Better Substrates for Bacterial Transglycosylases

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Resistance to antibiotics has been increasing rapidly in recent years and now represents a threat to public health. The need for new antibiotics to treat resistant infections has led to a resurgence of interest in the structure and function of essential bacterial enzymes. The enzymes that synthesize the peptidoglycan layers surrounding bacterial cell membranes have received special attention because many known antibiotics function by blocking peptidoglycan synthesis.[1] Among these enzymes, the bacterial transglycosylases (TGases) represent some of the most promising targets.[2,3] TGases are located on the external surface of the bacterial membrane where they polymerize Lipid II (Figure 1), a disaccharide anchored to the membrane by a 55 carbon undecaprenyl chain. Although the TGases were first identified decades ago, their structures and mechanisms are not well understood.[3a,4]

Some of the difficulties in studying TGases are related to problems obtaining and handling Lipid II. Because the 55 carbon chain aggregates, assays utilizing Lipid II, which can be isolated only in small quantities from bacterial membranes, must include organic solvents, detergents, and other additives.[3a,5] Results can be variable, and it is difficult to determine whether problems are due to the enzymes or to the substrate. Better substrates would facilitate the study of TGases. To identify better TGase substrates, we have synthesized natural Lipid II as well as a set of analogues containing different lipid chains. These compounds have been tested for their ability to function as TGase substrates. The results show that bacterial TGases have clear preferences with regard to the structure of the lipid chain, but they do not require the 55 carbon undecaprenyl moiety. In fact, we have identified a compound with a shorter lipid chain that is a much better TGase substrate than natural Lipid II.

We have previously reported the synthesis of a Lipid I analogue (Scheme 1, **7**) containing a 10 carbon citronellyl chain in place of the undecaprenyl chain.[6−8] This Lipid I analogue allowed us to purify active MurG, the enzyme that converts Lipid I to Lipid II in the biosynthetic pathway to peptidoglycan (Figure 1).[7,9] Using purified MurG, we converted **7** to the corresponding Lipid II analogue **13**.[10] Preliminary studies revealed, however, that TGases do not accept **13**, making it necessary to examine other Lipid II derivatives, including those containing double bonds allylic to the pyrophosphate. Because the method used to activate citronellyl phosphate for coupling to sugar phosphate **6** in the synthesis of **7**[6,7] caused extensive decomposition of the less stable allylic phosphates, we used the 1,1′-carbonyldiimidazole (CDI) method[11]

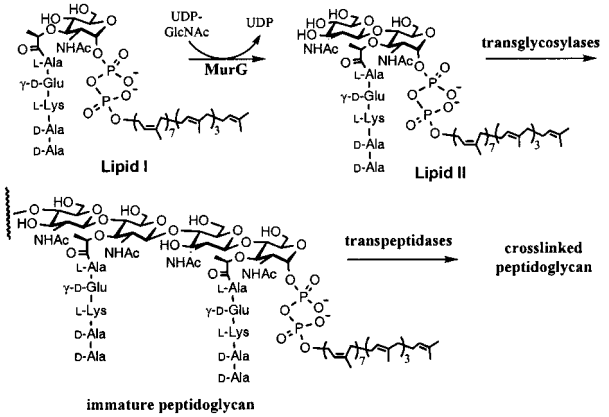
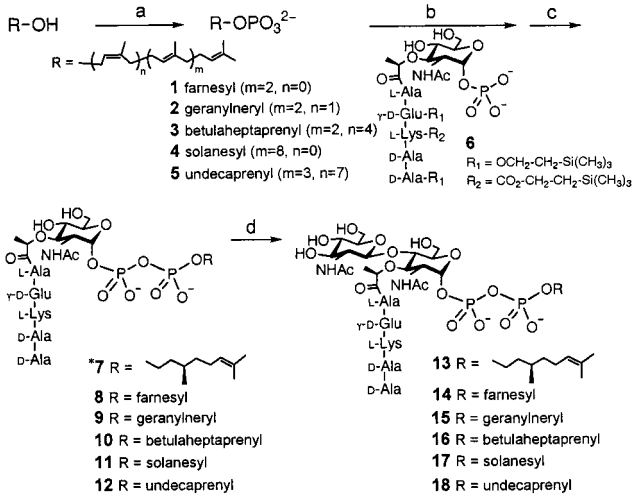


Figure 1. Later steps in peptidoglycan biosynthesis.

Scheme 1. Synthesis of Lipid II and Analogues



(a) (i) Bis(2-cyanoethoxyl)(diisopropylamino)phosphine, CH_2Cl_2, tetrazole, 0 °C to room temperature, 1 h; (ii) $NaIO_4$, Py; (iii) NaOMe, MeOH. (b) 1,1′-carbonyl diimidazole, THF, room temperature, 0.5 h. Then **6**, DMSO−THF, room temperature, 24 h. (c) TBAF, DMF, room temperature, 4 h. Yield over three steps: **8** = 28%, **9** = 28%, **10** = 23%, **11** = 15%, **12** = 11%. (d) MurG, 2 equiv of UDP-(^{14}C)-GlcNAc, 288 mCi/mmol, 50 mM HEPES-5 mM $MgCl_2$, pH 7.9, room temperature, 1 h. All glucosamine carbons are ^{14}C labeled. ***7** was prepared by the route outlined previously.[6,7]

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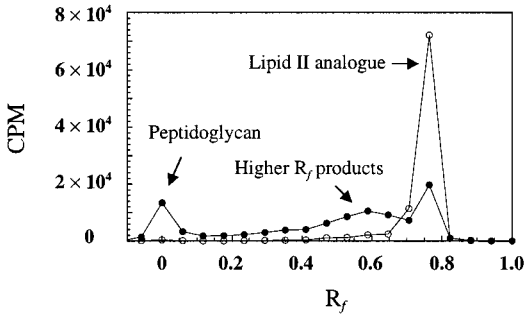

Figure 2. Transglycosylase assay results for **16** in the presence of detergent. 400 pmol of radiolabeled **16** in 5 mL of CH_3OH was added to *E. coli* membranes[15] (50 mg total protein) in 30 mL of reaction buffer containing 50 mM Tris (pH 8.0), 42 mM $Mg(OAc)_2$, 208 mM KCl, and 0.1% sodium deoxycholate (in the presence or absence of moenomycin). After 2 h, 35 mL of solubilization buffer (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.2% Triton X-100) was added and half of the reaction mixture was spotted on Whatman 3MM chromatography paper (1 × 20 cm). The strips were developed in isobutyric acid/1 N NH_4OH (5:3 v/v). After the solvent front migrated to the top, each strip was cut into 1 cm squares and counted for radioactivity. Results were plotted as cpm versus R_f (the relative mobility to the solvent front). ○ and ● represent the reactions with and without the addition of moenomycin (a known transglycosylase inhibitor), respectively.

to make natural Lipid I (**12**) and derivatives **8**−**11**.[12] These compounds vary with respect to the structure and to the length of the lipid chain; both the natural *cis* and unnatural *trans* geometries at the allylic position of the lipid are represented.

Compounds **7**−**12** (Scheme 1) were converted to the corresponding radiolabeled Lipid II analogues **13**−**18** by reaction with UDP-(^{14}C)-GlcNAc with purified *E. coli* MurG as the catalyst.[13] MurG accepted all the Lipid I analogues, allowing us to explore a range of different Lipid II derivatives for testing simultaneously. Nevertheless, there were significant differences in the reactions. For example, compound **12**, although it is the natural glycosyl acceptor for MurG, reacted slowly and gave the lowest amount of product **18**—apparently because it aggregates in a manner that precludes reaction in solution. Following reaction, the radiolabeled Lipid II analogues were isolated from excess UDP-(^{14}C)-GlcNAc by C18 chromatography.[14] Each analogue was incubated with *E. coli* membranes[15] in transglycosylase reaction buffer for 2 h and the reaction mixtures were then subjected to paper chromatography to quantitate peptidoglycan formation (Figure 2).

The results, summarized in Table 1, merit a few comments. First, substrate recognition does not show a simple dependence on lipid chain length. Compounds **13**−**15**, which have much shorter lipid chains than the natural C55 substrate (**18**), do not react, and neither does compound **17**, which has a 45 carbon chain; however, compound **16**, with a 35 carbon chain, is an excellent substrate. The results also indicate that the TGases have distinct preferences with regard to the double bond geometry of some of the isoprene units. For example, compounds **14** and **17** both contain the unnatural *trans* double bond geometry at the allylic position and they both failed to function as substrates for the TGases. Nevertheless, it is not sufficient simply to have a lipid chain containing a *cis*-allylic double bond because compound **15**, with only one *cis* double bond in a 20 carbon chain, does not yield peptidoglycan whereas **16**, which contains four successive *cisoid* isoprene units in a 35 carbon chain, is the best transglycosylase substrate under all conditions examined.[16]

Table 1. Percentage of Peptidoglycan (cpm baseline material/total cpm) Formed from Radiolabeled Compounds **13**−**18** and *E. coli* Membranes in Assay Buffer Containing the Indicated Additives[a]

	% peptidoglycan (% S.M.)		
	15% CH_3OH/ 0.1% deoxycholate	15% CH_3OH	5% CH_3OH
13	<1	<1	n.d.
14	<1	<1	n.d.
15	<1	<1	n.d.
16	18.1 (34.0)	20.8 (55.2)	18.9 (61.0)
17	<1	<1	n.d.
18	12.6	2.4	1.7

[a] Compound **16** also yields moenomycin-sensitive products that do not run at the baseline (see Figure 2). Therefore, the % starting material remaining (% S.M.) after reaction is shown for **16** because it provides a more accurate measure of the extent of reaction. More nonbaseline products form in the presence of detergent, suggesting that detergent affects the polymerization reaction.[17]

For the study of bacterial TGases, the most significant result here is the identification of a substrate, **16**, that is much better than natural Lipid II for monitoring TGase activity. Natural Lipid II (**18**) reacts poorly unless detergent is added to the reaction. Even with detergent, it does not react to the same extent as **16**, which forms similar amounts of peptidoglycan under a range of conditions (Table 1). Compound **16** thus has all the structural features required for recognition but with more desirable physical properties than natural Lipid II. Therefore, it can be used to monitor TGase activity in the absence of detergent. This may be an advantage because detergent alters the products that form with both **16** and **18**, giving significant amounts of moenomycin-sensitive products that do not run at the baseline (see Figure 2 and Table 1, % S.M.).[17]

The work described provides an efficient chemoenzymatic approach to making Lipid II and analogues for studying bacterial TGases. A comparison of natural Lipid II and several analogues as substrates for TGases provides some insight into structural features required for recognition and also demonstrates that the long chain on natural Lipid II is a disadvantage because it facilitates aggregation. Appropriate shorter chain analogues such as **16** are better than the natural substrate for studying TGases because they react more effectively, which enhances sensitivity, and because they do not require specialized conditions involving detergents to function, which permits flexibility in formatting assays. With better substrates now available through synthesis, we can begin to develop reliable TGase assays using purified enzymes.

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Supporting Information Available: Experimental details, ^{1}H NMR spectra for **7**−**13**, ^{31}P NMR spectra for **9**−**12**, and mass spectra for **7**−**18** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) Compounds **7**−**12** were characterized by NMR and electrospray MS. Lipid II analogues **13**−**18** were prepared in nonradioactive form, subjected to C18 chromatography (Extract-Clean Solid-Phase Extraction Column, Alltech), and characterized by electrospray MS. Spectral data are available in the Supporting Information.

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