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Synthesis and Evaluation of a New Fluorescent Transglycosylase Substrate: Lipid II-Based Molecule Possessing a Dansyl-C20 Polyprenyl Moiety

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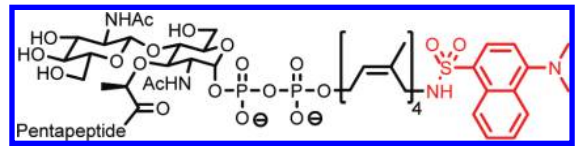
ABSTRACT



The preparation of a novel fluorescent lipid II-based substrate for transglycosylases (TGases) is described. This substrate has characteristic structural features including a shorter lipid chain, a fluorophore tag at the end of the lipid chain rather than on the peptide chain, and no labeling with a radioactive atom. This fluorescent substrate is readily utilized in TGase activity assays to characterize TGases and also to evaluate the activities of TGase inhibitors.

Due to serious antibiotic drug resistance, such as VRE (vancomycin resistant enterococcus) and MRSA (methicillin-resistant *Staphylococcus aureus*), it is urgent for scientists to search for new antimicrobial targets for the development of effective antibiotics.[1] In bacterial cell-wall biosynthesis, transglycosylases (TGases), one class of glycosyl-transferases, catalyze the transfer of the sugar moiety from the activated polymeric peptidoglycan (a glycosyl donor) to the specific hydroxyl group (4-OH) of Lipid II (**1**, a glycosyl acceptor) with concomitant release of an undecaprenyl pyrophosphate moiety (Figure 1).[2] These bacterial enzymes are attractive antibiotic targets since their functions are essential, their structures are conserved, and they are located on the external surface of bacterial membranes to allow easy

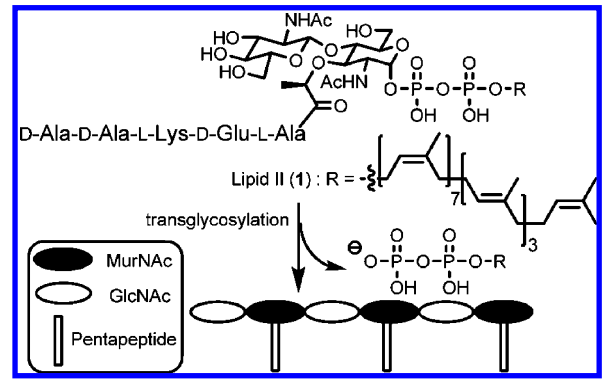


Figure 1. Lipid II as the substrate in transglycosylation for bacterial peptidoglycan formation.

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access by inhibitors. Furthermore, the polysaccharide backbone of peptidoglycan remains conserved in methicillin-resistant strains; thus, antibiotics targeting the transglycosylation (TG) step may therefore be less liable to resistance.[3] However, the study of transglycosylation for drug discovery has been hampered by the difficulty in acquirement and modification of the TGase substrate **1**. Isolation of **1** from bacterial sources is extremely difficult and tedious due to its low natural abundance and its inherent structural complexity.[4] Fortunately, synthetic approaches toward **1** and its analogues via chemical or chemo-enzymatic methods have shed light on this study.[5−7] For example, the Lipid II analogue **2** bearing a fluorophore of the dansyl group at the lysine residue of the pentapeptide chain has been prepared (Figure 2).[8] A chemo-enzymatic approach has culminated

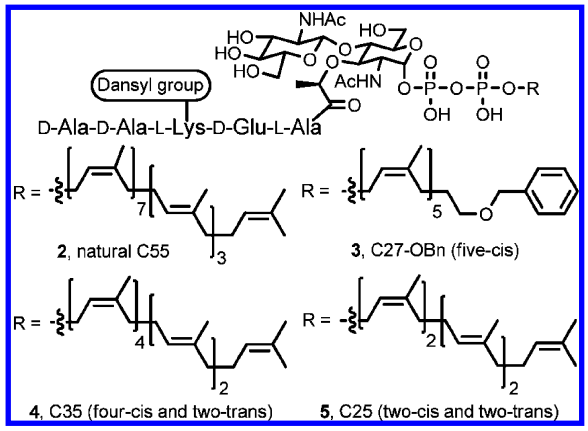

Figure 2. Structures of Lipid II analogues **2**−**5** bearing a fluorescent tag at the ε-NH_2 site of lysine.

in the syntheses of radioactive Lipid II and several analogues with all glucosamine carbons having ^{14}C-labeling.[9] The assay systems for evaluation of the TGase activity have thus been established with the assistance of the fluorescent or radioactive Lipid II substrates.[8,9]

Walker and co-workers have shown, in their assay using radioactive substrate, that a Lipid II analogue containing the betulaheptaprenyl lipid chain (C35) with defined double bond configurations is a better TGase substrate than the natural Lipid II with the C55 lipid chain.[9] The transglycosylation products usually contain mixtures of immature peptidoglycans with varying degrees of polymerization that are not easily quantified. The formed immature peptidoglycan can be subjected to enzymatic cleavage by *N*-acetylmuramidase to release the GlcNAc-MurNAc-pentapeptide molecules, the truncated Lipid II without the moiety of undecaprenyl diphosphate.[8,10]

During the course of our research on the development of new antibiotics that target TGase in bacteria,[11,12] we explored an efficient method for the preparation of polyprenyl alcohols with varying chain lengths and double bond configurations via solution- and solid-phase organic synthesis.[13] We have also prepared the fluorescent Lipid II (**2**)[8] and its analogues **3**−**5** carrying a dansyl tag at the terminal ε-NH_2 site of lysine (see Supporting Information). In accord with our expectations, the dansyl-labeled Lipid II (**2**) and analogue **4**, bearing a betulaheptaprenyl chain, are active substrates for TGase. Compound **3** bearing a benzene ring at the end of the lipid chain is still an active substrate toward transglycosylation, whereas compound **5** with a pentaprenyl chain is inert to TGase. Inspired by this result, we proposed that compound **7** would be a possible substrate that could further improve the TGase assay (Figure 3). Compound **7** incorporates a

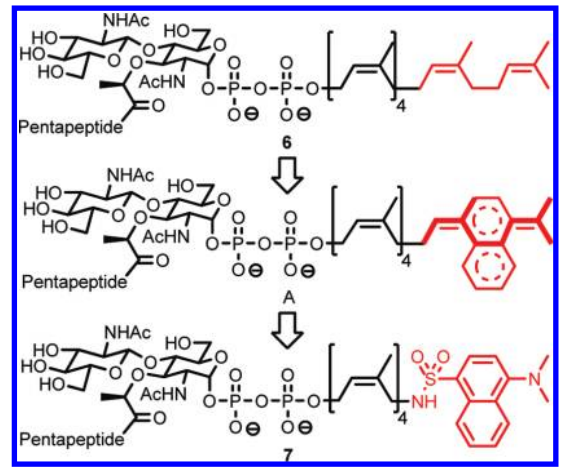

Figure 3. A schematic diagram for design of the fluorescent TGase substrate **7** by mimicking a truncated Lipid II analogue **6** via an imaginary transformation **A**.

dansyl group into the lipid chain for facile detection of the progress of transglycosylation. The dansyl group can be

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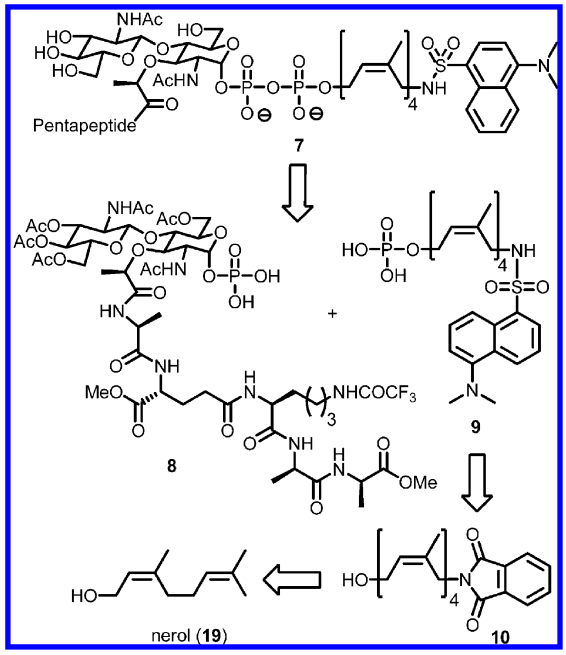
visualized as a mimic of the diprenyl unit, i.e. the lipid moiety in **7** can be considered equivalent to the hexaprenyl chain in **6**. Though the biological function of the polyprenyl chain in Lipid II is not fully understood, it is generally thought to promote penetration into the hydrophobic bacterial membrane.[10] Lipid II analogues having a proper fluorophore on the lipid chain, instead of the peptide chain,[8] might not interfere with substrate−enzyme recognition. In comparison, the conventional use of a Lipid II analogue with a dansyl group on the peptide chain, e.g., **2**, in TG assay could result in many fluorescent elongation products that could complicate the HPLC analysis. On the other hand, the fluorescent moiety of lipid diphosphate, e.g., in **7**, would not be incorporated into the elongation products, so that the HPLC analysis would be much simplified.

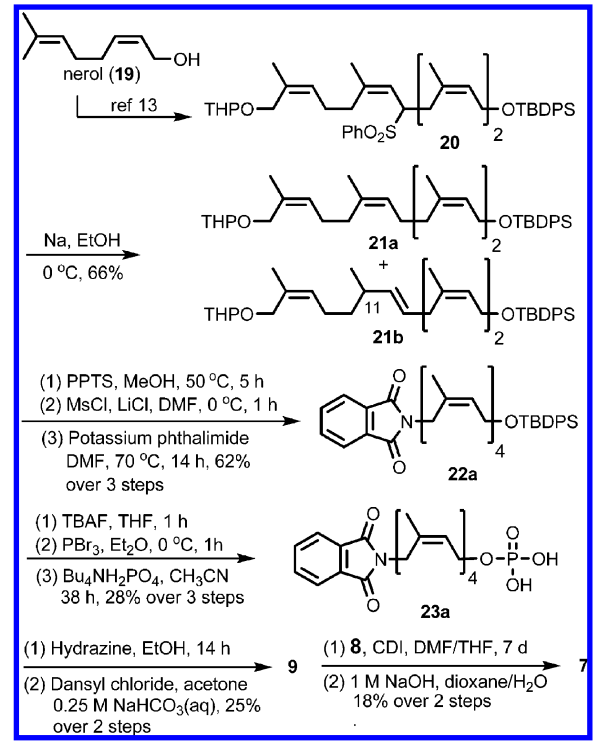
We herein describe the synthesis of compound **7** and its use in a functional assay for the TGase represented by a full-length penicillin-binding protein (PBP). To circumvent the problem in handling the acid labile α-glycosyl diphosphate group,[14] the diphosphate moiety in **7** was constructed at the final stage by conjugation of a disaccharyl pentapeptide **8** with the dansyl-lipid phosphate **9** as illustrated in the retrosynthetic route (Scheme 1).

Scheme 1. Retrosynthetic Analysis of **7** Bearing a Dansyl Label in the Lipid Chain



Compound **8** was prepared by using the reported procedure[5] with slight modifications, and syntheses of the intermediate compounds **11**−**18** are described in the Supporting Information. For the preparation of **9**, nerol (**19**) was converted to a key intermediate **20** according to our previously described procedures.[13] However, an unexpected problem was encountered in the desulfonation of **20**. After several unsuccessful attempts at using $LiEt_3BH/PdCl_2(dppp)$,[15] SmI_2/HMPA,[16] Na/naphthalene, Li/naphthalene,[17] or $Mg/HgCl_2$[18] as the reducing agents, we finally carried out the desulfonation reaction with Na/EtOH at 0 °C to give a 66% yield of an inseparable mixture containing **21a** and isomer **21b** in a ratio of 7:3 as shown by the ^1H NMR analysis. Isomer **21b**, presumably from double bond migration of the allylic radical intermediate,[13] was characterized by the C_{11}-methyl group showing as a doublet at δ 0.94 ppm. The isomeric mixture was carried through the sequence of *O*-THP deprotection, potassium phthalimide substitution, and phosphorylation to give compound **23** (Scheme 2) The

Scheme 2. Synthesis of the Probe Molecule **7**[a]



[a] The structures of isomers **22b** and **23b** are omitted for clarity.

phthaloyl group in **23** was removed by hydrazine, and a subsequent reaction with dansyl chloride under basic conditions delivered **9**. The coupling reaction of **8** and **9** in the presence of 1,1′-carbonyldiimidazole (CDI),[5] followed by global deprotection under basic conditions and purification by reverse-phase HPLC, afforded the desired probe molecule **7**.

The feasibility of **7** for a TGase substrate was demonstrated by HPLC-based TGase assays. As we had anticipated, **7**

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was recognized by both TGases from *C. difficile* and *E. coli*. The transglycosylation reaction catalyzed by *C. difficile* TGase (3 μig, 0.3 mg/mL) proceeded more efficiently, consuming ~90% of **7** (7.5×10^{-4} μmol, 75 μM) in 4 h at 25 °C (Figure 4).

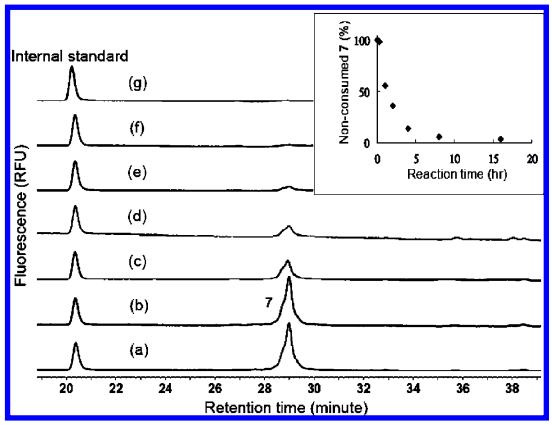

Figure 4. Measurement of the transglycosylation progress by HPLC. The reactions (a−g) are at t = 0, 0.25, 1, 2, 4, 8, and 16 h. (see details in the Supporting Information).

We further demonstrated the use of the fluorescent substrate **7** in the characterization of TGase inhibitors. Moenomycin A (**24**) is a potent natural inhibitor against TGases,[19−21] though its poor bioavailability has limited its development as an antibacterial drug. According to our binding assay fluorescence anisotropy method,[12] moenomycin A and its analogue **25** (see the structures in the SI) exhibit IC_{50} values of 0.36 and 2.10 μM against TGase, respectively. In agreement with this result, the TGase inhibition assays with **7** as the substrate also showed that the two inhibitors could be easily distinguished at concentrations between 0.3 and 1 μM (Figure 5). The TGase activity was completely inhibited when the concentrations of **24** and **25** were higher

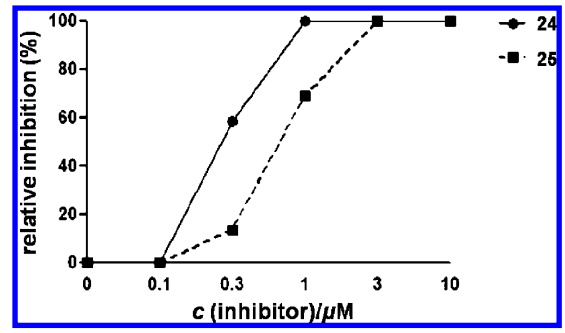

Figure 5. Relative TGase inhibition percentage of moenomycin A (**24**) and its analogue **25** via HPLC analysis with substrate **7**.

than 1 and 3 μM, respectively. In contrast, when concentrations were below 0.1 μM **24** or 0.3 μM **25**, no inhibitory activities were observed. In the presence of 0.3 μM **24** or **25**, the TG inhibition was 59% or 14%, corresponding to 76% or 50% of nonconsumed **7**, respectively.

In conclusion, we have designed a new fluorescent TG substrate **7** by incorporating a dansyl group to mimic the diprenyl moiety in the lipid chain. We have also explored a convergent approach to synthesize this probe molecule. Unlike the Lipid II analogue **2** bearing the dansyl group at the peptide site, substrate **7** could be readily used in the functional assay of TGases without an additional treatment with *N*-acetylmurmidase. The assay system with substrate **7** was also able to screen TGase inhibitors, as exemplified by distinguishing the inhibitor potency of moenomycin A versus its analogue in this study. In principle, one can also modify compound **7** to construct a high throughput TG probe by installation of another chromophore at the peptide site as the FRET donor or acceptor to the dansyl group at the lipid site. We are currently investigating the feasibility of this research approach.

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Supporting Information Available: Experimental procedures, characterization of the synthetic compounds, and HPLC analysis of transglycosylation. This material is available free of charge via the Internet at http://pubs.acs.org.

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