Supporting Information Material for:

A Nanomolar Multivalent Ligand as Entry Inhibitor of the Hemagglutinin of Avian Influenza

Moritz Waldmann,[†] Raffael Jirmann,[†] Ken Hoelscher,[†] Martin Wienke,[†] Felix C. Niemeyer,[†] Dirk Rehders,[‡] and Bernd Meyer*[,†]

†Department of Chemistry, University of Hamburg, 20146 Hamburg, Germany

‡Joint Laboratory for Structural Biology of Infection and Inflammation, Institute of Biochemistry and Molecular Biology, University of Hamburg and Institute of Biochemistry, University of Luebeck, c/o Deutsches Elektronen-Synchrotron (DESY), Notkestrasse 85, 22607 Hamburg, Germany

* To whom correspondence should be addressed

E-mail: bernd.meyer@chemie.uni-hamburg.de

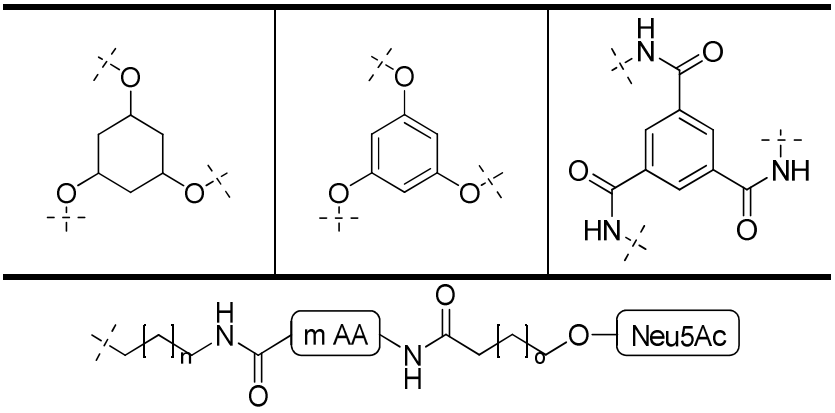
Content

Modeling

Sybyl X1.1 was used for molecular modeling and docking experiments. Gasteiger-Marsili charges, Tripos force fields and a dielectric constant of 15 were used. The crystal structure of 1JSO [1] shows hemagglutinin co-crystallized with sialic acid at a good resolution while 2IBX [2] shows the apo protein with a lower resolution. The crystal structures were aligned, hydrogen atoms added and defects in the protein structure were repaired. The ligands were prepositioned, the complex energetically minimized and the binding energies calculated. Binding energy was calculated by following equation: $E_{binding} = E_{PL}-E_P-E_L$, with E_{PL} = energy of protein-ligand complex, E_P = energy of the protein and E_L = energy of the ligand

The optimized ligand complexes were used to identify structural requirements. Results of all docked structures are summarized in the following table.

Table S 1. Summary of the *in silico* results. Different scaffolds as well as different peptidic linker length (m) and alkyl chain length (n and o) were docked and binding energies calculated by the equation $E_{PL}-E_P-E_L$, with E_{PL} = energy of protein-ligand complex, E_P = energy of the protein and E_L = energy of the ligand. The energies are expressed relative to the ligand with the best binding energy.



calc. binding energy		calc. binding energy		calc. binding energy	
n = 3 m = 8 o = 1	7.0 [kcal/mol]	n = 3 m = 8 o = 1	8.5 [kcal/mol]	n = 4 m = 8 o = 1	18.0 [kcal/mol]
n = 3 m = 8 o = 2	14.0 [kcal/mol]	n = 3 m = 8 o = 2	15.7 [kcal/mol]	n = 4 m = 8 o = 2	17.0 [kcal/mol]
n = 3 m = 9 o = 2	1.1 [kcal/mol]	n = 3 m = 9 o = 2	1.3 [kcal/mol]	n = 4 m = 9 o = 2	0.0 [kcal/mol] ligand **1**
n = 2 m = 9 o = 2	1.5 [kcal/mol]	n = 2 m = 9 o = 2	2.3 [kcal/mol]	n = 3 m = 9 o = 2	3.4 [kcal/mol]
n = 4 m = 9 o = 2	0.2 [kcal/mol]	n = 4 m = 9 o = 2	1.1 [kcal/mol]	n = 5 m = 9 o = 2	4.4 [kcal/mol]

For MD simulation of H7 a trimeric structure (pdb code: 3M5G) [3] was loaded and repaired using the "Preparation Wizard" implemented in the Schrodinger suite. Artifacts of the crystal structure, i. e. polyethylene glycols, were deleted. The molecular

dynamic (MD) simulations with ligand **1** and H5 and H7, respectively, were performed using Desmond as part of the Schrodinger suite. The protein/ligand complex was embedded into a water box containing 150 mM sodium chloride. After minimization of the water box the MD was run for 10 ns at 300 K. The MD showed for both protein/ligand complexes almost exclusively movement at the core, linker and the spacer while all three binding epitopes remain in the binding pocket during the whole simulation. Movie "mov1" shows 2 ns MD from H5 with ligand 1 and movie "mov2" shows 2 ns MD from H7 with ligand **1**. Ligand interaction plot was also prepared using Maestro.

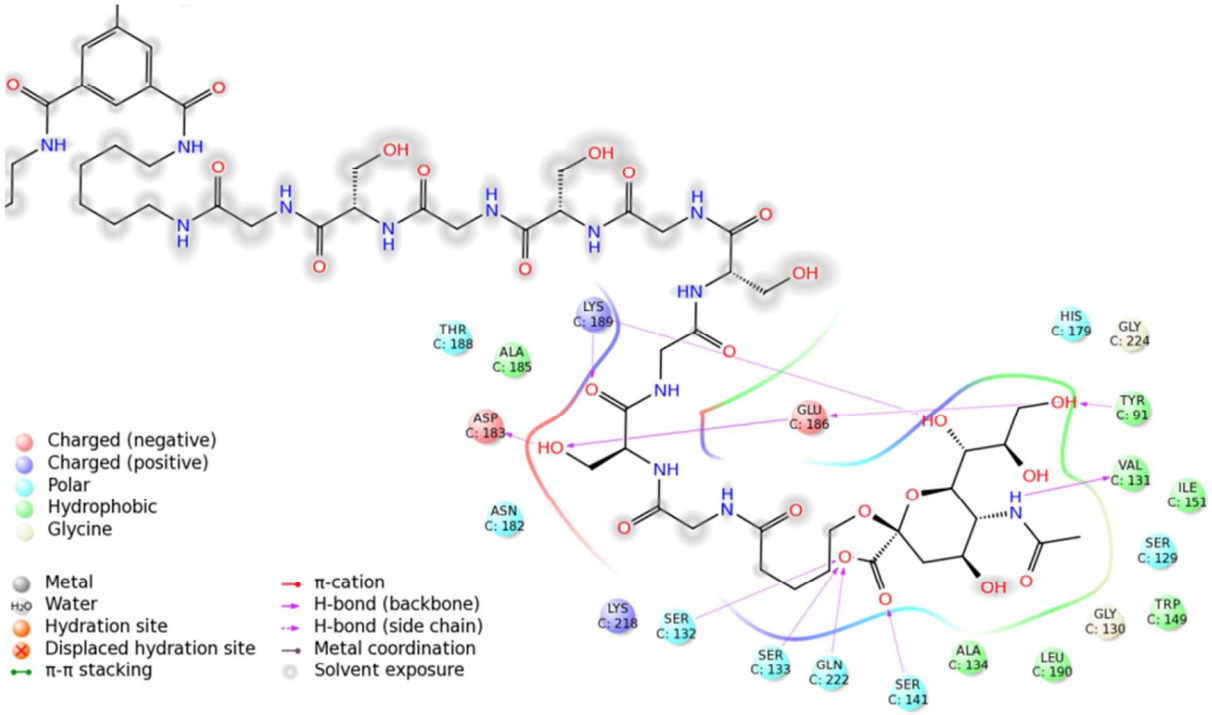


Figure S 1. Ligand interaction plot between compound **1** and the hemagglutinin H5. The main interaction between the ligand and the protein is found at the sialic acid epitope, which is in good accordance with the X-ray crystal structure of monovalent sialic acid complexed with H5. Few more interactions are found between the pentyl chain and the *N*-terminal amino acids.

SPR-Measurements

SPR data was acquired on a Biacore T100 using standard protocols. [4] Commercially obtained H5 (Protein Sciences Corporation; A/Vietnam/1203/2004) H5 was immobilized on a EDC/NHS activated CM5 sensor chip (Biacore) at flow rate of 10 µL/min. Immobilization was carried out in maleate buffer (pH 5.5) with 20 mM Neu5Acα2Me at an approximate protein concentration of 5 µg/mL. 20-25 fmol H5 were immobilized. HBS-EP was used as running buffer at a flow rate of 10 µL/min and different ligand concentrations were analyzed. Contact time was 120 s and dissociation time was 600 s. The system was validated using known binder Neu5Acα2Me. All experiments were carried out twice. As we observed a short half life time of the protein on the chip that might be due to shear stress we abstained from priming and harsh regeneration conditions.

Thermodynamic binding constants of trimeric ligands **11**-**14** (Fig. S 5 – Fig. S 8) were all in the same range as for monovalent ligand **9** (Fig. S 4) indicating that the gain of affinity when compared to the known binder Neu5Acα2Me is due to structural

properties and not because of a multivalent effect. The observed strong affinity of those ligands for H5 was in context with the competitive STD-NMR experiments quite unusual. In order to analyze the origin of these tight binding fragments **6** and **7** were also tested by SPR (Fig. S 2 and Fig. S 3). Derivatization of the sialic acid with pentyl spacer yielded a by factor 2-3 stronger binding for H5 which is quite good concerning the depth of the binding pocket. The increased affinity could be ascribed to the peptidic linker and was also confirmed by STD-NMR titration (Figure S 14). Ligand **1** showed significantly better binding (Fig. 3).

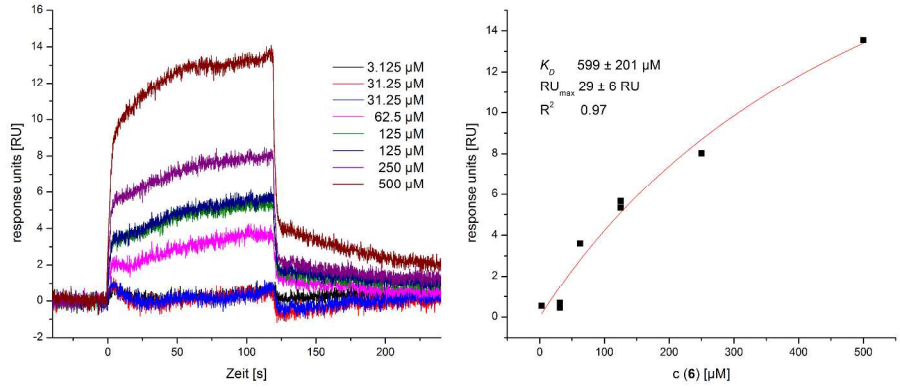


Figure S 2. SPR analysis of ligand **6**.

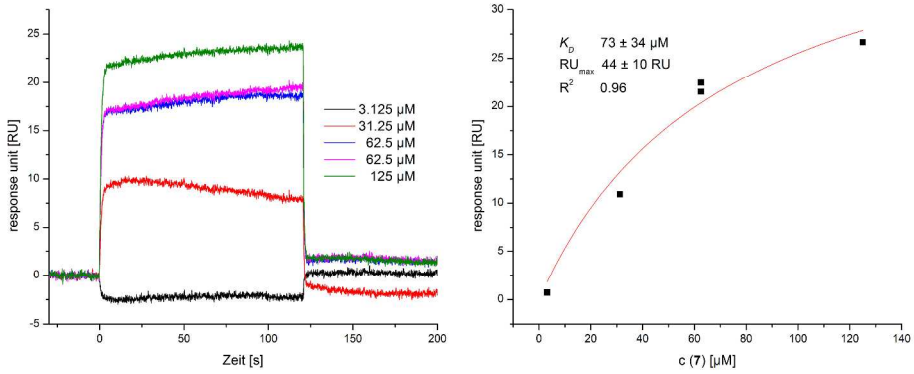


Figure S 3. SPR analysis of ligand **7**.

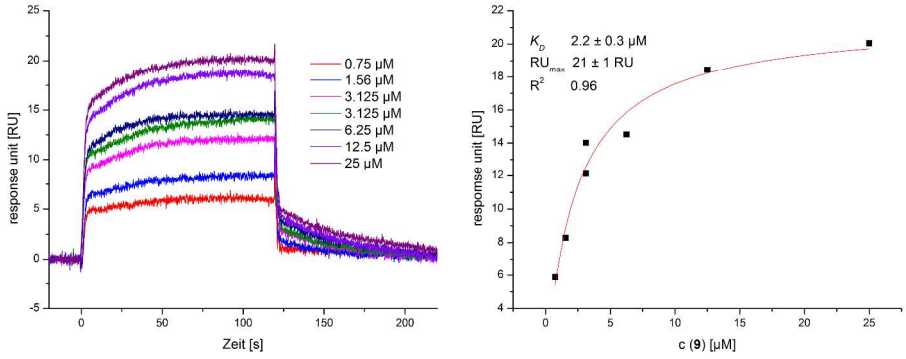


Figure S 4. SPR analysis of ligand **9**.

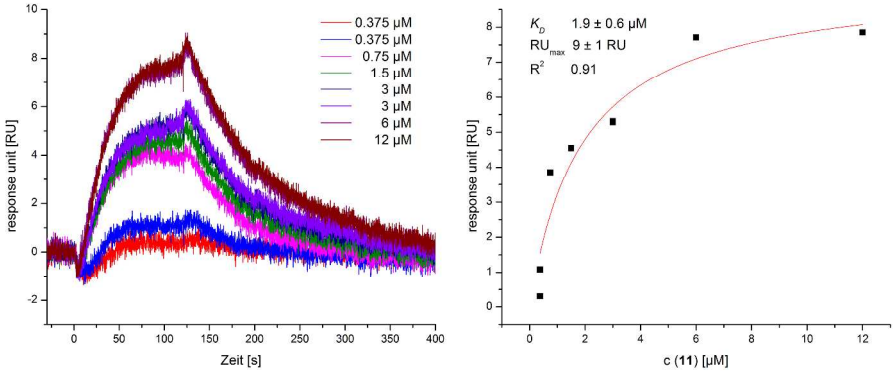


Figure S 5. SPR analysis of ligand **11**.

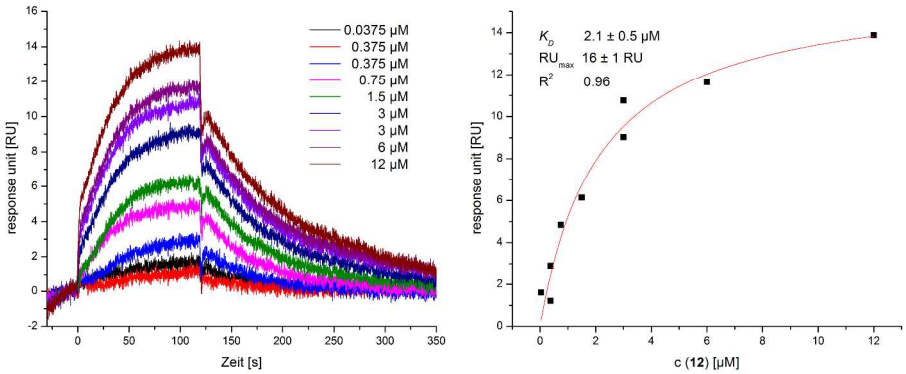


Figure S 6. SPR analysis of ligand **12**.

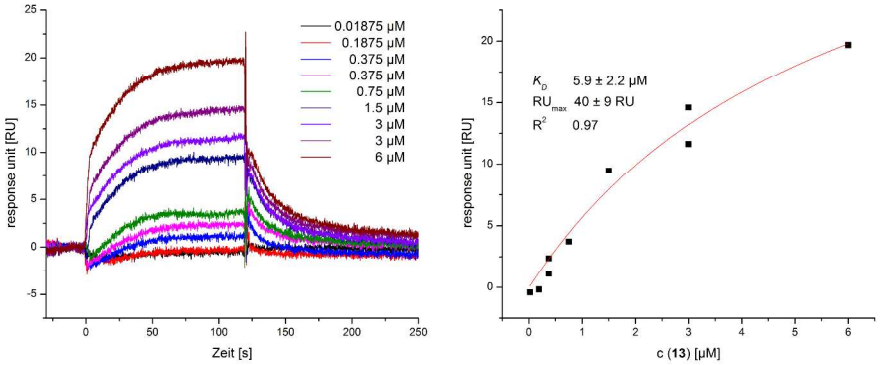


Figure S 7. SPR analysis of ligand **13**.

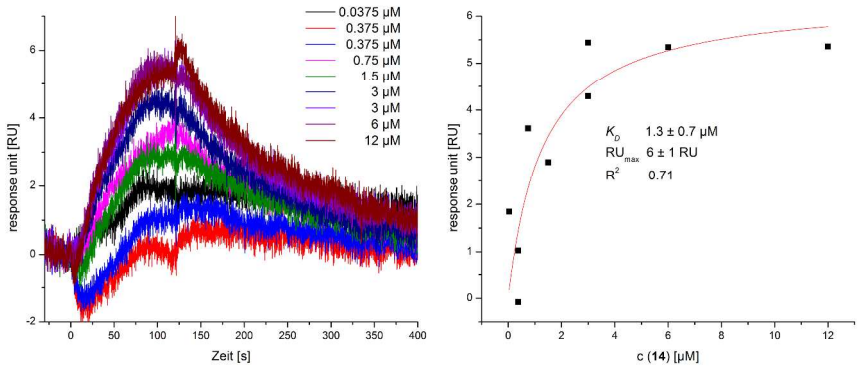


Figure S 8. SPR analysis of **14**.

STD-Measurements

Competitive STD-NMR experiments were performed on a Bruker 700 MHz Avance spectrometer with cryo probe. 0.5 – 1 µg soluble H5 was mixed with Neu5Acα2Me in 180 µL in deuterated PBS (*d*PBS; 150 mM NaCl, 10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 10 mM NaN_3) yielding a final concentration of 20 mM Neu5Acα2Me, corresponding to a protein occupancy of 91%. Ligand concentrations were analyzed by stepwise addition of solved ligand. Spectra were acquired with 256/512 scans and frequencies for saturation pulses were 40 ppm and -0.5 ppm (P12: 2000 µs, SP1: 34.5 dB). Receptor occupancy *r* was calculated by following equations [5]:

$$r = \frac{[PL]}{[P]_0} \quad \text{and} \quad [PL] = \tfrac{1}{2}(K_D + [P]_0 + [L]_0) - \sqrt{\tfrac{1}{4}(K_D+[P]_0+[L]_0)^2 - [L]_0[P]_0}.$$

Competitor integrals were integrated (abs. STD%) and after normalization plotted against the concentration (left side) for a trend analysis or plotted as abs. STD% against the logarithm of the ligand concentration for determination of the IC_{50} value (right). This value was transformed by the Cheng-Prusoff equation into the inhibitor constant K_I. [6]

In the competitive STD-NMR experiments only **1** and **13** showed distinct competitive behavior in the analyzed concentration range (Fig. 3 and Fig. S 12). We abstained from analyzing higher ligand concentrations because as the structure of the competitor is also found in the structure of the ligand problems for the assignment of the origin of an observed signal were expected. By comparison of binding with ligand **9**, which showed no competitive behavior for the primary carbohydrate recognition domain (CRD) (Fig. S 9), the multivalent effect was demonstrated.

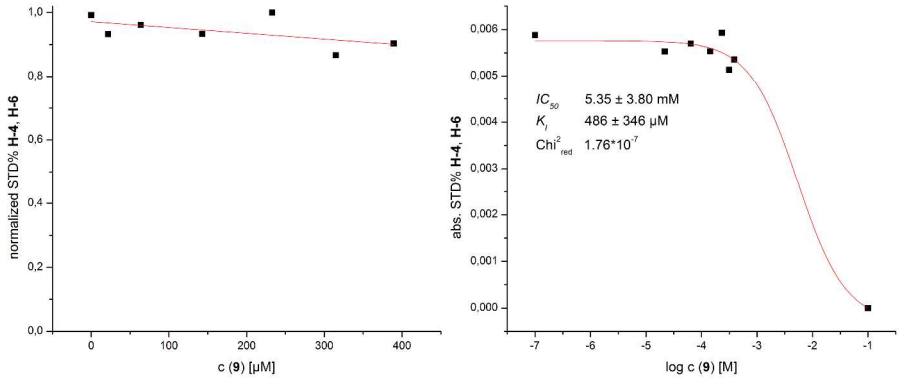


Figure S 9. Competitive STD analysis of ligand **9** with H5 and Neu5Acα2Me as competitor.

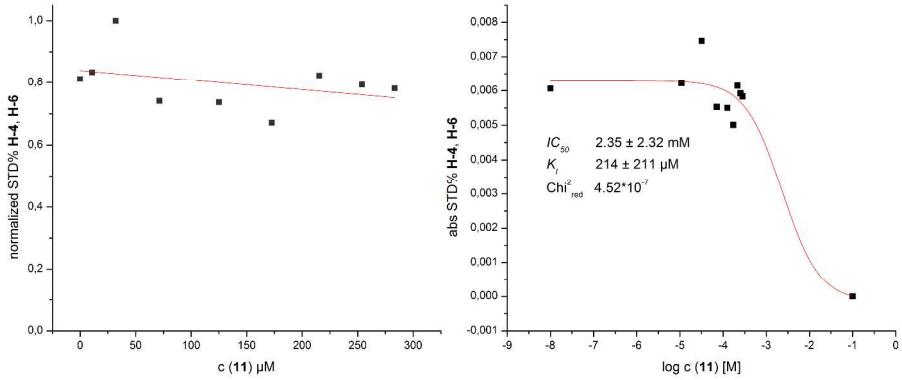


Figure S 10. Competitive STD analysis of ligand **11** with H5 and Neu5Acα2Me as competitor.

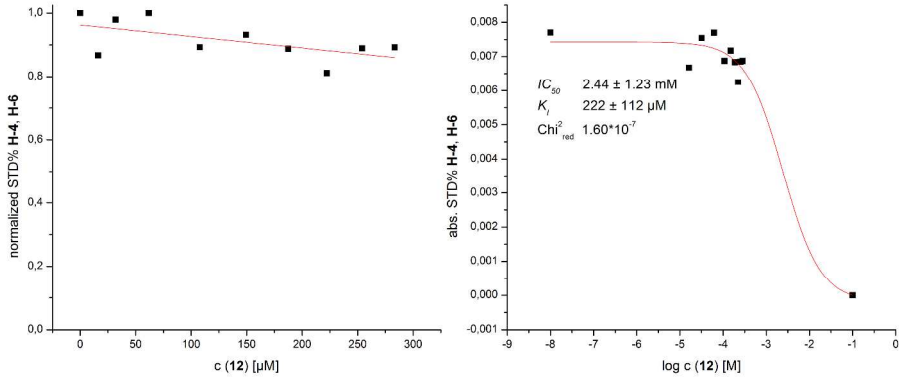


Figure S 11. Competitive STD analysis of ligand **12** with H5 and Neu5Acα2Me as competitor.

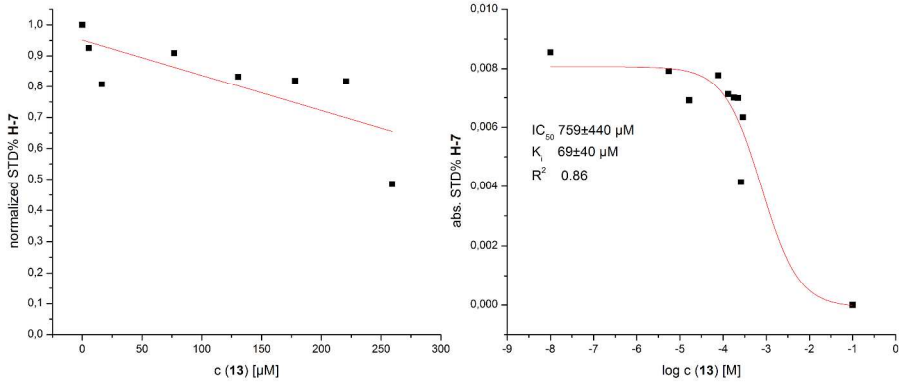


Figure S 12. Competitive STD analysis of ligand **13** with H5 and Neu5Acα2Me as competitor.

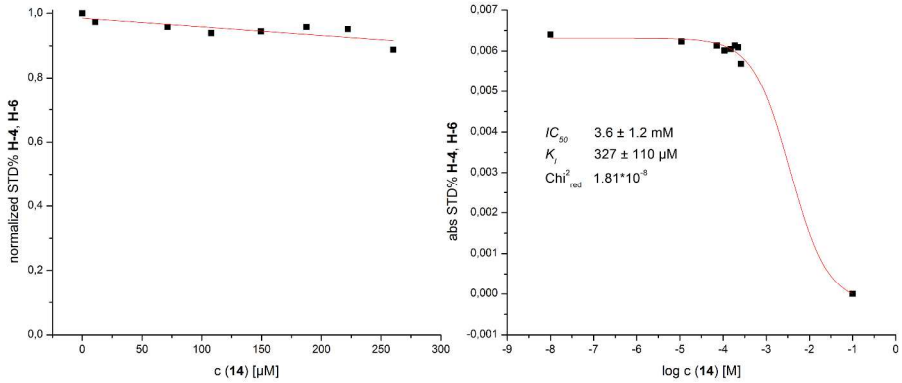


Figure S 13. Competitive STD analysis of ligand **14** with H5 and Neu5Acα2Me as competitor.

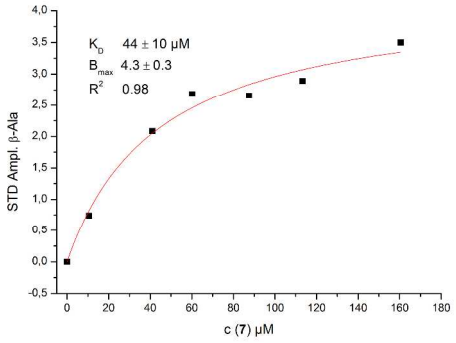


Figure S 14. STD titration of ligand **7**. In a competitive assay with Neu5Acα2Me ligand **7** showed no competitive behavior for the primary CRD.

Synthesis

HR-MS spectra were recorded on an Agilent 6224 LC/ESI TOF MS. NMR spectra were recorded on a Bruker DRX 500 or a Bruker Avance 700 with cryo probe. NMR-spectra of the compounds **1**, **6**-**14** were acquired in H_2O/D_2O 9:1, pH 3 at 298 K. *RP*-HPLC purification was performed on a preparative Agilent 1200 system equipped with DAD and Quadrupole ESI-MS at 20 mL/min flow (Macherey & Nagel, Nucleodur Isis, 250x21 mm, 5 µm, 300 Å). HPLC solvents A (95% H_2O, 5% acetonitrile, 0.1% formic acid) and B (95% acetonitrile, 5% H_2O, 0.1% formic acid) were used.

Ligand **1** was synthesized by a convergent approach. Synthesis of building block **3** was performed in analogy to a literature protocol [7] by kinetic controlled reaction of 1,6-diaminohexane with sub stoichiometric amounts of di-*tert*-butyl dicarbonate to result in just one Boc protected amino group. The mono-Boc protected diamine was reacted with benzene-1,3,5-tricarboxylic acid chloride to give the Boc-protected precursor of **2.** [7-8] **3** was generated just prior to use in the synthetic procedure shown in (Fig. S 15a). The sialic acid chloride glycosyl donor for the synthesis of building block **5** was prepared by standard procedure. [9] The glycosylation reaction was performed with the glycosyl acceptor 5-hexene-1-ol as solvent and silver salicylate as promoter. The alkene function was oxidized under cleavage of the terminal double bond by ruthenium (III) chloride yielding building block **5**, which was also used for synthesis of **6** (Fig. S 15b). Synthesis of the peptide was carried out by standard protocol for microwave assisted automated SPPS using a glycine preloaded 2-chloro-trityl resin. The yield was determined by photometric analysis of the final Fmoc cleavage. The sialic acid building block **5** was coupled to the nascent peptide on the solid support. The glycoconjugate was cleaved from the resin with simultaneous removal of all protecting groups of the peptide side chain. This was essential to assure solubility and allowing purification of the compounds by *RP*-HPLC. Compound **3** was reacted with 4.5 equivalents of **8** to give the trivalent glycoconjugate **10**, which was purified by *RP*-HPLC. The latter two reactions are very sensitive to the excess and concentration of **5** respectively **8**. Re-isolation of unreacted educts was only possible in limited quantities. The final deprotection is a critical step at the end of a 20 step synthesis. Deacetylation was performed under Zemplén conditions with an apparent pH of 9.5 (wet pH paper). The hydrolysis of the methyl ester was performed under precise observance of the pH in aqueous NaOH at pH 10.5 (Fig. 2d). A slightly reduced pH resulted in an incomplete removal of the methyl ester even after 36 h as examined by HSQC experiments. During *RP*-HPLC purification of **1** we could observe two other components eluting slightly after **1**, corresponding to modifications of **1** that lack one or two sialic acids, respectively. In the NMR spectra the data set of a single branch was observed indicating a symmetric substitution of the aromatic core.

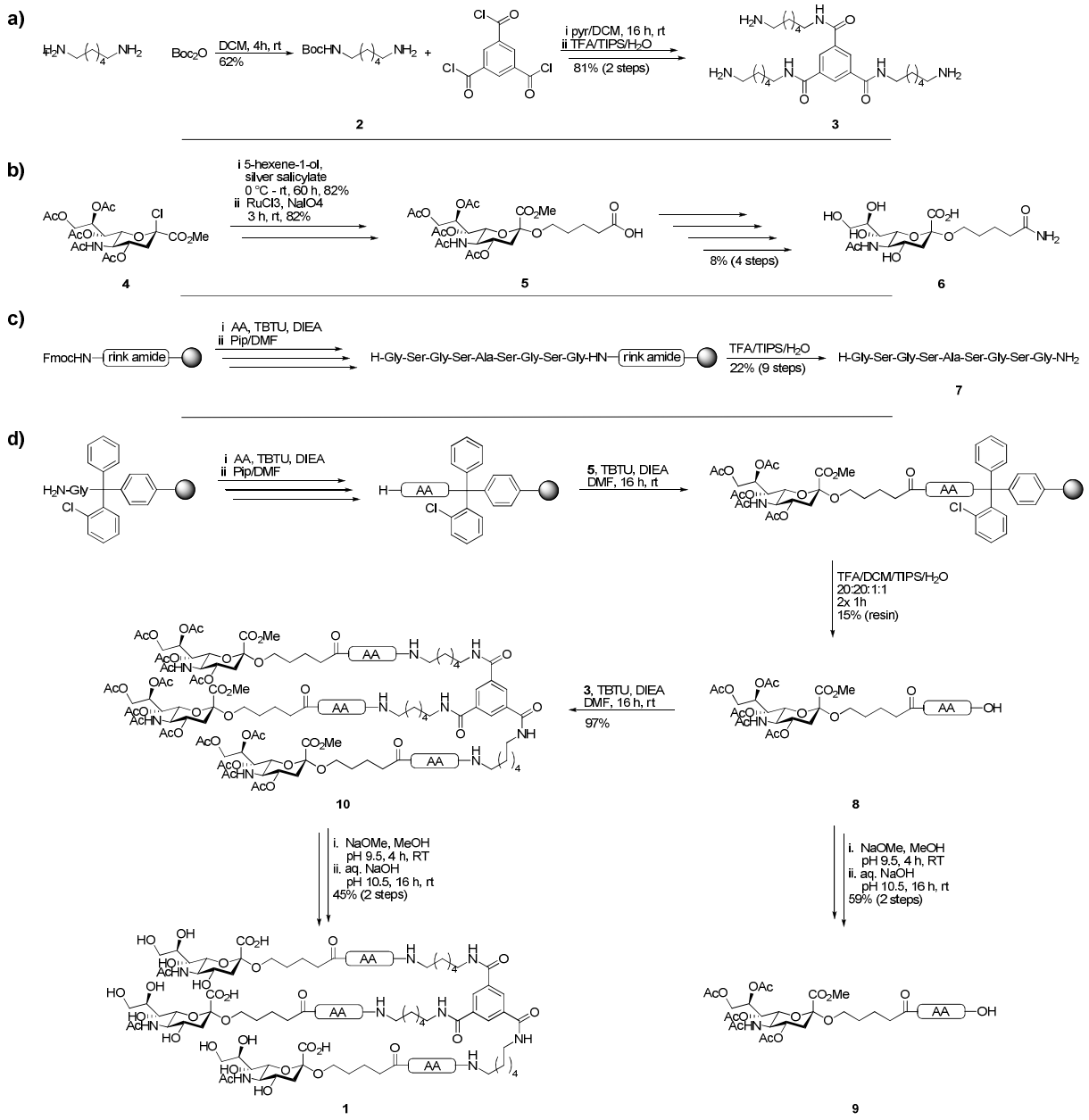


Figure S 15. Schematic presentation of the synthesis of ligand **1**. a) Building block **3** was prepared by a slightly modified literature protocol. [7-8] b) Synthesis of building block **5**, which was also prepared by Knowles *et al.* with a different strategy. [10] This building block was also used for synthesis of fragment **6**. c) Synthesis of the peptide fragment by standard protocol for microwave assisted SPPS. d) Synthesis of ligand **1** using building blocks **3** and **5**.

In order to get a better insight into the structural influences of the multivalent effect as well as evaluating the computer model the synthesis of fragments of **1**, i.e. compound **6**, **7** and **9** (Fig. S 15b, c, d) as well as a small substance library was initiated. We synthesized analogs of **1** with 9, 10 and 11 amino acids (ligands **11**-**14**, Fig. S 16). Ligands **11**-**14** were prepared analog to **1** whereat a decrease of yield was observed the longer the peptide sequence was. Deprotection of **8** yielding **9** was performed analog to the deprotection of **10**. Peptide synthesis for glycoconjugate **8** and peptidic fragment **7** was performed using microwave peptide

synthesizer (CEM Discover & Liberty) and standard protocol (double coupling, capping) with TBTU and DIEA as activator and activator base. **7** was prepared by using a rink amide resin.

Ligands **11**-**14** were prepared analog to **1** whereat a decrease of yield was observed the longer the peptide sequence was. Deprotection of **8** yielding **9** was preformed analog to the deprotection of **10**.

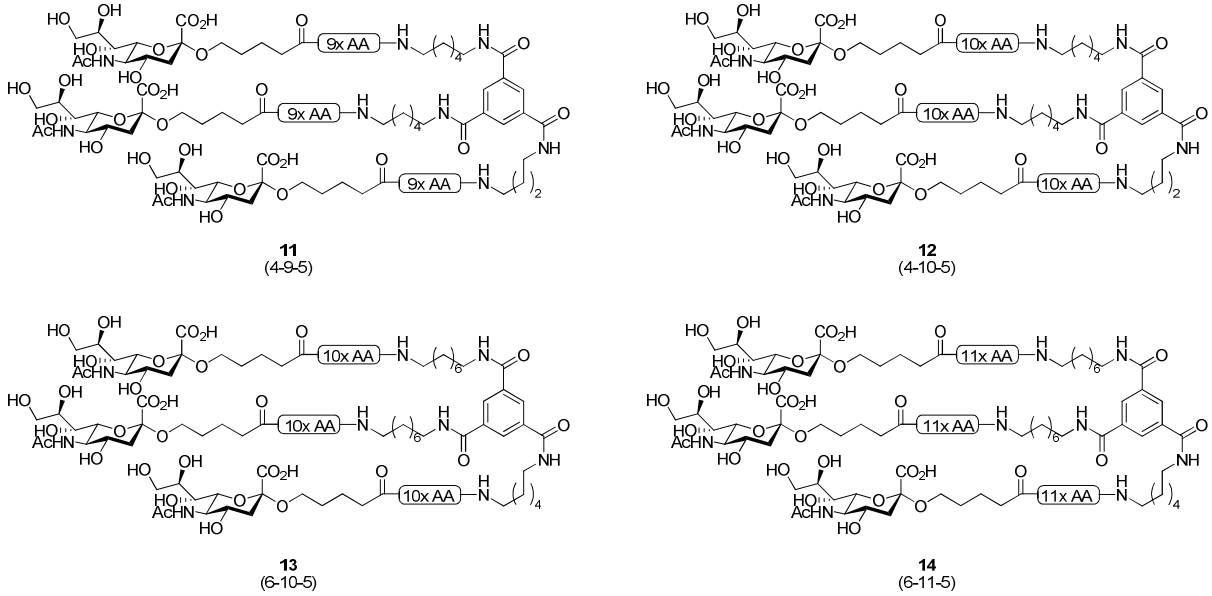


Figure S 16. Structures of the other ligands synthesized by the same strategy as for ligand **1**. The numbers in brackets indicate the structural properties: first number = number of carbons in the core proximal alkyl chain; second number = number of amino acids in the peptidic linker; third number = number of carbons in the sialic acid proximal alkyl chain.

Synthesis of 1:

14.5 mg (3.50 µmol) **10** were deacetylated in a nitrogen atmosphere at room temperature in 15 mL freshly prepared sodium methoxide solution (pH 9-9.5; wet pH paper) for 4 h. The mixture was neutralized with acetic acid (1 M), diluted with water and freeze dried. Hydrolysis of the methyl ester was achieved in 200 mL aquatic sodium hydroxide solution (pH 10.5) during 16 h at room temperature. The mixture was neutralized with Amberlite IR120 H^+. The ion exchanger was extensively washed with water and the solution freeze dried. The product was purified twice by *RP*-HPLC. After purification 5.7 mg (1.6 µmol) **1** were obtained (yield: 45%).

R_t: 11.2 min (0-15 min, 0-25% B)

HR-ESI-MS: *m/z* = calculated: 1810.7956 $[M+2H]^{2+}$, found.: 1810.8134 $[M+2H]^{2+}$

^{1}H-NMR (500 MHz, H_2O/D_2O 9:1, pH 3):

System	δ NH [ppm]	δ H_α [ppm]	δ H_β [ppm]
Gly 1	8.299	3.928	
Ser 2	8.372	4.412	3.838
Gly 3	8.525	3.945	
Ser 4	8.178	4.392	3.808
Ala 5	8.409	4.324	1.357
Ser 6	8.237	4.363	3.818
Gly 7	8.347	3.954	
Ser 8	8.261	4.353	3.828
Gly 9	8.446	3.828	
sialic acid-alkyl	3.687 (**H**-5'a); 3.398 (**H**-5'b); 2.279 (**H**-2'); 1.575 (**H**-3'); 1.517 (**H**-4')		
sialic acid	8.048 (N**H**Ac); 3.804 (**H**-9a); 3.796 (**H**-8); 3.762 (**H**-5); 3.658 (**H**-6); 3.630 (**H**-4); 3.576 (**H**-9b); 3.524 (**H**-7); 2.666 (**H**-3eq); 1.971 (NHAc); 1.612 (**H**-3ax)		
core	8.568 (N**H**-1''); 8.186 (**H**-arom); 7.683 (N**H**-8''); 3.350 (**H**-2''); 3.140 (**H**-7''); 1.570 (**H**-3''); 1.437 (**H**-6''); 1.319 (**H**-4''); 1.283 (**H**-5'')		

$^2J_{\text{H-3ax, H-3eq}}$ = 13.0 Hz, $^3J_{\text{H-3eq, H-4}}$ = 4.9 Hz, $^3J_{\text{H-4', H-5'a}}$ = 6.6 Hz, $^3J_{\text{H-4, H-5'b}}$ = 6.8 Hz, $^2J_{\text{H-5'a, H-5'b}}$ = 9.9 Hz, $^3J_{\text{NH-1'', H-2''}}$ = 6.0 Hz, $^3J_{\text{H-7'', NH-8''}}$ = 6.0 Hz.

^{13}C-NMR (125 MHz, H_2O/D_2O 9:1, pH 3): **δ** [ppm] = 128.7 (**C**-H-arom); 72.6 (**C**-6); 71.7 (**C**-8); 68.5 (**C**-7); 68.1 (**C**-4); 63.9 (**C**-5'); 63.1 (**C**-9); 61.3 (β-Ser); 56.0, 55.7 (α-Ser); 52.1 (**C**-5); 50.0 (α-Ala); 42.8 (α-Gly's); 40.2 (**C**-2''); 40.1 (**C**-3); 39.1 (**C**-7''); 35.0 (**C**-2'); 28.4 (**C**-4'); 28.2 (**C**-3''); 28.2 (**C**-6''); 25.5 (**C**-4''); 25.2 (**C**-5''); 22.6 (NHCO**C**H_3); 21.6 (**C**-3'); 16.6(β-Ala).

Synthesis of Methyl [*n*-hex-5-enyl (5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-didesoxy-d-glycero-α-d-galacto-2-nonulopyranosid)]onat (5a):

In a nitrogen atmosphere 1.37 g (2.69 mmol) 4 were suspended in 18 mL freshly distilled 5-hexene-1-ol together with 1.5 g molecular sieve (3 Å) and dried calcium chloride. At -10 °C and under exclusion of light 2.64 g (10.8 mmol) silver salicylate were added and the reaction mixture was stirred for 60 h. The mixture was diluted with dichloromethane, filtered and the solvent removed under reduced pressure. Crude product was purified by flash chromatography using a mixture of petroleum ether/ ethyl acetate 1:2 yielding 1.30 g (2.20 mmol) 3a (yield 82%).

$[\alpha]_D^{25}$ = -10.0 ° (c = 1, $CHCl_3$)

R_f: 0.13 (PE/EE 1:2)

HR-ESI-MS: *m/z* = calculated: 574.2499 $[M+H]^+$, found: 574.2496 $[M+H]^+$

^{1}H-NMR (400 MHz, $CDCl_3$): **δ** [ppm] = 5.80 (*ddt*, 1H, **H**-5´); 5.43-5.35 (*m*, 1H, **H**-8); 5.32 (*dd*, 1H, **H**-7); 5.13 (*d*, 1H, N**H**Ac); 5.01 (*ddt*, 1H, **H**-6'*E*); 4.94 (*ddt*, 1H, **H**-6'*Z)*; 4.84 (*ddt*, 1H, **H**-4); 4.31 (*dd*, 1H, **H**-9a); 4.13-4.04 (*m*, 3H, **H**-5, **H**-6, **H**-9b); 3.79 (*s*, 3H, COOC**H**$_3$); 3.76 (*dt*, 1H, **H**-1'a); 3.23 (*dt*, 1H, **H**-1'b); 2.56 (*dd*, 1H, **H**-3eq); 2.14, 2.13, 2.03, 2.02 (4x *s*; 4x 3H, 4x OAc); 2.09-2.03 (*m*, 2H, **H**-4'); 1.94 (*dd*, 1H, **H**-3ax); 1.88 (*s*, 1H; NHAc); 1.61-1.51 (*m*, 2H, **H**-2'); 1.48-1.39 (*m*, 2H, **H**-3'). $^2J_{H\text{-}3ax,\ H\text{-}3eq}$ = 12.9 Hz, $^3J_{H\text{-}3ax,\ H\text{-}4}$ = 12.3 Hz, $^3J_{H\text{-}3eq,\ H\text{-}4}$ = 4.6 Hz, $^3J_{H\text{-}4,\ H\text{-}5}$ = 9.6 Hz, $^3J_{H\text{-}6,\ H\text{-}7}$ = 1.7 Hz, $^3J_{H\text{-}7,\ H\text{-}8}$ = 8.3 Hz, $^3J_{H\text{-}8,\ H\text{-}9a}$ = 2.6 Hz $^3J_{H\text{-}8,\ H\text{-}9b}$ = 5.5 Hz, $^2J_{H\text{-}9a,\ H\text{-}9b}$ = 12.4 Hz, $^2J_{H\text{-}1'a,\ H\text{-}1'b}$ = 9.3 Hz, $^3J_{H\text{-}1a',\ H\text{-}2'}$ = 6.3 Hz, $^3J_{H\text{-}1'b,\ H\text{-}2}$ = 6.3 Hz.

^{13}C-NMR (101 MHz, $CDCl_3$): **δ** [ppm] = 171.2, 170.8, 170.3, 170.3, 170.1 (**C**=O); 168.7 (**C**-1); 138.9 (**C**-5'); 114.6 (**C**-6'); 98.9 (**C**-2); 72.4 (**C**-6); 69.3 (**C**-4); 68.8 (**C**-8); 67.6 (**C**-7); 65.0 (**C**-1'); 62.5 (**C**-9); 52.8 (CO_2**C**H_3); 49.7 (**C**-5); 38.3 (**C**-3); 33.4 (**C**-4'); 29.2 (**C**-2'); 25.3 (**C**-3'); 23.3 (NHCO**C**H_3); 21.2, 21.0, 21.0, 20.9 (OCO**C**H_3).

Synthesis of Methyl [*n*-pent-5-yl acid (5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-didesoxy-d-glycero-α-d-galacto-2-nonulopyranosid)]onat (5):

4.00 g (6.98 mmol) 5a were stirred with 175 mg (~ 0.70 mmol) $RuCl_3$*xH_2O and 5.97 g (28.0 mmol) $NaIO_4$ in 50 mL of a mixture of CCl_4/MeCN/H_2O (2:2:3) for 3 h at room temperature. The solvent was removed under reduced pressure and the crude product was purified by flash chromatography using a gradient of toluene/acetone/TEA 1:1:0.01 → toluene/acetone/acetic acid 1:1:0.01 yielding 3.37 g (5.70 mmol) 3 (yield 82%).

$[\alpha]_D^{25}$ = -10.6 ° (c = 1, $CHCl_3$)

R_f: 0.48 (toluene/acetone/AcOH 1:1:0.01)

HR-ESI-MS: *m/z* = calculated: 592.2241 $[M+H]^+$, found: 592.2242 $[M+H]^+$

^{1}H-NMR (400 MHz, $CDCl_3$): **δ** [ppm] = 5.45-5.36 (*m*, 1H, **H**-8); 5.30 (*dd*, 1H, **H**-7); 5.14 (*d*, 1H N**H**Ac); 4.85 (*ddd*, 1H, **H**-4); 4.32 (*dd*, 1H, **H**-9a); 4.14-4.01 (*m*, 3H, **H**-5, **H**-6, **H**-9b); 3.79 (*s*, 3H, COOC**H**$_3$); 3.78 (*dt*, 1H, **H**-5'a); 3.25 (*dt*, 1H, **H**-5'b); 2.57 (*dd*, 1H, **H**-3eq); 2.39 (*dt*, 2H, **H**-2'); 2.15, 2.14, 2.04, 2.02 (4x *s*, 4x 3H, 4x OAc); 1.95 (*dt*, 1H, **H**-3ax); 1.85 (*s*, 3H, NHAc); 1.79-1.66 (*m*, 2H, **H**-3'); 1.66-1.59 (*m*, 2H, **H**-4'). $^2J_{H\text{-}3ax,\ H\text{-}3eq}$ = 12.5 Hz, $^3J_{H\text{-}3ax,\ H\text{-}4}$ = 12.4 Hz, $^3J_{H\text{-}3eq,\ H\text{-}4}$ = 4.6 Hz, $^3J_{H\text{-}4,\ H\text{-}5}$ = 9.6 Hz, $^3J_{H\text{-}6,\ H\text{-}7}$ = 1.7 Hz, $^3J_{H\text{-}7,\ H\text{-}8}$ = 8.5 Hz, $^3J_{H\text{-}8,\ H\text{-}9a}$ = 2.5 Hz, $^2J_{H\text{-}9a,\ H\text{-}9b}$ = 12.4 Hz, $^2J_{H\text{-}2',\ H\text{-}2'}$ = 1.6 Hz $^3J_{H\text{-}2',\ H\text{-}3'}$ =7.6 Hz, $^3J_{H\text{-}4',\ H\text{-}5'a}$ = 6.3 Hz, $^3J_{H\text{-}4',\ H\text{-}5'b}$ = 6.3 Hz, $^2J_{H\text{-}5'a,\ H\text{-}5'b}$ = 9.6 Hz.

^{13}C-NMR (101 MHz, $CDCl_3$): **δ** [ppm] = 171.2, 171.1, 170.4, 170.4, 170.3 (**C**=O); 168.6 (**C**-1); 98.7 (**C**-2); 72.4 (**C**-6); 69.1 (**C**-4); 68.5 (**C**-8); 67.4 (**C**-7); 64.2 (**C**-5'); 62.6 (**C**-9); 52.7 (CO_2**C**H_3); 49.5 (**C**-5); 38.0 (**C**-3); 33.2 (**C**-2'); 28.7 (**C**-4'); 23.4 (NHCO**C**H_3); 21.2 (**C**-3'); 21.1, 20.8, 20.8; 20.7 (OCO**C**H_3).

Synthesis of 6:

110 mg (100 µmol) of a Fmoc rink amide resin were deprotected by standard procedure. The resin was washed with DMF and DCM. 59.1 mg (100 µmol) 5, 152 mg (400 µmol) HATU and 87 µL (500 µmol) DIEA were solved in 2 mL DMF and immediately given to the resin. The reaction mixture was shaken for 14 h at room temperature. Cleavage from the resin was achieved using TFA/TIPS/H_2O 95:5:2 twice for 1 h. The product was purified using acetone/toluene 2:1 (R_f: 0.15; HR-ESI-MS: *m/z* = calculated: 613.2221 $[M+Na]^+$, found: 613.2222 $[M+Na]^+$). The purified compound was deprotected analog to 1 and purified by size exclusion chromatography (Biogel P2) giving 3.2 mg (7.8 µmol) 7 (yield: 8%, 4 steps).

HR-ESI-MS: *m/z* = ber.: 407.1666 $[M-H]^-$, gef.: 407.1658 $[M-H]^-$

^{1}H-NMR (400 MHz, D_2O): **δ** [ppm] = 3.86-3.78 (m, 2H, **H**-8, **H**-9a); 3.78-3.73 (m, 1H, **H**-5); 3.73-3.61 (m, 3H, **H**-5‘a, **H**-6, **H**-4); 3.61-3.55 (m, 1H, **H**-9b); 3.55-3.49 (m, 1H, **H**-7); 3.43 (dt, 1H, **H**-5’b); 2.67 (dd, 1H, **H**-3eq); 2.23 (t, 2H, **H**-2’); 1.98 (s, 3H, NHAc); 1.63 (dd, 1H, **H**-3ax); 1.60-1.48 (m, 4H, **H**-3’, **H**-4‘). $^2J_{H\text{-}3ax,\ H\text{-}3eq}$ = 12.5 Hz, $^3J_{H\text{-}3ax,\ H\text{-}4}$ = 12.4 Hz, $^3J_{H\text{-}3eq,\ H\text{-}4}$ = 4.5 Hz, $^3J_{H\text{-}2‘,\ H\text{-}3‘}$ =7.3 Hz, $^3J_{H\text{-}4‘,\ H\text{-}5‘b}$ = 6.3 Hz, $^2J_{H\text{-}5‘a,\ H\text{-}5‘b}$ = 9.4 Hz.

^{13}C-NMR (101 MHz, D_2O): **δ** [ppm] = 72.7 (**C**-6); 71.5 (**C**-8); 68.2 (**C**-7); 68.0 (**C**-4); 64.3 (**C**-5‘); 62.7 (**C**-9); 51.9 (**C**-5); 40.1 (**C**-3); 34.6 (**C**-2’); 28.3 (**C**-4’); 22.0 ($NHCO\mathbf{C}H_3$); 21.7 (**C**-3‘).

Synthesis of 8:

The peptide was prepared by standard protocol for microwave assisted SPPS using a glycine preloaded 2-chloro trityl resin in a 250 µmol scale. Rate of yield was assessed by photometric assignment of the last Fmoc value and was used as reference for the following reaction (146 µmol, 1 eq). 145 mg (245 µmol, 1.6 eq) 5 were solved with 193 mg (600 µmol) TBTU and 131 µL (750 µmol) DIEA in 2.4 mL DMF, added to the resin and reacted for 16 h at room temperature. The excess of reagents was removed and the glycoconjugate 8 cleaved from the resin with TFA/DCM/TIPS/H_2O 20:20:1:1 twice for 1 h. The crude product was purified with *RP*-HPLC yielding 45 mg (36 µmol) 8.

HR-ESI-MS: *m/z* = calculated: 1239.4752 $[M+H]^+$, found: 1239.4747 $[M+H]^+$

R_t: 11.3 min (0-20 min, 0-50% B)

^{1}H-NMR (500 MHz, H_2O/D_2O 9:1, pH 3):

System	δ NH [ppm]	δ H_α [ppm]	δ H_β [ppm]
Gly 1	8.277	4.015	
Ser 2	8.396	4.507	3.910
Gly 3	8.567	4.029	
Ser 4	8.225	4.470	3.892
Ala 5	8.443	4.425	1.442
Ser 6	8.290	4.469	3.900
Gly 7	8.391	4.058	
Ser 8	8.254	4.532	3.909
Gly 9	8.372	4.016	
alkyl	3.785 (**H**-5’a); 3.438 (**H**-5‘b); 2.377 (**H**-2’); 1.677 (**H**-3’); 1.622 (**H**-4’)		
sialic acid	8.120 (N**H**Ac); 5.412 (**H**-8); 5.382 (**H**-7); 4.914 (**H**-4); 4.363 (**H**-9a); 4.254 (**H**-6); 4.231 (**H**-9b); 3.923 (**H**-5); 3.880 ($COOC\mathbf{H}_3$); 2.733 (**H**-3eq); 1.960 (**H**-3ax)		
acetyl groups	2.220, 2.182, 2.104, 2.065 (4x OAc), 1.930 (NHAc)		

$^2J_{H\text{-}3ax,\ H\text{-}3eq}$ = 13.6 Hz, $^3J_{H\text{-}3ax,\ H\text{-}4}$ = 12.2 Hz, $^3J_{H\text{-}3eq,\ H\text{-}4}$ = 4.8 Hz, $^3J_{H\text{-}6,\ H\text{-}7}$ = 1.7 Hz, $^3J_{H\text{-}7,\ H\text{-}8}$ = 8.7 Hz, $^3J_{H\text{-}8,\ H\text{-}9a}$ = 2.4 Hz, $^2J_{H\text{-}9a,\ H\text{-}9b}$ = 12.7 Hz, $^3J_{H\text{-}2‘,\ H\text{-}3‘}$ = 7.6 Hz, $^3J_{H\text{-}4‘,\ H\text{-}5‘a}$ = 6.7 Hz, $^3J_{H\text{-}4‘,\ H\text{-}5‘b}$ = 6.6 Hz, $^2J_{H\text{-}5‘a,\ H\text{-}5‘b}$ = 10.0 Hz.

^{13}C-NMR (125 MHz, D_2O): **δ** [ppm] = 99.7 (**C**-2); 72.3 (**C**-6); 70.1 (**C**-4); 69.1 (**C**-8); 68.0 (**C**-7); 65.6 (**C**-5‘), 63.0 (**C**-9); 61.9, 61.8, 61.7, 61.7 (β-Ser); 56.4, 56.3, 56.2, 56.1 (α-Ser); 54.1 ($COO\mathbf{C}H_3$); 50.6 (α-Ala); 49.5 (**C**-5); 43.3, 43.2, 43.1 (α-Gly); 37.6 (**C**-3); 35.6 (**C**-2‘); 29.0 (**C**-4‘); 22.4 ($NHCO\mathbf{C}H_3$); 22.3 (**C**-3‘); 21.2, 20.9, 20.8 ($OCO\mathbf{C}H_3$); 17.0 (β-Ala).

Synthesis of 10:

In a nitrogen atmosphere 10.6 mg (8.55 µmol, 4.5 eq.) 4, 5.4 mg (17 µmol, 9 eq.) TBTU and 2.8 µL (17 µmol, 9 eq.) DIEA were dissolved in 1.7 mL DMF and immediately given to 0.95 mg (1.9 µmol, 1 eq.) 2. The solvent was removed under reduced pressure; the crude product was suspended in H_2O/MeCN 7:3 and freed from particles. The product was purified by *RP*-HPLC yielding 7.7 mg (1.8 µmol) 5 (yield: 97%).

R_t: 20.2 min (0-30 min, 0-50% B)

HR-ESI-MS: *m/z* = calculated: 1389.5909 $[M+3H]^{3+}$, found: 1389.5870 $[M+3H]^{3+}$

^{1}H-NMR (500 MHz, H_2O/D_2O 9:1, pH 3):

System	**δ NH [ppm]**	**δ H_α [ppm]**	**δ H_β [ppm]**
Gly 1	8.260	4.011	
Ser 2	8.389	4.497	3.910
Gly 3	8.565	4.028	
Ser 4	8.212	4.470	3.888
Ala 5	8.424	4.403	1.428
Ser 6	8.248	4.444	3.889
Gly 7	8.373	4.035	
Ser 8	8.283	4.440	3.905
Gly 9	8.465	3.908	
alkyl	3.766 (**H**-5'a); 3.407 (**H**-5'b); 2.358 (**H**-2'); 1.655 (**H**-3'); 1.613 (**H**-4')		
sialic acid	8.104 (N**H**Ac); 5.413 (**H**-8); 5.374 (**H**-7); 4.904 (**H**-4); 4.351 (**H**-9a); 4.222 (**H-**6); 4.201 (**H**-9b); 3.927 (**H**-5); 3.867 (COOC**H**$_3$); 2.703 (**H**-3eq); 1.941 (**H-**3ax)		
acetyl groups	2.205, 2.168, 2.092, 2.050 (4x OAc), 1.929 (NHAc)		
core	8.581 (N**H**-1''); 8.281 (**H**-arom); 7.719 (N**H**-8''); 3.429 (**H**-2''); 3.214 (**H**-7''); 1.645 (**H**-3''); 1.519 (**H**-6''); 1.385 (**H**-4''); 1.365 (**H**-5'')		

$^2J_{\text{H-3ax, H-3eq}}$ = 13.6 Hz, $^3J_{\text{H-3eq, H-4}}$ = 4.7 Hz, $^3J_{\text{H-6, H-7}}$ = 1.6 Hz, $^3J_{\text{H-7, H-8}}$ = 8.7 Hz, $^3J_{\text{H-2', H-3'}}$ = 7.9 Hz, $^3J_{\text{H-4', H-5'a}}$ = 6.5 Hz, $^2J_{\text{H-5'a, H-5'b}}$ = 9.9 Hz, $^3J_{\text{NH-1'', H-2''}}$ = 6.4 Hz, $^3J_{\text{H-7'', NH-8''}}$ = 6.1 Hz.

^{13}C-NMR (125 MHz, H_2O/D_2O 9:1, pH 3): **δ** [ppm] = 128.9 (**C**-H-arom); 71.9 (**C**-6); 68.6 (**C**-8); 67.5 (**C**-7); 65.2 (**C**-5'); 62.5 (**C**-9); 61.5 (β-Ser); 56.2 (α-Ser); 53.6 (COO**C**H$_3$); 50.4 (α-Ala); 49.1 (**C**-5); 43.1 (α-Gly); 40.5 (**C**-2''); 39.5 (**C**-7''); 37.1 (**C**-3); 35.2 (**C**-2'); 28.6, 26.0, 22.0 (**C**-2', **C**-3', **C**-2'', **C**-3'', **C**-4'', **C**-5''); 22.0 (NHCO**C**H$_3$); 20.8, 20.8, 20.7, 20.3 (4x OCO**C**H$_3$); 16.8 (β-Ala).

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