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Note

Differing rates of galactosylation of homologous spacer-modified disaccharides are an indication for two separate GlcNAc binding-sites in galactosyltransferase

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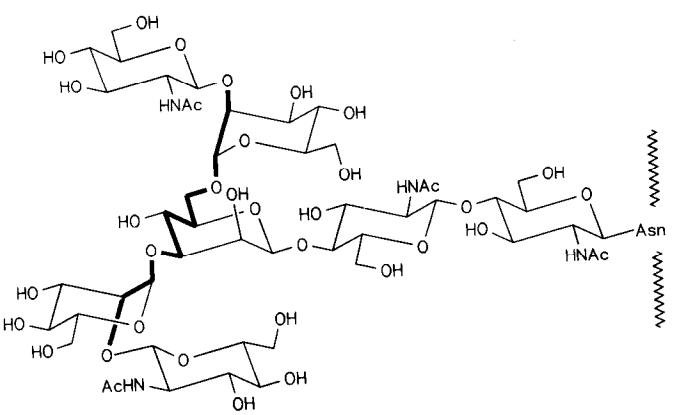
The biantennary N-linked core-heptasaccharide **1** is a natural acceptor for enzymic (1 → 4)-β-D-galactosylation, preferentially of the 3-linked antenna[1]. This site-specificity and the difference in K_M of one order of magnitude between the nongalactosylated acceptor **2** and the corresponding monogalactosylated glycopeptide[2] **3** may be taken as indications for two separate recognition- and binding-subsites (A and B) for the terminal GlcNAc residues. One (A) is also the site of galactosylation (Fig. 1).



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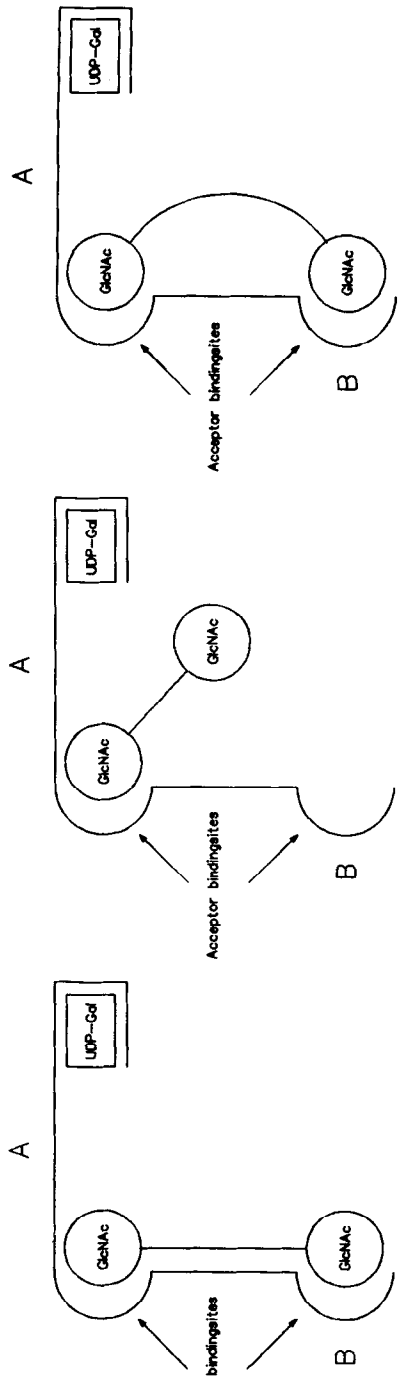
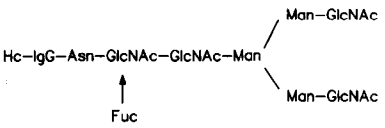


Fig. 1. Schematic presentation of Gal-T-acceptor complexes I, II, and III. The relative rate of galactosylation is optimal with the fitting spacer-modified disaccharide **4** (I). Shortening the spacer, as in compounds **7** and **8** (II), decreases relative rates drastically. Lengthening the spacer, as in compound **6** (III) has only a minor effect. A, galactosylation site; B, additional GlcNAc binding-site.



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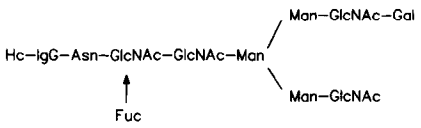

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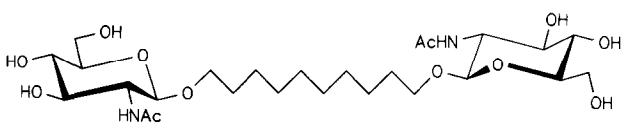
This "mechanical" model requires that the two GlcNAc end groups of an acceptor substrate be kept at the "right" distance for optimal binding to occur. In an earlier publication, we demonstrated that the carbohydrate structure linking the end groups in the natural ligand may be replaced by an acyclic flexible spacer of the right length – in this case 10 atoms, not including the glycosidic oxygen atoms[3] (structure **4**). Increasing the hydrophobicity of an acceptor by introducing a lipophilic spacer normally increases the rate of galactosylation relative to GlcNAc *. This so called hydrophobic effect, however, is not solely responsible for the very high relative rate of galactosylation of **4**. Pentyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**5**) having the same relative hydrophobicity as compound **4** (because it has exactly half its structure), is an inferior substrate[4].



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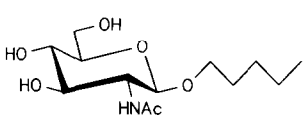

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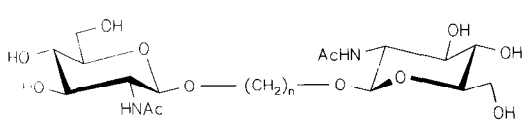

6 n = 12
7 n = 8
8 n = 6

We have now synthesised three spacer-modified disaccharides (**6**, **7**, and **8**), as homologues of **4**, differing in the length of the spacer. Both shorter homologues (**7**

* The V_{max} values for the galactosylation of all GlcNAc derivatives do not differ significantly. Relative rates of galactosylation with GlcNAc as a competing acceptor are therefore a measure for relative affinities.

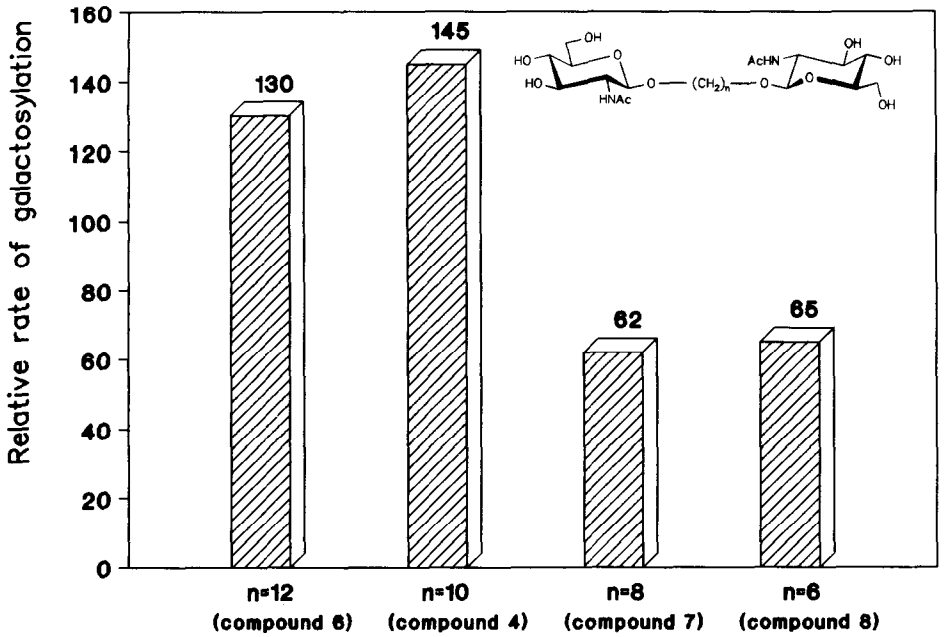

Fig. 2. Relative rates of galactosylation were measured in the presence of GlcNAc as a competing acceptor.

and **8**) showed highly diminished relative rates of galactosylation, pointing to a decreased affinity of the ligands. Lengthening the chain by two methylene groups (compound **6**) has only a minor effect on the relative rate of galactosylation. Apparently the insufficient length of the spacer is responsible for this effect (Fig. 2). These findings further support the model of a binding area in galactosyltransferase with two subsites for two GlcNAc residues, which must be placed at a certain distance for optimal binding and high relative rate of galactosylation (Fig. 1).

EXPERIMENTAL

General methods.—All reactions were monitored by TLC on Silica Gel 60 F_{254} (Merck). Scanning for radioactivity after TLC was carried out with a Berthold TLC-linear analyzer LB 282. Flash-column chromatography[5] was performed on ICN-silica 32–63 (ICN Biomedicals). Melting points were measured with a Büchi apparatus and are uncorrected. Optical rotations were obtained with a Schmidt & Haensch Polartronic I polarimeter. ^{1}H NMR spectra (250 MHz) were recorded with a Bruker WM 250 spectrometer for solutions in $CDCl_3$ (internal Me_4Si). Elemental analyses were obtained with a Perkin–Elmer 240 analyser.

TABLE I

^{1}H NMR data [a] (chemical shift, δ)

Compound	9	11	13
H-1	5.37	5.36	5.28
H-2	4.33	4.35	4.27
H-3	5.97	5.80	5.77
H-4	5.18	5.20	5.17
H-5	3.87	3.86	3.86
H-6a	4.33	4.35	4.34
H-6b	4.17	4.18	4.17
OCH_2	3.84, 3.43	3.87, 3.35	3.67, 3.25
OAc	2.12, 2.03, 1.87	2.13, 2.06, 1.88	2.12, 2.04, 1.87
Aryl H	7.87, 7.78	7.86, 7.74	
CH_2	1.42(2), 1.03(4) 0.94(4)	1.4(2), 0.95(3) 0.75	1.1(2), 0.84(2)

[a] $J_{1,2}$ 8.7, $J_{2,3}$ 10.5, $J_{3,4}$ 9, $J_{4,5}$ 10.5, $J_{5,6a}$ 3, $J_{5,6b}$ 4.5, $J_{6a,6b}$ 12 Hz.

β-D-Galactosyltransferase (UDP-galactose : *N*-acetylglucosamine-1,4-β-D-galactosyltransferase; EC 2.4.1.22) from bovine milk (4 U/mg) was purchased from Sigma, and UDP-D[^{14}C]Gal (300 mCi/mmol) from Amersham Buchler.

1,12-Bis(3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyloxy)dodecane (**9**).—To a solution of 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranose[6] (5 g, 10.47 mmol) in CH_2Cl_2 (100 mL), $SnCl_4$ (1.18 mL, 10 mmol) was added under anhydrous conditions. The mixture was stirred for 15 min at room temperature, 1,12-decanediol (0.71 g, 3.5 mmol) was added and stirring was continued for 8 h. The mixture was poured into ice–water (250 mL) with stirring, the organic layer was separated, the aqueous layer was extracted with $CHCl_3$ (3 × 60 mL), the organic layers were combined, neutralised with satd aq $NaHCO_3$ (200 mL), dried (Na_2SO_4), and concentrated to yield an oil which crystallised from EtOH to give **9** (2.8 g, 77%); mp 146–147°C; $[\alpha]_D^{20}$ +22° (*c* 1.1, $CHCl_3$); R_f 0.22 (1:1 cyclohexane–EtOAc); Anal. Calcd for $C_{52}H_{64}N_2O_{20}$: C, 60.22; H, 6.22; N, 2.7. Found: C, 60.21; H, 6.24; N, 2.67. For ^{1}H NMR data see Table I.

1,12-Bis(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyloxy)dodecane (**10**).—To a solution of **9** (2 g, 1.93 mmol) in EtOH (100 mL), $BuNH_2$ (20 mL) was added, and the solution was boiled under reflux for 25 h, concentrated in vacuo, and methanol (2 × 50 mL) then toluene (2 × 50 mL) were evaporated from the residue. Pyridine (40 mL) and Ac_2O (20 mL) were added at 0°C and the mixture was kept overnight at 25°. When TLC showed the reaction to be complete (R_f 0.26, 5:1 toluene–EtOH), the mixture was concentrated in vacuo. Flash-column chromatography of the residue gave a colourless oil, which crystallised from EtOH to give **10** (1.2 g, 72%); mp 168–70°, $[\alpha]_D^{23}$ −20° (*c* 1.0, $CHCl_3$, R_f 0.26 (5:1 toluene–EtOH); For ^{1}H NMR data see Table II. Anal. Calcd for $C_{40}H_{64}N_2O_{18}$: C, 55.80; H, 7.49; N, 3.23. Found: C, 56.00; H, 7.31; N, 3.23.

TABLE II

^{1}H NMR data [a] (chemical shift, δ)

Compound	10	12	14
H-1	4.70	4.73	4.65
H-2	3.83	3.84	3.83
H-3	5.32	5.34	5.30
H-4	5.06	5.06	5.00
H-5	3.72	3.71	3.62
H-6a	4.27	4.28	4.26
H-6b	4.13	4.13	4.13
OCH_2	3.85, 3.47	3.84, 3.48	3.66, 3.25
*H*NAc	5.84	5.93	5.89
NAc	2.03	2.0	2.03
OAc	2.04, 2.03, 1.94	2.10, 2.0, 1.95	2.03, 2.01, 1.96
CH_2	1.56(4), 1.37(6)	1.54(2), 1.25(4)	1.15(2), 0.84(2)

[a] $J_{1,2}$ 8.7, $J_{2,3}$ 9, J_{34} 9.75, $J_{4,5}$ 9.75, $J_{5,6a}$ 2.25, $J_{5,6b}$ 4.5, $J_{6a,6b}$ 12 Hz.

*1,12-Bis(2-acetamido-2-deoxy-β-*D*-glucopyranosyloxy)dodecane* (**6**).—To a suspension of **10** (350 mg, 0.4 mmol) in MeOH (20 mL), M methanolic NaOMe (0.1 mL) was added. After 5 min the mixture became clear. After 15 min crystals appeared, and after 1 h TLC (R_f 0.46; 4:2:1 EtOAc–MeOH–H_2O) showed the reaction to be complete. The mixture was made neutral with 30% aq AcOH and evaporated in vacuo. The residue, a white solid, was crystallised from MeOH to give **6** (200 mg, 82.5%); mp 202°C; $[\alpha]_D^{20}$ −28°C (*c* 1, MeSO. Anal. Calcd for $C_{28}H_{52}N_2O_{12}$: C, 55.25; H, 8.61; N, 4.60. Found: C, 55.12; H, 8.46; N, 4.54.

*1,8-Bis(3,4,6-Tri-*O*-acetyl-2-deoxy-2-phthalimido-β-*D*-glucopyranosyloxy)octane* (**11**).—To a solution of 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranose (2.0 g, 4.47 mmol) in CH_2Cl_2 (50 mL) was added $SnCl_4$ (0.5 mL, 4.26 mmol) under anhydrous conditions. After 15 min 1,8-octanediol (218 mg, 1.49 mmol) was added, and the reaction was continued as described for **9**. Flash-column chromatography (1:1 cyclohexane–EtOAc) of the product yielded a colourless oil, which was crystallised from EtOH to give **11** (1.16 g, 78%); R_f 0.2 (1:1 cyclohexane–EtOAc); mp 110–112°C; $[\alpha]_D^{23}$ +25° (*c* 1.1 $CHCl_3$). For ^{1}H NMR data see Table I. Anal. Calcd for $C_{48}H_{54}N_2O_{20}$: C, 58.77; H, 5.75; N, 2.85. Found: C, 58.18; H, 5.54; N, 5.11.

*1,8-Bis(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-*D*-glucopyranosyloxy)octane* (**12**).—To a solution of **11** (1 g, 1.02 mmol) in EtOH (50 mL) was added $BuNH_2$ (10 mL), and the solution was treated as described for **10**. Flash-column chromatography (5:1 toluene–EtOH) of the product yielded **12** isolated as a colourless oil (620 mg, 75.7%); R_f 0.35 (5:1 toluene–EtOH). For ^{1}H NMR data see Table II.

*1,8-Bis(2-acetamido-2-deoxy-β-*D*-glucopyranosyloxy)octane* (**7**).—A solution of **12** (500 mg, 0.62 mmol) in MeOH (10 ml) was *O*-deacetylated as described for **6**. Flash-column chromatography (7:2:1 EtOAc–MeOH–H_2O) of the product yielded a colourless oil, which was crystallised from EtOAc to give **7** (310 mg,

90.5%); R_f 0.34 (4:2:1 EtOAc–MeOH–H_2O); mp 190–192°C; $[\alpha]_D^{23}$ −26° (*c* 1.0, H_2O). Anal. Calcd for $C_{24}H_{44}N_2O_{12}$: C, 52.16; H, 8.03; N, 5.07. Found: C, 51.45; H, 7.96; N, 5.04.

*1,6-Bis(3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-*D*-glucopyranosyloxy)hexane* (**13**).—To a solution of 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranose (1.0 g, 2.1 mmol) in CH_2Cl_2 (10 mL) was added $SnCl_4$ (0.4 mL, 3.33 mmol) under anhydrous conditions. After 15 min 1,6-hexanediol (83 mg, 0.7 mmol) was added, and the reaction was continued as described for **9**. Flash-column chromatography (1:1 cyclohexane–EtOAc) of the product yielded a colourless oil, which was crystallised from EtOH to give **13** (0.4 g, 62%); R_f 0.15 (1:1 cyclohexane–EtOAc); mp 141°C; $[\alpha]_D^{23}$ +23° (*c* 1.0, $CHCl_3$). For ^1H NMR data see Table I. Anal. Calcd for $C_{46}H_{52}N_2O_{20}$: C, 57.98; H, 5.50; N, 2.94. Found: C, 59.62; H, 5.56; N, 3.19. M: 952.67.

*1,6-Bis(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-*D*-glucopyranosyloxy)hexane* (**14**).—To a solution of **13** (5.0 g, 5.25 mmol) in EtOH (100 mL) was added $BuNH_2$ (20 mL), and the solution was treated as described for **10**. Flash-column chromatography (5:1 toluene–EtOH) of the product yielded **14**, isolated as a colourless oil (3.4 g, 83%); R_f 0.3 (5:1 toluene–EtOH). For ^1H NMR data see Table II.

*1,6-Bis(2-acetamido-2-deoxy-β-*D*-glucopyranosyloxy)hexane* (**8**).—A solution of **14** (3.4 g, 4.37 mmol) in MeOH (20 ml) was *O*-deacetylated as described for **6**. Flash-column chromatography (7:2:1 EtOAc–MeOH–H_2O) of the product yielded a colourless oil, which was crystallised from MeOH–EtOAc to give **8** (2.0 g, 87%); R_f 0.27 (4:2:1 EtOAc–MeOH–H_2O); mp 198°C; $[\alpha]_D^{23}$ −35° (*c* 1.0, H_2O). Anal. Calcd for $C_{22}H_{40}N_2O_{12}$: C, 50.37; H, 7.69; N, 5.34. Found: C, 50.02; H, 7.40; N, 5.61.

Competitive galactosylations.—Assays involved 5 mM solutions of the spacer-modified oligosaccharides (SMO) **7** or **8** in buffer (10 μL, 50 μmol), 0.5 M solution of GlcNAc in buffer (10 μL, 5 mmol), aq 85.6 μM UDP[D-[^{14}C]Gal 10 μL, 0.86 nmol), and pure buffer (10 μL). Each mixture was incubated with enzyme solution in buffer (10 μL, 10 μg/400 μL) at 37°C and the reactions were monitored by TLC (6:4:3 1 BuOH–pyridine–H_2O). Because of the low solubility of the SMO **6** in the buffer, a 0.25 mM solution (20 μL, 5.0 μmol) of this SMO competing with a 50 mM solution of GlcNAc (10 μL, 0.5 mmol) in buffer was measured. The results are shown in Fig. 2.

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REFERENCES

1 M.R. Paquet, S. Narasimhan, H. Schachter, and M.A. Moscarello, *J. Biol. Chem.*, 259 (1984) 4716–4721.

2 A.K. Rao and J. Mendicino, *Biochemistry*, 17 (1978) 5632–5638.
3 J. Lehmann and S. Petry, *Carbohydr. Res.*, 204 (1990) 141–144.
4 S.-C. Ats, J. Lehmann, and S. Petry, *Carbohydr. Res.*, 233 (1992) 125–139.
5 W.C. Still, M. Khan, and A. Mitra, *J. Org. Chem.*, 43 (1978) 2923–2925.
6 R.U. Lemieux, T. Takeda, and B.Y. Chang, *ACS Symp. Ser.*, 39 (1976) 90–115.