Lipid II: Total Synthesis of the Bacterial Cell Wall Precursor and Utilization as a Substrate for Glycosyltransfer and Transpeptidation by Penicillin Binding Protein (PBP) 1b of *Eschericia coli*

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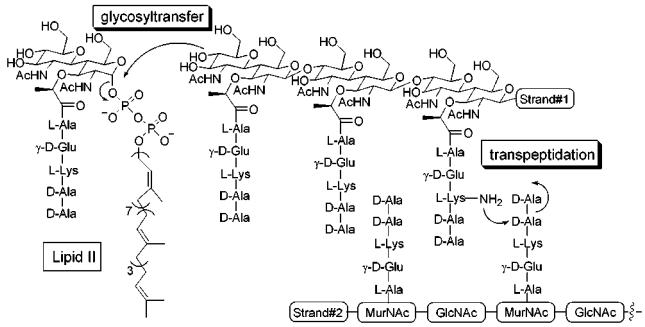
Abstract: An essential feature in the life cycle of both gram positive and gram negative bacteria is the production of new cell wall. Also known as murein, the cell wall is a two-dimensional polymer, consisting of a linear, repeating *N*-acetylmuramic acid (MurNAc) and *N*-acetylglucosamine (GlcNAc) motif, cross-linked via peptides appended to MurNAc. The final steps in the maturation of murein are catalyzed by a single, bifunctional enzyme, known as a high MW, class A penicillin binding protein (PBP). PBPs catalyze polymerization of the sugar units (glycosyltransfer), as well as peptide cross-linking (transpeptidation) utilizing Lipid II as substrate. Detailed enzymology on this enzyme has been limited, due to diffculties in obtaining sufficient amounts of Lipid II, as well as the availability of a convenient and informative assay. We report the total chemical synthesis of Lipid II, as well as the development of an appropriate assay system and the observation of both catalytic transformations.

Introduction

Bacterial cell walls consist of a repeating, two-dimensional array of peptidoglycan known as murein.[1] Structurally, murein is composed of linear polysaccharide chains of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc), cross-linked via peptides appended to MurNAc (Scheme 1). The final steps in the biosynthesis of murein occur extracellularly, associated with the cell membrane, and are catalyzed by high molecular weight, class A penicillin-binding proteins (PBPs), such as *Eschericia coli* PBP 1b.[2,3] This bifunctional enzyme catalyzes both sugar polymerization (glycosyltransfer) and peptide cross-linking (transpeptidation) utilizing the cognate monomer, Lipid II, as substrate (Scheme 1).

Inhibition of either activity of PBP1b, and the analogous enzymes in other bacterial species, is regarded as an excellent opportunity for antimicrobial intervention. Natural products which inhibit both reactions in vitro are known; flavomycin (moenomycin, bambermycin) blocks the glycosyltranser step, while penicillin (and all β-lactams) blocks transpeptidation.[1] Though these compounds are potent antimicrobial agents, they suffer serious clinical problems, as flavomycin has poor bioavailability,[4] while resistance to β-lactam drugs develops quickly and is widespread.[5] Recent efforts at analoging flavomycin[4] and vancomycin[6] to produce better inhibitors of the glycosyltransfer reaction have been encouraging; however, to drive drug discovery access to novel classes of compounds is desirable. Research in this respect has been slow primarily due to the lack of a prerequisite understanding of the glycosyltransfer and transpeptidation reactions. In turn, this is due to an inability to obtain sufficient quantities of substrate, and the lack of an informative, yet unencumbered, system for studying the enzyme. Thus, we have developed a total chemical synthesis of Lipid II, and developed a homogeneous assay format with recombinant PBP1b that can be used for detailed enzymology of both glycosyltransfer and transpeptidation.

Scheme 1



Synthesis of Lipid II

The synthesis of Lipid II began with the commercially available MurNAc precursor **1**, which was coupled with alanine, deprotected, and selectively monoacetylated to generate the glycosyl acceptor **2** (Scheme 2). Alanine was introduced at this step to avoid the unwanted cyclization of compound **2**, which occurred when the lactate was protected as an ester. Compound **2** was coupled with the donor **3**, utilizing AgOTF to activate the glycosyl bromide.[7] The phthalimido group in the 2 position

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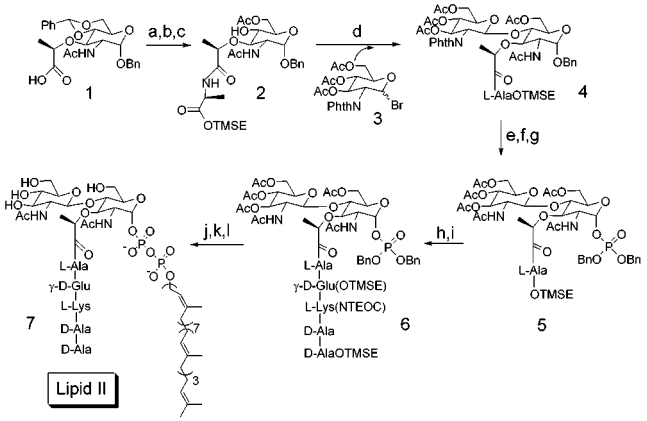
Scheme 2



[a] HAlaOTMSE, DIEA, HOBt, PyBOP, 1:1 THF:CH_2Cl_2, room temperature, 2 h, 81%. [b] Catalyst TsOH, MeOH, 75 °C, 30 min, 89%. [c] Pyridine, 1 equiv of AcCl, −30 °C, 15 min, then warm to room temperature, 80%. [d] 3 equiv of AgOTF, 2 equiv of compound **3**, 4 Å sieves, CH_2Cl_2, −40 °C, drybox, 3 h, then O.N., 60%. [e] Amino-modified resin, 4 Å sieves, butanol, 85 °C, 24 h, then pyridine, Ac_2O, room temperature, O.N., 53%. [f] 10%Pd/C, H_2, MeOH, 1 h, 93%. [g] Tetrazole, $(BnO)_2PN(i\text{-}Pr)_2$, CH_2Cl_2, −30 °C to room temperature, 1 h, then *m*-CPBA, −40 °C to room temperature, 1 h, 55%. [h] TBAF, THF, room temperature, 45 min, 87%. [i] H-γ-D-Glu(OTMSE)-Lys(TEOC)-D-Ala-D-AlaOTMSE, DIEA, HOBt, PyBOP, 1:1 THF:CH_2Cl_2, room temperature, 2 h, 61%. [j] 10%Pd/C, H_2, MeOH, 1 h, 100%. [k] CDI, dry DMF, room temperature, 4 h, then MeOH, then undecaprenyl phosphate, room temperature, 48 h, 39%. [l] TBAF, DMF, room temperature, 24 h, then 3% NaOMe, MeOH, 0 °C, 1 h, 35%.

of compound **3** directs attack predominantly on the β face, to give the desired product **4**. Following coupling, the phthalimido group was deprotected utilizing an ethylenediamine-derivatized resin,[8] and the amino group re-acetylated with acetic anhydride. This compound was then converted to the desired phosphate by successive anomeric deprotection and phosphorylation to yield compound **5**. The phosphorylation is done in a one-pot, two-step procedure by initial formation of the phosphite, followed by in situ oxidation with *m*-CPBA.[9,10] The carboxyl protecting group of alanine was removed, and the resulting acid was coupled to protected tetrapeptide to yield the desired glycopeptide (**6**). The phosphoryl group was freed, activated with CDI, and coupled to undecaprenyl phosphate. Finally, removal of the peptidyl and hydroxyl protecting groups provided Lipid II in 12 steps and 0.7% overall yield. This report constitutes the first published total chemical synthesis of Lipid II;[11] the only previously published synthesis of Lipid II is by Walker and co-workers, using the enzyme MurG to convert the monosaccharide (and biogenic) precursor Lipid I to the desired material.[9]

It may be useful to compare the synthetic and chemoenzymatic routes to Lipid II. The total yield of material appears to be comparable for each method (milligram quantities), and it is expected that both should scale without difficulty. One difference in these approaches may be in their abilities to generate Lipid II analogues. While MurG is noted to have

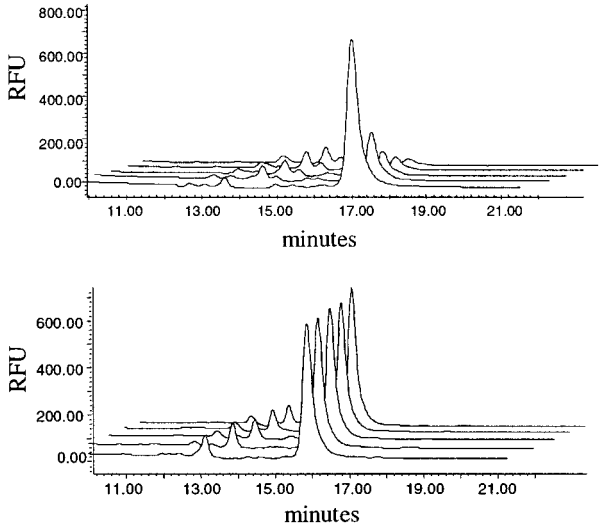


Figure 1. Measurement of the glycosyltransferase reaction. (Top) Lipid II (2 μM) was incubated at 30 °C with PBP1b (60 nM) in 50 mM HEPES buffer (pH 7.5) containing 0.085% decyl PEG, 10% DMSO, and 10 mM $MgCl_2$. Aliquots were removed, labeled, and injected on an anion-exchange HPLC column. Labeled Lipid II elutes at 16−17 min. Samples are at $t = 0$, 10, 20, 30, and 40 min (time increasing front to back). (Bottom) Lipid II was incubated with enzyme as in the top panel, with the addition of 10 μg/mL of flavomycin. Samples are at the same reaction times.

specificity preferences with respect to both the sugar[12] and lipid moieties,[9] it is expected that the synthetic route will not be limited in this manner. We will pursue generation of these analogues in the future, for both mechanistic and screening purposes.

Observation of Glycosyltransfer Reaction

For almost 20 years, studies of the glycosyltransfer reaction have required cumbersome and lengthy paper chromatographic analysis of radiolabled substrates, incubated with either ether-permeabilized whole bacterial cells or recombinant PBP1b as a source of enzyme. Though some elegant work has been done by Suzuki,[13] Ghuysen,[14] and van Heijenoort[15] to study the glycosyltransfer reaction using this methodology, it is unsuitable for detailed enzymology, as throughput is low and product analysis is unavailable.

Using the ready supply of Lipid II afforded by the chemical synthesis, we constructed an HPLC-based assay to interrogate the glycosyltransfer reaction. Lipid II contains only a single primary amino group, making it suitable for derivatization with fluorescamine. Importantly, this can be done postreaction, removing any concerns about the effects of labels on the reaction progress. Incubation of Lipid II with reconstituted PBP1b in a simple detergent system led to the observation of a time-dependent depletion of substrate (Figure 1, top). It is worth pointing out that the system is homogeneous, with both enzyme and substrate presumably partitioning into detergent micelles. DMSO (10%) was included in the assays, as it was found to accelerate the enzymatic reaction, as reported previously.[14]

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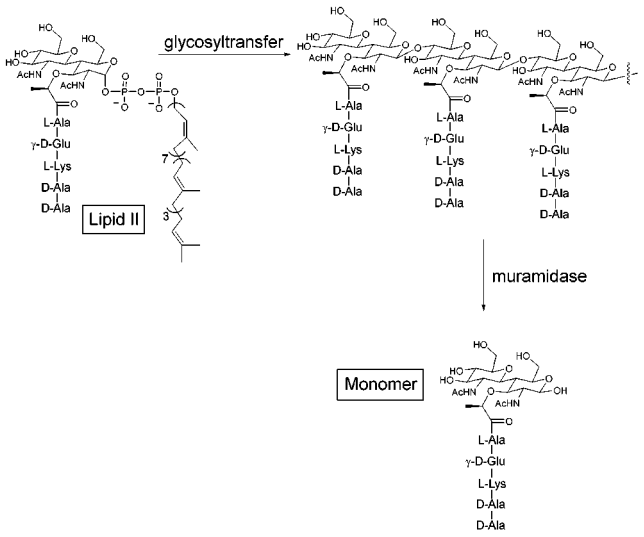
Scheme 3



Flavomycin (or Moenomycin) is a known glycosyltransferase inhibitor of PBP1b,[16] and has been used in past studies to verify the proposed source of observed activity in assays. The addition of flavomycin at 10 μg/mL to our reaction led to complete inhibition of substrate depletion (Figure 1, bottom).

Polymeric Nature of Glycosyltransferase Reaction

To verify that the reaction observed between Lipid II and PBP1b was the result of glycosyltransfer, attempts were made to analyze the enzymatic product. Bacterial muramidases are enzymes capable of hydrolyzing un-cross-linked murein at the β-1,4 linkage between MurNAc and GlcNAc, producing monomeric material[17] (Scheme 3). Thus, the addition of muramidase to our in vitro reaction with PBP1b was expected to yield a new chemical species, amenable to the same labeling reaction as Lipid II.

To produce a homogeneous product, and thus simplify the analysis, reactions were conducted in the presence of penicillin G, which has been shown to specifically inhibit cross-linking of murein. Important to this analysis, the addition of penicillin G was found not to affect the glycosyltransfer reaction (Figure 2).

When reactions were carried out in the presence of muramidase, the disappearance of Lipid II was accompanied by the appearance of a new peak (Figure 3). Consistent with the possibility that this species was labeled monomer, the new peak was equal in intensity to Lipid II (monomer and Lipid II are both labeled through lysine), and eluted more rapidly off of the anion-exchange HPLC column (monomer has fewer negative charges than Lipid II as the lipid diphosphate moiety is lost during the enzymatic reaction). Importantly, this new peak was not generated by the action of muramidase itself on Lipid II, as control reactions carried out in the absence of PBP1b showed no turnover (data not shown).

To obtain the identity of the muramidase-dependent species, unlabeled product from a reaction containing both PBP1b and muramidase was isolated and subjected to mass spectral analysis. Material was obtained by first carrying out a complete reaction with Lipid II and PBP1b, followed by removal of all small (<10 000 MW) species by dialysis. Muramidase was then added, followed by a second dialysis, in which digested polymer was collected. This material was found to have a MW of 966.4, as predicted for the un-cross-linked monomer, and when labeled was shown to correspond with the new, muramidase-dependent HPLC peak. Thus, the in vitro enzymatic glycosyltransfer reaction produces polymeric peptidoglycan.

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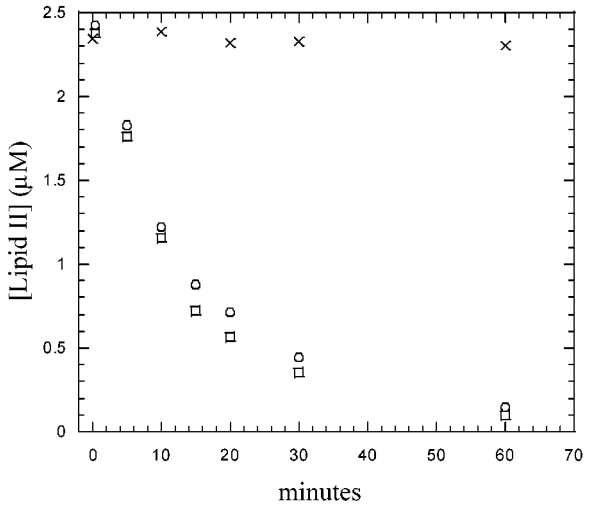

Figure 2. Effect of inhibitors on the glycosyltransferase reaction. Enzymatic reactions were carried out as indicated in Figure 1, with either no inhibitor (□), 100 μM penicillin G (○), or 10 μg/mL of flavomycin (×)

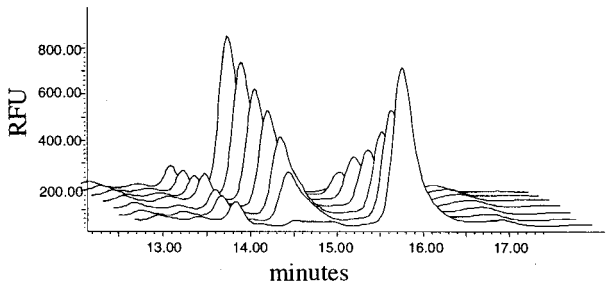

Figure 3. Glycosyltransferase reaction in the presence of muramidase. The enzymatic reaction was carried out under similar conditions to Figure 1, with the addition of 0.02 mg/mL of muramidase. Aliquots were removed, labeled, and injected on an anion-exchange HPLC column. Labeled monomer elutes at 14.5 min and labeled Lipid II elutes at 16 min. Samples are at t = 0, 2, 6, 10, 15, 30, and 60 min (time increasing front to back).

Observation of Transpeptidation Reaction

As PBP1b is a bifunctional enzyme, reactions with Lipid II should yield not only polymerized sugars, but also D-Ala as the result of interstrand peptide cross-linking. It has not been possible to quantitatively study this reaction, as short peptides are not utilized as substrates in transpeptidation.[18] Qualitatively, it has been possible to establish the transpeptidation reaction by digesting mature peptidoglycan with lysozyme, and analyzing the resultant products.[3] For kinetic measurements of transpeptidation, the hydrolysis of thioester peptide analogues has been used to emulate the natural reaction.[11] Though thioester hydrolysis is likely to be a useful approximation of the transpeptidation reaction, as penicillin irreversibly inhibits both reactions, questions about the substrate specificity and processivity of PBP1b cannot be answered with this methodology.

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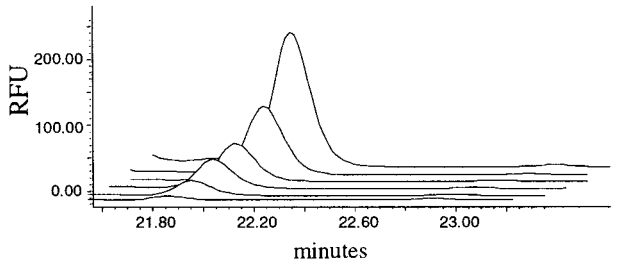

Figure 4. Measurement of the transpeptidase reaction. Lipid II (2 μM) was incubated at 30 °C with PBP1b (60 nM) in 50 mM HEPES buffer (pH 7.5) containing 0.085% decyl PEG, 10% DMSO, and 10 mM $MgCl_2$. Aliquots were removed, labeled, and injected on a reverse-phase C18 HPLC column. Labeled D-Ala elutes at 21.8 min. Samples are at $t = 0$, 2, 6, 10, 20, and 40 min (time increasing front to back). The largest peak corresponds to 0.28 μM D-Ala.

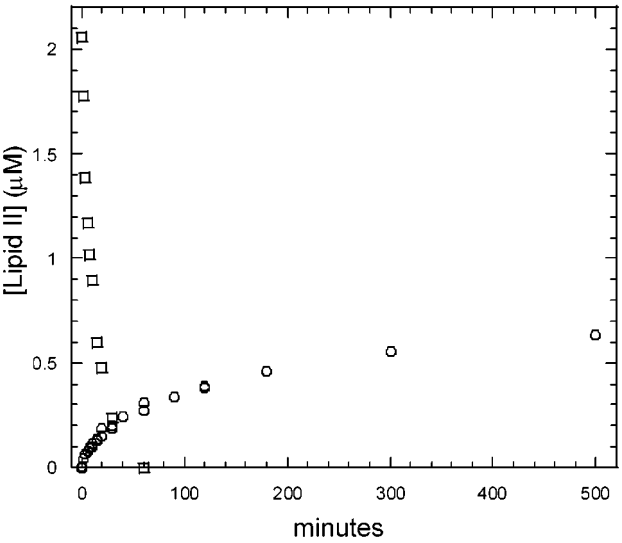

Figure 5. Relative rates of glycosyltransfer (□) and transpeptidation (○) reactions. Conditions for analyses of the two reactions are the same as in Figure 1 and Figure 4.

An advantage of the current assay system is that D-Ala can also be visualized with the same labeling reaction, as fluorescamine will react with the amino group of amino acids. As shown in Figure 4, a time-dependent formation of D-Ala is observed upon incubation of PBP1b with Lipid II. As mentioned previously, penicillin G is known to inhibit the transpeptidation reaction, by chemical modification of a key active site serine residue.[11] The addition of penicillin G to the in vitro reaction catalyzed by PBP1b resulted in a lack of detectable D-Ala formation (data not shown), confirming the enzymatic source of this product. To our knowledge, this is the first direct observation of the transpeptidation reaction, and will allow future study of this activity.

It is interesting to compare the timecourses for the glycosyltransfer and transpeptidation reactions (Figure 5). While the glycosyltransfer reaction is virtually complete in 30 min, the transpeptidation reaction occurs on a longer time scale. Mechanistically, this indicates that in vitro, the active sites of PBP1b do not act in concert, as transpeptidation is still occurring after completion of the glycosyltransfer reaction. Based on the inability of penicillin G to inhibit this reaction (Figure 2), it appears that both active sites can function independently. The relevance of these results in vivo is not known; however, it should be noted that bacteria possess enzymes capable of catalyzing each of these reactions singly (monofunctional glycosyltransferases[19] and class B, high MW PBPs[18]), so there exists in vivo precedence for the results obtained with PBP1b.

Attempts were made to analyze the muramidase-digested products of this reaction, as with un-cross-linked material. However, an identifiable species could not be found, possibly due to the heterogeneous nature of the products.

Conclusions

Using a synthetically prepared Lipid II, a recombinant form of *E. coli* PBP1b, and a simple HPLC analytical system, we have observed both glycosyltransferase and transpeptidase activities in a simple in vitro system. Products of the observed glycosyltransfer reaction were shown to be un-cross-linked murein by the isolation of polymeric product, and subsequent digestion by muramidase to yield the expected monomeric material.

There are many crucial, yet currently unexplored mechanistic aspects involved in the polymerizations carried out by PBP1b, and the analogous enzymes in other bacterial species. These include the possible processivity of these enzymes, as well as the effects of detergents, metals, organic cosolvents, and inhibitors. Using the system described here, we will begin to study these questions and support future drug discovery efforts.

Experimental Section

General Procedures. ^{1}H NMR spectra were recorded on dilute solutions in $CDCl_3$, CD_3OD, or DMSO-d_6 at 300 MHz on Varian Unity instruments. Chemical shifts are reported in parts per million (δ) downfield from tetramethylsilane. Low-resolution mass spectral analyses were performed on an Micromass Platform II (ESI) instrument. High-resolution mass spectra were obtained on a Finnigan MAT95S (ESI) instrument. Reactions were performed under an atmosphere of dry nitrogen or argon in flame-dried glassware and were monitored for completeness by thin-layer chromatography (TLC) by using silica gel 60 F-254 (0.25 mm) plates. Visualization of TLC plates was accomplished by I_2 vapor, phosphomolybdic acid in ethanol, ceric ammonium molybdate in aqueous methanol, or UV light absorption at 254 nm. Flash column chromatography was performed by the method of Still, using 230−400 mesh silica gel (E Merck). Tetrahydrofuran was distilled from potassium/benzophenone ketyl immediately prior to use. Other solvents and reagents were purchased from commercial sources and were used without further purification.

Cloning, Expression ,and Purification of PBP1b. The gene encoding PBP1b was amplified from the *Streptococcus pneumonia* genomic DNA library (American Type Culture Collection) by PCR. A *Nde I* site was included at the 5′ end of the sense primer, and a *Xho I* site was included at the 5′ end of the antisense primer. The PCR product was subsequently inserted into a pet-21a expression vector (Novagen) between the *Nde I* and *BamH I* sites. As a result, a hexahistidine tag was introduced to the c-terminus of the protein. *E. coli* cells (BL21−DE3) transformed with the vectors showed moderate level expression of PBP1b (5−7 mg/L in LB medium). After harvesting, cells were suspended in loading buffer (100 mM NaCl, 50 mM NaPi, pH 8.0) and disrupted in a French Press apparatus. After brief centrifugation, PBP1b protein was found in pellets. The pellets were dissolved in loading buffer containing 1% CHAPS, and loaded onto a Ni-agarose column. The column was washed with 5× and 3× bed volumes of load buffer and loading buffer containing 25 mM imidazole, respectively. The protein was then eluted by applying loading buffer containing 500 mM imidazole to the column (1% CHAPS was included in all column steps). The purity of the protein was >95%, as judged by SDS-page analysis.

Assay of Transglycosylase Activity. Assays consisted of 270 μL of buffer (0.085% $C_{10}E_6$ PEG, 50 mM HEPES, pH 7.5), 10% DMSO, and 2 μM Lipid II, and were conducted at 30 °C. Reactions were

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initiated by the addition of recombinant PBP1b (60nM final concentration). Aliquots (25 μL) were removed at various times and analyzed by adjusting the pH to >9, followed by addition of fluorescamine (1 mM) and HPLC separation on an anion-exchange column (SAX1, Supelco Co.). A linear gradient of 20 mM to 1 M ammonium acetate (in methanol) was used as eluant. *N*-Acetyllysine was used as a standard to quantitate the fluorescent signal observed. Reactions involving the observance of monomer were carried out identically, with the exception of added muramidase from *Streptomyces globisporus* (Calbiochem) at 0.02 mg/mL.

Assay of Transpeptidase Activity. Assay conditions were identical with those of the transglycosylase assays. Analysis, however, was carried out by using a Reverse-phase C-18 HPLC column. A linear gradient of water (+0.1% TFA) and acetonitrile (+0.1% TFA) was used as eluant. Authentic material was used as a standard for identification and quanitation of product.

Isolation of Cell Wall Monomer for Mass Spectral Analysis. Enzyme (60 nm) was preincubated in reaction buffer for 30 min with penicillin G (100 μM). Lipid II (2 μM) was added, and after 1 h the reaction was placed in a Slide-A-Lyzer (Pierce) with a 10 000 MW cutoff and dialyzed against water for 4 h (3 × 200 mL). To the resultant polymeric material was added 0.02 mg of muramidase from *S. globisporus* (Calbiochem) and the mixture was allowed to react for 30 min at 30 °C. The crude reaction was spun through a microconcentrator (Amicon) with a 10 000 MW cutoff, and the flowthrough was lyopholized for analysis. ESI-MS for $C_{39}H_{66}N_8O_{20}$: calcd [M + H$^+$] 967.4, found 967.4

Reaction A. To a solution of protected muramic acid **1** (3.88 g, 8.23 mmol) in 60 mL of 1:1 THF:CH_2Cl_2 was added HAlaOTMSE (1.5 g, 7.9 mmol). To this mixture was added DIEA (2.84 mL, 16.4 mmol), HOBt (1.14 g, 8.46 mmol), and PyBOP (4.4 g, 8.46 mmol). The reaction was stirred under N_2 for 2 h, after which solvent was removed. The residue was redissolved in 400 mL of EtOAc and washed 3 times with 150 mL of 0.01 M HCl and once with 150 mL of brine. The organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo. The crude reaction was purified by silica gel chromatography, with 2:1 EtOAc:Hex as eluant (R_f = 0.41), to yield a white powder after extensive drying (4.15 g, 81%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.40−7.45 (m, 2H), 7.22−7.36 (m, 8H), 6.88 (d, 1H, J = 7.3 Hz), 6.24 (d, 1H, J = 8.8 Hz), 5.54 (s, 1H), 4.95 (d, 1H, J = 4.0 Hz), 4.57 (ABq, 2H, J_{AB} = 11.7 Hz, $\Delta\nu$ = 67 Hz), 4.41 (t, 1H, J = 7.4 Hz), 4.15−4.30 (m, 4H), 4.10 (q, 1H, J = 6.7 Hz), 3.80−3.87 (m, 1H), 3.60−3.77 (m, 3H), 1.91 (s, 3H), 1.38 (d, 3H, J = 6.9 Hz), 1.36 (d, 3H, J = 6.6 Hz), 0.93−0.99 (m, 2H), 0.00 (s, 9H). ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 172.67, 172.66, 170.51, 137.13, 136.87, 129.01, 128.63, 128.28, 128.24, 128.12, 125.94, 101.40, 97.50, 81.73, 78.20, 77.45, 70.05, 68.86, 63.77, 63.17, 53.16, 48.08, 23.34, 19.45, 17.93, 17.28, −1.48. HRMS (FAB) for $C_{33}H_{46}N_2O_9Si$: calcd [M + H$^+$] 643.3051, found 643.3064.

Reaction B. To a 250 mL RB flask containing 4.15 g of Ala-coupled MurNAc (6.45 mmol) was added 100 mL of methanol and 120 mg of TsOH. The reaction was warmed to 75 °C, and after 30 min the reaction was judged complete by the disappearance of s.m. by TLC (2:1 EtOAc: Hex). The solvent was removed from the crude reaction, and the resulting oil was purified by silica gel chromatography, with 5:1 EtOAc: MeOH as eluant (R_f = 0.62). After removal of solvent, a foamy solid was obtained (3.2 g, 89%). ^{1}H NMR (300 MHz, CD_3OD) δ 7.20−7.37 (m, 5H), 4.83 (d, 1H, J = 3.7 Hz), 4.58 (ABq, 2H, J_{AB} = 12.3 Hz, $\Delta\nu$ = 72 Hz), 4.28 (q, 1H, J = 7.3 Hz), 4.27 (q, 1H, J = 6.7 Hz), 4.14−4.20 (m, 2H), 3.91 (dd, 1H, J = 10.4, 3.5 Hz), 3.78 (d, 1H, J = 9.9 Hz), 3.57−3.69 (m, 3H), 3.45 (t, 1H, J = 9.2 Hz), 1.85 (s, 3H), 1.35 (d, 3H, J = 7.3 Hz), 1.33 (d, 3H, J = 6.3 Hz), 0.94−1.00 (m, 2H), 0.00 (s, 9H). ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 173.53, 173.06, 170.82, 137.11, 128.54, 128.07, 127.97, 97.00, 79.75, 77.22, 72.04, 69.95, 69.73, 63.89, 61.73, 52.66, 48.13, 23.19, 19.39, 17.57, 17.24, −1.51. HRMS (FAB) for $C_{26}H_{42}N_2O_9Si$: calcd [M + H$^+$] 555.2738, found 555.2757.

Reaction C. Diol (3.2 g, 5.8 mmol) was dissolved in 100 mL of dry CH_2Cl_2, and cooled to −30 °C in a three-neck flask with an addition funnel. Pyridine (1.0 mL, 12.4 mmol) was added to the reaction. Acetyl chloride (0.464 mL) was dissolved in 30 mL of dry CH_2Cl_2 and placed in the addition funnel. The acid chloride solution was added dropwise to the reaction over 15 min. The reaction was allowed to warm slowly to 0 °C, after which it was diluted with 200 mL of CH_2Cl_2 and transferred to a separatory funnel. The organic layer was washed 2 times with 150 mL of 0.01 M HCl and once with 150 mL of brine. The organic layer was then dried over $MgSO_4$, filtered, and concentrated in vacuo. The crude material was purified by silica gel chromatography, with EtOAc as eluant (R_f = 0.47). Upon removal of EtOAc, a foamy solid (compound **2**) was obtained (2.8 g, 80%). ^{1}H NMR (300 MHz, CD_3OD) δ 8.22 (d, 1H, J = 8.4 Hz), 7.20−7.36 (m, 5H), 4.82 (d, 1H, J = 3.6 Hz), 4.56 (ABq, 2H, J_{AB} = 12.1 Hz, $\Delta\nu$ = 61 Hz), 4.25−4.33 (m, 3H), 4.14−4.21 (m, 3H), 3.87−3.95 (m, 1H), 3.75−3.91 (m, 1H), 3.60 (dd, 1H, J = 10.6, 8.8 Hz), 3.46 (t, 1H, J = 9.3 Hz), 2.04 (s, 3H), 1.85 (s, 3H), 1.35 (d, 3H, J = 7.4 Hz), 1.33 (d, 3H, J = 6.9 Hz), 0.94−1.00 (m, 2H), 0.00 (s, 9H). ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 172.93, 172.75, 171.67, 170.39, 136.99, 128.57, 128.15, 128.08, 97.18, 79.94, 77.67, 70.47, 69.92, 69.77, 63.78, 63.12, 52.55, 48.08, 23.26, 20.85, 19.17, 17.73, 17.27, −1.51.

Reaction D. In a drybox, compound **2** (2.8 g, 4.7 mmol) was placed in 25 mL of dry CH_2Cl_2 and stirred for 20 min with 4 Å sieves. The solution was cooled to −40 °C, and silver triflate (3.6 g, 14.1 mmol) was added. 1-Bromo-2-deoxy-2-*N*-phthalimido-3,4,6-tri-*O*-acetyl-D-glucopyranoside was dissolved in 30 mL of dry CH_2Cl_2 and added dropwise over 3 h via addition funnel. After addition, the reaction was allowed to continue overnight at −40 °C, followed by slow warming to room temperature. The crude reaction was diluted with 200 mL of CH_2Cl_2 and washed 2 times with 50 mL of $NaHCO_3$ and once with 50 mL of brine. The organic layer was dried, filtered, and concentrated in vacuo to yield a crude yellow oil. The oil was purified by silica gel chromatography in 3:1 toluene:acetone (R_f = 0.17) to yield the desired coupled disaccharide as a white solid (2.85 g, 60%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.86−7.91 (m, 2H), 7.75−7.79 (m, 2H), 7.15−7.27 (m, 5H), 6.94 (d, 1H, 7.7 Hz), 6.76 (d, 1H, J = 7.3 Hz), 5.82 (dd, 1H, J = 10.6, 9.1 Hz), 5.38 (d, 1H, J = 8.5 Hz), 5.20 (t, 1H, J = 9.7 Hz), 4.99 (d, 1H, J = 3.7 Hz), 4.46 (ABq, 2H, J_{AB} = 12.4 Hz, $\Delta\nu$ = 43 Hz), 4.39−4.49 (m, 2H), 4.10−4.31 (m, 7H), 4.01−4.08 (m, 1H), 3.94 (t, 1H, J = 9.6 Hz), 3.82−3.88 (m, 1H), 3.45−3.64 (m, 3H), 2.13 (s, 3H), 2.07 (s, 3H), 2.02 (s, 3H), 1.92 (s, 3H), 1.85 (s, 3H), 1.49 (d, 3H, J = 6.9 Hz), 1.45 (d, 3H, J = 7.4 Hz), 0.97−1.02 (m, 2H), 0.03 (s, 9H). ^{13}C NMR (100.52 MHz, DMSO-d_6) δ 173.52, 172.10, 169.81, 169.60, 169.55, 169.33, 169.20, 137.37, 134.96, 128.02, 127.29, 127.08, 123.64, 96.81, 95.59, 76.29, 75.99, 75.07, 70.53, 70.05, 68.45, 68.35, 68.12, 62.61, 62.11, 61.35, 54.66, 53.54, 47.47, 22.50, 20.30, 20.26, 19.92, 18.57, 17.00, 16.52, −1.65. HRMS (FAB) for $C_{48}H_{63}N_3O_{19}Si$: calcd [M + H$^+$] 1014.390, found 1014.389.

Reaction E. Disaccharide (3.29 g, 3.24 mmol) was placed in a 250 mL round-bottom flask with 16 g of diaminoethylene-derivatized Merrifield resin (ref), 4 Å sieves, and 150 mL of dry butanol. The mixture was heated at 85 °C under dry atmosphere for 24 h, at which time the majority of starting material was gone as judged by TLC. After being cooled to room temperature, the reaction was filtered and concentrated to yield a syrupy oil. The oil was redissolved in 12 mL of pyridine, to which 12 mL of acetic anhydride was added. The reaction was stirred at room temperature overnight, after which it was concentrated and purified by silica gel chromatography in 4:3 toluene acetone (R_f = 0.26) to yield the peracetylated sugar as a white solid (1.58 g, 53%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.28−7.38 (m, 52H), 7.21 (d, 1H, J = 7.3 Hz), 6.99 (d, 1H, J = 6.9 Hz), 6.15 (d, 1H, J = 9.5 Hz), 5.05−5.16 (m, 3H), 4.59 (ABq, 2H, J_{AB} = 12.3 Hz, D_n = 42 Hz), 4.48 (t, 1H, J = 7.3 Hz), 4.39−4.43 (m, 2H), 4.29−4.37 (m, 2H), 3.98−4.22 (m, 6H), 3.77 (d, 2H, J = 5.2 Hz), 3.56−3.66 (m, 2H), 2.15 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H), 2.03 (s, 3H), 1.96 (s, 3H), 1.94 (s, 3H), 1.42 (d, 3H, J = 6.3 Hz), 1.39 (d, 3H, J = 6.6 Hz), 0.96−1.02 (m, 2H), 0.03 (s, 9H). ESI-MS for $C_{42}H_{63}N_3O_{18}Si$: calcd [M + H$^+$]: 926.4, found 926.8.

Reaction F. To a round-bottom flask containing benzyl-protected sugar (1.58 g, 1.71 mmol) was added 300 mg of 10% Pd/C and 50 mL of methanol. The reaction was stirred vigorously under an atmosphere of hydrogen until reaction was judged complete by TLC. The reaction was then filtered through Celite and concentrated in vacuo to yield the desired compound as a white solid (1.32 g, 93%). ^{1}H NMR (300 MHz,

$CDCl_3$) δ 7.43−7.46 (m, 2H), 6.27 (d, 1H, J = 5.50 (br s, 1H)), 5.13 (t, 1H, J = 9.4 Hz), 5.08 (t, 1H, J = 8.8 Hz), 4.59 (q, 1H, J = 6.6 Hz), 4.41−4.50 (m, 2H), 4.34 (dd, 1H, J = 12.5, 4.0 Hz), 4.07−4.28 (m, 6H), 3.96−4.00 (m, 1H), 3.81−3.88 (m, 2H), 3.69 (dd, 1H, J = 10.8, 8.7 Hz), 3.60−3.66 (m, 1H), 2.14 (s, 3H), 2.06 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H), 2.01 (s, 3H), 1.99 (s, 3H), 1.46 (d, 3H, J = 7.3 Hz), 1.39 (d, 3H, J = 6.6 Hz), 1.00−1.06 (m, 2H), 0.05 (s, 9H). ESI-MS for $C_{35}H_{57}N_3O_{18}Si$: calcd [M + Na^+] 858.3, found 858.6.

Reaction G. To a 500 mL round-bottom flask containing the product of reaction E (1.32 g, 1.58 mmol) was added 338 mg of tetrazole (4.725 mmol) and 50 mL of dry CH_2Cl_2. After the mixture was cooled to −30 °C, dibenzyl(*N*,*N*-diisopropyl)phosphoramidite (1.07 mL, 3.1 mmol) was added dropwise via syringe, and the reaction was slowly warmed to room temperature, followed by stirring for 1 h. The reaction was then cooled to −40 °C, and 2 g of *m*CPBA was added. The reaction was then allowed to continue for 30 min at 0 °C, and 30 min with slow warming to room temperature. The reaction was then diluted with 200 mL of CH_2Cl_2, and washed successively 2 times with 50 mL of 10% sodium thiosulfate, 2 times with 50 mL of concentrated Sodium bicarbonate, and 2 times with 50 mL of water. The organic layer was then dried, filtered, and concentrated in vacuo. The crude material was then purified by silica gel chromatography in 3:2 toluene:acetone (R_f = 0.18) to yield compound **5** as a foamy solid (0.95 g, 55%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.42 (d, 1H, J = 5.9 Hz), 7.33−7.36 (m, 10H), 7.19 (d, 1H, J = 7.3 Hz), 5.95−5.99 (m, 2H), 5.02−5.17 (m, 6H), 4.46−4.55 (m, 2H), 4.40 (d, 1H J = 8.5 Hz), 4.30 (td, 1H, J = 13.3, 3.8 Hz), 4.04−4.22 (m, 6H), 3.82−3.99 (m, 3H), 3.51−3.61 (m, 2H), 2.07 (s, 3H), 2.06 (s, 3H), 2.04 (s, 3H), 2.02 (s, 3H), 1.97 (s, 3H), 1.83 (s, 3H), 1.43 (d, 3H, J = 7.4 Hz), 1.40 (d, 3H, J = 7.0 Hz), 0.84−0.95 (m, 4H), 0.01 (s, 9H). ^{13}C NMR (150.8 MHz, $CDCl_3$) δ 173.87, 172.54, 171.06, 170.99, 170.75, 170.48, 169.24, 135.65, 128.64, 128.59, 127.92, 127.88, 100.04, 95.85, 95.81, 76.87, 75.05, 74.27, 72.45, 72.13, 71.13, 69.59, 69.55, 69.45, 69.42, 68.12, 63.77, 61.89, 61.60, 54.53, 53.66, 53.61, 48.17, 23.21, 22.83, 20.82, 20.61, 20.57, 20.54, 18.77, 17.88, 17.19, −1.56. HRMS (FAB) for $C_{49}H_{70}N_3O_{21}PSi$: calcd [M + Na^+] 1118.390, found 1118.388.

Reaction H. In a 100 mL round-bottom flask was added compound **5** (770 mg, 0.70 mmol) in 20 mL of THF. Tetrabutylammonium fluoride solution (2.6 mL, 1 M in THF) was added, and the reaction was followed by TLC. After 45 min, THF was removed in vacuo, and the crude reaction was redissolved in 200 mL of EtOAc. After extracting the EtOAc solution 2 times with 20 mL of 1 M HCl, the organic layer was dried, filtered, and concentrated to yield a clear oil. The oil was azeotroped 3 times with 2 mL of toluene to yield the desired deprotected compound as a white solid (610 mg, 87%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.85 (d, 1H, J = 7.4 Hz), 7.80 (d, 1H, J = 3.6 Hz), 7.25−7.39 (m, 10H), 6.18 (d, 1H, J = 9.9 Hz), 5.99 (dd, 1H, J = 5.9, 2.5 Hz), 4.96−5.17 (m, 6H), 4.75 (q, 1H, J = 6.6 Hz), 4.43−4.50 (m, 2H), 4.06−4.35 (m, 5H), 3.96 (dd, 1H, J = 9.9, 8.4 Hz), 3.86−3.90 (m, 1H), 3.60−3.79 (m, 3H), 2.07 (s, 3H), 2.05 (s, 3H), 2.04 (s, 3H), 2.02 (s, 3H), 1.99 (s, 3H), 1.84 (s, 3H), 1.52 (d, 3H, J = 7.4 Hz), 1.36 (d, 3H, J = 6.6 Hz). ESI-MS for $C_{44}H_{58}N_3O_{21}P$: calcd [M + Na^+] 1018.3, found 1018.5.

Reaction I. In a 25 mL round-bottom flask was added the phosphodisaccharide from reaction H (70 mg, 0.092 mmol) in 5 mL of a 1:1 THF:CH_2Cl_2 solution. Protected tetrapeptide (139 mg, 0.14 mmol) (purchased from New England Peptide, Inc., Fitchburg, MA) was added, followed by diisopropylethylamine (48.4 μL, 0.28 mmol), HOBt (20.3 mg, 0.15 mmol), and finally PyBOP (78 mg, 0.15 mmol). The reaction was stirred for 3 h, after which solvent was removed and the crude residue was redissolved in 30 mL of CH_2Cl_2 and transferred to a separatory funnel. The organic layer was washed 3 times with 5 mL of dilute HCl, dried over magnesium sulfate, filtered, and concentrated in vacuo. The resulting oil was purified by silica gel chromatography in 9:1 EtOAc:MeOH (R_f = 0.43) to yield compound **6** as a white solid (98 mg, 61%). ^{1}H NMR: (300 MHz, CD_3OD) δ 7.28−7.35 (m, 10H), 5.76 (dd, 1H, J = 6.1, 3.1 Hz), 5.21 (t, 1H, J = 9.7 Hz), 3.45−4.67 (m, 23H), 2.98−3.07 (m, 2H), 2.16−2.32 (m, 2H), 1.57−2.06 (m, 24H), 1.23−1.47 (m, 16H), 0.78−1.01 (m, 6H), 0.00 (s, 9H), −0.01 (s, 9H), −0.03 (s, 9H). HRMS (FAB) for $C_{77}H_{123}N_8O_{29}PSi_3$: calcd [M + H^+] 1739.752, found 1739.750.

Reaction J. A solution of the phosphotriester from reaction I (252 mg, 0.14 mmol) in 10 mL of absolute ethanol was treated with 10% Pd/C (100 mg) and placed under an atmosphere of H_2. The mixture was stirred 1 h, treated with anhydrous $MgSO_4$, and filtered. The solid was rinsed with ethanol, and the combined filtrates were treated with triethylamine (100 μL), concentrated under reduced pressure, and azeotroped twice with 5 mL of dry toluene to afford 240 mg (100%) of the triethylammonium phosphate salt as a glassy foam. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.10−8.55 (m, 4H), 8.03 (d, 1H, J = 8.4 Hz), 7.80−7.89 (m, 1H), 6.88−6.95 (m, 2H), 5.30−5.35 (m, 1H), 5.20 (t, 1H, J = 10.5 Hz), 4.83 (t, 1H, J = 10.6 Hz), 4.66 (d, 1H, J = 8.4 Hz), 3.90−4.48 (m, 15H), 3.83−3.87 (m, 1H), 3.54−3.78 (m, 4H), 3.80−3.94 (m, 8H), 2.05 (s, 3H), 1.93 (s, 3H), 1.91 (s, 3H), 1.89 (s, 3H), 1.78 (s, 3H), 1.73 (s, 3H), 1.70−1.95 (m, 4H), 1.05−1.60 (m, 27H), 0.82−0.96 (m, 6H), −0.010 (s, 18H), −0.023 (s, 9H). HRMS (FAB) for $C_{63}H_{111}N_8O_{29}PSi_3$ + Na^+: calcd [M + Na^+] 1581.640, found 1581.641.

Reaction K. Under dry argon, a solution of the phosphate salt from reaction J (75 mg, 0.045 mmol) in 0.4 mL of DMF was treated with carbonyldiimidazole (38 mg, 0.23 mmol). The solution was stirred 4 h at ambient temperature, treated with anhydrous MeOH (17 μL, 0.42 mmol), and stirred an additional 0.5 h. To this solution was added a solution of undecaprenyl phosphate triethylammonium salt (38 mg, 0.036 mmol) in 1.5 mL of CH_2Cl_2. The volume of the solution was reduced to 1 mL under a stream of dry argon, and the reaction was stirred for 48 h at ambient temperature. The mixture was concentrated under reduced pressure and purified by anion exchange chromatography (Bio-Rad High Q, elution with NH_4OAc in MeOH). The appropriate fractions were concentrated under reduced pressure and lyophilized twice from water−MeOH to afford 34 mg (39%) of the pyrophosphate as a white powder. ^{1}H NMR (300 MHz, CD_3OD) δ 5.31−5.43 (m, 3H), 5.27 (t, 1H, J = 9.9 Hz), 4.99−5.15 (m, 13H), 4.68−4.90 (m, 2H), 4.41−4.61 (m, 3H), 4.23−4.39 (m, 5H), 4.01−4.19 (m, 8H), 3.63−3.90 (m, 4H), 3.04 (t, 2H, J = 6.5 Hz), 2.27 (t, 2H, J = 5.7 Hz), 1.79−2.22 (m, 60H), 1.50−1.76 (m, 42H), 1.30−1.45 (m, 12H), 0.87−1.02 (m, 6H), 0.00 (s, 18H), −0.01 (s, 9H). ESI-MS for $C_{118}H_{200}N_8O_{32}P_2Si_3$: calcd [M − $2H^{2-}$] 1192.6, found 1193.4.

Reaction L. A solution of the pyrophosphate from reaction K (21 mg, 0.0088 mmol) in 2 mL of DMF was treated with a 1 M solution of tetra(*n*-butyl)ammonium fluoride in THF (1 mL, 1.0 mmol). The solution was stirred 24 h, concentrated under reduced pressure, and purified by anion exchange chromatography (Bio-Rad High Q, elution with NH_4OAc in MeOH). The appropriate fractions were concentrated under reduced pressure and lophilized twice from water−MeOH to afford a mixture of tetra- and triacetates. The mixture was dissolved in 1 mL of CH_2Cl_2 and treated with 0.5 M NaOMe in MeOH (0.16 mL, 0.08 mmol). The solution was stirred 1 h, treated with 0.16 mL more NaOMe in MeOH, and stirred 1 h longer. The mixture was concentrated under reduced pressure and purified by anion exchange chromatography (Bio-Rad High Q, elution with NH_4OAc in MeOH). The appropriate fractions were concentrated under reduced pressure and lyophilized twice from water−MeOH to afford 6 mg (35%) of Lipid II tris-(ammonium) salt as a white powder. ^{1}H NMR (300 MHz, CD_3OD) δ 5.48−5.56 (m, 1H), 5.42 (t, 1H, J = 7.5 Hz), 5.03−5.19 (m, 13H), 4.43−4.56 (m, 3H), 4.16−4.42 (m, 7H), 4.04−4.12 (m, 1H), 3.79−3.94 (m, 3H), 3.59−3.75 (m, 3H), 3.48 (dd, 1H, J = 10.2, 8.1 Hz), 2.93 (t, 2H, J = 6.0 Hz), 1.91−2.38 (m, 48H), 1.48−1.89 (m, 44H), 1.33−1.45 (m, 12H). HRMS (FAB) for $C_{94}H_{156}N_8O_{26}P_2$: calcd [M − $2H^{2-}$] 936.5225, found 936.5216.

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