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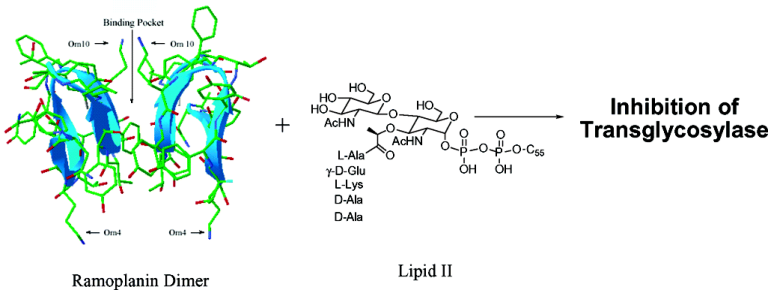

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Ramoplanin Inhibits Bacterial Transglycosylases by Binding as a Dimer to Lipid II

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Genes that confer resistance to the glycopeptide antibiotic vancomycin have begun to spread at an alarming rate among nosocomial pathogens. Because many such pathogens are already resistant to most other antibiotics, the emergence of vancomycin resistance is a serious problem that has fueled a search for new antimicrobial agents. The cyclic lipoglycodepsipeptide antibiotic ramoplanin (**1**, Figure 1a) has excellent activity against a wide range of Gram-positive bacteria, including vancomycin resistant enterococci (VRE)[1], and it is currently in Phase III clinical trials as a therapy to prevent vancomycin-resistant infections in at-risk hospitalized patients.[2] A good understanding of the mechanism of action of ramoplanin is essential given the potential importance of this natural product.

Early studies on ramoplanin led to the proposal that it blocks peptidoglycan biosynthesis at the MurG step by binding to Lipid I (Figure 1b).[3] However, the inhibition of MurG by ramoplanin is too weak to explain the biological activity; furthermore, inhibition does not require binding to Lipid I.[4] We suggested that ramoplanin inhibits the transglycosylases that form the glycan chains of peptidoglycan by binding to Lipid II on the external surface of the bacterial membrane.[4,5] Preliminary studies in crude bacterial membranes confirmed that ramoplanin can block transglycosylation,[5] but we were unable to determine the mode of inhibition because appropriate assays were not available.[6] We recently developed an assay for the major synthetic transglycosylase in *E. coli*, PBP1b,[6a,d] and we report here that ramoplanin does, in fact, inhibit bacterial transglycosylases by binding to Lipid II. The inhibition curves provide new insight into the recognition event, revealing that the inhibitory species has a stoichiometry of 2:1 ramoplanin:Lipid II. On the basis of this finding, we propose a model in which Lipid II binds in a cleft formed by the dimerization of two ramoplanin molecules.

Figure 2 shows velocity versus substrate concentration curves for PBP1b in the presence of 0, 6, and 8 μM ramoplanin. In the absence of ramoplanin, PBP1b displays Michaelis−Menten kinetics. In the presence of ramoplanin, the reaction rate is negligible at low substrate concentrations but jumps when the substrate concentration exceeds a certain critical value. Inhibition is overcome at high substrate concentrations. These sigmoidal inhibition curves are consistent with a mechanism in which ramoplanin binds Lipid II, sequestering it so that it cannot be processed by PBP1b.[7]

Two features of the inhibition curves are worth noting. First, the apparent K_d for the interaction of ramoplanin and Lipid II must be low because there is essentially no reaction at low substrate concentrations, indicating that there is no free Lipid II in solution. Second, the reaction rate increases rapidly when the substrate concentration exceeds one-half the concentration of ramoplanin. One interpretation of this result is that ramoplanin binds as a dimer to Lipid II.[8]

In previous studies on ramoplanin, we showed that the ornithine 4 amine can be modified without eliminating substrate binding or

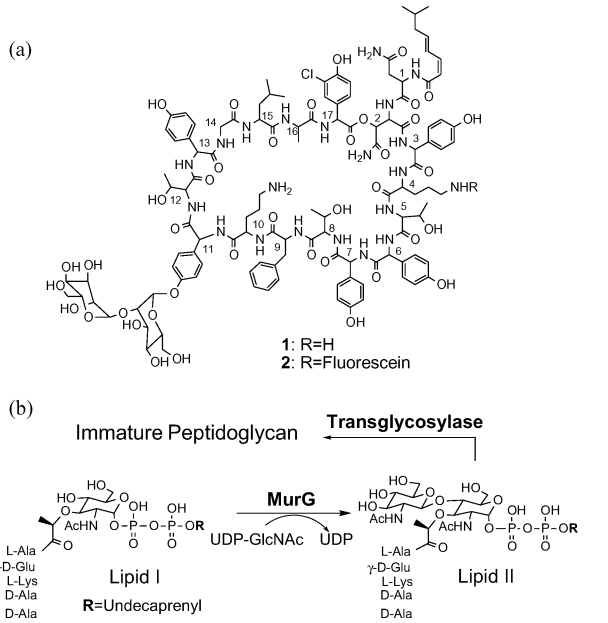

Figure 1. (a) Ramoplanin (**1**) and the fluorescent derivative (**2**) used here. (b) MurG converts Lipid I to Lipid II, which is translocated across the membrane and then polymerized by the transglycosylases.

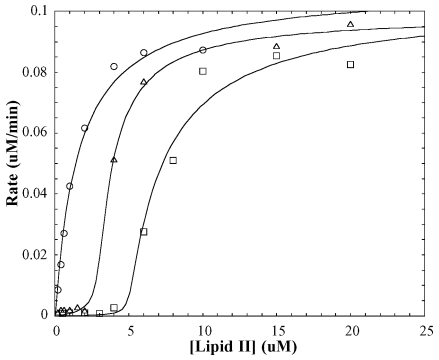

Figure 2. Rate of PBP1b versus the concentration of heptaprenyl Lipid II in the absence (○) and presence of ramoplanin (△ = 6 μM **1**; □ = 8 μM **1**).

biological activity.[4] Thus, we attached a fluorescein probe to ornithine 4 (**2**, Figure 1a) to examine Lipid II binding. We monitored the fluorescence change during a Job titration of compound **2** and Lipid II at a total concentration of 2 μM.[9] The maximum change occurs at a ramoplanin mole fraction of 0.66 (Supporting Information), confirming the kinetic results showing that ramoplanin binds in a 2:1 ratio to Lipid II.

To evaluate the affinity of ramoplanin for Lipid II, we monitored the fluorescence of Orn4F (**2**) at a range of concentrations as a function of the Lipid II concentration. A titration carried out at 20 nM **2** shows that Lipid II binds to ramoplanin at nanomolar

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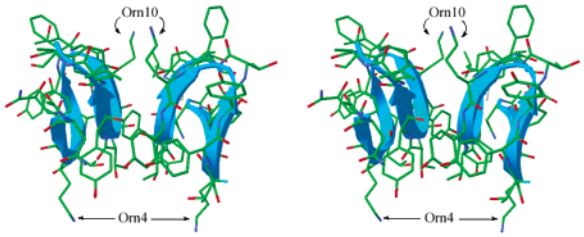

Figure 3. Stereoview of the NMR solution structure of the ramoplanin dimer in methanol. The dimer interface is formed by contacts between antiparallel β strands corresponding to amino acids 10−14 in each monomer. Dimerization forms a cleft, flanked by ornithine 10, that provides a possible binding site for Lipid II.[13]

concentrations. The apparent K_d calculated from the titration data is in the 10−100 nM range, which agrees well with the values calculated from the inhibition curves (Supporting Information).[8] The apparent K_d is consistent with ramoplanin's MIC, which is $\sim 5 \times 10^{-8}$ M against typical enterococcal strains.[4]

The affinity of ramoplanin and Lipid II is remarkable given the nature of these molecules: by way of comparison, the K_d of vancomycin for Lipid II is about 1 μM.[10] We have determined that the length of the lipid chain on Lipid II has a negligible effect on the apparent K_d's of ramoplanin. Instead, the polar headgroup of Lipid II constitutes the recognition epitope.[4,5,11]

Ramoplanin's ability to bind a charged carbohydrate so tightly is unusual and requires an explanation. We have previously shown that ramoplanin self-assembles to form fibrils at high concentrations in the presence of Lipid II,[5] and it seemed possible that tight binding to Lipid II might be coupled to the cooperative formation of higher order structures even at nanomolar concentrations. To evaluate this possibility, we monitored the anisotropy of a 5:1 mixture of ramoplanin and Orn4F ramoplanin (**2**) upon the addition of Lipid II. The anisotropy increases dramatically during the titration, plateauing when the concentration of Lipid II is one-half that of the ramoplanin mixture (Supporting Information). This result reveals that the complexes do associate at submicromolar concentrations, which may help to explain the high apparent affinity.

Association of the ramoplanin:Lipid II complexes greatly complicates structural analysis.[12] We discovered, however, that ramoplanin can adopt two alternative structures depending on the environment.[13−15] In membrane mimetic solvents such as methanol, it forms a dimer in which backbone strands spanning residues 10−14 from different molecules hydrogen bond in an antiparallel fashion. Dimerization forms a cleft flanked by two ornithine 10 residues (Figure 3).[13]

This solution structure provides a testable model for how Lipid II might bind to a ramoplanin dimer. Consistent with Lipid II binding in the cleft formed by dimerization and flanked by Orn10, we have found that both substrate binding and biological activity are greatly reduced when ornithine 10 is acylated.[4] The total synthesis of ramoplanin was recently reported and should enable the construction of additional analogues to probe the recognition event.[16] For example, covalent dimers of ramoplanin might show enhanced affinity for Lipid II. Alternatively, if ramoplanin could be modified so that the complexes do not associate, both structural and thermodynamic analyses of Lipid II binding would be greatly facilitated.[17]

In closing, we have shown that ramoplanin inhibits bacterial transglycosylases by binding with a stoichiometry of 2:1 to Lipid II at concentrations that are consistent with reported MICs, and we propose that transglycosylase inhibition is the likely cause of bacterial cell death. We note that the transglycosylase assay used here to characterize ramoplanin could be a useful tool for assessing the binding characteristics of many other putative substrate binders because it provides information on both the stoichiometry and the apparent affinity of substrate binding.

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Supporting Information Available: Experimental details for the synthesis of **2**, the transglycosylase assay conditions, the titration procedures, and the anisotropy data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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