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Total Synthesis

Total Synthesis of the Glycopeptide Recognition Domain of the P-Selectin Glycoprotein Ligand 1**

*Katharina Baumann, Danuta Kowalczyk, and Horst Kunz**

During the recruitment of leukocytes into inflamed tissues in acute and chronical inflammatory processes, the carbohydrate-recognizing receptors P- and E-selectin exposed on the activated endothelium and L-selectin on the leukocytes play most important roles.[1] A similar situation holds true for the metastasis of tumor cells.[2] The intrusion of malign T cells into the skin could be more readily influenced through ligands of P-selectin than ligands of E-selectin. Therefore, in this case the inhibition of P-selectin provided the more promising antitumor strategy.[3] In contrast to the natural ligands of E-selectin,[4] it is known for the P-selectin glycoprotein ligand 1 (PSGL-1) that in addition to the essential tetrasaccharide sialyl Lewis^x the N-terminal peptide sequence significantly contributes to the recognition epitope.[5] Within the PSGL-1 the binding site for binding to P-selectin has been identified by biochemical and molecular biological methods (Figure 1).

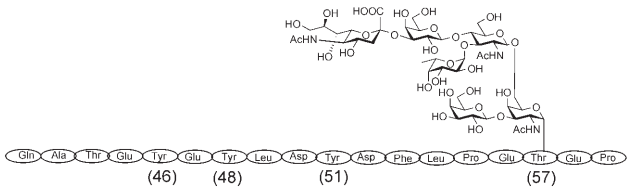

Figure 1. Binding site of P-selectin glycoprotein ligand 1 (PSGL-1) for binding to P-selectin.

It is located in the N-terminal portion, which, after cleavage of a signal peptide, begins with Gln^{42}. At least one of the tyrosine residues Tyr^{46}, Tyr^{48}, and Tyr^{51} should be O-sulfated. The threonine in position 57 carries the *O*-glycan side chain, which consists of a combination of a sialyl Lewis^x ligand and the core 2 structure of *O*-glycoproteins (mucins).

Chemoenzymatic syntheses of partial sequences[6] and of the entire binding site of PSGL-1[7] have been described. Wong et al.[6] synthesized the glycopeptide Tyr^{51}–Glu^{58} which was O-glycosylated at Thr^{57} with the disaccharide GlcNAcβ-(1–6)-αGalNAc. After chemical sulfation of the tyrosine, the sialyl Lewis^x structure was assembled at the GlcNAc residue by enzymatic glycosylation reactions. Cummings et al.[7] conducted the chemical solid-phase synthesis of the glycopeptide Gly^{41}–Leu^{63} carrying an αGalNAc residue, and they subsequently performed all glycosylation reactions and the final sulfation using the corresponding enzymes. They report a high affinity of the glycopeptide ligand, which was obtained in micrograms quantities, to P-selectin ($K_d = 350$ nM). This affinity nearly equalled that measured for PSGL-1 isolated from neutrophiles (300 nM). These results give evidence that the recognition site of PSGL-1 constitutes a particularly interesting structure for detailed model studies of cell adhesion phenomena. This holds even more so, as it was recently found that the bacterium *Anaplasmosis phagocytophylum*, which causes human granulocytoplasmosis (Ehrlichiosis), expresses a receptor analogous to P-selectin which binds the PSGL-1 ligand in the non-sulfated form.[8] In addition, it was shown that the separate presentation of partial structures of PSGL-1 as sialyl Lewis^x and *O*-sulfated tyrosine in conjugates of polyacrylamide can result in a high affinity to L-selectin.[9] Considering these significant functions of the natural P-selectin ligand in the regulation of cell adhesion and in their aberrant forms causing severe diseases, we deemed that a chemical synthesis of the recognition domain of PSGL-1 on a preparative scale would be desirable. Such a synthesis would provide sufficient amounts of exactly specified compounds for model investigations of cell adhesion processes and for enzymatic modifications, for example the O-sulfation of tyrosine, as well as for structural investigations. Furthermore, the chemical synthesis would pave the way for the preparation of structural mimics that are more resistant towards biological degradation, as was recently shown for glycopeptide ligands of E-selectin.[10]

The synthesis of the protected sialyl Lewis^x–core2–threonine building block **I** certainly is the most challenging problem in the chemical construction of the recognition site of PSGL-1. According to the retrosynthetic analysis (Scheme 1) **I** should be accessible from the sialyl Lewis^x donor **II** and the partially deprotected T antigen–threonine conjugate **III**, which is known from syntheses of tumor-associated glycopeptide antigens.[11]

The N-protecting group of the glucosamine is decisive for the β-selective block glycosylation of acceptor **III** with donor **II**. Based on previous experience,[12] the 2,2,2-trichloroethoxycarbonyl (Troc) group was applied; it should prevent the formation of a less reactive oxazoline. Sufficient reactivity of the donor should be guaranteed by the trichloroacetimidate group according to Schmidt and Michel.[13a] The regioselective glycosylation at the 6-OH group can be expected in agreement with numerous previous examples.[11,14] The axial 4-OH group is of low reactivity, in particular, in molecules carrying a

[*] Dr. K. Baumann, D. Kowalczyk, Prof. Dr. H. Kunz
Institut fuer Organische Chemie
Johannes Gutenberg-Universitaet Mainz
Duesbergweg 10–14, 55128 Mainz (Germany)
Fax: (+49) 6131-392-4786
E-mail: hokunz@mail.uni-mainz.de

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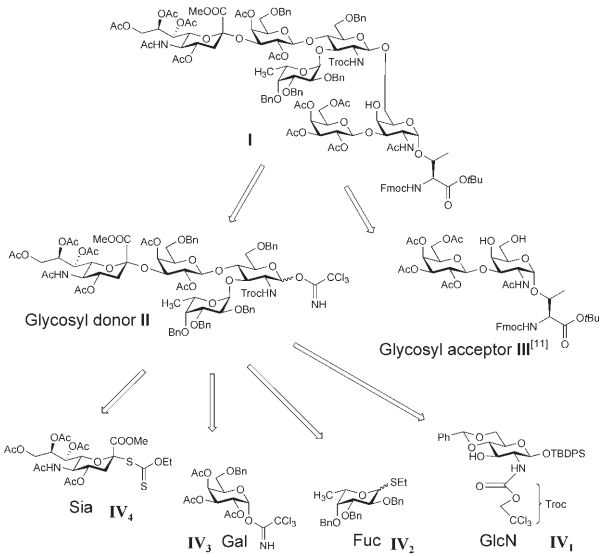
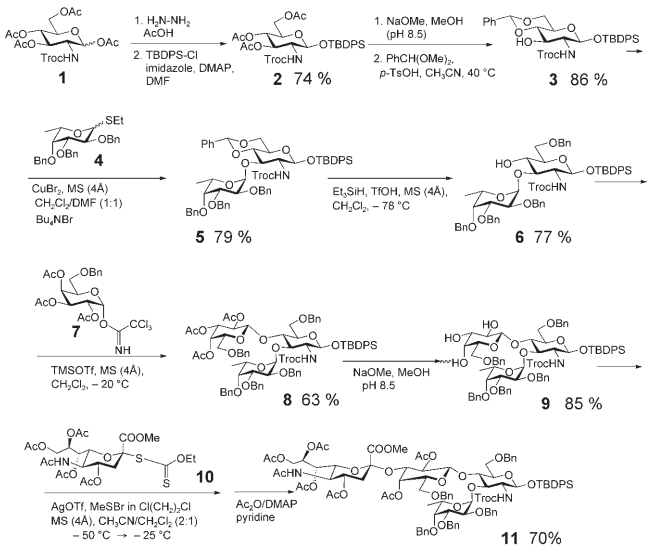


Scheme 1. Retrosynthesis of the sialyl Lewis^x—T antigen–threonine building block **I**. Bn = benzyl, Fmoc = 9-fluorenylmethoxycarbonyl, Troc = trichlorethoxycarbonyl, TBDPS = *tert*-butyldiphenylsilyl.



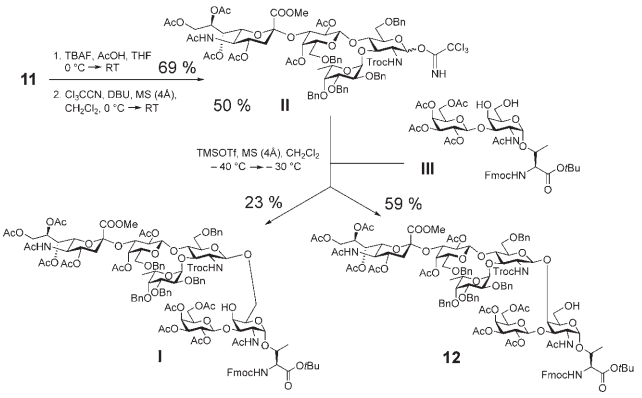
Scheme 2. Synthesis of the sialyl Lewis^x glycosyl donor. DMAP = 4-dimethylaminopyridine, TMSOTf = trimethylsilyl trifluoromethansulfonate.

large substituent at O-3. The protecting group at the anomeric oxygen of the glucosamine building block $\mathbf{IV_1}$ is also of importance. This group must be stable under acidic (during glycosylation reactions) and basic conditions (during removal of *O*-acetyl groups). However, finally it must be selectively removable and, thus, make it possible to introduce the trichloroacetimidate, giving **II**. The *tert*-butyldiphenylsilyl group[15] was supposed to meet these requirements. The other monosaccharide building blocks $\mathbf{IV_2}$–$\mathbf{IV_4}$ have already been used successfully in syntheses of sialyl Lewis^x derivatives.[10]

For the construction of the sialyl Lewis^x donor **II** (Scheme 2), peracetylated *N*-trichloroethoxycarbonyl glucosamine **1** was deacetylated at O-1 by treatment with hydrazine acetate.[12,16] The product was subsequently silylated with *tert*-butyldiphenylsilyl chloride (TBDPS-Cl)[15] to give **2**. After removal of the *O*-acetyl groups by mild transesterification with catalytic NaOMe in methanol, the 4,6-*O*-benzylidene acetal was introduced. Product **3** served as the glycosyl acceptor in the reaction with thiofucoside[17] **4** under modified in situ anomerization conditions.[18] Regioselective opening of acetal **5** was performed with triethylsilane/trifluoromethanesulfonic acid[19] to give **6**, which was subsequently glycosylated with galactosyl trichloroacetimidate[13] **7** to furnish the Lewis^x trisaccharides **8**.

The *O*-acetyl groups were removed from **8** by mild transesterification in methanol (see above). The sialic acid moiety was regio- and stereoselectively introduced to the resulting Lewis^x derivative **9** according to a previously described method[10,11,20] using xanthate **10**.[21] Acetylation of the 2- and 4-OH groups of the galactose portion furnished sialyl Lewis^x tetrasaccharide **11**. Remarkably, the TBDPS group remained unaffected during all these conversions (Scheme 2).

In order to transform tetrasaccharide **11** into a glycosyl donor, the TBDPS group was removed by treatment with tetrabutylammonium fluoride (TBAF) in tetrahydrofuran/acetic acid (100:1). Subsequent reaction with trichloroacetonitrile[13] in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) resulted in formation of sialyl Lewis^x trichloroacetimidate **II** (Scheme 3). Both reactions proceeded slowly with the sterically demanding substrate.

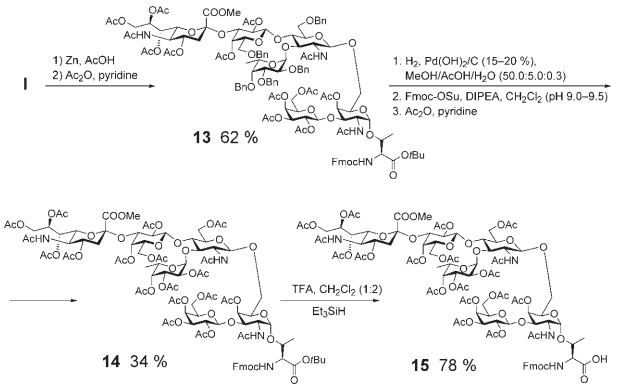


Scheme 3. Block glycosylation of a T antigen–threonine conjugate with a sialyl Lewis^x trichloroacetimidate.

The block glycosylation of the partially deprotected βGal(1–3)αGalNAc–threonine derivative **III**,[11] carrying the Fmoc/*t*Bu ester protecting-group combination, with SLe^x trichloroacetimidate **II** constitutes the key step of the overall synthesis. It led to products **I** and **12**, which have identical molecular weight, in a total yield of more than 80 %. The isomers could be separated by flash chromatography. Two-dimensional NMR spectroscopy (HSQC and HMQC spectra) revealed, in particular through the position of the ^{13}C signal for C-6 of the GalNAc moiety ($\delta = 69.2$ ppm for **I**, $\delta =$ 59.2 ppm for **12**), that the major product **12** had been

formed by an entirely unexpected glycosylation at the axial 4-OH group of T antigen–threonine derivative **III**. This regioselectivity is in contradiction to every previous observation[11,14,20] and shows that unexpected effects can arise in the course of slowly proceeding glycosylation reactions between large building blocks. Fortunately, the desired compound **I** was isolated in a sufficient amount (0.66 g) so that the synthesis of the glycopeptide of the recognition domain (Figure 1) could be continued.

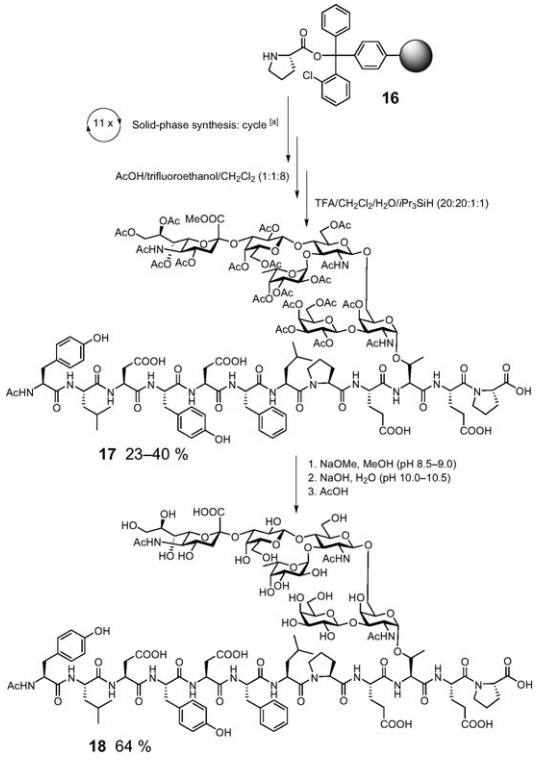
Starting from **I**, an Fmoc-protected glycosyl threonine building block is not directly accessible since its α-fucoside structure is sensitive even to dilute trifluoracetic acid in dichloromethane. It is indispensable to stabilize this structure towards acids by an exchange of the *O*-benzyl for the *O*-acetyl protecting groups.[10,22] Fist, however, the removal of the *N*-Troc group had to be carried out by reductive elimination with zinc,[12] otherwise, the Troc group would lose chloro substituents during the hydrogenation reaction. After N-acetylation, the benzyl ethers of product **13** were hydrogenolytically cleaved. Monitoring this conversion indicated that a simultaneous loss of the Fmoc protecting group occurred. Therefore, selective re-introduction of the Fmoc group using Fmoc-*O*-hydroxysuccinimide (Fmoc-OSu) was necessary prior to the O-acetylation to give **14** (Scheme 4).[23]



Scheme 4. Protecting-group manipulations giving an acid-stable Fmoc-glycosyl–threonine building block for the solid-phase synthesis.

Compound **14** is sufficiently stable towards acids, and its *tert*-butyl ester can be cleaved selectively with trifluoroacetic acid (TFA). The Fmoc(sialyl Lewis^x–T antigen)–threonine building block **15**, suitable for the solid-phase synthesis of the glycopeptide was obtained in a pure form. Compound **15** was used for the solid-phase synthesis of the sequence Tyr^{48}–Pro^{59} of PSGL-1 which was performed according to the Fmoc strategy starting from polystyrene resin **16**,[24] which was loaded with proline attached by means of the Barlos linker.[25]

For Fmoc-amino acids, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt),[26] and diisopropylethylamine (DIPEA) in DMF served as coupling reagents, whereas the more reactive mixture *O*-(7-azabenotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU), 7-aza-1-hydroxybenzotriazol (HOAt),[27] and *N*-methylmorpholine (NMM) in *N*-methylpyrrolidinone (NMP) was applied for activation of the demanding Fmoc-glycosyl–threonine **15** (Scheme 5). Coupling of **15** required a reaction time of 8 h. The amino acids Glu, Pro, and Leu following the glycosyl threonine were attached in a double coupling reaction (one repetition). After completion of the glycopeptide sequence



Scheme 5. Solid-phase synthesis of the *O*-glycopeptide of the P-selectin binding site of PSGL-1. [a] Solid-phase synthesis cycle: 1) Fmoc removal: piperidine/NMP (1:4); 2) amino acid coupling: Fmoc-AA-OH, HBTU, HOBt, DIPEA, DMF; in the case of **15**: HATU, HOAt, NMM, NMP; 3) Capping: Ac_2O, DIPEA, HOBt, NMP.

and final N-acetylation, the linker was cleaved with acetic acid/trifluoroethanol in dichloromethane[25] without affecting the *tert*-butyl esters and ethers. These groups were subsequently removed by treatment with trifluoroacetic acid to yield glycopeptide **17**. Preparative HPLC was employed to separate the C-terminal dipeptide and the glycosyltripeptide as the by-products from **17**, which was isolated as the main fraction (23 %) besides compounds identical in molecular weight and showing very similar retention times (17 %). Since re-injection of these compounds gave an HPLC profile very similar to that of crude **17**, they are probably conformers or different salt forms of **17**. The main fraction was treated with catalytic NaOMe in methanol (pH > 9), but this did not result in complete removal of the *O*-acetyl groups. During the subsequent hydrolysis of the sialic acid methyl ester with NaOH in water at pH 10.5[20] (the pH has to be maintained carefully, otherwise β elimination of the entire glycan may occur), the remaining *O*-acetyl groups were removed. After neutralization with acetic acid, the pure sialyl Lewis^x–T antigen–glycopeptide **18**[28] of the binding site of P-selectin

ligand PSGL-1 was isolated as 31 mg of a colorless lyophilisate.

In the total synthesis of PSGL-1 binding epitope **18**, problems accumulate that complicate the chemical glycopeptide synthesis. In order to stereoselectively install the α-fucoside structure, a benzyl ether protected fucosyl donor must be used. However, the resulting fucoside bond is acid-labile and not compatible with the conditions needed for the cleavage of *tert*-butyl groups during the solid-phase synthesis. In turn, the necessary exchange of the *O*-benzyl for *O*-acetyl protecting groups does not allow protection of the sialic acid, whose chemistry already is distinctly difficult, as a benzyl ester.[29] Hence, the sialic acid methyl ester must be hydrolyzed after the long synthesis in basic media without effecting a β elimination of the entire glycan.[30]

The value of the synthesis of such a complex and sensitive compound as **18** is not only that sufficient amounts of material are provided for the investigation of its structure and for its use in biological model studies; such a total synthesis also discloses a strategy for the synthesis of structural analogues that may have modified effects and potentially improved biological stability. These options are generally not provided by enzymatic syntheses. In the case of the PSGL-1 binding site glycopeptide **18**, its use as a substrate for tyrosine *O*-sulfatyl transferases and for the determination of the inhibitory potential of both the unsulfated and the sulfated compounds toward P-selectin will be investigated.

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[23] **14**: Yield: 165 mg (34% based on **13**); colorless amorphous solid; $[\alpha]_D^{23} = -16\ \mathrm{deg\,cm^3\,g^{-1}\,dm^{-1}}$ ($c = 1.0\ \mathrm{g\,cm^{-3}}$, $CHCl_3$); $R_f =$ 0.54 (CH_2Cl_2/MeOH, 25:2), $R_f = 0.19$ (CH_2Cl_2/MeOH, 50:3); Analyt. HPLC: $R_t = 35$ min (Phenomenex Luna C18, gradient: CH_3CN/H_2O 5:95→100:0 in 40 min; $\lambda = 214$ nm). ^{1}H NMR [^{1}H-^{1}H COSY, TOCSY] (400 MHz, $CDCl_3$): $\delta = 5.69$ (d, 1H, $^3J_{NH,Thr\alpha} = 8.6$ Hz, NH-Thr), 5.50 (m_c, 1H, H8-Sia), 5.44 (dd, 1H, $^3J_{H7,H6} = 2.7$ Hz, $^3J_{H7,H8} = 9.7$ Hz, H7-Sia), 5.36–5.34 (m, 2H, H1-Fuc {5.36, d, $^3J_{H1,H2} = 3.9$ Hz}, H4-Gal′ {5.34, d, $^3J_{H4,H3} =$ 3.1 Hz}), 5.31 (d, 1H, $^3J_{H4,H3} = 2.4$ Hz, H4-Fuc), 5.25 (s_b, 1H, H4-GalN), 5.20 (dd, 1H, $^3J_{H3,H2} = 10.9$ Hz, $^3J_{H3,H4} = 3.2$ Hz, H3-Fuc), 5.12 (d, 1H, $^3J_{NH,H5} = 10.2$ Hz, NH-Sia), 5.06 (dd, 1H, $^3J_{H2,H1} = 7.7$ Hz, $^3J_{H2,H3} = 10.5$ Hz, H2-Gal′), 5.00–4.84 (m, 6H, H2-Fuc {4.98}, H4-Gal {4.94}, H5-Fuc {4.93}, H3-Gal′ {4.92}, H2-Gal {4.89}, H4-Sia {4.87}), 4.80 (d, 1H, $^3J_{H1,H2} = 2.9$ Hz, H1-GalN), 4.70 (d, 1H, $^3J_{H1,H2} = 8.0$ Hz, H1-Gal), 4.63 (d, 1H, $^2J_{H6a,H6b} = 11.1$ Hz, H6a-GlcN), 4.56–4.43 (m, 6H, H1-GlcN {4.55}, H1-Gal′ {4.55}, H3-Gal {4.53}, CH_{2a}-Fmoc {4.53}, H2-GalN {4.48}, CH_{2b}-Fmoc {4.45}), 4.36 (dd, 1H, $^3J_{H6a,H5} = 6.7$ Hz, $^2J_{H6a,H6b} = 11.5$ Hz, H6a-Gal), 3.43 (m_c, 1H, H6b-GalN), 2.57 (dd, 1H, $^2J_{H3eq,H3ax} = 12.6$ Hz, $^3J_{H3eq,H4} = 4.6$ Hz, $H3_{eq}$-Sia), 1.27 (d, 3H, $^3J_{Thr\gamma,Thr\beta} = 5.9$ Hz, Thr$^\gamma$), 1.16 ppm (d, 3H, $^3J_{H6abc,H5} = 6.2$ Hz, H6a,b,c-Fuc). ^{13}C NMR [BB, HSQC, HMBC] (100.6 MHz,$CDCl_3$): [$\delta = 100.67$ (C1-GlcN, C1-Gal′), 99.84 (C1-Gal), 99.25 (C1-GalN), 96.72 (C2-Sia), 95.22 (C1-Fuc), 83.08 ($C(CH_3)_3$), 75.60 (Thr$^\beta$), 67.79 (C6-GalN), 58.92 (Thr$^\alpha$), 18.58 (Thr$^\gamma$), 15.80 ppm (C6-Fuc). ESI-MS (positive): $m/z =$ 1148.5 ($[M+2Na]^{2+}$, calcd.: 1148.4), 2273.9 ($[M+Na]^+$, calcd.: 2273.8).

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[28] **18**: Yield: 31 mg (64 %); colorless lyophilisate; $[\alpha]_D^{23} = -23\ \mathrm{deg\,cm^3\,g^{-1}\,dm^{-1}}$ ($c = 0.96\ \mathrm{g\,cm^{-3}}$, DMSO); Analyt. HPLC: $R_t = 13$ min (Phenomenex Jupiter C18, gradient: CH_3CN/H_2O + 0.1 % TFA 10:90→100:0 in 40 min; $\lambda = 214$ nm). ^{1}H NMR [^{1}H-^{1}H COSY, TOCSY] (400 MHz, [D$_6$]DMSO): [$\delta = 8.32$ (db, 1H, $^3J_{NH,Glu1\alpha} = 6.0$ Hz, NH-Glu$_1$), 8.23–8.19 (m, 2H, NH-Leu$_1$ {8.22, d, $^3J_{NH,Leu1\alpha} = 8.4$ Hz}, NH-Asp$_1$ {8.20, d, $^3J_{NH,Asp1\alpha} = 7.8$ Hz}), 8.14 (d, 1H, $^3J_{NH,Asp2\alpha} = 7.4$ Hz, NH-Asp$_2$), 8.07 (d, 1H, $^3J_{NH,Glu2\alpha} = 7.4$ Hz, NH-Glu$_2$), 8.03–7.93 (m, 4H, NH-GlcN {8.02, d, $^3J_{NH,H2} = 8.8$ Hz}, NH-Tyr$_1$ {7.98, d, $^3J_{NH,Tyr1\alpha} = 8.2$ Hz}, NH-Leu$_2$ {7.98, d, $^3J_{NH,Leu2\alpha} = 8.2$ Hz}, NH-Sia {7.94, d, $^3J_{NH,H5} = 7.6$ Hz}), 7.85 (d, 1H, $^3J_{NH,Thr\alpha} = 8.2$ Hz, NH-Thr), 7.70–7.64 (m, 2H, NH-Tyr$_2$ {7.69, d, $^3J_{NH,Tyr2\alpha} = 7.6$ Hz}, NH-Phe {7.65, d, $^3J_{NH,Phe\alpha} = 7.2$ Hz}), 4.80 (d, 1H, $^3J_{H1,H2} = 2.9$ Hz, H1-Fuc), 4.71–4.65 (m, 2H, H1-GalN {4.70, d, $^3J_{H1,H2} = 3.3$ Hz}, H5-Fuc {4.67}), 1.07 (d, 3H, $^3J_{Thr\gamma,Thr\beta} = 5.6$ Hz, Thr$^\gamma$), 0.97 (d, 3H, $^3J_{H6abc,H5} = 6.2$ Hz, H6a,b,c-Fuc), 0.88–0.81 ppm (m, 12H, Leu$_1^{\delta}{}_{abc}$, Leu$_2^{\delta}{}_{abc}$). 100.6 MHz-^{13}C NMR [BB, HSQC] (100.6 MHz, [D$_6$]DMSO): [$\delta = 104.79$ (C1-Gal′), 102.52 (C1-Gal), 100.92 (C1-GlcN), 99.06 (C1-Fuc), 98.98 (C1-GalN), 98.61 (C2-Sia), 76.00 (Thr$^\beta$), 59.25 (Pro$_1^\alpha$), 58.56 (Pro$_2^\alpha$), 56.09 (Thr$^\alpha$), 55.21 (C2-GlcN), 54.50 (Tyr$_1^\alpha$), 54.25 (Tyr$_2^\alpha$), 53.45 (Phe$^\alpha$), 52.22 (C5-Sia), 51.78 (Glu$_1^\alpha$/Glu$_2^\alpha$), 51.05 (Leu$_2^\alpha$), 49.85, 49.77, 48.79 (Asp$_1^\alpha$, Asp$_2^\alpha$, Leu$_1^\alpha$, Glu$_1^\alpha$/Glu$_2^\alpha$), 18.80 (Thr$^\gamma$), 16.64 ppm (C6-Fuc). HR-ESI-MS (positive, + 0.1 % TFA): $m/z = 1356.5509$ ($[M+1+2H]^{2+}$, calcd.: 1356.5550).
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