Preparation and Conformational Analysis of Vancomycin Hexapeptide and Aglucovancomycin Hexapeptide

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The preparations of the hexapeptides (**3**) and (**6**) derived from the antibiotic vancomycin (**1**), are described. The conformation of the *N*-terminal regions of (**3**) and (**6**) are discussed and compared to previous observations made on vancomycin. The hexapeptides lack any significant binding capability relative to vancomycin, and the reasons for this are discussed.

The glycopeptide antibiotic vancomycin (**1**) has been in clinical use for over 20 years, but is now assuming growing importance owing to its effectiveness in the treatment of methicillin-resistant *Staphylococcus aureus* infections and of *Clostridium difficile*-induced pseudomembranous colitis.[1] Since there is as yet no unequivocal evidence of bacterial resistance to vancomycin, it is likely to become of still greater therapeutic importance in the light of the increasing incidence of Gram-positive bacteria resistant to other antibiotics.[2] Vancomycin acts to inhibit bacterial cell-wall biosynthesis by binding tightly and specifically to cell-wall mucopeptide precursors terminating in D-Ala-D-Ala.[3] This interaction may be modelled *in vitro* using short cell-wall precursor analogues such as di-Ac-L-Lys-D-Ala-D-Ala or Ac-D-Ala-D-Ala, and the results studied by highfield ^{1}H n.m.r. and difference u.v. spectroscopy. Analysis of these results has led to a detailed binding model for the interaction of vancomycin with its substrate peptides, and this constitutes arguably the most thoroughly characterised example of specific small peptide-small peptide binding.[4] In light of this, study of the binding behaviour of vancomycin appears to offer an excellent opportunity to test current theories of molecular recognition.

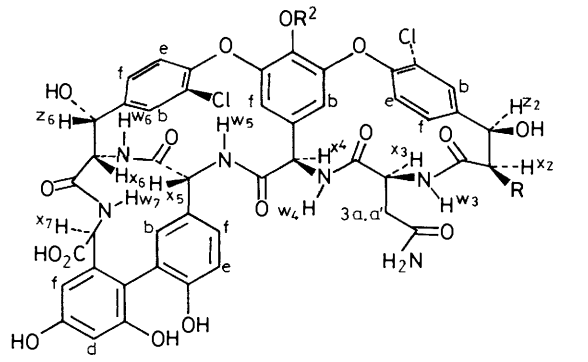
Although the synthetic modification of naturally derived antibiotics is a common tool in the search for new drugs,[5] the opportunity rarely exists for direct study of the interaction at the molecular level of the drug with its receptor. If the structure of vancomycin could be systematically altered in some manner, the resulting changes in binding energy and specificity could be studied in unique detail. Up to the present time, the major drawback to exploiting this opportunity has been the complexity of the structure of vancomycin. The high degree of cross-linking of the peptide backbone has restricted synthetic modification to the essentially trivial preparation of the aglycone,[6] iodovancomycin,[7] and of the mono- and di-dechloro derivatives.[8] It occurred to us that the *N*-methyl-D-leucine residue of vancomycin should be amenable to removal *via* an Edman-type degradation since it possesses a reactive amino group, and is unencumbered by cross-linking to other residues in the vancomycin backbone. This was attractive for our purpose, since previous studies have shown that a major portion of the binding ability of vancomycin is centred about a 'binding pocket' (see below) formed by the *N*-terminal portion of the molecule.[9] Hence, by removing the *N*-terminal residue and replacing it with different amino acids, we are directly affecting the principal site of binding. Additionally, the conformation of this region has been analysed in great detail with respect to structural features believed to be important in the formation of this binding pocket,[10] and we were interested in the effect on this conformation of removing the *N*-terminal residue. We describe below the preparation of vancomycin hexapeptide and aglucovancomycin hexapeptide,[11] and a preliminary conformational analysis of their *N*-terminal regions.

Preparation of Vancomycin Hexapeptide.—The Edman degradation is a well-known and valuable procedure which allows the stepwise cleavage of the *N*-terminal residue from a peptide or protein molecule.[12] It has previously been applied to the vancomycin group of antibiotics as an aid to the structural assignment of avoparcin.[13] In this case however, only the phenylthiohydantoin derivatives from the cleavage steps were characterised. We reasoned that the Edman procedure could also be applied to vancomycin, and that isolation of the other cleavage product, which we name vancomycin hexapeptide, should be possible.

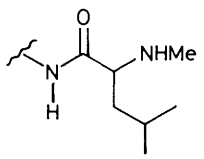
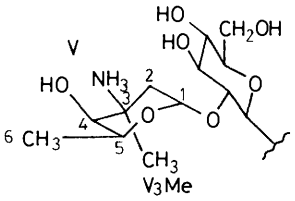
Accordingly, vancomycin hydrochloride was treated with phenyl isothiocyanate (1.2 equiv.) in pyridine–water (1:1), to afford a single new compound by reversed-phase h.p.l.c. analysis. A 250 MHz n.m.r. spectrum of this material showed a downfield shift of the *N*-methyl group of *N*-methyl-leucine by 0.75 p.p.m., which together with a molecular weight of 1582 as determined by f.a.b.m.s. confirmed that selective reaction had occurred at the *N*-terminus of vancomycin to afford the mixed thiourea (**2**).

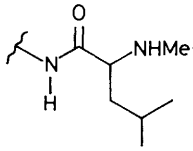
Optimum conditions for the cleavage of (**2**) were found to be treatment with trifluoroacetic acid in dichloromethane for 1–2 min, to afford a single new compound by reversed-phase h.p.l.c. analysis. Longer reaction times led to loss of the sugar residues of (**2**). F.a.b.m.s. analysis of this compound indicated a molecular weight of 1230, and this, together with a complete assignment of the 400 MHz n.m.r. spectrum of the material in [$^2H_6$]-DMSO solution (see below) indicated that the desired cleavage of the *N*-methyl-leucine had indeed occurred to afford (**3**).

An interesting feature of the n.m.r. spectrum of (**3**) was the change in chemical shift and coupling constants of the asparagine β-protons in going from native vancomycin (**1**) to the hexapeptide (**3**). In (**1**), in DMSO solution these occur at 2.20 and 2.45 p.p.m.; both with a coupling constant of 7 Hz to the α-CH of the asparagine residue (designated *x*3 in our previously described nomenclature).[10] These coupling constants indicate equal population of the possible rotamers during fast rotation about the C(α)–(Cβ) bond of residue 3. In the hexapeptide (**3**), the β protons (designated 3a and 3a′) occur at δ2.15 and 2.90, with coupling constants of 8 and <1Hz to *x*3, respectively, indicating that the equilibrium populations of the rotamers are no longer equal, *i.e.* the side chain displays some conformational preference. Disturbingly, the chemical shifts of these β-protons were very close to those observed in CDP-1 (**4**), a well-known degradation product of vancomycin, which is devoid of antibiotic activity due to disruption of the binding pocket region (see below). In CDP-1, 3a and 3a′ are incorporated into a

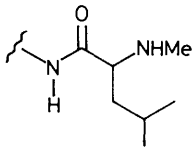


Compound: R^1 R^2

(1)  

(2)  NHMeC(S)NHPh as in (1)

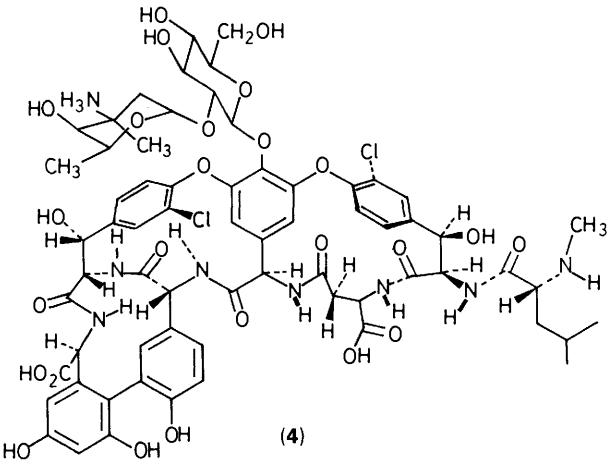
(3) NH_3^+ as in (1)

(5)  H

(6) NH_3^+ H

Figure 1. The structure of vancomycin (**1**) and derivatives, including the code used to designate ^1H n.m.r. resonances

large cyclophane ring *via* an asparagine–isoaspartate rearrangement and become diastereotopic, hence their widely differing chemical shifts. Three pieces of evidence militated against compound (**3**) having undergone CDP-1-like rearrangement however. First, two resonances at δ 7.15 and 7.52 could be assigned to the asparagine NH_2 protons in the 400 MHz n.m.r. spectrum of (**3**). Second, the coupling constants of protons 3a and 3a′ to *x*3 in CDP-1 have been shown to be approximately 3 Hz each[14] as compared with 8 and <1 Hz in (**3**). This could perhaps have been accounted for by a change in the con-



(**4**)

formation of the cyclophane ring on removal of the *N*-terminal amino acid however. Third, CDP-1 has been shown to exist in two interconverting atropisomeric forms,[14] owing to rotation of the aromatic ring of residue 2 about its axis. The half-life of this interconversion process is several hours, allowing separation of the two isomers by h.p.l.c. Despite extensive experimentation, compound (**3**) could not be induced to separate into two isomers by h.p.l.c.

Unequivocal proof that (**3**) had not undergone rearrangement was obtained by preparation of CDP-1 hexapeptide. CDP-1, prepared by a literature method,[6] was treated under conditions identical with those used for vancomycin, to afford CDP-1 hexapeptide. The resultant compound displayed two peaks on h.p.l.c. analysis which slowly interconverted on a time scale of several hours. In the n.m.r. spectrum of the major CDP-1 isomer the isoaspartate β-protons occurred at 1.95 and 3.00 p.p.m. and in the minor isomer at 2.10 and 2.97 p.p.m.; all clearly different from the shifts observed in (**3**). Furthermore, neither of the CDP-1 isomers was co-eluted with (**3**) on h.p.l.c. analysis, confirming that no arrangement of the latter had taken place. The reasons for the anomalous n.m.r. behaviour of compound (**3**) are detailed below.

Starting from aglucovancomycin (**5**), the hexapeptide (**6**) was also prepared in an essentially identical manner to that described above.

Conformational Analysis.—The ^1H n.m.r. spectra of samples of purified vancomycin hexapeptide (**3**) and aglucovancomycin hexapeptide (**6**) were recorded in [$^2H_6$]-DMSO, and resonance assignments made with the aid of 2-dimensional COSY[15] and NOESY[16] techniques. The observed chemical shifts are listed in the Table. Since (**3**) and (**6**) were purified using h.p.l.c. solvents containing 0.1% trifluoroacetic acid as modifier, it was assumed that they were present in the form protonated at both amino groups. This was confirmed by observation of significant changes in the spectra of (**3**) and (**6**) on addition of 1 equiv. of NaOD (see below). The spectrum of (**6**) in the presence of NaOD was also assigned *via* COSY and NOESY techniques (see Table). For purposes of comparison the chemical shifts observed in vancomycin in both the protonated ('acid' form) and deprotonated ('basic' form) states are also listed.

It is readily apparent from the Table that the major differences between the spectra of vancomycin and its hexapeptide derivative lie in the region of residue 3. Both the chemical shifts and coupling constants of 3a and 3a′ indicate that residue 3 exhibits a substantially different conformational bias in the two molecules. This is of great interest from the viewpoint of understanding the binding behaviour of vancomycin, since this

Table. Chemical shifts (p.p.m.) of vancomycin and derivatives

Proton	Vancomycin hexapeptide (acid form)	Aglucovancomycin-hexapeptide (acid form)	Vancomycin (acid form)	Vancomycin (base form)	Aglucovancomycin-hexapeptide (base form)
v6	1.10		1.07		
v3 Me	1.36		1.35		
v2	1.75		1.75		
v2′	1.92		1.90		
3a	2.15	2.12	2.15	2.16	2.10
3a′	2.90	2.90	2.53	2.32	2.35
v4	3.20		3.2		
x3	4.10	4.06	4.30	4.42	4.55
x6	4.20	4.15	4.17	4.18	4.20
x2	4.49	4.52	4.92	4.90	3.75
x5	4.49	4.40	4.53	4.42	4.46
x7	4.49	4.40	4.43	4.36	4.46
v5	4.69		4.68		
4f	5.17	5.10	5.20	5.22	5.15
z6	5.20	5.10	5.12	5.12	5.05
z2	5.29	5.15	5.21	5.14	5.05
v1	5.30		5.22		
4b	5.68	5.62	5.58	5.50	5.47
x4	5.87	5.32	5.27	5.76	5.75
7f	6.28	6.26	6.25	6.34	6.20
7d	6.40	6.40	6.44	6.34	6.40
w3	6.46	6.59	6.59	6.78	
w6	6.50	6.70	6.68	6.62	6.80
5e	6.70	6.75	6.72	6.70	7.15
5f	6.78	6.75	6.77	6.74	6.75
Asn NH	7.10	7.32	7.02	6.92	
5b	7.15	7.15	7.17	7.16	7.15
2e	7.20	7.30	7.20	7.24	7.15
6e	7.30	7.26	7.35	7.32	7.16
6f	7.49	7.47	7.47	7.44	7.45
2f	7.53	7.49	7.59	7.52	7.60
Asn NH	7.53	7.65	7.49	7.36	
2b	7.63	7.60	7.46	7.34	7.15
6b	7.88	7.84	7.87	7.88	7.85
w2	8.25	8.25	8.59	8.11	
w7	8.50	8.60	8.53	8.46	8.48
w4	8.65	8.40	8.2	8.22	8.30
w5	8.66	8.77	8.66	8.62	8.65

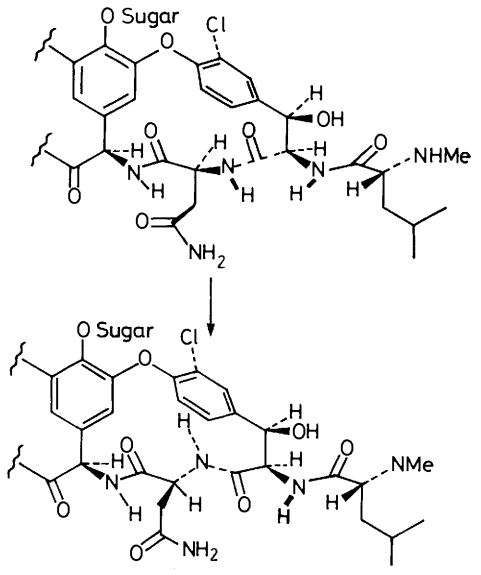


Figure 2. A schematic representation of the rotation of the w_3 amide unit of vancomycin, with the amide proton passing from the front face, as in (a), to the rear face, as in (b)

asparagine residue is involved directly in the 'binding pocket' of vancomycin as mentioned earlier. This unusual structural feature consists of the adjacent backbone amide protons (w2, w3, and w4) of residues 2, 3, and 4, all pointing inwards on one face of the molecule (defined henceforth as the front face of the molecule) in the presence of bound substrate. In the absence of substrate it has previously been shown that the w3 amide unit flips rapidly between this binding conformation and one in which w3 is at the rear of the molecule, with a barrier to rotation of 12—14 kcal mol^{-1} (see Figure 2). The normally energetically unfavourable feature of having three adjacent amide protons pointing in the same direction is believed to be stabilized in vancomycin by the effect of residue 3 bearing the opposite absolute configuration to those of residues 1, 2, and 4, and also by the cross linking of the side chains of residue 2 and 4. These features combine to destabilize the more common β-pleated sheet type conformation found in peptides, and stabilize the binding conformation.[10] Clearly it is important to be able to rationalize the change in conformation of residue 3 in going from (**1**) to (**3**) if we are truly to understand what makes vancomycin such a strong and substrate-specific binding molecule.

The coupling constants of 8 and <1 Hz of 3a and 3a′ to *x*3 respectively, together with the observation of relatively strong

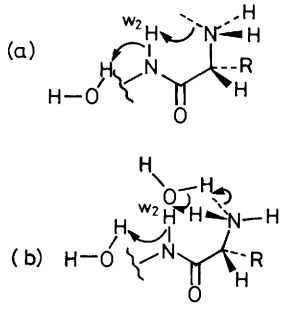


Figure 3. Schematic representation of possible mechanisms for the catalysis of exchange of the amide proton w2 and water in the solvent by the neutral *N*-terminal amine (a) directly and (b) *via* a molecule of water

n.O.e.s between 3a and w4 and between 3a′ and x3, are compatible with a conformation in which the dihedral angle between 3a and x3 is *ca.* 200° and that between 3a′ and x3 is *ca.* 90°. Weak n.O.e.s are also seen from 3a′ and w3 and from 3a to x3, but these are probably due either to spin diffusion or to the transmission of n.O.e. effects *via* the strong (15 Hz) geminal coupling.[17] An n.O.e. is seen from w3 to 2b, the aromatic proton of residue 2 adjacent to chlorine, on the back face of the molecule, but none is observed from w3 to w4. This indicates that the w3 amide unit in (**3**) does not adopt the conformation in which w3 sits at the front of the molecule, but adopts exclusively that conformation where the carbonyl of the amide unit sits in the binding pocket. Examination of CPK molecular models indicates that two possible conformations of the asparagine side-chain satisfy the appropriate dihedral angles required to produce the characteristic coupling constants of 3a and 3a′. There is no obvious reason for the unusual chemical shift of 3a′. Hence it appears that changing the weakly positive charge of the residue 2 amide function in vancomycin to the full positive charge of the protonated amino group in (**3**) increases the electrostatic repulsion felt by w3 when occupying a position in the binding pocket to such an extent that this conformation is not populated.

In order to probe further whether the close proximity of the new protonated *N*-terminus does, in fact, change the conformation of residue 3, the conformation of (**6**) was studied in [$^2H_6$]-DMSO solution containing one equivalent of NaOD, to generate the 'free base', in which the *N*-terminus is unprotonated. Upon addition of base, the resonance due to w3 broadened to such an extent that it could no longer be detected. No significant broadening of the other amide NH resonances was observed. This finding was consistent with the now-basic *N*-terminus of the molecule catalysing proton exchange between w2 and residual water in the solvent, in a manner analogous to that found in vancomycin[10] (see Figure 3). This exchange broadening of the resonance of the amide proton adjacent to the *N*-terminus appears to be a general phenomenon in peptides when the *N*-terminus is a free amine.[18] In the case of (**6**), however, examination of molecular models indicates that such catalysis is impossible if w3 remains on the back face of the molecule, since it is then spatially remote from the *N*-terminus. The catalytic process is only possible if the w3 unit rotates around into the binding pocket on the front face of the molecule, and this would therefore suggest that, in the absence of a positive charge on the *N*-terminus, compound (**6**), and by analogy, compound (**3**), may adopt a conformation similar to the binding conformation found in vancomycin. Further examination of the spectrum of (**6**) under basic conditions revealed substantial broadening of the resonances assigned to 3a′, x2, x3, z2, 2b, and the side-chain NH_2 protons, indicating that these protons were involved in some exchange process, occurring at a rate intermediate between fast and slow exchange (on the n.m.r. timescale). Since in the fast exchange limit the degree of broadening of a resonance is greater the greater the chemical-shift difference between the two environments, no broadening was observed for the resonance of proton 3a, which shifted only 0.02 p.p.m. on addition of base. Additionally, the resonance of 3a′ shifted upfield to 2.35 p.p.m. (*cf.* 2.32 in vancomycin). On warming the n.m.r solution all these resonances sharpened, consistent with the exchange phenomenon moving towards the fast exchange limit. Resonance 3a′ sharpened to a sufficient extent that a coupling constant of *ca.* 7 Hz to x3 could be observed, implying that free rotation had been restored to the asparagine side-chain. All these results are entirely consistent with the 'free-base' form of compound (**6**) displaying essentially identical motional phenomena about the amide unit of residue 3 to those depicted in Figure 1 for vancomycin itself.[10] The exchange broadening of the resonances of residues 2 and 3 reflects chemical-shift differences for the same resonances between the conformers where w3 is at the front and back faces of the molecule. Unfortunately, the exchange broadening effect precluded the observation of any n.O.e.s from w3 to either or both of 2b and w4, which would have provided confirmatory evidence for the rotation of the amide unit.

It is pertinent with reference to the solution conformation of vancomycin hexapeptide that both (**3**) and (**6**) display negligible binding to model peptides such as Ac-D-Ala-D-Ala, as determined by both difference u.v. and n.m.r. spectroscopy. This is not surprising, given that at the usual pH for these measurements (pH 5.6), the *N*-terminus would be protonated, and hence, as described above, the binding pocket conformation would not be populated to an extent significant enough to promote binding. In principle, formation of the binding pocket could be induced by the electrostatic field associated with the carboxylate anion of the cell wall analogue, as in vancomycin itself (where pocket formation is complete in the complex, but not in the free state[10]). However, in the case of the hexapeptides, the lack of significant binding ($K \leqslant 50\ M^{-1}$) establishes that the free energy of product formation is unfavourable, even in the presence of the cell-wall analogue.

Conclusions.—Cleavage of the *N*-terminal residue from vancomycin (**1**) and aglucovancomycin (**5**) to yield the hexapeptide derivatives (**3**) and (**6**) has been achieved in an efficient manner. The conformations of the *N*-terminal regions of (**3**) and (**6**) have been studied by highfield n.m.r. spectroscopy. When the *N*-terminus of either is deprotonated, the residue 2-residue 3 *trans* amide unit exhibits a fast 180° flipping motion, from having the amide proton w3 at the front of the molecule, to having it at the rear. This motion is essentially identical with that found in vancomycin. When the *N*-termini of the hexapeptides are protonated, a single conformation of the molecule is seen, with w3 at the back of the molecule. This change in conformation on protonation of the *N*-terminus is due to the additional electrostatic repulsion felt within the w2-w3-w4 binding pocket region. This outweighs those influences which help to favour the formation of the binding pocket, as previously discussed.[10] Hence the presence or absence of a proximal ionic charge upon the dipole associated with a peptide backbone can exert a powerful influence upon the conformation of that backbone. This has clear implications for conformation-dependent binding phenomena such as drug-receptor interactions and protein folding.

Experimental

Vancomycin hydrochloride was obtained as a gift from Eli Lilly and Co., and used without further purification. Aglucovancomycin and CDP-1 were prepared by the method of Marshall.[6] Reversed-phase h.p.l.c. was carried out using a Waters 6000A

solvent-delivery system; C-18 columns were employed with 0.1% aqueous TFA–acetonitrile mixtures as eluants. ^{1}H n.m.r. spectra of solutions in [$^2H_6$]-DMSO were run on Bruker WM250, AM400 and WH400 spectrometers using quadrature detection. One-dimensional spectra were obtained using spectral widths of *ca.* 4000 Hz, and 16K data points were recorded. Two-dimensional spectra—double quantum filtered COSY and NOESY experiments—were run in the phase-sensitive mode with time-proportional phase incrementation. Typically 2K data points were recorded in f2 and 512 data points in f1. All NOESY experiments employed a 20 ms z-filter. In all cases the data matrices were zero-filled in f1 and subjected to Lorentzian-Gaussian multiplication prior to transformation. F.a.b. spectra were obtained using thioglycerol/glycerol matrices on a Kratos MS50 mass spectrometer.

All hexapeptide derivatives were prepared in identical fashion. The following serves as a general procedure:

Preparation of Vancomycin Hexapeptide (**3**).—To a solution of vancomycin hydrochloride (100 mg, 67 μmol) in pyridine (0.2 ml) and water (0.2 ml), was added a solution of phenyl isothiocyanate (10 mg, 74 μmol) in DMF (100 μl). The mixture was stirred overnight and evaporated to dryness. Water (1 ml) was added and the mixture lyophilised. To the resultant buff powder was added dichloromethane (1 ml), followed by trifluoroacetic acid (1 ml). The mixture was stirred for 2 min and evaporated to dryness. Water (5 ml) was added and the resultant suspension extracted with diethyl ether (2 × 5 ml). The aqueous layer was lyophilised, and resuspended in water (1 ml). This suspension was filtered and the filtrate purified by reversed-phase h.p.l.c. to yield pure vancomycin hexapeptide as a white solid (30 mg, 33%).

Acknowledgements

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