Published in final edited form as:

J Am Chem Soc. 2013 March 13; 135(10): 3776–3779. doi:10.1021/ja4000933.

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

Christian M. Gampe[†], Hirokatsu Tsukamoto[†], Emma H. Doud[‡], Suzanne Walker^{‡,*}, and Daniel Kahne^{†,¶,*}

[†]Department of Chemistry and Chemical Biology, Harvard University, Cambridge, MA 02138, USA

[‡]Department of Microbiology and Immunobiology, Harvard Medical School, Boston, MA 02115, USA

[¶]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, USA

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 analog 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 (TP) active site.² Additionally, some bacteria possess monofunctional peptidoglycan glycosyltransferases (PGTs) that form polysaccharide strands, which are then crosslinked 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 have focused on targets involved in

*Corresponding Author: suzanne_walker@hms.harvard.edu, kahne@chemistry.harvard.edu.

Supporting Information. Details about rational probe design and PBP/PGT homology. Experimental details for the synthesis of **2** and model studies, protein expression, assay development, assay validation, screening, *in vitro* PGT inhibition, and MIC test. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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

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 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} Peracetyl 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 glucoconfiguration) 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 peptidoglycan formation *in vitro* with IC₅₀ values of 12 μ M and 70 nM against *E. coli* PBP1b¹⁴ 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 (GalNAz), to the C4 hydroxyl of *N*-acetylglucose derivatives.¹⁶ Installation of galactosamine at a model disaccharide proceeded smoothly;⁴ however, we found that incubation of **8** and UDP-GalNAz 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 CuSO₄/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 **2** inhibits *E. coli* PBP1b and *S. aureus* SgtB with IC₅₀ 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 0.18 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 2 bottom). 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 probe compound to PGTs so that we could identify PGT inhibitors with low micromolar potency in a FP-based displacement assay (*cf.* **8**; IC₅₀(*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 endpoint. Ca. 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 II¹⁹ by two PGTs of *S. aureus*, as well as PGTs of *E. faecalis* and *E. coli* was inhibited with IC₅₀ values in the low micromolar range (Figure 3c). These findings indicate that hit compound **10**, identified in our screen using *S. aureus* SgtB, is not only able to inhibit 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 analog 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 IC₅₀ 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institutes of Health (R01GM066174 to D.K., R01GM076710 to S.W. and D.K., 1P01AI083214 to S.W.), a fellowship for C.M.G within the Postdoc-program of the German Academic Exchange Service (DAAD), and a research fund of the New England Regional Center for Excellence in Biodefense and Emerging Infectious Disease Research (U54 AI057159). We are grateful to Drs. Qasba and Ramakrishnan (NIH) for providing us with GalT. Dr. B. Kraybill (Merck & Co., Inc.) is acknowledged for helpful discussions. We thank the staff of the ICCB/NERCE BEID at Harvard Medical School (Dr. S. Chiang, D. Flood, J. Nale, Dr. R. Ross, B. Seiler, Dr. A. Onderdonk, C. Anderson) for their help with setting up and carrying out the screening campaign. We acknowledge Pfizer for their donation of the overexpression plasmid for *S. aureus* SgtB.

References

1. (a) Van Heijenoort J. *Glycobiology*. 2001; 11:25R–36R. (b) Vollmer W, Blanot D, De Pedro MA. *FEMS Microbiol Rev*. 2008; 32:149. [PubMed: 18194336] (c) Vollmer W, Bertsche U. *Biochim Biophys Acta*. 2008; 1778:1714. [PubMed: 17658458] (d) Vollmer W, Seligman SJ. *Trends Microbiol*. 2010; 18:59. [PubMed: 20060721] (e) Lovering AL, Safadi SS, Strynadka NC. *Annu Rev Biochem*. 2012; 81:451. [PubMed: 22663080]
2. Sauvage E, Kerff F, Terrak M, Ayala JA, Charlier P. *FEMS Microbiol Rev*. 2008; 32:234. [PubMed: 18266856]
3. (a) Schwartz B, Markwalder JA, Seitz SP, Wang Y, Stein RL. *Biochemistry*. 2002; 41:12552–12561. [PubMed: 12369847] (b) Chen L, Walker D, Sun B, Hu Y, Walker S, Kahne D. *Proc Natl Acad Sci USA*. 2003; 100:5658–5663. [PubMed: 12714684] (c) Barrett DS, Chen L, Litterman NK, Walker S. *Biochemistry*. 2004; 43:12375–12381. [PubMed: 15379577] (d) Barrett D, Leimkuhler C, Chen L, Walker D, Kahne D, Walker S. *J Bacteriol*. 2005; 187:2215–2217. [PubMed: 15743972] (e) Born P, Breukink E, Vollmer W. *J Biol Chem*. 2006; 281:26985–26993. [PubMed: 16840781] (f) Barrett D, Wang TS, Yuan Y, Zhang Y, Kahne D, Walker S. *J Biol Chem*. 2007; 282:31964–31971. [PubMed: 17704540] (g) Yuan Y, Barrett D, Zhang Y, Kahne D, Sliz P, Walker S. *Proc Natl Acad Sci USA*. 2007; 104:5348–5353. [PubMed: 17360321] (h) Perlstein DL, Zhang Y, Wang TS, Kahne DE, Walker S. *J Am Chem Soc*. 2007; 129:12674–12675. [PubMed: 17914829] (i) Wang T, Manning S, Walker S, Kahne D. *J Am Chem Soc*. 2008; 130:14068–14069. [PubMed: 18834124] (j) Bertsche U, Breukink E, Kast T, Vollmer W. *J Biol Chem*. 2008; 283:38096–38101. (k) Bouhss A, Trunkfield AE, Bugg TDH, Mengin-Lecreulx D. *FEMS Microbiol Rev*. 2008; 32:208–233. [PubMed: 18081839] (l) Perlstein D, Wang TA, Doud E, Kahne D, Walker S. *J Am Chem Soc*. 2010; 132:48–49. [PubMed: 20017480]
4. See supporting information for details.

5. (a) Walsh, C. In *Antibiotics: Actions, Origins, Resistance*. ASM Press; Washington, DC: 2003. p. 1-9. (b) Silver LL. *Clin Microbiol Rev*. 2011;71–109. [PubMed: 21233508]
6. (a) Goldman RC, Gange D. *CurrMed Chem*. 2000; 7:801. (b) Ostash B, Walker S. *Curr Opin Chem Biol*. 2005; 9:459. [PubMed: 16118062] (c) Halliday J, McKeveney D, Muldoon C, Rajaratnam P, Meutermans W. *Biochem Pharmacol*. 2006; 71:957. [PubMed: 16298347] (d) Silver LL. *Ann N Y Acad Sci*. 2013; 1277:29–53. [PubMed: 23278681]
7. (a) Welzel P. *Chem Rev*. 2005; 105:4610. [PubMed: 16351056] (b) Ostash B, Walker S. *Nat Prod Rep*. 2010; 27:1594. [PubMed: 20730219]
8. (a) Hentschel S, Kusch D, Sinell HJ. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, 1 Abteilung, Originale, Reihe B: Hygiene, Betriebshygiene, präventive Medizin*. 1979; 168:546. (b) Butaye P, Bevriese LA, Haesebrouck F. *Antimicrob Agents Chemother*. 2001; 45:1374. [PubMed: 11302798]
9. Cheng T-J, Rachel, Sung M-T, Liao H-Y, Chang Y-F, Chen C-W, Huang C-Y, Chou L-Y, Wu Y-D, Chen Y-H, Cheng Y-SE, Wong C-H, Ma C, Cheng W-C. *Proc Natl Soc Sci USA*. 2008; 105:431.
10. (a) Anderson JS, Matsushashi M, Haskin MA, Strominger JL. *Proc Natl Acad Sci USA*. 1965; 53:881–889. [PubMed: 14324547] (b) Wang TSA, Lupoli TJ, Sumida Y, Tsukamoto H, Wu Y, Rebets Y, Kahne DE, Walker S. *J Am Chem Soc*. 2011; 133:8528. [PubMed: 21568328]
11. Lovering AL, de Castro LH, Lim D, Strynadka NCJ. *Science*. 2007; 315:1402. [PubMed: 17347437] Yuan YQ, Fuse S, Ostash B, Sliz P, Kahne D, Walker S. *ACS Chem Biol*. 2008; 3:429. [PubMed: 18642800] Heaslet H, Shaw B, Mistry A, Miller AA. *J Struct Biol*. 2009; 167:129. [PubMed: 19416756] Sung MT, Lai YT, Huang CY, Chou LY, Shih HW, Cheng WC, Wong CH, Ma C. *Proc Natl Acad Sci USA*. 2009; 106:8824. [PubMed: 19458048] for a review, see: Lovering AL, Gretes M, Strynadka NC. *Curr Opin Struct Biol*. 2008; 18:534. [PubMed: 18721881]
12. (a) Adachi M, Zhang Y, Leimkuhler C, Sun B, LaTour JV, Kahne DE. *J Am Chem Soc*. 2006; 128:14012. [PubMed: 17061868] (b) Fuse S, Tsukamoto H, Yuan Y, Wang TSA, Zhang Y, Bolla M, Walker S, Sliz P, Kahne D. *ACS Chem Biol*. 2010; 5:701. [PubMed: 20496948]
13. (a) Taylor JG, Li X, Oberthür M, Zhu W, Kahne DE. *J Am Chem Soc*. 2006; 128:15084. [PubMed: 17117848] (b) Zhang Y, Fechter EJ, Wang TSA, Barrett D, Walker S, Kahne DE. *J Am Chem Soc*. 2007; 129:3080. [PubMed: 17323951] (c) Gampe CM, Tsukamoto H, Wang TSA, Walker S, Kahne D. *Tetrahedron*. 2011; 67:9771. [PubMed: 22505780]
14. Paradis-Bleau C, Markovski M, Uehara T, Lupoli TJ, Walker S, Kahne DE, Bernhardt TG. *Cell*. 2010; 143:1110–1120. [PubMed: 21183074]
15. Heaslet H, Shaw B, Mistry A, Miller AA. *J Struct Biol*. 2009; 167:129–135. [PubMed: 19416756]
16. (a) Ramakrishnan B, Shah PS, Qasba PK. *J Biol Chem*. 2001; 276:37665. [PubMed: 11485999] (b) Ramakrishnan B, Qasba PK. *J Biol Chem*. 2002; 277:20833. [PubMed: 11916963]
17. In comparison, the probe compound described in ref. 9 shows a K_D with PBP1b (*E. coli*) of 54 nM.
18. Non-specific 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 non-specifically to inhibit PGTs. For details, see supporting information.
19. For synthetic and semi-synthetic methods to access lipid II, see: Men H, Park P, Ge M, Walker S. *J Am Chem Soc*. 1998; 120:2484–2485. Ha S, Chang E, Lo MC, Men H, Park P, Ge M, Walker S. *J Am Chem Soc*. 1999; 121:8415–8426. Ye X, Lo M, Brunner L, Walker D, Kahne D, Walker S. *J Am Chem Soc*. 2001; 123:3155–3156. [PubMed: 11457035] Schwartz B, Markwalder JA, Wang Y. *J Am Chem Soc*. 2001; 123:11638–11643. [PubMed: 11716719] VanNieuwenhze M, Mauldin S, Zia-Ebrahimi M, Winger B, Hornback W, Saha S, Aikins J, Blaszcak L. *J Am Chem Soc*. 2002; 124:3656–3660. [PubMed: 11929255] Chen L, Men H, Ha S, Ye XY, Brunner L, Hu Y, Walker S. *Biochemistry*. 2002; 41:6824–6833. [PubMed: 12022887] Liu CY, Guo CW, Chang YF, Wang JT, Shih HW, Hsu YF, Chen CW, Chen SK, Wang YC, Cheng TJR, Ma C, Wong CH, Fang JM, Cheng WC. *Org Lett*. 2010; 12:1608–1611. [PubMed: 20187630] Meng FC, Chen KT, Huang LY, Shih HW, Chang HH, Liang PH, Cheng TJR, Wong CH, Cheng WC. *Org Lett*. 2011; 13:5306–5309. [PubMed: 21913698] Shih HW, Chang YF, Li WJ, Meng FC, Huang CY, Ma C, Cheng TJR, Wong CH, Cheng WC. *Angew Chem Int Ed*. 2012; 51:10123–10126.

20. CLSI. Methods for Dilution Antimicrobial Susceptibility Test for Bacteria That Grow Aerobically; Approved Standard-Eighth Edition. Wayne, PA: Clinical and Laboratory Standards Institute; 2009. CLSI document M07-A8
21. Compound **10** was not active against the Gram-negative *E. coli* presumably due to inability to penetrate the outer membrane

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 grey. IC₅₀ values are given for *in vitro* PGT inhibition.^{3b,10}

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 μ M) was observed in the uninhibited reaction: *S. aureus* SgtB (50 nM), *S. aureus* PBP2 (1.2 μ M),^{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 a procedure by the Clinical and Laboratory Standard Institute.²⁰

Scheme 1. Synthesis of **2**.^a

^a Reagents and conditions: a) *p*-MeOC₆H₄OH, BF₃·OEt₂, DCM, rt, 24 h; b) cat. NaOMe, MeOH, rt, 2 h; c) PhCH(OMe)₂, cat. PTSA, CH₃CN; d) ClCO₂Ph, pyridine, -40 °C, 2 h, 75% (over 3 steps); e) **4**, Tf₂O, DTBMP, ADMB, mol. sieves 4 Å, DCM, -78 °C, 1.5 h, 56%; f) Et₃SiH, TfOH, mol. sieves 4 Å, DCM, -78 °C; g) Tf₂O, pyridine, DCM, -40 °C to rt, 2 h; h) CsOAc, 18-crown-6, PhMe, rt, 14 h, 55% (over 3 steps); i) H₂, 10% Pd/C (1 wt %), Cl₃CCO₂H/MeOH, rt, 45 min; j) TEMPO, PhI(OAc)₂, DCM/H₂O, (2/1), rt, 2 h; k) ClCO₂iBu, *N*-methyl morpholine, THF, -40 °C, 5 min; then NH₃, ⁴PrOH, rt, 24 h, 46% (over 3 steps); l) CAN, MeCN/H₂O (4/1), rt, 1.5 h; m) **7**, tetrazole, mol. sieves 3 Å, MeCN, 0 °C, 2 h; then ⁴BuO₂H, 0 °C, 1 h; then P(OMe)₃, rt, 0 °C, 47 % (over 2 steps); n) Zn, Ac₂O, AcOH, THF, rt, 10 h; o) LiOH, THF/H₂O₂ (8/1), 0 °C, 2 h, 63% (over 2 steps); p) **8** (0.1 mM); GalT (Y289L) (5 mol%); UDP-GalNAz (1.3 mM), TRIS (16 mM, pH = 8.0), MnCl₂ (16 mM), CIP (0.2 U/μL), 37 °C, 60 h, > 90%; q) fluorescein 6-propargyl carboxamide, CuSO₄, Na-ascorbate, DMF, rt, 48 h, 87 % (over 2 steps). PTS: *para*-toluene sulfonic acid; ADMB: allyldimethoxy benzene; CIP: calf intestinal phosphatase; Troc: 2,2,2-trichloroethoxycarbonyl; CEO: cyanoethyl; IC₅₀ values are given for *in vitro* inhibition.^{3c,10}