Glycobiology vol. 7 no. 1 pp. 79–93, 1997

Selectin inhibition: synthesis and evaluation of novel sialylated, sulfated and fucosylated oligosaccharides, including the major capping group of GlyCAM-1

Andrea Koenig[3], Rakesh Jain[1,3,4], Rakesh Vig[1], Karin E.Norgard-Sumnicht, Khushi L.Matta[1] and Ajit Varki[2]

Glycobiology Program, UCSD Cancer Center, and the Division of Cellular and Molecular Medicine, University of California San Diego, La Jolla, CA 92093, USA and [1]Gynecologic Oncology Research, Roswell Park Cancer Institute, Buffalo, NY 14263, USA

[2]To whom correspondence should be addressed at: Cancer Center, 0687, UCSD School of Medicine, La Jolla, CA 92093–0687

[3]The contributions of the first two authors should be considered equivalent.

[4]Present address: Trans Cell Technology Inc., Monmouth Junction, NJ 08852, USA

Inquiries regarding chemical synthesis should be directed to K.L.M.

Selectins interact with glycoconjugate ligands in important normal and pathological situations. While high affinity recognition of natural ligands is associated with α1–3(4)fucosylated, α2–3sialylated (and/or sulfated) lactosamine sequences, small oligosaccharides that potently inhibit the selectins have not been found. One possibility suggested by other investigators is that high affinity may require unusual sequences not yet tested, for example, the "major capping group" (6′-sulfo-sialyl Le^x) of the L-selectin ligand GlyCAM-1. To explore this possibility, we synthesized a spectrum of novel synthetic and semisynthetic oligosaccharides related to those on natural ligands. In studying these molecules, we noted that binding of recombinant soluble selectins to immobilized sialyl Le^x or 3′-sulfo-Le^x is markedly inhibited by concentrations of chloride above the physiological range. This indicates the ionic nature of the interactions, and shows that buffers typically used in screening assays for inhibitors are not optimal. Using parameters that more closely approximate physiological conditions, we confirmed that α2–3-linked sialic acids, and α1–3(4)fucosylation are important for recognition. Similar results obtained with both types of immobilized targets for the three selectins indicated that the binding sites for sialic acid and sulfate are very close, or identical. While O-sulfate esters mostly improved L- and P-selectin recognition, effects depended upon their position and number. Furthermore, sulfation can also impart some "negative" specificity: the major capping group does not interact with E-selectin. The branched Core 2 sequence seemed to enhance L- and P-selectin binding, however, the best inhibitors still appeared to be sialyl Le^a and 3′-sulfo-Le^x, with the aglycone group of the latter affecting binding. Of particular note, the "major capping group" of GlyCAM-1 was not an unusually potent nor highly selective inhibitor of L-selectin, even when studying the interaction of L-selectin with native GlyCAM-1 itself.

Key words: lectins/structure/binding/ELISA

Introduction

The selectins are mammalian lectins that mediate the early steps of the recruitment of leukocytes from the blood stream in a variety of normal and pathologic situations (see Varki, 1992, 1994; Bevilacqua and Nelson, 1993; Rosen and Bertozzi, 1994; Lasky, 1995; McEver *et al.*, 1995; for reviews). Several natural glycoconjugate ligands of the selectins have been described, many of which share the biochemical properties of sialomucins (reviewed in Rosen and Bertozzi, 1994; Varki, 1994; Lasky, 1995; McEver *et al.*, 1995). GlyCAM-1 is the prototypic member of the "peripheral addressins," a group of sialylated sulfated mucin-like ligands for L-selectin synthesized by the specialized high endothelial venules (HEV) of lymphatic tissues (Imai *et al.*, 1991; Lasky *et al.*, 1992), which includes CD-34 (Imai *et al.*, 1991; Baumhueter *et al.*, 1993), the mucosal addressin-cell adhesion molecule-1 (MAdCAM-1) (Berg *et al.*, 1993), and an as yet unidentified 200 kDa ligand (Rosen and Bertozzi, 1994). The O-linked glycans on GlyCAM-1 are sialylated, fucosylated, and sulfated (Imai and Rosen, 1993; Hemmerich and Rosen, 1994; Hemmerich *et al.*, 1994), and the sialic acids and sulfate esters are required for binding by L-selectin. With all three ligands, only glycoforms expressed by HEV cells are able to bind L-selectin, indicating the importance of tissue-specific glycosylation in recognition. Likewise, the polypeptides PSGL-1 (P-selectin glycoprotein ligand-1) and ESL-1 (E-selectin ligand-1) can generate high affinity ligands for P- and E-selectin only when expressed in the correct cell types (Moore *et al.*, 1992; Norgard *et al.*, 1993b; Sako *et al.*, 1993). In the case of PSGL-1, recent data indicates that high affinity recognition is mediated at least in part by the combined recognition of sialylated oligosaccharides and tyrosine sulfate residues (Pouyani and Seed, 1995; Sako *et al.*, 1995; Wilkins *et al.*, 1995).

All three selectins are known to selectively recognize the tetrasaccharide sialyl-Le^x (sLe^x; Siaα2–3Galβ1–4(Fucα1–3)GlcNAcβ1-R) and its isomer sialyl-Le^a (sLe^a; Siaα2–3Galβ1–3(Fucα1–4)GlcNAcβ1-R). Many studies indicate that the sialic acid and the fucose residues are essential for recognition, and that a sulfate ester can replace sialic acid in some instances (Yuen *et al.*, 1994). However, the affinity of the selectins for these oligosaccharides and for some of their derivatives appears to be poor (most studies report values in the high micromolar to low mM range, based upon IC_{50}s and binding-inhibition assays, see Brandley *et al.*, 1993; Nelson *et al.*, 1993a,b; Narasinga Rao *et al.*, 1994; Yoshida *et al.*, 1994). Recently, the directly measured K_d for the interaction of soluble, monomeric E-selectin with the tetrasaccharide Siaα2–3Galβ1–4(Fucα1–3)Glc was reported to be 120 μM (Jacob *et al.*, 1995). In contrast, the physiological ligands seem to have much better affinities, showing binding interactions that can survive stringent washing under a variety of conditions. Direct measurements have confirmed the better affinities, for ex-

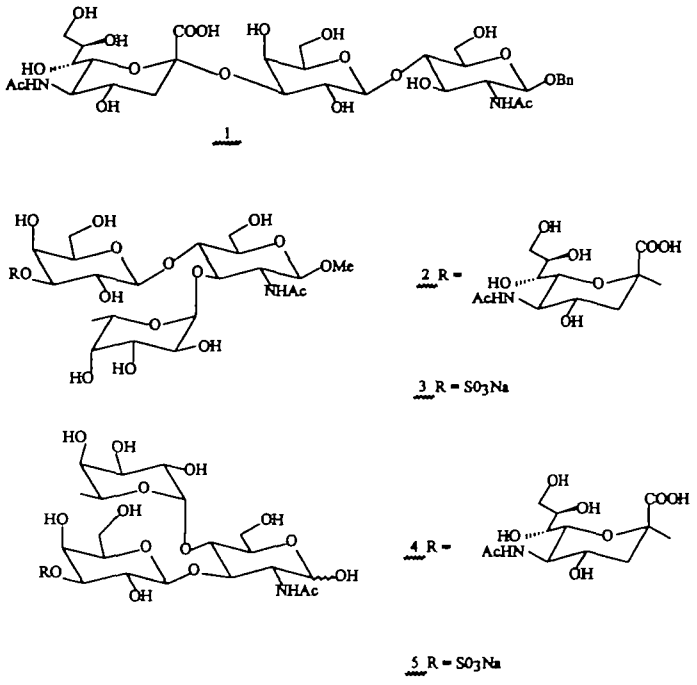


Fig. 1. Some target molecules for synthesis. Sialyl lactosamine 1; sialyl, sulfated Le^x (2 and 3); Sialyl, sulfated Le^a (4 and 5).

ample, a K_d of 70 nM for monomeric, soluble P-selectin interacting with its native ligand on HL-60 cells (Ushiyama *et al.*, 1993), and a similar affinity predicted for monovalent E-selectin (Hensley *et al.*, 1994).

There are currently several theories to explain the disparity between the high affinities of the selectins for their natural ligands and the relatively poor affinities of the common sialylated/sulfated fucosylated lactosamines. Simple multivalency of both oligosaccharide and selectin on intact cell surfaces or by presentation on a polypeptide backbone could enhance avidity. Indeed, while the IC_{50} of dimeric SLe^x for E-selectin showed only a fivefold improvement over the monomeric form (DeFrees *et al.*, 1995), a tetravalent form showed considerable improvement in inhibiting L-selectin (Turunen *et al.*, 1995). However, P- and L-selectin do not bind to some cell types that express considerable amounts of SLe^x, and cell recognition is usually destroyed by the mucin-degrading enzyme O-sialoglycoprotease (Steininger *et al.*, 1992; Norgard *et al.*, 1993b; Ushiyama *et al.*, 1993; Mannori *et al.*, 1995), even when the vast majority of cell surface SLe^x remains intact after this treatment (Norgard *et al.*, 1993b). Thus, simple ligand multivalency seems insufficient to explain biologically relevant selectin binding. Another possibility is the multivalent aggregation of selectins. However, the high affinity binding of soluble monomeric E- and P-selectin to cell surfaces (Ushiyama *et al.*, 1993) indicates that this is not essential, and there is no published evidence to date for naturally occurring multimerization of selectins. The possibility of multiple binding sites within a single selectin lectin domain is unlikely based on epitope mapping, mutagenesis, and crystallographic data, which indicate that the binding sites for carbohydrates is quite small (Erbe *et al.*, 1992, 1993; Hollenbaugh *et al.*, 1993; Graves *et al.*, 1994).

An alternate hypothesis is that the natural selectin ligands carry rare structural variants of the common sialylated fucosylated oligosaccharides, which are responsible for the high affinity interaction. The best candidate to date is unusual oligosaccharide 6′-sulfo-SLe^x, which was found on GlyCAM-1 (Hemmerich *et al.*, 1994; Hemmerich and Rosen, 1994), carrying an O-sulfate ester at the 6′-position of the galactose residue in SLe^x. In a recent study, it was concluded that "this structure can account for all of the known carbohydrate requirements in the binding of L-selectin to its cognate HEV ligands" (Hemmerich *et al.*, 1995). Our recent successful synthesis of this compound (Jain *et al.*, 1994) prompted us to examine if this molecule is indeed a superior ligand for L-selectin. In pursuing this matter, we have demonstrated the ionic nature of selectin binding, optimized the conditions for *in vitro* inhibitor screening assays, and synthesized for comparison several other related sulfated sialylated fucosylated oligosaccharides.

Results and discussion

Rationale for selection of synthetic oligosaccharide targets as potential inhibitors of the selectins

Most prior studies of the inhibitory properties of sialyloligosaccharides have focused upon a single selectin or upon a limited range of inhibitors. We decided to directly compare all three selectins against a wide range of potential inhibitors in

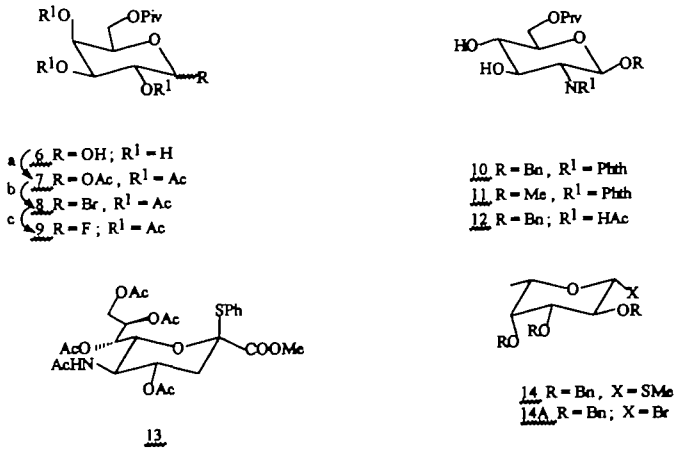


Fig. 2. Key intermediates (6–14A) involved in the synthesis of target compounds (1–5). **Scheme 1.** Reagents and conditions: (a) 1.4 equiv. of 9, 1.2 equiv. of AgOTf, 1.2 equiv. of $SnCl_2$, 4Å molecular sieves, CH_2Cl_2-toluene, 5:1 (v/v), −15 to 20°C, 4 h, 15 (17%), 16 (50%), 17 (21%), 18 (48%); (b) MeOH-CH_2Cl_2, 1:1 (pH 10), 2 h, 0°C, 78%; (c) 2 equiv. of 13, 3 equiv. of NIS, triflic acid in propionitrile, −45°C, 2 h, 53%; (d) 8 equiv. of LiI in pyridine, 120°C, 3 h, 75%; (e) MeOH-hydrazine hydrate, 5:1 (v/v), 80°C, 7 h, (f) Ac_2O (excess), MeOH-CH_2Cl_2 (1:1), 0°C, 1 h; MeOH-MeONa, 48 h, 53% from 19. **Scheme 2.** Reagents and conditions: (a) 2 equiv. of 14A, 1 equiv. of 18, 2 equiv. of AgOTf, 1.8 equiv. of 2,6-di-tertbutyl-4-methyl-pyridine, 4Å molecular sieves, CH_2Cl_2-toluene, 2:3 (v/v), −35°C, 3 h, 79%; (b) EtOH-hydrazine hydrate, 9:1 (v/v), 100°C, 6 h, MeOH-Et_3N-Ac_2O, 4:2:1 (v/v) 0 to 20°C, 2 h, 62%; (c) 2.5 equiv. of 13, 3 equiv. of NIS-triflic acid in propionitrile, −75°C, 2 h, 66%; (d) 6 equiv. of SO_3-pyridine complex in pyridine, 5°C, 16 h; (e) MeOH, 10% Pd-C, MeOH-MeONa, 72 h, H_2O, 5 h, 2 (96%), 3 (37% from 21). **Scheme 3.** Reagents and conditions: (a) 1.5 equiv. of 8, 1.0 equiv. of 12, 1.5 equiv. of $Hg(CN)_2$ in benzene-nitromethane, 1:1 (v/v), 55°C, 16 h, 65%; (b) 1.0 equiv. of 23, 2.0 equiv. of 14, 3.0 equiv. of $CuBr_2$, 3.0 equiv. of Bu_4NBr, $ClCH_2CH_2Cl$-DMF, 5:1 (v/v), 4Å molecular sieves, 16 h, 56%; (c) 2.5 equiv. of 13, 3.0 equiv. of NIS-triflic acid in propionitrile, −70°C, 2 h, 54%; (d) 6 equiv. of SO_3-pyridine complex in pyridine, 5°C, 16 h; (e) MeOH-10% Pd-C, MeOH-MeONa, 72 h, H_2O, 5 h, 4 (66%), 5 (50% from 25).

ELISA assays that used two different immobilized ligands. The selection of oligosaccharide targets for synthesis was based mainly on three considerations: (1) existing knowledge of natural oligosaccharide structures containing fucose, sulfate and sialic acid; and (2) knowledge of the specificity characteristics of fucosyl, sialyl, and sulfotransferases involved in their synthesis; and (3) some limitations on the ability to chemically synthesize all possible structures. Biosynthetic pathways indicate that sialylation occurs before fucosylation. Thus, Neu5Acα2–3Galβ1-(3)4GlcNAcβ- sequences are acceptors for α-fucosyltransferases (Howard *et al.*, 1987; Foster *et al.*, 1991) to give sialyl-Le^x and sialyl-Le^a (see Figure 1). In contrast, Neu5Acα2–6Galβ1–4GlcNAcβ- is not an acceptor for α1–3-fucosyltransferase (Paulson *et al.*, 1978) and synthetically prepared Neu5Acα2–6Galβ1–4(Fucα(1–3)GlcNAcβ1- does not bind to selectins. One of us (Chandrasekaran *et al.*, 1995) first employed synthetic sulfated oligosaccharides to examine the specificity of α-fucosyltransferases. For example, we found that SE-3Galβ1–4GlcNAc is an acceptor for α1–3-fucosyltransferase to give SE-3Galβ1–4(Fucα1–3)GlcNAc (Chandrasekaran *et al.*, 1995). We and others (Nicolaou *et al.*, 1992; Lubineau *et al.*, 1993; Chandrasekaran *et al.*, 1995) also developed the chemical synthesis of this molecule, and showed that it can act as a ligand for selectins. Also, in examining the specificity of α2–3-sialyltransferase with a series of sulfated saccharides, we found that Galβ1–4(SE-6GlcNAc) is an effective acceptor for 2–3-sialyltransferase to give Neu5Acα2–3Galβ1–4(SE-6)GlcNAc (Chandrasekaran *et al.*, 1995; unpublished observations). The resulting 2,3-sialylated compounds can be fucosylated by α1–3-fucosyltransferase to give 6-sulfosialyl-Le^x,which others have recently shown to have a moderate affinity for L-selectin (Scudder *et al.*, 1994). This compound is an isomer of the "major capping group" on GlyCAM-1 (6′-sulfated sialyl Le^x) (Hemmerich and Rosen, 1994). Thus, certain sialylated and/or sulfated saccharides are capable of acting as acceptors for the α1–3(4)-fucosyltransferases to yield products which can act as a ligand for selectins. More recently, others reported some bisulfated molecules to be moderately good L-selectin inhibitors (Bertozzi *et al.*, 1995; Manning *et al.*, 1995)

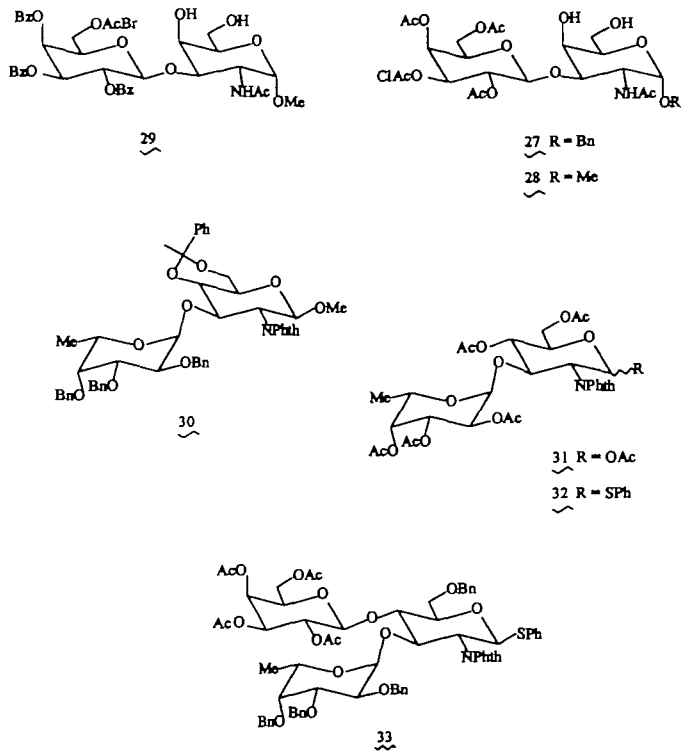
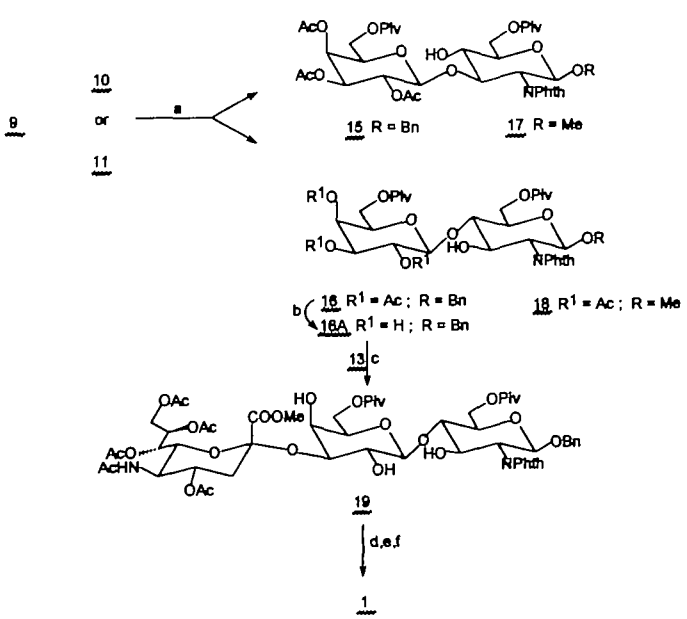


Fig. 3. Key intermediates (27–33) involved in the synthesis of some target compounds. **Scheme 4.** Reagents and conditions: (a) NIS-Triflic acid, –30°C; (b) Thiourea-2,6-lutidine, CH_2Cl_2-EtOH (1:1, v/v), 70°C, 6 h; (c) SO_3-pyridine complex, DMF, 5°C, 16 h; (d) EtOH-Hydrazine hydrate (4:1, v/v), 100°C, 3 h; (e) Ac_2O-MeOH-Et_3N, 0°(20°C, 2 h. **Scheme 5.** Reagents and conditions: (a) NIS-triflic acid/CH_2Cl_2, –60°C, 0.5 h; (b) thiourea-2,6-lutidine/EtOH-CH_2Cl_2 (1:1, v/v), 70°C, 6 h; (c) SO_3-pyridine complex/DMF, 0°C, 6 h; (d) EtOH-hydrazine hydrate (4:1, v/v), 100°C, 3 h; (e) Ac_2O-MeOH-Et_3N, 0°C (20°C, 2 h; (f) MeOH-H_2O (4:1, v/v), 10% Pd-C, 48 h. **Scheme 6.** Reagents and conditions: (a) NIS-Triflic acid/CH_2Cl_2, –60°C, 0.5 h; (b) thiourea-2,6-lutidine/EtOH-CH_2Cl_2 (1:1, v/v), 70°C, 6 h; (c) SO_3-pyridine complex/DMF, 0°C, 6 h; (d) EtOH-hydrazine hydrate (4:1, v/v), 100°C, 3 h; (e) Ac_2O-MeOH-Et_3N, 0°C (20°C, 2 h; (f) MeOH-H_2O (4:1, v/v), 10% Pd-C, 48 h.

Scheme 1

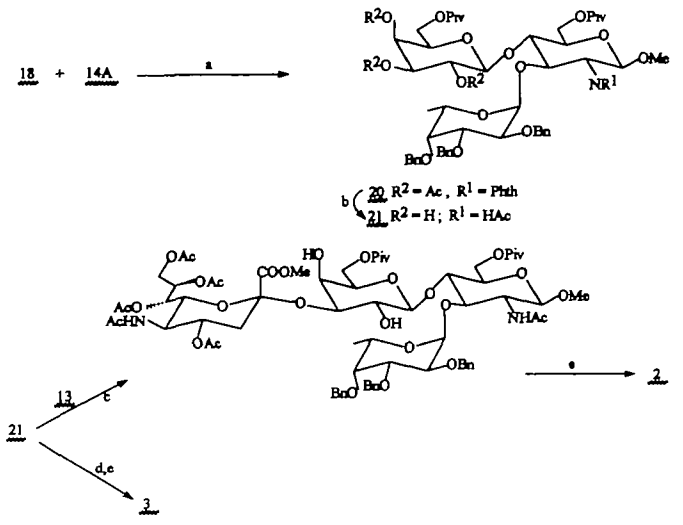


Synthesis of compound **42** was based upon our recent findings on the specificity of 3-O-sulfotransferase from ovarian, colon, and breast tissues (Kuhns *et al.*, 1995; Chandrahekaran *et al.*, unpublished observations). This enzyme, particularly from ovarian and breast tissue, is highly specific for C-3 position of galactose in the Galβ1–3GalNAc sequence. Corresponding to this finding, the synthetic analog of **42** without the sulfate functional group is a very good acceptor for the transfer of sulfate onto **42** via 3-O-sulfotransferase. In addition to these linear structures, many selectin ligands are characterized by O-linked mucin core structures, which can be branched. In the case of GlyCAM-1,the inner core structure Galβ1–4GlcNAcβ1–6(Galβ1–3)GalNAcα1- is further modified by sialic acid, sulfate, and fucose to give one of the larger oligosaccharide moieties on this protein (Hemmerich *et al.*, 1995). Thus, we also focused our attention on trying to synthesize some of these branched molecules.

Synthesis of oligosaccharides as potential inhibitors of the selectins

Based on the above considerations, a variety of new potential oligosaccharide inhibitors for the selectins were synthesized as

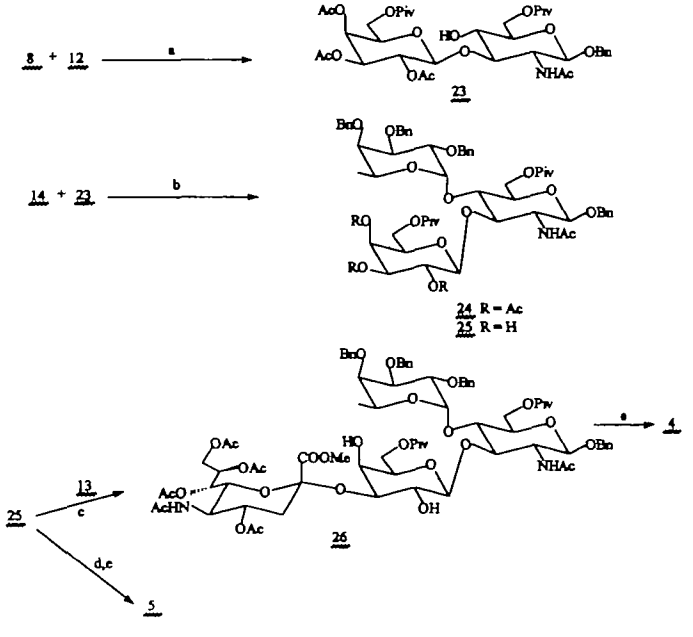
Scheme 2



described in *Materials and methods* (compounds **1–42**, see also Figures 2, 3).

Synthetic methods used included the use of 2,3,4-tri-O-acetyl-6-O-pivaloyl-α/b-galactopyranosyl halide for the syntheses of both 3-O-sialyl- or 3-O-sulfo-Le^x and Le^a type structures (Jain *et al.*, 1996) Further details of the synthesis of compounds **44, 46, 47,** and **50** will be published elsewhere (unpublished observations). Compounds **48** and **49** were synthesized as described elsewhere (Jain *et al.*, 1994; Vig *et al.*, 1995). The structure and purity of each compound was confirmed by TLC, NMR, and FAB mass spectroscopy. As seen in Figures 1–3, many of these compounds are based upon previously known natural structures (**2, 4, 5, 46, 49;** see Table I), but are synthesized with various modifications at the reducing termini. Others are structures that have not been shown in nature, but do carry features that are typical of mucin-type O-linked

Scheme 3

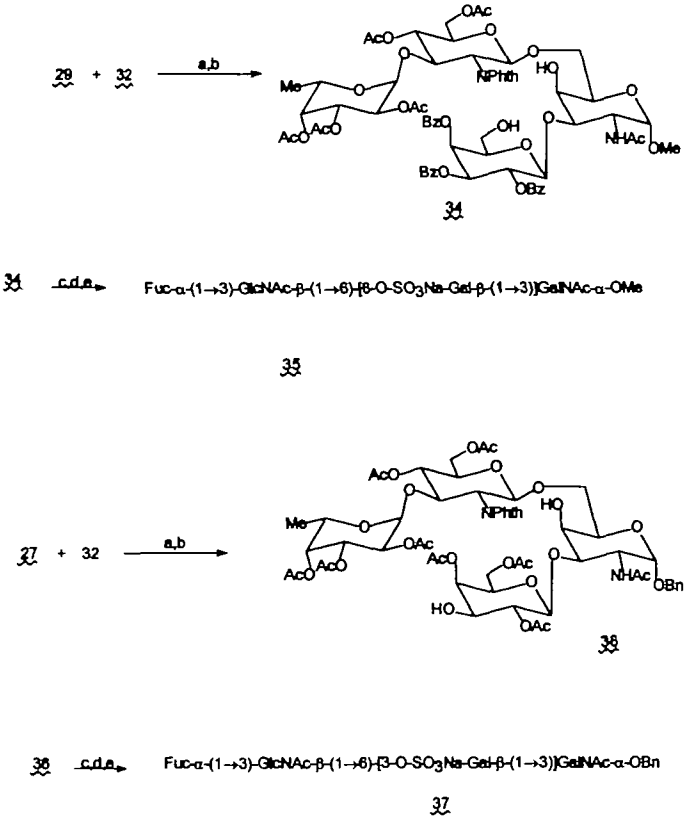


chains (Capon *et al.*, 1992; Mawhinney *et al.*, 1992; Strous and Dekker, 1992; Lo-Guidice *et al.*, 1994), for example, the Core 2 structure found in oligosaccharides **35, 37, 40, 42, 50.** In addition, several of these compounds (**35, 37, 40, 42, 48,** and **50**) contain modifications suggested as important for selectin recognition, such as the sulfate groups and α1–3(4)fucosyl residues.

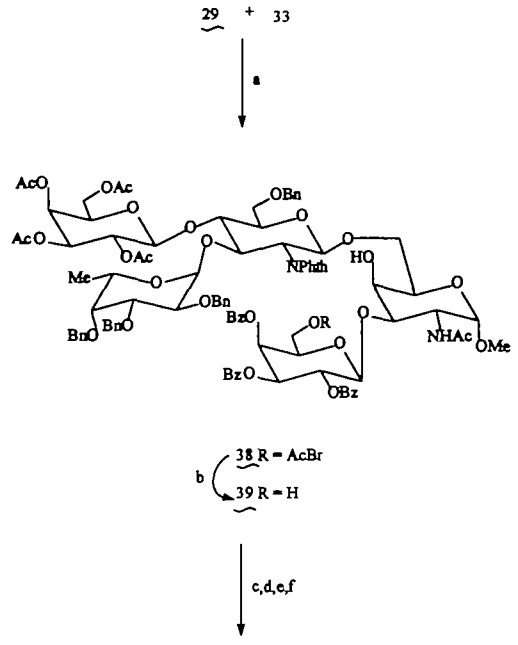
Effects of ionic strength, pH, and temperature on the binding of selectins to immobilized sialyl Le^x and 3′-sulfo Le^x

The screening evaluation of potential selectin inhibitors is typically carried out by ELISA assays, monitoring the binding of recombinant soluble selectins to immobilized ligands such as SLe^x. The solutions typically used for the initial binding and subsequent washing steps in such assays contain 150 mM NaCl (''normal'' saline), a buffer (typically 20 mM HEPES, MOPS or Tris), as well as 2–5 mM each of $CaCl_2$ and $MgCl_2$, bringing the total anion milliequivalents to about 178–190 mM (assuming the buffers are used within their ideal pK_a range and contribute one anion milliequivalent per mM buffer). The total anion equivalents in normal human plasma is about 150 mM (contributing to normal osmolality of ~290 mOsm/Kg), and pH is tightly controlled between 7.35 and 7.45, and temperature between 36–38°C. Despite the voluminous extant literature on the binding of selectins to ligands, there appears to be no published study of the importance of these parameters in such assays. Before evaluating the synthetic molecules described above, we examined the effects of varying these parameters on the binding of recombinant E-, P-, or L-selectin to immobilized SLe^x and P- or L-selectin. (Recombinant E-selectin did not show detectable binding to 3′sulfo-Le^x immobilized on polyacrylamide, despite the fact that it interacts with this compound

Scheme 4



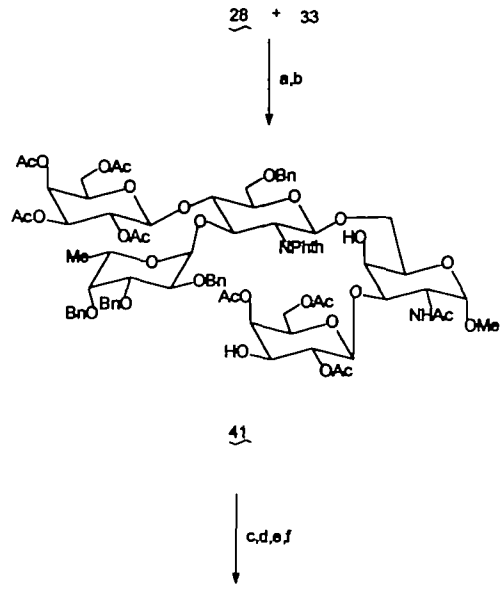
Scheme 5



Fuc-α-(1→3)-[Gal-β-(1→4)]-GlcNAc-β-(1→6)-[6-O-SO_3Na-Gal-β-(1→6)]GalNAc-α-OMe

40

Scheme 6



Fuc-α-(1→3)-[Gal-β-(1→4)]-GlcNAc-β-(1→6)-[3-O-SO_3Na-Gal-β-(1→3)]GalNAc-α-OMe

42

in solution.) As shown in Figure 4, increasing chloride concentrations (at pH 7.45 and 4°C) had a substantial inhibitory effect upon the binding of all three selectins to SLe^x. These results indicate that selectin-ligand binding likely involves ionic interactions. This fits well with the fact that the C-type lectin binding pockets of the selectins are rich in lysine residues (Erbe *et al.*, 1992, 1993; Hollenbaugh *et al.*, 1993; Graves *et al.*, 1994), while the natural ligands are often polyanionic. It is notable that under the conditions typically used in previous studies (e.g., 150 mM NaCl in addition to the standard components of the buffer), selectin binding to immobilized SLe^x would be decreased by almost 50%. As NaCl concentration is further increased, the effect is more substantial (Figure 4), for example, 100 mM NaCl above physiological buffer conditions almost completely abolishes binding. Thus, many experimental conditions previously used may not have not been optimal for studying E-, L-, and P-selectin binding to such immobilized ligands. Furthermore, variations in pH from 7.1–7.7 also affected selectin-ligand interaction, with maximal binding seen at pH 7.1 and 75% of this value obtained at pH = 7.7 (data not shown). Based on these studies, we lowered the NaCl concentration in our ELISA buffer to 125 mM, used 2 mM each of $CaCl_2$ and $MgCl_2$ (close to their physiological range in plasma), and kept the buffer concentration at 20 mM HEPES (pH adjusted to normal plasma value pH of 7.45). The effects of temperature was next studied under these optimized conditions (total anion concentration = 153 mM). Increasing temperatures gave some decrease in binding, with binding at 37°C being 45–50% of that at 4°C (data not shown). However, increasing temperatures also markedly increased the nonspecific (EDTA insensitive) background level of binding. Thus, we chose to carry out further studies at 4°C, using the "physiological" buffer and pH conditions listed above.

General features of the inhibitory properties of the synthetic oligosaccharides

The panel of synthetic oligosaccharides were analyzed for their potential selectin inhibitory properties. A few of these have been previously studied, for example, compounds **2–5**, and are included for comparison. As summarized in Table I (see also examples of the data in Figures 5–6) various combinations of α2–3-linked sialic acid residues, sulfate esters and α1–3(4)fucosylation seem to contribute to binding. In general, sulfate esters at the 6-position seemed to improve binding when they were in the internal position but not at the terminal position (compare compounds **44** and **46** vs **45** and **48** in Table I). In contrast, and as indicated from prior work (Yuen *et al.*, 1994), sulfate esters at the 3 position of terminal galactose residues increased binding efficiency (see compounds **3** and **5** in Table I). However, compound **50**, which contains two sulfate esters linked via the 6-position, demonstrates poor inhibitory properties, indicating that the positions of the sulfate groups are important for selectin recognition and that sulfate multivalency alone does not necessarily improve selectin binding. As also shown in Table I, some 3′sulfo-$Le^{x/a}$–based compounds are able to inhibit the binding of all three selectins to SLe^x.

Similar inhibitory results obtained with immobilized SLe^x or 3′sulfo-Le^x as targets

The binding of the recombinant E-, P-, and L-selectin to immobilized SLe^x and P- or L-selectin to 3′sulfo-Le^x requires calcium, indicating that in each case, the calcium-dependent C-type lectin domain is involved. Prior reports have raised the possibility that the selectins may have partially or completely

Table I. Relative inhibitory concentrations of oligosaccharides against recombinant selectin binding to immobilized SLE^x, or 3′-sulfo-Le^x

No.	Structure	IC_{50} values (μM) against SLe^x			3′-sulfo-Le^x		Notes
		L	P	E	L	P	
43	Fucα1$_4$ Galβ1-3GlcNAcβ1-OBn	>1000	>1000	>1000	>1000	>1000	Le^a-OBn
4	Fucα1$_4$ NeuAcα2-3Galβ1-3GlcNAcβ1-OH	280	240	160	400	300	Sialyl Le^a
2	Fucα1$_3$ NeuAcα2-3Galβ1-4GlcNAcβ1-OMe	600	520	540	670	630	Sialyl Le^x-OMe
44	SE-6Galβ1-4Glc-OH	>1000	>1000	>1000	>1000	>1000	6′-Sulfo-lactose
45	Fucα1$_4$ Galβ1-3(SE-6)GlcNAcβ1-OMe	750	400	510	520	350	6-Sulfo-Le^a
46	Fucα1$_3$ SE-6Galβ1-4GlcNAcβ1-OBn	>1000	>1000	>1000	>1000	>1000	6′-Sulfo-Le^x
5	Fucα1$_4$ SE-3Galβ1-3GlcNAc-OH	400	370	260	450	350	3′-Sulfo-Le^a
3	Fucα1$_3$ SE-3Galβ1-4GlcNAc-OMe	330	320	>1000	370	280	3′-Sulfo-Le^x-OMe
47	Fucα1$_4$ SE-3Galβ1-3GlcNAc-O-pNP	>1000	>1000	380	>1000	>1000	3′-Sulfo-Le^a-O-pNP
48	Fucα1$_4$ NeuAcα2-3(SE-6)Galβ1-3GlcNAcβ1-OMe	440	>1000	>1000	420	>1000	6′-Sulfo-sialyl Le^a
49	Fucα1$_3$ NeuAcα2-3(SE-6)Galβ1-4GlcNAcβ1-OMe	250	220	>1000	230	270	6′-Sulfo-sialyl Le^x; GlyCAM-1 major capping group
40	Fucα1$_3$ Galβ1-4GlcNAcβ1$_6$ SE-6Galβ1-3GalNAcα1-OMe	300	250	>1000	290	200	Branched Core 2 structure with 6′-sulfo group
42	Fucα1$_3$ Galβ1-4GlcNAcβ1$_6$ SE-3Galβ1-3GalNAcα1-OMe	320	270	>1000	240	160	Branched Core 2 structure with 3′-sulfo group
35	Fucα1$_3$ GlcNAcβ1$_6$ SE-6Galβ1-3GalNAcα1-OMe	470	400	>1000	670	220	Branched Core 2 structure with 6′-sulfo group. 1 Gal less than **40**
37	Fucα1$_3$ GlcNAcβ1$_6$ SE-3Galβ1-3GalNAcα1-OBn	>1000	>1000	800	670	620	Branched Core 2 structure with 3′-sulfo group. 1 Gal less than **42**
50	SE$_6$ Galβ1-4GlcNAcβ1$_6$ SE-6Galβ1-3GalNAcα1-OMe	>1000	>1000	>1000	>1000	>1000	Branched Core 2 structure with two 6′-sulfo groups

The compounds shown were tested in an ELISA competition assay against the binding of recombinant selectins as described under *Materials and methods*. IC_{50} values (μM) against immobilized SLe^x and 3′-sulfo-Le^x are the mean values of two to five determinations, and were calculated as described under *Materials and methods*. SE, Sulfate ester.

independent binding sites for sialic acids and sulfate esters (Hollenbaugh *et al.*, 1993; Chou, 1995). However, as shown in Table I, the relative inhibitory properties of the various oligosaccharides for each selectin did not seem to be markedly different when comparing results between the two different immobilized target structures. One interpretation of these data is that the binding sites for the sialylated and the sulfated oligosaccharides are very close, or identical. This does not preclude possible additional sulfate dependent interactions that are calcium-independent.

Potential importance of oligosaccharide branching in enhancing recognition by P- or L-selectin

At first glance, it appears that no other structural pattern predicts the best inhibitory properties of the compounds studied. However, as shown in Figure 6, two branched, fucosylated structures **40** and **42** with sulfated galactose residues had some of the best inhibitory effects against P- and L-selectin, despite not carrying sialic acid residues (see Table I for IC_{50} values of each). These two molecules are in fact similar to some

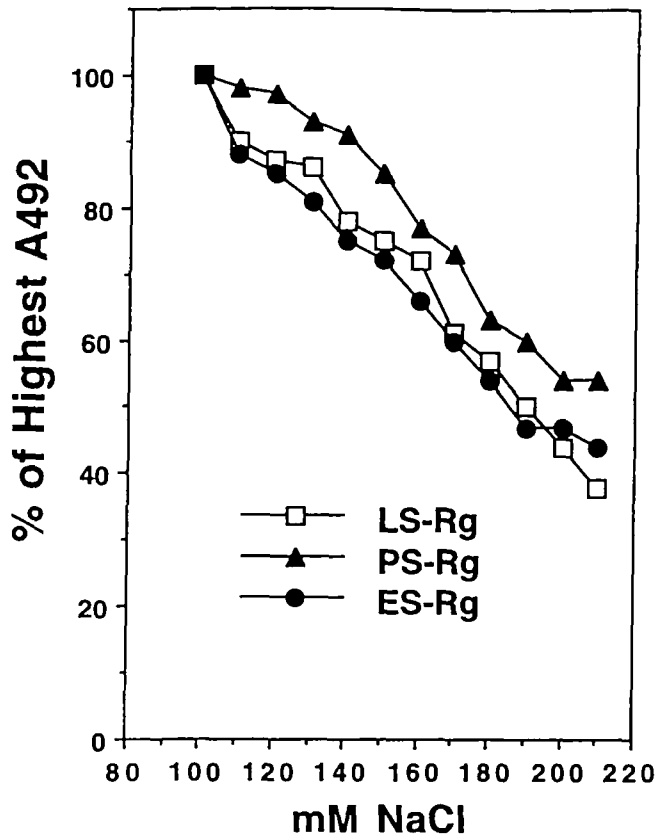


Fig. 4. Effects of ionic strength on the binding of recombinant soluble selectins to immobilized SLe^x. An ELISA assay was used to study the effects of ionic strength on the binding of recombinant soluble selectins to immobilized SLex. NaCl was added in 10 mM increments to a standard ELISA buffer and the binding of the three selectins to immobilized SLe^x was studied as described under *Materials and methods*. Data was applied to the formula: ([(average of duplicates) – (average of negative controls)]/[(average of highest Abs492 duplicates) – (average of negative controls)]) × 100, where the highest Abs492 values contained 100 mM NaCl, and negative controls were assayed in the presence of 5 mM EDTA. The graph is one example of three separate experiments. Similar results were also obtained for P- and L-selectin binding to 3'sulfo-Le^x (data not shown).

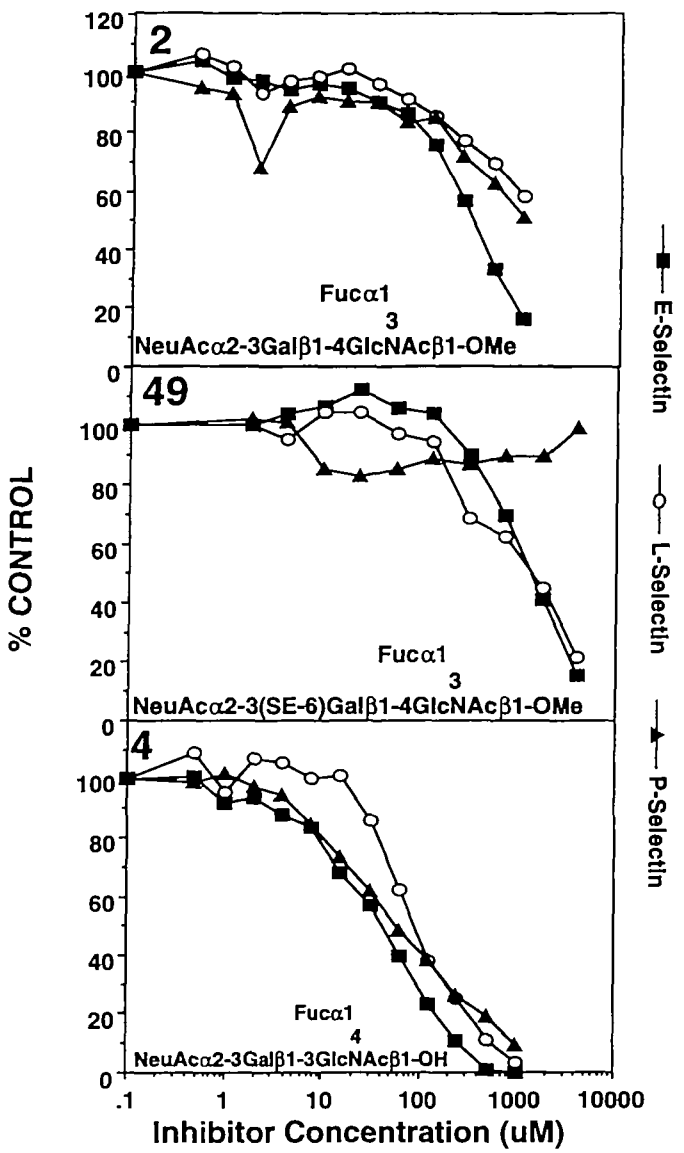


Fig. 5. Comparison of the inhibitory properties of the major capping group of GlyCAM-1 with that of sialyl Le^x and sialyl Le^a. The oligosaccharides were tested for their inhibitory properties in ELISA assays of all three selectin chimeras binding to immobilized SLe^x, as described under *Materials and methods*. Data was applied to the formula: ([(average of duplicates) – (average of negative controls)]/[(average of positive controls) – (average of negative controls)]) × 100, where the positive controls were without inhibitors, and negative controls contained 5 mM EDTA. The graphs represent one example of an inhibition ELISA experiment; points on the graphs are the means of duplicates. Each inhibition experiment was performed 2–5 times, and the averaged IC_{50} values for each oligosaccharide are presented in Table I.

branched O-GalNAc-linked oligosaccharides that can be found on sulfated mucins, including GlyCAM-1 (Hemmerich *et al.*, 1995). Indeed, a comparison of the results obtained with compounds **40** and **42**, versus **35** and **37** shows the importance of the Galβ1–4 residue on the GlcNAcβ1–6 branched arm in enhancing the inhibitory effects towards P- and L-selectin (see Table I). In this regard, it is interesting that recent studies from others have suggested that branched O-linked chains containing the same Galβ1–3(GlcNAcβ1–6)GalNAc-O-Ser/Thr Core 2 sequence may have a role in enhancing binding to L- and P-selectin (Maaheimo *et al.*, 1995; Li *et al.*, 1996).

The major capping group of GlyCAM-1 is neither a superior nor a selective ligand for L-selectin

While natural ligands demonstrate binding affinities for the selectins in the nanomolar range, many common sialylated fucosylated oligosaccharides show IC_{50} values in the micromolar to millimolar range (Varki, 1994). This general observation is confirmed and extended in this study for a wide range of compounds. One possible explanation for this is that the natural ligands bear unique oligosaccharide structures, for example, the natural L-selectin ligand GlyCAM-1 has the unusual oligosaccharide 6'-sulfo-SLe^x, in which a O-sulfate ester is attached to the 6-position of the galactose residue in SLe^x (Hemmerich and Rosen, 1994; Hemmerich *et al.*, 1994, 1995). These studies concluded by predicting that this "major capping group" might be a much better ligand for L-selectin, thus explaining the high affinity binding of the parent glycoprotein. Although the enzymatic pathway for the synthesis of this structure has not yet been clearly elucidated, one of us has succeeded in its complete chemical synthesis (Jain *et al.*, 1994). We therefore studied the inhibitory properties of this compound

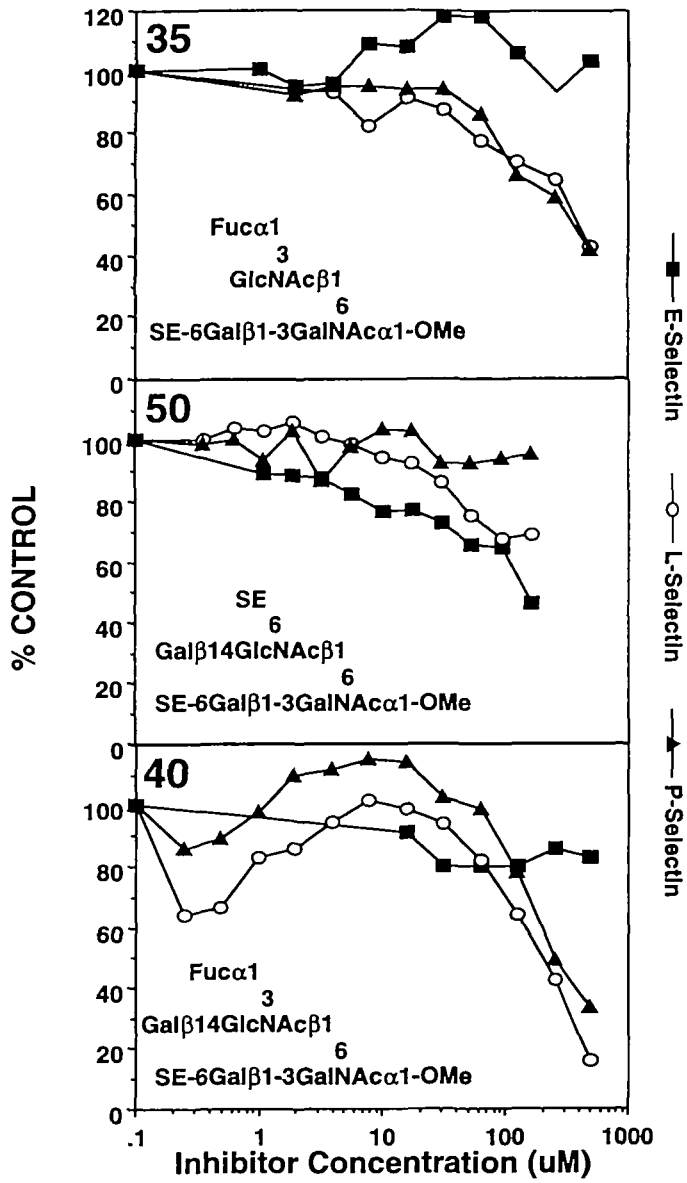


Fig. 6. Comparison of the inhibitory properties of some branched sulfated, fucosylated oligosaccharides. The oligosaccharides were tested for their inhibitory properties in ELISA assays of all three selectin chimeras binding to immobilized Sle^x, and the data analyzed and presented as described in the Figure 5 caption. The averaged IC_{50} values for each oligosaccharide are presented in Table I.

(49) towards the binding of recombinant E-, P-, and L-selectin to immobilized SLe^x and P- or L-selectin to 3′sulfo-Le^x. As shown in Figure 5, this compound gave an IC_{50} value for L-selectin of ~250 μM, and also inhibits P-selectin. However, unlike nonsulfated SLe^x, it does not inhibit E-selectin, indicating that the sulfate group can impart some "negative" specificity among the selectins. In this regard, the sialyl-Le^a-based isomer of this compound (48) was not as effective an inhibitor.

The major capping group of GlyCAM-1 not a superior inhibitor of L-selectin binding to GlyCAM-1 itself

The lack of superiority of 6′-sulfo-SLe^x as an L-selectin inhibitor could also be explained by the fact that the target structures in the ELISA assay were polyacrylamide-based SLe^x, and 3′-sulfo-SLe^x, rather than the native ligands. We therefore studied the inhibitory properties of this compound against the direct binding of L-selectin to the natural glycoconjugate ligand GlyCAM-1 (see *Materials and methods* for details of this assay). The IC_{50} for this interaction was 560 μM (mean of two determinations, see example in Figure 7), somewhat worse than that with the artificial ligands used for the earlier ELISA assays. Furthermore, the inhibitory property of SLe^a against this interaction was similar to that of the major capping group (IC_{50} = 590 μM, mean of four determinations, see example in Figure 7), again somewhat worse than against the artificial ligands (compare Figures 5 and 7). Interestingly, the rest of the

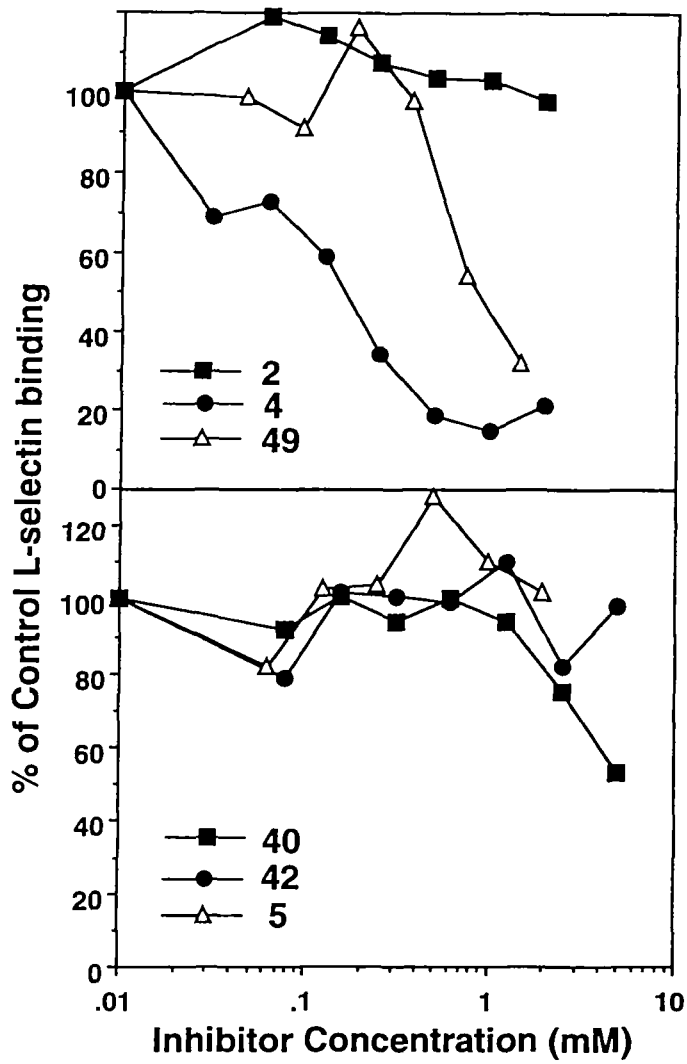


Fig. 7. Inhibitory properties of the major capping group of GlyCAM-1 and other oligosaccharides against native GlyCAM-1 binding to L-selectin. GlyCAM-1 was partially purified from mouse serum by L-selectin affinity chromatography and captured by polyclonal anti-GlyCAM-1 antibodies coated on the wells of microtiter plates as described under *Materials and methods*. The oligosaccharides in serial dilution were mixed with the LS-Rg -secondary antibody complex prior to addition to the wells. Nonspecific binding was determined by adding 5 mM EDTA during the initial binding. The absorbance at 492 nm was read, and the IC_{50} values were generated by a non-linear least squares curve fit equation. Each point shown is: [(average of duplicates) – (negative control)]/[(positive control) – (negative control)] expressed as percentage. All oligosaccharides listed in Table I were tested, and the examples shown are: 2, SLe^x; 4, Sle^a; 49, GlyCAM-1 major capping group; 40 and 42, branched oligosaccharides; 5, 3′HSO_3Le^x (see Table I for structural details).

oligosaccharides (including sialyl-Le^x) that had showed moderate inhibitory properties towards L-selectin interactions with immobilized sialyl Le^x/3′-sulfo-Le^x (see Table I) failed to show such properties against the binding with native GlyCAM-1 (all IC_{50} values greater than 2 mM, data not shown). This assay should be more physiologically relevant in identifying inhibitors of natural L-selectin interactions. Thus, the discrepancies with the results of conventional ELISA assays utilizing artificial ligands raise caution about the use of the latter in screening therapeutic compounds.

Regardless, it appears that the major capping by itself is insufficient to explain the high affinity interaction of L-selectin with GlyCAM-1. Eventually, it will be important to study the inhibitory properties of a completely sialylated and sulfated branched compound such as the one previously reported by others (Lo-Guidice *et al.*, 1994) and recently described by Hemmerich and Rosen on GlyCAM-1 (Crommie and Rosen, 1995):

$$\begin{array}{l} \qquad\qquad \text{Fuc}\alpha 1_3 \\ \text{Sia}\alpha 2\text{-}3\text{Gal}\beta 1\text{-}4(\text{SE-}6)\text{GlcNAc}\beta 1_6 \\ \qquad\qquad\quad \text{Sia}\alpha 2\text{-}3\text{Gal}\beta 1\text{-}3\text{GalNAc}\alpha 1\text{-OMe} \end{array}$$

or a similar compound incorporating a SE-3Galβ1–3GalNAcα1-OMe sequence. The complete large scale synthesis of such compounds is a challenging task that we are currently pursuing.

Superior properties of sialyl-Le^a in inhibiting all three selectins

A surprising finding arising from all of the above data is that none of the compounds tested (including sialyl-Le^x) were superior to the unsulfated molecule Sialyl-Le^a, which inhibited all three selectins with IC_{50} values in the range of 160–400 μM (see Figures 5 and 7 and Table I). In general, sialyl-Le^x with the type 2 backbone Galβ1–4GlcNAc has been the motif most associated with natural selectin ligands (Tamatani *et al.*, 1995), while the type 1 backbone of sialyl-Le^a (Galβ1–3GlcNAc) is mainly associated with epithelial surfaces. For this reason, and because it is easier to synthesize, sialyl-Le^x has received more attention than sialyl-Le^a. However, sialyl-Le^a was noted early on to be recognized by the selectins (Berg *et al.*, 1991), and in at least one instance (cardiac allograft rejection in the rat), there is evidence that it is synthesized in a relevant cell type (endothelium) (Turunen *et al.*, 1995). It should also be noted that sialyl-Le^a is found in the mucins of malignant epithelia such as colon carcinomas (Mannori *et al.*, 1995) that would become exposed to selectins during tumor invasion.

Implications of these studies for the nature of high affinity recognition of natural ligands by the selectins

There is little doubt that highly multivalent displays of structures such as sialyl-Le^x on either protein (Welply *et al.*, 1994) or oligosaccharide (Turunen *et al.*, 1995) backbones can generate avid binding to multivalently presented selectins. However, this does not seem to explain the high-affinity interactions of monovalent selectins with their natural ligands. Scudder and colleagues (Scudder *et al.*, 1994) recently synthesized a structural isomer of the major capping group of GlyCAM- 1 (6-sulfo-SLe^x, in which a O-sulfate ester is attached to the 6-position of the GlcNAc residue in SLe^x), and showed an IC_{50} of only 0.8 mM for the interaction of L-selectin with lymph node derived peripheral addressins (representing a fourfold improvement over SLe^x in inhibiting the same interaction). However, they raised the possibility that the high affinity interactions might require the sulfate to be at the 6-position of the galactose residue as in the native ligand GlyCAM-1. The present results appear to negate that possibility. Likewise, synthetic variations of some of the branched O-linked structures expected to be found on GlyCAM-1 did not reveal an oligosaccharide with clearly superior binding. In keeping with this, we have recently found that while subsets of sialylated sulfated mucins of diverse origins can bind to L-selectin, O-linked chains released from these mucins do not contain a high-affinity subset of chains (Crottet *et al.*, 1996). These studies, together the data reported here, suggest that rare structural variants of the common sialylated fucosylated oligosaccharides may not explain the high-affinity recognition of natural selectin ligands. Of course, we were not able to synthesize every possible sialylated, sulfated fucosylated structure that might be found among the O-linked chains of selectin ligands. Thus, as discussed earlier, it is possible that some complex form of branched Core 2 containing oligosaccharide might impart improved binding. Furthermore, some other unknown mechanism might operate *in vivo* to improve the binding affinity of any of these types of oligosaccharides.

Another possibility is the combination of a basic oligosaccharide sequence and an adjacent peptide, acting either by presenting a composite recognition site, or by forcing a monovalent oligosaccharide into an unusual conformation favored for recognition. Although the persistence of high affinity recognition following extensive denaturation or proteolytic digestion (Imai *et al.*, 1992; Lasky *et al.*, 1992; Moore *et al.*, 1992; Norgard *et al.*, 1993b; Norgard-Sumnicht *et al.*, 1993) seems to be against this, recent studies suggest that tyrosine sulfate residues adjacent to sialylated oligosaccharides may create such a composite binding site for P-selectin on the natural ligand PSGL-1 (Pouyani and Seed, 1995; Sako *et al.*, 1995; Wilkins *et al.*, 1995). A more complex variation involves recognition of a "clustered saccharide patch" (Norgard *et al.*, 1993b) created by a polypeptide carrying multiple oligosaccharides. In this scenario, oligosaccharides packed closely together, such as in mucins, and having limited mobility could present a discontiguous saccharide epitope generated by multiple oligosaccharides. It is important to note that in all these instances, high affinity recognition would be lost if the arrangement is disrupted, and the individual sugar chains may not have detectable affinity for the selectin. This might explain why most reported high affinity selectin ligands are heavily glycosylated macromolecules, and the finding that free oligosaccharides released from such ligands do not bind with recognizable affinity to the same selectin (Imai *et al.*, 1992; Norgard-Sumnicht *et al.*, 1993; Crottet *et al.*, 1996). Such recognition is distinct from the improved binding of certain other lectins interacting with multiantennary N-linked oligosaccharides (Rice *et al.*, 1990, 1991), or with glycopeptides carrying widely spaced O-linked oligosaccharides (Lee, 1992). This type of enhancement results from correct spacing of terminal sugar residues, each of which are still recognized as distinct components of individual chains by separate lectin domains of multidomain and/or clustered lectins.

It should also be pointed out that E-selectin may be a partial exception to these considerations. Recent studies have shown that a tetrantennary N-linked oligosaccharide with a sialylated polyfucosylated polylactosamine borne on the α1–3Man

linked arm of the sugar chain can bind with high affinity to immobilized E-selectin (Patel *et al.*, 1994). Interestingly, related structures can also be found on "myeloglycan," a family of sialylated polyfucosylated polylactosamine glycolipids from neutrophils (Stroud *et al.*, 1995, 1996). Another complexity for consideration is the finding that P- and L-selectin can bind selectively and strongly to some heparin/heparan sulfate chains, despite being a very different class of glycoconjugates that do not carry sialic acids and fucose (Skinner *et al.*, 1989; Nelson *et al.*, 1993a; Norgard-Sumnicht *et al.*, 1993). Indeed, small heparin-derived oligosaccharides are capable of competing with L- and P-selectin binding to sLe^x (Nelson *et al.*, 1993a), suggesting that the two classes of molecules (sialylated, fucosylated oligosaccharides and heparin oligosaccharides) might compete for the same binding site. One possible explanation is that these sulfated, uronic acid–rich polysaccharides are capable of mimicking the anionic surface of the recognition site for selectins that is created on the surface of certain mucin-type molecules. This is supported by our recent findings that only subsets of heparins and mucins are selectively recognized by L-selectin, and that oligonucleotides of defined structure can mimic these high-affinity ligands for L-selectin (O'Connell *et al.*, 1996). Further work is obviously needed to elucidate the nature of the natural high-affinity ligands for the selectins. Meanwhile, the systematic comparisons of the three selectins presented here indicate that the explanation for high affinity may not lie solely in the superior binding properties of unusual sulfated oligosaccharide structures. Rather, the branching, arrangement, and/or clustering of relatively common acidic oligosaccharides many produce unique binding motifs, with or without a direct contribution from the polypeptide carrier. This fits with recent studies showing that a sulfated tyrosine containing peptide can also be selectively recognized by P-selectin in the context of a sialylated, fucosylated oligosaccharide (Pouyani and Seed, 1995; Sako *et al.*, 1995; Wilkins *et al.*, 1995; Li *et al.*, 1996).

Implications of these studies for the therapeutic blockade of the selectins

While sialyl-Le^x shows differential reactivity among the selectins (being a much poorer inhibitor of L- and P-selectin than of E-selectin), the present study shows that sialyl-Le^a uniformly inhibits all three selectins with IC_{50} values in the low micromolar range. Sialyl-Le^x in large quantities appears to be effective in various animal models of selectin-mediated pathologies (Albelda *et al.*, 1994; Bevilacqua *et al.*, 1994; Lefer *et al.*, 1994), and is currently in clinical trials for certain reperfusion states in humans. Based on the present work, it appears that strong consideration should be given to using sialyl-Le^a in a similar fashion. On the other hand, despite the large amount of effort reported here in synthesizing many related sialylated, fucosylated and/or sulfated oligosaccharides, none so far have emerged as clearly superior inhibitors. Further efforts to synthesize more complete branched sialylated sulfated O-linked chains are worthwhile, since they may prove to be more potent. However, parallel efforts should be directed towards generating artificial inhibitors of the selectins, such as those based on peptides (Martens *et al.*, 1995), oligonucleotides (O'Connell *et al.*, 1996), and unrelated organic compounds from pharmacore searching (Narasinga Rao *et al.*, 1994). Of course, if the structural basis for high-affinity recognition of the natural selectin ligands could be elucidated with precision, this could lead to a more rational drug design of synthetic inhibitors.

Materials and methods

General methods for oligosaccharide synthesis

Optical rotations were measured at ~25°C with a Perkin-Elmer 241 Polarimeter. TLC was conducted on glass plates precoated with a 0.25 mm layer of silica gel 60F-254 (Analtech GHLF uniplates). The compounds were located by exposure to UV light and (or) by spraying with 5% H_2SO_4 in EtOH and charring on a hot plate. The silica gel used for column chromatography was Baker Analyzed (60–200 mesh). NMR spectra were recorded at ~25°C; ^{1}H-spectra with a Varian EM-390 at 90 MHz and with a Bruker AM-400 at 400 MHz and ^{13}C-spectra with a Bruker AM-400 at 100.6 MHz. All chemical shifts were referenced to tetramethylsilane. Solutions in organic solvents were generally dried with anhydrous sodium sulfate. Dichloromethane, N,N-dimethylformamide, and 1,2-dichloroethane were kept dried over 4 Å molecular sieves. Elemental analyses were performed by Robertson Laboratory, Madison, NJ. All new compounds gave satisfactory elemental analysis.

Preparation and characterization of synthetic oligosaccharides

The compounds **48** and **49** (see Table I) were synthesized as described elsewhere (Jain *et al.*, 1994; Vig *et al.*, 1995). The following describes the intermediates and methods of syntheses of most of the remaining compounds used in this study. Some details regarding compounds **43–47** and **50** will be published elsewhere.

Synthesis of 2,3,4-tri-O-acetyl-6-O-pivaloyl-α/β-D-galactopyranosyl bromide (**8**) and fluoride (**9**)

Treatment of 1,2,3,4-di-O-isopropylidene-α-D-galactopyranose (10 g; 38.5 mmol) with pivaloyl chloride (3.45 ml; 46.1 mmol) in pyridine (100 ml) at room temperature (RT) followed by the hydrolysis with 80% aqueous acetic acid provided the intermediate **6**. Acetylation of this compound with pyridine-acetic anhydride, furnished compound **7** (10.45 g) 1,2,3,4-tetra-O-acetyl-6-O-pivaloyl-D-galactopyranose in 83% yield; $[\alpha]_D$ +97° (c 1.5, $CHCl_3$); ^{1}H-NMR (CD_2Cl_2): δ 6.37 (d, J = 2.8 Hz, H-1), 5.48 (d, J = 3.0 Hz, 1 H, H-4), 5.34 (dd, 1 H, H-2), 4.35 (dd, 1 H, H-3), 4.09 (d, 2 H, H-6), 2.15 (s, 6 H, 2×OAc), 2.02 (s, 3 H, OAc), 1.99 (s, 3 H, OAc), 1.71 (s, 9 H, CMe_3); ^{13}C-NMR: δ 89.55 (C-1), 68.53 (C-5), 67.29 (C-3), 67.17 (C-2), 66.36 (C-4), 60.70 (C-6), 28.86 (C-Me).

Compound **7** (10.45 g; 24.2 mmol) was readily converted, in high yield, the bromide **8** by treatment with 31% hydrogen bromide in glacial acetic acid (50 ml) containing a little acetic anhydride (2.5 ml) in dichloromethane (100 ml); $[\alpha]_D$ +162° (c 1.1, $CHCl_3$); ^{1}H-NMR (CD_2Cl_2): δ 6.73 (d, J = 3.9 Hz, 1 H, H-1), 5.48 (d, J = 3.0 Hz, 1 H, H-4), 5.37 (dd, 1 H, H-2), 5.05 (dd, 1 H, H-3), 2.13, 2.08 and 1.98 (each s, 9 H, 3×OAc), 1.17 (s, 9 H, CMe_3).

The bromide **8** (9.8 g) was treated with silver fluoride (Hall *et al.*, 1969) (4.5 g) in acetonitrile (50 ml) with the protection of light and moisture for 16 h. The resulting solution was filtered, dissolved in chloroform and washed with saturated sodium chloride solution, dried over Na_2SO_4. Concentration and purification over silica gel using hexane-EtoAc 4:1 (v/v) as eluent provided fluoride **9** (6.6 g) in 77% yield; $[\alpha]_D$ +8° (c 1.1, $CHCl_3$); ^{1}H-NMR (CD_2Cl_2): δ 5.40 (d, J = 3.4 Hz, H-1), 5.34 (d, J = 7.1 Hz, H-1β), 5.31 (d, H-1, H-4), 5.04 (dd, 1 H, H-3), 2.15, 2.07, and 1.97 (each s, 9 H, 3×OAc), 1.17 (s, 9 H, CMe_3).

Synthesis of benzyl O-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→3)-O-(β-D-galactopyranosyl)-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (**1**)

Glycosylation of benzyl 2-deoxy-2-phthalimido-6-O-pivaloyl-β-D-glucopyranoside (4.0 g; 8.3 mmol) with fluoride **9** (4.6 g; 11.6 mmol) under Mukaiyama's conditions [$SnCl_2$ (1.8 g; 9.94 mmol)-AgOTf (2.54 g; 9.94 mmol)] (Mukaiyama *et al.*, 1981) in 5:1 (v/v) dichloromethane-toluene (120 ml) in the presence of 4 Å molecular sieves (7 g) and purification over silica gel using hexane-EtoAc (3:2 → 1:1 v/v) as eluent afforded major β1→4 linked disaccharide **16** in 40% yield; $[\alpha]_D$ +0.5° (c 1.7, $CHCl_3$); ^{1}H-NMR (CD_2Cl_2): δ 7.74–7.72 (m, 4 H, arom.), 7.14–7.08 (m, 5 H, arom.), 5.31 (d, J = 2.5 Hz, 1 H, H-4'), 5.23 (d, J = 8.6 Hz, 1 H, H-1), 4.63 (d, J = 8.0 Hz, 1 H, H-1'), 2.11, 2.07 and 1.95 (each s, 9 H, 3×OAc), 1.26, and 0.98 (each s, 18 H, 2×CMe_3).

Selective de-O-acetylation of **16** (3 g) in 1:1 (v/v) MeOH-CH_2Cl_2 (60 ml) with MeOH-MeONa (pH 10) at 0°C for 2 h provided **16A** in 78% yield; $[\alpha]_D$ −17° (c 1.7, $CHCl_3$); ^{1}H-NMR (CD_2Cl_2): δ 7.77–7.69 (m, 4 H, arom.), 7.15–7.09 (m, 5 H, arom.), 5.23 (d, J = 8.6 Hz, 1 H, H-1), 4.31 (d, J = 7.5 Hz, 1 H, H-1'), 1.24, and 0.99 (each s, 18 H, 2×CMe_3).

Glycosylation of **16A** (0.22 g; 0.3 mmol) with the sialic acid donor **13**(Marra and Sinaÿ, 1989) (0.35 g; 0.6 mmol) under N-iodosuccinimide (0.26 g; 0.9 mmol) triflic acid in propionitrile (Veeneman *et al.*, 1990) (10 ml) at −45°C for

2 h and purification over silica gel utilized 25% acetone in chloroform as eluent gave **19** in 61% yield; $[\alpha]_D$ −8°C (c 0.4, $CHCl_3$); ^{1}H-NMR (CD_2Cl_2): δ 7.74–7.73 (m, 4 H, arom.), 7.16–7.11 (m, 5 H, arom.), 5.25 (d, J = 8.7 Hz, 1 H, H-1), 4.47 (d, J = 7.8 Hz, 1 H, H-1'), 3.81 (s, 3 H, OMe), 2.68 (dd, J = 4.6 Hz, H-3‴e), 2.11, 2.10, 2.02, 2.01, and 1.86 (each s, 15 H, 4×OAc and NAc), 1.24, and 1.01 (each s, 18 H, 2×CMe_3).

A solution of **19** (0.18 g; 0.15 mmol) in pyridine (10 ml) containing LiI (Nicolaou *et al.*, 1992) (0.15 g; 1.1 mmol) was stirred at 120°C for 3 h. The solvent was removed under reduced pressure and passed through small silica gel column using 4:1 (v/v) chloroform–methanol as an eluent to provide the acid derivative which on treatment with 5:1 (v/v) methanol–hydrazine hydrate (60 ml) at 80°C for 7 h and followed by N-acetylation in 1:1 (v/v) MeOH-CH_2Cl_2 (30 ml) with excess acetic anhydride at 0°C for 1 h and de-O-acetylation with methanol–MeONa for 48 h provided **1** in 53% yield after purification over silica gel using $CHCl_3$-MeOH-H_2O (4:5:1; v/v) as an eluent; $[\alpha]_D$ −21° (c 0.5; H_2O); ^{1}H-NMR (D_2O): δ 7.52–7.42 (m, 5 H, arom.), 4.94 (d, J = 12.2 Hz, H-1), 4.59 (d, J = 8.1 Hz, H-1'), 2.81 (dd, J = 4.6 Hz, H-3‴e), 2.08 and 1.97 (each s, 2×NAc), 1.84 (t, J = 12.1 Hz, H-3‴a); ^{13}C-NMR: δ 101.56 (C-1'), 98.85 (C-1), 98.81 (C-2‴), 77.43 (C-3'), 77.29 (C-4), 61.59 (C-9‴), 59.98 (C-6'), 59.01 (C-6), 54.02 (C-2), 50.68 (C-5‴), 38.63 (C-3‴); MS *m/z*: 765.3 $[M+H]^+$, 786.8 $[M+Na]^+$.

Synthesis of methyl O-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→3)-O-(β-D-galactopyranosyl)-(1→4)-O-[α-L-fucopyranosyl-(1→3)-O]-2-acetamido-2-deoxy-β-D-glucopyranoside (2)

Glycosylation of **11** (4.9 g; 12.02 mmol) with **9** (6.5 g; 16.8 mmol) under condition similar to those described for the preparation of **16** gave β1→4 linked disaccharide **18** after silica gel column chromatography (hexane:ethyl acetate; 1:1) in 48% yield; $[\alpha]_D$ +16 (c 1.1, $CHCl_3$); ^{1}H-NMR (CD_2Cl_2): δ 7.84–7.73 (m, 4 H, arom.), 5.37 (d, J = 3.3 Hz, 1 H, H-4'), 5.20 (d, J = 8.0 Hz, 1 H, H-1), 4.64 (d, J = 8.1 Hz, 1 H, H-1'), 3.38 (s, 3 H, OMe), 2.12, 2.07, and 1.95 (each s, 9 H, 3×OAc), 1.24 and 0.98 (each s, 18 H, 2×CMe_3).

Reaction of **18** (3.0 g; 3.85 mmol) with 2,3,4-tri-O-benzyl-α-L-fucopyranosyl bromide (Dejter-Juszynski and Flowers, 1971; Lemieux *et al.*, 1975) (3.8 g; 7.7 mmol) in the presence of silver triflate (1.98 g; 7.7 mmol) and 2,6-di-O-(tert-butyl)-4-methyl pyridine (Nilsson and Norberg, 1988) (1.58 g; 7.7 mmol) in 2:3 (v/v) CH_2Cl_2-toluene (60 ml) at −35°C for 3 h gave fully protected trisaccharide **20** in 79.1% yield, which after removal of the phthalimido and O-acetyl group with 9:1 (v/v) EtOH-hydrazine hydrate (100 ml) at 100°C for 6 h and N-acetylation in MeOH-Et_3N-Ac_2O (4:2:1; v/v) provided **21** in 62% yield; $[\alpha]_D$ −34° (c 1.1, $CHCl_3$); ^{1}H-NMR (CD_2Cl_2): δ 7.39–7.24 (m, 15 H, arom.), 5.96 (d, J = 7.2 Hz, 1 H, NH), 5.09 (d, J = 3.2 Hz, 1 H, H-1‴), 4.92 (d, J = 10.9 Hz, 1 H, H-1), 3.36 (s, 3 H, OMe), 1.69 (s, 3 H, NAc), 1.21 and 1.20 (each s, 18 H, 2×CMe_3), 1.19 (d, J = 6.6 Hz, 3 H, H-6‴).

Glycosylation of **21** (0.23 g; 0.23 mmol) with same sialic acid donor **13** (0.35 g; 0.64 mmol) under N-iodosuccinimide (0.29 g; 1.3 mmol) triflic acid in propionitrile (15 ml) for 2 h at −75° afforded compound **22** in 66% yield; $[\alpha]_D$ −25° (c 0.7, $CHCl_3$); ^{1}H-NMR (CD_2Cl_2): δ 7.39–7.23 (m, 15 H, arom.), 5.87 (d, J = 7.6 Hz, 1 H, NH), 5.26 (d, J = 9.7 Hz, 1 H, NH), 5.13 (d, J = 2.6 Hz, 1 H, H-1‴), 4.92 (d, J = 11.0 Hz, 1 H, H-1), 3.80 (s, 3 H, OMe), 3.35 (s, 3 H, OMe), 2.69 (dd, J = 4.6 Hz, H-3⁗e), 2.11, 2.09, 2.02, 2.00, 1.86, and 1.61 (each s, 18 H, 4×OAc and 2×NAc), 1.21 and 1.20 (each s, 18 H, 2×CMe_3), 1.16 (d, J = 6.4 Hz, 3 H, H-6⁗). Hydrogenolytic cleavage of the benzyl groups of **22** in the presence of 10% Pd-C/methanol, followed by de-O-acetylation with methanolic sodium methoxide (0.02 M) for 72 h and addition of water to the reaction mixture to hydrolyze methyl ester to acid provided title compound **2** in 96% yield after silica gel chromatography using $CHCl_3$-MeOH-H_2O (4:5:1, v/v) as eluent; $[\alpha]_D$ −38° (c 0.4, H_2O); ^{1}H-NMR (D_2O): δ 5.09 (d, J = 3.9 Hz, H-1‴), 4.81 (d, J = 7 Hz, H-1), 4.76 (d, J = 7 Hz, H-1'), 3.50 (s, OMe), 2.76 (dd, J = 4.6 Hz, H-3⁗e), 2.03 and 2.02 (each s, 2×NAc), 1.79 (t, J = 12.1 Hz, H-3⁗a), 1.16 (d, J = 6.6 Hz, H-6⁗); ^{13}C-NMR: δ 100.74 (C-1'), 100.65 (C-1), 98.67 (C-2⁗), 97.56 (C-1‴), 74.67 (C-3'), 74.30 (C-3), 73.88 (C-4), 61.61 (C-9⁗), 60.44 (C-6'), 58.67 (C-6), 56.10 (OMe), 54.59 (C-2), 50.70 (C-5⁗), 14.24 (C-6⁗); MS: m/z 833.3 $[M-Na]^-$.

Synthesis of methyl O-(3-O-sulfo-β-D-galactopyranosyl sodium salt)-(1→4)-[α-L-fucopyranosyl-(1→3)-O]-2-acetamido-2-deoxy-β-D-glucopyranoside (3)

Regioselective sulfation of **21** (0.45 g; 0.5 mmol) with SO_3-pyridine complex (0.48 g; 3 mmol) in pyridine (20 ml) at 5°C for 16 h and followed by the removal of protecting groups as described for the preparation of **2** from **22** gave title compound **3** in 37% yield after silica gel column chromatography ($CHCl_3$-MeOH-H_2O; 4:5:1, v/v); $[\alpha]_D$ −45° (c 0.6, H_2O); ^{1}H-NMR (D_2O): δ 5.15 (d, J = 4.4 Hz, H-1‴), 4.62 (d, J = 7.8 Hz, H-1'), 3.54 (s, OMe), 2.07 (s, NAc), 1.21 (d, J = 6.6 Hz, H-6‴); ^{13}C-NMR: δ 100.72 (C-1'), 100.45 (C-1), 97.54 (C-1‴), 79.20 (C-3'), 74.24 (C-3), 73.82 (C-4), 60.31 (C-6'), 58.70 (C-6), 56.11 (OMe), 54.62 (C-2), 14.23 (C-6‴); MS: m/z 622.3 $[M-Na]^-$.

Synthesis of O-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→3)-O-(β-D-galactopyranosyl)-(1→3)-O-[α-L-fucopyranosyl-(1→4)-O]-2-acetamido-2-deoxy-D-glucopyranose (4)

Glycosylation of **12** (3.6 g; 8.8 mmol) with bromide **8** (5.3 g; 11.3 mmol) in the presence of $Hg(CN)_2$ (2.8 g; 11.3 mmol) at 55° afforded compound **23** in 65% yield; $[\alpha]_D$ −15° (c 0.8, $CHCl_3$; ^{1}H-NMR (CD_2Cl_2): δ 7.33–7.27 (m, 5 H, arom.), 5.82 (d, J = 7.7 Hz, 1 H, NH), 5.37 (d, J = 3.1 Hz, 1 H, H-4'), 5.17 (dd, J = 8.0 Hz, 1 H, H-2'), 2.14, 2.05, 1.95, 1.94 (each s, 12 H, 3×OAc and NAc), 1.22, 1.17 (each s, 18 H, 2×CMe_3). Compound **23** (0.8 g; 1 mmol) on reaction with thiomethyl donor **14** (Sato *et al.*, 1987; Jain and Matta, 1990) (1.15 g; 2.5 mmol) in 5:1 (v/v) dichloroethane-DMF (48 ml) in the presence of $CuBr_2$ (0.85 g; 3.5 mmol) and Bu_4NBr (Sato *et al.*, 1986) (1.12 g; 3.5 mmol) and 4 Å molecular sieves (5 g) for 16 h afforded crude compound **24** which after de-O-acetylation with methanolic sodium methoxide gave compound **25** in 56% yield; $[\alpha]_D$ −62° (c 0.6, $CHCl_3$, ^{1}H-NMR (CD_2Cl_2): δ 7.40–7.27 (m, 20 H, arom.), 6.60 (d, J = 8.3 Hz, 1 H, NH), 5.06 (d, J = 3.6 Hz, 1 H, H-1‴), 4.84 (d, J = 10.7 Hz, 1 H, H-1), 4.38 (d, J = 7.3 Hz, 1 H, H-1'), 1.67 (s, 3 H, NAc), 1.22 (s, 9 H, CMe_3), 1.17 (d, J = 6.4 Hz, 1 H, H-6‴), 1.12 (s, 9 H, CMe_3).

Glycosylation of **25** (0.53 g; 0.5 mmol) with the sialic acid donor **13** (0.58 g; 1 mmol) under reaction condition described for the preparation of **22** afforded **26** in 54% yield; $[\alpha]_D$ −47° (c 0.5, $CHCl_3$, ^{1}H-NMR (CD_2Cl_2): δ 7.40–7.26 (s, 20 H, arom.), 6.61 (d, J = 8.3 Hz, 1 H, NH), 5.19 (d, J = 9.2 Hz, 1 H, NH), 5.05 (d, J = 3.6 Hz, 1 H, H-1‴), 4.83 (d, J = 11.0 Hz, 1 H, H-1), 4.71 (d, J = 7.5 Hz, 1 H, H-1'), 3.81 (s, 3 H, OMe), 2.62 (dd, J = 4.6 Hz, H-3⁗e), 2.09, 2.02, 2.00, 1.85, 1.71, 1.58 (each s, 18 H, 4×OAc and 2×NAc), 1.22 (s, 9 H, CMe_3), 1.16 (d, J = 6.4 Hz, 3 H, H-6⁗), 1.13 (s, 9 H, CMe_3).

De-O-acetylation of compound **26** with methanolic sodium methoxide in methanol, followed by removal of O-benzyl protecting groups as described for the preparation of compound **2** gave title compound **4** in 66% yield; $[\alpha]_D$ −36° (c 0.8, H_2O); ^{1}H-NMR (D_2O): δ 5.11 (d, J = 3.0 Hz, 1 H, H-1‴), 4.56 (d, J = 7.7 Hz, 1 H, H-1), 4.52 (d, J = 7.7 Hz, H-1'), 2.77 (dd, J = 4.6 Hz, H-3⁗e), 2.04 and 2.03 (each s, 6 H, 2×NAc), 1.76 (t, J = 12.1 Hz, H-3⁗a), 1.17 (d, J = 6.6 Hz, H-6⁗); ^{13}C-NMR: δ 101.77 (C-1'β), 98.39 (C-1‴α), 98.35 (C-2⁗), 96.99 (C-1⁗), 93.73 (C-1β), 89.96 (C-1α), 75.07 (C-3β), 74.64 (C-3'), 74.58 (C-3α), 73.71 (C-4β), 73.58 (C-4α), 61.27 (C-9⁗), 60.61 (C-6'β), 60.58 (C-6'α), 58.76 (C-6β), 58.71 (C-6α), 55.85 (C-2β), 52.95 (C-2α), 50.67 (C-5⁗), 39.02 (C-3⁗), 14.33 (C-6⁗); MS: m/z 819.3 $[M-H]^-$.

O-(3-O-sulfo-β-D-galactopyranosyl sodium salt)-(1→3)-[α-L-fucopyranosyl-(1→4)-O]-2-acetamido-2-deoxy-D-galacto-pyranose (5)

The procedure used for preparing known **5** (Lubineau *et al.*, 1993) (from **25**) were essentially the same as described for the preparation of **3** from **21**; $[\alpha]_D$ −41° (c 0.9, H_2O); reported (Lubineau *et al.*, 1993) −38° (c 0.5, MeOH); ^{1}H-NMR (D_2O): δ 5.06 (d, J = 3 Hz, H-1⁗), 2.11 (s, NAc), 1.22 (d, J = 6.6 Hz, H-6⁗); ^{13}C-NMR: δ 101.59 (C-1'β), 99.44 (C-1'α), 97.01 (C-1⁗), 93.77 (C-1β), 89.94 (C-1α), 79.33 (C-3'), 79.22 (C-3β), 75.21 (C-3α), 74.58 (C-4β), 73.50 (C-4α); MS: m/z 608.3 $[M-Na]^-$.

Synthesis of benzyl O-(2,4,6-tri-O-acetyl-3-O-chloroacetyl-β-D-galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-α-D-galactopyranoside (27)

Glycosylation of benzyl 2-acetamido-2-deoxy-4,6-O-methoxybenzylidene-α-D-galactopyranoside (Piskorz *et al.*, 1984) (3.2 g; 8.0 mmol) with 2,4,6-tri-O-acetyl-3-O-chloroacetyl-α-D-galactopyranosyl bromide (Jain and Matta, 1994) (5.5 g; 11.9 mmol) in the presence of $Hg(CN)_2$ (2.9 g; 11.9 mmol) at 55°C and followed by the removal of acetal ring with 60% aq. acetic acid at 70°C afforded **27** (2.7 g) in 50% yield; $[\alpha]_D$ +88°C (c 1.0, $CHCl_3$); ^{1}H-NMR (CD_2Cl_2): δ 7.38–7.32 (m, 5 H, arom.), 5.55 (d, J = 9.9 Hz, 1 H, NH), 5.37 (d, J = 3.2 Hz, 1 H, H-4'), 5.20 (dd, J = 7.9 Hz, 1 H, H-2'), 5.05 (dd, 1 H, H-2), 4.93 (d, J = 3.7 Hz, 1 H, H-1), 4.62 (d, J = 7.9 Hz, 1 H, H-1'), 3.98 (s, 2 H, $COCH_2Cl$), 2.14, 2.02, 2.01, 1.90 (each s, 12 H, 3×OAc and NAc).

Synthesis of methyl O-(2,4,6-tri-O-acetyl-3-O-chloroacetyl-β-D-galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-α-D-galactopyranoside (28)

A similar glycosylation of methyl 2-acetamido-2-deoxy-4,6-O-methoxybenzylidene-α-D-galactopyranoside (1.4 g; 3.97 mmol) with bromide (2.8 g; 6.0 mmol) as described for the preparation of **27** afforded **28** (1.1 g) in 46% yield: $[\alpha]_D$ +61° (c 1.4, $CHCl_3$): ^{1}H-NMR (CD_2Cl_2): δ 5.66 (d, J = 9.7 Hz, 1

H, NH), 5.39 (d, J = 3.1 Hz, 1 H, H-4'), 5.19 (dd, J = 7.9 Hz, 1 H, H-2'), 5.08 (dd, 1 H, H-2), 4.67 (d, J = 3.7 Hz, 1 H, H-1), 4.65 (d, J = 7.9 Hz, 1 H, H-1'), 3.99 (s, 2 H, $COCH_2Cl$), 3.37 (s, 3 H, OMe), 2.15, 2.05, 2.04, and 1.94 (each s, 12 H, 3×OAc and NAc).

Synthesis of methyl O-(2,3,4-tri-O-benzoyl-6-O-bromoacetyl-β-D-galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-α-D-galactopyranoside (**29**)

Glycosylation of methyl 2-acetamido-2-deoxy-4,6-O-methoxybenzylidene-α-D-galactopyranoside (4.0 g; 11.3 mmol) with 2,3,4-tri-O-benzoyl-6-O-bromoacetyl-α-D-galactopyranosyl bromide (Kovac *et al.*, 1985) (8.5 g; 12.5 mmol) under a similar reaction condition as described for the preparation of **27** gave compound **29** in 72% yield; $[\alpha]_D$ +91°C (c 1.2, $CHCl_3$); ^{1}H-NMR (CD_2Cl_2): δ 8.04–7.21 (m, 15 H, arom.), 5.87 (d, J = 3.3 Hz, 1 H, H-4'), 5.79 (dd, J = 7.9 Hz, 1 H, H-2'), 5.60 (dd, 1 H, H-2), 5.49 (d, J = 9.4 Hz, 1 H, NH), 5.04 (d, J = 8.0 Hz, 1 H, H-1''), 4.67 (d, J = 3.7 Hz, 1 H, H-1), 3.90 (s, 2 H, $COCH_2Br$), 3.33 (s, 3 H, OMe), 1.26 (s, 3 H, NAc).

Synthesis of phenyl O-(2,3,4-tri-O-acetyl-α-L-fucopyranosyl)-(1→3)-4,6-di-O-acetyl-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside (**32**)

Methyl 4,6-O-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranoside (3.0 g; 7.3 mmol) on reaction with methyl 2,3,4-tri-O-benzyl-1-thio-β-L-fucopyranoside (Sato *et al.*, 1987; Jain and Matta, 1990) (4.2 g; 9.05 mmol) in 5:1 (v/v) dichloromethane-DMF in the presence of $CuBr_2$ (3.15 g; 13.5 mmol) and Bu_4NBr (Sato *et al.*, 1986) (4.35 g; 13.5 mmol) and 4 Å molecular sieves (8 g) for 16 h afforded compound **30** (5.9 g) in 97% yield; $[\alpha]_D$ −18°C (c 1.3, $CHCl_3$); ^{1}H-NMR (CD_2Cl_2): δ 7.70–7.14 (m, 24 H, arom.), 5.56 (s, 1 H, PhCH), 5.29 (d, J = 2.0 Hz, 1 H, H-1'), 5.21 (d, J = 8.5 Hz, 1 H, H-1), 4.76 (dd, J = 11.0 and 3.3 Hz, 1 H, H-2'), 4.62 (dd, J = 8.3 Hz, 1 H, H-2), 3.42 (s, 3 H, OMe), 0.83 (d, J = 6.6 Hz, 3 H, CMe).

Hydrogenolytic cleavage of the benzyl groups of **30** in the presence of 10% Pd-C/glacial acetic acid, followed by the acetolysis with H_2SO_4-AcOH-Ac_2O (1:12:11.5; v/v) at 5°C for 16 h provided fully acetylated derivative **31**; $[\alpha]_D$ +2°C (c 1.0, $CHCl_3$); ^{1}H-NMR (CD_2Cl_2): δ 7.87–7.74 (m, 4 H, arom.), 6.37 (d, J = 8.9 Hz, 0.8 H, H-1), 6.15 (d, J = 2.0 Hz, 0.2 H, H-1), 2.14–1.49 (cluster of s, 18 H, 6×OAc), 1.07–1.01 (m, 3 H, CMe).

Treatment of **31** (2.4 g; 3.4 mmol) with BF_3.ethereate (2.15 ml; 15.4 mmol) and thiophenol (Ferrier and Furneaux, 1980) (0.6 ml; 5.5 mmol) in dichloromethane (50 ml) at RT for 4 h provided compound **32** (2.0 g) in 78% yield; $[\alpha]_D$ −3°C (c 1.6, $CHCl_3$); ^{1}H-NMR (CD_2Cl_2): δ 7.87–7.24 (m, 9 H, arom.), 5.62 (d, J = 10.6 Hz, 1 H, H-1), 5.16 (d, J = 2.9 Hz, 1 H, H-4'), 5.15 (d, J = 2.9 Hz, 1 H, H-1'), 2.07, 2.06, 2.05, 1.78, and 1.43 (each s, 15 H, 5×OAc), 1.03 (d, J = 6.5 Hz, 3 H, CMe).

Synthesis of methyl O-(α-L-fucopyranosyl)-(1→3)-O-(2-acetamido-2deoxy-β-D-glucopyranosyl)-(1→6)-O-[6-O-sulfo-β-D-galactopyranosyl sodium salt (1→3)-O-]-2-acetamido-2-deoxy-α-D-galactopyranoside (**35**)

Glycosylation of **29** (1.0 g; 1.19 mmol) with donor **32** (1.04 g; 1.37 mmol) under N-iodosuccinimide (0.94 g; 4.2 mmol) triflic acid in CH_2Cl_2 (60 ml) for 1 h at −30°C and followed by the removal of bromoacetyl group with thiourea-2,6-lutidine afforded compound **34** (1.1 g) in 46% yield; $[\alpha]_D$ +105°C (c 0.7, $CHCl_3$); ^{1}H-NMR (CD_2Cl_2): δ 8.07–7.22 (m, 19 H, arom.), 5.80 (d, J = 3.4 Hz, 1 H, H-4'), 5.75 (dd, J = 7.9 Hz, 1 H, H-2'), 5.55 (dd, 1 H, H-2), 5.28 (d, J = 8.6 Hz, 1 H, H-1''), 5.17 (d, J = 3.1 Hz, 1 H, H-4'''), 5.16 (d, J = 3.2 Hz, 1 H, H-1'''), 4.99 (d, J = 3.1 Hz, H-1), 4.90 (d, J = 7.9 Hz, 1 H, H-1'), 2.86 (s, 3 H, OMe), 2.08, 2.05, 1.79, 1.73, and 1.48 (each s, 15 H, 5×OAc), 1.20 (s, 3 H, NAc), 1.04 (d, J = 6.5 Hz, 3 H, CMe).

Sulfation of **34** (0.65 g; 0.47 mmol) with SO_3-pyridine complex (0.16 g; 1 mmol) in DMF (15 ml) at 5°C for 16 h and followed by the removal of phthalimido group (EtOH-hydrazine hydrate; 4:1, 100°C, 3 h) and N-acetylation with Ac_2O-MeOH-Et_3N gave title compound **35** (0.19 g) in 47% yield after silica gel column chromatography ($CHCl_3$-MeOH-H_2O; 5:4:1, v/v); $[\alpha]_D$ −12°C (c 0.9, H_2O); ^{1}H-NMR (D_2O): δ 4.99 (d, J = 2.9 Hz, 1 H, H-1'''), 4.76 (d, J = 3.0 Hz, 1 H, H-1), 4.55 (d, J = 8.3 Hz, 1 H, H-1''), 4.46 (d, J = 7.1 Hz, 1 H, H-1'), 3.54 (s, 3 H, OMe), 2.01 and 2.00 (each s, 6 H, 2×NAc), 1.16 (d, J = 6.6 Hz, H, CMe); ^{13}C-NMR: δ 103.48 (C-1''), 100.26 (C1'''), 98.91 (C-1'), 97.13 (C-1), 79.44 (C-3''), 76.38 (C-3), 67.91 (C-6), 67.68 (C-6'), 59.81 (C-6''), 54.29 (OMe), 53.89 (C-2''), 47.46 (C-2), 21.27 and 21.01 (2×NAc), 14.18 (C-6'''); m/z: 825.8 $[M-Na]^-$.

Benzyl O-(α-L-fucopyranosyl)-(1→3)-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→6)-O-[3-O-sulfo-β-D-galactopyranosyl-(1→3)-O]-2-acetamido-2-deoxy-α-D-galactopyranoside (**37**)

Glycosidation of **27** (0.41 g; 0.6 mmol) with **32** (0.50 g; 0.66 mmol) under similar reaction condition as described for the preparation of **34** (from **29**) provided **36** (0.32 g) in 43% yield; $[\alpha]_D$ +25°C (c 1.1, $CHCl_3$); ^{1}H-NMR (CD_2Cl_2): δ 7.73–7.15 (m, 9 H, arom.), 5.43 (d, J = 9.7 Hz, 1 H, H-1''), 5.42 (d, J = 3.4 Hz, 1 H, H-4'), 5.24 (d, J = 3.6 Hz, 1 H, H-4'''), 5.15 (d, J = 3.1 Hz, 1 H, H-1'''), 5.04 (dd, J = 9.0 Hz and 8.7 Hz, 1 H, H-2'), 5.01 (d, J = 3.2 Hz, 1 H, H-1), 2.13, 2.08, 2.06, 2.05, 2.04, 1.99, 1.85, and 1.77 (each s, 24 H, 8×OAc), 1.46 (s, 3 H, NAc), 1.04 (d, J = 6.5 Hz, 3 H, CMe).

Sulfation of **36** (0.25 g; 0.2 mmol) with SO_3-pyridine complex (0.16 g; 0.9 mmol) in DMF (10 ml) at 0°C for 6 h and followed by the removal of protecting groups as described for the preparation of **35** from **34** gave title compound **37** (0.08 g) in 43% yield; $[\alpha]_D$ +11°C (c 0.5, H_2O); ^{1}H-NMR (D_2O): δ 7.49–7.45 (m, 5 H, arom.), 5.01 (d, J = 4.0 Hz, 1 H, H-1'''), 5.00 (d, J = 3.8 Hz, 1 H, H-1), 4.57 (d, J = 8.2 Hz, 1 H, H-1''), 4.56 (d, J = 7.7 Hz, 1 H, H-1'), 1.98 (bs, 6 H, 2×NAc), 1.18 (d, J = 6.6 Hz, H, CMe); ^{13}C-NMR: δ 103.27 (C-1'), 100.28 (C-1''), 98.92 (C-1'''), 95.24 (C-1), 79.55 (C-3''), 79.17 (C-3'), 76.37 (C-3), 59.85 (C-6''), 59.78 (C-6'), 54.23 (C-2''), 47.48 (C-2), 21.33 and 20.92 (2×NAc), 14.17 (C-6'''); m/z: 901.4 $[M-Na]^-$.

Methyl O-(α-L-fucopyranosyl)-(1→3)-O-[β-D-galactopyranosyl-(1→4)-O]-(2-aceta-mido-2-deoxy-β-D-glucopyranosyl)-(1→6)-O-[6-O-sulfo-β-D-galactopyra-nosyl sodium salt-(1→3)-O]-2-acetamido-2-deoxy-α-D-galactopyranoside (**40**)

Glycosylation of **29** (1.1 g; 1.3 mmol) with donor **33** (1.4 g; 1.13 mmol) under similar N-iodosuccinimide-triflic acid condition at −60°C for 0.5 h afforded compound **38** (1.3 g) in 51% yield; $[\alpha]_D$ +71°C (c 0.8, $CHCl_3$); ^{1}H-NMR (CD_2Cl_2): δ 8.05–7.08 (m, 39 H, arom.), 6.90 (d, J = 7.1 Hz, 1 H, NH), 5.83 (d, J = 3.3 Hz, 1 H, H-4'), 5.71 (dd, J = 8.0 Hz, 1 H, H-2'), 5.53 (d, J = 3.4 Hz, 1 H, H-4'''), 5.49 (d, J = 3.5 Hz, 1 H, H-1'''), 5.09 (t, J = 7.7 Hz and 8.4 Hz, 1 H, H-2''), 5.03 (dd, J = 8.3 Hz, 1 H, H-2'''), 4.82 (d, J = 2.7 Hz, 1 H, H-1), 4.79 (dd, 1 H, H-2), 4.51 (d, J = 8.4 Hz, 1 H, H-1'''), 4.48 (d, J = 7.7 Hz, 1 H, H-1'), 3.70 (s, 2 H, $COCH_2Br$), 2.80 (s, 3 H, OMe), 2.02, 2.01, 1.94, 1.93 (each s, 12 H, 4×OAc), 1.58 (s, 3 H, NAc), 1.22 (d, J = 6.5 Hz, 3 H, CMe).

Removal of bromoacetyl group from **39** (1.14 g) was accomplished with thiourea-lutidine procedure to give compound **39** (0.9 g) in 84% yield; $[\alpha]_D$ +75°C (c 1.5, $CHCl_3$); ^{1}H-NMR (CD_2Cl_2): δ 8.05–7.08 (m, 39 H, arom.), 6.91 (d, J = 7.1 Hz, 1 H, NH), 5.80 (d, J = 3.4 Hz, 1 H, H-4'), 5.76 (dd, J = 8.0 Hz, 1 H, H-2'), 5.59 (d, J = 3.4 Hz, 1 H, H-4'''), 5.56 (d, J = 3.5 Hz, 1 H, H-1'''), 5.36 (d, J = 8.3 Hz, 1 H, H-1''), 5.26 (d, J = 3.2 Hz, 1 H, H-1), 5.08 (d, J = 8.4 Hz, 1 H, H-1'), 5.01 (dd, 1 H, H-2), 4.91 (d, J = 7.9 Hz, 1 H, H-1'''), 2.82 (s, 3 H, OMe), 2.00, 1.98, 1.94, 1.93 (each s, 12 H, 4×OAc), 1.87 (s, 3 H, NAc), 1.21 (d, J = 6.5 Hz, 3 H, CMe).

Sulfation of compound **39** (0.44 g) with SO_3-pyridine complex and followed by removal of phthalimido group, N-acetylation and removal of O-benzyl group (10% Pd-c) afforded the target compound **40** in 46% yield; $[\alpha]_D$ −6°C (c 1.0, H_2O); ^{1}H-NMR (D_2O): δ 5.16 (d, J = 4.0 Hz, 1 H, H-1'''), 4.81 (d, J = 3.7 Hz, 1 H, H-1), 4.61 (d, J = 8.1 Hz, 1 H, H-1''), 4.51 (d, J = 7.8 Hz, 1 H, H-1'''), 4.50 (d, J = 7.7 Hz, 1 H, H-1'), 3.41 (s, 3 H, OMe), 2.06 (bs, 6 H, 2×NAc), 1.22 (d, J = 6.6 Hz, 3 H, CMe); ^{13}C-NMR: δ 103.47 (C-1'''), 100.81 (C-1'), 100.30 (C-1''), 97.60 (C-1'''), 97.12 (C-1), 76.38 (C-3), 74.32 (C-3''), 73.94 (C-4''), 67.93 (C-6), 67.34 (C-6'), 60.43 (C-6''), 58.82 (C-6'''), 54.78 (OMe), 53.92 (C-2''), 47.46 (C-2), 21.28 and 20.99 (2×NAc), 14.26 (C-6'''); m/z: 987.5 $[M-Na]^-$.

Methyl O-(α-L-fucopyranosyl)-(1→3)-O-[β-D-galactopyranosyl-(1→4)-O]-(2-aceta-mido-2-deoxy-β-D-glucopyranosyl)-(1→6)-O-[3-O-sulfo-β-D-galactopyranosyl sodium salt (1→3)-O-]-2-acetamido-2-deoxy-α-D-galactopyranoside (**42**)

Glycosylation of **28** (0.25 g; 0.42 mmol) with same glycosyl donor **33** (0.62 g; 0.5 mmol) under N-iodosuccinimide triflic acid condition and followed by the removal of chloroacetyl group as described for the preparation of **39** from **29** gave compound **41** (0.3 g) in 44% yield; $[\alpha]_D$ +22°C (c 0.9, $CHCl_3$); ^{1}H-NMR (CD_2Cl_2): δ 7.70–6.89 (m, 24 H, arom.), 5.42 (d, J = 9.7 Hz, H-1''), 5.27 (d, J = 2.9 Hz, 2 H, H-4. and H-4'''), 5.25 (d, J = 3.3 Hz, 1 H, H-1'''), 5.05 (dd, J = 8.5 Hz, 1 H, H-2''), 4.89 (dd, J = 8.0 Hz, 1 H, H-2'), 4.75 (d, J = 3.5 Hz, 1 H, H-1), 4.40 (d, J = 7.9 Hz, 2 H, H-1, and H-1'''), 2.81 (s, 3 H, OMe), 2.13, 2.06, 2.01, 2.00, 1.94, 1.93, and 1.87 (each s, 21 H, 7×OAc), 1.62 (s, 3 H, NAc), 1.21 (d, J = 6.4 Hz, 3 H, CMe).

The procedure used for preparing **42** (from **41**) were essentially the same as described for the preparation of **41** from **40**; $[\alpha]_D$ −4°C (c 0.5, H_2O); ^{1}H-NMR (D_2O): δ 5.16 (d, J = 4.0 Hz, 1 H, H-1'''), 4.81 (d, J = 3.7 Hz, 1 H, H-1), 4.62 (d, J = 8.1 Hz, 1 H, H-1''), 4.61 (d, J = 7.8 Hz, 1 H, H-1'''), 4.50 (d, J = 7.8 Hz, 1 H, H-1'), 3.41 (s, 3 H, OMe), 2.06 and 2.05 (each s, 6 H, 2×NAc), 1.22 (d, J = 6.6 Hz, 6 H, CMe); ^{13}C-NMR: δ 103.33 (C-1'''), 100.83 (C-1'), 100.36 (C-1''), 97.62 (C-1'''), 97.19 (C-1), 79.24 (C-3'), 76.52 (C-3), 74.35 (C-3''), 73.95 (C-4''), 60.45 (C-6''), 59.85 (C-6'), 58.79 (C-6'''), 54.71 (OMe), 53.93 (C-2''), 47.43 (C-2), 21.27 and 21.01 (2×NAc), 14.28 (C-6'''); m/z: 987.5 $[M-Na]^-$.

Materials for biological assays

Cell culture reagents were obtained from CORE Cell Culture Facility, San Diego, CA. Unless otherwise stated, most of the materials used were obtained from the Sigma Chemical Co. and were reagent grade or better. Other materials used are described in the text below.

Recombinant soluble selectins

The recombinant selectin receptor globulins (Rgs) used contain the extracellular domains of human selectins fused to the hinge and F_c portion of human IgG. The L-selectin (LS-Rg) contains of the entire extracellular domain of a human L-selectin and human IgG_2F_c domain (Norgard *et al.*, 1993a); the E- and P-selectin receptor globulin (ES-Rg and PS-Rg) contain the signal sequence, C-type lectin domain, EGF domain, and six or two of the complement regulatory-like domains, respectively, attached to a human IgG_1F_c domain (Nelson *et al.*, 1993b). The E- and P-selectin chimeric proteins were constructed in pcDM8 vectors, and L-selectin in the vector pcDNA1. Each was stably expressed in 293 cells with G418 selection, and purified from the culture media using Protein A–Sepharose (PAS) as previously described (Nelson *et al.*, 1993b).

Enzyme-linked immunosorbent assays (ELISA) for the lectin properties of the selectins

Inhibition assays. Sterile polystyrene 96 well ELISA plates (no. 25801, Corning, Corning, NY) were coated with 200 ng of polyacrylamide-SLe^x or -HSO_3Le^x (no. 18205PA and no. 18605PA, respectively, Syntesome, Gesellschaft fur Medizinische Biochemie m.b.H., Munich, Germany) by overnight incubation at 4°C in 100 μl of 50 mM sodium carbonate/bicarbonate buffer, pH 9.5. Plates were then blocked with 200 μl per well of assay buffer: 20 mM Hepes (no. 16926, U.S. Biochemical, Cleveland, OH), 125 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1% protease-free BSA (no. 82–045–1, Pentex, Miles, Inc., Kankakee, IL), pH 7.45 (osmolality 290 milliosmoles, determined with a Vapor Pressure Osmometer, model no. 5500XR, Wescor Inc., Logan, UT) for a minimum of 2 h at 4°C. During the blocking step, the selectin chimeras were separately preincubated at 4°C with the secondary antibody, peroxidase-conjugated goat anti-human IgG (no. 109–035–098, Jackson Immunoresearch Laboratories, West Grove, PA) in assay buffer for approximately 1 h. Final selectin-Rg concentration was 1 nM, and the optimal secondary antibody dilution was determined to be 1:1000 for the particular serum used. Potential inhibitors were serially diluted in assay buffer, at 2× the final required concentration. The selectin-Rg/secondary antibody stock was aliquoted into tubes containing an equivalent volume of inhibitor solution; buffer alone for the positive control, or buffer with 10 mM Na_2EDTA, pH 7.5, for the negative control (final concentration 5 mM EDTA). These tubes were preincubated at 4°C for 30 min, and then added to ELISA plates, in duplicates, at final well volume of 100 μl. After 4 h of plate incubation at 4°C, plates were washed three times with 200 μl per well of assay buffer at 4°C, followed by development with 150 μl per well of OPD solution at RT: 0.002 mg *o*-phenylenediamine dihydrochloride (OPD)/ml in 50 mM sodium citrate, 50 mM disodium phosphate buffer, pH 5.2 containing 1 μl/ml 30% H_2O_2. Using a timer, each well was sequentially quenched with 40 μl of 4 M H_2SO_4 after a fixed time of peroxidase reaction. Softmax software and a microplate reader (Molecular Devices, Menlo Park, CA) determined and recorded absorbance at 492 nm. Prior to curve fitting, the data was changed into percentages for comparative purposes, using the formula: ([(average of duplicates) – (negative control)]/ [(positive control) – (negative control)]*100) again with the Softmax software. For determination of IC_{50} values, the data were iteratively fit (Nelson *et al.*, 1993b) to a nonlinear least squares plot using SigmaPlot (Jandel Scientific, San Rafael, CA).

Effects of pH on selectin binding. Plates were prepared with immobilized polyacrylamide ligands and blocked as described above. Twelve tubes of assay buffer with increasing pH increments were prepared so that when added in an equivalent volume to the original assay buffer (pH 7.45) containing selectin-antibody complexes, the final pH in the ELISA plate wells ranged from 7.1 to 7.7. While carrying out the preblocking in pH 7.45 assay buffer, Eppendorf tubes containing the final pH assay buffer and selectin chimera-secondary antibody were mixed and incubated for 30 min at 4°C, and then added to ELISA plates, in duplicates, at final well volume of 100 μl. After 4 h of 4°C incubation, plates were washed (each well with buffer of the appropriate pH) and developed as described above.

Effects of ionic strength on selectin binding. Plates were prepared with immobilized polyacrylamide ligands and blocked as described above. Twelve tubes of assay buffer: 20 mM Hepes, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1% protease-free BSA, pH 7.45, with increasing NaCl concentrations were prepared so that when added in an equivalent volume to assay buffer containing 100 mM NaCl (and 20 mM Hepes, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1% protease-free BSA) and precomplexed selectin chimera-secondary antibody, the final NaCl concentration ranged from 100 mM NaCl to 210 mM, with increasing increments of 10 mM. Tubes containing the final mixtures were mixed and incubated for 30 min at 4°C, and then added to ELISA plates, in duplicate, at final well volume of 100 μl. After 4 h of 4°C incubation, plates were washed three times with 200 μl/well of assay buffer containing the NaCl concentrations corresponding to that originally present in each well. Binding was detected by developing with OPD solution as described above.

Effects of temperature on selectin binding. Plates were prepared with immobilized polyacrylamide ligands and blocked at 4°C as described above. During the last 30 min of blocking, plates were incubated at 4°C at RT: 20–22°C, or 37°C. In addition vials prepared as stated above with positive and negative controls (±5 mM EDTA) or serial dilutions of inhibitor (SLe^a, compound 4). These were also preincubated in the appropriate temperature for 30 min. Contents of the tubes were aliquoted into ELISA plate wells in 100 μl volume and in duplicates. After 4 h incubation at 4°C, RT, or 37°C incubation, plates were washed with 200 μl/well of assay buffer preincubated at the corresponding temperature. OPD development and following steps were done as described above.

Preparation of ^{35}S-labeled GlyCAM-1. The labeling and purification of secreted murine lymph node L-selectin ligands was carried out by a modification (Crottet *et al.*, 1996) of previously reported methods (Imai *et al.*, 1991; Lasky *et al.*, 1992). Briefly, lymph nodes slices were labeled with [^{35}S] Na_2SO_4, the supernatant was passed over wheat germ agglutinin Sepharose, and the material eluted with 0.2 M GlcNAc was subjected to affinity chromatography on LS-Rg-PAS. Bound ligands (primarily ^{35}S-labeled GlyCAM-1) were eluted with 2 mM EDTA.

Isolation of L-selectin ligands from mouse serum. It is known that GlyCAM-1 is secreted by lymph node HEV into the mouse bloodstream (Hoke *et al.*, 1995). Soluble L-selectin ligands, including GlyCAM-1, were isolated from sterile mouse serum (Pel-Freez Biologicals, no. 35126–3, Rogers, AR). Serum (5 ml, trace hemolyzed), was spiked with ~3000 c.p.m. of ^{35}S-labeled GlyCAM-1 (see protocol above) and loaded directly onto an 0.6 mg/ml LS-Rg-PAS column (equilibrated in 20 mM Hepes, 125 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.45), followed by washing with the same buffer. Specifically bound material was eluted with 2 mM EDTA, 20 mM Hepes, 125 mM NaCl, pH 7.45, and was followed by counting 10% of each fraction collected. Complete binding of the radioactivity ensured that the column was not overloaded, and complete recovery ensured that elution was complete. This process was repeated six times (30 ml total of mouse serum ran over the LS-Rg-PAS column). The fractions containing the majority of the EDTA-eluted material were pooled and used for inhibition assays described below. As expected, this material included GlyCAM-1 (detected by dot blotting using anti-GlyCAM-1 antibody, data not shown).

Assay for L-selectin binding to GlyCAM-1. The following steps were performed at 4°C unless otherwise stated. ELISA plate (Corning, no. 25801, Corning, NY) wells were coated with 0.6 μg of rabbit monospecific polyclonal anti-GlyCAM-1 antibody (a kind gift from Susan Watson, Genentech) in 100 μl of 50 mM $Na_2CO_3/NaHCO_3$ buffer, pH 9.5 per well at 22°C for 1–1.5 h. The wells were then blocked with 200 μl per well of ELISA assay buffer containing BSA (see above) for 2 h. Mouse serum L-selectin ligands (see above for isolation) were added (0.6 μg per 100 μl of ELISA assay buffer per well), incubated overnight to allow specific capture of GlyCAM-1, and wells washed once with 200 μl per well of ELISA assay buffer. The addition of serially diluted oligosaccharide inhibitors mixed with the LS-Rg-secondary antibody complexes to wells was done exactly as described for the ELISA inhibition assays above. Loss of binding in the absence of added mouse serum ligands, or in the presence of 5 mM EDTA, confirmed the specificity of binding. Binding was read, data was converted to percentages and IC_{50} values were generated by fitting data to a nonlinear least-squares curve, exactly as described above for the ELISA inhibition assays.

Acknowledgments

We thank Young Kim and Conrad Piskorz for helpful discussions, and for their careful review of the manuscript. This research was supported by Grants CA38701 (to A.V.) and CA63213 (to K.L.M.).

Abbreviations

GlyCAM-1, Glycosylation-dependent cell adhesion molecule-1; SLe^x, Sialyl Lewis x, Siaα2–3Galβ1–4(Fucα1–3)GlcNAcβ1-R; SLe^a, Sialyl Lewis a, Siaα2–3Galβ1–3(Fucα1–4)GlcNAcβ1-R; $3'SO_3Le^x$, 3-O-SO_3Galβ1–4(Fucα1–3)GlcNAcβ1-R; 6′SO3Le^x, 6-O-SO_3Galβ1–4(Fucα1–3)GlcNAcβ1-R; SE, sulfate ester; IC_{50}, concentration giving 50% inhibition; ELISA, enzyme-linked immunosorbent assay; ES-Rg, E-selectin receptor globulin; LS-Rg, L-selectin receptor globulin; PS-Rg, P-selectin receptor globulin; LN, lymph nodes; BSA, bovine serum albumin; PAS, Protein A–Sepharose.

References

Albelda,S.M., Smith,C.W. and Ward,P.A. (1994) Adhesion molecules and inflammatory injury. *FASEB J.*, **8**, 504–512.

Baumhueter,S., Singer,M.S., Henzel,W., Hemmerich,S., Renz,M., Rosen,S.D. and Lasky,L.A. (1993) Binding of L-selectin to the vascular sialomucin CD34. *Science*, **262**, 436–438.

Berg,E.L., Robinson,M.K., Mansson,O., Butcher,E.C. and Magnani,J.L. (1991) A carbohydrate domain common to both sialyl Le^a and sialyl Le^X is recognized by the endothelial cell leukocyte adhesion molecule ELAM-1. *J. Biol. Chem.*, **266**, 14869–14872.

Berg,E.L., McEvoy,L.M., Berlin,C., Bargatze,R.F. and Butcher,E.C. (1993) L-Selectin-mediated lymphocyte rolling on MAdCAM-1. *Nature*, **366**, 695–698.

Bertozzi,C.R., Fukuda,S. and Rosen,S.D. (1995) Sulfated disaccharide inhibitors of L-selectin: deriving structural leads from a physiological selectin ligand. *Biochemistry*, **34**, 14271–14278.

Bevilacqua,M.P. and Nelson,R.M. (1993) Selectins. *J. Clin. Invest.*, **91**, 379–387.

Bevilacqua,M.P., Nelson,R.M., Mannori,G. and Cecconi,O. (1994) Endothelial-leukocyte adhesion molecules in human disease. *Annu. Rev. Med.*, **45**, 361–378.

Brandley,B.K., Kiso,M., Abbas,S., Nikrad,P., Srivasatava,O., Foxall,C., Oda,Y. and Hasegawa,A. (1993) Structure–function studies on selectin carbohydrate ligands. Modifications to fucose, sialic acid and sulphate as a sialic acid replacement. *Glycobiology*, **3**, 633–639.

Capon,C., Laboisse,C.L., Wieruszeski,J.-M., Maoret,J.-J., Augeron,C. and Fournet,B. (1992) Oligosaccharide structures of mucins secreted by the human colonic cancer cell line CL.16E. *J. Biol. Chem.*, **267**, 19248–19257.

Chandrasekaran,E.V., Jain,R.K., Larsen,R.D., Wlasichuk,K. and Matta,K.L. (1995) Selectin ligands and tumor-associated carbohydrate structures: Specificities of α2,3-sialyltransferases in the assembly of 3′-sialyl-6-sulfo/sialyl Lewis a and x, 3′-sialyl-6′-sulfo Lewis x, and 3′-sialyl-6-sialyl/sulfo blood group T-hapten. *Biochemistry*, **34**, 2925–2936.

Chou,K.-C. (1995) The convergence-divergence duality in lectin domains of selectin family and its implications. *FEBS Lett.*, **363**, 123–126.

Crommie,D. and Rosen,S.D. (1995) Biosynthesis of GlyCAM-1, a mucin-like ligand for L-selectin. *J. Biol. Chem.*, **270**, 22614–22624.

Crottet,P., Kim,Y.J. and Varki,A. (1996) Subsets of sialylated, sulfated mucins of diverse origins are recognized by L-selectin. Lack of evidence for unique oligosaccharide sequences mediating binding. *Glycobiology*, **6**, 191–208.

DeFrees,S.A., Kosch,W., Way,W., Paulson,J.C., Sabesan,S., Halcomb,R.L., Huang,D.-H., Ichikawa,Y. and Wong,C.-H. (1995) Ligand recognition by E-selectin: synthesis, inhibitory activity, and conformational analysis of bivalent sialyl Lewisx analogs. *J. Am. Chem. Soc.*, **117**, 66–79.

Dejter-Juszynski,M. and Flowers,H.M. (1971) *Carbohydr. Res.*, **18**, 219–226.

Erbe,D.V., Wolitzky,B.A., Presta,L.G., Norton,C.R., Ramos,R.J., Burns,D.K., Rumberger,J.M., Rao,B.N.N., Foxall,C., Brandley,B.K. and Lasky,L.A. (1992) Identification of an E-selectin region critical for carbohydrate recognition and cell adhesion. *J. Cell Biol.*, **119**, 215–227.

Erbe,D.V., Watson,S.R., Presta,L.G., Wolitzky,B.A., Foxall,C., Brandley,B.K. and Lasky,L.A. (1993) P- and E-selectin use common sites for carbohydrate ligand recognition and cell adhesion. *J. Cell Biol.*, **120**, 1227–1235.

Ferrier,R.J. and Furneaux,R.H. (1980) 1,2-Trans-1-thioglycosides. *Methods Carbohydr. Chem.*, **8**, 251–253.

Foster,C.S., Gillies,D.R.B. and Glick,M. C. (1991) Purification and characterization of GDP-L-Fuc-*N*-acetyl-β-D-glucosaminide α1(3fucosyltransferase from human neuroblastoma cells. Unusual substrate specificities of the tumor enzyme. *J. Biol. Chem.*, **266**, 3526–3531.

Graves,B.J., Crowther,R.L., Chandran,C., Rumberger,J.M., Li,S., Huang, K.-S., Presky,D.H., Familletti,P.C., Wolitzky,B.A. and Burns,D.K. (1994) Insight into E-selectin/ligand interaction from the crystal structure and mutagenesis of the lec/EGF domains. *Nature*, **367**, 532–538.

Hall,L.D. Manville,J.F. and Bhacca,N.S. (1969) Studies of specifically fluorinated carbohydrates. Part I. Nuclear magnetic resonance studies of hexopyranosyl fluoride derivatives. *Can. J. Chem.*, **47**, 1–17.

Hemmerich,S. and Rosen,S.D. (1994) 6′-sulfated sialyl Lewisx is a major capping group of GlyCAM-1. *Biochemistry*, **33**, 4830–4835.

Hemmerich,S., Bertozzi,C.R., Leffler,H. and Rosen,S.D. (1994) Identification of the sulfated monosaccharides of GlyCAM-1, an endothelial-derived ligand for L-selectin. *Biochemistry*, **33**, 4820–4829.

Hemmerich,S., Leffler,H. and Rosen,S.D. (1995) Structure of the *O*-glycans in GlyCAM-1, an endothelial-derived ligand for L-selectin. *J. Biol. Chem.*, **270**, 12035–12047.

Hensley,P., McDevitt,P.J., Brooks,I., Trill,J.J., Feild,J.A., McNulty,D.E., Connor,J.R., Griswold,D.E., Kumar,N.V., Kopple,K.D., Carr,S.A., Dalton,B.J. and Johanson,K. (1994) The soluble form of E-selectin is an asymmetric monomer. Expression, purification, and characterization of the recombinant protein. *J. Biol. Chem.*, **269**, 23949–23958.

Hoke,D., Mebius,R.E., Dybdal,N., Dowbenko,D., Gribling,P., Kyle,C., Baumhueter,S. and Watson,S.R. (1995) Selective modulation of the expression of L-selectin ligands by an immune response. *Curr. Biol.*, **5**, 670–678.

Hollenbaugh,D., Bajorath,J., Stenkamp,R. and Aruffo,A. (1993) Interaction of P-selectin (CD62) and its cellular ligand: analysis of critical residues. *Biochemistry*, **32**, 2960–2966.

Howard,D.R., Fukuda,M., Fukuda,M.N. and Stanley,P. (1987) The GDP-fucose:N-acetylglucosaminide 3-alpha-L-fucosyltransferases of LEC11 and LEC12 Chinese hamster ovary mutants exhibit novel specificities for glycolipid substrates. *J. Biol. Chem.*, **262**, 16830–16837.

Imai,Y. and Rosen,S.D. (1993) Direct demonstration of heterogeneous, sulfated *O*-linked carbohydrate chains on an endothelial ligand for L-selectin. *Glycoconjugate J.*, **10**, 34–39.

Imai,Y., Singer,M.S., Fennie,C., Lasky,L.A. and Rosen,S.D. (1991) Identification of a carbohydrate-based endothelial ligand for a lymphocyte homing receptor. *J. Cell Biol.*, **113**, 1213–1222.

Imai,Y., Lasky,L.A. and Rosen,S.D. (1992) Further characterization of the interaction between L-selectin and its endothelial ligands. *Glycobiology*, **2**, 373–381.

Jacob,G.S., Kirmaier,C., Abbas,S.Z., Howard,S.C., Steininger,C.N., Welply,J.K. and Scudder,P. (1995) Binding of sialyl Lewis X to E-selectin as measured by fluorescence polarization. *Biochemistry*, **34**, 1210–1217.

Jain,R.K. and Matta,K.L. (1990) Synthesis of the sodium salts of methyl 2-O-α-L-fucopyranosyl-α-L-fucopyranoside 3- and 4-sulfate. *Carbohydr. Res*, **208**, 51–58.

Jain,R.K. and Matta,K.L. (1994) Synthesis of sulfated and sialic acid analogs of Galβ1,3GalNAcα-OR as acceptors for sulfotransferases. XIIIth International Carbohydrate Symposium. July 17–22, Ottawa, Canada, p. 443.

Jain,R.K., Vig,R., Locke,R.D., Mohammad,A. and Matta,K.L. (1996) Selectin ligands: 2,3,4-tri-O-acetyl-6–0-pivaloyl-α/β-galactopyranosyl halide as novel glycosyl donor for the synthesis of 3–0-sialyl and 3–0-sulfo Le^x and Le^a type structures. *J. Chem. Soc. Chem. Commun.*, 65–67.

Jain,R.K., Vig,R., Rampal,R., Chandrasekaran,E.V. and Matta,K.L. (1994) Total synthesis of 3′-*O*-sialyl, 6′-*O*-sulfo $Lewis^x$, NeuAcα2→3(6-OSO_3Na)Gal1β1→4(Fucα1→3)-GlcNAcβ-OM e: a major capping group of GLYCAM-I. *J. Am. Chem. Soc.*, **116**, 12123–12124.

Kovac,P., Glaudemans,C.P., Guo,W. and Wong,T.C. (1985) Synthesis of methyl O-(3-deoxy-3-fluoro-beta-D-. *Carbohydr. Res.*, **140**, 299–311.

Kuhns,W., Jain,R.K., Matta,K.L., Paulsen,H., Baker,M.A., Geyer,R. and Brockhausen,I. (1995) Characterization of a novel mucin sulphotransferase activity synthesizing sulphated *O*-glycan core 1,3-sulphate-Galβ1–3GalNAcα-R. *Glycobiology*, **5**, 689–697.

Lasky,L.A. (1995) Selectin-carbohydrate interactions and the initiation of the inflammatory response. *Annu. Rev. Biochem.*, **64**, 113–139.

Lasky,L.A., Singer,M.S., Dowbenko,D., Imai,Y., Henzel,W.J., Grimley,C., Fennie,C., Gillett,N., Watson,S.R. and Rosen,S.D. (1992) An endothelial ligand for L-selectin is a novel mucin-like molecule. *Cell*, **69**, 927–938.

Lee,Y.C. (1992) Biochemistry of carbohydrate-protein interaction. *FASEB J.*, **6**, 3193–3200.

Lefer,A.M., Weyrich,A.S. and Buerke,M. (1994) Role of selectins, a new family of adhesion molecules, in ischaemia-reperfusion injury. *Cardiovasc. Res.*, **28**, 289–294.

Lemieux,R.U., Bundle,D.R. and Baker,D.A. (1975) The properties of a "synthetic" antigen related to the human blood-group Lewis a. *J. Am. Chem. Soc.*, **97**, 4076–4083.

Li,F., Wilkins,P.P., Crawley,S., Weinstein,J., Cummings,R.D. and McEver,R.P. (1996) Post-translational modifications of recombinant P-selectin glycoprotein ligand-1 required for binding to P- and E-selectin. *J. Biol. Chem.*, **271**, 3255–3264

Lo-Guidice,J.-M., Wieruszeski,J.-M., Lemoine,J., Verbert,A., Roussel,P. and Lamblin,G. (1994) Sialylation and sulfation of the carbohydrate chains in respiratory mucins from a patient with cystic fibrosis. *J. Biol. Chem.*, **269**, 18794–18813.

Lubineau,A., Le Gallic,J. and Lemoine,R. (1993) First synthesis of the 3′-sulfated Lewis trisaccharide, putative ligand for the leucocyte homing receptor. *J. Chem. Soc. Chem. Commun.*, **1993**, 1419–1420.

Maaheimo,H., Renkonen,R., Turunen,J.P., Penttila,L. and Renkonen,O. (1995) Synthesis of a divalent sialyl Lewisx O-glycan, a potent inhibitor of lymphocyte-endothelium adhesion—evidence that multivalency enhances the saccharide binding to L-selectin. *Eur. J. Biochem.*, **234**, 616–625.

Manning,D.D., Bertozzi,C.R., Pohl,N.L., Rosen,S.D. and Kiessling,L.L. (1995) Selectin–saccharide interactions: revealing structure–function relationships with chemical synthesis. *J. Organic Chem.*, **60**, 6254–6259.

Mannori,G., Crottet,P., Cecconi,O., Hanasaki,K., Aruffo,A., Nelson,R.M., Varki,A. and Bevilacqua,M.P. (1995) Differential colon cancer cell adhe-

sion to E-, P-, and L-selectin: role of mucin type glycoproteins. *Cancer Res*, **55**, 4425–4431.

Marra,A. and Sinaÿ,P. (1989) Stereoselective synthesis of 2-thioglycosides of *N*-acetylneuraminic acid. *Carbohydr. Res.*, **187**, 35–42.

Martens,C.L., Cwirla,S.E., Lee,R.Y.W., Whitehorn,E., Chen,E.Y.F., Bakker,A., Martin,E.L., Wagstrom,C., Gopalan,P., Smith,C.W., Tate,E., Koller,K.J., Schatz,P.J., Dower,W.J. and Barrett,R.W. (1995) Peptides which bind to E-selectin and block neutrophil adhesion *J. Biol. Chem.*, **270**, 21129–21136.

Mawhinney,T.P., Adelstein,E., Gayer,D.A., Landrum,D.C. and Barbero,G.J. (1992) Structural analysis of monosulfated side-chain oligosaccharides isolated from human tracheobronchial mucous glycoproteins. *Carbohydr. Res.*, **223**, 187–207.

McEver,R.P., Moore,K.L. and Cummings,R.D. (1995) Leukocyte trafficking mediated by selectin–carbohydrate interactions. *J. Biol. Chem.*, **270**, 11025–11028.

Moore,K.L., Stults,N.L., Diaz,S., Smith,D.F., Cummings,R.D., Varki,A. and McEver,R.P. (1992) Identification of a specific glycoprotein ligand for P-selectin (CD62) on myeloid cells. *J. Cell Biol.*, **118**, 445–456.

Mukaiyama,T., Murai,Y. and Shoda,S. (1981) An efficient method for glucosylation of hydroxy compounds using glucopyranosyl fluoride. *Chem. Lett.*, 431–432.

Narasinga Rao,B.N., Anderson,M.B., Musser,J.H., Gilbert,J.H., Schaefer,M.E., Foxall,C. and Brandley,B.K. (1994) Sialyl Lewis X mimics derived from a pharmacophore search are selectin inhibitors with anti-inflammatory activity. *J. Biol. Chem.*, **269**, 19663–19666.

Nelson,R.M., Cecconi,O., Roberts,W.G., Aruffo,A., Linhardt,R.J. and Bevilacqua,M.P. (1993a) Heparin oligosaccharides bind L- and P-selectin and inhibit acute inflammation. *Blood*, **82**, 3253–3258.

Nelson,R.M., Dolich,S., Aruffo,A., Cecconi,O. and Bevilacqua,M.P. (1993b) Higher-affinity oligosaccharide ligands for E-selectin. *J Clin. Invest.*, **91**, 1157–1166.

Nicolaou,K.C., Hummel,C.W. and Iwabuchi,Y. (1992) Total synthesis of sialyl dimeric Le^x. *J. Am. Chem. Soc.*, **114**, 3126–3128.

Nilsson,M. and Norberg,T. (1988) Synthesis of a dimeric Lewis X hexasaccharide derivative. *Carbohydr. Res.*, **183**, 71–82.

Norgard,K.E., Han,H., Powell,L., Kriegler,M., Varki,A. and Varki,N.M. (1993a) Enhanced interaction of L-selectin with the high endothelial venule ligand via selectively oxidized sialic acids. *Proc. Natl. Acad. Sci. USA*, **90**, 1068–1072.

Norgard,K.E., Moore,K.L., Diaz,S., Stults,N.L., Ushiyama,S., McEver,R.P., Cummings,R.D. and Varki,A. (1993b) Characterization of a specific ligand for P-selectin on myeloid cells. A minor glycoprotein with sialylated *O*-linked oligosaccharides. *J. Biol. Chem.*, **268**, 12764–12774.

Norgard-Sumnicht,K.E., Varki,N.M. and Varki,A. (1993) Calcium-dependent heparin-like ligands for L-selectin in nonlymphoid endothelial cells. *Science*, **261**, 480–483.

O'Connell,D., Koenig,A., Jennings,S., Hicke,B., Han,H.-L., Fitzwater,T., Chang,Y.-F., Varki,N., Parma,D. and Varki,A. (1996) Calcium-dependent oligonucleotide antagonists specific for L-selectin. *Proc. Natl. Acad. Sci USA*, **93**, 5883–5887.

Patel,T. P., Goelz,S.E., Lobb,R.R. and Parekh,R.B. (1994) Isolation and characterization of natural protein-associated carbohydrate ligands for E-selectin. *Biochemistry*, **33**, 14815–14824.

Paulson,J.C., Prieels,J.P., Glasgow,L.R. and Hill,R.L. (1978) Sialyl- and fucosyltransferases in the biosynthesis of asparaginyl-linked oligosaccharides in glycoproteins. Mutually exclusive glycosylation by beta-galactoside alpha2–6 sialyltransferase and N-acetylglucosaminide alpha1–3 fucosyltransferase. *J. Biol. Chem.*, **253**, 5617–5624.

Piskorz,C.F., Abbas,S.A. and Matta,K.L. (1984) Synthetic mucin fragments: benzyl 2-acetamido-6-O-(2-acetamido-2-deoxy-beta-D-glucopyranosyl) -2-deoxy-3-O-beta-D-galactopyranosyl-alpha-D-gal actopyranoside and benzyl-2-acet amido-6-O-(2-acetamido-2-deoxy-beta-D-glucopyranosyl)- 3-O-[6-O-(2-acetamide-2-deoxy-beta-D-gluco pyranosyl)-beta-D-galac topyranosyl]-2-deoxy-alpha-D-galactopyranoside. *Carbohydr. Res.*, **126**, 115–124.

Pouyani,T. and Seed,B. (1995) PSGL-1 recognition of P-selectin is controlled by a tyrosine sulfation consensus at the PSGL-1 amino terminus. *Cell*, **83**, 333–343.

Rice,K.G., Weisz,O.A., Barthel,T., Lee,R.T. and Lee,Y.C. (1990) Defined geometry of binding between triantennary glycopeptide and the asialoglycoprotein receptor of rat hepatocytes. *J. Biol. Chem.*, **265**, 18429–18434.

Rice,K.G., Wu,P., Brand,L. and Lee,Y.C. (1991) Interterminal distance and flexibility of a triantennary glycopeptide as measured by resonance energy transfer. *Biochemistry*, **30**, 6646–6655.

Rosen,S.D. and Bertozzi,C.R. (1994) The selectins and their ligands. *Curr. Opin. Cell Biol.*, **6**, 663–673.

Sako,D., Chang,X.-J., Barone,K.M., Vachino,G., White,H.M., Shaw,G., Veldman,G.M., Bean,K.M., Ahern,T.J., Furie,B., Cumming,D.A. and Larsen,G.R. (1993) Expression cloning of a functional glycoprotein ligand for P-selectin. *Cell*, **75**, 1179–1186.

Sako,D., Comess,K.M., Barone,K.M., Camphausen,R.T., Cumming,D.A. and Shaw,G.D. (1995) A sulfated peptide segment at the amino terminus of PSGL-1 is critical for P-selectin binding. *Cell*, **83**, 323–331.

Sato,S., Mori,M., Ito,Y. and Ogawa,T. (1986) An efficient approach to O-glycosides through $CuBr_2$-Bu_4NBr mediated activation of glycosides. *Carbohydr. Res.*, **155**, C6–C10.

Sato,S., Ito,Y., Nukada,T., Nakahara,Y. and Ogawa,T. (1987) Total synthesis of X hapten, III3 Fuc alpha-. *Carbohydr. Res.*, **167**, 197–210.

Scudder,P.R., Shailubhai,K., Duffin,K.L., Streeter,P.R. and Jacob,G.S. (1994) Enzymatic synthesis of a 6′-sulphated sialyl-Lewis^x which is an inhibitor of L-selectin binding to peripheral addressin. *Glycobiology*, **4**, 929–933.

Skinner,M.P., Fournier,D.J., Andrews,R.K., Gorman,J.J., Chesterman,C.N. and Berndt,M.C. (1989) Characterization of human platelet GMP-140 as a heparin-binding protein. *Biochem. Biophys. Res. Commun.*, **164**, 1373–1379.

Steininger,C.N., Eddy,C.A., Leimgruber,R.M., Mellors,A. and Welply,J.K. (1992) The glycoprotease of *Pasteurella haemolytica* A1 eliminates binding of myeloid cells to P-selectin but not to E-selectin. *Biochem. Biophys. Res. Commun.*, **188**, 760–766.

Stroud,M.R., Handa,K., Ito,K., Salyan,M.E.K., Fang,H., Levery,S.B., Hakamori,S., Reinhold,B.B. and Reinhold,V.N. (1995) Myeloglycan, a series of E-selectin-binding polylactosaminolipids found in normal human leukocytes and myelocytic leukemia HL60 cells. *Biochem. Biophys. Res. Commun.*, **209**, 777–787.

Stroud,M.R., Handa,K., Salyan,M.E.K., Ito,K., Levery,S.B., Hakomori,S., Reinhold,B.B. and Reinhold,V.N. (1996) Monosialogangliosides of human myelogenous leukemia HL60 cells and normal human leukocytes. 2. Characterization of E-selectin binding fractions, and structural requirements for physiological binding to E-selectin. *Biochemistry*, **35**, 770–778.

Strous,G.J. and Dekker,J. (1992) Mucin-type glycoproteins. *CRC Crit. Rev. Biochem. Mol Biol.*, **27**, 57–92.

Tamatani,T., Suematsu,M., Tezuka,K., Hanzawa,N., Tsuji,T. and Ishimura,Y. (1995) Recognition of consensus CHO structure in ligands for selectins by novel antibody against sialyl Lewis X. *Am. J. Physiol. Heart Circ. Physiol.*, **269**, H1282-H1287.

Turunen,J.P., Majuri,M.L., Seppo,A., Tiisala,S., Paavonen,T., Miyasaka,M., Lemström,K., Penttilè,L., Renkonen,O. and Renkonen,R. (1995) *De novo* expression of endothelial sialyl Lewis^a and sialyl Lewis^x during cardiac transplant rejection: superior capacity of a tetravalent sialyl Lewis^x oligosaccharide in inhibiting L-selectin-dependent lymphocyte adhesion. *J. Exp. Med.*, **182**, 1133–1141.

Ushiyama,S., Laue,T.M., Moore,K.L., Erickson,H.P. and McEver,R.P. (1993) Structural and functional characterization of monomeric soluble P-selectin and comparison with membrane P-selectin. *J. Biol. Chem.*, **268**, 15229–15237.

Varki,A. (1992) Selectins and other mammalian sialic acid-binding lectins. *Curr. Opin. Cell Biol.*, **4**, 257–266.

Varki,A. (1994) Selectin ligands. *Proc. Natl. Acad. Sci. USA*, **91**, 7390–7397.

Veeneman,G.H., Van Leeuwen,S.H. and Van Boom,J.H. (1990) Iodonium ion promoted reactions at the anomeric centre. II. An efficient thioglycoside mediated approach toward the formation of 1,2-trans linked glycosides and glycosidic linkages. *Tetrahedron Lett.*, **31**, 1331–1334.

Vig,R., Jain,R.K., Piskorz,C.F. and Matta,K.L. (1995) Selectin ligands: synthesis of 3′-O-sialyl-6′-O-sulfo Lewis^a, NeuAcα2→3(6-O-SO_3Na)Galβ1→3 (Fucα1→4) GlcNAcβ-OMe. *J. Chem. Soc. Chem. Commun.*, **1995**, 2073–2074.

Welply,J.K., Abbas,S.Z., Scudder,P., Keene,J.L., Broschat,K., Casnocha,S., Gorka,C., Steininger,C., Howard,S.C., Schmuke,J.J., Graneto,M., Rotsaert,J.M., Manger,I.D. and Jacob,G.S. (1994) Multivalent sialyl-LeX: potent inhibitors of E-selectin–mediated cell adhesion; reagent for staining activated endothelial cells. *Glycobiology*, **4**, 259–266.

Wilkins,P.P., Moore,K.L., McEver,R.P. and Cummings,R.D. (1995) Tyrosine sulfation of P-selectin glycoprotein ligand-1 is required for high affinity binding to P-selectin. *J. Biol. Chem.*, **270**, 22677–22680.

Yoshida,T., Fennie,C., Lasky,L.A. and Lee,Y.C. (1994) A liquid-phase binding analysis for L-selectin—a strong dependency on highly clustered sulfate groups. *Eur. J. Biochem.*, **222**, 703–709.

Yuen,C.-T., Bezouska,K., O'Brien,J., Stoll,M., Lemoine,R., Lubineau,A., Kiso,M., Hasegawa,A., Bockovich,N.J., Nicolaou,K.C. and Feizi,T. (1994) Sulfated blood group Lewis^a. A superior oligosaccharide ligand for human E-selectin. *J. Biol Chem.*, **269**, 1595–1598.

Received on June 12, 1996; revised on August 1, 1996; accepted on August 11, 1996