# Ligands which Bind Weakly to Vancomycin: Studies by $^{13}C$ NMR Spectroscopy

**Clive M. Pearce,[a] Ute Gerhard[b] and Dudley H. Williams*[,b]**
[a] *AgrEvo UK Ltd., Chesterford Park, Saffron Walden, Essex, UK CB10 1XL*
[b] *Cambridge Centre for Molecular Recognition, University Chemical Laboratory, Lensfield Road, Cambridge, UK CB2 1EW*

$^{13}C$ NMR chemical shifts of vancomycin have been used to obtain the association constants for a number of ligands which bind weakly to the antibiotic. The change in these $^{13}C$ chemical shifts upon stepwise addition of ligands provides evidence that the ligands bind in a manner analogous to natural cell-wall precursor analogues. The binding constants obtained are in good agreement with those determined earlier by other methods. Where the ligands are amino acids (glycine or alanine), a potential alkylammonium to amide carbonyl interaction does not promote binding.

Vancomycin group antibiotics are a large family of heptapeptides with extensively cross-linked sidechains and with a variety of sugar residues attached to the peptide backbone. They bind to the peptide unit of cell wall precursor molecules terminating in an L-lysyl-D-alanyl-D-alanine sequence (Fig. 1). This recognition process is due to a combination of polar and hydrophobic interactions, and is promoted by good complementarity of the van der Waals surfaces of the ligand and binding pocket.[1-3] The binding constant for the above natural ligand to vancomycin[4] is *ca.* $10^6$ dm$^3$ mol$^{-1}$. Cell-wall precursor analogues such as *N*-acetyl-D-alanyl-D-alanine (NADADA) bind with somewhat smaller binding constants (in the region of $10^4$ to $10^5$ dm$^3$ mol$^{-1}$).[4-7] The majority of ligands studied in this way are truncated derivatives of the natural substrate; this allows a part of the same complementarity with the binding site that has evolved for the natural ligand. The fit of cell wall precursor analogues into the antibiotic binding pocket was studied in earlier work by $^1H$ NMR spectroscopy.[8,9] The geometry of the bimolecular complex formed by the antibiotics with cell-wall precursor analogues is well known from $^1H$ NMR studies and based on intermolecular nuclear Overhauser effects (NOEs). Comparison of the thermodynamic parameters for binding of modified ligands such as *N*-acetyl-D-alanine (NADA) or *N*-acetylglycyl-D-alanine (NAGDA) with those for NADADA can yield estimates of the strengths of extra hydrogen bonds formed or the magnitude of the hydrophobic effect of methyl groups.[2] These estimates can be used as guides for rough predictions of overall binding constants for non-covalent bimolecular interactions. The binding of a number of weakly interacting ligands (Fig. 2) to vancomycin has now been studied by $^{13}C$ NMR spectroscopy;[10] the results, and their analysis, form the basis of this paper. The ligands were selected as further truncated analogues of natural cell-wall precursor analogues binding into the binding pocket of vancomycin group antibiotics.

## Results and Discussion

*Analysis of Carbon Chemical Shift Changes of Vancomycin in $^{13}C$ NMR Titrations.*—In order to obtain the association constants, the chemical shifts of all vancomycin carbon atoms (assigned in the preceding paper)[11] were monitored as a function of ligand concentration. The majority of $^{13}C$ resonances experienced no significant chemical shift changes (< 5 Hz) upon association with the ligands. The final calculation of a binding constant was based on those signals which showed changes in limiting chemical shifts of more than 20 Hz and remained well resolved over the entire titration. The association constants were extracted from a curve-fitting routine and Scatchard plots and were averaged for each ligand over all monitored $^{13}C$ signals.[10] The result of this analysis is summarised in Table 1 and the Scatchard plot for the binding of acetate is shown in Fig. 3.

**Table 1** Binding constants and free energies of binding (at 300 K) for truncated cell-wall precursor analogues interacting with vancomycin

| Ligand | $K$/dm$^3$ mol$^{-1}$ | $-\Delta G$/kJ mol$^{-1}$ |
|---|---|---|
| Acetate (**2**) | 30 | 8.5 |
| Glycine (**3**) | < 10 | < 5.7 |
| D-Alanine (**4**) | 10–20 | 5.7–7.5 |
| D-Lactate (**5**) | 80 | 10.9 |
| *N*-Acetylglycine (**6**) | 80 | 10.9 |
| *N*-Acetyl-D-alanine (**7**) | 300 | 14.2 |

*Structural Information on the weakly Associated Bimolecular Complexes from $^{13}C$ Chemical Shift Changes.*—In earlier work,[1] the characteristic upfield chemical shift of the methyl group protons of the *C*-terminal D-amino acid of weakly binding ligands supported a similar position of the methyl group in the complex to that found for analogues of extended cell wall precursors (*e.g.*, *N,N*-diacetyl-L-lysyl-D-alanyl-D-alanine). In cases of ligands lacking such a probe (*e.g.*, acetate or glycine), the assumption of a similar complex geometry was based on similar changes in the UV absorbances of the antibiotic chromophore on binding. By considering the identity of the carbon resonances which shift significantly on binding as well as the extent and the direction of the shift (upfield and downfield), the location of the ligand in the binding pocket can now be traced. The carbon atoms experiencing significant chemical shift changes are illustrated in Fig. 4. From this diagram it is apparent that the largest chemical shift changes are found where the ligands are expected to bind according to the existing binding model (Fig. 1). Further, the trend in chemical shift change is for all ligands (with the exception of the very weakly binding glycine) the same. For example, Table 2 shows the limiting $^{13}C$ chemical shift changes upon ligand binding for the cases of acetate and NADA. Additionally, it should be noted that the signal of carbon $X_5$ (Fig. 1) is not shifted significantly in the acetate complex, which is in accord with the fact that the additional hydrogen bond formed in the NADA complex between the carbonyl group of residue 4 and the NH of the ligand is not present. This provides evidence that acetate binds, even though weakly, in a defined manner to the antibiotic as predicted from the existing knowledge of the structure of the

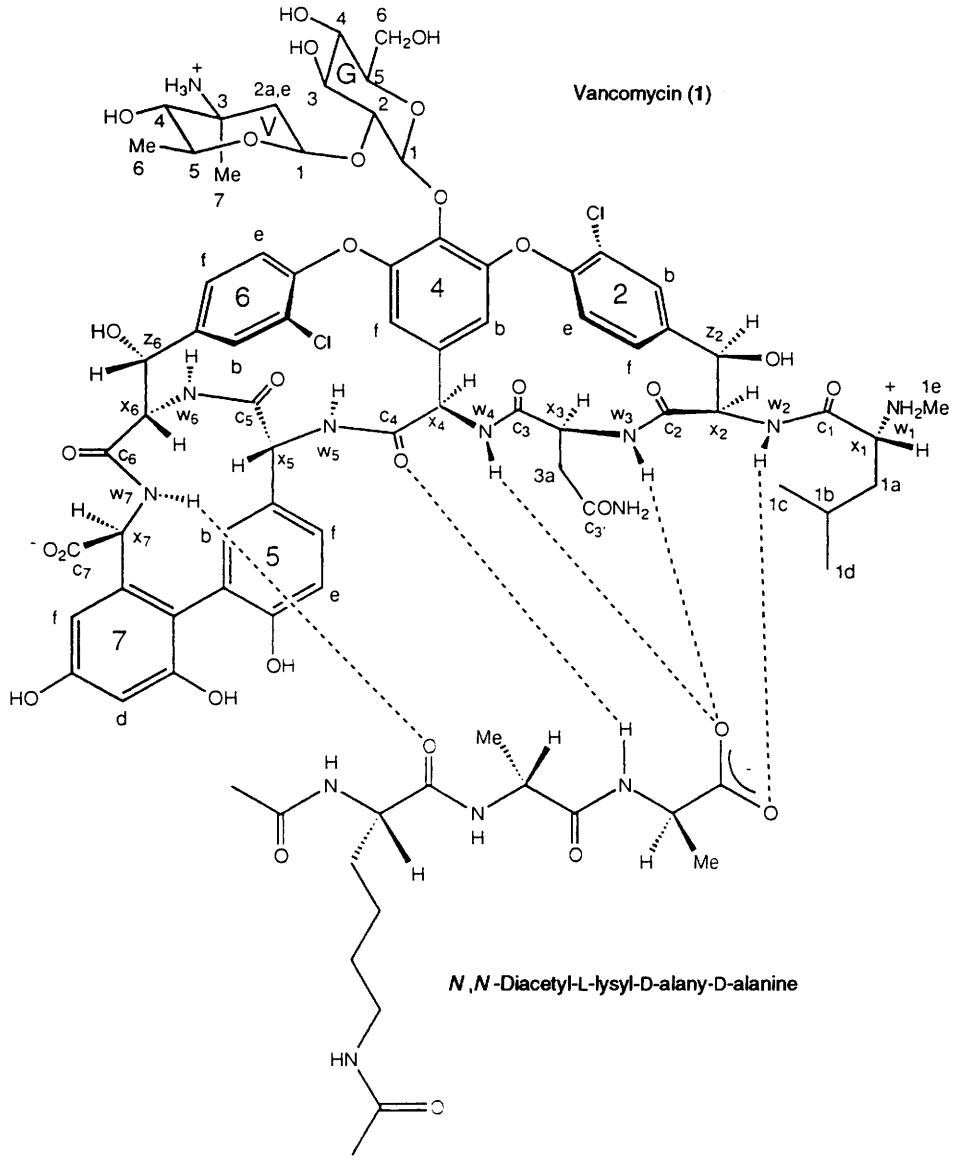


**Fig. 1** Bimolecular complex formed between vancomycin (**1**) and the cell-wall precursor analogue *N*,*N*-diacetyl-L-lysyl-D-alanyl-D-alanine. Dotted lines indicate hydrogen bonds.

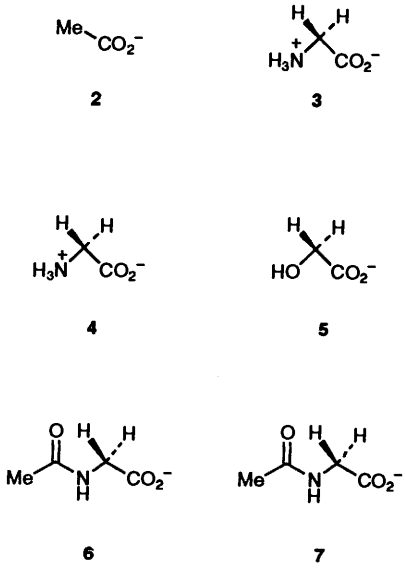


**Fig. 2** Ligands studied by $^{13}C$ NMR spectroscopy binding to **1**

binding pocket. The analysis of the $^{13}C$ chemical shift changes induced by the other ligands confirmed this view. Only in the case of glycine are the results ambiguous due to the extremely weak interaction with the antibiotic. It is evident from the data of Table 2 that the limiting chemical shifts are on average smaller in the case of acetate than for NADA, indicating a less tight interaction. This result is consistent with the relative exothermicities for the binding of these two ligands to vancomycin: $-24$ kJ mol$^{-1}$ for the binding to acetate, and $-36$ kJ mol$^{-1}$ for the binding to NADA.[12] Some of the data can however be used to infer specific differences between the complexes. For example, it appears that the position of residue 1 in the case of acetate binding (Fig. 4) is different from its position in the stronger NADA complex. This is suggested by the $^{13}C$ chemical shift changes of residue 1 carbon atoms (see Table 2): carbon atom 1c shifts to a similar extent in both complexes whereas carbon 1a undergoes a significant shift (56 Hz) upon NADA addition, but not with acetate.

*Contribution of Ammonium Groups in Ligands to the Overall Binding Constant.*—The free energies of binding for glycine (**3**) and D-alanine (**4**) were found to be significantly *lower* than the

corresponding *N*-acetyl amides (**6** and **7**), D-lactate (**5**) and even acetate (**2**). This is a striking observation since glycine and D-alanine could feature additional interactions with the receptor compared with acetate: an amide/ammonium hydrogen bond (compare Fig. 1, where this hydrogen bond would replace that

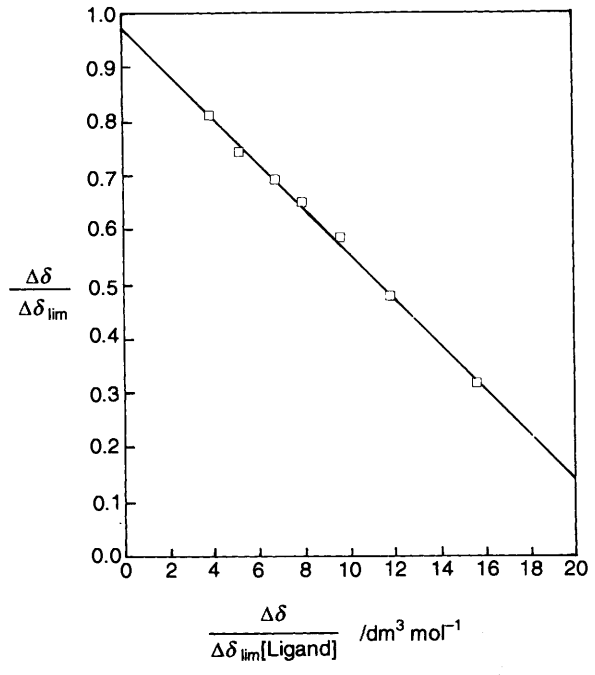


**Fig. 3** Scatchard plot of the average $\Delta\delta/\Delta\delta_{lim}$ values for the **1**/**3** complex

to the amide NH of *N*,*N*-diacetyl-L-lysyl-D-alanyl-D-alanine), and the hydrophobic interaction of a methyl group in the case of D-alanine, the latter unambiguously known to promote binding.[1] From this superficial analysis, the amino acids would therefore be expected to form *stronger* complexes than acetate. From protein engineering experiments,[13] charge–dipole hydrogen bonds have been shown to be generally stronger than dipole–dipole hydrogen bonds. The amide/ammonium hydrogen bond would be expected to promote the equilibrium constant for binding by about a factor of 10 over the hydroxyamide (D-lactate) hydrogen bond. However, the data show lower net binding energy when the ammonium ions are present (Table 1). We speculate that this observation may reflect two factors. First, the difficulty of simultaneous solvation by water of the tetrahedral alkyl ammonium ion when forming the hydrogen bond to the antibiotic amide carbonyl group (perhaps due to steric effects). Second, the loss of stabilisation, found only in the *free* amino acid, due to intramolecular interaction (perhaps mediated by solvent) of the $NH_3^+$ and $CO_2^-$ groups. Of these two possible factors, the first could reduce the net binding energy from the ammonium/amide hydrogen bond if formed; and the second could reduce the net binding energy even if the putative hydrogen bond were not formed (*e.g.* owing to the first effect). Whatever the cause, the weak binding of the amino acids **3** and **4** is striking and unambiguous.

## Conclusions

The $^{13}C$ analysis allows four conclusions concerning the weak interaction of highly truncated cell wall precursors binding to vancomycin. First, where comparisons can be made, the binding constants are very similar to those found by other methods.[1]

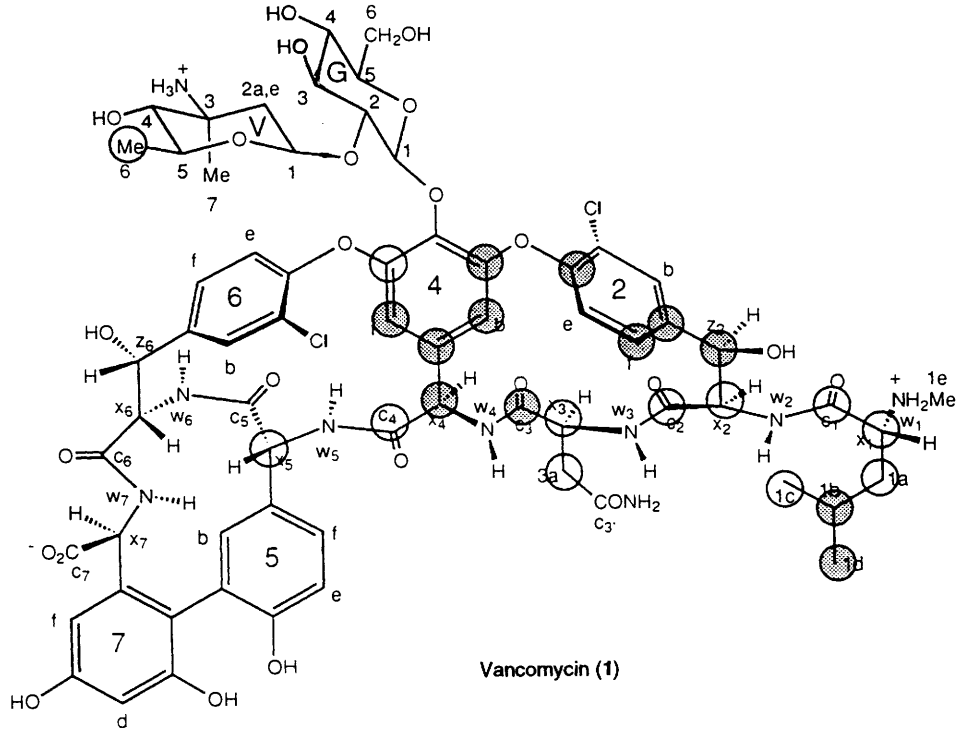


**Fig. 4** Schematic illustration of the chemical shift changes to the $^{13}C$ resonances of **1** upon addition of ligands **2** to **8**

**Table 2** Limiting chemical shift changes of $^{13}C$ resonances ($\Delta\delta$) of vancomycin (**1**) carbon atoms upon addition of ligands **2** and **7**

| Carbon | Extrapolated limiting chemical shift changes ($\Delta\delta$) upon addition of **2**/Hz | Extrapolated limiting chemical shift changes ($\Delta\delta$) upon addition of **7**/Hz |
|---|---|---|
| $C_1$ | *a* | 53 |
| $X_1$ | 30 | 38 |
| 1a | *b* | 56 |
| 1c | 27 | 23 |
| 1d | *a* | 86 |
| $C_2$ | 105 | 123 |
| $X_2$ | 44 | 42 |
| $Z_2$ | 35 | 39 |
| 2a | 85 | 99 |
| 2d | 70 | 86 |
| 2f | 36 | 49 |
| $C_3$ | 97 | 104 |
| $X_3$ | 34 | 50 |
| 3a | 116 | 165 |
| $C_4$ | 93 | 95 |
| $X_4$ | *a* | 66 |
| 4a | 48 | 51 |
| 4c | 44 | 44 |
| 4f | 37 | 74 |
| $X_5$ | *b* | 68 |

[a] $^{13}C$ Signal obscured. [b] No significant shift in the $^{13}C$ resonance.

Secondly, by comparing trends in $^{13}C$ chemical shifts, the geometry of these bimolecular complexes is shown to be relatively specific, and comparable to complexes with larger association constants. Thirdly, chemical shift changes suggest the formation of a somewhat tighter complex to NADA than to acetate, in accord with the larger exothermicity for the formation of the complex to the former ligand. Fourthly, the ammonium ion in D-alanine and glycine does not contribute favourably to the binding constant of the ligands.

## Experimental

*Chemicals.*—Vancomycin was supplied as the HCl salt by Eli Lilly (Indianapolis) and was used without further purification. $D_2O$ was obtained from Aldrich and had an isotopic purity of 99.96 atom% D; other chemicals were obtained from either Sigma or Aldrich.

*$^{13}C$ NMR.*—NMR spectra were recorded on a Bruker instrument, model AC 300 (75 MHz $^{13}C$ frequency). All $^{13}C$ spectra were acquired into 32 K data points with a spectral width of 200 ppm ($\delta$ 0–200) and using composite pulse proton decoupling. The temperature was maintained at 303 K during all acquisitions.

*Preparation of NMR Samples and Solutions.*—All binding studies were carried out in a partially deuteriated (10%) aqueous solution buffered to pH 6. This was prepared by taking 50 $cm^3$ of 0.2 mol $dm^{-3}$ $KH_2PO_4$ (27.2 g $cm^{-3}$), 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.[14] 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.

*Titrations.*—The titrations were performed by keeping the concentration of **1** constant at 20 mmol $dm^{-3}$ (20 mg of **1** in 650 $cm^3$ of the aqueous buffer) and incrementing the amount of ligand present. To accomplish this, weighed portions of the ligand were dissolved sequentially in the 650 $cm^3$ solution following the acquisition of each $^{13}C$ spectrum, so as to achieve ligand concentrations of 0–200 mmol $dm^{-3}$ at 20 mmol $dm^{-3}$ intervals (for the more strongly binding ligands **5**, **6** and **7**, a 10 mmol $dm^{-3}$ solution was also used). The starting solution with no ligand present acted as the reference from which any $^{13}C$ chemical shift changes, brought about by the ligand, were measured.

*Calculation of Binding Constants.*—For each binding process studied, limiting chemical shift changes ($\Delta\delta_{lim}$) were obtained from a curve-fitting computer program called EZ-FIT.[15] Binding constants were calculated from plots of $\Delta\delta/\Delta\delta_{lim}$ *vs.* $\Delta\delta/\Delta\delta_{lim}$[ligand], which are straight-line graphs known as Scatchard plots.[16] By taking the numerical average of binding constants calculated for individual $^{13}C$ signals, overall representative binding constants were obtained for each ligand–vancomycin complex (these are the values which appear in Table 2).

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