Dissection of the Contributions toward Dimerization of Glycopeptide Antibiotics

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Abstract: A procedure for the determination of association constants in aqueous solution using hydrogen–deuterium exchange has been developed and used to measure the dimerization constant, K_{dim}, for a number of strongly dimerizing glycopeptide antibiotics. These values provide further insight into the thermodynamic contributions of various structural epitopes to the dimerization of these antibiotics. Consideration of ligand binding affinities together with dimerization potentials provides evidence that dimerization is implicated in the physiological mode of action of these antibiotics.

Introduction

The vancomycin group of antibiotics includes several members which are clinically important in the treatment of post-surgical staphylococcal infections.[1] The most important of these is vancomycin itself (Figure 1), which has found clinical use for 35 years[2] and to which bacterial resistance has only recently been reported.[3-6] Members of this group share similar heptapeptide backbones which usually carry one or more sugar substituents of various types.[7] Despite this nascent resistance, vancomycin is still the main line of defence against (otherwise frequently lethal) attacks of methicillin resistant *Staphylococcus aureus* (MRSA).

The molecular basis for the antibacterial activity of the group has been studied extensively[8-10] and arises from specific binding of the glycopeptide to nascent bacterial cell wall bearing the C-terminal sequence -D-Ala-D-Ala. Synthetic oligopeptides (two to five residues in length) have been used to probe the binding site of these antibiotics and have been shown to exhibit binding constants to the glycopeptides of up to 10^6 M^{-1}.[9] However, although the affinity of these antibiotics for bacterial cell wall analogues does correlate directly with *in vitro* antibiotic activity in some cases,[10] it has recently been shown that this is not always the case.[11] Eremomycin (Figure 1), a compound with a structure very similar to that of vancomycin, is consistently more active than vancomycin against a range of staphylococcal strains, despite the former having a lower affinity for the cell wall analogue di-*N*-acetyl-L-Lys-D-Ala-D-Ala (DALAA).

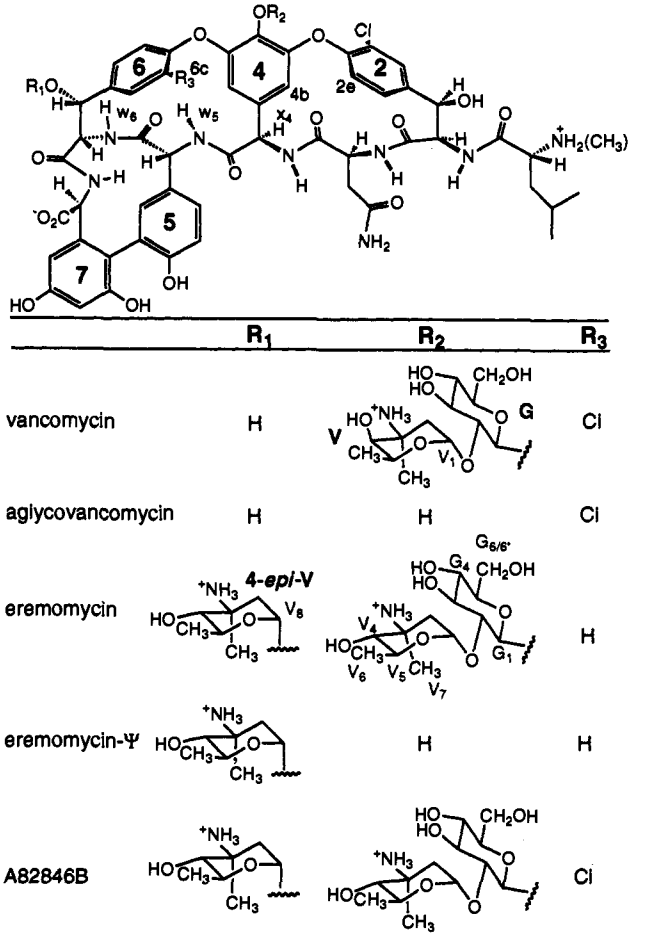

Figure 1. The structures of vancomycin, aglycovancomycin, eremomycin, eremomycin-Ψ, and A82846B (V = vancosamine; 4-*epi*-V = 4-*epi*-vancosamine; G = glucose). Protons which are referred to in the text are labeled.

Recently, self-association in aqueous solution was observed for the complexes formed both between ristocetin A (Figure 2) and DALAA and between ristocetin pseudoaglycone (ristocetin-Ψ, Figure 2) and DALAA.[12] In both cases, a dimer is formed in which a number of hydrogen bonds are proposed to be made between the nonbinding faces of two glycopeptide molecules

* Abstract published in *Advance ACS Abstracts,* April 15, 1994.

(1) Recent papers on clinical uses of vancomycin include the following: (a) McHenry, M. C.; Gavan, T. L. *Pediatr. Clin. North Am.* **1982**, *66*, 175. (b) Geraci, J. E.; Herman, P. E. *Mayo Clin. Proc.* **1983**, *58*, 88. (c) Symposium on Vancomycin Therapy. In *Journal of Antimicrobial Chemotherapy*; Wise, R., Reeves, D., Eds.; 1984; Vol. 14, Suppl. D.

(2) See, for example: Foldes, M.; Munro, R.; Sorrell, T. C.; Shankar, S.; Toohey, M. *J. Antibiot. Agents Chemother.* **1983**, *11*, 21.

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(7) For examples of the range of antibiotics in this class, see: (a) Kalman, J. R.; Williams, D. H. *J. Am. Chem. Soc.* **1976**, *99*, 2768. (b) Spiri-Nakagawa, P.; Tanaka, Y.; Oiwa, R.; Tanaka, H.; Omura, S. *J. Antibiot.* **1979**, *32*, 995. (c) Hunt, A. H.; Vernon, P. D. *J. Antibiot.* **1981**, *34*, 469. (d) Bardone, M. R.; Paternoster, M.; Coronelli, L. *J. Antibiot.* **1978**, *31*, 170.

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(12) Waltho, J. P.; Williams, D. H. *J. Am. Chem. Soc.* **1989**, *111*, 2475.

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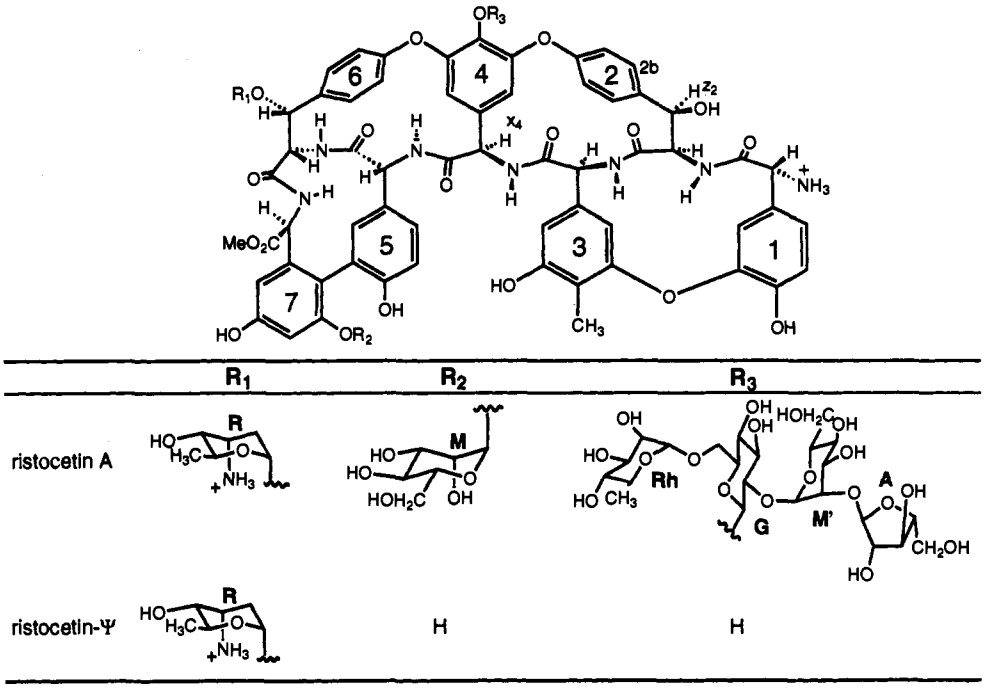

Figure 2. The structures of ristocetin A and ristocetin-Ψ (G = glucose; M = M′ = mannose; Rh = rhamnose; A = arabinose; R = ristosamine).

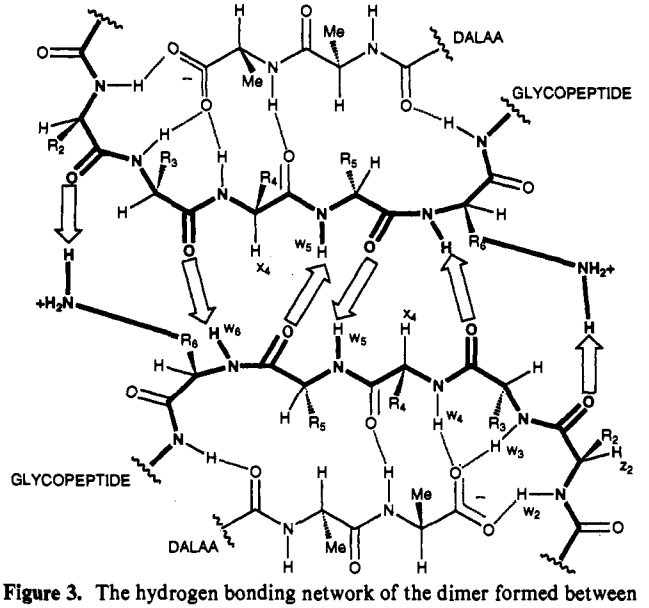

Figure 3. The hydrogen bonding network of the dimer formed between two molecules of ristocetin A when bound to DALAA. The arrows represent hydrogen bonds formed between the two ristocetin A molecules and the broken lines indicate hydrogen bonds made to the ligand. R_2–R_6 represent the side chains of residues 2–6, respectively.

(Figure 3). Other members of the vancomycin group have since been shown to form analogous dimers with values of K_{dim} ranging between 10 M^{-1} and greater than 10^5 M^{-1}, and a preliminary study of the roles of a number of structural epitopes in promoting dimerization has been carried out.[13] At the outset of the present work, it was intended to expand on the earlier investigation by first measuring the dimerization constant for a number of strongly dimerizing antibiotics such as eremomycin (which cannot be measured using ^1H NMR chemical shift titrations, since at the concentrations used for NMR experiments, monomer is not significantly populated), and then to draw comparisons with related compounds. In the following paper in this issue, the possible interplay between ligand binding and glycopeptide dimerization is examined, and conclusions are drawn about the mode of action of these antibiotics.[14]

Materials and Methods

Eremomycin and vancomycin were donated by SmithKline Beecham and Eli Lilly respectively as their hydrochloride salts. 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. Vancomycin hexapeptide was prepared using a previously published procedure.[15] Dechloroeremomycin was prepared using the method described previously for the preparation of monodechlorovancomycin.[16] Eremomycin (100 mg) afforded 62 mg of dechloroeremomycin (93% pure by analytical reverse phase HPLC).

NMR Spectroscopy. Glycopeptides, their derivatives, and ligands were typically prepared for NMR by lyophilization twice from D_2O, followed by dissolution in a pD 7.0 buffer containing KD_2PO_4 (0.05 M) and NaOD (0.029 M). Samples at pD 3.7 for hydrogen–deuterium exchange measurements were prepared by adjusting the pD of a solution of KD_2PO_4 (50 mM) to *ca.* pD 3 using solutions of NaOD and DCl. Following addition of the glycopeptide, the pD was adjusted to pD 3.7. All pH and pD sample readings were measured with a Corning pH meter 125 equipped with a combination glass electrode. The pD readings quoted throughout are pH meter readings, and no corrections have been made for isotope effects or for the variation of pH with temperature.

NMR spectra were recorded using Bruker AM 400, AM 500, and AMX 500 spectrometers. One-dimensional spectra were recorded with either 8K or 16K data points. Chemical shifts were measured with respect to internal dioxan (δ = 3.74 ppm). For variable-temperature NMR work, probe temperatures were calibrated using samples of either methanol or ethylene glycol, using the method of van Geet.[17]

Two-dimensional spectra (DQFCOSY and NOESY) were recorded in phase-sensitive mode using time proportional phase incrementation (TPPI)[18] to give quadrature detection in f_1. Routinely, 2048 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 between 50 and 400 ms, and a z-filter was employed to suppress zero

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quantum artefacts. Suppression of the H_2O or HOD resonance was routinely achieved by irradiation during the recycle delay and during the mixing time of NOESY spectra.

Dimerization constants were measured where indicated by monitoring the change in the chemical shift of the proton x_4 (see Figures 1–3) over a range of concentrations, then using a simplex least-squares curve-fitting program.[19] 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.

In spectra recorded for the observation of hydrogen–deuterium exchange of the amide protons, the residual HOD signal was suppressed using a Jump and Return selective excitation sequence[20] to ensure no attenuation of the amide proton resonances took place through transfer of magnetization from the water signal. Samples were equilibrated for *ca.* 10 min at the temperature of the probe before the first acquisition of the experiment. Typically, spectra were acquired with spectral widths of 10 ppm and 16K data points. Spectra were recorded in 1 min (24 transients). The delay between successive transients was 1.5 s and that between successive experiments was 5–120 min. Data sets were exponentially multiplied by a broadening function of 2 Hz prior to Fourier transformation.

The hydrogen–deuterium amide proton exchange rates were determined by monitoring the decrease in intensity of the NMR signal with time for individual hydrogens following dissolution of the antibiotic in D_2O containing KD_2PO_4 (50 mM). In each case, the entire experiment took place in the NMR spectrometer probe, and typically 25–40 spectra were collected in sequence at suitable intervals such that, where possible, spectra were recorded over at least 3 half-lives of decay. The loss in amide proton intensity during the acquisition of the spectra was assumed to be linear as the time taken to record each spectrum was considerably shorter than the half-life for amide proton exchange. The intensities of the resonances, measured as peak heights from the spectra relative to a nonexchangeable signal, were plotted *vs* time, and exponential functions were fitted to the resulting data sets. The time increments between successive data points were taken as the interval between the middle of acquisition of each spectrum.

UV Spectrophotometry. The determination of binding constants between glycopeptides and DALAA by UV spectrophotometry was carried out on a UVIKON 940 dual beam spectrophotometer. Both antibiotic and DALAA solutions were buffered with KH_2PO_4 (0.05 M)/NaOH (0.029 M), pH 7. The initial concentration of glycopeptide solutions was 0.05–0.13 mM, and the ligand solution was 8.0–30.0 mM. The λ_{max} for each glycopeptide and its complex with DALAA was determined from a four cell tandem arrangement in which the two cells in the reference beam contained the antibiotic and ligand solutions, respectively, while the two cells in the sample beam contained a mixture of these two solutions and buffer, respectively. Antibiotic concentrations in different cells were the same, as were ligand concentrations. Typically the λ_{max} to be monitored fell in the region 280–294 nm. In the binding titration, antibiotic solution was in the sample beam and buffer in the reference beam; ligand was added in aliquots to the antibiotic solution only. The solution was stirred after each addition, and the absorbance at both 281 and 293 nm was measured repeatedly until stable. The data at 281 nm were subtracted from those at 293 nm, and the resulting dataset was analyzed using a simplex nonlinear least-squares curve-fitting program[19] which fits the data to a simple 1:1 binding isotherm.

Results and Discussion

Determination of K_{dim} Values Using Hydrogen–Deuterium Exchange Rates. Recently, dimerization constants have been determined for a number of vancomycin antibiotics and for derivatives lacking some or all of the pendant sugar substituents commonly found in this class of compounds.[13] These equilibrium constants were measured either by isothermal microtitration calorimetry or by monitoring the change in chemical shift with antibiotic concentration of a proton at the dimerization interface. Both of these techniques require that a range of solutions of the antibiotic can be prepared with different proportions of dimer present. Consequently, neither of these techniques are suitable for the measurement of very large dimerization constants where extremely low antibiotic concentrations are necessary for significant population of the monomer. The dimerization constant of eremomycin was estimated to be $>10^5$ M^{-1} from the lack of evidence of any monomeric antibiotic by ^1H NMR at *ca.* 1 mM, and until now a more accurate measurement has been precluded on the above grounds.

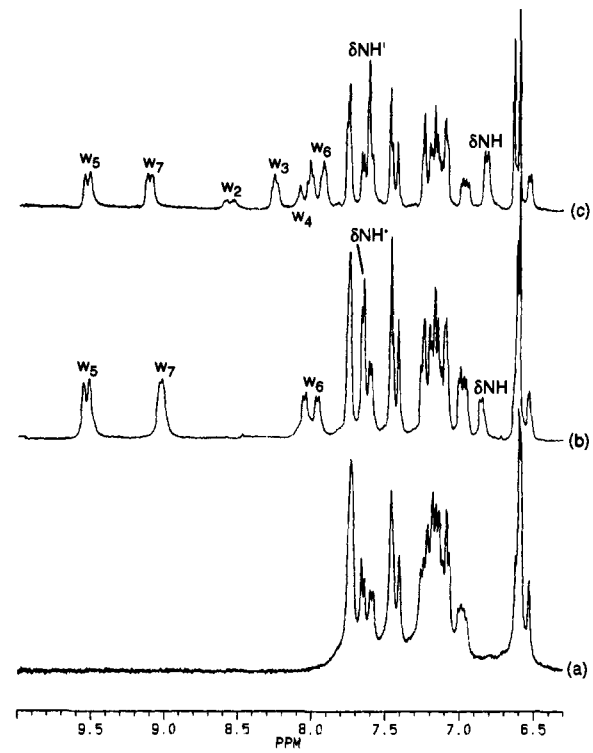

Figure 4. The aromatic and amide proton region of the ^1H NMR spectrum of eremomycin (a) in D_2O, pD 7, 50 mM KD_2PO_4; (b) in H_2O/D_2O (9/1), pH 7, 50 mM KH_2PO_4; and (c) in H_2O/D_2O (9/1), pH 4, 50 mM KH_2PO_4. All spectra were recorded at 285 K using a Jump and Return sequence to suppress the water signal.

It has previously been noted that the proton w_5 of eremomycin has, at very acidic pH, a half-life for deuteration of 24 h or more,[21] and this was interpreted as a consequence of its involvement in an intermolecular hydrogen bond in the dimer. It was therefore proposed that quantitative hydrogen–deuterium exchange data may allow calculation of the dimerization constant of eremomycin. Initially, the ^1H NMR spectrum of eremomycin in H_2O/D_2O (9/1) buffered at pH 7 was recorded (Figure 4b). A number of resonances not seen in the corresponding spectrum recorded in D_2O (Figure 4a) are apparent. Each of the three most downfield resonances is split into two signals corresponding to the two forms of the glycopeptide dimer, and these were assigned using a standard combination of NOESY and DQFCOSY experiments. The geminal amide protons of the residue three asparagine side chain were also observed, and the assignments of all these protons are given on the spectrum. It was notable that signals due to the protons w_2, w_3, and w_4 were absent, presumably because they were in fast exchange with the water signal. A spectrum was also recorded at pH 4 (Figure 4c), where exchange with water is significantly slower; w_2, w_3, and w_4 could then be observed and were assigned using NOESY and DQFCOSY data (Figure 5). In addition, a signal was observed at 8.05 ppm which, from NOESY data, was assigned as the primary amino group of the ring 6 amino sugar. This proton exhibits intermolecular NOEs to z_2 and 2b (Figure 1), providing further evidence for the proposed structure of the dimer.

A sample of eremomycin in D_2O (7.5 mM) containing 50 mM KD_2PO_4 was adjusted to pD 3.7, and a series of 30 spectra were recorded over a 24-h period (Figure 6a). Plots of signal intensity

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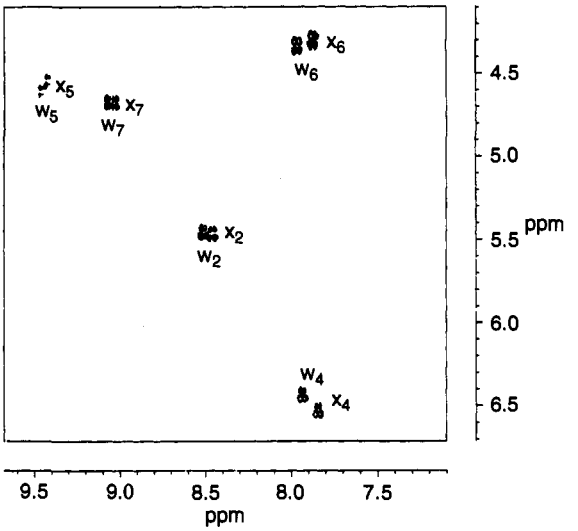

Figure 5. Portion of a DQFCOSY spectrum of eremomycin, showing the NH-C$^{\alpha}$H region used in the assignment of the amide NH protons. A w_3–x_3 correlation is not observed since x_3 lies underneath the water signal—w_3 was assigned from NOESY data. The spectrum was recorded at 280 K in H_2O/D_2O (9:1) containing KH_2PO_4 (50 mM), pH 4, on a Bruker AM 500 spectrometer.

versus elapsed time for protons w_5 (see Figure 6b) and w_6 yielded pseudo-first-order rate constants (k_{obs}) of 4.8×10^{-5} and 1.6×10^{-5} s^{-1}, respectively. The exchange rates of w_5 and w_6 are both significantly reduced compared to the rates expected for solvent-exposed amide protons (see below). This is a consequence of their involvement in intermolecular hydrogen bonds in the antibiotic dimer (Figure 3). Clearly w_5 is the more suitable of the two protons for quantitative studies as it is completely resolved in the ^{1}H NMR spectrum.

The next question to be addressed was that of the mechanism of exchange—is dissociation of the dimer a prerequisite for exchange of w_5 with solvent or is a breathing mechanism possible, whereby the hydrogen bond involving w_5 is broken and the proton is available for exchange without complete dissociation of the dimer? The latter mechanism was rejected on the grounds that the observed pseudo-first-order rate constant for exchange is seen to decrease with increasing antibiotic concentration (see below). This observation implicates the dimerization process in the exchange mechanism. In addition, the rigidity of the peptide backbones comprising the dimer makes such a local breathing event unlikely; such fluctuations in protein secondary structure typically involve the disruption of structure of approximately eight or more residues,[22] and the antibiotics are significantly less flexible than a linear polypeptide backbone. Thus the exchange mechanism is represented as follows:

$$(\text{A-H})_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{A-H} \xrightarrow[D_2O]{k_3} \text{A-D}$$

where A-H represents the antibiotic monomer carrying a proton at position w_5, $(A-H)_2$ is the corresponding dimer, A-D is the deuterium-exchanged monomer, k_1 and k_2 are the rate constants for dissociation and association of the dimer, respectively, and k_3 is the intrinsic exchange rate of solvent-exposed w_5 with water. Two possible kinetic limiting cases can then be envisaged, depending on the relative values of k_2 and k_3: (1) $k_3 \gg k_2$. The observed exchange rate is given by

$$k_{obs} = k_1 \tag{1}$$

In this case, an exchange event occurs every time the dimer dissociates, and k_{obs} depends only on the off-rate for the dimer. (2) $k_2 \gg k_3$. k_{obs} is given by

$$k_{obs} = k_3 f([\text{A-H}]) \tag{2}$$

where $f([A-H])$ is the fraction of monomer present in solution, derived from the concentration balance of the solution

$$[\text{A}]_t = [\text{A-H}] + 2[(\text{A-H})_2] \tag{3}$$

$$\Rightarrow f([\text{A-H}]) = \frac{[\text{A-H}]}{[\text{A}]_t} \tag{4}$$

$[A]_t$ corresponds to the total concentration of antibiotic present in solution as either monomer or dimer.

In case (2), the on-rate for formation of the dimer is greater than the intrinsic exchange rate, and the dimer dissociates and reassociates many times before exchanging. These two cases are similar to the description of hydrogen exchange in proteins given

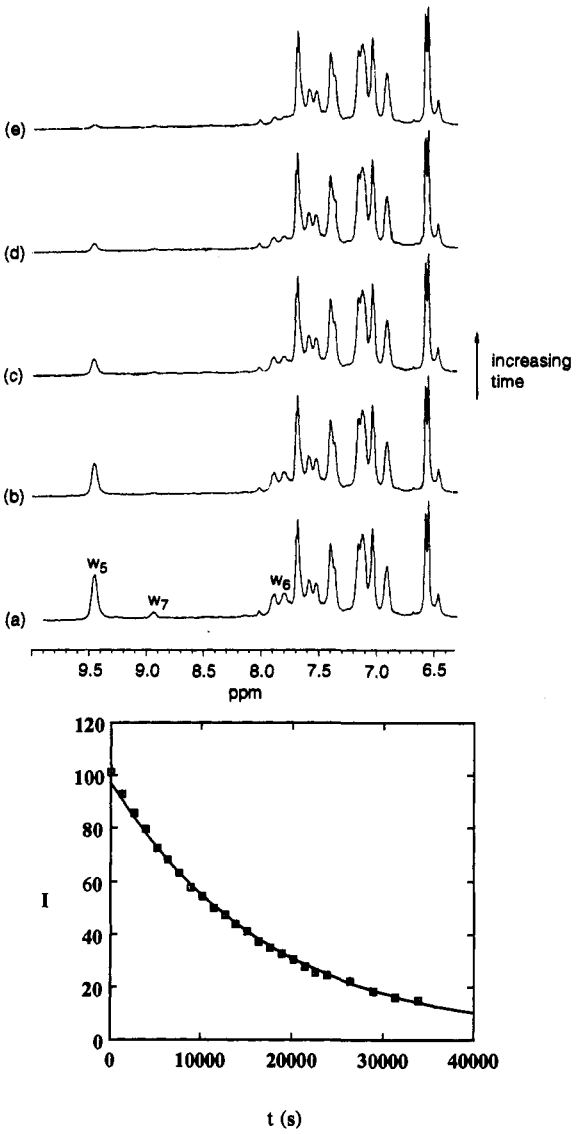

Figure 6. (a) Partial ^{1}H NMR spectra of eremomycin illustrating the decay of the signal due to the protons w_5, w_6, and w_7 with time. Spectra were recorded at 298 K and pD 3.7; (b) plot of signal intensity (I) against time (t,s) for the exchange of the w_5 proton of eremomycin with solvent D_2O. The conditions of the experiment were [eremomycin] = 7.5 mM in 50 mM KD_2PO_4, pD 3.72, 298 K. Data were collected over 3 half-lives of decay and fitted to an exponential function ($y = 97.347 \times 10^{-2.50\times10^{-5}x}$) with $R^2 = 0.998$.

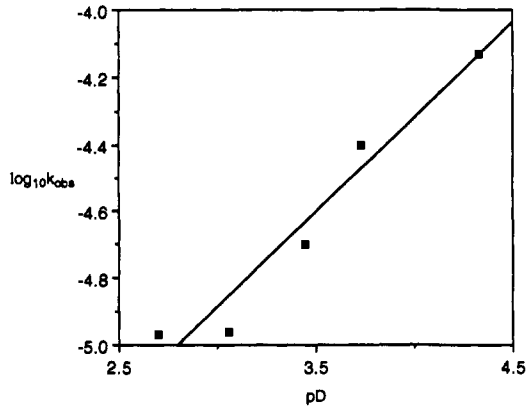

Figure 7. Plot of log k_{obs} *vs* pD for the proton w_5 of eremomycin. All experiments were carried out at 5.3 mM eremomycin and 298 K. The first-order dependence of log k_{obs} on pD supports an EX_2 type mechanism for the exchange of w_5.

by Hvidt and Nielsen,[23] where they use the descriptors EX_1 and EX_2 to indicate cases (1) and (2), respectively. The same descriptors are used in the following analysis.

In order to determine which exchange mechanism is operating, the pD dependence of amide proton exchange may be considered. The observed rate constant, k_{obs}, in an EX_1 mechanism will vary with pD only if there is an effect on the off-rate for the dimer (k_1 above). Similarly, in an EX_2 mechanism, a change in the dimerization constant (and hence in the fraction of monomer) and pD will alter k_{obs}. However, the reaction rate in an EX_2 mechanism is also limited by the chemical exchange event, k_3, which is known to have a primary dependence on pD through eq 5.[24]

$$k_3 = k_{D^+}[D^+] + k_{DO^-}[DO^-] + k_0 \qquad (5)$$

k_{D^+} and k_{DO^-} are second-order rate constants for acid- and base-catalyzed exchange, and k_0 is the first-order rate constant for pD independent exchange. For backbone amide protons in linear peptides, k_3 has been shown to follow a V-shaped dependence on pH, with a minimum at *ca.* pH 2.8.[25]

Thus the influence of pD on the rate constant k_{obs} was determined for eremomycin over the pD range 2.5–4.3 at a constant NMR probe temperature of 298 K. The logarithm–logarithm plot of Figure 7 demonstrates that the pseudo-first-order rate constant k_{obs} follows a first-order dependence on base concentration. A similar plot was obtained for eremomycin-Ψ (data not shown). This dependence is in accord with eq 5 and thus the kinetic limit described by eq 2 (EX_2).

However, as noted above, the pD dependence of the EX_1 and EX_2 mechanisms may also be modulated by the possible pD dependence of k_1 and K_{dim}, respectively. Consequently, the dimerization constant was determined at pD 3.7 for both eremomycin-Ψ and vancomycin by following the change in chemical shift with concentration of the proton x_4 (2×10^4 and 420 M^{-1}, respectively). The small decrease observed in the case of vancomycin (K_{dim} = 700 M^{-1} at pD 7) may be attributed to the protonation of the C-terminal carboxylate of the glycopeptide at low pD. The larger errors associated with the measurement of K_{dim} for eremomycin-Ψ (only part of the titration curve can be sampled because of the relatively high dimerization constant, and the value at 298 K must be extrapolated from measurements at higher temperatures because of the broadness of the lines at room temperature) may mask an analogous effect. This small dependence of K_{dim} on pD translates to a decrease in k_{obs} with increasing pD for both the EX_1 and the EX_2 mechanism.

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(25) Wüthrich, K. *NMR of Proteins and Nucleic Acids*; Wiley: New York, 1986; p 24.

Table 1. Values of K_{dim} at pH 3.7 for Several Glycopeptides Calculated from Data Obtained at Different Antibiotic Concentrations

glycopeptide	[glycopeptide] (mM)	k_{obs} ($\times 10^{-5}$ s^{-1})	K_{dim} ($\times 10^6$ M^{-1})
eremomycin	1.9	8.5	2.9
eremomycin	3.5	6.3	2.9
eremomycin	5.3	5.2	2.7
eremomycin	7.5	5.4	1.9
eremomycin	8.0	4.8	2.1
eremomycin	15.0	2.4	4.5
A82846B	5.3	13	0.44
A82846B	7.1	21	0.13
A82846B	7.9	18	0.15
MM47761	5.8	3.1	7.1
MM47761	6.0	3.3	6.1
dechloroeremomycin	6.0	38	0.044
dechloroeremomycin	6.3	39	0.040

Consequently, the observed *increase* in k_{obs} with pD (Figure 7) must be attributable to the pD dependence of k_3 in eq 2, and the data are therefore consistent with the EX_2 kinetic limit of exchange. Further strong support for this conclusion exists in that k_{obs} is observed to increase with decreasing antibiotic concentration (Table 1). This implies that k_{obs} is sensitive to the fraction of monomer present, in line with the EX_2 mechanism. EX_2 kinetics also appears to be more likely when approximate values of k_2 and k_3 are considered. k_3 has been shown to be around 5×10^{-3} s^{-1} at pH 4 for amide protons in model peptides[26] and may vary (either larger or smaller) by a factor of about 10. The on-rate for dimer formation, k_2, will be slower than the diffusion controlled rate (*ca.* 10^{10} s^{-1} M^{-1}), but will almost certainly still be much larger than 5×10^{-3} s^{-1}, and this is the requirement for EX_2 kinetics.

By combining eq 3 with the expression for the dimerization constant (K_{dim}, eq 6) and rearranging, eq 2 provides eq 7:

$$K_{dim} = [A\text{-}H]_2/[A\text{-}H]^2 \qquad (6)$$

$$k_{obs} = k_3\left(\frac{-1 + \sqrt{1 + 8K_{dim}[A]_t}}{4K_{dim}[A]_t}\right) \qquad (7)$$

Thus, to calculate a value for K_{dim} the value of the intrinsic exchange rate for the proton w_5 (k_3) must be measured. Values of k_3 can be computed for sequences of natural amino acids using the rules determined by Molday *et al.*[26] for sequence-dependent inductive contributions. It is found that 80% of all peptide NH exchange rates fall within a factor of ten of the exchange rate of $CH_3C(O)N\mathit{H}CH(CH_3)C(O)NHCH_3$ (the amide NH concerned is italicized), although adjacent positive charges can generate rates which are 10^2 faster.[22,26] Poly-D,L-alanine has been studied in detail as a model for the unhindered polypeptide group.[27,28] Under the conditions used in the current study (pD 3.7, 298 K), the hydrogen–tritium exchange rate for this polymer corresponds to a k_3 value of 5.4×10^{-3} s^{-1}. A correction factor for the tritium isotope effect of 1.2 should be applied when considering hydrogen–deuterium exchange, but this is probably small compared to other experimental uncertainties.

Thus, the observed hydrogen–deuterium exchange rate for an amide proton will depend on the structures of the two flanking residues, in addition to that of the residue carrying the proton of interest. Residues 4 and 5 of eremomycin are hydroxylated derivatives of phenylglycine, and residue 6 is a hydroxylated derivative of tyrosine. To some extent it is possible to estimate the effects of these unnatural amino acids by comparing them

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Table 2. Dimerization Constants for a Number of Vancomycin Group Antibiotics and Their Derivatives

compound	K_{dim}[a] (M^{-1}, 298 K)
eremomycin[b]	$(3 \pm 1) \times 10^6$
eremomycin-Ψ[c]	$(1.5 \pm 0.4) \times 10^4$
ristocetin A[d]	*ca.* 300 ± 100
ristocetin-Ψ[c]	50 ± 10
vancomycin[c]	700 ± 170
A82846B[b]	$(1.8 \pm 0.6) \times 10^5$
MM47761[b]	$(6.6 \pm 2) \times 10^6$
dechloroeremomycin[b]	$(4 \pm 1) \times 10^4$
vancomycin hexapeptide[e]	*ca.* 700 ± 200

[a] Errors in dimerization constants estimated as 25% for those determined by chemical shift titration in ref 13, and 30% for those determined in the present work using the amide proton exchange protocol and those determined by other methods. [b] Dimerization constants measured in the present work. [c] Dimerization constants measured in 50 mM KD_2PO_4/NaOD buffer at pD 7 (ref 13). [d] Preliminary result obtained using isothermal microtitration calorimetry by successively diluting a solution of ristocetin A (Cooper, A., unpublished results). [e] Estimated by comparison of ^1H NMR spectra of vancomycin at similar concentrations (D.A.B., unpublished results).

with natural amino acids with similar side chains and known inductive effects, for example, tyrosine. However it is possible that hydroxyphenylglycine would show an effect different to that of tyrosine as the α-carbon is benzylic in the former case. An approach was therefore employed whereby k_{obs} was measured for w_5 of eremomycin-Ψ using the ^1H NMR method described above (at 2.0 mM, $k_{obs} = 9.3 \times 10^{-4}$ s^{-1}). K_{dim} of eremomycin-Ψ at pD 3.7 was then measured at several temperatures in the range 318–338 K by following the change in chemical shift of the proton x_4 with antibiotic concentration, and a van't Hoff plot was constructed. Extrapolation gave a value for K_{dim} at 298 K of 2.0×10^4 M^{-1}. Thus a value for k_3 can be calculated using eq 6, which gives $k_3 = 8.9 \times 10^{-3}$ s^{-1}, in good agreement with the previously measured value for poly-D,L-alanine. A similar treatment using vancomycin yielded a value of 1.3×10^{-2} s^{-1} for k_3. This is consistent with the above data given that, in the case of vancomycin, a small amount of overlap of w_5 with another, more rapidly exchanging, proton was seen and therefore monitoring the decay of the NMR signal peak height with time leads to the determination of increased values of k_3. Consequently, the value of k_3 obtained from eremomycin-Ψ was used in subsequent calculations.

Because of the near identity of the backbone structures of eremomycin and eremomycin-Ψ, this value of k_3 for eremomycin-Ψ should also be applicable to eremomycin. Values of k_{obs} for eremomycin have been measured over a range of antibiotic concentrations, and values for K_{dim} have thus been calculated using eq 6. These values are in good agreement and are listed in Table 1. The dimerization constants for A82846B[29] (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, Figure 1), MM47761[30] (identical to eremomycin, except that the amino sugar of the disaccharide of eremomycin is replaced by a rhamnose unit), and dechloroeremomycin have been calculated by the same method, and the results are also given in Table 1 (it has been assumed that the inductive effect of the 3-chloro-β-hydroxytyrosine side chain of A82846B is comparable to that of its dechloro analogue).

Partitioning Contributions to Dimerization Affinities. Dimerization constants for eremomycin and related antibiotics are summarized in Table 2.[13] The contribution of a number of structural elements to dimerization can be estimated from the following results.

(i) A factor of *ca.* 100 in dimerization constant (*ca.* 11 kJ mol^{-1} at 298 K) can be attributed to the ring 4 disaccharide of eremomycin, by comparison with the dimerization constant of eremomycin-Ψ. Thus a number of favorable interactions must be formed between the two disaccharides and/or between the disaccharides and the peptide backbones of the dimer. These could be hydrogen bonds involving the hydroxyl groups of the sugars, van der Waals complementarity, hydrophobic interactions, or most likely some combination of these. Based on this number, a dimerization constant of less than 10 would be predicted for vancomycin aglycon ($K_{dim} = 700$ M^{-1} for vancomycin), and indeed ^1H NMR spectra of vancomycin aglycon are consistent with such a prediction, showing little evidence of dimer formation at millimolar concentrations. In contrast, removal of the disaccharide from vancomycin reduces its binding constant to DALAA by only 3 kJ mol^{-1}.[10] The combination of these two results leads to the prediction that vancomycin aglycon should be less active than vancomycin. Experimental data indicate that the *in vitro* activity is in fact similar but that the *in vivo* activity is 5 times less.[31] Collectively, the data show that (1) the disaccharide enhances dimerization, and (2) this dimerization may contribute to the antibiotic activity of vancomycin, although the evidence on this latter point is ambiguous in this particular case.

(ii) The dimerization constant of A82846B is a factor of 250 larger than that of vancomycin (at pD 3.7), and the two compounds essentially differ only in the presence of a ring 6 amino sugar in A82846B. Thus this sugar contributes 14 kJ mol^{-1} to the free energy for dimerization of A82846B. Part of this is due to the intermolecular hydrogen bond formed between the amino group of the sugar and the carbonyl group of residue 2 of the complementary half of the dimer (Figure 3). The remainder can be attributed to van der Waals complementarity and/or hydrophobic interactions. The ligand binding constant of A82846B with DALAA was also measured (1.3×10^6 M^{-1}). Its similarity to the corresponding value for vancomycin[9] (1.5×10^6 M^{-1}) suggests that the ring 6 sugar does not contribute significantly to the binding of ligand and may actually oppose binding.[14] This provides evidence that the specific role of the ring 6 sugar is to promote dimerization, and thus that dimerization is likely to be biologically relevant, as the synthesis and incorporation of such a heavily modified sugar presumably involves considerable metabolic complexity. It should be noted that comparisons of ligand binding constants between glycopeptides with different dimerization constants must be made with care; this is discussed further in the following paper in this issue.

(iii) The substitution of rhamnose for 4-*epi*-vancosamine on the disaccharide of eremomycin (to give MM47761) has little effect (around a factor of 2) on the dimerization potential of the glycopeptide. However, point (i) above shows that the disaccharide contributes 11 kJ mol^{-1} to dimerization. Neither does the sugar substitution alter the affinity of the glycopeptide for DALAA. The binding constant, measured by UV, between MM47761 and DALAA is 3.5×10^4 M^{-1}, compared to 3.9×10^4 M^{-1} for eremomycin to the same ligand.[11] However, MM47761 exhibits similar *in vitro* antibacterial activity to vancomycin[30] and is thus 2–5 times less active than eremomycin.[11,32] Therefore, considering the similarities between MM47761 and eremomycin in both dimerization and ligand binding affinity, it appears that the amino sugar on the disaccharide of eremomycin has some further effect to enhance the *in vitro* activity of eremomycin. The nature of this effect is unknown but may result from selectivity conferred in binding ligand.[10]

(iv) It has previously been noted[13] that the ring 2 chlorine of eremomycin fits very precisely into a pocket at the dimer interface. Removal of this chlorine and the consequent creation of a cavity at the dimer interface reduces K_{dim} by a factor of 80. This is in

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line with previous observations that glycopeptides lacking this chlorine are significantly elss efficacious *in vitro* than their chloro analogues, despite only small differences in ligand binding affinities.[13,14,29,33]

(v) The presence of the ring 6 chlorine substituent of A82846B appears to **decrease** its ability to dimerize by an order of magnitude compared to eremomycin (*ca.* 6 kJ mol^{-1} at 298 K), despite being oriented **away** from the interface of the dimer. The precise origin of this effect is not clear. It is significant however that this chlorine substituent substantially favors ligand binding—the binding constant of DALAA to A82846B is 1.0×10^6 M^{-1}, 26-fold higher than that to eremomycin. Despite their differing dimerization constants and ligand binding affinities, eremomycin and A82846B exhibit the same level of *in vitro* antibacterial activity (both are around five times more active than vancomycin). Thus there exists an extremely subtle balance between ligand binding and dimerization in these compounds; vancomycin binds DALAA as strongly as A82846B but has a 250-fold lower dimerization constant, consistent with its lower *in vitro* activity.

(vi) Vancomycin hexapeptide has a dimerization constant comparable to vancomycin (Table 2). This constitutes further evidence for the proposed structure of the dimer, as molecular models reveal no likely intermolecular interactions involving either residue 1 or the amide group linking residues 1 and 2 of intact vancomycin. The balance between ligand binding and dimerization is also evident here; the binding constant between vancomycin hexapeptide and DALAA was found to be 1300 M^{-1}. This is only a factor of *ca.* 30 lower than that for eremomycin, and yet vancomycin hexapeptide shows no antibacterial activity at all.[34] Presumably the difference in dimerization constants between eremomycin and vancomycin hexapeptide is the other determining factor in this lack of activity.

(vii) Teicoplanin-A_2-1, teicoplanin-A_2-2, and teicoplanin-A_2-3, which are deglycosylated derivatives of the glycopeptide teicoplanin A_2[34,36] (Figure 8), exhibit no evidence of dimer formation at millimolar concentrations. The chemical shift of the proton x_4, a sensitive probe of the presence of dimer, remains essentially invariant for each of these compounds at 5.60–5.64 ppm in the temperature range 290–340 K. This is consistent with values previously observed for the monomeric forms of a range of glycopeptides (J.P.M., U.G., D.A.B., data not shown).[12] Teicoplanin itself forms a micellar structure in aqueous solution at concentrations greater than *ca.* 0.2 mM,[37] owing to the presence of a C_{10} fatty acid side chain which is connected *via* an amide bond to the ring 4 sugar of the antibiotic. Consequently, the ^{1}H NMR spectrum of teicoplanin-A_2 in D_2O is poorly resolved. However, under conditions which disrupt micelles (*e.g.*, the addition of 15% (v:v) acetonitrile) to yield sharp lines in the NMR spectrum, no evidence for the formation of a dimer is found (note that ristocetin A dimerizes in D_2O/CD_3CN mixtures[12]). Chemical shifts are comparable to those of the deglycosylated derivatives in D_2O. In addition, molecular models show that the bulk of this side chain could seriously hinder the formation of a dimer. However, despite the absence of dimer formation, teicoplanin has an antibacterial activity 2–8 times greater than that of vancomycin,[38] and the possible function of the fatty acid side chain is discussed in the following paper in this issue.[14]

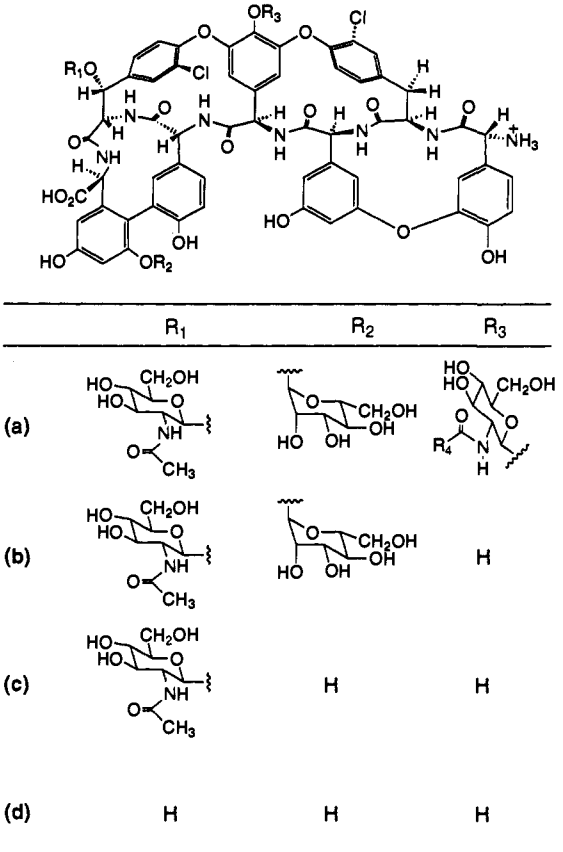

Figure 8. The structure of (a) teicoplanin-A_2, (b) teicoplanin-A_2-1, (c) teicoplanin-A_2-2, and (d) teicoplanin-A_2-3. R_4 represents the C_{10} fatty acid side chain of teicoplanin.

Conclusion

It has been demonstrated that both the chlorine and the sugar substituents common to many vancomycin antibiotics significantly affect the stability of the homodimers formed by these compounds. The differences in dimerization potential between the various members of the group permit rationalization of their relative *in vitro* activities, which previously has not been possible on the basis of DALAA affinities alone. The interactions formed in these dimers are highly specific, and it is remarkable that a molecule of such modest molecular mass carries **two** sophisticated molecular recognition sites. This specificity of the interactions at the dimer interface provides further support for the proposal that the dimer has some function in the mode of action of the glycopeptides. It is an extremely subtle balance between the two processes (dimerization and ligand binding) which determines the actual antibacterial activity of each individual glycopeptide. This idea is pursued further in the following paper in this issue,[14] but certain trends are already evident. In particular, we conclude that although dimerization of the antibiotics is not a prerequisite for antibiotic action, high *in vitro* activity seems to be promoted by one of the two following requirements: either (i) if the dimerization tendency is only moderate (K_{dim} *ca.* 10^2–10^3 M^{-1}), then binding to the cell wall analogue DALAA must be strong (at, or approaching 10^6 M^{-1}) or (ii) if binding to this cell wall analogue falls to slightly greater than 10^4 M^{-1}, then dimerization must be strong (K_{dim} *ca.* 10^6 M^{-1}).

Teicoplanin does not form an analogous dimer, presumably as a result of the modification (relative to the antibiotics depicted in Figures 1 and 2) of sugars attached to residue 4. However, this compound is an effective antibacterial agent, and the hydrophobic side chain which is linked to residue 4 is proposed in the following paper in this issue to serve as a membrane anchor.

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Such an anchor can promote antibiotic action in a manner which can compensate for the lack of dimer formation.[14]

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