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Chemoenzymatic synthesis of dendritic sialyl Lewis^x

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Abstract

Traditional structure activity relationship studies (SAR) have led to the development of numerous sialyl Lewis^x analogs in the search for potential antiinflammatory agents. However, these methods do not take into account cluster or multivalent effects. Reported herein is the chemoenzymatic synthesis of di-, tetra-, and octa-valent sLe^x ligands scaffolded on dendrimers. Hypervalent L-lysine cores with covalently attached 2-acetamido-2-deoxy-D-glucose (*N*-acetylglucosamine, GlcNAc) residues were chemically prepared and enzymatically transformed into sLe^x-containing dendrimers so that multivalency, and its role in selectin–sLe^x interactions may be evaluated. This work constitutes another successful enzymatic synthesis of sLe^x and represents the first example of GlcNAc elongation on a synthetic dendrimer scaffold. These sLe^x dendrimers are currently being investigated as selectin antagonists. © 1998 Elsevier Science Ltd.

Keywords: Sialyl Lewis^x; Dendrimers; Enzymes; L-Lysine

1. Introduction

L, P, and E selectins are a family of transmembrane glycoproteins responsible for the adhesion of leukocytes to vascular endothelium in the early cascade of events leading to inflammation [1–3]. Sialylated and fucosylated oligosaccharides related to sialyl Lewis^x tetrasaccharide (α-Neu*p*5Ac-(2 → 3)-β-D-Gal*p*-(1 → 4)-[α-L-Fuc(1 → 3)]-β-D-Glc*p*NAc-OR, sLe^x) are key ligands expressed on leukocytes and on endothelial cell surfaces that are initially recognized by L-selectin on leukocytes [4]. Following timely events, these ligands are then captured by P- and E-selectins. Therefore, sLe^x and related oligosaccharides represent lead compounds for the pharmaceutical industry interested in the development of drugs for inflammatory diseases.

Traditional structure–activity relationship (SAR) strategies have led to the synthesis of a number of sLe^x analogs [5]. These monovalent sLe^x mimetics generally showed modest inhibitory potencies in vitro (IC_{50}s ≈ 1–5 mM) that do not compare favorably with the much better in vivo IC_{50}s (≈ 1 μM) activities as exemplified in lung injury protection in rat experiments [6]. Unfortunately, SAR studies neglect

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the cluster or multivalent effect obtained from either glycolipids or neoglycoproteins. That is, SAR methods alone do not allow for the amplification of affinity via cooperative carbohydrate protein interactions.

Given that the biological forms of selectins [3,7–9] and the natural ligands themselves, i.e., GLYCAM-1/Sgp50, CD34/Sgp90, MAdCAM-1 for L-selectin and PSGL-1 for P-selectin [8,10] are likely to have clustered arrangements, it is logical to look to multivalent sLe^x conjugates as viable selectin antagonists.

In fact, small clusters containing sLe^x antigens have been prepared [11–15]. Sialyl Lewis^x enzymatically scaffolded on an ethyl β-D-galactoside core and sLe^x tethered to both 1,4-butanediol and 1,5-pentanediol [11,12] resulted in divalent sLe^x conjugates with increased activity. Chemically and enzymatically constructed clusters of sLe^x ligands based on both an *N*-acetylglucosamine-linked asparagine building block [13] and on fixed cyclic peptides [14,15] also exhibited lower IC_{50} values over monomeric sLe^x when tested against E-selectin-Ig chimera. Further exploiting multivalency, sialyl Lewis^x–containing polymers [16,17] and liposomes [18,19] have been similarly reported and these too showed drastically increased inhibitory potentials.

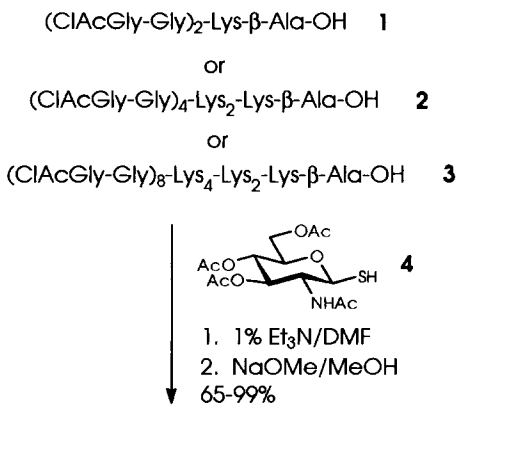
To systematically explore multivalency requirements in sLe^x–selectin interactions, series of chemically well-defined, structurally similar, dendritic sLe^x conjugates, varying only in valency are necessary. Glycodendrimers [20] based on L-lysine [20–24], Starburst™ PAMAM [25–27], a 1,3,5-benzene tricarbonyl derivative [28], and 3,3′-iminobis(propylamine) cores [29,30] have been previously described. These novel neoglycoconjugates with demonstrated cluster effects have so far been limited to simple structures incorporating mono- to tri-saccharides. The most complex glycodendrimer reported contained a simpler analog of sLe^x, 3′-sulfo-Lewis^x-(Glc), residues [24]. These preliminary results combined glycomimetic and multivalent strategies in the search for selectin antagonists.

Recently, dendritic *N*-acetyllactosamine conjugates based on an L-lysine core have been synthesized using a chemoenzymatic approach [31]. A chemoenzymatic strategy is attractive owing to the stereo- and regio-selectivity in the formation of glycosidic linkages by glycosyl transferases [32], and the efficient approach reported provides an entry into the synthesis of dendritic sLe^x. We report herein the chemoenzymatic synthesis of sialyl Lewis^x-containing dendrimers.

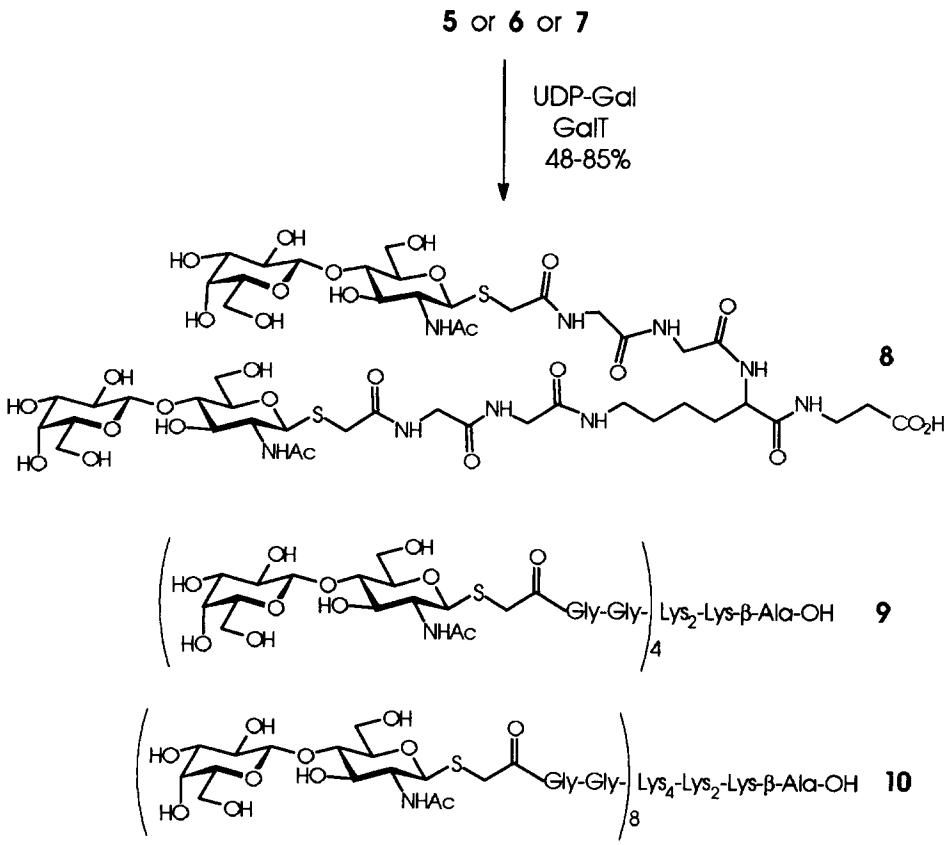
2. Results and discussion

The enzymatic scaffolding of dendritic sialyl Lewis^x ligands onto synthetic multivalent GlcNAc cores was initiated from *N*-chloroacetylated glycylglycine-ending clusters **1–3** (Scheme 1). These 'seeding' molecules were prepared on solid phase as previously described using Wang resin and Fmoc protecting groups [22,31]. Briefly, the chemical strategy entailed the rational scaffolding of L-lysine core structures on solid-phase. The convergent strategy involved the solid phase synthesis of *N*-chloroacetylated glycylglycine-capped, hyperbranched L-lysine as already reported [20]. Each *N*-chloroacetyl group was substituted with a peracetylated 1-thio-GlcNAc derivative (**4**). Hydrolysis from the resin (95% aqueous TFA), followed by O-acetyl protecting group removal (NaOMe, MeOH), afforded the starting series of glycodendrimers **5–7** (Scheme 1) [22,31]. *N*-Acetylglucosamine-containing dendrimers with valencies of two (**5**), four (**6**), and eight (**7**) were thus synthesized for the next enzymatic glycosylations.

These GlcNAc-based dendrimers were then enzymatically galactosylated using UDP-galactose and bovine milk β-(1 → 4) galactosyltransferase (GalT; EC 2.4.1.38) in cacodylate buffer (Scheme 2). The reactions were monitored by MALDI–MS. Upon reaction completion, the desired *N*-acetyllactosamine-containing dendrimers were purified via centrifugation, followed by gel-permeation chromatography (GPC) using Biogel P-4 for divalent **8** and tetravalent **9** or Bio-Rad AG50W × 8 (Na^+ form) for octavalent



Scheme 1.

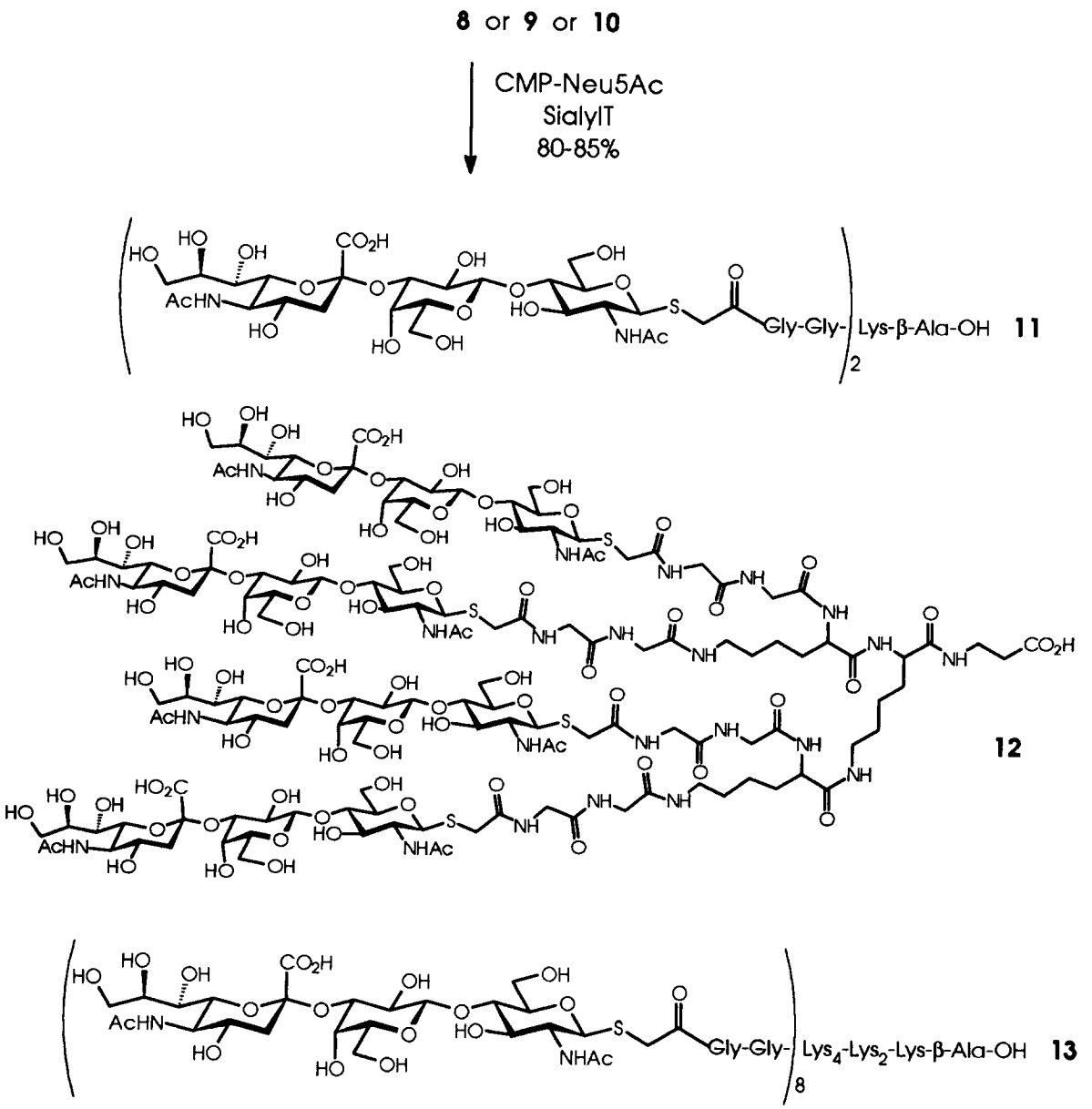


Scheme 2.

10. Isolated yields were fair to excellent (48–85%). NMR analyses (deuterium oxide) confirmed L-lysine dendritic backbone stability and complete galactosyl incorporation as measured by the relative integrations of the β-alanyl α-CH_2 and the lysyl ε-CH_2 at δ 2.38 and 3.21 ppm, respectively, and the H-1 galactose signal at δ 4.48 ppm relative to the NAc and H-1 signals of the GlcNAc residues at δ 2.03 and 4.70 ppm, respectively.

The LacNAc-based dendrimers **8–10** were further elongated by *N*-acetylneuraminic acid using CMP-Neu5Ac and a cloned α-(2 → 3) sialyltransferase from rat liver (SialylT; EC 2.4.99.6) in cacodylate buffer (Scheme 3). Again, monitoring of the reaction was performed by MALDI–MS. Purification as above afforded di- (**11**), tetra- (**12**), and octa- (**13**) valent sialyl *N*-acetyllactosamine (sialyl LacNAc) containing dendrimers in first through third generations, respectively. Glycoconjugates **11–13** were obtained as lyophilized powders in excellent yields (80–85%). Again, NMR spectral characterization revealed complete sialylation with the emergence of new signals corresponding to the newly covalently attached Neu5Ac residue (δ 1.80 and 2.76 ppm for Neu5Ac H-3ax and H-3eq signals in deuterium oxide, respectively).

In a third enzymatic transformation, sialyl LacNAc dendrimers **11–13** were fucosylated using GDP-fucose and a mixture of human milk α-(1 → 3/4) fucosyltransferase (FucT; EC 2.4.1.65 and EC 2.4.1.152) in HEPES buffer (Scheme 4). Monitoring by MALDI–MS and purification via GPC as above, afforded fully deprotected sialyl Lewis^x-containing dendrimers **14–16** with valencies of two, four, and eight. Isolated yields were 74 to 88%. Characteristic fucose signals at δ 1.17, 4.83, and 5.12 ppm (methyl, H-5, and H-1, respectively) were always observed in NMR spectra (deuterium oxide) and undeniably confirmed complete fucosylation had taken place when compared to the L-lysine, *N*-acetylglucosamine, galactose, and sialic acid NMR signals as listed above. Fig. 1 shows the ^{1}H NMR (500 MHz, deuterium oxide) spectrum of octavalent sLe^x **16**. Furthermore, the purity of the synthesized glycodendrimers was assured by careful analyses of these NMR spectra. The mass spectra were frequently very



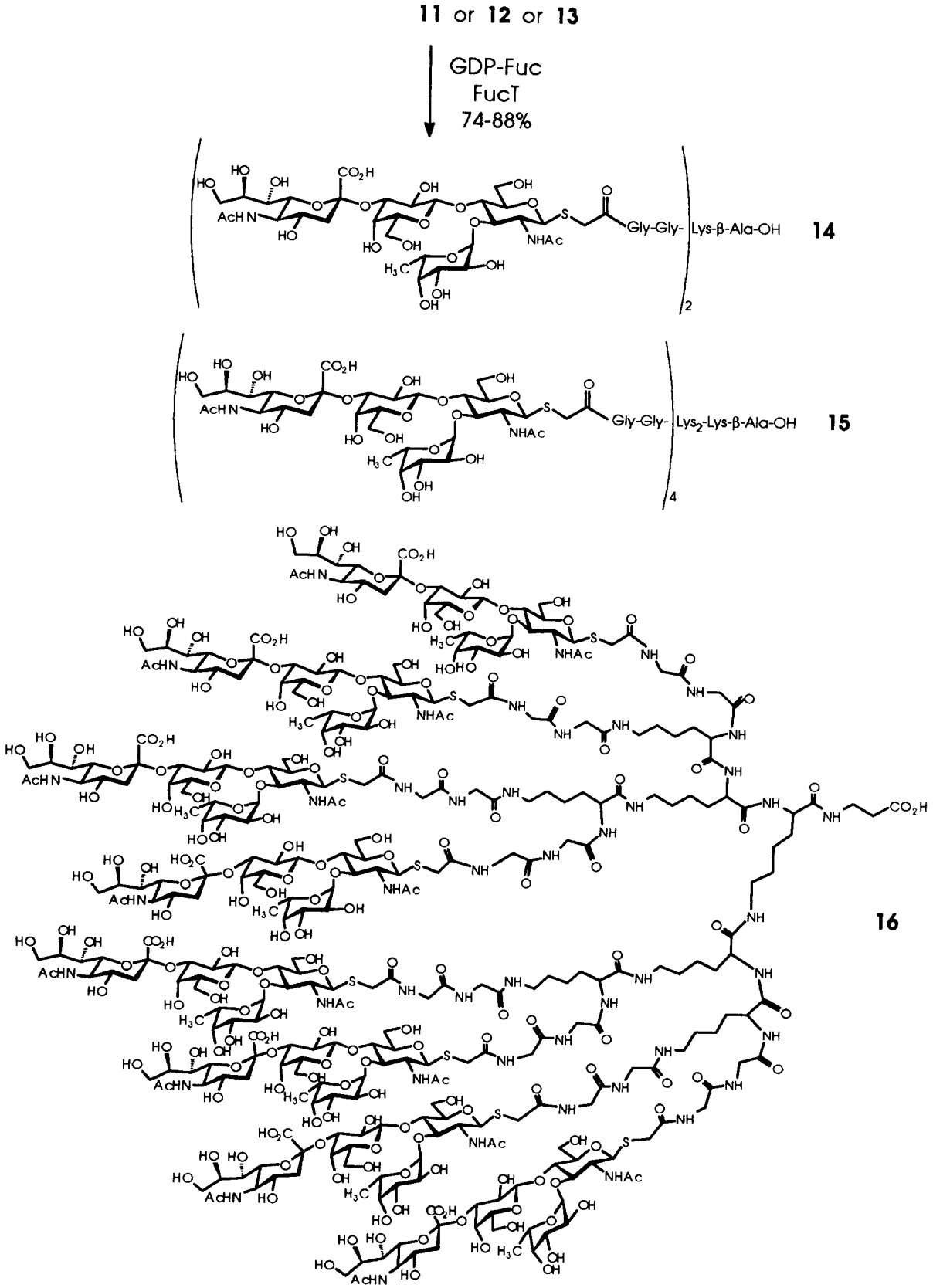
Scheme 3.

complex. In addition to the ions reported, ions with sodium substitution were also observed and confirmed the expected molecular weights.

In conclusion, all chemical and enzymatic yields were good to excellent, indicating that this chemoenzymatic approach is an effective and viable route to well-defined, structurally similar series of dendritic sLe^x differing only in valency. In addition, the results indicate that even the shorter arms of the dendritic L-lysine core were sufficiently long and free of steric hindrance to be amenable to complete enzymatic transformations. This leads to the possibility of using enzymatic manipulation for further glycosylations of existing glycodendrimers. This work represents the first reported achievement of the complete enzymatic conversion of GlcNAc to sialyl Lewis^x on synthetic dendrimers. Di- (**14**), tetra- (**15**), and octa- (**16**) valent sLe^x-containing dendrimers are currently undergoing biological investigation as selectin antagonists.

3. Materials and methods

Materials.—UDP-Galactose (UDP-Gal), bovine serum albumin (BSA), 2,5-dihydroxybenzoic acid (DHB) and 3-aminoquinoline (AQ) were purchased from Sigma Chemical. Calf intestinal alkaline phosphatase (CIAP; EC 3.1.3.1) and CMP-*N*-


11 or 12 or 13
GDP-Fuc
FucT
74-88%
Gly-Gly-Lys-β-Ala-OH
14
Gly-Gly-Lys2-Lys-β-Ala-OH
15
16

Scheme 4.

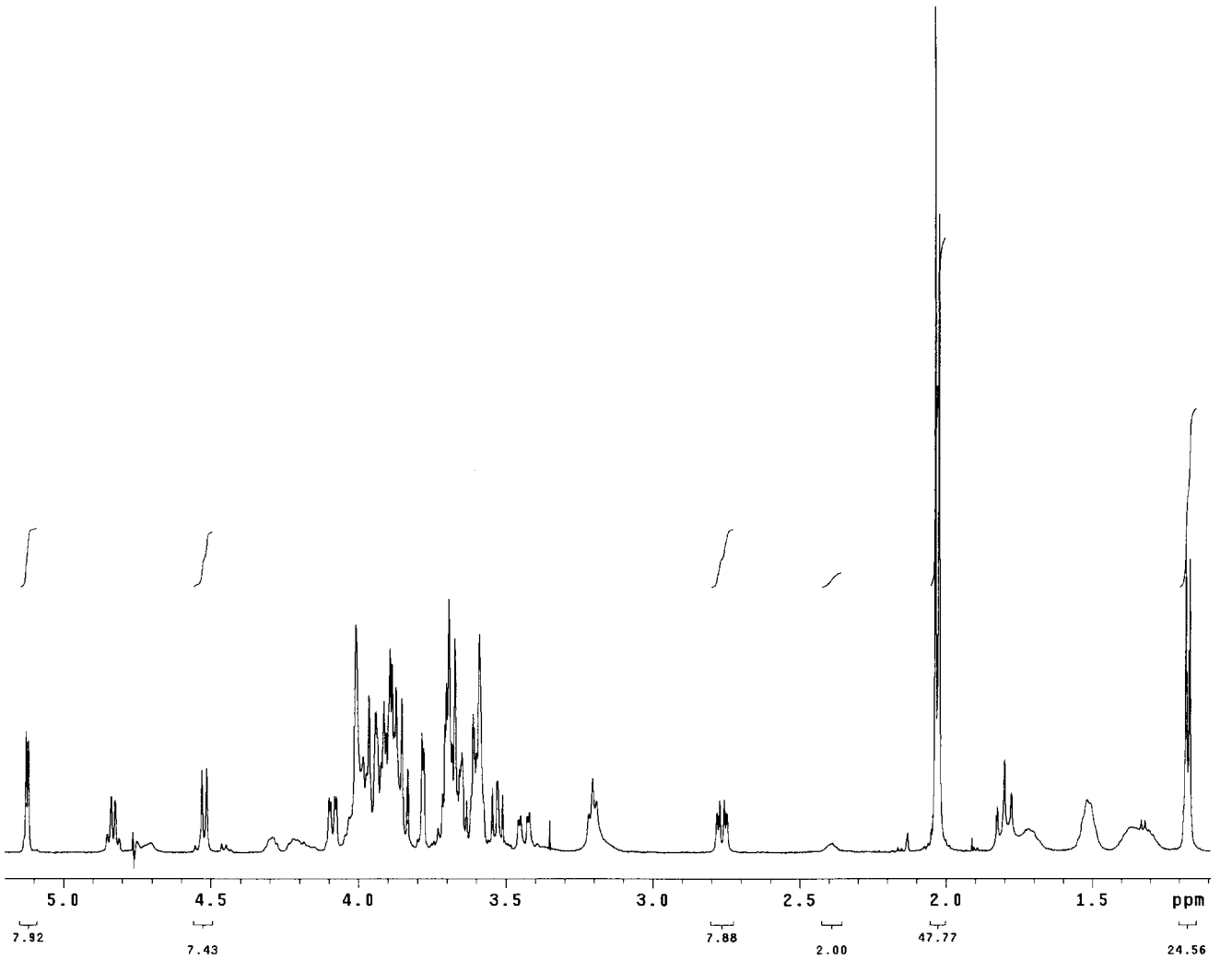


Fig. 1. ^{1}H NMR spectrum (500 MHz, deuterium oxide) of octavalent sialyl Lewis^x **16**.

acetylneuraminic acid (CMP-Neu5Ac) were obtained from Boehringer–Mannheim. GDP-Fucose (GDP-Fuc) was a gift from Dr. R. Öhrlein, Ciba–Geigy. Bio-Gel P-4, P-10, and Bio-Rad AG 50 W × 8 resins were from Bio-Rad. Millex-GV (0.22 mm) filter units were purchased from Millipore and Sep-Pak C_{18} reversed-phase cartridges from Waters Associates. Microsep centrifugal concentrators were from Filtron. β-(1 → 4)-Galactosyltransferase (GalT; EC 2.4.1.38) was isolated from bovine milk by high-speed centrifugation of defatted milk to remove casein [33], followed by chromatography on a UDP-hexanolamine column eluting with GlcNAc and EDTA [34]. A mixture of α-(1 → 3/4)-fucosyltransferases (FucT; EC 2.4.1.65 and EC 2.4.1.152) were isolated from human milk by a modification [35] of literature procedures [36,37]. Briefly, enzyme in defatted milk was adsorbed onto SP-Sephadex C-50, then eluted with a linear gradient of NaCl. After concentration by ultrafiltration and dialysis, the enzyme was applied to a GDP-hexanolamine column and eluted with NaCl and guanosine monophosphate. Cloned α-(2 → 3)-sialyltransferase (SialylT; EC 2.4.99.6, accession number M97754) was purified on an S-Sepharose fast-flow column [38,39]. The enzyme was a soluble form of the α-(2 → 3)-sialyltransferase from rat liver cloned and expressed in baculovirus infected Sf9 cells [39]. A unit of enzyme is the amount that converts one micromole of substrate to product in one minute under standard radiochemical assay conditions [35,39]. *N*-Acetylglucosamine dendrimers were synthesized as previously described [22,31].

General methods.—All ^{1}H NMR spectra were recorded on a Varian Unity 500 (500 MHz) spectrometer as solutions in deuterium oxide (external acetone reference, δ 2.225 ppm) at 30 °C. Due to the repetitive nature of the neoglycoconjugates, selected NMR data are reported. All data were in accordance

with the proposed structures. Mass spectrometric analyses were performed using negative-ion electrospray ionization on a Micromass ZabSpec Hybrid Sector-TOF (ESI–MS). The liquid carrier used was a 1% solution of ammonium hydroxide in 1:1 water–acetonitrile. The calculated molecular weights reported were monoisotopic. The observed molecular weights reported were based on the detection of singly- or multiply-charged ions as noted. Ions with sodium substitution were also observed and confirmed the expected molecular weights. Mass spectrometry with a Kratos Kompact MALDI I (MALDI–MS) was used to monitor enzyme reaction progress. DHB was used as a matrix for monitoring the synthesis of LacNAc and sLe^x dendrimers, while AQ was used a matrix for monitoring the synthesis of sialyl LacNAc dendrimers.

LacNAc dimer (**8**).—GlcNAc dimer **5** (5.9 mg, 5.9 μmol), UDP-Gal (7.4 mg, 11.8 μmol), BSA (0.5 mg), CIAP (10 units), and GalT (1 unit) were dissolved in 50 mM sodium cacodylate buffer (0.9 mL, pH 7.5) containing 5 mM magnesium chloride, 25 mM manganese chloride, and 0.2 mM dithiothreitol. The mixture was incubated at ambient temperature with rotation on a hematology mixer for 28 h. Additional UDP-Gal (3.7 mg, 5.9 μmol total) was added in equal portions at 18 and 22 h. The reaction progress was monitored by MALDI–MS with DHB as matrix. When conversion was complete, the reaction mixture was filtered through a 0.2 μm Millex-GV filter, and the proteins were removed by centrifugation in a 4-mL Microsep concentrator (30 kDa MW cut off) at 3400 rpm. The filtrate was concentrated in vacuo. The residue was further purified on a Bio-Gel P-4 column (1.5 × 110 cm) using 10% ethanol in water as eluent. After concentration and lyophilization LacNAc-based **8** was obtained as a fluffy, white powder in 48% yield (3.8 mg, 2.9 μmol). ^{1}H NMR: δ 1.26–1.40 (m, 2 H, lysyl γ-CH_2), 1.48–1.56 (m, 2 H, lysyl δ-CH_2), 1.66–1.84 (2m, 2 H, lysyl β-CH_2), 2.03 (s, 6 H, NAc × 2), 2.38 (t, 2 H, *J* 7.2 Hz, β-alanyl α-CH_2), 3.21 (t, 2 H, *J* 7.3 Hz, lysyl, ε-CH_2), 4.23 (dd, 1 H, *J* 5.5, 8.9 Hz, lysyl α-CH), 4.48 (d, 2 H, *J* 7.9 Hz, Gal H-1 × 2), 4.69 (d, 1 H, *J* 10.4 Hz, GlcNAc H-1), 4.70 (d, 1 H, *J* 9.6 Hz, GlcNAc H-1). ESIMS: Calcd for $C_{49}H_{81}N_9O_{29}S_2$ 1323.5; Found 1322.1 (M − 1).

LacNAc tetramer (**9**).—The enzymatic reaction was carried out as for **8** using GlcNAc tetramer **6** (5.6 mg, 2.7 μmol) and UDP-Gal (6.9 mg, 11 μmol) as substrates with the addition of UDP-Gal (3.8 mg, 5.5 μmol) during incubation. Monitoring and purification were identical to the procedures followed for **8**. Tetravalent **9** was obtained as a fluffy, white powder (6.2 mg, 9.4 μmol, 85%). ^{1}H NMR: δ 2.03 (s, 24 H, NAc × 4), 2.38 (t, 2 H, *J* 6.8 Hz, β-alanyl α-CH_2), 3.15–3.24 (m, 6 H, lysyl ε-CH_2), 4.18-4.32 (2m, 3 H, lysyl α-CH_2), 4.48 (d, 4 H, *J* 7.8 Hz, Gal H-1 × 4), 4.70 (d, 2 H, *J* 10.2 Hz, GlcNAc H-1 × 2), 4.71 (d, 2 H, *J* 10.5 Hz, GlcNAc H-1 × 2). ESI–MS: Calcd for $C_{101}H_{167}N_{19}O_{57}S_4$ 2686.0; Found: 2684.6 (M − 1), 1341.9 (doubly charged).

LacNAc octamer (**10**).—The enzymatic reaction and monitoring was carried out as described for **8** using GlcNAc octamer **7** (8.2 mg, 2.0 μmol) and UDP-Gal (10.0 mg, 16 μmol) at reaction onset with the addition of UDP-Gal (6 mg, 9.6 μmol total) during the incubation. After 28 h, the mixture was filtered through two 0.22 μm Millex-GV filters, and the filtrate was applied to three Sep-Pak C_{18} cartridges (preconditioned by washing with 20 mL of MeOH and 20 mL of water). The cartridges were washed with water (100 mL), and the product was eluted with 30% MeOH in water (80 mL). The eluate was concentrated and lyophilized to give a powder that was dissolved in 1 mL of water and passed over 1 mL of Bio-Rad AG 50W × 8 resin (Na^+ form). The column was washed with 100 mL of water to elute the desired product, which was further purified on two Sep-Pak C_{18} cartridges as described above. After concentration and lyophilization, octavalent **10** was obtained as a fluffy, white powder in 80% yield (8.7 mg, 1.6 μmol). ^{1}H NMR: δ 2.03 (s, 24 H, NAc × 8), 2.38 (t, 2 H, *J* 7.0 Hz, β-alanyl α-CH_2), 3.15–3.24 (m, 14 H, lysyl ε-CH_2), 4.15–4.32 (m, 7 H, lysyl α-CH), 4.47 (d, 8 H, *J* 7.8 Hz, Gal H-1 × 8), 4.69 (d, 2 H, *J* 10.4 Hz, GlcNAc H-1 × 2), 4.70 (d, 2 H, *J* 10.4 Hz, GlcNAc H-1 × 2), 4.70 (d, 4 H, *J* 10.4 Hz, GlcNAc H-1 × 4). ESIMS: Calcd for $C_{205}H_{339}N_{39}O_{113}S_8$ 5411.0. Found doubly and triply charged ions as broad peaks enveloping the corresponding isotopic distribution, centered at 2707 and 1804.

Sialyl LacNAc dimer (**11**).—Divalent LacNAc **8** (3.2 mg, 2.4 μmol), CMP-Neu5Ac (3.0 mg, 4.5 μmol), CIAP (5 units), and SialylT (0.1 unit) were dissolved in 30 mM sodium cacodylate buffer (0.5 mL, pH 6.5) containing 0.6 M NaCl, 1 mg/mL BSA and 50% glycerol. The reaction was incubated at 37 °C with rotation on a hematology mixer for 48 h. Additional CMP-Neu5Ac (4 mg, 6.0 μmol total) was added in equal portions after 8 and 20 h. The sample was purified as described for **8**. Sialyl LacNAc dimer **11** was obtained as a fluffy, white powder in 85%

yield (3.9 mg, 2.0 μmol). ^{1}H NMR: δ 1.25–1.40 (m, 2 H, lysyl γ-CH_2), 1.48–1.56 (m, 2 H, lysyl δ-CH_2), 1.65–1.82 (m, 2 H, lysyl β-CH_2), 1.80 (t, 2 H, *J* 11.6 Hz, Neu5Ac H-3ax × 2), 2.03 (s, 12 H, NAc × 4), 2.38 (t, 2 H, *J* 6.6 Hz, β-alanyl α-CH_2), 2.76 (dd, 2 H, *J* 5.0, 13.2 Hz, Neu5Ac, H-3eq × 2), 3.21 (t, 2 H, *J* 7.4 Hz, lysyl ε-CH_2), 4.11 (dd, 2 H, *J* 3.2, 9.9 Hz, Gal H-3 × 2), 4.23 (dd, 1 H, *J* 5.5, 8.9 Hz, lysyl α-CH), 4.55 (d, 1 H, *J* 7.9 Hz, Gal H-1 × 2), 4.70 (d, 1 H, *J* 10.4 Hz, GlcNAc H-1), 4.71 (d, 1 H, *J* 10.5 Hz, GlcNAc H-1). ESIMS: Calcd for $C_{71}H_{115}N_{11}O_{45}S_2$ 1905.6. Found singly charged ion observed as a broad peak enveloping the corresponding isotopic distribution, centered at 1906.

Sialyl LacNAc tetramer (**12**).—The enzymatic reaction mixture was carried out as for **11** using LacNAc tetramer **9** (4.1 mg, 1.5 μmol) and CMP-Neu5Ac (5.0 mg, 7.6 μmol). Additional CMP-Neu5Ac (4 mg, 6.1 μmol total) was added during the incubation, and the purification was carried out identically to that used for **8**. Tetravalent **12** was obtained as a fluffy, white powder (4.8 mg, 1.2 μmol, 80%). ^{1}H NMR: δ 1.80 (t, 4 H, *J* 11.8 Hz, Neu5Ac H-3ax × 4), 2.03 (s, 24 H, NAc × 8), 2.38 (t, 2 H, *J* 7.1 Hz, β-alanyl α-CH_2), 2.76 (dd, 4 H, *J* 5.2, 12.3 Hz, Neu5Ac H-3eq × 4), 3.16–3.24 (m, 6 H, lysyl ε-CH_2), 4.11 (dd, 4 H, *J* 3.0, 10.0 Hz, Gal H-3 × 4), 4.18–4.32 (m, 3 H, lysyl α-CH), 4.55 (d, 4 H, *J* 7.7 Hz, Gal H-1 × 4), 4.69 (d, 2 H, *J* 10.0 Hz, GlcNAc H-1 × 2), 4.70 (d, 2 H, *J* 10.0 Hz, GlcNAc H-1 × 2). ESIMS: Calcd for $C_{145}H_{235}N_{23}O_{89}S_4$ 3850.3; Found doubly and triply charged ions as broad peaks enveloping the corresponding isotopic distribution, centered at 1929 and 1286.

Sialyl LacNAc octamer (**13**).—The enzymatic reaction conditions and monitoring were identical to those described for **11** using LacNAc octamer **10** (6.1 mg, 1.1 μmol) and CMP-Neu5Ac (6.0 mg, 9.0 μmol). Additional CMP-Neu5Ac (9 mg, 13.5 μmol total) was added in equal portions after 8, 20, and 29 h. Purification was the same as for **8** except that a Bio-Gel P-10 (1.5 × 110 cm) column was used. Sialyl LacNAc octamer **13** was obtained as a fluffy, white powder in 83% yield (7.2 mg, 0.91 μmol). ^{1}H NMR: δ 1.80 (t, 8 H, *J* 12.5 Hz, Neu5Ac H-3ax × 8), 2.03 (s, 48 H, NAc × 16), 2.56 (t, 2 H, *J* 7.7 Hz, β-alanyl α-CH_2), 2.76 (dd, 8 H, *J* 4.6, 12.3 Hz, Neu5Ac H-3eq × 8), 3.16–3.24 (m, 14 H, lysyl ε-CH_2), 4.11 (dd, 8 H, *J* 3.1, 10.8 Hz, Gal H-3 × 8), 4.15–4.32 (m, 7 H, lysyl α-CH), 4.55 (d, 8 H, *J* 7.8 Hz, Gal H-1 × 8), 4.69 (d, 4 H, *J* 10.4 Hz, GlcNAc H-1 × 4), 4.70 (d, 4 H, *J* 10.4 Hz, GlcNAc H-1 × 4). ESIMS: Calcd for $C_{293}H_{475}N_{47}O_{177}S_8$ 7739.7. Found triply and quadruply charged ions as broad peaks enveloping the corresponding isotopic distribution, centered at 2581 and 1936.

Sialyl Lewis^x dimer (**14**).—Sialyl LacNAc dimer **11** (2.9 mg, 1.5 μmol), GDP-Fuc (2.1 mg, 3.5 μmol), and CIAP (2 units) were dissolved in 50 mL of concentrated incubation buffer (200 mM Hepes, pH 7.0, 200 mM manganese chloride and 2% BSA) containing 50 milliunits of FucT (in 500 μL sodium cacodylate buffer (25 mM, pH 6.5) with 5 mM manganese chloride and 25% glycerol). GDP-Fuc (1 mg, 1.7 μmol total) was added in equal portions after 8 and 20 h. The reaction was incubated at room temperature with rotation using a hematology mixer for 48 h. Monitoring and isolation was as described for compound **8**. Divalent **14** was obtained as a fluffy, white powder (3.1 mg, 1.3 μmol, 88%). ^{1}H NMR: δ 1.17 (d, 6 H, *J* 6.5 Hz, Fuc-CH_3 × 2), 1.25–1.40 (m, 2 H, lysyl γ-CH_2), 1.48–1.56 (m, 2 H, lysyl δ-CH_2), 1.65–1.82 (m, 2 H, lysyl β-CH_2), 1.80 (t, 2 H, *J* 10.8 Hz, Neu5Ac H-3ax × 2), 2.02 (s, 6 H, NAc × 2), 2.04 (s, 6 H, NAc × 2), 2.38 (t, 2 H, *J* 6.5 Hz, β-alanyl α-CH_2), 2.76 (dd, 2 H, *J* 4.7, 12.3 Hz, Neu5Ac H-3eq × 2), 3.21 (t, 2 H, *J* 6.6 Hz, lysyl ε-CH_2), 4.08 (dd, 2 H, *J* 3.1, 9.3 Hz, Gal H-3 × 2), 4.23 (dd, 1 H, *J* 5.5, 8.6 Hz, lysyl α-CH), 4.52 (d, 2 H, *J* 7.8 Hz, Gal H-1 × 2), 4.68–4.80 (m, 2 H, GlcNAc H-1 × 2), 4.83 (dd, 2 H, *J* 6.4, 13.3 Hz, Fuc H-5 × 2), 5.12 (d, 2 H, *J* 3.8, Fuc H-1 × 2). ESI–MS: Calcd $C_{83}H_{135}N_{11}O_{53}S_2$ 2197.8. Found singly charged ion as a broad peak enveloping the corresponding isotopic distribution, centered at 2198.

Sialyl Lewis^x tetramer (**15**).—The enzymatic reaction was carried out as for **14** using sialyl LacNAc tetramer **12** (3.5 mg, 0.90 μmol) and GDP-Fuc (2.8 mg, 4.8 μmol). Additional GDP-Fuc (2 mg, 3.4 μmol total) was added after 8 and 20 h. The reaction was monitored and purified as described for **8** except that a Bio-Gel P-10 column (1.5 × 110 cm) was used. Sialyl Le^x tetramer **15** was obtained as a fluffy, white powder in 75% yield (3.0 mg, 0.67 μmol). ^{1}H NMR: δ 1.17 (d, 12 H, 7.0 Hz, Fuc-CH_3 × 4), 1.80 (t, 4 H, *J* 12.0 Hz, Neu5Ac, H-3ax × 4), 2.02 (s, 12 H, NAc × 4), 2.03 (s, 12 H, NAc × 4), 2.38 (t, 2 H, *J* 7.0 Hz, β-alanyl α-CH_2), 2.76 (dd, 4 H, *J* 4.6, 12.7 Hz, Neu5Ac H-3eq × 4), 3.15–3.24 (m, 6 H, lysyl ε-CH_2), 4.08 (dd, 4 H, *J* 3.0, 9.7 Hz, Gal H-3 × 4), 4.18–4.32 (m, 3 H, lysyl α-CH), 4.52 (d, 4 H, *J* 7.9 Hz, Gal H-1 × 4), 4.68–4.79 (m, 4 H, GlcNAc H-1 × 4), 4.83 (dd, 4 H, *J* 6.6, 13.3 Hz, Fuc H-5 × 4), 5.12 (d, 4 H, *J* 4.0 Hz, Fuc H-1 × 4). ESIMS: Calcd

for $C_{169}H_{275}N_{23}O_{105}S_4$ 4434.6. Found doubly and triply charged ions as broad peaks enveloping the corresponding isotopic distribution, centered at 2221 and 1480.

Sialyl Lewis^x octamer (16).—The enzymatic reaction contained sialyl LacNAc octamer **13** (4.9 mg, 0.63 μmol), GDP-Fuc (3.8 mg, 6.4 μmol), CIAP (5 units), concentrated incubation buffer (60 μL) and FucT [60 milliunits in 25 mM sodium cacodylate buffer (600 μL, pH 6.5) containing 5 mM manganese chloride and 25% glycerol]. Additional GDP-Fuc (1 mg, 1.7 μmol) was added after 8 and 20 h, and the desired product was purified as described for **15**. Octavalent sLe^x **16** was obtained as a fluffy, white powder in 74% yield (4.2 mg, 0.47 μmol). ^{1}H NMR: δ 1.18 (d, 24 H, *J* 7.1 Hz, Fuc-CH_3 × 8), 1.80 (t, 8 H, *J* 12.4 Hz, Neu5Ac H-3ax × 8), 2.02 (s, 24 H, NAc × 8), 2.04 (s, 24 H, NAc × 8), 2.36–2.43 (m, 2 H, β-alanyl α-CH_2), 2.76 (dd, 8 H, *J* 4.6, 12.4 Hz, Neu5Ac H-3eq × 8), 3.12–3.24 (m, 14 H, lysyl ε-CH_2), 4.09 (dd, 8 H, *J* 2.8, 9.7 Hz, Gal H-3 × 8), 4.14–4.32 (m, 7 H, lysyl α-CH), 4.53 (d, 8 H, *J* 7.9 Hz, Gal H-1 × 8), 4.68–4.78 (m, 8 H, GlcNAc H-1 × 8), 4.83 (dd, 8 H, *J* 6.6, 13.6 Hz, Fuc H-1 × 8), 5.12 (d, 8 H, *J* 4.0Hz, Fuc H-1 × 8). ESIMS: Calcd for $C_{341}H_{555}N_{47}O_{209}S_8$ 8908.2. Found quadruply charged ion as a broad peak enveloping the corresponding isotopic distribution, centered at 2227.

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