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Tuning the Moenomycin Pharmacophore To Enable Discovery of Bacterial Cell Wall Synthesis Inhibitors

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Supporting Information

ABSTRACT: New antibiotic drugs need to be identified to address rapidly developing resistance of bacterial pathogens to common antibiotics. The natural antibiotic moenomycin A is the prototype for compounds that bind to bacterial peptidoglycan glycosyltransferases (PGTs) and inhibit cell wall biosynthesis, but it cannot be used as a drug. Here we report the chemoenzymatic synthesis of a fluorescently labeled, truncated analogue of moenomycin based on the minimal pharmacophore. This probe, which has optimized enzyme binding properties compared to moenomycin, was designed to identify low-micromolar inhibitors that bind to conserved features in PGT active sites. We demonstrate its use in displacement assays using PGTs from *S. aureus*, *E. faecalis*, and *E. coli*. 110,000 compounds were screened against *S. aureus* SgtB, and we identified a non-carbohydrate based compound that binds to all PGTs tested. We also show that the compound inhibits *in vitro* formation of peptidoglycan chains by several different PGTs. Thus, this assay enables the identification of small molecules that target PGT active sites, and may provide lead compounds for development of new antibiotics.

The supporting structure of the bacterial cell wall is a layer of polysaccharide strands containing peptide cross bridges, termed peptidoglycan (PG). This polymer protects the cell membrane from rupture in harsh environments. The final stage of the extracellular biosynthesis of PG proceeds in two steps: In the transglycosylation step, the disaccharide phospholipid lipid II is polymerized to form polysaccharide strands, and in the subsequent transpeptidation step, these strands are cross-linked (Figure 1a).[1] These transformations are catalyzed by bifunctional penicillin binding proteins (PBPs) that have both a glycosyltransferase (GT) and a transpeptidase active site.[2] Additionally, some bacteria possess monofunctional peptidoglycan glycosyltransferases (PGTs) that form polysaccharide strands, which are then cross-linked by PBPs.[3] All GT domains, whether found within bifunctional PBPs or in monofunctional enzymes, contain a set of invariant residues that both bind substrate and catalyze the polymerization of lipid II.[4] In bacteria, proper synthesis of PG is required for cell viability, and inhibition of PG synthesis leads to cell death. For decades, development of new antibiotics has focused on targets involved in the cell wall synthesis and remodeling.[5] However, direct inhibition of PGT activity has so far not been exploited for the development of antibiotics.[6]

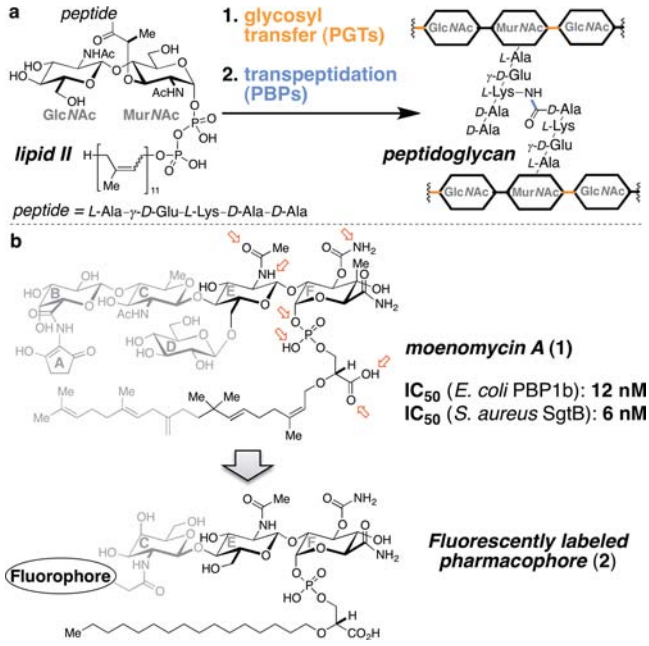

Figure 1. Probe compound **2** was designed to identify compounds that bind to the conserved features of the PGT active site. (a) The final stage of the biosynthesis of peptidoglycan. (b) The moenomycin pharmacophore is represented in black. Red arrows mark the functional groups that form crucial hydrogen bonds to conserved active site amino acid residues of PGTs. Parts of the molecule that do not contribute significantly to protein binding are shown in gray. IC_{50} values are given for *in vitro* PGT inhibition.[3b,10]

The only known active site inhibitor of the PGTs is the natural product moenomycin A (Figure 1b).[6,7] Its desirable properties include extraordinary potency without development of resistance,[8] but its clinical use is prevented due to physical properties that result in poor oral bioavailability and long serum half-life. However, moenomycin is potentially useful for discovering other structural classes of molecules that target the same active site. In one example of such a strategy, a fluorophore was directly attached to the A-ring of the natural product, and displacement of

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this probe was monitored to discover new PGT inhibitors.[9] The beauty of this approach is its simplicity. However, because moenomycin is a low-nanomolar inhibitor of PGTs, it cannot be displaced by low-affinity binders found in typical compound or fragment collections. Thus, we set out to design a probe based on moenomycin that shows weaker binding affinity but retains the structural features that determine the specificity for compounds that bind to the active site of the PGTs, which is conserved across all pathogens.

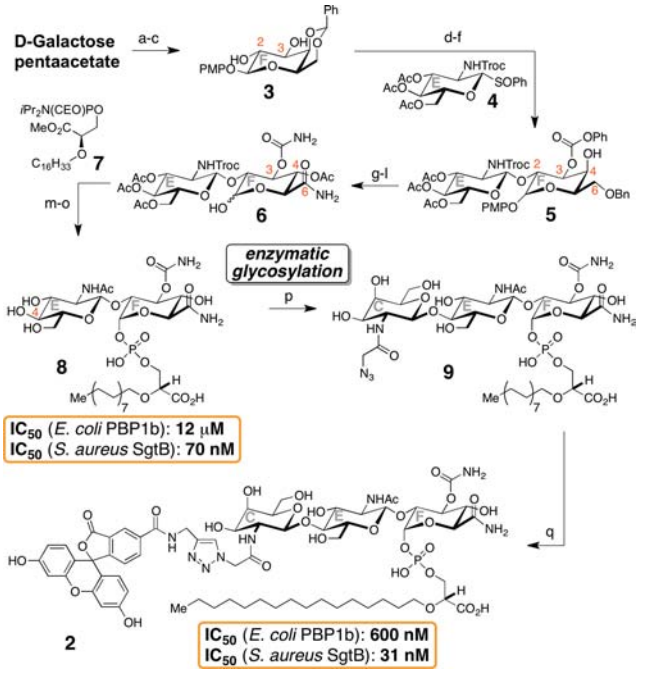
Four crystal structures of moenomycin bound to PGTs showed that the E,F-disaccharide and the phosphoglycerate engage in hydrogen bonds to several conserved, catalytically essential amino acid residues in the active site (Figure 1b).[11] It was also reported that a lipid side chain of at least 10 carbon atoms in length is required for enzyme inhibitory activity.[7,11e,12] Thus, we needed to design a probe that retained these structural features of moenomycin and contained a site that could be easily derivatized for installation of a fluorophore. The crystal structures indicated that a fluorescent label attached to the C-ring *N*-acetyl group could be accommodated, since it points out of the enzyme binding pocket. Thus, we targeted trisaccharide phosphoglycerate **2** as a probe compound (Figure 1b) that would allow us to identify low-affinity active site inhibitors.

The synthesis of probe **2** was achieved using a variety of synthetic methods developed in our group (Scheme 1).[12,13] Peracyl galactose was first converted into F-ring building block **3** in three steps (75% yield). Following carboxylation of the C3-hydroxyl of **3**, glycosylation with sulfoxide **4** and reductive opening of the benzylacetal provided disaccharide **5**. In a sequence of six steps, the F-ring was equipped with the moenomycin substituents crucial for contacting the active site. First, the configuration at C4 was inverted (galacto to gluco configuration) and the C3-carbamate as well as the C6-amide were installed. Deprotection at the anomeric center then provided disaccharide **6**. Installation of phosphoglycerate **7** proceeded smoothly, and, following global deprotection, we obtained the moenomycin pharmacophore **8**. To our delight, we noted that disaccharide **8** inhibits PG formation *in vitro* with IC_{50} values of 12 μM and 70 nM against *E. coli* PBP1b[14] and *S. aureus* SgtB,[10b,15] respectively.[3b,10]

Next, we had to selectively attach the fluorescently labeled C-ring to the unprotected disaccharide phosphoglycerate **8**. To this end, we took advantage of the bovine glycosyltransferase GalT (Y289L), which was previously engineered to selectively transfer a range of *N*-acetyl galactosamine derivatives, including *N*-azidoacetyl galactosamine (Gal*N*Az), to the C4 hydroxyl of *N*-acetylglucosamine derivatives.[16] Installation of galactosamine at a model disaccharide proceeded smoothly;[4] however, we found that incubation of **8** and UDP-Gal*N*Az with GalT (Y289L) generated trisaccharide **9** only in yields below 10%. We speculated that either **8** or product **9**, being derivatives of moenomycin A, might inhibit GalT. Gratifyingly, we found that reducing the concentration of **8** by 10-fold while increasing the amount of GalT to 5 mol% allowed the glycosyl transfer to proceed in >90% yield after 60 h (>4 mg isolated). Treatment of trisaccharide **9** with $CuSO_4$/Na-ascorbate and fluorescein 6-propargyl carboxamide in DMF provided **2** in 87% yield (>4 mg, 17 steps overall).

In order to assess the potential of trisaccharide phosphoglycerate **2** to be used as a probe in the proposed displacement assay, we examined its ability to bind to bacterial PGTs using three different methods. First, using a well described biochemical assay that monitors PGT formation *in vitro*,[3b,10] we showed that

Scheme 1. Synthesis of 2[a]



[a]Reagents and conditions: (a) *p*-$MeOC_6H_4OH$, $BF_3{\cdot}OEt_2$, DCM, rt, 24 h; (b) cat. NaOMe, MeOH, rt, 2 h; (c) $PhCH(OMe)_2$, cat. PTSA, CH_3CN; (d) $ClCO_2Ph$, pyridine, −40 °C, 2 h, 75% (over 3 steps); (e) **4**, Tf_2O, DTBMP, ADMB, mol. sieves 4 Å, DCM, −78 °C, 1.5 h, 56%; (f) Et_3SiH, TfOH, mol. sieves 4 Å, DCM, −78 °C; (g) Tf_2O, pyridine, DCM, −40 °C to rt, 2 h; (h) CsOAc, 18-crown-6, PhMe, rt, 14 h, 55% (over 3 steps); (i) H_2, 10% Pd/C (1 wt%), Cl_3CCO_2H/MeOH, rt, 45 min; (j) TEMPO, $PhI(OAc)_2$, DCM/H_2O (2/1), rt, 2 h; (k) $ClCO_2i$Bu, *N*-methylmorpholine, THF, −40 °C, 5 min; then NH_3, ^{i}PrOH, rt, 24 h, 46% (over 3 steps); (l) CAN, MeCN/H_2O (4/1), rt, 1.5 h; (m) **7**, tetrazole, mol. sieves 3 Å, MeCN, 0 °C, 2 h; then ^{t}BuO_2H, 0 °C, 1 h; then $P(OMe)_3$, rt, 0 °C, 47% (over 2 steps); (n) Zn, Ac_2O, AcOH, THF, rt, 10 h; (o) LiOH, THF/H_2O_2 (8/1), 0 °C, 2 h, 63% (over 2 steps); (p) **8** (0.1 mM); GalT (Y289L) (5 mol%); UDP-Gal*N*Az (1.3 mM), Tris (16 mM, pH 8.0), $MnCl_2$ (16 mM), CIP (0.2 U/μL), 37 °C, 60 h, >90%; (q) fluorescein 6-propargyl carboxamide, $CuSO_4$, Na-ascorbate, DMF, rt, 48 h, 87% (over 2 steps). Abbreviations: PTSA, *p*-toluenesulfonic acid; ADMB, allyldimethoxybenzene; CIP, calf intestinal phosphatase; Troc, 2,2,2-trichloroethoxycarbonyl; CEO, cyanoethyl. IC_{50} values are given for *in vitro* inhibition.[3b,10]

2 inhibits *E. coli* PBP1b and *S. aureus* SgtB with IC_{50} values of 600 nM and 31 nM, respectively. By comparison the parent natural product moenomycin A shows low-nanomolar inhibition of both enzymes. Second, we measured binding of **2** to PGTs based on fluorescence polarization (FP) readout (Figure 2a) and obtained K_D values ranging from 0.15 to 0.38 μM (75 nM **2**) for enzymes from three different pathogens (*E. coli*, *S. aureus*, and *E. faecalis*;[3i] Figure 2b). Lastly, we verified that **2** can be displaced from *S. aureus* SgtB by moenomycin (**1**) and disaccharide **8**, which is a weak inhibitor of PGTs (Figure 2c). Similar behavior was seen when *E. coli* PBP1b and *E. faecalis* PBP2a were used. In contrast, no significant drop in FP was observed when the detergents Tween-20 and dodecyl maltoside were used instead of moenomycin. Likewise, using bovine serum albumin instead of PBPs did not result in a significant change of FP.[4]

Taken together these results suggest that **2** binds to PGTs at the same site as moenomycin A (**1**), i.e., the active site of PGTs. Furthermore, we had successfully attenuated the affinity of the

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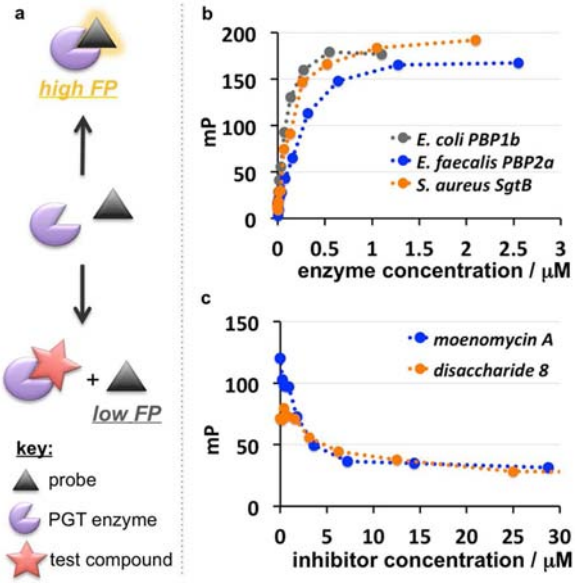

Figure 2. Probe **2** can be used in fluorescence polarization assays to screen for PGT inhibitors. (a) Schematic representation of the fluorescence polarization (FP) assay. (b) Probe **2** binds to PGTs as observed by an increase of FP of **2** (75 nM) when exposed to PBP1b (*E. coli*), PBP2a (*E. faecalis*), and SgtB (*S. aureus*); K_D(*E. coli* PBP1b) = 0.15 μM; K_D(*E. faecalis* PBP2a) = 0.38 μM; K_D(*S. aureus* SgtB) = 0.18 μM. (c) Probe **2** (75 nM) is displaced from *S. aureus* SgtB (0.2 μM) by addition of either moenomycin (**1**) or the weaker PGT inhibitor **8**, as evidenced by reduction of FP. K_i(**1**) = 0.64 μM; K_i(**8**) = 3.17 μM. mP, millipolarization; K_D, dissociation constant; K_i, inhibitor constant.

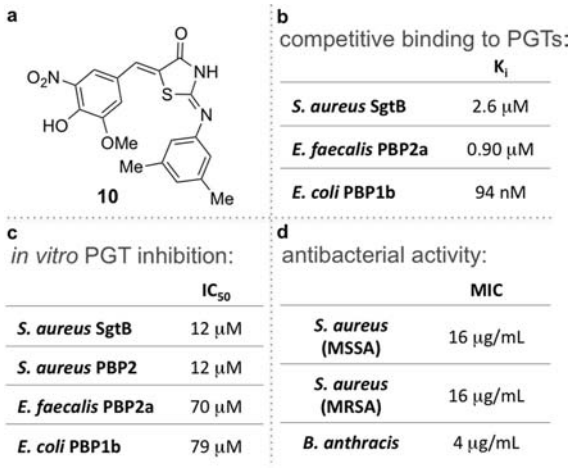

Figure 3. Hit compounds found in a screen against *S. aureus* SgtB also inhibit PGTs of other pathogens. (a) Molecular structure of the characterized hit compound **10** (b) Dose-dependent displacement of probe **2** (75 nM) from PGTs of three pathogens by **10** was determined by FP readout. (c) Inhibition of the polymerization of lipid II by PGTs of different organisms. The following concentrations of enzymes were chosen so that 30–40% conversion of lipid II (4 μM) was observed in the uninhibited reaction: *S. aureus* SgtB, 50 nM; *S. aureus* PBP2, 1.2 μM;[3d] *E. faecalis* PBP2a, 50 nM; and *E. coli* PBP1b, 50 nM. (d) Minimum inhibitory concentrations of **10** against *S. aureus* ATCC 29213 (MSSA), *S. aureus* USA 300 (MRSA), and *B. anthracis* ANR-1 were determined following the CLSI procedure.[20]

probe compound to PGTs so that we could identify PGT inhibitors with low micromolar potency in a FP-based displacement assay (cf. **8**, IC_{50}(*E. coli* PBP1b) = 12 μM).[17] At the same time, probe **2** binds tightly enough to PGT enzymes so that the amount of protein required for the assay is limited.

With the assay in hand, we set out to screen for new structural classes of inhibitors using *S. aureus* SgtB. This enzyme is from a relevant pathogen, accessible in sufficient quantities by heterologous expression in *E. coli* (7 mg/L culture), and it can be obtained as a well-behaved, stable monomer. We adjusted the assay to a 1536 well plate format and screened 110,000 compounds of the ICCB library collection at Harvard Medical School (Z' = 0.78). Wells that showed 90% reduction of FP in duplicate (as compared to controls) were scored as hits. The initially obtained 186 hits (hit rate = 0.17%) were retested with the same displacement assay, this time measuring a dose–response curve rather than an end point. About 47% of primary hits showed dose-dependent displacement of the probe and were reconfirmed; 21% of the initial hits were found to be fluorescent and were not further evaluated.

The PGT active site is conserved throughout prokaryotes, and the assay is based on probe displacement from the active site. Thus, hit compounds obtained in this screen, which uses *S. aureus* SgtB, may not only show binding affinity to *S. aureus* PGTs but also to PGTs of other organisms, provided they contact conserved active site features. To examine their selectivity, hit compounds were tested for their PGT inhibitory activity in two orthogonal assays. In competitive binding studies, using PGTs from the pathogens *S. aureus*, *E. faecalis*, and *E. coli*, we found that hit compound **10** displaces probe **2** in a dose-dependent fashion from all three enzymes. The corresponding inhibitor constants K_i ranged from 2.6 μM to 94 nM (Figure 3).[18] Compound **10** showed similarly pan-selective inhibitory activity in an established assay that monitors PGT activity *in vitro*.[3b,10] Polymerization of lipid II[19] by two PGTs of *S. aureus*, as well as PGTs of *E. faecalis* and *E. coli* was inhibited with IC_{50} values in the low micromolar range (Figure 3c). These findings indicate that hit compound **10**, identified in our screen using *S. aureus* SgtB, is able to inhibit not only the enzymatic activity of *S. aureus* PGTs but also the activity of PGTs from other pathogens.

In this paper we have described the development of a displacement assay that uses a truncated moenomycin analogue with attenuated binding affinity to enable the identification of compounds that bind to PGTs. By using a natural product based probe that contacts invariant residues in the active site, we hoped to discover compounds that likewise contact conserved active site features. We report a compound that binds to several different PGTs and also inhibits their enzymatic activities. The enzyme binding affinities do not allow accurate prediction of the order of IC_{50} values for enzyme inhibition, but it is worth noting that the conformations of PGTs in the process of polymerizing lipid II may be different from those bound to moenomycin. This phenomenon highlights the need for both enzyme inhibition and binding assays to guide the development of PGT inhibitors. Finally, we note that compound **10** has MICs of 4–16 μg/mL against methicillin-sensitive and -resistant *S. aureus* strains as well as *B. anthracis*,[21] consistent with the PGT inhibitory activity (Figure 3d). Further studies are underway to determine whether the mechanism of cell killing is due to PGT inhibition.

■ ASSOCIATED CONTENT

Supporting Information

Rational probe design, PBP/PGT homology, and experimental details. 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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(17) In comparison, the probe compound described in ref 9 shows K_D = 54 nM for *E. coli* PBP1b.

(18) Nonspecific denaturation of the target protein by compounds that aggregate in solution can lead to false positives in biochemical assays. In order to assess whether **10** aggregates in solution we performed the assay in the presence of detergents. Probe displacement was not affected by the detergents, consistent with an interpretation that **10** does not act nonspecifically to inhibit PGTs. For details, see Supporting Information.

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(21) Compound **10** was not active against the Gram-negative *E. coli*, presumably due to inability to penetrate the outer membrane.