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Chemoenzymatic Synthesis of a New Class of Macrocyclic Oligosaccharides

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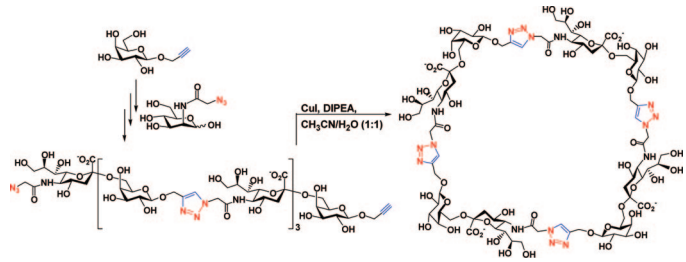

A novel and highly efficient chemoenzymatic method has been developed for the preparation of structurally defined macrocyclic oligosaccharides of varied sizes. This method involves chemical or chemoenzymatic synthesis of oligosaccharides containing a galactose at the nonreducing end and a propargyl group at the reducing end as sialyltransferase acceptors. Introducing an azido-containing sialic acid to the nonreducing end of the galactosides through a sialyltransferase-catalyzed enzymatic reaction followed by copper(I)-catalyzed Huisgen's 1,3-dipolar cycloaddition of alkyne and azide provides size-defined macrocyclic carbohydrates. The produced negatively charged macrocycles have high solubility in water and interact with hydrophobic small molecules in a size-dependent manner.

Introduction

Macrocyclic carbohydrates have found widespread and diverse applications such as building blocks in supramolecular chemistry,[1,2] drug carrier systems,[3,4] molecular reactors,[5] and artificial receptors.[6] Cyclodextrins are a family of such macrocyclic oligosaccharides which contain α1,4-linked glucopyranoside units and are produced from starch by enzymatic conversion in nature.[7] The three most common cyclodextrins are α-cyclodextrin, *β*-cyclodextrin, and *γ*-cyclodextrin with six, seven, and eight glucose residues, respectively. Cyclodextrins are capable of forming inclusion complexes with a variety of molecular and ionic compounds.[8,9] The ability of cyclodextrins to bind hydrophobic molecules within their cavity while still dissolved in aqueous solution has led to intensive studies of their inclusion complexes.[10,11] However, naturally occurring macrocyclic carbohydrates such as cyclodextrins (CDs) have relatively low water solubility and are difficult to be functionalized. Selective modification of naturally occurring CDs has been successful but limited to certain positions on the CD macrocycles.[12] A common alternative approach to selectively functionalize CD macrocycles and their analogues is by stepwise synthesis from monosaccharides.[13,14] Such a method involves a lengthy synthetic pathway with a low overall yield. Despite the remarkable advances in modifying naturally occurring

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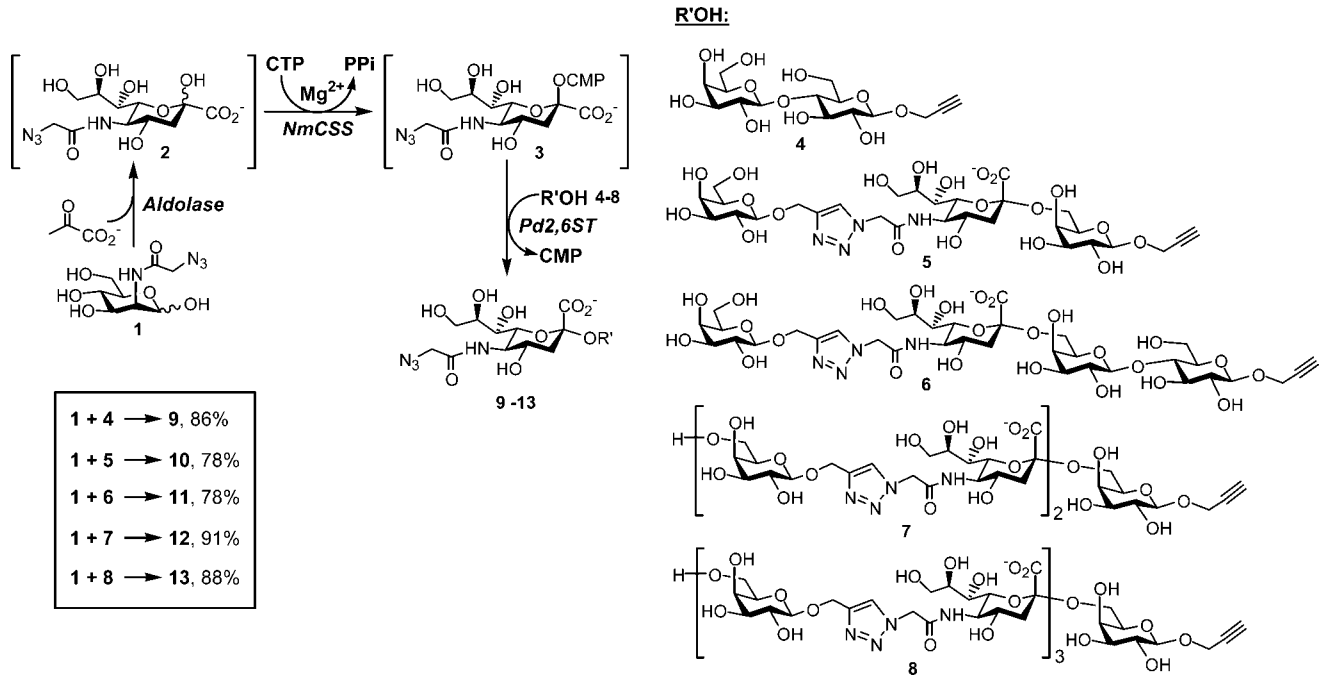
SCHEME 1. Synthesis of Acyclic Sialic Acid-Containing Oligosaccharides of Different Sizes



cyclodextrins, selective functionalization of macrocyclic carbohydrates remains challenging.

Cu(I)-catalyzed Huisgen's 1,3-dipolar cycloaddition ("click chemistry") of an azide and an alkyne has been increasingly used in the carbohydrate research field such as chemical labeling of biomolecules[15−17] as well as preparation of oligosaccharide analogues,[18,19] glycodendrimers,[20] scaffolds,[21] and carbohydrate microarrays.[22,23] The synthesis of macrocyclic carbohydrates has been recently achieved by the click chemistry of the azido and the alkyne groups presented on the same oligosaccharide molecules. Briefly, this method involves the chemical synthesis of bifunctional molecules that have an azido group at one end and a terminal alkyne group at the other end, followed by cyclodimerization of the bifunctional molecules using Cu(I)-catalyzed 1,3-dipolar cycloaddition to form triazole-containing macrocycles.[24−26] Despite the presence of mannose and triazole in the cyclodextrin analogue synthesized by this method, the obtained macrocyclic carbohydrate showed a similar binding affinity with 8-anilino-1-naphthalene sulfonate as that of β-cyclodextrin.[25]

Our experience in chemoenzymatic synthesis of sialoside derivatives and size-defined sialic acid containing polysaccharides[27−30] provides us an efficient chemoenzymatic approach to produce sialic acid containing structurally defined macrocyclic oligosaccharides of different sizes. Our strategy to obtain macrocyclic carbohydrates of different sizes is to chemoenzymatically synthesize acyclic oligosaccharides of varied lengths containing an azide and an alkyne group, respectively, at two termini, followed by the macrocycle formation using Cu(I)-catalyzed 1,3-dipolar cycloaddition of the terminal azido and alkyne groups.[31,32] The chemoenzymatic approach is superior in efficiently obtaining complex oligosaccharides of defined sizes. The presence of sialic acid residues enhances the solubility of the macrocyclic structures significantly.

Results and Discussion

The process to obtain sialic acid-containing macrocyclic carbohydrates started with chemical or chemoenzymatic synthesis of oligosaccharides containing a galactose at the nonreducing end and a propargyl group at the reducing end of **4**−**8** (Scheme 1) as sialyltransferase acceptors accordingly to the procedures reported previously.[28] Briefly, disaccharide **4** was chemically synthesized by the coupling of peracetylated lactosyl trichloroacetimidate and propargyl alcohol followed by deacetylation. Galactose-terminated trisaccharide **5** was synthesized by a recombinant *Photobacterium damsela* α2,6-sialyltransferase (Pd2,6ST)-catalyzed block transfer of a disaccharide derivative

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(AcGal-triazole-Sia).[28] Starting from *N*-azidoacetylmannosamine (ManNAz), an azido derivative of CMP-sialic acid was synthesized using a one-pot two-enzyme system followed by click chemistry to a peracetylated propargyl-β-D-galactopyranoside to give CMP-activated disaccharide (CMP-AcGal-triazole-Sia). In the presence of Pd2,6ST, AcGal-triazole-Sia was transferred from its CMP-activated form to a propargyl-β-D-galactopyranoside followed by deprotection of the acetyl groups to give trisaccharide **5**. Repeating this process using the newly formed product as a sialyltransferase acceptor resulted in the formation of pentassaccharide **7** and heptasaccharide **8**. Tetrasaccharide **6** was synthesized in the same way except that a propargyl-β-D-lactoside was used as the acceptor for the Pd2,6ST-catalyzed block transfer of the AcGal-triazole-Sia disaccharide analogue.

With propargyl-containing galactosides of different sizes **4**−**8** in hand, sialosides **9**−**13** with an azido and an alkyne group at two termini, respectively, were readily synthesized by a highly efficient chemoenzymatic sialylation approach.[27−30] In this approach, an azido-containing ManNAc derivative, ManNAz (*N*-azidoacetylmannosamine **1**), was chemically synthesized as a sialic acid precursor. ManNAz **1** was then used in a one-pot three-enzyme system[30] to produce sialosides of different sizes without the isolation of intermediates. As shown in Scheme 1, a sialic acid analogue, *N*-azidoacetylneuraminic acid (Neu5NAz) **2**, produced from ManNAz **1** catalyzed by a recombinant *Escherichia coli* K-12 sialic acid aldolase was activated in situ by a recombinant *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS) to form CMP-Neu5NAz **3**. The CMP-Neu5NAz **3** was then transferred in situ by Pd2,6ST to galactosides **4**−**8** to form different sizes of acyclic sialosides which have an azido group at one end and a terminal alkyne group at the other end (Scheme 1, **9**−**13**). The one-pot three-enzyme system was very efficient in producing sialosides **9**−**13** with excellent yields ranging from 78 to 91%. Intermediates **2** and **3** were generated in situ and were not isolated.

Macrocyclic carbohydrates **14**−**19** (Scheme 2) were prepared from azide/alkyne-bifunctionalized sialosides **9**−**13** by copper(I)-catalyzed Huisgen's 1,3-dipolar cycloaddition (click chemistry) of alkyne and azide.[31,32] The reactions were carried out at room temperature for 14−16 h in water/acetonitrile (1:1, v/v) in the presence of diisopropylethylamine (DIPEA) and CuI. Sialosides **9**−**13** were used at 1 mM for the 1,3-dipolar cycloaddition to limit the formation of polymer byproduct. As shown in Scheme 2, intramolecular cyclization of acyclic sialosides **9**, **12**, and **13** led to the formation of macrocyclic trisaccharide **14** (91%), hexasaccharide **17** (70%), and octasaccharide **18** (70%), respectively. Treatment of acyclic tetrasaccharide **10** with CuI and DIPEA afforded the desired macrocyclic tetrasaccharide **15** in 63% yield and macrocyclic octasaccharide **18** in 31% yield. Similarly, cycloaddition of acyclic pentasaccharide **11** resulted in a macrocyclic pentasaccharide **16** (59% yield) and a macrocyclic decasaccharide **19** (30% yield). These macrocyclic carbohydrates were characterized by NMR, MALDI-TOF, and high-resolution mass spectrometry (HRMS). Other byproducts of the cycloaddition reactions were not isolated.

Small-scale cyclization reactions tested using increased concentrations (5 and 10 mM) of monomers showed the formation of multiple products of larger sizes. In contrast to the α2,6-linked sialotrisaccharide **9**, which formed a cyclic monomer after click when 1 mM concentration of acyclic monomer was used as the starting material, the click of an α2,3-linked sialotrisaccharide (1 mM) synthesized using a similar method as described in Scheme 1, except that the Pd2,6ST was replaced by a multifunction *Pasteurella multocida* α2,3-sialyltransferase (PmST1),[29,33,34] gave mainly a cyclic dimer (data not shown). These experimental results indicate that the ring size of macrocyclic oligosaccharides formed may be dependent on the concentration, the length, and the stereochemistry or topology of the acyclic oligosaccharide substrates. For all of the oligosaccharides examined here, the click macrocyclization favored the formation of cyclic monomers over cyclic dimers when the acyclic oligosaccharide monomers were used at a concentration of 1 mM. For the acyclic trisaccharide **9**, hexasaccharide **12**, and octasaccharide **13**, cyclic monomers were the only products isolated. In the case of acyclic tetrasaccharide **10** and pentasaccharide **11**, the ratio of cyclic monomer versus cyclic dimer formed was around 2:1. This is different from what has been previously reported for the head-to-tail cyclodimerization of oligosaccharides[25] and peptides.[35,36] The formation of cyclic monomers has also been reported for the synthesis of polymers via click chemistry.[37,38] The difference may be largely due to the conformational preferences and the concentrations of these compounds.

Comparing the ^{1}H NMR spectra of acyclic oligosaccharides (Figure 1) and cyclic oligosaccharides (Figure 2), it is apparent that macrocyclic carbohydrates **15** (cyclic tetrasaccharide), **17** (cyclic hexasaccharide), and **18** (cyclic octasaccharide) are C_2-, C_3-, and C_4-symmetric, respectively. In the ^{1}H NMR spectra of acyclic tetrasaccharide **10**, hexasaccharide **12**, and octasaccharide **13**, a peak is shown for each triazole proton, while only a single peak is shown for all of the triazole protons in the cyclic products of these oligosaccharides. In comparison, macrocyclic pentasaccharide **16** has two triazole proton peaks, one for each triazole. It is also important to note that there is significant downfield shift of triazole protons upon macrocyclization. The triazole protons in the acyclic oligosaccharides have a chemical shift range of 7.9−8.2 ppm, where the macrocyclic oligosaccharides are more deshielded (8.2−8.4 ppm). The protons on carbon 3 in the sialic acids of the acyclic and cyclic oligosaccharides also indicate the presence of the symmetry of macrocyclic carbohydrates **15**, **17**, and **18**. For example, the equatorial protons on C3 (2.75 ppm) are shown as multiplets for acyclic oligosaccharides (**10**, **12**, **13**), but double doublets for the cyclic oligosaccharides (**15**, **17**, **18**). Similarly, the axial protons on C3 ($\sim$1.70 ppm) are shown as multiplets for acyclic oligosaccharides but triplets for cyclic oligosaccharides (**15**, **17**, **18**).

The chemoenzymatic synthetic approach described above is highly efficient for the preparation of macrocyclic carbohydrates of different sizes in a controlled manner from simple monosaccharide and disaccharide derivatives. For application purposes, it is important to determine whether these macrocycles can be used as potential host molecules.

Cyclodextrins and their analogues are known to form inclusion complexes with a number of hydrophobic molecules. For

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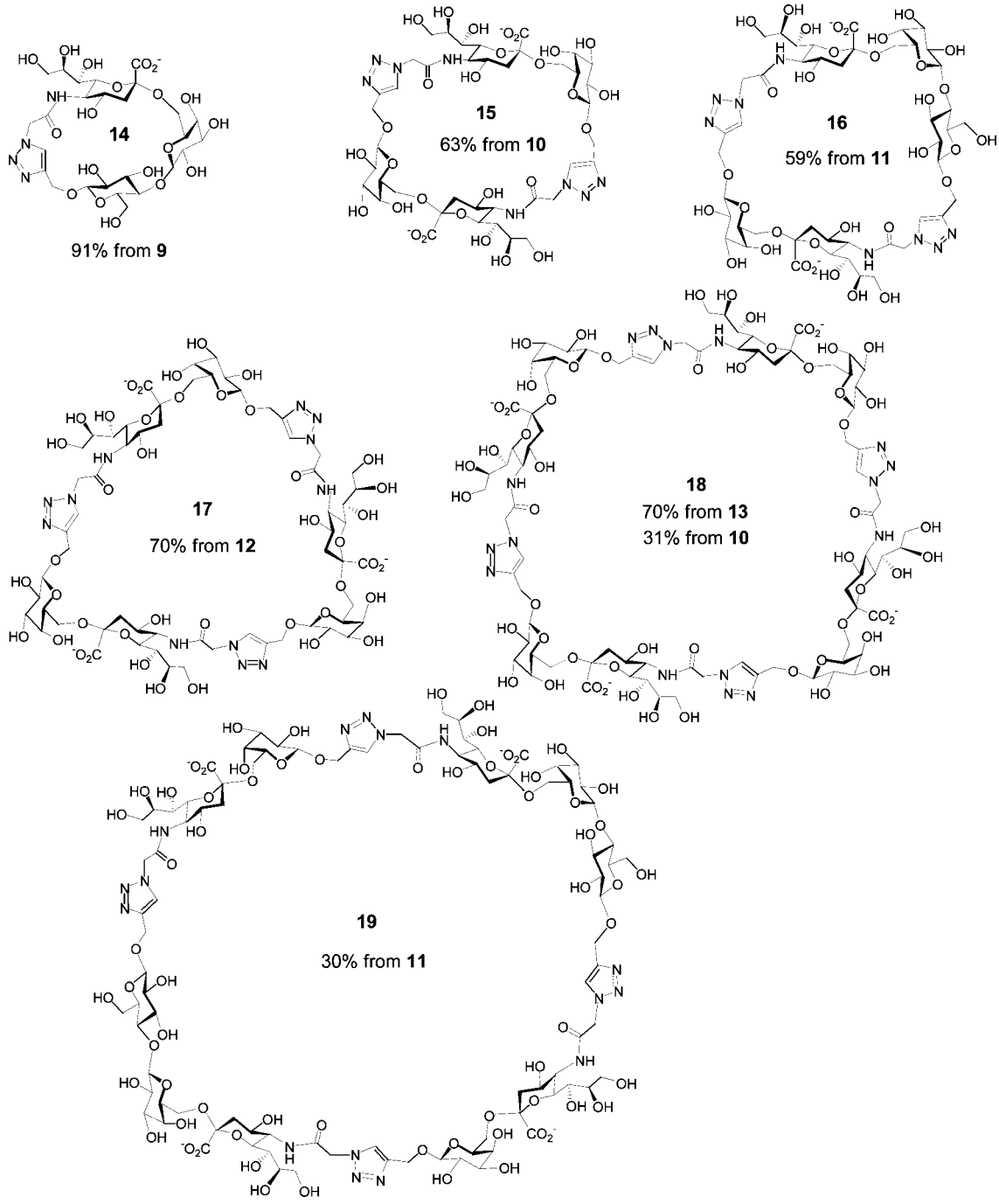
SCHEME 2. Macrocyclic Carbohydrates Synthesized by Cu(I)-Catalyzed 1,3-Dipolar Cycloaddition Reactions



example, β-CD has been reported to be able to form an inclusion complex with *para*-methylbenzoate (*p*MB), *para-tert*-butylbenzoate (*pt*BB), and *para*-toluenesulfonate (*p*TS) (Scheme 3).[39−41] We tested the ability of the synthetic macrocyclic carbohydrates in forming complexes with these compounds using capillary electrophoresis (CE) analysis. The binding constants of these compounds and the macrocyclic carbohydrates were estimated and compared to those obtained with β-CD. It is important to note that all macrocyclic oligosaccharides reported here have high solubility (>200 mM) in aqueous solutions in comparison to β-CD (16 mM) at 25 °C.

There are a number of CE methods that have been used to estimate the binding constants of host and guest molecules.[42,43] Here we use the affinity capillary electrophoresis (ACE) method,[39,44,45] which is the most commonly used CE method for estimating binding constants. In order to validate the ACE method, the binding constants of β-CD with *p*MB, *p*TS, and *pt*BB were measured and calculated. The concentration of analytes was kept constant (0.25 mM), while the concentrations of β-CD were varied (1.00−8.00 mM). The binding constants were obtained by nonlinear regression analysis of the data using

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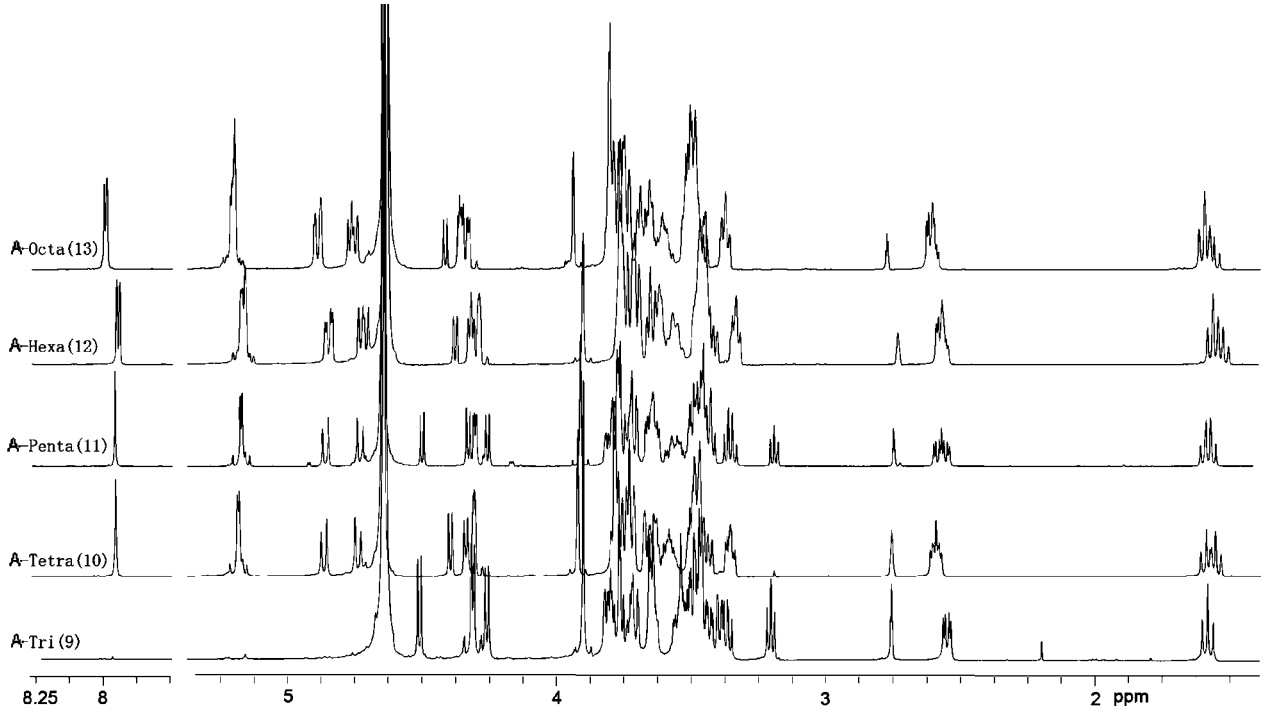

FIGURE 1. [1]H NMR of acyclic oligosaccharides with an azide group at one end and a terminal alkyne group at the other end: A-Octa (13), acyclic octasaccharide **13**; A-Hexa (12), acyclic hexasaccharide **12**; A-Penta (11), acyclic pentasaccharide **11**; A-Tetra (10), acyclic tetrasaccharide **10**; and A-Tri (9), acyclic trisaccharide **9**.

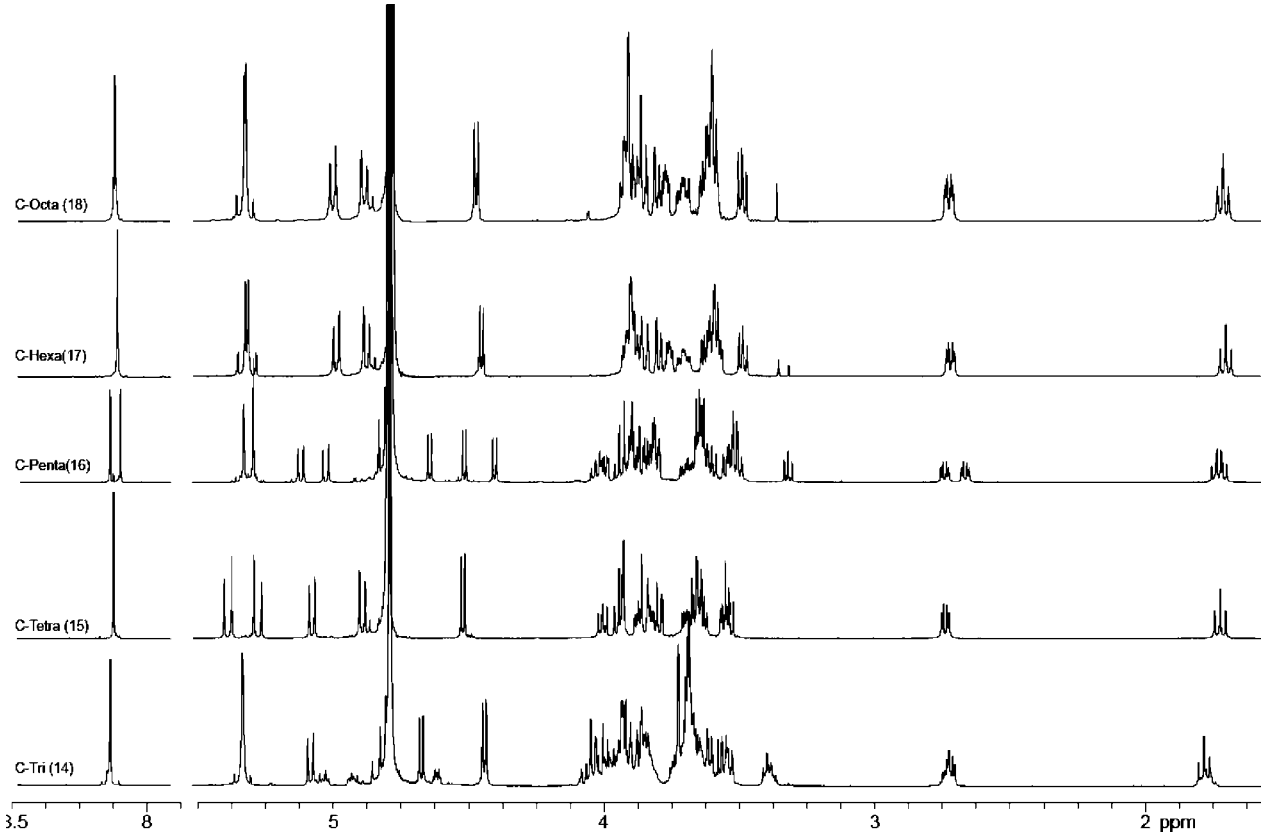

FIGURE 2. [1]H NMR of macrocyclic oligosaccharides: C-Octa (18), cyclic octasaccharide **18**; C-Hexa (17), cyclic hexasaccharide **17**; C-Penta (16), cyclic pentasaccharide **16**; C-Tetra (15), cyclic tetrasaccharide **15**, and C-Tri (14), cyclic trisaccharide **14**.

the GraFit 5 software. As summarized in Table 1, the binding constants obtained are in good agreement with those reported.[39−41]

Using the same method as described above for β-CD, the binding constants of macrocyclic carbohydrates **14**−**18** and *p*MB (Figure 3b), *p*TS (Figure 3c), and *pt*BB (Figure 3d) were obtained. For comparison, the binding constant for the acyclic tetrasaccharide derivative **10** was also attained. The preliminary experiments using ACE were encouraging and showed interac-

SCHEME 3. Structures of *para*-Methylbenzoate (*p*MB), *para*-Toluenesulfonate (*p*TS), and *para-tert*-Butylbenzoate (*pt*BB)

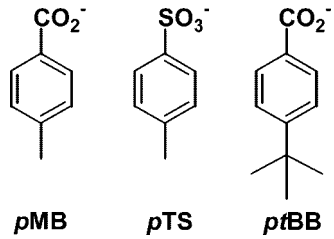

TABLE 1. Binding Constants of Macrocyclic or Acyclic Carbohydrates and *p*MB, *p*TS, or *pt*BB Obtained by Affinity Capillary Electrophoresis (ACE)

K_a (M^{-1})	*p*MB	*p*TS	*pt*BB
β-CD reported	66−110[40,41]	95 ± 6[39]	18400[40]
β-CD measured	81 ± 7	84 ± 2	$(1.0 \pm 0.1) \times 10^4$
cyclic-octasac **18**	$(6.3 \pm 0.4) \times 10^2$	$(1.9 \pm 0.1) \times 10^2$	$(7.7 \pm 1.2) \times 10^2$
cyclic-hexasac **17**	$(4.3 \pm 0.3) \times 10^2$	80 ± 5	$(3.6 \pm 0.1) \times 10^2$
cyclic-pentasac **16**	$(1.0 \pm 0.1) \times 10^2$	13 ± 4	$(2.0 \pm 0.1) \times 10^2$
cyclic-tetrasac **15**	$(1.6 \pm 0.2) \times 10^2$	56 ± 4	$(1.3 \pm 0.2) \times 10^2$
cyclic-trisac **14**	$(2.4 \pm 0.3) \times 10^2$	24 ± 11	$(1.0 \pm 0.2) \times 10^2$
acyclic-tetrasac **10**	31 ± 8	45 ± 11	54 ± 20

tions between the macrocyclic carbohydrates and the hydrophobic small molecules. From Table 1, we can see clearly that macrocyclic carbohydrates **14**−**18** synthesized here have the ability to form complexes with hydrophobic molecules such as *p*MB and *pt*BB. In general, the binding affinity tends to increase when the size of the macrocyclic carbohydrate increases. Apparently, *p*TS is a weak guest molecule for β-CD and the macrocyclic oligosaccharides **14**−**17**. This may be due to its high solubility in aqueous solution. Among the macrocyclic carbohydrates tested, macrocyclic octasaccharide **18** has the strongest binding for *p*TS. Overall, compared to β-CD, macrocyclic carbohydrates **14**−**18** have a better binding affinity to guest molecule *p*MB, but a weaker binding affinity to a bigger guest molecule *pt*BB. This indicates that macrocyclic carbohydrates **14**−**18** may have different types of cavity or binding mode compared to β-CD. The binding constants of acyclic tetrasaccharide **10** to *p*MB and *pt*BB are lower than those of the macrocyclic tetrasaccharide **15**, indicating that the cyclic structure of macrocyclic carbohydrates does contribute to their increased interaction with guest molecules.

To investigate the mode of interaction of macrocyclic oligosaccharides and hydrophobic molecules, fluorescence emission spectra of 8-anilino-1-naphthalenesulfonate (ANS) or fluorescein isothiocyanate (FITC) in the presence and the absence of β-CD, α-CD, cyclic oligosaccharides, or acyclic oligosaccharides were measured and compared (Figure 3). ANS has been shown to form complexes with cyclodextrin and its analogues.[25] FITC is commonly used in biological studies and exists in different protolytic forms in aqueous solutions of different pH values.[46,47] At pH 3.0, the fluorescence intensities of ANS increased significantly with the addition of β-CD. In comparison, the fluorescence intensities only increased slightly with the presence of α-CD or macrocyclic oligosaccharides. Interestingly, in the case of FITC, the fluorescence intensities increased considerably upon the addition of α-CD and moderately with macrocyclic oligosaccharides but decreased slightly in the presence of β-CD. The fluorescence emission spectra of FITC for acyclic oligosaccharides were also obtained. Under the pH condition used, the FITC should mainly be in the neutral form.

The concentration of FITC used for all the measurements was 0.01 mM, and the concentrations of β-CD, α-CD, cyclic oligosaccharides, and acyclic oligosaccharides were 5.0 mM. The excitation wavelength of FITC was set at 440 nm. The changes in the fluorescence intensity of FITC upon the addition of β-CD, α-CD, cyclic oligosaccharides, and acyclic oligosaccharides are illustrated in Figure 3. The fluorescence intensity of FITC was slightly lower in the presence of β-CD and was increased more than two-fold in the presence of α-CD. Among the cyclic oligosaccharides, macrocyclic octasaccharide **18** had the strongest fluorescence signal enhancement for FITC, which was followed by cyclic hexasaccharide **17**. Macrocyclic pentasaccharide **16** and tetrasaccharide **15** had similar effect, and cyclic trisaccharide had the weakest enhancement. As controls, the fluorescence spectra of β-CD, α-CD, and cyclic oligosaccharides were measured, which all displayed similar spectra to that of the background spectrum of the phosphate solution used for the measurements.

For the acyclic oligosaccharides, the fluorescence intensity of FITC was increased by more than two-fold in the presence of acyclic octasaccharide **13**. This considerable increase in the fluorescence intensity of FITC may be due to the open cavity and more flexible structure that acyclic octasaccharides have. The fluorescence intensity of FITC increased slightly in the presence of acyclic hexasaccharide **12** and tetrasaccharide **10** and decreased slightly in the presence of acyclic pentasaccharide **11** and trisaccharide **9**. The increase in the fluorescence of FITC by cyclic oligosaccharides and some acyclic oligosaccharides indicates the ability of these molecules to interact with hydrophobic molecules. It is worth noting that, at pH 7.4, only small increases of the fluorescence intensity were seen for ANS and FITC upon the addition of the macrocyclic oligosaccharides.

The fluorescence measurement data indicate that some of the macrocyclic oligosaccharides and acyclic oligosaccharides form inclusion complex-like structures with FITC at pH 3. Acyclic octasaccharide **13** may have a more suitable cavity than cyclic octasaccharide **18** in forming inclusion complexes with FITC at pH 3.

In conclusion, we have demonstrated a novel and highly efficient chemoenzymatic method for the preparation of size-defined macrocyclic carbohydrates. Chemoenzymatic approach reduces the use of protecting groups, shortens the synthetic routes, and enables the utilization of mild conditions to carry out the reactions. These macrocyclic carbohydrates tend to bind to hydrophobic molecules with size-dependent affinities. Negatively charged sialic acid residues in these macrocyclic carbohydrates may contribute to their high solubility in aqueous solution.

Experimental Section

Propargyl-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (Propargyllactose) (4). Lactosyl trichloroacetimidate (3.13 g, 4.01 mmol) was dissolved in anhydrous CH_2Cl_2 (40 mL) under argon. Then propargyl alcohol (2.4 mL, 40.7 mmol) was added, and the mixture was stirred for 30 min. TMSOTf (0.15 mL, 0.83 mmol) diluted with CH_2Cl_2 (1 mL) was slowly added at −40 °C, then allowed to warm up to room temperature and stirred overnight. Et_3N (0.3 mL) was added to the mixture and allowed to stir for 30 min. The mixture was filtered over Celite and concentrated in vacuo. The residue was dissolved in MeOH (25 mL) containing a catalytic

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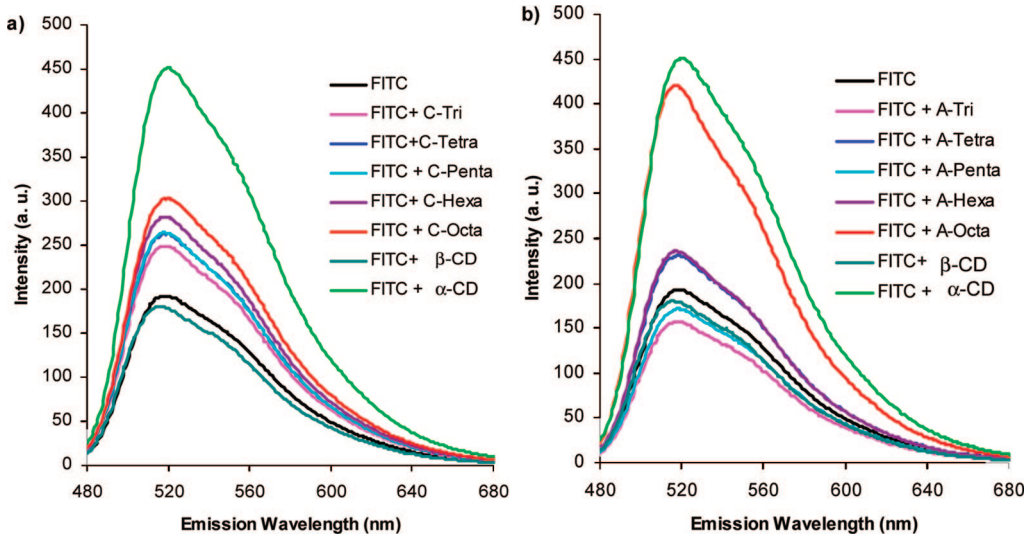

FIGURE 3. Fluorescence emission spectra of FITC (0.01 mM) in the presence and absence of β-CD, α-CD, cyclic oligosaccharides, and acyclic oligosaccharides (5.0 mM).

amount of NaOMe. The mixture was stirred at room temperature overnight. The reaction mixture was then neutralized with DOWEX HCE-W2 (H^+) resin, filtered, and concentrated to give propargyllactose **4** as a white solid (1.37 g, 90%): ^{1}H NMR (300 MHz, D_2O) δ 4.51 (d, 1H, J = 7.8 Hz), 4.32 (t, 2H, J = 2.1 Hz), 4.28 (d, 1H, J = 7.8 Hz), 3.82 (dd, 1H, J = 1.8 and 12.6 Hz), 3.76 (d, 1H, J = 3.3 Hz), 3.67−3.46 (m, 8H), 3.38 (dd, 1H, J = 7.5 and 9.9 Hz), 3.18 (m, 1H), 2.77 (t, 1H, J = 2.7 Hz); ^{13}C NMR (75 MHz, D_2O) δ 103.1, 100.5, 78.9, 78.4, 76.6, 75.5, 75.0, 74.5, 72.7, 72.6, 71.1, 68.7, 61.2, 60.1, 56.8; MALDI (TOF) *m/z* calcd for $C_{15}H_{24}NaO_{11}$ (M + Na) 403.1, measured 403.1.

Synthesis of Tetrasaccharide (6). Tetrasaccharide **6** was prepared from a CMP-activated disaccharide and propargyl-β-D-lactopyranoside **4** in the presence of *Photobacterium damsela* α2,6-sialyltransferase (Pd2,6ST) as previously reported:[28] Yield, 72%; white foam; ^{1}H NMR (600 MHz, D_2O) δ 8.10 (s, 1H), 5.29 (s, 2H), 4.99 (d, J = 12.6 Hz, 1H), 4.87 (d, J = 12.0 Hz, 1H), 4.63 (d, J = 7.8 Hz, 1H), 4.47−4.39 (m, 5H), 3.96−3.55 (m, 23H), 3.50 (t, J = 7.8 Hz, 2H), 2.88 (t, J = 2.4 Hz, 1H), 2.69 (dd, J = 4.8 and 12.6 Hz, 1H), 1.73 (t, J = 12.0 Hz, 1H); ^{13}C NMR (150 MHz, D_2O) δ 173.4, 168.5, 143.9, 127.0, 103.4, 102.0, 100.3, 79.6, 78.8, 76.5, 75.4, 74.8, 74.7, 73.8, 72.8, 72.6, 72.5, 72.4, 71.9, 70.9, 70.8, 68.7, 68.43, 68.36, 63.7, 62.7, 61.9, 61.1, 60.3, 56.6, 52.2, 52.2, 40.2; HRMS (ESI) *m/z* calcd for $C_{35}H_{54}N_4NaO_{25}$ (M + H) 953.2969, measured 953.2969.

General Procedures for Preparation of α2,6-Linked Acyclic Sialosides (9−13) in a One-Pot Three-Enzyme System. The one-pot three-enzyme reactions were carried out as previously reported.[29,30] All reactions were carried out in a 50 mL centrifuge tube in 5−10 mL of Tris-HCl buffer (100 mM, pH 8.5) containing a sialyltransferase acceptor substrate (galactoside) (50−150 mg), *N*-azidoacetylmannosamine (**1**) as a donor (1.5 equiv), sodium pyruvate (5 equiv), CTP (1.5 equiv), and $MgCl_2$ (20 mM). Before adding the enzymes, the pH of the mixtures was adjusted to 8.5 with 2 M NaOH. The appropriate amount of aldolase, NmCSS, and Pd2,6ST were added. The reaction mixtures were incubated at 37 °C for 3 h with agitating at 120 rpm. The reactions were monitored by TLC using EtOAc/MeOH/H_2O/HOAc = 5:2:1:0.2 (v/v) or *n*-PrOH/H_2O/NH_4OH = 7:2:1 to 4:2:1 (v/v) and the *p*-anisaldehyde stain solution. The reaction mixtures were quenched with equal volume of 95% EtOH and centrifuged to remove insoluble precipitates. The supernatants were concentrated and filtered through a silica gel column and a BioGel P-2 gel filtration column to obtain the desired product. The sialoside products were characterized by ^{1}H NMR, ^{13}C NMR, MALDI-TOF, and high resolution mass spectrometry (HRMS).

Acyclic Trisaccharide (9). This compound was prepared from propargyllactose **4** with *N*-azidoacetyl-D-mannosamine **1** in a one-pot three-enzyme system as described above to give **9** as a white solid (0.132 g, 86%): ^{1}H NMR (600 MHz, D_2O) δ 4.51 (d, 1H, J = 7.8 Hz), 4.31 (d, 2H, J = 6.0 Hz), 4.26 (d, 1H, J = 7.8 Hz), 3.90 (s, 2H), 3.82−3.63 (m, 8H) 3.57−3.35 (m, 10H), 3.20 (t, 1H, J = 9.0 Hz), 2.76 (t, 1H, J = 2.4 Hz), 2.55 (dd, 1H, J = 4.2 and 12.0 Hz), 1.58 (t, 1H, J = 12.6 Hz); ^{13}C NMR (150 MHz, D_2O) δ 173.6, 171.2, 103.4, 100.43, 100.38, 79.7, 78.9, 76.5, 74.9, 74.7, 73.8, 72.7, 72.5, 72.3, 72.0, 70.9, 68.6, 68.5, 68.3, 63.7, 62.7, 60.3, 56.7, 52.1, 52.0, 40.3; HRMS (ESI) *m/z* calcd for $C_{26}H_{40}N_4NaO_{19}$ (M + H) 735.2179, measured 735.2168.

Acyclic Tetrasaccharide (10). Compound **10** was synthesized from *N*-azidoacetyl-D-mannosamine **1** and trisaccharide **5** in a similar method described for preparing **9**: Yield, 78%; white foam; ^{1}H NMR (600 MHz, D_2O) δ 8.11 (s, 1H), 5.33 (d, J = 3.6 Hz, 2H), 5.01 (d, J = 12.6 Hz, 1H), 4.88 (d, J = 12.6 Hz, 1H), 4.54 (d, J = 7.8 Hz, 1H), 4.48 (d, J = 7.8 Hz, 1H), 4.46−4.45 (m, 2H), 4.06 (s, 2 H), 3.94−3.56 (m, 24H), 3.51−3.48 (m, 2H), 2.89 (t, J = 2.4 Hz, 1H), 2.75−2.71 (m, 2H), 1.74−1.67 (m, 2H); ^{13}C NMR (150 MHz, D_2O) δ 173.64, 173.58, 171.4, 168.7, 143.9, 127.0, 102.2, 101.4, 100.64, 100.61, 79.1, 76.4, 73.70, 73.68, 72.7, 72.6, 72.48, 72.45, 71.97, 71.96, 70.7, 70.6, 68.74, 68.71, 68.32, 68.26, 68.2, 63.5, 63.5, 62.8, 62.7, 62.2, 57.0, 52.3, 52.2, 52.1, 52.1, 40.3; HRMS (ESI) *m/z* calcd for $C_{40}H_{59}N_8Na_2O_{28}$ (M + H) 1145.3229, measured 1145.3229.

Acyclic Pentasaccharide (11). Compound **11** was synthesized from *N*-azidoacetyl-D-mannosamine **1** and tetrasaccharide **6** in a similar method described for preparing **9**: Yield, 78%; white foam; ^{1}H NMR (600 MHz, D_2O) δ 8.11 (s, 1H), 5.32 (d, J = 4.2 Hz, 2H), 5.01 (d, J = 12.6 Hz, 1H), 4.88 (d, J = 12.6 Hz, 1H), 4.65 (d, J = 8.4 Hz, 1H), 4.48−4.40 (m, 4H), 4.06 (s, 2 H), 3.96−3.56 (m, 30 H), 3.52−3.48 (m, 2H), 3.34 (t, J = 9.0 Hz, 1H), 2.89 (t, J = 2.4 Hz, 1H), 2.74−2.69 (m, 2H), 1.75−1.69 (m, 2H); ^{13}C NMR (150 MHz, D_2O) δ 173.62, 173.60, 171.3, 168.7, 143.9, 127.0, 103.4, 102.2, 100.6, 100.46, 100.37, 79.6, 78.8, 76.5, 74.8, 74.7, 73.8, 73.7,72.6, 72.5, 72.44, 72.38, 72.0, 70.9, 70.7, 68.7, 68.6, 68.6, 68.4, 68.3, 68.2, 63.7, 63.5, 62.8, 62.7, 62.1, 60.3, 56.7, 52.3, 52.2, 52.1, 52.0, 40.3, 40.2; HRMS (ESI) *m/z* calcd for $C_{46}H_{69}N_8Na_2O_{33}$ (M + H) 1307.3757, measured 1307.3757.

Acyclic Hexasaccharide (12). Compound **12** was synthesized from *N*-azidoacetyl-D-mannosamine **1** and pentasaccharide **7** in a similar method described for preparing **9**: Yield, 91%; white foam; ^{1}H NMR (600 MHz, D_2O) δ 8.11 (s, 1H), 8.10 (s, 1H), 5.33−5.32 (m, 4H), 5.00 (dd, J = 4.2 and 13.2 Hz, 2H), 4.89−4.86 (m, 3H), 4.53 (d, J = 7.8 Hz, 1H), 4.49−4.44 (m, 4H), 4.06 (s, 2H),

3.92−3.56 (m, 35H), 3.49−3.47 (m, 3H), 2.88 (t, J = 2.4 Hz, 1H), 2.74−2.70 (m, 3 H), 1.73−1.66 (m, 3H); ^{13}C NMR (150 MHz, D_2O) δ 173.7, 173.61, 173.59, 171.3, 168.7, 143.88, 143.86, 127.0, 102.2, 102.2, 101.4, 100.7, 100.62, 100.59. 79.1, 76.4, 73.69, 73.68, 73.66, 72.7, 72.6, 72.5, 72.4, 72.0, 71.9, 70.7, 70.6, 68.73, 68.70, 68.4, 68.31, 68.27, 68.24, 68.21, 63.51, 63.48, 62.8, 62.7, 62.7, 62.2, 62.1, 57.0, 52.3, 52.2, 52.1, 52.0, 40.3, 40.2; HRMS (ESI) m/z calcd for $C_{60}H_{87}N_{12}Na_4O_{42}$ (M + Na) 1739..4626, measured 1739.4548.

Acyclic Octasaccharide (13). Compound **13** was synthesized from *N*-azidoacetyl-D-mannosamine **1** and heptasaccharide **8** in a similar method described for preparing **9**: Yield, 88%; white foam; ^{1}H NMR (600 MHz, D_2O) δ 8.15−8.14 (m, 3H), 5.37−5.35 (m, 6H), 5.06−5.03 (m, 3H), 4.93−4.90 (m, 4H), 4.57 (d, J = 7.8 Hz, 1H), 4.52−4.48 (m, 5H), 4.10 (s, 2H), 3.97−3.60 (m, 47H), 3.52−3.51 (m, 4H), 2.93 (t, J = 2.4 Hz, 1H), 2.79−2.74 (m, 4H), 1.77−1.70 (m, 4H); ^{13}C NMR (150 MHz, D_2O) δ 173.7, 173.6, 171.4, 168.7, 143.9, 127., 102.3, 102.23, 102.22, 101.5, 100.72, 100.69, 79.2, 76.4, 73.74, 73.73, 73.70, 72.72, 72.69, 72.53, 72.49, 72.01, 72.02, 70.8, 70.7, 68.78, 68.75, 68.4, 68.34, 68.31, 68.3, 63.6, 63.5, 62.82, 62.79, 62.76, 62.2, 62.1, 57.0, 52.4, 52.3, 52.14, 52.10, 40.34, 40.32, 40.28; HRMS (ESI) m/z calcd for $C_{80}H_{117}N_{16}Na_4O_{56}$ (M + H) 2289.6385, measured 2289.6297.

General Procedures for Preparing Macrocyclic Sialosides (14−18) via Click Chemistry. All the reactions were carried out in CH_3CN/H_2O (1:1, v/v) at room temperature. The sialoside product (50−150 mg) was first dissolved in water. Equal volume of acetonitrile was added followed by adding diisopropylethylamine (1 equiv), and the solution was mixed for 5 min. Copper(I) iodide powder (0.3−1.0 equiv) was added to the mixture, and the solution was stirred at room temperature for 14−16 h. The reaction was monitored by TLC using n-PrOH/H_2O/NH_4OH = 7:2:1−4:2:1 (v/v) as developing solvent and p-anisaldehyde stain solution for sugar staining. The mixture was filtered over Celite and concentrated in vacuo. The residues separated and purified by silica gel flash chromatography followed by a BioGel P-2 filtration column. The products were then characterized by ^{1}H NMR, ^{13}C NMR, and HRMS. The specific rotations of macrocyclic carbohydrates **14−18** were also measured.

Synthesis of Macrocyclic Trisaccharide (14). Macrocyclic trisaccharide was synthesized from acyclic trisaccharide **9** by Huisgen's 1,3-dipolar cycloaddition as described above to give **14**: Yield, 91%; ^{1}H NMR (300 MHz, D_2O) δ 8.10 (s, 1H), 5.30 (s, 2H), 5.05 (d, 1H, J = 9.4 Hz), 4.64 (d, 1H, J = 6.0 Hz), 4.41 (d, 1H, J = 6.0 Hz), 4.05−3.36 (m, 19H), 3.36 (t, 1H, J = 6.0 Hz), 2.76 (t, 1H, J = 6.0 Hz), 2.69 (dd, 1H, J = 3.6 and 9.0 Hz), 1.75 (t, 1H, J = 9.3 Hz); ^{13}C NMR (150 MHz, D_2O) δ 173.5, 168.3, 143.6, 127.0, 103.4, 101.4, 100.4, 80.1, 74.83, 74.80, 74.2, 72.8, 72.44, 72.38, 72.0,70.9, 69.7, 69.0, 68.8, 68.7, 62.8, 61.8, 52.1, 52.0, 40.4; $[\alpha]^{25}_D$ = −11.8 (c = 3.9 H_2O); HRMS (ESI) m/z calcd for $C_{26}H_{39}N_4O_{19}$ (M) 711.2209, measured 711.2194.

Synthesis of Macrocyclic Tetrasaccharide (15). Macrocyclic tetrasaccharide **15** was synthesized from acyclic tetrasaccharide **10** in a similar method described for preparing **14**: Yield, 63%; white foam; ^{1}H NMR (600 MHz, D_2O) δ 8.12 (s, 2H), 5.38 (d, J = 16.8 Hz, 2H), 5.27 (d, J = 16.8 Hz, 2H), 5.07 (d, J = 12.6 Hz, 2H), 4.89 (d, J = 12.6 Hz, 2H), 4.51 (d, J = 7.8 Hz, 2H), 4.40−3.51 (m, 26H), 2.73 (dd, J = 4.2 and 12.0 Hz, 1H), 1.71 (t, J = 12.0 Hz, 1H); ^{13}C NMR (75 MHz, D_2O) δ 173.8, 168.5, 144.1, 126.8, 101.9, 100.6, 74.1, 72.8, 72.7, 72.1, 70.9, 69.0, 68.6, 68.3, 64.2, 62.7, 62.3, 52.4, 52.2, 40.6; $[\alpha]^{25}_D$ = −19.2 (c = 5.6 H_2O); HRMS (ESI) m/z calcd for $C_{40}H_{58}N_8Na_3O_{28}$ (M + Na) 1167.3054, measured 1167.3091.

Synthesis of Macrocyclic Pentasaccharide (16). Compound **16** was synthesized from acyclic pentasaccharide **11** in a similar method described for preparing **14**: Yield, 59%; white foam; ^{1}H NMR (600 MHz, D_2O) δ 8.14 (s, 1H), 8.12 (s, 1H), 5.33 (s, 2H), 5.30 (s, 2H), 5.12 (d, J = 11.4 Hz, 1H), 5.03 (d, J = 12.6 Hz, 1H), 4.65 (d, J = 7.8 Hz, 1H), 4.52 (d, J = 7.8 Hz, 1H), 4.41 (d, J = 8.4 Hz, 1H), 4.05−3.80 (m, 17H), 3.72−3.50 (m, 16H), 3.32 (t, J = 8.4 Hz, 1H), 2.75 (dd, J = 4.2 and 12.6 Hz, 1H), 2.67 (dd, J = 4.8 and 12.6 Hz, 1H), 1.76−1.71 (m, 2H); ^{13}C NMR (150 MHz, D_2O) δ 173.6, 173.5, 168.6, 168.3, 144.2, 127.1, 126.7, 105.0, 103.5, 102.1, 101.4, 100.4, 80.4, 74.7, 74.2, 74.1, 72.7, 72.7, 72.5, 72.4, 72.3, 72.0, 70.8, 70.8, 69.8, 69.7, 69.2, 68.9, 68.7, 68.6, 68.4, 68.3, 64.6, 62.7, 62.2, 61.9, 60.4, 52.3, 52.2, 40.6, 40.3; $[\alpha]^{25}_D$ = −16.8 (c = 5.1 H_2O); HRMS (ESI) m/z calcd for $C_{46}H_{68}N_8Na_3O_{33}$ (M + Na) 1329.3582, measured 1329.3548.

Synthesis of Macrocyclic Hexasaccharide (17). Compound **17** was synthesized from acyclic hexasaccharide **12** in a similar method described for preparing **14**: Yield, 70%; white foam; ^{1}H NMR (600 MHz, D_2O) δ 8.11 (s, 3H), 5.33 (d, J = 6.6 Hz, 6H), 5.00 (d, J = 12.6 Hz, 3H), 4.89 (d, J = 12.6 Hz, 3H), 4.46 (d, J = 8.4 Hz, 3H), 3.94−3.79 (m, 23H), 3.65−3.57 (m, 13 H), 3.51−3.48 (m, 3H), 2.73 (dd, J = 4.8 and 12.6 Hz, 3H), 1.72 (t, J = 12.0 Hz, 3H); ^{13}C NMR (150 MHz, D_2O) δ 173.6, 168.6, 143.7, 127.1, 102.0, 100.6, 73.8, 72.7, 72.5, 72.0, 70.7, 68.8, 68.4, 68.3, 63.7, 62.7, 61.9, 52.3, 52.2, 40.3; $[\alpha]^{25}_D$ = −10.8 (c = 5.6 H_2O); HRMS (ESI) m/z calcd for $C_{60}H_{89}N_{12}Na_3O_{42}$ (M + 2H) 1718.4890, measured 1718.4780.

Synthesis of Macrocyclic Octasaccharide (18). Compound **18** was synthesized from acyclic octasaccharide **13** in a similar method described for preparing **14**: Yield, 78%; white foam. Compound **18** was also isolated from the cyclodimerization of acyclic tetrasaccharide **10** in 31% yield: ^{1}H NMR (600 MHz, D_2O) δ 8.12 (s, 4H), 5.33 (d, J = 3.6 Hz, 8H), 5.00 (d, J = 12.6 Hz, 4H), 4.89 (d, J = 12.6 Hz, 4H), 4.48 (d, J = 7.8 Hz, 4H), 3.94−3.58 (m, 48H), 3.51−3.48 (m, 4H), 2.74 (dd, J = 4.2 and 12.6 Hz, 4H), 1.72 (t, J = 12.0 Hz, 4H); ^{13}C NMR (150 MHz, D_2O) δ 173.6, 168.6, 143.9, 127.1, 102.2, 100.7, 73.7, 72.7, 72.5, 72.0, 70.7, 68.8, 68.4, 68.3, 63.6, 62.7, 62.1, 52.4, 52.3, 40.3; $[\alpha]^{25}_D$ = −12.4 (c = 3.6 H_2O); HRMS (ESI) m/z calcd for $C_{80}H_{118}N_{16}Na_4O_{56}$ (M + 2H) 2290.6468, measured 2290.6368.

Synthesis of Macrocyclic Decasaccharide (19). Compound **19** was obtained from head-to-tail cyclodimerization of acyclic pentasaccharide **11**: Yield, 30%; white foam; ^{1}H NMR (600 MHz, D_2O) δ 8.11 (s, 2H), 8.10 (s, 2H), 5.31 (s, 8H), 5.00 (d, J = 12.6 Hz, 4H), 4.87 (m, 4H), 4.58 (d, J = 7.8 Hz, 2H), 4.47 (d, J = 7.8 Hz, 2H), 4.40 (d, J = 7.8 Hz, 2H), 3.99−3.47 (m, 62H), 3.34 (t, J = 8.4 Hz, 2H), 2.75−2.68 (m, 4H), 1.75−1.71 (m, 4H); ^{13}C NMR (150 MHz, D_2O) δ 173.6, 168.6, 168.5, 143.9, 143.8, 127.0, 127.0, 103.4, 102.2, 101.4, 100.6, 100.4, 79.8, 74.0, 74.7, 73.9, 73.7, 72.8, 72.7, 72.5, 72.4, 72.0, 70.9, 70.7, 68.8, 68.5, 68.4, 68.3, 63.9, 63.6, 62.8, 62.7, 62.1, 60.4, 52.4, 52.3, 52.2, 52.1, 40.3; HRMS (ESI) m/z calcd for $C_{92}H_{136}N_{16}Na_5O_{66}$ (M + Na) 2635.7261, measured 2635.7404.

General Method for Capillary Electrophoresis (CE) Binding Studies. The ACE method involves varying the concentration of one component (host or guest molecule) in the CE running buffer and measuring the change in electrophoretic mobility of the injected component whose concentration is kept constant. Dimethyl sulfoxide (DMSO) is added to the injected samples and used as internal electroosmotic flow (EOF) marker. The effective electrophoretic mobility (μ_e) of the analytes is calculated from the migration times according to eq 1:[48]

$$\mu_e = \frac{L_d L_t}{V}\left(\frac{1}{t_i} - \frac{1}{t_f}\right) \quad (1)$$

where L_t and L_d are the total capillary length and the length of the capillary to the detector, respectively. V is the run voltage, and t_i and t_f are the migration time of analyte and migration time of the neutral marker, respectively.

All solutions were prepared in borate buffer (20 mM, pH 9.2). β-Cyclodextrin, sodium borate, 4-methylbenzoic acid, 4-*tert*-

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butylbenzoic acid, and 4-toluenesulfonic acid were purchased and used without further purification. Dimethyl sulfoxide (DMSO) was used as the electroosmotic flow (EOF) marker. The separation buffers contain 20 mM borate buffer at pH 9.2. All solutions were prepared in 20 mM borate buffer and filtered with 0.20 μm filters. Separation was carried out in an untreated fused silica capillary with a total length of 60.2 cm and effective length of 50 cm to the detector. New capillary was first conditioned with methanol for 10 min, HCl (1 N) for 10 min, NaOH (1 N) for 10 min, HCl (0.1 N) for 10 min, NaOH (0.1 N) for 10 min, deionized water for 10 min, and separation buffer for 10 min. Capillary was washed daily with methanol for 10 min, NaOH (0.1 N) for 10 min, deionized water for 10 min, and separation buffer for 10 min. Between runs, the capillary was washed with NaOH (0.1 N) for 2 min, deionized water for 2 min, and separation buffer for 2 min at high pressure purge (20 psi). Samples (in duplicates) were injected to the capillary in 5 s at 0.5 psi and applied voltage of 25 kV. The capillary temperature was maintained at 25 °C, and the absorbance was measured at 214 nm.

General Method for Fluorescence Measurements. All solutions were prepared in 50 mM phosphate at pH 3.0 or at pH 7.4. The concentration of FITC used for all the measurements was 0.01 mM. The concentrations of β-CD, α-CD, cyclic oligosaccharides, and acyclic oligosaccharides were 5.0 mM. Stock solutions of FITC (0.02 mM), β-CD (10.0 mM), α-CD (10.0 mM), individual macrocyclic oligosaccharides (10.0 mM), and individual acyclic oligosaccharides (10.0 mM) were first prepared in 50 mM phosphate solution. The test solutions were then obtained by combining the same volume of the stock solutions of FITC and oligosaccharide sample (using buffer only to replace the solution of oligosaccharide sample for control). The background fluorescence spectra for β-CD, α-CD, macrocyclic oligosaccharides, and acyclic oligosaccharides solutions were also obtained using the mixture of the stock solution of oligosaccharide sample and buffer only. The fluorescence emission spectra were obtained by scanning the emission monochromator from 480 to 700 nm while exciting at a fixed wavelength of 440 nm.

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Supporting Information Available: Nonlinear plots of CE data, ^{1}H, and ^{13}C NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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