Assignment of the Carbon-13 Spectrum of Vancomycin and its Derivatives

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The antibiotic vancomycin and its derivatives CDP-I and aglucovancomycin have been studied by ^{13}C n.m.r. spectroscopy. The spectra have been partially assigned by means of model compounds, pH titrations, addition of lanthanide ions, selective $\{^1H\}$ decoupling, and ^{13}C labelling experiments, allowing all but four of the 66 carbon atoms in vancomycin to be assigned. Full details of the assignment are presented.

VANCOMYCIN (I) is a glycopeptide antibiotic isolated from *Streptomyces orientalis*,[1] which has been shown to interfere with the biosynthesis of bacterial cell walls. Perkins [2] has shown that it binds strongly to mucopeptide precursor molecules containing the terminal D-Ala-D-Ala fragment and it has been postulated that formation of such complexes could interfere with the incorporation of the peptides into the cell walls, thus providing an explanation for the antibiotic action of vancomycin.

The structure of vancomycin was established by *X*-ray analysis of a derivative CDP-I (II) allowing a possible site of binding to be proposed.[3] This binding site has recently been confirmed by ^1H n.m.r., which also suggests a possible conformational change on binding.[4]

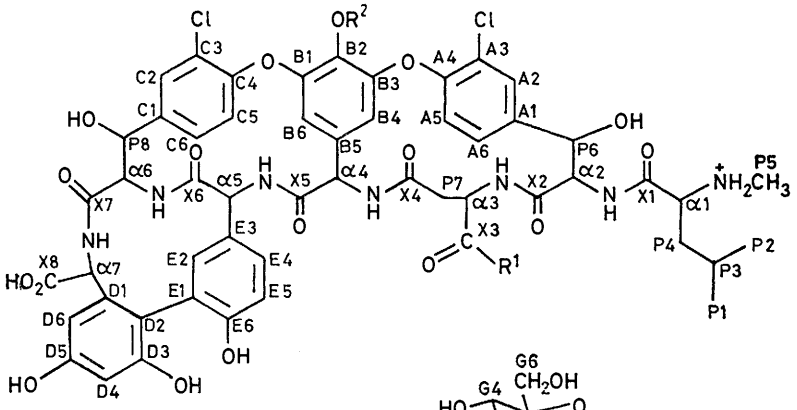
We present here a detailed assignment of the ^{13}C spectrum of vancomycin and its derivative CDP-I and aglucovancomycin (III). It is hoped that this assignment will serve three functions: first that it will allow further ^{13}C studies to throw more light on the conformational changes involved in binding to D-Ala-D-Ala; secondly, that it will enable biosynthetic studies to be carried out using ^{13}C-labelled precursors; and thirdly, that it will facilitate the structural elucidation and ^{13}C assignment of other antibiotics in the vancomycin class.

EXPERIMENTAL

Vancomycin hydrochloride was supplied by Eli Lilly and used without further purification. Aglucovancomycin was prepared by the method of Marshall [5] and CDP-I by the method of Johnson.[6] $[^2H_6]$DMSO was obtained from Nuclear Magnetic Resonance Ltd., and ^2H_2O from Norsk Hydroelectrisk.

Solutions were generally examined at 80 °C and with concentrations up to 0.15M in DMSO or 0.07M in ^2H_2O.

Titrations were performed at 60 °C in ^2H_2O at a concentration of 0.1M. The pH was measured on a Titrator TTT2 meter using a Radiometer combination glass electrode, and adjusted using a 1M solution of NaOH in ^2H_2O and a 4M solution of HCl in ^2H_2O.



The Gd^{3+} broadening experiments were made by adding Gd^{3+} solution to a 70mM solution of vancomycin in ^2H_2O at 65 °C.

Most ^{13}C spectra were obtained at 25.2 MHz on a Varian XL-100 instrument operating in the Fourier transform mode. Typical spectra had a spectral width of 5 000 Hz with an acquisition time of 0.8 s and collected in 8K data points. Some spectra were obtained at 62.5 MHz using a Bruker WP250 instrument.

RESULTS AND DISCUSSION

Proton noise-decoupled ^{13}C spectra of vancomycin (I) and its derivatives CDP-I (II) and aglucovancomycin

(III) were obtained in DMSO at 80 °C and their chemical shifts, measured relative to tetramethylsilane, are presented in the Table. The ^{13}C 62.5 MHz spectrum of vancomycin hydrochloride (0.15M) in DMSO at 80 °C is

^{13}C Chemical shifts (p.p.m. from tetramethylsilane) of vancomycin and its derivatives

Signal	Multiplicity	V–D_2O	V–DMSO	AGV–DMSO	CDP-I–DMSO	Calculated	Assignment	Method of assignment
1	s	170.2	172.2	172.6	172.7		X1	*e*
2	s	177.3	172.0	171.6	172.0		X8	*h*
3	s	175.1	171.1	170.0	173.4		X3	*b*
4	s	171.9	170.1	169.3	170.5		X2	*b*
					170.8			
5	s	171.2	169.0	168.1	168.9		(X5,X6)	
6	s	169.8	168.8	167.5	168.4		(X5,X6)	*f*
7	s	169.0	167.2	167.0	167.2		X7	*h*
8	s	168.7	166.8	166.0	166.3		X4	*g*
9	s	157.3	156.9	157.1	157.0	154.5	D5	*a,h*
10	s	155.8	156.2	156.4	156.3	156.4	D3	*a,h*
11	s	155.0	154.8	154.9	154.8	152.6	E6	*c*
12	s	153.6	151.9	148.5	152.4	143.0	B1	*a,b*
13	s	152.2	151.0	147.9	150.8	143.0	B3	*a,b,i*
14	s	151.4	149.9	150.2	148.8	158.8	A4	*c*
15	s	149.8	148.9	149.6	148.4	158.8	C4	*c*
16	s	141.2	142.1	141.9	142.2	136.3	C1	*g,c*
17	s	139.5	139.6	139.3	140.6	136.3	A1	*g,b,c*
18	s	138.5	136.1	136.1	136.2	144.1	D1	*e,h*
19	d	136.5	135.5	135.8	135.6	130.8	E2	*a,c*
20	s	135.9	134.2	134.2	137.0	131.5	B5	*a*
21	s	133.4	131.8	128.9	131.6	138.7	B2	*a*
22	d	129.6	128.3	128.5	127.4	127.6	A2	*c*
23	d	128.7	127.2	128.2	127.4	129.9	C2	*g*
24	d	128.1	127.1	127.5	127.2	127.6	(A6,C6)	*g*
25	s	128.1	127.0	127.5	127.1	126.7	E3	*a*
26	d	129.1	126.9	126.8	127.0	129.9	(A6,C6)	*g*
						127.6		
27	s	127.5	126.0	126.20	126.4	126.7	A3	*a*
28	s	127.1	126.0	126.15	126.1	126.7	C3	*a*
29	d	127.3	125.3	125.5	125.3	129.9	E4	*c*
30	d	125.3	123.9	124.5	124.2	121.3	A5	*c*
31	d	124.7	122.9	123.1	123.0	121.3	C5	*c*
32	s	122.2	121.6	121.7	121.7	126.4	E1	*h,a*
33	s	118.5	117.8	118.0	117.8	118.5	D2	*h,a*
34	d	118.9	116.2	116.4	116.3	115.4	E5	*a,c*
35	d	109.0	107.4	109.4	107.0	114.9	B4	*c*
				107.3				
36	d	109.1	106.1	106.2	106.5	102.6	D6	*e,h*
37	d	106.2	104.6	106.5	104.7	114.9	B6	*c*
				104.5				
38	d	103.9	102.5	102.8	102.5	108.7	D4	*e,h*
39	d	102.4	100.8		100.5	102.3	G1	*b,a,*
40	d	98.5	96.3		96.8	90.4	V1	*b,a*
41	d	80.1	77.6		77.7	79.0	G2	*a*
42	d	77.5	77.2		77.3	77.4	G5	*a*
43	d	76.9	76.7		76.9	75.8	G3	*a*
44	d	72.9	71.3	71.5	71.4		P6	*c*
45	d	72.4	71.3	71.5	71.4		P8	*c*
46	d	71.8	70.7		70.7	70.8	V4	*a*
47	d	70.3	70.6		73.0	70.7	G4	*e*
48	d	64.9	63.0		63.2	67.3	V5	*a,c*
49	d	64.1	61.8	62.0	61.8		α6	*c*
50	d	61.7	61.6	(59.8) †	62.3		α1	*e*
51	t	61.6	61.5		61.3	62.4	G6	*c*
52	d	59.6	58.8	(59.5) †	57.4		α2	*c*
53	d	60.4	56.8	56.8	57.1		α7	*e,h*
54	d	56.0	54.9	55.0	53.8		α4	*c*
55	s	55.6	54.1	54.5	53.0	59.5	V3	*c*
56	d	54.9	53.6	53.9	54.1		α5	*c*
57	d	52.5	51.2	51.3	48.2		α3	*b*
58	t	39.6	40.4	39.7	41.8	~40	P4	*a*
59	t	36.5	36.9	38.6	36.7	36.3	P7	*g*
60	t	34.0	33.3		33.9	36.4	V2	*b*
61	q	32.9	32.8	31.2	34.0		P5	*a*
62	d	24.9	24.1	23.9	24.2	24.4	P3	*c*
63	q	23.1	22.7	22.9	22.8	22.2	P1	*g*
64	q	22.8	22.5		22.7	18.4	V7	*b,e*
65	q	22.8	22.4	22.4	22.2	22.2	P2	*g*
66	q	17.1	16.6		16.3	17.1	V6	*b,e*

V = Vancomycin, AGV = aglucovancomycin, s = singlet, d = doublet, t = triplet, q, quartet. † These assignments can be reversed.

[a] Chemical shift calculations. [b] Comparison with derivatives. [c] {^1H} decoupling. [d] pH Titration. [e] ^{13}C Labelling. [f] Logical exclusion. [g] Paramagnetic ion addition. [h] Ac-D-Ala-D-Ala addition.

shown in Figure 1; at this frequency separate signals are resolved for each of the 66 carbon atoms in the molecule. Spectra were also obtained for vancomycin hydrochloride in ^2H_2O and in mixtures of ^2H_2O and [$^2H_6$]DMSO from which the ^2H_2O and DMSO spectra can be correlated, allowing assignments in one solvent to be transferred to the other. The ^{13}C chemical shift data measured in ^2H_2O solution are included in the Table. A series of ^{13}C spectra were recorded at different pH* values in ^2H_2O (pH* is the uncorrected pH meter reading); the variation of chemical shifts with pH* is shown in Figure 2. No data are presented between pH* 7 and 9 as vancomycin is insoluble in this pH range. Vancomycin begins to decompose rapidly above pH* 10.

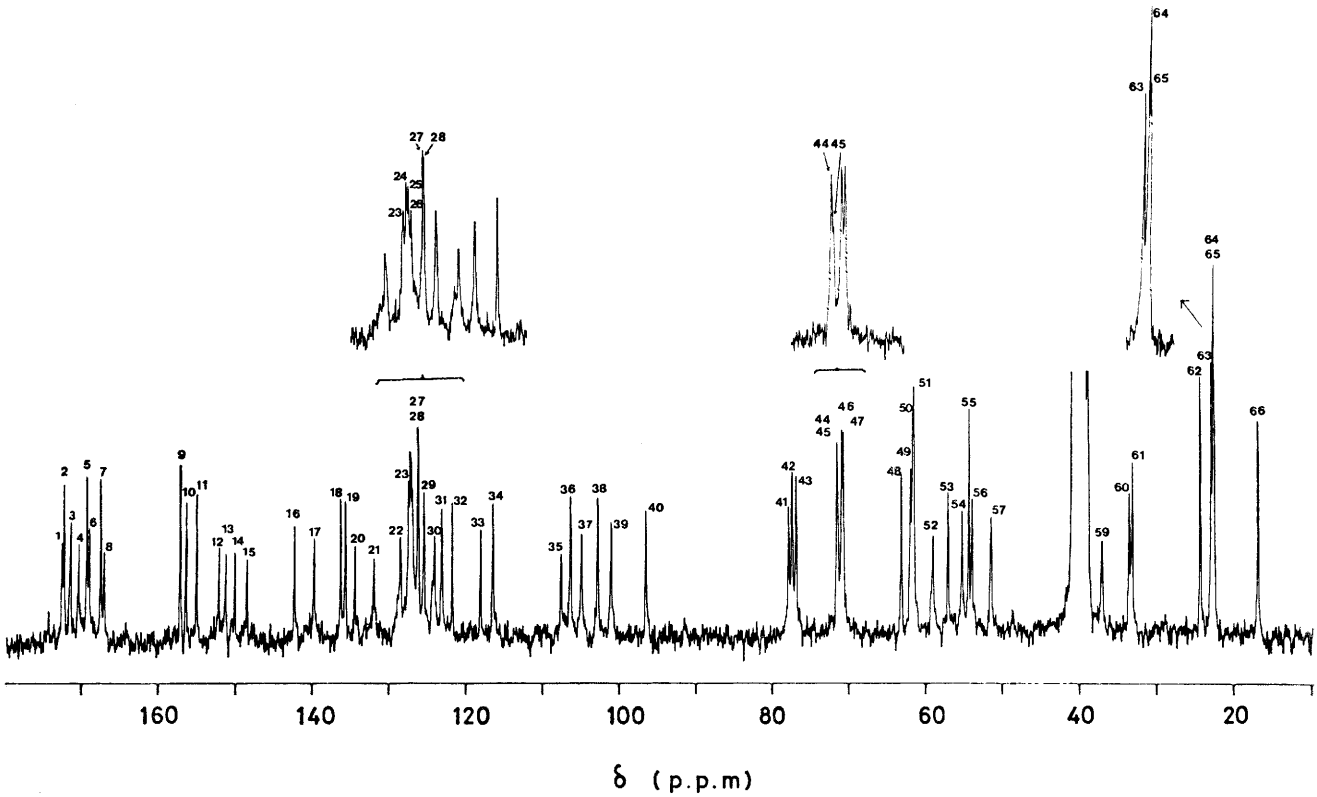


FIGURE 1 The 62.5 MHz ^{13}C spectrum of 0.15M-vancomycin hydrochloride in [$^2H_6$]DMSO at 80 °C. Chemical shifts are given in p.p.m. relative to tetramethylsilane

The first step in the assignment of the ^{13}C spectrum of vancomycin is a rough calculation of the various chemical shifts using empirical rules and model compounds.[7] The empirical rules used were taken from Wehrli and Wirthlin.[8] Suitable model compounds for the sugars were glucobioses [9] and *N*-acetyl-*O*-methylvancosamine [10] while good models for the aromatic carbons were provided by ristocetin A and its ψ-aglycone.[11] (Ristocetin A is a peptide antibiotic in which the major differences from vancomycin are that *N*-methyl-leucine and β-asparagine are replaced by aromatic amino-acids and the sugar moieties are different.[12])

The differences in chemical shifts between vancomycin, aglucovancomycin, and CDP-I were also very useful in making the assignments.

Further assignments can be made by using data from pH titrations, from addition of lanthanide reagents such as Gd^{3+} (broadening reagent) or Eu^{3+} (shifting reagent), from relaxation studies, and from ^1H selective decoupling experiments. The latter assignments were made either by single specific irradiations at the corresponding ^1H frequencies or by graphical interpolation of the results from a series of such experiments. The ^1H assignments used in these experiments were those reported by Williams and Kalman.[13] The multiplicities of the signals in the ^{13}C–{^1H} off-resonance decoupled spectra indicate the numbers of protons attached to the corresponding carbon atoms, and are included in the Table. Details of the assignments are presented below.

Carboxy and Carbonyl Carbons (*Signals* 1—8).—The spectral perturbations caused by addition of Gd^{3+} and Eu^{3+} ions allow the immediate assignment of X8 (signal 2) and X7 (signal 7) which are both close to the expected binding site of these ions (the free carboxylate anion): pH titration experiments further confirm these assignments. The pH titration also shows that X1 corresponds to signal 1, while comparison with the CDP-I spectrum indicates that X3 should be assigned to signal 3. A sample of vancomycin isolated from a medium containing [2-^{13}C]tyrosine was found to be enriched with ^{13}C at signals 49 and 52 (corresponding to α6 and α2) and 5 and 6.[14] These two latter signals presumably arise from the carbonyls of the two *p*-hydroxyphenylglycine units (X5 and X6), which are assumed to be biosynthesised

from tyrosine by a route similar to that proposed by Hosoda *et al.*[15] Thus carbons X5 and X6 correspond to signals 5 and 6. Signal 4 is a doublet in the CDP-I spectrum as are the peaks corresponding to A1 and P6; these splittings are thought to result from epimerisation at the P6 or α6 positions during the preparation of CDP-I (prepared from vancomycin at 70 °C and pH 4.2 for 5 days [6]). This suggests that signal 4 corresponds to X2 which is the unassigned carbonyl closest to these nuclei. The other unassigned carbonyl X4 can therefore be assigned to signal 8.

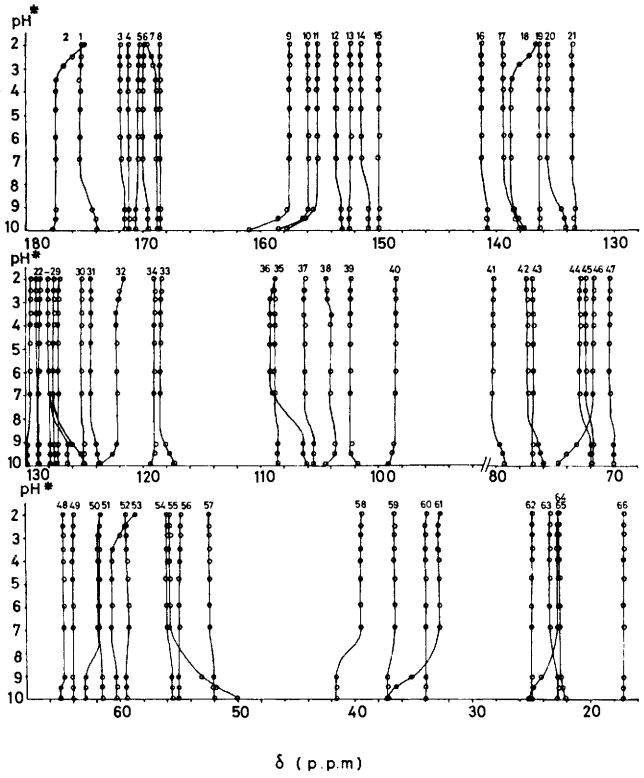


FIGURE 2 The variation with pH* of ^{13}C resonances in the vancomycin spectrum (0.1M-vancomycin in ^2H_2O, 65 °C)

Low-field Aromatic Carbons (*Signals* 9—15).—Empirical chemical-shift calculations indicate that the group of signals immediately to high field of the carbonyl resonances correspond to seven quaternary aromatic carbons bonded to oxygen (all the oxygen-bearing aromatics except for B2). In the single resonance ^{13}C spectrum where all the ^{13}C–^1H spin–spin splittings are retained only signals 11, 14, and 15 show three-bond coupling splittings and these are therefore assigned to A4, C4, and E6. It is easily shown that A4 and C4 give 14 and 15 by using selective ^1H decoupling experiments and by observing the significant shifts of these signals between vancomycin and aglucovancomycin. Selective specific ^1H decoupling experiments also suggest that A4 is 14 and C4 is 15 although this evidence is not conclusive.

Chemical-shift calculations lead us to predict that signals 12 and 13 come from B1 and B3 and this is confirmed by their large shifts on removal of the sugars. These carbons are relatively remote from their nearest proton neighbours and consequently they relax slowly. Under the rapid pulsing conditions used in the experiments these carbons give less intense signals than other nuclei with shorter relaxation times. [The low intensity of signal 21 (from B2) can be similarly explained.] The assignment of B3 to signal 13 is made by the changes in chemical shift observed on addition of Ac-D-Ala-D-Ala to vancomycin (in 60% ^2H_2O–40% [$^2H_6$]DMSO, pH* 3.6, 80 °C). Sequential addition of the peptide has a significant effect on the chemical shifts of signals 13, 14, and 35, the latter two being already assigned to A4 and B4. The observed changes in shift are consistent with a rotation of ring A about the A1—A4 axis induced by the peptide binding. This rotation would be expected to affect the ^{13}C chemical shifts of carbons in rings A and B, particularly B3 and B4 which are close to the chlorine atom of ring A. The shift changes of signals 13 and 35 are two of the largest observed in the whole spectrum on peptide binding and thus allow a reasonably confident assignment of signals 13 to B3.

The remaining carbon nuclei D5 and D3 are assigned to signals 9 and 10 on the basis of their chemical shifts and Gd^{3+} line broadening experiments in which signal 9 is broadened much more than signal 10.

Remaining Aromatics and Anomeric Carbons (*Signals* 16—40).—Off resonance ^1H decoupling experiments allow us to differentiate between signals from quaternary carbons (singlets) and those of proton-bearing carbons (multiplets) and it is simpler to consider these two groups separately.

Quaternary Aromatic Carbons.—Chemical-shift calculations, while of relatively little value for detailed assignments are very useful for indicating which nuclei are resonating in a particular region of the spectrum. Such calculations indicate that the five signals at low field (16—18, 20, and 21) correspond to the aromatic quaternary carbons A1, C1, B5, D1, and B2. Carbon B2 is readily assigned to 21 by its large shift (*ca* 3.1 p.p.m.) on removal of the sugar moiety. Carbon D1 is also relatively simply assigned to 18 by its pH dependence and its broadening on addition of Gd^{3+}. Comparison with model compounds suggests that B5 should resonate at *ca.* δ 131.5 p.p.m and is thus assigned to 20 (δ 134.2 p.p.m.), leaving C1 and A1 as 16 and 17 (in good agreement with chemical shift calculations). The agreement between calculated and observed shift for B5 is not very good, but this is only to be expected from the crowded nature of the site. C1 is probably 16, as it shows no change in chemical shift between vancomycin and CDP-I. Moreover, low-power specific ^1H decoupling at the frequency of the proton attached to α6 leaves 16 as a broad singlet, but 17 with a measurable small splitting.

There are five remaining signals from quaternary aromatics, two of which (32 and 33) broaden on addition of Gd^{3+} and must therefore be E1 and D2. These cannot be convincingly differentiated on the basis of chemical shift comparisons, and the assignment of D2 to 33 is

based on the ^{13}C spectrum of ristocetin A and its ψ-aglycone.[11] Ristocetin A has a sugar (mannose) attached to the phenolic oxygen at the D3 carbon which is not present in the ψ-aglycone.[12] Only one ^{13}C quaternary signal shifts appreciably on removal of the sugar; it moves upfield by 3.1 p.p.m. to δ 117.4 p.p.m. and is therefore assigned to D2 rather than to E1. There is a small change in chemical shift of 32 on protonation of the carboxy-group and this resonance is also broader than 33 in the presence of Gd^{3+}: examination of a molecular model of vancomycin indicates that the carboxylate ion is closer to E1 than to D2 which is in agreement with the assignment.

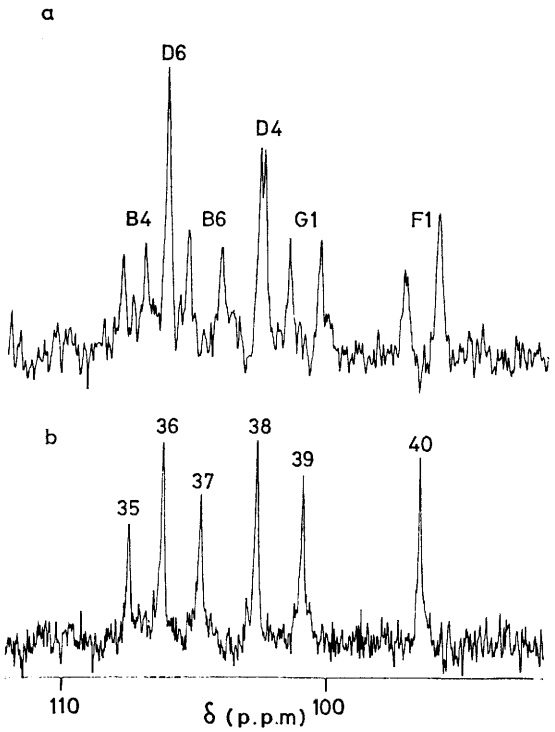


FIGURE 3 (*a*) Part of the 62.5 MHz ^{13}C spectrum of vancomycin, with specific ^1H decoupling at the frequency of the proton attached to D6. (*b*) The same region of the spectrum, with proton noise decoupling

The three aromatic quaternary carbons remaining can only be distinguished by their chemical shifts. Two give almost coincident signals at δ 126.0 p.p.m., and are assigned to C3 and A3, while the third, at δ 127.0 p.p.m., is assigned to E3.

Protonated Aromatic Carbons.—There are nine protonated carbons with signals in the region δ 116—136 p.p.m., three of which (23, 24, and 26) have very similar chemical shifts and are fully resolved only at 62.5 MHz. Except for two of these three, all signals from the protonated aromatic carbons could be assigned by a combination of selective ^1H decouplings and graphical interpolation of ^1H decoupled ^{13}C spectra recorded at 62.5 MHz.

Anomeric and High-field Aromatic Carbons (*Signals* 35—40).—Chemical-shift calculations indicate that the six signals between δ 96 and 108 p.p.m. belong to the four aromatic carbons D4, D6, B4, and B6 and two anomeric carbons G1 and V1. The anomerics are clearly identified as 39 and 40 by comprison with aglucovancomycin and chemical-shift calculations show that 39 is from G1 and 40 is from V1. B4 and B6 are readily assigned to 35 and 37 respectively by selective ^1H decoupling, as they are coupled to remarkably high-field protons. D6 may be assigned as 36 by its pH dependence and the fact that it is broadened more than D4 (38) by addition of Gd^{3+}. This is further confirmed by selective ^1H decoupling and model compounds. Figure 3a shows the selective irradiation experiments in which the irradiation at the frequency of the proton attached to D6 causes collapse of the D6 doublet.

Backbone and Sugar Carbons (*Signals* 41—57).—All signals in this region (δ 50—80 p.p.m.) are doublets under off-resonance decoupling conditions, except for one singlet V3 and one triplet G6 (see Table). Comparison with aglucovancomycin allows the signals from the other sugar carbons to be identified as removal of the sugars makes very little difference to the signals of other carbon nuclei. The only signals which could be confused with sugar carbon signals are 44 and 45 and these can be shown to be P6 and P8 by selective ^1H irradiation. Of the sugar carbons, V5 is expected to be at high field (see Table), and is therefore taken as peak 48. This can be confirmed by specific ^1H decoupling. V4 may be expected to show a pH dependence because of its proximity to a primary amino-group, and is assigned as signal 46, the only peak in this region which shifts significantly on titration (see Figure 2). The other sugar assignments must be made on the basis of chemical shift calculations (see Table).

The other seven signals in this region are the α-carbons from the seven amino-acid residues. Three are easily assigned; line 50 (α1) has a marked pH dependence (see Figure 2) with a pK_a of *ca.* 8, line 57 (α3) shifts 3.0 p.p.m. between vancomycin and CDP-I, and line 53 (α7) is pH dependent with a pK_a of *ca.* 2.6, is broadened by addition of Gd^{3+}, and shifted by Eu^{3+}. The other signals can then be assigned by off-resonance decoupling, as all appropriate ^1H shifts are far enough apart to allow convincing decoupling experiments to be made.

High-field Carbons (*Signals* 58—66).—Nine nuclei fall into this category, comprising five methyl, three methylene, and one methine carbon (P3). The latter is unambiguously assigned to signal 62 by its doublet multiplicity in the off-resonance decoupled spectra, and the *N*-methyl group (P5) and the β-carbon of *N*-methylleucine (P4) to signals 61 and 58, respectively, by their chemical shifts. Both also show the expected pH dependence. Of the other two methylene carbons, V2 and P7, one, signal 60, is not present in aglucovancomycin allowing it to be assigned as V2 and 59 to P7 as expected from model compounds. The two vancosamine methyl carbons, V6 and V7, may be identified by comparison with aglucovancomycin; V7 can be shown to be the low-field of the two by its pH dependence. This leaves signals 63 and 65 as the two leucine side-chain methyls

P1 and P2. These assignments have all been confirmed by selective ^1H decoupling experiments.

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