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Semiquantitation of Cooperativity in Binding of Vancomycin-Group Antibiotics to Vancomycin-Susceptible and -Resistant Organisms

DANIEL A. BEAUREGARD,[1]† ALISON J. MAGUIRE,[1]‡ DUDLEY H. WILLIAMS,[1]* AND PETER E. REYNOLDS[2]

Cambridge Centre for Molecular Recognition, University Chemical Laboratory, Cambridge CB2 1EW,[1] and Department of Biochemistry, Cambridge CB2 1QW,[2] England

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The association of vancomycin group antibiotics with the growing bacterial cell wall was investigated by using the cell wall precursor analog di-*N*-acetyl-Lys-D-Ala-D-Ala in competition binding experiments. The affinities of the antibiotics for the -D-Ala-D-Ala-containing cell wall precursors of *Bacillus subtilis* ATCC 6633 (a model for vancomycin-susceptible gram-positive bacteria) and for the -D-Ala-D-Lac-containing cell wall precursors of *Leuconostoc mesenteroides* (a model for vancomycin-resistant strains of *Enterococcus faecium* and *Enterococcus faecalis*) were determined by a whole-cell assay. The binding of strongly dimerizing antibiotics such as eremomycin to the bacterial surface was thus shown to be enhanced by up to 2 orders of magnitude (relative to the binding in free solution) by the chelate effect, whereas weakly dimerizing antibiotics like vancomycin and antibiotics carrying lipid tails (teicoplanin) benefited less (ca. 1 order of magnitude). The affinity measured in this way correlates well with the MIC of the antibiotic, and a consequence of this is that future design of semisynthetic vancomycin-group antibiotics should attempt to incorporate chelate effect-enhancing structural features.

Vancomycin and teicoplanin are used to treat methicillin-resistant *Staphylococcus aureus* infections, with vancomycin being recognized in the clinic as the drug of choice. However, vancomycin resistance is now common in *Enterococcus faecalis* and *Enterococcus faecium*, and it appears possible that it will be transferred to *S. aureus* (22). Synthetically modified vancomycin-group antibiotics which are impressively active against vancomycin-resistant enterococci are under active development (19).

More than 200 naturally occurring vancomycin-group antibiotics are known (7), and their common mode of action (which is shared by several synthetically modified glycopeptide antibiotics [1]) involves binding of the antibiotic to the -D-Ala-D-Ala motif of the growing cell wall and membrane-bound cell wall precursors of gram-positive bacteria (21) by forming specific hydrogen bonds and other noncovalent interactions (Fig. 1) (15, 27). The resulting disruption of the transglycosylase and/or transpeptidase activity of enzymes responsible for polymerization and cross-linkage of the cell wall results in bacteriostasis or bacterial cell death (25). It has been shown that LY307599, a semisynthetic vancomycin-group antibiotic, binds to analogs of the cell wall precursors of vancomycin-resistant enterococci (which contain a -D-Ala-D-Lac motif) in the same way that it binds to -D-Ala-D-Ala analogs (9, 23).

These binding interactions were elucidated by nuclear magnetic resonance experiments which investigated the binding interactions between antibiotics and cell wall analogs such as di-*N*-Ac-Lys-D-Ala-D-Ala (where Ac is acetyl). However, it is apparent that the binding of a vancomycin-group antibiotic to this type of cell wall analog in solution does not accurately reflect the affinity of the antibiotic for the cell wall precursors on the outer surface of the cytoplasmic membrane of a bacterium. This is suggested by the fact that association constants between antibiotics and peptide cell wall analogs in solution do not correlate well with the MICs of the antibiotics (10).

Two other factors are now known to be at least partly responsible for the enhanced activity of this class of antibiotics. Dimerization of the vancomycin-group antibiotics (Fig. 2) results in an enhanced affinity of antibiotic for cell wall analogs in free solution (16, 17), and it has also been shown that dimerization allows a chelate effect enhancement of affinity for the bacterial cell wall peptidoglycan (4). These two factors were shown to be important for antibacterial activity because antibiotics which dimerize weakly are less active than strongly dimerizing antibiotics unless they have an initially greater affinity for bacterial cell wall analogs. In a manner similar to that permitted by dimerization, the presence of lipid anchors on antibiotics such as teicoplanin permits a chelate effect enhancement of antibacterial activity.

The purpose of this work was to develop a method for the quantitative measurement of the affinities of vancomycin-group antibiotics for bacterial cell wall precursors in intact bacteria since the affinities of the antibiotics for synthetic cell wall analogs do not accurately reflect the antibacterial activity of the antibiotics. Vancomycin-susceptible (*Bacillus subtilis* ATCC 6633) and vancomycin-resistant (*Leuconostoc mesenteroides*) bacteria were used to develop models of the binding of vancomycin-group antibiotics to cell wall precursors containing -D-Ala-D-Ala and -D-Ala-D-Lac, respectively. The microbiological experiments allowed for the calculation of the contribution of the chelate effect to the binding of glycopeptides which dimerized or had membrane anchors.

MATERIALS AND METHODS

Antibiotics and cell wall analogs. Vancomycin, LY264826, and LY307599 were obtained as gifts from Eli Lilly & Co (Indianapolis, Ind.). SmithKline

* Corresponding author. Mailing address: Cambridge Centre for Molecular Recognition, University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, England. Phone: 44-1223-336368. Fax: 44-1223-336913. E-mail: dhw1@cam.ac.uk.

† Present address: Department of Biochemistry, Cambridge CB2 1QW, England.

‡ Present address: Clinical Microbiology and Public Health Laboratory, Level 6, Addenbrooke's Hospital, Cambridge CB2 2QW, England.

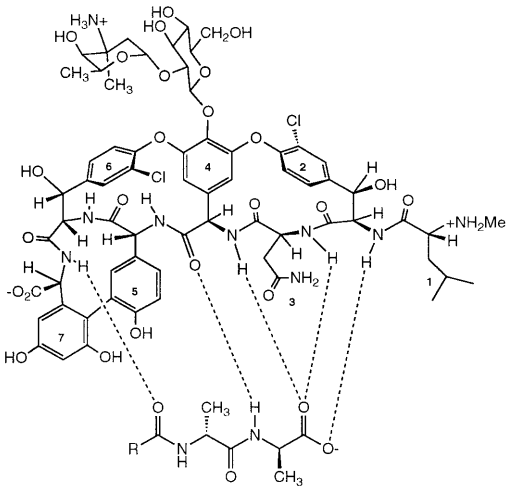


FIG. 1. Structure of vancomycin (top) showing hydrogen bonds (dashed lines) made to a piece of bacterial cell wall precursor (bottom). The heptapeptide backbone of the antibiotic runs through the center of the molecule, from left to right; the residues are numbered. Note the high degree of cross-linking of the side chains of these residues. The hydrogen bonds are made between the amide NH and CO groups of the binding pocket along the peptide backbone and those (including the free carboxylate) of the -D-Ala-D-Ala motif of the growing cell wall. Me, methyl.

Beecham (Brockham Park, United Kingdom) provided eremomycin and decaplanin; and MMDRI-Lepetit Research Center (Gerenzano, Italy) provided teicoplanin and teicoplanin A_3-1.

Phenylbenzyleremomycin was prepared by the previously published procedure (8), and ristocetin-ψ was provided by H. Yan. Di-*N*-Ac-Lys-D-Ala-D-Ala was purchased from Sigma.

Organisms, growth media, and MICs. *B. subtilis* ATCC 6633 was purchased as a standardized spore suspension (Difco), and for the experiments it was incubated at 37°C in Oxoid Nutrient Broth No. 2 or Difco Bacto Antibiotic Medium 1 agar, both of which were prepared according to the manufacturer's directions. *L. mesenteroides* R91/891 was a clinical isolate provided by the Public Health Laboratory Service, London, United Kingdom; it was incubated at 30°C in broth (BHY broth) prepared from Difco brain heart infusion to which Difco yeast extract (0.5%; wt/vol) was added or in agar (BHY agar) prepared in the same way but to which Difco Bacto Agar (1.6%; wt/vol) was added. Sterile glucose (to give 1% [wt/vol]) was added to BHY broth or agar immediately prior to use rather than before sterilization of the medium to avoid charring. The MICs (in micrograms per milliliter) of the vancomycin-group antibiotics were determined by serial dilution in tubes containing broth (4 ml). The inocula were spore suspension (*B. subtilis* ATCC 6633, 30 μl) or a 6- to 8-h culture of *L. mesenteroides* (20 μl).

Agar diffusion assays. Paper disk diffusion assays were based on those described by Rake et al. (24). The agar culture plates (10 by 10 cm square; 1-mm agar depth) were prepared from 10 ml of agar which was inoculated at 49°C with 30 μl of spore suspension (*B. subtilis* ATCC 6633) or 20 μl of a 6- to 8-h culture of *L. mesenteroides*. Dried paper disks to which antibiotic and ligand solutions had been added were then applied to the surface of the agar, and the plates were incubated for 16 h. The antibiotic caused a circular inhibition zone around the paper disk, the diameter of which was measured to the nearest 0.1 mm by using calipers. Dose-response calibration curves (logarithmic plots) were made by measuring the inhibition zone diameters produced by each antibiotic (in the range of 0 to 20 μg) in the absence of di-*N*-Ac-Lys-D-Ala-D-Ala. The relative potencies (10) of the antibiotic–di-*N*-Ac-Lys-D-Ala-D-Ala combinations were calculated as the ratio of the mass of the antibiotic on the paper disk to the apparent mass of the antibiotic when ligand was added; the apparent mass of the antibiotic when ligand was added was determined from the calibration curve. Control experiments showed that 500 μg or more of di-*N*-Ac-Lys-D-Ala-D-Ala on a paper disk gave rise to an inhibition zone, presumably because this interfered with normal cross-linking (10). In the cases in which the amount of di-*N*-Ac-Lys-D-Ala-D-Ala needed to reduce antibiotic activity to 50% of that in the absence of this antagonist was greater than 200 μg, the plot of relative potency of the antibiotic against amount of added di-*N*-Ac-Lys-D-Ala-D-Ala was extrapolated to give an estimate of this value (eremomycin and LY264826).

RESULTS AND DISCUSSION

In addition to the bimolecular association between the nascent bacterial cell wall and antibiotic, dimerization of vancomycin-group antibiotics is an important factor affecting antibiotic activity (17). Dimerization can promote binding of antibiotic to cell wall in at least two ways: (i) because the association constant of a dimeric antibiotic for cell-wall analogs in free solution is greater than that of a monomer, and (ii) because the dimeric antibiotic can take advantage of the chelate effect (Fig. 3) (4). Thus, the actual affinity of antibiotic for cell wall precursors [K_{assoc}(cell)] is the result of a combination of factors, which may be summarized as follows: actual binding affinity [K_{assoc}(cell)] = (bimolecular association with monomer [K_{assoc}]) × (enhancement by cooperativity [$\rho_{cooperativity}$]). We can break this down further, as follows: actual binding affinity [K_{assoc}(cell)] = (bimolecular association with monomer [K_{assoc}]) × (enhancement by dimerization [$\rho_{dimerization}$]) × (enhancement by chelate effect [$\rho_{chelate}$]). Here, the bimolecular association constant (K_{assoc}) is that between the monomeric antibiotic and the cell wall analog measured in vitro (by UV absorption spectophotometric titration). The enhancement by dimerization ($\rho_{dimerization}$) is that, also determined in vitro, by which the binding of cell wall analogs to dimeric antibiotics in free solution is greater than that to the corresponding monomers, the latter of which is derived from experiments which measured antibiotic dimerization in the presence and absence of cell wall analogs (17). The third term ($\rho_{chelate}$), the chelate-effect enhancement of binding of antibiotic to the nascent bacterial cell wall, has been shown to be important for the activity of vancomycin-group antibiotics (4), but it has not

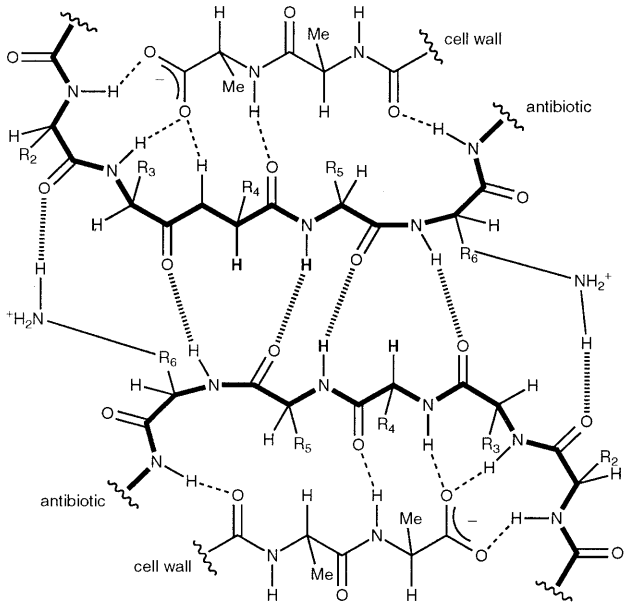


FIG. 2. Hydrogen-bonding network of the dimer formed between two antibiotic molecules when they are bound to the cell wall. Bold lines trace the antibiotic peptide backbones; R_2 to R_6 represent the side chains of residues 2 through 6, respectively. The heavy dashed lines represent hydrogen bonds formed between the two antibiotic molecules, and the broken lines indicate hydrogen bonds made to the cell wall. Note that the residue 6 amino sugar which forms a hydrogen bond to the residue 2 amide carbonyl is not present in all vancomycin-group antibiotics and that the residue 6 amino sugar of ristocetin-ψ makes a hydrogen bond with the carbonyl of residue 3 rather than residue 2 of the other half of the dimer. Upon dimerization, the association constant of the antibiotic for cell wall precursors increases because the peptide backbones are more constrained and so form better hydrogen bonds with the cell wall.

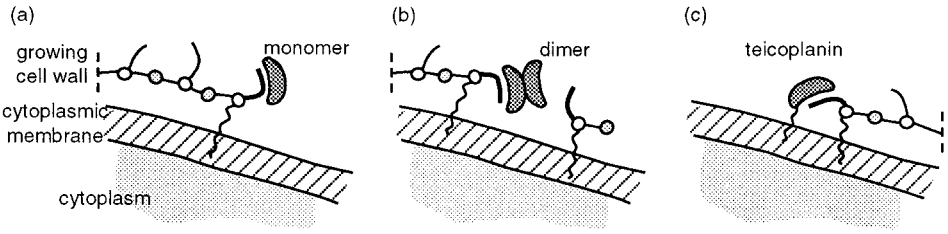


FIG. 3. Model for the enhancement of binding of dimeric and lipid-containing antibiotics to bacterial cell wall. The cell wall is made from polymerized subunits composed of a *N*-acetylmuramyl-*N*-acetylglucosamine disaccharide (open and shaded circles, respectively), from which branch the undecaprenyl lipid carrier (inserted into the cytoplasmic membrane; wavy line) and the -peptidyl-D-Ala-D-Ala pentapeptide (bold line). (a) Monomeric antibiotic binding to growing cell wall. (b) Dimeric antibiotic binding to growing cell wall. Binding of the dimer is enhanced relative to that of the monomer because the second binding event occurs with little loss in translational and rotation freedom of the tethered antibiotic (the chelate effect). (c) Association with the growing cell wall of lipid-containing (wavy line) antibiotics such as teicoplanin, which can simultaneously associate with the cytoplasmic membrane, is also enhanced by the chelate effect.

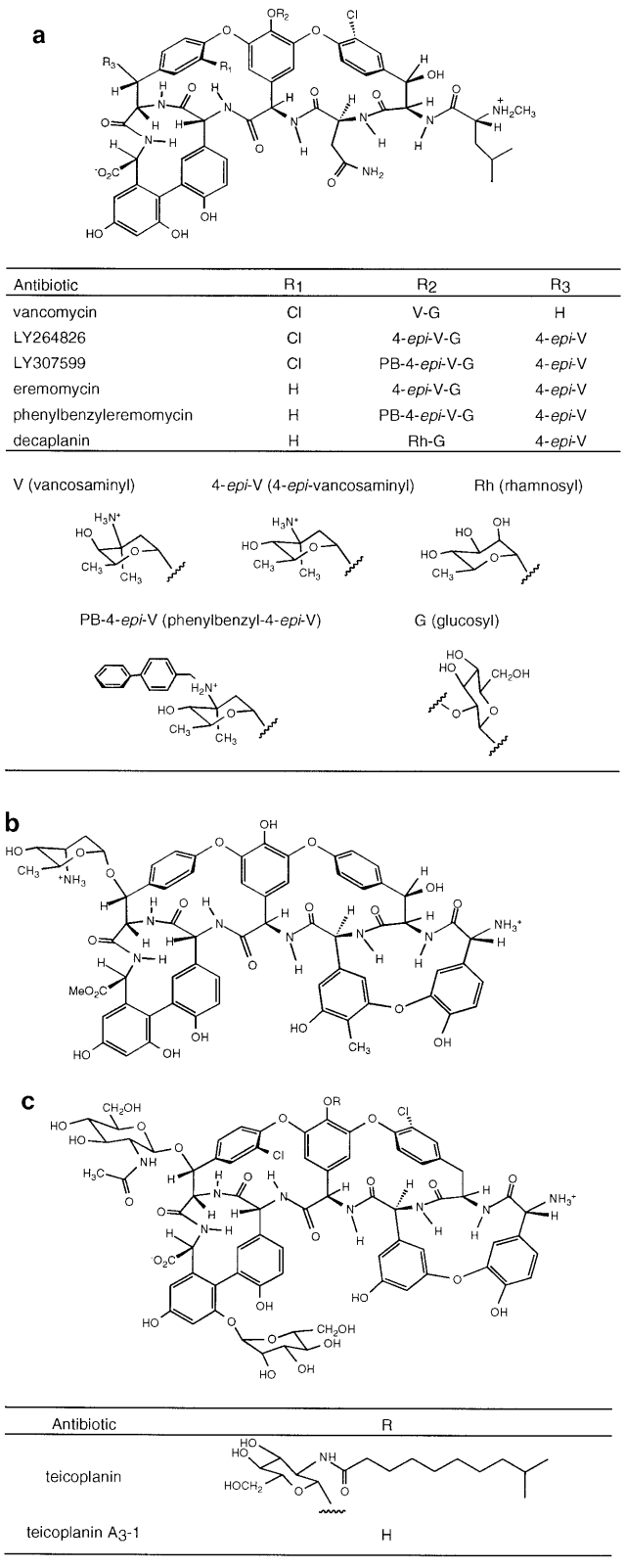
been quantified until now. The work described here demonstrates that the chelate effect can enhance the association between strongly dimerizing antibiotics and late cell wall precursors by a factor of up to 10^2. Furthermore, the actual binding affinity which is determined when all three factors are known correlates very well with the antibacterial activity of a given antibiotic, in contrast to the poor correlation which is seen between the (bimolecular) association constant of antibiotic for cell wall analog and antibacterial activity (MIC).

The K_{assoc} between di-*N*-Ac-Lys-D-Ala-D-Ala and nine vancomycin-group antibiotics (Fig. 4) taken from the literature are given in Table 1. The factors by which dimerization of these antibiotics enhances binding to this cell wall analog ($\rho_{dimerization}$) are given. The level of enhancement of antibiotic affinity for di-*N*-Ac-Lys-D-Ala-D-Ala due to dimerization is by a factor of 1 to 12 (Table 1). The affinity of dimeric antibiotic for cell wall analogs is in part increased over that found for monomer when a salt bridge mediated by a polarizable amide unit is formed between the carboxylate of the cell wall and the amino group of the residue 6 amino sugar of the other half of the dimer, which is present in some antibiotics (11, 23, 28).

The contribution of the chelate effect to the binding of vancomycin-group antibiotics to the cell wall of growing bacteria was determined by competition assays. In these, the antibacterial activities of the antibiotics were disrupted by the addition of di-*N*-Ac-Lys-D-Ala-D-Ala, which competes with bacterial cell wall precursors for the binding sites of antibiotic. Antibiotics which take advantage of dimerization and the chelate effect to enhance binding to cell wall through a unimolecular step (Fig. 3b and c) relative to binding through a bimolecular step (Fig. 3a) are more difficult to displace with di-*N*-Ac-Lys-D-Ala-D-Ala. This is because the latter binds to the antibiotic in a bimolecular step, and it is difficult to disrupt a unimolecular binding process with a competing bimolecular process. However, one of the antibiotics tested, teicoplanin A_3-1, does not dimerize and lacks an acyl chain, and thus, it always binds to the bacterial cell wall in a bimolecular step (another nondimerizing antibiotic, teicoplanin, is discussed below). The ratio of the amount of di-*N*-Ac-Lys-D-Ala-D-Ala needed to reverse the activity of a given antibiotic relative to the amount needed to reverse the activity of teicoplanin A_3-1 ($\rho_{cooperativity}$) is a measure of the extent to which the antibiotic benefits from dimerization (expressed in free solution) and the chelate effect (expressed at the surface of a bacterium). This ratio is composed of $\rho_{dimerization}$ and $\rho_{chelate}$, in which the latter is the extent to which the chelate effect enhances binding of the antibiotic to the bacterial cell wall. The values of $\rho_{dimerization}$ are known, and the values of $\rho_{cooperativity}$ determined in the antagonism assays allowed us to isolate the contribution of the chelate effect ($\rho_{chelate}$) to the binding of antibiotics to bacterial cell wall precursors.

There were two parts to the antagonism assay, the first involving *B. subtilis* ATCC 6633, a model gram-positive organism, and the second involving the use of *L. mesenteroides*, a gram-positive organism which serves as a model for vancomycin-resistant enterococci (discussed below). The ratio of the amount of di-*N*-Ac-Lys-D-Ala-D-Ala needed to reduce the activity of an antibiotic against *B. subtilis* ATCC 6633 to 50% of its activity in the absence of this antagonist relative to the amount needed for the nondimerizing teicoplanin A_3-1, which we take as a measure of the extent to which the antibiotic benefits from dimerization and the chelate effect, is given in Table 1 ($\rho_{cooperativity}$). This value represents the degree to which it is more difficult to disrupt an intramolecular process (binding of a dimeric and/or lipid-anchored antibiotic to the cell wall) relative to an intermolecular process (binding of a monomeric antibiotic to the cell wall). We may partition this into the contributions from dimerization and the chelate effect in which the enhancement of binding of cell wall analogs to dimeric antibiotics relative to that to monomeric antibiotics ($\rho_{dimerization}$) is known. The result of this analysis ($\rho_{chelate}$) is presented in Table 1 and indicates that the promotion of the binding of a strongly dimerizing antibiotic to the bacterial cell wall attributable to the chelate effect may be as great as 10^2. The actual binding affinity of the antibiotics for the bacterial cell wall can be semiquantitated as a result of these analyses, and values are presented as K_{assoc}(cell) (Table 1). Figure 5 illustrates that the values thus calculated for the affinity of antibiotics for cell wall precursors in situ reflect the antibacterial activity (MIC) of the antibiotics. This is not the case for the values for the association constant between di-*N*-Ac-Lys-D-Ala-D-Ala and the antibiotic, as may be seen from the K_{assoc} and MIC data presented in Table 1.

Vancomycin-resistant enterococci produce modified cell wall precursors for which vancomycin has a low affinity (6). The strains of *E. faecalis* and *E. faecium* which are being isolated with increasing frequency in hospitals synthesize cell wall precursors which terminate in -D-Ala-D-Lac rather than the -Ala-D-Ala of their susceptible counterparts. It has been reported that some antibiotics including LY307599 possess activity against such vancomycin-resistant enterococci (19); therefore, a model organism was selected to study the involvement of dimerization and the chelate effect in the activities of such antibiotics. Vancomycin-resistant enterococci were not used because clinical strains produce cell wall precursors of which a proportion terminate in -D-Ala-D-Ala (3). *L. mesenteroides* was selected from a number of candidates including lactobacilli and pediococci because *L. mesenteroides* produces cell wall precur-



Antibiotic	R_1	R_2	R_3
vancomycin	Cl	V-G	H
LY264826	Cl	4-*epi*-V-G	4-*epi*-V
LY307599	Cl	PB-4-*epi*-V-G	4-*epi*-V
eremomycin	H	4-*epi*-V-G	4-*epi*-V
phenylbenzyleremomycin	H	PB-4-*epi*-V-G	4-*epi*-V
decaplanin	H	Rh-G	4-*epi*-V

Antibiotic	R
teicoplanin	
teicoplanin A_3-1	H

FIG. 4. (a) Structures of vancomycin, LY264826, eremomycin, decaplanin, and synthetic analogs. Modifications to the basic heptapeptide motif which enhance dimerization (vancomycin, residue 4 disaccharide; eremomycin, residue 4 disaccharide and residue 2 chlorination; LY264826, residue 6 amino sugar) are illustrated. (b) Structure of ristocetin-ψ. Me, methyl. (c) Structures of teicoplanin and teicoplanin A_3-1. The modification of the basic heptapeptide motif which enhances membrane anchorage (lipoyl chain on residue 4 saccharide) is illustrated.

TABLE 1. Binding data and MICs of vancomycin-group antibiotics for *B. subtilis*[a]

Antibiotic	K_{assoc} (M^{-1})	$\rho_{dimerization}$	$\rho_{chelate}$	$\rho_{cooperativity}$	K_{assoc} (cell) (M^{-1})	MIC (μg/ml)
Vancomycin	1.5×10^6	3	6	20	3×10^7	0.25
LY264826	1.6×10^5	8	50	400	7×10^7	0.03
LY307599	2.7×10^5	12	27	300	9×10^7	0.008
Eremomycin	4.9×10^3	8	200	2,000	1×10^7	0.03
Phenylbenzyl-eremomycin	2.0×10^5			400	8×10^7	0.015
Decaplanin	9.8×10^3	4	30	100	1×10^6	0.5
Ristocetin-ψ	6.4×10^5	4	5	20	1×10^7	0.5
Teicoplanin	1.6×10^6	1	5	5	8×10^6	0.5
Teicoplanin A_3-1	1.2×10^6	1	1	1	1×10^6	4

[a] Association constants for the monomeric antibiotics with the cell wall analog di-*N*-Ac-Lys-D-Ala-D-Ala (K_{assoc}) and for antibiotics with cell wall at the bacterial surface [K_{assoc}(cell)] are given, with a breakdown of contributions to binding from dimerization ($\rho_{dimerization}$), the chelate effect ($\rho_{chelate}$), and the combined cooperativity from these factors ($\rho_{cooperativity}$). Association constants for di-*N*-Ac-Lys-D-Ala-D-Ala (K_{assoc}s) with the monomers of vancomycin (20), LY264826 (16), LY307599 (26), eremomycin (10), decaplanin (12), ristocetin-ψ (14), teicoplanin (18), and teicoplanin A_3-1 (18) are from the literature. In the cases of LY264826, LY307599, eremomycin, and decaplanin, the values were calculated from the association constant of di-*N*-Ac-Lys-D-Ala-D-Ala with dimeric antibiotic by using values for the extent to which binding of antibiotics to di-*N*-Ac-Lys-D-Ala-D-Ala is enhanced by dimerization ($\rho_{dimerization}$) (17, 26). The values of $\rho_{dimerization}$ are from previous reports (17, 26). Values for the extent to which binding of antibiotics to the bacterial cell wall is enhanced by the chelate effect ($\rho_{chelate}$) and the combined action of dimerization and the chelate effect ($\rho_{cooperativity}$) were determined as described in the text and were used to calculate the association constant for the antibiotics with the bacterial cell wall in situ [K_{assoc}(cell)]. MICs are for *B. subtilis* ATCC 6633, determined in duplicate experiments after incubation for 12 h at 37°C.

sors which contain solely -D-Ala-D-Lac (13) and because it was found to grow satisfactorily in the agar diffusion assays.

The assay conditions were modified to take into account the decreased susceptibility of *L. mesenteroides* to vancomycin-group antibiotics relative to that of *B. subtilis*. Four antibiotics were found to possess some activity against *L. mesenteroides*, and the results of an analysis of their dependence on dimerization and the chelate effect are given in Table 2. Di-*N*-Ac-Lys-D-Ala-D-Ala was used as a competitive inhibitor of -D-Ala-D-Lac binding to antibiotic, because the two cell wall precursor motifs bind to vancomycin-group antibiotics with the same geometry (although with one fewer hydrogen bond in the case of the -D-Lac-containing peptide) (9). Smaller amounts of di-*N*-Ac-Lys-D-Ala-D-Ala were needed to reverse the activities of these antibiotics against *L. mesenteroides* because the affinities of the antibiotics for di-*N*-Ac-Lys-D-Ala-D-Ala relative to their affinities for cell wall precursors containing -D-Ala-D-Lac are proportionately higher than those in the *B. subtilis* experiments, but the trend in the results was the same. It has been reported that the addition of a very large excess of di-*N*-Ac-Lys-D-Ala-D-Lac to vancomycin and LY264826 results in a loss of antibacterial activity against *Micrococcus luteus* (2). This should be a consequence of the low affinity of the antibiotic for di-*N*-Ac-Lys-D-Ala-D-Lac and indicates that even very large quantities of this cell wall analog are unlikely to give the reversal of activity in the agar diffusion assays which are necessary to determine the contribution of dimerization and the chelate effect in the activities of vancomycin-group antibiotics.

To determine the values of $\rho_{cooperativity}$, the activity of teicoplanin was used as a reference point because teicoplanin A_3-1 showed no activity against *L. mesenteroides*. The binding of teicoplanin to the cell wall already benefits from the chelate

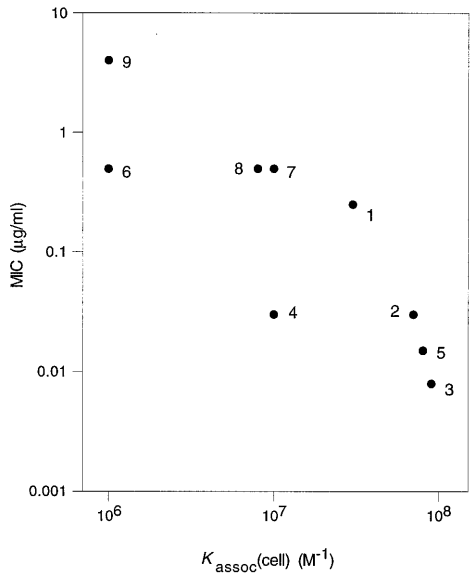


FIG. 5. Plot of the abilities of a range of antibiotics to kill bacteria. Antibiotics are vancomycin (point 1), LY264826 (point 2), LY307599 (point 3), eremomycin (point 4), phenylbenzyleremomycin (point 5), decaplanin (point 6), ristocetin-ψ (point 7), teicoplanin (point 8), and teicoplanin A_3-1 (point 9). There exists a correlation between the MIC (representing the actual binding affinity of the antibiotics for cell wall precursors) and the actual binding affinity, as determined from the combination of data from binding experiments with monomeric and dimeric antibiotic and in vitro measurement of the contribution of dimerization and the chelate effect to antibiotic activity.

effect, and so use of this antibiotic as a reference for semiquantitation of the chelate effect may lead to values which are a factor of 5 lower (on the basis of the experiments with *B. subtilis*). After taking into consideration dimerization and the chelate effect, the affinities of the antibiotics for the modified cell wall are low. However, these factors act to increase the association constant to a level sufficient to exhibit antibacterial activity in the case of LY307599, which has a MIC of 32 μg/ml for *L. mesenteroides*. While this would not make LY307599 a clinically useful compound against this organism, it is clear that future semisynthetic antibiotics which are to be active against vancomycin-resistant organisms should have a large $\rho_{cooperativity}$, in addition to having a large association constant for di-*N*-Ac-Lys-D-Ala-D-Lac. This should probably also be true for antibiotics which are to be active against vancomycin-resistant organisms which produce other cell wall motifs, for example, *Enterococcus gallinarum*, which makes cell wall precursors terminating with -D-Ala-D-Ser (5).

TABLE 2. Binding data for an analog (di-*N*-Ac-Lys-D-Ala-D-Lac) of cell wall precursors of vancomycin-resistant organisms (K_{assoc}) and for cell wall [K_{assoc}(cell)] of a vancomycin-resistant organism (*L. mesenteroides* Colindale R89/891)[a]

Antibiotic	K_{assoc} (M^{-1})	$\rho_{cooperativity}$	K_{assoc}(cell) (M^{-1})	MIC (μg/ml)
Vancomycin	500	6	3×10^3	1,024
LY264826	1,400	8	1.1×10^4	256
LY307599	1,400	8	1.1×10^4	32
Teicoplanin		1		>512

[a] Association constants of di-*N*-Ac-Lys-D-Ala-D-Lac for vancomycin and LY264826 have been reported elsewhere (2); the value for LY307599 is an estimate. Values for the extent to which binding of antibiotics to bacterial cell wall is enhanced by the combined action of dimerization and the chelate effect ($\rho_{cooperativity}$) were determined as described in the text. MICs are for *L. mesenteroides*, determined in duplicate experiments after incubation for 12 h at 30°C.

The fact that the MIC of LY307599 is eight times lower than that of LY264826, despite identical derived K_{assoc}(cell) values, may mean that (i) the assumption that the K_{assoc} of 1,400 M^{-1} is the same for LY307599 as the experimental value for LY264826 is invalid and/or (ii) a variable which has not been considered can additionally affect MICs. The probability that the first assumption is invalid is certainly raised by the observation that di-*N*-Ac-Lys-D-Ala-D-Ala binds more strongly to the monomer of LY307599 than to that of LY264826 by a factor of 2 (Table 1).

In conclusion, it has been demonstrated by the use of antagonist binding assays that the binding of vancomycin-group antibiotics to bacterial cell wall precursors involved in peptidoglycan synthesis can benefit from the chelate effect by up to 2 orders of magnitude. In the case of a vancomycin-resistant organism (*L. mesenteroides*), the enhancement of activity by the chelate effect was smaller but was sufficient to allow the binding of LY307599 to the lactate-containing cell wall precursors.

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