Complete Assignment of the ^{13}C NMR Spectrum of Vancomycin

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The antibiotic vancomycin has been studied by NMR spectroscopy using modern, two-dimensional, ^{1}H–^{13}C correlation techniques. Working with the fully assigned ^{1}H spectrum it has been possible, by a series of experiments designed to detect direct (one-bond) and indirect (multiple-bond) ^{1}H–^{13}C couplings, to assign all 66 carbons of the antibiotic. The assignment was carried out in a DMSO solution and the results then translated to an aqueous solution by titration.

Vancomycin (**1**) is a clinically important antibiotic and is probably the most studied member of a large family of glycopeptide antibiotics.[1] Members of this closely related group of compounds inhibit bacterial cell-wall biosynthesis by binding to the *C*-terminal peptide sequence L-lysyl-D-alanyl-D-alanine in mucopeptide precursor molecules. Much work has been carried out in recent years to study the receptor/antibiotic binding interactions using cell-wall receptor analogues such as *N*,*N*-diacetyl-L-lysyl-D-alanyl-D-alanine and *N*-acetyl-D-alanyl-D-alanine. As a result of this research, we now have a relatively detailed picture of the binding processes involved in these particular bimolecular associations.[2]

The structure of vancomycin was established over ten years ago through a combination of ^{1}H NMR experiments, in which NOE evidence played a significant part,[3,4] as did also the X-ray analysis of a degradation product.[5] From this early work, a binding site was proposed; and further ^{1}H NMR studies then confirmed many details of this site and also suggested a possible conformational change on binding.[6,7] The complementarity between vancomycin and the cell-wall peptide has allowed the system to be used as a model system to study molecular recognition processes; in particular, work has been carried out aimed at estimating binding constants for peptide–peptide interactions in aqueous solutions.[2] These investigations have required the use of a variety of ligands. Binding constants are most often obtained from UV and/or CD measurements, with ^{1}H NMR, and in particular NOEs, being used to report on the specific interactions involved. In some cases, it has been necessary to work with very small ligands such as acetate, glycine and D-alanine which have few protons with which to probe the interactions made with the antibiotic. The way forward was seen to lie with ^{13}C NMR. Changes in the chemical shifts of the carbons of both vancomycin and ligand would reflect the extent of any interactions and therefore could be used to follow the binding process. This has proved a successful approach,[8] but it first required the complete and unambiguous assignment of all 66 carbon atoms of vancomycin.

The only published ^{13}C assignments for members of the vancomycin family were the result of work carried out in support of the structure elucidation of these molecules. In two papers,[9,10] ^{13}C assignments relating to vancomycin, ristocetins A and B, and a few derivatives were given. However, because the work predated the use of heteronuclear (^{1}H–^{13}C) correlation spectroscopy, it was necessary to use a variety of methods to include all the carbon signals. All of the following methods contributed to these assignments: chemical shift calculations; off-resonance ^{1}H decoupling; relaxation studies; logical exclusion; pH titration; ^{13}C labelling; addition of paramagnetic ions; and addition of *N*-acetyl-D-alanyl-D-alanine. By a combination of all these methods, assignments were achieved in both $(CD_3)_2SO$ (hereafter written as DMSO) and D_2O solutions. However, a number of the assignments could not be rigorously proved and we therefore report here the complete ^{13}C assignment of vancomycin.

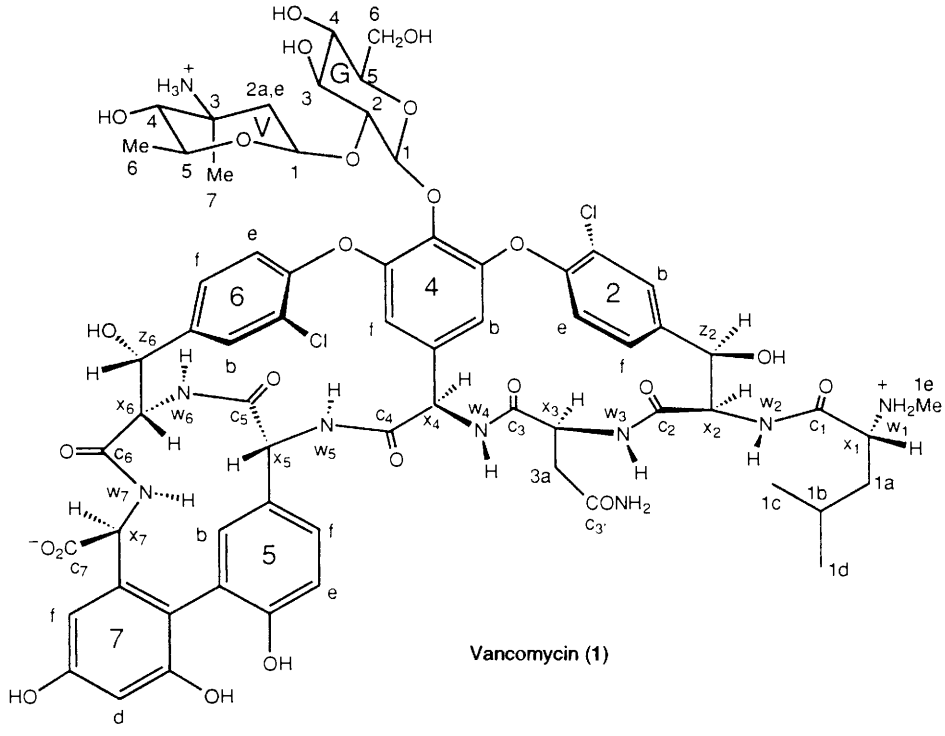
Results and Discussion

Preliminary survey spectra were acquired on 50 mmol dm^{-3} solutions of vancomycin in DMSO and D_2O. In both solvents, exchange broadening occurred; this was more pronounced in D_2O (affecting some peaks more than others), and therefore DMSO was the solvent of choice for the start of the investigation.

As the ^{13}C assignment was to be based on ^{1}H–^{13}C correlation experiments, it was imperative that a fully assigned ^{1}H spectrum, acquired under conditions comparable to those which were to be used for the correlation spectra, be used as the starting point. Hence, the same 50 mmol dm^{-3} solution of vancomycin in DMSO was used throughout. ^{1}H assignments have appeared in a previous report,[11] but in the present work a proton assignment was carried out *de novo*. The proton spectrum relevant to this assignment is shown in Fig. 1(*a*). Sufficient information to complete the task was gathered from just three homonuclear two-dimensional experiments; a phase-sensitive DQF-COSY, a phase-sensitive NOESY, and a ROESY experiment. The two sets of NOE data complemented each other and differentiated saturation transfer between labile signals from true NOEs. Table 1 summarises the scalar and dipolar connections shown in these spectra and provides a ^{1}H assignment based on the numbering system shown for **1**. We shall not discuss the reasoning behind these assignments, as the principles behind such an operation are nowadays regarded as standard practice.

There are four proton signals, all originating from labile protons, that are not observed in these spectra. Presumably, the reason for this is that they are in fast exchange with each other and with the residual water signal (no attempt was made to improve this situation by using drier DMSO as their observation was not crucial to the ^{13}C assignment). By logical exclusion these missing signals correspond to the following protons: the carboxylic acid proton of residue 7; one of the phenolic hydroxy protons from rings 5 or 7; and the amine protons of residue 1 and the vancosamine moiety.

The goal of ^{13}C assignment of the molecule [see Fig. 1(*b*)] involved an extensive programme of ^{1}H–^{13}C correlation experiments carried out in both conventional (^{13}C-detected) and reverse (^{1}H-detected) modes. The conventional mode gave better resolution of the 66 carbon signals and the reverse mode offered the advantage of better sensitivity. Working in the conventional mode at a field strength of 7.0 T (300 MHz ^{1}H; 75



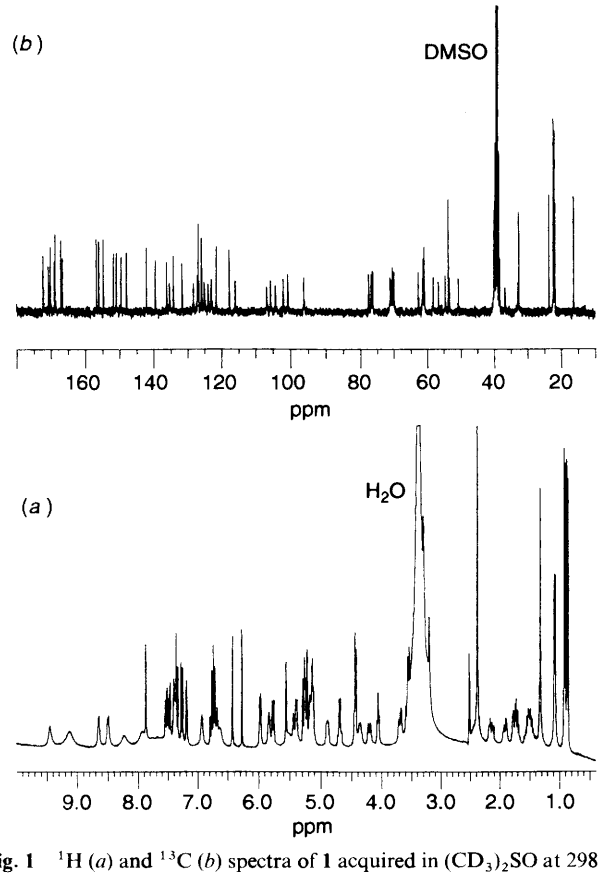


Fig. 1 ^{1}H (*a*) and ^{13}C (*b*) spectra of **1** acquired in $(CD_3)_2SO$ at 298 K

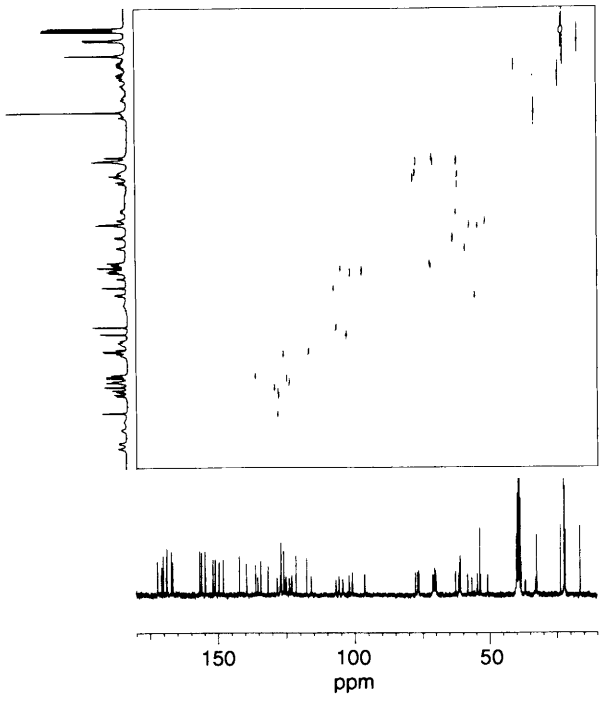


Fig. 2 Conventional-mode ^{13}C-^{1}H correlation spectrum of **1** acquired in $(CD_3)_2SO$ at 75 MHz ^{13}C frequency (303 K). Delays in the pulse sequence have been set so as to optimise direct (one-bond) couplings (150 Hz). All the carbon-bearing protons of **1** (40 in total) can be identified from this one spectrum.

MHz ^{13}C) and setting pulse sequence delays so as to optimise 150, 15, 10, 7 and 4 Hz couplings, it was possible to assign the majority of the ^{13}C signals, either by their direct (one-bond) or their indirect (multiple-bond) couplings, to the previously assigned protons. Fig. 2 shows the spectrum obtained from the experiment where the delays have been optimised for 150 Hz (one-bond); all 40 proton-bearing carbons can be assigned from this one experiment. However, even after such an extensive programme of experiments some quaternary carbons remained unassigned, including four of the eight carbonyls. For their assignment, it was necessary to go to a higher field strength of 11.7 T (500 MHz ^{1}H; 125 MHz ^{13}C). All the remaining carbon signals were then assigned from just one further experiment in which 10 Hz couplings were optimised. The full plot of this spectrum is shown in Fig. 3, and an expanded view of the carbonyl correlations appears in Fig. 4.

Table 1 ^{1}H NMR data for **1** in $(CD_3)_2SO$ at 298 K

Proton	δ_H (multiplicity, J/Hz)[a]	Protons connected by scalar coupling	Protons connected by dipolar coupling
OH	9.44 (br s)		
OH	9.12 (v br s)		
W_5	8.64 (br d, 2.8)	X_5	X_4, 4f, 5f
W_7	8.48 (br d, 5.6)	X_7	X_6, X_7, Z_6, 4f, 5b, 6b, 7f
W_4	8.25 (v br s)	X_4	4b
W_2	7.93 (v br s)	X_2	sat. trans to water
6b	7.86 (s)	6e	X_6, Z_6, 4f
2f	7.52 (d, 8.5)	2b, 2e	1d, 1e, 2e
6f	7.47 (dd, 8.1, 1.0)	6b, 6e	W_6, Z_6, Z_6-OH, 4f, 6e
2b	7.39 (br s)	2f	X_2, Z_2
$CONH_2$	7.37 (o)	$CONH_2$	$CONH_2$
6e	7.34 (d, 8.5)	6f	G_1, V_1, V_6, V_7, 4f, 6f
2e	7.26 (d, 8.5)	2f	G_1, V_1, V_6, V_7, 2f, 4b
5b	7.18 (br s)	5f	W_7, X_5, X_6
$CONH_2$	6.92 (br s)	$CONH_2$	$CONH_2$
5f	6.77 (dd, 8.5, 1.0)	5b, 5e	W_5, X_7, 5e
5e	6.72 (d, 8.5)	5f	X_7, 5f
W_6	6.67 (br s)	X_6	X_7, 6f
W_3	6.62 (v br s)	X_3	
7d	6.42 (d, 2.1)	7f	X_7
7f	6.26 (d, 2.1)	7d	V_4, X_7
Z_6-OH	5.96 (d, 6.4)	Z_6	Z_6, 6f, sat. trans. to water
Z_2-OH	5.82 (br s)	Z_2	Z_2, sat. trans. to water
X4	5.75 (d, 8.1)	4b, 4f	W_5, 4b, 4f
4b	5.55 (br s)	X_4, 4f	W_4, X_4, 2e
V_4-OH	5.43 (br s)		sat. trans. to water
G_3-OH	5.38 (d, 4.9)	G_3	sat. trans. to water
G_1	5.27 (d, 7.8)	G_2	G_3, G_5, V_5, 2e, 6e
V_1	5.24 (d, 3.5)	V_{2ax}, V_{2eq}	G_2, G_3, V_{2ax}, V_{2eq}
4f	5.21 (d, 1.4)	X_4, 4b	W_5, X_4, X_5, 6e
Z_2	5.16 (br s)	X_2, Z_2-OH	X_6, Z_2-OH, 2b
Z_6	5.13 (br s)	X_6, Z_6-OH	X_6, Z_6-OH, 6b
G_4-OH	5.11 (br s)	G_4	sat. trans. to water
X_2	4.88 (br m)	Z_2, W_2	Z_2, 2b
V_5	4.68 (q, 6.4)	V_4, V_6	G_1, G_2, V_4, V_6, V_7
X_5	4.43 (d, 2.8)	W_5	X_6, 4f, 5b
X_7	4.42 (d, 5.6)	W_7, 7f	W_6, W_7, 5e, 5f, 6f, 7d, 7f
X_3	4.35 (br q, 5.6)	W_3, 3a, 3a′	
X_6	4.19 (d, 11.6)	W_6, Z_6	W_7, X_5, Z_6, 5b, 6b
G_6-OH	4.05 (t, 5.3)	G_{6a}, $G_{6a'}$	sat. trans. to water
G_{6a}	3.68 (dd, 10.9, 3.2)	G_5, $G_{6a'}$, G_6-OH	G_4, G_5, $G_{6a'}$
G_2	3.59 (t, 8.5)	G_1, G_3	G_4, V_1, V_5
$G_{6a'}$	3.57 (o)	G_5, G_{6a}, G_6-OH	G_4, G_5, G_{6a}
G_3	3.50 (t, 8.5)	G_2, G_3-OH, G_4	G_1, G_5
G_5	3.31 (o)	G_{6a}, $G_{6a'}$	G_3, G_{6a}, $G_{6a'}$
G_4	3.31 (o)	G_3, G_4-OH	G_2, G_{6a}, $G_{6a'}$
X_1	3.31 (o)	1a, 1a′	1e
V_4	3.23 (br s)	V_5	V_5, V_6, V_7, 7f
3a	2.42 (o)	X_3, 3a′	3a′
1e	2.37 (s)		X_1, 2f
3a′	2.14 (dd, 15.5, 5.6)	X_3, 3a	3a
V_{2ax}	1.90 (br d, 10.6)	V_1, V_{2eq}	V_1
V_{2eq}	1.75 (br d, 10.6)	V_1, V_{2ax}	V_1
1b	1.72 (non, 7.1)	1a, 1a′, 1c, 1d	1a, 1a′, 1c, 1d
1a	1.51 (quin, 7.1)	X_1, 1a′, 1b	1a′, 1b, 1c, 1d
1a′	1.47 (quin, 7.1)	X_1, 1a, 1b	1a, 1b, 1c, 1d
V_7	1.32 (s)		V_1, V_{2eq}, V_4, V_5, 2e, 6e
V_6	1.07 (d, 6.4)	V_5	V_4, V_5, 2e, 6e
1c	0.91 (d, 6.7)	1b, 1d	1a, 1a′, 1b
1d	0.86 (d, 6.7)	1b, 1c	1a, 1a′, 1b, 2f

[a] Multiplicity abbreviations: br = broad; d = doublet; m = multiplet; non = nonet; o = obscured; quin = quintet; s = singlet; t = triplet; v br = very broad; q = quartet.

Table 2 shows the assignments made for all 66 carbons and also the indirect (2–3 bond) connections which each ^{13}C resonance displays. When these assignments are compared with the ones made in the previous work,[9] there is remarkable consistency, bearing in mind the more indirect methods used in the earlier assignment. This good agreement is reflected in Table 2 by indicating the previous assignments in parentheses only where we now propose amendment. As the major discrepancy arises in the assignment of the carbonyl signals, we discuss the reason behind our new deductions.

By use of Fig. 4 (in conjunction with inspection of the appropriate f_2 traces), the following correlations can be made: the almost coincident C_1 and C_7 signals couple to protons 1a and 1a′ and to X_7; C_3 couples to protons X_4 and 3a′; C_3′ couples to proton 3a′; C_4 couples to proton X_4; C_5 couples to protons X_5 and X_6; C_6 couples to protons X_6 and X_7; and finally C_2

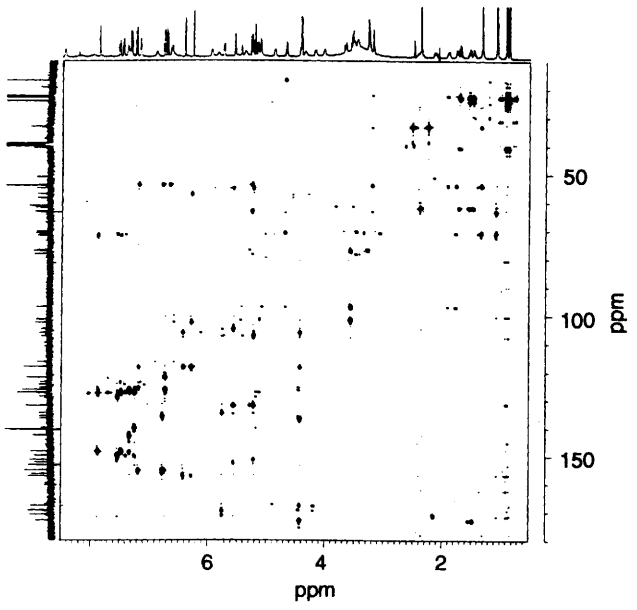


Fig. 3 Reverse-mode ^1H–^{13}C correlation spectrum of **1** acquired in $(CD_3)_2SO$ at 500 MHz ^1H frequency (303 K). Delays in the pulse sequence have been set so as to optimise indirect (multiple-bond) couplings (10 Hz). From this spectrum all the remaining ^{13}C assignments can be made. A portion of this spectrum is reproduced in Fig. 4 and shows the connections given by the eight carbonyl carbons.

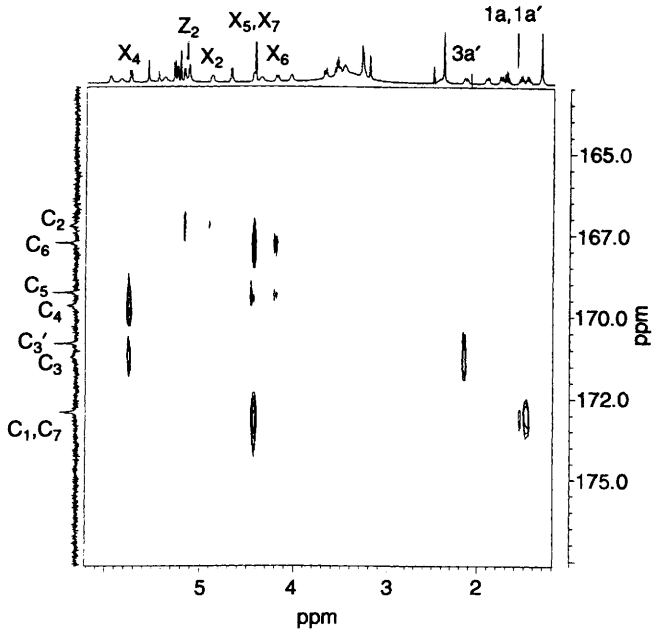


Fig. 4 Expanded view of the carbonyl connectivities observed in the ^1H–^{13}C correlation spectrum of **1** (Fig. 3). The couplings seen in this spectrum lead to the unambiguous assignment of all eight carbonyl resonances.

couples to X_2 and Z_2. It is clear from these results that not all the correlations which might be expected are present; there are no correlations to protons X_1, X_3 or 3a, probably as a result of the multiplicity of these resonances. However, the correlations which are observed are sufficient to assign unambiguously all eight carbonyls. By similar close scrutiny of other regions in the spectrum, the remaining quaternary carbons can be assigned.

For binding studies conducted by monitoring the ^{13}C resonances of vancomycin, we required an aqueous solution buffered (phosphate) to pH 6, with the antibiotic present at 20 mmol dm^{-3}. Therefore, the DMSO assignment was translated by titration to one relevant to these aqueous conditions.

Table 2 ^{13}C NMR data for **1** in $(CD_3)_2SO$ and pH 6 phosphate buffer at 303 K

Carbon[a]	$(CD_3)_2SO$		pH 6 aq. buffer		Indirectly coupled protons (determined in DMSO)
C_1	1	173.4	5	170.7	1a,1a'
C_7	2	172.5	1	177.8	X_7
$C_3(C_{3'})$	3	171.1	2	175.1	X_4, 3a'
$C_{3'}(C_2)$	4	170.6	3	171.9	3a
C_4	5	169.5	6	170.3	X_4
C_5	6	169.1	4	171.3	X_5, X_6
C_6	7	167.6	7	169.0	X_6, X_7
$C_2(C_3)$	8	167.1	8	168.9	X_2, Z_2
7e	9	157.1	9	157.2	7d, 7f
7c	10	156.4	10	155.6	7d
5d	11	155.0	11	154.9	5b, 5f
4c	12	152.1	12	153.4	4b
4e	13	151.3	13	152.4	4f
2d	14	149.8	14	151.0	2b, 2e, 2f
6d	15	148.3	15	149.8	6b, 6e, 6f
6a	16	142.4	16	140.8	6e
2a	17	139.8	17	139.0	Z_2, 2e
7a	18	136.2	18	138.5	X_7
5b	19	135.6	19	136.1	5f
4a	20	134.5	20	135.3	X_4, 4b, 4f
4d	21	131.9	21	133.3	G_1, 4b, 4f
2b	22	128.6	22	129.7	Z_2, 2f
6b	23	127.3	24/25	128.6	Z_6, 6f
6f(2f/6f)	24	127.3	24/25	127.6	Z_6, 6b
2f(5a)	25	127.2	23	128.7	Z_2, 2b
2c(2f/6f)	26	127.1	27	126.9	2e
6c	27	126.2	26	127.2	6b, 6e
5a(2c)	28	126.2	29	126.5	X_5, 5e
5f	29	125.4	28	126.9	X_5, 5b
2e	30	124.2	30	125.1	
6e	31	123.3	31	124.2	
5c	32	121.6	32	122.1	5e
7b	33	118.0	34	118.3	X_7, 5b, 7d, 7f
5e	34	116.2	33	118.7	
4b	35	107.1	36	107.7	4f
7f	36	105.8	35	108.6	X_7, 7d
4f	37	104.6	37	105.8	X_4, 4b
7d	38	102.3	38	103.5	7f
G_1	39	101.2	39	102.1	G_2
V_1	40	97.6	40	98.3	G_2, V_{2ax}, V_{2eq}, V_5
G_2	41	78.0	41	80.0	G_3, V_1
$G_3(G_5)$	42	77.0	43	76.9	G_2
$G_5(G_3)$	43	76.7	42	76.9	G_1, G_4
$Z_6(Z_2)$	44	71.5	45	72.0	6b, 6f
$Z_2(Z_6)$	45	71.1	44	72.4	2b, 2f
V_4	46	70.7	46	71.4	V_{2eq}, V_5, V_6, V_7
G_4	47	70.2	47	69.8	G_3
V_5	48	63.1	48	64.5	V_1, V_4, V_6
X_6	49	61.9	50/51	61.3	
X_1	50	61.8	49	63.8	1a, 1a', 1b, 1e
G_6	51	61.2	50/51	61.3	
X_2	52	58.3	53	59.3	
X_7	53	56.7	52	60.2	7f
X_4	54	54.9	54	55.5	4b, 4f
V_3	55	53.9	55	55.1	V_1, V_{2ax}, V_{2eq}, V_4, V_7
X_5	56	53.7	56	54.8	5b, 5f, 5e
X_3	57	51.0	57	52.2	3a'
1a	58	40.7	58	39.3	1b, 1c, 1d
3a	59	37.2	59	36.3	
V_2	60	33.3	61	32.4	V_4, V_7
1e	61	33.2	60	33.6	
1b	62	24.1	62	24.4	1a, 1a', 1c, 1d
1c	63	22.9	64/65	22.3	1a, 1a', 1b, 1d
1d(V_7)	64	22.5	64/65	22.2	1a, 1a', 1b, 1c
V_7(1d)	65	22.2	63	22.9	V_{2ax}, V_4
V_6	66	16.8	66	16.9	V_5

[a] Assignments in parentheses indicate where a different interpretation has been given in ref. 9.

Diluting a 50 mmol dm^{-3} DMSO solution to a 20 mmol dm^{-3} DMSO solution results in no significant change in the ^{13}C

spectrum of vancomycin. However, by following the spectrum in a series of mixed solvent solutions (DMSO–pH 6 buffer), in which the aqueous component was present at 3, 7, 10, 12.5, 15, 20, 25, 33, 50, 66, 75 and 87.5% (v/v), all signals were seen to experience chemical shift changes and several resonances changed their relative positions. An interesting observation from these titrations is that the largest chemical shift changes occur between solutions where the aqueous percentage is low; in solutions above 33% of deuteriated buffer, there are only small changes in chemical shifts and no changes to the relative position of peaks. Table 2 gives the chemical shifts of all the carbons of vancomycin and also shows their relative peak positions (1–66) in both the DMSO and aqueous solutions. A full and unambiguous assignment of the carbon spectrum of the antibiotic is now therefore available for binding studies in aqueous solution (see the accompanying paper).

Experimental

Preparation of Solutions.—Vancomycin was supplied as the HCl salt by Eli Lilly (Indianapolis) and was used without further purification. For all spectra used in the ^1H and ^{13}C assignments the same 50 mmol dm^{-3} solution of vancomycin (50 mg) in DMSO (650 cm^3) was used. The solvent was obtained from Aldrich and had an isotopic purity of 99.96 atom% D. No special precautions were taken to reduce the size of the water peak. When the ^{13}C assignments were checked at 20 mmol dm^{-3}, 20 mg of vancomycin was dissolved in 650 cm^3 of DMSO.

The aqueous buffer solution was prepared by taking 50 cm^3 of 0.2 mol dm^{-3} KH_2PO_4 (27.2 g dm^{-3}), adding 11.2 cm^3 of 0.2 mol dm^{-3} NaOH and 10 cm^3 of D_2O, and then diluting to 100 cm^3 with water according to a published procedure.[12] The pH of this stock solution was measured with a Corning pH meter 125 equipped with a combination glass electrode. To bring the pH to exactly 6.0 a few drops of the 0.2 mol dm^{-3} NaOH were added from a Pasteur pipette.

The ^{13}C assignments were transferred from the 20 mmol dm^{-3} DMSO solution to the 20 mmol dm^{-3} pH 6 buffer solution by means of 12 mixed solvent solutions. These were prepared by taking 20 mg portions of vancomycin and dissolving them separately in the following solvent mixtures (pH 6 buffer: DMSO (v/v): 20:655; 50:630; 70:610; 85:590; 100:575; 135:540; 170:505; 225:450; 340:340; 445:230; 505:170 and 590:85. These solvent mixtures contain approximately 3, 5, 7, 10, 12.5, 15, 20, 25, 33, 50, 66, 75 and 87.5% water, respectively.

NMR Spectroscopy.—NMR spectra were recorded on Bruker Instruments, models AC 300 and AMX 500. One-dimensional ^1H spectra were acquired into 16 K data points with a spectral width of 9.5 ppm (δ 0.5–10.0). One dimensional ^{13}C spectra were recorded over 32 K data points with a spectral width of 200 ppm (δ 0–200) and using composite pulse proton decoupling. A temperature range of 298 to 303 K was used for all one- and two-dimensional experiments.

The phase-sensitive DQF-COSY, phase-sensitive NOESY and ROESY experiments were all acquired using standard procedures.[13] The same spectral widths as used in the one-dimensional experiments were employed (δ 0.5–10.0), and 64 transients (DQF-COSY and NOESY) or 16 transients (ROESY) were acquired into 2 K data points in f_2. All three experiments used 512 increments and the data sets were zero-filled to 1 K in f_1 before Fourier transformation. Various Gaussian and sinebell weighting functions were applied in each dimension and none of the spectra were symmetrised.

The heteronuclear (^1H–^{13}C) correlation experiments were performed in both conventional, ^{13}C-detected, and reverse, ^1H-detected modes. At a field strength of 7.0 T it was preferable to work in the conventional mode (^{13}C–^1H), with the ^{13}C spectrum in f_2, because of the large number of resonances with similar chemical shifts. The ^{13}C–^1H correlation experiments used a data size of 8 K × 256 points (zero-filling once in f_1) and between 96 and 192 transients per t_1 increment were collected. No weighting functions were applied prior to Fourier transformation and evolution delays were set to optimise 150, 15, 10, 7 and 4 Hz couplings (J_{CH}). The spectra each required between 16 and 30 h of acquisition time. At a field strength of 11.7 T it was preferable to work in reverse mode (^1H–^{13}C) to take advantage of the better sensitivity offered by this experiment. A single ^1H–^{13}C correlation spectrum was acquired at this field strength, employing a data size of 2 K × 512 points (zero-filling once in f_1), and 144 transients per t_1 increment were collected. The evolution delay was set to optimise 10 Hz couplings and shifted sinebell weighting functions were, overall, the most suitable for both dimensions, however, some correlations were best observed using various Gaussian and sinebell combinations. For both the conventional and reverse mode experiments, the same ^1H and ^{13}C spectral widths as used in the one-dimensional experiments were employed and, in both cases, the correlations were achieved using standard pulse programs.[13]

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