Transglycosylase assays:

_E. coli_ PBP1b was expressed and purified as described in reference 6(d). Assays were carried out as described by separately incubating varying amounts of $^{14}$C-GlcNAc-labeled heptaprenyl Lipid II (specific activity = 273 cpm/pmol) and ramoplanin (concentrations are indicated in the figure legend) in eppendorf tubes containing 9 µL of buffer (50 mM HEPES at pH 7.5, 10 mM CaCl₂, 1000 U/mL penicillin G, 0.2 mM decyl PEG, and 11% DMSO), and 1 µL PBP1b (from a solution freshly prepared by diluting a 50% glycerol stock of PBP1b 20-fold into 5 mM Tris buffer, pH 8.0, containing 8 mM decyl PEG) for 10 minutes. Reactions were started by adding 1 µL of the above PBP1b mixture to the substrate solution (for a final volume of 10 µL and an enzyme concentration of 30 nM), and were typically stopped after 15 minutes by adding 10 µL ice cold 10 mM Tris (pH 8.0) containing 0.2% Triton X-100. Reactions were left on ice until spotted on cellulose chromatography paper (3MM Whatman chromatography paper). Products and starting material were separated using chromatography (isobutyric acid:1N NH₄OH = 5:3) and quantitated by scintillation counting.

![Graph](image-url)

Figure 2. Initial velocity of PBP1b as a function of heptaprenyl Lipid II concentration in the absence (O) and presence of ramoplanin (∆=6 µM ramoplanin, =8 µM ramoplanin). A K₅ of 16 nM for the ramoplanin-lipid II complex was calculated (Kaleidograph) based upon a curve fit to the kinetic data assuming a 2:1 binding stoichiometry and using the following equation for substrate depletion: v=Vmax *(((0.5*[I]-[S]+Kd)2+4*Kd*[S]))0.5-(0.5*[I]-[S]+Kd))/ 2/(Km+(((0.5*[I]-[S]+Kd)2+4*Kd*[S]))0.5-(0.5*[I]-[S]+Kd))/2). See reference 7. The data cannot be fit to a 1:1 binding model.
**Job titrations:**

Job titrations were carried out in 96-well microplates using a Perkin-Elmer HTS 700 Plus Bio-Assay Plate Reader. An appropriate amount of Orn4-fluorescein-labeled ramoplanin (2) from a 20 µM stock solution (prepared in 2% DMSO/H₂O) was added to wells containing 90 µL buffer (prepared by combining 5 µL 1 M HEPES, pH = 7.5, 10 µL 2 mg/mL BSA in H₂O and 75 µL H₂O) and allowed to equilibrate for 10 minutes. The fluorescence of each well was then measured (λₓₛ = 492 nm, λₑₓₙ = 535 nm). The appropriate amount of undecaprenyl Lipid II was added from a 20 µM stock solution (prepared in H₂O and DMSO) and the fluorescence was measured after a 10-minute equilibration period. The combined volume of Lipid II plus Orn4F (2) added to each well was 10 µL. The fluorescence change for each measurement was plotted against the mole fraction of ramoplanin to obtain the stoichiometry.

![Graph](image)

Figure 4. Job titration of Orn4F (2) and undecaprenyl Lipid II in 50 mM HEPES buffer (pH=7.5) at a total concentration of 2 uM.
**Determination of binding parameters:**
The affinity of Lipid II for Orn4-fluorescein-labeled ramoplanin (2) was determined by monitoring fluorescence intensity in the presence of increasing concentrations of Lipid II. Fluorescence measurements were made using an Aminco-Bowman® Series 2 Luminescence Spectrometer ($\lambda_{ex}= 492$ nm, $\lambda_{em}= 525$ nm). 1 µL of 20 µM Orn4-fluorescein-labeled ramoplanin (2) was added to a quartz cuvette containing 1 mL 50 mM HEPES buffer (pH=7.5) and allowed to equilibrate 2 minutes prior to the initial reading. Aliquots of a 2 µM undecaprenyl Lipid II stock solution (prepared in 2% DMSO/H$_2$O) were added to achieve the final concentrations shown in Figure 4. Before each measurement, the solution was allowed to equilibrate 2 minutes.

![Figure 5](image.png)

*Figure 5. Fluorescence of a 20 nM solution of (2) (50 mM HEPES buffer, pH=7.5) as a function of [undecaprenyl Lipid II]. $K_d$'s of $10^{-7}$ M$^2$ and $10^{-9}$ M were calculated when the titration data in Figure 4 was fit to either a 2:1 binding model with ramoplanin monomer (2R + L->R2L) or a 1:1 binding model with a ramoplanin dimer (R2 + L -> R2L) respectively. The data cannot be fit to a 1:1 binding model (R + L->RL).*
Anisotropy analysis showing association of ramoplanin-Lipid II complexes: 
The anisotropy of Orn4F (2) was monitored as a function of Lipid II concentration using an Aminco-Bowman® Series 2 Luminescence Spectrometer (λ<sub>ex</sub> = 492 nm, λ<sub>em</sub> = 525 nm). 2 µL of 20 µM Orn4-fluorescein-labeled ramoplanin (2) (in 2% DMSO/H<sub>2</sub>O), and 10 µL of 20 µM ramoplanin (1) (in H<sub>2</sub>O) was added to a quartz cuvette containing 1 mL 50 mM HEPES buffer (pH=7.5) and allowed to equilibrate 2 minutes prior to the initial reading. The final concentration of ramoplanin + 2 was 240 nM. Aliquots of a 20 µM undecaprenyl Lipid II stock solution (in 2%DMSO/H<sub>2</sub>O) were added to achieve the final concentrations shown in Figure 6. Before each measurement, the solution was allowed to equilibrate 2 minutes.

![Anisotropy change of 5:1 mixture of ramoplanin and Orn4F ramoplanin (2) upon the addition of Lipid II.](image)

Figure 6. Anisotropy change of 5:1 mixture of ramoplanin and Orn4F ramoplanin (2) upon the addition of Lipid II.
**Preparation of Orn4F (2):** Ramoplanin (30 mg, 11.7 µmoles) was dissolved in a 2:1 mixture of aqueous NaHCO₃ (0.1M) and dioxane. Fluorescein-NHS (6.1mg, 12.9 µmoles) was added as a solution in 2 mL of dioxane. The reaction was monitored by analytical HPLC and was complete within 2 hours. Separation of Orn4F, Orn10F and Orn4+Orn10F ramoplanin derivatives was carried out using reverse phase HPLC (Phenomenex Luna 5µ C18(2), 250 mm × 21.2 mm. Solvent A: H₂O/0.1% AcOH, Solvent B: CH₃CN/0.1% AcOH). The following gradient was used for the purification: t=0, %B=20, t=5, %B=20, t=85, %B=60). The sites of modification were determined by comparing diagnostic chemical shifts to those in the fully assigned parent compound, ramoplanin:

Ramoplanin (1) (¹H NMR, D₂O, 500 MHz): Orn4γCH²= 2.47 & 2.68, Orn10γCH²= 3.05 & 3.10
Orn10F (¹H NMR, 80% D₂O/20% DMSO d₆, 500 MHz): Orn4γCH²= 2.44 & 2.67, Orn10γCH²= 3.46 & 3.53
Orn4F (2) (¹H NMR, 80% D₂O/20% DMSO d₆, 500 MHz): Orn4γCH²= 3.4-3.6, Orn10γCH²= 3.05 & 3.16; ESI MS⁺ of Orn4F (2)= 2950.2 (calculated mass for Orn4FK⁺ is 2949.1).