Function of the Amino Sugar and N-Terminal Amino Acid of the Antibiotic Vancomycin in Its Complexation with Cell Wall Peptides

Rajamoorthi Kannan,[†] **Constance M. Harris,**[†] **Thomas M. Harris,***[†] **Jonathan P. Waltho,**[‡] **Nicholas J. Skelton,**[‡] **and Dudley H. Williams***[‡]

Contribution from the Chemistry Department, Vanderbilt University, Nashville, Tennessee 37235, and University Chemical Laboratory, Lensfield Road, Cambridge, CB2 1EW, U.K. *Received June 18, 1987*

Abstract: Four N-acetylated derivatives of vancomycin (1) were prepared: the derivative at the N-terminus (**2**, NAcV), the *N*-acetyl derivative on the vancosamine residue (**3**, N'AcV), the N,N'-bis derivative (**4**, DiAcV), and the N-terminal derivative of the aglycon (**6**, NAcAGV). In addition, hydrolysis products involving (1) loss of vancosamine, desvancosaminylvancomycin (**7**, DVV), and (2) the total aglycon, aglycovancomycin (**5**, AGV), were prepared. Binding studies with Ac_2-L-Lys-D-Ala-D-Ala, which is an analogue of the vancomycin binding site in bacterial peptidoglycan, revealed that both hydrolysis products and all of the acetyl derivatives complexed with peptide, although none as effectively as the parent antibiotic. It is concluded that the vancosamine residue, as well as the N-terminal amino acid of the antibiotic *N*-methyl-D-leucine, plays an important role in the binding process. High-field NMR studies suggest that their roles are similar, in helping to define a hydrophobic pocket while simultaneously placing the amino group with maximum exposure to solvent.

Vancomycin, a glycopeptide antibiotic produced by the actinomycete *Nocardia orientalis*,[1] has received considerable attention in recent years both for its clinical applications in the treatment of methicillin-resistant *Staphylococcus aureus* infections[2] and because its complexes with small peptides provide an excellent system for studying substrate–receptor interactions. Vancomycin exerts its antibacterial action by binding preferentially to peptide intermediates involved in biosynthesis of bacterial cell wall peptidoglycan.[3] In elegant studies carried out before the structure of any member of this family of glycopeptide antibiotics was known, Nieto and Perkins prepared an extensive series of peptides and studied their binding to vancomycin and ristocetin.[4] Subsequently, there have been several high-field NMR investigations of complexes involving various vancomycin group antibiotics with the peptides Ac-D-Ala-D-Ala (dipeptide) and Ac_2-L-Lys-D-Ala-D-Ala (tripeptide).[5-7] These studies have led to the proposal and refinement of a model in which the complex (Figure 1) is stabilized by a series of intermolecular hydrogen bonds between the amides in the antibiotic and the amides and carboxylate anion of the peptide. In addition, electrostatic interaction between the COO^- of the peptide and the amino terminus of the antibiotic has been postulated, although in models of the complex the distance between the anion and cation is too great for strong interactions.[8] Data on the binding of peptides to vancomycin as a function of pH have been obtained employing classical methods[4a] and ^{1}H NMR;[6c] the results show (1) binding decreases steeply below pH 3, indicating the need for an ionized carboxyl group in the peptide, and (2) binding decreases above pH 8, suggesting involvement of one or more amino groups (or possibly phenolic groups) in stabilization of the complex. Perkins[9] reported that acetylated vancomycin (of unknown structure) still bound cell wall precursors. However, recently di-N-acetylated vancomycin was shown to have no detectable binding to dipeptide by UV difference spectroscopy.[8] It was concluded that the initial interaction between the peptide carboxylate and a protonated (either the N-terminus or the vancosamine) amine on the vancomycin is advantageous for efficient formation of the complex. There was supporting evidence for the importance of the N-terminal amino group from studies on N-acetylated derivatives of ristocetin A and teicoplanin,[10] which are structurally similar to vancomycin. In addition, recent studies[11] on N-terminally modified aglycoristocetins revealed that acetylation of the N-terminus reduced the binding constant to Ac_2-L-Lys-D-Ala-D-Ala by a factor of approximately 4. We have reinvestigated the acetylation of vancomycin and herein report the preparation and characterization of the isomeric mono-*N*-acetyl derivatives as well as the diacetyl derivative and the *N*-acetyl derivative of aglycovancomycin. Complexes of these derivatives have been studied by UV spectroscopy and NMR, and a possible role for the vancosamine in stabilizing the peptide complex has been discovered.

[†] Vanderbilt University.
[‡] University Chemical Laboratory.

Experimental Section

General Procedures. Vancomycin was obtained as a gift from Eli Lilly. The peptides used in this study were Ac_2-L-Lys-D-Ala-D-Ala and Ac-D-Ala-D-Ala, which were synthesized as described by Nieto and Perkins;[4b] the former was also purchased from Serva, Inc. Reversed-phase HPLC was carried out using a Spectra-Physics SP 8700, an IBM

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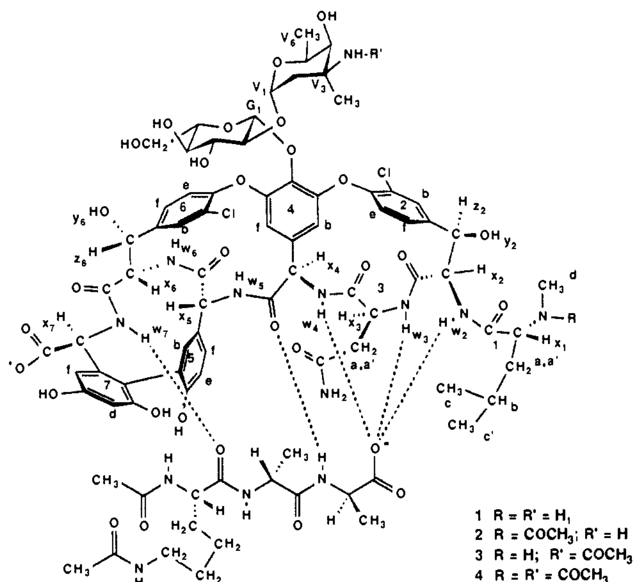

Figure 1. Vancomycin and its acetylated derivatives showing the proton nomenclature. Proposed model for binding of peptidoglycan analogues to vancomycin. Dashed lines indicate hydrogen bonds.

9533, or a Varian 5000 liquid chromatograph; C-18 columns were employed with aqueous ammonium formate–acetonitrile mixtures or 0.1% aqueous TFA–acetonitrile mixtures as eluants. Low-pressure reversed-phase chromatography was carried out on a 15 × 500 mm column (Altex) packed with Whatman Partisil 40 ODS-3. UV spectra were obtained on a Varian 210 spectrophotometer. ^{1}H NMR spectra were obtained on Bruker AM-500, AM-400, and WH-400 spectrometers. FAB spectra were obtained with use of thioglycerol–glycerol, glycerol–Me_2SO, or Magic Bullet matrices on VG 70-250 and Kratos MS50 mass spectrometers. The calculated value represents species containing ^{12}C and ^{35}Cl.

***N*-Acetylvancomycin (2; Figure 1).** Vancomycin was acetylated at the N-terminus by the method of Perkins,[9] and we believe the product to be identical with the unidentified *N*-acetyl derivative he obtained. To vancomycin hydrochloride (150 mg) in H_2O (1.4 mL) at 0 °C, was added saturated $NaHCO_3$ solution (0.6 mL), followed by Ac_2O (0.6 mL, slow addition). The solution was stored at 0 °C for 1 h followed by room temperature for 0.5 h and then evaporated in vacuo. The residue was brought to pH 10 with aqueous NH_3 and incubated at 37 °C for 1 h to remove any *O*-acetyl groups. The product was purified by low-pressure reversed-phase chromatography employing elution with 20% acetonitrile–0.1% TFA. *N*-Acetylvancomycin was also prepared by acetylation of vancomycin hydrochloride in 4:1 MeOH–Ac_2O for 3 h at room temperature followed by evaporation to dryness. The product was purified as described above. In the ^{1}H NMR spectrum the acetyl signal appeared at 2.1 ppm, the *N*-Me resonance was shifted downfield to 2.86 ppm, and the α-proton (x_1) resonance was shifted downfield to 5.22 ppm. FABMS: 1490 (calcd for (MH$^+$) $C_{68}H_{78}Cl_2N_9O_{25}$, 1490.4).

***N*-(Carbobenzyloxy)vancomycin.** Vancomycin hydrochloride (200 mg) was dissolved in MeOH (4 mL) and treated with benzyl chloroformate (0.1 mL) for 30 min at room temperature; the pH was maintained above 7 by addition of dilute NaOH. The reaction was stopped by addition of a few drops of aqueous NH_3, and the mixture was evaporated to dryness in vacuo. Purification by low-pressure reversed-phase chromatography as described above on a column packed in 25% acetonitrile–0.1% TFA and developed by gradient elution with 25% acetonitrile–0.1% TFA (350 mL) in the first chamber and 30% acetonitrile–0.1% TFA (350 mL) in the second gave 63 mg of the carbobenzyloxy derivative. Fractions were assayed by HPLC. ^{1}H NMR of the derivative showed new signals due to the benzylic phenyl and methylene groups at 7.34 and 5.22 ppm, respectively, and the *N*-Me group and α-proton (x_1) of the D-leucine residue at 2.8 and 4.8 ppm, respectively.

***N'*-Acetyl-*N*-Cbz-vancomycin.** *N*-Cbz-vancomycin (63 mg) was acetylated by treatment with MeOH–Ac_2O (4:1, 4 h, room temperature) followed by evaporation to dryness. The product (19 mg) was isolated as described for Cbz-vancomycin. The NMR spectrum of *N'*-acetyl-*N*-Cbz-vancomycin was similar to that of Cbz-vancomycin except it showed additional peaks at 1.7 (CH_3CO) and 7.1 ppm (NH of vancosamine).

***N'*-Acetylvancomycin (3; Figure 1).** A mixture of *N'*-acetyl-*N*-Cbz-vancomycin (19 mg) and Pd/C (10%, 20 mg) in MeOH (4 mL) was treated with H_2 (3 atm) in a Parr apparatus for 1.5 h. The reaction was followed by HPLC. The reaction mixture was filtered through Celite and evaporated to dryness. The product was purified by low-pressure reversed-phase chromatography as described earlier by gradient elution with 5% acetonitrile–0.1 M ammonium formate and 10% acetonitrile–0.1 M ammonium formate. After lyophilization, the sample was loaded once again on the reversed-phase column, which was washed with water to remove ammonium formate and eluted with 30% acetonitrile to give 13 mg of purified vancosamine-acetylated vancomycin (3, N'AcV). In the ^{1}H NMR the new acetyl group appeared at 1.7 ppm; there was also an additional signal at 7.1 ppm, assignable to the new amide proton, which showed an NOE to the signal at 1.38 ppm (V_3Me) and to the acetyl group. The resonances of the *N*-Me group and x_1 appeared unchanged. FAB mass spectrometry confirmed the introduction of only one acetyl group. FABMS: 1513 (calcd for (MNa$^+$) $C_{68}H_{77}Cl_2N_9O_{25}Na$, 1512.4).

***N,N'*-Diacetylvancomycin (4; Figure 1).**[12] Vancomycin hydrochloride (200 mg) in aqueous 0.5 N acetic acid was placed on a Dowex AG1-X8 ion-exchange column (200–400 mesh, acetate form, 5 g) and eluted with aqueous 0.5 N acetic acid. UV-absorbing material was dried in vacuo over P_2O_5 and NaOH, forming vancomycin free base. The free base (180 mg), suspended in MeOH (4 mL), was treated with Ac_2O (1 mL) for 4 h at room temperature. The reaction was followed by HPLC (20% acetonitrile–0.1 M ammonium formate). The mixture was evaporated to dryness, and the product was purified by low-pressure reversed-phase chromatography with 15% acetonitrile–0.1 M ammonium formate as eluant to give 28 mg of **4**. The ^{1}H NMR spectrum showed the chemical shift changes seen in the two monoacetyl compounds; i.e., there were signals for two acetyl groups (2.1 and 1.7), the *N*-Me and x_1 protons were at 2.86 and 5.20 ppm, respectively, and there was a new amide proton at 7.0 ppm. FABMS: 1532 (calcd for (MH$^+$) $C_{70}H_{80}Cl_2N_9O_{26}$, 1532.4).

Aglycovancomycin (5; Figure 2). Aglycovancomycin was prepared by mild acid treatment of vancomycin.[13] Vancomycin hydrochloride (1.0 g) was dissolved in 25 mL of 1 N HCl and heated in a boiling water bath for 2 min. The mixture was cooled and filtered. Crude **5** was purified by low-pressure reversed-phase chromatography (20% acetonitrile–0.1% TFA). The loss of sugar units was confirmed by FABMS and NMR. FABMS: 1143 (calcd for (MH$^+$) $C_{53}H_{53}Cl_2N_8O_{17}$, 1143.2).

***N*-Acetylaglycovancomycin (6; Figure 2).** Aglycovancomycin (48 mg) and *N*-ethylmorpholine (50 μL) were stirred in MeOH–Ac_2O (4:1) for 3 h at room temperature. The reaction mixture was evaporated to dryness and purified by low-pressure reversed-phase chromatography (25% acetonitrile–0.1% TFA) to give 11 mg of purified material. The ^{1}H NMR signals for the *N*-Me group and x_1 were shifted downfield to 2.8 and 5.22 ppm, respectively. FABMS: 1185 (calcd for (MH$^+$) $C_{55}H_{55}Cl_2N_8O_{18}$, 1185.2).

Desvancosaminylvancomycin (7; Figure 2). A mixture of vancomycin hydrochloride (100 mg), CH_2Cl_2 (5 mL), and TFA (5 mL) was stirred for 1 h at room temperature. The solvents were removed under reduced pressure, and the crude product was lyophilized. Purification was carried out by reversed-phase HPLC using water–acetonitrile gradients with TFA comodifier. The reaction mixture yielded 41 mg of **7** in addition to unreacted vancomycin and aglycovancomycin. FABMS: 1305 (calcd for (MH$^+$) $C_{59}H_{63}Cl_2N_8O_{22}$, 1305.3).

UV Difference Spectroscopy. Binding constants of Ac_2-L-Lys-D-Ala-D-Ala with vancomycin and its derivatives were measured essentially as

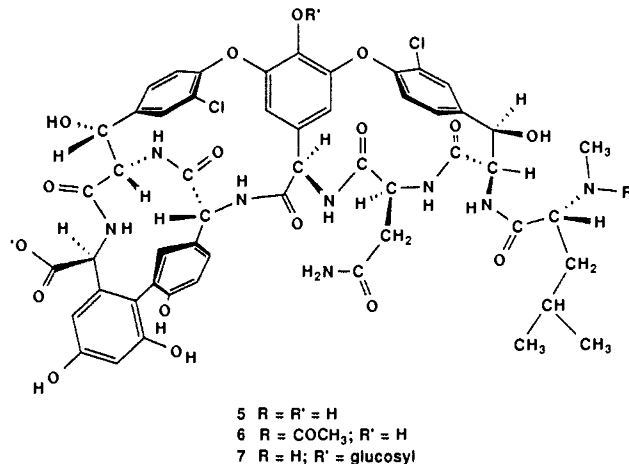

Figure 2. Desvancosaminylvancomycin derivatives.

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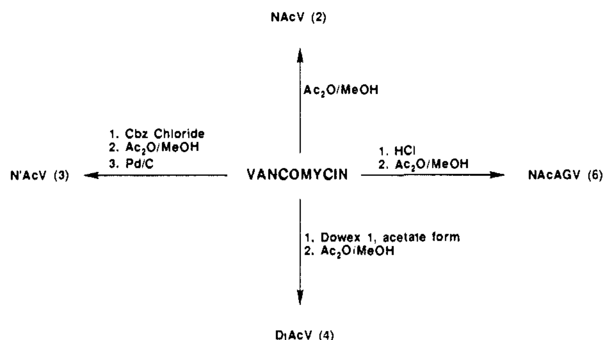
Scheme I



described by Nieto and Perkins,[4a,b] except that the tandem arrangement of cells was not required since the tripeptide had no significant absorption in the 230–320-nm range. Cells with a 1-cm path length were used. Solutions (2 mL) containing antibiotic (0.05–0.2 mg/mL in 0.02 M sodium citrate buffer, pH 5.1) were placed in the sample and reference cells, and the difference in absorbance that developed upon addition of peptide (5–100 μL of 0.0025 M solution) to the sample cell and buffer to the reference cell was measured with an accuracy of ±0.0003 absorbance unit. The temperature was 25 ± 2 °C. Association constants were determined by means of a nonlinear least-squares program based on the assumption of a simple V + P ⇄ VP equilibrium. Binding constants of 10^4 or less were obtained with standard deviations of ±5–10%; the standard deviations were greater (±10–20%) for peptide–antibiotic complexes with association constants of 10^5 or higher on account of the steepness of the titration curve.[4b] Antibiotic concentrations were determined by UV with a value of $\epsilon^{1\%}$ = 45.0 at 280 nm for vancomycin and its derivatives.

NMR Studies. NMR experiments on vancomycin and its derivatives were performed on 10 mg/mL solutions in Me_2SO-d_6. For study of peptide complexes a solution of antibiotic and peptide (~20 mM in antibiotic and 20–40 mM in peptide) in Me_2SO-d_6 containing ~30% CCl_4 was used. The complex was in fast exchange with its free components at ambient temperature, but at 2–5 °C dissociation of the complex is slow on the NMR time scale. One-dimensional spectra were obtained employing spectral widths of 4000–5000 Hz and 16K of data points. NOE difference spectra were obtained by subtraction of two fids: one accumulated with the decoupler set on a peak of interest and the second with the decoupler set outside the spectral range. The decoupler was gated off during data acquisition, and the minimum feasible irradiation times and decoupler power were employed to minimize spin diffusion and the spread of the decoupler frequency. Sufficient transients were accumulated to assure a good signal-to-noise ratio after subtraction and transformation. Exponential line broadenings of approximately 2 Hz were used. High-resolution, double-quantum-filtered COSY experiments (DQFCOSY) were run in the phase-sensitive mode. Typically 2K data points were recorded in F2 with 512 in F1, over a spectral width of 4000 Hz. Normal low-resolution COSY spectra were recorded with a 45° read pulse and without phase discrimination. NOESY spectra were recorded in both phase-sensitive and magnitude modes with mixing times of 0.1–0.5 s. A 20–30-ms Z-filter was employed. In all cases, the data matrices were zero-filled in F1 prior to transformation. Phase-sensitive data were subjected to Lorentzian–Gaussian manipulation and other data to sine bell manipulation. CAMELSPIN[14] spectra were recorded in the phase-sensitive mode. A 2-kHz spin-lock field was applied for 200 ms.

Antibiotic Assays. Antibiotic assays were carried out by disc diffusion on agar with *Bacillus subtilis* (Difco) as the test organism.[15,16] Assays were run in triplicate with vancomycin standard on each plate. A graph of the diameter of inhibition zone versus ln *c* was plotted for each case, and the concentration required for an inhibition zone of 12-mm diameter was calculated for comparison purposes.

Results

N-Acetyl derivatives of vancomycin were prepared selectively by taking advantage of the fact that the vancosamine amino group is more basic than the *N*-methyl group of the terminal *N*-methyl-D-leucine (Scheme I). Thus, under conditions of monoprotonation the vancosamine amino group is protected by cation formation, so that acetylation occurs predominantly at the N-terminus to give **2**. Indeed, N,N'-diacetylation could not be achieved with the monohydrochloride. To obtain the isomeric *N'*-acetyl derivative **3**, the N-terminus was protected as the carbobenzyloxy group by treatment of the monohydrochloride salt with carbobenzyloxy chloride prior to acetylation of the free base. The Cbz group was then removed by hydrogenolysis. Preparation of the *N,N'*-diacetyl derivative **4** required use of the free base of the antibiotic. It is possible that partial acetylation of the free base would have formed the *N'*-acetyl derivative **3** selectively, but we did not explore this approach. The *N*-acetyl derivative **6** of the aglycon of vancomycin was prepared by acetylation of the aglycon.

Table I. ^{1}H NMR Chemical Shifts of Selected Protons in Vancomycin and Its Derivatives[a]

proton	vancomycin (1)	NAcV (2)	N'AcV (3)	DiAcV (4)	AGV (5)	NAcAGV (6)
w_7	8.46	8.30	8.50	8.50	8.58	8.52
w_4	8.22	8.00	8.16	7.99	8.00	7.94
w_2	7.80	6.84	7.74	6.82[b]	8.67	6.80[b]
2b	7.34	7.25	7.32	7.24	7.51	7.26
6e	7.32	7.34	7.31	7.33	7.24	7.16
2e	7.24	7.04	7.22	7.02	7.27	7.26
w_3	6.78	6.74	6.88	7.08[b]	6.72[b]	7.02[b]
4b	5.50	5.50	5.52	5.52	5.61	5.51
V_1	5.25	5.21	5.14	5.16		
x_2	4.90	4.78	4.88	4.78	4.90	4.75
x_3	4.42	4.50	4.38	4.47	4.31	4.50
x_7	4.36	4.35	4.31	4.40	4.42	4.45
x_1	3.14	5.22	3.05	5.20	3.01	5.22
N-Me	2.32	2.86	2.30	2.86	2.58	2.85
3a'	2.32	2.20	2.30	2.20	2.14	2.10
V_2	1.90	1.92	2.02	2.00		
1b	1.70	1.44	1.70	1.42	1.64	1.55
1a	1.50	1.60	1.50	1.60	1.64	1.55
1a'	1.41	1.48	1.40	1.48	1.54	1.42
V_3Me	1.26	1.26	1.38	1.40		

[a] Spectra were obtained in Me_2SO-d_6 at 298 K; only signals changing by more than 0.1 ppm are listed. [b] Assignments were made at 320 K.

The structures of the acetyl derivatives were established by ^{1}H NMR. Comparison of the spectra with those of the unacetylated antibiotic and aglycon revealed the sites at which modifications had occurred. Assignments of the acylated derivatives were made by comparison with the spectrum of vancomycin and by specific decoupling and NOE difference experiments and from COSY spectra. Assignments for protons exhibiting significant chemical shift changes (>0.1 ppm) are shown in Table I. The designation of protons is analogous to the system used previously for teicoplanin[17] and is shown in Figure 1.

Peptide Binding Studies by UV. Affinity constants for the binding of derivatives of vancomycin with Ac_2-L-Lys-D-Ala-D-Ala were measured essentially as described by Nieto and Perkins[4b] and are tabulated in Table II. As noted by Nieto and Perkins, in this system experimental difficulties exist in the measurement of accurate binding constants when K_A values are greater than 10^5. These errors arise because of the lack of variation in the curvature of binding curves as a function of the binding constant when the binding process is very effective. Whereas this problem can be circumvented with other antibiotics, such as ristocetin, by carrying out titration at lower concentrations of peptides and antibiotic, this option is limited in the present case by the magnitude of the absorbance change, which is much smaller for vancomycin than that for ristocetin and other antibiotics in this group. In spite of this difficulty, significant trends can be observed in the data.

N-Acetylvancomycin (**2**) and tripeptide form a complex with a binding constant only 5% of that of vancomycin, but which is readily observed (a decrease in binding energy of 1.8 kcal mol^{-1}).

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Table II. Association Constants[a] and Antibiotic Activity[b] for Vancomycin and Its Derivatives

	association const		antibiotic act.	
antibiotic	K_A ($-\Delta G$, kcal/mol)	rel affinity, %	concn, µg/mL	rel act., %
vancomycin (**1**)	1.5×10^6 (8.4)	100	24	100
N-acetylvancomycin (**2**)	7.6×10^4 (6.6)	5	416	6
N'-acetylvancomycin (**3**)	4.4×10^5 (7.7)	29	204	12
N,N'-diacetylvancomycin (**4**)	2.9×10^4 (6.0)	2	1350	2
aglycovancomycin (**5**)	4.6×10^5 (7.7)	31	220	11
N-acetylaglycovancomycin (**6**)	5.0×10^3 (5.0)	<1	5600	<1
desvancosaminylvancomycin (**7**)	4.9×10^5 (7.7)	32	120	20

[a] Association constants with Ac_2-L-Lys-D-Ala-D-Ala were determined by UV difference spectroscopy. [b] Antibiotic activity was assayed by filter paper disc diffusion on agar with *B. subtilis* as the test organism. Results are reported in terms of the concentration required for a 12-mm inhibition zone.

This observation agrees with Perkins' observation, made by TLC, that compound **2** complexes with cell wall precursors.[9] Therefore, it appears that a free N-terminus on the antibiotic is not required for complex formation, although acetylation of it significantly destabilizes the complex. The reduction in the binding energy of *N*-acetylaglycovancomycin (**6**) compared with aglycovancomycin (**5**) (2.7 kcal mol^{-1}) and of *N,N'*-diacetylvancomycin (**4**) compared with *N'*-acetylvancomycin (1.7 kcal mol^{-1}) is in line with this. Removal of the vancosamine moiety from residue 4 also caused a substantial, but smaller, decrease in the peptide binding constant; **7** had less than one-third the binding affinity of the parent antibiotic. Clearly, vancosamine is playing an important role in the binding process.

The binding constant of the *N'*-acetyl derivative **3** of vancomycin was measured in order to probe in greater depth the influence of the vancosamine and was found to be almost identical with that of **7**; i.e. N'-acetylation and removal of vancosamine cause the same reduction in the binding constant. Therefore, the role of the vancosamine in binding is negated, in an as yet undefined manner, by N'-acetylation. The binding constant for the total aglycon **5** was also found to be of approximately the same magnitude. The glucose unit therefore has no independent contribution to binding, and it is likely that its role with respect to the binding constant is merely to position the vancosamine optimally relative to the aglycon portion. It should be noted that although the K_A for *N,N'*-diacetylvancomycin–tripeptide is small in comparison to that of vancomycin and the monoacetylated derivatives, it is still approximately the same as that of vancomycin–dipeptide. Previously, it had been established that N,N'-diacetylation of vancomycin reduced the binding constant of dipeptide beyond the range observable by UV difference methods, but some binding was detected by ^1H NMR in Me_2SO-d_6 solution.[8] The use of tripeptide brings the K_A value to within the region observable by UV difference and allows a more accurate comparison of the effects of acetylation.

Antibiotic Activity. Decreases in antibiotic activity parallel decreases in binding constant for the acetyl derivatives (Table II). The antibiotic activities were measured by disc diffusion on agar with *B. subtilis* as the test orgranism.[15,16] The concentration of the derivatives needed to produce an inhibition zone of 12 mm was compared with the vancomycin concentration needed to produce the same inhibition zone.

Peptide Binding Studies by NMR. The peptide binding studies carried out by UV spectroscopy implicate vancosamine in the binding process. Examination of space-filling models suggested that the disaccharide could have sufficient mobility so that the amino group of the vancosamine would be able to lie near the binding cleft in the antibiotic and provide electrostatic stabilization for the peptide carboxylate anion. To investigate this possibility, NOE studies were carried out on vancomycin–peptide complexes. The studies were initially attempted in D_2O solution, since the UV studies of peptide binding had been carried in aqueous buffers. Vancomycin alone gave relatively high-quality spectra in D_2O such that all nonexchangeable protons could be assigned by COSY and NOESY spectra and by comparison with previous results.[6c] However, the same procedure could not be extended to the spectrum of the antibiotic in the presence of di- or tripeptide.

Table III. ^1H NMR Chemical Shifts of Selected Protons of Vancomycin in Its Peptide Complexes[a]

antibiotic	1	1 + dipeptide[b]	1 + tripeptide[b]
w_5	8.64	8.76	8.90
w_7	8.46	8.74	9.15
w_4	8.22	9.00	9.00
w_2	7.80	11.70	11.80
6b	7.89	7.26	7.16
2f	7.50	7.62	7.60
2b	7.46	7.17	7.16
2e	7.20	6.90	6.88
5b	7.20	7.77	7.82
w_3	6.78	8.30	8.34
x_4	5.74	5.88	5.90
x_2	4.86	5.18	5.17
4b	5.52	5.40	5.40
x_5	4.38	4.64	4.64
x_3	4.38	4.70	4.70
x_7	4.38	4.40	4.50
x_1	3.20	4.14	4.16
x_6	4.20	4.50	4.40
N-Me	2.40	2.64	2.60
3a, 3a'	2.38, 2.12	2.16	2.10
1b	1.70	1.65	1.38
1a, a'	1.50, 1.30	~1.38	1.40
V_6	1.06	1.02	1.00
1c	0.90	0.70	0.68
1c'	0.86	0.70	0.68

[a] Spectra were obtained in Me_2SO-d_6–CCl_4 at 275–278 K; only signals changing by more than 0.1 ppm are listed. [b] See Figure 3.

Broad signals were obtained, many of which were buried in the base line. Raising and lowering the temperature did not improve the spectrum, nor did addition of CH_3OH-d_4, CH_3CN-d_3, Me_2SO-d_6 or DMF-d_7. Other workers also had not been able to make complete assignments in studies of aqueous vancomycin–peptide complexes.[6c] Furthermore, the solubility of the complexes is limited, restricting the signal to noise ratio.

Above room temperature the free and bound forms of vancomycin complexes are in fast exchange in Me_2SO solutions, leading to spectra in which only the average positions for free and bound components are seen. When substrate–receptor interactions are studied, it is desirable, although not necessary, to have slow enough exchange between free and bound species so that separate signals can be observed for each. Other antibiotics in this group do not suffer from this problem.[5] For example, complexes of ristocetin are in much slower exchange because the peptide binding site is more rigidly structured. Studies of ristocetin and avoparcin complexes with peptides[5,7] suggest that conformations of complexes involving dipeptide do not change substantially upon moving from water into Me_2SO-d_6. The vancomycin–peptide complex can be studied in mixtures of Me_2SO-d_6 and CCl_4 at 0–5 °C,[5c] the CCl_4 acting effectively as an antifreeze. Under these conditions spectra of adequate resolution are obtained with predominantly slow exchange and negative NOEs.

The ^1H assignments of the *N*-acetyl-D-Ala-D-Ala complex with vancomycin have been reinvestigated employing specific decoupling, NOE difference, COSY, and NOESY experiments. Se-

Table IV. ^{1}H NMR Chemical Shifts of the Peptides in Their Vancomycin Complexes[a]

peptide proton	1 + dipeptide[b] free	1 + dipeptide[b] bound	peptide proton	1 + tripeptide[b] free	1 + tripeptide[b] bound
A_CMe	1.28	0.50	A_CMe	1.28	0.50
A_CCH	4.14	3.90	A_CCH	4.16	3.90
A_CNH	8.16	7.84	A_CNH	8.10	7.84
A_NMe	1.18	0.92	A_NMe	1.20	0.92
A_NCH	4.30	4.70	A_NCH	4.30	4.52
A_NNH	8.06	8.06	A_NNH	8.24	8.02
CH_3CO	1.82	1.82	KαCH	4.18	4.18
			$K\epsilon CH_2$	3.00	3.06
			KαNH	8.10	7.48
			KεNH	7.84	7.94

[a] Spectra were obtained in $Me_2SO-d_6-CCl_4$ at 275–278 K. [b] See Figure 3.

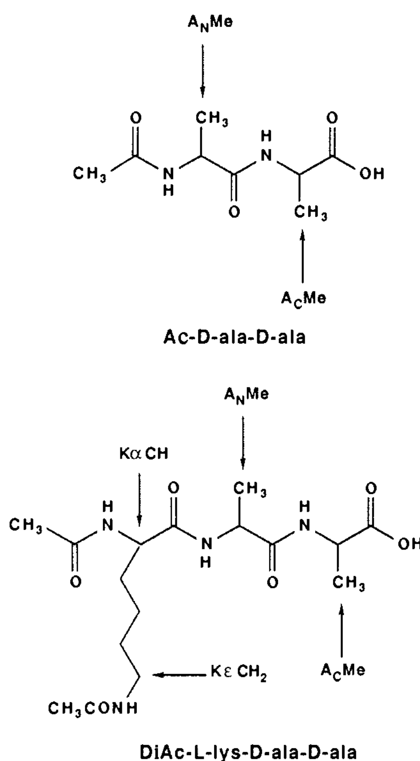

Figure 3. Peptide models of bacterial peptidoglycan.

lected assignments of the vancomycin resonances in the complex are compared in Table III with those of free antibiotic (acquired under the same conditions). In general, the signal assignments are in agreement with those made previously[5c] with the exception of 3a, 3a′, which we assign as both being at 2.16 ppm rather than one at 2.14 and the other at 2.60, and 1a, 1a′, which we assign as being at 1.38 rather than 1.69. Neither of these assignments is important in the present study. Previously unreported assignments include the vancosamine protons V_2, $V_{2'}$, at 1.70 and 1.85, V_4 at 3.14, and V_5 at 4.62 ppm. Anomeric protons V_1 and G_1 appear at 5.22 and 5.23 ppm, respectively. None of these sugar protons undergo a significant shift on complex formation and hence do not appear in Table III. Chemical shifts of the free and bound peptides are listed in Table IV, the nomenclature for which is shown in Figure 3.

NOE measurements were carried out in order to identify sites of close approach between antibiotic and peptide in this complex. These studies confirmed observations previously reported.[5c] NOEs involving the carbohydrate moiety were also seen. The methyl group V_6, as well as showing intrasugar NOEs, shows an NOE to 2e, and vice versa. Although small (<5%), exponential growth may be observed as the irradiation time is increased, indicating the close approach of these protons. A similar NOE may be observed between V_6 and A_CMe (bound) on careful irradiation of either resonance. Although these resonances are close in

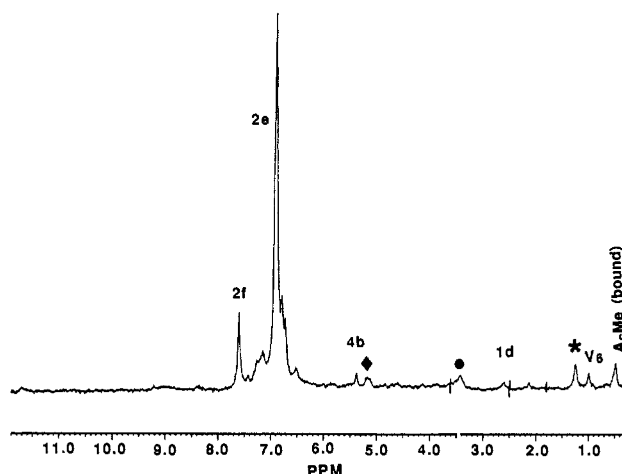

Figure 4. NOE difference spectrum obtained upon irradiation of 2e proton for 0.2 s in the vancomycin–Ac-D-Ala-D-Ala complex in $Me_2SO-d_6-CCl_4$ (10:3) solution at 278 K. Key: *, V_3Me and A_CMe (free) protons overlap and are not resolved; ●, Me_2SO peak; ♦, overlapping peaks prevented unambiguous assignment of NOE.

Table V. Selected NOEs Observed in Vancomycin–Ac-D-Ala-D-Ala Complex

resonance irradiated	resonances reduced in intensity
A_CMe bound	2e, V_6
2e	2f, 4b, A_CMe free, A_CMe bound, V_6
6e	6f, 4f, V_3Me
V_5	V_4, V_3Me, V_6
w_2	w_3, 2f, x_1, N-Me
w_3	w_2, w_4, 4b, x_3, 3a,a′
N-Me	x_1, 2f, A_CCH bound

chemical shift, the possibility of a spread of the decoupler power may be eliminated owing to the lack of other peaks in the vicinity of the decoupler frequency. As previously reported,[5c] an intermolecular NOE is also observed between 2e and A_CMe (bound), indicating the proximity of each of the protons V_6, 2e, and A_CMe (bound) to the other two. The NOE difference spectrum obtained upon irradiation of 2e is shown in Figure 4. The peaks at 1.00 and 0.55 ppm can be assigned to the V_6 and A_CMe (bound) protons. The difference peak at 1.28 ppm is ascribed to the A_CMe (free) and is due to saturation transfer from A_CMe (bound). The peaks at 7.6 and 5.4 ppm are due to NOEs to 2f (ortho to the 2e proton) and the nearby 4b proton, respectively. The second sugar methyl group, V_3Me, shares a NOE with 6e, but no other protons outside the disaccharide. The anomeric proton resonance of glucose shows no NOEs to the aglycon portion.

Appropriate NOESY cross peaks may be observed for all the above NOEs. However, the cross peak between V_6 and 2e in the antibiotic–peptide complex is very weak and would be unconvincing in the absence of NOE difference data. Selected NOEs observed in the vancomycin–dipeptide complex are listed in Table V. The equivalent intramolecular NOEs, i.e., 2e ↔ V_6 and 6e ↔ V_3Me, are not unambiguously observable by NOE difference or NOESY experiments in a sample of vancomycin hydrochloride in Me_2SO-d_6 solution in the absence of the peptide. However, both NOEs are observable on protonation of the N-terminus with an inorganic acid and in a CAMELSPIN spectrum of the original monohydrochloride solution. This indicates that it is a motional and not a positional phenomenon that prevents the observation of the above NOEs by conventional means in the absence of peptide or inorganic acid. Further NOEs observed in the CAMELSPIN spectra and on protonation of the N-terminus are V_3Me ↔ 2e, V_3Me ↔ 6b, V_6 ↔ 6e, G_1 ↔ 2e, and G_1 ↔ 6e.

The complex between vancomycin and the tripeptide Ac_2-L-Lys-D-Ala-D-Ala was investigated to seek confirmation of the spatial relationship between the vancosamine and the peptide binding site in the presence of peptide. Assignments of protons in the tripeptide complex were made by single-frequency decou-

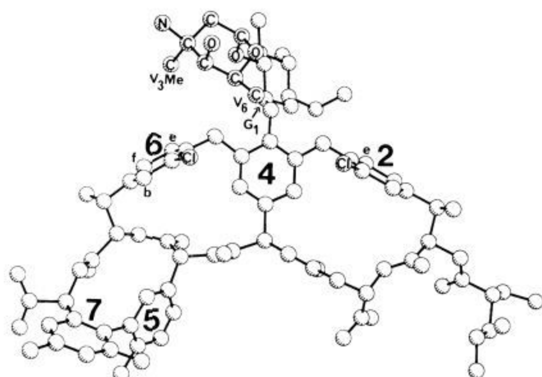

Figure 5. Crystal structure of CDP-I.

Table VI. Selected NOEs Observed in the N,N'-Diacetylvancomycin–Ac_2-L-Lys-D-Ala-D-Ala Complex

resonance irradiated	resonances reduced in intensity
A_CMe bound	2e, V_6
2e	2f, 4b, A_CMe free, A_CMe bound, V_6, G_1
6e	6f, 4f, V_3Me, G_1
G_1	G_3, G_5, 2e, 6e
N-Me	x_1, N-CH_3CO
N-CH_3CO	x_1, N-Me, 2f, A_CCH bound
1c,c′	x_1, N-Me, N-CH_3CO, A_CCH bound

pling, COSY, NOE difference, and NOESY spectra. The presence of the lysine residue increases the complexity of the upfield region of the spectrum. Chemical shifts (Table III) of most of the antibiotic protons in the tripeptide complex are very similar to those seen in the dipeptide complex. Notable exceptions are w_5, w_7, 6b, x_6, and x_7, which are near the site occupied by the lysine residue. The tripeptide complex, which is more stable than that of the dipeptide, gave more intense NOEs, including V_3Me ↔ 6e and V_6 ↔ 2e. However, no new NOEs involving V_3Me, V_6, or G_1 were observed.

To investigate the effects of N-acetylation, the conformations of N,N'-diacetylvancomycin (**4**) in Me_2SO-d_6 and in D_2O–CH_3CN-d_3 mixtures were studied along with those of its complex with tripeptide in Me_2SO-d_6–CCl_4 solution. In Me_2SO-d_6 solution and in the absence of peptide, the NOEs involving vancosamine observed in a CAMELSPIN spectrum were almost identical with those reported above in the CAMELSPIN spectrum of vancomycin monohydrochloride. The coupling constants of the vancosamine proton resonances indicated no change in the ${}^1C_4 \rightleftarrows {}^4C_1$ equilibrium position between Me_2SO-d_6 and D_2O whether or not the vancosamine was acetylated. On complexation with tripeptide, the NOEs V_3Me ↔ 6e and V_6 ↔ 2e were again observed. However, unlike in the vancomycin–tripeptide complex, NOEs now connected G_1 with 2e and 6e. Selected NOEs observed in the N,N'-diacetylvancomycin–tripeptide complex are listed in Table VI. In the N-terminal region the NOEs N-Me ↔ 2f, N-Me ↔ x_1, and N-Me ↔ A_CCH observed in the vancomycin–tripeptide complex are superseded by the NOEs CH_3CO ↔ 2f, CH_3CO ↔ x_1, CH_3CO ↔ A_CCH, and CH_3CO ↔ N-Me in the N,N'-diacetylvancomycin–tripeptide complex (N-Me ↔ x_1 is still observable but is of low intensity). The proximity of the leucine methyl groups of the N-terminal amino acid side chain (1c,c′) to the binding site and in particular the peptide carboxylate anion is evidenced by the NOEs to A_CCH, x_1, CH_3CO, and N-Me groups.

Discussion and Conclusion

The NOEs observed between the disaccharide and the aglycon portion of vancomycin in the absence of peptide cannot be satisfied by a single conformation. The NOEs V_6 ↔ 2e, V_3Me ↔ 6e, and G_1 ↔ 6e are consistent with the population of a conformer of the sugar region similar to that observed in the crystal structure of CDP-I[18] (see Figures 5 and 6a). The NOEs V_6 ↔ 6e, V_3Me ↔ 2e, and G_1 ↔ 2e are indicative of a conformer that differs from the one above by a rotation through approximately 180° about the glucose ring 4 glycosidic linkage (see Figure 6b). The presence of the NOE V_3Me ↔ 6b is a consequence of such a rotation. There is no obvious reason why rotation between these two conformers should be restricted in one direction more than the other, and so it is likely that the entire disaccharide rotates relatively freely about the glucose ring 4 glycosidic linkage from conformers with G_1 on the front face (binding pocket side) to those with G_1 on the rear face. The absence of NOEs between the disaccharide and the aglycon at 400 MHz is suggestive of a time period between rotations of the order of 10^{-8} s and hence a barrier to rotation of approximately 6 kcal mol^{-1}. Addition of acid reduces the rate of this rotation although it does not change the charge state of the sugar, which is already protonated in the monohydrochloride. An analogous reduction in molecular motion on protonation of the N-terminus of vancomycin has been observed in mobile parts of the aglycon portion.[19] It is interesting in the case of the monohydrochloride that negative NOEs are observable within both the aglycon and the disaccharide portions and yet the NOEs between them are approximately zero because of their relative motional correlation time and, furthermore, that protonation at a remote site significantly reduces this relative motion.

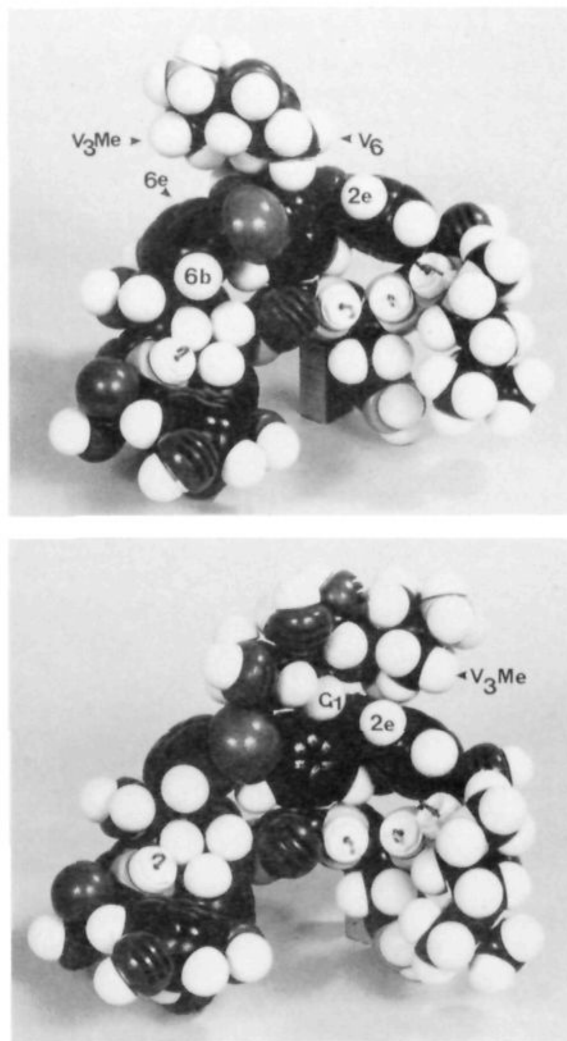

Figure 6. Structure of vancomycin, showing two different conformations of sugar unit: (a) V_6 facing front; (b) V_6 facing rear (although not seen in figure, it is closer to 6e).

(18) Sheldrick, G. M.; Jones, P. G.; Kennard, O.; Williams, D. H.; Smith, G. A. *Nature (London)* **1978**, *271*, 223.

(19) Waltho, J. P.; Williams, D. H.; Skelton, N. J.; Stone, D. J. M., submitted for publication in *J. Am. Chem. Soc.*

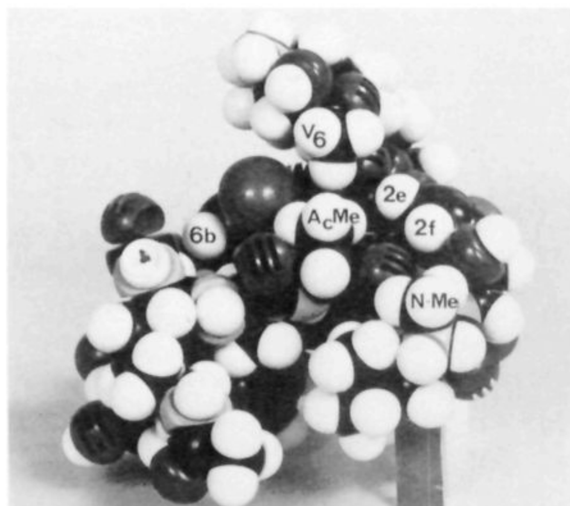

Figure 7. Structure of vancomycin–tripeptide complex, showing the preferred conformation of the vancosamine.

The NOEs observed within vancosamine at 400 MHz are characteristic of a $^1C_4$ conformation, and there is no evidence of a significant population of the $^4C_1$ conformer (the intra-glucose NOEs are indicative of a $^4C_1$ conformer for the β-glucopyranoside ring). Acetylation of vancosamine does not significantly perturb the motion or conformations of this system—the same NOEs are observed and their intensities are comparable. The conformation of the N-terminal region on acetylation has been described.[19]

On addition of di- or tripeptide to vancomycin the observed disaccharide NOEs, namely $V_6 \leftrightarrow$ 2e, $V_6 \leftrightarrow A_C$Me, and V_3Me $\leftrightarrow$ 6e, can be satisfied by a single conformation of the disaccharide with respect to the binding site. This conformation would be similar to that occupied in the crystal structure of CDP-I where the center of the vancosamine ring lies above the ring 4–ring 6 ether linkage, the V_3Me group is close to 6e on the rear face, and the methyl group V_6 occupies the break in the hydrophobic A_CMe pocket between ring 2 (2e) and the ring 6 chlorine substituent (see Figure 7). A small oscillation of vancosamine about this central position would bring the NOEs $V_6 \leftrightarrow$ 2e and V_3Me $\leftrightarrow$ 6e into the fast-building NOE region. There is no evidence of a significant population of the 180° rotamer of the above conformation, which is observed in the absence of peptide, but the size of the observed NOEs does not allow a small population of such a state to be excluded. The absence of NOEs between G_1 and the aglycon is unlikely to be the result of a motional phenomenon and probably reflects the proximity of G_1 to the ring 2 chlorine. With vancosamine in the observed conformer, the amino group is pointed away from the binding cleft. Consequently, the charged group is available for maximum solvation but is by no means optimized for direct electrostatic interaction with the peptide carboxylate anion.

The main factor in the reduction of 0.7 kcal mol^{-1} in binding energy on removal of vancosamine is probably the loss of the extension of the hydrophobic pocket for A_CMe provided by V_6. When another part of this hydrophobic pocket is independently removed—namely the ring 6 chlorine substituent—a similar loss in binding energy (0.8 kcal mol^{-1}) to tripeptide results.[20] However, an electrostatic contribution to the binding energy cannot be disregarded; with a charge separation between the vancosamine amine and the peptide carboxylate anion of approximately 15 Å, the attraction energy may be as high as 0.3 kcal mol^{-1} (estimated from the fall in binding energy of 0.9 kcal mol^{-1} on N-acetylation of aglycoristocetin[11]—see below).

Acetylation of the amino group of vancosamine causes a reduction in the binding constant similar to that observed upon removal of the sugar. NMR studies reveal the population of a conformer of the disaccharide in the *N,N'*-diacetylvancomycin–tripeptide complex similar to that observed prior to acetylation (i.e., by the NOEs $V_6 \leftrightarrow$ 2e, $V_6 \leftrightarrow A_C$Me, and V_3Me $\leftrightarrow$ 6e), but population of other conformers (observed, e.g., by the NOE $G_1 \leftrightarrow$ 2e) is also observed. Although a hydrophobic interaction between the V_6 methyl group and the A_CMe group is still possible, energetically it appears to be less favorable in the absence of the charged amine.

Electrostatic attraction appears to play a more important part in the role of the N-terminal group in binding. It has been proposed that deprotonation of the N-terminal amino group leads to approximately 1.4 kcal mol^{-1} reduction in binding energy.[6a] Acetylation of this amino group leads to a similar reduction (1.8 kcal mol^{-1}). These values are not strictly comparable because of the likelihood of partial ionization of the phenolic groups in the former experiment. In ristocetin, the N-terminal amine is conformationally restricted by the cross-linkage between residues 1 and 3; its geometry in peptide complexes has also been investigated.[21] N-Acetylation of aglycoristocetin leads to a smaller reduction in binding energy (0.9 kcal mol^{-1}) than in the case of aglycovancomycin (2.7 kcal mol^{-1});[11] 0.9 kcal mol^{-1} is probably a good estimate of the contribution of the electrostatic attraction between the protonated amine and the carboxylate anion to the binding constant in the ristocetin complex (a 5-Å charge separation). In a vancomycin–peptide complex, NOEs from the *N*-methyl group to 2f and x_1 indicate (see below) that although the δ^+ NH protons are oriented away from the binding site for at least some of the time, the N-terminal cation is of a comparable distance from the peptide carboxylate anion as that observed in ristocetin complexes. Consequently it would be expected that the charge–charge electrostatic contribution to K_A from the N-terminus of vancomycin would be similar to that for aglycoristocetin (i.e., ~1 kcal mol^{-1}).

In free solution the conformation of the N-terminal region of *N*-acetylvancomycin differs significantly from that of vancomycin only in the orientation of the N-terminal nitrogen;[19] i.e., their relative energies are comparable. In both cases fast rotation of parts of the N-terminal amino acid is observed.

In a vancomycin–peptide complex the NOEs between *N*-Me, x_1, and 2f together with the absence of NOEs between *N*-Me and other residue 2 protons indicate that the cationic amine is mainly oriented such that the hydrophobic methyl group, and not the δ^+ N–H protons, is adjacent to the peptide carboxylate anion (see Figure 7). In this way, the hydrophobic pocket around the peptide carboxylate anion has another boundary, and, probably more significantly, the cationic charge is available for maximum stabilization by the solvent. This arrangement appears to be energetically favored over the alternative in which the amino groups would be pointed toward the binding cleft in position for the formation of a stronger salt bridge but having restricted opportunity for solvation. It is noteworthy that both (at residue 1) epiristocetin aglycon[11] and epiavoparcin[22] show diminished binding even though models suggest that the N-terminal amino group in the epi compounds is in a better position for electrostatic interaction with the peptide carboxylate anion than in the natural isomer.

In a *N,N'*-diacetylvancomycin–peptide complex, it is the *N*-Ac methyl group that shares intense NOEs with x_1, 2f, and A_CCH. Unlike in free solution, the NOE between the *N*-Ac methyl group and the *N*-Me group is small, suggesting that the trans arrangement of these groups may be more populated. Both methyl groups have NOEs to leucine methyl groups (1c,c'), but only the *N*-Me group has NOEs to the leucine methylene protons (1a,a'). The *N*-Me group retains a small NOE with x_1. Again all the

(20) Harris, C. M.; Kannan, R.; Kopecka, H.; Harris, T. M. *J. Am. Chem. Soc.* **1985**, *107*, 6652.

(21) Studies by both Williamson et al.[8] and Fesik et al.[7b] on structures of Ac_2-L-Lys-D-Ala-D-Ala–ristocetin and ristocetin–Ψ-aglycon complexes show however that the N-terminal region of the antibiotic is not as rigid as the C-terminus; no single conformation for rings 1 and 3 could be determined because the aromatic rings of residues 1 and 3 apparently are oscillating even in the complex.

(22) Ellestad, G. K.; Leese, R. A.; Morton, G. O.; Barbatschi, F.; Gore, W. E.; McGahren, W. J.; Armitage, I. M. *J. Am. Chem. Soc.* **1981**, *103*, 6522.

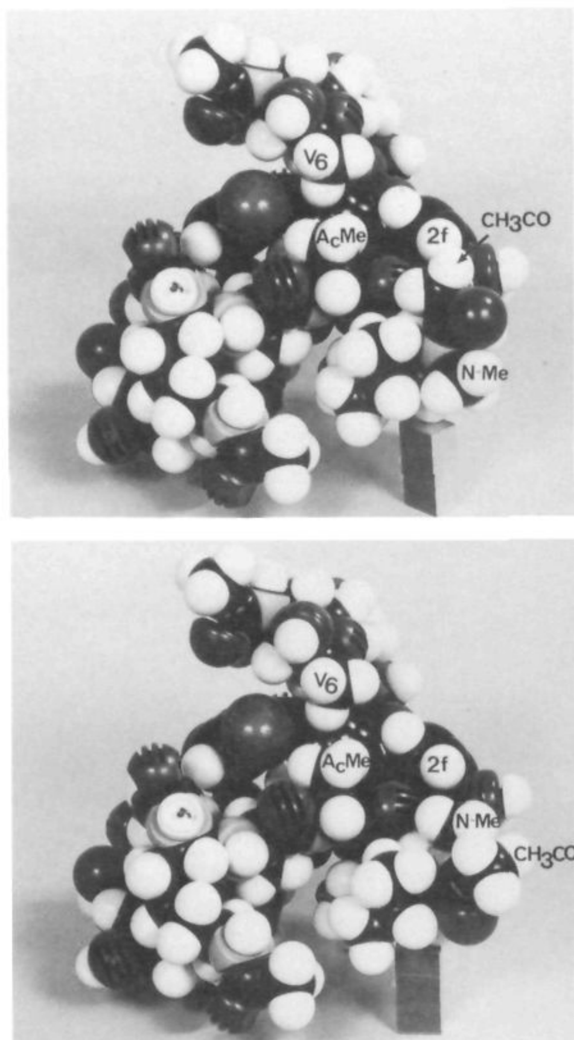

Figure 8. Structure of *N,N′*-diacetylvancomycin–tripeptide complex, showing two different conformations of the N-terminus of the antibiotic: (a) major conformer with A_CMe close to 2f and trans to *N*-Me; (b) minor conformer with *N*-Me close to 2f and cis to A_CMe.

observed NOEs cannot be satisfied within a single conformation. However, the relative intensities of the NOEs are consistent with the major conformer having the *N*-Ac methyl group providing the extension of the hydrophobic wall of the peptide carboxylate anion binding pocket in conjunction with x_1 and the leucine methyl groups (see Figure 8a). A trans arrangement of the *N*-Ac methyl and *N*-Me groups ensures that the *N*-Ac carbonyl group is not adjacent to the peptide carboxylate anion. There is also significant population of a minor conformer formed by a 180° rotation of the bond attaching the N-terminal nitrogen to the peptide backbone. Spatially this replaces the *N*-Ac methyl group near the *N*-Me group (see Figure 8b). In this conformer a cis arrangement of the above two methyl groups would explain the small NOE between them (relative to that of **4** in free solution) and the lack of NOEs between the *N*-Ac methyl group and the leucine methylene protons (1a,a′).

Energetically, the fall of 1.8 kcal mol^{-1} in binding energy on acetylation of the N-terminus of vancomycin is considerably larger than the estimated electrostatic contribution (~1 kcal mol^{-1}), and yet the same sort of nonelectrostatic interactions are available to both acetylated and nonacetylated systems. The same is true in the case of vancosamine versus *N*-acetylvancosamine. The underlying common factor is that the gain in energy through the presence of a hydrophobic group adjacent to the peptide is greater in the presence of a charge. The observation that N-acetylation of aglucovancomycin has a greater effect on K_A than the equivalent modification of vancomycin is indicative of the subtle interrelationship of the factors that influence the energetics of binding.

The arrangement of the amino groups of vancomycin appears to be governed by a desire to retain maximum solvation of the cationic groups. In the case of vancosamine, this is complemented in the antibiotic–peptide complex by a hydrophobic interaction between the V_5, V_6 region and the A_CMe group of the peptide. At the N-terminus, the *N*-methyl group extends the hydrophobic boundary of the carboxylate binding pocket and there is electrostatic stabilization of the carboxylate anion by the cation while the latter retains maximum solvation. The above model is consistent with observations of the solution structures of other peptides and proteins. For example, salt bridges are relatively uncommon in globular proteins. Lysyl residues and other groups of high polarity commonly lie on the surface of proteins where they have good access to solvent. On the other hand, hydrophobic residues aggregate in the interior.

All the previous studies have shown that acetylation of the N-terminus of members of this group of antibiotics, e.g. vancomycin,[8] ristocetin,[10,11] and teicoplanin,[10] significantly reduces, but does not destroy, binding of the aforementioned model peptides. The charged N-terminus contributes to the binding constant in all the cases studied although the amount of this contribution varies between members of the group. For ristocetin, the binding constant to the tripeptide may be maintained or even increased by the replacement of the N-terminal amine and α-CH by a carbonyl group or an oxime.[11] It appears that stabilization of the peptide carboxylate anion by a sympathetically polarizable group may be energetically similar to the electrostatic interaction of a protonated amine.

It is unlikely that the role of vancosamine in nature is solely to increase the binding constant to the target peptide to such a small extent. The amino sugar undoubtedly affects the solubility and aggregation properties of vancomycin in aqueous solution and may also have a role in the selectivity of the size of the C-terminal amino acid side chain of binding peptides, in conjunction with the chlorine on ring 6. It has been proposed[8] that the N-terminal cation of vancomycin aids the kinetics of the binding process by first attracting the carboxylate anion of the aliphatic peptide and then directing the peptide into the binding cleft. Vancosamine possibly plays a similar role. The results obtained in the present study together with, for example, those obtained previously on hydrophobic interactions in binding,[23] and with dechloro analogues of vancomycin,[20] increase our understanding of how various structural features of the vancomycin molecule contribute to its biological activity. The decreased antibacterial activity when the vancosamine is acetylated or removed is in line with the decrease observed when ristosamine is removed from ristocetin but in contrast to the increase in activity observed when the neutral ring 4 sugars and the mannose on ring 7 are removed from the latter.[11]

Acknowledgment. Some FAB spectra were obtained with the help of Dr. Brian Sweetman, Department of Pharmacology, Vanderbilt University (Grant No. GM 27557 from the USPHS). We thank Dr. Steve Bunkhall, Cambridge Crystallographic Data Center, for the diagram of CDP-I. Funds for the support of this work were provided by the U.S. Public Health Service (Grant GM 25793), the SERC (U.K.), and Smith Kline and French Research Ltd.

Registry No. 1, 1404-90-6; **1**·HCl, 1404-93-9; **1** (*N*-Cbz), 113585-98-1; **1** (*N′*-Ac-*N*-Cbz), 113585-97-0; **2**, 113598-01-9; **3**, 113585-94-7; **4**, 113585-95-8; **5**, 82198-76-3; **6**, 113585-96-9; **7**, 101485-50-1; Ac_2-L-Lys-D-Ala-D-Ala, 24570-39-6; Ac-D-Ala-D-Ala, 19993-26-1.

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