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General Tolerance of Galactosyltransferases toward UDP-galactosamine Expands Their Synthetic Capability

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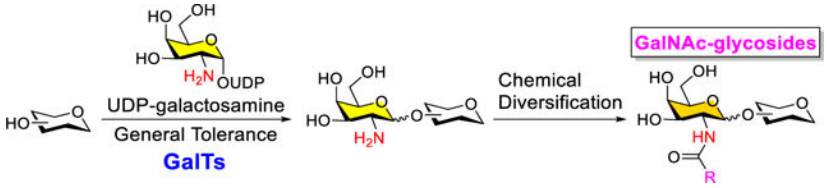
Abstract

Accessing large numbers of structurally diverse glycans and derivatives is essential to functional glycomics. We showed a general tolerance of galactosyltransferases toward uridine-diphosphate-galactosamine (UDP-GalN), which is not a commonly used sugar nucleotide donor. The property was harnessed to develop a two-step chemoenzymatic strategy for facile synthesis of novel and divergent *N*-acetylgalactosamine (GalNAc)-glycosides and derivatives in preparative scales. The discovery and the application of the new property of existing glycosyltransferases expand their catalytic capabilities in generating novel carbohydrate linkages, thus prompting the synthesis of diverse glycans and glycoconjugates for biological studies.

Graphical Abstract



Preparation of diverse glycans is essential to glycobiology, while synthetic-useful enzymes for this purpose are limited. This study discovered a general tolerance of galactosyltransferases (GalTs) toward uncommon donor UDP-galactosamine. A two-step strategy was devised to harness this property for the facile synthesis of GalNAc-glycosides. The newly identified catalytic properties of GalTs would greatly expand their utilization in chemical glycobiology.

Keywords

galactosyltransferase; GalNAc-glycosides; general tolerance; UDP-galactosamine; synthesis

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Conflict of Interest

The authors declare no conflict of interest.

Supporting information for this article is given via a link at the end of the document.

The preparation of large collections of structural-defined glycans, glycoconjugates, and their derivatives, collectively known as “synthetic glycome”,[1] is essential for their structural and functional studies to understand glycan-involved biological processes. In the past two decades, numerous chemical, enzymatic, and chemoenzymatic strategies have been developed to expand the structural diversity of the synthetic glycome.[1–2] Comparing to chemical approaches, enzyme-catalyzed glycan syntheses feature high stereo/regio-selectivity, and permit the use of unprotected donors and acceptors, which are superior especially in the preparation of complex structures. Enzyme catalysts used for glycan synthesis include glycosidases, their engineered mutants (glycosynthases and glycoligases),[3] and glycosyltransferases (GTs). GTs are natural enzymes responsible for the assembly of glycans, thus have been most widely employed. GTs catalyze the transfer of a monosaccharide residue from its activated sugar nucleotide to an acceptor (glycan or other biomolecules) with the formation of a specific glycosidic bond. In recent years, dozens of GTs from microorganisms have been discovered and applied to synthesize diverse N-glycans,[2b] O-GalNAc glycans,[4] O-mannosyl glycans,[5] glycosphingolipids,[6] and human milk oligosaccharide,[7] etc.

Despite continuous efforts in GTs discovery[8] and recent advances in mammalian GT expression,[9] the choice of catalytically robust and synthetically useful GTs is still limited.[10] Some GTs of bacterial origin were found to be promiscuous in substrate specificity and had been employed to access diverse glycan structures and their derivatives. For example, taking advantage of the extremely flexible donor substrate specificity of *Photobacterium damsela* α2–6-sialyltransferase (Pd2,6ST), a panel of sialoside derivatives with modifications on C4, C5, C7, C8, or C9 of the sialic acid residue were prepared.[11] Still, most natural GTs have a rather limited substrate specificity in tolerating substrate modifications. Structure-guided engineering was applied to coerce GTs to catalyze the transfer of desired substrates. For example, wild-type bovine β1–4-galactosyltransferase I (GalT1) could transfer *N*-acetylgalactosamine (GalNAc) from UDP-GalNAc to an *N*-acetylglucosamine (GlcNAc) acceptor, but with only 0.1% of its GalT activity. A single mutant Y289L enhanced its *N*-acetylgalactosaminyltransferase (GalNAcT) activity to an equal level of the GalT activity.[12] However, protein engineering requires substantial efforts, and the acquirement of a successful GT mutant is typically accompanied by dozens of fails. A general strategy to expand the synthetic capabilities of existing wild-type GTs would represent a significant addition to the current synthetic glycobiology toolbox.

Recently, Xu and coworkers developed an enzymatic strategy harnessing a glucosyltransferase to synthesize N-glycopeptides.[13] A key step involves the transfer of a glucosamine (GlcN) residue from the uncommon sugar donor UDP-GlcN onto a peptide acceptor catalyzed by a mutant of *Actinobacillus pleuropneumoniae N*-glucosyltransferase, which was then subjected to C2 acylation and endoglycosidase-catalyzed transglycosylation to produce the intact glycopeptide. This work inspired us to test whether other hexosyltransferases, particularly GalTs, could recognize the uncommon donor UDP-galactosamine (UDP-GalN).

Galactosides and GalNAc-glycosides are ubiquitous in mammalian and bacterial glycomes. They not only participate in biological processes via glycan-protein interactions, but more

importantly, constitute the stem structures of complex glycan epitopes.[14] There are six major types of galactosides found in mammalian glycomes, each with a different glycosidic linkage, including Galα1–3Gal in the α-Gal epitope, Galα1–3(Fucα1–2)Gal in human blood B-antigen, Galα1–4Gal in Globo-series glycans, type 1 (Galβ1–3GlcNAc) and type 2 (Galβ1–4GlcNAc) *N*-acetyllactosamine disaccharides that are common complex glycan precursors, as well as Galβ1–3GalNAc found in glycosphingolipids and *O*-glycans (Figure 1A). Robust GalTs have been discovered for the preparative-scale synthesis of all six types of galactosides, for example, bovine α1–3GalT (bα3GalT) for the synthesis of α-Gal epitope,[15] and human GTB for the synthesis of blood group B-antigen,[16] *etc*. Some GalNAcTs were also exploited to synthesize common GalNAc-glycosides (Figure 1B). For example, human GTA and bacterial homolog BgtA are efficient in catalyzing the synthesis of blood group A-antigen,[16] GalT1 Y289L could efficiently catalyze the formation of LacdiNAc (GalNAcβ1–4GlcNAc),[12] and an α1–3GalNAcT from *Pasteurella multocida* Pm70 was used to prepare the Forssman antigen (GalNAcα1–3GalNAc).[17] So far, no GT has been identified to synthesize the GalNAcβ1–3GalNAc linkage, which is present in the *para*-Forssman antigen. We intend to explore the tolerance of UDP-GalN by synthetically useful GalTs and harness them to synthesize novel GalN-glycosides and GalNAc-glycosides.

UDP-GalN is needed in adequate amounts to evaluate whether GalTs could tolerate this uncommon sugar nucleotide. A conventional synthetic approach to obtain UDP-GalN is by using a hexose-1-phosphate uridylyltransferase (GALT, EC 2.7.7.12) as the catalyst and UDP-glucose (UDP-Glc) and galactosamine-1-phosphate (GalN-1-P) as the starting materials.[18] However, gram-scale separation of UDP-GalN from UDP-Glc and the byproduct Glc-1-P poses a challenge. One-pot three-enzyme (OP3E) systems had been developed to obtain grams of UDP-sugars and derivatives starting from the corresponding monosaccharides.[19] These systems usually contain a kinase to generate the monosaccharide-1-phosphate from the monosaccharide and adenosine triphosphate (ATP), a UDP-sugar pyrophosphorylase to generate the sugar nucleotide, and a pyrophosphatase (PpA) to drive the reaction forward by breaking down the pyrophosphate byproduct. However, although GalN-1-P can be prepared using galactokinase (GalK),[20] it is not recognized by UDP-sugar pyrophosphorylases (e.g., BLUSP[19b] and AGX1[19c]). We envisioned that UDP-GalN would be obtained from UDP-*N*-trifluoroacetylgalactosamine (UDP-GalNTFA)[21] by simple hydrolysis of the trifluoroacetyl (TFA). UDP-GalNTFA is a UDP-GalNAc derivative that can be readily prepared from GalNTFA[21] using an OP3E system with *N*-acetylhexosamine kinase NahK,[22] AGX1,[19c] and PpA (Scheme S1). Accordingly, we prepared 1.2 g of UDP-GalNTFA, which was subsequently treated under a mild basic condition (pH 10, 30 °C, 2 h) to form 1 g of UDP-GalN in quantitative yield (Supporting Information).

With UDP-GalN in hand, we tested the activities of eight most commonly used GalTs toward UDP-GalN and compared them with those using UDP-Gal as the donor in synthetic scale reactions under the same reaction conditions. Surprisingly, UDP-GalN was tolerated by all GalTs tested (Table 1). Remarkably, all three α-GalTs could recognize UDP-GalN very well, as evidenced by comparable yields of the reactions using UDP-GalN to those using the natural donor UDP-Gal. For example, the two α1–3GalTs from bovine (bα3GalT)

and human (GTB) efficiently catalyzed the formation of GalNα1–3Galβ1–4GlcβProN_3 (**5**) and GalNα1–3(Fucα1–2)Galβ1–4GlcβProN_3 (**8**) in high yields of 79% and 87%, respectively, close to those of the reactions for the formation of the corresponding galactosides **4** (α-Gal epitope, 95% yield) and **7** (blood group B-antigen, 93% yield). With the high activity and expression level, *Neisseria meningitidis* α1–4GalT (NmLgtC) had been widely applied to synthesize globo-series glycans and the P1 antigen.[23] Our results showed that NmLgtC could accommodate UDP-GalN, catalyzing the synthesis of GalNα1–4Galβ1–4GlcβProN_3 (N-Gb3, **10**, 265 mg) in an excellent yield of 95%, the same to that of the reactions using UDP-Gal as a donor to produce Gb3 (**9**). This was in consistent with a recent observation that another α1–4GalT from *N. waeveri* (NwLgtC) tolerated UDP-GalN, although with a lower relative activity (32%) compared to that with UDP-Gal as the donor.[24]

The β-GalTs tested also showed a general tolerance to UDP-GalN (Table 1). *Chromobacterium violaceum* β1–3GalT (Cvβ3GalT) is a recently discovered enzyme that enabled multigram-scale synthesis of human milk oligosaccharide lacto-*N*-tetraose (LNT, **12**) and its fucosylated forms.[25] Our results revealed that Cvβ3GalT is highly active toward UDP-GalN, catalyzing the formation of GalNβ1–3GlcNAcβ1–3Galβ1–4GlcβProN_3 (**13**, 10.9 mg) in a yield of 87%. Similarly, *Campylobacter jejuni* β1–3GalT (CjCgtB),[26] which has been used to prepare ganglioside GM1 (**15**) and other Galβ1–3GalNAc-containing glycans (e.g., globopentaose), also accommodates UDP-GalN. As shown in Table 1, CjCgtB exhibited moderate reactivities to both UDP-Gal (72% conversion rate) and UDP-GalN (57% conversion rate) when using GM2βProN_3 (**14**) as an acceptor. On the other hand, three β1–4GalTs tested could tolerate UDP-GalN but had much lower product yields than those with UDP-Gal as the donor substrate. For example, bacterial β1–4GalTs from *N. meningitidis* (NmLgtB) and *Helicobacter pylori* (HpLgtB) catalyzed the synthesis of LacNAc disaccharide (**18**) in excellent yields of 91% and 89%, respectively, whereas the conversion rates declined to 29% and 23% when UDP-GalN was used as the donor. The bovine β1–4GalT (GalT1) showed relatively higher activity with a moderate conversion rate of 49%.

The general tolerance of UDP-GalN by the eight GalTs was further quantified by kinetics studies. As reflected by the k_{cat}/K_m values shown in Table 1, the catalytic efficiencies of all GalTs tested decreased when UDP-GalN instead of UDP-Gal was used as the donor substrate. For instance, the catalytic efficiency of NmLgtB for UDP-GalN (k_{cat}/K_m = 9.5±0.5 $min^{-1}mM^{-1}$) was about 17% of that for UDP-Gal (k_{cat}/K_m = 56.9±3.1 $min^{-1}mM^{-1}$), and the catalytic efficiency of bα3GalT for UDP-GalN (k_{cat}/K_m = 3.1±0.2 $min^{-1}mM^{-1}$) was around 23% of that for UDP-Gal (k_{cat}/K_m = 13.2±1.4 $min^{-1}mM^{-1}$). Specifically, NmLgtC was equally efficient in recognizing UDP-Gal (k_{cat}/K_m = 34.6±1.3 $min^{-1}mM^{-1}$) and UDP-GalN (k_{cat}/K_m = 31.6±7.1 $min^{-1}mM^{-1}$). The catalytic efficiencies of other GalTs for UDP-GalN were 27–52% of those for UDP-Gal. Nevertheless, all tested GalTs could utilize UDP-GalN for the preparative-scale synthesis (4.4 to 165 mg) of corresponding GalN-glycosides with 23–95% yields. These results suggest a possible general tolerance of GalTs to UDP-GalN, which may be attributed to the similar size and reactivity of the C2 hydroxyl group in Gal and the C2 amino group in GalN. Interestingly,

UDP-GalN was also tolerated by enzymes that are responsible for the biosynthesis of UDP-Gal, including GALT[18] and UDP-Gal 4'-epimerase (GalE).[27]

We then performed molecular docking based on the crystal structures of bα3GalT (PDB: 1G93[28]) and GalT1 (PDB: 1O0R[29]), both in complex with UDP-Gal, to rationalize the substrate tolerance of GalTs to UDP-GalN (Supporting Information). As shown in Figure S1B and S2B, UDP-GalN fits nicely into the donor binding pocket of bα3GalT and GalT1, with calculated minimized binding energy and orientation similar to those of UDP-Gal. In bα3GalT, the Gal and GalN residues are surrounded by Arg202, Asp225, Trp314, Asp316, and Glu317, forming 6 and 5 hydrogen bonds, respectively, with these residues in the binding pocket (Figure S1C, S1D). In GalT1, the Gal and GalN residues are surrounded by Asp252, Lys279, Gly292, Trp314, and Glu317, forming 5 and 6 hydrogen bonds, respectively, with these residues (Figure S2C, S2D). In both structures, an acidic amino acid (Asp225 in bα3GalT, Asp252 in GalT1) forms one hydrogen bond with either C2-OH in Gal or C2-NH_2 in GalN. Noting that the C2-NH_2 can be protonated under reaction conditions, which may contribute to the relatively lower activity of the GalTs in using UDP-GalN as the donor substrate compared to UDP-Gal.

Having demonstrated the general tolerance of GalTs to UDP-GalN, we developed a two-step GalT-catalyzed strategy for the synthesis of novel GalNAc-glycosides and derivatives. As illustrated in Figure 2A, the strategy involves the synthesis of GalN-glycosides by wild-type GalTs (Step 1), followed by selective acylation of the amine to access diverse GalNAc-glycosides (Step 2). The approach is superior to conventional methods of generating and using UDP-GalNHR from GalNHR directly (Figure 2B) because 1) wild-type GalTs can be readily used to generate GalN-glycosides with novel carbohydrate linkages that are not accessible by known GalNAcTs, and 2) selective acylation of the amine in the products readily installs various functional groups without being limited by the substrate specificity of GalTs, forming diverse *N*-substituted GalNAc-glycosides. In contrast, conventional methods rely on the relaxed substrate specificities of GalNAcTs as well as substrate promiscuity of related sugar donor biosynthetic enzymes such as HexNAc kinases and UDP-sugar pyrophosphatases for *in situ*-generation of UDP-GalNAc derivatives, which are not always achievable.

To showcase the efficiency of the strategy, we carried out the two-step synthesis of Shiga toxin 2 ligand GalNAcα1–4Galβ1–4Glc (NAc-Gb3) and derivatives. Shiga toxins 1 and 2 (Stx1 and Stx2) are major virulence factors of Shiga toxin-producing *Escherichia coli* (STEC). While Stx1 recognized Gb3 (**9**), it was observed that Stx2 preferably binds to NAc-Gb3, a chemically prepared Gb3 derivative.[30] A corresponding α1–4GalNAcT for generating the GalNAcα1–4Gal linkage in NAc-Gb3 has not been discovered. The two-step strategy would enable us to access GalNAcα1–4GalβOR and derivatives with a terminal GalNHR featuring different N-acyl groups. As shown in Scheme 1, selective acylation of the amine in N-Gb3βProN_3 (**10**) generated by NmLgtC-catalyzed reaction with UDP-GalN as the donor produced the Stx2 ligand NAc-Gb3βProN_3 (**20**) and derivatives (**21**−**28**). For example, selective acylation of **10** using acetic anhydride in MeOH afforded NAc-Gb3βProN_3 (**20**) in an excellent yield (97%). Similarly, acylation of **10** using ethyl trifluoroacetate in the presence of Na_2CO_3

generated *N*-trifluoroacetylgalactosamine-analog of Gb3 (NTFA-Gb3βProN_3, **21**) in 91% yield. In addition, various carboxylic acids were used to produce C2-substituted Gb3βProN_3 derivatives with a terminal *N*-azidoacetylgalactosamine (NAz-Gb3βProN_3, **22**), diazirine-modified GalNHR (DAz-Gb3βProN_3, **23**), *N*-propanoylgalactosamine (NPr-Gb3βProN_3, **24**), and *N*-butanoylgalactosamine (NBu-Gb3βProN_3, **25**) in 72−95% yields. Due to steric hindrance and limitations in substrate specificity, installing large chemical groups onto glycans by using conventional GTs is often not possible. Significantly, we succeeded in the installation of biorthogonal groups or fluorescent probes (e.g. biotin, a fluorescent probe Atto495, and Fmoc-N-amido-dPEG_4) to GalN-glycoside **10** via *N*-hydroxysuccinimide (NHS)-amine crosslinking to form compounds **26**−**28** in excellent yields (95–99%). Collectively, by surpassing the substrate specificity limitations of GTs and other enzymes, this two-step GalT-catalyzed approach could achieve facile syntheses of a large variety of novel GalNAc-glycosides and derivatives in a highly efficient manner.

To explore the application of the novel GalN-glycosides and GalNAc-glycosides prepared here, we evaluated their recognition by GBPs (Figure S3). As expected, Gal-specific lectins including *Ricinus communis* agglutinin I (RCA-I) and *Griffonia simplicifolia* lectin I isolectin B4 (GS-I-B4) could recognize GalN-glycosides, but with significantly reduced signals compared to their Gal-glycoside counterparts. On the other hand, GalNAc-specific lectins soybean agglutinin (SBA) and *Helix pomatia* agglutinin (HPA) could tolerate various *N*-substituted GalNAc-glycosides and ligands. Interestingly, cholera toxin subunit B (CTB) showed comparable binding signals to GM1 (**15**) and its GalN-substitution (**16**) (Figure S3). Binding profiles of other GBPs are detailed in the Supporting Information.

To showcase the application of the two-step approach, we developed a practical synthetic route to access the *para*-Forssman antigen (GalNAcβ1–3GalNAcβ1–3Galα1–4Galβ1–4Glc), which is among the many glycolipid antigens identified from human erythrocyte membrane[31] but has yet been studied in detail. One report described the observation of significantly higher anti-*para*-Forssman IgG levels in patients with gastric and colorectal cancer than controls,[32] suggesting a potential altered expression of *para*-Forssman glycolipid in pathogenic conditions. Total chemical synthesis of protected *para*-Forssman pentasaccharide was achieved previously, but with a very low overall yield (<3%).[33] In addition, no GT has been found to catalyze the synthesis of the GalNAcβ1–3GalNAc linkage. The two-step approach enabled efficient chemoenzymatic synthesis of the *para*-Forssman antigen. As elaborated in Scheme 2, Gb4βProN_3 (**29**) was enzymatically prepared in excellent yields from LacβProN_3 (**3**) in two steps by reactions catalyzed by NmLgtC and *Haemophilus influenza* β1–3GalNAcT (HiLgtD)[23a] respectively. Pentasaccharide GalNβ1–3GalNAcβ1–3Galα1–4Galβ1–4GlcβProN_3 (**30**) was then generated in a moderate yield of 36% from **29** and UDP-GalN via CjCgtB-catalyzed reaction. Subsequent selective acetylation of the free amine on the terminal GalN residue afforded the desired *para*-Forssman antigen (**31**) quantitively. The yield over the four steps was 31%.

In conclusion, we demonstrated the general tolerance toward the uncommon donor UDP-GalN by all synthetically useful GalTs tested here. The newly discovered property was harnessed to develop an efficient two-step chemoenzymatic strategy to generate novel GalN-glycosides and GalNHR-glycosides with novel glycosidic linkages not accessible by known

GalNAcTs. Although further investigation is needed, the tolerance of UDP-GalN could be shared by many other GalTs. A panel of GalN-glycosides were successfully prepared with yields ranging from 57−95% (NmLgtB had a lower yield of 29%), which were comparable to the 72−96% yields obtained for the reactions with the natural donor UDP-Gal. The obtained GalN-glycosides were readily derivatized by selective acylation in high yields, generating corresponding GalNAc-glycosides and *N*-substituted derivatives. Significantly, large fluorescent groups or affinity handles such as biotin can be efficiently installed on GlaN loaded glycans (e.g., **26**−**28**). The two-step GalT-catalyzed strategy is efficient for the synthesis of novel GalNAc-glycosides as demonstrated by the preparation of *para*-Forssman antigen with an overall yield of 31% over four steps using known GalTs. Such a new application of existing glycosyltransferase catalysts could readily be used to expand the synthetic glycome. In addition, synthesized GalN-glycosides, GalNAc-glycosides, and their derivatives are novel tools to probe the ligand specificity of GBPs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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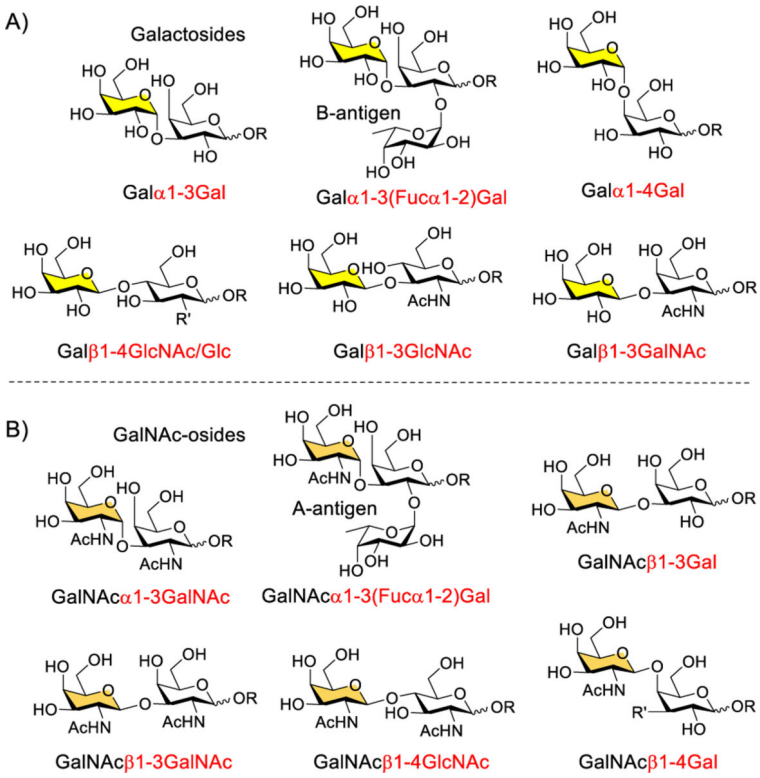


Figure 1.
Common mammalian galactosides (A) and GalNAc-glycosides (B) with glycosidic linkage information.

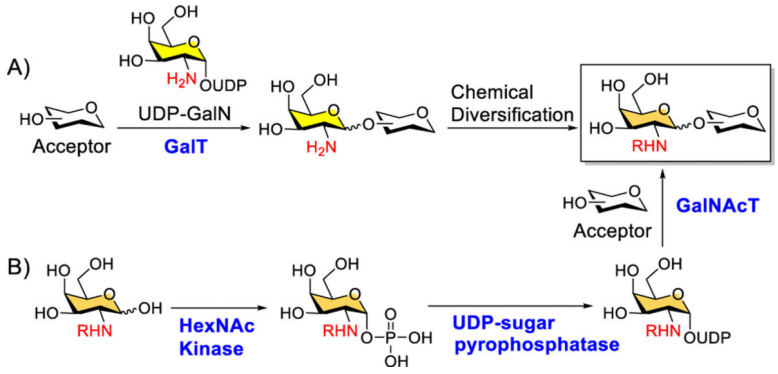
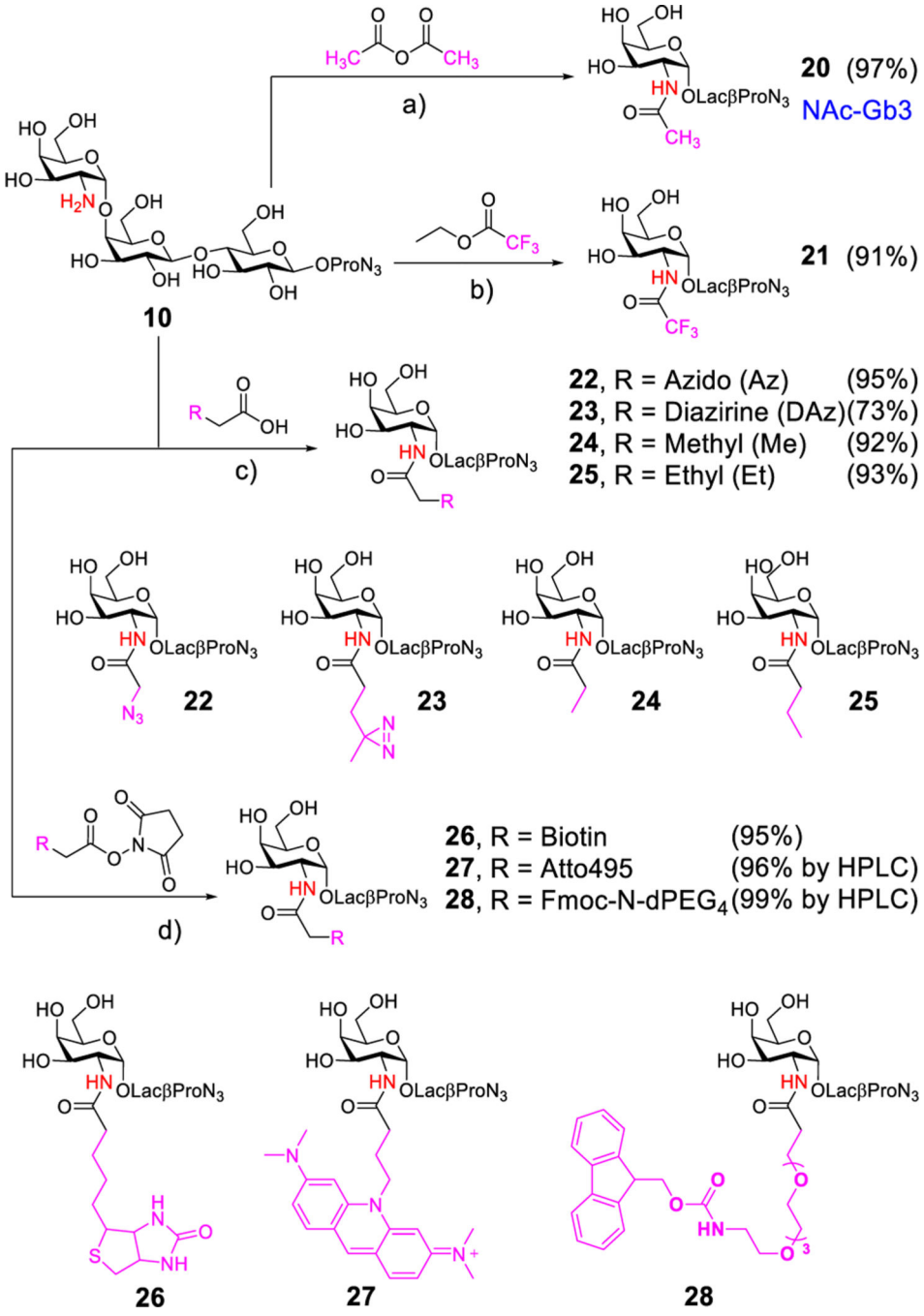


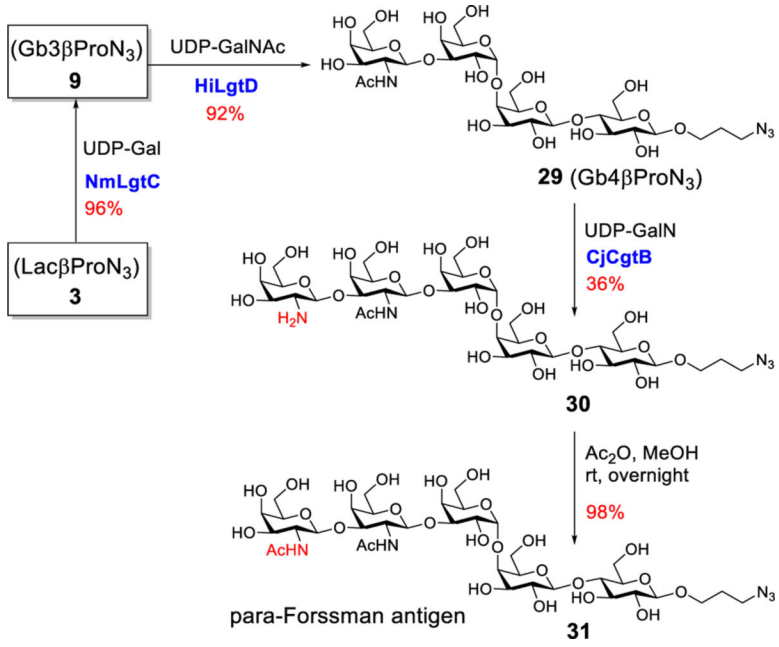
Figure 2.

Two-step GalT-catalyzed approach (A) versus the conventional approach (B) to access diversified GalNAc-glycosides and derivatives.



Scheme 1.

Chemical diversification of compound **10** at the amino group of GalN. a) MeOH, rt, overnight, b) Na_2CO_3, MeOH, rt, overnight, c) EDCI, HOBT, DMF, rt, overnight, d) H_2O: DMF = 1:1 (v/v), rt, overnight.

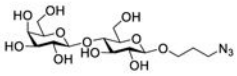
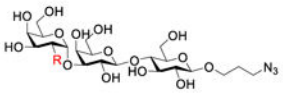
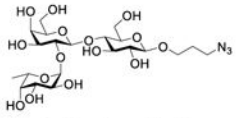
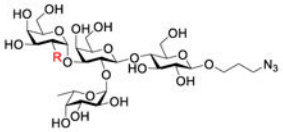
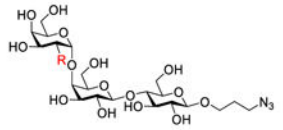
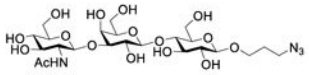
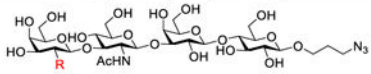
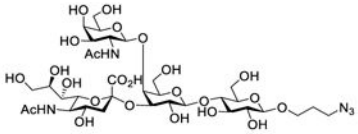
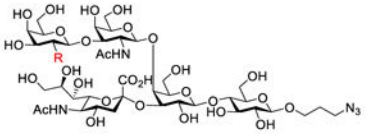
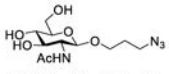
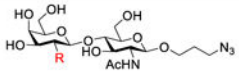


Scheme 2.

Chemoenzymatic synthesis of the *para*-Forssman antigen. HiLgtD, β1–3GalNAcT from *Haemophilus influenza*.

Table 1.

Synthesis of various galactosides and their 2-amino-counterparts using wild-type GalTs.

GalTs	Acceptor	Product	Yield (%)		k_{cat}/K_m ($min^{-1}mM^{-1}$)	
			Donor **1** UDP-Gal	Donor **2** UDP-GalN	Donor **1**	Donor **2**
bα3GalT	 **3**, LacβProN$_3$	 **4** (R =OH); **5** (R = NH$_2$)	**4** (95%) 13.0 mg	**5** (79%) 10.9 mg	13.2±1.4	3.1±0.2
GTB	 **6**, H-antigen-ProN$_3$	 **7** (R =OH); **8** (R = NH$_2$)	**7** (93%) 11.9 mg	**8** (87%) 11.2 mg	64.3±8.6	18.4±1.6
NmLgtC	**3**, LacβProN$_3$	 **9** (R =OH); **10** (R = NH$_2$)	**9** (96%) 13.3 mg	**10** (95%) 265 mg	34.6±1.3	31.6±7.1
Cvβ3GalT	 **11**, GlcNAcβ1-3LacβProN$_3$	 **12** (R =OH); **13** (R = NH$_2$)	**12** (97%) 12.2 mg	**13** (87%) 10.9 mg	89.5±8.1	46.5±0.3
CjCgtB	 **14**, GM2βProN$_3$	 **15** (R =OH); **16** (R = NH$_2$)	**15** (72%) 8.4 mg	**16** (57%) 6.7 mg	24.4±0.6	6.8±0.5
NmLgtB	 **17**, GlcNAcβProN$_3$	 **18** (R =OH); **19** (R = NH$_2$)	**18** (91%) 13.9 mg	**19** (29%) 4.4 mg	56.9±3.1	9.5±0.5
HpLgtB	**17**, GlcNAcβProN$_3$	**18**; **19**	**18** (89%)[a]	**19** (23%)[a]	30.1±0.8	8.1±0.3
GalT1	**17**, GlcNAcβProN$_3$	**18**; **19**	**18** (89%)[a]	**19** (49%)[a]	30.0±0.1	10.9±0.8

[a]Yields were determined by HPLC.