An NMR Study of the Interaction between the Antibiotic Ristocetin A and a Cell Wall Peptide Analogue. Negative Nuclear Overhauser Effects in the Investigation of Drug Binding Sites

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Abstract: An analysis of the ^{1}H NMR spectrum of a ristocetin A/acetyl-D-alanyl-D-alanine complex in dimethyl sulfoxide is reported. The spectra of both the bound and free antibiotic and peptide having been assigned, the resonances in both series of spectra were then correlated by coalescence and transfer of saturation experiments. Information on the conformation of the bound peptide was derived from negative intramolecular nuclear Overhauser effects (NOEs) and $J_{\alpha,NH}$ coupling constants. The nature of the antibiotic–peptide binding site was then deduced from the temperature coefficients and chemical shifts of amide NH resonances, in conjunction with model-building studies. Finally, the nature of the binding site was confirmed by the observation in the spectrum of four intermolecular NOEs between the protons of the bound peptide and ristocetin A. The binding site is similar to that proposed for vancomycin, but strengthened by bonding of the carboxyl terminus of Ac-D-Ala-D-Ala to several NH protons of ristocetin A.

Introduction

In the preceding paper,[1] on the basis of NMR evidence, we have proposed a structure (ambiguous at only one asymmetric center) for the aglycone of the glycopeptide antibiotic ristocetin A. In the present paper, we report a similar study on a complex of ristocetin A with the peptide Ac-D-Ala-D-Ala. The exchange rate of the complex with its free components in Me_2SO solution is sufficiently slow at 30 °C that, for most proton resonances, discrete signals are observed for the complex and the free components if peptide and antibiotic are present in, say, a 1:2 or 2:1 ratio.[2] The study of the complex is of interest since the antibiotic is known to act by binding to cell-wall mucopeptides terminating in the sequence -D-Ala-D-Ala.[3] Cross-linking of the cell wall is thus inhibited during the process of biosynthesis, leading to a cessation of growth and the eventual destruction of the cell by lysis. The NMR study has allowed us to map out the ristocetin A binding site for Ac-D-Ala-D-Ala, and thus to define the molecular basis for the antibiotic action. In this work, the occurrence of negative nuclear Overhauser effects (NOEs) has provided important information on the details of binding.

Discussion

Assignment of the Free Peptide Resonances. The methyl resonances of the peptide have previously been assigned in D_2O solution.[4] The complete assignments in Me_2SO-d_6 solution for the present work were made from the upfield shifts of the resonances of the C-terminal residue upon addition of solid NaOMe to a Me_2SO-d_6 solution of the peptide; the conclusions (Table I) are in accord with decoupling experiments. The resonances are subsequently referred to by addition of the subscript F (to indicate "free" peptide, e.g., Ala_1NH_F).

Assignment of the Bound Peptide Resonances. Addition of 0.5 molar equiv of ristocetin A to a Me_2SO-d_6 solution of the peptide gives rise to two sets of peptide peaks due to bound and free peptide. The correlation between methyl resonances due to bound and free peptide was made by transfer of saturation experiments. The methyl resonances due to bound peptide occur at unusually high field (0.38 and 0.83 ppm); in separate experiments, these were irradiated and the intensity changes in the other *C*-methyl resonances observed (Table II). Peaks A and D are secondary methyl resonances of ristocetin A sugar residues, whereas peaks B and C are methyl resonances of free peptide (cf. Table I). Peak A is used as a peak intensity reference, while D overlaps C, and the sum of their intensities is given.

These experiments establish that $Ala_2\beta Me_F$ shifts to 0.38 ppm on binding and that $Ala_1\beta Me_F$ shifts to 0.83 ppm. Thus, the methyl resonances of the peptide "cross over" on binding; thus the methyl protons of the carboxyl terminal alanine are remarkably shielded (by 0.89 ppm) in the complex. This result parallels the situation found in the antibiotic vancomycin,[4] and it is probable that in the bound state the Me group of the C-terminal alanine lies over an aromatic ring of ristocetin. Further, from these experiments, all the chemical shifts of the bound peptide can be assigned (Table III) from decoupling experiments.

Assignment of the Spectrum of Bound Ristocetin A. Examination of spectra recorded at a variety of temperatures between 24 and 70 °C, while following the changes in resonance positions of exchangeable protons and taking into account decoupling experiments at 23, 34, and 50 °C and the results of D_2O exchange, enables the protons in the ranges δ 4–12 and 0–2.5 ppm to be assigned (Table IV and **2**, where **2** is the aglycone structure assigned in the preceding paper).[1] The letter codes used for the antibiotic in the preceding paper are different, since the correlation between "free" and "bound" chemical shifts requires many further experiments (detailed subsequently). A ^{1}H spectrum (270 MHz) of ristocetin A in Me_2SO-d_6 solution at 30 °C, in the presence of approximately 2 molar equiv of Ac-D-Ala-D-Ala, is reproduced in Figure 1. An expansion of the δ 6–8.4 ppm region at a higher temperature (43 °C) is given in Figure 2.

A. Phenolic OH Protons. The phenolic OH protons are distinguished from amide NH protons by being (1) readily broadened upon increasing the temperature of the sample, (2) rapidly exchanged on D_2O addition, (3) reduced in intensity by ca. 80% on irradiation of the residual water peak, and (4) readily sharpened to singlets or broadened according to pH, and occurring in the δ 8.5–10 ppm region.[5] Four such protons (coded b_1–b_4) are visible in spectra taken at 30 °C (δ 9.69, 9.47, 9.40, and 8.94 ppm). This observation supports our conclusion in the previous paper[1] that ristosamine is not bound to a phenolic oxygen.

B. Amide -CONHCH- Protons. These are distinguished (1) as doublets coupled to an α-CH proton in the region δ 3.5–6 ppm, (2) as resonances which do not readily accept transfer

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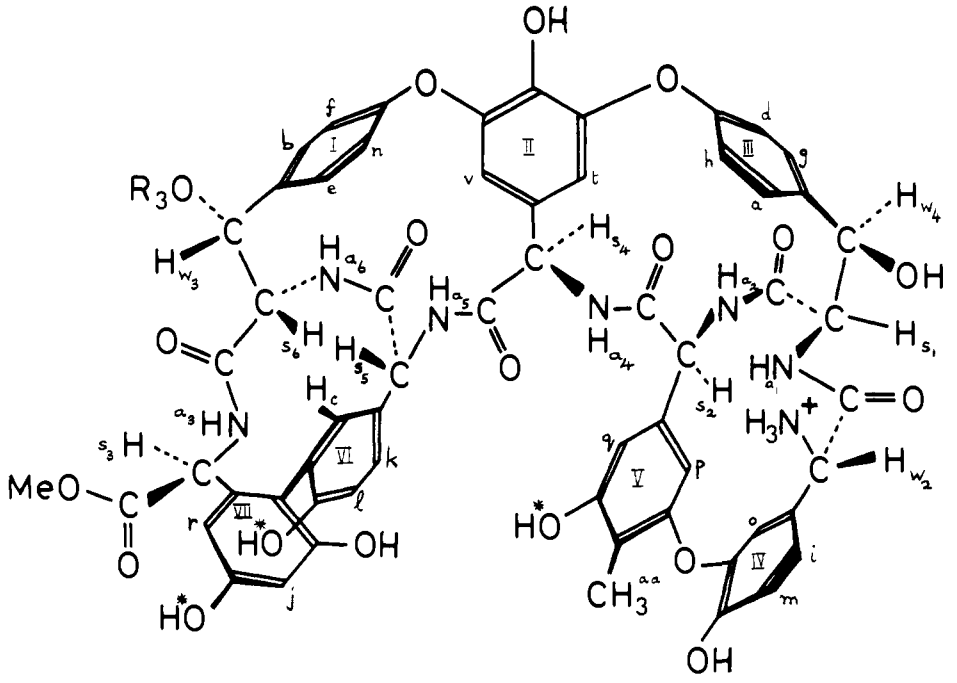

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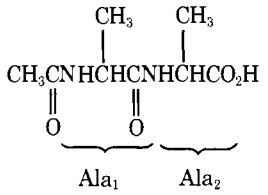
Table I. Assignment of the Proton Resonances of Ac-D-Ala-D-Ala (**1**) in Me_2SO-d_6

	β-Me	α-CH	NH	CH_3CO
Ala_1	1.18	4.30	7.99	1.81
Ala_2	1.27	4.17	8.13	



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of saturation on irradiation of the H_2O peak, but do exchange (in a period of minutes or less) the proton for deuterium on addition of D_2O. There are six such protons, coded a_1–a_6.

C. Aliphatic O*H* Protons. These resonances are characterized by strong temperature dependence, broadening on heating of the sample, D_2O exchange, and reduction in intensity on irradiation of the HOD peak. They all occur upfield of δ 6.2 ppm, and are numerous in the region δ 4–6 ppm. They are coded A_n in Table IV.

D. Aromatic Protons. On the basis of structure **2**, there should be 18 such protons in the δ 6–8 ppm region. The number of aromatic protons found in the δ 6.2–8.4 region is indeed 18, and these are coded a–r (Table IV). Decoupling experiments, the results of which are illustrated in Figure 2, establish the ring substitution patterns given in **3–8.** The assignment of **3–8** to rings I–VII is discussed subsequently.

E. The "α-CH Region" δ 4–6. This region should contain the signals due not only to α-CH protons but also to the two high-field aromatic protons and the anomeric protons of the sugars. The α-CH protons (s_1–s_6) were identified as those coupled to NH protons (a_1–a_6, respectively). In each case, the α-CH resonance was seen to sharpen (or change from a doublet to a singlet) on D_2O exchange of the NH resonances. The resolution of the α-CH region was improved in spectra obtained on complete D_2O exchange, effected by controlled addition of KOD–D_2O. These spectra established the mutual coupling (4.5 Hz) of s_1 and w_4. In addition, irradiation of s_6 led to a sharpening of w_3. Thus, the pairs s_1/w_4 and s_6/w_3 represent the nonexchangeable protons of the two -CH(OR)CHNH systems. The one well-resolved doublet (δ 4.92, x) not affected by D_2O exchange, but coupled to a resonance at δ 3.58, is assigned to the glucose anomeric proton,[1]

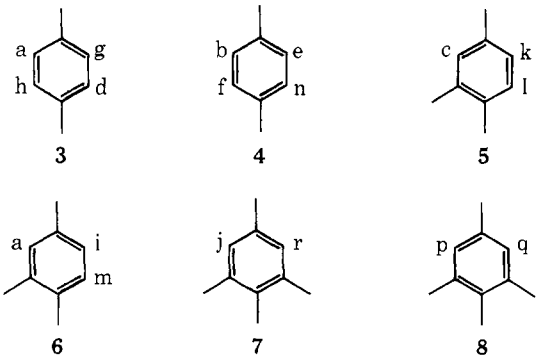
Table II. Transfer of Saturation Experiments on Alaβ-Me Resonances

	obsd peak heights, mm[a]		
peak irradiated	A (1.05 ppm)	B (1.18 ppm)	C + D (~1.27 ppm)
no irradiation	50	84	128
bound Alaβ-Me (0.38 ppm)	53	90	107
bound Alaβ-Me (0.83 ppm)	48	64	133

[a] Quoted values are the average of two experiments.

Table III. Assignment of the Proton Resonances of Bound Ac-D-Ala-D-Ala

	β-Me	α-CH	NH	CH_3CO
Ala_1	0.83	4.85	8.28	1.81
Ala_2	0.38	3.84	7.84	



3 **4** **5**

6 **7** **8**

Table IV. ^{1}H Chemical Shifts and Coupling Constants for the Ristocetin A–Ac-D-Ala-D-Ala Complex in Me_2SO-d_6 at 24 °C

chemical shift, δ ppm	intensity	multiplicity	J, Hz	code
11.88	1	d	10.3 ± 0.5	a_1
9.83	1	d	10.2	a_2
9.66	1	broad s		b_1
9.43	1	d	5	a_3
9.41	2	broad s		$b_2 + b_3$
9.15	1 + 1	d + d	8.5 (a_4), 5 (a_5)	$a_4 + a_5$
~9.00	1	broad s		b_4
8.25	1	d	8.5	Ala_1NH_B
7.81	1	d	8.5	Ala_2NH_B
7.81	1	d	~8.0	a
7.40	1	d	11.0	a_6
7.33	1	d	~8.0	b
7.29	1	broad s		c
7.24	1	d	~8.0	d
7.24	1	d	~8.0	e
7.15	1	d	8.0	f
7.01	1	d	7.6	g
6.91	1	d	7.5	h
6.83	1	d	8–9	i
6.82	1	s		j
6.78	1	d	8–9	k
6.72	1	d	8.0	l
6.63	1	d	~8	m
6.59	1	d	~8	n
6.47	1	s		o
6.44	1	s		p
6.40	1	s		q
6.23	1	s		r
6.02	1			A_1
5.80	1	d	8.5	s_4
5.72	1			A_2
5.69	1	s		t
5.46	1	s		u
5.37	1	s		v
5.28	1	d	10.2	s_2
5.20	2		4.5 (J_{s_1,w_4})	$w_1 + s_1$
5.12	3		4.5 (J_{s_1,w_4})	$w_2 + w_3 + w_4$
5.03	?			A_3
4.97	?			A_4
4.92	1	d	7.3 ± 0.2	x
4.87	3			$s_5 + y_1 + y_2$
4.83	1			$Ala_1\alpha CH_B$
4.74				A_5
4.67	≫3			A_6
4.62				A_7
4.53	2			$s_3 + s_6$
4.30	1	s		z
3.9–2.8				$Ala_2\alpha CH_B$; sugar C*H* and O*H* protons; COOCH_3
2.23	1	d	15	ristosamine H-2 or H-2′
2.07	3	s		aa
2.03	1	d	15	ristosamine H-2 or H-2′
1.83	3	s		Ala CH_3CO_B
1.27	3	d		bb
1.02	3	d		cc
0.83	3	d	~7	$Ala_2\beta Me_B$
0.38	3	d	~7	$Ala_1\beta Me_B$

diaxially coupled to H-2. Irradiation of y_1 and y_2 leads to the decoupling of the ristosamine H-2 which is partly obscured by the aromatic methyl group at 2.07 ppm. The ristosamine anomeric proton is therefore one of the y_1, y_2 pair.

F. NH_2 Protons. Two basic amino groups exist in ristocetin A.[1] In the complex, these protons occur as a broad resonance centered under the low-field aromatic protons at about 7.44 ppm. The chemical shift and the dependence of the line width on pH are consistent with the assignment to the amino-group protons.

Further detailed assignments rely on relating resonances of bound and unbound ristocetin A, and on studying the observed negative nuclear Overhauser effects (NOEs).[1] Some relevant NOEs which have been observed during the course of decoupling experiments carried out on the complex are summarized in Table V.

Important conclusions are derived from negative NOEs observed upon irradiation of phenolic OH protons. Separate experiments involving irradiation of the phenolic OH protons b_1, b_2, and b_3 lead to reduction of the intensity of the aromatic resonances (r + j), q, and l, respectively. Since both r and j are reduced in intensity upon irradiation of b_1, its assignment is unambiguous (see H* on ring VII in **2**[11]). Moreover, since irradiation of the anomeric proton of a mannose residue leads to a reduction in intensity of resonance j, then the second hydroxyl function of ring VII in the aglycone (**2**) carries mannose in ristocetin A. Collectively, the NOEs observed upon irradiation of phenolic OH protons establish that the three oxygens indicated by asterisks in **2** do not carry sugars in ristocetin A.

Further discussion of structural information which is available from the NOEs is deferred until methods of correlating the spectra of bound and free ristocetin A have been considered.

Correlating the Spectra of Bound and Free Ristocetin A. Such a correlation is desirable because, where prior assignments to both bound and free states are made, these assignments may then be checked for consistency. Much more importantly, chemical-shift changes in NH resonances between bound and free states give information about the nature of the peptide–antibiotic binding site. Two experimental methods for establishing correlations between bound and free chemical shifts conveniently utilize a solution containing only 0.5 molar equiv of peptide. For this solution at 30 °C, superimposed spectra of bound and free antibiotic are obtained.

The first method involves the examination of coalescence of "bound" and "free" resonances as a function of temperature. Some resonances are already broadened at 40 °C, due to intermediate exchange rates; these all sharpen on heating the solution to 80 °C, and represent the resonances whose chemical shifts are least affected on complexation. Further heating of the solution to 100 °C results in the coalescence and sharpening of a number of other resonances, but even at this temperature a completely averaged spectrum is not obtained owing to the high barrier to dissociation (~18 kcal mol^{-1})[2] and the large shift differences suffered by some of the protons.

On the basis of coalescence of a_B (resonance "a" in the bound state) and a_F (resonance "a" in the free state—see assignments given in the preceding paper[1]), the resonances of aromatic protons a_B, h_B, g_B, and d_B can be assigned to ring III. Similarly, the correspondences of all the other rings **4–8** can be established unambiguously from the coalescences of the pairs of resonances t_B/t_F (ring II), b_B/c_F (ring I), q_B/p_F (ring V), k_B/k_F and l_B/n_F (ring VI), and r_B/r_F and j_B/l_F (ring VII). The appropriate assignments of the aromatic resonances in the bound state of the antibiotic are given in **2.** In addition, the coalescence phenomena directly establish the correspondence of some of the lesser shifted αCH resonances (s_{4B}/s_{3F} (ring II), s_{3B}/s_{1F} (ring VII), and s_{2B}/s_{5F} (ring V)) and of the sugar resonances u_B/u_F, y_{1B}/aa_F, y_{2B}/bb_F, and the sugar methyl groups.

Some of the correlations have been checked by transfer of saturation experiments. The solution at 50 °C containing equimolar amounts of "free" and "bound" antibiotic was ir-

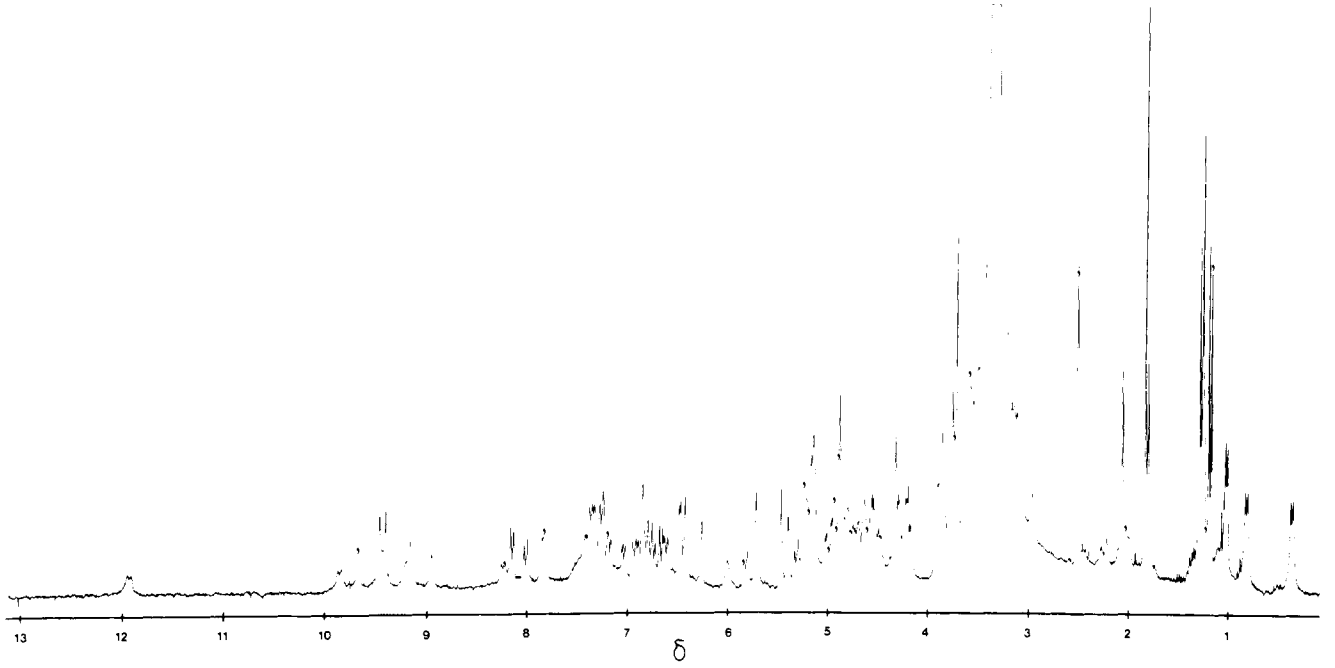


Figure 1. ^{1}H NMR spectrum of ristocetin A (12 mM in Me_2SO-d_6 solution) at 270 MHz, in the presence of Ac-D-Ala-D-Ala (24 mM) at 30 °C.

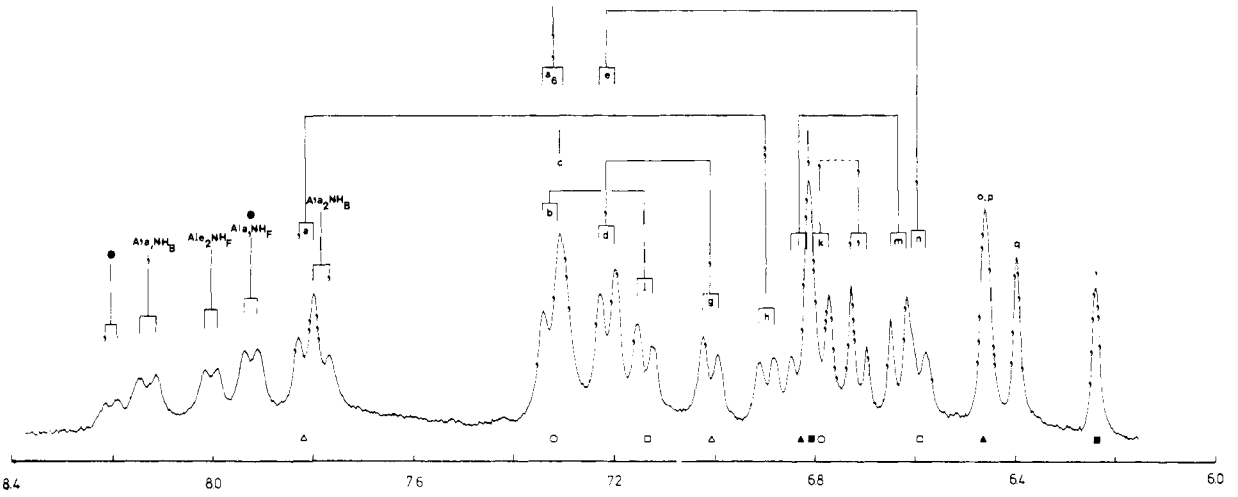


Figure 2. The δ 6–8.4 ppm region of ^{1}H spectrum of ristocetin A in the presence of 2 molar equiv of Ac-D-Ala-D-Ala (concentrations as for Figure 1, but at 43 °C). Meta-coupled pairs of resonances are indicated by the symbols under the spectrum. The full circles indicate NH resonances due to Ac-D-Ala-D-AlaOCH_3, present as a 16% impurity in the sample of peptide used in this experiment.

Table V. NOEs Observed in 270- and 360-MHz ^{1}H Spectra of the Ristocetin A–Ac-D-Ala-D-Ala Complex

NOE no.	proton irradiated	resonance reduced in intensity	NOE no.	proton irradiated	resonance reduced in intensity
1	s_4	a_5	10	$s_3 + s_6$	c
2	$s_1 + w_1$	j	11	Ala CH_3CO_B	Ala_1 NH_B
3	$s_5 + y_1 + y_2$	c	12	b_1	r + j
4	$Ala_1\alpha CH_B$	c and s_6	13	b_2	q
5	$Ala_2\beta Me_B$	h	14	b_3	l
6	$Ala_1\beta Me_B$	c	15	s_5	s_6
7	a_1	a or Ala_2NH_B	16	o + p	s_2
8	Ala_2NH_B or a	$Ala_1\alpha CH_B$	17	s_6	$Ala_1\alpha CH_B$
9	s_1 or w_1	j	18	$w_2 + w_3 + w_4$	g

radiated using gated decoupling in order to minimize distortion of the spectrum. For example, upon irradiation at 7.2 ppm, both resonances a_{1B} and a_{2B} decreased in intensity by about 40%. The high-field NH resonances of the free antibiotic occur at 7.36 (a_{4F}), 7.29 (a_{6F}), and 7.23 ppm (a_{5F}); examination of a spectrum of the "free" antibiotic alone under similar conditions (continuous irradiation at 7.26 ppm) shows that all three resonances are affected (as judged by off-resonance effects on the corresponding α-CH resonances). Thus, the two lowest field NH resonances, a_{1B} and a_{2B} (11.9 and 9.8 ppm),

Table VI. NMR Parameters of αCH--NH Units of "Bound" and "Free" Ristocetin A and of Ac-D-Ala-D-Ala at 40 °C

code (ring no.)		$J_{\alpha,NH}$, Hz		$\delta_{\alpha CH}$		δ_{NH}		$\Delta\delta/\Delta T$[a]	
free	bound	free	bound	free	bound	free	bound	free	bound
s_1/a_1 (VII)	s_3/a_3	5	5	4.51	4.55	9.17	9.46	−3	−0.7
s_2/a_2 (VI)	s_5/a_5	6.5	5	4.70	4.88	8.68	9.06	−6	−6
s_3/a_3 (II)	s_4/a_4	8.2	8.5	5.64	5.79	7.68	9.13	−3	−0.9
s_5/a_4 (V)	s_2/a_2	10	10	5.19	5.30	7.36	9.78	−0.5	−3.1
s_4/a_5 (III)	s_1/a_1	9.3	10.3	5.05	5.21	7.26	11.9	−1.5	−1.1
s_6/a_6 (I)	s_6/a_6	12	11	4.28	4.55	7.26	7.33	−5.5	−5.5
Ala_1[b]	Ala_1[b]	6.6	8.5	4.30	4.85	7.99	8.28	−5.5	−6.2
Ala_2[b]	Ala_2[b]	7	8.5	4.17	3.84	8.13	7.84	−6.2	−1.5

[a] Temperature coefficients of amide NH resonances (in units of 10^{-3} ppm/K). [b] Data obtained at 23 °C.

Table VII. Binding Constants (K_A) for Peptides to Ristocetin A[a]

peptide	K_A	peptide	K_A
Ac_2Lys-D-Ala-D-Ala	5.9×10^5	Ac_2Lys-D-Ala-D-Ala	5.9×10^5
Ac_2Lys-D-Ala-Gly	2.2×10^4	Ac_2Lys-Gly-D-Ala	1.6×10^5
Ac_2Lys-D-Ala-D-Leu	6.1×10^5	Ac_2Lys-D-Leu-D-Ala	5.8×10^4
Ac_2Lys-D-Ala-L-Ala	no combination	Ac_2Lys-L-Ala-D-Ala	no combination

[a] Data from ref 10.

of bound ristocetin A correspond to two of the three high-field NH resonances of the free antibiotic. Since a_{1B}, a_{5F}, and a_{6F} are known to be incorporated in -CH(OR)CHNH- groups, a_{2B} and a_{4F} must correspond, confirming the conclusion from coalescence experiments that s_{2B} and s_{5F} correspond.

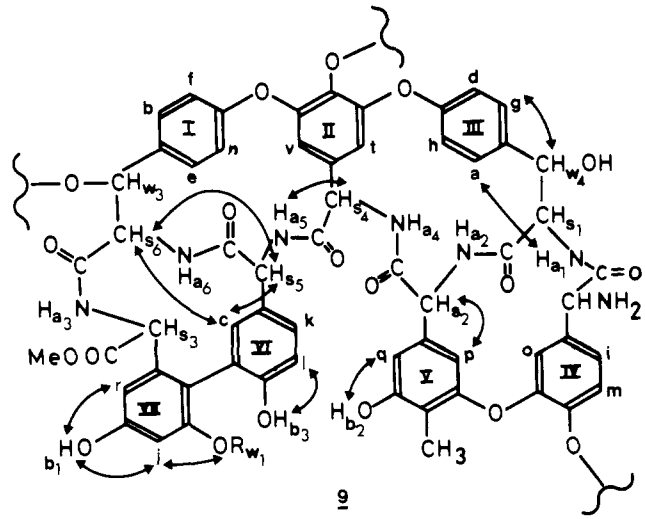
The correspondence of the αCH-NH pair s_{1B}/a_{1B} with s_{4F}/a_{5F} is inferred from the coupling constants $J_{\alpha\beta}$ to the vicinal -C*H*(OH)- protons y_F and w_{4B} (5.0 and 4.5 Hz). Therefore, the s_{1B}/a_{1B} pair is attached to ring III, and this assignment then allows us to place the s_{6B}/a_{6B} pair on ring I. In the light of the earlier attachment of αCH resonances in the bound form to rings II, V, and VII (see coalescence experiments detailed above), s_{5B} is assigned to ring VI by a process of elimination. These assignments all conform to the NOEs which are listed in Table V. The close correspondences in $J_{\alpha,NH}$ coupling constants and αCH chemical shifts can be seen in Table VI.

Of the sugar anomeric resonances, x_B and y_{2B} are identified as due to the anomeric protons of glucose and ristosamine, on the basis of their coupling; and u_B and y_{1B} are defined by their coalescence with u_F and aa_F, respectively. The remaining singlets in the δ 4-6 ppm region are matched on the basis of their chemical shifts (ppm): w_F (5.29) with w_{1B} (5.21); cc_F (4.70) with w_{2B} (5.14); dd_F (4.55) with z_B (4.35). A possible ambiguity in these assignments is removed by the observation of the strong NOE $w_{1B} \rightarrow j_B$ (Table V); this NOE is equivalent to $w_F \rightarrow l_F$ reported in the preceding paper.[1] It is noteworthy that the downfield shift of the proton (cc_F, w_{2B}) bound to the same carbon atom as the free amino group is consistent with the complete protonation of this amino group after the addition of the acidic peptide Ac-D-Ala-D-Ala (to give the complex).

All the correspondences between the resolvable resonances of bound and free ristocetin A are listed in Table VIII. It is of course necessary that the assignments of the resonances in the spectrum of the bound antibiotic be consistent with the intramolecular NOEs given in Table V. That this is indeed the case may be seen from the NOEs (indicated by double-headed arrows) summmarized in **9**.

It is noteworthy that the family of mutual NOEs s_5, s_6, c has analogies not only in the unbound antibiotic,[1] but also in vancomycin.[5,6] These observations clearly point to a common structural feature in ristocetin A and in vancomycin.

Investigation of the Ristocetin A-Peptide Binding Site. The main evidence for the molecular nature of the binding site of



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ristocetin A for the cell wall peptide analogue has come from (1) the temperature dependence and chemical shifts of NH resonances and (2) intramolecular and intermolecular NOEs in the antibiotic-peptide complex.

The NMR parameters of the αCH-NH units of "bound" and "free" ristocetin A and of the peptide Ac-D-Ala-D-Ala are given in Table VI. Relevant NOEs have already been detailed in Table V.

It is clear from the values of $J_{\alpha,NH}$ for the free and bound antibiotic (Table VI) that no major conformational change (such as peptide bond rotation or cis-trans isomerization) takes place on binding. The conformation of the antibiotic in its bound state must be essentially as previously determined for its free state.[1]

The free peptide serves as a standard which shows that the temperature coefficients of amide NH protons exposed to solvent are in the −5 to −6 ($\times 10^{-3}$) range. From the temperature coefficients of the NH resonances in the bound form, it is evident (Table VI) that Ala_1NH_B ($\Delta\delta/\Delta T$ −6.2) is exposed to solvent, but that Ala_2NH_B ($\Delta\delta/\Delta T$ −1.5) is not. These data indicate that the peptide forms a hydrogen bond to ristocetin A by the Ala_2NH but not by the Ala_1NH. Similarly, the reduced accessibility of the ristocetin NH a_{1F}/a_{3B} to solvent, and the substantial downfield shifts of the a_{3F}, a_{4F}, and a_{5F} resonances on binding (Table VI), strongly suggests a hydrogen-

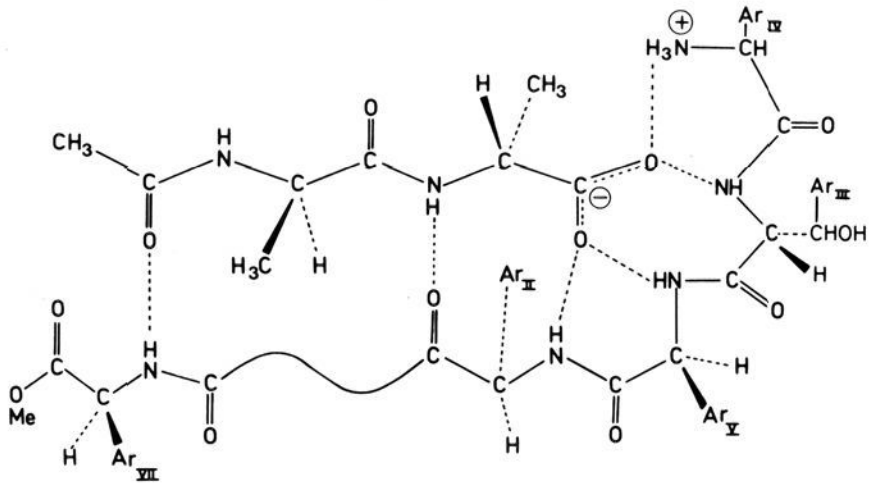


Figure 3. Illustration of the binding of Ac-D-Ala-D-Ala to ristocetin A. A portion of the peptide backbone of ristocetin A which is not involved in binding Ac-D-Ala-D-Ala by hydrogen bonding is indicated by a curved line to simplify the diagram.

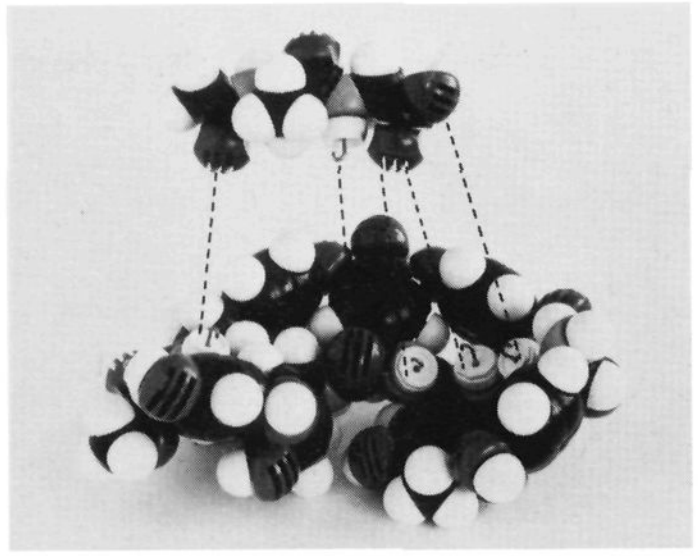
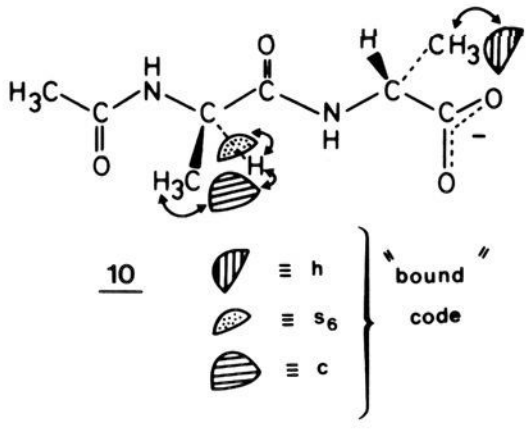


Figure 4. A CPK model giving an exploded view of the binding site of ristocetin A for Ac-D-Ala-D-Ala. Hydrogen bonds which are formed in the complex are indicated by broken lines.

bonding role for these protons in the complex. The other two antibiotic NH resonances (a_{2F}/a_{5B} and a_{6F}/a_{6B}) undergo little change on binding in either chemical shifts or temperature coefficients. On the basis of the former data, we conclude that these two protons are not involved in hydrogen bonding to the peptide, and from the latter that they are still exposed to solvent in the antibiotic–peptide complex. It is these two NH protons which have already been shown to reside at the "back" of the antibiotic.[1] The four other NH protons (a_{1F}, a_{3F}, a_{4F}, and a_{5F}) are known to be at the "front" of ristocetin A (see hydrogen atoms with attached hooks in Figure 4 of the preceding paper[1]).

On the basis of the foregoing data, we propose that in the ristocetin A–peptide complex the antibiotic NH proton a_{1F}/a_{3B} and the "pocket" of NHs formed by a_{3F}/a_{4B}, a_{4F}/a_{2B}, and a_{5F}/a_{1B} act as H-bond donors to respectively the acetyl C=O and the carboxylate group of Ac-D-Ala-D-Ala, and that the ring II C=O group is hydrogen bonded to the Ala_2-NH (see Figure 3). Although the stereochemistry at the α-carbon atom of ring IV remains the only unknown element of the aglycone structure, it is noteworthy that, if the absolute configuration at this center is *S*, then the close approach of the carboxylate anion of the peptide to the $-N^+H_3$ group of the antibiotic will provide additional Coulombic stabilization of the complex and, possibly, further hydrogen bonding. The arrangement is illustrated in a CPK model (Figure 4), where the peptide is shown above its binding site in order to clarify the nature of the binding.

That Figures 3 and 4 do correctly define the binding site of ristocetin A for Ac-D-Ala-D-Ala has been confirmed by the observation of intermolecular NOEs between protons of the antibiotic and the peptide in the complex (Table V, NOEs 4, 5, 6, and 17). From CPK models, it may be seen that the binding results in a situation where those protons of the antibiotic indicated in **10** are in contact with, or very close to, protons of the peptide.

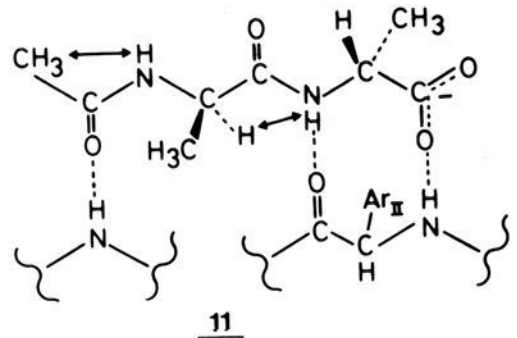


10

In fact, negative NOEs are observed between the αCH proton of the N-terminal alanine and both c and s_6 (Table V, NOEs 4 and 17). Additionally, the methyl protons of this alanine residue exhibit a negative NOE with c (NOE 6). Thus, in the complex, this aromatic proton of ring VI (see **2**) lies between the geminally bonded CH_3 and αCH proton of Ala_1, as demanded by models based on the proposed binding site. The proposed binding site also demands that the protons of the methyl group of the carboxyl terminal alanine are buttressed against proton h of ring III (see **10**). The close approach of the relevant protons is established by NOE 5 (Table V).

The above model not only accommodates all the data already discussed, but allows for a close fit of the *C*-methyl group of the C-terminal alanine residue immediately over the $aryl_{II}$ benzene ring. Thus, the large upfield shift (0.89 ppm, Tables I and III) of this methyl resonance upon binding to peptide is also explained.

The rigidity of the binding is indicated first by the nonaveraged values of $J_{\alpha,NH}$ for the two alanine residues of the peptide (Table VI). According to the Bystrov equation,[8] these *J* values are typical for the stable "extended form" adopted by the peptide chain (see Figure 4). The same conformation of the bound peptide is also indicated by two negative NOEs due to intramolecular interactions in the peptide (Table V); these establish the proximity of the NH of the carboxyl terminal alanine and of the $Ala_1\alpha$CH (NOE 8) and of the methyl group of the acetyl residue to the NH of the N-terminal alanine (NOE 11). These proximities are indicated by double-headed arrows in **11.**



11

The trans-extended geometry of the peptide of course allows one NH of the backbone to interact with the receptor surface while leaving the other NH exposed to solvent, as deduced earlier. It is noteworthy that the geometry of the bound peptide indicated in **11** is similar to that found in an X-ray structure of L-Ala-L-Ala-L-Ala.[9] Such a trans-extended geometry is that found in β-pleated sheets, and is favorable for intermolecular hydrogen-bonding interactions. Finally, we note that of the hydrogen bonds indicated in Figure 3, the three also indicated in **11** have previously been proposed to occur analogously in the binding of vancomycin to Ac-D-Ala-D-Ala.[7] The proposals with regard to the mode of action of vancomycin, based upon

Table VIII. Effect of Binding on the Ristocetin A Spectrum. Translation Table for Bound and Unbound Resonances[a]

code		shift		ring or	code		shift		ring or
free	bound	free	bound	sugar	free	bound	free	bound	sugar
b	e	7.49	7.20		h	i	7.12	6.84	
c	b	7.36	7.32		j	m	6.98	6.64	
m	n	6.80	6.60		o	o	6.53	6.47	IV
g	f	7.17	7.14	I	cc	w_2	4.81	5.14	
z	w_3	5.12	5.14						
s_6	s_6	4.35	4.56		f	c	7.20	7.31	
a_6	a_6	7.17	7.21		k	k	6.83	6.79	
					n	l	6.72	6.70	VI
t	t	5.81	5.72		s_2	s_5	4.70	4.89	
v	v	5.38	5.37	II	a_2	a_5	8.56	8.87	
s_3	s_4	5.62	5.74						
a_3	a_4	7.64	9.10		l	j	6.81	6.81	
					r	r	6.27	6.25	
a	a	7.81	7.80		s_1	s_3	4.51	4.50	VII
d	d	7.24	7.20		a_1	a_3	9.26	9.39	
e	h	7.19	6.89						
i	g	7.06	7.01	III	bb	y_2	4.84	4.89	ristosamine
y	w_4	5.16	5.14		w	w_1	5.29	5.18	$mannose_1$
s_4	s_1	5.05	5.18		x	x	5.23	5.00	glucose
a_5	a_1	7.19	11.83		aa	y_1	4.87	4.89	$mannose_2$
					u	u	5.45	5.44	arabinose
p	q	6.40	6.40		dd	z	4.53	4.36	rhamnose
q	p	6.36	6.47						
s_5	s_2	5.20	5.30	V	ee_1		2.26	2.25	
a_4	a_2	7.35	9.68		ee_2		2.02	2.03	
ff	aa	2.02	2.06		gg	bb	1.27	1.28	
					hh	cc	1.11	1.05	

[a] Shifts refer to spectra obtained at ca. 70 °C for ~15 mM solutions in Me_2SO-d_6 solution.

model-building and limited NMR data, become more secure in the light of the present detailed work on ristocetin A. However, details of the carboxylate binding site of vancomycin may be incomplete; further work on this point is in progress.

Binding Specificity of Ristocetin A. The studies of Nieto and Perkins[10] have shown the limits within which the amino acid residues of mucopeptide analogues can be changed and yet permit binding to ristocetin A and vancomycin. Some of their data for ristocetin A are reproduced in Table VII.

For ristocetin A, they found that a larger side chain of a D residue can be accommodated at the C terminus of the peptide (Table VII, columns 1 and 2), but not at the next position on the chain, and further, that on substituting a glycine for D-Ala at the C terminus decreased binding is observed, and that an L residue in either of the last two positions in the peptide prevents binding. Inspection of a CPK model of the bound antibiotic provides ready rationalizations of these observations. The side chain of the C-terminal D-alanine projects above ring II; there is more room to accommodate a larger group. In contrast, in vancomycin[7] there are bulky chlorine atoms on either side of the upper face of ring II, and therefore less room to accommodate a larger side chain of a C-terminal D residue—exactly as observed by Nieto and Perkins.[10]

The $Ala_1\beta Me$ fits snugly against ring VI without much room for a larger side chain. The $Ala_1\alpha CH$ is in a very crowded environment, and evidently not even a methyl group can be fitted in that site (Table VII, last entry in columns 3 and 4).

The $Ala_2\alpha CH$ is not near any part of the antibiotic aglycone, but is held rigidly in a conformation favorable for D but not L residues, namely, with the αCH almost eclipsing the C=O group of Ala_1.

Conclusion

The binding of Ac-D-Ala-D-Ala (a model of the bacterial cell wall mucopeptide) to ristocetin A occurs through hydrogen bonding involving five (or possibly six) sites. The binding resembles that proposed[7] for the same peptide with vancomycin but with additional binding of the peptide carboxyl group. The elucidation of the nature of the binding site was made possible by the assignments of the separate spectra of bound and unbound antibiotic and peptide, and has been greatly facilitated by the observation of negative NOEs. This method should be of general applicability for complexes of molecular weight >1300 studied by high-field ^1H NMR if the solvent employed is slightly viscous (e.g., Me_2SO).

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