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Sulfated Disaccharide Inhibitors of L-Selectin: Deriving Structural Leads from a Physiological Selectin Ligand†

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ABSTRACT: The selectins are a family of three adhesion molecules (L-, P-, and E-) that direct the interaction of circulating leukocytes with endothelial cells during the first step in recruitment to tissue sites. Their involvement in inflammatory disease makes the selectins attractive targets for anti-inflammatory therapy. The sialyl Lewis x tetrasaccharide binds weakly to all three selectins and has demonstrated anti-inflammatory activity *in vivo*. However, the synthetic difficulties inherent to sialylated and fucosylated oligosaccharides motivate the search for alternative antagonists. Here we demonstrate that information gained from the biochemical analysis of a physiological selectin ligand can provide new leads for small molecule design. Previous structural analysis of the oligosaccharide chains on GlyCAM-1, an endothelial-derived ligand for L-selectin, revealed two novel structures: 6′-sulfo sialyl Lewis x and 6-sulfo sialyl Lewis x. The sulfate esters on these structures are thought to be essential for high-affinity binding to L-selectin. By incorporating sulfate esters on the analogous positions of the disaccharide lactose, we generated a simple small molecule (lactose 6′,6-disulfate) with greater inhibitory potency for L-selectin than sialyl Lewis x.

Leukocyte-endothelial interactions are a prerequisite for the recruitment of leukocytes from the blood vascular compartment into inflammatory sites and lymphoid organs (Springer, 1995). The first step in the recruitment process is the transient "rolling" interaction of leukocytes along the endothelial cells that line the blood vessel, an event mediated by a family of adhesion molecules called the selectins [see Bevilacqua and Nelson (1993), Rosen and Bertozzi (1994), Lasky (1995), and Tedder et al. (1995a) for recent reviews]. There are three members of the selectin family, L-, P-, and E-selectin. While P- and E-selectin are induced on the endothelial surface in response to inflammatory signals, L-selectin is constitutively expressed on all classes of circulating leukocytes and interacts with cognate ligands on endothelial cells. In addition to its role in leukocyte trafficking to sites of inflammation, L-selectin also participates in lymphocyte binding to high endothelial venules (HEV)[1] of lymph nodes during the process of lymphocyte recirculation. Molecular cloning of the three selectins has revealed the presence of an N-terminal domain with homology to a family of calcium-dependent carbohydrate-binding proteins termed C-type lectins (Drickamer, 1988). Accordingly, the selectins function as lectins in recognizing carbohydrate-based ligands on opposing cells.

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[1] Abbreviations: HEV, high endothelial venule; sLe^x, sialyl Lewis x; sLe^a, sialyl Lewis a; NeuAc, *N*-acetylneuraminic acid; Gal, galactose; Fuc, fucose; GlcNAc, *N*-acetylglucosamine; Sia, sialic acid; THF, tetrahydrofuran; DMF, dimethylformamide; LSIMS, liquid secondary ion mass spectrometry; MS, mass spectum; DEAE, diethylaminoethyl; ELISA, enzyme-linked immunosorbent assay; lac, lactose; β-OMe, β-methyl glycoside; pyr-OAc, pyridinium acetate; M6P, mannose 6-phosphate.

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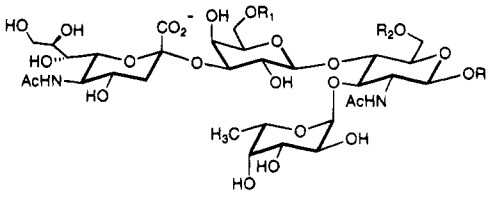


1: $R_1 = R_2 = H$, sialyl Lewis x (sLe^x)
2: $R_1 = SO_3^-$, $R_2 = H$, 6'-sulfo sLe^x
3: $R_1 = H$, $R_2 = SO_3^-$, 6-sulfo sLe^x
4: $R_1 = R_2 = SO_3^-$, 6',6-disulfo sLe^x

FIGURE 1: Oligosaccharide structures found on GlyCAM-1.

The broad participation of the selectins in inflammatory disease (Rosen & Bertozzi, 1994; Carlos & Harlan, 1994; Tedder et al., 1995b) has stimulated tremendous interest in the nature of their carbohydrate ligands as leads for the development of anti-inflammatory agents. All three selectins share a common recognition motif, the sialyl Lewis x [NeuAcα2,3Galβ1,4(Fucα1,3)GlcNAc, **1**, Figure 1] and the related sialyl Lewis a [NeuAcα2,3Galβ1,3(Fucα1,4)GlcNAc] tetrasaccharides (Feizi, 1993; Varki, 1994). Sialylation and fucosylation of the core *N*-acetyllactosamine disaccharide are essential for conferring binding activity. Although the interaction of the selectins with sLe^x oligosaccharides is fairly weak, with equilibrium dissociation constants in the millimolar range (Cooke et al., 1994; Jacob et al., 1995), sLe^x has proven to be an effective antagonist of selectin-mediated adhesion in several animal models of acute inflammation (Mulligan et al., 1993a,b; Buerke et al., 1994; Seekamp et al., 1994).

Two central problems in the development of sLe^x derivatives as anti-inflammatory agents are the lack of an inexpensive and expedient synthesis and the susceptibility of the sialic acid and fucose residues to cleavage in the bloodstream (Uchiyama et al., 1995). The chemical syntheses of sLe^x are laborious, involving difficult glycosylation reactions with sialic acid and fucose (Nicolaou et al., 1991, 1992, Danishefsky et al., 1992a,b; Yoshida et al., 1993; Stahl et al., 1994). The enzymatic synthesis of sLe^x is more efficient but requires the use of enzymes and cofactors that are either not generally available or very expensive (Dumas et al., 1991; Ichikawa et al., 1992; Ball et al., 1992; de Vries et al., 1993; Palcic, 1994). These difficulties have prompted several groups to explore sLe^x mimetics with reduced structural complexity and with functional alternatives to sialic acid and fucose. For example, Feizi and co-workers and Brandley et al. have demonstrated that replacement of sialic acid in sLe^x with a sulfate ester results in equivalent or enhanced selectin binding activity (Brandley et al., 1993; Chen et al., 1994). Wong and co-workers designed sLe^x mimetics possessing the minimal functional groups (i.e., hydroxyl and anionic groups) required for E-selectin binding activity (Uchiyama et al., 1995). In an alternative approach to this problem, Rao et al. (1994) used sLe^x-derived pharmacophores as the basis for a computer-aided structure search, from which emerged a noncarbohydrate natural product with selectin binding activity.

A heretofore unexplored source for new leads in the design of selectin antagonists lies in the characterization of the biological macromolecules that support selectin adhesion *in vivo*. Accumulating evidence suggests that each selectin binds to a discrete glycoprotein ligand or set of ligands on cognate cells and that features unique to these ligands confer selectin specificity (Varki, 1994; Rosen & Bertozzi, 1994; McEver et al., 1995). Three HEV-associated ligands for L-selectin have been identified as mucin-like glycoproteins: GlyCAM-1 (Lasky et al., 1992), CD34 (Baumhueter et al., 1993), and MAdCAM-1 (Briskin et al., 1993). Discrete physiological ligands for P-selectin (PSGL-1) (Norgard et al., 1993; Sako et al., 1993) and E-selectin (ESL-1) (Steegmaier et al., 1995) on myeloid cells have also been described. Presumably, these glycoproteins present the proper carbohydrate-based epitopes for optimal selectin binding *in vivo*.

GlyCAM-1, the best characterized among the three known L-selectin ligands, is a secreted component that is present in serum and may function as a signaling molecule that acts through L-selectin. GlyCAM-1 is modified with dense clusters of sulfated and sialylated O-linked oligosaccharide chains. Sulfation and sialylation are crucial for high-avidity binding to L-selectin (Imai et al., 1993; Crommie & Rosen, 1995). We have recently performed a detailed structural analysis of these sulfated oligosaccharides, revealing two novel structures: Siaα2,3(6-SO_3)Galβ1,4(Fucα1,3)GlcNAc (6'-sulfo sLe^x, **2**, Figure 1) and Siaα2,3Galβ1,4(Fucα1,3)-(6-SO_3)GlcNAc (6-sulfo sLe^x, **3**) (Hemmerich et al., 1994, 1995; Hemmerich & Rosen, 1994). 6',6-Disulfo sLe^x (**4**) may also be a determinant on GlyCAM-1, and direct evidence for this structure is currently being pursued. On the basis of the known sulfation requirement for L-selectin binding to GlyCAM-1, it has been proposed that the sulfate esters in structures **2**−**4** enhance the affinity of sLe^x for L-selectin. Indeed, Jacob and co-workers have recently reported that 6-sulfo-sLe^x (**3**) shows enhanced *in vitro* L-selectin binding activity compared to sLe^x (Scudder et al., 1994).

These observations suggested a strategy for the generation of simple small molecules with L-selectin binding activity. We reasoned that the incorporation of sulfate esters at key positions on a core disaccharide might impart significant binding activity for L-selectin without need for sialylation or fucosylation. Here we report the synthesis of a series of sulfated lactose derivatives and their relative L-selectin binding activities. The compounds were assayed using a novel inhibition ELISA based on the binding of L-selectin to GlyCAM-1, which enables a direct comparison of the L-selectin-binding activities of different compounds. The use of a physiological ligand as a binding substrate for L-selectin ensures that only functionally relevant interactions are probed. We found that lactose 6',6-disulfate, which is sulfated at the positions suggested by the GlyCAM-1 oligosaccharides, is more potent than lactose 3'-sulfate, 6'-sulfate, 3',6'-disulfate, sLe^x-β-OMe, and sLe^a. Lactose 6',6-disulfate can be readily prepared without any of the difficulties associated with sialylated and fucosylated oligosaccharides.

EXPERIMENTAL PROCEDURES

General. *p*-Methoxybenzaldehyde dimethylacetal and dibutyltin oxide were from Fluka and were used without further purification. *p*-Nitrophenylphosphate and lactose were from Sigma. Benzoyl chloride was distilled onto 3 Å molecular sieves/K_2CO_3 prior to use, and anhydrous solvents were obtained as Sure-Seal reagents (Aldrich) and stored over 3 Å molecular sieves. Mouse serum was obtained from Pel-

Freez. Microtiter plates were from Dynatech (Immulon 2). Biotinylated-goat anti-human Fc ($F(ab')_2$) and streptavidin-alkaline phosphatase were from Caltag. The L-selectin–IgG chimera (Watson et al., 1990) and the anti-GlyCAM-1 polyclonal antibody CAM02 (Lasky et al., 1992) were generated as previously described. All other reagents were obtained from Aldrich and were used without further purification. ^{1}H NMR spectra were recorded at 300 MHz, and ^{13}C NMR spectra were recorded at 75 MHz on a General Electric NMR Spectrometer. NMR data are tabulated as chemical shifts in ppm (δ) downfield of tetramethylsilane, followed by multiplicity (s, singlet; d, doublet; t, triplet; app, apparent; br, broad), integration, and coupling constant(s) (J) in Hz. Liquid secondary ion mass spectra (LSIMS) were obtained on a VG-70SE spectrometer at the UCSF Mass Spectrometry Facility.

*6′-O-tert-Butyldimethylsilyl-1-β-O-allyl Lactose (**6**).* A solution of 0.69 g (1.8 mmol) of 1-β-*O*-allyl lactose (**5**) (Sato et al., 1988) and 0.5 g (2.0 mmol) of dibutyltin oxide in 35 mL of anhydrous methanol was heated at reflux for 9 h in the presence of 3 Å molecular sieves. The solution was cooled to room temperature, filtered through Celite and concentrated. The resulting residue was suspended in anhydrous THF under an argon atmosphere, and 520 μL (2.0 mmol) of *tert*-butyldimethylsilyl chloride was added via syringe. After 24 h, the solution was concentrated and purified by silica gel chromatography eluting with 20:1 CH_2Cl_2/methanol to afford 0.79 g (71%) of a white solid. Only one regioisomer was detected by NMR analysis; ^{1}H NMR (CD_3OD) δ 0.98 (s, 9 H), 3.18 (app t, 1 H, J = 8.4), 3.35 (m, 1 H), 3.40–3.53 (m, 4 H), 3.59 (app t, 1 H, J = 6.5), 3.75–3.83 (m, 4 H), 4.06 (dd, 1 H, J = 12.8, 5.9), 4.23–4.31 (m, 4 H), 5.08 (dd, 1 H, J = 10.3, 1.0), 5.24 (dd, 1 H, J = 17.2, 1.0), 5.87 (m, 1 H), 7.31–7.35 (m, 6 H), 7.60–7.65 (m, 4 H); ^{13}C NMR δ 19.96, 27.34, 61.73, 63.53, 69.66, 71.08, 72.42, 74.77, 76.12, 76.34, 76.59, 80.22, 103.18, 104.92, 117.46, 128.83, 128.89, 130.93, 134.32, 134.38, 135.61, 136.66. The position of silylation (6′) was assigned by proton decoupling analysis of the corresponding peracetylated derivative.

*6′-O-tert-Butyldimethylsilyl-3′,4′-O-p-methoxybenzylidene-1-β-O-allyl Lactose (**7**).* A solution of 0.25 g (0.4 mmol) of compound **6**, 205 μL (1.2 mmol) of *p*-methoxybenzaldehyde dimethylacetal, and a catalytic amount of *p*-toluenesulfonic acid in 7 mL of anhydrous DMF was stirred over 4 Å sieves. The solution was heated to 50 °C under aspirator pressure for 9 h, cooled to room temperature, and neutralized with triethylamine. The solution was concentrated and the product purified by silica gel chromatography eluting with 60:1 CH_2Cl_2/methanol to afford 0.21 g (71%) of a colorless syrup. The product was obtained as an inseparable mixture of diastereomers (3:1 ratio by ^{1}H NMR analysis), which was carried on to the next step without further characterization.

*6′-O-tert-Butyldimethylsilyl-3′,4′-O-p-methoxybenzylidene-2′,2,3,6-tetra-O-benzoyl-1-β-O-allyl Lactose (**8**).* Compound **7** (0.63 g, 0.85 mmol, diastereomeric mixture) was dissolved in 10 mL of anhydrous CH_2Cl_2 under an argon atmosphere. Diisopropylethylamine (1.47 mL, 8.5 mmol) and pyridine (1.37 mL, 17 mmol) were added via syringe, and the solution was cooled to 0 °C. Benzoyl chloride (0.98 mL, 8.5 mmol) was added dropwise over a 10-min period, and the solution was warmed to room temperature overnight. The reaction was quenched with a saturated solution of $NaHCO_3$ and then concentrated. The residue was dissolved in ether, washed with water, 0.2 M HCl, saturated $NaHCO_3$ and brine, and dried over $MgSO_4$. The product was purified by silica gel chromatography eluting with 5:1 hexanes/ethyl acetate to give 0.99 g (100%) of a white foamy solid. ^{1}H NMR analysis indicated a mixture of diastereomeric tetrabenzoates, which was taken on to the next step without further characterization.

*2′,2,3,6-Tetra-O-benzoyl-1-β-O-allyl Lactose (**9**).* A solution of anhydrous HCl in methanol was generated by the addition of 0.4 mL of acetyl chloride to 10 mL of anhydrous methanol. This solution (6 mL) was added to a solution of 0.2 g (0.17 mmol) of compound **8** in 6 mL of anhydrous ether. The reaction was stirred at room temperature for 24 h and then diluted with ether and washed with water, saturated $NaHCO_3$ and brine. After drying over $MgSO_4$, the solution was concentrated and the product purified by silica gel chromatography eluting with 50:1 CH_2Cl_2/methanol to afford 91 mg (67%) of a white solid; ^{1}H NMR (50:1 $CDCl_3$/CD_3OD) δ 2.98–3.04 (m, 1 H), 3.07–3.13 (m, 1 H), 3.20 (app t, 1 H, J = 6.0), 3.58 (dd, 1 H, J = 10.0, 3.4), 3.74–3.80 (m, 2 H), 3.98–4.12 (m, 2 H), 4.21 (dd, 1 H, J = 13.2, 4.7), 4.44 (dd, 1 H, J = 12.0, 4.9), 4.53 (m, 2 H), 4.71 (d, 1 H, J = 7.8), 5.03 (app d, 1 H, J = 10.4), 5.11 (app d, 1 H, J = 17.9), 5.23 (dd, 1 H, J = 9.7, 8.3), 5.37 (app t, 1 H, J = 9.5), 5.62–5.75 (m, 2 H), 7.26–7.61 (m, 12 H), 7.88–8.02 (m, 8 H).

*6′-Sulfo-1-β-O-allyl lactose (**10**).* A solution of compound **9** (20.7 mg, 0.026 mmol) and sulfur trioxide–trimethylamine complex (SO_3–NMe_3, 22 mg, 0.052 mmol) in 1 mL of anhydrous DMF was stirred at room temperature. Selective sulfation of the primary 6′-OH was monitored by anion exchange HPLC (Rainin Hydropore-5-AX) eluting with a linear gradient of 90% methanol to 1 M NH_4OAc in 90% methanol, with detection at 280 nm. After 2.5 h, compound **9** was converted to the corresponding monosulfate along with a small amount of disulfate. The reaction was stopped by addition of 250 μL of methanol, and the products were purified on a column of Sephadex LH-20 (2.8 × 100 cm) eluting with DMF. The fractions containing the product were pooled and concentrated. The residue was dissolved in 5 mL of methanol, combined with 5 mL of aqueous NH_4OH (10 M), and heated at 55 °C for 12 h. The crude deprotected product was purified by anion exchange chromatography on DEAE-Sepharose eluting with a linear gradient of 2 mM to 1 M pyr-OAc (pH 5). Fractions containing the monosulfate (eluting at 150–200 mM pyr-OAc) were pooled and lyophilized twice from water. The pyridinium salt was converted to the sodium salt by passage down a column of BioRad AG50W-X4 resin (Na^+ form), and the eluate was lyophilized to afford 7 mg (55%) of the desired 6′-monosulfate (only one regioisomer was observed by ^{1}H NMR); ^{1}H NMR (D_2O) δ 3.35 (m, 1 H), 3.52–3.71 (m, 5 H), 3.77–3.82 (m, 1 H), 3.96–4.00 (m, 3 H), 4.20-4.26 (m, 3 H), 4.39 (dd, 1 H, J = 12.7, 5.7), 4.47 (d, 1 H, J = 7.7), 4.54 (d, 1 H, J = 8.0), 5.29 (app d, 1 H, J = 10.4), 5.38 (app d, 1 H, J = 17.0), 5.97 (m, 1 H). MS (LSIMS, negative mode): calcd for $C_{15}H_{26}O_{14}S$, 462; found, 461 (M − H).

*3′,6′-Disulfo-1-β-O-allyl Lactose (**11**).* A solution of compound **9** (37 mg, 0.046 mmol) and SO_3–NMe_3 (19 mg, 0.14 mmol) was heated at 37 °C for 5 h. The progress of sulfation was monitored by anion exchange HPLC as described for compound **10**, and the reaction was stopped

by addition of methanol after approximately 50% conversion to the disulfate, the remainder being largely monosulfate with a small amount of trisulfate. Purification by Sephadex LH-20 chromatography and deprotection with methanolic NH_4OH were performed as described for compound **10**. The mixture of deprotected mono- and disulfates was separated by anion exchange chromatography on DEAE-Sepharose eluting with a linear gradient of 2 mM to 1 M pyr-OAc (pH 5). The monosulfate eluted at 150−200 mM pyr-OAc, and the disulfate eluted at 400−500 mM pyr-OAc. The products were converted to their Na^+ salts as described for compound **10**. The monosulfated product (8 mg, 35% yield) was identical to compound **10**, with no other monosulfate regioisomers detectable by ^1H NMR. The disulfated product **11** (17 mg, 62%) was also a single isomer by ^1H NMR. Sulfation at the 3′- and 6′-positions was assigned based on the downfield shifts of the adjacent protons in the ^1H NMR spectrum; ^1H NMR (D_2O) δ 3.34 (m, 1 H), 3.62−3.71 (m, 4 H), 3.80 (dd, 1 H, J = 12.1, 4.4), 3.96−4.04 (m, 2 H), 4.20−4.25 (m, 3 H), 4.33−4.42 (m, 3 H), 4.53 (d, 1 H, J = 8.0), 4.58 (d, 1 H, J = 7.9), 5.27 (app d, 1 H, J = 10.4), 5.37 (app d, 1 H, J = 16.2), 5.97 (m, 1 H); MS (LSIMS, negative mode): calcd for $C_{15}H_{26}O_{17}S_2$, 542; found, 563 (M − 2H + Na).

6′,6-Di-O-tert-butyldimethylsilyl-1-β-O-allyl Lactose (12). To a solution of 1-β-*O*-allyl lactose (**5**) (0.5 g, 1.3 mmol) in 4 mL of anhydrous DMF were added diisopropylethylamine (905 μL, 5.2 mmol), DMAP (32 mg, 0.26 mmol), *tert*-butyldimethylsilyl chloride (750 μL, 2.9 mmol), and 3 Å molecular sieves. The solution was stirred for 10 h at room temperature, quenched with methanol, and concentrated. The residue was dissolved in CH_2Cl_2 and washed three times with water, once with 0.1 M HCl, and once with saturated $NaHCO_3$. The organic layer was dried and the product was purified by silica gel chromatography eluting with 4:1 ethyl acetate/hexanes to give 0.46 g (43%) of **12**; ^1H NMR ($CDCl_3$) δ 1.05 (s, 18 H), 2.57 (s, 1 H), 2.73 (s, 1 H), 2.85 (s, 1 H), 2.87 (s, 1 H), 2.95 (s, 1 H), 3.35−3.69 (m, 6 H), 3.83−4.11 (m, 6 H), 4.24−4.40 (m, 4 H), 5.21 (app d, 1 H, J = 10.1), 5.31 (app d, 1 H, J = 17.1), 5.95 (m, 1 H), 7.50 (m, 12 H), 7.66−7.74 (m, 8 H); ^{13}C NMR δ 19.12, 26.60, 26.78, 62.25, 63.07, 68.11, 69.87, 71.76, 73.54, 73.68, 74.60, 74.87, 74.95, 80.76, 101.12, 103.73, 117.85, 127.59, 127.74, 127.87, 129.76, 129.97, 133.83, 135.49, 135.56, 135.83; MS (LSIMS, positive mode): calcd for $C_{47}H_{62}O_{11}Si_2$, 858.4; found, 881.4 (M + Na).

6′,6-Di-O-tert-butyldimethylsilyl-3′,4′-O-p-methoxybenzylidene-1-β-O-allyl lactose (13). A solution of compound **12** (0.23 g, 0.28 mmol), *p*-methoxybenzaldehyde dimethylacetal (0.14 mL, 0.84 mmol), and a catalytic amount of *p*-toluenesulfonic acid in 1 mL of anhydrous DMF was heated at 55 °C over 4 Å molecular sieves. After 7 h, the solution was neutralized with diisopropylethylamine, diluted with ether, and washed with water and brine. After drying over $MgSO_4$, the product was purified by silica gel chromatography eluting with 2:1 hexanes/ethyl acetate to give 0.12 g (44%) of a 5:1 mixture of diastereomers (determined by ^1H NMR analysis). The mixture was carried on to the next step without further purification.

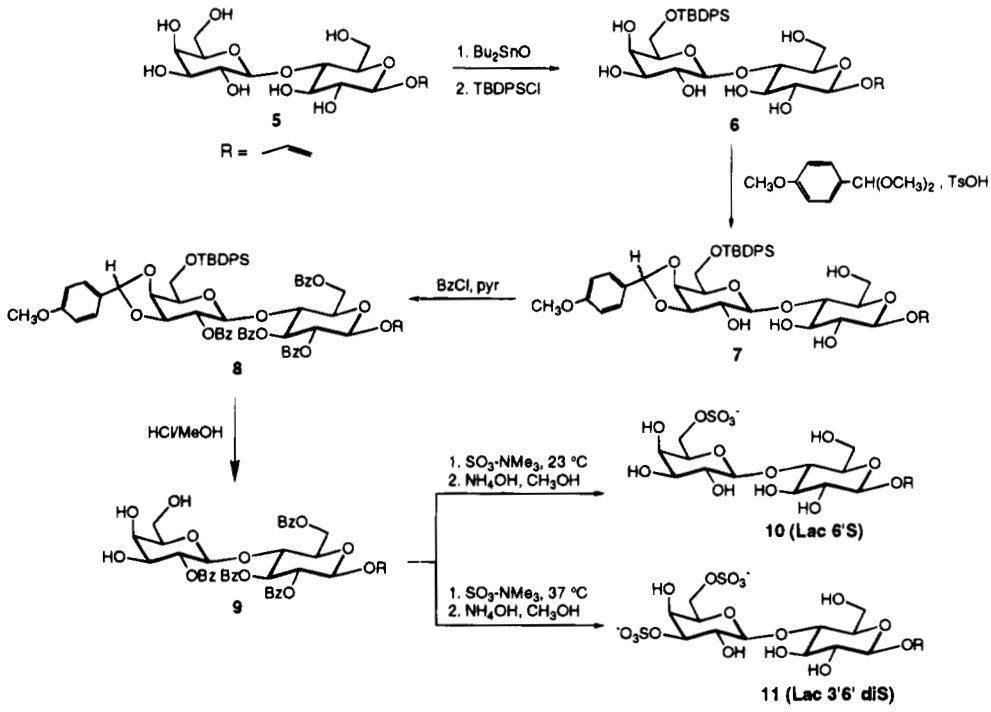
6′,6-Di-O-tert-butyldimethylsilyl-3′,4′-O-p-methoxybenzylidene-2′,2,3-tri-O-benzoyl-1-β-O-allyl Lactose (14). Benzoylation of **13** was accomplished as described for compound **8**, with 0.12 g (0.12 mmol) of **13**, 127 μL of diisopropylethylamine (0.73 mmol), 116 μL of pyridine (1.44 mmol), and 85 μL of benzoyl chloride (0.73 mmol) in 2 mL of anhydrous CH_2Cl_2. Purification by silica gel chromatography eluting with 5:1 hexanes/ethyl acetate gave 154 mg (100%) of a white solid. The mixture of diastereomeric tribenzoates was characterized by ^1H NMR and carried on to the next step without further separation.

2′,2,3-Tri-O-benzoyl-1-β-O-allyl Lactose (15). Methanolysis was accomplished as described for compound **9**, with 154 mg of compound **14** (0.119 mmol). Purification by silica gel chromatography eluting with 40:1 chloroform/methanol gave 40 mg (48%) of a white solid; ^1H NMR ($CDCl_3$) δ 2.03 (br s, 2 H), 2.27 (br s, 2 H), 3.08−3.14 (m, 1 H), 3.20−3.25 (m, 1 H), 3.33−3.54 (m, 3 H), 3.69−3.87 (m, 3H), 4.04 (dd, 1 H, J = 13.2, 6.1), 4.17 (app t, 1 H, J = 9.3), 4.26 (dd, 1 H, J = 13.2, 4.7), 4.67−4.71 (m, 2 H), 5.08 (app d, 1 H, J = 10.4), 5.16 (app d, 1 H, J = 17.3), 5.26 (app t, 1 H, J = 8.7), 5.38 (app t, 1 H, J = 9.4), 5.60 (app t, 1 H, J = 9.4), 5.71 (m, 1 H), 7.33−7.60 (m, 9 H), 7.90 (d, 2 H, J = 7.7), 7.98 (d, 2 H, J = 7.7), 8.08 (d, 2 H, J = 7.7); ^{13}C NMR δ 60.36, 61.77, 69.57, 70.17, 71.73, 72.75, 73.67, 73.77, 74.22, 75.06, 75.34, 76.91, 99.61, 101.07, 117.71, 128.32, 128.48, 128.58, 128.71, 129.26, 129.49, 129.57, 129.73, 129.86, 133.17, 133.27, 133.38, 165.79, 166.27; MS (LSIMS, positive mode): calcd for $C_{36}H_{38}O_{14}$, 694.2; found, 695.3 (M + H).

6′,6-Disulfo-1-β-O-allyl Lactose (16) and 3′,6′,6-Trisulfo-1-β-O-allyl Lactose (17). A solution of 20 mg (0.029 mmol) of compound **15** and 24 mg (0.172 mmol) of SO_3−NMe_3 in 1 mL of anhydrous DMF was stirred at 37 °C over 4 Å molecular sieves. The progress of sulfation was monitored as described for compounds **10** and **11**. After 20 h, HPLC analysis indicated that compound **15** had been converted into a ≈ 1:1 mixture of di- and trisulfates along with a small amount of tetrasulfate. Purification of the crude products on a column of Sephadex LH-20 and removal of the benzoate esters were accomplished as described for compounds **10** and **11**. Chromatography on DEAE-Sepharose eluting with a linear gradient of 2 mM to 2 M pyr-OAc (pH 5) afforded separation of the disulfated (eluting at 0.5−0.6 M pyr-OAc) and trisulfated (eluting at 1.25−1.6 M pyr-OAc) derivatives. The pyridinium salts were converted to sodium salts yielding 10.4 mg (60%) of compound **16** and 5.6 mg (28%) of compound **17**. Only one regioisomer was observed for each product by ^1H NMR analysis: Compound **16**: ^1H NMR (D_2O) δ 3.39 (app t, 1 H, J = 8.4), 3.56 (app t, 1 H, J = 9.8), 3.64−3.74 (m, 3 H), 3.83 (m, 1 H), 4.00 (m, 2 H), 4.22−4.33 (m, 4 H), 4.38−4.44 (m, 2 H), 4.54 (d, 1 H, J = 8.0), 4.57 (d, 1 H, J = 8.1), 5.30 (app d, 1 H, J = 10.4), 5.40 (app d, 1 H, J = 17.1), 6.01 (m, 1 H); MS (LSIMS, negative mode) calcd for $C_{15}H_{26}O_{17}S_2$: 542; found 563 (M-2H+Na). Compound **17**: ^1H NMR (D_2O) δ 3.40 (app t, 1 H, J = 8.4), 3.66−3.76 (m, 3 H), 3.86 (m, 1 H), 4.07 (app t, 1 H, J = 6.1), 4.24−4.32 (m, 4 H), 4.38−4.48 (m, 4 H), 4.58 (d, 1 H, J = 8.0), 4.65 (d, 1 H, J = 7.8), 5.31 (app d, 1 H, J = 10.4), 5.41 (app d, 1 H, J = 17.2), 6.01 (m, 1 H); MS (LSIMS, negative mode): calcd for $C_{15}H_{26}O_{20}S_3$, 622.5; found, 643 (M − 2H + Na).

3′-Sulfo-1-β-O-allyl Lactose (18). A solution of compound **5** (50 mg, 0.13 mmol) and dibutyltin oxide (36 mg, 0.14 mmol) in 2 mL of anhydrous methanol was heated at reflux with 3 Å molecular sieves for 8 h. The solution was filtered through Celite and the filtrate concentrated. The

Scheme 1



residue was suspended in 2.5 mL of anhydrous acetonitrile and SO_3−NMe_3 (18 mg, 0.13 mmol) was added. The solution was stirred at room temperature under an argon atmosphere for 72 h. The solution was diluted with water (10 mL), filtered through Celite, and concentrated. Purification on DEAE-Sepharose eluting with a linear gradient of 2 mM to 1 M pyr-OAc (pH 5) gave the monosulfate, which eluted at 100−150 mM pyr-OAc. Conversion to the sodium salt followed by lyophilization afforded 37 mg (59%) of the desired product (one regioisomer by ^{1}H NMR analysis); ^{1}H NMR (D_2O) δ 3.37 (app t, 1 H, J = *8.4*), 3.64−3.88 (m, 8 H), 4.02 (m, 1 H), 4.27 (dd, 1 H, J = *12.5, 6.2*), 4.32−4.46 (m, 3 H), 4.57 (d, 1 H, J = *8.1*), 4.60 (d, 1 H, J = *7.9*), 5.32 (app d, 1 H, J = *10.3*), 5.41 (app d, 1 H, J = *16.9*), 6.01 (m, 1 H); ^{13}C NMR δ 58.03, 58.82, 64.77, 67.01, 68.57, 70.74, 72.33, 72.64, 72.82, 76.35, 77.95, 99.00, 100.47, 116.63, 131.27; MS (LSIMS, negative mode): calcd for $C_{15}H_{26}O_{14}S$, 462; found, 483 (M − 2H + Na).

Preparation of GlyCAM-1 for Inhibition Binding Assay. Mouse serum (100 mL) was extracted with four volumes of 2:1 chloroform/methanol, and the layers were separated by centrifugation at 2000*g*. The aqueous layer (top) was separated from the organic and precipitated protein layers, concentrated to 50 mL by boiling, and dialyzed against 2 × 1 L of Dulbecco's PBS at 4 °C. This preparation was then diluted to 100 mL with PBS and used as a source of semipure GlyCAM-1 for the ELISA described below. A detailed description of the purification and quantification of GlyCAM-1 from mouse serum will be reported elsewhere.

L-Selectin−GlyCAM-1 Inhibition ELISA. A 96-well microtiter plate was coated overnight at 4 °C with a polyclonal antibody specific for the peptide core of GlyCAM-1 (CAM02, 100 μL/well, 1.6−1.9 μg/mL in PBS/0.1% NaN_3). The plate was washed with PBS/0.1% Tween 20 and blocked with a 3% solution of BSA in PBS (200 μL/well) for 2 h at room temperature. GlyCAM-1 was captured on the wells by incubation with 100 μL/well of the crude GlyCAM-1 preparation described above for 1 h at room temperature. The plate was then washed with PBS/Tween in preparation for incubation with L-selectin−IgG and soluble inhibitors.

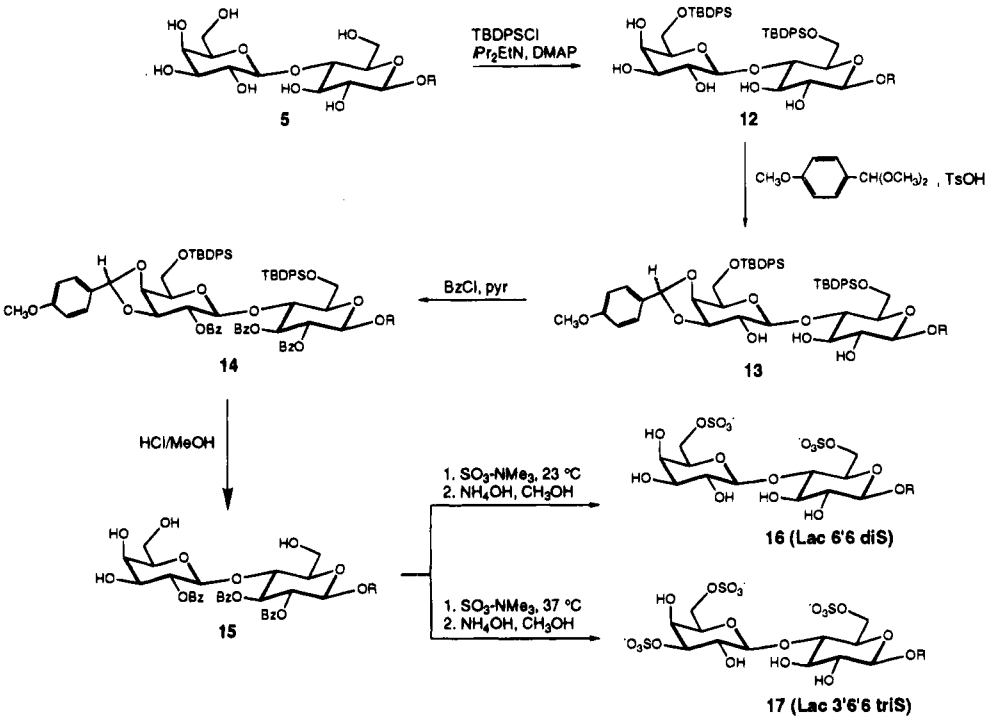
L-Selectin−IgG (2 μg/mL) was preincubated with biotinylated goat anti-human Fc ($F(ab')_2$, 2/1000 dilution) and streptavidin−alkaline phosphatase conjugate (2/1000 dilution) in PBS with 0.2% BSA for 25 min at room temperature. Normal rabbit serum (5% v/v) was added, and the solution was incubated for an additional 5 min. This solution (70 μL) was combined with 70 μL of a solution of soluble inhibitor in PBS and then incubated for 30 min at 4 °C. The mixture (100 μL) was transferred to the GlyCAM-1-coated wells and incubated for 30 min at room temperature. Finally, the plate was washed quickly with PBS/Tween, and the presence of bound L-selectin−IgG was detected by the addition of phosphatase substrate (*p*-nitrophenyl phosphate, 1 mg/mL in diethanolamine/$MgCl_2$, 100 μL/well) followed by optical density determination at 405 nm (OD 405). Details on the development of this assay will be reported elsewhere.

RESULTS AND DISCUSSION

We examined a series of lactose derivatives with sulfate esters on key positions suggested by the GlyCAM-1 oligosaccharides (6′- and 6-positions) and by previous work (3′-position) (Brandley et al., 1993; Chen et al., 1994). Lactose β-allyl glycoside (Galβ1,4Glcβ1-OAll, **5**, Scheme 1) (Sato et al., 1988) was chosen as a readily available core disaccharide on the basis of the observation that replacement of GlcNAc by glucose in sLe^x has no detrimental effect on selectin binding activity (Nelson et al., 1993). In addition, the allyl group allows facile conjugation to lipids, proteins, or other scaffolds.

An efficient synthetic strategy was designed to provide several sulfated disaccharides from a minimal set of precursor molecules. Lactose 6′-sulfate (lac 6′-S, **10**) and lactose 3′,6′-

Scheme 2



disulfate (lac 3′,6′-diS, **11**) were synthesized as depicted in Scheme 1. Selective protection of the 6′-hydroxyl group of **5** was achieved by stannylene-mediated silylation with *tert*-butyldiphenylsilyl chloride (TBDPSCl), as described by Leigh and co-workers, to afford compound **6** (Glen et al., 1993). The 3′- and 4′-hydroxyl groups were protected as the *p*-methoxybenzylidene acetal (**7**), and the remaining hydroxyl groups were protected as benzoate esters (**8**). Removal of the TBDPS and benzylidene groups by methanolysis (Nashed & Glaudemans, 1987) gave compound **9** in which the 3′-, 4′-, and 6′-hydroxyl groups are available for sulfation. Treatment of compound **9** with SO_3−NMe_3 at room temperature resulted in sulfation at the primary hydroxyl group (6′) with good selectivity, affording, after deprotection, lac 6′-S (**10**). When the temperature of the sulfation reaction was increased to 37 °C, disulfate **11** was formed with good selectivity. In this reaction, the equatorial 3′-hydroxyl group is sulfated preferentially over the less reactive axial 4′-hydroxyl group.

A related strategy was applied to the synthesis of lactose 6′,6-disulfate (lac 6′,6-diS, **16**) and lactose 3′,6′,6-trisulfate (lac 3′,6′,6-triS, **17**) (Scheme 2). Selective protection of the 6′- and 6-hydroxyl groups of **5** with TBDPSCl yielded compound **12**. Selective protection of the 3′- and 4′-hydroxyls (**13**) followed by benzoylation of the remaining hydroxyl groups provided compound **14**, which was subjected to methanolysis to give the selectively protected derivative **15**. Sulfation of **15** at room temperature followed by deprotection gave lac 6′,6-diS (**16**) with good selectivity, while sulfation at 37 °C and deprotection gave lac 3′,6′,6-triS (**17**). Finally, lactose 3′-sulfate (lac 3′-S, **18**) was synthesized in two steps from **5** by stannylene-directed sulfation with SO_3−NMe_3 (Guilbert et al., 1994; Lubineau & Lemoine, 1994).

Compounds **10**, **11**, and **16**−**18** were tested as inhibitors of the binding of a recombinant L-selectin−IgG chimera (Watson et al., 1990) to immobilized GlyCAM-1 in an

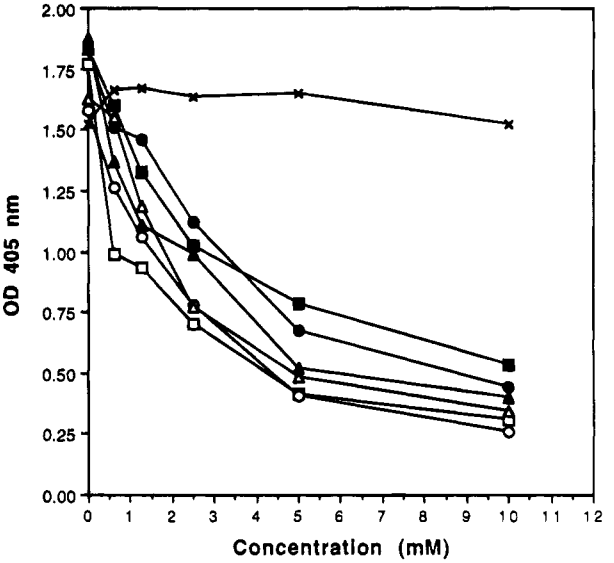


FIGURE 2: Inhibition of L-selectin−IgG binding to GlyCAM-1 by sulfated disaccharides. Lac 6′-S (■), lac 3′-S (●), lac 3′,6′-diS (▲), lac 6′,6-diS (□), lac 3′,6′,6-triS (○), M6P (△) and lactose (×) were assayed for inhibitory activity against the binding of L-selectin−IgG to immobilized GlyCAM-1 in an ELISA. The concentrations required for 50% inhibition (IC_{50}) were calculated using a value for 100% inhibition derived from the amount of bound L-selectin−IgG in the presence of 20 mM M6P.

ELISA. The synthetic compounds were compared to the known L-selectin-binding carbohydrates sLe^x-β-OMe, sLe^a, and mannose 6-phosphate (M6P) (Imai et al., 1992). A set of binding curves for the synthetic compounds and M6P is shown in Figure 2. The concentrations required for 50% inhibition (IC_{50}) were all found to be in the low millimolar range (0.8−4 mM), yet significant differences in potency were observed (*vida infra*). In order to compare data from several experiments, IC_{50} values were normalized for each experiment, with lac 3′,6′,6-triS being assigned a value of 1.0. Figure 3 shows the mean relative IC_{50} values obtained

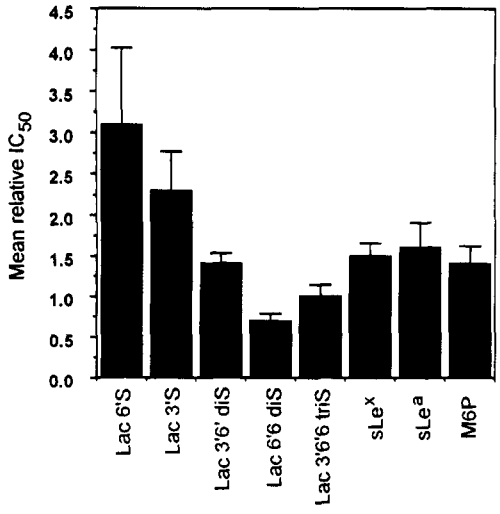


FIGURE 3: Mean relative IC_{50} values for synthetic lactose sulfates and comparison compounds. In order to compare data from several experiments, IC_{50} values obtained in each experiment were normalized, with lac 3′,6′,6-triS being assigned a value of 1.0. Mean relative IC_{50} values were calculated for each compound, and error bars represent the standard error of the mean. The error associated with the mean relative IC_{50} of lac 3′,6′,6-triS was calculated as follows. IC_{50} values for lac 3′,6′,6-triS were normalized to those for lac 6′,6-diS in each experiment. A standard error of the mean was calculated and then scaled to the assigned mean relative IC_{50} value for lac 3′,6′,6-triS of 1.0. The significance of differences in mean relative IC_{50} values were evaluated using the two-tailed Student *t*-test: lac 6′-S vs lac 3′,6′-diS ($p < 0.14$); lac 3′-S vs lac 3′,6′-diS ($p < 0.15$); lac 3′,6′-diS vs lac 6′,6-diS ($p < 0.0018$); lac 6′,6-diS vs sLe^x ($p < 0.0048$); lac 6′,6-diS vs sLe^a ($p < 0.0073$).

from several experiments. The statistical significance of differences in relative IC_{50} were evaluated by application of the two-tailed Student *t*-test to pairs of data sets.

The parent disaccharide lactose showed no inhibitory activity at a concentration of 25 mM, and the IC_{50} value was estimated to be >100 mM (not shown). However, the addition of one sulfate ester at either the 6′- (lac 6′-S, **10**) or 3′-position (lac 3′-S, **18**) confers weak but measurable binding activity. The activity of lac 3′-S is reminiscent of the previously observed interaction of L-selectin with sulfatides (Imai et al., 1990) and 3′-sulfo Lewis x/a oligosaccharides (Brandley et al., 1993; Green et al., 1992), both of which contain 3′-sulfated galactosides. The relative IC_{50} values for lac 6′-S and lac 3′-S were 2.2- and 1.6-fold higher, respectively, than that of lac 3′,6′-diS (**11**). These differences did not achieve a statistical level of significance ($p < 0.14$ and 0.15, respectively) with the relatively low number of replicate determinations (4 for lac 3′,6′-diS, 5 for lac 6′-S and lac 3′-S). Nonetheless, the observed trend is strongly suggestive of an enhancement in binding of the disulfate vs the monosulfates. Suzuki et al. (1993) have reported that L-selectin binds lipid-linked 3′- or 6′-monosulfated, and 3′,6′-disulfated galactosides in a direct binding assay but relative binding potencies were difficult to ascertain due to the potential variation in packing density of different immobilized lipid substrates, an intrinsic limitation of this type of assay.

When sulfate esters were simultaneously present at the 6′- and 6-positions, the resulting derivative (lac 6′,6-diS, **16**) was 3−5 times more potent than the monosulfates ($p < 0.029$ for lac 6′-S; $p < 0.011$ for lac 3′-S) and 2-fold more potent than lac 3′,6′-diS ($p < 0.0018$). Although lac 6′,6-diS and lac 3′,6′-diS are both disulfated, our results establish that the positions of sulfation are important for dictating inhibitory potency. Notably, lac 6′,6-diS was greater than 2-fold more potent than sLe^x-β-OMe ($p < 0.0048$) or sLe^a ($p < 0.0073$), despite the absence of functionalities corresponding to sialic acid and fucose. SLe^x-β-OMe and sLe^a showed inhibitory activities comparable to M6P.

To our surprise, the presence of an additional sulfate ester at the 3′-position, yielding lac 3′,6′,6-triS (**17**), did not enhance the inhibitory potency relative to lac 6′,6-diS. This result contrasts with the apparent trend observed with lac 6′-S and lac 3′,6′-diS. It is possible that cooperative interaction of the 6′- and 6-sulfates with L-selectin precludes a favorable, simultaneous interaction with the 3′-sulfate.

In summary, we have synthesized sulfated disaccharides which inhibit the binding of L-selectin to the physiological ligand GlyCAM-1. Judicious placement of sulfate esters on the lactose core, guided by the oligosaccharide structures on GlyCAM-1, results in the generation of a simple molecule (lac 6′,6-diS) with greater L-selectin inhibitory potency than sLe^x-β-OMe. Unlike sLe^x, lac 6′,6-diS can be synthesized in only a few steps from an inexpensive disaccharide and can be easily generated on a large scale. Also, we have designed the molecule to allow facile coupling to a broad array of different scaffolds. The inhibitory activity of these sulfated lactose derivatives *in vivo* and the kinetics of their interaction with L-selectin (Alon et al., 1995) are of considerable interest for future investigations. Similar leads in the development of P- and E-selectin inhibitors may derive from a detailed biochemical analysis of their respective physiological ligands.

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