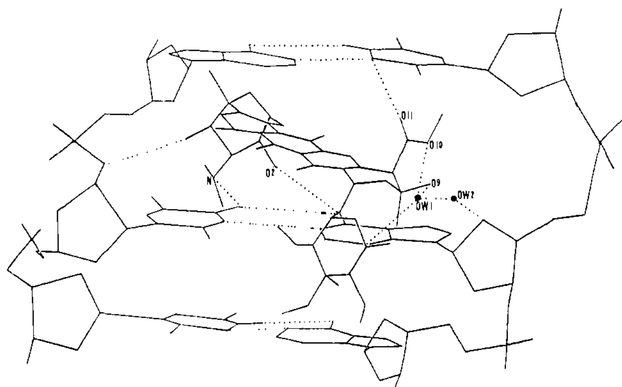

Figure 9. Possible mode of interaction of nogalamycin with DNA. Only base pairs AT, TA, and TA are shown.

dichroism studies[9] have shown that nogalamycin binds firmly to calf thymus DNA. This result is also supported by the results from the melting point increase technique. The DNA unwinding angle has a value of 8.1°.

Only one study[23] of an anthracycline (daunomycin) and DNA (CpGpTpApCpG) by crystallography has been done. It confirmed that daunomycin intercalates into DNA and that the amino sugar lies in the narrow groove. Fluorescence studies[8] have shown that nogalamycin has a preference for the A-T rather than G-C base pair. A study by DuVarney et al.[11] on anthracyclines revealed that stereospecificity of the carbomethoxy group at C(10) is very important for antitumor activity and DNA binding; i.e., it must be axial. Using the above information, along with the stereochemistry and molecular geometry of nogalamycin found in the present study and making use of Kendrew and CPK models of antibiotic and DNA, we have postulated a possible model for nogalamycin-DNA interaction in Figure 9. The base pairs AT, TA, and TA are shown. It has been assumed that water molecules are involved in antibiotic-DNA interaction. The primary interaction causing the binding of nogalamycin to DNA is of course, intercalation. Rings A and D will stick out (as in daunomycin), and the amino sugar and nogalose will lie in the wide and narrow grooves, respectively. The secondary forces of interaction will be hydrogen bonding involving (a) O(11) of the carbomethoxy group with O(2) of thymine in the top base pair, (b) the hydoxyl O(9), O(10), and backbone in the narrow groove through water molecules, and (c) O(2′) and nitrogen of amino sugar with thymine of the middle base as well as with the backbone in the wide groove. The nogalose may be involved in hydrogen bonding (since the oxygens of methoxyls are involved in hydrogen bonding in the crystal structure).

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Registry No. Nogalamycin, 1404-15-5.

Supplementary Material Available: Bond lengths, angles, and isotropic thermal parameters (2 pages). Ordering information is given on any current masthead page.

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Detailed Binding Sites of the Antibiotics Vancomycin and Ristocetin A: Determination of Intermolecular Distances in Antibiotic/Substrate Complexes by Use of the Time-Dependent NOE

Dudley H. Williams,* Michael P. Williamson, David W. Butcher, and Stephen J. Hammond

Contribution from University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, U.K.
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Abstract: The binding site of the antibiotic vancomycin for the peptide cell-wall analogue Ac-D-Ala-D-Ala has been studied by nuclear Overhauser effect difference spectroscopy (NOEDS). Intramolecular nuclear Overhauser effects (NOEs), observed between protons of the antibiotic in the ^{1}H spectrum of the vancomycin/Ac-D-Ala-D-Ala complex, confirm the formation of a carboxylate binding pocket in the bound state of the antibiotic. This pocket is not present in the X-ray structure of a compound, CDP-I, closely related to vancomycin; it appears to be induced by binding. The spectrum of the complex shows three intermolecular NOEs that define further the overall picture of the binding site, and one establishes that the hydrophobic side chain of N-terminal *N*-methylleucine is folded in to form a pocket that accommodates the carboxyl group of Ac-D-Ala-D-Ala. Additionally, by measuring the rate of buildup of the NOE, it is possible to measure intermolecular as well as intramolecular distances. This technique has been applied to a complex of ristocetin A with Ac_2-L-Lys-D-Ala-D-Ala, and intermolecular proton-proton distances have been determined. These studies also confirm the recent proposal that the N-terminal amino acid of ristocetin A has the *R* absolute configuration. Relative to the binding of Ac-D-Ala-D-Ala, the lysine residue is more weakly bound to both vancomycin and ristocetin, and its binding site less precisely defined.

In earlier work, we have determined the X-ray structure and conformation of a compound, CDP-I, obtained by conversion of a primary amide in the antibiotic vancomycin to a carboxyl group.[1] This structure was initially interpreted as representing the structure and conformation of vancomycin, except for the conversion noted above.[1] However, it has been shown subsequently that two other changes occur in the conversion of vancomycin to CDP-I. One is that the ring bounded by curved arrows in Figure 1 is rotated through ca. 180°;[2a] this brings both chlorine atoms to the same side ("top face") in CDP-I. The second is that the third amino

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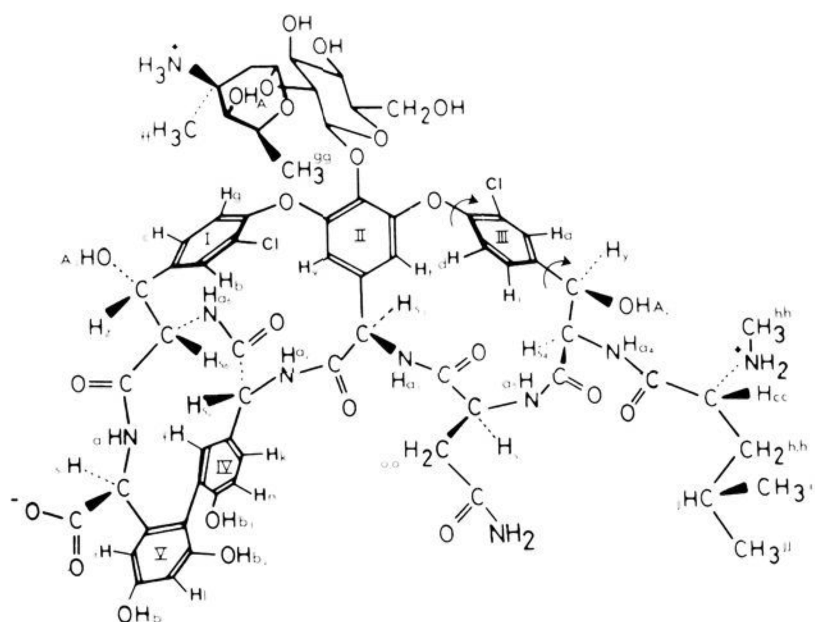

Figure 1. Structure of vancomycin, showing the letter code used to assign the major part of the ^{1}H NMR spectrum.

acid from the N terminus isomerizes from asparagine to isoasparagine.[2b] A bond-breaking process, which is necessary[2a] for the reorientation of the chlorine-containing ring, appears likely to occur in the isomerization of asparagine to isoasparagine.[2b] Thus, the structure of vancomycin that satisfies the currently available evidence is that reproduced in Figure 1.

If the conformation of vancomycin is otherwise assumed to be that found for the crystals of CDP-I, it is possible, on the basis of limited proton NMR evidence and model-building studies, to propose[1] a binding site for vancomycin to a peptide analogue, Ac-D-Ala-D-Ala, of the portion of cell wall to which vancomycin is known to bind.[3] We have recently suggested[4] that this proposal, while correct in the interactions proposed, omits additional interactions that arise when vancomycin changes its conformation upon binding; from an initial conformation that is assumed (with supporting evidence) to be similar to that found for CDP-I, to one fundamentally modified in the orientations of the *N*-methylleucine, asparagine, and the β-hydroxychlorotyrosine, which is bounded by the first two mentioned amino acids. The proposed binding site of vancomycin is strikingly similar to that of ristocetin A for the same cell-wall analogue.[5] A CPK model of the proposed binding site of vancomycin for Ac-D-Ala-D-Ala is reproduced in Figure 2.[4] It can be seen that the extended form of the antibiotic amide backbone, found for the three N-terminal residues in the X-ray structure (approximately conveyed in Figure 1), has disappeared in Figure 2. Instead, the four NH groups of the N-terminal residues (*N*-methylleucine, β-hydroxychlorotyrosine, asparagine, and (*p*-hydroxyphenyl)glycine) are clustered together to form a pocket to bind the carboxylate anion of the C-terminal D-alanine. The proposed conformational change was largely based upon changes in proton chemical shifts of vancomycin upon binding of the peptide;[4] most importantly, upon binding, the chemical shift of a_4 changes from 8.00 to 11.75 ppm. This is consistent only with the formation of a strong hydrogen bond from this NH proton to a carbonyl oxygen of Ac-D-Ala-D-Ala. The formation of such a hydrogen bond is not possible while a conformation of vancomycin similar to that found for crystalline CDP-I, is maintained. However, this shift, and several others, are accommodated by the binding site illustrated in Figure 2.

In the first part of the present paper, we use nuclear Overhauser difference spectra (NOEDS) to establish further the binding site illustrated in Figure 2 and to refine it by showing that the hydrophobic side chain of N-terminal *N*-methylleucine is folded in to form a hydrophobic wall to the pocket that binds the carboxylate anion of Ac-D-Ala-D-Ala.

The second part of the paper deals with the interaction of the antibiotic ristocetin A with the cell-wall peptide analogues Ac-D-Ala-D-Ala and Ac_2-L-Lys-D-Ala-D-Ala.[5] Ristocetin A,[6,7] like

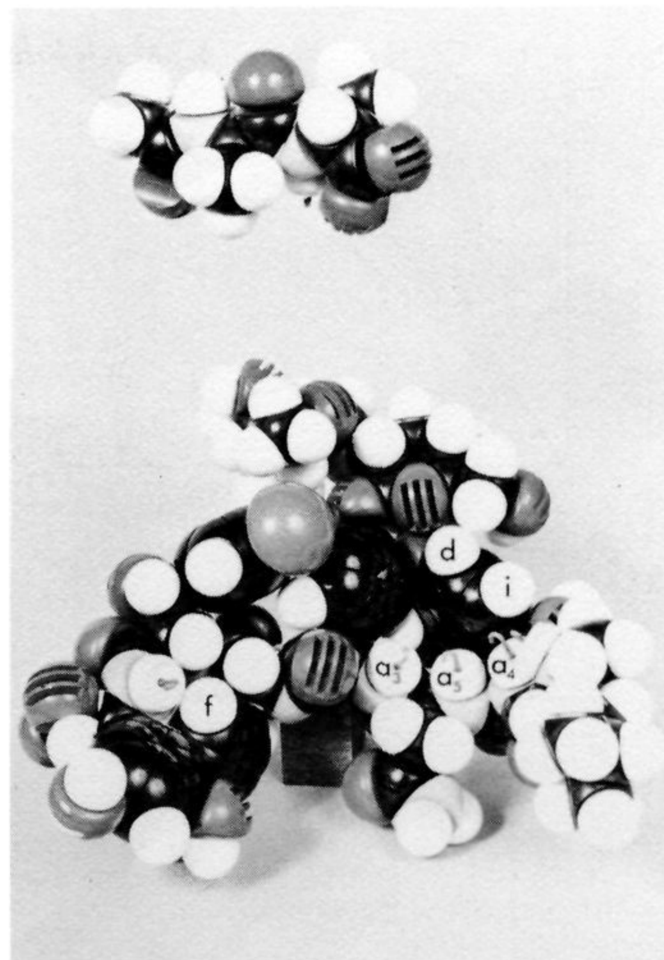

Figure 2. An exploded view of a proposed CPK model of the binding site of vancomycin for the cell-wall peptide analogue, Ac-D-Ala-D-Ala.

vancomycin, is a glycopeptide antibiotic known to exert its physiological action by binding to bacterial cell-wall mucopeptides. The structure of the mucopeptide varies according to the species,[8] but prior to cross-linking in the final stage of its synthesis, it invariably contains the C-terminal sequence -L-R_3-D-Ala-D-Ala. The residue R_3 is commonly L-lysine, L-ornithine, or *meso*-diaminopimelic acid. UV studies[9] have shown that the major contribution to the binding energy with ristocetin B (which differs from ristocetin A only in lacking two of the neutral sugars) arises from the terminal dipeptide. NOEs that occur in the complex formed between ristocetin A and Ac_2-L-Lys-D-Ala-D-Ala are consistent with the recent conclusion of Harris and Harris[6] that the N-terminal amino acid of ristocetin A has the *R* stereochemistry at the α-carbon. We show that the binding sites of ristocetin A and vancomycin are very similar despite major differences in both structure and solution conformation in the absence of the cell-wall analogues.

In the final part of the paper we deal with the less-well-defined binding of both antibiotics to the lysine side chain of Ac_2-L-Lys-D-Ala-D-Ala.

Experimental Section

Vancomycin was purchased as "Vanococin HCl", a commercial product of Eli Lilly, Indianapolis, and ristocetin A was a gift from Lundbeck, Copenhagen. Ac-D-Ala-D-Ala was prepared by acetylation of D-Ala-D-Ala (Bachem Inc., U.S.A.) using acetic anhydride in water. Ac_2-L-Lys-D-Ala-D-Ala was prepared by coupling of dicarbobenzoxy-L-Lys with the benzyl ester of D-Ala-D-Ala in weakly basic 1,2-dimethoxyethane. The resulting ester was crystallized (mp 166–167 °C) from dilute acetic acid, and the protecting groups were removed by hydrogenolysis. The peptide was acetylated (acetic anhydride/water) and the product freed of α-Ac-L-Lys-D-Ala-D-Ala by chromatography on zeo-Carb 225 cation exchange resin. The resulting aqueous solution was lyophilized to give the desired α,ϵ-Ac_2-L-Lys-D-Ala-D-Ala, which was used without further purification. This compound had the expected ^{1}H NMR spectrum at 400 MHz and gave molecular ions in both positive and negative ion modes in its fast atom bombardment mass spectra (MH^+ at m/z 373 and $(M - H)^-$ at m/z 371).

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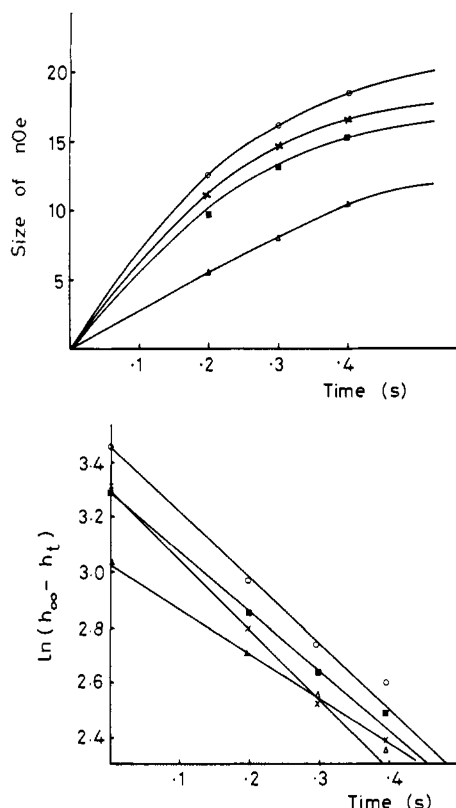

Figure 3. (a, Upper) Examples of the rate of buildup of the NOE after irradiation of bound alanine methyl protons in the ristocetin A (Figure 9)/tripeptide complex: O {Ala$_1\beta$} → e; □ {Ala$_2\beta$} → x; X {Ala$_1\beta$} → j; Δ {Ala$_1\beta$} → w. The irradiation power was sufficient to saturate the irradiated peak essentially instantaneously. The size of NOE is measured in arbitrary units. (b, Lower) Plot of the data in (a) as ln ($h_\infty - h_t$) against time; h_t is the size of the NOE shown above at time t. The gradient of the straight line gives the rate at which the NOE is building up, which is proportional to r^{-6}. Points at later times are given a lower weighting.

Concentrations of the complexes in solution were approximately 7 mM; ristocetin A/peptide complexes in [$^2H_6$]Me_2SO, and vancomycin/peptide complexes in [$^2H_6$]Me_2SO to which 30% CCl_4 had been added. NOEs in the spectra of the ristocetin/peptide complexes were measured at ambient temperature and those in the spectra of vancomycin/peptide complexes at 0 °C.

The NMR spectra were obtained by using a Bruker WH 400 spectrometer (^{1}H, 400 MHz) equipped with an Aspect 2000 computer in the Fourier transform mode with quadrature detection and phase alternation. A spectral width of 4000–5200 Hz was used with digitization over 8K data points. Exponential line broadenings of about 2 Hz were used for difference NOE spectra. The standard Bruker gated NOE pulse sequence was used with minor modifications, and a 4-s delay before each pulse allowed for complete relaxation. Sizes of NOEs were estimated by measuring the heights of peaks in the difference NOE spectra and by comparison with heights in the normal spectrum. Wherever possible NOEs were measured in both directions, although some reverse NOEs, especially to methyl groups, were not observed (see text).

Buildup of the NOE to a steady state (usually about 5 s) was followed to obtain distance measurements (see, for example, Figure 3, top). If only a direct NOE is involved, this growth is exponential and a plot of ln ($h_\infty - h_t$) vs. t, where h_t is the size of the NOE at time t, should give a straight line of gradient $-k$, where $k \propto r^{-6}$. The constant of proportionality was evaluated from protons of known separation (ortho aromatic protons and CH_3–CH groups) and was such that for k = 4.5, r = 2.15 Å. An alternative method, based on theoretical calculations,[10] gives similar values. We consider that this method for the measurement of interproton distances is adequate for all cases to which the simple two-spin approach is applicable,[11] that is, in which there is no third proton intervening between irradiated and observed protons.[12]

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Table I. Assignment of the ^{1}H NMR Spectrum of Vancomycin/Ac-D-Ala-D-Ala[a]

δ	assignment	δ	assignment
0.51	Ala$_1\beta$ bound	5.47	s_4
0.69	ii/jj	5.76	A_2
0.93	Ala$_2\beta$ bound	5.86	s_3
1.02	gg	5.98	A_3
1.18	Ala$_2\beta$ free	6.20	r
1.26	ff	6.31	l
1.28	Ala$_2\beta$ free	6.69	a_6
1.38	j	6.73	n
1.69	h/h'	6.81	k
1.85	Ala CH_3CO	6.92	d
2.14	o	7.17	a
2.60	o'	7.28	b
2.65	hh	7.32	g
3.14–3.66	Sugar protons	7.46	c
3.86	Ala$_1\alpha$ bound	7.63	i
4.16	cc; Ala$_1\alpha$ free	7.75	f
4.30	Ala$_2\alpha$ free	8.02	Ala$_2$ NH (bound + free)
4.41	s_1	8.10	Ala$_1$ NH free
4.50	s_6	8.37	a_5
4.61	s_2	8.75	a_1
4.68	Ala$_2\alpha$ bound	8.79	a_2
4.74	s_5	9.00	a_3
5.13	z	9.05	b_2
5.19	y	9.15	b_3
5.22	v/m/bb	9.28	b_1
5.24		11.75	a_4
5.36	A_1(?)		
5.40	t		

[a] Vancomycin/Ac-D-Ala-D-Ala (1:2) in [^{2}H]Me_2SO/CCl_4 (10:3) at 0 °C and 400 MHz. Resonances not listed in the table have not been assigned; the assignment of the resonance at 5.36 ppm is uncertain.

Discussion and Results

The Vancomycin/Ac-D-Ala-D-Ala Complex. The principle involved in the present work is that an NOE to a proton, upon irradiation of another, indicates the spatial proximity of the two protons. Details of our approach (see also Experimental Section) and the care necessary in interpretation of the data have been given in an earlier paper.[2a] Since an NOE manifests itself as an intensity change of a proton resonance, the effects are most clearly observed in difference spectra.[13] In such spectra, the intense peak due to the irradiated proton is due to saturation of its resonance upon irradiation, and other peaks (usually, in our work, 5–50% of the intensity of the irradiated peak) reflect NOEs. Obviously, a series of such experiments, involving different protons, can give very detailed information as to molecular geometry. Effects due to decoupling are avoided by employing gated decoupling. Since the NOE is transmitted according to the same principles either in a single molecule or in a molecular complex, NOEs are a powerful method of either determining molecular structure[7,13,14] or mapping out drug-binding sites,[5] as in the present work.

To use the above techniques to define the binding site of vancomycin for Ac-D-Ala-D-Ala, it was first necessary to assign fully the proton spectrum of the antibiotic/peptide complex under conditions where dissociation of the complex is slow on the NMR time scale. Such slow dissociation of the complex was induced by recording spectra in a mixed solvent ([$^2H_6$]Me_2SO/CCl_4 in the ratio 10:3) at 0–5 °C.[4] Since the spectrum of vancomycin had been previously assigned in [$^2H_6$]Me_2SO solution[15] and changes little upon addition of 30% CCl_4, it was not difficult to assign it in the above solvent mixture, using the previously described techniques where necessary. The spectrum of the vancomycin/Ac-D-Ala-D-Ala complex was then assigned by using a combination of (i) conventional spin decoupling studies, (ii)

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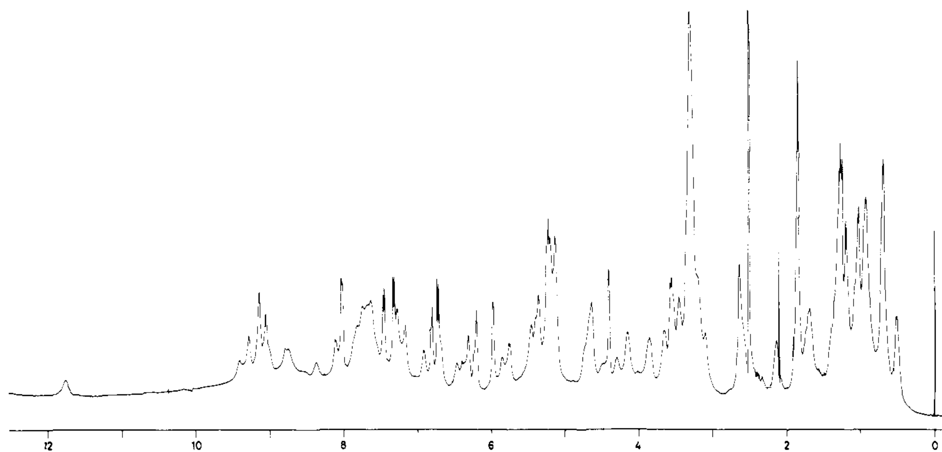


Figure 4. Proton 400-MHz spectrum of the vancomycin/Ac-D-Ala-D-Ala complex (~10 mM) in $[^2H_6]Me_2SO/CCl_4$ (10:3) solution at 0 °C. Chemical shifts are referenced to internal DSS.

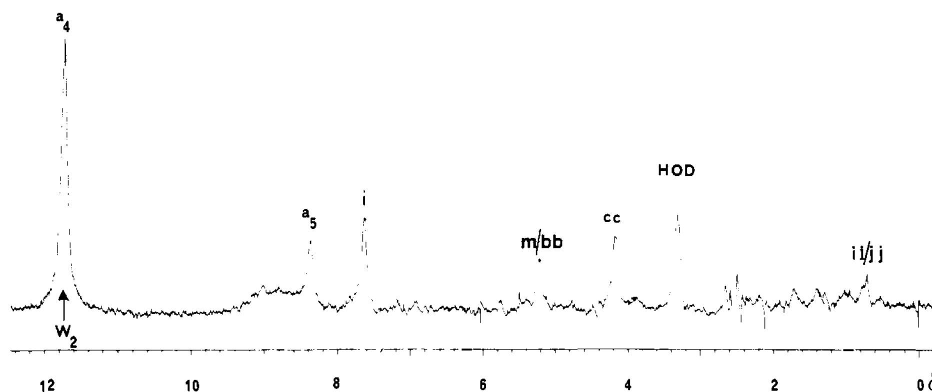
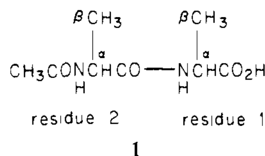


Figure 5. NOE difference spectrum obtained upon irradiation of proton a_4 in the vancomycin/Ac-D-Ala-D-Ala complex (~10 mM) in $[^2H_6]Me_2SO/CCl_4$ (10:3) solution at 5 °C. Irradiation duration was 0.1 s.

transfer of saturation studies as previously described for the ristocetin A/Ac-D-Ala-D-Ala complex,[5] and (iii) observation of NOEs. The spectrum is reproduced in Figure 4 and the assignments are given in Table I; they were made in the presence of a molar excess of Ac-D-Ala-D-Ala over that necessary for complex formation. The code employed for the peptide is given as **1**.



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With the spectrum of the complex assigned, large numbers of NOEDS were acquired by irradiation of selected protons. Since the complex is relatively large and has a moderately long correlation time for rotation, the observed NOEs are negative, and limited spin diffusion occurs.[2a] Where necessary, the complications due to spin diffusion could be differentiated from direct NOEs by determining the time dependence of the NOE buildup,[2a,12] as described in the Experimental Section. Those direct NOEs that are useful in defining the conformation of the vancomycin/Ac-D-Ala-D-Ala complex are given in Table II.

The NOED spectrum obtained upon irradiation of a_4 is reproduced in Figure 5. The peak at 3.25 ppm arises due to transfer of saturation from the irradiated NH proton into HOD. The peak at 5.2 ppm is due to the pair of sugar anomeric protons m and bb. That the NOEs to these protons are not a direct phenomenon is indicated by the slower initial buildup of the signal at 5.2 relative to the signals at 4.16 (cc), 7.63 (i), and 8.37 (a_5). The effects to m and bb may involve transfer of saturation from the a_4 NH proton to exchangeable protons of the sugars, followed by an NOE to m and bb from such protons. Figure 6 shows the time dependence of a number of NOEs after irradiation of a_4; the sigmoid

Table II. Selected NOES Observed for the Vancomycin/Ac-D-Ala-D-Ala Complex[a]

resonance irradiated	resonance(s) reduced in intensity, %
a_4	i(25), a_5(20), cc(20)
a_5	a_4(19), a_3(30), i(17), o(15)
$Ala_1\beta$ bound	d(7)
$Ala_2\beta$ bound	f(7), ii/jj(30)
Ala_2 CH_3CO	Ala_2 NH bound (15)
Ala_2 NH bound	Ala_2 CH_3CO (20)
ii/jj	$Ala_2\beta$ bound (25)

[a] Solvent mixture as in Table I; data recorded at 400 MHz and 5 °C.

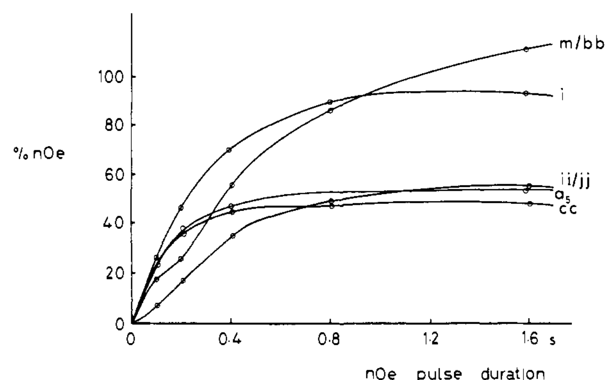

Figure 6. Time dependence of NOEs seen after irradiation of proton a_4. Experimental conditions as for Figure 5.

shape of the m/bb and ii/jj buildup is characteristic of nondirect NOEs. The fast buildup of the NOEs to a_5, i, and cc (Figure 6)

Figure 7. Enlarged and front view (taken from Figure 2) of the carboxylate anion binding pocket of vancomycin in the bound state.

establishes that these three protons are near a_4. This is not the case in the X-ray structure of CDP-I (cf. vancomycin in this conformation, Figure 1), but it is the case in the proposed vancomycin/peptide complex shown in Figure 2. Since a_4 is known to be hydrogen bonded to the carboxylate anion of Ac-D-Ala-D-Ala, the experiment establishes that the NH of asparagine (a_5) has rotated from its position at the "back" of the molecule in the X-ray structure[1] to the "front" (i.e., the same side as the peptide) in the antibiotic/peptide complex. It is noteworthy that this asparagine NH (a_5) does spend some time at the "back" in free vancomycin also, since in this state it gives an NOE to s_4 (see Figure 1). Thus, the concept of a conformational change in vancomycin on binding is supported.

The data also clearly show that in the bound state in solution, a_4 is adjacent to i, as in Figure 7 (which shows an enlarged view of the carboxylate pocket present in Figure 2). Thus, the β-hydroxyl group of the β-hydroxychlorotyrosine unit must move out from its position in the solid state CDP-I structure (cf. Figure 1) to allow the NH a_4 to move inward in proximity to i. There now exists, therefore, direct experimental evidence to support a conformation (Figures 2 and 7) based upon chemical shift arguments and analogy to the ristocetin A structure.[4] Further details of the conformation shown in Figures 2 and 7 are supported by the NOED spectrum obtained upon irradiation of a_5 (Table II). The NOEs to both a_4 and a_3 show that in the bound state a_5 lies close to both these NH protons (Figure 7), whereas it is relatively remote from both these protons in a conformaation (Figure 1) based upon the X-ray structure of CDP-I.

Two further intramolecular NOEs help to define the conformation of bound Ac-D-Ala-D-Ala. Upon irradiation of the acetyl methyl resonance of the peptide, there is a large NOE to the Ala_2 NH, and vice versa (Table II). It is therefore concluded that in the complex (**2**) the acetyl methyl group and the Ala_2 NH are

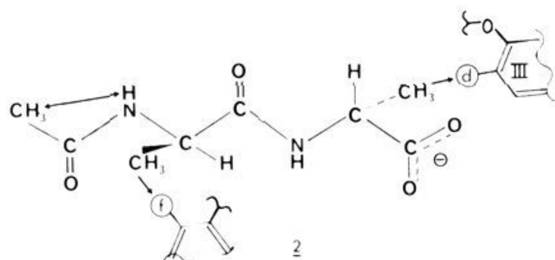

Figure 8. CPK model of the vancomycin/Ac-D-Ala-D-Ala complex. Protons of the dipeptide are shown shaded for clarity, and those of the antibiotic $NH_2CH_3^+$ group are marked by small open circles.

approximately cis to each other. This is in accord with our original[1] and present proposals.

The orientation of portions of the peptide cell-wall analogue with respect to the antibiotic can be checked by the observation of two intermolecular NOEs in the difference spectra (Table II). Irradiation of the methyl protons of the C-terminal Ala residue in its bound form gives an NOE to d. This result shows that this methyl group, in its position over the face of central benzene ring II,[1,16] is placed so that it also lies against the "front" edge of ring III (**2**). Additionally, irradiation of the methyl protons of the Ala_2 residue in its bound form gives an NOE to f. Thus, this methyl group extends over the biphenyl portion of the antibiotic (**2**).

Finally, the large NOEs between ii/jj and Ala β_2 bound (Table II) can only occur if the isobutyl side chain of N-terminal *N*-methylleucine is folded in and back along the antibiotic peptide backbone to bring it close to the methyl group of the Ala_2 residue in the complex. A space-filling model of Ac-D-Ala-D-Ala bound to vancomycin, and incorporating this feature of the binding site, is reproduced in Figure 8. The remarkably tight fit of the peptide to the antibiotic is clearly evident from this figure, as are the hydrophobic walls (comprising aromatic rings II and III and the isobutyl group) of the pocket, which receives the carboxylate anion at the peptide. We presume that this hydrophobic environment serves to strengthen the hydrogen bonding of the CO_2^- to no less than four NH groups with which it can interact. Since the attractive forces in hydrogen bonding can be regarded as largely electrostatic in nature, these attractions will be increased in an environment of low dielectric constant. The location of the isobutyl side chain in the complex is further supported by the intermolecular NOEs observed from the asparagine methylene proton o to ii/jj (10%) and from the asparagine NH (a_5) to ii/jj (9%) in the complex of vancomycin with the tripeptide Ac_2-Lys-D-Ala-D-Ala.

Now that the spectrum of the bound form of the antibiotic has been assigned (Table I), it can be seen that an appreciable downfield shift of a_5 does occur upon binding (6.59→8.37 ppm). This is in accord with its involvement in the formation of a hydrogen bond. The complex illustrated in Figure 8 is consistent with all the accumulated evidence and accommodates the points recently made by Convert et al.[16] The *N*-methyl group of the terminal *N*-methylleucine residue is of course protonated under the conditions of our experiments. The protons of the $-NH_2CH_3^+$ group are indicated by small open circles (Figure 8); their proximity to one of the oxygens of the peptide CO_2^- group can

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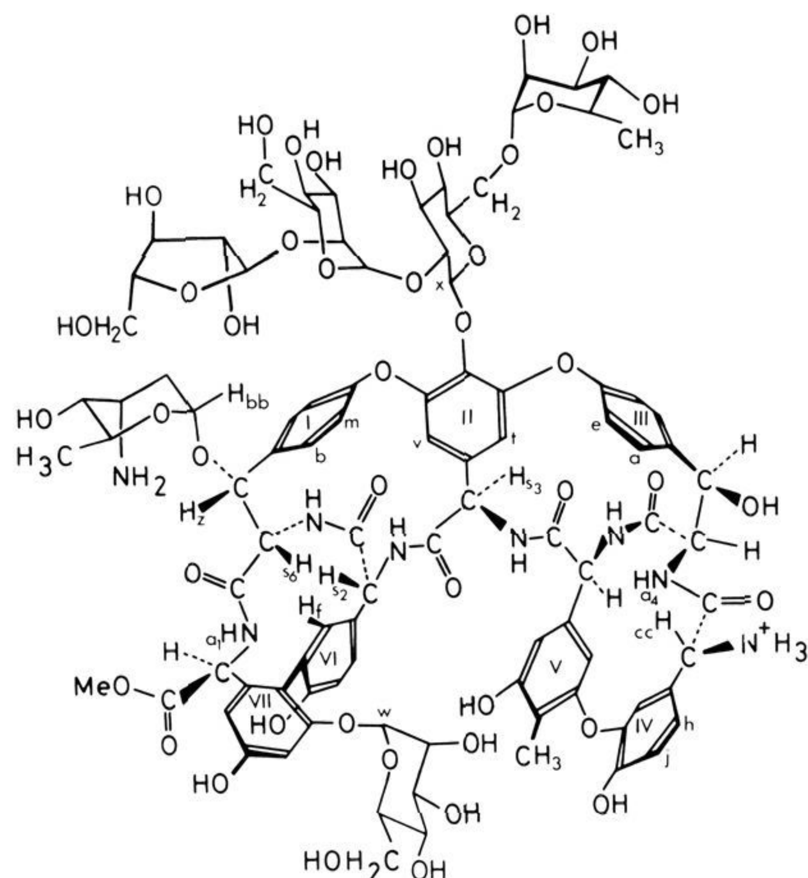

Figure 9. Structure of ristocetin A showing letter code used.

clearly be seen. Discussion of the binding site for the lysine side chain is deferred until the binding of ristocetin A for –D-Ala-D-Ala has been considered.

Ristocetin A and Its Binding Site for –D-Ala-D-Ala. The structure of ristocetin A is reproduced in Figure 9. Ristocetin A exhibits a larger barrier to the dissociation of antibiotic/dipeptide complex, or antibiotic/tripeptide complex (where "tripeptide" is Ac_2-Lys-D-Ala-D-Ala), than does vancomycin.[17] It was therefore decided to use the ristocetin A/tripeptide system to obtain NOE-derived intermolecular proton–proton distances. This system is convenient because the complex and free components are in slow exchange on the NMR time scale well above room temperature.

The rate of buildup of the NOE is proportional to r^{-6}, where r is the distance between the two nuclei. If the constant of proportionality can be determined, the rate of buildup may be used to measure interproton distances, and because of the r^{-6} dependence, the distance measurements can be quite precise.[2,14] There are two sources of potential error that must be avoided. First, spin diffusion will lead to spurious results, and second, vibration or other periodic distance variation will tend to produce a smaller distance than the true equilibrium value. For these reasons, the technique is preferably applied over distances of less than 3 Å, and in reasonably rigid systems. Ristocetin A seems to satisfy the last criterion well. Indeed, the present study has refined and extended earlier work[5] on ristocetin A/peptide binding sites. It is shown that (i) the two alanine residues are firmly bound and intermolecular proton distances can be calculated, (ii) aromatic ring IV (see Figure 9) is folded in in the complex to provide a hydrophobic wall for the peptide carboxylate anion pocket (cf. vancomycin), and (iii) the lysine is less firmly bound, and NOE-derived distances are not reliable. We have also been able to show that the data from NOE difference spectra are consistent with the results of Harris and Harris,[6] showing that, contrary to our earlier conclusion,[18] the configuration of the N-terminal residue is *R*.

(i) The Stereochemistry of the N-Terminal Residue. The assignment and nature of the proton spectra of ristocetin A and of the ristocetin A/dipeptide complex have previously been re-

(17) Williams, D. H.; Rajanada, V.; Williamson, M. P.; Bojesen, G. "Topics in Antibiotic Chemistry", Sammes, P., Ed.; Horwood: Chicester, U.K., 1980, Vo. 5, Part B.
(18) Williams, D. H.; Rajanada, V.; Bojesen, G.; Williamson, M. P. *J. Chem. Soc., Chem. Commun.* **1979**, 906.

Figure 10. A portion of the binding site of ristocetin A for the tripeptide cell-wall analogue, with indication of some antibiotic protons to which distances from peptide protons have been measured. As previously deduced,[5] in this environment the C-terminal D-Ala carboxylate anion can potentially hydrogen bond to three NH protons of secondary amides. Although the *R* configuration at the N terminus of the antibiotic (see text) does not permit salt-bridge formation between its NH_3^+ group and the carboxylate anion of the peptide, the electrostatic attraction of the two groups probably does have a role in binding the peptide.[6] A newly deduced feature of the binding site, the "folding in" of the aromatic ring carrying proton j, is discussed in the text.

Table III. NOE-Derived Intermolecular Distances for the Ristocetin A/Tripeptide Complex[a]

proton pair	distance, Å	proton pair	distance, Å
$Ala_1 \beta$–e	2.3	$Ala_2 \beta$–j	2.4
$Ala_1 \beta$–a	>2.5	$Ala_2 \beta$–w	2.2
$Ala_1 \beta$–x	2.3	$Ala_2 \beta$–f	2.1
$Ala_1 \beta$–m	2.3	$Ala_2 \alpha$–s_2	2.5
$Ala_1 \alpha$–j	2.8	Lys ϵ-CH_3CO–bb	>2.6

[a] Distances are ±0.1 Å. In each case the first named proton of the pair was irradiated and the NOE buildup to the second observed. In most cases the reverse NOE could not be measured; this is not surprising as NOES to methyl groups would be expected to be small.

ported.[5,7] The proton spectrum of the ristocetin A/tripeptide complex was assigned by using the previously described techniques. Internuclear distances may be obtained from truncated Overhauser effects (TOEs) as described previously[2] (see Experimental Section).

In their NMR determination of the structure of ristocetin A, Kalman and Williams[7] were unable to determine the stereochemistry of the N-terminal residue. It was subsequently reported[18] that the stereochemistry was *S*, largely on the basis of an NOE to proton a upon irradiation of the NHCOMe proton at the N terminus of an acetylated derivative of ristocetin A. This configurational assignment was in contradiction to the findings of Lomakina et al.[19] In recent work, Harris and Harris[6] have reinvestigated this point and, on the basis of chemical experiments, reached the conclusion that the configuration of the (*p*-hydroxyphenyl)glycine residue at the N terminus is *R*. The NOE mentioned above was observed before quantitative experiments, utilizing difference spectra, could be performed to uncover NOEs that might arise through spin diffusion. The experiments have been repeated with use of short preirradiation times to avoid spin diffusion. These experiments gave ambiguous results, probably because rings IV and V can undergo large-scale oscillations in free ristocetin. However, on addition of peptide, ring IV "folds in" over the carboxylate ion of the peptide as described below, and the conformation becomes locked. Large NOEs can then be

(19) Lomakina, N. N.; Zenkova, V. A.; Bognar, R.; Sztariczkai, F.; Sheinker, Y. N.; Turchin, K. F. *Antibiotiki (Moscow)* **1968**, *13*, 675.

observed, from which the distances cc–h = 2.45 ± 0.1 Å and cc–a_4 = 2.2 ± 0.1 Å can be calculated. These distances are only consistent with the *R* stereochemistry at the carbon bearing proton cc (see Figure 10) and are consistent with the conclusion reached on the basis of chemical degradation.[6] It is therefore clear that the NOE observed previously[18] was due to spin diffusion. Another conformation of ristocetin as described by Harris[6] may also contribute, although there is no evidence for it in time-course NOEs.[20]

(ii) Intermolecular NOE-Derived Distances. Intermolecular distances obtained for the ristocetin A/tripeptide (3) complex are

```
                                          βCH3      βCH3
         ε   δ    γ    β   α               |         |
CH3CONHCH2CH2CH2CH2CHCO-NHCHCO-NHCHCO2H
                       |        α         α
                       NH
                       |
     Lys               COCH3  Ala2      Ala1
```

3

given in Table III. (The ristocetin nomenclature is the same as that used by Kalman and Williams for free ristocetin.[7]) It should be stressed that these are "weighted" values in that they are not $\langle r \rangle$, but $\langle r^{-6} \rangle^{-1/6}$; they will therefore tend to be lower limits for the equilibrium distances.

It was anticipated that these distances should be fairly accurate because of the r^{-6} dependences. The binding constant between ristocetin A and tripeptide is large (roughly 6 × 10^5 L/mol in aqueous solution[9]), and the ristocetin should be 98.5% complexed at the concentrations used here. The on–off process can therefore be ignored; the only problem is whether the peptide is held tightly enough so that the distances are meaningful and do not merely reflect apparently short distances generated by occasional close approaches of one proton to another in the course of large amplitude oscillations. Construction of a space-filling CPK model, based on these distances and those derived from other intramolecular NOEs seen, is possible and indicates that the binding site for the -D-Ala-D-Ala terminus is very tight and that many of the protons are in van der Waals contact (see Table III). While this does not provide unequivocal proof of the correctness of the distances, it is at least self-consistent,[21] and the binding site produced is in accord with all other data.

In contrast to the alanine residues, which gave a number of large NOEs, very few NOEs were seen involving the Ac_2-L-Lys protons. The only NOE seen for the Lys side chain in the ristocetin A complex was from the ε-acetyl methyl to the anomeric proton of ristosamine bb. This gave an apparent distance of 2.6 Å; the reverse NOE was not seen. Although it is quite possible to construct models giving this internuclear distance, there seems to be no good reason why the Lys side chain should stay in any of these configurations. In view of the general paucity of NOEs in this part of thee molecule, we must conclude that the lysine side chain is fairly flexible. This NOE only allows us to say that the ε-acetyl methyl is within 2.6 Å of bb for some of the time, and further discussion of the binding of the lysine residue is deferred. The appropriate conclusion would seem to be that NOE-derived distances should be treated with caution. They are most reliable when the derived distances are <2.5 Å, and the structure being analyzed is relatively rigid.

(iii) The Binding Site for -D-Ala-D-Ala. This study has confirmed the previous proposals[5] for the binding site of ristocetin A for cell-wall peptide analogues but has refined these and uncovered several additional features. The intermolecular distances of Table III can, for the binding of -D-Ala-D-Ala, be divided into four groups: distances of the antibiotic protons from those of (i) the C-terminal Ala methyl group, $Ala_1\beta$, (ii) the α-CH proton of this residue, $Ala_1\alpha$, (iii) the in-chain Ala methyl group, $Ala_2\beta$, and (iv) the α-CH proton of this residue, $Ala_2\alpha$. These groups

(20) Williamson, M. P., Thesis, University of Cambridge, 1981.

(21) Any large scale oscillation would almost certainly lead to an impossible distance map; see the example of the s_3–v–t system in ref 2.

Figure 11. (a, Upper) The binding site of ristocetin A for the in-chain D-Ala methyl group. (b, Lower) The binding site in (a) has been "opened up" to expose protons (w, j, and f) whose distances from the in-chain D-Ala methyl group have been measured from NOEs.

of distances are now considered in pairs (i, ii and iii, iv).

The C-terminal Ala methyl group is deduced to be at a distance of ca. 2.3 Å from the aromatic protons e and m and from the glucose anomeric proton x. These interactions are summarized in Figure 10. In this figure, the only portion of the tetrasaccharide shown is the glucose residue, since the sugars do not have a major role in binding the peptide. Although we conclude that the glucose anomeric proton spends at least some time on the "binding side" of ristocetin A ("top face" as shown in Figure 9), we do not preclude sufficient conformational mobility of the sugar side chain to allow this proton to rotate to the opposite face of ristocetin A (where the corresponding proton of vancomycin lies in the X-ray structure of CDP-I[1]). However, it is possible that when the glucose residue occupies the conformation shown in Figure 10, it can contribute to hydrophobic bonding of the C-terminal Ala methyl group (three axial C–H protons of glucose lie close to this methyl group). The Ala_1 α-CH proton approaches to ca. 2.8 Å of proton j of the antibiotic. Until these experimental data were available, we had no secure evidence that the aromatic ring IV (Figure 9) could fold in to provide an extended hydrophobic pocket to encompass the carboxyl group when it interacts with the various NH protons of the antibiotic (see legend to Figure 10 for details). However, this feature had appeared to be a possibility from ^{13}C data.[22] The advantage of this position of the aromatic ring IV in the binding peptide is presumably to place the interactions between the CO_2^- and various NH groups in a hydrophobic environment. This will reduce the effective dielectric constant in the region of the interactions and thereby increase the attractive forces. It is noteworthy that the same effect is achieved in vancomycin, but that in the vancomycin case the effect has a different molecular basis (see earlier).

The Ala_2 methyl group is very close to protons j, w, and f of the antibiotic (Table III). Although the $Ala_2\beta$–f interaction had been previously noted,[5] the other two interactions had not been

(22) Williamson, M. P.; Williamson, D. H. *J. Chem. Soc., Perkin Trans. 1* **1981**, 1483.

uncovered. This reflects the advantage of using difference spectroscopy in the present work. The relevant part of the binding site is reproduced in Figure 11, which may be related to Figure 10 by the occurrence of protons j and $Ala_1\alpha$ in both figures. The close approach of $Ala_2\alpha$ and j confirms that aromatic ring IV is folded in the complex to give a compact structure. The studies of Nieto and Perkins[9] had indicated that the binding site for this D-alanine residue is very restricted. The proximities of the methyl group to j, w, and f show the reason for this. Additionally, we observed an NOE indicating a distance of 2.1 Å from $Ala_2\beta$ to an unidentified proton, resonating as a broad singlet at 2.94 ppm, presumably a mannose hydroxyl proton.

The Binding Site for L-Lys in Vancomycin and Ristocetin A. The conclusions with regard to the binding site of the L-lysine residue of the tripeptide are less clear cut than for the binding of the D-Ala residues and are conventiently dealt with together for both antibiotics. The binding of the lysine residue is too weak to allow any distance measurements, but the collective data give a good indication of its position. The only protons that change chemical shift significantly on changing the peptide from Ac-D-Ala-D-Ala to Ac_2-L-Lys-D-Ala-D-Ala are s_6, b, and a_1 (see Figure 9). Relative to its position in the Ac-D-Ala-D-Ala/ristocetin A complex, the NH proton a_1 is deshielded by 0.73 ppm, suggesting stronger hydrogen bonding to the Lys carbonyl oxygen than to the acetyl carbonyl oxygen of Ac-D-Ala-D-Ala. The shifts of b and s_6 suggest that the lysine side chain is lying in the direction of ring I rather than in the direction of ring VII (see Figure 9). This conclusion is reinforced by a number of NOEs (seen after a 0.3-s preirradiation); in ristoscetin A, Lys ϵ-$CH_3CO \rightarrow$ bb, and in vancomycin, Lys ϵ-$CH_2 \rightarrow$ z.

The extension of the hydrophobic portion of the lysine side chain over ring I is reasonable in light of the hydrophobic nature of this area. The fact that the side chain is free to adopt a large number of conformations makes the binding more favorable in terms of entropy. Additionally, it implies that the antibiotics can bind with similar strengths to the mucopeptides of a number of bacterial species, irrespective of the variable nature of the antepenultimate residue.

Conclusion

The binding of both vancomycin and ristocetin A to Ac-D-Ala-D-Ala is remarkably efficient. In the case of vancomycin, the most striking result of the present work is to establish the formation of a "carboxylate anion binding pocket" upon complexation with Ac-D-Ala-D-Ala. This pocket has hydrophobic walls on two sides, formed from aromatic and aliphatic hydrocarbon groups, thus strengthening the hydrogen bonds that occur within it. An analogous pocket is established to occur in the complex between ristocetin A and Ac_2-L-Lys-D-Ala-D-Ala. However, in this case, both walls of the pocket are formed from aromatic hydrocarbon groups.

Such is the efficiency of both antibiotics in binding the cell-wall analogues that it seems highly probable that the structures have been refined for this purpose by the pressures of natural selection. The necessary pressures would have operated if the organisms producing the antibiotics (*Streptomyces orientalis and Nocardia lurida*) derived an advantage by an ability to kill Gram-positive bacteria in their immediate environment.

It is clear that, in cases where proton NMR spectra of both a drug and its receptor can be analyzed, NOEDs provide a powerful method for establishing the molecular basis of drug action, permitting in favorable cases the calculation of interproton distances in the complexes.

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Registry No. Vancomycin, 1404-90-6; ristocetin A, 11021-66-2; Ac-D-Ala-D-Ala-OH, 19993-26-1; α,ϵ-Ac_2-L-Lys-D-Ala-D-Ala-OH, 24570-39-6; vancomycin/Ac-D-Ala-D-Ala complex, 84174-46-9; vistocetin A/α,ϵ-Ac_2-L-Lys-D-Ala-D-Ala complex, 84174-47-0; Z_2-L-Lys-OH, 405-39-0; D-Ala-D-Ala-OCH_2Ph, 82748-54-7; Z_2-L-Lys-D-Ala-D-Ala-OCH_2Ph, 84192-54-1; H-L-Lys-D-Ala-D-Ala-OH, 33755-56-5; α-Ac-L-Lys-D-Ala-D-Ala-OH, 28845-97-8.

Solvent Effects on Equilibria of Addition of Nucleophiles to Acetaldehyde and the Hydrophilic Character of Diols

Roger Bone, Paul Cullis, and Richard Wolfenden*

Contribution from the Department of Biochemistry, University of North Carolina, Chapel Hill, North Carolina 27514. *Received July 12, 1982*

Abstract: Equilibria of addition of water, methanol, methanethiol, ammonia, methylamine, nitromethane, and ethylene glycol to acetaldehyde have been compared in water and in chloroform, and the partition coefficients of reactants and products between the two solvents have been estimated by direct and indirect methods. Single additions of oxygen nucleophiles were found to proceed equally favorably in either solvent, whereas single additions of sulfur, nitrogen, and carbon nucleophiles proceeded much further toward completion in water than in chloroform. Equilibria of acetal formation, involving methanol or ethylene glycol, were somewhat more favorable in chloroform than in water. Reexamination of the vapor pressures of ethylene glycol and related compounds over water indicated that their hydrophilic character was greater than had been supposed.

Enzymatic transformations of carbonyl compounds and other unsaturated molecules commonly involve attack by nucleophiles at sp_2-hybridized carbon. During the action of papain, chymotrypsin, and triosephosphate dehydrogenase, for example, tetrahedral intermediates are believed to be formed during the generation and breakdown of an acyl–enzyme intermediate. With a slightly different strategy, adenosine and cytidine deaminases apparently catalyze substrate hydrolysis in part by stabilizing tetrahedral intermediates formed by direct addition of water across a C=N bond of the substrate. These enzymes are inhibited by small molecules that can adopt structures resembling these intermediates at the active site (for a recent review, see ref 1).

When substrates and inhibitors are bound at the active sites of enzymes, these small molecules are presumably stripped of much of the solvent water with which they were in contact. In considering catalytic devices that might be employed by enzymes whose reactions are thought to proceed through tetrahedral in-

(1) Wolfenden, R. In "Transition States of Biochemical Processes"; Gandour, R. D. Schowen, R. L., Eds., Plenum New York, 1978; pp 555–578.

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