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A substrate tagging and two-step enzymatic reaction strategy for large-scale synthesis of 2,7-anhydro-sialic acid

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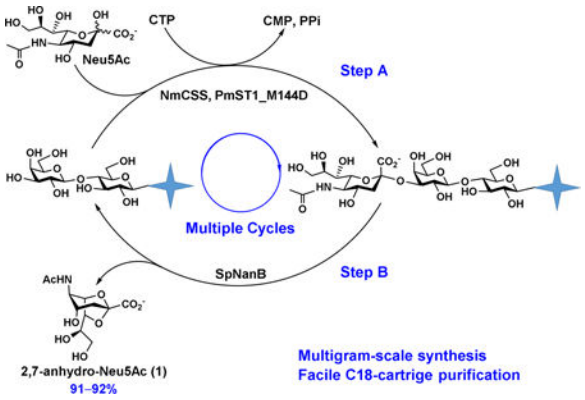
Abstract

A sialyltransferase acceptor tagging and two-step enzymatic reaction strategy has been developed for multigram-scale chemoenzymatic synthesis of 2,7-anhydro-*N*-acetylneuraminic acid (2,7-anhydro-Neu5Ac), a compound that can serve as a sole carbon source for the growth of *Ruminococcus gnavus*, a common human gut commensal. Different approaches of introducing hydrophobic UV-active tags to lactose as well-suited sialyltransferase acceptors have been explored and a simple two-step high-yield chemical synthetic procedure has been identified. The UV-active hydrophobic tag facilitates monitoring reaction progress and allows facile product purification by C18-cartridges. A two-step enzyme-catalyzed reaction procedure has been established to combine with C18 cartridge-based purification process for high-yield production of the desired product in multigram scales with the recycled use of chromophore-tagged lactoside starting material and sialoside intermediate. This study demonstrated an environmentally friendly highly-efficient synthetic and purification strategy for the production of 2,7-anhydro-Neu5Ac to explore its potential functions.

Graphical abstract



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Keywords

2,7-anhydro-*N*-acetylneuraminic acid; chemoenzymatic synthesis; sialic acid; sialidase; sialyltransferase; substrate tagging

1. Introduction

2,7-Anhydro-*N*-acetylneuraminic acid (2,7-anhydro-Neu5Ac, **1**) (Fig. 1) is a unique naturally existing non-reducing sialic acid form. Its methyl ester was reported as the methanolysis by-product of *N*-acetylneuraminic acid (Neu5Ac) [1], the most common sialic acid form [2]. In nature, 2,7-anhydro-Neu5Ac was found in rat urine [3] and human wet cerumen [4]. It was the product released from sialosides by a novel intramolecular *trans*-sialidase (IT-sialidase L) from *Macrobdella decora* (the leech) [5, 6], Gram-positive human pathogenic bacterium *Streptococcus pneumoniae* sialidase SpNanB [7], and Gram-positive human gut commensal *Ruminoccocus gnavus* sialidase RgNanH [8]. 2,7-Anhydro-Neu5Ac, but not Neu5Ac, was shown to serve as a sole carbon source for the growth of *Ruminoccocus gnavus* [9]. Therefore, the activity of *Ruminoccocus gnavus* sialidase RgNanH in producing 2,7-anhydro-Neu5Ac provides the bacterium a competitive growth advantage in sialic acid-rich host gut environment [9]. A recent study reported by us also indicates that 2,7-anhydro-Neu5Ac derivatives can be potential selective sialidase inhibitors against *Streptococcus pneumoniae* sialidases SpNanB and SpNanC [10]. To further explore the potential applications of 2,7-anhydro-Neu5Ac, efficient methods for high-yield large-scale synthesis are of great interest.

2,7-Anhydro-Neu5Ac has been produced by chemical [11–13] and enzymatic strategies [14]. For example, it was synthesized by intramolecular glycosidation of thiosialosides using silver triflate palladium (II) salt as a promoter [11]. Recently, an improved chemical synthetic procedure was reported for the synthesis of 2,7-anhydro-Neu5Ac from Neu5Ac through methyl ester formation, per-*O*-trimethylsilylation, followed by intramolecular anomeric protection and deprotection steps [13]. Furthermore, instead of forming the 2,7-anhydro-ring by chemical derivatization of Neu5Ac or Neu5Ac-containg structures, *de novo* construction of the 6,8-dioxabicyclo[3.2.1]-octane ring in 2,7-anhydro-Neu5Ac prior to its conversion to Neu5Ac was achieved by direct ketalization of diene-diol and ketal followed by catalytic ring-closing olefin metathesis [15, 16]. Nevertheless, even with the improved synthetic routes [13], chemical synthetic methods require multistep protection and deprotection steps with purification processes using large amounts of environmentally unfriendly organic solvents. IT-sialidase-dependent enzymatic methods using either RgNanH [14] or SpNanB [10] have advantages of avoiding protection and deprotection processes and allowing the reactions to be carried out in environmentally friendly aqueous solutions. Compared to the use of sialylglycoprotein fetuin as the source of sialoside [14] which limited the scale of the 2,7-anhydro-Neu5Ac production due to the high cost and the limited availability of sialylated glycoproteins, the use of in-situ generated sialyl oligosaccharides as the substrates for SpNanB-catalyzed reaction in a one-pot multienzyme (OPME) reaction scheme allowed gram-scale production of the desired 2,7-anhydro-Neu5Ac using readily accessible less expensive materials [10]. The latter strategy also

allowed the use of 0.25–0.50 equivalent of the sialyltransferase acceptor as it was regenerated in the sialidase-catalyzed process in the same reaction mixture. Nevertheless, the product purification of the OPME strategy using lactose as the sialyltransferase acceptor was challenging and required multiple chromatography purification steps including a Bio-Gel P-2 Gel filtration column, a silica column, and a C18 reverse phase column [10].

To meet the need of large scale production of 2,7-anhydro-Neu5Ac (**1**) to explore its potential applications, we report here a sialyltransferase acceptor substrate tagging and two-step enzymatic reaction strategy that allow easy C18-based product purification. The strategy leads to high yield product formation with recycling of chromophore-tagged lactoside starting material and sialoside intermediate.

2. Results and discussion

2.1 Synthesis of chromophore-tagged sialyltransferase acceptors.

A hydrophobic chromophore or fluorophore was designed to attach to lactose to form well-suited tagged sialyltransferase acceptors to simplify product purification by using a single C18-cartridge and to allow the re-use of the sialyltransferase acceptor. Both carboxybenzyl (Cbz) and fluorenylmethyloxycarbonyl (Fmoc) tags were tested. As shown in Scheme 1, Cbz-protected lactosyl propylamine (LacβProNHCbz, **3**, 65% yield) was synthesized from LacβProN_3 (**2**) [17] by catalytic hydrogenation of the terminal azido group to form a primary amino group followed by conjugation with Cbz using benzyl chloroformate (CbzCl) and sodium carbonate (Na_2CO_3) [18–20]. Product purification was achieved easily using a C18 reverse phase column. Similarly, Fmoc-protected lactosyl propylamine (LacβProNHFmoc, **4**) was synthesized in 70% yield as a potential acceptor for sialyltransferases [21].

Between the two sialyltransferase acceptors synthesized, the Cbz-protected LacβProNHCbz (**3**) was more stable during enzymatic reactions and purification processes. It was chosen for large-scale production of 2,7-anhydro-Neu5Ac (**1**) using a two-step enzymatic reaction cycle.

2.2 Two-step enzymatic production of 2,7-anhydro-Neu5Ac (1) using LacβProNHCbz (3) as a substrate

As shown in Scheme 2A, sialoside Neu5Acα2–3LacβProNHCbz (**5**) was synthesized in the first step from LacβProNHCbz (**3**) by a one-pot two-enzyme (OP2E) sialic acid activation and transfer system (Step A, Scheme 2A) [22]. In this system, *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS) [23] was responsible for the activation of Neu5Ac in the presence of cytidine 5'-triphosphate (CTP) to form cytidine 5'-monophosphate-*N*-acetylneuraminic acid (CMP-Neu5Ac) *in situ*, providing the donor substrate for *Pasteurella multocida* sialyltransferase 1 M144D mutant (PmST1_M144D) [17, 24] for the formation of α2–3-linked sialoside Neu5Acα2–3LacβProNHCbz (**5**) from LacβProNHCbz (**3**). In order to achieve maximal turnover of Neu5Ac, it was used as a limiting reagent (0.95 equivalent of sialyltransferase acceptor). The OP2E sialylation reaction using LacβProNHCbz (**3**) (1.0 g, 1 eq.) led to the total consumption of the Neu5Ac (0.55 g, 0.95 eq.) for the formation of

sialoside Neu5Acα2– 3LacβProNHCbz (**5**). As both sialyltransferase acceptor LacβProNHCbz (**3**) and the product Neu5Acα2–3LacβProNHCbz (**5**) were tagged with a chromophore, they bound to the C18-cartridge and were easily separated from other more polar/charged molecules in the reaction mixture which were washed out using water. The negatively charged product Neu5Acα2– 3LacβProNHCbz (**5**) (1.51 g, 99% yield) was then readily separated from the remaining neutral sialyltransferase acceptor LacβProNHCbz (**3**) using 15% acetonitrile in water. LacβProNHCbz (**3**) (0.05 g) was recovered by eluting the C18-cartridge with 50% acetonitrile in water.

The obtained sialoside Neu5Acα2–3LacβProNHCbz (**5**) (1.51 g) was used in the second step (Step B, Scheme 2B) for the formation of the desired 2,7-anhydro-Neu5Ac (**1**) by a SpNanB-catalyzed reaction. LacβProNHCbz (**3**) was regenerated at the same time. The reaction process was monitored by thin-layer chromatography (TLC) and the reaction time was controlled carefully for the maximal production of 2,7-anhydro-Neu5Ac (**1**) and minimal hydrolysis. 2,7-Anhydro-Neu5Ac (**1**) (0.51 g) was produced in an excellent yield (91%) after passing the concentrated reaction mixture through a C18-cartridge followed by washing with water. The remaining Neu5Acα2–3LacβProNHCbz (**5**) (0.06 g) was recovered by eluting the C18-cartridge with 15% acetonitrile in water. LacβProNHCbz (**3**) (0.90 g) was obtained by eluting the C18-cartridge with 50% acetonitrile in water and the fraction was combined with the LacβProNHCbz (**3**) (0.05 g) recovered from the first step reaction for the next round of production.

The two-step enzymatic production of 2,7-anhydro-Neu5Ac was carried out for additional four cycles. Each cycle used LacβProNHCbz (**3**) recovered from the previous cycle as the reusable sialyltransferase acceptor. The Neu5Acα2–3LacβProNHCbz (**5**) recovered from the previous cycle was also combined with the Neu5Acα2–3LacβProNHCbz (**5**) formed in the first step in the new cycle for the production of 2,7-anhydro-Neu5Ac (**1**) in the second step of the new cycle. As shown in Table 1, cycle 1 produced 0.51 g of 2,7-anhydro-Neu5Ac (**1**) from 1.00 g of LacβProNHCbz (**3**) and 0.55 g of Neu5Ac with the recovery of 0.95 g of LacβProNHCbz (**3**) (0.05 g from step A and 0.90 g from step B) and 0.06 g of Neu5Acα2–3LacβProNHCbz (**5**) which were applied to the second cycle. Cycle 2 produced another 0.50 g of 2,7-anhydro-Neu5Ac (**1**) from 0.95 g of LacβProNHCbz (**3**) and 0.06 g of Neu5Acα2–3LacβProNHCbz (**5**) recovered from cycle 1. At the same time, 0.94 g of LacβProNHCbz (**3**) (0.06 g from step A and 0.88 g from step B) and 0.07 g of Neu5Acα2–3LacβProNHCbz (**5**) were recovered and applied to cycle 3. All five cycles were carried out using the same strategy. Overall, excellent yields (95–99%) were achieved in the step A of all five cycles for the production of Neu5Acα2– 3LacβProNHCbz (**5**). The yields (87–92%) of the production of 2,7-anhydro-Neu5Ac (**1**) in the step B of all five cycles were also high. In total for five cycles, 2.38 g (92%) of 2,7-anhydro-Neu5Ac (**1**) were produced from 2.55 g of Neu5Ac and 1.00 g of LacβProNHCbz (**3**). Meanwhile, a total of 0.81 g of LacβProNHCbz (**3**) and 0.12 g of Neu5Acα2–3LacβProNHCbz (**5**) were recovered and could be used for additional cycles for the production of the desired product 2,7-anhydro-Neu5Ac (**1**). The purification procedures were straightforward using a single C18-cartridge (51 g, 50 μm, 120 Å) eluting with isocratic solutions containing only water and acetonitrile. This was possible for the first step reaction as the product and the sialyltransferase acceptor were readily separated from

each other and from other hydrophilic components in the reaction mixture. For the second step reaction, it was important to monitor the reaction process to control the reaction time, minimizing the hydrolysis of 2,7-anhydro-Neu5Ac (**1**) to form Neu5Ac to achieve high efficient purification of 2,7-anhydro-Neu5Ac (**1**) by the simple C18-cartridge purification process.

2.3 Imporved synthesis of a chromophore-tagged sialyltransferase acceptor via a protection group-free approach.

To further improve the process for large-scale production of 2,7-anhydro-Neu5Ac (**1**), a protection group-free approach was developed for the synthesis of a tagged sialyltransferase acceptor in a multi-gram scale. As shown in Scheme 3, lactose was incubated with ammonia bicarbonate in ammonia hydroxide to form lactosylamine (LacβNH_2) readily [19]. After removing the solvent, the product was directly coupled with benzyl chloroformate (CbzCl) [19] in the presence of a base (Na_2CO_3) and a mixed solvent of H_2O and CH_3CN to form the desired product LacβNHCbz (**6**, 5.07 g, 73% yield over two steps) which was readily purified by a C18 cartridge. The yield for the formation of LacβNHCbz (**6**) from lactose was further improved to 80% by using anhydrous methanol as the solvent and *N,N*-diisopropylethylamine as the base (see ESI for details).

2.4 Two-step enzymatic production of 2,7-anhydro-Neu5Ac (1) using LacβNHCbz (6) as a substrate

To our delight, LacβNHCbz (**6**) was a well suited acceptor for PmST1_M144D [24]. To demonstrate the efficiency of the two-step enzymatic reaction procedure with a tagged sialyltransferase acceptor strategy in larger scale synthesis of 2,7-anhydro-Neu5Ac (**1**), two cycles of production were carried out. Five grams of LacβNHCbz (**6**) was used as the starting material in the first cycle. The corresponding α2–3-sialylated product Neu5Acα2–3LacβNHCbz (**7**) (Scheme 2) and the LacβNHCbz (**6**) recovered from cycle 1 were used in the next cycle. A larger C18 cartridge (140 g, 50 μm, 120 Å) was used for purification. Neu5Acα2–3LacβNHCbz (**7**) was eluted with 10% acetonitrile in water and LacβNHCbz (**6**) was eluted with 40% acetonitrile in water. As shown in Table 2, cycle 1 produced 2.75 g of 2,7-anhydro-Neu5Ac (**1**) from 3.09 g of Neu5Ac (0.95 equivalent, the limiting reagent) with the recovery of 4.81 g of LacβNHCbz (**6**) and 0.20 g of Neu5Acα2–3LacβNHCbz (**7**). Cycle 2 produced another 2.81 g of 2,7-anhydro-Neu5Ac (**1**) from 2.97 g of Neu5Ac (0.95 equivalent, the limiting reagent) with the recovery of 4.65 g (93%) of LacβNHCbz (**6**) and 0.17 g (2%) of Neu5Acα2–3LacβNHCbz (**7**). A total of 5.56 g of 2,7-anhydro-Neu5Ac (**1**) was obtained from 6.06 g Neu5Ac in two cycles of reactions, resulting in a 91% net yield. Again, sialylations in step A for both cycles were achieved in excellent 95–99% yields while the yields (92–93%) for SpNanB-catalyzed productions of 2,7-anhydro-Neu5Ac (**1**) in step B were also excellent.

Compared to the use of sialylated glycoprotein fetuin as a starting material in a membrane-enclosed enzymatic synthetic system for the production of 2,7-anhydro-Neu5Ac production [14], the current method uses less expensive starting materials that can be accessed in large amounts, therefore making the multigram-scale production feasible. Compared to one-pot multienzyme production of 2,7-anhydro-Neu5Ac in a single reaction process which requires

tedious multiple column purification steps [10], the current method separates the sialoside synthesis and the 2,7-anhydro-Neu5Ac production steps to allow facile C18-cartridge-based purification, simplifying the product purification process significantly.

3. Conclusions

In conclusion, a sialyltransferase acceptor substrate tagging and two-step enzymatic reaction cycle strategy has successfully developed for highly efficient production of 2,7-anhydro-Neu5Ac (**1**) from Neu5Ac in high yields with a facile C18 cartridge purification scheme. Carboxybenzyl (Cbz) group was found to be a well suited tag to derivatize sialyltransferase acceptors, allowing easy purification of products and intermediates by C18-cartidges. In addition, a high-yield protection group-free derivatization strategy was established to convert low-cost commercially available lactose in two steps to form LacβNHCbz as a suitable tagged sialyltransferase substrate for multigram-scale production of the desired product. The reactions and purification processes are environmentally friendly. The methodology presents a highly effective strategy to access 2,7-anhydro-Neu5Ac (**1**) in large scales to allow the exploration of its potential applications. The strategy can be extended to the synthesis and purification of other carbohydrates.

4. Experimental

4.1. Materials and general methods

All chemicals were obtained from commercial suppliers and used without further purification. ^{1}H NMR (400 or 800 MHz) and ^{13}C NMR (100 or 200 MHz) spectra were recorded on a Bruker Avance-400 Spectrometer or a Avance-800 Spectrometer. High resolution electrospray ionization (ESI) mass spectra were obtained using a Thermo Electron LTQ-Orbitrap Hybrid MS at the Mass Spectrometry Facility in the University of California, Davis. Column chromatography was performed using a Redi*Sep* Rf silica gel column (24 g Flash Column, CV 33 mL, 35 mL/min) or ODS-SM columns (51 g or 140 g, 50 μm, 120 Å, Yamazen) on a CombiFlash® Rf 200i system. Analytical thin-layer chromatography was performed on silica gel plates 60 GF_{254} (Sorbent technologies) using anisaldehyde stain for detection. Recombinant NmCSS [23], PmST1_M144D [24], and SpNanB [10, 25] were expressed as described previously.

4.2. Chemical synthesis of chromophore-tagged sialyltransferase acceptors 3 and 4.

4.2.1 Chemical synthesis of benzyl 3-[β -D-galactopyranosyl-(1→4)-β-D-glucopyranosyl] propyl carbamate (LacβProNHCbz, 3)—A catalytic amount (10%) of palladium in charcoal (Pd/C) was added to a solution of LacβProN_3 (**2**, 0.50 g, 1.18 mmol) [17] in H_2O (10 mL). The mixture was stirred under a hydrogen atmosphere for 4 h. When the reaction was completed, MeOH (10 mL) was added to dilute the solution before passing the mixture through a filter to remove palladium and charcoal [24]. The solvent was removed *in vacuo*. The obtained LacβProNH_2 was used directly in the following reaction without further purification. A sodium carbonate solution (10%, 10 mL) was used to dissolve LacβProNH_2 in a round-bottom flask. Benzyl chloroformate (CbzCl) (1.00 g, 5.88 mmol, 5 equiv.) in acetonitrile (10 mL) was added to the mixture in the flask immersed in an

ice-water bath [19]. After stirring the reaction mixture for 10 min, a saturated sodium carbonate solution was used to adjust the pH of the reaction mixture to 8.0–10.0. The reaction was then stirred at room temperature for overnight. After the reaction was completed, solvent was removed. The residue was re-dissolved in H_2O and the solution was passed through a ODS-SM column (51 g, 50 μm, 120 Å, Yamazen) using CombiFlash® Rf 200i system. LacβProNHCbz (**3**, 0.41 g, 0.77 mmol) was obtained as a white powder. ^{1}H NMR (800 MHz, D_2O) δ 7.44–7.20 (m, 5H), 5.00 (s, 2H), 4.36–4.30 (m, 2H), 3.89–3.79 (m, 3H), 3.71–3.55 (m, 6H), 3.54–3.49 (m, 2H), 3.49–3.40 (m, 2H), 3.27–3.07 (m, 3H), 1.70 (p, J = 6.7 Hz, 2H). ^{13}C NMR (200 MHz, D_2O) δ 158.38, 136.55, 128.76, 128.32, 127.61, 102.91, 102.04, 78.38, 75.32, 74.71, 74.33, 72.77, 72.49, 70.92, 68.51, 67.71, 66.77, 60.99, 60.05, 37.27, 28.83. HRMS (ESI-Orbitrap) *m/z*: $[M+Cl]^-$ Calcd for $C_{23}H_{35}NO_{13}Cl$ 568.1802; found 568.1792.

4.2.2 Chemical synthesis of 9-fluorenylmethyl 3-[β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl] propyl carbamate (Lac ProNHFmoc, 4)—

A catalytic amount (10%) of palladium in charcoal (Pd/C) was added to a solution of LacβProN_3 (**2**, 30 mg, 0.07 mmol) [17] in H_2O (3 mL). The mixture was stirred under a hydrogen atmosphere for 2 h. When the reaction was completed, MeOH (3 mL) was added to dilute the mixture before passing it through a filter to remove palladium and charcoal [24]. The solvent was removed in *vacuo*. The obtained LacβProNH_2 was used directly in the following reaction without further purification. A sodium carbonate solution (10%, 3 mL) was added to dissolve LacβProNH_2 in a round-bottom flask. 9-Fluorenylmethoxycarbonyl chloride (28 mg, 0.11 mmol, 1.5 equiv.) in 3 mL acetonitrile was added to the mixture in the flask immersed in an ice-water bath. The reaction was stirred then at room temperature for 1 h. After the reaction was completed, solvent was removed. The residue was purified using a Redi*Sep* Rf silica gel column chromatography (24 g Flash Column, CV 33 mL, 35 mL/min) using ethyl acetate: methanol = 1:0 to 1:1 (by volume) as the eluants and an ODS-SM reverse phase column (51 g, 50 μm, 120 Å, Yamazen) chromatography on the CombiFlash® Rf 200i system prior to small-scale enzymatic assays. LacβProNHFmoc (**4**, 31 mg, 0.05 mmol) was obtained in 70% yield as a white powder. ^{1}H NMR (800 MHz, D_2O) δ 7.43–6.62 (m, 8H), 4.39 (d, J = 7.8 Hz, 1H), 4.28–4.11 (m, 1H), 4.04–3.84 (m, 3H), 3.79 (dd, J = 12.1, 7.3 Hz, 2H), 3.76–3.71 (m, 2H), 3.71–3.43 (m, 7H), 3.41– 3.10 (m, 3H), 3.06–2.64 (m, 2H), 1.62–1.18 (m, 2H). ^{13}C NMR (200 MHz, D_2O) δ 157.39, 143.63, 140.73, 127.42, 126.93, 124.90, 119.60, 102.96, 102.14, 78.38, 75.31, 74.58, 74.37, 72.73, 72.60, 71.00, 70.93, 68.52, 67.34, 60.99, 60.06, 46.63, 37.28, 29.10. HRMS (ESI-Orbitrap) *m/z*: $[M+H]^+$ Calcd for $C_{30}H_{40}NO_{13}$ 622.2500; found 622.2515.

4.3 Small-scale assays for testing LacβProNHCbz (3) and LacβProNHFmoc (4) as sialyltransferase acceptors.

LacβProNHCbz (**3**) and LacβProNHFmoc (**4**) were tested as sialyltransferase acceptors in small-scale assays. The reactions were monitored by thin-layer-chromatography (TLC) and analyzed by high resolution electrospray ionization (ESI) mass spectra (ESI-HRMS). Each reaction mixture had a total volume of 10 μL containing a Tris-HCl buffer (100 mM, pH = 7.0, 7.5, 8.0, or 8.5), LacβProNHCbz (**3**, 10 mM) or LacβProNHFmoc (**4**, 10 mM), Neu5Ac (10 mM), $MgCl_2$ (20 mM), CTP (15 mM), appropriate amounts of NmCSS (3 μg) and

PmST1_M114D (5 μg). The reactions were carried out at 30 °C for 4–6 h. After the TLC showed the nearly complete consumption of the acceptor, the reaction mixtures were analyzed by ESI-HRMS.

4.4 Two-step enzymatic production of 2,7-anhydro-Neu5Ac (1) using LacβProNHCbz (3) as a substrate.

Cycle 1

Step A. One-pot two-enzyme synthesis of Neu5Acα2–3LacβProNHCbz (5).: The reaction was carried out at 30 °C with an agitation at 120 rpm in a reaction mixture (40 mL) containing LacβProNHCbz (**3**, 1.0 equiv. 1.0 g, 1.88 mmol), Neu5Ac (0.95 equiv. 0.55 g, 1.78 mmol), CTP (1.1 equiv.), $MgCl_2$ (20 mM), and appropriate amounts of NmCSS (5 mg) and PmST1_M144D (12 mg) in Tris-HCl buffer (100 mM, pH = 8.5). The reaction was monitored by thin-layer chromatography (TLC) using a plate developed with solvent of EtOAc:MeOH:H_2O = 5:2:1 (by volume) and stained with a *p*-anisaldehyde sugar stain solution. When Neu5Ac was completely consumed, the reaction was quenched by adding the same volume (40 mL) of pre-chilled ethanol and the reaction mixture was centrifuged to remove precipitates. The supernatant was concentrated and passed through a C18 cartridge (ODS-SM column, 51 g, 50 μm, 120 Å, Yamazen) on CombiFlash® Rf 200i system followed by washing with water. Neu5Acα2–3LacβProNHCbz (**5**, 1.51 g, 99% yield) and the remaining LacβProNHCbz (**3**, 0.05 g) were eluted using 15% and 50% acetonitrile in water, respectively. Neu5Acα2–3LacβProNHCbz (**5**) ^{1}H NMR (800 MHz, D_2O) δ 7.47–7.36 (m, 5H), 5.09 (s, 2H), 4.42 (d, *J* = 8.0 Hz, 1H), 4.40 (d, *J* = 8.2 Hz, 1H), 3.97 (dd, *J* = 10.4, 8.3 Hz, 1H), 3.95–3.89 (m, 3H), 3.89–3.83 (m, 3H), 3.82–3.78 (m, 1H), 3.77–3.73 (m, 2H), 3.70–3.61 (m, 6H), 3.58–3.49 (m, 4H), 3.30 (t, *J* = 8.7 Hz, 1H), 3.25–3.18 (m, 2H), 2.69 (dd, *J* = 12.6, 4.6 Hz, 1H), 2.01 (d, *J* = 1.2 Hz, 3H), 1.83–1.74 (m, 3H). ^{13}C NMR (200 MHz, D_2O) δ 174.88, 172.59, 158.39, 136.57, 128.76, 128.32, 127.62, 103.20 (2C), 101.92, 79.65, 74.57, 73.59, 72.68, 72.61, 72.34, 71.45, 70.72, 68.46, 68.36, 68.05, 67.70, 66.77, 63.49, 62.73, 60.22, 59.28, 51.72, 39.71, 37.29, 28.82, 22.03. HRMS (ESI-Orbitrap) *m/z*: $[M-H]^-$ Calcd for $C_{34}H_{51}N_2O_{21}$ 823.2990; found 823.2975.

Step B. Intramolecular trans-sialidase (IT-sialidase) SpNanB-catalyzed production of 2,7-anhydro-Neu5Ac.: SpNanB (5 mg) sample in Tris-HCl buffer (20 mM, pH = 7.5) containing 10% glycerol was dialyzed against 1 L of water (4 °C, 1 h × 2) before being added to the reaction system. The reaction was carried out at 30 °C in a buffer-free aqueous solution (40 mL, pH = 7) containing Neu5Acα2–3LacβProNHCbz (**5**, 1.51 g, 1.78 mM) obtained from Step A above and SpNanB (5 mg). The reaction progress was monitored by thin-layer chromatography (TLC). The plate was developed using solvent EtOAc:MeOH:H_2O = 5:2:1 (by volume) and stained with a *p*-anisaldehyde sugar stain solution. After 2–4 h, the reaction was quenched by adding the same volume of pre-chilled ethanol and the reaction mixture was centrifuged to remove precipitates. The supernatant was concentrated and passed through a C18 column and washed with water to obtain pure 2,7-anhydro-Neu5Ac (**1**, 0.51 g, 1.63 mM, 91% yield, >98% purity). LacβProNHCbz (**3**, 0.90 g) and Neu5Acα2–3LacβProNHCbz (**5**, 0.06 g) were eluted using 15% and 50% acetonitrile in water, respectively. The total amount of LacβProNHCbz (**3**) recovered was 0.95 g (0.05 g from Step A and 0.90 g from Step B) with a 95% recovery yield. It was used

in the Step A of the next cycle for the production of sialoside. In addition, Neu5Acα2–3LacβProNHCbz (**5**, 0.06 g) was recovered and was used in the Step B of the next cycle for the production of 2,7-anhydro-Neu5Ac (**1**). 2,7-Anhydro-Neu5Ac (**1**) ^{1}H NMR (400 MHz, D_2O) δ 4.57 (s, 1H), 4.46 (d, *J* = 7.6 Hz, 1H), 3.97 (d, *J* = 5.3 Hz, 1H), 3.94 (s, 1H), 3.80–3.75 (m, 1H), 3.65–3.51 (m, 2H), 2.19 (dd, *J* = 15.4, 5.5 Hz, 1H), 2.05 (d, *J* = 1.3 Hz, 4H). ^{13}C NMR (100 MHz, D_2O) δ 174.06, 173.56, 105.57, 77.15, 76.65, 71.96, 66.84, 62.28, 51.97, 35.30, 21.80. HRMS (ESI-Orbitrap) *m/z*: $[M-H]^-$Calcd for $C_{11}H_{16}NO_8$ 290.0881; found 290.0874.

Cycles 2–5—As described for Cycle 1, the same strategy was applied for the following cycles 2–5 to efficiently convert Neu5Ac into 2,7-anhydro-Neu5Ac (**1**). After each cycle, recovered LacβProNHCbz (**3**) was used in the following sialoside formation reaction in Step A while recovered Neu5Acα2–3LacβProNHCbz (**5**) was used in the following SpNanB-catalyzed reaction in Step B. In total of 5 cycles of reactions, Neu5Ac (2.55 g, 8.24 mmol) was converted to 2,7-anhydro-Neu5Ac (**1**, 2.38 g, 7.60 mmol, 92%) with the recovery of LacβProNHCbz (**3**, 0.81 g, 1.52 mmol, 81%) and Neu5Acα2–3LacβProNHCbz (**5**, 0.12 g, 0.14 mmol, 7%).

4.5 Synthesis of chromophore-tagged sialyltransferase acceptor benzyl β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl carbamate (LacβNHCbz, 6) via a protection group-free approach.

4.5.1 Chemical synthesis of benzyl β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl carbamate (LacβNHCbz, 6) [19].—

Lactose (5 g, 14.62 mmol) and ammonia bicarbonate (1.2 g, 15.19 mmol) were dissolved in ammonia hydroxide (20 mL) and the mixture was stirred at 45–50 °C for 24 h. Solvent was removed *in vacuo* and the residue was dried under vacuum for 3–4 h. The product was used directly for the following reaction without purification. Sodium carbonate aqueous solution (10%, 30 mL) was used to dissolve $LacβNH_2$ (5.00 g, 14.62 mmol) in a 250 mL round flask, benzyl chloroformate (CbzCl) (12.20 g, 71.52 mmol) in 30 mL of acetonitrile was added to the reaction mixture in a flask submerged in an ice-water bath. The pH was adjusted and kept at 8.0–10.0 by adding sodium carbonate during the reaction process. The reaction was stirred at room temperature for overnight. LacβNHCbz (**6**) was purified by an ODS-SM column (51 g, 50 μm, 120 Å, Yamazen) and obtained as a white powder (5.07 g, 10.67 mmol, 73% over two steps). ^{1}H NMR (800 MHz, D_2O) δ 7.46–7.35 (m, 5H), 5.23–5.06 (m, 2H), 4.79 (d, *J* = 9.2 Hz, 1H), 4.41 (dd, *J* = 7.9, 1.4 Hz, 1H), 3.88 (d, *J* = 3.7 Hz, 2H), 3.80–3.73 (m, 2H), 3.71 (dd, *J* = 11.9, 3.9 Hz, 1H), 3.70–3.67 (m, 1H), 3.66–3.57 (m, 4H), 3.51 (t, *J* = 8.9 Hz, 1H), 3.39 (t, *J* = 8.7 Hz, 1H). ^{13}C NMR (200 MHz, D_2O) δ 158.09, 135.99, 128.77, 128.47, 127.82, 102.84, 81.64, 77.76, 76.12, 75.32, 75.02, 72.46, 71.40, 70.91, 68.52, 67.39, 61.01, 59.84. HRMS (ESI-Orbitrap) *m/z*: $[M+Cl]^-$ Calcd for $C_{20}H_{29}NO_{12}Cl$ 510.1384; found 510.1363.

4.5.2. Improved chemical of LacβNHCbz (6).—

Lactose (3.00 g, 8.77 mmol) and ammonia bicarbonate (0.72 g, 9.11 mmol) was dissolved in 15 mL of ammonia hydroxide and the mixture was stirred under 45–50 °C for 20 h. Solvent was removed *in vacuo* and the residue was dried under vacuum for 3–4 h. $LacβNH_2$ (2.991 g, 8.77 mmol, quant.) was obtained and was used directly for the following reaction without purification. Anhydrous

methanol (125 mL) was used to dissolve LacβNH$_2$ (1.00 g, 2.93 mmol) and benzyl chloroformate (CbzCl) (1.79 g, 10.51 mmol) was added to the reaction mixture in a flask submerged in an ice-water bath. The pH was adjusted and kept at 8.0–10.0 by adding *N,N*-diisopropylethylamine. The reaction was stirred at room temperature for overnight. LacβNHCbz (**6**) was purified by ODS-SM column (37 g, 50 μm, 120 Å, Yamazen) and obtained as a white powder (1.11 g, 2.34 mmol, 80% over two steps).

4.6. Two-step enzymatic production of 2,7-anhydro-Neu5Ac (1) using LacβNHCbz (6) as a substrate

Cycle 1

Step A. One-pot two-enzyme-catalyzed formation of Neu5Acα2–3LacβNHCbz (7).: The reaction was carried out at 30 °C with agitation at 120 rpm in a reaction mixture (250 mL) containing Tris-HCl buffer (100 mM in 250 mL of H_2O, pH = 8.5), LacβNHCbz (**6**, 1.0 equiv., 5.0 g, 10.52 mmol, 42.08 mM) and Neu5Ac (0.95 equiv., 3.09 g, 9.99 mmol), CTP (1.1 equiv.), $MgCl_2$ (20 mM), NmCSS (20–25 mg), and PmST1_M144D (40–50 mg). The reaction was monitored by thin-layer chromatography (TLC). The plate was developed using a solvent of EtOAc:MeOH:H_2O = 5:2:1 (by volume) and was stained with a *p*-anisaldehyde sugar stain solution. After 6 h when the complete consumption of Neu5Ac or CMP-Neu5Ac was observed, the reaction was quenched by adding the same volume of pre-chilled ethanol and the reaction mixture was centrifuged to remove precipitates. The supernatant was concentrated and passed through an ODS-SM column (140 g, 50 μm, 120 Å, Yamazen). After washing with water, Neu5Acα2–3LacβNHCbz (**7**, 7.50 g, 95% yield) and the remaining LacβNHCbz (**6**, 0.44 g) were eluted using 10% and 25% acetonitrile in water. Neu5Acα2–3LacβNHCbz (**7**) ^{1}H NMR (800 MHz, D_2O) δ 7.41–7.29 (m, 5H), 5.09 (m, 2H), 4.44 (dd, *J* = 8.0, 1H), 4.02 (dd, *J* = 9.9, 3.2 Hz, 1H), 3.86 (d, *J* = 3.2 Hz, 1H), 3.85–3.72 (m, 5H), 3.69–3.63 (m, 3H), 3.60 (m, 4H), 3.57–3.53 (m, 3H), 3.52–3.47 (m, 2H), 3.33 (t, *J* = 8.3 Hz, 1H), 2.70–2.63 (dd, *J* = 12.4, 4.7 Hz, 1H), 1.94 (s, 3H), 1.74–1.68 (t, *J* = 12.0 Hz, 1H). ^{13}C NMR (200 MHz, D_2O) δ 174.98, 173.87, 158.08, 136.00, 128.77, 128.47, 127.82, 102.57, 99.76, 81.67, 77.66, 76.12, 75.45, 75.15, 74.99, 72.85, 71.76, 71.42, 69.33, 68.33, 68.07, 67.44, 62.55, 61.02, 59.84, 59.36, 51.65, 39.62, 22.01. HRMS (ESI-Orbitrap) *m/z*: [M-H]$^-$ Calcd for $C_{31}H_{45}N_2O_{20}$ 765.2571; found 765.2546.

Step B. SpNanB-catalyzed production of 2,7-anhydro-Neu5Ac (1).: SpNanB (20 mg) sample in Tris-HCl buffer (20 mM, pH = 7.5) containing 10% glycerol was dialyzed against 1 L of water (4 °C, 1 h × 2) before being added to the reaction system. The reaction was carried out at 30 °C in a buffer-free aqueous solution (250 mL, pH = 7) containing Neu5Acα2–3LacβNHCbz (**7**, 7.50 g, 9.52 mM) obtained from Step A above and SpNanB (20 mg). The reaction progress was monitored by thin-layer chromatography (TLC). The plate was developed using solvent EtOAc:MeOH:H_2O = 5:2:1 (by volume) and stained with a *p*-anisaldehyde sugar stain solution. After 2–4 h, the reaction was quenched by adding the same volume of pre-chilled ethanol and the reaction mixture was centrifuged to remove precipitates. The supernatant was concentrated and passed through a C18 column and washed with water to obtain pure 2,7-anhydro-Neu5Ac (**1**, 2.75 g, 8.79 mM, 92% yield, >98% purity). LacβNHCbz (**6**, 4.37 g) and Neu5Acα2–3LacβNHCbz (**7**, 0.20 g) were eluted using 10% and 40% acetonitrile in water, respectively. The total amount of

LacβNHCbz (**6**) recovered was 4.81 g (0.44 g from Step A and 4.37 g from Step B) with a 96% recovery yield. It was used in the Step A of the next cycle for the production of sialoside. In addition, Neu5Acα2–3LacβNHCbz (**7**, 0.2 g) was recovered and was used in the Step B of the next cycle for the production of 2,7-anhydro-Neu5Ac (**1**).

Cycle 2—As described in Cycle 1, the same strategy was applied in the Cycle 2 to efficiently convert Neu5Ac into 2,7-anhydro-Neu5Ac (**1**). The LacβNHCbz (**6**) recovered from Cycle 1 was used in the sialoside formation reaction in Step A of Cycle 2 while the Neu5Acα2–3LacβNHCbz (**7**) recovered from Cycle 1 was used in SpNanB-catalyzed reaction in Step B of Cycle 2. In total of 2 cycles of reactions, Neu5Ac (6.06 g, 19.61 mmol) was converted to 2,7-anhydro-Neu5Ac (**1**, 5.56 g, 17.76 mmol, 91%) with the recovery of LacβNHCbz (**6**, 4.65 g, 9.78 mmol, 93%) and Neu5Acα2–3LacβNHCbz (**7**, 0.17 g, 0.21 mmol, 2%).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

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Highlights:

- High-yield multigram synthesis and facile purification of 2,7-anhydro-sialic acid
- Demonstrated recycled use of chromophore-tagged lactoside and sialoside
- Designed and synthesized several chromophore-tagged sialyltransferase acceptors
- Improved protection group-free synthesis of a tagged sialyltransferase acceptor
- Developed an environmentally free high-yield two-step enzyme reaction cycle

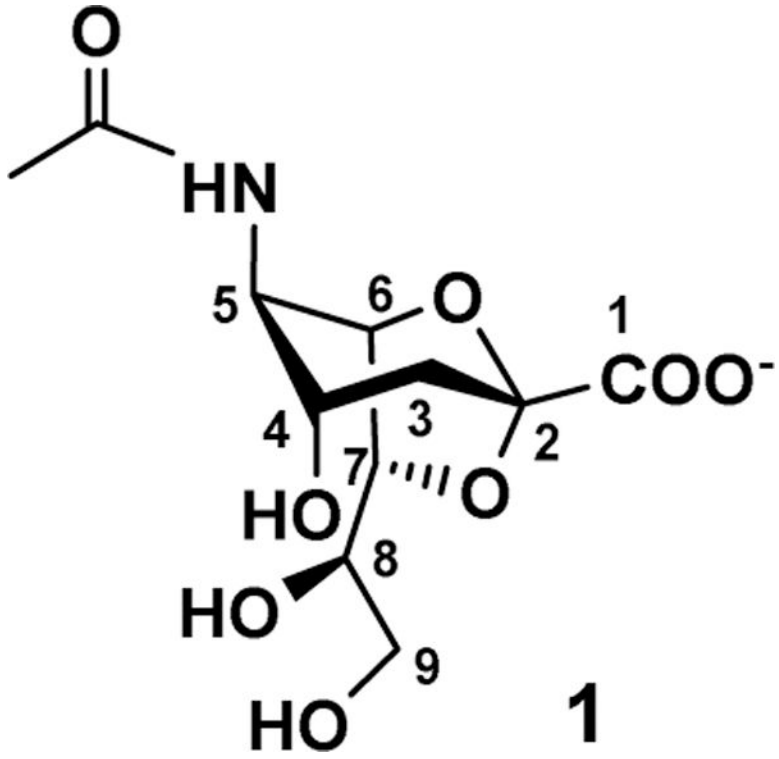
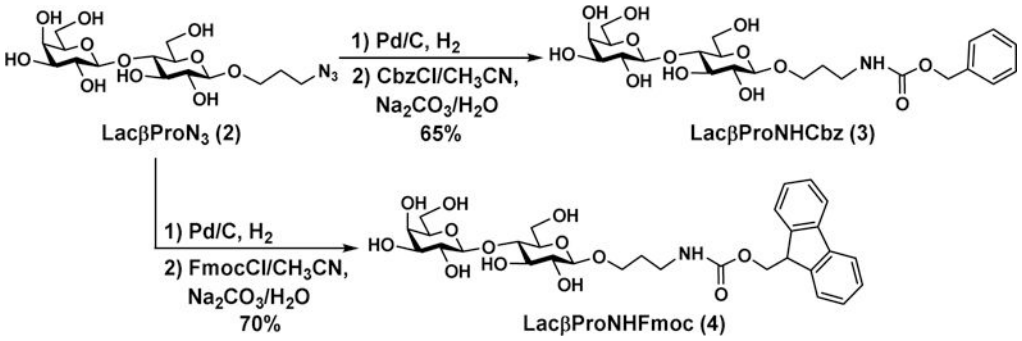
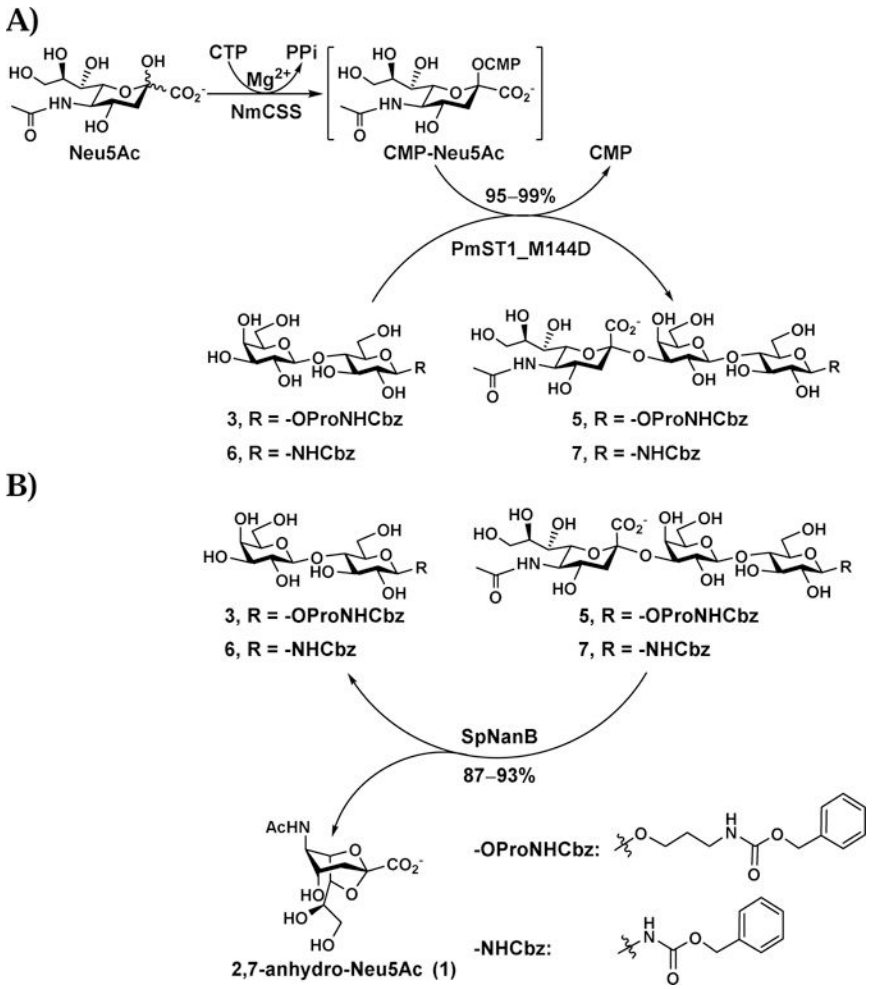


Fig. 1.
The structure of 2,7-anhydro-Neu5Ac (**1**).



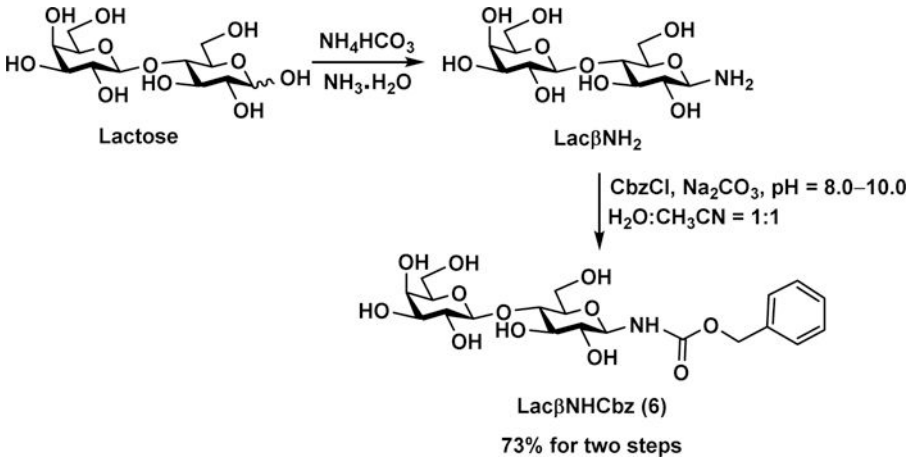
Scheme 1.

Synthesis of LacβProNHCbz (**3**) and LacβProNHFmoc (**4**) from LacβProN_3 (**2**).



Scheme 2.

Two-step enzymatic reaction cycle for the production of 2,7-anhydro-Neu5Ac from Neu5Ac. A) Step A, OP2E α2–3-sialylation system containing NmCSS and PmST1_M144D for the formation of Neu5Acα2–3LacβProNHCbz (**5**) or Neu5Acα2–3LacβNHCbz (**7**) from LacβProNHCbz (**3**) or LacβNHCbz (**6**) in the presence of Neu5Ac and CTP; and B) Step B, SpNanB-catalyzed formation of 2,7-anhydro-Neu5Ac (**1**) from Neu5Acα2–3LacβProNHCbz (**5**) or Neu5Acα2–3LacβNHCbz (**7**) produced in Step A.



Scheme 3.

Multigram (5 g)-scale protection group-free two-step synthesis of LacβNHCbz (**6**) from lactose.

Table 1.

Amounts and yields for the production of 2,7-anhydro-Neu5Ac (**1**) from 1.00 gram of LacβProNHCbz (**3**) via the formation of Neu5Acα2–3LacβProNHCbz (**5**) using two-step enzymatic reaction processes in five cycles.

Cycle/Step	Neu5Ac input (g)	3 input (g)	5 formed (g)	3 formed (g)	1 produced (g)
1/A	0.55	1.00	1.51 (99%)	0.05	
1/B			0.06	0.90	0.51
2/A	0.52	0.95	1.41 (99%)	0.06	
2/B			0.07	0.88	0.50
3/A	0.52	0.94	1.37 (96%)	0.06	
3/B			0.09	0.84	0.48
4/A	0.50	0.90	1.29 (95%)	0.07	
4/B			0.11	0.77	0.45
5/A	0.46	0.84	1.25 (99%)	0.06	
5/B			0.12	0.75	0.44
Total	**2.55**	**1.00**	**0.12 (7%)**	**0.81 (81%)**	**2.38 (92%)**

Table 2.

Amounts and yields of the production of 2,7-anhydro-Neu5Ac (**1**) from 5.00 grams of LacβNHCbz (**6**) via the formation of Neu5Acα2–3LacβNHCbz (**7**) using a two-step enzymatic process in two cycles of reactions.

Cycles/Step	Neu5Ac input (g)	6 input (g)	7 formed (g)	6 formed (g)	1 produced (g)
1/A	3.09	5.00	7.50 (95%)	0.44	
1/B			0.20	4.37	2.75
2/A	2.97	4.81	7.43 (99%)	0.31	
2/B			0.17	4.34	2.81
Total	**6.06**	**5.00**	**0.17 (2%)**	**4.65 (93%)**	**5.56 (91%)**