

Using substrate engineering to harness enzymatic promiscuity and expand biological catalysis

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Despite their unparalleled catalytic prowess and environmental compatibility, enzymes have yet to see widespread application in synthetic chemistry. This lack of application and the resulting underuse of their enormous potential stems not only from a wariness about aqueous biological catalysis on the part of the typical synthetic chemist but also from limitations on enzyme applicability that arise from the high degree of substrate specificity possessed by most enzymes. This latter perceived limitation is being successfully challenged through rational protein engineering^{1,2} and directed evolution efforts^{3–6} to alter substrate specificity. However, such programs require considerable effort to establish. Here we report an alternative strategy for expanding the substrate specificity, and therefore the synthetic utility, of a given enzyme through a process of 'substrate engineering'. The attachment of a readily removable functional group to an alternative glycosyltransferase substrate induces a productive binding mode, facilitating rational control of substrate specificity and regioselectivity using wild-type enzymes.

An excellent test case for this approach is that of oligosaccharide synthesis, as the chemical synthesis of such molecules remains arduous. In stark contrast to the situation for peptides and oligonucleotides, a lack of synthetic capacity has delayed our understanding of the important roles that glycan structures have in various biological phenomena. Glycosyltransferases are the anabolic enzymes responsible for glycan synthesis in nature. Glycosyl moieties from activated donor sugar substrates (typically nucleotide sugars) are transferred to acceptor substrates, including other oligosaccharides, antibiotics, proteins and lipids, with either retention or inversion of configuration at the anomeric reaction center^{7–9}. This class of enzyme is generally believed to be highly selective for both the donor and acceptor substrates, although some exceptions have recently been reported^{10–17}.

The retaining α -1-4-galactosyltransferase from *Neisseria meningitidis*, LgtC, transfers galactose from a uridine 5'-diphosphogalactose (UDP Gal, **1**) donor substrate to the axial 4'-OH of terminal lactose-containing acceptor substrates (Fig. 1)^{18,19}. Previous studies had shown that D-galactose (**3**) also serves as an acceptor, though with greatly reduced efficiency²⁰. Notably, as shown in Table 1 and

confirmed by ESI-MS (data not shown), we found that a range of D-aldose sugars and even *myo*-inositol (**10**) could act as acceptor substrates for LgtC at rates comparable to that for D-galactose. This wide acceptor range surprised us, especially given that some of these sugars possess only equatorial hydroxyl groups yet LgtC transfers to an axial hydroxyl in its natural substrate. To determine whether LgtC was indeed capable of transferring to an equatorial hydroxyl, we investigated the product formed when *p*-nitrophenyl β -D-glucopyranoside (pNP Glc, **18**) acts as acceptor. Analysis of the single disaccharide product (**19**) by ESI-MS and two-dimensional ¹H NMR spectroscopy (Supplementary Fig. 1 online) clearly revealed the formation of a Gal- α -(1-4)-Glc linkage (Fig. 2), indicating that pNP Glc adopts the same basic binding mode as lactose, but transfer occurs to the equatorial 4-hydroxyl.

This finding of relatively broad specificity led to an initial hope that we could use wild-type LgtC as a catalyst in a range of useful galactosylation reactions. We therefore acetylated, purified and subjected to ESI-MS and NMR structural analysis the disaccharide products derived from two of the alternative acceptor monosaccharides studied, D-mannose (**8**) and D-xylose (**9**). In both cases, although MS analysis revealed masses consistent with that of a disaccharide product, ¹H NMR analysis showed that the purified products were mixtures of regioisomers. This finding was disappointing because a catalyst that yields a mixture of regioisomers is clearly of limited utility. However, the fact that we had obtained a single regioisomer when pNP Glc was used as acceptor suggested a strategy whereby appending a conveniently installed and removed substituent onto the acceptor might control its active site binding orientation in a useful

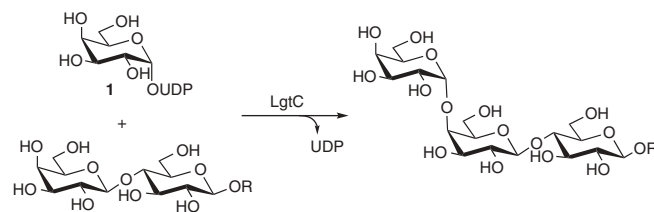


Figure 1 Galactosylation reaction catalyzed by LgtC.

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Table 1 Kinetic parameters for various LgtC acceptor substrates

Acceptor	$k_{\text{cat}} / K_{\text{m}}$ ($\text{min}^{-1} \text{mM}^{-1}$)
Lactose (2)	240
D-Galactose (3) ^a	0.3
D-Lyxose (4) ^a	0.3
D-Arabinose (5) ^a	0.1
D-Glucose (6) ^a	0.2
D-Allose (7) ^a	0.05
D-Mannose (8) ^a	0.3
D-Xylose (9) ^a	1.8
Myo-inositol (10) ^a	0.2
Benzyl β -D-cellobioside (11)	1.7
Benzyl β -D-xyloside (12)	40
Octyl β -D-glucoside (13) ^a	1.4
Octyl β -D-galactoside (14) ^a	3.8
Octyl β -D-xyloside (15) ^a	22
6-OBz-D-Mannose (16)	5.0
4-OBz-D-Xylose (17) ^a	12

^aNo saturation of enzyme activity was observed up to near saturated concentrations. Reliable k_{cat} and K_{m} values were therefore only attainable for lactose ($k_{\text{cat}} = 160 \text{ s}^{-1}$, $K_{\text{m}} = 40 \text{ mM}$), benzyl- β -D-cellobioside ($k_{\text{cat}} = 1.0 \text{ s}^{-1}$, $K_{\text{m}} = 35 \text{ mM}$), benzyl- β -D-xyloside ($k_{\text{cat}} = 10 \text{ s}^{-1}$, $K_{\text{m}} = 15 \text{ mM}$) and 6-OBz Mannose ($k_{\text{cat}} = 2.5 \text{ s}^{-1}$, $K_{\text{m}} = 30 \text{ mM}$).

manner. In fact, the three-dimensional structures of the sugar-binding sites from many carbohydrate-using proteins contain aromatic residues that provide a “hydrophobic platform” onto which a sugar ring face can bind²¹. Inspection of the three-dimensional structure of LgtC reveals the aromatic side chain of Phe132 positioned to function in such a role, providing a hydrophobic platform to which the glucose ring of the lactose acceptor or aromatic moieties may bind (Fig. 3a)¹⁹.

We investigated the generality of this modification approach amongst glycosyltransferases using an inverting α -2-3/8-sialyltransferase from *Campylobacter jejuni* (Cst II)²² and a retaining bovine α -1-3-galactosyltransferase (α 3GalT)²³. Cst II catalyzes the transfer of sialic acid (NeuAc) from cytidine 5'-monophospho-*N*-acetylneuraminic acid (CMP NeuAc, 20) to terminal lactose- or 3'-sialyllactose-containing glycoconjugates, yielding α -(2-3)- and α -(2-8)-linked products,

respectively. Owing to the high rate of enzyme-catalyzed hydrolysis of the donor substrate²², we did not observe any product formation when underivatized D-galactose was used as acceptor. However, when the D-galactose was derivatized with an aromatic substituent (*p*-nitrophenyl β -D-galactopyranoside, pNP Gal, 21), quantitative product formation occurred in a completely regioselective manner (Supplementary Fig. 2 online). The natural role of α 3GalT is to transfer galactose from UDP Gal to terminal *N*-acetylglucosamine-containing glycoconjugates, thereby producing Gal- α -(1-3)-Gal- β -(1-4)-GlcNAc epitopes. We therefore investigated the ability of α 3GalT to use D-galactose as an acceptor, and as we had observed with LgtC, transfer to the underivatized monosaccharide led to the formation of a mixture of regioisomers of disaccharide products, as determined by ¹H NMR analysis (~60:40 ratio), whereas in the presence of an aromatic anomeric substituent (pNP Gal as acceptor), we observed exclusive formation of a pure α -(1-3)-linked product (22) (Supplementary Fig. 3 online). As is the case with LgtC, α 3GalT possesses an aromatic side chain (Trp249) that serves as a platform that is critical for binding the GlcNAc residue of LacNAc acceptors²⁴.

We further explored this process of substrate engineering in more detail using LgtC. The most convenient appendage at the anomeric center is a benzyl group, which can be installed by classical glycoside formation chemistry and can be removed by hydrogenation. Accordingly, we synthesized and tested benzyl β -D-cellobioside (11) and benzyl β -D-xyloside (12) as acceptors. TLC and ESI-MS analysis clearly indicated that these compounds both functioned well in this role, resulting in the formation of a single product as had been seen with pNP Glc. Measurement of reaction rates revealed that not only did the inclusion of an aromatic substituent control regiochemistry, but it also resulted in substantially improved reaction rates: the $k_{\text{cat}}/K_{\text{m}}$ value of benzyl β -D-xyloside was over 20-fold higher than that of its parent sugar and within a factor of 4 of the value for the natural acceptor, lactose (2; Table 1). Our analysis of the product (23) formed from benzyl β -D-xyloside by two-dimensional ¹H NMR spectroscopy revealed the exclusive formation of an α -1-3-linkage (Supplementary Fig. 4 online), indicating that xylose adopts an alternative binding mode such as that shown in Figure 2. The formation of 1-3-linkages to xylosides has been observed in transglycosylation reactions with several glycosidases and glycosynthases that otherwise show preferences for 1-4-linkages, providing precedent for the existence of this alternative binding mode^{25,26}.

We next went on to test whether other types of chemical modifications could be used for substrate engineering by replacing the aromatic group with simple alkyl substituents. We tested octyl β -D-glucoside (13), octyl β -D-galactoside (14) and octyl β -D-xyloside (15) as alternative acceptors for LgtC. As we had found with aromatic substituents, appending an alkyl group facilitates the exclusive formation of a single pure regioisomer and results in an increase in rates of transfer (Table 1). Notably, ¹H-COSY NMR spectroscopy revealed that, in contrast to the Gal- α -(1-4)-Glc linkage observed when pNP Glc was used as acceptor, the single product (24) obtained from octyl β -D-glucoside possesses a Gal- α -(1-3)-Glc linkage (Fig. 2 and Supplementary Fig. 5 online). Thus, although chemical modification of the alternative substrate with either an aromatic or an alkyl substituent provides control of regioselectivity, the nature of the substituent facilitates alternative productive binding modes resulting in the production of different regioisomers. In the case of octyl β -D-galactoside, presumably interactions with the natural galactose moiety dominate, resulting in the exclusive formation of a Gal- α -(1-4)-Gal linkage (25, Fig. 2). As the aromatic derivative of xylose had already been found to adopt an alternative binding

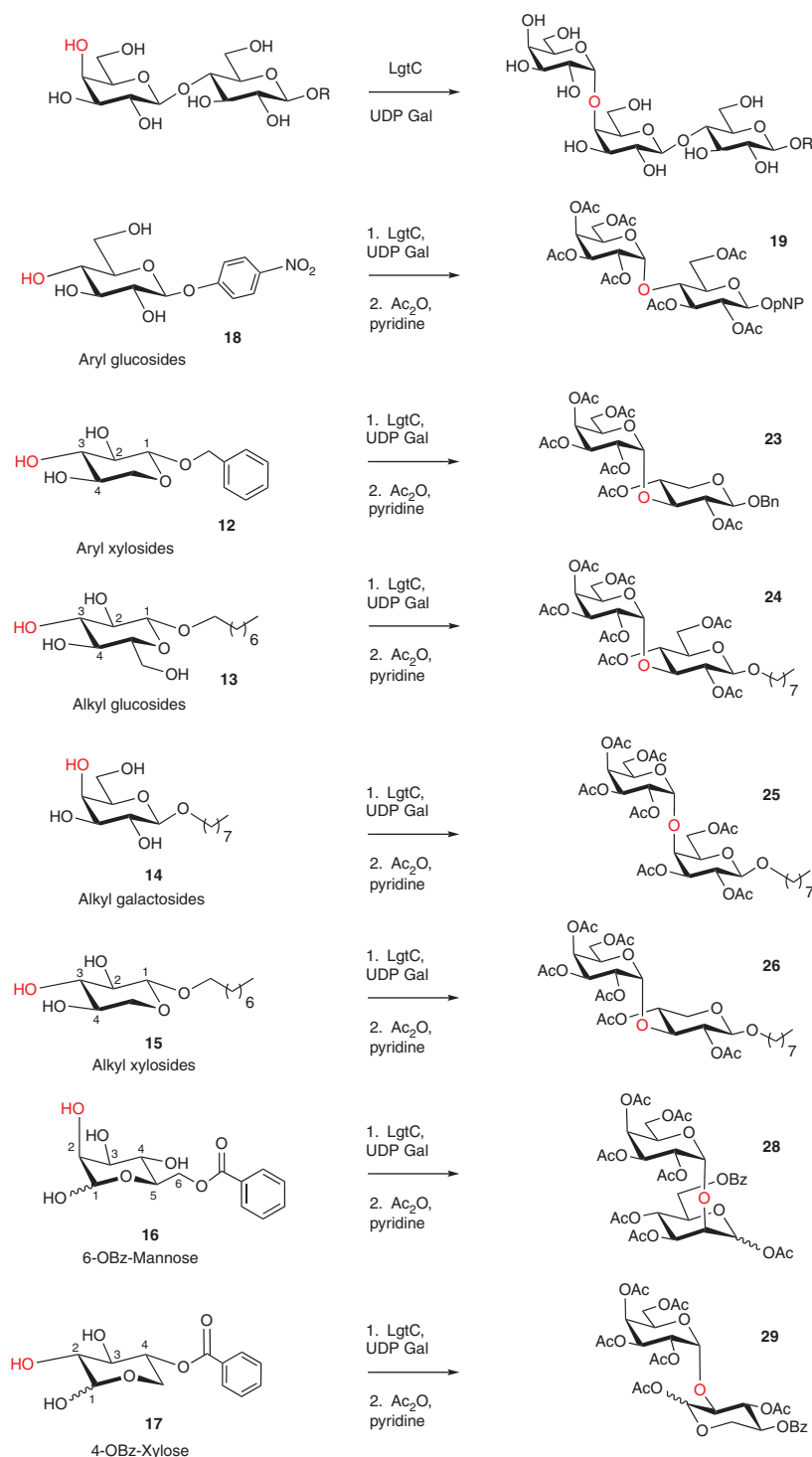


Figure 2 Substrate engineering strategy applied to various alternative LgtC acceptor substrates. Derivatization of alternative acceptors is used to generate substrates that mimic the lactose binding mode for LgtC-catalyzed galactosyl transfer. All products were characterized as their acetylated derivatives. Abbreviations: pNP, *para*-nitrophenyl; Bz, benzoyl; Bn, benzyl; Ac, acetyl.

mode, it was not unexpected that octyl β -D-xyloside also yielded a Gal- α -(1-3)-Xyl-linked product (**26**, Fig. 2). The ability of a glycosyltransferase to use these alkylated lipid-like derivatives could prove useful in combinatorial oligosaccharide synthesis²⁷ and in the production of unusual glycolipids.

This flexibility in sugar-binding mode opened up the intriguing possibility that the regiochemical outcome could be further controlled by appending an aryl group at other positions on the sugar ring, as has been reported for glycosynthases²⁸. For aldohexoses, the most convenient substituent is a benzoate ester, which can be installed selectively at the 6-position at low temperatures, with limiting quantities of acylating agent, and removed under a variety of mild conditions. A binding mode for a 6-O-benzoyl sugar could be envisioned in which the sugar 2-hydroxyl adopts the same position as the 4-hydroxyl of the galactose and the aromatic substituent takes the place of the glucose ring of lactose (Fig. 3b). Indeed, in the case of 6-O-benzoyl-D-mannose (6-OBz Man, **16**), the hydroxyl at this position also has the axial configuration. In fact the molecular mechanics (MM2) energy-minimized structure of 4'-deoxylactose (**27**) bound to LgtC, with the 2-OH of mannose being oriented in the position that would be occupied by the 4'-OH that undergoes glycosylation in the LgtC catalyzed reaction (Fig. 3c). We readily prepared 6-OBz Man in one step from D-mannose by low-temperature benzylation and tested it as an alternative acceptor substrate for LgtC; kinetic analysis revealed that the addition of the benzoyl group to mannose improved k_{cat}/K_m by approximately 20-fold (Table 1). To determine whether the appended aromatic functional group also provides control of regioselectivity, we acetylated the single product formed, purified it and subjected it to product analysis as described. ESI-MS revealed the expected mass for a disaccharide product, and ¹H NMR analysis confirmed the presence of a single, pure regioisomer. We used ¹H-COSY NMR analysis to assign all proton signals, identifying the newly formed glycosidic bond as the predicted Gal- α -(1-2)-Man linkage (**28**; Fig. 2 and Supplementary Fig. 6 online).

To further test the predictability of our model, we subjected the relatively symmetric xylose moiety to further derivatization. With a knowledge of the alternative binding mode of xylosides in hand (Fig. 2), we expected that aromatic derivatization at C4 should allow a binding mode that leads to the formation of an α -(1-2)-linkage. We readily prepared 4-O-benzoyl-D-xylose (4-OBz Xyl, **17**)²⁸ and tested this as an alternative acceptor substrate for

LgtC-catalyzed galactosylation. Appending the aromatic group again resulted in an increase in the observed rate constant (~ 7 -fold) (Table 1), and product analysis (ESI-MS and ¹H, ¹³C and ¹H-COSY NMR) revealed exclusive production of the anticipated α -1-2-linked disaccharide (**29**; Fig. 2 and Supplementary Fig. 7 online).

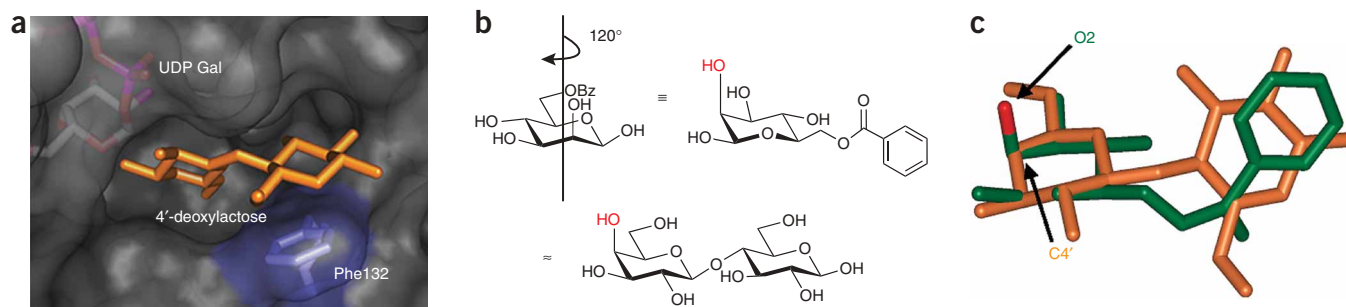


Figure 3 A model for the substrate-engineering strategy using 6-OBz Man. (a) Acceptor binding site of LgtC containing a hydrophobic platform (Phe132) for binding the glucose sugar ring of lactose. (b) Two representations of equivalent (\equiv) 6-OBz Man illustrating the way that 6-OBz Man can approximate (\approx) a lactose-binding mode. (c) Overlay of the MM2 minimized structure of 6-OBz Man (green) with the structure of 4'-deoxylactose (orange) bound within the active site of LgtC (PDB 1GA8).

Previous attempts to broaden the substrate specificity of enzymes by substrate modification required changes in both the substrate and the catalyst^{29,30}. In contrast, by the simple expedient of transiently attaching an alkyl or aryl substituent to the acceptor sugar, we were able to broaden the substrate specificity of wild-type LgtC to allow exclusive formation of α -1-2, α -1-3 or α -1-4 linkages at synthetically useful rates. Beside demonstrating an alternative strategy for expanding the synthetic utility of enzymatic catalysis by transient chemical modification (substrate engineering), our findings here illustrate four significant points. First, they highlight the need for caution in assigning the natural role of glycosyltransferases by using unnaturally modified acceptors. Second, they indicate that the acceptor substrate specificity of glycosyltransferases is not quite as rigid as previously believed, an observation that has significant implications for their synthetic utility. Third, they show that this promiscuity can be harnessed by modification of the acceptor sugar with a readily removable aromatic substituent or a simple alkyl chain that facilitates a productive and predictable binding mode providing both control of regioselectivity and an increase in observed rates. Finally, the strategy described here provides facile access to glycosidic linkages for which no 'wild-type' enzymatic activities have yet been identified. Further investigations into the broad applicability of this approach are ongoing.

METHODS

General. We recorded ^1H and ^{13}C NMR spectra on Bruker Avance-400 or Avance-600 spectrometers. Chemical shifts are reported in δ units (p.p.m.) using the residual ^1H and ^{13}C signals of deuterated solvents as reference: δ_{H} (CDCl_3) 7.27, δ_{C} (CDCl_3) 77.23. Specific assignments of individual ^1H peaks were unambiguously determined using two-dimensional ^1H COSY NMR. We made the assignments by examining the couplings within the ring system and determined the locations of glycosidic linkages on the basis of chemical shift. Because the acetate group is more electron withdrawing than a glycoside substituent, the proton attached to the carbon involved in the glycosidic bond resonates further upfield (<4.50 p.p.m.) in a region clearly distinct from anomeric protons or protons attached to carbons bearing an acetate group. We used $J_{1,2}$ coupling constants to assign the configuration of the anomeric linkage (α or β). We expressed and purified LgtC¹⁸ and Cst II²² as described previously. We cloned $\alpha 3\text{GalT}$ by RT-PCR from the cell line FB3.SP/THY Bovine Spleen/Thymus pool, normal (ATCC CRL 6039), into the pCW plasmid using standard molecular biology techniques and expressed and purified it as a MalE fusion protein.

LgtC-catalyzed galactosylation of modified alternative acceptor substrates. We incubated the acceptor substrate (5 mM), UDP galactose (7.5 mM) and LgtC (~ 0.5 mg ml^{-1}) for ~ 16 h at room temperature in 20 mM HEPES buffer

(pH 7.5) containing 50 mM NaCl, 5 mM MnCl_2 and 5 mM dithiothreitol. Upon completion, we partially purified the reactions with SepPak C₁₈Plus Cartridges (0.39 g, Waters) by washing with HPLC-grade water (10 ml) and eluting with HPLC-grade MeOH (10 ml). We then subjected the lyophilized products to standard per-O-acetylation with pyridine/ Ac_2O followed by a final purification by column chromatography (10:1 to 3:1 toluene/ EtOAc). We combined all fractions containing disaccharide products and analyzed them by ^1H NMR. In contrast to the cases where underivatized monosaccharides were used as acceptors and mixtures of regioisomers were observed, when we used derivatized acceptors, the reactions were completely regioselective to beyond the limit of ^1H NMR detection. Of potential utility, in the cases where pNP Glc, octyl Glc and octyl Gal were used as acceptors, we detected a trisaccharide product (confirmed by ESI MS) accounting for the lack of near-quantitative yields. Detailed chemical characterization data for LgtC-derived products (19, 23–26, 28, 29) are available as **Supplementary Methods** online.

Cst II-catalyzed sialylation of alternative acceptor substrates. We incubated the acceptor substrate (10 mM), CMP NeuAc (15 mM) and Cst II (~ 0.5 mg ml^{-1}) at room temperature in 100 mM HEPES buffer (pH 7.5) containing 10 mM MgCl_2 . Reactions were monitored by TLC (7:2:1:0.1 (v/v/v/v) $\text{EtOAc}/\text{MeOH}/\text{H}_2\text{O}/\text{AcOH}$). When galactose was used as acceptor, we detected only hydrolysis of the donor substrate and no product formation, even after extensive incubation. When pNP Gal was used as acceptor, we observed near-quantitative conversion to product after a 6-h incubation. We partially purified this product using a SepPak C₁₈Plus Cartridge by fractionated elution with water and characterized it by high-resolution ESI-MS ($\text{C}_{23}\text{H}_{32}\text{N}_2\text{O}_{16}\text{Na}^+$ expected, $m/z = 615.1650$; found, $m/z = 615.1648$ [$\text{M} + \text{Na}$] $^+$). We confirmed regioselectivity by treating 5 nmol of the reaction product with a selective α -2-3-sialidase (New England Biolabs). Under a unit defined time condition, we observed complete reversion of the product to pNP Gal.

$\alpha 3\text{GalT}$ -catalyzed galactosylation of alternative acceptor substrates. We incubated the acceptor substrate (10 mM), UDP galactose (15 mM) and $\alpha 3\text{GalT}$ (~ 0.5 mg ml^{-1}) for ~ 16 h at room temperature in 50 mM HEPES buffer (pH 7.5) containing 10 mM MnCl_2 , 0.1% BSA and 15 U of bovine alkaline phosphatase. Upon completion, we purified the reactions as described for LgtC. The chemical characterization of 22 is detailed in **Supplementary Methods**.

Kinetic analysis. We used a continuous coupled assay similar to that described previously¹⁸, in which the release of UDP is coupled to the oxidation of NADH ($\lambda = 340$ nm, $\epsilon = 6.22$ $\text{mM}^{-1}\text{cm}^{-1}$), to monitor the activity of LgtC. We obtained absorbance measurements using a Cary 4000 UV-VIS spectrophotometer equipped with a Peltier temperature controller. We used GraFit version 4.0 to calculate kinetic parameters by direct fit of initial rates to the respective equations. The error range of the data was between 5% and 15%.

Note: Supplementary information is available on the Nature Chemical Biology website.

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AUTHOR CONTRIBUTIONS

L.L. and A.W. conceived of this project, which was refined with input from S.W. and W.W. L.L. performed essentially all of the experimental work and, in conjunction with S.W., wrote the manuscript with input from A.W. and W.W.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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