who found the release energy to be 0.96 eV with a slight contribution from a low-energy release channel. It is impossible to determine the heat of formation of the $C_7H_7O^+$ ion from the derived dissociation limit because we do not know the fraction of the reverse activation energy that appears as kinetic energy of the departing fragments. However, we can determine an upper limit to the heat of formation for the resulting $C_6H_4CH_3O^+$ ion by subtracting the kinetic energy release from the NO loss dissociation limit. This yields a $\Delta H_f°_0 < 910$ kJ/mol, which translates to a 298 K value of 890 kJ/mol.

It is interesting to compare this derived heat of formation with that of the similar ion, $C_6H_5O^+$, which is the product of the NO loss from the nitrobenzene ion.[48] The 298 K heat of formation of $C_6H_5O^+$ was calculated to be 874 kJ/mol from the ionization energy (8.56 eV) of the phenoxy radical[49] and its neutral radical heat of formation (46 kJ/mol).[50] We can estimate the effect of adding a methyl group to a benzene ring from the difference in the heats of formation of the benzene ion (975 kJ/mol) and the toluene ion (901 kJ/mol).[44] Thus, we estimate that the $\Delta H_f°_{298}(C_6H_4CH_3O^+)$ should be about 874 - 74 = 800 kJ/mol. Thus, the upper limit of 890 kJ/mol for the heat of formation of $C_6H_4CH_3O^+$ obtained in this study is indeed an upper limit. However, the discrepancy of 90 kJ/mol suggests that the release energy represents only 50% of the total reverse activation energy.

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V. Conclusions

The PEPICO experiments on the dissociative ionization of benzyl bromide ions have confirmed the previously reported heat of formation of the $C_7H_7^+$ benzyl ion on the basis of the ionization energy of the benzyl radical. The benzyl ion heat of formation at 298 K can now be considered established at 896 ± 4 kJ/mol. The PEPICO measurements of the *m*- and *p*-nitrotoluene ion dissociation rates modeled with the statistical theory (RRKM/QET) have been used to establish for the first-time experimental values for the tolyl ion heats of formation. The meta and para isomers were found to have 298 K heats of formation of 1054 and 1074 ± 10 kJ/mol, respectively. No experimental information about the stability and thermochemistry of the *o*-tolyl ion is available.

Aside from the *o*-tolyl ion, the major mystery in the $C_7H_7^+$ story yet to be solved is the heat of formation of the tropyl ion. This must await an experiment in which the dissociation limit of a precursor such as the tropylium tetrafluoroborate ion is measured. However, such a study will also require the heat of formation of the neutral salt.

Acknowledgment. We thank John Holmes (Ottawa University) and his students for many helpful discussions and sharing of results on the structure of the $C_7H_7^+$ product ions. We are also indebted to Odile Dutuit for allowing us to measure the benzyl bromide breakdown diagram on the PEPICO experiment at the synchrotron radiation facility (LURE—University of Paris, Orsay) and Christian Rolando (Ecole Normal Superieur) for rushing the sample to us during the Paris rush hour. Finally, we thank the National Science Foundation and the Department of Energy for financial support of this work.

Intramolecular Determinants of Conformation and Mobility within the Antibiotic Vancomycin

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Abstract: The conformations of the N-terminal region of the glycopeptide antibiotic vancomycin, and *N*-acetylvancomycin, in DMSO solution are discussed. A 180° rotation of the central amide unit of the carboxylate binding pocket is observed. It is shown that the anomalously rapid exchange of the N-terminal amide proton with the residual water within the solvent is dependent upon the basic N-terminal amine.

The structure of the clinically important glycopeptide antibiotic vancomycin (**1**) has been elucidated by a combination of methods. Following the solution of the crystal structure of the degradation product, CDP-I,[1] the proposed structure of vancomycin was revised with high-field ^1H NMR[2] and chemical studies.[3] The geometry of the binding of vancomycin to bacterial cell wall precursor analogues di-*N*-acetyl-L-Lys-D-Ala-D-Ala (tripeptide) and *N*-acetyl-D-Ala-D-Ala (dipeptide) has also been studied by ^1H NMR.[4-6] Within these structural studies, the solution conformation of the rigid C-terminal region of vancomycin has been well-defined, but little has been reported about the more flexible N-terminal region. This paper will deal with our observations concerning the conformations and motion of the N-terminal region as studied by ^1H NMR. For convenience, we define the binding pocket side of vancomycin as the "front" face and the nonbinding side as the "back" face. The code used to designate the protons of vancomycin is that used previously[6] and is shown in Figure 1. The constituent amino acid residues are numbered 1-7 starting from the N-terminus. The observed chemical shifts of the proton resonances are listed in Table I, and the nuclear Overhauser effects (NOEs) mentioned in the text are shown in Table II. It should

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Table I. ^{1}H NMR Chemical Shifts in Vancomycin and *N*-Acetylvancomycin in DMSO Solution

proton	vanco (base)	vanco (acid)	*N*-Acvanco (**2**)
N-Me	2.32	2.62	2.86
w_1, w_1'		9.05, 9.95	
x_1	3.14	3.92	5.22
1a,a′	1.41, 1.50	~1.7	1.48, 1.60
1b	1.70	~1.7	1.44
1c,c′	0.84, 0.88	0.86, 0.92	0.80, 0.88
w_2	8.11	8.59	6.84
x_2	4.90	4.92	4.78
z_2	5.14	5.21	5.16
2b	7.34	7.46	7.25
2e	7.24	7.20	7.04
2f	7.52	7.59	7.52
w_3	6.78	6.59	6.74
x_3	4.42	4.30	4.50
3a,a′	2.16, 2.32	2.15, 2.53	2.10, 2.20
3-NH_2	6.92, 7.36	7.02, 7.49	6.61, 7.21
w_4	8.22	~8.2	8.00
x_4	5.76	5.27	5.72
4b	5.50	5.58	5.50
4f	5.22	5.20	5.20
w_5	8.62	8.66	8.60
x_5	4.42	4.43	4.46
5b	7.16	7.17	7.18
5e	6.70	6.72	6.70
5f	6.74	6.77	6.73
w_6	6.62	6.68	6.58
x_6	4.18	4.17	4.17
z_6	5.12	5.12	5.12
6b	7.88	7.87	7.88
6e	7.32	7.35	7.34
6f	7.44	7.47	7.46
w_7	8.46	8.53	8.30
x_7	4.36	4.43	4.35
7d	6.34	6.44	6.38
7f	6.34	6.25	6.32
V_1	5.25	5.22	5.21
V_2, V_2'	1.72, 1.90	1.75, 1.90	1.70, 1.92
V_3Me	1.26	1.35	1.26
V_4	3.20	3.19	3.20
V_5	4.68	4.68	4.70
V_6	1.06	1.07	1.07
G_1	5.25	5.25	5.24

Table II. Selected NOEs Observed within the *N*-Terminal Region of Vancomycin and *N*-Acetylvancomycin[a]

residue 1	residue 2	residue 3	residue 4
N-Me ↔ x_1	w_2 ↔ 2f	3a,a′ ↔ x_3	w_4 ↔ 4b
1c,c′ ↔ 1b	x_2 ↔ z_2		
1a,a′ ↔ 1b	z_2 ↔ 2b		
1a,a′ ↔ x_1			
x_1 ↔ w_2	x_2 ↔ w_3	3a,a′ ↔ w_4	
N-Me ↔ 2f	z_2 ↔ w_3	w_3 ↔ w_4	
N-Me ↔ w_2[b]	2b ↔ w_3	w_3 ↔ 4b	
N-Me ↔ AcMe[c]	w_2 ↔ w_3		
AcMe ↔ 2f[c]	2f ↔ w_3		

[a] Where the NOEs involve protons from different residues, they are listed according to the residue with the lower number. [b] Unconfirmed as a direct NOE owing to saturation-transfer phenomena. [c] Observed in only in *N*-AcV (**2**).

be noted that although reference will be made to the conformation of a region, it is not necessarily the result of the exclusive population of one energy minimum but, more likely, the population of a range of mimina, which lead to the same general orientation of the specified protons. In many cases, the population of drastically different conformations cannot be excluded, but the absence of expected NOEs suggests that the average population of these states is low.

We have used various ^{1}H NMR phenomena for the estimation of the rates of molecular processes. High-frequency processes have been distinguished as above or below ca. 10^8 Hz according to the sign of the NOEs between protons involved in the process. The

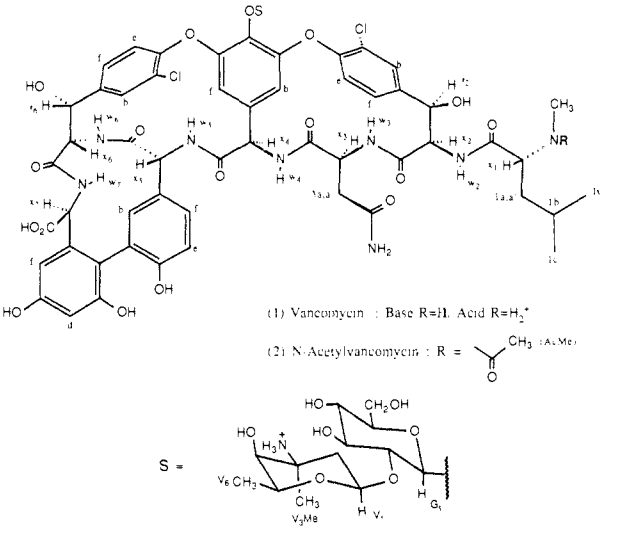

Figure 1. Structure of vancomycin (**1**) and *N*-acetylvancomycin (**2**) including the code used to designate ^{1}H NMR resonances.

percentage size (η) of the NOE between two proton spins is given by the equation

$$\eta = \frac{(5 - 4\omega^2\tau_c^2)(\omega^2\tau_c^2 + 1)}{4\omega^4\tau_c^4 + 23\omega^2\tau_c^2 + 10}$$

where ω is the Larmor frequency of the protons (400 MHz in our case) and τ_c is the motional molecular correlation time. One may consider τ_c as the reciprocal of the rate at which the vector between the two spins varies, and so it can be used as a measure of the frequency of the relative motion of two protons. The above relationship between η and τ_c defines that η passes through a zero point when $\omega\tau_c \cong 1.1$. At 400 MHz the NOE will be positive when $\tau_c < 3 \times 10^{-9}$ s but negative when $\tau_c > 3 \times 10^{-9}$ s. From the rate of buildup of NOEs between protons of known distance separation, the molecular correlation time for the overall rotation of vancomycin in DMSO at 35 °C has been estimated[7] to be 1.0×10^{-8} s. This estimation was made by using the above equation in conjunction with the largest steady-state NOE seen[2] for vancomycin. Thus, at 400 MHz and in the absence of internal rotations, the NOEs would be negative.

A second means that we have used to estimate motion occurs when the frequency of the motion is lower than the difference of the Larmor frequencies of the different proton environments created by the motion. Under these conditions, the slow-exchange region, two (or more) resonances will be observed for each proton sufficiently perturbed. Increasing the frequency of motion will cause these resonances to broaden (intermediate-exchange region) and coalesce. At this point, the frequency (k) of the motion can be estimated from the relationship

$$k = \pi\delta\nu/\sqrt{2}$$

where $\delta\nu$ is the frequency separation of the ^{1}H resonances in the slow-exchange region. Further increasing of the frequency of motion causes the time-averaged line to sharpen to its normal line width (fast-exchange region). In the slow-exchange region the frequency of motion may be estimated by a saturation-transfer experiment.[8]

Experimental Section

Vancomycin was obtained as a gift from Eli Lilly, and *N*-acetylvancomycin was prepared as described previously.[6] ^{1}H NMR spectra of either 5 or 10 mM solutions in DMSO-d_6 were run on Bruker AM400 and WH400 spectrometers. One-dimensional spectra were obtained in

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the quadrature detection mode, recording 16K data points over spectral widths of approximately 4000 Hz. NOE difference/saturation-transfer experiments were performed as described previously.[6,8] Resonance assignment of the various systems under investigation was achieved with the aid of double-quantum-filtered COSY, NOESY, and/or CAMELSPIN spectra. The strategy used has been described.[9] The NOEs involved in the conformational arguments presented in the text were confirmed with NOE difference experiments. All two-dimensional spectra—double-quantum-filtered COSY, NOESY, and CAMELSPIN experiments—were run in the phase-sensitive mode with time-proportional phase incrementation (TPPI). Typically, 2K data points were recorded for $f2$ and 512K or 1K data points for $f1$. In all cases the data matrices were zero-filled in $f1$ and subjected to Lorentzian–Gaussian multiplication prior to transformation. All NOESY experiments employed a 20-ms z-filter.[10] CAMELSPIN experiments were carried out with a centrally placed 2-kHz spin-locking field. Possible ambiguity of CAMELSPIN correlations owing to Hartmann–Hahn transfer was investigated through rerunning experiments with different spin-locking carrier frequencies (typically on resonance with one of the J-coupled spins involved in the relevant NOE).

Results and Discussion

In order that direct comparison could be made with previous studies, the present work was carried out in DMSO-d_6 solution. In this solvent, the N-terminal amine of vancomycin hydrochloride (as prepared for clinical use) is uncharged. On addition of 1 equiv of trifluoroacetic acid, the resonances of the N-methyl group (N-Me) and proton x_1 (see Figure 1) move downfield 0.30 and 0.78 ppm, respectively (see Table I). In contrast, the same resonances showed no change in chemical shift on addition of 1 equiv of sodium methoxide.

Residues 1 and 2. The N-terminal amino acid residue, N-methylleucine (see Figure 1), appears to exhibit considerably more molecular motion than the majority of the C-terminal region. When unprotonated at the N-terminus, the side-chain methyl groups and their adjacent methine proton share positive NOEs (see Table II). Moving toward the peptide backbone, the methylene protons do not show any NOEs even though it is impossible for them to be distant from all other protons. The absence of NOEs may be attributed to a reduction in the molecular motion in this region, i.e. for these protons $\omega\tau_c \cong 1$. In support of this, the adjacent α CH proton (x_1) may be linked to the protons of the N-Me group by a negative NOE. The relevance of the latter NOE to the solution conformation is discussed below. The molecular motion of the system is altered on protonation of the N-terminal amine by the addition of HCl or TFA; the NOEs involving the isobutyl side chain become negative. In general, when species in polar solvents become charged, their rotational correlation time increases. This is a result of the greater attraction between the substrate and the solvent, increasing its effective moment of inertia. The increase in τ_c for the isobutyl side chain may be attributed to a general and/or a local restriction of motion. In favor of a contribution from a general restriction of molecular motion, NOEs between vancosamine (the sugar attached to C-2 of glucose) and the aglycon are not observed when the N-terminus is deprotonated but become negative on addition of TFA (the sugar amine is protonated in both cases). However, the concentration of ions in the solvent, for example, may also influence the motion of the sugar. In a CAMELSPIN spectrum of vancomycin in the absence of acid, where the effective field for proton relaxation is of the order of 10^3 Hz, positive NOEs may be observed along the isobutyl side chain from the methyl groups to the α CH proton, confirming that it is a motional phenomenon that controls the NOE intensity in conventional NOE spectra.

The motion of residue 2 is restricted by the peptide backbone and the oxidative coupling of its side chain to that of residue 4. Consequently, all the NOEs involving residue 2 are negative. An intense, fast-building NOE between w_2 and 2f places the amide proton on the front face of the molecule adjacent to ring 2 (see Figure 2a). The coupling constant between w_2 and x_2 (9 Hz) is indicative of an approximately trans relationship of these protons (see Figure 2b). Fast-building NOEs $x_2 \leftrightarrow z_2$ and $z_2 \leftrightarrow$ 2b confirm the proximity of these protons. A coupling constant of 4 Hz between x_2 and z_2 may be accommodated along with the above data by rotating the two β-carbon substituents z_2 and z_2OH, from their positions in the crystal structure of CDP-1 (see Figure 3) toward the back face of the molecule so that z_2 lays between x_2 and 2b. The z_2–x_2 bond angle is thus approximately −50° compared with +50° in CDP-I, and w_2 is adjacent to 2f. A similar rotation of a smaller magnitude has been proposed for the conformation of vancomycin when bound to dipeptide.[4]

The relative orientation of residues 1 and 2 may be deduced by two inter-residue NOEs, namely $x_1 \leftrightarrow w_2$ and N-Me ↔ 2f (see Figure 4). Therefore, both x_1 and the N-Me group occupy the front face of the molecule, which allows the isobutyl side chain

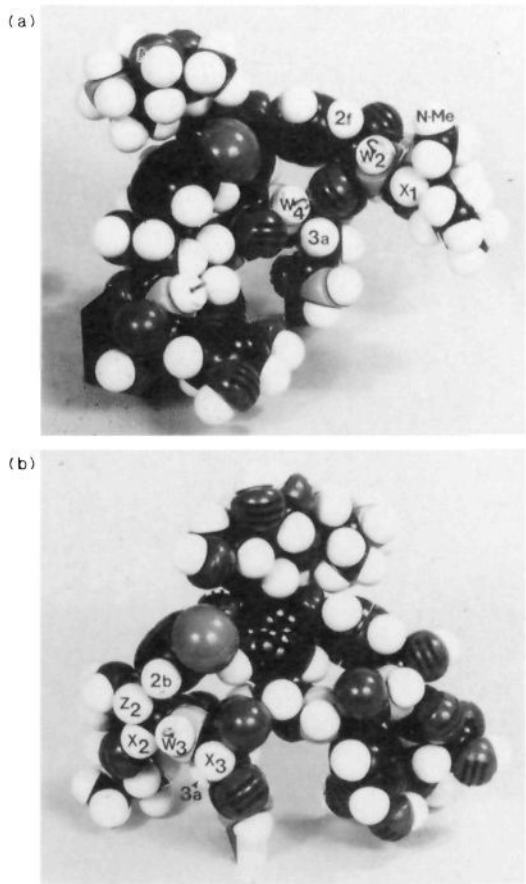

Figure 2. Solution conformation of vancomycin (a) showing the front face and (b) showing the back face. Note that the N-terminal amine is oriented such that the N-Me group is adjacent to proton x_1 and its lone pair is adjacent to proton w_2. The w_3 amide group is oriented such that proton w_3 is on the back face of the molecule and so protons w_2 and w_4 are separated by a carbonyl group.

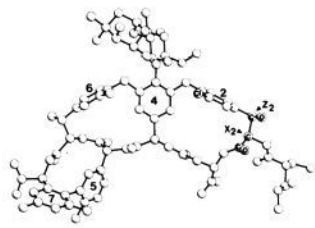

Figure 3. Crystal structure of CDP-I. The hydrogens have been omitted as information concerning their positions is not available. The likely orientation of protons x_2 and z_2 is shown.

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(10) See, for example: Kumar, A.; Wagner, G.; Ernst, R. R.; Wuthrich, K. *J. Am. Chem. Soc.* **1981**, *103*, 3654.
(11) For example, it is the conformation observed in a β pleated sheat within proteins and in crystal structures of small peptides. See: Creighton, T. E. *Proteins, Structure and Principles*; Freeman: New York, 1983.

Figure 4. Solution conformation of vancomycin showing the front face. The N-terminal amine is inverted relative to its position in Figure 2a, the *N*-Me group now being adjacent to proton 2f. The w_3 amide unit is now oriented such that proton w_3 is on the front face; cf. Figure 2a.

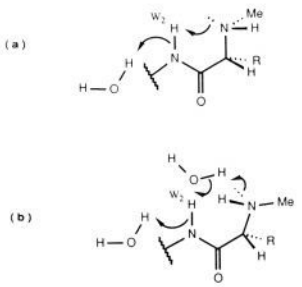

Figure 5. Schematic representation of possible mechanisms for the catalysis of exchange of the amide proton w^2 and water in the solvent by the neutral N-terminal amine (a) with direct exchange and (b) via a molecule of water.

of residue 1 to project freely into the solvent, consistent with the molecular motion identified above. Inspection of CPK molecular models suggests a relatively small barrier to rotation about the α C–carbonyl C bond of residue 1. However, the relative motion of x_1 and w_2 must be at a lower frequency than 400 MHz because the NOE between them is negative. Furthermore, the intensity of the above NOEs, combined with the absence of other interresidue NOEs, is evidence that the rotamer with x_1 and *N*-Me on the front face is the most heavily populated.

Preirradiation of w_2 (e.g. for 100 ms) in a saturation-transfer experiment shows that, unlike *all* other amide protons in vancomycin, it exchanges with the protons of the residual water in the solvent on the time scale of the experiment. At room temperature, the resonance of w_2 is very broad, owing to its proximity to atoms involved in a dynamic process of an "intermediate" rate involving w_3 (see below). Proton w_4 is also adjacent to the atoms involved in this process, and it is informative to compare the behavior of the resonances of w_2 and w_4 as the temperature is increased. On heating, the resonances of both w_2 and w_4 sharpen as the aforementioned dynamic process approaches its fast-exchange limit (see Figure 6). However, above 315 K the resonance of w_2 begins to broaden again as the frequency of its exchange with the solvent water moves from the slow-exchange region into the intermediate-exchange region. Reducing the preirradiation time of the saturation-transfer experiment allows an estimate of the rate of exchange of w_2 with the solvent water. At 310 K where the line width of w_2 is close to its minimum, the w_2-exchange rate is approximately 300–400 Hz. The corresponding exchange rate of the other amide protons of vancomycin at this temperature is less than 1 Hz; i.e., the exchange rate of w_2 is over 2 orders of magnitude faster than that of all other amide protons in this system. A rationale of this phenomenon based simply on solvent accessibility is clearly insufficient since the asparagine primary amide group of vancomycin, which is in a sterically uncrowded

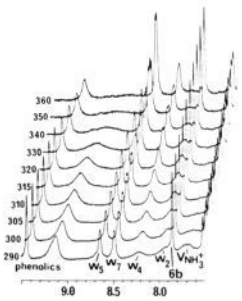

Figure 6. Variation of the line widths of selected proton resonances with increasing temperature.

part of the molecule, also exchanges 2 orders of magnitude more slowly than w_2.

On addition of 1 equiv of TFA, the N-terminal amine is protonated, and saturation-transfer experiments with irradiation for 100 ms show that w_2 is no longer exchanging with the solvent water on this time scale at 310 K. Indeed, the new exchange rate is less than 1 Hz and thus comparable with those of the other amide protons. Investigation of the spectra of *N*-acetylvancomycin (**2**), prepared by the acetylation of the N-terminal amine with acetic anhydride in water, reveals that in this system the exchange rate of w_2 with the solvent water is similar to that observed in the vancomycin + TFA system. The conformation of the N-terminal region of *N*-acetylvancomycin will be discussed below.

In summary, the exchange rate of the amide proton w_2 with the residual water in the solvent is greater than 2 orders of magnitude faster than those of all other amide protons in the system. On protonation or acetylation of the N-terminal amine, this differential is lost. Therefore, it appears that the uncharged, basic N-terminal amine is assisting the exchange of w_2. The NOEs *N*-Me ↔ 2f and w_2 ↔ 2f indicate that, for at least some of the time, the N-terminal amine and w_2 are in close proximity. Mutual relaxation of *N*-Me and w_2 is observed, but identification of this as a direct NOE is confused by the transfer of saturation between w_2 and the amine NH protons and the NOEs of the latter protons with those of the *N*-Me group. Inspection of CPK molecular models suggests that this region can adopt a conformation, which is consistent with all the NMR data, where the lone pair of the N-terminal amine can assist the exchange of w_2 (see Figure 4). This may occur either by direct methods (see Figure 5a) or via a molecule of solvent (see Figure 5b); the NMR data cannot be used to distinguish between the two mechanisms. The positively polarized nature of w_2, relative to a similar amide proton unperturbed by the N-terminus, is displayed by the upfield shift of 1.27 ppm on acetylation of the N-terminal amine. Figure 4 shows only one form of the N-terminal amino nitrogen; the NH and *N*-Me groups may swap positions by rotation of the α-C–N bond and inversion of the amino nitrogen to the N-terminal orientation shown in Figure 2a. Hence, the *N*-Me group shows NOEs to both 2f and x_1.

The high degree of homology in the NMR data concerning residues 1 and 2 of vancomycin before (basic form) and after addition of HCl or TFA (acidic form) suggests that the conformations adopted by these residues are remarkably similar in both systems. NOEs equivalent to those observed in the nonprotonated form, i.e. *N*-Me ↔ x_1, *N*-Me ↔ 2f, and x_1 ↔ w_2, are observed in the acidic form with similar intensities. The resonances of x_1, w_2, and *N*-Me move downfield 0.78, 0.48, and 0.30 ppm, respectively, on addition of TFA. In comparison, the resonance of x_1 in ristocetin pseudoaglycon moves downfield by 0.89 ppm on protonation of the N-terminus. Ristocetin has a rigidly held N-terminus, and hence the downfield shift of the resonance of x_1 in this system is predominantly electronic in nature. A similar downfield shift for the x_1 resonance of vancomycin is consistent with the N-terminal region of vancomycin *not* undergoing any major conformational changes on protonation of the N-terminal amine.

Residue 3. Residue 3 is bonded to its two neighbors on the peptide backbone, but the side chain is not chemically cross-linked.

Previous studies[3] have shown that $J_{x_3,3a}$ and $J_{x_3,3a'}$ are identical (7 Hz) and that this condition is satisfied by free rotation about the α-C–β-C bond of residue 3. In the basic form of vancomycin the NOEs 3a,a′ ↔ x_3 on the back face (see Figure 2b) and 3a,a′ ↔ w_4 on the front face (see Figure 2a) are consistent with the above conclusion. No NOEs are observed between 3a,a′ and the AsnNH$_2$ protons. An intense NOE between w_3 and x_2, in conjunction with the NOEs w_3 ↔ z_2 and w_3 ↔ 2b, indicates that w_3 is on the back side of the molecule (see Figures 2a and 2b). However, w_3 also has NOEs to w_2, w_4, and 2f, indicating that at some time w_3 is on the front face of the molecule (see Figure 4). Proton w_2 was shown above to occupy the front face, giving the intense NOE w_2 ↔ 2f. An intense NOE between w_4 and 4b, but not between w_4 and x_4, indicates that w_4 is also on the front face.

The presence of w_3 on both the front and back faces of the molecule on the NMR time scale may be rationalized in terms of a fast 180° flipping of the residue 2–residue 3 trans amide unit, from having w_3 in its position in the binding pocket (see ref 4) to having w_3 adjacent to x_2. Inspection of CPK molecular models suggests that sterically such a rotation is plausible if it is the NH (w_3) and not the carbonyl group that passes directly under ring 2. An intense NOE between w_3 and 4b is consistent with the close approach required between these protons in the course of the above rotation. Experience from the investigation of the rotation of ring 2 in CDP-I[3] tells us that CPK models overestimate the van der Waals contribution to rotational barriers of this nature. The observation that the NH group but not the carbonyl group of the amide unit may pass under ring 2 in a CPK model is nevertheless indicative of a considerable kinetic advantage to the direction of rotation proposed above.

The rate of rotation of the w_3 amide unit is such that it may be studied by observing the line width of adjacent proton resonances as the temperature is varied (see Figure 6). At room temperature the resonances of protons w_2, w_3, and w_4 are very broad compared with those of w_5, w_6, and w_7 and have almost zero peak height. On heating, the resonances of w_2 (initially at least), w_3, and w_4 sharpen, with those of w_3 and w_4 attaining approximately the same line width as those of w_5 and w_7 at ca. 350 K. Above 310 K, the exchange of w_2 with traces of water in the solvent becomes the primary determinant of the line width of its resonance. Similar, though less dramatic, sharpening is observed for the resonances of x_2, x_3, and z_2 but not for other α-CH and β-CH protons. Clearly, the protons thus affected would be adjacent to a carbonyl group in one form and a NH group in the other. This is reflected by chemical shift differences for the same resonance in the two forms.

Unfortunately, the rate of rotation is too rapid to isolate the resonances of the two forms on the NMR time scale; i.e., the dynamic process is on the fast-exchange side of the coalescence point of the component resonance frequencies. Cooling below room temperature, which requires a cosolvent for DMSO, leads only to extensive broadening of all resonances. It is therefore impossible to obtain an accurate value for the frequency of the rotation, but the fact that, at room temperature, the resonances of w_2 and w_4 are close to their coalescence points allows a "ballpark" estimation of the frequency involved. The following arguments all refer to a spectrometer frequency of 400 MHz. In the case of ristocetin, acetylation of the N-terminal amine places proton x_1 in a 1,3-relationship to a carbonyl group. This causes a downfield shift of 480 Hz. Consequently, we can estimate a conservative *upper* limit of approximately 1000 Hz for the difference in frequency for the resonances of w_2 and w_4 when adjacent to a carbonyl group compared with being adjacent to an amide NH group (i.e. compare Figures 2a and 4). Therefore, at room temperature, it is likely that the frequency of rotation of the w_3 amide unit is lower than approximately 2000 Hz.

A *lower* limit to the frequency of rotation may be estimated from the change in chemical shift of the resonances of x_2 and x_3 on addition of dipeptide. Although on the opposite face to the binding site, the resonances of x_2 and x_3 move downfield by 250 and 150 Hz, respectively, for vancomycin, compared with 40 Hz and less than 10 Hz for ristocetin. The large changes in chemical

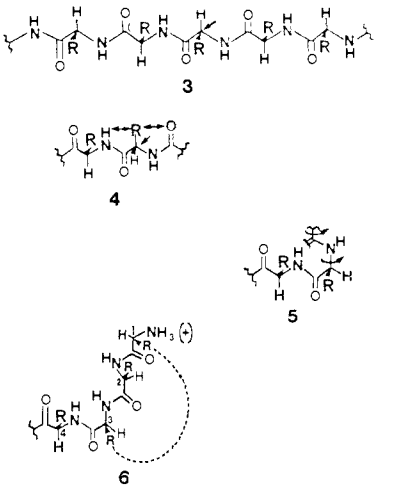

Figure 7. Proposed conformational changes for a linear peptide with a single amino acid of opposite stereochemistry.

shift observed for vancomycin are a result of protons x_2 and x_3 being separated by a carbonyl group when vancomycin is bound to dipeptide (where the NOE w_3 ↔ x_2 is not observed) as opposed to the time average of a carbonyl group and an amide NH group (w_3) when vancomycin is in the free form. Therefore, in the free form a reasonable *minimum* frequency difference for the resonance of x_2 between the conformer with proton w_3 on the back face (as in Figure 2) and that with w_3 on the front face (as in Figure 4) is 100 Hz. Because, at room temperature, the resonance of x_2 is on the fast-exchange side of the coalescence point, the rate of rotation must be greater than approximately 200 Hz. Hence, application of the Boltzmann equation produces an estimate of the barrier to the rotation of the w_3 amide unit of the range 12–14 kcal mol^{-1}. From NOE and line-width studies, the addition of TFA to protonate the N-terminal amine has no measurable effect on the rate of rotation of the w_3 amide unit.

In order to effectively bind the terminal carboxylate anion of its target peptide, the bound conformer of vancomycin has three adjacent amide NH groups. However, in solution, the central amide group exists for part of the time with its carbonyl group in the binding pocket (as shown above). It is important for vancomycin that this latter conformation is not strongly favored energetically with respect to the conformation with three adjacent NH groups because the energy required to overcome such a difference is energy removed from the binding process. Vancomycin achieves similar energies for the two states by having the opposite stereochemistry at the α position of residue 3 compared with that of residues 1, 2, and 4. In a peptide chain of a single stereochemistry, an up–down–up–down alternation of amide NH groups is an energy minimum conformation[11] (see **3** in Figure 7). In this conformation, sterically unfavorable 1,3-interactions between side chain and backbone are minimized, the side chains all being "pseudoequatorial". However, inverting the stereochemistry of one center places one side chain in a "pseudoaxial" orientation as shown in **4**. The resultant 1,3-repulsions can be relieved by rotation to **5**. In **5**, however, new repulsive interactions are introduced. These may in turn be relieved by rotation of the amide bond unit bounded by arrows through 180° to form **6**. This final conformation has three adjacent NH groups and no unfavorable 1,3-interactions involving side chains. In vancomycin, linkage of the side chains numbered 2 and 4 restricts the reorientation of most of this peptide fragment.

A further consequence of the opposite stereochemistry of residue 3 is that, in the above conformation, **6**, its side chain is on the opposite side of the peptide backbone to those of residues 2 and 4. This, in conjunction with the side chain of residue 1, enables these antibiotics to produce a hydrophobic pocket around the carboxylate binding site rather than just a hydrophobic wall. In, for example, ristocetin and teicoplanin, the cross-linkage of the side chains of residues 1 and 3 appears to prevent the rotation of the w_3 amide group; there is no evidence of the population of

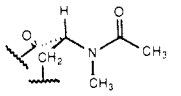

Figure 8. Solution conformation of the backbone region of residue 1 of *N*-acetylvancomycin.

the conformation with w_3 on the back faces of these molecules.

All the antibiotics of the vancomycin group reported to date have the same *R,R,S,R* backbone stereochemistry starting from the N-terminus. Base-catalyzed epimerization of residue 3 occurs readily in teicoplanin,[12] resulting in a *R,R,R,R* system, which has exclusively an alternating up–down–up conformation for the amide NH groups of residues 2–4. This is indicative of the considerable stability of the alternating backbone for single stereochemistry peptides. For non-1,3-cross-linked antibiotics, which are not conformationally restricted with respect to the rotation of the w_3 amide group, it appears that the population of the conformer with three adjacent NH groups is energetically favored by residue 3 having *S* stereochemistry relative to a similar conformation when residue 3 is *R*.

***N*-Acetylvancomycin (2).** Acetylation of the N-terminal amine in vancomycin reduces the binding constant to tripeptide by a factor of 20,[6] which is considerably greater than the corresponding factor for aglycoristocetin (4.5).[13] From inspection of molecular models, it appears that the distances between the protonated N-terminus and the peptidal carboxylate anion are similar and so the charge–charge contribution to the binding constant should be similar. However, it was proposed[6] that vancomycin has the additional advantage that the *N*-Me group is positioned such that it further removes the peptidal carboxylate anion from solvation, hence increasing the binding constant. N-Acetylation causes a reorientation of the *N*-Me group such that it is no longer available to reduce solvent accessibility to the peptide. The relative binding constants are dependent on the energy of the free species in solution as well as changes in interaction energy in the complex. Therefore, in conjunction with the studies of the preferred conformations of the N-terminal region of unbound vancomycin in DMSO solution, it was decided to investigate whether any major changes in conformation occurred on acetylation of the N-terminal amine.

The NOEs involving proton w_3 (see Figure 1) and the variation of the line width of its resonance with temperature remained unchanged from those of vancomycin, above, indicating that the w_3 amide unit was still undergoing a 180° rotation. The resonance of proton w_2 no longer showed saturation transfer with the residual water in the solvent and had moved upfield by 1.27 ppm (see Table I); i.e., the deshielding influence of the N-terminal amine is removed by acetylation. Intense NOEs were again observable from w_2 to 2f and x_1, confirming that these protons remain close in space after acetylation.

The only significant differences observed upon N-acetylation were within residue 1, the most mobile region of vancomycin. In *N*-acetylvancomycin, an intense NOE is observed between the acetyl methyl group (Ac Me) and the *N*-Me group, indicating that it is predominantly the *N*-Me group and not the *N*-alkyl group that is trans to the *N*-acetyl carbonyl oxygen. The large NOE observed between the *N*-Me group and x_1 in vancomycin is virtually lost, reflecting an approximately trans relationship of these groups. The disappearance of the NOE *N*-Me ↔ 2f and the appearance of the NOE Ac Me ↔ 2f is supportive of a rotation of the *N*-Me group away from the binding pocket on *N*-acetylation.

The conformation of the backbone region of residue 1 described above is shown in Figure 8 and would necessitate a close 1,3-relationship of x_1 with the acetyl carbonyl group. This is reflected by a downfield shift of x_1 by 2.08 ppm on N-acetylation (compared with 0.78 ppm on protonation of the N-terminus). A similar downfield shift (1.2 ppm) is observed on N-acetylation of ristocetin pseudoaglycon,[14] where the residue 1 side chain is more conformationally restricted. As in vancomycin, CPK models suggest a relatively small barrier to rotation about the α-C–carbonyl C bond of residue 1, but again no evidence exists for the significant population of drastically different rotamers to that/those described above. The motion of the side chain of residue 1 appears to be slower after N-acetylation. The Leu CH_2 group (1a,a′) shares negative NOEs with x_1 and the *N*-Me group and the Leu Me groups (1c,c′) with x_1.

Conclusion

In solution, the N-terminal region of vancomycin is considerably more mobile than its C-terminal region. The terminus of the isobutyl side chain of residue 1 rotates at a frequency in excess of 3×10^8 Hz, i.e. at least 1 order of magnitude faster than the overall rotation of vancomycin. The rotational frequency of this side chain is considerably reduced on protonation of the N-terminal amine. As a free base, the N-terminal amine catalyzes the exchange of the first amide NH group (w_2) with water in the solvent, increasing the exchange rate by greater than 2 orders of magnitude. This phenomenon is removed by protonation or acetylation of the N-terminal amine. The amide bond unit connecting residues 2 and 3 (which contains proton w_3) rotates approximately 180° between a conformer with three adjacent NH groups in a similar orientation to that observed in the vancomycin–tripeptide complex and a conformation where the carbonyl group of the w_3 amide unit separates protons w_2 and w_4. Proton w_3 passes under the face of ring 2 and close to proton 4b, giving a barrier to rotation of 12–14 kcal mol^{-1}. The above two conformers are of comparable energy, which we propose is a consequence of the stereochemistry of residue 3 being opposite to that of residues 1, 2, and 4. This similarity in energy is in stark contrast to the overwhelming population of an alternating up–down–up conformation of NH groups observed in *epi*-teicoplanin where the stereochemistries of residues 1–4 are all *R*.

The only significant differences in the solution conformation of *N*-acetylvancomycin compared with vancomycin is that the *N*-Me group is no longer close to proton x_1 but is positionally replaced by the acetyl group oxygen. This brings the Ac Me group and the *N*-Me group close in space.

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