3.09 Controlling Glycosyltransferase Activity: Inhibition and Enzyme Engineering

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3.09.1 Introduction

Glycosyltransferases (GTs) are enzymes that catalyze the assembly of glycans and glycoconjugates, and play many diverse roles in biology. GTs catalyze the transfer of sugar residues from activated sugar donor substrates to acceptor molecules, forming glycosidic bonds.[1] The acceptor nucleophiles can be either other sugars or non-sugar aglycones such as proteins, lipids, nucleic acids, or small molecules (e.g., antibiotics and other secondary metabolites).[2,3] Although the most common glycosylated products are O-linked, GTs can also produce N-, C-, and S-linked glycosides.[3] Considering the biological significance of glycans, there is great potential to control the activity of the enzymes that assemble them, which would be useful for both medicinal and industrial applications. This could be achieved by blocking GTs by identifying small molecules that act as inhibitors and engineering GTs to generate improved biocatalysts. The manipulation of GT function through these strategies is facilitated by an understanding of the enzyme structure and mechanism, which allows researchers to target the key residues involved in GT function.

3.09.1.1 Leloir versus non-Leloir GTs and their donor substrates

Activated sugar donors used by GTs can be in the form of nucleoside diphosphosugars (e.g., UDP-glucose, UDP-galactose, and GDP-mannose), nucleoside monophosphosugars (e.g., CMP-*N*-acetyl-neuraminic acid), lipid phosphosugars (e.g., dolichol phosphomannose, decaprenol phosphoarabinose), lipid diphosphosugars (e.g., dolichyl-diphosphooligosaccharide), sugar-1-phosphates (e.g., glucose-1-phosphate), or sugar-1-diphosphates (e.g., phosphoribosyl pyrophosphate) (Fig. 1).[3–7] Enzymes that use nucleotide-activated sugar donors are the most prevalent, consisting of approximately 65% of known GTs[8] and are termed Leloir GTs (after Nobel laureate, Luis F. Leloir, who was the first to discover a nucleotide-sugar[9]). Conversely GTs that use other sugar donors are defined as non-Leloir GTs, and those that use sugar-1-phosphates and sugar-1-diphosphates also categorized as phosphorylases and pyrophosphorylases, respectively.[3,6,7]

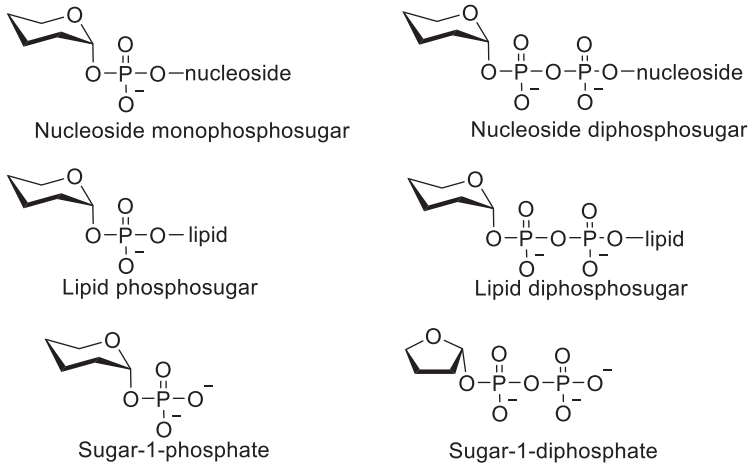

Fig. 1 Sugar donors utilized by Leloir and non-Leloir GTs. Leloir GTs use nucleotide activated donors and non-Leloir GTs use other donors.

3.09.1.2 Sequence-based CAZy families and structural categorization of GTs

In 1997, Henrissat and colleagues classified GTs into families based on the similarities of their amino acid sequences.[1] As of 2020, >750,000 GTs have been classified into >110 GT families that are regularly updated in the Carbohydrate-Active enZymes (CAZy) database (www.cazy.org). Additionally, >17,000 non-classified sequences are also reported. Of these families, only families GT2 and GT4 are represented in primitive archaea, suggesting that these might be the ancestral families from which all other GT families had evolved.[2]

Despite the great number of GT families, the protein topology is limited to only a few structurally different folds, which in addition to the GT-A, GT-B, and GT-C folds includes lysozyme-type, and 5-bladed propeller folds.[10–12] Structural analysis of Leloir GTs has revealed that GT-A and GT-B folds possess Rossmann-like folds. The two β/α/β Rossmann-like domains are compactly associated in GT-A fold enzymes, and thus often referred as a single Rossmann-like domain.[2,3,10] Generally, the catalytic activity of GT-A enzymes requires a divalent metal cation, such as Mg^{2+} or Mn^{2+}, which is coordinated along with the nucleotide-activated donor by a DXD (Asp-Xaa-Asp) amino acid sequence motif.[10,13–15] However, some GT-A enzymes do not rely on a metal ion for activity, and not all GT-A enzymes contain the DXD motif, nor is this motif diagnostic for GTs.[2,3]

The two Rossmann-like domains of GT-B fold enzymes face one another and are less compactly linked. The catalytic activity of these enzymes is metal independent, thus does not require a DXD motif.[2,3] Nevertheless, both GT-A and GT-B folds contain separate acceptor and nucleotide binding domains and these two folds have been observed to have either inverting or retaining mechanisms (Section 3.09.1.3).[2]

Based on sequence searches, Liu and Mushegian proposed a third GT-fold, called GT-C, that has multiple transmembrane α-helices.[16] GTs that use lipid phosphate- and lipid diphosphate-activated sugars possess this fold and so far, all known GT-C enzymes operate with an inverting mechanism (Section 3.09.1.3).[3,17] Considering these structural folds and the stereochemistry of glycosylated products, GT families can be categorized into clans (Fig. 2).

3.09.1.3 Mechanism of GTs

The glycoside products of GT action can have the same (retained) or opposite (inverted) anomeric configuration, relative to the sugar donor. Based on the product stereochemistry, GTs are classified as inverting or retaining (Fig. 3). The difference in the stereochemical outcome is related to the reaction mechanism.[3]

3.09.1.3.1 Inverting GT mechanisms

Most inverting GTs use a single displacement S_N2 (substitution nucleophilic bimolecular) substitution mechanism through an oxocarbenium ion-like transition state that results in inversion of configuration at the anomeric carbon of the sugar donor (Fig. 4A). A side chain of an active site aspartic acid or glutamic acid assists as a general base catalyst, deprotonating the nucleophilic hydroxyl group of the acceptor molecule. At the same time, the nucleophile attacks the anomeric carbon of the donor and the nucleotide moiety leaves from the opposite side. Most inverting GT-A enzymes use a divalent metal ion to stabilize the negative charge of the phosphate leaving group, and in metal-independent inverting GT-B enzymes a positively-charged amino acid side-chain performs this function.[3,18]

Not all inverting GTs contain an amino acid general base. In some cases the β-phosphate oxygen atom of the donor molecule is proposed to fulfil this function. Examples include POFUT1 and FUT1, which are proposed to use an S_N1 (substitution nucleophilic unimolecular) reaction mechanism as illustrated by Fig. 4B. Herein, glycosidic bond cleavage would result in a transient

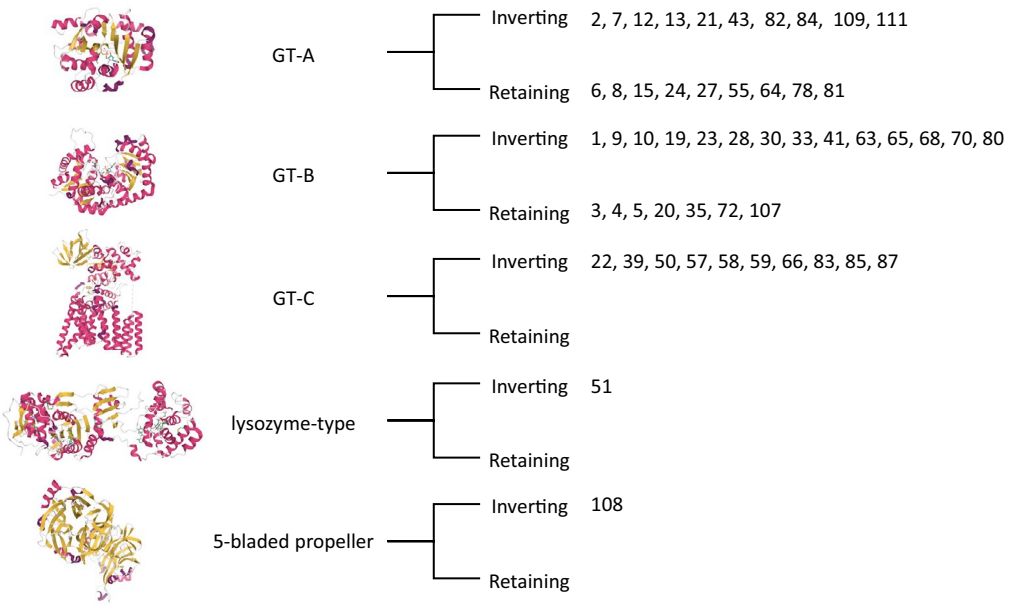

Fig. 2 GT families are divided into clans based on fold and reaction mechanism (data from the CAZy database). GT-A, GT-B, GT-C, lysozyme-type, and 5-bladed propeller folds are represented by protein structures of LgtC from *Neisseria meningitidis* (PDB: 1G9R), VvGT1 from *Vitis vinifera* (PDB: 2C1X), and PglB from *Campylobacter lari* (PDB: 3RCE), Pbp2 from *Staphylococcus aureus* (PDB:2OLV), and MTP2 from *Leishmania mexicana* (PDB: 6Q4X), respectively.

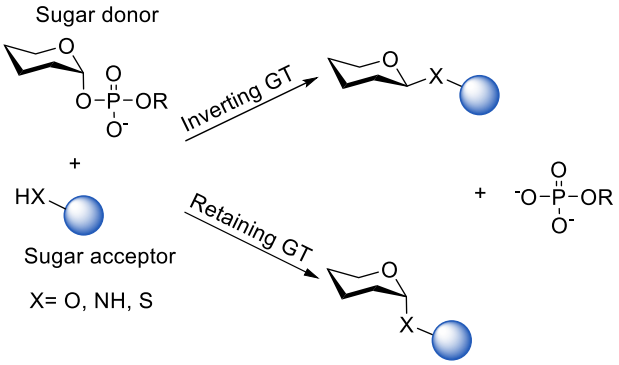

Fig. 3 The two stereochemical products of GT catalyzed reactions. With respect to the sugar donor, an inverting GT gives a product with inversion of the anomeric configuration while the anomeric configuration is retained by a retaining GT.

oxocarbenium ion. The close association of the β-phosphate group of the donor with the oxocarbenium ion leads to the attack of the incoming acceptor from the opposite direction.[19–21]

3.09.1.3.2 Retaining GT mechanisms

Through detailed structural and kinetic studies, compelling evidence of retaining glycoside hydrolases supports a double-displacement substitution reaction involving a covalently linked glycosyl-enzyme intermediate.[22] The same mechanism has been suggested for retaining GTs.[3,18] As shown by Fig. 5A, this proposed two-step double-displacement mechanism (going through two transition states with oxocarbenium character) requires a properly situated active site nucleophile to attack the anomeric carbon of the donor molecule.[3] Structural studies of several GTs have suggested that the leaving phosphate group may act as the base catalyst to activate the incoming acceptor nucleophile.[23,24] Similar to inverting GTs, a divalent cation or a positively charged side chain assists the removal of the leaving group in many retaining GT-A or GT-B enzymes, respectively.[3]

In 2004, Withers and colleagues observed the first covalent intermediate of a mutant retaining GT, but were unable to demonstrate a covalent intermediate for the wildtype enzyme.[25] As of 2020, confirmation of a covalent glycosyl-enzyme intermediate has not been obtained for any wild-type retaining GT. Moreover, the relative location of a catalytic enzyme nucleophile seems poorly conserved among many retaining GTs suggesting an alternative mechanism for at least some of these enzymes.[3,17]

An S_Ni (substitution nucleophilic internal)-like reaction with an oxocarbenium ion-like transition state has been proposed for some retaining GTs. In this mechanism, the leaving group deprotonates the nucleophilic group of the acceptor and the retention of the stereochemistry is an outcome of the same side departure of the leaving group and attack by the nucleophile (Fig. 5B). It is

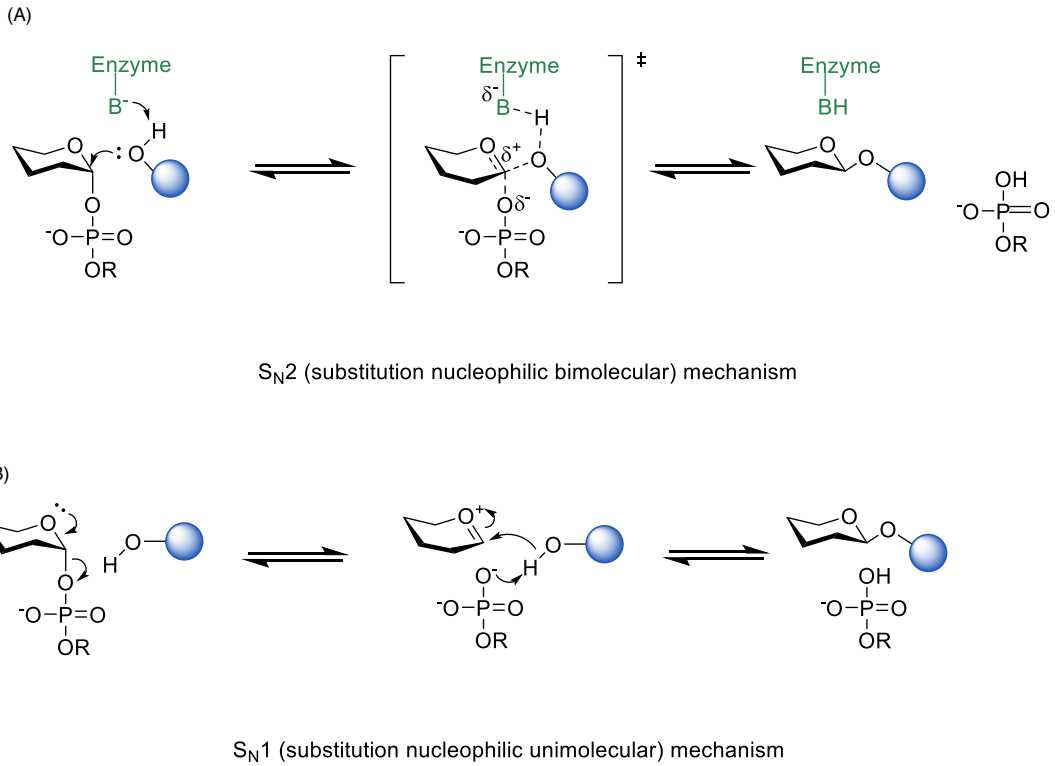

Fig. 4 Inverting GT reaction mechanisms. (A) S_N2 reaction mechanism, (B) S_N1 mechanism.

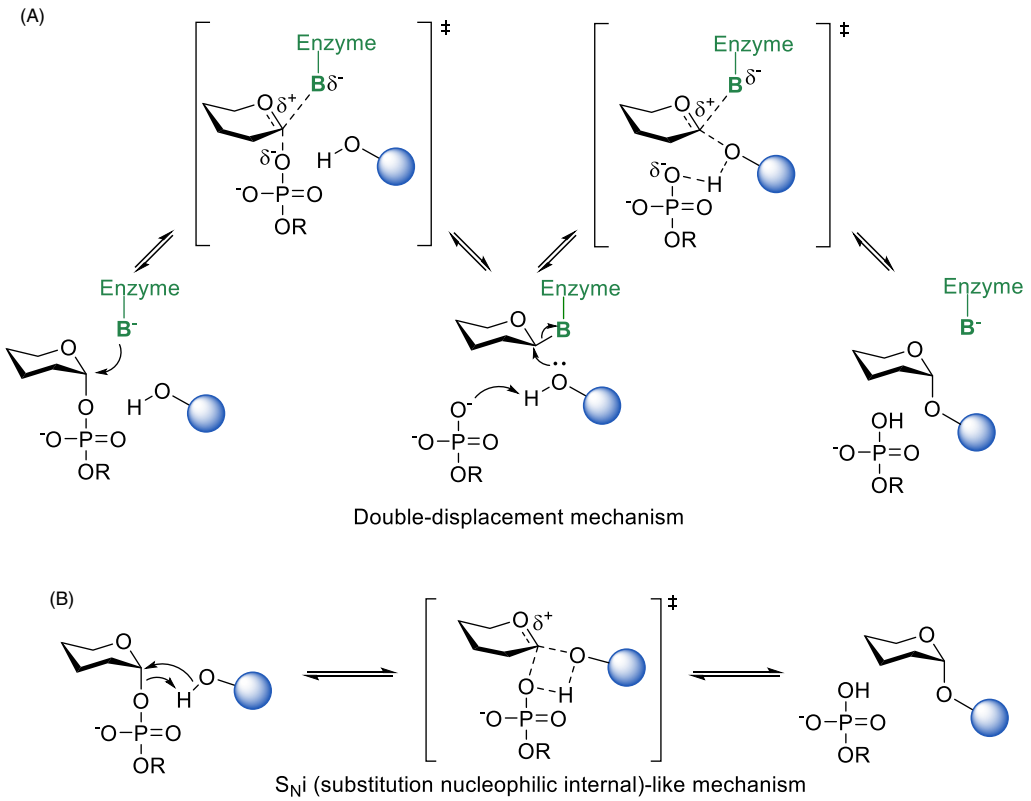

Fig. 5 Retaining GT reaction mechanisms. The proposed (A) double-displacement reaction mechanism with a covalently linked enzyme-glycosyl intermediate (and two oxocarbenium ion-like transitions states) and (B) S_Ni-type mechanism with an oxocarbenium ion-like transition state; whether this reaction occurs in a concerted or non-concerted manner is an open question.

proposed that the front-face attack by the acceptor nucleophile is supported by hydrogen bonds between acceptor nucleophile and the leaving group, as first proposed for UDP-Glc dependent trehalose-6-phosphate synthase from *E. coli*.[3,26,27] This mechanism has since been assigned to retaining GTs including human polypeptide *N*-acetylgalactosaminyltransferase 2 (GalNAc-T2),[28] mammalian xyloside xylosyltransferase 1 (XXYLT1),[29] and mycobacterial glucosyl-3-phosphoglycerate synthase (GpgS)[30] by crystallographic, molecular dynamic, and mechanistic studies.

The elucidation of GT structure and mechanism have assisted the discovery of small molecule inhibitors and informed enzyme engineering efforts, as discussed in the following sections.

3.09.2 Inhibition of GT activity

The carbohydrate products of GTs are involved in a multitude of cellular processes. These include intercellular interactions within an organism as well as interspecies interactions such as those between host and pathogen. Glycans coat the cells of all organisms and GT-catalyzed assembly of polysaccharides is essential for the structural integrity of cell walls in plants and fungi, and the cell envelopes of bacteria and archaea, and for pathogens to assist immune evasion. Many natural products identified through screens for antimicrobial activity target various GTs in bacteria and fungi, as elaborated in Section 3.09.2.1.3. Glycosylation of macromolecules including proteins, lipids, and nucleic acids, catalyzed by GTs, has a multitude of effects on their biological roles. The function and dysfunction of GTs are very important in health and disease. Over-expression of cell-surface glycans and aberrant glycosylation of proteins is linked to cancer metastasis and progression. Abnormal glycosylation has been linked to diseases including Gaucher's disease, the autoimmune disease lupus, and inflammatory disorders.[31,32] Targeting glycosylation has emerged as potential therapeutic approach and therefore it has inspired a quest to find inhibitors. This has led to rational design of inhibitors against GTs (Sections 3.09.2.1.1 and 3.09.2.1.2). In the section below we have classified inhibitors based on their structural similarity, type of scaffolds, origin, and mechanism of action.

3.09.2.1 Types of GT inhibitors

Glycosyltransferase enzymes have complex reaction mechanisms. Activity requires the interaction of two substrates (a donor and an acceptor) with the enzyme—in some cases together with a metal ion—to catalyze the transfer of a sugar unit with the stereospecific retention or inversion of stereochemistry. Careful consideration of the enzyme mechanism and protein structure can guide the design of GT inhibitors.[33–36] As the complex between the GT and its substrates traverses its path along the reaction coordinates, the reactants overcome an energy barrier, forming a transition state at their highest potential energy, then proceed to form reaction products—the glycoside and leaving group of the donor, which are finally released by the enzyme. The enzyme can form a complex with either the acceptor or the donor substrate as a binary complex, or with both as a ternary complex, in addition to making very tight interactions with the transition state formed by the reactants. There are multiple types of inhibitors that can interrupt catalysis through different interactions and in these sections, we discuss substrate analogs, transition state analogs, glycomimetics, natural products, and structurally-diverse synthetic small molecules; some compounds fall under more than one category.

3.09.2.1.1 GT substrate analogs and transition state analogs

One approach to design enzyme inhibitors is based on substrate binding and recognition. In the case of GTs, substrate analog inhibitors can be divided into two major classes: (a) nucleotide-sugar donor analogs and (b) modified carbohydrates that mimic acceptor substrates (Fig. 6). The first approach focuses on the donor substrate (Fig. 6A), and for Leloir GTs, which make relatively strong interactions with the nucleotide portions of such substrates, these are typically bound more tightly than the acceptor substrate. Donor substrate analogs can include different modifications either on the nucleotide or sugar portions.

Nucleotide-modified inhibitors have one or more substituent groups on the nucleoside mono- or di-phosphate portion that result in binding of the inhibitors to the GT with high affinity in such a way that results in a catalytically unproductive complex. Many early studies on GT inhibitors were performed on mammalian β1,4-galactosyltranferase I (β4Gal-T1) from the GT7 family, which is the most widely studied glycosyltransferase as it can be obtained from cow's milk and has been commercially available for many years. The first donor analog inhibitors reported for this enzyme were nucleotide-modified analogs, uridine 5′-phosphoric α-D-galactopyranosylphosphonic anhydride (**1**) and uridine 5′-[(α-D-galactopyranosylhydroxyphosphonyl)methyl]phosphonate (**2**), synthesized by Vaghefi et al., which differ from the natural UDP-Gal donor in the pyrophosphate portion in a way that prevents transfer of the galactosyl residue.[37,38] Applying a similar concept, Allen et al. designed the prodrug fucostatin II (**3a**) to target fucosyltransferases.[39] Upon entry into cells, fucostatin II is turned over by intracellular esterases to produce the deprotected fucostatin II derivative (**3b**), which is subsequently converted by the fucose salvage pathway into the guanylyl fucostatin II derivative (**3c**). As an analog of GDP-Fuc, modified in the pyrophosphate portion of the nucleotide, this donor mimic inhibits fucosyltransferases and blocks fucosylation in the production of glycosylated antibodies in cell culture, showing utility in tailoring the glycosylation patterns of therapeutic proteins. Other examples of nucleotide-modified donor substrate analogs focus on the base portion of the nucleotide. Structural analysis of mammalian galactosyltransferases led Wagner and colleagues to design a derivative of UDP-Gal with a formylthienyl substituent at the 5 position of the uracil base (5-FT UDP-Gal; **4**), which inhibited β4Gal-T1, the dual-specificity A/B blood group antigen-synthesizing α-(1,4)-glycosyltransferase AA(*Gly*)B, and other galactosyltransferases.[40,41] Pesnot et al. studied the structural basis for inhibition and showed that this analog blocks the closure of an active-site flexible loop

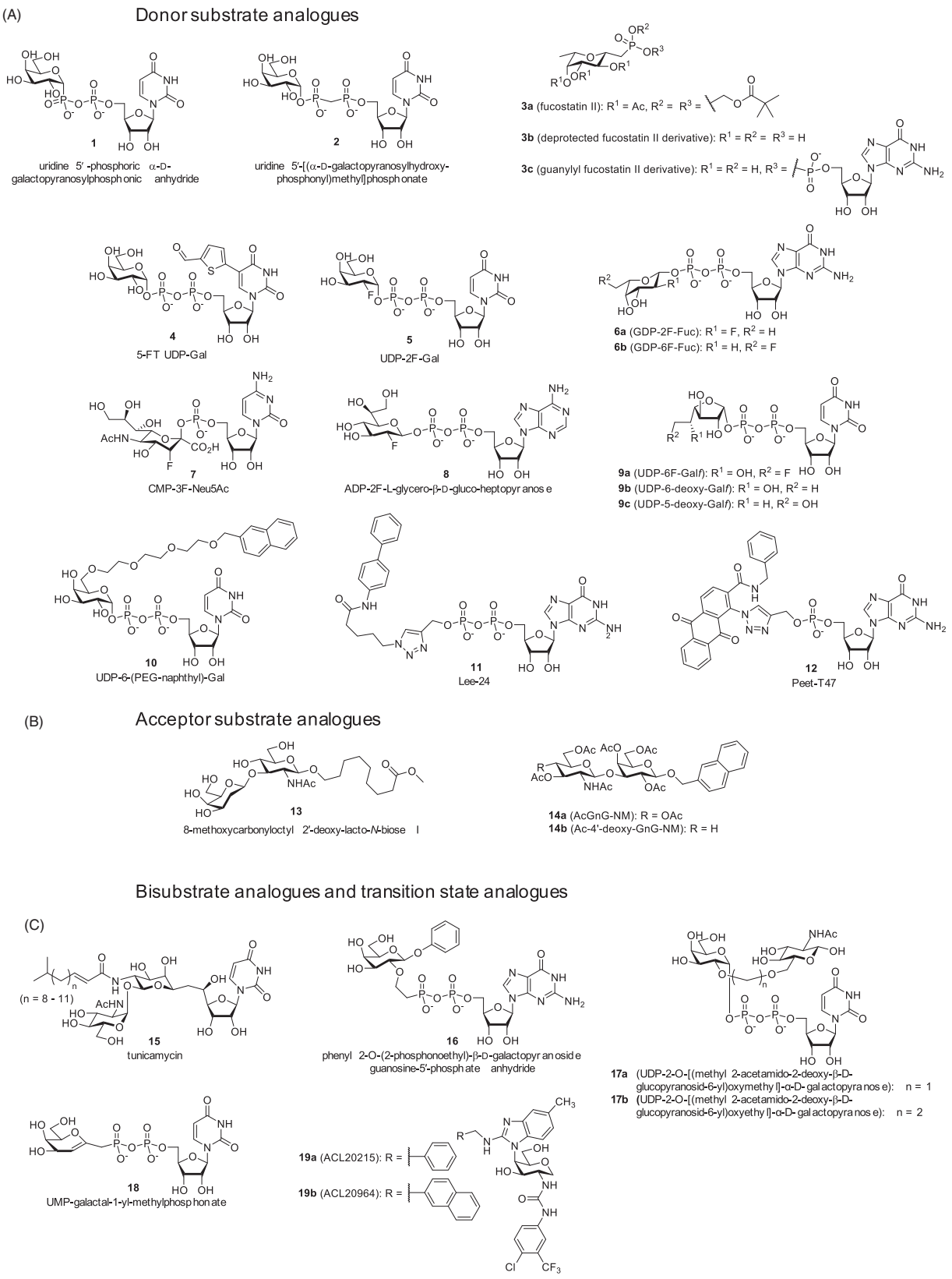

Fig. 6 GT substrate and transition state analogs. Inhibitors designed to mimic (A) GT donor substrates, (B) acceptor substrates, or (C) both as bisubstrate or transition state analogs.

fixing it in an unproductive conformation and rendering the enzyme catalytically inactive.[40] These studies highlight the importance of structural insights on the design of substrate analog inhibitors.

Sugar-modified donor analogs have also been studied as inhibitors. Wong and coworkers reported several studies in which they modified the sugar groups in the donor substrates of galactosyltransferases,[42] fucosyltransferases,[43] and sialyltransferases[44] (UDP-Gal, GDP-Fuc, and CMP-Neu5Ac), installing fluorine atoms and replacing hydroxyl groups. In each of these derivatives—UDP-2F-Gal (**5**), GDP-2F-Fuc (**6a**), GDP-6F-Fuc (**6b**), and CMP-3F-Neu5Ac (**7**)—the electronegative fluorine atom destabilizes formation of an oxocarbenium-like transition state (Section 3.09.1.3) resulting in a donor analog that can form an unreactive enzyme complex. Adopting this strategy, Ducruix and colleagues synthesized a fluorinated donor analog, ADP-2F-L-glycero-β-D-gluco-heptopyranose (**8**), as an inhibitor of the lipopolysaccharide-synthesizing heptosyltransferase in Gram-negative bacteria.[44] Daniellou, Mikušová, and colleagues reported a series of synthetic UDP-furanoses with side chains fluorinated and deoxygenated at the 5 and 6 positions as analogs of UDP-galactofuranose (UDP-Gal*f*), the donor substrate of the mycobacterial galactofuranosyltransferases, GlfT1 and GlfT2. These GTs are involved in the synthesis of the galactan component of polysaccharides found in the cell wall of *Mycobacterium tuberculosis* and related species.[45] The analogs, UDP-6F-Gal*f* (**9a**), UDP-6-deoxy-Gal*f* (**9b**), and UDP-5-deoxy-Gal*f* (**9c**) were assessed as inhibitors of these enzymes. These compounds inhibited galactan assembly by *M. smegmatis* enzymes *in vitro* and the mode of action suggested the incorporation of modified galactofuranose residues into the galactan polymer, causing chain termination.

Other reports have studied the installation of more complex functional groups onto the sugar portion in the synthesis of donor analogs. Nishimura and coworkers synthesized novel UDP-Gal analogs as candidates for mechanism-based inhibitors of β4Gal-T1 and identified one compound that contains a naphthyl group attached by a long polyethylene glycol linker to the 6 position of the galactose residue of the donor analog, UDP-6-(PEG-naphthyl)-Gal (**10**). Tanaka et al. hypothesized that the mode of inhibition involves hydrophobic interaction of the naphthyl group with a key Trp-310 residue of β4Gal-T1, which prevents the conformational change of a flexible loop, locking the enzyme in an inactive state that prevents binding of the acceptor substrate.[46] Further examples of donor substrate analogs that include linker-tethered functional groups—which make specific interactions beyond the donor binding-site of the GT—dispense entirely with the sugar portion of the donor. Wong and colleagues created a diverse library of compounds each made up of a hydrophobic group tethered to a nucleoside diphosphate by a linker of variable length. Using click chemistry the authors prepared a library of molecules composed of a hydrophobic moiety attached through an amide bond to an acyl chain connected to GDP through a triazole to mimic the GDP-fucose substrate of the enzyme. This library was screened for inhibition of a human α1,3-fucosyltransferase. Using a high-throughput screening approach (Section 3.09.2.2.1), Lee et al. identified a compound, herein referred to as Lee-24 (**11**),[47] that acted as a potent fucosyltransferase inhibitor.[47,48] Adopting this combinatorial strategy, Williams and coworkers used similar techniques to discover inhibitors of *Leishmania* β1,2-mannosyltransferases from a focused library of 47 GMP-triazole compounds. Triazole Peet-T47 (**12**), was a potent inhibitor of the target enzyme from the parasite that causes leishmaniasis.[49]

While donor analog inhibitors can bind target GTs with high affinity, due to their mimicry of the tight interactions between the natural nucleotide-sugar and enzyme, they often lack specificity since many GTs share the same donor. On the other hand, analogs that mimic the native acceptor can provide specificity (Fig. 6B), although their affinity tends to be weaker than that of the donor analogs. In early studies, Hindsgaul, Palcic, and coworkers evaluated several deoxygenated oligosaccharide acceptor analogs as specific inhibitors of several GTs. 8-Methoxycarbonyloctyl 2′-deoxy-lacto-*N*-biose I (**13**), an acceptor analog that lacks the hydroxyl group to which a fucose would normally be transferred, was identified as a competitive inhibitor of mammalian α1,2-fucosyltransferase.[50] In contrast, some acceptor analogs are effective acceptor substrates and inhibit transfer of a sugar onto the native acceptor of a GT by acting as a competitive substrate (sometimes termed a decoy), accepting the sugar that would otherwise be transferred to the natural acceptor substrate, thereby inhibiting product formation. Esko and colleagues synthesized a cell-permeable disaccharide, per-*O*-acetylated GlcNAcβ1,3Galβ-*O*-naphthalenemethanol (AcGnG-NM; **14a**), which upon entry into the cell is first deacetylated by endogenous cellular esterases, and then the newly formed disaccharide inhibits the assembly of the cell-surface carbohydrate, sialyl Lewis^X, on tumor cells by competing with its precursors as a GT acceptor substrate.[51] A potential drawback of this approach is that glycosylation of the decoy can result in accumulation of unnatural free glycans in the cell, the effect of which is unknown. In this case, modification of the disaccharide to produce a 4′-deoxygenated acceptor analog, per-*O*-acetylated 4-deoxy-GlcNAcβ1,3Galβ-*O*-naphthalenemethanol (Ac-4′-deoxy-GnG-NM; **14b**), yielded a compound that upon intracellular deacetylation acts as a competitive GT inhibitor, rather than a decoy.[52]

Inhibitor compounds that include covalently attached donor and acceptor analogs are called bisubstrate analogs. These compounds take advantage of the high affinity and high specificity that each component contributes, respectively.[53] Bisubstrate analog inhibitors can mimic the interactions made with a GT enzyme in the ternary complex, yet some of these may also be considered analogs of the transition state, which has a combined character derived from both the donor and acceptor substrates (Fig. 6C). The natural product tunicamycin (**15**) (Section 3.09.2.1.3), is a microbial secondary metabolite that acts as a bisubstrate analog-type inhibitor of eukaryotic GlcNAc-1-phosphotransferase and oligosaccharyltransferase, which are enzymes involved in protein *N*-glycosylation.[54–57] Inspired by Nature, chemists have synthesized bisubstrate analogs as GT inhibitors. Structural and mechanistic considerations are important in designing bisubstrate and transition state analogs,[10,15] but the limited amount of GT structural information and the sometimes enigmatic enzyme mechanisms can present a challenge to designing inhibitors with high specificity and potent inhibition. Nonetheless, many bisubstrate and transition state analogs have been synthesized and some have shown to have strong inhibition. Palcic, Hindsgaul, and colleagues, reported the first chemical synthesis of a bisubstrate or transition state analog that had been designed as a GT inhibitor; despite having no structure of the target enzyme, phenyl

2-*O*-(2-phosphonoethyl)-β-D-galactopyranoside guanosine-5′-phosphate anhydride (**16**) was designed to inhibit mammalian α-1,2-fucosyltransferase and was shown to be a competitive inhibitor with respect to both the donor and acceptor substrates.[58] By replacement of the hydrophilic diphosphate group, bisubstrate analogs with different linkers such as malondiamide or ethylene were designed to increase membrane permeability.[59,60] Taking into account speculation on the mechanistic aspects of GT catalysis, Hashimoto and coworkers designed tricomponent, bisubstrate analogs—which consist of the sugar component of the acceptor substrate, and the sugar and nucleotide components of the donor substrate. These were designed to mimic the putative S_N2-like transition state (Section 3.09.1.3.1) of β4Gal-T1. UDP-2-*O*-[(methyl 2-acetamido-2-deoxy-β-D-glucopyranosid-6-yl)oxymethyl]-α-D-galactopyranose (**17a**) and UDP-2-*O*-[(methyl 2-acetamido-2-deoxy-β-D-glucopyranosid-6-yl)oxyethyl]-α-D-galactopyranose (**17b**) have the sugar components of the acceptor and donor linked by a methylene or ethylene tether, respectively. Interestingly, these inhibitors act by different modes with the length of the linker playing a role in determining the mode of inhibition: the methylene-linked analog was a competitive inhibitor and the ethylene-linked analog was a non-competitive inhibitor of β4Gal-T1.[61,62] The proposed oxocarbenium ion-like character of GT transition states has also served as the basis for the design of transition state analogs that mimic the distorted, partially planar structure that the donor sugar is likely to transiently adopt (Section 3.09.1.3). Schmidt and Frische designed UMP-galactal-1-yl-methylphosphonate (**18**) as an inhibitor to β4Gal-T1.[63] Though it is an inhibitor that lacks an acceptor analog moiety, and thus is not a bisubstrate analog, it is a transition state mimic wherein the donor sugar analog, which is linked to the nucleotide portion by a non-cleavable C—C glycosidic bond, adopts a half-chair conformation, bearing conformational analogy to the proposed transition state. Schmidt and colleagues designed several very potent sialyltransferase inhibitors based on transition state analogs of the sialyl donor.[64]

Just as tunicamycin has inspired the design of bisubstrate analogs as GT inhibitors, the moenomycin family of antibiotic natural products (Section 3.09.2.1.3) has served as a template for the construction of a collection of compounds that bind both donor and acceptor sites of the bacterial peptidoglycan GT. Centered on a monosaccharide-based scaffold, Cooper, Muetermans and colleagues synthesized several such compounds as inhibitors of the peptidoglycan GT targeted by moenomycins. Compounds ACL20215 (**19a**) and ACL20964 (**19b**) were identified as the strongest inhibitors.[65] Though the scaffold for these compounds bears functional groups that bind in the acceptor and donor sites, these groups are chemically distinct from either donor or acceptor, and the scaffold itself, which resembles a monosaccharide, is missing the exocyclic oxygen on what would be the anomeric center. Thus, these analogs can each be classified as a mix of glycomimetic (Section 3.09.2.1.2) and natural product-inspired, structurally diverse small molecule GT inhibitors (Sections 3.09.2.1.3 and 3.09.2.1.4).

While useful experimental tools, most of the substrate and transition state analogs discussed thus far suffer a lack of drug-like qualities (e.g., cell membrane permeability, chemical stability, and practical synthetic routes) and have not found application as pharmaceutical interventions beyond limited academic studies. Thus, investigation of other GT inhibitor types like glycomimetics, natural products, and structurally diverse small molecules is of interest for discovery of drug-like GT inhibitors.

3.09.2.1.2 Glycomimetic inhibitors of GTs

Glycomimetics are a class of molecules that resemble the carbohydrate scaffold and can mimic the properties of natural GT substrates. This is an overlapping category under which some substrate and transition state mimics can be categorized, but unlike most of the analogs described above, which can contain sugar groups that may be modified, glycomimetics typically contain moieties that resemble sugars, but are chemically distinct in some key aspect that distinguishes them from being classified as sugars (Fig. 7). These sugar-like groups are typically composed of a quasi-carbohydrate structure, wherein either the ring oxygen or the glycosidic oxygen is replaced with carbon, nitrogen, sulfur, etc., and can further be classified as carbasugars, iminosugars, thiosugars, or *C*-glycosides, *N*-glycosides, and thioglycosides, depending on the substituent atom. Glycomimetics have been designed to increase drug-like characteristics of inhibitors with higher cell penetration efficiency.

Carbasugar glycomimetics (Fig. 7A)—in which a sugar ring oxygen is replaced with carbon (methylene)—have been extensively studied as inhibitors of glycosidases, and there are several carbasugar-based glycosidase inhibitors that are in clinical use.[66,67] Carbasugars have been studied for inhibition of GTs including fucosyltransferases, galactosyltransferases, and sialyltransferases. Carbasugar derivatives of the natural donor substrates (GDP-Fuc, UDP-Gal, and CMP-Neu5Ac) are potent inhibitors *in vitro*. Replacement of the fucose ring oxygen in GDP-Fuc and the galactose ring oxygen in UDP-Gal yields GDP-5a-carba-Fuc (**20**) and UDP-5a-carba-Gal (**21**), respectively, which are donor analogs from which oxocarbenium ions cannot be formed. These compounds are inhibitors of fucosyltransferases and galactosyltransferases, respectively.[68,69] The nucleotide-carbasugar, CMP-quinic acid (**22**), and its derivatives are effective inhibitors of sialyltransferases.[70] Whereas replacement of a sugar's ring oxygen with a carbon generates a carbasugar glycomimetic, replacement of the glycosidic oxygen yields a C-glycoside, which is sometimes also classified as a glycomimetic. C-glycosides, such as fucostatin II (**3a**), tunicamycin (**15**), and UMP-galactal-1-yl-methylphosphonate (**18**), are also substrate and transition state analog inhibitors, and were described in Section 3.09.2.1.1.

Other GT substrate mimics fall under the iminosugar classification of glycomimetics (Fig. 7B), with nitrogen substituting the ring oxygen atom in the carbohydrate ring. Like carbasugars, iminosugars were also initially developed as glycosidase inhibitors. They are an attractive inhibitor class due to their metabolic stability which is advantageous as potential therapeutics. The positive charge that the iminosugar nitrogen bears at physiological pH also acts as an electrostatic mimic of an oxocarbenium ion. Iminosugars are the most successful among glycomimetic GT inhibitors in terms of their clinical application. A notable example is the therapeutic drug miglustat (**23**), which is a synthetic *N*-alkylated iminosugar mimic of glucose, used in treating the lysosomal storage disorders, Gaucher's disease and Niemann-Pick disease. It acts as a competitive inhibitor of human glucosylceramide synthase (GCS), a GT that catalyzes the glycosylation of ceramide (a sphingolipid) to produce glucosylceramide. It does this by mimicking the ceramide

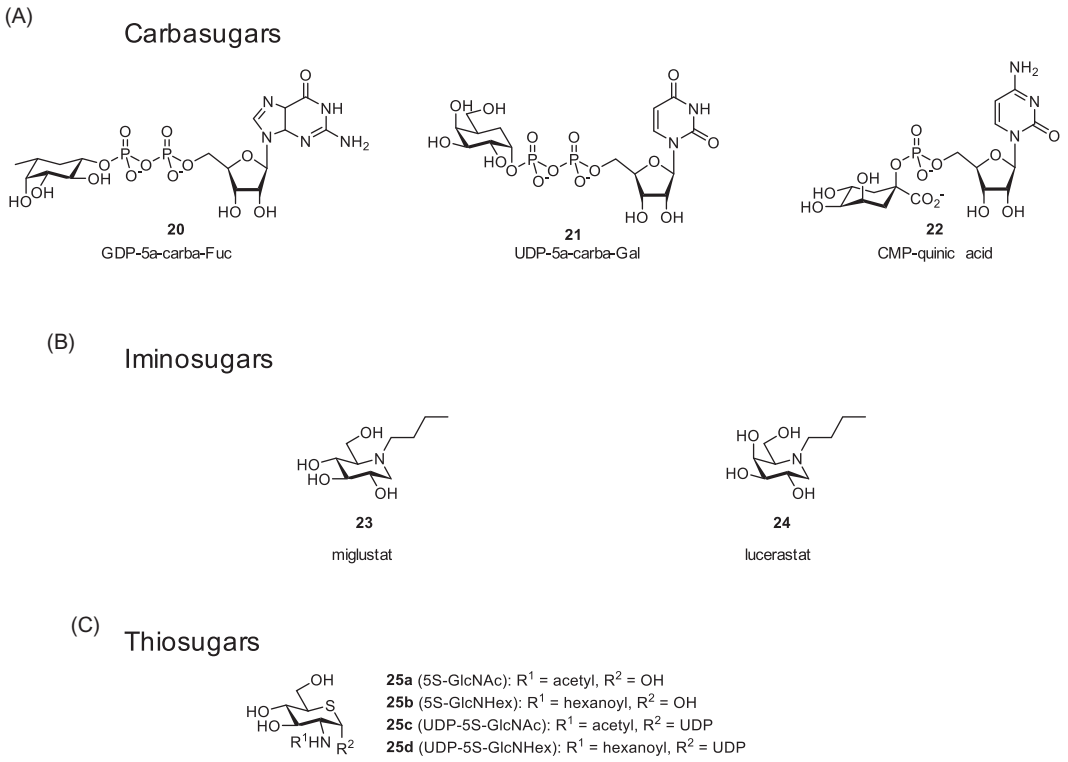

Fig. 7 Glycomimetics as GT inhibitors. Inhibitors falling under carbasugar (A), iminosugar (B), and thiosugar (C) classifications.

acceptor substrate through its alkyl chain, along with the donor glucose through the iminosugar portion. Treatment with miglustat prevents the accumulation of glycosphingolipids like glucosylceramide in lysosomal storage disorders that result in serious illness.[71] Recent studies have investigated the effect of a similar iminosugar, lucerastat (**24**) in inhibiting GCS as a potential treatment for Fabry disease, another lysosomal storage disorder, and it has been shown to reduce the accumulation of glycosphingolipids in clinical trials.[72] Other iminosugars like pyrrolines and pyrolidines have also been studied as inhibitors for β4Gal-T1, but they exhibit weak inhibition.[73–75]

Thiosugars, in which the sugar ring oxygen is replaced with a sulfur atom, have been explored as GT inhibitors (Fig. 7C), and in one case have found utility as tools to study post-translational protein modification by glycosylation *in vivo*. The O-GlcNAc modification involves the attachment of GlcNAc residues onto the hydroxyl groups of serine and threonine, and mediates a multitude of critical cellular processes. Transient O-GlcNAc protein modifications are ubiquitous in multicellular eukaryotes with several hundred proteins being targets for O-GlcNAc transferase (OGT), the GT that transfers GlcNAc from UDP-GlcNAc onto these amino acid side chains. To develop tools to probe O-GlcNAc function, Vocadlo and coworkers synthesized thiosugar analogs of GlcNAc, 5S-GlcNAc (**25a**), and derivatives with improved membrane permeability like 5S-GlcNHex (**25b**), which can be delivered into cells and metabolized to generate corresponding UDP-activated thiosugars, UDP-5S-GlcNAc (**25c**) and UDP-5S-GlcNHex (**25d**) that inhibit OGT.[76,77]

There is scope to develop potential therapeutic drugs from GT inhibitors based on carbohydrate and glycomimetic scaffolds. However, challenges remain in translational studies and few of these molecules have found application as drugs, although there are some notable successes like miglustat (**23**). Broadly, the development of substrate analog and glycomimetic GT inhibitors follow the principles of rational design of ligands. Therefore, the design of substrate mimics and analogs depends heavily on the availability of crystal structures of GTs and access to details of enzyme mechanism, constituting a bottleneck in rapid drug discovery. Most enzyme inhibitors that are in clinical use have been identified from vast compound libraries using classical screening approaches applied to discover antibiotics and more contemporary high-throughput screens against specific enzyme targets. In the following sections we discuss different classes of GT inhibitors that include non-carbohydrate compounds that have been identified by screening compound libraries.

3.09.2.1.3 Natural products as GT inhibitors

Traditional approaches in the discovery of antibiotics have mainly involved phenotypic screening of natural compounds. Subsequent elucidation of the mode of action for many of these natural products demonstrated that they target GT activity in microbes and halt vital cell processes, such as protein glycosylation or cell-wall biosynthesis (Fig. 8).

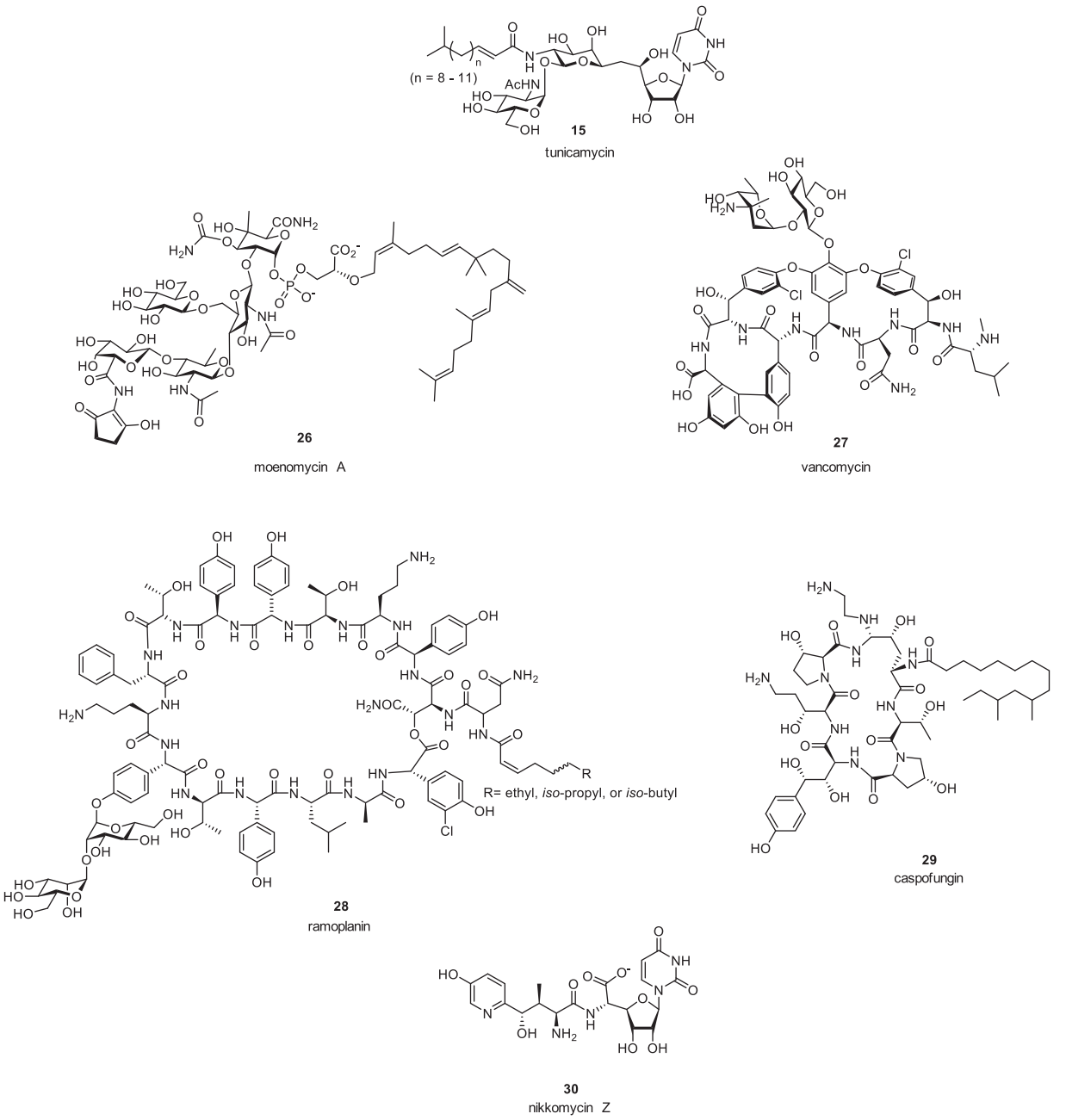

Fig. 8 Natural product GT inhibitors.

Tunicamycin (**15**) is a natural product from *Streptomyces* that acts upon multiple molecular targets.[54] Notably it was the first compound discovered to target eukaryotic protein N-glycosylation. It acts on the first enzyme in protein N-glycosylation, GlcNAc-1-phosphotransferase, which catalyzes the transfer of GlcNAc-1-phosphate from UDP-GlcNAc to dolichol phosphate.[78,79] It also inhibits the GT oligosaccharyltransferase, which catalyzes transfer of the 14-residue oligosaccharide core unit ($Glc_3Man_9GlcNAc_2$) from a dolichol pyrophosphate-activated donor onto the asparagine of a nascent glycoprotein. Thus, tunicamycin inhibits both the synthesis and attachment of N-linked glycans to glycoproteins (it is a bisubstrate and transition state analog inhibitor, as described in Section 3.09.2.1.1). Tunicamycin is also a weak inhibitor of the bacterial enzyme MraY, a phospho-*N*-acetylmuramoyl-pentapeptide transferase involved in bacterial peptidoglycan biosynthesis.[80]

Moenomycin A (**26**), a member of the moenomycin family of antibiotics, is an antibacterial agent, consisting of a lipid-linked pentasaccharide, that inhibits peptidoglycan GTs.[81] The moenomycins contain a phosphoglycerate that links a glycan portion to a polyprenyl chain, and competitively inhibit GT transglycosylases by mimicking the disaccharide–pyrophosphate–prenol linkage

of the donor, lipid II. Moenomycins are used in animal husbandry, where they are added to cattle feed in combination with other drugs.[82] They are not used in humans due to poor pharmacokinetic properties attributed to the lengthy lipid chain. However, the moenomycin structure has inspired the development of analogs in the design of transition state mimics as reported by Zuegg et al. (Section 3.09.2.1.1).[65]

Peptide-based GT inhibitors have been used as drugs to treat bacterial and fungal infections. Naturally occurring macrocyclic peptides (MCPs) often have favorable pharmacological properties, are metabolically stable, target-specific, and have high affinity with the ability to modulate interactions with proteins, thus making them excellent drug candidates.[83,84] Examples include the glycopeptide natural product, vancomycin (**27**), which is a clinically used antibiotic that inhibits peptidoglycan GT and transpeptidase and disrupts cell-wall biosynthesis in bacteria.[85] Ramoplanin (**28**) is an MCP that targets peptidoglycan synthesis by inhibiting transglycosylation.[86–89] Echinocandins are a class of antifungal drugs discovered by natural product screening and are also classified as MCPs.[90] They target 1,3-β-glucan synthase, which is a glucosyltransferase involved in 1,3-β-glucan synthesis in fungal cell wall.[91] Caspofungin (**29**), a representative of the echinocandin family, is an approved antifungal drug that inhibits cell wall biosynthesis (e.g., by *Aspergillus* and *Candida* species).[92] The successful use of these drugs highlights that the exploration of macrocyclic scaffold libraries as potential GT inhibitors is a promising avenue for future research. The natural product nikkomycin Z (**30**) is a peptide-based inhibitor used as an antifungal medication, which targets fungal chitin synthase, but unlike the previous examples it is a short linear peptide linked to a nucleoside.[93,94]

3.09.2.1.4 Structurally diverse synthetic small molecules as GT inhibitors

With the advent of high-throughput screening methods, inhibitors can be discovered from vast compound libraries. Structurally diverse drug-like small molecules are keenly sought due to their simple synthesis pathways, cell penetration capabilities, and bioactivity.[95] Small molecule inhibitors have been identified as antagonists of GTs (Fig. 9).

Ethambutol (**31**), a diamine molecule identified from a screen of over 2000 compounds,[96] is a synthetic small molecule drug that targets a GT. It is an anti-mycobacterial drug prescribed as a treatment for tuberculosis. Ethambutol inhibits the enzyme arabinosyltransferase involved in the assembly of the arabinogalactan layer in the polysaccharide component of the cell wall in mycobacteria. Efforts to discover ethambutol analogs based on the core diamine structure led to the discovery of SQ109 (**32**) which inhibits cell wall biosynthesis, but it was later revealed that its molecular target is different.[97] Nonetheless, it provides an alternate scaffold for potential GT inhibitors.[98]

The search for effective antibacterial drugs has motivated high-throughput screening efforts to discover new inhibitors of GTs involved in cell wall biosynthesis. Walker and coworkers applied a novel screening strategy described in Section 3.09.2.2.3.

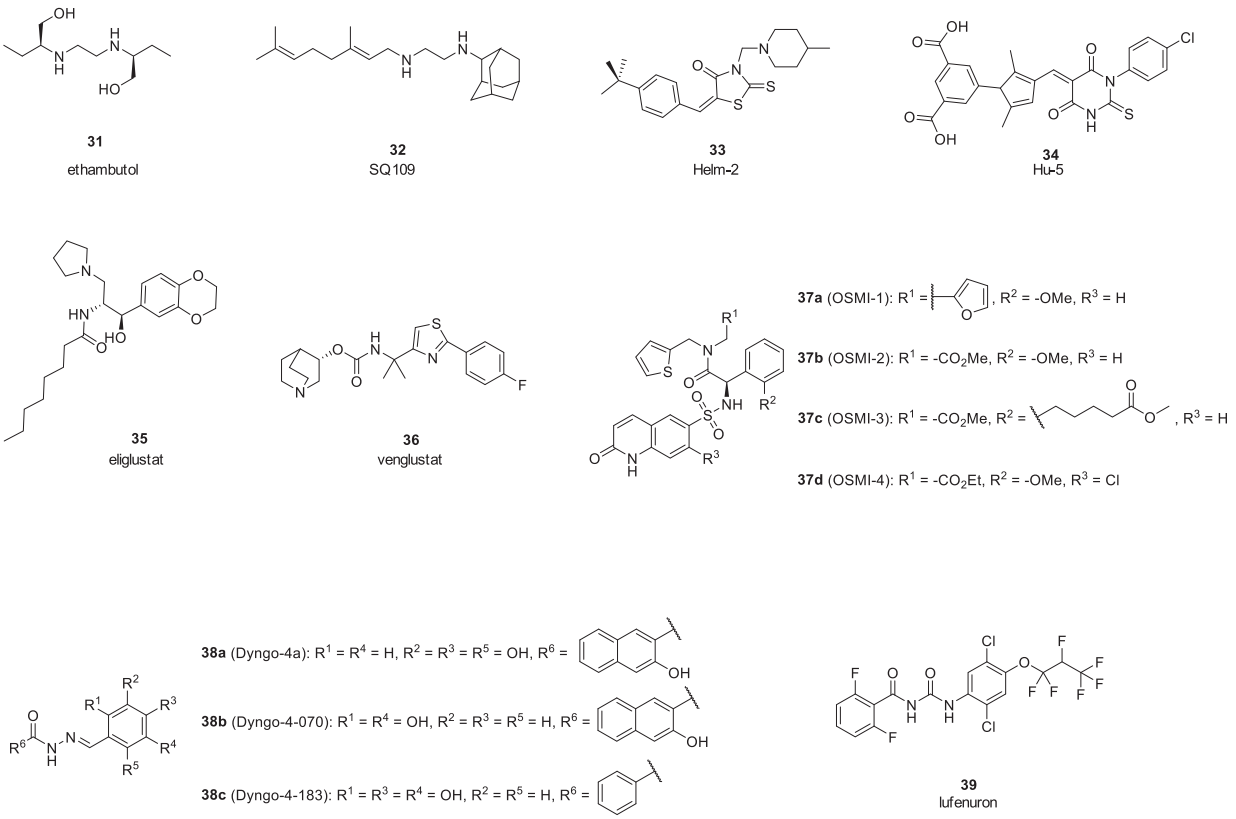

Fig. 9 Structurally diverse synthetic small molecule inhibitors of GTs.

Targeting MurG, a GT involved in the biosynthesis of bacterial peptidoglycan, a thiazolidinone-based library of 48,877 compounds was screened leading to the identification of several inhibitors with a common five membered ring core structure, represented by the molecule referred to herein as Helm-2 (**33**).[99] Using the same approach, Hu et al. identified structurally diverse compounds with inhibitory activity against MurG, with the molecule herein referred to as Hu-5 (**34**) being the most potent and selective.[100]

As exemplified by miglustat (**23**), one of the major clinical successes of synthetic GT inhibitors is in the treatment of lysosomal storage disorders, which result in the failure to break down and recycle glycosphingolipids. Miglustat targets human GCS, a GT responsible for glucosylceramide synthesis—the first step in converting sphingolipids into glycosphingolipids—to prevent glycosphingolipid accumulation. Abnormalities of GCS activity have also been linked with renal disease, Parkinson's disease, and cancer.[101–103] Many studies have focused on developing structurally diverse, small molecule GCS inhibitors. Eliglustat (**35**) is a carboxamide GCS inhibitor approved for use as a long-term treatment drug for Gaucher's disease.[104–106] Venglustat (**36**) is a carbamate GCS inhibitor that is currently under investigation as a therapeutic for disorders resulting from the accumulation of glycosphingolipids, including Gaucher's and Parkinson's diseases.[107]

Other human GT enzymes hold promise as therapeutic targets. Aberrant activity of the OGT enzyme, which is responsible for post-translational O-GlcNAc modification of several proteins, is implicated with many diseases including cancer, neurodegeneracy, cardiovascular disease, and obesity. Inhibition of its activity, the transfer of GlcNAc onto serine and threonine residues of proteins from a UDP-activated donor, has gained interest as a potential therapy following insights into the enzyme's function.[76,77] Walker and colleagues explored several strategies to discover inhibitors of OGT.[108–111] As part of these efforts (using the same approach that the group had used to identify MurG inhibitors), Ortiz-Meoz et al., reported a small molecule OGT inhibitor, OSMI-1 (**37a**), developed from a high-throughput screening hit identified from a small library of 1280 compounds. OSMI-1 is cell-permeable and inhibits protein O-GlcNAc modification in several mammalian cell lines without qualitatively altering other types of protein glycosylation.[110] Guided by crystal structures of OGT, Martin et al. refined this molecular scaffold resulting in OSMI-2 (**37b**), OSMI-3 (**37c**) and OSMI-4 (**37d**).[111] Vocadlo and coworkers also employed a high-throughput screening strategy (Section 3.09.2.2.4) to identify several OGT inhibitors, Dyngo-4a (**38a**), Dyngo-4-070 (**38b**), and Dyngo-4-183 (**38c**) from a library of 4480 compounds.[112]

Other structurally diverse synthetic small molecule GT inhibitors have found commercial applications apart from use as therapeutics for human diseases. These include use in veterinary medicine and agriculture as antifungals, antiparasitics, pesticides, and insecticides. For example, lufenuron (**39**) is a small molecule inhibitor of chitin synthase that is used to treat flea and heartworm infestations as well as fungal infections in pet animals. It is also used in agriculture to control pests and fungal plant pathogens due to its broad specificity, targeting chitin synthases in a variety of organisms.[113]

Despite thorough investigation, only a handful of GT inhibitors have made it to market, including the examples miglustat (**23**), eliglustat (**35**), vancomycin (**27**), caspofungin (**29**), nikkomycin Z (**30**), and ethambutol (**31**) as clinically approved drugs; and moenomycin A (**26**) and lufenuron (**39**) have found use in agriculture, animal husbandry, and veterinary medicine. Given the varied structures of the GT inhibitors with drug-like properties that are on the market, under trials, or used as chemical and biological tools, the need for efficient strategies to screen diverse small molecule libraries has motivated the development of several high-throughput assay techniques, as discussed in the following sections.

3.09.2.2 High-throughput screening strategies to identify GT inhibitors

Classically, GTs have been assayed by radiochemical, HPLC-based, and immunoprecipitation-based assays. However, these formats are time consuming and challenging to implement in high-throughput screens. Additionally, designing the reagents needed for these assays can become a challenging task. Here, we discuss more recently developed GT assays that have been applied in high throughput screens to identify GT inhibitors.

3.09.2.2.1 Coupled enzyme assays that measure GT activity by nucleotide release

In most cases, glycoside bond formation catalyzed by a GT does not generate a conveniently measurable optical readout like a change in absorbance or fluorescence. This has led to the development of coupled enzyme assays to detect Leloir GT-catalyzed reactions by coupling the release of a nucleotide to downstream enzyme-catalyzed conversions that can be measured optically (Fig. 10). Fitzgerald et al.[114] devised a continuous coupled spectrophotometric assay to detect β4Gal-T1 activity. Transfer of Gal from UDP-Gal onto Glc forming lactose results in release of UDP, which was coupled to oxidation of NADH through enzyme reactions involving pyruvate kinase and lactate dehydrogenase. Pyruvate kinase catalyzes the transfer of a phosphate from phosphoenolpyruvate to UDP generating UTP and releasing pyruvate. Lactate dehydrogenase catalyzes reduction of pyruvate to form lactate with the concomitant oxidation of NADH to form NAD^+. The rate of decrease in absorbance at 340 nm due to NADH oxidation is directly proportional to the amount of galactosyltransferase activity. Palcic and colleagues extended this assay to mammalian glycosyltransferases that use different nucleotide donors. Pyruvate kinase can also utilize GDP, CDP and ADP, and it was found that the coupled enzyme assay could be applied to quantify fucosyltransferase and *N*-acetylglucosaminyltransferase activity, which release GDP and UDP, respectively. In order to assay sialyltransferases that use CMP-Neu5Ac acid and release CMP, an additional reaction is required to use this approach. Coupling of CMP release to nucleoside monophosphate kinase catalyzes the transfer of a phosphate from ATP to CMP, forming ADP and CDP, both of which are consumed in downstream reactions resulting in NADH oxidation. These coupled assays have been used for GTs from animals, plants, and bacteria. For example, Lowary, Daniellou, Mikušová, and colleagues used this approach to study mycobacterial cell wall-synthesizing GTs and characterize synthetic

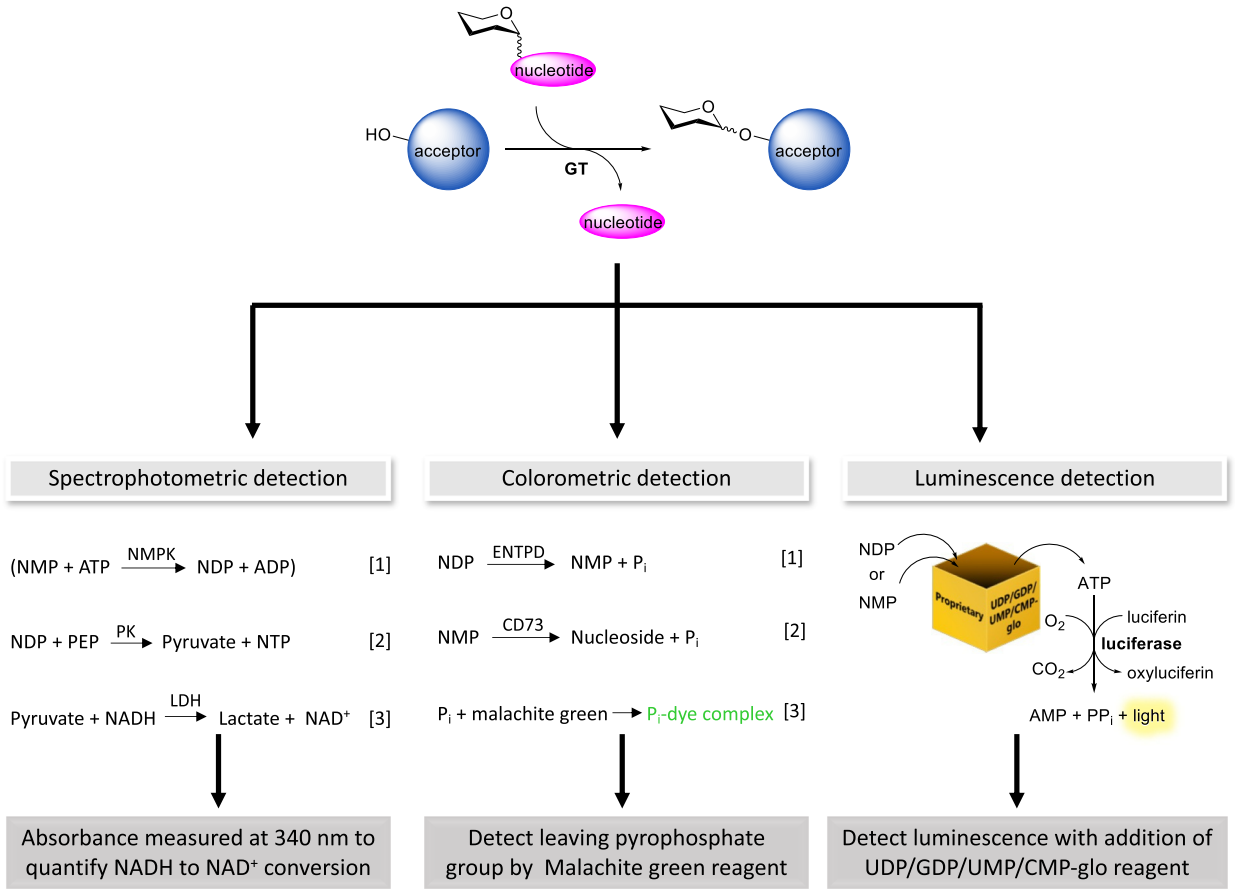

Fig. 10 Coupled enzyme assays to measure GT activity by detecting nucleotide release.

inhibitors.[45,115] Wong and coworkers have applied this assay to fucosyltransferases to identify inhibitors from a screen of a combinatorial compound library.[47]

Alternative coupled enzyme approaches have been developed to detect nucleotide release from Leloir GT-catalyzed reactions. R&D Systems Incorporated devised the Malachite Green Phosphate Detection kit as part of a universal colorimetric assay for Leloir GTs, which quantifies the release of inorganic phosphate when nucleoside mono- and di-phosphates are cleaved using specific phosphatases.[116] The β-phosphate of a nucleoside diphosphate, such as UDP or GDP, is cleaved by an ectonucleoside triphosphate diphosphohydrolase, such as CD39L3 or ENTPD3, whereas the α-phosphate of a nucleoside monophosphate, such as CMP, is cleaved by a 5′-nucleotidase, such as CD73. The inorganic phosphate released reacts with malachite green, which produces a characteristic color that is detected by absorbance at 620 nm. This assay has been used in high-throughput screen, as demonstrated by Wagner and colleagues.[117]

Promega Corporation have developed a universal assay to detect nucleotide release from Leloir GTs.[118,119] This uses a one-step detection technique that measures activity by quantifying the amount of UDP, GDP, UMP, or CMP released during the reaction. After the GT reaction is complete, the samples are incubated with a proprietary reagent (as part of kits marketed as UDP-Glo, GDP-Glo, and UMP/CMP-Glo) that uses the nucleoside mono- or di-phosphate to generate ATP, which is then consumed in a luciferase-catalyzed reaction, producing light. The assay has been used in high-throughput screens for bacterial GT inhibitors.[120]

While the assay kits that detect GT activity through nucleotide release by coupled enzyme reactions can be highly sensitive, a key drawback is that the transfer of a sugar onto an acceptor molecule is not observed, and these assays are sensitive to donor nucleotide-sugar hydrolysis or contaminating nucleotides or phosphate,[121] leading to false positives or interference with the assay. Furthermore, these strategies cannot be applied for the assay of non-Leloir GTs, which do not release nucleotides and this has motivated the development of alternatives.

3.09.2.2.2 Carbohydrate microarray-based GT assays

Wong and colleagues developed a strategy to assay GT activity and inhibition using carbohydrate microarrays (Fig. 11). Eleven azide-conjugated oligosaccharides were displayed on microtiter plates by attaching them to the surfaces of alkyne-functionalized wells using copper(I)-catalyzed azide-alkyne cycloaddition.[122] Subsequently, α1,3-fucosyltransferase and GDP-fucose was added to

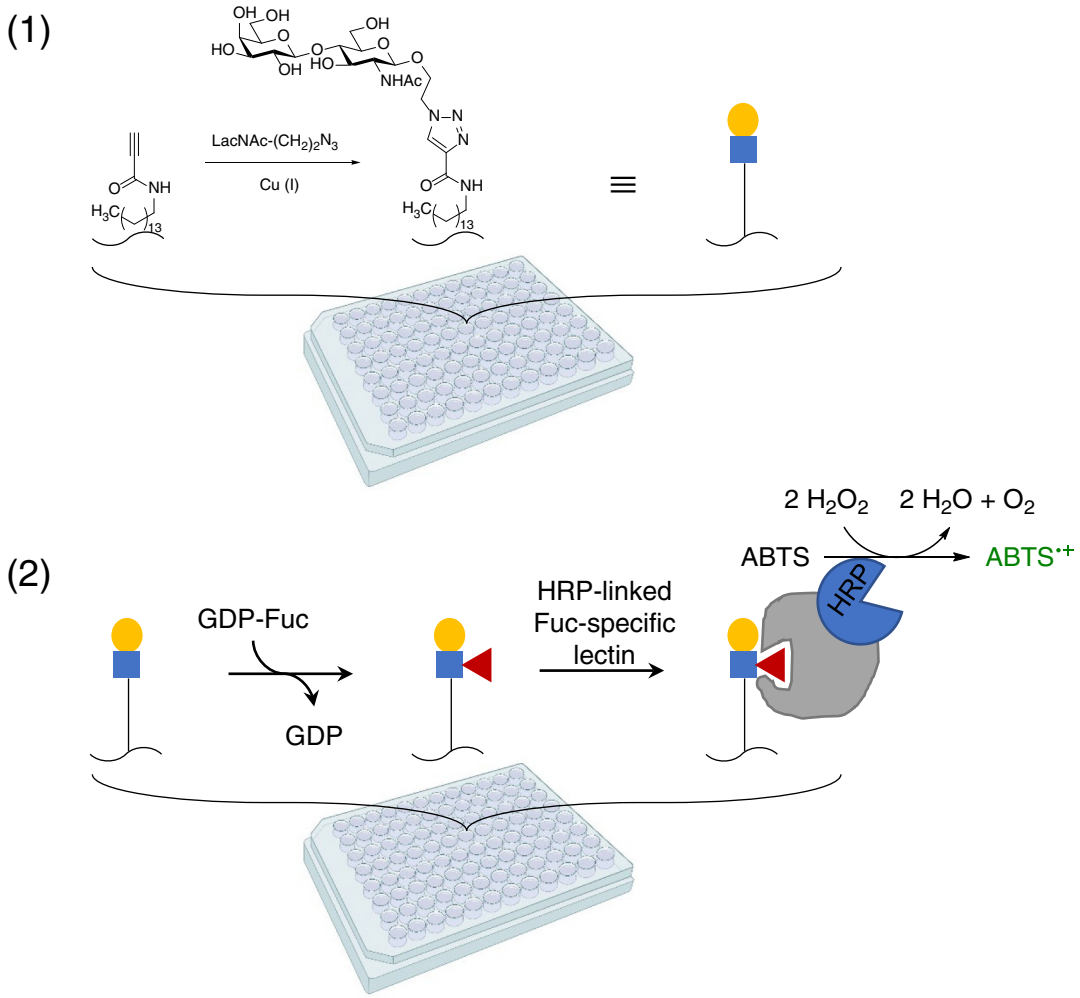

Fig. 11 Carbohydrate microarray-based GT activity and inhibition assay. (1) Azide modified acceptor oligosaccharide glycoconjugates are immobilized onto plates coated with an alkyne linked to a long lipid chain. (2) GT-catalyzed transfer of a sugar onto immobilized glycans is detected by a lectin linked to horseradish peroxidase (HRP) in an enzyme-linked lectin assay (ELLA) that results in a color change from the HRP-catalyzed oxidation of ABTS.

examine these oligosaccharides as acceptor substrates and study the acceptor substrate specificity of the enzyme. An enzyme-linked lectin assay (ELLA) was used to detection the GT product, by binding of a fucose-specific lectin (from *Tetragonolobus purpureas*) that is conjugated to a peroxidase, which catalyzes oxidation of ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) to produce a color change in the well if fucose was successfully transferred. Bryan et al. applied this strategy in a high-throughput screen to identify inhibitors of human α1,3-fucosyltransferase from library comprising 85 GDP-triazole compounds.[48] It was noted that inhibitor screening using the carbohydrate microarray-based assay was 70% cheaper than the coupled assay using pyruvate kinase and lactate dehydrogenase to detect nucleotide release, which they had previously applied.[47,48] GT activity can also be detected with a fluorophore-linked lectin rather than an enzyme-linked lectin,[122] and variations of this approach have been reported to detect the activity of other GTs (e.g., the GT in *Neisseria meningitidis* that synthesizes the pathogen's anionic cell wall polysaccharide), using GFP-tagged recombinant glycan-binding proteins.[123]

3.09.2.2.3 Fluorescence polarization-based GT assays

The principles of fluorescence polarization (FP) have been widely implemented to develop assays for screening compound libraries (Fig. 12). The general concept of FP is that when a fluorophore is attached to a small molecule and excited by plane-polarized light, a change in polarization occurs between the bound and unbound states of the molecule. If the fluorophore is immobilized by binding to an enzyme or large macromolecule, the emitted light will retain its polarization, because the orientation of the slowly-rotating, large complex has not changed sufficiently during the fluorescence lifetime (i.e., the short time between excitation and emission), whereas if the fluorophore is free it rapidly tumbles in solution within the span of its fluorescence lifetime, resulting in emission of light in different planes from that of the initial excitation and loss of polarization.[124,125]

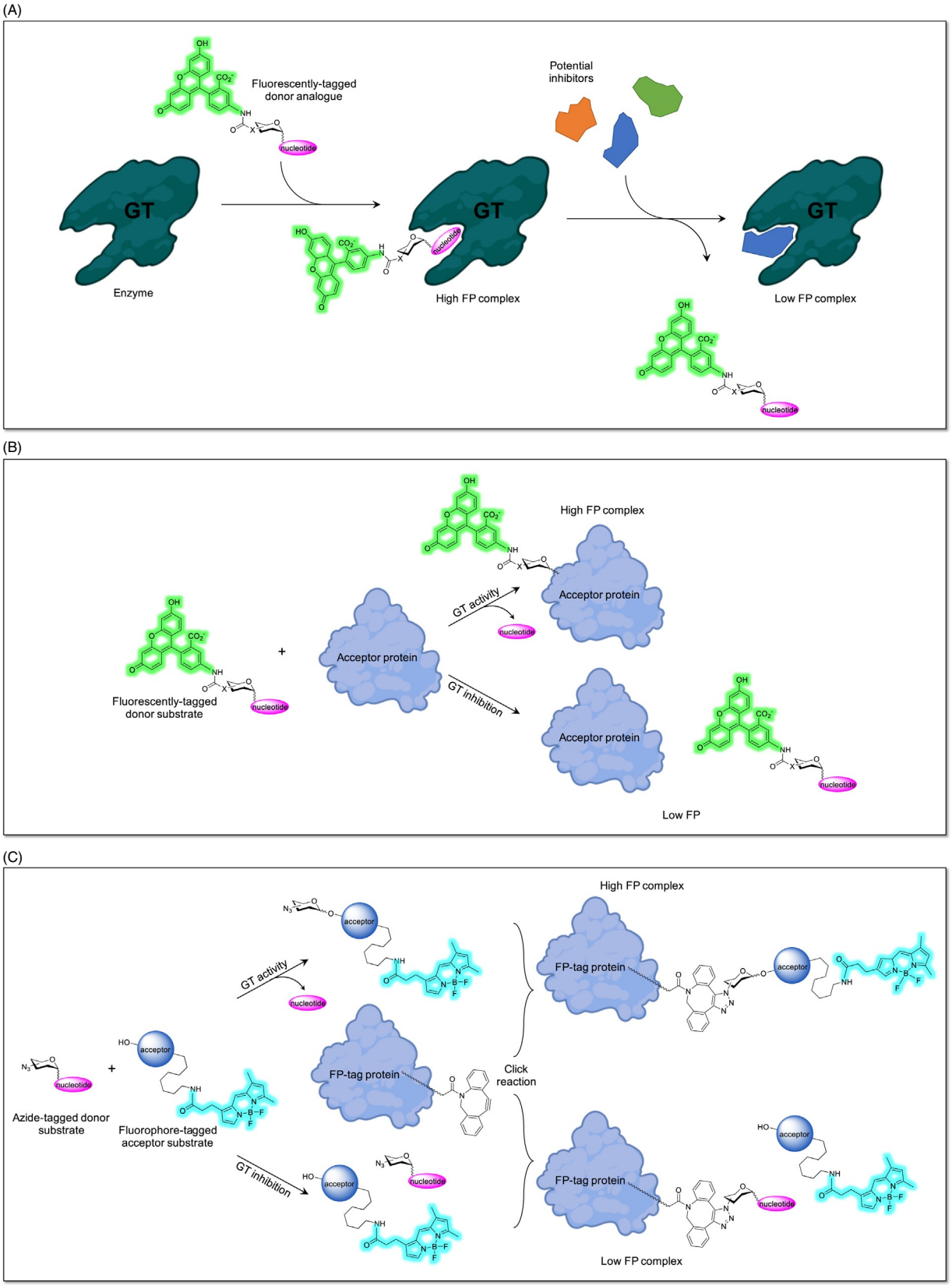

Fig. 12 Fluorescence polarization (FP)-based assay. (A) Fluorophore displacement assay, (B) catalytic FP assay, and (C) FP-tag-based assay.

The Walker laboratory pioneered an FP-based GT assay to screen for inhibitors (Fig. 12A). A fluorophore-labeled substrate analog was used in a ligand displacement assay to study the activity of nucleotide glycosyltransferase MurG involved in bacterial peptidoglycan biosynthesis.[99] A fluorescein tag was attached to the substrate, UDP-GlcNAc, to give UDP-GlcN(5-fluorescein). When the UDP-GlcN(5-fluorescein) binds to the enzyme, FP increases due to the decrease in molecular rotation. The dissociation constant was calculated by recording the change in FP as a function of MurG concentration. Applying the principles of competitive binding, an inhibitor displaces the fluorophore-tagged ligand from its binding site resulting in a decrease in fluorescence anisotropy. This assay was used to screen a library of 48,877 compounds for inhibition of MurG. The group established a similar assay to screen inhibitors for OGT.[108,110] However, upon binding of UDP-GlcN(5-fluorescein) to OGT, no significant change in fluorescence was observed, and this required the design and synthesis of two new probes with a longer linker between the fluorophore and UDP-GlcNAc to increase binding efficiency. Fluorescein-tagged donor analogs have allowed for rapid screening of inhibitors for GTs but pose a challenge of the large size of the fluorophore impeding binding of the molecules to the target enzyme. To address this issue, Pesnot et al. developed auto-fluorescent derivatives by modifying the uracil base of the donor, UDP-Glc, and these derivatives were used in a ligand displacement assay to screen for inhibitors of GTs.[126]

Paulson and colleagues devised a strategy to screen for GT activity and inhibition using an FP-based assay wherein the change in FP is measured when a fluorophore-tagged sugar is transferred from the nucleotide-activated donor to a glycoprotein (Fig. 12B). Modified analogs of CMP-NeuAc and GDP-Fuc were made by tagging them with fluorescein isothiocyanate (FITC), and the derivatives were used as substrates for the enzymes sialyltransferase and fucosyltransferase, respectively. The FITC-tagged sugars were transferred to asialo-fetuin or fetuin by sialyltransferases or fucosyltransferases, respectively, generating a high FP signal. Inhibitors were identified by a lowering of FP anisotropy.[127]

A variation of the catalytic FP assay was developed by Withers and coworkers to increase the adaptability in screening different GTs (Fig. 12C). The intensity of FP depends on the molecular size of the ligand and the degrees of freedom of the fluorophore. Larger molecules generate higher FP due to low tumbling and high stability. This principle was applied for an "FP-tag" assay of the GT KpsC, which is responsible for bacterial capsular polysaccharide synthesis. The acceptor—a β2,7-linked disaccharide consisting of two ketodeoxyoctonic acid (Kdo) units—was tagged with a fluorophore (BODIPY).[128] The sugar of the donor substrate, CMP-Kdo, was modified with an azide at the 8-position, which could be linked to a cycloalkyne-modified biotin via strain-promoted azide-alkyne cycloaddition (SPAAC), and subsequently bound to streptavidin. Upon KpsC-catalyzed transfer of $8N_3$-Kdo from its CMP-activated donor onto the BODIPY-labeled, β2,7-linked di-Kdo acceptor, the oligosaccharide product is tagged by SPAAC with biotin, and the product bearing both a BODIPY label and biotin binds to streptavidin resulting in a large, fluorescently labeled complex with high FP. The addition of an inhibitor in the GT reaction lowers the FP in the assay, which was used to conduct a high throughput screen of 1000 natural product extracts from marine sponges, from which two inhibitors were identified. Gao and coworkers improved this strategy, producing a more economical version of the "FP-tag" assay by using bovine serum albumin (BSA) functionalized with cycloalkyne groups. The GT product, a glycoside bearing an azide modification from the donor sugar and a fluorescent tag from the acceptor, was conjugated to the modified BSA. This produces a large, fluorescently labeled complex exhibiting high FP, without the need for a biotin linker or the use of streptavidin, which is more expensive than BSA. Yin et al. used this advanced strategy to screen a library of 260 compounds for inhibitors of OGT and identified one hit. The authors noted a 1000-fold cost advantage compared to the original method.[129]

3.09.2.2.4 A direct fluorescent assay for GT activity using fluorophore-tagged sugar donors

A direct fluorescence assay was designed by Vocadlo and coworkers to overcome the complexity of FP-based assays that indirectly measure fluorescence (Fig. 13). OGT catalyzed transfer of a fluorophore-labeled sugar, UDP-GlcN-BODIPY, to a biotinylated peptide acceptor derived from casein kinase II (CKII). The OGT-modified glycopeptide and unmodified peptide are captured using streptavidin-coated microplate wells or beads, followed by washing steps and finally the measurement of fluorescent signal. A library of 4480 bioactive compounds were screened using this direct fluorescent assay and several OGT inhibitors were identified. This technique could be readily applied to other classes of GTs as well as non-Leloir GTs, which cannot be assayed with coupled enzyme reactions that detect nucleotide diphosphate reaction products. Additionally, this approach can detect weak binders, and the authors note that screens can be performed with low concentrations of enzyme, donor, and acceptor making high-throughput screening possible with limited reagents.

One major limitation of using fluorescently labeled donor substrate analogs in FP-based assays or in a direct fluorescent assay format is the requirement for modifications to the donor that must be accommodated by the GT. In screens using such assays, inhibitors are identified for the GT-catalyzed transfer of a derivative of the natural sugar donor, which may not be possible for all GTs.

3.09.2.2.5 A glycosidase-dependent fluorescent coupled GT assay

Our research group has developed a very sensitive high-throughput GT screening assay in which a change in fluorescent signal results from the transfer of an unmodified sugar from its natural donor substrate onto a fluorogenic acceptor substrate (Fig. 14). The strategy relies upon the ability of certain glycosidases to distinguish the glycoside acceptor substrates from the glycosylated products of GT-catalyzed reactions, cleaving the former but not the latter. In this approach, the substrate is used in a sequential coupled assay, which involves the GT-catalyzed reaction and a subsequent glycosidase-catalyzed reaction. GT-catalyzed transfer of a sugar onto the glycoside probe prevents its cleavage by glycosidases in the subsequent hydrolytic reaction, which would otherwise result in the release of a fluorescent reporter molecule. GT activity is inversely related to the fluorescence released in the coupled assay (Fig. 14A).

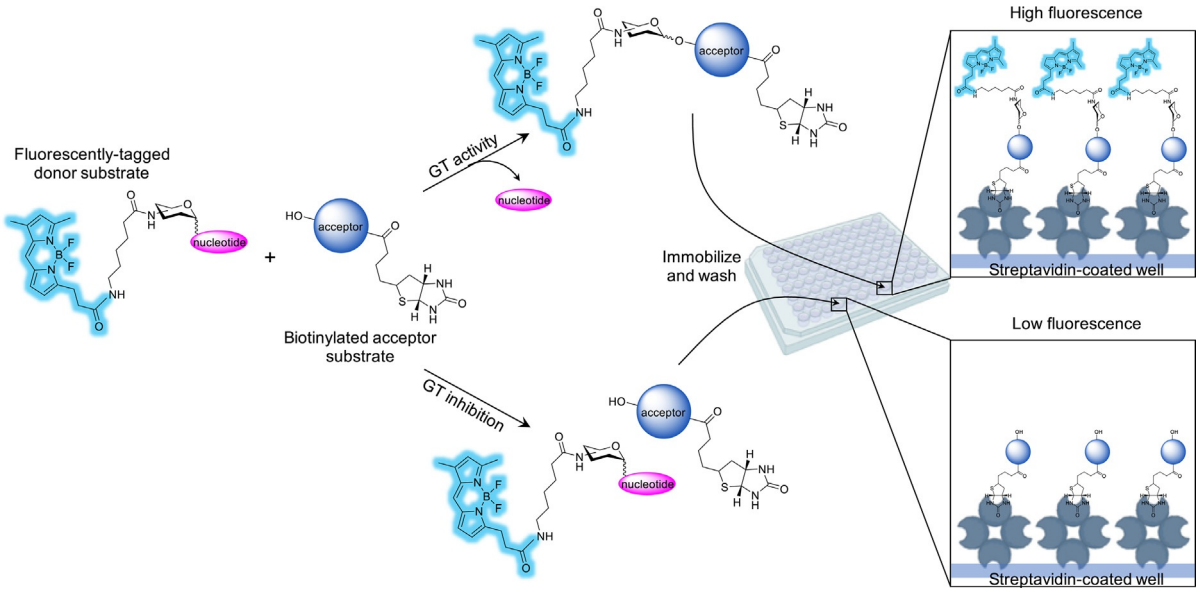

Fig. 13 Direct fluorescent assay to screen GT inhibitors. The transfer of sugar from UDP-GlcN-BODIPY to biotinylated peptide acceptor by OGT can be measured by measuring the fluorescence of the captured modified peptide onto streptavidin coated beads or micro titer well plates.

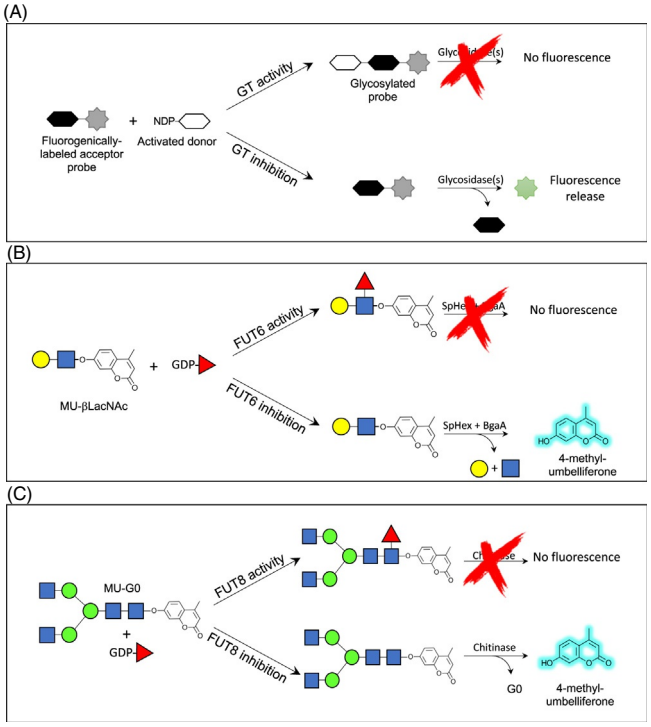

Fig. 14 Glycosidase-dependent fluorescent coupled GT assay. GT catalyzed transfer of sugar onto fluorophore labeled acceptor substrate prevents subsequent glycosidase reaction preventing probe digestion and thus, generating no fluorescence. In presence of an inhibitor, the reporter probe can be released, thus reconstituting fluorescent signal. (A) Generalized scheme for the assay strategy. (B) Activity and inhibition assay of FUT6: hydrolytic glycosidases BgaA and SpHex can act together to digest MU-βLacNAc and release fluorescent 4-methylumbelliferone, but not if it is first fucosylated by FUT6. (C) Activity and inhibition assay of FUT8: in presence of an inhibitor, chitinase acts on the non-fucosylated MU-G0 releasing 4-methylumbelliferone. Red triangle = fucose.

We used this approach to assay the activity and inhibition of human fucosyltransferase VI (FUT6), an α1,3-fucosyltransferase (Fig. 14B).[130] The labeled disaccharide, 4-methylumbelliferyl β-*N*-acetyllactosamine (MU-βLacNAc), was used as a fluorogenic probe. Hydrolytic glycosidases BgaA and SpHex act together to digest MU-βLacNAc and release fluorescent 4-methylumbelliferone, but not if it is fucosylated by FUT6. While FUT6 activity blocks the release of the fluorescent reporter in this assay, inhibitors of FUT6

maintain the fluorescent signal. Using this assay, we screened a small collection of molecules derived from a previously reported FUT6 inhibitor and quantified their potency.

This approach can be generalized to other GTs, provided a fluorogenically labeled acceptor substrate can act as a probe and that a glycosidase (or glycosidases) can be identified that will cleave the substrate, but not cleave the product of glycosyl transfer. Soroko and Kwan demonstrated this approach with the GT fucosyltransferase VIII (FUT8), which is responsible for core fucosylation of protein *N*-glycans (Fig. 14C).[131] The natural acceptor substrate for FUT8 is a heptasaccharide attached to glycoproteins referred to as G0, to which the enzyme transfers a fucose from GDP-Fuc. Using the 4-methylumbelliferyl glycoside of G0 as a substrate (MU-G0) and a chitinase that digests the substrate to release the fluorophore, but does not act on the fucosylated product, we assayed the activity and inhibition of FUT8.

The advantage of the coupled strategy is that it allows for the use of the native donor substrate of the GT, and very sensitive assays can be performed with minimal amounts of the enzyme and the donor and acceptor substrates, enabling screening of very large libraries with very little material. Furthermore, it is specific in that the fluorescence signal depends upon transfer of a sugar unit from the donor to the acceptor, allowing for measurement with little background interference even in complex biological samples.

3.09.3 GT activity engineering

GTs are increasingly used as biocatalytic tools for the synthesis of valuable and diverse glycosylated compounds. These include oligosaccharides and glycans found in nature as well as synthetic analogs.[132] Robust recombinant expression of GTs allows for their use as biocatalysts in multi-step reactions to generate products on milligram to multi-gram scales. Yet, many wild-type GTs do not have optimal characteristics for synthetic processes, with limitations in terms of activity, stability, heterologous expression yield, substrate flexibility, regioselectivity, and unwanted side-reactions.[133] It is highly desirable to optimize GTs to enhance these specific properties to produce desired glycosides. In principle this can be achieved by enzyme engineering approaches, such as rational design and directed evolution.

3.09.3.1 Modifying GT activity using rational protein design

A better understanding of GT mechanism and a greater depth of structural information have paved the way for engineering these enzymes through sequence- and structure-guided rational design strategies. These include strategies involving precise, site-specific mutations and the exchange of domains between closely related GTs to construct GT chimeras.[133]

3.09.3.1.1 Targeted mutagenesis of GTs

The function of GTs can be enhanced or modified by designing mutants at sites identified based on mechanistic insights into catalytic residues and other important amino acid positions. This may be guided by information available from sequence similarity and phylogeny or from 3D protein structures. In this section, we highlight a few examples of engineered GTs obtained by targeted mutagenesis, focusing primarily on enzymes that assemble glycans found on mammalian cell surfaces, which are involved in a broad range of biological interactions and are the most common targets of synthesis.[132] Prominent cases include the human blood group A antigen-synthesizing α1,3-*N*-acetylgalactosaminyltransferase from the GT6 family, mammalian β1,4-galactosyltransferase I (β4Gal-T1) from the GT7 family, and bacterial sialyltransferases (STs) from the GT80 family.

Human A-specific α1,3-*N*-acetylgalactosaminyltransferase (GTA) and B-specific α1,3-galactosyltransferase (GTB) are retaining GTs that catalyze the addition of the terminal sugar to give the blood group A and B antigens, respectively. Both enzymes use the same site on the same acceptor, the 3-hydroxyl of the galactose residue of Fucα1,2-Galβ-R, the epitope of the H-antigen found on blood group O cells. GTA transfers *N*-acetylgalactosamine (GalNAc) from UDP-GalNAc, while GTB transfers galactose (Gal) from UDP-Gal. The sequence homology between human GTA and GTB is high and they differ at only four amino acid residues (Arg/Gly-176, Gly/Ser-235, Leu/Met-266, and Gly/Ala-268).[134,135] In 1997, Narang and coworkers carried out one of the earliest studies to engineer the activity of a GT, systematically mutating GTA to obtain blood group B antigen-synthesizing variants with α1,3-galactosyltransferase activity. They observed that the donor specificity was unaltered in the R176G single point mutant of GTA, but there was an 11-fold increase in the k_{cat} value compared to the wild-type GTA. The triple mutant R176G/G235S/L266M showed the ability to use UDP-GalNAc and UDP-Gal sugar donors, highlighting its significance as a functional blood group A/B antigen-synthesizing hybrid GT.[136]

Bovine β4Gal-T1 is readily available from cow's milk, and is the most widely used GT in biocatalysis.[132] The enzyme catalyzes the transfer of a Gal moiety from UDP-Gal to GlcNAc-terminated acceptor substrates (through an inverting mechanism) to generate Galβ1,4-GlcNAc-R (*N*-acetyllactosamine; LacNAc) structures found on mammalian cell surfaces.[137] β4Gal-T1 is also involved in the synthesis of various other galactosides including lactose, which is stimulated in the mammary gland by allosteric regulation of the enzyme upon binding of α-lactalbumin, a mammary gland-specific calcium binding protein.[138,139] β4Gal-T1 also possesses a low *N*-acetylgalactosaminyltransferase activity, using a UDP-GalNAc donor and a GlcNAc acceptor. Based on structural observations of bovine β4Gal-T1 bound with UDP-GalNAc, three single point mutants Y289L, Y289N, and Y289I were designed and generated by site-directed mutagenesis to disrupt the hydrogen bond between the *N*-acetyl group of GalNAc and the Tyr-289 residue of β4Gal-T1, which hampers the *N*-acetylgalactosaminyltransferase activity. These three mutations resulted in improved *N*-acetylgalactosaminyltransferase activity, without affecting its galactosyltransferase activity. The Y289L mutant gave the highest

N-acetylgalactosaminyltransferase activity, which was almost the same as the galactosyltransferase activity of the mutant. Furthermore, the mutants displayed *N*-acetylglucosaminyltransferase activity (using UDP-GlcNAc as a donor) that was not observed for the wild-type β4Gal-T1.[140]

Sialic acid-containing glycans mediate a multitude of biological interactions, making them attractive synthetic targets. Many of the challenges that hamper their synthesis by conventional approaches can be circumvented through enzymatic biocatalysis.[141] STs catalyze the enzymatic synthesis of sialosides by transferring a sialic acid moiety, most commonly *N*-acetylneuraminic acid (Neu5Ac), from CMP-sialic acid (CMP-Neu5Ac) to an acceptor.[142] Family GT80 is comprised solely of bacterial GTs and these are the useful catalysts for the enzymatic and chemoenzymatic production of α2,3- and α2,6-sialosides (such as those in glycoprotein biologics) because of their high expression yields in *E. coli*, high rates of catalysis, and broad substrate flexibility.[143,144] *Pasteurella multocida* α2,3-sialyltransferase 1 (PmST1) is a multifunctional enzyme which, apart from its main catalytic activity as an inverting α2,3-sialyltransferase, is also a weak α2,6-sialyltransferase, an α2,3-sialidase, and an α2,3-transsialidase.[143] The α2,6-sialyltransferase and α2,3-transsialidase activity of PmST1 can be minimized by adjusting reaction conditions. However, extreme care needs to be taken with respect to reaction conditions to prevent its activity as an α2,3-sialidase, which results in hydrolysis of the product thereby reducing yields. Yet, these conditions are difficult to control during glycoprotein sialylation. Using 3D crystal structures of PmST1 (in complex with an acceptor substrate and donor analog) and knowledge of the α2,6-sialyltransferase and α2,3-sialidase mechanisms, Chen and coworkers applied site-directed mutagenesis to engineer mutants with low α2,3-sialidase catalytic activity.[142] Two single point mutants E271F, R313Y, and one double mutant, E271F/R313Y, were constructed to enhance the hydrophobicity of the substrate binding site, thus making it less accessible to water molecules without interfering with the ST acceptor binding. In comparison to the wild-type PmST1, the double mutant E271F/R313Y exhibited a dramatic decrease (6333-fold) in unwanted α2,3-sialidase activity without affecting the desired α2,3-sialyltransferase activity.[142]

Detailed mechanistic investigation of the sialidase and transsialidase activities of GT80 STs showed that they derive from the tendency of enzymes in this family to catalyze hydrolysis of CMP-Neu5Ac.[145] PmST1 exhibits donor substrate hydrolysis activity when a poor acceptor is used, resulting in a low sialylation yield. For example, Lewis^X is a poor acceptor, so competitive hydrolysis of CMP-Neu5Ac by PmST1 results in a low yield of sialyl Lewis^X product. Chen and colleagues analyzed the 3D crystal structure of PmST1 and designed mutants to reduce donor hydrolysis activity.[146] A single point mutant was generated, PmST1-M144D, in which a charged aspartate replaced the hydrophobic methionine at a position near the catalytically important Asp-141. Compared to the wild-type, PmST1-M144D had 20-fold decreased rate of CMP-Neu5Ac donor hydrolysis, 5500-fold reduced rate of α2,3-sialidase activity, yet retained full α2,3-sialyltransferase activity in the presence of a poor acceptor such as Lewis^X. PmST1-M144D is a useful mutant for the synthesis of a variety of different sialyl Lewis^X-containing glycans.[146]

The GT80 α2,3-sialyltransferase from *Pasteurella dagmatis* (PdST) has a strong preference for α2,3-sialylation and produces trace amounts of α2,6-sialylated products. Nidetzky and coworkers found that substituting Pro-7 of PdST with a histidine favored the α2,6-sialylated product, yet retained some α2,3-sialylation activity. A complete switch of PdST regioselectivity from α2,3 to α2,6 was achieved with the double mutant P7H/M117A, highlighting the importance of structure-based active-site engineering.[147]

Site-directed mutagenesis has been used to alter the sugar donor specificity of *Photobacterium* sp. α2,6-sialyltransferase. Gilbert and coworkers engineered the A235M mutant to transfer the sialic acid analog legionaminic acid (Leg5Ac7Ac) instead of Neu5Ac.[148] In comparison to the wild-type enzyme, the mutant showed significant increase in α2,6 transferase activity with CMP-Leg5Ac7Ac, thus making this mutant a useful tool for synthesizing Leg5Ac7Ac glycoconjugates. Remarkably, the catalytic efficiency of the mutant was 10-fold greater than wild-type using CMP-Neu5Ac as donor, emphasizing the importance of Met-235 at this position. However, hydrolysis of both CMP-Neu5Ac and CMP-Leg5Ac7Ac by the A235M variant was faster than for the wild-type.[148] Chen and coworkers reported that the A366G mutant of *Photobacterium* sp. α2,6-ST has a $>$ 2-fold increase in soluble protein expression level, and an enhanced ability to transfer CMP-Neu5Ac to α-GalNAc containing acceptors, enabling the synthesis of sialyl-Tn antigens. The Ala-366 residue is located near the acceptor binding pocket and was mutated to a small glycine residue to create more room to accommodate these acceptors.[149]

3.09.3.1.2 Domain swapping to generate GT chimeras

Domain swapping is another design approach that has been mostly applied for engineering natural product-tailoring GTs to produce modified natural products with different sugars and aglycones (Fig. 15). Success with this approach relies heavily on high sequence identity between parental genes and the selected crossover point.[133] The natural product-modifying GTs possess the GT-B fold, in which the acceptor and sugar donor substrates primarily interact with residues in the N- and C-terminal domains, respectively.[2] The distinct domains for donor and acceptor substrate interaction facilitates mix-and-match of GT domains to provide functional chimeras (Fig. 15A).

An important example of domain swapping in GTs involved those responsible for the biosynthesis of the vancomycin group of glycopeptide antibiotics, including chloroemomycin, teicoplanin, and vancomycin itself. Due to their ability to treat infections caused by multi-drug resistant pathogens, the vancomycin-type antibiotics are considered "drugs of last resort." Yet, the emergence of dangerous pathogens resistant even to vancomycin has highlighted the need to develop strategies to generate novel derivatives. One such strategy, called "glycodiversification" uses GTs to modify natural product scaffolds with alternative sugar groups, but the stringent substrate specificity of many GTs hampers their use in glycodiversification. To address this challenge, Spencer and colleagues used a structure-guided approach to engineer chimeras from GTs encoded in the chloroemomycin and teicoplanin biosynthetic gene clusters from *Amycolatopsis orientalis* and *Actinoplanes teichomyceticus*, respectively.[150] GtfB from the chloroemomycin pathway[151] and Orf10* from the teicoplanin pathway[152] are sequence homologous with 70% identity. They transfer either

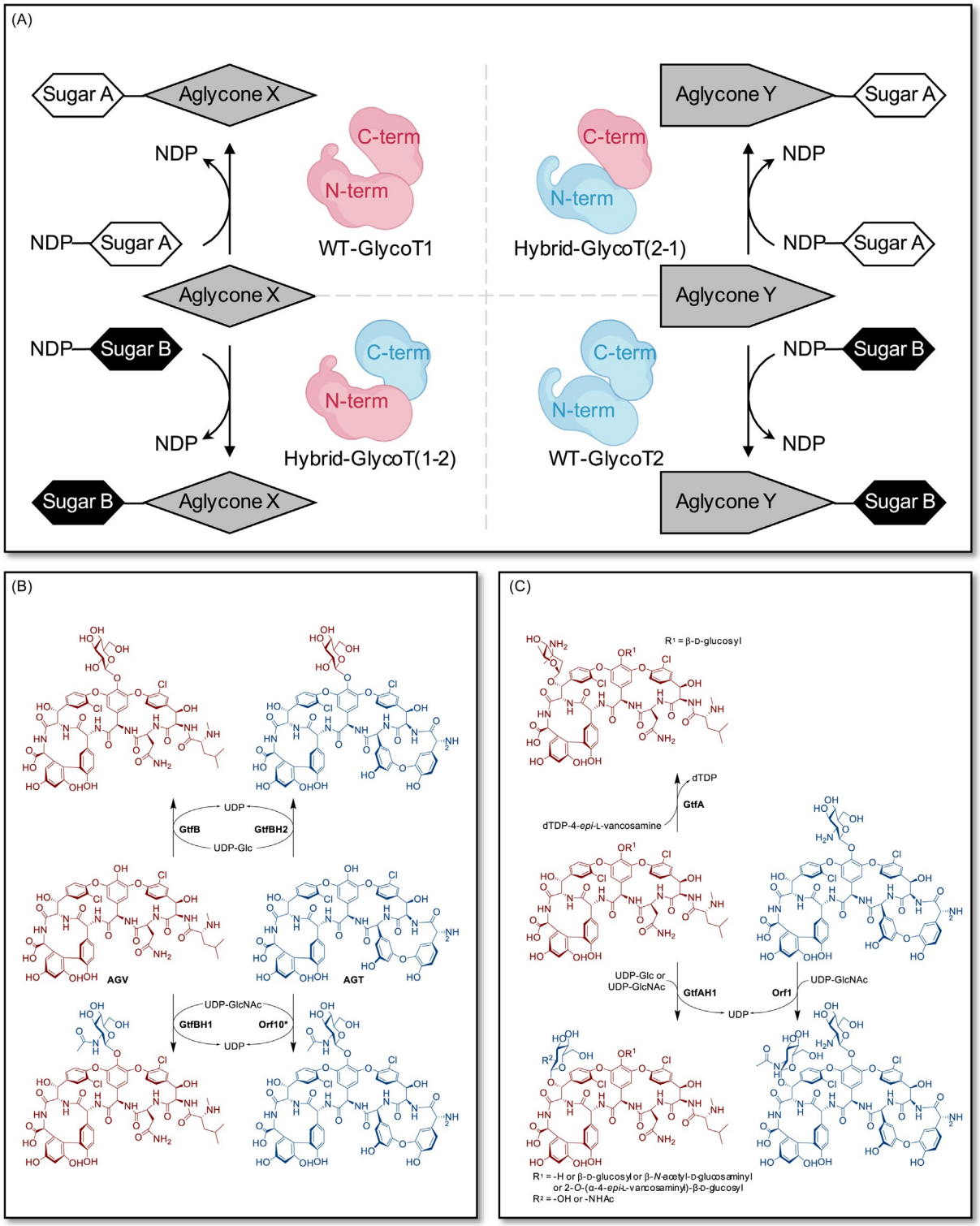

Fig. 15 Domain swapping of GTs. (A) Generalized strategy for GT domain swapping, combining acceptor-binding N-terminal, and donor-binding C-terminal domains from different GTs. (B) Chimeras produced from GtfB from the chloroeremomycin pathway and Orf10* from the teicoplanin pathway. (C) A chimera produced from GtfA from the chloroeremomycin pathway and Orf1 from the teicoplanin pathway.

glucose (in the case of GtfB) or *N*-acetylglucosamine (in the case of Orf10*) from UDP-donors onto the 4-hydroxyphenylglycine residue of closely related macrocyclic peptide acceptor substrates from their respective pathways: aglyco-vancosamine (AGV) in the case of GtfB, and aglyco-teicoplanin (AGT) in the case of Orf10*. By exchanging the N- and C-terminal domains of these enzymes, two hybrid GTs were generated with swapped acceptor and donor specificities relative to their parents: GtfBH1 transferring

N-acetylglucosamine from UDP-GlcNAc onto AGV, and GtfBH2 transferring glucose from UDP-Glc onto AGT (Fig. 15B).[150] These authors also generated a chimera from the GtfA enzyme from the chloroemomycin pathway[153] and Orf1 from the teicoplanin pathway,[152] two GTs that share 66% sequence identity. These enzymes glycosylate the 3-chloro-β-hydroxytyrosine residue of their respective macrocyclic peptide acceptor substrates: desvancosaminyl vancomycin (DVV) for GtfA, and teicoplanin glucosaminyl-pseudoaglycone for Orf1; with GtfA transferring L-β-4-epi-vancosamine and Orf1 transferring *N*-acetylglucosamine from dTDP- and UDP-donors, respectively. The GtfAH1 chimera, a hybrid GT consisting of the GtfA N-terminal domain and Orf1 C-terminal domain, can transfer *N*-acetylglucosamine from UDP-GlcNAc or glucose from UDP-Glc onto DVV as well as several related macrocyclic peptides (Fig. 15C).[150] Interestingly, this hybrid GT possessed a substrate scope beyond that of its parents for the acceptors and donors that it can utilize, and can synthesize a variety of hybrid vancomycin derivatives. Obtaining a substrate-flexible GtfAH1 chimera by fusing domains of parent GTs that have strict substrate specificity is an interesting development with respect to the potential for glycodiversification.

Efforts have also been made to build GT chimeras to enable glycodiversification of polyene macrolide antibiotics. Caffrey and coworkers used trial and error to determine an appropriate crossover point for domain swapping, as structural data was not available for the parental GTs, AmphDI and PerDI from *Streptomyces nodosus* and *S. aminophilus*, respectively. Despite the structural similarity of their native substrates, PerDI does not recognize the acceptor substrate of AmphDI (amphoteronolide), and the glycosylation of amphoteronolides by AmphDI was extremely inefficient when the PerDI sugar donor (GDP-perosamine) was used. A functional chimera constructed from the AmphDI N-terminal region and the PerDI C-terminal region produced a perosaminylated amphoteronolide in good yield.[154] These studies demonstrated that the acceptor and donor specificity in these two parent enzymes is mainly governed by the N- and C-terminal domains, respectively.[150,154]

Landomycins, produced by various *Streptomyces*, are glycosylated antibiotics that consist of a tetracyclic, phenolic scaffold modified by a saccharide component made up of either a single monosaccharide unit or a chain of multiple sugar residues that can measure up to a hexasaccharide in length. *Streptomyces cyanogenus* S136 produces landomycin A, which has a hexasaccharide chain, consisting of two repeats of the trisaccharide L-rhodinose-D-olivose-D-olivose. LanGT1 is one of four GTs in *S. cyanogenus* S136 responsible for the assembly of the hexasaccharide of landomycin A, adding D-olivose at the second and fifth position in the sugar chain.[155] *S. globisporus* 1912 produces landomycin E, which has only the L-rhodinose-D-olivose-D-olivose trisaccharide as its sugar component. LndGT1, one of the three GTs in *S. globisporus* 1912 that glycosylate landomycin E, catalyzes addition of D-olivose at the second position of the trisaccharide.[156] LanGT1 and LndGT1 share 75% amino acid sequence identity, but despite their similarity, LndGT1 lacks the iterative bifunctional activity and does not replace LanGT1's function (i.e., addition of the fifth sugar) in a *S. cyanogenus* S136 *ΔlanGT1* knockout strain.[157] Sequence comparison of these two GTs revealed that the amino acid difference is mostly situated between 50 and 170 amino acids at N-terminal region. By studying 10 chimeric genes constructed from the *lanGT1* and *lndGT1* parental genes, Bechthold and coworkers identified the region of the protein responsible for the iterative activity and they identified the functional motifs within the N-terminal domain that determine acceptor specificity of LanGT1. Some of the chimeric genes were found to introduce the iterative activity of LanGT1 into LndGT1, allowing for the biosynthesis of extended landomycins.[157]

The above studies showed that, in GT-B fold enzymes, acceptor and donor substrate recognition are independently determined by the N- and C-terminal domains, respectively. In contrast, Park and coworkers showed that the sugar donor specificity of hybrid pteridine glycosyltransferases (PGTs) is not exclusively determined by the C-terminal domain, and instead both N- and C-terminal regions are involved. CY-007 PGT from *Arthrospira platensis* and CY-049 PGT from *A. maxima*, which share 93% sequence identity, glycosylate the same pteridine acceptor by transferring glucose and xylose, respectively, from UDP-activated donors. Construction of chimeras by swapping N- and C-terminal domains resulted in two hybrids that each transfer both glucose and xylose donor moieties.[158]

Even though amino acid sequence identity is a key factor for obtaining functionally active chimeras via domain swapping, a study of GT1 family enzymes by Hansen and colleagues demonstrated that this strategy is not limited strictly to GTs with high sequence identity.[159] Twenty different GT hybrids were constructed using domains from seven plant GTs UGT71C1, UGT71C2, UGT71E1, UGT85C1, UGT85B1, UGT88B1, and UGT94B1. Out of the 20 chimeras, 12 were catalytically active and comprised domain swapping of closely as well as distantly related GTs—some hybrids resulting from parent proteins that shared as little as 22% sequence identity. These authors obtained chimeras with modified acceptor substrate specificities, enhanced catalytic efficiency, and altered regiospecificity. The catalytic activity of the chimeras toward acceptor substrates was inconsistent and unpredictable, and the acceptor specificities of the hybrids were defined by both N- and C-terminal domains.[159] Thin-layer chromatography of radiolabeled products was used to identify functional enzymes, limiting the number of chimeras that could be screened.

One approach to deal with large numbers of domain swap variants is to employ high-throughput screening methods. Kim and coworkers sourced parental GTs templates from the kanamycin (KanF) and the vancomycin (GtfE) biosynthetic pathways. An incremental truncation strategy was used to generate a hybrid library of GTs with varying crossover points of the KanF N-terminal region and the GtfE C-terminal region. The GT chimeras were screened by a plate-based pH-dependent color assay wherein release of an acidic nucleotide upon sugar transfer from an NDP-activated donor is detected by the absorbance shift of a pH-indicator. A GT chimera was obtained that showed increased catalytic activity in the presence of the KanF acceptor aglycone (2-deoxystreptamine) and the sugar donor recognized by GtfE (dTDP-glucose). This chimera exhibited enhanced activity with other sugar donors, highlighting the potential for high-throughput screening of domain swap libraries for engineering functional GT hybrids.[160]

3.09.3.2 High-throughput screening strategies and their application to discover and engineer GT activity

High-throughput enzyme screening platforms are valuable tools in identifying useful activities from naturally diverse pools of enzymes, exploring substrate scope and combinatorial synthetic activity in promiscuous enzymes, and in evolving existing enzymes by directed evolution.[161] However, development of a screening approach for GTs is difficult, since the formation of glycosidic bonds does not produce a conveniently measurable signal like a change in absorbance or fluorescence.[162] Several high-throughput assays have been reported and herein we discuss some of the new GT screening strategies. Although, many of these methods were originally developed for directed evolution, these powerful tools can be applied in prospecting for novel enzyme activities from nature (e.g., in functional metagenomic screening) and in high-throughput strategies to survey the landscapes of promiscuous catalytic reactivity for combinatorial enzymatic synthesis.

3.09.3.2.1 A plate-based fluorescence quenching strategy for the directed evolution of natural product GTs

Certain natural product GTs are inherently substrate flexible supporting the use of non-native donor and acceptor molecules to allow natural product glycodiversification. However, most GTs have a restricted substrate specificity, limiting their use in glycodiversification. One strategy to alter and/or broaden the substrate scope of GTs is to apply directed evolution.[163] While it can be laborious to screen a very large libraries by plate-based methods, it remains a good option for small to medium size libraries.[164] Thorson and colleagues developed a plate-based fluorescence assay to screen a small mutant library derived from a GT (OleD) that is active on the antibiotic macrolide natural product, oleandomycin, produced by *Streptomyces antibioticus*.[163] OleD glycosylates oleandomycin and a range of other macrolides as part of a resistance mechanism to the antibiotic in the same organism that produces it. While the native acceptor of OleD is oleandomycin[165] it can also use the fluorescent coumarin 4-methylumbelliferone as an acceptor, with loss of fluorescence upon glycosylation allowing for the establishment of a fluorescence-quenching assay (Fig. 16).[166] Using error-prone PCR, a mutant OleD library was built and crude extracts of around a thousand mutant colonies were screened using the fluorescence quenching assay. The best mutants were further analyzed, and a triple mutant was designed by combining the amino acid substitutions from active mutants via site-directed mutagenesis. Compared to the wild-type, the triple mutant showed a 60-fold improvement in efficiency in glucosylating the fluorescent acceptor with UDP-Glc. Furthermore, the triple mutant resulted in a broad substrate specificity toward a range of non-native acceptor and donor molecules.[163]

3.09.3.2.2 Intracellular fluorescence entrapment to screen GT activity by FACS

Withers and colleagues used fluorescently tagged acceptors to screen the activity of a bacterial ST mutant library by fluorescence-activated cell sorting (FACS) (Fig. 17).[162] Using FACS, millions of mutants can be screened per hour. CstII is an ST from *Campylobacter jejuni* that uses CMP-activated Neu5Ac as the sugar donor.[167] The authors used an engineered *E. coli* strain that lacks the ability to catabolize Neu5Ac and lactose that also encoded a CMP-Neu5Ac synthetase to ensure synthesis of CMP-Neu5Ac, the donor for ST's, within the cell.[168] These cells were shown to be permeable to exogenously provided substrates and substrate precursors: Neu5Ac (the precursor to CMP-Neu5Ac) and fluorescently-tagged galactoside or lactoside acceptors, which freely cross the cell membrane using transporters expressed within the host strain.[162] A CstII mutant library was constructed by random

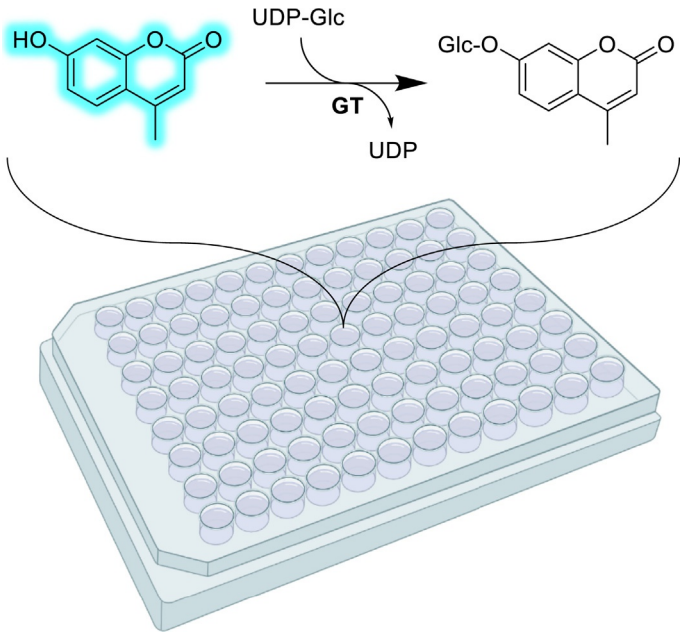

Fig. 16 Plate-based fluorescence quenching assay for OleD activity. The enzyme activity is quantified based on fluorescence quenching of coumarin 4-methylumbelliferone acceptor upon glycosylation.

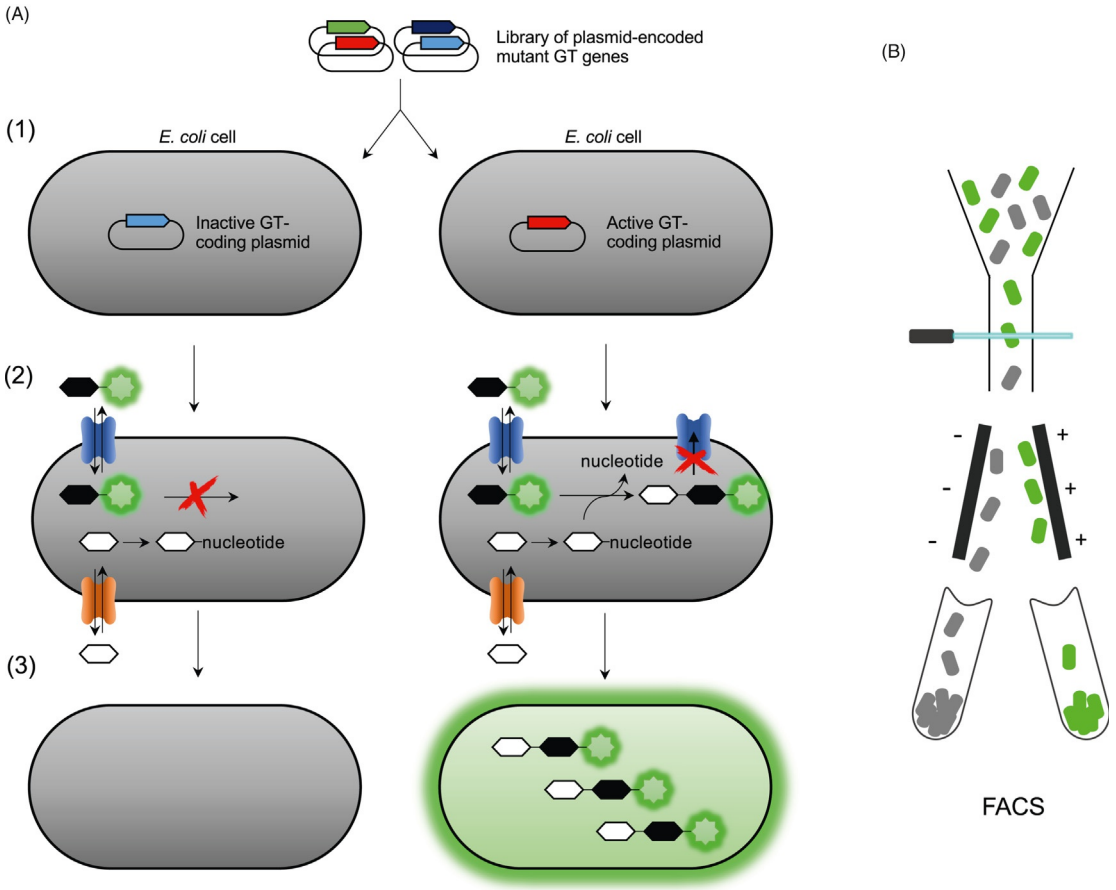

Fig. 17 Cell entrapment assay for GT activity. (A) *E. coli* cells are transformed with a mutant GT gene library (1). GT proteins are expressed, and sugar donor precursor and fluorescently labeled acceptor are supplied exogenously. The sugar donor is formed within the cell and glycosylated products are synthesized by active GTs (2). The unreacted fluorescent acceptors are washed away while the glycosylated fluorescent products are trapped in cells (3). (B) Cells are analyzed by FACS.

mutagenesis and mutant proteins were expressed in the modified *E. coli* cells. Functional CstII mutant enzymes catalyze the synthesis of a negatively-charged sialylated fluorescent product that is not a substrate for membrane transport proteins, and therefore gets entrapped within the cell linking the genotype (the gene encoding the mutant GT) and the resulting fluorescent phenotype. To minimize background noise and to allow the detection of lower CstII activity, the unreacted fluorescent tagged acceptors were washed away from cells. Using this high-throughput method, the authors screened over one million mutants and evolved an improved CstII mutant with an increased catalytic efficiency up to 400-fold for a variety of non-native, fluorescently tagged acceptors. X-ray crystallographic analysis of the CstII mutant revealed that the enhanced activity is due to the evolution of a binding pocket for the fluorescent tag.[162]

Withers and colleagues reported an improvement to the previous screening approach and applied it for engineering a β-1,3--galactosyltransferase (CgtB) from *C. jejuni*. To minimize the bias toward selecting CgtB variants with improved activity on the tagged acceptor due to evolving a fluorophore-binding site, Yang et al. used two acceptor substrates: *N*-acetyl-D-galactosamine tagged with two structurally different fluorophores. While CgtB transfers the neutral galactose, the FACS method remained effective owing to an increase in polarity of the entrapped product, which is not recognized by membrane transport proteins. From a screen of over 10 million CgtB mutants a variant was discovered with increased substrate specificity and with catalytic activity increased 300-fold compared to the wild-type enzyme.[169]

In a further development, Yang and colleagues extended this screening approach for the directed evolution of α1,3-fucosyltransferase (FutA) from *Helicobacter pylori*. Again, two LacNAc acceptor derivatives were synthesized by tagging two different fluorophores. To prevent galactoside cleavage, the *lacZ* gene of the used *E. coli* strain was deleted. The fluorescently-labeled LacNAc acceptors and fucose enter the cell through transporters. Once in the cell, fucose is converted to GDP-fucose by GDP-fucose synthetase (FKP) expressed from a plasmid. Active FutA mutants catalyze the synthesis of fucosylated fluorescent products that accumulate within the cell and can be sorted by FACS. Three rounds of directed evolution led to a functional FutA mutant with 6-fold and 14-fold higher catalytic efficiency toward Lewis^X and 3′-fucosyllactose synthesis, respectively.[170] Compared to the traditional microtiter plate-based assays, these FACS based methods enable ultra-high throughput and require amounts of lower reagents saving time and cost.[162,169,170]

3.09.3.2.3 *Glycan-binding proteins to screen GT activity in plate- and particle-based* in vitro *assays and FACS-based* in vivo *assays*

In another screening approach, the GT acceptor substrate is linked to a nanoparticle or immobilized on a plate or other surface. Upon successful glycosylation of the acceptor, the product can be detected using a fluorescently-tagged or untagged glycan-binding protein that binds to the product (Fig. 18). If the assembled glycan is instead attached to the surface of a cell, a glycan-binding protein can be used to label cells displaying the saccharide synthesized by an intracellular GT, and cell-based FACS used to isolate clones that encode GT variants with the desired activity from a pool of millions of variants.

Gold nanoparticles (AuNPs) have unique quantum and spectral properties, and when functionalized on their surfaces can be used as biosensors for screening enzyme activities that modify the surface. Lin and colleagues described an AuNP-based colorimetric assay for detection of the activity of an α2,8-polysialyltransferase (α2,8-PST) from *Neisseria meningitidis* (Fig. 18A).[171] PSTs catalyze the synthesis of the polysaccharide, polysialic acid (PSA), a negatively charged linear homopolymer. The enzyme polymerizes PSA by transferring sialic acid (Neu5Ac) from CMP-Neu5Ac to the nonreducing end of the growing PSA chain.[172,173] Yu et al. bound the acceptor oligosaccharides (ganglioside GD3 analogs) to AuNPs, and incubated the glyco-nanoparticles with α2,8-PST and the CMP-Neu5Ac.[171] The resulting AuNPs with attached PSA were incubated with a catalytically-inactive K1F endosialidase double mutant (EndoNF DM), which is an engineered trimeric glycan-binding protein that caused aggregation of the nanoparticles.[174] PST activity was detected by the decrease in the characteristic plasmon resonance absorption peak intensity of the AuNP that occurs upon aggregation. Considering its scalability, high specificity, easy detection, and the ability to detect PST activity in cell lysates, the authors propose that this would be an attractive assay for directed evolution of sialyltransferases.[171]

Withers and colleagues developed a high-throughput, plate-based screening method that involves immobilization of GT acceptor substrates to the wells of microtiter plates (Fig. 18B). This was used to screen a library of α2,8-PST mutants from *N. meningitidis* where activity was measured by detection of the PSA product through binding of a fluorescently-tagged glycan binding protein, which consisted of a green fluorescent protein (GFP)-fused to EndoNF DM (GFP-EndoNF DM). An acceptor substrate for α2,8-PST, consisting of a trisialyl lactoside (GT3 oligosaccharide) conjugated to an azide-functionalized alkyl chain, was immobilized in wells that were coated with alkyne groups using click chemistry. These plates were incubated with purified α2,8-PST or crude α2,8-PST from cell lysates along with CMP-Neu5Ac. Subsequently, α2,8-PST activity was measured using GFP-EndoNF DM, which binds to the elongated PSA product immobilized on the plate. Through this sensitive and robust high-throughput plate-based assay, directed evolution can be carried out with libraries consisting of several thousand mutants.[123] Yet, screening large mutant libraries that number millions or more variants using this approach is challenging.[175]

Wakarchuk and coworkers developed a screening strategy for the directed evolution of α2,8-PST using detection of PSA with a fluorescently-tagged binding protein in a cell-based *in vivo* assay (Fig. 18C). Using a FACS based high-throughput approach, over a million α2,8-PST mutants were screened.[175] α2,8-PST activity was detected by α2,8-PST knockout complementation in a non-pathogenic *E. coli* EV36 strain (*E. coli* EV36_NeuS^{KO}) in which the gene coding for the native PSA-synthesizing enzyme, NeuS, was deleted.[175,176] Active variants were enriched by FACS using GFP-EndoNF DM to label cells by binding to the synthesized and secreted capsular PSA. Subsequently, the activity of 400 FACS-enriched variants were verified in a secondary screen using a plate-based assay. In this secondary screen, acceptor substrates were immobilized on a microtiter plate and PSA production by the active variants were probed using GFP-EndoNF DM. This combined platform led to the identification of mutants with enhanced activity and temperature stability emphasizing its potential as a screening platform in directed evolution.[175]

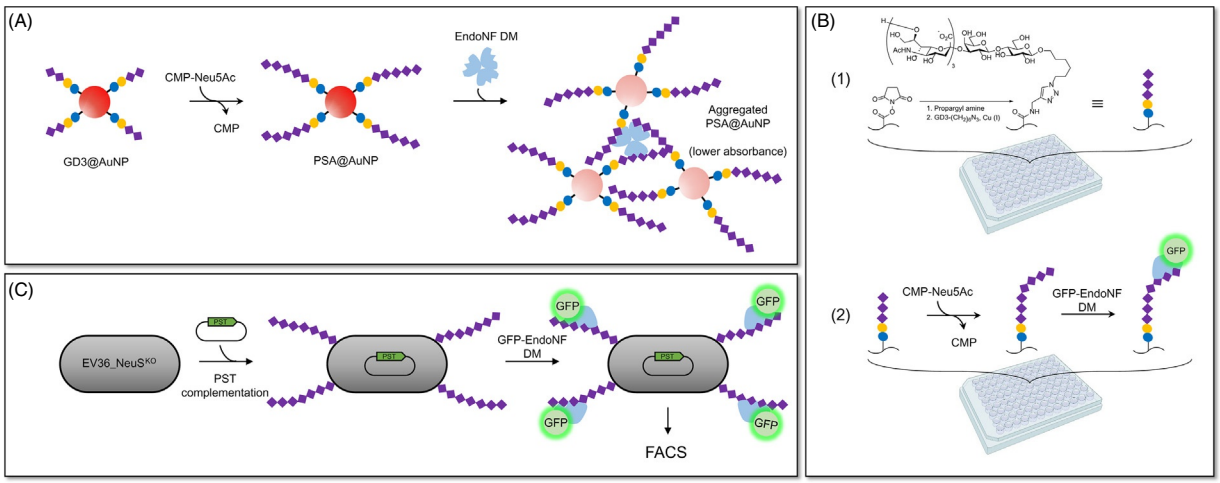

Fig. 18 PST activity screening using EndoNF DM PSA binding protein. (A) Neu5Ac is transferred to the acceptor oligosaccharides bound to AuNP by PST. Synthesized PSA@AuNP is aggregated using EndoNF DM cross linker, whereupon the AuNP absorbance decreases owing to quenching of surface plasmon resonance effects. (B) On microtiter plates, PST acceptor substrates are immobilized. PST activity is detected by GFP-EndoNF DM bound to PSA. (C) A PST plasmid library is transformed into a PST knockout strain. Cells are incubated with GFP-EndoNF DM and PST complemented cells are analyzed by FACS.

3.09.4 Conclusions

Glycans are an incredibly diverse and important class of molecules with a multitude of roles in biology that make the glycosyltransferases that catalyze their assembly attractive as both targets for pharmaceutical intervention, and as tools for biocatalysis. Our growing understanding of the catalytic mechanisms and structures of these enzymes has supported the development of small molecule inhibitors and efforts to alter catalytic activity through protein engineering. High-throughput screening platforms have been developed to support the discovery of GT inhibitors, and the evolution of novel GTs. Future investigations that reveal ever finer details of glycosyltranferase mechanism and function, and continuing developments in novel screening strategies will together contribute to more specific and potent inhibitors that will be useful as therapeutic agents, and to newer generations of engineered biocatalysts for synthesizing valuable glycans and glycoconjugates.

See Also: 3.07 Biosynthesis of Bacterial Polysaccharides

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