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Effect of the Lipid II Sugar Moiety on Bacterial Transglycosylase: the 4-Hydroxy Epimer of Lipid II is a TGase Inhibitor

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Lipid II analogues bearing major modifications on the second sugar (GlcNAc) were synthesized and evaluated for their substrate activity toward TGases. Unexpectedly, *N*-deacetyled Lipid II decreased its activity dramatically, and the C4-axial OH Lipid II became an inhibitor (IC_{50} = 8 μM) with an approximately 14-fold increase in binding affinity toward TGase (25 vs. 27).

The rise of antibiotic drug resistant bacteria such as MRSA (methicillin-resistant *Staphylococcus aureus*) has stimulated the development of new antibiotics.[1] Peptidoglycan, a major component in the bacterial cell wall, is composed of long glycan chains cross-linked by short peptides to form a mesh-like structure, and is essential for bacterial shape and growth.[2] Among those enzymes involved in bacterial cell-wall (peptidoglycan) biosynthesis, transglycosylases (TGases) catalyze the transfer of the sugar moiety from the activated Lipid II or polymeric peptidoglycan such as Lipid IV (a glycosyl donor) to the specific hydroxyl group (4-OH) of Lipid II (a glycosyl acceptor), with concomitant release of an undecaprenyl pyrophosphate moiety (Figure 1).[3] These enzymes are attractive antibiotic targets since their functions are essential and; their structures are conserved; and they are located on the external surface of bacterial membranes, which should allow easy access by inhibitors.[2] To date, moenomycin A (MoeA) and its analogues are the only known naturally occurring antibiotics that target the glycosyl donor site in TGase and disable peptidoglycan biosynthesis, but its poor pharmacokinetic properties preclude its clinical use.[4]

Lipid II is a structurally unique and complex molecule, consisting of a disaccharide (GlcNAc-MurNAc); a pyrophosphate; an undecaprenol lipid tail; and a pentapeptide moiety. Structure-activity relationship studies of Lipid II towards TGase are important to both establish minimum structural requirements of Lipid II for transglycosylation, and inspire novel, substrate-based, TGase inhibitors.[5-7] To date, only straightforward and easy structural modifications on Lipid II, including MurNAc (the first sugar), lipid chain, peptide chains, have been studied previously by us and others.[5,6,8,9] Their TGase substrate studies have shown that Lipid II derivatives bearing a shorter peptide (only L-alanine) or a shorter lipid chain containing a fluorophore tag can still be recognized by substrates with reasonable activities.[5,6] These preliminary results suggest that not only the complex structure of natural Lipid II is readily amenable to simplification, but also we are able to use this simplified Lipid II as a molecule template to investigate the difficult but important task: *what is the effect of GlcNAc (the second sugar moiety) in Lipid II toward TGase?*

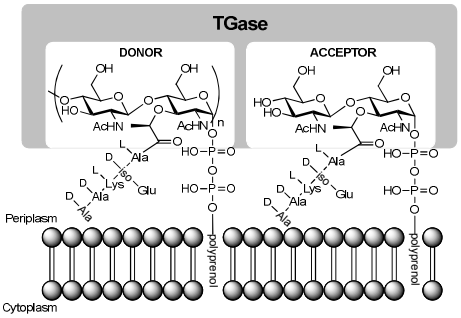

Fig. 1 TGase-catalyzed formation of peptidoglycan from Lipid II.

Bespoke Lipid II analogues are a prerequisite for the investigation of TGase substrate specificity, but known chemo-enzymatic approaches are still limited.[10,11] Chemical synthesis, in contrast, allows the systematic generation of analogues for testing.[11-13] We have a long-standing interest in developing the synthetic methodology necessary to prepare peptidoglycan fragments and precursors as well as investigating their biological functions.[5,14,15] Herein, we describe the design and synthesis of a series of Lipid II analogues wherein the structure of the second sugar (GlcNAc) moiety is varied. These modified

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Lipid II derivatives were tested, to see if they still function as TGase substrates, and/or are suitable for the development of TGase probes or inhibitors.

Figure 2 depicts Lipid II analogues **1**–**5**, which consist of a disaccharide (modified GlcNAc-MurNAc), a pyrophosphate, a shorter C27 polyprenol (five *cis*-isoprene units) bearing a fluorescent group (NBD) instead of an undecaprenyl lipid tail (C55)[8], and the simplified peptide, L-alanine alone.[5] The fluorophore NBD was deliberately incorporated at the end of the lipid to allow quantitative monitoring of substrate consumption in our HPLC-based TGase activity assay.[8,12]

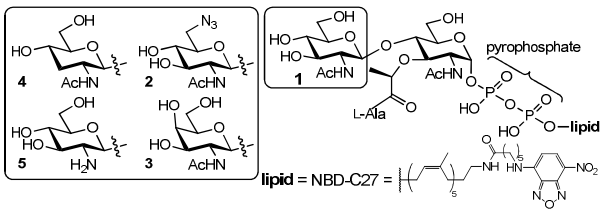
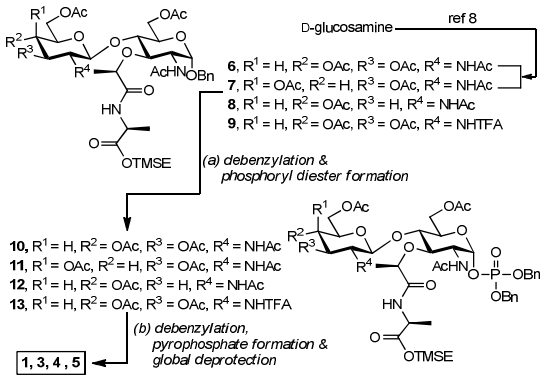


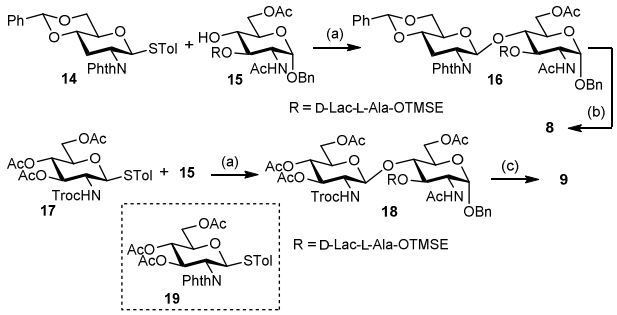
Fig. 2 Synthetic Lipid II analogues for this substrate activity study.

A general route to prepare Lipid II analogues **1**, and **3**–**5** from the corresponding key intermediates **6**–**9** was shown in Scheme 1. Debenzylation of **6**–**9**, followed by the phosphoryl diester formation got **10**–**13**, respectively. And, a sequence of debenzylation, pyrophosphate formation[16,17] (conjugation with a fluorophore-labeled lipid phosphate (NBD-C27P), and global deprotection by catalytic hydrogenation of **10**–**13** was performed to furnish the desired Lipid II analogues **1**, and **3**–**5** (Scheme 1).



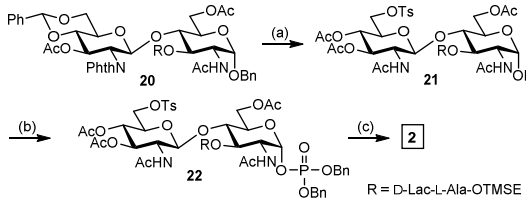
Scheme 1 A General Route for the Synthesis of Lipid II Analogues **1**, and **3**–**5**. Reagents and conditions: (a) i. $Pd(OH)_2/C$, H_2, MeOH, ii. $(i\text{-}Pr)_2NP(OBn)_2$, 1*H*-tetrazole, CH_2Cl_2, iii. *t*-BuOOH, -78 °C, 62-87% over three steps; (b) i. $Pd(OH)_2/C$, H_2, MeOH, 95%, ii. NBD-C27P, CDI, CH_2Cl_2, 1*H*-tetrazole, DMF, iii. LiOH, MeOH, 41–47% over three steps.

Notably, key intermediates **6** and **7** were synthesized from D-glucosamine following the similar approach previously described.[8] Preparation of other two disaccharides **8** and **9** was clearly described in Scheme 2. Glycosylation between donor **14**[18] and acceptor **15** mediated by TMSOTf afforded the corresponding disaccharide **16** (86%), followed by deprotection of the benzylidene and *N*-phthalimide moiety and then global acetylation to give **8**. To prepare disaccharide **9**, the *N*-Troc protected donor **17** was applied instead of *N*-Phth protected **19** in order to selectively manipulate the amino group in the subsequent transformations. Glycosylation between donor **17**[19] and acceptor **15** gave disaccharide **18** in a reasonable yield (70%). Selective *N*-Troc deprotection of **18** was performed under mild conditions (Zn/acetic acid),[20] followed by *N*-acetylation (TFAA in pyridine) to obtain disaccharide **9** in 82% over two steps. With *N*-TFA protected **9** in hand, Lipid II analogue **5** was smoothly prepared (35%, from **9** over six steps, Scheme 1).



Scheme 2 Preparation of disaccharides **8** and **9**. Reagents and conditions: (a) NIS, TMSOTf, CH_2Cl_2, -78 °C, 2 h, 86% for **16,** 70% for **18**; (b) i. TFA, CH_2Cl_2, ii. hydrazine acetate, MeOH, 60 °C, iii. Ac_2O, pyridine, 53% over three steps; (c) i. Zinc dust, HOAc, CH_2Cl_2, ii. TFAA, pyridine, 82% over two steps.

We next turned our attention to the preparation of azido-Lipid II **2**. As depicted in Scheme 3, **20** was converted to **21** with a sequence of steps including removal of the benzylidene moiety, selective tosylation at the C6 position,[21] de-phthalimidation, and acetylation. After debenzylation of **21**, followed by phosphitylation/oxidation, **22** was obtained in 67% over three steps. To avoid azido reduction during catalytic hydrogenation,[21] installation of the azide moiety on **22** was performed after debenzylation. The azido-disaccharide intermediate was conjugated with NBD-C27P, and deprotected under basic conditions to furnish **2**.



Scheme 3 Preparation of Azido-Lipid II **2**. Reagents and conditions: (a) i. PTSA, MeOH, 60 °C, 0.5 h; ii. TsCl, pyridine, 0 °C, 8 h, iii. hydrazine acetate, MeOH, 80 °C, 15 h; iv. Ac_2O, pyridine, rt. 4 h, 43% over four steps; (b) i. $Pd(OH)_2/C$, H_2, MeOH, ii. $(i\text{-}Pr)_2NP(OBn)_2$, 1*H*-tetrazole, CH_2Cl_2, iii. *t*-BuOOH, -78 °C, 67% over three steps; (c) i. $Pd(OH)_2/C$, H_2, MeOH, 95%, ii. NaN_3, DMF, 78%, iii. NBD-C27P, CDI, CH_2Cl_2, 1*H*-tetrazole, DMF, iv. LiOH, MeOH, 25% over four steps.

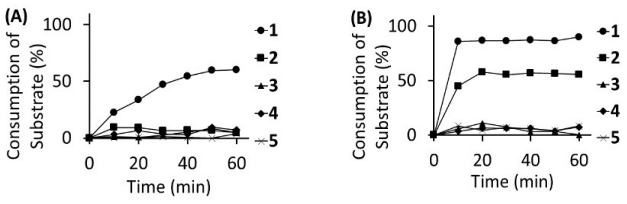


Fig. 3 Evaluation of the substrate activity of Lipid II analogues **1**–**5** toward TGase. Time course of consumption of **1**–**5** (30 μM) by *C. difficile PBP1b* (2.5 μg/mL) without (A) or with (B) Lipid IV (20 μM).

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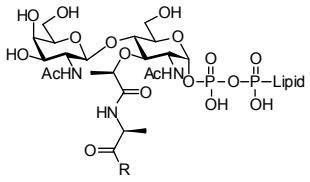
With fluorescent Lipid II analogues **1**–**5** in hand, we embarked on the study of their TGase (*C. difficile* PBP1b) substrate activity using an HPLC-based activity assay to measure the initial rate of the consumption of Lipid II in the absence or presence of Lipid IV. According to our previous study, Lipid IV binds only at the TGase donor site (*C. difficile* PBP1b) and it alone cannot undergo self-elongation in transglycosylation.[12] In contrast, an enzymatic reaction occurs only when the reaction mixture contains Lipid II alone or a mixture of both Lipid II and Lipid IV.[22] As expected, when the reaction mixture contained a structurally simplified Lipid II analog **1** alone, it functioned as a TGase substrate, with an initial consumption rate of 0.35 μM/min. The consumption rate increased to 2.60 μM/min when Lipid IV was added (Lipid IV / **1** = 1:1.5).

To our delight, analog **2** was also recognized as a TGase substrate with a consumption rate of 0.87 μM/min in the presence of Lipid IV, which implies **2** incorporates into peptidoglycan biosynthesis without affecting an oligopeptide or cross-linking peptides.[23] Analog **4**, the C3-dehydroxylated **1**, was significantly less active than **1**, even after a prolonged period of time (2 h), or with prior incubation with Lipid IV. Analog **5**, without the *N*-acetyl group at C2 on GlcNAc of **1**, resulted in significant loss of its TGase substrate activity. *N*-Deacetylated peptidoglycans have been reported to resist degradation by the host lysozyme, but it is still unclear when *N*-deacetylation of the second sugar (GlcNAc) occurs.[24] Since **5**, the *N*-deacetylated Lipid II, is an unsuitable TGase substrate, we reasoned that *N*-deacetylation should occur after the transglycosylation stage in the peptidoglycan forming process.

Compound **3**, the GalNAc-MurNAc typed analog, was not itself a TGase substrate, even in the presence of Lipid IV (see SI, Fig S1). To confirm this, the analog **S4** (GalNAc-MurNAc type) bearing a longer polyprenyl lipid chain was applied (see in SI, Fig S3). Our result showed that it alone cannot be a TGase substrate. Besides, it could not be consumed in the presence of normal Lipid II (up to 1000 μM) in our HPLC-based TGase activity analysis, suggesting it does not bind to the glycosyl donor site during the peptidoglycan elongating process (see in SI, Fig S3).

Since **3** turned out not to be a TGase substrate, we were curious whether it could function as a TGase inhibitor. Four GalNAc-MurNAc typed analogues **23**–**26** bearing different lipid chain lengths (C20 or C55) and oligopeptides (pentapeptide or monopeptide) were synthesized (see detailed experiments in SI) to examine their inhibitory activities against *E. coli* TGase (Table 1). All four were found to be moderate to potent inhibitors of TGase, with IC_{50} values ranging from 8 to 80 μM, with inhibitors bearing longer lipid chains exhibiting the best inhibitory potency. For example, **23** or **24** bearing a longer C55 lipid were more potent inhibitors than **25** or **26** bearing a shorter C20 lipid. The IC_{50} value of **23** was 8 μM, about 9-fold more potent than that of its cognate **25** (IC_{50} = 77 μM). Similar activity relationships were also observed between **24** and **26.** In contrast, when the peptide chain of **23** or **25** was truncated to give the corresponding **24** or **26**, respectively, no significant decrease in inhibitory activity was observed. Since TGases are highly conserved in many bacterial species,[25-27] the inhibition study of **23**–**26** against *C. difficile* TGase (PBP1b) were also performed. Our results show that the lipid modification more significantly affects inhibitory potency than the peptide part (Table 1).

Table 1 Inhibition activity of Lipid II analogues against TGases.



23, Lipid = undecaprenyl, R = D-iso-Glu-L-Lys-D-Ala-D-Ala
24, Lipid = undecaprenyl, R = OH
25, Lipid = tetraprenyl, R = D-iso-Glu-L-Lys-D-Ala-D-Ala
26, Lipid = tetraprenyl, R = OH

Compound		**23**	**24**	**25**	**26**
IC_{50} (μM)[a]	*E. coli* TGase	8	15	77	80
	C.difficile TGase	18	34	248	246

[a] Experiments were performed in 0.085% decyl-PEG, 50 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$, 10% DMSO, 15% MeOH, 10 μM NBD-Lipid II and *E. coli* PBP1b or *C. difficile* PBP1b at 37 °C.

To quantitatively measure the impact of C4-OH epimerization on the second sugar in Lipid II analogues, a surface plasmon resonance (SPR) analysis was used to determine the difference in binding affinity resulting from structural modifications. Inspired from our previous structural studies, we planned to use MoeA to occupy the glycosyl donor site and evaluate the relative binding affinity of the molecules tested at the acceptor site.[27,28] To verify our hypothesis, a preliminary molecular competitive examination was performed by a fluorescence polarization (FP) assay with using FITC-labeled MonA analog as the probe against TGase.[18] The results show that FITC-moenomycin probe cannot be replaced by **25** at high concentration (up to 1 mM), importantly indicating that **25** binds to the acceptor site but not the donor site of TGase.

For the SPR study, to reduce non-specific hydrophobic interactions, the shorter C20 lipid was employed,[5] and **25**, **28**, **29** (GalNAc-MurNAc type) and **27** (GlcNAc-MurNAc type) were synthesized using the procedure above.[29] Our results are shown in Table 2 (also see in SI). Analog **25** with a C4 axial OH (the C4 epimer of **27)**, had a K_D value with 33 μM. The K_D value of **27** was 445 μM. The results show that changing the orientation of the C4-hydroxy group on GalNAc in Lipid II not only mechanically interrupts the transglycosylation progress, but also significantly increases the binding affinity (14-fold increase) to TGase. Analog **28**, lacking the pyrophosphate-lipid moiety, exhibited a very weak binding affinity (K_D = 778 μM) to the enzyme. In addition, no binding signal was observed for **29**, lacking both the pyrophosphate-lipid moiety and oligopeptide of **25**. This quantitative data demonstrates the importance of the orientation of the hydroxyl group on the second sugar in Lipid II for enzyme recognition. We also illuminated the important role played by the pyrophosphate-lipid moiety in Lipid II-enzyme binding, even though this information cannot be inferred from the previous co-crystal structure.[27]

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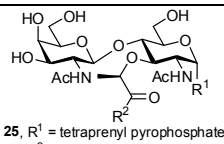
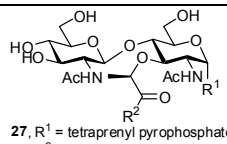
Table 2 Dissociation constant (K_D) values for Lipid II analogues toward *C. difficile* TGase (PBP1b).



25, R^1 = tetraprenyl pyrophosphate; R^2 = L-Ala-D-iso-Glu-L-Lys-D-Ala-D-Ala



27, R^1 = tetraprenyl pyrophosphate; R^2 = L-Ala-D-iso-Glu-L-Lys-D-Ala-D-Ala

28, R^1 = OH; R^2 = L-Ala-D-iso-Glu-L-Lys-D-Ala-D-Ala

29, R^1 = OH; R^2 = L-Ala

Compound	**25**	**27**	**28**	**29**
K_D (μM)[a]	33 ± 6.5	445 ± 19.9	778 ± 21.1	NB[b]

[a] Experiments were performed in the presence of 1 μM MonA (K_D = 600 nM) in triplicate at 25 °C, and the K_D values were determined by using steady-state affinity. [b]NB refers no significant binding signal was observed.

In summary, a series of Lipid II analogues were designed and synthesized to investigate the effect of the GlcNAc moiety of Lipid II on TGase. Special attention was given to structural modifications at the second sugar GlcNAc. The capacity of these molecules to serve as TGase substrates was tested, and several important results were obtained. Firstly, the *N*-acetyl group on GlcNAc was found to play an important role in substrate activity – without it, activity was found to dramatically decrease. Secondly, the Lipid II analog bearing a modification at the C6-position of GlcNAc still can be recognized as a TGase substrate. This finding suggests it could be qualified as a chemical probe for further bacterial cell wall studies. Thirdly, a C4-epimer of Lipid II analog alone is not a TGase substrate. In contrast, it is identified as an inhibitor and the epimerization of the C4-OH group from GlcNAc to GalNAc in Lipid II results in a 14-fold increase of binding affinity in our study. Fourthly, this study resulted in the discovery of **23**, the most potent TGase inhibitor with the IC_{50} value of 8 μM. Taken together, these results not only illuminate the structure activity relationships of Lipid II and TGase, but also provide a direction for both probe and inhibitor design; and validate the TGase acceptor site as a target for novel antibiotics. New applications derived from molecules discovered in this work are ongoing, and the results will be published in due course.

Acknowledgements

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