# Supplementary Information

## Unambiguous identification of α-Gal epitopes in intact monoclonal antibodies by NMR spectroscopy

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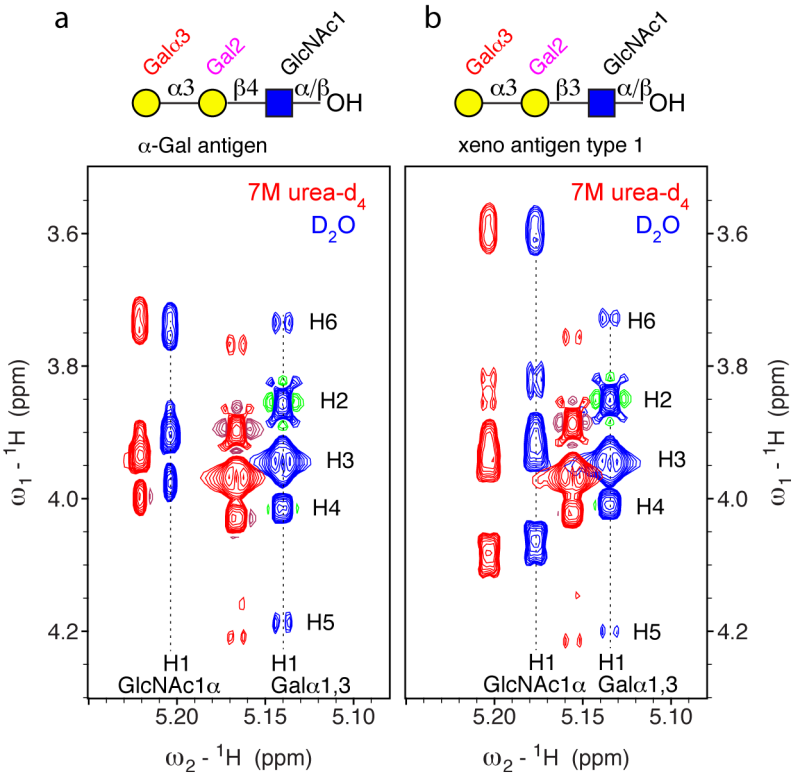
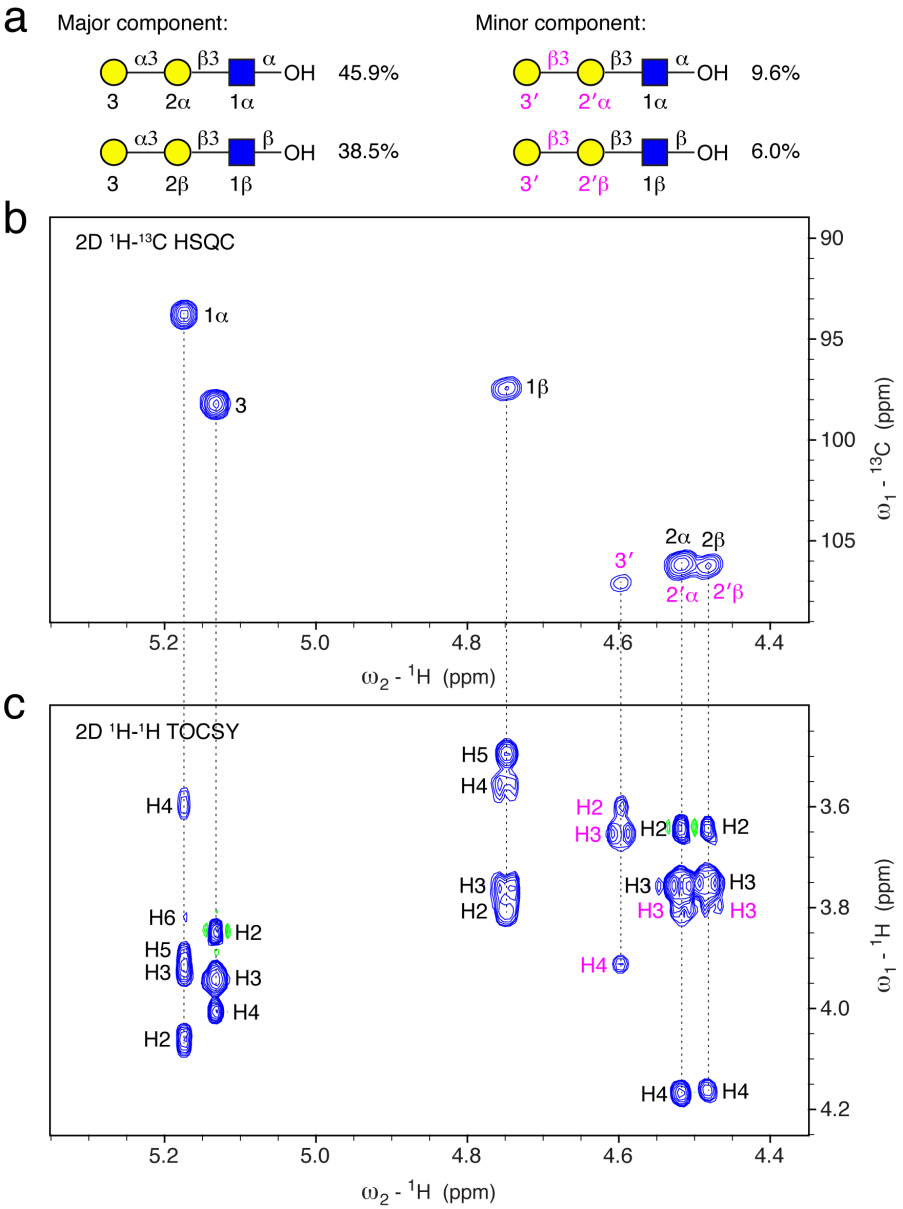
**Supplementary Figures**




**Supplementary Figure S1 caption**: Influence of the presence of 7 M urea on the 2D $^{1}$H-$^{1}$H TOCSY correlations of the α-Gal epitope trisaccharide (Galβ1,3Galβ1,4GlcNAc) and the xeno antigen type 1 trisaccharide (Galβ1,3Galβ1,3GlcNAc). (a) 2D $^{1}$H-$^{1}$H TOCSY spectrum of α-Gal epitope trisaccharide measured either in 7 M urea-$d_4$ pH* 7.4 (red) or plain $D_2O$ (blue) at 298 K. The acquisition parameters were 4 transients, a recycle delay of 1 sec and 4096×512 points. (b) 2D $^{1}$H-$^{1}$H TOCSY spectrum of xeno antigen type 1 trisaccharide measured either in 7 M urea-$d_4$ pH* 7.4 (red) or plain $D_2O$ (blue) at 298 K using comparable acquisition parameters as for the α-Gal epitope trisaccharide.

**Supplementary Figure S1 Alt Text**: Overlay of 2D $^{1}$H-$^{1}$H TOCSY correlations of the α-Gal epitope and the xeno antigen type 1 trisaccharides in the presence and absence of 7 M urea. Shown is the region in which the anomeric H1 resonance shows correlations to all protons within the moiety. The H1 resonances of terminal Gal and the terminal GlcNAc shift significantly approx. by 0.03 ppm.

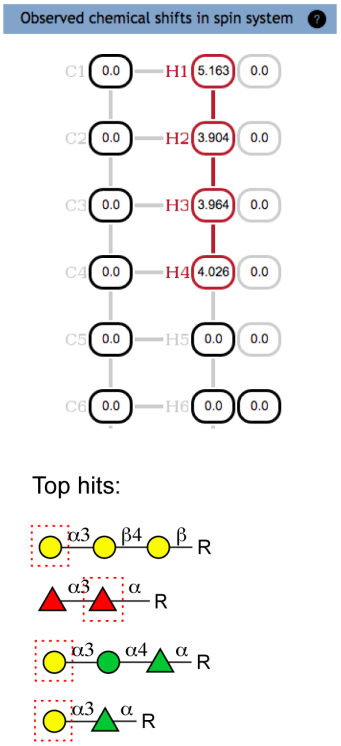



**Supplementary Figure S2 caption**: Assignment of the minor component Galβ1,3Galβ1,3GlcNAc, which was found to be present in the xeno antigen type 1 trisaccharide (Galα1,3Galβ1,3GlcNAc) from Elicityl (GLY74-1) with 16% abundance. a) Symbolic representations of the major and minor component. b) $^{1}$H-$^{13}$C HSQC spectrum with the anomeric signals of the minor component labeled in magenta. c) $^{1}$H-$^{1}$H TOCSY spectrum showing intra-monosaccharide correlations of the anomeric H1 resonances. Isolated signals of the minor component are labeled in magenta.

**Supplementary Figure S2 Alt Text**: 2D $^{1}$H-$^{13}$C HSQC and 2D $^{1}$H-$^{1}$H TOCSY correlations of the xeno antigen type 1 trisaccharide at low contour level. Signals of a impurity are visible, which could be identified as Galβ1,3Galβ1,3GlcNAc.

a

Input




Top hits:




Output

Top 1000 matches out of 16465 database records (result generated in 33 ms.)

| LinucsID | Residue | Linkage | Match |
|---|---|---|---|
| 26613 | α-D-Galp | 3, 4, 3 | 99.921 |

| | H | | | | | | | | | H' | | | | | | | | | C | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ID | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| Sugar | 5.186 | 3.888 | 3.966 | 4.026 | 4.194 | 3.740 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Query | 5.163 | 3.904 | 3.964 | 4.026 | | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Error | 0.023 | 0.016 | 0.002 | 0.000 | | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

| LinucsID | Residue | Linkage | Match |
|---|---|---|---|
| 12634 | α-L-Fucp | 4,6 | 99.861 |

| | H | | | | | | | | | H' | | | | | | | | | C | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ID | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| Sugar | 5.174 | 3.897 | 3.930 | 4.034 | 4.155 | - | - | - | - | - | - | - | - | 1.236 | - | - | - | | 100.930 | 67.630 | 75.480 | 69.070 | 66.750 | 16.160 | - | - | - |
| Query | 5.163 | 3.904 | 3.964 | 4.026 | | - | - | - | - | - | - | - | - | - | - | - | - | | - | - | - | - | - | - | - | - | - |
| Error | 0.011 | 0.007 | 0.034 | 0.008 | | - | - | - | - | - | - | - | - | - | - | - | - | | - | - | - | - | - | - | - | - | - |

| LinucsID | Residue | Linkage | Match |
|---|---|---|---|
| 9207 | α-D-Galp | 2,4 | 99.836 |

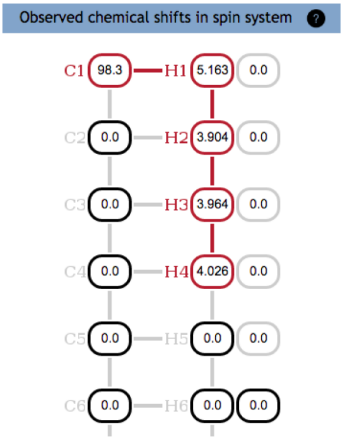
| | H | | | | | | | | | H' | | | | | | | | | C | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ID | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| Sugar | 5.174 | 3.898 | 3.946 | 4.060 | 4.091 | 3.690 | - | - | - | - | - | - | - | - | 3.750 | - | - | - | - | - | - | - | - | - | - | - | - |
| Query | 5.163 | 3.904 | 3.964 | 4.026 | | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Error | 0.011 | 0.006 | 0.018 | 0.034 | | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

| LinucsID | Residue | Linkage | Match |
|---|---|---|---|
| 9737 | α-D-Galp | 3,2,3,2 | 99.730 |

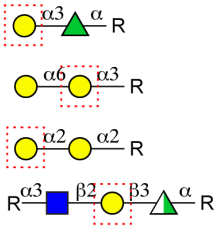
| | H | | | | | | | | | H' | | | | | | | | | C | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ID | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| Sugar | 5.140 | 3.860 | 3.950 | 4.020 | 4.210 | 3.730 | - | - | - | - | - | - | 3.730 | - | - | - | | | 96.200 | 69.100 | 70.200 | 70.000 | 71.600 | 61.700 | - | - | - |
| Query | 5.163 | 3.904 | 3.964 | 4.026 | | - | - | - | - | - | - | - | - | - | - | - | | | - | - | - | - | - | - | - | - | - |
| Error | 0.023 | 0.044 | 0.014 | 0.006 | | - | - | - | - | - | - | - | - | - | - | - | | | - | - | - | - | - | - | - | - | - |

b

Input




Top hits:




Output

Top 1000 matches out of 16465 database records (result generated in 33 ms.)

| LinucsID | Residue | Linkage | Match |
|---|---|---|---|
| 9737 | α-D-Galp | 3,2,3,2 | 99.640 |

| | H | | | | | | | | | H' | | | | | | | | | C | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ID | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| Sugar | 5.140 | 3.860 | 3.950 | 4.020 | 4.210 | 3.730 | - | - | - | - | - | - | 3.730 | - | - | - | | | 96.200 | 69.100 | 70.200 | 70.000 | 71.600 | 61.700 | - | - | - |
| Query | 5.163 | 3.904 | 3.964 | 4.026 | | - | - | - | - | - | - | - | - | - | - | - | | | 96.500 | - | - | - | - | - | - | - | - |
| Error | 0.023 | 0.044 | 0.014 | 0.006 | | - | - | - | - | - | - | - | - | - | - | - | | | 0.300 | - | - | - | - | - | - | - | - |

| LinucsID | Residue | Linkage | Match |
|---|---|---|---|
| 26448 | α-D-Galp | 3 | 98.698 |

| | H | | | | | | | | | H' | | | | | | | | | C | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ID | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| Sugar | 5.171 | 3.892 | 3.951 | 4.106 | 4.300 | 3.882 | - | - | - | - | - | - | 3.752 | - | - | - | | | 97.290 | 70.720 | 72.190 | 71.870 | 71.380 | 68.910 | - | - | - |
| Query | 5.163 | 3.904 | 3.964 | 4.026 | | - | - | - | - | - | - | - | - | - | - | - | | | 96.500 | - | - | - | - | - | - | - | - |
| Error | 0.008 | 0.012 | 0.013 | 0.080 | | - | - | - | - | - | - | - | - | - | - | - | | | 0.790 | - | - | - | - | - | - | - | - |

| LinucsID | Residue | Linkage | Match |
|---|---|---|---|
| 22508 | α-D-Galp | 6, 5, 3, 3, 2, 2 | 98.566 |

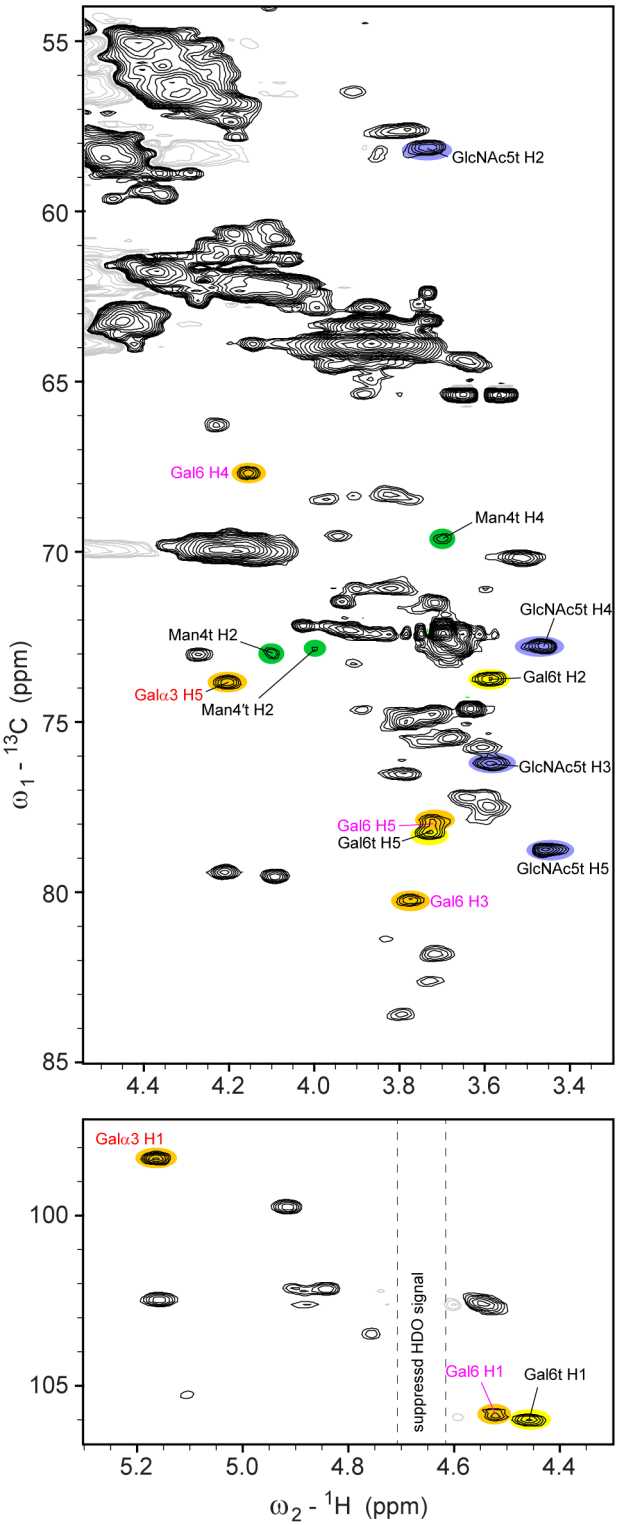
| | H | | | | | | | | | H' | | | | | | | | | C | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ID | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| Sugar | 5.247 | 3.840 | 3.943 | 3.983 | 4.138 | 3.700 | - | - | - | - | - | - | - | - | - | - | | | 96.200 | 68.540 | 69.720 | 70.790 | 71.550 | 61.500 | - | - | - |
| Query | 5.163 | 3.904 | 3.964 | 4.026 | | - | - | - | - | - | - | - | - | - | - | - | | | 96.500 | - | - | - | - | - | - | - | - |
| Error | 0.084 | 0.064 | 0.021 | 0.043 | | - | - | - | - | - | - | - | - | - | - | - | | | 0.300 | - | - | - | - | - | - | - | - |

| LinucsID | Residue | Linkage | Match |
|---|---|---|---|
| 9346 | α-D-Galp | 3,2,3,until | 97.615 |

| | H | | | | | | | | | H' | | | | | | | | | C | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ID | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| Sugar | 5.310 | 3.860 | 3.970 | 4.010 | 4.180 | 3.750 | - | - | - | - | - | - | 3.730 | - | - | - | | | 96.470 | 79.350 | 69.120 | 70.430 | 71.370 | 61.750 | - | - | - |
| Query | 5.163 | 3.904 | 3.964 | 4.026 | | - | - | - | - | - | - | - | - | - | - | - | | | 96.500 | - | - | - | - | - | - | - | - |
| Error | 0.147 | 0.044 | 0.006 | 0.016 | | - | - | - | - | - | - | - | - | - | - | - | | | 0.030 | - | - | - | - | - | - | - | - |

**Supplementary Figure S3 caption**: Using the chemical shifts obtained from the terminal Galα1,3 moiety for a search in GlycoNMRSearch. a) Search using four connected $^{1}H$ chemical shifts as input (left) and the top four results on the right. b) Search using four connected $^{1}H$ chemical shifts together with the $^{13}C$ chemical shift for C1 as input (left) and the top four results on the right.

**Supplementary Figure S3 Alt Text**: The input mask of the web application GlycoNMRSearch is shown with the chemical shifts of the terminal Galα1,3 moiety. Panel a shows values for H1, H2, H3 and H4 as input and the corresponding output. The first, the third and fourth hit shows oligosaccharides with a terminal α1,3-linked galactose. In panel b the input was extended by the value of C1. The top and the third result shows an oligosaccharide with a terminal α1,3-linked galactose.




**Supplementary Figure S4 caption**: Semiquantitative analysis of the terminal residues observed in a $^{1}$H-$^{13}$C HSQC spectrum of cetuximab. The spectrum is identical to the one presented in Figures 3 and 4. The signals used for integration are color-coded: terminal GlcNAc in blue, terminal α-Gal epitopes in orange (consisting of the terminal Gal with the red label and the preceding Gal labeled in magenta) and terminal Man in green.

**Supplementary Figure S4 Alt Text**: $^{1}H$-$^{13}C$ HSQC spectrum of cetuximab with highlighted signals that were used for the quantification of the terminal glycan residues. All signals were completely isolated except two with slight overlap (indicated in the following by an asterisk): H3*, H4, H5 of terminal GlcNAc the completely isolated signals of H4 and H5; H2 and H4 of terminal Man; H2 of terminal Man4′; H1 and H2 of Gal (β1,3-linked to GlcNAc); H1 and H5 of terminal α1,3-linked Gal; H1, H3, H4, H5* of the underlying Gal of the α-Gal epitope.

**Supplementary Table S1:** Observed chemical shifts of xeno antigen type 1 trisaccharide (Galα1,3Galβ1,3GlcNAc) measured either in 7 M urea-$d_4$/$D_2O$ pH* 7.4 (indicated by U) or in $D_2O$ (indicated by D). The data was measured at 298 K and referenced to internal DSS. A comparison to the entry 2615 "Xeno antigen type 1 / Galα3 LacNAc type 1" in the Glyco3D database measured in $D_2O$ [42] is given as well.

| Moiety | H1 | H2 | H3 | H4 | H5 | H6 | H6' | H8 | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gal3 (U) | 5.132 | 3.847 | 3.941 | 4.005 | 4.198 | 3.726 | 3.726 | — | 98.2 | 70.9 | 72.1 | 71.9 | 73.5 | 63.8 | — | — | this work |
| Gal3 (D) | 5.132 | 3.847 | 3.941 | 4.005 | 4.198 | 3.726 | 3.726 | — | 98.2 | 70.9 | 72.1 | 71.9 | 73.5 | 63.8 | — | — | this work |
| | 5.13 | 3.84 | 3.92 | 4.00 | 4.20 | — | — | — | 97.6 | 71.1 | 72.2 | 72.1 | 73.8 | 63.9 | — | — | Glyco3D[b] |
| Gal2(α)[a] (U) | 4.517 | 3.639 | 3.757 | 4.166 | 3.719 | 3.775 | 3.775 | — | 106.2 | 72.1 | 80.2 | 67.5 | 77.7 | 63.8 | — | — | this work |
| Gal2(α)[a] (D) | 4.517 | 3.639 | 3.757 | 4.166 | 3.719 | 3.775 | 3.775 | — | 106.2 | 72.1 | 80.2 | 67.5 | 77.7 | 63.8 | — | — | this work |
| Gal2(β)[a] (U) | 4.481 | 3.642 | 3.749 | 4.161 | 3.719 | 3.775 | 3.775 | — | 106.2 | 72.1 | 80.2 | 67.5 | 77.7 | 63.8 | — | — | this work |
| Gal2(β)[a] (D) | 4.481 | 3.642 | 3.749 | 4.161 | 3.719 | 3.775 | 3.775 | — | 106.2 | 72.1 | 80.2 | 67.5 | 77.7 | 63.8 | — | — | this work |
| | 4.48 | 3.62 | 3.73 | 4.16 | 3.70 | — | — | — | 106.4 | 72.3 | 80.3 | 67.7 | 81.9[c] | 63.9 | — | — | Glyco3D[b] |
| GlcNAcα (U) | 5.174 | 4.062 | 3.922 | 3.591 | 3.893 | 3.828 | 3.816 | 2.020 | 93.8 | 55.5 | 83.6 | 71.6 | 73.9 | 63.3 | 177.3 | 24.9 | this work |
| GlcNAcα (D) | 5.174 | 4.062 | 3.922 | 3.591 | 3.893 | 3.828 | 3.816 | 2.020 | 93.8 | 55.5 | 83.6 | 71.6 | 73.9 | 63.3 | 177.3 | 24.9 | this work |
| GlcNAcβ (U) | 4.747 | 3.811 | 3.763 | 3.558 | 3.494 | 3.901 | 3.757 | 2.017 | 97.4 | 58.22 | 86.03 | 71.5 | 78.17 | 63.48 | 177.4 | 24.9 | this work |
| GlcNAcβ (D) | 4.747 | 3.811 | 3.763 | 3.558 | 3.494 | 3.901 | 3.757 | 2.017 | 97.4 | 58.22 | 86.03 | 71.5 | 78.17 | 63.48 | 177.4 | 24.9 | this work |
| | 4.74 | 3.79 | 3.76 | 3.57 | 3.49 | — | — | 2.01 | 98.4 | 58.4 | 86.2 | 71.7 | 78.3 | 63.7 | 177.4 | 24.9 | Glyco3D[b] |

[a] The chemical shifts depend on the anomeric form at the GlcNAc at the reducing end. (α) stands for GlcNAcα at the reducing end and (β) stands for GlcNAcβ at the reducing end.

[b] The reported values showed an offset. For better comparison, a correction of -0.06 ppm was subtracted from the database $^{1}H$ values and +1.9 ppm was added to the $^{13}C$ values

[c] Deviation

**Supplementary Table S2:** Observed chemical shifts of the impurity Galβ1,3Galβ1,3GlcNAc in $D_2O$ measured at 298 K and referenced to internal DSS.

| Moiety | H1 | H2 | H3 | H4 | H5 | H6 | H6' | H8 | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gal3 | 4.597 | 3.601 | 3.648 | 3.908 | 3.679 | 3.789 | 3.789 | — | 107.1 | 73.8 | 75.2 | 71.4 | 77.8 | 63.8 | — | — |
| Gal2(α)[a] | 4.516 | 3.666 | 3.796 | 4.190 | 3.719[b] | 3.745 | 3.745 | — | 106.2[b] | 72.6 | 84.9 | 71.1 | 77.7[b] | 63.8[b] | — | — |
| Gal2(β)[a] | 4.478 | 3.666 | 3.796 | 4.183 | 3.719[b] | 3.745 | 3.745 | — | 106.2[b] | 72.6 | 84.9 | 71.1 | 77.7[b] | 63.8[b] | — | — |
| GlcNAcα | 5.174 | 4.062 | 3.922 | 3.591 | 3.893 | 3.828 | 3.816 | 2.020 | 93.8[b] | 55.5[b] | 83.6[b] | 71.6[b] | 73.9[b] | 63.3[b] | 177.3[b] | 24.9[b] |
| GlcNAcβ | 4.747 | 3.811 | 3.763 | 3.558 | 3.494 | 3.901 | 3.757 | 2.017 | 97.4[b] | 58.2[b] | 86.0[b] | 71.5[b] | 78.2[b] | 63.5[b] | 177.4[b] | 24.9[b] |

[a] The chemical shifts depend on the anomeric form at the GlcNAc at the reducing end. (α) stands for GlcNAcα at the reducing end and (β) stands for GlcNAcβ at the reducing end.

[b] Red values are indistinguishable from Galα1,3Galβ1,3GlcNAc

## References


42. Perez S, Sarkar A, Rivet A, Breton C, Imberty A. Glyco3D: a portal for structural glycosciences. Methods Mol Biol 2015; 1273:241-58. doi: 10.1007/978-1-4939-2343-4_18.