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ScienceDirect
Bioorganic & Medicinal Chemistry 14 (2006) 7187–7195
Bioorganic & Medicinal Chemistry

Characterization of a transglycosylase domain of *Streptococcus pneumoniae* PBP1b

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Received 23 May 2006; revised 16 June 2006; accepted 23 June 2006
Available online 25 July 2006

Abstract—Inhibitors of transglycosylases may serve as potent antibiotics that are less prone to resistance development in bacterial pathogens. To facilitate the search of such compounds, a transglycosylase (TGase) domain of the membrane integral multidomain *Streptococcus pneumoniae* PBP1b was cloned and expressed. This TGase domain was characterized by a substrate-dependent fluorescence coupled enzyme assay and an inhibitor-tethered surface plasmon resonance binding assay. Both assays show that the catalytic efficiency of the domain is comparable to that of the monofunctional transglycosylases, and it is fully active in the absence of other domains. The isolation of the active TGase domain makes it possible to screen for potential antibiotics targeting transglycosylases.

1. Introduction

After six decades of widespread antibiotic use, bacterial pathogens of human and animal origin are becoming increasingly resistant to many antimicrobial agents.[7] Many common bacterial pathogens such as *Staphylococcus aureus, Streptococcus pneumoniae*, and *Enterococcus faecalis* have become resistant. Methicillin-resistant *S. aureus* (MRSA), penicillin-resistant *S. pneumoniae*, and vancomycin-resistant *E. faecalis* are now commonplace pathogens that are proving difficult to treat effectively.[21] To date, even vancomycin, the last resort of antibiotics active against *S. aureus* and some other Gram-positive bacteria, has encountered resistance,[31] and this new public health crisis has renewed interest in antibacterial development.[5] Current strategies for tackling the problem of antibiotic resistance generally involve chemical modification of existing antibiotics, discovery of new targets, and development of new types of antibiotics. Our interest in the field is to develop new antibiotics that target unique carbohydrates or carbohydrate-utilizing enzymes in bacteria and evade the resistance development.

Bacterial cell-wall (peptidoglycan) biosynthesis is both essential and unique to bacteria and is a proven target for identifying compounds as new antibiotics.[6] Among the different steps in the biosynthesis of peptidoglycan, transglycosylation is the most interesting target for the development of new antibiotics for two reasons. First, the transglycosylase is fully exposed to the cell surface and thus easily accessible to drugs as no transfer across the plasma membrane is required. Second, the polysaccharide backbone of peptidoglycan remains intact in wild-type and resistant strains, whereas the peptide side chain shows a high frequency of change. Antibiotics targeting the transglycosylation step may therefore be less prone to resistance development.

In the final step of peptidoglycan biosynthesis, Lipid II, the substrate for the transglycosylation, is polymerized and crosslinked into peptidoglycan by transglycosylation and transpeptidation (Scheme 1).[12] Two major enzymatic activities in the periplasmic space responsible for the formation of peptidoglycan are the transglycosylase (TGase) and the transpeptidase (TPase). A group of bifunctional high-molecular-weight (HMW) penicillin-binding proteins (PBPs) possessing both TGase and TPase activity have been identified in both

Keywords: Enzyme catalysis; Lipid II; Transglycosylase; *Streptococcus pneumoniae*.
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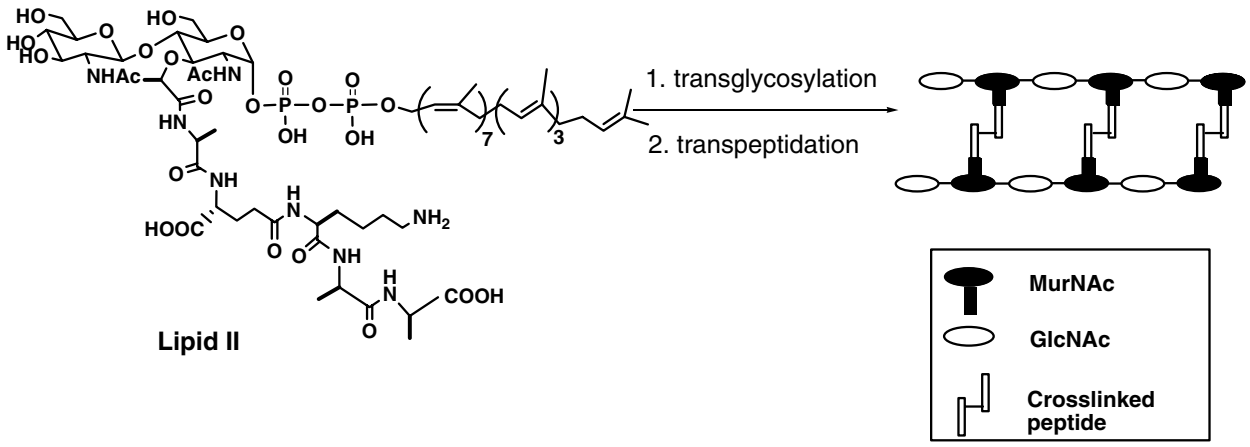
doi:10.1016/j.bmc.2006.06.058



Scheme 1. Formation of peptidoglycan by transglycosylation and transpeptidation.

Gram-positive and Gram-negative bacteria.[22] These HMW PBPs are multidomain molecules, consisting of a short N-terminal cytoplasmic region, a single transmembrane domain, and a large C-terminal periplasmic region. The N-terminal domain of the periplasmic region possesses TGase activity, while the C-terminal domain contains TPase and penicillin-binding activity.[9,26] Monofunctional enzymes with only TGase or TPase activity have also been identified. Low molecular mass PBPs function mostly as the D, D-carboxypeptidase and are believed to regulate the amount of crosslinking within the peptidoglycan mesh.[8] Non-PBP-related TGase or monofunctional TGase (MTG) has been reported recently in *Escherichia coli*, *S. aureus*, and *Micrococcus luteus*.[20,10] These proteins have the characteristic fingerprints found in the N-terminal TGase domains of HMW PBPs. In addition, the solubilized membrane fraction of *E. coli* cells overexpressing the *mtg* gene contains a significantly increased peptidoglycan activity.[10] The roles of PBPs and MTGs in cellular peptidoglycan synthesis, however, are not known.

Investigation of the mode of function of transglycosylases, in particular the development of synthetic inhibitors, has been hampered by difficulties in the development of an efficient assay system. Some difficulties in studying transglycosylases are related to problems in obtaining and handling the substrate Lipid II. Isolating Lipid II from bacteria is extremely difficult due to its low natural abundance and its inherent structural complexity. The total amount of Lipid II present in bacteria is very small and has been determined to be no higher than 2000 molecules per cell.[28] To separate this miniscule amount of Lipid II from cell membrane and other cellular lipid components is tedious and requires enrichment of radiolabeled precursors.[19] Lipid II can be alternatively prepared by using MurG and its natural substrate Lipid I, but the yield is low due to the detergent-like property of the substrate.[33] Recently, the development of improved assay systems for monitoring transglycosylation has been the subject of many research efforts. The chemical[11] and chemo-enzymatic[16] syntheses of the Park nucleotide as well as several synthetic routes to Lipid I and Lipid II[18,33,30,24] afford milligram quantities of the advanced cell-wall precursors. These discoveries made it possible to set up efficient enzymatic assays for transglycosylases.

We previously reported the characterization and assay development of bacterial cell-wall stage II enzyme MurG.[15] Now we have extended our program into transglycosylases, the last enzymes involved in the peptidoglycan biosynthesis. Here we describe the cloning and expression of a TGase domain of *S. pneumoniae* PBP1b and the characterization of the domain by fluorescence coupled enzyme assay and surface plasmon resonance (SPR) binding assay. Kinetic characterization of a MTG from *S. aureus* by fluorescence coupled enzyme assay was also performed. The initial reaction rates of several MTGs from different species were compared. The enzymatic characterization will be useful for future structure determination and antibiotic development.

2. Results and discussion

To facilitate the mechanistic and structural studies of transglycosylases, we cloned and overexpressed a TGase domain of PBP1b from common bacterial pathogen *S. pneumoniae* and several MTGs from *S. aureus* and *E. coli*. Full-length Pbp1b from *S. pneumoniae* which has both the TGase and the TPase domains contains 821 amino acids (90 kDa). The proposed topology of this enzyme predicted from a domain software globplot 2 consists of a cytoplasmic tail, a hydrophobic transmembrane helix (60–82), and a periplasmic region with a TGase domain (106–279) and a TPase domain (422–696). A plasmid to express the full-length protein was constructed, but initial attempts to solubilize membrane-associated full-length *S. pneumoniae* PBP1b using various detergents including Triton and CHAPS failed. So a truncated mutant which has only the TGase domain terminating at amino acid 300 was prepared. The termination site was chosen to locate in the proposed junction between TGase and TPase. Inclusion of the transmembrane domain in expression would complicate protein purification due to its hydrophobicity. So our final TGase domain of PBP1b (amino acid

82–300) does not have the transmembrane domain for easy purification. After cloning and expression, TGase domain of the enzyme was obtained with a yield of 1.5 mg/L. Recently the TGase domain of *E. coli* PBP1b and PBP2 from *S. aureus* were cloned and expressed.[3,2] To compare the enzymatic activities of this TGase domain with other MTGs, we also cloned and expressed three MTGs including a truncated MTG from *S. aureus*,[32] a full-length and a truncated (without the transmembrane domain) MTG from *E. coli*. Their expression levels are summarized in Table 1.

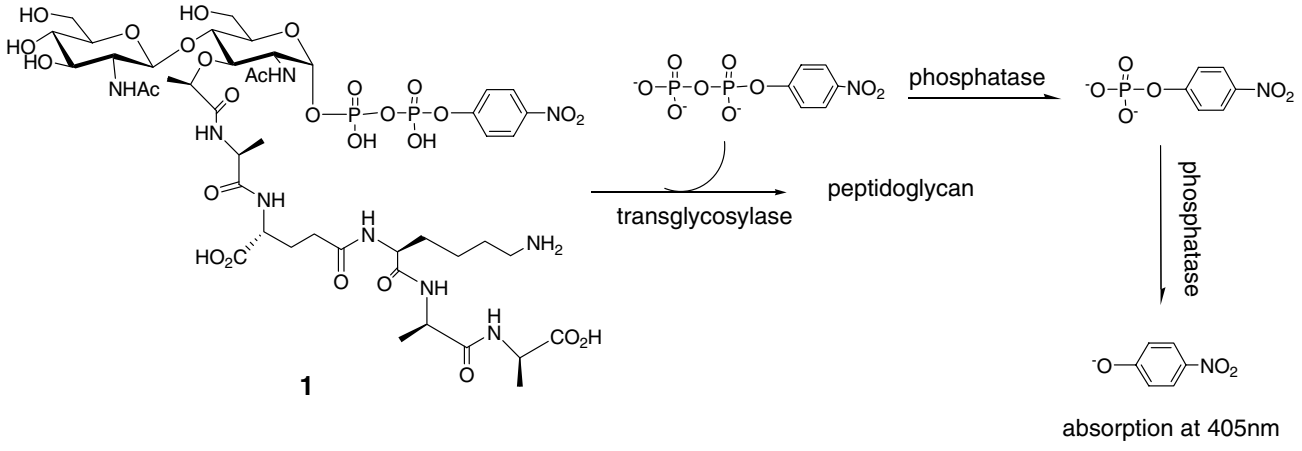
To characterize the isolated TGase domain and the MTGs, we need access to the natural substrate Lipid II. Lipid II, the final monomeric unit in the bacterial cell-wall biosynthesis pathway, is a disaccharide anchored to the membrane by a 55-carbon undecaprenyl chain with a *cis*-allylic pyrophosphate linkage to the sugar moiety. The long chain of Lipid II together with the allylic pyrophosphate linkage complicates the synthesis, purification, and handling. Therefore, we wish to find an analogue of Lipid II that is active for this enzyme and can be manipulated conveniently.

A Lipid II nitrophenol analogue which can be used to set up an efficient optical assay for transglycosylase activity was synthesized. *para*-Nitrophenyl phosphate (*p*-NPP) is a chromogenic substrate for most phosphatases such as alkaline phosphatases, acid phosphatases, protein tyrosine phosphatases, and serine/threonine phosphatases. The reaction yields *para*-nitrophenol, which becomes an intense yellow soluble product under alkaline conditions and can be conveniently measured at 405 nm on a spectrophotometer. This chromogenic reaction has been utilized for direct characterization of enzyme activities of, for example, protein phosphatase,[17] α-glucosidase,[14] and manosidase.[4] We adapted it here to assay transglycosylase activity as shown in Scheme 2. In this assay, the transglycosylase polymerizes the sugar part of the Lipid II nitrophenol analogue **1** and releases the nitrophenol diphosphate. Another enzyme, alkaline phosphatase, hydrolyzes nitrophenol diphosphate to give nitrophenol, which absorbs at 405 nm. So the transglycosylase activity can be monitored continuously by checking absorbance at 405 nm.

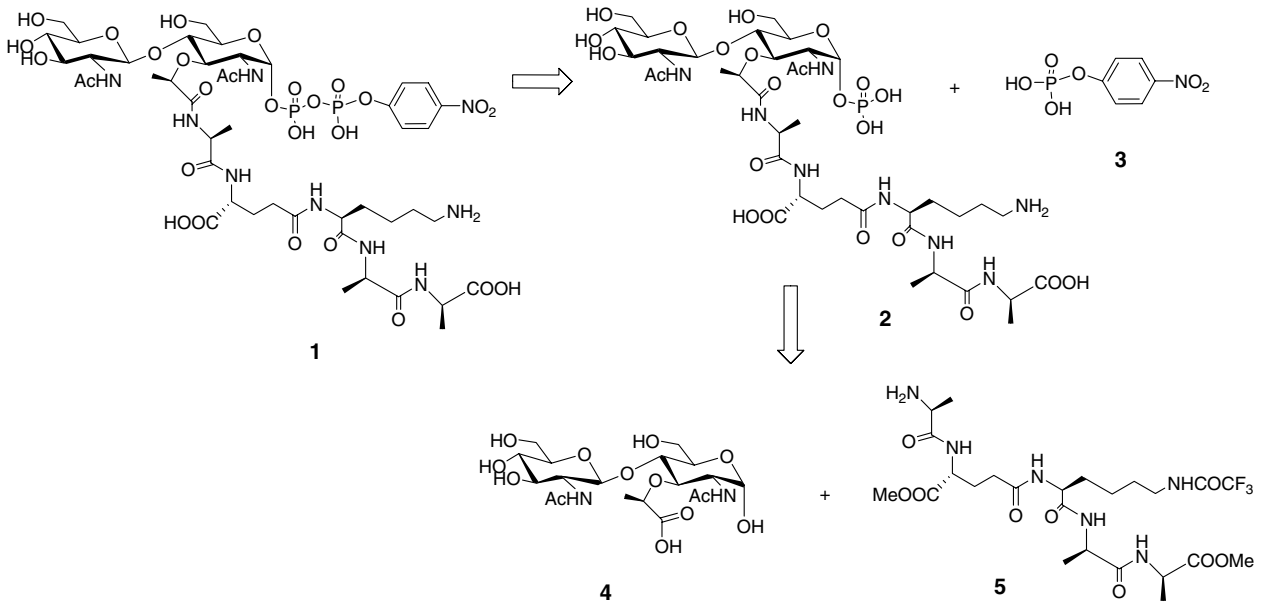
Table 1. Transglycosylases expressed and purified from *Escherichia coli*

Expressed protein	Yield (mg/L)
Full-length PBP1b from *S. pneumoniae*	n.a.
Amino acid 82–300 TGase domain of Pbp1b from *S. pneumoniae*	1.5
Amino acid 51–250 MTG from *S. aureus*	1.4
Amino acid 37–241 MTG from *E. coli*	0.34
Full-length MTG from *E. coli*, periplasmic expression	2.0

Our synthetic target, Lipid II nitrophenol analogue **1**, consists of three parts: the disaccharide central core **4**, the nitrophenol phosphate group **3**, and the pentapeptide moiety **5** (Scheme 3). In analogy to the Lipid I nitrophenol analogue synthesis,[15,30] the first disconnection reveals disaccharide monophosphate **2** and the nitrophenol-linked phosphate **3**. Disaccharide monophosphate **2** could be derived from coupling protected pentapeptide **5** with the disaccharide monophosphate **4**. Since we were successful in synthesizing Lipid I nitrophenol analogue, the challenge of synthesizing this molecule would be access to multiple gram quantities of orthogonally protected *N*-acetyl-(2-deoxy-2-aminoglycopyranosyl)-β-[1,4]-*N*-acetylmuramyl acid **11** (NAG-NAM). The synthesis of the disaccharide subunit **11** started from conversion of 1-α-*O*-benzyl-*N*-acetyl-4,6-benzylindene muramic acid **6** to the desired benzoyl-acceptor **8** in three steps. *N*-Troc-protected donor **9** was prepared from glucosamine **7** in three steps for 80% yield. Reaction of donor **9** with acceptor **8** in the presence of *N*-iodosuccinimide and trifluoromethanesulfonic acid gave the desired β-glycoside **10** in 56% yield after optimization. At this stage, the disaccharide **10** was unable to be separated from the acceptor **8**. After working up the reaction, the glycosylation mixture was dissolved in acetic acid and zinc dust was then added to reveal the free amine, which was acetylated with acetic anhydride in pyridine to provide glycosylation product **11**. Pure glycosylation product **11** can be separated by flash column (Scheme 4). Next reactions are to introduce the penta-



Scheme 2. Optical assay of transglycosylase with nitrophenol Lipid II analogue **1**.

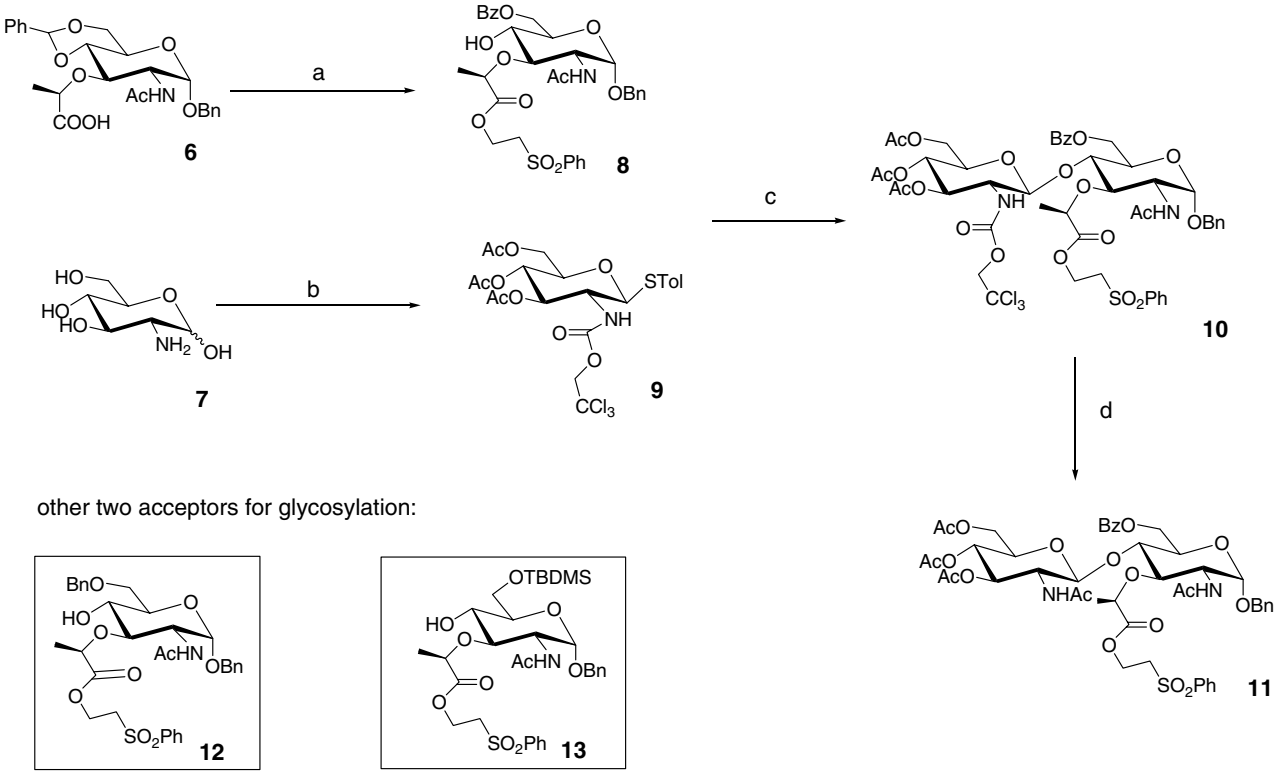


Scheme 3. Retrosynthetic route for Lipid II nitrophenol analogue **1**.

peptide part and the nitrophenol phosphate which are similar with the procedures for synthesizing Lipid I nitrophenol analogues. The final product was purified by reverse-phase HPLC.

For comparison, the natural substrate of the enzyme Lipid II was also synthesized. The synthetic strategy is similar as that of nitrophenol analogue except for the diphosphate coupling reaction. We utilized phosphoroimidazolidate method that was exploited in the Lipid I and Lipid II total syntheses.[15,30,29]

Measurements of enzymatic activity were conducted next with the synthesized Lipid II and TGase domain.



Scheme 4. Synthesis of disaccharide subunit **11**. Reagents and conditions: (a) i—2-phenolsulfonic acid, DMAP, EDC; ii—H_2O, acetic acid; iii—benzyl chloride, pyridine, 70% for three steps; (b) i—Troc-Cl, $NaHCO_3$; ii—acetic anhydride, pyridine; iii—Tol-SH, $BF_3{\cdot}Et_2O$, 80% for three steps; (c) NIS, triflic acid, molecular sieves AW300, 56%; (d) i—zinc dust, acetic acid; ii—acetic anhydride, pyridine, 40% for two steps.

At the outset, an HPLC-based assay was used to identify the glycosyltransfer reaction.[24] Direct detection of polymerized Lipid II was confirmed by following the procedures published by Schwartz and coworkers.[24] After positive results were obtained from both the HPLC assay and the direct detection of enzymatic product, dansyl-Lipid II derivative was synthesized and a fluorescence coupled enzyme assay was used to evaluate the isolated TGase domain (Fig. 1).[23] This coupled fluorescence assay involves the use of a substrate labeled with dansyl moiety, whose fluorescence intensity decreases when the surroundings of the fluorophore change from a less polar environment to a more polar one. In this assay, incubation of dansyl Lipid II with the transglycosylase releases the lipid phosphate and produces polymerized Lipid II, the uncrosslinked peptidoglycan. Muramidase then hydrolyzes the polymer to produce the dansyl disaccharide derivative. Enzymatic removal of the lipid phosphate exposes the dansyl fluorophore to a less polar environment resulting in decrease of fluorescence intensity, which is monitored to measure the enzyme kinetics. Under the optimized reaction conditions, the TGase obeys Michaelis–Menten kinetics, and the K_m for dansyl-Lipid II was 62 μM and the k_{cat} was 0.76 s^{-1}. The MTG from *S. aureus*[32] was also characterized using this approach and a K_m of 2 μM and a k_{cat} of 0.01 s^{-1} were obtained as shown in Figure 2. A similar construct of *S. aureus* MTG was characterized using a radioactive assay and *meso*-[^{14}C]A_2pm-labeled C55 Lipid II while this paper was under review.[27] The

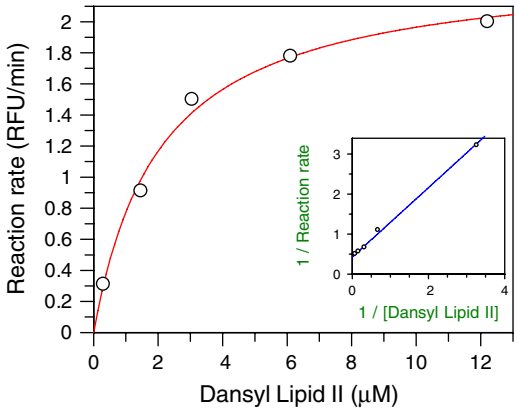


Figure 2. Michealis–Menten plot of kinetic characterization of *Staphylococcus aureus* MTG. The inset shows a double reciprocal plot.

results are comparable with ours obtained from the fluorescence coupled enzyme assay.

Using the fluorescence coupled enzyme assay, we characterized and compared the initial reaction rates of other MTGs we cloned and expressed. Assays were performed under identical conditions and the results are shown in Table 2. Although all enzymes are active, they showed different initial reaction rates and the TGase domain from *S. pneumoniae* was the most active enzyme. The full-length enzyme from *E. coli* is less active than the truncated one probably

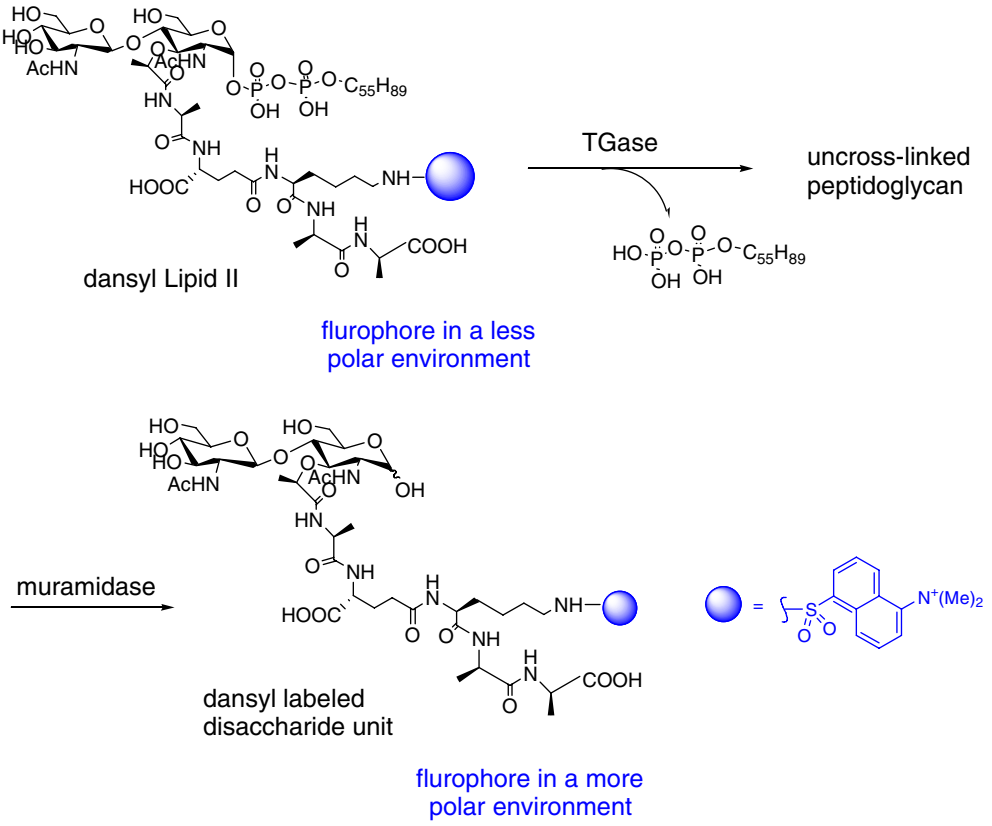


Figure 1. Coupled fluorescence assay for transglycosylase.

Table 2. Reaction rates (RFU/min) and the relative reaction rates of different transglycosylases

Transglycosylases (60 nM)	Reaction rates[a] (RFU/min)	Relative rates (U/min)
S. pneumoniae TGase domain of PBP1 b	17	6.1
Truncated MTG from *E. coli*	13	4.8
Full-length MTG from *E. coli*	5.0	1.8
Truncated MTG from *S. aureus*	2.7	1

[a] The reaction rates of different enzymes were measured under identical conditions at a dansyl-Lipid II concentration of 2 μM.

due to its propensity to aggregate in solution. Unfortunately, the Lipid II nitrophenol analogue we synthesized was not active for all enzymes we expressed including the TGase domain from *S. pneumoniae* PBP1b, MTGs from *S. aureus* and *E. coli*.

We compared the amino acid sequences of MTGs from *S. aureus* and *E. coli*, the TGase domains from *S. pneumoniae* and *E. coli* PBP1b, and *S. aureus* PBP2. The alignment result is shown in Figure 3. It has been proposed the glutamate residue of the first conserved motif, EDXXFXXH, is highly conserved in all known TGases.[27] This residue was found to be essential for catalysis by site-directed mutagenesis, suggesting it acts as the key catalytic residue in the active site of the TGase. About the substrate specificity, we notice that although the nitrophenol analogue of Lipid I works efficiently with MurG,[15] the nitrophenol analogue of Lipid II is not active toward all MTGs we tested. Because Lipid I and Lipid II share the same lipid chain, these results suggest MurG and transglycosylases recognize the lipid part of their substrates differently and the TGase can recognize at least part of the lipid chain. We think the difference is partly due to that the catalytic reaction of TGase is taking place at the carbon (1) residue of Lipid II which is close to the undecaprenol moiety, while the reaction of MurG is taking place at the carbon (4) residue of Lipid I which is far from the lipid chain.

We next used a different method to evaluate TGase domain that does not rely on a substrate. In this assay, TGase is bound to a known inhibitor, and potential inhibitors are evaluated based on their ability to compete for the binding, which is monitored by surface plasmon resonance (SPR). Moenomycin, a known transglycosylase inhibitor which binds to the enzyme effectively at low concentration, can be used in the assay.[13] It was derivatized with an amine moiety and

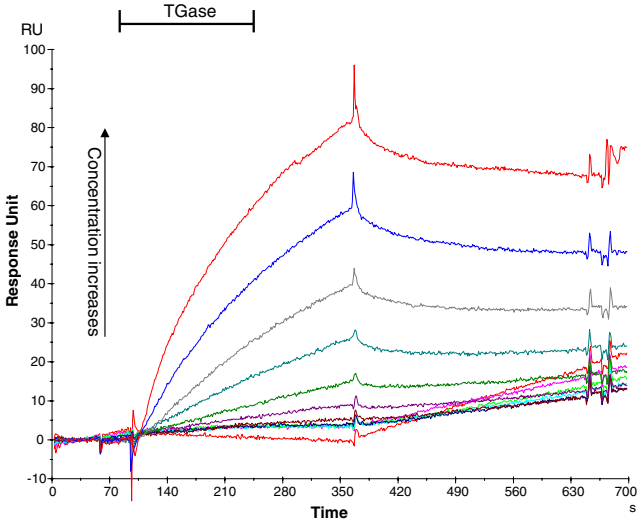


Figure 4. Binding curve for different concentrations of *Streptococcus pneumoniae* TGase domain of PBP1b to moenomycin.

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TGase domain 1  91 ----------------------------SDLLRTSISSEQISENLKKAIIATEDEHFKEH
MTG 1            1 --MSVAPVPFSAVMVERQVSAWLHGNFRYVAHSDWVSMDQISPWMGLAVIAAEDQKFPEH
MTG 2           72 ------------------------ELRKIENKSSFVSADNMPEYVKGAFISMEDERFYNH
TGase domain 2 181 ATIVNMENNRQFGFFRLDPRLITMISSPNGEQRLFVPRSGFPDLLVDTLLATEDRHFYEH
PBP2            90 ----------------------------NGQRHEHVNLKDVPKSMKDAVLATEDNRFYEH
Conserved motif 1                                                  EDXXFXXH

TGase domain 1  123 KGVVPKAVIRATLGKFVGLGSSSGGSTLTQQLIKQQVVGDAPTLARKAAEIVDALALERA
MTG 1            59 WGFDVASIEKALAHNERNENRIRGASTISQQTAKNLFLWDGRSWVRKGLEAGLTLGIETV
MTG 2           108 HGFDLKGTTRALFSTI-SDRDVQGGSTITQQVVKNYFYDNDRSFTRKVKELFVAHRVEKQ
TGase domain 2  241 DGISLYSIGRAVLANLTAGRTVQGASTLTQQLVKNLFLSSERSYWRKANEAYMALIMDAR
PBP2            122 GALDYKRLFGAIGKNLTGGFGSEGASTLTQQVVKDAFLSQHKSIGRKAQEAYLSYRLEQE
Conserved motif 2                                                  RKXXE

TGase domain 1  183 MNKDEILTTYLNVAPFGRNNKGQN----IAGARQAAEGIFGVDASQLTVPQAAFLAGLPQ
MTG 1           119 WSKKRILTVYLNIAEF-----GDG----VFGVEAAAQRYFHKPASKLTRSEAALLAAVLP
MTG 2           167 YNKNEILSFYLNNIYF-----GDN----QYTLEGAANHYFGTTVNK--------------
TGase domain 2  301 YSKDRILELYMNEVYL-----GQSGDNEIRGFPLASLYYFGRPVEELSLDQQALLVGMVK
PBP2            182 YSKDDIFQVYLNKIYY-----SDG----VTGIKAAAKYYFNKDLKDLNLAEEAYLAGLPQ
Conserved motif 3     KXXXLXXYXN

TGase domain 1  239 SPITYSPYENTGELKS----------------------------------------------
MTG 1           170 NPLRFKVSSPSGYVRSRQAWILRQMYQLGGEPFMQQHQLD--------------------
MTG 2           204 ----------------------------------------------------------
TGase domain 2  356 GASIYELQAKLGDKVK----------------------------------------------
PBP2            233 VPNNYNIYDHPKAAEDRKNTVLYLMHYHKRITDKQWEDAKKIDLKANlVNRTAEERQNID
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Figure 3. Alignment of amino acid sequences of TGases and MTGs from different species. The three conservative motifs are highlighted in dark. TGase domain 1 is TGase domain of *S. pneumoniae* PBP1b, MTG 1 is MTG from *Staphylococcus aureus*, MTG 2 is MTG from *Escherichia coli*, TGase domain 2 is TGase of *E. coli* PBP1b, and PBP2 is PBP2 from *S. aureus*.

then immobilized on the sensor chip CM5.[1] When a solution of the isolated TGase domain passes over the chip, the enzyme binds to the chip through specific interaction with moenomycin. Initial binding experiments indicated the interaction between the enzyme and immobilized moenomycin was concentration dependent, as shown in Figure 4. The dissociation constant K_d of 12 μM was obtained from these experiments.

This binding assay can be used to analyze potential inhibitors by flowing them over the chip to observe the dissociation of the enzyme. If the inhibitor cannot compete with moenomycin for binding with the enzyme, a large SPR signal will be observed. If the inhibitor competes with moenomycin for binding with the enzyme, a small signal will be observed. A mock competition experiment in solution was performed using moenomycin as a competitive inhibitor. The elution of the enzyme from the chip was observed. As the concentration of flowing moenomycin increased, more and more enzyme was eluted from the chip. We found that when the concentration of moenomycin reached at 160 μM, 50% dissociation of TGase domain from the chip occurred. This result is in good agreement with the value determined previously using PBP1b from *E. coli*.[25] This SPR binding assay is now being used to identify new inhibitors.

3. Conclusion

We have characterized several MTGs including a TGase domain from *S. pneumoniae* PBP1b. The TGase domain can be expressed and purified from *E. coli* with good yield, and it is fully active in the absence of the transmembrane helix and the TPase domain. To set up an efficient enzyme assay for evaluating inhibitors of transglycosylase, we synthesized a nitrophenol and a dansyl analogue of Lipid II. The dansyl Lipid II analogue was used in a coupled fluorescence assay to evaluate the TGases we expressed. An SPR binding assay using moenomycin as the tether also works well for the TGase domain. This assay does not require the substrate which is difficult to synthesize.

The high activity of the isolated TGase domain from the full-length Pbp1b of *S. pneumoniae* and its compatibility with the above two assays should enable us to search for inhibitors of transglycosylases, which may serve as new antibiotics that are less prone to resistance development. In the meantime, we are trying to solve the crystal structure of the TGase domain to guide our inhibitor search on a more rational basis.

4. Experimental

4.1. Generation of transglycosylase expression constructs

The locations of potential transmembrane domains in *S. pneumoniae*, *S. aureus*, and *E. coli* transglycosylases were predicted using PSORT and TMpred software analysis programs. The genes of transglycosylase were cloned from *S. pneumoniae*, *S. aureus*, and *E. coli* genomic DNA by PCR and inserted into vector pET 26(b) and pET 16(b) (Novagen). A His6 tag was added to its C-terminus or N-terminus. After expression, enzyme was purified by Ni^{2+} affinity chromatography according to the manufacturer's protocol (Qiagen).

4.2. Synthesis of benzoylated monosaccharide acceptor 8

Benzyl-*N*-acetyl muramic acid phenylsulfonyl ester **6** (415 mg, 0.75 mmol) was dissolved in 7 mL CH_2Cl_2. To this solution, benzoyl chloride (0.3 mL, 1.5 mmol) and pyridine (0.27 mL) were added at −40 °C and the reaction mixture was warmed to 0 °C and stirred for 1 h. The reaction mixture was diluted with CH_2Cl_2 (50 mL) and washed with ammonia chloride once and then brine twice. The organic layer was dried and concentrated in vacuo. Purification with flash chromatography on silica gel eluting with EtOAc/hexanes (4:1) yielded compound **8** 0.369 g (75%) as a white solid: $R_f = 0.2$ (EtOAc/hexanes 4:1); ^1H NMR ($CDCl_3$, 500 MHz) δ 8.09 (d, $J = 7.92$, 2H), 7.96 (d, $J = 7.86$, 2H), 7.73 (m, 1H), 7.62–7.54 (m, 3H), 7.48–7.45 (m, 2H), 7.35–7.26 (m, 5H), 5.34 (d, $J = 3.30$, 1H), 4.91 (dd, $J = 2.95$, 12.1, 1H), 4.73 (d, $J = 11.8$, 1H), 4.56–4.51 (m, 3H), 4.47 (m, 1H), 4.22 (dd, $J = 2.20$, 12.5, 1H), 3.81–3.76 (m, 2H), 3.64–3.56 (m, 2H), 3.45 (m, 3H), 1.99 (s, 3H), 1.24 (d, $J = 6.95$, 3H); HR-FTMS (pos) calcd for $C_{33}H_{37}NO_{11}S$ $[M+Na]^+ = 678.198$, found 678.1991.

4.3. Synthesis of *tert*-butyldimethylsilyl monosaccharide acceptor 13

Benzyl-*N*-acetyl muramic acid phenylsulfonyl ester **6** (166 mg, 0.30 mmol) was dissolved in 7 mL pyridine. To this solution, *tert*-butyl, dimethylsilylchloride (54.4 mg, 0.36 mmol) was added at 0 °C and the reaction mixture was warmed to room temperature and stirred for overnight. The reaction mixture was diluted with CH_2Cl_2 (50 mL) and washed with brine twice. The organic layer was dried and concentrated in vacuo. Purification with flash chromatography on silica gel eluting with EtOAc/hexanes (4:1) yielded compound **13** 0.369 g (75%) as a white solid: $R_f = 0.5$ (EtOAc/hexanes 4:1); ^1H NMR ($CDCl_3$, 600 MHz) δ 7.91 (d, $J = 7.44$, 2H), 7.66 (d, $J = 7.44$, 2H), 7.56 (t, $J = 7.44$, 2H), 7.33 (m, 4H), 7.23 (m, 1H), 5.26 (d, $J = 3.06$, 1H), 4.65 (d, $J = 11.8$, 1H), 4.52 (m, 4H), 3.84 (m, 1H), 3.77 (m, 1H), 3.72–3.45 (m, 10H), 1.98 (s, 3H), 1.25 (d, $J = 7.02$, 3H), 0.91 (s, 9H), 0.10 (d, $J = 3.48$, 6H); HR-FTMS (pos) calcd for $C_{32}H_{47}NO_{10}SSi$ $[M+Na]^+ = 688.2582$, found 688.2568.

4.4. Synthesis of benzylated monosaccharide acceptor 12

Benzyl-*N*-acetyl-4,6-benzylidine-muramic acid phenylsulfonyl ester **6** (500 mg, 0.78 mmol) was dissolved in 3.1 mL THF with 1 M sodium boroncyanide hydride. To this solution, 4 N HCl in dioxane was added to make the solution acidic and the reaction mixture was stirred for 8 h. The reaction mixture was concentrated in vacuo and then diluted with CH_2Cl_2 (50 mL) and washed with brine twice. The organic layer was dried and

concentrated in vacuo. Purification with flash chromatography on silica gel eluting with EtOAc/hexanes (4:1) yielded compound **12** 0.426 g (85%) as a white solid: $R_f = 0.3$ (EtOAc/hexanes 4:1); ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.90 (d, $J = 7.44$, 2H), 7.65 (t, $J = 7.44$, 1H), 7.55 (d, $J = 7.86$, 2H), 7.33–7.24 (m, 11H), 5.28 (d, $J = 3.54$, 1H), 4.66 (d, $J = 11.8$, 1H), 4.60 (d, $J = 11.8$, 1H), 4.47–4.43 (m, 5H), 3.82 (m, 1H), 3.72 (m, 3H), 3.63 (m, 3H), 3.62 (m, 2H), 3.47 (m, 2H), 3.33 (m, 1H), 1.98 (s, 3H), 1.24 (d, $J = 7.02$, 3H); HR-FTMS (pos) calcd for $C_{33}H_{39}NO_{10}S$ $[M+Na]^+ = 664.2187$, found 664.2165.

4.5. Glycosylation

Donor **9** (233 mg, 0.40 mmol), acceptor **8** (130 mg, 0.20 mmol) and molecular sieve AW300 (3 g) were dried under vacuo overnight. The mixture was dissolved in dried CH_2Cl_2 (30 mL) and NIS (141 mg, 0.60 mmol) was added at −40 °C. To this mixture, triflic acid (597 μL 0.5 M in ether) was added and the reaction mixture was stirred for 1 h. The reaction mixture was filtered and concentrated in vacuo and then diluted with CH_2Cl_2 (50 mL) and washed with brine twice. The organic layer was dried and concentrated in vacuo. Purification with flash chromatography on silica gel eluting with EtOAc/hexanes (3:2) to EtOAc/hexanes (1:3) yielded compound **10** 0.194 g (65%) as a white solid: $R_f = 0.5$ (EtOAc/hexanes 3:1); ^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.09 (d, $J = 7.50$, 2H), 7.96 (d, $J = 7.44$, 1H), 7.73 (t, $J = 7.44$, 2H), 7.66–7.62 (m, 4H), 7.52 (t, $J = 7.86$, 2H), 7.32 (m, 3H), 6.20 (d, $J = 9.66$, 1H), 5.33 (d, $J = 3.54$, 1H), 5.09 (t, $J = 9.6$, 1H), 4.98 (t, $J = 10.08$, 1H), 4.64 (d, $J = 12.3$, 1H), 4.56–4.50 (m, 4H), 4.47 (q, $J = 6.12$, 1H), 4.33–4.28 (m, 3H), 4.12 (q, $J = 9.66$, 1H), 3.92 (m, 1H), 3.85–3.80 (m, 1H), 3.79 (m, 1H), 3.56–3.47 (m, 2H), 3.35 (m, 1H), 2.03 (s, 3H), 2.01 (s, 3H), 2.00 (s, 3H), 1.99 (s, 3H), 1.98 (s, 3H), 1.21 (d, $J = 7.02$, 3H); HR-FTMS (pos) calcd for $C_{47}H_{56}N_2O_{19}S$ $[M+Na]^+ = 1007.309$, found 1007.3059.

4.6. Disaccharide acetate 11

Disaccharide **10** (147 mg, 0.15 mmol) was dissolved in acetic acid (10 mL) and zinc dust (1.48 g) was added and the reaction mixture was stirred for 2 h. The reaction mixture was filtered and concentrated in vacuo and then was dissolved in 5 mL pyridine and 5 mL acetic anhydride was added. The reaction mixture was stirred for overnight. The reaction mixture was quenched by adding MeOH and was concentrated in vacuo. Purification with flash chromatography on silica gel eluting with (EtOAc/hexanes 3:1) yielded the titled compound **11** 0.150 g (85%) as a white solid: $R_f = 0.5$ (EtOAc/hexanes 3:1); ^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.02–7.34 (m, 15H), 5.44 (d, $J = 3.96$, 1H), 5.09 (t, $J = 11.5$, 1H), 4.96 (m, 2H), 4.75–4.61 (m, 2H), 4.59–4.53 (m, 2H), 4.50–4.49 (m, 2H), 4.44–4.40 (m, 2H), 4.15–4.05 (m, 1H), 3.99–3.82 (m, 3H), 3.63–3.15 (m, 5H), 3.47 (m, 1H), 2.13 (s, 3H), 2.12 (s, 3H), 2.11 (s, 3H), 2.04 (s, 3H), 1.80 (s, 3H), 1.26 (d, $J = 7.02$, 3H); HR-FTMS (pos) calcd for $C_{40}H_{50}N_2O19S$ $[M+Na]^+ = 917.2621$, found 917.2616.

4.7. Disaccharide phosphate

Disaccharide acetate **11** (233 mg, 0.02 mmol) was hydrogenolyzed in MeOH overnight. Then the reaction mixture was filtered and concentrated in vacuo. 1*H*-Tetrazole (6.5 mg 0.09 mmol) was added and the mixture was coevaporated with toluene three times. Then the mixture was dissolved in dried CH_2Cl_2 (4 mL) and cooled to −30 °C. To this solution, dibenzyl diethylphosphoramidate (65 uL, 0.03 mmol) was added. The reaction mixture was warmed to room temperature and stirred for 30 min. Then the reaction mixture was cooled to −40 °C and *m*-CPBA (52 mg, 0.2 mmol) was added and the reaction was stirred at 0 °C for 30 min. The reaction mixture was diluted with CH_2Cl_2 (50 mL) and washed with sodium sulfite, sodium bicarbonate, and brine twice, respectively. The organic layer was dried and concentrated in vacuo. Purification with flash chromatography on silica gel eluting with EtOAc/hexanes (2:3) yielded the titled compound 0.015 g (65%) as a white solid: $R_f = 0.5$ (EtOAc/hexanes 3:2); ^{1}H NMR (CDCl3, 500 MHz): characteristic peaks: δ 8.12–7.30 (m, 15H), 5.86 (m, 1H), 4.42 (d, $J = 6.60$, 2H), 4.33 (m, 1H), 4.09 (d, $J = 9.90$, 2H), 3.98 (t, $J = 9.15$, 1H), 2.16 (s, 3H), 2.09 (s, 3H), 2.07 (s, 3H), 2.03 (s, 3H), 1.57 (d, $J = 7.0$, 3H), 1.52 (d, $J = 6.6$, 3H), 1.49 (d, $J = 7.0$, 3H), 1.46 (d, $J = 7.35$, 3H); HR-FTMS (pos) calcd for $C_{70}H_{92}F_3N_8O_{28}S$ $[M+Na]^+ = 1603.5603$, found 1603.5689.

4.8. Nitrophenol Lipid II analogue 1

The procedure was similar as the preparation of nitrophenol Lipid I analogue. ^{1}H NMR (D_2O, 600 MHz) δ 8.27 (d, $J = 8.76$, 2H), 7.32 (d, $J = 8.76$, 2H), 5.41 (m, 1H), 4.50 (t, $J = 7.86$, 1H), 4.25–4.11 (m, 6H), 4.14 (t, $J = 7.02$, 1H), 3.99 (m, 1H), 3.85 (m, 4H), 3.79 (d, $J = 11.88$, 1H), 3.68 (m, 5H), 3.48 (t, $J = 8.76$, 1H), 3.33 (m, 2H), 2.92 (t, $J = 6.96$, 2H), 2.27 (m, 2H), 1.92 (s, 3H), 1.86 (s, 3H), 1.71 (m, 2H), 1.61 (m, 2H), 1.34 (m, 14H); ESI (neg) calcd for $C_{45}H_{71}N_9O_{28}P_2$ $[M-H]^- = 1246$, found 1246.

4.9. Fluorescence coupled enzyme assay

Reactions were carried out in a Quartz cuvette in 200 μL volume and the decrease in fluorescence was monitored at 520 nm using Hitachi F-2000. Each reaction mixture contained MTG reaction buffer (50 mM PIPES, pH 6.1, 50 mM $MgCl_2$, 10 mM $CaCl_2$, 10% DMSO, and 0.2 mM decyl-PEG), 0.6 μg *N*-acetylmuramidase, and an appropriate amount of dansyl-labeled Lipid II analogue and 1 μL enzyme. All of the components except for the enzyme were premixed in an Eppendorf tube and incubated at room temperature for 10 min. Then the enzyme was added and the fluorescence was monitored. The negative control was done by adding 1 μL buffer instead of 1 μL enzyme to the reaction mixture.

4.10. Surface plasmon resonance assay

The SPR measurements were carried out with Biacore 3000 with research grade sensor chips. (1) Coupling of

moenomycin–amine derivative to a CM5 sensor chip: The immobilization procedure was carried out according to the BIACORE's protocol. The immobilization buffer is HBS-Ep buffer biacertified, and moenomycin–amine derivative at 0.5 mg/mL was used for immobilization. Ethanol amine was used as the negative control for immobilization. (2) After the immobilization, the chip was rinsed thoroughly with buffer A (10 mM Tris–maleate, pH 6.6, 150 mM NaCl, and 1% Triton X-100). A solution of TGase domain from *S. pneumoniae* PBP1b in buffer A at constant concentrations (25 μM, 12.5 μM, 6.24 μM, 3.12 μM, 1.51 μM, 0.76 μM, 0.38 μM, 0.19 μM, 0.095 μM, and 0.048 μM) was injected and bound to the chip. The interaction was investigated under constant conditions at 125 μL of protein solution and 25 μL/min flow rate. In the elution experiments, moenomycin was dissolved in buffer A and injected at different concentrations (1 mM, 500 μM, 30 μM, 3 μM, 300 nM, 30 nM, and 3 nM) to elute the bound protein.

Acknowledgments

This research was supported by the National Institutes of Health (GM 44154). We thank Thomas K. Ritter for providing moenomycin amine derivative.

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