Selective $^{13}$C-Labels on Repeating Glycan Oligomers to Reveal Protein Binding Epitopes through NMR: Polylactosamine Binding to Galectins

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1. Methods

Enzymatic reactions were monitored by mass spectrometry recorded on an Autoflex III Smartbeam MALDI TOF/TOF Bruker Daltonics using 2,5-dihydroxybenzoic acid (DHB) as a matrix. Normal phase column chromatography was performed on silica gel G60 (Silicycle, 60–2000 µm, 60Å). Thin layer chromatography (TLC) was conducted on Silica gel 60 F254 (EMD Chemicals, Inc.) with detection by UV-absorption (254 nm) where applicable. Visualization of TLC plates were accomplished by spraying with 10% sulphuric acid in ethanol and Hanessian’s Stain, followed by charring at ~150 °C. Reagents were purchased from commercial sources and used without further purification. Gel filtration chromatography was performed using a column (75 x 1.5 cm) packed with Bio-Gel P-2 media (BIO-RAD), eluted with H2O eluent.

2. Materials

The production of sugar nucleotides was prepared in one-pot enzymatic synthesis.[1] The enzymes for the production of sugar nucleotides: N-acetylglucosamine 1-phosphate uridylyltransferase (PmGlmU, from Pasteurella multocida), N-acetylhexosamine 1-kinase (NahK, from Bifidobacterium longum), galactose-1-phosphate uridylyltransferase (GLUSP, from Bifidobacterium longum), and galactokinase (GalK, from Bifidobacterium Infantis); and the H. pylori β1,3-N-acetylglucosaminyltransferase (HP-39, from Helicobacter pylori) and bacterial β1,4-galactosyltransferase (LgtB, from Neisseria meningitis) plasmids were provided from Boons group. All enzymes were expressed in BL21 (D3) bacterial cells and prepared in large scale as described elsewhere.[2,3]

2.1 Expression and purification of β3GlcNAcT (HP-39)[4] and β1,4-galactosyltransferase (LgtB)[5]

β3GlcNAc Transferase and β1,4-galactosyltransferase were expressed in BL21 bacterial cells grown in LB broth media with Ampicillin (150 µg/mL) until cells reached an OD600 between 0.6-0.8. Protein expression was induced with 1mM and 0.5 mM isopropylthio galactopyranoside (IPTG), respectively, and culture continued at 20 °C with rapid shaking (200 rpm) for 20 hours. Cells were harvested by spinning at 5500 rpm for 20 minutes at 4 °C. The obtained pellets were purified as described: The HP-39 pellet was resuspended 1:1 (1 mg cell pellet: 1 mL buffer) in loading buffer of 20 mM Tris-HCl pH 7.5, 200 mM NaCl, 5 mM β-mercapto-ethanol (BME), and 1 mM EDTA. Enzyme was released by sonication (8 x 30 s, with 1 min intervals between each burst). Extract was centrifuged at 8500 rpm for 30 minutes at 4 °C and supernatant was diluted 1:1 with loading buffer and loaded onto a 5 mL Amylose resin column. Protein was purified with elution buffer of 20 mM pH 7.5 Tris-HCl, 200 mM NaCl, 5 mM BME, 1 mM EDTA, and 10 mM Maltose. Purified enzyme was stored in 10% glycerol at -80 °C. The LgtB pellet was resuspended 1:1 (1 mg cell pellet: 1 mL buffer) in lysis buffer (50 mM Tris-HCl pH 8 and 0.01% Triton). Enzyme was released by sonication in presence of 1 mM of PMSF and 1 tablet of
EDTA-free SIGMAFAST™ protease inhibitor cocktail. Cells were subsequently disrupted by eight 30 s, 10W sonication pulses applied at 60 s intervals. Extract was centrifuged at 35000 rpm for 30 minutes at 4 ºC and supernatant was loaded onto a Ni-NTA affinity chromatography pre-equilibrated with buffer A (50 mM Tris-HCl pH 7.5, 150 mM NaCl and 4 mM BME). The column was washed with a step gradient of 0 and 25 mM imidazole added to buffer A, at ten column volumes each. The His6-LgtB eluted with ten column volumes of buffer A plus 500 mM imidazole. Purified enzyme was dialyzed against buffer 20 mM Tris-HCl pH 7.5 and stored in 10% glycerol at -80 ºC.

2.2 Construct design, expression and purification of human lectins.

The carbohydrate recognition domains (CRD) of galectin-3 (residues 114-248, P17931), the N-terminal domain of galectin-9 (galectin-9Nter, residues 1-148, O00182) and the full length galectin-1 (residues 2-135, P09382), galectin-7 (residues 1-136, P47929) and galectin-8 (residues 1-317, O00214) were cloned into pET-21a (+) (GenScript), pGEX-4T-2 (Riken DNA Bank), pET-21a (GenScript), pET-22b (+) (GenScript) and pET-11a (GenScript) vectors, respectively. Expression and purification of protein was performed as described elsewhere.[6]

3. General Procedures for Enzymatic Synthesis

3.1 General procedure for the installation of β1,3 GlcNAc using HP-39

LacNAc acceptor and UDP-GlcNAc (2.0 eq per GlcNAc added) were dissolved at a final acceptor concentration of 10 mM in a HEPES buffer solution (50 mM, pH 7.3) containing KCl (25 mM), MgCl₂ (2 mM) and DTT (1mM). Calf intestine alkaline phosphatase (CIAP, 10 U µL⁻¹, Invitrogen) and HP-39 (100 µg per µmol acceptor) were added, and the reaction mixture was incubated overnight at 37 ºC with moderate shaking. Reaction progress was monitored by MALDI-TOF MS, and if starting material remained after 20 h another portion of HP-39 was added until no starting material could be detected. The reaction mixture was centrifuged using a Vivaspin® Sartorius ultrafiltration device (10 kDa MWCO) to remove enzymes and the supernatant subjected to gel filtration chromatography (see Section 1.a.). Fractions containing product were combined and lyophilized to give the desired product.

3.2 General procedure for the installation of β1,4 Gal using LgtB to form Type II LacNAc moieties

Acceptor and UDP-Gal (2.0 eq per Gal added) were dissolved at a final acceptor concentration of 10 mM in a Tris buffer solution (100 mM, pH 7.9) containing MnCl₂ (10 mM) and BSA (0.1% wt/wt). CIAP (10 U µL⁻¹) and LgtB (75 µg per µmol acceptor) were added, and the reaction mixture was incubated overnight at 37 ºC with moderate shaking. Reaction progress was monitored by MALDI MS, and if starting material remained after 20 h another portion of LgtB was added until no starting material could be detected. The reaction mixture was centrifuged using a Vivaspin® Sartorius ultrafiltration device (10 kDa MWCO) to remove enzymes and BSA, and the supernatant subjected to gel filtration
chromatography (see Section 1.a.). Fractions containing product were combined and lyophilized to give the desired product.

4. NMR Spectroscopy

All NMR experiments were recorded at 298 K on BRUKER AVANCE III spectrometers operating at 600 MHz (equipped with a PA-TXI probe) or 800 MHz (equipped with a TCI cryoprobe) under TopSpin 3.2.7 (BRUKER BioSpin). To minimise problems with H2O signal suppression, all samples were dissolved in deuterated (> 99%) phosphate-buffered saline (50 mM sodium phosphate, 150 mM NaCl, pD 7.5). Chemical shifts are reported in parts per million (ppm) relative to 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt (TSP) as the internal standard. NMR data is represented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, dd = doublet of doublets, m = multiplet and/or multiple resonances, bs. = broad signal), J coupling (Hz), peak integral and intensity. NMR signals were assigned on the basis of 1D 1H NMR, 2D 1H 13C HSQC, and 2D STD-1H, 13C-HSQC spectra.

2D 1H,13C-HSQC with constant-time evolution. All 2D 1H,13C-HSQC experiments were recorded with residual water suppression by highly selective presaturation (2 μW) during the interscan recovery delay (d1 = 1 s), broadband 13C decoupling during acquisition (AQ = 153.6 ms) using GARP4 (Bdec = 2.52 kHz), and States-TPPI type complex data sampling in the indirect 13C(F1) dimension. The latter was implemented with editing by attached CHn proton multiplicity, resulting in an inversion of signals from methylene groups (i.e. C6). For 13C enriched moieties like 13C6-labelled β-galactose, homonuclear 1J,C,C coupling (≈ 40 – 45 Hz in saccharides) produces additional signal broadening (for a sampled resolution > 1J,C,C) or splitting (for a sampled resolution < 1J,C,C) in the 13C frequency domain. In indirect 13C frequency dimensions, this can be suppressed by constant time evolution during 1/1J,C,C ≈ 23 ms, which also causes a sign modulation by (-1)n according to the multiplicity n of connected 13C neighbour spins. Thus, signals from terminal 13C atoms (e.g., C1 and C6 in hexapyranoses) with only one attached 13C neighbour appear inverted.

2D STD-1H,13C-HSQC. The 2D STD-1H,13C-HSQC is a combination of the conventional 1H',1H-STD with a 2D 1H,13C-HSQC. The latter read-out module was implemented in the same way as described above, with optional constant-time evolution for 13C, but without residual water signal suppression by presaturation during d1 (= 3 s) to avoid a detrimental saturation of coincident protein HA signals. This was unproblematic due to our choice of a deuterated solvent, but could otherwise be implemented by 13C,1H coherence selective echo/antiecho gradients[7] or an Excitation Sculpting module[8] in the refocussing INEPT prior to acquisition. The initial 1H',1H-STD module was implemented with saturation by a train of 4 × 90º PC9_4 shaped pulses (with 1 ms separation) during d9 = 2 s, applied at the methyl
1H peak (0.84 ppm) with the non-saturation cut-off set at 1.18 ppm (i.e. distance Δν = 0.34 ppm), resulting in a PC9_4 pulse length of 33 ms at 800 MHz field strength. Protein saturation was alternated with off-resonant irradiation (at -25 ppm) in successive scans, prior to phase cycling and t1(13C) incrementing, and stored in distinct memory buffers to produce a pseudo 3D spectrum with the 3rd pseudo dimension indexing for either the on-resonant saturated or off-resonant irradiated 2D STD-1H,13C-HSQC. The STD spectrum was then constructed by simple subtraction of both 2D 1H,13C-HSQC spectra.

For analysis by 2D STD-1H,13C-HSQC we used mixtures with a triLacNAc: galectin ratio of 30:1, a galectin concentration of 30 µM, and a total volume of 180 µL in 3 mm NMR tubes.

2D 1H,1H ROESY with 13C decoupling. The 2D 1H,1H ROESY was implemented as EASY-ROESY\(^9\) variant to suppress concomitant 1H,1H TOCSY transfer by means of a jump-symmetrised spinlock pair (BSL = 6 kHz) for ROE mixing, with framing adiabatic projection pulses to minimise the offset dependence. Furthermore, COSY artefacts were suppressed by framing the ROE mixing time (150 ms) with an asymmetric pair of ZQC suppression filters implementing slice selective dephasing by concomitant application of adiabatic sweeps (CHIRP shape with 20% truncation, 15 vs. 20 ms duration, 40 kHz sweep) and matched z-gradient pulses (< 5 G/cm strength).\(^{10}\) For residual water suppression, highly selective presaturation (10 µW) was applied during the interscan recovery delay (d1 = 1.5 s).

Finally, 13C decoupling was implemented in both t1, using a centered 13C BIP pulse\(^{11}\) (300 us, BW/BRF = 1.2) and during FID acquisition, using a 13C BUSS (123.2 ms duration) pulse\(^{12}\) to ensure minimal power decoupling.

For analysis by 2D ROESY we used mixtures of a triLacNAc: galectin ratio of 4:1, a galectin concentration of 450 µM, and a total volume of 500 µL in 5 mm NMR tubes.

4.1 Structural characterization of unlabeled compound 5 and labeled compounds 5a, 5b and 5c. Monosaccharide residue assignments of the compounds 5 were made by numbering each sugar of the unlabeled N-acetyllactosamine starting from the reducing terminus and continuing in sequential order as indicated below:

Gal(β1,4)GlcNAc(β1,3)Gal(β1,4)GlcNAc(α/β)-OH (5)

\(^1\)H NMR (800 MHz, D\(_2\)O): δ (ppm) 5.13 (d, J = 2.11 Hz, 1H, H-1\(_{a1}\)), 4.65 (d, J = 8.2 Hz, 1H, H-1\(_{\beta1}\)), 4.63 (d, J = 8.3 Hz, 2H, H-1\(_{\beta3,4}\)), 4.41-4.37 (m, 3H, H-1\(_{\beta2,4,6}\)), 4.10 (d, J = 2.5
SUPPORTING INFORMATION

Hz, 2H, H-4$_{2,4}$), 3.91-3.86 (m, 4H, H-6$_{1,3,5}$), 3.85 (d, J = 3.11 Hz, 1H, H-4$_6$), 3.83-3.62 (m, 26 H, H-2$_{1,3,5}$, H-3$_{1,3,5}$, H-3$_{2,4}$, H-5$_{2,4,6}$, H-6'$_{1,3,5}$, H-6$_{2,4,6}$), 3.59 (dd, J = 10.2, 3.3 Hz, 1H, H-3$_6$), 3.53-3.49 (m, 5H, H-2$_{2,4}$, H-4$_{1,3,5}$), 3.5 (t, J = 8.95 Hz, 1H, H-2$_6$), 1.98-1.95 (m, 9H, CH$_3$CO$_{1,3,5}$). $^{13}$C form HSQC (201 MHz, D$_2$O): $\delta$ (ppm) 102.9 (C-1$\beta$_2,4,6), 102.7 (C-1$\beta$_3,5), 94.9 (C-1$\beta$_1), 90.5 (C-1$\alpha$_1), 82.0 (C-3$_{2,4}$), 75.1 (C-5$_{2,4,6}$), 74.5 (C-4$_{1,3,5}$), 72.4 (C-3$_6$), 72.1 (C-3$_{1,3,5}$), 70.9 (C-2$_6$), 69.9 (C-2$_{2,4}$), 68.4 (C-4$_6$), 68.2 (C-4$_{2,4}$), 60.9 (C-6, C-6'$_{2,4,6}$), 60.0, 59.8 (C-6, C-6'$_{1,3,5}$), 55.2 (C-2'$_{1,3,5}$), 22.1 (CH3CO$_{1,3,5}$).

MALDI TOF-MS m/z calcd for C$_{42}$H$_{71}$N$_3$O$_3$Na (M + Na)$^+$ exact 1136.3964, found 1136.3912.

Figure S1. Assigned 1D $^1$H NMR spectrum of unlabelled polyLacNAc 5 (800 MHz, 298 K, D$_2$O).

Figure S2. Assigned 2D $^1$H, $^{13}$C HSQC spectrum of unlabelled polyLacNAc 5.

Gal(β1,4)GlcNAc(β1,3)[U-$^{13}$C]Gal(β1,4)GlcNAc(β1,3)Gal(β1,4)GlcNAc(α/β)-OH (5b)
MALDI TOF-MS m/z calec for C$_{36}$H$_{71}$N$_{3}$O$_{31}$Na (M + Na)$^+$ exact 1142.4165, found 1142.6227.

**Figure S3.** Assigned $^1$D $^1$H NMR spectrum ($^{13}$C decoupled) of compound 5b (800 MHz, 298 K, D$_2$O).

**Figure S4.** Assigned 2D $^1$H,$^{13}$C HSQC spectrum of 5b, with constant-time evolution during CT$_1 = 23$ ms and resulting $^{13}$C($^{13}$C)$_n$ carbon multiplicity editing resulting in inverted sign for C(C)$_1$ groups.

$[^{13}$C]$\text{Gal(}\beta_{1,4}\text{GlcNAc(}\beta_{1,3}\text{)]}[^{13}$C]$\text{Gal(}\beta_{1,4}\text{GlcNAc(}\beta_{1,3}\text{)]}[^{13}$C]$\text{Gal(}\beta_{1,4}\text{GlcNAc(}\alpha/\beta\text{-)}\text{OH}$ (5a)
MALDI TOF-MS m/z calcd for C_{24}\textsuperscript{13}C_{18}H_{71}N_{3}O_{31}Na (M + Na)\textsuperscript{+} exact 1154.4568, found 1154.5757.

**Figure S5.** Assigned 1D \textsuperscript{1}H NMR spectrum (\textsuperscript{13}C decoupled) of compound of 5a (800 MHz, 298 K, D\textsubscript{2}O).

**Figure S6.** Assigned 2D \textsuperscript{1}H,\textsuperscript{13}C HSQC spectrum of 5a, with constant-time evolution during CT\textsubscript{1} = 23 ms and resulting \textsuperscript{13}C(\textsuperscript{13}C)\textsubscript{n} carbon multiplicity editing resulting in inverted sign for C(C)\textsubscript{i} groups.

Gal(\beta1,4)GlcNAc(\beta1,3)Gal(\beta1,4)GlcNAc(\beta1,3)[U-\textsuperscript{13}C]Gal(\beta1,4)GlcNAc(\alpha/\beta)-OH (5c)
MALDI TOF-MS m/z calcd for C\textsubscript{36}\textsuperscript{13}C\textsubscript{6}H\textsubscript{71}N\textsubscript{3}O\textsubscript{31}Na (M + Na)	extsuperscript{+} exact 1142.4165, found 1142.5394.

**Figure S7.** Assigned 1D $^1$H NMR spectrum ($^{13}$C decoupled) of compound of 5c (800 MHz, 298 K, D\textsubscript{2}O).

**Figure S8.** Assigned 2D $^1$H,$^{13}$C HSQC spectrum of 5c, with constant-time evolution during CT\textsubscript{1} = 23 ms and resulting $^{13}$C($^{13}$C)\textsubscript{n} carbon multiplicity editing resulting in inverted sign for C(C)\textsubscript{1} groups.
Figure S9. STD-$^1$H,$^{13}$C-HSQC experiments with Gal-1: left, reference spectrum; right, STD-$^1$H,$^{13}$C-HSQC spectrum. A, with ligand 5c. B, with ligand 5b. C, with ligand 5a.
Figure S10. STD-$^1$H,$^{13}$C-HSQC experiments with Gal-3 CRD: left, reference spectrum; right, STD-$^1$H,$^{13}$C-HSQC spectrum. A, with ligand 5c. B, with ligand 5b. C, with ligand 5a. The off-resonance spectrum is shown in the middle, with 5-fold intensity to highlight the signals of the bound form.
Figure S11. STD-$^1$H,$^{13}$C-HSQC experiments with Gal-7: left, reference spectrum; right, STD-$^1$H,$^{13}$C-HSQC spectrum. A, with ligand 5c. B, with ligand 5b. C, with ligand 5a.
Figure S12. STD-$^1$H, $^{13}$C-HSQC experiments with Gal-8 FL: left, reference spectrum; right, STD-$^1$H, $^{13}$C-HSQC spectrum. A, with ligand 5c. B, with ligand 5b. C, with ligand 5a.
Figure S13. STD-$^1$H,$^{13}$C-HSQC experiments with Gal-9 Nter: left, reference spectrum; right, STD-$^1$H,$^{13}$C-HSQC spectrum. A, with ligand 5c. B, with ligand 5b. C, with ligand 5a.
Figure S14. Analysis of STD-HSQC NMR experiments for 5a with the different galectins. The plots represent the STD intensity (in%) ((cross-peak integral in the STD spectrum/cross-peak intensity in the reference spectrum)×100) of every CH cross-peak of the Gal residues (represented in the X axis). * Not reported due to too low resolution (F1) to appropriately distinguish signals from A and (B+C).
**Figure S15.** Schematic interaction of Gal-3 CRD with tri-LacNAc 5a (with $^{13}$C$_6$ labelling of galactose residues). (B) 2D EASY-ROESY spectrum (with 6 kHz spin locking during 150 ms and $^{13}$C decoupling) of 5a in the presence of Gal-3 CRD (molar ratio 4:1), showing positive exchange signals (red arrows) between free and bound forms of the ligand signals. (C) 2D $^1$H, $^{13}$C- HSQC spectrum (with F1($^{13}$C) frequency evolution during 23 ms constant time) of the same sample showing the cross-peaks of the β-galactose in the bound form. The red arrows indicate their up-field shift relative to the free form. All spectra were acquired at 298 K, 800 MHz.
**Figure S16.** Comparison of LacNAc recognition by Gal-3, Gal-9N and Gal-7 according to X-ray crystallography. The perspective is the same for all of them with respect to the conserved Trp residue at the binding site (W181 in Gal-3, W82 in Gal-9N and W69 in Gal-7). Only the b-galactopyranose and aminoacids at the binding site are shown. On the left, the complex of Gal-3 with LacNAc (pdb 1a3k), in the middle, the complex of Gal-9N with tri-LacNAc (pdb 2zhm), and on the right, the complex of Gal-7 with LacNAc (pdb 5gal).
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