^{1}H N.M.R. Studies of the Structure of Ristocetin A and of its Complexes with Bacterial Cell Wall Analogues in Aqueous Solution

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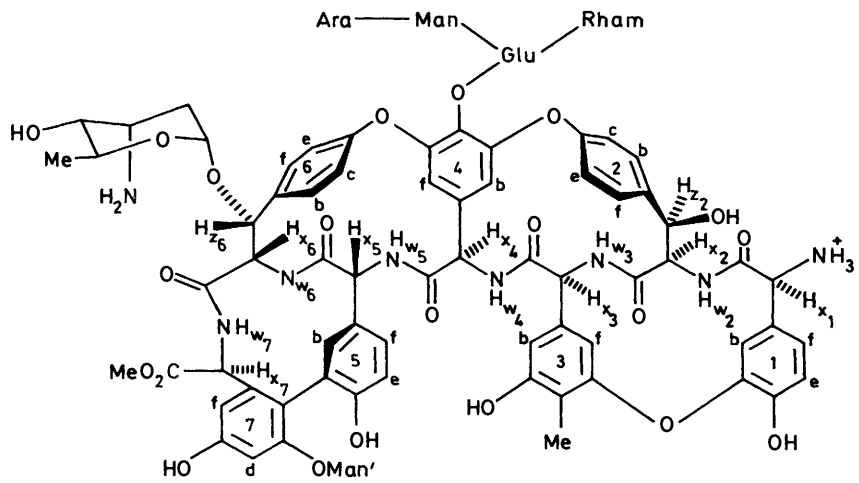
The conformation of ristocetin A in aqueous solution is shown to be very similar to that previously found in dimethyl sulphoxide solution. In particular, there is a cleft on one face which binds the carboxylate anion of bacterial cell wall analogues, and which is not fully accessible to the solvent. Binding studies with the analogues *N*-acetyl-D-alanyl-D-alanine and *N,N*-diacetyl-L-lysyl-D-alanyl-D-alanine indicate that the structures of the complexes with ristocetin A are also very similar to those previously found in dimethyl sulphoxide solution; again, there is very unusual behaviour of some amide protons. Two-dimensional nuclear Overhauser effect studies in D_2O show that the lysyl side chain is in a different orientation in aqueous solution to that found in dimethyl sulphoxide.

Ristocetin A is a glycopeptide antibiotic of the vancomycin class[1] and has the structure (**1**).[2,3] It acts by binding to bacterial cell wall precursors, which in many species have structures ending at the C terminus with -L-Lys-D-Ala-D-Ala. The peptides Ac-D-Ala-D-Ala and Ac_2-L-Lys-D-Ala-D-Ala (**2**) are good models for studying the interactions of the vancomycin class of antibiotics with cell walls, as they have been shown by u.v. difference spectroscopy to bind to the antibiotics almost as tightly as the intact cell wall.[4] Extensive studies have already been made by n.m.r. in [$^2H_6$]dimethyl sulphoxide solution on the structure of ristocetin[5] and its complexes with the above-mentioned peptides,[3,6] in particular using ^{1}H chemical shifts,



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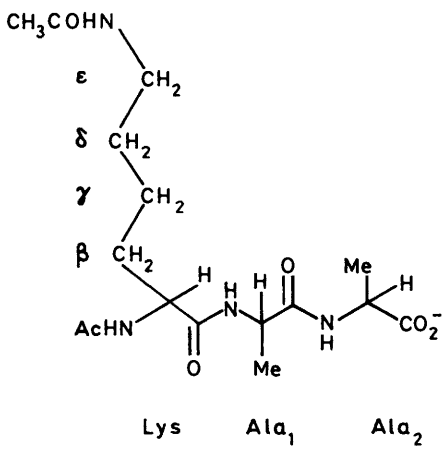

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nuclear Overhauser effects (n.O.e.s) and NH temperature coefficients. The results of these studies have been able to provide a satisfactory explanation for the observed binding constants to a variety of peptides in aqueous solution, and to provide a *model for the kinetic* processes involved in binding.[7] However, it was felt that, as the results were obtained in a non-aqueous solvent, their relevance to physiological conditions was suspect, and accordingly we report here a study of the antibiotic and of its interactions with peptides in aqueous solution. It will be shown that the results obtained in aqueous solution almost exactly parallel those found in dimethyl sulphoxide, with the exception of the lysyl sidechain in the complex between ristocetin A and Ac_2-L-Lys-D-Ala-D-Ala, which adopts a strikingly different conformation in aqueous solution.

Experimental

Ristocetin A was a gift from Lundbeck (Copenhagen). Ac-D-Ala-D-Ala and Ac_2-L-Lys-D-Ala-D-Ala were synthesized as previously described.[3] Samples were made up in 0.05M-NaCl solution and the pH was adjusted using 0.1—0.5M-NaOD and DCl. The pH was measured using a Corning pH meter 125 equipped with a combination glass electrode and the reported values are uncorrected for isotope effects. pH Values were checked before and after each set of measurements. Sample concentrations were roughly 15×10^{-3}M, and the temperature was calibrated using ethylene glycol.[8] Measurements were made on Bruker WM400 and AM500 spectrometers equipped with Aspect 2000 computers. Chemical shifts were measured with respect to internal [$^2H_4$]trimethylsilyl propionate (TSP).

Solvent suppression in H_2O was carried out by presaturation of the solvent resonance for 0.5—1.0 s: similar results could be obtained by multipulse techniques, but with poorer solvent suppression and no gain in information. N.O.e. and transfer of saturation experiments in H_2O were carried out by irradiating alternately the H_2O resonance for 50 ms and the desired resonance for 25 ms, over a period of 0.9 s before signal acquisition, at which point the decoupler was gated off. Absorption mode NOESY experiments in D_2O were carried out using the time-proportional phase incrementation scheme described previously[9] and processed using a modified version of the Bruker program FTNMR2D.

Results

Assignment of Exchangeable Protons.—The exchangeable protons of ristocetin A and its complexes with Ac-D-Ala-D-Ala

Table 1. Chemical shift assignments[a] (δ/p.p.m.) of free and bound ristocetin A; T = 20 °C, pH 4.5

Proton	Free compound	Complex w. Ac_2-K-A-A	Proton	Free compound	Complex w. Ac_2-K-A-A
x_1	4.87	4.97	6b	7.53	7.25
w_2	7.94	11.61	6c	6.94	6.98
x_2	5.18	5.18	6e	7.23	7.21
z_2	5.44	5.38	6f	7.42	7.34
w_3	7.44	9.72	7d	6.94	6.91
x_3	5.52	5.53	7f	6.48	6.49
w_4	8.33	9.43	3Me	2.07	2.03
x_4	5.67	5.93	G_1	5.18	5.18
w_5	9.24	9.23	A_1	5.59	5.70
x_5	5.18	5.29	Rh_6	1.07[b]	1.08
x_6	4.36	4.75	Ri_1	5.42	5.29
z_6	5.59	5.50	Ri_2	2.04	2.15
w_7	9.55	9.58	Ri_2'	2.31	2.30
x_7		4.75	M_1'	5.44	5.39
1b	6.94	6.78	Ala_1Me	1.40	0.91
1e	7.06	6.91	Ala_2Me	1.42	0.59
1f	7.16	7.10	Ala_1CH	4.38	4.94
2b	7.23	7.12	Ala_2CH	4.32	4.19
2c	7.42	7.28	Ala_1NH	8.33	8.14
2e	7.36	7.19	Ala_2NH	8.45	8.57
2f	7.94	7.85	LysαNH	8.40	8.32
3b	6.52	6.70	LysβCH_2	1.73	1.63
3f	6.51	6.65	LysγCH_2	1.47	1.38
4b	5.85	5.87	LysδCH_2	1.52	1.63
4f	5.54	5.51	LysεCH_2	3.19	3.26
5b	7.06	7.20	LysεNH	8.20	8.08
5e	6.50	6.87			
5f	6.94	6.99			

[a] Values are ±0.02 p.p.m., with respect to internal [$^2H_4$]trimethylsilyl propionate. [b] At low temperature, splits into two signals at 1.28 and 0.98 p.p.m. (see text).

Table 2. Temperature coefficients of amide protons [10^{-3} δ/T(p.p.m./K)$^{-1}$]; pH 4—5, T = 20 °C[a]

Proton	Free compound	Complex w Ac-A-A	Complex w Ac_2-K-A-A
w_2	−3.5	−2.2	−1.4
w_3	*b*	−0.5	−0.6
w_4	−11.5	−0.5	−1.0
w_5	−7.5	−7.0	−7.0
w_7	−5.5	−1.5	−0.7
Ala_1NH	−8.5	−5.6	*b*
Ala_2NH	−11.5	−5.5	−1.0
LysαNH	−8.5		−5.0
LysεNH	−9.0		*b*

[a] Taken as the average value over the range 10—30 °C. A negative sign indicates a decrease in chemical shift as the temperature is increased (Figure 1). [b] Not observed.

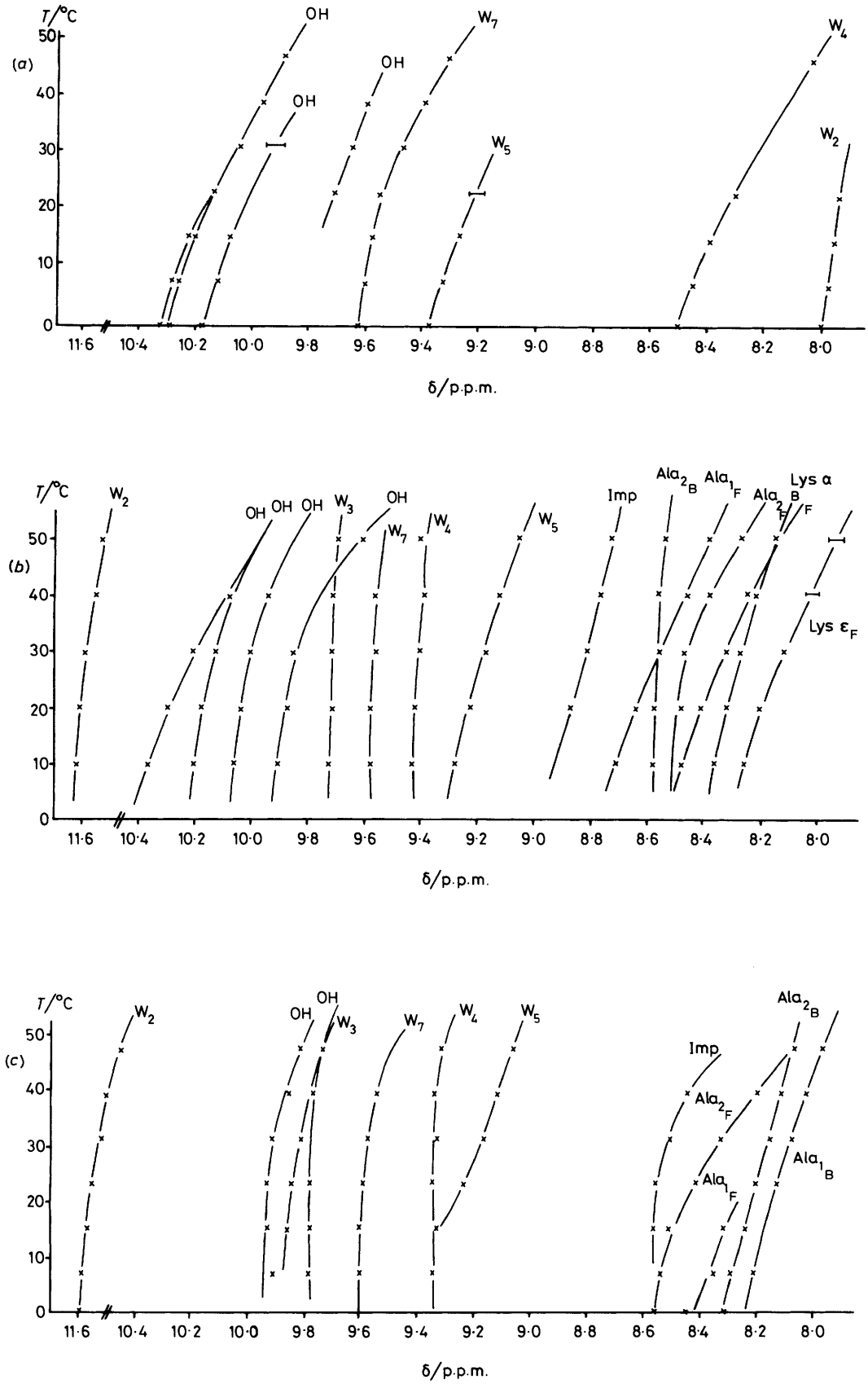

Figure 1. Temperature dependence of NH and OH chemical shifts at pH 4.5. (*a*) Ristocetin A; (*b*) ristocetin A/Ac_2-L-Lys-D-Ala-D-Ala complex; (*c*) ristocetin A/Ac-D-Ala-D-Ala complex

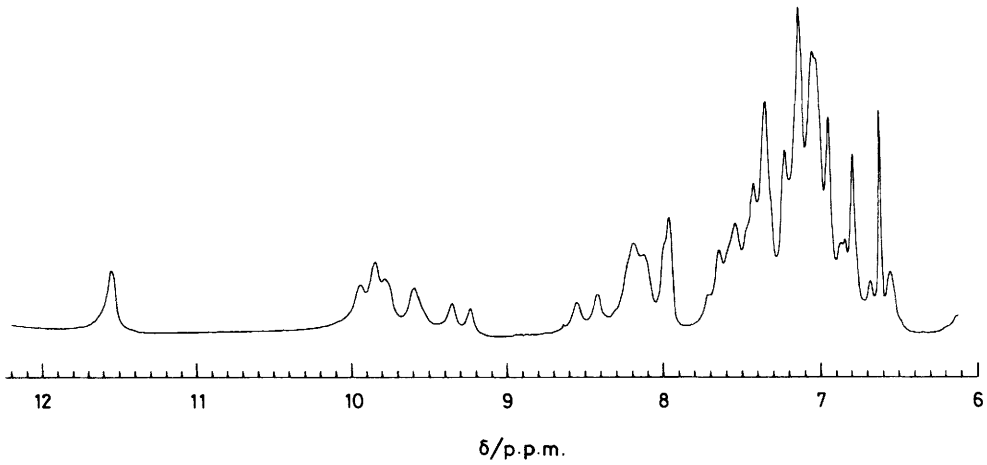

Figure 2. Aromatic, NH, and OH protons in the spectrum of ristocetin A + Ac-D-Ala-D-Ala (1 equiv.), pH 5.99, T = 293 K

Table 3. Proton exchange rates

(*a*) Free ristocetin, T = 276 K, pH 2.34

Proton	OH	OH	OH	w_7	w_5
Exch. rate (10^4 k/s^{-1})	7.0	8.8	17	0.8	0.7

(*b*) Ristocetin/Ac-D-Ala-D-Ala complex, T = 286 K, pH 4.23

Proton	w_2	w_3	w_7	w_4
Exch. rate (10^4 k/s^{-1})	5.5	3.8	3.5	4.5

Table 4. N.O.e.s seen involving exchangeable protons of ristocetin A

Irr.	Obs.	Irr.	Obs.
w_2	w_3[a,b]	w_5	5f[a]
w_2	x_1[b]	w_5	3Me[a]
w_2	2f[a,b]	w_5	3cOH[a,b,c]
w_3	w_2[a,b]	w_7	5b[a]
w_3	2f[b]	w_7	z_6[b]
w_4	w_3[a]	Ala_1NH	AcMe[a]
w_4	Ala_2Me[a]		

[a] Seen in the complex with Ac-D-Ala-D-Ala. [b] Seen in the complex with Ac_2-Lys-D-Ala-D-Ala. [c] At 9.84 p.p.m., T = 20 °C, pH 4.3.

and Ac_2-L-Lys-D-Ala-D-Ala were studied at a range of different temperatures and pH values between T = 0—70 °C and pH 2.5—7.0. Typical results are shown in Figures 1 and 2. The final assignments of the protons are given in Table 1, and temperature coefficients in Table 2. Variation of chemical shift with temperature was by no means linear, as shown in Figure 1. It proved to be surprisingly difficult to distinguish amide NH and phenolic OH protons, as their exchange rates were not sufficiently dissimilar to permit ready differentiation by line width measurements or transfer of saturation from the solvent signal, and the line-width of the signals was such that decoupling experiments gave no meaningful results. Nevertheless at higher temperature and pH, the OH protons were in general broadened before the NH protons, although this could not be relied on as a method of assignment. In particular, the signal assigned to the amide proton w_5 broadened out into the baseline at significantly lower temperature and pH than several of the OH protons. In some cases exchange rates could be measured directly by dissolving the sample in D_2O and watching the decay of the signal with time: the results are given in Table 3. Protons exchanging at rates less than 10^{-4} s^{-1} at pH 2.5 or 10^{-3} s^{-1} at pH 4.3 can be identified as NH protons. Comparison of the spectra of ristocetin A and its complexes was a further guide. Final assignments of the exchangeable protons could be made by n.O.e. and transfer of saturation experiments.

Transfer of saturation experiments were carried out on a sample containing ristocetin–Ac-D-Ala-D-Ala (2 mol:1 mol) and permitted the pairwise assignments of the free and bound signals of five of the six NH protons of ristocetin A. The sixth NH proton is hidden under the aromatic envelope in both spectra, and was never found. Bound signals in the complex with Ac_2-L-Lys-D-Ala-D-Ala were similar enough to those in the complex with Ac-D-Ala-D-Ala to be assigned by comparison of chemical shifts and temperature coefficients. No transfer of saturation was observed for phenolic protons, presumably because of the greater exchange rate of these protons with the solvent.

N.O.e. experiments were carried out on ristocetin A and on the two complexes, and some of the n.O.e.s observed are listed in Table 4. These n.O.e. results should be compared with the space-filling model of ristocetin and Ac_2-L-Lys-D-Ala-D-Ala shown in Figure 3, on which most of the relevant protons are indicated. The n.O.e.s were sufficient to provide tentative assignments for all of the observed NH protons, and one of the OH protons. Confirmation came from comparing chemical shifts of the free and bound ristocetin A and peptides, and all assignments were confirmed by comparing the shifts in H_2O with those in dimethyl sulphoxide, which in most cases (particularly in the complexes) were very similar.

Assignments of Non-exchangeable Protons.—This part of the work was carried out in D_2O. Two-dimensional correlated spectroscopy (COSY[10]) was used to provide coupling information, and absorption mode two-dimensional n.O.e. spectroscopy (NOESY[9]) to provide n.O.e. information. The n.O.e.s seen were essentially identical with those observed previously in dimethyl sulphoxide solution, notably the very strong n.O.e.s among the group of protons x_6, z_6, x_5, 6b, 5b, and, in the complexes, Ala_1CH (see Figure 3). The assignments are listed in Table 1. Most chemical shifts are very similar to those found in dimethyl sulphoxide solution.[6,11] The NOESY spectrum used to provide n.O.e.s for the ristocetin A/Ac_2-L-Lys-D-Ala-D-Ala complex is shown in Figure 4, and the corresponding one-dimensional spectrum in Figure 5. Two significant n.O.e.s which proved particularly useful in defining the orientation of the lysyl side chain are indicated by arrows in Figure 4, and will be discussed below.

Figure 3. Space-filling model of the complex between Ac_2-L-Lys-D-Ala-D-Ala and ristocetin A, exploded view. The molecules are shown in the conformations indicated by this study, except that ristocetin A has been slightly opened out for clarity. Hydrogen bonds formed in the complex are indicated by dashed lines. The protons marked are discussed in the text. The two n.O.e.s marked by solid arrows, between LysδCH_2 and Ala_1Me, and LysεCH_2 and M_1', were of crucial importance in establishing the orientation of the lysyl side chain in the complex

Discussion

The Structure of Ristocetin A in Aqueous Solution.—Previous studies of ristocetin A have been carried out in dimethyl sulphoxide solution, because the amide protons could be readily observed. As an added bonus the line-widths are lower in this solvent. We have previously assumed[3,7,12] that the structures observed in dimethyl sulphoxide would be maintained in aqueous solution, and this has been shown to be true for vancomycin.[13] Studies on avoparcin in aqueous solution show that the structures of its complexes with cell wall analogues are very similar to those found for ristocetin and vancomycin in dimethyl sulphoxide.[14,15] The studies described here show that the structure for ristocetin A is very similar in the two solvents, as expected. We are particularly interested in the solvent accessibility of the amide protons in ristocetin A, as from the dimethyl sulphoxide studies three of the protons appear to be in a cleft in the molecule which is involved in binding to the carboxylate anion of the cell wall analogue.[7] The chemical shift is not a good guide to solvent accessibility, especially here as there are so many aromatic rings present. A fairly good guide to

accessibility is, however, provided by the temperature coefficients of the amide protons,[16] which for ristocetin A in dimethyl sulphoxide were found to vary between -6×10^{-3} p.p.m./K for the most exposed proton and -0.5×10^{-3} p.p.m./K for the least exposed. The results given in Table 2 show that in H_2O the coefficients are larger but show the same trends, and in particular that the proton w_2, which is in the carboxylate-binding pocket, is rather shielded from the solvent. Proton w_3 is also expected to be in the carboxylate-binding pocket (see Figure 3), but the temperature coefficient of this proton could not be measured with sufficient accuracy as it is obscured by aromatic protons and could only be located by transfer of saturation experiments. The third proton in the carboxylate-binding pocket is w_4, which in dimethyl sulphoxide shows a low temperature coefficient, indicating reduced solvent accessibility. However in H_2O the temperature coefficient is consistent with full solvent accessibility. Proton w_7, which is also involved in binding to the peptide, has a low temperature coefficient, and is therefore somewhat shielded from the solvent, as found in dimethyl sulphoxide.

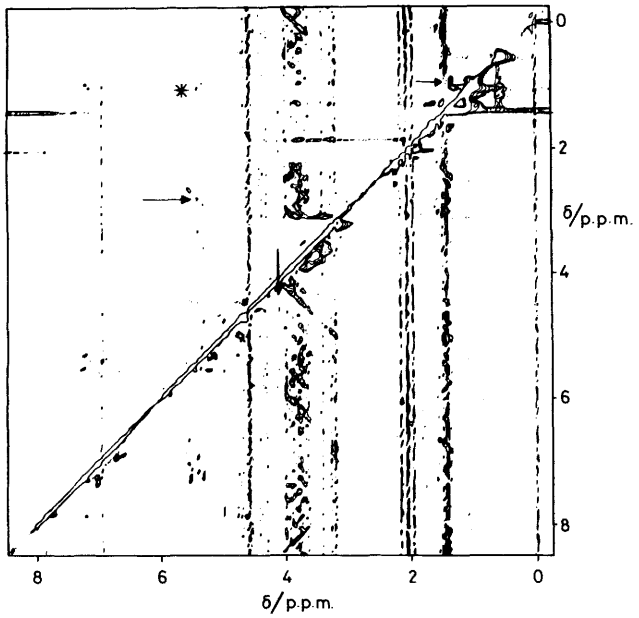

Figure 4. Absorption mode NOESY spectrum of the complex between ristocetin A and Ac_2-L-Lys-D-Ala-D-Ala, pH 4.5, T = 318 K. A faint image diagonal can be seen running from top left to bottom right, arising from inaccurate 90° phase shifting. A number of intense bands of t_1 noise are also evident, which arise mainly from instability of the variable temperature unit. They are particularly marked as many of the n.O.e.s are rather weak, and low contour levels are plotted. The two n.O.e.s indicated by arrows and that marked by an asterisk are discussed in the text

The Structures of the Complexes between Ristocetin A and Cell Wall Analogues.—Once again, the structures in aqueous solution and in dimethyl sulphoxide are closely similar. The changes both in chemical shift and in temperature coefficient observed parallel those seen in dimethyl sulphoxide, with very striking large changes observed particularly for w_2 and w_3, the two protons in the bottom of the carboxylate-binding cleft. The chemical shifts of w_2 and w_3 (11.61 and 9.72 p.p.m. respectively) are most unusual and are indicative of the strength of the hydrogen bonding to the carboxylate anion of the peptide. The charge on the carboxylate and the hydrophobic nature of the carboxylate-binding pocket both contribute to the strength of the bonding.[7]

It has previously been observed[17] in the spectra of ristocetin A in D_2O that a sugar methyl group at 1.07 p.p.m., now identified[5] as the rhamnose methyl proton Rh_6, can exist in two environments which are in slow exchange on the n.m.r. timescale, with a rate constant for the exchange process of *ca.* 40 s^{-1} at 24 °C. A number of other protons have now also been observed to show similar splitting, including the aromatic proton 4b. On addition of one equivalent of Ac-D-Ala-D-Ala or Ac_2-L-Lys-D-Ala-D-Ala the Rh_6 signal sharpens to a single peak, as do the other protons involved. This is an indication that, in the absence of cell wall analogue, the rhamnose can

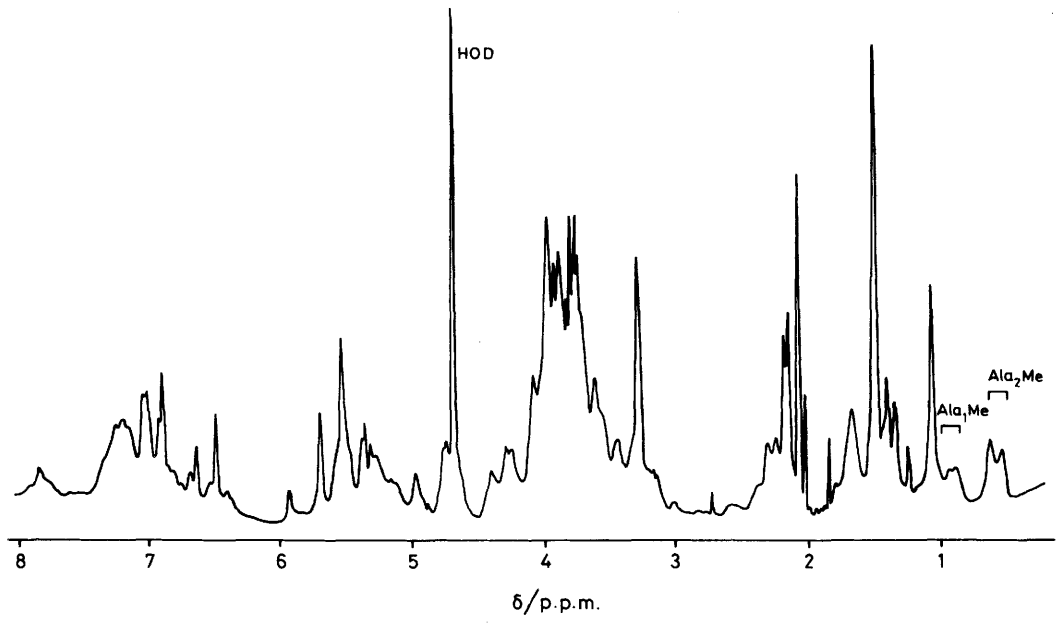

Figure 5. Ristocetin A/Ac_2-L-Lys-D-Ala-D-Ala complex, pH 4.5. The temperature was lowered to 313 K, to bring the two alanyl methyl groups fully into the slow exchange regime

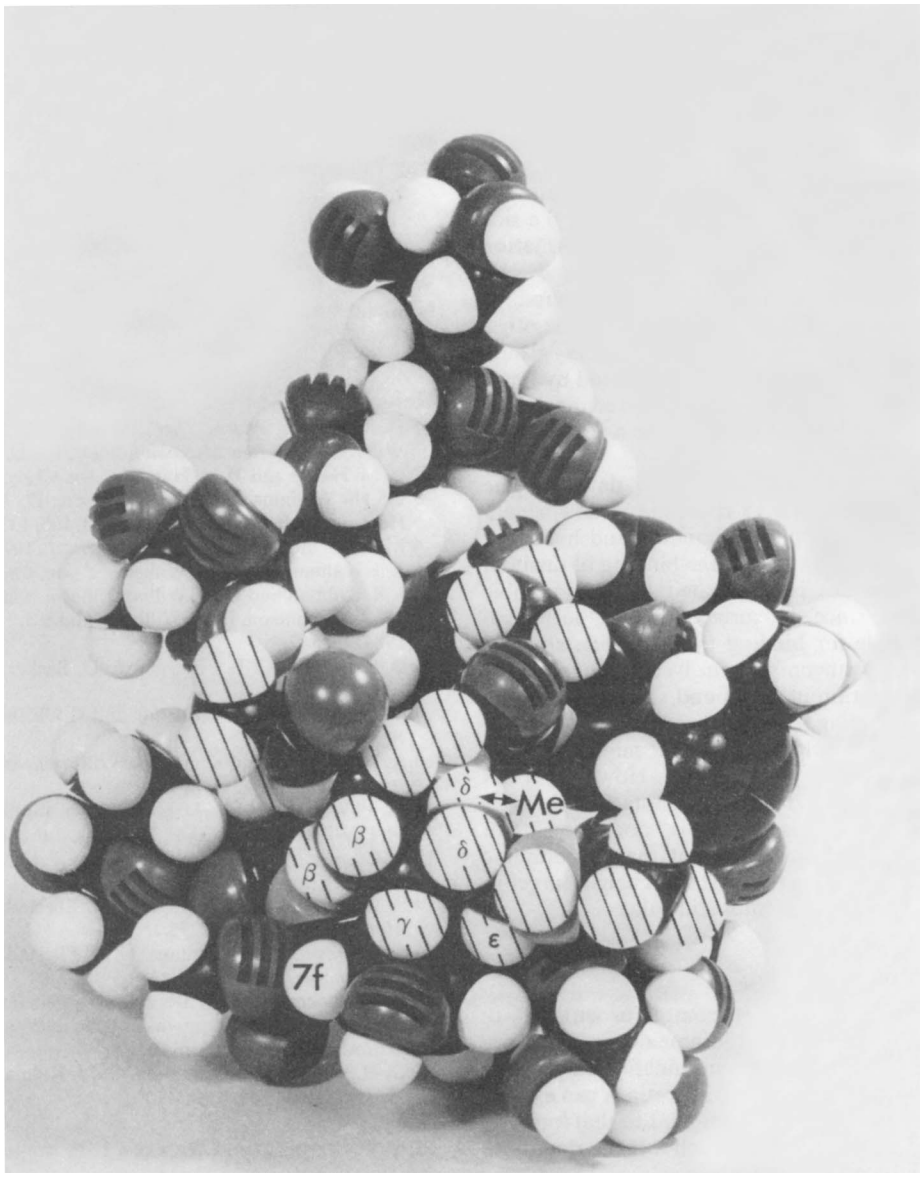

Figure 6. Space-filling model of the complex between Ac_2-L-Lys-D-Ala-D-Ala and ristocetin A. Protons of the tripeptide are shaded. Note that the carboxylate pocket has been closed up relative to its position in Figure 3

adopt a position somewhere in the binding site for the cell wall analogue, probably near the carboxylate binding site. The fact that this behaviour was not observed in dimethyl sulphoxide may indicate that hydrophobic forces predominate in this interaction.

Another interesting conformational feature of both complexes is that there is clear evidence for two different conformations of the complex: in the complexes both with Ac-D-Ala-D-Ala and with Ac_2-L-Lys-D-Ala-D-Ala, two sets of bound alanyl methyl signals can be seen, which are in slow exchange, one being about 1.5 times the intensity of the other. This is most clearly seen in Figure 5, where the two pairs of alanyl methyls are indicated, and is also evident in Figure 4. The n.O.e. indicated by an arrow at the top right of the figure is between LysδCH_2 and Ala_1Me, and would be merely a circular blob in normal circumstances. It is however clearly elongated in the vertical direction, as a consequence of the n.O.e. going to both Ala_1Me signals, at 0.94 and 0.88 p.p.m. This indicates that LysδCH_2 is close to Ala_1Me in both bound conformations. By contrast, there is an n.O.e. to Ala_1Me from the anomeric proton of the mannose residue M′ (M_1′ at 5.39 p.p.m.), indicated by an asterisk in Figure 4, which only involves the lower field Ala_1Me signal. This indicates that M_1′ is close to Ala_1Me only in one of the two bound conformations, and suggests that the difference between the two bound conformations may involve the orientation of the mannose M′. In Figure 3, mannose M′ is positioned such that M_1′ will be in contact with the bound Ala_1Me.

The Orientation of the Lysyl Side Chain.—In the complexes between Ac_2-L-Lys-D-Ala-D-Ala and vancomycin or ristocetin A in dimethyl sulphoxide, the lysyl side chain lies along the side of ring 6.[3] However, recent work on the similar antibiotic avoparcin has shown that for this antibiotic in aqueous solution the lysyl side chain lies over the face of ring 7.[15] This latter conformation is close to the standard extended peptide

backbone conformation, and is therefore the more populated conformation for free peptide. It is thus of particular interest to examine the conformation of the lysyl side chain in the complex with ristocetin A in aqueous solution, to see whether the different conformation found for avoparcin is a peculiar feature of avoparcin or is more general. The lysyl conformation is indicated by the two n.O.e.s marked by arrows in Figures 3 and 4, which are between LysεCH_2 and M_1', and LysδCH_2 and the Ala_1 methyl group. These give a clear indication that the side chain is indeed lying over ring 7, and therefore its conformation changes on changing the solvent.

Figure 6 shows how the peptide looks when bound to ristocetin. The LysδCH_2–Ala_1Me n.O.e. is indicated; the other n.O.e. is not marked, as M_1' is obscured by the LysεCH_2 group. The conformation shown is consistent with that found by Fesik *et al.*[15] for the avoparcin complex; they observed an n.O.e. between Lysβ and/or γ and 7f (see Figure 6). The n.O.e. was not seen in the present work.

We have previously used thermodynamic data for the binding of Ac_2-L-Lys-D-Ala-D-Ala to ristocetin A and vancomycin in a discussion of hydrophobic bonding and hydrogen bond strength.[12] There we showed that the binding of the lysyl side chain to the antibiotics is dominated by hydrophobic forces, and argued that, since the surface area of the hydrophobic region responsible for binding the lysyl side chain is larger in ristocetin than in vancomycin, the lysyl side chain must have more freedom of motion in the bound state in ristocetin. At that time we were assuming that the lysyl side chain lies over ring 6, as found in dimethyl sulphoxide, and it might therefore be felt that our argument is now invalidated. However, it has previously been shown [3] that the mannose M′ attached to ring 7 has a hydrophobic face. This sugar is not present in vancomycin, and thus it remains true that the hydrophobic surface area available for binding the lysyl side chain is larger in ristocetin, and our previous argument is still valid.

Conclusions

The structure of ristocetin A and of its complexes with Ac_2-L-Lys-D-Ala-D-Ala and Ac-D-Ala-D-Ala in aqueous solution are very similar to those previously found in dimethyl sulphoxide solution. In the absence of the peptides, ristocetin A can exist in two conformations, one of which is very similar to that found in dimethyl sulphoxide and the other of which has the rhamnose sugar occupying part of the peptide binding site. The complexes can also exist in two conformations, which may differ in the orientation of the mannose M′. The most significant difference between the dimethyl sulphoxide conformation and the aqueous conformation is in the position of the lysyl side chain, which is along the side of ring 6 in dimethyl sulphoxide but over ring 7 in aqueous solution.

Acknowledgements

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