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Synthesis and Biological Activity of Novel Sialyl-Lewis^X Conjugates

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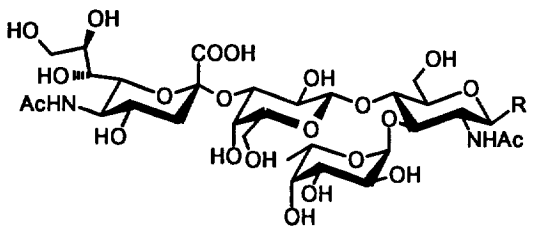
Abstract: Novel sialyl Lewis^X conjugates have been synthesized and evaluated as inhibitors of E- and P-selectin mediated cell adhesion in cell culture assays. The most potent conjugate in the static inhibition assays exhibited a significant and dose-dependent pharmacological potency as inhibitor of the endotoxin-induced leukocyte adhesion to the endothelium of postcapillary venules in rats.

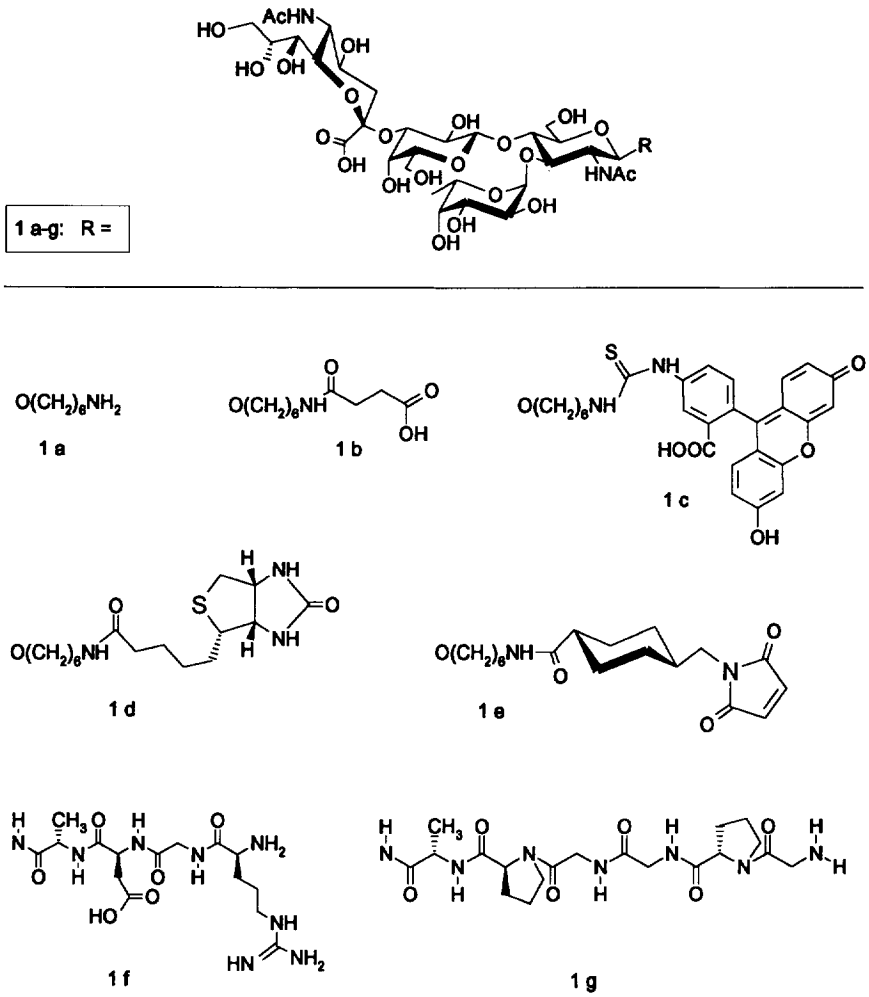
The excessive accumulation of leukocytes in inflammed tissues is accompanied by increased leukocyte adhesion to the endothelium of postcapillary venules.[1] This recruitment of blood lymphocytes into sites of inflammation involves a dynamic and well coordinated sequence of events in which cellular adhesion molecules (CAM's) and chemotactic mediators (cytokines) play an active role. The selectin family of leukocyte adhesion molecules is recognized to play key roles in the recruitment of neutrophils and other leukocytes to sites of inflammation and tissue injury, and to mediate trafficking of lymphocytes to peripheral lymph nodes during recirculation between the blood and lymph. The selectins are transmembrane glycoproteins expressed on platelets (P-selectin), leukocytes (L-selectin) and on endothelial cells (E- and P-selectins) which mediate early rolling interactions on the endothelial vessel wall in normal and in excessive inflammatory response to cytokines and to other inflammatory mediators. Glycoproteins and -lipids bearing the sialyl Lewis^X tetrasaccharide **1** (SLe^X) are the primary ligands for this selectin mediated cell adhesion.[2] The selectins and their saccharide ligands provide new targets for therapeutics for a broad array of inflammatory disorders.[3] Glycomimetics that functionally mimic the



1 (R = OH) Sialyl Lewis^X (SLe^X)
Selectin-Ligands: R = O-Glycoprotein/-lipid moiety

natural ligand **1** are currently being evaluated as potential anti-adhesive and anti-inflammatory drugs.[4] A pentasaccharide derivative of **1** has already shown promising efficacy in animal models of acute lung inflammation[5a] and of myocardial ischaemia and reperfusion injury.[5b]

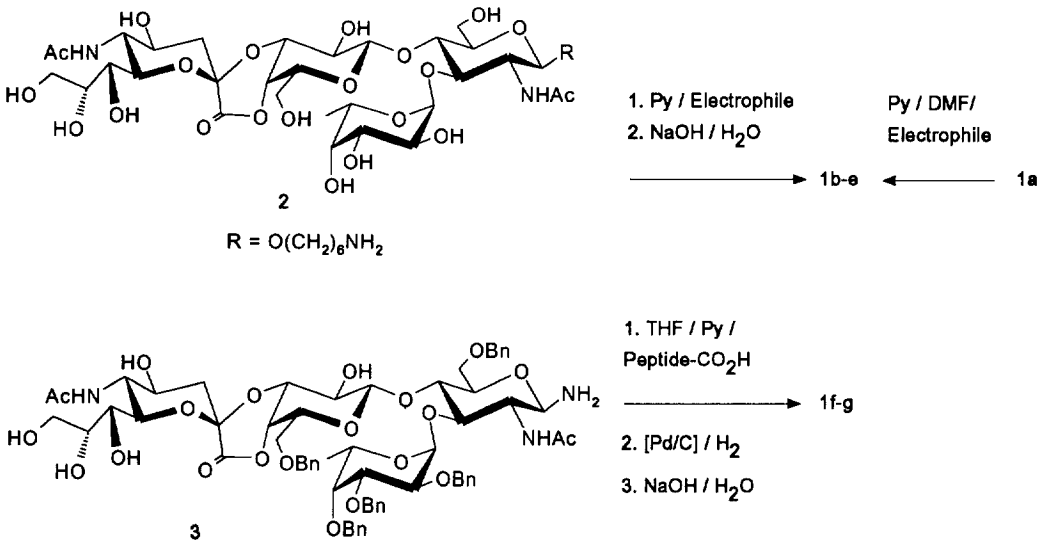
Inherent weak binding of selectins to the carbohydrate ligand **1** is well documented in different assay types with half-inhibition concentrations (IC_{50}) approximately in the 1 mmolar range. The *in vitro* efficacy of sLe^X -based glycomimetics reported thus far, however, showed no substantial improvement in cell-based adhesion assays.[6]



Scheme 1: Structures of the Sialyl Lewis^X Conjugates 1a-g

Conformational energy computations, NMR-studies and the X-ray structure of E-selectin indicated that most functional elements related to the reducing end of the saccharide **1** are not essential for binding to E-selectin.[7] The true functional binding situation *in vivo*, however, remains unclear. Recently we described the highly active ligand **1f** exhibiting an IC_{50}-value of 26 μM in a cell adhesion test in which competitive binding on P-selectin-IgG relative to the SLe^X-ligands expressing HL60 tumor cell line was determined.[4d] This encouraging result initiated a more systematic evaluation *in vitro* and *in vivo* of the reducing end moiety (R) in **1**.

The new sLe^X-conjugates **1b-1e** and **1f-1g** shown in Scheme 1 were prepared from the already described, unprotected tetrasaccharide precursors **1a** or **2**,[4b,c] and from the partially protected lactone **3**,[4d] respectively, as outlined in Scheme 2.[8] Two alternative methods could be applied for the preparation of the long-chain, O-linked conjugates **1a-e**. According to the first method, **1a** was stirred with 1.1 equivalent of the respective electrophile in pyridine / DMF at 20°C until complete consumption of **1a**, as indicated by TLC analysis. After concentration of the reaction mixtures the products were isolated in pure form by chromatography on Biogel P2. Likewise, 0.5 g of the fluorescein conjugate **1c** was obtained in 88% yield. Alternatively, the more soluble lactone precursor **2** of saccharide **1a** gave the succinate **1b** (92%), the biotinylated **1d** (83%) and the conjugate **1e** (81%). The latter reaction was preferably carried out starting from **1a** since in one experiment the maleimide



Electrophiles: **1b**: Succinic anhydride; **1c**: Fluorescein isothiocyanate; **1d**: N-Hydroxysuccinimido-biotin; **1e**: Succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate

Scheme 2: Preparation of the Sialyl Lewis^X Conjugates 1a-g

moiety was partly hydrolyzed during the saponification of the sLe^X-lactone. The synthesis of the RGDA-conjugate **1f** from the glycosylamine **3** has already been described[4d] and the hydrophobic peptide conjugate **1g** was obtained analogously in 65 % overall yield (coupling and deprotection).[9] The ^1H- and ^{13}C- NMR signals of the Fuc-, Gal- and NAcNeu of all conjugates proved to be very similar to the reported[10] spectra of sLe^X, indicating that the conformation of the tetrasaccharide ligand is not influenced by the aglycon moiety.

The bioassay for cell binding to immobilized selectin receptor globulins was performed as previously described.[4e] 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 immunosorbent assay) plates. Adhesion of labelled HL60 tumor cells was quantitatively measured in a cytofluorometer and the specific cell binding in the presence of inhibitors **1a-g** was calculated compared with nonspecific binding to the CD4-IgG fusion protein. The concentrations of the inhibitors required to block adhesion of 50% of the HL60 cells ranged from 5.0 mM to 0.026 mM, as is shown in Table 1.

inhibitor	IC_{50} [mM] E-selectin	IC_{50} [mM] P-selectin
1a	1.0	2.0
1b	1.15	1.50
1c	0.65	0.90
1d	0.59	0.60
1e	0.28	0.38
1f	>1.0	0.026
1g	2.5-5.0	-

Table 1: Inhibition of HL60 Cell Adhesion to Recombinant E- and P-Selectin-IgG Fusion Proteins on Plates

IC_{50}-values are concentrations of inhibitors required to block adhesion of 50% of the cells compared with the non-specific binding to the CD4-IgG fusion protein as negative control.[4e]

Conversion of the basic functionality at the reducing end of **1a** into the acidic functionality in **1b** gave no significant change. Interestingly, the introduction of structurally very different residues like the fluorescein dye in **1c**, biotin in **1d** and the 4-(N-maleimidomethyl)cyclohexane moiety in **1e** gave significantly improved IC_{50}-values for both selectins. The simple conjugate **1e** gave a better than 3-fold and 5-fold improvement with E- and P-selectin, respectively. These results should be compared with those recently obtained in the same cell assay system with much more complex inhibitors designed as polyvalent selectin antagonists: The IC_{50}- value of the best dimeric ligand reported was 0.2 mM for E-selectin, compared with 0.8 mM for a monomeric sLe^X-pentasaccharide.[11] Furthermore, with well defined trivalent sLeX-ligands, the maximum binding enhancement per SLe^X-ligand of a factor of 3 (E-) and 5 (P-selectin) was obtained[4e] which may support our finding that all

improvements in binding affinity performed were *not* sufficient to support the concept of multivalent interaction between selectins and their sialyl Lewis^X -ligands. These results may rather reflect secondary binding effects caused by the aglycon moieties. The low IC_{50}-value attained with the RGDA-conjugate **1f** in the P-selectin assay suggests a different binding mode of this special conjugate, in particular when compared with the low selectin affinity of the sLe^X-glycopeptide **1g**.

The inhibitory effects of the most active sLeX-conjugate **1e** and of the standard ligand **1a** in the static assays were then investigated in an animal model for the assessment of inflammatory effects of endotoxins on the adherence of leukocytes on the endothelium. In this model the effect of lipopolysaccharide (LPS, 15 mg/kg i.v.) induced leukocyte adhesion in the microcirculation in rat mesentery was measured by intravital microscopy and an analogous video image processing system as previously described in detail.[4e,12] Briefly, the mesentery of the anaesthetized rats was exposed on a thermocontrolled window of the microscope stage and the ileo-caecal portion of the mesentery was fixed in position. The experiment lasted 2 hours. For each experiment, the rats femoral artery and vein were cannulated for monitoring blood pressure and heart rate or intravenous injection of LPS or drugs. Sixty minutes after surgery LPS (from *E.coli* 15 mg/kg) was injected. Following a 30 min postsurgical equilibration period during which the tissue was allowed to stabilize, leukocyte adhesion was measured in postcapillary venules of 10-30 µm diameter and 100 µm length by using a Zeiss inverted camera microscope ICM 401. Leukocytes adhering to the endothelium were counted in 2-3 segments of postcapillary venules at 10 minute intervals. A leukocyte was considered adherent to the endothelium if it was stationary for more than 30 seconds. The mean values of the numbers of adhering cells between 10 and 30 minutes after surgery were calculated and served as control for the model. The drug was administered 30 min after surgery and the LPS-endotoxin 30 min thereafter. All data between the 70th and 120th minute after surgery were taken for calculations, whereby the untreated LPS-controls were compared with the drug treated animals.

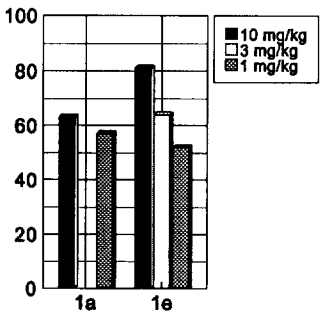


Figure 1: %-Inhibition of leukocyte adhesion in rat mesenteric venules by application of compounds **1a** or **1e** at doses from 10 mg/kg to 1 mg/kg in response to lipopolysaccharide (LPS) induced adhesion (**1a**: 63%, 57%; **1e**: 81%, 64%, 52%)

The reference ligand **1a** attenuated the leukocyte adhesion to the endothelium of postcapillary venules, as shown in Figure 1, at 10 mg/kg i.v. equally as well as a very similiar ligand used in a *Clostridium difficile* toxin A (Tx-A) induced microvascular dysfunction.[13] The more potent selectin antagonist *in vitro* **1e** was more potent *in vivo* than **1a**, at least at the higher 10 mg/kg dose, suggesting a correlation of the *in vitro* with the *in*

vivo data. Clearly, the difference obtained with the low 1 mg/kg dose is less significant and slightly reversed in favour of **1a**, indicating different bioavailabilities and intravascular half lives of both conjugates.

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9. ^{1}H-NMR of **1g** (300 MHz, D_2O): δ = 1.12 (d, 3 H, 6-H^{Fuc}), 1.30 (d, 1 H, α-Ala), 1.74 (pseudo-t, 1 H, 3-$H^{NANA/ax}$), 1.93, 1.98 (s, 6 H, $C\underline{H}_3^{NHAc}$), 2.72 (dd, 1 H, $J_{3eq,3ax}$ = 12.5, $J_{3eq,4}$ = 4.3 Hz, 3-$H^{NANA/eq}$), 3.90, 3.94 (α-Gly), 4.18 (α-Ala), 4.49 (d, 1 H, 1-H^{Gal}, $J_{1,2}$ = 8 Hz), 5.0-5.1 (2d, 2 H, $J_{1,2}$ = 4 Hz, 1-H^{Fuc}, $J_{1,2}$ = 8 Hz, 1-H^{GlcNAc}); ^{13}C-NMR (75.09 MHz, D_2O): δ = 15.38 (6-C^{Fuc}), 16.5 (β-Ala), 22.27, 22.14 (2NHAc), 40.80 (α-Gly), 41.87 (α-Gly), 42.47 (α-Gly), 50.11 (β-Ala), 78.61 (1-C^{GlcNAc}), 98.91 (1-C^{Fuc}), 99.89 (2-C^{NANA}), 101.88 (1-C^{Gal}), 166.96, 169.75, 171.84, 174.25, 174.54, 174.70, 175.11, 175.42, 175.97 (1-Gly/-Pro/-Ala, 1-$\underline{C}$=O^{NANA}, $\underline{C}$=O^{NHAc}). ESI-MS: $[MH^+]$ = 1256.8 $C_{50}H_{81}N_9O_{28}$ (1256.24).
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