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Efficient enzymatic synthesis of the sialyl-Lewis^X tetrasaccharide

A ligand for selectin-type adhesion molecules

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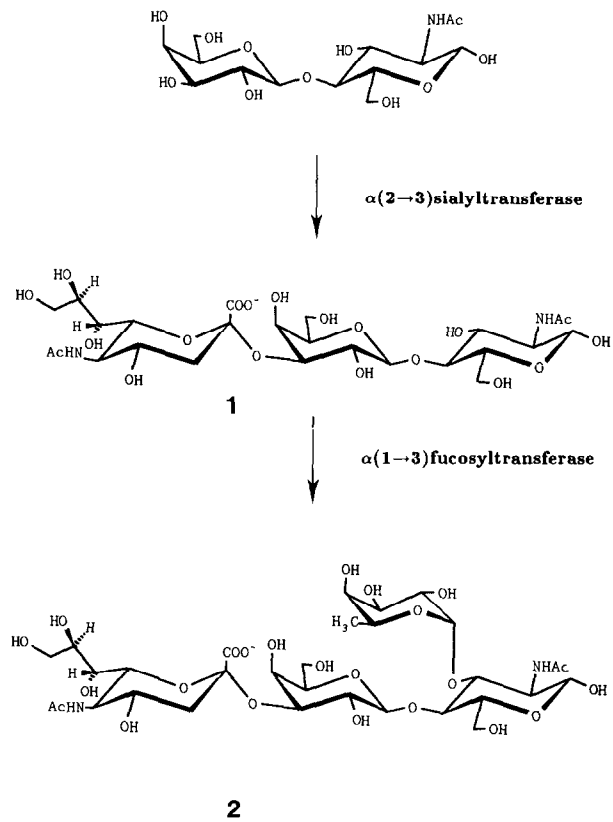
Sialyl-Lewis^X (NeuAcα2→3Galβ1→4[Fucα1→3]GlcNAc] has been identified as a ligand for E-selectin, P-selectin and recently also for L-selectin. We have synthesized the sialyl-Lewis^X tetrasaccharide by total enzymatic synthesis from *N*-acetyllactosamine using a placental α2→3-sialyltransferase specific for type-2 chain acceptors, followed by a cloned human α1→3-fucosyltransferase (FucTV, the 'plasma-type' enzyme). This procedure resulted in the tetrasaccharide in a 61% overall yield.

Sialyl-Le^X; α1→3-Fucosyltransferase; α2→3-Sialyltransferase; Selectin

1. INTRODUCTION

Several groups have identified the sialyl-Le^X determinant (NeuAcα2→3Galβ1→4[Fucα1→3]GlcNAc-R, Scheme 1, structure **2**) as a functional ligand for E-, P- and L-selectin [1–8]. E- and P-selectin play a role in inflammation and clotting by mediating adhesion of leukocytes to endothelium and platelets [9,10], while L-selectin is involved in lymphocyte homing to peripheral lymph nodes [7,8]. To further establish the nature of the adhesive properties and to explore the use of sialyl-Le^X-containing oligosaccharides as anti-inflammatory drugs it is necessary to test a wide series of such oligosaccharides. Therefore a simple and economical production procedure is essential. Sialyl-Le^X oligosaccharides can be obtained by total chemical synthesis, total enzymatic synthesis or a combination of the two. Two groups have reported the total chemical synthesis of sialyl-Le^X, [11,12] which is a multistep synthesis and results in a < 10% yield. The enzymatic approach to the synthesis of sialyl-Le^X, however, proceeds stereoselectively in one step per linkage and is applicable to other, more complex, sialyl-Le^X-containing structures. The biosynthetic pathway of the sialyl-Le^X determinant is summarized in Scheme 1. Sialylation has to occur be-



Scheme 1. Synthesis of 3′-sialyl-*N*-acetyllactosamine (**1**) and the sialyl-Le^X tetrasaccharide (**2**).

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Abbreviations: α_1-AGP, α_1-acid glycoprotein; α3-ST, α2→3-sialyltransferase; CMP-NeuAc:Galβ1→4GlcNAc→R, α2→3-sialyltransferase; α3-FT, α1→3-fucosyltransferase; GDP-Fuc:Galβ1→4GlcNAc→R, α1→3-fucosyltransferase; sialyl-Le^X, sialyl-Lewis^X.

fore fucosylation because none of the known sialyltransferases can accept fucosylated structures [13–15]. Recently, the total enzymatic synthesis of sialyl-Le^x was reported [16]. The $\alpha 2 \rightarrow 3$-sialyltransferase that was used for the synthesis of sialyl-*N*-acetyllactosamine, however, has a preference for type-1 chain (Galβ1→3GlcNAc) substrates and acts only slowly on type-2 chains (Galβ1→4GlcNAc). The goal of our study was to use glycosyltransferases with the proper acceptor specificity for the efficient synthesis of sialyl-Le^x, resulting in a simple and economical production procedure. The $\alpha 2 \rightarrow 3$-sialyltransferase of human placenta [17] has been identified as an enzyme specific for type-2 acceptors [18]. In this report, we have used the latter enzyme for the synthesis of the 3′-sialyl-*N*-acetyllactosamine. The product of this reaction was converted into the sialyl-Le^x tetrasaccharide in a high overall yield using a particularly efficient recombinant human $\alpha 1 \rightarrow 3$-fucosyltransferase.

2. MATERIALS AND METHODS

2.1. *Materials*

Human placenta was obtained from the Department of Obstetrics and Gynecology, Academic Hospital of the Vrije Universiteit, Amsterdam, and stored frozen until use. Plasmid pPROTA-α1,3/1,4-FTc containing the human $\alpha 1 \rightarrow 3/4$-fucosyltransferase (Fuc-TIII) gene [19], and plasmid pcDNA1-Fuc-TV containing the human $\alpha 1 \rightarrow 3$-fucosyltransferase (Fuc-TV) gene [20] were kindly provided by Dr. John Lowe (Howard Hughes Medical Institute, The University of Michigan Medical School, Ann Arbor, MI) Galβ1→4GlcNAcβ1-O-$(CH_2)_8COOCH_3$ was the kind gift of Dr. Ole Hindsgaul (University of Alberta, Edmonton, Alberta) *N*-Acetyllactosamine was obtained from Sigma, St. Louis, MO and unlabeled CMP-NeuAc was either purchased from Sigma or synthesized using a calf brain CMP-sialic acid synthetase preparation as described previously [21]. GDP-Fuc was provided by Dr. Jacques van Boom, University of Leiden, The Netherlands and was synthesized as described [22]. CMP-[^{3}H]NeuAc (18900 Ci/mol), CMP-[^{14}C]NeuAc (1.7 Ci/mol) and GDP-[^{14}C]Fuc (225 Ci/mol) were purchased from DuPont-New England Nuclear and diluted with the unlabeled nucleotide sugar to the desired specific radioactivity. α_1-Acid glycoprotein was prepared from human plasma Cohn fraction V supernatant as described [23] and desialylated by mild acid hydrolysis (0.1 N trifluoroacetic acid, 1 h at 80°C). All other chemicals were obtained from commercial sources and were of the highest purity available.

2.2. *CMP-NeuAc Galβ1→4GlcNAc $\alpha 2 \rightarrow 3$-sialyltransferase*

Human placenta was used as a source for α3-ST [17]. Placenta membranes were prepared as described [18]. Sialyltransferase activity was determined using asialo-α_1-AGP as an acceptor [18].

2.3. *GDP-Fuc. Galβ1→4GlcNAcβ-R $\alpha 1 \rightarrow 3$-fucosyltransferase*

A protein A fusion of Fuc-TV was generated in frame by replacing the Fuc-TIII sequence in the pPROTA vector with the corresponding sequence of the closely related fucosyltransferase V gene. The *Bam*HI site in Fuc-TIII, which is at the junction of the protein A fusion [19] is conserved in Fuc-TV. Thus, vector fragments [5300 bp *Bam*HI(partial) *Xho*I and 600 bp *Eco*RI (blunted)–*Xho*I] from pPROTA-α(1,3/1.4)FTc were ligated to the Fuc-TV fragment (1680 bp *Bam*HI *Sma*I) from pcDNA1-Fuc-TV, generating plasmid pPROTA-Fuc-TV.

Monolayers of COS-7 cells were grown in 100 mm dishes containing 10 ml of Dulbecco's Modified Eagle media plus 10% fetal bovine serum (Gibco/BRL) in a 37°C humidified 5% CO_2 atmosphere. At 50% confluency, cells were transfected with pPROTA-Fuc-TV DNA (20 μg per dish) using a $CaPO_4$ procedure [24]. After a 48 h expression period media were harvested daily for up to 14 days, and cells were replenished with additional media. The cells remained adherent during this time, and fucosyltransferase assays showed that Fuc-TV was continuously expressed. Media were filtered (0.45 μm) and stored at 4°C until use

Fuc-TV was collected by passing the harvested media through IgG-Sepharose 6 fast flow beads (Pharmacia), which had been prepared according to the manufacturer's instructions. After absorption of the enzyme, the beads were washed, resuspended in a 50:50 slurry of 50 mM Tris-HCl, pH 7.4 and stored at 4°C Fucosyltransferase assays were performed as described [13] using Galβ1→4GlcNAcβ1-O-$(CH_2)_8COOCH_3$ as an acceptor

2.4. *Determination of optimal reaction conditions for sialylation on a preparative scale*

To optimize conditions for sialylation on a preparative scale, small volumes containing each of the following ingredients were first mixed and lyophilized: 50 nmol CMP-[^{3}H]NeuAc (1.1 Ci/mol), 2 μmol sodium cacodylate buffer pH 7.0; asialo-α_1-AGP (220 nmol theoretical acceptor sites) and 80 nmol ATP. To the dry material 15 mg of freshly thawed placenta membrane pellet was added with a spatula together with 5 μl of H_2O. Before incubation at 37°C the components were thoroughly mixed with a small plastic stirring rod. The effects of detergent concentration on the activity of the enzyme was studied by varying the Triton X-100 concentration from 0 to 0.5% (w/v) and by following product formation with time. The effect of EDTA on product formation was tested at optimal Triton X-100 concentration and incubation time. The optimal incubation conditions were used for sialylation on a preparative scale.

2.5. *Preparation of 3′-sialyl-N-acetyllactosamine* (1)

N-Acetyllactosamine (9.6 mg, 25 μmol) and CMP-NeuAc (3.1 mg, 5 μmol) were lyophilized from small volumes together with 0.2 mmol sodium cacodylate (pH 7.0), 8 μmol ATP, 20 μmol EDTA and 4 mg Triton X-100, α3-ST (1.5 g, 39 mU) in the form of placenta membranes was added and mixed with the other ingredients and 0.5 ml distilled water as described in the previous paragraph, and the mixture was incubated at 37°C for 3 h. After incubation the reaction mixture was diluted with distilled water to 12 ml and centrifuged for 15 min at 30,000 × *g*. The supernatant was collected and the pellet was washed with another 10 ml of distilled water and centrifuged again. The second supernatant was combined with the first, and this was lyophilized to dryness, taken up in a volume of 1 ml in H_2O and applied to a column (1.6 × 200 cm) of Bio-Gel P-6 (200–400 mesh) equilibrated and eluted at a flow of 8 ml/h with 50 mM ammonium acetate, pH 5.2, at 45°C. Fractions (4 ml) were collected and monitored for sialic acid by applying the thiobarbituric acid procedure [25] on acid-hydrolysed aliquots. Fractions containing the product were pooled as indicated in Fig. 1, lyophilized and desalted on a column (1 × 40 cm) of Bio-Gel P-2 (200–400 mesh) run in water The fractions containing the sialyloligosaccharide were pooled and lyophilized This resulted in the title compound as a white powder, yield 2.1 mg (3.1 μmol; 62% calculated from CMP-NeuAc). In order to obtain enough material for the next step of the reaction the preparation of sialyl-*N*-acetyllactosamine was repeated twice.

2.6. *Preparation of sialyl-Le^x tetrasaccharide* (2)

A mixture of 3′-sialyl-*N*-acetyllactosamine (1) (4.44 mg, 6.58 μmol), GDP-fucose (4.78 mg, 8.11 μmol), $MnCl_2 \cdot 4H_2O$ (11.87 mg, 60 μmol), sodium cacodylate (48.0 mg, 0.3 mmol, pH 6.5) and 490 mU α3-FT (immobilized onto 0.6 ml packed volume IgG-sepharose) in 6 ml total volume aqueous solution was incubated for 96 hours at 22°C with agitation by gentle rocking of the reaction vessel. The solids were removed by filtration and the resulting filtrate (about 5.7 ml) was applied to a column (1.6 × 196 cm) of Bio-Gel P-2 (200–400 mesh) equilibrated and eluted at a flow of 6.5 ml/h with 500 mM ammonium

acetate, pH 6.83, at 25°C. Three ml fractions were collected and counted for radioactivity. Fractions containing the product were pooled and lyophilized. To remove residual ammonium acetate, two additional lyophilizations from H_2O were performed. This resulted in a yield of 5.80 mg (6.54 μmol, ~ 99% calculated from sialyl-*N*-acetyllactosamine).

2.7. *^1H NMR spectroscopy*

Prior to ^1H NMR spectroscopic analysis, the oligosaccharides were repeatedly exchanged in D_2O at room temperature with intermediate lyophilization. Finally, each sample was redissolved in 400 μl D_2O (99.96 atom% D, Aldrich, Milwaukee, WI). ^1H NMR spectroscopy (0.01% internal acetone, δ = 2.225 ppm) was performed on a Bruker MSL 400 spectrometer (Facility at the Department of Physical and Theoretical Chemistry, Vrije Universiteit, Amsterdam) operating at 400 MHz at a probe temperature of 300 K. Assignments of sialyl-Le^x oligosaccharide were essentially the same as reported before [26].

3. RESULTS

3.1. *Optimal sialylation conditions*

Optimal activity of the placenta α3-ST which is a relatively unstable enzyme [17] was obtained at a Triton X-100 concentration of 0.2% (w/v). Increase of the detergent concentration in the assay mixtures to a final concentration of 0.3% or higher slightly inhibited the enzyme activity and at the same time increased incorporation into endogenous acceptors. EDTA which has previously been shown to enhance placental sialyltransferase activity [27] was added to a concentration of 10 mM. This addition appeared to stimulate the activity of the sialyltransferase 1.4-fold. Under these conditions product was formed at an almost constant rate up to 3 h. Further incubation did not yield more product and led eventually to product degradation possibly due to endogenous sialidase activity.

3.2. *Preparation of 3′-sialyl-N-acetyllactosamine* (1)

The acceptor substrate *N*-acetyllactosamine was used in large excess (5-fold) over CMP-NeuAc. Sufficient enzyme was used to give a calculated 1.5-fold excess based on a linear enzyme activity over an incubation period of 3 h. After incubation the product was isolated by gel filtration (Fig. 1, panel A). Of different batches the overall yield based on CMP-NeuAc as the limiting substrate ranged from 35 to 65% with an average of 53%. The ^1H NMR spectrum of 3′-sialyl-*N*-acetyllactosamine obtained by this procedure indicated a high degree of purity (95%) with minor resonances of unreacted *N*-acetyllactosamine still present (< 5%).

3.3. *Preparation of sialyl-Le^x oligosaccharide* (2)

GDP-fucose was used in moderate excess (1.2-fold) over the acceptor sialyl-*N*-acetyllactosamine. However the reaction was performed long enough (96 hours) so that most of the acceptor was converted to sialyl-Le^x and a yield of 99% was obtained (calculated from the carbohydrate acceptor). The elution profile on Bio-Gel P-2 is shown in Fig. 1, panel B. Examination of the profile revealed that while 90% of the available fucose

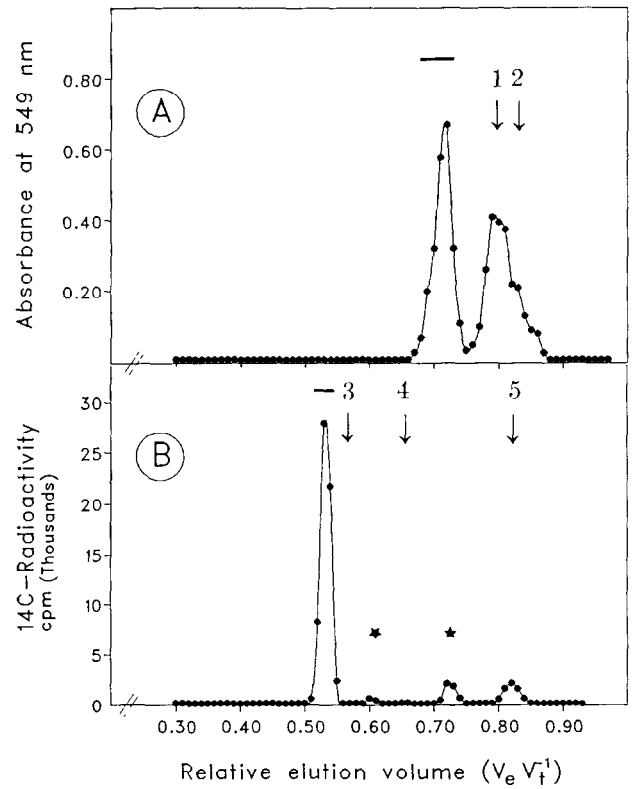

Fig. 1 Isolation of oligosaccharide products by gel filtration. (A) 3′-Sialyl-*N*-acetyllactosamine (compound **1**) was synthesized by enzymatic catalysis using a human placenta α2→3-sialyltransferase preparation as described in section 2. The product was isolated by Bio-Gel P-6 chromatography as described in section 2. Fractions of 4 ml volume were collected and monitored for sialic acid. The arrows indicate the elution positions of free sialic acid (1) and *N*-acetyllactosamine (2). Fractions indicated by the bar were pooled to give the product which was desalted in a subsequent gel filtration step. (B) Sialyl-Le^x oligosaccharide (**2**) was synthesized enzymatically using recombinant plasma type α1→3-fucosyltransferase. The product was isolated by chromatography on a column of Bio-Gel P-2 as described in section 2. Fractions of 3 ml volume were collected and monitored for radioactivity. The arrows indicate the elution positions of 3′-sialyl-*N*-acetyllactosamine (**1**) (3), 3-fucosyl-*N*-acetyllactosamine (4), GDP-fucose and free fucose (co-eluting in this chromatographic system (5). Peaks marked with an asterisk indicate products of contaminating acceptors. Fractions indicated by the bar were pooled to give the product which was desalted by lyophilizing from water several times.

was transferred to acceptors, four distinct fucose transfer products were obtained, representing 80.7, 1.9, 0.5 and 7.4%, respectively, of the total fucose present in the reaction mixture. The major peak, representing 80.7% of the total fucose present, was identified as sialyl-Le^x by ^1H NMR analysis (see Fig. 2 and Table I).

4. DISCUSSION

So far, several distinct sialyltransferase activities have been described that are capable of catalyzing the transfer of sialic acid from CMP-NeuAc to Gal-R acceptors to form a NeuAcα2→3Gal-R linkage. The α2→3sia-

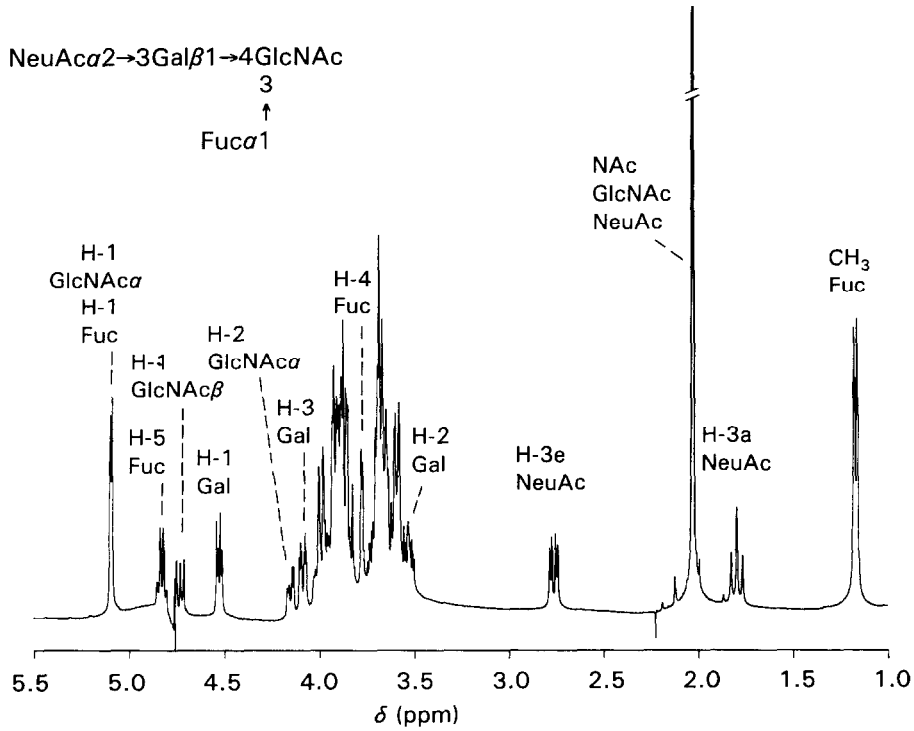

Fig. 2. 400 MHz ^{1}H NMR spectrum of the sialyl-Le^x tetrasaccharide

lyltransferase (EC 2.4.99.4) specific for Galβ1→3GalNAcα1-Ser/Thr has been purified from porcine submaxillary glands [28] and human placenta [29], and is commercially available. This enzyme has been demonstrated to be effective in the synthesis of sialylated type 1 chains (NeuAcα2→3Galβ1→3GlcNAc-R), but not type 2 structures (NeuAcα2→3Galβ1→4GlcNAc-R) [13]. Recently a cDNA for this enzyme has been cloned and expressed using a construct in COS-1 cells, producing an active sialyltransferase [30]. A second enzyme is the Galβ1→3/4GlcNAc:CMP-NeuAc α2→3-sialyltransferase (EC 2.4.99.-), which has been purified to homogeneity from rat liver [31], recently leading to its cloning as a cDNA [32]. This sialyltransferase catalyzes sialic acid transfer to both type 1 and type 2 acceptors, but has a strong preference for type 1 structures [32,33]. The utility of this enzyme for synthesis of sialyl-*N*-acetyllactosamine (**1**) has been demonstrated [34], although the yields were not high (14 to 47% with an average of 27% when CMP-NeuAc was the limiting substrate). Recently, a recombinant form of this sialyltransferase [32] was used for a combined chemical and enzymatic synthesis of sialyl-Le^x [16]. However, these authors employed an elaborate in situ nucleotide-sugar recycling scheme, which makes this production procedure for sialyl-Le^x not a simple one. A third sialyltransferase activity is the Galβ1→4GlcNAc:CMP-NeuAc α2→3-sialyltransferase (EC 2.4.99.6) detected in fetal calf liver, embryonic chicken brain, human placenta and several other tissues [17]. Most of these tissues also contain high levels of Galβ1→4GlcNAc:CMP-NeuAc α2→6-sialyltransferase. Placenta, however, appears to have only a minimal level (< 3%) of α2→6-sialyltransferase activity [35]. Furthermore, the enzyme in placenta appears to have a pronounced preference for type-2 structures over type-1 structures [18]. Therefore placenta was the tissue of choice as a source for this sialyltransferase in the synthesis of the sialyl-Le^x oligosaccharide. Efforts to purify this enzyme have so far not succeeded. Being membrane bound, this enzyme needs to be extracted with Triton X-100 or other detergents in order to be active in vitro, but it is at the same time inactivated once extracted from its membrane environment [29]. However, we demonstrated that a placenta-derived membrane fraction is a very useful source of α2→3-sialyltransferase in the synthesis of sialylated *N*-acetyllactosamine. Reaction of *N*-acetyllactosamine with CMP-NeuAc in the presence of placenta membranes for only 3 h at 37°C produced the sialylated trisaccharide **1** in good yield (35–65%), which was conveniently isolated by means of gel filtration.

Several α3-fucosyltransferase activities have been described to date (see [36,37] for review). With the exception of the myeloid enzyme all of them can efficiently catalyze fucose transfer to sialyl-*N*-acetyllactosamine (**1**) to form sialyl-Le^x (**2**). Palcic et al. [13] showed that

Table I

400 MHz ^{1}H NMR chemical shift values and J constants of structural reporter group protons of the constituent monosaccharides of 3′-sialyl-*N*-acetyllactosamine and sialyl-Lewis^x

Reporter group	Residue	NeuAcα2→3Galβ1→4GlcNAc	NeuAcα2→3Galβ1→4[Fucα1→3]-GlcNAc
		ppm (Hz)	
H-1	GlcNAcα	5.206 ($J_{1,2}$ 2.5)	5.092 ($J_{1,2}$ 3.6)
	GlcNAcβ	4.718 ($J_{1,2}$ 8.1)	4.722 ($J_{1,2}$ 8.1)
	Galα	4.556 ($J_{1,2}$ 7.9)	4.531 ($J_{1,2}$ 7.8)
	Galβ	4.551 ($J_{1,2}$ 7.9)	4.522 ($J_{1,2}$ 7.8)
	Fucα		5.097 ($J_{1,2}$ 4.0)
	Fucβ		5.092 ($J_{1,2}$ 3 6)
H-3e	NeuAc	2.757 ($J_{3e,4}$ 4.6, $J_{3e,3a}$−12 1)	2.764 ($J_{3e,4}$ 4.6; $J_{3e,3a}$−12.5)
H-3a	NeuAc	1.831 ($J_{3a,4}$ and $J_{3a,3e}$−12.1)	1.798 ($J_{3a,4}$ and $J_{3a,3e}$−12.5)
H-5	Fucα,β		4 828 ($J_{5,6}$ 6.7)
H-6	Fucα		1.171 ($J_{5,6}$ 6.7)
	Fucβ		1.166 ($J_{5,6}$ 6.7)
NAc	GlcNAcα	2.040	2.032
	GlcNAcβ	2.039	2 032
	NeuAc	2.032	2.028

a fucosyltransferase preparation isolated from human milk was able to catalyze the synthesis of analogs of **2**. Fucosylation reactions using this enzyme preparation typically proceeded to near-completion (> 95%) within 72 h at 37°C using 2.2 mU of enzyme and 3.4 equivalents of GDP-fucose. Another group used a recombinant form of the Lewis α1→3/4-fucosyltransferase to transfer fucose to analogs of **1** [38]. It was shown that the relative rate for fucosylation of **1** was 9.7% compared to the better substrate, the type 1 acceptor Galβ1→3GlcNAc [38]. Recently the cDNA thought to code for the plasma type α1→3-fucosyltransferase was cloned [20]. This enzyme was shown to be restricted in its action to type 2 sialylated or neutral acceptor molecules. With the recombinant Lewis α1→3/4-fucosyltransferase (data not shown) we observed severe product inhibition at only about 100 μM GDP. In our work, with the recombinant plasma α1→3-fucosyltransferase described in this paper, there was little evidence of product (GDP) inhibition, and fucosylation reactions routinely proceeded to near completion in the presence of >mM concentrations of GDP. Thus the plasma type fucosyltransferase is far superior for the practical synthesis of fucosylated *N*-acetyllactosamine (Le^x) bearing structures. This advantage has recently also been exploited by others in the synthesis of sialyl-Le^x containing structures [16].

Finally, the use of enzymes as tools for the synthesis of oligosaccharides has the great advantage of flexibility in producing other more complex variants of sialyl-Le^x determinants. For instance, di- and tri-antennary structures, with sialyl-Le^x determinants on each branch, as well as elongated polylactosaminoglycan chains with a sialyl-Le^x determinant and/or internal fucose residues can be synthesized by relatively simple extension of the method described here for the synthesis of the sialyl-Le^x tetrasaccharide. Synthesis of these more complex types of sialyl-Le^x bearing structures is currently in progress.

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