Structural and Mode of Action Studies on the Antibiotic Vancomycin. Evidence from 270-MHz Proton Magnetic Resonance

Dudley H. Williams* and John R. Kalman

Contribution from the University Chemical Laboratory, Cambridge, CB2 1EW, United Kingdom. *Received June 14, 1976*

Abstract: The antibiotics vancomycin and aglucovancomycin have been studied by proton magnetic resonance at 270 MHz. The study has established the presence of seven amide bonds in vancomycin, six of which are secondary (-CONH-) and one primary ($CONH_2$). The sugars vancosamine and glucose are attached as the α and β anomers, respectively. The site of attachment of the nitrogen atom of aspartic acid has been determined, and a carboxyl group in the molecule is shown to be part of a biphenyl-2,3′-diyldiglycinate unit. The binding of Eu^{3+} and Pr^{3+} to the carboxylate group, nuclear Overhauser effects, and the binding of the Ac-D-Ala-D-Ala to vancomycin, while not allowing detailed structural proposals, do permit some general conclusions on the shape of the vancomycin molecule and the parts of vancomycin which may be intimately involved in binding Ac-D-Ala-D-Ala.

Although the glycopeptide antibiotic vancomycin was first isolated 20 years ago,[1] and structural studies have been carried out by numerous groups since that time,[2-5] the gross structure of the antibiotic (which acts by binding to mucopeptides terminating in the sequence D-Ala-D-Ala[6]) remains unknown. However, we have recently described experiments which appear to account for the whole skeleton of aglucovancomycin[2] in terms of four units: N-terminal D-*N*-methylleucine (1), aspartic acid (2), (3), and (4).[7,8] Although we have provided tentative evidence[8] that appropriate interconnection of these units through amide bonds [such as to leave one free carboxy (5) and one primary amide group (6)] would account for the structure of aglucovancomycin, firm evidence for such proposals has been lacking. We have therefore undertaken a 270-MHz ^1H NMR study of vancomycin and aglucovancomycin and of the interaction of the former with Ac-D-Ala-D-Ala. This study has provided evidence for (i) seven amide bonds in vancomycin, (ii) the stereochemistry of attachment of the sugars vancosamine and glucose, (iii) the sequence of attachment of some amino acid residues, and (iv) portions of the molecule which are important in binding Ac-D-Ala-D-Ala.

Discussion

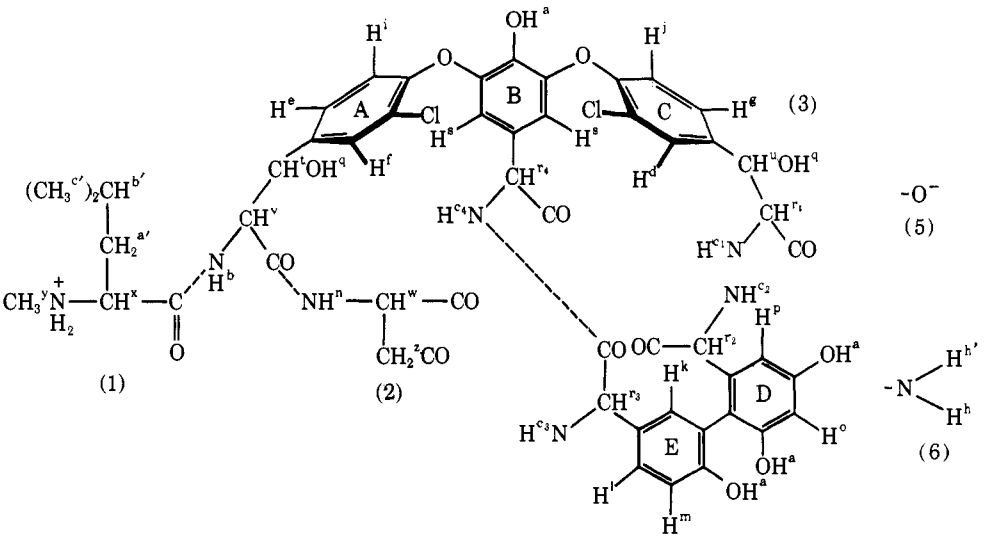
To ensure that the study would provide useful information, it was first necessary completely to assign the spectrum of aglucovancomycin in terms of the four proposed structural units mentioned above. This proved impossible at 100 MHz, since aglucovancomycin contains some 52 protons; the spectrum was therefore obtained at 270 MHz and assignments (Table I, Figure 1) were largely made on the basis of chemical shifts and homonuclear spin decoupling in the Fourier transform mode.

Some of the assignments merit immediate comment, whereas the reasons for others will be discussed later.

Assignment of Phenolic OH Protons (9.30, 9.30, 8.95, and 8.92). Upon addition of 0.3 mol equiv of sodium methoxide in dimethyl sulfoxide to the solution, the exchange rate of these protons is decreased, and in spectra then obtained at ambient temperature, the resonances appear as four sharp singlets (superimposed trace, Figure 1). The sharpening of the resonances appears to be due simply to the decreased acidity of the medium.

Assignment of -CON*H*CH- Protons. These are recognizable as protons which (i) are exchanged upon addition of DCl but are still in slow exchange at 80 °C and (ii) occur as doublets due to coupling to amino acid α-CH protons resonating in the 4–6-ppm region. There are six such resonances (8.67, 8.50, 8.50, 8.04, 6.57, and 6.48), giving the first direct evidence for the previously postulated[8] existence of six secondary amide groups in aglucovancomycin.

Assignment of $CONH_2$ Protons. The previous evidence for



(1) (2) (3) (4) (5) (6)

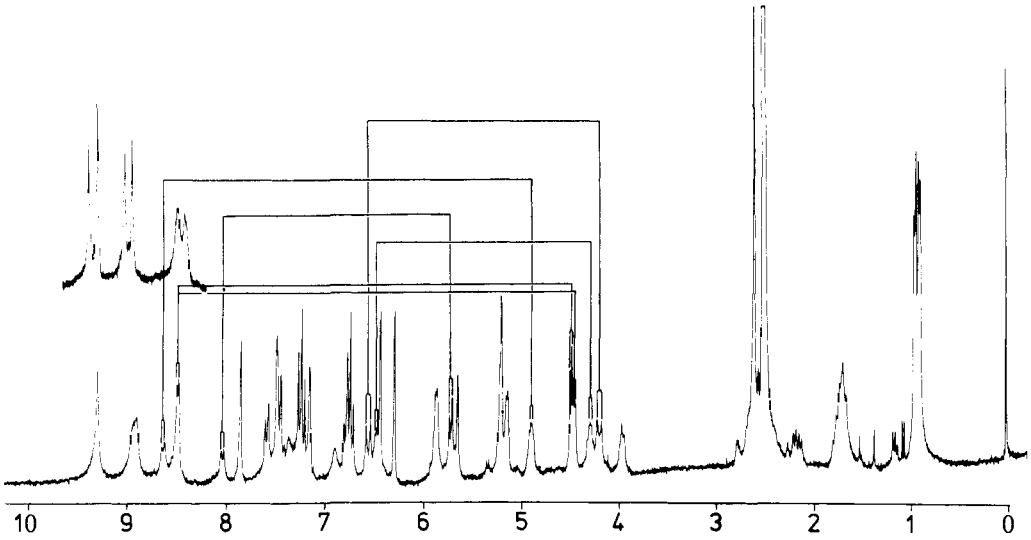


Figure 1. 270-MHz ^{1}H NMR spectrum of aglucovancomycin (~0.1 M) in Me_2SO-d_6 at 60 °C; the bridged peaks are coupled partners.

Table I. Assignment of the ^{1}H NMR Spectrum (Figure 1) of Aglucovancomycin[a]

δ, ppm	Intensity	Multiplicity[b]	J, Hz	Code
9.30	1	s		a
9.30	1	s		a
8.95	1	s (br)		a
8.92	1	s (br)		a
8.67	1	d	9	b
8.50	1	d	5	c_2
8.50	1	d	5	c_4
8.04	1	d	8	c_3
7.86	1	s		d
7.60	1	d	8	e
7.49	1	s		f
7.48	1	d	8	g
7.36	1	s (br)		h′
7.26	1	d	8	i
7.23	1	d	8	j
7.16	1	s		k
6.90	1	s (br)		h
6.81	1	dd	8, ~1	l
6.76	1	d	8	m
6.57	1	d	12	c_1
6.48	1	d	~7	n
6.45	1	d	2	o
6.30	1	d	2	p
5.88	2	~d (br)	~6	q
5.72	1	d	8	r_3
5.65	1	s		s
5.22	1	~d	5	t
5.20	1	s		s
5.14	1	d	6	u
4.92	1	dd	9, 5	v
4.49	1	d	5	r_2
4.47	1	d	5	r_4
4.31	1	m	~7, ~7, ~7	w
4.22	1	d	12	r_1
3.99	1	m		x
2.65	3	s		y
~2.50	1			z
2.18	1	dd	16, 7	z
1.75	3	br		a′, b′
0.94	3	d	6	c′
0.90	3	d	6	c′

[a] Spectrum recorded for 0.1 M aglucovancomycin in Me_2SO-d_6 at 60 °C using sodium 3-(trimethylsilyl)propanesulfonate as internal reference. [b] s = singlet, d = doublet, dd = double doublet, m = multiplet, br = broad.

such a group is that a neutral group in vancomycin is hydrolyzed under relatively mild conditions (pH 4.2, 80–90 °C, 40 h) to give 1 mol of ammonia and an anionic group.[3,7] If such a primary amide group exists in aglucovancomycin then, at an appropriate temperature, rotation about the C–N bond should occur at such a rate as to allow irradiation of one NH to lead to saturation of the other. In fact, upon irradiation of the one-proton resonance at 7.36, the one-proton resonance at 6.90 is removed due to saturation. Such a saturation phenomenon is not observable upon irradiation of any of the other exchangeable protons.

Assignment of Secondary Alcohol OH Protons. The two-proton resonance centered at 5.88 is removed by exchange broadening either on decreasing the pH of the solution or raising the temperature to 90 °C; one of these protons is coupled (J = 6 Hz) to the one-proton resonance at 5.14 (–CHOH group).

The above assignments place all the protons directly bound to O or N in the structural units (1)–(6) except the two associated with the nitrogen atom of the *N*-methylleucine (1). In aglucovancomycin isolated in the published manner,[2] this nitrogen is clearly protonated from the chemical shift of the attached methyl group (2.60) and from experiments to be described subsequently. The two nitrogen-bound protons are not discernible in the 60 °C spectrum, due to intermediate exchange rates (resulting in extremely broad resonances), but evidence for their sharpening is obtained by integration of the 8.3–9.8 region in spectra obtained at ca. 20 °C. In such experiments, the integrated intensity in this region increases from 7 protons (at 60 °C) to 8.3 protons (at 20 °C); further useful cooling of the solution was prevented by the relatively high freezing point of Me_2SO-d_6.

All resonances ascribed to nitrogen or oxygen bound protons are removed from the spectrum upon addition of DCl, as are the appropriate splittings of vicinal protons to which they are coupled.

Differentiation of the Chlorine-Substituted Aromatic Rings. On the basis of mass spectrometric evidence,[8] we have previously concluded that the terminal *N*-methylleucine is attached through an amide bond to one of the β-hydroxytyrosine units [see broken line connecting (1) and (3)]. Thus the protonated *N*-methylleucine group must be near one of the chlorine-substituted aromatic rings. This conclusion is now reinforced by experiments in which the basic nitrogen atom is deprotonated by the addition of ca. 1.5 mol equiv of sodium methoxide in Me_2SO-d_6 to the solution used for the spectral

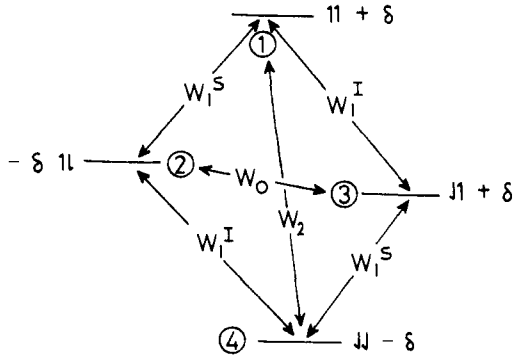


Figure 2. Energy levels and transition probabilities for a two-spin system of nuclei I and S.

Table II. Upfield Shifts (ppm) Observed on Deprotonation of *N*-Methylleucine

Proton	Upfield shift	Proton	Upfield shift
x	>0.5[a]	f	0.14
b	0.42	v	0.10
a′	0.25	e	0.08
y	0.25	t	0.06

[a] Obscured by H_2O resonance in this sample in the shifted spectrum.

determinations; relatively large upfield shifts are observed for the protons listed in Table II. These experiments establish that the positive pole is on average near protons v, t, f, and e and probably on average nearer f than e.

The above data are consistent with the decoupling data which independently establish vicinal coupling between v and t (5 Hz).

The second chlorine-substituted aromatic ring is associated with resonances d, g, and j. These assignments are not only from the typical coupling pattern of a trisubstituted benzene ring but also from the proximity of protons d and u. Proton u (5.14, -C*H*OH) is assigned from its vicinal coupling (6 Hz) to a hydroxyl proton but is not significantly coupled to r, presumably because the dihedral angle is near 90 °C. However, upon irradiation of proton d (various spectra obtained in the range 30-50 °C), the intensity of proton u is decreased by a negative nuclear Overhauser effect; a similar effect is observed upon irradiating u and observing d. Therefore, d and u are probably nearly eclipsed.

Assignment of the Biphenyl Aromatic Protons. The chemical evidence[7,8] shows that there are three trisubstituted benzene rings in aglucovancomycin. Since two such systems have already been assigned, the third system of 1,2,4 protons of a benzene ring evident in Figure 1 must be associated with the biphenyl system [protons k, l, and m in (4)]. Two protons (o, p) undergoing only meta coupling are associated with the tetrasubstituted ring of the biphenyl unit, the specific assignments being justified from experiments to be described subsequently.

Assignment of the "α-CH Region" (4-6 ppm). This region is anticipated to contain the nine methine protons, attached to carbons also bonded to N or O, which are postulated from chemical evidence. It also contains the two aromatic protons (s, s) of the pyrogallol ring. These are identified as the only protons in the 4-6-ppm region which have a single very small coupling (~1.5 Hz). The mutual coupling of broadened singlets at 5.65 and 5.20 is uncovered by decoupling experiments. These unusually high chemical shifts for aromatic protons are probably due to two reasons: (i) the ring carrying them bears three electron-donating oxygen substituents and (ii) the conformation of the three ring system (3) may be similar to that found in thyroxine and related compounds,[9] in which the protons analogous to s are shielded by the ring currents[10] of adjacent rings. Estimation of the chemical shift value of the s protons leads to values in the 5-6-ppm region.

The *N*-methylleucine α-CH (3.99) is assigned from its upfield shift on deprotonation of the *N*-methyl group (see above). The $-CH^t-CH^v-$ system is assigned from the vicinal coupling and proton u from its coupling to a hydroxyl proton and its nuclear Overhauser effect (see above). The aspartic acid α-CH (4.31, w) is assigned by decoupling from one of the β-CH_2 group protons (2.18, z). This leaves four doublets (5.72, 4.49, 4.47, and 4.22) which are due to three phenylglycine α-CH protons and the α-CH proton of one of the β-hydroxytyrosine units. As noted previously, this proton presumably subtends a ~90 °C dihedral angle with respect to proton u. These four protons are not unambiguously assigned at this stage in the analysis and are designated r in (3) and (4).

Assignment of the High-Field Region (0-3 ppm). The assignments given in Table I are supported by double resonance experiments where applicable.

Nuclear Overhauser Effects. These were observed in numerous spectra recorded between room temperature and 50 °C using the same decoupling power as in the spin-decoupling experiments. All the observed nuclear Overhauser effects (NOE's) were negative, and the intensity reductions varied between 15 and 50%. An understanding of the NOE is facilitated by considering the work of Solomon.[11] The origin of the negative NOE which is relevant to this case has been discussed previously,[12] and applications have been reported.[12,13] Briefly, if we consider as a qualitative model a simple two-spin system (Figure 2), when nucleus S is saturated by the decoupling frequency, then relative to the equilibrium populations, the energy levels will initially be augmented or depleted in population by an amount δ as shown in Figure 2. The intensity of the observed nucleus I will be dependent on the difference in populations between the sum in levels 1 and 2 and the sum in levels 3 and 4. This population difference is not affected by transitions associated with the transition probabilities W_1^I, since the population changes caused by the irradiation are to deplete *both* levels 2 and 4 by δ and to augment *both* levels 1 and 3 by δ. However, the above population difference will be affected by transitions associated with the transition probabilities W_0 and W_2. The relaxation process 1 → 4 is associated with a relatively large energy difference and is accordingly promoted by relatively high frequency components of molecular motion. Hence this process is relatively important in small molecules (fast tumbling), and the effect of the decoupling frequency is in this case to increase the number of 1 → 4 transitions; thus the intensity of the observed nucleus I is increased (positive NOE). In contrast, the relaxation process 2 → 3 is associated with a relatively small energy difference and is accordingly promoted by relatively low-frequency components of molecular motion; it is more important for large molecules (vancomycin will tumble relatively slowly). In this case, the effect of the decoupling frequency is to increase the number of 3 → 2 transitions, i.e., the intensity of the observed nucleus I is decreased (negative NOE). As expected, the NOE's disappear or are greatly reduced in spectra recorded in the temperature range 60-90 °C. The observed NOE's are summarized in Table III.

The following conclusions are drawn from the NOE's.

(i) Since irradiation of the aspartic acid N*H*(n) causes a reduction in intensity of resonance v, it is concluded that the N terminus of the aspartic acid is attached to the C terminus of the β-hydroxytyrosine residue which also carries the *N*-methylleucine [see dotted line joining (1) and (2)]. H_n and H_v must be held in an approximately eclipsed orientation (7), in accord with NOE's which have previously permitted the in-

Table III. Negative NOE's Observed in Spectra of Aglucovancomycin in Me_2SO-d_6

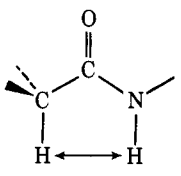
Irradiated	Intensity reduced	Irradiated	Intensity reduced
a (8.95/8.92)	p	d	u[a]
c_2	k[a], r_2[a], r_1[a]	k	r_1[a], r_2
c_4	r_3[a], s	n	v[a]

[a] In these cases, the NOE is also observed when the irradiated and observed protons are reversed.

ference of sequence information in other peptide antibiotics.[13]

(ii) Protons d and u must be in, or near, an eclipsed orientation (a trans orientation of these protons is excluded).

(iii) All of the remaining NOE's of potential structural utility involve either one of the four r protons or one of the four c protons. Three of the r protons are α-CH resonances of phenylglycines, which should have δ values of ca. 5.3 ppm in the absence of unusual shielding or deshielding effects (5.34 in a synthetic sample of *N*-acetylphenylglycine); the fourth is an α-CH of a β-hydroxytyrosine (estimated chemical shift 4.6 ppm). The observed values (Table I) are 5.72, 4.49, 4.47, and 4.22. It therefore appears highly probable that one phenylglycine α-CH is somewhat deshielded (5.72), while two are strongly shielded (by 0.8–1.1 ppm). It is clear that r_4 [see (3)] could be shielded by such an amount due to its spatial relationship to the Cl-substituted benzene rings; r_2 should also be shielded since the benzene rings of the biphenyl unit cannot be planar (steric interactions), and in a nonplanar conformation, the bonds attaching NH and CO groups to the same carbon as r_2 are likely to be orientated away from the trisubstituted benzene ring (8), leaving r_2 in a region of shielding.



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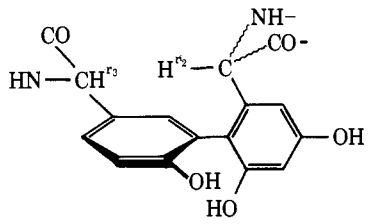


(8)

On the basis of the above reasoning, r_3 gives rise to the signal at 5.72. Irradiation of the NH which is coupled to the α-CH occurring at 4.47 at 60 °C causes (at room temperature) 10% intensity reduction in the aromatic resonance which occurs at 5.20 (s) at 60 °C; therefore, the signal at 4.47 is assigned to r_4 (3). Reasons for the assignments of the 4.49 and 4.22 resonances to r_2 and r_1, respectively, are given subsequently.

One of the strongest NOE's observed in the spectra is that observed on irradiating c_4 when, in spectra obtained at room temperature, the 5.72 resonance (r_3) falls to 50% of its original intensity. We therefore tentatively conclude that (3) and (4) are connected through an amide bond possessing the geometry (7).

Proton k has NOE's to r_2, r_1, and c_2; k is therefore close both to the α-CH of the dihydroxylated phenylglycine unit of (4) and to the α-CH of the tyrosine unit which is not carrying the *N*-methylleucine. These conclusions are supported by metal ion binding studies to be described below.

Binding of Eu^{3+} to Aglucovancomycin. It has earlier been reported that vancomycin (which has a free carboxyl group) forms a copper salt.[2] In an attempt to locate this carboxyl group, Me_2SO-d_6 solutions were monitored following stepwise additions of anhydrous $EuCl_3$ in the same solvent.

Table IV. Selective Broadening of Resonances in the ^1H NMR Spectrum of Aglucovancomycin upon Addition of $EuCl_3$

First order broadening[a]	Second order broadening[a]	Second order broadening[a]
r_2	r_1	p
	u	c_2

[a] These resonances are broadened to a similar relative extent in the spectrum of vancomycin, and a more quantitative impression can be obtained from Figure 3.

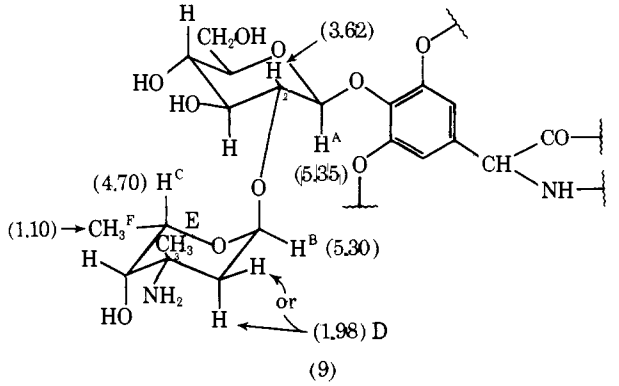
After addition of $EuCl_3$ (5 μmol) to aglucovancomycin (0.1 mM) in Me_2SO-d_6, shifts of resonances in the spectrum were small ($\geq$0.1 ppm), but marked selective broadening of resonances occurred as summarized in Table IV.

Since Eu^{3+} is normally more effective in producing shifts than broadening effects at these concentrations,[14,15] it appeared likely that the observed broadening was not a consequence of the paramagnetism of Eu^{3+} but rather due to slow exchange between aglucovancomycin and its Eu^{3+} salt. This possibility was confirmed in the case of vancomycin by a variable temperature study in the presence of Eu^{3+} or Pr^{3+}, which uncovered the occurrence of resonances near both the fast and slow exchange limits (see later). It is deduced from the data that the carboxyl group of aglucovancomycin is attached to the carbon atom which bears r_2.

Experiments with Vancomycin. The ^1H NMR spectrum of vancomycin obtained in Me_2SO-d_6 solution at 70 °C is reproduced in Figure 3. In the light of the prior analysis of the spectrum of aglucovancomycin, it is also possible to make an almost complete analysis (Table V) of this spectrum (except for the 3–4-ppm region, in which some protons of the sugars glucose[2] and vancosamine[4,5] occur).

One striking difference between Figures 1 and 3 is that those protons which suffer an upfield shift on deprotonation of the *N*-methylleucine of aglucovancomycin (Table II) also suffer an upfield shift on passing from aglucovancomycin to vancomycin. The latter shifts (followed by the shifts from Table II in parentheses) are x >0.3 (>0.5); b 0.67 (0.42), y 0.24 (0.25), f 0.07 (0.14), v 0.06 (0.10), e 0.03 (0.08), t 0.07 (0.06). From these data, it is clear that under these conditions the *N*-methylleucine residue of vancomycin is not protonated.

A comparison between Figures 1 and 3 also shows that in the spectrum of vancomycin only three extra proton resonances occur in the δ 4–6-ppm region, and these are due to the anomeric protons of glucose (A, 5.35), vancosamine (B, 5.30), and the C-5 methine proton of vancosamine (C, 4.70). The assignments are all confirmed by spin decoupling experiments in which the protons indicated by arrows in (9) were decoupled from A, B, and C.



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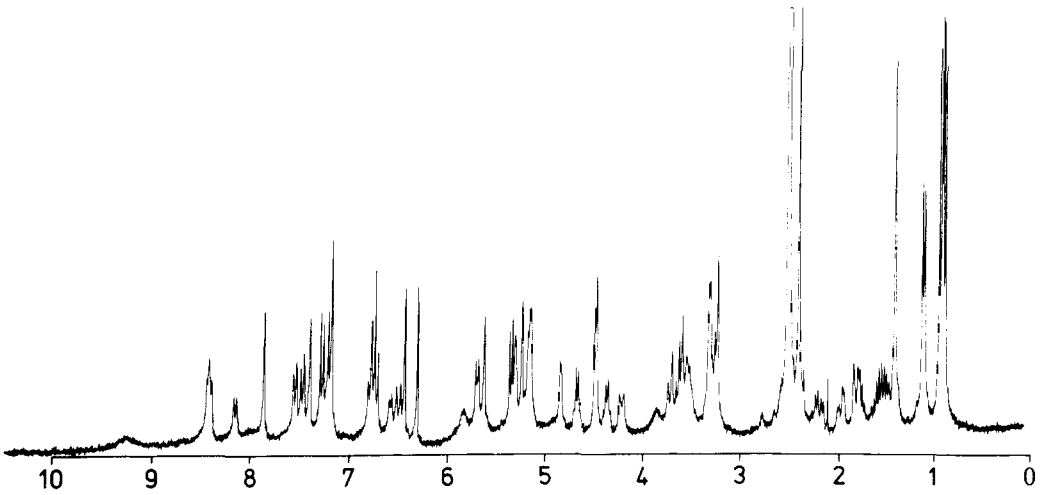

Figure 3. 270-MHz ^{1}H NMR spectra of vancomycin (~0.1 M) in Me_2SO-d_6 at 70 °C.

Table V. Partial Assignment of the ^{1}H NMR Spectrum (Figure 3) of Vancomycin[a]

ppm	Intensity	Multiplicity[b]	J, Hz	Code
9.27	*c*	(br)		a
8.43	1	d	6	c_4
8.39	1	d	7	c_2
8.14	1	d	8	c_3
8.00	1	(br)		b
7.87	1	s		d
7.57	1	d	8	e
7.48	1	d	8	g
7.42	1	s		f
7.28	1	d	8	j
7.20	1	d	8	i
7.19	1	s		k
6.78	1	dd	8, ~1	l
6.73	1	d	8	m
6.59	1	d	7	n
6.50	1	d	12	c_1
6.44	1	d	2	o
6.30	1	d	2	p
5.85	2	br		q
5.71	1	d	8	r_3
5.63	1	s		s
5.35	1	d	8	A
5.30	1	dd	2.5, 4.5	B
5.21	1	s		s
5.15	1	d	4	t
5.13	1	s		u
4.86	1	d	4	v
4.70	1	q	~7	C
4.50	1	d	7	r_2
4.50	1	d	6	r_4
4.38	1	q	~7, ~7, ~7	w
4.22	1	d	12	r_1
2.41	3	s		y
2.20	1	dd	16, 7	z
1.98	1	dd	14, 4.5	D
1.41	3	s		E
1.10	3	d	7	F
0.93	3	d	7	c′
0.90	3	d	7	c′

[a] Spectrum recorded for 0.1 M vancomycin in Me_2SO-d_6 at 70 °C. [b] Abbreviations as in Table I. [c] Signal extremely broad due to exchange.

Table VI. Negative NOE's Observed in the Spectra of Vancomycin Obtained at 30 or 40 °C in Me_2SO-d_6

Irradiated	Intensity reduced	Irradiated	Intensity reduced
r_1	k, c_2; d, u	r_2 and r_4	c_2, k, s (5.21); d, u
v	t, f	c_2	k
r_3	c_4	d	u and r_1
n	v	c_4	r_3, s (5.21)

The splitting of A by 8 Hz establishes that glucose must be present as the β anomer, while the splitting of B (4.5 and 2.5 Hz) establishes that the C-2 oxygen of glucose[7] must be axially attached to the anomeric carbon of vancosamine as shown in (9). Thus vancosamine is present as the α anomer and not as the β anomer as was previously thought probable on the basis of chemical shift arguments (J. P. Brown, personal communication; see footnote in ref 7). The relatively low-field chemical shift of C (4.70) is in accord with its 1,3-diaxial interaction with oxygen; this reasoning is supported by the fact that the chemical shift of C in the α anomer of free vancosamine is 4.36 ppm but only 4.00 in the corresponding β anomer. The arguments based on coupling constants are of course the more convincing, and the earlier tentative suggestions (based on the shifts of the C-3 methyl proton shifts, i.e., 1.41 in vancomycin and 1.48 and 1.62 ppm in the respective β and α anomers of free vancosamine) appear to be in error.

Three further sets of experiments carried out with vancomycin gave further structural insights: (i) NOE effects, (ii) experiments with Pr^{3+} and Eu^{3+}, and (iii) experiments with Ac-D-Ala-D-Ala, and these are now described.

(i) NOE Effects. As in the case of aglucovancomycin, all observed NOE's were negative, and in order to maximize the intensity decreases, the relevant spectra were obtained at 40 °C or normal probe temperature (~30 °C) in Me_2SO-d_6. However, where it is necessary to refer to specific chemical shifts, the ones quoted refer to 70 °C spectra, so that they may be clearly related to Figure 3. The data are summarized in Table VI; intensity reductions were in the range 15–50%.

Many of the NOE's are the same as those observed in aglucovancomycin (Table III). In particular, the reduction in intensity of the s resonance upon irradiation of c_4 supports the assignment of the coupled system c_4/r_4 as being attached to the trioxygenated aromatic ring (3). Other assignments are supported by the evidence for the proximity of v, t, and f and also of r_1, d, and u. The data also establish that the β-hydroxychlorotyrosine unit which is not linked to the terminal *N*-methylleucine residue is in close proximity to the biphenyl ring system (4). Furthermore, the present assignments of the proton spectrum, and previous work,[8] demand that the antibiotic be

Table VII. Selective Broadening of Resonances in the ^1H NMR Spectrum of Vancomycin upon Addition of $EuCl_3$

First order	Second order	Third order
c_2, r_2	l, m	
k, r_1	c_1, d	o, r_3, c_3
p, u	r_4	c_4, g

tricyclic; interconnection of (2), (3), and (4) in a tricyclic system that can satisfy the NOE's of Tables III and VI produces a globular structure. In this structure, the aromatic protons e, i, j, g, l, m, o, and p are not in sterically crowded environments, whereas f, d, and especially k are crowded.

(ii) Experiments with Pr^{3+} and Eu^{3+}. As with aglucovancomycin, highly selective broadening of certain resonances in the vancomycin spectrum occurs upon stepwise addition of anhydrous $PrCl_3$ and $EuCl_3$. Since the selective broadenings observed with Eu^{3+} and Pr^{3+} were qualitatively the same (except insofar as Pr^{3+} produced associated downfield shifts of a few hertz, whereas Eu^{3+} produced corresponding upfield shifts), only the selective broadening by Eu^{3+} is reported (Table VII). Some of these highly selective effects may be seen in Figure 4.

With the notable exceptions of protons p and r_2, the protons listed in Table VII, after broadening by $PrCl_3$, resharpen rapidly upon increasing the temperature of the solution from 40 to 90 °C. In contrast, p and r_2 broaden further upon increasing the temperature in the quoted range. Thus, p and r_2 are in the slow exchange limit, whereas the other broadened protons are near the fast exchange limit. To account for this phenomenon in the binding of Pr^{3+} (or Eu^{3+}) to the carboxylate group of vancomycin requires that the differences in the bound and free shifts of p and r_2 be much greater than the corresponding differences for the other protons.[16] It is concluded that since p and r_2 have their chemical shifts perturbed more drastically than the other protons upon the binding of Pr^{3+} or Eu^{3+}, that these protons are very near to the carboxylate group, which is therefore attached to the dihydroxylated ring of the biphenyl unit (4) (see also earlier discussion). These data also allow the assignments of o and p to the lower and higher field meta-coupled protons, respectively, although the reverse assignment had previously been made on the assumption[8] that the resonance (6.30) which was removed more quickly by exchange in CF_3COOD would correspond to the proton between the two hydroxyl groups. This assumption has now been shown to be false by examining the exchange deuteration of 3,5-dihydroxytoluene in $CDCl_3$ containing ~5% CF_3COOD; in this model, the 2, 4, and 6 protons all undergo exchange at comparable rates.

The selective broadenings establish the proximity of rings C, D, and E (a conclusion independently reached on the basis of NOE's) and the proximity of the carboxyl group to these rings. Conversely, the sugars, *N*-methylleucine and aspartic acid units, and ring A are remote from the carboxyl group. The results further indicate that the protons of the $CONH_2$ group are as remote, or more remote, from the carboxyl group as those protons which undergo third-order broadenings (Table VII). This result is a little surprising, since the proximity of COOH and $CONH_2$ groups could account for mild conditions[2] required for the conversion of vancomycin to CDPI (via intramolecular catalysis).[17]

The conclusion that ring D carries the carboxyl group is in accord with the observation of Nieto and Perkins[18] that electromeric titration of a carboxyl group in vancomycin is accompanied by a progressive change in the UV extinction coefficient at 294 nm, a band appropriate for the biphenyl system.

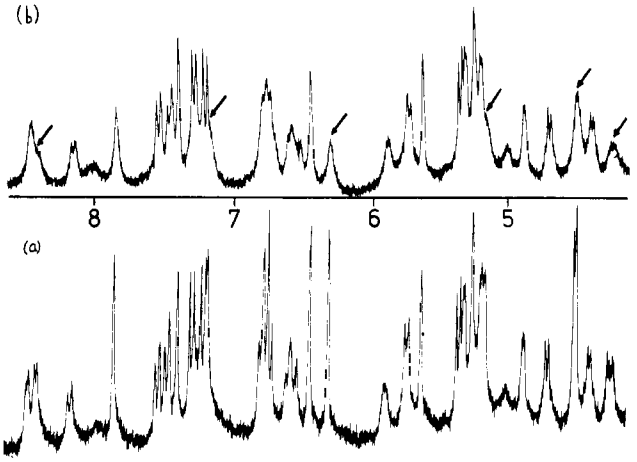

Figure 4. Partial 270-MHz ^1H NMR spectra of vancomycin (~0.1 M) in Me_2SO-d_6 at 60 °C: (a) normal spectrum; (b) after addition of anhydrous $EuCl_3$ (molar ratio of vancomycin-$EuCl_3$ ≃ 100:1). Six protons undergoing first-order broadening are indicated by arrows.

Table VIII. Selective Shifts[a] in the 270-MHz ^1H NMR Spectrum of Vancomycin upon Addition of ~1 mol equiv of Ac-D-Ala-Ala at 70 °C

Proton	Shift, Hz	Proton	Shift, Hz
c_3	−51	n	−15
d	+36	r_4	−13
f	+14	v	−27
i	+19	w	−23
k	−31	x	−18
		y	−18

[a] A negative sign indicates a downfield shift.

(iii) Experiments with Ac-D-Ala-D-Ala. Since mucopeptide precursors containing the terminal D-Ala-D-Ala fragment bind strongly to vancomycin,[6,19] it is clearly of interest to examine the binding of Ac-D-Ala-D-Ala (which forms a complex with vancomycin, with an association constant of 1.4×10^4 M^{-1})[20] to vancomycin. Such an analysis has previously been carried out by monitoring only the signals of Ac-D-Ala-D-Ala and the *N*-methylleucine residue,[20,21] since the vancomycin spectrum could not be completely assigned at that time. The present analysis was carried out in Me_2SO-d_6, since it is in this solvent that the vancomycin has been assigned.

Ac-D-Ala-D-Ala was prepared by acetylation of D-Ala-D-Ala with acetic anhydride in methanol (1:4) containing a few drops of water. Upon addition of 1 mol equiv to the solution of vancomycin in Me_2SO-d_6 at 70 °C, the formation of a vancomycin-peptide complex was evident from selective broadening and shifts of some proton resonances in the vancomycin spectrum. The largest shifts observed in spectra recorded at 70 °C are given in Table VIII.

Much of the remainder of the spectrum is not perturbed, and in particular there seems to be no strong interaction of the peptide with the sugar residues or the "outer" portion of the structural unit (4) (protons l, m, o, p). As the temperature is lowered to 40 °C, all the carbon-bound proton resonances listed in Table VIII (with the exception of f, whose resonance cannot clearly be followed) are broadened. It is likely, but not certain, that the protons whose resonances are most perturbed and selectively broadened are the ones near to the associated Ac-D-Ala-D-Ala. On this assumption, the downfield shifts of the *N*-methylleucine *N*-Me and α-CH resonances (Table VIII) probably reflect the formation of a zwitterionic salt bridge between the carboxyl group of the peptide and the *N*-meth-

ylleucine of the antibiotic. Indeed at 45 °C, the downfield shift of the *N*-Me resonance of the *N*-methylleucine caused by 1 mol equiv of Ac-D-Ala-D-Ala is 60 Hz, i.e., to a position near to that observed in aglucovancomycin, in which it is known to be protonated. This finding is in accord with the situation in aqueous media.[20] The data further suggest that proton k, the aspartic acid residue, and the terminal aromatic rings of the structural unit (3) are involved in binding the peptide unit.

One doublet methyl resonance of the Ac-D-Ala-D-Ala, in the presence of vancomycin, moves from 1.27 ppm at 120 °C to 0.85 ppm at 50 °C, while undergoing extensive broadening. Over the same temperature range, the upfield shift of the other Ac-D-Ala-D-Ala doublet methyl resonance (which is not extensively broadened) is only 0.11 ppm. The methyl resonance which suffers the large high-field shift can be assigned to the carboxyl terminal CH_3 resonance of Ac-D-Ala-D-Ala,[20] which is probably shielded by an aromatic ring near the *N*-methylleucine residue (e.g., ring A).

Conclusions

Although the data are insufficient to provide a unique structure for vancomycin, or a detailed picture of the binding of Ac-D-Ala-D-Ala to vancomycin, the present study of the problem has advanced our knowledge in the following ways.

(1) The units (1)–(4) are interconnected through six secondary amides, such that the relative positions of the units approximate those given. The structure is completed by the presence of a free carboxyl group attached to the α-carbon of ring D and a primary amide group ($-CONH_2$).

(2) The sugars, vancosamine and glucose, attached to ring B as previously deduced,[7] are present as the α and β anomers, respectively.

(3) Vancomycin is tricyclic, and Ac-D-Ala-D-Ala must bind by inserting into the cavity of this tricyclic structure such that the carboxylate group can bind to the *N*-methyl groups of the leucine (see also ref 20 and 21). In the complex in Me_2SO, the protons listed in Table VIII appear to be close to the Ac-D-Ala-D-Ala.

Experimental Section

Vancomycin was a generous gift from Eli-Lilly and Professor Howard Whitlock. Aglucovancomycin was prepared by the published procedure.[2] NMR spectra were recorded on a Brucker 270-MHz instrument operating in the Fourier transform mode.

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