

Improvement of the transglycosylation efficiency of a lacto-*N*-biosidase from *Bifidobacterium bifidum* by protein engineering

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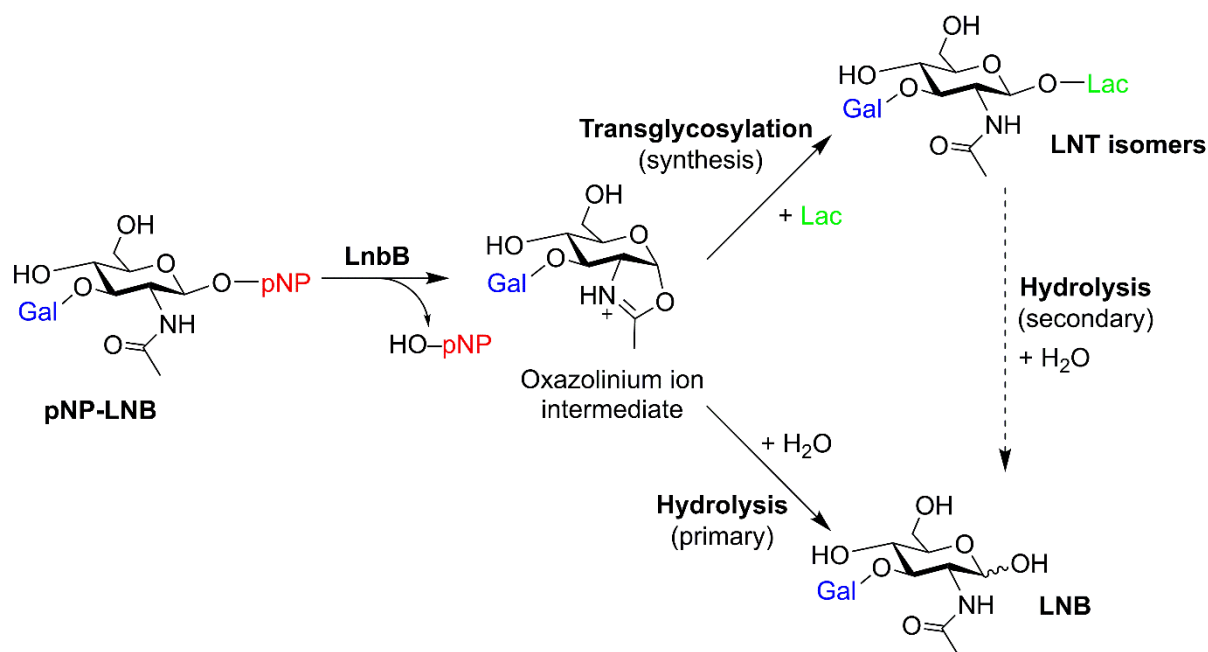
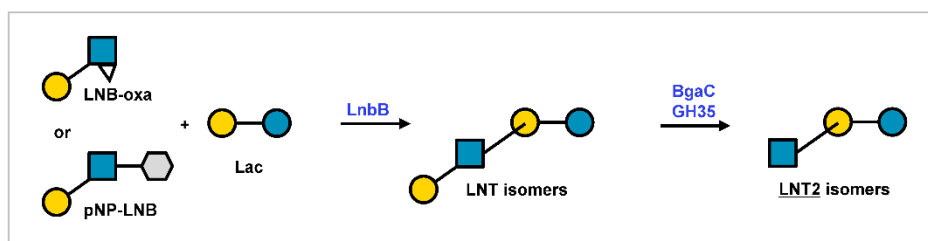


Figure S1. Transglycosylation takes place in competition with hydrolysis of substrate and product (primary and secondary hydrolysis). The reaction is shown with pNP-LNB as donor substrate. With LNB-oxazoline as donor substrate, the substrate is already similar to the reaction intermediate, but can also undergo both transglycosylation and hydrolysis. For details on the LNT isomer products, please refer to Figure 1.



Theoretically possible isomers before and after BgaC treatment:

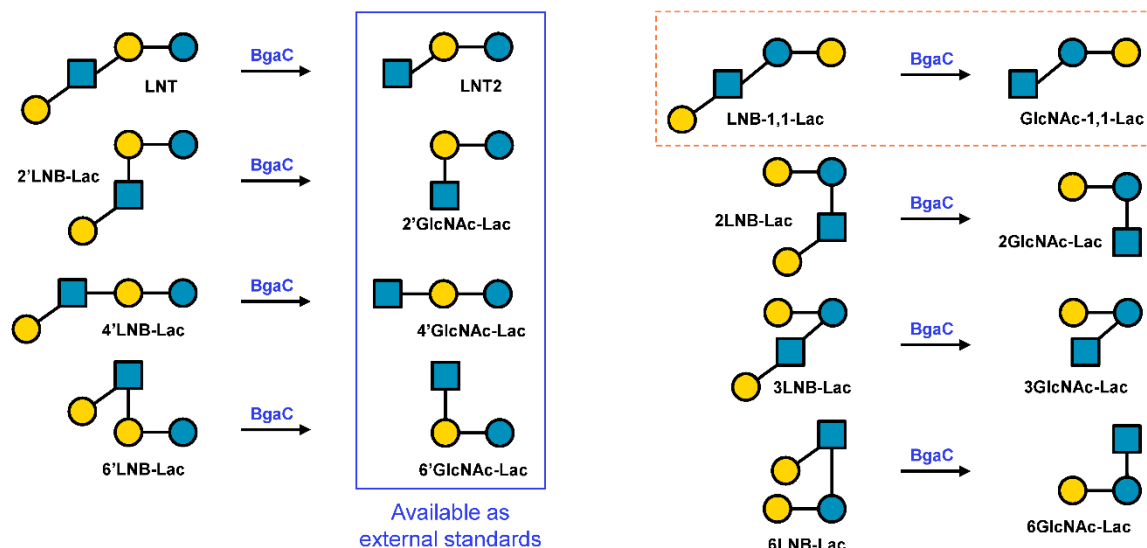


Figure S2. The LnbB-catalyzed transglycosylation products (LNT isomers) were degraded by the GH35 β 1,3-galactosidase BgaC (grey box) to yield LNT2 isomers for which we have external standards (blue box) for structural elucidation. Additional isomers include the non-reducing LNB-1,1-Lac (orange dashed box; detectable by NaBH_4 reduction analysis) as well as isomers with LNB 1,2/3/6-linked to the reducing end Glc moiety for which no external standards were available. The figure uses the Symbol Nomenclature for Glycans (SNFG).

Table S1. Forward (_f) and reverse (_r) primers used for site-directed mutagenesis in LnbB. Mutated codons are underlined.

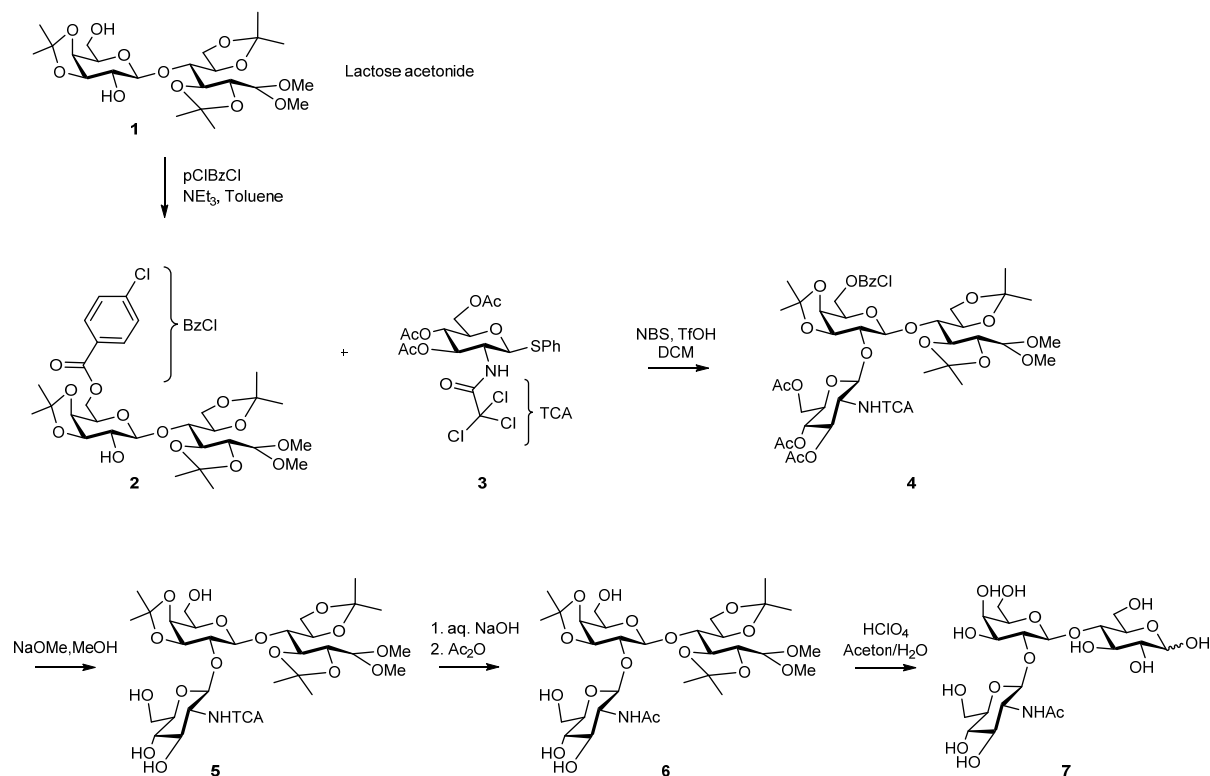
Primer	Sequence
N259T_f	C GTT ATT CCG GAA ATC <u>ACC</u> AGC CCG GGC CAC ATG
N259T_r	GAT TTC CGG AAT AAC GTC GAT ACC GTA GTT GTT CG
D320E_f	C TGG CAC ATG GGT GCG <u>GAA</u> GAG TAT ATG ATC GGC ACC AG
D320T_f	C TGG CAC ATG GGT GCG <u>ACC</u> GAG TAT ATG ATC GGC ACC AG
D320E_r	CGC ACC CAT GTG CCA GTA TTT GGT GGT AAA CAC GCC ATC
W373H_f	GT AAA CAA CTG CGT ATT <u>CAC</u> AAC GAC GGC ATC GTT AAC
W373F_f	GT AAA CAA CTG CGT ATT <u>TTC</u> AAC GAC GGC ATC GTT AAC
W373_r	AAT ACG CAG TTG TTT ACC CTT CGC TTT AAC GTA C
W394H_f	GAT ATC GTT ATT GAG TAC <u>CAC</u> TAT GGT GCG GGC CGT AAG
W394F_f	GAT ATC GTT ATT GAG TAC <u>TTC</u> TAT GGT GCG GGC CGT AAG
W394_r	GTA CTC AAT AAC GAT ATC TTT GTT CAG GCT CAC GTT C
G398R_f	G TAC TGG TAT GGT GCG <u>CGC</u> CGT AAG CCG CAG GAA CTG
G398_r	CGC ACC ATA CCA GTA CTC AAT AAC GAT ATC TTT GTT CAG
ΔG398_f	G TAC TGG TAT GGT GCG CGT AAG CCG CAG GAA CTG
Y419N_f	GCG ACC CAA GCG CTG <u>AAC</u> TGG AGC CGT AGC GCG CAA G
Y419_r	CAG CGC TTG GGT CGC GTT CAT CAG GGT GTA ACC GTC
W465H_f	GCG AAG GTT AGC ATC <u>CAC</u> CCG GAC AGC AGC TAC
W465F_f	GCG AAG GTT AGC ATC <u>TTC</u> CCG GAC AGC AGC TAC
W465_r	GAT GCT AAC CTT CGC GCC GGT CAG TTT ATC ATA G
D467N_f	GTT AGC ATC TGG CCG <u>AAC</u> AGC AGC TAC TTT CAG ACC G
D467N_r	CGG CCA GAT GCT AAC CTT CGC GCC GGT CAG

Section S1:

Chemical synthesis of LNT2 isomers 2'GlcNAc-Lac, 4'GlcNAc-Lac, and 6'GlcNAc-Lac

Synthesis of 2'GlcNAc-Lac:

4-O-(2'-O-(2-Acetamido-2-deoxy- β -D-glucopyranosyl))- β -D-galactopyranosyl-D-glucose (7): The synthesis method is presented in Scheme S1 and described below.



Scheme S1. Overview of the synthesis of 7. Molecular weight (MW) 1: 508.56 g/mol; MW 2: 647.11 g/mol; MW 3: 542.81 g/mol; MW 4: 1079.74 g/mol; MW 5: 815.08 g/mol; MW 6: 711.76 g/mol; MW 7: 545.49 g/mol.

2: Acceptor synthesis: 4-O-(3,4-O-isopropylidene-6-O-para-chlorobenzoyl- β -D-galactopyranosyl)-2,3;5,6-di-O-isopropylidene-D-glucose dimethyl acetal: 5.0 g (9.8 mmol) lactose acetonide 1 [1] were dissolved in 50 mL toluene. 2.75 mL (19.8 mmol) triethylamine and 1.6 mL (12.5 mmol) p-chlorobenzoyl chloride were added. The mixture was stirred at room temperature for 24 hours. The reaction progress was monitored by TLC (*R_f* (2): 0.50 (Tol/Ac 4:1 v/v)). 0.5 mL NH₃(aq.) and 10 mL water were added. The phases were separated, and the organic phase was washed with 10 mL HCl solution (2.5 mL cCHCl and 7.5 mL water), 10 mL water, NaHCO₃ solution (0.5 g NaHCO₃ and 10 mL water) and 10 mL water. The organic phase was concentrated to approx. 50% of its volume ($\approx$ 22 g solution).

4: Glycosylation of 2 and 3: (4-O-(2-O-(2-Trichloroacetamido-2-deoxy-3,4,6-tri-O-acetyl- β -D-glucopyranosyl))-3,4-O-isopropylidene-6-O-para-chlorobenzoyl- β -D-galactopyranosyl)-2,3;5,6-di-O-isopropylidene-D-glucose dimethyl acetal: 11 g of the acceptor solution ($\approx$ 2.5 g acceptor; $\approx$ 3.9 mmol) were mixed with donor 2 [2] (4.1 g, 7.5 mmol), dissolved in 30 mL toluene and 20 mL DCM. 1.2 g N-bromosuccinimide (1.2 g, 6.7 mmol) were added and the solution cooled to 0-5 °C. 40 μ L (0.45 mmol) TfOH (trifluoromethanesulfonic acid) were added. The cooling bath was removed, and the mixture was stirred for 1.5 hours. The reaction was stopped with 2 mL NH₃(aq.) and 30 mL water. The phases were separated, the organic phase was washed twice with 30 mL water each and 30 mL brine. The organic phase was concentrated, and the product was purified by column chromatography on silica gel (Tol/Ac 9:1). The fractions contained the product (TLC: *R_f*: 0.21 Tol/Ac 7:1) were combined and concentrated. The coupling product was used in the next step without further analysis.

5: Zemplen-deprotection: (4-O-(2-O-(2-Trichloroacetamido-2-deoxy-β-D-glucopyranosyl))-3,4-O-isopropylidene-β-D-galactopyranosyl)-2,3;5,6-di-O-isopropylidene-D-glucose dimethyl acetal: The coupling product was dissolved in 20 mL MeOH and NaOMe was added as powder until pH was at 9. The mixture was stirred at room temperature for 18 hours. MeOH was removed under reduced pressure and the residual syrup was used for the next step without further purification.

6: TCA-deprotection / NHAc-protection: (4-O-(2-O-(2-Acetamido-2-deoxy-β-D-glucopyranosyl))-3,4-O-isopropylidene-β-D-galactopyranosyl)-2,3;5,6-di-O-isopropylidene-D-glucose dimethyl acetal: 20 mL water and 10 mL MeOH were added to the syrup. 800 μL 2M NaOH were added and the mixture stirred overnight. Afterwards 1.0 mL Ac₂O and after 15 minutes 10 mL 1M NaOH were added to neutralize the mixture. The product was purified by column chromatography on silica gel (Eluent: Ethylacetate (EA) 100%). In total 650 mg of product were obtained. MS showed still starting material **5**, thus TCA-deprotection and N-acetylation were repeated. The material was dissolved in 2 mL water and 1 mL 2M NaOH was added. The mixture was stirred at 50 °C for 2 hours. After cooling, AcOH was added (155 μL) and stirred for 15 minutes followed by the addition of 1.1 mL 2M NaOH to reach a pH > 9. The formation of compound **6** was confirmed by MS: 710.346 [M-H]⁻.

7: Acetal hydrolysis: 4-O-(2-O-(2-Acetamido-2-deoxy-β-D-glucopyranosyl))-β-D-galactopyranosyl-D-glucose: The mixture with compound **6** was diluted with 3 mL water and 300 μL HClO₄ (70%) were added (pH < 3). The solution was stored in the fridge for 24 hours. Afterwards, the solution was passed through a strongly acidic/weakly basic resin pair to desalt and neutralize the solution. Finally, the solution was freeze dried to give the product **7** (2'-GlcNAc-Lactose; TLC: R_f: 0.23 ACN/H₂O/NH₃(aq). 6:3:1). MS: 544.199 [M-H]⁻.

¹H-NMR (800 MHz, D₂O):

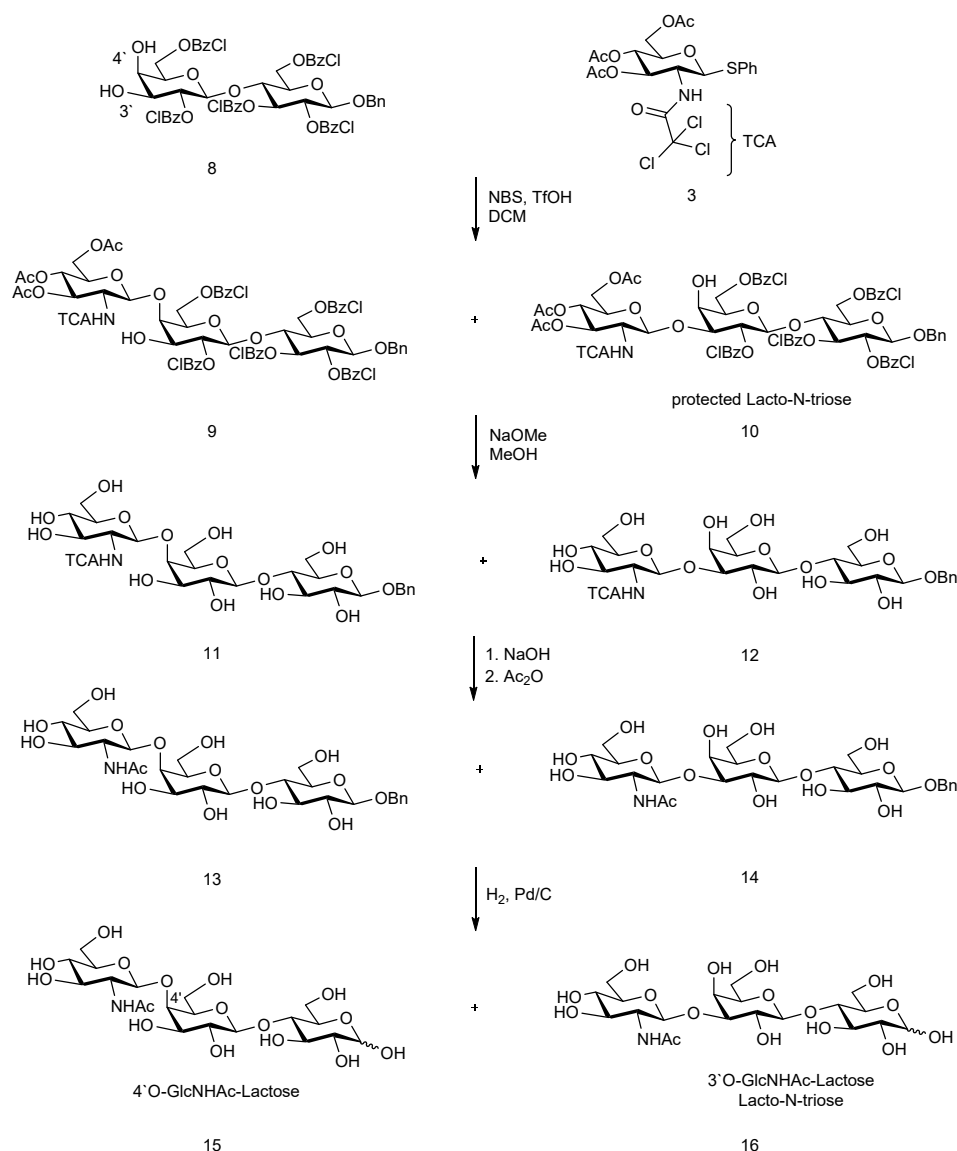
α-Glc: 5.22 (H-1, ³J = 3.8 Hz), 3.57 (H-2, ³J = 3.8 Hz, ³J = 9.6 Hz), 3.86 (H-3, ³J = 9.6 Hz), 3.62 (H-4), 3.96 (H-5), 3.98 (H-6); β-Glc: 4.66 (H-1, ³J = 8.0 Hz), 3.28 (H-2, ³J = 8.0 Hz, ³J = 9.0 Hz), 3.64 (H-3), 3.61 (H-4), 3.62 (H-5), 4.07, 3.91 (H-6, ²J = 12.3 Hz, ³J = 1.9 Hz); β-Gal: 4.51 (H-1), 3.71 (H-2), 3.71 (H-3), 3.86 (H-4), 3.71 (H-5), 3.77, 3.74 (H-6); β-GlcNAc: 4.84 (H-1, ³J = 8.4 Hz), 3.72 (H-2), 3.40 (H-3), 3.42 (H-4), 3.55 (H-5), 3.92, 3.74 (H-6), 2.05 (CH₃).

¹³C-NMR (200 MHz, D₂O):

α-Glc: 94.5 (C-1), 73.8 (C-2), 74.1 (C-3), 82.4 (C-4), 72.8 (C-5), 62.9 (C-6); β-Glc: 98.4 (C-1), 76.5 (C-2), 77.0 (C-3), 82.2 (C-4), 77.3 (C-5), 63.0 (C-6); β-Gal: 104.3 (C-1), 80.2 (C-2), 78.0 (C-3), 71.6 (C-4), 76.5 (C-5), 63.6 (C-6); β-GlcNAc: 177.7 (C=O), 104.1 (H-1), 58.8 (H-2), 78.5 (H-3), 72.7 (H-4), 76.5 (H-5), 63.7 (H-6), 25.0 (CH₃).

Synthesis of 4'GlcNAc-Lac and LNT2 mixture:

4-O-(4-O-(2-Acetamido-2-deoxy-β-D-glucopyranosyl))-β-D-galactopyranosyl-D-glucose (**15**) and 4-O-(3-O-(2-Acetamido-2-deoxy-β-D-glucopyranosyl))-β-D-galactopyranosyl-D-glucose (LNT-II) (**16**): The method used for preparing this mixture is shown in Scheme S2 and described below.



Scheme S2. Overview of the synthesis of a mixture of **15** and **16** (**16**: LNT-II). MW **8**: 1125.17 g/mol; MW **3**: 542.81 g/mol; MW **9,10**: 1696.35 g/mol; MW **11,12**: 738.94 g/mol; MW **13,14**: 635.62 g/mol; MW **15,16**: 545.49 g/mol.

9+10: Glycosylation of 8 and 3: Benzyl (4-O-(4-O-(2-Trichloroacetamido-2-deoxy-3,4,6-tri-O-acetyl- β -D-glucopyranosyl))-2,6-di-O-para-chlorobenzoyl- β -D-galactopyranosyl)-2,3,6-tri-O-para-chlorobenzoyl-D-glucopyranoside (**9**) and Benzyl (4-O-(3-O-(2-Trichloroacetamido-2-deoxy-3,4,6-tri-O-acetyl- β -D-glucopyranosyl))-2,6-di-O-para-chlorobenzoyl- β -D-galactopyranosyl)-2,3,6-tri-O-para-chlorobenzoyl-D-glucopyranoside (**10**): 5.5 g (4.9 mmol) Acceptor **8** [2] and 4.0 g (7.4 mmol) donor **3** [2] were dissolved in 50 mL DCM. 1.5 g (8.4 mmol) N-bromosuccinimide and afterwards 20 μ L (0.23 mmol) TfOH (trifluoromethanesulfonic acid) were added. After 3 hours stirring at room temperature the reaction was stopped with 2 mL $\text{NH}_3(\text{aq.})$ and 30 mL water. The organic phase was washed three times with 30 mL water each and twice with 20 mL brine each. The organic phase was concentrated, and the product was purified by column chromatography on silica gel (Tol/EA 1:2 $\rightarrow$ EA). The fractions contained the product were combined and concentrated. The product mixture was used in the next step without further analysis.

11+12: Zemplen-deprotection: Benzyl (4-O-(4-O-(2-Trichloroacetamido-2-deoxy- β -D-glucopyranosyl))- β -D-galactopyranosyl)-D-glucopyranoside (**11**) and Benzyl (4-O-(3-O-(2-Trichloroacetamido-2-deoxy- β -D-glucopyranosyl))- β -D-galactopyranosyl)-D-glucopyranoside (**12**): **9+10** were dissolved in 100 mL MeOH and NaOMe was added as powder until pH was at 9. The mixture was stirred at room temperature for 18 hours. MeOH was removed under reduced

pressure and the residual syrup was taken up in water and ethyl acetate. The aqueous phase was used for the next step (TLC R_f: 0.56 EA/MeOH/H₂O 7:3:0.5; MS: 736.126 and 738.122 [M-H]⁻).

13+14: TCA-deprotection / NHAc-protection: Benzyl (4-O-(4-O-(2-Acetamido-2-deoxy-β-D-glucopyranosyl))-β-D-galactopyranosyl)-D-glucopyranoside (**11**) and Benzyl (4-O-(3-O-(2-Acetamido-2-deoxy-β-D-glucopyranosyl))-β-D-galactopyranosyl)-D-glucopyranoside (**12**): The starting material (**11+12**, 640 mg) were dissolved in 2 mL H₂O, 1 mL 2M NaOH were added and the mixture was stirred at 45 °C for 1.5 hours. After cooling down 150 μL Ac₂O were added and 1.5 hours later 500 μL 2M NaOH were added. The mixture was diluted with 3 mL water and was desalted on Amberlite IR120 (H⁺) and Dowex66 resin pair. The solution was freeze dried. ¹H-NMR measurement showed two benzyl protected compounds with a ratio of approx. 1:1.

15+16: Hydrogenation: 4-O-(4-O-(2-Acetamido-2-deoxy-β-D-glucopyranosyl))-β-D-galactopyranosyl-D-glucopyranoside (**15**) and 4-O-(3-O-(2-Acetamido-2-deoxy-β-D-glucopyranosyl))-β-D-galactopyranosyl-D-glucopyranoside (**16**): The starting mixture **13+14** (345 mg) was dissolved in 10 mL MeOH. 50 mg Pd/C, suspended in 1 mL water, were added and the mixture stirred under H₂-atmosphere for 18 hours. The catalyst was filtered off and the filtrate was concentrated, water added and freeze dried.

¹H-NMR (400 MHz, D₂O) – **15**:

α-Glc: 5.22 (H-1, ³J = 3.7 Hz), 3.57 (H-2), 3.83 (H-3), 3.63 (H-4), 3.94 (H-5), 3.96, 3.82 (H-6); β-Glc: 4.66 (H-1, ³J = 8.0 Hz), 3.28 (H-2), 3.64 (H-3), 3.63 (H-4), 3.60 (H-5), 3.96, 3.82 (H-6); β-Gal: 4.43 (H-1, ³J = 7.8 Hz), 3.39 (H-2), 3.75 (H-3, ³J = 2.9 Hz), 4.08 (H-4, ³J = 2.9 Hz), 3.60 (H-5), 3.77 (H-6); β-GlcNAc: 4.70 (H-1, ³J = 8.5 Hz), 3.73 (H-2), 3.55 (H-3), 3.45 (H-4), 3.44 (H-5), 3.90, 3.78 (H-6), 2.05 (CH₃).

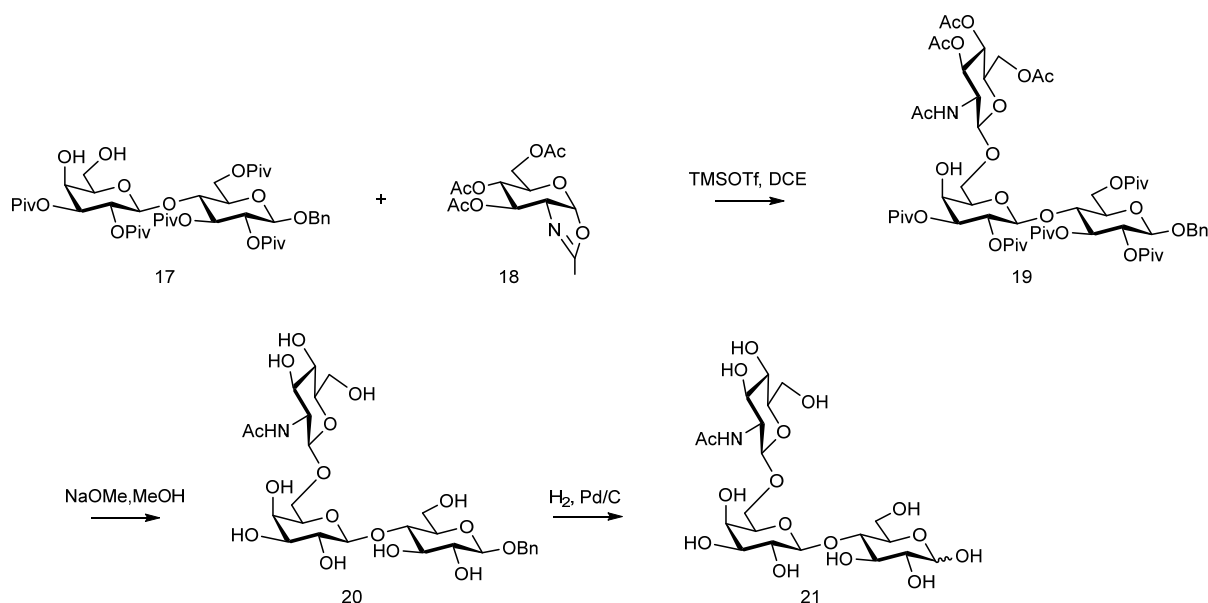
¹³C-NMR (100 MHz, D₂O) – **15**:

α-Glc: 92.4 (C-1), 72.0 (C-2), 72.1 (C-3), 79.0 (C-4), 70.8 (C-5), 60.6 (C-6); β-Glc: 96.4 (C-1), 74.4 (C-2), 75.0 (C-3), 79.1 (C-4), 75.4 (C-5), 60.7 (C-6); β-Gal: 103.6 (C-1), 71.7 (C-2), 73.1 (C-3), 76.9 (C-4), 75.1 (C-5), 61.5 (C-6); β-GlcNAc: 175.4 (C=O), 102.8 (H-1), 56.3 (H-2), 74.5 (H-3), 70.6 (H-4), 76.2 (H-5), 61.4 (H-6), 23.0 (CH₃).

Data for **16** were confirmed identical to those of the external LNT2 standard (data not shown).

Synthesis of 6'GlcNAc-Lac:

4-O-(6-O-(2-Acetamido-2-deoxy-β-D-glucopyranosyl))-β-D-galactopyranosyl-D-glucose (**21**): Synthesis of **21** with different protecting groups and other glycosylation conditions was described in [3]. The method used in the current work is presented in Scheme S3 and described below.



Scheme S3. Overview of the synthesis of **21**. MW **17**: 853.01 g/mol; MW **18**: 329.31 g/mol; MW **19**: 1182.32 g/mol; MW **20**: 635.62 g/mol; MW **21**: 545.49 g/mol.

19: Glycosylation of 17 and 18 (PC177-157): Benzyl (4-O-(6-O-(2-Acetamido-2-deoxy-3,4,6-tri-O-acetyl- β -D-glucopyranosyl))-2,3-di-O-pivaloyl- β -D-galactopyranosyl)-2,3,6-tri-O-pivaloyl-D-glucopyranoside (**19**): 50.0 g of the acceptor **17** [4] were mixed with 40.2 g oxazoline donor **18** [2,5] in 550 mL DCE (dichloroethane). 50 mL DCE were removed under reduced pressure. TMSOTf (2.1 mL) were added and the mixture heated to 80-85 °C for 4 hours. The mixture was cooled down to 0 °C and 200 mL NaHCO₃ (5%) were added and stirred for 45 minutes. After separation of the layers the aqueous layer was extracted with DCM. The pooled organic fractions were washed with brine and concentrated to a brown foam. 250 mL iPrOH were added and the mixture was heated up to 70 °C, then cooled down to 5 °C. the product was filtered off, washed with cold iPrOH and dried to get 30.7 g product.

20: Deprotection (PC177-159): Benzyl (4-O-(6-O-(2-Acetamido-2-deoxy- β -D-glucopyranosyl))- β -D-galactopyranosyl)-D-glucopyranoside (**20**): The protected product was dissolved in 125 mL MeOH and 5.3 mL NaOMe (25 wt.% in MeOH) were added. The mixture was stirred at 60-65 °C for 20 hours. After cooling the mixture was quenched with Amberlite IR120 H⁺, the resin was filtered off and the product crystallized from MeOH/EtOH to obtain 11.2 g **20**.

21: Hydrogenation (PC188-2): 4-O-(6-O-(2-Acetamido-2-deoxy- β -D-glucopyranosyl))- β -D-galactopyranosyl-D-glucose (**21**): 16.9 g of the final intermediate were dissolved in THF/H₂O 1:1 and 0.85 Pd(10%)/C were added. The mixture was stirred under H₂-atmosphere (atm pressure) for 24 hours (TLC EtOAc/MeOH/H₂O 7:3:1; starting material: R_f (**20**) = 0.33; R_f (**21**) = 0.08. The mixture was diluted with water (25 mL) and the catalyst was filtered off. THF was removed under reduced pressure and the residual aqueous solution was freeze dried. 15.0 g product were obtained.

¹H-NMR (400 MHz, D₂O):

α -Glc: 5.22 (H-1), 3.58 (H-2), 3.84 (H-3), 3.62 (H-4), 3.94 (H-5), 3.94, 3.87 (H-6); β -Glc: 4.66 (H-1), 3.29 (H-2), 3.63 (H-3), 3.62 (H-4), 3.62 (H-5), 3.94, 3.82 (H-6); β -Gal: 4.44 (H-1), 3.52 (H-2), 3.65 (H-3), 3.91 (H-4), 3.84 (H-5), 3.99, 3.85 (H-6); β -GlcNAc: 4.61 (H-1), 3.70 (H-2), 3.56 (H-3), 3.46 (H-4), 3.46 (H-5), 3.93, 3.77 (H-6), 2.06 (CH₃).

¹³C-NMR (100 MHz, D₂O):

α -Glc: 92.4 (C-1), 71.9 (C-2), 72.0 (C-3), 79.6 (C-4), 70.6 (C-5), 60.5 (C-6); β -Glc: 96.4 (C-1), 74.5 (C-2), 75.0 (C-3), 79.4 (C-4), 75.4 (C-5), 60.7 (C-6); β -Gal: 103.7 (C-1), 71.5 (C-2), 73.0 (C-3), 69.1 (C-4), 74.1 (C-5), 68.9 (C-6); β -GlcNAc: 175.2 (C=O), 101.7 (H-1), 56.1 (H-2), 74.4 (H-3), 70.5 (H-4), 76.5 (H-5), 61.3 (H-6), 23.0 (CH₃).

References for the section on chemical synthesis:

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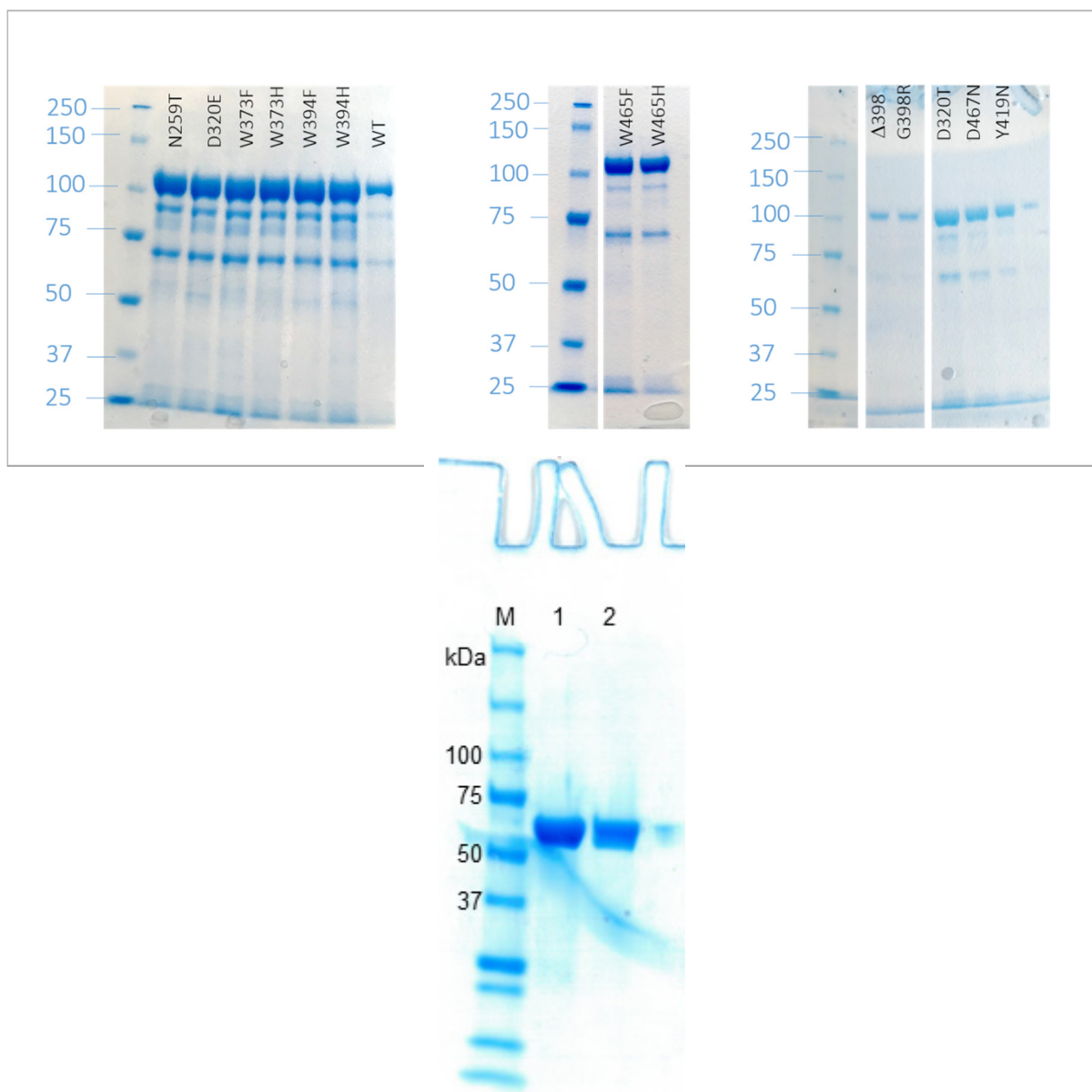


Figure S3. SDS-PAGE of the purified LnbB WT and variants (top) and of the GH35 β 1,3-galactosidase BgaC from *Bacillus circulans* ATCC 31382 (GenBank BAA21669.1) used for LNT isomer degradation (bottom; lanes 1-2). First lane in all gels is the kDa marker (M).

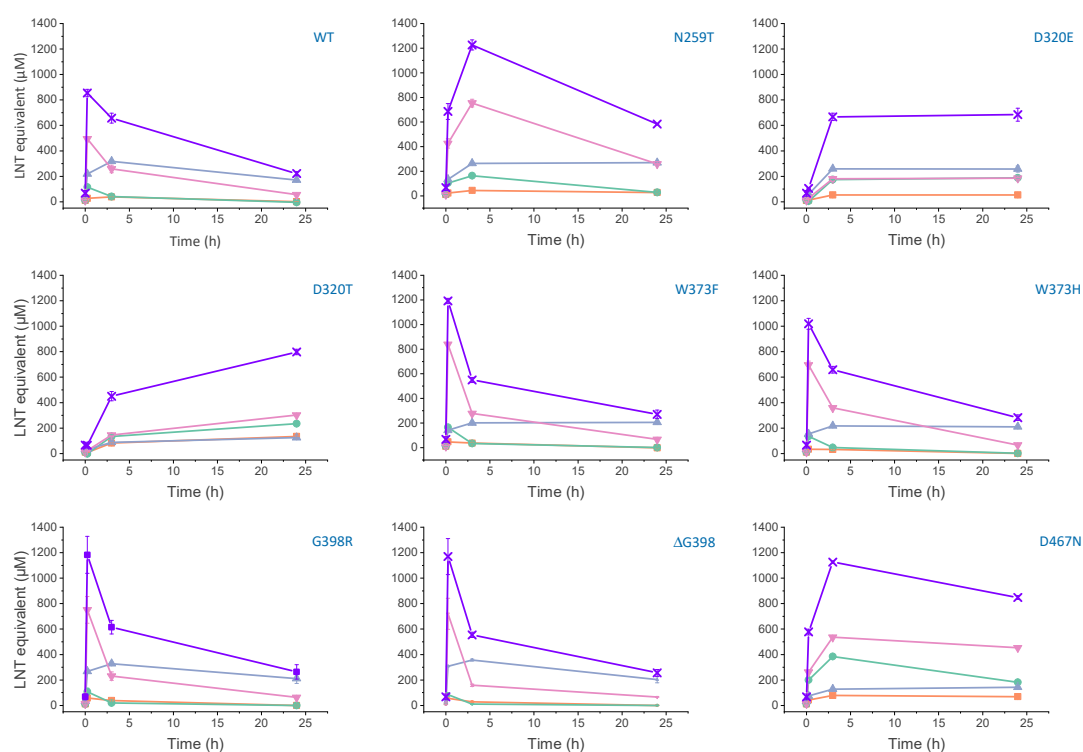


Figure S4. Transglycosylation reactions using LNB-oxazoline as donor and lactose as acceptor at pH 7.4 and 37°C for WT LnbB and variants N259T, D320E, D320T, W373F, W373H, G398R, ΔG398, and D467N. Similar plots for five best variants (W394F, W394H, Y419N, W465F, W465H) are shown in Figure 4. The formation of trans-glycosylation products was followed over 24h with LC-MS and quantified as LNT equivalents. Purple crosses represent the total formation of all LNT isomers, while pink triangles represent formation of LNT (LNB-β1,3-Lac), orange squares LNB-β1,2-Lac, green circles LNB-1,1-Lac, and blue triangles LNB-β1,6-Lac.

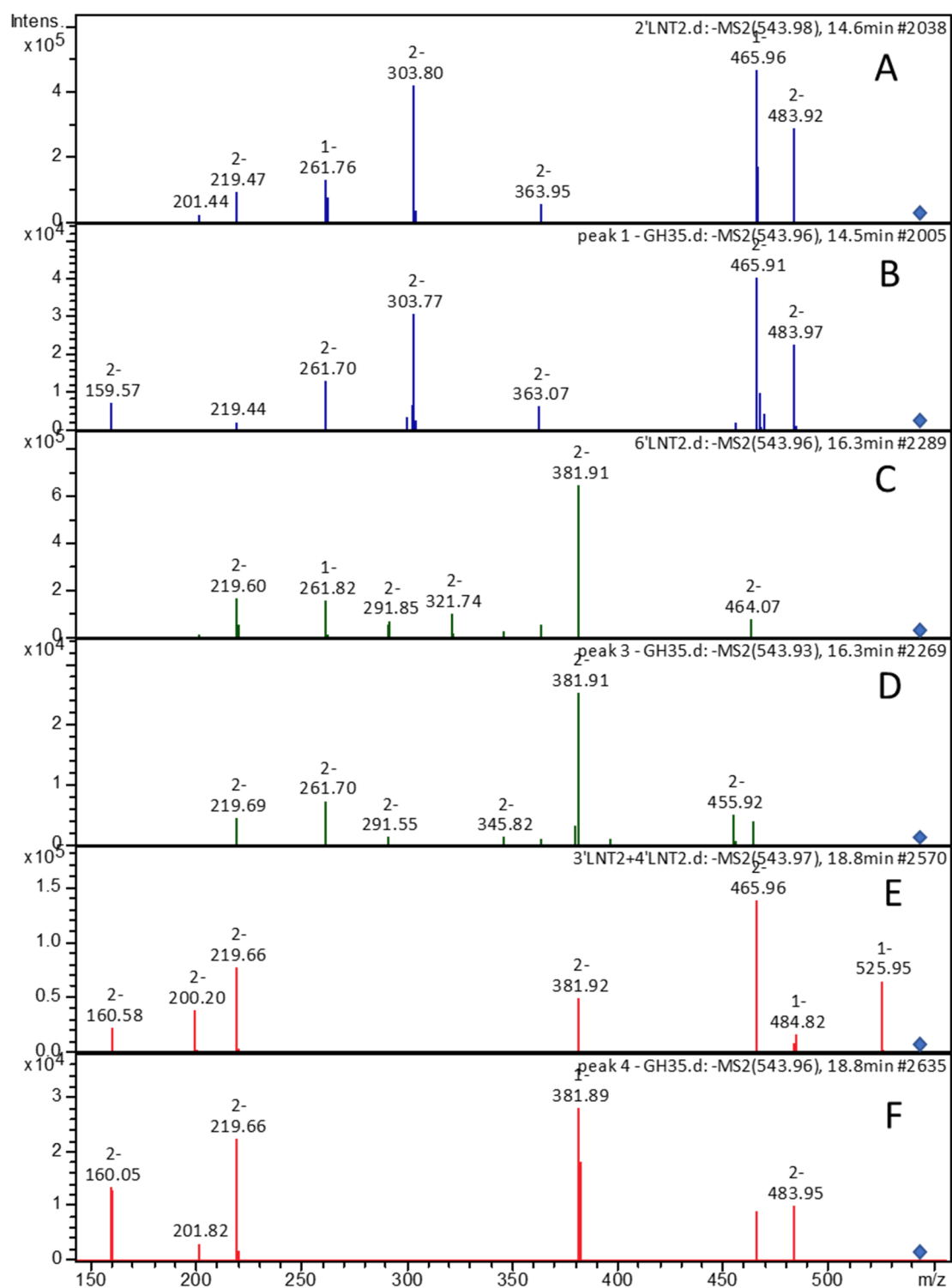


Figure S5. GH35 product fragmentation. A, C, E: MS² fragmentation pattern of 2'LNT2, 6'LNT2, and 3'LNT2. B, D, F: MS² fragmentation pattern of m/z 544 in chromatograms E, I, and K in Figure 7. Blue diamond indicates precursor ion.