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Pitfalls in the Synthesis and Biological Evaluation of Sialyl-Lewis^X Mimetics as Potential Selectin Antagonists

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Abstract: The lower molecular weight analog **12** of sialyl Lewis^X was prepared and effected equal binding affinity to E- and P-selectin compared to the parent tetrasaccharide. If **12** was prepared using acidic ion exchange resins, false positive test were produced, especially binding to P-selectin seemed to be considerably enhanced. Traces of polyanions released from the resins which are difficult to detect by routine analysis were identified to be highly potent selectin inhibitors, probably by their action on a non-carbohydrate binding site.

Introduction

A large number of structurally very different compounds has been prepared and published during the last 4 years which were designed as mimetics to replace the sialyl Lewis^X tetrasaccharide (sLe^X, Figure 1) as potentially novel antiinflammatory drugs.[1] The sLe^X structure found on the termini of glycolipids and glycoproteins is considered to be the minimal determinant recognized by the selectins,[2] a group of cell surface lectins with a distinct carbohydrate recognition domain (CRD). This binding event mediates the initial adhesion of several groups of leukocytes to areas of inflammation. Antagonists of this process are therefore potential agents to prevent leukocyte adhesion and their subsequent migration to the affected tissues in several acute and chronic inflammatory diseases.[3] The variation of functional groups of sLe^X has led to a more detailed knowledge about structure-activity relationships, for instance the acid function present in the sialic acid moiety is essential for binding. However, the lower molecular weight sLe^X mimetics thus far reported[4] exhibited receptor affinities equal or lower than the natural tetrasaccharide ligand in cell-based selectin adhesion

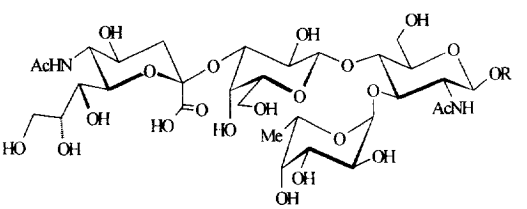

Figure 1: Sialyl Lewis^X

assays. Frequently cell-free assays have been applied to detect binding of inhibitors and of the reference ligand sLe^X at reasonable concentration levels.[5,6] The overall results obtained in different assay systems as well as our efforts to settle some inconsistency in the biological data, have led us to report here some important observations made during our synthesis program[7] directed towards more efficient mimetics of sLe^X. In fact, we found that the same compounds obtained from different work-up procedures apparently had

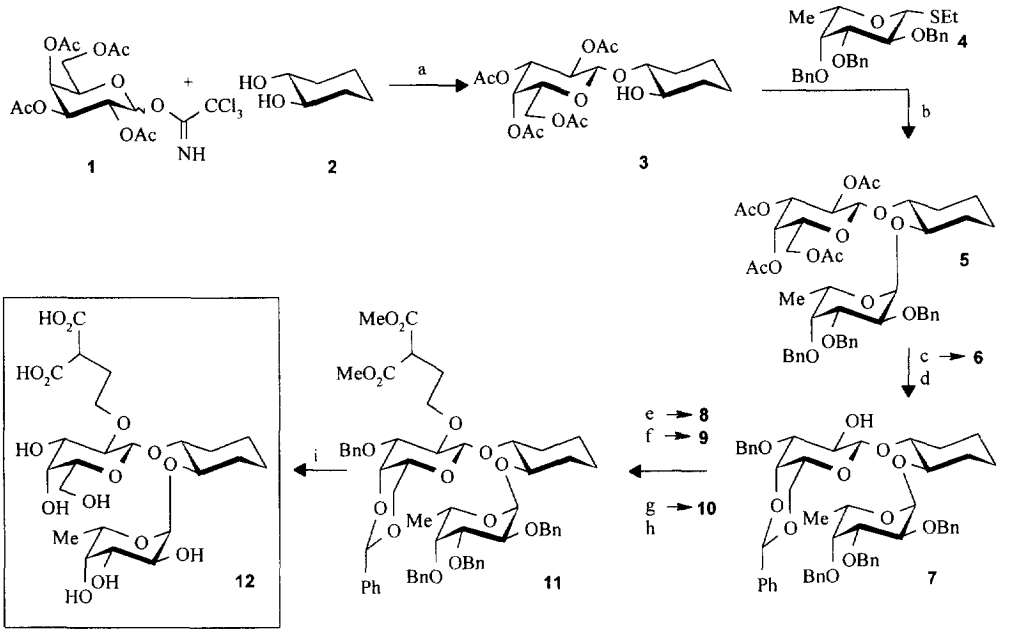

Figure 2: Synthesis of the sLe^X mimetic **12**. a) cat. TMSOTf; b) TBABr/$CuBr_2$, DMF/CH_2Cl_2 (1:1); c) i.: NaOMe/MeOH; **6**); ii.: Bu_2SnO/MeOH reflux, iii: BnBr, TBAI 45 °C; d) benzaldehyde dimethylacetal, p-TosOH (89%); e) NaH, $AllO(CH_2)_2OTos$/DMF (→ **8**); f) *Wilkinson* cat. C_2H_5OH/H_2O 9:1 reflux (→ **9**); g) PPh_3, NEt_3, 1,2-dibromo-tetrachloro-ethane (→ **10**); h) dimethyl malonate, K_2CO_3, dibenzo-18-crown-6, toluene, 95°C, 5 h; i) i: Pd/C, H_2, CH_3OH/dioxane/ AcOH (10:1:0,5), ii: NaOMe (1 M), then acidification with AcOH (method A) or an acidic ion exchange resin (method B); then Biogel P-2 chromatography.

very different inhibitory potencies (IC_{50} values) in selectin inhibition assays. To illustrate this observation, we report here the synthesis of the sLe^X mimetic **12**, which contains the core structure O-α-fucosyl-(1R,2R)-1,2-cyclohexanediol. The fucose and galactose were retained as carbohydrate moieties, and malonic acid attached in the 2-position of the latter *via* a two-carbon spacer was chosen to replace the carboxyclic acid group of N-acetyl-neuraminic acid. Molecular modeling suggested that the cyclohexane system provides the proper arrangement of the critical functional groups in selectin binding.

Preparative Results

The sLe^X mimetic **12** was synthesized from the readily available building blocks **1** and **4** activated as trichloroacetimidates[12] and thioglycosides[13], respectively (Figure 2). Galactosylation of the diol **2** and subsequent fucosylation afforded the Le^X mimetic **5** which was deacetylated by sodium methoxide. Selective benzylation in 3-position of the galactose was successfully achieved *via* the stannylene method and the 4,6-positions were protected with benzaldehyde. After alkylation of the free hydroxyl group with 2-allyloxy-1-tosyloxy-ethane[7e] in dimethylformamide and selective deallylation with the *Wilkinson* catalyst the primary hydroxyl group was replaced by the dimethyl malonate moiety *via* bromination and alkylation reactions. The fully deprotected test compound **12** was obtained after hydrogenolytic cleavage of the benzyl groups and saponification of the two carboxyl groups by aqueous base. The acidification step was performed by two alternative procedures: by the often employed[6] addition of acidic ion exchange resins (method B)[8], or by addition of acetic acid and strict avoidance of any contact with such resins (method A). After chromatography through a column filled with Biogel P-2, for method A as well as for method B, the purified compound **12** was obtained. By this very efficient synthesis sequence (method A), multigram amounts of the sLe^X mimetic **12** were prepared and investigated in biological assays.

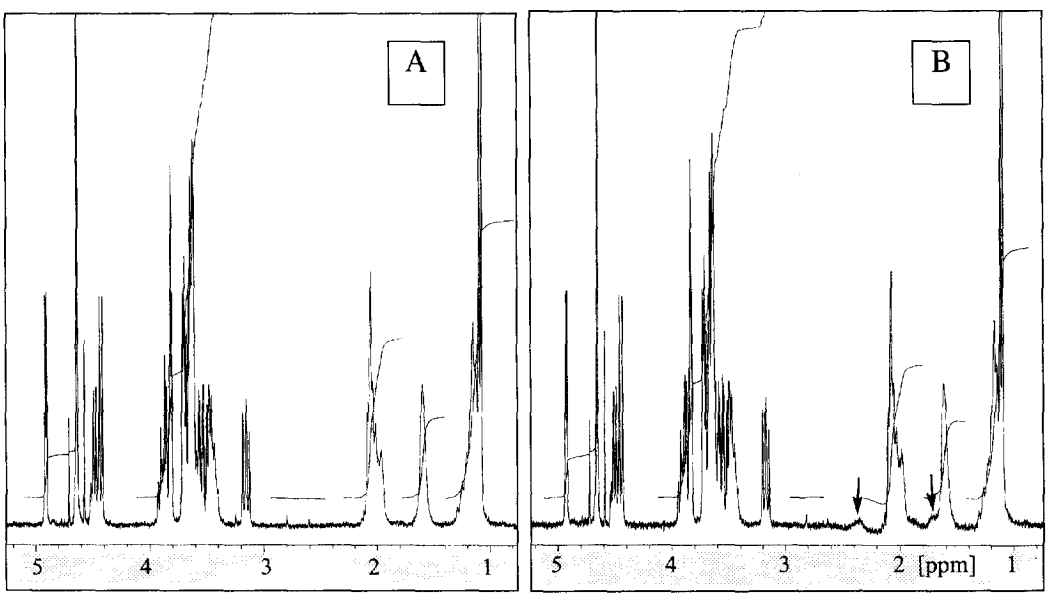

Figure 3: NMR spectra of pure **12** (A) and of a sample of **12** containing approximately 5% of extract derived from IRC-84 resin (B). The indicated signals at 1.7 and 2.0 ppm are assigned to polyacrylates by comparison with the spectrum of the neat extract (not shown).

Biological Results

Compound **12** prepared either by method A or B was then examined for its inhibitory potency towards E- and P-selectin. The bioassay for cell binding to immobilized selectin receptor globulins was performed as previously described.[7d] The concentrations of the inhibitors required to block adhesion of 50% of the HL60 cells are shown in Table 1. Surprisingly, the samples of **12** obtained by either method exhibited very different biological activities, whereas all analytical data routinely determined (NMR; IR, MS, TLC not shown) did not provide any obvious distinction. We then found out, that traces of material released from ion exchange resins under the aqueous neutralization conditions which obviously had not been removed by chromatography (Figure 3) produced micromolar IC_{50}'s, especially in the P-selectin receptor assay.[9] The effect on E-selectin was much weaker in the case of compound **12**, but the results produced by some resins indicate that false positive IC_{50}'s at higher concentration levels (1 mM and above) cannot be ruled out in this system.

Table 1: Inhibition of HL60 cell adhesion to recombinant E- and P-selectin-IgG fusion proteins by mimetic **12**, by extracts from acidic ion exchange resins and by commercially available polyacrylates of different molecular weight.

	E-selectin IC_{50} [μM]	P-selectin IC_{50} [μM]
reference compound[7d]		
sLe^X-1β–$O(CH_2)_6NH_2$	1000[7d]	2000[7d]
sLe^X mimetic		
12 (method A)	800	1600
12 (method B)	1400	7.5
resin extracts		
Amberlite IR 120	190[a]	830[a]
Amberlite IRC-84	>2000[a]	0.3[a]
polyacrylates[b]		
995	>2000	165
4100	>2000	21
62900	>2000	0.009

IC_{50} values are concentrations of inhibitors required to block adhesion of 50% of the cells compared with the negative control.[7d] a) calculated based on a fictitious molecular weight of 500 g/mol. b) Sodium salts of indicated MW (Fluka).

The results reported here may be interpreted in the light of a recently discovered second binding domain in the P-[10] (and L-)[11] selectins for anionic substrates (Figure 4). P-Selectin binding requires the P-selectin

glycoprotein ligand 1(PSGL-1) as well as O-linked sLe^X glycan determinants. The carbohydrate recognition domain (CRD), the short segment comprising sulfated tyrosines (Y) and further acidic residues (E,D) are critical for binding.[10] Both P- and L-selectin bind the anionic polymers fucoidan, dextran sulfate and heparin. The corresponding site distinct from the CRD in L-selectin was recently reported.[11] The binding properties of polyanions derived from ion exchange resins reported here are very likely related to the same selectin binding domain. Traces of this material contaminating test compounds in selectin assays which very likely may produce false positive results cannot be excluded if the compounds had contacted acidic resins during synthesis. Up to 2% of this material, sufficient to produce IC_{50}'s at 1 mM or below, may not be detected spectroscopically by routine analysis (Figure 3).

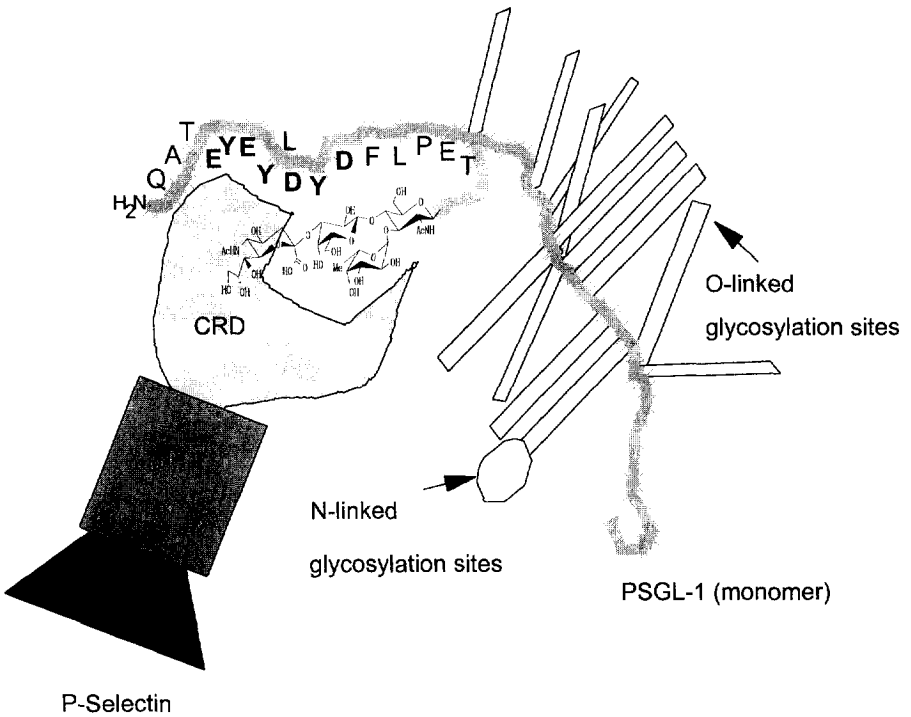

Figure 4: Schematic illustration of the possible binding contacts of P-selectin and its counterligand PSGL-1.

In summary, this study provides the demonstration that simplified mimetics of sLe^X can be prepared, however, any use of acidic ion exchange resins in synthesizing these compounds should be strictly avoided. Our relevant findings, which are much less surprising now from a retrospective point of view, should be considered while interpreting some encouraging improvements in the design of sLe^X mimetics, especially for the P- and L-selectins, which recently have been reported.[6]

EXPERIMENTAL PART

General: Thin layer chromatography (TLC) was carried out on precoated Kieselgel 60 F_{254} plates (0.25 mm thickness, E. Merck) with the specified solvent mixtures and spots were visualized by spraying the plates with sulfuric acid/anisaldehyde reagent, followed by heating. E.Merck silica gel (60, particle size 0.040-0.063 mm) was used for flash column chromatography. Yields refer to chromatographically (TLC) and spectroscopically (NMR) homogeneous materials. Optical rotations were measured using a Perkin Elmer 241 polarimeter. NMR spectra were recorded on a Bruker WT 300 (300 MHz). NMR chemical shifts are given as δ-values with reference to tetramethylsilane (TMS) as internal standard, if not otherwise noted. The spectra recorded in D_2O as solvent were locked to deuterium, unless otherwise stated. Mass spectra were recorded on a TSQ 700, Finnigan/MAT, electrospray ionization (ESI), and MS/MS daughter ion scan.

Preparation of the sLe^X mimetic 12

(1R,2R)-trans-1,2-Cyclohexanediol-O-2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside **(3)**. A 1 M trimethylsilyl-trifluoromethanesulfonate solution (2.3 ml) was added to a solution of 11.29 g (22.9 mmol) O-(2,3,4,6-tetra-O-acetyl-D-galactopyranosyl)-trichloroacetimidate **(1)**[12] and 4.00 g (34.3 mmol) **2** in dichloromethane/diethyl ether (100/200 ml). After 1 h, the mixture was neutralized with sodium hydrogencarbonate (1.0 g) , filtered and concentrated *in vacuo*. Chromatography of the residue (toluene/acetone 5/1) yielded 7.4 g of **3** (72 %). ^{1}H NMR ($CDCl_3$): 1.98, 2.06, 2.06, 2.16 (4 s, 12 H, 4 OAc), 3.28 (m, 1 H), 3.44 (m, 1 H), 4.54 (d, 1 H, 1-H), 5.05 (dd, 1 H, 3-H), 5.20 (dd, 1 H, 2-H), 5.38 (dd, 1 H, 4-H).

(1R,2R)-trans-1,2-Cyclohexanediol-O-(2,3,4-tri-O-benzyl-α-L-fucopyranosyl)-2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside **(5)**. A mixture of 4.0 g (9.0 mmol) **3**, 6.45 g (13.5 mmol) thioethyl-O-2,3,4-tri-O-benzyl-β-L-fucopyranoside **(4)**[13] and 0.66 g (2.07 mmol) tetrabutylammonium bromide in dichloromethane (300 ml) and DMF (60 ml) was stirred with molecular sieves (4 Å) for 1 h. Then 3.62 g (16.2 mmol) copper(II) bromide were added. After 24 h, the mixture was filtered through Celite and washed with saturated aqueous sodium hydrogen carbonate solution and then with brine. The organic phase was dried over magnesium sulfate and concentrated *in vacuo*, and the residue was chromatographed (hexane/ethyl acetate 3/2 → 2/1). Yield: 7.39 g (95%) of compound **5**. ^{1}H NMR ($CDCl_3$): 1.15 (d, 3 H, 6-H_{fuc}), 1.92, 1.96, 2.02, 2.02 (4 s, 12 H, 4 OAc), 4.50 (d, 1 H, 1-H_{gal}), 5.33 (dd, 1 H, 4-H_{gal}).

(1R,2R)-trans-1,2-Cyclohexanediol-O-(2,3,4-tri-O-benzyl-α-L-fucopyranosyl)-β-D-3-O-benzyl-galactopyranoside **(6)**. A solution of 7.39 g (8.55 mmol) of compound **5** in methanol (360 ml) was treated with a 2 M

methanolic sodium methoxide solution (1.4 ml). After 3 h, the mixture was neutralized with acetic acid, filtered and concentrated, and the residue was chromatographed (dichloromethane/methanol 25/1 → 20/1). 5.83 g (98 %) of the deacetylated product **6** were obtained. 24.0 g (34.5 mmol) of **6** and 10.3 g (41.4 rmol) dibutyltin oxide in methanol (375 ml) were boiled under reflux. After 18 h, the mixture was concentrated and coevaporated with toluene. The residue was dissolved in toluene (330 ml), treated with 8.17 ml (69.0 mmol) benzyl bromide and 15.9 g (43.1 mmol) tetrabutylammonium iodide and warmed to 45 °C. After 17 h, the mixture was concentrated and chromatography using dichloromethane/methanol (25/1) gave 23.8 g (88 %) of the intermediate compound **6**. ^{1}H NMR ($CDCl_3$): 1.08 (d, 3 H, 6-H_{fuc}), 1.15-1.40 (m, 4 H, 2 CH_2-cyclohex), 1.68, 2.00 (2 m, 4 H, 2 CH_2-cyclohex), 4.30 (d, 1 H, 1-H_{gal}), 4.40 (m, 1 H), 7.20-7.43 (m, 15 H, 3 Ph).

(1R,2R)-trans-1,2-Cyclohexanediol-O-(2,3,4-tri-O-benzyl-α-L-fucopyranosyl)-β-D-3-O-benzyl-4,6-O-benzylidene-galactopyranoside (**7**). To a solution of **6** (23.8 g, 30.3 mmol) in acetonitrile (350 ml) and 6.8 ml (45.5 mmol) benzaldehyde dimethylacetal were added 80 mg of p-toluenesulfonic acid. After stirring for 1 h, the reaction was stopped by the addition of potassium carbonate (1 g). The mixture was filtered, concentrated, and chromatographed (hexane/ethyl acetate 3/1 → 2/1 → 1/1). Yield: 23.54 g (89 %) of **7**. ^{1}H NMR ($CDCl_3$): 1.03 (d, 3 H, 6-H_{fuc}), 1.10-1.40 (m, 4 H, 2 CH_2-cyclohex), 1.70, 2.01 (2 m, 4 H, 2 CH_2-cyclohex), 2.40 (d, 1 H, OH), 3.66 (m, 1 H), 4.13 (*br*d, 1 H), 4.41 (d, 1 H), 5.51 (s, 1 H, C<u>H</u>Ph), 7.10-7.62 (m, 20 H, 4 Ph).

(1R,2R)-trans-1,2-Cyclohexanediol-O-(2,3,4-tri-O-benzyl-α-L-fucopyranosyl)-β-D-2-O-(2-allyloxyethyl)-3-O-benzyl-4,6-O-benzylidene-galactopyranoside (**8**). To a solution of 17.5 g (20.0 mmol) **7** in DMF (150 ml) 0.72 g (30.0 mmol) NaH were added with stirring. After 1 h, 6.70 g (26.0 mmol) 2-allyloxy-1-p-toluenesulfonyloxyethane[7e], dissolved in DMF (20 ml), were added dropwise. After 18 h, the mixture was diluted with water (300 ml). Extraction with diethyl ether (3 x 300 ml), washing of the combined organic phases with water (3 x 300 ml), evaporation of the solvents and flash chromatography (hexane/ethyl acetate 3:1 → 2:2 → 1:1) yielded 16.0 g (84 %) of **8**. ^{1}H NMR ($CDCl_3$): 1.03 (d, 3 H, 6-H_{fuc}), 1.10-1.40 (m, 4 H, 2 CH_2-cyclohex), 1.69, 2.06 (2 m, 4 H, 2 CH_2-cyclohex), 5.20 (m, 2 H, O-CH_2-CH=C<u>H</u>$_2$), 5.51 (s, 1 H, C<u>H</u>Ph), 5.90 (m, 1 H, O-CH_2C<u>H</u>=CH_2), 7.10-7.62 (m, 20 H, 4 Ph).

(1R,2R)-trans-1,2-Cyclohexanediol-O-(2,3,4-tri-O-benzyl-α-L-fucopyranosyl)-β-D-2-O-(2-hydroxyethyl)-3-O-benzyl-4,6-O-benzylidene-galactopyranoside (**9**). A mixture of 16.0 g (16.7 mmol) of **8** and 1.54 g (1.67 mmol) of *Wilkinson* catalyst was boiled under reflux for 3 h in ethanol/water (9/1, 380 ml) and then concentrated *in vacuo*. The residue was taken up in tetrahydrofuran (330 ml) and water (150 ml) and treated with 4.20 g (33.4 mmol) of iodine for 30 min. After addition of dichloromethane (500 ml), the water layer was extracted with dichloromethane (2 x 300 ml). The combined organic extracts were washed with aqueous

sodium thiosulfate solution, dried, filtered and concentrated *in vacuo*. Flash chromatography (toluene/acetone 4:1) gave 12.8 g (83 %) of the intermediate compound **9**. ^{1}H NMR ($CDCl_3$): 1.06 (d, 3 H, 6-H_{fuc}), 1.10-1.40 (m, 4 H, 2 CH_2-cyclohex), 1.69, 2.06 (2 m, 4 H, 2 CH_2-cyclohex), 5.52 (s, 1 H, C<u>H</u>Ph), 7.10-7.61 (m, 20 H, 4 Ph).

(1R,2R)-trans-1,2-Cyclohexanediol-O-(2,3,4-tri-O-benzyl-α-L-fucopyranosyl)-β-D-2-O-(2-bromoethyl)-3-O-benzyl-4,6-O-benzylidene-galactopyranoside **(10)**. Compound **9** (12.8 g, 14 mmol) was dissolved in dichloro-methane (250 ml). At 0 °C, 7.3 g (28 mmol) triphenylphosphine, 7.8 ml (56 mmol) triethylamine and 9.1 g (28 mmol) 1,2-dibromo-tetrachloroethane) were added. After stirring for 2 h, the mixture was concentrated and purified by means of flash chromatography (hexane/ethyl acetate 2/1 → 1/1). Yield: 13.0 g (95 %) of the bromide **10**. ^{1}H NMR ($CDCl_3$): 1.06 (d, 3 H, 6-H_{fuc}), 1.10-1.40 (m, 4 H, 2 CH_2-cyclohex), 1.70, 2.06 (2 m, 4 H, 2 CH_2-cyclohex), 5.53 (s, 1 H, C<u>H</u>Ph), 7.10-7.62 (m, 20 H, 4 Ph).

(1R,2R)-trans-1,2-Cyclohexanediol-O-(2,3,4-tri-O-benzyl-α-L-fucopyranosyl)-β-D-2-O-(2-dimethylmalonyl-ethyl)-3-O-benzyl-4,6-O-benzylidene-galactopyranoside **(11)**. Compound **10** (13.0 g, 13.26 mmol) was treated with dimethyl malonate (116 ml), potassium carbonate (6 g) and dibenzo-18-crown-6 (1.6 g) at 95 °C. After 5 h, the mixture was cooled and diluted with dichloromethane (1 l). The organic layer was washed with water and treated with dry ice until the washing water became neutral. The organic phase was dried over sodium sulfate, filtered and concentrated *in vacuo*. Flash chromatography (hexane/ethyl acetate 3/1 → 2/1 → 1/1) yielded compound **11** (12.6 g, 92 %). ^{1}H NMR ($CDCl_3$): 1.03 (d, 3 H, 6-H_{fuc}), 1.10-1.40 (m, 4 H, 2 CH_2-cyclohex), 1.70, 2.02 (2 m, 4 H, 2 CH_2-cyclohex), 2.18 (m, 2 H, C<u>H</u>$_2$ CH [CO_2Me]$_2$), 4.03 (*br*d, 1 H), 4.38 (d, 1 H), 5.50 (s, 1 H, C<u>H</u>Ph), 7.10-7.62 (m, 20 H, 4 Ph).

(1R,2R)-trans-1,2-Cyclohexanediol-O-(α-L-fucopyranosyl)-β-D-2-O-(2-malonyl-ethyl)-galactopyranoside **(12)**. A mixture of the diester **11** (12.1 g, 11.7 mmol) and palladium on carbon catalyst (10 %, 12.1 g) in methanol (1.2 l), dioxane (120 ml) and acetic acid (66 ml) was hydrogenated under normal pressure in a hydrogen atmosphere for 24 h. The catalyst was filtered off, and the concentrated filtrate was stirred with a 1 M sodium hydroxide solution (715 ml). After 2 h, the mixture was neutralized with acetic acid (<u>method A</u>) or with an acidic ion exchange resin, e.g. one of the resin types which are listed in Table 1 (<u>method B</u>), concentrated in vacuo and purified through Biogel P-2 (for <u>method A</u> and for <u>method B</u>). 5.20 g (80 %) of the sLe^X mimetic **12** were obtained by using either method. $[\alpha]^{22}_D$ -92° (c 1.0, H_2O); ^{1}H NMR (D_2O, see Figure 3): 1.10 (d, $J_{5,6}$= 6.6 Hz, 3 H, 6-H_{fuc}), 1.16 (m, 4 H, 2 CH_2-cyclohex), 1.61 (m, 2H, CH_2), 2.07 (m, 4 H, 2 CH_2-cyclohex), 3.16 (dd, 1 H, $J_{1,2}$= 7.8 Hz, $J_{2,3}$= 9.5 Hz, 2-H_{gal}), 3.44-3.93 (m, 12 H), 4.43 (d, $J_{1,2}$= 7.8 Hz, 1 H, 1-H_{gal}), 4.49 (q, $J_{5,6}$= 6.6 Hz, 1 H, 5-H_{fuc}), 4.91 (d, $J_{1,2}$= 3.9 Hz, 1 H, 1-H_{fuc}). ^{13}C NMR (D_2O, H,H- and H,C-COSY): 18.26 (6-C_{fuc}), 25.89, 25.94, 31.46, 32.31 (CH_2-cyclohex), 31.84 (OCH_2

$\underline{C}H_2$), 51.68 ($\underline{C}H[COOH]_2$), 64.21 (6-C_{gal}), 69.30 (5-C_{fuc}), 70.90 (4-C_{fuc}), 71.58 (4-C_{gal}), 72.54 (3-C_{fuc}), 73.15 ($O\underline{C}H_2$ CH_2), 74.91 (2-C_{fuc}), 75.19 (3-C_{gal}), 77.61 (5-C_{gal}), 79.25 ($\underline{C}H_{Ogal}$-CH_{Ofuc}), 80.93 (CH_{Ogal}-$\underline{C}H_{Ofuc}$), 82.67 (2-C_{gal}), 97.77 (1-C_{fuc}), 102.56 (1-C_{gal}), 176.57, 176.76 (2 COOH). ESI-MS: m/e = 553 $[M-H]^-$; 509, 423, 277, 261, 247, 161. IR (KBr [cm^{-1}]): 3427, 2937, 1721, 1370, 1249, 1168, 1077, 972.

Preparation of the resin extracts

Acidic anion exchange resins, e.g. the resin types listed in Table 1, were thoroughly washed with methanol and water and then used to neutralize an aqueous 1 M NaOH solution. Then the filtrate was lyophilized. Typically, about 10-50 mg amounts of colourless solid material could be obtained from 30 g of the resins.

Bioassay for cell binding to immobilized selectin receptor globulins

The bioassays for cell binding to immobilized selectin receptor globulins were performed as previously described.[7d] Briefly, the soluble recombinant E- and P-selectin-IgG fusion proteins which contain the signal sequence, the lectin-like domain, the EGF (epidermal growth factor) repeat domain and six (E-selectin) and two (P-selectin) of the CR-like (complement regulatory) domains obtained from transfected COS cells were adsorbed on anti-human-IgG-antibodies immobilized on ELISA (enzyme-linked immuno-sorbent assay) plates. Adhesion of labelled HL60 tumor cells was quantitatively measured in a cyto-fluorometer and the specific cell binding in the presence of a potential inhibitor, e.g. **12**, was calculated compared with nonspecific binding to the CD4-IgG fusion protein.

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8. Resins were thoroughly washed with methanol and water before use. The solution of neutralized **12** was then filtered through a one-way syringe membrane (0.2 μM) before chromatography.
9. The discrepancy in biological activity was observed fortuitously after a synthesis batch had been reproduced on a larger scale according to method A. Attempts to exclude trace impurities released from the anion exchange resins by means of techniques to separation according to molecular size (Biogel, Sephadex) may not be fully successful, since the material released from the resins exhibits a rather broad molecular weight distribution, including small polyanionic fragments (gel permeation chromatography).
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