Two Conformers of the Glycopeptide Antibiotic Teicoplanin with Distinct Ligand Binding Sites

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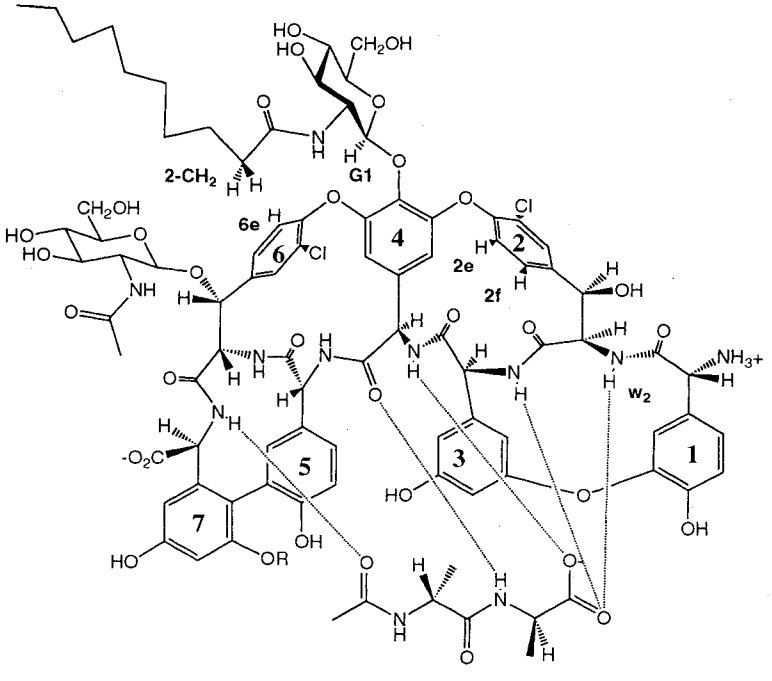
The clinically important vancomycin group glycopeptide antibiotics, which act by blocking cell wall synthesis, are crucial in the treatment of methicillin resistant *Staphylococcus aureus*. All of the group members studied so far, with the apparent exception of teicoplanin, enhance their antibiotic action by the formation of an asymmetric homodimer. Teicoplanin exists in two main conformers which differ by a rotation of approximately 180° of a sugar residue. From NMR studies and molecular modelling, we present structures for the two conformers and conclude that they have different binding affinities for cell wall analogues. The two conformers of teicoplanin are closely analogous to those adopted by each half of the asymmetric dimers of the other vancomycin group antibiotics.

The vancomycin family of glycopeptide antibiotics[1~4] are clinically important compounds, being active against Gram-positive bacteria. The emerging methicillin resistant *Staphylococcus aureus* (MRSA), which are responsible for many pneumonia and post surgical infections, are typically resistant to penicillin and ampicillin and also other antibiotics such as erythromycin, tetracycline and sulphonamides.[5] Today, in excess of 95% of *S. aureus* are resistant to penicillin and ampicillin[5] and a large fraction are resistant to all antibiotics except those of the vancomycin group. Consequently, these antibiotics have become a last line of defence against post-surgical infections. Recently, some resistance to vancomycin has emerged and the mechanism of resistance is under investigation.[6~8]

The antibiotic activity of the vancomycin group arises

Fig. 1. Exploded structure of teicoplanin complex with the cell wall analogue *N*-acetyl-D-Ala-D-Ala.



The hydrogen bonds that are formed in the complex are shown. Note the decanoylglucosamine residue attached to ring 4 as referred to in the text. The atom labels w_2, 2e, 2f, G1, 6e, and 2-CH_2 are also for reference from the text.

from their ability to block cell wall synthesis through the recognition of immature cell wall terminating in the sequence -L-Lys-D-Ala-D-Ala[9~11] (Fig. 1). The activity is further enhanced by homodimerization of the antibiotics which, with the exception of ristocetin A, is cooperative with the binding of peptides terminating in -L-Lys-D-Ala-D-Ala.[12~15] The concave face of each antibiotic molecule forms a pocket which binds cell wall peptides through several specific, non-covalent interactions, while the back faces of each molecule are able to interact in a highly complementary head-to-tail geometry.[12,13]

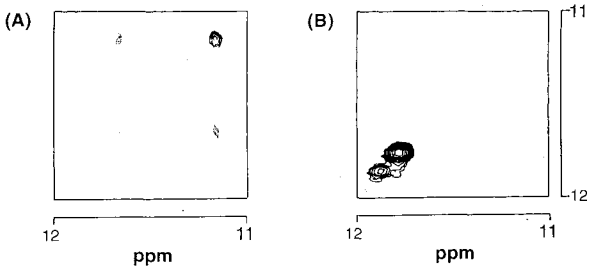
Recent work in this laboratory has shown that when the head-to-tail dimers of the vancomycin group glycopeptide antibiotics are formed, the sugars on ring 4 interact head-to-head thus forming an asymmetric dimer with no rotational axis of symmetry.[16] As has been described previously[15] one member of this group of antibiotics, teicoplanin, shows no measurable dimerization and has a decanoyl chain attached to the ring 4 glucosamine (Fig. 1) which is postulated to bury itself in the bacterial cell membrane, and thus act as a membrane anchor.[15] Despite this lack of dimerization, we now report that teicoplanin shows a property characteristic of the dimeric structures insofar as it exists in two predominant conformations differing by a rotation of the decanoylglucosamine relative to the peptide backbone; these two conformers have different binding affinity for bacterial cell wall analogues. We also show that, as postulated, teicoplanin does indeed anchor itself into a model cell membrane.

Results and Discussion

Observations in NMR Spectra of Free and Ligand Bound Teicoplanin

The existence of two forms of teicoplanin is apparent in Fig. 2. In the NMR spectra of the complexes of teicoplanin with di-*N*-acetyl-D-Ala and *N*-acetyl-D-Ala-D-Ala, the only signal to appear in the 11 ~ 12 ppm region is that of the amide proton labelled w_2 in Fig. 1. All of the signals from the NH protons of the glycopeptide antibiotics involved in hydrogen bonding within the complex undergo downfield shifts compared to their free value, but the shift of w_2 is the greatest.[17,18] Despite this assertion that only one signal appears in the 11 ~ 12 ppm region, two signals are apparent along the diagonal of the NOESY spectra of the complexes of teicoplanin with di-*N*-acetyl-D-Ala and *N*-acetyl-D-Ala-D-Ala (Figs. 2A and 2B respectively). It could be inferred

Fig. 2. The 11 ~ 12 ppm region of the NOESY spectra of the teicoplanin-cell wall analogue complexes.



Only the NH proton w_2 gives a signal in this region. (A) The complex with *N*-acetyl-D-Ala which shows some chemical exchange between the two diagonal peaks indicating that there are two distinct environments for the proton w_2 and, (B) the complex with *N*-acetyl-D-Ala-D-Ala.

that this is a result of impurities in the ligand, or even the antibiotic, but the crosspeaks due to chemical exchange (which are clearest in Fig. 2A) show that this is not the case. In a NOESY spectrum, if a proton exchanges its environment with another due to the change in chemical environment, a crosspeak can be seen as the magnetization is transferred from one signal to another; this is not an NOE crosspeak but a chemical exchange crosspeak. Hence, we conclude that there are two forms of the teicoplanin-peptide complexes exchanging quickly enough to give chemical exchange crosspeaks in the NOESY experiments. It has also been shown that the absolute shift of the signal from the NH proton w_2 is related to the binding constant of the ligand to the antibiotic.[17,18] Thus, we can also conclude that the two forms of the teicoplanin-peptide complex with either *N*-acetyl-D-Ala or *N*-acetyl-D-Ala-D-Ala have different binding constants because they have different values of the chemical shift of the NH proton w_2.

Teicoplanin is known to form non-specific aggregates in aqueous solution[19,20] which are evidenced in the ^1H NMR spectrum (Fig. 3A) by the appearance of broad lines. On the addition of *N*-acetyl-D-Ala-D-Ala, the signals become sharp (Fig. 3) indicating that the micelle has dispersed into individual molecules. It may be that one of the two conformers of teicoplanin is preferred for ligand binding, but the other conformer is preferred for micelle formation. Thus, on the addition of ligand, the teicoplanin changes from one conformation (forming micelles) to the other (binding ligand).

To investigate further the two forms of teicoplanin that were apparent from the two chemical shifts of w_2 and further suggested by the dispersion of the teicoplanin micelles, more detailed structural analysis was required.

In the NOESY spectra of teicoplanin alone and complexed with *N*-acetyl-D-Ala-D-Ala, the crosspeaks include two that cannot be satisfied simultaneously by a single conformer of teicoplanin. These NOE enhancements are for the protons G1 to 2e, and 6e to the first methylene group of the decanoyl chain (labelled in Fig. 1). For the G1→2e crosspeak to be satisfied, the hydrophobic face of the glucose on ring 4 must lie over the ligand binding pocket; for 6e→acyl 2-CH_2 the opposite face of the glucose ring must lie over the ligand binding pocket. Extra ligand binding energy may be attained in the first case, since the hydrophobic face of the glucose is buried against the methyl group of the C-terminal alanine residue of *N*-acetyl-D-Ala-D-Ala, enhancing ligand binding through the hydrophobic effect.

Fig. 3. Titration of *N*-acetyl-D-Ala-D-Ala into a solution of teicoplanin (*ca.* 4 mM) in D_2O.

The approximate ratio of *N*-acetyl-D-Ala-D-Ala/teicoplanin is (A) 0 : 1, (B) 0.5 : 1, (C) 1 : 1, (D) 1.5 : 1, (E) 2 : 1.

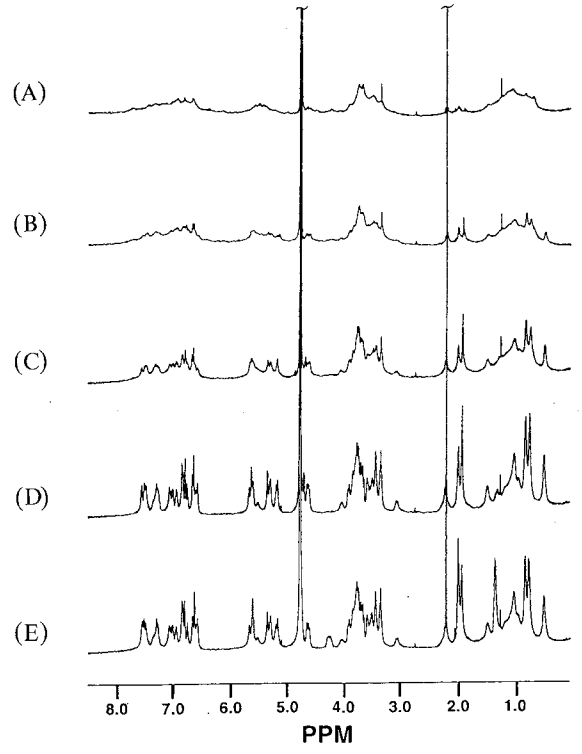
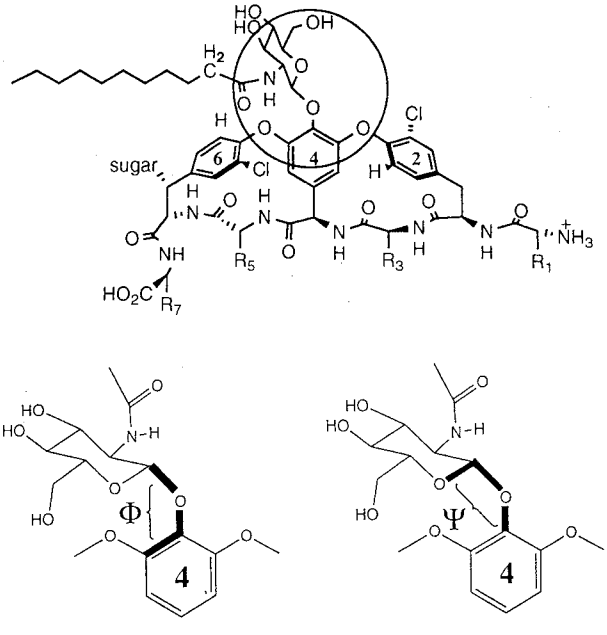


Fig. 4. The dihedral angles Φ and Ψ between the peptide residue 4 and the pendant sugar residue as used in the molecular modelling.



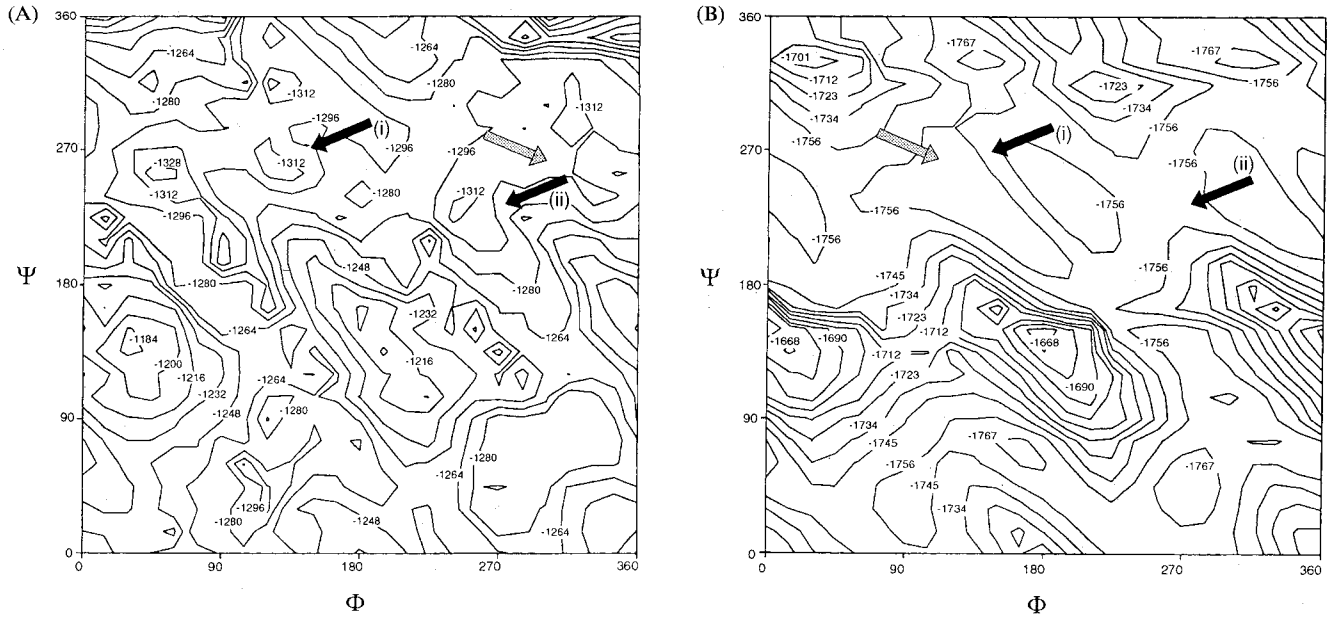
Relative interatomic distances of the atoms which give the above mutually exclusive NOE enhancements were estimated by the NOE build-up rate relative to that of 2e→2f, separated by 2.5 Å. The G1→2e distance was estimated as 3.2 Å in free teicoplanin differing little from the 3.5 Å estimated in the complex with *N*-acetyl-D-Ala-D-Ala. However, the 6e→acyl 2-CH_2 distance *apparently* increases significantly from 3.8 Å to 6.5 Å on the addition of the same ligand. Clearly, the *apparent* distance of 6.5 Å in reality reflects a time-averaged situation where, in the complex of teicoplanin with *N*-acetyl-D-Ala-D-Ala, the conformation satisfying the G1→2e NOE enhancement is favored over that which satisfies the 6e→acyl 2-CH_2 NOE. That is, the latter conformation is much less populated.

Use of Molecular Modelling to Explore the Conformational Space of the Ring 4 Sugar Residues

In view of the evidence above for two predominant forms of teicoplanin with one favored for ligand binding, molecular modelling was used to investigate the effect of changing the dihedral angles, Φ and Ψ (Fig. 4) on the overall energy of free teicoplanin and the complex with cell wall analogue peptide. Thus Ramachandran-style plots were produced (Fig. 5) for the energy minimised structures, in 15° increments, where the contours represent energy levels which vary with angles Φ and Ψ. The Ramachandran-style plots show that there are distinct regions of low energy where preferred conformations may be expected for both bound and free teicoplanin. The values of Φ and Ψ which give the conformers with minimum separation of the protons that give mutually exclusive NOE enhancements are shown by solid black arrows in Fig. 5; (i) G1—2e at $\Phi = 146°$, $\Psi = 266°$, and (ii) 6e—acyl 2-CH_2 at $\Phi = 274°$, $\Psi = 234°$. The points (i) and (ii) are close to regions of low energy in the Ramachandran plots and more detailed analysis of the region in which they lie ($\Phi = 0 \sim 360°$ in 15°

Fig. 5. Ramachandran-style plots showing the energy contours for the conformational space available to the ring 4 sugar residue.

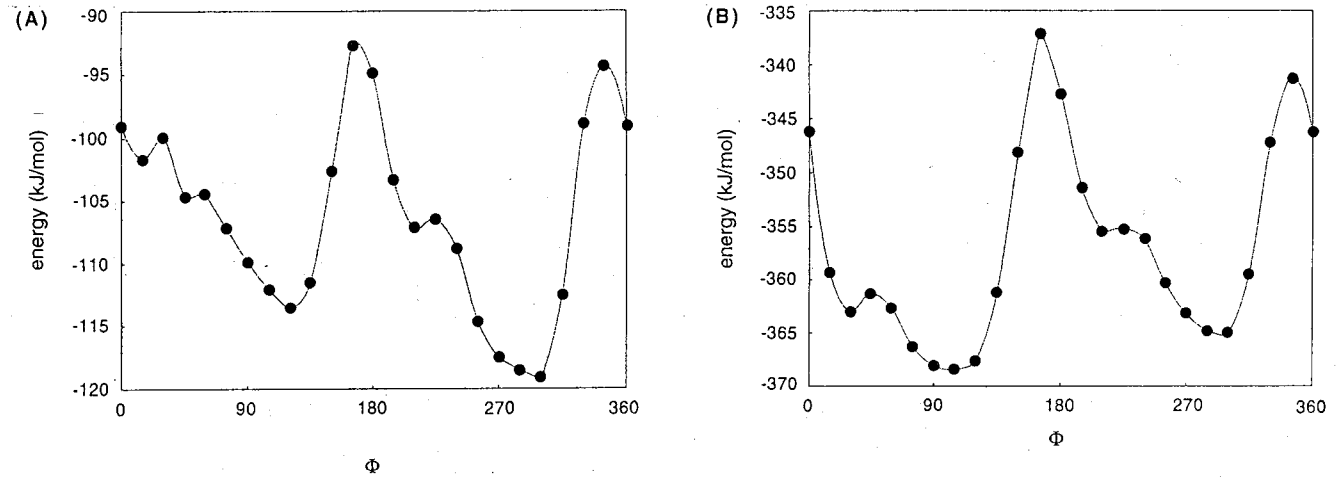
(A) Free teicoplanin, the contours representing the energies $-1328\,kJ\,mol^{-1}$ to $-1184\,kJ\,mol^{-1}$ in 9 increments of $16\,kJ\,mol^{-1}$. (B) The teicoplanin-*N*-acetyl-D-Ala complex, the contours representing the energies $-1767\,kJ\,mol^{-1}$ to $-1668\,kJ\,mol^{-1}$ in 9 increments of $11\,kJ\,mol^{-1}$.



The black arrows show the conformers which are consistent with the closest distance of approach of the atoms which give mutually exclusive NOE enhancements (G1 to 2e and 6e to the acyl 2-CH_2; see text and Fig. 1). The grey arrows show local minima from a more detailed analysis of the region $\Psi = 255 \sim 270°$ in 2.5° increments (see text).

Fig. 6. Slices through the Ramachandran-style plots at $\Psi = 262.5°$.

(A) Free teicoplanin and, (B) the teicoplanin-*N*-acetyl-D-Ala complex.

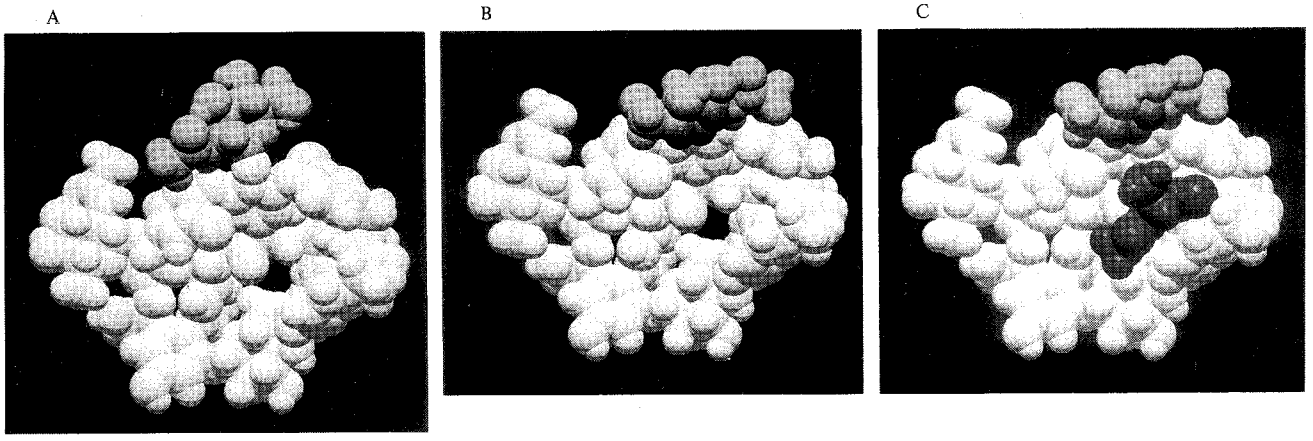


The two wells in each plot appear at approximately the same values of $\Phi = 104°$ and $\Phi = 299°$ but the minimum for free teicoplanin is at $\Phi = 299°$ and for the complex the minimum occurs at $\Phi = 104°$. The absolute energies are different from those in Fig. 5, but the relative energies are similar and are of better resolution.

increments, $\Psi = 255 \sim 270°$ in 2.5° increments; not shown) gave local minima at $\Phi = 104°$, $\Psi = 267°$ (grey shaded arrow in Fig. 5A) and $\Phi = 299°$, $\Psi = 262°$ (grey shaded arrow in Fig. 5B) for ligand bound and free teicoplanin respectively. The detailed analysis is illustrated in Fig. 6.

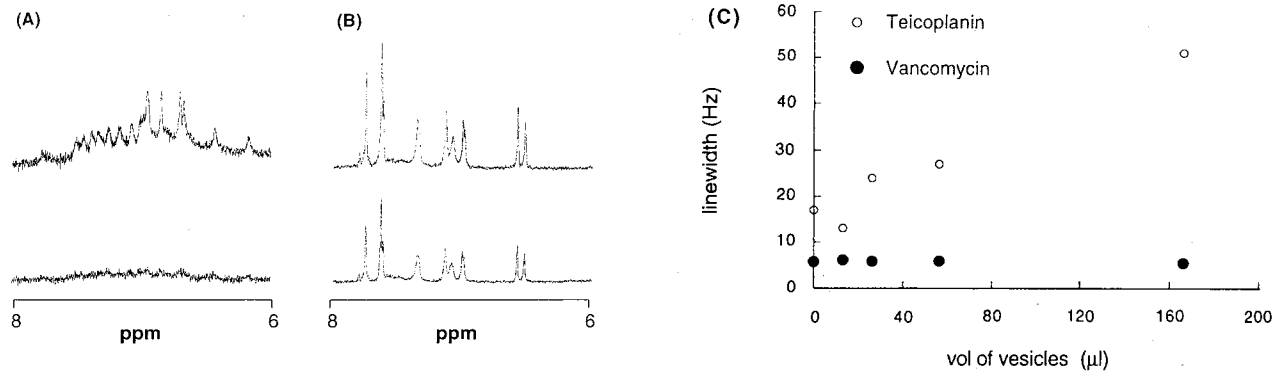
The two calculated minima can be seen to involve an approximate 180° rotation of the ring 4 glucose residue ($\Delta\Phi = 195°$) but the free antibiotic is lowest in energy at $\Phi = 299°$ and the antibiotic complex with *N*-acetyl-D-Ala-D-Ala at $\Phi = 104°$. Thus from molecular modelling

Fig. 7. The two conformers of teicoplanin.



The structures which satisfy both the NMR data and the molecular modelling are shown as (A) and (B) with the body of the antibiotic shown in white and the ring 4 acylglucosamine in grey. (B) favors ligand binding and is shown as (C) with *N*-acetyl-D-Ala (shown in black) in the binding pocket. In this conformation, the hydrophobic face of the glucose ring (black face of the grey acylglucosamine) can interact with the methyl group of the D-alanine residue and increase binding through the hydrophobic effect.

Fig. 8. Changes in the NMR spectra of 1 mM (A) teicoplanin and (B) vancomycin on the addition of vesicles.



No vesicles above, 10 mM phophatidylcholine below. (A) The teicoplanin signals broaden to be barely visible above the noise. (B) There is no significant change to the vancomycin signals. There is poorer signal-to-noise for teicoplanin due to its propensity to self aggregate to a small extent (C) Plot of added vesicles (20 mM phosphatidylcholine to 500 ml of 1 mM antibiotic) against linewidth for both antibiotics.

there are two conformers of teicoplanin, which satisfy the NMR data, having similar Ψ values but distinct Φ angles differing by approximately 180°; the existence of two similar conformers is also consistent with the NMR data.

The Two Conformers of Teicoplanin

Fig. 7 shows the two conformers of teicoplanin from NMR and molecular modelling. In the presence of cell wall analogue, the form shown in Figs. 7B and 7C is preferred. Thus, the more intense w_2 peaks (at higher field) in both Figs. 2A and 2B are assigned to this preferred conformation. In the bound conformer, the hydrophobic face of the glucosamine can be buried against the methyl group of the C-terminal alanine of the cell wall analogue, conferring extra binding affinity to this conformer due to an extra contribution from the hydrophobic effect.

The Acyl Chain of Teicoplanin Acts as a Membrane Anchor

We have already noted[14,15] that antibiotic dimerization allows the location of a second molecule of antibiotic at the site of cell wall biosynthesis with which the antibiotic interferes and, that where that dimerization is absent in teicoplanin, the C_{10} acyl sidechain may locate the antibiotic at the same site of biosynthesis if the C_{10} chain acts as a membrane anchor. An analysis of the

linewidths of the ^1H NMR signals of teicoplanin with vesicles shows that the C_{10} sidechain can indeed act as a membrane anchor. On the addition of vesicles (formed from bilayers of the lipid phosphatidylcholine) to an aqueous solution of teicoplanin, the ^1H NMR signals broaden until they are barely visible above the noise (Fig. 8A and C). As a control the experiment was repeated with vancomycin, which does not posses a C_{10} acyl sidechain, and no broadening of the ^1H NMR signals was apparent (Fig. 8B and C). The broadening of the lines is due to the teicoplanin taking on the slow tumbling rate of the vesicle when it is anchored into the lipid bilayer; the vancomycin does not become attached to the vesicle and retains its own intrinsic tumbling rate.

Materials and Methods

The antibiotic teicoplanin was kindly provided by Gruppo Lapetite S.p.A., Milan, Italy. *N*-acetyl-D-Ala, *N*-acetyl-D-Ala-D-Ala and L-α-phosphatidylcholine (Type XVI-E from fresh egg yolk) were purchased from Sigma and used without further purification.

NMR Spectroscopy

NOESY data (collected at various mixing times—75 ms, 100 ms, 200 ms, 300 ms, 500 ms) and COSY data (to aid with assignment, not shown) were collected at 500 MHz on Bruker AM500, DRX500 and Varian Unity spectrometers using standard 2D NMR pulse sequences and processing software. Linewidth experiments were performed at 500 MHz using a Varian Unity spectrometer.

Molecular Modelling

All molecular modelling was performed on a Silicon Graphics Iris Indigo color graphics workstation using MacroModel software.[21] To simplify the calculations, the ligand used was *N*-acetyl-D-Ala and the long decanoyl chain of teicoplanin was replaced by an acetyl group. Initial structures of teicoplanin both free and bound with the ligand *N*-acetyl-D-Ala were subjected to conjugate-gradient energy minimisation, employing AMBER empirical energy functions,[22,23] to relieve bad contacts between non-bonded atoms and to optimise bond lengths and geometries. A continuum dielectric model was used appropriate to water. Ramachandran-style plots were produced in MacroModel by energy minimization after each systematic incrementation of the two torsion angles attaching the acylglucosamine to ring 4 of the antibiotic. No restraints from the NMR data were used in the Macro Model calculations.

Vesicles

0.08 g of L-α-phosphatidylcholine was dissolved in 20 ml $CHCl_3$ (EtOH removed in a column of activated Alumina) which was dried as a thin layer by rotary evaporation. Residual solvent was removed at high vacuum for 30 minutes. The film was then dispersed in 2 ml D_2O by mechanical shaking for 30 minutes to give a 50 mM suspension of multilamellar vesicles. Unilamellar vesicles were obtained from the multilamellar vesicles by sonicating for 90 minutes. This suspension was diluted with D_2O to give the required vesicle concentrations.

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