

A Novel Three-Enzyme Reaction Cycle for the Synthesis of *N*-Acetyllactosamine with *in Situ* Regeneration of Uridine 5'-Diphosphate Glucose and Uridine 5'-Diphosphate Galactose

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Abstract: A new three-enzyme reaction cycle consisting of sucrose synthase, UDP glucose 4'-epimerase, and human β -1,4-galactosyltransferase was established for the synthesis of *N*-acetyllactosamine (LacNAc) with *in situ* regeneration of UDP galactose. We found that UDP glucose 4'-epimerase is reductively inactivated in the presence of UMP and acceptor substrates of β -1,4-galactosyltransferase. Reactivation of UDP glucose 4'-epimerase by the transition state analogues dUDP or dTDP 6-deoxy-D-xylo-4-hexulose in combination with the repetitive batch technique enabled us to use the native enzymes for 11 days in this cycle. With 10 U of sucrose synthase, 5 U of UDP glucose 4'-epimerase, and 1.25 U of β -1,4-galactosyltransferase, 594 mg of LacNAc could be synthesized. *N*-Acetyllactosamine was also subsequently converted to Neu5Ac α 2,6Gal β 1,4GlcNAc with α -2,6-sialyltransferase and CMP-Neu5Ac.

Introduction

The realization that the oligosaccharide moieties of glycoconjugates are involved in important intra- and intercellular communication events leads to an increasing demand for oligosaccharides.^{1a-f} Compared to chemical syntheses with many protection and deprotection steps, enzymatic syntheses using glycosyltransferases and glycosidases give stereo- and regioselectively glycosidic bonds in one step without the need for protection.^{2a-d}

Glycosyltransferases require the very expensive nucleotide sugars as substrates which can chemically^{3a-8} and enzymatically^{3h-o} be prepared. In order to avoid product inhibition

of transferases by nucleotides as well as large-scale preparation and isolation of nucleotide sugars for stoichiometric reactions, *in situ* regeneration cycles for nucleotide sugars have been established.⁴ Previously published enzymatic syntheses of LacNAc with *in situ* regeneration of UDP-Gal comprised six enzymes.^{5,6}

We present here the synthesis of *N*-acetyllactosamine (LacNAc, **1**) with a three-enzyme reaction cycle using human milk β -1,4-galactosyltransferase (GalT, EC 2.4.1.38) and the concomitant *in situ* regeneration of UDP-Gal by two enzymes: sucrose synthase (SuSy, EC 2.4.1.13) and UDP glucose 4'-epimerase (epimerase, EC 5.1.3.2) (Figure 1).

The unique character of sucrose synthase to generate activated glucoses by the cleavage of sucrose with nucleoside diphosphates makes this plant glycosyltransferase favorable over pyrophosphorylases.⁷⁻⁹ Compared to previously published *in situ* regeneration cycles for UDP-Gal,^{5,6} our novel three-enzyme reaction cycle does not afford phospho(enol)pyruvate (PEP) for nucleoside triphosphate regeneration nor does it generate phosphate (from pyrophosphate by pyrophosphatase) avoiding inhibition. To ensure enzyme availability we have purified sucrose synthase from rice grains in a pilot scale.¹⁰

In this paper, we present results following three objectives: (i) optimized conditions for all three enzymes with respect to high enzyme productivities (g of **1**/U of enzyme), (ii) reuse of the three native enzymes with the repetitive batch technique in order to increase enzyme productivity and decrease enzyme costs, (iii) reactivation of reductively inactivated UDP-Glc 4'-epimerase appearing in the presence of UMP and free monosaccharides (acceptor substrates of GalT) in the reaction mixture.

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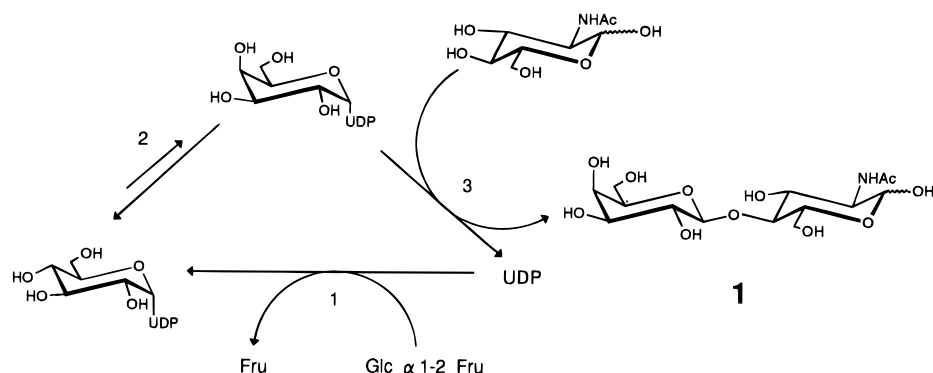


Figure 1. Three-enzyme reaction cycle for LacNAc synthesis with (1) sucrose synthase, (2) UDP-Glc 4'-epimerase, and (3) β -1,4-galactosyltransferase.

Table 1. Results for LacNAc Synthesis under Starting and Optimized Conditions

	starting conditions	optimized conditions
yield	95.6% after 24 h	100% after 15 h
space-time yield ($\text{g L}^{-1} \text{d}^{-1}$)	1.8	6.1
cycle no. ^a	4.8	10
g of LacNAc/U of GalT	0.018	0.077
g of LacNAc/U of SuSy	0.002	0.010
g of LacNAc/U of epimerase	0.009	0.019

^a Cycle number means mol of UDP-Glc/mol of **1**.

Results and Discussion

Optimization of LacNAc Synthesis. The synthesis of **1** was optimized considering data from literature^{11–13} which were included in sequential and parallel strategies.¹⁴ Table 1 demonstrates that under optimized conditions a yield of 100% for 10 mM acceptor substrate could be obtained. The space-time yield and cycle number of UDP-Glc were increased by 2-fold. The productivity of GalT and SuSy (g of **1**/U of enzyme) could be increased by 4–5-fold.

LacNAc Synthesis with Repetitive Batch Technique. In an attempt to increase enzyme productivity further, we tried to reuse the native enzymes in subsequent batches. In the first batch the yield of **1** was 95% after 12 h. After separation of the enzymes from the product solution by diafiltration, new substrates were added to the enzyme solution. The second batch synthesis resulted in only 6.2% yield after 8 h. Detailed investigations revealed that the epimerase was no longer active in the second batch. Addition of fresh epimerase increased the yield of **1** to 67.1% after 14 h, demonstrating that SuSy and GalT were still active. The repetitive addition of UDP-Glc 4'-epimerase (\$6.3/U) appeared too costly and inefficient for large-scale synthesis. We therefore investigated the stability of epimerase in more detail.

Stability of UDP-Glc 4'-Epimerase. The reason for the rapid loss of UDP-Glc 4'-epimerase activity is a "suicide" mechanism by which the enzyme is inactivated.

UDP-Glc 4'-epimerase from yeast or *Escherichia coli* is a homodimeric enzyme with one molecule NAD^+ per native enzyme molecule. The cofactor is tightly bound to the enzyme

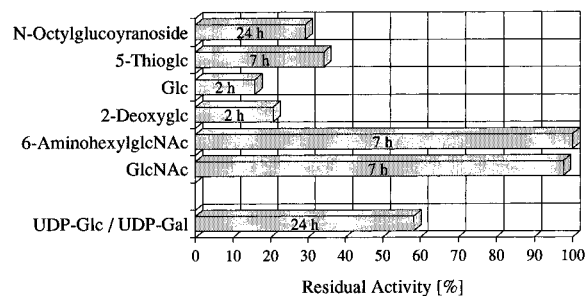


Figure 2. Reductive inactivation of UDP-Glc 4'-epimerase in the presence of different acceptors and donors of GalT. Epimerase was incubated in the presence of 1 mM UDP-Glc alone and in the presence of 0.1 mM UMP with 50 mM GlcNAc, 10 mM *O*-(6-aminohexyl)-2-acetamido-2-deoxy- β -D-glucopyranoside, 50 mM *O*-*n*-octyl- β -D-glucopyranoside, 50 mM D-Glc, 50 mM 2-deoxy-D-Glc, and 50 mM 5-thio-D-Glc, respectively. The incubation period is indicated in the bars.

by noncovalent forces.^{13,15} Epimerization at C-4 occurs via a UDP 4-ketoglucose transition state with concomitant formation of NADH. The cofactor NAD^+ is then regenerated intramolecularly by reduction of the C-4 oxo group. The enzyme suffers from reductive inactivation in the presence of uridine nucleotides (UMP) and different sugars as well as in the presence of UDP-Glc and UDP-Gal.^{15,16} Binding of UMP increases the reactivity of NAD^+ toward reducing sugars by an induced conformational change of the epimerase. The oxidized sugar quickly leaves the enzyme, and the enzyme-bound NADH cannot be regenerated.^{15,16}

During synthesis of **1**, UMP is formed as a byproduct by the Mn^{2+} -catalyzed decomposition of UDP-Glc and UDP-Gal.¹⁷ We found that in the presence of 0.1 mM UMP and 500 mM sucrose in 200 mM Hepes-NaOH (pH 7.2) the epimerase activity is drastically reduced after 8 h (15% residual activity). Buffer, sucrose, or UMP alone do not affect epimerase activity.

Since UMP is certainly also a byproduct in previously published LacNAc cycles,⁵ we investigated whether epimerase inactivation also occurs in the presence of UMP and monosaccharides. Figure 2 clearly demonstrates that glucose derivatives, which can serve as acceptors for GalT, inactivate epimerase in the presence of UMP. With GlcNAc and its derivatives the epimerase was not inactivated after 7 h. In the presence of UDP-Glc or UDP-Gal alone, 40% of the epimerase activity was lost after 24 h.

In conclusion, the epimerase is inactivated in LacNAc cycles where UMP and Glc or derivatives thereof as well as UDP-Glc

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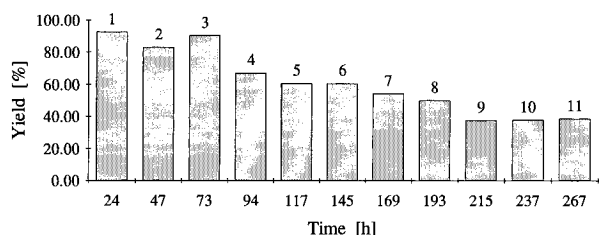


Figure 3. Preparative synthesis of **1** with repetitive batch technique (11 repeated batches) and with reactivation of epimerase using dUDP 6-deoxy-D-xylo-4-hexulose.

and UDP-Gal are present. Since sucrose synthase still contains 0.05% invertase activity, Glc is probably also present in the three enzyme reaction cycle.

Reactivation of UDP-Glc 4'-epimerase can be achieved by incubation of reductively inactivated epimerase with transition state analogues like dTDP 6-deoxy-D-xylo-4-hexulose,^{16,18} myo-inosose-2, or 2-ketoglucose.¹⁹

We have enzymatically synthesized dUDP and dTDP-6-deoxy-D-xylo-4-hexulose on a preparative scale (0.1–0.6 g) from dUMP²⁰ and dTDP glucose²¹ and used them for reactivation and stabilization of UDP-Glc 4'-epimerase. Under inactivating conditions (50 mM Gal, 0.1 mM UMP, 3% residual epimerase activity) the addition of the transition state analogue (0.1 mM or 1 mM dUDP 6-deoxy-D-xylo-4-hexulose) induces a quick reactivation (full activity within 1 h) and a prolonged enzyme stability over 1–5 days, respectively. With this improved epimerase stability it is now possible to synthesize lactose analogues and analogues of **1** with reuse of all enzymes and improved enzyme productivities.²²

Preparative LacNAc Synthesis. With the improved epimerase stability we used the three-enzyme reaction cycle for preparative synthesis of **1** by applying the repetitive-batch-technique (Figure 3). In 11 batches (11 days) 594 mg (1.55 mmol) of **1** was produced from 2.7 mmol of GlcNAc using 5 U of epimerase, 10 U of SuSy, and 1.25 U of GalT. For the reactivation of epimerase, 0.027 mmol (14.3 mg) of dUDP 6-deoxy-D-xylo-4-hexulose was consumed. The addition of this transition state analogue had no influence on the activity of SuSy, GalT, and other glycosyltransferases. The overall average yield of **1** was 57.4%. The drop in yield after 3 days is probably due to the repetitive filtration of the enzyme solution. Table 2 compares the effectiveness of 11 parallel batches (based on optimized conditions in Table 1) versus one batch with 10 repetitions (sum: 11 batches) as performed in our experiment. The productivities of all enzymes are significantly increased when the repetitive batch technique is used. These figures reveal that for the production of 1 g of **1**, 13.1 U of GalT is needed in parallel batches and only 2.1 U of GalT in the repetitive batch mode. Although the cycle number, space-time yield, and

Table 2. Comparison of Parallel and Repetitive Batches for LacNAc Synthesis

	parallel batches	repetitive batches
batches/repetitions/volume (mL)	11/0/25	1/10/25
GalT (U)	13.75	1.25
epimerase (U)	55	5
SuSy	110	10
average yield (%)	100	57.4
space-time yield (g L ⁻¹ day ⁻¹)	6.1	2.2
cycle no.	10	5.7
LacNAc synthesized (g)	1.05	0.594
g of LacNAc/U of GalT	0.077	0.475
g of LacNAc/U of epimerase	0.019	0.119
g of LacNAc/U of SuSy	0.004	0.059
estimated cost \$/g of LacNAc	4124	712

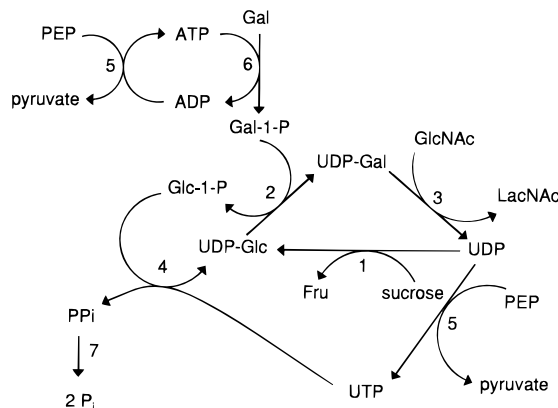


Figure 4. Multi-enzyme system for the synthesis of **1** with (1) sucrose synthase, (2) Gal-1-phosphate uridylyltransferase, (3) β -1,4-galactosyltransferase, (4) UDP-Glc pyrophosphorylase, (5) pyruvate kinase, (6) galactokinase, and (7) pyrophosphatase.

average yield are lower, the product costs are reduced by 6-fold based on consumed chemicals, GalT and epimerase.

Product isolation started with the cleavage of excess sucrose by invertase [EC 3.2.1.26] from yeast. The protein was separated from the product solution by ultrafiltration. In order to destroy traces of LacNAc-cleaving activities the commercial invertase preparation was preincubated for 2 h at 45 °C. D-Fructose and most of GlcNAc and Glc could be separated from the product by chromatography using AG50W-X8 in Ca²⁺ form. After separation from buffer and polar substances by anion exchange, **1** was further purified by gel filtration (356 mg of **1**, overall yield 34.4%).

Synthesis of Neu5Ac α 2,6Gal β 1,4GlcNAc. On a smaller scale, a solution of **1** obtained after separation of the enzymes by ultrafiltration was directly used for the synthesis of Neu5Ac α 2,6Gal β 1,4GlcNAc with α -2,6-sialyltransferase [EC 2.4.99.1] from rat liver.^{23a,b} The CMP formed was cleaved with calf intestinal alkaline phosphatase [EC 3.1.3.1].²⁴ The product formation was analyzed with HPLC. Neu5Ac α 2,6Gal β 1,4GlcNAc (7.36 mg) was isolated (overall yield 64.3%). By this combination the laborious isolation of **1** can be avoided.²⁵

Replacement of UDP Glucose 4'-Epimerase by Galactose 1-Phosphate Uridyltransferase. An alternative way to overcome the obvious disadvantages of epimerase utilization is to replace the enzyme by Gal-1-phosphate uridylyltransferase [EC 2.7.7.12] (Figure 4). By starting from Gal-1-phosphate omitting

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(25) Work is in progress in our laboratory to combine the novel three-enzyme reaction cycle with *in situ* regeneration of CMP-Neu5Ac.

Glc-1-phosphate recycling, **1** was synthesized in 63% yield after 24 h. However, recycling of Glc-1-phosphate to UDP-Glc and ADP to ATP as well as PEP consumption in both cases makes this cycle more complex.

Conclusion

In summary, we have developed a novel three-enzyme reaction cycle with *in situ* regeneration of UDP-Glc and UDP-Gal for the synthesis of **1**. We have clearly demonstrated that reductive inactivation of UDP-Glc 4'-epimerase is a serious problem in all cycles where at least UDP-Glc/UDP-Gal or UMP and glucose derivatives as acceptor substrates of GalT are present. The reactivation of UDP-Glc 4'-epimerase by dNDP 6-deoxy-D-xylo-4-hexulose and the utilization of the repetitive batch technique enabled us to increase the productivity of all three enzymes. Further work is in progress in our laboratory to exploit the novel LacNAc cycle for the synthesis of **1**, as well as analogues of lactose and **1**, and to combine it with other glycosyltransferases employing *in situ* regeneration cycles for CMP-NeuAc and GDP-Fuc.

Experimental Section

Materials. Sucrose synthase (SuSy) was purified from rice grains.¹⁰ CMP-Neu5Ac was kindly provided by U. Kragl. β -1,4-GalT [EC 2.4.1.38] from human milk, calf intestinal alkaline phosphatase [EC 3.1.3.1], and α -2,6-sialyltransferase [EC 2.4.99.1] from rat liver were from Boehringer Mannheim (Mannheim, Germany). UDP-Glc 4'-epimerase [EC 5.1.3.2] from *Saccharomyces cerevisiae*, Gal-1-phosphate uridylyltransferase [EC 2.7.7.12] from yeast, invertase [EC 3.2.1.26] from yeast, bovine serum albumine BSA, nucleotides, nucleotide sugars, GlcNAc, and *O*-*n*-octyl- β -D-glucopyranoside were supplied by Sigma (Deisenhofen, Germany). Hepes and NAD^+ were from Biomol (Hamburg, Germany). *S*-*n*-Octyl- β -D-thioglucofuranoside was from Aldrich (Steinheim, Germany). *O*-(6-Aminoheptyl)-2-acetamido-2-deoxy- β -D-glucopyranoside was a gift from the Hoechst AG (Frankfurt am Main, Germany). 5-Thio-D-Glc, 2-deoxy-D-Glc, *p*-anisaldehyde, and dithiothreitol (DTT) were from Fluka (Neu-Ulm, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany).

Analytical Methods. The activity of SuSy was determined for the cleavage reaction with UDP.¹⁰ One enzyme unit (U) corresponds to the formation of 1 μmol of UDP-Glc/min using standard conditions.

Optimization of the LacNAc Synthesis. The concentrations of **1** and GlcNAc were analyzed by HPLC using two connected Aminex HPX-87H columns (300 $\times$ 7.8 mm each, Biorad, München, Germany). Compounds were separated using 4 mM H_2SO_4 as the eluent at a flow rate of 0.55 mL/min at 65 °C and quantified by UV detection at 205 nm.

Starting conditions for synthesis of **1**: 1 mM UDP-Glc, 1 mM MnCl_2 , 5 mM GlcNAc, 500 mM sucrose, 0.1 U/mL GalT, 0.2 U/mL epimerase, and 0.8 U/mL SuSy were incubated for 24 h in 200 mM Hepes-NaOH (pH 7.2, 25 mM KCl, 0.01% BSA, 1 mM DTT) at 30 °C.

Because of the high stability of SuSy in the buffer we selected 200 mM Hepes-NaOH (pH 7.2) for synthesis of **1** including 25 mM KCl, 0.01% BSA which activates GalT,¹¹ and 1 mM dithiothreitol (DTT) which prevents the oxidation of SH groups of cysteine in the epimerase.^{12,13} The experiments were carried out with native enzymes at 30 °C.

In optimization experiments,¹⁴ the pH and the concentrations of GlcNAc, MnCl_2 , UDP-Glc, SuSy, GalT, epimerase, and BSA were varied.

Optimized conditions for synthesis of **1**: 1 mM UDP-Glc, 1 mM MnCl_2 , 10 mM GlcNAc, 500 mM sucrose, 0.05 U/mL GalT, 0.2 U/mL epimerase, and 0.4 U/mL SuSy were incubated for 15 h in 200 mM Hepes-NaOH (pH 7.2, 25 mM KCl, 0.1% BSA, 1 mM DTT) at 30 °C.

LacNAc Synthesis with Repetitive Batch Technique. To test the repetitive use of the native enzymes,²⁶ synthesis of **1** was carried out

under optimized conditions in a 1 mL scale. After 12 h, 750 μL of the product solution was separated from the enzymes by ultrafiltration using a centricon 10 cartridge (cutoff 10 kDa, Amicon, Beverly U.S.A.) and analyzed by HPLC. After diafiltration of the enzyme solution with 3 $\times$ 250 μL of buffer (without BSA), a fresh substrate solution containing UDP-Glc, GlcNAc, MnCl_2 , and sucrose was added. After adjustment of the volume to 1 mL with buffer, the second batch was incubated for 8 h and analyzed again by HPLC. To prove that UDP-Glc 4'-epimerase was not active in the second batch, 0.18 U of epimerase was added and incubation continued for further 14 h.

Stability of UDP-Glc 4'-Epimerase. Photometrical Enzyme Assay. The reaction mixture consisted of 893 μL of 0.1 M glycine-NaOH buffer (pH 8.8), 20 μL of 5 mM UDP-Gal, 20 μL of 50 mM NAD^+ , and 33.3 μL of UDP-Glc dehydrogenase (2 U/mL). The reaction was started by the addition of 33.3 μL epimerase diluted in buffer. The initial reaction rate was followed by the increase of absorbance at 340 nm (DE/min) at 25 °C. One unit is the amount of enzyme which forms 1 μmol of UDP-Glc/min.

Inactivation of UDP-Glc 4'-Epimerase in the Presence of Sucrose and UMP. Epimerase (0.13 mg (0.13 U)) was incubated in 500 μL of buffer A (200 mM Hepes-NaOH (pH 7.2), 1 mM DTT, 25 mM KCl, 0.1% BSA), in buffer A with 0.1 mM UMP, in buffer A with 500 mM sucrose, and in buffer A with 500 mM sucrose and 0.1 mM UMP, respectively. After 8 h of incubation at 30 °C, the epimerase activity was determined and expressed as residual activity (100% residual activity at $t = 0$).

Inactivation of UDP-Glc 4'-Epimerase in the Presence of Nucleotide Sugars and Different Acceptor Substrates of β -1,4-GalT. Epimerase (0.13 mg (0.13 U)) was incubated at 30 °C in 500 μL of buffer A containing 0.1 mM UMP with 50 mM GlcNAc, 50 mM 2-deoxy-D-Glc, 50 mM D-Glc, 50 mM 5-thio-D-Glc, 50 mM *O*-*n*-octyl- β -D-glucopyranoside, and 10 mM *O*-(6-aminohexyl)-2-acetamido-2-deoxy- β -D-glucopyranoside, respectively. The epimerase activity was determined after various incubation periods and expressed as residual activity (100% residual activity at $t = 0$).

Reactivation of Reductively Inactivated UDP-Glc 4'-Epimerase. Epimerase (0.25 mg/mL (0.25 U/mL)) was inactivated in the presence of 50 mM Gal and 0.1 mM UMP at 30 °C in 2 h, yielding 3% residual activity. Inactivated epimerase (160 μL) was incubated with 40 μL of 0.1 or 1 mM dUDP and dTDP 6-deoxy-D-xylo-4-hexulose, respectively. dUDP and dTDP 6-deoxy-D-xylo-4-hexulose were prepared from dUMP- or dUDP-Glc²⁰ and dTDP-Glc^{9,21} by the combination of sucrose synthase and recombinant dTDP glucose 4,6-dehydratase [EC 4.2.1.46].

Preparative Synthesis of LacNAc. The repetitive batch technique (11 repeated batches) was also applied for the synthesis of **1** in a preparative scale. Each batch (10 with a final volume of 25 mL and one with 20 mL) contained 1 mM UDP-Glc, 10 mM GlcNAc, 1 mM MnCl_2 , 500 mM sucrose, and 0.1 mM dUDP 6-deoxy-D-xylo-4-hexulose and 0.1% (w/v) BSA in 200 mM Hepes-NaOH (pH 7.2, 1 mM DTT, 25 mM KCl). Starting with the first batch, the solution (25 mL) was incubated with 1.25 U of GalT (0.05 U/mL), 5 U of epimerase (0.2 U/mL), and 10 U of SuSy (0.4 U/mL) at 30 °C. After 22.5 h, the product and nonreacted substrates were separated from the enzymes by ultrafiltration in a stirred ultrafiltration cell (Amicon, model 8050, equipped with a membrane YM 10, cutoff 10 kDa). The solution was brought to a volume of 50 mL with buffer and concentrated to 25 mL. Filtration was repeated three times with 20 mL buffer for the last step. The second batch (25 mL) was started by addition of concentrated substrates in 5 mL buffer solution. After sterile filtration the second batch was incubated at 30 °C for 24 h. The synthesis consisted of 10 batches with a 25 mL and 1 batch with a 20 mL reaction volume employing incubation times between 21 and 30 h. Each batch was followed by HPLC to determine the yield of each batch. Overall, 183 mg of UDP-Glc (0.3 mmol), 597 mg of GlcNAc (2.7 mmol), 46.2 mg of MnCl_2 , 46.2 g of sucrose (135 mmol), 14.3 mg of dUDP 6-deoxy-D-xylo-4-hexulose (0.025 mmol), 25 mg of BSA, 1.25 U of GalT, 5 U of epimerase, and 10 U of SuSy were used. Overall 594 mg of **1** (1.55 mmol) was synthesized from 2.7 mmol of GlcNAc (yield 57.4%).

Product isolation was started by enzymatic cleavage of sucrose. Invertase (25 000 U/mL) was preincubated in buffer (200 mM Hepes-NaOH (pH 7.2, 500 mM sucrose, 1 mM DTT, 0.1% BSA, 25 mM KCl)) 2 h at 45 °C. The reaction was started by addition of 10 μL of

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invertase solution/mL of product solution at 45 °C. Cleavage of sucrose was followed by a polarimeter (241 polarimeter, Perkin-Elmer). After 2 h the invertase was separated from the product solution by ultrafiltration (see above). In order to facilitate product isolation the final solution was divided into five batches. Each batch was concentrated at 20–25 mbar and 30–35 °C to a volume of 30 mL. After chromatography on AG50W-X8 (Ca²⁺ form, column 5 × 35 cm, 0.5 mL/min bidistilled water as the eluent), **1** was further purified on an anion-exchange column (2.6 × 26.5 cm, Dowex 1x2, 100–200 mesh, Cl[−] form). Prior to loading the pH of the solution was brought to 8.5. The sugars were eluted with bidest. water (flow rate 3.5 mL/min). The fractions containing **1** were pooled and subsequently adjusted to pH 7.0. The product solution was concentrated at 20–25 mbar and 30–35 °C to a volume of 9 mL. After a gel filtration on Biogel P2 (2.6 × 82 cm, 0.5 mL/min bidest. water as the eluent), the resulting product solutions of all five batches were combined. After repeating the anion-exchange chromatography and the gel filtration once and subsequent lyophilization, 462 mg of product containing 77% of **1** (356 mg, 0.93 mmol, overall yield 34.4%), 4.2% GlcNAc, and 18.7% NaCl according to HPLC analysis was found. For NMR analysis the residual GlcNAc and salt was separated from **1** (20 mg) by gel filtration. The NMR data correspond exactly to those previously reported.^{5,27}

NMR analysis of LacNAc: ¹³C-NMR (75 MHz, D₂O) δ 177.6 (C=O α, β), 105.8, 105.7 (Gal, C-1, (α), (β) anomerization of GlcNAc), 97.7 (GlcNAc, C-1, β), 93.4 (GlcNAc, C-1, α), 81.7 (GlcNAc, C-4, α), 81.3 (GlcNAc, C-4, β), 78.2 (Gal, C-5), 77.7 (GlcNAc, C-5, β), 75.4 (Gal, C-3), 73.8 (Gal, C-2), 73.1 (GlcNAc, C-5, α), 72.1 (GlcNAc, C-3, α, β), 71.4 (Gal, C-4), 63.8 (Gal, C-6), 62.8 (GlcNAc, C-6, α, β), 59.1 (GlcNAc, C-2, β), 56.6 (GlcNAc, C-2, α), 25.0 (CH₃−, β), 24.8 (CH₃−, α); ¹H-NMR (300 MHz, D₂O) δ 5.21 (d, 1 H, GlcNAc, H-1, α, ³J_{H-1a,H-2} = 2.2 Hz), 4.73 (d, 1 H, GlcNAc, H-1, β, ³J_{H-1b,H-2} = 8 Hz), 4.478 (d, 1 H, Gal (α), H-1, ³J_{H-1,H-2} = 8 Hz), 4.476 (d, 1 H, Gal (β), H-1, ³J_{H-1,H-2} = 8 Hz), 4.03–3.69 (m, 12 H), 2.055 (s, 3 H, CH₃−).

Synthesis of Neu5Acα2,6Galβ1,4GlcNAc. Synthesis of **1** was carried out under optimized conditions (see above) on a 5 mL scale. After ultrafiltration (see above), 2 mL of product solution, 50 μL of

α-2,6-sialyltransferase (0.1 U), 6 μL of alkaline phosphatase (6 U), 2 mg of BSA, and 13.7 mg of CMP-Neu5Ac (10 mM) were incubated at 37 °C for 24 h. The reaction was analyzed by HPLC as described above for **1**. The concentration of CMP-Neu5Ac during the synthesis was followed by ion pair HPLC.⁷

The product was isolated from a 1.7 mL solution as described above. After cleavage of sucrose and ultrafiltration, Neu5Acα2,6Galβ1,4GlcNAc was separated off by gel filtration on Biogel P2. After lyophilization, 7.36 mg of product (overall yield 64.3 %) was isolated and analyzed by HPLC (100% Neu5Acα2,6Galβ1,4GlcNAc) and NMR spectroscopy.

The NMR spectrum corresponds to that previously reported.²⁸

NMR analysis of NeuAcα2,6Galβ1,4GlcNAc: ¹H-NMR (300 MHz, D₂O) δ 5.25 (d, 1 H, GlcNAc, H-1, α, ³J_{H-1a,H-2} = 2.2 Hz), 4.54 (d, 1 H, Gal, H-1, ³J_{H-1,H-2} = 8 Hz), 2.74 (dd, 1 H, 3e-H-Neu5Ac, ²J_{H-3a,H-3e} = 13 Hz, ³J_{H-3e,H-4} = 5 Hz), 2.13 (s, 3 H, NAc, GlcNAc), 2.09 (s, 3 H, NAc, Neu5Ac), 1.77 (dd, 3a-H-Neu5Ac, ²J_{H-3a,H-3e} ≈ ³J_{H-3a,H-4} = 12 Hz).

Replacement of UDP-Glc 4'-Epimerase by Galactose 1-Phosphate Uridyltransferase. Galactose 1-phosphate (10 mM), 10 mM GlcNAc, 1 mM UDP-Glc, 1 mM MnCl₂, 0.2 U/mL Gal-1-phosphate uridyltransferase, 0.4 U/mL SuSy, and 0.05 U/mL GalT were incubated in 200 mM Hepes-NaOH (pH 7.2, 500 mM sucrose, 1 mM DTT, 25 mM KCl, 0.1% BSA) at 30 °C. The formation of **1** was followed by HPLC.

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