Synthetic Methods

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Enzymatic Synthesis of Lipid II and Analogues**

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Dedicated to Professor George M. Whitesides on the occasion of his 75th birthday

Abstract: *The emergence of antibiotic resistance has prompted active research in the development of antibiotics with new modes of action. Among all essential bacterial proteins, transglycosylase polymerizes lipid II into peptidoglycan and is one of the most favorable targets because of its vital role in peptidoglycan synthesis. Described in this study is a practical enzymatic method for the synthesis of lipid II, coupled with cofactor regeneration, to give the product in a 50–70% yield. This development depends on two key steps: the overexpression of MraY for the synthesis of lipid I and the use of undecaprenol kinase for the preparation of polyprenol phosphates. This method was further applied to the synthesis of lipid II analogues. It was found that MraY and undecaprenol kinase can accept a wide range of lipids containing various lengths and configurations. The activity of lipid II analogues for bacterial transglycolase was also evaluated.*

The increasing problem of antibiotic resistance has stimulated interest in the development of antibiotics with new mechanisms of action. In our effort toward this goal, we are targeting the synthesis of the universal polysaccharide backbone present within bacterial cell walls. Specifically, we are targeting the polymerization of lipid II, which is catalyzed by the transglycosylase (TGase) domain of the penicillin-binding protein (PBP), and the subsequent crosslinking of the peptide moiety, a step catalyzed by the transpeptidase domain of PBP.[1] The inhibitors that target the PBP of the peptidoglycan assembly can act through two modes of action: inhibiting the enzymatic activity, such as moenomycin, and interfering with substrate binding, such as vancomycin (Figure 1).[2]

Although moenomycin is an effective inhibitor of TGase and has been used in animals, it has not been developed further as antibiotics for use in humans. The development of antibiotics that target TGase requires sufficient quantities of the substrate lipid II for extensive studies on enzyme inhibition. The isolation of lipid II from bacteria is impractical since lipid II is estimated to be less than 2000 molecules per bacterial cell.[3] Chemical synthesis of lipid II has been reported. These methods involve selective linkage of two saccharides and coupling of lipid phosphate with sugar phosphate from MurNAc or from benzyl *N*-acetyl-4,6-benzylidinemuramic acid.[4] Walker and co-workers further developed a chemoenzymatic method in which lipid I was chemically synthesized and converted into lipid II by using the enzyme MurG to escape the chemical linkage of two saccharides.[5] Alternatively, in vitro enzymatic synthesis of lipid II has been proposed by following the bacterial cell wall synthesis pathway (Figure 1). Most groups use membrane extracts from *Micrococcus flavus* as an enzyme source for the small-scale synthesis of lipid II from UDP-MurNAc pentapeptide, undecaprenyl phosphate, and UDP-GlcNAc.[6]

In this study, we report a practical synthesis of lipid II from UDP-MurNAc pentapeptide and undecaprenol by using the enzymes MraY, MurG, and undecaprenol kinase (UK). UDP-MurNAc pentapeptide was prepared using the cytosolic enzymes MurA–F (Figure 2A).[7] To avoid the use of expensive UDP-GlcNAc, *N*-acetylhexosamine kinase (NahK) and GlcNAc-1-phosphate uridyl transferase (GlmU) were incubated with GlcNAc (**1**) in the presence of ATP and UTP to prepare UDP-GlcNAc (**2**).[8] The reaction mixture was then directly incubated with MurA and MurB (see Figure S1 in the Supporting Information) to produce UDP-MurNAc (**3**) in the presence of glucose dehydrogenase for regeneration of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH).[9] Subsequently, the mixture was incubated with MurC–F and the respective amino acids in one pot overnight. In addition, recombinant D-ala:D-ala ligase (Ddl), used to produce D-ala-D-ala from D-alanine, and a nucleotide regeneration system consisting of pyruvate kinase and PEP, were included in the reaction mixture.[10] After an overnight reaction at room temperature, the reaction mixture was subjected to an adsorption chromatography to obtain UDP-MurNAc pentapeptide (**4**) in a final yield of 60–80%.

After the production of **4**, the aim was to use MraY and MurG to catalyze the transformation of **4** into lipid I (**5**) and then to lipid II (**6**; Figure 2B). MraY, which catalyzes the

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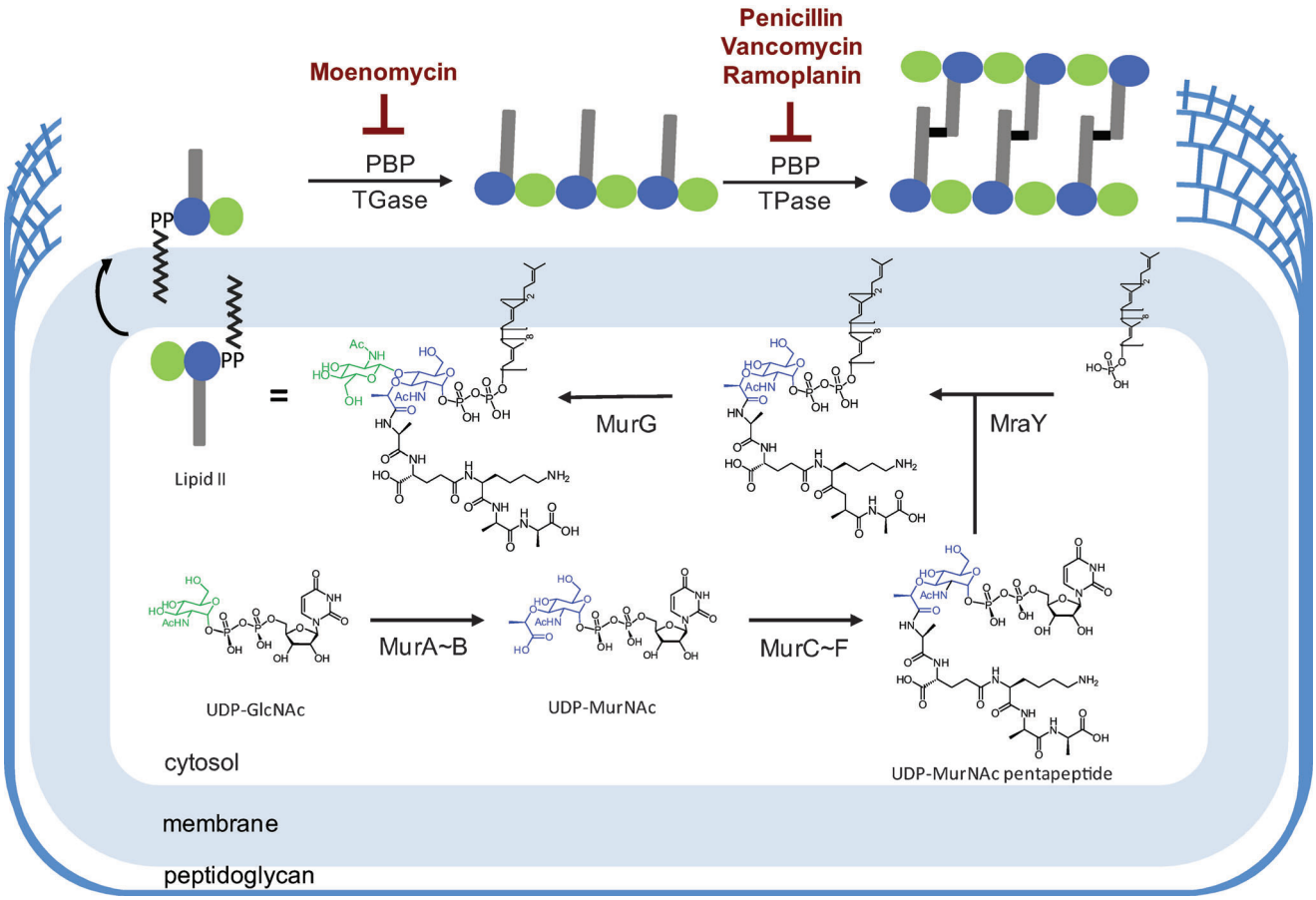


Figure 1. Biosynthesis of the bacterial cell wall.

translocation of MurNAc pentapeptide onto the carrier lipid present in the cytoplasmic membrane, is a protein with ten transmembrane domains.[11] Prior to the work by Bouhss et al. (2004), MraY could not be successfully purified to appear as a single band on SDS-PAGE.[12] Our initial attempt to purify MraY with a $(His)_6$ tag was not successful. Therefore, we tested the expression of MraY when fused to another protein, such as thioredoxin or maltose-binding protein (MBP), or with various tags, including T7, Strep, and Mistic tags on either the N or C terminus. The fusion of MraY to MBP resulted in a higher expression of the enzyme. Consistent with our finding, the group of Lee independently used MBP-fused MraY to determine the first crystal structure of MraY.[11b] Our investigation further revealed that a periplasmic signal could slow down protein degradation, thus resulting in a higher yield of full-length MraY. Ultimately, we were able to prepare MraY at the level of 1 $mg\,L^{-1}$ of culture (see Figure S1).

Another critical factor for the synthesis of lipid II is the preparation of undecaprenyl phosphates. The chemical methods used to convert lipid alcohol into lipid phosphates often produce byproducts.[13] Therefore, an enzymatic process that phosphorylates isoprenol was considered (Figure 2B). Diacylglycerol kinase A from *Streptococcus mutans* (DGKA), recently observed to be an undecaprenol kinase (UK),[14] was prepared and tested. It was found that in a neutral environment and in the presence of magnesium ions, the enzyme activity was optimal. However, only ATP, and not other nucleotides, could be used for the reaction (see Figure S2A–C). The phosphorylation of undecaprenol required a large amount of detergents for lipid solubilization. Therefore, we measured the UK activities in buffers containing undecaprenol and various detergents. As was expected, the measured UK activities varied significantly in different conditions (see Figure S2D), and lauryldimethylamine-oxide (LDAO) was finally chosen to be included in the reaction to transform undecaprenol into undecaprenyl phosphate at a rate of (5.1 ± 0.2) $\mu mole\,min^{-1}\,mg^{-1}$. Surprisingly, when the reaction contained only detergents, UK activities were observed in the presence of Tween 20, Triton X-100, or NP-40, and it was later confirmed that these detergents are substrates for UK (see Figure S3).

The undecaprenyl phosphate produced was incubated with MraY, MurG, and UDP-MurNAc pentapeptide to form lipid II. Regeneration of UDP-GlcNAc was performed by using pyruvate kinase, GlmU, NahK, and inorganic pyrophosphatase (IPP) as previously described.[8,10] The kinetic parameters of individual enzymes were determined (see Table S1). The reaction was halted by addition of the organic solvent butanol in the presence of acid[5a,15] to separate lipid II from other cytosolic components. In fact, the process can be simplified by incubating three recombinant enzymes, UK, MraY ,and MurG, with UDP-MurNAc pentapeptide, unde-

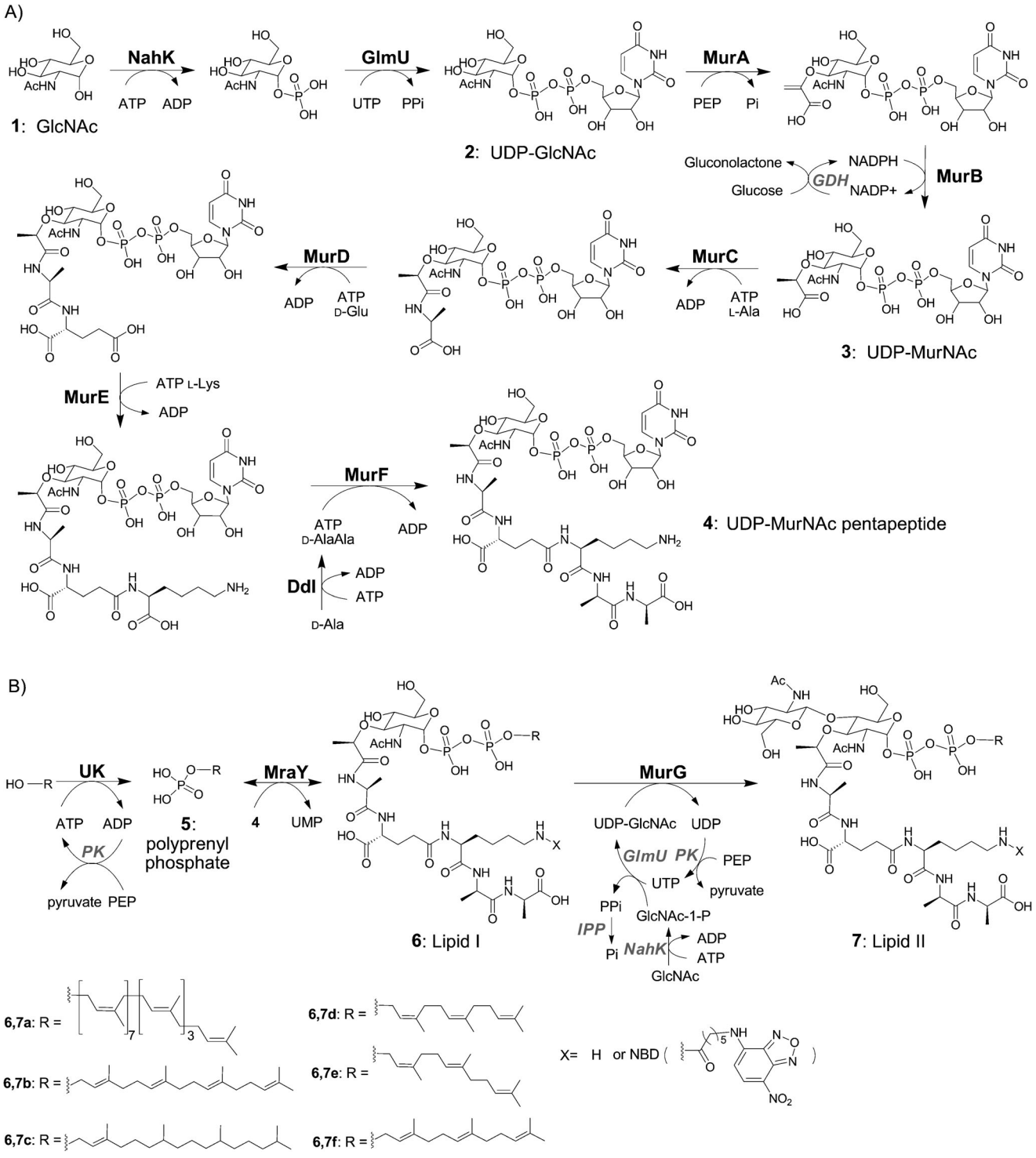


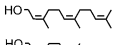
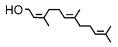
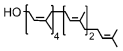
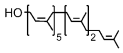
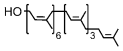
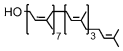
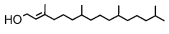
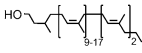
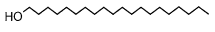
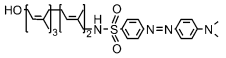
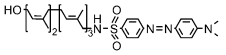
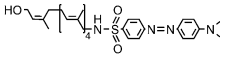
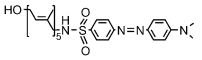
Figure 2. The enzymatic synthesis of lipid II. A) Synthesis of UDP-MurNAc pentapeptide from GlcNAc in one pot. B) Synthesis of lipid II from undecaprenol. The enzymes used for the synthesis are noted in bold and the enzymes used for regeneration are noted in italic.

caprenol, and other components to synthesize lipid II in a one-pot reaction with a 50–70% overall yield.

We further applied the same method to synthesize lipid II analogues containing various lipid moieties. As the first step, the UK activity was tested against a series of lipids (Table 1), and the results showed that this enzyme has broad substrate specificities. Isoprenols, in general, are good substrates. In addition, short isoprenols such as geraniol, nerol, farnesol, and geranylgeraniol are better than undecaprenol as substrates for UK. Most of the polyprenols tested were phosphorylated in a few hours (see Figure S4 A).

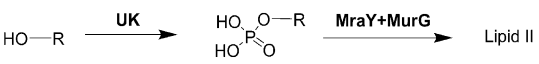
It was surprising to note that precipitation occurred during the reaction with short and all-*trans* polyprenyl phosphates (see Figure S4 B). The precipitate was confirmed

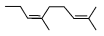
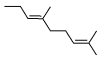
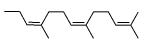
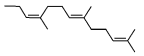
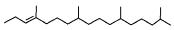
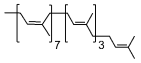
Table 1: The UK activity of different lipid substrates.

Substrate	Structure	Activity[a] [μmole min^{-1} mg^{-1}]	Mg^{2+}[b]
C10OH (*Z*,ω)		8.7 ± 0.3	+
C10OH (*E*,ω)		6.2 ± 0.1	+
C15OH (*Z*,*Z*,ω)		21.2 ± 0.4	+
C15OH (*Z*,*E*,ω)		5.4 ± 0.4	+
C15OH (*E*,*E*,ω)		11.5 ± 0.2	+
C20OH (*E*,*E*,*E*,ω)		9.3 ± 1.0	+
Heptaprenol C35OH (Z_4,E_2,ω)		8.8 ± 0.2	−
Octaprenol C40OH (Z_5,E_2,ω)		3.8 ± 0.1	−
Solanesol C45OH (E_8,ω)		4.9 ± 0.3	+
Decaprenol C50OH (Z_6,E_3,ω)		3.8 ± 0.1	−
Undecaprenol C55OH (Z_7,E_3,ω)		5.1 ± 0.2	−
Phytol (isomer mixture)		5.6 ± 0.8	+
Dolichol		4.5 ± 0.2	−
Undecanol (C11OH-alkyl)		2.1 ± 0.1	+
Hexadecanol (C16OH-alkyl)		1.9 ± 0.1	+
Eicosanol (C20OH-alkyl)		1.3 ± 0.1	
Dabsyl C27OH (Z_3,E_2)		4.1 ± 0.1	+/−
Dabsyl C27OH (Z_2,E_3)		4.0 ± 0.4	+/−
Dabsyl C27OH (Z,E_4)		5.9 ± 0.0	+
Dabsyl C27OH (E_5)		3.3 ± 0.1	+

[a] The activity was determined as described in the methods within the Supporting Information. All phosphorylated products were confirmed by ESI-mass spectrometry. [b] Precipitation with Mg^{2+}. +/−: part of phosphorylated products precipitated.

Table 2: The overall yields for the synthesis of polyprenyl phosphate and lipid II.



R=	UK[a] Substrate	Yield [%]	MraY + MurG[b] Substrate	Yield [%]
	C10OH(*Z*,ω)	53	C10P(*Z*,ω)	< 5
	C10OH(*E*,ω)	62	C10P(*E*,ω)	< 5
	C15OH(*Z*,*Z*,ω)	76	C15P(*Z*,*Z*,ω)	65
	C15OH(*Z*,*E*,ω)	84	C15P(*Z*,*E*,ω)	30
	C15OH(*E*,*E*,ω)	89	C15P(*E*,*E*,ω)	20
	C20OH(*E*,*E*,*E*,ω)	87	C20P(*E*,*E*,*E*,ω)	58
	Phytol (isomer mixture)	82	Phytol-P (isomer mixture)	52
	C55OH (Undecaprenol)	62	C55P	55–70

[a] The yields of polyprenyl phosphate synthesis using undecaprenol kinase and the specified polyprenol. [b] The yields for the synthesis of lipid II analogues from NBD-labeled UDP-MurNAc pentapeptide and polyprenyl phosphate.

to contain isoprenyl phosphate and Mg^{2+}. The precipitation thus provided a convenient alternative for the isolation of polyprenyl phosphates from unreacted components, by centrifugation followed by a brief wash and organic solvent extraction. For nonprecipitated isoprenyl phosphate, the isolation needs to be performed with extraction followed by HPLC purification. The yield of isoprenyl phosphate using UK ranged from 50 to 90% (Table 2). Interestingly, the structure of undecaprenyl phosphate was predicted to contain a central coiled portion, flanked by one *cis* and one *trans* isoprene arm.[16] The precipitation with Mg^{2+} for the short and all-*trans* polyprenyl phosphates indicated that such structures may provide stability in detergent micelles and membranes.

Moreover, dimethylamino-azobenzenesulfonyl (dabsyl)-conjugated isoprenols[4e] and dolichol were also tested as substrates for UK (Table 1). The rates for the phosphorylation of dabsyl-C27 and dolichol were similar to unlabeled isoprenols. Dolichyl phosphate is an essential glycosyl-carrier lipid in the assembly of N-linked glycoproteins, glycosylphosphatidylinositol anchors, and in the C and O mannosylation of proteins in eukaryotic cells.[17] Unlike dolichol kinase in eukaryotes, which catalyzes CTP-mediated phosphorylation,[18] bacterial UK uses ATP as the phosphate source, and the method described here provides an alternative for the preparation of dolichyl phosphate.

The phosphorylated isoprenols were then assembled with UDP-MurNAc pentapeptide in the presence of MraY and MurG to form lipid II analogues. It was observed that MraY and MurG accepted all polyprenyl phosphates tested in this study, but the reaction rate for geraniol and nerol was very low (Table 2), similar to previously reported results.[5a] After purification, the overall yield of each product was in the range of 20–65%. The activities of lipid II analogues as bacterial TGase substrates were evaluated by using *Escherichia coli* PBP1b and *Clostridium difficile* PBP (Figure 3). After an overnight incubation, the results surprisingly showed that PBPs can accept the lipid II analogue containing isoprene units in all *trans* forms (C20 (*E*,*E*,*E*,ω); lipid II-C20), and the analogue with partially saturated phytol (Figure 3B). *E. coli* PBP1b showed lower activity in polymerizing a lipid II analogue containing phytol. It was reported that an *E. coli* membrane fraction would not act on lipid II in the unnatural *trans* configuration.[6,19] The discrepancy may be due to the longer incubation used in this particular study. In contrast, *C. difficile PBP* was observed to completely convert lipid II

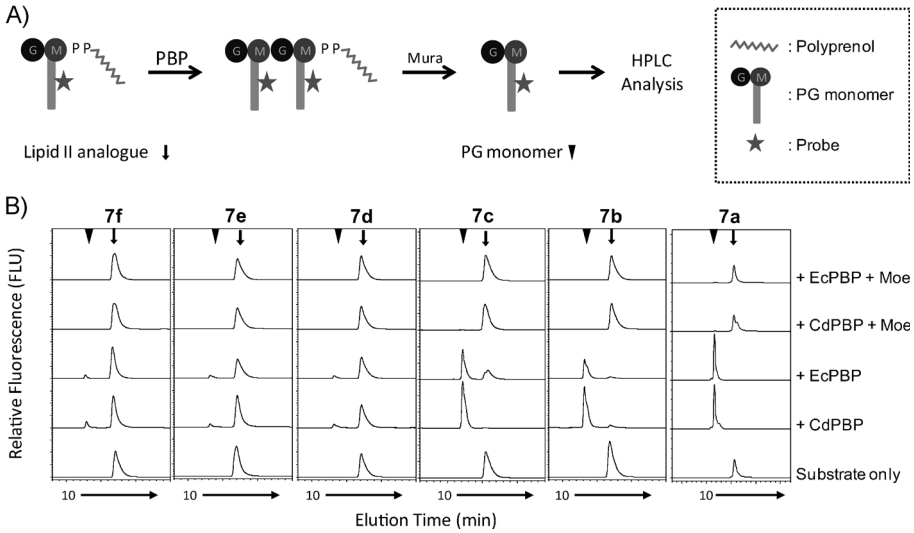


Figure 3. Transglycosylase assay of lipid II and analogues. A) Methodology of the TGase activity assay.[5f] B) Transglycosylation of lipid II analogues containing various lipid moieties (noted on the top of the chromatographs) by *E. coli* PBP1b (EcPBP) and *C. difficile* PBP (CdPBP). The reactions were conducted for overnight under the reaction conditions described in the Supporting Information. Shown are chromatograms of the HPLC analysis. Mura: muramidase; Moe: Moenomycin.

analogues containing genranylgeranyl lipid and phytol. We further compared the relative rates of lipid II and lipid II-C20 for *C. difficile* PBP. Both substrates can be accepted by *C. difficile* PBP at a concentration as low as 5 nM. In general, lipid II-C20 reacts slightly slower than lipid II (see Figure S5). A time-dependent study further showed lipid II reacts faster than lipid II-C20 (see Figure S6). It is possible that *C. difficile* PBP has broader substrate specificities and higher activities. Nonetheless, geranylgeraniol and phytol can be obtained commercially and may provide an alternative to preparing lipid II substrates.

In summary, an enzymatic method for the synthesis of lipid II, using purified enzymes MraY, MurG, and UK, coupled with nucleotide regeneration systems, was established. The system can be applied to the synthesis of lipid II analogues with various lengths of lipid tails, including dolichyl phosphate. It was also found that UK accepts a wide range of lipids and allows convenient preparation of polyprenyl phosphate with high efficiency. The identification of a lipid II analogue containing geranylgeranyl lipid as a possible substrate for bacterial transglycosylase is interesting because it provides a simpler alternative to TGase substrate.

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