

A ligand-mediated dimerization mode for vancomycin

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Background: Vancomycin and related glycopeptide antibiotics exert their antimicrobial effect by binding to carboxy-terminal peptide targets in the bacterial cell wall and preventing the biosynthesis of peptidoglycan. Bacteria can resist the action of these agents by replacing the peptide targets with depsipeptides. Rational efforts to design new agents effective against resistant bacteria require a thorough understanding of the structural determinants of peptide recognition by vancomycin.

Results: The crystal structure of vancomycin in complex with *N*-acetyl-D-alanine has been determined at atomic resolution. Two different oligomeric interactions are seen in the structure: back-to-back dimers, as previously described for the vancomycin–acetate complex, and novel face-to-face dimers, mediated largely by the bound ligands. Models of longer, naturally occurring peptide ligands may be built by extension of *N*-acetyl-D-alanine. These larger ligands can form an extensive array of polar and nonpolar interactions with two vancomycin monomers in the face-to-face configuration.

Conclusions: A new dimeric form of vancomycin has been found in which two monomers are related in a face-to-face configuration, and bound ligands comprise a large portion of the dimer interface. The relative importance of face-to-face and back-to-back dimers to the antimicrobial activity of vancomycin remains to be established, but face-to-face interactions appear to explain how increased antimicrobial activity may arise in covalent vancomycin dimers.

Introduction

Glycopeptide antibiotics such as vancomycin and teicoplanin are often used for the treatment of severe life-threatening infections when no other agents are effective or available. Unfortunately, high level bacterial resistance has significantly eroded the effectiveness of these ‘drugs of last resort’ over the past decade [1]. There is an urgent need for new antimicrobial agents in this class to overcome emerging resistance, and this need is propelling new investigations into the mode and site of action of glycopeptide antibiotics.

An important insight into the process by which glycopeptide antibiotics recognize their target ligands in bacterial cell walls was provided through nuclear magnetic resonance (NMR) investigations that suggested that these antibiotics formed dimers [2–5] and that dimerization could significantly enhance ligand affinity [6–14]. It was concluded that vancomycin dimerized in a ‘back-to-back’ configuration, with segments of antiparallel β structure forming both at the ‘back’ of each monomer, across the dimer interface between heptapeptide backbones, and at the ‘front’ of each monomer, between the drug and its ligand. X-ray crystallography has since verified this back-to-back antiparallel β configuration in balhimycin and vancomycin crystals, and has also shown that the aromatic rings and the sugar residues in these compounds engage in an extensive array of interactions across the dimer interface [15–17].

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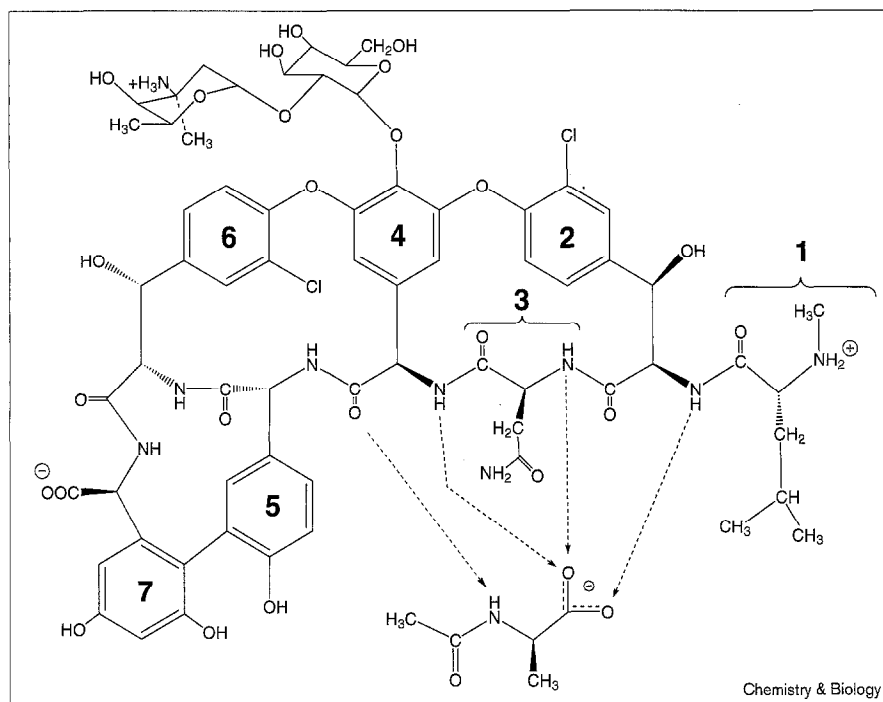
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Dimerization is believed to promote antimicrobial action because the binding of one monomer to the bacterial cell wall brings a second monomer into proximity with other peptidoglycan ligands, leading to the formation of a ‘chelate’ with the peptidoglycan [8,14]. Back-to-back dimerization also increases the affinity between ligands and individual monomers, however [6,12,14]. At least three mechanisms have been suggested to explain this allosteric effect. First, dimerization may induce conformational changes, or suppress thermomolecular deformations, and thereby yield a more favorable site for ligand binding [12,17]. Second, dimerization may position positively charged groups such that the negatively charged carboxyl terminus of a ligand is attracted into the binding site [12]. Third, antiparallel β structure in a dimer may polarize the backbone peptide groups involved in ligand binding and strengthen their hydrogen-bonding potential [17]. These mechanisms are mutually compatible, and all may contribute to ligand affinity.

Recent investigations demonstrate that covalently linked vancomycin monomers can show markedly increased affinity for depsipeptide ligands and significant antimicrobial activity against vancomycin-resistant bacteria [18,19]. These properties, and the requirement for rather long molecular linkages between vancomycin monomers (> 10 Å), suggest that covalent linkage does not enhance

Figure 1



Chemical structures of vancomycin and AcDA. The seven amino acid residues in vancomycin are indicated (1–7).

activity by promoting the same types of interactions found in noncovalent back-to-back dimers. In this report, we describe the crystal structure of vancomycin in complex with *N*-acetyl-D-alanine (AcDA). This complex illustrates the ability of vancomycin to form 'face-to-face' dimers, and suggests that face-to-face interactions may account for the enhanced ligand affinity and antimicrobial efficacy of covalently linked monomers.

Results and discussion

Nomenclature

Residues in the vancomycin–AcDA structure are referred to in a similar manner to those described previously by Loll *et al.* [17]. The four independent molecules of vancomycin are designated V1–V4, with the seven individual amino acid residues within each monomer designated V1:1–V1:7, V2:1–V2:7, and so on (Figure 1). The glucose and vancosamine residues are indicated by 'G' or 'V' in place of a residue number. The corresponding ligands are designated AcDA1–AcDA4, and there are four chloride ions, Cl-1–Cl-4 in each asymmetric unit.

Structure description

There are four copies each of vancomycin, AcDA and Cl⁻ in the asymmetric unit of vancomycin–AcDA crystals, implying that four counterions remain unidentified. These molecules form two asymmetric dimers (V1–V3 and V2–V4), similar to those seen in the ureido-balhimycin and vancomycin–acetate structures (Figure 2). V1 and V2, along with their ligands, are nearly identical

and may be superimposed with a root mean square (rms) difference of < 0.2 Å. The configuration of their disaccharides corresponds to that seen in the ligand-bound monomer of the vancomycin–acetate complex (V2), that is the glucose residue overhangs the ligand-binding site. Likewise, V3 and V4 are nearly identical and may be superimposed with an rms difference of < 0.2 Å. The configuration of their disaccharides, however, corresponds to that of the ligand-free monomer of the vancomycin–acetate complex (V1), so the vancosamine residue overhangs the ligand-binding site. Apart from opposite orientations for the disaccharides, V1 and V2 are quite similar to V3 and V4, and may be superimposed with an rms difference of < 0.4 Å.

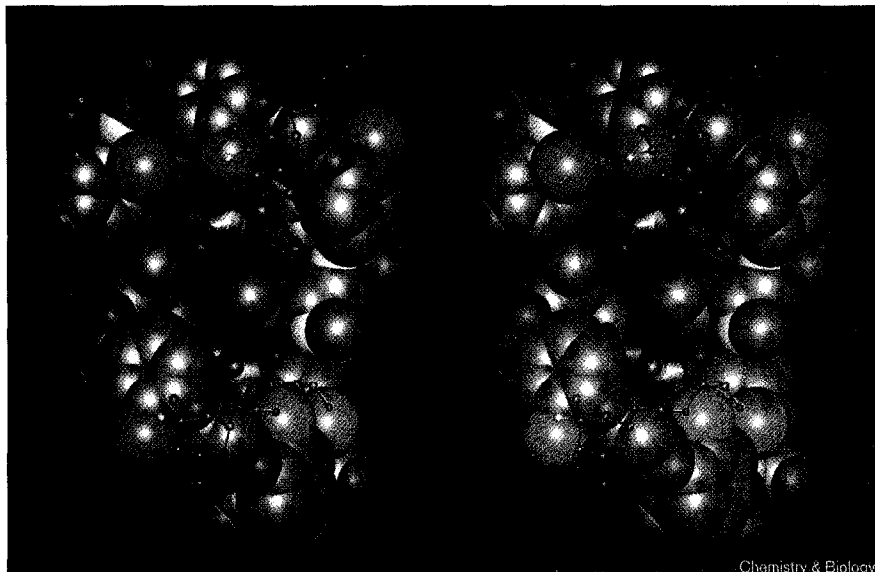
The charged amino groups of V1:V and V2:V hydrogen bond with the backbone carbonyl oxygen atoms of V2:2 and V1:2, respectively. These relationships are analogous to those found across the dimer interface in the crystal structure of vancomycin between the charged amino group of vancosamine on residue 6 and the backbone carbonyl of residue 2. It is noteworthy that the carbonyl groups of V3:2 and V4:2 are not similarly bound, but that electron densities assigned to water molecules 8 and 20 may be sodium cations in analogous positions.

Ligand binding

The ligand in V1 and V2 of the AcDA complex is situated slightly higher in the binding site than in V3 and V4 (when oriented with the disaccharide upwards). The chief

Figure 2

Stereoview of the vancomycin–AcDA face–face dimer. Atoms of vancomycin are space-filling models, except for the disaccharide units which are ball-and-stick. Atoms of AcDA are represented as tubes. Blue, carbon; green, chlorine; red, oxygen; yellow, nitrogen.



interaction causing this difference appears to be steric repulsion between the C₆ methyl of vancosamine in V3/V4 and the alanyl methyl group of the ligand. The ligands adopt essentially the same configuration in all four binding sites, however, with backbone ϕ angles in the range of -22.7 to -26.2° [20].

The vancomycin–AcDA complex adopts a different crystal form than either the acetate or *N*-acetyl-glycine complexes ([17]; P.J.L., unpublished observations), which reflects the differences in affinity between the drug and these different ligands. The ratio of vancomycin:ligand is 1:1 in the AcDA complex, whereas it is 2:1 in the acetate and glycine complexes. Molecule V1 in the acetate and glycine complexes is bound to a symmetry-related copy of itself (residue V1:1) instead of to the ligand. Thus, AcDA presumably has a higher affinity for the binding site than V1:1, thereby precluding formation of the crystal form seen in acetate and glycine crystals.

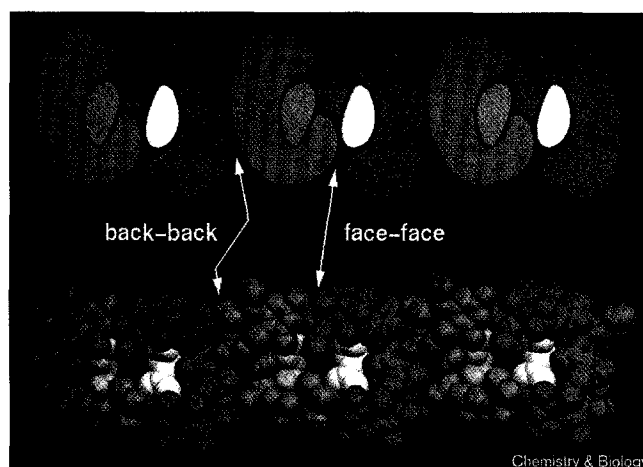
Dimer formation

Vancomycin–AcDA forms back-to-back dimers as seen previously in the crystal structures of balhimycin [15] and vancomycin–acetate [16,17]. Of interest, however, is the observation that V1, in addition to forming the back-to-back dimer with V3, also forms a face-to-face dimer with a symmetry-related copy of V3 (Figure 3). The same relationships are observed between V2 and V4. The bound AcDA ligands comprise a significant portion of these face-to-face dimer interfaces. Direct contact between V1 and V3 is limited to hydrophobic interactions between V1:1 and V3:1 (similarly for V2:1 and V4:1), but the ligand bound to V1 makes more extensive contact with V3,

including a hydrogen bond between the acetate carbonyl of AcDA1 and the quaternary nitrogen of V3:1 (similarly for AcDA2 and V4:1).

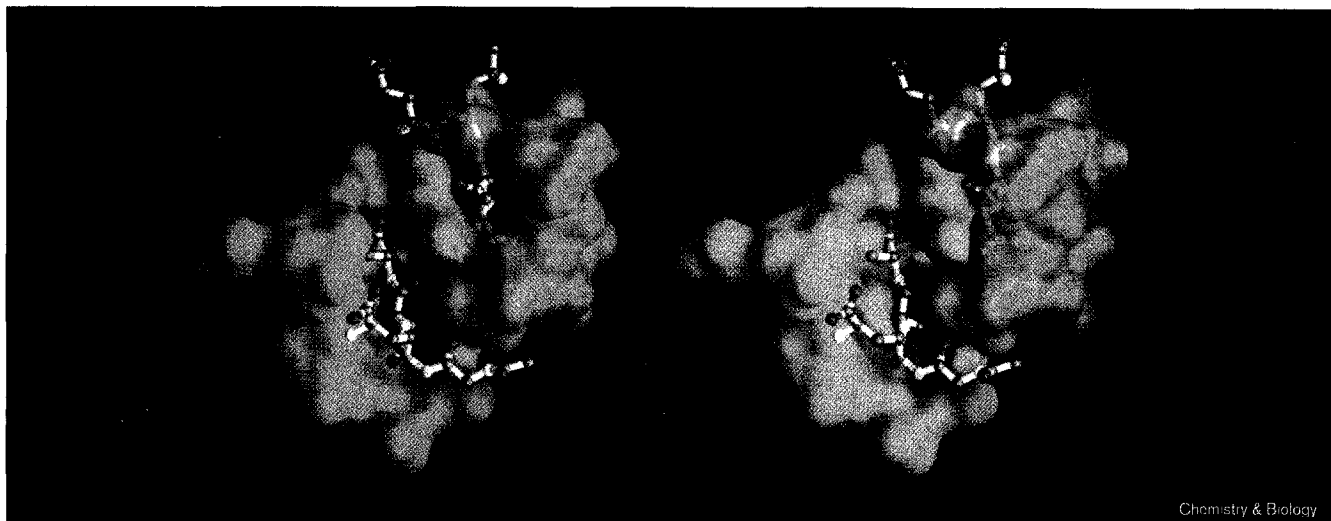
Natural ligand model

The natural peptidoglycan target of vancomycin in bacterial cell walls terminates in $-D\text{-Glu-L-Dap-D-Ala-D-Ala}$ (Dap, meso- α,ϵ -diaminopimelate). Two copies of

Figure 3

Schematic (above) and actual (below) views of the infinite chains of vancomycin monomers that comprise the crystal lattice. These chains are made up of alternating back-to-back and face-to-face contacts between monomers. V1 are blue and V3 are pink; their respective AcDA ligands are orange and yellow. There are two such chains of monomers found in this crystal form. Monomers in both chains pack in an essentially identical fashion, even though the chains are crystallographically independent. Only one chain is shown for clarity.

Figure 4



Stereoview of the vancomycin face-to-face dimer with the two AcDA ligands extended to illustrate a plausible binding mode for two Ac-D-Glu-L-Dap-D-Ala-D-Ala ligands. The position of the carboxy-terminal D-alanine residue is unchanged from the vancomycin-AcDA crystal structure.

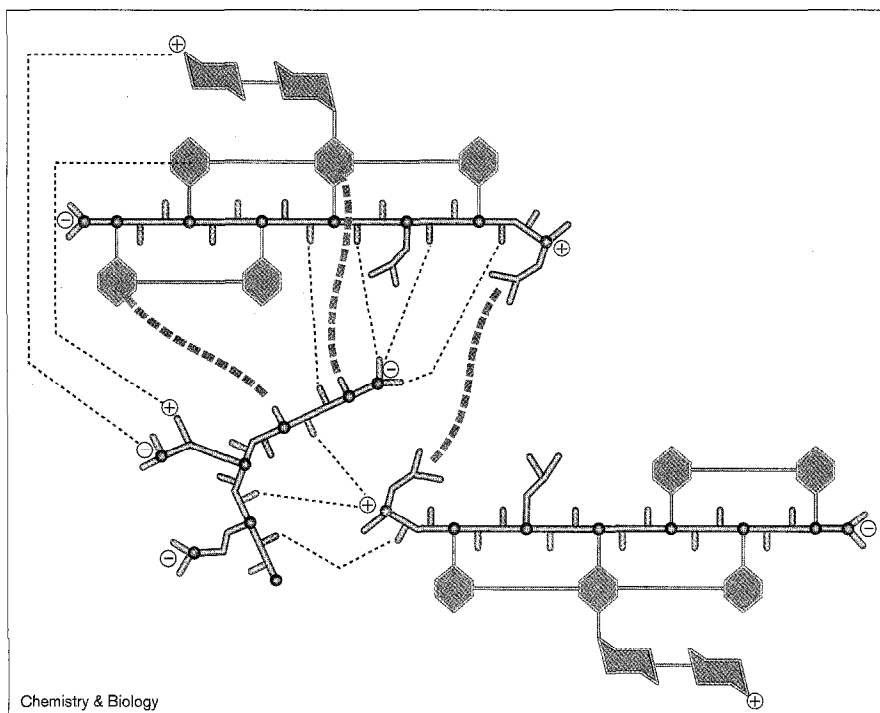
this natural ligand were modeled into the face-to-face vancomycin-AcDA structure as extensions of the AcDA ligands (Figure 4). In the model, the D-Glu-L-Dap-D-Ala-D-Ala ligands form a plausible and extensive interface between two of the vancomycin molecules designated V1 and V3. The nature and number of interactions possible across a face-to-face dimer interface compare

favorably with those seen across the back-to-back dimer interface (Figure 5).

Significance

Bacterial resistance to glycopeptide antibiotics such as vancomycin is emerging; a greater knowledge of the mode and site of action of such antibiotics is needed to

Figure 5



Schematic view of the molecular interactions between one ligand and two vancomycin monomers in the face-to-face dimer. Thin dashed lines (dark blue) represent polar interactions; thick dashed lines (light blue) represent nonpolar contact. Electropositive groups are blue (peptide hydrogens and charged nitrogens); electronegative groups (carbonyl and carboxylate oxygens) are red. Note that the positively charged amino group of L-Dap interacts with the electronegative aromatic π -electron system of residue 6.

combat this resistance. Here, the determination of the crystal structure of vancomycin in complex with *N*-acetyl-D-alanine (AcDA) demonstrates that vancomycin forms ligand-mediated face-to-face dimers as well as the ligand-independent back-to-back dimers previously observed by nuclear magnetic resonance.

The results cannot prove that face-to-face dimerization is important for the antimicrobial activity of vancomycin, but our finding that two nearly identical crystallographically independent copies of this face-to-face interface exist in the vancomycin–AcDA structure suggests that the interaction is not simply a fortuitous crystal contact and that the face-to-face configuration is thermodynamically favorable for the vancomycin–AcDA complex. Furthermore, it is clear that far more extensive face-to-face interactions are possible with longer natural ligands, and that this may increase the thermodynamic advantage of this configuration.

The distance between the charged amino groups of vancomamine in molecules V1 and V3, and in V2 and V4, is 13.6 Å, which corresponds precisely to the minimum effective length for a linker segment joining these sites in covalently linked monomers [19], suggesting that the covalent linkages facilitate face-to-face rather than back-to-back interactions. This observation further suggests that the increased antimicrobial efficacy of covalently linked monomers is a result of the formation of a face-to-face complex rather than bringing a second monomer close to a second ligand.

Materials and methods

Materials

Pharmaceutical grade vancomycin hydrochloride (Vancocin[®], Eli Lilly) was used without further purification. *N*-acetylated D-alanine was obtained from Sigma; other materials were of the highest purity available commercially. Crystals were grown by hanging drop vapor diffusion. Equal volumes of aqueous vancomycin hydrochloride (40 mg ml⁻¹) and reservoir buffer were mixed and equilibrated over reservoir buffer at 20°C; the reservoir buffer was 2.1 M NaCl in 0.1 M *N*-acetyl-D-alanine, pH 4.6. Prior to data collection, crystals were transferred to a solution of 30% (v/v) glycerol in reservoir buffer, mounted in fiber loops, and flash-cooled by plunging into liquid N₂. Crystals were maintained at -175°C in a stream of N₂ gas during data collection.

Data collection

High-resolution data were collected at beamline X12-B at NSLS, using a MAR 30 cm image plate detector. Because of overloading in the intense low resolution data, further data were taken from the same AcDA crystal using a MAR 30 cm detector mounted on a home source. These data were merged with the high resolution synchrotron data and used during refinement. Data were processed with DENZO and SCALEPACK; details are given in Table 1:

Data processing

Normalized structure factor magnitudes ($|E|$ s) were obtained using LEVY/EVAL [21]. A total of 150000 triples were generated from the largest 4000 E-magnitudes between resolution limits of 10.0 and 1.0 Å. 1000 trial structures were generated, each consisting of 100 randomly placed atoms. Each of the 1000 trial structures was then subjected to

Table 1

Data collection and refinement statistics.

| | |
|-------------------------------------|--|
| Contents of unit cell | |
| Vancomycin | 4 × C ₆₆ H ₇₅ Cl ₂ N ₉ O ₂₄ |
| Ligand | 4 × C ₅ H ₉ NO ₃ |
| Cl ⁻ ions | 4 |
| Solvent H ₂ O molecules | 96 |
| Temperature | 98 K |
| Wavelength | 0.978 Å/1.542 Å |
| Spacegroup | P1 |
| Unit cell: | |
| a | 21.4(1) |
| b | 24.3(1) |
| c | 24.9(1) |
| α | 64.5(2)° |
| β | 90.2(2)° |
| γ | 81.0(2)° |
| Unit cell volume | 11,544 Å ³ |
| Maximum resolution | 0.97 Å |
| No. of observations | 91,923 |
| No. of independent reflections | 21,253 |
| Completeness of data: | |
| 8.0–1.0 Å | 83% |
| 1.1–1.0 Å | 68% |
| R _{merge} | 0.046 |
| No. of restraints in refinement | 22,169 |
| No. of least-squares parameters | 4921 |
| R ₁ | 0.123 |
| wR ₂ | 0.299 |
| goodness of fit on F ² | 1.04 |
| min/max peaks in difference Fourier | -1.09/1.05 e ⁻ /Å ³ |

500 cycles of the cyclic *Shake-and-Bake* procedure [22,23], using a pre-release of *SnB* v2.0 [24] (R. Miller and C.M. Weeks, unpublished observations). Each *Shake-and-Bake* cycle consists of phase refinement, Fourier filtering, peak picking, and then a structure factor routine that is used to generate phases for the next cycle. Each instance of phase refinement consisted of making one complete pass through the 4000 phases, where each phase was, in turn, perturbed by at most two 90° shifts. For each phase, the original and perturbed phase values were evaluated in terms of the minimal function [22], which was used as a figure of merit. The phase value yielding the smallest value of this objective function was used to replace the current value of the phase under consideration. The number of peaks picked during the 500 cycles varied. During the first 100 cycles, only 20 peaks were selected in an attempt to anchor the heavier chlorine atoms. During cycles 101–200, 50 peaks were picked; during cycles 201–300, 100 peaks were picked; and finally, during cycles 301–500, 150 peaks were picked.

After processing several hundred trials, there was a small, but distinctive, break in the range of the final minimal function values. From past experience [22,23], we know that a bimodal histogram of final minimal function values is a strong indication of the presence of a solution. The single trial yielding the smallest minimal function value was then used as a starting point in an effort to obtain a more refined set of phases. We applied several rounds of Fourier recycling to this structure, using both *SnB* v1.5 [25] and the pre-release of *SnB* v2.0. During this recycling, we used 40000 triples generated from the top 4000 E-magnitudes. The parameter shift phase refinement procedure used phase shifts that were smaller than the 90° shifts used to obtain this starting set of phases. Further, during the Fourier recycling, we gradually extended the number of peaks from the 150 input peaks to 404 final peaks.

Manual editing of the peaks produced by *SnB* yielded an initial model comprising 293 of the 404 vancomycin atoms in the unit cell. This structure was refined with SHELXL [26] using alternate rounds of atom

placement into difference maps and refinement. Minimization was performed against F^2 using restraints on geometry and atomic displacement parameters; anisotropic displacement parameters were included at later stages in the refinement. Conjugate gradient refinement was used throughout, except for the final cycles, where blocked least-squares methods were used.

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