CHEMBIOCHEM

DOI: 10.1002/cbic.201000013

A Novel Fluorescent Probe for Retaining Galactosyltransferases

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Glycosyltransferases (GTs) are a large class of carbohydrate-active enzymes that are involved, in both pro- and eukaryotic organisms, in numerous important biological processes, from cellular adhesion to carcinogenesis. GTs have enormous potential as molecular targets for chemical biology and drug discovery. For the full realisation of this potential, operationally simple and generally applicable GT bioassays, especially for inhibitor screening, are indispensable tools. In order to facilitate the development of GT high-throughput screening assays for the identification of GT inhibitors, we have developed novel, fluorescent derivatives of UDP-galactose (UDP-Gal) that are recognised as donor analogues by several different retaining galactosyltransferases (GalTs). We demonstrate for one of these derivatives that fluorescence emission is quenched upon specific binding to individual GalTs, and that this effect can be used as the read-out in ligand-displacement experiments. The novel fluorophore acts as an excellent sensor for several different enzymes and is suitable for the development of a new type of GalT bioassay, whose modular nature and operational simplicity will significantly facilitate inhibitor screening. Importantly, the structural differences between the natural donor UDP-Gal and the new fluorescent derivatives are minimal, and the general assay principle described herein may therefore also be applicable to other GalTs and/or proteins that use nucleotides or nucleotide conjugates as their cofactor.

Introduction

Glycosyltransferases (GTs) are a large class of enzymes that are required, in both pro- and eukaryotic organisms, for the biosynthesis of complex carbohydrates and glycoconjugates.[1–4] GTs catalyse the transfer of a mono- or oligosaccharide from a glycosyl donor, generally a sugar nucleotide, to a diverse range of acceptor substrates, including saccharides, proteins, lipids and secondary metabolites.[1–5] GTs are involved in numerous fundamental biological processes, from cellular adhesion to carcinogenesis and neurobiology,[6–8] and it has been estimated that this class of enzymes accounts for up to 1% of ORFs across all sequenced genomes.[3] However, compared to other enzyme classes of similar size and biological significance (e.g., the protein kinases), the considerable potential of GTs as molecular targets for chemical biology and drug discovery has yet to be fully realised. Towards this goal, operationally simple GT bioassays, for example, for studies on donor/acceptor selectivity, function analysis and inhibitor screening, are indispensable tools, and considerable progress has recently been made in the development of such assays.[9–13]

In view of the size of the GT enzyme family, a considerable need remains for assay formats that are generally applicable with different GTs. A general GT ligand-displacement assay, for example, will allow the rapid screening of multiple enzymes in parallel and the efficient selectivity profiling of substrate analogues and inhibitors. Fluorescence-based formats are particularly attractive for such applications,[14] due to their operational simplicity and their adaptability for high-throughput screening (HTS). In pioneering work in this area, the Walker group have developed UDP-GalNAc/fluorescein conjugates as fluorophores for ligand-displacement assays with two *N*-acetyl glucosamine (GlcNAc) transferases.[15,16] However, the considerable steric demand of the fluorescein moiety necessitated the design of a separate fluorophore for each enzyme and limits the general applicability of these probes. In view of these results, the development of a generally applicable fluorophore that can be used for the simultaneous screening of an entire group of GTs (e.g., several galactosyltransferases) in a single experiment is an attractive proposition. Herein, we describe, for the first time, a broadly applicable GT fluorophore that might have the potential to be used in such a general assay.

UDP-α-D-galactose (UDP-Gal, Table 1) is the general sugar-nucleotide donor for all Leloir-type galactosyltransferases (GalTs).[17] Individual GalTs have been identified as molecular targets in a number of therapeutic areas, including cancer and infection.[18,19] For example, the inhibition of bacterial GalTs involved in the biosynthesis of lipopoly- and lipooligosaccharides of the Gram-negative cell envelope is a promising new strat-

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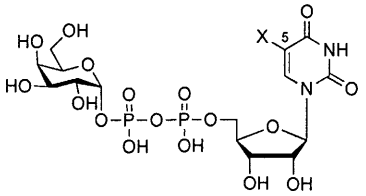
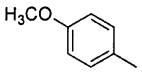
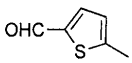
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201000013.

Table 1. Fluorescence and enzymological properties of UDP-Gal and base-modified UDP-Gal derivatives **1a–d**.



Cmpd	X	Quantum yield Φ	*B. taurus* α-(1→3)-GalT K_m [μM]	k_{cat} [s^{-1}]
UDP-Gal	H	4.5×10^{-5}	118±14	0.98
1a		<0.01	96±8	2.1×10^{-3}
1b		0.024	82±11	4.0×10^{-3}
1c		0.04	69±13	4.4×10^{-3}
1d		0.26	13±1	1.9×10^{-3}

egy for anti-bacterial drug discovery.[19] Known GalT inhibitors are generally donor or acceptor substrate analogues with only limited "drug-likeness", which could compromise their applicability for, for example, cellular studies.[20] The identification of novel, drug-like GalT-inhibitor chemotypes would be greatly facilitated by a general GalT-screening assay. Recently, fluorescent derivatives of uracil nucleotides have been generated, as probes for nucleic acid chemistry, by installation of a compact, heteroaromatic substituent in position 5 of the uracil base.[21] We reasoned that this phenomenon could also be harnessed for the development of autofluorescent UDP-Gal derivatives, as a novel type of fluorophore for GalT bioassays. We anticipated that such base-modified UDP-Gal derivatives might be broadly recognised by a range of different GalTs, due to the minimal steric demand of the fluorogenic substituent and the strongly conserved architecture of the nucleotide binding domain in different GalTs.[2–4]

The implementation of this strategy has now resulted in the identification of the novel UDP-Gal derivative **1d** (Table 1). Compound **1d** is a strong fluorescence emitter and is recognised as a high-affinity ligand by various GalTs. Importantly, the fluorescence of **1d** is quenched upon binding to protein, and we have exploited this effect for the development of an operationally simple GalT ligand-displacement assay. This new GalT assay format allows for screening against bacterial and mammalian enzymes in parallel, and thus the evaluation of inhibitor candidates for their potency and selectivity in the same experiment. To the best of our knowledge, this is the first example of the use of this type of fluorophore in a ligand-displacement assay, and the general assay principle we describe herein might also be applicable to other (sugar)-nucleotide-dependent proteins.

Results and Discussion

Using our previously developed synthetic methodology for the Suzuki–Miyaura cross-coupling of unprotected sugar nucleotides,[22,23] we prepared a series of novel UDP-Gal derivatives with an additional aromatic or heteroaromatic substituent in position 5 of the uracil base (Table 1, **1a–d**). In contrast to the practically nonfluorescent parent UDP-Gal, the 5-(hetero)aryl-substituted derivatives **1a–d** are fluorescence emitters, and their fluorescence properties can be modulated by the nature of the 5-substituent. Crucially, while the phenyl- and furyl-substituted derivatives **1a–c** showed only moderate to weak fluorescence, thienyl derivative **1d** was much more strongly fluorescent. The quantum yield of **1d** is 25 times greater than that of **1a**, and almost 6000 times greater than the quantum yield of the parent UDP-Gal (Table 1). We anticipated that, with these fluorescence characteristics, **1d** might be a suitable fluorophore for a fluorescence-based GalT ligand-displacement assay, provided that **1d** was recognised as a ligand by the target GalTs.

In order to assess the influence of the additional substituent in position 5 on GalT recognition and binding, we carried out enzymological studies with donor analogues **1a–d** and a representative bovine α-(1→3)-GalT (Table 1). Pleasingly, we found that although the turnover of **1a–d** was considerably lower than for UDP-Gal, the Michaelis–Menten constant of the base-modified analogues was of a similar order of magnitude as for UDP-Gal. These results suggested that the additional substituent in position 5 is not detrimental for binding of these donor analogues at α-(1→3)-GalT, a key criterion for their potential application in a GalT ligand binding assay. This interpretation has subsequently been confirmed by structural studies with **1d** and a blood-group galactosyltransferase.[24] As the most promising analogue, with regard to both its strong binding affinity and pronounced fluorescence, the thienyl-substituted derivative **1d** was selected for proof-of-principle investigations into the suitability of this novel type of fluorophore for assay development.

The fluorescence emission of a given fluorophore is modulated by its microenvironment.[25] We therefore speculated that, upon binding to a target GalT, the fluorescence emission of **1d** would either be enhanced or attenuated, and that the difference in fluorescence between protein-bound and free fluorophore could be used as the read-out for a GalT ligand-displacement assay. In order to test this hypothesis, we first carried out titration experiments with a fixed concentration of **1d** and with bovine α-(1→3)-GalT as a representative target enzyme. We found that the fluorescence of **1d** is indeed quenched in the presence of enzyme in a concentration-dependent manner (Figure 1A, circles).

To assess the specificity of this quenching effect, we next performed a range of control experiments. Like many other GTs, *B. taurus* α-(1→3)-GalT requires a divalent metal such as Mn^{2+} to bind the sugar-nucleotide donor.[26] Importantly, in the absence of Mn^{2+}, no fluorescence quenching was observed upon titration of fluorophore **1d** with α-(1→3)-GalT (Figure 1A, squares). We concluded from this result that binding of

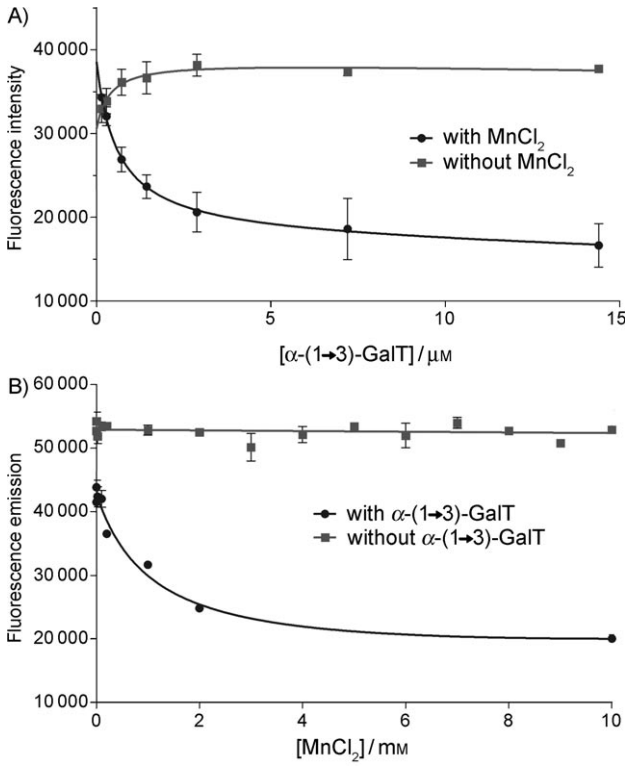


Figure 1. A) Fluorescence emission of fluorophore **1 d** upon titration with α-(1→3)-GalT in the presence or absence of 10 mM $MnCl_2$. B) Fluorescence emission of fluorophore **1 d** upon titration with $MnCl_2$, in the presence or absence of 0.58 μM α-(1→3)-GalT. Conditions: 200 nM **1 d**, 50 mM Tris buffer pH 7, 30 °C, incubation 15 min.

1 d occurs specifically at the donor binding site of α-(1→3)-GalT. This implies that the observed fluorescence-quenching effect also is specific and not due to, for example, nonspecific binding of **1 d** on the protein surface. In keeping with this interpretation, no significant fluorescence quenching was observed upon titration of **1 d** with bovine serum albumin (BSA), a protein lacking a binding site for UDP sugars (Figure S1 in the Supporting Information). To investigate the possibility that the reduced fluorescence in the presence of Mn^{2+} might be due to direct quenching by the divalent metal,[27] we also repeated the initial titration experiment with variable concentrations of Mn^{2+}, at fixed concentrations of **1 d** and α-(1→3)-GalT. Significantly, fluorescence quenching was only observed in the presence of all three binding partners, **1 d**, Mn^{2+}, and α-(1→3)-GalT (Figure 1 B, circles). In contrast, fluorescence emission remained strong upon titration of **1 d** with increasing concentrations of Mn^{2+} but in the absence of enzyme (Figure 1 B, squares). Taken together, these results provided strong support for the notion that the observed fluorescence quenching was due to the specific binding of **1 d** at the donor binding site of α-(1→3)-GalT.

This interpretation was further substantiated by the finding that the fluorescence of **1 d** could be restored by titration with nonfluorescent, competitive α-(1→3)-GalT ligands, including UDP-Gal (Figure 2 A). While these experiments confirmed the specificity of the binding, and concomitant fluorescence quenching, of **1 d**, they also allowed the determination of IC_{50} values for these known GalT ligands (Table 2). The order of potency observed for UDP-Gal, UDP, UMP and uridine was in agreement, qualitatively and quantitatively, with literature data for inhibition of GalTs by these ligands.[20] We saw these results as an important validation of our assay design and evidence for its reliability. As our experimental protocol allowed us to discriminate between α-(1→3)-GalT binders with strong (UDP-Gal, UDP), moderate (UMP) and poor (uridine) affinity, we concluded that, in principle, this assay set-up might also be suitable for inhibitor screening.

Before carrying out library screening experiments, we decided to assess the generality of this new GT assay principle. We therefore performed the requisite fluorescence quenching and control experiments with three other GalTs. This panel of enzymes covered a variety of GalT activities, including the mammalian blood-group enzymes GTB and AA(Gly)B, and the bacterial α-(1→4)-GalT LgtC. AA(Gly)B is a dual-specificity enzyme that can utilise either UDP-Gal or UDP-GalNAc as a donor substrate, producing either blood group A or B structures.[24] Significantly, we consistently observed a strong fluorescence-quenching effect for **1 d** with all of these enzymes (Figure S2). As in the case of bovine α-(1→3)-GalT, titration with UDP-Gal, UDP and, to a lesser extent, UMP also restored the fluorescence of **1 d** in the presence of the human enzymes GTB and AA(Gly)B, as well as the bacterial enzyme LgtC (Figure 2 B–D). On the other hand, uridine was, as expected, not an effective competitive binder for **1 d** at these enzymes. Notably, the IC_{50} values obtained for UDP-Gal in these competition experiments were in good agreement, for all four enzymes, with K_m values determined in other assays (Table 2). Thus, these findings demonstrated not only the reliability, but also the broad applicability of our ligand-displacement assay protocol.

With a novel fluorophore for different GalTs in hand, we investigated its suitability for the identification and selectivity profiling of new GalT inhibitors. In a proof-of-concept experiment, we screened a small library of drug-like inhibitor candidates in parallel against three different enzymes. For this screen, we selected a structurally diverse set of thiazolidinones as inhibitor candidates (Table 3), as thiazolidinones had previously been reported as inhibitors for other GTs.[15,28] Candidate compounds, at a concentration of 50 μM, were co-incubated on a single microplate with fluorophore **1 d** and three different GalTs (i.e., the model α-(1→3)-GalT from *B. taurus*, the *H. sapiens* α-(1→3)-GalT GTB, and the *N. meningitidis* α-(1→4)-GalT LgtC; Figure 3 A). As expected, the competitive displacement of **1 d** from the GalT donor binding site by high-affinity binders resulted in an increase in fluorescence. To quantify the displacement of fluorophore by individual binders, the fluorescence increase observed for the natural donor UDP-Gal was used as a reference. Using this procedure, we were able to establish an order for the inhibitor candidates according to their binding affinity and, at the same time, to assess their GalT-selectivity profile. To validate this approach, we determined complete binding curves with all three GalTs for the representative thiazolidinone inhibitor **2 b** (Figure 3 B). The IC_{50} values extracted from these binding curves suggest a slightly greater affinity

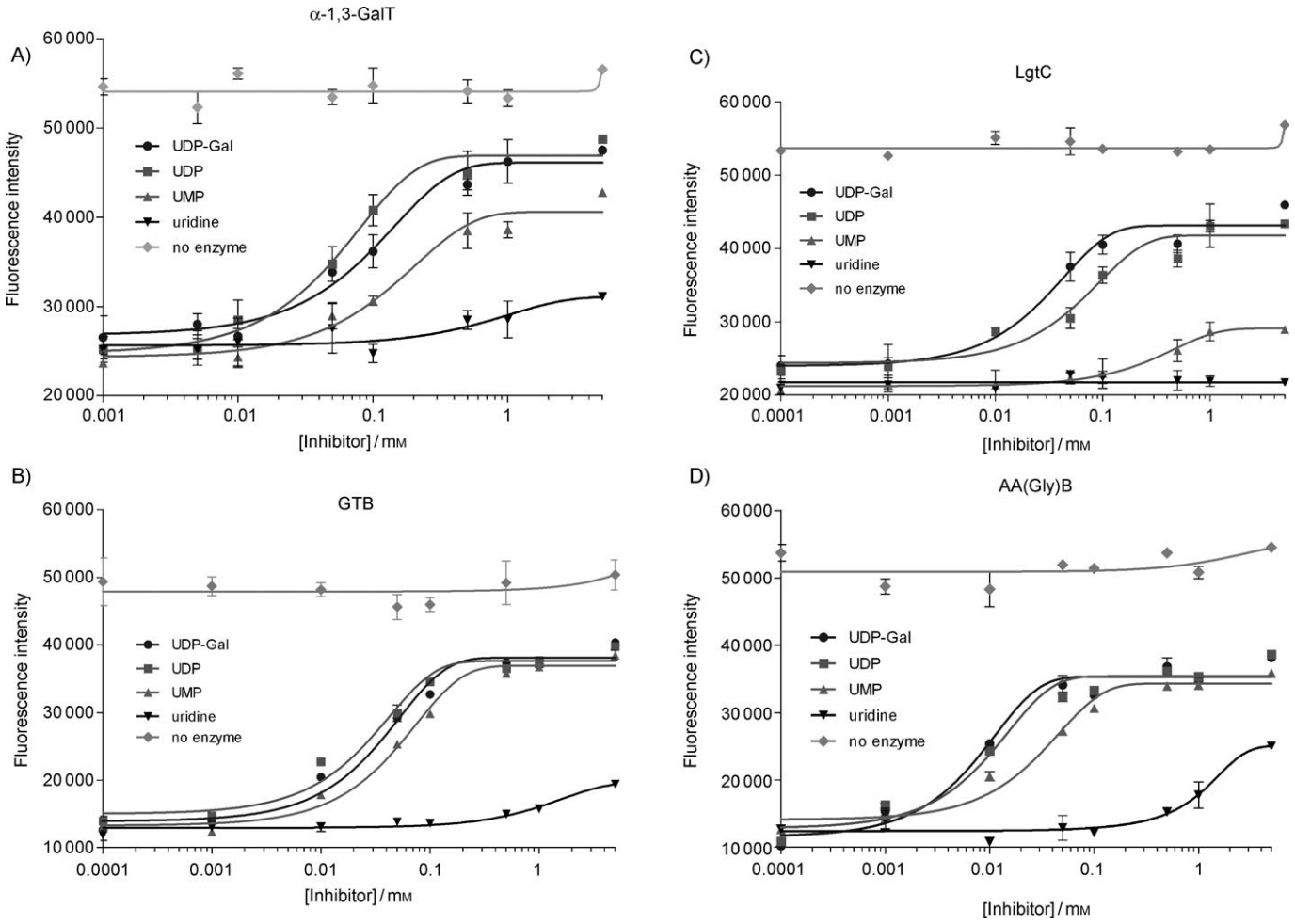


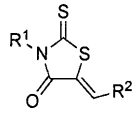
Figure 2. Titration of **1 d** (200 nM) and A) α-(1→3)-GalT, B) GTB, C) LgtC and D) AA(Gly)B with UDP-Gal, UDP, UMP and uridine, plus control experiments without enzyme.

Table 2. IC_{50} values for different GalTs/GalT ligands, determined with fluorophore **1 d**. K_m values for UDP-Gal are given for direct comparison.

GalT	K_m [μM]	IC_{50} [μM]			
	UDP-Gal	UDP-Gal	UDP	UMP	uridine
B. taurus α-(1→3)-GalT	118 ± 14[a]	109 ± 20	74 ± 30	240 ± 110	> 1000
GTB	27[b]	28 ± 5	18 ± 7	48 ± 5	> 1000
AA(Gly)B	0.7 ± 0.1[a]	7 ± 6	12 ± 5	22 ± 3	> 1000
LgtC	18[c]	26 ± 8	83 ± 49	293 ± 91	> 1000

[a] This study, HPLC-based assay. [b] Ref. [33]. [c] Ref. [19].

Table 3. Molecular structures of the thiazolidinone inhibitor library.



Code	R^1	R^2	Code	R^1	R^2
2 a	CH_2CO_2H	3-pyr	**2 g**	CH_2–CH=CH_2	3-pyr
2 b	CH_2CO_2H	4-BnO-C_6H_4	**2 h**	H	4-BnO-C_6H_4
2 c	CH_2–CH=CH_2	4-pyr	**2 i**	CH_2–CH=CH_2	2-pyr
2 d	CH_2CO_2H	4-pyr	**2 j**	CH_2–CH=CH_2	4-BnO-C_6H_4
2 e	H	Ph	**2 k**	Ph	3,4-(BnO)-C_6H_3
2 f	CH_2CO_2H	2-pyr	**2 l**	NH_2	4-BnO-C_6H_4

of **2 b** for α-(1→3)-GalT and LgtC than for GTB. Significantly, these results are in keeping with the selectivity profile observed for **2 b** in the single-concentration screen, thus confirming the suitability of the experimental set-up chosen for the library-screening experiment. Taken together, results from this screen provide a proof-of-principle that this experimental set-up allows the reliable discrimination between low- and high-affinity GalT binders. Our protocol therefore offers an extremely rapid and simple method for the identification and target profiling of novel, drug-like GalT inhibitors.

In summary, we have developed the new and broadly applicable fluorophore **1 d** for the HTS of retaining GalTs. We have demonstrated that **1 d** is suitable for the screening of several different enzymes in parallel and allows the simultaneous profiling of inhibitor candidates for both potency and selectivi-

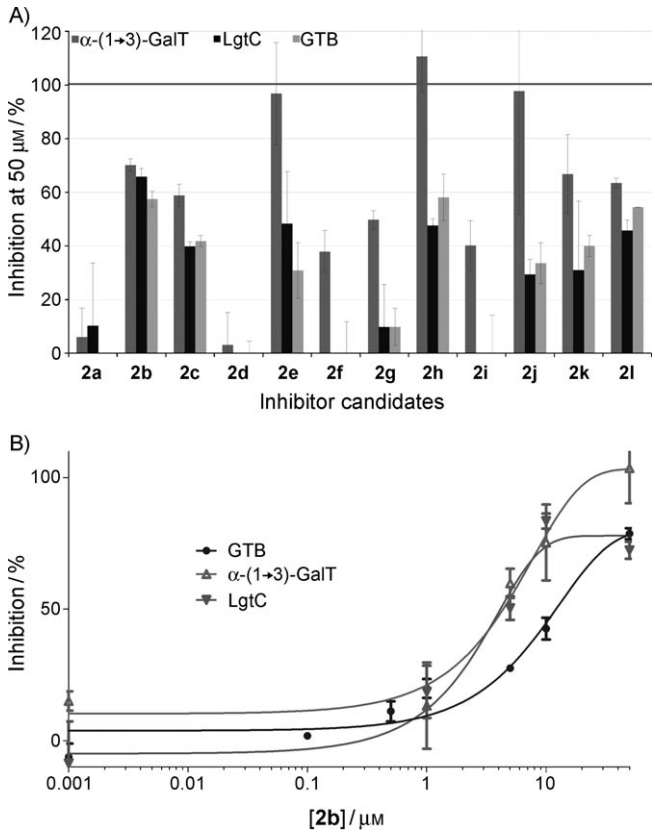


Figure 3. A) Screening of small molecular inhibitor candidates against three different GalTs, using fluorophore **1 d**. The potency of the inhibitors is given relative to that of UDP-Gal (indicated with a line). See Table 3 for inhibitor structures **2 a**–**l**. B) Displacement of fluorophore **1 d** from α-(1→3)-GalT, GTB and LgtC by thiazolidinone inhibitor **2 b**.

ty. This assay design significantly facilitates the identification of novel GalT inhibitor chemotypes, for example, for antibacterial drug discovery, and obviates the need for the time-consuming evaluation of candidate molecules in separate GT bioassays.[29] Moreover, the modular nature of this screening format allows, in principle, the continuous addition of new enzymes to this assay. These enzymes may include other retaining and, potentially, inverting GalTs as well as other UDP-Gal-dependent enzymes, such as the epimerase GalE.[30] While not all of these enzymes might tolerate the additional fluorogenic substituent at the uracil base of **1 d** as well as the GalTs used in this study, this potential limitation can very likely be addressed by generating mutants of the proteins in question. Beyond carbohydrate-active and glycoprocessing enzymes, the general assay principle described herein could also be applicable to other proteins that use nucleotides or nucleotide conjugates as their cofactor. Studies exploring the scope of the new fluorophore and some of its derivatives for such applications are ongoing.

Experimental Section

Synthetic chemistry: All chemicals and reagents were obtained commercially and used as received unless stated otherwise. Thiazolidinone inhibitors **2 a**–**l** were prepared as previously described.[28] Fluorophore **1 d** was prepared by Suzuki–Miyaura cross-coupling of 5-iodo UDP-α-D-galactose and (5-formylthien-2-yl)boronic acid, as previously described.[24] UDP-Gal derivatives **1 a**–**c** were prepared in analogous fashion, and full synthetic details will be reported elsewhere. All target compounds were purified by ion-pair and/or ion-exchange chromatography, on Lichroprep RP-18 or Macro Prep 25Q resin, respectively, and characterised analytically by ^{1}H, ^{13}C and ^{31}P NMR and HR ESI-MS (for the analytical characterisation of **1 a**–**c** see the Supporting Information). Chemical shifts (δ) are referenced to methanol (δ_H 3.34, δ_C 49.50 for solutions in D_2O).

5-(5-Formylthien-2-yl)-UDP-α-D-galactose (1 d): ^{1}H NMR (400 MHz, D_2O): $\delta = 1.27$ (2.1 equiv of TEA, t, $J = 6.8$ Hz), 3.19 (2.1 equiv of TEA, q, $J = 6.8$ Hz), 3.66–3.72 (m, 2H; H-6″), 3.72–3.76 (m, 1H; H-2″), 3.84 (dd, $J = 3.2$, 10.2 Hz, 1H; H-3″), 3.95 (d, $J = 3.0$ Hz, 1H; H-4″), 4.10–4.13 (m, 1H; H-5″), 4.28–4.31 (m, 2H; H-5′), 4.32–4.34 (m, 1H; H-4′), 4.40–4.48 (2t, $J = 5.1$, 5.1 Hz, 2H; H-2′, H-3′), 5.62 (dd, $J = 3.4$, 7.1 Hz, 1H; H-1″), 6.04 (d, $J = 4.9$ Hz, 1H; H-1′), 7.74 (d, $J = 4.2$ Hz, 1H; Th), 8.01 (d, $J = 4.1$ Hz, 1H; Th), 8.46 (s, 1H; H-6), 9.79 (s, 1H; CHO); ^{13}C NMR (125 MHz, D_2O): $\delta = 9.0$ (TEA), 47.5 (TEA), 61.7, 65.7 (d, $J_{C,P} = 4.6$ Hz), 69.0 (d, $J_{C,P} = 6.7$ Hz), 69.7, 70.0, 70.3, 72.6, 74.9, 84.3 (d, $J_{C,P} = 7.3$ Hz), 89.7, 96.4 (d, $J_{C,P} = 5.4$ Hz), 109.6, 126.0, 139.2, 140.3, 142.0, 144.8, 151.2, 163.5, 187.8; ^{31}P NMR (121.5 MHz, D_2O): $\delta = -11.2$ (d, $J_{P,P} = 22.5$ Hz), -12.7 (d, $J_{P,P} = 21.2$ Hz); MS (ESI): *m/z* $C_{20}H_{25}N_2O_{18}P_2S_1$: calcd 675.0304 $[M-H]^-$, found: 675.0305.

Biochemistry: Proteins were expressed and purified as previously described.[24,31] For donor kinetics, *B. taurus* α-(1→3)-GalT, UDP-Gal or 5-substituted UDP-Gal derivatives **1 a**–**d** (0.6–400 μM), lactose (2 mM) and $MnCl_2$ (10 mM) in Tris/HCl buffer (50 mM, pH 7.5) were incubated at 37 °C (total volume 100 μL, all concentrations are final concentrations). Enzyme concentrations and reaction times were chosen so as to avoid depletion of donor in excess of 10% (see Table 4). After the appropriate time, the reactions were stopped by cooling in dry ice, and samples were analysed immediately by HPLC.

Table 4. GalT activities and incubation times for enzyme kinetics.

Cmpd	5-Substituent R	α-(1→3)-GalT [mU]	t_{inc} [min]
UDP-Gal	H	0.16	5
1 a	phenyl	3.2	30
1 b	4-MeO-C_6H_4	3.2	60
1 c	2-furanyl	3.2	60
1 d	5-(2-formyl)thienyl	8	10

HPLC analyses were performed on a PerkinElmer Series 200 machine equipped with a column oven, a diode array detector and a Supelcosil LC-18-T column (5 μm, 25 cm × 4.6 mm). Each sample (injection volume 40 μL) was eluted at 30 °C, at a flow rate of 1.5 mL min^{-1}, with a gradient of methanol (2–15%) against phosphate buffer (0.5 M, adjusted to pH 8 with triethylamine). The depletion of donor (UDP-Gal, **1 a**–**d**) and the formation of nucleoside diphosphate, the secondary product of the glycosylation reaction, were monitored at 430 nm. K_m and v_{max} values were determined by fitting data points to a Michaelis–Menten curve ($v = v_{max} \times S \times (K_m + S)^{-1}$) by using GraFit 5.0.10. To assess the hydrolytic stability of **1 d**, two separate control experiments were carried out in the absence of either 1) enzyme (to account for potential chemical hydrolysis) or 2) acceptor (to account for potential enzymatic glycohydrolase activity). No significant degree of hydrolysis was observed in these experiments over a period of 24 h.

Photophysical experiments

General: UV absorbance spectra were recorded on a PerkinElmer Lambda 25 UV/Vis spectrometer at ambient temperature in FarUV quartz cells (path length = 1.0 cm). Fluorescence spectra were recorded on a PerkinElmer LS-45 spectrometer at ambient temperature in a quartz micro fluorescence cell (path length = 1.0 cm).

Quantum yields: UDP-Gal derivatives **1a–d** were serially diluted in H_2O (10, 20, 30, 40, and 50 μM for absorbance measurements, and 0.2, 0.4, 0.6, 0.8, 1 μM for fluorescence measurements), and UV absorbance and fluorescence emission (with λ_{max} absorbance = λ_{ex} fluorescence) were recorded for all samples. To determine quantum yields,[32] for each absorbance and fluorescence spectrum the area under the curve (AUC) was calculated by numerical integration, applying the midpoint rule. For each compound, AUC_{abs} and AUC_{fluo} were then plotted over compound concentration according to $AUC_{abs} = A \times [conc] + B$ and $AUC_{fluo} = A' \times [conc] + B'$. From these linear plots, the gradients A and A' were extracted, and for each compound the specific quantum yield Φ_s, under these experimental conditions, was calculated as the ratio A'/A. Quantum yields determined with this protocol for two reference compounds, 2-aminopyridine (0.60) and L-tryptophan (0.14) were in exact agreement with literature values.[32] The quantum yields for reference compounds were used to calculate the general quantum yield Φ_g for each compound **1a–d**, according to $\Phi_g = \Phi_{ref} \times (A'/A)/(A'/A)_{ref}$.

Microplate assays: Fluorescence-intensity measurements with **1d** were carried out in black NUNC F96 MicroWell polystyrene plates on a BMG LABTECH PolarStar microplate reader equipped with a 350 ± 5 nm excitation filter and a 430 ± 5 nm emission filter. The number of flashes per well was set to 50, the gain to 2240, and the position delay to 0.2 s. Prior to readings, the plates were incubated for 10 min at 30 °C. Prior to fluorescence readings, shaking of the microplate was performed in a double orbital for 10 s (shaking width 4 mm). The gain adjustment for fluorescence readings was performed on the entire microplate. Results were visualised with BMG LABTECH data-analysis software Mars 1.10 and analysed with GraphPad Prism 5.

Assay protocol: Samples were pipetted into the requisite wells of a black NUNC 96-well plate as shown in Tables S1–S3. Key: **B**: Tris/HCl buffer (50 mM, pH 7; 40 μL); **F**: fluorophore **1d** (200 nM in Tris/HCl buffer; 40 μL); **M**: $MnCl_2$ (10 mM in Tris/HCl buffer; 80 μL); **E**: galactosyltransferase (in Tris/HCl buffer; 40 μL); **I**: inhibitor (in Tris/HCl buffer; 40 μL); total volume per well: 200 μL; all concentrations are final concentrations per well. For the calculation of IC_{50} values, requisite data points were fitted to a four-parameter curve with GraphPad Prism 5. All experiments were carried out in triplicate, unless indicated otherwise.

Protocol for library screening: Samples were pipetted into individual wells of a black NUNC 96-well plate as shown in Table S4. Key: **M**: $MnCl_2$ (10 mM in Tris/HCl buffer; 80 μL); **F**: fluorophore **1d** (200 nM in Tris/HCl buffer; 40 μL); **T1–T12**: thiazolidinone inhibitors **2a–l** (50 μM in Tris/HCl buffer containing 5% DMSO; 40 μL); **U**: UDP-Gal (5 mM in Tris/HCl buffer; 40 μL); total volume per well: 160 μL; all concentrations are final concentrations per well. The microplate was incubated for 10 min at 30 °C, and the fluorescence emission was recorded (first reading). Tris/HCl buffer (**B**), α-(1→3)-GalT (**E1**), LgtC (**E2**) or GTB (**E3**; 40 μL) was then added to the requisite wells, as shown in Table S5. The microplate was incubated for another 10 min at 30 °C, and a second fluorescence emission reading was taken (second reading). The relative increase in fluorescence from first to second reading was attributed to the degree of binding of **1d** at the individual GalT in the presence or absence of inhibitor. Thus, for all wells, fluorescence intensity after GalT addition (second reading) was subtracted from fluorescence intensity before GalT addition (first reading) to give ΔFI. For each GalT, the maximum (no displacement of **1d**, e.g., wells A1 and A2) and minimum (displacement of **1d** by UDP-Gal, e.g., wells A3 and A4) changes in fluorescence (ΔFI_{max} and ΔFI_{min}) were calculated. The change in fluorescence in the presence of individual thiazolidinones (ΔFI_T, wells C1–H12) was used to quantify the displacement of **1d** from each GalT, by each thiazolidinone, relative to the displacement of **1d** by UDP-Gal from the same enzyme, according to the following Equation (1):

$$\% \text{ inhibition} = \frac{1-\Delta FI_T}{\Delta FI_{max}-\Delta FI_{min}} \times 100\,\% \quad (1)$$

Acknowledgements

We thank Durita Djurhuus, Dr. Dietlind Adlercreutz and Mette H. Bien (Copenhagen) for the isolation of enzymes, and Sebastian Gehrke, Adam Wiscombe and Ben Young (Norwich) for the synthesis of the thiazolidinone compound library. The plasmid for the N. meningitidis *α-(1→4)-GalT was a generous gift from Dr. Warren W. Wakarchuk (Ottawa). This work was supported by the EPSRC (First Grant EP/D059186/1, to G.K.W.), the MRC (Discipline Hopping Award G0701861, to G.K.W.), the Royal Society (research grant to G.K.W.) and the Danish Natural Science Research Council (to M.M.P.). G.K.W. is a Leverhulme Trust Research Fellow (RF/4/RFG/2008/0544). We thank the EPSRC National Mass Spectrometry Service Centre, Swansea, for the recording of mass spectra.*

Keywords: bioassays · enzymes · fluorescent probes · glycobiology · glycosyltransferases

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Received: January 7, 2010
Published online on June 8, 2010

