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New Continuous Fluorometric Assay for Bacterial Transglycosylase Using Förster Resonance Energy Transfer

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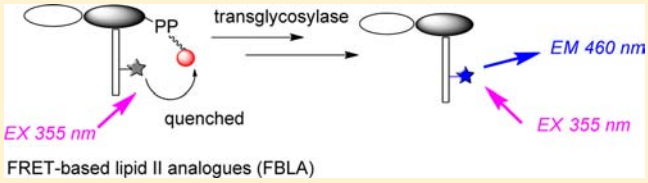
Supporting Information

ABSTRACT: The emergence of antibiotic resistance has prompted scientists to search for new antibiotics. Transglycosylase (TGase) is an attractive target for new antibiotic discovery due to its location on the outer membrane of bacteria and its essential role in peptidoglycan synthesis. Though there have been a few molecules identified as TGase inhibitors in the past thirty years, none of them have been developed into antibiotics for humans. The slow pace of development is perhaps due to the lack of continuous, quantitative, and high-throughput assay available for the enzyme. Herein, we report a new continuous fluorescent assay based on Förster resonance energy transfer, using lipid II analogues with a dimethylamino-azobenzenesulfonyl quencher in the lipid chain and a coumarin fluorophore in the peptide chain. During the process of transglycosylation, the quencher-appended polyprenol is released and the fluorescence of coumarin can be detected. Using this system, the substrate specificity and affinity of lipid II analogues bearing various numbers and configurations of isoprene units were investigated. Moreover, the inhibition constants of moenomycin and two previously identified small molecules were also determined. In addition, a high-throughput screening using the new assay was conducted to identify potent TGase inhibitors from a 120,000 compound library. This new continuous fluorescent assay not only provides an efficient and convenient way to study TGase activities, but also enables the high-throughput screening of potential TGase inhibitors for antibiotic discovery.



FRET-based lipid II analogues (FBLA)

INTRODUCTION

Discovery of new antibiotics is of current interest due to the emergence of drug-resistant pathogenic microorganisms.[1] Peptidoglycan (PG), a main component of the bacterial cell wall, is essential for bacterial growth, and therefore the relevant enzymes have been recognized as targets for antibiotic discovery. PG is formed by polymerization of lipid II via transglycosylation followed by cross-linking of the pentapeptide side chain via transpeptidation. The process is catalyzed by a bifunctional penicillin-binding protein (PBP)[2] which contains a transglycosylase (TGase) domain for the transglycosylation and a transpeptidase domain for the transpeptidation reaction (Figure 1A).[3] Because PBP catalyzes the last step in PG formation and is located on the bacterial surface, it has been a target for antibiotic development. Over the past 50 years, many antibiotics such as β-lactam derivatives have been developed to target the transpeptidase. In contrast, with the exception of the well-known moenomycin for animal use, only a few small molecules have been identified to inhibit the transglycosylation process,[4] and none of them have been successfully developed as an antibiotic for humans. One of the reasons for the slow progress in development of new antibiotics targeting TGase is the lack of convenient assays for discovery of new TGase inhibitors.

Currently, limited approaches are available for the evaluation of small molecules targeting bacterial TGase or bacterial cell wall synthesis. The classical TGase assays using radioactive lipid II combined with thin-layer chromatography are useful for enzymatic characterization of TGase but difficult to obtain quantitative results.[5,6] Alternatively, the PG formed by polymerization of [^{3}H]-labeled lipid II can be captured by wheat germ agglutinin-coated scintillation proximity assay beads, and the binding event can be translated into a quantitative measurement.[7] Nonetheless, the assays rely on radioactive lipid II which cannot be operated in a general laboratory without special setup. Recently, surface plasmon resonance (SPR)-based methods have been developed to identify TGase inhibitors either by measuring the competition of a given molecule for moenomycin[8] or by measuring the direct binding affinities of small molecules to PBP.[9] Though SPR can provide quantitative analysis, it is a low-

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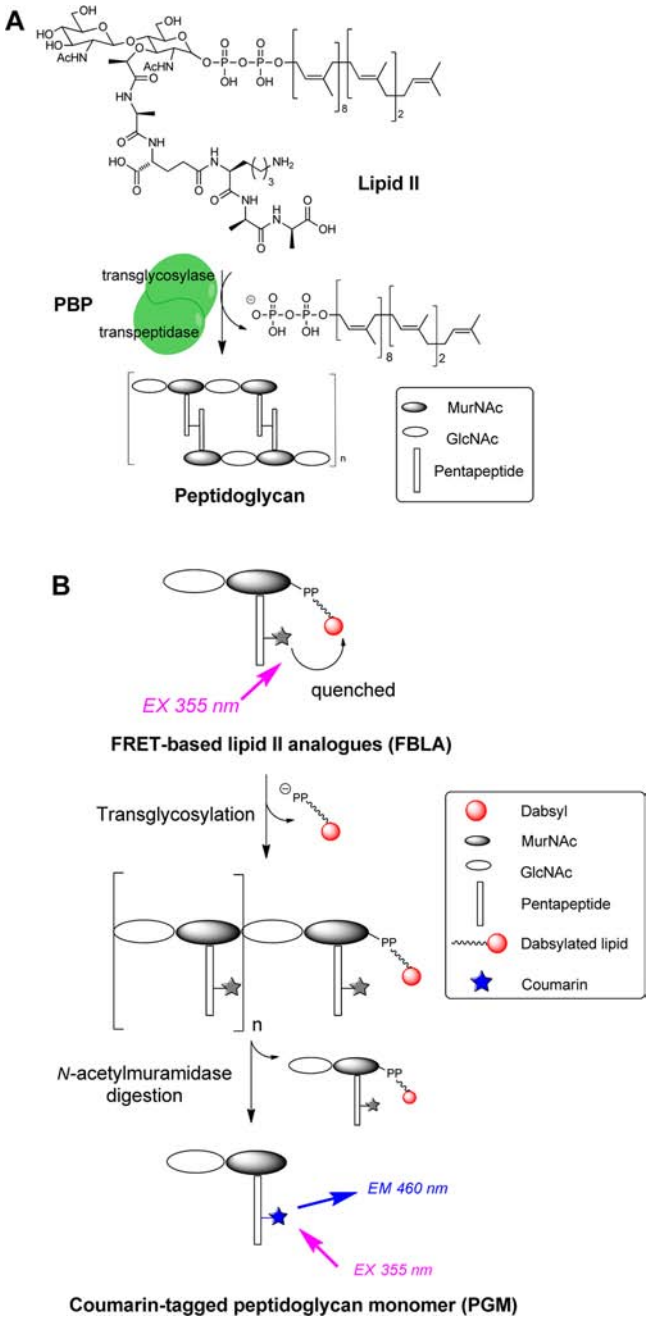

Figure 1. Peptidoglycan biosynthesis in bacterial cells (A) and the proposed FRET-based TGase activity assay (B). (A) Biosynthesis of peptidoglycan catalyzed by bifunctional penicillin-binding protein (PBP). The polymerization unit lipid II has a disaccharide GlcNAc-MurNAc unit with a undecaprenyl pyrophosphate and a pentapeptide stem linked to MurNAc. Lipid II is polymerized into peptidoglycan by PBP containing individual transglycosylase and transpeptidase domains for transglycosylation and transpeptidation, respectively. (B) Schematic representation of FRET-based TGase activity assay. FBLAs are designed by attaching a fluorophore to the peptide stem and a quencher to the lipid chain. In intact FBLAs, the fluorescence would be quenched by the quencher and showed low background. Upon polymerization of FBLAs in the presence of active TGases, dabsylated lipid is liberated and the fluorescence is no longer quenched. After digestion of the peptidoglycan (PG) polymer by *N*-acetylmuramidase, the coumarin-tagged PG monomers show fluorescence at 460 nm upon irradiation with 355-nm light.

to-medium throughput assay, and often requires interventions and considerable time to complete analysis of a small group of compounds. For enzymatic activity analysis, it is common to use fluorophore-tagged lipid II as TGase substrates. The polymerized products were then analyzed by HPLC[10] or by slight fluorescence changes that might be monitored with a detector.[11] However, the signal changes are often not robust enough to be adapted for high-throughput analysis. Development of a sensitive high-throughput and quantitative analysis of TGase is thus necessary in order to facilitate the identification of TGase inhibitors.

Fluorescence anisotropy (FA)-based high-throughput assay that measures the FA change of fluorescent moenomycin[9] or moenomycin pharmacophore[12] upon binding to TGase were reported and applied for inhibition screening. Although these methods can be used to screen for inhibitors, they are limited to identification of the inhibitors which compete with moenomycin for binding to PBP. The detailed analysis of TGase inhibition remains a bottleneck for inhibitor discovery. To solve this problem, we conceived a Förster resonance energy transfer (FRET)-based method to monitor the event of lipid II polymerization (Figure 1B). The *F*RET-*B*ased *L*ipid II *A*nalogue (FBLA) is designed to incorporate a fluorophore in the pentapeptide chain and a quencher in the lipid chain to absorb the fluorescence emission; i.e., the FBLA alone would not be fluorescent. When FBLAs undergo TGase-catalyzed polymerization, the lipid-diphosphate moiety carrying the quencher will be released, and the formed oligomer or polymer becomes fluorescent. The oligomer or polymer can then be digested with *N*-acetylmuramidase to release monomers (PGM),[13] resulting in an increase in fluorescence intensity at 460 nm. Moreover, labeling of the pentapeptide at the ε-NH_2 of the lysine residue would inhibit the transpeptidation and therefore minimize the interference from the inherent transpeptidase activities of PBP.[14,15] Herein, we reported our design and study to optimize the isoprenol portion of lipid II, including appropriate positioning of the fluorophore and quencher for the development of a sensitive and convenient TGase activity assay. This new FRET assay was used for determination of the inhibition constants (K_i) of moenomycin and other known inhibitors, and for the high-throughput screen of a structurally diverse library of 120,000 compounds for new inhibitors.

RESULTS

Design and Synthesis of FRET-Based Lipid II Analogues (FBLAs). To develop a FRET substrate for TGase activity assay that can be used in a high-throughput mode, the acceptor–quencher system should have a low background in order to generate a high signal-to-background ratio for real-time enzymatic assays. Two criteria for a successful FRET system were considered: (1) the optimum fluorescence emission of the donor molecule should overlap with the excitation wavelength of the acceptor, and (2) the distance between the two probes should be within 20–80 Å, depending on the nature of the pair.[16] Förster critical distance (R_0), the distance at which 50% of internal quenching occurs, can be optimized by using the donor–acceptor pair of long-wavelength dyes; for example, R_0 for fluorescein/tetramethylrhodamine is about 49–56 Å.[17] The fluorescein/tetramethyl–rhodamine pair may be ideal for FBLAs as the estimated length of the undecaprenol moiety in lipid II is about 50 Å.[18] However, we found that lipid II labeled with a longer wavelength dye, such as fluorescein or pyrene, in the pentapeptide chain was not a good substrate for bacterial TGases

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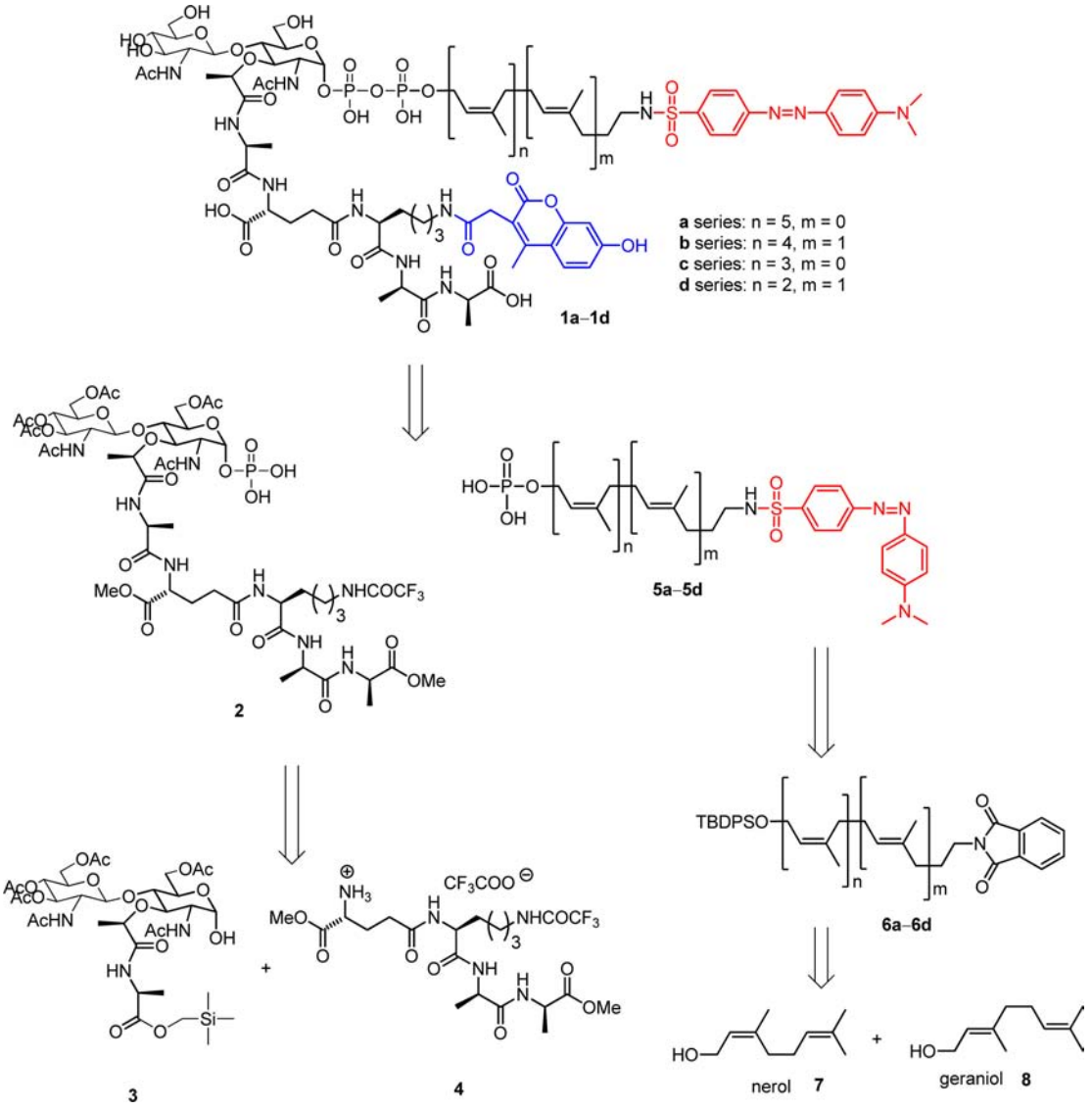
Scheme 1. Retrosynthetic Analysis of the FRET-Based Lipid II Analogues 1a−1d Bearing a Dabsyl Quench Group on the Lipid Chain and a Coumarin Fluorophore Attached to the Lysine Residue of the Pentapeptide



in our previous studies. In addition, lipid II analogues containing a dansyl or dabsyl moiety in the lipid chain were found to be recognized by TGase for transglycosylation.[19] Although the dabsyl group is commonly paired with 5-[(2-aminoethyl)-amino]naphthalene-1-sulfonic acid (EDANS) to have the R_0 value of 33 Å,[16] we utilized 7-hydroxy-4-methyl coumarinyl acetate (coumarin for abbreviation) to increase the R_0 value slightly in order to accommodate a longer lipid chain as well as higher flexibility for FBLAs. Thus, a FBLA was designed by incorporation of the dabsyl group into the lipid chain as quencher and the coumarin group at the ε-NH_2 group of the lysine residue as donor (Scheme 1). In addition, we decided to use FBLAs with lipids containing up to 5 isoprene units, since the length of isoprenol containing 5 isoprene units was reported to be about 35 Å, a value within the R_0 value of the chosen pair.[20−22] The questions remaining to be addressed are the optimal length and geometry of lipids to be used for TGase assay. We have demonstrated previously that a lipid II analogue with all isoprene units in the *cis* configuration connected to a dansyl group is adequate for transglycosylation.[15] To determine the optimal length and geometry of lipids in TGase assay, we prepared FBLAs with 3 or 5 isoprene units in *cis* form or with one of the isoprene units in the *trans* configuration (**1a−1d**, Scheme 1). As illustrated in the retrosynthetic analysis of FBLAs **1a−1d**, compound **2** can be assembled by a convergent approach from two major building blocks, a disaccharide (**3**) and a pentapeptide fragment (**4**), using our previously described procedure.[19] On the other hand, dabsyl-terminated lipid monophosphates **5a−5d** can be prepared from nerol (**7**) and geraniol (**8**).[23]

Scheme 2 shows the total synthesis of FBLAs. To circumvent the problem of inseparable mixture from double bond migration during desulfonation, we first synthesized building blocks **11** and **12** with saturated alkyl groups at the tail of lipid. The sulfonyl compounds **11** and **12** were then coupled with **9** and **10**, respectively, to afford the building blocks **13−16** containing different lengths of lipids.[24] The silyl group in **13−16** was removed by tetrabutylammonium fluoride (TBAF) to avoid destruction of the C−O bond.[25] The subsequent desulfonation was successfully carried out with $LiEt_3BH$/[$PdCl_2$(dppp)] at 0 °C to afford **17a−17d** in 72−76% yields. Alcohols **17a−17d** were reprotected with *tert*-butyl-diphenylchlorosilane. The THP ethers were hydrolyzed to yield the corresponding alcohols,

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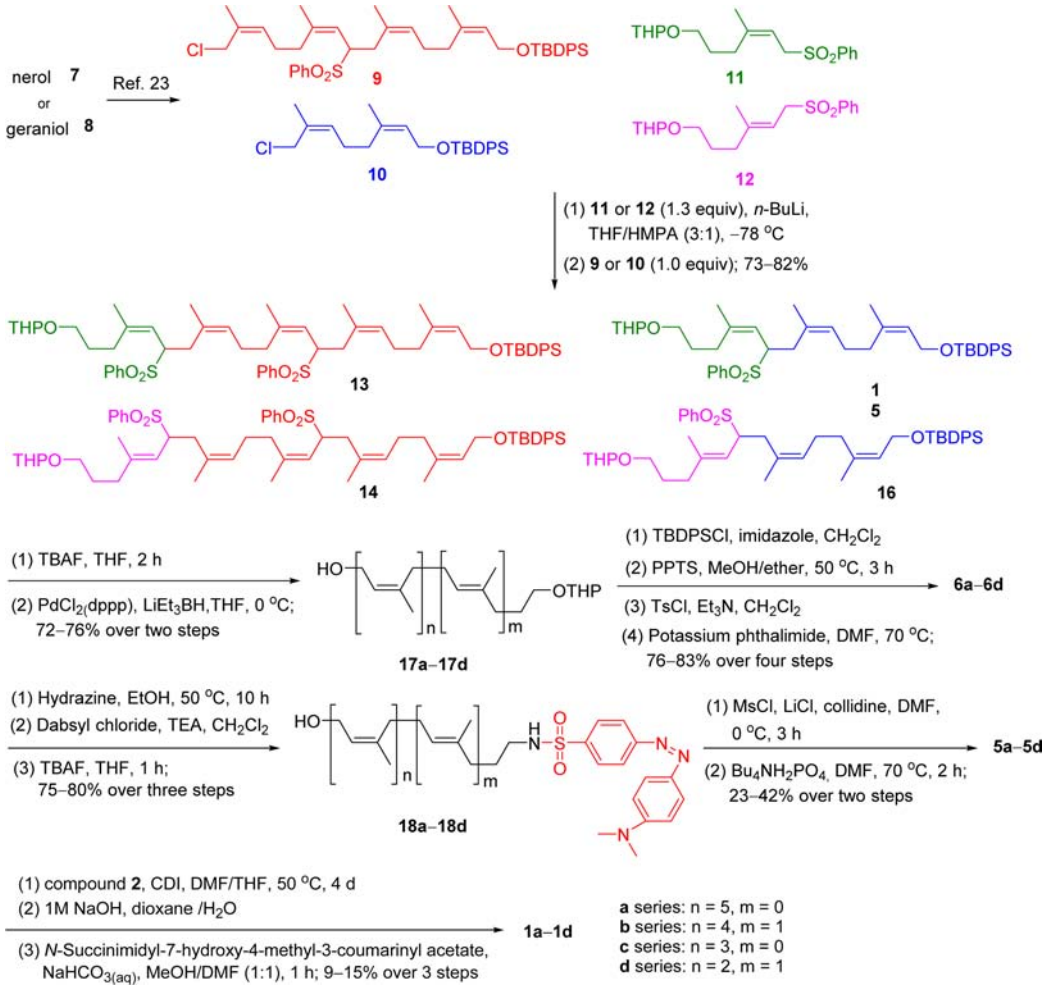
Scheme 2. Synthetic Schemes of FBLAs 1a−1d from Lipid Intermediates 13−16



which underwent tosylation and substitution with potassium phthalimide to give **6a−6d** in 76−83% overall yields. The phthaloyl group in **6a−6d** was removed by hydrazine to reveal an amino group for coupling with dabsyl chloride under basic conditions to give 75−80% of **18a−18d** after deprotection of the silyl group. Compounds **18a−18d** were treated with lithium chloride and methanesufonyl chloride to afford the corresponding allyl chlorides, which were reacted with tetrabutylammonium monophosphate in DMF to give dabsylated-lipid phosphates **5a−5d**. The monophosphate group was activated with 1,1′-cabonyldiimidazole and the coupling reactions with **5a−5d** were carried out at 50 °C for 4 days. After global deprotection under basic conditions, the ε-NH_2 group of the lysine residue was labeled with the coumarin fluorophore to afford the desired FBLAs **1a−1d**.

FBLAs as Bacterial Transglycosylase Substrates. Next, we tested whether these FBLAs could be recognized and polymerized by TGases. As shown in Figure 2A, incubation of **1b** with TGase in the presence of *N*-acetylmuramidase for 2 h produced a new peak (trace a), corresponding to the coumarin-labeled PGM that is released from polymerized FBLA by *N*-acetylmuramidase. As expected, the reaction would not proceed in the presence of moenomycin, a potent bacterial TGase inhibitor (trace b in Figure 2A). The reaction was proven to be specific to TGase, as the PGM peak would not be produced if TGase was replaced with other glycosyltransferases such as galactosyl transferase (trace c in Figure 2A). The emission spectrum of the reaction was also monitored upon excitation at 355 nm to ensure the FRET phenomenon. The reaction initially showed little emission at 460 nm upon excitation of 355 nm, indicating that the fluorescence of coumarin in the pentapeptide was quenched by the dabsyl group in the lipid chain (Figure 2B). When FBLAs were incubated with TGases in the presence of *N*-acetylmuramidase, the fluorescence signal at λ_{max} 460 nm showed a time-dependent increase (Figure 2B).

To investigate if the inclusion of *N*-acetylmuramidase in the reaction mixture is necessary for fluorescence increase, we compared the transglycosylation of FBLAs in the presence and absence of *N*-acetylmuramidase. It was observed that higher fluorescence changes occurred in the presence of *N*-acetylmuramidase (Figure 2C and D), indicating that the fluorescence of coumarin-tagged PGM is higher than that of coumarin-tagged polymerized lipid II. The result was reasonable as coumarin is a self-quencher and its fluorescence would decrease upon oligomerization of coumarin-tagged substrates.[26]

These data demonstrated that the designed FBLAs with structural modifications mainly in the lipid portion can be used for TGase-catalyzed transglycosylation to generate expected fluorescence signals. The fluorescence changes can be further increased with the addition of *N*-acetylmuramidase to release PGM for measurement. Although the TGase activity assay without *N*-acetylmuramidase might sound straightforward, kinetics measurement of such a reaction would become complicated as the degree of polymerization increased and the

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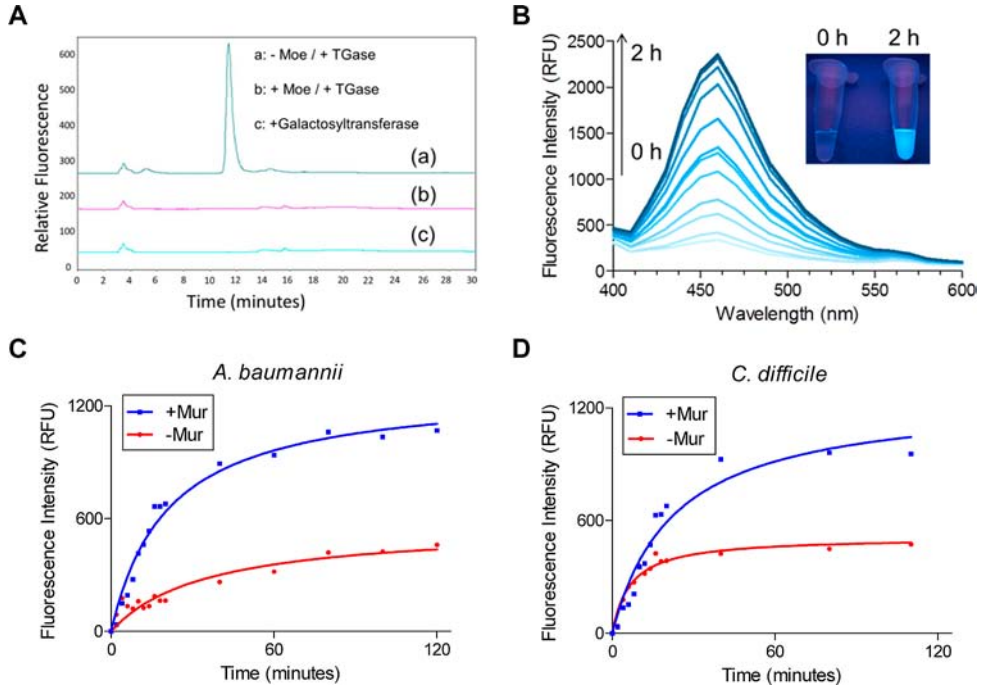

Figure 2. Characterization of FBLAs as substrates for TGase. (A) A solution of **1b** (10 μM) was incubated with *C. difficile* PBP (2.5 μg/mL) in a TGase buffer (0.85% decyl-PEG, 50 mM Tris-HCl, 10 mM $CaCl_2$, pH 8.0, 10% DMSO, and 15% MeOH) and *N*-acetylmuramidase (10 μg/mL) for 2 h in the absence (trace a) or presence (trace b) of moenomycin (Moe). The reaction mixture was analyzed using HPLC and the emergence of the fluorescent peptidoglycan monomer was monitored with λ_{ex} 355 nm/λ_{em} 460 nm. For a control experiment, galactosyl transferase instead of bacterial TGase was included in the reaction (trace c). (B) A solution of **1b** (10 μM) was incubated with TGase as described above. The reaction was monitored for 2 h by collecting the emission spectra upon excitation at 355-nm wavelength at 10-min intervals after the addition of TGase. Inset showed a photograph of the fluorescence of the reaction mixture before (left tube) and after (right tube) incubation with TGase for 2 h. The fluorescence was excited under a hand-held UV lamp (365 nm). (C, D) Time-dependent changes of fluorescence intensity (λ_{ex} = 355 nm, λ_{em} = 460 nm) in a mixture of TGase and **1b** (10 μM) in a TGase buffer with or without 10 μg/mL *N*-acetylmuramidase for 120 min in the wells of a 384-well plate. *A. baumannii* PBP1b (20 μg/mL) (C) and *C. difficile* PBP (2.5 μg/mL) (D) were used for the reaction. Mur: *N*-acetylmuramidase.

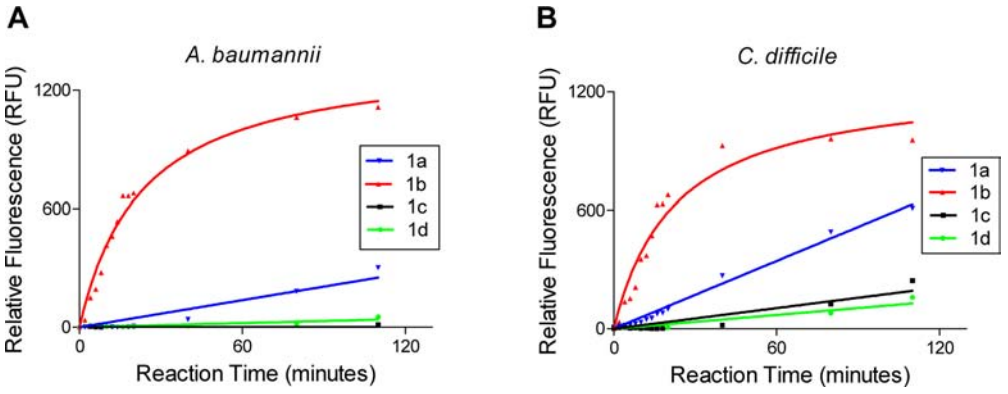

Figure 3. Progression curves of FBLAs **1a**−**1d** incubated with bacterial TGases. A solution of FBLA (**1a**−**1d**, 10 μM) was incubated with various bacterial TGases in TGase buffer containing 10 μg/mL *N*-acetylmuramidase for 120 min in the wells of a 384-well plate. The reactions were monitored with fluorescence (λ_{ex} = 355 nm, λ_{em} = 460 nm). *A. baumannii* PBP1b (20 μg/mL) (A) and *C. difficile* PBP (2.5 μg/mL) (B) were used.

polymerized FBLAs become TGase substrates. Addition of excess muramidase in the reaction promotes digestion of any polymerized products to produce disaccharide pentapeptide (or peptidoglycan monomer).[27] Moreover, the release of PGM by *N*-acetylmuramidase would result in a higher fluorescence change during the reaction and enable kinetics measurements as well as large-scale screening. Therefore, a coupled reaction of transglycosylation with *N*-acetylmuramidase cleavage was consistently used throughout the studies.

Substrate Specificity and Kinetics Parameters of Transglycosylases from Different Bacteria Using FBLAs. All synthesized FBLAs **1a**−**1d** with various modifications in lipids were further tested as substrates for transglycosylation. TGase was incubated with FBLAs **1a**−**1d** in the wells of microtiter plates and the fluorescence (λ_{ex} = 355 nm, λ_{em} = 460 nm) was monitored for 120 min (Figure 3A and B). When the FBLA containing 5 isoprene units in all *cis*-form (**1a**) or having one of the isoprene units in the *trans* configuration (**1b**) was used as substrate, the transglycosylation was observed with a time-dependent increase of the fluorescence at 460 nm (Figure 3). As the lipid chain was shortened to 17 carbons containing three *cis*-isoprene units (**1c**) or two *cis*- and one *trans*-isoprene units (**1d**),

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it was a better substrate for *C. difficile* PBP than *A. baumannii* PBP1b (Figure 3). When we further compared the progression curves for **1a** and **1b** that have the same numbers yet different configurations of the isoprene units, an increase in fluorescence signal was observed with **1b**. The results indicated that one isoprene unit in the *trans* configuration may be critical for the enzymatic reaction.

To understand the contribution of the *trans*-isoprene unit(s) to TGase reactions, kinetic parameters for each FBLA as substrate for the TGase from various bacteria species (including *A. baumannii* PBP1b, *C. difficile* PBP, *Escherichia coli* (*E. coli*) PBP1b, *Klebsiella pneumoniae* (*K. pneumoniae*) PBP1b, and *Mycobacterium tuberculosis* (*M. tuberculosis*) PonA1) were determined as reported.[28] To do this, the concentration of *C. difficile* PBP was kept constant (2.5 μg/mL), and the enzyme was incubated with varying concentrations of **1b** and the data were treated by following a reported method (Figure 4).[11,29] The

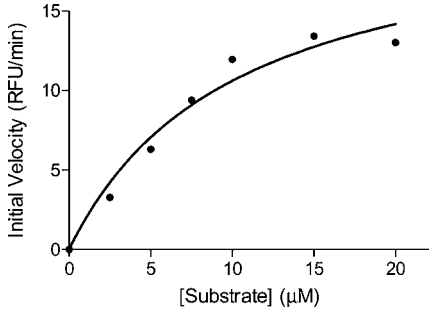

Figure 4. Transglycosylase reaction with various concentrations of FBLAs. *C. difficile* PBP (2.5 μg/mL) was incubated with various concentrations of FBLA **1b** (0−40 μM) in a buffer containing 10 μg/mL *N*-acetylmuramidase for 120 min in the wells of a 384-well plate. The reactions were monitored with fluorescence intensity (λ_{ex} = 355 nm, λ_{em} = 460 nm). Initial velocities of the reaction, V_o, were plotted against substrate concentrations and the K_m and k_{cat} values were determined using the equation $V_o/[\text{enzyme}] = k_{cat} \times [\mathbf{1b}]/(K_m + [\mathbf{1b}])$.[11,26]

apparent K_m ($^{app}K_m$) and apparent k_{cat} ($^{app}k_{cat}$) values of **1b** toward *C. difficile* TGase were thus determined to be 4.2 μM and 3.6 s^{-1}, respectively (Table 1). Following similar procedures, kinetic parameters were determined for different TGases using FBLAs **1a**−**1d** (Table 1). It was interesting to note that *C. difficile* PBP showed the highest apparent k_{cat} and k_{cat}/K_m values compared to other TGases, almost 20-fold higher. The affinities of FBLAs for *C. difficile* PBP were only 1−7-fold higher than the enzyme from other bacterial species, yet *C. difficile* PBP showed much higher catalytic activity than other TGases. When we further compared the kinetic parameters obtained from **1a** and **1b** using various TGases, it was shown that **1b** exhibited better affinity (lower K_m values) for all tested TGases. The results obtained from **1a** and **1b** indicated that a higher number of *cis* isoprene units would reduce the affinity for the enzyme but the catalytic efficiency was slightly increased, and a higher apparent k_{cat}/K_m of **1b** than **1a** for most bacterial TGases was observed, except the enzyme from *M. tuberculosis*. The $^{app}K_m$ of **1b** for *E. coli* PBP1b was determined to be 2.4 ± 0.6 μM, similar to the affinity of nature lipid II for bacterial TGases,[30] indicating that **1b**, with four *cis*- and one *trans*-isoprene units, retained the binding affinity of lipid II toward most bacterial TGases.

On the other hand, the FBLAs containing 3 isoprene units, i.e., **1c** and **1d**, showed a 5−10-fold lower reactivity than **1a** and **1b** toward *C. difficile* PBP and were not recognized by other TGases.

Table 1. Kinetic Parameters of Various TGases with FBLAs

	1a			**1b**			**1c**			**1d**		
TGases	k_{cat}^{a} (10^{-3} s^{-1})	K_m^{a} (μM)	k_{cat}/K_m^{a} (10^{-3} M^{-1} s^{-1})	k_{cat} (10^{-3} s^{-1})	K_m (μM)	k_{cat}/K_m (10^{-3} M^{-1} s^{-1})	k_{cat} (10^{-3} s^{-1})	K_m (μM)	k_{cat}/K_m (10^{-3} M^{-1} s^{-1})	k_{cat} (10^{-3} s^{-1})	K_m (μM)	k_{cat}/K_m (10^{-3} M^{-1} s^{-1})
A. baumannii PBP1b	47 ± 1.4[b]	4.1 ± 0.1	11.5	35.3 ± 5	1.5 ± 0.4	25.9	*n.d.*[c]	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
C. difficile PBP	3800 ± 20	6.1 ± 0.9	630.0	3600 ± 160	4.2 ± 0.2	867.4	835 ± 39	21 ± 4.5	34.8	389 ± 40	13.3 ± 9.9	29.4
E. coli PBP1b	157 ± 12	5.7 ± 1.2	28.3	120 ± 7	2.4 ± 0.6	51.6	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
K. pneumoniae PBP1b	540 ± 13	27.8 ± 9.7	20.2	445 ± 52	15.5 ± 5.3	31.4	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
M. tuberculosis PonA1	357 ± 38	31.2 ± 5.4	11.6	31.3 ± 1.2	28.1 ± 2.3	1.1	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*

[a]Shown are apparent values. [b]The values shown are the mean ± standard deviation of the data generated from three independent experiments. [c]n.d.: not determined.

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It is not surprising, as *C. difficile* PBP has the highest transglycosylation activity among all the TGases tested in this study. A previously developed lipid-II based probe with a 20-carbon lipid containing four *cis* isoprene units also can only react with *C. difficile* PBP, but not *E. coli* PBP1b.[19] Taken together, it is suggested that a minimum set of four *cis*-isoprene units is required for an efficient transglycosylation by TGases.

Determination of Inhibition Constant (K_i) of TGase Inhibitors. To further confirm the feasibility of the FRET-based assay for inhibitor screening, we set out to monitor the inhibition as well as to determine the K_i value of the well-known TGase inhibitor moenomycin using **1a** and **1b**. The assays were performed in a similar manner as in the determination of kinetic parameters, except that FBLAs (0−40 μM) and moenomycin (0−20 nM) were added prior to TGase to initiate the reaction. It was observed that moenomycin at 20 nM completely inhibited the TGase activity toward both **1a** and **1b**. The progression curves in the presence of various concentrations of moenomycin and substrate **1b** were obtained. The initial velocity was derived from the linear portion of the curves to calculate the K_i values. As shown in Table 2, the determined K_i of moenomycin is in the nanomolar range, which is in close proximity to the reported IC_{50} values of 2−14 nM for PBP1b.[3,28,31] It was also noticed that *M. tuberculosis* TGase appeared to be less sensitive to moenomycin than other TGases (Table 2). In addition, we tried to determine the inhibition mechanism of moenomycin by using Dixon's plot.[32] Thus, the reciprocals of the initial velocities of the reactions were plotted against moenomycin at various concentrations of **1b**. Unfortunately, the mode of inhibition was not very clear.

Table 2. Inhibition Constants (K_i) of Moenomycin for TGase Activity with FBLAs 1a and 1b

	K_i (nM)[a]	
TGases	**1a**	**1b**
A. baumannii PBP1b	4.9 ± 2.4	2.9 ± 0.1
C. difficile PBP	1.5 ± 0.1	0.7 ± 0.2
E. coli PBP1b	1.1 ± 0.4	1.2 ± 0.2
K. pneumoniae PBP1b	2.2 ± 0.6	*n.d.*[b]
M. tuberculosis PonA1	11.9 ± 1.3	11.5 ± 3.5

[a]Shown are the mean ± standard deviation of the data generated from three independent experiments. [b]n.d.: not determined.

We also tried to determine the K_i values as well as the inhibition mode for the two synthetic compounds **19** and **20** (Figure 5) identified from an earlier screening.[33] Since the K_i of moenomycin was similar to that of **1a** or **1b**, only **1b** was used for the analysis due to its relatively higher activity compared to **1a**. Various concentrations of compounds were incubated with TGase and 5 μM of **1b** and the time-dependent fluorescence increase was monitored (Figure 6A and B). The results clearly showed that the fluorescence intensity at 460 nm decreased as the concentration of the compounds increased, demonstrating the feasibility of the FRET-based assay for inhibition studies as well as inhibitor screening at a single concentration. To determine the inhibition mode, TGase was incubated with various substrate concentrations in the presence of varied inhibitor concentrations. The initial velocity was derived from individual progression curves and the data were treated by Dixon (Figure 6C and D)[30] and Cornish-Bowden (Figure 6E and F)[34] plots. The analysis revealed that using **1b** as substrate, the inhibition mode of **19** and **20** was competitive with K_i = 22.5 μM and 16.1 μM, respectively. The K_i values of the compounds for other bacterial TGases were determined in a similar manner and were shown in Table 3. Consistent with our previous studies using HPLC analysis for TGase inhibition measurement,[33] compound **20** indeed showed better inhibition activities than compound **19**.

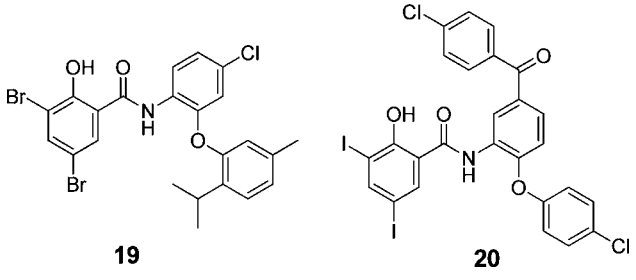

Figure 5. Chemical structures of TGase inhibitors **19** and **20**.

High Throughput Screening (HTS) for TGase Inhibitors. The new FRET-based TGase assay was used to screen for new structural classes of TGase inhibitors. The concentrations of **1b** and TGase were optimized to have the average fluorescence value of 94.8 ± 6.3, with the Z' factor of 0.68 in a 1536-well plate format. A 120,000 compound library containing FDA-approved drugs, bioactive molecules, and synthetic molecules were screened at 20 μM in 1536-well plates and a total of 25 primary hits were obtained. Subsequent confirmation with dose-dependent studies using both traditional HPLC- and FRET-based assays confirmed six hits as transglycosylation inhibitors (Figure 7). Successful identification of a known transglycosylase inhibitor, moenomycin, and glycopeptide antibiotics, teicoplanins (**21** and **22**) and vancomycin (**23**), validated the high-throughput screening method since no prior knowledge of compound identities in the compound plates were provided. The determined K_i values of glycopeptide antibiotics (**21**-**23**) (Table 4) were found to be similar to the reported IC_{50} values of 0.5−2 μM for the transglycosylation step.[28,31,35,36] Compound **24** was an analogue of previously identified TGase inhibitors **19** and **20**, and showed slightly better TGase inhibition activities. Surprisingly, **25** with a naphthalene chemical skeleton has low-micromolar inhibition activities comparable to that of glycopeptide inhibitors (Table 4). The antibacterial activities of these identified hits were also investigated and the minimal inhibitory concentrations (MIC) were determined using standard broth-dilution methods. All hits showed activities against the growth of *S. aureus* and *M. smegatis*, but not against the Gram-negative bacteria used in this study (Table 5).

■ DISCUSSION

In this study, we described the design and synthesis of several FRET-based lipid II analogues for development of a continuous TGase activity assay. Our study represents not only the first FRET for transglycosylation measurement, but also a new continuous monitoring of TGase-catalyzed polymerization of lipid II analogues. The FRET system combines the advantages of rapid and highly sensitive real-time fluorescence measurements and allows the determination of kinetic parameters of substrates and inhibitors in a high-throughput mode.

FRET is currently one of the most commonly used methods.[37,38] The distance-dependent transfer of energy can be applied to fluorophore/fluorophore, fluorophore/quencher, or fluorescence/luminescence pairs. It has been used to elucidate

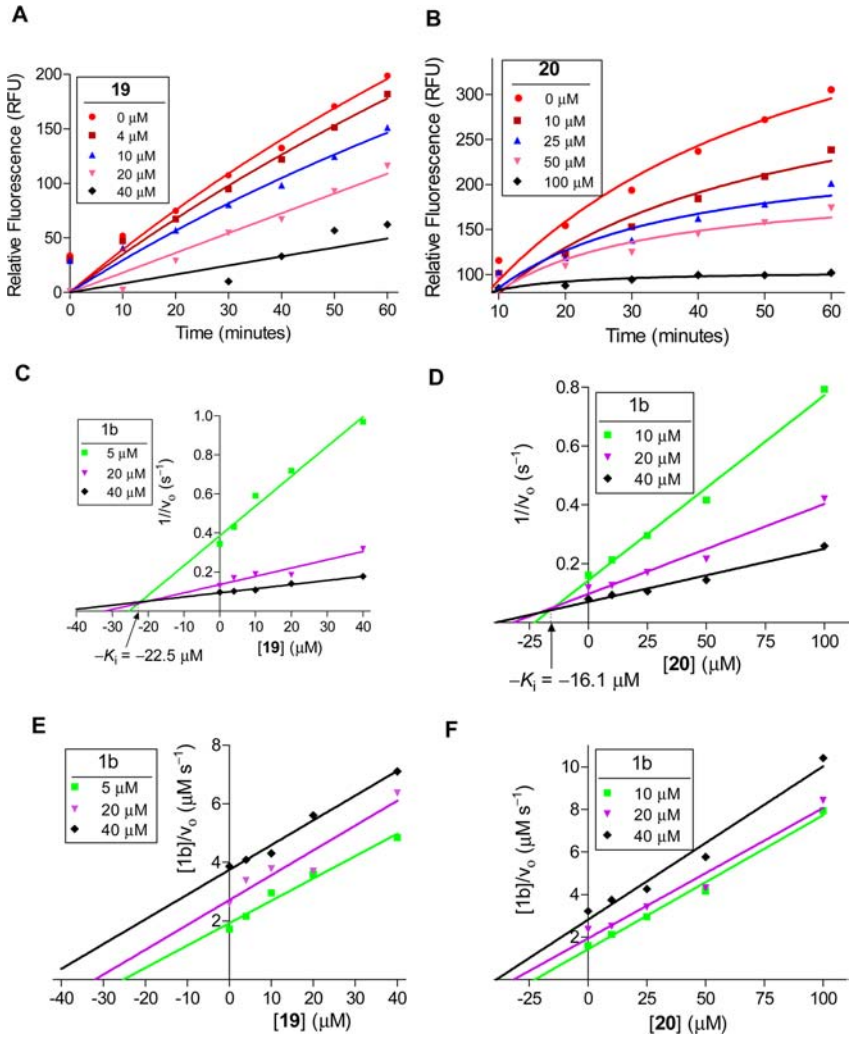

Figure 6. Competitive inhibition of compounds **19** and **20** against transglycosylation of by *E. coli* PBP1b using **1b**. *E. coli* PBP1b (12 μg/mL) was incubated with **19** (0−40 μM) or **20** (0−100 μM) in the presence of various concentrations of FBLA **1b**. The reaction was monitored by fluorescence (λ_{ex} = 355 nm, λ_{em} = 460 nm) for 2 h at 37 °C. (A, B) Shown are the progression curves of **1b** (5 μM) incubated with *E. coli* TGase in the presence of various concentrations of **19** (A) and **20** (B). (C, D) The initial velocities (V_0) of each reaction were derived from the linear portion of the progression curves. (C, D) The reciprocal of V_0 was plotted as a function of inhibitor concentration to generate the Dixon's plot for **19** (C) and **20** (D). (E, F) Cornish-Bowden's plots, where the multiplication of substrate concentration **1b** and the reciprocals of initial velocity (s/V_0) were plotted against concentrations of the inhibitors **19** (E) and **20** (F). Parallel lines indicated competitive inhibition.

Table 3. Inhibition Constants (K_i) of Compounds 19 and 20 against Various TGases Using 1b as Substrate

	K_i (μM)[a]	
TGases	**19**	**20**
C. difficile	5.3	28.3
E. coli	22.5	16.1
K. pneumonia	16.6	10.2

[a]Shown are the mean values of the data generated from three independent experiments.

the dynamics of proteins, to probe the binding/interaction status of molecules, and to monitor the enzyme reaction that can disturb the distance between the donor and the acceptor, and so forth.[39−42] In general, it is easier to design FRET substrates for the enzymes, such as proteases, that cleave the substrate into two parts to release the fluorophore for sensitive fluorescence measurement.[43,44] Most of these substrates can be designed simply by attaching desired fluorescence pairs onto the respective ends of the working substrates and have little impact on enzyme activity. However, fewer examples were reported for sugar transfer reactions, especially for direct, continuous, and quantitative measurement of glycosyltransferase activities. Recently, Maeda and Nishimura reported a FRET assay for fucosyltransferase,[45] where a sugar nucleotide with a fluorogenic probe, naphthalene, was used as donor and a dansylated sialyl-α2,3-LacNac derivative as acceptor to enable FRET analysis. Our methods reported here provide an alternative approach to the enzyme that uses substrates containing undecaprenol, such as MraY and MurG, and the principle can be expanded to other glycosyltransferases that use lipid(s), e.g., dolichol, as leaving group(s).

Walker and co-workers have predicted that lipid II with four successive *cis* isoprene units in a 35-carbon chain is the best transglycosylase substrate.[46] This study further confirmed that FBLAs bearing a lipid chain containing five isoprene units, either in all-*cis* form or with one in the *trans* configuration, can be recognized universally by bacterial TGase. The activity of this

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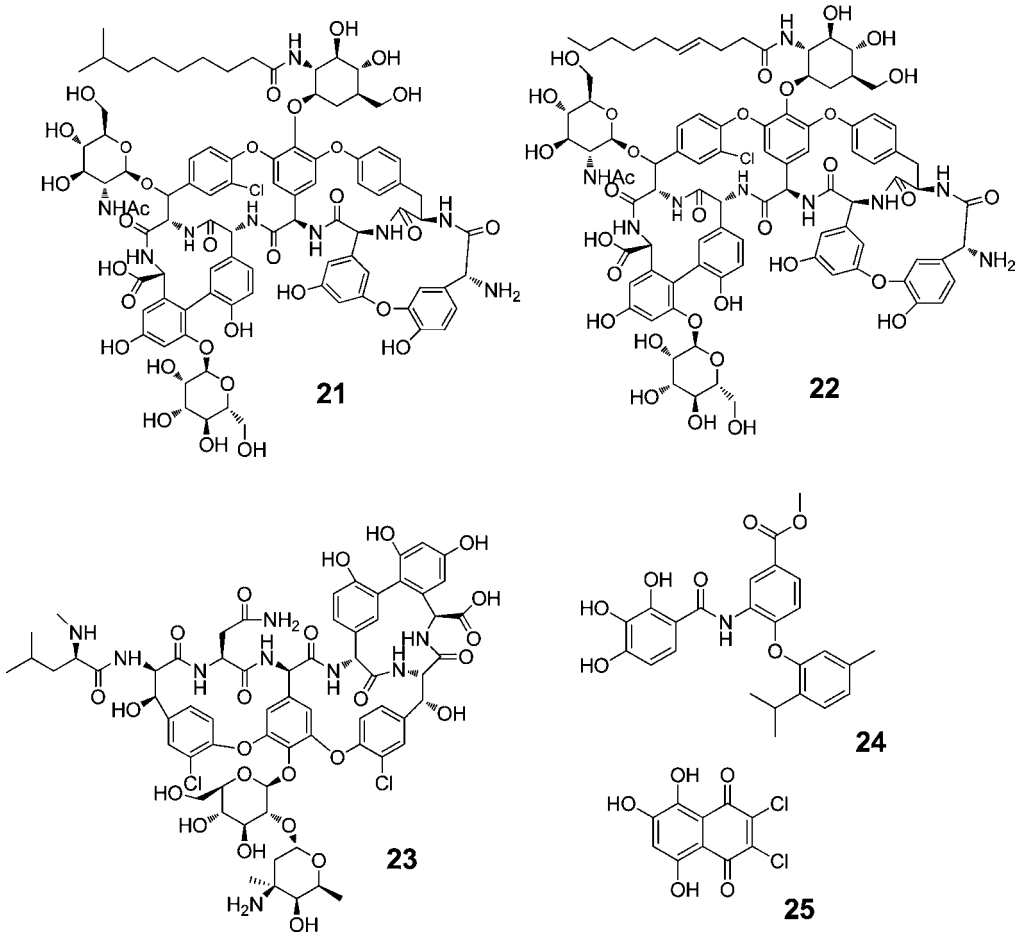

Figure 7. Chemical structures of identified hits **21**−**25** from high-throughput screening. **21**: teicoplanin A_2-2; **22**: teicoplanin A_2-1; **23**: vancomycin; **24** and **25**: synthetic compounds.

Table 4. K_i Value of HTS Hits against Various TGase

	K_i (μM)[a]				
TGases	**21**	**22**	**23**	**24**	**25**
A. baumannii	0.1 ± 0.01	0.6 ± 0.2	1.9 ± 0.6	4.1 ± 1.2	2.3 ± 0.5
C. difficile	0.6 ± 0.2	0.5 ± 0.1	2.4 ± 0.1	2.3 ± 0.3	2.7 ± 0.1
E. coli	0.4 ± 0.2	0.5 ± 0.1	1.3 ± 0.4	>15	4.6 ± 1.4

[a]Shown are the mean ± standard deviation of the data generated from three independent experiments.

Table 5. MIC Values of HTS Hits against Various Bacteria

	MIC (μg/mL)				
species	**21**	**22**	**23**	**24**	**25**
A. baumannii	>256	>256	128	256	>32
E. coli	>256	>256	256	>256	>32
MRSA	1 (0.5)[a]	1 (0.5)	2 (1.6)	1 (2)	32 (116)
P. aeruginosa	>256	>256	256	>256	>32
M. smegmatis	0.13 (0.01)	0.13 (0.1)	0.25 (0.2)	2 (4)	4 (15)
S. aureus	0.25 (0.1)	0.25 (0.1)	0.5 (0.4)	2 (4)	16 (58)

[a]Shown in parentheses are the MIC values in μM.

FBLA was also compared with the activities of NBD-labeled lipid II bearing an undecaprenyl lipid. The HPLC analysis results confirmed that complete consumption of both substrates can be observed in 120 min (Supporting Information Figure s1).

It is worth noting that the configuration of isoprene units played an important role for transglycosylation (Figure 3). FBLAs **1c** and **1d** remained reactive to the enzyme from *C. difficile*, but showed very little reactivity toward the TGase from *A. baumannii*, *E. coli*, or *M. tuberculosis* (Table 1). It is noteworthy that *C. difficile* PBP has a shorter transmembrane domain (predicted to be 23 amino acid long by TMHMM, Center for Biological Sequence Analysis, Denmark) compared to *E. coli*

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PBP1b with a 31-amino-acid transmembrane domain.[47,48] Whether the high catalytic activity of *C. difficile* TGase comes from changes in the active site or from minimizing lipid interactions by a shorter transmembrane domain or both will be interesting to investigate.

We were not able to conclude the mode of inhibition by moenomycin. It is possible that moenocinol, the 25-carbon lipid of moenomycin, complicated the study due to its hydrophobic nature. Walker's group also reported that they could not clearly confirm the competitive mode of inhibition of moenomycin even though moenomycin, theoretically, should be a competitive inhibitor of bacterial TGase.[28] Nonetheless, **19** and **20** identified from our screening efforts[33] were confirmed to be competitive inhibitors and more studies are ongoing.

The application of FRET-based TGase assay to HTS was validated with screening against 120,000 compounds. Moenomycin and glycopeptide antibiotics were identified as hits and the determined inhibition constants of those compounds were comparable to the reported IC_{50} value of 0.5−2 μM for the transglycosylation step.[28,31,35,36] Glycopeptide antibiotics inhibit bacteria growth by abolishing the maturation of peptidoglycan through interaction with the terminal D-Alanyl-D-Alanine (D-Ala-D-Ala) dipeptide moiety of lipid II.[49,50] While the binding of glycopeptides to the D-Ala-D-Ala in lipid II is well evidenced in NMR[51] and X-ray crystallography,[50] whether the glycopeptide inhibitors can also bind to transglycosylase directly to interfere with the transglycosylase step is still not clear. Kahne et al. reported that chlorobiphenyl vancomycin, but not vancomycin, can inhibit transglycosylase directly.[28] Since the FRET-based TGase assay measured overall transglycosylation, molecules that interfere with substrate availability or directly inhibit enzymatic activities would appear as inhibitors. Therefore, it is not surprising that D-ala-D-ala binding glycopeptides were identified as hits in our HTS campaign. Meanwhile, the identification of glycopeptide inhibitors as hits validated the HTS platform for bacterial transglycosylation inhibitors.

Compounds **24** and **25** represented active small molecule inhibitors of TGase in our screening and showed moderate antibacterial activities against Gram-positive bacteria and *M. smegatis*. Compound **24** is an analogue of previously identified hits and has a salicylanilide core structure. It was reported that salicylanilides block bacterial growth through inhibition of isocitrate lyase.[52,53] However, **24** showed no inhibition against isocitrate lyase in our studies. The aromatic esters of 2-(isopropyl)-5-methyl benzoate on salicylic acid may be critical for the activity against TGase.

■ CONCLUSION

In summary, we have developed a high-throughput FRET based activity assay for bacterial transglycosylase. Of the FBLA substrates developed, compound **1b** with the lipid moiety containing four *cis*- and one *trans*-isoprene units was best recognized by all TGases, and the continuous fluorescent TGase activity assay has proven useful for kinetic analysis and inhibitor identification. This method was further used in a high-throughput screen of 120,000 compounds to identify new inhibitors. With the X-ray crystal structure and mechanism of some transglycosylases available,[47,48] we begin to conduct structure-based optimization of the hits identified in this study.

■ EXPERIMENTAL SECTION

Materials. The genomic DNA of *Acinetobacter baumannii* (AYE) and of *Klebsiella pneumonia* (MGH78578) were purchased from American Type Culture Collection. The primers used for amplification were provided by Purigo (Taiwan) and DNA sequencing service for amplified genes was provided by Mission Biotech (Taiwan). Anti-$(His)_6$ antibodies were from Qiagen. All chemicals were purchased as reagent grade and used without further purification. All solvents were anhydrous grade unless indicated otherwise. All nonaqueous reactions were performed in oven-dried glassware under a slight positive pressure of argon unless otherwise noted. Nitrobenzoxadiazole-lipid II (NBD-lipid II) was prepared as reported.[54] Moenomycin was isolated according to the procedure described previously[33,55] and synthetic molecules **19** and **20** were obtained from ChemDiv Inc. Teicoplanins **21** and **22** were obtained from InterBioScreen Ltd. and MicroSource Discovery Systems Inc., respectively. Molecules **24** were synthesized in house and **25** were purchased from Ambinter Inc.

FRET-Based Lipid II Analogues 1a-1d (FBLAs). Compound **2**[18] (9 mg, 0.007 mmol) was added to a solution of 1,1′-carbonyldiimidazole (CDI, 5 mg, 0.03 mmol) in anhydrous DMF (0.3 mL). After stirring for 2 h, anhydrous MeOH (3 μL) was added and the mixture was stirred for another 40 min to destroy excess CDI. A solution of monophosphate compound **5a**−**5d**, 0.005 mmol in anhydrous THF (1.2 mL), was added. The mixture was concentrated in vacuum to remove THF and MeOH. The residue was dissolved in anhydrous THF/DMF (2:1, 3 mL), followed by addition of 1*H*-tetrazole (1 mg, 0.017 mmol). The mixture was heated to 50 °C, stirred under an atmosphere of argon for 4 d, and then concentrated by rotary evaporation under reduced pressure. The residue was dissolved in 1,4-dioxane/H_2O (1:1, 2 mL), and 1 M NaOH (0.11 mL) was added. The mixture was stirred for 1 h until TLC analysis showed that the trifluoroacetyl group was completely cleaved to give the desired amine product. The amine product was directly purified by reversed-phase HPLC on a semipreparative ZORBAX RX-8 column (5 μm, 9.4 × 250 mm) using a gradient elution with 50 mM aqueous NH_4OAc and MeOH (50:50 to 0:100 over 60 min) followed by 100% MeOH for 15 min at a flow rate of 2 mL/min. Lyophilization of the target fractions gave a dabsyl-lipid II analogue, in 18−23% overall yields. To a solution of dabsyl-lipid II analogue (0.35 μmol) in a mixture of MeOH (0.4 mL), DMF (0.4 mL) and sat. $NaHCO_{3(aq)}$ (0.05 mL) was added *N*-succinimidyl-7-hydroxy-4-methyl-3-coumarinyl acetate (coumarin-OSu, 5 mg, 15 μmol). The turbid red solution was stirred for 1 h and concentrated to dryness. The mixture was dissolved in MeOH and purified by reverse-phase HPLC on a semipreparative ZORBAX RX-C8 column (9.4 mm × 250 mm, 5 μm) with a gradient eluent of 0.05 M NH_4OAc/MeOH solution (50:50 to 0:100) over 60 min at a flow rate of 2.0 mL/min using a diode array detection at 460 nm. The retention time was about 46 min for both FBLAs **1a** and **1b** and was about 36 min for FBLAs **1c** and **1d**. **1a**: ESI−HRMS calculated for $C_{92}H_{133}N_{12}O_{32}P_2S$: 2012.8370. Found: m/z 2012.8639 $[M - H]^-$; **1b**: ESI−HRMS calculated for $C_{92}H_{133}N_{12}O_{32}P_2S$: 2012.8370. Found: m/z 2012.8629 $[M - H]^-$; **1c**: ESI−HRMS calculated for $C_{82}H_{117}N_{12}O_{32}P_2S$: 1875.7087. Found: m/z 1875.7291 $[M - H]^-$; **1d**: ESI−HRMS calculated for $C_{82}H_{117}N_{12}O_{32}P_2S$: 1875.7087. Found: m/z 1875.7307 $[M - H]^-$.

Cloning, Expression, and Purification of Full-Length Bifunctional Penicillin-Binding Proteins from Various Bacterial Species. Recombinant PBPs were prepared based on a previous reported method with slight modifications.[9] For *A. baumannii* PBP1b and *K. pneumoniae* PBP1b, the full-length gene was amplified from genomic DNA and cloned into the expression vector pET15b (Novagen). The plasmids were transformed into BL21(DE3) *E. coli* competent cells, and the transformants were grown overnight at 37 °C in LB broth supplemented with 100 μg/mL ampicillin. Each overnight broth culture was diluted 1:100 in fresh LB medium with antibiotics, and then incubated at 37 °C until OD_{600} reached 0.6. Protein expression was induced with 0.1 mM isopropyl β-D-1-thiogalatopyranoside (IPTG) overnight at 16 °C with vigorous shaking. Bacterial cells were harvested by centrifugation, and cell pellets were resuspended in starting buffer (20 mM Hepes, pH 7.0, 200 mM NaCl). Cells were lysed by Microfluidizer (Microfluidics) and the lysate was clarified by centrifugation at 12,000× *g* for 30 min at 4 °C. The supernatant was subjected to ultracentrifugation at 40,000 rpm for 1 h at 4 °C. The pellet containing the recombinant protein fused with an *N*-terminal $(His)_6$ tag was extracted

with starting buffer containing 1% *N*-dodecyl-maltopyranoside (DDM; Anatrace) for 2 h at 4 °C, and the extract was further purified by nickel chelation chromatography (GE) following manufacture's procedure. Purified recombinant PBPs were confirmed by immunoblots, concentrated, and the detergent was exchanged to 0.015% DDM using Amicon Ultrafiltration Unit (Millipore).

Transglycosylase Activity Analysis by High Performance Liquid Chromatography. A solution containing FBLAs (10 μM) or NBD-lipid II (4 μM) in TGase buffer (50 mM Tris, pH 8.0, 10 mM $CaCl_2$, 0.085% Decyl PEG50), 10% DMSO, 15% MeOH) containing 10 μg/μL *N*-acetylmuramidase was mixed at 1000 rpm at 25 °C. The reaction was initiated by addition of TGase (2.5 μg/mL) and terminated by addition of 100 μM moenomycin A in 1/10 volume. The reaction mixtures were then analyzed by a HPLC system (Hitachi) equipped with an anion-exchange column (5 μm, 4.6 × 250 mm, SAX1, Supelco Co.) using a linear gradient of NH_4OAc/MeOH (20 mM to 1 M) at a flow rate of 1 mL/min. The signals were detected with an online fluorimeter at λ_{ex} = 355 nm and λ_{em} = 460 nm.

Kinetic Analysis of FRET-Based lipid II Analogues. To determine the kinetic parameters of FBLAs for various bacterial TGases, the same amount of active TGase is preferred to be used in the reaction. To do this, we first determined the amount of TGase needed to complete the polymerization of NBD-lipid II into peptidoglycan in one hour at 37 °C using HPLC analysis. The enzyme in twice amounts was then incubated with various concentrations of FBLAs (0−40 μM) in TGase buffer in the wells of a 384-well microtiter plate at 25 °C. The reaction was followed by fluorescence at 460 nm (excitation wavelength of 355 nm) at 2-min intervals for 2 h using a SpectraMax M5 (Molecular Devices). The initial velocity (V_o) was calculated using the linear portion of the progression curves fit to the equation $V_o/[\text{enzyme}] = k_{cat} \times [\text{substrate}]/(K_m + [\text{substrate}])$[11,28] using Prism 5.0 (GraphPad).

Determination of K_i Values of Transglycosylase Inhibitors. The K_i values of moenomycin and synthetic TGase inhibitors were determined using FRET-based assays. The reaction was performed and monitored as described above with various concentrations of FBLAs (0−40 μM) in the presence of 0−20 nM of moenomycin or 0−100 μM synthetic inhibitors **19** and **20**. The K_i values of HTS hits were performed with various concentrations of **1b** in the presence of 0−0.25 μM of **21**, 0−0.2 μM of **22**, 0−5 μM of **23**, 0−10 μM of **24**, and 0−20 μM of **25**. Initial velocity for each progression curve was calculated and used for K_i determination. The K_i value for moenomycin and compounds **21**−**25** was determined with the competitive mode by using GraphPad Prism 5 (GraphPad Softwares Inc.), while the K_i values of **19** and **20** were determined using Dixon's and Bowden's plots (see those in the text). The data shown were the mean value from three independent experiments.

Ultra High-Throughput Screening (uHTS) of Transglycosylase Inhibitors. High-throughput screening (HTS) was performed in 1536-well plates at the Genomics Research Center of Academia Sinica in Taiwan using the uHTS system manufactured by GNF systems. The HTS system is integrated with dispensers, a 1536-pin transferring tool, incubators and a ViewLux plate reader (Perkin-Elmer Inc.). For TGase inhibitor screening, to the *C. difficile* PBP (5 μg/mL) in buffer (50 mM Tris, pH 8.0, 10 mM $CaCl_2$, 0.085% Decyl PEG50) containing 10% DMSO, 15% MeOH, 1 μg/μL *N*-acetylmuramidase, and 5 μM **1b** in 1536-well plates the compound to reach at a final volume of 5 μL per well into 1536-well plates followed by two compound transfers using a 1536-pin transferring device with 50-nL slots (V&P Scientific, San Diego, CA, USA). After covering with GNF-designed lids to minimize edge effects from evaporation, the plates were incubated in the environmentally controlled incubators (37 °C, 80% humidity) for 2 h. The fluorescence was monitored with the Viewlux reader using excitation at 355 nm and emission at 460 nm. The interested primary hits were hit-picked with a hit-picking system (GNF systems) for subsequent tests. The compound identities were further confirmed with LC−MS analysis (Bruker). The compounds that showed correct mass and >90% purity were used for activity analysis using the same protocol described above.

Determination of Minimal Inhibition Concentration (MIC). *Acinetobacter baumannii* (BCRC 10591), *Escherichia coli* (BCRC 11509), *Mycobacterium smegatis* (BCRC 11565), *Pseudomonas aeruginosa* (BCRC 11864), *S. aureus* (BCRC 11863), and MRSA (Methicillin-resistant *Staphylococcus aureus*, ATCC 33592) were obtained from ATCC or BCRC (Bioresource Collection and Research Center, Taiwan). The minimal inhibitory concentration of tested compounds was determined following the NCCLS standard. The experiments were conducted in 96-well microtiter plates using 16-point 2-fold dilutions in Muller-Hilton broth. Exponentially growing cells at 5×10^5 cells/mL were incubated with test compounds at various concentrations in a final volume of 100 μL. After an 18 to 24 h incubation at 37 °C, the minimal concentration of the compound that prevents at least 95% bacterial growth was determined as the MIC value of a given molecule.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Results and the chemical synthesis of compounds **5a**−**5d**, **6a**−**6d**, **17a**−**17d**, **18a**−**18d**, and **24** as well as NMR spectra (^1H and ^{13}C) of new compounds were described. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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