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Construction of multivalent sialyl Le^x from the type Ia group B *Streptococcus* capsular polysaccharide

Wei Zou,* JianJun Li, Suzon Larocque, Harold J. Jennings

Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6

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Abstract

The type Ia group B *Streptococcus* (GBSIa) capsular polysaccharide was specifically degraded by partial Smith oxidation of 2,3-diol of the Glc in the backbone to fragments representing asialo core repeating units. Sialylation of these oligomers furnished GBSIa multiple repeating units. One, two and three repeating units of GBSIa were obtained pure, and the higher oligomers were obtained as mixtures. After enzymatic fucosylation oligosaccharides carrying bivalent, trivalent and other multivalent sialyl Le^x epitopes presented as appendages on an oligolactoside scaffold were obtained.

Keywords: Oligosaccharide synthesis; Multivalent sialyl Le^x; Group B streptococcal polysaccharide; Degradation; Enzymatic glycosylation

1. Introduction

The development of carbohydrate-based anticancer[1] and antibacterial[2] vaccines is in need of more efficient methods for the synthesis of complex carbohydrate antigens. Although notable progress has been made in chemical and chemo-enzymatic synthesis,[3] solid-phase synthesis[4] and programmed robotic synthesis,[5] complex carbohydrates of biological significance are still very difficult to make. This difficulty is compounded by the fact that most biological interactions between carbohydrates and proteins are multivalent,[6] thus requiring, for maximum efficiency, the synthesis and presentation of multiple carbohydrate epitopes with defined structures. For example, sialyl $Le^{x/a}$ is a carbohydrate ligand for selectins and contributes to the hematogenous metastasis of cancer,[7] and enhanced expression of sialyl $Le^{x/a}$ on epithelial mucins is correlated to the progression and poor prognosis of carcinomas.[8] However, monovalent sialyl $Le^{x/a}$ binds with low affinity to the selectins, and recognition of mucin ligands by selectins requires the multivalent presentation of sialyl $Le^{x/a}$ epitopes.[9]

Multivalent carbohydrate epitopes can be chemically or chemo-enzymatically synthesised.[10] But, the methods widely used for the synthesis of oligosaccharides are at the present time consuming, difficult, and expensive. All share a common strategy; i.e., an oligosaccharide has to be built up step by step from monosaccharides and/or building blocks. The multiple steps involved in even obtaining various monovalent carbohydrate epitopes impose limits on their efficient synthesis, and obtaining them in a multivalent form adds a further degree of difficulty.

* Corresponding author. Tel.: +1-613-9910855; fax: +1-613-9529092.

E-mail address: wei.zou@nrc.ca (W. Zou).

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Our goal is to develop a simple and comprehensive approach to the preparation of important multivalent carbohydrate epitopes. Group B streptococcal (GBS) polysaccharides share extensive homology with human carbohydrate antigens. For example, the trisaccharide side chains of the GBSIa/Ib polysaccharides,[11] α-NeuAc(2→3)-β-Gal(1→4/3)-β-GlcNAc(1→, should easily be converted to their respective sialyl $Le^{x/a}$ analogs by enzymatic fucosylation of GlcNAc at the 3-*O*- or 4-*O*-position. This would provide multivalent sialyl $Le^{x/a}$ presentation on a polylactoside backbone. Using this strategy we were able to develop a synthetic approach to multivalent sialyl $Le^{x/a}$ antigens. For example, the GBSIa polysaccharide was depolymerized by partial Smith degradation to oligosaccharide fragments representing repeating units of the asialo core. Enzymatic sialylation of these oligomers then furnished GBSIa multiple repeating units. Finally, fucosylation of these GBSIa oligosaccharides afforded oligosaccharides containing multiple sialyl Le^{x} epitopes.

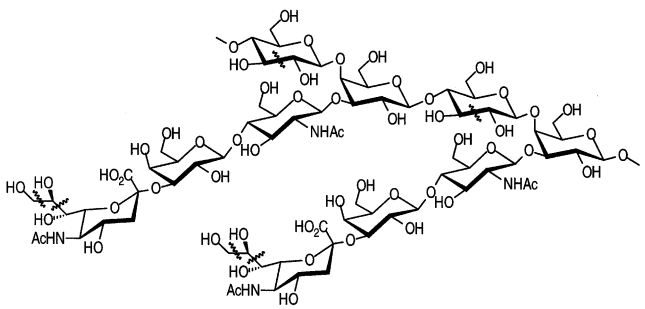

Fig. 1. Two repeating units of GBSIa capsular polysaccharide. The vicinal-diols as indicated are sensitive to periodate oxidation.

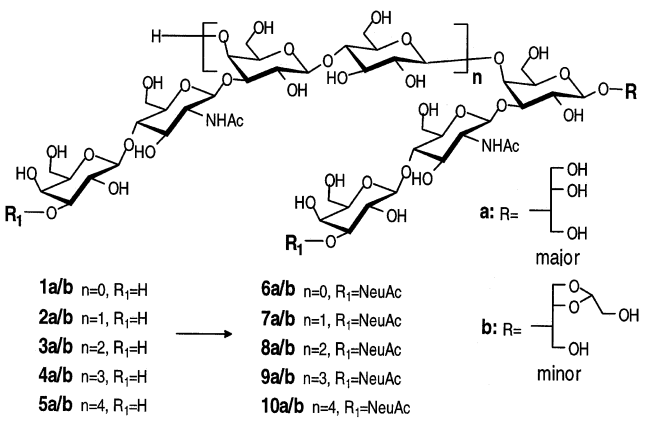

Fig. 2. GBSIa oligosaccharide repeating units obtained by controlled Smith degradation. Core oligosaccharides were enzymatically sialylated.

2. Results and discussion

GBSIa polysaccharide has three potential sites in each repeating unit that are susceptible to periodate oxidation as shown in Fig. 1. The vicinal-triol exocyclic chain of NeuAc can be preferentially oxidized with the first two equivalents of sodium periodate. Further oxidation results in the cleavage of 2,3-diol of the Glc in the backbone. Thus, GBSIa polysaccharide was treated with sodium periodate (2.5–3.0 equivalents) to achieve complete removal of C_8–C_9 of the sialic acid residues and partial 2,3-cleavage in the Glc residues. The degree of oxidation of Glc residues was estimated by ^{1}H NMR spectroscopy by integration of the H-2 resonance of the remaining intact Glc residues at 3.2 ppm. Reduction of the resultant aldehydes with sodium borohydride was followed by subsequent acid hydrolysis with 0.5 M HCl. Because free sialic acid exists exclusively as the β isomer, the hydrolysis could be monitored by the disappearance of the resonance at 2.65 ppm, corresponding to H-3e of the α-(2→3)-linked NeuAc, and the appearance of a resonance at 2.30 ppm indicative of H-3e of free sialic acid.[†] One, two and three repeating units of the core GBSIa oligosaccharides (**1a**/**b** through **3a**/**b**) were purified (see Fig. 2). The higher oligomers (**4a**/**b** and **5a**/**b**) were also obtained as mixtures.

The acid-catalyzed Smith degradation proceeded by two routes to afford two different products (**a** and **b**).[12] Complete hydrolysis of the anomeric acetal gave the major product (**a**) with D-threitol as an aglycon, whereas the minor product of partial hydrolysis containing 1,2-di-*O*-hydroxyethylidene D-threitol at the reducing end (**b**) was also obtained. The two forms of oligomers, **a** and **b**, were not separa-

[†] Most truncated sialic acid (NeuAc-7) linkages survived the mild hydrolysis (0.5 M HCl, 16 h at 4 °C). However, partial removal of sialic acid in multi-repeating units occurred and made separation of these oligomers difficult. We obtained NeuAc (C-7) analogs of **6a**/**b** that were also fucosylated to afford sialyl Le^{x} analogs.

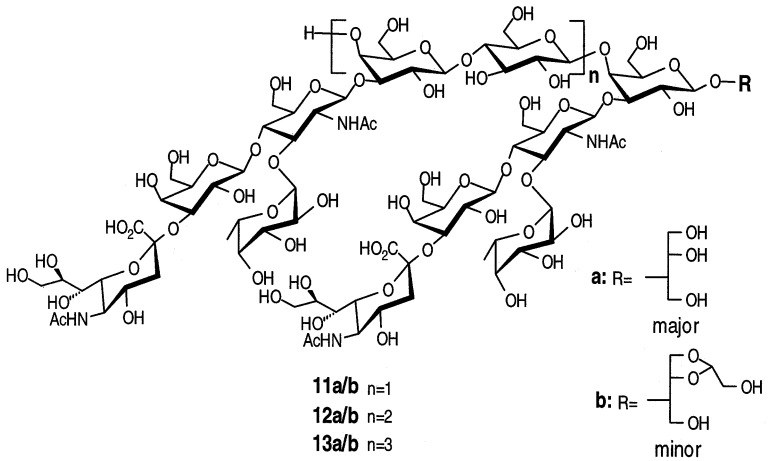

Fig. 3. Oligosaccharides carrying multivalent sialyl Le^x epitopes.

ble by gel-permeation chromatography, but their presence was confirmed by ESIMS and MS–MS analysis. The presence of ($>CHCH_2OH$) was confirmed by the observation of a mass difference of 42 throughout MS analysis. The ratio of **a** to **b** was estimated about 3:1 based on the molecular peak height by MS spectroscopy.

Sialylation[13] of **1**–**5a**/**b** was performed using a combination of NeuAc-CMP synthetase and α-(2→3)-sialyltransferase to furnish the respective **6**–**10a**/**b**, which represent from one to five repeating units of GBSIa (see Fig. 2). One, two and three repeating units of GBSIa (**6**–**8a**/**b**) were obtained pure, and the higher oligomers were obtained as mixtures. In addition to being precursors of multiple sialyl Le^x epitopes, these oligosaccharides will also be useful in future experiments to define the conformational epitope[14] of GBSIa polysaccharide.

Fucosylation[13] of **7**–**10a**/**b** afforded oligosaccharides carrying bivalent (**11a**/**b**), trivalent (**12a**/**b**), tetravalent (**13a**/**b**), and other multivalent sialyl Le^x epitopes. Fig. 3 illustrates these structures in which sialyl Le^x epitopes are presented as appendages on an oligolactoside scaffold, and their observed chemical shifts are very similar as shown in Fig. 4. The chemical shifts of Fuc and Gal in the different chains (**11**–**12a**/**b**) all overlapped as did H-3 of all their NeuAc residues. However, a slight difference was observed in the chemical shifts of their GlcNAc residues, probably due to the unique linkage position of each GlcNAc to the backbone. Fucosylation of the larger oligosaccharides was more sluggish. In a mixture of **8**–**10a**/**b** full fucosylation was achieved on **8a**/**b** and **9a**/**b** to afford **12a**/**b** and **13a**/**b**, respectively, but only tri- instead of penta-fucosylation was observed in **10a**/**b**, as indicated by MS analysis. We speculate that fucosylation takes place in a stepwise manner and proceeds first at both termini to afford α,ω-bivalent sialyl Le^x epitopes. Internal fucosylation follows more slowly because the formation of the enzyme–substrate complex is less favored. With further increase of saccharide chain length, fucosylation becomes even slower, as a result of which we have not been able to obtain fully fucosylated GBSIa polysaccharide.

In summary, a new approach for the construction of complex carbohydrates from a polysaccharide has been successfully demonstrated in the synthesis of GBSIa oligosaccharides as well as their multivalent sialyl Le^x analogs. With the increasing availability of various glycosyltransferases, this procedure could become a more cost-effective method for the construction of other biologically important carbohydrates. We are also investigating whether these oligosaccharides function as better immunogens in raising anti-sialyl Le^x specific antibodies and as better inhibitors with more effective binding to selectins.

3. Experimental

General methods.—^{1}H NMR spectra were recorded with INOVA-500 instrument at 293 K unless otherwise noted. Chemical shifts are given in ppm relative to the signal of internal acetone (δ_H 2.225 in D_2O for ^{1}H NMR spectra). ESIMS and LC–MS were performed with QUATTRO (MICROMASS) and CRYSTAL CE System (Themo BioAnalysis) instruments, respectively. Fucosyltransferase was purchased from CalBiochem, La Jolla, CA. GDP-β-L-Fuc was prepared in house.[15] Other chemicals were products of Aldrich Chemical Co.

Controlled Smith degradation.—To a solution of GBSIa polysaccharide (100 mg, 0.1 mmol) in 0.1 M NaOAc (10 mL, pH 6.0) was added 0.5 M $NaIO_4$ (0.5–0.6 mL, 0.25–0.3 mmol). After 3 days in the dark, the solution was dialyzed, treated with $NaBH_4$ (16 mg), dialyzed again, and lyophilized to a white powder (70–80 mg). The above material was treated with 0.5 N HCl (10 mL) at rt for 4 days. The solution was passed through a Dowex 1 × 8 (HCO_3^-) column with water as eluent to remove sialic acid and HCl and lyophilized to a powder. The final separation was performed on a Bio-Gel P-6 column using 0.03 M NH_4HCO_3 as eluent. Fractions were collected and lyophilized to afford pure **1a**/**b** (9–18 mg), **2a**/**b** (6–13 mg) and **3a**/**b** (2–4 mg), a mixture of **4a**/**b** and **5a**/**b** (4 mg), and higher oligomers.

^{1}H NMR (D_2O): **1a**/**b** δ 2.008 (3 H, s, NHAc), 4.130 (1 H, bs, H-4 of Gal), 4.466 (1 H, d, H-1 of Gal, $J_{1,2}$ 7.5 Hz), 4.482 (1 H, d, H-1 of Gal's, $J_{1,2}$ 7.5 Hz), 4.708 (1 H, d, H-1 of GlcNAc, $J_{1,2}$ 8.0 Hz) ppm; **2a**/**b** δ 2.001 and 2.011 (6 H, 2s, 2 × NHAc), 3.301 (1 H, dd, H-2 of Glc, $J_{1,2}$ 7.5, $J_{2,3}$ 9.0 Hz), 4.129 (1 H, bs, H-4 of Gal), 4.357 (1 H, bs, H-4 of Gal), 4.414 (1 H, d, H-1 of Gal, $J_{1,2}$ 8.0 Hz), 4.455 (2 H, d, 2 × H-1 of Gal's, $J_{1,2}$ 8.0 Hz), 4.470 (1 H, d, 2 H-1 of Gal, $J_{1,2}$ 8.0 Hz), 4.683 (1 H, d, H-1 of GlcNAc, $J_{1,2}$ 7.5 Hz), 4.697 (1 H, d, H-1 of GlcNAc, $J_{1,2}$ 8.5 Hz), 4.904 (1 H, d, H-1 of Glc, $J_{1,2}$ 7.5 Hz) ppm; **3a**/**b** δ 2.030 (9 H, s, 3 × NHAc), 3.313 (2 H, bdd, 2 × H-2 of Glc), 4.145 (1 H, bs, H-4 of Gal), 4.385 and 4.400 (1 H each, 2 × bs, 2 × H-4 of Gal), 4.416 (1 H, d, H-1 of Gal, $J_{1,2}$ 8.0 Hz), 4.435 (1 H, d, H-1 of Gal, $J_{1,2}$ 8.0 Hz), 4.456 (3 H, d, 3 × H-1 of Gal's, $J_{1,2}$ 8.0 Hz), 4.496 (1 H, d,

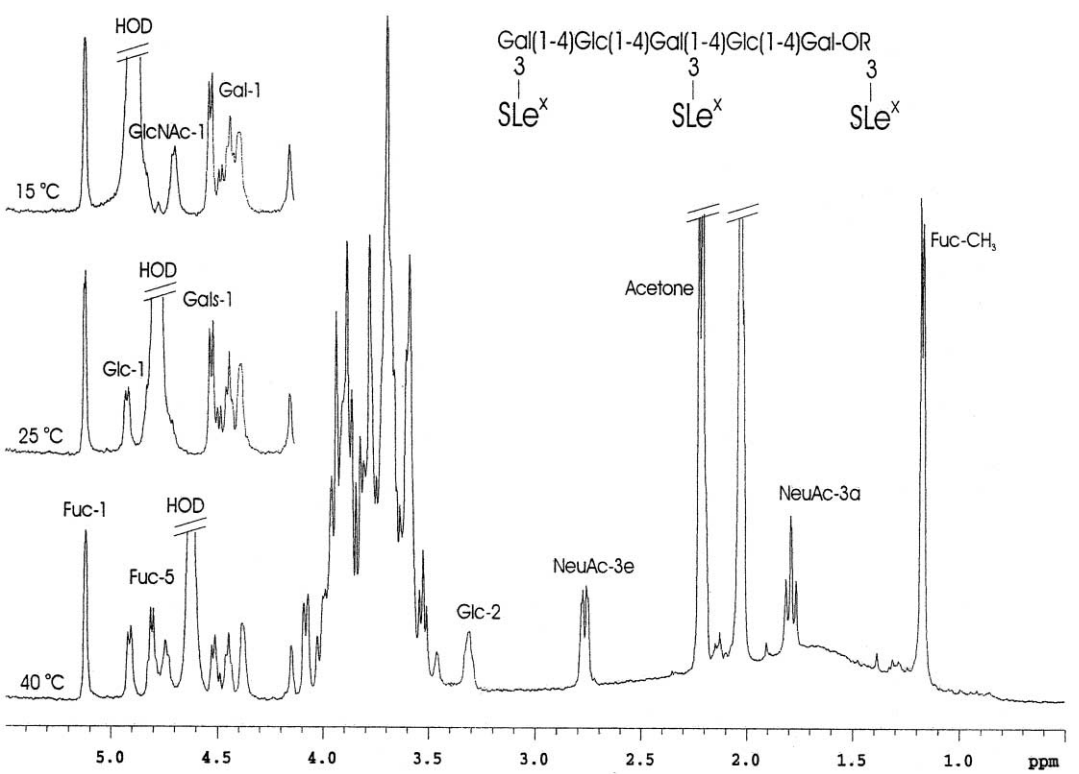

Fig. 4. 500-MHz ^{1}H NMR spectra of oligosaccharide **12a**/**b** with three sialyl Le^x epitopes in D_2O (Gal*s*, Gal of side chain).

H-1 of Gal, $J_{1,2}$ 8.0 Hz), 4.710 (3 H, bd, 3 × H-1 of GlcNAc, $J_{1,2}$ 7.5 Hz), 4.910 (2 H, d, 2 × H-1 of Glc, $J_{1,2}$ 8.0 Hz) ppm. ESIMS: **1a**/**b** Calcd for $C_{24}H_{43}NO_{19}$/$C_{26}H_{45}NO_{20}$ (649.60/691.64); Found 650.3/692.3 (M + 1) and 672.2/714.3 (M + Na); **2a**/**b** calcd for $C_{50}H_{86}N_2O_{39}$/$C_{52}H_{88}N_2O_{40}$ (1339.22/1381.26); Found 1338.5/1381.8; **3a**/**b** Calcd for $C_{76}H_{129}N_3O_{59}$/$C_{78}H_{131}N_6O_{60}$ (2028.84/2070.88); Found 2028.1/2071.2.

Sialylation.—To a solution of oligosaccharide (**1**–**5a**/**b**) (2 mg each), NeuAc (4 mg), CTP (4 mg) in water (1 mL) were added 0.1 M $MgCl_2$ (50 μL) and 0.1 M $MnCl_2$ (10 μL), and the pH was adjusted to pH 7.5 by the addition of 1 N sodium cacodylate. To the above solution were added alkaline phosphatase (2 U), NeuAc-CMP synthetase (0.5 U) and α-(2 → 3)-sialyltransferase (40 mU), respectively. Again the pH was adjusted to 7.5, and the mixture was incubated for 3 days at rt. Purification on a Bio-Gel P-6 column, using 0.03 M NH_4HCO_3 as eluent, afforded sialyl oligosaccharides (**6**–**10a**/**b**) (ca. 2.0 mg each), respectively.

^{1}H NMR (D_2O): **6a**/**b** δ 1.771 (1 H, dd, H-3a of NeuAc, $J_{3a,4} = J_{3e,3a}$ 11.5 Hz), 2.003 (6 H, s, 2 × NHAc), 2.730 (1 H, dd, H-3e of NeuAc, $J_{3e,4}$ 4.0 Hz), 4.469 (1 H, d, H-1 of Gal, $J_{1,2}$ 7.5 Hz), 4.557 (1 H, d, H-1 of Gal's, $J_{1,2}$ 7.5 Hz), 4.705 (1 H, d, H-1 of GlcNAc, $J_{1,2}$ 8.0 Hz) ppm; **7a**/**b** δ 1.775 (2 H, dd, 2 × H-3a of NeuAc, $J_{3a,4} = J_{3e,3a}$ 11.5 Hz), 2.005 (12 H, s, 4 × NHAc), 2.732 (2 H, dd, 2 × H-3e of NeuAc, $J_{3e,4}$ 4.0 Hz), 3.293 (1 H, dd, H-2 of Glc, $J_{1,2}$ 7.5, $J_{2,3}$ 9.0 Hz), 4.410 (1 H, d, H-1 of Gal, $J_{1,2}$ 8.0 Hz), 4.469 (1 H, d, H-1 of Gal, $J_{1,2}$ 8.0 Hz), 4.533 (2 H, d, 2 × H-1 of Gal's, $J_{1,2}$ 8.0 Hz), 4.684 (1 H, d, H-1 of GlcNAc, $J_{1,2}$ 7.5 Hz), 4.691 (1 H, d, H-1 of GlcNAc, $J_{1,2}$ 8.5 Hz), 4.890 (1 H, d, H-1 of Glc, $J_{1,2}$ 7.5 Hz) ppm; **8a**/**b** (22 °C) δ 1.800 (3 H, dd, 3 × H-3a of NeuAc, $J_{3a,4} = J_{3e,3a}$ 11.5 Hz), 2.030 (18 H, s, 6 × NHAc), 2.756 (3 H, dd, 3 × H-3e of NeuAc, $J_{3e,4}$ 4.0 Hz), 3.315 (2 H, bdd, 2 × H-2 of Glc), 4.115 (3 H, dd, 3 × H-3 of Gal's, $J_{2,3}$ 9.5, $J_{3,4}$ 3.0 Hz), 4.154 (H, bs, H-4 of Gal), 4.386 and 4.401 (1 H each, 2 × bs, 2 × H-4 of Gal), 4.441 (1 H, d, H-1 of Gal, $J_{1,2}$ 8.0 Hz), 4.457 (1 H, d, H-1 of Gal, $J_{1,2}$ 8.0 Hz), 4.496 (1 H, d, H-1 of Gal, $J_{1,2}$ 8.0 Hz), 4.558 (3 H, d, 3 × H-1 of Gal's, $J_{1,2}$ 8.0 Hz), 4.708 (3 H, bd, 3 × H-1 of GlcNAc, $J_{1,2}$ 7.5 Hz), 4.916 (2 H, d, 2 × H-1 of Glc, $J_{1,2}$ 8.0 Hz) ppm. ESIMS: **6a**/**b** Calcd for $C_{35}H_{60}N_2O_{27}$/$C_{37}H_{62}N_2O_{28}$ (940.85/982.89); Found 941.0/982.0; **7a**/**b** Calcd for $C_{72}H_{120}N_4O_{55}$/$C_{74}H_{122}N_4O_{56}$ (1921.73/1963.77); Found 1922.0/1964.0; **8a**/**b** Calcd for $C_{109}H_{180}N_6O_{83}$/$C_{111}H_{182}N_6O_{84}$ (2902.61/2944.65); Found 2901.6/2943.6; **9a**/**b** and **10a**/**b** Calcd for $C_{146}H_{240}N_8O_{111}$/$C_{148}H_{242}N_8O_{113}$ (3883.49/3925.53) and $C_{183}H_{300}N_{10}O_{139}$/$C_{185}H_{302}N_{10}O_{140}$ (4864.37/4906.41); Found 3882.0/3924.0 and 4863.0/4905.0.

Fucosylation.—To a solution of GBSIa oligosaccharides (ca. 2.0 mg) and GDP-β-L-Fuc (2.0 mg) in HEPES–NaOH buffer (0.5 mL, pH 7.5, 50 mM, 20 mM $MnCl_2$) was added α-(1 → 3)-fucosyltransferase VI (20 mU). More GDP-β-L-Fuc (2.0 mg) was added after 24 h, and the mixture was kept at 37 °C for 5 days. The purification was performed on a Bio-Gel P-6 column, using 0.03 M NH_4HCO_3 as eluent, to afford sialyl Le^x oligosaccharides (**11**–**13a**/**b**) (ca. 2.0 mg), respectively.

^{1}H NMR (D_2O): **11a**/**b** (15 °C) δ 1.151 (6 H, d, 2 × 6-CH_3 of Fuc, $J_{5,6}$ 6.5 Hz), 1.782 (2 H, dd, 2 × H-3a of NeuAc, $J_{3a,4} = J_{3e,3a}$ 12.0 Hz), 1.994 and 2.013 (3 H and 9 H, 2s, 4 × NHAc), 2.747 (2 H, dd, 2 × H-3e of NeuAc, $J_{3e,4}$ 3.5 Hz), 3.291 (1 H, dd, H-2 of Glc, $J_{1,2}$ 8.0, $J_{2,3}$ 9.0 Hz), 4.073(2 H, bd, 2 × H-3 of Gal's, $J_{2,3}$ 9.5 Hz), 4.142 (1 H, d, H-4 of Gal), 4.376 (1 H, bs, H-4 of Gal), 4.412 (1 H, d, H-1 of Gal, $J_{1,2}$ 8.0 Hz), 4.471 (1 H, d, H-1 of Gal, $J_{1,2}$ 7.5 Hz), 4.520 (2 H, d, H-1 of Gal's, $J_{1,2}$ 7.5 Hz), 4.690 (2 H, d, 2 × H-1 of GlcNAc, $J_{1,2}$ 8.0 Hz), 4.820 (2 H, q, 2 × H-5 of Fuc, $J_{5,6}$ 6.5 Hz), 4.921 (1 H, d, H-1 of Glc, $J_{1,2}$ 7.5 Hz), 5.108 (2 H, d, 2 × H-1 of Fuc, $J_{1,2}$ 3.5 Hz) ppm; **12a**/**b** (40 °C) δ 1.169 (9 H, d, 3 × 6-CH_3 of Fuc, $J_{5,6}$ 6.5 Hz), 1.787 (3 H, dd, 3 × H-3a of NeuAc, $J_{3a,4} = J_{3e,3a}$ 12.0 Hz), 2.016 and 2.033 (3 H and 15 H, 2s, 6 × NHAc), 2.766 (3 H, dd, 3 × H-3e of NeuAc, $J_{3e,4}$ 3.5 Hz), 3.305 (2 H, bdd, 2 × H-2 of Glc), 4.079 (3 H, bd, 3 × H-3 of Gal's, $J_{2,3}$ 10.5 Hz), 4.146 (1 H, d, H-4 of Gal), 4.371 and 4.383 (2 H, bs, 2 × H-4 of Gal), 4.439 (1 H, d, H-1 of Gal, $J_{1,2}$ 8.0 Hz), 4.471 (1 H, d, H-1 of Gal, $J_{1,2}$ 7.5 Hz),

4.496 (1 H, d, H-1 of Gal, $J_{1,2}$ 8.0 Hz), 4.519 (3 H, d, 3 × H-1 of Gal's, $J_{1,2}$ 8.5 Hz), 4.708 (3 H, m, 3 × H-1 of GlcNAc), 4.805 (3 H, q, 3 × H-5 of Fuc, $J_{5,6}$ 6.5 Hz), 4.911 (2 H, d, 2 × H-1 of Glc, $J_{1,2}$ 7.5 Hz), 5.117 (3 H, d, 3 × H-1 of Fuc, $J_{1,2}$ 3.5 Hz) ppm. ESIMS: **11a**/**b** Calcd for $C_{84}H_{140}N_4O_{63}$/$C_{86}H_{142}N_4O_{64}$ (2214.02/2256.06); Found 2214.0/2256.0; **12a**/**b** Calcd for $C_{127}H_{210}N_6O_{95}$/$C_{129}H_{212}N_6O_{96}$ (3341.04/3383.08); Found 3340.9/3383.9; **13a**/**b** Calcd for $C_{170}H_{280}N_8O_{127}$/$C_{172}H_{282}N_8O_{128}$ (4468.07/4510.10); Found 4467.0/4509.0.

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