Combinatorial Chemoenzymatic Synthesis and High-Throughput Screening of Sialosides

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Sialic acids are a family of diverse nonulosonic acids. Naturally occurring modifications on sialic acids, such as acetylation, lactylation, methylation, and sulfation, lead to >50 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 hemagglutinins 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 superfamily lectins primarily expressed on cells of the immune system. Their binding to sialic acid-containing structures is believed to play important roles in regulating the immune system (4).

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 because of 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.

ABSTRACT Although the vital roles of structures containing sialic acid 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 α2,6-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 whereas Sambucus nigra Lectin binds to α2,6-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.
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 homogeneous 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 α2,3- or α2,6-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 >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, >1,000 compounds are needed as the linkage between the sialic acid and the underlying oligosaccharide can be different as well (typical linkages are α2,3-, α2,6-, α2,8-, and α2,9-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 that can be directly applied, without product purification, to a high-throughput screening platform for quickly 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 laboratory 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 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 unreacted biotinylated sialyltransferase acceptors would be able to bind to the NeutrAvidin on the plates. After being washed 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.
tin 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-principle purposes, we chose 18 ManNAc, mannoside, 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 hexaethylene glycol (HEG) linker (Scheme 3, 24, 28, 26, and 27) were synthesized and used as sialyltransferase acceptors. The HEG linker was introduced to minimize nonspecific binding (11). To synthesize HEG-linked biotin 23, one of the two hydroxyl groups in hexaethylene 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-hydroxysuccinimide (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-acetylgalactosamine (GlcNAc) 25 using a reaction catalyzed by a β1,4-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. damselα α,2,6-sialyltransferase (Scheme 4) (5).

**Determine Reaction Yields by Erythrina cristagalli Lectin (ECA) Binding Assays.** Fluorescein isothiocyanate (FITC)-conjugated 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

![Scheme 2. Structures of ManNAc/mannose derivatives 1–18* used as the precursors for sialic acid analogs in one-pot three-enzyme synthesis of α,2,6-linked sialosides. *Abbreviations and names of compounds 1–18: 1, ManNAc (N-acetyl-D-mannosamine); 2, ManNAc6Ac (6-O-acetyl-N-acetyl-D-mannosamine); 3, ManNAc6L (6-O-lactyl-N-acetyl-D-mannosamine); 4, ManNAc6N (6-azido-6-deoxy-N-acetyl-D-mannosamine); 5, ManNCbzGly (N-benzyloxyacrylamidoacetyl-D-mannosamine); 6, ManNCPg (N-propargyloxycarbonyl-D-mannosamine); 7, ManNAcN (N-azidoacetyl-D-mannosamine); 8, ManNgc (N-glycolylmannosamine); 9, ManNgcMe (N-methoxyacetyl-D-mannosamine); 10, ManNgcAc (N-acetoxyacetyl-D-mannosamine); 11, Man (mannose); 12, Man6Ac (6-O-acetyl-D-mannose); 13, Man6N (6-azido-6-deoxy-D-mannose); 14, Man2Ac (2-O-acetyl-D-mannose); 15, Man2Me (2-O-methyl-D-mannose); 16, Man2N (2-azido-2-deoxy-D-mannose); 17, Man2,6Ac (2,6-di-O-acetyl-D-mannose); 18, Lyx (p-lyxose).](attachment:image)
Scheme 3. Synthesis of biotinylated sialyltransferase acceptors 24, 28, 26, and 27

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 Galβ1,4GlcNAc6Sβ-Biotin 24 (Figure 2, panel a) or Galβ1,4GlcNAcβ-Biotin 28 (Figure 2, panel b) is stronger than that of ECA to Galβ1,3GlcNAcβ-Biotin 26 (Figure 2, panel c) or Galβ1,4Glcβ-Biotin 27 (Figure 2, panel d). 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 μM) used in the assay.

Combinatorial Chemoenzymatic Synthesis of Sialosides and Reaction Yield Determination. Using 18 different sialic acid precursors 1–18 and four biotinylated sialyltransferase acceptors 24, 26–28 described above, seventy two α2,6-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 (Supplementary 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, sialylations of Galβ1,4Glcβ-Biotin 27 (blue bars) with different sialic acids (yields 70–100%) were the most efficient ones except that when N-acetyl-d-mannosamine (ManNAc) 1 or N-benzylxoycarbonylmalodacetyl-0-mannosamine (Man2CbzGly) 5 was used as the sialic acid precursor, Galβ1,4GlcNAc6Sβ-Biotin 24 (green bars) was the best acceptor. Galβ1,3GlcNAcβ-Biotin 26 (yellow bars) was a poorer acceptor compared with 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 Galβ1,4GlcNAc6Sβ-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 (Supplementary 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 SNA, a lectin from elderberry bark that recognizes sialic acids α2,6-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 (Supplementary Figure 2S), 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 4, panel a).

As shown in Figure 4, panel a, SNA was able to bind to all sialosides that have been tested, indicating the promiscuity of the interaction of SNA and α2,6-linked sialosides. Some preferences do exist for SNA binding. For example, variation of the terminal sialic acid structures can result in up to 2-fold differences in SNA binding. Variation of the underlying glycans can also result in up to 2-fold differences in SNA binding. The combination of changes on the sialic acid structure and underlying glycans can lead to up to 3-fold differences in SNA binding. In general, SNA preferentially binds to α2,6-linked sialylated LacNAc containing a sulfate at the C-6 of GlcNAc in LacNAc (sialylated 24, green bars) or α2,6-sialylated Galβ1,3GlcNAc 26 (yellow bars) compared to α2,6-sialylated LacNAc 28 (red bars) and α2,6-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 a recent study, which has shown the tolerance of SNA toward

Scheme 4. One-pot three-enzyme synthesis of Neu5Ac-linked biotinylated sialoside standards 29–32
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 sialosides containing α2,6-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) 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 sialosides containing α2,6-linked 4,6-bis-epi-KDO (synthesized from 5-carbon monosaccharide D-lyxose 18 as a sialic acid precursor), 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 the α2,6-linked sialoside structures (18) which has 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 72 biotinylated sialosides obtained in this study.

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 α2,6-sialosides. a) Galβ1,4GlcNAc6Sβ-Biotin 24 and Neu5Acα2,6Galβ1,4GlcNAc6Sβ-Biotin 29; b) Galβ1,4GlcNAcβ-Biotin 28 and Neu5Acα2,6Galβ1,4GlcNAcβ-Biotin 30; c) Galβ1,3GlcNAcβ-Biotin 26 and Neu5Acα2,6Galβ1,3GlcNAcβ-Biotin 31; d) Galβ1,4Glcβ-Biotin 27 and Neu5Acα2,6Galβ1,4Glcβ-Biotin 32.
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 (Supplementary Figure 2S) 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 4, panel b, human CD22 is quite specific toward the structures of the sialic acids in sialosides. Except for the sialoside containing 5-azido-5-deoxy-ketodeoxy-nonulosonic acid (KDN5N3, entry 16), all other sialosides containing KDN and KDN-derived sialic acids (Figure 4, panel b, entries 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 4, panel b, entry 2) and 9-O-lactylation (Figure 4, panel b, 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 4, panel b, entry 4). As expected (23), human CD22 has little preference for Neu5Gc (Figure 4, panel b, entry 8) over Neu5Ac-based sialosides (Figure 4, panel b, entry 1). It is interesting to notice that a big benzoxycarbonylamido group and a propargyloxy group on the N-acyl group of Neu5Ac do not change the binding of the sialosides (Figure 4, panel b, entries 5 and 6) to human CD22. However, the addition of an azido or a methoxy group to the N-acyt of Neu5Ac of sialosides (Figure 4, panel b, entries 7 or 9) decreases their binding to human CD22 moderately. Acetylation of the N-glycolyl hydroxyl group of Neu5Gc (Figure 4, panel b, entry 10) decreases the binding of the sialosides significantly.

In accordance with previous reports (19), human CD22 prefers α2,6-linked sialylated LacNAC containing a sulfate at the C-6 of GlcNAc in LacNAC (sialylated 24, green bars) over α2,6-sialylated LacNAC (sialylated 28, red bars), indicating the importance of the sulfation on the GlcNAc residue as an important recognition element (24). The data in Figure 4, panel b also show that human CD22 has slight preference in binding to sialylated type 1 disaccharides (α2,6-sialylated Galβ1,3GlcNAc 26, yellow bars) compared to sialylated type 2 disaccharides (α2,6-sialylated LacNAC 28, red bars). In addition, α2,6-sialylated lactosides (sialylated 27, blue bars) are not good ligands for human CD22. Among 18 sialylated lactosides tested, only Neu5Acα-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 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 bio- tinylated 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 a 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 chemo-enzymatic synthetic approach coupled with high-throughput screening format, we have shown here that SNA binds to α2,6-linked sialosides with an extreme flexibility in terms of the sialic acid structures and the underlying glycans. This is significant because 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 high selectivity for a number of sialic acid structures (Neu5Ac, Neu5Gc, KDN5N3, and limited C-5 modified Neu5Ac derivatives) and the underlying glycans (Galβ1,4GlcNAc6S-Biotin and Galβ1,3GlcNAcβ-Biotin were preferred compared to Galβ1,4GlcNAcβ-Biotin and Galβ1,4Glcβ-Biotin) in its sialoside ligands. The combinatorial chemoenzymatic synthesis and the screening method reported herein are valuable tools for initial identification of 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 that catalyze 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 laboratories. 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 antimouse IgG Alexa Fluor 488 used for detecting human CD22 was purchased from Invitrogen. Chemicals were purchased and used without further purification. NeutρAvidin-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 and 528 nm, respectively. 1H NMR and 13C NMR spectra were recorded on a 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 an 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 × 2.5 cm) packed with BioGel P-2 Fine (Bio-Rad). Re- combinant 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), MgCl2 (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 α2,6-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), MgCl2 (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 thin layer of paraffin and incubated in an isothermal 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 E. Cristagalli Lectin (ECA) and S. nigro Lectin (SNA). To each well of NeutrAvidin-coated plates (coating volume of 100 μL, Pierce Biotechnology) was added to individual wells 110 μL of each sialoside (10 μM, 400-fold dilution of the original reaction mixture) in duplicate, and the plate was incubated for 1 h at RT. The plate was then washed with 3 rounds of 1 × PBS buffer containing 0.05% Tween 20. To each set of duplicate samples for each sialoside, 100 μL of human CD22 (1 μg mL−1) premixed in a 1:3 weight ratio with goat antimouse 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. A 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 μg mL−1 in PBS buffer. For the construction of standard curves, NeutrAvidin-coated wells were incubated with a mixture (total concentration of 10 μM) containing different ratios (0, 20%, 40%, 60%, 80%, and 100%) of the biotinylated sialyltransferase acceptors and their corresponding biotinylated Neu5Ac-containing α2,6-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 to determine the yields 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 thawed 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 A406 using a Beckman P/ACE MDQ system equipped with a UV–vis detector and fused-silica capillary (60 cm × 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 α2,6-linked sialosides.

High-Throughput Screening of Biotinylated Saccharides and Sialosides Using Human Siglec-2 (CD22). To each well of NeutrAvidin-coated plates (coating volume of 100 μL, Pierce Biotechnology) was added to individual wells 110 μL of each sialoside (10 μM, 400-fold dilution of the original reaction mixture) in duplicate, and the plate was incubated for 1 h at RT. The plate was then washed with 3 rounds of 1 × PBS buffer containing 0.05% Tween 20. To each set of duplicate samples for each sialoside, 100 μL of human CD22 (1 μg mL−1) premixed in a 1:3 weight ratio with goat antimouse 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.

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REFERENCES


