Glycopeptides

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Sulfated and Non-Sulfated Glycopeptide Recognition Domains of P-Selectin Glycoprotein Ligand 1 and their Binding to P- and E-Selectin**

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A specific inhibition of leukocyte infiltration into inflamed tissue is of importance for the therapy of postoperative inflammations. During the recruitment of leukocytes, adhesion processes on the endothelial cells of blood vessels play an important role. At their start, the carbohydrate-recognizing receptors P- and E-selectin interact with their respective ligands located on the leukocytes.[1] Therefore, it is desirable to inhibit the binding of P- and E-selectin to their ligands, P-selectin glycoprotein ligand 1 (PSGL-1)[2] and ESL-1,[3] respectively, and to elucidate the structure of efficient inhibitors to these selectins. This is even more desirable, as selectins act similarly in the metastasis of tumor cells.[4] In addition, PSGL-1[5] expressed on leukocytes also is involved in granulocyte plasmosis induced by bacteria (ehrlichiosis)[6] and in acute phases of multiple sclerosis.[7] The glycopeptide of the binding region of human PSGL-1[5] (Figure 1) has already been synthesized chemoenzymatically in microgram quantities.[8] The glycopeptide showed high binding affinity only when the tyrosine residues of the N-terminal peptide are *O*-sulfated.[8]

We have developed a chemical total synthesis of the recognition domain of PSGL-1,[9] which afforded the glycopeptide **1** in milligram amounts and, thus, enable further investigations. One positive aspect of chemical total syntheses is that they pave the way for syntheses of mimics of the natural structure inaccessible by enzymatic reactions. Herein, we describe the chemical synthesis of the mimics **2** of the PSGL-1 binding site (Figure 2), in which the enzymatically cleavable *N*-actetyl neuraminic acid in **1** has been replaced by the mimic (*S*)-cyclohexyllactic acid,[10] and we concomitantly report the first purely chemical synthesis of *O*-sulfated glycopeptides of this complex type.

The preparation of the glycosyl threonine **A** (Scheme 1) is based on the strategy established in the described total synthesis.[9] Building block **A** should be formed by block glycosylation of the T-antigen threonine conjugate **III**, known from syntheses of tumor-associated antigens,[11] with the cyclohexyllactyl-Lewis^x trichloroacetimidate **B**. The donor **B** is obtained from Lewis^x trisaccharide **3**[9] by an S_N^2 reaction with the *O*-triflate derivative of methyl (*R*)-cyclohexyllactate.[10] The Lewis^x trisaccharide **3** has already been described; however, in that case the anomeric OH function is blocked as the *tert*-butyldiphenylsilyl

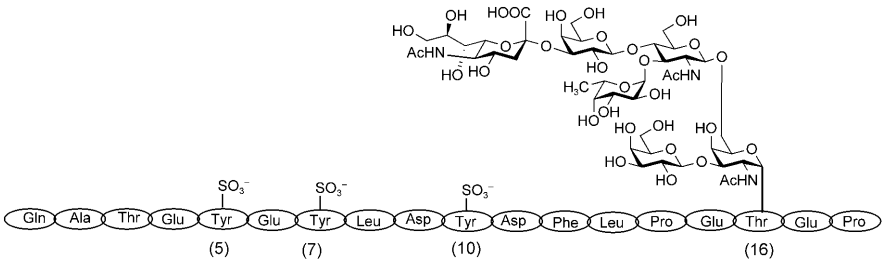

Figure 1. Recognition domain of the P-selectin glycoprotein ligand 1.

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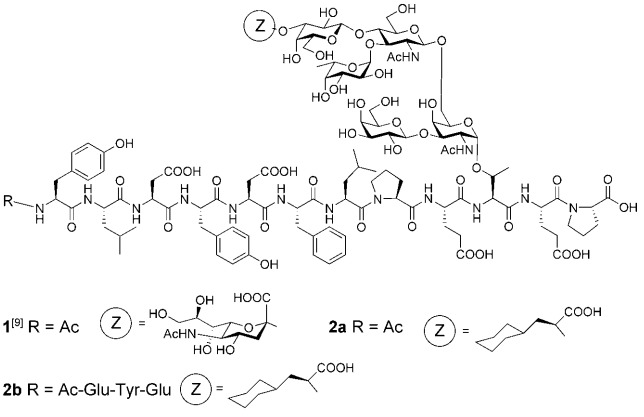
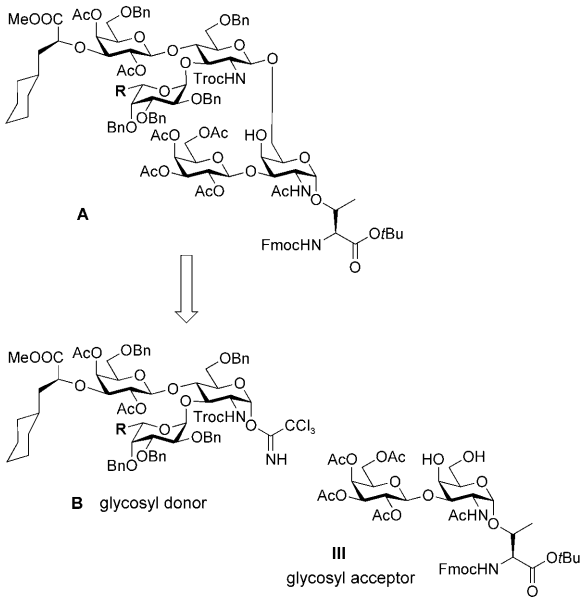
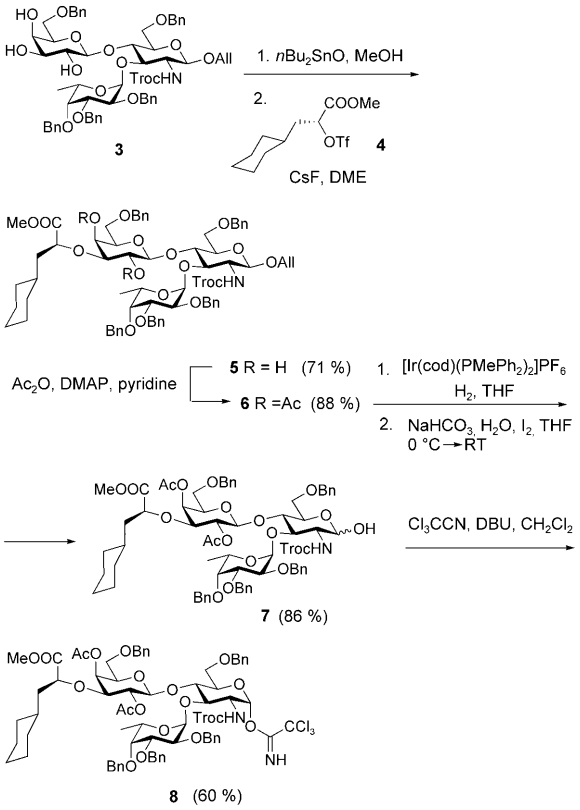


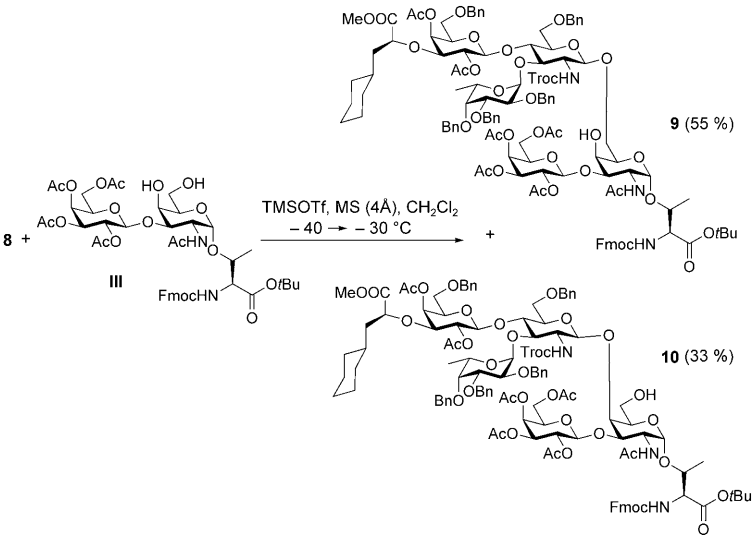
Figure 2. Synthetic glycopeptides from the recognition domain of PSGL-1.



Scheme 1. Retrosynthesis of the (*S*)-cyclohexyllactyl Lewis^x-threonine building block **A**. Bn = benzyl, Fmoc = 9-fluorenylmethoxycarbonyl, Troc = trichlorethoxycarbonyl.



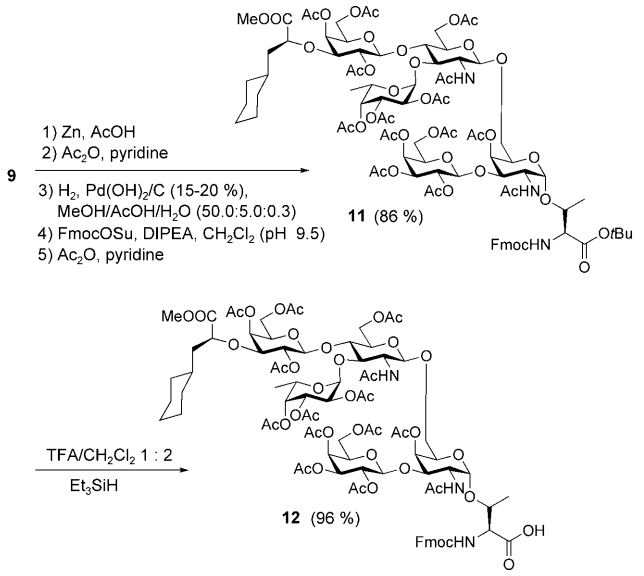
Scheme 2. Synthesis of the sialyl Lewis^x tetrasaccharide mimic **8**. cod = 1,5-cyclooctadiene, DMAP = 4-dimethylaminopyridine, DME = 1,2-dimethoxyethane, DBU = 1,8-diazabicycloundecene-7.



Scheme 3. Block glycosylation of the T-antigen threonine conjugate with the tetrasaccharide mimic.

ether instead of the allyl ether[12] required here. The *N*-trichloroethoxycarbonyl (Troc) group in **3** enables the stereoselective formation of the β-glycoside bond in **A** as a result of neighboring group participation. In the first step of the synthesis the (*S*)-cyclohexyllactyl residue was introduced in **3** using the *O*-triflate derivative **4** of methyl (*R*)-cyclohexyllactate according to the stannylene method[13] to yield the protected sialyl Lewis^x mimic **5** (Scheme 2). After acetylation of the 2- and 4-hydroxy groups of the galactose part, allyl glycoside **6** was cleaved by isomerization[16] catalyzed by 1,5-cyclooctadienebis(methyldiphenylphosphine)iridium hexafluorophosphate[15] and subsequent oxidation with iodine. Reaction of **7** with trichloroacetonitrile yielded the trichloroacetimidate[17] **8** required for the block glycosylation.[9]

The block glycosylation of the partially deblocked T-antigen threonine conjugate **III**[11] with sialyl Lewis^x mimic **8** is the key step of the entire synthesis. After activation of **8** using trimethylsilyltriflate at −40 °C both regioisomeric mimics **9** and **10** of the hexasaccharide threonine conjugates were formed with high β selectivity in an overall yield of 88 % (Scheme 3). Separation of these similar compounds succeeded efficiently by flash chromatography. In contrast to the synthesis of the sialyl Lewis^x core2 threonine building block,[9] the major product (55 %) was the desired regioisomer, as proven by the C6-GalNAc signal in the HSQC NMR spectrum of **9** ($\delta = 69.5$ ppm). However, the amount of the unexpected regioisomer **10** ($\delta = 59.2$ ppm) is surprisingly high compared to the results of analogous reactions between components of lower structural complexity.[18] The conversion of **9** into a building block that is sufficiently acid stable for solid-phase syntheses was achieved by reductive elimination of the *N*-Troc group, subsequent *N*-acetylation, hydrogenolysis of the *O*-benzyl groups, and, after re-introduction of the Fmoc group, *O*-acetylation (Scheme 4). By this exchange of protecting groups the α-fucoside bond is stabilized towards acids. The *tert*-butyl



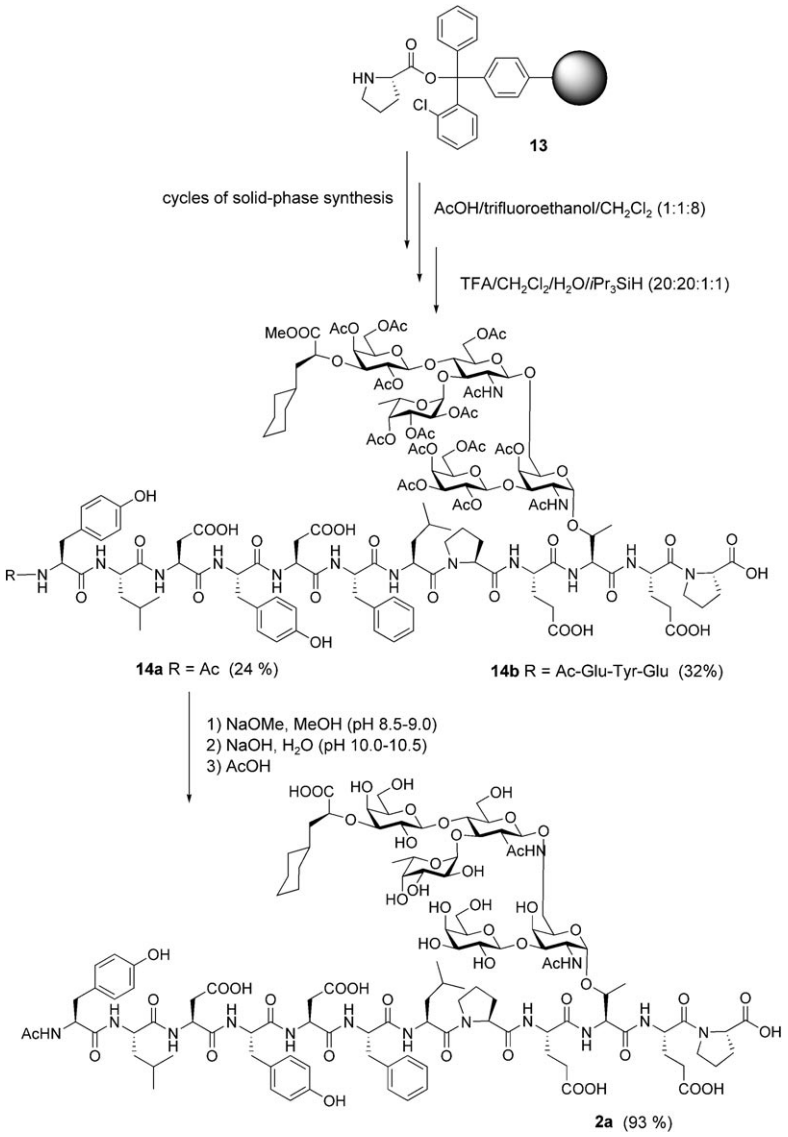
Scheme 4. Exchange of protecting groups yielding the Fmoc glycosyl threonine building block. DIPEA = *N*,*N*-diisopropylethylamine, TFA = trifluoroacetic acid.

ester **11** can now be cleaved with trifluoroacetic acid to give the pseudo-hexasaccharide threonine building block **12**.

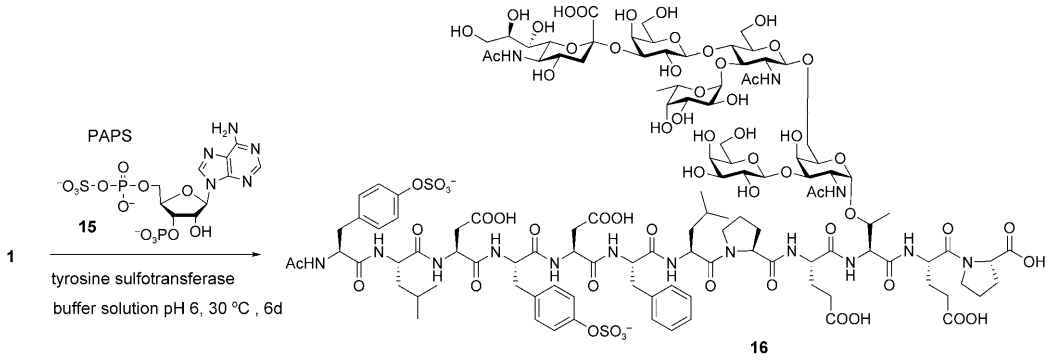
The solid-phase syntheses of the glycopeptides **2** of the binding site of PSGL-1 was carried out following the described protocol[9] according to the Fmoc strategy starting from proline linked through the Barlos anchor[19] to polystyrene[20] (Scheme 5). The linker was then cleaved with acetic acid in trifluoroethanol/dichloromethane. The *tert*-butyl groups were removed from the glycopeptide using trifluoroacetic acid. The glycopeptide **14a** still protected in the carbohydrate portion was isolated through preparative reversed-phase (RP) HPLC in an overall yield of 24%. PSGL-1 glycopeptide **14b**, which is N-terminally elongated by Glu-Tyr-Glu and contains all three tyrosine residues, was obtained in a yield of 32%. To remove the *O*-acetyl groups, **14a** was treated with a catalytic amount of NaOMe in methanol at pH 9. Remaining *O*-acetate groups and the methyl ester were hydrolyzed with aqueous NaOH at pH 10–10.5[21] within 36 h. Careful control of the pH is indispensable in order to prevent β elimination of the entire glycan. Neutralization with acetic acid and isolation by preparative RP-HPLC yielded pure glycopeptide **2a**[22] from the binding site of PSGL-1 containing the mimic of sialyl Lewis^x (45 mg).

For selective sulfation of the tyrosine OH groups in glycopeptides such as **1**[9] enzymatic procedures must be applied.[8] Therefore, the sialyl Lewis^x PSGL-1 peptide **1** was treated with phosphoadenosyl phosphosulfate (PAPS, **15**) in the presence of a recombinant tyrosine sulfotransferase[23] as catalyst at pH 6 and 30°C for 6 days (Scheme 6). The problem with the enzymatic sulfation is that both the sulfotransferase and the substrate PAPS are unstable in water at temperatures above 20°C, but the reaction should be carried out at 30°C. Furthermore, PAPS is expensive and can be used only in small-scale reactions (3 mg **1**) and in only slight excess (5 mg **15**, content 60%). Nevertheless, PSGL-1-sialyl Lewis^x glycopeptide **16** *O*-sulfated at both tyrosine residues[24] was obtained and isolated by RP-HPLC (3 mg).

Because of the preparative effort and the uncertainty of the enzymatic method, a chemical procedure was investigated as an alternative. Glycopeptides with deprotected carbohydrates, such as **1** or **2a**, cannot be used for this purpose.

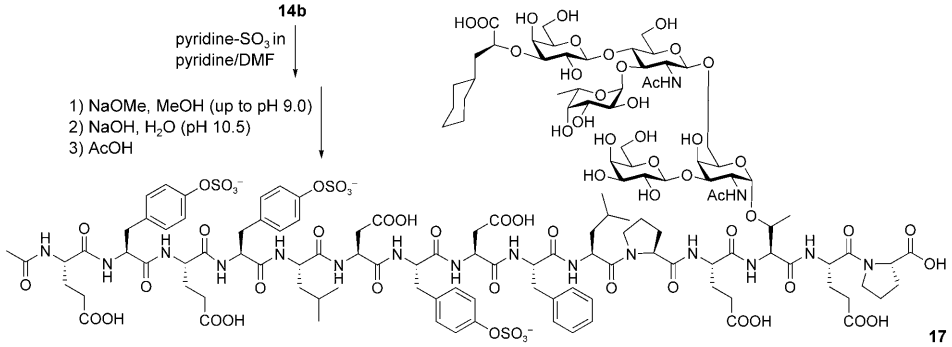


Scheme 5. Solid-phase synthesis: Cycle: 1) Fmoc cleavage: piperidine/NMP (1:4), 2) amino acid coupling: Fmoc-Xaa-OH, HBTU, HOBt, NMM, NMP; with **12**: HATU, HOAt, NMM, NMP; 3) capping: Ac_2O, DIPEA, HOBT, NMP. HATU = *O*-(7-azobenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluorophosphate, HOAt = 7-aza-1-hydroxy-1*H*-benzotriazole, HOBt = 1-hydroxy-1*H*-benzotriazole, NMM = *N*-methylmorpholine, NMP = *N*-methylpyrrolidine.



Scheme 6. Enzymatic tyrosine *O*-sulfation of the PSGL-1 glycopeptide **1**.

Therefore, the glycopeptide **14b** was carefully reacted with pyridine–SO_3 complex in pyridine/DMF.[25] After a reaction time of 18 h, analytical RP-HPLC showed the formation of two polar intermediates; after the addition of sodium carbonate solution (pH 6.5) and workup , the sulfated product was obtained. This product was carefully treated with catalytic amounts of NaOMe in methanol (pH 9) and subsequently with aqueous NaOH at pH 10.5[21] (42 h). After neutralization with acetic acid, the PSGL-1 sulfoglycopentadecapeptide mimic **17**, in which all three tyrosines are *O*-sulfated (Scheme 7), was isolated through preparative RP-HPLC (6 mg).[26] Indicative of the *O*-sulfation are the



Scheme 7. Chemical tyrosine *O*-sulfation of the PSGL-1 glycopeptide mimic **14b**.

signals of the tyrosine aryl protons in the ^{1}H NMR spectrum of **17** ($\delta = 7.12$ ppm), which are significantly shifted to lower field compared to the aryl-2,6 ($\delta = 6.95$ ppm) and aryl-3,6 protons ($\delta = 6.65$ ppm) of the tyrosine residues in **2a**.

With glycopeptide **1**,[9] its sulfated derivative **16**, the cyclohexyllactyl Lewis^x glycopeptide **2a**, and its derivative **17** bearing three *O*-sulfated tyrosines in hand, we could study the inhibition of the leukocyte adhesion to P-selectin. Flow cytometric analyses[3d,27] showed that the adhesion of murine neutrophiles (32DCl3 cell line) and granulocytes freshly prepared from mice to a murine P-selectin–IgG construct are not inhibited by the sialyl Lewis^x PSGL-1 glycopeptide **1**, its cyclohexyl lactyl mimic **2a**, and the sialyl Lewis^x sulfoglycopeptide **16** up to a concentration of 1 mM. Glycopeptides **1** and **2a** also did not inhibit the adhesion of human granulocytes to the P-selectin–IgG construct resembling the sialyl Lewis^x tetrasaccharide as the standard. The doubly *O*-sulfated sialyl Lewis^x PSGL-1 glycopeptide **16** showed low inhibition of the adhesion of human granulocytes to the murine P-selectin–IgG construct ($IC_{20} = 0.75$ mM). The small effects of the glycopeptides in these experiments may be traced back to the fact that the binding domain of human PSGL-1 differs from the one of murine PSGL-1 in the amino acid sequence.[28] It is astonishing that the chemically synthesized PSGL-1 sulfoglycopeptide **17**, which bears three *O*-sulfated tyrosines and (*S*)-cyclohexyllactic acid instead of neuraminic acid, efficiently inhibited the binding of all three cell lines—the 32DCl3 neutrophiles ($IC_{50} = 39$ μM), the murine granulocytes ($IC_{50} = 20$ μM), and the human granulocytes, the latter particularly strongly ($IC_{50} = 5$ μM).

The effect of the PSGL-1 glycopeptides on the binding of murine granulocytes to an E-selectin–IgG construct[27] was also investigated. The non-sulfated sialyl Lewis^x PSGL-1 glycopeptide **1** turned out to be a strong inhibitor of E-selectin ($IC_{50} = 40$ μM) compared to sialyl Lewis^x, which had 25 times lower activity ($IC_{50} \approx 1$ mM). Even higher affinity was shown by the PSGL-1 glycopeptide **2a** containing the (*S*)-cyclohexyllactic acid residue ($IC_{50} = 10$ μM); it reached the potency of the glycopeptides constructed according to the natural E-selectin ligand ESL-1.[3d] This affinity towards E-selectin was neither increased nor significantly lowered by *O*-sulfation of the tyrosines, as shown by the high inhibitory effect of the PSGL-1 sulfoglycopeptide **17** ($IC_{50} = 15$ μM).

These experiments with the fully synthetic glycopeptide selectin ligands give detailed insight into the role of the glycan, the peptide sequence, and the sulfation on the binding and selectivity of the selectin ligands. They pave the way to mimics of high affinity and to understanding the differences in the recognition processes of the different species. These experiments will be extended to binding studies on human P- and E-selectin.

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[22] **2a** $[\alpha]_D^{23} = -28$ ($c = 0.86$, DMSO); analytical HPLC: $t_R = 16$ min (Jupiter-C18, gradient: CH_3CN/H_2O [+ 0.1% TFA] 10:90→100:0 in 40 min, $l = 214$ nm). ^{1}H NMR [^{1}H-^{1}H-COSY, TOCSY] (400 MHz, [D$_6$]DMSO): $\delta = 7.21$–7.11 (m, 5H, H$_{Ar}$-Phe), 7.01, 6.96 (2 × d, 4H, $2 \times {}^3J = 8.4$ Hz, H$_{Ar\text{-}3,5}$-Tyr$_1$, H$_{Ar\text{-}3,5}$-Tyr$_2$), 6.84 (d, 1H, $^3J_{NH,H2} = 8.4$ Hz, NH-GalN), 6.62–6.59 (m, 4H, H$_{Ar\text{-}2,6}$-Tyr$_1$, H$_{Ar\text{-}2,6}$-Tyr$_2$), 4.80 (d, 1H, $^3J_{H1,H2} = 3.2$ Hz, H1-Fuc), 4.71–4.63 (m, 2H, H1-GalN {4.70, d, $^3J_{H1,H2} = 3.3$ Hz}, H5-Fuc {4.65}), 4.52–3.12 (m, Phe$^\alpha$ {4.52}, Leu$_1^\alpha$ {4.51}, Asp$_1^\alpha$ {4.51}, Asp$_2^\alpha$ {4.49}, Glu$_2^\alpha$ {4.48}, Glu$_1^\alpha$ {4.47}, Pro$_1^\alpha$ {4.42}, Thr$^\alpha$ {4.41}, Tyr$_1^\alpha$ {4.39}, H1-GlcN {4.37}, Tyr$_2^\alpha$ {4.34}, C*H*COOH {4.32}, H1-Gal {4.28}, Leu$_2^\alpha$ {4.28}, H1-Gal′ {4.19}, Pro$_2^\alpha$ {4.14}, H2-GalN {4.08}, Thr$^\beta$ {4.05}, H6a-GalN {3.85}, H4-GalN {3.80}, H4-Gal {3.78}, H2-GlcN {3.66}, Pro$_1^{\delta}{}_a$, Pro$_1^{\delta}{}_b$, Pro$_2^{\delta}{}_a$, Pro$_2^{\delta}{}_b$ {3.63–3.54, 3.46}, H3-GlcN {3.58}, H3-Gal' {3.58}, H3-GalN {3.56}, H3-Fuc {3.52}, H4-Fuc {3.45}, H6b-GalN {3.45}, H2-Gal {3.42}, H2-Fuc {3.37}, H2-Gal′ {3.30}, H3-Gal {3.13}), 1.23–1.06 (m, 6H, CH_2-Cyclo, Thr$^\gamma$ {1.07, d, $^3J_{Thr\gamma,Thr\beta} = 5.9$ Hz}), 0.98 (d, 3H, $^3J_{H6abc,H5} = 6.1$ Hz, H6a,b,c-Fuc), 0.90–0.78 ppm (m, 14H, CH_2-Cyclo {0.89, 0.81}, Leu$_1^{\delta}{}_{abc}$, Leu$_2^{\delta}{}_{abc}$). ^{13}C NMR [BB, HSQC] (100.6 MHz, [D$_6$]DMSO): $\delta =$ 176.57 (CHCOOH), 174.47, 174.23 (Glu$_1^\delta$, Glu$_2^\delta$), 173.53 (COOH-Pro), 172.30, 172.05 (Asp$_1^\gamma$, Asp$_2^\gamma$), 26.30, 26.09, 25.87 (CH_2-Cyclo), 24.79, 24.57 (Pro$_1^\gamma$, Pro$_2^\gamma$), 24.28, 24.16 (Leu$_1^\gamma$, Leu$_2^\gamma$), 23.37, 23.28 (Leu$_1^\delta$, Leu$_2^\delta$, CH_3-Ac), 22.70 (CH_3-Ac), 22.01, 21.73 (Leu$_1^\delta$, Leu$_2^\delta$), 18.81 (Thr$^\gamma$), 16.62 ppm (C6-Fuc). HR-ESI-MS (positive, + 0.1% TFA): $m/z = 1288.0548$ ([$M + 1 + 2$H]$^{2+}$, calcd.: 1288.0570).

[23] We thank Dr. K. L. Moore, University of Oklahoma, USA, for the tyrosine sulfotransferase coding plasmid; a) Y. Ouyang, W. S. Lane, K. L. Moore, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 2896.

[24] RP-HPLC in MeCN/0.1N NH_4OAc (Gradient 5-15-50 in 30-40-45 min): $R_t = 4.1$ min. ESI-MS (positive, + 0.1% TFA): $m/z =$ 1437.17 ([$M + 1 + 2$H]$^{2+}$, calcd.: 1435.91).

[25] A solution of pyridine/SO_3 (122 mg) in pyridine/DMF (2:1, 6 mL) at 0°C was charged with **14b** (50 mg) were added. After the reaction mixture had been stirred for 18 h at room temperature, MeOH (0.5 mL) was added. The solvents were removed in high vacuum. The remainder was dissolved in Na_2CO_3 solution (pH 6.5), stirred for 1 h, and lyophylized. Purification was carried out by preparative RP-HPLC in MeCN/0.1N NH_4OAc (gradient 5-35-50 in 100-120-140 min), $t_R = 66.5$ min; $[\alpha]_D^{23} = -52.8$ ($c = 0.11$, H_2O). For a sulfation of a GlNAc-GalNAc octapeptide, see: K. M. Koeller, M. E. B. Smith, C.-H. Wong, *J. Am. Chem. Soc.* **2000**, *122*, 742.

[26] ESI-MS (positive, + 0.1% TFA): $m/z = 1077.87$ ([$M + 1 +$ 3H]$^{3+}$, calcd.: 1078.03).

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