1. Introduction

One of the most important steps in fighting the microbial pathogens that pose a serious threat to human health came with the improvement of sanitation in the cities, by improving systems for drainage and removal of human waste. A second crucial step was the beginning of the antibiotic era in the 1930s, for example, the use of the sulfonamides against pneumonia, and the use of penicillin at the time of the Second World War. These antibiotics and their successors proved extremely effective in eradicating infectious diseases that had previously been potentially lethal. However, selectional processes can work rapidly in bacteria because of their short generation time and their ability to pass genetic information between species through a process called conjugation (Figure 1). These properties led to the widespread development of resistance to many of the new antibiotics, including penicillin, and by the late 1950s up to 85% of clinical isolates of staphylococci (disease-causing bacteria) were found to be penicillin-resistant. Since then, despite the isolation and development of new antibacterial agents (including naturally-occurring, semi-synthetic and fully synthetic compounds), bacterial strains resistant to virtually all known antibiotics have emerged. In the past decade the problem has become more acute with resistance to even the antibiotics of last resort, the vancomycin group, developing in certain species, appear to be able to account at least in part for the remarkable activity of a new semi-synthetic glycopeptide—developed at Eli Lilly—against bacteria that are resistant even to vancomycin. A full understanding of these devices is important, since occurrences of the “nightmare scenario” that clinicians have feared over recent years—that resistance to vancomycin will appear in new forms of MRSA—have recently been reported.

Keywords: antibiotics · cooperative effects · dimerizations · resistant bacteria · vancomycin

Figure 1. Drug resistance can be passed horizontally between species of bacteria. For example, a nonpathogenic, but antibiotic-resistant bacterium can physically join with a disease-causing bacterium and pass on the genetic information required for antibiotic resistance through a process called conjugation. With the advantage of drug resistance, the pathogenic bacterium can then proliferate into an untreatable, disease-causing colony.
and the prospect of a return to the pre-antibiotic days, with some infections being effectively untreatable, is becoming a reality.[1–3] It is against this background that we will describe the role that the vancomycin group of glycopeptide antibiotics have played in therapy during the last 40 years and how, in the research group in Cambridge, we elucidated the molecular basis for their mode of action. We then discuss how, in recent times, bacterial resistance to members of this group has developed and how the principles of action of a new semi-synthetic glycopeptide, which is active against these bacteria, emerged from our work. We also discuss the biosynthetic origins of these antibiotics and how, in the future, it may be possible to engineer the gene clusters encoding the enzymes responsible for this biosynthesis in order to produce novel, and possibly more efficient, antibiotics. Finally, we will discuss aspects of cooperativity in the mode of action of the glycopeptides that we have identified, and which can be used as a model for cooperative binding in more complex biological systems. The present review complements that produced by one of us (D.H.W.) in 1996.[4]

2. The Vancomycin Group of Antibiotics

Vancomycin was first isolated from a soil sample collected by the American pharmaceutical company Eli Lilly in the mid-1950s.[5] It is produced by the microorganism Amycolatopsis orientalis and was first used clinically in 1959. Its early use was somewhat limited, however, by side effects that resulted from imperfections in the purification process.[6] For example, when vancomycin was administered intravenously phlebitis would sometimes occur near the point of injection. Its use was also not recommended in patients with any history of hearing difficulties or kidney failure.[7] When improved purification techniques for vancomycin became available in later years, these side-effects were much reduced and it became more widely prescribed, particularly in combating infections arising from Staphylococcus aureus, the majority of clinically-isolated strains of which were resistant to penicillin. The acquisition by methicillin-resistant Staphylococcus aureus (MRSA, a serious pathogen commonly found in hospitals and responsible for large numbers of deaths) of resistance to virtually all antibiotics in clinical use, including cephalosporins, tetracyclines, aminoglycosides, erythromycin, and the sulfonamides, has propelled the vancomycin group of glycopeptide antibiotics to the forefront of the fight against these bacteria. Two members of the group are in clinical use today, vancomycin[8] and teicoplanin,[9] and these glycopeptides, along with gentamycin, are currently the antibiotics of last resort in our hospitals.

3. Glycopeptide Structure Determination

The first attempt to determine the structure of vancomycin, reported in 1965, indicated the presence of N-methylleucine, glucose, and chlorophenols within the molecule.[10] Further progress was slow, however, because the solving of such a complex structure required techniques that had not been developed at this point. Our efforts to solve the structure of vancomycin began in 1970 and utilized developing techniques, such as NMR spectroscopy, to achieve the target. In particular, the use of the negative nuclear Overhauser effect (NOE) in proton NMR spectroscopy to derive structural information represented a major advance at the time.[11] We were able to use this technique to obtain approximate interproton distances in the solution conformation of vancomycin for protons that were located close to each other in the vancomycin structure and were thus able to obtain a partial structure of vancomycin by 1977.[12] A further advance was then provided by solution of the crystal structure of CDP-1, a degradation product of vancomycin.[13] The resulting putative structures for vancomycin were incorrect, however, in that they mis-

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Ben Bardsley was born in Norwich in 1972 and received his bachelor degree in Natural Sciences at the University of Cambridge in 1994. He gained his PhD degree under the supervision of Dudley Williams at the University of Cambridge in 1998, studying aspects of cooperativity in molecular recognition and, in particular, cooperativity in the mode of action of the vancomycin group antibiotics. He is currently undertaking further research in this area with Dudley Williams.
represented the conformation of a chlorinated aromatic ring and incorporated an isoasparagine residue (rather than an asparagine residue). The former error was corrected by ourselves\[14\] and the latter by Harris and Harris\[15\] to give the now-accepted structure 1.

The increasing power of proton NMR spectroscopy meant that other structural determinations of members of the vancomycin group could be carried out relatively quickly compared to the pioneering work on vancomycin. In the structure determination of ristocetin A (2), important work on the sugars was carried out in Hungary\[16\] but the structure of the peptide portion was determined in our own group\[17\].

This was followed by the structure determination of teicoplanin (3), which was carried out in our group and completed in 1984.\[18\] Of these two antibiotics, teicoplanin is important as the only other member of the group apart from vancomycin in current clinical use. Ristocetin A was introduced into some hospitals in the late 1950s but was withdrawn soon afterwards because of the deaths from side-effects of a number of patients being treated with it. These two antibiotics typify the structural variation within the group: ristocetin A is the only member of the group so far discovered to possess a tetrasaccharide (as opposed to a disaccharide or monosaccharide) attached to the sidechain of the fourth amino acid residue of the peptide backbone, whereas a C11 acyl side chain attached to a glucosamine residue is incorporated at the equivalent position of teicoplanin. This last feature confers favorable pharmacological properties on teicoplanin (see later for details). At the time of writing, the structures of over 100 glycopeptide antibiotics have been established.

All the known glycopeptide structures consist of a peptide backbone constituted from seven amino acids. If these are numbered 1 to 7 from the N- to the C-terminus, then residues 4 and 5 are always constituted from \(\alpha\)-hydroxyphenylglycines, and residues 2 and 6 from tyrosine, whereas residue 7 is 3,5-dihydroxyphenylglycine. In many of the antibiotics described to date, residues 1 and 3 are both aliphatic amino acids. For example, residue 1 is commonly N-methylleucine, and residue 3 is asparagine (for example, vancomycin (1)). On the other hand, both these residues may occur as aromatic amino acids, as in the case of ristocetin A (2) and of teicoplanin (3); in these cases, residue 1 is again \(\alpha\)-hydroxyphenylglycine and residue 3 is 3,5-dihydroxyphenylglycine (also \(p\)-methylated in the case of ristocetin A). Where residues 1 and 3 are aromatic, they may, or may not, be cross-linked. In all cases where the stereochemistry has been examined, the stereochemistry at the \(\alpha\)-C centers of the amino acids 1 to 7 are R.R.S.R.R.S.S. Other structural features of note, which vary within different members of the group, include chlorine atoms attached to the aromatic rings of residues 2 and/or 6 (for example, 1 and 3), a \(\beta\)-hydroxy group on residues 2 and 6 (for example, 1 and 2) and sugar residues attached to the sidechains of residue 4 (for example, 1–3), residue 6 (for example, 2 and 3), and residue 7 (for example, 2 and 3). The combination of unusual structural features exhibited by these antibiotics means that as well as being of crucial clinical importance they provide a formidable challenge to synthetic chemists. Recently, however, total syntheses...
of the aglycone of vancomycin have been completed and described by the groups of Evans\cite{19} and Nicolaou.\cite{20}\cite{a}


An examination of the structures 1–3 allows some reasonable speculation as to their biosynthetic origins. It is a plausible expectation that in 2 and 3 the cross-linking of all the amino acid side chains (1→3, 2→4, 4→6, and 5→7) occurs by means of phenolic oxidative coupling. The first two of these hypothesized couplings are of oxygen to carbon atoms, but the 5→7 coupling is carbon to carbon. The biosynthetic experiments that we performed on vancomycin and ristocetin A are consistent with these proposals. To aid these studies, we first assigned the 13C NMR spectra of vancomycin\cite{21} and ristocetin A.\cite{22} A fermentation of the vancomycin-producing organism was then supplemented with [1,2,3\textsuperscript{13}C\textsubscript{2}]acetate. The 13C spectrum of the derived product revealed enrichment with [1,2,3\textsuperscript{13}C\textsubscript{2}]acetate at all eight carbon atoms of residue 7 in a manner that was consistent with its derivation from four units of acetate.\cite{23} A similar feeding experiment with the producing organism was then supplemented with [1,2-13C\textsubscript{2}]acetate. The labeled tyrosine and phenylglycine. Analysis of the products established that:

1) residues 2 and 6 are, in both cases, derived from tyrosine
2) residues 4 and 5 are, in both cases, also derived from tyrosine (as is residue 1 of ristocetin A).

Evidently, the producing organisms can derive the p-hydroxyphenylglycine residues from tyrosine. The study on vancomycin also established that the benzylic hydroxy groups found in residues 2 and 6 are introduced with retention of configuration.\cite{23}

A consequence of the sequence of R,R,S,R,R,S,S stereochemistries at the α-C centers of residues 1 to 7 is that the peptidic backbone is not of the β-sheet type often found in extended backbones of polypeptides where all residues have the S stereochemistry. As we will see later, natural selection has moulded this backbone into something much more subtle.

5. Sequencing and Analysis of the Genes Involved in the Biosynthesis of a Vancomycin Group Antibiotic

It is clear from the above-described experiments that the biosynthesis of vancomycin group antibiotics is complex, and involves the incorporation of units derived both from a polyketide (acetate) pathway and from tyrosine. Also, the amino sugars such as vancosamine, 4-epi-vancosamine and ristosamine present on some members of the group (for example, 1 and 2) would have to be biosynthesized by the producing organism prior to attachment to the antibiotic heptapeptide backbone. It is pertinent to enquire whether the genes that code for the biosynthesis of these amino sugars are clustered with those coding for the biosynthesis of the core heptapeptide, and to enquire as to the nature of the enzymes that catalyze the cross-linking of the side chains by means of phenol oxidative coupling and those that catalyze the regiospecific introduction of chlorine atoms. Additionally, through the cloning of selected enzymes that are involved in antibiotic biosynthesis, or direct engineering of the cluster of biosynthetic genes, it may become possible to produce novel, and possibly more efficient, antibiotics that are designed to incorporate structural features that have been shown to be beneficial in their mode of action. This method may be especially useful since selective chemical modifications of the antibiotic structures are not trivial. It was therefore desirable to sequence the DNA that codes for the biosynthesis of at least one of the glycopeptide antibiotics.

The first problem in attaining the above goal was to locate the genes that are responsible for glycopeptide biosynthesis. The key steps towards solving this problem were taken by Zmijewski and Briggs at Eli Lilly, who removed the vancosamine and glucose sugars from residue 4 of vancomycin (by a well-established acid-catalyzed cleavage), to give aglucovancomycin.\cite{25} A cell-free extract of enzymes from the vancomycin-producing strain was prepared and this mixture of enzymes then subjected to chromatographic fractionation. An enzyme (a glucosyl transferase) of the antibiotic biosynthetic pathway was then located by identifying the fraction that was able to catalyze the addition of glucose to residue 4 of aglucovancomycin. A limited amount of the peptide sequence of this purified enzyme was determined, and cDNA probes corresponding to this sequence were then synthesized and used to locate the biosynthetic cluster of genes for the production of vancomycin. The genes coding for the production of chloroeremomycin (4, also known as chloroorienticin, LY264826, and A82846B) were subsequently similarly locat-
ed. At Eli Lilly in Indianapolis, the genes for chloroeremomycin production were then isolated in two cosmids. Workers at Eli Lilly used one of these cosmids to sequence 5.7 kilobases of DNA that codes for the production of three enzymes. These enzymes (collectively known as glycosyl transferases) are responsible for the addition of the three sugars to the heptapeptide core of chloroeremomycin to give the fully glycosylated antibiotic. Eli Lilly then kindly made these cosmids available to our research group and, in collaboration with the Sanger Centre at Hinxton Hall, Cambridge, we have determined the sequence of 72 kilobases of DNA that codes for essentially the complete biosynthetic pathway of chloroeremomycin.

Analysis of these 72 kilobases of genomic DNA (for example, by identification of “start” and “stop” codons, and by analogy to known homologous protein sequences) led to the location of 39 putative genes (Figure 2). Of the 39 genes, 33 are found on the same DNA strand, whereas six of the genes (numbered as 17, 32, and 36–39 in Figure 2) are located on the opposite strand. About half of the anticipated protein products of these genes have clearly implicated roles in the biosynthesis of the antibiotic. Thus, natural selection has led to the clustering of genes that code for the production of the many enzymes in this biosynthetic pathway. These enzymes, constituted from the order of 10^4 amino acids, catalyze the biosynthesis of molecules with molecular weights in the range of approximately only 1400–2300 Daltons, which surely indicates the importance of the biosynthetic product to the producing strain. The roles of some of these enzymes are now considered.

6. Peptide Synthetases: Synthesis of the Linear Heptapeptide Core

Conclusions concerning the biosynthetic pathway for the formation of the heptapeptide core can be deduced because the proteins that are implicated show a significant homology to known peptide synthetases. These peptide synthetases are a class of large multi-functional enzymes that catalyze the formation of small peptides (for example, of the secondary metabolites gramicidin and surfactin) by a nonribosomal route. In the case of chloroeremomycin biosynthesis these enzymes contain a total of approximately 9000 amino acids and they are responsible for the biosynthesis of the heptapeptide backbone of the antibiotic prior to the addition of sugars, and very probably also prior to any cross-linking.

The biosynthesis involves the condensation of seven amino acids and proceeds from the N- to the C-terminus by a process known as the multi-enzyme thiotemplate mechanism. In the first step, the component amino acids are activated as aminoacyl adenylates. Each amino acid is then covalently linked to an enzyme-attached 4'-phosphopantetheine cofactor by thioesterification. Peptide synthesis then occurs by amide bond formation at the contact sites of the activating domains. The growing peptide chain is transferred from the 4'-phosphopantetheine of one module to the next, each time increasing in length by one amino acid.

Within the three multi-functional peptide synthetase enzymes, seven “modules” can be recognized (Figure 3). Each of these “modules” is responsible for the recognition of a specific amino acid, its activation (prior to attachment to another amino acid), inversion of its stereochemistry at the α-carbon atom where necessary, and its condensation (through amide bond formation) to give peptides of increasing length. The domains that recognize the amino acids show homologies to known domains in other peptide synthetases, and in some cases to each other (4, 5, and 7 to each other; 2 to 6). This evidence, taken in conjunction with the previously described feeding experiments, allows us to infer that the amino acids that are utilized for residues 2 to 7 are, respectively, (S)-tyrosine, (S)-asparagine, (S)-p-hydroxyphenylglycine, (S)-p-hydroxyphenylglycine, (S)-tyrosine, and (S)-3,5-dihydroxyphenylglycine. However, until further experiments are carried out, we cannot preclude the possibility that the (S)-tyrosine residues are hydroxylated and/or chlorinated prior to incorporation. The domain that recognizes the N-terminal amino acid does not show good homology to other (S)-leucine-recognizing domains, but does show 49% identity with the (S)-aminobutyric acid-recogniz-

![Figure 2. Pictorial representation of the genes located on cosmids pCZA363 (1–9) and pCZA361 (10–39) and putative enzymatic roles for their protein products in the biosynthesis of chloroeremomycin (4). (We thank Dr. P. F. Leadlay for pointing out that the product of gene 16 shows the characteristics expected of an N-methylase (putatively involved in the N-methylation of leucine). In the light of this observation, and sequence homology, the product of gene 14 is likely to be the C-methyltransferase responsible for C-methylation of 4-epi-vancosamine (see also Scheme 1), and not a hydroxylase as originally suggested by Solenberg et al.)](image-url)
ing domain (which is also associated with an epimerization domain) involved in pristinamycin biosynthesis.[24] In conjunction with other evidence (see below), it is possible that the substrate for residue 1 is (R)-leucine (or its N-methyl analogue).

Since the stereochemistry of the backbone is R,R,S,R,R,S,S, it might be expected that modules 1, 2, 4, and 5 would contain an enzyme for inverting the stereochemistry from S to R. In fact, this is true for modules 2, 4, and 5, but not for module 1 (Figure 3).[27] Two points of interest are evident from these findings. First, that although the phenylglycines at positions 4 and 5 can be biosynthesized from tyrosine, they must initially be available as S isomers (it has been concluded that epimerization generally occurs at the peptidyl stage for (R)-amino acids within the chain).[29, 35] Second, it appears that (R)-leucine, or its N-methyl analogue, (residue 1) may be directly loaded on to the biosynthetic apparatus (see above).[27]

A further point to note is that the multi-functional enzymes are three in number (genes 3, 4, and 5 in Figure 2, where they are also annotated as CepA, CepB, and CepC). This finding could not have been predicted, and is perhaps a quirk of the evolution of the biosynthetic apparatus. The first (CepA) is responsible for the biosynthesis of the N-terminal tripeptide. The condensation domain that catalyzes the condensation of the tripeptide to the next residue in the chain is located at the beginning of the following multi-enzyme (CepB). Overall, the second (CepB) is responsible for extending the tripeptide to a hexapeptide. The condensation domain that converts the hexapeptide to the final heptapeptide is located at the beginning of the third (CepC). CepC is also responsible for the recognition and activation of the seventh amino acid, and for the cleavage of the heptapeptide from the biosynthetic apparatus (to which it is attached as a thioester) through the action of a thioesterase.

7. Oxidative Reactions

Conversion of the linear heptapeptide, derived as described above, into the chloroeremomycin nucleus which lacks sugars, requires seven oxidative processes. These are the introduction of β-hydroxy groups onto the tyrosine residues 2 and 6, coupling of rings 5 → 7, 4 → 6, and 4 → 2, and introduction of chlorine atoms on to rings 2 and 6. The DNA sequence in fact shows evidence for eight enzymes (genes 7–10, 18, and 20–22) that might be involved in oxidative processes.[27] Of these, genes 10 and 18 show homology to non-heme haloperoxidases, and therefore appear likely to be involved in the introduction of the two chlorine atoms into the antibiotic. As indicated above, it cannot be precluded that the hydroxylation and/or chlorination may precede linear heptapeptide production.

8. Biosynthesis of 4-epi-Vancosamine and Attachment of Sugars to the Oxidized and Cyclized Heptapeptide Core

Once the cyclized and oxidized peptide core is available, two molecules of 4-epi-vancosamine and one of glucose must be added to it. The 4-epi-vancosamine is an L-amino-2,6-dideoxy sugars. Although L-amino-2,6-dideoxy sugars are relatively common in secondary metabolites, they are otherwise not widespread in nature. It was therefore likely that the genes that code for its biosynthesis would be clustered with those of the other biosynthetic genes, and this has indeed proved to be the case.[27] Based on analogy to the biosynthesis of other 6-dideoxy sugars such as daunosamine and mycarose,[36–38] the biosynthesis should proceed through an NDP-4-keto-6-deoxyglucose intermediate (NDP = nucleotide diphosphate). Enzymes for the production of this key intermediate were not identified in the cluster, but it is possible that general cellular pools of this precursor are utilized for the biosynthesis of 4-epi-vancosamine, an idea recently postulated by Summers et al. for the biosynthesis of the mycarose and desosamine sugars of erythromycin.[39]

Given NDP-4-keto-6-deoxyglucose as a precursor, homologies to enzymes identified in the biosynthetic pathways for daunosamine and mycarose[36, 38, 39] allowed a probable route to 4-epi-vancosamine to be deduced (Scheme 1).[27] Five putative enzymes were identified. The protein corresponding to gene 26 was assigned as a 3,5-epimerase on the basis of its strong homology to DnmU (60% identity) of the daunosamine pathway of Streptomyces peucetius[40] and EryBVII (55% identity) of the mycarose pathway of Saccharopolyspora erythraea.[36]

The enzyme corresponding to gene 23 is closely related to DnmT (49% identity) from the daunosamine pathway of S. peucetius[39] and EryBV (45% identity) from the mycarose...
pathway of *S. erythraea*, both of which have been linked with C-2 deoxygenation. In particular, EryBVI was assigned as a 2,3-dehydratase and a similar role is proposed for the product of gene 23. The product of gene 25 has a high homology (72% identity) to DnrJ, an enzyme found in daunosamine biosynthesis and implicated in the introduction of an amine functionality,[37, 40] probably with the aid of pyridoxamine or glutamic acid as a cofactor. The recent confirmation of EryBIII as a C-3 methyltransferase in mycarose biosynthesis[41] suggests that the product of gene 14, which has 28% identity to this enzyme, is responsible for this step of the pathway. The proposed sugar biosynthesis is completed by C-4 ketone reduction with the enzyme corresponding to gene 24, which possesses strong homology only to EryBIV (49% identity), the 4-ketoreductase of the mycarose pathway of *S. erythraea*.[36] Finally, the attachment of two molecules of 4-epi-vancosamine, and one of glucose, at three distinct sites on the antibiotic requires three glycosyl transferase enzymes, and the genes that code for these enzymes are 11–13.[26, 27]

9. The Complex of the Glycopeptides with Bacterial Cell-Wall Precursors

An important finding, even before the structure of vancomycin was known, was that of Perkins who showed that the antibiotic binds to bacterial cell wall mucoprotein precursors that terminate in the sequence L-lysyl-d-alanyl-d-alanine.[42] This peptide is described as a mucoprotein precursor because it is an intermediate in the process of bacterial cell-wall biosynthesis. Bacterial cell wall is built up on the outside of the cell in two main steps.[43] First, disaccharide units with pendant peptides are exported from the cytoplasm to the outside of the cell where they are joined together by a transglycosylase enzyme (Figure 4). Second, for mechanical strength, these long polysaccharide chains are cross-linked through their peptide chains by a transpeptidase enzyme. This enzyme recognizes the sequence d-alanyl-d-alanine at the end of the peptide chain, cleaves off the terminal alanine, and joins the remainder to a peptide chain from an adjacent polysaccharide. At the time of Perkins’ finding (1969), little about the molecular basis of action of vancomycin could be inferred because the structure of the antibiotic was as yet unknown.

When the structure of vancomycin was finally elucidated, the molecular basis for the binding of the antibiotic to mucoprotein precursors terminating in Lys-d-Ala-d-Ala could then be determined. This was achieved using proton NMR spectroscopy of the complexes formed between the antibiotic and analogues of bacterial mucopeptide precursors (N-acetyl-d-alanyl-d-alanine (Ac-dAdA) or N-α-acetyl-N-ε-acetyl-lysyl-d-alanyl-d-alanine (Ac2-KdAdA)). A number of key experiments were important in this determination. The first involved measurement of the chemical shifts of the methyl groups of Ac-dAdA when free in solution and when bound to antibiotic. The upfield changes in chemical shift

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**Scheme 1.** Postulated reaction scheme for the biosynthesis of NDP-4-epi-vancosamine starting from NDP-glucose, showing the corresponding putative enzymes involved by reference to the genes indicated in Figure 2.

**Figure 4.** Schematic outline of the biosynthesis of peptidoglycan. In (A), the disaccharide precursor is transported across the membrane after which it is appended to the growing polysaccharide chain by a transglycosylase (B). Mechanical strength is conferred to the cell wall through the cross-linking of adjacent polysaccharide chains (C). The cross-linking step proceeds with a transpeptidase enzyme removing the C-terminal d-alanine residue of the pentapeptide. The carbohydrate group released by this process then forms a peptide bond to the free N-terminal group of the bridging residue R. The constitution of the pendant peptide shown is that found in *Staphylococcus aureus*.

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observed for these resonances in the bound state indicated the methyl groups were located over aromatic rings of the antibiotics. Second, the antibiotic amide NH protons involved in hydrogen bonding to the ligand carboxylate were determined by observation of the chemical shifts in the NMR spectra, with the amide NH resonances suffering a downfield shift in the antibiotic–ligand complex characteristic of the formation of such a hydrogen bond. The other antibiotic amide NH groups involved in hydrogen bonding to ligand were determined by measurement of the solvent exposure of each of the amide NH protons. Finally, approximate interproton distances for antibiotic protons that lie close in space to ligand protons were determined using the NOE. This appears to have been the first use of the intermolecular NOE to determine the structure of a bimolecular complex. Such determinations were carried out for the binding of d-Ala-d-Ala-terminating peptides to ristocetin A, vancomycin, and teicoplanin in the early 1980s and led to the model of the binding interaction shown in Scheme 2. This model, with

1) the binding of the carboxylate anion of the C-terminal d-alanine into the pocket of the three amide NH groups of residues 2–4 of the antibiotic (the formation of this pocket is crucially dependent on the R,R,S,R stereochemistries of residues 1–4).

2) the formation of two amide–amide hydrogen bonds between the acetylated dipeptide and the antibiotic.

3) the hydrophobic interactions formed by the alanine methyl groups in their contacts with hydrocarbon portions of the antibiotic.

The relative importance of these contributions can only be addressed in a semi-quantitative manner because of the complex nature of cooperativity between weak interactions. However, from our studies of the problem it is clear that productive binding is crucially dependent on binding of the carboxylate (factor 1 above). It is this interaction that provides the main source of exothermicity (20–30 kJ mol⁻¹) in the association. The stronger binding of the carboxylate group by the pocket of the three antibiotic NH groups than by water seems likely to find its origin in the fact that the three repulsive NH ends of the associated CONH dipoles can be pushed together by the expenditure of energy during the process of antibiotic biosynthesis. Together, they are better able to solvate the negative charge of the carboxylate ion than water molecules (which cannot have their oxygen atoms held closely together in the absence of a template to hold them in such a position) without paying a cost in enthalpy. Given the binding of the carboxylate, the two amide–amide hydrogen bonds do little to directly promote further binding affinity, that is, their intrinsic binding affinities are probably close to zero. This is evidenced, for example, by the fact that the binding of N-acetylglycine is greater than that of acetate by a factor of only approximately three. Indirectly, however, they do play important roles. First, in orienting the acetylated dipeptide so that the hydrophobic interactions of the two Ala methyl groups can promote binding by a further factor of about 10⁴. Second, by virtue of the energy barriers that exist towards breaking these two amide–amide hydrogen bonds, the motion of the peptide ligand in its antibiotic binding site is reduced and the binding of the carboxylate anion into its binding site is thereby increased.

Evidence for the last effect mentioned above is found from ¹H NMR spectroscopic studies. The chemical shift of the amide NH (w₂, Scheme 2) of residue 2 of the antibiotics is moved markedly downfield upon binding the carboxylate oxygen atom of di- and tripeptide cell wall precursor analogues. The assumption is made that the extent of this downfield shift (measured for the completely bound state of an antibiotic to a given cell-wall analogue) increases with increased binding of the carboxylate group to w₂. Experimentally, we find that the limiting downfield shift of w₂ increases as shown in Figure 5 for a variety of ligands (chosen as a series of increasing affinity for the antibiotic, which is represented by the vertical axis and which records the Gibbs free energies of binding for the ligands). It is clear from these data that as the ligands are changed in the series acetate → N-acetyl-d-alanine (Ac-dA) → Ac-dAdA → Ac-KdAdA, the chemical shift of w₂ in the fully bound state increases dramatically. This is because the making of the adjacent
10. Dimerization of the Glycopeptides

In 1989 we discovered that the antibiotic ristocetin A forms a dimer in aqueous solution.\(^{[60]}\) We were also able to elucidate the relative orientations of the two monomer units of this dimer and the hydrogen bonding network formed between them. In the dimer structure of chloroeremomycin (4) bound to Ac2-KoAda, (Scheme 3), the peptide backbones of two antibiotic molecules are bound together by four amide—amide hydrogen bonds, and the ammonium ion of the amino sugar \(\text{Ac-epi-vancosamine}\), which is attached to residue 6 of the antibiotic, forms a hydrogen bond to an amide carbonyl group in the other half of the dimer to give a total of six hydrogen bonds at the dimer interface.\(^{[60]}\) In vancomycin, where this amino sugar is not present, there are only four intermolecular hydrogen bonds at the dimer interface. The overall structure of the dimer that we determined by proton NMR spectroscopy has now been independently confirmed by X-ray crystallography.\(^{[57, 46, 57, 58]}\) Of all the glycopeptides so far examined, except for ristocetin A, dimerization constants are greater when bacterial cell wall precursor analogue is bound to the antibiotic than when it is not. That is, dimerization is cooperative with ligand binding.\(^{[60]}\) Similarly, antibiotic dimers bind ligands more strongly than antibiotic monomers. This can be seen from a thermodynamic cycle showing the formation of a ligand-bound dimer from its constituent parts, that is, two antibiotic monomers and two bacterial cell wall precursor analogue molecules.\(^{[60]}\) An example is the dimerization of chloroeremomycin (4). The dimerization constant in the presence of the cell wall precursor analogue Ac2-KoAda is a factor of about 100 greater than the value in the absence of ligand.\(^{[60]}\) Such a value implies that two cell wall precursor analogues binding with equal affinity to an antibiotic dimer should each bind to a dimer approximately ten times more strongly than to a monomer.

A number of interactions appear to be important in the expression of cooperativity between ligand binding and dimerization. First, the binding of ligand to antibiotic monomer (or dimerization of free antibiotic) should result in increased polarization of the antibiotic amide groups involved in hydrogen bonding. Since the same amide (CONH) groups are involved in hydrogen bonding at the dimer interface (or ligand binding interface), they should form stronger hydrogen bonds as a consequence of their increased polarization and resulting stronger dipoles. Second, the presence of an initial hydrophobic interactions\(^{[54]}\) and of the adjacent amide—amide hydrogen bonds\(^{[55]}\) increases the binding affinity of the carboxylate anion into its antibiotic receptor pocket. This phenomenon is probably one of general relevance to weak interactions—the addition of neighboring interactions can strengthen an existing interaction by limiting the dynamic motion of a ligand at its receptor site. Thus, cooperativity derives not only from the entropic advantage of putting several interactions on the same template (the chelate effect), but also the bonding (enthalpic) advantage of doing this in a strain-free system. Not only can an enthalpic barrier to breaking a bond restrict the motion at an adjacent site, but by so doing, it can improve the bonding at that adjacent site.

11. Cooperativity Between Dimerization and the Binding of Cell Wall Precursor Analogues

An interesting feature of antibiotic dimerization is that for all the glycopeptides so far examined, except for ristocetin A, dimerization constants are greater when bacterial cell wall precursor analogue is bound to the antibiotic than when it is not. That is, dimerization is cooperative with ligand binding.\(^{[60]}\) Similarly, antibiotic dimers bind ligands more strongly than antibiotic monomers. This can be seen from a thermodynamic cycle showing the formation of a ligand-bound dimer from its constituent parts, that is, two antibiotic monomers and two bacterial cell wall precursor analogue molecules.\(^{[60]}\) An example is the dimerization of chloroeremomycin (4). The dimerization constant in the presence of the cell wall precursor analogue Ac2-KoAda is a factor of about 100 greater than the value in the absence of ligand.\(^{[60]}\) Such a value implies that two cell wall precursor analogues binding with equal affinity to an antibiotic dimer should each bind to a dimer approximately ten times more strongly than to a monomer.

A number of interactions appear to be important in the expression of cooperativity between ligand binding and dimerization. First, the binding of ligand to antibiotic monomer (or dimerization of free antibiotic) should result in increased polarization of the antibiotic amide groups involved in hydrogen bonding. Since the same amide (CONH) groups are involved in hydrogen bonding at the dimer interface (or ligand binding interface), they should form stronger hydrogen bonds as a consequence of their increased polarization and resulting stronger dipoles. Second, the presence of an initial
set of hydrogen bonds with the antibiotic backbone (for example, from ligand binding) should have the effect of restricting the rocking motions of the peptide dipoles. This motional restriction should lead to stronger bonding at the second interface. Third, it can be seen from Scheme 3 that the process of dimerization brings the ammonium ion of the 4-epi-vancosamine residue into the proximity of the carboxylate anion of a bacterial cell-wall precursor bound to the other half of the dimer. The electrostatic attraction between these groups, which is mediated by a peptide bond, can be thought of as a pseudo-salt bridge. This last interaction can only occur in antibiotics that possess an amino sugar attached to residue 6 (for example, chloroeremomycin (4) and ristocetin A (2), but not vancomycin (1)).

Our work on quantitation of antibiotic dimerization has established that dimerization is promoted both by the saccharides that are frequently attached to residue 4 of the antibiotics, and by the amino sugars that are often attached to the benzylid hydroxy group of residue 6. If the view is taken that the antimicrobial action of the glycopeptides under discussion represents the evolved function of these substances, then it seems reasonable to view the sugars as adornments added to the peptide backbones to improve this function. Given this outlook, the observation that the sugars are of such structures and so positioned as to promote dimerization leads to the proposal that dimerization may well be important in antibacterial action. This view is supported by the fact that some glycopeptides possess a chlorine atom attached to residue 2 and that these antibiotics can dimerize more strongly than the equivalent antibiotics that do not have this feature. These observations are consistent with the idea of a selectional pressure to produce antibiotics that dimerize, a selectional pressure that would only operate if dimerization had a functional role in aiding the survival of the producing strain.

12. Asymmetry of the Glycopeptide Dimers

A remarkable feature of all the vancomycin group glycopeptide dimer structures so far examined (by both proton NMR spectroscopy and X-ray crystallography) is that although the peptide backbones of the two constituent antibiotic molecules are head-to-tail relative to each other, the sugars attached to residue 4 are arranged in a head-to-head manner. This feature accounts for the fact that the dimers are asymmetric. For example, in the ristocetin A dimer (Figure 6), the 6-methyl resonance of the rhamnose, which is part of the tetrasaccharide attached to residue 4, occurs with two distinct chemical shifts (δ = 0.86 and 1.27). This is because the rhamnose sugar of one half of the dimer is in a different environment to the rhamnose in the other half of the dimer; one rhamnose is remote from the N-terminus of the antibiotic of which it is part, whereas the other is much closer to the N-terminus of the antibiotic of which it is part (Figure 6). This result implies that the dimers have two distinct binding sites for bacterial cell wall mucopolysaccharide precursors (Figure 6). However, for binding of free cell wall peptide analogues in aqueous solution, it is only in the case of ristocetin A that we have so far been able to demonstrate that these two sites have significantly different affinities. In the cases of the other antibiotics that we have examined, any differences in binding affinities of these two sites in aqueous solution are very small.

It is interesting to speculate as to the evolutionary basis for the asymmetry of the dimer structures and whether this is related to the structure and orientation of strands of immature peptidoglycan cell wall. Schäfer et al. have postulated previously that the head-to-tail nature of the peptide backbones in an antibiotic dimer and the consequent binding of two cell-wall precursors in a head-to-tail manner relative to each other suggest that vancomycin is inserted between different immature peptidoglycan strands. By implication, the adjacent strands of peptidoglycan bound by the two halves of the dimer must also be oriented head-to-tail (antiparallel) relative to each other. We would like, however, to put forward an alternative hypothesis. Previous evidence regarding the binding of glycopeptides to cell wall precursor analogues indicates that the N-terminal portion, -l-alanyl-g-glutamyl-, of the pentapeptide projects towards the sugars attached to residue 4 of the antibiotic. This would imply that the antibiotic binds with these sugar residues oriented towards the bacterial surface relative to the peptide portion of the molecule. Herein may lie the origin of the dimer asymmetry. Since the strands of peptidoglycan are constituted of polysaccharide chains there is likely to be some favorable sugar–sugar interaction between these strands and the antibiotic residue 4 saccharides. The parallel orientation of interacting saccharides on the antibiotic dimer would thus require a similar parallel orientation of the polysaccharide strands for a repeated interaction between the two saccharides of an antibiotic dimer and two polysaccharide strands. Such interactions between the polysaccharide of immature peptidoglycan and the antibiotic saccharides have not been investigated but they may be an influential factor in either location of the antibiotic at its site of action or in promoting the binding of the antibiotic to its target peptide. Thus, although no direct evidence for the parallel or antiparallel nature of adjacent immature strands of cell wall has been produced, sugar–sugar interactions between antibiotic dimers and cell-wall polysaccharides and the parallel nature of the sugars in the dimers suggests that the strands of cell wall may also be biosynthesized with a parallel orientation relative to each other.

Figure 6. Schematic illustration of the ristocetin A dimer, with a molecule of a peptide cell-wall analogue in each of its two binding sites. Each of the two sets of four colinear circles represents a tetrasaccharide (Ar = arabinose, M = mannose, G = glucose, Rh = rhamnose) attached to amino acid number 4 of the peptide backbone (see 2). Note that the sugars are arranged head-to-head, in contrast to the head-to-tail arrangement of the peptide backbone. This property, found for all ring 4-glycosylated antibiotics so far examined, means that the dimers are asymmetric.
13. Roles for Dimerization and Membrane Anchoring in the Action of Glycopeptides

When the glycopeptides were first shown to dimerize, we postulated that dimerization might provide an enhancement in activity through the simultaneous delivery of two antibiotic molecules to the site of action.\textsuperscript{[56]} Additionally, in 1990, Good et al. showed that the antibiotic eremomycin, which binds \textit{A} to the cell wall is a simple bimolecular association (such that externally-added ligand can replace the cell-wall peptide). B) The antibiotics dimer can utilize either dimerization (Figure 7 B) or membrane anchoring (Figure 7 C) to enhance their antibacterial activity.

Figure 7 A)\textsuperscript{[60, 69]} This hypothesis was extended to consider the activity of teicoplanin (3), which does not dimerize measurably. Instead, teicoplanin possesses a C\textsubscript{11} acyl chain, which may be able to insert into the bacterial membrane itself, both locating this antibiotic at its site of action and facilitating the formation of an “intramolecular” complex at the cell surface (Figure 7 C).\textsuperscript{[60, 69]} Hence, the vancomycin group of antibiotics can utilize either dimerization (Figure 7 B) or membrane anchoring (Figure 7 C) to enhance their antibacterial activity.

We first began to investigate whether any binding enhancement could be achieved from dimerization or membrane anchoring, but this was only observed in the mid-1990s.\textsuperscript{[60]} This involved attempting to antagonize antibacterial action against bacteria (\textit{Bacillus subtilis}) on agar plates by the addition of external \textit{A}.

The cooperative binding enhancement achieved from the binding of the dimer at the bacterial surface relative to the binding of a monomer.\textsuperscript{[69]}

Further experiments have shown that there is in fact a striking correlation between the amount of external \textit{A} required to antagonize antibiotic action and the antibiotic dimerization constant.\textsuperscript{[69]} The increases in the former quantity, for more strongly dimerizing antibiotics (Figure 9), measure
14. Cooperativity in Antibiotic Binding to Micelle-Associated Peptides

Once we had shown that an advantage was gained in antibacterial activity for antibiotics that were able to dimerize or anchor to membranes, we attempted to determine the extent to which binding was enhanced when it occurred “intramolecularly” at a membrane surface (templated binding) relative to the equivalent binding event occurring intermolecularly. Our first experiments to measure this enhancement employed a crude model system for the bacterial surface that consisted of sodium dodecyl sulfate micelles as an analogue of the bacterial membrane (Figure 10A) and N-decanoylated cell wall precursor analogues.

![Figure 10. Schematic representation of A) a micelle, B) Dec-dAdA using its membrane anchor, and C) an antibiotic dimer bound to Dec-dAdA at the surface of a micelle.](image-url)

The decanoyl chain of these precursor analogues was designed to be able to insert into the hydrophobic interior of the micelles with the polar C-terminus of the peptide projecting into solution (Figure 10B). Note that since the hydrocarbon side chains that form the interior of the micelle are able to pass each other (that is they can behave in the manner of a liquid crystal), N-decanoylated peptides that are inserted into the micelle are able to change their relative positions on the micelle. Therefore, if an antibiotic dimer now binds a micelle-bound peptide at one of its binding sites, then a second micelle-bound peptide has the possibility to adjust its position so that in principle it can bind “intramolecularly” into the second binding site of the dimer (Figure 10C). The binding affinity of the antibiotic to the peptide in such a templated assembly should be greater than that for the corresponding association in the absence of the micelle, providing this assembly can be formed in a relatively strain-free manner.

The above conjectures have been confirmed experimentally. The binding constant of free Ac-dAdA to ristocetin A in solution is $4.1 \times 10^4 \text{M}^{-1}$ (in the presence of SDS micelles, which are passive in this case, but present to give the necessary control experiment), whereas the binding constant for membrane-templated N-decanoyl-d-alanyl-d-alanine (Dec-dAdA, as in Figure 10C) is $1.6 \times 10^7 \text{M}^{-1}$, an increase in the binding constant of approximately 400.$^{[71]}$ Moreover, the chemical shift of $\delta_2$ (which was shown earlier to occur at lower field the more strongly the ligand was bound to it) in the latter assembly occurs further downfield ($\delta = 11.65$) than for the former association ($\delta = 11.43$).$^{[71]}$ Similar results were achieved for teicoplanin binding to the peptides Ac$_2$-KdAdA and N-$\alpha$-decanoyl-N-$\epsilon$-acetyl-lysyl-d-alanyl-d-alanine (Dec-KdAdA) in the presence of micelles. As for ristocetin A binding to Dec-dAdA and Ac-dAdA, the binding constant of teicoplanin to Dec-KdAdA was greater than that to Ac$_2$-KdAdA and $\delta_2$ was shifted further downfield for the binding of Dec-KdAdA than for the binding of Ac$_2$-KdAdA.$^{[71]}$ Thus, in the formation of templated assemblies, not only is there the advantage of a relatively small adverse entropy (the binding being effectively intramolecular), but the motional restriction associated with binding at a template (the micelle surface) is also associated with stronger bonding of the peptide carboxylate into its receptor pocket.

We have thus been able to demonstrate the operation of two distinct forms of cooperativity in the binding of bacterial cell wall precursor analogues to antibiotic dimers. First, an intrinsic form of cooperativity that arises from the influence of the two binding events (ligand binding and dimerization) on each other. This cooperativity occurs when all the components are free in solution (as opposed to being membrane-bound). Second, a form of cooperativity that arises from the restriction of motion of the component parts of binding as a consequence of their simultaneous location at the bacterial membrane.

15. Resistance to Vancomycin: A New Species of “Superbug”

Since its introduction into clinical use in the 1950s, vancomycin was notable among antibacterial agents in use because of the lack of resistance developed against it by bacteria. This lack of resistance helped it reach the forefront of the fight against infections due to MRSA, an increasingly prevalent pathogen. In 1988, however, the first report of vancomycin-resistant enterococci (VRE) appeared.$^{[72]}$ Although enterococci are not normally pathogenic, such infections can be lethal if they are contracted by immunodeficient patients. Causes of immuno-deficiency include chemotherapy, AIDS, or simply weakness caused by old age, severe illness, or a recent operation. Such infections are becoming increasingly common and there is a possibility that the genes that code for resistance could be transferred to more deadly bacterial species such as MRSA (Figure 1).

The genetics and enzymology of vancomycin resistance have been elucidated in respective major contributions from the groups of Courvalin in Paris and Walsh in Cambridge, Massachusetts.$^{[73, 74]}$ It appears that resistant enterococci have been able to obtain genes from other bacteria such that the precursors from which their cell wall is built no longer terminate in -d-alanyl-d-alanine, but rather terminate in -d-alanyl-d-lactate (-d-Ala-d-Lac).$^{[73, 74]}$ Resistance is associated with the expression of five genes.$^{[74]}$ In the absence of vancomycin, cell wall can be built in the normal way, using precursors terminating in -d-Ala-d-Ala. In the presence of vancomycin, the five genes for resistance (named vanS, vanR, vanH, vanA, and vanX) are switched on.$^{[74]}$ The product (VanS) of the gene vanS becomes phosphorylated on a histidine residue. This phosphorylated protein then interacts with a protein (VanR) which is the product of the second gene, vanR, and this protein in turn becomes phosphorylated.
Phosphorylated VanR causes transcriptional activation of the other three genes (\textit{vanH}, \textit{vanA}, and \textit{vanX}). The product of \textit{vanH} is an \( \alpha \)-keto reductase, which can reduce pyruvate to d-lactate. This d-lactate is then used as a substrate (along with d-alanine and ATP as co-factor) by the enzyme VanA (the product of expression of the gene \textit{vanA}). The enzymatic activity of VanA produces d-Ala-d-Lac, which is then used in the bacterial cell-wall precursors (instead of d-Ala-d-Ala).

The fifth gene (\textit{vanX}) codes for an enzyme (VanX), which acts as a d-Ala-d-Ala peptidase, so that the d-Ala-d-Ala that is produced to provide a constituent of normal cell wall biosynthesis can now be broken down to provide d-Ala as a precursor for d-Ala-d-Lac. The arrangement of these genes, and the functions of their products, are indicated in Figures 11 and 12, respectively.

As a consequence of the expression of the five genes associated with resistance, and the biosynthesis of cell-wall precursors that terminate in -d-Ala-d-Lac, the hydrogen bond which is normally made between the NH of the terminal d-alanine group of the precursors and a carbonyl group of the antibiotic is replaced by a repulsive interaction between the oxygens of the C-terminal d-lactate group and the carbonyl group of the antibiotic (Scheme 4). The affinity of glycopeptide antibiotics for precursor analogues terminating in -d-Ala-d-Lac is thus decreased by a factor of the order of 1000 relative to the binding to precursors terminating in -d-Ala-d-Ala.\textsuperscript{[76]} As a consequence of this much lower binding constant, vancomycin shows little activity against bacteria that biosynthesize such precursors.\textsuperscript{[76]} Inhibition of VRE requires 100–1000 times as much vancomycin as is required to inhibit the growth of sensitive organisms. Vancomycin is not, therefore, a viable antibiotic in these cases.

\textbf{16. Proposed Operation of Dimerization and Membrane Anchoring in the Action of a New Antibiotic Active Against VRE}

The nature of the difference between resistant and sensitive bacteria, and the reduction in affinity of vancomycin for precursors from resistant bacteria that results, suggests that the glycopeptide antibiotics may not be able to exhibit useful activity against such bacteria. However, attempts have been made at Eli Lilly in Indianapolis and Gruppo Lepetit-SpA in Gerenzano, Italy, to modify naturally-occurring glycopeptides to produce new antibiotics with increased activity against vancomycin-resistant bacteria.\textsuperscript{[77]} At Eli Lilly, scientists have recently taken the antibiotic chloroeremomycin (4) and added to this a hydrophobic p-chloro-p-phenylbenzyl group to give the resulting structure 5, which is known as LY333328.\textsuperscript{[78]} This new molecule shows remarkable activity against VRE, being typically 50 times more active than vancomycin.\textsuperscript{[79]} It is also more active, by a factor of 4–8 in vitro, than vancomycin against MRSA and its in vivo potency is comparable with its in vitro potency.\textsuperscript{[79]} The similar program at Gruppo Lepetit-SpA has also yielded a glycopeptide that shows promising activity, both in vitro and in vivo, against vancomycin-resistant enterococci.\textsuperscript{[80]}

An answer to the question of how the modified antibiotic LY333328 is able to kill resistant bacteria was not immediately obvious. This was especially so, given that LY333328 (5) and vancomycin both have low affinity (binding constants in the range 300–500 M\(^{-1}\)) for the precursor analogue of resistant bacteria N-\( \alpha \)-acetyl-N-\( \varepsilon \)-acetyl-lysyl-d-alanyl-d-lactate (Ac2-
KdAdLac) in aqueous solution. These values are in contrast to the binding constant of 1.5 × 10^6 M^-1 for vancomycin binding to Ac2-KdAdA. This similarity in binding affinity of Ac2-KdAdLac for vancomycin and for LY33328 was not entirely surprising, however, given that the semi-synthetic modification to LY33328 was made at a position remote from the ligand binding pocket of the antibiotic. We therefore hypothesized that LY33328 was gaining extra binding affinity for β-lactate-terminating ligands, relative to vancomycin, through the operation of the cooperative phenomena outlined earlier in this article. It was felt that the weak binding interaction between LY33328 and Ac2-KdAdLac might be enhanced at the surface of a bacterium through the simultaneous operation of both dimerization (LY33328 is derived from the antibiotic chloroeremomycin, which dimerizes strongly) and membrane anchoring (by the hydrophobic p-chloro-p-phenylbenzyl chain inserting into the bacterial membrane).

17. 1H and 13C NMR in Measurements of Binding Enhancements at Membrane Surfaces

We recently attempted to accumulate evidence in support of the above hypothesis through 1H and 13C NMR spectroscopic studies of membrane-anchored peptides terminating in β-lactate. In these experiments we have used phosphatidylcholine vesicles to mimic the bacterial membrane rather than SDS micelles. Vesicles are much larger lipid assemblies than micelles, and consist of lipid bilayers in which the polar head groups exist both in the inside and the outside of the spherical structure. Cell wall precursor peptides possessing N-docosanoyl (a linear C22 chain) lipid chains were then synthesized such that the docosanoyl chains could insert into the vesicles, as with the decanoylated peptides used earlier, in order to mimic the surface of the bacterium. Such vesicle assemblies have the advantage of being closer in size to bacteria than micelles, and the possibility of greater cooperativity being expressed therefore exists. Yet this advantage is bought at a price—the vesicles are so large that if an antibiotic becomes bound to these bacterial mimics, its proton NMR signals are no longer observable. This is because the bound antibiotic takes on the slow tumbling time that is characteristic of a large molecular assembly, and its protons then undergo fast nuclear relaxation, with a consequent severe broadening of the lines, so much so that they effectively disappear. Therefore, the w1 resonance, whose chemical shift can be used as a semi-quantitative measure of binding affinity (Figure 5), cannot be observed in the bound state.

However, this line-broadening effect can be put to advantage in competition experiments. Suppose a β-Lac-terminating peptide, possessing a docosanoyl group at its N-terminus, is anchored into the vesicle. It may therefore form the vesicle-templated assembly shown in Figure 13A. According to our hypotheses the antibiotic/peptide complex should be stronger in this assembly than it would be if both components were free in solution (as exemplified by the association between free chloroeremomycin and Ac2-KdAdLac in solution, where the binding constant is approximately 240 M^-1). If there is indeed extra binding affinity in the vesicle-attached assembly, then it should be possible to compete with such binding by the addition of a β-Ala-terminating peptide that is only able to bind in solution, for example, Ac2-KdAdA which lacks a membrane anchor. In such a competition experiment it is possible to adjust the concentration of added Ac2-KdAdA until it is sufficiently high to “steal” the antibiotic (Figure 13B) from its vesicle-attached state (Figure 13A) where it is bound to N-docosanoyl-glycyl-l-alanyl-β-glutamyl-l-lysyl-l-alanyl-l-lactate (Docos-GAdeEKdAdLac; an extended cell wall precursor analogue, where the glycine residue is used as a spacer unit in lieu of the sugar unit that is present in the natural cell-wall precursors). Removal of the antibiotic from its vesicle-attached state means that its 1H NMR signals are no longer severely line-broadened and resonances arising from the antibiotic/Ac2-KdAdA complex can be observed in 1D 1H NMR spectra. Integration of the resultant complex signals allows quantitation of the binding constant for the antibiotic binding to the vesicle-anchored ligand in a templated complex. We have carried out such a competition experiment using the strongly dimerizing antibiotic chloroeremomycin and the cell wall analogue components mentioned above; details of this experiment are given in Figure 14. It is found that a 5.7-fold excess of Ac2-KdAdA over vesicle-attached Docos-GAdeEKdAdLac is required to approximately equi-partition the binding of chloroeremomycin between these two peptides. Qualitatively, it can therefore be seen that the templated β-Lac-terminating peptide binds the antibiotic approximately six times more strongly than does Ac2-KdAdA. Since Ac2-KdAdA has a binding constant of approximately 10^6 M^-1 to free chloroeremomycin in solution, this corresponds to a binding constant of approximately 6 × 10^5 M^-1 for this strongly dimerizing antibiotic to the model of a vancomycin-resistant bacterium (a more quantitative treatment of the data gives a binding constant of 7 × 10^5 M^-1). The binding constant of the equivalent non-docosanoylated ligand, N-acetyl-glycyl-l-alanyl-β-glutamyl-l-lysyl-l-alanyl-l-lactate (Ac-GAdeEKdAdLac), was measured by a different NMR method to be 2000 M^-1. The binding constant to Docos-GAdeEKdAdLac thus corresponds to a remarkable cooperative enhancement of the binding of the antibiotic to the membrane-anchored precursor analogue of VRE of approximately 3500 over the binding observed in the absence of the docosanoyl membrane anchor.
The Vancomycin Group of Antibiotics

Figure 14. Competition experiment (see Figure 13) at pH 7 to determine the binding constant of chloroeremomycin (4) to a model of the surface of VRE. A) Proton resonances of chloroeremomycin when bound to the nonmembrane-bound cell wall precursor analogue Ac2-KdAdA in the presence of phosphatidylcholine vesicles. B) The severe broadening of the same resonances when the antibiotic is bound to the -d-Lac-terminating-hexapeptide cell wall precursor analogue Docos-GAeKdAdLac that is anchored into phosphatidylcholine vesicles. C) The solution from (B) to which Ac2-KdAdA has been added to a concentration roughly equal to that of the membrane-bound Docos-GAeKdAdLac. Note that most of the antibiotic is still bound to the membrane-bound -d-Lac-terminating peptide (from the contrasting signal intensities in (A) and (C)). D) The solution from (C), but to which more Ac2-KdAdA has been added. In (D), comparable amounts of antibiotic are bound to the free -d-Ala terminating peptide in solution and to the membrane-bound -d-Lac-terminating peptide. E) A large excess of Ac2-KdAdA over the membrane-bound Docos-GAeKdAdLac is required for most of the antibiotic to bind to the former.

We have employed a second technique that utilizes competition experiments to provide a further measurement of the binding enhancement that can be achieved through templated binding at a vesicle surface. This involved competing the antibiotic molecules from their vesicle-templated complexes with Docos-GAeKdAdLac using the ligand N-α-acetyl-N-ε-trifluoroacetyl-lysyl-d-alanyl-d-alanine (TFAc-KdAdA). This ligand is labeled with a [19F]trifluoroacetyl group at a position not intended to interfere significantly with the binding of the ligand to the antibiotic. [19F]NMR spectroscopy can therefore be used to examine and quantify the binding to antibiotics. This method has the advantage over 1H NMR spectroscopy that only two signals are seen in the 19F NMR spectra during the competition experiments; one signal from free (unbound) TFAc-KdAdA and one from TFAc-KdAdA complexed with antibiotic not on the vesicle surface (Figure 15). Integration of the free and bound peaks, and comparison to the integral of an external reference peak allows quantitation of the apparent binding constant of chloroeremomycin bound to Docos-GAeKdAdLac in a templated complex on the vesicle surface. The value thus obtained using this method, for the apparent binding constant of chloroeremomycin to vesicle-bound Docos-GAeKdAdLac was 2.5 × 10^4 M^-1, which is, as with the 1H NMR-derived value, much greater than the solution binding constant of chloroeremomycin to free Ac2-GAeKdAdLac (2000 M^-1), which confirms that a large enhancement to the binding of -d-Lac-terminating ligands can be achieved at a surface as a result of antibiotic dimerization.[30]

Recall that it is proposed that the cooperativity which can be gained by simultaneous strong dimerization and membrane anchoring of the antibiotic may provide the basis for the remarkable activity of LY333328 (5) against bacteria utilizing d-Lac-terminating peptides in their cell-wall biosynthesis. The experiments described thus far, although demonstrating the benefit of binding at a surface provided by dimerization, fail to quantify the cooperativity gained by simultaneous operation of these two effects. Two problems have been encountered in attempts to obtain this number. First, when micelle-associated peptides are used to bind 5, the solution containing both these components becomes cloudy, presumably because of aggregation, and a binding constant cannot be obtained by UV difference spectroscopy. Second, the competition experiments, described above for chloroeremomycin with vesicles, cannot be simply used with 5, since the p-chloro-p-phenylbenzyl group will influence the antibiotic towards always remaining vesicle-bound and thus direct NMR measurements of the populations of bound species are not possible. Nevertheless, the templated-enhancement to binding of a factor of the order of 10^4, cited above, from dimerization alone, is sufficiently dramatic to suggest that the remarkable activity of 5 against VRE can be accounted for, not by a different binding mode of 5 compared to vancomycin, but because of the simultaneous exercise of the cooperative benefits of dimerization and membrane-anchoring. This value of 10^4 is likely to represent a lower limit for the enhancement that will be experienced by 5, which should also be able to achieve a benefit as a result of membrane anchoring on top of the benefit from dimerization. The operation of the two

Figure 15. 19F NMR spectra from a titration of a solution of TFAc-KdAdA into a solution containing 0.2 mM chloroeremomycin (4), 1.0 mM Docos-GAeKdAdLac, and 10 mM phosphatidylcholine at 298 K. The concentrations of added TFAc-KdAdA are shown at the right-hand side of each of the corresponding 1D spectra. The peaks labeled (A) are those of unbound TFAc-KdAdA, while the peaks labeled (B) are those of TFAc-KdAdA complexed with chloroeremomycin not attached to the surface of a vesicle. The TFAc-KdAdA (binding constant to chloroeremomycin of 77 × 10^4 M^-1) competes effectively with Docos-GAeKdAdLac for the binding sites of chloroeremomycin. The binding constant of Docos-GAeKdAdLac to chloroeremomycin calculated from analysis of this data is 2.5 × 10^4 M^-1. Chemical shift values are relative to an external reference of trifluoromethanesulfonic acid (δ = -3.60).

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effects together may also result in a further cooperative enhancement from the mutual motional restriction caused by the two membrane-locating devices.

A note of caution must be expressed here, however. Although the model membrane systems that we have used have unambiguously allowed the expression of enhanced binding affinities in these systems, and the dimerization propensity of the antibiotics has been correlated with the difficulty of antagonizing antibiotic action against *B. subtilis*, we cannot preclude the additional operation of as yet undiscovered factors in the remarkable activity of 5. For example, the possibility that antibiotic dimerization can affect antibacterial activity through the cross-linking of adjacent cells may merit further investigation. Additionally, it must be borne in mind that where the antibiotics incorporate hydrocarbon groups that enhance binding at model membranes containing mucopptide precursors, we can so far only surmise that identical effects operate at the surfaces of bacteria. For example, we cannot yet preclude that the hydrocarbon groups of the antibiotics are alternatively associating with hydrophobic clusters in membrane-bound proteins, and thereby deriving cooperative binding energy from such interactions. Nor can we yet understand the often quite marked differences in activities of the various glycopeptide antibiotics against the wide diversity of Gram-positive pathogens that are found in the clinic. The crucial message for drug design, however, is that if binding efficacy is lost as a result of a mutation in a binding site, binding of similar efficiency can be restored in principle without making any modifications directly in the binding site. A direct corollary of this conclusion is of course that the prediction of binding constants in complex networks of interactions is at best a formidably difficult task! In the present case important antibacterial activity has been recouped from a situation that may initially have appeared hopeless.

18. Implications for the Functions and Evolution of Secondary Metabolite Structures

The occurrence of two complex binding sites within the glycopeptide antibiotics (one for binding cell wall peptide precursors and the other for dimerization) is remarkable. It is a feature that strongly suggests that the evolutionary origin of the glycopeptide structures (at least in the origins of their most recently selected molecular features) lies in the antimicrobial action described. In several articles we have argued that secondary metabolites (which are defined as having no known role in the internal economy of the producer, and are typically excreted from the producer) have evolved to increase the survival fitness of the producer. This increase in survival fitness can be expressed in principle by either symbiotic or antagonistic interactions with other organisms. In either case, a receptor site at another organism is necessary. This very frequently involves a specific molecular interaction and, where the details of such interactions have been elucidated (as in the case presently described), they rival enzyme/substrate interactions in their sophistication.

Against the above view, it is sometimes argued that remarkably complementary interactions of secondary metabolites to other molecules in a second organism may be found, but yet it might be difficult initially to accept that these interactions are the ones that have evolved through natural selection. A case in point is found in the molecular basis of action of the immunosuppressive compounds cyclosporin A, FK506, and rapamycin. It certainly seems impossible to justify that these three secondary metabolites, the first produced by a fungus and the last two by *Streptomyces* bacteria, have evolved to inhibit the activation or proliferation of human T cells! Yet,

1) this inhibition occurs through the binding of the secondary metabolites to intracellular receptor proteins, and in the cases of cyclosporin A and FK506, the resulting complexes then interact with calcineurin.

2) exactly the same sequence of events occurs in yeast cells, through which the secondary metabolites exercise antifungal properties.

Thus, we see in the second point a possible selectional force for the production of the secondary metabolites by the producing strains, because these producers have a weapon to combat other microorganisms with which at some stage they may have competed. The same pathways for activity against T cells may exist because of the common origins of T cells and yeast cells, with the consequence that their primitive signaling pathways may be the same.

One of the most cogent points in favor of the key importance of secondary metabolites to the survival of their producers lies in the impressively large amount of DNA that is frequently necessary to code for their production. The pioneering work of Hopwood in investigating this DNA, and the details of its functions, is now being extended by others in many parts of the world. The use of several tens of enzymes in the production of an antibiotic is not uncommon, and thus their production is coded within many tens of kilobases of DNA. The vancomycin group of glycopeptides are no exception in this remarkable feat of evolution.

19. Comments on Cooperativity: the Enthalpic Chelate Effect

During the course of our work on the mode of action of the glycopeptide antibiotics, we have been able to identify and comment upon a number of features regarding cooperativity in molecular recognition that have a general relevance to studies of binding processes in nature. In this respect, the glycopeptides have proved to be a simple and useful model for understanding aspects of binding that may be more difficult to study in more complex systems.

We have recently commented upon the fact that the decreased motion of a ligand in a binding site works to improve the electrostatic interactions that are formed in that binding site. In a reciprocal manner, features that improve the electrostatics of binding in a ligand/receptor interaction likewise clearly reduce the degree of motion in the binding site. This is a demonstration of the property of enthalpy–entropy compensation; the two effects can be
regarded as working iteratively on each other. For example, if A binds in isolation to part of a binding site with an exothermicity $X$, and B binds in isolation to another part of the binding site with an exothermicity $Y$, then attachment of A to B so that they can simultaneously bind into the binding site in a strain-free manner (that is as the entity $A-B$) is not only advantageous through the operation of the classical chelate effect (an entropic benefit), but leads to an exothermicity of binding greater than $X+Y$. This effect we call the enthalpic chelate effect.\[^{[55]}\]

An example of the enthalpic chelate effect was shown earlier with the increasing strength of the hydrogen bond between the carboxylate anion of a bacterial cell wall precursor analogue and the amide NH proton $w_2$ of a glycopeptide antibiotic as extra functional groups were added to the ligand (Figure 5). The extra functional groups and corresponding extra hydrogen bonds between ligand and antibiotic, relative to the binding of, say, acetate, result in an increased downfield chemical shift of $w_2$ upon ligand binding. Although the extra functional groups are remote from $w_2$ and therefore do not exert a direct influence on its chemical shift, they serve to anchor the ligand into its binding site and thus reduce the motion of the ligand carboxylate relative to the antibiotic, leading to a stronger (more exothermic) hydrogen bond to $w_2$ and a more downfield resonance.\[^{[55]}\]

### 20. Equilibrium Constants and the Parameters Describing Bound States

The experiments described above for measuring the chemical shift of $w_2$ illustrate a principle that we considered might be both general and important in terms of its implications for studies of other molecular recognition events. This is that a parameter representing the bound state of a system (for example, the limiting chemical shift of $w_2$) may vary according to the equilibrium constant for the association (Figure 5). Further, if we could examine the interface for a specified association, a relatively high value for the equilibrium constant would result in strongly bound states at the interface with correspondingly short bond lengths; the interface could be described as “tight”. Conversely, a weak association would be characterized by long interfacial bond lengths; the interface could be described as “loose”. Accordingly, we attempted to measure the “tightness” of the dimer interfaces of the glycopeptide antibiotics with respect to their dimerization constants. All glycopeptides, despite exhibiting a wide range of dimerization constants, share a common arrangement of at least four hydrogen bonds at the dimer interface (Scheme 2). A proton, $x_4$ (Figures 5 and 7), at the dimer interface undergoes a relatively large downfield shift upon dimerization and the extent of this limiting downfield shift upon dimerization ($\Delta \delta_{x4}$) was used to probe the tightness of the dimer interface as a function of the dimerization constant. The change in chemical shift of $x_4$ on dimerization ($\Delta \delta_{x4}$) is much larger for strongly dimerizing antibiotics than for weakly dimerizing antibiotics (Figure 16).\[^{[55]}\]

In summary, the data relating the chemical shifts of $w_2$ and $x_4$ to ligand binding and dimerization constants, respectively, indicate that as the equilibrium constants for the respective associations increase, the associating entities come into more intimate contact. A general conclusion to be derived from this data is given in Figure 17. This finding has implications for the study of transitions between bound (or folded) and free (or unfolded) states that are considered to occur by means of a two-state process. Such studies typically involve measurement of a parameter and comparison of this to values expected for the bound (or folded) and free (or unfolded) states. The measured value then allows calculation of the relative proportions of the states. Typically, the parameter values representing free (or unfolded) states may be relatively simply defined. For example, in considering ligands that bind to a glycopeptide antibiotic, the free state is that where no ligand is bound (free antibiotic) and a parameter ($w_2$) representing this state can be easily measured. Our work shows, however, that there is no single parameter value that represents the bound (or folded) state.\[^{[96]}\]

As the equilibrium constant for the association increases so does the parameter representing the bound (or folded) state, for example, the limiting chemical shift of $w_2$. Systematic errors can arise from the above complication. An example is the calculation of the fractional population of a short peptide (known to form an $\alpha$-helix when part of a full-length protein) occupying an $\alpha$-helical conformation in aqueous solution. The equation used to calculate this fraction $x$ is generally of the form (1), where $P_{\text{tot}}$ is a parameter

$$x = \frac{P_{\text{tot}} - P_{\text{ref}}}{P_{\text{ref}} - P_{\text{tot}}}$$

\[^{[1]}\]
characteristic of the fully unfolded ("random coil") state of the peptide, $p_{\text{fold}}$ is the parameter characteristic of the fully folded (helical) state of the peptide, and $p_{\text{obs}}$ is the measured value of the parameter under the conditions of interest (for example, aqueous solution).

In order to obtain an estimate for $p_{\text{fold}}$, it is common to introduce trifluoroethanol (TFE) as a solvent, which encourages the formation of $\alpha$-helices. However, the very act of diluting the TFE solution with water would be expected, by analogy to our work on glycopeptides, to lead not only to a gradually increasing population of the unfolded state, but simultaneously to a folded state which becomes increasingly loose relative to that existing prior to dilution. Thus, the parameter $p_{\text{fold}}$ will be reduced in magnitude as the dilution proceeds. The error inherent in such analyses therefore generally results in an underestimate of the population of the (increasingly dynamic) folded state.


Returning to the experiments described above which indicate the variation of the tightness of the dimer interface (identified by the magnitude of $\Delta \delta_{x4}$) with the variation of the dimerization constant, we have extended these studies further to probe the interfacial origins of cooperative binding energy within systems of ligand-bound glycopeptide dimers.

Our hypothesis was that if one of the interfaces (dimer interface or antibiotic–ligand interface) was initially loose (small association constant), then formation of a cooperative tetrameric assembly (two ligands bound to an antibiotic dimer) would potentially result in a significant tightening of that interface. Conversely, if one of the interfaces was initially tight (large association constant), then formation of a tetrameric assembly would allow little scope for further tightening through cooperative interactions. If this hypothesis is true, then if the dimer interface, for example, is loose in the absence of ligand (small dimerization constant $K_{\text{dim}}$), an important contribution to the increase in $K_{\text{dim}}$ in the presence of ligand will come from changes associated with the tightening of the dimer interface (which can be observed by an increase in limiting chemical shift of $x_4$). Alternatively, if the dimer interface is tight even in the absence of ligand (large value of $K_{\text{dim}}$), then the major portion of the favorable Gibbs free energy change that causes an increase in $K_{\text{dim}}$ in the presence of ligand should actually come from changes associated with the tightening of the ligand–antibiotic interface, and there should be little accompanying change in the limiting chemical shift of $x_4$. The way in which the cooperative Gibbs free energy of dimerization can be partitioned into changes associated with the dimer interface or with the ligand–antibiotic interface is indicated by sets of hypothetical points in Figure 18.[95] The expectation is that a weakly dimerizing antibiotic ristocetin-$\psi$ dimerizes more strongly in the presence of Ac$_2$KdAdA than in its absence essentially because the Gibbs free energy of binding associated with changes at the dimerization interface is more favorable. The more strongly dimerizing antibiotics chloroeremomycin, $p$-phenylbenzylchloroeremomycin (LY307599), and eremomycin-

![Figure 18](image-url)

**Figure 18.** A) Hypothetical plots of $-\Delta G_{\text{dim}}$ versus $\Delta \delta_{x4}$ for the dimerization of four antibiotics W, X, Y, and Z when free ($\circ$) and as antibiotic–ligand complexes ($\bullet$). The arrows connect the hypothetical points for a given antibiotic, and the series of antibiotics W$\rightarrow$X$\rightarrow$Y$\rightarrow$Z have increasing dimerization constants. B) Since the Gibbs free energy of dimerization associated with changes at the dimer interface is defined by the curve connecting the points ($\circ$) for the dimerization of antibiotic alone, the extent to which $\bullet$ lies vertically above this curve gives the Gibbs free energy of dimerization associated with changes in the ligand–antibiotic interfaces.

![Figure 19](image-url)

**Figure 19.** Combined plot of the Gibbs free energy of dimerization ($-\Delta G_{\text{dim}}$ [KJ mol$^{-1}$]) versus $\Delta \delta_{x4}$ for free ($\circ$) and ligand-bound ($\bullet$) glycopeptide antibiotics. For any one antibiotic the arrows represent the changes in $-\Delta G_{\text{dim}}$ and $\Delta \delta_{x4}$ that occur upon ligand binding. In order of increasing magnitude of $\Delta G_{\text{dim}}$ (for free antibiotics), the points represent the dimerization of the following antibiotics: ristocetin-$\psi$, monodechlorovancomycin, vancomycin, chloroeremomycin, eremomycin, and $p$-phenylbenzylchloroeremomycin.
cin dimerize more strongly in the presence of Ac2-KdAdA than in its absence largely because the Gibbs free energy of binding associated with changes at the antibiotic–ligand interface is more favorable in the ligand-bound dimer than in the ligand-bound monomer. In this case, there is little change in the Gibbs free energy of binding associated with changes at the dimer interface. The behavior of vancomycin is between these two extremes with the cooperative Gibbs free energy expressed partially at each of the ligand binding and dimerization interfaces.

These findings have implications for the study of protein–protein interactions and for drug design. In both areas it is common practice (though not of course uniquely the practice) to seek the origins of binding affinity at the interface formed between the associating entities. Our data (Figure 19) show that the increase in the equilibrium constant for dimerization of weakly-dimerizing antibiotics in the presence of ligand (relative to when the ligand is absent) arises largely from changes associated with the tightening of the interaction between ligand and antibiotic. By analogy, where proteins (or, more specifically, receptors) have loose structures prior to binding another protein (or in the specific case of a receptor, its natural ligand or a drug), then a portion of the binding affinity can be derived by the tightening of the internal structures of the proteins in the resulting bound state. Based on this reasoning the thermodynamic parameters for protein–protein associations, which are perplexing when analyzed in terms of interfacial interactions, are realized to have much more complex origins.

22. Summary and Outlook

Despite the advances detailed in this article regarding our increased knowledge of glycopeptide activity, there is no room for complacency as evidenced by recent reports37,99,100 of the appearances in hospitals of S. aureus infections that are resistant to vancomycin. Although the resistance in MRSA so far seen is at a low level (and appears to involve an augmented rate of cell-wall synthesis, rather than the synthesis of a d-Ala-d-Lac precursor99), there is still the possibility that more high-level resistance to vancomycin will develop in S. aureus. It is clear than any approach that opens new possibilities for increasing the activity of glycopeptides is of the utmost importance.101

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