The Roles of Dimerization and Membrane Anchoring in Activity of Glycopeptide Antibiotics against Vancomycin-Resistant Bacteria

Gary J. Sharman, Andrew C. Try, Robert J. Dancer, Younghoon R. Cho, Thomas Staroske, Ben Bardsley, Alison J. Maguire, Matthew A. Cooper, Dominic P. O'Brien, and Dudley H. Williams*

Contribution from the Cambridge Centre for Molecular Recognition, University Chemical Laboratory, Lensfield Road, Cambridge, CB2 1EW, United Kingdom

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Abstract: The mode of action of a semisynthetic glycopeptide active against vancomycin-resistant bacteria has been investigated. It is shown that the antibiotic, biphenylchloroeremomycin or LY307599, dimerizes strongly and anchors to membranes. It is hypothesized that these two locating devices, previously identified by us when acting separately, might combine to give enhanced binding at a cell surface. This hypothesis is tested experimentally by showing that glycopeptides can bind cell-wall precursor analogues from resistant bacteria (terminating in −D-lactate) in a similar manner to those from susceptible bacteria (terminating in −D-alanine) and by using model cell surfaces where the benefits of dimerization can be expressed and studied. These model systems use vesicles to represent the cell membrane, to which cell wall analogues are anchored *via* a docosanoyl chain, so mimicking the arrangement encountered at the cell surface. Using ^{1}H NMR spectroscopy, we demonstrate enhanced binding due to dimerization and propose that this enhancement will act cooperatively with membrane anchoring in biphenylchloroeremomycin.

Introduction

Vancomycin has proved a valuable weapon in the war against pathogenic bacteria which are increasingly resistant to other antibiotics.[1,2] It functions by binding to cell-wall peptidoglycan precursors terminating in −Lys-D-Ala-D-Ala, preventing cross-linking and thus causing cell death.[3−6] However, in recent years resistance to vancomycin has appeared, leading to increasing concern about treatment of bacterial infection in the future.[7−10] This resistance is the result of a deceptively simple change of an amide bond to an ester bond in the growing bacterial cell wall.[11−14] The change, conferred by substitution of the terminal D-alanine of the cell-wall precursors with D-lactate (D-Lac), results in a repulsive interaction within the binding pocket of the antibiotic and consequently a large decrease in affinity, rendering the antibiotic therapeutically useless.

Recently, however, several new semisynthetic glycopeptides have been discovered which show useful activity against resistant strains of bacteria, with increases in activity over vancomycin of *ca.* 500 times.[15,16] One such antibiotic, LY307599 or biphenylchloroeremomycin (BCE), is shown in Figure 1. This antibiotic, produced by scientists at Eli Lilly in Indianapolis, is synthesized by the condensation of a biphenyl-4-carboxaldehyde with the amino sugar on ring 4 of the antibiotic chloroeremomycin (CE), followed by reduction of the resultant imine with sodium cyanoborohydride.[15] This modification is remote from the known site of interaction with cell wall peptides, and it is therefore not obvious why it would have such an effect on the ability of the antibiotic to kill bacteria. However, we have shown in recent work that the affinity for the −Lys-D-Ala-D-Ala sequence is not the only factor determining antibiotic efficacy.[17,18] Membrane anchoring in teicoplanin and dimerization in eremomycin dramatically increase the effectiveness of these antibiotics. This enhancement of activity arises through

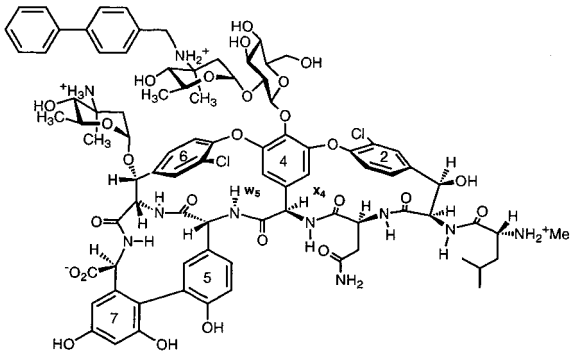

Figure 1. Structure of LY307599 or BCE. Protons referred to in the text are labeled.

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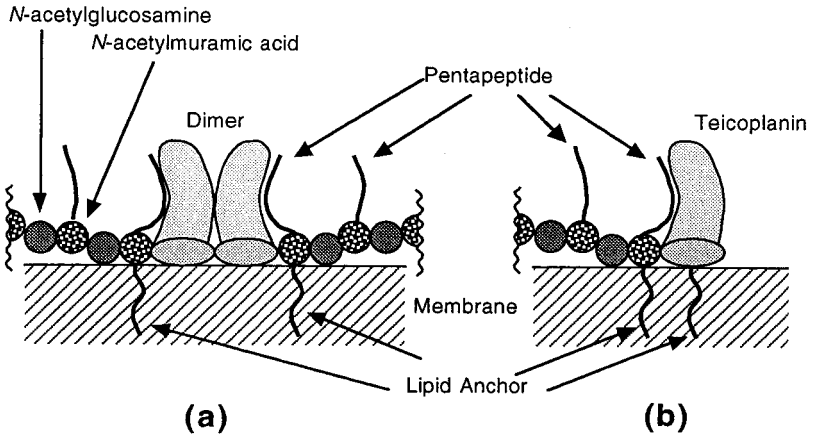

Figure 2. Schematic diagram showing how the devices of (a) dimerization or (b) membrane anchoring locate vancomycin group antibiotics at the cell surface, so increasing their activity.

the ability of these features to locate the antibiotic at its site of action, the cell surface. For an antibiotic dimer, once one half of the dimer has bound to a cell-wall precursor, the second binding event is effectively intramolecular (Figure 2(a)). In a similar fashion, membrane anchoring can also increase the effective concentration of the antibiotic at the cell surface (Figure 2(b)). In the case of the new antibiotic, BCE, it seemed possible that the biphenyl moiety could act as a membrane anchor, and being derived from a strongly dimerizing antibiotic, BCE would also be expected to dimerize. We therefore hypothesized, as had others[19] in the light of our previous work, that the activity of this antibiotic was due to membrane anchoring *and* dimerization acting cooperatively to greatly enhance the low intrinsic affinity for −D-Lac terminating ligands found at the surface of vancomycin-resistant bacteria, and we have recently demonstrated this using *in vitro* bacterial assays.[20] We now present evidence that BCE does indeed use these locating devices: firstly, that the biphenyl moiety does function as a membrane anchor; secondly, that vancomycin group antibiotics bind cell wall analogues terminating in −D-Lac with essentially the same geometry as those terminating in −D-Ala; and thirdly, that in the presence of model cell membranes and anchored ligands, cooperativity gives dramatically enhanced binding for peptides terminating in −D-Lac when the antibiotic dimerizes strongly.

Results and Discussion

BCE Dimerizes Strongly and Has a Membrane Anchor. We wanted to demonstrate that BCE does indeed dimerize strongly and that the biphenyl moiety can act as a membrane anchor. The question of dimerization was addressed first. CE, the parent compound of BCE, dimerizes strongly, but it was not clear what effect (if any) the biphenyl moiety would have on dimerization. The head-to-head arrangement of the sugars in glycopeptide antibiotic dimers[21,22] means that the biphenyl groups are adjacent to each other, allowing the possibility of hydrophobic and/or π-stacking interactions which might raise the dimerization constant or possibly bad steric interactions which would lower it. Dimerization constants were thus measured for the new antibiotic. Two methods were used: the first employed ^{1}H NMR spectroscopy to follow the exchange of the amide proton of residue 5 of the antibiotic (w_5, Figure 1) with deuterium of D_2O.[23] This proton is shielded from solvent in the dimer and can only exchange *via* monomer. The observed exchange rate is thus dramatically reduced for antibiotics that dimerize strongly and can be used to calculate dimerization constants. The results indicate that BCE does indeed dimerize strongly and that the dimerization constant of BCE is approximately the same as for the parent compound CE, both for free antibiotic and for the complex with the cell-wall analogue di-*N*-acetyl-Lys-D-Ala-D-Ala (di-*N*-ac-KDADA) (Table 1). The second method also employed ^{1}H NMR, in this case to measure directly proportions of monomer and dimer, using the α-proton of residue four of the antibiotic (x_4, Figure 1) as a probe. Like w_5, x_4 is located at the dimer interface and is useful because its shift changes dramatically (from *ca.* 5.5 ppm to *ca.* 6.4 ppm) on dimerization.[24] Given that the shift of x_4 is different in monomer and dimer, peak integrals are a measure of the different populations of monomer and dimer, and by measuring these differences at various concentrations a dimerization constant can be calculated. The results are also shown in Table 1. The result for BCE is in close agreement with that measured by the exchange method, although the value for CE is lower by this method. The origin of this difference is not clearly understood, but, in the present context, the important conclusion is that both methods indicate that BCE dimerizes strongly. This conclusion is further supported by results from researchers at Eli Lilly & Co., using capillary electrophoresis.[19,25] The value so obtained for BCE (3×10^5 M^{-1}) is very similar to that given in Table 1, though again, the value for CE so obtained is rather lower (7.6×10^3 M^{-1}).

Table 1. Dimerization Constants of BCE and CE in the Presence and Absence of the Tripeptide Cell-Wall Analogue Di-*N*-ac-Lys-D-Ala-D-Ala[a]

	dimerization constant (M^{-1})	
antibiotic	free antibiotic	tripeptide complex
BCE (w_5)	$1.6 \pm 0.2 \times 10^5$	$3 \pm 1 \times 10^7$
(x_4)	$2 \pm 1 \times 10^5$	
CE (w_5)	$1.8 \pm 0.6 \times 10^5$	$1.1 \pm 0.4 \times 10^7$
(x_4)	$1.6 \pm 0.2 \times 10^4$	

[a] w_5 or x_4 indicates method as described in the text.

The question of whether the biphenyl groups act as a membrane anchor was then addressed. This was achieved by

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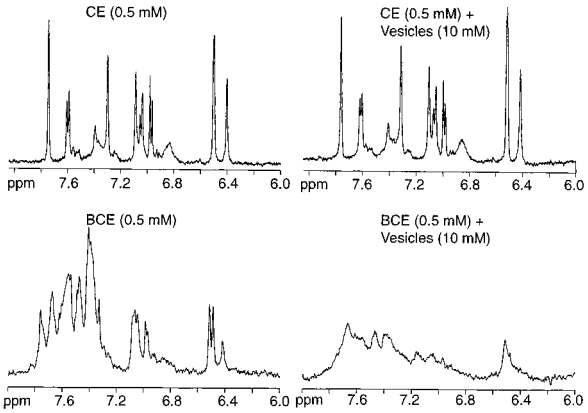

Figure 3. Aromatic region of the ^{1}H NMR spectra of CE and BCE in the presence and absence of phosphatidylcholine vesicles, 500 MHz, 300 K, D_2O, pD 6.2. The line width of protons for CE is unaffected by the presence of the vesicles. However, BCE broadens significantly, indicating it is associating with the vesicles.

observing the line width of the aromatic protons of the antibiotic in the presence and absence of phosphatidylcholine vesicles. Vesicles are a good mimic of cell membranes, which are composed of similar lipids. They are very large objects on the molecular scale, being typically 0.1−1 μm in diameter. Their slow tumbling and long correlation times mean that any solute which associates with the surface of the vesicle effectively takes on the same tumbling properties as the vesicle and would thus have very broad NMR resonances.[26] For the parent compound, CE, the line width in the presence and absence of vesicles is almost the same (Figure 3), but for BCE, there is a dramatic increase in line width on the addition of vesicles (Figure 3). This demonstrates that the biphenyl moiety can act as a membrane anchor. This conclusion was further supported by looking at T_1 relaxation times in the above systems. Shortened T_1 values have been used as evidence of association of a hydrophobic tripeptide with vesicles.[27] For CE, the T_1 of peaks in the aromatic region was essentially the same (1.55 and 1.49 s, with and without vesicles, respectively). However, for BCE, the T_1 value was dramatically shortened from 1.42 to 0.64 s in the presence of vesicles, again suggesting the biphenyl moiety can act as a membrane anchor.

We have also assayed the affinity of BCE for a model membrane surface using surface plasmon resonance. Small unilamellar vesicles constituted from phosphatidylcholine were layered on the surface of a hydrophobic association (HPA) chip from Pharmacia BIAsensor to form a supported lipid monolayer.[28] Solutions of antibiotics at 10 μM were then injected over this surface, and the surface plasmon resonance angle (which can be converted to response units, a measure of the amount of material associated with the lipid surface) was measured (Figure 4). Clearly, BCE associates with the model membrane to a much larger extent than CE. By fitting these response curves using a nonlinear least squares algorithm, an association constant of 4×10^6 M^{-1} was measured for BCE with the monolayer, whereas the value for CE was less than the detection limit for the instrument (*i.e.*, less than 10^3 M^{-1}). Since the only difference in structure between the two antibiotics is the biphenyl moiety, this further supports the hypothesis that it is acting as a membrane anchor.

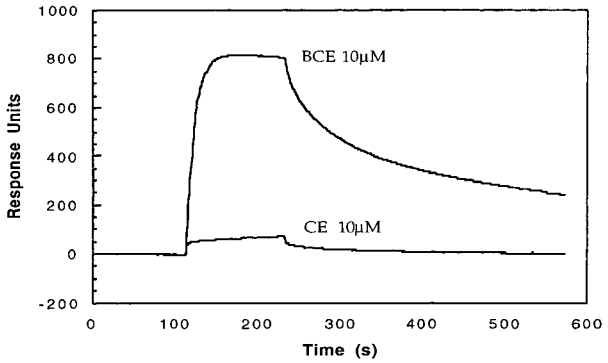

Figure 4. Surface plasmon resonance response curves for the binding of biphenylchloroeremomycin (BCE) and chloroeremomycin (CE) to a lipid monolayer.

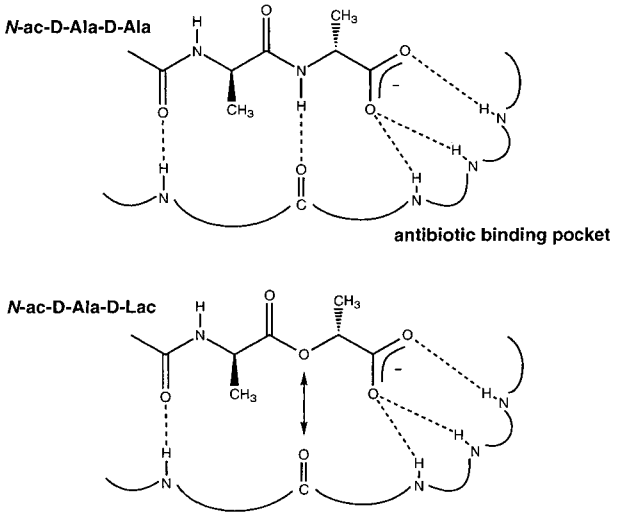

Figure 5. Schematic diagram of the antibiotic binding pocket with −D-Ala and −D-Lactate terminating ligands. The hydrogen bond between the amide proton of D-Ala and a carbonyl of the antibiotic backbone is replaced by a repulsive O−O interaction.

CE Binds −D-Lactate Terminating Peptides in Essentially the Same Conformation as Those Terminating in D-Alanine. The repulsive interaction between the lactate oxygen and the amide carbonyl of residue 4 of the antibiotic (Figure 5) results in a large decrease in affinity between the ligand and antibiotic (see below). However, we believed that the activity of this new antibiotic against resistant bacteria could be explained by its ability to both dimerize and membrane anchor, assuming that it could bind D-lactate-terminating ligands in essentially the same conformation as D-Ala-terminating ligands and that no additional hypotheses regarding the mode of lactate binding were required. We attempted to demonstrate this using the complex between CE and di-*N*-acetyl-Lys-D-Ala-D-Lac (di-*N*-ac-KDADLac), a cell-wall analogue of resistant bacteria. UV spectrophotometry was used to determine the binding constant for the association which was found to be 240 ± 10 M^{-1},[29] similar to that reported earlier by workers at Lilly[19] and dramatically lower than that of di-*N*-ac-KDADA which is 1.3×10^6 M^{-1}.[23] ^{1}H NMR spectroscopy was then used to investigate the conformational details of the complex. Because of the low binding constant, a high ligand concentration was used to ensure a high population of bound antibiotic. The first indication of ligand binding was provided by the chemical shift of the amide proton of residue 2 (w_2). In complexes with ligands terminating in −D-Ala, w_2 forms a hydrogen bond to the ligand carboxylate, which leads

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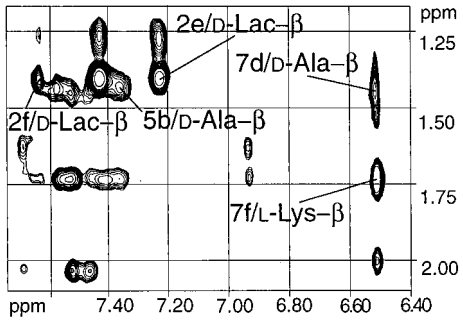

Figure 6. Part of the NOESY spectrum of the complex between CE and di-*N*-ac-Lys-D-Ala-D-Lac, 500 MHz, 300 K, H_2O:D_2O 9:1, pH 4.5. Key cross-peaks which define the structure of the complex are highlighted.

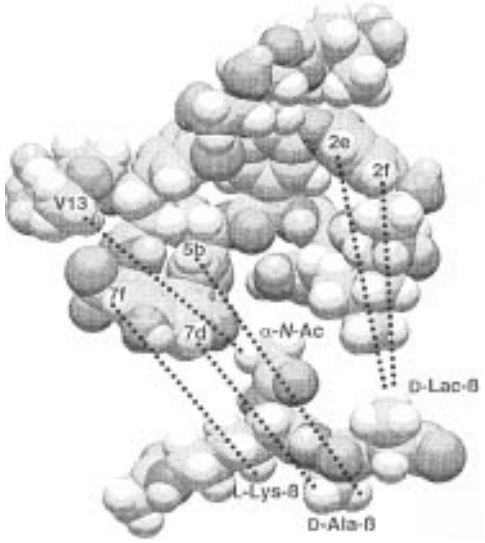

Figure 7. Exploded view of the complex between CE and di-*N*-ac-Lys-D-Ala-D-Lac. Dotted lines indicate observed NOE enhancements.

to a large downfield shift for the proton.[30,31] In this case, the ligand terminating in −D-Lac induced a similar downfield shift in w_2, from 8.48 ppm in the free antibiotic to 11.20 ppm in the complex, indicating that the carboxylate is bound in the "binding pocket". Two-dimensional NOESY experiments provided evidence of a number of key close contacts which defined the structure further. Some of the observed cross-peaks are shown in Figure 6, and the contacts they indicate are shown diagrammatically in Figure 7. They locate the methyl group of the −D-Lac residue over the face of ring 4, the methyl group of the −D-Ala residue over ring 7 and adjacent to ring 5, the side chain of the −Lys residue over ring 7, and the methyl group of the *N*-α-acetyl group adjacent to the equatorial methyl group of the residue 6 *epi*-vancosamine (V_{13}). In combination, these demonstrate that the gross conformation of the ligand−antibiotic complex is essentially the same as that previously reported for complexes of glycopeptide antibiotics with cell-wall analogues terminating in −D-Ala.[21,22,32−34]

Use of a Model Cell-Wall System To Demonstrate Greatly Enhanced Binding of Glycopeptide Antibiotics to −D-Lac Terminating Peptides on a Surface Compared to in Free Solution. We had thus shown that BCE dimerizes strongly and anchors to membranes and that a member of the vancomycin group of antibiotics could bind ligands terminating in −D-Lac in the same manner as those terminating in −D-Ala. It remained to be demonstrated that BCE could combine these two features to give a sufficiently high binding constant at the surface of the bacterium to account for its activity against −D-Lac-producing bacteria. We have recently demonstrated through competition experiments in bacterial assays that an effective binding constant of greater than *ca.* 10^7 M^{-1} at the surface of a bacterium is required for useful antibiotic action.[20] In the case of vancomycin-susceptible bacteria, enhancements due to membrane-anchoring[20,35] or dimerization[20,35,36] at a surface are sufficient to raise their effective binding constants from *ca.* 10^6 M^{-1} in a bimolecular association in solution to a value above this threshold. However, given the lower intrinsic binding constants for CE binding to −D-Lac-terminating peptides compared to −D-Ala-terminating peptides (240 M^{-1} for di-*N*-ac-KDADLac compared to 1×10^6 M^{-1} for di-*N*-ac-KDADA), evidence for a substantial increase in binding in a templated system was required to confirm that BCE could indeed obtain a sufficiently large binding constant at a surface to be therapeutically useful.

In previous work, we have shown that it is possible to make useful model membrane surfaces analogous to those found in vancomycin-susceptible bacteria using a combination of phosphatidylcholine (PC) vesicles as the membrane surface and cell-wall analogue peptides anchored to the surface of the vesicle *via* a hydrophobic tail.[36] Furthermore, the binding constants of chloroeremomycin with full-length cell-wall analogue peptides in such systems correlate well with those found on the surface of a bacterium (*ca.* 10^8 and 7×10^7 M^{-1}, respectively).[20,36] Therefore, we decided to measure the binding constant of CE in an analogous system using anchored peptides terminating in −D-Lac in order to determine what enhancements due to templating could be achieved in vancomycin-resistant bacteria.

The anchored depsipeptide used in this study was *N*-α-docosanoyl-Gly-L-Ala-D-γ-Glu-*N*-ϵ-acetyl-Lys-D-Ala-D-Lac (doc-hex-Lac). The docosanoyl (C_{22}) group was used here in preference over the decanoyl (C_{10}) group used in previous studies because of its greater propensity to anchor in a membrane (data not shown). This depsipeptide was synthesized using solid-phase peptide synthesis.[37] Direct NMR observation of complexes bound to vesicles is difficult because of the broadening caused by slow vesicle tumbling, as described above. Therefore, competition experiments were used, in which the antibiotic was displaced from an anchored complex on the vesicle into free solution by the addition of a nonanchored cell wall analogue (Figure 8). The results of such an experiment are shown in Figure 9. The aromatic region of the ^{1}H NMR spectrum of the complex of CE and di-*N*-ac-KDADA is shown in Figure 9(a). The binding constant of CE to di-*N*-ac-KDADA, as determined by UV difference spectrophotometry, is 1.0×10^6 M^{-1},[18] giving >95% bound antibiotic at the stated concentrations. The spectrum is sharp, as the antibiotic is not associated with the vesicle. Figure 9(b) shows the spectrum of the complex with doc-hex-Lac, which is almost completely broadened due to association with the vesicle. Figure 9(c) shows

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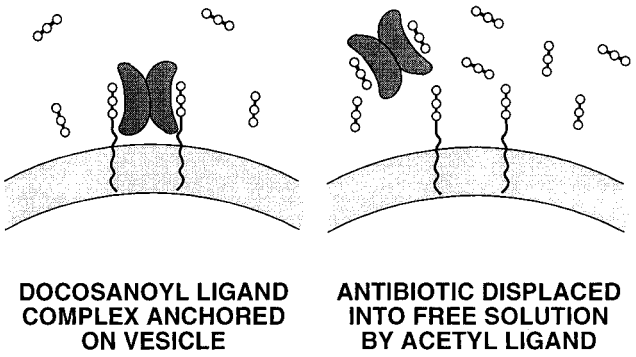

Figure 8. Schematic diagram illustrating the basis of the competition experiments used in this study. Binding enhancements at the vesicle surface result in anchored complex being favored even though unanchored ligand is in excess. Only a large excess of unanchored ligand can displace the anchored complex.

the spectrum when di-*N*-ac-KDADA and doc-hex-Lac are present in approximately equal concentrations. Clearly, the antibiotic is still predominantly associated with the vesicle, and therefore the binding constant to doc-hex-Lac at the surface of the vesicle must be higher than that to di-*N*-ac-KDADA in free solution (*i.e.*, greater than 1.0×10^6 M^{-1}). As the concentration of di-*N*-ac-KDADA is increased, the antibiotic is gradually competed off the surface of the vesicle (Figure 9(d),(e)).

For a more quantitative determination of the binding constant to doc-hex-Lac, an external reference of 3-trimethylsilyl-2,2,3,3-d_4-propionic acid, sodium salt (TSP) was used as an integration standard. Integrals of the aromatic doublet at approximately δ 7.1 ppm in Figure 9(a),(b) were taken as representing antibiotic fully off and fully on the vesicle, respectively. Therefore, by using the corresponding integrals from Figure 9(c)−(e), the concentration of antibiotic bound to di-*N*-ac-KDADA in free solution (A_C in the following equations) could be calculated.

Considering the two equilibria of interest, we have

$$K_L = \frac{A_L}{A \cdot L} \tag{1}$$

and

$$K_C = \frac{A_C}{A \cdot C} \tag{2}$$

where A is the concentration of free antibiotic, L is the concentration of free ligand anchored to the vesicle (doc-hex-Lac), C is the concentration of free competing ligand in solution (di-*N*-ac-KDADA), A_L is the concentration of antibiotic bound to doc-hex-Lac on the vesicle, A_C is the concentration of antibiotic bound to di-*N*-ac-KDADA in solution, and finally K_L and K_C are the equilibrium constants for antibiotic binding to doc-hex-Lac on a surface and di-*N*-ac-KDADA in solution, respectively. Additionally, we have

$$A_T = A_L + A_C + A \tag{3}$$

$$L_T = A_L + L \tag{4}$$

$$C_T = A_C + C \tag{5}$$

where A_T, L_T, and C_T are the total concentrations of antibiotic, doc-hex-Lac and di-*N*-ac-KDADA, respectively. Rearranging eq 2 in terms of A and substituting for C from eq 5 we have

$$A = \frac{A_C}{K_C(C_T - A_C)} \tag{6}$$

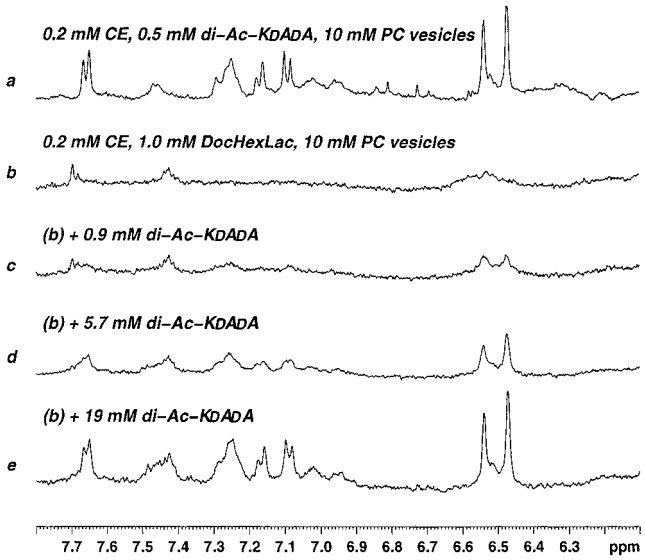

Figure 9. Portion of the ^{1}H NMR spectra of CE with di-*N*-ac-Lys-D-Ala-D-Ala and/or doc-hex-Lac, 500 MHz, 300 K, D_2O, pD 7.0. See main text for explanation.

Also, taking eq 1 and substituting for L from eq 4 we have

$$K_L = \frac{A_L}{A(L_T - A_L)} \tag{7}$$

Substituting for A_L from eq 3 we have

$$K_L = \frac{A_T - (A_C + A)}{A[L_T - A_T + (A_C + A)]} \tag{8}$$

Thus, we have an expression for K_L in terms of known values, where A is found from eq 6.[38]

Using this equation, it was determined that the binding constant for CE to a model membrane surface incorporating doc-hex-Lac is $(6.6 \pm 2.1) \times 10^6$ M^{-1}. This binding constant, somewhat less that the putative "therapeutic threshold" of $>10^7$ M^{-1} on the surface of the bacterium, is consistent with biological data, which shows that although CE is active against vancomycin-resistant bacteria, it is not sufficiently active to be therapeutically useful.

It is instructive to compare the binding constants of CE to di-*N*-ac-KDADA (1.0×10^6 M^{-1}) and to dec-hexa-Ala/vesicles (*ca.* 10^8 M^{-1})[36] on one hand, with those to di-*N*-ac-KDADLac (250 M^{-1}) and to doc-hex-Lac/vesicles (6.6×10^6 M^{-1}) on the other. In the case of the Ala-terminating peptides, an enhancement of *ca.* 100-fold is found from templating at a surface, whereas in the case of the Lac-terminating peptides, an enhancement of >10 000-fold is observed. An explanation for the dramatic increase in cooperative enhancement is clearly required. We have previously demonstrated that the binding of peptide ligands to the glycopeptide antibiotics is a cooperative process,[38] and this current result demonstrates an important principle concerning cooperativity and molecular recognition, *i.e.*, enthalpic cooperativity is an intrinsic property of systems of weak interactions, and therefore enthalpic enhancements due

(38) As for other cited antibiotic/ligand surface binding constants, K_L provides an important quantitative reference relative to the solution binding constant K_C (which has dimensions of M^{-1}). However, we note that the true dimensions of K_L are not M^{-1} and that its value is actually dependent upon the peptide ligand-to-lipid ratio.

to cooperative interactions are likely to be greater when the initial complex is weaker.[39]

Unfortunately, due to problems of vesicle aggregation we have been unable to repeat this experiment using BCE. However, as mentioned previously, we have shown that the presence of a single membrane anchor in these glycopeptide antibiotics leads to an enhancement of *ca.* 10-fold at the surface of a bacterium.[20] It would seem that a reasonable estimate for the total enhancement could therefore be obtained by taking the product of the binding enhancements observed for teicoplanin[20] and CE at a surface. This would suggest an enhancement of *ca.* 2×10^5 over the binding of di-*N*-ac-KDADLac, giving an estimated association constant of 5×10^7 M^{-1} at a bacterial surface, well over the therapeutic threshold. Furthermore, cooperativity between dimerization and membrane anchoring may well raise the association constant further, and 5×10^7 M^{-1} could reasonably be regarded as a lower limit to the actual value. It therefore seems that BCE can bind very effectively to −D-Lac-terminating cell-wall peptides by the simultaneous, cooperative operation of its two locating devices, allowing it to kill resistant bacteria, despite its low intrinsic affinity for −D-Lac-terminating ligands in free solution.

Conclusions

We have demonstrated that BCE dimerizes strongly and possesses a membrane anchor and that the mode of binding of −D-Lac-terminating peptides is essentially the same as that of −D-Ala-terminating peptides, albeit much reduced in strength in free solution. The association constant of a −D-Lac-terminating peptide to the related antibiotic, CE, on a model bacterial membrane surface, is much higher than to an analogous peptide in free solution, due to a cooperative enhancement resulting from antibiotic dimerization. Simultaneous operation of this enhancement due to dimerization with that previously reported for an antibiotic possessing a membrane anchor is likely to give an association constant at a bacterial surface sufficient to explain the activity of BCE against vancomycin-resistant bacteria, as we have reported previously for analogous antibiotic derivatives.[40] Understanding how this new antibiotic functions could assist in the design of further semisynthetic glycopeptides with activity against resistant bacteria. Furthermore, this work has general implications for the design of drugs: the drug considered in this paper has been modified at a site remote from the binding interface but is dramatically more active. In studying molecular recognition, it is tempting to concentrate on the interface between drug and receptor, but the example of BCE demonstrates that alterations remote from this interface can still confer significant increases in binding affinity due to cooperativity.

Experimental Section

Preparation of Phosphatidylcholine Vesicles. Type XV1-E L-α-Phosphatidylcholine from fresh egg yolk (Sigma) (80 mg) was dissolved in chloroform (20 mL) which had been rendered ethanol-free by passage through a column of activated alumina. The solution was then evaporated under reduced pressure to yield a thin film on the wall of the flask. The flask was flushed with argon, and D_2O or pH 6.2 50 mM NaH_2PO_4 buffer (5 mL) was added. The mixture was shaken for 20 min and then sonicated for 90 min to yield a slightly turbid suspension of vesicles, phosphatidylcholine concentration 20 mM.

For NMR competition experiments, this suspension was then passed 17 times through a 100 nm polycarbonate filter before dilution with D_2O to yield a clear suspension of vesicles, phosphatidylcholine concentration 10 mM. The samples were adjusted to pH 7.0 using $NaOD/D_2O$ and DCl/D_2O solutions. All quoted pD values were measured using a pH meter, and no attempt was made to correct for isotope effects.

Purification of BCE by Reverse Phase HPLC. Biphenylchloroeremomycin was purified by reverse phase HPLC on a 150 mm × 19 mm ODS-2 column (Waters), using H_2O/0.1% TFA as eluent with a gradient of acetonitrile/0.1% TFA running from 0 to 75% over 35 min. The flow rate was 9 mL min^{-1}. Approximately 10 mg, dissolved in 0.5 mL of H_2O, was purified in each run. The BCE fraction eluted after approximately 20 min. These fractions were pooled and evaporated under reduced pressure before being redissolved in water and lyophilized to yield an off-white, fluffy powder.

NMR Spectroscopy. All NMR experiments were performed on a Bruker DRX-500 spectrometer at 300 K. Suppression of solvent was achieved using WATERGATE in the case of samples dissolved in H_2O or by presaturation for samples dissolved in D_2O. One-dimensional spectra were recorded using 32k complex data points. In two-dimensional experiments, 4k complex points were acquired in f_2, with 512 increments in f_1. TPPI was used to achieve quadrature detection in the indirect dimension. Data was processed with XWIN-NMR software, using a sine-squared window function and zero filling in f_1 up to 1k points. NOESY experiments typically employed a mixing time of 120 ms and were used in all cases to check w_2 assignments. T_1 experiments were acquired using a standard π-delay-$\pi/2$-acquire pulse sequence. All spectra were referenced to 3-trimethylsilyl-2,2,3,3-d_4-propionic acid, sodium salt (TSP, $\delta = 0.00$ ppm). Because of solubility problems with BCE in phosphate buffers, all samples were prepared using ultrapure water or D_2O, the required pH being achieved by adjusting with HCl(DCl) or NaOH(NaOD). In experiments involving vesicles, the ligand was added to the vesicle solution, and the mixture was mixed using agitation only; no sonication was used. In the competition experiment, the unanchored ligand solution was prepared from a solution identical to that in the NMR tube, so as not to change the pH or concentration of the other components in the tube (vesicles, antibiotic, anchored ligand) and to allow accurate concentrations to be achieved on the addition of the appropriate volume.

Measurement of Dimerization Constants Using NH Exchange. The required quantity of antibiotic (and ligand where appropriate) was weighed out in an eppendorf such that the desired concentration would be achieved on the addition of 1 mL of D_2O. The sample was then adjusted to pD 3.7 using DCl and NaOD solutions. ^1H NMR spectra were then recorded at appropriate intervals until the resonance due to the proton w_5 had almost completely exchanged out, this typically taking a period of 6−12 h. The pseudo-first-order rate constant for the exchange process is given by the gradient of a plot of the natural logarithm of the integral of w_5 versus time, taken as the start time for the acquisition of each FID. The dimerization constant, K_{dim}, was then calculated using the equation

$$K_{dim} = \frac{(k_{obs}k_3 - k_3^2)}{2[A_t]k_{obs}^2}$$

where k_{obs} is the observed pseudo-first-order rate constant and k_3 is the intrinsic exchange rate of w_5 (8.9×10^{-3} s^{-1}). The derivation of this equation and justification for its use have been previously described.[23] Each K_{dim} determination was carried out in triplicate, each of the three experiments at different concentrations. The quoted values are the average of the three experiments, the error being calculated from the result most deviant from this average.

Measurement of Dimerization Constants Using x_4 Probe. Antibiotic samples were typically prepared for NMR by lyophilizing twice from D_2O. The solutions (10 μM to 5 mM) were adjusted to *ca.* pD 3.7 with DCl and NaOD. The signals due to x_4 in dimeric antibiotic were assigned by two-dimensional NOESY spectra, and, where ambiguous, the x_4 signal due to monomer was assigned by preirradiation of the dimer signal. Dimerization constants were determined by integration of the x_4 signals due to monomer and dimer. The concentration of dimer was plotted against total concentration of

(39) Searle, M. S.; Sharman, G. J.; Groves, P.; Benhamu, B.; Beauregard, D. A.; Westwell, M. S.; Dancer, R. J.; Maguire, A. J.; Try, A. C.; Williams, D. H. *J. Chem. Soc., Perkin Trans. 1* **1996**, 2781−2786.

(40) Sharman, G. J.; Williams, D. H. *Chem. Commun.* **1997**, 723−724.

antibiotic, and the resulting curve analyzed using the proprietary nonlinear curve-fitting routine of Abelbeck software's *Kaleidagraph* version 2.1.3.

Surface Plasmon Resonance. SPR analysis was carried out on a BIAcore 2000 instrument supplied by Pharmacia BIAsensor UK. Filtered, degassed 0.1 M phosphate buffer at pH 7.4 was used both as eluent and for serial dilutions of antibiotics and ligands. Small unilamellar vesicles were prepared from egg yolk L-α-phosphatidylcholine by extrusion through a polycarbonate membrane (pore size 50 nm). Vesicles at a concentration of 1 mM suspended in phosphate buffer were loaded onto an HPA chip following a 5 min injection of 40 mM octyl β-D-glucopyranoside. One 30 μL injection of lipid at a flow rate of 2 μL min^{-1} was generally sufficient to coat the surface of a chip to a level of 1000−1500 response units. An HPA chip fully coated with lipid showed no nonspecific binding to bovine serum albumin (BSA) at 0.1 mg mL^{-1}. The surface of the chip was then washed with 2 × 10 μL injections of 10 mM sodium hydroxide at a flow rate of 100 μL min^{-1} to remove any multiple lipid layers and partially fused liposomes. Antibiotics (10 μM) were then injected as 50 μL aliquots at a flow rate of 10 μL min^{-1} to give optimum binding curves. After the system had attained equilibrium the antibiotic solution was replaced by buffer to enable the complex to dissociate, and then the surface was regenerated by injection of 10 mM hydrochloric acid.

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Supporting Information Available: The details of the synthesis of the peptides used in this study (5 pages). See any current masthead page for ordering and Internet access instructions.

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