The Role of the Sugar and Chlorine Substituents in the Dimerization of Vancomycin Antibiotics

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Abstract: Evidence is presented for the formation of dimers in aqueous solutions of the glycopeptide antibiotics eremomycin, A82846B, vancomycin, and eremomycin-Ψ. The dimerization constant K_{dim} is determined by ^1H NMR spectroscopy for the last two compounds and also for the related compound ristocetin-Ψ, for which dimerization has previously been reported in mixed solvents. Values of K_{dim} are obtained for these compounds over a range of temperatures, and thus ΔH_{dim} and ΔS_{dim} are calculated. In addition, a lower limit for K_{dim} in the case of eremomycin is calculated (10^5 M^{-1}). This is a remarkably large value, and it may be that dimerization is implicated in antibiotic action. The possibility that natural selection has led to adaptations which promote dimerization (such as the nature and sites of attachment of the sugars and a ring 2 chlorine atom) is discussed.

Introduction

The vancomycin group of antibiotics consists of a growing number of compounds which share a very similar heptapeptide backbone and vary mainly in the number, type, and placement of sugar substituents attached to the peptide nucleus.[1] The compounds in the group are clinically important as antibacterial agents which act against Gram-positive bacteria.[2] Vancomycin (Figure 1) itself is the drug of choice for the treatment of post-surgical infections, especially those caused by penicillin-resistant staphlococci.[3] Resistance to vancomycin has only recently been reported,[4] and the possible molecular basis of this resistance has been addressed.[5]

The molecular basis for the antibacterial activity of the group has been studied extensively[6-8] using vancomycin itself and ristocetin A (Figure 2), another well-characterized member of the group, and arises from the specific binding of the glycopeptide to bacterial cell wall precursors terminating in the sequence -D-Ala-D-Ala. Mode-of-action studies have consequently been carried out using small peptides (2-5 residues) containing this or a related sequence, and in a number of cases the antibiotics are found to bind almost as tightly to such peptides as to the native bacterial cell wall.[7]

Recently, self-association was observed for the complexes formed both between ristocetin A and the tripeptide di-*N*-acetyl-L-Lys-D-Ala-D-Ala (DALAA) and between ristocetin pseudoaglycon (ristocetin-Ψ, Figure 2) and DALAA.[9] In both cases, a dimer is formed in which a number of hydrogen bonds are made between the nonbinding faces of two glycopeptide molecules (Figure 3). The dimerization constant K_{dim} was estimated for the ristocetin-DALAA dimer to be ca. 2000 M^{-1} (298 K, D_2O-CD_3CN solution). This dimerization was seen to occur in water, water-acetonitrile mixtures, and DMSO solution, and interestingly, two sets of dimer resonances were observed in the proton NMR spectra of the ristocetin-DALAA dimer, which could be rationalized in terms of either an asymmetric dimer or, perhaps, two interconverting symmetric dimers. Some evidence of aggregation had also been previously noted in aqueous solutions of vancomycin,[7] and dimerization may explain the observation made by Heald and co-workers of more than one conformer of the related compound teicoplanin A_2 by ^1H NMR spectroscopy.[10]

It was postulated that this dimer may have a role in the in vivo action of ristocetin,[9] and it was thus decided to investigate the dimerization phenomenon more thoroughly. In particular, it was thought that the sugars of these antibiotics, which may function by providing selectivity in binding cell wall precursors terminating in -D-Ala-D-Ala,[11] may also be involved in the stabilization of the dimer. The studies were initially carried out using the recently isolated member of the vancomycin group eremomycin (Figure 1),[12-14] which shares some of the fine structural features characteristic of both vancomycin and ristocetin and was shown to form a particularly stable dimer.[15] Subsequently, eremomycin-Ψ (Figure 1), ristocetin-Ψ, and vancomycin were investigated (analogous studies using the aglycon of vancomycin were not possible due to solubility problems), and we have obtained quantitative evidence that the attached sugars stabilize these glycopeptide dimers significantly.

Materials and Methods

NMR spectra were recorded on Bruker AM400 and AM500 spectrometers equipped with Aspect 3000 computers. Normal one-dimensional spectra were recorded with either 8K or 16K data points. Chemical shifts were measured with respect to internal dioxan. For variable temperature NMR work, the temperature of the probe was calibrated using samples of either methanol or ethylene glycol, according to the method of van Geet.[16] This was done at the time of the experiment for every temperature at which spectra were run and for individual probeheads. Two-dimensional spectra (DQFCOSY, ROESY, and NOESY) were typically recorded in the phase-sensitive mode using time-propor-

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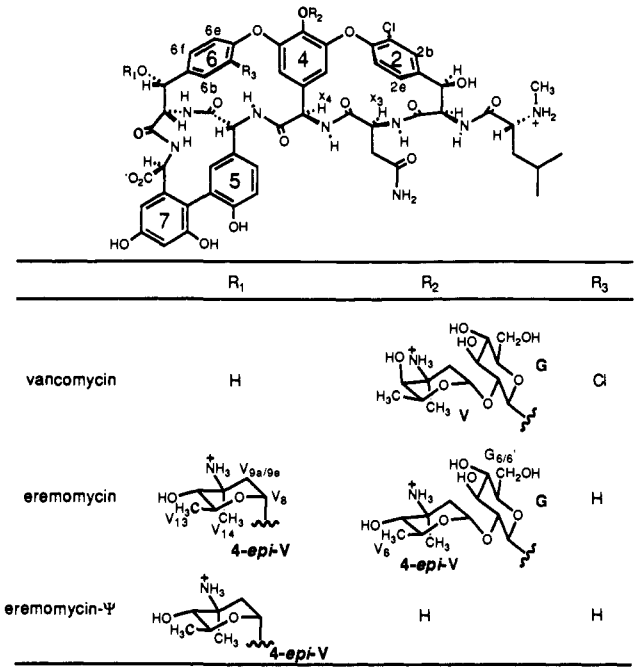

Figure 1. Structures of vancomycin, eremomycin, and eremomycin-Ψ (V = vancosamine; 4-*epi*-V = 4-*epi*-vancosamine; G = glucose).

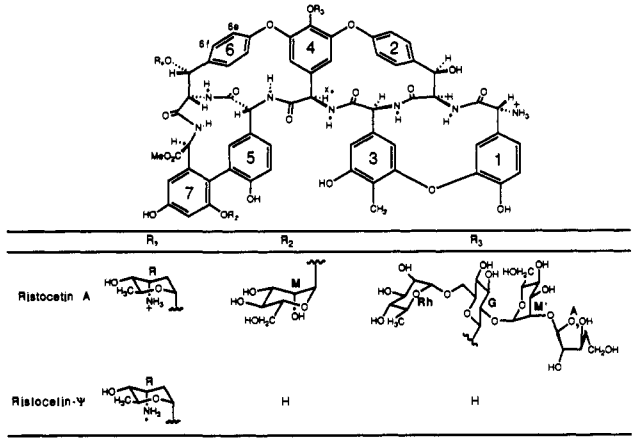

Figure 2. Structures of ristocetin A and ristocetin-Ψ (G = glucose; M = M′ = mannose; Rh = rhamnose; A = arabinose; R = ristosamine).

tional phase incrementation (TPPI)[17] to give quadrature detection in f_1. Routinely, 2K data points were recorded in f_2 and 512 in f_1, with either 16 or 32 transients collected at each value of t_1. NOESY spectra were recorded with mixing times of 50, 150, 250, and 400 ms, and a z-filter was employed to suppress zero quantum artefacts. The ROESY spectrum of eremomycin (6 mM in D_2O) was recorded using a 2-kHz spin-locking field applied for 300 ms.

Eremomycin and vancomycin were donated by SmithKline Beecham and Eli Lilly, respectively, as their hydrochloride salts. Ristocetin A was donated by both Abbott Laboratories and Lundbeck as its sulfate salt. Eremomycin-Ψ and A82846B were donated by SmithKline Beecham and Eli Lilly, respectively, as their acetate salts. These compounds were all used without further purification.

Glycopeptides and their derivatives were typically prepared for NMR by lyophilization twice from D_2O, followed by dissolution in a pH 7.0 buffer containing KD_2PO_4 (0.05 M) and NaOD (0.029 M). The deuterated buffer was prepared by successive dissolution and lyophilization of KD_2PO_4 with D_2O, addition of a 30% (w/w) solution of NaOD in D_2O, and dilution in D_2O. The pH was measured with a Corning pH meter 125 equipped with a combination glass electrode, and the values quoted are the meter readings with no correction made for the primary isotope effect.

Binding constants were obtained where indicated by monitoring the change in the chemical shift of the proton x_4 (Figures 1 and 2) over a range of concentrations, and then using a Simplex least-squares curve-fitting program.[18] Values for ΔH_{dim} and ΔS_{dim} were calculated by determining K_{dim} over a range of temperatures and then constructing van't Hoff plots.

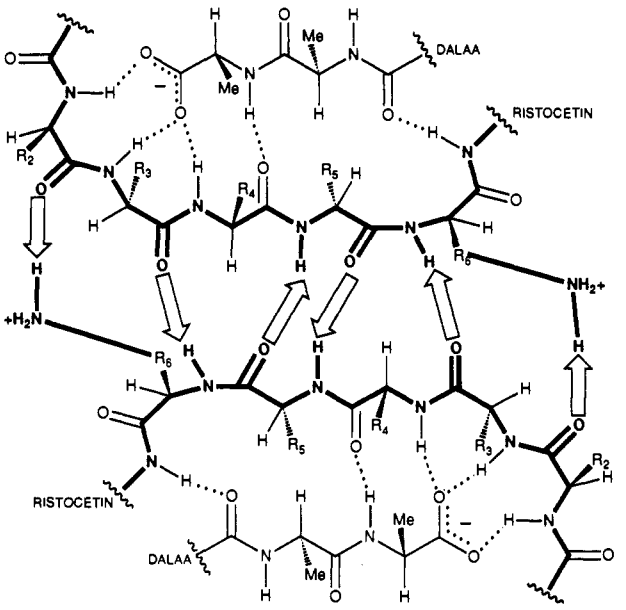

Figure 3. Hydrogen-bonding network of the dimer formed between two molecules of ristocetin when bound to DALAA. Arrows represent hydrogen bonds formed between the two ristocetin molecules, and broken lines indicate hydrogen bonds made to the ligand.

Preparation of Ristocetin-Ψ. In a typical experiment, a solution of ristocetin (1.3 g, 0.6 mM) in hydrogen chloride in methanol (5% w/w, 65 mL) was heated under reflux for 1 h. The methanolysate was evaporated to dryness under reduced pressure, and the residue was dissolved in water (45 mL). Dropwise addition of potassium carbonate (1 M) to pH ≈ 7.5 precipitated crude ristocetin-Ψ (460 mg), which was isolated by centrifugation, followed by drying under vacuum. Gel filtration (Sephadex G-25-50, 1 cm × 60 cm, 27 mL h^{-1}) using acetic acid (6% v/v) as eluent afforded pure ristocetin-Ψ (43%) as a white powder.

Results and Discussion

Initially, the ^1H NMR spectrum of eremomycin (6 mM) alone was examined for the presence of a dimer analogous to that formed by the ristocetin complexes. The spectra were recorded in buffer as described in the experimental section at constant ionic strength (provided by the buffer) in order to simulate physiological conditions. The spectrum contained many more signals than eremomycin has nonequivalent protons, and a number of the signals appeared to exist in pairs exhibiting the same multiplicity, line shape, and intensity (Figure 4a). On warming, these pairs were seen to broaden and coalesce and eventually reappear as single signals, indicating the existence of some chemical exchange process which was moving from the slow-exchange to the fast-exchange regime (Figure 4b–e). In addition, these pairs of signals exhibited chemical exchange crosspeaks in the ROESY spectrum of eremomycin (Figure 5). Similar observations have been made for the aforementioned ristocetin dimers.[9] In all of these cases, the formation of a dimer is accompanied by dramatic chemical shift changes for a few protons. For example, in the ristocetin–DALAA dimer, the proton 6e (Figure 2) is shifted upfield by ca. 2.3 ppm relative to the monomer, as it lies directly over the aromatic ring 4 of the other half of the dimer, and thus experiences a strong ring current effect. A similar but smaller effect ($\Delta\delta$ ca. 0.7) is observed for the proton ortho to 6e (6f). The proton x_4 becomes flanked by two intermolecular hydrogen bonds in the dimer (Figure 3) and is shifted downfield by 0.8 ppm. Quantitative studies of dimerization are made most conveniently by following the chemical shift change of this latter proton, as it is largely found in a region

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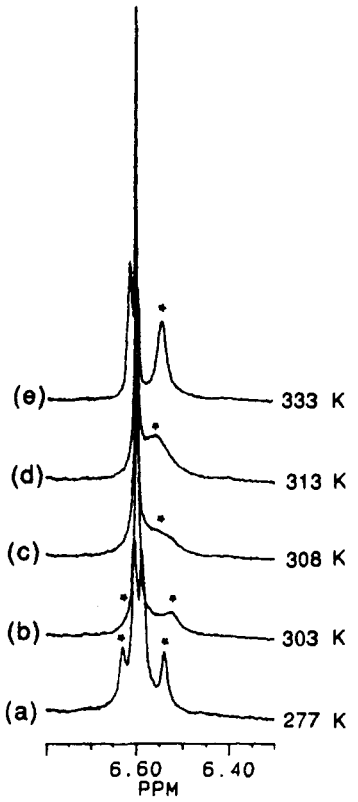

Figure 4. Variable temperature ^{1}H NMR spectra of eremomycin in the region 6.3–6.8 ppm showing the behavior of the proton x_4 (marked with *): (a) the slow exchange regime, where x_4 exists in two distinct forms; (b–e) the coalescence of these two signals, which then appear as a single resonance at an averaged chemical shift as the system moves into fast exchange. The slight upfield shift of the time-averaged x_4 chemical shift at 333 K is due to a small increase in the population of the monomer ($\delta(x_4)_{monomer} \approx 5.6$ ppm in eremomycin-Ψ), which is in fast exchange with the dimer. The other two signals seen correspond to the protons 7d and 7f, one of which exhibits an analogous downfield shift at high temperature.

of the spectrum which is otherwise devoid of signals. In the case of the ristocetin–DALAA complex, three resonances are observed for x_4: one at 5.73 ppm (monomeric ristocetin–DALAA complex) and two others farther downfield at 6.52 and 6.55 ppm, corresponding to the two distinct forms of the dimeric complex. Eremomycin displays only two broad signals for this proton, found at 6.52 and 6.61 ppm. No third signal (or chemical exchange crosspeak corresponding to such a signal) was observed when both NOESY and ROESY spectra of eremomycin were examined.

These and similar observations, together with the existence of a number of intermolecular NOEs in the NOESY spectrum of eremomycin which were analogous to those detected for the ristocetin dimers, strongly suggest that eremomycin exists solely (>90%) as a dimer at millimolar concentrations. It is worth noting that for most of the protons which both display two signals and give rise to intermolecular NOEs (viz. G_6, $G_{6'}$, 6f, and V_8), both of the observed signals give rise to the same NOEs. This supports the hypothesis that both sets of resonances correspond to dimeric species.

The absence of observable monomer signals translates to a dimerization constant in excess of 10^5 M^{-1}. The size of K_{dim} has thus far precluded a more accurate determination by NMR spectroscopy, and the absence of a discernible difference between the UV absorption profiles of monomer and dimer ruled out the use of UV difference spectroscopy. Currently, further NMR studies are in progress, and, in addition, both fluorescence spectroscopy and isothermal microtitration calorimetry are being investigated.

In view of the postulated influence of the sugars on dimerization, eremomycin-Ψ was investigated. This compound was found to form an analogous dimer with a dimerization constant of 1.5 × 10^4 M^{-1} at 298 K, *at least* an order of magnitude lower than the parent antibiotic. Figure 6 shows the plot of the chemical shift of x_4 versus antibiotic concentration used to determine K_{dim}, and

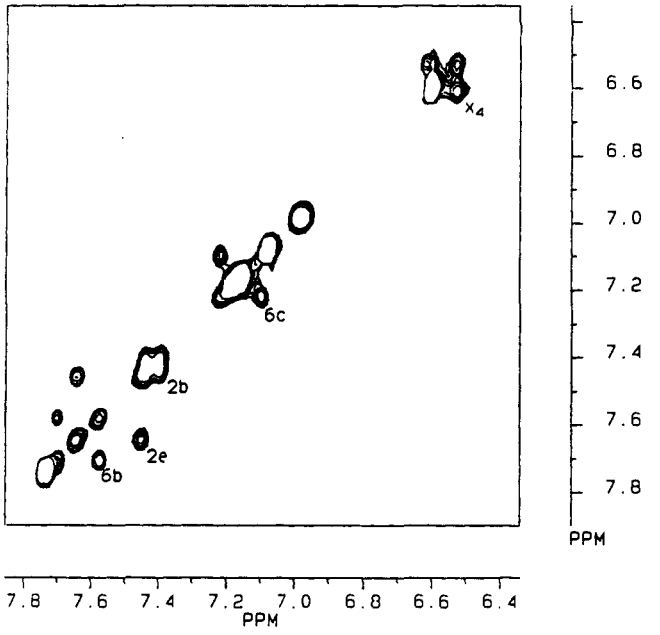

Figure 5. Portion of the ROESY spectrum of eremomycin illustrating the chemical exchange crosspeaks observed between exchanging pairs of signals. These crosspeaks can be distinguished from NOEs by virtue of their opposite phase (NOEs exhibit negative phase with respect to a positive diagonal in a ROESY spectrum). The spectrum was recorded at 500 MHz and 275 K with a 300-ms spin-locking pulse.

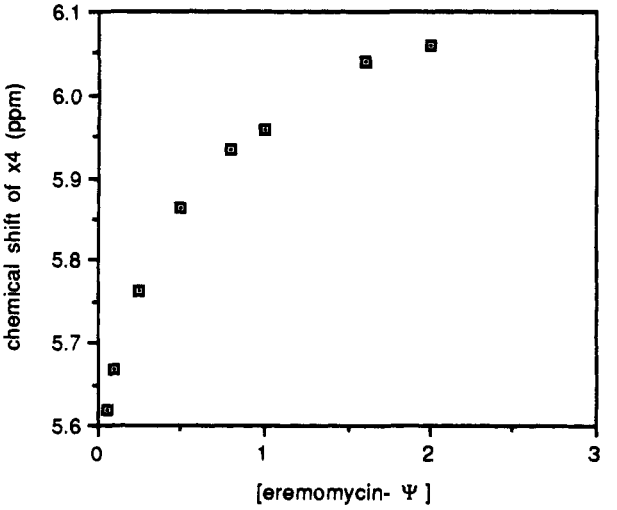

Figure 6. Plot of the chemical shift (δ, ppm) of the proton x_4 against the concentration of eremomycin-Ψ (mM) at 348 K.

Table I. Dimerization Constants for a Number of Different Glycopeptide Antibiotics and Their Derivatives[a]

compd	K_{dim} (M^{-1}, 298 K)	ΔH_{dim} (kJ mol^{-1})	$T\Delta S_{dim}$[b] (kJ mol^{-1} K^{-1})
eremomycin	≥100000[c]		
eremomycin-Ψ	15000	−51 ± 3	−27 ± 3
ristocetin	ca. 300[d]		
ristocetin-Ψ	50	−37 ± 6	−28 ± 7
vancomycin	700	−36 ± 2	−20 ± 5
A82846B	≥100000[c]		

[a] ΔH_{dim} and ΔS_{dim} values, where given, were calculated from van't Hoff plots of ln K_{dim} against $1/T$. [b] Calculated at 298 K. [c] Estimated from the absence of any signals assignable to monomer in the ^{1}H NMR spectra of the compound at ca. 1 mM. [d] Preliminary result obtained using isothermal microtitration calorimetry by successively diluting a solution of ristocetin A (Cooper, A., unpublished results).

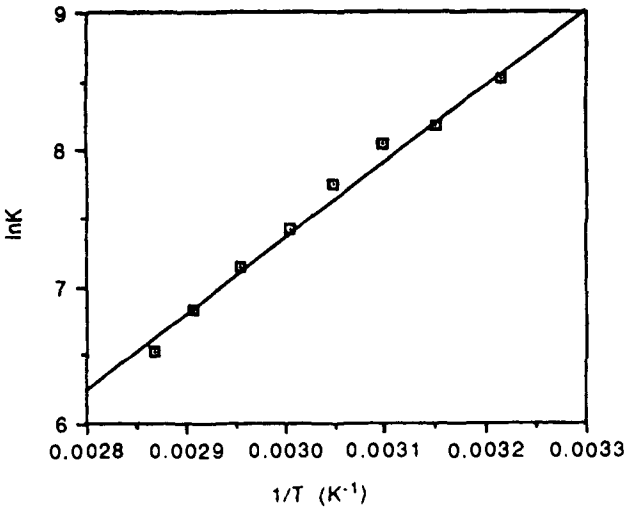

Figure 7. van't Hoff plot of ln K_{dim} against $1/T$ (K^{-1}) for eremomycin-Ψ. K_{dim} values were determined from the change in chemical shift of the proton x_4 over a range of temperatures.

the van't Hoff plot used to determine ΔH_{dim} and ΔS_{dim} is illustrated in Figure 7.

Similar studies were made of both ristocetin-Ψ and vancomycin, which yielded dimerization constants of 50 and 700 M^{-1}, respectively, at 298 K. These results are summarized in Table I. A lower limit for the dimerization constant of A82846B[19] (a compound which is identical to eremomycin except for the presence in the former of a ring 6 chlorine substituent analogous to that of vancomycin) is also given.

Implication of the Large ΔH for Dimerization. It is perhaps surprising to see that ΔG for dimerization contains a large negative enthalpy term. In previous work,[20] ΔG for the formation of a bimolecular complex has been divided into a number of favorable and unfavorable contributions as follows (assuming that the molecules are complementary in shape and that no significant strain is introduced in the formation of the complex):

$$\Delta G = \Delta G_{(trans+rot)} + \Delta G_{rotors} + \sum \Delta G_p + \Delta G_{vdW} + \Delta G_H \quad (1)$$

The first term is unfavorable for binding, arising from the loss of translational and rotational free energy which occurs when two molecules are brought together to make, in effect, one, and is composed largely of entropy. The second term, where present, is also unfavorable and largely entropy-based and is due to the restriction of free rotation about any σ-bonds on formation of the complex.

The third term, $\sum \Delta G_p$, refers to the sum of the intrinsic free energies associated with the specific electrostatic interactions formed between the two species, e.g., hydrogen bonds or salt bridges, and is clearly favorable for binding. Amide–amide hydrogen-bond formation may be associated with an exothermicity of ca. 4 kJ mol^{-1} in α-helix formation,[21] but in the formation of neutral–neutral hydrogen bonds in aqueous solution, exothermicities are generally believed to be small.[22] It should be noted, however, that little is known of the relative enthalpic and entropic contributions to a charge–dipole hydrogen bond such as the $NH_3^+ \cdots O{=}C$ bond proposed for both the ristocetin and eremomycin dimers (Figure 3), and these bonds may exhibit a somewhat larger exothermicity than neutral–neutral hydrogen bonds.

Electrostatic interactions leading to exothermicity may also arise due to orthogonal σ–π stacking (in particular where the C–H bonds of one aromatic ring are inserted into the π-electron cloud of another aromatic ring). Such stacking is apparent in molecular models of the dimers examined in this work and is evidenced by the chemical shift changes of the protons 6e and 6f discussed above.

Table II. Selected NOEs Observed in the NOESY Spectrum[a] of Eremomycin[b]

V_{13}–G_6	V_{13}–$G_{6'}$	6f–V_8	6f–V_{9e}
x_3–V_{9a}	x_3–V_{9e}	x_3–V_{14}	2e–V_6

[a] NOESY spectra were recorded at 500 MHz at both 275 K and 298 K (in order to observe resonances obscured by the residual HOD signal) with mixing times of 50, 150, 250, and 400 ms to account for the possibility of spin diffusion. [b] These NOEs are consistent with the ring 6 chlorine substituent lying in a pocket formed by the aromatic ring 4, the disaccharide, and the peptide backbone of one half of the dimer (the half carrying the chlorine in question), and the ring 6 amino-sugar and the aromatic ring 6 of the other half of the dimer. The atom codes used are illustrated in Figure 1).

ΔG_{vdW} corresponds to the formation of van der Waals interactions between nonpolar groups and is normally favorable in complexes exhibiting good complementarity. In such cases, this term comprises an exothermicity and only a small entropy contribution. Finally, ΔG_H, the third favorable term, is a measure of the hydrophobic surface area which is removed from solvent on formation of the complex and placed in a nonpolar environment in the complex. This term promotes binding in aqueous media and has been proposed to be mainly entropy-driven at room temperature as a result of the release of ordered water from a hydrophobic face to bulk water.[23-25]

Thus, the large exothermicity of dimerization may arise from a combination of factors. These include favorable van der Waals interactions and enthalpy terms associated both with the formation of the intermolecular hydrogen bonds (especially, perhaps, the protonated amine–amide hydrogen bonds) and with orthogonal σ–π interactions. There may be other contributions, and studies are in progress to characterize the various contributions to ΔH_{dim} more precisely.

Influence of the Sugars. In the case of both eremomycin and ristocetin, the removal of the sugar unit from residue 4 results in a substantial decrease in K_{dim}. Given that the presence of the sugars in the parent antibiotics may increase slightly the magnitudes of some terms opposing dimerization (i.e., $\Delta G_{(trans+rot)}$ and ΔG_{rotors}), this change in K_{dim} indicates that a number of favorable interactions must be formed between the two disaccharides and/or between the disaccharides and the peptide portions of the dimer; i.e., that the disaccharide in one half of the dimer exhibits complementarity to the other half of the dimer.

The role of the amino-sugars found on residue 6 of both eremomycin and ristocetin (4-*epi*-vancosamine and ristosamine, respectively) in stabilizing these dimers appears to be even more significant, as is evidenced by a consideration of the structural differences between eremomycin and vancomycin and their potential contributions to K_{dim}. These differences are as follows: (a) vancomycin possesses an additional chlorine substituent, found on ring 6, (b) the amino-sugars forming part of the ring 4 disaccharide in both compounds have opposite configurations at position 4, and (c) eremomycin alone has an amino-sugar in the β-position of residue 6. The presence in vancomycin of the ring 6 chlorine appears to have little or no negative effect on the dimerization constant, as judged from the temperature and concentration dependence of the ^1H NMR spectra of A82846B. A82846B exhibits the same behavior under such conditions as described for eremomycin above (Table I). The epimerization of the ring 4 amino-sugar may have some effect on dimerization, but inspection of molecular models reveals no obvious cause for such an effect. Therefore, it is likely that the bulk of the *at least* 100-fold difference between K_{dim} for vancomycin and eremomycin is due to interactions formed by the ring 6 amino-sugar of the

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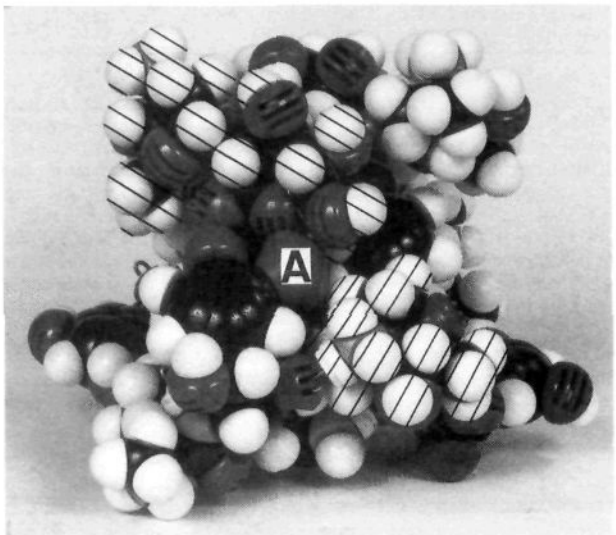

Figure 8. Complementarity of the two halves of the eremomycin dimer as shown by CPK models. Note the position of the ring 2 chlorine substituent (labeled A) in a cleft formed by a number of groups on both halves of the dimer. The protons of the disaccharide of one half of the dimer and of the monosaccharide of the other are indicated by different hatching schemes.

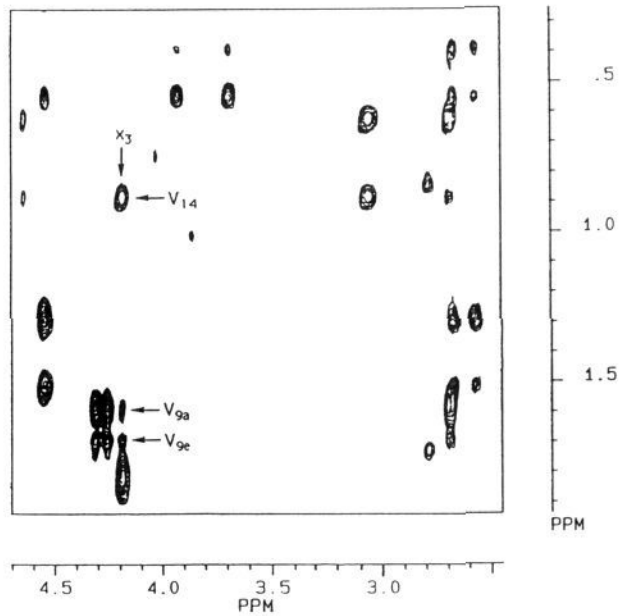

Figure 9. Portion of the NOESY spectrum of eremomycin (400 MHz, 298 K, 400-ms mixing time) illustrating three of the observed intermolecular NOEs which characterize the dimer.

Table III. Chemical Shifts of Selected Protons of Eremomycin

proton	δ (ppm)	proton	δ (ppm)
V_{13}	1.38	V_{9e}	2.47
G_6[a]	3.09/3.57	x_3	5.03
G_6'[a]	3.59/3.92	V_{14}	1.65
6f	6.86/6.90	2e	7.36/7.55
V_8	5.03/5.29	V_6	1.17/1.32
V_{9a}	2.37		

[a] These two protons cannot be distinguished from each other, and thus the chemical shifts given are interchangeable.

latter compound. This is in reasonable accord with the dimer structure postulated for ristocetin, which includes a hydrogen bond between the protonated amino group of the sugar and the carbonyl oxygen of residue 2 of the other half of the dimer.

Influence of the Ring 2 Chlorine. Inspection of a molecular model of the eremomycin dimer reveals a region of astonishing complementarity in which a small "gap", bordered partly by the ring 6 amino-sugar and the aromatic ring 6 of one half of the dimer and partly by the aromatic ring 4, the disaccharide unit, and the peptide backbone of the other half of the dimer, is closely filled by the ring 2 chlorine substituent of the latter molecule (marked A in Figure 8). This conformation is supported by a number of NOEs in the NOESY spectra of eremomycin (Table II). The chemical shifts of the protons involved in these NOEs are given in Table III, and a portion of the 400-ms NOESY of eremomycin is given in Figure 9, illustrating several of these NOEs. Furthermore, preliminary examination of the NMR spectra of dechloroeremomycin suggests that this compound dimerizes somewhat less strongly than eremomycin itself.[26]

In summary, the ring 2 chlorine atom may well promote dimerization, and it is noted subsequently that this atom appears also to promote in vitro antibiotic activity.

Eremomycin vs Ristocetin. The difference in K_{dim} between ristocetin and eremomycin and also between the pseudoaglycons of the two compounds is a factor of greater than 100 M^{-1}. Here, all four species possess a ring 6 amino-sugar, and the difference in K_{dim} may be the result of some interactions involving the residue 3 asparagine side chain of eremomycin (for which no specific function has previously been ascribed), the presence of a ring 2 chlorine atom in eremomycin (as described above), and/or the three additional sugars found in ristocetin. A more quantitative comparison between eremomycin and ristocetin cannot be made because of the differences between the two glycopeptides in backbone structure.

The Possible Biological Role of Dimerization. It appears from these studies and from earlier work done in this group[9] that many of the structural motifs observed in these glycopeptides may be quite sophisticated in promoting dimerization. For example, the possible roles of the deoxygenated amino-sugars common to many members of the group have not previously been considered, yet their biosynthesis must require the production of a number of enzymes and the expenditure of metabolic energy. Similarly demanding in terms of the length of the biosynthetic pathway is the extensive cross-linking of the antibiotics, which imparts to them a high degree of conformational restraint and favors dimerization (and binding to cell wall mucopeptide precursors) by reducing the number of rotors needing to be restricted on formation of the dimer. These features, which help to facilitate dimerization, would presumably not evolve unless they impart some selectional advantage to the organism possessing them.[27]

In this context, the results of a number of recent studies on the activity of glycopeptides and their derivatives lend support to the hypothesis that the efficacy of these antibiotics may be contributed to by the formation of such homodimers. For instance, it has been shown that although vancomycin (K_{dim} = 700 M^{-1}) binds peptide cell wall analogues somewhat more strongly than does eremomycin, eremomycin ($K_{dim} > 10^5$ M^{-1}) is actually more effective at inhibiting bacterial cell wall growth.[12] The removal of the disaccharide from vancomycin reduces its in vitro activity by a factor of 10, although its binding constant to DALAA is reduced only by a factor of 3.[8] A reliable value for the dimerization constant of vancomycin aglycon has not been obtained to date due to the compounds very low affinity for dimerization, but it is certainly a factor of at least 10 lower than vancomycin itself.[28] A similar but smaller effect is observed for the partially deglycosylated derivative of vancomycin, desvancosaminylvancomycin (R_2 = -β-D-glucosyl in Figure 1).[8] Thus, a reduction in K_{dim} could account, at least partially, for the reduced in vitro activity of aglucovancomycin. Furthermore, experiments conducted on the in vitro activities of partially deglycosylated derivatives of A82846B compared to vancomycin have demonstrated

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that the ring 6 amino sugar of A82846B (the same sugar that promotes dimerization in eremomycin) increases antibiotic activity by 2–10-fold.[29]

The in vitro activities of glycopeptides differing only in their patterns of chlorination have also been examined. The activity of vancomycin falls by 30% when its ring 2 chlorine substituent is removed reductively.[30] MM47761, a compound identical to eremomycin except that the ring 4 amino sugar of eremomycin is replaced by rhamnose, is consistently more active by a factor of 2–4 than its 2-dechloro analogue, MM49721.[31] Furthermore, eremomycin and A82846B have been shown to be 10-fold more active in vitro than A82846C and orienticin A, their respective 2-dechloro derivatives.[32] These results are consistent with the putative involvement of glycopeptide dimers in antibiotic activity, given the probable role of this chlorine atom in stabilizing such dimers.

These results all suggest that the mechanism of action of vancomycin antibiotics involves more than simply the binding of the monomeric antibiotic to a peptide receptor, but it should be noted that dimerization is undoubtedly only one of a number of factors which may combine to give rise to the observed activity of a glycopeptide. Consequently, direct correlations between antibiotic activity and dimerization potential are not generally straightforward, since even small structural changes can affect more than one of these factors simultaneously.

Thus, the adaptations promoting the formation of these dimers point toward their possible involvement in the mechanism of action of the antibiotics. Although no such role has been demonstrated experimentally, there are a number of sensible possibilities. For example, it may be that a dimer binds to cell wall precursors in the growing bacterial cell wall and that the association of one half of the dimer to nascent cell wall promotes the binding of the other half to another site, presuming such precursors are concentrated locally.[9] The latter peptide binding would, in effect, be intramolecular, resulting in a stronger effective association as a relatively small or negligible $\Delta G_{(trans+rot)}$ contributes to the overall ΔG for binding. In this connection, three further points are of interest. First, since the dimer has a pseudo-two-fold axis, it is possible that the growing cell wall structure also possesses a two-fold axis; such an axis is not evident in currently accepted structures for completed cell walls[33] but could also conceivably arise between *successive* layers of peptidoglycan in the multilayered cell wall structure. Second, it is striking that cell wall fragments can be induced to aggregate even by the weakly dimerizing vancomycin.[34] This behavior could be rationalized by an antibiotic dimer, able to provide two binding pockets within one structure, associating with cell wall fragments containing two or more peptide side chains to which the antibiotics can bind. Third, the antibacterial activity of eremomycin on agar plates has been shown to be *increased* by the presence of the cell wall analogue DALAA,[12] where a decrease would be expected for a simple monomeric antibiotic–substrate interaction. Such a decrease is indeed, observed for vancomycin under identical conditions.[12] This observation may be rationalized by the cooperative binding of a single molecule of DALAA to one half of an eremomycin dimer, which then binds the growing bacterial cell wall with increased efficiency. The activity of the antibiotic would be predicted to plateau and then decrease as the DALAA concentration is further increased, as both binding sites in the dimer become occupied by DALAA, and this is indeed observed.[12] The behavior exhibited by vancomycin is consistent with its comparatively low dimerization constant.

Alternatively, it is thought that the inhibition mechanism may have a steric component. Cross-linking of the growing cell wall occurs both at the D-Ala-D-Ala-terminating peptide chain and at the disaccharide unit to which the peptide is covalently attached. Thus, inhibition of cell wall synthesis by glycopeptides may be at least partially due to the steric hindrance of carbohydrate linking enzymes by the antibiotic which is bound to the peptide nearby.[35] A dimer could well achieve this more effectively than a single molecule.

A third hypothesis involves the putative hydrogen bond formed between the charged amino group of the residue 6 sugars found in both ristocetin and eremomycin and $(C{=}O)_2$ of the other half of the dimer. This hydrogen bond may polarize the residue 2 amide group in the latter molecule such that it forms a stronger hydrogen bond with the carboxyl group of the cell wall precursor (Figure 3). Such a configuration may be viewed as a $NH_3^+{-}^-O_2C$ salt bridge mediated through an amide functionality. This interaction implies that cell wall analogues terminating in D-Ala-D-Ala may promote antibiotic dimerization—a possibility which is currently under investigation. Such an explanation, however, cannot be applied to vancomycin, which lacks the residue 6 amino-sugar.

Conclusion

It has been demonstrated that vancomycin, eremomycin, A82846B, ristocetin-Ψ, and eremomycin-Ψ all form dimers in aqueous solution in the absence of any peptide ligand. These dimers have a structure analogous to that previously put forward for complexes of both ristocetin and ristocetin-Ψ with the tripeptide cell wall mimic, DALAA.

The values for K_{dim} vary considerably, and a number of intermolecular interactions have been postulated, both here and in prior work by this group, to account for these differences. It appears that the various sugar functionalities found on these antibiotics make important contributions toward dimerization, in addition to their previously assigned roles of increasing both the aqueous solubility of the antibiotics and the selectivity of their binding to cell wall analogues terminating in -D-Ala-D-Ala.

In addition, the ring 2 chlorine substituent found on some of these antibiotics may act to increase the stability of such dimers by forming a hydrophobic interaction with the other half of the dimer.

The process of dimerization displays a large exothermicity (ranging from −36 to −51 kJ mol^{-1} for the antibiotics investigated), which may be partially accounted for by the formation of favorable van der Waals interactions and π–σ interactions between aromatic rings of the glycopeptides. The remainder of this enthalpy may arise from a number of different factors, which are currently under investigation. Dimerization may play an active part in the biological action of these glycopeptide antibiotics, and a number of studies are currently under way with the aim of pinpointing this possible role.

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