^{19}F NMR in the measurement of binding affinities of chloroeremomycin to model bacterial cell-wall surfaces that mimic VanA and VanB resistance

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Background: The emergence of bacteria that are resistant to vancomycin, the drug of choice against methicillin-resistant *Staphylococcus aureus*, has made the study of the binding characteristics of glycopeptides to biologically relevant depsipeptides important. These depsipeptides, terminating in -D-alanyl–D-lactate, mimic the cell-wall precursors of resistant bacteria.

Results: The use of ^{19}F-labelled ligands in the study of the therapeutically important vancomycin series of antibiotics is demonstrated. The substantial simplification of spectra that occurs when such labelled ligands are employed is used in the measurement of binding affinities of depsipeptides to chloroeremomycin (CE). Large enhancements of binding affinities are found at a model bacterial cell-wall surface (constituted from depsipetides that are anchored into vesicles) relative to those measured in free solution.

Conclusions: Surface-enhanced binding, previously shown for strongly dimerising glycopeptide antibiotics to normal -D-alanyl–D-alanine-terminating cell-wall precursors, is now demonstrated for CE to the surface of models of VanA- and VanB-resistant bacteria. The effect of depsipeptide chain length is shown to be critically important in producing and maximising this enhancement.

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Key words: ^{19}F NMR, glycopeptides, model bacterial cell-wall surfaces, VanA and VanB resistance

Received: **13 March 1998**
Revisions requested: **17 April 1998**
Revisions received: **28 April 1998**
Accepted: **6 May 1998**

Published: **4 June 1998**

Chemistry & Biology June 1998, **5**:329–337
http://biomednet.com/elecref/1074552100500329

Introduction

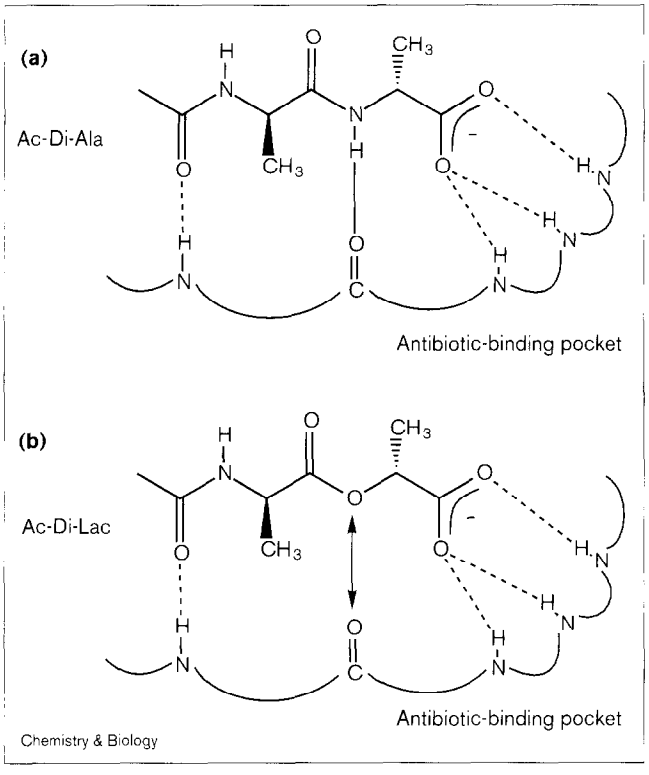
The clinically important vancomycin group of antibiotics bind to the cell-wall precursor C_{55}-NAG-NAM-alanyl–D-glutamyl–lysyl–D-alanyl–D-alanine on the surface of gram-positive bacterial membranes, inhibiting cell-wall biosynthesis, leading to the death of the bacterium [1,2]. They are now the last line of defence against many strains of pathogenic bacteria, especially methicillin-resistant *Staphylococcus aureus* (MRSA) [3,4].

These antibiotics, with the exception of teicoplanin, have been shown to dimerise along the opposite face of the antibiotic to that involved in ligand binding [5–7]. For most of those antibiotics that dimerise, it has been shown that the binding of a ligand to these antibiotics increases their dimerisation constants and that dimerisation leads to higher binding constants, that is, dimerisation is cooperative with ligand binding and *vice versa* [5].

Previous work in this group [8,9] has demonstrated that there is an increase in the free energy of binding of glycopeptide antibiotics to cell-wall precursors when they are bound to a model cell-wall system rather than cell-wall precursors in free solution. We define the complex formed between an antibiotic dimer bound to two surface-bound ligands as a templated complex. When a dimeric antibiotic binds to the cell surface, a significant portion of the entropic cost of binding is paid in the first binding event and hence the second binding event becomes effectively intramolecular. This chelate-like enhancement results in stronger binding to ligand and hence an increase in biological activity [5,9,10]. We have previously demonstrated an excellent correlation between binding constants measured in such model systems with the corresponding binding constants on the actual bacterial surface [8,11].

It is known that bacteria become resistant to vancomycin through the modification of the terminal D-alanine (D-Ala) residue in their growing cell wall to D-lactate (D-Lac). The transformation of an amide bond to an ester replaces an attractive carbonyl–NH hydrogen bond with a repulsive interaction between the ester oxygen from the D-Lac and the same carbonyl group within the binding pocket of the antibiotic (Figure 1) [12,13]. This substantially reduces the binding affinity of the antibiotic in solution (K_b moves from 1.3×10^6 M^{-1} for chloroeremomycin (CE) bound to di-*N*-acetyl–lysyl–D-alanyl–D-alanine (Ac-Tri-Ala) to 920 M^{-1} bound to di-*N*-acetyl–lysyl–D-alanyl–D-lactate (Ac-Tri-Lac)). It is this reduction in binding that appears to render the antibiotic, and the closely related antibiotic vancomycin, therapeutically useless against VanA- and VanB-resistant bacteria [14–16]. Hence, the

Figure 1



Resistance to vancomycin. In vancomycin-resistant enterococci, the terminal D-alanine residue of the immature cell wall has been replaced by D-lactate. This change drastically reduces the binding affinity to glycopeptides, as an NH group **(a)**, which can form a hydrogen bond, is replaced by an oxygen atom **(b)**.

binding affinities of lactate-terminating ligands at a surface are of great interest. We now demonstrate a useful and novel method for the determination of binding constants of vancomycin-group antibiotics to model bacterial surfaces in general, and in particular to those bearing D-Lac-terminating ligands.

Results and discussion

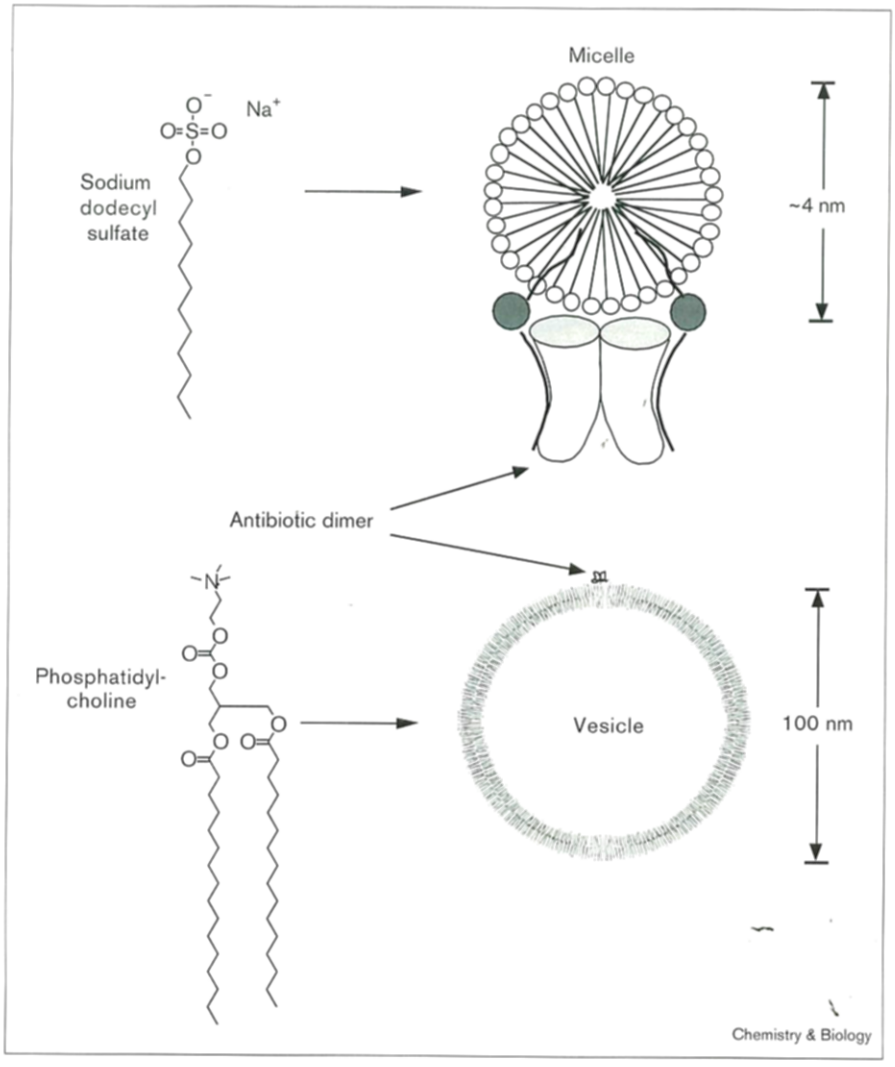
Micelles formed from sodium dodecyl sulphate (SDS) have been used as a model cell-membrane system to demonstrate the enhancement of activity through templated binding [9,17]. The system suffers from a number of distinct disadvantages, however. The high negative-charge density on the surface and small radius of curvature of the micelles is unlikely to allow the maximum possible enhancement of activity. Vesicles, prepared from phosphatidylcholine (PC), mimic the actual cell membrane more closely. PC, which is a component of cell membranes, forms a bilayer structure rather than the single layer of lipid found in micelles. The significant increase in volume of vesicles over their micellular counterparts, with concomitant increase in surface area and reduction in radius of curvature, should considerably reduce the strain within the ligand–dimer complex as the surface of the vesicle is closer to planarity, and therefore more similar to the surface of a bacterium. Additionally, the net charge associated with the vesicle is zero. For these reasons, vesicles have now replaced micelles in our experiments demonstrating templated binding at cell-wall surfaces (Figure 2) [8].

Cell-wall-precursor analogue peptides have been synthesised previously with hydrophobic decanoyl and docosanoyl chains attached to the amino terminus [9,18] and they have been shown to anchor to model cell membranes such as vesicles [8,9]. The arrangement of vesicle and anchored cell-wall analogue is similar to the bacterial cell surface, where the cell-wall precursors are anchored to the bilayer by a C_{55} chain. Because of the large size of the vesicles used (typically 100 nm diameter), any species associated with a vesicle will take on the slow tumbling properties (for rotation) of the vesicle itself, resulting in almost total broadening of nuclear magnetic resonance (NMR) signals. This effect can be used to determine whether complexes are anchored to the vesicle surface [8].

In previous ^{1}H NMR competition experiments, the antibiotic was antagonised from the surface-bound ligand under study by another ligand of known binding affinity to the antibiotic [8,9]. The proportion of antibiotic bound to the vesicle in these experiments was determined by integration of certain peaks corresponding to aromatic protons on the antibiotic. This analysis is complicated by neighbouring overlapping resonances and a variation in the degree of broadening of those resonances. Furthermore, it is difficult to obtain accurate integrals of small peaks when much larger peaks, such as residual water, are present in the spectra. The use of ^{19}F NMR and a fluorinated ligand has a number of distinct advantages over the previous methods. The spectra are far simpler and easier to obtain than ^{1}H NMR spectra because of the absence of any other ^{19}F signals in the sample. For example, there is no need for solvent suppression and the difficulties associated with receiver gain in the observation of small signals in a mixture are significantly reduced. This results in spectra that are easier to interpret and leads to more accurate integrals. Second, with the use of a fluorinated ligand as the antagonist, the only observed signals would be those for fluorinated ligand bound, and not bound, to antibiotic. The bound signal would represent fluorinated ligand that had successfully competed antibiotic from the vesicle (Figure 3). A fluorinated species of known concentration was used as an external reference, and the integrals of the signals were used as a direct measure of the concentration of the relevant species present, allowing the calculation of the binding affinities of the anchored ligands. The ^{19}F label was readily introduced into the required ligands by trifluoroacetylation of the

Figure 2

The size of an antibiotic dimer compared to both a sodium dodecyl sulfate (SDS) micelle and a phosphatidylcholine (PC) vesicle. It is clear that the vesicle will much more closely approximate the flatter surface found on a bacterial membrane than an SDS micelle.

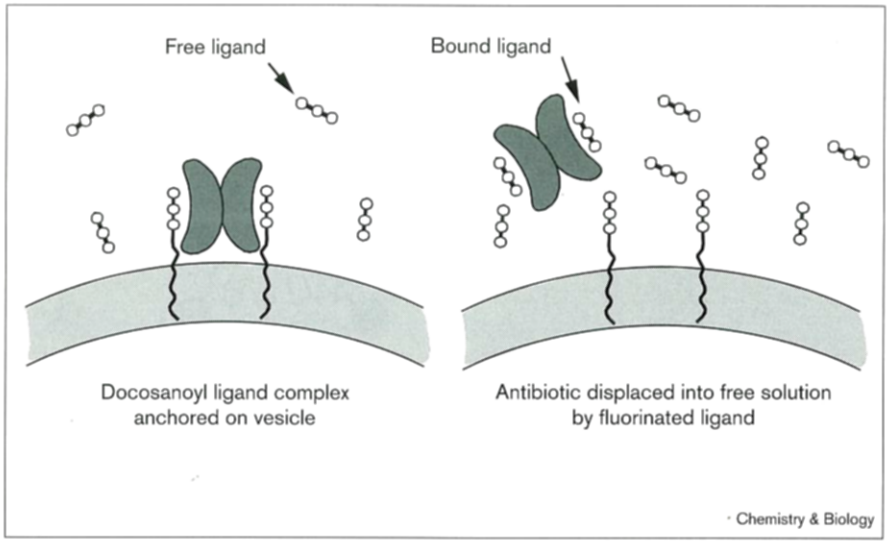


lysine residue, a method used successfully in previous work [19].

To obtain useful results from competition experiments, the competing ligand must have a binding affinity of similar magnitude to that of the templated complex. The peptide *N*-α-acetyl–*N*-ε-trifluoroacetyl–lysyl–D-alanyl–D-alanine (TFAc-Tri-Ala) was therefore synthesised, as it was expected to have a binding affinity similar to that of the nonfluorinated tripeptide Ac-Tri-Ala. The binding affinity of this ligand to CE, as determined by ultraviolet (UV) difference spectrophotometry [20], was $7.7 \pm 0.1 \times 10^5\ M^{-1}$, which is similar to the value of $1.3 \times 10^6\ M^{-1}$ for Ac-Tri-Ala [10]. As antibiotic dimerisation is an important factor in producing a strongly templated complex, the antibiotic chosen for this study was CE, which has a high dimerisation constant ($K_{dim} = 1.1 \times 10^7\ M^{-1}$ when bound, $K_{dim} = 1.8 \times 10^5\ M^{-1}$ when free) [5], and a strong affinity for cell-wall-precursor analogues (Figure 4) [10].

Previous work has demonstrated that the use of a decanoyl chain attached to depsipeptides can lead to templated binding enhancements, but only with peptides long enough to allow the alkyl chain to insert into the membrane [9]. For this reason we chose to study a series of ligands, investigating the effect of chain length on templated binding enhancements. Five depsipeptide ligands were studied: *N*-α-acetyl–*N*-ε-acetyl–lysyl–D-alanyl–D-lactate (Ac-Tri-Lac), *N*-α-acetyl–D-γ-glutamyl–*N*-ε-acetyl–lysyl–D-alanyl–D-lactate (Ac-Tetra-Lac), *N*-α-decanoyl–D-γ-glutamyl–*N*-ε-acetyl–lysyl–D-alanyl–D-lactate (Dec-Tetra-Lac), *N*-α-docosanoyl– D-γ-glutamyl–*N*-ε–acetyl–lysyl–D-alanyl–D-lactate (Doc-Tetra-Lac), and *N*-α-docosanoyl–glycyl–alanyl–D-γ-glutamyl–*N*-ε-acetyl–lysyl–D-alanyl–D-lactate (Doc-Hexa-Lac). The

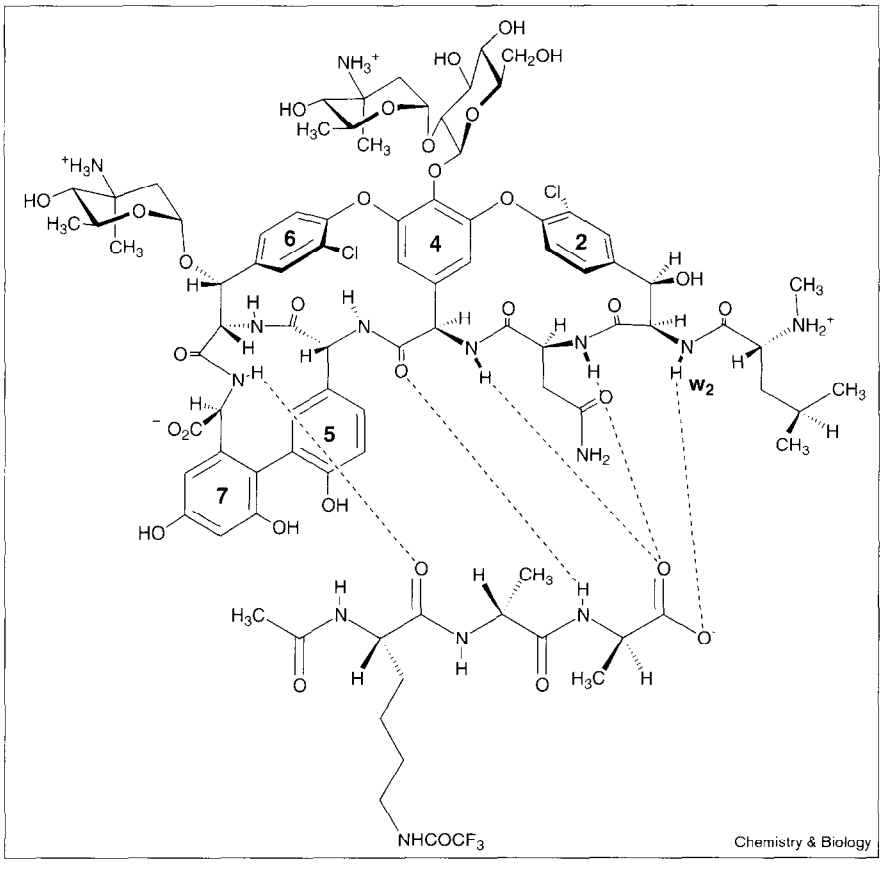
Figure 3



The concept behind the ^{19}F NMR competition experiments. A higher concentration of unanchored fluorinated ligand is required to displace more strongly bound antibiotic dimers. Shown are the species responsible for the free and bound signals in the ^{19}F NMR experiments.

affinities of Ac-Tri-Lac and Ac-Tetra-Lac were measured in solution by the method of monitoring the chemical shift of the w_2 proton (Figure 4) with increasing ligand concentration [21,22]. The remaining ligand affinities were all measured using the ^{19}F NMR method in the presence of vesicles. Note that the broadness of antibiotic

Figure 4



Exploded view of the complex formed between CE and the fluorinated ligand TFAc-Tri-Ala. The w_2 proton is indicated.

resonances when bound to a vesicle precludes the use of the w_2 method in these cases.

In all ^{19}F NMR experiments, an external reference of trifluoromethanesulfonic acid (TfOH) of known concentration was used to convert relative integrals to concentrations. The concentration of the TfOH reference was calibrated from a spectrum of the external reference with a TFAc-Tri-Ala solution of known concentration. To calculate the binding affinities of the ligands to CE, an equation was derived from a consideration of the known species and the equilibria involved.

The two competing equilibria are:

$$f + a \Leftrightarrow a_f \quad K_f = \frac{[a_f]}{[f][a]} \tag{1}$$

and:

$$l + a \Leftrightarrow a_l \quad K_l = \frac{[a_l]}{[l][a]} \tag{2}$$

Equation 1 describes the equilibrium existing between bound and unbound fluorinated ligand, where f represents free TFAc-Tri-Ala, a represents free antibiotic, and a_f represents TFAc-Tri-Ala bound to antibiotic. Equation 2 describes the same situation for the ligand anchored to the vesicle, where l represents unbound anchored ligand, and a_l represents anchored ligand bound to antibiotic. These lead to expressions for K_f and K_l, which are the corresponding binding constants. Also relevant are the mass balance expressions relating total antibiotic and anchored ligand concentrations. These are:

$$[a_t] = [a_l] + [a_f] + [a] \tag{3}$$

$$[l_t] = [l] + [a_l] \tag{4}$$

where a_t and l_t are the total concentrations of antibiotic and anchored ligand respectively. From a consideration of these equations, it is possible to obtain an expression for K_l (see Supplementary material):

$$K_l = \frac{x + [l_t] - [a]}{[a]([a] - x)} \tag{5}$$

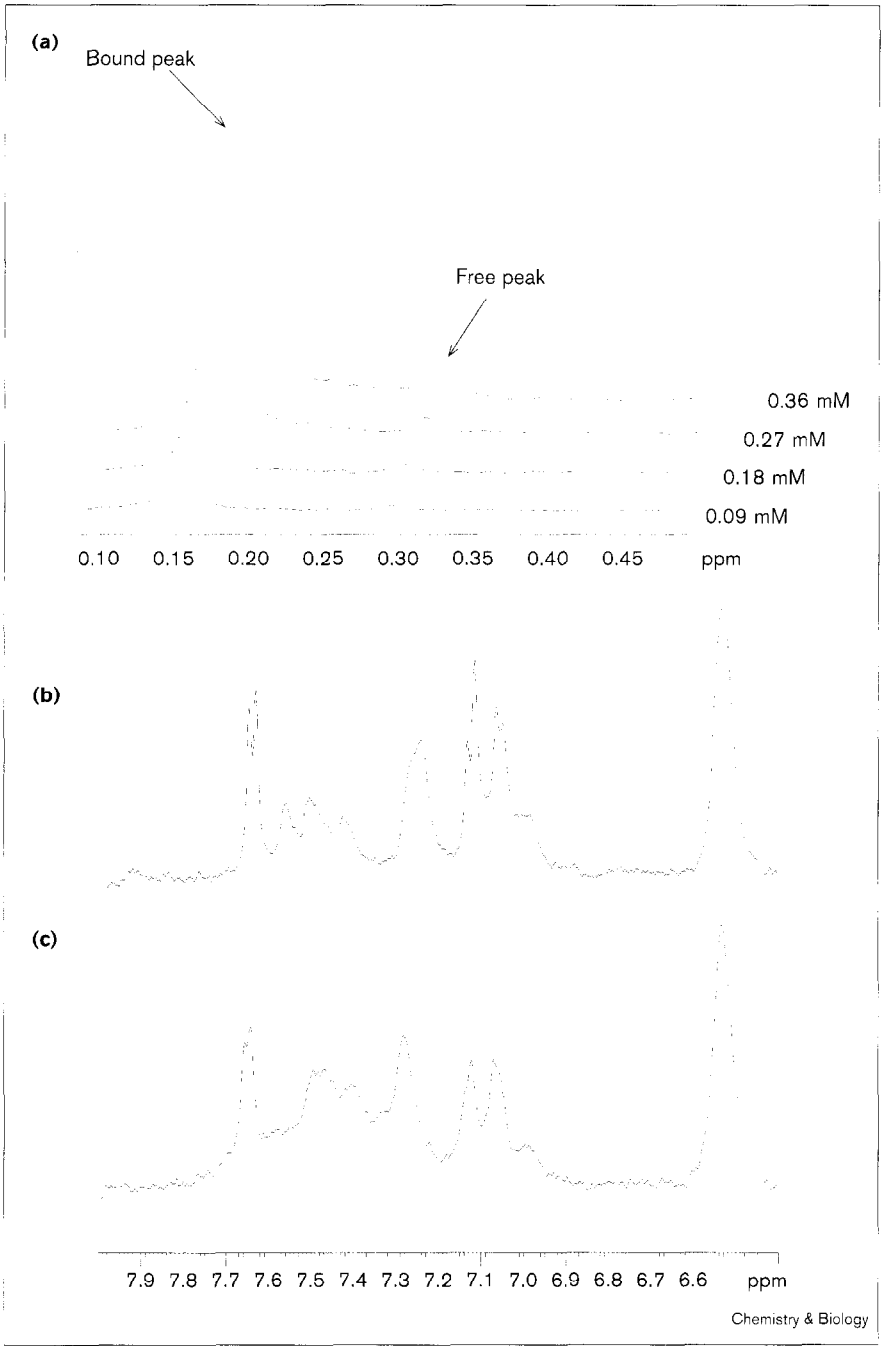
$$\text{where } x = [a_t] - [a_f] - [l_t], \text{ and } [a] = \frac{[a_f]}{[f]K_f}$$

It should be noted that the true dimensions of the surface-binding constant K_l are not M^{-1} but quoting them as such provides a useful quantitative reference to solution binding constants [8].

Figures 5 and 6 show the results of two ^{19}F NMR titration experiments with the depsipeptides Dec-Tetra-Lac and Doc-Hexa-Lac in the presence of vesicles, and a portion of the spectra from the corresponding ^{1}H NMR broadening experiments. Also shown is the relevant portion of the ^{1}H NMR control spectrum — that of CE and ligand with no vesicles. The first series of spectra are those for the study of binding to Dec-Tetra-Lac (Figure 5). Note that in the ^{19}F NMR spectra only two signals are present — those representing free ligand and ligand bound to antibiotic (Figure 5a). Using the equation derived previously, the affinity of Dec-Tetra-Lac for the antibiotic is calculated to be 1.0×10^4 M^{-1}. Although this value is greater than that for Ac-Tri-Lac (920 M^{-1}), within experimental error it is the same as that for Ac-Tetra-Lac (Table 1). An important conclusion can be derived from the above data: even in free solution Ac-Tetra-Lac binds to CE more strongly than does Ac-Tri-Lac by a factor of ~ 13. The difference between the tri- and tetra-depsipeptides is that of a D-glutamate (D-Glu) residue in the latter compound. It must be concluded that the D-Glu residue promotes much stronger binding to CE. It is striking that, from a ^{1}H NMR study of the binding of a cell-wall analogue terminating in alanyl–D-glutamyl–*N*-acetyl–lysyl–D-alanyl–D-alanine to CE [23], the γ-carboxyl group of the D-Glu residue is concluded to be in a close proximity to the NH^{3+} group of the 4-*epi*-vancosamine, which is attached to residue 4 via glucose. Although the study did not propose that significant binding might derive from such an interaction, we suggest that in the case of the binding of Ac-Tetra-Lac to CE, the interaction is probably largely the origin of the increased affinity relative to Ac-Tri-Lac. If the Dec-Tetra-Lac system were templated on the surface of the vesicles then one would expect to observe almost total broadening of signals in the ^{1}H NMR spectrum (Figure 5c) [8]. The distinct lack of broadening in the spectrum with vesicles (relative to the spectrum in the absence of vesicles; compare Figure 5b and 5c) is in agreement with the ^{19}F NMR result in so much as it confirms that little or no templating is occurring. The binding affinity was also calculated, in the absence of vesicles, using the method of following the chemical shift of the w_2 proton with a range of ligand concentrations [21,22], and the result was in excellent agreement with the ^{19}F NMR method, again indicating that, in the ^{19}F NMR experiment, no templated binding was occurring (Table 1).

The second series of spectra are for the ligand Doc-Hexa-Lac (Figure 6). Note the large increase in the binding affinity of the anchored docosanoyl ligand for CE, apparent from the peak for free TFAc-Tri-Ala being significantly more intense than the bound peak for all concentrations of TFAc-Tri-Ala (Figure 6a). In this case, the system benefits from the cooperative enhancement to be gained from templating that results from the ligand being long enough to allow insertion of the docosanoyl

Figure 5

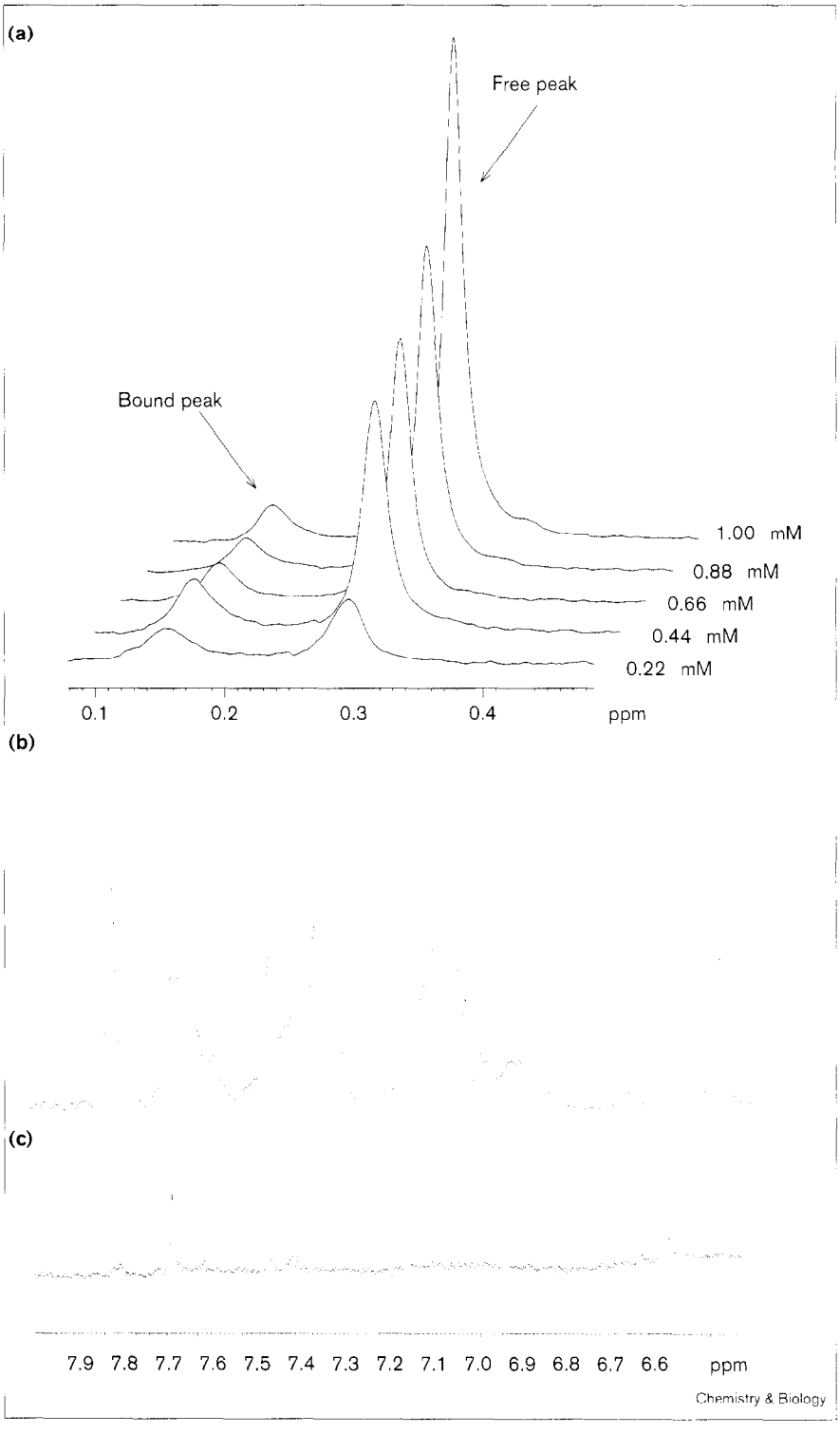


NMR spectra showing lack of efficient templated binding to Dec-Tetra-Lac. **(a)** Plot showing the ^{19}F NMR spectra from a titration of TFAc-Tri-Ala into a solution of CE (0.5 mM), Dec-Tetra-Lac (1.5 mM), and 100 nm 10 mM PC vesicles in 9:1 0.1 M phosphate buffer:D_2O at 298 K. Note that the bound peak dominates the spectra for all concentrations of TFAc-Tri-Ala (shown for each titration point), indicating a relatively low binding affinity of the depsipeptide for CE. **(b)** Portion of the ^1H NMR spectrum of 0.2 mM CE and 1.0 mM Dec-Tetra-Lac in the absence of vesicles. **(c)** Portion of the ^1H NMR spectrum of 0.2 mM CE and 1.0 mM Dec-Tetra-Lac in the presence of 100 nm 10 mM PC vesicles. The lack of extensive broadening in the spectrum relative to (b) is indicative of a nontemplated system.

chain into the membrane. This system gives the largest enhancement seen for a depsipeptide ligand as determined using the ^{19}F NMR method, the binding affinity being calculated as $2.5 \times 10^5\ M^{-1}$. The ^1H NMR data (Figure 6b,c) are again in agreement with the ^{19}F NMR data in that there is extensive broadening of the signals in the presence of vesicles (Figure 6c), indicative of a templated system. There is a notable difference between Figure 5b and Figure 6b, however. The former spectrum is characteristic of CE bound to cell-wall analogues in solution, whereas the latter is characteristic of CE in its free state. The ^1H NMR spectrum of CE in Figure 6b illustrates that as 1 mM Doc-Hexa-Lac is above its critical micelle concentration (0.10 mM) the Doc-Hexa-Lac is not available to bind CE (presumably due to steric crowding of many depsipeptide termini at the surface of the micelle). Table 1 summarises the results obtained.

Figure 6

NMR spectra illustrating templated binding to Doc-Hexa-Lac. **(a)** Plot showing the ^{19}F NMR spectra from a titration of TFAc-Tri-Ala into a solution of CE (0.2 mM), Doc-Hexa-Lac (1.0 mM), and 100 nm 10 mM PC vesicles in 9:1 0.1 M phosphate buffer:D_2O at 298 K. Now the appearance of the titration is reversed relative to Figure 5, the free peak dominating each titration point at all concentrations of TFAc-Tri-Ala. **(b)** Portion of the ^{1}H NMR spectrum of 0.2 mM CE and 1.0 mM Doc-Hexa-Lac in the absence of vesicles. **(c)** Portion of the ^{1}H NMR spectrum of 0.2 mM CE and 1.0 mM Doc-Hexa-Lac in the presence of 100 nm 10 mM PC vesicles. The total broadening relative to (b) indicates a templated system with this depsipeptide.



Significance

For the vancomycin group of antibiotics, there is likely to be an intrinsic upper limit for their binding affinities to cell-wall analogues [8,11]. In the case of strongly binding D-alanine (D-Ala) terminating ligands this limit is likely to be approached in solution, so there is less to gain through the addition of extra interactions such as templating. The binding of the ligand Doc-Tri-Ala to chloroeremomycin (CE) on a surface has been measured using surface plasmon resonance, giving a value of 2×10^7 M^{-1}

Table 1

Binding constants of various depsipeptides to CE in the presence of PC vesicles, from ^{19}F NMR competition experiments, and in the absence of vesicles, from the w_2 method.

Ligand	^{19}F NMR (M^{-1})	w_2 (M^{-1})
AcKDADLac		920 ± 50
AcDEKDADLac		$1.2 \times 10^4 \pm 2000$
decDEKDADLac	$1.0 \times 10^4 \pm 1900$	$9.0 \times 10^3 \pm 2000$
docDEKDADLac	$2.2 \times 10^4 \pm 2400$	
docGADEKDADLac	$2.5 \times 10^5 \pm 3 \times 10^4$	

The abbreviations used for the ligands are different from those used in the text. Single-letter amino-acid code is used here for the amino acids in the ligands.

[24] compared to 1.3×10^6 M^{-1} for Ac-Tri-Ala in solution [10] — a 15-fold enhancement. Increasing the number of residues after lysine has been shown to offer no significant enhancement in binding affinity to the glycopeptides for D-Ala-terminating ligands [2]. We can therefore take this value of 2×10^7 M^{-1} as being close to the upper limit measurable within our model system. Indeed, the binding of D-Ala-terminating ligands seems to limit near to 10^8 M^{-1} on the surface of bacteria [11]. In the case of the D-lactate (D-Lac) terminating ligands, however, there is a greater potential to gain binding affinity through cooperative enhancements such as templating. Thus, there is an enhancement of over 250-fold for Doc-Hexa-Lac (2.5×10^5 M^{-1}) over the weak binding of Ac-Tri-Lac (920 M^{-1}), demonstrating that binding can increase significantly for peptides longer than three residues. Care must be taken to distinguish between the enhancement from templating and from other effects. For example, the increase in affinity from Ac-Tri-Lac to Ac-Tetra-Lac is a result of the addition of the glutamate residue. It is unlikely that further residues will affect the binding in free solution to the same degree because they do not interact significantly with the antibiotic [23]. The experiments illustrate the general principle that cooperative enhancements are likely to be relatively greater when the initial complex is loose.

Recently, derivatives of CE have been developed bearing hydrophobic groups on the 4-*epi*-vancosamine on the residue-4 disaccharide [25], and these hydrophobic groups have been shown to act as membrane anchors [8,26]. These derivatised antibiotics show significant activity against vancomycin-resistant gram-positive bacteria [26,27] through strengthened binding on the bacterial surface [8,26], although any increase in activity against vancomycin-sensitive bacteria is less marked [27]. In combination with our previous work [8,9], the illustration here of the greater cooperative enhancement possible from initially looser complexes offers a self-consistent explanation for this phenomenon.

Materials and methods

Materials

Type XV1-E L-α-Phosphatidylcholine (99%, from fresh egg yolk) was purchased from Sigma. Solvents were purified according to standard procedures [28].

Preparation of phosphatidylcholine vesicles

Small unilamellar vesicles (SUVs) were prepared in 0.1 M phosphate buffer at pH 7 by extrusion [29]. Type XV1-E L-α-phosphatidylcholine from fresh egg yolk (Sigma, 64 mg, 80 mmol) was dissolved in ethanol-free chloroform (10 ml) in a 250 ml round bottom flask and deposited as a thin film after removal of the solvent under reduced pressure. The lipid was dried under high vacuum for 2 h, then phosphate buffer (8 ml) was added to give a 10 mM suspension. The lipid was shaken for 30 min, and was sonicated for 30 min to give a translucent solution. It was then passed between 17 and 21 times through a 100 nm pore size polycarbonate filter in an Avestin Lipofast Basic™ extrusion apparatus. This solution was then made up to a 9:1 mixture of SUV:D_2O.

Mass spectrometry

Electrospray mass spectra were recorded on a VG Bio-Q machine using a source temperature of 70°C, a capillary potential of 4 kV and a cone voltage of 40 V. The eluent was a 1:1 mixture of water and acetonitrile with a flow rate of 4 μl min^{-1}.

NMR spectroscopy

All ^1H NMR experiments were performed on a Bruker DRX-500 spectrometer at 298 K. One-dimensional spectra were recorded using 32k complex data points.

All ^{19}F NMR experiments were performed on a Bruker AM-400 spectrometer equipped with a $^{19}F/^1H$ probe at 298 K. One-dimensional spectra were typically recorded at 376.47 MHz using 8k complex data points over a spectral width of 6.9 ppm.

General procedure for NMR titrations

Titrations with vesicles. All titrations were performed at 298 K. The antibiotic concentration used in all of the titrations was 0.2 mM, and the depsipeptide concentration 1.0 mM, with the exception of the titration for Dec-Tetra-Lac in which they were 0.5 mM and 1.5 mM respectively. The concentration of TFAc-Tri-Ala used was dependent upon the estimated binding constant of the ligand: the higher the expected binding constant, the higher the concentration of TFAc-Tri-Ala. During the titration, a total of up to 50 μl of TFAc-Tri-Ala was added in 10 μl aliquots to 500 μl of the antibiotic/anchored ligand solution. All samples (antibiotic, ligand, TFAc-Tri-Ala) were prepared by dissolution in phosphate buffer (pH 7.4) with adjustment to pH 7.4 as required. These samples were lyophilised. The antibiotic and decanoyl/docosanoyl ligand were dissolved in a 9:1 solution of SUV:D_2O (without sonication), and TFAc-Tri-Ala was dissolved in 9:1 phosphate buffer:D_2O.

A sample of TfOH in water was used as an external reference (in a glass insert in the NMR tube) for both chemical shift and relative peak integrals. The TfOH concentration used (~ 1×10^{-4} M^{-1}) was such that its corresponding peak in the ^{19}F spectrum was of similar intensity to the other ^{19}F resonances to be observed. The relative integral of the TfOH peak was calibrated from a spectrum of TFAc-Tri-Ala of known concentration containing this TfOH insert. In order to adjust for the loss of any ^{19}F signal from TFAc-Tri-Ala due to non-specific binding of TFAc-Tri-Ala to the vesicle/ligand complex, an experiment was performed in which TFAc-Tri-Ala solution was titrated into a solution of ligand and vesicles (*i.e.*, in the absence of antibiotic). Any such loss of ^{19}F signal was below the limits of observation, however (< 4%). This control was performed for each different ligand examined. Experiments demonstrating the non-association of CE for vesicles has previously been published [8].

Measurement of association constants by UV difference spectrophotometry

All experiments were performed using a dual beam 940 spectrophotometer equipped with a thermocirculator maintaining a constant temperature of 25°C. Two samples, each 2.5 ml of a 25 μM solution of chloroeremomycin dissolved in pH 6 0.10 M phosphate buffer, were placed in cuvettes, one being the sample cell and the other the reference cell. A spectrum from 200 nm to 300 nm was then recorded. A ligand solution was prepared by dissolving ligand in the cuvette in the antibiotic solution, so as to keep the concentration of antibiotic in the cuvette constant, at a ligand concentration such that (using an estimate for the binding constant) 1 ml of this solution added to the antibiotic solution would give rise to 95% of the antibiotic bound. 1 ml of this solution was then added to the cuvette containing the antibiotic. The maximum changes in absorbance were observed to be at 264 and 231 nm, and so these wavelengths were monitored throughout the subsequent titration. The titration was then carried out with a fresh antibiotic sample, with ligand solution being added in 10 μl aliquots, and the difference in absorbance between the two wavelengths noted. The association constant was determined by curve-fitting a plot of change in absorbance against ligand concentration using *Kaleidagraph* (Abelbeck Software). Determinations were always carried out in triplicate, the average result being quoted, with the error being derived from the result with the largest deviation from this average.

Supplementary material

The details of the synthesis of all peptides used in this study and the determination of an expression for K_i are available on the internet.

Acknowledgements

Eli Lilly and Co. are thanked for generously providing CE. The BBSRC and Roussel (R.M.H.E.), the Wellcome Trust (R.J.D.), Unilever (D.P.O), Xenova (A.C.T.), and the George Murray Foundation (M.A.C.), are thanked for financial support. We thank Mike Gradwell and Tom Frenkiel of the NMR Laboratory, NIMR, Mill Hill, London, for helpful discussions and access to NMR equipment.

References

1. Perkins, H.R. (1969). Specificity of combination between mucopeptide precursors and vancomycin or ristocetin. *Biochem. J.* **111**, 195-205.
2. Nieto, M. & Perkins, H.R. (1971). Modifications of the acyl-D-alanyl-D-alanine terminus affecting complex-formation with vancomycin. *Biochem. J.* **123**, 780-803.
3. Foldes, M., Munro, R., Sorrell, T.C., Shankar, S. & Toohey, M. (1983). *In vitro* effects of vancomycin, rifampicin, fusidic acid, alone and in combination, against methicillin resistant *Staphylococcus aureus*. *J. Antimicrob. Chemother.* **11**, 21-26.
4. Neu, H.C. (1992). The crisis in antibiotic resistance. *Science* **257**, 1064-1073.
5. Mackay, J.P., Gerhard, U., Beauregard, D.A., Westwell, M.S., Searle, M.S. & Williams, D.H. (1994). Glycopeptide antibiotic acitivity and the possible role of dimerization: A model for biological signaling. *J. Am. Chem. Soc.* **116**, 4581-4590.
6. Waltho, J.P. & Williams, D.H. (1989). Aspects of molecular recognition: solvent exclusion and dimerization of the antibiotic ristocetin when bound to a model bacterial cell wall precursor. *J. Am. Chem. Soc.* **111**, 2475-2480.
7. Gerhard, U., Mackay, J.P., Maplestone, R.A. & Williams, D.H. (1993). The role of the sugar and chlorine substituents in the dimerization of vancomycin antibiotics. *J. Am. Chem. Soc.* **115**, 232-237.
8. Sharman, G.J., *et al.*, & Williams, D.H. (1997). The roles of dimerization and membrane anchoring in activity of glycopeptide antibiotics against vancomycin-resistant bacteria. *J. Am. Chem. Soc.* **119**, 12041-12047.
9. Try, A.C., Sharman, G.J., Dancer, R.J., Bardsley, B., Entress, R.M.H. & Williams, D.H. (1997). Use of model cell membranes to demonstrate templated binding of vancomycin group antibiotics. *J. Chem. Soc., Perkin Trans.* **1**, 2911-2917.
10. Mackay, J.P., Gerhard, U., Beauregard, D.A., Maplestone, R.A. & Williams, D.H. (1994). Dissection of the contributions toward dimerisation of glycopeptide antibiotics. *J. Am. Chem. Soc.* **116**, 4573-4580.
11. Beauregard, D.A., Maguire, A.J., Williams, D.H. & Reynolds, P.E. (1997). Semiquantitation of cooperativity in binding of vancomycin-group antibiotics to vancomycin-susceptible and resistant organisms. *Antimicrob. Agents Chemother.* **41**, 2418-2423.
12. Wright, G.D. & Walsh, C.T. (1992). D-Alanyl-D-alanine ligases and the molecular mechanism of vancomycin resistance. *Accounts Chem. Res.* **25**, 468-473.
13. Walsh, C.T., Fisher, S.L., Park, I.-S., Prahalad, M. & Wu, Z. (1996). Bacterial resistance to vancomycin: five genes and one missing hydrogen bond tell the story. *Chem. Biol.* **3**, 21-28.
14. Courvalin, P. (1990). Resistance of enterococci to glycopeptides. *Antimicrob. Agents Chemother.* **34**, 2291-2296.
15. Hospital Infection Control Practices Advisory Committee (1995). Recommendations for preventing the spread of vancomycin resistance. *Infect. Control Hosp. Epidemiol.* **16**, 105-113.
16. Johnson, A.P., Uttley, A.H.C., Woodford, N. & George, R.C. (1990). Resistance to vancomycin and teicoplanin: an emerging clinical problem. *Clin. Microbiol. Rev.* **3**, 280-291.
17. Westwell, M.S., Bardsley, B., Dancer, R.J., Try, A.C. & Williams, D.H. (1996). Cooperativity in ligand binding expressed at a model cell membrane by the vancomycin group antibiotics. *Chem. Commun.*, 589-590.
18. Cho, Y.R., Entress, R.M.H. & Williams, D.H. (1997). Synthesis of cell-wall analogues of vancomycin-resistant enterococci using solid phase peptide synthesis. *Tetrahedron Lett.* **38**, 5229-5232.
19. Dancer, R.J., Try, A.C. & Williams, D.H. (1998). Ligand-induced dissociation of the asymmetric homodimer of ristocetin A monitored by ^{19}F NMR. *Chem. Eur. J.* **4**, 740-744.
20. Searle, M.S., Williams, D.H. & Gerhard, U. (1992). Partitioning of free energy contributions in the estimation of binding constants: residual motions and consequences for amide-amide hydrogen bond strengths. *J. Am. Chem. Soc.* **114**, 10697-10704.
21. Bardsley, B. & Williams, D.H. (1997). Measurement of the different affinities of the two halves of glycopeptide dimers for acetate. *Chem. Commun.* 1049-1050.
22. Searle, M.S., *et al.*, & Williams, D.H. (1996). Enthalpic (electrostatic) contribution to the chelate effect: a correlation between ligand binding constant and a specific hydrogen bond strength in complexes of glycopeptide antibiotics with cell wall analogues. *J. Chem. Soc., Perkin Trans.* **1** 2781-2786.
23. Prowse, W.G., Kline, A.D., Skelton, M.A. & Loncharich, R.J. (1995). Conformation of A82846B, a glycopeptide antibiotic, complexed with its cell wall fragment: an asymmetric homodimer determined using NMR spectroscopy. *Biochemistry* **34**, 9632-9644.
24. Cooper, M.A., Williams, D.H. & Cho, Y.R. (1997). Surface plasmon resonance analysis of glycopeptide antibiotic activity at a model membrane surface. *Chem. Commun.* 1625-1626.
25. Cooper, R.D.G., *et al.*, & Thompson, R.C. (1996). Reductive alkylation of glycopeptide antibiotics: synthesis and antibacterial activity. *J. Antibiot.* **49**, 575-581.
26. Allen, N.E., LeTourneau, D.L. & Hobbs Jr, J.N. (1997). The role of hydrophobic sidechains as determinants of antibacterial activity of semisynthetic glycopeptide antibiotics. *J. Antibiot.* **50**, 677-684.
27. Nicas, T.I., *et al.*, & Cooper, R.D.G. (1996). Semisynthetic glycopeptide antibiotics derived from LY264826 active against vancomycin-resistant enterococci. *Antimicrob. Agents Chemother.* **40**, 2194-2199.
28. Perrin, D.D., Armarego, W.L.F. & Perrin, D.R. (1980). *Purification of Laboratory Chemicals.* (2nd), Pergamon Press, Oxford.
29. Macdonald, R.C., Macdonald, R.I., Menco, B.P., Takeshita, K., Subbarao, N.K. & Hu, L. (1991). Small-volume extrusion apparatus for preparation of large, unilamellar vesicles. *Biochim. Biophys. Acta* **1061**, 297-303.

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