A ^{1}H Nuclear Magnetic Resonance Study of the Interactions of Vancomycin with *N*-Acetyl-D-alanyl-D-alanine and Related Peptides

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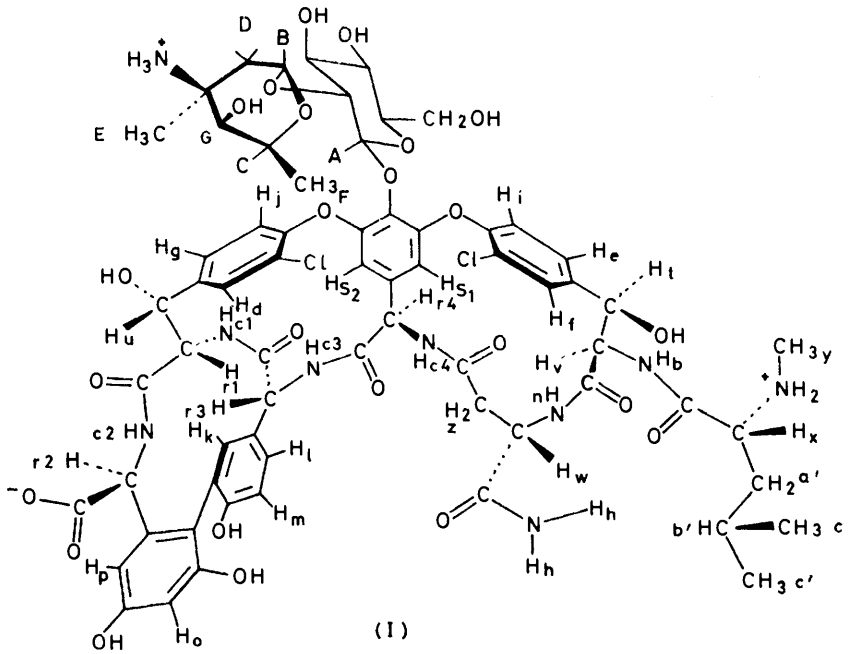
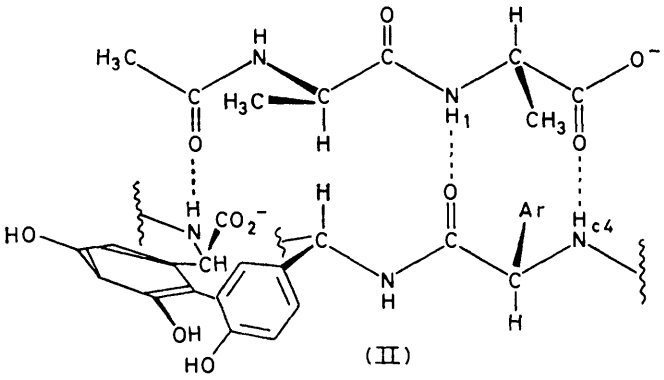
We have assigned the 270 MHz ^{1}H n.m.r. spectrum of vancomycin hydrochloride in aqueous solution and measured the changes in chemical shifts caused by its complexation with *N*-acetyl-D-Ala, *N*-acetyl-Gly-D-Ala, *N*-acetyl-D-Ala-D-Ala, and diacetyl-L-Lys-D-Ala-D-Ala. We have also measured the ^{1}H chemical shifts for the complex of diacetyl-L-Lys-D-Ala-D-Ala and vancomycin in [^{2}H]dimethyl sulphoxide where the NH protons can be directly observed. The observed shifts strongly support the model proposed by Sheldrick and his co-workers for D-Ala-D-Ala peptide binding to vancomycin. On peptide binding we observe large downfield chemical shifts for the NH proton in the Ala-Ala peptide bond and also for the vancomycin NH proton labelled c_4: this is strong evidence for the proposed hydrogen bonds involving these protons in the model. Ring-current chemical-shift calculations of the shifts in the bound peptides are consistent with the proposed model. We also present evidence for some induced conformational changes in the vancomycin molecule when the peptides bind. In particular, the flexible side-chain of the *N*-methyl-leucine residue probably undergoes considerable conformational rearrangement to allow the positively charged *N*-methyl group to interact favourably with the terminal carboxylate anion of the bound peptide.

VANCOMYCIN, an antibiotic isolated from *Streptomyces orientalis* n.sp., is active against gram-positive bacteria and has been shown to interfere with the biosynthesis of bacterial cell-walls. Perkins [1] has shown that vancomycin binds strongly to mucopeptide precursor molecules containing the terminal D-Ala-D-Ala fragment and he concluded that the formation of such complexes would prevent the incorporation of the precursor molecules into the bacterial cell wall thus providing an explanation for the antibiotic action of vancomycin. In a detailed study, Nieto and Perkins [2,3] have examined the extent to which the association constants for a series of peptides bound to vancomycin depend on the length and composition of the peptide chains. The most stable complexes result for peptides (i) with three amide linkages, (ii) a terminal carboxy-group, (iii) two *C*-terminal residues being either glycine or in D-configurations (with D-Ala being especially favourable), and (iv) a third residue in an L-configuration. Brown and her co-workers [4,5] have examined vancomycin–peptide complexes using n.m.r. to monitor shielding changes of the peptide protons which occur on binding. In the complex with Ac-D-Ala-D-Ala, the proton resonances of all three methyl groups showed upfield shifts on binding which indicated that they are bound in the proximity of the aromatic rings in vancomycin. From an analysis of the pH dependence of the association constants, a favourable interaction was detected between the terminal carboxyl-



(I)

ate anionic group on the peptide with the cationic group on the *N*-methyl-leucine of vancomycin (1.4 kcal mol^{-1}). In the complexes with a series of other peptides with different residues at positions 2 and 3, the shielding of the methyl protons in the *C*-terminal D-Ala(1) was perturbed in essentially the same way for the different peptides indicating that this residue remains in the same binding site in the different complexes. (The peptide residues are numbered from the *C*-terminus). The bound shifts of other protons, such as those in the methyl group of D-Ala(2) are affected more by substitution in other parts of the molecule indicating that their positions in the binding site are less constant. In these early n.m.r. studies only limited information was obtained concerning the effects of peptide binding on vancomycin proton resonances mainly because of the complexity of the unassigned vancomycin spectrum at 100 MHz. Recently the complete structure of vancomycin [see structure (I)] has been determined using chemical methods [6-9] and *X*-ray analysis [10] of a degradation product of vancomycin CDP-1 (formed from vancomycin with loss of one ammonia molecule) and the 270 MHz ^{1}H spectrum of vancomycin has been assigned in detail.[9] On the basis of the crystal structure of vancomycin CDP-1 and the limited n.m.r. data available on the complex of Ac-D-Ala-D-Ala with vancomycin, Sheldrick and his co-workers [10] have postulated a tentative model for this complex as illustrated in structure (II).



(II)

In this paper we report more detailed measurements of vancomycin ^{1}H chemical shifts in its complexes with a series of peptides related to Ac-D-Ala-D-Ala and examine the compatibility of the proposed model [structure (II)] with these results. The availability of the crystal structure data (the atomic co-ordinates were kindly provided by Dr. O. Kennard) on CDP-1 and the detailed assignments of the 270 MHz ^{1}H spectrum of vancomycin in DMSO solution [9] were of considerable help in analysing the data reported here.

EXPERIMENTAL

Materials.—Vancomycin hydrochloride was kindly supplied by Lilly Research Centre Ltd. Vancomycin base was prepared by adding 10% sodium hydroxide solution to a concentrated solution of vancomycin hydrochloride until pH 8.1 at 25 °C.[11]

Generous gifts of Ac-D-Ala, Ac-Gly-D-Ala and vancomycin base were obtained from Dr. J. P. Brown, Ac-D-Ala-D-Ala from Dr. D. H. Williams, and Di-Ac-L-Lys-D-Ala-D-Ala from Professor H. R. Perkins and Dr. M. Nieto. The samples were all used without further purification. The vancomycin and peptide samples were made up in a CD_3-COOD–NaOD buffer solution in ^2H_2O at pH* 5.5. The pH was measured with a glass electrode using a Radiometer model 26 pH meter and the reported values are meter readings with no allowance made for deuterium isotope effects. ^2H_2O (99.8% deuterium) was obtained from Norsk Hydro and [$^2H_6$]dimethyl sulphoxide from Ciba-Geigy Ltd.

N.m.r. Measurements.—The ^{1}H 270 MHz spectra were recorded using a Bruker WH270 spectrometer equipped with a Nicolet 1180 computer. All spectra were obtained using the Fourier transform technique; typically 2 000 transients were acquired using a spectral width of 3 600 Hz collected in 8 192 data points (acquisition time 1.1 s). Because there is rapid exchange between the free and bound species in the equilibria of almost all the complexes studied the observed chemical shifts are averaged values of those in the free (δ_V) and bound (δ_{VP}) species. As the amount of peptide is increased the vancomycin signals shift progressively towards the chemical shifts of the complexed species. The observed vancomycin shifts ($\delta_{obs.}$) can be expressed in terms of the free and bound shifts and the fraction of bound vancomycin in the equilibrium mixture using relationship (1) where [VP]

$$\delta_{obs.} = \delta_V + [VP](\delta_{VP} - \delta_V)/[V_T] \quad (1)$$

is the concentration of the complex and [V_T] is the total concentration of vancomycin.

The concentration of the complex can be expressed by equation (2) where K_a is the association constant and [P_T] is the total concentration of peptide.

$$[VP] = \tfrac{1}{2}([V_T] + [P_T] + 1/K_a - \{([V_T] + [P_T] + 1/K_a)^2 - 4[VT][P_T]\}^{\frac{1}{2}}) \quad (2)$$

We have measured the observed averaged vancomycin shifts for various concentrations of vancomycin and peptide at 45° and by using these in conjunction with previously determined values of the association constants (Perkins and Nieto; [2] Brown and her co-workers [5]) we have calculated the bound shifts using equations (1) and (2). In a typical set of experiments, ^{1}H spectra were obtained for a series of solutions formed by adding measured volumes of the peptide solution to a 0.25mM solution of vancomycin. It was necessary to work with this low concentration of vancomycin to avoid problems of self-association.[4]

At the highest concentrations of peptide used in the titrations >90% vancomycin was present in the bound form for all complexes except that with Ac-D-Ala where 69% of vancomycin was in this complexed form. Some errors (±10%) in the absolute values of the bound shifts are expected because the K_a values used to calculate the bound fraction at 45° were extrapolated from values measured at 30 and 35°.[2,5]

RESULTS AND DISCUSSION

Assignment of the ^{1}H Spectrum of Vancomycin in Aqueous Solution.—Williams and Kalman [9] have reported a detailed study of the assignments of the ^{1}H 270 MHz spectrum of vancomycin in DMSO solution. We have used their assignments (given in Table 1) to assist in the assignment of the spectrum from aqueous solutions of

vancomycin adopting all their reported assignments except those for the α-protons r_3 and r_4 which need to be reversed (Williams and Kalman, personal communication; Bongini and Feeney, unpublished results). A consideration of the *X*-ray structure (not available when the original assignments were made) indicates that r_4 is not shielded by an aromatic ring but is deshielded by a carbonyl group while r_1, r_2, and r_3 are all shielded by aromatic rings: thus r_4 is assigned to the signal at δ 5.70 and r_3 to that at 4.49. The NOE effect observed by Williams and Kalman [9] at the signal of the NH proton coupled to the CH proton at δ 4.49 is also in agreement with the assignment of r_3 to the signal at δ 4.49. Furthermore, it allows the signal at δ 5.27 to be assigned to the aromatic proton s_2 and thus the signal at δ 5.61 is from s_1. The 270 MHz ^{1}H spectrum of an aqueous solution of vancomycin (0.25 mM; 45 °C) is shown in Figure 1 and is found to be considerably different from that obtained in DMSO solution. It was therefore not possible to transfer the assignments of Williams and Kalman [9] immediately to the spectrum obtained on the aqueous solution of vancomycin. To connect the assignments in the two solvents we have examined vancomycin in mixtures of DMSO and D_2O (10mM solutions) as the fraction of D_2O was increased the signals moved progressively from their positions in DMSO solution to the positions in D_2O solution. In this way all the signals in the aqueous solution of vancomycin were assigned and the results are given in Table 1. We have also made the assignments for less concentrated aqueous solutions of vancomycin (0.25, 0.5, and 2.0mM) by following the small progressive shifts accompanying dilution of the 10mM sample; the results for 0.25mM-vancomycin are included in Table 1.

TABLE 1

^{1}H Chemical shifts * and assignments for vancomycin in DMSO and D_2O solutions

Proton	Vancomycin base 10mM, DMSO (60°)	Vancomycin HCl 10mM, D_2O (60°), pH *ca.* 3	Vancomycin HCl 0.25mM, D_2O (45°), pH 5.5
d	7.92	7.72	7.72
f	7.39	7.67	7.56
e	7.56	7.58	7.57
g	7.50	7.58	7.57
i	7.26	7.25	{7.38}
j	7.31	7.25	{7.26}
k	7.21	7.14	7.1
l	6.79	6.90	7.07
m	6.74	6.90	6.96
o	6.43	6.55	{6.54}
p	6.43	6.55	{6.53}
s_1	5.61	5.83	5.66
r_4	5.70	5.98	5.73
s_2	5.27	5.57	5.56
u	5.19	5.57	5.56
A	5.37	5.52	5.41
t	5.19	5.43	5.46
v	4.86	5.43	
B	5.29	5.37	5.33
C	4.68	4.86	
r_3	4.49	{4.74}	
r_2	4.41	{4.55}	
w	4.41	4.51	
r_1	4.20	4.23	
x	3.69	4.08	
Glu 6-H	3—3.45	3.9—3.5	
G	3.16	3.48	3.47
y	2.34	2.79	2.79
z′	2.45	2.70	2.69
z	2.20	2.28	2.46
D	1.7 and 1.9	2.07	2.04
a′	1.5 and 1.7	1.75	
b′	1.40	1.58	
E	1.32	1.47	1.41
F	1.11	1.18	1.19
c′	0.95	0.88	0.92
c	0.90	0.84	0.88

* The chemical shifts are referenced to hexamethyldisiloxane (for DMSO) and to sodium 4,4-dimethyl-4-silapentanesulphonate (for D_2O).

^{1}H *Shifts in the Complexes of Vancomycin with Peptides.*—Using the procedure outlined earlier, we have measured the bound shifts for the protons of vancomycin in its complexes with Ac-D-Ala, Ac-Gly-D-Ala, Ac-D-Ala-D-Ala, and Ac_2-L-Lys-D-Ala-D-Ala in aqueous solution and the results are presented in Table 2. No data for NH protons are given because they are exchanged for deuterium. We have also measured chemical shifts for the complex of Ac_2-L-Lys-D-Ala-D-Ala with vancomycin in [$^2H_6$]dimethyl sulphoxide where the NH signals from both peptide and antibiotic can be detected: these are reported in Table 3.

(i) *Vancomycin and* Ac-D-Ala. The binding of the relatively small molecule, Ac-D-Ala, causes extensive shielding effects on the vancomycin protons. From the data given in Table 2 it is seen that the shifts of at least 21 protons, widely distributed over the vancomycin structure, are influenced by the binding. From the crystal structure of vancomycin CDP-1 [10] the molecule is seen to form a crescent shaped disc structure with a cleft on the side bearing the two chlorine atoms (designated ' upper ' face) and a less well defined cleft on the other side (the ' lower ' face). Some of the protons influenced by the binding such as c′ (γ-methyl protons in *N*-methyl-leucine), E (methyl group in amino sugar fragment), and the aromatic proton k are at opposite extremities of the vancomycin structure. If the Ac-D-Ala is binding at a single binding site then its relatively small size would not allow it to make direct contacts with all the nuclei affected. The marked specificity for a terminal D-amino-acid residue to achieve strong binding suggests that here we are dealing with a single-binding site problem. Thus it seems likely that some of the vancomycin protons are influenced by induced conformational changes which accompany the binding of the peptide. The presence of five aromatic rings in this compact molecule results in most of the protons being influenced to some extent by the anisotropic shielding effects of ring currents. Many of the small shielding changes observed on binding are <0.05 p.p.m. (14 Hz) and could easily result from minor movements in the relative positions of the observed protons and the aromatic rings (<0.1 Å)

The largest shifts observed are at the methylene protons (z′, z) and the aromatic protons (e, f, s_1, m, and k) and for the methyl protons (c′) of the *N*-methyl-

TABLE 2

The bound ^1H chemical shifts (Hz at 270 MHz) for vancomycin hydrochloride † protons in complexes with peptides in aqueous solution (pH *5.5, 45 °C)

Proton	Position	Ac-D-Ala	Ac-Gly-D-Ala	Ac-D-Ala-D-Ala	Ac_2-L-Lys-D-Ala-D-Ala
c′		+29	+21	+40	+32
c		+29	+24	+41	+33
y		−13	−11	−6	−11
z′	Upper	+52	+39	+50	+34
z	Upper	−48	−29	−25	−34
f	Upper	+62	+48	+57	+60
e	Lower	−35 (−4)	+9	+16 (−8)	+22 (−3)
s_1	Upper	+44	+33	+31	+38
k	Upper	+23	−90	−151	−203
d	Upper	+15	+4	+69	+68
r_4	Lower	−22	−11		−11
l	Lower	{−16}	{−9}		{−7}
m	Lower	{−23}	{−4}		{−14}
i	Lower	+10	+16	+19	+14
s_2		+9	−18	−8	+9
j	Lower	+10	+16	+19	+14
g	Lower	−4 (+35)	+9	−8 (+16)	−3 (+22)
o	Upper	+3	{+4}	{+12}	{+16}
p	Upper	+2	{+9}	{+18}	{+20}
E		+10	+11		+26
G		+13	+13	+18	+11
F		+7	+9	+9	+9
B		+3	+6	0	+4

Upfield shifts positive.

† Vancomycin concentration was 0.25mM except for Ac-D-Ala titration where a 1.0mM concentration was used.

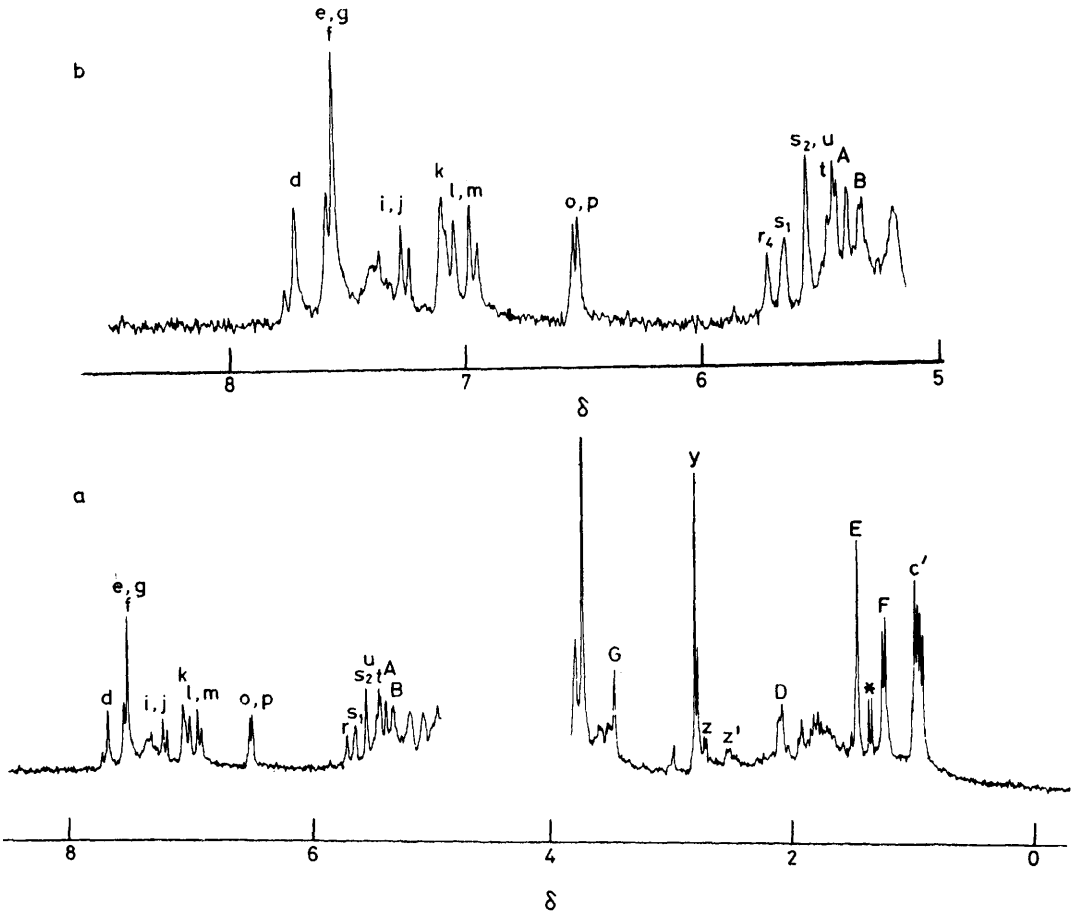


FIGURE 1 The 270 MHz ^1H resonance spectrum of 0.25mM-vancomycin hydrochloride in CD_3CO_2D–NaOD–D_2O buffer solution at pH 5.5 and 45 °C (* signal from decomposition product)

leucine groups. Some fairly major perturbation has taken place at the Asn methylene group (z′, z) where large opposing shielding effects are observed. All the protons which show large shifts (except for e and m) are positioned on or close to the ' upper ' face of the overall structure as shown in Figure 2. This suggests that the peptide is binding on this side of the ring at site(s) near to the protons affected. In addition to the large shifts

TABLE 3

The ' bound ' ^{1}H chemical shifts (Hz at 270 MHz) for vancomycin hydrochloride protons in its complex with Ac_2-L-Lys-D-Ala-D-Ala in DMSO solution (70 °C)

Proton	Chemical shift (Hz)	Proton	Chemical shift (Hz)
c′	+15	E	+28
c′	+15	G	+5
y	+18	F	+8
z′	+28	B	+10
z	−5	A	+6
f	+28	C	+5
e	−9	r_1	−11
s_1	0	w	~−60
k	−25	c_1	+9
d	Broadened	c_4	>−40
r_4	0	n	>−25
l	0	c_2	0
m	+4	c_3	−13
i	+23	r_2	−20
s_2	+15	r_2	−13
j	+5	ν	−22
g	+5		
o	+10		
p	−21		

Upfield shifts are positive.

already mentioned, smaller shifts are seen for other aromatic protons and for the sugar protons: these are all distant from the proposed binding region and could arise from minor conformational changes accompanying the binding.

(ii) *Vancomycin and* Ac-Gly-D-Ala. The ^{1}H spectrum of 0.25mM-vancomycin in the presence of Ac-Gly-D-Ala is shown in Figure 3. Several of the bound shifts in this complex have a broadly similar pattern to that seen in the complex with Ac-D-Ala. This is seen, for example, in the shifts of the *N*-methyl-leucine protons, the Asn methylene protons z′ and z, and the aromatic protons f and s_1. This suggests that the D-Ala(1) residue is interacting with the region of the molecule near to these protons. In contrast, the aromatic proton k experiences a major perturbation compared with that in the Ac-D-Ala complex (−90 compared to +23 Hz) which indicates that the second peptide residue is extending in the direction of proton k away from the *N*-methyl-leucine moiety (probably down the pronounced cleft seen in the *X*-ray structure on this side of the molecule). The small shifts seen in the sugar protons are similar to those seen in the complex with Ac-D-Ala: clearly there have been no additional interactions which perturb the conformation of the sugar rings with respect to the overall structure.

(iii) *Vancomycin and* Ac-D-Ala-D-Ala. The binding of Ac-D-Ala-D-Ala influences the shielding of the *N*-methyl-leucine protons (c′, y), the Asn methylene protons z′ and z, and the aromatic protons f and s_1, in a manner broadly similar to that seen for the complexes with Ac-D-Ala and Ac-Gly-D-Ala. As was observed in the latter complex, the aromatic proton k, on the ' upper ' face of the ring, is again perturbed, strongly confirming that the second residue is extending down the cleft away from the *N*-methyl-leucine residue. The aromatic proton d, which is also located on the ' upper ' face of the molecule, shows a large perturbation which was not seen for the other two complexes; this finding should assist in locating the approximate binding site of the D-Ala(2) residue. The vancomycin protons in the sugar and aromatic rings show similar small shifts to those observed in the previous complexes. Thus, since these are seen in all complexes it appears that the conformational change producing these is induced by the binding of the terminal RCO-D-Ala(1) fragment to vancomycin.

(iv) *Vancomycin and* Ac_2-L-Lys-D-Ala-D-Ala. We see a similar pattern of bound shifts as observed for Ac-D-Ala-D-Ala binding. Again proton d experiences a large shielding effect as was seen in the Ac-D-Ala-D-Ala complex which confirms that it is related to interactions with the D-Ala(2) residue. The aromatic proton k shows an even larger deshielding perturbation than that seen in previous complexes (signals from k and f have

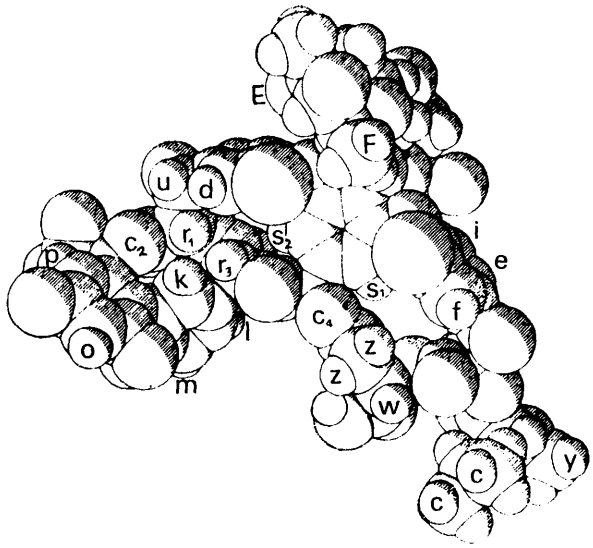


FIGURE 2 View of the ' upper ' face of the space-filling model of the vancomycin analogue (CDP–1) constructed by Sheldrick and his co-workers from *X*-ray crystal co-ordinates (reproduced with permission from ref. 10)

slow exchange characteristics in this complex and are very broad).

A moderately large shift change is also noted for the CH_3(E) protons in the amino-sugar ring: this is much larger than that observed in previous complexes and could indicate that the L-Lys residue is extending in the direction of the sugar moiety.

(v) *Vancomycin and* Ac_2-L-Lys-D-Ala-D-Ala in [$^2H_6$]-*dimethyl sulphoxide solution.* The ' bound ' shifts for this complex are given in Table 3. These were obtained from a 5mM solution of vancomycin hydrochloride in the

presence of 9mM-Ac_2-L-Lys-D-Ala-D-Ala. Under these conditions virtually all the vancomycin will be in the complexed state if the binding is similar to that measured in aqueous solutions. Unfortunately some decomposition of the peptide always occurred during the experiments and gave rise to extra peptide signals: these signals remained sharp and unshifted when the relative concentration of vancomycin was changed, indicating that the decomposition product does not bind to vancomycin in the presence of an excess of Ac_2-L-Lys-D-Ala-D-Ala.

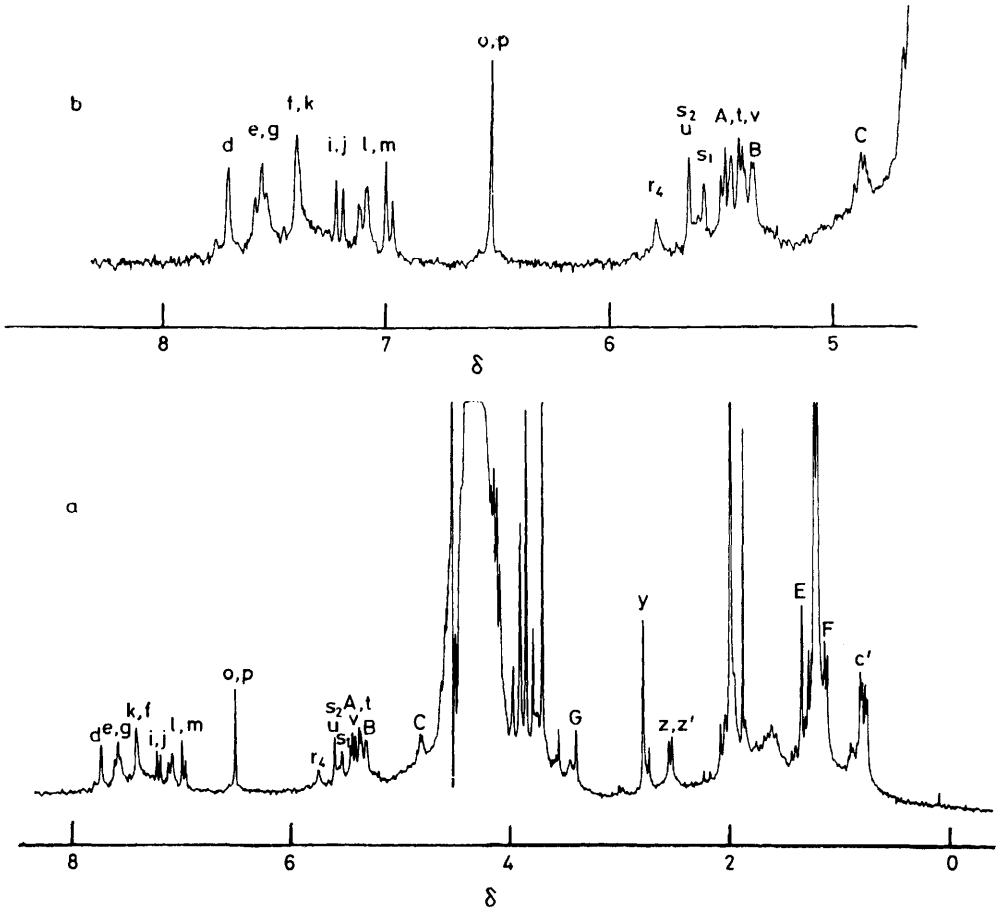


FIGURE 3 The 270 MHz ^1H resonance spectrum of 0.25mM-vancomycin hydrochloride in CD_3CO_2D–NaOD–D_2O buffer solution in the presence of 0.8mM-*N*-acetyl-Gly-D-Ala at pH 5.5 and 45 °C

The main advantage of examining this complex in dimethyl sulphoxide solution is that the NH proton signals can now be detected. Williams and Kalman [9] have previously examined the complex of vancomycin and Ac-D-Ala-D-Ala in this solvent and noted that the vancomycin Asn NH_n proton is affected by the binding. In the complex with Ac_2-L-Lys-D-Ala-D-Ala we also observe a large perturbation (> -25 Hz) for the NH_n proton and an even larger one for c_4 NH proton (> -40 Hz). These two NH protons are on opposite faces of the vancomycin molecule. Another NH proton (c_2 or c_3) also shows an appreciable shift (−13 Hz) on peptide binding.

We have also measured the ^1H shifts of the peptide NH protons in the complex. The NH(1) proton (in the D-Ala-D-Ala peptide bond) shows a very large downfield shift (>100 Hz) on binding while the other NH protons have much smaller shifts (> -12, 11, 5 Hz).

Structure of the Complexes.—The ^1H chemical shift changes observed in ligand binding clearly indicate which protons are influenced by the binding. As in all studies of this type, it is very difficult to distinguish between a change in shielding which results from a direct interaction with the ligand from one caused by an induced conformational effect. This uncertainty limits the usefulness of the bound shifts in helping to define the peptide binding site. However, by assuming that the largest shifts arise either from direct effects or from induced conformational changes near to the sites of direct interaction we can deduce some features of the overall conformation of the peptide–vancomycin complex.

(i) *The peptides bind on the 'upper' face of vancomycin.* Examination of the data given in Table 2 shows that although bound vancomycin ^1H chemical shifts are seen for protons on both faces of the molecule, by far the larger number of strongly perturbed protons are on the

' upper ' face of the disc. This strongly indicates that the peptide is binding on this face of the molecule as suggested earlier by Sheldrick and his co-workers.[10]

(ii) *There are some conformational changes in vancomycin on binding to peptides.* The binding of the relatively small molecule Ac-D-Ala causes chemical-shift changes for more vancomycin protons than would be expected from direct interactions. Thus some of these shifts must be caused by induced conformational changes. Furthermore, in the complex of vancomycin with Ac_2-L-Lys-D-Ala-D-Ala in DMSO, NH protons on opposite faces of the vancomycin molecule are perturbed by the binding: the shielding of at least one of these protons must be influenced by a conformational change.

(iii) *The* D-Ala(1) *residue binds near to the* Asn *and* N-*methyl-leucine residues of vancomycin. The other peptide residues are located down the vancomycin cleft in the direction of the* 2,4-*dihydroxyaromatic ring.* Earlier work [5] has indicated that the D-Ala(1) residue binds in a similar binding site in complexes with various peptides and that the D-Ala(1) carboxylate anion is sufficiently close to the *N*-methyl-leucine residue to interact directly with this cationic site. In the present study, the *N*-methyl-leucine δ-protons (c', c), the Asn methylene protons (z, z'), and the aromatic protons f and s_1 have a similar pattern of shifts for all the complexes in D_2O suggesting that these protons are close to the binding site for the D-Ala(1) residue which is present in all the peptides examined: these protons are all located around the Asn residue and near to the *N*-methyl-leucine end of the molecule. This can be seen in Figure 2 which shows a model of vancomycin with its ' upper ' face on view and with the *N*-methyl-leucine residue on the extreme right of the structure and the 2,4-hydroxyaromatic ring on the left of the structure.

As the peptides become longer the shielding of the aromatic protons d and k (and to a lesser extent o and p) becomes increasingly influenced, suggesting that the peptides are extending towards these protons away from the *N*-methyl-leucine end of the molecule. This would be consistent with the picture of the peptide extending down the cleft on the upper face of the structure as previously suggested by Sheldrick and his co-workers.[10]

(iv) *The* D-Ala(2) *residue is located near to proton d.* The bound shift of this proton is markedly different in the complexes with Ac-Gly-D-Ala and Ac-D-Ala-D-Ala which indicates the approximate location of the D-Ala(2) residue in these complexes.

(v) *The* ^1H *shifts of the bound peptides are consistent with the proposed model* [*structure* (II)]. Any model for peptide binding to vancomycin must also be consistent with the ^1H shifts of the bound peptides reported by Brown and her co-workers [5] and reproduced in Table 4.

There are five aromatic rings in vancomycin and it is reasonable to expect that they will contribute substantially to the shielding of the protons in the bound peptides. As a preliminary to understanding these shifts we have estimated the total ring current shielding contributions for all positions near to the vancomycin molecule using the Johnson–Bovey [12] equation. Figure 4b shows a typical contour map for the shielding above the ' upper ' side of the vancomycin molecule (Figure 4) in a plane parallel to ring B and 3.5 Å from its centre. If we assume that there are no major changes in vancomycin backbone conformation on peptide binding then such contour maps give us a reasonable picture of the possible shielding contributions for protons in the bound peptide. From a CPK model of the complex of Ac-D-Ala-D-Ala and vancomycin constructed according to model (II) (with the linear NH · · · OC hydrogen bonds having an N · · · O separation of 2.9 Å) we have estimated the approximate positions of the atoms in the bound peptide: the projections of some of the important atoms are indicated in Figure 4. The α-proton and the β-carbon at residue 1 are *ca.* 5.5 Å above the plane of ring B while the corresponding atoms in residue 2 are *ca.* 3 Å above the plane. The positions of the bound protons are only approximate estimates because of the uncertainty in the ϕ and ψ angles in the bound peptide. However, consideration of the contour map in Figure 4 and of similar maps in different planes indicates that the observed bound shifts could be adequately explained in terms of ring current shifts and the proposed model. The methyl protons of Ala(1) are in a shielding region of the map which could provide the observed 0.57 p.p.m. upfield shift while the α-CH of Ala(1) is in a region of very low shielding as required by the small observed

TABLE 4

The ^1H chemical shift changes (p.p.m.) in the proton signals of peptides bound to vancomycin in aqueous solution (taken from ref. 5)

	Residue 1				Residue 2				Residue 3
	a		b		a		b		
	CH_3	H	CH_3	H	CH_3	H	CH_3	H	CH_3
Ac-D-Ala-D-Ala	+0.57			+0.04	+0.20			−0.23	+0.09
Ac-L-Ala-D-Ala	+0.60			−0.07		+0.20	0		0
Ac-Gly-D-Ala	+0.55			+0.07		+0.31		−0.46	+0.18
Ac-D-Ala-Gly		+1.32		−0.07	+0.20			−0.45	+0.05
Ac-Gly-Gly		+1.80		−0.33		+0.47		−0.80	+0.26
Ac-D-Ala	+0.56			−0.14	+0.05 *				

* Acetyl methyl protons.

' bound ' shift. The shielding of the methyl protons of Ala(2) and the deshielding of its α-proton are also satisfactorily explained since they fall in shielding and deshielding zones of the contour map. The observed bound shifts for Gly α-protons in the Gly-containing peptides are usually much larger than those seen for protons in other residues and while the pattern of shifts can be rationalised in terms of the ring current contributions it is clear that other factors are involved. In these calculations no allowance has been made for shielding contributions from other anisotropic groups (in particular from carbonyl groups in the peptide bonds which could make large contributions to the α-proton shifts). A more rigorous attempt to define the conformation of the peptide–vancomycin complex from considerations of ' bound ' shifts would need to include such contributions and would require a more exhaustive search of the many possible conformations.

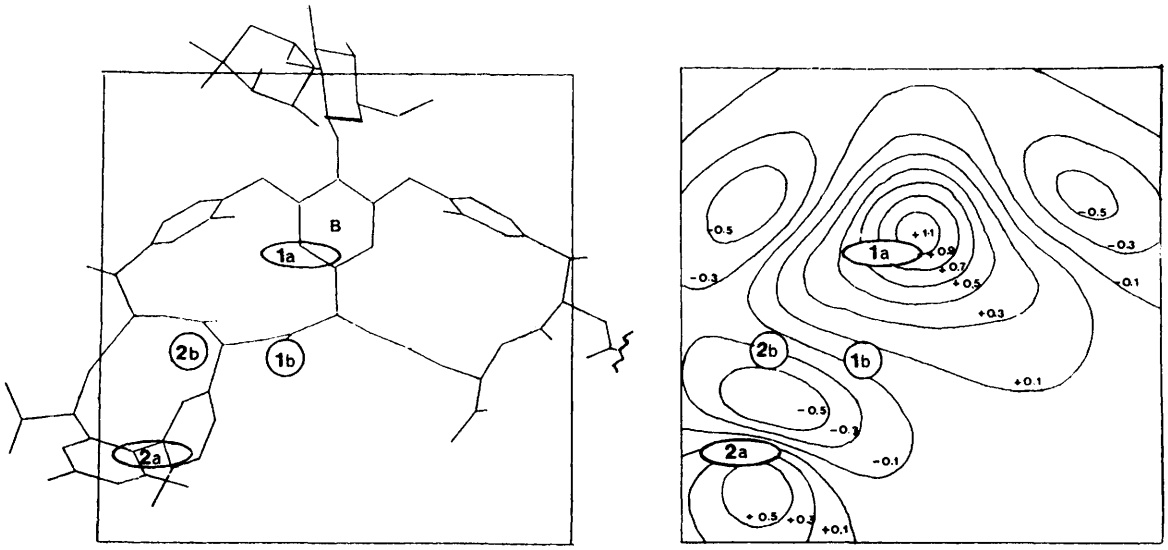


FIGURE 4 The calculated chemical shift contributions (p.p.m.) from the ring currents of the aromatic rings in vancomycin in a plane 3.5 Å above that of ring B in the projection of the structure shown on the left. The estimated projections of the methyl protons (1a, 2a) and α-protons (1b, 2b) in bound *N*-acetyl-D-Ala-D-Ala are also indicated

(vi) *The peptide* NH(1) *proton is directly involved on interaction with the vancomycin.* In the complex of vancomycin with Ac_2-L-Lys-D-Ala-D-Ala, the peptide NH(1) proton (in the D-Ala-D-Ala peptide linkage) is the one most influenced by the interaction: the observed shift is consistent with this NH proton forming a hydrogen bond with an oxygen atom on the vancomycin. This gives further support for the model [structure (II)] suggested by Sheldrick and his co-workers;[10] previously there was no direct evidence implicating specific peptide NH protons in the binding. It is also noted that the c_4 NH proton of vancomycin experiences a large shift as would be expected if it is hydrogen bonded to the adjacent D-Ala(2) carboxy oxygen.

Conclusions.—The foregoing results give information about the general location of the binding site which is consistent with that proposed in the model of Sheldrick and his co-workers[10] [structure (II)]. However, some modifications of this model are required in order to explain one of the features of the binding referred to in the Introduction, namely that the proposed model does not offer an immediate explanation for the increased binding of *N*-Ac-D-Ala-D-Ala when its terminal carboxylate group is in the anionic state and the vancomycin *N*-methyl-leucine is in its cationic state.[2-4] In the complex shown in structure (II) the D-Ala(1) carboxylate anion is *ca.* 9 Å from the positively charged *N*-methyl protons which is too far away to produce the observed favourable electrostatic interaction[3] (ΔH 1.4 kcal mol^{-1}). An alternative model in which the D-Ala(1) carboxylate group is interacting more directly with the *N*-methyl-leucine and where the peptide NH(1) proton is hydrogen bonded to the phenylglycine amide oxygen has been considered. However, this model does not readily explain the large upfield shift of the D-Ala(1) methyl protons nor the extensive shift changes observed for the aromatic protons d and k when the peptides bind. A more reasonable model is obtained by postulating that the peptide binds as in structure (II) but induces a conformational change in the flexible *N*-methyl-leucine side-chain which brings the interacting charged groups closer together: this is illustrated in Figure 5. Such a conformational change, together with some changes in the torsion angles of the Asn residue could reduce the inter-charge separation to *ca.* 5 Å where the electrostatic interaction energy would be sufficiently large to account for the observed increase in pK of *N*-methyl-leucine when the peptide binds to vancomycin. Such conformational changes would also be consistent with the bound shifts observed for the γ-methyl protons of *N*-methyl-leucine and the methylene and NH protons of the Asn residue.

It is also of interest to consider if the proposed model can account for the requirement that residues 1 and 2 be

D-amino-acids in the strongly binding peptides. For residue 2 (where the D–L specificity is lower) the proposed model would indeed lead to severe steric interactions between the side-chain protons of an L-amino-acid and parts of the vancomycin structure, while a D-amino-acid would have no such interactions. For an L-residue at residue 1, the steric interactions are less obvious although there could be some unfavourable interactions between the L-residue side-chain protons and those of the methylene group of the Asn residue. However, the D–L specificity could also arise if the peptide ϕ and/or ψ angles required for optimum binding are allowed only in the case of the D residue.

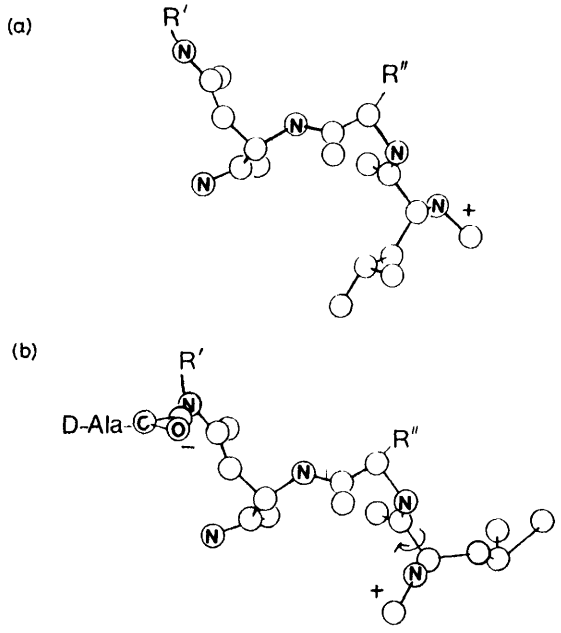


FIGURE 5 (a) The conformation of the *N*-methyl-leucine fragment of vancomycin as found in the crystal structure of CDP-1.[10] (b) The proposed change in conformation of the *N*-methyl-leucine induced by binding *N*-Ac-D-Ala-D-Ala

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