derivatives of amoxicillin, ampicillin, and penicillins G and V, stabilized with Zn^{2+} , were obtained from a methanolic medium. The enzymatic kinetic results show that these derivatives elicit reversible inhibition of the enzyme metallo- β -lactamase from *Bacillus cereus*, with inhibition constant values determined at pH 7.0 and 25°C.

Bacteria have developed various strategies to deactivate β -lactam antibiotics, including the production of β -lactamase enzymes. These have been grouped in four classes (6, 10). Classes A, C, and D contain a catalytic serine residue in their active sites, and several have inhibitors utilized in therapeutics (4, 15, 16). Class B metalloenzymes require one or two zinc ions to carry out the hydrolysis of the β -lactams (3) and act on a wide range of β -lactam antibiotics, including carbapenems and inhibitors of the serine-dependent enzymes (12). Over the past 10 years, several inhibitors of these enzymes have been discovered, for instance, two esters of benzyloxycarbonyl-methyl-6-aminopenicillanic acid (17), a group of α -amido-trifluoromethyl alcohols and ketones (18, 19), a series of thiol ester derivatives of mercaptoacetic and mercaptophenylacetic acids (7, 8, 13, 14), and derivatives of β -methylcarbapenem (11). More recently, derivatives of cysteinyl peptides have also been tested (1).

Because thiol derivatives inhibit the metallo- β -lactamases (1, 2), the present study tests the enzymatic inhibition of the metallo- β -lactamase of *Bacillus cereus* by the Zn^{2+} -stabilized penamaldic derivatives obtained from ampicillin, amoxicillin, and penicillins G and V. The type of inhibition is determined, and its parameters are calculated.

The penamaldic derivatives (Fig. 1) stabilized with Zn^{2+} as 2:1 (ligand-metal ion) complexes were obtained by the procedure described in a previous paper (9). The results of the chemical analysis of the polycrystalline solids obtained and the infrared and ^1H -nuclear magnetic resonance data were compatible with the formation of such complexes. According to the thermogravimetric analysis data, the structures of all solids feature two water molecules of crystallization, and the derivatives of ampicillin and amoxicillin have a molecule of NaCl.

The spectra of the penamaldic derivatives in the 50 mM MOPS (morpholinepropanesulfonic acid) medium, at pH 7 and 25°C, scarcely varied over time. Generally, the degradation of the four penamaldic derivatives produced an insignificant change in the rate of absorbance over time compared with that

for the degradation of substrate in the enzymatic reaction. This may be attributed to the fact that Zn^{2+} -stabilized penamaldic derivative compounds have a high constant of formation in an aqueous medium. Moreover, the slight instability of the Zn^{2+} -stabilized compounds in aqueous solution, presumably because of the enamine moiety, can tautomerize to imines, which undergo ester hydrolysis (5). In the presence of enzymes the spectra of the penamaldic derivatives showed the same changes over time as those observed for the derivatives separately.

For the kinetic study, cephaloridine was used as a substrate in concentrations that ranged from 2×10^{-5} to 2×10^{-4} M, while inhibitor concentrations were from 2×10^{-4} to 10×10^{-4} M. The reaction medium was a 50 mM MOPS buffer solution at pH 7.0 and 25°C. The total concentration of Zn in each assay mixture was that corresponding to the penamaldic derivative plus that, not specified in quantitative terms, for the commercial enzyme, which is balanced primarily with Zn salts. The enzyme solution was prepared by dissolving the contents of a commercial vial of β -lactamase II in 1 ml of buffer. The volume of the enzyme solution varied from 7 to 25 μl . After hydrolysis of the substrate, the decrease in absorbance at 255 nm was measured.

The incubation time of the inhibitor-enzyme mixture did not significantly modify the inhibition process, as demonstrated in kinetic experiments in which the enzyme and the inhibitor were incubated for different times (0, 5, and 10 min). At the end of the reaction, when the same concentration of substrate as that initially used was again added to the reaction medium, degradation of the substrate occurred at a similar rate. This finding points to the reversibility of the enzyme-inhibitor interaction. Furthermore, the value of apparent V_{max} is unaltered by the addition of the penamaldic derivatives—unlike the apparent K_m value—indicating that these act as competitive inhibitors of the metallo- β -lactamase from *B. cereus*.

Since the β -lactamase II of the commercial *B. cereus* used was stabilized with bovine serum albumin, we chose to determine whether the enzymatic activity was affected by the albumin's presence. A known inhibitor of this β -lactamase, captopril, with an inhibition constant (K_i) of $41.6 \pm 9 \mu\text{M}$ (1), was used. Under conditions described previously and using the commercial enzyme, we found the same inhibition constant (33

* Corresponding author. Mailing address: Department of Physical Chemistry, School of Pharmacy, University of Granada, E-18071 Granada, Spain. Phone: 34-58-243827. Fax: 34-58-244090. E-mail: mpng@platon.ugr.es.

R	Compound
	Penamaldic derivative of amoxicillin
	Penamaldic derivative of ampicillin
	Penamaldic derivative of penicillin G
	Penamaldic derivative of penicillin V

FIG. 1. Scheme of the penamaldic derivatives.

μM), indicating that the presence of albumin does not protect the enzyme from the inhibitory activity of captopril.

Based on the absorbance-time kinetic curves, the values of the initial rate of absorbance change over time, V_{ap} , were calculated for several substrate concentrations in the absence of the inhibitor and at different concentrations of the inhibitor and then plotted according to the equation

$$\frac{1}{V_{\text{ap}}} = \frac{1}{\Delta\epsilon V_{\text{max}}} + \frac{K_m \left(1 + \frac{[I]}{K_I}\right)}{\Delta\epsilon V_{\text{max}}} \frac{1}{[S]_0} \quad (1)$$

where $\Delta\epsilon$ is the change in the absorption coefficient during the enzymatic reaction, $[I]$ is the concentration of the inhibitor, and $[S]_0$ is the initial substrate concentration.

Equation 1 gives a straight line for each inhibitor concentration. When the slope of each line is plotted against the inhibitor concentration, the abscissa value where the line converges on the x axis gives the K_I . Values of 425 ± 11 , 615 ± 10 , 373 ± 12 , and $522 \pm 9 \mu\text{M}$ were obtained for the derivatives of amoxicillin, ampicillin, and penicillins G and V, respectively.

Of the four derivatives assayed, the best inhibitor was that of penicillin G.

The inhibition constants for the derivatives of ampicillin and amoxicillin were also calculated with absorbance data from the complete kinetic curves corresponding to the degradation of the substrate, when $[S]_0 \ll K_m$ and the enzymatic reaction takes place according to a first-order process with respect to the substrate, represented by the equation

$$\ln[A_t - A_\infty] = \ln[A_0 - A_\infty] - \frac{V_{\text{max}}}{K_m \left(1 + \frac{[I]}{K_I}\right)} t \quad (2)$$

The inverse values of the slopes of each straight line plotted against the inhibitor concentration (Fig. 2) give K_I . The values found were $406 \pm 8 \mu\text{M}$ for the amoxicillin derivative and $624 \pm 15 \mu\text{M}$ for the ampicillin derivative.

This method could not be used in for the penamaldates of penicillins G and V since the total change in absorbance was not that expected for the degradation of the substrate in the enzymatic reaction.

The penamaldic derivatives do not exert their inhibitory activity by chelating the Zn^{2+} bound to the enzyme, as the activity of the enzyme is not reestablished by the supplementary addition of external Zn ions in the form of zinc nitrate ($50 \mu\text{M}$). In view of this result, it is difficult to establish a mechanism for the interaction between the inhibitor and the β -lactamase. However, since the presence of a thiol group is considered indispensable (1) for a compound to bind with an enzyme and inhibit the metallo- β -lactamase, one possibility is that equilibrium is established between the penamaldic derivative bonded to the metal ion and that bound to the enzyme through the thiol group. This means that the enzyme and Zn compete to bind the inhibitor. Bearing in mind the large size of the molecule of the 2:1 chelate of the penamaldic derivative, this mechanism would be the most probable one.

FIG. 2. Plot of the data according to equation 2 for the calculation of K_I following the entire course of the reaction. Eight microliters of enzyme solution (equivalent to 0.176 mg of the commercial sample) was used. The substrate concentration was $5 \times 10^{-4} \text{ M}$, and the total volume was 0.8 ml.

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