New Insights into Glycopeptide Antibiotic Binding to Cell Wall Precursors using SPR and NMR Spectroscopy

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New Insights into Glycopeptide Antibiotic 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 multidrug-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, studies of the interaction of teicoplanin, teicoplanin-like A40926, and of their semisynthetic derivatives (mideplanin, MDL63,246, dalbavancin) with peptide analogues of cell-wall precursors by NMR spectroscopy and surface plasmon resonance (SPR) are reported. NMR spectroscopy 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 spectroscopy results parallel those deduced in the chip with respect to 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. Kinetic analysis of the interaction between the glycopeptide antibiotics and immobilized AcKDAlaDAlaOH by SPR suggest a dimerization process that was not observed by NMR spectroscopy in DMSO solution. Moreover, in SPR, all 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. In conclusion, SPR provides a tool 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 spectroscopy analysis.

Introduction

Glycopeptide antibiotics, such as vancomycin and teicoplanin, are frequently used to treat life-threatening infections caused by multidrug-resistant Gram-positive pathogens. They are drugs of last resort against multiresistant 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 (Figure 1). The D-Ala-D-Ala complex with vancomycin is stabilized by an array of hydrophobic van

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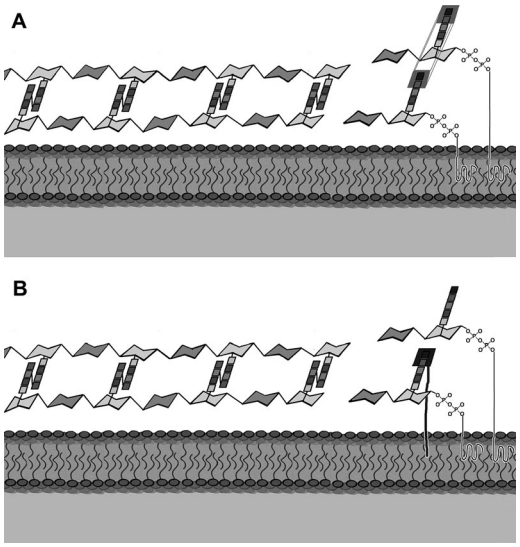


Figure 1. Schematic view of peptidoglycan structure and mechanism of action of glycopeptide antibiotics. Glycopeptides inhibit transglycosylation and transpeptidation by binding to the C-terminal D-Ala-D-Ala of the late PG precursors. A) Vancomycin-type glycopeptide activity is based on dimerization, 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.

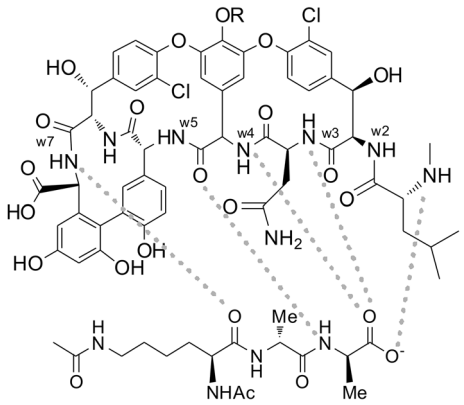


Figure 2. Schematic view of the key intermolecular hydrogen bonds described for vancomycin (PDB ID: 1FVM) and ligand terminating in D-Ala-D-Ala and kept for the molecular mechanics calculations of the different complexes studied herein. Chemical-shift perturbations at w2 of the antibiotic and at the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

der Waals contacts and five hydrogen bonds lining the antibiotic binding pocket (Figure 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 from a repulsive lone-pair interaction between the vancomycin residue 4 carbonyl and D-Ala-D-Lac ester oxygen atoms (Figure 2).

Previous NMR spectroscopy 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, balhimycin) 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]

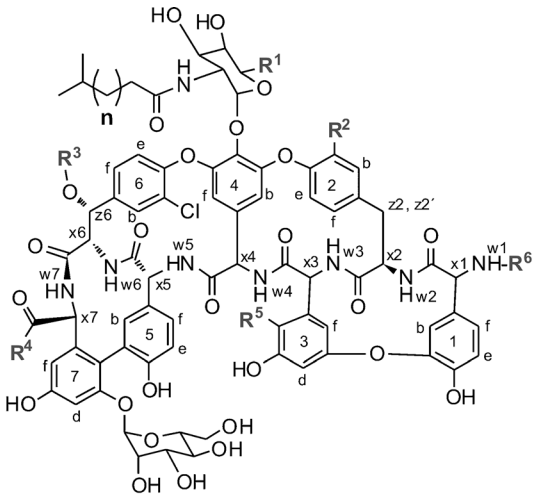
Crystal structure of the complexes teicoplanin–D-Ala-D-Ala and dalbavancin–D-Ala-D-Ala (a semisynthetic derivative) have been recently described, showing that the interaction of these lipoglycopeptides with the dipeptide is produced forming the same hydrogen bonds as vancomycin (Figure 2).[10] For teicoplanin deglycosylated derivatives, no evidence of dimer formation at millimolar concentrations was reported in past studies,[11] but teicoplanin's lipid chain (an extra aliphatic acyl side chain on the 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 semisynthetic 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 the PG terminus.[2b,13]

The aim of this study was to investigate the interaction of teicoplanin, teicoplanin-like A40926,[14] and the semisynthetic derivatives mideplanin,[15] MDL63,246,[16] dalbavancin[15,17] with peptide analogues of cell-wall precursors by NMR and SPR spectroscopy, the latter being an adequate system to predict biological activity. It is worth noting that dalbavancin is currently in phase III clinical trials for the treatment of acute bacterial skin and skin structure infections caused by susceptible Gram-positive bacteria (http://www.duratatherapeutics.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 spectroscopy 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 with the PG precursor analogues Ac2KDAlaDAlaOH and Ac2KDAla-DLac acid was monitored (Figure 3).

The obtained data will be presented in detail for MDL63,246 in comparison to teicoplanin. Nevertheless, analogous conclusions were deduced for all the studied glycopeptides. In all cases, no evidence for glycopeptide dimerization was inferred under these experimental conditions, especially from the DOSY experiments (see below).



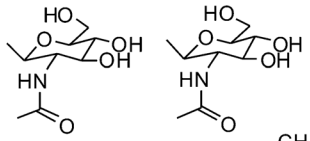
	Teicoplanin	Mideplanin	MDL63,246	Dalbavancin	A-40926
n =	5	5	7	7	7
R^1 =	$-CH_2\cdot OH$	$-CH_2\cdot OH$	$-CH_2\cdot OH$	$-COOH$	$-COOH$
R^2 =	$-Cl$	$-Cl$	$-H$	$-H$	$-H$
R^3 =			$-H$	$-H$	$-H$
R^4 =	$-OH$	$-NH-(CH_2)_3-N(CH_3)_2$	$-NH-(CH_2)_3-N(CH_3)_2$	$-NH-(CH_2)_3-N(CH_3)_2$	$-OH$
R^5 =	$-H$	$-H$	$-Cl$	$-Cl$	$-Cl$
R^6 =	$-H$	$-H$	$-CH_3$	$-CH_3$	$-CH_3$

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.

Nuclear magnetic resonance

Previous works[18] have focused on the solution conformation of teicoplanin as well as on its interactions with the di- and tripeptide 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 chain) derivatives of teicoplanin in a variety of solvents, as an attempt 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 spectroscopy experiments were performed in dimethyl sulfoxide (DMSO). As mentioned in the Experimental Section, there are two reported key structural evidences that permit the monitoring of the existence (or not) of stable intermolecular complexes between the antibiotics and the PG precursor Ac2KDAlaDAlaOH. Thus, chemical-shift perturbations at w2 (Figure 2) of the antibiotic and at the two methyl groups of the tripeptide were monitored to prove the existence of the molecular recognition process.[4,19]

The chemical shifts of the w2 protons were easily identified through the TOCSY crosspeak to the corresponding Hα. For teicoplanin free w2/x2 (7.45/4.85) was found to be similar to that previously reported (7.41/4.97);[18] for MDL63,246 a similar value was obtained (7.54/4.93).

Fittingly, when Ac2KDAlaDAlaOH (3 mM) was added to the NMR tube containing 2 mM of glycopeptide, the existence of drastic chemical-shift changes for several protons was evident. Interestingly, in all cases a new broad NH signal appeared, at very low field, between 12–13 ppm, corresponding to w2 NH, indicating that this proton is perturbed in more than 4 ppm when the corresponding glycopeptide forms the complex with Ac2KDAlaDAlaOH. In general this signal was found to be rather broad, split into two signals, permitting distinction of two cross-peaks in the TOCSY spectrum (Figure S1 in Supporting Information). The existence of these two new cross-peaks strongly suggests the existence of two different (although structurally similar) bound species in slow exchange in the chemical-shift timescale with the free form. Important shifts were also observed for protons w3 and w4 (ca. 2 ppm). This downfield shift for NH protons is usually related to participation in hydrogen bonding.

From the Ac2KDAlaDAlaOH viewpoint, for all the complexes a new signal (a doublet) appeared at high field (0.4 ppm), which corresponds to the methyl group of Ala-3. 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. Interestingly, chemical exchange cross-peaks were found in the ROESY spectra for most of the Ac2KDAlaDAlaOH protons (Figure 4). Again, this was an indication of the existence of one major and one minor species, accounting for the existence of two very similar bound species.

The analysis of the ROESY spectra of the complexes afforded valuable information, since intermolecular NOEs between protons of the glycopeptides and of Ac2KDAlaDAlaOH were identified. The ROESY spectrum for the complex with MDL63,246 is

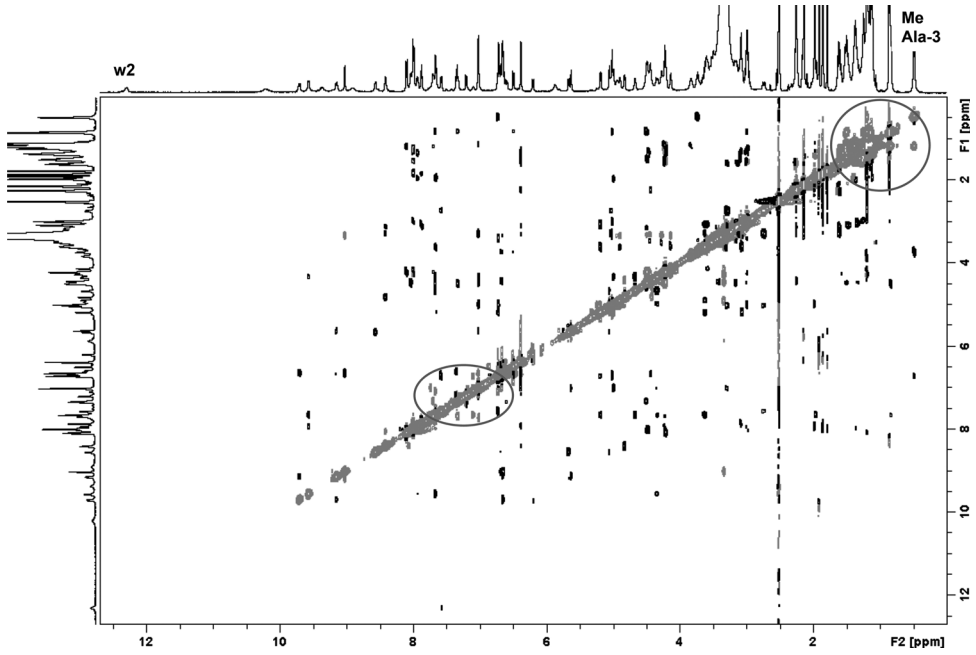


Figure 4. ROESY spectrum of the mixture of MDL63,246/Ac2KDAlaDAlaOH (1:1.5 molar ratio). Protons w2 of the glycopeptides and Me of Ala-3 are annotated in the 1D trace. The circled areas highlight chemical exchange cross-peaks corresponding to Ac2KDAlaDAlaOH methyl groups (at high field) and the NHs (at low field) between two bound forms.

shown in Figure 5. In particular NOEs between the methyl protons of Ala-3 of the cell-wall precursor with 2e of the glycopeptide, methyl of Ala-2 with 1e and 5b and Ha of Ala-2 with 1f were identified.

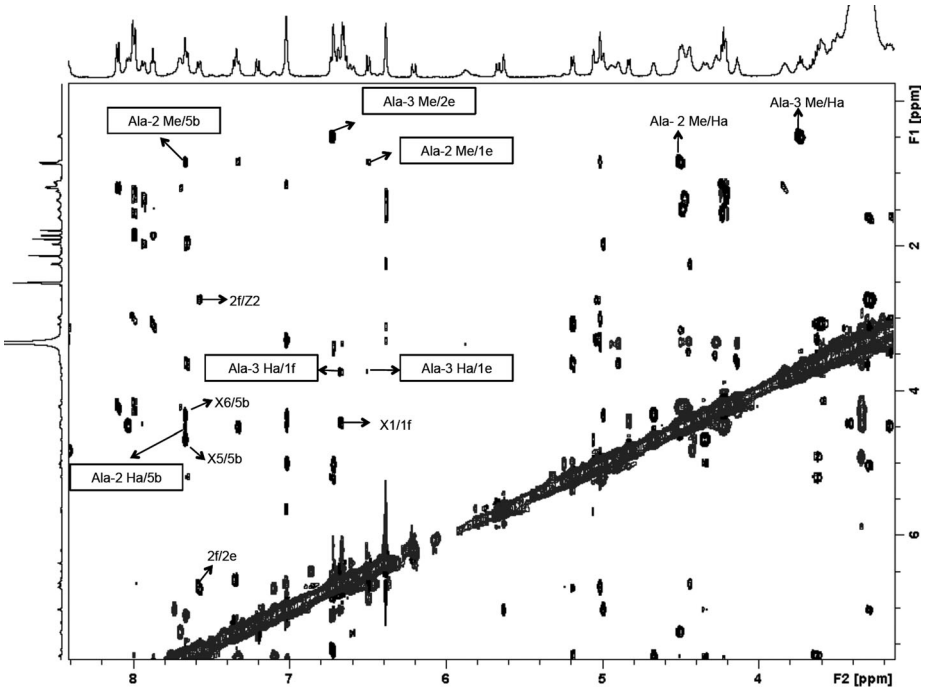


Figure 5. ROESY spectrum of the mixture of MDL63,246/Ac2KDAlaDAlaOH (1:1.5 molar ratio). Key intermolecular NOEs are annotated with squares. Some intramolecular NOEs of both MDL63,246 and Ac2KDAlaDAlaOH are also indicated.

Additional indication of the existence of interaction came from the analysis of DOSY spectra (Figure 6A). The comparison of the diffusion coefficients measured for free Ac2KDAlaDAlaOH and in the presence of MDL63,246 (MDL63,246/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 MDL63,246 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 assumed. The chemical-shift data suggest 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.

In contrast, when Ac2KDAlaDLac acid (3 mM) was added to the NMR tube containing MDL63,246, 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, the same apparent diffusion coefficients were observed both for free Ac2KDAlaDLac acid and for this compound in the presence of MDL63,246 (Figure 6B). These results indicate that, for this ligand, the interaction with MDL63,246 is much weaker than that described above for Ac2KDAlaDAlaOH.

The 3D structure of the formed complexes was derived using molecular modeling meth-

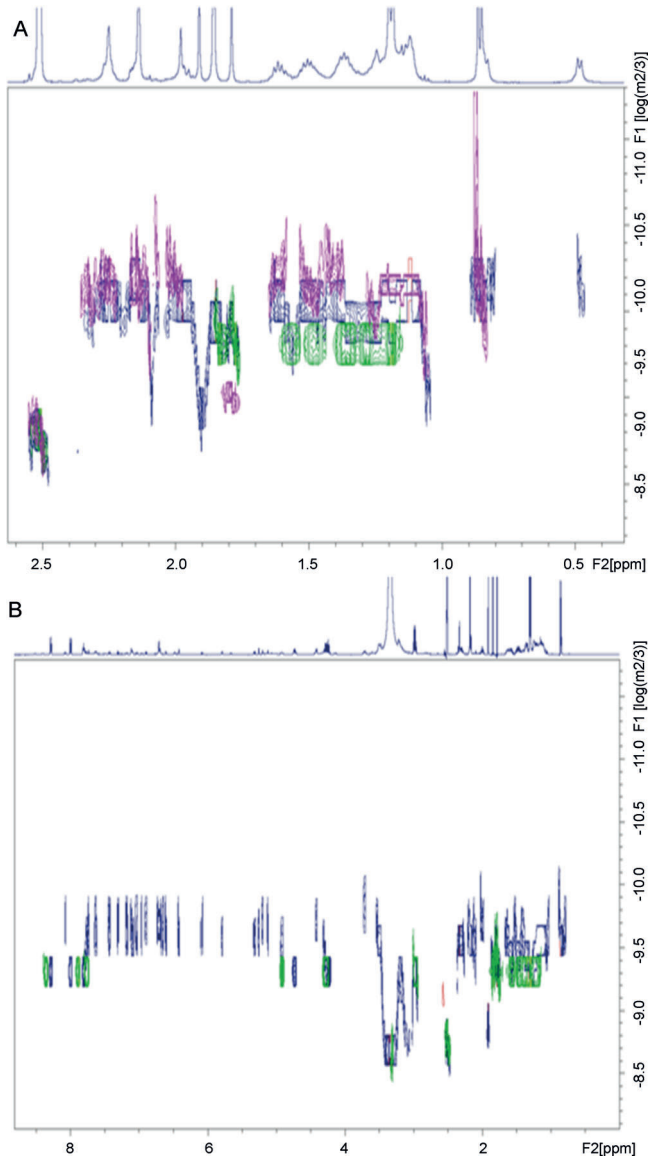


Figure 6. DOSY spectra: A) in blue free MDL63,246; in purple MDL63,246/Ac_2KDAlaDAlaOH at ratio 1:1.5; in green Ac2KDAlaDAlaOH. Only the aliphatic region is shown. The diffusion coefficient of Ac2KDAlaDAlaOH in the presence of the glycopeptides is smaller than in the free-state; B) in blue: MDL-63,246/Ac_2KDAlaLac acid at ratio 1:1.5; in green Ac2KDAlaDLac acid. The diffusion coefficient of Ac_2KDAlaDLac acid is the same in the absence and in the presence of the glycopeptide.

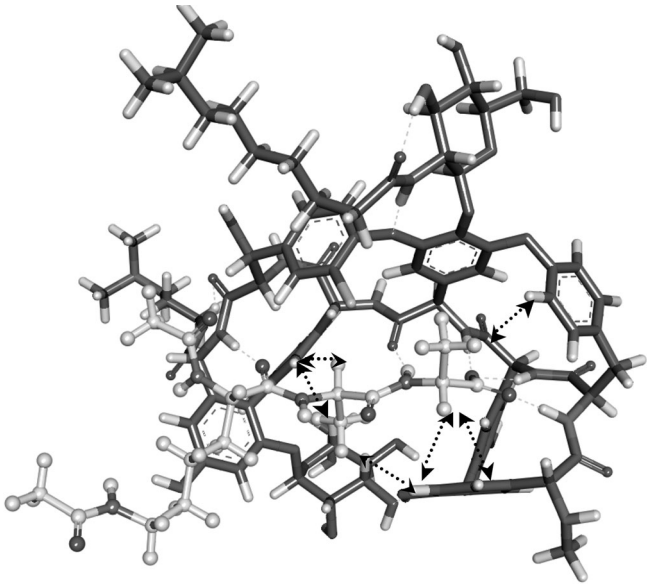


Figure 7. The complex formed between MDL63,246 and the Ac2KDAlaD-AlaOH tripeptide according to molecular modeling. The global minimum structure (the Ac2KDAlaDAlaOH is in ball and sticks representation) after MD (1 ns) simulations followed by energy minimization. The key hydrogen bonds are indicated with dashed lines. These hydrogen bonds (three involving the carboxylate group of D-Ala-3 and one involving D-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. The dashed arrows indicate the proton pairs showing intermolecular NOE. The orientation of the methyl group of D-Ala-3 packed toward the aromatic ring of residue 4 of MDL63,246 is in agreement with the observed chemical-shift perturbation data.

Table 1. Chemical shifts for the major bound conformer of Ac2KDAlaD-AlaOH complexed to MDL63,246, intermolecular NOEs and corresponding distance in minimized structure.

Proton	δ [ppm]	MDL63,246 proton[a]	d[b] [Å]
Ala-3			
NH	7.66		
Ha	3.75	1f	2.74
		1e	3.20
Me	0.47	2e	2.53
Ala-2			
NH	7.69		
Ha	4.50	5b	2.75
Me	0.79	1e	3.00
		5b	2.36
Lys			
NH	7.98		
Ha	4.21		

[a] Intermolecular NOE to MDL63,246 proton. [b] Proton–proton distance in modeled minimized structure [Å].

ods, 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 MDL63,246 and Ac2KDAlaDAlaOH is in agreement with those previously proposed for a variety of antibiotic–PG precursor analogue pairs (Figure 7).[18] More importantly this structure complies with the experimental data described above: the interatomic distances for the atom pairs exhibiting intermolecular NOE are within 2.3 and 3.2 Å (Table 1, Figure 5 and Figure 7). The hydrogen bonding pattern as well as the stacking interaction of the D-Ala-3 methyl group with the aromatic ring of residue 4 explains the corresponding chemical-shift perturbations.

A second ligand conformation was also detected, with a relative energy of about 2 kcal mol^{-1} above that of the global minimum. Herein, a hydrogen bond between the phenolic ring 3 of the glycopeptides and the carbonyl group of the acetyl group of the Lys moiety takes place. This acetyl group is in

turn hydrogen bonded to the NH group of D-Ala-2. This structural pattern replaces the most stable one formed by the amide nitrogen of 7 and the carbonyl oxygen of Lys (Figures 2 and 7). Still, this complex maintains the hydrogen bond pattern as well as the stacking interaction of the D-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 IDs: 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 complexes were evident, as seen for teicoplanin in Figure 8. The Ac2KDAlaDLac acid molecule dissociated from the glycopeptide 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 about 2 kcal mol^{-1} less stable than that obtained for the teicoplanin/Ac2KDAlaDAlaOH complex.

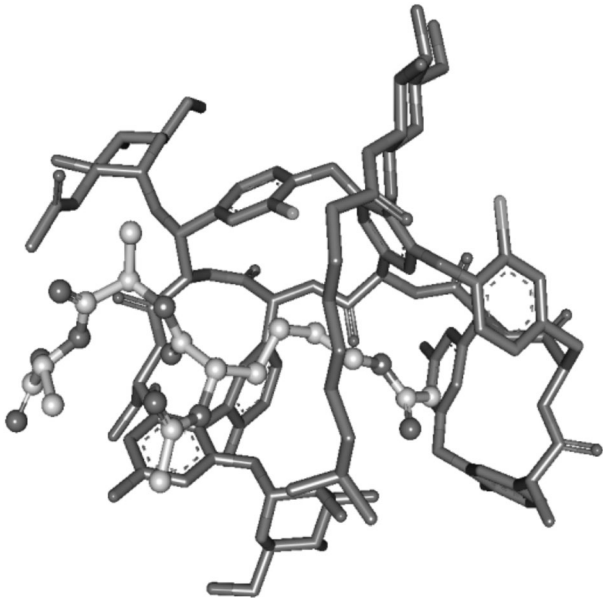


Figure 8. The complex formed between teicoplanin and the Ac2KDAlaDLac acid analogue (in ball and sticks representation). The key intermolecular interactions present in the tripeptide analogue (see above) are destroyed after the MD equilibration period. No intermolecular hydrogen bonds are formed and therefore, no complex is present in solution.

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

The NMR experiments indicated the existence of interactions between the antibiotics and Ac2KDAlaDAlaOH in all cases. Indeed, the acquired ROESY spectra are very similar to those presented in Figures 4 and 5 for MDL63,246. Similar features were always observed, including the presence of cross-peaks that suggest the existence of one major and one minor bound species in slow exchange in the chemical-shift timescale. Similar intermolecular NOEs were found for all the complexes (Figures S1–S5 and Tables S2 and S3 in the Supporting Information). Shielding of the methyl group of D-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 D-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 teicoplanin, 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 the 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 dalbavancin 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]

A superimposition of the global minima of all complexes derived from the molecular modeling protocol is displayed in Figure 9.

In contrast, when the Ac2KDAlaDLac acid analogue was employed, very minor chemical shifts were evident, no slow-ex-

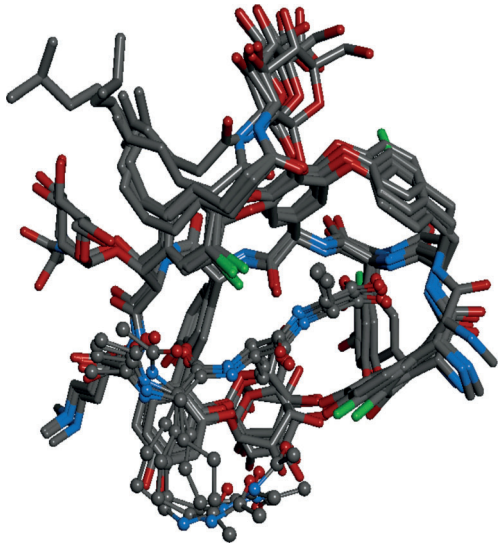


Figure 9. Superimposition of the complexes formed between MDL63,246, A40926, teicoplanin, mideplanim, and dalbavancin with the tripeptide (in ball and sticks representation) according to molecular modeling. The optimization involved MD (1 ns) simulations followed by energy minimization. The key hydrogen 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.

change 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 (Figure 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 Ac2KDAlaDAlaOH surface showed mass transport limitations that could be likely caused by an excessive ligand density on this surface (Figure S6 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 Figure 10. 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 on surfaces is stronger. Binding constants are provided in Table 2. None of the glycopeptides produced detectable binding signals in the AcKDAlaDLac acid surface, confirming the NMR spectroscopy data on the weakness of interaction with the PG precursor analogue terminating with the depsipeptide. SPR identical results were obtained in chip CM4 using the Ac2KDAlaDAlaOH and Ac2KDAlaDLac tripeptide analogues used in NMR spectroscopy experiments (data not show), as expected considering that the N-terminal region is not involved in ligand binding.

Table 2. Binding constants for the glycopeptide–AcKDAlaDAlaOH interactions determined at 25 °C.[a]

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	$2.36\ (7)\times10^{5}$	$8.2\ (2)\times10^{-1}$	$3.8\ (2)\times10^{-5}$	$4.2\ (2)\times10^{-3}$	3466	110
teicoplanin	$5.52\ (9)\times10^{5}$	$9.1\ (2)\times10^{-2}$	$6.0\ (2)\times10^{-5}$	$4.1\ (1)\times10^{-3}$	165	68
mideplanin	$2.77\ (7)\times10^{5}$	$1.06\ (2)\times10^{-1}$	$3.5\ (1)\times10^{-5}$	$2.93\ (7)\times10^{-3}$	383	83
dalbavancin	$8.9\ (3)\times10^{5}$	$2.9\ (1)\times10^{-1}$	$9.6\ (8)\times10^{-5}$	$7.1\ (4)\times10^{-3}$	327	74
A40926	$2.42\ (5)\times10^{4}$	$2.53\ (2)\times10^{-1}$	$2.06\ (7)\times10^{-6}$	$6.1\ (1)\times10^{-3}$	10455	2981
MDL63,246	$1.70\ (5)\times10^{5}$	$1.21\ (2)\times10^{-1}$	$2.0\ (1)\times10^{-5}$	$4.1\ (1)\times10^{-3}$	712	200

[a] Values in parentheses are standard errors.

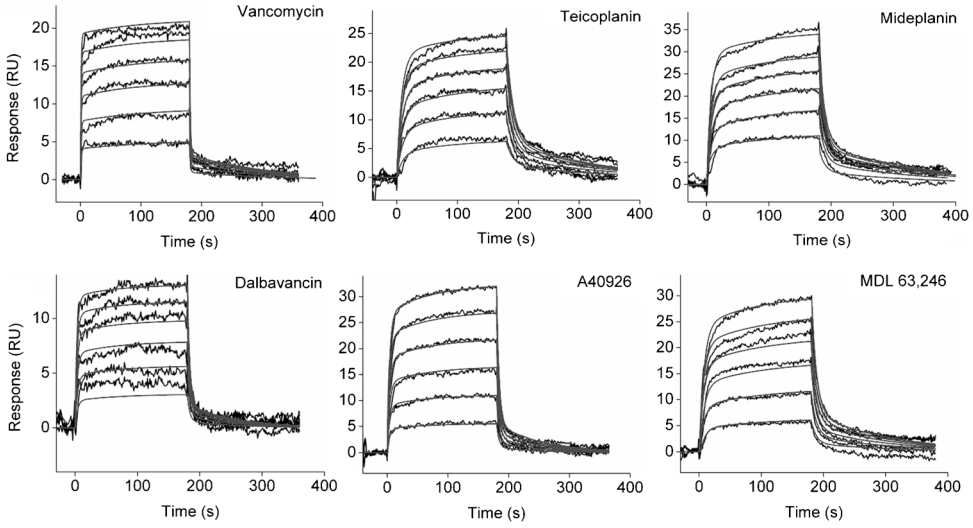


Figure 10. Binding responses of the glycopeptide antibiotics with AcKDAlaDAlaOH immobilized on a CM4 chip (straight lines) showing association and dissociation phases. Responses were reference subtracted and blank corrected. Binding responses were fit to a bivalent interaction model.

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 moderately higher K_{D1} and K_{D2}, indicating lower binding (Table 2).

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 suggest that all the glycopeptides analyzed presented stronger interaction through the hydrophobic surface in comparison with vancomycin (Figure 11). 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 semisynthetic derivatives, particularly MDL63,246, may correlate with the lower MICs observed for these molecules versus coagulase negative staphylococci, streptococci and enterococci.[15–17]

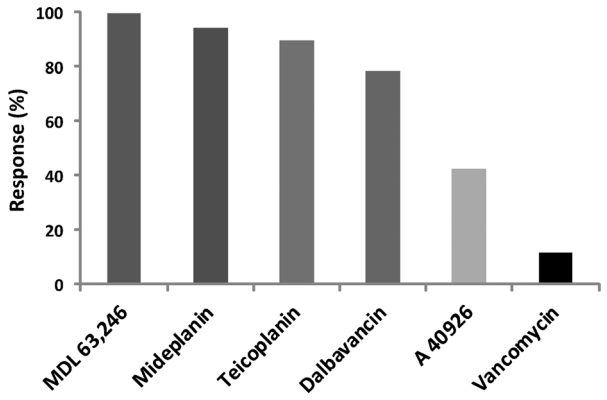


Figure 11. Binding responses [%] of the glycopeptide antibiotics on hydrophobic surface (HPA chip).

Conclusions

This work examined the binding of different glycopeptide antibiotics (vancomycin, 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 to be related to the improved antimicrobial activity of teicoplanin and analogues against Gram-positive cocci.

The NMR spectroscopy data were obtained in DMSO solution due to the poor solubility of these molecules in water. The analysis of the experimentally obtained NMR spectroscopy 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 with respect to 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 vancomycin, teicoplanin and A40926) and those chemically introduced in the semisynthetic 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 glycopeptides' poor biological activity versus vancomycin resistant vanA enterococci and staphylococci, that replace D-Ala with D-Lac.

We showed that SPR is well suited for real-time analysis of binding interactions at a model surface and provides tools to differentiate between vancomycin, teicoplanin, A40926 and other glycopeptide 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 spectroscopy in DMSO solution.

The NMR spectroscopy data indicate that the glycopeptides used in this study in DMSO solution do not dimerize and this is in agreement with previous data generated by NMR spectroscopy. Recent crystallographic studies show that teicoplanin and dalbavancin do not form the intimate hydrogen bonded back-to-back dimers seen with vancomycin-type glycopeptides, since the fatty acyl chain (absent in vancomycin but present in teicoplanin-like molecules) sterically blocks the close back-to-back approach of the monomers and prevents dimerization. The same studies also indicate that the fatty acid chain lies on the back of the antibiotic molecules, that is, the side opposite to the ligand binding site, and that the conformations of the fatty acid acyl chains differ suggesting a certain degree of flexibility for this group.[10] The same authors also report that two molecules of dalbavancin (in their cases interacting with tripeptides linked to proteins) associate loosely back-to-back via their fatty acid moieties. When glycopeptide interaction is studied in SPR, the role of the fatty acid chain in the localization of the molecules on the surface becomes a relevant factor as in the in vivo interaction between the antibiotic and its molecular target (the PG precursor) located on the external surface of the plasmatic membranes. In this situation the flexible fatty acid tails anchor the glycopeptide molecules to the membrane and force them to interact with the multiple exposed PG precursors. So it is possible that dimerization, cooperative binding, synergic activity become evident only in the SPR and not in NMR spectroscopy and in crystallography, since SPR better mimics the in vivo interaction which is the result of the primary mode of action (hydrogen bonds with D-Ala-D-Ala) and the secondary interactions with the target environment. Our data 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.

Experimental Section

Materials

All commercial products were used without further purification steps. 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 (>90%) on an HPLC Agilent 1100 with UV/Vis detector using a Mediterraneasea 18 15 cm×0.46 5 mm column (Teknokroma). A40926, dalbavancin, MDL63,246 and mideplanin were analyzed using the conditions determined by Gandolfi.[23] The solvent system consisted of an aqueous solution of trifluoroacetic acid (0.1%) and acetonitrile. Teicoplanin was analyzed using the analytical method of Borghi.[24] The mobile phase was: A, 0.02 M NaH_2PO_4/CH_3CN (95:5); B, 0.02 M NaH_2PO_4/CH_3CN (25:75).

NMR spectroscopy

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 [D_6]DMSO to obtain 100 mM stock solutions and further diluted to 3 mM. Glycopeptide antibiotics were dissolved to 2 mM in [D_6]DMSO. First, standard ^{1}H 1D NMR spectroscopy, 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 spectroscopy, 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 (Figure 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 $\delta = 1.8$ 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 programs (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 IDs: 1FVM, 1QD8, 2WDX, and 3MG9). The side chains of the different antibiotics were prepared from these structures as required. For the modeling the complexes with Ac2KDAlaDAlaOH, the starting geometry of the tripeptide 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 (Figure 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 assess 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 2°. 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 about 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 glycopeptides 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 54×54×54 points for the box dimensions, and a total of 200 Lamarckian genetic algorithm runs were performed, using 2×10^7 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

SPR experiments were performed at 25°C 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 whereas HBS (10 mM HEPES pH 7.4, 150 mM NaCl) was used for HPA experiments. AcKDAlaDAlaOH and AcKDAlaDLac acid acetate were covalently bound to the sensor surface through its primary amino groups. The carboxymethylated dextran surface of the CM-5 sensor chip was first activated using an injection pulse of an equimolar mix of NHS and EDC (35 μL, 5 μL min^{-1}, final concentration 0.05 M; mixed immediately prior to injection). Then AcKDAlaDAlaOH and AcKDAlaDLac acid acetate were diluted to 2 mg mL^{-1} in 10 mM phosphate buffer pH 8 and immobilized in separate flowcells of a CM5 sensor chip. Unoccupied active sites on the sensor surfaces were then blocked with an injection (35 μL) of 1 M ethanolamine. Immobilization response was 166 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 (Matrix: similar to Sensor Chip CM5 but with a lower degree of carboxymethylation). Immobilization response was 83 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 $\mu L\,min^{-1}$ for 180 s and dissociation was registered for 180 s. Chip CM5 concentration series were: 25, 50, 100, 150, 200 and 250 nM for vancomycin; 5, 10, 15, 20, 25, 30 nM for teicoplanin and A40926; 0.5, 1.0, 2.0, 5.0, 7.5 and 10.0 nM for mideplanin; and 1.0, 2.0, 5.0, 7.5, 10.0 and 12.5 nM for dalbavancin and MDL63,246. CM4 concentration series were: 100, 200, 300, 400, 500 and 600 nM for vancomycin; 5, 10, 15, 20, 25 and 30 nM for teicoplanin; 10, 20, 30, 40, 50 and 60 nM for mideplanin, dalbavancin and MDL63,264; and 20, 40, 80, 120, 160 and 200 nM for A40926. Finally, solutions (10 μM) of all glycopeptides were injected on a HPA chip at a flow rate of 5 $\mu L\,min^{-1}$ for 180 s and dissociation was registered for 180 s. Data processing and analysis were carried out using BiaEvaluation v.4.1.1 (GE Healthcare). All signals were blank subtracted, reference corrected and globally adjusted to an adequate kinetic model to obtain binding parameters.

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Keywords: antibiotics · glycopeptides · NMR spectroscopy · surface plasmon resonance · teicoplanin

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