Supplementary Information

Lacto-*N*-tetraose synthesis by wild-type and glycosynthase variants of β-*N*-hexosaminidase from *Bifidobacterium bifidum*

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SUPPLEMENTARY METHODS

Chemicals and materials

Media components and chemicals were of reagent grade from Sigma Aldrich/Fluka (Vienna, Austria), Roth (Karlsruhe, Germany) or Merck (Vienna, Austria). HisTrap FF 5 mL column was from GE Healthcare (Vienna, Austria). Minisart® NML syringe membrane filter (0.45 μm) and Vivaspin® Turbo 15 centrifugal concentrators (30 kDa) were from Sartorius (Goettingen, Germany). GlcNAc and succinonitrile were from Sigma Aldrich (Vienna, Austria). 4-Nitro-phenyl-β-LNB (LNB-β-*p*NP), lacto-*N*-triose II (LNT II), lacto-*N*-tetraose (LNT, purity ≥90%), mixture of LNT and lacto-*N*-neotetraose (LNnT), lacto-*N*-biose (LNB) and α-D-galactose-1-phosphate dipotassium salt hydrate (Gal 1-P) were from Carbosynth (Compton, Berkshire, UK). Chromabond Flash FM 70/10C C18 ac adsorbent was von Macherey Nagel (Düren, Germany). Acetonitrile (HPLC gradient grade) was from Chem-Lab NV (Zedelgem, Belgium).

Cloning and expression of LnbB enzymes in *E.coli*

Synthetic LnbB genes codon-optimised for the expression in *Escherichia coli* were produced by BioCat GmbH (Heidelberg, Germany). Production of the enzymes (without signal peptide and membrane anchor) and their purification were done according to protocols from literature.[1] Briefly, synthetic LnbB genes (wild-type lacto-*N*-biosidase from *B. bifidum* JCM1254 (GenBank: EU281545.1, aa 35-1064)[1b] and D320E, D320A, Y419F variants) codon-optimised for *E. coli* expression were ligated into *Nde*I-*Xho*I-cut pET24b(+) plasmid (BioCat GmbH, Heidelberg, Germany). Residue numbering of full-length enzymes is used. All inserts were confirmed by DNA sequencing. LnbB enzymes were expressed in *E. coli* BL21(DE3) following an auto-induction protocol[2] in LB medium with 50 μg mL^{-1}, kanamycin, 25 mM Na_2HPO_4, 25 mM K_2HPO_4, 50 mM NH_4Cl, 5 mM Na_2SO_4, 2 mM $MgSO_4$,

0.5% glycerol, 0.05% glucose and 0.2% lactose at 110 rpm and 30°C for 20 h.[3] Each enzyme was produced as C-terminal His_6-tag fusion protein.

Protein purification by His_6-tag affinity chromatography

For protein purification, cell pellet from 1 L cell culture was resuspended in 25 – 30 mL binding buffer (20 mM sodium phosphate, 150 mM NaCl, 15 mM imidazol, pH 7.4) and frozen at -20°C overnight. 35 mL aliquots of thawed cell suspension were ultrasonicated on an ice bath at 60% amplitude for 6 min (2 s pulse on and 4 s pulse off) using a Sonic Dismembrator (Ultrasonic Processor FB-505; Fisher Scientific, Austria) equipped with a 1.27 cm probe for cell disruption. Cell lysates were centrifuged at 4°C and 21,130 g for 1 h (Eppendorf centrifuge 5424R; Eppendorf, Hamburg, Germany) and filtered *via* 0.45 µm cellulose-acetate syringe filters. Target proteins were purified from the cell-free extract *via* their C-terminal His_6-tag using an ÄktaPrime plus system (GE Healthcare, Vienna, Germany) at 4°C. The cleared cell lysate was loaded onto a HisTrap FF 5 mL column (GE Healthcare, Vienna, Austria) at a flow rate of 2 mL min^{-1}. The column had been equilibrated with binding buffer. After a washing step of 15 column volumes (CVs), the enzyme was eluted with 300 mM imidazol within 6 CVs at a flow rate of 4 mL min^{-1}. Target protein containing fractions were pooled. Eluted enzyme was concentrated and buffer exchanged to 20 mM sodium phosphate, 150 mM NaCl, pH 7.4 using Vivaspin® Turbo 15 centrifugal concentrators (30 kDa, 3645 g, 4°C). SDS PAGE was used to confirm purity of enzyme preparations. The enzyme preparations used were (almost) pure by the criterion of migration as single protein band in SDS PAGE. Protein concentrations were measured with a DeNovix SA-11+ spectrophotometer (DeNovix Inc, Wilmington, US) at 280 nm. ~120 mg of LnbB enzymes were typically obtained per liter of culture medium. Purified enzymes were aliquoted and stored at -70°C.

Preparation of lacto-*N*-biose (LNB)

LNB was synthesized from Gal 1-P and GlcNAc by LNB phosphorylase from *Bifidobacterium longum* JCM 1217, previously described by Kitaoka and co-workers.[4]

LNB phosphorylase production. Production of the LNB phosphorylase and purification were done according to protocols from literature.[5] Briefly, synthetic LNB phosphorylase gene (GenBank: AB181926.1, aa 20-2275) not codon-optimised for *E. coli* expression was ligated into *Nde*I-*Xho*I-cut pET30a(+) plasmid (GenScript, Piscataway, USA). Insert was confirmed by DNA sequencing. LNB phosphorylase was expressed in *E. coli* BL21(DE3) at 30°C for 20 h by induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), using LB-medium supplemented with 50 mg L^{-1} kanamycin. LNB phosphorylase was produced as C-terminal His_6-tag fusion protein. Enzyme purification was done by single-step His_6-tag affinity chromatography (as described above). The following buffers were used: binding buffer (20 mM MOPS, 500 mM NaCl, 15 mM imidazol, pH 7.4), elution buffer (20 mM MOPS, 500 mM NaCl, 300 mM imidazol, pH 7.4), storage buffer (20 mM MOPS, 150 mM NaCl, pH 7.5). ~50 mg of LNB phosphorylase were typically obtained per liter of culture medium. The enzyme preparation used was (almost) pure by the criterion of migration as single protein band in SDS PAGE.

Enzymatic synthesis of LNB. Reaction was performed in a total volume of 40 mL using 5.4 mmol Gal 1-P (1.82 g) and 1.8 mmol GlcNAc (0.40 g) dissolved in water. The pH was adjusted to 6.8 with 4 M HCl and the reaction was started by adding 0.05 mg mL^{-1} (0.6 μM) LNB phosphorylase. The conversion was performed in a 50 mL Sarstedt tube (diameter 2.8 cm, height 11.5 cm) under magnetic stirring (stir bar: 18 × 5 mm; 500 rpm) at 37°C. For temperature control, the Sarstedt tube was placed in a water bath. The pH was constantly monitored and manually controlled by adding 4 M HCl (within first 1.5 h). Incubation was

for 3.5 h. Samples were taken at certain times and analyzed by HPLC. The reaction yield was 92% (42 mM, 16 g L^{-1}).

Downstream processing (DSP). Major task of the DSP was the removal of Gal 1-P (93 mM) from the LNB (42 mM). Only a small amount of GlcNAc (3 mM) was present. Gal 1-P was removed from the mixture by anion-exchange chromatography (AEC) after enzyme-removal by ultra-filtration (Vivaspin concentrators 30 kDa, 4000 rpm, 20°C). AEC was performed at pH 7.5. To allow efficient removal of Gal 1-P by binding to the anion-exchange (AEX) column, the filtrate was 8-fold diluted to an ionic strength of ~3.6 mS cm^{-1} with ultra-pure water. LNB and remaining GlcNAc are not ionized at pH 7.5 and elute in the flow-through. AEC was performed on an ÄktaPrime plus system (GE Healthcare, Germany) at room temperature. A self-packed Proteus 20 mL FliQ column (100 x 16.0 mm, Generon, UK) containing about 15 mL of Toyopearl SuperQ-650M was applied. Ultra-pure water (mobile phase A) and 1 M potassium chloride in ultra-pure water (mobile phase B) were used for binding and elution, respectively. Column was equilibrated with mobile phase A at 4 mL min^{-1} (5 CVs). 40 mL sample were loaded at a flow rate of 2 mL min^{-1} using mobile phase A. LNB eluted together with GlcNAc within 5 CVs. Gal 1-P was eluted with mobile phase B at 4 mL min^{-1} (5 CVs). Detection was by conductivity. Complete removal of Gal 1-P from LNB was verified by TLC analysis. LNB containing fractions were pooled. Sample was concentrated under reduced pressure at 40°C, frozen in liquid nitrogen under rotary motion before freeze-drying overnight (Christ Alpha 1-4, B. Braun Biotech International, Melsungen, Germany). The final product (80% isolated yield) was analyzed by HPLC (Fig. S4, ESI). 5% (w/w) of GlcNAc were detected in the final product.

Preparation of LNB-oxa

LNB-oxa was prepared as described previously.[6] LNB (1 equiv.) was dissolved in water (370 equiv.). Na_3PO_4*$12H_2O$ (7.5 equiv.) was added and the resulting solution was cooled to 0 –

3°C. CDMBI (3 equiv.) was added to the solution in portions within 15 min, and the mixture was stirred for 1 h at the same temperature.

For DSP of the LNB-oxa, the reaction mixture was flash-filtered over Chromabond Flash FM 70/10C C18 ac adsorbent (Macherey Nagel, Düren, Germany) (10 g per g of LNB used) and washed twice with ice-cold water (15 mL per g of LNB used). The filtrates were combined and immediately frozen in liquid nitrogen under rotary motion before freeze-drying overnight (AdVantage Pro BenchTop, SP Scientific, New York, USA). The lyophilized crude product was stable at -20°C for at least one month. LNB-oxa was synthesized on 0.5 mmol scale in a yield of 79%. NMR data are shown in Figures S5 and S6. ^{1}H NMR (500 MHz, D_2O): δ (ppm) 6.14 (d, J = 7.4 Hz, 1H), 4.62 (d, J = 7.9 Hz, 1H), 4.34 (m, 1H), 4.18 (t, J = 2.7 Hz, 1H), 3.97 (d, J = 3.4 Hz, 1H), 3.93 – 3.61 (m, 7H), 3.54 (dd, J = 9.9, 7.9 Hz, 1H), 3.39 (m, 1H), 2.09 (d, J = 1.7 Hz, 3H) (Fig. S6a). ^{13}C NMR (126 MHz, D_2O): δ (ppm) 169.31, 169.13, 103.34, 101.26, 80.02, 76.26, 73.50, 73.27, 71.57, 69.49, 68.63, 64.97, 62.39, 61.93, 14.03 (Fig. S6b). The yield was determined by quantitative ^{1}H NMR spectroscopy (internal standard: succinonitrile; Fig. S5).

For desalting, extraction with acetonitrile (9.5 g per g lyophilized crude product) under magnetic stirring at room temperature for 1 h was used. The solid was removed via filtration and washed twice with acetonitrile (3 g per g lyophilized crude product). The filtrates were combined and concentrated under reduced pressure (45 – 4 mbar) at 25°C. LNB-oxa was dried under oil-pump vacuum and stored at -20°C, where it is stable for at least two weeks. LNB-oxa was obtained on 0.082 mmol scale. Note that the yield of extraction into acetonitrile is rather low (~10%).

Synthetic evaluation of enzymes

Reactions were performed in 300 – 400 µL total volume using 50 mM sodium phosphate buffer, pH 7.5 (with LNB-oxa) or pH 5.8 (with LNB-β-*p*NP). Reactions contained 12 mM

LNB-oxa, 600 mM lactose or 20 mM LNB-β-*p*NP, 15% DMSO, 600 mM lactose and 0.5 – 20 µM enzyme. Temperature was 37°C. An agitation rate of 650 rpm was used (Thermomixer comfort; Eppendorf, Hamburg, Germany). Samples taken at certain times were heat-treated (10 min, 99°C) and precipitated protein was removed by centrifugation (13,200 rpm, 10 min). The supernatant was analyzed by HPLC and TLC. LNT, LNB and LNT II were measured from reactions with LNB-oxa. LNT and *p*NP were measured from reactions with LNB-β-*p*NP.

Enzyme activities for formation and hydrolysis of LNT were obtained from the reaction time courses. For reactions with LNB-oxa, the parameter R_{TH} was obtained from the ratio of the LNT and LNB concentrations measured at maximum LNT yield (see Fig. 2 of the main text). For reactions with LNB-β-*p*NP, R_{TH} was obtained from the ratio enzyme activities for trans-glycosylation (i.e., LNT formation) and donor substrate hydrolysis (i.e., LNB formation). The LNB formation rate was calculated from the difference of total LNB-β-*p*NP consumption rate and trans-glycosylation (LNT formation) rate. The activity of total LNB-β-*p*NP consumption (based on pNP release) was obtained from the reaction time courses. Note: due to requirements of the analytical procedures (LNB-oxa is not stable during the analysis), the approaches of R_{TH} determination differed slightly between reactions using LNB-oxa and LNB-β-*p*NP as the donor substrate. However, the resulting R_{TH} values can be compared directly with each other.

Analytics

LNT, LNB, LNT II, GlcNAc and *p*NP were analysed by HILIC-HPLC using a Luna® NH_2 column (3 µm, 100 Å, 250 × 4.6 mm; Phenomenex, Germany). HPLC analysis was performed at 30°C with a mobile phase of 75% acetonitrile and 25% water at an isocratic

flow rate of 1 mL min^{-1}. UV-detection at 195 nm was used for quantification of LNT, LNB, LNT II, GlcNAc and *p*NP.

TLC was performed on silica gel 60 F_{254} aluminium sheet (Merck, Germany). The plate was developed in a solvent system of 1-butanol – acetic acid – water (2/1/1 by volume). TLC plates were analyzed under UV light (254 nm). Then carbohydrates were visualized by heating the plate after spraying it with thymol – sulfuric acid reagent.

LNT, LNB, LNT II, GlcNAc, *p*NP and lactose were used as authentic standards.

A Varian (Agilent) INOVA 500-MHz NMR spectrometer (Agilent Technologies, Santa Clara, California, USA) and the VNMRJ 2.2D software were used for all NMR measurements. Succinonitrile was used as internal standard for quantitative ^{1}H NMR measurement. 11.41 mg of LNB-oxa (lyophilized crude product) and 12.85 mg succinonitrile were dissolved in D_2O. ^{1}H NMR spectra (499.98 MHz) were measured on a 5 mm indirect detection PFG-probe, while a 5 mm dual direct detection probe with z-gradients was used for ^{13}C NMR spectra (125.71 MHz). Standard pre-saturation sequence was used: relaxation delay 2 s; 90° proton pulse; 2.048 s acquisition time; spectral width 8 kHz; number of points 32 k. ^{13}C NMR spectra were recorded with the following pulse sequence: standard ^{13}C pulse sequence with 45° carbon pulse, relaxation delay 2 s, Waltz decoupling during acquisition, 2 s acquisition time. Mnova 9.0 was used for evaluation of spectra.

SUPPLEMENTARY MATERIAL

Amino acid sequences of wild-type LnbB and the D320E, D320A, Y419F variants thereof.

Mutation sites are highlighted in green.

>truncated LnbB_WT(ABZ78855.1, aa 35-1064)
MADDSAAGYSATAPVNLTRPATVPSMDGWTDGTGAWTLGEGTRVVSSDALAARAQSLASELTKFTDVDIKAATGS
ATGKDISLTLDASKKAELGDEGFKLNIGSKGLEVIGATDIGVFYGTRSVSQMLRQGQLTLPAGTVATKPKYKERG
ATLCACQINISTDWIDRFLSDMADLRLNYVLLEMKLKPEEDNTKKAATWSYYTRDDVKKFVKKANNYGIDVIPEI
NSPGHMNVWLENYPEYQLADNSGRKDPNKLDISNPEAVKFYKTLIDEYDGVFTTKYWHMGADEYMIGTSFDNYSK
LKTFAEKQYGAGATPNDAFTGFINDIDKYVKAKGKQLRIWNDGIVNTKNVSLNKDIVIEYWYGAGRKPQELVQDG
YTLMNATQALYWSRSAQVYKVNAARLYNNNWNVGTFDGGRQIDKNYDKLTGAKVSIWPDSSYFQTENEVEKEIFD
GMRFISQMTWSDSRPWATWNDMKADIDKIGYPLDIREYDYTPVDAGIYDIPQLKSISKGPWELITTPDGYYQMKD
TVSGKCLALFTGSKHLDVVTQVGARPELRNCADVSVGQDQRNTANERNTQKWQIRADKDGKYTISPALTQQRLAI
ATGNEQNIDLETHRPAAGTVAQFPADLVSDNALFTLTGHMGMSATVDSKTVNPASPSKITVKVRAASNANTGDVT
VTPVVPEGWEIKPGSVSLKSIPAGKAAIAYFNVVNTTGTGDATVQFKLTNTKTGEELGTTSVALTGSLTKDVEAS
DYAASSQETTGEHAPVGNAFDKNANTFWHSKYSNPSANLPHWLAFKASPGEGNKIAAITHLYRQDKLNGPAKNVA
VYVVAASDANSVADVTNWGEPVATAEFPYTKELQTIALPNTIPSGDVYVKFQINDAWGLTETSAGVTWAAVAELA
ATAKATPVELTEPEQPKDNPEVTETPEATGVTVSGDGVANGALSLKKGTTAQLTAKVAPDDADQAVTWASSDDKV
VTVDKTGKVTAVAKGVAKVTATTANGKSASVTVTVTEDSEVPGPTGPTEPTKPGTELEHHHHHH

>truncated LnbB_D320E
MADDSAAGYSATAPVNLTRPATVPSMDGWTDGTGAWTLGEGTRVVSSDALAARAQSLASELTKFTDVDIKAATGS
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NSPGHMNVWLENYPEYQLADNSGRKDPNKLDISNPEAVKFYKTLIDEYDGVFTTKYWHMGAFEYMIGTSFDNYSK
LKTFAEKQYGAGATPNDAFTGFINDIDKYVKAKGKQLRIWNDGIVNTKNVSLNKDIVIEYWYGAGRKPQELVQDG
YTLMNATQALYWSRSAQVYKVNAARLYNNNWNVGTFDGGRQIDKNYDKLTGAKVSIWPDSSYFQTENEVEKEIFD
GMRFISQMTWSDSRPWATWNDMKADIDKIGYPLDIREYDYTPVDAGIYDIPQLKSISKGPWELITTPDGYYQMKD
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>truncated LnbB_D320A
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VTPVVPEGWEIKPGSVSLKSIPAGKAAIAYFNVVNTTGTGDATVQFKLTNTKTGEELGTTSVALTGSLTKDVEAS
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>truncated LnbB_Y419F
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NSPGHMNVWLENYPEYQLADNSGRKDPNKLDISNPEAVKFYKTLIDEYDGVFTTKYWHMGADEYMIGTSFDNYSK

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TVSGKCLALFTGSKHLDVVTQVGARPELRNCADVSVGQDQRNTANERNTQKWQIRADKDGKYTISPALTQQRLAI
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SUPPLEMENTARY FIGURES

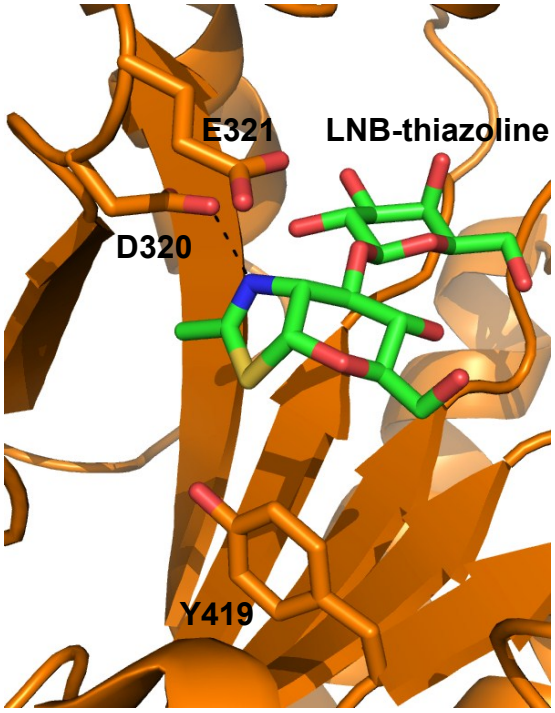

Fig. S1. Close-up view of the enzyme active site in the experimental structure of LnbB in complex with LNB-thiazoline (PDB-code 4JAW)[1a]**.** Key active-site residues (Asp320, polarising residue; Glu321, acid/base; Tyr419, stabilization of oxazolinium reaction intermediate, probably by hydrogen bonding) are drawn in sticks. LNB-thiazoline (drawn with green-colored carbon atoms) is shown.

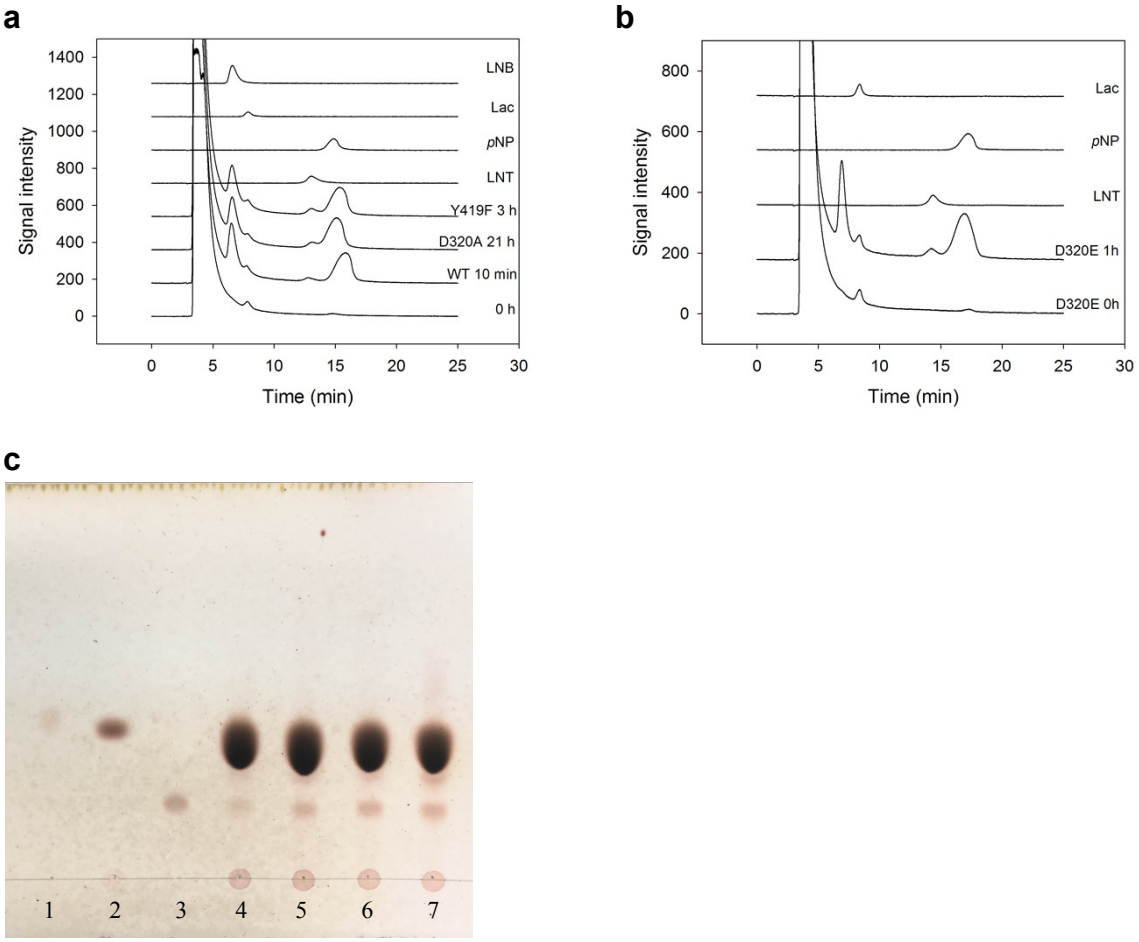

Fig. S2. LNT synthesis from LNB-β-*p*NP by wild-type LnbB and variants thereof. (a – b) HPLC analysis. Overlay of HPLC-chromatograms showing maximum LNT formation obtained by various enzyme variants of LnbB. Reaction mixtures contained 20 mM LNB-β-*p*NP, a 30-fold excess of lactose, 15% DMSO and 0.5 – 20 µM of the enzymes. Samples were 5-fold diluted. UV-detection at 195 nm was used. First peak is LNB-β-*p*NP in DMSO. Note, a new HPLC column caused slightly different retention times in HPLC analyses (a – b). (c) TLC-analysis. Reaction mixtures were 5-fold diluted. Carbohydrates were visualised by thymol-sulfuric acid reagent. Lane 1, LNB; lane 2, lactose; lane 3, LNT; lane 4, wild-type reaction after 10 min; lane 5, D320E reaction after 1 h; lane 6, Y419F reaction after 3 h; lane 7, D320A reaction after 21 h.

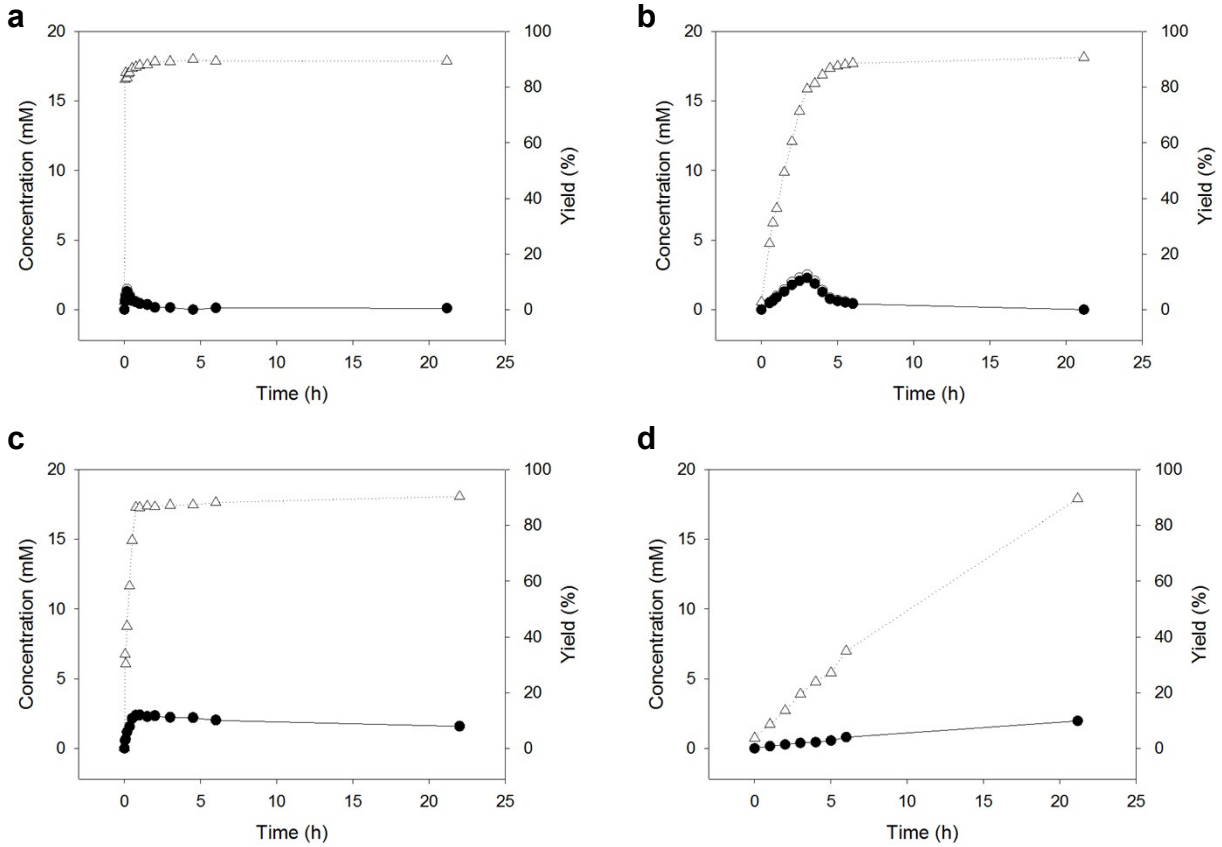

Fig. S3. LNT synthesis from LNB-β-*p*NP by wild-type LnbB and variants thereof. Time courses show synthesis from 20 mM LNB-β-*p*NP in the presence of 15% DMSO, using a 30-fold excess of lactose. (a) Wild-type, 0.5 µM; (b) Y419F, 4 µM; (c) D320E, 10 µM; (d) D320A, 20 µM; LNT, filled circles; yield, open circles; *p*NP released, open triangles.

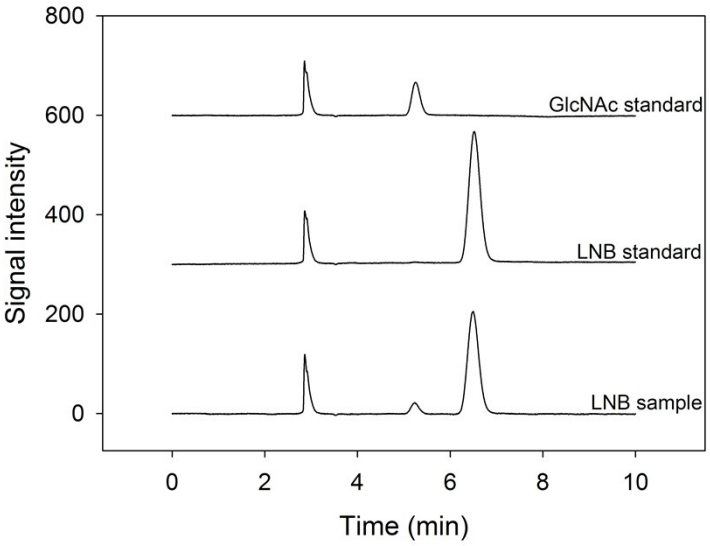

Fig. S4. Final purity of LNB after downstream processing. Superposition of HILIC-HPLC elution profiles. UV-detection at 195 nm was used.

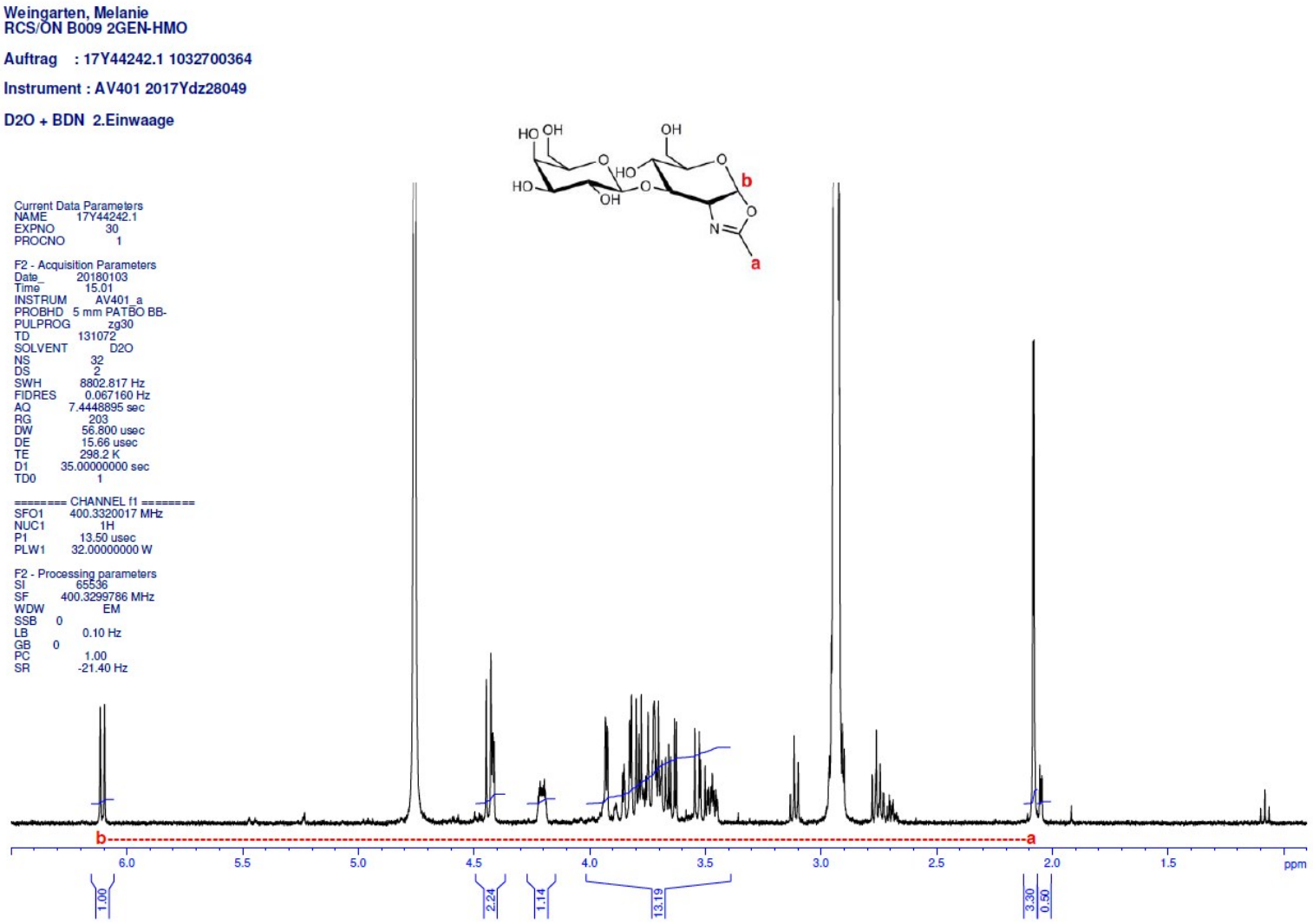

Fig. S5. Quantitative ^{1}H NMR spectrum of LNB-oxa (lyophilised crude product). 11.41 mg of lyophilized crude product and 12.85 mg succinonitrile (internal standard) were dissolved in D_2O.

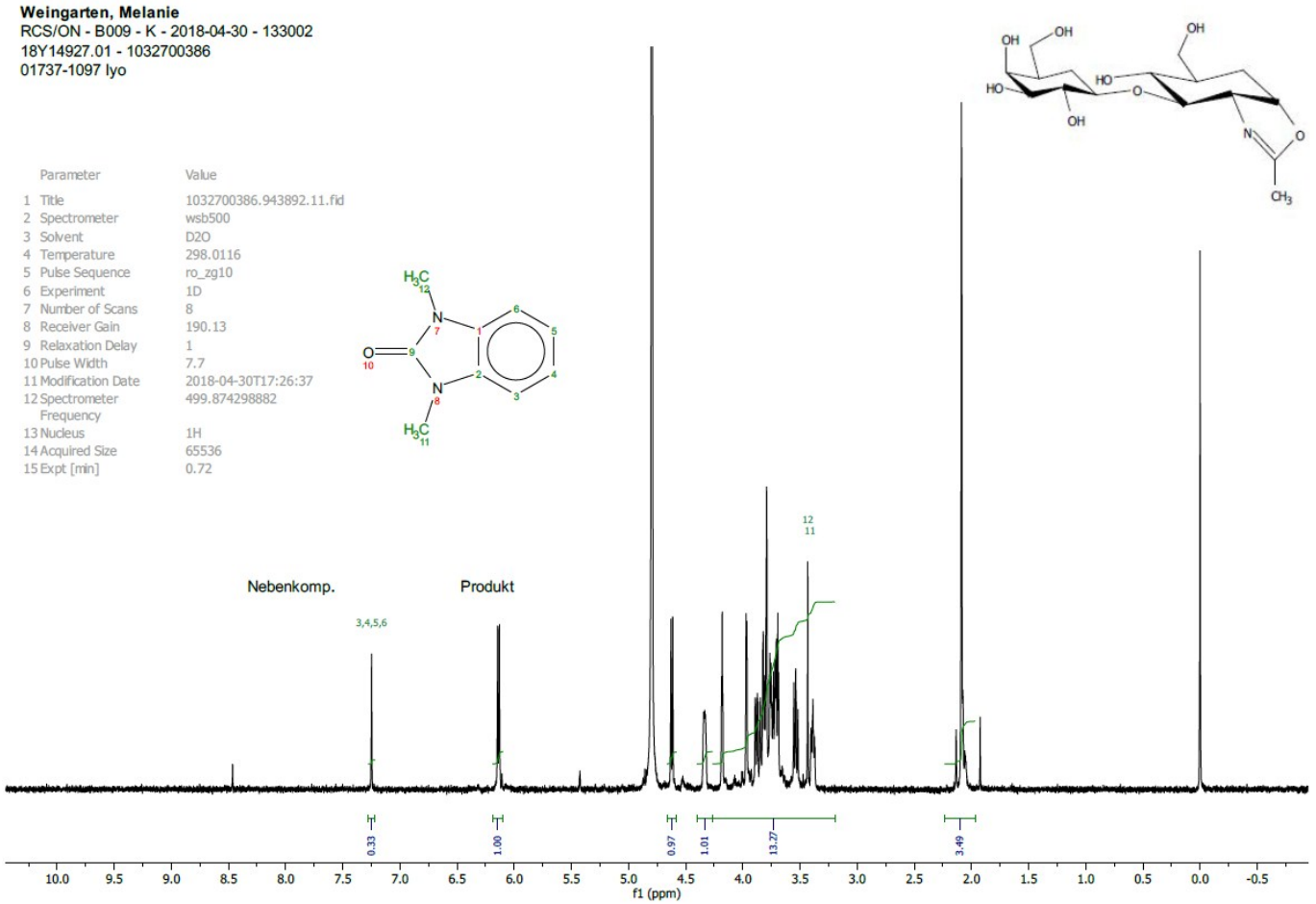

Fig. S6a. ^{1}H NMR spectrum of LNB-oxa (lyophilised crude product). Ratio of LNB-oxa to 1,3-dihydro-1,3-dimethyl-2*H*-benzimidazol-2-one (DMBI) is ~9 : 1. Spectrum is in accordance with previously published data.[6] ^{1}H NMR (500 MHz, D_2O): δ (ppm) 6.14 (d, *J* = 7.4 Hz, 1H), 4.62 (d, *J* = 7.9 Hz, 1H), 4.34 (m, 1H), 4.18 (t, *J* = 2.7 Hz, 1H), 3.97 (d, *J* = 3.4 Hz, 1H), 3.93 – 3.61 (m, 7H), 3.54 (dd, *J* = 9.9, 7.9 Hz, 1H), 3.39 (m, 1H), 2.09 (d, *J* = 1.7 Hz, 3H).

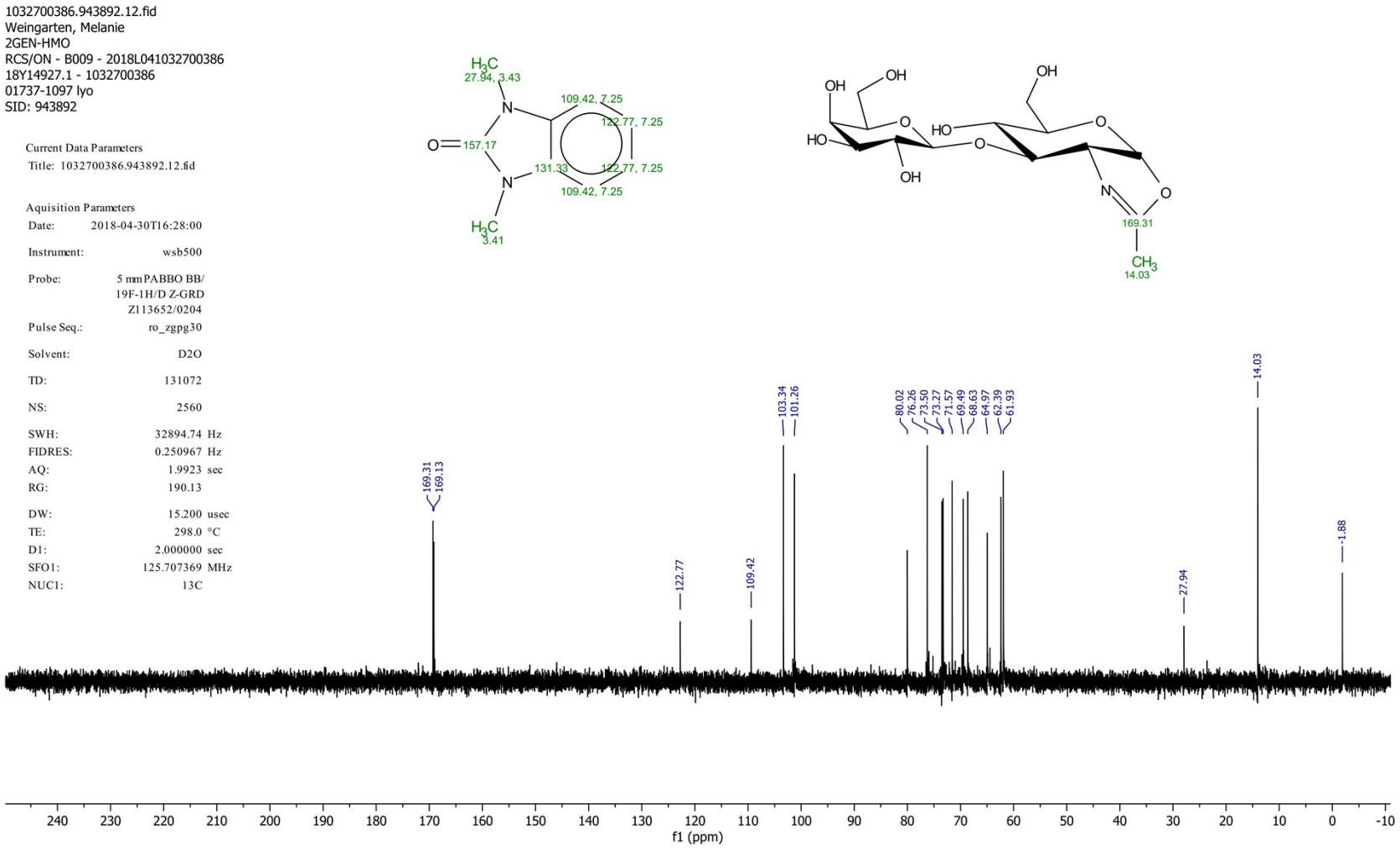

Fig. S6b. ^{13}C NMR spectrum of LNB-oxa (lyophilised crude product). Ratio of LNB-oxa to 1,3-dihydro-1,3-dimethyl-2*H*-benzimidazol-2-one (DMBI) is ~9 : 1. ^{13}C NMR (126 MHz, D_2O): δ (ppm) 169.31, 169.13, 103.34, 101.26, 80.02, 76.26, 73.50, 73.27, 71.57, 69.49, 68.63, 64.97, 62.39, 61.93, 14.03.

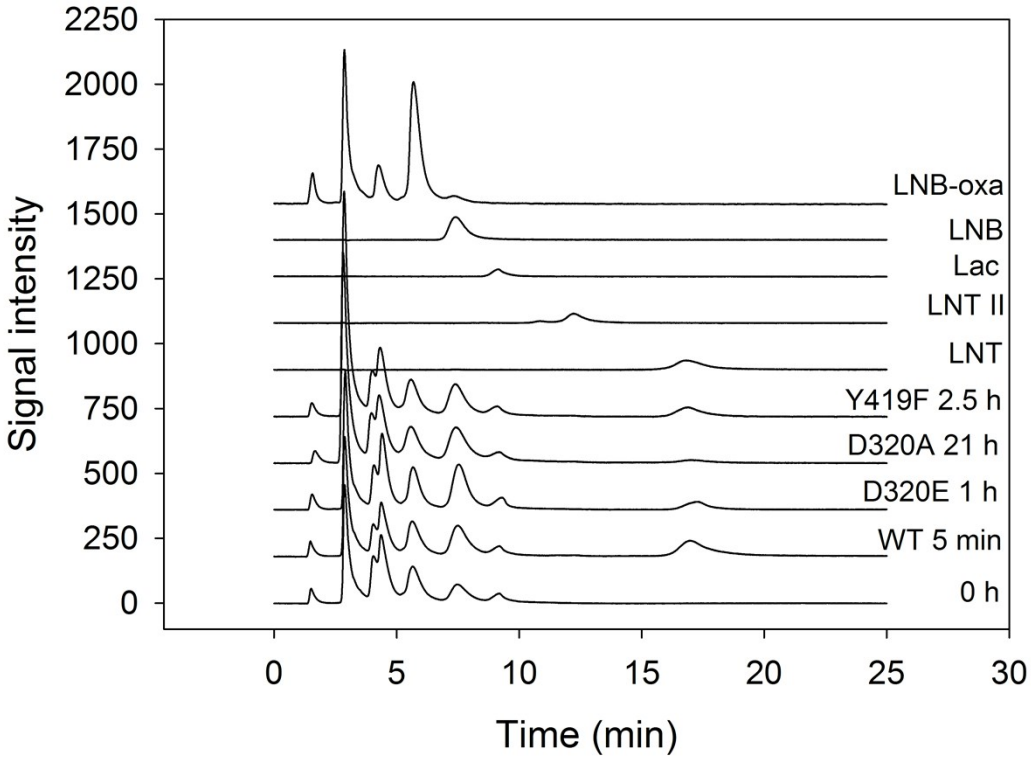

Fig. S7. HPLC analysis of LNT synthesis from LNB-oxa by wild-type LnbB and variants thereof. Overlay of HPLC-chromatograms showing maximum LNT formation obtained by various enzyme variants of LnbB. Reaction mixtures contained 12 mM LNB-oxa, a 50-fold excess of lactose and 0.5 – 20 μM of the enzymes. Samples were 5-fold diluted, thus ~2.4 mM of LNB-oxa substrate were applied. Concentrations of authentic standards were 1 mM LNB, 100 mM Lac, 0.7 mM LNT II, 1 mM LNT. UV-detection at 195 nm was used.

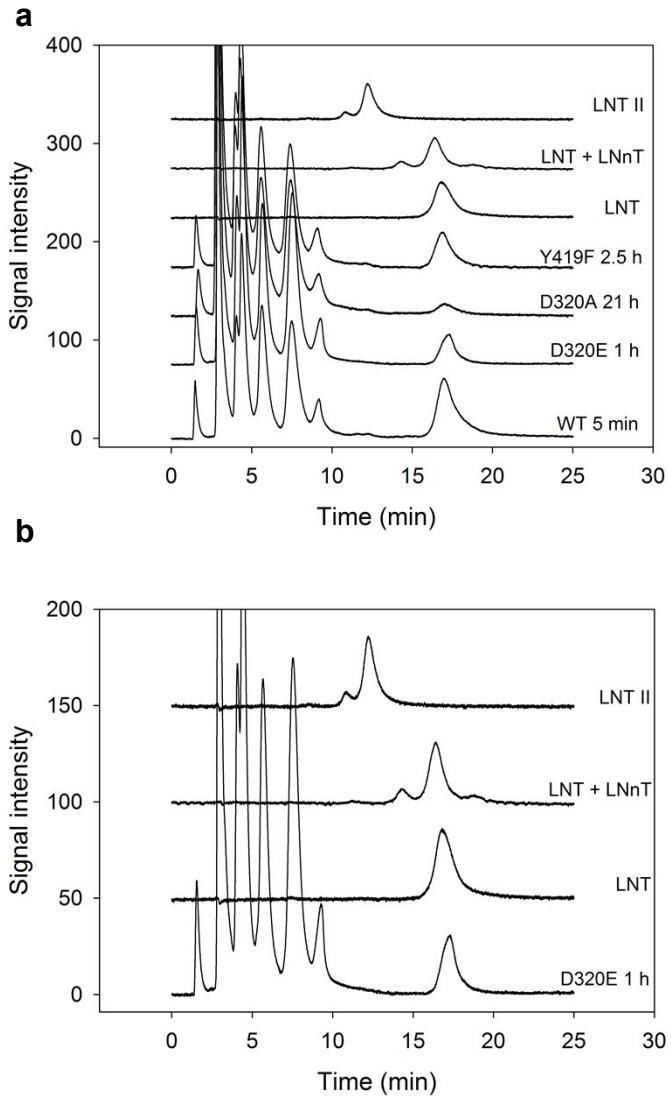

Fig. S8. HPLC analysis to confirm formation of LNT by LnbB enzymes. Overlay of HPLC-chromatograms showing maximum LNT formation obtained by various enzyme variants of LnbB. Full (a) and zoomed views (b) of HPLC-chromatograms are shown. Reaction mixtures contained 12 mM LNB-oxa, a 50-fold excess of lactose and 0.5 – 20 µM of the enzymes. Samples were 5-fold diluted. UV-detection at 195 nm was used. Authentic LNT (Carbosynth, OL162665; Gal-β1,3-GlcNAc-β1,3-Gal-β1,4-Glc) and a mixture of authentic LNT and lacto-*N*-neotetraose (LNnT, Gal-β1,4-GlcNAc-β1,3-Gal-β1,4-Glc) (Carbosynth, OL05683) were used for product identification. Clear separation of the regioisomers LNT and LNnT was achieved. Note, wild-type LnbB is highly specific for degrading LNT into LNB and lactose.[1b] However, engineering into glycosynthases could cause a change in enzyme selectivity. Thus, authentic LNT II (Carbosynth, OL09898; GlcNAc-β1,3-Gal-β1,4-Glc) was used to detect hydrolysis of LNT into LNT II. Hydrolysis of LNT into LNT II was not observed.

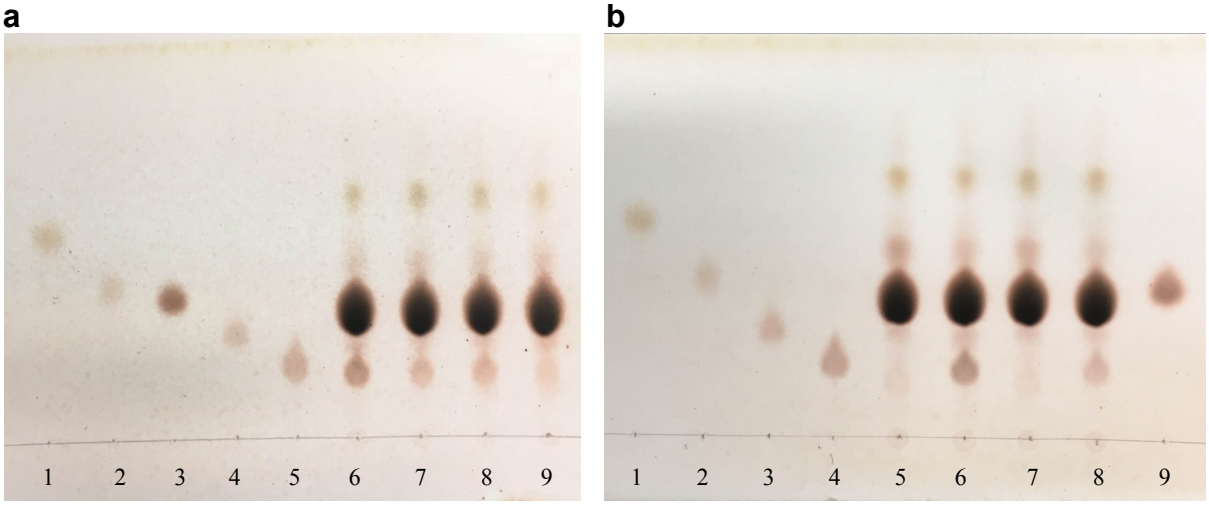

Fig. S9. TLC analysis of LNT synthesis from LNB-oxa by wild-type LnbB and variants thereof. 12 mM LNB-oxa, a 50-fold excess of lactose and 0.5 – 20 µM of the enzymes were used. Reaction mixtures were 5-fold diluted. Carbohydrates were visualized by thymol-sulfuric acid reagent. (a) Lane 1, GlcNAc; lane 2, LNB; lane 3, lactose; lane 4, LNT II; lane 5, LNT; lane 6, wild-type reaction after 10 min; lane 7, D320E reaction after 20 min; lane 8, Y419F reaction after 2.5 h; lane 9, D320A reaction after 21 h. (b) Lane 1, GlcNAc; lane 2, LNB; lane 3, LNT II; lane 4, LNT; lane 5, wild-type reaction after 0 min; lane 6, wild-type reaction after 10 min; lane 7, D320E reaction after 0 h; lane 8, D320E reaction after 1 h; lane 9, lactose.

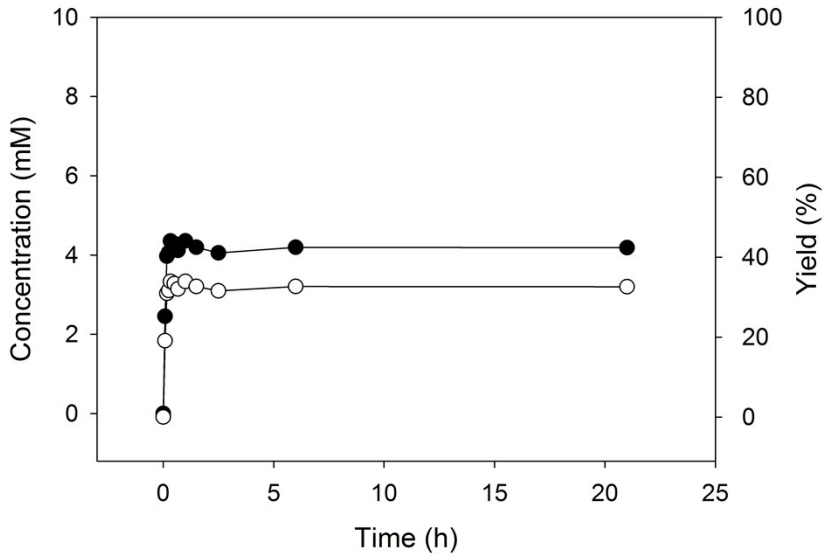

Fig. S10. Synthesis of LNT from LNB-oxa by the LnbB D320E variant. Reaction mixture contained 12 mM LNB-oxa, a 50-fold excess of lactose and 20 µM D320E. LNT, filled circles; yield, open circles.

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