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Combinatorial Chemoenzymatic Synthesis and High-throughput Screening of Sialosides

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Abstract

Although the vital roles of sialic acid-containing structures in biomolecular recognition are well documented, limited information is available on how sialic acid structural modifications, sialyl linkages, and the underlying glycan structures affect the binding or the activity of sialic acid-recognizing proteins and related downstream biological processes. A novel combinatorial chemoenzymatic method has been developed for the highly efficient synthesis of biotinylated sialosides containing different sialic acid structures and different underlying glycans in 96-well plates from biotinylated sialyltransferase acceptors and sialic acid precursors. By transferring the reaction mixtures to NeutrAvidin-coated plates and assaying for the yields of enzymatic reactions using lectins recognizing sialyltransferase acceptors but not the sialylated products, the biotinylated sialoside products can be directly used, without purification, for high-throughput screening to quickly identify the ligand specificity of sialic acid-binding proteins. For a proof-of-principle experiment, 72 biotinylated α 2,6-linked sialosides were synthesized in 96-well plates from 4 biotinylated sialyltransferase acceptors and 18 sialic acid precursors using a one-pot three-enzyme system. High-throughput screening assays performed in NeutrAvidin-coated microtiter plates show that while *Sambucus nigra* Lectin binds to α 2,6-linked sialosides with high promiscuity, human Siglec-2 (CD22) is highly selective for a number of sialic acid structures and the underlying glycans in its sialoside ligands.

Sialic acids are a family of diverse nonulosonic acids. Naturally occurring modifications on sialic acids, such as acetylation, lactylation, methylation, sulphation, lead to more than fifty structurally distinct sialic acids primarily found as the terminal residues on carbohydrate moieties of glycolipids and glycoproteins on mammalian cell surfaces, and as components of bacterial capsular polysaccharides and lipopolysaccharides (1,2). As the outermost carbohydrate residues, sialic acids are critical recognition elements in a number of biologically important processes including cell-cell interaction, bacterial and viral infection, and tumor metastasis (1). For example, binding of influenza virus haemagglutinins to host cell surface sialic acids is the first step in the viral infection (3). Also, influenza virus neuraminidases are believed to catalyze the removal of sialic acid from the surface of infected host cells to release the newly formed progeny virus (3). Siglecs are sialic acid binding immunoglobulin super family lectins primarily expressed on cells of immune system. Their binding to sialic acid containing structures is believed to play important roles in regulating the immune system (4).

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Sialyloligosaccharides (or sialosides) are recognized by a number of vertebrate lectins such as complement factor H, selectins, Siglecs, and others (1). Although the vital roles of sialic acid-containing structures in biomolecular recognition are well documented, the understanding of the biological significance of naturally occurring sialic acid modifications is limited due to the difficulties in obtaining compounds containing these structures and the lack of techniques in studying them (5). Despite recent advances, stereoselective formation of α -sialyl linkage by chemical sialylation is still considered as one of the most difficult glycosylation reactions due to the hindered tertiary anomeric center and the lack of a participating auxiliary functionality in sialic acids (6). In addition, it is challenging to isolate diverse sialosides from natural sources in homogenous forms without losing the naturally existing sialic acid modifications, and in sufficient amount for characterization and biological studies (7). We reported previously a highly efficient one-pot three-enzyme chemoenzymatic approach for the synthesis of diverse sialosides containing structurally modified sialic acids (5,8). In this method, *N*-acetyl-D-mannosamine (ManNAc) and D-mannose (Man) derivatives were chemically or enzymatically synthesized and used as sialic acid precursors for direct production of sialosides using a reaction catalyzed by three bacterial enzymes, including a sialic acid aldolase, a CMP-sialic acid synthetase, and a sialyltransferase (Scheme 1). Depending on the specificity of the sialyltransferase used, either α 2,3- or α 2,6- linked sialosides can be synthesized with typical yields of over 70%. Each sialoside was individually synthesized and purified before being used in bioassays (5,8–10).

In nature, sialosides constitute a large family of carbohydrates. In addition to more than 50 naturally occurring sialic acids, the number of the naturally existing underlying oligosaccharide structures that are linked to sialic acid goes beyond 15. In order to generate a sialoside library representing the majority of the naturally existing sialosides, more than 1,000 compounds are needed as the linkage between the sialic acid and the underlying oligosaccharide can be different as well (typical linkages are α 2,3-, α 2,6-, α 2,8-, and α 2,9- sialyl linked to structures containing a terminal galactose, *N*-acetyl galactosamine, or another sialic acid residue). Introducing non-natural modifications on sialic acid and the underlying glycan structures can further significantly increase the size of the sialoside library. Synthesizing all of these sialosides one by one followed by individual purification is time consuming and unnecessary for initial screening of sialic acid binding proteins to identify their suitable ligands. Here, we describe the development of a novel combinatorial chemoenzymatic synthetic approach which can be directly applied, without product purification, to a high-throughput screening platform for quick identifying the ligand(s) for carbohydrate-binding proteins.

RESULTS AND DISCUSSION

Overview of the strategy

The strategy of combinatorial chemoenzymatic synthesis and high-throughput screening is illustrated in Figure 1. Biotinylated sialyltransferase acceptors were chemically or chemoenzymatically synthesized and used in the one-pot three-enzyme system developed in our lab for the production of biotinylated sialosides in 96-well plates. Enzymatic reactions in different wells contained either a different sialic acid precursor or a different sialyltransferase acceptor. When the reactions proceeded to optimal conversions as identified by thin layer chromatography (TLC) analysis, the reaction mixtures were transferred to NeutrAvidin-coated 96-well plates with each reaction mixture generating two sets of triplicate samples in six wells. Only the produced biotinylated sialosides and un-reacted biotinylated sialyltransferase acceptors would be able to bind to the NeutrAvidin on the plates. After washing with PBS buffer containing 0.05% Tween 20, two sets of triplicate samples for each reaction were separately assayed for binding to a fluorescent-labeled sialic acid-binding lectin and a fluorescent-labeled acceptor-binding lectin, respectively. The readouts of the assays with the

acceptor-binding lectin gave the efficiency of the in-plate enzymatic reactions, and the readouts of the sialic acid-binding lectin gave the raw data on the interaction of sialosides and the lectin, which can then be adjusted by the enzymatic reaction efficiency to give the real comparison of the sialoside structure-dependent binding efficiency of the sialic acid-binding lectin. This 96-well plate format allows for efficient screening and multivalent presentation of the oligosaccharide ligands, which is important for studying carbohydrate-protein interactions.

Sialic acid precursors

For proof-of-the-principle purpose, we chose eighteen ManNAc, mannose, and their analogs containing *N*-glycolyl, methyl, acetyl, lactyl, alkynyl, azido, or other substituents at C-2 and/or C-6 as sialic acid precursors (Scheme 2, **1–18**) (5,10).

Biotinylated sialyltransferase acceptors

Four biotinylated disaccharides linked through a hexa-ethylene glycol (HEG) linker (Scheme 3, **24**, **28**, **26** and **27**) were synthesized and used as sialyltransferase acceptors. The HEG linker was introduced to minimize non-specific binding (11). To synthesize HEG-linked biotin **23**, one of the two hydroxyl groups in hexa-ethylene glycol **19** was tosylated and displaced by an azido group to form **20**. The remaining hydroxyl group was reacted with *tert*-butyl bromoacetate in dry dimethylformamide (DMF) and sodium hydride to afford **21**. The azido group in **21** (12) was reduced to an amine, which was coupled to *N*-hydroxysuccinamide (NHS) activated biotin to form **22**. The *tert*-butyl protecting group was then removed to give a carboxylic acid **23**, which can be conjugated with oligosaccharides containing a propylamine aglycone to give biotinylated oligosaccharides **24–27** (Scheme 3). Biotinylated *N*-acetyllactosamine (LacNAc) **28** was enzymatically synthesized from biotinylated *N*-acetylglucosamine (GlcNAc) **25** using a reaction catalyzed by a β 1,4-galactosyltransferase (LgtB) cloned from *N. meningitidis*.

Biotinylated sialoside standards

As sialylated product standards, four α 2,6-linked Neu5Ac-containing sialosides **29–32** were synthesized on a preparative scale in high yields (81–91%) from ManNAc and the corresponding biotinylated sialyltransferase acceptors **24**, **28**, **26** and **27**, respectively, using the one-pot three-enzyme system containing an *E. coli* aldolase, an *N. meningitidis* CMP-sialic acid synthetase, and a *P. damsela* α 2,6-sialyltransferase (Scheme 4) (5).

Determine reaction yields by *Erythrina cristagalli* Lectin (ECA) binding assays

Fluorescein isothiocyanate (FITC) conjugated *Erythrina cristagalli* Lectin (ECA) was chosen as the acceptor-specific lectin. ECA specifically recognizes terminal galactose/GalNAc in glycans (13) and sialylation of these glycans abolishes their binding to ECA. Therefore, ECA serves well for the purpose of specifically recognizing only the biotinylated sialyltransferase acceptors but not their corresponding sialylated products. Standard curves obtained for FITC-ECA binding to biotinylated acceptors assayed using the mixtures of an acceptor and the corresponding Neu5Ac-sialylated product (Figure 2) indicated that the binding of ECA to Gal β 1,4GlcNAc6S β -Biotin **24** (Figure 2A) or Gal β 1,4GlcNAc β -Biotin **28** (Figure 2B) is stronger than that of ECA to Gal β 1,3GlcNAc β -Biotin **26** (Figure 2C) or Gal β 1,4Glc β -Biotin **27** (Figure 2D). Nevertheless, all of the sialyltransferase acceptors used showed a good signal to noise ratio for ECA binding and the fluorescence intensity was in a linear range for the varied acceptor concentrations (0–10 μ M) used in the assay.

Combinatorial chemoenzymatic synthesis of sialosides and reaction yield determination

Using eighteen different sialic acid precursors 1–18 and four biotinylated sialyltransferase acceptors **24**, **26–28** described above, seventy two α 2,6-linked sialosides were individually

synthesized in 96-well plates using the one-pot three-enzyme system and directly transferred to NeutrAvidin-coated plates with each reaction mixture generating two sets of triplicate samples in six wells. One set of triplicate samples were assayed using FITC-ECA to determine the degree of the completion of enzymatic reaction for each sialoside (Figure 3) by comparing FITC-ECA assay data (Figure 1S) to the standard curves shown in Figure 2. As shown in Figure 3, most one-pot three-enzyme reactions carried out in 96-well plates were quite efficient. The yields were in the range of 31–100%. In general, sialylation of Gal β 1,4Glc β -Biotin **27** (blue bars) with different sialic acids (yields ranging 70–100%) was the most efficient ones except that when *N*-acetyl-D-mannosamine (ManNAc) **1** or *N*-benzoxycarbonylamidoacetyl-D-mannosamine (Man2CbzGly) **5** was used as the sialic acid precursor, Gal β 1,4GlcNAc6S β -Biotin **24** (green bars) was the best acceptor. Gal β 1,3GlcNAc β -Biotin **26** (yellow bars) was a poorer acceptor than others for most of the ManNAc/Man analogs used except for 2,6-di-*O*-acetyl-D-mannose (Man2,6Ac₂) **17** and D-lyxose (Lyx) **18**, for which sialylation of Gal β 1,4GlcNAc6S β -Biotin **24** (green bars) was the least efficient. Compared to other ManNAc/Man analogs used, mannose derivatives 6-*O*-acetyl-D-mannose (Man6Ac) **12**, 2-*O*-methyl-D-mannose (Man2Me) **15**, and 2,6-di-*O*-acetyl-D-mannose **17**, as well as 5-carbon monosaccharide D-lyxose **18** were generally poorer sialic acid precursors for the one-pot three-enzyme reactions.

Comparison of reaction yields determined by ECA and capillary electrophoresis (CE) assays

To verify the accuracy of the yields of the one-pot three-enzyme reactions determined by the ECA assay described above, a capillary electrophoresis (CE) assay was developed based on the UV absorbance of Neu5Ac-containing structures at 200 nm (14). Standard curves (data not shown) of the CE assay were obtained using mixtures containing varied concentrations (0–1 mM) of biotinylated sialyltransferase acceptor and the corresponding Neu5Ac-sialylated product. Four one-pot three-enzyme reactions were carried out in 96-well plates using ManNAc as the sialyltransferase donor precursor and four different biotinylated sialyltransferase acceptors. The yields of the enzymatic reactions were determined by both ECA and CE assays and the results were compared. The sialoside yields obtained for these four reactions from ECA and CE assays matched well (Table 1S). This demonstrated the reliability of the ECA binding method described herein for determining the reaction yields of sialosides in the one-pot three-enzyme reactions.

High-throughput screening of *Sambucus nigra* Lectin (SNA)-binding to sialosides

Fluorescein-labeled *Sambucus nigra* Lectin (SNA), a lectin from elderberry bark that recognizes sialic acids α 2,6 linked to galactose/GalNAc (15), was selected as a model system to test the relationship of the protein binding and the variation of the sialoside structures including the structures of the terminal sialic acids and the underlying carbohydrates. Another set of triplicate samples in NeutrAvidin-coated plates were assayed using fluorescein-SNA to determine the raw data on the interaction of SNA and the sialosides (Figure 2SA), which can be adjusted by the enzymatic reaction yields obtained for different sialosides shown in Figure 3 to give the real comparison of the sialoside structure-dependent binding efficiency of SNA (Figure 4A).

As shown in Figure 4A, SNA was able to bind to all sialosides that have been tested, indicating the promiscuity of the interaction of SNA and α 2,6-linked sialosides. Some preferences do exist for SNA binding. For example, variation of the terminal sialic acid structures can result in up to two-fold differences in SNA binding. Variation of the underlying glycans can also result in up to two-fold differences in SNA binding. The combination of changes on the sialic acid structure and underlying glycans can lead to up to three-fold differences in SNA binding. In general, SNA preferentially binds to α 2,6-linked sialylated LacNAc containing a sulphate at the C-6 of GlcNAc in LacNAc (sialylated **24**, green bars) or α 2,6-sialylated Gal β 1,3GlcNAc

26 (yellow bars) compared to α 2,6-sialylated LacNAc **28** (red bars) and α 2,6-sialylated lactose **27** (blue bars). SNA was also able to bind sialosides with a number of modifications at C-5 and C-9 of sialic acid residue. These results corroborate well with a recent study, which has shown the tolerance of SNA towards binding sialosides with a number of *N*-acyl substituents at C-9 of sialic acid (16). As expected (17), SNA has no preference in binding to α 2,6-linked *N*-glycolylneuraminic acid (Neu5Gc, synthesized from *N*-glycolylmannosamine **8** as a sialic acid precursor) or Neu5Ac (synthesized from ManNAc **1** as a sialic acid precursor)-containing sialosides irrespective of the acceptor used. Previous studies have indicated that mild periodate treatment of sialosides containing an intact glycerol side chain on the sialic acid residue abolished SNA binding to these sialosides (15, 17). Interestingly, SNA binds well to α 2,6-linked 4,6-*bis*-*epi*-KDO (synthesized from 5-carbon monosaccharide D-lyxose **18** as a sialic acid precursor)-containing sialosides, indicating that C-9 hydroxyl group of sialic acid is not important for the sialoside recognition by SNA.

High-throughput screening of human Siglec-2 (CD22)-binding to sialosides

The application of the method in screening ligands for sialic acid-binding proteins has also been demonstrated for human Siglec-2. Human Siglec-2 (CD22) is expressed on B-cells. It specifically recognizes α 2,6-linked sialoside structures (18) which have been implicated to be important for regulating B cell signaling (4,19). A number of ligand specificity studies for human CD22 have been carried out which have shown the importance of C-5 and C-9 substituents on sialic acids in modulating binding (16,20,21). A systematic ligand specificity study for human CD22 using sialosides varying in both terminal sialic acid structure and the internal glycans has been lacking. We present here the ligand specificity study of human CD22 using the seventy-two biotinylated sialosides obtained by one-pot three-enzyme synthesis in 96-well plates described above.

Similar to that described above for SNA binding assays, the raw data on the interaction of human CD22 and the sialosides (Figure 2SB) can be adjusted by the enzymatic reaction yields obtained for different sialosides shown in Figure 3 to give the real comparison of the sialoside structure-dependent binding efficiency of human CD22. As shown in Figure 4B, human CD22 is quite specific towards the structures of the sialic acids in sialoside. Except for the sialoside containing 5-azido-5-deoxy-ketodeoxynonulosonic acid (KDN5N₃, entry **16**), all other sialosides containing KDN and KDN derived sialic acids (Figure 4B, entry **11–15, 17–18**) are not recognized by human CD22, indicating the importance of the presence of a nitrogen at C-5 of sialic acids for the recognition of sialosides by human CD22. As observed before (22, 23), 9-*O*-acetylation (Figure 4B, entry **2**) and 9-*O*-lactylation (Figure 4B, entry **3**) on Neu5Ac abolish the binding of human CD22 to the sialosides. In addition, substituting the C-9 hydroxyl group in Neu5Ac of sialosides with an azido group also prevents the binding of human CD22 (Figure 4B, entry **4**). As expected (23), human CD22 has little preference for Neu5Gc (Figure 4B, entry **8**) over Neu5Ac based sialosides (Figure 4B, entry **1**). It is interesting to notice that a big benzoxycarbonylamido group and a propargyloxy group on *N*-acyl group of Neu5Ac do not change the binding of the sialosides (Figure 4B, entries **5** and **6**) to human CD22. However, the addition of an azido or a methoxy group to the *N*-acetyl of Neu5Ac of sialosides (Figure 4B, entries **7** or **9**) decreases their binding to human CD22 moderately. Acetylation of the *N*-glycolyl hydroxyl group of Neu5Gc (Figure 4B, and **10**) decreases the binding of the sialosides significantly.

In accordance with previous reports (19), human CD22 prefers α 2,6-linked sialylated LacNAc containing a sulphate at the C-6 of GlcNAc in LacNAc (sialylated **24**, green bars) over α 2,6-sialylated LacNAc (sialylated **28**, red bars), indicating the importance of the sulphation on the GlcNAc residue as an important recognition element (24). The data in Figure 4B also show that human CD22 has slight preference in binding to sialylated type 1 disaccharides (α 2,6-sialylated

Gal β 1,3GlcNAc **26**, yellow bars) compared to sialylated type 2 disaccharides (α 2,6-sialylated LacNAc **28**, red bars). In addition, α 2,6-sialylated lactosides (sialylated **27**, blue bars) are not good ligands for human CD22. Among eighteen sialylated lactosides tested, only Neu5Ac α -2,6-linked lactosides shows moderate binding. The data obtained provide useful information for designing high affinity sialoside probes for studying Siglec-2 function.

Compared to the sialoside microarray assay for CD22 reported recently by Blixt and Paulson et al. (16), the combinatorial chemoenzymatic synthesis of biotinylated sialoside combined with high-throughput screening approach described here has the advantage of generating a much larger sialoside library without the tedious product purification. With the expansion of the library of sialic acid derivatives or their precursors and the collection of biotinylated sialyltransferase acceptors, the method will allow an easy access to a large collection of sialosides which will significantly simplify the process of identifying novel ligands for sialic acid-binding proteins and finding novel sialic acid-binding proteins.

Conclusion

Combinatorial chemoenzymatic synthesis of sialosides using biotinylated sialyltransferase acceptors presents a simple and effective approach that can be combined with convenient microtiter plate based high-throughput screening method for quick identification of the ligand specificity of sialic acid-binding proteins. The approach requires the synthesis of only nanomole quantities of complex sialosides and no product purification procedure is necessary. Therefore, it is extremely useful in characterizing the ligand specificity of sialic acid-binding proteins or identifying unknown sialic acid-binding proteins using a large sialoside library. Using the efficient combinatorial chemoenzymatic synthetic approach coupled with high-throughput screening format, we have showed here that SNA binds to α 2,6-linked sialosides with an extreme flexibility in terms of the sialic acid structures and the underlying glycans. This is significant since SNA has been broadly used as a histochemical tool for glycan analysis of tissues, organs, cells, glycoproteins, and other biological samples. In contrast, human Siglec-2 showed highly selectivity for a number of sialic acid structures (Neu5Ac, Neu5Gc, KDN5N3, and limited C-5 modified Neu5Ac derivatives) and the underlying glycans (Gal β 1,4GlcNAc6S β -Biotin and Gal β 1,3GlcNAc β -Biotin were preferred compared to Gal β 1,4GlcNAc β -Biotin and Gal β 1,4Glc β -Biotin) in its sialoside ligands. The combinatorial chemoenzymatic synthesis and the screening method reported herein are valuable tools for initial identifying high affinity sialoside ligands for sialic acid-binding proteins. The ligands identified will aid in elucidating the biological functions of sialic acid-binding proteins. Increasing the number of sialic acid precursors and the number of sialyltransferase acceptors as well as using different sialyltransferases which synthesize the formation of different sialyl linkages will greatly expand the scope of the structures of the sialosides for identifying ligand specificity of sialic acid-binding proteins in a high throughput format.

MATERIALS AND METHODS

Materials

FITC-ECA was purchased from EY labs. Fluorescein labeled SNA was purchased from Vector Laboratories. Human CD22/Siglec-2 4D (1 C2-set and 3V-set domains) fused to mouse IgG was obtained as described previously (25). Goat anti-mouse IgG Alexa Fluor 488 used for detecting human CD22 was purchased from Invitrogen. Chemicals were purchased and used without further purification. NeutrAvidin coated 96 well plates were purchased from Pierce Biotechnology. Clear 96 well plates used for the combinatorial chemoenzymatic synthesis were purchased from Corning. Biotek synergy HT microplate reader was used to measure fluorescence from the 96 well plates. For FITC or Alexa Fluor 488 labeled lectins or antibodies, the excitation and emission wavelengths used were 485 nm and 528 nm, respectively. ¹H NMR

and ^{13}C NMR spectra were recorded on Mercury-300, Varian Inova-400, or Varian-600 spectrometer. High and low resolution electrospray ionization (ESI) mass spectra were obtained at the Mass Spectrometry Facility in the University of California-Davis, or recorded on ABI 4700 MALDI TOF/TOF mass spectrometer. Silica gel 60 Å (200–425 mesh, Sorbent technologies) was used for flash column chromatography. Thin-layer chromatography (TLC) was performed on silica gel plates 60 GF254 (Sorbent technologies) using anisaldehyde sugar stain or 5% sulfuric acid in ethanol stain for detection. Gel filtration chromatography was performed with a column (100 cm $\times$ 2.5 cm) packed with BioGel P-2 Fine (Bio-Rad). Recombinant *E. coli* aldolase (26), *N. meningitidis* CMP-sialic acid synthetase (26), and Pd2,6ST (5,27) were expressed and purified as reported previously.

Preparative-scale synthesis of sialosides 29–32 using biotinylated sialyltransferase acceptors 24, 28, 26 and 27 in one-pot three-enzyme system

A biotinylated saccharide selected from 24, 28, 26 or 27 (20–30 mg, 1.0 equiv.), ManNAc (1.5 equiv.), pyruvate (7.5 equiv.), and CTP (1.5 equiv.) were dissolved in a solution containing Tris-HCl buffer (100 mM, pH 8.5), MgCl_2 (20 mM), *E. coli* aldolase (2–5 mg), *N. meningitidis* CMP-sialic acid synthetase (2–5 mg), and Pd2,6ST (2–4 mg) in a 50 ml centrifuge tube. The reaction mixture was incubated at 37 °C with agitation at 125 rpm for 2 h. The product formation was monitored by thin layer chromatography (TLC) developed with EtOAc:MeOH:H₂O:HOAc = 4:2:1.5:0.2 (v/v) and stained with *p*-anisaldehyde sugar stain. The reaction was stopped by adding the same volume of ice-cold EtOH and incubating at 4 °C for 30 min. The mixture was centrifuged to remove precipitates and the supernatant was concentrated before passed through a BioGel P-2 gel filtration column to obtain the corresponding biotinylated sialosides **29–32**.

One-pot three-enzyme combinatorial chemoenzymatic synthesis of α 2,6-linked sialosides in 96-well plates

Reactions were carried out in a final volume of 35 μL in individual wells in 96-well plates containing a prospective biotinylated sialyltransferase acceptor **24**, **28**, **26** or **27** (4 mM), a precursor of sialic acid or its analog **1–18** (10 mM), sodium pyruvate (50 mM), CTP (12 mM), MgCl_2 (20 mM) in a solution of Tris-HCl buffer (0.1 M, pH 7.5 for acetylated/lactylated precursors and 0.1 M, pH 8.8 for others), *E. coli* sialic acid aldolase, *N. meningitidis* CMP-sialic acid synthetase, and Pd2,6ST. The plates were covered by a parafilm and incubated in an Isotherm incubator for 12–24 h at 37 °C. After the incubation, the plates were frozen at –20 °C before lectin binding assays.

High-throughput screening of biotinylated saccharides and sialosides using *Erythrina cristagalli* Lectin (ECA) and *Sambucus nigra* Lectin (SNA)

To each well of NeutrAvidin coated plate (coating volume of 100 μL , Pierce Biotechnology), 110 μL of each sialoside reaction mixture (10 μM , 400-fold dilution of the original reaction mixture) was added to individual wells to generate two sets of triplicate samples in six wells and the plate was incubated for 1 h at rt. The plate was then washed with 3 rounds of 1 $\times$ PBS buffer containing 0.05% Tween 20. To one set of triplicate samples for each sialoside, 100 μL of ECA (FITC conjugated *Erythrina cristagalli* Lectin) at a concentration of 50 $\mu\text{g mL}^{-1}$ in PBS buffer was added and incubated at RT for 2 h. The wells were then washed twice with PBS buffer containing 0.05% Tween 20 and once with DI water prior to the measurement of fluorescence. Similar procedure was followed for SNA assays using the other set of the triplicate samples, except that SNA was used at a concentration of 20 $\mu\text{g mL}^{-1}$ in PBS buffer. For the construction of standard curves, NeutrAvidin coated wells were incubated with a mixture (total concentration is 10 μM) containing different ratios (0, 20%, 40%, 60%, 80%, and 100%) of the biotinylated sialyltransferase acceptors and their corresponding biotinylated

Neu5Ac-containing α 2,6-linked sialosides (2 sets of triplicate samples) before being assayed for ECA and SNA binding. Lectin binding assays of one-pot three-enzyme reaction samples using the same biotinylated sialyltransferase acceptor were run in the same plate. Assays for obtaining the standard curve of the corresponding biotinylated acceptor were run for each plate.

Verification of yields obtained by ECA assays using capillary electrophoresis assays

One-pot three-enzyme synthesis reactions were carried out similarly to those described above for combinatorial chemoenzymatic synthesis except that 0.5 ml centrifuge tubes were used as the reaction containers and ManNAc **1** was used as the only precursor of sialic acid (10 mM). After the incubation, the reactions were frozen at -20°C before analysis. The reaction mixtures were thaw and analyzed by both the ECA assay as described above and the capillary electrophoresis assay as described below.

Capillary electrophoresis (CE) assay to determine the reaction yields of one-pot three-enzyme combinatorial chemoenzymatic synthesis

The reaction mixtures were diluted 4-fold and kept on ice until they were analyzed by applying 25 kV at 25°C and monitored at $A_{200\text{ nm}}$ using a Beckman P/ACE MDQ system equipped with a UV/Vis detector and fused-silica capillary (60 cm $\times$ 75 μm i.d.). Sodium borate solution (25 mM, pH 10.8) was used as the running buffer. Standard curves were obtained in duplicate by analyzing mixtures with a total concentration of 1 mM containing different ratios (0, 20%, 40%, 60%, 80%, and 100%) of the biotinylated sialyltransferase acceptors and their corresponding biotinylated Neu5Ac-containing α 2,6-linked sialosides.

High-throughput screening of biotinylated saccharides and sialosides using human Siglec-2 (CD22)

To each well of NeutrAvidin coated plate (coating volume of 100 μL , Pierce Biotechnology), 110 μL of each sialoside (10 μM , 400-fold dilution of the original reaction mixture) was added to individual wells in duplicate and the plate was incubated for 1 h at RT. The plate was then washed with 3 rounds of $1 \times$ PBS buffer containing 0.05% Tween 20. To each set of duplicate samples for each sialoside, 100 μL of human CD22 (1 $\mu\text{g ml}^{-1}$) pre-mixed in a 1:3 weight ratio with goat anti-mouse IgG Alexa Flour 488 in PBS buffer was added and incubated at RT for 2 h. The wells were then washed twice with PBS buffer containing 0.05% Tween 20 and once with DI water prior to the measurement of fluorescence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Angata T, Varki A. Chemical diversity in the sialic acids and related alpha-keto acids: An evolutionary perspective. *Chem Rev* 2002;102:439–469. [PubMed: 11841250]
2. Schauer R. Achievements and challenges of sialic acid research. *Glycoconj J* 2000;17:485–499. [PubMed: 11421344]
3. Suzuki Y. Sialobiology of influenza molecular mechanism of host range variation of influenza viruses. *Biol Pharm Bull* 2005;28:399–408. [PubMed: 15744059]
4. Crocker PR, Paulson JC, Varki A. Siglecs and their roles in the immune system. *Nat Rev Immunol* 2007;7:255–266. [PubMed: 17380156]

5. Yu H, Huang S, Chokhawala H, Sun M, Zheng H, Chen X. Highly efficient chemoenzymatic synthesis of naturally occurring and non-natural alpha-2,6-linked sialosides: A *P. damsela* alpha-2,6-sialyltransferase with extremely flexible donor-substrate specificity. *Angew Chem, Int Ed* 2006;45:3938–3944.
6. Boons GJ, Demchenko AV. Recent advances in *O*-sialylation. *Chem Rev* 2000;100:4539–4566. [PubMed: 11749357]
7. Chappell MD, Halcomb RL. Enzyme-catalyzed synthesis of oligosaccharides that contain functionalized sialic acids. *J Am Chem Soc* 1997;119:3393–3394.
8. Yu H, Chokhawala HA, Huang S, Chen X. One-pot three-enzyme chemoenzymatic approach to the synthesis of sialosides containing natural and non-natural functionalities. *Nat Protoc* 2006;1:2485–2492. [PubMed: 17406495]
9. Chokhawala HA, Yu H, Chen X. High-throughput substrate specificity studies of sialidases by using chemoenzymatically synthesized sialoside libraries. *Chembiochem* 2007;8:194–201. [PubMed: 17195254]
10. Yu H, Chokhawala H, Karpel R, Yu H, Wu B, Zhang J, Zhang Y, Jia Q, Chen X. A multifunctional *Pasteurella multocida* sialyltransferase: A powerful tool for the synthesis of sialoside libraries. *J Am Chem Soc* 2005;127:17618–17619. [PubMed: 16351087]
11. Mrksich M, Chen CS, Xia Y, Dike LE, Ingber DE, Whitesides GM. Controlling cell attachment on contoured surfaces with self-assembled monolayers of alkanethiolates on gold. *Proc Natl Acad Sci U S A* 1996;93:10775–10778. [PubMed: 8855256]
12. Svedhem S, Hollander CA, Shi J, Konradsson P, Liedberg B, Svensson SCT. Synthesis of a series of oligo(ethylene glycol)-terminated alkanethiol amides designed to address structure and stability of biosensing interfaces. *J Org Chem* 2001;66:4494–4503. [PubMed: 11421767]
13. Turton K, Natesh R, Thiagarajan N, Chaddock JA, Acharya KR. Crystal structures of *Erythrina cristagalli* lectin with bound N-linked oligosaccharide and lactose. *Glycobiology* 2004;14:923–929. [PubMed: 15201215]
14. Li Y, Yu H, Cao H, Lau K, Muthana S, Tiwari VK, Son B, Chen X. *Pasteurella multocida* sialic acid aldolase: a promising biocatalyst. *Appl Microbiol Biotechnol* 2008;79:963–970. [PubMed: 18521592]
15. Shibuya N, Goldstein IJ, Broekaert WF, Nsimbalubaki M, Peeters B, Peumans WJ. The elderberry (*Sambucus nigra* L) bark lLectin recognizes the Neu5Ac(alpha-2-6)Gal GalNAc sequence. *J Biol Chem* 1987;262:1596–1601. [PubMed: 3805045]
16. Blixt O, Han S, Liao L, Zheng Y, Hoffmann J, Futakawa S, Paulson JC. Sialoside analogue arrays for rapid identification of high affinity Siglec ligands. *J Am Chem Soc* 2008;130:6880–6881.
17. Brinkman-Van der Linden ECM, Sonnenburg JL, Varki A. Effects of sialic acid substitutions on recognition by *Sambucus nigra* agglutinin and *Maackia amurensis* hemagglutinin. *Anal Biochem* 2002;303:98–104. [PubMed: 11906157]
18. Powell LD, Sgroi D, Sjoberg ER, Stamenkovic I, Varki A. Natural ligands of the B-cell adhesion molecule CD22-beta carry N-linked oligosaccharides with alpha-2,6-linked sialic acids that are required for recognition. *J Biol Chem* 1993;268:7019–7027. [PubMed: 8463235]
19. Walker JA, Smith KGC. CD22: an inhibitory enigma. *Immunology* 2008;123:314–325. [PubMed: 18067554]
20. Blixt O, Head S, Mondala T, Scanlan C, Huflejt ME, Alvarez R, Bryan MC, Fazio F, Calarese D, Stevens J, Razi N, Stevens DJ, Skehel JJ, van Die I, Burton DR, Wilson IA, Cummings R, Bovin N, Wong CH, Paulson JC. Printed covalent glycan array for ligand profiling of diverse glycan binding proteins. *Proc Natl Acad Sci U S A* 2004;101:17033–17038. [PubMed: 15563589]
21. Blixt O, Collins BE, van den Nieuwenhof IM, Crocker PR, Paulson JC. Sialoside specificity of the Siglec family assessed using novel multivalent probes -Identification of potent inhibitors of myelin-associated glycoprotein. *J Biol Chem* 2003;278:31007–31019. [PubMed: 12773526]
22. Sjoberg ER, Powell LD, Klein A, Varki A. Natural ligands of the B-cell adhesion molecule CD22-beta can be masked by 9-*O*-acetylation of sialic acids. *J Cell Biol* 1994;126:549–562. [PubMed: 8034751]

23. Kelm S, Schauer R, Manuguerra JC, Gross HJ, Crocker PR. Modifications of cell-surface sialic acids modulate cell-adhesion mediated by sialoadhesin and CD22. *Glycoconj J* 1994;11:576–585. [PubMed: 7696861]
24. Kimura N, Ohmori K, Miyazaki K, Izawa M, Matsuzaki Y, Yasuda Y, Takematsu H, Kozutsumi Y, Moriyama A, Kannagi R. Human B-lymphocytes express alpha 2-6-sialylated 6-sulfo-*N*-acetylactosamine serving as a preferred ligand for CD22/Siglec-2. *J Biol Chem* 2007;282:32200–32207. [PubMed: 17728258]
25. Powell LD, Varki A. The oligosaccharide binding specificities of CD22 beta, a sialic acid-specific lectin of B cells. *J Biol Chem* 1994;269:10628–10636. [PubMed: 8144652]
26. Yu H, Yu H, Karpel R, Chen X. Chemoenzymatic synthesis of CMP-sialic acid derivatives by a one-pot two-enzyme system: comparison of substrate flexibility of three microbial CMP-sialic acid synthetases. *Bioorg Med Chem* 2004;12:6427–6435. [PubMed: 15556760]
27. Sun M, Li Y, Chokhawala HA, Henning R, Chen X. N-terminal 112 amino acid residues are not required for the sialyltransferase activity of *Photobacterium damsela* α 2,6-sialyltransferase. *Biotechnol Lett* 2008;30:671–676. [PubMed: 17989925]

Figure 1.
The combinatorial chemoenzymatic synthetic strategy for direct high-throughput screening of sialosides in 96-well plates.

Figure 2. Standard curves of *Erythrina cristagalli* Lectin (ECA) binding to solutions containing different percentages of biotinylated sialyltransferase acceptors and their corresponding biotinylated Neu5Ac-containing α 2,6-sialosides

A, Gal β 1,4GlcNAc6S β -Biotin 24 and Neu5Ac α 2,6Gal β 1,4GlcNAc6S β -Biotin 29; B, Gal β 1,4GlcNAc β -Biotin 28 and Neu5Ac α 2,6Gal β 1,4GlcNAc β -Biotin 30; C, mixtures of Gal β 1,3GlcNAc β -Biotin 26 and Neu5Ac α 2,6Gal β 1,3GlcNAc β -Biotin 31; D, mixtures of Gal β 1,4Glc β -Biotin 27 and Neu5Ac α 2,6Gal β 1,4Glc β -Biotin 32.

Figure 3. Reaction yields of sialosides chemoenzymatically synthesized in 96-well plates.^a

^aObtained by comparing the fluorescence intensity of *Erythrina cristagalli* Lectin (ECA) binding to the remaining biotinylated sialyltransferase acceptors in the one-pot three-enzyme reaction mixtures to the ECA standard curves in Figure 2.

A.

B.

Figure 4. Adjusted fluorescence intensities (arbitrary units, adjusted using reaction yields shown in Figure 3) of A, *Sambucus nigra* Lectin (SNA); and B, human CD22 binding to NeutrAvidin plates containing one-pot three-enzyme reaction mixtures for the synthesis of sialosides.^a

Sialic acid residues in different sialosides: 1) Neu5Ac; 2) Neu5,9Ac₂; 3) Neu5Ac9Lt; 4) Neu5Ac9N₃; 5) Neu5CbzGly; 6) Neu5CPg; 7) Neu5AcN₃; 8) Neu5Gc; 9) Neu5GcMe; 10) Neu5GcAc; 11) KDN; 12) KDN9Ac; 13) KDN9N₃; 14) KDN5Ac; 15) KDN5Me; 16) KDN5N₃; 17) KDN5,9Ac₂; 18) 4,6-bis-epi-KDO; 19) negative controls with biotinylated sialyltransferase acceptors 24, 28, 26, and 27. ^aRefer to Scheme 2 for abbreviations and structures of substituents.

Scheme 1.
One-pot three-enzyme synthesis of sialosides.

Scheme 2. Structures of ManNAc/mannose derivatives 1–18^a used as the precursors for sialic acid analogs in one-pot three-enzyme synthesis of α 2,6-linked sialosides

^aAbbreviations and names of compounds 1–18: 1, ManNAc (*N*-acetyl-D-mannosamine); 2, ManNAc6Ac (6-*O*-acetyl-*N*-acetyl-D-mannosamine); 3, ManNAc6Lt (6-*O*-lactyl-*N*-acetyl-D-mannosamine); 4, ManNAc6N₃ (6-azido-6-deoxy-*N*-acetyl-D-mannosamine); 5, ManNCbzGly (*N*-benzoxycarbonylamidoacetyl-D-mannosamine); 6, ManNCPg (*N*-propargyloxycarbonyl-D-mannosamine); 7, ManNAcN₃ (*N*-azidoacetyl-D-mannosamine); 8, ManNGc (*N*-glycolylmannosamine); 9, ManNGcMe (*N*-methoxyacetyl-D-mannosamine); 10, ManNGcAc (*N*-acetoxyacetyl-D-mannosamine); 11, Man (D-mannose); 12, Man6Ac (6-*O*-acetyl-D-mannose); 13, Man6N₃ (6-azido-6-deoxy-D-mannose); 14, Man2Ac (2-*O*-acetyl-D-mannose); 15, Man2Me (2-*O*-methyl-D-mannose); 16, Man2N₃ (2-azido-2-deoxy-D-mannose); 17, Man2,6Ac₂ (2,6-di-*O*-acetyl-D-mannose); 18, Lyx (D-lyxose).

Scheme 3.

Synthesis of biotinylated sialyltransferase acceptors 24, 28, 26, and 27.

Scheme 4.

One-pot three-enzyme synthesis of Neu5Ac-linked biotinylated sialoside standards 29–32.