Infrared spectroscopic studies of vancomycin and its interactions with *N*-acetyl-D-Ala-D-Ala and *N*,*N*′-diacetyl-L-Lys-D-Ala-D-Ala

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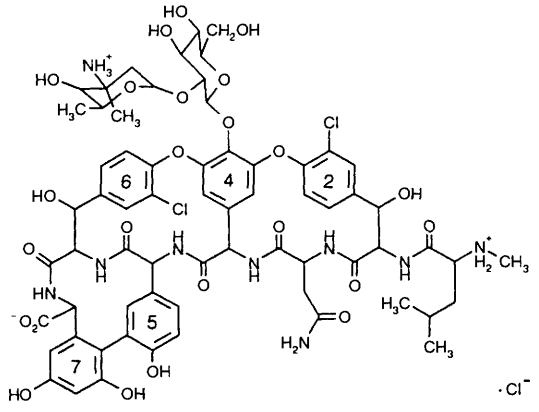
The infrared (IR) spectrum of vancomycin, in D_2O solution, has been assigned by recording spectra at different pD values and by comparing them with the spectrum in H_2O at pH 5. The effects of self-association on the spectrum of vancomycin at pD 5 have also been investigated. Details of underlying components of the broad bands in the spectra were revealed using resolution enhancement and second derivatives. The IR spectra of two peptide models, *N*-Ac-D-Ala-D-Ala and *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala, have also been assigned in D_2O solution. The interactions of these peptides with vancomycin, in pD 5 solution, have been studied by infrared spectroscopy and the above assignments used to interpret the observed spectral changes; the solubilities of the vancomycin–peptide complexes at pD 5 were determined to facilitate these studies. The IR spectra of the complexes show substantial increases in intensity of a component at about 1588 cm^{-1}. Using ^{13}C labelled *N*-Ac-D-Ala-D-Ala this was found to be due to the asymmetric stretch of the carboxylate group of the peptide, showing that this group undergoes a substantial perturbation on binding to vancomycin.

Vancomycin (**1**) is a naturally occurring glycopeptide antibiotic which is active against Gram-positive bacteria.[1] Although largely superseded by the β-lactam antibiotics, it still finds applications in severe staphylococcal infections and has renewed importance in the light of increasing β-lactam resistance of infections. The three dimensional structure of vancomycin was derived from the X-ray crystal structure of a derivative[2] and from NMR studies.[3,4] The structure contains a heptapeptide chain of which both the amino and carboxy termini are free. In addition to these two ionisable centres there are also three weakly acidic aryl hydroxy groups and a basic vancosamine. Both vancomycin itself and a related glycopeptide antibiotic, ristocetin A, have been extensively used in studies into the structure and mechanism of this class of antibiotic.[1]

Evidence to date indicates that the antibiotic activity of these glycopeptides arises from their interactions with part of the bacterial cell wall. In Gram-positive bacteria the cell walls are composed of peptidoglycan, which consists of polysaccharide chains substituted with pentapeptides. Development of the cell wall involves cross-linking of these pentapeptides by pentaglycine, brought about by a transpeptidase. Early studies have shown that vancomycin will form a complex with the peptidoglycan precursor UDP-*N*-acetylmuramylpentapeptide.[5] Subsequent work has indicated that the essential region of this peptide for forming the complex is the D-alanyl-D-alanine terminus.[6] Accordingly, the antibiotic activity has been inferred to be due to the glycopeptide forming a complex with the D-alanyl-D-alanine terminus of the cell wall peptidoglycan, which would prevent the terminal D-alanine residue from being replaced with a pentaglycine by the transpeptidase. Consequently, the bacterial cell wall cannot develop normally.

Vancomycin has been extensively studied by NMR spectroscopy,[3,4] but no detailed infrared (IR) spectroscopic studies have been reported. The IR spectra of this glycopeptide, in the region 1800 cm^{-1} to 1300 cm^{-1}, will contain information on the amide groups of the peptide backbone, the terminal carboxy group and the aromatic side chains. Changes in the spectrum, brought about by the binding of peptides containing the D-alanyl-D-alanine terminus, should provide information regarding functional groups in the antibiotic, and in the peptide, which are affected by the binding process.

The purpose of the current studies was therefore to use IR spectroscopy to investigate the binding of two peptides, *N*-Ac-D-Ala-D-Ala and *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala, to vancomycin in aqueous solution. The assignment of the IR spectra of vancomycin and the two peptides was of primary importance. In the case of vancomycin, deuterium exchange was used to identify the amide I and amide II bands, while spectra recorded in solutions at different pD values were used to assign bands arising from ionisable groups. To assign the amide I′ bands of *N*-Ac-D-Ala-D-Ala, spectra in D_2O and in pD 5 buffer were compared. The spectrum of *N*-Ac-D-Ala-D-Ala labelled with ^{13}C at the carboxy group was recorded to assign unequivocally the bands due to carboxy stretching vibrations.



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From preliminary experiments, the complexes formed between vancomycin and the two peptides were found to have substantially lower solubilities in D_2O at pD 5, than the individual components. Consequently, the solubilities of the complexes were measured in order to find the maximum concentration which could be used for IR spectroscopy. The effect of concentration on the IR spectrum of vancomycin was also investigated, since there is evidence from optical rotation[7] and NMR[8] studies that these glycopeptide antibiotics can form non-covalent dimers in solution.

Experimental

Vancomycin and peptides

Vancomycin hydrochloride and *N*-Ac-D-Ala-D-Ala were supplied by Dr D. H. Williams of the Cambridge Centre for Molecular Recognition. *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala, and an additional batch of vancomycin hydrochloride, were purchased from Sigma. *N*-Ac-D-Ala-D-Ala labelled with ^{13}C at the carboxy group was supplied by Dr G. Batta of The Hungarian Academy of Sciences. All of these materials were used without further purification. Vancomycin was analysed by thermogravimetric analysis and microanalysis in order to determine its water content and concentrations were corrected accordingly. The stereochemical purity of the ^{13}C labelled peptide was determined by hydrolysis and subsequent analysis by capillary electrophoresis, showing the peptide to contain 76% D-alanine and 24% L-alanine.

Solvents and reagents

The water used was deionised, glass-distilled and then passed through a Millipore MilliQ system before preparing the buffers, to avoid bacterial contamination. D_2O of ≥99.8 atom% D purity was obtained from Aldrich. DCl, NaOD and KOD were obtained from Sigma as 40% solutions in D_2O. HCl and NaOH were Convol concentrated volumetric solutions from BDH, used either undiluted (approximately 10 mol dm^{-3}) or diluted to the required concentration with water. All other reagents and buffer salts were obtained commercially and were of the highest purity available.

Infrared spectra

IR spectra were recorded on a Perkin-Elmer 1720X FTIR spectrometer or on a Perkin-Elmer 1760 FTIR spectrometer, both fitted with a TGS detector, using a medium Norton–Beer apodisation function. The instruments were purged with dry air to remove water vapour and carbon dioxide. These are single beam spectrometers, fitted with sample shuttles which can be operated in interleave mode to simulate double beam operation. All solution spectra were recorded in interleave mode, but with nothing in the reference side of the sample shuttle. Separate spectra were recorded of the relevant solvents, using the same scan parameters and temperature as for the analyte solutions.

For each spectrum a number of scans were accumulated to give the required signal/noise ratio. For 1×10^{-2} mol dm^{-3} solutions in a 50 μm pathlength cell it was sufficient to accumulate 64 scans, but at 5×10^{-4} mol dm^{-3} it was necessary to accumulate 4000 scans. For 1×10^{-2} mol dm^{-3} solutions in a 6 μm cell, 1000 scans were accumulated. All of the solution spectra were recorded at 4 cm^{-1} resolution, using cells fitted with calcium fluoride windows. For the 50 μm pathlength a sealed cell was used, which was fitted with a removable luer fitting for filling with a syringe. The 6 μm pathlength cell was demountable and was filled by placing 30 μl of solution on one window, fitted with the 6 μm spacer, and then placing the other window on top, taking care to avoid air bubbles forming between the windows. The cell was then assembled and the windows clamped together. Both types of cell were held in a water jacket, in the spectrometer, through which constant temperature water was passed to control the temperature of the cell.

Data processing

Spectra recorded on the Perkin-Elmer 1720X spectrometer were transferred to a Perkin-Elmer 7000-series computer, running CDS-3 software. Spectra recorded on the Perkin-Elmer 1760 spectrometer were acquired directly onto a similar computer. Subsequent processing of the spectra was carried out using the functions of CDS-3. Second derivative spectra were produced using the *Deriv* function, with a *Width* factor of 3. Resolution enhancement was carried out using the *Enhance* function, with a *Width* of 10.0 and a *Factor* of 2.0. For conversion into ε, each spectrum was multiplied by a factor determined from the molar concentration (calculated using the molecular weight of 1485.7 for the hydrochloride salt) and pathlength.

Solvent baselines were subtracted from solution spectra using the *Interactive Difference* function, varying the subtraction factor to effect complete removal of bands due to the solvent. D_2O solutions also required the subtraction of a spectrum of HOD, using the same approach. The HOD spectrum was generated by recording the spectrum of a dilute solution of H_2O in D_2O and then subtracting a D_2O solvent baseline. HOD spectra were generated at several concentrations, because the shapes of the bands change with concentration. The spectrum closest in intensity to the HOD bands in the analyte spectrum was then used for the final subtraction.

Measurement of pH and pD

pH and pD measurements were carried out using a pH meter equipped with a semi-micro combination glass electrode, calibrated using standard pH 4 and pH 9 buffers. pD values were taken as the pH meter reading +0.40.[9]

IR spectra of vancomycin in solution

The following buffers were prepared by dissolving each salt in D_2O, to give the required concentration, and then adjusting to the correct pD by adding either DCl or NaOD solution: pD 1.5, KCl (5.0×10^{-2} mol dm^{-3}); pD 5.0, 2-morpholinoethanesulphonic acid [MES] (5.0×10^{-2} mol dm^{-3}); pD 7.9, KH_2PO_4 (5.0×10^{-2} mol dm^{-3}); pD 9.1, KCl (5.2×10^{-2} mol dm^{-3}) + H_3BO_3 (5.2×10^{-2} mol dm^{-3}); pD 10.0, KCl (5.0×10^{-2} mol dm^{-3}) + H_3BO_3 (5.0×10^{-2} mol dm^{-3}); pD 11.1, Na_2HPO_4 (2.5×10^{-2} mol dm^{-3}); pD 12.0, KCl (5.0×10^{-2} mol dm^{-3}). pH 5.0 buffer consisted of MES (5.0×10^{-2} mol dm^{-3}) in water, adjusted with 10 mol dm^{-3} NaOH. All buffer salts were selected to have minimal absorption in the 1800–1300 cm^{-1} region of the IR spectrum. Solutions of vancomycin hydrochloride were prepared to the required molarities and the pD readjusted, if necessary, using NaOD or DCl; the pH of the solution in H_2O MES buffer was adjusted to 5.0 using 1.0 mol dm^{-3} NaOH. The spectra of the buffers were recorded for subtraction from the spectra of the corresponding solutions of vancomycin.

Solubilities of the vancomycin–peptide complexes

Separate solutions of vancomycin hydrochloride (1.9×10^{-2} mol dm^{-3}) and *N*-Ac-D-Ala-D-Ala (2.0×10^{-2} mol dm^{-3}) were prepared in pD 5.0 buffer and equal volumes of them mixed. The resulting solution was adjusted to pD 5.0 with NaOD (4.0% and 0.2% w/v solutions in D_2O) and DCl (0.5% w/v solution in D_2O) and thoroughly mixed. The solution was centrifuged to remove the precipitate and an aliquot of the supernatant removed for analysis by UV spectrometry, diluting as necessary, with the buffer, to obtain a measurable absorbance. The solubility was then calculated from the measured absorbance, the dilution factor and the extinction coefficient of the complex ($\varepsilon = 6.0 \times 10^3$ dm^3 mol^{-1} cm^{-1} at $\lambda_{max} = 280$ nm).[10] This procedure was repeated using solutions of vancomycin hydrochloride (1.8×10^{-2} mol dm^{-3}) and *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala (2.0×10^{-2} mol dm^{-3}); the solubility of the resulting complex was calculated from the measured absorbance of the diluted supernatant, the dilution factor and the extinction coefficient ($\varepsilon = 6.2 \times 10^3$ dm^3 mol^{-1} cm^{-1} at $\lambda_{max} = 280$ nm).[10]

IR spectra of peptide models

In each case a weighed quantity of peptide was dissolved in a suitable volume of pD 5.0 buffer, to give the required

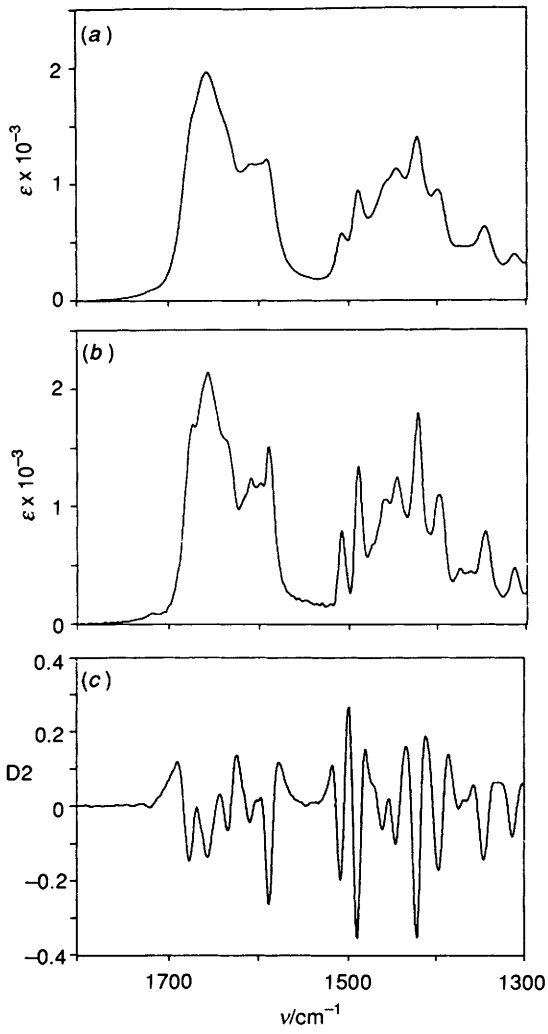

Fig. 1 IR spectrum of vancomycin hydrochloride (9.1×10^{-3} mol dm^{-3}) in D_2O MES (5.0×10^{-2} mol dm^{-3}) buffer at pD 4.9, 25 °C: (*a*) absorption spectrum; (*b*) resolution enhanced; (*c*) second derivative

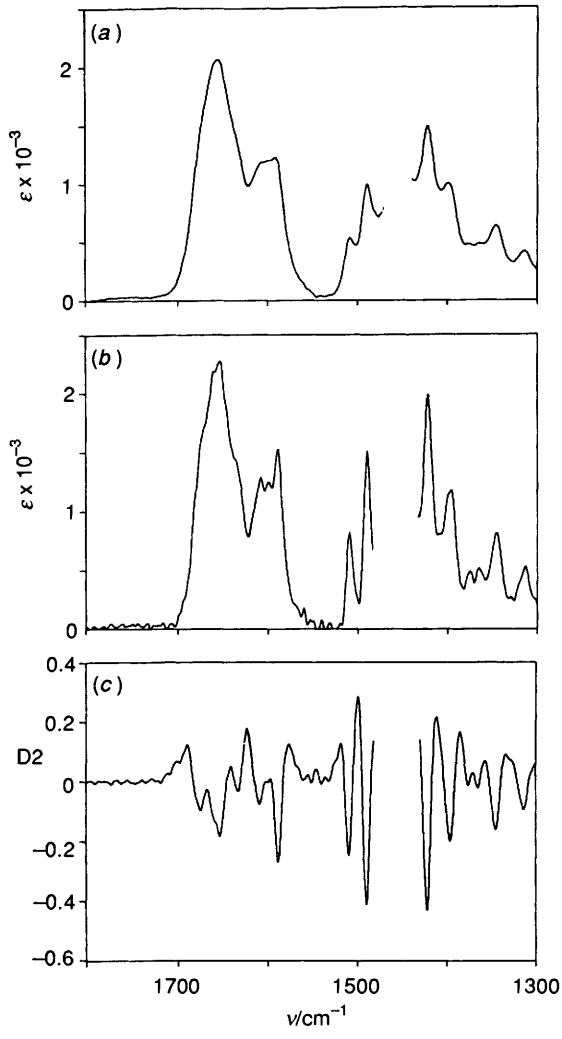

Fig. 2 IR spectrum of vancomycin hydrochloride (4.5×10^{-4} mol dm^{-3}) in D_2O MES buffer at pD 4.9, 25 °C: (*a*) absorption spectrum; (*b*) resolution enhanced; (*c*) second derivative. Part of the spectrum, from 1480 cm^{-1} to 1430 cm^{-1}, has been omitted because of distortion caused by noise.

concentration. The solutions were then readjusted to pD 5.0, if necessary, with a solution of NaOD in D_2O; the concentration of the NaOD varied from 0.2% to 4% w/v and was selected to minimise the volume required to adjust the pD with sufficient accuracy. *N,N'*-Ac_2-L-Lys-D-Ala-D-Ala and *N*-Ac-D-Ala-D-Ala, including the ^{13}C labelled material, were examined at 5.0×10^{-4} mol dm^{-3} concentration. The spectrum of *N*-Ac-D-Ala-D-Ala was also recorded at 9.8×10^{-3} mol dm^{-3} in pD 5.0 buffer and at 1.0×10^{-2} mol dm^{-3} in D_2O.

IR spectra of vancomycin–peptide complexes

Separate solutions of vancomycin hydrochloride (9.1×10^{-4} mol dm^{-3}) and *N*-Ac-D-Ala-D-Ala (1.0×10^{-3} mol dm^{-3}) were prepared in pD 5.0 buffer, the latter solution requiring some readjustment to pD 5.0 with 0.2% w/v NaOD. Equal volumes of these solutions were mixed, giving a clear solution of pD 4.9, which was used to record the spectrum. This procedure was repeated with ^{13}C labelled *N*-Ac-D-Ala-D-Ala and with *N,N'*-Ac_2-L-Lys-D-Ala-D-Ala, to obtain solutions of the corresponding complexes, and the IR spectra were recorded.

Results and discussion

IR spectrum of vancomycin in D_2O solution at pD 5

The IR spectrum of vancomycin hydrochloride (9.1×10^{-3} mol dm^{-3}) in D_2O MES buffer, at pD 4.9, is shown in Fig. 1(*a*). The dominant feature is a broad envelope of overlapping bands from about 1700 cm^{-1} to 1560 cm^{-1}. The resolution enhanced spectrum [Fig. 1(*b*)] and the second derivative of the original spectrum [Fig. 1(*c*)] help in locating the underlying components. The band centred at 1656 cm^{-1} in Fig. 1(*a*) has three components, which are at 1676, 1656 and 1633 cm^{-1} in the second derivative. Similarly, the lower frequency part of the band envelope is composed of at least three underlying components, at 1609, 1598 and 1588 cm^{-1}. Resolution enhancement and second derivative also reveal more detail below 1550 cm^{-1}. The only definite assignments that can be made, on the basis of previously reported data,[11,12] are for the components at 1676 cm^{-1} and 1656 cm^{-1}, which can be assigned as amide I'. The component at 1633 cm^{-1} may also be an amide I', but could alternatively be an aromatic ring mode; this is further addressed below.

At the concentration used to record the spectrum, vancomycin will be largely in its self-associated form.[7] To ascertain whether information could be obtained regarding the association process, the IR spectrum was also recorded using a more dilute solution of vancomycin (4.5×10^{-4} mol dm^{-3}) in the same buffer. The resulting spectrum [Fig. 2(*a*)] shows only minor differences from that recorded at higher concentration, although the second derivative [Fig. 2(*c*)] indicates a difference

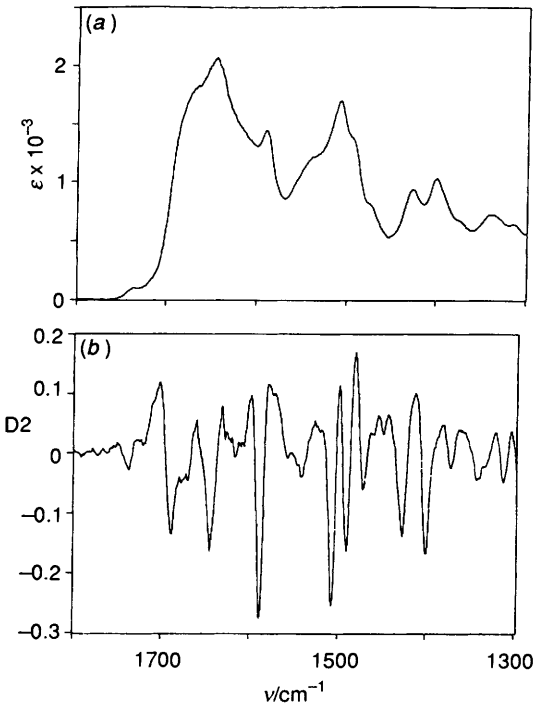

Fig. 3 (*a*) IR spectrum and (*b*) second derivative of vancomycin hydrochloride (9.0×10^{-3} mol dm^{-3}) in H_2O MES (5.0×10^{-2} mol dm^{-3}) buffer at pH 5.0, 25 °C

in the relative intensities of the 1676 and 1656 cm^{-1} amide I′ components, which may be related to a change in the environment of one or more amide groups as a result of the association process.

IR spectrum of vancomycin in H_2O solution at pH 5

The spectrum of vancomycin hydrochloride (9.0×10^{-3} mol dm^{-3}) in H_2O MES buffer, at pH 5.0, is shown in Fig. 3. The most notable differences between this spectrum and the spectrum of the sample recorded in the corresponding pD 4.9 buffer (Fig. 1) are the changes in the shape of the broad feature above 1600 cm^{-1}, the substantial increase in intensity at 1507 cm^{-1} and loss of the band at 1445 cm^{-1}. These differences are due to frequency shifts of some components, caused by deuterium exchange in D_2O solution, and can be used to assign the amide vibrations in the second derivatives of the spectra. On this basis the components at 1460 and 1445 cm^{-1} in D_2O, and at 1541 cm^{-1} in H_2O, are assigned as amide II′ and amide II, respectively. The components at 1676 and 1656 cm^{-1} in D_2O are also shifted, confirming their previous assignment as amide I′. However, these latter components in the second derivative do not appear to undergo the simple frequency shift expected for deuterium exchange; instead, a different pattern is observed, with components at 1688, *ca.* 1673 and 1644 cm^{-1} in the H_2O solution. This may be due to interference from the NH_3^+ deformation,[13] or may suggest that D_2O causes some conformational changes in vancomycin, presumably as a result of the deuterium exchange of the amide groups.

The effect of pD on the IR spectrum of vancomycin

To make further assignments, IR spectra were recorded of vancomycin hydrochloride (1×10^{-2} mol dm^{-3}) in D_2O buffers of various pD values. The buffers were chosen to give different ionisation states of vancomycin in solution, based on the assumption that the pK_as of vancomycin in D_2O solution are similar to the reported values in H_2O, which are about 2.9, 7.2, 8.6, 9.6, 10.5 and 11.7.[7] This assumption appears to be reasonable, since the pK_as of the hydroxylated rings in D_2O

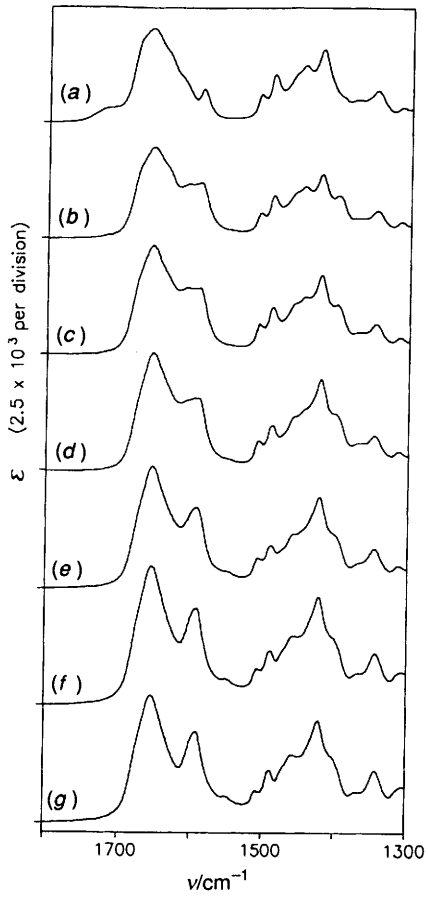

Fig. 4 IR spectra of vancomycin hydrochloride (1×10^{-2} mol dm^{-3}) in D_2O solution at pD values of (*a*) 1.5; (*b*) 4.9; (*c*) 7.9; (*d*) 9.1; (*e*) 10.0; (*f*) 11.1; (*g*) 12.0. The spectra were all recorded at 25 °C.

solution were found to be approximately 9.6 and 10.6,[10] which are in good agreement with the above values in H_2O (9.6 and 10.5).

The pK_as at 9.6 and 11.7 have been assigned as the acidic pK_as of the hydroxy groups on ring 7, while the pK_a of 10.5 has been ascribed to the hydroxy group on ring 5.[10] The lowest value, 2.9, can be assigned as the acidic pK_a of the terminal carboxy group, by comparison with acetylglycine (pK_a = 3.67).[14] Consequently, the values of 7.2 and 8.6 must correspond to the basic pK_as of the amino groups, which is reasonable by comparison with glycinamide (pK_a = 7.95) and adriamycin (pK_a of the daunosamine group = 8.2);[14] however, there is insufficient information to say which of these is due to the vancosamine and which is due to the *N*-methylleucine side chain.

The spectra of the solutions at different pD values are shown in Fig. 4, with resolution enhancement in Fig. 5 and as second derivatives in Fig. 6. By comparing the effects of pD on the spectra it has been possible to assign most of the vibrational components, as described below (these assignments are summarised in Table 1).

1676 cm^{-1}. As discussed above, this component has been assigned as an amide I′, based on its frequency alone. The intensity decreases as the pD is increased from 1.5 to 9.1 and then remains fairly constant. This variation correlates with changes in the ionisation of vancomycin and, by comparison with the changes associated with concentration, may be related to a decrease in the extent of self-association.

1656 cm^{-1}. This has been assigned as another amide I′. Unlike the 1676 cm^{-1} component, this amide I′ increases in intensity as the pD is increased, and this continues up to pD 11.1. The likely

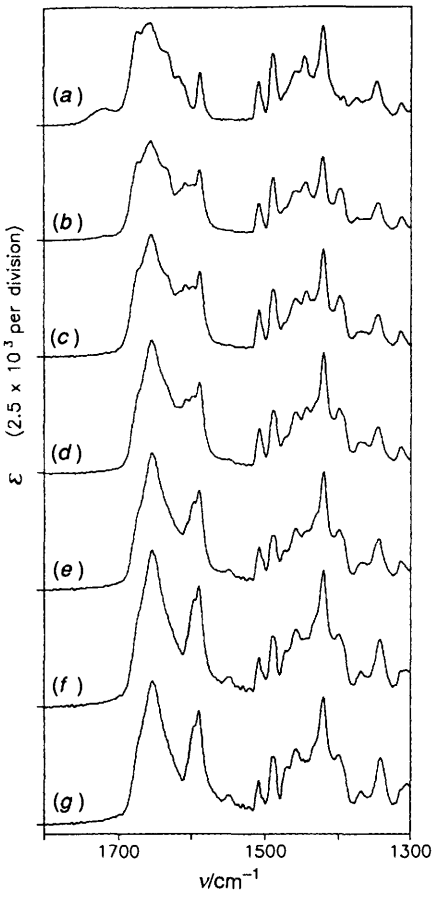

Fig. 5 Resolution enhanced IR spectra of vancomycin hydrochloride (1×10^{-2} mol dm^{-3}) in D_2O solution at pD values of (*a*) 1.5; (*b*) 4.9; (*c*) 7.9; (*d*) 9.1, (*e*) 10.0; (*f*) 11.1; (*g*) 12.0

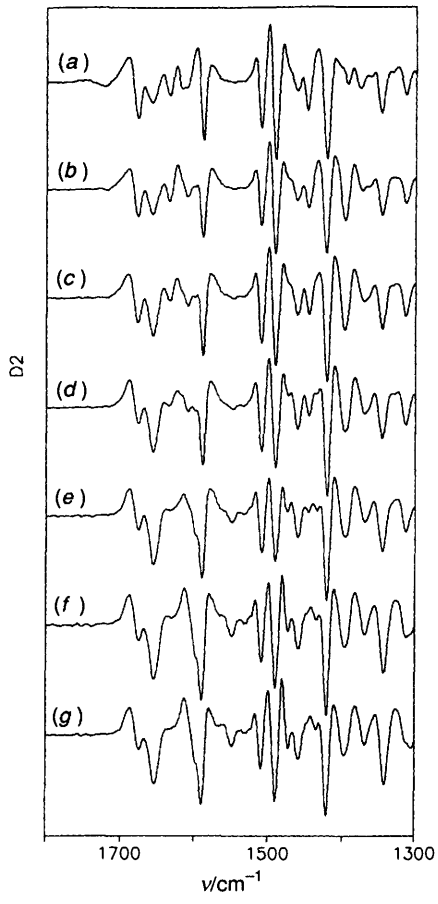

Fig. 6 Second derivatives of the IR spectra of vancomycin hydrochloride (1×10^{-2} mol dm^{-3}) in D_2O solution at pD values of (*a*) 1.5; (*b*) 4.9; (*c*) 7.9; (*d*) 9.1; (*e*) 10.0; (*f*) 11.1; (*g*) 12.0

causes of these changes are as discussed for the 1676 cm^{-1} component.

1633 cm^{-1}. This component starts to reduce in intensity at pD 7.9 and is completely absent at pD 10.0. However, it is unlikely to arise from an ionisable aromatic ring, since the lowest phenolate pK_a is 9.6.[10] Consequently, it has been assigned as another amide I′, possibly arising from the terminal *N*-methylleucine residue containing an NH_2 group which will have a pK_a of ca. 8.0.[15]

1618 cm^{-1}. This is seen only as an unresolved feature in the resolution enhanced spectrum at pD 1.5, and in the corresponding second derivative. Although this component is absent from the second derivatives at higher pD values, it appears from the resolution enhanced spectra that this is due to interference from the component at 1609 cm^{-1}. It is therefore likely that this is an aromatic ring mode.

1609 cm^{-1}. This component starts to decrease in intensity at pD 9.1 and is virtually absent at pD 10.0. Consequently it has been assigned as an aromatic ring mode originating from ring 7, which has a pK_a of approximately 9.6.[10]

1598 cm^{-1}. This component, which is most clearly seen in the resolution enhanced spectra, is absent at pD 1.5 but present in all of the other spectra. Therefore, it is assigned as the asymmetric stretch of the terminal carboxylate anion which has a pK_a of about 2.9.[7]

1588 cm^{-1}. There are virtually no changes in position or intensity of this component over the full pD range and consequently it is assigned as an aromatic ring mode from a non-ionisable ring, *i.e.*, ring 2, 4 or 6.

1508 cm^{-1} and 1489 cm^{-1}. The behaviour and assignment of these components are the same as for the 1588 cm^{-1} component.

Table 1 Assignments of bands in the IR spectra of vancomycin in D_2O solutions at different pD values

ν/cm^{-1}	Assignment
1676	amide I′
1656	amide I′
1633	amide I′
1618	aromatic ring mode (tentative)
1609	aromatic ring mode (ring 7)
1598	CO_2^- asymmetric stretch
1588	aromatic ring mode (ring 2, 4 or 6)
1508	aromatic ring mode (ring 2, 4 or 6)
1489	aromatic ring mode (ring 2, 4 or 6)
1472	amide II′ (tentative)
1460	amide II′
1445	amide II′
1421	aromatic ring mode (ring 2, 4 or 6) and/or CH_2/CH_3 deformation
1396	CO_2^- symmetric stretch

1472 cm^{-1}. Up to pD 7.9 this component appears in the resolution enhanced spectra only as a shoulder. However, it intensifies as the pD is increased and is eventually seen as a peak in both the resolution enhanced spectra and the second derivatives. The intensification occurs up to pD 12, rather than over a limited pD range, suggesting that it does not arise from an ionisable aromatic ring. Since the amide I′ at 1656 cm^{-1} also shows intensity changes over a wide pD range, this 1472 cm^{-1} component can be tentatively assigned as an amide II′.

1460 cm^{-1}. This component increases in intensity throughout the increase in pD, in a similar manner to the amide I′ at 1656 cm^{-1}; it is therefore assigned as an amide II′.

1445 cm^{-1}. The intensity of this component decreases with

increasing pD until it disappears at pD 11.1. This is again largely consistent with the changes observed in the 1676 and 1656 cm^{-1} amide I′ components. On this basis it is assigned as another amide II′.

1421 cm^{-1}. This is one of the most intense components in the spectra. In the resolution enhanced spectra its intensity increases with pD, except for an initial decrease between pD 1.5 and 4.9. However, the intensity in the second derivative remains relatively constant throughout, suggesting that the changes observed in the resolution enhanced spectra may arise primarily from surrounding components. Since there appears to be little effect of pD on this component it is assigned as a ring mode of a non-ionisable aromatic ring and/or a CH_2/CH_3 deformation.

1396 cm^{-1}. This component is absent at pD 1.5 but present in all of the other spectra. Consequently, it is assigned as the symmetric stretch of the terminal carboxylate anion. This is in agreement with published data on some amino acids and small peptides.[16]

Solubilities of the vancomycin–peptide complexes

The supernatant from a saturated solution of the vancomycin–*N*-Ac-D-Ala-D-Ala complex was diluted tenfold with buffer, giving an absorbance of 0.889 at 280 nm using a 1.0 cm pathlength cuvette. From this absorbance value, the dilution factor and the extinction coefficient of 6.0×10^3 dm^3 mol^{-1} cm^{-1} at 280 nm for the complex,[10] the solubility of the complex in D_2O MES buffer at pD 5.0 has been determined to be *ca.* 1.5×10^{-3} mol dm^{-3}. A similar determination was carried out for the vancomycin–*N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala complex. The supernatant was again diluted tenfold with buffer, giving an absorbance of 1.222 at 280 nm using a 1.0 cm pathlength cuvette. From this absorbance, the dilution factor and the extinction coefficient of 6.2×10^3 dm^3 mol^{-1} cm^{-1} at 280 nm,[10] the solubility of the complex can be calculated as *ca.* 2.0×10^{-3} mol dm^{-3}.

The solubility of vancomycin is substantially reduced by the binding of these peptides. This is consistent with the proposal that the peptides occupy sites in vancomycin that would otherwise be occupied by water molecules.[17] Owing to the low solubility of the vancomycin–peptide complexes, the IR spectra of the complexes were recorded at 5×10^{-4} mol dm^{-3} concentration.

IR spectra of peptide models

The spectrum of *N*-Ac-D-Ala-D-Ala (9.8×10^{-3} mol dm^{-3}) in D_2O MES buffer, at pD 5.1, [Fig. 7(*a*)] shows four main bands which can be assigned on the basis of published work on labelled glycylglycine.[18] These assignments are given in Table 2. From the second derivative of the spectrum [Fig. 7(*b*)], the amide I′ band at 1632 cm^{-1} has two components at 1644 cm^{-1} and 1629 cm^{-1}. The spectrum was also recorded in unbuffered D_2O [Fig. 8(*a*)] to effect assignment. The intensified band at 1715 cm^{-1}, which is the stretching vibration of the non-ionised carboxy group,[19] indicates the presence of substantially more of the non-ionized peptide in the D_2O solution. The second derivative of the spectrum [Fig. 8(*b*)] shows amide I′ components at 1650 and 1629 cm^{-1}. The latter is at the same frequency at pD 5.1, indicating that it is not affected by changing the ionisation of the carboxy group; however, the component at 1644 cm^{-1} at pD 5.1 is shifted to 1650 cm^{-1} in D_2O. The 1644 cm^{-1} component is therefore assigned as the amide I′ of the amide group adjacent to the carboxy, while the 1629 cm^{-1} component is the amide I′ of the terminal acetyl amide. This is in agreement with the reported assignments for *N*-Ac-Gly-Gly, which were established by labelling with ^{13}C at the acetyl carbonyl;[19] the component at 1664 cm^{-1} in the second derivative of unlabelled *N*-Ac-Gly-Gly is assigned as the amide I′ of the Gly at the carboxy terminus, while the component at 1636 cm^{-1} is assigned to the terminal acetyl amide.

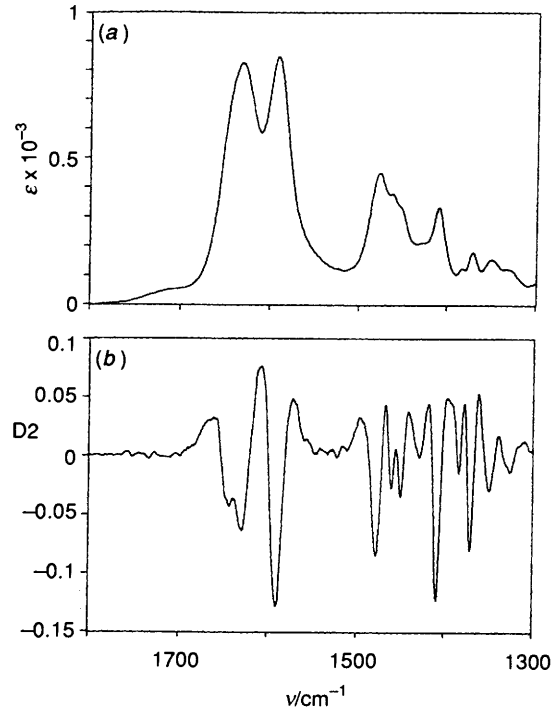

Fig. 7 (*a*) IR spectrum and (*b*) second derivative of *N*-Ac-D-Ala-D-Ala (9.8×10^{-3} mol dm^{-3}) in D_2O MES buffer at pD 5.1, 25 °C

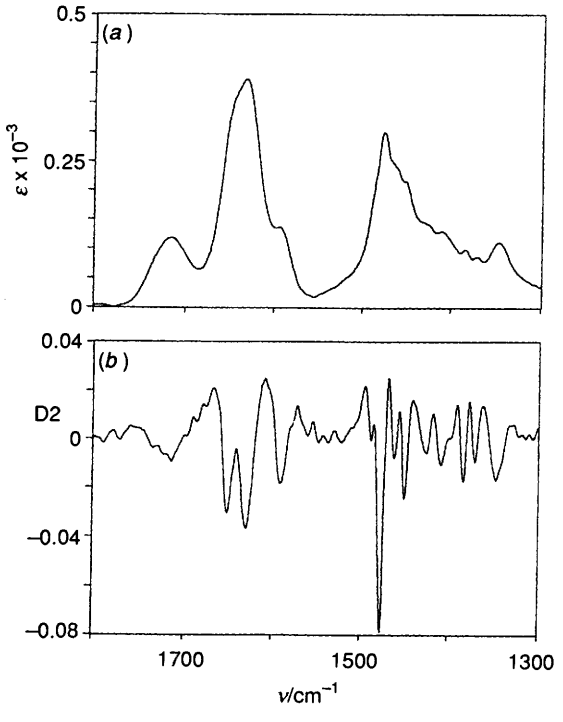

Fig. 8 (*a*) IR spectrum and (*b*) second derivative of *N*-Ac-D-Ala-D-Ala (1.0×10^{-2} mol dm^{-3}) in D_2O at 25 °C

Table 2 Assignments for the IR spectrum of *N*-Ac-D-Ala-D-Ala in D_2O buffer at pD 5.1 (Fig. 7)

ν/cm^{-1}	Assignment
1632	amide I′
1591	CO_2^- asymmetric stretch
1476	amide II′
1409	CO_2^- symmetric stretch

A more dilute solution of *N*-Ac-D-Ala-D-Ala (5.0×10^{-4} mol dm^{-3}) in D_2O MES buffer, at pD 5.0, was also analysed for comparison with the vancomycin–peptide complex described below. The resulting spectrum shows no significant difference from that of the more concentrated solution. Also for comparison with the vancomycin–peptide complex, the spectrum was recorded of ^{13}C labelled *N*-Ac-D-Ala-D-Ala in D_2O MES buffer at pD 4.9. The ^{13}C substitution is in the carboxylate group, as a result of which the stretching vibrations of the carboxylate group are shifted to lower frequency, allowing them to be readily assigned to the CO_2^- asymmetric stretch at 1550 cm^{-1} and the symmetric stretch at 1393 cm^{-1}.

The spectrum of *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala in D_2O MES buffer, at pD 4.9 (Fig. 9) shows five bands in the 1700–1300 cm^{-1} region, for which the assignments are given in Table 3. As expected the two additional amide groups in this peptide cause the extinction coefficients of the amide I′ and II′ bands to be approximately twice those in the spectrum of *N*-Ac-D-Ala-D-Ala [Fig. 7(*a*)]. However, owing to the low signal/noise ratio, resulting from the low concentration of the solution, it was not possible to generate a meaningful second derivative to separate the components of these bands.

IR spectra of vancomycin–peptide complexes

Fig. 10(*a*) shows the spectrum of a solution containing vancomycin (4.5×10^{-4} mol dm^{-3}) and *N*-Ac-D-Ala-D-Ala (5.0×10^{-4} mol dm^{-3}) in D_2O MES buffer at pD 4.9. Based on the reported association constant of 1.99×10^4 dm^3 mol^{-1},[6] approximately 70% of the vancomycin in the solution will be complexed with the peptide under these conditions. Comparison of this spectrum with a composite [Fig. 10(*b*)], produced by summing the spectra of the individual species, shows there to be some substantial differences as a result of the interactions in the complex. Most notably, there is a large increase in the intensity of a band at 1588 cm^{-1} and a number of changes in the bands above 1600 cm^{-1}.

From the assignments in Tables 1 and 2, the band at 1588 cm^{-1} in the complex is composed of the asymmetric CO_2^- stretching vibration of the peptide and an aromatic ring mode of vancomycin. To determine which of these underlying components causes the increase in intensity at 1588 cm^{-1}, a spectrum was recorded of an identical solution prepared using the ^{13}C labelled *N*-Ac-D-Ala-D-Ala [Fig. 11(*a*)]. In this case there is no significant intensification of the band at 1588 cm^{-1}, compared with the corresponding composite spectrum [Fig. 11(*b*)], but there is an increase in intensity of the carboxylate asymmetric stretching band, which is slightly shifted from 1550 cm^{-1} in the spectrum of the peptide alone to 1546 cm^{-1} in the complex. The intensification of the 1546 cm^{-1} carboxylate band is not as great as the increase at 1588 cm^{-1} in Fig. 10, which can be attributed to the fact that the ^{13}C labelled peptide was not stereochemically pure and therefore would not all bind to the vancomycin. The intensity increase at 1588 cm^{-1} in the spectrum of the complex with unlabelled peptide [Fig. 10(*a*)] can therefore be attributed to changes in the carboxylate group of the peptide when it binds to vancomycin.

The spectrum of the complex [Fig. 10(*a*)] also shows additional features at 1610 and 1622 cm^{-1} compared with the composite spectrum [Fig. 10(*b*)]. The 1610 cm^{-1} component is almost certainly an aromatic ring mode of vancomycin, assigned previously, which appears because of the intensification of the 1588 cm^{-1} component; in the spectrum of the complex formed with the ^{13}C labelled peptide [Fig. 11(*a*)], in which the 1588 cm^{-1} component is not intensified, this 1610 cm^{-1} feature is apparent only as a shoulder. The component at 1622 cm^{-1}, though, is a new feature resulting from the formation of the complex. Secure assignment of this component was not feasible, but from its position it is likely to be either an aromatic ring mode of vancomycin or an amide I′ component.

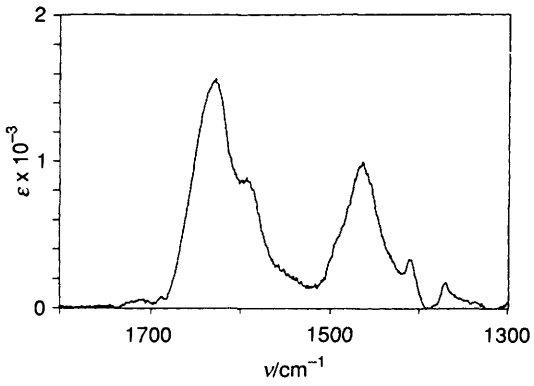

Fig. 9 IR spectrum of *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala (5.0×10^{-4} mol dm^{-3}) in D_2O MES buffer at pD 4.9, 25 °C

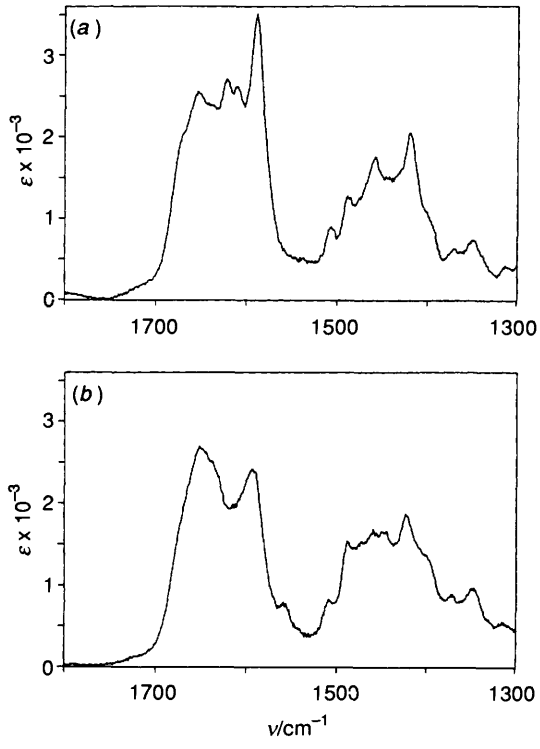

Fig. 10 (*a*) IR spectrum of a solution containing vancomycin hydrochloride (4.5×10^{-4} mol dm^{-3}) and *N*-Ac-D-Ala-D-Ala (5.0×10^{-4} mol dm^{-3}) in D_2O MES buffer at pD 4.9, 25 °C; (*b*) composite produced by summing of the individual spectra of vancomycin and *N*-Ac-D-Ala-D-Ala normalised to the composition in (*a*)

Table 3 Assignments for the IR spectrum of *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala in D_2O buffer at pD 4.9 (Fig. 9)

ν/cm^{-1}	Assignment
1629	amide I′
ca. 1593	CO_2^- asymmetric stretch
1464	amide II′
1410	CO_2^- symmetric stretch
1370	CH_3 deformation

Fig. 12(*a*) shows the spectrum resulting from the corresponding solution of vancomycin (4.5×10^{-4} mol dm^{-3}) containing *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala (5.0×10^{-4} mol dm^{-3}). In this case the reported association constant is 1.5×10^6 dm^3 mol^{-1},[6] as a result of which approximately 96%

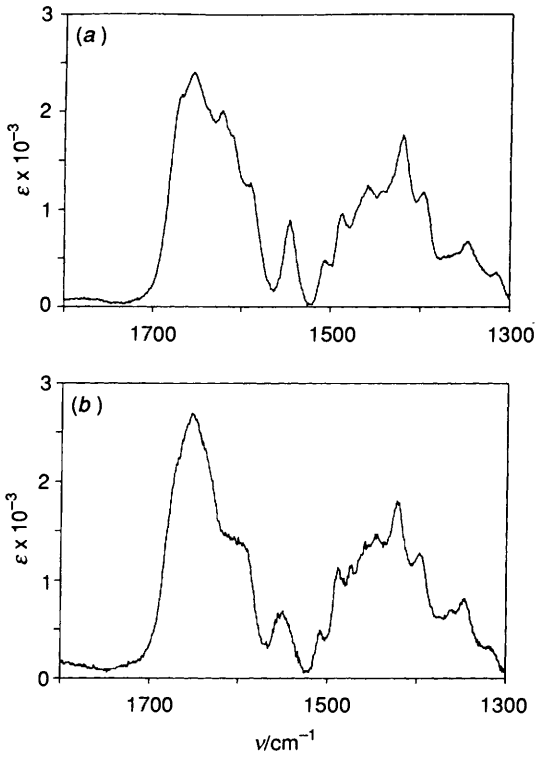

Fig. 11 (*a*) IR spectrum of a solution containing vancomycin hydrochloride (4.5×10^{-4} mol dm^{-3}) and ^{13}C labelled *N*-Ac-D-Ala-D-Ala (5.0×10^{-4} mol dm^{-3}) in D_2O MES buffer at pD 4.9, 25 °C; (*b*) composite produced by summing of the individual spectra of vancomycin and ^{13}C labelled *N*-Ac-D-Ala-D-Ala normalised to the composition in (*a*)

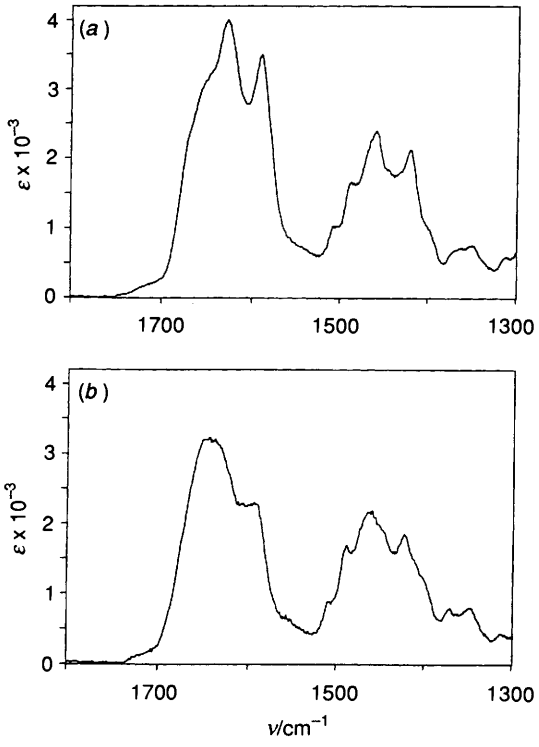

Fig. 12 (*a*) IR spectrum of a solution containing vancomycin hydrochloride (4.5×10^{-4} mol dm^{-3}) and *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala (5.0×10^{-4} mol dm^{-3}) in D_2O MES buffer at pD 4.9, 25 °C; (*b*) composite produced by summing of the individual spectra of vancomycin and *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala normalised to the composition in (*a*)

of the vancomycin will be complexed with the peptide under the conditions used. The spectrum again shows substantial differences compared with a composite [Fig. 12(*b*)] produced by summing the spectra of the individual species. Like the *N*-Ac-D-Ala-D-Ala–vancomycin complex there is a large increase in intensity of a component at 1590 cm^{-1}. Based on the discussion above, this can be attributed to interactions of the peptide carboxylate group in the complex. In this case, though, there is also an increase in intensity of a component at 1628 cm^{-1}, which corresponds to the frequency of the amide I′ band of the peptide. The intensification may therefore result from an interaction involving one of the terminal acetyl groups of the peptide. Alternatively, it may be related to the new component which appears at 1622 cm^{-1} in the complex with *N*-Ac-D-Ala-D-Ala. Further studies with isotopically labelled peptides are required to resolve this.

Conclusions

Resolution enhancement and second derivatives reveal details of overlapping components in the 1700–1400 cm^{-1} region of the IR spectra of vancomycin in D_2O solutions. By altering the pD of the solutions, and thereby changing the ionisation state of the vancomycin, it has been possible to assign most of the vibrational components of the IR spectra. These assignments, summarised in Table 1, have subsequently been used in interpreting the changes which occur in the IR spectrum of vancomycin when it binds the peptide models, *N*-Ac-D-Ala-D-Ala and *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala.

Substantial intensity changes occur in the IR spectrum when *N*-Ac-D-Ala-D-Ala binds to vancomycin in aqueous solution. The largest of these changes, at 1588 cm^{-1}, has been attributed to an intensification of the carboxylate asymmetric stretching band of the peptide, clearly indicating that this group is involved in interactions with vancomycin in the complex. On using ^{13}C labelled peptide a small shift of the corresponding $^{13}CO_2^-$ band to lower frequency was also observed, suggesting that the carboxylate group is tightly hydrogen bonded in the complex. This is consistent with NMR studies, which show the formation of a carboxylate binding pocket in the bound state of the antibiotic; within this pocket, the carboxylate group can form hydrogen bonds with three amide NH protons of vancomycin.[20]

Additionally, a new component is observed a 1622 cm^{-1} in the spectrum of the *N*-Ac-D-Ala-D-Ala–vancomycin complex. This is either an aromatic ring mode of vancomycin, or an amide I′ component, which is affected by the binding process. However, this component cannot be definitively assigned from the available information as it does not coincide with any of the components in the spectra of the individual species.

The IR spectrum of the *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala–vancomycin complex shows an intensification at 1590 cm^{-1}, which is again attributed to the interactions of the peptide carboxylate group in the complex. In addition, there is a significant intensification of a component at 1628 cm^{-1}, which may be due to an interaction involving one of the terminal acetyl groups of the peptide.

In conclusion, this paper illustrates that IR spectroscopy offers a good means of studying vancomycin–peptide interactions in aqueous media at values of pH and concentration where vancomycin exhibits little self-association. Unique information is provided on the role of terminal carboxylate groups in the binding process.

Acknowledgements

We thank Dr D. H. Williams, of the Cambridge Centre for Molecular Recognition, for useful discussions and advice, and

Dr G. Okafo, of SB Pharmaceuticals, for determining the stereochemical purity of the ^{13}C labelled *N*-Ac-D-Ala-D-Ala.

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Paper 5/04361A
Received 5*th July* 1995
Accepted 21*st July* 1995