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Transglycosylases in peptidoglycan biosynthesis: advances in structure, function, and antimicrobial development

Xiao-Lin Zhang, Han Ding, Aoxin Guo & Xue-Wei Liu*

School of Chemistry, Chemical Engineering and Biotechnology, Nanyang Technological University, Singapore 637371, Singapore

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Peptidoglycan (PG), the essential exoskeleton in most bacteria, is synthesized through the action of bacterial transglycosylases (TGases), positioning these enzymes as elegant and desirable targets for antibiotic discovery. This review covers the major TGases involved in PG biogenesis, including TGases from the glycosyltransferase family 51 (GT51) and the newly discovered shape, elongation, division, sporulation (SEDS) family. We discuss the distinct roles of these two TGases during PG synthesis and emphasize the structural and catalytic differences, highlighting their coordination in PG assembly. Moreover, we summarize recent advances in TGase-involved antimicrobial strategies, including substrate-mimicking TGase inhibitors, PG terminators, and TGase-related immunological therapy targeting TGase from the GT51 family, and the first non-substrate-like TGase inhibitor against the SEDS protein. These valuable insights pave the way for the further development of novel TGase-related antimicrobial agents.

bacterial transglycosylase, antibiotic, glycosylation, peptidoglycan biosynthesis, lipid II

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1 Introduction

The rise of antimicrobial resistance poses a global health threat, driving the ongoing search for novel antibiotic agents [1]. Peptidoglycan (PG), the major structural component of bacterial cell walls, is crucial for maintaining cellular integrity against osmotic pressure. Present in most bacterial species yet absent in eukaryotes, PG biosynthetic pathways provide multiple desirable antibiotic targets [2,3]. Structurally, PG consists of linear glycan strands formed from alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc), each MurNAc bearing a branched peptide chain. In the final stages of PG assembly, transglycosylases (TGases) catalyze the incorporation of incoming glycosyl acceptor, Lipid II, into the reducing end of the growing glycan strand (glycosyl donor) to form the β-1,4-glycosidic bond and extend the glycan strands, while the mesh-like architecture can be forged through cross-linkages between peptide chains on neighbouring strands via transpeptidases (TPases) (Figure 1) [4,5]. Essential for cell viability and located at readily accessible cell surfaces, TPases have been extensively targeted by various antibiotics in use, most notably β-lactams, including the revolutionary penicillin discovered by Alexander Fleming in 1928 [6,7]. Nevertheless, bacteria have evolved increasing resistances against TPase-targeting antibiotics, necessitating the development of novel antibiotics with distinct mechanisms of action.

For decades, class a penicillin-binding proteins (aPBPs), the well-characterized members of glycosyltransferase family 51 (GT51), were acknowledged as the primary cellular machinery for synthesizing PG sacculus [2]. However, recent studies have led to the wide recognition of the principal roles

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*Corresponding author (email: xuewei@ntu.edu.sg)

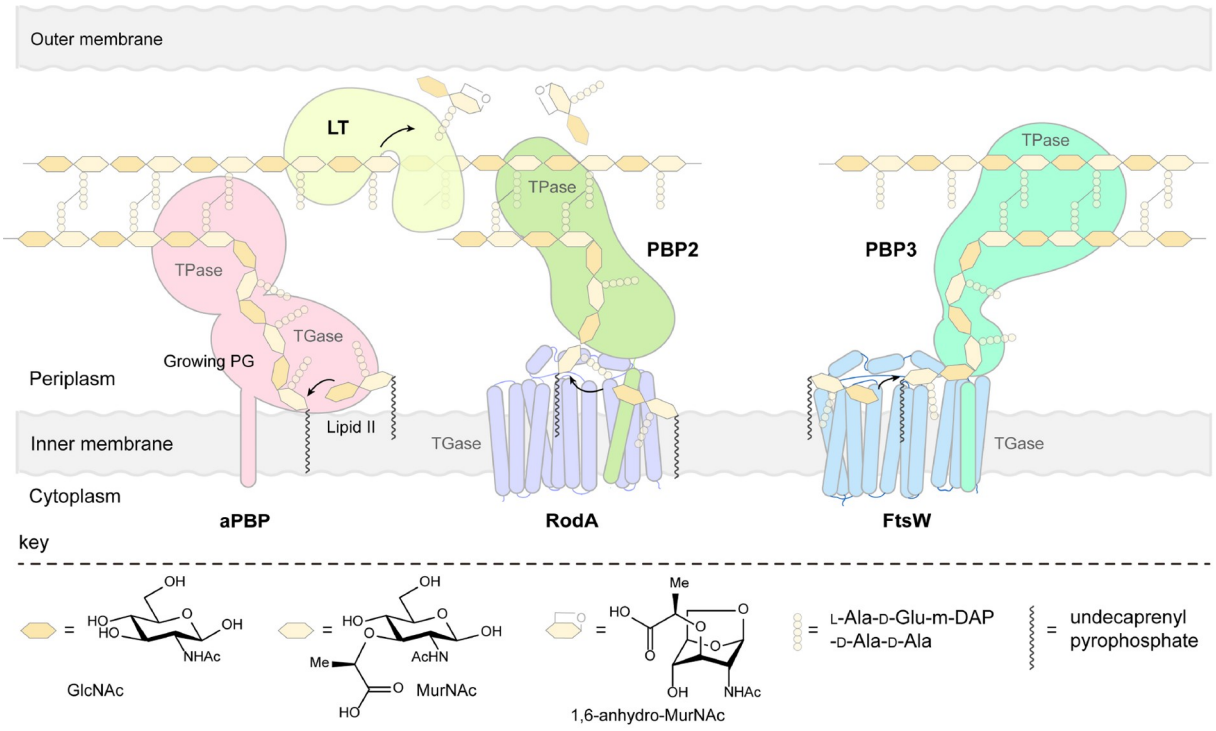


Figure 1 (Color online) Schematic of bacterial PG assembly in *Escherichia coli* (*E. coli*). PG glycan elongation is catalyzed by TGases in aPBPs and SEDS in elongasome and divisome (e.g., RodA and FtsW), meanwhile, TPases in aPBPs and cognate bPBPs (e.g., PBP2 and PBP3) crosslink the adjacent PG strands. During PG remodelling, lytic transglycosylases (LTs) catalyze the formation of soluble GlcNAc-1,6-anhydro-MurNAc-peptides.

of shape, elongation, division, and sporulation (SEDS) family TGases (Figure 1), which can drive the biosynthesis of scaffold and septal PG [8]. Unlike the well-explored antimicrobial target, TPase, the TGase-targeting antimicrobial agents are still under exploration, with limited reported antibiotics or inhibitors. To date, the moenomycin (Moe) family is the sole commercially available antibiotic, acting as the inhibitor that competitively binds to the TGase domain of PBPs and monofunctional transglycosylases (MGTs) from the GT51 family [9]. Nevertheless, Moe's poor pharmacokinetic profile, partially due to the long fatty acid chain, limits its utilization as a mere animal food additive rather than a drug for human use [10]. Meanwhile, few inhibitors against SEDS have been reported to date. Notably, TGases conserve among most bacterial species and rare resistance has been observed, suggesting the potential of TGases as promising alternatives to TPase targets. To advance TGase-specific antibiotics, enormous efforts have been devoted to understanding the TGase catalytic mechanisms and potential inhibitory modes. While previous reviews have extensively covered the structural activity and inhibition of aPBP TGases [2,11,12], this review will focus on recent advances in the coordination of aPBP TGases and newly discovered SEDS, summarize synthetic progress of TGase substrate mimics and derivatives, and emphasize the identification of TGase-related antimicrobial candidates based on action modes.

2 Bacterial transglycosylase in PG biosynthesis

In most bacteria, the biosynthesis of PG relies on two independent systems, aPBPs, and the SEDS family in complex with the partner TPases, class b penicillin-binding proteins (bPBPs), like RodA-PBP2 and FtsW-PBP3 pairs [13–17]. Recently, a new paradigm in PG synthesis has emerged, proposing that SEDS and their bPBPs partners are the principal PG synthases for constructing the cell wall exoskeleton complexes and directing cell elongation and division [18]. In this section, we discuss the distinct structures, catalytic modes, and functions of aPBPs and SEDS proteins in PG synthesis, highlighting their significance as potential antibiotic targets.

2.1 Class a penicillin-binding proteins

Structurally, aPBPs possess an *N*-terminal TGase domain that synthesizes long glycan chains. Meanwhile, the *C*-terminal domain binding to penicillin provides TPase activity necessary for cross-linking the PG strands [19]. Though the newly found SEDS family has forced us to reconsider the precise role of aPBPs in PG biosynthesis, it is worth mentioning that aPBPs remain essential for maintaining PG integrity during bacterial growth. For instance, Cho *et al*. [20] reported that the inactivation of aPBP activity reduced overall cell wall synthesis by approximately 80% in

Escherichia coli (*E. coli*), and showed that collaboration between PG synthases ensured the construction of a robust PG sacculus. This paradigm not only exists in rod-like Gram-negative bacteria but also presents in an oval-shaped Gram-positive bacterium, *Streptococcus pneumoniae* (*S. pneumoniae*), as reported by Straume *et al*. [21] who found that aPBPs (PBP1a and PBP2a) in *S. pneumoniae* functioned as an autonomous and independent machine to fully mature the primary PG synthesized by FtsW-PBP2X, rather than serving as the main enzyme for the nascent PG synthesis during the cell division and elongation. Beyond their basic functions, aPBPs can also repair cell wall defects and respond to stress. For instance, Vigouroux *et al*. [22] utilized fluorescent microscopy to real-time track the PBP1b expression, demonstrating that PBP1b in *E. coli* sensed and localized to damaged sites of cell wall, inserting PG in a need-based manner. In addition, Murphy *et al*. [23] probed the PG synthesis of *Vibrio cholerae* (*V. cholerae*) with endopeptidase (EP) insufficiency and found that aPBPs were required to maintain structural integrity and prevent cell lysis of the EP-insufficient *V. cholerae* cells.

It is important to note that not all bacterial species adhere to the general model of PG synthesis abovementioned. For instance, in *Acinetobacter baumannii* (*A. baumannii*), the PBP1A contributes to proper cell division; its absence induces cell chaining and multiple septal sites. Recently, Kang and co-workers [24] localized PBP1A in *A. baumannii* by fluorescence microscopy and demonstrated that PBP1A localized at the midcell during growth, likely coordinating with divisome proteins to synthesize septal PG. Notably, the PBP1A TGase mutant prevented its midcell localization, which implied the necessity of TGase activity for PBP1A in septum formation. In addition, their recent work indicated that the direct interactions between PBP1A and the divisome complex PBP3 supported septal PG synthesis in *A. baumannii*, potentially impacting the efficacy of the PBP3-targeting antibiotic therapies [25]. The role of aPBPs in cell divisome is also supported by Shrestha *et al*.'s recent work [26] on the endosporeforming bacteria, *Clostridioides difficile* (*C. difficile*) which demonstrated that unlike divisome pairs (FtsW-PBP3) in *E. coli* and *Bacillus subtilis* (*B. subtilis*), *C. difficile* utilized the bifunctional aPBPs (PBP1) as the core divisome PG synthase during sporulation and vegetative growth, indicating a unique target for potential antibiotics. Future work needs to focus on investigating the role of aPBPs and further testing models presented above.

2.2 Shape, elongation, division, and sporulation proteins

Rod-shaped bacteria employ two distinct PG synthesis pathways throughout the cell cycle: the elongasome and divisome, in which SEDS proteins serve as core TGases responsible for glycan strand extension, exemplified by RodA and FtsW in *E. coli* and *B. subtilis* (Figure 2) [14]. Decades ago, the SEDS protein, RodA, was first identified as contributing to the synthesis of PG sacculus pairing with PBP2 in *E. coli* PBP1b mutant by Ishino *et al*. [27]. However, in this work, instead of being identified as a type of new TGase, RodA was thought to regulate PBP2 TGase activity or combine with PBP2 acting as a PG synthase complex. Hence, the RodA system was neglected until recent studies revealed the pivotal role of the SEDS proteins as missing bacterial TGases in PG biosynthesis. Noticing the rod shape of *B. subtilis* unperturbed lacking aPBPs, Meeske *et al*. [18] reevaluated the biological role of SEDS proteins and examined the structural similarities between SEDS and known bacterial polymerases by homology and structural predictions, which demonstrated high structural similarity between SEDS and *O*-antigen ligases, the TGases that utilize undecaprenyl-PP-linked oligosaccharides for the lipopolysaccharide (LPS) synthesis in Gram-negative bacteria, suggesting the potential of RodA acting as PG TGases to utilize Lipid II. Notably, the overexpression of RodA ameliorated the growth defect of *B. subtilis* lacking all aPBPs, which was also observed by Emami *et al*. [28]. Instead of the structural prediction, Emami and co-workers employed a candidate gene approach and identified that RodA shared several conserved regions with other SEDS proteins. Remarkably, the *in vitro* enzymatic analysis of purified RodA with ~60% purity by Meeske *et al*. [18] strongly supported RodA as a PG TGase. In addition, the TGase activity of SEDS in *E. coli* was co-currently reported by Cho *et al*. [20], in which a novel *in vivo* assay for tracking PG polymerization was developed, demonstrating that RodA was the crucial PG polymerase in the Rod system, while aPBPs functioned outside of PG scaffold complexes. Moreover, the RodA homologue protein, FtsW, essential for septal PG synthesis during cell division, was recently purified by Taguchi *et al*. [29]. The *in vitro* assay depicted that the purified FtsW polymerized Lipid II into PG glycans when paired with its cognate bPBPs. Meanwhile, Reichmann also observed two SEDS-bPBPs cognate pairs, RodA-PBP3 and FtsW-PBP1 in *S. aureus*, which were responsible for sidewall and septal PG elongation, respectively [8]. The balance between these two cognate pairs is critical for maintaining bacterial morphology. These findings light up the role of SEDS in PG synthesis and revolutionize the paradigm of bacterial cell wall assembly.

Recent studies of the exemplary *E. coli* RodA-PBP2 functional pair structures, resolved by single-molecule FRET, cryo-electron microscopy (cryo-EM) and single-particle cryo-EM, have provided critical insights into their architecture and binding modes [14,30]. The resolved structures revealed that RodA is embedded in the inner membrane (IM) with 10 transmembrane (TM) helices and 3 small periplasmic juxta-membrane helices (PHs), while PBP2 features a single TM helix in the IM and a major

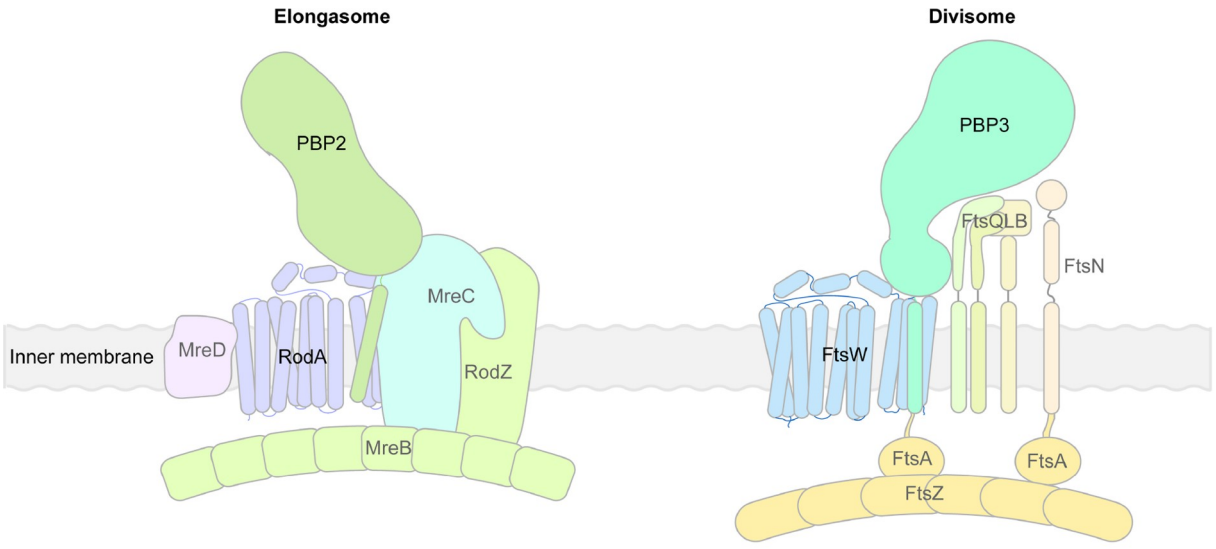


Figure 2 (Color online) Proposed models of the RodA-PBP2-directed elongasome complex (left) and FtsW-PBP3-directed divisome complex (right). In the elongasome complex, the cytoskeleton protein MreB and MreC sequester RodA-PBP2 pair and other accessory proteins, including MreD and RodZ, to form the complex. In the divisome complex, the PG biosynthesis is organized by the Z ring, FtsZ filaments, which promotes the recruitment and further activation of other divisome components like FtsA, FtsN and FtsQLB. In most bacteria, the elongasome complex and divisome complex collaborate to construct the cell wall exoskeleton.

extracellular domain with TPase activity protruding outward in the periplasm. The outer part of PBP2 shows significant mobility, transitioning from a compact apo form (40–50 Å from IM) to an extended open conformation (70–80 Å from IM) during catalysis. The TM helix in PBP2 interacts with the RodA TM helices (TM 8 and 9) side by side (interface 1), and the periplasmic main domain of PBP2 interacts with the top site PHs of RodA (interface 2) in the closed conformation suppresses RodA's TGase activity. The periplasmic domain is highly mobile and can adopt an open conformation, in which a pedestal region of the PBP2 interacts with MreC. The elongasome complex, comprising a RodA-PBP2 pair and accessory proteins like MreC, MreD, and RodZ, orchestrates bacterial cell elongation (Figure 2) [31]. The RodA-PBP2 assembles upon the RodZ, a bitopic IM protein, interacting with the cytoplasmic MreB cytoskeleton protein. This interaction enables the coupling of the elongasome to MreB filaments, which rotates dynamically along the cell circumference underneath the IM guided by RodA-PBP2 activity [32]. This movement guides sites of PG synthesis, which enables the elongasome to processively produce PG strands. In addition to PG elongation, septal PG synthesis is primarily carried out by the divisome, like FtsW-PBP3 pairs organized by the Z ring composed of tubulin-like FtsZ filaments (Figure 2) [33,34]. Structurally, FtsW is a TM protein with a central cavity facing the periplasm. This cavity, surrounded by PHs setting on the top of 10 TM helices, forms the plausible active site, where Lipid II is polymerized. Mutational studies have identified key conserved residues in these PHs that are critical for FtsW function. Interestingly, FtsW's TGase activity appears to be allosterically regulated through interactions with PBP3 and other divisome components, such as FtsN and the FtsQLB complex [35]. These interactions induce conformational changes that activate the polymerase function of FtsW, ensuring the synchronized synthesis and cross-linking of septal PG.

2.3 Structural and catalytic differences between aPBPs and SEDS-bPBP pairs

While functionally homologous, SEDS proteins and TGases in aPBPs are structurally distinct (Table 1, Figure 3). In general, both SEDS and aPBP TGases are partially immersed in IM and have separate binding sites for the Lipid II and the growing PG chain. However, aPBP TGase is anchored in IM by a single TM helix, while the main body of the SEDS is comprised of 10 TM helices anchored in IM, with additional 3 PHs. Moreover, the structures of substrate-binding cavities are distinct between aPBP TGases and SEDS as well. In aPBP TGases, the binding sites are located at the "jaw" region of a periplasmic globular domain composed of multiple α-helices and 5 conserved motifs. An α-helix separates the donor binding site from the TM helix, while the acceptor site, accessible from the IM surface, is located farther away from the TM helix in the "jaw" region, with a "flap" helix beneath. By contrast, this globular domain is largely absent in SEDS, replaced by smaller PHs. The donor and acceptor sites in SEDS proteins are separated cavities with sugar moieties of PG and Lipid II positioned on the top of TM helices, and the lipid tails interacting with different sides of the TM helices embedded in the IM.

Combined experimental and theoretical investigations

Table 1 Overview and comparison of three main TGases in PG biosynthesis

Category	aPBP TGase	RodA	FtsW
PDB ID	3VMA [19], 5HLB [36]	8TJ3 [14], 6PL5 [30], 6PL6 [13]	8BH1 [33], 8P1U [35]
Protein complex	PBP1b	Elongasome	Divisome (FtsWIQLB)
Bacterial species	*E. coli*	*E. coli* [14], *Thermus thermophilus* [13,30]	*P. aeruginosa*
Function in PG biosynthesis	Elongation repair and gap filling	Elongation scaffold	Division scaffold
Cell location	IM-anchored; Gaps in cell wall	Polytopic IM protein; Directed by MreB	Polytopic IM protein; Cell septum region
Cognate TPase	PBP1b TPase	PBP2	PBP3 (FtsI)
Contacting interfaces with cognate TPase	One between globular TGase and TPase domains	Two at the top and side of RodA, important for activity	Two at the top and side of FtsW, presumably important for activity
TM helices	One	Ten	Ten
Periplasmic Domains	Large globular head with multiple α-helices	3 PHs	3 PHs
Conserved motifs	Five motifs in the globular head	Five motifs in and between two binding sites	Presumably homologous to RodA, M257-I263 loop
Substrate binding sites	Adjacent but separate donor and acceptor sites at the globular head	Separate donor and acceptor sites	N.A., presumably separate donor and acceptor sites
Reducing end sugars of a donor substrate	Not directly in contact with TM helix; Pyrophosphate/phosphoglycerate and GlcNAc-MurNAc essential	In contact with the top regions of TM helices	N.A. Presumably in contact with the top regions of TM helices
Disaccharide of acceptor substrate	Not in contact with and further away from TM helix; AnhydroMurNAc analogue binding with comparable affinity [39]	In contact with the top regions of TM helices at a different site	N.A. Presumably in contact with the top regions of TM helices at a different site
Lipid tail of donor substrate	Interacting with TM helix; >20 C length [40]	Interacting with TM helices; >30 C length [41]	Interacting with TM helices; >30 C length [41]
Lipid tail of acceptor substrate	Not directly interacting with TM helix; >16 C length [42]	Interacting with TM helices; >20 C length [41]	Interacting with TM helices; >20 C length [41]
Peptide of donor substrate	Lactoyl group essential; pentapeptide not directly involved in binding interactions	NA	N.A.
Peptide of acceptor substrate	Nonessential, dipeptide or peptide-free analogues binding with comparable affinities [39,42]	NA	N.A.
Donor site key interacting amino acids (AAs)	Arg^{286} and Lys^{274}	Arg^{210}	N.A.
Acceptor site key interacting AAs	Arg and Lys residues	Arg^{48}, Arg^{109}	N.A.
Essential catalytic residue	Glu^{233}, conserved at the acceptor site, stabilized by nearby Lys^{355} and universally conserved Arg^{237}	Asp^{262} at periplasmic loop 4, conserved and located between two binding sites	Asp^{275} located in a deep, highly conserved cleft
Prosthetic metal cation	Mg^{2+}	None	None
Elongation kinetics	Processive; *E. coli* PBP1a might also involve distributive mechanism [43]	Processive	N.A. Presumably processive;
Specific inhibitors	Moe and Lipid II analogues	None known to date	Compound **5-6** tested on *S. aureus* FtsW [44]

suggest that both SEDS and TGase domains in aPBPs synthesize PG in a processive manner [30,36,37], meaning that after adding disaccharide units of Lipid II, to the growing PG chain, the elongated PG chain translocates within the TGase complex to the donor site to free up the acceptor site for new incoming Lipid II acceptors to repeat the cycle, without dissociating from the TGase in each step. In aPBPs, TGase reaction depends on prosthetic Mg^{2+} cations coordinated

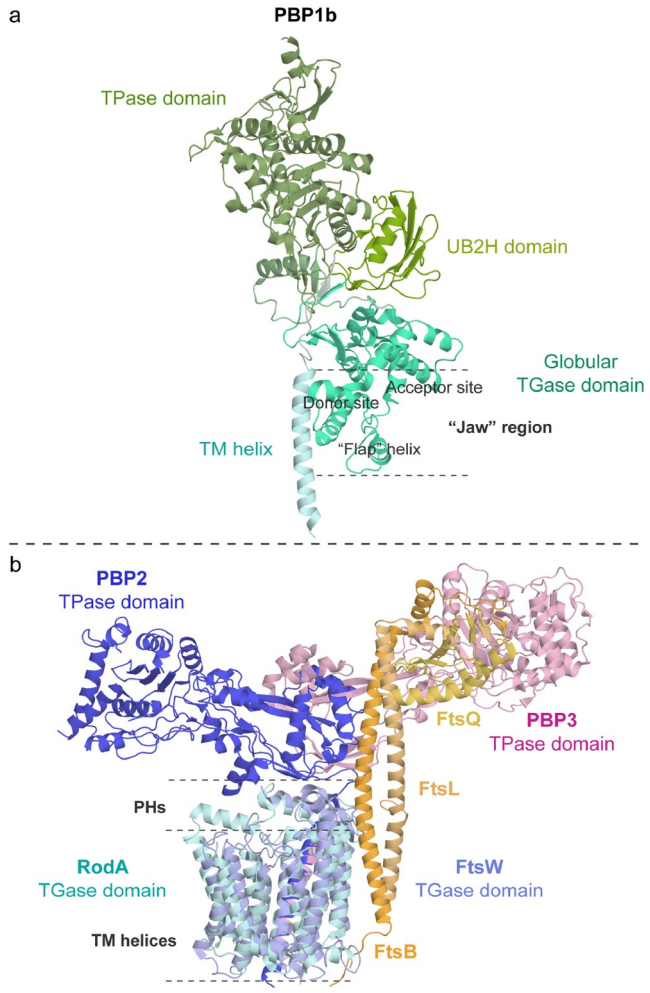


Figure 3 (Color online) Structural comparison of aPBP and SEDS-bPBP pairs. (a) Structure of PBP1b, an essential aPBP in *E. coli*, consisting of one IM-inserted TM helix in palecyan, and periplasmic regions comprising a globular TGase domain in greencyan, a TPase domain in smudge, and a PBP1b regulator binding domain, UB2H, in splitpea. In the "jaw" region of the globular TGase domain, the glycosyl donor and acceptor site are separated by an α-helix. (b) Structures of RodA-PBP2 and FtsW-PBP3 pairs. The TGase proteins, RodA in palecyan and FtsW in lightblue insert in IM and share similar structural features, such as 10 TM helices and 2–3 PHs, while the periplasmic regions, bPBPs, including PBP2 in blue and PBP3 in lightpink, are structurally distinct. Additionally, FtsQLB interacts with FtsW to regulate its TGase activity. All protein structures are shown as a ribbon.

within the binding cavity, with the deprotonated glutamate residue in the acceptor site or possibly a Mg^{2+}-bound hydroxide anion acting as the general base to deprotonate MurNAc 4-OH and promote the nucleophilic attack to form the glycosidic linkage [36,38]. However, SEDS TGases are metal-independent, using Asp^{262} in a conserved motif on PH adjacent to the acceptor site featuring the general base (Table 1) [14]. The mechanistic distinctions between SEDS and TGases in aPBPs highlight their complementary roles in PG synthesis, though details of the catalytic cycle are still being explored.

3 Advances in TGase-involved antimicrobial strategies

TGase-related antibiotics have been developed for decades due to the conservation of TGases among various bacteria and absence in eukaryotes. Due to misconceptions about the role of SEDS TGases, TGase-targeting inhibitors explored to date have mainly focused on TGases from GT51, like aPBPs and MGTs, including Moe and Lipid II substrate analogues, and non-sugar inhibitors identified by high-throughput screening, the effectiveness of which has been well-discussed in several reviews [2,3]. Here we summarize recent advances in antibiotic candidates towards these essential bacterial TGases, including their synthesis, antimicrobial efficacies and plausible bacteria-killing modes.

3.1 Substrate-like TGase inhibitors

According to the structure similarity and biological source, reported substate-mimicking TGase inhibitors are divided into two clusters: (1) Moe and its analogues; (2) Lipid II analogues. Since no literature has been reported on substrate-like inhibitors of TGases from the SEDS family, TGases discussed in this section belong to the GT51 family, unless specified.

3.1.1 Moe and its analogues

Moe are phospholipid antibiotics first found in secondary metabolites of *Streptomyces ghanaensis* (*S. ghanaensis*) [45,46], which exhibit excellent antimicrobial effects on multiple-resistant bacteria, such as methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *Enterococcus faecalis* (*E. faecalis*) [10]. Despite the achievements of chemical total synthesis [47], Moe is mainly produced through submerged fermentation by *Streptomyces* in industry due to its complex structure. Several attempts have been made to improve Moe yields. For example, the recent work by Li *et al*. [48] recruited an adaptive optimization strategy to refactor the strong promoter and overcome the physiological damage in *Streptomyces albus*, enhancing Moe production to 40.0 mg/L. Moreover, Makitrynskyy *et al*. [49] recently utilized genetic engineering approaches to optimize the growth of *Streptomyces ghanaensis*, demonstrating the Moe accumulation.

In the past few years, the structural-activity relationship (SAR) studies of Moe have been thoroughly explored for elucidating its antimicrobial core. These findings have guided the design of numerous Moe analogues aiming at overcoming the poor pharmacokinetics and complex preparation, as extensively summarized in many reviews [2,10]. Nevertheless, the limited resolved structures of Moe analogue-bound TGase structure remain a key obstacle for further mechanistic investigations and clinical application of Moe.

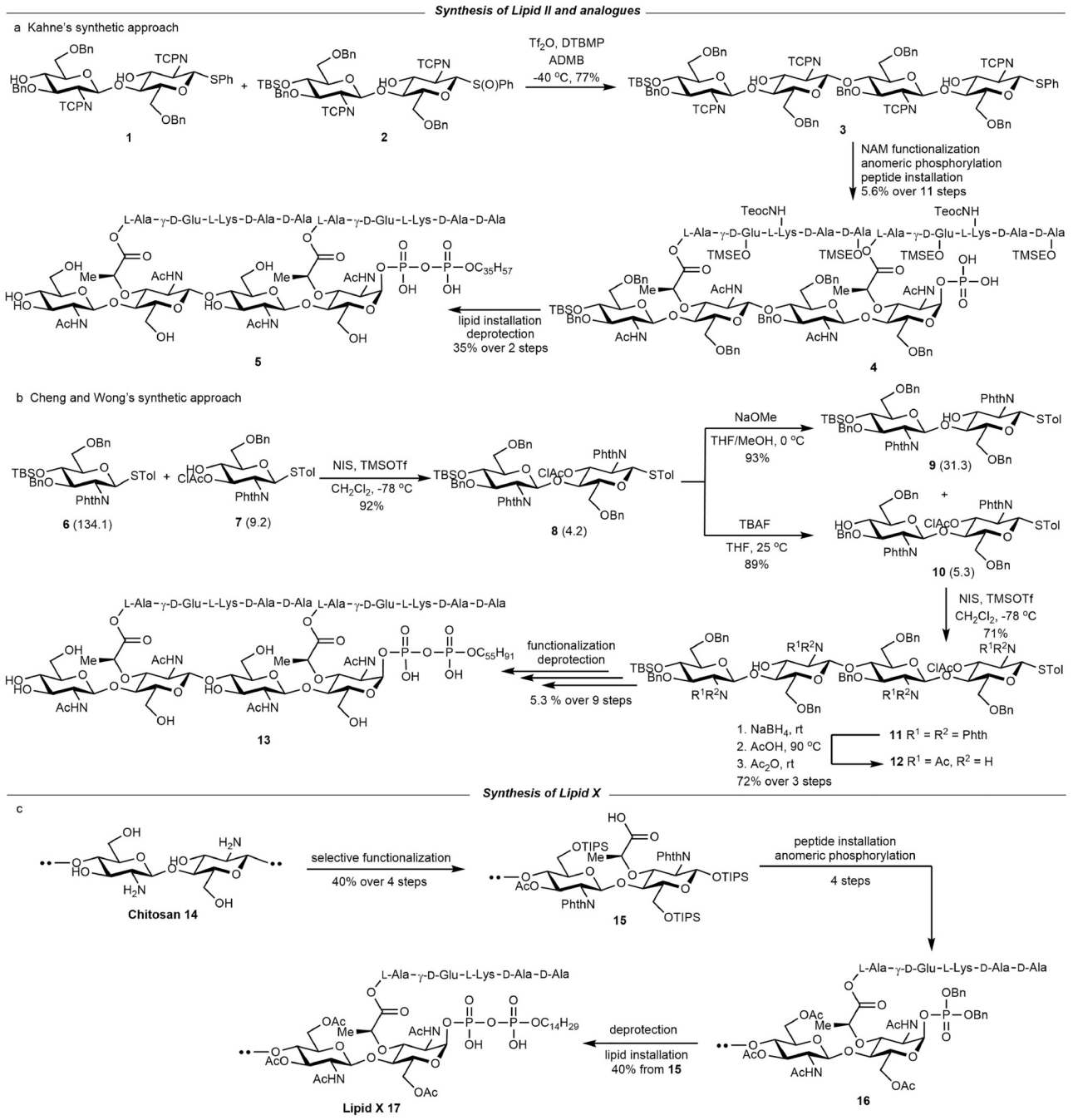
Till now, the inhibitory mechanism of Moe on TGase has been explored by several researchers. Due to the similar structure between Moe and Lipid II, Moe was initially presumed to perform as the Lipid II competitor and bind to the acceptor site of PG TGases, preventing the PG glycan biosynthesis. Surprisingly, Moe was found to occupy the donor site, the same as Gal-Lipid IV which overcame the Moe inhibitory activity *in vitro*, as reported by Gampe *et al*. [50]. This finding was also supported by the resolved TGase-Moe crystal structure, which confirmed that MoeA bound to the donor site of MGT [51]. It was reasonable that Moe inhibitory activity was rescued by a high concentration of Lipid IV rather than Lipid II, because Lipid II required a longer time to activate TGase and initiate the PG elongation than Lipid IV reported by Wang *et al*. [52]. Moreover, Gampe *et al*. [50] also found that Moe would not interfere with the PG glycan distribution through sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, implying that Moe blocked the TGase reaction initiation rather than elongation. Taken together, Moe can competitively bind to the TGase donor site with growing PG strands and inhibit the glycan chain initiation though some debates still exist. Arbeloa *et al*. [53] observed Moe resistance in an *E. faecalis* mutant lacking non-essential *ponA* and *pbpF*, suggesting that Moe interfered the aPBPs-PG complexes rather than merely inhibiting TGases activity. Despite these fantastic results about the inhibiting mechanisms of Moe, the detailed binding mode of Moe to TGase remains undetermined and calls for further investigation.

3.1.2 Lipid II analogues

Another attractive direction for developing potential TGase inhibitors is the Lipid II and Lipid IV mimics, which are proposed to be recognized by TGase and interfere with PG glycan elongation. Given the important role of Lipid II in antibiotic development, the complex PG subunit has garnered significant synthetic interest. Schwartz and co-workers [54] were the first to achieve the total synthesis of Lipid II, which they accomplished in 12 steps with an overall yield of 0.7%, starting from a protected MurNAc. This method introduced alanine to the lactic acid moiety for successful glycosylation, preventing the formation of lactone side products. Moreover, the phthalimido group (PhthN) ensured complete β-selectivity as a stereodirecting element. In the same year, van Nieuwenhze and co-workers [55,56] also published their works on the assembly of the GlcNAc-MurNAc disaccharide, completing the synthesis of Lipid II with similar transformations. Addressing challenges posed by the aggregation tendency of natural Lipid II caused by the C55 undercaprenyl chain, Ye *et al*. [57] developed a chemoenzymatic method to synthesize natural Lipid II and analogues with different modified lipid chains by an *E. coli* MurG-catalyzed glycosylation reaction of various Lipid I derivatives using radiolabeled UDP-GlcNAc as the donor.

The synthesis of Lipid IV is notably difficult due to challenges in forming the β-1,4-glucosaminyl bond with the 2-deoxy-2-glucosaminyl acceptor, which acts as a weak nucleophile. Early work by Mobashery *et al*. [58] employed a stepwise, bidirectional strategy, enabling the assembly of GlcNAc-MurNAc tetrasaccharides and ultimately leading to more complex octasaccharide structures. Kahne *et al*. [59] achieved the first total synthesis of heptaprenyl Lipid IV (Scheme 1a). The synthesis employed the Kahne glycosylation method, utilizing glycosyl sulfoxide donors to form β-1,4-glycosaminyl linkages and assemble a tetrasaccharide core [43]. In 2011, Wong *et al*. [60] introduced an alternative route to the key tetrasaccharide, utilizing the concept of relative reactivity values (RRV), which was controlled by the protecting group pattern of thioglycosides and enabled efficient tetrasaccharide synthesis and Lipid IV assembly (Scheme 1b) [61]. More recently, our group developed a strain-release glycosylation method using glycosyl ortho-2,2-dimethoxycarbonylcyclopropylbenzoates (CCBz) donors catalyzed by $Sc(OTf)_3$. We applied this method to the formal synthesis of Lipid IV, utilizing a [(1 + 1) + 2] glycosylation strategy and permitting gram-scale synthesis of Lipid IV tetrasaccharides with minimal side reactions [62]. The direct utilization of biomass to generate structurally well-defined molecules holds significant promise for accelerating the production of these valuable compounds. In this context, our group developed a top-down synthesis of biohybrid peptidoglycan oligomers (PGOs) from the inexpensive and readily available chitosan (Scheme 1c). We devised a nine-step process to convert chitosan into PGOs mimicking the natural structure of PG with alternating GlcNAc-MurNAc pattern [63]. These easily obtained PGOs can be recognized and incorporated into bacterial cell walls, enabling bacterial imaging with high specificity.

Several series of Lipid II to Lipid X analogues have been prepared by the aforementioned approaches for developing novel TGase inhibitors, as described in related reviews [2,3]. These modifications primarily adjust the lipid tail, disaccharide, and stem peptide moieties (Figure 4a). The hydrophobic lipid tail on Moe and Lipid II analogues essential for substrate binding renders their poor pharmacokinetics. It was found that longer sugar moieties compensated for the reduced binding affinity caused by the shortened lipid tail and restored their antimicrobial efficacy, indicating complex sugar modifications may represent a new avenue for optimizing lipid-like antibiotics [42,63]. Moreover, a Lipid II analogue with the C4-epimerized GlcNAc was found to effectively inhibit *E. coli* PBP1b and *C. difficile* PBP1b, as reported by Chen *et al*. [64]. Beyond modifying Lipid II, Wang and co-workers [65] designed an iminosugar mimic of the Lipid II intermediate at the transition state during the TGase reaction, which demonstrated good inhibition of



Scheme 1 Summary of strategies for synthesizing Lipid IV (a, b) and Lipid X analogues (c). More detailed information can be referred to the substrate-like TGase inhibitors section of the review.

A. baumannii TGase. These findings underscore the promise of substrate-like TGase inhibitors and offer valuable insights for the development of TGase-targeting antibiotics.

3.2 Substrate-like PG terminator

Over the years, investigations into TGase-specific antibiotics have predominantly focused on two key objectives: (1) Moe pharmacokinetics optimization; (2) the design of new saccharide or non-saccharide TGase inhibitors. Recently, our group found non-canonical TGase acceptors as the PG terminators, GlcNAc-1,6-anhydro-MurNAc-pentapeptide and its analogue without peptide, which were recognized by MGT and incorporated into the growing PG strands *in vitro* though lacking the reducing end and the phospholipid chain (Figure 4b) [39]. GlcNAc-1,6-anhydro-MurNAc-peptides are natural PG fragments produced by lytic transglycosylases (LTs) during PG recycling in Gram-negative bacteria (Figure 1) [66–68]. Such GlcNAc-1,6-anhydro-MurNAc-peptides and their analogues have attracted significant synthetic in-

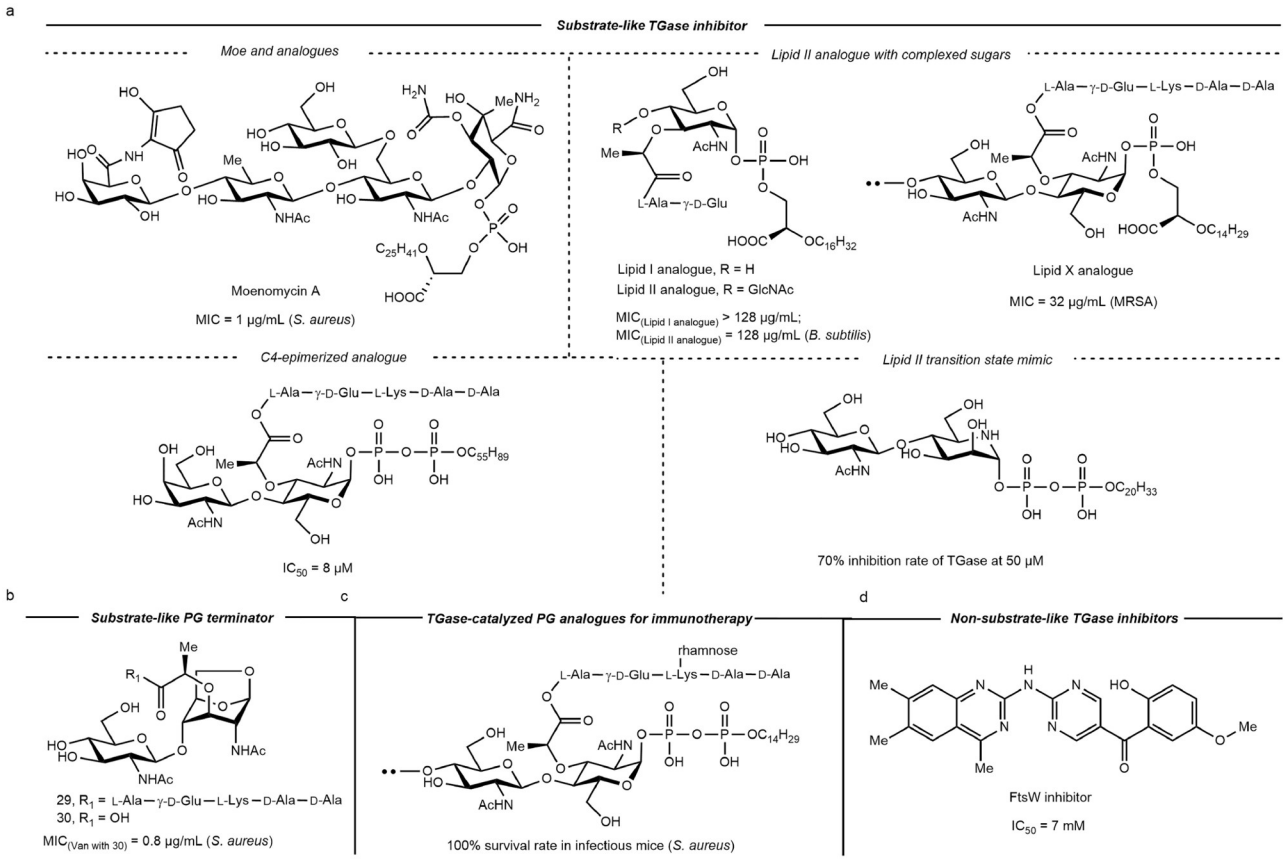
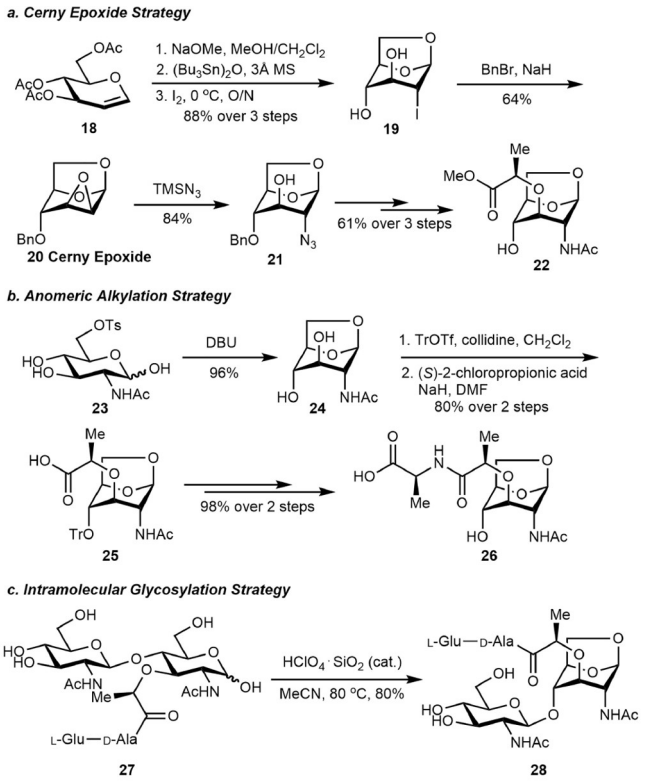


Figure 4 Summary of TGase-involved antimicrobial candidates. (a) Substrate-like aPBP and MGT TGase inhibitors. Moenomycin A is the typical compound from the Moe family. (b) Substrate-like PG terminator. (c) TGase-catalyzed PG analogues for immunotherapy. (d) The only reported FtsW inhibitor. Brief descriptions of these compounds on their antimicrobial activities are shown in the figure. More detailed information can be referred to the advances in antimicrobial strategies section of the review.

terest, with their important role in bacterial β-lactam resistance and antibiotic development [69–71]. Access to these constructs has often relied on the Černý epoxide, a pivotal intermediate in carbohydrate synthesis developed by Černý and co-workers [72]. The Černý epoxide has facilitated the synthesis of GlcNAc-1,6-anhydro-MurNAc bearing a stem *L*-Ala-*D*-Gln dipeptide reported by Paulsen *et al*. [73,74]. This approach was further improved by Schmidt and co-workers, and such advanced modifications have further been used by Mobashery, Fukase, and our group to synthesize these valuable molecules (Scheme 2a) [39,75,76]. In addition to the widely utilized Černý epoxide strategy, the 1,6-anhydro-MurNAc ring can also be alternatively formed by intramolecular glycosylation and anomeric alkylation. Nikitushkin's group [77] achieved 1,6-anhydro muropeptides through an intermolecular cyclization reaction by employing a dehydrative cyclization mechanism (Scheme 2b). Very recently, Grimes detailed the synthesis of 1,6-anhydro-MurNAc-peptides, in which 1,6-anhydro GlcNAc was efficiently prepared in a single step through anomeric alkylation using DBU as the base with *O*-6 tosylate (Scheme 2c) [78].

Although prior studies of anhydromuropeptides emphasized their important roles in PG recycling and immune regulation, our group very recently identified GlcNAc-1,6-anhydro-MurNAc-peptides and its analogue lacking the pendant peptide motif as potential antimicrobial agents [39]. We utilized an *in vitro* enzymatic assay to investigate the ability of GlcNAc-1,6-anhydro-MurNAc-pentapeptide as the substrate of PG synthases. Considering the equipment of pentapeptide, we employed the GlcNAc-1,6-anhydro-MurNAc-pentapeptide and extracted Lipid II for TPase-catalyzed cross-linking. As confirmed by liquid chromatography-high resolution mass spectrometry (LC-HRMS) analysis, the anhydromuropeptide bearing 1,6-anhydro-moiety was recognized by TPase and cross-linked with the growing PG strand. Surprisingly, a glycosylated product, a tetrasaccharide fragment containing anhydromuropeptide and Lipid II, was also detected, which indicated that GlcNAc-1,6-anhydro-MurNAc-pentapeptide can be recognized and utilized by TGase to incorporate into the PG strand. Further SAR study and docking analysis depicted that even anhydrodisaccharide without pentapeptide was recruited by TGase with higher reactive activity, of which the Michaelis-Menten constant (K_m) value was 217.9 μM, indicating rea-



Scheme 2 (Color online) Summary of strategies for synthesis of 1,6-anhydro-MurNAc analogues.

sonable TGase activity. Due to lacking the necessary reducing end for continued Lipid II coupling, we hypothesized that the anhydrodisaccharide might terminate the PG glycan strand elongation, which was supported by SDS-PAGE analysis of the glycan strand distribution, demonstrating that the ratio of longer glycan strand was reduced from 60% to 48%, dependent with the increasing addition of the anhydrodisaccharide. More importantly, the preliminary antimicrobial evaluation was also attempted by investigating the adjunctive function of this anhydrodisaccharide with a well-known glycopeptide antibiotic, vancomycin, which inhibits the formation of cross-linkages. *In vitro* co-incubation analysis demonstrated that the anhydrodisaccharide decreased the minimum inhibitory concentration (MIC) value of vancomycin (1.6 to 0.8 μg/mL) at a high concentration. Unlike Moe inhibiting the TGase initiation, these findings indicate the potential of GlcNAc-1,6-anhydro-MurNAc as an effective PG terminator for further antibiotic development.

3.3 TGase-catalyzed PG analogues for immunotherapy

Inspired by our previous work that the synthetic Lipid X successfully incorporated into various bacterial cell walls by TGases [63], we continued to design antibody-recruiting subtle PG analogues featuring haptens attached to the amine group of *L*-Lys in the stem peptide, aiming to congregate antibodies to the cell surface (Figure 4c) [79]. As expected, these PG analogues effectively engineered cell wall surfaces of pathogenic bacteria by TGases, including *S. aureus* and *Pseudomonas aeruginosa* (*P. aeruginosa*), and the flow cytometry analysis demonstrated the efficient binding of FITC-IgG from human serum to bacterial surfaces, which promoted the phagocytosis and subsequent bacterial clearance with the assistance of macrophages. In addition, similar results were observed in the mice model, where the *S. aureus* modified with PG analogues was successfully cleared from different organs by leveraging the pre-inducing antibodies, without stimulating excessive immune responses. Notably, treatment with a PG analogue bearing *L*-rhamnose significantly improved survival rates in the infected mice, which rescued all infectious mice and outperformed the commonly used kanamycin. While comprehensive pharmacokinetic profiles have yet to be fully evaluated, these PG analogues depicted remarkable safety profiles with the negligible toxicity to mammalian cells *in vitro*, suggesting their good bioavailability for further clinical evaluation. These findings underscore the potential of PG analogues-based immunotherapeutic strategies for combating pathogenic bacterial infections.

3.4 Non-substrate-like TGase inhibitors

Increasing studies for the SEDS family has recently boomed out as several reports unveil the importance of SEDS in PG biosynthesis and its potential as a new antibiotic target since 2016. Notably, in 2003, McPherson and Popham [80] demonstrated that a *B. subtilis* mutant lacking all aPBPs showed resistance to Moe, indicating the distinct catalytic pockets between aPBPs and RodA. This finding was later supported by Meeske and co-workers [18], who depicted that the RodA induction restored Moe resistance in the Moe-sensitive *B. subtilis* mutant, further confirming the intrinsic resistance of RodA to Moe. These insights underscore the need to move beyond traditional Moe-like TGase inhibitor and explore alternative antimicrobial strategies for advancing the discovery of novel RodA-targeting antibiotics.

Despite recent progress in their structural basis, only one SEDS-specific inhibitor has been identified by Park *et al.* [44] so far (Figure 4d). They developed a fantastic time-resolved Förster resonance energy transfer (FRET) assay for high-throughput screening TGase activity of FtsW and identifying its inhibitors *in vitro*. The time-resolved FRET assay utilized two Lipid II derivatives, Lipid II-2,4-dinitrophenyl (Lipid II-DNP) and Lipid II-biotin, and generated the FRET signals by the addition of DNP antibody and streptavidin XL665. By this approach, they screened 900 compounds known to inhibit *S. aureus* growth and recognized ten compounds that decreased TGase activities of FtsW to <30% of normal. Further binding analysis indicated

that one of these compounds competitively bound the donor site of FtsW with Lipid II. However, the detailed binding mode was unclear, and the *in vitro* antimicrobial efficacy and the FtsW-inhibiting mode were not assessed. Despite these limitations, this report provides a promising direction for further optimization of SEDS-targeting inhibitors. Future efforts should focus on elucidating molecular interactions and enhancing their antimicrobial potency *in vivo*, paving the way for facilitating the clinical translation of these inhibitors.

4 Summary and outlooks

In this review, we discuss two major bacterial TGases involved in PG assembly, including TGases in aPBPs from the GT51 family and the newly discovered SEDS proteins. We compare their distinct structures, catalytic modes and functional roles during PG biogenesis, emphasizing their complementation in synthesizing strong PG sacculus. In addition, we describe the synthetic strategies of TGase substrates and analogues for SAR studies and the potential antimicrobial agents search and summarize recent advances in TGase-involved antimicrobial candidates based on their action modes, including substrate-like TGase inhibitors, substrate-like PG terminators, and TGase-related immunological therapy targeting TGase from GT51 family, and the sole reported non-substrate-like TGase inhibitor against the SEDS protein. These valuable insights provide the fundamental understanding for further designing attractive TGase-related antimicrobial strategies with new mechanisms.

Unlike the widely used TPase-targeting antibiotics, the discovery of TGase-related antibiotics remains an unmet challenge, with no clinically approved drugs to date. Endeavours have extended beyond the optimization of TGase substrate-like inhibitors, such as Moe and Lipid II analogues, to exploring innovative strategies, including identifying novel PG terminators, combining TGase reaction with immunotherapy, as well as screening new inhibitors for newly discovered SEDS TGases. Although the development of TGase-targeting antibiotics has seen remarkable progress including improving their pharmacokinetic properties and involving novel antimicrobial mechanisms, translating these advances into clinical therapies remains challenging. The progress of substrate-like TGase antibiotics is constrained by the lack of detailed understanding of TGase-inhibitor binding interactions, which are essential for advanced antibiotic optimization. Both TGase domains in aPBPs and SEDS are TM proteins with one to ten TM helices which are indispensable for mimicking the natural catalytic structures of TGases but complicate crystallographic studies due to their flexibility. Furthermore, most reported TGase inhibitors and PG terminators exhibit moderate binding affinities, resulting in low occupancy and further poor structural characterization. This, in turn, limits their antimicrobial efficacy, posing significant obstacles to advanced clinical trials.

In addition, although a few *apo* forms and complex structures of TGases have been resolved by X-ray diffraction and cryo-EM, the dynamic catalytic process of TGases also remains incompletely understood, impeding the further improvements of TGase inhibitors and PG terminators. For example, despite TGases being widely recognized to function in a processive manner, their conformation changes during catalytic circles remain speculative. Moreover, the flap region in aPBP TGases has been proposed to push the glycosylated products to the donor site due to its flexibility reported by Walker *et al.*; however, the poor structural resolution of this region in the crystal hampers a comprehensive understanding of its role. These insights into the catalytic circle are critical for guiding the design of novel antibiotics. By leveraging the catalytic mechanism, we can develop intermediate compounds that inhibit the TGase conformational change or restrict flap movement, thereby impeding bacterial cell wall synthesis.

The realization of the principal role of SEDS family TGases has revolutionized our understanding of peptidoglycan biogenesis, raising new questions about how these enzymes work in coordination with aPBPs during cell elongation and division. Meanwhile, the coexistence of two distinct TGase families suggests that the inhibitor designs based solely on one family may inadvertently promote the emergence of new antibiotic resistance. Hence, detailed structural studies of both TGases are required to achieve a more comprehensive understanding of their collaboration and provide valuable insights for antibiotic development.

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