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Conversion of GalNAcβ(1–4)GlcNAcβ-OMe into GalNAcβ(1–4)-[Fucα(1–3)]GlcNAcβ-OMe using human milk α3/4-fucosyltransferase

Synthesis of a novel terminal element in glycoprotein glycans

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Incubation of GalNAcβ(1–4)GlcNAcβ-OMe with GDP-Fuc in the presence of human milk α3/4-fucosyltransferase resulted in the formation of GalNAcβ(1–4)[Fucα(1–3)]GlcNAcβ-OMe. Under conditions that led to complete α3-fucosylation of Galβ(1–4)GlcNAcβ-OEt, GalNAcβ(1–4)GlcNAcβ-OMe was fucosylated for more than 85%. For the identification of the isolated fucosylated products one- and two-dimensional ^{1}H-NMR spectroscopy was applied. In vacuo molecular dynamics simulations of Galβ(1–4)[Fucα(1–3)]GlcNAcβ-OEt and GalNAcβ(1–4)[Fucα(1 3)]GlcNAcβ-OMe using the CHARMm based force field CHEAT, demonstrated only small differences between the conformations of these compounds. This illustrates the minor conformational influence of the substituent at C-2′, i.e. a hydroxyl function versus a *N*-acetyl group.

Fucosyltransferase; GalNAc-containing N-glycan; Conformational analysis; Lewis x

1. INTRODUCTION

A structural variant of the Le^x oligosaccharide element Galβ(1–4)[Fucα(1–3)]GlcNAcβ, namely GalNAcβ(1–4)[Fucα(1 3)]GlcNAcβ, has been demonstrated to occur in O-linked oligosaccharides of the sea squirt H-antigen [1]. Recently, this structural variant was also identified as a terminal element in N-linked carbohydrate chains derived from human urinary-type plasminogen activator [2], from *Schistosoma mansoni* glycoproteins [3], from honeybee venom phospholipase A_2 [4] and from bovine pro-opiomelanocortin [5].

The biosynthesis of the Le^x determinant is known to proceed by the ordered addition of βGal in (1–4)-linkage and αFuc in (1–3)-linkage to a terminal GlcNAc residue (for a review see [6]). Human milk has been demonstrated to contain at least two different α3/4-fucosyltransferase activities, which both are capable of catalyzing the synthesis of the Le^x sequence [6]. Human milk α3/4-fucosyltransferase preparations have been used in the synthesis of the Le^y epitope [7], the α(2–3)-sialylated Le^x determinant (SLe^x) [8], the α(1–3)-galactosylated Le^x tetrasaccharide [9], and the 3′-*O*-sulfated Le^x trisaccharide (T. De Vries and D.H. Van den Eijnden, unpublished results), indicating that substitution at C-2 or C-3 of the Gal moiety in the Galβ(1–4)GlcNAcβ acceptor substrate does not impair the transfer of Fuc to C-3 of the GlcNAc residue. In view of this acceptor property it was of interest to investigate whether the human milk preparation could also function in the synthesis of the GalNAc variant of Le^x.

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Abbreviations: Fuc, fucose; Neu5Ac, *N*-acetylneuraminic acid; Le^x, Lewis x; 1D, one dimensional; 2D, two-dimensional; COSY, correlation spectroscopy; HOHAHA, homonuclear Hartmann–Hahn; MLEV, composite pulse devised by Malcom Levitt; r.O.e., rotating frame nuclear Overhauser enhancement; ROESY, rotating frame nuclear Overhauser enhancement spectroscopy; CHEAT, carbohydrate hydroxyls represented by extended atoms; CHARMm, Molecular Simulations Inc. version of Chemistry Harvard macromolecular mechanics; MD, molecular dynamics.

2. MATERIALS AND METHODS

2.1. *Materials*

GalNAcβ(1–4)GlcNAcβ-OMe (**GNGn**) was a generous gift of Dr. J.G.M. Van der Ven [10]. Galβ(1–4)GlcNAcβ-OEt (**GGn**) and GDP-Fuc were obtained from BioCarb AB, Lund, Sweden. GDP-[^{3}H]Fuc (7 Ci/mmol) was purchased from DuPont NEN (Boston, MA), and was diluted with unlabelled GDP-Fuc. Partially purified human milk α3/4-fucosyltransferase, kindly provided by Mrs. T. De Vries, was isolated from pooled human milk as described in [11] with minor modifications [12]. The resulting preparation contained the enzyme in a 225-fold purified form (1.4 mU/ml) in a solution of 50 mM sodium cacodylate buffer, pH 7.2, containing 50% (by vol.) glycerol, 100 mM NaCl, 5 mM $MgCl_2$ and 0.05% sodium azide.

2.2. *Enzymic conversions and isolation of the products*

The incubation mixtures used for fucosylation of **GNGn** and **GGn** contained in a volume of 1 ml: 1 μmol acceptor oligosaccharide, 1.5 μmol GDP-[^{3}H]Fuc (0.5 Ci/mol), 50 μmol 3-(*N*-morpholino)-

propanesulfonate (MOPS/NaOH) buffer pH 7.5, 4 μmol ATP, 10 μmol $MnCl_2$, and 1.4 mU of the α3/4-fucosyltransferase preparation mentioned above. The mixtures were incubated for 44 h at 37°C, and passed over a column (2.5 ml) of Dowex 1-X8 (Cl^- form). In each case, the columns were washed with 3 bed volumes of H_2O, and the combined eluate and washes were lyophilized. Then, the samples were fractionated on a column (1.6 × 200 cm) of Bio-Gel P-4 (200 400 mesh, Bio-Rad) equilibrated in and eluted with 0.05 M ammonium acetate buffer, pH 5.2, at a flow rate of 17 ml/min and 27°C. Fractions of 4 ml were collected and small aliquots were assayed for ^{3}H-radioactivity by liquid scintillation counting. Radioactive fractions were further analyzed by high-pH anion-exchange chromatography (HPAEC) in a system consisting of a Dionex Bio-LC gradient pump, a CarboPac MA-1 column (4.0 × 250 mm) and a Model PAD 2 detector. The pulse potentials and durations used for the pulsed- amperometric detection (PAD) were E_1 = 0.05 V (t_1 = 480 ms); E_2 = 0.60 V (t_2 = 120 ms); E_3 = −0.60 V (t_3 = 60 ms); response time 1s. Isocratic elution was performed using 0.1 M NaOH at a flow rate of 0.4 ml/min. Bio-Gel P-4 fractions that appeared to contain ^{3}H- labelled carbohydrate material and no unconverted substrate were pooled, lyophilized and desalted on a column (1.0 × 45 cm) of Bio-Gel P-2 (200–400 mesh), eluted with H_2O.

2.3. *Molecular dynamics simulations*

Molecular dynamics simulations were performed using CHEAT, a CHARMm based force field for carbohydrates [13], in which carbohydrate hydroxyl groups are represented by extended atoms to prevent the formation of intramolecular hydrogen bonds. This force field was adapted (Dr. L.M.J. Kroon-Batenburg, Department of Crystal and Structural Chemistry, Utrecht University, unpublished data) to be used with the molecular modelling package INSIGHT/DISCOVER (Biosym Technologies Inc.). The two fucosylated carbohydrate structures were created graphically with the initial interglycosidic torsion angles conform those of the minimum energy conformations of the Le^x trisaccharide [14]. The atoms of the methyl, ethyl and *N*-acetyl substituents were implemented in these models on basis of the existing general valence force field (CHARMm). After initial energy minimisation, the in vacuo dynamics simulations were run for 1 ns, with a time step of 0.001 ps at a constant temperature of 27°C. The first 100 ps of each run were considered to be the equilibration time, then data were stored every 1 ps. The calculations were performed on a Silicon Graphics Iris Indigo R3000. The interglycosidic torsion angles Φ and Ψ are defined as O_5–C_1–O_x–C_x and C_1–O_x–C_x–C_{x-1}, respectively, where O_x, C_x and C_{x-1} are aglyconic atoms (IUPAC convention [15]).

2.4. *^{1}H-NMR spectroscopy*

1D 500- and 600-MHz ^{1}H-NMR spectra were recorded as described [16,17]. In the case of 2D NMR experiments, data matrices of 512 × 2048 points were recorded at temperatures as indicated in section 3, using Bruker NMR-software and the time-proportional phase increment method. The $^1HO^2H$ signal was presaturated for 1 s during the relaxation delay. 500-MHz 2D HOHAHA spectra of **GNGn** and GalNAcβ(1–4)[Fucα(1–3)]GlcNAcβ-OMe (**GN(F)Gn**) were recorded using a MLEV-17 mixing sequence of 120 ms, and a spin-lock field-strength corresponding to a 90° ^{1}H pulse-width of 28.1 μs. For **GNGn** the data matrix represented in each dimension a spectral width of 3106 Hz, and for **GN(F)Gn** of 3817 Hz. The 2D HOHAHA spectrum of Galβ(1–4)[Fucα(1 3)]GlcNAcβ-OEt (**G(F)Gn**) was recorded at 600 MHz, using a MLEV-17 mixing sequence of 120 ms, a spin-lock field-strength corresponding to a 90° ^{1}H pulse-width of 28.2 μs, and a data matrix representing a spectral width of 4505 Hz in each dimension. The 500-MHz 2D ROESY spectra of **GNGn** and **GN(F)Gn** were acquired, using a spin-lock mixing pulse of 200 ms at a field-strengh corresponding to a 90° ^{1}H pulse-width of 112.3 μs. The data matrices represented a spectral width of 3106 Hz and 4274 Hz in each dimension for **GNGn** and **GN(F)Gn**, respectively. In the case of **GNGn**, the carrier-frequency was placed on the $^1HO^2H$-line at 4.94 ppm, whereas for **GN(F)Gn** it was placed 200 Hz upfield from the Fuc H-1 signal. The 500-MHz double quantum filtered ^{1}H–^{1}H 2D COSY spectra of **GNGn** and **GN(F)Gn** were obtained with a spectral width of 3106 Hz for **GNGn**, and 3816 Hz for **GN(F)Gn**, respectively, in each dimension. The 600-MHz double quantum filtered ^{1}H–^{1}H 2D COSY spectrum of **G(F)Gn** was acquired with a spectral width of 4506 Hz in each dimension. The time domain data of the COSY, HOHAHA and ROESY experiments, were zero-filled to 1024 × 2048 data matrices prior to multiplication with a squared-bell function, phase shifted by π/3.

3. RESULTS

Incubation of GalNAcβ(1–4)GlcNAcβ-OMe (**GNGn**) with GDP-Fuc in the presence of human milk α3/4-fucosyltransferase afforded GalNAcβ(1–4)[Fucα(1–3)]GlcNAcβ-OMe (**GN(F)Gn**). In a similar way, incubation of Galβ(1–4)GlcNAcβ-OEt (**GGn**) yielded the Le^x trisaccharide Galβ(1–4)[Fucα(1–3)]GlcNAcβ-OEt (**GN(F)Gn**). Under the conditions that resulted in complete conversion of **GGn**, more than 85% of **GNGn** was transformed. Isolation of the products was achieved by Bio-Gel P-4 filtration (Fig. 1), using HPAEC-PAD as a monitoring system to allow the identification of fractions containing only product (**G(F)Gn**, retention time (t_e) 4.8 min; **GN(F)Gn**, t_e 5.0 min) and no starting material (**GGn**, t_e 5.3 min; **GNGn**, t_e 7.7 min).

Complete unravelling of the 1D ^{1}H-NMR spectra of **GN(F)Gn** (Fig. 2) and **GNGn** was carried out by apply-

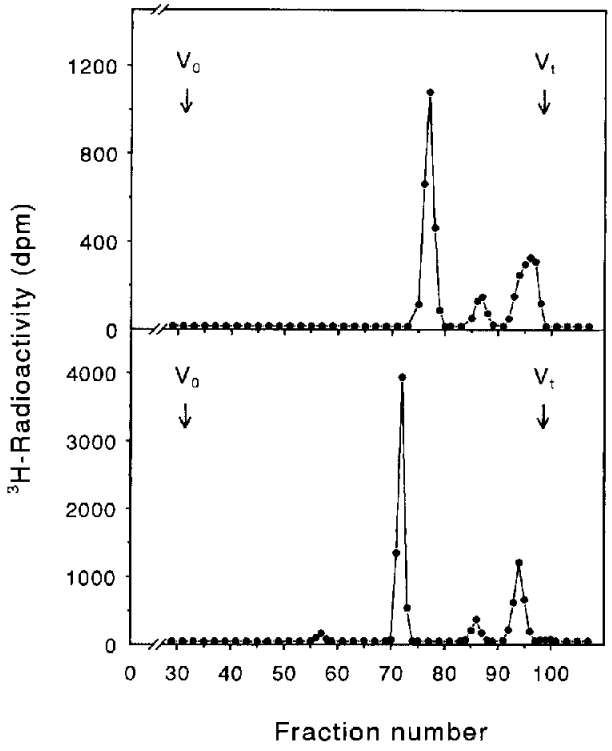

Fig. 1. Isolation of the products of α3-fucosylation catalyzed by the human milk enzyme of **G(F)Gn** (upper panel) and **GN(F)Gn** (lower panel) by Bio-Gel P-4 chromatography. Of the radioactive fractions number 75–78 (upper panel) and 71–72 (lower panel), respectively, were pooled, lyophilized and the materials were analyzed by ^{1}H-NMR spectroscopy. The radioactive materials eluting in fractions 85 88 and 92–98 are degradation products of GDP-[^{3}H]Fuc.

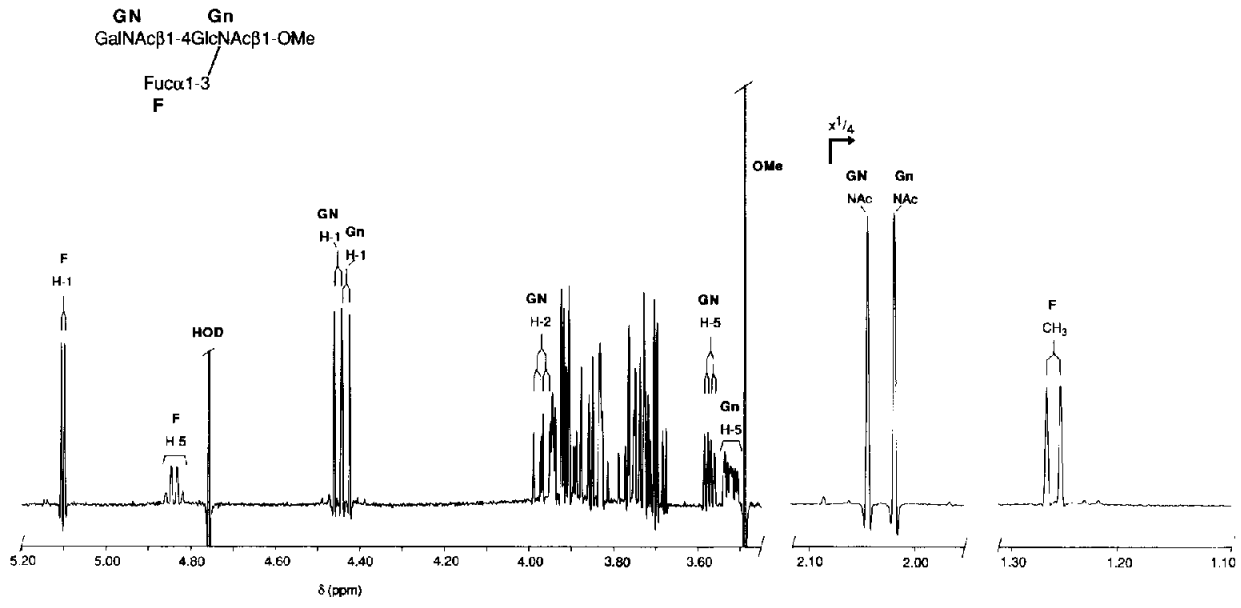

Fig. 2. Resolution-enhanced 500-MHz ^{1}H-NMR spectrum at 300 K of GalNAcβ(1–4)[Fucα(1–3)]GlcNAcβ-OMe. The letters and numbers refer to the residues in the structure and to the protons in that residue.

ing 2D COSY, HOHAHA and ROESY experiments. In order to obtain an increased intensity for the r.O.e. cross-peaks, the 2D ROESY spectra were measured at 285 K. COSY and HOHAHA spectra were measured at 285 K to attain identical chemical shifts for the corresponding protons in each 2D spectrum, and at 300 K for comparison with standard structural-reporter-group data. The spectral data are listed in Table I. For both compounds the assignment of the H-1 signals of GalNAc and GlcNAc is based on the appearance in the HOHAHA spectrum of the unique spin-coupling systems for GalNAc (H-1–H-4) and for GlcNAc (H-1–H-6/6′), respectively. Starting at H-1, all individual proton signals of GlcNAc could be assigned via the ^{1}H–^{1}H cross-peaks in the COSY spectrum. Furthermore, the GalNAc H-1,2,3,4 and H-5,6,6′ spin systems were traced, and the two sets were correlated via an intraresidual r.O.e. cross-peak between GalNAc H-1 and GalNAc H-5 in the 2D ROESY spectrum. For the full assignment of the proton signals of the Fuc residue in **GN(F)Gn**, the typical set of H-1, H-5 and CH_3 signals formed the starting point [16]. The cross-peaks in the ROESY spectrum which correlate Fuc H-1 ($^3J_{H\text{-}1,H\text{-}2}$, 4 Hz) with GlcNAc H-3 (strong) and Fuc H-1 with GlcNAc H-4 (very weak), demonstrate that the Fuc residue is α(1–3)-linked exclusively to the GlcNAc residue. The ^{1}H-NMR data of **G(F)Gn** (Table I) are in good agreement with those reported for Galβ(1–4)[Fucα(1–3)]GlcNAcβ(1–3)Galβ(1–4)Glc [14,18] and Galβ(1–4)[Fucα(1–3)]GlcNAc [19].

In the 2D ROESY spectrum of **GN(F)Gn**, a weak r.O.e. cross-peak between Fuc H-5 and GalNAc H-2 was observed. A similar r.O.e. contact has been found for Galβ(1–4)[Fucα(1–3)]GlcNAcβ(1–3)Galβ(1–4)Glc, i.e. Fuc H-5 $\leftrightarrow$ Gal H-2, originating from a rigid conformation of the Le^x trisaccharide element [14,20]. The presence of similar r.O.e. interactions in the Le^x and in the GalNAc variant of the Le^x element initiated a comparative conformation study of **G(F)Gn** and **GN(F)Gn**, which was focused on the influence of the replacement of OH by NAc at C-2′. The applied CHEAT force field has the advantage that no explicit solvent molecules have to be included in the calculation, because the average effect of inter- and intramolecular hydrogen bonding is covered by the potential energy function of the extended atom type for the hydroxyl groups. Two molecular dynamics (MD) simulations were performed for **G(F)Gn**, starting with the interglycosidic torsion angles of the minimum energy conformations **A** and **B** (Table II) of reference Galβ(1–4)[Fucα(1–3)]GlcNAcβ-OMe, respectively, as presented in [14]. As can be deduced from the time-averaged interglycosidic torsion angles, Φ_G/Ψ_G and Φ_F[G(F)Gn]/Ψ_F[G(F)Gn] describing the Galβ(1–4)GlcNAc and the Fucα(1–3)GlcNAc linkages in **G(F)Gn**, respectively, the two MD runs for **G(F)Gn** produced essentially the same results (Table II). The time-averaged torsion angles match those of reference **MD1** and **MD2** [14], resulting from the MD simulations of minimum energy conformations **A** and **B**, respectively.

One MD simulation was performed for **GN(F)Gn** (Fig. 3) using the interglycosidic torsion angles of the lowest energy conformation **A** [14] as start parameters. Projections of the time-averaged conformations are depicted in Fig. 4. The time-averaged interglycosidic torsion angles, Φ_{GN}/Ψ_{GN} and Φ_F[GN(F)Gn]/Ψ_F[GN(F)Gn]

Table I

^{1}H-NMR chemical shifts of **GNGn, G(F)Gn** and **GN(F)Gn**. Chemical shifts are given at 285 K and at 300 K, as indicated, in ppm downfield from internal acetone in ^2H_2O (δ 2.225) [16]. Compounds are represented by short-hand symbolic notation: (□), L-Fuc; (●), D-GlcNAc; (■), D-Gal; (◇), D-GalNAc; Et, ethyl; Me, methyl. n.d., not determined.

Residue	Proton	Chemical shifts (ppm) in					
		G(F)Gn	G(F)Gn	GNGn	GN(F)Gn	GNGn	GN(F)Gn
Temperature (K)		285	300	285	285	300	300
GlcNAc	H-1	4.552	4.561	4.433	4.422	4.438	4.433
	H-2	n.d.	3.87	3.733	3.915	3.712	3.905
	H-3	n.d.	3.86	3.714	3.822	3.682	3.832
	H-4	3.929	3.920	3.628	3.882	3.617	3.876
	H-5	3.585	3.584	3.521	3.524	3.520	3.523
	H-6	3.996	3.996	3.865	3.939	3.861	3.933
	H-6′	3.855	3.852	3.668	3.747	3.664	3.733
	NAc	2.028	2.028	2.026	2.018	2.027	2.019
OMe/OEt	CH_3	1.157	1.157	3.497	3.489	3.498	3.489
	CH_2/CH_2'	n.d.	3.90/3.67	–	–	–	–
Fuc	H-1	5.107	5.104	–	5.107	–	5.102
	H-2	3.681	3.688	–	3.683	–	3.691
	H-3	3.907	3.902	–	3.939	–	3.931
	H-4	3.792	3.790	–	3.832	–	3.830
	H-5	4.856	4.830	–	4.871	–	4.839
	CH_3	1.173	1.174	–	1.264	–	1.260
Gal/GalNAc	H-1	4.454	4.451	4.512	4.451	4.517	4.453
	H-2	3.496	3.494	3.928	3.985	3.928	3.970
	H-3	3.654	3.651	3.745	3.708	3.747	3.712
	H-4	3.890	3.899	3.935	3.902	3.940	3.909
	H-5	3.595	3.595	3.719	3.573	3.720	3.573
	H-6	3.740	3.742	3.799	3.768	3.797	3.769
	H-6′	3.731	3.724	3.757	3.718	3.765	3.713
	NAc	–	–	2.069	2.044	2.069	2.045

describing the GalNAcβ(1–4)GlcNAc and the Fucα(1–3)GlcNAc linkages in **GN(F)Gn**, respectively, correspond with those of **G(F)Gn** (Table II). Like the Fuc and Gal residues in **G(F)Gn**, the Fuc and GalNAc residues in **GN(F)Gn** form a stacking complex, wherein the averaged distance between Gal/GalNAc H-2 and Fuc H-5 is 2.6 Å (Fig. 4), which is a realistic value for nuclear Overhauser effects. It should be noted that the trajectories of Φ_F of reference Galβ(1–4)[Fucα(1–3)]GlcNAcβ-OMe shows a rapid change between two different dihedrals [14], whereas the trajectory of Φ_F of **G(F)Gn** and of **GN(F)Gn** (Fig. 3) show basically one dihedral. Possibly, this difference in flexibility stems from the use of united atoms for the hydroxyl groups in the CHEAT forcefield.

4. CONCLUDING REMARKS

Functionally purified preparations of human milk α3/4-fucosyltransferase have been used in the enzyme assisted synthesis of α(1 3)-fucosylated oligosaccharides, including Le^x [7–9,12]. Here it is demonstrated that the enzyme can also be used to produce the GalNAc-containing variant of Le^x in high yield. This finding implies that for a Galβ(1–4)GlcNAcβ1-R specific fucosyltransferase the OH group at C-2′ can be replaced by a NAc group without a gross effect on the enzyme activity. Interestingly, it has been found previously that the Galβ(1–4)GlcNAcβ1-R α(2–6)-sialyltransferase whether isolated from rat liver [10,21] or bovine colostrum [22] also can act on GalNAcβ(1–4)GlcNAcβ1-R with high efficiency. In view of these results it will be of interest to investigate whether other Galβ(1–4)GlcNAcβ1-R specific glycosyltransferases such as α(2 3)-sialyltransferase [23], α3-galactosyltransferase [24] and β3-*N*-acetylglucosaminyltransferase [25] are similarly capable of acting on GalNAcβ(1–4)GlcNAcβ1-R.

The average dihedrals and the corresponding fluctuations in the trajectories, and therefore the time-averaged

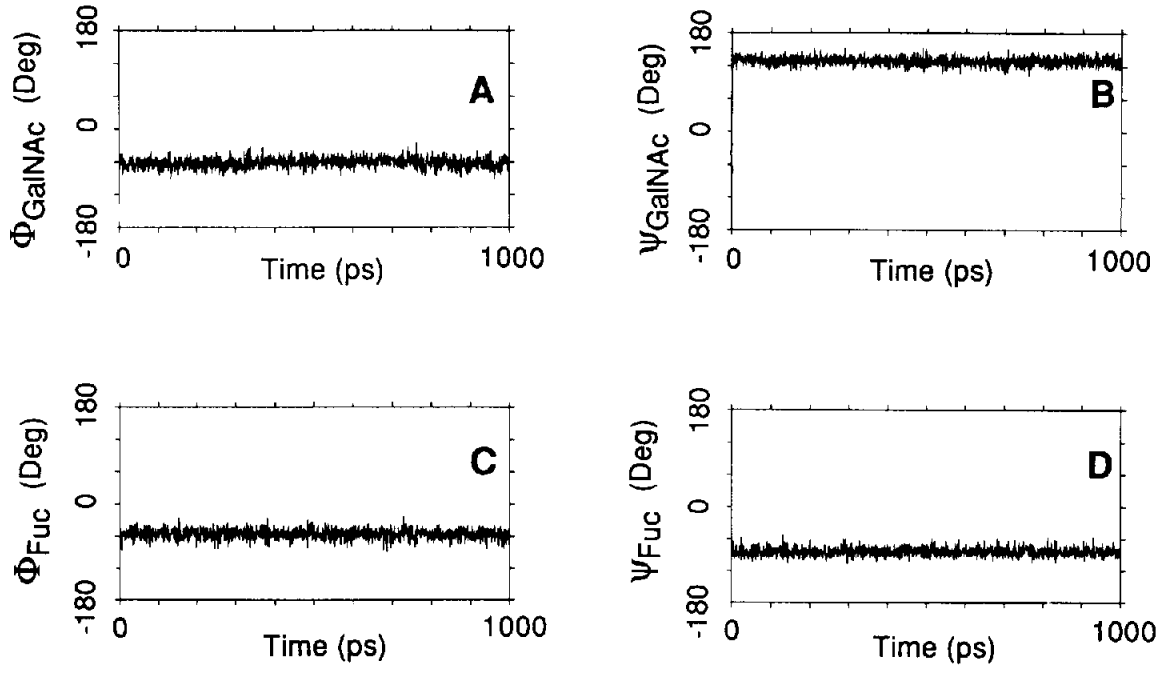

Fig. 3. The 1-ns trajectories of the interglycosidic torsion angles in **GN(F)Gn**: A, Φ_{GN}; B, Ψ_{GN}; C, Φ_F; D, Ψ_F.

conformations of **G(F)Gn** and **GN(F)Gn** obtained from the MD simulations, show great similarity. Consequently, it can be concluded that the substitution of a hydroxyl function by a *N*-acetyl group, converting Gal into GalNAc, does not have a profound influence on the conformation of the trisaccharides. So far, the GalNAc variant of Le^x, carried on oligosaccharide alditols, released from sea squirt H-antigen [1], has been shown to induce allergic skin reactions in humans [26]. However, no role in interaction processes, as found for Le^x [27] and carbohydrate ligands having the Le^x epitope as structural element (sialyl-Le^x, VIM-2 structure, sulfo-Le^x) [28–30], has been reported. In this context, it should be mentioned that comparison of the time-averaged conformations of **G(F)Gn** and **GN(F)Gn** shows that Gal OH-2 and GalNAc NAc are oriented to the outside of the models. Since these functional groups seem to be explicitely present on the surface of the oligosaccharides, the replacement of Gal OH-2 by a NAc group in the Le^x sequence may affect the binding with carbohydrate-recognizing macromolecules. Whether (and if so to what extent) such a replacement has indeed a modulating effect on interactions events, mediated by Le^x-containing ligands, remains to be established.

Table II

Interglycosidic dihedral angles of the time-averaged conformations of **G(F)Gn** and **GN(F)Gn** given in degrees, together with those of reference Galβ(1–4)[Fucα(1–3)]GlcNAcβ-OMe

Compound	Φ_G/Φ_{GN}	Ψ_G/Ψ_{GN}	Φ_F	Ψ_F
G(F)Gn (run 1)	−65 (± 9)	131 (± 8)	−61 (± 11)	−86 (± 9)
G(F)Gn (run 2)	−65 (± 9)	131 (± 7)	−61 (± 11)	−86 (± 10)
GN(F)Gn	−62 (± 10)	129 (± 8)	−56 (± 10)	−84 (± 9)
Reference:				
MD1	−61 (± 9)	128 (± 7)	−59 (± 20)	−88 (± 10)
MD2	−64 (± 10)	128 (± 8)	−64 (± 21)	−88 (± 11)
conformer **A**	−57	122	−45	−96
conformer **B**	−65	132	−83	−97

Conformations of **G(F)Gn** and **GN(F)Gn** were calculated from 1 ns molecular dynamics simulations using the force field CHEAT. The data of reference Galβ(1–4)[Fucα(1–3)]GlcNAcβ-OMe were collected from conformational energy calculations (conformer **A** and **B**) and from 300 ps molecular dynamics simulations (**MD1** and **MD2**) using the force field CHARMm [14]. Interglycosidic torsion angles are defined as follows: Φ_G/Φ_{GN} = Gal/GalNAc O-5–Gal/GalNAc C-1–GlcNAc O-4–GlcNAc C-4; Ψ_G/Ψ_{GN} = Gal/GalNAc C-1–GlcNAc O-4–GlcNAc C-4–GlcNAc C-3; Φ_F = Fuc O-5 Fuc C-1–GlcNAc O-3–GlcNAc C-3; Ψ_F = Fuc C-1–GlcNAc O-3–GlcNAc C-3 GlcNAc C-2. The values given in brackets are the root-mean-square fluctuations in the angles observed during the simulations.

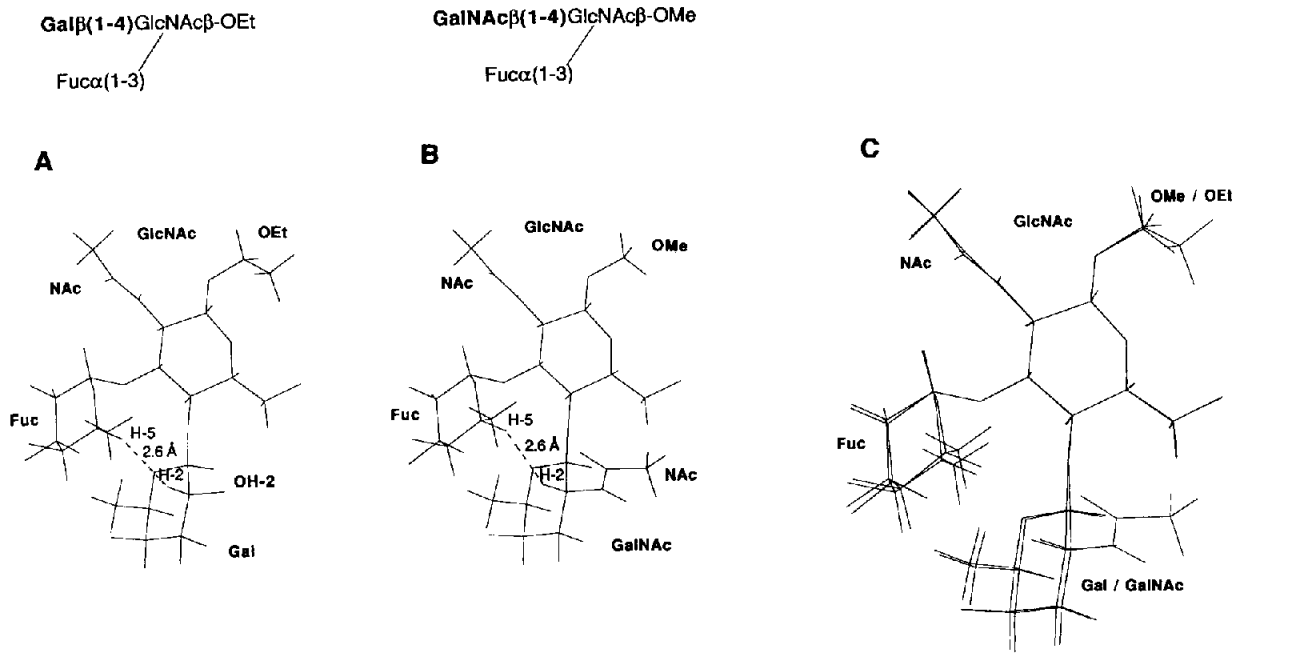

Fig. 4. Diagram of the time-averaged conformations of (A) **G(F)Gn**, (B) **GN(F)Gn** and (C) superimposition of both models (cf. Table II). The ring-skeletons of the GlcNAc residues are presented from the same view-point.

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