Carbohydrate scaffolds as glycosyltransferase inhibitors with in vivo antibacterial activity

Johannes Zuegg1,2, Craig Muldoon2, George Adamson2, Declan McKeveney2, Giang Le Thanh2, Rajaratnam Premraj2, Bernd Becker2, Mu Cheng1, Alysha G. Elliott1, Johnny X. Huang1, Mark S. Butler1, Megha Bajaj1, Joachim Seifert2, Latika Singh2, Nicola F. Galley3, David I. Roper3, Adrian J. Lloyd3, Christopher G. Dowson3, Ting-Jen Cheng4, Wei-Chieh Cheng4, Dieter Demon5, Evelyne Meyer5, Wim Meutermans2 & Matthew A. Cooper1

The rapid rise of multi-drug-resistant bacteria is a global healthcare crisis, and new antibiotics are urgently required, especially those with modes of action that have low-resistance potential. One promising lead is the liposaccharide antibiotic moenomycin that inhibits bacterial glycosyltransferases, which are essential for peptidoglycan polymerization, while displaying a low rate of resistance. Unfortunately, the lipophilicity of moenomycin leads to unfavourable pharmacokinetic properties that render it unsuitable for systemic administration. In this study, we show that using moenomycin and other glycosyltransferase inhibitors as templates, we were able to synthesize compound libraries based on novel pyranose scaffold chemistry, with moenomycin-like activity, but with improved drug-like properties. The novel compounds exhibit in vitro inhibition comparable to moenomycin, with low toxicity and good efficacy in several in vivo models of infection. This approach based on non-planar carbohydrate scaffolds provides a new opportunity to develop new antibiotics with low propensity for resistance induction.
Peptidoglycan glycosyltransferases (GT) and transpeptidases (TP) are two key enzymes in the final steps of peptidoglycan (PG) biosynthesis essential for bacterial cell wall integrity and stability. GTs catalyse the polymerization of lipid II disaccharide units, forming a long chain of alternating β-1,4-linked N-acetylmuramic acids and N-acetylmuramic acid, leading to a linear glycan chain and the release of undecaprenyl-pyrophosphate carrier. These carbohydrate chains are further crosslinked by TP enzymes, forming linkages between the peptide chain and the D-alanine of a neighboring unit (Fig. 1). GT and TP enzymes are unique to bacteria and are expressed either as individual domains, monofunctional GT (MGT) and penicillin-binding proteins (PBP), respectively or as bifunctional proteins that possess both GT and TP domains (class A PBP).

Inhibition of extracellular bacterial cell wall synthesis has been a very successful strategy in the development of many important antibacterial agents, with teixobactin, one of the most recently reported. The β-lactam class, which includes cephalosporins, monobactams and carbapenems, inhibit PG crosslinking by covalently binding to the TP enzyme, while glycopeptides such as vancomycin bind directly to the lipid II unit and sterically inhibit further polymerization and crosslinking of PG. Bacteria developed resistance to cell wall inhibitors via β-lactamases, thickened cell walls and modification of the lipid II site of PBP2 with reduced affinity for moenomycin as well as some peculiarities of their cell wall organization. Further, moenomycin is primarily accumulated inside of the cells, while its target is located on the cell surface. In vitro-induced resistance with S. aureus showed mutations in the binding site of PBP2 with reduced affinity for moenomycin as well as its ligand, resulting in strains with shorter PG polymers and major cell division defects. The lack of a specific resistance mechanism and the paucity of antibiotics that specifically mimic the carbohydrate portion of bacterial lipid II suggest that direct GT inhibition remains an attractive strategy for the development of novel antibacterial agents with low potential for resistance development.

Moenomycin A is a highly functionalized pentasaccharide attached via a phosphoglycerate linkage to a polyprenyl chain (Fig. 1) that binds competitively to GT enzymes by mimicking the disaccharide–pyrophosphate–prenol linkage of the donor lipid.

**Figure 1 | Overview of PG cell wall synthesis and inhibitors.** (a) PG synthesis in bacteria from lipid II with subsequent GT and TP catalysis, with A: L-Ala, a: D-Ala, e: D-iGln, X: either D-Lys(Ala5) in case of Staphylococcus, or mDap in case of Bacillus. (b) Structure and in vitro activity of moenomycin A, indicating the different moieties with A to G. (c) Structure and in vitro activity of moenomycin’s disaccharide degradation product.
Although the E and F rings and the phosphoglycerate (G) portion of moenomycin A are important for GT inhibitory activity, analogues of this pharmacophore subunit did not maintain whole cell antibacterial activity. Attempts to mimic the EFG structural fragment with disaccharide derivatives resulted in compounds (such as TS30153) with cell-based activity, that is, minimum inhibitory concentration (MIC) of 3.12 and 12.5 µg ml⁻¹ against staphylococci and enterococci, with no in vivo activity. Compound TS30153 has three hydrophobic binding elements that mimic the acyl and alkoxy moieties of moenomycin A. Attempts to mimic directly lipid II or, the ring F of moenomycin, with monosaccharide scaffolds gave compounds with only low to medium activity (MIC = 60 µM against Bacillus cereus). More recently, de novo inhibitors for GT discovered using high-throughput screening or in silico methods, were shown to have improved in vitro activity (MIC = 0.25 µM against MRSA), but no in vivo activity.

In this study, we explore novel chemistry based on a monosaccharide scaffold to mimic the essential structure features of moenomycin and to improve the drug-like properties, in particular reduced molecular weight and hydrophobicity. Compared with other scaffolds, the monosaccharide scaffold approach provides structural diversity using up to five chiral attachment points within a small volume. This allows for more efficient pharmacophore optimization, while still enabling the generation of a broad structural diversity to scope and improve activity and physicochemical properties. Here we use the approach to produce moenomycin-focused libraries and select compounds with in vitro antibacterial activity and in silico potential to inhibit the GT enzyme. We demonstrate the strength of this strategy with two of the most promising candidates showing inhibition of GT and PG synthesis in in vitro assays, as well as in vivo efficacy in eliminating S. aureus infection from a mouse mammary gland.

Results
Design and synthesis. We synthesized a small library of compounds by replacing the phosphoglycerate/phosphate moieties (G, Fig. 1) with simpler lipophilic substituents (that is, phenyl, biphenyl or naphthyl groups linked via a urea) and changing the orientation and nature of ring F. This approach yielded compounds like ACL19378 (Fig. 2a, Supplementary Figs 1–8) and ACL19333 (Supplementary Fig. 1), with MICs against Gram-positive bacteria in the range of 2 µg ml⁻¹, but with limited activity in the presence of 50% serum. In the second stage, we used the disaccharide structure–activity relationship information to design more synthetically feasible and smaller monosaccharide molecules. A versatile solid-phase method was developed to rapidly synthesize the representatives of three different core chemotypes M1 to M3, starting from a single monosaccharide building block, that is, 1,5-anhydro-galactitol (see Fig. 3). Chemotype M1 explored the option of using only two of the hydrophobic elements, whereas the other two, M2 and M3, used a benzimidazole moiety as the third hydrophobic group (Fig. 3).

Five hundred compounds were thus synthesized combinatorially on solid-phase resin, purified using high-performance liquid chromatography, and tested for their MIC activity against two Gram-positive staphylococcal strains (methicillin-sensitive (MSSA) and methicillin-resistant S. aureus (MRSA)), three enterococcal strains and Escherichia coli as a Gram-negative control. Although most compounds were inactive against E. coli, many compounds displayed activity against the Gram-positive strains. The derivatives with Gram-positive antibacterial activity generally contained a lipophilic substituent such as an alkyl moiety (minimum length of 10 carbon atoms) or a biaryl, and one or two electron-deficient aryl groups. All active compounds were then tested for haemolytic activity and, after filtering out the haemolytic compounds, a series of compounds of chemotype M3 containing substituted amino-benzimidazoles were selected for further study (Supplementary Table 1). Two compounds (Fig. 4), ACL20215 (Supplementary Figs 10–15) and ACL20964 (Supplementary Figs 16–20), showed broad activity against a range of drug resistant S. aureus strains, including MRSA, GISA (glycopeptide-intermediate S. aureus), VRSA (vancomycin-resistant S. aureus) and DRSA (daptomycin-resistant S. aureus) and multi-drug-resistant S. pneumoniae, with low haemolytic activity against human red blood cells (Table 1). ACL20215 was assayed for resistance potential and showed a spontaneous mutation frequency against S. aureus (ATCC 13709; Smith strain) of less than $2.5 \times 10^{-10}$ at four times its MIC value.
Evaluation of mode of action. To elucidate the mode of action of the inhibitors, we conducted various GT enzyme inhibition assays. We first examined the inhibitory effect of the compounds in a bacterial membrane environment, comparing ACL20215 and ACL20964 in an in vitro assay for bacterial PG biosynthesis, using crude *Bacillus megaterium* membrane preparations. This assay monitors [14C]UDP-GlcNAc incorporation into lipid II and mature PG, in the presence of different concentrations of antibiotics. Owing to the sequential nature of the glycosyltransfer and transpeptidation, it is not possible to determine at which step PG biosynthesis is blocked in this assay. The low concentration of lipid II can be detected by thin-layer chromatography (TLC) separation and subsequent phosphorimaging of pre-solubilized membranes, which was incubated with the cytoplasmic PG precursors UDP-MurNAc-pentapeptide and UDP-[14C]GlcNAc. As shown in Fig. 5 (and Supplementary Table 2 and Fig. 21), both ACL20215 and ACL20964 caused an inhibition of mature PG production at a concentration of 200 μg ml⁻¹, to a similar extent as the controls, vancomycin and moenomycin A. Both inhibitors also caused an accumulation of lipid II, although to a lesser extent compared with vancomycin or moenomycin A.

**Figure 3 | Design of monosaccharide libraries.** The figure illustrates the common starting building block, the three different chemotypes (M1, M2 and M3) and corresponding diversification at each substitution point.

**Figure 4 | Structures of ACL20215 and ACL20965.** ACL20215 and ACL20965 are two of the most active monosaccharide compounds.

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Membrane-disruption experiments were performed using *S. aureus* (ATCC 25923) in combination with membrane potential-sensitive cyanine dye diSC₃5 (ref. 43). Neither compounds showed membrane disruption (Supplementary
Table 1 | In vitro activity data of ACL20215 and ACL20964.

| Organism   | Strain/type            | Vancomycin | Moenomycin A | ACL20215 | ACL20964 |
|------------|------------------------|------------|--------------|-----------|-----------|
| S. aureus  | MSSA, ATCC 25923       | 1          | 4            | 4         | 4         |
|            | MRSA, ATCC 43300       | 1          | 4            | 4         | 8         |
|            | Newbould 305           |            |              | 2         | 1         |
|            | NRS 17—GISA            | 8          | 16–32        | 8         | 32        |
|            | NRS 1—GISA             | 4          | 1            | 4         | 16        |
|            | VRS 1                  | >64        | 8            | 4         | 8         |
|            | mMRSA, DRSA, ci        | 4          | 16           | 4         | 8         |
| E. faecium | ATCC 35667             |            |              | 16        | 2         |
|            | VanA, ATCC 51559       | >64        | 32           | 8         | >64       |
| E. faecalis| ATCC 29212             |            |              |           |           |
|            | VanA, ci               | >64        | >64          | 8         | 64        |
| S. pneumoniae | ATCC 700677         | 2          | 8–16         | 4         | 8–16      |
| E. coli    | ATCC 25922             | >64        | >64          | >64       | >64       |

Mutation frequency

| S. aureus (at 4 × MIC) | ATCC 13709 | 2.5 × 10⁻¹⁰ |
|------------------------|------------|--------------|
| Human                  | RBC        | 74           | >100        |
| IC₅₀ (µg ml⁻¹) S. aureus | MGT        | 17.1         | 11.1        |

ci, clinical isolate; DRSA, daptomycin-resistant S. aureus; GISA, glycopeptide-intermediate S. aureus; HC₅₀, half maximal haemolytic concentration; IC₅₀, half maximal inhibitory concentration; MDR, multi-drug-resistant; MGT, monofunctional glycosyltransferase; MIC, minimum inhibitory concentration; mMRSA, multi-drug-resistant methicillin-resistant S. aureus; Moenomycin A (Sigma, 32404); MRSA, methicillin-resistant S. aureus; MSSA, methicillin-sensitive S. aureus; RBC, red blood cells; Vancomycin (Sigma, 861987); VRS, vancomycin-resistant S. aureus. All values are µg ml⁻¹.

Figure 5 | Inhibition of PG synthesis. The inhibitory effect in bacterial PG biosynthesis within a crude B. megaterium membrane is shown for (a) vancomycin, (b) moenomycin A, (c) ACL20215 and (d) ACL20964, showing the relative change of lipid II and PG isolated from the crude membrane by TLC, after 3 h, compared with non-antibiotic treatment. Error bars show s.d. for n = 3.
bars show s.d. for the transformation rate of fluorescent lipid II analogue and comparing it with the maximum inhibitory effect of moenomycin A at 50 μM or 79.2 μg ml⁻¹. Error bars show s.d. for n = 4.

Fig. 22) compared with a positive control Citropin 1.1 (refs 44–46), which suggested that the GT inhibitors disrupt PG biosynthesis without disrupting the cell membrane.

We then monitored the transformation of fluorescent NBD-lipid II by two different GT enzymes, PBP1 from *Clostridium difficile* and MGT from *S. aureus*. The single concentration test at 200 μg ml⁻¹ revealed that both inhibitors showed an inhibitory effect against MGT *S. aureus*, while showing only moderate effect against PBP1 *C. difficile* (Supplementary Fig. 23). Confirmative dose–response assays were subsequently conducted with continuous fluorescent activity assay using a dansyl-labelled, lysine-lipid II substrate⁴⁷. The assay were subsequently conducted with continuous fluorescent activity (Supplementary Fig. 23). Confirmative dose–response assays while showing only moderate effect against PBP1 inhibitors showed an inhibitory effect against MGT *S. aureus* species. One main feature of the structures is a binding site loop showing a high structural similarity between the difference species. One main feature of the structures is a binding site loop (MGT *S. aureus* Phe₁₂₀–Gly₁₃₀; PBP2 *S. aureus* Gly₁₃₄–Gly₁₄₅)⁵⁴ located between the donor-binding site, occupied by moenomycin, and the acceptor binding site occupied by the incoming lipid II molecule. This binding site loop is highly flexibly and partly disordered in most of the crystal structures (see Supplementary Table 3). Even when the loop is resolved, it can occupy different conformations, either separating the donor from the acceptor sites or opening a groove between the sites (see Supplementary Fig. 24 and Supplementary Note 1). We have carried out in *silico* virtual docking with the monosaccharides ACL20215 and ACL20964, choosing the receptor model (and loop conformation), which best reproduced the binding orientation of moenomycin. A receptor model based on the crystal structure of MGT from *S. aureus* with a loop conformation blocking the access to the acceptor site, 3HZS⁵⁴, was thereby selected (see Supplementary Fig. 25). As shown in Fig. 7, the benzimidazole group of both inhibitors was located similarly to portion G of moenomycin, with one of the other hydrophobic groups located in the donor-binding site (similar to ring E of moenomycin), and the other was located towards the acceptor site not occupied by moenomycin. While the virtual docking experiments were able to reproduce the binding orientation of moenomycin, a degree of uncertainty remained due to the flexibility of the binding site loop (Fig. 7), and its ability to adopt different conformations depending on the ligand.⁴⁹ However, the docking experiments clearly indicated the potential of the inhibitors to extend to the acceptor binding site not occupied by moenomycin.

**Virtual docking.** Several crystal structures of GT domains have been reported for Gram-positive (MGT⁴⁸,⁴⁹ and PBP2 (refs 50,51) from *S. aureus*) and Gram-negative bacteria (PBP1 (ref. 52) from *E. coli* and PGT¹¹,⁵³ from *Aquifex aeolicus*), showing a high structural similarity between the difference species. One main feature of the structures is a binding site loop (MGT *S. aureus* Phe₁₂₀–Gly₁₃₀; PBP2 *S. aureus* Gly₁₃₄–Gly₁₄₅)⁵⁴ located between the donor-binding site, occupied by moenomycin, and the acceptor binding site occupied by the incoming lipid II molecule. This binding site loop is highly flexibly and partly disordered in most of the crystal structures (see Supplementary Table 3). Even when the loop is resolved, it can occupy different conformations, either separating the donor from the acceptor sites or opening a groove between the sites (see Supplementary Fig. 24 and Supplementary Note 1). We have carried out in *silico* virtual docking with the monosaccharides ACL20215 and ACL20964, choosing the receptor model (and loop conformation), which best reproduced the binding orientation of moenomycin. A receptor model based on the crystal structure of MGT from *S. aureus* with a loop conformation blocking the access to the acceptor site, 3HZS⁵⁴, was thereby selected (see Supplementary Fig. 25). As shown in Fig. 7, the benzimidazole group of both inhibitors was located similarly to portion G of moenomycin, with one of the other hydrophobic groups located in the donor-binding site (similar to ring E of moenomycin), and the other was located towards the acceptor site not occupied by moenomycin. While the virtual docking experiments were able to reproduce the binding orientation of moenomycin, a degree of uncertainty remained due to the flexibility of the binding site loop (Fig. 7), and its ability to adopt different conformations depending on the ligand.⁴⁹ However, the docking experiments clearly indicated the potential of the inhibitors to extend to the acceptor binding site not occupied by moenomycin.

**In vivo studies.** ACL20215 testing with *in vitro* metabolic stability assays showed no degradation of the compound using both human and mouse liver microsomes. The *in vivo* pharmacokinetic properties of ACL20215 and ACL20964 were investigated using intravenous (i.v.) administration at 3.5 mg kg⁻¹ to male rats (Table 2 and Supplementary Table 4). Both compounds had a high apparent half-life (t₁/₂) of 27.2 and 33.8 h, respectively. They also showed a very high volume of distribution (V₁) and a high clearance (Cl₅₀₅). No urinary excretion was detected for either compound, and no metabolites were observed in plasma. The maximum tolerated dose for both compounds (see Supplementary methods) was determined following intraperitoneal (i.p.) administration of the compounds to mice, and showed good tolerance up to 60 mg kg⁻¹, with no mortality up to 100 mg kg⁻¹. Both compounds induced some minor changes to central/
ACL20215 and ACL20964 were subsequently tested in a mouse model of septicemia, using 10 male CD-1 (Crl)-derived mice, inoculated i.p. with a LD90–100 of S. aureus (Smith; 3.7 × 10⁵ c.f.u. per mouse). Both compounds administered i.p. 10 min after inoculation at 50 mg kg⁻¹ resulted in 100% survival rate after 7 days. However, same studies with i.v. administration of the compounds (4 mg kg⁻¹, 10 min after inoculation) showed no antibacterial effect, resulting only in a 10% survival rate, compared with 90% for ampicillin (0.1 mg kg⁻¹). The lack of efficacy following i.v. administration is most likely due to a lower dose (4 mg kg⁻¹, limited by solubility) combined with the high volume of distribution and serum-binding properties of the compounds, which effectively lowers the free drug concentration at the site of infection. When a higher dose (50 mg kg⁻¹) was suspended and administered i.p. at the site of infection, the high local concentration of the drug ensures effective clearance of the bacterial infection. Further optimization of compound properties, dose or formulation is required for parenteral administration.

In vivo efficacy studies were conducted with ACL20215 and ACL20964 using a mouse mammary gland infection (mastitis) model with intraductal inoculation of S. aureus (Newbould 305, ATCC 29740). Each compound was instilled at different doses into the teat canal of both contralateral glands from the fourth mammary gland pair of lactating mice at 4 h after bacterial inoculation. Mice were killed at 14 h post infection and both glands were analyzed for c.f.u. counts (Fig. 8, Table 2 and Supplementary Tables 5 and 6). The effective dose to reduce the bacterial load by 2 × log(c.f.u.) (ED₂log) was 730 and 510 µg per gland, for ACL20215 and ACL20964, respectively, indicating that both compounds cleared 99% of the staphylococci from the infected mammary gland at a moderate dose. Similarly, the protective doses to clear all bacteria in 50% (PD₅₀) and 100% (PD₁₀₀) of the glands, respectively, indicated that a high dose of ACL20964 very efficiently cleared S. aureus from the infected glands (Table 2). In contrast, no PD values could be determined for ACL20215 as the latter compound was unable to eradicate all bacteria in 50 or 100% of the glands in the analyzed doses to at least the detection limit of the assay.

### Table 2 | Pharmacokinetic properties and in vivo efficacy of ACL20215 and ACL20964.

|                          | ACL20215 | ACL20964 |
|--------------------------|----------|----------|
| **Pharmacokinetic properties** |          |          |
| Metabolic stability (in vitro) | No degradation | ND |
| Rat (i.v.) | Dose (mg kg⁻¹) | 3.5 | 3.5 |
| t₁/₂ (h) | 27.2 | 33.8 |
| Plasma Cl₅₀ (ml min⁻¹ kg⁻¹) | 42.1 | 17.9 |
| Blood Cl₅₀ (ml min⁻¹ kg⁻¹) | 48.9 | 21.5 |
| V₅ (l kg⁻¹) | 97.2 | 53.0 |
| Mice (i.p.) | MTD (mg kg⁻¹) | 100 | 100 |
| **Survival 7 days (%)** | 100 | 100 |
| **Survival 7 days (%)** | 10 | 10 |
| **ED₂log c.f.u. (µg per gland)** | 730 | 510 |
| **ED₁₀₀ c.f.u. (µg per gland)** | 1,400 | 770 |
| **PD₂₀ (µg per gland)** | >1,000 | 800-1,000 |
| **PD₁₀₀ (µg per gland)** | >1,000 | >1,000 |

Cl, clearance; ED₂log, effective dose to reduce bacterial load by 2 × log(c.f.u.); ED₁₀₀, effective dose to reduce bacterial load by 4 × log(c.f.u.); i.p., intraperitoneal injection; i.v., intravenous injection; MTD, maximal tolerated dose; ND, not determined; PD₂₀, 50% protective dose; PD₁₀₀, 100% protective dose; t₁/₂, half-life; V₂, volume of distribution.

Autonomic responses at the higher dose of 100 mg kg⁻¹. No adverse effects were observed in a single-dose study (4 mg kg⁻¹) following i.v. administration.

**Discussion**

Using moenomycin A and previously reported GT inhibitors, we designed and synthesized a small library of disaccharide-based compounds with a smaller, more drug-like, hydrophobic tail. These compounds (such as ACL19378) showed good in vitro antibacterial activity but had unfavourable physicochemical properties that limited their in vivo application. Nevertheless, this set of active compounds gave valuable structure–activity relationship information, which was used to design libraries of compounds based on a smaller monosaccharide scaffold. This strategy also reduced compound hydrophobicity and chemical complexity, enabling the synthesis of the first reported direct GT inhibitors with in vivo efficacy against bacteria.

A reductionist approach, moving from disaccharide mimics of the moenomycin EFG fragment to a smaller monosaccharide scaffold, maintains the key pyranose scaffold and the substitution pattern derived from the disaccharide actives. Chemical chirality inherent in the pyranose scaffold ensures a rigid three-dimensional positioning of substituents that is maintained in the series. Second, the solid-phase synthetic method allowed us to make substantial libraries of chemotypes designed to mimic the disaccharide series. In this way, we identified a series of compounds, corresponding to the amino-benzimidazole chemo-type, which showed clear antibacterial activity against a range of drug-resistant Gram-positive bacteria. While the cell-based activity suggests a preference for more hydrophobic substituents, some structural variations are not reflected in their activity, such as the difference in activity between a 2- and 1-naphthyl group and the lack of activity of the corresponding biphenyl compound.

The two most promising compounds from this monosaccharide library, that is, ACL20215 and ACL20964, showed good in vitro antibacterial activity against a range of Gram-positive bacteria, including those resistant to common antibiotics, that is, MRSA, GISA and VanA enterococci. PG biosynthesis assay data, taken together, suggests that both compounds trigger an accumulation of lipid II and a decrease of mature PG, as is the...
case for moenomycin A. Compounds can inhibit the function of GT with IC50 values similar to that determined for moenomycin.

The virtual docking experiment suggests that the compounds are able to bind in the catalytic site of the GT by occupying part of the donor lipid II-binding site (similar to moenomycin A) as well as part of the acceptor lipid II-binding site (not occupied by moenomycin A).

Both hit compounds can be tolerated in mice up to a dose of 100 mg kg⁻¹, while showing good metabolic stability in rats. Even though the library design aimed to reduce the lipophilicity, it is apparent that GT inhibitory compounds require a certain degree of hydrophobicity to be active in vitro and in vivo.

The monosaccharide scaffold is an excellent scaffold for drug design, as it is able to present various substituents or binding elements (in this case, three hydrophobic elements) in diverse spatial orientation using up to five chiral attachment points. The scaffold is also able to present those substituents in a conformational rigid form, indicated by the fact that both monosaccharide compounds, ACL20215 and ACL20964, existed as two atropisomers (see Supplementary Fig. 1 and Supplementary Note 1), conformational restricted isomers or rotamers, which would not occur if the carbohydrate scaffold itself was flexible. Virtual docking experiments show both atropisomers among the top ranked poses. It is reasonable to assume that one isomer will be the preferred binding partner for the GT active site, but our in silico and in vitro experiments were unable to distinguish them.

The membrane-associated nature of the GT enzyme and the hydrophobicity of its natural substrate lipid II necessitates a certain degree of lipophilicity for a compound with an inhibitory effect. While serum binding could not be eliminated in this pilot series, ACL20215 and ACL20964 showed effect. While serum binding could not be eliminated in this pilot case, the degree of lipophilicity for a compound with an inhibitory effect. While serum binding could not be eliminated in this pilot case, the degree of lipophilicity for a compound with an inhibitory effect. While serum binding could not be eliminated in this pilot case, the degree of lipophilicity for a compound with an inhibitory effect.

Figure 8 | in vivo efficacy in mastitis mouse model. S. aureus c.f.u. counts (c.f.u. g⁻¹ gland) at 14 h post treatment in infected mouse mammary glands treated with increasing doses of ACL20215 (a) and ACL20964 (b). Significance compared with control (paraffin oil) are given as P < 0.0001 (****) and P < 0.0001 (****), calculated by one-way analysis of variance followed by Holm–Sidak post hoc test. Dashed line represents the detection limit at 1.7 log10 c.f.u. Data values are given in Supplementary Table 5.

### Methods

#### Solid-phase synthesis

All monosaccharide compounds were synthesized on solid-phase using an orthogonally protected galactitol-building block attached to WANG resin. The synthesis of ACL20215 and ACL20964 is given in Supplementary Fig. 9 and below as a representative example.

**DTPM removal:** The resin was treated with a solution of 5% hydrazine hydrate in dimethylformamide (DMF; 10 ml g⁻¹ of resin), shaking (1 h, RT), drained and washed (3 × DMF, 3 × DCM, 3 × DMF). Urea formation: the resin was treated with a solution of 4-chloro-3-trifluoromethyl-phenyl isocyanate (0.15 M) in DMF (10 ml g⁻¹ of resin), shaken (O/N, RT), drained and washed (3 × DMF, 3 × DCM). The resin was taken up in a solution of sodium methoxide (0.15 M) in MeOH (5 ml g⁻¹ of resin) and tetrahydrofuran (20 mg l⁻¹ of resin), shaken (3 h, RT), drained and washed (3 × tetrahydrofuran, 3 × MeOH, 3 × DCM, 3 × DMF). Azide reduction: the resin was treated with a solution of lithium tert-butoxide (0.2 M) and DL-dithiothreitol (DTT, 0.2 M) in DMF (15 ml g⁻¹ of resin), shaken (O/N, RT), drained and washed (3 × DMF, 3 × MeOH, 3 × DCM, 3 × DMF).

To form the benzyldiazole, the resin was treated with Dipea (0.5 M) in DMF (10 ml g⁻¹ of resin), and heated at 50 °C. The resin was drained and washed (3 × DMF, 3 × DCM, 3 × DMF). Reduction the nitro group: the resin was treated with a solution of SnCl₂.2H₂O in DMF (2.0 M, 10 ml g⁻¹ of resin), shaken (O/N, RT), drained and washed (3 × DMF, 3 × MeOH/MeOH 1:1, 3 × DCM, 3 × DMF/MeOH 1:1, 3 × DCM).

Analytical data for ACL20215: The analytical data for ACL20125 are given as 1H-NMR, temperature dependent 1H-NMR, 13C-NMR, COSY, edCOSY and HMBC NMR spectra in Supplementary Figs. 10–15, respectively. The structure of saturated ammonia in methanol (1.0 ml) and left to stand at RT for 2 h, and treated with a solution of SnCl₂.2H₂O in DMF (2.0 M, 10 ml g⁻¹ of resin), shaken (O/N, RT), drained and washed (3 × DMF, 3 × MeOH, 3 × DCM). The structure and conformation of ACL20215 indicate restricted torsional rotation of the C4–NBenzimidazole bond, due to size of the benzimidazole group. Energy barrier calculation indicate an upper range of 25 kcal/mol for this rotational barrier (see Supplementary Fig. 3), which, in relation to the GT active site, but our in vitro experiments were unable to detect these compounds. Transition between the two isomers, or restricted torsional rotation of the C4–NBenzimidazole bond, due to size of the benzimidazole group. Energy barrier calculation indicate an upper range of 25 kcal/mol for this rotational barrier (see Supplementary Fig. 3), which, in relation to the GT active site, but our in vitro experiments were unable to detect these compounds.

#### Analytical data for ACL20215

The analytical data for ACL20125 are given as 1H-NMR, temperature dependent 1H-NMR, 13C-NMR, COSY, edCOSY and HMBC NMR spectra in Supplementary Figs. 10–15, respectively. The structure and conformation of ACL20215 indicate restricted torsional rotation of the C4–NBenzimidazole bond, due to size of the benzimidazole group. Energy barrier calculation indicate an upper range of 25 kcal/mol for this rotational barrier (see Supplementary Fig. 3), which, in relation to the GT active site, but our in vitro experiments were unable to detect these compounds.
(m, 1H, H-1x), 3.27 (dd, J = 8.8, 8.8, 11.2 Hz, 1H, H-6b);13C-NMR (150 MHz, DMSO-d6): major rotamer δ 158.9 (C-15), 154.9 (C-7), 143.0 (C-16), 140.1 (C-24), 139.9 (C-8), 137.4 (C-21), 131.8 (C-12), 128.0 (C-26, C-28), 126.8 (C-29, C-31), 124.8 (C-27), 125.5 (q, JCF = 271 Hz, C-22), 122.9 (q, JCF = 273 Hz, C-14), 122.6 (q, JCF = 271 Hz, C-12), 123.3 (C-13), 121.1 (J = 12.0 Hz, C-5, J = 11.6 Hz, C-6), 121.0 (J = 12.0 Hz, C-5, J = 11.6 Hz, C-6), 127.5 (C-21), 127.5 (C-13), 121.5 (C-12), 121.0 (q, J CF = 31 Hz, C-13), 116.1 (C-31), 114.6 (C-9), 77.7 (C-7), 70.3 (C-5), 68.8 (C-13), 60.3 (C-15). 53.2 (C-4), 48.1 (H-1x), 48.0 (H-1x). Minor rotamer δ 155.8 (C-15), 155.0 (C-24), 150.6 (C-12), 149.1 (C-9), 139.0 (C-20), 131.9 (C-12), 128.4 (C-26, C-28), 127.5 (C-25, C-29), 127.0 (C-27), 125.5 (q, JCF = 271 Hz, C-22), 122.9 (q, JCF = 273 Hz, C-14), 122.6 (q, JCF = 271 Hz, C-12), 123.3 (C-13), 121.1 (C-12), 121.0 (q, J CF = 31 Hz, C-13), 116.1 (C-31), 114.6 (C-9), 77.7 (C-7), 70.3 (C-5), 68.8 (C-13), 60.3 (C-15). 53.2 (C-4), 48.1 (H-1x), 48.0 (H-1x). 3H2Cl2F6N5O4, 658.1650; found, 658.1659.

Analytical data for A CL20964. The analytical data for A CL20964 are given as 1H-NMR, 13C-NMR, COSY, edCOSY and HMBC NMR spectra in Supplementary Figs 16–20, respectively. Similarly to ACL20215, ACL20964 exists as two conformational isomer, due to rotational restriction of the C4–NBenzimidazole bond, caused by a disulfide group.

Inhibition of glycolysransferase. Inhibition experiments were carried out using a fluorescence detection method by adding 1 μM of S. aureus MGT to 1.45 μM fluorescent dansyl-Lys Lipid II and different concentration of inhibitors, all in a buffer of 50 mM Tris pH 8 containing 10 mM MnCl2, 0.08% (w/v) decyl PEG, 10% (v/v) DMSO, 100 μg·mL−1 hen egg white lysozyme, in 96-well microtiter plates. Initial rates were measured as a decrease in fluorescence (ex/em: 340/521 nm) and calculated as a percentage compared with no inhibitor as negative control (0% of rate inhibition) and moenomycin A (Sigma, Cat. no.: 32404) as positive control (100% of rate inhibition). The data were fitted to a simple Michaelis-Menten model of inhibitor activity. IC50 values were extracted. Moenomycin A showed an IC50 of 5 μM in this assay.

Minimal inhibitory concentration (MIC) determination. The compounds along with standard antibiotics were serially diluted twofold across the wells of 96-well standard polystyrene non-treated plates (Corning 3370). Compounds and standard antibiotic controls ranged from 1.28 mg·mL−1 to 0.06 μg·mL−1 with final volumes of 50 μL per well. Bacteria were cultured in Brain–Heart Infusion (Bacto laboratories, Cat. no. CM1153B) at 37°C overnight. A sample of each culture was then diluted 40-fold in fresh brain–heart infusion broth and incubated at 37°C for 2–3 h. The resultant mid-log phase cultures were diluted to 5 × 105 c.f.u.·mL−1 then 50 μL was added to each well of the compound-containing 96-well plates giving a final compound concentration range of 64 μg·mL−1 to 0.03 μg·mL−1 in 2.5-fold dilutions. The plates were dried, exposed to phosphorimaging screen (1 week), scanned by Typhoon 8600 and calculating the value of each compound's MIC. Values of PG or lipid II were calculated as a percentage for MIC (for more details see Supplementary Methods).

In vivo PG biosynthesis. The cell-free particulate fraction of B. megaterium KM (ATCC13632), capable of catalysing the polymerization of PG from UDP-linked precursors was performed as described previously. B. megaterium was grown in standard medium, harvested and washed with Tris buffer by centrifugation. Resuspended bacteria were subjected to three freeze/thaw cycles (5 min dry ice, followed by 10 min at RT), homogenized by the glass homogenizer and centrifuged all at 4°C, leaving most of the cell wall in the pellet. Resuspended pellet was combined with UDP-N-acetylglucosamine-pentapeptide, [14]D/UDP-N-acetylgalactosamine and individual compounds or antibiotics (that is, vancomycin hydrochloride or moenomycin A), and incubated at 37°C at RT, placed in a boiling water bath for 3 min to inactivate enzymes and to prevent any further lipid II transformation, and analysed by TLC on silica gel plates. After separation, plates were dried, exposed to phosphorimaging screen (1 week), scanned by Typhoon 8600 and calculating the value of each compound's MIC. Values of PG or lipid II were calculated as a percentage for MIC (for more details see Supplementary Methods).

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Author contributions

C.M., G.A., D.M., G.L.T., R.P., B.B., L.S. and J.S. developed the chemistry and performed the synthesis, with J.Z. contributing to the design of the library. M.C., A.G.E., J.X.H., M.S.B., T.-J.C., W.-C.C., N.F.G., D.I.R., A.I.L. and C.G.D. developed, performed or supervised the various biochemical assays including analytical methods. D.D. and E.M. performed the in vivo assays. J.Z. and M.B. performed the in silico experiments. J.Z., C.M. and M.S.B. contributed to the writing of the manuscript. W.M. and M.A.C. supervised the studies, with W.M. establishing the project.

Additional information

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