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An automated platform for the enzyme-mediated assembly of complex oligosaccharides

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Abstract

An automated platform that can synthesize a wide range of complex carbohydrates will greatly increase their accessibility and should facilitate progress in glycoscience. Here we report a fully automated process for enzyme-mediated oligosaccharide synthesis that can give easy access to different classes of complex glycans including poly-*N*-acetyllactosamine derivatives, human milk oligosaccharides, gangliosides and *N*-glycans. Our automated platform uses a catch and release approach in which glycosyltransferase-catalyzed reactions are performed in solution and product purification is accomplished by solid phase extraction. We developed a sulfonate tag that can easily be installed and enables highly efficient solid phase extraction and product release using a single set of washing conditions regardless of the complexity of the glycan. Using this custom-built synthesizer, as many as 15 reaction cycles can be performed in an automated fashion without a need for lyophilization or buffer exchange steps.

Automated synthesis has made it possible for non-specialists to prepare almost every possible peptide[1] and oligonucleotide[2] sequence and the broad availability of the resulting compounds has been central to the proteomic and genomic revolution. These approaches are based on solid-supported chemical synthesis in which a growing peptide or oligonucleotide

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Author contributions

T.L., L.L., K.W.M., and G.-J.B. designed research; T.L., L.L., N.W., J.-Y. Y. and D. G. C. performed research; J.-Y. Y. and D. G. C. contributed new reagents; T.L., L.L., and G.-J.B. wrote the paper.

Data availability

All data related with this study are included in this article and the Supplementary Information, and also available from the authors upon request.

Competing interests

The authors declare no competing interests.

is attached to an insoluble resin allowing purification by simple filtration and washing steps. The introduction of mild and efficient coupling chemistries, mechanically stable solid supports with appropriate swelling behavior and flexible linkers for anchoring of a starting material and release of final product makes it possible to perform multiple coupling steps under standardized conditions with high fidelity providing reliable access to oligomeric compounds[3]. It has been estimated that automation of peptide synthesis is as much as a 50-times less labor intensive compared to traditional solution phase chemistry[4].

A number of platforms have been introduced to automate the chemical synthesis of several other classes of biologically important compounds. For example, glycans have been assembled on solid support in an automated fashion by anchoring a monosaccharide onto a resin, which can then be extended into an oligomeric structure by sequential removal of a temporary protecting group followed by chemical glycosylation[5]. A commercial glycan synthesizer is now available (Glyconeer 2.1) that employs a linker-functionalized polystyrene solid support, several optimized monosaccharide building blocks, and standardized purification and quality-control protocols[6]. Polymer-supported oligosaccharide synthesis requires relatively large excesses of expensive glycosyl donors and to address this issue an automation platform has been introduced in which chemical glycosylations are performed in solution and product purification is accomplished by solid phase extraction[7]. The latter was possible by modifying a growing oligosaccharide chain with a light-fluorous tag allowing selective capture on fluorous silica gel and product release could then be accomplished by washing with an organic solvent such as methanol or acetonitrile. Recently, 14 distinct classes of small molecules were prepared in an automated fashion by sequential assembly of bifunctional *N*-methyliminodiacetic acid boronates[8], and in this case product purification was accomplished by selective capture of a product on silica gel and byproducts could be removed by elution with a solvent mixture of methanol and diethyl ether. Next, the product could be released by washing with tetrahydrofuran (THF) to give a solution that could immediately be used in the next coupling step. Several automation platforms have been described in which thousands of organic coupling reactions can be performed in parallel allowing facile optimization of reaction conditions or provide many different products for bio-testing[9–11]. These platforms enable high-throughput experimentation at nanomole scale but are not designed for multi-step syntheses.

Enzyme-catalyzed reactions, which often proceed in a regio- and stereo-selective manner at ambient temperature, offer exciting opportunities to synthesize highly complex organic compound[12]. Although various methods have been introduced to facilitate the purification of enzyme-catalyzed reactions, no methods have been reported to automate iterative enzyme-mediated coupling reactions. The introduction of such methods would in particular be relevant for the preparation of oligosaccharides of biological importance, which often are structurally very complex requiring many coupling steps to access specific targets[13].

A number of features make oligosaccharides assembly by glycosyltransferases attractive for automation. These reactions proceed in a regio- and stereo-specific manner and can be driven to completion by the use of a phosphatase that hydrolyzes the nucleotide products that can act as a product inhibitors[14]. Many microbial and mammalian derived glycosyltransferases have been identified that can readily be expressed in *E. coli* or in

mammalian cell culture allowing the installation of a wide range of glycosidic linkages.[15,16] Sugar nucleotide donors are readily available by convenient enzyme-based methods, or can be generated *in-situ* by coupled enzymatic transformations[17]. Furthermore, universal acceptors have been described that can give access to a wide array of glycans having complex branched architectures[18,19]. As a result, it is possible to prepare almost every animal derived glycan by glycosyltransferase-catalyzed transformations but labor intensive and time-consuming purification protocols have still been an impediment for the implementation of these approaches.

We report here an automation platform for enzyme-mediated oligosaccharide synthesis based on an approach in which glycosyltransferase-catalyzed reactions are performed in solution and product purification is accomplished by selective capture onto a resin followed by appropriate washing steps and then expeditious release for a subsequent enzymatic transformation (Fig. 1). The catch and release of oligosaccharides is facilitated by a sulfonate tag that can easily be installed and allows retrieval using a diethylaminoethyl (DEAE) ion exchange resin. Product release can be accomplished by washing with aqueous ammonium bicarbonate (NH_4HCO_3) and simply adjusting the pH of the resulting solution with acetic acid will give a buffer appropriate for the next enzymatic transformation. A liquid handling system has been developed that can easily be implemented in other laboratories, and it has been shown that as many as 15 reaction cycles can be performed in an automated fashion to give easy access to various classes of highly pure complex glycans.

A number of unique challenges are associated with the design and implementation of a fully automated method for enzyme-mediated oligosaccharide assembly. Although the solid phase paradigm has been extended to glycosyltransferase-catalyzed ractions[20], the scope is limited because these reactions are slow when an acceptor is immobilized to a resin leading to substantial hydrolysis of employed sugar nucleotide donors. As a result, only simple tri- and tetra-saccharides have been prepared by the polymer supported paradigm[21,22], which has not led to effective automation. A catch and release approach in which glycosyltransferase-catalyzed reactions are performed in solution and product purification is achieved by solid phase extraction can potentially overcome these difficulties. The successful implementation of such an approach for automated multi-step synthesis will, however, need to meet stringent requirements such as a tagging method that allows capture and release of a wide range of oligosaccharide acceptors in aqueous solution with high efficiency using a single set of washing conditions. It should be possible to perform multiple reaction cycles without a need of lyophilization, and finally an automation platform needs to be developed that can perform all liquid handling steps without human interference. Apolar[23], ionic-liquid-based[24] and light fluorous tags[25,26] have been used to streamline the purification of intermediates of enzyme-catalyzed oligosaccharide synthesis by catch and release using reversed-phase and fluorous silica gel, respectively. These methods employ organic solvents for product release, and thus the resulting solutions cannot be directly employed in the next reaction cycles. Furthermore, they require different elution conditions depending on the complexity of glycans and the tags may cause aggregation in aqueous media, which are impediments for the implementation of automated synthetic procedures. Immobilization of a glycosyl acceptor on a thermo-responsive polymeric support that exhibits inverse temperature-dependent solubility made it possible to perform enzymatic transformations in solution and

accomplish purification by filtrations[27]. This method has only been used to prepare simple tri- and tetra-saccharides. Dendrimer-supported oligosaccharide synthesis[28], which exploits ultrafiltration for purification, is the only enzyme-based approach that has been performed in an automated fashion. This platform does not allow removal of enzymes and exhibits low conversions and the preparation of the tetra-saccharide sialyl Lewis^x in a low 16% overall yield (3 steps) is the only reported compound prepared by this procedure. In an earlier study, heparan sulfate was immobilized on magnetic nanoparticles and enzymatically modified in a droplet-based digital microfluidic device by recombinant 3-OST-1[29]. Although this report represented the first step toward the construction of an artificial Golgi organelle, the relatively low modification with 3-O-sulfate moieties (~5%) highlighted the challenge to enzymatically modify immobilized saccharide chains.

Results

Catch and release system for glycosyltransferase-mediated oligosaccharide assembly.

To identify a versatile strategy for capture and release of oligosaccharides that is amenable to automation, we examined several sulfonate-bearing tags (**1a**, **1b** and **1c**). It was anticipated that saccharides equipped with such a moiety can be captured on an anion exchange resin and released by washing with aqueous NH_4HCO_3 (Fig. 1a). Adjustment of the pH of the released oligosaccharide would then give a buffer solution that can be used for the next reaction cycle without a need for lyophilization (Fig. 1b). Furthermore, it was expected that a carefully selected sulfonated tag would allow capture and release using a single set of conditions regardless of oligosaccharide complexity.

Compounds **1a**-**c**, having one, two or three sulfonate moieties, respectively were synthesized from readily available starting materials (Supplementary Figs. 1–3). These compounds are equipped with a methyl hydroxylamine moiety, which can be reacted with the anomeric center of reducing sugars to give exclusively β-linked cyclic structures[30]. At the end of a sequence of enzymatic transformations, the tag can be removed under mild acidic conditions to give complex glycans having a free reducing end. Thus, treatment of *N*-acetylglucosamine (GlcNAc) with tags **1a**-**c** in sodium acetate buffer (0.1 M, pH 4.2) at 37 °C gave, after purification by DEAE ion exchange resin, compounds **2a**-**c** as only the β-anomer. The latter derivatives were treated with β–1,4-galactosyltransferase 1 (B4GalT1), uridine 5′-diphosphogalactose (UDP-Gal) in the presence of $MnCl_2$, calf intestine alkaline phosphatase (CIAP) and bovine serum albumin (BSA) for 16 h to give LacNAc derivatives **3a**-**c**. Disaccharide **3b**, which is modified by a tag bearing two sulfonate moieties, could quantitatively be captured on a DEAE anion exchange resin, and all reaction components including enzymes and excess sugar nucleotide could be removed by washing with water followed by low concentration of aqueous ammonium bicarbonate (60 mM NH_4HCO_3). A subsequent washing step with a small volume of 0.3 M NH_4HCO_3 resulted in quantitative release of **3b**. Solid phase extraction of disaccharides **3a** and **3c** did not meet our stringent requirements resulting either in too strong binding to the resin requiring very high concentrations and large volumes of NH_4HCO_3 for release or too weak binding and washing with low concentration of NH_4HCO_3 (20 mM) lead to release of **3a** contaminated with excess UDP-Gal. The general application of the catch and release approach requires that a

wide range of glycosyltransferases have high activity in ammonium acetate buffer. Gratifyingly, all examined mammalian enzymes including β–1,4-galactosyltransferase 1 (B4GalT1), β–1,3-*N*-acetylglucosaminyltransferase 2 (B3GNT2), *N*-acetyllactosaminide β –1,6-*N*-acetylglucosaminyltransferase (GCNT2), ST6 β-galactoside α–2,6-sialyltransferase 1 (ST6Gal1), ST3 β-galactoside α–2,3-sialyltransferase 4 (ST3Gal4), ST8 α-*N*-acetyl-neuraminide α–2,8-sialyltransferase 1 (ST8Sia1), α-fucosyltransferases 1, 3 and 5 (FUT1, FUT3, FUT5), and bacterial enzymes such as *Pasteurella multocida* α–2,3-sialyltransferase 1 (PmST1), *Campylobacter jejuni* α–2,8-sialyltransferase (CstII), *Campylobacter jejuni* β –1,4-*N*-acetylgalactosaminyltransferase (CgtA), *Campylobacter jejuni* β–1,3-galactosyltransferase (CgtB) were active in ammonium acetate buffer.

Development of a glycosynthesizer.

A glycosynthesizer was built based on ISYNTH AI SWING workstation from ChemSpeed Technologies (Fig. 1c). It is equipped with a robotic arm to move a 4-needle head that is independently controlled by high-resolution syringe pumps of different capacity for volumetric liquid handling and sampling. The robotic arm is further modified with a pH needle for measuring and adjusting pH values. The platform contains a rack that can be cooled for reagent storage, a temperature controlled reactor block with vortexing ability for mixing and an additional rack that can hold solid phase extraction (SPE) cartridges for compound purification. The AutoSuite controlling software provides full control of the platform, and allows to set-up workflows in a flexible manner.

Glycans **4**-**8** were synthesized in an automated fashion using the glycosynthesizer to establish whether multiple reaction cycles can be performed with fidelity using standardized conditions to give compounds of high purity in good yields (Fig. 2). Thus, vials containing sugar nucleotides, such as UDP-Gal, uridine 5′-diphospho-*N*-acetylglucosamine (UDP-GlcNAc), guanosine 5′-diphospho-β-L-fucose (GDP-Fuc) and cytidine-5′-monophospho-*N*-acetylneuraminic acid (CMP-Neu5Ac), enzymes (B4GalT1, B3GNT2, ST6Gal1, ST3Gal4, FUT1 and CIAP), and additives (BSA, $MnCl_2$ and $MgCl_2$) were placed in rack D that was cooled at 4 °C. Vials (60 mL) containing water, aqueous solutions of NH_4HCO_3 (60 mM and 0.3 M) and acetic acid in water (3 M) were placed in rack G, and SPE cartridges (3 mL) containing activated DEAE (0.7 mL) were positioned in rack F.

Compound **2b** (6 μmol) in a 15 mL tube was placed in the reaction block (C) that was heated at 37 °C. The needle head driven by a 10 mL syringe pump was programmed to transfer 0.3 M NH_4HCO_3 (5 mL, pH = 8.7) to the reaction vessel, which was followed by several additions of 3 M acetic acid (200 μL each time by 1 mL syringe pump) with intermitted pH measurements to obtain an ammonium acetate buffer of pH = 7. Next, the needle head administered in an automated fashion appropriate amounts of UDP-Gal, B4GalT1, CIAP, BSA and $MnCl_2$. After incubating the resulting reaction mixture for 16 h at 37 °C with shaking, a small sample (10 μL) was transferred to a vial in rack H for analysis by LC-MS. Next, the pH of the reaction mixture was adjusted to 6 by addition of aqueous acetic acid, and the resulting mixture was transferred to an SPE cartridge containing DEAE resin. Subsequently, the resin was washed with water (4 mL) and 60 mM NH_4HCO_3 (2.5 mL) to remove salts, enzymes, excess UDP-Gal and other components, which was followed by

product release by elution with 0.3 M NH_4HCO_3 (5 mL). The collected solution was transferred to a reaction vessel in rack C for the next reaction cycle.

All liquid handling steps were performed by the needle head in an automated fashion controlled by the AutoSuite software and details of the programming can be found in Supplementary Section 7. The program was set-up to perform nine alternating transformations catalyzed by B4GalT1 and B3GNT2 to give, after purification by size exclusion column chromatography using Bio-Gel P4, decamer **4** in an overall yield of 15% (average yield of 81% per enzymatic transformation) (Fig. 2a). The samples taken after each enzymatic transformation were analyzed by LC-MS (Fig. 2b), which demonstrated that each transformation had been performed with high efficiency.

Compound **4** was further diversified by selective α−2,6-sialylation, α−2,3-sialylation and α−1,2-fucosylation using ST6Gal1, ST3Gal4 or FUT1, respectively to afford derivatives **5**-**7** (Fig. 2c). The resulting compounds were purified by HPLC using a HILIC column (XBridge® Amide 5 μm, 10 mm × 250 mm, Waters) to give highly pure compounds. We also prepared compound **8** (Fig. 2d), which is a 16-mer LacNAc derivative by 15 alternating reactions catalyzed by B4GalT1 and B3GNT2. The successful preparation of various compounds highlights that the capture and release approach is very efficient and regardless of the complexity of the compounds, a single set of conditions can be employed for automated compound purification (Supplementary Fig. 16).

The syntheses can be performed in a fully automated fashion in which all reactions are performed consecutively and analysis of the reaction intermediates is performed after completion of the target. Alternatively, the system can be programmed in such a way that different follow-up steps can be chosen depending on the results of the analysis. This approach offers the possibility to repeat a reaction step in case it has not proceeded to completion. This feature was important for the preparation of target **8** because it was observed that the B3GNT2 catalyzed transformations were slow for compounds larger than a 12-mer and these transformations needed to be repeated to achieve full conversion.

Automated synthesis of different classes of complex glycans.

To demonstrate the broad scope of the technology, we prepared in an automated fashion a number of biologically important glycans including several human milk oligosaccharides (HMOs, **15**, **18** and **19**), the gangliosides GT1a **20** and GT1b **21** (Fig. 3) and *N*-linked glycan **22** (Fig. 4).

Human milk oligosaccharides are structurally very diverse and it has been postulated that specific compounds can shape the intestinal microbiome, serve as soluble decoys for receptors of viral, bacterial or *protozoan* parasite pathogens, and have immuno-modulatory properties[31]. Well-defined HMOs in sufficient quantity are needed to elucidate the biological mechanisms by which these compounds exert their properties. The synthesis of the HMOs **15**, **18** and **19** started from tag modified lactose **9**, which was extended to tetrasaccharide **11** by B3GNT2 and B4GalT1 catalyzed reaction cycles (Fig. 3a). Next, a branching point was installed by employing GCNT2, which selectively transferred a GlcNAc moiety of UDP-GlcNAc to the internal galactoside of **11** to give bi-antennary glycan **12**. The terminal

galactoside of the β3-antenna of **12** was modified by a sialoside using ST6Gal1 to provide **13**. The resulting α2,6-sialoside is resistant to modifications by mammalian glycosyltransferases, and therefore it was possible to selectively extend the β6-antenna by subsequent reaction cycles using B4GalT1 and FUT5 to give **15** having a Lewis^x epitope at the upper arm in an overall yield of 37% (average yield of 85% for each enzymatic step, 5 cycles) after purification by HPLC. Compound **19** having a Lewis^y epitope at the upper arm was prepared by a similar approach by extension of the upper arm of **14** using B3GNT2, B4GalT1, FUT1 and FUT3. Homogeneous final product was obtained in an overall yield of 16% (average yield of 82% for each enzymatic step, 9 cycles) after purification by HPLC using a HILIC column. In addition, the gangliosides GT1a **20** and GT1b **21** (Fig. 3b), which are compounds implicated in neurodevelopment and nervous system disease[32], could also be prepared from starting material **9** highlighting that the methodology can be extended to targets modified by several sialosides (Supplementary Fig. 23).

Finally, *N*-glycan **22** was prepared starting from bi-antennary glycan **24** that is derived from a glycopeptide isolated from egg yolk powder[33] demonstrating that complex naturally derived starting materials can be modified by the sulfonated tag **1b** and elaborated in an automated fashion into complex compounds (Fig. 4). Thus, treatment of **24** with **1b** in sodium acetate buffer (0.1 M, pH 4.2) at 37 °C gave, after purification by size exclusion column chromatography using Bio-Gel P2 and then DEAE ion exchange resin, compounds **25** in a yield of 82%. Selectively α2,6-sialylated of the Galβ(1,4)GlcNAcβ(1,2)Manα(1,3) arm with ST6Gal1[34] afforded mono-sialylated *N*-glycan **26**. Further modifications of this arm by mammalian glycosyltransferases were blocked by the presence of the α2,6-sialoside, and therefore it was possible to extend the Galβ(1,4)GlcNAcβ(1,2)Manα(1,6) arm by subsequent reactions with B3GNT2, B4GalT1, ST3Gal4 and FUT5. In addition to the required product **22**, the crude product contained a byproduct that had resulted from bis-sialylation of **25**. The two compounds could readily be separated by semi-preparative HPLC on a HILIC column to give highly pure **22** in an overall yield of 21% (average yield of 73% for each enzymatic step, 5 cycles) (Supplementary Fig. 31). The anomeric tag of **22** could be removed by treatment with 0.25% trifluoroacetic acid (TFA) in water for 3 h to provide reducing glycan **23** in 92% yield without affecting acid sensitive sialic acid and fucose residues. These results demonstrate the robustness of the automation platform and highlight that a wide variety of complex glycans can be prepared in a fully automated fashion yielding products of high purity.

Discussion

Complex glycans have been implicated in almost every human disease[35], are structurally very diverse and cannot easily be obtained from natural sources in pure forms. Large collections of well-defined glycans are needed to develop comprehensive glycan arrays to examine protein-glycan interactions, as standard to determine exact structures of glycans in complex biological samples and to study the molecular details of their biosynthesis[36–39]. Efforts to accelerate the preparation of complex glycans have mainly been focused on chemical synthesis using programmable one-pot[40] and automated synthesis on solid support[6,7,41]. Although these methods make it possible to streamline the preparation of

specific types of glycans, they have limitations due to a lack of general chemical glycosylation protocols[13,42]. Furthermore, chemical oligosaccharide synthesis requires global deprotection steps that can be tedious to perform requiring a high level of expertise.

Oligosaccharide assembly using glycosyltransferases provides an attractive alternative to chemical synthesis[43]. Despite many attractive traits, labor-intensive and time consuming purification protocols of synthetic intermediates are an impediment for the preparation of large collections of glycans by enzymatic procedures. The automation platform described here greatly speeds up glycan assembly by removing tedious purification protocols. A key feature is the use of a sulfonate tag that facilitates solid phase extraction and after compound release, a solution is obtained that immediately can be used in a subsequent enzymatic reaction. It made it possible to prepare in a convenient manner oligo-LacNAc derivatives composed of as many as ten to sixteen monosaccharides, which could be diversified by α−2,6-sialylation, α−2,3-sialylation and α−1,2-fucosylation. These derivatives are important constituents of *N*- and *O*-linked glycans that have been implicated in a wide range of biological processes such as immune responses[44], viral infections[45] and cancer metastasis[46]. The synthesis of complex well-defined LacNAc derivatives has been described[45], but can now conveniently be assembled on a synthesizer using standardized protocols.

The broad scope of the methodology is demonstrated by the preparation of highly complex and structurally differing oligosaccharides including human milk oligosaccharides, gangliosides and *N*-linked glycans. Recently, we introduced a strategy to prepare asymmetrically branched HMOs such as compounds **15**, **18** and **19** by strategically introducing a branching point that allowed selective extension of each antenna[19]. In this study, conventional size exclusion column chromatography and HPLC were used for compound purification, which was followed by lyophilization of product containing fractions requiring approximately 3–4 days for each step. These time demanding procedures were an impediment for the facile synthesis of a large collection of compounds. The newly described catch and release approach reduces the respective purification steps to less than 2 h to give a solution that immediately can be used in the next enzymatic transformation. It does not need any human intervention thereby greatly increasing the speed of oligosaccharide assembly. The enzymatic transformations take 16–24 h, which is considerable longer than typical chemical glycosylations. The speed of glycosyltransferase-catalyzed transformations is directly proportional to enzyme activity and sugar donor used. Thus, oligosaccharide assembly can be considerably speed up by employing larger quantities of these reagents. As the cost of enzymes will go down through commercial production, the implementation of such an approach will become feasible.

To further demonstrate the potential of the technology, glycans of a number of gangliosides were prepared that have been implicated in neurodevelopment, nervous system disease and cancer metastasis[32]. For example, the ganglioside GT1a (**20**) is associated with Guillain-Barré syndrome[47] and GT1b (**21**) serves as a brain metastatic maker[48]. These oligosaccharides have been chemically synthesized by time-consuming protocols involving more than 50 chemical steps including difficult sialylations that gave mixtures of anomers requiring tedious separation by silica gel column chromatography[49,50]. Gangliosides, such as GM1, GD1b and fucosyl GM1, have been synthesized by elegant (chemo)enzymatic

approaches, but purification relied on time consuming size exclusion column or reverse phase column chromatography followed by lyophilization of product containing fractions[51–53]. Finally, the successful preparation of *N*-glycan **22** (Fig. 4), which was found on trophoblast cells[54], demonstrated that complex naturally derived starting materials can be modified by the sulfonated tag **1b** and enzymatically extended in an automated fashion to give glycans having complex architectures.

The oligosaccharides were prepared in milligram quantities, which is ample for serving as standards for structural studies, glycan array development and cell-based structure-activity relationship studies. The current platform can, however, easily be adapted to reaction scales as large as a 100 mg by changing the size of the reaction vessel and by performing reactions and purifications in parallel mode. Another attractive feature of the platform is that samples can be taken from the reaction mixture for analysis by LC-MS. In the current configuration, this analysis is performed offline, however, this process can easily be automated by installing a commercially available robotic module that can transfer a sample vial from the synthesizer to an auto-sampler of an LC-MS system. It was observed that regardless of the complexity of a glycan, one set of washing conditions could be used for solid phase extraction, which facilitated standardization of the protocols. However, a number of biologically important glycans such as heparan sulfates are modified by highly charged sulfate moieties, which may complex more tightly with a DEAE resin, and thus the release of this class of compounds may need alternative washing conditions.

Although many glycosyltransferases are now readily available,[15,16] not every glycosidic bond can yet be installed enzymatically. Technologies such as genome sequencing, gene synthesis and recombinant proteins expression offer opportunities to identify and produce many more glycosyltransferases thereby expanding the capabilities of enzyme-mediated oligosaccharide assembly. In addition, automated chemical and enzyme-mediated syntheses are highly complementary and especially the combined use of these methodologies will greatly expand the chemical space that can be assessed by synthesis. Many other complex natural products have been prepared by so-called total biosynthesis[12], in which all transformations are performed by enzyme-mediated catalysis. It is expected that the platform described here can be adapted to such syntheses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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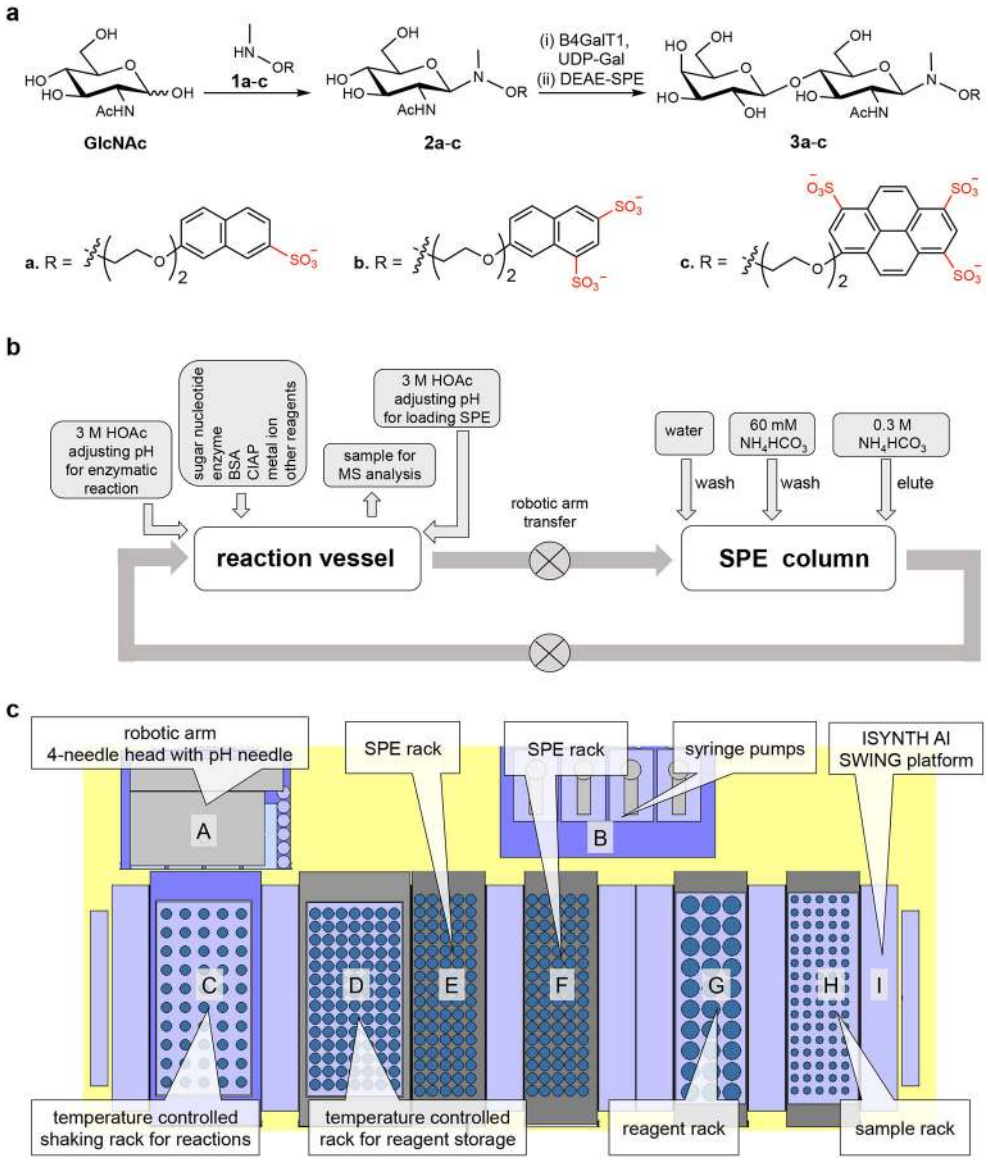

Fig. 1 |. Automation of enzyme-mediated oligosaccharide assembly.

a, Sulfonated tags **1a-c** bearing an methyl hydroxylamine moiety can be condensed with reducing glycans to give compounds such as **2a-c**. After a glycosyltransferase-catalyzed reaction to give compounds such as **3a-c**, the sulfonate moieties of the tag allow product purification by DEAE solid phase extraction (SPE). **b**, Various steps for automated glycosyltransferase-mediated oligosaccharide assembly. The addition of reagents, pH adjustment, withdrawal of samples, transfer of reaction mixtures, and SPE are performed by a 4-needle head mounted on a robotic arm. **c**, Schematic illustration of the automation platform. A. 4-needle head with pH needle mounted on a robotic arm for liquid handling. B. Four syringe pumps of different capacity to drive liquid handling. C. Temperature controlled shaking rack to hold reaction vessels (50 × 15 mL vials). D. Temperature controlled rack (4 °C) for reagent storage (96 × 8 mL vials). E and F. SPE racks for compound purification (80 × 1 mL cartridges and 80 × 3 mL cartridges). G. Reagent rack (30 × 60 mL vials). H.

Sample rack for analysis intermediate compounds (160 × 2 mL vials). I. ISYNTH AI SWING platform.

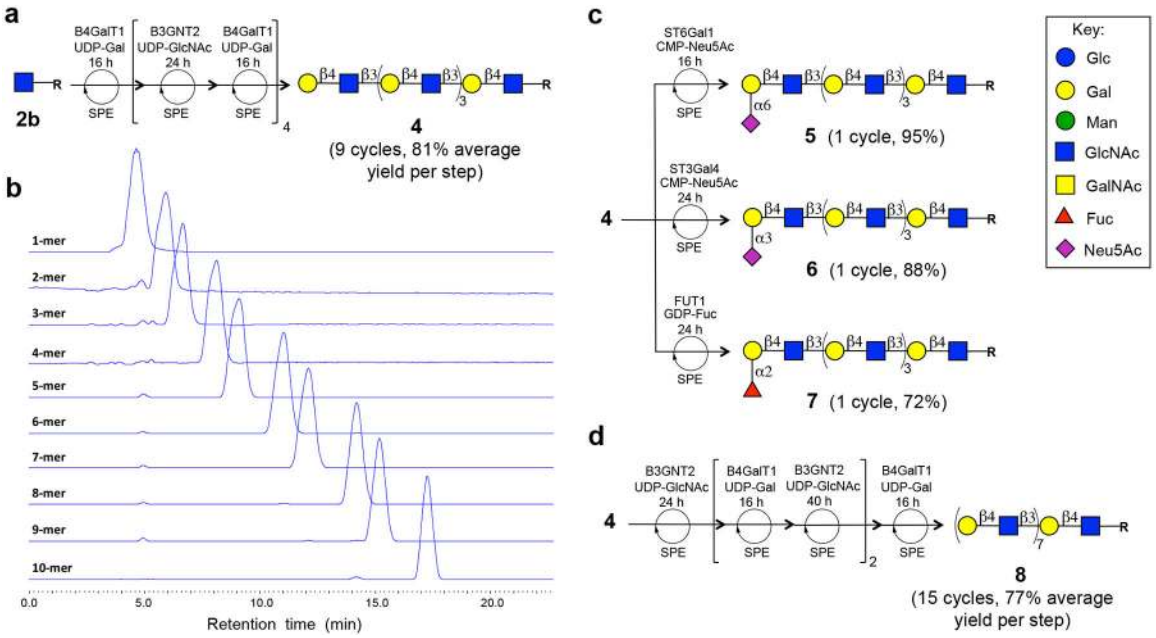

Fig. 2 |. Automated enzymatic synthesis of poly-LacNAc and derivatives thereof.

a, Preparation of decamer **4** using nine alternating transformations catalyzed by B4GalT1 and B3GNT2. R is sulfonate tag derived from **1b** (Fig. 1). **b**, LC-MS profiles of crude reaction products after each cycle during the poly-LacNAc elongation to give decamer **4**. **c**, Further diversification of decamer **4** by selective α−2,6-sialylation, α−2,3-sialylation and α−1,2-fucosylation using ST6Gal1, ST3Gal4 or FUT1, respectively to afford derivatives **5**-**7**. **d**, Fifteen alternating reactions catalyzed by B4GalT1 and B3GNT2 gave 16-mer LacNAc **8**.

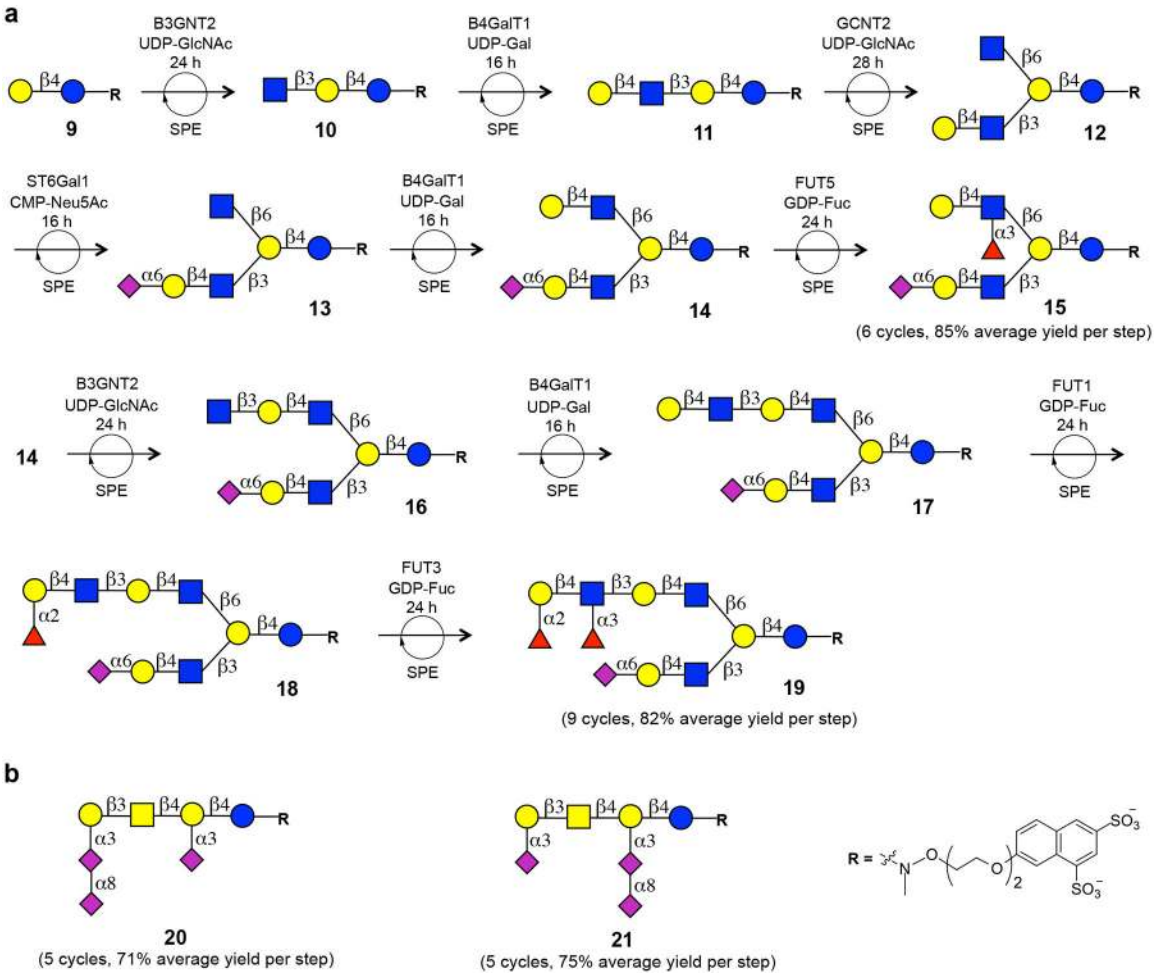

Fig. 3 |. Automated synthesis of human milk oligosaccharides and gangliosides.

a, Tag-modified lactose **9** was converted into human milk oligosaccharides **15**, **18** and **19**. Compound **15** was prepared by extension of **9** using B3GNT2, B4GalT1, GCNT2, ST6Gal1, B4GalT1 and FUT5 catalyzed reaction cycles. Human milk oligosaccharide **18**, having an H-epitope, was prepared by further extension of **14** by B3GNT2, B4GalT1, and FUT1 catalyzed reaction cycles. Selective fucosylation of **18** by a FUT3 catalyzed reaction cycle afforded **19** having a Lewis^y epitope at one of the termini. These transformations demonstrate that different oligosaccharides can be prepared from a common precursor by differentially modifying termini of an antenna **b**, Ganglioside oligosaccharides GT1a (**20**) and GT1b (**21**) were prepared by automated synthesis starting from **9** demonstrating that compounds having several sialic acids can be prepared in an automated fashion using a sulfonate tag (see Supplementary Fig. 23 for synthetic details).

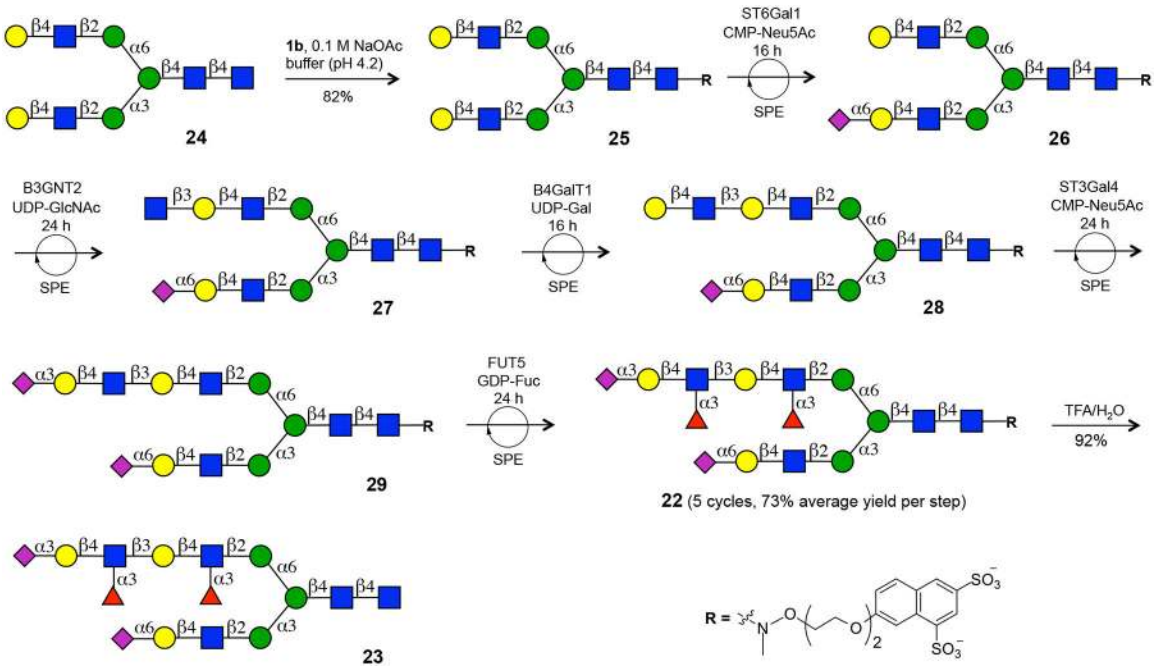

Fig. 4 |. Automated synthesis of asymmetrical *N*-glycan 22 and removal of tag to afford 23.

The preparation of complex *N*-glycan **22** started from **24**, which was obtained from a naturally derived oligosaccharide. Installation of the sulfonate tag (**24**→**25**) followed by ST6Gal1, B3GNT2, B4GalT1, ST3Gal4, and FUT5 catalyzed reaction cycles gave complex glycan **22**; removal of the sulfonate tag of **22** was accomplished by treatment with 0.25% TFA in water at room temperature for 3 h without affecting acid sensitive sialosides and fucosides to provide a reducing *N*-glycan **23** in 92% yield.