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SYMPOSIUM MECHANISMS OF RESISTANCE TO BETA-LACTAM ANTIBIOTICS

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PERMEABILITY OF THE OUTER MEMBRANE OF GRAM-NEGATIVE BACTERIA
FOR β -LACTAM ANTIBIOTICSHiroshi Nikaido

Gram-negative bacteria such as Escherichia coli or Pseudomonas aeruginosa are surrounded by an extra membrane layer that is not present in the cells of Gram-positive bacteria. This membrane, called outer membrane, constitutes a significant diffusion barrier for various kinds of solutes including antibiotics, and contributes very significantly to the frequently observed, higher resistance of Gram-negative organisms toward antibiotics and chemotherapeutic agents. In organisms such as E. coli, the lipid bilayer continuum of the outer membrane appears to exhibit an unusually low permeability toward hydrophobic or lipophilic solutes (1). This property has been ascribed to the extremely asymmetric distribution of component lipids in this bilayer, with lipopolysaccharides present only in the outer leaflet and glycerophospholipid molecules present essentially only in the inner leaflet of the bilayer (2). Irrespective of the validity of this explanation, this makes the basic continuum of the outer membrane very poorly permeable to both hydrophobic and hydrophilic molecules, because, like any other lipid bilayer structure, the outer membrane bilayer shows low permeability toward hydrophilic molecules in general.

The outer membrane, however, must still allow the passage of molecules of nutrients needed by the cell. Some years ago, we discovered that this function is performed by a special class of proteins, which we named "porins" (3). Porins so far examined usually have molecular weights in the range of 35,000 to 45,000, contain large amount of β -sheet structure, and have a characteristic property to form water-filled, transmembrane, diffusion channels both in intact cells and in reconstituted, liposome systems. By using mutants producing only traces of the porin protein, we have been able to show that β -lactam antibiotics, and presumably most of other antibiotics as well, enter E. coli cells through these porin channels (4). Because of the crucial role porins play in the influx of antibiotics, it becomes very important to understand and characterize the properties of the porin channel. To date we have performed work along these lines with porins of E. coli, and to a limited extent with porins from P. aeruginosa, Neisseria gonorrhoeae, various Brucella species, and some other nonpathogenic Gram-negative organisms.

Two approaches were used in determining the permeability of solutes through the porin channel. In one method, an in vitro reconstitution is used to produce phospholipid vesicles containing porin molecules within the phospholipid bilayer. Such vesicles are

INTRINSIC RESISTANCE TO β -LACTAM ANTIBIOTICS AT THE LEVEL OF THE
ENZYME ACTIVE SITES. MANY CHALLENGES, SOME ACHIEVEMENTS.

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Exposure of bacteria to penicillin and other β -lactams leads to the formation of stable adducts between the antibiotic molecules and several membrane-bound proteins. These proteins are known as penicillin binding proteins or PBPs.

Those PBPs which have been shown to possess enzymatic activity are peptidases exhibiting unique optical specificity. They catalyse attack of peptide bonds located in α position to a free carboxyl group and extending between two carbon atoms having the D configuration. Depending on whether the acceptor is a water molecule or an amino compound, the catalysed transfer reaction is hydrolysis or transpeptidation of the carbonyl donor peptide. Some DD-peptidases act solely as hydrolases (strict DD-carboxypeptidases), others catalyse concomitantly and on a competitive basis, both carboxypeptidation and transpeptidation reactions (DD-carboxypeptidases/transpeptidases), and others catalyse exclusively peptide bond synthesis by transpeptidation (strict DD-transpeptidases).

Mechanistically, one strict DD-carboxypeptidase is known which performs liganding catalysis via a Zn^{++} ion cofactor, in a way similar to the metallo (Zn^{++}) peptidases, carboxypeptidase A and thermolysin. Several DD-carboxypeptidases/transpeptidases are also known which perform covalent catalysis via an active serine residue. Depending on the available nucleophiles and as observed with the mammalian serine peptidases, α -chymotrypsin and trypsin, the enzyme activity can be channelled through hydrolysis and aminolysis (transpeptidation) by partitioning at the level of the acyl enzyme intermediate. The strict DD-transpeptidases probably also operate via formation of an acyl enzyme but the nature of the postulated intermediate and the reason why catalysis is effective only in the presence of a suitable amino compound, remain to be elucidated. The strict DD-peptidases appear not to have any counterpart in the eukaryotic world.

The DD-peptidases are involved in wall peptidoglycan metabolism. The attachment of the nascent peptidoglycan to the preexisting wall peptidoglycan requires the concerted action of both transglycosylase activity (catalysing extension of the glycan strands) and DD-transpeptidase activity (catalysing crosslinking and insolubilization of the expanding network). Transpeptidation and transglycosylation seem to be catalysed by distinct polypeptide entities in some bacteria, but in *E. coli*, there is an assortment of membrane-bound proteins which, to all appearances, are bifunctional DD-transpeptidases-transglycosylases. In this organism, these "bi-headed" enzymes fulfil specialized functions related to wall elongation, cell septation and perhaps, shape maintenance, respectively. Following the incorporation of newly

formed peptidoglycan strands, further reactions occur which lead to an increased extent of peptide crosslinking and permit constant remodelling of the wall peptidoglycan throughout the bacterial life cycle. These reactions are under the control of various DD-carboxypeptidases and DD-carboxypeptidases/transpeptidases (as well as glycosidases) which, somehow, work in a well coordinated manner. The last stages of wall peptidoglycan biosynthesis thus rely on a complex interplay between a set of DD-peptidases. Such an enzyme assortment is species specific; great variations are observed when taxonomically unrelated bacteria are compared to each other.

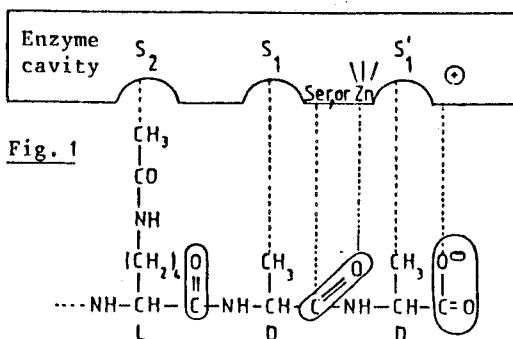
That the DD-carboxypeptidases/transpeptidases are PBPs is well understood at the molecular level. Indeed, both β -lactams and D-Ala-D-Ala terminated peptides suitably complement the surface of the serine DD-peptidases. With the peptides, the reaction flux is to reaction products but with the β -lactams, the serine-linked acyl enzyme intermediates are highly stable and the reaction flux stops at this level. By analogy, penicillin binding to the PBPs of unknown enzymatic activity is thought to proceed through a similar suicide process, thus implying that all the PBPs function in vivo as DD-peptidases. That some of them lack activity in vitro would be due to the inadequacy of the carbonyl donor and acceptor cosubstrates used so far. In the case of the bifunctional DD-transpeptidases-transglycosylases, penicillin binding concerns only the peptidase active site, while the transglycosylase site remains functional. Depending on the β -lactam and the PBP or DD-peptidase considered, the efficacy of the binding or inactivating process varies widely.

Penicillin action on sensitive bacteria somehow unleashes the cell autolytic system and causes bacterial death by cellular lysis. However, selective inactivation (or selective alteration or deletion by genetic manipulations) of some PBPs (DD-peptidases) may not have any apparent harmful effect on bacterial growth (at least under laboratory conditions) or may induce reversible (at least during a limited period of time) morphological abnormalities. These observations have led to the concept of PBPs (DD-peptidases) of varying dispensability. Non-dispensable PBPs (DD-peptidases) are important (lethal) targets for β -lactam action. Recently, clinical isolates have led to the characterization of bacterial strains exhibiting highly increased intrinsic resistance to β -lactams. Resistance could be related to modifications at the level of the PBPs (DD-peptidases) pattern (T. Dougherty; this symposium).

In order to understand the mechanism of intrinsic resistance at the level of the enzyme active sites, the spatial structure and mode of catalysis of the DD-peptidases (PBPs) must be known at the atomic resolution. Such knowledge can be obtained only by crystallography and X-ray diffraction studies. Since membrane-bound proteins are inherently difficult to crystallize, the strategy has been to make use of the fact that some Streptomyces and Actinomadura strains secrete water-soluble DD-peptidases during growth. The G (Mr : 22,000), R61 (Mr : 38,000) and R39 (Mr : 53,000) DD-peptidases, isolated from culture filtrates, catalyse transfer of the electrophilic group Ac_2-L-

Lys-D-alanyl of the aminoacyl amide $\text{Ac}_2\text{-L-Lys-D-Ala-D-Ala}$ (peptidase activity) or the aminoacyl ester $\text{Ac}_2\text{-L-Lys-D-Ala-D-lactic acid}$ (esterase activity) to a nucleophile. The G and R61 enzymes have been crystallized. The G DD-peptidase is a metallo (Zn^{++}) enzyme and both R61 and R39 DD-peptidases are serine enzymes.

Initial recognition of the carbonyl donor substrate, binding energy and proper alignment of the scissile bond with regard to the catalytically active groups of the DD-peptidases rely on the complementation of at least three enzyme binding subsites (Fig. 1). Subsites



S_1' and S_1 accommodate the two methyl groups in the D configuration that occur on both sides of the scissile bond. Subsite S_1' also contains a cationic side chain (most likely an Arg residue at least in the case of the G and R61 enzymes) involved in charge pairing with the C-terminal carboxylate of the substrate. Subsite S_2 accommodates a long side chain that protrudes from the L

centre located on the amino side of the C-terminal D-Ala-D-Ala. Complementation of S_2 may not be essential for initial binding but is crucial for subsequent catalysis. Subsite S_2 is highly enzyme specific (Table 1).

Carbonyl donor peptide	Catalytic efficiency (turnover number/ K_m : $\text{M}^{-1}\text{s}^{-1}$) for hydrolysis of peptide substrates by the DD-peptidases:		
	G	R61	R39
$\text{N}^{\alpha, \text{E}}\text{-Ac}_2\text{-L-Lys-D-Ala-D-Ala}$	7600	4600	21900
$\text{N}^{\alpha}\text{-Ac-L-Lys-D-Ala-D-Ala}$	80	17	160000

Following accommodation of a carbonyl donor substrate in a peptidase active site, cleavage of the scissile peptide (ester) bond requires the concerted action of several functional groups of the enzyme cavity. An electrophile or anion hole polarizes the bond $\text{C}=\text{O}$, a nucleophile performs attack of the carbon atom and a proton donor achieves proton donation to the nitrogen (oxygen) atom. Most likely, the DD-peptidases obey the same rule.

The active site of the Zn^{++} G DD-peptidase (1,2,4,5)

The amino acid sequence (212 residues) and three-dimensional structure at 1.8 Å resolution of the G DD-peptidase are known. The G DD-peptidase, thermolysin and carboxypeptidase A show complete lack of structural relatedness. The DD-peptidase has two domains connected by a rather unusual single link. The large C-domain is of the α/β type structure and contains the active site one side of which is a

mixed sheet of the five β -strands.

The coordination around Zn^{++} , within the enzyme active site, involves three histidine residues (His 153, His 194 and His 196) and most likely, a water molecule as the fourth ligand. (In thermolysin and carboxypeptidase A, the protein ligands are two His and one Glu.) Near Zn^{++} , there are two residues which, to all appearances, are catalytically important: His 191 is thought to facilitate proton donation (the same role is played by a His in thermolysin and a Tyr in carboxypeptidase A) and Ser 151 is thought to orient an hydroxyl ion (in alcohol dehydrogenase, a similar role is played by the dyad His 31... Ser 48 while in thermolysin and carboxypeptidase A, a Glu acts as proton abstractor heightening the nucleophilicity of the water molecule).

The hand and geometry of the zinc environment is unvariant in thermolysin, carboxypeptidase A, carbonic anhydrase, alcohol dehydrogenase and the G DD-peptidase, with regard to the three protein ligands, the water position, the nucleophile (proton abstractor or OH^- orientor) and the carbonyl donor binding site. This similarity in geometry suggests similarity in catalysis. Upon binding to the active site of the G DD-peptidase, the carbonyl donor becomes the fifth ligand of Zn^{++} in a transient pentagonal complex in which Zn^{++} itself acts as an anion hole and where the carbonyl carbon is pyramidalized in the form of a tetrahedral adduct. Collapse of this intermediate by proton donation and re-entry of a water molecule permits release of the reaction products. At any time during the process, no covalent intermediate is formed with the carbonyl donor substrate so that partitioning of the enzyme activity between alternate nucleophiles (H_2O and an amino compound) cannot occur. The G DD-peptidase as well as thermolysin and carboxypeptidase A do not catalyze transpeptidation reactions; they are strict hydrolases. Note however that the G DD-peptidase differs from these two metallo-peptidases with respect to the hand of the reactive intermediates. In thermolysin and carboxypeptidase A, the nucleophile points to the carbonyl carbon from below the plane formed by the triad CON while the proton donor points to the nitrogen atom from above that plane. A reverse disposition of these two catalytic groups occurs in the G DD-peptidase.

Models show that accommodation of the carbonyl donor substrate Ac₂-L-Lys-D-Ala-D-Ala in the active site of the G DD-peptidase leads to a close interaction between the C-terminal carboxylate and the guanidinium side chain of Arg 137 involved in charge pairing and, simultaneously, to a positioning of the scissile CON bond such that the oxygen atom is oriented toward the zinc atom while the nitrogen atom is oriented toward the imidazole ring of His 191. When the tripeptide is thus aligned, then the methyl group of the C-terminal D-Ala points to a large subsite S₁. This subsite can accommodate side chains much larger than a methyl group (a property which is compatible with the peptidoglycan hydrolase activity of the G DD-peptidase) but changing the D configuration of the methyl group to an L configuration, suppresses all possible interaction with the enzyme

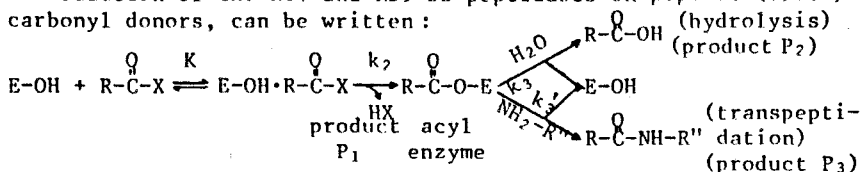
cavity. In turn, the methyl group of the penultimate D-alanine finds its place in a subsite S_1 of small size. Large side chains cannot be accommodated and a methyl group with an L configuration is excluded since it would collide with the zinc ligands and other residues of the active site. This property well explains the strict requirement shown by this DD-peptidase for the occurrence of a D-alanine at this position. Finally, the side chain at the L centre of the carbonyl donor peptide complements subsite S_2 . Subsite S_2 is a largely open cavity and Arg 203, located on the edge of the protein, offers a possible site of interaction.

The G DD-peptidase shows very high intrinsic resistance to β -lactam action; it behaves as a PBP only under drastic conditions (high antibiotic concentrations and prolonged incubation times). Cephaloglycine and β -iodo-benzyl-7-amino-cephalosporinate act essentially as competitive inhibitors. Study at 4.5 Å resolution has provided direct evidence that this latter compound binds to the enzyme active site. With classical penicillins and cephalosporins, models show that it is not possible to align simultaneously both the β -lactam amide bond with the zinc ion and the C-terminal carboxylate with Arg 137 because the fused ring system would collide with His 191. With 6- β -iodopenicillanate (which has a very short side chain), irreversible inactivation of the G DD-peptidase occurs slowly (the first order rate constant has a limit value of $7 \times 10^{-4} \text{ s}^{-1}$). The difference Fourier synthesis at 2.8 Å of the crystal enzyme derivative shows that the inactivator molecule is located just in front of Zn^{++} and superimposes His 191, suggesting that in this case His 191 (which acts as a proton donor during catalysis of a bound peptide substrate) acts as a nucleophile by its lone pair of the nitrogen and undergoes alkylation by 6- β -iodopenicillanate with loss of the iodine.

It thus follows that the high intrinsic resistance of the G DD-peptidase is caused by a particular geometry of the enzyme active site. Productive enzyme-ligand association occurs only with carbonyl donor peptides. The β -lactams are little predisposed to align correctly with regard to the active functional groups. Consequently, the enzyme functions as a β -lactamase of very poor efficiency (in contrast to the Zn^{++} - β -lactamase II of B. cereus) and has little opportunity to commit suicide.

The active site of the serine R61 and R39 DD-carboxypeptidases/transpeptidases (1,3,4,6,7)

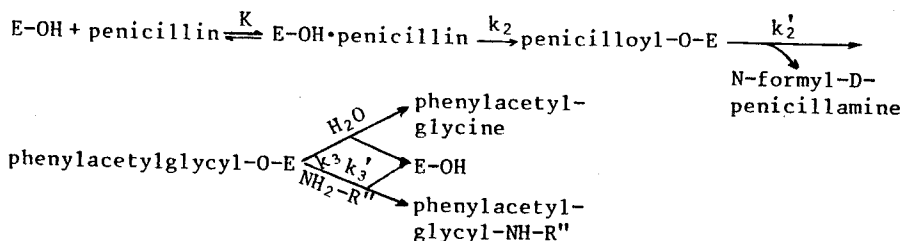
A precise picture of how these enzymes perform catalysis cannot yet be proposed. But, by analogy with the mammalian serine peptidases and giving E-OH = the enzyme with the active serine residue; $\text{X} = \text{NH-R}'$ or $\text{O-R}'$; K = dissociation constant; k_2 and k_3 = first order rate constants, the reaction of the R61 and R39 DD-peptidases on peptide (ester) carbonyl donors, can be written:



In agreement with the above pathway : i) an intermediate is present at the steady state during hydrolysis of the ester carbonyl donor Ac₂-L-Lys-D-Ala-D-lactic acid, suggesting that deacylation is not much faster than acylation; ii) penicillin effectively immobilizes the enzyme in the form of a serine ester-linked penicilloyl derivative (detectable as a PBP) and strong experimental evidence suggests that the same serine residue is involved in the formation of the intermediate which is trapped from Ac₂-L-Lys-D-Ala-D-lactic acid; iii) no intermediate can be trapped at the steady state during hydrolysis of the peptide carbonyl donor Ac₂-L-Lys-D-Ala-D-Ala, suggesting that enzyme acylation is rate determining; iv) increasing concentrations of NH₂-R'' does not increase the maximal rate of peptide carbonyl donor consumption but aminolysis (i.e. transpeptidation) occurs at the expense of hydrolysis on a competitive basis, suggesting that the alternate nucleophile NH₂-R'' reacts only on the acyl enzyme, i.e. after the leaving group P₁ has diffused away.

The serine DD-peptidases, however, are peculiar. Kinetic studies suggest that the amino acceptor binds first to the enzyme. Moreover, some complex amino acceptors behave as regulators of both hydrolysis and transpeptidation pathways. In addition, at least the R39 DD-peptidase appears to possess an additional peptide binding site to which binding of peptides that do not function as acceptors or carbonyl donors, modulates the ratio of hydrolysis over transpeptidation. The activities of the serine DD-peptidases are susceptible to exquisite modulation. Similar mechanisms might be involved in the control of the peptidoglycan crosslinking in living bacteria.

Much has been written on the inactivation of the serine DD-peptidases by the penicillins, cephalosporins and monobactams. Essentially, the underlying mechanism is that the corresponding acyl enzyme "intermediate" has, in most cases, a very long half-life. Since the scissile amide bond of the β-lactam ring is endocyclic, what should be regarded as the leaving group (P₁) during acyl enzyme formation cannot leave the enzyme active site which remains occupied. Enzyme deacylation, however, may slowly occur. In some cases, rupture of the C₅-C₆ linkage in the enzyme-bound penicilloyl moiety with formation of phenylacetyl-glycyl enzyme is the rate determining step of enzyme deacylation. Once formed, this new intermediate is immediately attacked by water or a suitable amino compound (on a competitive basis) with regeneration of an active enzyme



Since k₂' is rate determining, NH₂-R'' does not accelerate the rate of enzyme reactivation. In other cases, enzyme deacylation may slowly

occur without prior fragmentation of the bound acyl moiety. When this occurs, the DD-peptidase functions as a classical β -lactamase of very weak efficiency.

The higher the bimolecular rate constant of enzyme acylation and the smaller the rate of enzyme deacylation, the more potent the β -lactam as a DD-peptidase inactivator. Various side chains and various bi (or mono) cyclic structures can be accommodated by the serine DD-peptidases but depending on both the enzyme and the β -lactam (Table 2), the k_2/K values thus generated vary widely.

β -Lactam	Bimolecular rate constant (k_2/K ; $M^{-1}s^{-1}$) for the inactivation (acylation) of the DD-peptidases (at 37° unless otherwise stated)	
	R61	R39
6-APA	0.2	900
Benzylpenicillin	14,000 (25°)	300,000
7-ACA	14	200
Nitrocefin	500	3,000,000
Sulfazecin	40	6,500
Monobactam SQ26234	70	8,300

The data of Table 2 show that when compared to the serine R39 DD-peptidase, the serine R61 DD-peptidase has a pronounced intrinsic resistance to β -lactam action. In order to fully understand these observations, it would be valuable to have knowledge of the exact, atomic level structures of these two penicillin targets. At present, the solution of the R61 enzyme's structure has proceeded to a resolution of 2.8 Å, showing via difference Fourier maps the enzyme binding site of cephalosporin C and 6,6-dichloro-4-desaza-2,2-didesmethylpenicillanic acid. On the basis of these studies, it has been possible to fit reasonably well a serine residue whose O_γ is less than 3 Å away from the ring carbonyl carbon of the antibiotic. However, detailed interpretation of the interactions between this enzyme and the β -lactams (and substrate analogues) must await refinement of the structure (6).

While these studies are progressing (and attempts to crystallize the R39 serine DD-peptidase are being made), study of the molecular electrostatic potential maps generated by a set of peptide substrates and β -lactam inactivators has led to interesting observations. The D-Ala-D-Ala peptide bond in the substrates and the endocyclic amide bond in the β -lactams are susceptible to attack by the same enzyme active site functional groups, and thus are functionally equivalent. This may be related to the fact that, in all cases, the scissile CON bond and the two flanking reactive groups COOH (on one side) and CON (on the other), create zones of positive and negative electrostatic potentials around the common backbone $\begin{array}{ccccccc} \text{C} & \text{-N-C-C-N-C-C-OH} & & & & & \\ & \text{O} & & \text{O} & & \text{O} & \end{array}$. Depending on the peptide conformation, the presence of bulky side chains, the type of bi (or mono) cyclic framework in the β -lactams, the presence of electron-withdrawing

substituents, etc., important variations in the electrostatic environment of the molecules are observed. Although substrates and inactivators have common reactive properties around the carbonyl of the scissile bond, the observed variations in the relative spatial disposition and strength of the electrostatic potentials must be important for the orientation and reactivity of the whole molecules within the enzyme active site. They suggest multiple modes of binding leading to enzyme-ligand associations of highly varying complementarity and productiveness. They also suggest that variations in the geometry of the DD-peptidases' active sites do not have necessarily the same effects on peptide substrate activity and β -lactam inactivating potency (J. Lamotte-Brasseur, G. Dive and J.M. Ghuysen; Eur. J. Biochem., submitted).

REFERENCES

1. Charlier, P., Dideberg, O., Dive, G., Dusart, J., Frère, J.M., Ghuysen, J.M., Joris, B., Lamotte-Brasseur, J., Leyh-Bouille, M. and Nguyen-Distèche, M. : The active sites of the D-alanyl-D-alanine-cleaving peptidases, in The Murein Sacculus of Bacterial Cell Walls. Architecture and Growth. International FEMS Symposium. March 13-18, 1983 Berlin (West).
2. Dideberg, O., Charlier, P., Dive, G., Joris, B., Frère, J.M. and Ghuysen, J.M. : Structure at 2.5 Å resolution of a Zn⁺⁺-containing D-alanyl-D-alanine-cleaving carboxypeptidase. Nature 299: 469-470, 1982.
3. Frère, J.M., Kelly, J.A., Klein, D., Ghuysen, J.M., Claes, P. and Vanderhaeghe, H. : Δ^2 - and Δ^3 -Cephalosporins, penicillanate and 6-unsubstituted penems. Intrinsic reactivity and interaction with β -lactamases and D-alanyl-D-alanine-cleaving serine peptidases. Biochem. J. 203:223-234, 1982.
4. Ghuysen, J.M., Frère, J.M., Leyh-Bouille, M., Dideberg, O., Lamotte-Brasseur, J., Perkins, H.R. and De Coen, J.L. : Penicillins and Δ^3 -cephalosporins as inhibitors and mechanism-based inactivators of D-alanyl-D-Ala peptidases, in Topics in Molecular Pharmacology (A.S.V. Burgen and G.C.K. Roberts, eds), Elsevier/North-Holland Biomedical Press, 1981, 63-97.
5. Joris, B., Van Beeumen, J., Casagrande, F., Gerday, Ch., Frère, J.M. and Ghuysen, J.M. : The complete amino acid sequence of the Zn⁺⁺-containing D-alanyl-D-alanine-cleaving carboxypeptidase of Streptomyces albus G. Eur. J. Biochem. 130:53-69, 1983.
6. Kelly, J.A. : X-Ray structure of a penicillin target enzyme, in The Murein Sacculus of Bacterial Cell Walls (see ref. 1).
7. Kelly, J.A., Moews, P.C., Knox, J.R., Frère, J.M. and Ghuysen, J.M. : Structure of a penicillin target enzyme and location of the antibiotic binding site. Science 218:479-481, 1982.

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