Supplementary Material to:

Carbohydrate specificity of an insecticidal lectin isolated from the leaves of *Glechoma hederacea* towards mammalian glycoconjugates

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Supplementary Materials and Method:

Glycoproteins and polysaccharides

Hamster submaxillary glycoprotein (gp), one of the simplest gps among mammalian salivary mucins containing NeuAc-GalNAc (sialyl-**Tn**) and GalNAcα1-Ser/Thr (**Tn**) residues as carbohydrate side chains, was obtained by a modification of the methods previously described [45]. About two-thirds of the carbohydrate side chains of armadillo submandibular gland mucin (ASG-A) is GalNAcα1-Ser/Thr (**Tn**) and one third is Neu5Acα2-6GalNAcα1-*O*-Ser/Thr (sialyl **Tn**) [48,49]. Native ASG-**Tn** [46], a salivary glycoprotein of nine-banded armadillo (*Dasypus novemcinctus mexicanus*) containing only **Tn** as carbohydrate side chains, was isolated from the 0.01 M PBS, pH 6.8 gland extract after removal of ASG-A, which is one of the sialoglycoproteins in armadillo glands [48].

Tn and sialyl-**Tn** containing ovine (OSM), bovine (BSM), and porcine (PSM) sub-mandibular/salivary mucins (glycoproteins) were purified according to the method described earlier by Tettamanti and Pigman [50] with some modifications [48]. About 75% of the carbohydrate side chains of asialo OSM were GalNAcα1-Ser/Thr (**Tn**). Asialo PSM contains Galβ1-3GalNAcα1-Ser/Thr ($\mathbf{T}_\alpha$) [47] together with **Tn** and **A** (GalNAcα1-3Gal, human blood group A-specific disaccharide) sequences, as most of the outer fucosyl residues are cleaved by the mild acid hydrolysis.

The active (M.W. 10.5×10^3-21.0×10^3) and inactive (M.W. 2.6×10^3-3.8×10^3) anti-freeze gp from the Antarctic fish (*Trematomus borchgrevinki*), which contains exclusively Galβ1-3GalNAcα1- ($\mathbf{T}_\alpha$) as carbohydrate chains [51], was provided by Dr. R.E. Freeney (Department of Food Science and Technology, University of California, Davis, CA, USA) through the late Dr. E.A. Kabat (Columbia Medical Center, New York, USA).

Glycophorin A, a sialyl **T**$_\alpha$-containing glycoprotein, was prepared from the membranes of old human blood group O erythrocytes by phenol/saline extraction and was purified by gel filtration in the presence of SDS [52]. The **Tn**-glycophorin was obtained by removing the terminal galactose residues from asialo-glycophorin by periodate oxidation and mild acid hydrolysis [20].

Human α_1-acid glycoprotein (Sigma) contains tetra-, tri- and di-antennary complex type glycans in the ratio of 2:2:1 [53,54]. Fetuin (Gibco Laboratories, Grand Island, NY, USA), which is the major glycoprotein in fetal calf serum and has six oligosaccharide side chains per molecule, three *O*-glycosidically-linked to Ser/Thr and three *N*-glycosidically-linked to Asn [55] that contains tri- and di-antennary complex type glycans in the ratio of 1:2 [56].

ABH blood group active glycoproteins (Cyst Beach, MSS, Tighe, Mcdon and JS phenol insoluble) were prepared from human ovarian cyst fluid [42,57-60].

Desialylation of sialoglycoproteins was performed by mild acid hydrolysis in 0.01 N HCl at 80°C for 90 min and dialyzed against distilled H_2O for 2 days to remove small fragments [50].

The human blood group P_1-active substance, purified from sheep hydatid cyst glycoprotein, was kindly provided by late Dr. W.M. Watkins (Imperial College School of Medicine, Hammersmith Hospital London, UK) [61].

The *Pneumococcus* type 14 polysaccharide, isolated from *Streptococcus pneumoniae* capsule [62], was a gift from the late Dr. E.A. Kabat. Mannan from *Saccharomyces cerevisiae* was purchased from Sigma.

Lectinochemical assays

The assay was performed according to the procedures described by Duk et al. [20]. The volume of each reagent applied to the plate was 50 μl/well, and all incubations, except for coating, were performed at 20°C. The reagents, if not indicated otherwise, were diluted with Tris HCl buffered saline containing 0.05% Tween 20 (TBS-T). The TBS buffer or 0.15 M NaCl containing 0.05% Tween 20 were used for washing the plates between incubations.

The 96-well microtiter plates (Nunc, Maxisorp, Vienna, Austria) were coated with glycoproteins at the amount of 5 to less than 0.07 ng (see Figure 1a of manuscript), and

500 to less than 10 ng per well (see Figures 1c and 1d of manuscript) in 0.05 M carbonate buffer, pH 9.6, overnight at 4°C. After washing the plate, biotinylated lectins were added and incubated for 30 min. The plates were washed to remove unabsorbed lectin and the ExtrAvidin/alkaline phosphatase solution (Sigma, diluted 1:10,000) was added. After 1h the plates were washed at least four times and incubated with *p*-nitrophenyl phosphate (Sigma 104 phosphatase substrate 5 mg tablets) in 0.05 M carbonate buffer, pH 9.6, containing 1 mM $MgCl_2$ (1 tablet/5 ml). The absorbance was read at 405 nm in a microtiter plate reader, after 2h incubation with the substrate.

For inhibition studies, serially diluted inhibitor samples were mixed with an equal volume of lectin solution containing a fixed amount of lectin. The control lectin sample was diluted two-fold with TBS-T. After 30 min incubation at 20°C, samples were tested in the binding assay, as described above. The inhibitory activity was estimated from the inhibition curve and is expressed as the amount of inhibitor (ng or nmole/well) giving 50% inhibition of the control lectin binding.

All experiments were done in duplicate or triplicate, and data are presented as the mean value of the results. The standard deviation did not exceed 10% and in most experiments was less than 5% of the mean value. For the binding experiment, the control wells, where coating or addition of biotinylated lectin was omitted, gave low absorbance values (below 0.1). It showed that blocking the wells before lectin addition was not necessary when Tween 20 was present in the TBS. On the other hand, for the inhibition experiment, two controls were set up. The first control was treated under exactly the same condition as the experimental group except that the inhibitor was left out; the absorbance value was recorded at 2h was between 2.8-3.3. For the second control (coating only or negative control), both lectin and inhibitors were left out while other conditions were kept the same as in the experimental group, and the absorbance value of this control was below 0.1, which was used as the background value.

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Supplementary Figure 1. Results from the Gleheda-glycan binding array analysis. A total of 190 glycans were screened for binding along with positive and negative controls, as described under “Experimental”.

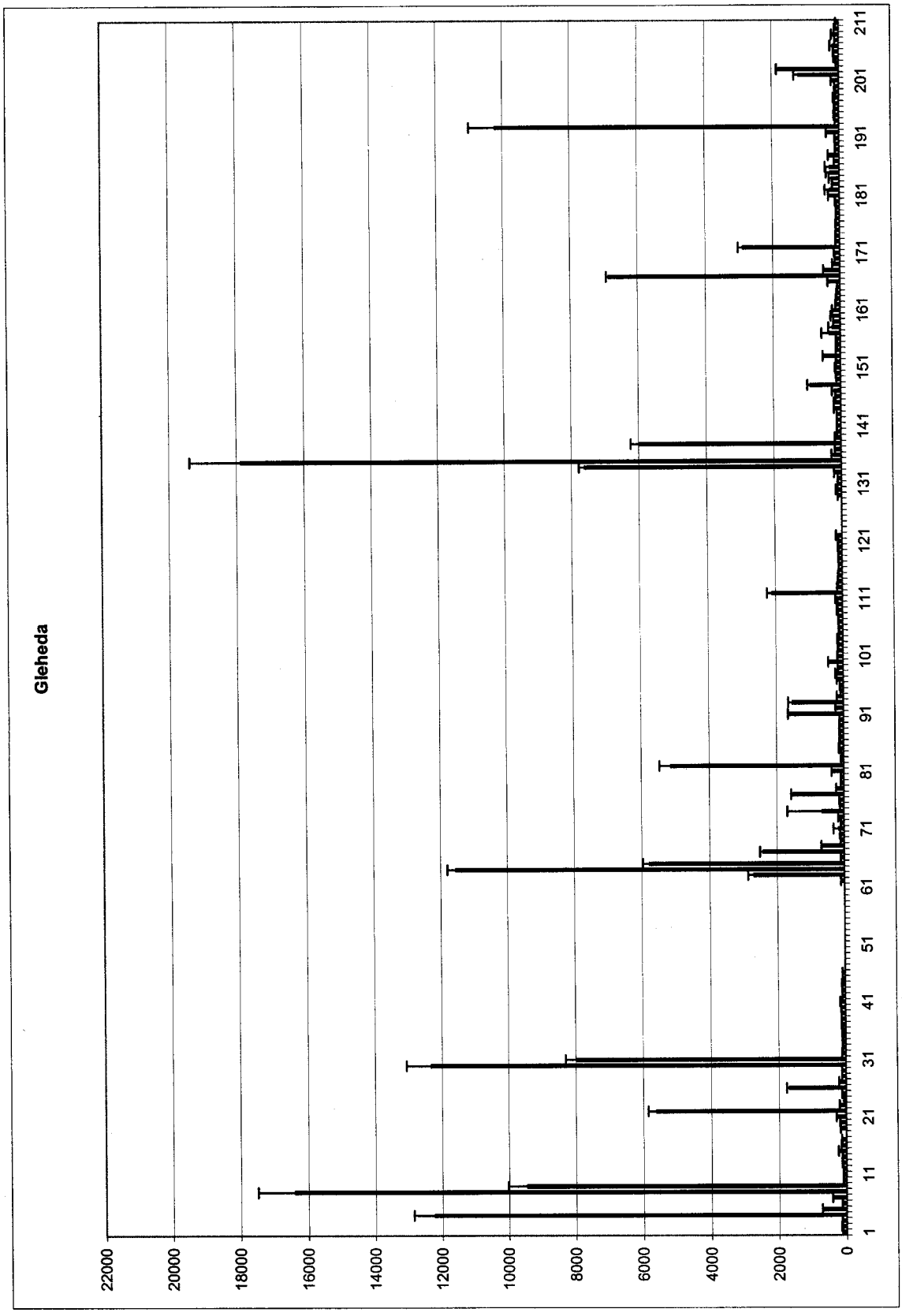
Gleheda
22000
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
1
11
21
31
41
51
61
71
81
91
101
111
121
131
141
151
161
171
181
191
201
211
Glycan No.

Fig. 1

Supplementary Table 1. Comparison of the binding properties of two Tn specific lectins, recognizing GalNAcα/β1→ glycotopes

#	Carbohydrate specificity	Lectins	
		Gleheda	SSL*
1	Monosaccharide and submolecular specificity	α- and β- anomer of Gal and enhanced by *N*-acetyl group at carbon-2 (GalNAcα1→) strongly	α- and β- anomer of GalNAc; *N*-acetyl group at carbon-2 and configuration of carbon-3 in GalNAc
2	a) Ratio of α and β anomer for *p*-nitrophenyl GalNAc glycosides††	1.3 (α ≥ β; curve 21 vs. 22)	1.7 (α ≤ β; curve 1 vs. 2)
	b) Ratio of α and β anomer for *p*-nitrophenyl Gal glycosides††	1.4 (α ≥ β; curve 26 vs. 27)	1.2 (α ≤ β; curve 6 vs. 7)
	c) Ratio of α and β anomer for methyl GalNAc glycosides††	11.2 (α > β; curve 20 vs. 24)	4.3 (α > β; curve 3 vs. 5)
	d) Ratio of α and β anomer for methyl Gal glycosides††	17.3 (α > β; curve 25 vs. 29)	3.0 (α > β; curve 8 vs. 14)
	e) Hydrophobicity for GalNAcα-anomer†	No (0.6; Ratio of curve 21 / curve 20)	No (1; Ratio of curve 2 / curve 3)
	f) Hydrophobicity for Galα-anomer†	No (1.0; Ratio of curve 26 / curve 25)	Yes (12.3; Ratio of curve 7 / curve 8)
	g) Hydrophobicity for GalNAcβ-anomer†	Yes (5.6; Ratio of curve 22 / curve 24)	Yes (7.4; Ratio of curve 1 / curve 5)
	h) Hydrophobicity for Galβ-anomer†	Yes (12.6; Ratio of curve 27 / curve 29)	Yes (44.4; Ratio of curve 6 / curve 14)
3	Reactivity toward mammalian structural units expressed in decreasing order (based on nanomoles comparison)	GalNAcα1-Ser/Thr (**Tn**) > GalNAcα1-3Galβ1-4Glc ($\mathbf{A}_L$) ≥ GalNAcα1-3Gal (**A**) > GalNAcα1-3(ʟFucα1-2)Gal ($\mathbf{A}_h$) > Galα1-3Gal (**B**) ≥ GalNAcα1-3GalNAc (**F**) ≥ GalNAcβ1-4Gal (**S**) ≥ GalNAcβ1-3Gal (**P**) > Galα1-4Gal (**E**) >> Galβ1-4Glc (**L**); while Galβ1-3GalNAc (**T**), Galβ1-3GlcNAc (**I**) and Galβ1-4GlcNAc (**II**) were inactive (Table 3)	GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glc (**F**p) ≥ GalNAcα1-3Galβ1-4Glc ($\mathbf{A}_L$) > GalNAcα1-3GalNAcβ1-Me ($\mathbf{F}_\beta$) ≥ GalNAcα1-3GalNAcα1-Me ($\mathbf{F}_\alpha$) > GalNAcα1-Ser/Thr (**Tn**) ≥ GalNAcα1-3Gal (**A**) > GalNAcα1-3GalNAc (**F**) ≥ GalNAcβ1-4Gal (**S**) > GalNAcβ1-3Gal (**P**); while Galβ1-4GlcNAc (**II**), Galβ1-3 GlcNAc (**I**), Galβ1-3GalNAc (**T**), Galα1-4Gal (**E**) and Galα1-3Gal (**B**) were inactive (Table 2)
4	The most active monomeric ligands	GalNAcα1-Ser/Thr (**Tn**) mainly	GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glc (**F**p), GalNAcα1-3 Galβ1-4Glc ($\mathbf{A}_L$), GalNAcα1-3GalNAcβ1-Me ($\mathbf{F}_\beta$) and GalNAcα1-Ser/Thr (**Tn**)
5	Ratio of glycotope clusters (simple multivalent form) / monomeric **Tn**	**Tn**-glycopeptides from asialo ovine submandibular glycoprotein (M.W. <3,000 Da) was 5 times more active than monomeric **Tn** (Table 3)	**Tn**-glycopeptides from asialo ovine submandibular glycoprotein (M.W. <3,000 Da) was only 1.6 times more active than monomeric **Tn** (Table 2)
6	Ratio of complex polyvalent glycotopes in macromolecules / monomeric **Tn**	3100 times more active than monomeric **Tn** (Table 2)	33 times more active than monomeric **Tn** (Table 3)
7	Proposed shape of combining site§	Small cavity type of GalNAcα1- as major combining site with shallow groove as subsite	Shallow groove type
8	The most complementary size	GalNAcα1-Ser/Thr (**Tn**)	One GalNAcα1-Ser/Thr (**Tn**) to three to five sugars ($\mathbf{A}_h$ or $\mathbf{A}_L$ and **F**p)
9	Biological Function	Insecticidal property	Unknown

Characterization no. ; *SSL (*Salvia sclarea* lectin), [41]; ††From Table 3 of manuscript; †Based on *p*-nitrophenyl > the corresponding methyl glycosides as Yes; *p*-nitrophenyl < the corresponding methyl glycosides as No, in Table 3 of manuscript; §The proposed combining site, whether specific for terminal nonreducing ends of chains (cavity-type sites) or for internal sequences for sugar chains (groove-type sites) based on the criteria of reference [63].