Aspects of Molecular Recognition: Solvent Exclusion and Dimerization of the Antibiotic Ristocetin When Bound to a Model Bacterial Cell-Wall Precursor

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Abstract: Proton–proton nuclear Overhauser effects (NOES) have been used to study the conformation and intermolecular interactions of the antibiotic ristocetin A and of ristocetin Ψ (which lacks five of the six sugars of ristocetin A). It is established that when ristocetin Ψ interacts with the peptide cell-wall analogue di-*N*-acetyl-L-Lys-D-Ala-D-Ala ("tripeptide"), the aromatic ring of residue 1 of the antibiotic folds in over the hydrogen bond network formed between the carboxylate anion of the cell-wall analogue and the amide backbone of the antibiotic. Reasons for this spontaneous folding of a hydrocarbon region over ionic interactions are discussed. It is also established that ristocetin A and ristocetin Ψ, in both the presence and absence of cell-wall analogues, form dimers. From line-broadening effects, intermolecular NOES, and model building, the main structural features of these dimers have been deduced. Dimer formation could have a role in promoting the biological activity of the antibiotics.

The vancomycin group of glycopeptide antibiotics is becoming increasingly important in the treatment of enterocolitis and penicillin-resistant staphylococcal infections.[1] One of the first discovered members of the group, ristocetin A (see Figure 1), has proven useful for the study of the mode of action of these antibiotics.[2-9] Specifically, this is the binding of peptides in bacterial cell walls with the C-terminal sequence -L-X-D-Ala-D-Ala where, for example, X = lysine, diaminopimelic acid, alanine, or homoserine. The model peptides *N*-acetyl-D-Ala-D-Ala ("dipeptide") and di-*N*-acetyl-L-Lys-D-Ala-D-Ala ("tripeptide") have been used extensively to study the intermolecular interactions that characterize complex formation (see Figure 2). Partial assignment of the ^{1}H NMR spectrum of ristocetin A and its complexes with di- and tripeptide in aqueous solution has been reported,[8] as have studies in DMSO[4] and methanol solution.[9] The studies in aqueous solution were hampered by large line widths of the proton resonances and spectral crowding. Owing to the narrow line widths obtained for the resonances of A40926 in D_2O–CD_3CN solvent mixtures,[10] it was decided to reinvestigate the solution conformation of ristocetin and its complexes in this pseudoaqueous medium. Initially, studies were centered on the pseudoaglycone of ristocetin (ristocetin Ψ) owing to its considerably simpler NMR spectrum that that of ristocetin A—for structures see Figure 1.

Although ristocetin is a relatively rigid molecule on account of the cross-linkages between its constituent amino acid side chains, it has been proposed that rings 1 and 3 (see Figure 1) undergo sizable oscillations in ristocetin A in DMSO solution[4] and in the ristocetin Ψ–tripeptide complex in methanol.[9] These conclusions are based on observations of the NOEs $x_3 \leftrightarrow$ 3b versus $x_3 \leftrightarrow$ 3f, and $x_1 \leftrightarrow$ 1b versus $x_1 \leftrightarrow$ 1f. Both studies calculated inter-proton distances from the rate of buildup of these NOEs, assuming a uniform molecular correlation time for the entire molecule. These distances were too short for both NOEs to be satisfied simultaneously while accommodating reasonable bond lengths for the systems involved. Thus, it was concluded that oscillatory motion was present. However, similar studies of the ristocetin A–dipeptide complex in DMSO solution[4] produced reasonable inter-proton distances, and it was proposed that this system exists predominantly as a single conformer in the ring 1–ring 3 region. In this conformer, ring 1 is adjacent to the carboxylate anion of the peptide. Molecular mechanics and dynamics studies based on the NMR studies in methanol solution led to the postulation[9] that, in its tripeptide complex, ring 3 of ristocetin Ψ is oscillating through approximately 90° between two energetically equivalent states (see Figure 3) with virtually no barrier to the rotation. In addition, it was proposed that this oscillation leads to smaller changes in the position of ring 1. However, these simulations do not take into account solvent effects which undoubtedly influence the population of the various conformers and thus the barrier to any rotational processes.

Methods

NMR experiments were perfomed with 10–15 mM solutions of ristocetin Ψ, 1:1 ristocetin Ψ–tripeptide, or 1:1 ristocetin A–tripeptide in D_2O with 0–29% acetonitrile-d_3 modifier. Spectra were recorded on Bruker AM500 and AM400 spectrometers. Normal one-dimensional spectra were acquired for approximately 1 s. NOE difference spectra were recorded after a selective irradiation of 0.2–1 s and exponential line broadenings of approximately 2 Hz were applied. Double quantum filtered COSY experiments (DQFCOSY) were run in the phase-sensitive mode using time proportional phase incrementation for quadrature detection in F_1. Typically 2K data points were recorded in F_2 with 512 or 1K in F_1, over a spectral width of ca. 4000 Hz. Phase-sensitive NOESY spectra were recorded similarly to DQFCOSY spectra with mixing times of 0.1–0.5 s. A 20-ms Z filter was employed during the mixing time. In all cases, the data matrices were zero filled once in F_1 and subjected to Lorentzian–Gaussian apodization prior to Fourier transformation. The proton resonances of the three systems above were assigned by using DQFCOSY and NOESY spectra. The assignment strategy has been described previously.[10]

Results

As in the case of A40926, addition of acetonitrile to aqueous solutions of ristocetin and its peptide complexes leads to a reduction in the line width of their proton resonances. The assignment of the proton resonances is given in Table I. In spectra of ristocetin Ψ and the ristocetin Ψ–tripeptide complex, the resonance of proton 6e was not observed and those of 6f and x_4 were very broad relative to other resonances. In NOESY and NOE difference spectra of free ristocetin Ψ, the resonances of residue 3 were involved in a small number of NOEs. Proton x_3 shares an intense NOE with proton 3b but no NOE with proton 3f. The rate of buildup of the NOE between x_3 and 3b corresponds to a distance between these protons of approximately 2.4 Å (assuming a correlation time equal to that between, e.g., protons 2e and 2f). The NOEs of the protons of residue 3 are not perturbed significantly by the addition of di- or tripeptide.

(1) See, for example: Foldes, M.; Munro, R.; Sorrell, T. C.; Shanker, S.; Toohey, M. *J. Antibiot. Agents Chemother.* **1983**, *11*, 21.
(2) Nieto, M.; Perkins, H. R. *Biochem. J.* **1971**, *124*, 845.
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(7) Herrin, T. R.; Thomas, A. M.; Perun, T. J.; Mao, J. C.; Fesik, S. W. *J. Med. Chem.* **1985**, *28*, 1371.
(8) Williamson, M. P.; Williams, D. H. *J. Chem. Soc., Perkin Trans. 1* **1985**, 949.
(9) Fesik, S. W.; O'Donnell, R. T.; Gampe, R. T.; Olejniczak, E. T. *J. Am. Chem. Soc.* **1986**, *108*, 3165.
(10) Waltho, J. P.; Williams, D. H.; Selva, E.; Ferrari, P. *J. Chem. Soc., Perkin Trans. 1* **1987**, 2103.

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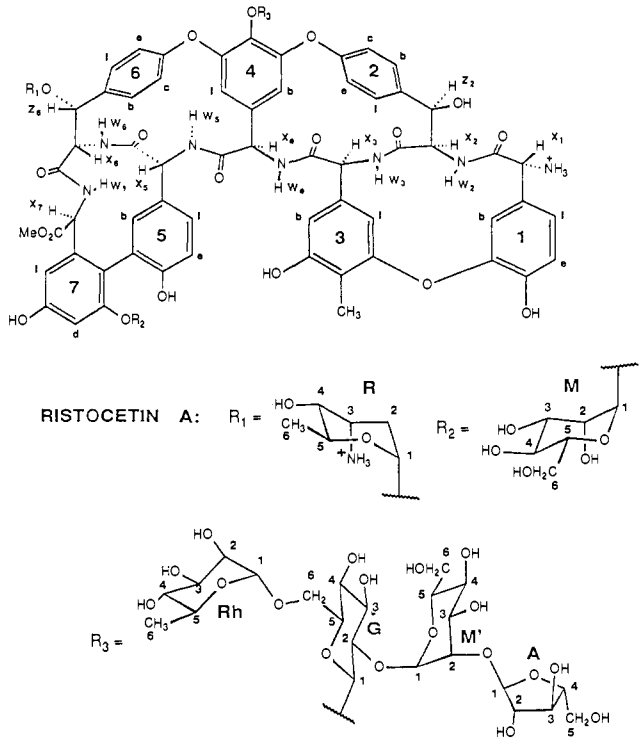

Figure 1. Structures of ristocetin A and ristocetin Ψ showing the nomenclature of its constituent hydrogen atoms.

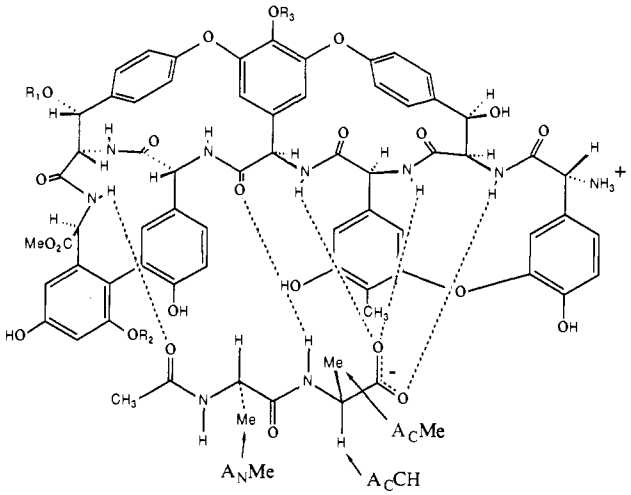

Figure 2. Schematic representation of the complex formed between ristocetin and a bacterial cell-wall peptide model, *N*-acetyl-D-alanyl-D-alanine. The broken lines indicate the position of intermolecular hydrogen bonds.

Of the NOEs of residue 1 of free ristocetin, the NOEs $x_1 \leftrightarrow$ 1f and $x_1 \leftrightarrow$ 1b are both small (see Figure 4a). This reflects a relatively large separation of these protons. In order to show that the lack of intensity of the NOE $x_1 \leftrightarrow$ 1f was not the result of a correlation time phenomenon, the intensity of this NOE was compared with others in the spectrum at different temperatures and no change in the ratios was apparent. Similarly, the small size is not the result of a line-width phenomenon. Both protons 1f and 1b are involved in other fast-building NOEs (with protons 1e and 3f, respectively). The NOE $x_1 \leftrightarrow$ 1f is shown in Figure 4a relative to the NOEs $z_2 \leftrightarrow$ 2b, $z_6 \leftrightarrow$ 2b, and $x_3 \leftrightarrow$ 3b. On formation of the tripeptide complex, the NOE $x_1 \leftrightarrow$ 1f increases vastly in buildup rate (see Figure 4b) whereas that between x_1 and 1b remains small.

In addition to NOEs characteristic of the previously defined conformation(s) of ristocetin,[2-9] "unusual" NOEs were observed

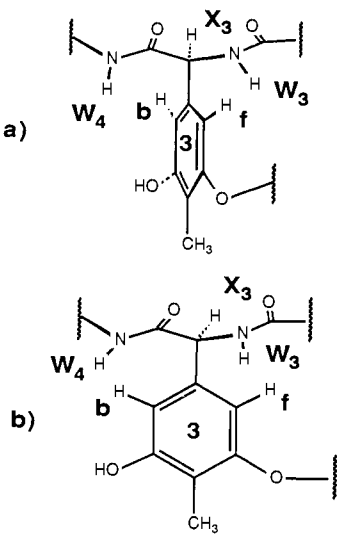

Figure 3. The two 90° rotamers considered for ring 3 of ristocetin.

Table I. Assigned Chemical Shift Values of Some Resonances of the Two Dimer Forms (Molecules A and B) and the Monomer (Molecule C) Present in the Spectrum of the Ristocetin A–Tripeptide Complex in 5:2 D_2O–CD_3CN Solution at 295 K[a]

	molecule				
	A	B	C	ref 8	RisΨ–AcAA
x_1	5.32	5.32		4.97	5.27
1b				6.78	6.77
1e	6.88	6.88		6.91	6.86
1f	7.14	7.14		7.10	7.10
x_2	5.50	5.50		5.18	5.4
z_2	5.42	5.48		5.38	5.4
2b	7.20	7.17		7.12	7.14
2c	7.39	6.98		7.28	7.00
2e	7.13	7.35		7.19	7.10
2f	7.81	7.86		7.85	7.94
x_3	5.50	5.53		5.53	5.46
3b	6.63	6.63		6.70	6.55
3f				6.65	6.51
3Me				2.03	2.05
x_4	6.52	6.55	5.73	5.93	6.08
4b	5.83	5.76		5.87	5.65
4f	5.15	5.04		5.51	5.05
x_5	4.90	4.83		5.29	4.81
5b	7.79	7.79		7.20	7.68
5e	6.93	6.93		6.87	6.90
5f	7.00	7.00		6.99	6.94
x_6	4.63	4.63		4.75	4.71
z_6	5.18	5.27		5.50	5.37
6b	7.16	7.28		7.25	7.28
6c	6.91	6.62		6.92	6.71
6e	5.09	5.18	7.38	7.21	
6f	6.81	6.70	7.51	7.34	
x_7	4.82	4.82		4.75	4.65
7d	6.88	6.88		6.91	6.53
7f	6.42	6.42		6.49	6.24
G_1	5.55	4.95	5.18	5.18	
R_1	5.31	5.24		5.29	5.09
R_2	2.20	2.20		2.15	2.13
$R_{2'}$	2.53	2.53		2.35	2.31
R_6	1.48	1.48			1.40
Rh_4	3.53	3.26			
Rh_5	3.62	2.66			
Rh_6	1.33	0.91	1.11	1.08	

[a] For comparative purposes, the assignment carried out in D_2O solution[8] and the assignment for the Risψ–dipeptide complex in 5:2 D_2O–CD_3CN are shown. Values for molecules A and B are interchangeable.

which do not fit the covalent structure of the molecule. These NOEs were observed for ristocetin Ψ in the presence and absence of tripeptide, and in the ristocetin A–tripeptide complex. The protons involved in these NOEs occupy the nonbinding face of

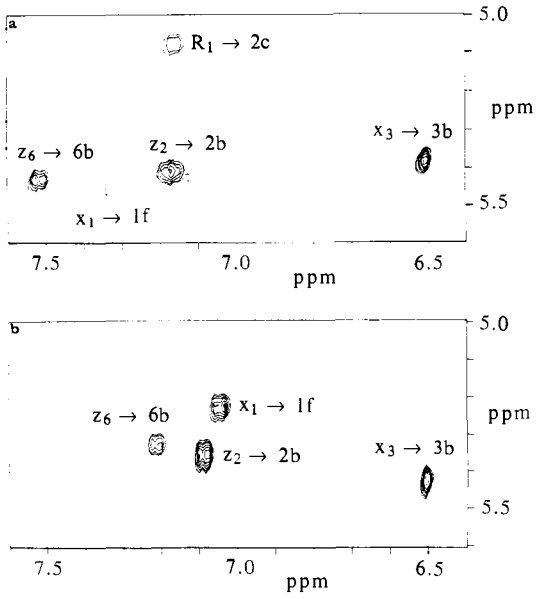

Figure 4. Sections of NOESY spectra of (a) ristocetin Ψ in 5:2 D_2O–CD_3CN solution and (b) the ristocetin Ψ–di-*N*-acetyl-L-Lys-D-Ala-D-Ala complex in the same solvent system. Some NOEs involving resonances of residues 1 and 3 of ristocetin may be compared with some of residues 2 and 6. In spectrum a, the NOE 1b ↔ x_1 falls below the lowest contour level shown.

Table II. NOEs Observed within Ristocetin A and Ristocetin ψ in Their Tripeptide Complexes in D_2O–CD_3CN Solution That Are Inconsistent with the Covalent Structure of the Antibiotic (R = Ristosaminyl Protons)

$R_{2,2'}$ ↔ 2c	R_3 ↔ x_3
$R_{2,2'}$ ↔ x_3	6f ↔ 4b

ristocetin and ristosamine and are listed in Table II.

Discussion

Oscillation of Ring 3. The NOEs observed involving the protons in the region of ring 3 in ristocetin Ψ in water–acetonitrile solution may be satisfied by a single conformer. In this conformer, the NOE-derived approximate distance between protons x_3 and 3b (2.4 Å) would reflect an almost eclipsed relationship of these protons—from molecular models the minimum distance between them is ca. 2.3 Å.[11] Such a conformer is shown in Figure 3a. However, if this conformer were populated for only 50% of the time and its 90° rotamer (see Figure 3b; x_3–3b distance = 3.1 Å[11]) for 50% of the time, then the *calculated* distance would be ca. 2.5 Å. Thus, the NOE-derived distance above does not rule out the possibility of the equal population of both conformers. However, if equal population were the case, then the rate of buildup of the NOE between x_3 and 3f would be ca. 7 times slower than that of the NOE between x_3 and 3b. The absence of this NOE in NOESY and NOE difference spectra requires that the conformer in Figure 3b is significantly higher in energy than the lower energy conformer, i.e., that shown in Figure 3a. The line widths of 3f and 3b are indistinguishable, thus eliminating the possibility of error in the calculation of NOE buildup rate as a result of differential T_2 relaxation rates. The conformational preferences of ring 3 are not affected by the addition of tripeptide to this system (compare the NOE x_3 ↔ 3b in Figure 4, parts and b).

Unmeasurable population of the rotamer shown in Figure 3b is similar to the situation observed for the ristocetin A–tripeptide complex in DMSO solution[4] but in contrast to that observed for the ristocetin Ψ–tripeptide in methanol.[9] This is an interesting example of the subtle solvent dependency of conformational preferences in this system.

(11) Obtained from a molecular model built with the COSMIC computational chemistry package of Smith Kline and French Research, U.K.; see: Vinter, J. G.; Davis, A.; Saunders, M. R. *J. CAMD.* **1987**, *1*, 31.

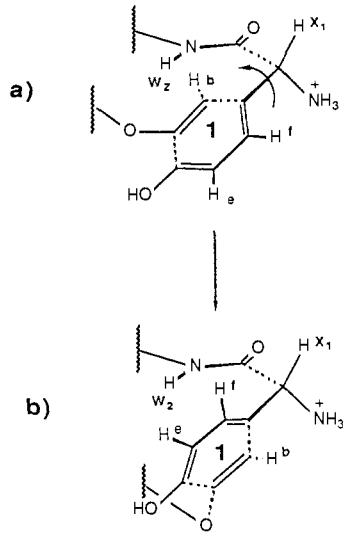

Figure 5. Orientation of ring 1 of ristocetin (a) in free solution (5:2 D_2O–CD_3CN) and (b) in the presence of di-*N*-acetyl-L-Lys-D-Ala-D-Ala. Note that in (a) the protons 1f and 1b are both relatively distant from proton x_1, whereas in (b) protons 1f and x_1 are adjacent.

Reorientation of Ring 1 on Binding. In contrast to the absence of any changes in the NOEs of residue 3 when tripeptide is added to ristocetin Ψ, the NOEs of residue 1 change significantly. In the absence of tripeptide, the NOEs x_1 ↔ 1f and x_1 ↔ 1b are both small. This suggests that the C–H bond involving proton x_1 is approximately perpendicular to the plane of ring 1 (see Figure 5a). The increase in the size of the NOE x_1 ↔ 1f on addition of tripeptide may be rationalized in terms of a reorientation of the time-averaged position of ring 1 with respect to the binding pocket bringing the face of the ring in closer to the peptide carboxylate anion (see Figure 5b). The folded-in nature of the residue 1 side chain of ristocetin in its tripeptide complex is reflected by the intermolecular NOEs A_cCH ↔ 1f and A_NMe ↔ 1e, as well as the intramolecular NOE x_1 ↔ 1f (see Figure 2).

It appears that restriction of solvent access to the binding pocket of ristocetin Ψ in free solution is unfavorable, whereas it is desirable on formation of the complex. In free solution, it is energetically more favorable for the solvent molecules solvating the binding pocket to be as accessible as possible to the bulk solvent, in particular because of the high entropy and enthalpy demands for isolating a water molecule from the bulk. In the complex, the entropy demands on the binding species are much lower because the carboxylate anion is a planar species, thus having, for example, its out-of-plane motion already restricted by its bonding characteristics. Comparing the benefits of having a hydrophobic boundary to the pocket versus water in the complex, restriction of solvation of the electrostatic intermolecular interactions is an enthalpically favorable process because the electrostatic component of the interaction energy is increased (ΔH becoming more negative). This is a result of the dielectric effect of the surrounding medium being reduced. Entropically, it should also be a favorable process. The organization of water about a polar interaction serves to destabilize the interaction through a negative ΔS contribution. Furthermore, the removal of one face of ring 1 (i.e., a hydrophobic region) from the solvent should contribute to the stability of the complex.

The folding-in of a hydrophobic group to shield the interaction between the peptide carboxylate group and the amide groups of the binding pocket has also been proposed for vancomycin,[4] and recent evidence supports the population of such a conformation.[12]

Dimerization of the Ristocetin A–Tripeptide Complex. A feature present in the spectra of ristocetin Ψ in D_2O–acetonitrile-d_3 solution, and one that had puzzled us previously in other spectra,

(12) Waltho, J. P.; Cavanagh, J.; Williams, D. H. *J. Chem. Soc., Chem. Commun.* **1988**, 707.

is that certain resonances, in particular that of x_4, are very broad. Further examples of broad resonances occur in the spectra of the ristocetin Ψ–tripeptide complex where, for example, the resonance of the A_CMe group is considerably broader than that of the A_NMe group and the resonance of 6e cannot be found. Such variations in line widths are often the result of an exchange process occurring at a rate of the order of the chemical shift differences of the environments created by the exchange process. Initially, our attention was drawn to the proposed oscillation of ring 3 discussed above[9] as the cause of the variation in line widths, i.e., the possibility that the barrier to such a rotation in the presence of solvent was considerably larger (i.e., of the order of 15 kcal mol^{-1}) than that predicted for the gas-phase species by computational methods.

It has been shown that certain resonances of ristocetin A[13] and the ristocetin A–tripeptide complex[8] in water are present as two forms in slow exchange. In the former system, it was noticed that at room temperature two almost equal intensity resonances were present for the 6-methyl group of rhamnose (δ 0.98 and 1.28)—see Figure 1. At 25 °C, the interconversion of one form to the other was calculated to be ca. 40 Hz.[13] Later studies[8] revealed that other proton resonances were perturbed by this exchange process and identified the involvement of proton 4b. In the latter system, i.e., the ristocetin A–tripeptide complex, two sets of bound alanine methyl signals in slow exchange at 40 °C were observed. The relative intensity of the two signals was estimated to be 3:2, and from the absence of a NOE from *one* of the two A_NMe groups to the mannose anomeric proton, it was suggested that the orientation of the ring 7 mannose residue may be the cause of the difference. If this exchange phenomenon is similar to the fast-exchange perturbation of the spectrum of the ristocetin Ψ–tripeptide complex (which does not have a ring 7 mannose), then the above rationalization is improbable. However, the change in the kinetics of the process on going from ristocetin A to ristocetin Ψ does implicate an involvement of the sugars in some part of the exchange process. As interconversion of the two forms of the ristocetin Ψ–tripeptide complex forms was on the fast-exchange side of the coalescence point, it was decided to investigate further the species involved in slow exchange within the larger (ristocetin A–tripeptide) system.

From the presence of saturation transfer cross-peaks between more than two resonances of certain protons (e.g. 2c, 4f, 6e, G_1, Rh_6), it was immediately apparent that more than two conformers were present. For some protons, the resonances of two of the forms had similar chemical shifts, while those of a third form were shifted considerably (e.g., x_4 δ 6.52, 6.55, 5.73). The resonance(s) of 6e, which was not found in the spectrum of the ristocetin Ψ–tripeptide complex in D_2O–acetonitrile-d_3, could now be assigned. Its signal also consisted of three resonances. Two resonances of 6e (δ 5.09 and 5.18) occurred close to those of 4f and so must be experiencing *large* upfield ring current shifts, whereas the resonance of the third form occurred as expected at 7.32 ppm. From this chemical shift evidence, in conjunction with the "unusual" NOEs listed in table II, it became clear that *inter*molecular processes (other than tripeptide binding) were involved.

The NOEs between protons of ristosamine and residues 2 and 3 were indicative of the formation of a dimer through the combination of the back faces of two ristocetin molecules (with tripeptide still occupying the binding site). On inspection of molecular models, it was noticed that there existed a high degree of complementarity of hydrogen bond donors and acceptors (as indicated in Figure 6) as two molecules were placed back to back, with w_5 opposite the carbonyl group of residue 5, $(CO)_5$, and w_6 opposite $(CO)_3$. Four intermolecular hydrogen bonds are made in total involving w_5 and w_6 from both molecules in the dimer (see Figure 6 and 7). The large chemical shift changes observed for the resonances of x_4 can be rationalized by the proton being flanked by two w_5–$(CO)_5$ hydrogen bonds in two forms of the tripeptide complex but not in the third. The conformation enforced by the above hydrogen bonds places the proton 6e of each molecule (and

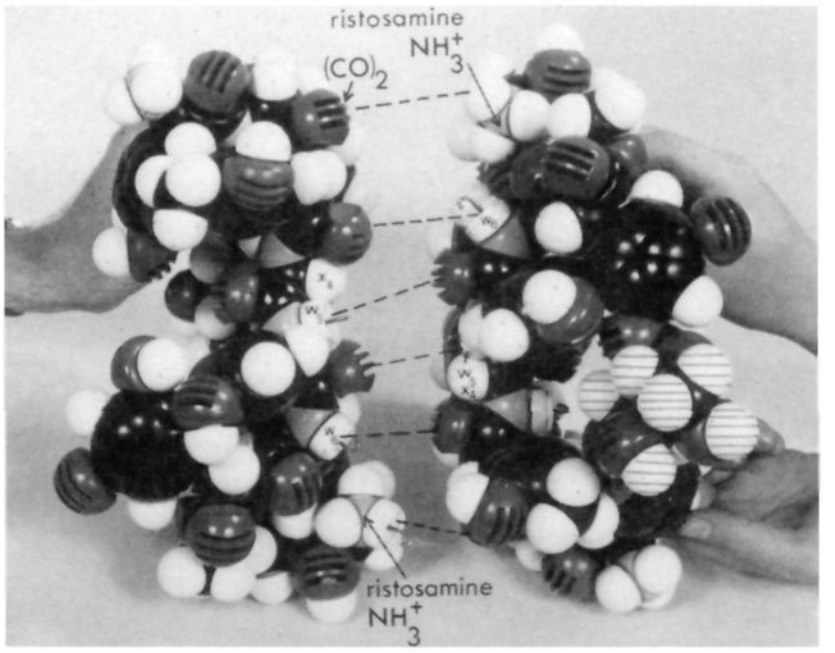

Figure 6. Complementarity of hydrogen bond donors and acceptors on the "back" faces of two molecules of ristocetin. Note the position of proton x_4, i.e., between two intermolecular hydrogen bonds. To simplify the picture, the sugars have been removed except for (i) ristosamine in each half of the dimer and (ii) mannose in the right half of the dimer only, with its protons indicated by hatching.

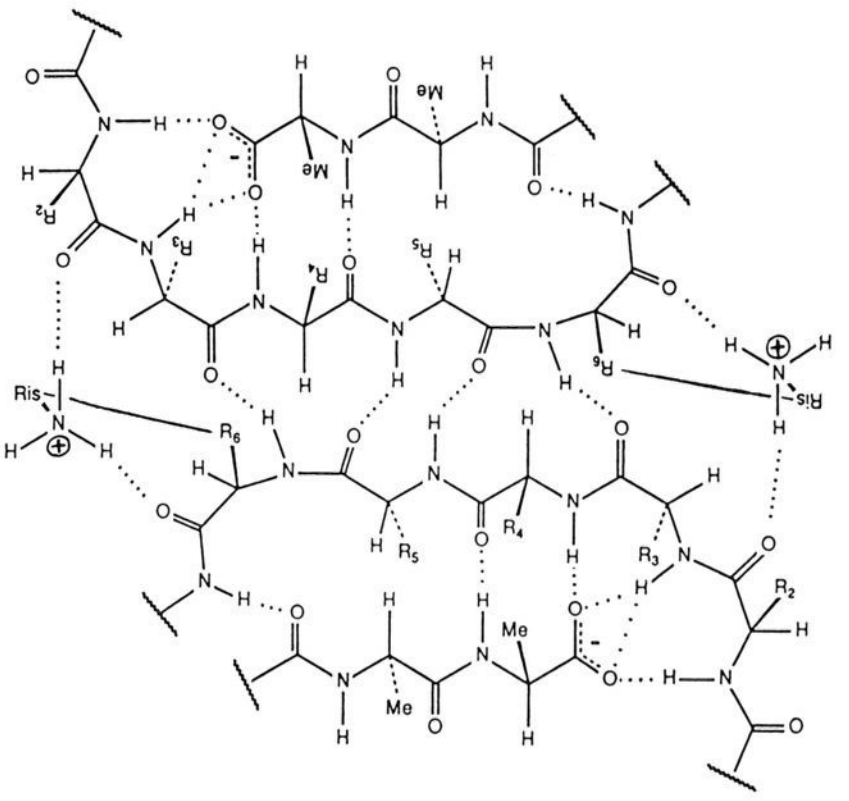

Figure 7. Hydrogen-bonding network of the dimer formed between two molecules of ristocetin when bound to tripeptide (this view is of the opposite face of the dimer to that shown in Figure 6).

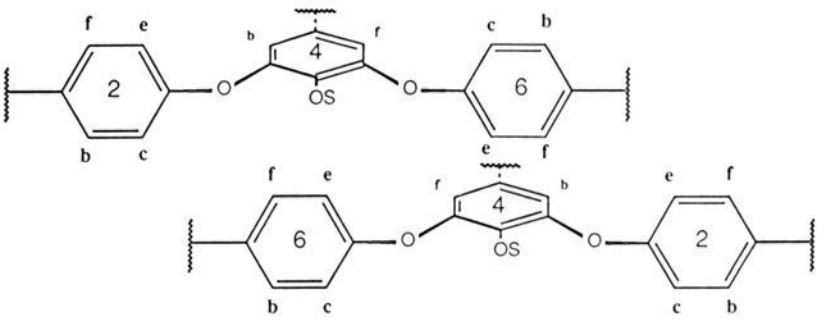

Figure 8. Orientation of the triaryl system of ristocetin in the dimer as dictated by the intermolecular hydrogen bonds shown in Figure 7. Notice how the protons 6e and 6f lie over the face of ring 4 of the dimer partner.

to a lesser extent 6f) over the face of ring 4 of the other molecule (see Figure 8), satisfying the observed upfield chemical shifts of their resonances. Indeed, all the unusual NOEs listed above may be satisfied by intermolecular proximity created by a dimer of this nature. This geometry also allows indirect interaction of the protonated amine of ristosamine of each molecule with the carboxylate anion of the peptide bound to the other molecule through the polarization of the carbonyl group, $(CO)_2$, which is part of

(13) Williams, D. H.; Rajananda, V.; Kalman, J. R. *J. Chem. Soc., Perkin Trans. 1* **1979**, 787.

an amide system involved in the carboxylate anion binding pocket (see Figures 6 and 7).

The ratio of the intensities/populations of the three forms observed in the normal one-dimensional spectrum at room temperature is approximately 5:5:1, using a 15 mM sample. The low-intensity form was shown to be the monomer by diluting the sample by a factor of 10. The ratio of the three forms was now approximately 1:1:4. These ratios indicate a binding constant of the order of 2×10^3 L M^{-1} for dimer formation. From saturation transfer experiments, the off-rate for the dimer was measured as ca. 8 Hz (and thus dimerization has an on-rate of ca. 2×10^4 L M^{-1}).

The observation of two sets of dimer resonances may be rationalized in terms of two different forms of the dimer of equal energy in slow exchange or asymmetry within the dimer with a slow interconversion rate between the two halves. It was not possible to distinguish between the exchange rates of the two sets of dimer resonances with the monomer by saturation transfer experiments. It was also not possible to distinguish between the intensities of the two sets of dimer resonances. This is suggestive, though not conclusive proof, that there exists a single, asymmetric dimer.

Whether an asymmetric dimer or two different symmetric dimers, the difference between the two forms of the dimer may be probed by examining the protons with the largest chemical shift differences of their dimer resonances. The largest differences (see Table I) are observed within the tetrasaccharide portio of the molecule, e.g., Rh_5 $\Delta\delta$ 0.96 ppm, G_1 $\Delta\delta$ 0.60 ppm, Rh_6 $\Delta\delta$ 0.48 ppm, with smaller changes for aglycone protons adjacent to the tetrasaccharide, e.g., 2c $\Delta\delta$ 0.41 ppm, 6c $\Delta\delta$ 0.29 ppm, 2e $\Delta\delta$ 0.22 ppm. It is likely that one major difference between the two forms is a result of the orientation of glucose with respect to the aglycone portion, specifically with respect to ring 4. The upfield glucose anomeric resonance, and the corresponding G_3 and G_5 resonances, share NOEs with the downfield A_CMe resonance, indicating that in this form the hydrophobic G_1, G_3, G_5 face of glucose lies above the A_CMe binding pocket (cf. the position of the hydrophobic face of vancosamine in vancomycin complexes[14]). A NOE confirming this is observed between G_1 (upfield) and 2e (downfield). The corresponding NOEs are not observed for the other dimer conformer. It is likely that in this latter form the G_1, G_3, G_5 face of glucose lies on the back side of the aglycone to which it is bonded, such that G_1 lies over the edge of ring 6 of the dimer partner. This would account for its relatively low-field resonance (δ 5.55 ppm). The anomalously upfield shifts of Rh_5 and Rh_6 in one form are indicative of it being over the face of an aromatic ring. The NOE between Rh_6 (upfield) and R_1 (downfield) is evidence that the aromatic ring in question is ring 6, although it is not known whether it is ring 6 of the same molecule or the dimer partner. Furthermore, it is not known which orientation of glucose with respect to ring 4 is populated when the 5,6-region of rhamnose is over ring 6.

Dimerization of the Ristocetin Ψ–Tripeptide Complex. Reinspection of the spectra of the ristocetin Ψ–tripeptide complex reveals that a similar dimer⇔monomer process is occurring in this system. The intermolecular NOEs $R_1 \leftrightarrow$ 2c, $R_2 \leftrightarrow$ 2c, and $R_3 \leftrightarrow x_3$ are observed, but the off-rate of the dimer is such that the separate proton resonances are not observed for dimer and monomer. The resonance most perturbed on dimer formation in the ristocetin A–tripeptide complex, 6e ($\Delta\delta$ ca. 2.2 ppm), is not observed in the ristocetin Ψ–tripeptide complex, presumably because its line width is too large. Other strongly perturbed resonances in the ristocetin A–tripeptide system, e.g., x_4 ($\Delta\delta$ ca. 0.8 ppm) and 6f ($\Delta\delta$ ca. 0.7 ppm), are present in the ristocetin Ψ–tripeptide spectra as single, *broad* resonances, indicating that they are only just on the fast-exchange side of the coalescence point. Therefore, the off-rate of the ristocetin Ψ–tripeptide dimer at room temperature must be greater than ca. 300 Hz, compared with ca. 8 Hz for the ristocetin A–tripeptide dimer. As a sample of the ristocetin Ψ–tripeptide complex is heated, the resonance of, for example, x_4 sharpens *and* moves upfield indicating that the equilibrium constant for dimerization falls with temperature. Thus, it has a negative ΔS component. It may be concluded that it is the tetrasaccharide attached to ring 4 that slows the kinetics of dimer separation (the ring 7 mannose being on the opposite face to the intermolecular interaction).

Dimerization of Other Species. The dimerization phenomenon is probably not unique to ristocetin complexes, aqueous solvents, or even ristocetin. For example, in the spectrum of ristocetin A in D_2O–CD_3CN solution, the resonances of the two forms observed for the Rh_6 methyl group[13] are of identical chemical shift to the two forms of the *dimer* in the ristocetin A–tripeptide complex; in the spectrum of ristocetin Ψ in D_2O–CD_3CN solution, the resonances of x_4 and 6e are again very broad; in that of ristocetin Ψ in DMSO solution,[15] the resonances of x_3 and 6f join those of x_4 and 6e in having large line widths; in that of A40926-PS in DMSO solution, the resonances of residue 3 are particularly broad.[15] Vancomycin appears to dimerize in aqueous solution but not in DMSO solution, although interpretation of line width phenomena is slightly confused by a dynamic process involving the w_3 amide unit.[16] Indeed, in studies of the specific rotation of vancomycin in 0.02 M citrate buffer,[17] it was noted that significant aggregation of the antibiotic occurred at millimolar concentrations. At 10 mM, aggregation was judged to be total, and although no geometry of the aggregated species was determined, an association constant of 8×10^2 L M^{-1} was calculated, assuming dimerization to have occurred. This figure is comparable with the association constant of 2×10^3 L M^{-1} calculated above for the ristocetin A–tripeptide complex. Dimerization may also explain the two conformers recently reported for teicoplanin A_2.[18] The downfield shift of x_4 ($\Delta\delta$ 0.61 ppm) is similar to that observed for ristocetin, but small changes for the resonance of 6e suggest that if a dimer is formed, its geometry is significantly different from that of ristocetin. The other resonances shifted considerably for teicoplanin are 3b ($\Delta\delta$ 0.23 ppm) and 5f ($\Delta\delta$ 0.23 ppm). The critical micelle concentration of teicoplanin (at pH 7.4) has been measured[19] to be 210 μM, suggesting that intermolecular interaction *is* prevalent in this system at the concentrations used in NMR studies.

It is possible that dimerization has a role in the biological function of ristocetin. For example, the affinity of one ristocetin molecule for another might promote the siting of a second antibiotic molecule with one already bound to a cell-wall precursor terminating in -D-Ala-D-Ala. If such cell-wall precursor molecules are concentrated locally, then the antibiotic could thereby be targeted more effectively. Alternatively, the same end might be achieved by delivery of antibiotic dimers to locally concentrated cell-wall precursors terminating in -D-Ala-D-Ala. There are indications that dimerization may be most strongly promoted in aqueous media, e.g., ristocetin A dimerizes in D_2O, but much less so (if at all) in DMSO.[13] If this is generally true, then the fact that the off-rate for the dimer of ristocetin A in D_2O (40 Hz) at room temperature is faster than that (8 Hz) for the dimer of the ristocetin A–tripeptide complex in 5:2 D_2O–CD_3CN implies an even greater difference for these rate constants if both were determinable in D_2O. The larger barrier to the off-process for the dimer of the complex (relative to that for the simple dimer) implies that dimerization is promoted by filling the antibiotic binding cleft with the cell-wall analogues. This implication is physically reasonable since it has already been noted that the carboxylate anion of the cell-wall analogue (bound to half of the antibiotic dimer) can indirectly interact with the protonated amine of ristoamine (in the other half of the antibiotic dimer). Additionally, the "back-faces" of ristocetin A that are involved in the dimerization (Figure 6) should have a greater mutual affinity the more remote

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are the hydrogen bonds formed between the faces from bulk water. That is, these hydrogen bonds are more buried from bulk water, and thereby strengthened, when the cell-wall analogues are bound. In a similar way, the hydrogen bonds formed deep in the interior of proteins should in general be stronger than those near the surface. In this sense, the aggregates discussed in this paper model some aspects of protein folding. In relation to a possible biological role for dimerization, it should be noted that, with a dimerization equilibrium constant of 2×10^3 L M^{-1} at ristocetin concentrations of less than ca. 1 mM, the concentration of monomer outweighs that of dimer. The minimum inhibitory concentration (MIC) values for ristocetin[20] are of the order of 5 μM, which means that the antibiotic will occur as the monomer in solution (assuming the $K_{\text{dimerization}}$ for unbound ristocetin is similar to that for the tripeptide complex). However, this is not a measure of the concentration of antibiotic at the active site which would be expected to be much higher. Ristocetin may dimerize when bound to the bacterial cell wall unless the pulling together of two peptidoglycan termini is energetically restricted.

Conclusions

For ristocetin Ψ in the pseudoaqueous solvent 5:1 D_2O–acetonitrile-d_6, there is no evidence of sizable oscillations of rings 1 and 3. On addition of tripeptide, the NOEs of ring 3 are unchanged, whereas the changes of those of ring 1 indicate a rotation of the time-averaged orientation of the aromatic ring with respect to the peptide backbone. The major consequence of this is that it is energetically favorable for ring 1 to prevent solvent access to the carboxylate anion of the bound tripeptide but not to the polar groups of the binding pocket in the absence of peptide. This is consistent with our general observation of binding processes within the vancomycin group that the prevention of solvent access to the intermolecular electrostatic interactions is an energetically advantageous process. Intermolecular NOEs appropriate to the folding-in of ring 1 on addition of tripeptide are observed, e.g., between 1f and A_CCH, and 1e and A_NMe.

The different forms observed in a ristocetin A–tripeptide complex in D_2O–acetonitrile-d_6 solution are *not* the result of some slow intramolecular motion but represent the formation of either an asymmetric dimer or two symmetric dimers, with an association constant of ca. 2×10^3 L M^{-1}. The geometry of the two forms of the dimer, as determined by chemical shift changes and intermolecular NOEs, is such that the backsides of two molecules come together forming hydrogen bonds along the antibiotic amide backbone. This causes ring 6 of each molecule to lie close to the face of ring 4 of the other. The difference between the two forms of the dimer appears to be a result of the orientation of the tetrasaccharide attached to ring 4 of ristocetin. Primarily, it appears that the hydrophobic G_1, G_3, G_5 face of glucose occupies one side of ring 4 or the other. Also, in one form, the Rh_5, Rh_6 region lays over the face of ring 6.

Similar dimerization is observed in the ristocetin Ψ–tripeptide complex, although the off-rate of the dimer is considerably faster. This further implicates the tetrasaccharide in a specific role in dimer formation in addition to causing asymmetry. It appears that dimerization also occurs, but to a lesser extent, in DMSO solution and *may* be responsible for some of the NMR phenomena observed for vancomycin, A40926, and teicoplanin A_2. The geometry of the dimer is such that the intermolecular hydrogen bonds are shielded from the solvent by the coming together of large hydrophobic regions of the amino acid side chains within the antibiotic. This is analogous to the binding of the target peptide on the opposite face of the antibiotic.

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A New Electronegativity Scale for the Correlation of Heats of Formation. 2. The Differences in Heats of Formation between Hydrogen and Methyl Derivatives

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Abstract: A new empirical equation $\Delta\Delta_f H°(CH_3X/HX)/p = a' + b'V_X$ (5) has been found where $\Delta\Delta_f H°(CH_3X/HX) = \Delta_f H°(CH_3X) - \Delta_f H°(HX)$, p is the number of hydrogen atoms in the HX molecule, V_X is the unshielded core potential of the atom in X attached to H, and a' and b' are constants. Heats of formation of some Si-, Ge-, Sn-, P-, As-, and Se-containing compounds are estimated with eq 5. Two sets of (a,b) constants are found to be very similar. One describes the relation when X is a monovalent atom and the other applies to polyvalent atoms.

Dating from the earliest efforts by Pauling in the 1930's to find quantitative relationships between heats of formation in homologous or otherwise related series of compounds and some measure of "electronegativity" of the atoms involved in bonding, there has developed a huge literature. Many of these efforts have been restricted to either single-bonded diatomic molecules or to substitutional effects in homologous compounds. Many of the authors have used different measures of electronegativity. Many of these efforts have been partially successful in correlating the empirical data in quantitative or semiquantitative terms. In recent years with greater facility by theoreticians in using various ab initio methods there has been a renewed effort to find empirical correlations for substituent effects in homologous series of organic compounds. Schleyer and co-workers have been among the most active in this field. Some of their findings and references to much of the earlier work will be found in a series of recent papers[1-3]

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