Ligand Recognition by E-Selectin: Synthesis, Inhibitory Activity, and Conformational Analysis of Bivalent Sialyl Lewis x Analogs

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Abstract: Several sialyl Lewis x dimers anchored onto a galactose template or attached to 1,4-butanediol or 1,5-pentanediol have been prepared chemoenzymatically and evaluated as inhibitors of E-selectin-mediated cell adhesion. Two monosaccharide units were simultaneously incorporated (i.e., Gal, NeuAc, Fuc) by a glycosyltransferase into a chemically synthesized core structure containing GlcNAc and Gal. Each of the galactose-anchored dimers had higher activity than the sialyl Lewis x pentasaccharide **1a**, with the general trend being 3,6-linked > 2,3 ≥ 4,6 ≥ 2,6 > monomer. The dimers linked to butanediol or pentanediol showed the same level of activity as the pentasaccharide monomer. Conformational analysis of these dimers with NMR indicated that each sialyl Lewis x domain of the dimers retains the same conformation as the monomer. The differences in activity of the dimers most likely derive from differences in the relative orientation and distance between the monomer domains, suggesting the importance of the linker used in the preparation of dimers.

Carbohydrate-mediated cell adhesion is an important event initiated by tissue injury or infection and is involved in cancer metastasis.[1] One such recently discovered adhesion process is the interaction between the glycoprotein E-selectin (formerly called endothelial leukocyte adhesion molecule-1 or ELAM-1[1e]) on the surface of activated endothelial cells and an oligosaccharide structure displayed on the surface of neutrophils (Figure 1). The ligand recognized by E-selectin has been identified as the tetrasaccharide sialyl Lewis x (SLe^x, **1a**).[2] Recent studies of the inhibition of E-selectin indicate that, in addition to SLe^x,[2a] several related structures including the glucose analog **1b**,[3a] sialyl Lewis A **2**,[3b] Le^x-3′-O-sulfate **3**,[3c] and sialyl Le^x glycal **4**[3d] (Figure 2a) have similar inhibition activities for E-selectin binding, with the IC_{50} values in the range of 1−2 mM[3d] based on an ELISA assay.

Conformational analysis of **1**−**4** indicates that these oligosaccharides possess a common topostructure in the space composed of Neu5Ac, Gal, and Fuc,[3d,4] suggesting that the E-selectin binding domain of sialyl Le^x may reside on the surface illustrated by **5**. The bivalent sialyl Le^x (**6**) is, however, about 5-fold better than SLe^x as an inhibitor of E-selectin, suggesting a possible multivalent ligand−receptor interaction.[3d] This is further supported by the observation in a preliminary study that the conformations[3d] of the two SLe^x units are essentially the same as monomeric SLe^x. Although the affinity of SLe^x for E-selectin is relatively weak *in vitro*, the IC_{50} *in vivo* for protecting against lung injury in rats is about 1 μM,[5] suggesting that the inhibition analysis *in vitro* does not reflect the activity *in vivo*. This high *in vivo* activity and the available large-scale chemo-enzymatic synthesis[4] of SLe^x, however, has led to the development of SLe^x as a potential new antiinflammatory agent.[5] Since the bivalent SLe^x derivative is more active than the monomer investigations of the activities of other multivalent analogs were undertaken with the hope that more effective antagonists could be developed. As part of our interest in further understanding the nature of E-selectin ligand recognition, we report here the syntheses of several bivalent SLe^x analogs with different spacer groups and analyses of their inhibition activities. Efforts to correlate

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(3) (a) Tyrrell, D.; James, P.; Rao, N.; Foxall, C.; Abbas, S.; Dasgupta, F.; Nashed, M.; Hasegawa, A.; Kiso, M.; Asa, D.; Kidd, J.; Brandley, B. K. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 10372. (b) Berg, E. L.; Robinson, M. K.; Mansson, O.; Butcher, E. C.; Magnani, J. L. *J. Biol. Chem.* **1991**, *266*, 14869. (c) Yuen, C.-T.; Lawson, A. M.; Chai, W.; Larkin, M.; Stoll, M. S.; Stuart, A. C.; Sullivan, F. X.; Ahern, T. J.; Feizi, T. *Biochemistry* **1992**, *31*, 9126. (d) DeFrees, S. A.; Gaeta, F. C. A.; Lin, Y.-C.; Ichikawa, Y.; Wong, C.-H. *J. Am. Chem. Soc.* **1993**, *115*, 7549.

(4) Ichikawa, Y.; Lin, Y.-C.; Dumas, D. P.; Shen, G.-J.; Garcia-Junceda, E.; Williams, M. A.; Bayer, R.; Ketcham, C.; Walker, L. E.; Paulson, J. C.; Wong, C.-H. *J. Am. Chem. Soc.* **1992**, *114*, 9283. Both galactosylation and sialylation have been carried out on 0.5−1 kg scales using galactosyltransferase and sialyltransferase coupled with regeneration of the corresponding nucleoside phosphates. The fucosylation step is currently performed chemically in large-scale processes. Work is in progress to prepare recombinant GDP-fucose pyrophosphorylase for the large-scale regeneration of GDP-fucose.

(5) Mulligan, M. S.; Paulson, J. C.; DeFrees, S.; Zheng, Z.-L.; Lowe, J. B.; Ward, P. A. *Nature* **1993**, *364*, 149.

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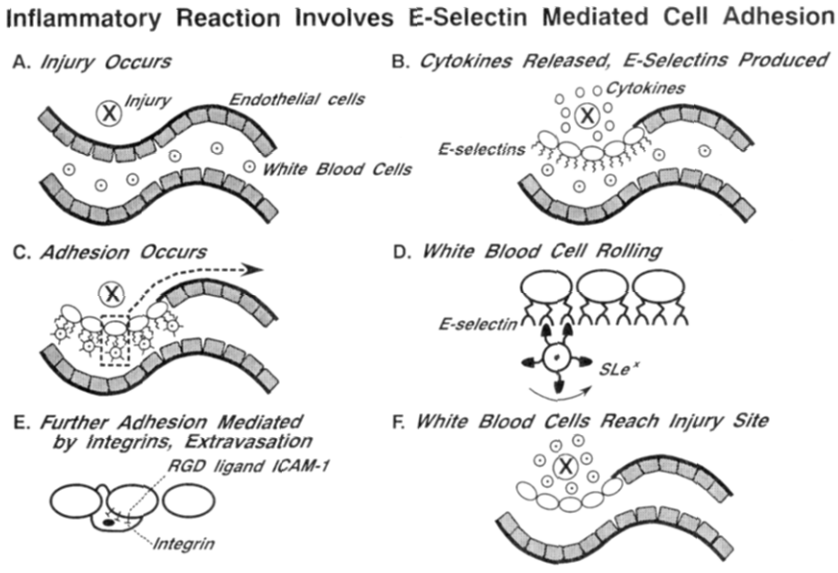

Figure 1. Inflammatory reaction involves E-selectin-mediated adhesion. When tissue injury occurs (A), cytokines are released to signal E-selectin synthesis (B). Adhesion then occurs by interaction of E-selectin and SLe^x on leukocytes. As leukocytes roll along the surface of endothelial cells, further adhesion occurs by interactions between integrins on leukocytes and the Arg-Gly-Asp (RDG)-containing ligand within intercellular adhesion molecule (ICAM-1) to enable leukocytes to reach the site of injury. If too many leukocytes are recruited to the site of injury, normal cells can also be damaged.

activity with 3-dimensional conformation, as determined by NMR, are also described.

Results and Discussion

A. Synthesis of Dimeric Sialyl Lewis X Derivatives. The dimeric sialyl Lewis X derivatives were synthesized using a combined chemical and enzymatic route.[3d,6] Small acceptor primers were chemically synthesized, and the peripheral carbohydrate residues were added enzymatically. The synthesis of the primer for the dimer **6**, which contains a somewhat rigid galactose unit as a template, is shown in Scheme 1. The protected galactose derivative **7**[7,8] was selectively benzoylated at O-2[9] in a three-step procedure to give **8**,[8] which was subsequently glycosylated with a phthalimido-protected glucosamine to give the disaccharide **9**. The benzylidene was hydrolyzed to give the diol **10**, which was regioselectively glycosylated at O-6 to give the trisaccharide **11**.[10,11] Hydrazinolysis of **11** to remove the phthalimide and ester protecting groups followed by acetylation gave compound **12**. Finally, saponification of the O-acetates provided **13**,[8] the primer for the following enzymatic glycosylations.

Scheme 2 illustrates the synthesis of the dimeric SLe^x **6**[3d] from **13**. Treatment of **13** with β-1,4-galactosyltransferase and 2 equiv of UDP-Gal gave the pentasaccharide **14**. In a similar manner, two sialic acid residues were added with N-type α-2,3-sialyltransferase (EC 2.4.99.6) to give **15** (80% isolated yield). Alkaline phosphatase was added to hydrolyze the product phosphoester CMP and thus drive the reaction to completion.[12] Monitoring the reaction by TLC suggested that the incorporation of the first sialic acid was rapid, but that the incorporation of the second was relatively slow. Finally, two fucose residues were added with α-1,3-fucosyltransferase V[13] to give **6** as the ammonium salt (84% isolated yield). The dimers **16**−**19** (Figure 2b) were prepared by doubly sialylating and fucosylating previously reported dimeric *N*-acetyllactosamine derivatives (see Experimental Section).[14] It is noted that fucosylation of **36** only gives a mixture of mono- and difucosylated products which are not separable. Compound **19** was therefore not evaluated for its activity.

The SLe^x dimers having flexible butanediol and pentanediol templates were synthesized in an analogous manner. The chemical synthesis of the requisite primers is shown in Scheme 3. Glycosylation of either 1,4-butanediol or 1,5-pentanediol with 2 equiv of acetobromogalactose gave **20a** and **20b**, respectively. Deacetylation afforded **21a** and **21b**, each of which was regioselectively acetylated at the 2- and 6-positions of galactose, *via* the intermediate isopropylidene,[14] to give **22a** and **22b**, respectively. Glycosylation of **22a** with 2 equiv of 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-D-glucopyranosyl bromide gave the bis(disaccharide) **23**. Similarly, glycosylation of **22b** with a protected lactosamine donor provided **24**.

Hydrazinolysis and acetylation of **23** gave **25**, which was then deacetylated to give **26** (Scheme 4). Sequential enzymatic

(6) For recent reviews on chemo-enzymatic synthesis of complex carbohydrates: (a) Tonne, E. J.; Simon, E. S.; Bednarski, M. D.; Whitesides, G. M. *Tetrahedron* **1989**, *45*, 5365. (b) Drueckhammer, D. G.; Hennen, W. J.; Pederson, R. L.; Barbas, C. F., III; Gautheron, C. M.; Krach, T.; Wong, C.-H. *Synthesis* **1991**, 499. (c) Hindsgaul, O. *Sem. Cell Biol.* **1991**, *2*, 319. (d) Look, G. C.; Ichikawa, Y.; Wong, C.-H. *Anal. Biochem.* **1992**, *202*, 215. (e) Ito, Y.; Gaudino, J. J.; Paulson, J. C. *Pure Appl. Chem.* **1993**, *65*, 753. (f) Wong, C.-H.; Halcomb, R. L.; Ichikawa, Y.; Kajimoto, T. *Angew. Chem., Int. Ed. Engl.*, in press.

(7) Wallenfels, von K.; Lehmann, J. *Liebigs Ann. Chem.* **1960**, *635*, 166.

(8) Lemieux, R. U.; Abbas, S. Z.; Chung, B. Y. *Can. J. Chem.* **1981**, *60*, 68.

(9) The position of the 2-benzoate in **8** was verified by adding trichloroacetyl isocyanate to the NMR sample ($CDCl_3$) to form **8a**. The spectrum contained a doublet of a doublet at 5.27 ppm (J = 8.0 and 2.3 Hz) typical of the H-3 of galactose.

(10) Lemieux, R. U.; Takeda, T.; Chung, B. Y. *ACS Symp. Ser.* **1976**, *39*, 90.

(11) The position of substitution was verified by reacting **11** with trichloroacetyl isocyanate in the NMR tube ($CDCl_3$) to produce **11a**. A downfield shift of a new peak to 5.39 ppm with two small coupling constants (J = 2.1 Hz) was observed indicating that the 4-position proton of the ethyl galactoside was unsubstituted.

(12) (a) Sabesan, S.; Paulson, J. C. *J. Am. Chem. Soc.* **1986**, *108*, 2068. (b) Unverzagt, C.; Kunz, H.; Paulson, J. C. *J. Am. Chem. Soc.* **1990**, *112*, 9308.

(13) Weston, B. W.; Nair, R. P.; Larsen, R. D.; Lowe, J. B. *J. Biol. Chem.* **1992**, *267*, 4152.

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(15) (a) Paulsen, H.; Paal, M. *Carbohydr. Res.* **1985**, *137*, 39. (b) Paulsen, H.; Paal, M.; Hadamczyk, D.; Steiger, K.-M. *Carbohydr. Res.* **1984**, *131*, C1.

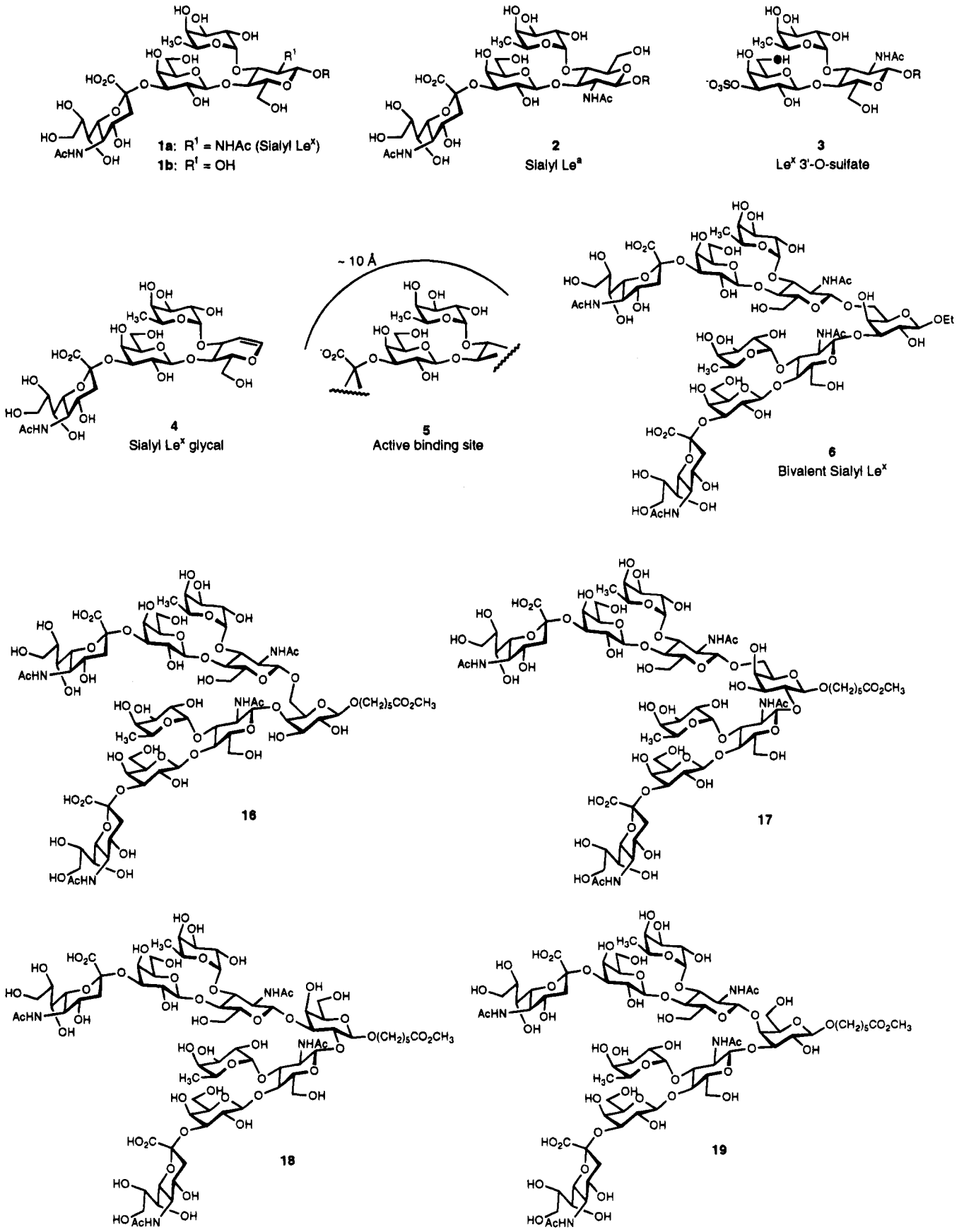
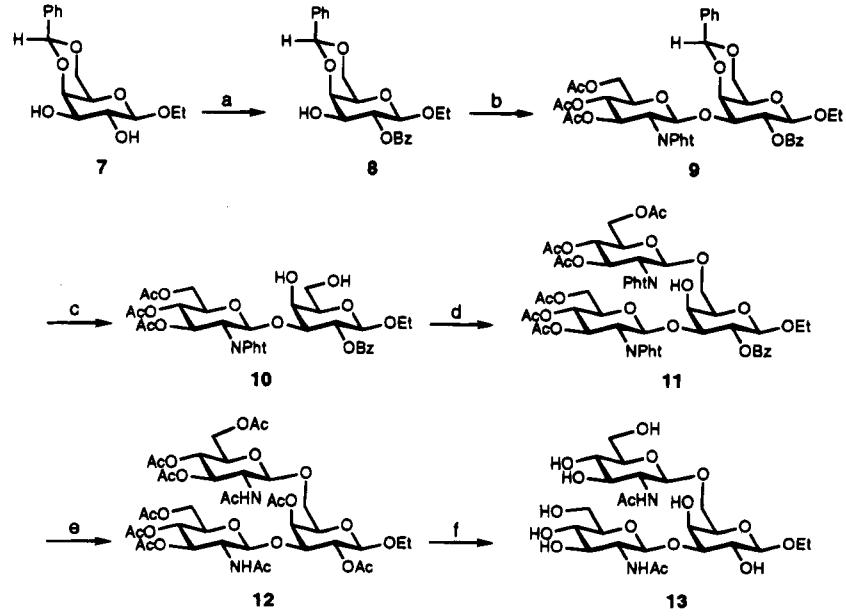


Figure 2. Ligands for E-selectin (**1-6**) and (b) Sialyl Lewis x dimers (**16**–**19**).

transfer of galactose, NeuAc, and fucose residues gave **29** via the intermediates **27** and **28**. Similarly, compound **24** was converted to the dimer **33** *via* the bis(tetrasaccharide) **32** (not shown) as outlined in Scheme 5.

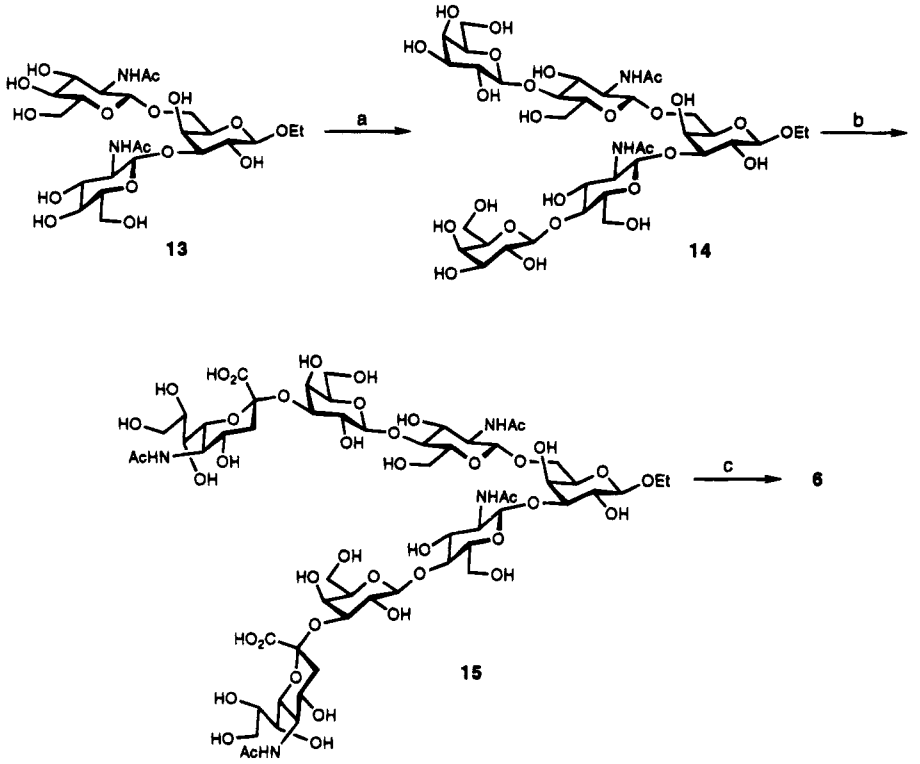
B. Biological Activity of SLe^x Dimers. A soluble form of E-selectin (sol-E-selectin) was prepared for inhibition assays of the dimeric SLe^x derivatives. A 1.67 kbp DNA fragment encoding a truncated structural gene for E-selectin was isolated

Scheme 1[a]



[a] Key: (a) (i) $(ClCH_2CO)_2O$, pyridine, CH_2Cl_2, −65 °C; (ii) PhCOCl, −65 to 25 °C; (iii) NH_3 in MeOH, MeOH, −30 °C (48% overall); (b) 2-deoxy-2-phthalimido-2,4,6-tri-*O*-acetyl-β-D-glycopyranosyl bromide, AgOTf, collidine, CH_2Cl_2, −20 °C (86%); (c) AcOH, H_2O, 80 °C (80%); (d) 2-deoxy-2-phthalimido-2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl bromide, AgOTf, collidine, CH_2Cl_2, −20 °C (86%); (e) (i) $H_2NNH_2-H_2O$, EtOH, reflux; (ii) Ac_2O, pyridine, DMAP (76%); (f) NaOMe, MeOH (70%).

Scheme 2[a]

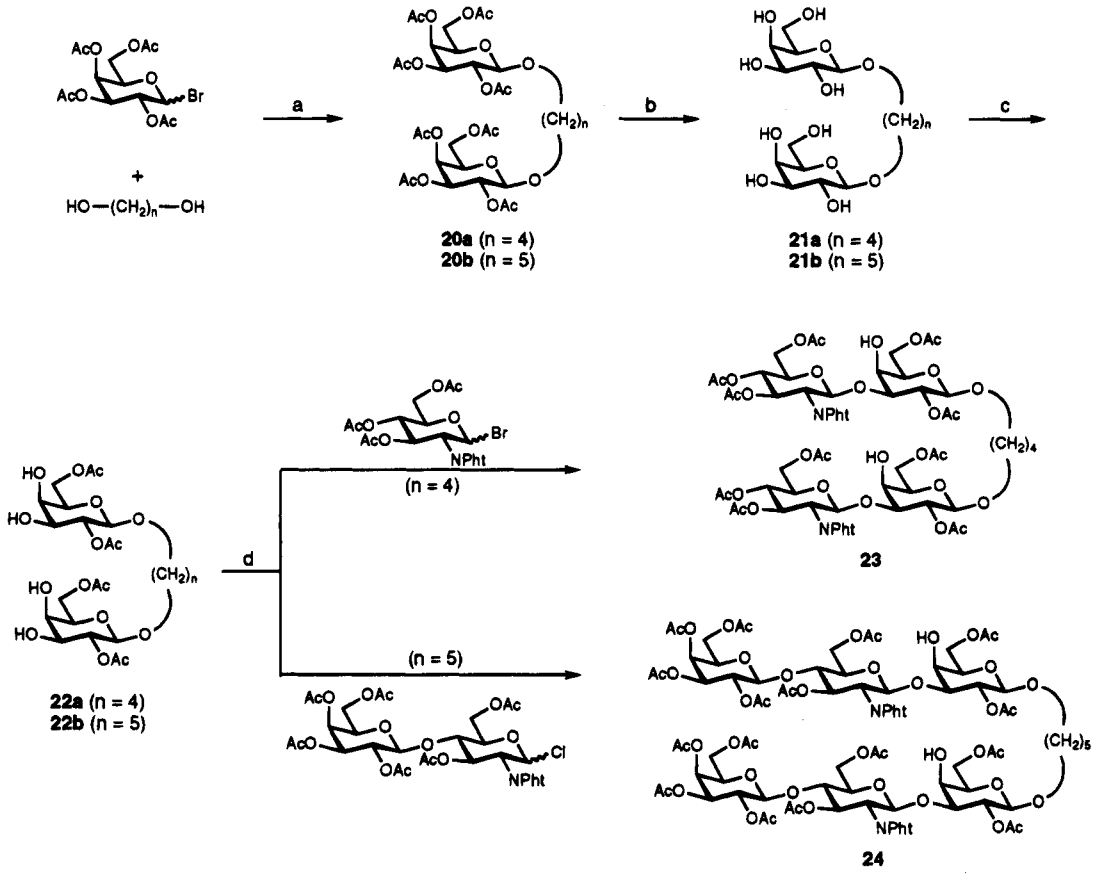


[a] Key: (a) UDP-glucose, uridine 5′-diiphosphogalactose 4′-epimerase, β-1,4-galactosyl transferase, sodium cacodylate (0.2 M, pH 7.5), $MnCl_2$ (85%); (b) CMP-*N*-acetylneuraminic acid, α-2,3-sialyltransferase, sodium cacodylate (0.2 M, pH 6.5), $MnCl_2$ (82%); (c) GDP-fucose, fucosyltransferase V, sodium cacodylate (0.2 M, pH 6.5), $MnCl_2$ (84%).

by PCR amplification of cDNA derived from messenger RNA that was isolated from IL-1 activated human endothelial cells. The cDNA was subcloned into the vector pBluescript II and was transfected into 293 cells. The clones were screened for the production of sol-E-selectin, and the clone 293#3 was selected as the stable cell line that produced the greatest amount of sol-E-selectin per cell. Sol-E-selectin was produced on a large scale from this line using a Nunc cell factory. Recombinant sol-E-selectin was isolated from the media using immunoaffinity chromatography.

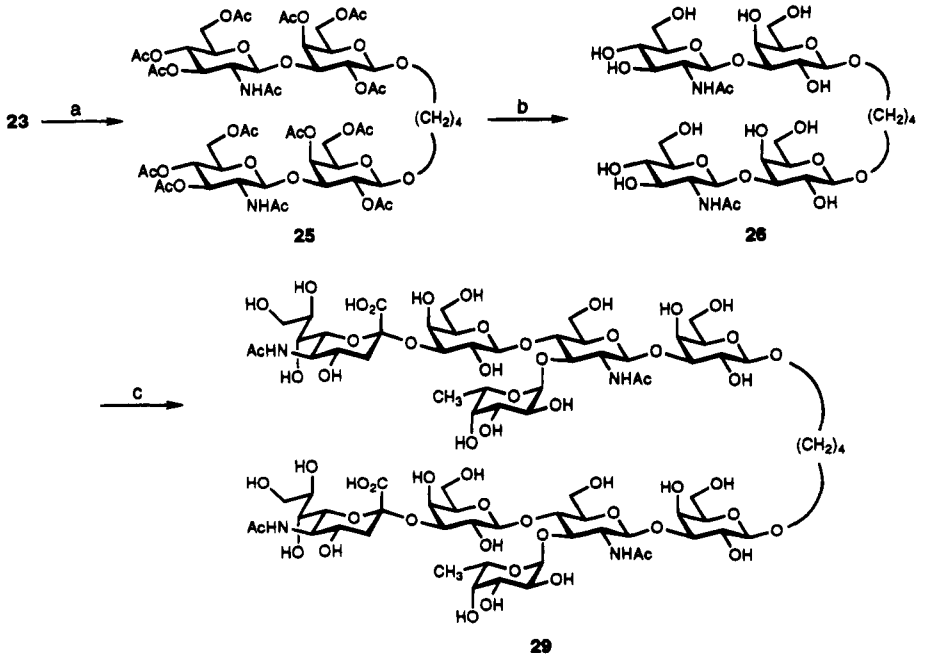
The SLe^x dimers were assayed for ability to block the adhesion of HL-60 cells to immobilized sol-E-selectin. Immobilized E-selectin was incubated first with inhibitor and then with HL-60 cells. The bound cells were lysed, and myeloperoxidase released from the bound cells was detected with *o*-phenylenediamine and hydrogen peroxide. The percentage

Scheme 3[a]



[a] Key: (a) Ag_2CO_3, CH_2Cl_2, −20 °C (68−73%); (b) NaOMe, MeOH (92%); (c) (i) 2,2-dimethoxypropane, acetone, DMF, reflux; (ii) Ac_2O, pyridine; (iii) AcOH, H_2O, reflux (28−38% overall); (d) AgOTf, CH_2Cl_2, −20 °C (**23**: 52%, **24**: 40%).

Scheme 4[a]

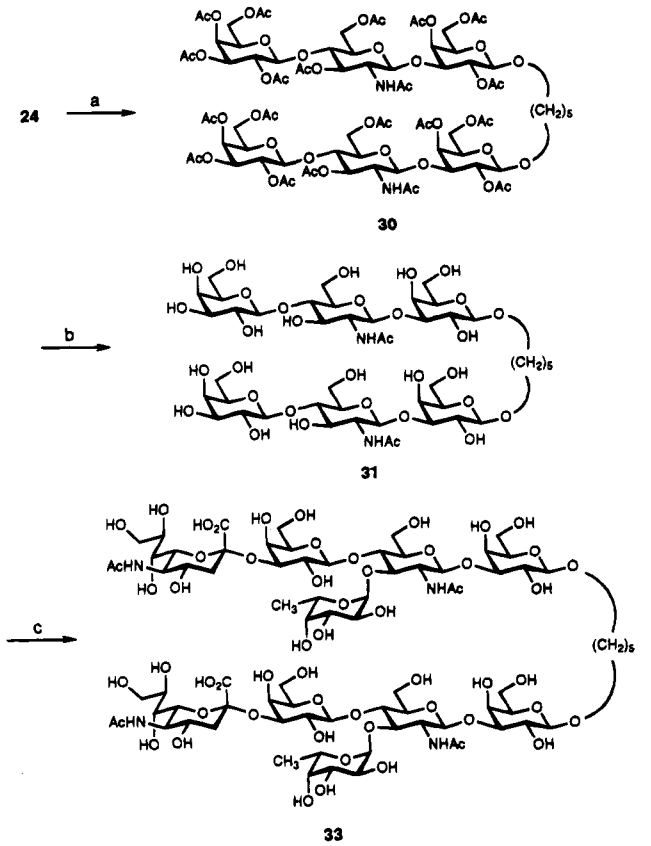


[a] Key: (a) (i) $H_2NNH_2-H_2O$, EtOH, reflux; (ii) Ac_2O, pyridine (57%); (b) NaOMe, MeOH (96%); (c) (i) 2 equiv of UDP-glucose, uridine 5′-diiphosphogalactose 4′-epimerase, β-1,4-galactosyl transferase (**27**: 91%); (ii) 2 equiv of CMP-*N*-acetylneuraminic acid, α-2,3-sialyltransferase (**28**: 93%); (iii) 2 equiv of GDP-fucose, α-1,3-fucosyltransferase (**29**: 93%).

inhibition was determined by comparing the absorbance of the resulting solution at 492 nm to that in wells containing no inhibitor.

Each dimer tested contained an SLe^x domain linked to each of two positions of a galactose template. A pentasaccharide consisting of an SLe^x linked to the 3 position of ethyl

Scheme 5[a]



[a] Key: (a) (i) $H_2NNH_2-H_2O$, EtOH, reflux; (ii) Ac_2O, pyridine (86%); (b) NaOMe, MeOH (96%); (c) (i) 2 equiv of CMP-*N*-acetylneuraminic acid, α-2,3-sialyltransferase (**32**: 97%); (ii) 2 equiv of GDP-fucose, α-1,3-fucosyltransferase (**33**: 89%).

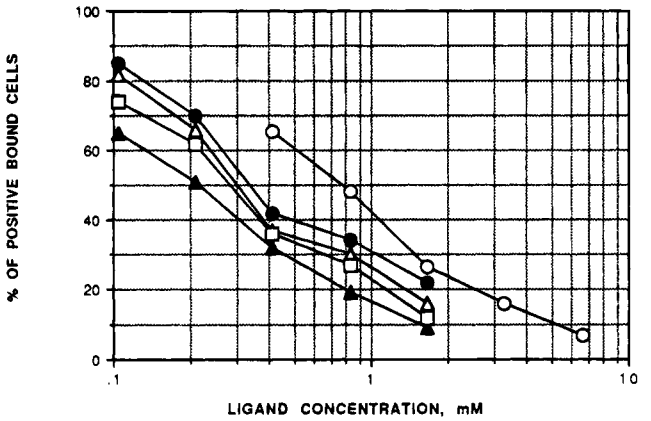


Figure 3. Inhibition of HL-60 adhesion to recombinant soluble E-selectin-coated plates: (○) pentasaccharide, NeuAcα2,3-Galβ1,4-(Fucα1,3-)GlcNAcβ1,4-GalβOEt; (▲) 3,6-dimer **6**; (△) 4,6-dimer **16**; (●) 2,6-dimer **17**; (□) 2,3-dimer **18**. Compounds **29** and **33** exhibited similar activities as the pentasaccharide.

β-galactoside was used as a monomer standard. As is shown in Figure 3, each of the five dimers was more potent than the monomer in blocking adhesion. The 3,6-linked SLe^x dimer **6** was the most potent, with the general trend being 3,6-linked > 2,3 ≥ 4,6 > 2,6 > monomer. The dimers attached to 1,4-butanediol (**29**) or 1,5-pentanediol (**33**) exhibited the same level of activity as the monomer. The concentrations of the dimers required to block adhesion of 50% of the cells ranged from 0.2 mM (for **6**) to 0.45 mM. As references, the value for the pentasaccharide monomer was 0.8 mM and that for the tetrasaccharide monomer was 1.2 mM.[3d,4]

As has been demonstrated in other systems,[14,16] multivalent display of carbohydrate ligands often results in higher affinities for target protein receptors. The same principle appears to be true for the SLe^x–E-selectin interaction. Dimeric SLe^x derivatives, as illustrated here, can have affinities for E-selectin which are up to 5-fold greater than that of the monomer. There are also subtle differences in the inhibitory properties within the series of dimers, so factors other than simple multivalency must also be considered. Perhaps some of the dimers (e.g., **29** and **33**) still exhibit monovalent activity. In the case of hemagglutinin inhibition, dimeric sialic acids exhibited 0–10-fold increased inhibitory activity, depending on the nature of the linker used in the design.[14] It is, however, noted that the assay used in hemagglutinin inhibition analysis is quite different from the E-selectin assay, which is not read out as a plus or minus for each data point. Each data point in the E-selectin assay is a direct measure of cells bound using a quantitative enzyme assay. The values were then plotted to give the titration curve and IC^{50} values. Five assays were performed for each inhibitor, and the results were highly reproducible. The standard deviation did not exceed ±6.3% for any data point. The IC_{50} value reported here is derived from all the data, not by a titration end point as in hemagglutinin.

C. Conformational Analysis of SLe^x Dimers by NMR. We have previously reported preliminary evidence that each SLe^x domain of **6** exists in the same conformation as in **1a**. The same phenomenon was predicted for each of the other dimers. Therefore, it was tempting to speculate that the differences in the inhibition properties of each dimer are due to differences in the relative orientation of the SLe^x domains in each respective dimer rather than changes in these domains. Validation of this hypothesis, however, requires a detailed conformational analysis of each compound. As a first step toward this objective, we have carried out NMR studies of selected dimers. We report the complete assignment of the proton and carbon resonances of **6** and some 2D NMR experiments with **16** and **17**.

Although the 1D ^1H NMR spectrum of the dimer **6** was quite complex, certain key proton resonances were sufficiently resolved so as to allow the use of various 2D techniques to make a complete proton and carbon assignment. Protons were assigned in a manner similar to that reported for the SLe^x monomer,[4] making use of PE-COSY, ROESY, TOCSY, HMBC, and HMQC techniques.

The anomeric proton of the template Gal was identified by the presence of an ROESY to one of the methylene protons of the aglyconic ethyl group. This permitted identification and assignment of all of the protons of this Gal spin system. Each of the GlcNAc anomeric protons was identified by the presence of ROE's to the template Gal residue, particularly an ROE between H-1 of the 3-*O*-GlcNAc and H-3 of the template Gal, and between H-1 of the 6-*O*-GlcNAc and H-6 of Gal. After identification of these key protons, all of the resonances of each GlcNAc spin system were assigned using HMBC, HMQC, and COSY (Figures 4 and 5). The protons of the Fuc, Gal, and NeuAc residues of the SLe^x domains were assigned in a similar manner, employing techniques previously used by our group

(16) (a) Spaltensin, A.; Whitesides, G. M. *J. Am. Chem. Soc.* **1991**, *113*, 686. (b) Glick, G. D.; Toogood, P. L.; Wiley, D. C.; Skehel, J. J.; Knowles, J. R. *J. Biol. Chem.* **1991**, 266, 23660. (c) Conolly, D. T.; Townsend, R. R.; Kawaguchi, K.; Bell, W. R.; Lee, Y. C. *J. Biol. Chem.* **1982**, *257*, 939. (d) Weis, W. J.; Drickamer, K.; Hendrickson, W. A. *Nature* **1992**, *360*, 127. (e) Kingery-Wood, J. E.; Williams, K. W.; Sigal, G. B.; Whitesides, G. M. *J. Am. Chem. Soc.* **1992**, *114*, 7303. (f) Spevak, W.; Nagy, J. O.; Charych, D. H.; Schaefer, M. E.; Gilbert, J. H.; Bednarski, M. D. *J. Am. Chem. Soc.* **1993**, *115*, 1147. (g) Roy, R.; Pon, R. A.; Tropper, F. D.; Andersson, F. O. *J. Chem. Soc., Chem. Commun.* **1993**, 264.

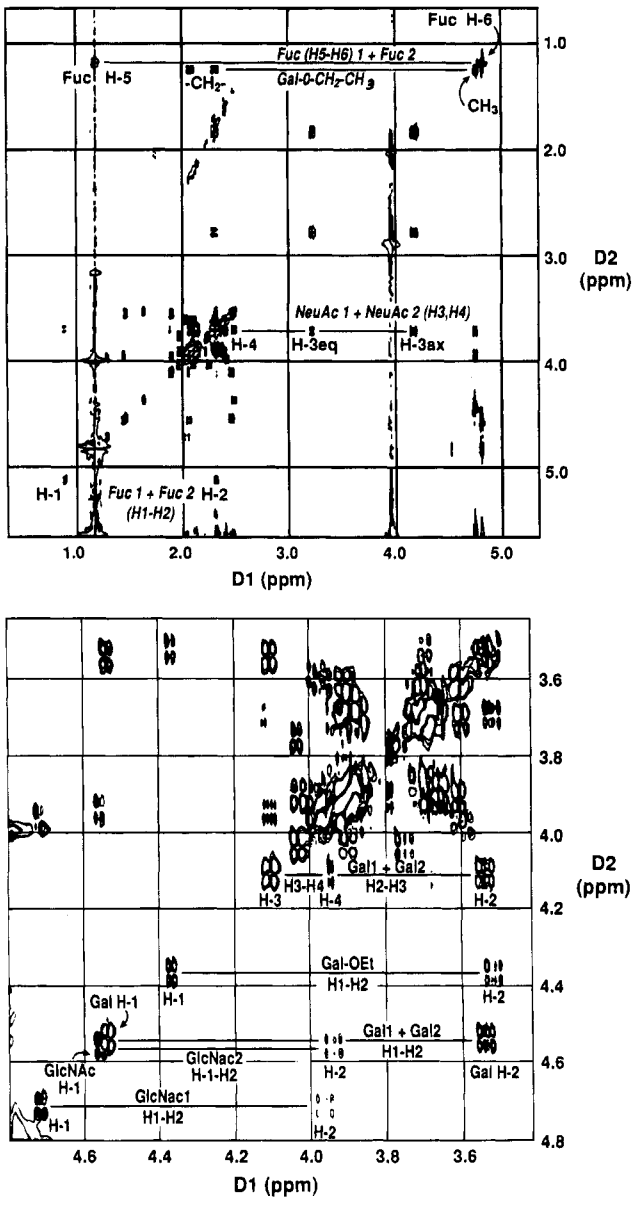


Figure 4. (Top) 2D phase-sensitive double quantum filter COSY (COSYDFPT) of **6**. (Bottom) expansion of 2D COSYDFPT of **6**.

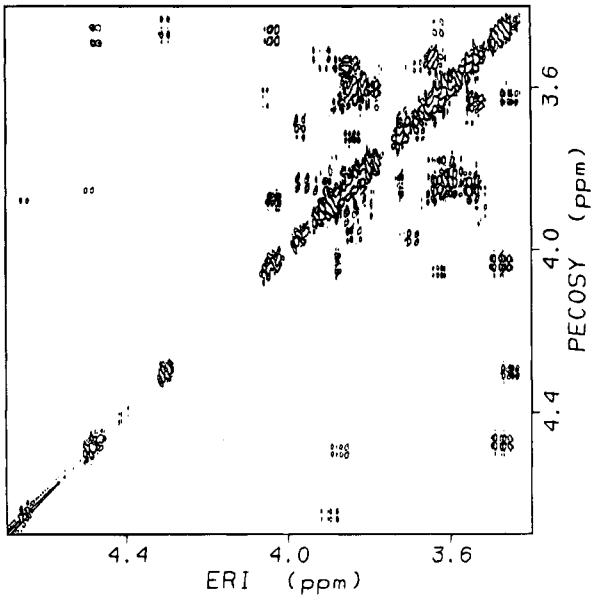


Figure 5. PECOSY spectrum of **6**.

to assign the monomer[4] (see Table 1 for complete assignment). No attempt was made to distinguish between the corresponding resonances of each of the two Fuc, Gal, and NeuAc residues due to the coincidence of most of the signals.

One conclusion that can be drawn upon examination of the ROESY spectrum of **6** (Figure 6) is that each SLe^x domain exists in basically the same conformation as monomeric SLe^x. The presence of cross peaks between Fuc H-5 and Gal H-2, between Fuc H-6 and Gal H-2, and between NeuAc H-2_{ax} and Gal H-3 are consistent with those observed for the monomer. Additionally, several ROESY cross-peaks from each GlcNAc to the template Gal were apparent. A detailed analysis of these cross peaks should permit the determination of the relative orientation and distance between each respective SLe^x domain and is the subject of current study.

We have carried out experiments with dimers **16** and **17**. The ROESY spectra of **16** and **17** (data not shown) suggest that the SLe^x regions of these compounds also exist in the same conformation as **1a**, thus supporting our hypothesis.

Summary. In conclusion, several SLe^x dimers were synthesized and evaluated as inhibitors of E-selectin-mediated cell adhesion. Each of the dimers had greater activity than **1a** according to the assay used, with the general trend being 3,6-linked > 2,3 ≥ 4,6 > 2,6 > monomer. No increase of activity was observed for the dimers linked by butane- or pentanediol. A general conclusion that can be drawn from these results is that multivalent display of the SLe^x ligand leads to increased activity in certain cases. There are, however, slight differences in the activity of each dimer. On the basis of NMR analysis, each SLe^x domain of **6** retains the same conformation as the monomer. The results of inhibitory and conformational analysis indicate that **6** interacts with two adjacent E-selectin molecules. The same situation is predicted for each of the other dimers based on our inhibitory analysis and preliminary NMR results. Therefore, the differences in activity of the dimers most likely derives from differences in the relative orientation and distance between the SLe^x domains. How changes in this orientation and spacing are manifested in differences in the mode of inhibition of cell adhesion remains to be determined, and are the subject of ongoing research.

Experimental Section

Synthesis. General. All reactions were monitored by thin layer chromatography carried out on 0.25 mm Whatman silica gel plates (60F-254) using UV light and anisaldehyde reagent as developing agent (Gordan, A. J.; Ford, R. A. *The Chemists Companion*; Wiley-Interscience: New York, 1972; p 377). E. Merck silica gel (60, particle size 0.040-0.063 mm) was used for flash column chromatography.

All reactions were carried out under an argon atmosphere and with anhydrous solvents from Aldrich unless otherwise noted. Yields refer to chromatographically and spectroscopically (^{1}H NMR) homogeneous materials unless otherwise stated.

The UDP-glucose, CMP-sialic acid, uridine 5′-diphosphogalactose 4-epimerase, and β1,4-galactosyltransferase were purchased from Sigma. The GDP-β-fucose was purchased from Oxford Glycosystems. The N-type α(2,3)-sialyl transferase and fucosyl transferase V enzymes were prepared at Cytel Corp.

Ethyl 4,6-*O*-Benzylidene-2-*O*-benzoyl-β-D-galactopyranoside (8) and Ethyl 4,6-*O*-Benzylidene-3-*O*-((trichloroacetyl)-*N*-carbamoyl)-2-*O*-benzoyl-β-D-galactopyranoside (8a). Ethyl 4,6-*O*-benzylidene-β-D-galactopyranoside (**7**)[7] (2.9 g, 9.8 mmol) was dissolved in CH_2Cl_2 (29 mL) and pyridine (5.4 mL) and cooled to −65 °C under an argon atmosphere. Chloroacetic anhydride (0.82 mL, 10.3 mmol) was then added and the reaction mixture stirred at −65 °C for 2 h. The first stage of the reaction was complete at this time, and thus benzoyl chloride (1.36 mL, 11.78 mmol) was added at −65 °C. The reaction

Table 1. Complete Chemical Shift Assignment of **6**

	NeuAc		Gal		Fuc		GlcNAc		branch Gal	
carbon no.	H	C	H	C	H	C	H	C	H	C
1		1.73	4.48	102.0	5.06	99.2	4.50	103.1	4.30	103.0
		1.73	4.48	102.1	5.05	99.2	4.65	103.1		
2		100.28	3.47	69.95	3.62	67.3	3.87	55.3	3.45	70.06
		100.28	3.47	69.95	3.62	67.3	3.89	55.3		
3	1.74, 2.70	40.3	4.03	76.3	3.83	68.8	3.83	74.5	3.63	82.86
	1.74, 2.70	40.3	76.3	3.83	68.8	3.83	74.5			
4	3.63	69.0	3.88	66.9	3.71	71.5	3.93	73.71	4.06	69.1
	3.63	69.0	3.88	66.9	3.72	71.5	3.93	73.71		
5	3.80	52.2	3.51	75.58	4.77	67.3	3.54	68.7	3.71	70.2
	3.80	52.2	3.51	75.58	4.77	67.3	3.54	68.7		
6	3.65	72.6	3.63	62.08	1.10	15.8	3.82, 3.85	60.2	3.70, 3.97	70.13
	3.65	72.6	3.63	62.08	1.10	15.8	3.82, 3.85	60.2		
7	3.59	67.7								
	3.59	67.7]								
8	3.90	71.4								
	3.90	71.4								
9	3.59, 3.82	63.7								
	3.59, 3.82	63.7								
									OCH_2CH_3	
CH_3	2.03	21.7					2.01	21.9	3.65	66.59
	2.03	21.7					2.01	21.9	3.88	
C=O		175						173.7	1.17	14.8
								173.4		

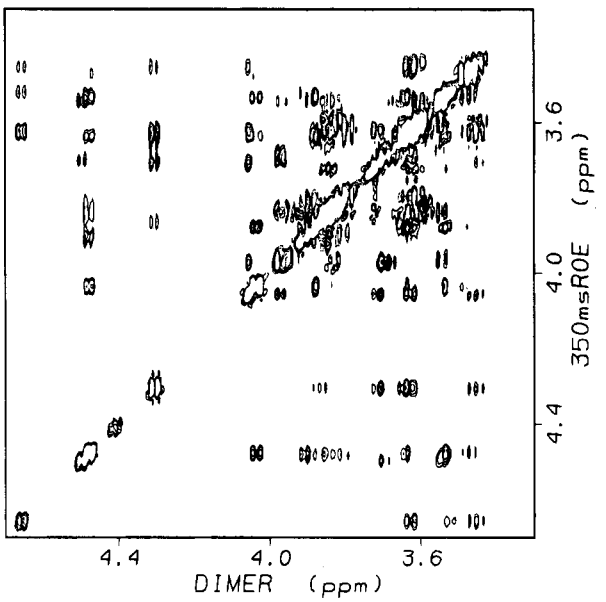


Figure 6. ROESY spectrum of **6** (mixing time 350 ms).

mixture was allowed to warm to room temperature. The mixture was stirred overnight and then diluted with CH_2Cl_2 (100 mL) and washed with 1 M citric acid (100 mL), water (50 mL), saturated $NaHCO_3$ (50 mL), water (50 mL), and saturated NaCl (50 mL). The organic layer was dried (Na_2SO_4), filtered, and concentrated to afford crude **8**. This material was then dissolved in methanol (50 mL) and cooled to −30 °C. A solution of 2 M NH_3 in methanol (6.0 mL) was added, and the reaction mixture was stirred at −30 °C for 4 h. The resulting mixture was poured into CH_2Cl_2 (100 mL) and washed with water (100 mL), saturated $NaHCO_3$ (100 mL), water (100 mL), and saturated NaCl (50 mL). The organic layer was dried (Na_2SO_4), filtered, concentrated, and chromatographed (silica, 80% ethyl acetate/hexane) to afford 1.7 g (48%) of **6** as a white solid: $R_f = 0.3$ (silica, ethyl acetate/hexane); ^{1}H NMR ($CDCl_3$) δ 8.07 (d, J = 7.5 Hz, 2H, arom), 7.55 (m, 3H, arom), 7.46 (d, J = 7.5 Hz, 2H, arom), 7.38 (m, 3H, arom), 5.59 (s, 1H, benzylidene), 5.36 (dd, J = 10.07, 8.2 Hz, 1H, H-2), 4.64 (d, J = 8.2 Hz, 1H, H-1), 4.39 (dd, J = 12.5, 1.1 Hz, 1H, H-6), 4.27 (d, J = 4.0 Hz, 1H, H-4), 4.12 (dd, J = 1.5, 12.5 Hz, 1H, H-6), 3.99−3.86 (multiple peaks, 2H, OCH_2CH_3, H-3), 3.61 (m, 1H, OCH_2CH_3), 3.56 (bs, 1H, H-5), 2.63 (d, J = 9.0 Hz, 1H, OH), 1.21 (t, 3H, CH_3). Trichloroacetyl isocyanate was then added to the NMR sample to form **8a**. A new low-field signal was observed at 5.27 ppm (J = 3.1, 9.7 Hz) typical for the H-3 of galactose. This confirmed that the H-3 position of **8** was unsubstituted.

Ethyl 4,6-*O*-Benzylidene-2-*O*-benzoyl-3-*O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-β-D-galactopyranoside (9). A suspension of molecular sieves (4 Å, 1 g), collidine (0.167 mL, 1.26 mmol), silver triflate (0.3 g, 1.16 mmol), and **8** (0.71 g, 0.974 mmol) in CH_2Cl_2 (10 mL) was stirred under argon for 1 h. The reaction mixture was then cooled to −20 °C and a solution of 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl bromide[3] (0.53 g, 1.07 mmole) in CH_2Cl_2 (1 mL) was added. The reaction mixture was stirred at −20 °C for 30 min, allowed to warm to room temperature, and filtered through a Celite pad. The filtrate was diluted with ethyl acetate (100 mL), washed with water (50 mL), 1 M citric acid (100 mL), water (50 mL), saturated $NaHCO_3$ (100 mL), water (50 mL), and saturated NaCl (50 mL), dried (Na_2SO_4), and concentrated. The residue was purified by chromatography (silica, 40% ethyl acetate/toluene) to afford 0.967 g (86%) of **9** as a white solid: $R_f = 0.4$ (silica, 70% ethyl acetate/hexane); ^{1}H NMR ($CDCl_3$) δ 7.65 (dd, J = 2.9, 7.5 Hz, 2H, arom), 7.54 (dd, J = 7.6, 1.8 Hz, 2H, arom), 7.50 (dd, J = 7.0, 7.0, 2H, arom), 7.45−7.31 (m, 6H, arom), 7.28 (d, J = 9.7 Hz, 1H, arom), 7.25 (d, J = 7.1 Hz, 1H, arom), 5.65 (dd, J = 9.4, 10.8 Hz, 1H, H-3 GlcN), 5.61 (d, J = 8.6 Hz, 1H, H-1 GlcN), 5.57 (s, 1H, benzylidene), 5.38 (dd, J = 8.0, 10.1 Hz, 1H, H-2 Gal), 5.16 (dd, J = 9.6, 9.6 Hz, 1H, H-4 GlcN), 4.52 (d, J = 8.0 Hz, 1H, H-1 Gal), 3.96 (dd, J = 3.6, 10.1 Hz, 1H, H-6 GlcN), 3.87−3.77 (m, 2H), 3.48−3.43 (m, 2H, OCH_2CH_3 and H-5 Gal), 2.07 (s, 3H, OAc), 2.01 (s, 3H, OAc), 1.76 (s, 3H, OAc), 0.951 (t, 3H, CH_2CH_3).

Ethyl 2-*O*-Benzoyl-3-*O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-β-D-galactopyranoside (10). The benzylidene derivative **9** (1.0 g, 1.22 mmoles) was dissolved in a 80% aqueous acetic acid (20 mL), and the reaction mixture was heated at 80 °C

aqueous acetic acid (20 mL) and then was heated at 80 °C for 30 min. The reaction mixture was the poured into a solution of saturated $NaHCO_3$ (200 mL), and solid $NaHCO_3$ was added until the pH was neutral. The solution was extracted with ethyl acetate (100 mL), and the organic layer was washed with water (2 × 100 mL) and saturated NaCl (20 mL). The organic layer was dried (Na_2SO_4), concentrated, and chromatographed (silica, 85% ethyl acetate/hexane) to afford 0.716 g (80%) of diol **10** as a white solid: $R_f = 0.23$ (silica, 80% ethyl acetate/hexane); ^1H NMR ($CDCl_3$) δ 7.58 (d, J = 7.9 Hz, 2H, arom), 7.43−7.25 (bm, 5H, arom), 7.18 (d, J = 7.7 Hz, 1H, arom), 7.15 (d, J = 7.7 Hz, 1H, arom), 5.66 (dd, J = 10.5, 9.3 Hz, 1H, H-3 GlcN), 5.59 (d, J = 8.5 Hz, 1H, H-1 GlcN), 5.29 (dd, J = 9.3, 9.3 Hz, 1H, H-2 Gal), 5.11 (dd, J = 9.6, 9.6 Hz, 1H, H-4 GlcN), 4.46 (d, J = 8.0 Hz, 1H, H-1 Gal), 4.38−4.32 (m, 2H), 4.29−4.14 (m, 2H), 4.01−3.75 (multiple peaks, 5H), 3.61 (bt, J = 5.6 Hz, 1H, H-5 GlcN), 3.45 (m, 1H, OCH_2-CH_3), 2.12 (s, 3H, OAc), 2.01 (s, 3H, OAc), 1.76 (s, 3H, OAc), 0.96 (t, 3H, CH_2CH_3).

Ethyl 3,6-Bis-*O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-2-*O*-benzoyl-β-D-galactopyranoside (11) and Ethyl 3,6-Bis-*O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-4-*O*-((trichloroacetyl)-*N*-carbamoyl)-2-*O*-benzoyl-β-D-galactopyranoside (11a). A suspension of molecular sieves (4 Å, 1 g), collidine (0.167 mL, 1.26 mmol), silver triflate (0.3 g, 1.16 mmol), and diol **10** (0.71 g, 0.974 mmol) in CH_2Cl_2 (10 mL) was stirred under an argon atmosphere for 1 h. The reaction mixture was then cooled to −20 °C, and a solution of 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl bromide (0.53 g, 1.07 mmole) in CH_2Cl_2 (1 mL) was added. The reaction mixture was stirred at −20 °C for 30 min and then allowed to warm to room temperature and filtered through a Celite pad. The filtrate was diluted with ethyl acetate (100 mL), washed with water (50 mL), 1 M citric acid (100 mL), water (50 mL), saturated $NaHCO_3$, (100 mL), water (50 mL), and saturated NaCl (50 mL), dried (Na_2SO_4), and concentrated. The residue was purified by chromatography (silica, 40% ethyl acetate/toluene) to afford 0.967 g (86%) of **11** as a white solid: R_f = 0.22 (silica, 40% ethyl acetate/benzene); ^1H NMR ($CDCl_3$) δ 7.95−7.76 (bd, 3H), 7.53 (dd, J = 7.0, 1.1 Hz, 2H, phthalimido), 7.41 (m, 2H, benzoyl), 7.35 (m, 3H, benzoyl), 7.15 (dd, J = 7.9, 7.9 Hz, 1H, phthalimido), 7.12 (dd, J = 7.9, 7.9 Hz, 2H, phthalimido), 5.76 (dd, J = 9.2, 10.5 Hz, 1H, H- 3 GlcN), 5.55 (dd, J = 9.2, 10.5 Hz, 1H, H-3 GlcN), 5.47 (d, J = 8.5 Hz, 1H, H-1 GlcN), 5.34 (d, J = 8.5 Hz, 1H, H-2 Gal), 4.38−4.06 (m, 8H), 3.94 (d, J = 2.8 Hz, 1H, H-4 Gal), 3.87 (m, 2H, H-6 Gal), 3.67 (dd, J = 3.1, 9.7 Hz, 1H, H-3 Gal), 3.54 (m, 1H, H-5 GlcN), 3.49−3.42 (m, 1H, H-5 Gal), 3.37 (m, 1H, OCH_2CH_3), 3.14 (m, 1H, OCH_2CH_3), 2.13 (s, 3H, OAc), 2.09 (s, 3H, OAc), 2.03 (s, 3H, OAc), 2.01 (s, 3H, OAc), 1.85 (s, 3H, OAc), 1.75 (s, 3H, OAc), 0.75 (t, J = 7.1 Hz, 3H, CH_2CH_3). Addition of trichloroacetyl isocyanate to the NMR sample formed compound **11a** and caused a shift of the 4-position hydrogen of galactose to 5.39 (d, J = 2.1 Hz, 1H) ppm.

Ethyl 3,6-Bis-*O*-(3,4,6-tri-*O*-acetyl-2-acetamido-2-deoxy-β-D-glucopyranosyl)-2,4-di-*O*-acetyl-β-D-galactopyranoside (12). A solution of trisaccharide **11** (0.9 g, 0.785 mmol) and hydrazine monohydrate (1.52 mL, 31.4 mmol) in ethanol (30 mL) was refluxed for 8 h. The reaction mixture was concentrated and the residue was dissolved in pyridine (40 mL). Acetic anhydride (20 mL) was then added and the reaction mixture stirred for 24 h. The reaction mixture was concentrated and the residue was dissolved in CH_2Cl_2 (100 mL). The solution was washed with saturated $NaHCO_3$ (100 mL). The aqueous layer was extracted again with CH_2Cl_2 (50 mL), and the combined organic layers were dried (Na_2SO_4) and concentrated. The residue was chromatographed (silica, 3% MeOH/ethyl acetate) to afford 0.57 g (76%) of **12** as white solid: R_f = 0.33 (silica, 5% MeOH/ethyl acetate); ^1H NMR ($CDCl_3$) δ 5.43−5.31 (m, 3H), 5.08−4.99 (m, 3H), 4.95 (d, J = 8.1 Hz, 1H, H-1 GlcNAc), 4.77 (d, J = 8.3 Hz, 1H, H-1 GlcNAc), 4.37 (d, J = 8.0 Hz, 1H, H-1 Gal), 4.31 (dd, J = 1.2, 12.7 Hz, 1H, CH_2-OAc), 4.24 (dd, J = 4.2, 12.4 Hz, 1H, CH_2OAc), 4.13−4.05 (m, 2H), 3.89−3.78 (m, 4H), 3.73−3.64 (m, 3H), 3.59−3.43 (m, 2H), 3.41 (m, 1H, OCH_2CH_3), 2.10 (s, 3H, OAc), 2.09 (s, 3H, OAc), 2.08 (s, 3H, OAc), 2.07 (s, 3H, OAc), 2.01 (s, 9H, OAc), 2.00 (s, 3H, OAc), 2.95 (s, 3H, NHAc), 1.90 (s, 3H, NHAc), 1.17 (t, J = 7.0 Hz, 3H, CH_2-CH_3).

Ethyl 3,6-Bis-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-galactopyranoside (13). Sodium methoxide (1.0 mL, 25% solution in MeOH) was added to a solution of trisaccharide **12** (0.366 g, 0.38 mmol) in MeOH (40 mL). The reaction mixture was stirred for 20 h during which time a precipitate formed. Water (~5 mL) was added until all of the solid had dissolved, and then prewashed BioGel AG 50W-X8 (hydrogen form) was added to the reaction mixture until the pH was neutral. The reaction mixture was then filtered, and the filtrate was concentrated. The residue was dissolved in water (10 mL) and filtered through a C-18 sep pack (Whatman), and the filtrate was concentrated to yield 0.167 g (70%) of **13** as a white solid: R_f = 0.47 (silica, 30% 1 M NH_4OAc/2-propanol); ^1H NMR (D_2O) δ 4.64 (d, J = 8.4 Hz, 1H, H-1 GlcNAc), 4.48 (d, J = 8.4 Hz, 1H, H-1 GlcNAc), 4.32 (d, J = 7.7 Hz, 1H, H-1 Gal), 4.06 (d, J = 3.3 Hz, 1H, H-4 Gal), 3.99 (d, J = 8.2 Hz, 1H), 3.91−3.83 (m, 3H), 3.74−3.61 (m, 8H), 3.59−3.38 (m, 7H), 1.99 (s, 3H, NHAc), 1.98 (s, 3H, NHAc), 1.19 (t, J = 7.7 Hz, 3H, CH_2CH_3); ^{13}C NMR (D_2O) δ 175.0 (C-0), 174.4 (C-0), 102.7 (C-1), 102.4 (C-1), 101.4 (C-1), 82.1, 75.8, 75.7, 73.8, 73.6, 73.4, 69.9, 69.7, 69.6, 69.4, 68.6, 66.1, 60.7, 60.5, 55.5, 55.5, 22.2 (2C, CH_3C=O), 14.3 (CH_2CH_3); MS (LSI MS^+) calcd for $C_{24}H_{42}N_2O_{16}$-Cs: 747.4840, found 747.4845.

Ethyl 3,6-Bis-*O*-[β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-β-D-galactopyranoside (14). Galactosyltransferase (Ec 2.4.1.22, 6 U) and uridine 5′-diphosphogalactose 4′-epimerase (EC 5.1.3.2, 8 U) were added to a solution containing sodium cacodylate (pH 7.5, 1 M, 0.4 mL), water (3.8 mL), BSA (5% solution, 88 μL), $MnCl_2$ (1M, 40 μL), alkaline phosphatase (EC 3.1.3.1, 1U/μL, 44 μL), uridine diphosphoglucose disodium salt (161 mg, 0.285 mmol), and the trisaccharide **13** (70 mg, 0.114 mmol). The reaction mixture was inverted several times and then allowed to sit at room temperature for 72 h. The mixture was filtered and purified by chromatography (BioGel P-2, 0.1 M NH_4HCO_3) to afford 100 mg (93%) of **14** as a white solid after lyophilization: R_f = 0.25 (silica, 30% 1M NH_4OAc/2-propanol); ^1H NMR (D_2O) δ 4.65 (d, J = 7.8 Hz, 1H, H-1 GlcNAc), 4.49 (d, J = 7.6 Hz, 1H, H-1 GlcNAc), 4.42 (d, J = 7.6 Hz, 1H, H-1 Gal), 4.41 (d, J = 7.8 Hz, 1H, H-1 Gal), 4.30 (d, J = 7.8 Hz, 1H, H-1 bridging Gal), 4.05 (d, J = 3.2 Hz, 1H, H-4 Gal), 3.99−3.43 (m, 31H), 1.97 (s, 6H, OAc), 1.18 (t, 3H, CH_2CH_3); ^{13}C NMR (D_2O) δ 174.9 (C=0), 174.4 (C=0), 102.9 (C-1), 102.8 (C-1), 102.6 (C-1), 102.4 (β-anomer), 101.3 (β-anomer), 82.2, 78.4, 78.1, 75.4, (2 C), 74.7, 74.6, 73.4, 72.5, (2 C), 72.4, 72.2, 70.1 (2 C), 69.71, 69.67, 69.4, 68.4, 68.5 (2 C), 66.0, 61.0, (2 C), 60.0, 59.8, 55.3, 55.0, 22.2 (CH_3C=O), 17.0 (CH_3C=O), 14.3 (CH_3); MS (ion spray) calcd for $C_{36}H_{62}N_2O_6$ 938, found 961 ($M + Na^+$), 977 ($M + K^+$).

Ethyl 3,6-Bis-*O*-[(ammonium 5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranuronate)-(2→3)β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-β-D-galactopyranoside (15). The N-type α(2−3)-sialyl transferase (EC 2.4.99.6, 1 U) was added to a solution of cytidine-5′-monophosphosialic acid (0.116 g, 0.166 mmol), BSA (5% solution, 0.12 mL), sodium cacodylate (pH 6.5, 1M, 1.8 mL), water (5.1 mL), $MnCl_2$ (1M, 0.6 mL), alkaline phosphatase (EC 3.1.3.1, 1U/μL), and the pentasaccharide **14** (52 mg, 55 μmol). The reaction mixture was tipped for 4 days. Since the reaction was not complete by TLC, addition cytidine-5′-monophosphosialic acid (0.116 g, 0.166 mmol), alkaline phosphatase (1 U/μL, 30 μL), $MnCl_2$ (1M, 0.2 mL), and N-type α(2−3)-sialyl transferase (1 U) were added and the reaction mixture tipped for another 5 days. The mixture was filtered and chromatographed (GioGel P-2, 0.1 M NH_4HCO_3) to afford 75 mg (86%) of **15** as a white solid after lyophilization: R_f = 0.15 (silica, 30% 1 M NH_4OAc/2-propanol); ^1H NMR (D_2O) δ 4.66 (d, J = 7.8 Hz, 1H, H-1 GlcNAc), 4.51 (bd, 3H, H-1apos of GlcNAc, Gal, Gal), 4.32 (d, J = 7.8 Hz, 1H, H-1 GlcNAc), 4.1−3.5 (m, 45H), 2.71 (dd, J = 12.3, 4.3 Hz, 2H, H-3(eq) sialic acid), 1.99 (s, 12H, NHAc), 1.76 (dd, J = 12.5 Hz, 2H, H-3(ax) sialic acid), 1.19 (t, 3H, CH_2CH_3); MS (ion spray) calcd for $C_{58}H_{93}N_4O_{42}$ 1520, found 1519 ($[M - H^+]^-$), 759 ($[M - 2H^+]^{2-}$).

Ethyl 3,6-Bis-*O*-[(ammonium 5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranuronate)-(2→3)-β-D-galactopyranosyl-(1→4)-(α-L-fucopyranosyl-(1→3))-2-acetamido-2-deoxy-β-D-glucopyranosyl]-β-D-galactopyranoside (6). The flucosyltransferase V (100 mU attached to beads) was added to a solution containing sodium cacodylate (pH 6.5, 0.5 mL), water (4.5 mL), $MnCl_2$ (1M, 0.2 mL),

alkaline phosphatase (32 U), GDP-fucose disodium salt (0.11 g, 0.18 mmol), and the heptasaccharide **15** (56 mg, 36 μmol). The reaction mixture was tipped for 48 h. Additional transferase (100 mU) and $MnCl_2$ (1M, 0.2 mL) were added, and the reaction mixture was tipped for another 4 days. The mixture was filtered and chromatographed (BioGel P-2, 0.1 M NH_4HCO_3) to afford 40 mg (60%) of **6** as a white solid after lyophilization: R_f = 0.38 (silica, 40% 1 M NH_4OAc/2-propanol); ^1H NMR (D_2O, 500 MHz) δ 5.13 (d, J = 3.5 Hz, 1H, H-1 Fuc), 5.12 (d, J = 3.5 Hz, 1H, H-1 Fuc), 4.72 (d, J = 8.0 Hz, 1H, H-1 GlcNAc), 4.59−4.51 (multiple peaks, 3H, H-1's of GlcNAc, Gal and Gal), 4.37 (d, J = 8.0 Hz, 1H H-1 bridging Gal), 4.10 (d, J = 2.9 Hz, 1H, H-3 Gal), 4.10 (d, J = 2.9 Hz, 1H, H-3 Gal), 4.03−3.47 (m, 54 H), 2.77 (dd, J = 12.4, 4.4 Hz, 2H, H-3(eq) sialic acids) 2.03 (s, 6H, NHAc), 2.01 (s, 6H, NHAc), 1.81 (dd, J = 12.4, 12.4 Hz, 2H, H-3(ax) sialic acids), 1.24 (t, 3H, CH_2CH_3), 1.18 (d, J = 6.5 Hz, 6H, CH_3-fucose); HRMS (LS1MS-) calcd for $C_{70}H_{117}O_{50}N_2$ ($M + H^+$) 1811.6579, found 1811.6693.

1,4-Bis((2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)oxy)butane (20a). A suspension of dry molecular sieves (4 Å, 5 g), CH_2Cl_2 (75 mL), silver carbonate (14 g, 51 mmol), and 1,4-butanediol (2.25 g, 25 mmol) was stirred under argon for 20 min. The reaction mixture was then cooled to −20 °C, and a solution of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (21 g, 51 mmol) in CH_2Cl_2 (50 mL) was added. The reaction mixture was then stirred at −20 °C overnight, allowed to warm to room temperature, and filtered through Celite. The filtrate was washed with water (50 mL), 0.1 M HCl (50 mL), water (50 mL), saturated $NaHCO_3$ (50 mL), water (50 mL), and brine (50 mL) and dried (Na_2SO_4). Concentration and chromatography (silica, ethyl acetate/hexane 1:1) 13.8 g (73%) of **20a** as an amorphous foam: R_f = 0.52 (silica, ethyl acetate/hexane 2:1); ^1H NMR ($CDCl_3$) δ 5.37 (d, J = 3.1 Hz, 2H, H-4apos), 5.17 (t, J = 7.9, 8.0 Hz, 2H, H_2's), 5.00 (t, J = 3.1, 7.9 Hz, 2H, H-3's), 4.44 (d, J = 7.9 Hz, 2H, H-1), 4.10 (m, 4H, H6 and H-5), 3.88 (m, 4H, H-6 and OC*H*), 3.45 (bm, 2H, OC*H*), 2.12 (s, 3H, OAc), 2.03 (s, 3H, OAc), 2.02 (s, 3H, OAc), 1.60 (m, 4H, CH-2's); ^{13}C NMR ($CDCl_3$) δ 170.3, 170.1, 170.0, 169.3, 101.1, (C-1), 70.8, 70.5, 69.5, 68.8, 66.9, 61.1, 25.7, 20.62, 20.55, 20.47.

1,5-Bis((2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)oxy)pentane (20b). A suspension of dry molecular sieves (4 Å, 5 g), CH_2Cl_2 (75 mL), silver carbonate (14 g, 51 mmol), and 1,5-pentanediol (2.6 g, 25 mmol) was stirred under argon for 20 min. The reaction mixture was cooled to −20 °C and a solution of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (21 g, 51 mmol) in CH_2Cl_2 (50 mL) added. This mixture was stirred at −20 °C overnight and then allowed to warm to room temperature before filtering through Celite. The filtrate was washed with water (50 mL), 0.1 M HCl (50 mL), water (50 mL), saturated $NaHCO_3$ (50 mL), water (50 mL), and brine (50 mL) and dried (Na_2SO_4). Concentration and chromatography (silica, ethyl acetate/hexane 1:1) afforded 13 g (68%) of **20b** as an amorphous foam: R_f = 0.5 (silica, ethyl acetate/hexane 2:1); ^1H NMR ($CDCl_3$) δ 5.37 (d, J = 3.0 Hz, 2H, H-4), 5.17 (t, J = 7.9, 8.1 Hz, 2H, H-2's), 5.00 (t, J = 3.0, 7.9 Hz, 2H, H-3), 4.44 (d, J = 8.0 Hz, 2H, H-1), 4.12 (m, 4H, H-6 and H-5), 3.85 (m, 4H, H-6 and OC*H*), 3.49 (bm, 2H, OC*H*), 2.14 (s, 3H, OAc), 2.04 (s, 3H, OAc), 2.03 (s, 3H, OAc), 1.61 (m, 4H, CH_2), 1.30 (m, 2H, CH_2); ^{13}C NMR ($CDCl_3$) δ 170.3, 170.2, 170.1, 169.3, 101.2 (C-1), 70.8, 70.5, 69.9, 68.8, 66.9, 61.1, 29.0, 22.1, 20.

1,4-Bis-((β-D-galactopyranosyl)oxy)butane (21a). Sodium methoxide (100 mg) was added to a solution of compound **20a** (13 g, 17.3 mmol) in MeOH (80 mL), and the reaction mixture was stirred for 2 h. The prewashed Biogel AG 50W-X8 (hydrogen form) was added to the mixture until neutral. The reaction mixture was filtered and concentrated. The residue was dissolved in methanol (25 mL) and **21a** crystallized by the addition of ether to yield 6.6 g (92%) as a white solid: R_f = 0.52 (silica, $CHCl_3$/MeOH/H_2O 60/50/15); ^1H NMR (CD_3OD) δ 4.19 (d, J = 7.0 Hz, 2H, H-1), 3.92−3.29 (m, 24H), 1.70 (m, 4H, CH_2's); MS (FAB) calcd for $C_{16}H_{30}O_{12}$ 414, found 437 ($M + Na^+$), 453 ($M + K^+$).

1,5-(Bis-((β-D-galactopyranosyl)oxy)pentane (21b). Sodium methoxide (100 mg) was added to a solution of compound **20b** (11.5 g, 15 mmol) in MeOH (80 mL), and the reaction mixture was stirred for 2 h. Then prewashed Biogel AG 50W-X8 (hydrogen form) was added to the mixture until the pH was neutral. The reaction mixture was then filtered and the filtrate concentrated. The residue was dissolved in methanol (25 mL) and **21b** crystallized by the addition of ether to yield 5.9 g (92%) as a white solid: R_f = 0.53 (silica, $CHCl_3$/MeOH/H_2O 60/50/15); ^1H NMR (D_2O) δ 4.33 (d, J = 7.9 Hz, 2H, H-1), 3.92−3.85 (m, 2H), 3.77−3.55 (m, 10H), 3.43 (m, 2H, OC*H*'s), 1.60 (m, 4H, CH_2's), 1.40 (m, 2H, CH_2's); MS (FAB) calcd for $C_{16}H_{30}O_{12}$ 414, found 451 ($M + K^+$)$^{2-}$.

1,4-Bis((2,6-di-*O*-acetyl-β-D-galactopyranosyl)oxy)butane (22a). Compound **21a** (6 g, 14.5 mmol) was suspended in 300 mL of dry acetone, 10 mL of 2,2-dimethoxypropane, and 10 mL of dry DMF. Five drops of concd sulfuric acid were added, and the mixture was refluxed for 3 h. Solid $NaHCO_3$ was added until neutral and the mixture filtered and concentrated. The syrupy residue was extracted with hexane and then dissolved in 50 mL of pyridine. Acetic anhydride (25 mL) was then added and the mixture stirred for 8 h. After concentration, the residue was dissolved in 75 mL of CH_2Cl_2, washed with water (50 mL), 0.1 M HCl (3 × 50 mL), water (50 mL), saturated $NaHCO_3$ (3 × 50 mL), water (50 mL), and brine (50 mL), and dried (Na_2SO_4). Concentration afforded a viscous oil which was refluxed for 3 h in 50 mL of acetic acid (70%). After concentration, the residue was crystallized from methanol/ether. Chromatography (silica, chloroform/methanol 5:1) and recrystallization in methanol/ether afforded 2.3 g (28%) of **22a** as white crystals: R_f = 0.38 (silica, chloroform/methanol 4:1); ^1H NMR ($CDCl_3$) δ 4.95 (t, J = 7.8, 8.1 Hz, 2H, H-2), 4.33 (m, 6H, H-1, H-6), 3.88 (m, 4H, H-4, OC*H*), 3.65 (m, 4H, H-3, H-5), 3.51 (bm, 2H, OC*H*), 2.85 (bs, 4H, OH), 2.12 (s, 6H, OAc), 2.10 (s, 6H, OAc), 1.63 (bm, CH_2's).

1,5-Bis((2,6-di-*O*-acetyl-β-D-galactopyranosyl)oxy)pentane (22b). Compound **21b** (5 g, 11.7 mmol) was suspended in 300 mL of dry acetone, 10 mL of 2,2-dimethoxypropane, and 10 mL of dry DMF. Five drops of concd sulfuric acid were added, and the mixture was refluxed for 3 h. Solid $NaHCO_3$ was added until neutral, the mixture filtered, and the filtrate concentrated. The syrupy residue was extracted with hexane and then dissolved in 50 mL of pyridine. Acetic anhydride (25 mL) was then added and the mixture stirred for 8 h. After concentration, the residue was diluted to 75 mL of CH_2Cl_2, washed with water (50 mL), 0.1 M HCl (3 × 50 mL), water (50 mL), saturated $NaHCO_3$ (3 × 50 mL), water (50 mL), and brine (50 mL), and dried (Na_2SO_4). Concentration afforded an oil which was refluxed for 3 h in 50 mL of acetic acid (70%). Concentration and crystallization from methanol/ether after a solid which was chromatographed (silica, chloroform/methanol 7:1−5:1) and then recrystallization in methanol/ether afforded 2.5 g (38%) of **22b** as white crystals: R_f = 0.49 (silica, chloroform/methanol 4:1); ^1H NMR ($CDCl_3$) δ 4.92 (d, J = 8.0, 8.2 Hz, 2H, H-2), 4.30 (m, 6H, H-1, H-6), 3.89 (d, J = 3.1 Hz, 2H, H-4), 3.82 (m, 2H, OC*H*), 3.65 (m, 4H, H-3, H-5), 3.46 (m, 2H, OC*H*), 3.32 (bs, 4H, OH), 2.10 (s, 3H, OAc), 2.07 (s, 3H, OAc), 1.55 (m, 4H, CH_2), 1.36 (m, 2H, CH_2). ^{13}C NMR ($CDCl_3$) δ 171.4, 171.1 (C-O), 100.8 (C-1), 72.9, 72.3, 72.0, 69.4, 68.8, 62.8, 28.9, 22.2, 21.0, 20.8.

1,4-Bis((3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→3)-2,6-di-*O*-acetyl-β-D-galactopyranosyl)oxy)butane (23). A suspension of molecular sieves (4 Å, 1 g), CH_2Cl_2 (30 mL), collidine (0.3 mL, 2.3 mmol), silver triflate (0.57 g, 2.2 mol), and compound **22a** (0.5 g, 0.876 mmol) was stirred under argon for 1 h. The reaction mixture was then cooled to −20 °C, and a solution of 2-deoxy-2-phthalimido-3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl bromide (1.06 g, 2.14 mmol) in CH_2Cl_2 (20 mL) was added. The reaction mixture was stirred at −20 °C for 30 min, warmed to room temperature overnight, and filtered through Celite. The filtrate was diluted with dichloromethane (100 mL), washed with water (50 mL), 0.1 M HCl (100 mL), water (50 mL), saturated $NaHCO_3$ (100 mL), water (50 mL), and brine (50 mL), and dried (Na_2SO_4). Concentration and chromatography (silica, acetone/toluene 1:2) afforded 0.650 (52%) of **23** as a white solid: R_f = 0.25 (silica, toluene/acetone 2:1); ^1H NMR ($CDCl_3$) δ 7.84 (m, 4H, Phth), 7.76 (m, 4H, Phth), 5.74 (dd, J = 10.6, 10.4 Hz, 2H, H-3 Glc), 5.50 (d, J = 8.3 Hz, 2H, H-1), 5.16 (dd, J = 8.3, 8.5 Hz, 4H, H-4 Glc, H-3 Glc), 4.92 (dd, J = 8.2, 8.2 Hz, 2H, H-2 Gal), 4.38−4.22 (m, 10H), 4.15 (d, J = 8.1 Hz, 2H, H-1 Gal), 3.99−3.84 (m, 4H), 3.71−3.58 (m, 4H), 3.28 (m, 2H, OC*H*), 2.11 (s, 12H, OAc), 2.07 (s, 6H, OAc), 2.04 (12H, OAc), 1.44 (m, 4H, CH_2); MS (ion spray) calcd for $C_{64}H_{72}N_2O_{34}$ 417, found 1439 ($M + Na^+$)$^-$, 1455 ($M + K^+$)$^{2-}$.

Acetylation of compound **23** with acetic anhydride in pyridine[15] caused a shift of the 4-H of the galactoses to 5.35 (d, $J = 3.5$ Hz, 2H) ppm.

1,4-Bis((2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→3)-2,6-di-*O*-acetyl-β-D-galactopyranosyl)oxy)pentane (24). A suspension of molecular sieves (4 Å, 1 g), CH_2Cl_2 (30 mL), collidine (0.27 mL, 2 mmol), silver triflate (0.53 g, 2 mmol), and **22b** (0.4 g, 0.68 mmol) was stirred under argon for 1 h. The reaction mixture was then cooled to −20 °C and a solution of 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-2-deoxy-2-phthalimido-3,6-di-*O*-acetyl-β-D-glucopyranosyl chloride (1.3 g, 1.7 mmol) in CH_2Cl_2 (20 mL) was added. The reaction mixture was stirred at −20 °C for 30 min, warmed to room temperature overnight and filtered through Celite. The filtrate was diluted with dichloromethane (100 mL), washed with water (50 mL), 0.1 M HCl (100 mL), water (50 mL), saturated $NaHCO_3$ (100 mL), water (50 mL), and brine (50 mL), and dried (Na_2SO_4). Concentration and chromatography (silica, acetone/toluene 1:1,5) afforded 0.550 g (40%) of **24** as a white solid: $R_f = 0.38$ (silica, toluene/acetone 1:1); ^{1}H NMR ($CDCl_3$) δ 7.84 (m, 4H, Phth), 7.76 (m, 4H, Phth), 5.69 (dd, $J = 10.2$, 8.5 Hz, 2H, H-3 Glc), 5.49 (d, $J = 8.4$ Hz, 2H, H-1 Glc), 5.31 (3, $J = 3.2$ Hz, 2H, H-4 Gal), 5.12 (dd, $J = 8.0$, 7.8 Hz, 2H, H-2 Gal), 4.99−4.87 (m, 4H, H-2 Gal, H-3 Gal′), 4.62−4.55 (m, 4H, H-6 Gal′,H-5 Gal′), 4.32−4.30 (d, $J = 8.2$ Hz, 2H, H-1 Gal′), 4.28−4.24 (m, 4H, H-2 Glc), 4.21 (d, $J = 2.9$ Hz, 2H, H-4 Gal), 4.18−3.94 (m, 10H), 3.92−3.76 (m, 6H), 3.70−3.63 (m, 2H, OC*H*), 2.14 (s, 6H, OAc), 2.13 (s, 6H, OAc), 2.07 (s, 6H, OAc), 2.06 (s, 6H, OAc), 2.03 (s, 12H, OAc), 1.96 (s, 6H, OAc), 1.90 (s, 6H, OAc), 1.44−1.30 (m, 4H, CH_2), 1.25−1.12 (m, 2H, CH_2); MS (ion spray) calcd for $C_{89}H_{110}O_{50}N_2$ 2008, found 2141 (M + Na^+), 2139 (M + $Na^+)^{2-}$.

Acetylation[15] of compound **24** with acetic anhydride in pyridine caused a shift of the galactose H-4 to 5.33 (d, $J = 2.0$ Hz, 2H) ppm.

1,4-Bis((2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl-(1→3)-2,3,6-tri-*O*-acetyl-β-D-galactopyranosyl)oxy)butane (25). A solution of compound **23** (0.1 g, 0.067 mmol), hydrazine monohydrate (1 mL, 20.6 mmol), and ethanol (20 mL) was refluxed for 8 h. The reaction mixture was concentrated and the residue dissolved in pyridine (15 mL). Acetic anhydride (7 mL) was added, and the mixture was stirred for 12 h and then concentrated. The residue was dissolved in CH_2Cl_2 (100 mL) and washed with saturated $NaHCO_3$ (100 mL). The aqueous layer was extracted with CH_2Cl_2 (50 mL), and the combined organic layers were dried (Na_2SO_4). Concentration and chromatography (silica, toluene/acetone 1:1) afforded 50 mg (57%) of **25** as white solid contaminated with phthalimido hydrazide (~10% by ^{1}H NMR), $R_f = 0.2$ (silica, toluene/acetone 1:1). This product was used directly for the next step: ^{1}H NMR ($CDCl_3$) δ 5.54 (dd, $J = 7.5$, 8.2 Hz, 2H), 5.36 (d, $J = 3.2$ Hz, 2H, H-4 Gal), 5.13−4.99 (m, 6H), 4.36−4.03 (m, 5H), 3.89−3.76 (m, 6H), 3.71−3.63 (m, 4H), 3.50−3.44 (m, 2H, OC*H*), 2.12 (s, 3H, OAc), 2.11 (s, 3H, OAc), 2.09 (s, 3H, OAc), 2.01 (s, 3H, OAc), 1.91 (s, 6H, OAc), 1.71−1.62 (m, 4H, CH_2).

1,5-Bis((2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl-(1→3)-2,3,6-tri-*O*-acetyl-β-D-galactopyranosyl)oxy)pentane (30). A solution of compound **24** (0.275 g, 0.137 mmol), hydrazine monohydrate (1 mL, 20.6 mmol), and ethanol (20 mL) was refluxed for 8 h. The reaction mixture was concentrated and the residue dissolved in pyridine (15 mL). Acetic anhydride (7 mL) was then added and the reaction mixture stirred for 12 h. After concentration the residue was dissolved in CH_2Cl_2 (100 mL) and washed with saturated $NaHCO_3$ (100 mL). The aqueous layer was extracted again with CH_2Cl_2 (50 mL), and the combined organic layers were dried (Na_2SO_4). Concentration and chromatography (silica, toluene/acetone 1:1) afforded 227 mg (86%) of **30** as a white solid containing some phthalamido hydrazide as a contaminant (~15% by ^{1}H NMR). This product was used without further purification: $R_f = 0.34$ (silica, toluene/acetone 1:1); ^{1}H NMR ($CDCl_3$) δ 5.41 (dd, $J = 8.6$, 8.6 Hz, 2H, H-3 Glc), 5.35 (bs, 2H), 5.21 (dd, $J = 9.4$, 9.4 Hz, 2H), 5.14−4.94 (m, 4H), 4.75−4.69 (m, 2H), 4.53 (d, $J = 7.9$ Hz, 2H, H-1 Glc), 4.33 (d, $J = 8.1$ Hz, 2H, H-1 Gal), 4.18−3.96 (m, 10H), 3.88−3.75 (m, 8H), 3.58−3.50 (m, 4H), 3.46−3.41 (m, 2H, OC*H*), 2.15 (s, 12H, OAc), 2.11 (s, 6H, OAc), 2.07 (s, 12H, OAc), 2.06 (s, 12H, OAc), 2.04 (s, 6H, OAc), 1.96 (s, 6H, OAc), 1.58−1.46 (m, 4H, CH_2), 1.33−1.25 (m, 2H, CH_2).

1,4-Bis((2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl)oxy)butane (26). Sodium methoxide (75 mg) was added to a solution of compound **25** (40 mg, 0.03 mmol) in MeOH (20 mL). The reaction was stirred for 20 h during which time a precipitate formed. Water (~5 mL) was added until all of the solid had dissolved, and then prewashed Biogel AG 50W-X8 (hydrogen form) was added until neutral. The reaction mixture was filtered and concentrated. The residue was dissolved in water (10 mL) and filtered through a C-18 sep peak (Whatman) and the filtrate concentrated to yield 24 mg (96%) of **26** as a white solid: $R_f = 0.15$ (silica, $CHCl_3$/MeOH/H_2O 60/50/15); ^{1}H NMR (D_2O) δ 4.69 (d, $J = 7.4$ Hz, 2H, H-1 Glc), 4.37 (d, $J = 7.9$ Hz, 2H, H-1 Gal), 4.12 (d, $J = 3.0$ Hz, 2H, H-4 Gal), 3.95−3.87 (bm, 4H), 3.80−3.44 (m, 20H), 2.02 (s, 6H, NHAc), 1.69 (bm, 4H, CH_2).

1,5-Bis((β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl)oxy)pentane (31). Sodium methoxide (0.1 g) was added to a solution of compound **30** (0.1 g, 0.05 mmol) in MeOH (20 mL). The reaction mixture was stirred for 20 h during which time a precipitate formed. Water (~5 mL) was added until the solid had dissolved, and then prewashed Biogel AG 50W-X8 (hydrogen form) was added to the reaction mixture until neutral. The reaction mixture was filtered, the filtrate concentrated, and the residue dissolved in water (10 mL). Filtration through a C-18 sep pack (Whatman) and concentration afforded 56 mg (96%) of **31** as a white solid: $R_f = 0.23$ (silica, $CHCl_3$/MeOH/H_2O 60/50.15); ^{1}H NMR (D_2O) δ 4.65 (d, $J = 7.8$ Hz, 2H, H-1 Glc), 4.42 (d, $J = 7.7$ Hz, 2H, H-1 Gal′), 4.32 (d, $J = 7.9$ Hz, 2H, H-1 Gal), 4.08 (d, $J = 3.0$ Hz, 2H, H-4 Gal), 3.95−3.45 (m, 38H), 1.97 (s, 6H, NHAc), 1.64−1.55 (m, 4H, CH_2), 1.39−1.37 (m, 2H, CH_2).

1,4-Bis((β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl)oxy)butane (27). Galactosyltransferase (1 U) and uridine 5′-diphosphogalactose 4′-epimerase (3 U) were added to a solution containing solution cacodylate (pH 7.5, 0.2 M, 0.4 mL), water (1 mL), BSA (5% solution, 20 μL), $MnCl_2$ (1M, 10 μL), alkaline phosphatase (1 U/μL, 10 μL), uridine diphosphoglucose disodium salt (28.3 mg, 0.05 mmol, 2.5 eq), and compound **26** (17 mg, 0.02 mmol). The reaction mixture was inverted several times and allowed to sit at room temperature for 72 h. Filtration and chromatography (Biogel P-4, 0.1 M NH_4HCO_3) afforded 21 mg (91%) of **27** as a white solid after lyophilization: $R_f = 0.08$ (silica, $CHCl_3$/MeOH/H_2O 60/50/15); ^{1}H NmR (D_2O) δ 4.70 (d, $J = 7.8$ Hz, 2H, H-1 Glc), 4.47 (d, $J = 7.6$ Hz, 2H, H-1 Gal′), 4.37 (d, $J = 8.0$ Hz, 2H, H-1 Gal), 4.13 (d, $J = 3.0$ Hz, 2H, H-4 Gal), 3.92−3.53 (m, 38H), 2.02 (s, 6H, NHAc), 1.69 (bm, 4H, CH_2); MS (ion spray) calcd for $C_{44}H_{26}O_{32}N_2$ 1145, found 1167 (M + $Na^{+)+}$.

1,4-Bis((ammonium 5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-nonulopyranosuronate)-(2→3)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl)oxy)butane (28). The α(2−3)-sialyl transferase (0.1 U) was added to a solution of cytidine 5′-monophosphate−sialic acid (27.5 mg, 0.04 mmol), BSA (5% solution, 10 μL), sodium cacodylate (pH 6.5, 0.2 M, 1.25 mL), water (1 mL), Triton (0.2 M, 25 μL), alkaline phosphatase (1 U/μL, 24 μL), and compound **27** (15 mg, 13 μmol). The reaction mixture was incubated at 37 °C for 2 days. Filtration and chromatography (Biogel P-4, 0.1 M NH_4HCO_3) afforded 21 mg (93%) of **28** as a white solid after lyophilization: $R_f = 0.53$ (silica, $CHCl_3$/MeOH/1 M NH_4OAc 1/4/1); ^{1}H NMR (D_2O) δ 4.65 (d, $J = 7.8$ Hz, 2H, H-1 Glc), 4.50 (d, $J = 7.8$ Hz, 2H, H-1 Gal′), 4.33 (d, $J = 7.9$ Hz, 2H, H-1 Gal), 4.08 (d, $J = 2.8$ Hz, 2H, H-4 Gal′), 4.06 (dd, $J = 2.8$, 10.1 Hz, 2H, H-3 Gal′), 3.92−3.45 (m, 50H), 2.71 (dd, $J = 12.3$, 4.4 Hz, 2H, H-3 SA), 1.97 (s, 12H, NHAc), 1.75 (dd, $J = 12.3$, 12.1 Hz, 2H, H-3 SA), 1.64 (bm, 4H, CH_2); MS (ion spray) calcd for $C_{66}H_{110}O_{48}N_4$ 1728, found 1726 $(M)^{2-}$.

1,5-Bis(((ammonium 5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-nonulopyranosuronate)-(2→3)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl)-oxy)pentane (32). The α(2−3)-sialyl transferase (0.135 U) was added to a solution of cytidine 5′-monophosphate−sialic acid (54.5 mg, 0.078 mmol), BSA (5% solution, 5 μL), sodium cacodylate (pH 6.5, 0.2 M, 2.56 mL), water (1.75 mL), Triton (0.2 M, 51.7 μL), alkaline

phosphatase (1 U/μL, 27 μL), and compound **31** (30 mg, 26 μmol). The reaction mixture was incubated at 37 °C for 2 days. Filtration and chromatography (Biogel P-4, 0.1 M NH_4HCO_3) afforded 44 mg (93%) of **32** as a white solid after lyophilization: R_f = 0.53 (silica, $CHCl_3$/MeOH/1 M NH_4OAc 1:4:1); ^1H NMR (D_2O) δ 4.70 (d, J = 6.8 Hz, 2H, H-1 Glc), 4.54 (d, J = 7.8 Hz, 2H, H-1 Gal'), 4.36 (d, J = 7.9 Hz, 2H, H-1 Gal), 4.12 (d, J = 2.9 Hz, 2H, H-4 Gal'), 4.09 (dd, J = 3.0, 10.3 Hz, 2H, H-3 Glc), 3.95−3.50 (m, 50H), 2.76 (dd, J = 12.3, 4.4 Hz, 2H, H-3 SA), 2.02 (s, 12H, NHAc), 1.79 (dd, J = 8.1, 12.1 Hz, 2H, H-3 SA), 1.67−1.62 (m, 4H, CH_2), 1.44−1.33 (m, 2H, CH_2): MS (ion spray) calcd for $C_{67}H_{112}O_{48}N_4$ 1742, found 870 $(M)^{2-}$.

1,4-Bis(ammonium 5-acetamido-3,5-dideoxy-α-D-*glycero*-D-galacto-nonulopyranosuronic acid)-(2→3)-β-D-galactopyranosyl-(1→4)-(6-deoxy-α-L-galactopyranosyl-(1→3))-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl)oxy)butane (29). The fucosyltransferase V (80 mU attached to beads) was added to a solution containing sodium cacodylate (pH 6.5, 0.2 M, 1.1 mL), water (1.1 mL), $MnCl_2$ (1 M, 0.2 mL), alkaline phosphatase (40 U), GDP-fucose disodium salt (15 mg, 0.025 mmol), and compound **28** (17.3 mg, 10 μmol). The reaction mixture was tipped for 48 h, filtered, and chromatographed (Biogel P-4, 0.1 M NH_4HCO_3) to afford 18.8 mg (93%) of **29** as a white solid after lyophilization: R_f = 0.24 (silica, 2-propanol/1 M NH_4OAc 2/1); ^1H NMR (D_2O) δ 5.06 (d, J = 3.6 Hz, 2H, H-1 Fuc), 4.65 (d, J = 7.9 Hz, 2H, H-1 Glc), 4.47 (d, J = 8.2 Hz, 2H, H-1 Gal'), 4.32 (d, J = 7.8 Hz, 2H, H-1 Gal), 4.08 (d, J = 2.3 Hz, 2H, H-4 Gal'), 4.03 (dd, 2H, H-3 Gal'), 3.94−3.44 (m, 56H), 2.71 (dd, J = 12.3, 4.4 Hz, 2H, H-3 SA), 1.97 (s, 12H, NHAc), 1.73 (dd, J = 12.1 Hz, 2H, H-3 SA), 1.65 (bs, 4H, CH_2), 1.10 (d, J = 6.3 Hz, CH_3-Fuc); MS (ion spray) calcd for $C_{78}H_{130}O_{56}N_4$ 2020, found 1008 $(M)_{1/2}^{2-}$.

1,5-Bis(((ammonium 5-acetamido-3,5-dideoxy-α-D-*glycero*-D-galacto-nonulopyranosuronic acid)-(2→3)-β-D-galactopyranosyl-(1→4)-(α-L-fucopyranosyl-(1→3)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl)oxy)pentane (33). The fucosyltransferase V (100 mU attached to beads) was added to a solution containing sodium cacodylate (pH 6.5, 0.2 M, 2 mL), water (2 mL), $MnCl_2$ (1M, 0.2 mL), alkaline phosphatase (60 U), GDP-fucose disodium salt (25 mg, 0.042 mmol), and compound **31** (30 mg, 17 μmol). The reaction mixture was tipped for 48 h. Filtration and chromatography (Biogel P-4, 0.1 M NH_4HCO_3) afforded 31 mg (93%) of **33** as white solid after lyophilization: R_f = 0.24 (silica, 2-propanol/1 M NH_4OAc 2/1); ^1H NMR (D_2O) δ 5.11 (d, J = 3.4 Hz, 2H, H-1 Fuc), 4.70 (d, J = 8.2 Hz, 2H, H-1 Glc), 4.51 (d, J = 8.2 Hz, 2H, H-1 Gal'), 4.32 (d, J = 7.8 Hz, 2H, H-1 Gal), 4.14 (d, J = 2.1 Hz, 2H, H-4 Gal'), 4.07 (dd, J = 3.0, 9.7 Hz, 2H, H-4 Gal'), 3.98−3.48 (m, 56H), 2.76 (dd, J = 12.3 Hz, 4.0 Hz, 2H, H-3 SA), 2.01 (s, 12H, NHAc), 1.79 (dd, J = 12.1, 12.1 Hz, 2H, H-3 SA), 1.64 (bm, 4H, CH_2), 1.41 (bm, 2H, CH_2), 1.15 (d, J = 5.7 Hz, 6H, CH_3-Fuc); MS (ion spray) calcd for $C_{79}H_{132}O_{56}N_4$ 2034, found 2032 $(M)^{2-}$, 1015 $(M)_{1/2}^{2-}$.

Enzymatic Sialylation for the Synthesis of 34-37. 5-(Methylcarbonyl)pentyl 4,6-Bis-*O*-[(ammonium 5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosuronate-(2→3)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-β-D-galactopyranoside (32). The N-type α(2−3)-sialyl transferase (EC 2.4.99.6, 1 U) was added to a solution of cytidine-5'-monophosphate-sialic acid (33 mg, 0.048 mmol), BSA (5% solution, 0.05 mL), sodium cacodylate (pH 6.5, 1 M, 0.9 mL), water (2.6 mL, $MnCl_2$ (1 M, 0.2 mL), alkaline phosphatase (EC 3.1.3.1, 1 U/μL, 30 μL), and 5-(methoxycarbonyl)-pentyl 4,6-bis-*O*-[β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-β-D-galactopyranoside[14] (16 mg, 16 μmol). The reaction mixture was allowed to stand for 4 days. Filtration and chromatography (Biogel P-2, 0.1 M NH_4HCO_3) of the filtrate afforded 18 mg (71%) of **32** as white solid after lyophilization: R_f = 0.21 (silica, 30% 1 M NH_4OAc/2-propanol); ^1H NMR (D_2O) δ 4.68 (d, J = 7.5 Hz, 1H, β-anomer Glc), 4.53 (bd, 3H, β-anomers of Glc, Gal, Gal), 4.29 (d, J = 8.0 Hz, 1H, β-anomer, bridging Gal), 4.08 (dd, J = 2.8 Hz, 1H, H-3 Gal), 4.05 (dd, J = 2.8 Hz, 1H, H-3 Gal), 3.97−3.49 (m, 46H), 3.31 (dd, J = 7.8, 9.6 Hz, 1H, OCH alkyl), 2.73 (dd, J = 12.3, 4.3 Hz, 2H, H-3(eq) sialic acid), 2.39 (t, J = 7.4 Hz, 2H, CH_2COOMe), 2.02 (s, 3H, NHAc), 2.01 (s, 6H, NHAc), 1.99 (s, 3H, NHAc), 1.78 (dd, J = 12.5 Hz, 2H, H-3(ax) sialic acid), 1.64−1.58 (m, 4H, CH_2 alkyl), 1.39 (m, 2H, CH_2 alkyl); MS (ion spray positive ion) calcd for $C_{63}H_{102}N_4O_{44}$ 1619, found 1641 $[(M^+ + Na^+])^{1-}$.

5-(Methoxycarbonyl)pentyl 2,6-Bis-*O*-[(ammonium 5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosuronate)-(2→3)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-β-D-galactopyranoside (35). Yield 18 mg (71%) of **35** a a white solid: R_f = 0.16 (silica, 30% 1 M NH_4OAc/2-propanol); ^1H NMR (D_2O) δ 4.66 (bd, 1H, β-anomer Glc), 4.53 (bd, 3H, β-anomers of Glc, Gal, Gal), 4.39 (d, J = 7.4 Hz, 1H, β-anomer, bridging Gal), 4.09 (dd, J = 2.4 Hz, 1H, H-3 Gal), 4.06 (dd, J = 2.4 Hz, 1H, H-3 Gal), 3.98−3.40 (m, 47H), 2.71 (dd, J = 12.3, 4.3 Hz, 2H, H-3(eq) sialic acid), 2.37 (t, J = 7.4 Hz, 2H, CH_2COOMe), 1.98 (s, 9H, NHAc), 1.97 (s, 3H, NHAc), 1.76 (dd, J = 12.5 Hz, 2H, H-3(ax) sialic acid), 1.64−1.54 (m, 4H, CH_2 alkyl), 1.37−1.32 (m, 2H, CH_2 alkyl); MS (ion spray, positive ion) calcd for $C_{63}H_{102}N_4O_{44}$ 1619, found 1619 (M^+), 1641 $([M^+ + Na^+])^{1-}$.

5-(Methoxycarbonyl)pentyl 3,4-Bis-*O*-[(ammonium 5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosuronate)-(2→3)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-β-D-galactopyranoside (36). Yield 18 mg (71%) of **36** as a white solid: R_f = 0.21 (silica, 30% 1 M NH_4OAc/2-propanol); ^1H NMR (D_2O) δ 4.58 (d, J = 8.4 Hz, 1H, β-anomer Glc), 4.50 (m, 3H, β-anomers of Glc, Gal, Gal), 4.30 (dd, J = 2.4 Hz, 1H, H-3 Gal), 4.06 (dd, J = 2.4 Hz, 1H, H-3 Gal), 3.95−3.43 (m, 45 H), 3.31 (t, J = 8.0 Hz, 1H, OCH alkyl), 2.70 (dd, J = 12.3, 4.3 Hz, 2H, H-3(eq) sialic acid), 2.34 (t, J = 7.3 Hz, 2H, CH_2COOMe), 1.98 (s, 3H, NHAc), 1.97 (s, 9H, NHAc), 1.74 (dd, J = 12.1 Hz, 2H, H-3(ax) sialic acid), 1.59−1.52 (m, 4H, CH_2 alkyl), 1.34−1.28 (m, 2H, CH_2 alkyl); MS (ion spray, positive ion) calcd for $C_{36}H_{102}N_4O_{44}$ 1619, found 1619 (M^+), 1641 $([M^+ + Na^+] + H^+)$.

5-(Methoxycarbonyl)pentyl 2,3-Bis-*O*-[(ammonium 5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosuronate)-(2→3)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-β-D-galactopyranoside (37). Yield 16 mg (64°) of **37** as a white solid: R_f = 0.29 (silica, 30% 1 M NH_4OAc/2-propanol); ^1H NMR (D_2O) δ 4.66−4.49 (multiple peaks, 5H, β-anomers Glc, Glc, Gal, Gal, bridging Gal), 4.09 (dd, J = 2.2 Hz, 1H, H-3 Gal), 4.06 (dd, J = 2.7 Hz, 1H, H-3 Gal), 3.98−3.41 (m, 46H), 2.72 (dd, J = 12.3, 4.3 Hz, 2H, H-3(eq) sialic acid), 2.38 (t, J = 7.4 Hz, 2H, CH_2COOMe), 2.07 (s, 3H, NHAc), 2.03 (s, 3H, NHAc), 1.99 (s, 6H, NHAc), 1.76 (dd, J = 12.1 Hz, 2H, H-3(ax) sialic acid), 1.64−1.55 (m, 4H, CH_2 alkyl), 1.39−1.34 (m, 2H, CH_2 alkyl); MS (ion spray, positive ion) calcd for $C_{63}H_{102}N_4O_{44}$ 1619, found 1619 (M^+), 1641 $([M^+ + Na^+])^{1-}$.

Enzymatic Fucosylation of 34-37. 5-(Methoxycarbonyl)pentyl 4,6-Bis-*O*-[(ammonium 5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosuronate)-(2→3)-b-D-galactopyranosyl-(1→4)-(α-L-fucopyranosyl-(1→3)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-β-D-galactopyranoside (16). The fucosyltransferase V (200 mU attached to beads) was added to a solution containing sodium cacodylate (pH 6.5, 1 M, 0.3 mL), water (1.8 mL), $MnCl_2$ (1 M, 0.1 mL), alkaline phosphatase (32 U), GDP-fucose disodium salt (34 mg, 55 μmol), and **34** (16 mg, 9.6 μmol). The reaction mixture was tipped for 5 days. Filtration and chromatography (Biogel P-2, 0.1 M NH_4HCO_3) of the filtrate afford 17 mg (98%) of **16** as white solid after lyophilization: Rf = 0.56 (silica, 40% 1 M NH_4OAc/2-propanol); ^1H NMR (D_2O) δ 5.07 (d, J = 3.5 Hz, 1H, α-anomer Fuc), 5.05 (d, J = 3.5 Hz, 1H, α-anomer Fuc), 4.63 (d, J = 7.8 Hz, 1 H, β-anomer Glc), 4.53−4.47 (bd, 3H, β-anomers Glc, Gal and Gal), 4.02 (d, J = 2.5 Hz, 1H, H-3 Gal), 3.99−3.44 (m, 42 H), 3.27 (t, J = 8.8 Hz, 1H, OCH alkyl), 2.71 (dd, J = 12.4, 4.4 Hz, 2H, H-3(eq) sialic acids), 2.36 (t, J = 7.4 Hz, 2H, CH_2 alkyl), 1.98 (s, 9 H, NHAc), 1.95 (s, 3H, NHAc), 1.74 (dd, J = 12.4, 12.4 Hz, 2 H, H-3(ax) sialic acids), 1.60−1.53 (m, 4H, CH_2 alkyl), 1.38−1.31 (m, 2H, CH_2 alkyl), 1.11 (d, J = 6.5 Hz, 6 H, CH_3-fucose); MS (ion spray) calcd for $C_{75}H_{122}N_4O_{52}$ 1912, found 1912 (M^+), 955 $(M^+ - 2NH_4)$.

5-(Methoxycarbonyl)pentyl 2,6-Bis-*O*-[(ammonium 5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranuronate)-(2→3)-β-D-galactopyranosyl-(1→4)-(6-deoxy-α-L-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-β-D-galactopyranoside (17). Yield 18 mg (85%) of **17** as white solid after lyophilization: R_f = 0.41 (silica, 40% 1 M NH_4OAc/2-propanol); ^1H NMR (D_2O) δ 5.07 (d, J = 3.5 Hz, 1H, α-anomer Fuc), 5.05 (bd, 2H, α-anomers Fuc), 4.64 (d, J = 8.4 Hz, 1H, β-anomer Glc), 4.51−4.44 (multiple peaks, 3H, β-anomers Glc, Gal and Gal), 4.38 (d, J = 7.4 Hz, 1H, β-anomer

bridging Gal), 4.06 (d, J = 2.7 Hz, 1H, H-3 Gal), 4.02 (d, J = 2.3 Hz, 1H, H-3 Gal), 3.99−3.38 (m, 41 H), 2.71 (dd, J = 12.4, 4.4 Hz, 2H, H-3(eq) sialic acids), 3.28 (t, J = 7.4 Hz, 2H, CH_2 alkyl), 1.98 (s, 9H, NHAc), 1.96 (s, 3H, NHAc), 1.74 (dd, J = 12.4, 12.4 Hz, 2H, H-3(ax) sialic acids), 1.62−1.55 (m, 4H, CH_2 alkyl), 1.37−1.32 (m, 2H, CH_2 alkyl), 1.11 (d, J = 6.5 Hz, 6H, CH_3-fucose); MS (ion spray) calcd for $C_{75}H_{122}N_4O_{52}$ 1912, found 1912 (M^+), 955.

5-(Methoxycarbonyl)pentyl 3,4-Bis-*O*[(ammonium 5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonate)-(2→3)-β)-D-galactopyranosyl-(1→4)-(6-deoxy-α-L-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-β-D-galactopyranoside (19). The fucosyl transferase V (200 mU attached to beads) was added to a solution containing sodium cacodylate (pH 6.5, 1 M, 0.3 mL), water (1.8 mL), $MnCl_2$ (1 M, 0.1 mL), alkaline phosphatase (32 U), GDP-fucose disodium salt (68 mg, 110 μmol), and **36** (16 mg, 9.6 μmol). The reaction mixture was tipped for 21 d. Filtration and chromatography (Biogel P-2, 0.1 M NH_4HCO_3) of the filtrate afford 12 mg (68%) of a mixture of mono- and difucosylated products as white solid after lyophilization: R_f = 0.54 (silica, 40% 1 M NH_4OAc/2-propanol); ^1H NMR (D_2O) δ 5.13−5.10 (m, 1H), 5.05−5.02 (d, J = 3.5 Hz, 1H), 4.95−4.87 (m, 2H), 4.58−4.54 (m, 2H), 4.51−4.41 (m, 3H), 4.29−4.25 (m, 2H), 4.24−4.20 (m, 2H), 4.07−3.44 (m, 48H), 3.30−3.22 (m, 1H), 2.71−2.66 (m, 2H), 2.32 (t, J = 7.4 Hz, 2H), 2.09−1.91 (m, 12H), 1.76−1.68 (m, 2H), 1.59−1.49 (m, 4H), 1.33−1.25 (m, 2H), 1.09 (d, J = 6.5 Hz, 6H); MS (ion spray) calcd for $C_{75}H_{122}N_4O_{52}$ (**19**) 1912, found 1912 (M^+), 1766.

5-(Methoxycarbonyl)pentyl 2,3-Bis-*O*-[(ammonium 5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranuronate)-(2→3)-β-D-galactopyranosyl-(1→4)-(α-L-fucopyranosyl)-(1→3)-2-acetamido-2-deoxy-β-D-galactopyranosyl]-β-D-galactopyranoside (18). The fucosyl transferase V (200 mU attached to beads) was added to a solution containing sodium cacodylate (pH 6.5, 1 M, 0.3 mL), water (1.8 mL), $MnCl_2$ (1 M, 0.1 mL), alkaline phosphatase (32 U), GDP-fucose disodium salt (68 mg, 110 μmol), and **37** (15 mg, 9.1 μmol). The reaction mixture was tipped for 15 d. Filtration and chromatography (Biogel P-2, 0.1 M NH_4HCO_3) of the filtrate afford 12 mg (74%) of **18** as white solid after lyophilization: R_f = 0.54 (silica, 40% 1M NH_4OAc/2-propanol); ^1H NMR (D_2O) δ 5.05−5.00 (m, 2H), 4.58−4.52 (m, 1H), 4.50−4.44 (m, 2H), 4.10−3.40 (m, 57H), 2.72−2.66 (m, 2H), 2.34 (t, 2H, J = 6.5 Hz), 2.05−1.97 (m, 6H), 1.95 (s, 6H), 1.76−1.68 (m, 2H), 1.60−1.52 (m, 4H), 1.36−1.28 (m, 2H), 1.12−1.08 (m, 6H); MS (ion spray), calcd for $C_{75}H_{122}N_4O_{52}$ 1912, found 1912 (M^+), 1766.

Production of Recombinant Soluble E-Selectin Cells and Plasmid DNA. The procedure for the preparation of recombinant E-selectin is essentially the same as described previously.[17] The adenovirus transformed human kidney cell line 293 was obtained from the ATCC (CRL-1573). The 293 cells were grown as adherent cultures in DMEM, obtained from Whittaker Bioproducts (Walkersville, MD), supplemented with 10% fetal calf serum, obtained from JRH Biochemical (Lenexa, KS). The plasmid pCDNAI, a derivative of pCDM8,[18] was obtained from Invitrogen (San Diego, CA). The plasmid pBluescript II was obtained from Stratagene (San Diego, CA). The plasmid pSV2-Neo[19] contains the *E. coli* gene encoding the aminoglycoside 3′-phosphotransferase gene. When pSV2-neo is introduced into mammalian cells, the transferred cells exhibit resistance to the antibiotic G418.

Recombinant DNA. A soluble form of E-selectin was prepared as described in the following. A 1.67 kbp DNA fragment encoding a truncated structural gene for E-selectin was isolated by polymerase chain reaction (PCR) amplification of cDNA derived from messenger RNA that was isolated from IL-1 activated human endothelial cells. The 5′-amplimer inserted a unique ClA I restriction site 28 nucleotides upstream from the initiation codon of the E-selectin structural gene. The 3′-amplimer inserted the termination codon TGA after amino acid residue 527 of the mature E-selectin, followed by a unique XhoI restriction site. The carboxy terminus of soluble E-selectin is located at the carboxy terminus of the sixth censensus repeat element, thereby deleting the transmembrane domain. The 1.67 Kbp PCR fragment was codigested with Cla I and Xho I restriction endonucleases and subcloned into the Cla I and Xho I restriction sites of the cloning vector pBluescript II, generating pBSII-sol-E-selectin. Soluble E-selectin is 527 amino acids in length and contains 11 potential N-glycosylation sites. A 1.67 Kbp DNA fragment containing the soluble E-selectin cDNA was isolated from pBSII-sol-E-selectin and sub-cloned into the EcoRV and Xho I sites of the expression vector pCDNAI generating pCDNAI-sol-E-selectin.

Generation of a Stable Cell Line Secretin Sol-E-selectin. pCDNAI-sol-E-selectin was cotransfected with pSV2-neo, via the calcium phosphate technique,[20] into 293 cells. At 48 h post-transfection, the transfected 293 cells were trypsinized and plated into DMEM, 10% FBS, and 600 μg/mL (potency) of G418 (Geneticin, Sigma). The selection media was changed every 3 days until a stable G418-resistant population was established. Single clones of G418-resistant cells were isolated by cloning cylinders. Isolated clones were screened for the synthesis of sol-E-selectin by enzyme-linked immunosorbent assay (ELISA) utilizing the anti-E-selectin monoclonal antibody CY1787 as the primary antibody. Positive clones were plated at 10^6 cells/100 mm dish, 24 hours later they were metabolically labelled with [^{35}S]-methionene for 5 h. Labelled sol-E-selectin was immunoprecipitated from the media with CY1787 and electrophoresed through a 10% PAGE gel, the gel dried and subjected to authoradiography. Clone 293#3 was selected as the stable cell line that produced the greatest amount of the 110-Kd sol-E-selectin protein/cell.

Large Scale Production of Sol-E-selectin. A 10-chambered Nunc Cell Factory (6250 cm^2 total surface area, Nunc) was seeded with 2.78 × 10^8 cells (Clone 293#3) in 850 mL in DMEM supplemented with 5% FBS and incubated at 37 °C for 72 h. The media was harvested and replaced with 850 mL of DMEM, 5% FBS. After the cell factory was incubated at 37 °C for 48 h the media was harvested a second time and replaced with 850 mL of DMEM 5% FBS. After the cell factory was incubated at 37 °C for 48 h the media was harvested a third (and final) time. After each harvest 0.02% sodium azide was added to the media. The media was clarified by centrifugation (5000*g*), passed through a 0.2 μm filter, and stored at 4 °C until further purification.

Immunoaffinity Column. Monoclonal antibody CY1787, anti E-selectin, was conjugated to protein-A Sepharose.[21] Briefly, 28 mg of CY1787 (5 mg/mL) in PBS was mixed with 5 mL of protein-A Sepharose for 30 min at room temperature. The beads were then washed four times by centrifugation with 25 mL of 0.1 M borate buffer, pH 8.2, followed by two washes with 10 mL of 0.2 M triethanolamine, pH 8.2. The resin was then suspended in 40 mL of 0.2 M triethanolamine buffer, pH 8.2, containing 0.02 M dimethyl pimelimidate. After reacting for 45 min at room temperature on a rotator, the resin was washed twice with 0.02 M ethanolamine, pH 8.2, followed with three washes with 10 mL of 0.1 M borate buffer, pH 8.2. Unbound antibody was removed by elution with 0.1 M sodium acetate buffer, pH 4.5. Approximately 89% of the antibody applied was conjugated to the protein-A Sepharose.

Isolation of Recombinant Soluble E-Selectin. Tissue culture supernatant (2.55 L) was passed through a 0.7 cm × 1.5 cm precolumn of protein-A Sepharose connected in series to a 1.5 cm × 3 cm affinity column of CY1787-protein-A-Sepharose at a flow rate of 20 mL/h. The columns were then disconnected, and the CY1787 affinity column was washed with 20 mM Tris buffer, pH 7.5, containing 150 mM NaCl and 2 mM $CaCl_2$ until the A280 of the eluate approached zero. Bound E-selectin was eluted with 0.1 M sodium acetate buffer, pH 3.5, containing 1 mM $CaCl_2$ using gravity flow. Fractions (1 mL) were collected into 300 μL of 2 M Tris, pH 10. Protein containing fractions were pooled and dialyzed against DPBS. Following concentration on an Amicon Centriprep 30 until the protein concentration was approximately 1 mg/mL, the purified E-selectin was aliquoted and stored at −80 °C. Purity was greater than 90% by SDS-PAGE. A total of 10 mg of E-selectin was purified from 2.5 L of cell culture media.

Inhibition of HL-60 Cell Binding to Sol-E-selectin. Ninety-six well Immunolon 2 plates (Dynatech Laboratories, Inc., Chantilly, VA,

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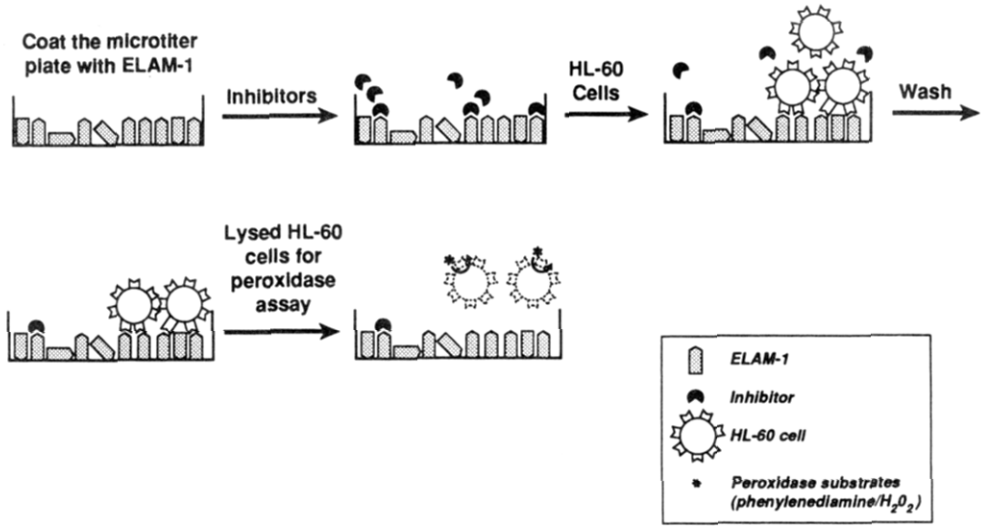

Figure 7. Schematic representation of the E-selectin inhibition analysis based on ELISA assay. For details see the Experimental Section.

catalog no. 011-0103655) were coated at room temperature with 50 μL of a 3 μg/mL solution (150 mg/well) of sol-E-selectin (recombinant soluble E-selectin) in DPBS (Dulbeco's phosphate buffered saline). The sol-E-selectin molecule was bound after 3 h at room temperature, the excess coating solution was removed by three washes with DPBS containing 1% bovine serum albumin (DPBS/1% BSA), and protein binding sites on the plate were blocked with 200 μL/well of DPBS/1% BSA for 1 h. Serial dilutions of inhibitors were prepared at an initial concentration of 10 mM in DPBS and diluted in Hank's balanced salt solution containing 20 mM HEPES, pH 7.27.4, 0.2% D-glucose and 1% BSA. After removing the blocking buffer, 40 μL of appropriately diluted inhibitor was added per well followed by 2×10^5 HL-60 cells (ATCC, CCL 240) in 20 μL. After 15 min at room temperature, the plate was washed three times with Hank's balanced salt solution containing 20 mM HEPES, pH 7.2−7.4, 0.2% D-glucose, 1% bovine serum albumin, and 1 mM calcium chloride using a Molecular Devices Microplate washer (Model no. 4845-02) adjusted for slow liquid delivery. Following addition of 50 μL of cell lysis buffer (24 mM citric acid, 51 mM sodium phosphate dibasic, 0.1% nonidet P-40), the plates were incubated for 5 min on a plate shaker at room temperature. Myeloperoxidase released from bound HL-60 cells was detected by the addition of 50 μL of substrate solution (24 mM citric acid, 51 mM dibasic sodium phosphate, 0.1% *O*-phenylenediamine and 0.03% hydrogen peroxide). The reaction was stopped with 10 min by the addition to each well of 40 μL of 4 N sulfuric acid. Absorbances at 492 nm were determined in a TiterTex plate reader. The percentage inhibition was determined based on the absorbance in positive control wells containing no inhibitor. For a schematic representation of the assay, see Figure 7.

NMR Experiments. Proton and carbon NMR experiments were conducted in D_2O at 295 K using a Bruker AMX-500 NMR spectrometer equipped with an X-32 computer and an ASPECT-3000 process controller. The sample was not spun. ^1H chemical shifts were referenced to internal HOD at 4.76 ppm, and ^{13}C chemical shifts referenced to external DMSO at 39.5 ppm. All NMR data were processed and analyzed with the Felix program (Hare Research, Woodinville, WA) run on a Sun SPARC station or a Silicon Graphics Indigo^2 workstation. For further experimental details, see ref 4.

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