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Article

GlcNAc-1,6-anhydro-MurNAc Moiety Affords Unusual Glycosyl Acceptor that Terminates Peptidoglycan Elongation

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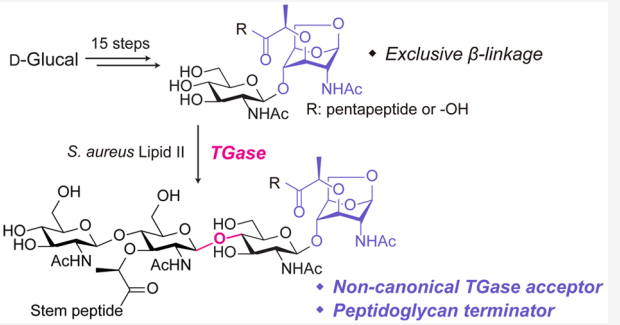
ABSTRACT: Peptidoglycan (PG), an essential exoskeletal polymer in bacteria, is a well-known antibiotic target. PG polymerization requires the action of bacterial transglycosylases (TGases), which couple the incoming glycosyl acceptor to the donor. Interfering with the TGase activity can interrupt the PG assembly. Existing TGase inhibitors like moenomycin and Lipid II analogues always occupy the TGase active sites; other strategies to interfere with proper PG elongation have not been widely exploited. Inspired by the natural 1,6-anhydro-MurNAc termini that mark the ends of PG strands in bacteria, we hypothesized that the incorporation of an anhydromuramyl-containing glycosyl acceptor by TGase into the growing PG may effectively inhibit PG elongation. To explore this possibility, we synthesized 4-*O*-(*N*-acetyl-*β*-D-glucosaminyl)-1,6-anhydro-*N*-acetyl-*β*-D-muramyl-L-Ala-*γ*-D-Glu-L-Lys-D-Ala-D-Ala, **1**, within 15 steps, and demonstrated that this anhydromuropeptide and its analogue lacking the peptide, **1-deAA**, were both utilized by bacterial TGase as noncanonical anhydro glycosyl acceptors *in vitro*. The incorporation of an anhydromuramyl moiety into PG strands by TGases afforded efficient termination of glycan chain extension. Moreover, the preliminary *in vitro* studies of **1-deAA** against *Staphylococcus aureus* showed that **1-deAA** served as a reasonable antimicrobial adjunct of vancomycin. These insights imply the potential application of such anhydromuropeptides as novel classes of PG-terminating inhibitors, pointing toward novel strategies in antibacterial agent development.



INTRODUCTION

The rising prevalence of antibiotic-resistant bacteria poses a severe threat to global health, stimulating increased efforts in the development of novel antibiotics.[1−3] Peptidoglycan (PG), which is the major component of the bacterial cell wall, is the target of many antibiotics. PG is composed of linear glycan backbones comprising repeating GlcNAc-MurNAc disaccharides, with a stem peptide covalently linked to the lactoyl group in each MurNAc residue.[4] In the final steps of PG assembly, transglycosylases (TGases) catalyze the successive incorporation of incoming glycosyl acceptor, Lipid II, into the reducing end of the growing glycan strand (glycosyl donor), thus elongating the PG polymer, whereas transpeptidases (TPases) further cross-link the stem peptides in adjacent glycan strands (Figure 1).[5−7] Bacterial TGases are conserved and essential enzymes in bacteria but are absent in mammalian cells, making them promising antibiotic targets.[8] Till now, the only known TGase-targeting antibiotic is moenomycin A (MoeA), a natural glycolipid product that binds to the glycosyl donor site of TGase and impedes PG synthesis. However, MoeA possesses a phosphoglycerate-polyprenyl moiety, a long lipid chain that renders its poor pharmacokinetics.[9,10] Other reported TGase inhibitors, including moenomycin analogues with aromatic branches, Lipid II analogues, and nonsaccharide compounds, have demonstrated potent antimicrobial efficiency; however, so far, none has advanced to clinical use due to adverse pharmacodynamics associated with the hydrophobic moiety.[11−14] Hence, novel classes of compounds that interfere with TGase-catalyzed PG elongation are highly desirable.

To develop novel classes of TGase inhibitors, insights into the structural requirements of potential glycosyl donors and acceptors for transglycosylation are crucial.[15] Previous studies with modified Lipid II have elucidated the structure−activity relationship of potential TGase substrates.[16−19] Cheng and co-workers discovered that Lipid II analogue with only an L-Ala in the stem peptide still shows reasonable binding affinity to TGases.[19] Additionally, Walker and co-workers reported that a Lipid II variant with a shorter lipid tail (a C35 chain) exhibits

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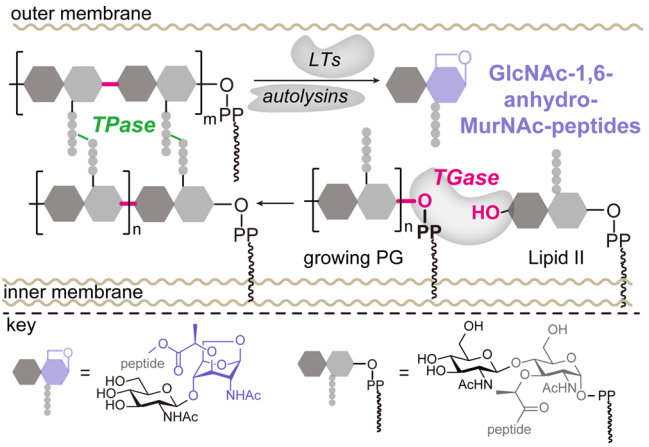


Figure 1. Schematic of bacterial peptidoglycan (PG) assembly by transglycosylases (TGases) and transpeptidases (TPases). Soluble GlcNAc-1,6-anhydro-MurNAc-peptides are generated by autolysins and lytic transglycosylases (LTs) during PG turnover.

enhanced reactivity *in vitro*.[18] Based on these studies, we reasoned that the disaccharide moiety in Lipid II is key for the TGase reaction, while the stem peptide and the lipid chain of Lipid II appear less important. We recognized that anhydromuropeptides with a 1,6-anhydro-MurNAc moiety fulfill such structural requirements. Notably, anhydromuropeptides are naturally generated from PG recycling by lytic transglycosylases (LTs) in bacteria (Figure 1).[20,21] Given the structural similarity of anhydromuropeptides to natural Lipid II molecules, we hypothesized that they may hijack the TGase-catalyzed glycosylation and the absence of a reducing end in anhydromuropeptides may effectively terminate the successive PG elongation. Thus, we set out to study the suitability of these anhydromuramyl-containing molecules as potential TGase substrates.

A substantial quantity of anhydromuropeptides (i.e., GlcNAc-1,6-anhydro-MurNAc peptide) is needed for biochemical studies. Although these anhydromuropeptides can be obtained via cell wall sacculi degradation, the process is often plagued with low yields and uncertain purity. Hence, chemical total syntheses of GlcNAc-1,6-anhydro-MurNAc-peptides are highly desirable, exemplified by the pioneering studies from Paulsen et al.,[22] Fukase et al.,[23] and Mobashery et al.[24] Notably, Mobashery and co-workers made a significant contribution to the synthesis of anhydromuropeptides. Nevertheless, the laborious protection and deprotection steps can still be shortened.[24] Herein, we present the shortest route toward 4-*O*-(*N*-acetyl-β-D-glucosaminyl)-1,6-anhydro-*N*-acetyl-β-D-muramyl-L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala **1** (Figure 2). We obtained anhydromuropeptide **1** and its disaccharide-only analogue **1-deAA** from D-glucal in only 15 steps followed by evaluating their suitability as potential substrates for PG assembly *in vitro*. Our work revealed new insights into the substrate requirement of TGase's acceptor domain and novel glycan strand terminators of PG elongation.

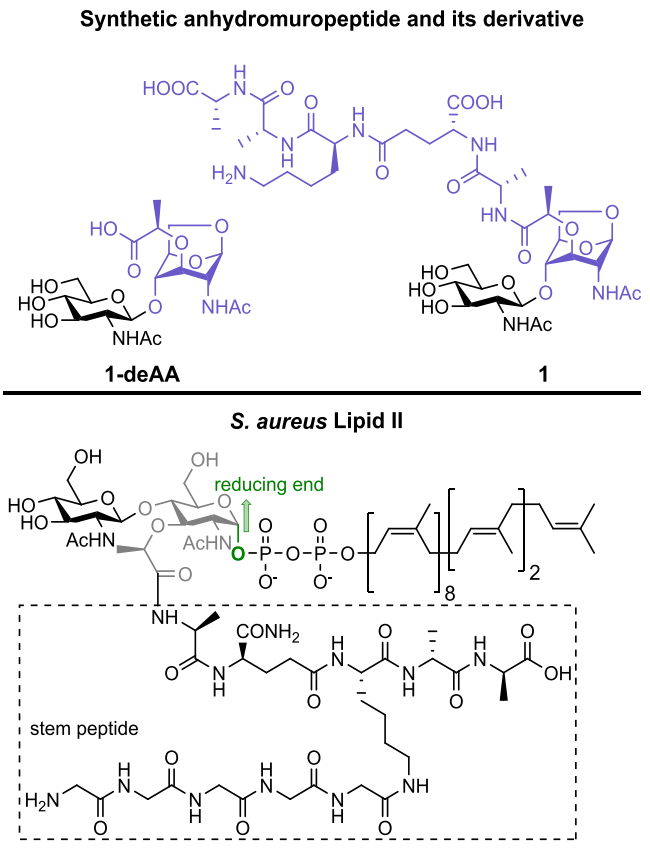
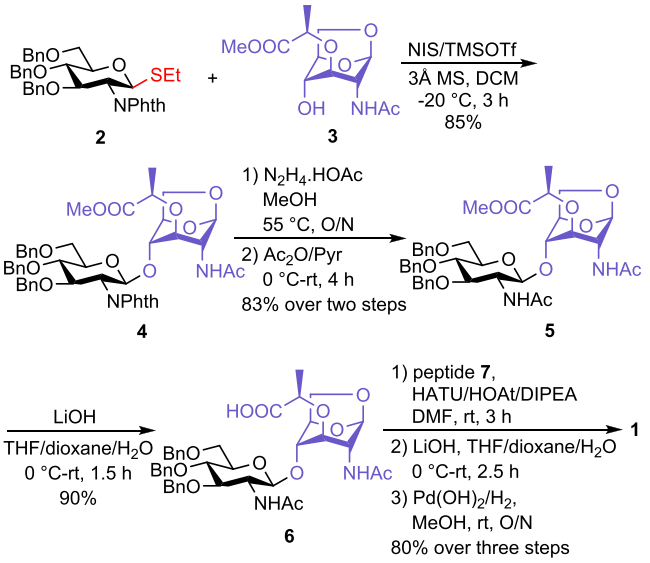


Figure 2. Structures of anhydromuropeptide analogues (**1** and **1-deAA**) and natural *S. aureus* Lipid II used in subsequent *in vitro* enzymatic assays.

Scheme 1. Synthesis of 4-*O*-(*N*-Acetyl-β-D-glucosaminyl)-1,6-anhydro-*N*-acetyl-β-D-muramyl-L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala (1)



RESULTS AND DISCUSSION

Our study commenced with the synthesis of our target molecule **1** (Scheme 1). The anhydromuropeptide **1** was prepared by a glycosylation reaction between **2**[25] and **3**, which was effectively promoted by the combination of 1.3 equiv of *N*-iodosuccinimide (NIS) and 0.3 equiv of trimethylsilyl trifluoromethanesulfonate (TMSOTf) producing the desired anhydrodisaccharide **4** as the β-anomer in 85% yield.[26] In contrast, when the disarmed acetylated thioglycoside **S3**[27] was glycosylated with the acceptor **3** under the promotion of NIS/TMSOTf, the target disaccharide **S4** was obtained in an inferior yield (67%, Scheme S1). Other mild activators such as

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1-benzenesulfinyl piperidine (BSP)/2,4,6-tri-*tert*-butylpyrimidine (TTBP)/trifluoromethanesulfonic anhydride (Tf_2O)[28] failed to afford the target disaccharide. Next, with disaccharide **4** in hand, we tested different reagents, such as ethylenediamine, $NaBH_4$,[29] and hydrazine acetate[30] to remove the *N*-phthalimido group in **4**, and finally successfully converted it into acetamide group by consecutively treating **4** with hydrazine acetate and acetic anhydride to give **5** in a satisfactory yield (83%) over two steps. Subsequently, the methyl ester group of the lactic acid in **5** was unmasked by the lithium hydroxide mediated hydrolysis to give **6**[23] followed by the installation of the key pentapeptide **7** in the presence of hexafluorophosphate azabenzotriazole tetramethyluronium (HATU), 1-hydroxy-7-azabenzotriazole (HOAt), and Hünig's base. The hydrolysis under basic conditions and the global hydrogenolysis[23] finally furnished the desired anhydromuropeptide **1** with an overall yield of 80% over 3 steps. Additionally, the disaccharide analogue 4-*O*-(*N*-acetyl-β-D-glucosaminyl)-1,6-anhydro-*N*-acetyl-β-D-muramyl acid **1-deAA** was also prepared by the similar synthetic route, which commenced with the glycosylation between acetylated thioglycoside **S3** and acceptor **3** followed by phthalimido group conversion to acetamide, and finally global deprotection (Scheme S1). Notably, the overall high efficiency of our synthesis was ensured by the early stage introduction of methyl lactate and the use of superarmed thioglycoside donor **2** as the glycosylating agent, enabling the high glycosylation efficiency and global deprotection in the final stage.

With anhydromuropeptide **1** in hand, we proceeded to investigate its suitability as a potential substrate for bacterial PG enzymes. Given the presence of a stem pentapeptide, we reasoned that **1** may be recognized and activated by TPase as a donor strand. To test this, we first set up a TPase-catalyzed terminal D-amino acid exchange reaction (Figure S2a). *S. aureus* PBP4, a low molecular weight TPase, is known to incorporate noncanonical D-amino acid into the terminal position of the stem peptide in PG precursors and muropeptides.[31−33] In the reaction, we incubated **1** with PBP4 in the presence of an excess amount of D-serine.[33] Indeed, LC-HRMS analysis of the reaction showed that the terminal D-Ala in **1** was effectively substituted by D-Ser, yielding the product with *m*/*z*: 965.4310 ($[M+H]^+$) (Figure S2b). Thus, this observation implied that **1** was effectively utilized for the PBP4-catalyzed terminal D-amino acid exchange reaction.

After attaining the desired results in the preceding TPase reaction, we set up a more intricate cross-linking reaction between **1** and the growing PG. In short, we incubated **1** and isolated *S. aureus* Lipid II (Figure S1)[6] with two enzymes, SgtB (monofunctional TGase)[34] and PBP4.[35] In the reaction, *S. aureus* Lipid II was first polymerized by SgtB to produce the growing PG strands, which possess pentaglycine branches that act as the incoming acceptor nucleophiles for peptide cross-linking with **1** (Figure 3a). To evaluate the products, we added lysozyme to the reaction mixture, which specifically hydrolyzes the β-1,4-glycosidic bond between MurNAc and GlcNAc in PG polymer, thus generating soluble muropeptides that are suitable for LC-HRMS analysis.[36] The putative cross-linking between synthetic **1** and growing PG will lead to muropeptides $\mathbf{X_{1-PG}}$ and $\mathbf{Y_{PG-PG}}$, which are indicative of cross-linked **1−PG** and **PG−PG**, respectively (Figure 3a). As expected, both cross-linked products ($\mathbf{X_{1-PG}}$, *m*/*z*: 1055.9791 $[M+2H]^{2+}$ and $\mathbf{Y_{PG-PG}}$, *m*/*z*: 805.3676 $[M+3H]^{3+}$) were detected by LC-HRMS (Figure 3b and Figures S2c−4), suggesting that **1** was readily utilized by PBP4 for cross-linking with the long PG polymer.

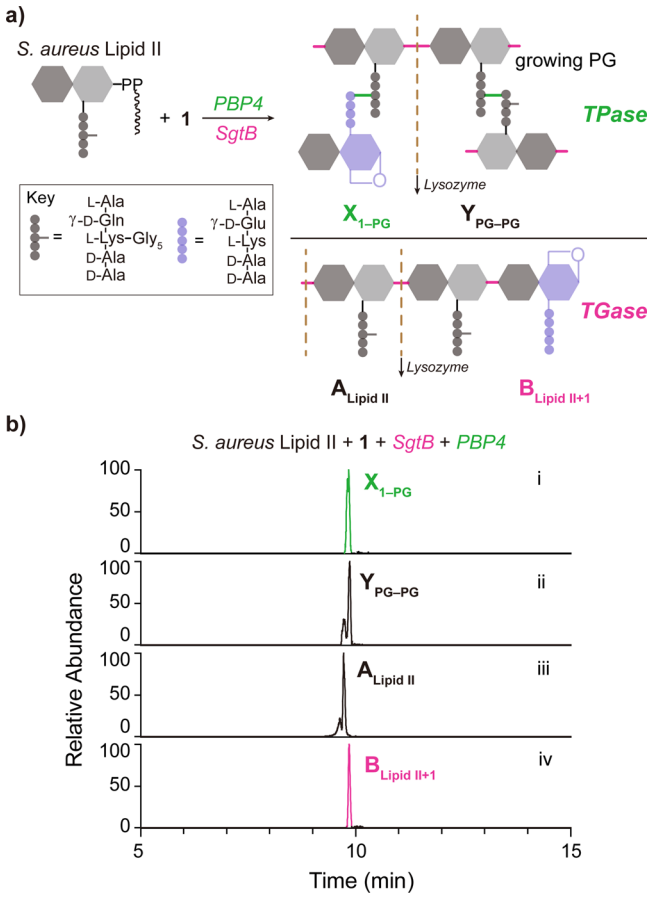

Figure 3. Synthetic **1** is employed by both bacterial TPase and TGase in the presence of *S. aureus* Lipid II. (a) Schematic of the assay monitoring the utilization of **1** by PBP4 for cross-linking with SgtB and *S. aureus* Lipid II, which leads to two cross-linking products after lysozyme digestion, $\mathbf{X_{1-PG}}$ and $\mathbf{Y_{PG-PG}}$. Meanwhile, two polymerized products, $\mathbf{A_{Lipid\ II}}$ and $\mathbf{B_{Lipid\ II+1}}$, are also detected by LC-HRMS, indicating that **1** reacts as a glycosyl acceptor of TGase. (b) LC-HRMS corresponding to extracted ion chromatograms (EICs) of the *in vitro* PBP4-SgtB reaction containing **1** and *S. aureus* Lipid II. $\mathbf{X_{1-PG}}$: 1055.9791 *m*/*z*; $\mathbf{Y_{PG-PG}}$: 805.3676 *m*/*z*; $\mathbf{A_{Lipid\ II}}$: 626.2886 *m*/*z*, $\mathbf{B_{Lipid\ II+1}}$: 1091.4977 *m*/*z*.

Surprisingly, apart from the expected products from TPase activity in the abovementioned enzymatic reaction containing anhydromuropeptide **1** and Lipid II, we also identified a novel glycosylated product, tetrasaccharide fragment $\mathbf{B_{Lipid\ II+1}}$, that is indicative of successful glycosylation between **1** and Lipid II (Figure 3b). As anhydromuropeptide **1** possesses a GlcNAc in the disaccharide backbone, we reasoned that **1** might be recognized by SgtB as a glycosyl acceptor to participate in glycosylation. The putative incorporation of **1** into the glycan backbone would lead to two different fragments, disaccharide fragment $\mathbf{A_{Lipid\ II}}$ and tetrasaccharide fragment $\mathbf{B_{Lipid\ II+1}}$ (Figure 3a). Consistently, both products were detected in LC-HRMS analysis (Figure 3b), and the identities of these fragments were further confirmed by MS/MS fragmentation (Figures S5b and S6). In addition, the formation of fragment $\mathbf{B_{Lipid\ II+1}}$ was fully inhibited by MoeA, supporting its formation in a TGase-dependent reaction (Figure S7).[37,38]

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Next, to investigate the structural requirement of pentapeptide in **1** as a glycosyl acceptor for TGase recognition, we did a docking study of SgtB (PDB ID 3VMT) with compound **1** and its analogue without pentapeptide **1-deAA**, respectively, based on the experimental resolved cocrystal structure between Lipid II analogues and SgtB (PDB ID 3VMT) reported by Wong and co-workers.[39] Docking results demonstrated comparable vina scores of −6.1 and −5.2 for **1** and **1-deAA**, respectively. Further inspection of docked complexes revealed that more polar interactions were observed between the GlcNAc-anhydro-MurNAc unit and SgtB glycosyl acceptor sites in **1-deAA** (Figure 4 and Figure S8), suggesting that **1-deAA** may

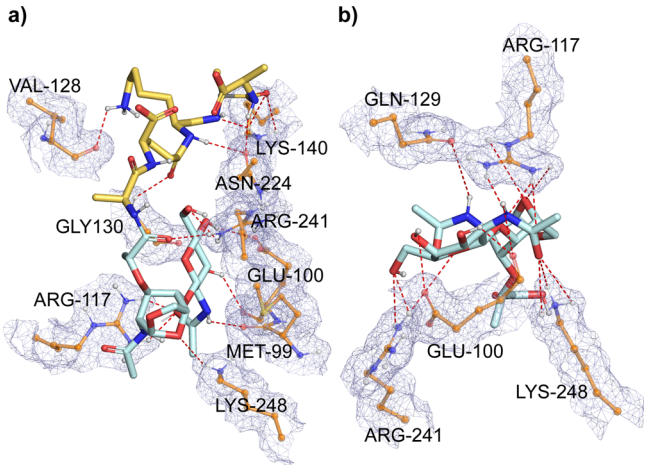

Figure 4. Synthetic **1** (a) and **1-deAA** without pentapeptide (b) can interact with the acceptor sites of SgtB (PDB ID 3VMT) based on the docking analysis. The red dashed lines mark the interactions between the compounds (**1** and **1-deAA**) and the SgtB residues. GlcNAc-1,6-anhydro-MurNAc: palecyan; pentapeptide: yellow; SgtB residues: orange.

also serve as an appropriate acceptor substrate of SgtB. Encouraged by the docking studies, we next evaluated **1-deAA** in the *in vitro* TGase reaction with SgtB (Figure 5a). As expected, **1-deAA** was readily incorporated into the glycan strand as a glycosyl acceptor, as evidenced by the formation of the tetrasaccharide fragment $\mathbf{C}_{\text{Lipid II+1-deAA}}$ (m/z: 856.3733 $[M+2H]^{2+}$) (Figure 5b and Figure S9), whose identity was further confirmed by MS/MS (Figure 5c and Figure S10). To explore how the removal of the pentapeptide in **1** affects its TGase reactivity, we compared relative amounts of representative tetrasaccharide fragment products, $\mathbf{B}_{\text{Lipid II+1}}$ and $\mathbf{C}_{\text{Lipid II+1-deAA}}$, in a time-course study. In brief, we quantified peak area ratios of the precursor ions of $\mathbf{B}_{\text{Lipid II+1}}$ and $\mathbf{C}_{\text{Lipid II+1-deAA}}$, to $\mathbf{A}_{\text{Lipid II}}$. The LC-HRMS analysis showed that the peak area ratio, $\mathbf{C}_{\text{Lipid II+1-deAA}}/\mathbf{A}_{\text{Lipid II}}$ (0.38) was 5 times higher than $\mathbf{B}_{\text{Lipid II+1}}/\mathbf{A}_{\text{Lipid II}}$ (0.07) at 60 min, indicating that **1-deAA** is a better glycosyl acceptor of TGase (Figure 5b and Figures S11 and S12).

Thus, we also conducted detailed kinetic studies with **1-deAA**, as it embodied a minimum structure as a TGase acceptor with better reactivity in the abovementioned time-course study. In Michaelis−Menten kinetic analysis, the product tetrasaccharide fragment $\mathbf{C}_{\text{Lipid II+1-deAA}}$ in the TGase reaction was quantified as the peak area of its precursor ion shown in LC-HRMS and the catalytic parameter of SgtB for synthetic **1-deAA** was determined to be K_m = 217.9 μM (Figure 6 and Figure S13). Given the lack of a reducing end in **1-deAA**, we inferred that its effective incorporation into growing PG may terminate the consecutive glycan elongation and shorten the glycan length of the PG strands (Figure 5a). To assess this, we visualized the glycan length distribution of PG polymers in TGase reactions by a Tris/Tricine SDS-PAGE system reported by Walker and co-workers.[40−42] We supplemented reactions with Rhodamine-labeled *S. aureus* Lipid II (Rho-Lipid II) to enable fluorescent detection of PG polymers.[19,43−45] The effective termination of transglycosylation by **1-deAA** will lead to a reduction in polymerized glycan strands. In the SDS-PAGE gel, the Rho-Lipid II migrated to the bottom of the gel (Figure 7, Lane 1), and glycan strands polymerized by TGase shifted from the bottom to higher regions (Figure 7, Lanes 2 to 5). To better evaluate the glycan distribution of the TGase reaction mixtures, we assigned the ratio of glycan strands in the Lipid II only reaction to 100% and the SDS-PAGE results demonstrated that the ratio of glycan strands dropped to 60% with the addition of 400 μM **1-deAA** and further declined to 48% as the concentration of **1-deAA** increased to 800 μM.

In consideration of the synergy effect of moenomycin and other cross-linkage-targeting antibiotics on pathogenic bacteria,[46,47] we conducted the synergy testing by coincubating **1-deAA** with vancomycin against *S. aureus*. Given the known inhibitory effect of vancomycin on the PG cross-linking formation, we hypothesized the synergistic interaction between these two compounds, potentially disrupting the bacterial PG biosynthesis. The synergy testing analysis demonstrated that the minimum inhibitory concentration (MIC) of vancomycin in *S. aureus* was effectively improved from 1.6 μg/mL to 0.8 μg/mL when coincubated with 512 μg/mL **1-deAA**.[48] In addition, the OD_{600} values of *S. aureus* showed a significant concentration-dependent decline (65% to 21%) with the addition of **1-deAA** (0 μg/mL to 256 μg/mL) when coincubated with 0.8 μg/mL of vancomycin (Figure S14). The preliminary antibacterial results implied reasonable antimicrobial activity of **1-deAA** against pathogenic bacteria when used synergistically with commercial antibiotics.

Overall, our observations indicate that synthetic anhydromuropeptide **1** is effectively activated by PBP4 as a donor substrate for D-amino acid exchange as well as cross-linking with the PG polymer, suggesting that the anhydroMurNAc moiety does not impede PBP4-catalyzed peptidoglycan transpeptidation. More importantly, our results show that SgtB also utilizes anhydromuropeptide **1** and its analogue **1-deAA** as glycosyl acceptors in the glycosylation reaction. Notably, the incorporation of **1** and **1-deAA** to the PG backbone via TGase-catalyzed glycosylation competes with the elongation of the glycan strand employing the natural Lipid II monomers. Our studies establish that synthetic anhydromuropeptides are suitable TGase acceptors for the first time, while most naturally occurring anhydromuropeptides are involved in the PG recycling pathway in Gram-negative bacteria, potentially impeding the reincorporation of these natural anhydromuropeptides into PG.[49,50] In the reported cocrystal structure of SgtB and a synthetic Lipid II,[39] the pyrophosphate moiety of Lipid II interacted with residues in the acceptor site of SgtB; meanwhile, the C6-OH group and the ester oxygen O1 of MurNAc unit bound to Mg^{2+}, which is crucial for stabilizing the glycosyl acceptor, Lipid II, in the TGase reaction. In our *in vitro* Mg^{2+}-free assay, compounds **1** and **1-deAA**, which lack both functional groups, can still be readily recognized and utilized by TGase as glycosyl acceptors.

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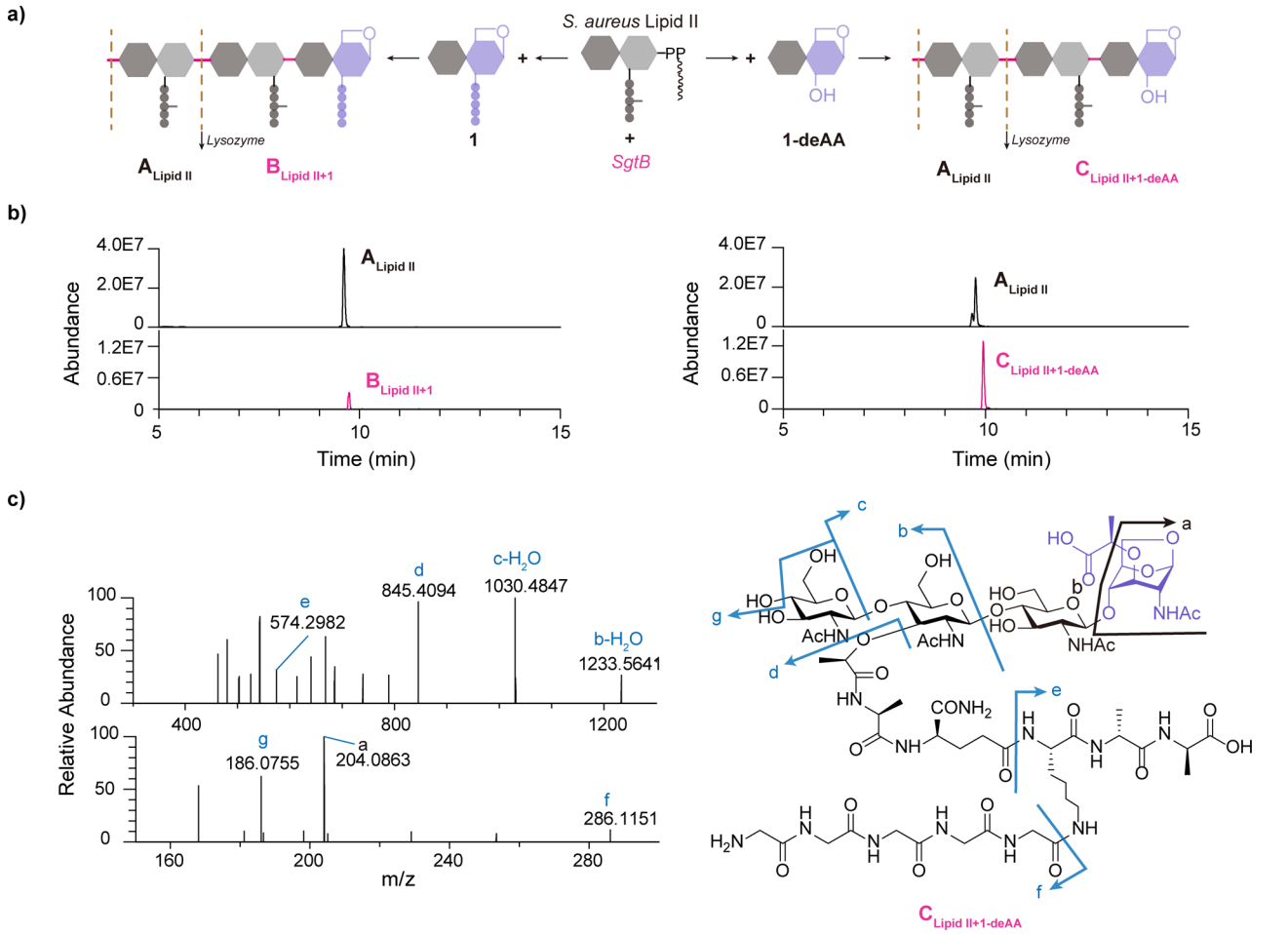

Figure 5. 1-deAA without pentapeptide reacts as a suitable glycosyl acceptor of bacterial TGase. (a) Schematic of the assay to analyze the structural requirement of the TGase acceptor. The SgtB can utilize both **1** and its analogue **1-deAA** without pentapeptide as acceptors, which leads to two degradation products $\mathbf{B}_{\text{Lipid II+1}}$ and $\mathbf{C}_{\text{Lipid II+1-deAA}}$. (b) LC-HRMS EICs of TGase reactions containing **1** (left) and **1-deAA** (right) with *S. aureus* Lipid II show significantly higher TGase reactivity of **1-deAA**. $\mathbf{A}_{\text{Lipid II}}$: 626.2886 m/z; $\mathbf{B}_{\text{Lipid II+1}}$: 1091.4977 m/z; $\mathbf{C}_{\text{Lipid II+1-deAA}}$: 856.3733 m/z. (c) MS/MS spectrum of $\mathbf{C}_{\text{Lipid II+1-deAA}}$. Fragment ions a and b represent **1-deAA** and dehydrated *S. aureus* Lipid II, respectively, which supports the structure of $\mathbf{C}_{\text{Lipid II+1-deAA}}$.

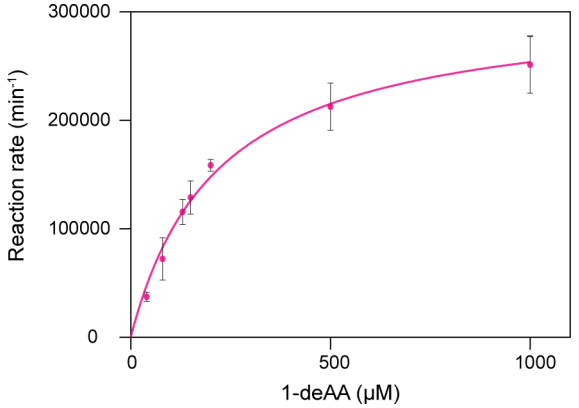

Figure 6. Reaction rate versus concentrations of **1-deAA** for SgtB. Curve fit was generated in GraphPad using the Michaelis–Menten equation: $V = (V_{max} \times S)/(K_m + S)$, where V_{max} is the maximum reaction rate, K_m is the Michaelis–Menten constant, and S represents the initial concentration of **1-deAA** available to SgtB: K_m = 217.9 μM.

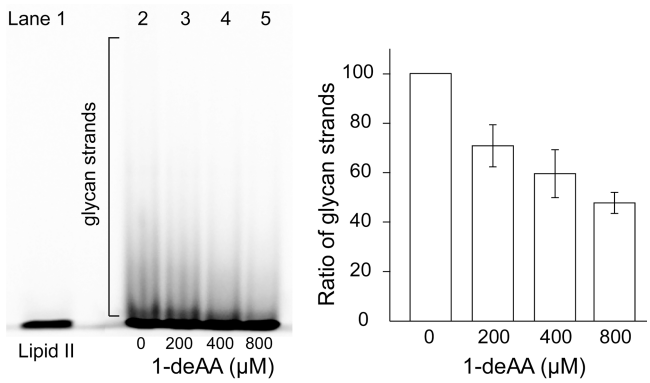

Figure 7. SDS-PAGE analysis of TGase reactions with Lipid II and **1-deAA**. Lane 1, Rho-Lipid II; Lanes 2 to 4, Lipid II and **1-deAA** (0 μM, 200 μM, 400 μM, and 800 μM) incubated with SgtB for 1 h followed by the addition of another aliquot of SgtB for another 1 h. The ratio of glycan strands decreased to 60% with the addition of **1-deAA** (400 μM) and further dropped to 48% at 800 μM of **1-deAA**.

Moreover, based on the stocking analysis, a few polar interactions exhibit between anhydro-MurNAc and the glycosyl acceptor site of SgtB, and the GlcNAc moiety maintains interactions with SgtB, with the position and conformation highly similar to the reported Wong's resolved structure, suggesting that the GlcNAc-anhydro-MurNAc unit

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still suffices for the recognition and utilization of TGase as glycosyl acceptors. In addition, the effective utilization of **1-deAA** by TGase reveals that pentapeptide is not necessary for the TGase acceptor, which is consistent with the reported cocrystal work that the D-lactoyl ether moiety of MurNAc but not the stem pentapeptide interacted with SgtB.[39] Previous structural requirement studies toward TGase also demonstrated that Lipid II analogues with various peptide, lipid, and sugar lengths were suitable substrates for bacterial TGases. For instance, *Aquifex aeolicus* PBP1A and *Escherichia coli* PBP1A can also utilize Lipid IV − a substrate with a longer glycan strand as a glycosyl acceptor *in vitro*.[40,51] Our results further provided new insights and expanded the scope of noncanonical acceptors utilized by bacterial TGases.

Interestingly, the preliminary enzymatic assay indicates **1-deAA** as a more reactive TGase acceptor than **1**. We postulate that the removal of the relatively large pentapeptide may mitigate the steric hindrance for TGase binding, enhancing the reactivity of **1-deAA** toward TGases. Further kinetic results revealed that the K_m value of **1-deAA** (217.9 μM) was approximately 2.4 times weaker than that of Lipid II analogues containing a nonconical tripeptide (90.2 μM) and 52-fold weaker than that of synthetic Lipid II (4.2 μM) as reported by Cheng et al.,[19] suggesting that **1-deAA** is a relatively appropriate substrate of TGase. Subsequent SDS-PAGE analysis reveals shortening of glycan length in PG after incorporation with **1-deAA**, marking the first revelation of an anhydro-PG fragment as a covalent glycan terminator of PG elongation that is distinct from the currently only TGase inhibitor MoeA. While we acknowledge that a high amount of **1-deAA** (400 μM) is required to terminate PG *in vitro*, it nevertheless provides a starting point for further improvement. For instance, Cheng and co-workers reported that C4-OH epimerization on GlcNAc significantly improved the binding affinity of Lipid II analogues to TGase.[16] We reason that it might be possible to modify the sugar unit of **1-deAA** to enhance its reactivity as a TGase glycosyl acceptor to block PG elongation. More importantly, the preliminary synergy testing analysis highlights not only the potential of **1-deAA** as an antibiotic adjunct but also offers a promising direction for the development of novel antibiotic treatments. Moreover, in contrast to the commercial MoeA with poor pharmacokinetics due to its lipid tail, **1-deAA** containing the anhydro terminus might exhibit improved pharmacokinetics.

CONCLUSIONS

In summary, we successfully performed the shortest total synthesis of compound **1**, an analogue of anhydro-PG fragments generated during cell wall recycling, in only 15 steps from D-glucal. The method gives a high overall yield with β-exclusive stereochemistry in **1** and its analogue without pentapeptide, **1-deAA**. This new methodology provides cost-effective and quick access to an invaluable anhydromuropeptide substrate for biochemical characterizations of bacterial PG enzymes. Following *in vitro* TPase enzymatic assays demonstrated that anhydromuropeptide **1** is an effective donor for transpeptidation. More importantly, TGase enzymatic studies revealed that both **1** and **1-deAA** are utilized by TGases as acceptors for glycosylation, which for the first time unveils these anhydro-PG fragments as potential glycosyl acceptors for bacterial TGases and expands our understanding of substrate requirements of TGases. Further kinetic study toward TGase implied that **1-deAA**, representing the structurally minimal TGase acceptor, possesses the appropriate TGase reactivity. Moreover, the SDS-PAGE analysis demonstrated that effectively incorporating **1-deAA** into growing PG polymers enabled the termination of the PG glycan strand elongation. Subsequent preliminary *in vitro* antibacterial studies of **1-deAA** against *S. aureus* revealed that **1-deAA** may serve as a potential antimicrobial adjunctive. These findings suggest that small molecule mimetics of anhydromuropeptides acting as PG elongation terminators may provide a new strategy to interfere with PG polymerization for further antimicrobial agent development.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.3c12526.

Experimental procedures (synthetic methods, LC-HRMS measurements, docking study, kinetic study, SDS-PAGE analysis, and antibacterial assay), all NMR spectra, and supplementary figures (LC-HRMS, docking, and antibacterial data) (PDF)

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Notes

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■ REFERENCES

(1) Lee, A. S.; de Lencastre, H.; Garau, J.; Kluytmans, J.; Malhotra-Kumar, S.; Peschel, A.; Harbarth, S. Methicillin-Resistant *Staphylococcus Aureus*. *Nat. Rev. Dis. Primers* **2018**, *4*, 18033.

(2) García-Solache, M.; Rice, L. B. The *Enterococcus*: A Model of Adaptability to Its Environment. *Clin. Microbiol. Rev.* **2019**, *32*, No. e00058.

(3) Murray, C. J. L.; Ikuta, K. S.; Sharara, F.; Swetschinski, L.; Aguilar, G. R.; Gray, A.; Han, C.; Bisignano, C.; Rao, P.; Wool, E.; Johnson, S. C.; Browne, A. J.; Chipeta, M. G.; Fell, F.; Hackett, S.; Haines-Woodhouse, G.; Hamadani, B. H. K.; Kumaran, E. A. P.; McManigal, B.; Achalapong, S.; Agarwal, R.; Akech, S.; Albertson, S.; Amuasi, J.; Andrews, J.; Aravkin, A.; Ashley, E.; Babin, F.-X.; Bailey, F.; Baker, S.; et al. Global Burden of Bacterial Antimicrobial Resistance in 2019: A Systematic Analysis. *Lancet* **2022**, *399*, 629–655.

(4) Wolf, A. J.; Underhill, D. M. Peptidoglycan Recognition by the Innate Immune System. *Nat. Rev. Immunol.* **2018**, *18*, 243–254.

(5) Lovering, A. L.; Safadi, S. S.; Strynadka, N. C. J. Structural Perspective of Peptidoglycan Biosynthesis and Assembly. *Annu. Rev. Biochem.* **2012**, *81*, 451–478.

(6) Qiao, Y.; Srisuknimit, V.; Rubino, F.; Schaefer, K.; Ruiz, N.; Walker, S.; Kahne, D. Lipid II Overproduction Allows Direct Assay of Transpeptidase Inhibition by β-Lactams. *Nat. Chem. Biol.* **2017**, *13*, 793–798.

(7) Kuru, E.; Radkov, A.; Meng, X.; Egan, A.; Alvarez, L.; Dowson, A.; Booher, G.; Breukink, E.; Roper, D. I.; Cava, F.; Vollmer, W.; Brun, Y.; VanNieuwenhze, M. S. Mechanisms of Incorporation for D-Amino Acid Probes that Target Peptidoglycan Biosynthesis. *ACS Chem. Biol.* **2019**, *14*, 2745–2756.

(8) Vacariu, C. M.; Tanner, M. E. Recent Advances in the Synthesis and Biological Applications of Peptidoglycan Fragments. *Chem.—Eur. J.* **2022**, *28*, No. e202200788.

(9) Ostash, B.; Walker, S. Moenomycin Family Antibiotics: Chemical Synthesis, Biosynthesis, and Biological Activity. *Nat. Prod. Rep.* **2010**, *27*, 1594–1617.

(10) Halliday, J.; McKeveney, D.; Muldoon, C.; Rajaratnam, P.; Meutermans, W. Targeting the Forgotten Transglycosylases. *Biochem. Pharmacol.* **2006**, *71*, 957–967.

(11) Kumar, S.; Mollo, A.; Kahne, D.; Ruiz, N. The Bacterial Cell Wall: From Lipid II Flipping to Polymerization. *Chem. Rev.* **2022**, *122*, 8884–8910.

(12) Chen, X.; Wong, C.-H.; Ma, C. Targeting the Bacterial Transglycosylase: Antibiotic Development from a Structural Perspective. *ACS Infect. Dis.* **2019**, *5*, 1493–1504.

(13) Zuegg, J.; Muldoon, C.; Adamson, G.; McKeveney, D.; Le Thanh, G.; Premraj, R.; Becker, B.; Cheng, M.; Elliott, A. G.; Huang, J. X.; Butler, M. S.; Bajaj, M.; Seifert, J.; Singh, L.; Galley, N. F.; Roper, D. I.; Lloyd, A. J.; Dowson, C. G.; Cheng, T.-J.; Cheng, W.-C.; Demon, D.; Meyer, E.; Meutermans, W.; Cooper, M. A. Carbohydrate Scaffolds as Glycosyltransferase Inhibitors with *in Vivo* Antibacterial Activity. *Nat. Commun.* **2015**, *6*, 7719.

(14) Derouaux, A.; Sauvage, E.; Terrak, M. Peptidoglycan Glycosyltransferase Substrate Mimics as Templates for the Design of New Antibacterial Drugs. *Front. Immunol.* **2013**, *4*, 1–13.

(15) Kwan, J. M. C.; Qiao, Y. Mechanistic Insights into the Activities of Major Families of Enzymes in Bacterial Peptidoglycan Assembly and Breakdown. *ChemBioChem* **2023**, *24*, No. e202200693.

(16) Chen, K.-T.; Lin, C.-K.; Guo, C.-W.; Chang, Y.-F.; Hu, C.-M.; Lin, H.-H.; Lai, Y.; Cheng, T.-J. R.; Cheng, W.-C. Effect of the Lipid II Sugar Moiety on Bacterial Transglycosylase: The 4-Hydroxy Epimer of Lipid II is a TGase Inhibitor. *Chem. Commun.* **2017**, *53*, 771–774.

(17) Liu, H.; Ritter, T. K.; Sadamoto, R.; Sears, P. S.; Wu, M.; Wong, C.-H. Acceptor Specificity and Inhibition of the Bacterial Cell-Wall Glycosyltransferase MurG. *ChemBioChem* **2003**, *4*, 603–609.

(18) Ye, X.-Y.; Lo, M.-C.; Brunner, L.; Walker, D.; Kahne, D.; Walker, S. Better Substrates for Bacterial Transglycosylases. *J. Am. Chem. Soc.* **2001**, *123*, 3155–3156.

(19) Shih, H.-W.; Chang, Y.-F.; Li, W.-J.; Meng, F.-C.; Huang, C.-Y.; Ma, C.; Cheng, T.-J. R.; Wong, C.-H.; Cheng, W.-C. Effect of the Peptide Moiety of Lipid II on Bacterial Transglycosylase. *Angew. Chem., Int. Ed.* **2012**, *51*, 10123–10126.

(20) Sakata, N.; Mukai, T. Production Profile of the Soluble Lytic Transglycosylase Homologue in *Staphylococcus Aureus* during Bacterial Proliferation. *FEMS Microbiol. Immunol.* **2007**, *49*, 288–295.

(21) Lee, M.; Hesek, D.; Dik, D. A.; Fishovitz, J.; Lastochkin, E.; Boggess, B.; Fisher, J. F.; Mobashery, S. From Genome to Proteome to Elucidation of Reactions for All Eleven Known Lytic Transglycosylases from *Pseudomonas Aeruginosa*. *Angew. Chem., Int. Ed.* **2017**, *56*, 2735–2739.

(22) Paulsen, H.; Himpkamp, P.; Peters, T. Bausteine von Oligosacchariden, LXIX. Synthese von 1,6-Anhydromuramylpeptiden. *Liebigs Ann. Chem.* **1986**, *1986*, 664–674.

(23) Kawasaki, A.; Karasudani, Y.; Otsuka, Y.; Hasegawa, M.; Inohara, N.; Fujimoto, Y.; Fukase, K. Synthesis of Diaminopimelic Acid Containing Peptidoglycan Fragments and Tracheal Cytotoxin (TCT) and Investigation of Their Biological Functions. *Chem.—Eur. J.* **2008**, *14*, 10318–10330.

(24) Hesek, D.; Lee, M.; Zhang, W.; Noll, B. C.; Mobashery, S. Total Synthesis of *N*-Acetylglucosamine-1,6-Anhydro-*N*-Acetylmuramylpentapeptide and Evaluation of Its Turnover by AmpD from *Escherichia Coli*. *J. Am. Chem. Soc.* **2009**, *131*, 5187–5193.

(25) Cirla, A.; McHale, A. R.; Mann, J. Synthesis of Analogues of Calicheamicin and Neocarzinostatin Chromophore. *Tetrahedron* **2004**, *60*, 4019–4029.

(26) Veeneman, G. H.; van Leeuwen, S. H.; van Boom, J. H. Iodonium Ion Promoted Reactions at the Anomeric Centre. II An Efficient Thioglycoside Mediated Approach toward the Formation of 1, 2-Trans Linked Glycosides and Glycosidic Esters. *Tetrahedron Lett.* **1990**, *31*, 1331–1334.

(27) Codée, J. D. C.; Litjens, R. E. J. N.; den Heeten, R.; Overkleeft, H. S.; van Boom, J. H.; van der Marel, G. A. Ph_2SO/Tf_2O: A Powerful Promotor System in Chemoselective Glycosylations Using Thioglycosides. *Org. Lett.* **2003**, *5*, 1519–1522.

(28) Crich, D.; Smith, M. 1-Benzenesulfinyl Piperidine/Trifluoromethanesulfonic Anhydride: A Potent Combination of Shelf-Stable Reagents for the Low-Temperature Conversion of Thioglycosides to Glycosyl Triflates and for the Formation of Diverse Glycosidic Linkages. *J. Am. Chem. Soc.* **2001**, *123*, 9015–9020.

(29) Shih, H.-W.; Chen, K.-T.; Cheng, T.-J. R.; Wong, C.-H.; Cheng, W.-C. A New Synthetic Approach toward Bacterial Transglycosylase Substrates, Lipid II and Lipid IV. *Org. Lett.* **2011**, *13*, 4600–4603.

(30) Liu, C.-Y.; Guo, C.-W.; Chang, Y.-F.; Wang, J.-T.; Shih, H.-W.; Hsu, Y.-F.; Chen, C.-W.; Chen, S.-K.; Wang, Y.-C.; Cheng, T.-J. R.; Ma, C.; Wong, C.-H.; Fang, J.-M.; Cheng, W.-C. Synthesis and Evaluation of a New Fluorescent Transglycosylase Substrate: Lipid II-Based Molecule Possessing a Dansyl-C20 Polyprenyl Moiety. *Org. Lett.* **2010**, *12*, 1608–1611.

https://doi.org/10.1021/jacs.3c12526
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(31) Alexander, J. A. N.; Chatterjee, S. S.; Hamilton, S. M.; Eltis, L. D.; Chambers, H. F.; Strynadka, N. C. J. Structural and Kinetic Analyses of Penicillin-Binding Protein 4 (PBP4)-Mediated Antibiotic Resistance in *Staphylococcus Aureus*. *J. Biol. Chem.* **2018**, *293*, 19854−19865.
(32) Maya-Martinez, R.; Alexander, J. A. N.; Otten, C. F.; Ayala, I.; Vollmer, D.; Gray, J.; Bougault, C. M.; Burt, A.; Laguri, C.; Fonvielle, M.; Arthur, M.; Strynadka, N. C. J.; Vollmer, W.; Simorre, J.-P. Recognition of Peptidoglycan Fragments by the Transpeptidase PBP4 from *Staphylococcus Aureus*. *Front. Microbiol.* **2019**, *9*, 3223.
(33) Qiao, Y.; Lebar, M. D.; Schirner, K.; Schaefer, K.; Tsukamoto, H.; Kahne, D.; Walker, S. Detection of Lipid-Linked Peptidoglycan Precursors by Exploiting an Unexpected Transpeptidase Reaction. *J. Am. Chem. Soc.* **2014**, *136*, 14678−14681.
(34) Karinou, E.; Schuster, C. F.; Pazos, M.; Vollmer, W.; Gründling, A. Inactivation of the Monofunctional Peptidoglycan Glycosyltransferase SgtB Allows *Staphylococcus Aureus* to Survive in the Absence of Lipoteichoic Acid. *J. Bacteriol.* **2018**, *201*, e00574-18.
(35) Atilano, M. L.; Pereira, P. M.; Yates, J.; Reed, P.; Veiga, H.; Pinho, M. G.; Filipe, S. R. Teichoic Acids are Temporal and Spatial Regulators of Peptidoglycan Cross-Linking in *Staphylococcus Aureus*. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 18991−18996.
(36) Vocadlo, D. J.; Davies, G. J.; Laine, R.; Withers, S. G. Catalysis by Hen Egg-White Lysozyme Proceeds via a Covalent Intermediate. *Nature* **2001**, *412*, 835−838.
(37) Hsu, T.-W.; Fang, J.-M. Effective Assay of Bacterial Transglycosylation by Molecular Turn-on Sensing and a Second-Order Scattering Effect. *Analyst* **2021**, *146*, 5843−5847.
(38) Hsieh, P.-Y.; Meng, F.-C.; Guo, C.-W.; Hu, K.-H.; Shih, Y.-L.; Cheng, W.-C. Harnessing Fluorescent Moenomycin A Antibiotics for Bacterial Cell Wall Imaging Studies. *ChemBioChem* **2021**, *22*, 3462−3468.
(39) Huang, C.-Y.; Shih, H.-W.; Lin, L.-Y.; Tien, Y.-W.; Cheng, T.-J. R.; Cheng, W.-C.; Wong, C.-H.; Ma, C. Crystal Structure of *Staphylococcus Aureus* Transglycosylase in Complex with a Lipid II Analog and Elucidation of Peptidoglycan Synthesis Mechanism. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 6496−6501.
(40) Barrett, D.; Wang, T.-S. A.; Yuan, Y.; Zhang, Y.; Kahne, D.; Walker, S. Analysis of Glycan Polymers Produced by Peptidoglycan Glycosyltransferases. *J. Biol. Chem.* **2007**, *282*, 31964−31971.
(41) Wang, T.-S. A.; Manning, S. A.; Walker, S.; Kahne, D. Isolated Peptidoglycan Glycosyltransferases from Different Organisms Produce Different Glycan Chain Lengths. *J. Am. Chem. Soc.* **2008**, *130*, 14068−14069.
(42) Schaefer, K.; Owens, T. W.; Page, J. E.; Santiago, M.; Kahne, D.; Walker, S. Structure and Reconstitution of a Hydrolase Complex that may Release Peptidoglycan from the Membrane after Polymerization. *Nat. Microbiol.* **2021**, *6*, 34−43.
(43) van't Veer, I. L.; Leloup, N. O. L.; Egan, A. J. F.; Janssen, B. J. C.; Martin, N. I.; Vollmer, W.; Breukink, E. Site-Specific Immobilization of the Peptidoglycan Synthase PBP1B on a Surface Plasmon Resonance Chip Surface. *ChemBioChem* **2016**, *17*, 2250−2256.
(44) Rebets, Y.; Lupoli, T.; Qiao, Y.; Schirner, K.; Villet, R.; Hooper, D.; Kahne, D.; Walker, S. Moenomycin Resistance Mutations in *Staphylococcus Aureus* Reduce Peptidoglycan Chain Length and Cause Aberrant Cell Division. *ACS Chem. Biol.* **2014**, *9*, 459−467.
(45) Wang, T.-S. A.; Lupoli, T. J.; Sumida, Y.; Tsukamoto, H.; Wu, Y.; Rebets, Y.; Kahne, D. E.; Walker, S. Primer Preactivation of Peptidoglycan Polymerases. *J. Am. Chem. Soc.* **2011**, *133*, 8528−8530.
(46) Yang, F.; Gao, S.; Yan, J.; Lin, X.; van der Veen, S. Moenomycin is Broadly Active against Multidrug-Resistant *Neisseria Gonorrhoeae* and Clears an Infection from a Murine Vaginal Tract Infection Model. *J. Antimicrob. Chemother.* **2022**, *77*, 2461−2469.
(47) Pinho, M. G.; de Lencastre, H.; Tomasz, A. An Acquired and a Native Penicillin-Binding Protein Cooperate in Building the Cell Wall of Drug-Resistant *Staphylococci*. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 10886−10891.
(48) Wiegand, I.; Hilpert, K.; Hancock, R. E. W. Agar and Broth Dilution Methods to Determine the Minimal Inhibitory Concentration (MIC) of Antimicrobial Substances. *Nat. Protoc.* **2008**, *3*, 163−175.
(49) Fujimoto, Y.; Pradipta, A. R.; Inohara, N.; Fukase, K. Peptidoglycan as Nod1 Ligand; Fragment Structures in the Environment, Chemical Synthesis, and Their Innate Immunostimulation. *Nat. Prod. Rep.* **2012**, *29*, 568−579.
(50) Acebrón, I.; Mahasenan, K. V.; De Benedetti, S.; Lee, M.; Artola-Recolons, C.; Hesek, D.; Wang, H.; Hermoso, J. A.; Mobashery, S. Catalytic Cycle of the *N*-Acetylglucosaminidase NagZ from *Pseudomonas Aeruginosa*. *J. Am. Chem. Soc.* **2017**, *139*, 6795−6798.
(51) Zhang, Y.; Fechter, E. J.; Wang, T.-S. A.; Barrett, D.; Walker, S.; Kahne, D. E. Synthesis of Heptaprenyl−Lipid IV to Analyze Peptidoglycan Glycosyltransferases. *J. Am. Chem. Soc.* **2007**, *129*, 3080−3081.

https://doi.org/10.1021/jacs.3c12526
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