THE SIGNIFICANCE OF D-ALANYL-D-ALANINE TERMINI IN THE BIOSYNTHESIS OF BACTERIAL CELL WALLS AND THE ACTION OF PENICILLIN, VANCOMYCIN AND RISTOCETIN

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ABSTRACT

D-Alanyl-D-alanine is a key structure in the biosynthesis of the peptidoglycans of bacterial cell walls. It is introduced as the last step in the assembly of the precursor nucleotide compound containing muramic acid and remains throughout the biosynthetic process until the terminal D-alanine residue is lost at the final transpeptidation reaction required to effect crosslinking. This transpeptidation reaction is a target of penicillin action, and soluble carboxypeptidase–transpeptidases are inhibited by the antibiotic. The action of vancomycin and ristocetin is also tied up with the same D-alanyl-D-alanine terminus, but in a different way. These antibiotics contain an aglycone made up of phenolic amino acid residues in such a way that the resulting structure recognizes an acyl-D-alanyl-D-alanine terminus and combines with it with high affinity. By this mechanism bound vancomycin or ristocetin can inhibit reactions in the final stages of peptidoglycan synthesis. Correspondingly, in the presence of peptides that combine with vancomycin, the inhibition brought about by the antibiotic in either growing cells or in membrane preparations synthesizing peptidoglycan is reversed. At the same time some antibiotic remains bound to the preparations in such a way that it is no longer inhibitory. Studies with synthetic peptides have provided a rational basis for these observations.

INTRODUCTION

The cell walls of almost all bacteria contain a polymer known as peptidoglycan (mucopeptide, murein) that consists of polysaccharide chains substituted by peptides, some of which are crosslinked one to another[1, 2, 3]. The polysaccharide is a 1,4β-linked polymer of *N*-acetylglucosamine (exactly like chitin) except that each alternate residue has at C-3 an ether-linked D-lactic acid residue. The complete substituted hexosamine residue is called muramic acid. In some Mycobacteria the *N*-acetyl substituent on muramic acid is replaced by an *N*-glycolyl residue.[4] The muramic acid carboxyl groups are rarely free, except for a proportion of the residues in certain Micrococci, but are linked to peptide chains that are characteristic of

the peptidoglycan. The sequence of these amino acid residues in the precursor molecules, from which the peptidoglycan is biosynthesized, is shown in *Table 1*. The sequence of configuration is always L (or glycine)-D (γ-link)-L-D-alanyl-D-alanine. It is the presence of the terminal D-alanine dipeptide that is the main topic of this report.

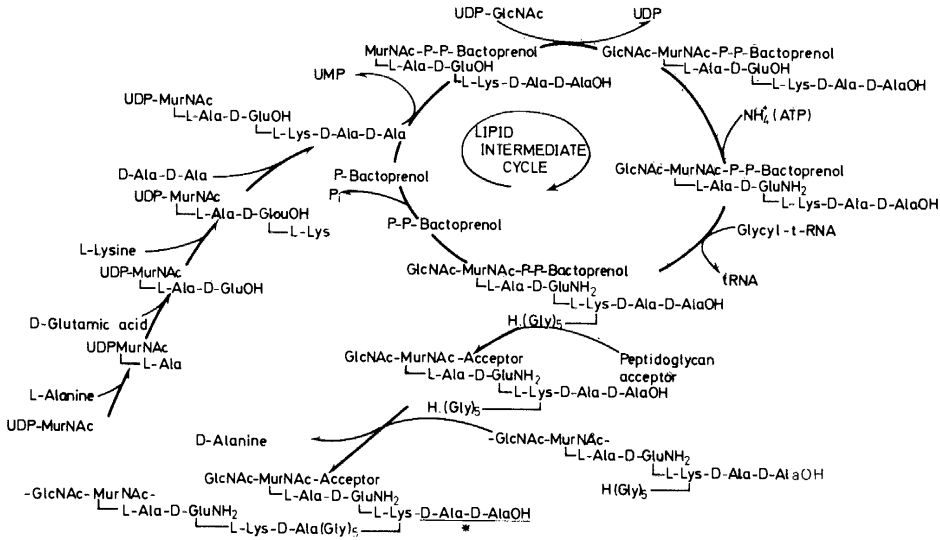
Table 1. Sequence of amino acid residues in the peptide portion of peptidoglycan precursors. The possible alternatives for each position are given, but not all combinations have so far been discovered.

Muramic acid → Aminoacid 1 → Amino acid 2				
		γ-link → Amino acid 3 →	Amino acid 4 →	Amino acid 5
L-alanine	D-glutamic	*meso*-diamino-	D-alanine	D-alanine
L-serine	acid	pimelic acid		
Glycine		(L-centre)		
		LL-diaminopimelic acid		
		L-lysine		
		L-ornithine		
		L-diaminobutyric acid		
		L-homoserine		
		L-glutamic acid		
		L-alanine		

The biosynthesis of the peptidoglycan, as at present understood, is shown in *Scheme 1*. It commences with the synthesis of the unique nucleotide precursor, specific for any particular organism, in which a UDP-*N*-acetylmuramylpentapeptide is built up. By the intervention of a lipid, bactoprenol[5], coupling to *N*-acetylglucosamine occurs and a lipid disaccharide-pentapeptide is assembled. Subsequently the crosslinking amino acids or peptides that occur in many organisms are added at the appropriate point on the primary peptide chain, e.g. in *Staphylococcus aureus* a pentaglycine unit is added to the ε-amino group of the L-lysine residue that occurs in the main chain[6]. Then the disaccharide units are polymerized to give glycan chains. After glycan synthesis a varying proportion of the D-alanyl-D-alanine termini are involved in a transpeptidation reaction, in which the terminal D-alanine residue is eliminated and the sub-terminal D-alanine residue forms a peptide bond with a free amino group on another chain. Depending upon the species, this free amino group may be at the D-centre of *meso*-diaminopimelic acid, the ω-amino group of L-lysine or L-ornithine, or the *N*-terminus of a crosslinking amino acid or peptide[2]. This crosslinking is considered to be essential for the stability of what Weidel and Pelzer called the 'murein sacculus'[7], the strong peptidoglycan network that surrounds the bacterium, the assumption being that by this means any newly synthesized glycan chain can be brought into covalent linkage with the rest of the network.

In many organisms, those D-alanyl-D-alanine termini that are not used for crosslinking as just outlined are then attacked by carboxypeptidases[8], either one or both residues being removed, so that in isolated cell-wall specimens the residual D-alanine is only that which is involved in crosslinks[9].

Scheme 1. Biosynthesis of cross-linked peptidoglycan in *Staphylococcus aureus**



* In some species, such as Bacilli, D-alanine residues in this situation are subsequently removed by carboxypeptidase.

The carboxypeptidase I attacks the terminal D,D linkage and removes a D-alanine residue, while carboxypeptidase II attacks an L,D linkage to remove the second D-alanine[10].

The foregoing account shows that the introduction and subsequent breakdown of the D-alanine dipeptide are key processes in the biosynthesis of the peptidoglycans of bacterial cell walls. It is hardly surprising, therefore, that these processes provide a target for the action of certain antibiotics. Those resembling D-cycloserine inhibit the enzymes involved in bringing the D-alanine dipeptide into the structure in the first place[11], but they are not our present concern. The crosslinking process and the removal of unwanted D-alanine residues are inhibited by penicillins on the one hand and by vancomycin and ristocetin on the other, by very different mechanisms, which we shall now consider further.

PENICILLIN

A connection between penicillin action and bacterial cell wall synthesis has been recognised for many years[12]. With the advent of cell-free systems synthesizing peptidoglycan, it became possible to study the system in detail. At first it was found that overall synthesis of peptidoglycan was not prevented by penicillin[13], except at very high concentrations, but later it became clear that in the presence of the antibiotic crosslinking was prevented[14]. Tipper and Strominger proposed a mechanism in which *N*-acyl-6-aminopenicillanic acid (penicillin) was supposed to be a structural analogue of *N*-acyl-D-alanyl-D-alanine[15]. By occupying a site on the transpeptidase that should have been

taken by the C-terminus of a peptide chain prior to crosslinking, the penicillin was thought to inhibit the enzyme action. Uncrosslinked peptidoglycan was therefore unable to link into the pre-existing network, so that faulty walls resulted. At the same time lytic enzymes, always present in the enzymic complex involved in wall synthesis and remodelling, might assist the death of the cell by breaking down parts of the wall that had been made prior to the addition of the antibiotic.

Izaki and Strominger[10] were able to show that *Escherichia coli* contained a carboxypeptidase I that was inhibited *in vitro* by penicillin, whereas carboxypeptidase II was unaffected. A further extension of the mode of action proposed by Strominger was that the transpeptidase, having accepted penicillin as a substrate analogue, should undergo penicilloylation so that it could no longer function even in the absence of penicillin. This conclusion was not supported by the results of Rogers, who found that *Staphylococcus aureus*, treated with penicillin and then washed free of external antibiotic, soon recovered the ability to synthesize peptidoglycan, even in the presence of chloroamphenicol that should have prevented the synthesis of new transpeptidase[16]. These ideas have been investigated extensively with penicillin-sensitive enzymes from *Streptomyces* species by Professor Ghuysen and ourselves. Covalent binding of penicillins to the *Streptomyces* carboxypeptidase–transpeptidase was not observed.

VANCOMYCIN AND RISTOCETIN

Both these antibiotics inhibit peptidoglycan synthesis and cause the accumulation of precursors in sensitive bacteria[17], and cell-free systems synthesizing peptidoglycan were 50 per cent inhibited by the same concentrations of vancomycin and ristocetin that were required for 50 per cent growth inhibition[18]. The formation of lipid intermediate (*Scheme 1*) was not inhibited, and was sometimes even enhanced, so that the antibiotic action had to occur at a later stage in peptidoglycan synthesis. The precise stage at which such inhibition might occur *in vivo* will be discussed after consideration of the probable chemical mechanism by which these antibiotics exert their action.

It was first observed in 1966 that vancomycin and ristocetin formed complexes with UDP-muramyl-pentapeptide precursors of peptidoglycan biosynthesis[19]. These complexes were formed *in vitro* and required the presence of the C-terminal D-alanine dipeptide; loss of even one of these D-alanine residues completely prevented complex formation[20]. Various alanine peptides were examined and it became clear that, first, complex formation was stoicheiometric, a given mass of antibiotic combining with one molecule of a suitable peptide and secondly, a D,D configuration and a free carboxyl group were essential for combination. Evidently, therefore, in the living cell vancomycin could combine with any of the precursors to which it had access, from the time of addition of the D-alanine dipeptide up to the final loss of one D-alanine residue during transpeptidation or by carboxypeptidase action. It was remarkable that a relatively small molecule like vancomycin should combine with such stereospecificity with a small peptide, and the system clearly merited further investigation. At this stage

relatively little was known about the chemistry of vancomycin, beyond the fact that it contained glucose, aspartic acid, *N*-methylleucine, phenols and chlorophenols[21].

For detailed study of the interaction we prepared an analogue of part of the uncrosslinked peptide chain of the peptidoglycan of *S. aureus* (or any other of the many organisms containing L-lysine at the central position in the pentapeptide), namely diacetyl-L-lysyl-D-alanyl-D-alanine. This substance combined very readily with vancomycin, thus facilitating physicochemical studies on the complex[22]. Vancomycin was titrated electrometrically and spectrophotometrically, and shown to contain groups with pK values of 2.9, 7.2, 8.6, 9.6, 10.5 and 11.7, the four last-mentioned being phenolic. The formation of the vancomycin–peptide complex under various conditions was studied by means of u.v. difference spectroscopy, since suitable peptides were known to produce a characteristic change in the absorption spectrum of vancomycin. In this way the association constant for the combination of antibiotic and peptide could be calculated. The stability of the complex in the range pH 1 to pH 13 indicated that the complex was formed only when carboxyl groups were ionized and phenolic groups were not, there being almost constant stability over the range pH 3 to pH 8, with a fairly sharp decrease on either side of those values. Furthermore, complex formation was not prevented by 8 M urea, 4 M KCl, 1 per cent sodium dodecyl sulphate or temperatures up to 60°C (*Table 2*). Although there was evidence that the

Table 2. Stability of the complex between vancomycin and diacetyl-L-lysyl-D-alanyl-D-alanine at pH 5 in the presence of various reagents.
Stability was calculated from the values for the association constant K_A, that in 0.02 M citrate buffer, pH 5.0 at 26°C without additions, being taken as 100%.

Substance added	Concentration	K_A ($l\ mol^{-1}$)	Stability (%)
None	—	1.5×10^6	100
KCl	0.2 M	1.1×10^6	73
	2.0 M	1.8×10^5	12
	4.0 M	7.0×10^4	5
Urea	4.0 M	3.7×10^5	25
	8.0 M	5.0×10^4	3
Sodium dodecyl sulphate	0.10%	4.0×10^5	27
	0.38%	1.3×10^5	9
	0.95%	2.5×10^4	2
	9.50%	1.4×10^5	9

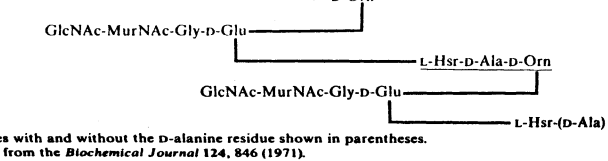
peptide carboxyl group was involved, the survival of the complex even in 4 M KCl seemed to weigh against a simple acid–base interaction being the main binding force. The results supported a minimum molecular weight for vancomycin of 1700–1800, but there were strong indications from optical rotatory dispersion and circular dichroism experiments that vancomycin molecules readily aggregated at higher concentrations, so that total aggregation was present at a concentration of 10 mg ml^{-1}. At the same time, the results suggested that vancomycin has only limited conformational flexibility.

We also studied the specificity of peptide structures that will form complexes

Table 3. Association constants and free enthalpy changes for the combination of vancomycin and ristocetin with peptides.
α-Ac-L-Lys-D-Ala-D-Ala-NH_2 and Ac_2-L-Lys-D-Ala-Gly-NHOH did not combine with either antibiotic.
Abbreviations: Aib, α-aminoisobutyric acid; MurNAc, *N*-acetylmuramic acid; Hsr, homoserine

Peptide	Ristocetin		Vancomycin	
	K_A(l mol^{-1})	ΔG(cal mol^{-1})	K_A(l mol^{-1})	ΔG(cal mol^{-1})
Changes in residue 1 (C-terminal)				
1. Ac_2-L-Lys-D-Ala-D-Ala	5.9×10^5	−7850	1.5×10^6	−8400
2. Ac_2-L-Lys-D-Ala-Gly	2.2×10^4	−5900	1.3×10^5	−6950
3. Ac_2-L-Lys-D-Ala-D-Leu	6.1×10^5	−7860	9.2×10^3	−5390
4. Ac_2-L-Lys-D-Ala-D-Lys	1.0×10^5	−6800	1.4×10^4	−5620
5. Ac_2-L-Lys-D-Ala-L-Ala	No combination		No combination	
6. Ac-D-Ala-D-Ala	7.2×10^4	−6600	2.0×10^4	−5840
7. Ac-D-Ala-Gly	1.9×10^3	−4470	5.4×10^3	−5070
Changes in residue 2				
8. Ac_2-L-Lys-Gly-D-Ala	1.6×10^5	−7070	9.4×10^4	−6760
9. Ac_2-L-Lys-D-Leu-D-Ala	5.8×10^4	−6470	2.9×10^5	−7420
10. Ac_2-L-Lys-L-Ala-D-Ala	No combination		No combination	
11. Ac_2-L-Lys-Aib-Gly	No combination		No combination	
12. Ac-Gly-D-Ala	4.9×10^4	−6390	1.1×10^4	−5500
Changes in residue 3				
13. Ac-Gly-D-Ala-D-Ala	1.6×10^5	−7070	9.4×10^4	−6760
14. Ac-L-Ala-D-Ala-D-Ala	2.2×10^5	−7270	3.1×10^5	−7450
15. *N*-Ac-L-Tyr-D-Ala-D-Ala	2.9×10^5	−7430	1.9×10^5	−7180
16. Ac-D-Ala-D-Ala-D-Ala	1.3×10^5	−6960	5.0×10^4	−6380
Influence of free amino groups				
17. L-Lys-D-Ala-D-Ala	8.2×10^3	−5320	1.2×10^4	−5510
18. α-Ac-L-Lys-D-Ala-D-Ala	1.9×10^5	−7200	4.7×10^5	−7700
Simultaneous changes in residues 1, 2 and 3				
19. Ac-L-Ala-Gly-Gly	2.5×10^3	−4620	4.9×10^3	−5200
20. Ac-Gly-Gly-Gly-Gly	8.0×10^2	−3950	1.5×10^3	−4300
21. Ac-L-Ala-D-Glu-Gly	6.8×10^2	−3850	4.8×10^5	−7720
22. *C. poinsettiae* dimer*	9.8×10^5	−8150	9.4×10^4	−6760

* The structure of this peptide is as follows:[26]



representing a mixture of molecules with and without the D-alanine residue shown in parentheses.

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with vancomycin and ristocetin, with the results summarized in *Table 3*[23, 24]. Certain similarities and differences emerged. Changes of amino acid at residue 1, in which the D-configuration was retained but the side-chain was increased from a methyl group (peptide 1) to more bulky groups (peptides 3 and 22) led to much lower affinity for vancomycin, whereas ristocetin continued to combine very well. Complete loss of carbon side-chain at residue 1 (glycine) led to a somewhat greater decrease in affinity for ristocetin than for vancomycin. Thus ristocetin imposes less exact steric restrictions for binding at position 1 of the peptide than does vancomycin. A positive charge on the side-chain at position 1 decreased combination with ristocetin but had the reverse effect with vancomycin (compare peptides 3 and 4). However, this latter enhancement of affinity was small compared with the deleterious effect for combination with vancomycin of a large side-chain at this position.

The effects of changes at residue 2 were particularly noticeable when the side chain was replaced by a hydrogen atom (peptides 1 and 8), causing considerable decrease in affinity for vancomycin but only a small change for ristocetin. On the other hand introducing a bulky side-chain decreased the affinity for ristocetin more than for vancomycin. With these results in mind, it is not surprising that peptide 21, with glycine at residue 1 and a bulky (and acidic) side chain at residue 2 (D-glutamic acid), should combine well with vancomycin but very poorly with ristocetin.

The fact that vancomycin will combine well with acetyl-L-alanyl-D-glutamyl glycine (peptide 21) and appreciably with the dimer from *Corynebacterium poinsettiae*, offers an explanation for another feature of the effect of the antibiotic on growing bacteria. It is known that vancomycin is rapidly absorbed by bacteria and that the isolated cell walls will take up considerable amounts[24]. Most peptidoglycans in fact contain, in addition to any remaining D-alanyl-D-alanine termini, other sequences that should combine with vancomycin or ristocetin, as can be seen by a comparison of the peptides in *Table 3* with known peptidoglycan structures. A few typical examples are given in *Table 4*. It is interesting that, as regards recognition of L-D-D carboxyl terminal sequences in addition to those ending in D-alanyl-D-alanine, vancomycin and ristocetin resemble the *Streptomyces* carboxypeptidases[26–28].

As shown above, the site on the vancomycin molecule that recognizes peptides contains phenolic groups. Similar groups are certainly present in ristocetin and ristomycin and other related antibiotics such as actinoidin[29], and the aglycones (deprived of their neutral sugars and also of the newly identified vancosamine[30] that may also be a common constituent of all these antibiotics), also act as antibiotics and combine *in vitro* with suitable peptides[24]. A partial structure was proposed for this aglycone, including a cyclic dipeptide composed of two diaminodicarboxylic acids, each consisting of two residues of hydroxylated phenylglycine joined by an ether link. Other hydroxyl groups were also present, so that all four aromatic rings retained pehnolic functions[31]. From this limited information, models could not be made with sufficient accuracy to clarify the mechanism of complex formation with peptides[24]. More chemical information will be needed before this problem can be finally solved.

Table 4. Some sequences found in crosslinked peptidoglycans and possible combination with vancomycin or ristocetin. The relevant residues are printed in italics.
N.B. Any D-alanine dipeptide termini that remain will also combine with both antibiotics.

Organism	Peptidoglycan structure	Proposed affinity for antibiotic	
		Vancomycin	Ristocetin
Micrococcus lysodeikticus	Disacc-L-*Ala*-D-*Glu*-*Gly* OH L-Lys-D-Ala-L-*Ala*-D-*Glu*-*Gly* OH L-Lys-D-Ala OH	Good	Poor
C. poinsettiae	Disacc-Gly-D-Glu L-*Hsr*-D-*Ala*-D-*Orn OH* Disacc-Gly-D-Glu L-Hsr-D-Ala OH	Quite good	Good
S. faecalis (fecium)	Disacc-L-Ala-D-Glu-NH_2 Disacc-L-Ala-D-Glu NH_2 L-Lys-D-Ala--- L-*Lys*-D-*Ala*-D-*Asp*-NH_2	None (terminates in amide)	None
B. megaterium KM	Disacc-L-Ala-D-Glu (L) D-Ala Disacc-L-Ala-D-Glu (Dap) (L) D-*Ala* OH (Dap) (D) OH (D)	Quite good	Good

COMPETITION FOR VANCOMYCIN BINDING SITES

In a cell-free system that was capable of peptidoglycan biosynthesis, the inhibitory action of vancomycin and ristocetin was reversed when large amounts of cell wall preparations that would absorb the antibiotics were added either simultaneously or after the inhibitory action had already manifested itself[32]. This reversal of vancomycin action was studied in more detail by the addition of solutions of a synthetic peptide that was also known to have affinity for the antibiotic[33]. Reversal of growth inhibition of *B. megaterium* was brought about by a molar ratio of peptide to antibiotic of 38, the time-lapse between addition of peptide and resumption of growth increasing with the time that the cells were left in contact with antibiotic alone. Similar results were observed with *S. aureus* and *M. lysodeikticus*. In parallel experiments a cell-free membrane preparation from *B. megaterium*, that could synthesize peptidoglycan *in vitro*, was also inhibited by vancomycin and again rapid reversal by added peptide was achieved. The fate of the antibiotic in these experiments was followed by the use of iodovancomycin labelled with ^{125}I[33, 34]. Experiments with growing cells and doses of iodovancomycin near the minimum inhibitory concentration showed that after growth inhibition had commenced some further uptake of antibiotic occurred, but the excess was released from the cells before rapid growth recommenced. The amount of antibiotic retained was about the same as when growth inhibition first occurred and hence it appeared that sensitive sites in the bacteria had been released from vancomycin inhibition. We concluded that perhaps in these limiting concentrations of vancomycin the antibiotic was being removed from inhibitory sites by the leakage of UDP-*N*-acetyl-muramylpentapeptide, with which it would combine. There was also considerable evidence from the experiments with whole cells and with membrane preparations that much of the antibiotic was sequestrated by being bound to sites that were not involved in biosynthesis. This conclusion fits admirably with the ideas on binding sites propounded in the previous section.

It seems likely, therefore, that in the living cell the vancomycin antibiotics will attach to any free D-alanine dipeptide termini that are available and by so doing will inhibit enzymes that bind to such fragments. Such an action was easily demonstrated with the soluble carboxypeptidase of *Streptomyces albus* G[35]. It must be borne in mind, however, that enzymes not immediately concerned with acting upon the D-alanine dipeptide itself will certainly have to recognize regions that contain it. Thus the enzyme that transfers a new disaccharide-pentapeptide unit from lipid-intermediate to preformed peptidoglycan may well have to recognize uncrosslinked pentapeptide side chains. If these side-chains were surrounded by vancomycin molecules recognition would be prevented, the acceptor could no longer be bound to the enzyme and chain extension could not occur. Proof of this hypothesis must await the unravelling of the complete enzyme chain of peptidoglycan synthesis.

CONCLUSION

D-Alanyl-D-alanine carboxyl termini are key structures in the biosynthesis of the crosslinked peptidoglycans of bacterial cell walls. Correspond-

ingly, antibiotics that either recognize and combine with these termini and thus interfere with biosynthesis (vancomycin and ristocetin) or those that inhibit enzymes, which in their turn recognize and modify the same termini (penicillins acting on carboxypeptidase–transpeptidases) are specific to bacteria and cannot react in the same way with host systems. Antibiotics of the vancomycin type that recognized only acyl-D-alanyl-Dalanine termini and showed low affinity for other structures in peptidoglycan should be effective at far lower concentrations than those presently known. A knowledge of the chemical structure of the binding site of vancomycin and ristocetin should go a long way on the path towards enabling such antibiotics to be synthesized. Consideration of the detailed effect of penicillins upon carboxypeptidase–transpeptidases, discussed in detail in Professor Ghuysen's paper[36], seems likely to throw new light both upon penicillin action and also upon the nature of the crosslinking reaction of peptidoglycan synthesis.

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