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# Characterization of a Galactosynthase Derived from *Bacillus circulans* β-Galactosidase: Facile Synthesis of D-Lacto- and D-Galacto-*N*-bioside

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Glycosynthases—retaining glycosidases mutated at their catalytic nucleophile—catalyze the formation of glycosidic bonds from glycosyl fluorides as donor sugars and various glycosides as acceptor sugars. Here the first glycosynthase derived from a family 35 β-galactosidase is described. The Glu→Gly mutant of BgaC from *Bacillus circulans* (BgaC-E233G) catalyzed regioselective galactosylation at the 3-position of the sugar acceptors with α-galactosyl fluoride as the donor. Transfer to 4-nitophenyl α-D-*N*-acetyl-glucosaminide and α-D-*N*-acetylgalactosaminide yielded 4-nitophenyl α-lacto-*N*-biose and α-galacto-*N*-biose, respectively, in high yields (up to 98%). Kinetic analysis revealed that the high affinity of the acceptors contributed mostly to the BgaC-E233G-catalyzed transglycosylation. BgaC-E233G showed no activity with β-(1,3)-linked disaccharides as acceptors, thus suggesting that this enzyme can be used in "one-pot synthesis" of LNB- or GNB-containing glycans.

Carbohydrates are essential components of a variety of important glycoconjugates, including glycolipids, glycoproteins, and nucleotide sugars. Therefore, they play a key role in numerous biological processes, such as signal transduction, tumor progression, and energy metabolism.[1] Of the various functional oligosaccharides and glycans, galacto-*N*-biose (GNB, Gal-β-(1,3)-GalNAc) and lacto-*N*-biose (LNB, Gal-β-(1,3)-GlcNAc) glycoconjugates (which contain a Gal-β-(1,3) linkage) have one of the more important carbohydrate structures. They occur in a variety of bioactive molecules, such as glycosphingolipids (e.g., GM1, GD1, and GT1), carbohydrate moieties of glycoproteins and glycolipids (e.g., type I glycans, Lewis a, sialyl Lewis a, and Lewis b antigens), human milk oligosaccharides (lacto-*N*-tetraose and its sialylated and fucosylated derivatives), and Thomsen–Friedenreich tumor-associated antigens (TF, or T-antigen).[2]

To understand the cellular functions of GNB- and LNB-containing oligosaccharides and glycoconjugates, tools are required for the efficient synthesis of Gal-β-(1,3)-HexNAc (GlcNAc or GalNAc) disaccharides. Because of the relatively simple and mild reaction conditions and the high stereospecificity of the glycosidic bonds, enzymatic synthesis has been considered as an alternative to conventional chemical methods.[3] Although naturally occurring galactosylations are performed by galactosyl transferases with activated nucleotide galactoside as the sugar donor, galactosidase-catalyzed galactosylations have also been reported. β-Galactosidase-catalyzed transglycosylations yield galacto-oligosaccharides containing usually β-1,4 or β-1,6 (but occasionally β-1,3) linkages.[4] In contrast, several β-galactosidases from glycoside hydrolase (GH) family 35, including a β-galactosidase from *Bacillus circulans* (BgaC; accession: BAA21669.1)[5] exhibit high specificity for the hydrolysis of terminal Gal β-(1,3)-HexNAc (GlcNAc or GalNAc) sugar linkages in gangliosides.[6] They also exhibit transglycosylation activity in the synthesis of galactosyl β-1,3-linked transfer products, but with modest yields because of the significant rehydrolysis of the transfer products by the enzyme.[7,8] Several successful attempts, with ionic liquid as the co-solvent to improve the yields of the reactions catalyzed by wild-type (WT) BgaC, resulted in accumulation of transfer products in the reaction mixtures, with high yields because of the low hydrolysis of the products.[9] In addition, coupled enzyme systems that used D-galactosyl-β-(1,3)-*N*-acetyl-D-hexosamine phosphorylase with enzymes that supply galactosyl-1-phosphate as the donor produced LNB and GNB with yields above 90%.[10] Despite the high efficiency of these enzymatic methods, there are still limitations, such as the high cost of the ionic liquids, the requirement for an expensive cofactor (adenosine triphosphate) for the kinase reaction, and different optimal conditions for each of the individual enzymes used in the coupled-enzyme system. As an engineered glycosidase, a thioglycoligase derived from a GH35 β-galactosidase from *Xanthomonas manihotis* was capable of catalyzing transglycosylation when using various thiosugars as acceptors to form galactosyl-*S*-β-(1,3)-linked disaccharides, including thio-linked LNB.[11]

In the last decade, glycosynthases have emerged as a powerful tool for the synthesis of oligosaccharides, polysaccharides, glycolipids, and glycopeptides.[12,13,14] Engineered glycosynthases are mutants of retaining glycosidases in which nucleophile residues are mutated to non-nucleophile residues (e.g., Ala, Ser, or Gly; Scheme 1).[15] They do not show obvious hydrolytic activity with the substrates of the parent enzyme, but show significant transglycosylation activity with glycosyl fluorides or glycosyl azides of the opposite stereochemistry to that of the original substrates. Lack of hydrolysis of the transfer products leads to high reaction yields.[14] Although glycosynthases offer a versatile strategy for the synthesis of oligosaccharides, there is no report of a successful GH35-derived glycosynthase that is able to form Gal-β-(1,3)-HexNAc linkages. Recently, a glycosyn-

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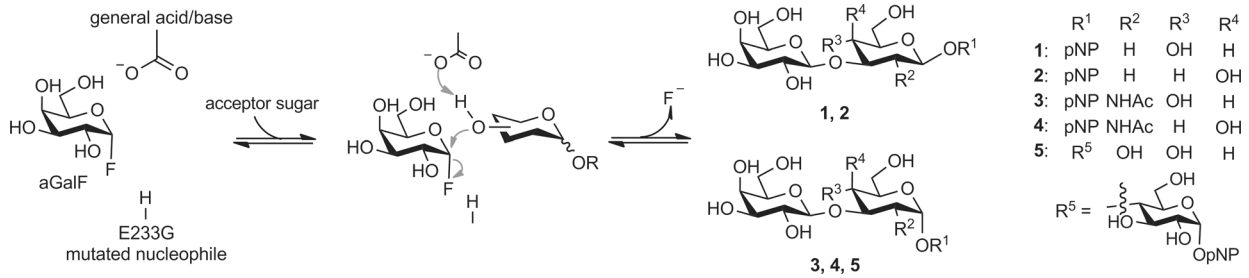


**Scheme 1.** Mechanism of a glycosynthase derived from BgaC (BgaC-E233G).

thase derived from a GH1 β-glycosidase from *Thermus thermophilus* was used in a chemoenzymatic strategy to synthesize LNB from a 2-deoxy-2-amino sugar acceptor, followed by specific *N*-acylation.[16] However this reaction required time-consuming multi-step protection and deprotection processes. In this study, we describe for the first time a galactosynthase derived from GH35 β-galactosidase. A BgaC mutant exhibited highly regioselective glycosynthase activity and yielded disaccharides containing a galactosyl-β-(1,3)-linkage, including LNB and GNB.

Ala, Gly, and Ser were used as candidate substitutions for the catalytic nucleophile (E233) of BgaC. All three mutants (BgaC-E233A, E233S, and E233G) were purified to homogeneity by Ni-NTA affinity chromatography. Transglycosylation reactions were then carried out with α-D-galactopyranosyl fluoride (αGalF) and 4-nitrophenyl β-D-glucopyranoside (pNPβGlc) as the sugar donor and acceptor, respectively, to investigate the catalytic properties of these BgaC mutants. All reactions were incubated for 5 h, and the products were then detected by TLC, with 4-nitrophenyl β-D-galactopyranosyl-β-(1,4)-D-glucopyranoside (pNPβLac) as the standard. With the Ala and Ser mutants, no detectable transfer products were detected, although galactose spots were evident (Figure 1, lanes 2 and 4).

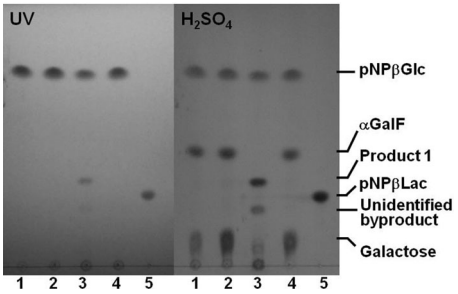


**Figure 1.** TLC analysis of the reaction mixtures catalyzed by BgaC nucleophile mutants. Lane 1, blank reaction; lane 2, reaction by BgaC-E233A; lane 3, reaction by BgaC-E233G; lane 4, reaction by BgaC-E233S; lane 5, pNPβLac (control).

The amounts of galactose produced by the Ala and Ser mutants (larger than for the blank reaction) suggest that these mutants hydrolyzed αGalF rather than using it as a donor substrate for transglycosylation. In contrast, the Gly mutant (BgaC-E233G) produced transfer products with $R_f$ values different from that of pNPβLac (Figure 1, lane 3). When the reaction mixture was assessed by LC-MS, there was no peak corresponding to trisaccharides (data not shown), thus suggesting that the transfer product was a disaccharide with glycosidic linkages different from that of pNPβLac.

Eighteen aryl sugar acceptors (see the Supporting Information) were evaluated to investigate the acceptor specificity of BgaC-E233G. (Ala and Ser mutants were also tested, but showed no detectable TLC UV-active spots expected for a transfer product; data not shown). The mixtures for all reactions catalyzed by BgaC-E233G were incubated at 25 °C for 5 h with αGalF as the sugar donor, and were monitored by TLC. Of the 18 aryl sugars, 5 glycosides (pNPβGlc, 4-nitrophenyl β-D-galactopyranoside (pNPβGal), 4-nitrophenyl α-D-2-*N*-acetyl-glucosaminide (pNPαGlcNAc), 4-nitrophenyl α-D-2-*N*-acetyl-galactosaminide (pNPαGalNAc), and 4-nitrophenyl α-D-maltoside (pNPαG2)) were identified as acceptors for BgaC-E233G after TLC analysis of the reaction mixtures (Table S1 and Figure S2).

Next, preparative-scale reactions catalyzed by BgaC-E233G were carried out with the five successful acceptors (pNPβGlc, pNPβGal, pNPαGlcNAc, pNPαGalNAc, and pNPαG2). The transfer products (**1**, **2**, **3**, **4**, and **5**, respectively, Scheme 1) were then purified by flash chromatography (isolation yields: 54, 42, 97, 98, and 92%, respectively), and structurally identified by NMR analysis ($^1$H COSY and $^1$H,$^{13}$C HSQC correlation spectra) and ESI-MS (Figure S3). All signals for the anomeric protons (including H-1, H-1′, and H-1″) appeared around 4.5–5.5 ppm as typical doublet peaks (Table 1). The coupling constant of H-1′ of the products (with the exception of **5**) is relevant to the synthetic sugar linkage catalyzed by BgaC-E233G. Because of the high values of the coupling constant $J_{1,2}$ (7.4–8.3 Hz), the anomeric configurations of the synthetic linkages within **1**, **2**, **3**, and **4** were β-type. H-1″ in the trisaccharide product **5** also exhibited a high coupling constant (up to 7.8 Hz), thus suggesting that the anomeric configuration of its synthetic linkage is also a β-type. Next, the regioselectivity of BgaC-E233G was determined by $^{13}$C NMR. Based on previous studies, the chemical shift of glycosylated C-3 (80–85 ppm) is significantly downfield relative to unmodified C-3 (75–80 ppm).[17] In our study, the chemical shift of C-3 in the standard pNPβLac was 77.21 ppm, whereas the chemical shifts of C-3 in the products (except **5**) appeared at 81–87 ppm, thus suggesting that these four products contained (1,3) rather than (1,4) or (1,6) linkages. The C-3′ of transfer product **5** (trisaccharide) exhibited a signal at 85.01 ppm, which also corresponds to a (1,3)-linkage. In addition, there was no significant change in the chemical shifts

**Table 1.** Structural identification of transfer products by NMR.

| Signal (ppm) | pNPβLac | Transfer product 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|---|
| H1 | 5.27 (*J*=7.9) | 5.31 (*J*=7.7) | 5.28 (*J*=8.2) | 5.85 (*J*=3.2) | 5.80 (*J*=1.2) | 5.82 (*J*=3.7) |
| H1′ | 4.73 (*J*=8.3) | 4.76 (*J*=7.9) | 4.71 (*J*=7.4) | 4.55 (*J*=7.8) | 4.51 (*J*=8.3) | 5.47 (*J*=3.6) |
| H1′′ | –[a] | – | – | – | – | 4.63 (*J*=7.8) |
| C3 | 77.21 | 86.90 | 85.64 | 82.49 | 81.74 | 75.43 |
| C3′ | – | – | – | | – | 85.01 |

[a] There is no corresponding atom.

towards C-2, C-4, and C-6 of the five transfer products (see the NMR data in the Supporting Information). Consequently, the synthesized sugar linkages of all five transfer products were confirmed as the expected β-(1,3) linkages.

However, the structure of a by-product in the reaction of pNPβGlc (Figure 1) was not analyzed, as the reaction yield (< 5%) was too low to be isolated by silica gel chromatography. Given the regioselectivity of the transglycosylation catalyzed by wild-type BgaC, the minor product was likely a Gal-β-(1,6)-linked disaccharide.[8] In contrast, there was no by-product with other types of linkages from the other reactions. It is worth noting that the WT BgaC-catalyzed transglycosylation yielded various regioisomers, depending on the acceptor.[8] One of the drawbacks of glycosynthases is oligomerization by the additional glycosylation of the transfer products.[12,18] However, HPLC and LC-MS analyses revealed that there was no trisaccharide (data not shown), thus suggesting that BgaC-E233G showed no activity with β-(1,3)-linked disaccharides as acceptors. Therefore, this enzyme can be used in "one-pot synthesis" of LNB or GNB-containing glycans.

To determine the substrate specificity and catalytic profile of the β-galactosynthase further, the apparent kinetic parameters were determined at a fixed concentration of either the donor or acceptor (Table 2 and Figure S4). The kinetic analysis revealed that BgaC-E233G showed higher catalytic efficiency ($k_{cat}/K_M$) for α-configured glycosides than for β-configured glycosides (5–16-fold). Of the acceptors, pNPβGal was the least efficient substrate: $k_{cat}=0.28\ min^{-1}$, an order of magnitude lower than for the other substrates (1.0–2.3 $min^{-1}$). Although the $k_{cat}$ for pNPβGlc was identical to that for pNPαGalNAc, its catalytic efficiency was much lower because of its high $K_M$ value. Therefore, high affinity of the acceptors contributed most strongly to the BgaC-E233G-catalyzed transglycosylation. When the donor sugar (αGalF) was varied, all acceptors showed $K_M$ values for αGalF of around 0.9 mM.

**Table 2.** Kinetics of BgaC-E233G-catalyzed transglycosylation reactions.

| Variable substrate[a] | Fixed substrate | $k_{cat}$ [$min^{-1}$] | $K_M$ [mM] | $k_{cat}/K_M$ [$min^{-1}\,mM^{-1}$] |
|---|---|---|---|---|
| pNPβGlc | αGalF | 1.5 ± 0.1 | 3.1 ± 0.2 | 0.49 |
| pNPβGal | αGalF | 0.28 ± 0.02 | 1.2 ± 0.1 | 0.22 |
| pNPαGlcNAc | αGalF | 1.8 ± 0.08 | 0.71 ± 0.08 | 2.4 |
| pNPαGalNAc | αGalF | 1.5 ± 0.07 | 0.41 ± 0.06 | 3.6 |
| pNPαG2 | αGalF | 1.9 ± 0.08 | 0.58 ± 0.06 | 3.3 |
| αGalF | pNPβGlc | 1.0 ± 0.05 | 0.93 ± 0.1 | 1.1 |
| αGalF | pNPβGal | 0.29 ± 0.02 | 0.91 ± 0.1 | 0.31 |
| αGalF | pNPαGlcNAc | 2.3 ± 0.1 | 0.89 ± 0.06 | 2.5 |
| αGalF | pNPαGalNAc | 2.0 ± 0.09 | 1.0 ± 0.1 | 1.9 |
| αGalF | pNPαG2 | 1.9 ± 0.08 | 0.82 ± 0.07 | 2.4 |

[a] Concentrations of αGalF and pNP glycosides were fixed at 2 mM.

Interestingly, the acceptor specificity of BgaC-E233G was different from that of WT BgaC, which produced galactosyl β-(1,3)-products from both α- and β-configured GlcNAc and GalNAc as acceptors, with a preference for HexNAc with the α-configuration.[8] Although BgaC-E233G exhibited the same preference as the WT enzyme, it had no activity towards pNPβGalNAc or pNPβGlcNAc. This preference is likely attributable to a hydrophobic pocket at the acceptor-binding site; this would prevent proper binding of the 1,2-*cis* configuration of pNP and 2-*N*-acetyl groups of acceptors. Structural analysis of a β-(1,3)-galactosidase from *Streptococcus pneumoniae* (based on a model structure) suggested that W240 and Y455 form hydrophobic interactions with the *N*-acetyl group and the sugar ring of GlcNAc.[19] These residues are conserved in BgaC (W235 and Y450). Although it remains unclear as to why pNPβGlcNAc and pNPβGalNAc were not substrates for the glycosynthase, they are also poor substrates for WT BgaC.[8] For galactosides, WT BgaC can use both α- and β-galactosides with similar reaction yields,[8] whereas only pNPβGal is used for the glycosynthase. Surprisingly, WT BgaC exhibited exclusive regioselectivity towards 6-OH of pNPβGal,[8] whereas BgaC-E233G retained β-1,3-regioselectivity for pNPβGal. In addition, there are no reports of transglycosylation of pNPβGlc by WT BgaC. Interestingly pNPαG2 (a glucoside with the α configuration) was a strong acceptor for BgaC-E233G. However this was unsurprising, because this is consistent with a study of the aglycone specificity of WT BgaC: maltotriose (which pNPαG2 mimics) was the best of 44 potential acceptor sugars.[20] This suggests that several hydrophilic interactions between the +2 subsite of BgaC and the second α-configured glucose unit could yield a productive binding mode comparable with pNPαGlc, which can generate only hydrophobic interactions between the +2 subsite of BgaC and the pNP group. Neither α- nor β-configured mannosides and xylosides (glucoside analogues) could be accommodated by BgaC-E233G (data not shown). Therefore, the equatorial 2-OH and 6-hydroxymethyl groups were important for BgaC-E233G-catalyzed transglycosylation.

In conclusion, we developed the first glycosynthase derived from GH35. BgaC-E233G exhibited transglycosylation activity with high regioselectivity for the formation of β-(1,3)-galactosidic bonds towards Glc, Gal, GlcNAc, and GalNAc. High yields of pNPαLNB and pNPαGNB were obtained (up to 98%). Because of the preference for α-configured HexNAc, this glycosynthase

could be used for the synthesis of glycopeptides containing the TF antigen.

## Experimental Section

**General experiments:** *Escherichia coli* TOP10 and BL21(DE3) were used for DNA manipulation and protein production, respectively. Plasmid pET29-BgaC, the template for the mutagenesis, was kindly provided by Professor Stephen G. Withers, University of British Columbia. Pwo polymerase was purchased from Roche Applied Science, and restriction enzymes were from Fermentas (Thermo Scientific). αGalF was synthesized as described previously.[21] 4-Nitrophenyl glycosides and silica gel (200–425 mesh) for flash chromatography were purchased from Sigma–Aldrich. HPLC was carried out with a YL9100 HPLC system (Younglin, Anyang, Korea) equipped with a Nova-Pak C18 column (3.9×150 mm; Waters, Milford, MA). The products were eluted at 0.8 mL min$^{-1}$ with acetonitrile/water (10:90, *v/v*) and analyzed with an ultraviolet (UV) detector (254 nm). $^{1}$H and $^{13}$C NMR spectra were recorded on a 400 MHz spectrometer (JEOL, Tokyo, Japan) with $D_2O$ as the solvent and sodium 2,2-dimethyl-2-silapentane-5-sulfonate as the internal standard. Mass spectra for transfer products were recorded with an LTQ XL linear ion trap mass spectrometer (Thermo Scientific). TLC was performed on aluminum-backed 0.2 mm silica gel 60$F_{254}$ sheets (Whatman/GE Healthcare) with ethyl acetate/methanol/water (7:2:1, *v/v/v*). The plates were visualized under UV (254 nm) and/or by exposure to sulfuric acid (10%) in methanol followed by charring.

**Construction of BgaC mutants:** Mutagenesis of the nucleophile residues of BgaC was conducted with pET29-BgaC[22] as the template by a four-primer method. For the introduction of the E233A mutation, two synthetic primers (T7 promoter primer, and BgaC-E233-rev (5′-CATGC ACATT AGAGG AGC-3′)) were used to amplify the front part of the gene fragment; BgaC-E233A-fw (5′-GCTCC TCTAA TGTGC ATGGC ATTTT GGCAT-3′, the mutated codon for E233A is underlined) and the T7 terminator primer were used for the rear gene fragment. The two PCR products were purified separately by using an agarose gel extraction kit (iNtRON Biotechnology, Seongnam, Korea). PCR products (20 ng each) were then combined for primerless assembly PCR. After seven cycles with Pwo polymerase, the T7 promoter and T7 terminator primers were added to the reaction mixture (50 μM each), and PCR was continued for an additional 25 cycles. The resulting PCR product was digested with NdeI and XhoI, and subcloned into pET29a (Novagen/Merck Millipore) to generate pET29-BgaC-E233A. Primers BgaC-E233G-fw (5′-GCTCC TCTAA TGTGC ATGGG ATTTT GGCAT-3′) and BgaC-E233S-fw (5′-GCTCC TCTAA TGTGC ATGAG CTTTT GGCAT-3′) replaced BgaC-E233A to construct pET29-BgaC-E233G and pET29-BgaC-E233S, respectively.

**Production and purification of BgaC mutants:** *E. coli* carrying recombinant pET29a (containing a *BgaC* variant) was cultured overnight in lysogeny broth (1 L; tryptone (1% *w/v*), yeast extract (0.5% *w/v*), NaCl (0.5% *w/v*)) supplemented with kanamycin (20 μg mL$^{-1}$) at 37 °C until $OD_{600}$ = 0.5. Isopropyl-β-D-thiogalactopyranoside (0.2 mM) was added to induce protein expression, and after further culturing (20 h, 20 °C with shaking), the cells were harvested, resuspended in Tris·HCl (50 mM, pH 7.5), and then disrupted in a CV334 sonicator (Sonic and Materials Inc., Newtown, CT). The crude enzyme solution was subjected to Ni-affinity chromatography in an AKTA prime plus system (GE Healthcare) equipped with a His-Trap column (GE Healthcare) as previously described.[23] The eluted target proteins were dialyzed against sodium phosphate (50 mM, pH7.5).

**Catalytic properties of BgaC mutants:** To identify active glycosynthases from BgaC, the BgaC variants (1 mg mL$^{-1}$) were treated with a substrate solution consisting of αGalF (5 mM) and pNPβGlc (10 mM) as the sugar donor and acceptor, respectively, in sodium phosphate (0.2 M, pH 7.0). The mixture was incubated (25 °C, 5 h), followed by TLC analysis to detect transfer products. To investigate substrate specificity, BgaC-E233G (1 mg mL$^{-1}$) was treated with sugar donor αGalF (5 mM) and one of 18 aryl glycosides sugar acceptors (10 mM) in sodium phosphate (0.2 M, pH 7.0). The mixtures were incubated (25 °C, 5 h), then subjected to TLC or HPLC analysis. For HPLC, a standard curve was generated by using the respective transfer product as the standard.

**Preparative scale reaction and structural analysis of transfer products:** A mixture of donor αGalF (9.1 mg, 50 μmol) and acceptor (100 μmol) in phosphate buffer (5 mL; 0.1 M, pH 7.0) was treated with BgaC-E233G (5 mg), and the mixture was incubated for 5 h at 25 °C. Aryl glycoside products were purified on a C18 SEP PAK cartridge (Waters), and the solvent was evaporated under reduced pressure. Transfer products were isolated by flash silica gel chromatography by solvent gradient elution (ethyl acetate/methanol/water, 17:2:1 to 7:2:1).

**Kinetic analysis of BgaC mutant-catalyzed transglycosylation:** All kinetic studies were carried out at 25 °C in sodium phosphate (0.2 M, pH 7.0). The amount of released fluoride ion was detected by using a fluoride electrode (Thermo Scientific). The concentration of the donor or acceptor sugar was fixed (2 mM), while that of the counterpart was varied. All enzymatic rates were corrected for the spontaneous hydrolysis rate of αGalF. $K_M$ and $k_{cat}$ values (strictly, apparent $K_M$ and $k_{cat}$, as they were determined at a fixed, nonsaturating, co-substrate concentration) were determined by fitting the initial velocity curves to the Michaelis–Menten equation by nonlinear regression in GraFit (version 7.0; Erithacus Software, Horley, UK).

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**Keywords:** beta-galactosidase · glycosylation · glycosynthase · regioselectivity · transglycosylation

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