Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supporting Information

A hybrid polymer to target blood group dependence of cholera toxin

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Table of Contents

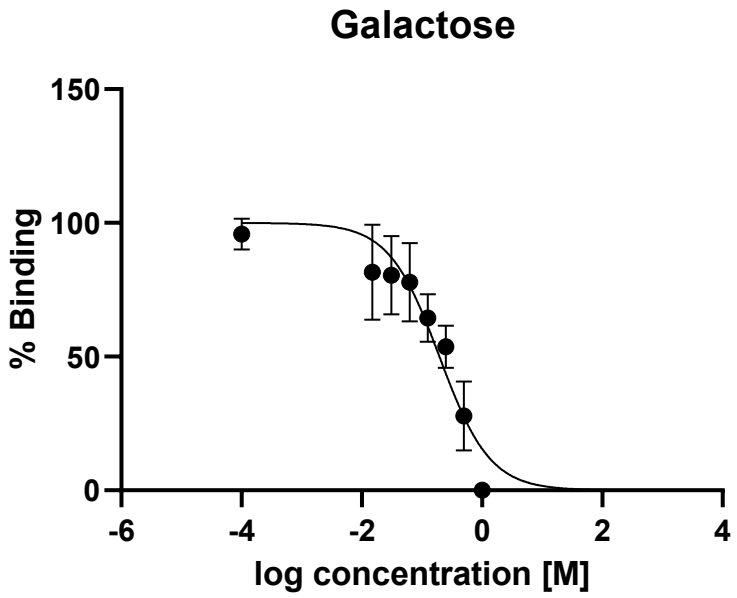
General

Chemicals were obtained from commercial sources and were used without further purification unless noted otherwise. Solvents were purchased from Biosolve (Valkenswaard, The Netherlands). All the other solvents were dried over molecular sieves 4 Å or 3 Å. TLC was performed on Merck precoated Silica plates. Spots were visualized by UV light and 10% H_2SO_4 in MeOH . Microwave reactions were carried out in a Biotage microwave Initiator (Uppsala, Sweden). The microwave power was limited by temperature control once the desired temperature was reached. Sealed vessels of 2-5 mL and 10-20 mL were used. 1H NMR, HSQC, COSY (600 MHz) and 13C (151 MHz) were performed on a Bruker 600 spectrometer. Infrared (IR) spectroscopy was performed using Universal Attenuated Total Reflectance (UATR) accessory of Perkin Elmer Spectrum Two FT-IR. Dextran (D-4876 Sigma, Avg Mn:: 150000) was purchased from Sigma-Aldrich.

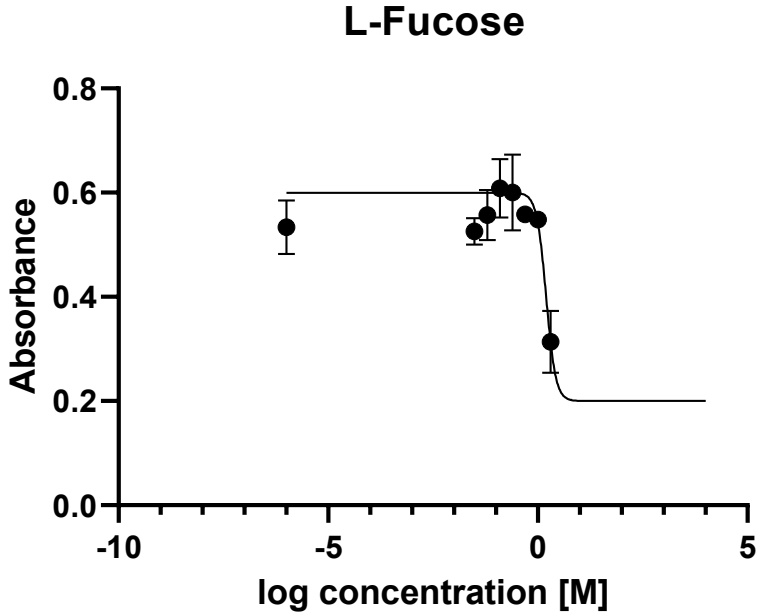
CTB5 inhibition assay (GM1 ELISA)

A 96-well plate (Nunc MaxiSorp™) was coated with a solution of GM1 (100 µL, 2 µg/mL) in phosphate buffered saline (PBS) overnight. Unattached ganglioside was removed by washing with PBS and the remaining binding sites of the surface were blocked with BSA (1%) which was followed by washing with PBS. Samples of toxin-biotin conjugate (CTB5-biotin; Sigma C9972, 40ng/ml) and inhibitor in PBS with BSA (0.1%) and Tween-20 (0.05%) were incubated at room temperature for 2 h and were then transferred to the GM1-coated plate. After 30 min of incubation the solution was removed and the wells were washed with BSA (0.1 %)/Tween-20 (0.05%) in PBS. HRP-streptavidin conjugate (1:10 000) was incubated for 1 h to detect biotinylated toxin. HRP activity was measured by using a freshly prepared solution of o-phenylenediamine/H_2O_2 in citrate buffer (100 µL) for a maximum of 15 min. After quenching with H_2SO_4, the absorbance in each well was measured at 490 nm. Inhibition data from at least two independent experiments with the exception of L-Fucose, were averaged and fitted in GraphPad Prism 8.0.

Inhibition Graphs

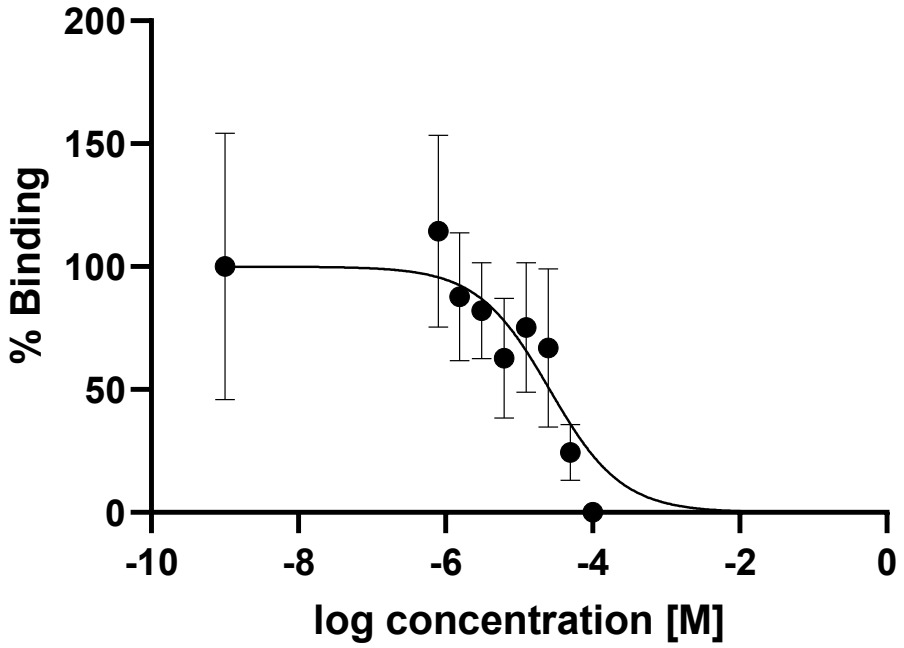


log(inhibitor) vs. response -- Variable slope (met IC50 errors)	
Best-fit values	
Bottom	= 0.000
Top	= 100.0
LogIC50	-0.7090
HillSlope	-1.036
IC50	0.1954
Span	= 100.0
Std. Error	
LogIC50	0.04680
HillSlope	0.1162
IC50	0.02106
95% CI (profile likelihood)	
LogIC50	-0.8052 to -0.6157
HillSlope	-1.325 to -0.8151
IC50	0.1566 to 0.2423
Goodness of Fit	
Degrees of Freedom	54
R squared	0.8165
Sum of Squares	10425
Sy.x	13.89
Constraints	
Bottom	Bottom = 0
Top	Top = 100
Number of points	
# of X values	80
# Y values analyzed	56



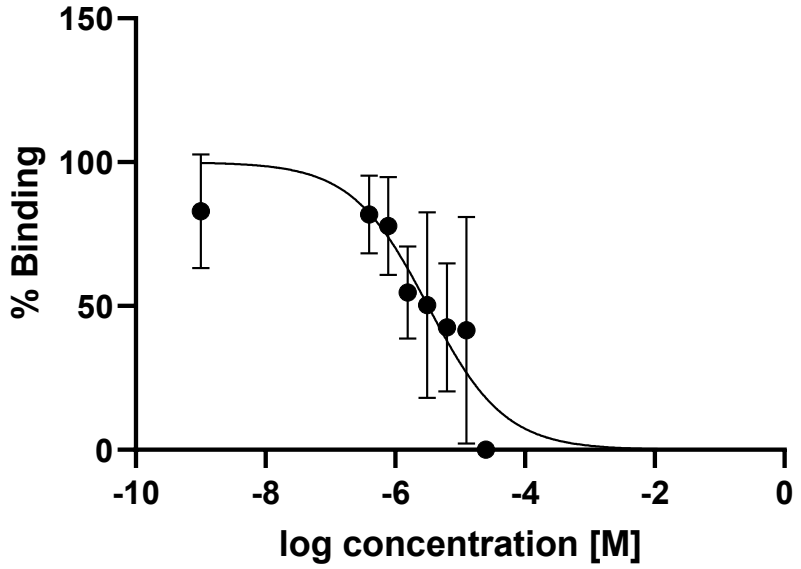
log(inhibitor) vs. response -- Variable slope (met IC50 errors)	
Best-fit values	
Bottom	= 0.2000
Top	= 0.6000
LogIC50	0.1988
HillSlope	-3.837
IC50	1.581
Span	= 0.4000
Std. Error	
LogIC50	0.04697
HillSlope	1.317
IC50	0.1710
95% CI (profile likelihood)	
LogIC50	0.09529 to 0.2886
HillSlope	??? to -1.586
IC50	1.245 to 1.944
Goodness of Fit	
Degrees of Freedom	14
R squared	0.6777
Sum of Squares	0.04419
Sy.x	0.05618
Constraints	
Bottom	Bottom = 0.2
Top	Top = 0.6
Number of points	
# of X values	24
# Y values analyzed	16

Compound 4



log(inhibitor) vs. response -- Variable slope (met IC50 errors)	
Best-fit values	
Bottom	= 0.000
Top	= 100.0
LogIC50	-4.584
HillSlope	-0.8865
IC50	2.605e-005
Span	= 100.0
Std. Error	
LogIC50	0.1754
HillSlope	0.3432
IC50	1.052e-005
95% CI (profile likelihood)	
LogIC50	-4.903 to -4.015
HillSlope	-2.305 to -0.3624
IC50	1.252e-005 to 9.651e-005
Goodness of Fit	
Degrees of Freedom	28
R squared	0.3971
Sum of Squares	24943
Sy.x	29.85
Constraints	
Bottom	Bottom = 0
Top	Top = 100
Number of points	
# of X values	54
# Y values analyzed	30

Compound 5



log(inhibitor) vs. response -- Variable slope (met IC50 errors)	
Best-fit values	
Bottom	= 0.000
Top	= 100.0
LogIC50	-5.495
HillSlope	-0.7364
IC50	3.199e-006
Span	= 100.0
Std. Error	
LogIC50	0.1277
HillSlope	0.1886
IC50	9.407e-007
95% CI (profile likelihood)	
LogIC50	-5.774 to -5.225
HillSlope	-1.187 to -0.3944
IC50	1.682e-006 to 5.963e-006
Goodness of Fit	
Degrees of Freedom	30
R squared	0.5213
Sum of Squares	16388
Sy.x	23.37
Constraints	
Bottom	Bottom = 0
Top	Top = 100
Number of points	
# of X values	32
# Y values analyzed	32

Polyacrylamide-linked L-fucose assay (PAA-fucose ELISA)

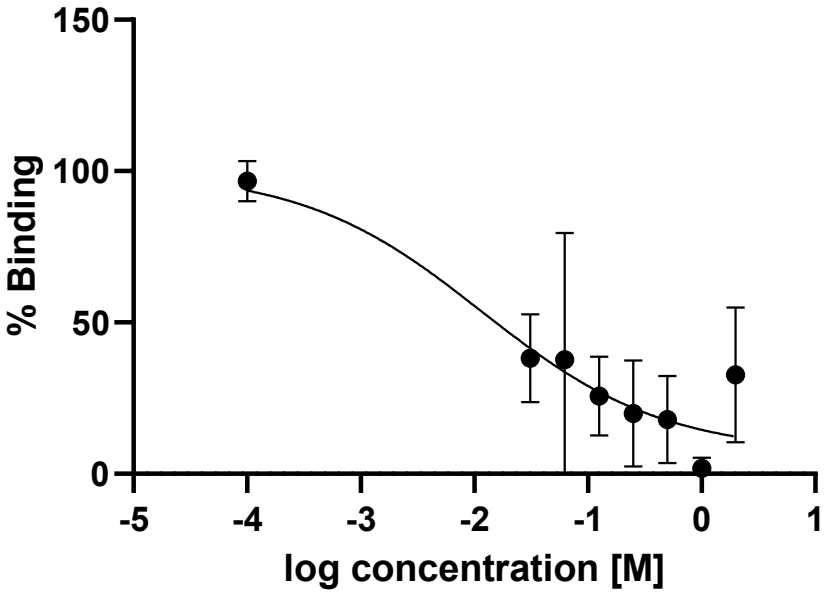
A 96-well plate (Nunc MaxiSorp™) was coated with a solution of PAA-fucose (GlycoNZ Cat. No. 0027-PA ;100 µL, 50 µg/mL) in carbonate buffer for 1h at 37°C. Unattached PAA-fucose was removed by washing with PBS and then blocked with BSA (3%) for 1h which was followed by washing with PBS. Samples of toxin-biotin conjugate (Thermo fischer C34779; Sigma C9972, 15.3 µg/mL) and inhibitor in PBS with Tween-20 (0.05%) were incubated at room temperature for 2 h and then transferred to the PAA-fucose-coated plate. After 1h of incubation, the solution was removed and the wells were washed with Tween-20 (0.05%) in PBS. HRP-streptavidin conjugate (1:10 000) was incubated for 1 h to detect biotinylated toxin. HRP activity was measured by using a freshly prepared solution of o-phenylenediamine/H_2O_2 in citrate buffer (100 µL) for a maximum of 15 min. After quenching with H_2SO_4, the absorbance in each well was measured at 490 nm. Ulex europeaus I lectin (Sigma L8146) was used as a control for PAA-fucose binding to the plate. Inhibition data from at least two independent experiments were averaged and fitted in GraphPad Prism 8.0.

Cell culture: T84 cells (ATCC) were cultured in DMEM/Ham's F12 (1:1) + FCS (v/v) at 37 °C, 5% carbon dioxide. For collagen coating of 96 well plates, rat tail collagen (Corning 354236) was diluted in 0.1% acetic acid and coated on 96 well plates in a final concentration of 3.5µg/well at 4 °C overnight.

T84 cell ELISA: T84 cells (25 000 cells/well) were cultured in DMEM/Ham's F12 + 5% FCS on collagen coated 96 well plates for 3 days at 37 °C / 5% carbon dioxide. Plates were then washed with 2X PBS and toxin-biotin conjugate was added and incubated on ice for 30 min. Excess toxin was washed with 3X PBS. The cells were fixed with 4% paraformaldehyde for 5 min. on ice and 25 min. at room temperature and then washed with 3X PBS. The cells were blocked with 1% BSA/PBS for 1h. HRP activity was measured by using a freshly prepared solution of o-phenylenediamine/H_2O_2 in citrate buffer (100 µL) for a maximum of 15 min. After quenching with H_2SO_4, the absorbance in each well was measured at 490 nm.

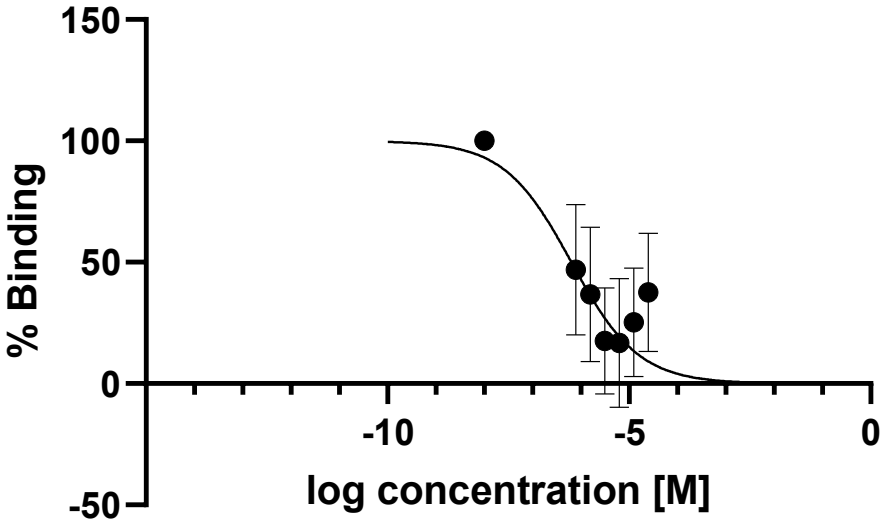
Inhibition Graphs

L-Fucose



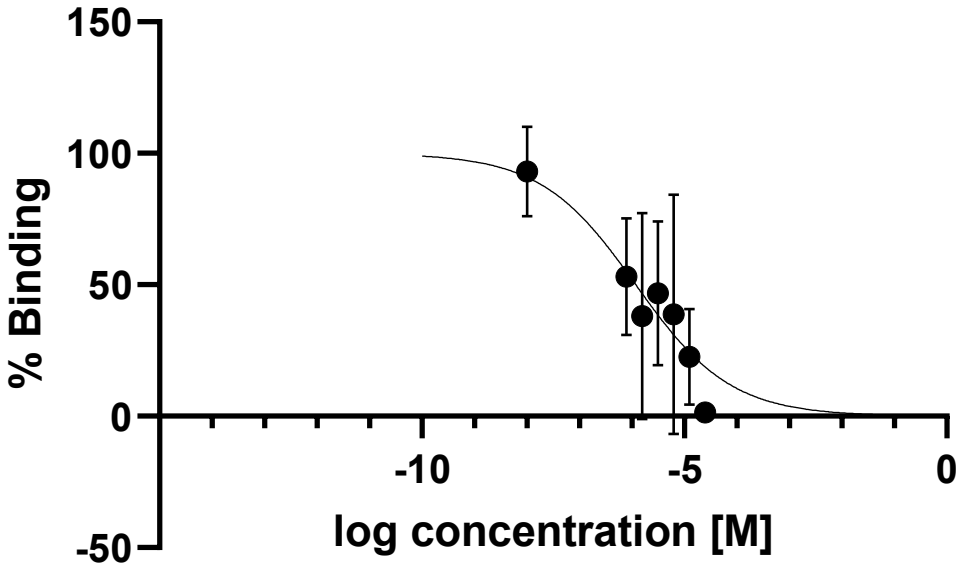
log(inhibitor) vs. response -- Variable slope (met IC50 errors)	
Best-fit values	
Bottom	= 7.000
Top	= 100.0
LogIC50	-1.931
HillSlope	-0.5461
IC50	0.01173
Span	= 93.00
Std. Error	
LogIC50	0.3361
HillSlope	0.1794
IC50	0.009079
95% CI (profile likelihood)	
LogIC50	-2.651 to -1.438
HillSlope	-1.259 to -0.3139
IC50	0.002231 to 0.03650
Goodness of Fit	
Degrees of Freedom	30
R squared	0.6181
Sum of Squares	12116
Sy.x	20.10
Constraints	
Bottom	Bottom = 7
Top	Top = 100
Number of points	
# of X values	32
# Y values analyzed	32

Compound 3



log(inhibitor) vs. response -- Variable slope (met IC50 errors)	
Best-fit values	
Bottom	= 0.000
Top	= 100.0
LogIC50	-6.196
HillSlope	-0.6262
IC50	6.372e-007
Span	= 100.0
Std. Error	
LogIC50	0.1995
HillSlope	0.1885
IC50	2.927e-007
95% CI (profile likelihood)	
LogIC50	-6.625 to -5.894
HillSlope	-1.240 to -0.3652
IC50	2.369e-007 to 1.277e-006
Goodness of Fit	
Degrees of Freedom	34
R squared	0.5847
Sum of Squares	18469
Sy.x	23.31
Constraints	
Bottom	Bottom = 0
Top	Top = 100
Number of points	
# of X values	48
# Y values analyzed	36

Compound 4



log(inhibitor) vs. response -- Variable slope (met IC50 errors)	
Best-fit values	
Bottom	= 0.000
Top	= 100.0
LogIC50	-5.926
HillSlope	-0.4817
IC50	1.185e-006
Span	= 100.0
Std. Error	
LogIC50	0.2272
HillSlope	0.1686
IC50	6.198e-007
95% CI (profile likelihood)	
LogIC50	-6.501 to -5.529
HillSlope	-0.9201 to -0.2393
IC50	3.153e-007 to 2.957e-006
Goodness of Fit	
Degrees of Freedom	36
R squared	0.4024
Sum of Squares	29199
Sy.x	28.48
Constraints	
Bottom	Bottom = 0
Top	Top = 100
Number of points	
# of X values	48
# Y values analyzed	38

Synthesis

General procedure for the deacetylation reaction

The protected compound is dissolved in anhydrous methanol, followed by addition of a catalytic amount of aqueous 1M NaOH solution and stirred at room temperature. The reaction is monitored by TLC. Upon the completion of the reaction, if required, the reaction mixture is neutralized by the addition of Dowex marathon resin. The solvent is evaporated and the crude mixture is purified by column chromatography (EtOAc: MeOH: H_2O- 4:2:1) to get the pure compound.

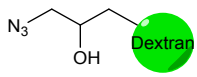
General Procedure for the Click Reaction

The azido polymer is dissolved in water followed by the addition of the alkyne ligand (1.3 equiv. or 0.65 equiv of each in case of the hybrid). 0.1 equiv. of copper sulphate pentahydrate is dissolved in water separately and added to the reaction mixture. 0.3 equiv. of sodium ascorbate is also dissolved in water separately and added to the reaction mixture. The reaction is carried out at 100°C in the microwave for 60 min. CupriSorb™ (Seachem®) resin was added to the reaction mixture and stirred, followed by filtration of the resin. The solvent is evaporated and the crude reaction mixture is purified by dialysis using a cellulose based dialysis cassette (MWCO: 2K) against deionized water for 3-4 days and freeze dried.

Compound 1 was synthesized according to the reported glycosylation procedure[1] and deprotected according to the above described deacetylation method, with the spectral data in agreement with the reported values.[2]

Compound 2 and **Compound 5** were synthesized according to the previously reported procedure.[3]

Dextran Azide



The azidation of dextran (Mn: 150000) was carried out according to a reported procedure[4] by first synthesizing 1-azido-2,3-epoxypropane which was then used for conjugation to dextran. The incorporation of the azide group was confirmed by the appearance of the characteristic azide stretching at 2105 cm^{-1} in the IR spectra. The degree of functionalization was calculated from the NMR spectra of the conjugated compound as described previously.[3,5]

Calculation for degree of functionalization:

Azide Dextran integrals-Native Dextran integrals = 8.36-6.53 = 1.83

Azide ligand has 5 protons ; 1.83 ÷ 5 = 0.366 ; 0.366 ÷ Dextran integrals = 0.402 ÷ 6.53 = 0.056 = 5.6 %

Number of functionalized monomers = 5.6% of 925.6 glucose monomers = 52 monomers

Compound 3

Compound **1** (1.3 equiv) was conjugated to the dextran azide (1 equiv) via the described click reaction to give a white solid compound. The disappearance of the azide stretching peak in the IR spectra of the final compound confirmed that all of the azido polymer was consumed. Yield: 75%

^{1}H NMR (600 MHz, D_2O-*d*) δ: 8.08-8.01(triazole), 4.96 (d, dextran H-1, 1H), 4.01-3.41 (m, dextran [5H], Fuc [4H]) , 1.11 (d, Fuc H-6, 3H)

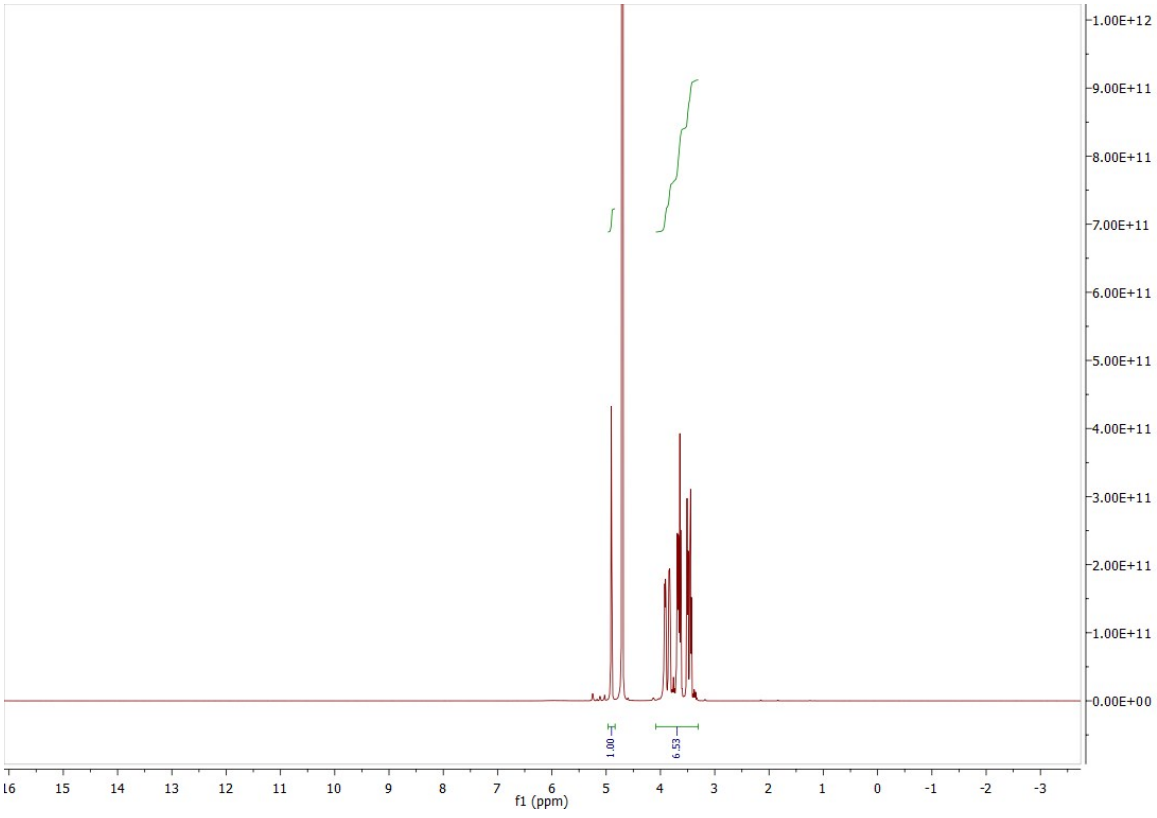
Compound 4

Compound **1** (0.65 equiv) and Compound **2** (0.65 equiv) was conjugated to the azido dextran via the described click reaction to give an off-white solid compound. The disappearance of the azide stretching peak in the IR spectra of the final compound confirmed that all of the azido polymer was consumed. Yield: 81%

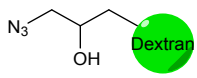
^{1}H NMR (600 MHz, D_2O-*d*) δ: 8.38 -7.87 (m, 3x aromatic [MNPG] , 2x triazole, 5H), 5.82 (MNPG. H-1, 1H), 4.96 (d, dextran H-1, 1H), 4.10-3.30 (m, dextran [5H], MNPG [7H], Fuc [4H]), 1.02 (d, Fuc H-6, 3H).

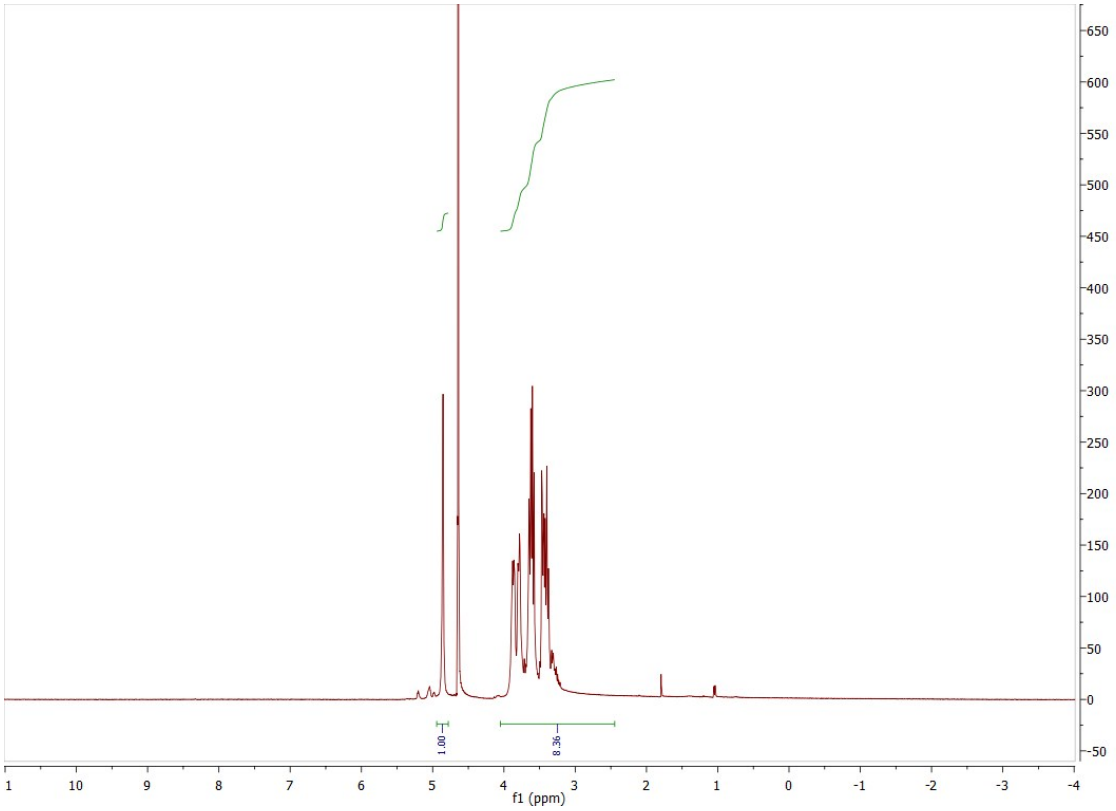
NMR spectra

Dextran (150,000)

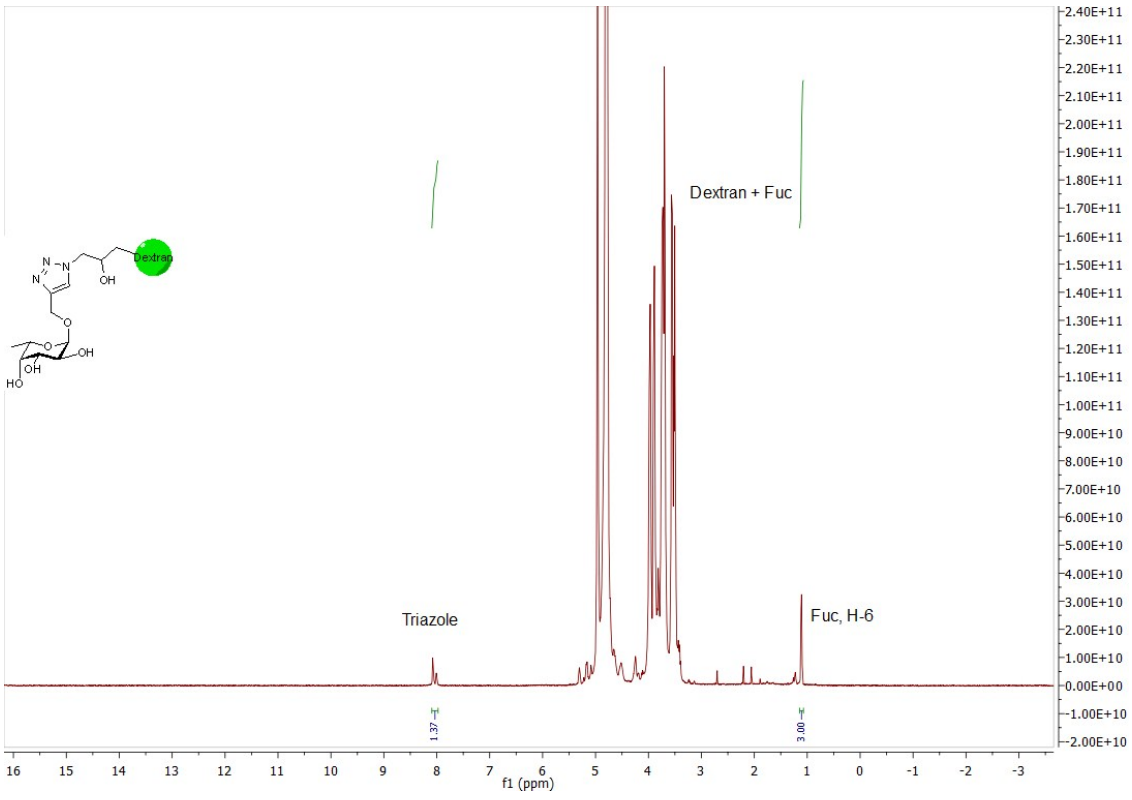


Dextran Azide

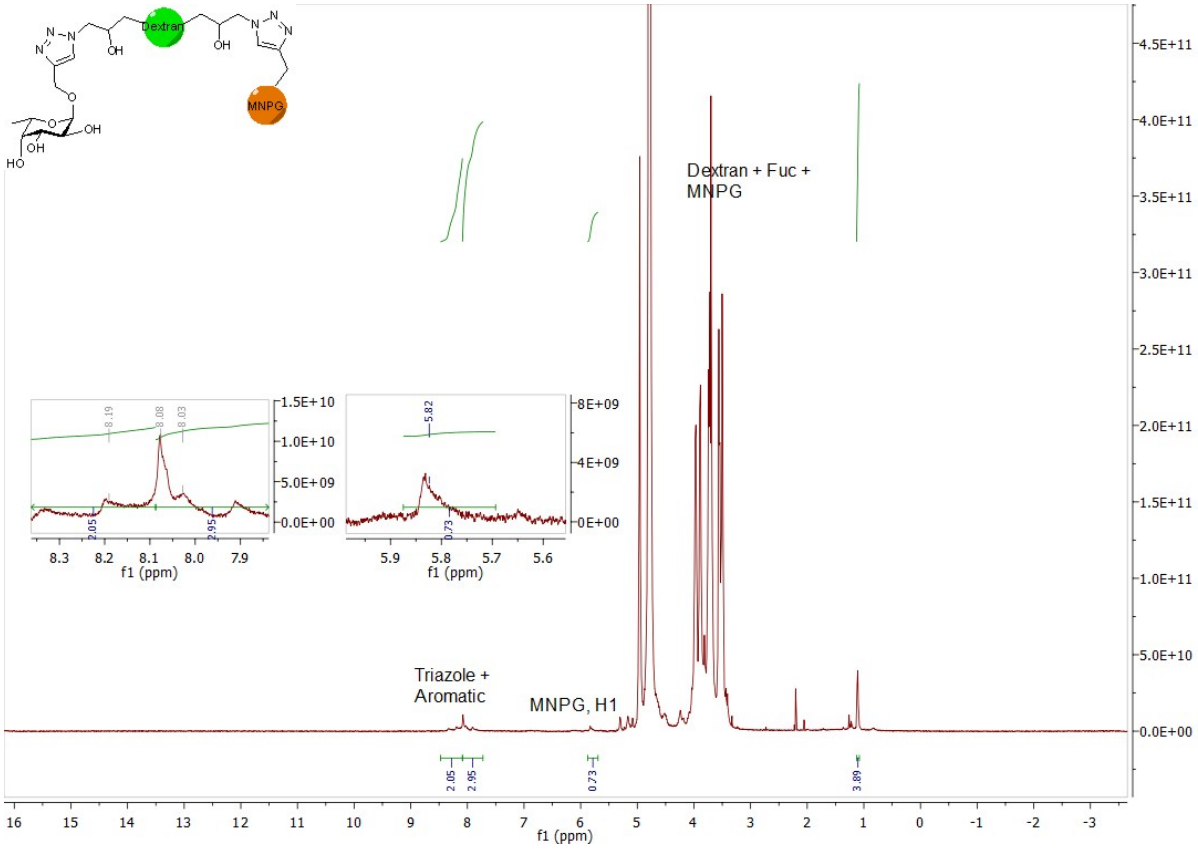




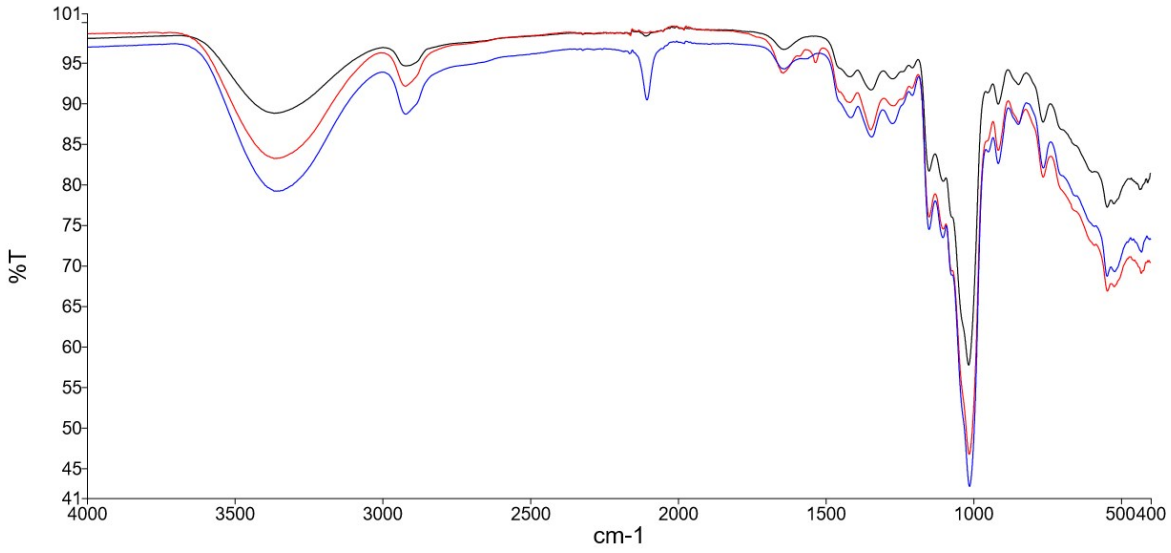
Compound 3



Compound 4


Dextran
MNPG
Dextran + Fuc +
MNPG
Triazole +
Aromatic
MNPG, H1
f1 (ppm)

IR spectra



Dextran azide ; Compound 3; Compound 4.

Bibliography

(1) Nierengarten, J. F.; Iehl, J.; Oerthel, V.; Holler, M.; Illescas, B. M.; Muñoz, A.; Martín, N.; Rojo, J.; Sánchez-Navarro, M.; Cecioni, S.; et al. Fullerene Sugar Balls. *Chem. Commun.* **2010**, *46* (22), 3860–3862.

(2) Fernandez-Megia, E.; Correa, J.; Rodríguez-Meizoso, I.; Riguera, R. A Click Approach to Unprotected Glycodendrimers. *Macromolecules* **2006**, *39* (6), 2113–2120.

(3) Haksar, D.; de Poel, E.; van Ufford, L. Q.; Bhatia, S.; Haag, R.; Beekman, J.; Pieters, R. J. Strong Inhibition of Cholera Toxin B Subunit by Affordable, Polymer-Based Multivalent Inhibitors. *Bioconjug. Chem.* **2019**, *30* (3), 785–792.

(4) Pahimanolis, N.; Vesterinen, A.-H.; Rich, J.; Seppala, J. Modification of Dextran Using Click-Chemistry Approach in Aqueous Media. *Carbohydr. Polym.* **2010**, *82*, 78–82.

(5) Heller, D. A.; Levi, Y.; Pelet, J. M.; Doloff, J. C.; Wallas, J.; Pratt, G. W.; Jiang, S.; Sahay, G.; Schroeder, A.; Schroeder, J. E.; et al. Modular "click-in-Emulsion" Bone-Targeted Nanogels. *Adv. Mater.* **2013**, *25*, 1449–1454.