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Synthetic Glycopeptides from the E-Selectin Ligand 1 with Varied Sialyl Lewis^x Structure as Cell-Adhesion Inhibitors of E-Selectin

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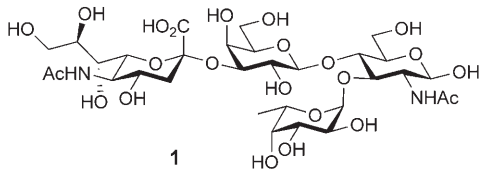
Dedicated to Professor Klaus Müllen on the occasion of his 60th birthday

During the recruitment and direction of leucocytes into inflamed tissues, carbohydrate-recognizing receptors P- and E-selectin play decisive roles at the beginning of a cell-adhesion cascade.[1] Inhibition of E-selectin would be a great benefit in the therapy of acute or chronic inflammation[2,3] or in blocking tumor metastasis.[4] The tetrasaccharide sialyl Lewis^x (**1**) was identified as the ligand of P- and E-selectin[1]



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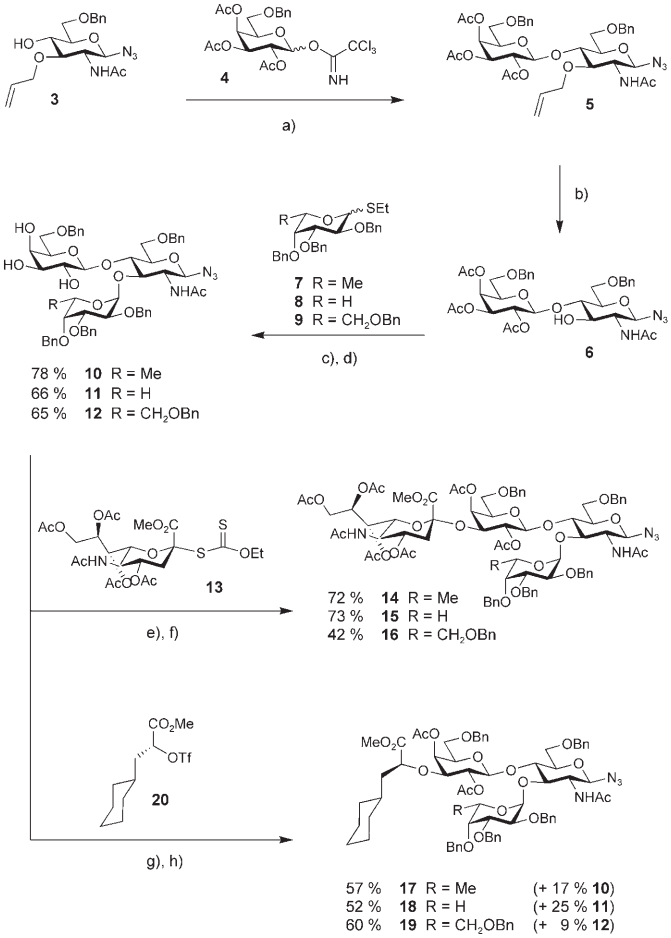
and, therefore, serves as the lead structure for the development of selectin inhibitors.[1e–h] Binding studies with variants of **1**,[5–7] transfer NOE NMR experiments,[8] and X-ray structure analysis[9] have shown that the hydroxy groups of fucose,[5] the 4- and 6-hydroxy groups[6] of galactose, and the carboxy function of sialic acid[7,9] are essential for an effective binding to E-selectin. The glucosamine residue is necessary for an optimal orientation of fucose and galactose.[10]

By experiments with synthetic sialyl Lewis^x glycopeptides it was revealed that affinity and selectivity of binding to P- and E-selectin depend upon the peptide part of these compounds.[11] In the natural ligand E-selectin ligand 1 (ESL-1)[12] **1** is presented at different positions. The amino acid sequence 672–681 **2** of ESL-1 containing one of five potential N-glycosylation sites[13] is highly conserved in E-selectin ligands from different species (MG160/rat/human; CFR/chicken).[14] A glycopeptide with this sequence had shown an increased affinity to E-selectin.[11c] Therefore, this peptide is considered a favorable anchor structure for systematically varied sialyl Lewis^x ligands.

G^{672}N*LTELESED^{681} **2**

Saccharide structure variants, especially in the fucose and sialic acid portions, would be valuable as anti-inflammatory agents. The biological half-time of the ligands is expected to increase by exchanging L-fucose for D-arabinopyranose[5a,11c] or L-galactose,[15] which do not occur in mammals. The same should be true for substitution of sialic acid by its mimic (*S*)-cyclohexyl lactic acid introduced by Ernst and co-workers.[16]

During the synthesis of such a set of sialyl Lewis^x glycopeptide selectin ligands, problems mainly caused by the acid lability of the fucoside bond should be solved. Because of this acid lability it has not been possible so far to



Scheme 1. a) Trimethylsilyltrifluoromethanesulfonate, CH_2Cl_2, molecular sieve (4 Å), −50 °C→20 °C, 70 %; b) 1. $[Ir^I(cod)(PMePh_2)_2]PF_6$, THF, H_2; 2. 4-toluenesulfonic acid, MeOH, 85 %; c) $CuBr_2$, nBu_4NBr, molecular sieve (4 Å), CH_2Cl_2/DMF (1:1); d) NaOMe, MeOH; e) MeSBr, AgOTf, molecular sieve (4 Å), CH_2Cl_2/CH_3CN (1:2), −50 °C→−30 °C; f) Ac_2O, dimethylaminopyridine (DMAP), pyridine; g) Bu_2SnO, MeOH, reflux; h) Ac_2O, DMAP, pyridine. cod = cycloocta-1,5-diene.

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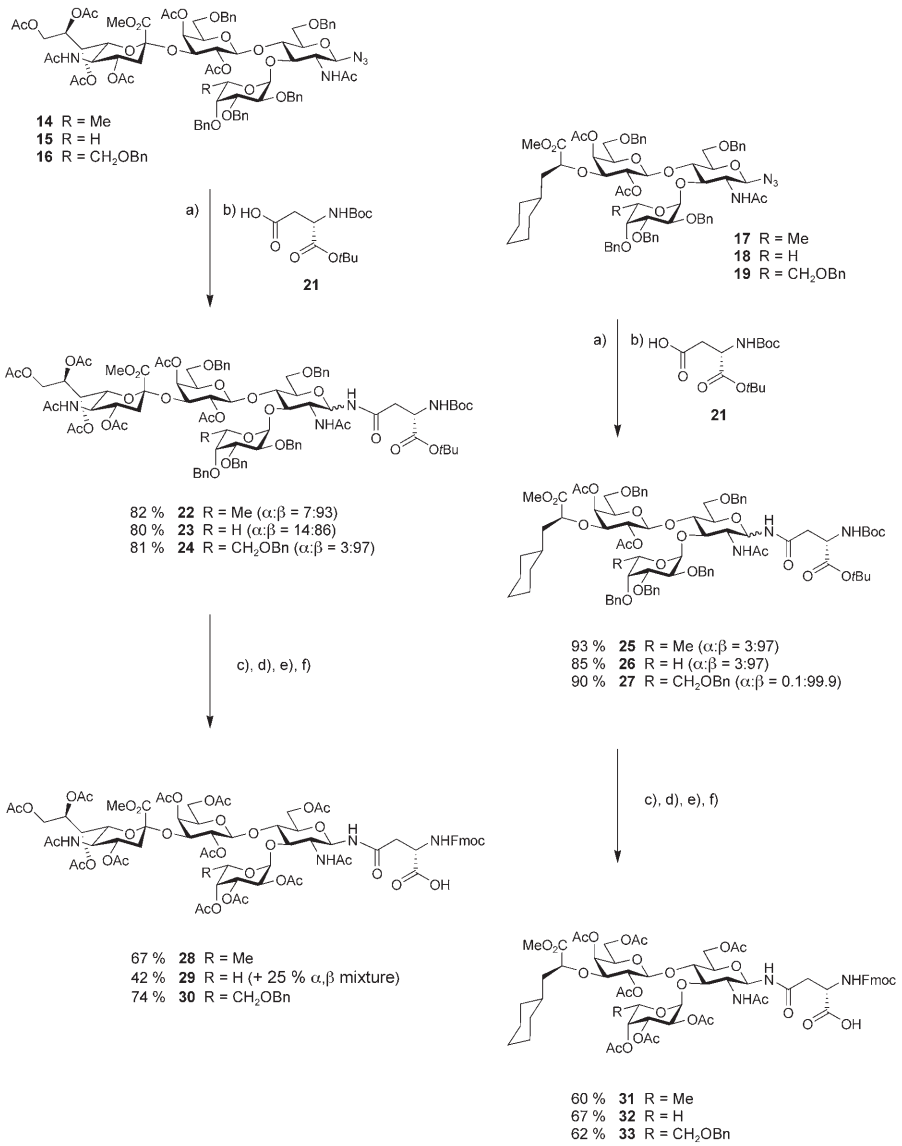
apply common acid-labile anchors and side-chain protecting groups in solid-phase syntheses of such glycopeptides.[11c,17] Benzyl protecting groups, on the other hand, have disadvantages owing to their final removal and serious side reactions (for example, aspartimide formation). Herein, we describe the synthesis of sialyl Lewis^x asparagine building blocks which can generally be used in solid-phase syntheses on acid-labile anchor groups according to Fmoc strategy.

The azido group served as the anomeric protecting group in glucosamine building block **3** in the synthesis of sialyl Lewis^x glycopeptides and mimetics.[11] Not the fucose but the β-galactosyl residue as its trichloroacetimidate **4**[18,19] was first introduced to furnish the lactosamine backbone **5** of all envisioned sialyl Lewis^x variants (Scheme 1). In this way, the steric hindrance at the 4-position of the acceptor and the acid-labile fucoside can be avoided.[11]

After catalytic removal of the allyl ether in **5**,[20] L-fucose **7**,[21] D-arabinose **8**,[11c] and L-galactose **9** building blocks were introduced under in situ anomerization conditions.[22,23] After removal of the *O*-acetyl groups, trisaccharides **10**–**12** were sialylated with sialic acid xanthate **13**.[24] Thus, tetrasaccharides **14**–**16**, which vary in the fucose part, were obtained after acetylation of the hydroxy groups. Regio- and stereoselective sialylation was achieved by activation of the xanthate **13** with methylsulfenyl triflate,[25] thereby exploiting the nitrile effect.[26] From Lewis^x azides **10**–**12**, tetrasaccharide mimetics **17**–**19** were obtained according to the stannylene methodology[27] by reaction with the cyclohexyl lactic acid triflate derivative **20**[1g,28] and subsequent acetylation.

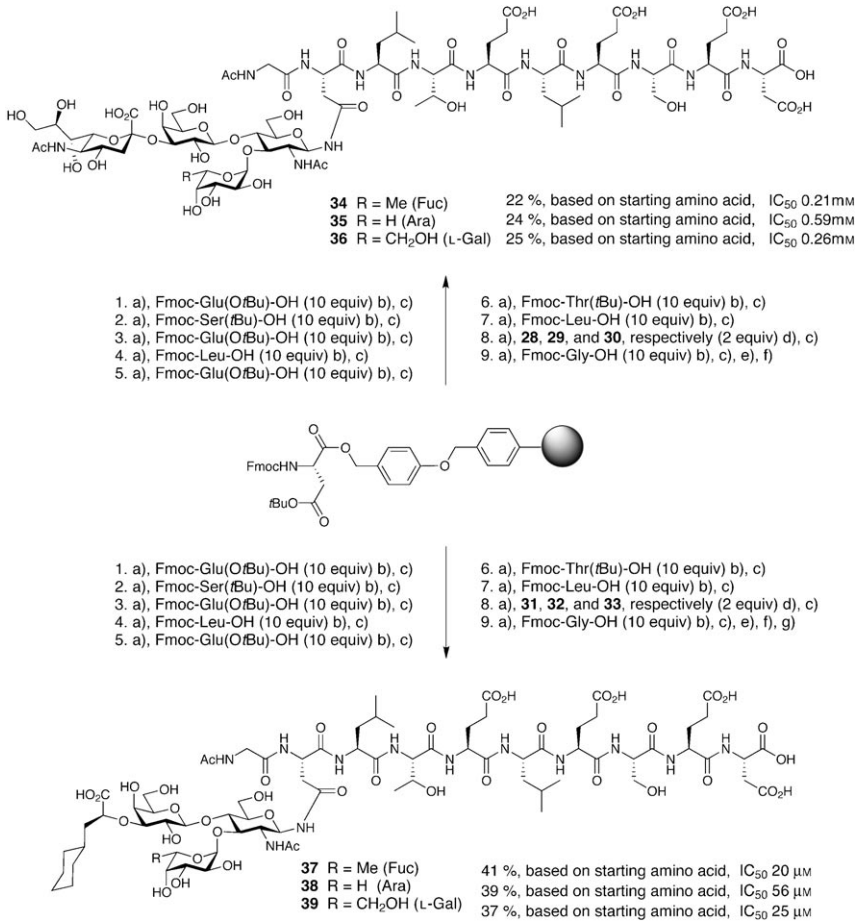


Scheme 2. a) Raney nickel, pH 7.0, isopropyl alcohol/H_2O (9:1), H_2; b) TBTU, HOBt, DMF, *i*Pr_2NEt, pH 6.5–7.5; c) Pd/C (10%), H_2, MeOH/AcOH (95:5); d) Ac_2O, DMAP, pyridine; e) TFA/CH_2Cl_2 (1:2), triethylsilane; f) FmocOSu, CH_2Cl_2, *i*Pr_2NEt, pH 9.0–9.5. TBTU = benzotriazol-1-yltetramethyluronium tetrafluoroborate; HOBt = 1-hydroxybenzotriazole; TFA = trifluoroacetic acid; FmocOSu = *N*-(fluorenylmethoxycarbonyloxy) succinimide.

For this purpose benzyl ethers were hydrogenolyzed over palladium on carbon (10%), and the resulting free hydroxy groups were acetylated to stabilize the glycosidic bonds.[29] Then, Boc and *tert*-butyl protecting groups were cleaved with trifluoroacetic acid without affecting the glycoside bonds. Selective N-acylation with FmocOSu[33] gave Fmoc(sialyl Lewis^x)asparagine **28** and variants **29**–**33**. Pure β-anomers of **28** and **31**–**33** were isolated after preparative reverse-phase HPLC, and those of compounds **29** and **30** were isolated after flash chromatography[34] and were used in the solid-phase syntheses.

Reduction of the glycosyl azides **14**–**19** through hydrogenation over neutral-washed Raney nickel[29] gave glycosyl amines, which immediately were coupled with *tert*-butyloxycarbonyl (Boc)[30] asparagin acid *tert*-butyl ester **21**[31] to furnish the protected glycosyl amino acids **22**–**27**[32] (Scheme 2). Analytical samples of pure β-anomers were isolated by using HPLC. Separation of the anomers by flash chromatography was only possible for **24**. However, separation through preparative reverse-phase HPLC was not necessary because an exchange of protecting groups was carried out to increase the acid stability of the compounds.

The automated syntheses were conducted according to the Fmoc strategy using TentaGel resin[35] loaded with aspartic acid (Scheme 3). Activation of Fmoc amino acids was performed with HBTU[36] and HOBt,[37] and activation of



Scheme 3. a) Piperidine/NMP (1:4); b) HBTU, HOBt, DMF, iPr$_2$NEt; c) Ac$_2$O, iPr$_2$NEt, cat. HOBt, NMP; d) HATU, HOAt, NMM, NMP; e) TFA/CH_2Cl_2 (1:1), TIS (2.5%), H_2O (2.5%); f) NaOMe, MeOH, pH 8.5–9.5; g) 1. $NaOH_{aq}$, pH 9.5–10.5; 2. $AcOH_{aq}$. HBTU = *O*-(benzotriazolyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HATU = *O*-(7-azabenzotriazolyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HOAt = 7-aza-1-hydroxybenzotriazole; TIS = triisopropylsilane; NMP = *N*-methylpyrrolidin-2-one; NMM = *N*-methylmorpholine.

glycosyl amino acids **28**–**33** with HATU[38] and HOAt.[39] The glycopeptides were detached from the resin by using TIS in trifluoroacetic acid/dichloromethane (1:1). During this procedure, all *tert*-butyl protecting groups of the side-chain functions were also cleaved. The *O*-acetyl groups of the carbohydrate side chains were removed under Zemplén conditions.[40] The methyl esters of sialic acid and of cyclohexyl lactic acid were saponified in aqueous sodium hydroxide (pH 9.5–10.5). After neutralization with acetic acid, the target compounds **34**--**39** were isolated by preparative reverse-phase HPLC in yields of 22–41% based on the resin-bound starting amino acid. The structures of all glycopeptides were confirmed by high-resolution mass spectrometry and one- and two-dimensional NMR spectroscopy.[41]

The inhibitory properties of these glycopeptides towards E-selectin were determined in flow-cytometry experiments as IC_{50} values in the inhibition of binding of an E-selectin-IgG construct[42] to the murine neutrophile cell line 32Dcl3.[43] The sialyl Lewis^x containing glycopeptide **34** showed an IC_{50} value of 0.21 mM, which is lower by a factor of 13 than that of sialyl Lewis^x **1** as the standard. Substitution of enzyme-labile fucose by L-galactoses that are more stable in mammals (**36**) caused almost no loss of affinity ($IC_{50} = 0.26$ mM). In the case of **35**, in which L-fucose is substituted by D-arabinose, inhibition is decreased by a factor of two ($IC_{50} = 0.59$ mM). Exchange of sialic acid (in **34**–**36**) by (*S*)-cyclohexyl lactic acid in glycopeptides **37**–**39** decreased the IC_{50} values by an additional factor of ten (**37**: $IC_{50} = 20$; **38**: $IC_{50} = 56$; **39**: $IC_{50} = 25$ µM). The glycopeptide **39** inhibits the adhesion between the murine neutrophile cell line 32Dcl3 expressed by E-selectin ligand[42] and E-selectin more than 100-fold stronger than sialyl-Lewis^x (**1**), although **39** contains the metabolically more stable L-galactose instead of fucose, which usually is thought to be essential.[5,8,9]

These properties are of interest for the development of practically applicable inhibitors of inflammatory processes and tumor metastasis. In the course of this work, a more economic synthesis for sialyl Lewis^x amino acids was developed by forming the common lactosamine backbone first. By means of an exchange of benzyl for acetyl protecting groups sufficiently acid-stable building blocks were synthesized that are generally applicable in solid-phase syntheses by the Fmoc strategy.

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[32] Pure β-anomer $[\alpha]_D^{23}$ [deg cm^3 g^{-1} dm^{-1}] ($c = 1.0$ g cm^{-3} in $CHCl_3$): **22**: −44; **23**: −57; **24**: −38; **25**: −56; **26**: −64; **27**: −44.

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[34] $[\alpha]_D^{23}$ [deg cm^3 g^{-1} dm^{-1}] ($c = 1.0$ g cm^{-3} in $CHCl_3$): **28**: −27; **29**: −35; **30**: −32; **31**: −32 (in MeOH); **32**: −43; **33**: −32; ^{1}H NMR (400 MHz, $CDCl_3$, ^{1}H-^{1}H-COSY): **28**: $\delta = 5.31$ (d, 1H, $J_{3,4} = 2.7$ Hz, Fuc-4), 4.98 (m, 1H, GlcNAc-1), 4.95 (d, 1H, $J_{3,4} = 2.7$ Hz, Gal-4), 2.55 ppm (dd, 1H, $J_{eq,ax} = 12.3$ Hz, $J_{3,4} = 3.7$ Hz, Sial-3eq); **29**: $\delta = 5.53$ (d, 1H, $J_{1,2} = 2.7$ Hz, Ara-1), 5.00 (t, 1H, $J_{1,2} \approx J_{1,NH} = 9.2$ Hz, GlcNAc-1), 4.70 (d, 1H, $J_{1,2} = 7.8$ Hz, Gal-1), 2.55 ppm (dd, 1H, $J_{eq,ax} = 12.1$ Hz, $J_{3,4} = 3.9$ Hz, Sial-3eq); **30**: $\delta = 5.67$–5.63 (m, 2H, L-Gal-4, L-Gal-1), 5.17 (m, 1H, GlcNAc-1), 4.95 (d, 1H, $J_{3,4} = 3.5$ Hz, Gal-4), 2.47 ppm (dd, 1H, $J_{eq,ax} = 12.1$ Hz, $J_{3,4} = 3.9$ Hz, Sial-3eq); **31**: $\delta = 5.58$ (m, 2H, Fuc-1, Gal-4); 4.93 (m, 1H, GlcNAc-1), 4.05 ppm (dd, $J_{vic} = 5.1$ Hz, $J_{vic} = 7.4$ Hz, C*H*-COOMe); **32**: $\delta = 5.56$ (m_{small}, 2H, Ara-1, Gal-4), 4.98 (m, 1H, GlcNAc-1), 4.93 (dd, 1H, $J_{1,2} = 3.1$ Hz, $J_{2,3} = 11.0$ Hz, Ara-2), 4.05 ppm (dd, 1H, $J_{vic} = 5.5$ Hz, $J_{vic'} = 7{,}0$ Hz, C*H*-COOMe); **33**: $\delta = 5.65$ (db, 1H, $J_{3,4} = 2.7$ Hz, L-Gal-4), 5.56 (db, 1H, $J_{1,2} = 3.1$ Hz, L-Gal-1), 5.07 (m, 1H, GlcNAc-1), 4.25 ppm (d, 1H, $J_{1,2} = 8.2$ Hz, Gal-1), 1.48 (m, 2H, CH_2-CHCOOMe); HR-ESI-MS: *m*/*z*: $[M+Na]^+$ (calcd): **28**: 1655.5310 (1655.5287); **29**: 1641.5093 (1641.5131); **30**: 1713.5386 (1713.5342); **31**: 1350.4924 (1350.4904); **32**: 1336.4713 (1336.4748); **33**: 1408.4906 (1408.4959).

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[41] Glycopeptide **34**–**39**: amount, $[\alpha]_D^{23}$ [deg cm^3 g^{-1} dm^{-1}] ($c = 0.5$ g cm^{-3} in H_2O), HR-ESI-MS (with 0.1% TFA) $[M+Na]^+$ (calcd): **34**: 42 mg, −54, 1972.7614 (1972.7634); **35**: 46 mg, −47, 1958.7511 (1958.7477); **36**: 49 mg, −45, 1988.7568 (1988.7583); **37**: 75 mg, −60, 1835.7638 (1835.7673); **38**: 70 mg, −29, 1799.7725 (1799.7698, $[M+H]^+$); **39**: 67 mg, −28 (in MeOH), 1829.7859 (1829.7803, $[M+H]^+$). The structures were confirmed by 600-MHz ^{1}H NMR and 150.9-MHz ^{13}C NMR spectroscopy.

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[43] In a dilution series (0–6 mM), the glycopeptides and the E-selectin-IgG construct[42] (25 μg mL^{-1}) were incubated in cell culture media (pH 7.4) for one hour at 4 °C. The murine neutrophile cell line 32Dcl3 was mixed with the glycopeptide/E-selectin-IgG solutions. After one hour at 4 °C, the cells were incubated with a phycoerythrin-linked anti-IgG antibody for 30 minutes at 4 °C. The bound E-selectin-IgG was analyzed by measuring the phycoerythrin fluorescence in a flow cytometer (FACSCanto, Becton-Dickinson). IC_{50} values were derived from binding curves.

