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Note

Enzyme-assisted synthesis of Asn-linked diantennary oligosaccharides occurring on glycodelin A[1]

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Abstract

The preparation of a series of sialylated and fucosylated *N,N'*-diacetyllactosediamine-type diantennary glycopeptides is reported. By sequential enzymatic action of jack bean β-galactosidase, snail β4-*N*-acetyl-galactosaminyltransferase, bovine colostrum α6-sialyltransferase and human milk α3-fucosyltransferase, a diantennary glycopeptide obtained from asialo fibrinogen was converted at a 5-μmol scale to a series of structures occurring on the glycoprotein glycodelin A, which potentially inhibit human sperm-egg binding.

Keywords: Glycosyltransferases; Enzymatic synthesis; *N,N'*-diacetyllactosediamine; Glycodelin

Human glycodelin A is a glycoprotein produced in the endometrium and hematopoietic tissues of the bone marrow and can be isolated from amniotic fluid. It has immunosuppressive properties and in addition it is a potent inhibitor of sperm-egg binding [1]. Many of the N-glycans occurring on glycodelin A carry fucosylated and/or sialylated GalNAc(β1–4)GlcNAc (LacdiNAc) elements rather than Gal(β1–4)GlcNAc (LacNAc) based determinants [2]. Although less common than LacNAc, the LacdiNAc fragment has been found on several human glycoproteins (for a review, see ref. [3]). The biological significance of most LacdiNAc-based determinants is not yet very well understood, but it has been hypothesized that the sialylated and fucosylated LacdiNAc- containing carbohydrate chains on glycodelin A may account for its contraceptive properties [2]. Within our research program studying the role of carbohydrates in mammalian sperm-egg interactions we aim to test this hypothesis and therefore we have carried out the enzyme-assisted synthesis of a series of LacdiNAc-containing Asn-linked diantennary carbohydrate chains occurring on glycodelin A. The starting compound **GP-F2**, a diantennary LacNAc-type glycopeptide isolated from asialo fibrinogen, was first enzymatically remodeled to a diantennary glycopeptide with two LacdiNAc branches. Subsequently, enzymatic sialylation and fucosylation of the LacdiNAc branches was carried out to yield the desired series of compounds (Scheme 1) which potentially are responsible for

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[1] Dedicated to Roger W. Jeanloz on the occasion of his 80th birthday.

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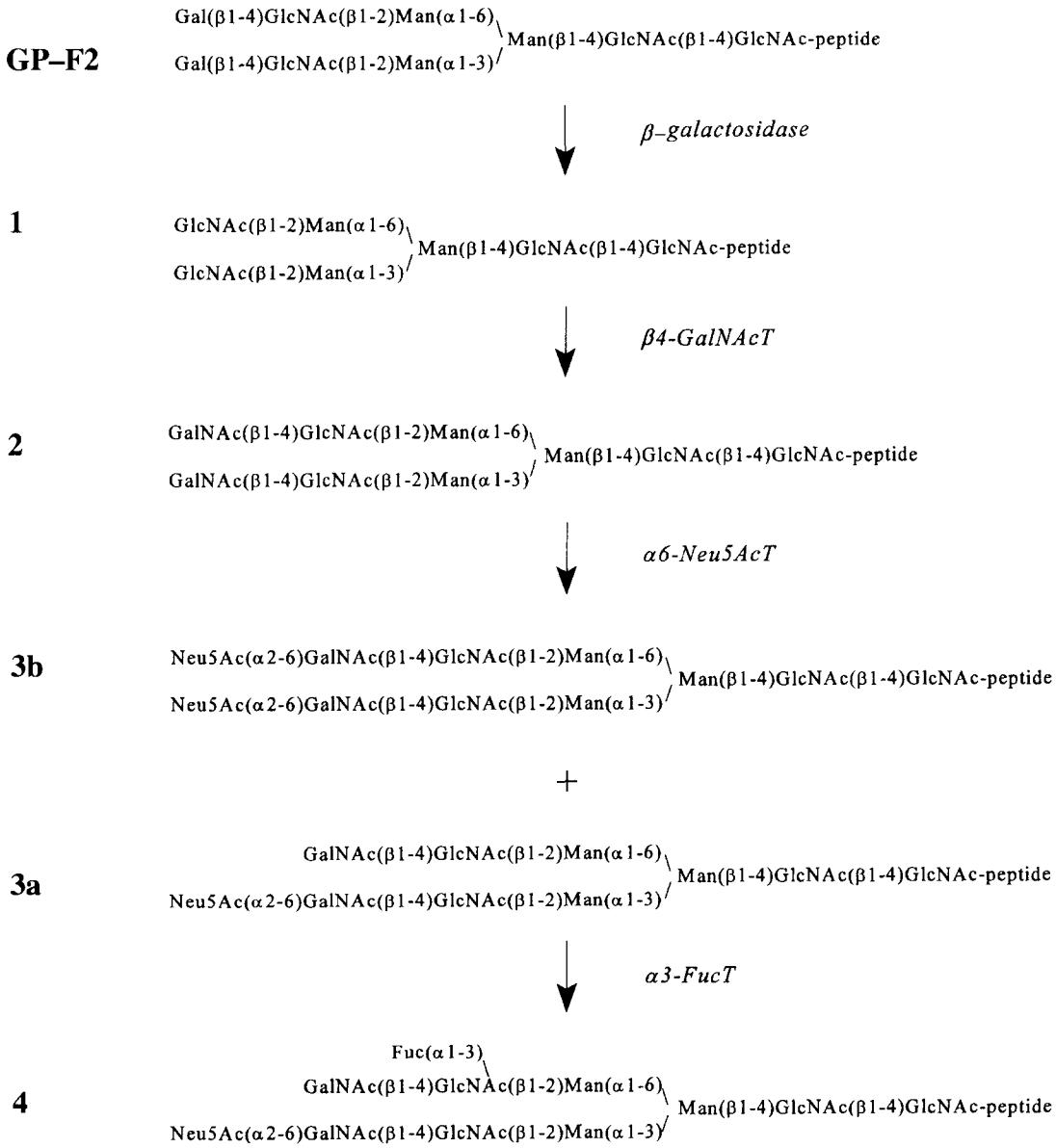
the contraceptive properties of glycodelin A. The ^{1}H NMR data of each of the synthesized compounds are given in Table 1.

The diantennary glycopeptide **GP–F2** was quantitatively converted at a 5 μmol scale to **1** by removal of the terminal Gal residues using jack bean β-galactosidase. The product was purified on Bio-Gel P-4 and identified by ^{1}H NMR spectroscopy. The ^{1}H NMR data of **1** match those of compound **1** in [4], except for slight differences in the chemical shift values of the structural-reporter groups of GlcNAc-**1** due to the presence of different amino acids.

Compound **1** was incubated with UDP-GalNAc: β-GlcNAc (β1–4)-*N*-acetyl-galactosaminyltransferase (β4–GalNAcT) and UDP-[^{3}H]GalNAc. The reaction product was isolated by Bio-Gel P-4 chromatography (Fig. 1(A)) to give **2** at high yield (4.5 μmol, 90%). The ^{1}H NMR data of **2** are in accordance to those of compound **IIa** in [4]. The utilization of β4-GalNAcT from *Lymnaea stagnalis* albumen glands for the synthesis of the LacdiNAc sequence has been reported previously [4–6].

Incubation of 2.5 μmol of **2** with CMP-Neu5Ac:Gal(β1–4)GlcNAc α6-sialyltransferase (α6-Neu5AcT, ST6Gal I) and CMP-[^{14}C]Neu5Ac gave a monosialylated and a disialylated product. Bio-Gel P-4 chromatography (Fig. 1(B)) of the incubation mixture resulted in the separation of the two products **3a** (1.0 μmol, 40%) and **3b** (1.0 μmol,



Scheme 1. Enzyme-assisted synthesis of diantennary LacdiNAc-containing glycopeptides.

40%). The ^{1}H NMR spectra of **3a** and **3b** are given in Fig. 2(A) and (B), respectively. The data of the monosialylated product **3a** match those of compound **rHPC #9** in [7], with the exception of the chemical shifts of protons in the proximity of GlcNAc-**1** which have different values because of the structural differences in this region of the compounds. The presence of the additional Neu5Ac residue in the Man(α1-6)-branch of the disialylated product **3b** is reflected by the shifts of the H-1 signals of Man-**4′**, GlcNAc-**5′** and GalNAc′ ($\Delta\delta$ 0.021, $\Delta\delta$ 0.023 and $\Delta\delta$−0.022, respectively) and of the GlcNAc-**5′** NAc signal ($\Delta\delta$ 0.025). Similar chemical shift differences are observed for the GlcNAc-**5** and GalNAc residue upon α6-sialylation of the GalNAc(β1–4)GlcNAc fragment in the Man(α1–3)-branch when going from **2** to **3a**. It has been reported previously that the bovine colostrum α6-Neu5AcT is capable of catalyzing the introduction of Neu5Ac residues to C-6 of GalNAc in smaller LacdiNAc-containing oligosaccharides [8]. The conditions for the present α6-Neu5AcT incubation were chosen such that the amount of CMP-Neu5Ac available was limiting, giving rise to the formation of both monosialylated and disialylated compounds, as desired. In the monosialylated product **3a**, the Neu5Ac residue was exclusively located in the Man(α1-3)- branch. The same branch specificity has been observed in the case of

Table 1
^{1}H Chemical shifts of the structural-reporter-group protons of the constituent monosaccharides of the **GP-F2** glycopeptide and enzymatically modified derivatives thereof. Chemical shifts are given at 32 °C and were measured in D_2O relative to acetone (δ 2.225 [15]). The numbering system of the monosaccharide residues is illustrated in Fig. 2(B)

Reporter group	Residue	Chemical shift (ppm) in				
		1	**2**	**3a**	**3b**	**4**
H-1	GlcNAc-**1**	5.034	5.045	5.045	5.045	5.044
	GlcNAc-**2**	4.610	4.617	4.616	4.614	4.612
	Man-**4**	5.118	5.109	5.131	5.128	5.13[a]
	Man-**4′**	4.919	4.916	4.918	4.939	4.904
	GlcNAc-**5**	4.556	4.555	4.583	4.584	4.583
	GlcNAc-**5′**	4.556	4.560	4.561	4.584	4.553
	GalNAc	—	4.515	4.498	4.498	4.500
	GalNAc′	—	4.523	4.524	4.502	4.460
	Fuc	—	—	—	—	5.132
H-2	Man-**3**	4.239	4.245	4.248	4.252	4.245
	Man-**4**	4.183	4.176	4.187	4.187	4.183
	Man-**4′**	4.095	4.093	4.096	4.108	4.075
H-3a	Neu5Ac	—	—	1.719	1.718	1.711
	Neu5Ac′	—	—	—	1.718	—
H-3e	Neu5Ac	—	—	2.659	2.659	2.662
	Neu5Ac′	—	—	—	2.665	—
H-5	Fuc	—	—	—	—	4.851
NAc	GlcNAc-**1**	1.998	2.012	2.012	2.011	2.011
	GlcNAc-**2**	2.077	2.080	2.080	2.081	2.078
	GlcNAc-**5**	2.044	2.046	2.073	2.075	2.068
	GlcNAc-**5′**	2.044	2.040	2.041	2.066	2.035
	GalNAc	—	2.066	2.069	2.069	2.068
	GalNAc′	—	2.073	2.069	2.069	2.046
	Neu5Ac	—	—	2.031	2.031	2.033
	Neu5Ac′	—	—	—	2.031	—
CH_3	Fuc	—	—	—	—	1.268

[a] Value given with two decimals because of spectral overlap.

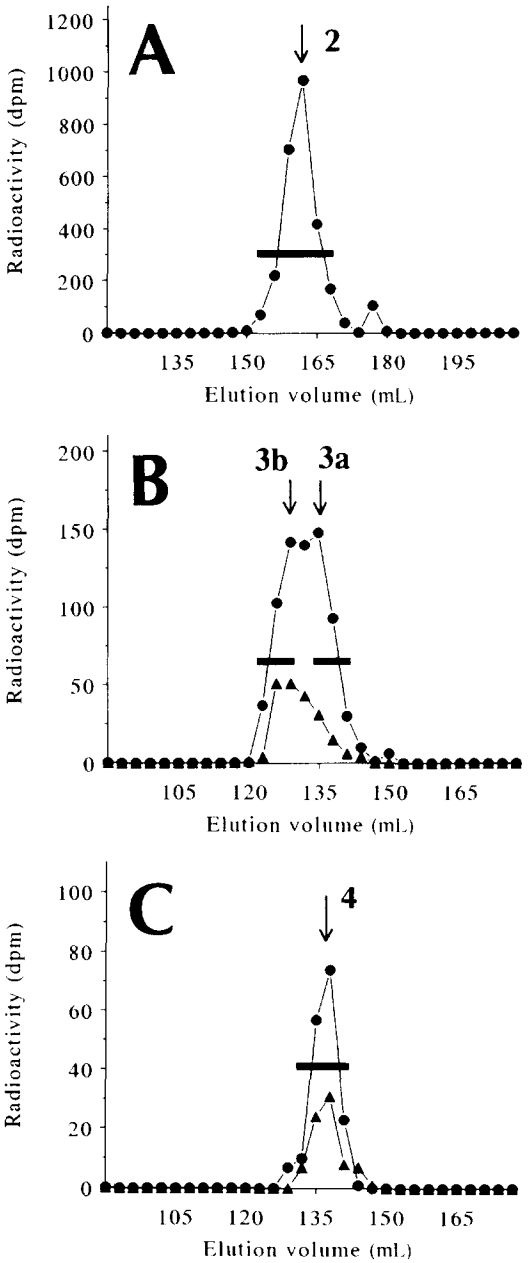


Fig. 1. Bio-Gel P-4 chromatography of the reaction mixtures of the incubation with β4-GalNAcT (A), α6-Neu5AcT (B) and α3-FucT (C). Fractions containing the products were pooled as indicated by the bars. ●, ^{3}H-radioactivity; ▲, ^{14}C-radioactivity.

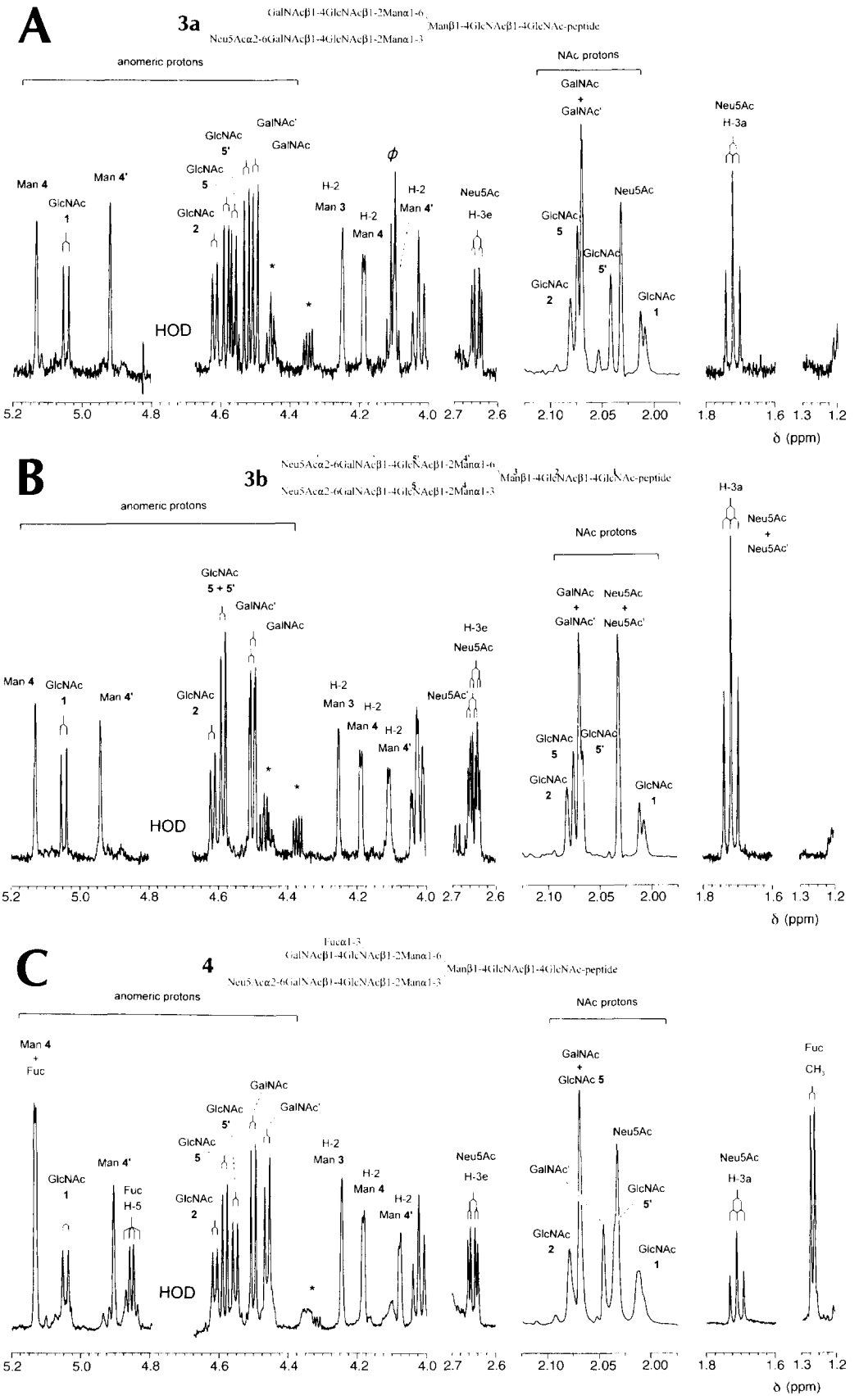


Fig. 2. Structural-reporter-group regions of the 600 MHz ^{1}H NMR spectra of the diantennary glycopeptides **3a** (A), **3b** (B) and **4** (C), recorded in D_2O at 32 °C. The relative scale of the NAc region differs from that of the rest of the spectrum. The asterisks (*) indicate H_α signals of amino acid residues. ϕ denotes impurity.

LacNAc-terminating diantennary oligosaccharides, in which the Man(α1–3)-branch is prefered more than 40 to 1 over the Man(α1–6)-branch [9]. This result confirms our earlier notion that the α6-Neu5AcT recognizes parts of the acceptor substrate well beyond the actual site of sialic acid attachment [9], and that this recognition is not interfered with by the *N*-acetyl group at C-2 of the GalNAc residues in the substrate.

A portion of **3a** (750 nmol) was further extended by incubation with GDP-Fuc:[Gal(β1–4)]GlcNAc (α1–3)-fucosyltransferase (α3–FucT, FucT VI [10]) and GDP-Fuc. The final product **4** (700 nmol, 93%) was isolated on Bio-Gel P-4 (Fig. 1(C)) and identified by ^{1}H NMR spectroscopy (Fig. 2(C)). The NMR data are essentially in accordance to those of compound **rHPC #7** in [7]. The addition of the Fuc residue in (α1–3)-linkage to GlcNAc-**5′** when going from **3a** to **4** is reflected by the shifts on the H-1 and NAc signals of the GalNAc and GlcNAc residues in the Man(α1–6)-branch. These shifts are similar to those observed upon fucosylation by human milk α3-FucT of GalNAc(β1–4)GlcNAcβ1—OMe [5]. The α6-sialyllacdiNAc element, like α6-sialyllacNAc, is not a substrate for α3-FucT, which is consistent with the HO-6 (but not the HO-2) group of Gal being a key polar group for the FucT [11]. The fucosylation of the non-sialylated LacdiNAc-containing Man(α1–6)-branch occurred quantitatively.

In summary, each of the reactions described above gave a high yield (>90%) of the desired product(s). Using highly specific and active β4-GalNAcT, α6-Neu5AcT and α3-FucT isolated from natural sources, LacdiNAc-based determinants can be conveniently synthesized. The presented method provides the possibility to obtain sufficient amounts of oligosaccharides that can not easily be obtained otherwise by chemical synthesis or isolation from natural sources. The compounds synthesized in this paper constitute the major portion of the LacdiNAc-containing glycans on glycodelin A and they will be useful to test as inhibitors in human sperm-egg binding assays.

1. Experimental

Materials.—UDP-2-acetamido-2-deoxy-D-galactose (UDP-GalNAc), CMP-*N*-acetyl-neuraminic acid (CMP-Neu5Ac) and jack bean β-galactosidase (EC 3.2.1.23) were from Sigma (St. Louis, MO, USA). GDP-L-fucose (GDP-Fuc) was a gift from Organon BV (Oss, The Netherlands). UDP-2-acetamido-2-deoxy-D-[^{3}H]galactose and CMP-*N*-acetyl-[^{14}C]neuraminic acid were from New England Nuclear Corp. (Boston, MA). Radiolabeled nucleotide-sugars were mixed with the corresponding non-labeled compounds to obtain the desired specific radioactivity. The glycopeptide **GP–F2** was obtained by pronase digestion of asialo human fibrinogen as described [12]. The peptide portion of **GP–F2** consists for more than 90% of Gly-Glu-Asn and Glu-Asn in a ratio of 3:2. β4-GalNAcT was isolated from albumen glands of the snail *L. stagnalis* as reported previously [4]. α6-Neu5AcT (EC 2.4.99.1) was purified from bovine colostrum according to [13]. α3-FucT was obtained from human milk as described [14]. One milliunit (mU) of transferase is defined as the amount of enzyme that catalyzes the transfer of 1 nmol/min of the monosaccharide from the corresponding donor to the corresponding acceptor under the assay conditions.

β-*Galactosidase digestion.*—Digestion of the glycopeptide **GP–F2** (5.1 μmol) with jack bean β-galactosidase (0.95 U) was carried out in 1 mL 0.1 M NaOAc (pH 4.0). The mixture was incubated for 48 h at 37 °C.

Glycosyltransferase reactions.—The β4-GalNAcT incubation was performed in a reaction mixture (5 mL) containing 1 mM **1**, 2.3 mM UDP-[^{3}H]GalNAc (0.075 Ci/mol), 125 mM MES (pH 7.0), 40 mM $MnCl_2$, 5 mM ATP, 0.5% Triton X-100, and 40 mU β4-GalNAcT. The mixture was incubated for 24 h at 37 °C. The α6-Neu5AcT incubation was performed in a mixture (2.5 mL) containing 1 mM **2**, 1.8 mM CMP-[^{14}C]Neu5Ac (0.027 Ci/mol), 100 mM sodium cacodylate (pH 6.8) and 21 mU α6-Neu5AcT. The incubation mixture was kept at 37 °C for 24 h. The α3-FucT reaction was carried out in a mixture (1 mL) containing 0.75 mM **3a**, 2 mM GDP-Fuc, 50 mM sodium cacodylate (pH 7.2), 100 mM NaCl, 5 mM $MnCl_2$, 5 mM ATP, 50% glycerol, and 5 mU α3-FucT. The mixture was incubated for 48 h at 37 °C.

Isolation of the reaction products.—All enzyme reactions were terminated by heating the reaction mixtures for 3 min at 100 °C. The mixtures were then applied to a column (200×1.5 cm) of Bio-Gel P-4 (200–400 mesh, Bio-Rad), eluted with 25 mM NH_4HCO_3 (pH 7.0). The eluent was monitored using a refractive index detector or by measuring the radioactivity when applicable. Appropriate

fractions containing the products were pooled and lyophilized. The products were identified by ^{1}H NMR spectroscopy.

^{1}H NMR spectroscopy.—Prior to ^{1}H NMR spectroscopic analysis, samples were exchanged twice in 99.9% D_2O with intermediate lyophilization. Finally, samples were dissolved in 500 μL 99.95% D_2O (Merck). ^{1}H NMR spectra were recorded at 600 MHz on a Bruker AMX2-600 spectrometer (NSR Center, University of Nijmegen, The Netherlands), at a probe temperature of 32 °C. Chemical shifts are expressed in ppm by reference to internal acetone (δ 2.225) [15]. ^{1}H NMR spectra were recorded using the WEFT sequence in combination with a selective 180° pulse for inversion of the D_2O signal [16]. The spectral width was 6000 Hz and 256–512 FIDs of 16 K complex data points were collected. Resolution enhancement was achieved by Lorentzian-to-Gaussian transformation and the spectra were baseline corrected with a polynomal function when necessary.

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