Noncovalent Bond Lengths and Their Cooperative Shortening: Dimers of Vancomycin Group Antibiotics in Crystals and in Solution

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Dedicated to Professor *Jack D. Dunitz* on the occasion of this 80th birthday

A wide range of dimerisation constants (K_{dim} *ca.* $10^1 - 10^6$ M^{-1}) for various glycopeptide antibiotics have been determined. We consider these dimerisation constants in the light of the published X-ray structures of the antibiotics, in particular, the relationship between K_{dim} and the length of a specified distance at the dimer interface. In the crystals, we find that this distance is smaller for strongly dimerising antibiotics and larger for weakly dimerising antibiotics. Thus, the dimerisation constant is correlated with tightness at the dimer interface. Despite the crystal-packing forces exerted between adjacent dimer molecules in the crystals, the noncovalent bond distances at the dimer interface are correlated with the distances in solution (inferred from solution NMR data). These observations can account for the benefits in enthalpy, and costs in entropy, associated with positively cooperative binding.

Introduction. – Glycopeptide antibiotics of the vancomycin group bind strongly to bacterial cell-wall-analogue precursors (ligands) such as di-*N*-Ac-Lys-D-Ala-D-Ala [1–3]. Additionally, the antibiotics typically form dimers [4]. For the two types of interfaces in the complex (*i.e.*, ligand interface and dimer interface, *Figs. 1* and *2*), we have recently inferred, using chemical-shift criteria (see below), that the structural tightness of these noncovalently bound interfaces increases with increasing thermodynamic stability of the complex [5–8]. In this paper, dimerisation constants of some antibiotics whose X-ray structures have been determined are measured. An analysis of the X-ray structures of the antibiotics has been made, and the structural tightening of the dimer interface is again observed, as evidenced by a distance reduction at the interface in the crystals.

In previous work, the tightness of the interface between the antibiotic and its ligand (ligand interface, see *Fig. 1*) was probed by the downfield chemical shift of the NH proton w_2 located in the ligand-binding pocket of the antibiotic. This NH proton forms a H-bond to the terminal carboxylate group of the ligand when the ligand binds to the antibiotic. In the formation of H-bonds, it is generally accepted that increased downfield chemical shifts of NH protons indicate shorter H-bond lengths [9–16].

In the complex of antibiotics with a series of ligands (acetate → *N*-Ac-D-Ala → *N*-Ac-D-Ala-D-Ala → di-*N*-Ac-Lys-D-Ala-D-Ala), we observed the chemical shift of NH proton w_2 as the binding constant changed. These observations showed that NH w_2 is shifted much further downfield in higher-affinity complexes, indicating shortening of its H-bond length [5][6]. Higher-affinity complexes are characterised by a larger number of hydrophobic interactions between Me groups of the ligands and aromatic rings in the

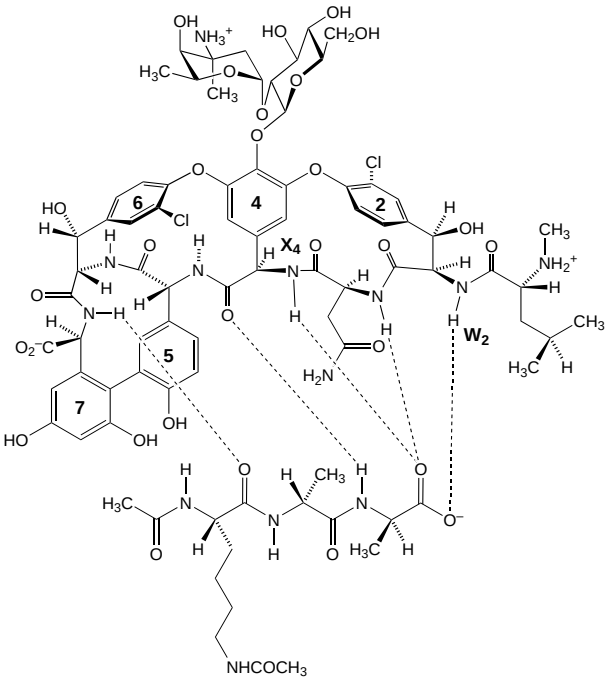


Fig. 1. *View of the binding interaction between the glycopeptide antibiotics* (in this case, vancomycin) *and the peptide ligand* (di-*N*-Ac-Lys-D-Ala-D-Ala). H-Bonds between the two are indicated by dotted lines. The amide NH proton w_2 and the α-CH proton x_4 (both mentioned in the text) are labelled and the aromatic rings are numbered.

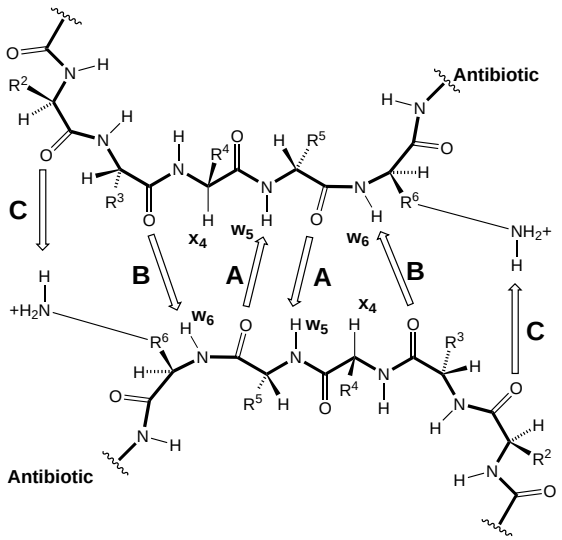


Fig. 2. *H-Bonding network of a dimer formed by two molecules of a vancomycin group antibiotic*. H-Bonds are shown as arrows and are labelled **A** or **B** according to whether the proton w_5 or w_6 is involved. H-Bonds from the ammonium ion on residue 6 are labelled as **C**. Also labelled are the x_4 protons, whose chemical shift moves downfield upon dimerisation.

antibiotic, and by additional H-bonding interactions. The added interactions are at variable distances from the site of the H-bond to w_2. Thus, as further interactions remote from this H-bond are added, the length of this H-bond is decreased. This cooperative bond shortening is depicted schematically in *Fig. 3*.

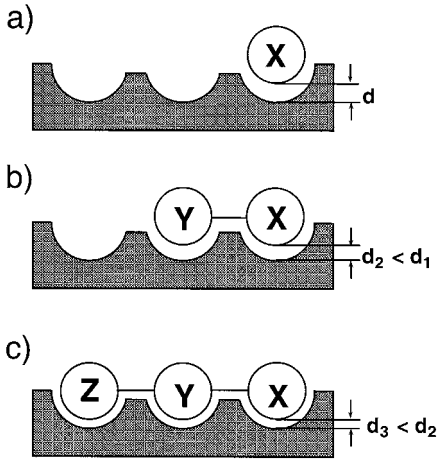


Fig. 3. *Schematic depiction of the effects of adding cooperative interactions to an interface.* In *a*), the receptor, shaded in grey (in this case, a glycopeptide antibiotic), has bound ligand X (*e.g.*, acetate) weakly, with a large interfacial distance (d_1) and relatively large amounts of residual motion in the complex (*i.e.*, loose binding). In *b*), the receptor has bound the ligand X–Y (*e.g.*, *N*-Ac-D-Ala) cooperatively, with a smaller interfacial distance (d_2) and less residual motion (*i.e.*, tighter binding). In *c*), the receptor has bound ligand X–Y–Z (*e.g.*, di-*N*-Ac-Lys-D-Ala-D-Ala) cooperatively, with still smaller interfacial distance (d_3) and still less residual motion.

A similar criterion for the downfield chemical shift of the α-CH proton x_4 [9–11] [17] was applied to study structural tightness at the various dimer interfaces (see *Fig. 2*). Upon dimerisation, the proton x_4, located near the centre of the dimer interface, can, in principle, experience a downfield shift for two reasons (*Fig. 4*). First, carbonyl group **a** is held with higher probability in the plane of x_4 in the dimer, compared to in the monomer, due to the geometric requirement imposed upon it by H-bonding to w_6. Second, carbonyl group **b** approaches x_4 upon dimerisation, causing a downfield shift of its resonance. Both these effects are magnified when the dimer is tighter and the approach of the two halves of the dimer is closer. Using this criterion, we found that the dimer interface of the antibiotics becomes tighter at the specified point as the number of adjacent noncovalent interactions is increased to give thermodynamically more-stable dimers [7][8].

The increasing tightness of the dimer interfaces observed in solution might persist in crystals of the dimers. The recent availability of a number of crystal structures of the dimeric antibiotics, given that their dimerisation constants are known or can be determined, allows the question to be addressed. The answer to the question is not self-evident. The reduced dynamic behaviour of crystals relative to their corresponding liquid states is evident from the observation that crystallisation is universally found to occur with a benefit in enthalpy and a cost in entropy. Thus, crystal-packing forces do reduce dynamic behaviour and improve noncovalent bonding. These forces can,

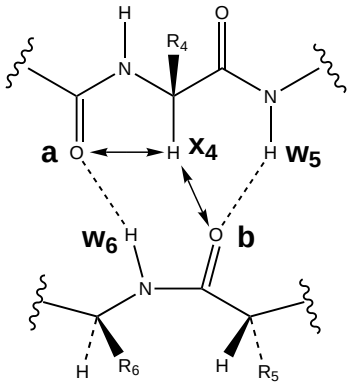


Fig. 4. *Section of the dimer interface of a vancomycin group antibiotic showing the two carbonyl groups that cause the downfield shift of the* ^{1}*H-NMR resonance of the proton* x_4

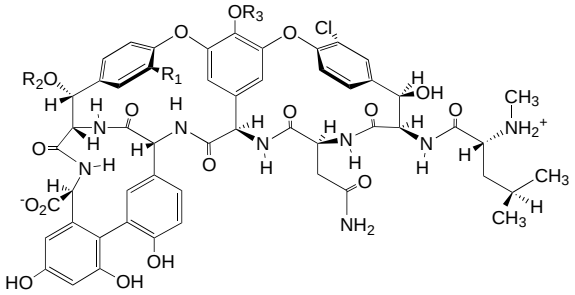
therefore, in principle, mask decreases in dynamic behaviour and increases in noncovalent bonding of complexes that give rise to positively cooperative binding in solution [8].

The above question is rendered particularly pertinent by a recent paper [18] that addresses the question of the origin of cooperative binding in the complexes of ligand-bound dimers of the antibiotics. It is suggested that the X-ray crystallographic structures of vancomycin demonstrate that neither the process of dimerisation, nor of ligand binding, significantly alters the structural framework common to glycopeptide antibiotics. It is also suggested that ligand binding does not shorten the H-bonds across the dimer interface.[18] Although, as discussed above, crystal-packing forces can mask the reduction in interfacial distances found in solution [8][19], we thought it desirable to examine dimer interfacial distances in crystals of dimers now available. We show that a specific interfacial distance is shorter in crystals of strongly dimerising antibiotics relative to their more weakly dimerising counterparts.

Results and Discussion. – *Thermodynamic Stability of the Dimers.* We have analysed various X-ray structures of the following glycopeptide antibiotics: decaplanin (also known as MM47761) [20], balhimycin bound to acetate and citrate [21], ureido-balhimycin [22], vancomycin bound to acetate [23][24], vancomycin bound to *N*-Ac-D-Ala [25], aglycovancomycin bound to acetate [26], and A-40926 aglycone [27]. The structures of these antibiotics are shown in *Fig. 5*. These crystal structures are in the form of asymmetric dimers. Some of the dimer structures were solved with one or two ligand molecule(s) in one or both of their two binding pockets.

The dimerisation constants of balhimycin, ureido-balhimycin, vancomycin bound to acetate, aglycovancomycin bound to acetate and A-40926 aglycone were determined by ^{1}H-NMR spectroscopy. The dimerisation constants of decaplanin and vancomycin bound to *N*-Ac-D-Ala were previously determined as described by *Mackay et al.* [28]. *Table 1* shows the dimerisation constants thus determined for these antibiotics.

Dimerisation constants of glycopeptide antibiotics are increased by positively cooperative binding of ligands [28]. The dimerisation constant of balhimycin in *Table 1* was determined without ligands, although the X-ray structure was solved with acetate and citrate in the binding pockets. The NMR spectrum of balhimycin at very low



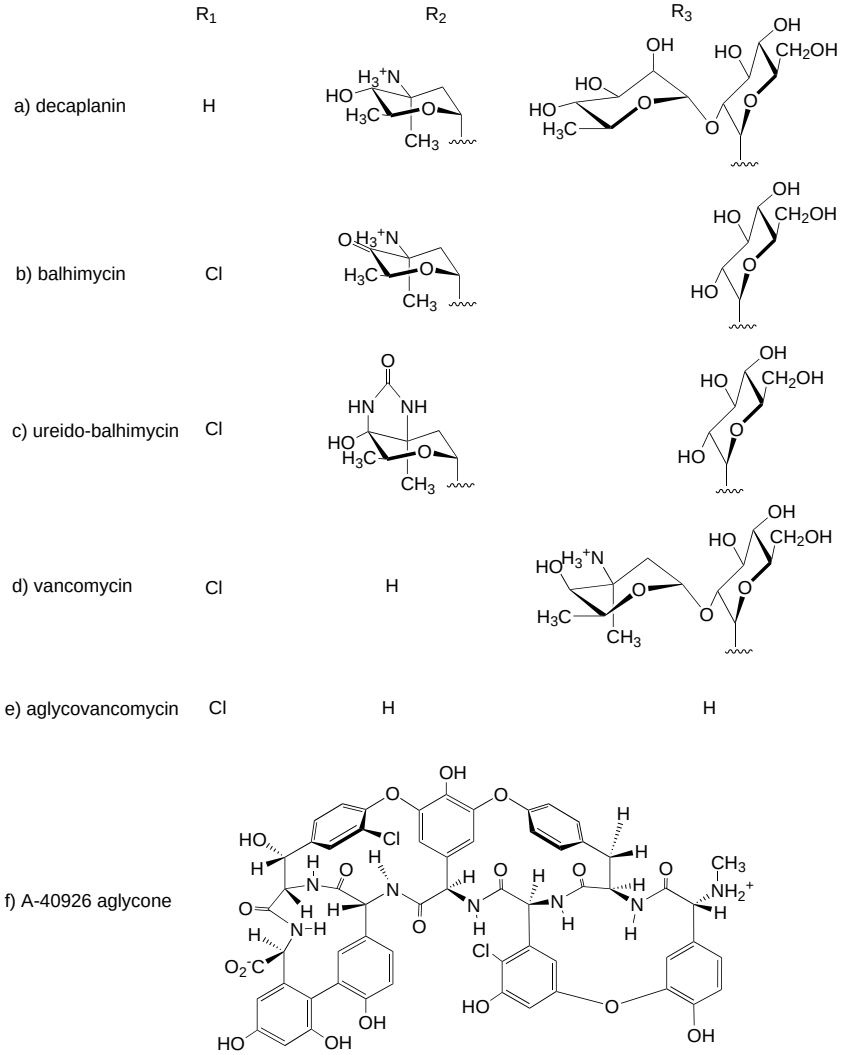


Fig. 5. *Structures of the antibiotics* a) *decaplanin,* b) *balhimycin,* c) *ureido-balhimycin,* d) *vancomycin,* e) *aglycovancomycin, and* f) *A-40926 aglycone*

Table 1. *Dimerisation Constants of the Glycopeptide Antibiotics in the Presence or Absence of Ligand*

Antibiotic	Ligand	K_{dim}/M^{-1} [a])
Decaplanin	None	5.0×10^{6} [b])
Balhimycin	None	5.5×10^{4} [c])
Ureido-balhimycin	None	5.4×10^{4} [c])
Vancomycin	Acetate	3.2×10^{3} [c])
Vancomycin	*N*-Ac-D-Ala	1.3×10^{3} [b])
Aglycovancomycin	Acetate	30 ± 20 [d])
A-40926 aglycone	None	30 ± 10 [d])

[a]) All dimerization constants were determined by NMR as described in the previous paper [8]. [b]) Measured in D_2O at 298 K, pD 7, data taken from [28]. [c]) Measured in D_2O at 300 K, pD 7 (present work). [d]) Measured in H_2O in the presence of 10% CD_3CN at 300 K, pH 8 (present work).

concentration (0.01 mM) shows the monomer resonance of the proton x_4 (see *Fig. 2*) only when the ligand is absent. A lower concentration of balhimycin bound to acetate would be necessary to obtain this dimerisation constant. The required concentrations are so low that good-quality NMR spectra are difficult to obtain, and we note that the value for balhimycin bound to ligands must be larger than the value determined for the uncomplexed balhimycin (*Table 1*). In the determination of the dimerisation constant of vancomycin in the presence of acetate, the concentration of acetate was such that approximately 90% of the vancomycin was bound by acetate (compared to 50% in the crystal). Since the dimerisation constant of vancomycin when free is 700 M^{-1} [29], the data show that dimerisation of vancomycin is cooperative with the binding of acetate by a factor of *ca.* 5 (*Table 1*).

The large dimerisation constants for decaplanin, balhimycin, and ureido-balhimycin emphasise the importance of an amino sugar at residue 6, commonly present in strongly dimerising antibiotics, to enhance dimerisation (*Fig. 5*, *a*–*c*). Their structures are similar to that of vancomycin (*Fig. 5*, *d*), the major difference being the *epi*-vancosamine, or closely related sugar, attached to residue 6. The ammonium ion of residue 6 can function as a H-bond donor to the carbonyl group of residue 2 in the opposite half of the dimer (*Fig. 2*, outer two arrows labelled **C**) [20–22]. It was shown that the extra H-bonds promote the dimerisation of the glycopeptide antibiotics that have amino-sugars on their residue 6 from comparison of the dimerisation constants with those of their aglyco-derivatives [29][30].

Sugars attached to the ring 4 are also known to promote the dimerisation of the antibiotics. Interactions between the two sugar units on residues 4 of the two halves of the dimer can explain the promotion of dimerisation [21–24][31][32]. In fact, the dimerisation constants of aglycovancomycin and A-40926 aglycone that do not have sugars bound to either residues 4 or 6 (*Fig. 5*, *e* and *f*), are very small (*Table 1*).

The dimerisation constants of aglycovancomycin and A-40926 aglycone were determined in the presence of 10% CD_3CN (in H_2O) at pH 8 because of their poor solubility in absolute aqueous solution at a neutral pH. We estimate that their dimerisation constants in aqueous solution at pD 7 will not be very different from the experimental values in *Table 1*. In fact, in the case of vancomycin, the reported dimerisation constant at pD 7 (in D_2O, 745 ± 15 M^{-1}) was only slightly greater than that

at pH 8 (in H_2O, 610 ± 70 M^{-1}) [33]. Additionally, in the case of another glycopeptide antibiotic ristocetin A, the dimerisation constant in aqueous solution was found to be smaller than that in the presence of 10% CD_3CN, but it varied only by a factor of 7 (500 *vs.* 3500 M^{-1}). Thus, the uncertainties associated with the above variables are small compared with the large variations in dimerisation constants (see *Fig. 6*, later) in reaching our key conclusion.

Distances across the Dimer Interface. The crystal structures were analysed to find the specified distances across the dimer interface (*Fig. 2*). These distances (formally, H-bond lengths) for each of the X-ray structures are summarised in *Table 2*. Because of the asymmetric feature of the dimers, the two bond lengths labelled **A**, or the two labelled **B**, in *Fig. 2* are usually different. Two values for each of these **A** and **B** distances were, thus, obtained, and the average values given in parentheses (see *Table 2*). For the cases of decaplanin, balhimycin with acetate/citrate, vancomycin with *N*-Ac-D-Ala, and aglycovancomycin with acetate, four values were reported for each type of H-bond (labelled **A** or **B**), because four independent monomers were found in the crystals that form two similar asymmetric dimers. Values greater than the threshold distance considered to be limiting for the formation of a formal H-bond are shown in italics.

Table 2. *H-Bond Lengths across the Dimer Interfaces in Crystals of Glycopeptide Antibiotics*

Antibiotic	Ligand 1	Ligand 2	T/K[a])	Distance **A**[b])/Å	Distance **B**[a])/Å
Decaplanin [20]	None	None[c])	100	2.914/2.863 (2.889)	3.105/3.035 (3.070)
Balhimycin [21]	Acetate	Citrate	100	2.867/2.831 (2.849)	3.018/3.006 (3.012)
Ureido-balhimycin [22]	H_2O	H_2O	293	2.994/2.970 (2.982)	3.230/3.145 (3.186)
Vancomycin [24]	Acetate	None[c])	125	2.921/2.843 (2.882)	3.103/3.046 (3.075)
Vancomycin [23]	Acetate	None[c])	98	2.910/2.865 (2.888)	3.133/3.054 (3.094)
Vancomycin [25]	*N*-Ac-D-Ala	*N*-Ac-D-Ala	125	2.94/2.91 (2.93)	3.09/3.04 (3.07)
Aglycovancomycin [26]	Acetate	Acetate	100	2.891/2.841 (2.866)	3.284/3.165 (3.225)
A-40926 aglycone [27]	H_2O	None	153	2.987/2.889 (2.938)	*3.708/3.603* (3.656)

[a]) The temperature used to collect the X-ray data. [b]) All values quoted are N···O distances, rather than direct H···O distances. Values in parentheses are the average of the two distances (see text). Values greater than the threshold distance for the formation of a formal H-bond are shown in italics. [c]) The second binding pockets of these dimers were occupied by the asparagine side chains of the antibiotics.

In *Table 2*, it can be seen that the H-bond distances to w_5 (distance **A**, *Fig. 2*) do not vary significantly (only 7% increase from the minimum, 2.80 Å, to the maximum, 2.99 Å). No significant differences of these H-bond distances are, thus, detected among strongly ($K_{dim} > 10^4$ M^{-1}), and moderately and weakly ($K_{dim} < 10^2$ M^{-1}) dimerising antibiotics (average value of 2.89 Å for all three groups of antibiotics).

The distances to w_6 (distance **B**, *Fig. 2*), however, are very different (27% increase from the minimum, 2.97 Å, to the maximum, 3.77 Å). The average values of these distances for strongly, moderately, and weakly dimerising antibiotics are 3.06, 3.09, and 3.44 Å, respectively. The longer distances are consistent with the dimerisation constants of aglycovancomycin and A-40926 aglycone being small (*Table 1*).

The H-bond lengths are unexpectedly long for the ureido-balhimycin dimer, despite its large dimerisation constant. This is very probably because the crystal structure was obtained at a temperature much higher (by *ca.* 140–195°) than that used for the other

crystal structures (*Table 2*). The higher internal thermal energy increases the average noncovalent bond length, and, for this reason, it is likely that the bond lengths are longer than they would have been had they been collected at a temperature comparable to that used for the other crystals.

Another factor that affects X-ray structures is crystal-packing forces. In some crystals of glycopeptide antibiotics, the usual dimer unit interacts with another dimer unit to form an oligomer such as a tetramer or an infinite chain. This higher-structure formation can, in principle, distort the usual dimer structure because of geometry requirements. Thus, in the oligomer, the H-bond lengths across the dimer interface in its X-ray structure could be different from those in its solution structure.

For example, in the case of X-ray structure of decaplanin, four independent molecules (A, B, C, and D) of the antibiotic are present in the asymmetric unit [20]. Molecules A and B form a normal 'back-to-back' dimer, as do C and D. However, A and C also interact with each other, as three new H-bonds could be detected. This interaction between molecules A and C probably affects the H-bond length at the usual dimer interface. In fact, in the X-ray structure of decaplanin, one of the four distances **B** in the dimers (3.11 Å) is considerably longer than the other three distances **B** (*ca.* 2.98–3.03 Å, see *Table 2*).

Another interaction, known as a 'face-to-face' interaction, could also affect X-ray structures of glycopeptide antibiotics. Oligomerisation through a 'face-to-face' interaction has never been found in solution structures studied by NMR, but it is widely observed in the X-ray structures [25][26][34]. Among the structures studied in the present work, this interaction can be found in the structures of vancomycin with *N*-Ac-D-Ala and aglycovancomycin with acetate. In the latter case, only two of the four independent molecules form an infinite chain; the other two form a simple 'back-to-back' dimer [26].

The 'face-to-face' interaction is largely mediated by bound ligands [25][26][34]. Thus, the interaction is relatively weak when the bound ligands are small. For example, in the structure of vancomycin with *N*-Ac-D-Ala it is mediated through only one H-bond and hydrophobic contacts [25], and in that of aglycovancomycin with acetate by only hydrophobic contacts [26].

In contrast, in the case of balhimycin with the longer ligand Lys-D-Ala-D-Ala, the 'face-to-face' interaction is mediated through four H-bonds between the 'back-to-back' dimer units [34]. We have not included this structure in our analysis, because the crystal-packing forces between the dimer units are expected to be strong and may distort the dimer structure. In fact, one of the two distances **B** for each dimer ranges between 3.05 and 3.08 Å, values comparable to those of the strongly dimerising antibiotics; the other, however, is longer and ranges between 3.12 and 3.20 Å.

With the exclusion indicated in the preceding analysis, we plot in *Fig. 6* the solution dimerisation constants against the average values of the distance **B** in the crystal structures of the antibiotics. The data point obtained at higher temperature for the ureido-balhimycin dimer is indicated by a filled circle. The data that may be affected by crystal-packing forces for the dimers of decaplanin, vancomycin with *N*-Ac-D-Ala, and aglycovancomycin with acetate, are indicated by crossed circles. Despite the potential for complications, *there is a good correlation between increasing dimerisation constant (over a large range) and shorter distance* **B**. *Additionally, the curvature of this correlation*

is similar to that of the previously found correlation [8] *between dimerisation constant and limiting chemical shift of* x_4. That is, both have a gentle slope for weakly dimerising antibiotics but a steep slope for strongly dimerising antibiotics.

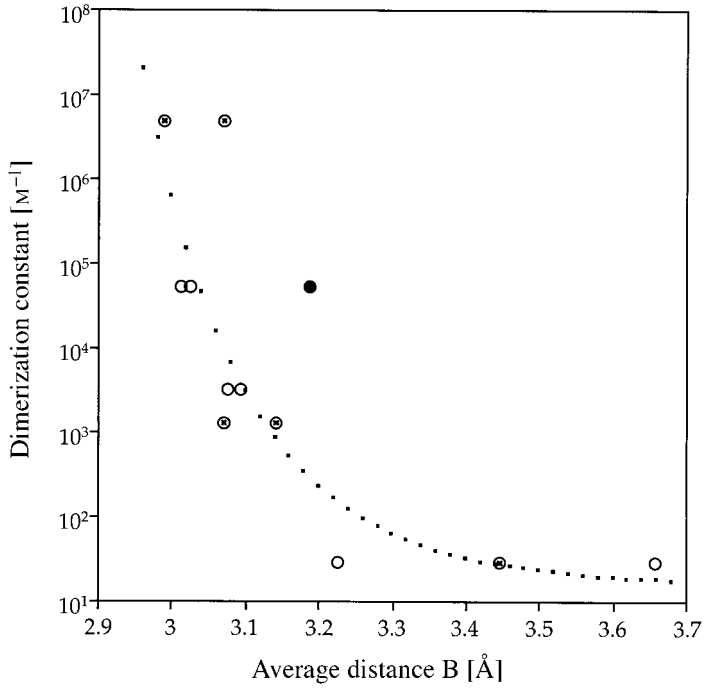


Fig. 6. *Plot of* K_{dim} (*Table 1*) vs. *the average distance* **B** *in the crystal structures* (*Table 2*). See text for details.

Cooperative Shortening of H-Bond Lengths. As stated above, the two sugars on residues 4 and 6 significantly promote the dimerisation of the antibiotics. In fact, the strongly dimerising antibiotics ($K_{dim} > 10^4$ M^{-1}) have both sugars, the moderately dimerising antibiotics have only the sugar at residue 4, and the weakly ($K_{dim} < 10^2$ M^{-1}) dimerising antibiotics have neither. These sugars can make additional interactions with the other half of the dimer, *i.e.*, the H-bonds **C** (see *Fig. 2*) that involve the sugar on residue 6, or the sugar-sugar interaction between the sugars on residue 4. Both of these additional interactions that cause shortening of the distance **B** are relatively remote from the **B** location. Thus, cooperative shortening of the distance **B** can be seen in the X-ray structures of the antibiotics.

Our earlier experiments by NMR indicate that cooperative tightening of the dimer interface is also caused by ligand binding [8]. From *Fig. 6*, it is clear that an increase in dimerisation constant due to ligand binding should produce a larger change in the average distance **B** when the antibiotic has a smaller dimerisation constant. This same prediction is made, and established, from NMR data [8]. However, no X-ray data is available for the same weakly dimerising antibiotic with or without a ligand.

In a recent publication, *Jusuf et al.* [18] comment that structural tightening cannot be detected in X-ray structures of glycopeptides. Specifically, it was observed that the

average length of a H-bond across the dimer interface of a vancomycin dimer singly bound to acetate is 2.17 Å, (O···H distance), while it is slightly longer (2.19 Å) for a vancomycin dimer bound to two molecules of *N*-Ac-D-Ala. However, in the present paper, we have determined the dimerisation constant for vancomycin bound to two molecules of acetate to be 3200 M^{-1} (*Table 1*). We can estimate that the dimerisation constant for vancomycin bound to one acetate is *ca.* 1500 M^{-1} (geometric mean of 3200 M^{-1} and 700 M^{-1}, the latter value being for vancomycin alone [29]). The value for vancomycin with *N*-Ac-D-Ala is 1300 M^{-1} [28]. Therefore, the average H-bond lengths cannot be anticipated to be different in the cited cases.

Molecular-dynamics simulations by *Jusuf et al.* [18] suggest that the cost in vibrational entropy upon dimerisation of vancomycin is less when the monomers are ligand-bound than when they are ligand-free. This conclusion seems reasonable, since ligand binding may reduce the internal motions of the monomers in a manner that aids formation of the dimer interface. The authors conclude that these favorable changes in vibrational entropy are of sufficient magnitude *by themselves* to account for the positive cooperativity between ligand binding and dimerisation. However, calorimetric experiments [33] establish that dimerisation of the ligand-bound antibiotic is *less* favorable in entropy, and more exothermic, than is dimerisation of the ligand-free antibiotic.

Thus, upon positively cooperative binding of ligand, the calorimetric measurements show the increased exothermicity consistent with improved noncovalent bonding at the interfaces of the complexes [33]. The calorimetric data are, therefore, consistent with the data from both NMR [8] and X-ray structures (present work). The positive cooperativity of ligand binding to dimers typically makes the entropy of dimerisation more *adverse*, not more favorable [33]. This finding is in accord with the supposition that the improvement in noncovalent bonding associated with positive cooperativity will cause increased order within the complex. This follows since it is improved bonding (favourable enthalpy change) that decreases the opposing motions (unfavourable entropy change), as evidenced by the phenomenon of enthalpy/entropy compensation [19][35]. Thus, it appears likely that it is because of enthalpy/entropy compensation that the experimental entropy changes typically found for the positively cooperative binding are negative [33]. The favorable entropy change identified by *Jusuf et al.* [18] as a source of positive cooperativity, therefore, appears to be outweighed by the enthalpy/entropy compensation that accompanies the improved noncovalent bonding at the interfaces of the complexes.

Conclusions. – We have studied a series of closely related compounds for which X-ray structures of the associated states are available. The distances **B** (see *Fig. 2*) in the crystals are found to be correlated with the dimerisation constants of the antibiotics (*Fig. 6*). This work supports our previous finding [8] by NMR that increasing tightness at the dimer interface (as evidenced from the downfield chemical shift of the proton x_4 that lies between the H-bonds **A** and **B**) correlates with increasing thermodynamic stability of the dimers. The greater structural tightness is associated with an overall benefit in enthalpy and a cost in entropy [19]. These conclusions are also in agreement with our recent considerations of melting and crystallisation [36]. The greater the restriction of internal motions of hydrocarbons upon crystallisation, the more positively cooperative the process of crystallisation, and the greater is the benefit in bonding, the

larger is the cost in entropy, and the larger is the volume reduction (increase in structural tightening). We conclude that benefits in enthalpy, costs in entropy, and (by implication) volume reductions, are typically consequences of positive cooperativity [37].

Experimental Part

Balhimycin, ureido-balhimycin, and A-40926 were donated by *Aventis Pharma* and *Biosearch Italia*. Vancomycin hydrochloride was purchased from *Aldrich*. Aglycovancomycin was prepared by the removal of the disaccharide at ring 4 of vancomycin by the mild hydrolysis technique described by *Kannan et al*. [38].

Preparation of A-40926 Aglycone. A-40926 Aglycone was prepared by a modified method of one previously described by *Selva et al*. [39] A-40926 (355.8 mg, 0.21 mmols) was dissolved in DMSO (5.5 ml), and conc. HCl (0.3 ml) was added. The resulting soln. was heated at 80° for 3 h. DMSO was removed from the soln. by *Kugelrohr* distillation to give a yellow solid. The product was purified by reverse-phase HPLC with a gradient system (0 to 20 min, 0 to 100% MeCN (0.1% TFA) and 100 to 0% H_2O (0.1% TFA)) and then lyophilised to give a white solid (83 mg, 38% yield). ESI-MS: 1212.24 ($[M+H]^+$, $C_{59}H_{48}Cl_2N_7O_{18}$; calc. 1212.24).

Determination of Dimerisation Constants. Dimerisation constants were determined at 300 K and pD 7 in D_2O unless otherwise indicated, by either of two methods described previously [8]. For weakly and moderately dimerising antibiotics, their monomer and dimer species were in fast exchange on the NMR time-scale. The chemical shift of x_4 was followed at different antibiotic concentrations. From a plot of $\delta(x_4)$ *vs.* conc., the dimerisation constant could be calculated. For strongly dimerising antibiotics, monomer and dimer species were in slow exchange on the NMR time-scale. At sufficiently low conc. of the antibiotics, both the monomer and dimer states were populated. Integration of x_4 signals of monomer and dimer yielded the dimerisation constants. The x_4 resonance of each antibiotic was assigned through its NOESY cross-peak to the w_5 resonance and its TOCSY cross-peak to the w_4 resonance in H_2O/D_2O 9:1. For the cases of strongly dimerising antibiotics, the x_4 resonance of the monomer was recognised by the transfer of saturation experiment to irradiate the x_4 resonance of the dimer. Dimerisation constants of acetate-bound antibiotics were determined in the presence of 2M of (D_3)NaOAc. The 3-9-19 WATERGATE [40][41] pulse sequence (in H_2O/D_2O) or presaturation (in D_2O) was used to suppress the H_2O signal.

The *BBSRC* (*B. B.*), the *EPSRC* (*R. Z.*), *Sankyo* (*H. S.*), and the *British Council* (*K. L. T.*) are thanked for financial support. We thank Dr. *L. Vértesy*, *Aventis Pharma*, Germany for a generous gift of decaplanin, balhimycin, and ureido-balhimycin, and Dr. *G. Panzone*, *Biosearch Italia S.p.A.*, Italy for the donation of A-40926. We also thank Prof. *George M. Sheldrick*, University of Göttingen, Germany for providing data on the crystal structure of decaplanin prior to publication.

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Received March 1, 2003