Structural Variations of the Cell Wall Precursor Lipid II and Their Influence on Binding and Activity of the Lipoglycopeptide Antibiotic Oritavancin

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Oritavancin is a semisynthetic derivative of the glycopeptide antibiotic chloroeremomycin with activity against Gram-positive pathogens, including vancomycin-resistant staphylococci and enterococci. Compared to vancomycin, oritavancin is characterized by the presence of two additional residues, a hydrophobic 4′-chlorobiphenyl methyl moiety and a 4-epi-vancosamine substituent, which is also present in chloroeremomycin. Here, we show that oritavancin and its des-_N_-methylleucyl variant (des-oritavancin) effectively inhibit lipid I- and lipid II-consuming peptidoglycan biosynthesis reactions _in vitro_. In contrast to that for vancomycin, the binding affinity of oritavancin to the cell wall precursor lipid II appears to involve, in addition to the D-Ala-D-Ala terminus, other species-specific binding sites of the lipid II molecule, i.e., the crossbridge and D-isoglutamine in position 2 of the lipid II stem peptide, both characteristic for a number of Gram-positive pathogens, including staphylococci and enterococci. Using purified lipid II and modified lipid II variants, we studied the impact of these modifications on the binding of oritavancin and compared it to those of vancomycin, chloroeremomycin, and des-oritavancin. Analysis of the binding parameters revealed that additional intramolecular interactions of oritavancin with the peptidoglycan precursor appear to compensate for the loss of a crucial hydrogen bond in vancomycin-resistant strains, resulting in enhanced binding affinity. Augmenting previous findings, we show that amidation of the lipid II stem peptide predominantly accounts for the increased binding of oritavancin to the modified intermediates ending in D-Ala-D-Lac. Corroborating our conclusions, we further provide biochemical evidence for the phenomenon of the antagonistic effects of _mecA_ and _vanA_ resistance determinants in _Staphylococcus aureus_, thus partially explaining the low frequency of methicillin-resistant _S. aureus_ (MRSA) acquiring high-level vancomycin resistance.

Glycopeptide antibiotics constitute a diverse group of natural products (1). The largest and most prominent class among the glycopeptide antibiotics is represented by the vancomycin group. The members of this class are characterized by an aglycone core consisting of seven amino acids forming a trimacrocyclic or tetramacrocyclic structure, synthesized by nonribosomal peptide synthetases. Additional structural heterogeneity within this class arises from the sugar moieties attached to the heptapeptide core, which vary in number, position, and chemical structure.

The glycopeptide antibiotic vancomycin was first isolated and described in the early 1950s (2). As a consequence of the worldwide emergence of methicillin-resistant _Staphylococcus aureus_ (MRSA) in the 1970s, vancomycin was increasingly considered the last resort for treatment of MRSA infections. For more than a decade, vancomycin was used successfully without a significant rise in resistance, until enterococcal strains with acquired resistance to vancomycin emerged in 1986 (3–5). Today, vancomycin-resistant enterococci (VRE) represent >30% of all clinical enterococcal isolates in the United States (6). In Europe, surveillance data show large variabilities between various countries with VRE ranging from <2% (Finland and Holland) to >25% (Ireland, Greece, Portugal, and United Kingdom) (7). Although high-level resistance to vancomycin in staphylococci is rare, the increased occurrence of vancomycin-intermediate _S. aureus_ (VISA) and heterogeneous VISA (hVISA) in the hospital setting is increasingly limiting treatment options.

The mode of action of vancomycin and other glycopeptide antibiotics relies on binding to the D-Ala-D-Ala terminus of the peptidoglycan (PG) cell wall precursor lipid II (8–10). Lipid II represents the central cell wall building block of PG biosynthesis. The precursor consists of the bactoprenol carrier, which is linked to the disaccharide unit _N_-acetylmuramyl-pentapeptide-_N_-acetylglucosamine via a pyrophosphate bridge (10). Binding of vancomycin to lipid II, which is mediated by formation of five hydrogen bonds between the glycopeptide and the D-Ala-D-Ala terminus of lipid II, locks the cell wall precursor in a stable complex, thereby blocking the entire peptidoglycan synthesis cycle. More precisely, binding to lipid II sterically shields the cell wall precursor substrate from the penicillin binding proteins (PBPs) that catalyze transglycosylation and transpeptidation (9, 11). In VRE and vancomycin-resistant _S. aureus_ (VRSA), the mechanism of resistance results from an alteration of the molecular target, i.e.,
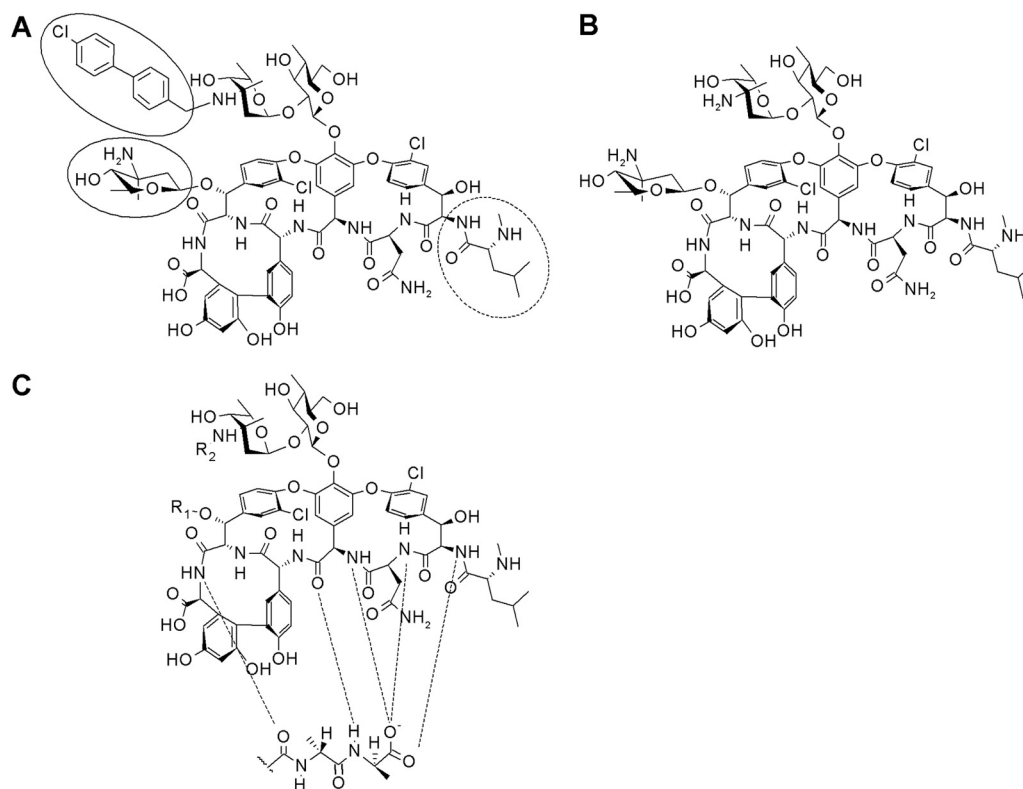
FIG 1 Chemical structure of oritavancin (A), chloroeremomycin (B), and the glycopeptide backbone (C). The hydrophobic 4′-chlorobiphenyl methyl moiety and the 4-epi-vancosamine substituent are highlighted. The leucyl residue missing in des-N-methylleucyl-oritavancin is marked by a dashed circle. Hydrogen bonds are marked by dashed lines.

the replacement of the D-Ala-D-Ala terminus by D-Ala-D-Lac (VanA/VanB) or D-Ala-D-Ser (VanC). The incorporation of D-lactate into peptidoglycan precursors results in the loss of one of the five hydrogen bonds, leading to a 1,000-fold decrease in vancomycin antibiotic activity, while decreased binding of vancomycin to the D-Ala-D-Ser termini of lipid II is due to steric hindrance (12–14). The emergence of vancomycin-resistant strains has revived the development of second-generation glycopeptides with improved activities against these pathogens (15–20). Oritavancin is the N-substituted 4′-chlorobiphenyl methyl synthetic derivative of the naturally occurring glycopeptide chloroeremomycin (Fig. 1A) (21–23). Chloroeremomycin (Fig. 1B) itself belongs to the eremomycin class, which differs from vancomycin by an additional monosaccharide moiety (4-epi-vancosamine) attached to the ring 6-amino acid residue and the substitution of the existing vancosamine at position 4 by 4-epi-vancosamine (24). In contrast to chloroeremomycin, oritavancin is rapidly bactericidal (25, 26) and displays antibacterial activity against MRSA, VISA, VRSA, daptomycin-nonsusceptible S. aureus, and VRE (22, 27, 28). Oritavancin was approved in 2014 for the treatment of complicated skin and skin structure infections caused by Gram-positive pathogens, including MRSA (29). The reasons for the enhanced antimicrobial activity of oritavancin even against vancomycin-resistant strains and its detailed mode of action are still a matter of debate. Like other glycopeptides, oritavancin inhibits the late steps of peptidoglycan biosynthesis (transglycosylation) by binding to the D-Ala-D-Ala terminus of lipid II. Oritavancin was proposed to additionally promote membrane depolarization and permeabili-

zation, leading to rapid cell death (30, 31). It has further been suggested that the hydrophobic substituent promotes dimerization of the antibiotic, resulting in increased binding affinity (32, 33). Moreover, membrane anchoring is thought to direct the antibiotic in close proximity to its membrane-standing target and to further stabilize the glycopeptide-lipid II interaction (34). The additional carbohydrate moiety present in oritavancin and chloroeremomycin has been reported to directly interfere with penicillin binding proteins, resulting in inhibition of peptidoglycan biosynthesis independent of D-Ala-D-Ala binding (35). Recent studies suggested that binding of oritavancin involves multiple interactions with its molecular target lipid II. Solid-state nuclear magnetic resonance (NMR) and computational modeling predicted secondary interactions of the 4′-chlorobiphenyl methyl side chain with the S. aureus pentaglycine crossbridge and the D-aspartate/D-asparagine (D-Asx) crossbridge in Enterococcus faecium (36–40). To better understand the subtleties of the mechanism of action of oritavancin, we characterized the binding to its target lipid II in vivo and in vitro using purified lipid II variants. A comprehensive comparison of oritavancin-binding parameters to variants of the natural occurring cell wall building block and its depsipeptide counterparts, ending in D-Ala-D-Lac, revealed that amidation of the lipid II stem peptide accounts for increased binding affinity of oritavancin in VRSA, rather than interaction with the pentaglycine crossbridge, which is almost absent in these strains (41). Corroborating these muropeptide analyses of VRSA (41), we provide the first biochemical evidence that lipid II termi-

nating in D-Ala-D-Lac is a poor substrate for the Fem peptidyl transferases of *S. aureus*.

MATERIALS AND METHODS

Susceptibility testing. MICs were determined by standard broth microdilution methods (CLSI) in a polypropylene microtiter plate using cation-adjusted Mueller-Hinton broth (MHB) (Oxoid) for *S. aureus* ATCC 29213 and brain heart infusion broth (BHIB) (Oxoid) for *E. faecium* BM4147. Bacteria in the exponential growth phase were diluted to give a final inoculum of 10^5 CFU. The MICs were read after 16 h at 37°C. Oritavancin and des-oritavancin (23, 42) were kindly provided by The Medicines Company and dissolved in 0.002% Tween 80 (vol/vol). A final concentration of 0.002% Tween 80 (vol/vol) was present in all dilution steps and assays performed in this study.

Analysis of the cytoplasmic peptidoglycan nucleotide precursor pool. *E. faecium* BM4147 was grown in BHI broth (0.002% Tween 80) to an optical density at 600 nm (OD_{600}) of 0.5 and supplemented with 80 mg/liter vancomycin to induce *vanA* expression. After 10 min, oritavancin or des-*N*-methylleucyl-oritavancin (des-oritavancin) was added at 10× MIC and incubated for 60 min. Cells were harvested and extracted with boiling water. The suspension was then centrifuged, and the supernatant was lyophilized. UDP-linked cell wall precursors were analyzed by high-performance liquid chromatography (HPLC) (43), and the corresponding fractions were confirmed by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry.

Cloning, overexpression, and purification of *Enterococcus faecium* Asl. *E. faecium* BM4147 *asl* was amplified using forward and reverse primers (Asl_for 5′-TAGGCTAGCATGAACAGTATTGAAAATG-3′ and Asl_rev 5′-TTACTCGAGGCCTTCTTTCACATGAAAATA-3′) and cloned into a pET21b vector (Novagen) using NdeI and XhoI restriction sites to generate C-terminal His_6 fusion proteins. *Escherichia coli* BL21(DE3) (Promega) cells transformed with the appropriate recombinant plasmid were grown in LB medium (50 µg/ml ampicillin) at 37°C. At an OD_{600} of 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a concentration of 0.75 mM to induce expression of the recombinant proteins. After 2 h, cells were harvested and resuspended in lysis buffer (50 mM Tris-HCl [pH 7.5], 300 mM NaCl, 10 mM imidazole). Aliquots of 200 mg/ml lysozyme, 100 mg/ml DNase, and 10 mg/ml RNase were added; cells were incubated for 30 min on ice and sonicated. Cell debris was spun down, and purification was performed as described for the MurG enzyme (44).

Analysis of enzyme activities. MurG activity assays were performed in a final volume of 30 µl containing 2.5 nmol purified lipid I, 25 nmol UDP-GlcNAc, or [^{14}C]UDP-GlcNAc in 200 mM Tris-HCl, 5.7 mM $MgCl_2$ [pH 7.5], and 0.8% Triton X-100 in the presence of 0.45 µg of purified MurG-His_6 enzyme. Reaction mixtures were incubated for 30 min at 30°C (44). The assay for synthesis of lipid II-Gly$_1$ catalyzed by FemX and lipid II-Gly$_3$ and lipid II-Gly$_5$ catalyzed by FemXA and FemXAB, respectively, was performed as described previously without any modifications (45); 5 nmol of lipid II or lipid II-D-Lac was used as a substrate. The enzymatic activity of PBP2 was determined by incubating 2.5 nmol [^{14}C]lipid II in 100 mM morpholineethanesulfonic acid (MES), 10 mM $MgCl_2$ [pH 5.5], and 0.1% Triton X-100 in a total volume of 50 µl. The reaction was initiated by the addition of 7.5 µg PBP2-His_6 and incubated for 1.5 h at 30°C (44). The Asl-catalyzed addition of D-[^{14}C]aspartate to lipid II was performed in a total volume of 50 µl containing 2.5 nmol lipid II, 25 nmol D-[^{14}C]aspartate in 120 mM Tris-HCl, 40 mM $MgCl_2$ [pH 8], 8 mM ATP, 0.4% Triton X-100, and 2 µg Asl-His_6 (37, 38). The synthesized lipid intermediates were extracted from the reaction mixtures with *n*-butanol-pyridine acetate (pH 4.2) (2:1, vol/vol) and analyzed by thin-layer chromatography (TLC) as described earlier (45). The analysis of lipid II conversion catalyzed by PBP2 was carried out by applying reaction mixtures directly onto TLC plates developed in solvent B (butanol-acetic acid-water-pyridine [15:3:12:10, vol/vol/vol/vol]). The quantitative analysis was performed by phosphorimaging. Antibiotics were

added at increasing molar ratios of 0.25 to 4 with respect to the amount of lipid precursor, respectively. The lantibiotic nisin was used as a control.

***In vitro* synthesis and purification of lipid II variants.** Large-scale synthesis and purification of the peptidoglycan precursors lipid I, lipid II, lipid II-Gly$_{1-5}$ and amidated lipid II were performed as described previously (45, 46). Lipid II-Asp was synthesized with purified lipid II and purified recombinant Asl-His_6 as described above using nonradioactive D-aspartate (37, 38). Purification was performed as described for unmodified lipid II (45, 47).

For synthesis of the lipid II variant containing the depsipeptide terminus (D-Ala-D-Lac), UDP-MurNAc-depsipeptide was purified from *Lactobacillus casei* ATCC 393. *L. casei* was grown in MRS broth to an OD_{600} of 0.6 and incubated with 65 µg/ml of chloramphenicol for 15 min. Intracellular accumulation was achieved by incubation with bacitracin (10× MIC, 40 µg/ml) in the presence of 1.25 mM zinc for another 60 min. The assay for synthesizing lipid II-D-Lac was performed in a final volume of 60 µl using 3 µg recombinant MraY-His_6 and 1.25 µg recombinant MurG-His_6 by incubating the UDP-MurNAc-depsipeptide, 5 nmol $C_{55}P$, and 150 nmol UDP-GlcNAc in 83.3 mM Tris-HCl (pH 7.5), 6.3 mM $MgCl_2$ and 0.5% Triton X-100. The reaction mixtures were incubated for 4 h at 30°C. Lipid II-D-Lac was extracted from the reaction mixtures with *n*-butanol-pyridine acetate (pH 4.2) (2:1, vol/vol), analyzed by TLC, and purified by HPLC.

Amidated lipid II and lipid II-D-Lac, containing D-isoglutamine in position 2 of the stem peptide, were synthesized and purified as described previously (46). Lipid II or amidated lipid II was used as a substrate in a Fem peptidyl transferase assay (45). Vancomycin, chloroeremomycin, or oritavancin was added in molar ratio with respect to the lipid precursors. The reaction products were extracted with BuOH-PyrAc (2:1) and separated by TLC in solvent Rick (chloroform, methanol, water, ammonium hydroxide [88:48:10:1, vol/vol]). The detection and quantification of radiolabeled products were carried out using a Storm PhosphorImager.

Complex formation of oritavancin and des-oritavancin with purified lipid II and lipid II variants. Complex formation of oritavancin and des-oritavancin with lipid II and lipid II variants was analyzed by incubating 2 nmol of each purified cell wall precursor with increasing concentrations of oritavancin or des-oritavancin in 50 mM Tris-HCl (pH 7.5) for 20 min at room temperature. The free precursors were extracted with *n*-butanol-pyridine acetate (pH 4.2) (2:1, vol/vol) and analyzed by TLC and subsequent phosphomolybdic acid (PMA) staining. The quantitative analysis of the lipid-containing precursors in the butanol phase was carried out using phosphorimaging.

Quartz crystal microbalance. To apply this mass-sensitive biosensor technique, a LiquiLab21 quartz crystal microbalance (QCM) device (ifake.V., Germany) was employed. Changes in the oscillation frequency of quartz sensors, representing binding events at the sensor surface equipped in a flow chamber, were followed in real time and used to calculate binding kinetic constants with appropriate software. The quartz sensors were cleaned and prepared with the covalent immobilization of a hexadecanthiol monolayer as described previously (48). The supported bilayers were completed by transferring a monolayer consisting of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC)-0.1 mol% lipid intermediate onto a covalently fixed $C_{16}H_{33}SH$ monolayer by the Langmuir-Blodgett technique. After equilibration of the quartz crystals under flow conditions until they reached a constant frequency, 1 µM peptide solution was added. The frequency curves allow the calculation of the kinetic binding constants as described previously (49, 50).

Statistics. All experiments were performed at least in triplicate, and the values are expressed as the means ± standard deviations (SD).

RESULTS

Oritavancin blocks individual cell wall biosynthesis reactions *in vitro*. We investigated the effect of oritavancin in various individual *in vitro* peptidoglycan biosynthesis assays using purified substrates and enzymes and included the lantibiotic nisin, which is
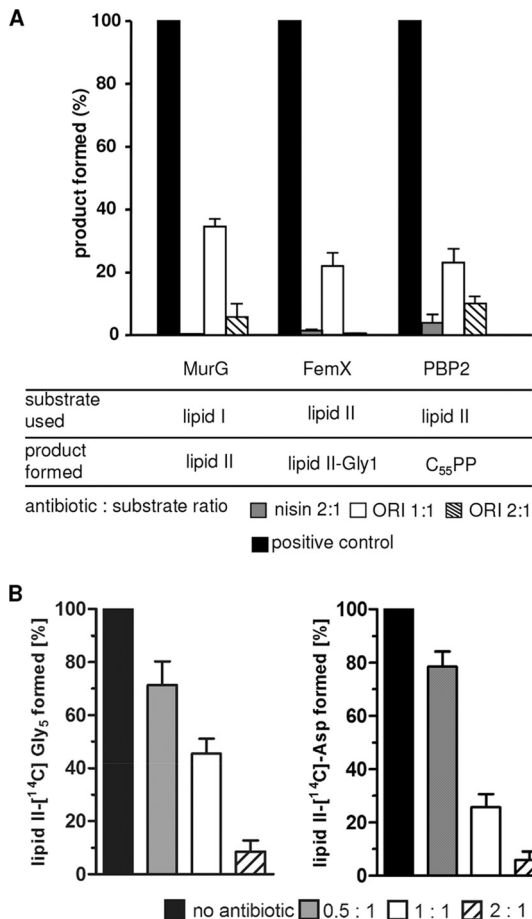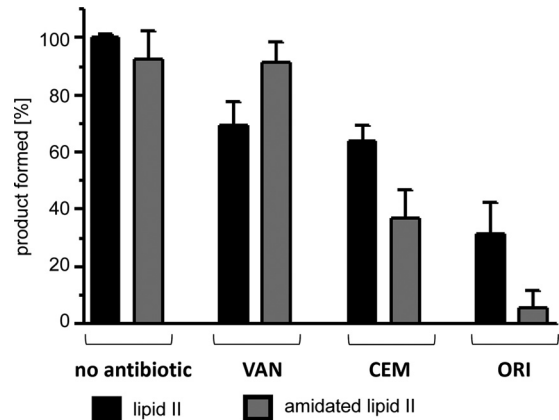
FIG 3 Impact of lipid II amidation on the inhibitory effect of different glyco-peptide antibiotics *in vitro*. Purified lipid II or amidated lipid II (D-isoglu-tamine instead of D-glutamate in position 2 of the stem peptide) was used as a substrate in a FemX *in vitro* assay (see Materials und Methods). The addition of [14C]glycine to the lipid precursor in the absence and presence of vancomycin, chloroeremomycin, or oritavancin was quantified using a Storm Phosphor Imager. Antibiotics were added in equimolar ratio (1:1) with respect to the lipid precursor. The mean values from three independent experiments are shown. All experiments were performed at least in triplicate, and data are presented as means ± standard deviations (SD). VAN, vancomycin; CEM, chloroeremomycin; ORI, oritavancin.

FIG 2 Inhibition of membrane-associated cell wall biosynthesis steps by ori-tavancin. (A) Synthesis and analysis of the individual biosynthesis steps were performed as described in Materials and Methods. The amount of reaction products synthesized in the absence of antibiotics was taken as 100%. The bactoprenol-containing products were analyzed by TLC. Radiolabeling was based on ^{3}H-labeled $C_{55}P$ (for lipid I), [14C]GlcNAc for lipid II, and [14C]gly-cine for lipid II-Gly$_1$. Antibiotics were added in molar ratios with respect to the bactoprenol-bound substrates as indicated. The lantibiotic nisin was used as a control. (B) Impact of oritavancin on the staphylococcal pentaglycine and enterococcal D-aspartate crossbridge formation. The addition of [14C]glycine by purified FemXAB-His$_6$ (*S. aureus*) or the formation of the [14C]aspartate crossbridge by Asl-His$_6$ (*E. faecium*) was assayed in the presence of increasing oritavancin concentrations (0.5 to 2 molar ratio with respect to lipid II). The reaction products synthesized in the absence of oritavancin were taken as the 100% level. The lipid intermediates were extracted from the reaction mixture and analyzed by TLC and subsequent phosphorimaging. The mean values from three independent experiments are shown. All experiments were per-formed at least in triplicate, and data are presented as means ± standard deviations (SD). ORI, oritavancin.

known to target lipid II and other bactoprenol-bound precursors, as a control (Fig. 2A) (51). In the presence of increasing concen-trations of oritavancin, inhibition of all enzymatic steps (MurG, FemX, PBP2) using lipid I or lipid II as a substrate was observed. Since the crossbridge has been proposed to be an additional bind-ing site for oritavancin (36, 40), we further analyzed the impact of oritavancin on both pentaglycine (*S. aureus*) and D-aspartate (*E. faecium*) crossbridge formation *in vitro*, as catalyzed by the puri-fied enzymes FemXAB and Asl (37, 38, 45), respectively (Fig. 2B). Compared to the control reactions in the absence of an antibiotic,

increasing concentrations of oritavancin in the reaction mixtures resulted in successive decreases of the respective products. An al-most complete inhibition of each reaction was observed when oritavancin was added in 2-fold molar excess with respect to the lipid intermediate (Fig. 2).

Influence of lipid II modifications on the binding of orita-vancin. For numerous Gram-positive pathogens, the amidation of the α-carbonyl group of D-glutamate in position 2 of the lipid II stem peptide is essential and has been shown to affect the interac-tion with antimicrobial compounds, such as plectasin (46). To investigate the impact of lipid II amidation on the interaction with selected glycopeptide antibiotics, either lipid II or amidated lipid II was used as a substrate in a FemX-catalyzed lipid II-Gly$_1$ syn-thesis assay (Fig. 3). The glycopeptides tested (vancomycin, orita-vancin, and chloroeremomycin) were added at a 1:1 molar ratio with respect to the lipid intermediate. Compared to the negative control, vancomycin inhibited the FemX-catalyzed addition of [14C]glycine to unmodified lipid II by 30%, whereas no significant inhibitory effect was observed when amidated lipid II was used as a substrate. Oritavancin, in contrast, showed a markedly increased affinity for the amidated lipid II variant, resulting in an almost complete inhibition (>95%) of the FemX-catalyzed reaction at an equimolar ratio. The inhibitory effect was reduced when unmod-ified lipid II was used as a substrate (62%), suggesting enhanced binding of oritavancin to amidated lipid II. Although the overall inhibitory effect was less pronounced with chloroeremomycin, enhanced binding to amidated lipid II was also observed (Fig. 3).

To further validate the data obtained from the *in vitro* assays, the binding parameters of oritavancin and vancomycin to selected lipid II variants (lipid II, lipid II-Gly$_5$, amidated lipid II, amidated lipid II-Gly$_5$, lipid II-D-Lac, and amidated lipid II-D-Lac) were determined using a quartz crystal microbalance (QCM) technique (48–50).

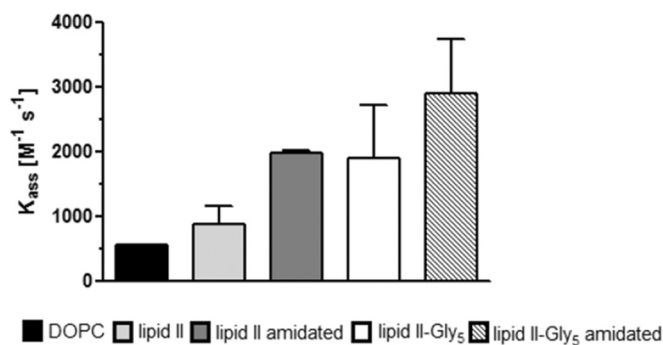QCM is a versatile mass-sensitive biosensor system based on

FIG 4 Association rate (K_{ass}) of oritavancin binding to different lipid II variants embedded in DOPC model membranes or pure DOPC as determined by QCM biosensor measurements.

the piezoelectric effect of quartz crystals. QCM inverses this effect, enabling time-resolved measurements of mass changes at the sensor surface, capable of measuring subnanogram levels. Covering the quartz surface by using supported lipid bilayers allows for determination of the membrane interaction of analytes, whereas covering by embedding specific targets into the lipid bilayer allows for detection of kinetic binding constants. Consequently, this approach combines the advantage of analyzing the interaction of the antibiotic with its membrane-bound target (lipid II) embedded in a lipid bilayer, mimicking its natural membrane environment. In agreement with the observations obtained from the *in vitro* assays, determination of the binding parameters revealed that vancomycin binds to unmodified lipid II (equilibrium constant [K_D] = $5.37 \times 10^{-7} \pm 0.41 \times 10^{-7}$ M), and this was unchanged for lipid II-Gly$_5$, while no binding to the amidated lipid II variant was detected. Oritavancin, in contrast, efficiently bound to lipid II ($K_D = 2.13 \times 10^{-7} \pm 0.60 \times 10^{-7}$ M), and the binding affinity is further increased by 1 order of magnitude ($K_D = 6.64 \times 10^{-8} \pm 1.62 \times 10^{-8}$ M) when the amidated lipid II variant is used for the binding approach. The association rates (K_{ass}) of oritavancin binding to the various lipid variants are meaningful indicators of successively increased affinity of oritavancin for its target, depending on the extent of lipid II modification (lipid II < amidated lipid II < lipid II-Gly$_5$ < amidated lipid II-Gly$_5$) (Fig. 4). A 3-fold increase in the association rate of oritavancin was observed for the fully modified cell wall precursor (amidated lipid II-Gly$_5$) (K_{ass} = 2,896.0 ± 994.0 M^{-1} s^{-1}) compared to that for lipid II lacking modifications ($K_{ass} = 890.11 \pm 275.16$ M^{-1} s^{-1}) (Fig. 4).

Taken together, these results demonstrate that the binding of oritavancin to the cell wall precursor involves, in addition to the D-Ala-D-Ala terminus of lipid II, the interaction with the pentaglycine crossbridge. Furthermore, a tertiary binding site, D-isoglutamine in position 2 of the stem peptide, significantly contributes to binding affinity.

In VRSA, the terminal D-Ala residue is exchanged to D-Lac, and it has been proposed that oritavancin compensates for the resulting loss of one critical hydrogen bond to this target through the interaction with additional binding sites on the lipid II molecule, i.e., the crossbridge (36, 39). However, *vanA*-type vancomycin-resistant *S. aureus* strains are characterized by a complete lack of or an incomplete chain length of the pentaglycine crossbridge (41), suggesting that in these strains yet another binding site on the lipid II molecule contributes to the high-affinity binding. Since the

amidation of D-Glu of the lipid II stem peptide proved to significantly enhance binding affinity by 1 order of magnitude, oritavancin binding to the unmodified and amidated depsipeptide counterparts, as present in *vanA*-type VRSA, was investigated (Fig. 5). Indeed, the binding of oritavancin to lipid II-D-Lac is comparatively low (Fig. 5D) compared to that of the other lipid II derivatives tested. Although the overall binding affinity ($K_D = 1.05 \times 10^{-7}$ M) is in the range of that of unmodified lipid II, tracking of frequency changes provides an interesting insight into the binding process. While target recognition is obviously represented by a low dissociation rate (k_{off}) (afferent part of the curve) in Fig. 5B, C, and D for lipid II, amidated lipid II, and amidated lipid II-D-Lac, respectively, the k_{off} rate of the nonamidated lipid II-D-Lac variant is evidently higher and comparable to that of oritavancin binding to the plain DOPC membrane (Fig. 5A). The data show that amidation of the cell wall precursor is a crucial factor for oritavancin target recognition of both lipid II and its depsipeptide counterpart (lipid II-D-Lac) as represented by decreased dissociation rates and increased binding affinity compared to those for the respective nonamidated variants (Fig. 5C and D).

Lipid II-D-Lac is a poor substrate for Fem peptidyl transferases of *S. aureus*. In *S. aureus*, the cell wall building block lipid II is characteristically modified by a pentaglycine crossbridge attached to the L-Lys of the stem peptide, which is catalyzed by FemXAB peptidyl transferases (45, 52).

Severin et al. hypothesized that Fem peptidyl transferases of *S. aureus* do not efficiently recognize the D-Lac-containing depsipeptide precursors as a substrate (41) to explain the pentaglycine bridge deficiency in *vanA*-type VRSA. Here we provide the first biochemical evidence that the Fem-catalyzed addition of glycine residues to lipid II-D-Lac is strongly hampered. Compared to the control reaction using lipid II-D-Ala, the addition of the first glycine catalyzed by FemX is strongly decreased (34%) when lipid II-D-Lac was used as a substrate (Fig. 6). This effect was even more pronounced in a coupled FemXA synthesis assay (17%), providing biochemical evidence that the depsipeptide precursor is a poor substrate for the Fem enzymes of *S. aureus*. Most likely, the modification of the stem peptide terminus interferes with substrate recognition.

Interactions of des-oritavancin with lipid II and lipid II variants. The chemical removal of the N-terminal amino acid of the glycopeptide aglycone core results in damage to the binding pocket, crucial for the tight binding of D-Ala-D-Ala residues of the lipid II molecule. Damage to the carboxylate binding pocket leads to a complete loss of vancomycin antibacterial activity (36). In contrast, des-*N*-methylleucyl-oritavancin (des-oritavancin), lacking the terminal leucyl residue (Fig. 1A), still retains significant antibiotic activity against vancomycin-resistant isolates (Table 1) (36). To further investigate the activity of des-oritavancin *in vivo*, we determined the level of the UDP-MurNAc-depsipeptide in the cytoplasm of VanA-type *E. faecium* BM4147 after treatment with oritavancin and des-oritavancin (Fig. 7A). Antibiotics that interfere with the late steps of peptidoglycan synthesis block the recycling of the undecaprenol lipid carrier and lead to accumulation of the soluble cell wall precursor (UDP-MurNAc-penta-/depsipeptide) in the cytoplasm of treated cells (42). As previously reported, no accumulation of UDP-MurNAc-depsipeptide was detected after treatment of VRE with vancomycin (53). Oritavancin, in contrast, led to a significant accumulation of UDP-MurNAc-depsipeptide at 10× MIC, in either the VanA-induced (80
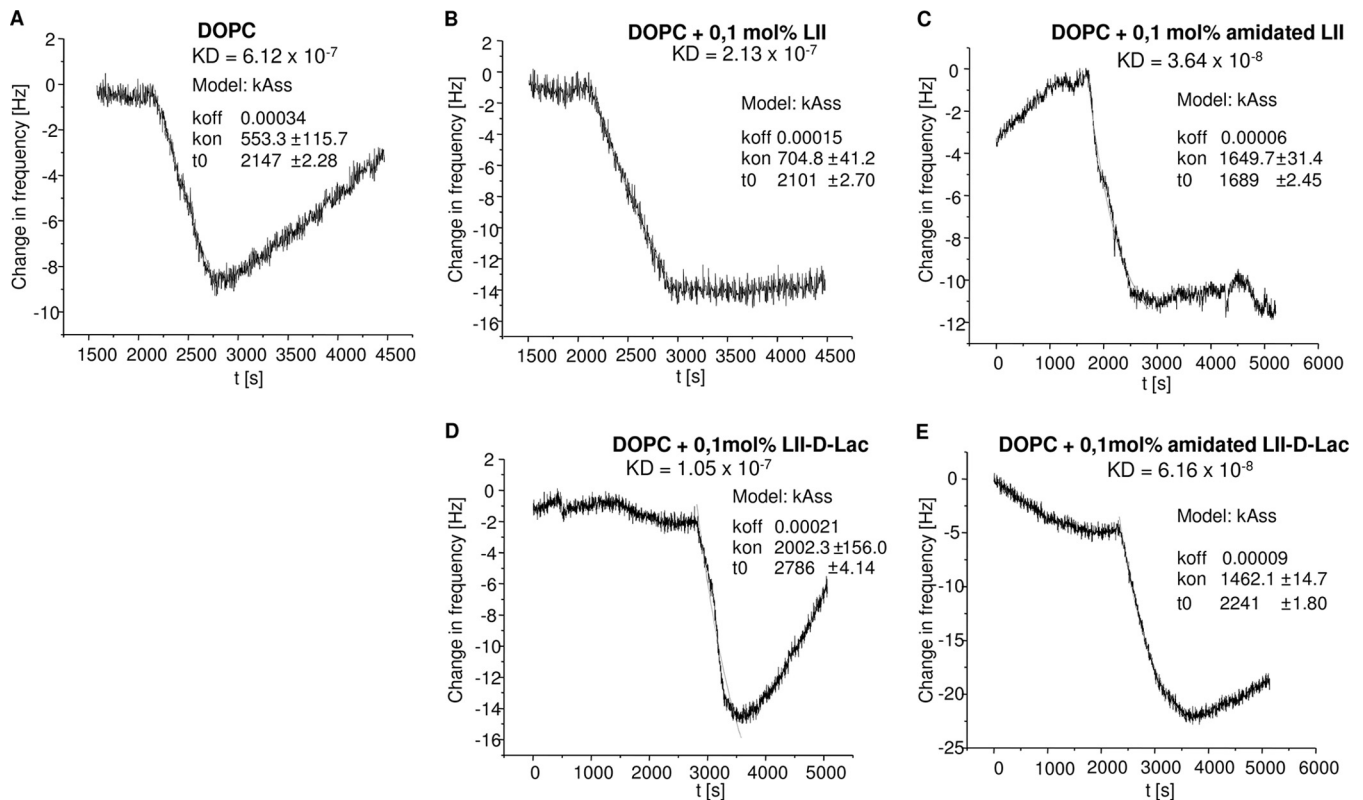
FIG 5 Frequency shifts of the QCM measurements of oritavancin binding to pure DOPC (A) or DOPC containing 0.1 mol% of lipid II derivative (B to E). The binding affinity K_D results from the ratio of k_{off} (afferent shift) and K_{ass} (slope). (A) Intrinsic affinity of oritavancin to membranes; (B, C, and E) target binding by a much slower dissociation tendency, which is superimposed on membrane affinity.

mg/liter vancomycin) or uninduced cells, comparable to that for chloroeremomycin (53). In line with its antibiotic activity against VRE, treatment with des-oritavancin (10× MIC) also led to accumulation of the D-Lac (UDP-MurNAc-depsipeptide)-containing soluble precursor, although the overall level of accumulation was markedly reduced compared to that in oritavancin-treated cells (Fig. 7A). Additionally, the antibiotic activities of both oritavancin and des-N-methylleucyl-oritavancin were antagonized by full-length lipid I and lipid II, as well as lipid II-Gly₅ and lipid II-Asp variants (data not shown). Since Ge et al. proposed a direct inter-
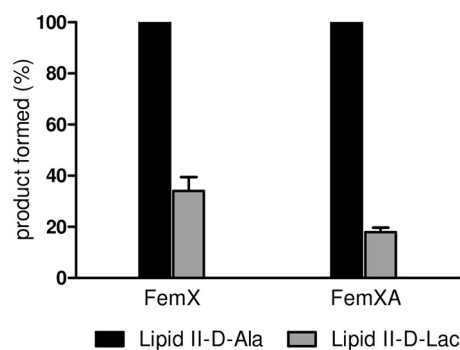
action of the 4-epi-vancosamine sugar attached to ring 6 of the peptide core with penicillin binding proteins, thereby inhibiting enzyme activity without binding to the D-Ala-D-Ala of the cell wall precursor (35), we investigated the effect of des-oritavancin on the PBP2-catalyzed transglycosylation *in vitro*. In this test system, the addition of des-methylleucyl-oritavancin was found to inhibit the polymerization of lipid II-Gly₅ in a dose-dependent manner (Fig. 7B), and an almost complete inhibition was observed at a molar ratio of 4:1 (antibiotic to lipid), suggesting substrate sequestration rather than a direct inhibition of enzyme activity. To further verify the binding of des-oritavancin to lipid II, the glycopeptide was incubated with the purified cell wall precursor at various molar ratios. In line with the *in vitro* assay (Fig. 7B), only traces of unbound lipid II (10%) were detectable at a 4:1 (antibiotic to lipid) molar ratio after extraction from the reaction mixtures and subsequent TLC analysis (Fig. 7C). In contrast to des-N-methylleucyl-vancomycin, which has no measurable affinity for lipid II (36),

FIG 6 Enzymatic activities of FemX and FemXA. The addition of [¹⁴C]glycine to the substrate lipid II-D-Ala or lipid II-D-Lac by FemX and FemXA was analyzed by TLC and quantified by phosphorimaging. Reaction products using unmodified lipid II were taken as the 100% level.

TABLE 1 MICs of test strains

Test strain	MIC (μg/ml) of test strain for[a]:			
	ORI	des-ORI	CEM	VAN
S. aureus ATCC 29213	0.08	2	0.15	0.5
E. faecium BM 4147	1.4	4	16	>512

[a] ORI, oritavancin; des-ORI, des-N-methylleucyl-oritavancin; CEM, chloroeremomycin; VAN, vancomycin.
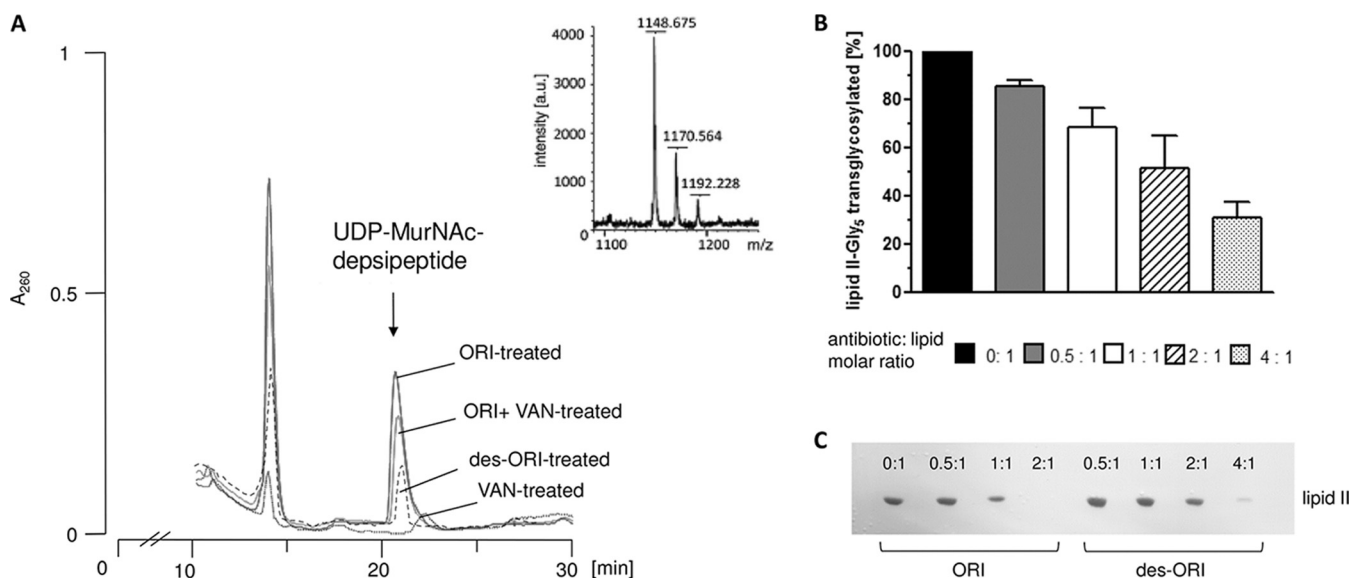
FIG 7 Intracellular accumulation of the soluble cell wall precursor UDP-MurNAc-depsipeptide in VanA-type *E. faecium*. (A) *E. faecium* BM4147 was grown in HHD broth (0.002% Tween 80) to an OD_{600} of 0.5 and supplemented with 80 mg/liter vancomycin (VAN) to induce *vanA* expression. After 10 min, oritavancin (ORI) or des-*N*-methylleucyl-oritavancin (des-ORI) was added at 10× MIC and incubated for 60 min. Cells were harvested and extracted with boiling water, and the intracellular nucleotide pool was analyzed by reversed-phase HPLC. UDP-MurNAc-depsipeptide (UDP-MurNAc-L-Ala-D-Gln-L-Lys-D-Ala-D-Lac) was identified by MALDI-TOF mass spectrometry. (B) Inhibition of the PBP2-catalyzed reaction by des-ORI *in vitro*. The conversion of [^{14}C]lipid II-Gly$_5$ into polymeric peptidoglycan in the presence of increasing concentrations of des-ORI was quantitatively analyzed by applying the reaction mixtures directly onto TLC plates and subsequent separation in solvent B (butanol-acetic acid-water-pyridine [15:3:12:10, vol/vol/vol/vol]) followed by the detection and quantification of residual [^{14}C]lipid II-Gly$_5$ using phosphorimaging. (C) Purified lipid II was incubated with increasing concentrations of ORI or des-ORI at molar ratios of 0.5 to 4:1 with respect to the substrate lipid II. The reaction mixtures were extracted, and unbound lipid was analyzed by TLC and visualized by PMA staining.

des-oritavancin is able to trap the unmodified lipid II in a stable complex, thereby preventing its extraction. Analysis of oritavancin binding to lipid II revealed a 2:1 binding stoichiometry (Fig. 7C), supporting dimerization of the heptapeptide (32).

DISCUSSION

Semisynthetic glycopeptide antibiotics, like oritavancin, represent promising agents for the treatment of infections caused by multidrug-resistant Gram-positive bacteria. Like vancomycin, the lipoglycopeptide antibiotics such as oritavancin and telavancin inhibit transglycosylation via binding to the D-Ala-D-Ala terminus of lipid II. In addition, structural modifications, like the 4-epi-vancosamine and the 4′-chlorobiphenyl methyl side chain of oritavancin, account for improved antibacterial activity against MRSA, VISA, VRSA, and VRE strains (22, 40, 54).

Here, we provide evidence at the molecular level that these structural modifications enable oritavancin, in contrast to vancomycin, to bind to further target sites of the lipid II peptidoglycan precursor other than the D-Ala-D-Ala terminus. With use of solid-state nuclear magnetic resonance (NMR) and rotational-echo double resonance (REDOR), a model for the oritavancin-PG complex has been proposed in which the crossbridge is bound in a cleft between the epi-vancosamine residue and the core of the oritavancin molecule (36, 39, 40). We now provide biochemical evidence that the crossbridge and the D-iso-glutamine in position 2 of the lipid II stem peptide represent crucial binding sites for oritavancin. *In vitro* assays and determination of binding parameters using a QCM-based approach (Fig. 4 and 5) with different purified lipid II variants embedded in a lipid bilayer revealed that a progressive increase in oritavancin binding affinity to lipid II

involves both lipid II modifications of *S. aureus*: the crossbridge and the amidation of the glutamate residue of the stem peptide (45, 46). In contrast, this increase in binding affinity was not observed for vancomycin. Rather, lipid II amidation appeared to strongly decrease vancomycin binding affinity, both in the *in vitro* system and as revealed by the determination of binding parameters. Earlier studies suggested a correlation between vancomycin resistance and the reduced degree of peptidoglycan amidation in *S. aureus* strain Mu50, and inducible amidation of D-iso-Glu has been reported in *Clostridium difficile* and *Streptomyces coelicolor* A3(2), but not in glycopeptide-resistant enterococci (55–61). Moreover, it was shown that the antimicrobial activity of vancomycin is antagonized more efficiently by short synthetic peptides (D-Glu/Gln-L-Lys-D-Ala-D-Ala) containing D-iso-Glu instead of D-iso-Gln (55, 62). It was concluded that the increase in the glutamine nonamidated muropeptide components in the peptidoglycan of Mu50 may efficiently trap a larger amount of vancomycin in the thickened cell wall.

In contrast to observations with vancomycin, enhanced binding affinity to amidated lipid II was also observed with chloroeremomycin, suggesting that the 4-epi-vancosamine residue, present in both oritavancin and chloroeremomycin but absent in vancomycin, is involved in the interaction with the amidated precursor.

Oritavancin exhibits antimicrobial activity against VRSA (27, 28), in which resistance to vancomycin is mediated by the exchange of the terminal D-Ala to D-Lac. Muropeptide analyses of *vanA*-type VRSA revealed that these strains are characterized by a complete lack of or at least severely shortened pentaglycine crossbridges (41). Thus, the interaction of oritavancin with the cross-

bridge appears less relevant in VRSA. Rather, as revealed by comparative analysis of binding parameters, enhanced binding affinity to lipid II-D-Lac relies on additional interactions of oritavancin with D-iso-Gln in position 2 of the stem peptide (Fig. 5) and thereby compensates for the loss of one hydrogen bond.

Up to now, the *vanA*-mediated mechanism of resistance has not been evolving or spreading rapidly in MRSA (63). It has been shown that the simultaneous expressions of chromosomally located *mecA* and plasmid-borne *vanA* resistance determinants are mutually antagonistic, and it was concluded that this phenomenon is based on the inefficient recognition of depsipeptide precursors by the alternative penicillin binding protein PBP2a (41, 64).

Biochemical analysis of the FemX- and FemXA-catalyzed reactions in this study clearly demonstrated that the D-Lac-containing cell wall precursors are poor substrates for the staphylococcal Fem peptidyl transferases, presumably due to interference with substrate recognition. In staphylococci, FemX, which initiates pentaglycine bridge formation by adding the first glycine residue, is essential (52). Although characterized by abnormal cell morphology, reduced cell wall turnover, and retarded cell separation, cells expressing at least monoglycine crossbridges are generally viable, but hypersusceptible to oxacillin (65).

Since pentaglycine bridge formation is severely hampered in VRSA, a reduction in FemX activity (34%) (Fig. 6) results in a synthetically lethal phenotype in the presence of oxacillin, because PBP2a′ is unable to cross-link monoglycyl-containing precursors (65, 66).

Interestingly, *Streptomyces coelicolor* produces an alternative Fem homologue, VanK, which is essential under conditions of *vanHAX* induction (67) to ensure the formation of the crossbridge that consists of a single glycine residue. This indicates that FemX of *S. coelicolor* is also unable to efficiently recognize lipid II-D-Lac, although biochemical evidence is lacking so far. FemX of enterococci, in contrast, appears to efficiently recognize depsipeptide precursors (68), which might partially explain the enhanced prevalence of VRE compared to that of VRSA.

The differences in the molecular interactions with the PG precursor lipid II between vancomycin and oritavancin are further reflected by the antibiotic activity of their respective Edman degradation products. Chemical removal of the N-terminal *N*-methylleucyl residue from the aglycone core of vancomycin results in damage to the D-Ala-D-Ala binding cleft, impairing binding to the peptidoglycan precursor, as evidenced by a total loss of its antimicrobial activity (36). Interestingly, the Edman degradation product of oritavancin (des-*N*-methylleucyl-oritavancin) retains good antimicrobial activity (Table 1) (36). Although species-specific lipid II modifications were shown to significantly contribute to oritavancin binding, its hexapeptide derivative lacking the carboxylate binding pocket is also able to bind to unmodified lipid II (Fig. 7), suggesting that additional features further compensate for the loss of one hydrogen bond. The hydrophobic substitutions on the vancosamine residue have been demonstrated to enhance the antibacterial activity of chloroeremomycin and oritavancin by promoting dimerization of the glycopeptides in solution (32, 33, 55). In contrast, vancomycin lacking these additional substitutions reaches its target site, where its antibacterial activity against susceptible bacteria is assumed to be a sole function of its binding to D-Ala-D-Ala-containing PG residues, predominantly as a monomer (32). Studies on various synthetic covalent dimers of vancomycin, which showed significantly improved antibacterial

activity against vancomycin-susceptible and -resistant bacteria, support the crucial role for dimerization as a determinant of antibacterial activity (69).

In the case of oritavancin, enhanced antibacterial activity appears to further rely on the ability to interact with the bacterial membrane, which may contribute to its rapid bactericidal activity (30, 70). Kim et al. have recently shown that the hydrophobic 4′-chlorobiphenyl methyl moiety of oritavancin promotes the interaction with isolated membrane protoplasts and increases membrane permeability in artificial liposomes composed of bacterial membrane phospholipids, when a certain threshold concentration is reached (70). The integration of oritavancin into the membrane might lead to changes in the physicochemical properties of the membrane and affect the organization of highly dynamic biosynthesis machineries and energy-generating systems. One can assume that trapping of the limited central bactoprenol-bound precursor, combined with integration of oritavancin into the cytoplasmic membrane, has a dramatic effect on the tightly controlled membrane-associated steps of cell wall biosynthesis, where proteins are in intimate contact. These pleiotropic effects have not been reported for chloroeremomycin nor for vancomycin, both of which lack alkyl side chains. More recently, it has further been shown that oritavancin prevents vegetative outgrowth and recovery of *C. difficile* spores probably due to direct binding to the spore surface (71) through the hydrophobic chlorobiphenyl side chain.

In conclusion, we here showed at the molecular level that the binding of oritavancin to its target involves the interaction with additional species-specific binding sites, namely, the crossbridge and D-iso-Gln in position 2 of the lipid II stem peptide. Interactions with D-iso-Gln in position 2 of the lipid II stem peptide in particular facilitate stronger intramolecular interactions with the PG precursor, thereby compensating for the loss of a crucial hydrogen bond in vancomycin-resistant strains.

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