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New Insights into the Glycopeptide Antibiotics Binding to Cell Wall Precursors using SPR and NMR spectroscopy

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Abstract: Glycopeptide antibiotics such as vancomycin and teicoplanin are used to treat life-threatening infections caused by multi-drug-resistant Gram-positive pathogens. They inhibit bacterial cell wall biosynthesis by binding to the D-Ala-D-Ala C-terminus of peptidoglycan precursors. Vancomycin resistant bacteria replace the dipeptide with the D-Ala-D-Lac depsipeptide, thus reducing the binding affinity of the antibiotics with their molecular targets. Herein, we report on our studies of the interaction of teicoplanin, teicoplanin-like A40926, and of their semi-synthetic derivatives (mideplanin, MDL63,246, dalbavancin) with peptide analogues of cell wall precursors by NMR spectroscopy and surface plasmon resonance (SPR). NMR revealed the existence of two different complexes in solution, when the different glycopeptides interact with Ac2KDAlaDAlaOH. Despite the NMR experimental conditions, which are different from those employed for the SPR measurements, the NMR results parallel those deduced in the chip regarding the drastic binding difference existing between the D-Ala and the D-Lac terminating analogues, confirming that all these antibiotics share the same primary molecular mechanism of action and resistance. The kinetic analysis of the interaction between the glycopeptide antibiotics and immobilized AcKDAlaDAlaOH by SPR suggest a dimerization process, that was not observed by NMR in DMSO solution. Moreover in SPR, all the glycopeptides with a hydrophobic acyl chain present stronger binding with a hydrophobic surface than vancomycin, indicating that additional interactions through the employed surface are involved. Concluding, SPR provide tools to differentiate between vancomycin and other glycopeptides and the calculated binding affinities at the surface seem to be more relevant to *in vitro* antimicrobial activity than the estimations from NMR analysis.

Keywords: Magnetic Resonance Nuclear (NMR), Surface Plasmon Resonance (SPR), Glycopeptide antibiotics, Teicoplanin, A40926, Dalbavancin

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Introduction

Glycopeptide antibiotics such as vancomycin and teicoplanin are frequently used to treat life-threatening infections caused by multi-drug-resistant Gram-positive pathogens. They are drugs of last resort against multi-resistant methicillin-resistant *Staphylococcus aureus* (MRSA), which is nowadays a major cause of community-acquired infections and determines high morbidity and mortality rates in hospital-acquired infections.[1] Vancomycin and teicoplanin are in clinical use since 1958 and 1988, respectively. The spread of resistance to glycopeptides in enterococci since 1988 and the recent emergence of high level of glycopeptide resistance in clinical isolates of MRSA have prompted the search for second-generation drugs belonging to this chemical class.[2]

This glycopeptide family is composed of heptapeptides, oxidatively linked among aromatic amino acids and decorated with chlorine atoms, glycosidic moieties, and (in the case of teicoplanin and teicoplanin-like molecules) lipid chains. These complex molecules inhibit bacterial cell wall synthesis by binding to the

dipeptide terminus D-Ala-D-Ala of the peptidoglycan (PG) precursors[3], sequestering the substrate from transpeptidation and transglycosylation reactions in the late extracellular stages of PG cross-linking (Fig.1). The D-Ala-D-Ala complex with vancomycin is stabilized by an array of hydrophobic van der Waals contacts and five hydrogen bonds (H-bonds) lining the antibiotic binding pocket (Fig. 2).[4] Bacteria resistant to glycopeptides remodel their PG precursor terminus from D-Ala-D-Ala to D-Ala-D-Lac.[5] The substitution of the initial amide moiety in D-Ala-D-Ala by an ester linkage in D-Ala-D-Lac strikingly reduces the binding (in aqueous solution) to vancomycin by 1000-fold and renders the antibiotic therapeutically useless.[5-6] The complex of vancomycin with D-Ala-D-Lac lacks the central H-bond and suffers of a repulsive lone pair interaction between the vancomycin residue 4 carbonyl and D-Ala-D-Lac ester oxygens (Fig.2).

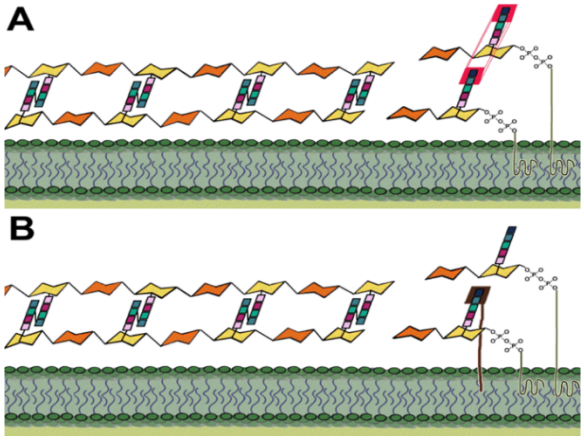

Figure 1. Schematic view of peptidoglycan structure and mechanism of action of glycopeptide antibiotics. Glycopeptides inhibit transglycosylation and transpeptidation by binding to the C-terminal D-Ala-D-Ala of the late PG precursors. (**A**) Vancomycin-type glycopeptide activity is based on dimerization, which enhances binding to the target peptide through both cooperative and allosteric effects. (**B**) Lipoglycopeptides (e.g. teicoplanin and its derivatives) have a fatty acyl chain anchored in the phospholipid bilayer that enhances the binding affinity to the target.

Previous NMR and X-ray studies[7] have indicated that vancomycin and similar natural products such as eremomycin have the ability to dimerize in aqueous solution, and that dimerization plays an important role in their biological activity. Surface Plasmon Resonance (SPR) has been also used to study the interaction between vancomycin (and related eremomycin, cloroeremomycin, balhimacyn) and tripeptide analogues of PG precursors terminating in D-Ala-D-Ala and D-Ala-D-Lac in lipid bilayers[8] and self assembled monolayers.[9]

Crystal structure of the complexes teicoplanin-D-Ala-D-Ala and dalbavacin-D-Ala-D-Ala (a semi synthetic derivative) have been recently described, showing that the interaction of these lipoglycopeptides with the dipeptide is produced forming the same hydrogen bonds than vancomycin (Fig.2).[10] For teicoplanin deglycosylated derivatives, no evidence of dimer formation at millimolar concentrations was reported in past studies[11], but the teicoplanin's lipid chain, (an extra aliphatic acyl side chain on glucosamine at residue 4) which is absent in vancomycin-like molecules, anchors the antibiotic to the lipid layer of the bacterial membrane[7b]. Lipoglycopeptides such as teicoplanin and its derivatives are reported to be more effective than vancomycin against Gram-positive cocci[12]. As a consequence, most of the second generation semi-synthetic glycopeptides have been prepared introducing hydrophobic moieties in the heptapeptide scaffold in order to confer increased membrane anchoring ability, which could conceivably lead to increased binding to PG terminus[2b, 13].

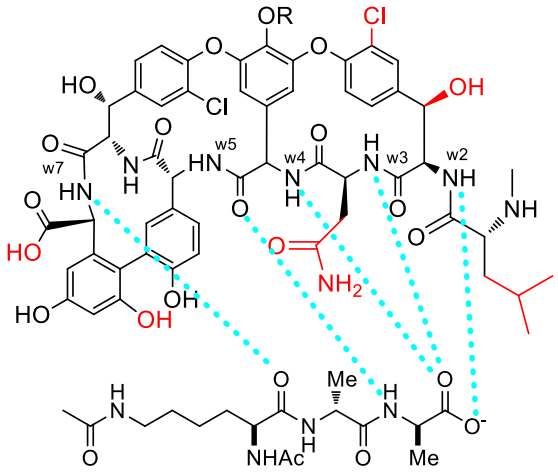

Figure 2. Schematic view of the key intermolecular hydrogen bonds described for vancomycin (pdb code 1FVM) and ligand terminating in D-Ala-D-Ala and kept for the molecular mechanics calculations of the different complexes studied herein. Chemical shift perturbations at w2 of the antibiotic and at the two methyl groups of the three-peptide were monitored to proof the existence of stable binding.

The aim of this study is to investigate the interaction of teicoplanin, teicoplanin-like A40926[14], and the semi synthetic derivatives mideplanin[15], MDL63,246[16], dalbavancin[15, 17] with peptide analogues of cell wall precursors by NMR and SPR spectroscopy, being the latter an adequate system to predict biological activity. It is worth noting that dalbavancin is currently completing phase III clinical trials to treat acute bacterial skin and skin structure infections caused by susceptible Gram-positive bacteria (http://www.durata therapeutics.com/). These studies may be also useful in the recent efforts to redesign glycopeptide antibiotics for the treatment of resistant microbial infections, including MRSA, and examine their future potential for providing a new class of antibiotics less prone to bacterial resistance.

Results and Discussion

NMR studies were performed to analyze the molecular recognition behavior of the different glycopeptides *versus* D-Ala-D-Ala and D-Ala-D-Lac terminating peptides. In particular, the interaction of the glycopeptide antibiotics mideplanin, teicoplanin, dalbavancin, A40926 and MDL63,246 (Fig. 3) with the PG precursor analogues Ac2KDAlaDAlaOH and Ac2KDAlaDLac acid was monitored.

The obtained data will be presented in detail for teicoplanin. Nevertheless, analogous conclusions were deduced for all the studied glycopeptides. In all cases, no evidences of glycopeptide dimerization were inferred under these experimental conditions, especially from the DOSY experiments (see below).

Nuclear Magnetic Resonance: teicoplanin model

Previous works[18] have focused on the solution conformation of teicoplanin as well as on its interactions with the di- and three-peptide PG precursor models. These previous works have been performed mostly on the aglycone (devoid of all the sugar moieties and consequently of the lipid chain) or pseudoaglycone (devoid of some sugar moieties and of the lipid chian) derivatives of

teicoplanin in a variety of solvents, trying to overcome the solubility problems inherent to some of these molecules[18]. All the molecules studied herein display a lipid chain attached at the glucosamine moiety and therefore their solubility in water is basically negligible. Thus, the NMR experiments were performed in dimethyl sulfoxide (DMSO). As mentioned in the experimental section, there are two key structural reporter evidences that permit to monitor the existence (or not) of stable intermolecular complexes between the antibiotics and the PG precursor Ac2KDAlaDAlaOH. Thus, chemical shift perturbations at w2 (Fig. 2) of the antibiotic and at the two methyl groups of the tripeptide were monitored to proof the existence of the molecular recognition process.[4, 19]

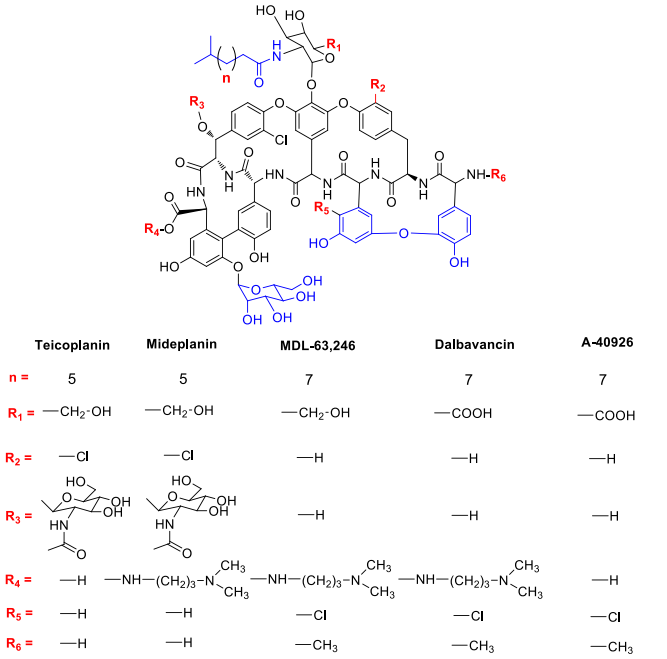

Figure 3. Structures of glycopeptide antibiotics used in this study. Teicoplanin and A40926 are natural microbial products[20]. Mideplanin and dalbavancin are the dimethylaminopropyl amide of teicoplanin and A40926, respectively[15]. MDL63,246 differs from dalbavancin for the reduction to alcohol of the carboxyl group in the *N*–acylglucuronic moiety..

For teicoplanin, the chemical shift of the w2 proton was identified through its TOCSY crosspeak to the corresponding Hα (7.45/4.85), and found to be similar to that previously reported (7.41/4.97).[18]

Fittingly, when Ac2KDAla DAlaOH (3mM) was added to the NMR tube containing 2 mM teicoplanin, the existence of drastic chemical shift changes for several protons was evident. Interestingly, a new broad NH signal appeared, at very low field, at ca. 13 ppm. This NH displayed a broad TOCSY cross peak with one Hα at 5.02 ppm (see Fig. S1 in supporting information), indicating that the w2 NH is perturbed in more than 5 ppm when teicoplanin forms the complex with Ac2KDAlaDAlaOH. The corresponding signal was found to be rather broad, splitted into two signals, permitting distinction of a major and a minor peak, and thus suggesting the existence of two different (although structurally similar) bound species.

From the Ac2KDAlaDAlaOH viewpoint, a new signal at high field (0.46 ppm) appeared. In the ROESY spectrum, it can be seen that this signal shows a chemical exchange crosspeak with one of the methyl resonances of the free species at 1.28 ppm (Fig. 4). Therefore, this signal can be readily identified as belonging to Ala-3 moiety. The important shielding (more than 0.8 ppm) of this methyl group suggests that, in the complex, it is surrounded by one or more of the aromatic rings of the antibiotic. Additional exchange cross peaks were found for the NH protons of Ala-2 and Ala-3 residues which were deshielded in ca. 0.7 and 0.3 ppm, respectively. Again, there was indication of the existence of one major and one minor species, accounting for the existence of two very similar bound species (as demonstrated in Fig. S1 in supporting information).

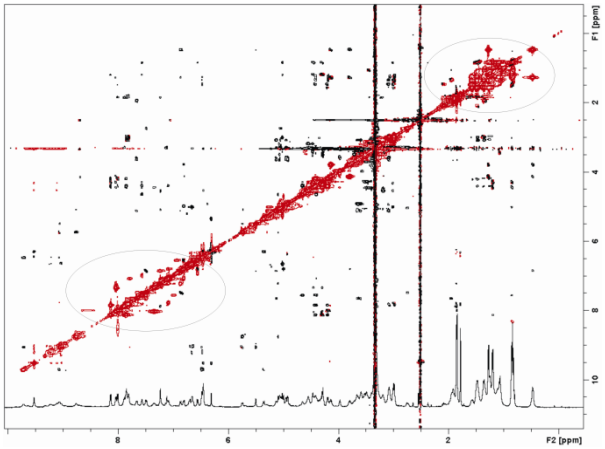

Figure 4. ROESY spectrum of the mixture of teicoplanin/Ac2KDAlaDAlaOH (1/1.5 molar ratio). The circled areas highlight chemical exchange cross peaks corresponding to the Ala-3 methyl group (high field) and the Ala NHs (low field) between its free and antibiotic-bound forms, as discussed in the text.

Additional indication of the existence of interaction came from the analysis of DOSY spectra (Fig. 5). The comparison of the diffusion coefficients measured for free Ac2KDAlaDAlaOH and in the presence of teicoplanin (teicoplanin/Ac2KDAlaDAlaOH in 1/1.5 molar ratio) indicated that the mixture showed an apparent diffusion coefficient significantly higher (ca. 0.4 units in log D) than that of free Ac2KDAlaDAlaOH. Thus, this experiment provided an additional indication of the existence of a strong intermolecular interaction between both species. No changes in the diffusion coefficient for the glycopeptide itself was observed in the presence of Ac2KDAlaDAlaOH, strongly suggesting that teicoplanin remains as monomer in DMSO solution even in the presence of the PG model peptide. Thus, according to these NMR experimental data, the existence of a molecular complex can be granted. The chemical shift data suggests that, very probably, the geometry already described for this family of antibiotics is indeed that present in DMSO solution. A 3D view of the complex will be presented below**.**

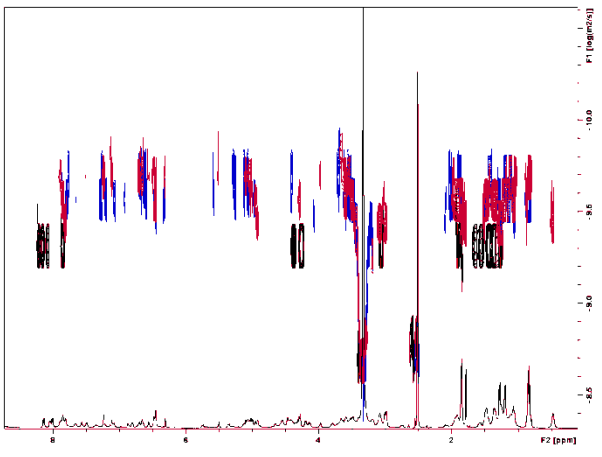

Figure 5. DOSY spectra: black: free teicoplanin; blue: teicoplanin+Ac2KDAlaDAlaOH; red: Ac2KDAlaDAlaOH.

In contrast, when Ac2KDAlaDLac acid (3 mM) was added to the NMR tube containing teicoplanin, only very minor chemical shift changes in some protons of teicoplanin and of Ac2KDAlaDLac acid were evident. Importantly, no protons at either low (ca. 12 ppm) or high (ca. 0.46 ppm) field were detected. Moreover, when the DOSY were compared, now the same apparent diffusion coefficients were observed both for free Ac2KDAlaDLac acid and for this compound in the presence of teicoplanin. These results indicate that, for this ligand, the interaction with teicoplanin is much weaker than that described above for Ac2KDAlaDAlaOH.

The 3D structure of the formed complexes was derived using molecular modelling methods, as described in the experimental section. The combination of docking protocols, molecular mechanics and molecular dynamics calculations provided different solutions to the structure of the complex. The most stable one formed between teicoplanin and Ac2KDAlaDAlaOH is displayed in Fig. 6, and is in agreement with those previously proposed for a variety of antibiotic-PG precursor analogue pairs[18]. In any case, a second ligand conformation was also detected, with a relative energy about 2 kcal/mol above that of the global minimum. Herein, a hydrogen bond between the phenolic ring of 3 and the carbonyl group of the acetyl group of the Lys moiety (K) takes place. This acetyl group is in turn hydrogen bonded to the NH group of Ala-2. This structural pattern (see Fig. S2 in supporting information) replaces the most stable one formed by the amide nitrogen of 5 and the carbonyl oxygen of Lys (Fig. 2 and Fig. 6). Both types of complexes comply with the experimental NMR data described above, since both maintain the hydrogen bond pattern as well as the stacking interaction of the Ala-3 methyl group. Recently, the X-ray structures of different teicoplanin analogues complexed with bacterial cell-wall peptides, using either MBP or ubiquitin as ligand carrier have been reported [10a]. The obtained structures (pdb codes 3VFJ and 3VFK) were strikingly similar to the major conformer presented herein.

In contrast, for the Ac2KDAlaDLac acid analogue, after the MD protocol, no intermolecular complex was evident (Fig. 7). The Ac2KDAlaDLac acid molecule dissociated from the teicoplanin binding site, indicating that the hydrogen bonds involving the carboxylate moiety were not strong enough to keep this molecule attached to teicoplanin. Indeed, after molecular mechanics optimization, the obtained structure was ca. 10 kJ/mol less stable than that obtained for the teicoplanin/Ac2KDAlaDAlaOH complex.

Nuclear Magnetic Resonance: teicoplanin family derivatives

Analogously, NMR experiments were performed to monitor the molecular recognition features of a variety of glycopeptide antibiotics with Ac2KDAlaDAlaOH and its lactic acid analogue. In particular, the interaction of these two tripeptides with mideplanin, MDL63,246, dalbavancin, and A40926 was monitored using chemical shift perturbation data and DOSY experiments.

The NMR experiments indicated the existence of interactions between the antibiotics and Ac2KDAlaDAlaOH in all cases and the acquired spectra are very similar to those presented in Figures 4 and 5 for teicoplanin. Similar features were always observed, including the presence of one very major and one minor bound species. Shielding of the methyl group of Ala-3 upon binding to the antibiotic was observed, together with the presence of a chemical exchange process which is slow in the chemical shift timescale. The antibiotic-complexed Ala-3 methyl group appeared always around 0.45 ppm, indicating a similar recognition motif in all cases. Moreover, deshielding of the w2 NH of MDL63,246, A40926, mideplanin, and dalbavancin in the presence of Ac2KDAlaDAlaOH was evident. The NH corresponding to the bound species appeared between 12 and 13 ppm. Besides, the change in the diffusion coefficient of Ac2KDAlaDAlaOH was always significant, varying in 0.3-0.4 units in log D value. These data indicate that, in all cases, there is a strong interaction between Ac2KDAlaDAlaOH and all the antibiotics, and suggest that, in solution, at the atomic level, the recognition features of MDL63,246, A40926, teicoplanin, mideplanin, and dalbavacin are fairly similar. Fittingly, our global minima structures are also very similar to the X-ray crystallographic structure recently obtained for dalbavancin using a carrier protein strategy [10b].

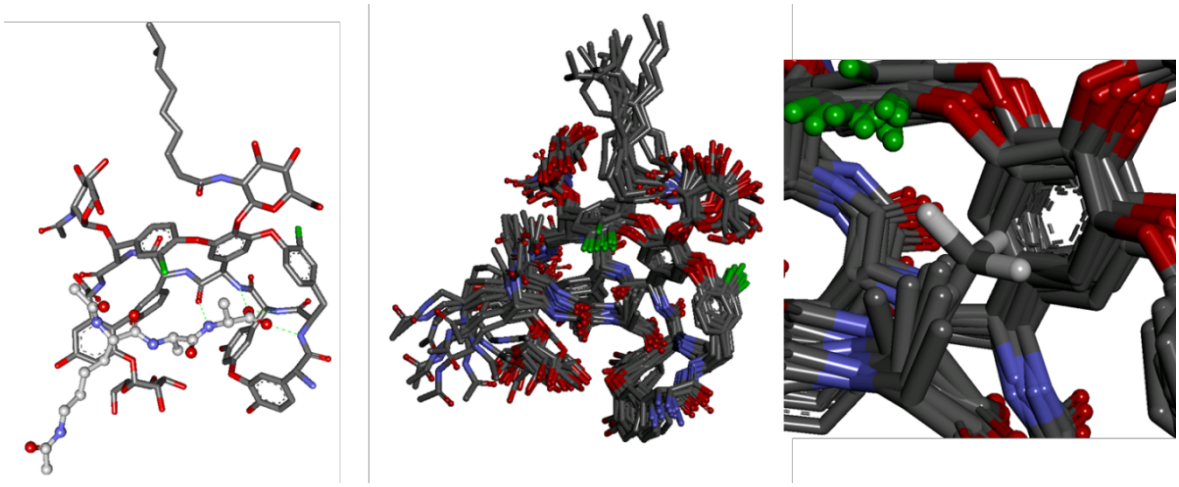

Figure 6. The complex formed between teicoplanin and the Ac2KDAlaDAlaOH peptide according to molecular modeling. Left, the global minimum structure (the Ac2KDAlaDAlaOH is in ball and sticks representation) after MD (1 ns) simulations followed by energy minimization. The key hydrogen bonds are indicated. These hydrogen bonds (three involving the carboxylate group of Ala-3 and one involving Ala-3 NH) remained for more than 90% of the time during the MD run. One additional hydrogen bond involving the CO of Lys-1 was present in the MD for more than 50% of the time. A superimposition of 10 snapshots at different times of the MD simulation is presented in the middle and

right panels. The structure of the complex is fairly well defined, with significant motion of the lysine side chain and the N-acyl lipid chain at the glucosamine moiety. The right panel highlights the packing of the methyl group of Ala-3 towards one of the aromatic rings of teicoplanin. This orientation perfectly matches with the observed chemical shift perturbation data.

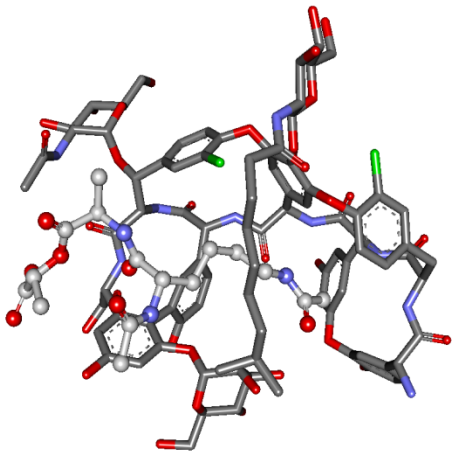

Figure 7. The complex formed between teicoplanin and the Ac2KDAlaDLac acid analogue (in ball and sticks representation). The key intermolecular interactions present in the three-peptide analogue (see above) are destroyed after the MD equilibration period. No intermolecular hydrogen bonds are formed and therefore, no complex is present in solution.

A superimposition of the global minima of all complexes derived from the molecular modeling protocol is displayed in Figure 8.

In contrast, when the Ac2KDAlaDLac acid analogue was employed, very minor chemical shifts were evident, no slow exchange process took place and no changes in the diffusion coefficient of the ligand were observed. Therefore, these experimental evidences strongly indicate that the interaction of the lactic acid terminating analogue with the tested antibiotics is rather weak.

Surface Plasmon Resonance Studies

The interaction of the glycopeptide antibiotics mideplanin, teicoplanin, dalbavancin, A40926 and MDL63,246 (Fig. 3) with PG precursor analogues AcKDAlaDAlaOH and AcKDAlaDLac acid immobilized on the surface were also analyzed in SPR by firstly using the CM5 sensor chip. Vancomycin was used a control.

Sensograms registering the binding of the glycopeptides to AcKDAlaDAlaOH surface showed mass transport limitations that could be likely caused by a excessive ligand density on this surface (see Fig. S3 in supporting information). To elude this problem a CM4 chip, with a lower degree of carboxymethylation, was prepared to obtain less ligand density. Binding responses of each glycopeptide antibiotic with AcKDAlaDAlaOH in chip CM4 are shown in Fig 9. Binding responses were fit to a bivalent interaction model[21]. This mechanism could be consistent with dimerization of the glycopeptide molecules at the surface, enhanced in the presence of the ligand[22]. This effect might explain the association signals obtained, which exhibit an initial increase in response to the binding of one glycopeptide molecule to an AcKDAlaDAlaOH molecule in the surface and a subsequent less steeped slope associated with the binding of a second glycopeptide molecule due to surface dimerization. On the other hand, dissociation profiles show a first rapid phase and a slower second phase that would be due to the fact that binding between the dimerized glycopeptides and AcKDAlaDAlaOH in surface being stronger. Binding constants are provided in Table 1. None of the glycopeptides produced detectable binding signals in the AcKDAlaDLac acid surface, confirming the NMR data on the weakness of interaction with the PG precursor analogue terminating with the depsipeptide. SPR identical results were obtained using the Ac2KDAlaDAlaOH and Ac2KDAlaDLac tripeptide analogues used in NMR experiments (data not show), as expected considering that the N-terminal region is not involved in ligand binding.

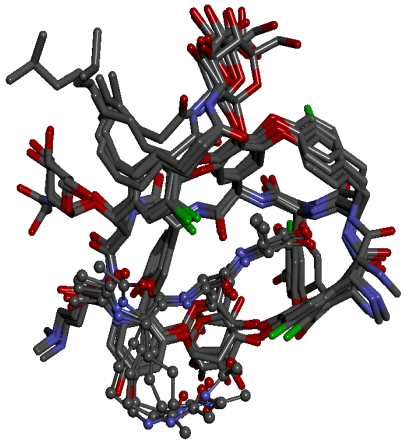

Figure 8. Superimposition of the complexes formed between MDL63,246, A40926, teicoplanin, mideplanim, and dalbavancin with the three-peptide (in ball and sticks representation) according to molecular modeling. The optimization involved MD (1 ns) simulations followed by energy minimization. The key hydrogen bonds are present in all cases for more than 90% of the time during the MD run. The shapes of the formed complexes are fairly similar. Different orientations of the pendant chains of the antibiotics as well as motion of the lysine side chain may be appreciated. In contrast, the recognition of the D-Ala-D-Ala dipeptide moiety is basically identical in the five cases.

The dissociation constants K_{D1} and K_{D2} calculated as k_{d1}/k_{a1} and k_{d2}/k_{a2} respectively, were found to be in a nanomolar range. Teicoplanin, mideplanin, dalbavancin and MDL63,246 exhibit a slightly lower K_{D1} and K_{D2}, which indicated a stronger binding to AcDAlaDAlaOH. In contrast, vancomycin and A40926 exhibit a moderate higher K_{D1} and K_{D2} indicating lower binding (Table 1).

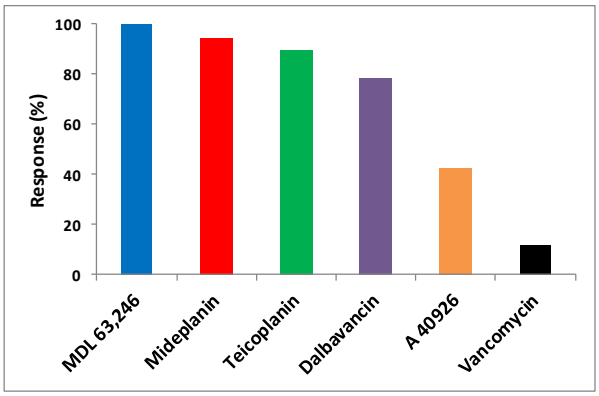

Figure 10. Binding responses (%) of the glycopeptide antibiotics on hydrophobic surface (HPA chip)

Vancomycin, mideplanin, teicoplanin, dalbavancin, A40926 and MDL63,246 were then injected in a hydrophobic surface (HPA chip) at 10 μM to analyze the interaction involving lipid chains. Responses shown in Fig. 10 suggest that all the glycopeptides analyzed presented stronger interaction through the hydrophobic surface in comparison with vancomycin. This fact could be attributed to the presence of a hydrophobic acyl chain on the heptapeptide scaffold, which can be responsible of the anchorage of the glycopeptides at the cell membrane.[7b] The stronger interactions of semi synthetic derivatives, particularly MDL63,246, may correlate with the lower MICs observed for these molecules versus coagulase negative staphylococci, streptococci and enterococci.[15-17]

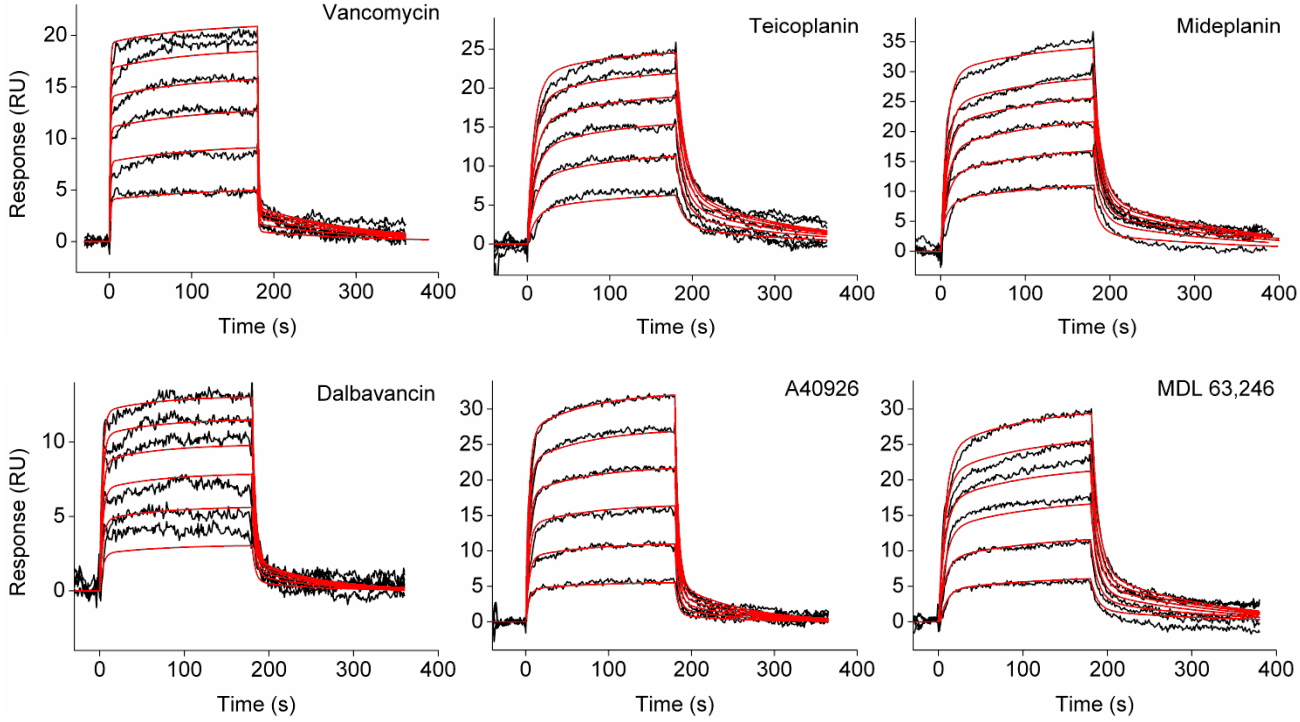

Figure 9. Binding responses of the glycopeptide antibiotics with AcKDAlaDAlaOH immobilized on a CM 4 chip (black lines) showing association and dissociation phases. Responses were reference subtracted and blank corrected. Binding responses were fit to a bivalent interaction model.

Table 1. Binding constants for the glycopeptide – AcKDAlaDAlaOH interactions determined at 25 ºC.

Glycopeptide	*k*a1 ($M^{-1}s^{-1}$)	*k*d1 (s^{-1})	*k*a2 ($RU^{-1}s^{-1}$)	*k*d2 (s^{-1})	*K*D1 (nM)	*K*D2 (RU)
Vancomycin	2.36 (7) × 105	8.2 (2) × 10-1	3.8 (2) × 10-5	4.2 (2) × 10-3	3466	110
Teicoplanin	5.52 (9) × 105	9.1 (2) × 10-2	6.0 (2) × 10-5	4.1 (1) × 10-3	165	68
Mideplanin	2.77 (7) × 105	1.06 (2) × 10-1	3.5 (1) × 10-5	2.93 (7) × 10-3	383	83
Dalbavancin	8.9 (3) × 105	2.9 (1) × 10-1	9.6 (8) × 10-5	7.1 (4) × 10-3	327	74
A40926	2.42 (5) × 104	2.53 (2) × 10-1	2.06 (7) × 10-6	6.1 (1) × 10-3	10455	2981
MDL63,246	1.70 (5) × 105	1.21 (2) × 10-1	2.0 (1) × 10-5	4.1 (1) × 10-3	712	200

Note: numbers between parentheses are standard errors.

Conclusion

This work examined the binding of different glycopeptides antibiotics (vamcomycin, teicoplanin, teicoplanin-like A40926, mideplanin, MDL63,246, dalbavancin) toward two types of cell wall precursor analogues (AcKDAlaDAlaOH/Ac2KDAlaDAlaOH and AcKDAlaDLac acid/Ac2KDAlaDLac acid) by employing solution NMR spectroscopy and SPR methods. In particular, we focused on the role of the hydrophobic fatty acid tail, which is considered related to the improved antimicrobial activity of teicoplanin and analogues against Gram-positive cocci.

The NMR data were obtained in DMSO solution due to the poor solubility of these molecules in water. The analysis of the experimentally obtained NMR parameters strongly suggests the

existence of two different complexes in solution for the glycopeptides when they interact with Ac2KDAlaDAlaOH. Although the NMR experimental conditions were markedly different to those employed for the SPR measurements, the NMR results paralleled those deduced in the chip regarding the drastic binding difference existing between the D-Ala and the D-Lac terminating analogues, confirming that all these antibiotics share the same primary molecular mechanism of action and resistance. In other terms structural modifications occurring among natural glycopeptides (such as vancomicyn, teicoplanin and A40926) and those chemically introduced in the semi-synthetic derivatives do not modify their mode of interaction with D-Ala-D-Ala or D-Ala-D-Lac termini of PG precursors. The dramatic differences in the antibiotic affinity between the tripeptide analogues terminating in D-Ala and D-Lac is in agreement with the glycopeptide poor biological activity *versus* vancomycin reistant vanA enterococci and staphylococci, that replace D-Ala with D-Lac.

We showed that SPR is well suited for the real time analysis of binding interactions at a model surface and provides tools to differentiate between vancomycin, teicoplanin, A40926 and other glycopeptides derivatives. Using a hydrophobic surface, it was possible to sort out the existence of a positive effect of the fatty acid chain for anchoring the antibiotics at the membrane. The observed differences under the SPR experimental conditions indicate that additional interactions through the employed surface are involved. Thus, the calculated binding affinities at the surface seem to be more relevant to *in vitro* antimicrobial activity than the estimations from NMR in DMSO solution.

Previous data about the absence of dimerization in the NMR studies of teicoplanin were confirmed by our data in DMSO solution. Considering the crystal structures previously described, which demonstrated that the interaction between lipoglycopeptides and cell wall precursors are established in the same way than vancomycin,[10], analysis by SPR showed that the binding of the glycopeptides to AcKDAlaDAlaOH on the surface was fit to a bivalent interaction model, which could be consistent with dimerization of the glycopeptide molecules. Thus, we provided evidences on the effect of cooperative binding of teicoplanin and its derivatives to bacterial cell surfaces. Our data suggest that dimerization and membrane anchoring are two interaction processes, which may synergically contribute to the activity of semi synthetic antibiotics and that can be further exploited in the search of better drugs to face resistant bacteria.

Experimental Section

Materials

All commercial products were used without any further purification step. Ac2KDAlaDAlaOH, AcKDAlaDAlaOH, Ac2KDAlaDLac acid acetate and AcKDAlaDLac acid acetate (PG precursor analogues) were purchased from Bachem. Vancomycin was purchased from Sigma-Aldrich. SPR sensor chips CM5 (carboxymethylated dextran), CM4 (low-density carboxymethylated dextran) and HPA (flat hydrophobic surface) and others reagents used in SPR experiments were purchased from GE Healthcare. All other chemicals were obtained from commercial sources.

Preparation of Glycopeptides

A40926 and teicoplanin were prepared by fermentation of the producing strains *Nonomuraea* sp. ATCC 39727 and *Actinoplanes teichomyceticus* ATCC 31121, respectively, as described elsewhere[20]. Mideplanin, MDL63,246 and dalbavancin were prepared by chemical modification of teicoplanin and A40926, respectively[15].

The purity of the glycopeptides was confirmed by HPLC (> 85%) on an HPLC Agilent 1100 with UV-vis detector using Mediterraneasea 18 15 cm x 0.46 5 mm column (Teknokroma). A40926, Dalbavancin, MDL63,246 and mideplanin were analysed using the conditions determined by Gandolfi.[23] The solvent system consisted of an aqueous solution of trifluoracetic acid (0.1%) and acetonitrile. Teicoplanin was analyzed using the analytical method of Borghi[24]. Mobile phase was: A 0.02M NaH_2PO_4-CH_3CN (95:5), B 0.02M NaH_2PO_4-CH_3CN (25:75).

Nuclear Magnetic Resonance

NMR spectra were recorded on a Bruker Avance 500 MHz spectrometer, equipped with a triple channel cryoprobe. Chemical shifts (δ) are expressed in parts per million (ppm). Two peptides that are PG precursor analogues (Ac2KDAlaDAlaOH and Ac2KDAlaDLac acid acetate) were dissolved in DMSO-d6 to obtain 100mM stock solutions and further diluted to 3 mM. Glycopeptide antibiotics were dissolved to 2 mM in in DMSO-d6. First, standard ^{1}H 1D NMR, 2D ROESY (mixing time 100 ms) and 2D TOCSY (mixing time 60 ms) spectra were recorded. Then, the PG precursor analogues were added to the NMR tube containing the antibiotic to give a final concentration of 3 mM. Therefore, the final antibiotic: ligand molar ratio was 1:1.5. For these samples, additional standard ^{1}H 1D NMR, 2D ROESY, and 2D TOCSY experiments, with the same mixing times were also acquired. All the experiments were recorded at 298 K. The existence of stable complexes was deduced by monitoring the chemical shift perturbation induced at the W2 proton (Fig. 2), at the "east side of the macrocyclic structure". When stable complexes are formed, this N-H establishes a strong intermolecular hydrogen bond with the carboxylate group of the ligand. Thus, this particular proton suffers a drastic downfield shift upon binding. At the same time, and from the perspective of the peptide, the methyl groups of the D-Ala-D-Ala moiety suffer a significant upfield shift, indicating that they are now located below the aromatic rings of the antibiotic.[4] DOSY experiments were also performed to obtain further information about the molecular recognition process. Experiments were performed for the isolated PG precursor analogues and for their final 1:1.5 samples with the different antibiotics. The DOSY spectra were recorded with the double stimulated spin echo (dstegp3s) pulse sequence, with convection compensation, with a linear gradient between 2% and 95%. Thirty-two 1D ^{1}H spectra were collected with a duration of δ = 1.8 ms and an echo delay of = 250 ms and processed using the standard Bruker software.

Conformational analysis

Molecular mechanics calculations for the antibiotics in the presence of the PG precursor analogues were performed using Macromodel 9.6, as implemented in the Maestro suite of programmes (version 8.5.110). The structures of the antibiotics were built using the coordinates for vancomycin, teicoplanin and other related antibiotics complexed to different PG precursor analogues deposited in the Protein Data Bank (pdb codes 1FVM, 1QD8, 2WDX, and 3MG9). The side chains of the different antibiotics were prepared from these structures as required. For the modelling the complexes with Ac2KDAlaDAlaOH, the starting geometry of the three-peptide in the 1FVM deposit was chosen. The Ac2KDAlaDLac analogues were built by simple modification of the affected atoms. Then, Ac2KDAlaDAlaOH or Ac2KDAlaDLac were manually docked to comply with the experimental intermolecular hydrogen bond pattern extensively described (Fig. 2) and the obtained complexes were minimized using the OPLS* force field, and a distance-dependence bulk dielectric constant of 70 debyes.

The minimized complexes were then submitted to 1 ns of MD simulations at 300 K, using the same force field, and no intermolecular restraints, to proof the conformational stability of the complexes. The equilibration time was 100 ps, the integration step was 1.5 fs, and the shake protocol was applied to the C-H bonds. In all cases, the temperature was stable within 2º. For the Ac2KDAlaDAlaOH complexes, the geometry of the complexes were found to be fairly stable during the whole MD run, keeping the intermolecular hydrogen bonds for more than 80% of the simulation time. Variations on the orientation and hydrogen bond pattern of the C-terminus of the Ac2KDAlaDAlaOH complexes were found, while the Ac2KDAlaDLac complexes dissociated after ca. 200 ps of simulation time, indicating their marginal stability.

Additionally, alternative geometries for the complexes were deduced by using AutoDock 4.2. Atom types and partial charges (Gasteiger-Hückel) for each nucleotide were calculated using the Sybyl 8.0 program, and the corresponding data were saved as a mol2 file. The starting geometries for the antibiotics were obtained from the Protein Data Bank, as described above, and optimized using the protein preparation wizard of Macromodel, as integrated in the Maestro package. In particular, after the hydrogen atoms were added, the structures were subsequently minimized with the OPLS-2005* force field, using Truncated Newton Conjugate Gradients. The ligands (if any) were fixed to their crystallographic positions. Then, the ligand was removed from the binding pocket and the resulting coordinates were saved as a new pdb file. For those cases with several molecules in the crystallographic unit cells, only one of them was considered as being rigid, to facilitate the docking process. For the Autodock calculations, grid maps (grid spacing: 0.375 Å) were constructed using 54 x 54 x 54 points for the box dimensions, and a total of 200 Lamarckian genetic algorithm runs were performed, using $2x10^7$ evaluations. The systematic analysis of the different binding poses was

performed by clustering the results using a r.m.s.d. of 1.8 Å. The pose obtained manually was always within the two best poses found by AutoDock.

Surface Plasmon Resonance

Surface Plasmon Resonance (SPR) experiments were performed at 25 ºC using Biacore 3000 (GE Healthcare). PBST (10 mM phosphate pH 7.4, 150 mM NaCl and 0.005% v/v surfactant P20) was used as running buffer for CM5 and CM4 experiments while HBS (10 mM HEPES pH 7.4, 150 mM NaCl) was used for HPA experiments. AcKDAlaDAlaOH and AcKDAlaDLac acid acetate (or Ac2KDAlaDAlaOH and Ac2KDAlaDLac acid acetate in early experiments) were diluted to 2 mg/mL in 10 mM phosphate pH 8 buffer and immobilized in separate flowcells of a CM5 sensor chip following amine coupling method according to the manufacturer's instructions. Immobilization response was 166 RU and 196 RU for AcKDAlaDAlaOH (flow cell 2) and AcKDAlaDLac acid acetate (flow cell 3), respectively. Sensor chip flow cell 1 was activated, blocked and used as a reference surface. The same immobilization procedure was carried out on a CM4 chip. Immobilization response was 83 RU and 69 RU for AcKDAlaDAlaOH (flow cell 2) and AcKDAlaDLac acid acetate (flow cell 3), respectively. Sensor chip flow cell 1 was activated, blocked and used as a reference surface. Blank samples and concentration series were injected on CM5 and CM4 chips at a flow rate of 50 µL/min for 180 s and dissociation was registered for 180 s. Chip CM5 concentration series were 25, 50, 100, 150, 200 and 250 nM of vancomycin; 5, 10, 15, 20, 25, 30 nM of teicoplanin and A40926; 0.5, 1.0, 2.0, 5.0, 7.5 and 10.0 nM of mideplanin and 1.0, 2.0, 5.0, 7.5, 10.0 and 12.5 nM of dalbavancin and MDL63,246. CM4 concentration series were 100, 200, 300, 400, 500 and 600 nM of vancomycin; 5, 10, 15, 20, 25 and 30 nM of teicoplanin; 10, 20, 30, 40, 50 and 60 nM of mideplanin, dalbavancin and MDL63,264 and 20, 40, 80, 120, 160 and 200 nM of A40926. Finally, 10 µM solutions of all glycopeptides were injected on a HPA chip at a flow rate of 5 µL/min for 180 s and dissociation was registered for 180 s. Data processing and analysis was carried out using BiaEvaluation v.4.1.1 (GE Healthcare). All signals were blank substracted, reference corrected and globally adjusted to an adequate kinetic model to obtain binding parameters.

Acknowledgements ((optional))

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Entry for the Table of Contents

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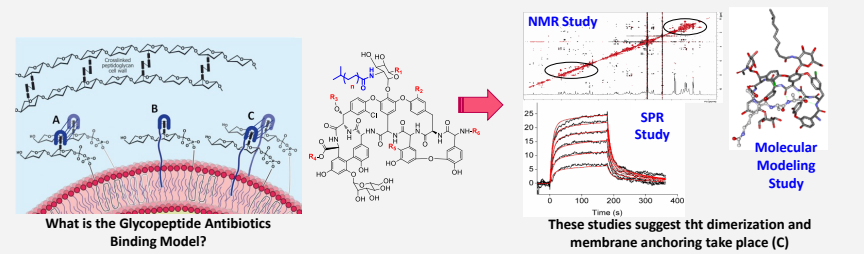
New Insights into the Glycopeptide Antibiotics Binding to Cell Wall Precursors using SPR and NMR spectroscopy



Binding analysis of different glycopeptide antibiotics toward two cell wall precursor analogues suggest that dimerization and membrane anchoring are two interaction processes which may synergically contribute to the activity of semisynthetic antibiotics and that can be further exploited in the search of better drugs to face resistant bacteria.

Supporting Information

New Insights into the Glycopeptide Antibiotics Binding to Cell Wall Precursors using SPR and NMR spectroscopy

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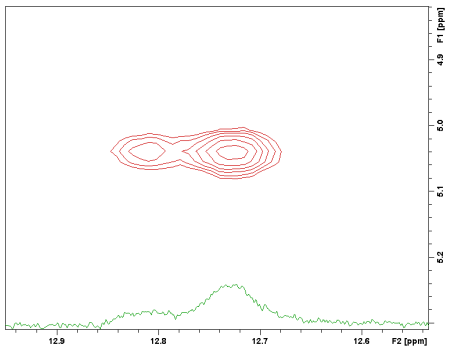

Fig. S1. TOCSY (60 ms) correlation of the W2 proton of teicoplanin upon addition of Ac2KDAlaDAlaOH. The chemical shift (> 12.5 ppm) indicates the presence of binding to the three-peptide and the existence of hydrogen bonding for this particular NH. The duplication of signals suggests the existence of two very similar bound species.

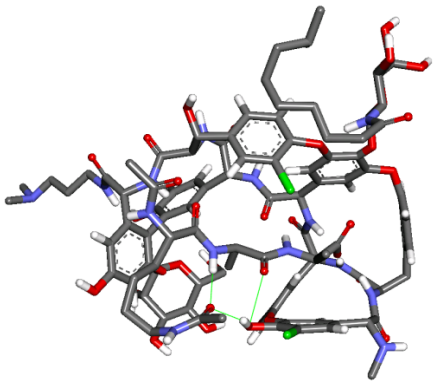

Fig. S2. The alternative secondary energy minimum of the complex of MDL63246 with Ac2KDAlaDAlaOH. The key hydrogen bonds that replace the regular ones present in the global minimum are highlighted in green in the south part of the figure.

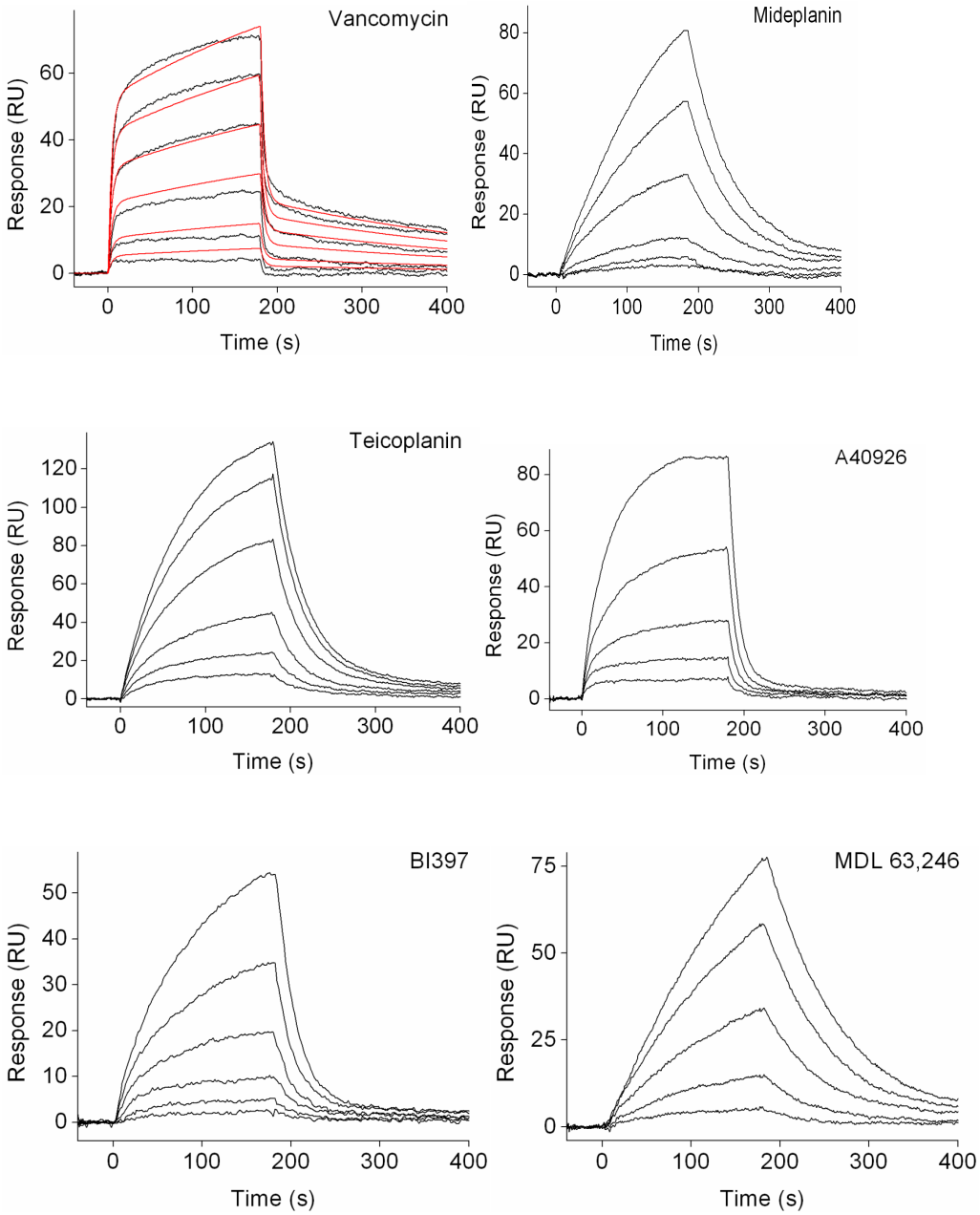

Fig. S3 Sensograms of Ac2KDAlaDAlaOH immobilized on a CM5 chip binding to vancomycin, mideplanin, teicoplanin, A40926, dalbavancin, and MDL63,246.