Bacterial transpeptidase and transglycosylase on the surface are essential for cell wall synthesis, and many antibiotics have been developed to target the transpeptidase; however, the problem of antibiotic resistance has arisen and caused a major threat in bacterial infection. The transglycosylase has been considered to be another excellent target, but no antibiotics have been developed to target this enzyme. Here, we determined the crystal structure of the *Staphylococcus aureus* membrane-bound transglycosylase, monofunctional glycosyltransferase, in complex with a lipid II analog to 2.3 Å resolution. Our results showed that the lipid II-contacting residues are not only conserved in WT and drug-resistant bacteria but also significant in enzymatic activity. Mechanistically, we proposed that K140 and R148 in the donor site, instead of the previously proposed E156, are used to stabilize the pyrophosphate-leaving group of lipid II, and E100 in the acceptor site acts as general base for the 4-OH of GlcNAc to facilitate the transglycosylation reaction. This mechanism, further supported by mutagenesis study and the structure of monofunctional glycosyltransferase in complex with moenomycin in the donor site, provides a direction for antibacterial drugs design.