

# *Penicillins and $\Delta^3$ -cephalosporins as inhibitors and mechanism-based inactivators of D-alanyl-D-Ala peptidases*

Jean-Marie GHUYSEN, Jean-Marie FRÈRE, Melina LEYH-BOUILLE,  
Otto DIDEBERG, Josette LAMOTTE-BRASSEUR, Harold R. PERKINS  
and Jean-Louis DE COEN

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## 1. Introduction

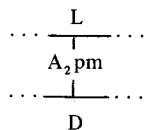
Penicillins,  $\Delta^3$ -cephalosporins and other  $\beta$ -lactam antibiotics interfere with the enzyme machinery responsible for crosslinking the wall peptidoglycan causing inhibition of cellular growth. Usually, this primary bacteriostatic effect is accompanied by cellular lysis and death (bacteriocidal effect) due to the unleashing or triggering of the autolytic system of the sensitive bacteria (for a recent review, see Ghuysen, 1980). These antibacterial effects have been related to the ability of the  $\beta$ -lactam antibiotics to form adducts with various membrane-bound proteins, adducts that are sufficiently stable to be analysed by gel electrophoresis in the presence of sodium dodecyl sulphate. If a  $^{14}\text{C}$ -labelled  $\beta$ -lactam is used, autoradiography of the gels permits visualization of these particular proteins. All bacteria possess four to ten or more membrane-bound proteins that are the specific targets of the  $\beta$ -lactam antibiotics. Their molecular weights range from 20 000 to 100 000 or more.

All the penicillin binding proteins which have been identified as enzymes are D-alanyl-D-Ala peptidases. On the other hand, D-alanyl-D-Ala peptidases exist which do not behave as penicillin-binding proteins. In fact, the D-alanyl-D-Ala peptidases exhibit widely varying affinities, and therefore sensitivities, to the  $\beta$ -lactam antibiotics.

The D-alanyl-D-Ala peptidases catalyse transfer of L-Ala- $\gamma$ -D-Glu-L-R-D-Ala\* from L-Ala- $\gamma$ -D-Glu-L-R-D-Ala-D-Ala pentapeptide units to a nucleophilic cosubstrate. R is a diamino acid residue whose  $\omega$ -amino group is either free or substituted by one or several additional amino acids. The term pentapeptide refers only to the size of the main backbone of the carbonyl donor substrate.

The terminal amino group of the lateral chain at the L-R position of another peptide may serve as nucleophile. The reaction products are D-Ala, i.e. the leaving group of the pentapeptide carbonyl donor, and a crosslinked peptide dimer (transpeptidase activity) (Fig. 1). Alternatively, water may be the attacking nucleophile in which case the reaction products are D-Ala and a tetrapeptide unit (carboxypeptidase activity) (Fig. 2). Finally, D-alanyl-D-Ala peptidases may accommodate D-centres other than D-Ala at the C-terminal position; crosslinked peptides may thus be cleaved at the peptide bond between a

\* Abbreviations:  $\text{Ac}_2\text{Lys}$ , ( $N^\alpha, N^\epsilon$ -diacetyl)Lys;  $\text{Suc}_2\text{Lys}$ , ( $N^\alpha, N^\epsilon$ -disuccinyl)Lys;  $\text{A}_2\text{pm}$ , diamino-pimelic acid. To indicate the stereochemistry of the two chiral centers of  $\text{A}_2\text{pm}$  in peptides the notation



is used.  $\gamma$ -Glu- indicates a peptide bond formed with the  $\gamma$ -carboxylate of Glu. Similarly,  $\epsilon$ -Lys indicates a peptide bond formed with the  $\epsilon$ -amino group of Lys.

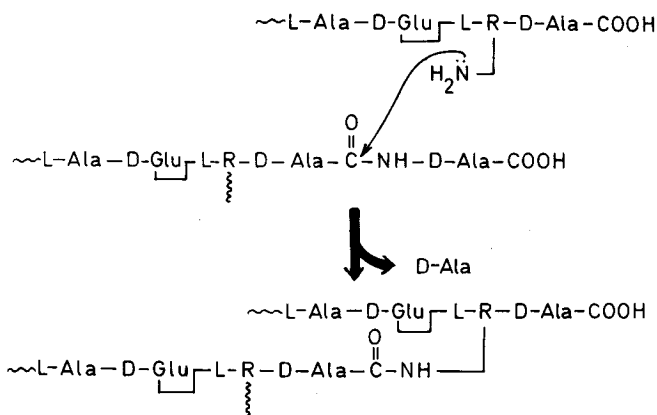


Fig. 1. Transpeptidase activity of D-alanyl-D-Ala peptidases.

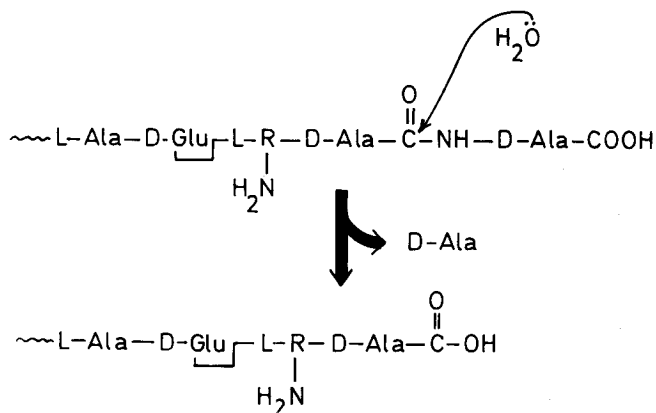
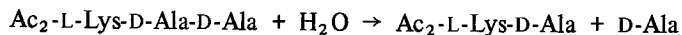


Fig. 2. Carboxypeptidase activity of D-alanyl-D-Ala peptidases.

D-Ala residue and another D-residue bearing a free carboxyl group. The reaction products are tetrapeptide units ("endopeptidase" activity) (Fig. 3). The D-alanyl-D-Ala peptidases thus perform antagonistic activities. They permit wall expansion by transpeptidating the nascent peptidoglycan strands as they emerge from the plasma membrane, to the pre-existing wall peptidoglycan. They also control the final extent of crosslinking by limiting the number of pentapeptide donor sites available for transpeptidation and by hydrolysing peptide cross-links previously made by transpeptidation.

On the basis that the C-terminal sequence L-R-D-Ala-D-Ala is that part of the carbonyl donor directly involved in substrate activity, the tripeptide  $\text{Ac}_2\text{-L-Lys-D-Ala-D-Ala}$  appeared to be the simplest peptide that may serve as substrate analogue of D-alanyl-D-Ala peptidases. Enzymes exhibiting carboxypeptidase activity have been isolated which catalyse the reaction:



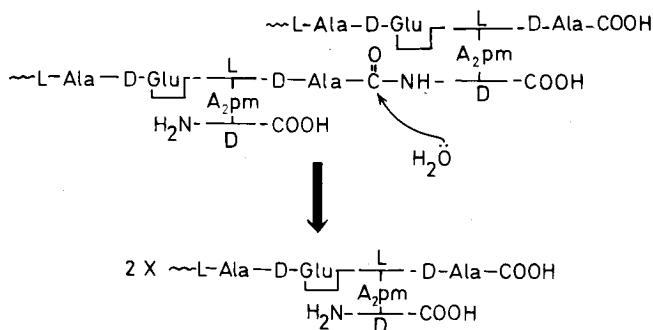
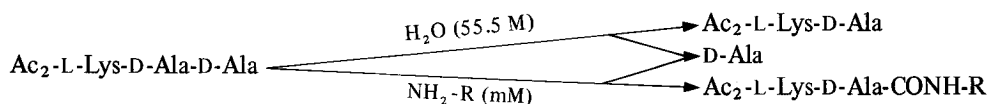


Fig. 3. "Endopeptidase" activity of D-alanyl-D-Ala peptidases.

In turn, mixtures containing  $\text{Ac}_2\text{-L-Lys-D-Ala-D-Ala}$  and a suitable  $\text{H}_2\text{N-R}$  compound have permitted isolation of enzymes catalysing concomitant hydrolysis and transpeptidation reactions on a competitive basis:



The fact that millimolar concentrations of the amino compound successfully compete with 55.5 M  $\text{H}_2\text{O}$  demonstrates that such bifunctional enzymes, whatever their exact physiological functions, possess a binding site for  $\text{H}_2\text{N-R}$  compounds of very high efficiency.

The usual peptidases and proteases fall into two distinct classes of enzymes. The serine and thiol proteases (e.g., chymotrypsin and papain) are thought to form well-defined acyl-enzyme intermediates with their substrates; the acid and metalloproteases (e.g., pepsin and carboxypeptidase A) do not. Similarly, the D-alanyl-D-Ala peptidases, which distinguish themselves by their ability to attack peptide bonds extending between two D-centres in an  $\alpha$  position to a free carboxyl group, can operate by different mechanisms. At this time, four serine and one zinc-containing D-alanyl-D-Ala peptidases are known.

A precise knowledge of the active centres of D-alanyl-D-Ala peptidases of different mechanistic properties and widely varying penicillin sensitivities should lead to the design of new and specific agents to selectively inactivate these important bacterial enzyme targets. In this review, the current state of our knowledge on the geometry and functioning of the active sites of the D-alanyl-D-Ala peptidases is presented.

## 2. The serine D-alanyl-D-Ala peptidases

During growth, *Actinomadura* R39 and *Streptomyces* R61 each excrete a bifunctional D-alanyl-D-Ala carboxypeptidase-transpeptidase. The enzyme from *Actinomadura*, known as the R39 enzyme, has a molecular mass of 53 000 and is highly penicillin-sensitive,

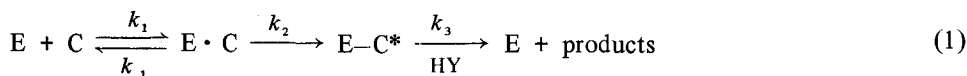
while the R61 enzyme from *Streptomyces*, with a molecular mass of 38 000, is only moderately penicillin-sensitive. Both enzymes have been purified to protein homogeneity and are available in a truly water-soluble form. Each of them consists of a single polypeptide chain (Frère et al., 1973a; Frère et al., 1974a).

The R61 enzyme is the only serine D-alanyl-D-Ala peptidase which has yet been crystallized (Knox et al., 1979). X-Ray structure analyses at 5 Å resolution of well-formed orthorhombic crystals (space group  $P2_12_12_1$ ) have shown that the unit cell dimensions are  $a = 51.1$  Å,  $b = 67.4$  Å and  $c = 102.9$  Å. The radius of gyration of the molecule in solution is  $20.8 \pm 0.5$  Å (at 20°C and a protein concentration of 16 mg/ml). If the molecule is considered spherical, this value translates to a diameter of 52 Å which is close to the  $a$  unit cell dimension of the crystals.

The R39 and R61 serine D-alanyl-D-Ala peptidases have been used as models for the discussion presented in this chapter. Two other penicillin-sensitive serine D-alanyl-D-Ala peptidases have also been isolated from the membranes of *Bacillus subtilis* and *B. stearothermophilus*, respectively, by Strominger and his colleagues (for a recent review on these enzymes, see Waxman et al., 1980). Their molecular weights are between 45 000 and 50 000. They have been purified to protein homogeneity, but are available only in solution containing a suitable detergent. Recently, however, one of them has been converted, by controlled proteolysis of the isolated membranes, into a shortened, water-soluble protein without affecting the enzyme activity or the penicillin binding capacity of the parent molecule (Waxman and Strominger, 1979).

The serine D-alanyl-D-Ala peptidases are inactivated by  $\beta$ -lactam compounds. The mechanism of this inactivation rests upon the ability of these enzymes to catalyse nucleophilic attack of the carbonyl carbon of the endocyclic amide bond of the  $\beta$ -lactam ring which is exposed on the relatively unhindered  $\alpha$  face of the molecule in a position roughly equivalent to that of the sensitive C-terminal D-Ala-D-Ala peptide bond in the carbonyl donor substrates (Fig. 4).

Whether the carbonyl donor substrate is a peptide terminating in D-Ala-D-Ala or a  $\beta$ -lactam compound, the general model for the reactions catalysed by the serine D-alanyl-D-Ala peptidases is:



where E is enzyme, C the carbonyl donor substrate,  $E \cdot C$  the Michaelis complex,  $E-C^*$  the acyl-enzyme intermediate and HY the nucleophile (water, or a suitable compound bearing an amino group). The dissociation constant of the first step is given by  $K = k_{-1}/k_1$ . Formation of the Michaelis complex  $E \cdot C$  is thought to be a rapid equilibrium process. Note that in this general model, there is no indication of the step in which HY binds to the enzyme.

Covalent catalysis involves, first, binding of the substrate with the relevant sensitive amide linkage positioned in the vicinity of a serine residue within the enzyme active site. This serine residue (Z in enzyme as shown in Figs. 5 and 6) then attacks the carbonyl carbon of the amide linkage to form a covalent ester-linked acyl-enzyme intermediate. The process, most likely, proceeds via a tetrahedral adduct. With peptides terminating

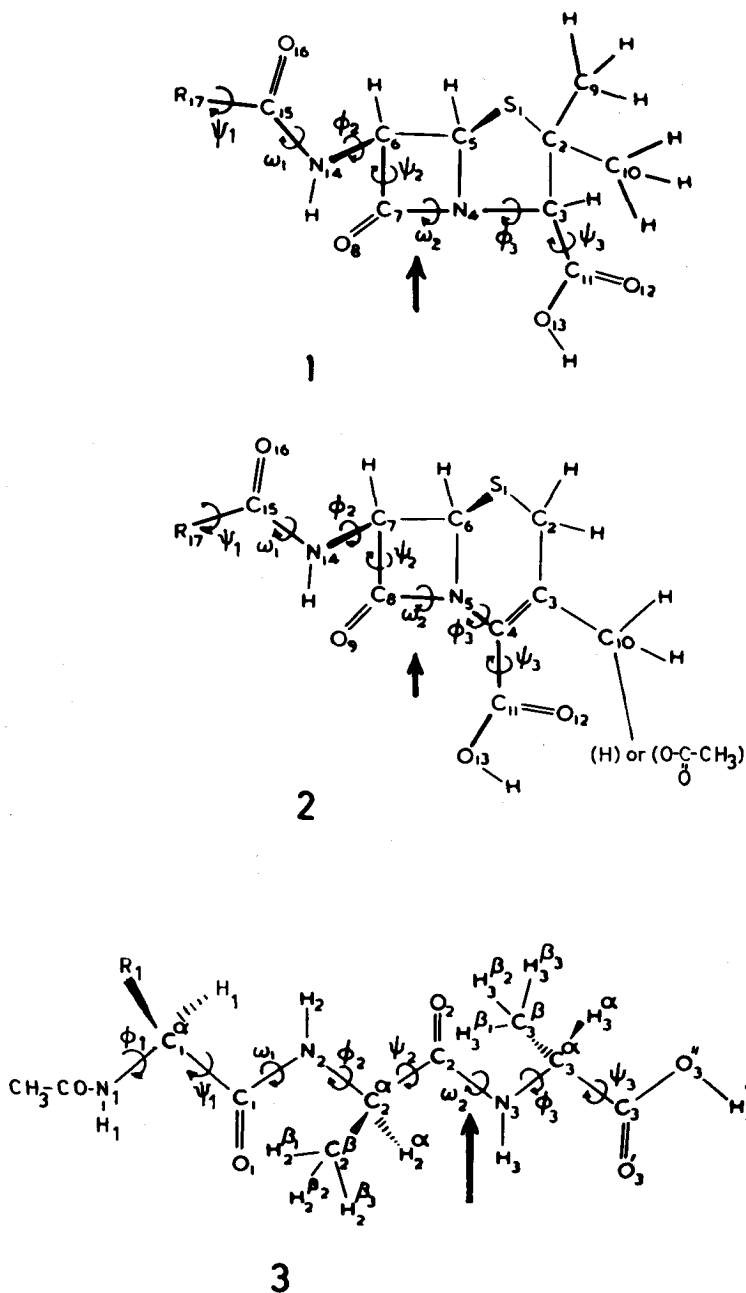


Fig. 4. Structure of penicillins (1),  $\Delta^3$ -cephalosporins (2) and Ac-L-R-D-Ala-D-Ala (3) showing the backbone dihedral angles and the amide linkages attacked by the D-alanyl-D-Ala peptidases (arrows). The  $\beta$ -lactam ring is fused to another ring, either thiazolidine forming the penam nucleus of penicillins, or dihydrothiazine forming the 3-cephem nucleus of  $\Delta^3$ -cephalosporins. The 6(7) $\beta$ -substituents in the penicillins and  $\Delta^3$ -cephalosporins are in the *cis* sense so that the  $\alpha$ -faces of the molecules are relatively unhindered.

in L-R-D-Ala-D-Ala, the leaving group (D-Ala) is released during formation of the acyl-enzyme intermediate. This intermediate is then attacked in a second step by an exogenous nucleophile HY (water or a suitable amino compound) to regenerate the active enzyme and to yield the reaction products. The covalent intermediate is very short-lived, i.e. the reaction flux is completely to reaction products, generating high turnover numbers (Fig. 5).

With penicillins (or  $\Delta^3$ -cephalosporins), the leaving group, i.e. the thiazolidine (or dihydrothiazine) ring, remains part of the acyl-enzyme intermediate and stabilizes it. The reaction flux stops at this abortive level, conferring on the  $\beta$ -lactam antibiotics their property to behave as enzyme inactivators (Fig. 6).

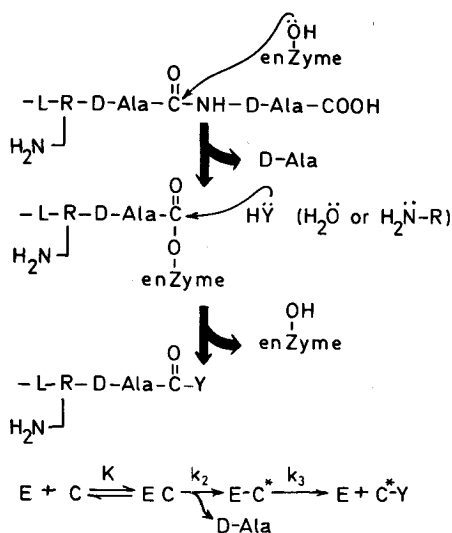


Fig. 5. Reaction of the serine D-alanyl-D-Ala peptidases with L-R-D-Ala-D-Ala terminated peptides.

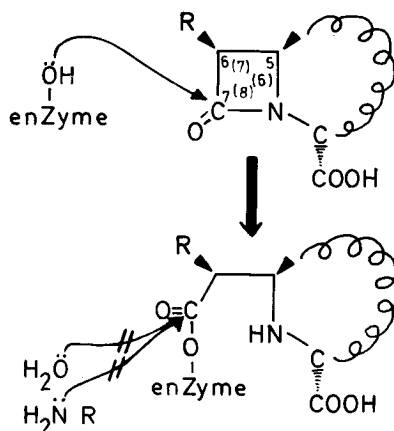


Fig. 6. Reaction of the serine D-alanyl-D-Ala peptidases with the penicillins and  $\Delta^3$ -cephalosporins.

Not only do the penicillins (or  $\Delta^3$ -cephalosporins) complement the enzyme active site sufficiently to bind to it but part of the normal catalytic mechanism occurs before the "irreversible" step takes place. Different names have been used to describe enzyme inactivators that work this way. Rando named them " $k_{\text{cat}}$  inhibitors"; Abeles and Maycock used the term "suicide enzyme inactivators", Walsh "suicide substrates", and Seiler et al. "enzyme-activated irreversible inhibitors" (cited in John, 1980). In turn, Fisher and Knowles (1980) speak of "(enzyme) inactivation by mechanism-based reagents".

The suicide step which occurs during reaction between the serine D-alanyl-D-Ala peptidases and penicillins (or  $\Delta^3$ -cephalosporins) leads to an unusual free energy profile of the reaction. The height of the energy barrier to enzyme acylation is negligible when compared to that which has to be overcome to achieve enzyme deacylation ( $k_2 \gg k_3$ ) (Fig. 7). Long-lived [ $^{14}\text{C}$ ]benzylpenicilloyl-enzyme intermediates are thus produced. They have been isolated and submitted to various proteolytic degradations. Studies of the isolated  $^{14}\text{C}$ -labelled peptide fragments have established the amino acid sequences around the active serine residue (Frère et al., 1976c; Yocum et al., 1979; Waxman et al., 1980; Duez et al., 1981a). NMR data (Degelaen et al., 1979) supports the idea that the penicillin is covalently bound as a penicilloyl moiety in the denatured intermediate.

With  $\text{Ac}_2\text{-L-Lys-D-Ala-D-Ala}$ , the reverse situation occurs: enzyme deacylation is more rapid than enzyme acylation, and the  $\text{Ac}_2\text{-L-Lys-D-alanyl-enzyme}$  intermediate cannot accumulate in the steady-state. Like chymotrypsin and other serine peptidases, however, the D-alanyl-D-Ala peptidases are more effective esterases than peptidases. With the depsi-peptide  $\text{Ac}_2\text{-L-Lys-D-Ala-D-lactate}$  (instead of  $\text{Ac}_2\text{-L-Lys-D-Ala-D-Ala}$ ), the deacylation rate of course remains unaffected but the acylation rate markedly increases and the  $\text{Ac}_2\text{-L-Lys-D-alanyl-enzyme}$  intermediate accumulates. With the two *Bacillus* enzymes (Rasmussen et al., 1978; Yocum et al., 1979; Waxman et al., 1980) and the R61 enzyme (R.R. Yocum and J.L. Strominger, personal communication), the  $\text{Ac}_2\text{-L-Lys-D-alanyl}$  group was shown to be covalently linked to the same serine residue of the enzyme as that involved in the penicilloyl-enzyme intermediate. (The R39 enzyme is under current investigation.)

The most basic questions that one can ask of the serine D-alanyl-D-Ala peptidases are: (i) how do L-R-D-Ala-D-Ala terminated peptides and penicillins (or  $\Delta^3$ -cephalosporins) overcome the energy barrier to enzyme acylation; (ii) why do the penicilloyl (cephalosporoyl)-enzyme intermediates exhibit such a high stability; and (iii) what is the mechanism underlying the transpeptidation reactions?

### 2.1 The serine D-alanyl-D-Ala peptidases as transpeptidases

The usual serine peptidases and proteases function only as hydrolases; they are unable to catalyse acyl transfer to a nucleophilic amino acceptor. In marked contrast, the serine D-alanyl-D-Ala peptidases can act as transpeptidases. This remarkable property was first reported in 1972 (Pollock et al., 1972). When  $\text{Ac}_2\text{-L-Lys-D-Ala-D-Ala}$  (1.5 mM) was used (as carbonyl donor substrate) in the presence of D- $^{14}\text{C}$  Ala (as the amino group containing acceptor and at "saturating" concentrations; molar ratio of acceptor to donor of 100:1), the time course of transpeptidation (formation of  $\text{Ac}_2\text{-L-Lys-D-Ala-D-}^{14}\text{C-Ala}$ ) catalysed by the R39 and R61 enzymes roughly paralleled the time course of hydro-



Free energy profiles for reactions of serine D-alanyl-D-Ala peptidases

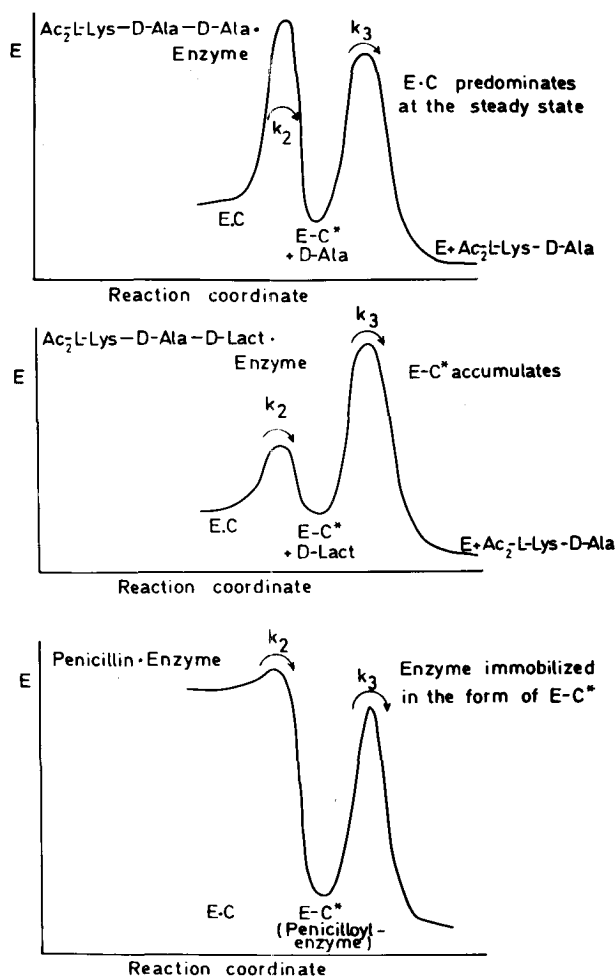
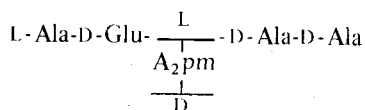


Fig. 7. Qualitative free-energy profile for reactions of D-alanyl-D-Ala peptidases with the tripeptide  $\text{Ac}_2\text{-L-Lys-D-Ala-D-Ala}$ , the depsipeptide  $\text{Ac}_2\text{-L-Lys-D-Ala-D-Lact}$  and the  $\beta$ -lactam penicillin. The energy profiles of reaction with the peptide or the depsipeptide are drawn at the same scale while the energy profile of reaction with penicillin is drawn at a smaller scale. Note that because of the distorted  $\beta$ -lactam nitrogen, the initial energy level is high.

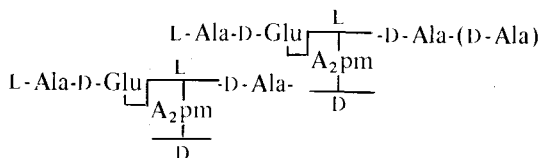
lysis (formation of  $\text{Ac}_2\text{-L-Lys-D-Ala}$ ) observed when no nucleophile other than water was present in the reaction mixture. In the following discussion, we shall refer to the two substrates of the transpeptidase reaction simply as "donor" and "acceptor"; when the acceptor is water — i.e. in hydrolysis — this will be explicitly stated.

Following this initial observation, experiments were carried out which led to the conclusion that the two enzymes possess acceptor binding sites of considerable specificity.

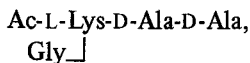
With Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala as donor substrate, only glycine and compounds whose amino group is on a D centre in an α-position to a free carboxyl group, are accepted for transpeptidation by the R39 enzyme. On the contrary, the R61 enzyme can use, with varying efficiency, a wide range of compounds as acceptors, including not only glycine and D-amino acids, but also D-cycloserine, 6-aminopenicillanic acid, ω-amino acids, aminohexuronic acids, and finally, peptides with N-terminal D-alanine or glycine (Perkins et al., 1973). The fact that, in parallel to this, the peptidoglycan cross-link is a C-terminal D-alanyl-(D)-*meso*-diamino-pimelic acid in *Actinomadura* R39 (which excretes the R39 enzyme) (Ghuysen et al., 1973) and is a D-alanyl-glycyl-LL-diaminopimelic acid in an endo-position in *Streptomyces* R61 (which excretes the R61 enzyme) (Leyh-Bouille et al., 1970a), shows that the specificity profiles of the two D-alanyl-D-Ala peptidases for acceptors reflect specific structural features of the wall peptidoglycans of the corresponding bacteria. When incubated with the pentapeptide



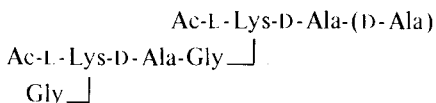
the R39 enzyme concomitantly catalyses hydrolysis into tetrapeptide and formation of the peptide dimer



(Ghuysen et al., 1973, 1974). The R39 enzyme has no action on the tetrapeptide



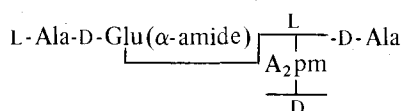
but the R61 enzyme concomitantly catalyses hydrolysis into tripeptide and formation of the peptide dimer



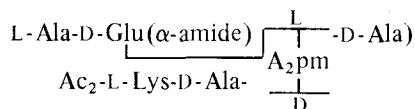
(Zeiger et al., 1975). Peptide trimer is also formed in small amount. Its synthesis is not a random process; it preferentially occurs by transpeptidation between a peptide monomer acting as carbonyl donor and a preformed peptide dimer acting as amino nucleophile (Frère et al., 1976a).

Kinetic studies were then used to unravel the mechanism of the transpeptidation reactions. Essentially, the acceptor is a noncompetitive inhibitor of the hydrolysis pathway. With simple acceptors such as D-Ala or *meso*-A<sub>2</sub>pm, the total enzyme activity (hydrolysis + transpeptidation) is only a function of the amount of carbonyl donor peptide present. Hydrolysis and transpeptidation occur in such a proportion that the increase in the rate of transpeptidation and the decrease in the rate of hydrolysis caused by increased concentrations of the acceptor are commensurate. When saturated by the carbonyl donor peptide, the enzyme has the same turnover number (11.5 s<sup>-1</sup> for the R39 enzyme and 55 s<sup>-1</sup> for the R61 enzyme on Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala) whether it works as a hydrolase or is engaged in a bimolecular transpeptidation reaction occurring concomitantly with the hydrolysis of the peptide donor. Replacing part of the water of the reaction mixture by glycerol and ethylene glycol causes a decreased total enzyme activity but, at the same time, a remarkable increase of the ratio of transpeptidation to hydrolysis (Frère et al., 1973b).

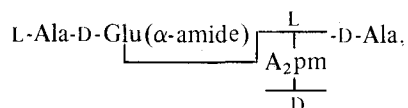
Complex acceptors related to wall peptidoglycan, however, do not act as simple non-competitive inhibitors of the hydrolysis pathway. As observed with the substrates Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala and Gly-L-Ala for the R61 enzyme (transpeptidation product: Ac<sub>2</sub>-L-Lys-D-Ala-Gly-L-Ala) (Frère et al., 1973b) or the substrates Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala and



for the R39 enzyme (transpeptidation product:



(Ghuysen et al., 1974), the picture is more complicated. First, the increase of the rate of transpeptidation caused by increasing concentrations of acceptor is less than can be accounted for by the decrease of the rate of hydrolysis. Hence, the acceptor produces an overall inhibition of attack on the donor peptide. The further the enzyme is from being saturated by the donor peptide, the more strongly is the total reaction inhibited by low concentrations of the acceptor. In addition, at high concentrations of acceptor, the transpeptidation itself is inhibited. For example (Fig. 8), with the R39 enzyme and co-substrates Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala and



one observes that at a fixed 0.27 mM concentration of Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala (which is close to the K<sub>m</sub> value), hydrolysis is progressively inhibited by increasing concentrations of the amidated tetrapeptide, whereas transpeptidation rises to a maximum at an acceptor concentration of about 0.8 mM. At higher concentrations, both transpeptidation and hydrolysis are progressively inhibited until, eventually, the tripeptide Ac<sub>2</sub>-L-Lys-D-Ala-

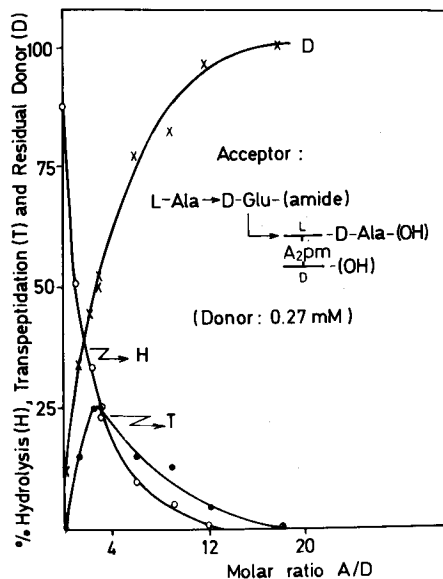
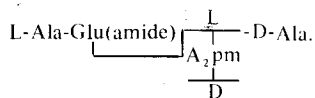


Fig. 8. Effect of variation of amino acceptor concentrations, in the presence of a fixed amount of Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala (0.27 mM), on hydrolysis (H) and transpeptidation (T) by the R39 D-alanyl-D-Ala peptidase. (D = residual tripeptide donor). The amino acceptor is the amidated tetrapeptide



D-Ala remains unaffected, i.e. the enzyme is frozen in a catalytically inactive state (Ghuysen et al., 1974). This phenomenon is dependent on the presence of the  $\alpha$ -amide group on the D-glutamic acid residue (as it occurs in the wall peptidoglycan of *Actinomadura* R39). High concentrations of the same tetrapeptide acceptor but lacking the amide substituent still inhibit overall attack on the peptide donor by inhibiting its hydrolysis but do not decrease the amount of transpeptidation product formed, which remains at a maximal value (Fig. 9). In addition, the same tetrapeptide but with the amide situated on the carboxyl group of the D-carbon of *meso*-A<sub>2</sub>pm is neither cosubstrate for transpeptidation nor inhibitor of the hydrolysis of the carbonyl donor.

Presumably efficient transpeptidation requires both the donor peptide and the acceptor to be immobilised in an optimal orientation at the enzyme's active centre. Bimolecular transpeptidation reactions occurring concomitantly with the hydrolysis of the carbonyl donor molecule may proceed through at least ten different mechanisms which, however, fall into three main groups: those where the donor binds first to the enzyme, those where the acceptor (or water) binds first to the enzyme, and, finally, the rapid equilibrium random mechanism (Frère, 1973). An examination of the effects of the concentrations of the donor peptide (C) and the acceptor (HY), respectively, on the  $\nu_T/\nu_H$  ratio (T = transpeptidation; H = hydrolysis) is diagnostic of the mechanism in-

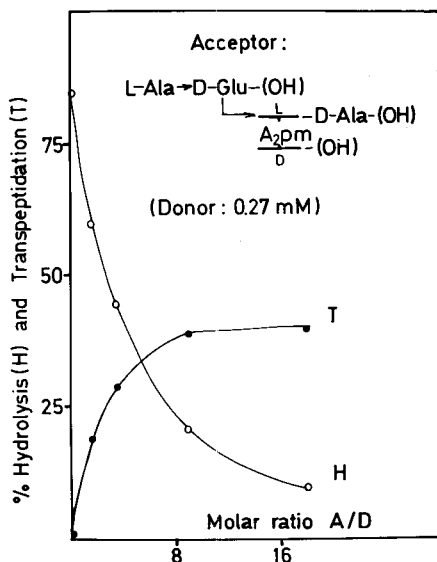
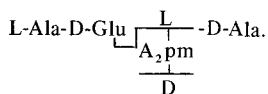
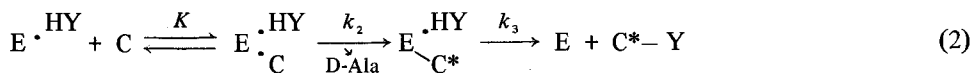


Fig. 9. Effect of variation of amino acceptor concentrations, in the presence of a fixed amount of  $\text{Ac}_2\text{-L-Lys-D-Ala-D-Ala}$  (0.27 mM) on hydrolysis (H) and transpeptidation (T) by the R39 D-alanyl-D-Ala peptidase. (D = residual tripeptide donor). The amino acceptor is the non-amidated tetrapeptide



involved. At a given  $[\text{HY}]$  value, the ratio  $\nu_T/\nu_H$  is independent of  $[\text{C}]$  if the reactions occur randomly or proceed through an ordered mechanism where the donor binds first to the enzyme, but, conversely, the ratio  $\nu_T/\nu_H$  is a function of  $[\text{C}]$  if the acceptor binds first. As the experiments carried out with the R61 enzyme and the system  $\text{Ac}_2\text{-L-Lys-D-Ala-D-Ala}$  and either *meso*- $\text{A}_2\text{pm}$  or  $\text{Gly-L-Ala}$  showed (Frère et al., 1973b), the ratios  $\nu_T/\nu_H$  significantly decrease as  $[\text{C}]$  increases suggesting an ordered pathway where the acceptor  $\text{HY}$  binds first to the enzyme:



In a first step, binding of  $\text{HY}$  to the enzyme's centre might involve transfer of a proton from the  $-\text{NH}_3^+$  group of the acceptor to a side chain of the enzyme; the amino group of the bound nucleophile would then possess the lone pair necessary to carry out immediate attack on the ester-linked acyl-enzyme intermediate formed in a second step. Such a covalent catalysis appears to be an efficient process for accommodating bond breaking and formation in a single active centre. That the usual serine peptidases and proteases do not use it as a means to catalyse transpeptidation reactions may reflect a lack of a

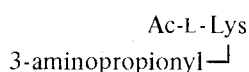
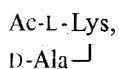
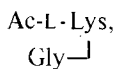
specific acceptor site. The acid protease pepsin, however, can function as a transpeptidase. In this case, the moiety normally regarded as the leaving group, i.e. the amine fragment, would be retained on the enzyme while, instead, the acyl fragment would depart so that the bound amino group could be transferred to a different acyl moiety.

The above studies concerning the effects of [C] and [HY] on the  $\nu_T/\nu_H$  ratios revealed another peculiarity of the R61 serine D-alanyl-D-Ala peptidase. Irrespective of the mechanism (random, or with the acceptor or the donor binding first), the  $\nu_T/\nu_H$  ratio at a given [C] value, should be directly proportional to [HY] if the enzyme does not bind more than one molecule of amino acceptor. The substrate pair Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala + *meso*-A<sub>2</sub>pm behaves in this way. But again, complex acceptors related to wall peptidoglycan behave differently: the ratio  $\nu_T/\nu_H$  is a function of [HY] according to

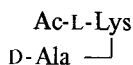
$$\frac{\nu_T}{\nu_H} = \frac{f [\text{HY}]}{e + g [\text{HY}]}$$

where *e*, *f* and *g* are constants at a given [C] value, suggesting that an additional acceptor binding site is present on the enzyme, leading, at high concentrations, to the formation of an E-(HY)<sub>2</sub>-C quaternary complex which is non-productive for transpeptidation (Frère et al., 1973).

Finally, the presence in the R39 enzyme of an additional peptide binding site distinct from the carbonyl donor and the acceptor sites, has also been demonstrated (Perkins et al., 1981). Simple peptides such as



or Gly-Gly-Gly do not function as acceptors for the R39 enzyme. But, when added to a system containing Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala as donor and either Gly or *meso*-A<sub>2</sub>pm as acceptors, they produce selective and extensive inhibition of the transpeptidation reaction while hydrolysis proportionally increases. While



acts in this way, its configurational isomer containing L-Ala is without effect. Gly-Gly-Gly is more effective than Gly-Gly-Gly-Gly, suggesting that the overall distance between the terminal amino and carboxyl groups might be of importance. The inhibitory peptides give noncompetitive kinetics relative to both carbonyl donor and amino acceptor, implying that they are not binding at the active centre of the enzyme but are rather involved in some allosteric action.

The antagonistic activities of the bifunctional serine D-alanyl-D-Ala peptidases thus appear to be susceptible to exquisite modulation. Modifying the polarity of the enzyme micro-environment, changing the donor and acceptor concentrations, altering some structural properties of these peptides (for example, amidation of specific carboxyl groups) and supplementing the enzyme—substrates system with other peptides that are neither

donor nor acceptor, profoundly modify the enzyme activity and/or its channelling to hydrolysis and transpeptidation. Similar mechanisms might be involved in the control of the peptide crosslinking of peptidoglycan in the living bacteria.

## 2.2 Formation of the serine ester-linked L-R-D-alanyl-enzyme intermediates

Peptides terminating in L-R-D-Ala-D-Ala readily overcome the energy barrier to enzyme acylation and the reaction flux of enzyme action is completely to hydrolysed peptides (when H<sub>2</sub>O is the only exogenous nucleophile present). It thus follows that the kinetic parameters that can be measured are  $K_m$  and turnover number. However, if in the model reaction (1), formation of the Michaelis complex E·C is a rapid equilibrium process, then  $K_m$  and turnover number are given by

$$K_m = \frac{k_3}{k_2 + k_3} K \text{ and turnover number} = \frac{k_2 k_3}{k_2 + k_3}$$

Consequently, the ratio turnover number/ $K_m$ , which expresses the enzyme catalytic efficiency, is equivalent to  $k_2/K$ , i.e. is a valid measure of the bimolecular rate constant which governs the formation of the serine ester-linked acyl-enzyme intermediate.

Kinetics of the hydrolysis of Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala and several peptide analogues by the R39 and R61 serine enzymes (Leyh-Bouille et al., 1971, 1972) show that catalytic efficiency (turnover number/ $K_m$ ), (i) requires a D-Ala at position 2 (i.e., the residue whose carbonyl carbon undergoes nucleophilic attack must be D-Ala; Table IA); (ii) is decreased but not abolished when Gly or a D-amino acid other than D-Ala is the leaving group at the C-terminal position 3. L-Ala at this position, however, abolishes substrate activity (Table IB); (iii) is influenced differently in the different enzymes by the presence of charged groups at the end of the long side chain at position 1 (Table IC); and (iv) is drastically decreased as the neutral side chain of the L residue at position 1 is progressively shortened (Table ID).

Peptides which are not substrates may behave as enzyme inhibitors (Table II). Ac-D-Ala-D-Ala and to a greater extent, Ac-D-Ala-D-Glu, Suc<sub>2</sub>-L-Lys-D-Ala-D-Glu and Ac<sub>2</sub>-L-Lys-D-Glu-D-Ala inhibit the hydrolysis of Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala by the R61 enzyme (Nieto et al., 1973a). Hence, elimination of (*N*<sup>ε</sup>-acetyl)-L-Lys, replacement of D-Ala at the penultimate position by D-Glu, replacement of D-Ala at the C-terminal position by D-Glu together with elimination of (*N*<sup>ε</sup>-acetyl)-L-Lys at position 1 or succinylation instead of acylation of the ε-amino group of the L-Lys residue, each abolishes substrate activity but not enzyme binding, thus conferring on the modified peptides their inhibitory activities.

As shown by the data of Table I, structurally related peptides differing from each other only by the nature of the leaving group or the size of the neutral side-chain at the L-centre do not have the same catalytic efficiency. This observation shows that the Ac<sub>2</sub>-L-Lys-D-alanyl-enzyme intermediate that is formed during hydrolysis of Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala, does so after the rate limiting step. If, as all these observations suggest, enzyme acylation is much slower than enzyme deacylation ( $k_2 \gg k_3$ ),  $K_m$  becomes equivalent to  $K$  and the catalytic efficiency becomes equivalent to  $k_2$ . The following

TABLE I

Efficiency of hydrolysis (release of the C-terminal residue)

Peptide	R61 serine enzyme			R39 serine enzyme			G Zn <sup>2+</sup> enzyme		
	<i>K<sub>m</sub></i> (mM)	Turnover number (s <sup>-1</sup> )	Catalytic efficiency (M <sup>-1</sup> · s <sup>-1</sup> )	<i>K<sub>m</sub></i> (mM)	Turnover number (s <sup>-1</sup> )	Catalytic efficiency (M <sup>-1</sup> · s <sup>-1</sup> )	<i>K<sub>m</sub></i> (mM)	Turnover number (s <sup>-1</sup> )	Catalytic efficiency (M <sup>-1</sup> · s <sup>-1</sup> )
(A) Ac <sub>2</sub> -L-Lys-D-Ala-D-Ala	12	55.2	4600	0.8	17.5	21 900	0.33	2.5	7600
Ac <sub>2</sub> -L-Lys-Gly-D-Ala	15	0.1	7	nonsubstrate			15	2.7	180
Ac <sub>2</sub> -L-Lys-D-Leu-D-Ala	10	0.6	60	nonsubstrate			nonsubstrate		
Ac <sub>2</sub> -L-Lys-D-Glu-D-Ala	nonsubstrate			nonsubstrate			nonsubstrate		
Ac <sub>2</sub> -L-Lys-L-Ala-D-Ala	nonsubstrate			nonsubstrate			nonsubstrate		
(B) Ac <sub>2</sub> -L-Lys-D-Ala-D-Lys	13	5.6	430	n.d.			0.80	2.16	2700
Ac <sub>2</sub> -L-Lys-D-Ala-D-Leu	10	3.1	310	0.7	11.4	16 300	0.33	0.82	2500
Ac <sub>2</sub> -L-Lys-D-Ala-Gly	36	12.6	350	2.5	5	2 000	2.50	1.5	600
Ac <sub>2</sub> -L-Lys-D-Ala-L-Ala	nonsubstrate			nonsubstrate			nonsubstrate		
(C) Ac-L-Lys-D-Ala-D-Ala	15	0.25	17	0.2	32	160 000	6.0	0.48	80
R <sub>1</sub> - $\begin{array}{c}   \\ \text{---} \\   \\ \text{A}_2 \text{ pm} \\   \\ \text{---} \\   \\ \text{D} \end{array}$ -D-Ala-D-Ala	11	0.19	17	0.25	20.8	83 000	0.4	0.48	625
(H) $\begin{array}{c}   \\ \text{---} \\   \\ \text{D} \end{array}$ (OH)									
R <sub>2</sub> -L-Lys-D-Ala-D-Ala	14	50.4	3600	0.30	22.1	73 800	0.28	0.25	830
(Gly) <sub>2</sub> - $\begin{array}{c}   \\ \text{---} \\   \\ \text{D} \end{array}$									
Relative substrate activity (Ac <sub>2</sub> -L-Lys-D-Ala-D-Ala = 100%) <sup>a</sup>									
(D) Ac <sub>2</sub> -L-Orn-D-Ala-D-Ala		45%		83%			n.d.		
Ac <sub>2</sub> -L-A <sub>2</sub> -bu-D-Ala-D-Ala		8%		15%			0.60	0.78	1300
R <sub>3</sub> -L-Hser-D-Ala-D-Ala		3%		7%			1.0	0.3	300
Ac-L-Ala-D-Ala-D-Ala		1.4%		<1%			3.3	0.01	3
Ac-Gly-D-Ala-D-Ala		n.d.		n.d.			1.1	0.03	30

At pH 7.5 and 37°C.

R<sub>1</sub> = UDP-MurNAc-L-Ala-γ-D-Glu, R<sub>2</sub> = N<sup>α</sup>(β-1,4-GlcNAc-L-Ala-γ-D-Glu(NH<sub>2</sub>);R<sub>3</sub> = UDP-MurNAc-Gly-γ-D-Glu;<sup>a</sup> Substrate activity measured at 0.5 mM concentration.

n.d., not determined.



TABLE II

## Peptides as enzyme inhibitors

Peptide	R61 serine enzyme		G Zn <sup>2+</sup> enzyme	
	Activity as substrate (%)	% Inhibition	Activity as substrate (%)	% Inhibition
Ac <sub>2</sub> -L-Lys-D-Ala-D-Ala	100	—	100	—
Ac <sub>2</sub> -L-Lys-D-Glu-D-Ala	0	35	0	87
Ac-D-Ala-D-Ala	1	9	0	10–20
Suc <sub>2</sub> -L-Lys-D-Ala-D-Ala	16	43	2–8	50
Ac <sub>2</sub> -L-Lys-D-Ala-D-Glu	10	—	100	—
Ac-D-Ala-D-Glu	0	25	0	88 <sup>a</sup>
Suc <sub>2</sub> -L-Lys-D-Ala-D-Glu	0	32–46	0	88–96

Activities as substrate were measured by incubating the peptides (at 5–7.5 mM concentrations) and comparing the extent of hydrolysis (release of the C-terminal residue) with that observed with Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala under identical conditions. Activities as inhibitor were measured by estimating the inhibitory effect of the peptides (at 5–7.5 mM concentrations) on the hydrolysis of 0.5 mM solution of Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala. None of the peptides listed inhibited the activity of the serine R39 enzyme.

<sup>a</sup> Competitive inhibitor:  $K_1 = 0.2$  mM.

induced fit model then best explains the underlying mechanism through which the enzymes undergo acylation by the L-R-D-Ala-D-Ala terminated peptides (Nieto et al., 1973a).

The initial binding of the donor peptide to the active site, measured by  $K$ , primarily depends on the residues at position 2 and 3, the sequence of D-Ala-D-Ala being a favourable but not the only efficient one. Following initial binding, formation of the acyl-enzyme intermediate ( $k_2$ ) requires D-Ala at position 2 but, very remarkably, the effectiveness of the process depends entirely on the size, structure, charge, orientation, etc. of the side chain at the L-centre of the peptide. By interacting with some amino acid(s) of the active site, a suitable side chain at this L position in the bound peptide induces conformational isomerization in the protein which in turn results in the establishment of the correct active geometry and converts the serine residue of the enzyme's cavity into a very reactive nucleophile. Conversely, the binding of a peptide terminating in L-R-D-Ala-D-Ala whose side chain at position 1 is lacking or incorrect, does not induce the correct conformational response, but may confer on the bound peptide the property of an enzyme inhibitor. The effect of the side chain at the L-centre may be highly enzyme-specific. The catalytic efficiency of the R61 enzyme on Ac-L-Lys-D-Ala-D-Ala (lacking the acetyl group on Lys- $\epsilon$ -NH<sub>2</sub>) is  $17 \text{ M}^{-1} \cdot \text{s}^{-1}$  while that of the R39 enzyme is  $1.6 \cdot 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ .

Conformational analysis (involving local as well as non-local, backbone to backbone, side-chain to backbone and side-chain to side-chain interactions) of Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala and the peptide analogues listed in Table I suggests that substrate activity is associated with a strong probability of four particular families of conformers (De Coen et al., 1981). Each family can be characterized by its own most probable conformer. These

conformers are called  $ee^*b^*$ ,  $be^*b^*$ ,  $eb^*b^*$  and  $bb^*b^*$ , respectively. The values of the dihedral angles of the peptide backbones are given in Table III and the  $\chi$  angles which define the most probable positions of the L-Lys side chain are  $\chi^1 = 60^\circ$  in conformers  $bb^*b^*$  and  $be^*b^*$ ,  $\chi^1 = -60^\circ$  in conformers  $ee^*b^*$  and  $eb^*b^*$ , and  $\chi^2$ ,  $\chi^3$ ,  $\chi^4$  and  $\chi^5 = 180^\circ$  in all conformers.

In all four conformers, the main peptide backbone shows a more or less pronounced tendency to fold back on itself and to form a loop-like structure. Remarkably, the  $N^\epsilon$ -acetylated side chain always protrudes at the L-centre of the peptide backbones, forming an angle of  $120^\circ$  (in conformers  $be^*b^*$  or  $bb^*b^*$ ) or  $180^\circ$  (in conformers  $ee^*b^*$  or  $eb^*b^*$ ) with the C-terminal D-Ala-D-Ala. The  $N^\epsilon$ -acetyl group of the L-Lys residue and the carbonyl carbon which undergoes attack by the enzyme's serine residue are separated from each other by a distance of 10 Å or more (Fig. 10).

### 2.3 Formation of the serine ester-linked penicilloyl ( $\Delta^3$ -cephalosporoyl)-enzyme intermediates

Penicillins and  $\Delta^3$ -cephalosporins also readily overcome the energy barrier to enzyme acylation but the reaction flux stops at the acyl-enzyme intermediate, permitting direct determination of the bimolecular rate constant of enzyme acylation ( $k_2/K$ ) and, in some favourable cases, the individual  $K$  and  $k_2$  values.

Two preliminary points deserve attention. (i) With the  $\beta$ -lactam compounds, the  $k_3$  term of the reaction is so small that the ratio  $k_2/K$  has no longer its usual physiological meaning of expressing the enzyme catalytic efficiency (turnover number/ $K_m$ ). If, for example, the interaction between the enzyme and a given antibiotic is characterized by  $K = 1$  mM,  $k_2 = 10$  s $^{-1}$  and  $k_3 = 10^{-3}$  s $^{-1}$ , then the "theoretical"  $K_m$  [ $k_3 \cdot K / (k_2 + k_3)$ ] is  $10^{-7}$  M and the "catalytic efficiency"  $k_2/K$  refers to the enzyme activity at an antibiotic concentration as low as  $10^{-8}$  M, i.e.,  $0.1 \cdot K_m$  (under these conditions  $k_2 \cdot [S] / K = 10^{-4}$  s $^{-1}$ ; it is lower than the  $k_3$  value and deacylation of the acyl-enzyme intermediate is not rate-limiting). (ii) Both rapid formation and high stability of the penicilloyl (cephalosporoyl)-enzyme intermediates are essential features of the process of enzyme inactivation. When used at a  $10$   $\mu$ M concentration (which is lower than the  $K$  value), a  $\beta$ -lactam compound that exhibits, for a given enzyme, a  $k_2/K$  value of  $1000$  M $^{-1}$ ·s $^{-1}$  or more, and a  $k_3$  value of  $10^{-4}$  s $^{-1}$  or less, immobilizes 99% or more of the enzyme in the form of the acyl-enzyme intermediate in the steady-state, and the time required for the acyl-enzyme to reach 95% of the steady state level is about 5 min or less. The efficacy of a  $\beta$ -lactam compound as an enzyme inactivator may be expressed by

$$K_i = \frac{k_3 K}{k_2 + k_3} \text{ or, since } k_3 \ll k_2,$$

by

$$K_i = \frac{k_3 K}{k_2}$$

TABLE III

Dihedral angles of the main backbone of Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala and of benzylpenicillin and cephaloglycine<sup>a</sup>

	Degrees of angle (°)							
	$\phi_1$	$\psi_1$	$\omega_1$	$\phi_2$	$\psi_2$	$\omega_2$	$\phi_3$	$\psi_3$
Ac <sub>2</sub> -L-Lys-D-Ala-D-Ala								
ee*b*	-80	80	180	80	-80	180	160	20
be*b*	-140	140	180	80	-80	180	160	20
eb*b*	-80	80	180	160	-160	180	160	20
bb*b*	140	140	180	160	-160	180	160	20
Benzylpenicillin			169	-93	-128	136	149	42
Cephaloglycine			175	-138	-120	154	36	55

<sup>a</sup> Calculated from X-ray data and as reported by Virudachalam and Rao (1977).

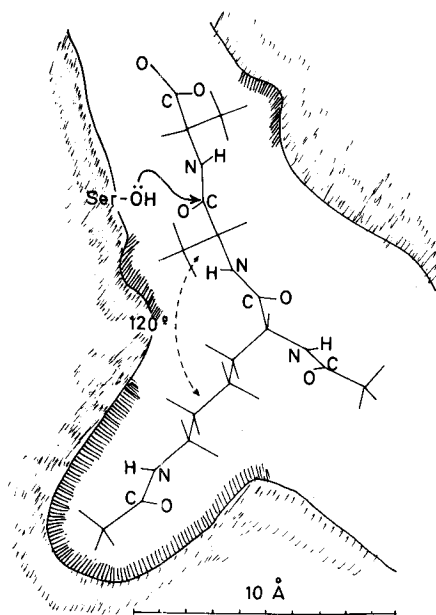


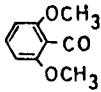
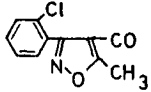
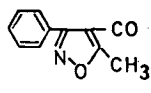
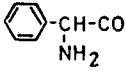
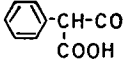
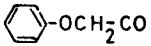
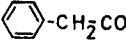
Fig. 10. Side chain to backbone arrangement found in the highly probable conformer bb\*b\* of the carbonyl donor Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala.

The lower the  $K_i$  value, the more potent is the inactivator. However, if  $k_3$  is extremely small,  $K_i$  is necessarily small even if  $k_2/K$  itself is small; hence,  $K_i$  becomes meaningless. Indeed, under these conditions, at an antibiotic concentration equivalent to  $K_i$ , the formation of the acyl-enzyme intermediate may be so slow that within the time used for the experiment, it may not occur at all, i.e. the enzyme may remain fully active.

The basic information gained by expressing in the form of  $k_2/K$ , the propensity of the R39 and R61 enzymes to undergo acylation by various comparable penams (Table IV) and 3-cephems (compounds 9–12 in Table V) can be summarized as follows (Frère et

TABLE IV

Bimolecular rate constants for the acylation of the serine R61 and R39 enzymes by penicillins (at pH 7.5 and 37°C, unless otherwise stated) and the hydrolysis of the same penicillins by OH<sup>-</sup> (at 37°C)

	R	R61 enzyme (M <sup>-1</sup> · s <sup>-1</sup> )	R39 enzyme (M <sup>-1</sup> · s <sup>-1</sup> )	OH <sup>-</sup> (M <sup>-1</sup> · s <sup>-1</sup> )
1	H	0.2	900	0.15
2		15	1 100 <sup>a</sup>	0.45
3		30	15 000	0.50
4		130	40 000	0.50
5		110	74 000 <sup>a</sup>	0.57
6		820	2 900 <sup>a</sup>	0.50
7		1 500	>70 000	0.85
8		14 000 <sup>b</sup>	300 000	0.50

1 = 6-APA; 2 = methicillin; 3 = cloxacillin; 4 = oxacillin; 5 = ampicillin; 6 = carbenicillin; 7 = phenoxy-methylpenicillin; 8 = benzylpenicillin.

<sup>a</sup> At 20°C.

<sup>b</sup> At 25°C.

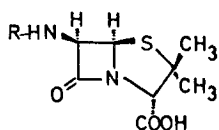
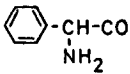
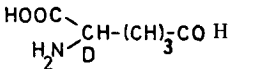
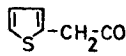
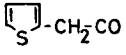
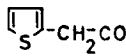
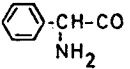


TABLE V

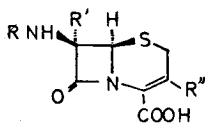
Bimolecular rate constants for the acylation of the serine R61 and R39 enzymes by  $\Delta^3$ -cephalosporins (at pH 7.5 and 37°C unless otherwise stated) and the hydrolysis of the same  $\Delta^3$ -cephalosporins by  $\text{OH}^-$  (at 37°C)

	R	R'	R''	R61 enzyme	R39 enzyme	$\text{OH}^-$
				( $\text{M}^{-1} \cdot \text{s}^{-1}$ )	( $\text{M}^{-1} \cdot \text{s}^{-1}$ )	( $\text{M}^{-1} \cdot \text{s}^{-1}$ )
9	H	H	$\text{CH}_2\text{-O-CO-CH}_3$	14	200 <sup>a</sup>	0.30
10		H	$\text{CH}_2\text{-O-CO-CH}_3$	22	74 000 <sup>a</sup>	0.90
11		H	$\text{CH}_2\text{-O-CO-CH}_3$	1 150	67 000 <sup>a</sup>	0.25
12		H	$\text{CH}_2\text{-O-CO-CH}_3$	3 000	> 70 000	0.75
13		$\text{OCH}_3$	$\text{CH}_2\text{-O-CO-CH}_3$	1 000	7 000	0.30
14		H	$\text{CH=CH-C}_6\text{H}_4\text{-NO}_2$	460 <sup>b</sup>	3 000 000 <sup>b</sup>	10
15		H	$\text{CH}_3$	4	3 000 <sup>a</sup>	0.10

9 = 7-ACA; 10 = cephaloglycine; 11 = cephalosporin C; 12 = cephalothin; 13 = cefoxitin; 14 = nitrocefepin; 15 = cephalixin.

<sup>a</sup> At 20°C.

<sup>b</sup> At 10°C.



al., 1974b, c, 1975a, b, 1980; Fuad et al., 1976; and unpublished results): (i) The very low acylating ability (low  $k_2/K$  values) exhibited by the unsubstituted 6-amino-penicillanic and 7-aminocephalosporanic acid, can be considerably enhanced (high  $k_2/K$  values) by the presence of a suitable 6(7)  $\beta$ -substituent. (ii) The observed effects cannot be attributed to variations in the chemical reactivity of the  $\beta$ -lactam compounds since, irrespective of the side chains, their bimolecular rate constants for hydrolysis by  $\text{OH}^-$  (at 37°C) show very little variation ( $0.15\text{--}0.9 \text{ M}^{-1} \cdot \text{s}^{-1}$ ). (iii) The effect exerted by a given side chain on the  $k_2/K$  value is enzyme specific. (iv) With all the  $\beta$ -lactam compounds tested, acylation of the R39 enzyme is always more effective than acylation of the R61

enzyme. (v) Finally, as shown by the interactions between the R61 enzyme and 6-aminopenicillanic acid, carbenicillin, ampicillin and benzylpenicillin, respectively (Table VI), the 6 $\beta$ -substituent appears to have relatively little influence on the efficiency of the initial binding ( $K$ ) but exerts an enormous effect on the rate of enzyme acylation ( $k_2$ ).

The conclusions seem to be obvious (Ghuysen et al., 1979, 1980). The part of the antibiotic molecule which is primarily involved in initial binding of the penicillins and  $\Delta^3$ -cephalosporins must be the bicyclic fused ring system; in turn, the part of the molecule which largely dictates the effectiveness of the acylation step must be the 6(7) $\beta$ -substituent of the  $\beta$ -lactam ring. The most likely interpretation is that by interacting with some amino acids in the active site of the enzyme, a suitable 6(7) $\beta$ -side chain in the bound  $\beta$ -lactam compound induces conformational isomerization in the protein which in turn results in the establishment of the correct active geometry.

That conformational changes occur concomitantly with penicilloylation of the R61 enzyme is suggested by fluorescence quenching and alterations in the CD spectrum of the protein (Nieto et al., 1973b). X-Ray crystallographic studies also support this view. On the basis of the different Fourier map obtained after diffusion of *o*-iodophenylpenicillin in a native crystal of the R61 enzyme, the site of interaction has been visualized as a 22 Å segment elongated in the  $y$  direction of the map. The map also shows another peak at similar  $x$  and  $y$  coordinates but a lower  $z$  coordinate which, most likely, is associated with a conformational change induced in the structure upon interaction with this penicillin (DeLucia et al., 1980).

To some extent, initial binding also depends on the structure of the 6(7) $\beta$ -substituent and, reciprocally, the acylation step is also controlled by the structure of the bicyclic fused ring system. Thus, for example:

(i) The  $K$  values for the binding to the R61 enzyme of the four penams considered in Table VI range between about 0.1 and 10 mM depending on the structure of the 6 $\beta$ -substituent. Conversely, the comparable pair ampicillin–cephalexin (compounds 5 and 15) shows that the penam is a better acylating agent than the 3-cephem, although the two compounds have the same 6(7) $\beta$ -substituent.

(ii) If the acaudal 3-methyl side chain of cephalexin (compound 15) is substituted by an acetoxy group, thus increasing the tendency of the C(3) substituent to withdraw

TABLE VI

Penicilloylation (cephalosporoylation) of the serine D-alanyl-D-alanine peptidases

Enzyme	Antibiotic	$K$ (mM)	$k_2$ ( $s^{-1}$ )
R61	6-APA	1	0.0002
	Carbenicillin	0.11	0.09
	Ampicillin	7	0.8
	Benzylpenicillin	13 <sup>a</sup>	180 <sup>a</sup>
R39	Cephalosporin C	0.19 <sup>b</sup>	13b

At pH 7.5 and 37°C unless otherwise stated.

<sup>a</sup> At 25°C.

<sup>b</sup> At 26°C.

electrons from the nucleus, the resulting 3-cephem cephaloglycine (compound 10) gains a much higher acylating ability. If the  $-\text{CH}_2-\text{O}-\text{CO}-\text{CH}_3$  group of cephalothin (compound 12) is replaced by the more potent electron-withdrawing dinitrostyryl group, the resulting 3-cephem nitrocefim (compound 14) becomes an acylating agent of the R39 enzyme of extraordinary efficacy. The  $k_2/K$  value is  $3 \cdot 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ , i.e. only 30–300-fold smaller than the bimolecular rate constant value ( $10^8 - 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ ) for the diffusional approach of a small molecule with a macromolecule such as an enzyme. With the R61 enzyme, however, nitrocefim and cephalothin are acylating agents of similar potency (at  $37^\circ \text{C}$ ).

(iii) It is generally accepted that a relatively unhindered  $\alpha$  face of the  $\beta$ -lactam molecule is required for antibacterial activity while sensitivity of  $\beta$ -lactamase is suppressed at least in part, by the presence of  $7\alpha$ -substituents in  $\Delta^3$ -cephalosporins. Compromises at this level are possible which confer on cephamycins such as cefoxitin, both low  $\beta$ -lactamase sensitivity and high antibiotic potency. In agreement with this view, comparison of cephalothin and cefoxitin (compounds 12 and 13) shows that, depending on the enzyme, the presence of a  $7\alpha$ -methoxy group causes only a 3–10-fold decrease in the  $k_2/K$  value.

In the induced fit model proposed for enzyme acylation by penicillins (or  $\Delta^3$ -cephalosporins) and by peptides terminating in L-R-D-Ala-D-Ala, respectively, the fused ring system or the D-Ala-D-Ala dipeptide on the one hand, and the  $6(7)\beta$ -substituent on the  $\beta$ -lactam ring or the side chain at the L-centre of the peptide on the other hand, appear to play similar functions. An analysis of the isoterism which may exist between the two carbonyl donors under consideration deserves attention.

Steric constraints within the fused bicyclic system of the penams and 3-cephems make it impossible for the four groups attached to the C–N bond of the  $\beta$ -lactam grouping to lie in a common plane. Consequently, the dihedral angles  $\omega_2$  in penicillins and  $\Delta^3$ -cephalosporins in their ground state conformations ( $135^\circ$  and  $155^\circ$ , respectively) are considerably smaller than that about the peptide bond ( $180^\circ$ ) of a D-Ala-D-Ala dipeptide. In addition, among the two sets of angles  $(\phi_2, \psi_2)$  and  $(\phi_3, \psi_3)$ ,  $\psi_2$  and  $\phi_3$  are fixed to specific values by the lactam and the thiazolidine (dihydrothiazine) ring. Hence the conformation depends on the two variable angles  $\phi_2$  and  $\psi_3$  which determine the orientation of the aminoacyl substituent and the carboxyl grouping, respectively (Virudachalam and Rao, 1977).

Fig. 11 a and b are stereoscopic views in which conformers  $ee^*b^*$  and  $bb^*b^*$ , respectively, of  $\text{Ac}_2\text{-L-Lys-D-Ala-D-Ala}$  have been superimposed on benzylpenicillin so that the corresponding carbonyl carbons which undergo attack by the active serine coincide (De Coen et al., 1981). As the views show, some structural resemblance exists between the D-Ala-D-Ala sequence of the peptide and the corresponding backbone of the penam nucleus. The 3-cephem nucleus, however, must show a poorer structural analogy because of the large differences that exist between its  $\phi_3$  angle (as determined from X-ray data) and that of D-Ala-D-Ala (Table III). Hence, although binding to the enzymes strictly requires the occurrence of D-amino acids at both the penultimate and C-terminal positions of the peptides (see above), it is clear that the active site may accommodate compounds exhibiting large differences in their overall geometry. The idea that penicillin in its ground state conformation would be perfectly isosteric with D-Ala-D-Ala in its transition state conformation (Tipper and Strominger, 1965; Lee, 1971) and would function as a true transition state analogue, is very unlikely. Indeed, the high dissociation

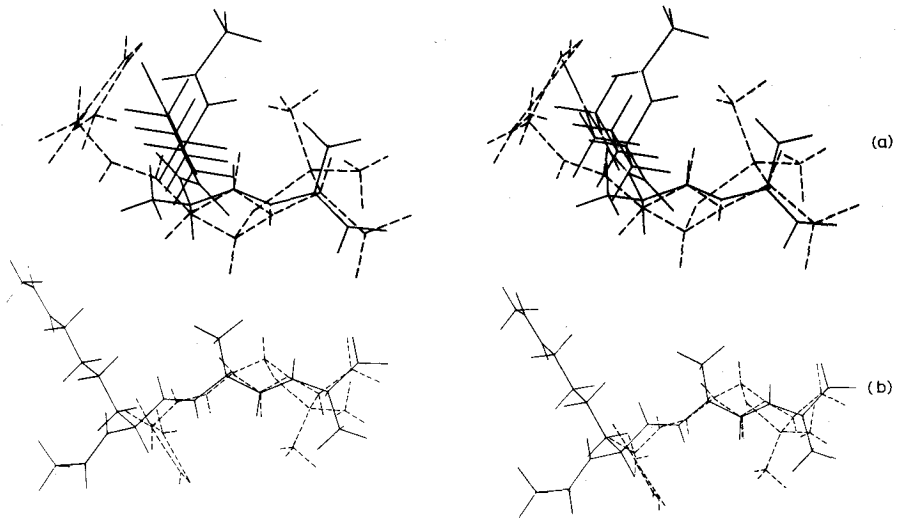
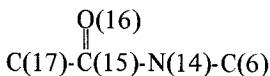


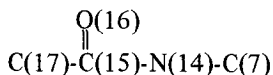
Fig. 11. Superimposition of benzylpenicillin (constructed on the basis of the crystallographic data of Dexter and van der Veen (1978)), and the D-Ala-D-Ala sequence of conformers ee\*b\* (a) and bb\*b\* (b) of Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala.

constants of the Michaelis complexes formed between the enzymes and the  $\beta$ -lactam antibiotics exclude such a possibility (the closer the analogue is to the transition state, the tighter should be the binding to the enzyme).

The atoms



in penicillins or



in  $\Delta^3$ -cephalosporins are coplanar. This plane, which comprises the exocyclic amide bond, includes three dihedral angles, one of which,  $\omega_1$ , is fixed at a value close to  $180^\circ$  as found in normal amide bonds while the two others  $\psi_1$  and  $\phi_2$  are variable. Average  $\phi_2$  values for active penicillins or  $\Delta^3$ -cephalosporins seem to be about  $-90^\circ$  to  $-140^\circ$  in the solid state (as determined by X-ray crystallography) or about  $-150^\circ$  in solution (as determined by NMR) (Virudachalam and Rao, 1977). The angle  $\psi_1$  can also vary. Side chains may have a high degree of rotational freedom around the C<sub>15</sub>-C<sub>17</sub> bond as in benzylpenicillin (Fig. 11). Conversely, steric hindrance and increased rigidity of this side chain can be produced by multiple substitution at C<sub>17</sub> or by incorporating C<sub>17</sub> in an aromatic or



heterocyclic nucleus. Such a situation occurs in methicillin, oxacillin and cloxacillin. The views of Fig. 11 fail to reveal any structural analogy between the side chain at the  $6\beta$ -position of benzylpenicillin and that at the L-centre of  $\text{Ac}_2\text{-L-Lys-D-Ala-D-Ala}$ . Moreover, on the basis of the data of Tables I, IV and V, side chains in the peptides or in penicillins ( $\Delta^3$ -cephalosporins) which generate equivalent turnover number/ $K_m$  or  $k_2/K$  values with a given enzyme are, to all appearances, structurally unrelated.

Although there is still a long way to go, we suspect that the serine D-alanyl-D-Ala peptidases which possess one common binding site able to accommodate D-Ala-D-Ala or the fused  $\beta$ -lactam ring systems (binding site 1), also possess two distinct sites specifically devised to bind the lateral chain of the bound peptide and the  $6(7)\beta$ -substituent of the bound  $\beta$ -lactam compound, respectively (binding sites 2). By interacting with their relevant binding sites 2, the peptide side chain and the  $6(7)\beta$ -lactam substituent would confer on the induced catalytic site a conformation specifically devised to operate on the amide linkage of D-Ala-D-Ala and that of the  $\beta$ -lactam ring, respectively (Ghuysen et al., 1979). Following this view, the penicillins ( $\Delta^3$ -cephalosporins) might be regarded as weak isosteric inhibitors of the serine D-alanyl-D-Ala peptidases (through their  $\beta$ -lactam fused ring system), that can be converted into powerful enzyme inactivators as a result of a correct interaction between the  $6(7)\beta$ -substituent and an "allosteric" subsite close to the active site.

#### 2.4 Stabilization of the serine ester-linked penicilloyl ( $\Delta^3$ -cephalosporoyl)-enzyme intermediates

The penicilloyl (cephalosporoyl)-enzyme intermediates are very stable. Table VII gives the half-lives of some of them (Frère et al., 1974b, c, 1975a, b, 1980; Fuad et al., 1976, and unpublished results). If one excepts 6-aminopenicillanic acid, those formed with the R39 enzyme are always more stable than those formed with the R61 enzyme. Since the R39 enzyme also has the highest propensity to undergo acylation (Tables IV and V), it follows that this enzyme is much more "sensitive" to  $\beta$ -lactam antibiotics than the R61 enzyme.

Extensive studies have been carried out on the acyl-enzyme intermediate formed between benzylpenicillin and the R61 enzyme (Frère et al., 1975c, 1976b, 1978a; Adriaens et al., 1978; Marquet et al., 1979). Study of the R39 enzyme (Frère et al., 1975c) and the *Bacillus* enzymes (Hammarström and Strominger, 1975, 1976) led to similar conclusions.

The serine ester-linked benzylpenicilloyl-R61 enzyme intermediate is not susceptible to attack by water or by a transpeptidation acceptor. Enzyme deacylation slowly occurs (half-life: 80 min at  $37^\circ\text{C}$ ), but remarkably, the penicilloyl group is not released as such (Fig. 12). In fact, enzyme deacylation requires prior fragmentation of the penicilloyl group, a reaction which slowly occurs by C(5)-C(6) cleavage and transfer of a proton from water to C(6). As a result of the fragmentation reaction, the original benzylpenicilloyl-enzyme intermediate is converted into a phenylacetyl-glycyl-enzyme intermediate which, once formed, is immediately attacked by water or a suitable  $\text{H}_2\text{N-R}$  compound. As a result, the enzyme is regenerated and phenylacetyl-glycine or a mixture of phenyl-

TABLE VII

Stability of the acyl-enzyme intermediates formed between penicillins and  $\Delta^3$ -cephalosporins, and the serine of R61 and R39 enzymes

Enzyme	Antibiotic (numbers refer to those of Tables IV and V)	$k_3$ ( $s^{-1}$ )	Half-life of the intermediate (min)
R61	6-aminopenicillanic acid (1)	$< 6 \times 10^{-5}$	$> 2000$
	ampicillin (5)	$1.4 \times 10^{-4}$	80
	carbenicillin (6)	$1.4 \times 10^{-4}$	80
	phenoxymethylpenicillin (7)	$2.8 \times 10^{-4}$	40
	benzylpenicillin (8)	$1.4 \times 10^{-4}$	80
	7-aminocephalosporanic acid (9)	$4.4 \times 10^{-3}$	2.5
	cephalglycine (10)	$3 \times 10^{-6}$	3800
	cephalosporin C (11)	$1 \times 10^{-6}$	10000
	cefoxitin (13)	$5 \times 10^{-5}$	220
	nitrocefin (14)	$3 \times 10^{-4}$	40
	R39	6-aminopenicillanic acid (1)	$5.6 \times 10^{-3}$
ampicillin (5)		$4.4 \times 10^{-6}$	2600
carbenicillin (6)		$5.4 \times 10^{-6}$	2125
benzylpenicillin (8)		$2.8 \times 10^{-6}$	4100
7-aminocephalosporanic acid (9)		$1.1 \times 10^{-6}$	11580
cephalglycine (10)		$8 \times 10^{-7}$	14000
cephalosporin C (11)		$3 \times 10^{-7}$	38000
cefoxitin (13)		$< 3 \times 10^{-5}$	$> 370$
nitrocefin (14)		$1.8 \times 10^{-6}$	7700
cephalexin (15)		$2.4 \times 10^{-6}$	4700

At 37°C and pH 7.5.  $k_3$  ( $s^{-1}$ ) and half-life (in s) are related to each other by half-life =  $(- \ln 0.5)/k_3$ .

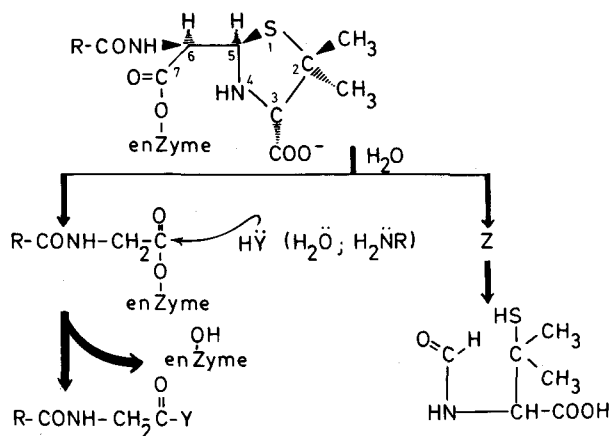


Fig. 12. Fragmentation of the benzylpenicilloyl-R61 enzyme intermediate.

acetylglycine and a phenylacetylgllycyl-NH-R compound (on a competitive basis) is released. The other fragmentation product which arises from the thiazolidine part of the penicilloyl group, is an unidentified compound Z. Compound Z has no detectable thiol groups but degrades further with a half-life of 10–15 min at 37°C to *N*-formyl-D-penicillamine.

A central feature of the mechanism of enzyme deacylation is that fragmentation of the enzyme-bound benzylpenicilloyl group is the rate-limiting step, i.e. causes immediate destabilization of the acyl–enzyme intermediate. The stability of the acyl–enzyme thus appears to be due to the monocyclic thiazolidine ring. The most likely conclusion (Ghuysen et al., 1979) is that formation of the ester linkage between C(7) [C(8)] of the penicillin ( $\Delta^3$ -cephalosporin) molecule and the serine residue (binding site 1) of the enzyme centre involves additional interactions not only between the 6(7) $\beta$ -substituent and an enzyme binding site 2, but also between the monocyclic thiazolidine (dihydrothiazine) ring and another binding site 3 (Fig. 13).

The three-dimensional disposition of the enzyme binding sites 1, 2 and 3 appears to be an important factor that governs the stability of the intermediate and the fate of the bound metabolite. This disposition depends on the conformation of the acyl–enzyme intermediate and therefore on both the enzyme and the  $\beta$ -lactam compound considered. Such a model explains the widely varying stabilities exhibited by the penicilloyl (cephalosporoyl)–enzyme intermediates (Table VII) and the fact that, depending on the enzyme and the conditions used, denaturation of the benzylpenicilloyl–enzyme intermediates may increase or decrease their half-lives (Table VIII). In all cases, however, breakdown of such denatured complexes results in the release of penicilloic acid. The degradation products arising from  $\Delta^3$ -cephalosporins have not been studied in detail but it is likely that the reaction pathway differs from that observed with benzylpenicillin. Nitrocefin is not fragmented as a result of its interaction with the R61 enzyme, but is converted to a product which to all appearances is identical to that obtained by  $\beta$ -lactamase action. The thiazolidine (dihydrothiazine) ring and the 6(7) $\beta$ -substituent exert a concerted action on the stability of the intermediate and the pathway through which its breakdown occurs. The intermediates formed between the unsubstituted 6-aminopenicillanic acid and the R61 and R39 enzymes are long- and short-lived, respectively; conversely, those formed between the unsubstituted 7-aminocephalosporanic acid and the same R61 and

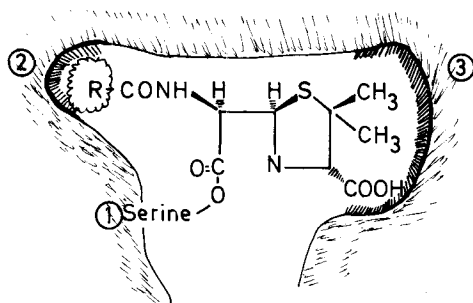


Fig. 13. Schematic view of a penicilloyl–enzyme intermediate showing the covalent attachment to the enzyme serine residue (binding site 1) and the additional enzyme binding sites 2 and 3.

R39 enzymes, are short- and long-lived, respectively (Table VII). In addition, breakdown of the intermediates formed between the R39 enzyme and 6-aminopenicillanic acid or the R61 enzyme and 7-cephalosporanic acid gives rise to several (4–6) ninhydrin-positive compounds. One of them is indistinguishable from the degradation products obtained as a result of  $\beta$ -lactamase action. None of them is glycine, the product which should have arisen by fragmentation of the acyl moiety (J. Kelly, J.M. Frère and J.M. Ghuysen, unpublished results).

### 2.5 2-Cephems, 2-penems and carba-2-penems

Noncoplanarity of the four groups attached to the C–N bond of the  $\beta$ -lactam grouping labilizes the system. Departure from coplanarity can be expressed by the distance  $h$  between the apex and the base of a trigonal pyramid where the  $\beta$ -lactam nitrogen is at the apex and its three substituents at the corners of the base (Woodward, 1980). The distance in the  $\Delta^3$ -cephalosporins is already considerable ( $h = 0.24$ – $0.32$  Å) and is greater in the penicillins ( $h = 0.38$ – $0.40$  Å).  $\beta$ -Lactam compounds exist which are both more and less reactive than the  $\Delta^3$ -cephalosporins and penicillins, and some of these have been investigated (Fig. 14).

The  $\Delta^2$ -cephalosporins have less ring strain ( $h = 0.06$ – $0.10$  Å) than the  $\Delta^3$ -cephalosporins. It has been proposed that the lack of antibacterial activity of the  $\Delta^2$ -cephalosporins is due to their inability to overcome the height of the barrier to react with the enzyme. Study of the comparable pair of  $\Delta^3$ - and  $\Delta^2$ -cephalosporins (compounds 16 and 17 in Fig. 14) shows that with the R39 enzyme, the 3- and 2-cephems have, as expected, fairly high ( $3300 \text{ M}^{-1} \cdot \text{s}^{-1}$ ) and low ( $50 \text{ M}^{-1} \cdot \text{s}^{-1}$ )  $k_2/K$  values, respectively. With the R61 enzyme, however, both compounds have the same low  $k_2/K$  value ( $60$ – $70 \text{ M}^{-1} \cdot \text{s}^{-1}$ ) demonstrating that with this particular enzyme, the differences in the  $h$  values of the two  $\beta$ -lactam rings considered have no influence on the rate of acylation. Moreover, the acyl-enzyme intermediates formed with the R39 enzyme are very stable. With the R61 enzyme, the intermediate formed with the 3-cephem is also very stable (half-life 40 h) but, surprisingly, that formed with the 2-cephem is fairly unstable (half-life 230 s).

TABLE VIII

Half-lives of benzylpenicilloyl-enzyme intermediates; effect of denaturation on the stability of the intermediates and the nature of the breakdown products

Benzyl- penicilloyl- R61 enzyme	Native	80 min	PAG + FPA
	Denatured by		
	heat ( $100^\circ\text{C}$ )	80 h	penicilloate
	heat + trypsin	35 h	penicilloate
	6 M guanidine-HCl in water	several days	penicilloate
	6 M guanidine-HCl in methanol	100 min	penicilloate ( $\alpha$ -methyl ester)
Benzyl- penicilloyl- R39 enzyme	Native	70 h	PAG (+ FPA?)
	Denatured by		
	heat ( $100^\circ\text{C}$ )	10 h	penicilloate
	heat + trypsin	very labile	penicilloate

PAG = phenylacetyl-glycine; FPA = *N*-formyl-D-penicillamine.

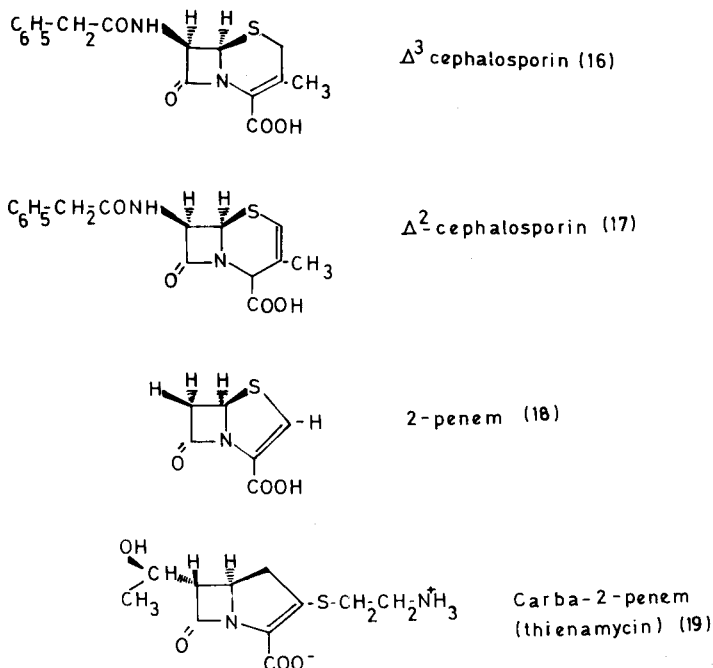


Fig. 14. Unusual  $\beta$ -lactam compounds. The  $\Delta^2$ - and  $\Delta^3$ -cephalosporins were gifts from Professor H. Vanderhaeghe, Rega Institute, Leuven, Belgium, the 2-penem from Drs. I. Ernst and H.R. Pfandler from the Woodward Research Institute, Basel, Switzerland, and the *N*-formimidoyl derivative of thienamycine from Merck Institute for Therapeutic Research, Rahway, NJ, U.S.A.

At the other extreme of the scale of  $\beta$ -lactam reactivity, the unsubstituted 2-penem shown in Fig. 14 (compound 18;  $h \approx 0.44 \text{ \AA}$ ) behaves as a substrate: the  $K_m$  app and  $k_{cat}$  values are  $85 \mu\text{M}$  and  $2.7 \text{ min}^{-1}$  with the R61 enzyme, and  $4 \mu\text{M}$  and  $0.42 \text{ min}^{-1}$  with the R39 enzyme. Conversely, the *N*-formimidoyl derivative of the carba-2-penem thienamycin (compound 19 in Fig. 14;  $h = 0.50 \text{ \AA}$ ) is a reasonably effective enzyme inactivator: the  $k_2/K$  and  $k_3$  values are  $1000 \text{ M}^{-1} \cdot \text{s}^{-1}$  and  $< 7 \cdot 10^{-6} \text{ s}^{-1}$ , respectively, with the R61 enzyme, and  $10\,000 \text{ M}^{-1} \cdot \text{s}^{-1}$  and  $< 2 \cdot 10^{-6} \text{ s}^{-1}$ , respectively, with the R39 enzyme. Note that thienamycin is unusual because of the occurrence of a hydroxyethyl group at the  $6\alpha$ -position (i.e. in a *trans* sense) and a cysteamine side chain at position 2.

Although the road to a full understanding of the mechanism of enzyme acylation is still unpaired in many places, we think that the process does not necessarily require a carbonyl donor whose amide bond is labilized in its ground state conformation (i.e., a distorted nitrogen atom).  $\text{Ac}_2\text{-L-Lys-D-Ala-D-Ala}$  is an excellent substrate and the nitrogen atom of the D-Ala-D-Ala peptide bond is planar. However, beyond a certain level of distortion, enzyme acylation is greatly facilitated by the presence of a suitable  $6(7)\beta$ -side chain on the  $\beta$ -lactam ring, or cannot occur unless the peptide possesses a suitable side chain at its L-centre. Side chains effective in promoting enzyme acylation have widely varying structures depending on whether the carbonyl donor is a peptide terminating in

D-Ala-D-Ala or a  $\beta$ -lactam compound with a 2-cephem, a 3-cephem or a penam nucleus. With  $\beta$ -lactam compounds that are sufficiently distorted (the 2-penems and carba-2-penems), the picture is different. These compounds may acylate the enzymes only by virtue of their highly electrophilic  $\beta$ -lactam carbonyl carbon. Of course, they must complement the active site and the enzyme itself is not inert, the entropic contribution that results from a correct positioning of the  $\beta$ -lactam compound in the enzyme cavity greatly contributing to the efficacy of the reaction. Finally, enzyme acylation may give rise to stable acyl-enzyme intermediates if the amine fragment of the carbonyl donor, i.e. the "leaving" group in the acylation reaction, cannot depart from the enzyme cavity — as happens with the  $\beta$ -lactam compounds. Yet the half-lives of these acyl-enzyme intermediates vary enormously and appear to be governed by a complex interplay between several sites on the enzyme, the bicyclic ring system itself and its substituents.

### 3. The zinc-containing D-alanyl-D-Ala peptidase

A highly penicillin-resistant D-alanyl-D-Ala peptidase of  $M_r$  20 000 is excreted by *Streptomyces albus* G. It functions solely as a hydrolase (Pollock et al., 1972) and is a potent bacteriolytic agent (Ghuysen et al., 1970). Like the R39 and R61 enzymes, the G enzyme has been purified to protein homogeneity (Duez et al., 1978); it is available in a truly water-soluble form and consists of a single polypeptide chain.

Hydrolysis of peptides terminating in L-R-D-Ala-D-Ala by the G enzyme involves an active centre-bound  $Zn^{2+}$  (Dideberg et al., 1980b). The apoprotein binds  $Zn^{2+}$  with an association constant of about  $2 \cdot 10^{14} M^{-1}$  (a value close to that found with EDTA),  $Zn^{2+}$  is required for carboxypeptidase activity and, to all appearances, it plays an essential catalytic function.  $Co^{2+}$  also binds stoichiometrically to the G apoenzyme, a feature which should facilitate study by NMR of the amino acid residues that must serve as ligands to the metal ion. The precise mechanistic properties of the G enzyme are unknown. In particular, it is not known whether the donor peptide, upon binding to the enzyme, itself becomes a ligand of the active site metal ion or whether a molecule of water does.

The induced fit model proposed for the serine D-alanyl-D-Ala peptidases also applies to the hydrolysis of peptides terminating in L-R-D-Ala-D-Ala by the G enzyme (see Table I). Initial binding depends on the C-terminal dipeptide sequence and the lateral chain at the L-centre of the peptide brings the enzyme-substrate system into the active conformation (Leyh-Bouille et al., 1970b). Peptide inhibitors of the hydrolysis of Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala by the R61 enzyme are even better inhibitors of the G enzyme (Table II) (Nieto et al., 1973a). Inhibition by the dipeptide Ac-D-Ala-D-Glu is competitive ( $K_i = 0.3$  mM).

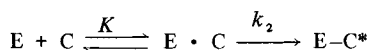
In contrast to the serine D-alanyl-D-Ala peptidases, the G enzyme does not possess any binding site for an acceptor; it cannot catalyse transpeptidation (Pollock et al., 1972). In addition, the G enzyme is very highly resistant to the penicillins and the  $\Delta^3$ -cephalosporins (Leyh-Bouille et al., 1970c). The propensity of the G enzyme to form a stable adduct with benzylpenicillin is  $10^6$ – $10^7$  times lower than that observed with the serine R61 and R39 enzymes, respectively, and only about 10–100 times higher than that

observed with lysozyme or insulin (Duez et al., 1981a). Yet, removal of  $Zn^{2+}$  from the G enzyme results in an apoprotein which is as resistant to penicillin action as any common protein or large polypeptide. The native adduct formed with benzylpenicillin (under forcing conditions) spontaneously breaks down in water with a half-life of 180 min at  $37^{\circ}C$  and pH 7.5 leading to enzyme regeneration and release of benzylpenicilloic acid. The adduct is thus probably a penicilloyl-enzyme derivative but the nature of the amino acid which undergoes penicilloylation (with great difficulty) is unknown. The G enzyme thus behaves as a  $\beta$ -lactamase of very low efficiency. Its turnover number for benzylpenicillin is about  $5 \cdot 10^6$ -fold lower than that observed with the true exocellular  $\beta$ -lactamase which is also excreted by *Streptomyces albus* G (Duez et al., 1981b).

Kinetically, the interaction between the G enzyme and phenoxymethylpenicillin, cephalothin and cephalosporin C proceeds according to the same three-step reaction (1) as that described for the serine D-alanyl-D-Ala peptidases (Frère et al., 1978b). The resistance of the G enzyme to  $\beta$ -lactam antibiotics is due neither to a lack of binding efficiency (at least with the three  $\Delta^3$ -cephalosporins examined; Table IX), nor to a lack of stability of the intermediates once they have been formed after prolonged incubations in the presence of high concentrations of antibiotics (Table X), but to the fact that, irrespective of the 6(7) $\beta$ -substituent of the  $\beta$ -lactam ring, the acylation step is always

TABLE IX

Reaction of the G  $Zn^{2+}$  enzyme with  $\beta$ -lactam antibiotics:



Antibiotic	K (mM)	$k_2$ ( $s^{-1}$ )	$k_2/K$ ( $M^{-1} \cdot s^{-1}$ )
Phenoxymethylpenicillin	150	$8 \times 10^{-4}$	0.005
<i>p</i> -Iodo- $\beta$ -phenylacetyl-aminocephalo- sporic acid	5	$6 \times 10^{-4}$	0.11
Cephalosporin C	1.6	$1 \times 10^{-4}$	0.06
Cephaloglycine	9.5	$5 \times 10^{-4}$	0.06

At pH 7.5 and  $37^{\circ}C$ .

$k_2/K$  values smaller than  $2 \times 10^{-3} M^{-1} \cdot s^{-1}$  have been found with 6-APA, benzylpenicillin, carbenicillin, methicillin, oxacillin, cloxacillin, 7-ACA, cephalothin, nitrocefin and cefoxitin.

TABLE X

Stability of the adducts formed between the G  $Zn^{2+}$  enzyme and  $\beta$ -lactam antibiotics

Antibiotic	Half-life of the adduct (min)
Benzylpenicillin	180
Phenoxymethylpenicillin	130
Cephalothin	350
Cephalosporin C	150

At pH 7.5 and  $37^{\circ}C$ .

very slow ( $1-5 \cdot 10^{-4} \text{ s}^{-1}$ ; Table IX) (Frère et al., 1978b). 7-Aminocephalosporanic acid behaves essentially as a competitive, reversible inhibitor ( $K_i = 0.2 \text{ mM}$ ; J. Kelly, J.M. Frère and J.M. Ghuysen, unpublished results). The reason why the acylation step is so slow is not understood. One possibility is that the  $\text{Zn}^{2+}$ -containing active site has no binding site able to complement 6(7)-substituents occurring on the  $\beta$ -face of the  $\beta$ -lactam ring and therefore is unable to adopt the geometry necessary for enzyme action.

The G enzyme has been crystallized (Dideberg et al., 1979). X-ray structure analysis at 4.5 Å resolution of well-formed prismatic crystals (space group  $P2_1$ ) has revealed that the enzyme molecule can be inscribed in a  $48 \times 34 \times 28 \text{ Å}$  ellipsoid and consists of two globular domains (Dideberg et al., 1980a). The larger domain possesses a region of very high electron density, most likely  $\text{Zn}^{2+}$ , in the vicinity of which occurs a deep cleft ( $20 \times 6 \times 6 \text{ Å}$ ) which roughly cuts the domain in two parts (Fig. 15). As shown by small X-ray angle scattering, the shape of the native enzyme in solution (5–20 mg/ml) is similar to that seen in the solid state (Labischinski et al., 1981).

Diffusion of the inhibitor dipeptide Ac-D-Ala-D-Glu into a native enzyme crystal has allowed the active site to be located (Dideberg et al., 1980a). On the basis of the difference Fourier map, the site of the interaction appears as a 12 Å segment elongated in the  $y$  direction of the map, inside the cavity and close to the  $\text{Zn}^{2+}$  site.

The  $\beta$ -lactam  $p$ -iodo- $\beta$ -phenylacetylaminoccephalosporanic acid has also been successfully diffused into a native enzyme crystal (Dideberg et al., 1980a). Although the difference Fourier map synthesis at 4.5 Å resolution is rather noisy, the highest peak found in the map is located in the enzyme cavity close to the  $\text{Zn}^{2+}$  site.  $p$ -Iodo- $\beta$ -phenylacetylaminoccephalosporanic acid competitively inhibits the hydrolysis of Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala (Labischinski et al., 1981). Surprisingly, cephalosporin C behaves as a noncompetitive inhibitor (Frère et al., 1978b); perhaps related to this observation, enzyme crystals soaked in mother liquor containing 0.1 mM (or less) cephalosporin C crack very rapidly. Moreover, small angle X-ray scattering studies suggest that in solution cephalosporin C causes aggregation of the enzyme (Labischinski et al., 1981).

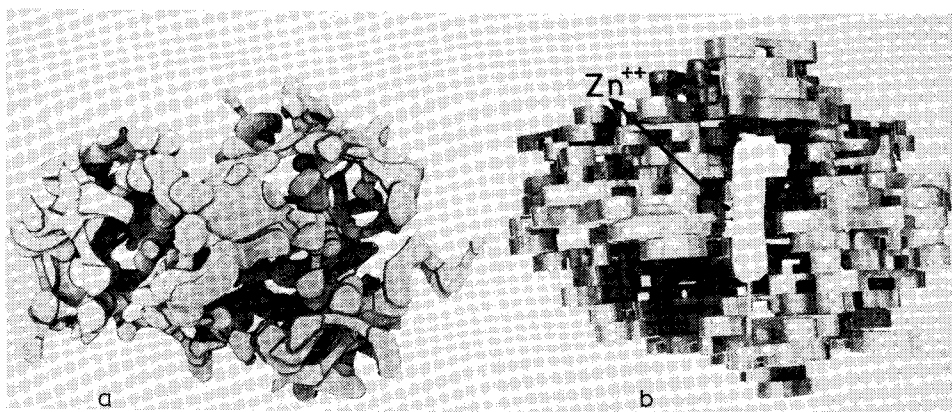


Fig. 15. Views of the model of the  $\text{Zn}^{2+}$  G enzyme constructed from the electron density map at 4.5 Å. (a) Along the  $b$  axis. (b) Along (101). The difference density observed with the peptide inhibitor is represented in white, inside the cleft (Dideberg et al., 1980).



#### 4. Concluding remarks

Incorporation of the nascent peptidoglycan into the preexisting wall peptidoglycan involves a series of enzymes which have the common property of acting on peptide bonds extending between two D centres in an  $\alpha$ -position to a free carboxyl group. For this purpose, bacteria have developed at least two types of D-alanyl-D-Ala peptidases. Their mechanistic properties are similar to those adopted by the eukaryotes for their own peptidases and proteases. The G enzyme is a  $Zn^{2+}$  metallopeptidase which catalyses hydrolysis of peptides terminating in L-R-D-Ala-D-Ala and of other C-terminal D-Ala-D peptide bonds. The R39 and R61 enzymes are bifunctional serine D-alanyl-D-Ala peptidases. Covalent catalysis by these enzymes permits transfer of the acyl moiety of the serine ester-linked L-R-D-alanyl-enzyme intermediates not only to water (hydrolysis) but also to suitable amino compounds (transpeptidation). In order to do so, the serine enzymes possess a specific and highly structured acceptor site situated in the vicinity of the relevant serine residue. The acceptor probably binds first to the active site so that when, in a second step, the L-R-D-alanyl-enzyme intermediate is formed, efficient transpeptidation can occur. Hydrolysis and transpeptidation can be exquisitely modulated by various factors.

Irrespective of their different mechanistic properties, the specificity of action of both the  $Zn^{2+}$  and serine D-alanyl-D-Ala peptidases on peptides terminating in L-R-D-Ala-D-Ala rests upon an induced fit mechanism whose main characteristic is that following the initial binding of the C-terminal D-Ala-D-Ala sequence of the donor peptide, the active site is, in a second step, made catalytically active as a result of a specific interaction between the lateral chain of the L-residue of the bound peptide and some amino acid(s) of the enzyme.

The C-terminal D-Ala-D-Ala sequence of the peptides and the equivalent portion of the backbone of the bicyclic fused ring system of penicillins ( $\Delta^3$ -cephalosporins) compete for the same binding sites on the metallo and serine D-alanyl-D-Ala peptidases. Binding of the  $\beta$ -lactam compound to the  $Zn^{2+}$  G enzyme is mostly reversible and has no inactivating effect (except under very drastic conditions). Conversely, with the serine enzymes, part of the normal catalytic mechanism occurs following the initial binding of penicillin ( $\Delta^3$ -cephalosporin). The active site is made catalytically active as a result of a specific interaction of the 6(7) $\beta$ -substituent of the  $\beta$ -lactam ring and some (allosteric?) enzyme grouping. However, what should be regarded as the leaving group in the acylation reaction, i.e. the thiazolidine (dihydrothiazine) ring, remains part of the acyl (penicilloyl or cephalosporoyl)-enzyme intermediate and stabilizes it, immobilizing the enzyme in an inactive form. Penicillins and  $\Delta^3$ -cephalosporins are thus mechanism-based inactivators of only the serine D-alanyl-D-Ala peptidases. Covalent catalysis, which confers on these enzymes the capacity of performing transpeptidations, also makes them vulnerable to  $\beta$ -lactam antibiotics.

#### 5. Acknowledgements

The work in Liège has been supported in part by grants to JMG from the Fonds de la

Recherche Scientifique Médicale, Brussels, Belgium (contract No. 3.4501.79), an Action Concertée with the Belgian Government (convention No. 79/48-II) and the National Institutes of Health, U.S.A. (contract No. 2 RO1 13364-04). H.R.P. is in receipt of a grant from the Medical Research Council, London, England.

## 6. References

- Adriaens, P., Meesschaert, B., Frère, J.M., Vanderhaeghe, H., Degelaen, J., Ghuysen, J.M. and Eysen, H. (1978) *J. Biol. Chem.* 253, 3660–3665
- De Coen, J.L., Lamotte-Brasseur, J., Ghuysen, J.M. and Frère, J.M. (1981) *Eur. J. Biochem.*, 121, 221–232
- Degelaen, J., Feeney, J., Roberts, G.C.K., Burgen, A.S.V., Frère, J.M. and Ghuysen, J.M. (1979) *FEBS Lett.* 98, 53–56
- DeLucia, M.L., Kelly, J.A., Mangion, M.M., Moews, P.C. and Knox, J.R. (1980) *Phil. Trans. R. Soc. Lond. B* 289, 374–376
- Dexter, D. and van der Veen, J. (1978) *J.C.S. Perkins I*, 185–190
- Dideberg, O., Frère, J.M. and Ghuysen, J.M. (1979) *J. Mol. Biol.* 129, 677–679
- Dideberg, O., Charlier, P., Dupont, L., Vermeire, M., Frère, J.M. and Ghuysen, J.M. (1980a) *FEBS Lett.* 117, 212–214
- Dideberg, O., Joris, B., Frère, J.M., Ghuysen, J.M., Weber, G., Robaye, R., Delbrouck, J.M. and Roelandts, I. (1980b) *FEBS Lett.* 117, 215–218
- Duez, C., Frère, J.M., Geurts, F., Ghuysen, J.M., Dierickx, L. and Delcambe, L. (1978) *Biochem. J.* 175, 793–800
- Duez, C., Joris, B., Frère, J.M. and Ghuysen, J.M. (1981a) *Biochem. J.* 193, 75–82
- Duez, C., Frère, J.M., Klein, D., Noël, M., Ghuysen, J.M., Delcambe, L. and Dierickx, L. (1981b) *Biochem. J.* 193, 83–86
- Fisher, J.F. and Knowles, J.R. (1980) In: *Enzyme Inhibitors as Drugs* (M. Sandler, ed.), pp. 209–219, The Macmillan Press Ltd.
- Frère, J.M. (1973) *Biochem. J.* 135, 469–481
- Frère, J.M., Ghuysen, J.M., Perkins, H.R. and Nieto, M. (1973a) *Biochem. J.* 135, 463–468
- Frère, J.M., Ghuysen, J.M., Perkins, H.R. and Nieto, M. (1973b) *Biochem. J.* 135, 483–492
- Frère, J.M., Moreno, R., Ghuysen, J.M., Perkins, H.R., Dierickx, L. and Delcambe, L. (1974a) *Biochem. J.* 143, 233–240
- Frère, J.M., Ghuysen, J.M., Reynolds, P.E., Moreno, R. and Perkins, H.R. (1974b) *Biochem. J.* 143, 241–249
- Frère, J.M., Leyh-Bouille, M., Ghuysen, J.M. and Perkins, H.R. (1974c) *Eur. J. Biochem.* 50, 203–214
- Frère, J.M., Ghuysen, J.M. and Iwatsubo, M. (1975a) *Eur. J. Biochem.* 57, 343–351
- Frère, J.M., Ghuysen, J.M. and Perkins, H.R. (1975b) *Eur. J. Biochem.* 57, 353–359
- Frère, J.M., Ghuysen, J.M., Degelaen, J., Loffet, A. and Perkins, H.R. (1975c) *Nature* 258, 168–170
- Frère, J.M., Ghuysen, J.M., Zeiger, A.R. and Perkins, H.R. (1976a) *FEBS Lett.* 63, 112–116
- Frère, J.M., Ghuysen, J.M., Vanderhaeghe, H., Adriaens, P. and Degelaen, J. (1976b) *Nature* 260, 451–454
- Frère, J.M., Duez, C., Ghuysen, J.M. and Vandekerckhove, J. (1976c) *FEBS Lett.* 70, 257–260
- Frère, J.M., Ghuysen, J.M. and DeGraeve, J. (1978a) *FEBS Lett.* 88, 147–150
- Frère, J.M., Geurts, F. and Ghuysen, J.M. (1978b) *Biochem. J.* 175, 801–805
- Frère, J.M., Duez, C., Dusart, J., Coyette, J., Leyh-Bouille, M., Ghuysen, J.M., Dideberg, O. and Knox, J.R. (1980) In: *Enzyme Inhibitors as Drugs* (M. Sandler, ed.), pp. 183–207, The Macmillan Press Ltd.,
- Fuad, N., Frère, J.M., Ghuysen, J.M., Duez, C. and Iwatsubo, M. (1976) *Biochem. J.* 155, 623–629
- Ghuysen, J.M. (1980) *Topics in Antibiotic Chemistry*, Vol. 5, pp. 9–117, Ellis Horwood
- Ghuysen, J.M., Leyh-Bouille, M., Bonaly, R., Nieto, M., Perkins, H.R., Schleifer, K.H. and Kandler, O. (1970) *Biochemistry* 9, 2955–2961

- Ghuysen, J.M., Leyh-Bouille, M., Campbell, J.N., Moreno, R., Frère, J.M., Duez, C., Nieto, M. and Perkins, H.R. (1973) *Biochemistry* 12, 1243–1251
- Ghuysen, J.M., Reynolds, P.E., Perkins, H.R., Frère, J.M. and Moreno, R. (1974) *Biochemistry* 13, 2539–2547
- Ghuysen, J.M., Frère, J.M., Leyh-Bouille, M., Coyette, J., Dusart, J. and Nguyen-Distèche, M. (1979) *Annu. Rev. Biochem.* 48, 73–101
- Ghuysen, J.M., Frère, J.M., Leyh-Bouille, M., Perkins, H.R. and Nieto, M. (1980) *Phil. Trans. R. Soc. Lond. B* 289, 285–301
- Hammarström, S. and Strominger, J.L. (1975) *Proc. Natl. Acad. Sci. U.S.A.* 72, 3463–3467
- Hammarström, S. and Strominger, J.L. (1976) *J. Biol. Chem.* 251, 7947–7949
- John, R.A. (1980) *Enzyme Inhibitors as Drugs*, pp. 73–114. (M. Sandler, ed.). The Macmillan Press Ltd.
- Knox, J.R., DeLucia, M.L., Murthy, N.S., Kelly, J.A., Moews, P.C., Frère, J.M. and Ghuysen, J.M. (1979) *J. Mol. Biol.* 127, 217–218
- Labischinski, H., Barnickel, G., Bradaczek, H., Dideberg, O., Charlier, P., Ghuysen, J.M. and Frère, J.M. (1981) in preparation
- Lee, B. (1971) *J. Mol. Biol.* 61, 463–469
- Leyh-Bouille, M., Bonaly, R., Ghuysen, J.M., Tinelli, R. and Tipper, D.J. (1970a) *Biochemistry* 9, 2944–2952
- Leyh-Bouille, M., Ghuysen, J.M., Bonaly, R., Nieto, M., Perkins, H.R., Schleifer, K.H. and Kandler, O. (1970b) *Biochemistry* 9, 2961–2970
- Leyh-Bouille, M., Ghuysen, J.M., Nieto, M., Perkins, H.R., Schleifer, K.H. and Kandler, O. (1970c) *Biochemistry* 9, 2971–2975
- Leyh-Bouille, M., Coyette, J., Ghuysen, J.M., Idczak, J., Perkins, H.R. and Nieto, M. (1971) *Biochemistry* 10, 2163–2170
- Leyh-Bouille, M., Nakel, M., Frère, J.M., Johnson, K., Ghuysen, J.M., Nieto, M. and Perkins, H.R. (1972) *Biochemistry* 11, 1290–1298
- Marquet, A., Frère, J.M., Ghuysen, J.M. and Loffet, A. (1979) *Biochem. J.* 177, 909–916
- Nieto, M., Perkins, H.R., Leyh-Bouille, M., Frère, J.M. and Ghuysen, J.M. (1973a) *Biochem. J.* 131, 163–171
- Nieto, M., Perkins, H.R., Frère, J.M. and Ghuysen, J.M. (1973b) *Biochem. J.* 135, 493–505
- Perkins, H.R., Nieto, M., Frère, J.M., Leyh-Bouille, M. and Ghuysen, J.M. (1973) *Biochem. J.* 131, 707–718
- Perkins, H.R., Frère, J.M. and Ghuysen, J.M. (1981) *FEBS Lett.* 123, 75–78
- Pollock, J.J., Ghuysen, J.M., Linder, R., Salton, M.R.J., Perkins, H.R., Nieto, M., Leyh-Bouille, M., Frère, J. and Johnson, K. (1972) *Proc. Natl. Acad. Sci. U.S.A.* 69, 662–666
- Rasmussen, J.R. and Strominger, J.L. (1978) *Proc. Natl. Acad. Sci. U.S.A.* 75, 84–88
- Tipper, D.J. and Strominger, J.L. (1965) *Proc. Natl. Acad. Sci. U.S.A.* 54, 1133–1141
- Virudachalam, R. and Rao, V.S.R. (1977) *Int. J. Peptide Protein Res.* 10, 51–59
- Waxman, D.J. and Strominger, J.L. (1979) *J. Biol. Chem.* 254, 4863–4867
- Waxman, D.J., Yocum, R.R. and Strominger, J.L. (1980) *Phil. Trans. R. Soc. Lond. B* 289, 257–275
- Woodward, R.B. (1980) *Phil. Trans. R. Soc. Lond. B* 289, 239–250
- Yocum, R.R., Waxman, D.J., Rasmussen, J.R. and Strominger, J.L. (1979) *Proc. Natl. Acad. Sci. U.S.A.* 76, 2730–2734
- Zeiger, A.R., Frère, J.M., Ghuysen, J.M. and Perkins, H.R. (1975) *FEBS Lett.* 52, 221–225