Eur. J. Biochem. 242, 674–681 (1996)

Exploring the substrate specificities of α-2,6- and α-2,3-sialyltransferases using synthetic acceptor analogues

Johannes A. L. M. VAN DORST[1], Jaana M. TIKKANEN[1], Christian H. KREZDORN[2], Markus B. STREIFF[3], Eric G. BERGER[2], J. Albert VAN KUIK[1], Johannis P. KAMERLING[1] and Johannes F. G. VLIEGENTHART[1]

[1] Bijvoet Center, Department of Bio-Organic Chemistry, Utrecht University, The Netherlands
[2] Institute of Physiology, University of Zürich, Switzerland
[3] CIBA Central Research Laboratories, Basel, Switzerland

(Received 20 June/25 September 1996) – EJB 96 0909/5

The acceptor specificities of rat liver Gal(β1-4)GlcNAc α-2,6-sialyltransferase, recombinant full-length human liver Gal(β1-4)GlcNAc α-2,6-sialyltransferase, and a soluble form of recombinant rat liver Gal(β1-3/4)GlcNAc α-2,3-sialyltransferase were studied with a panel of analogues of the trisaccharide Gal(β1-4)GlcNAc(β1-2)Man(α1-O)$(CH_2)_7CH_3$. These analogues contain structural variants of D-galactose, modified at either C3, C4 or C5 by deoxygenation, fluorination, O-methylation, epimerization, or by the introduction of an amino group. In addition, the enantiomer of D-galactose is included. The α-2,6-sialyltransferases tolerated most of the modifications at the galactose residue to some extent, whereas the α-2,3-sialyltransferase displayed a narrower specificity. Molecular dynamics simulations were performed in order to correlate enzymatic activity to three-dimensional structure. Ineffective acceptors for rat liver α-2,6-sialyltransferase were shown to be inhibitory towards the enzyme; likewise, the α-2,3-sialyltransferase was found to be inhibited by all non-substrates. Modified sialyloligosaccharides were obtained on a milligram scale by incubation of effective acceptors with one of each of the three enzymes, and characterized by 500-MHz ^{1}H-NMR spectroscopy.

Keywords: sialyltransferase; substrate specificity; enzymatic synthesis; glycoprotein.

Sialic acids that are located at the periphery of glycoconjugate glycans can be involved in a variety of biological phenomena, such as cell-cell and receptor-ligand interactions, cell differentiation, or tumor progression and metastasis [1–5]. The various types of sialylation patterns found in either glycolipid or glycoprotein glycans are the result of the action of at least 12 sialyltransferases, that can be readily distinguished by their strict *in vivo* specificity for acceptor substrates, including the type of linkage formed in the product [1]. However, in patients suffering from β-mannosidosis, we found the unusual trisaccharide Neu5Ac(α2-6)Man(β1-4)GlcNAc [6], and it has been suggested that this compound is formed by transfer of Neu5Ac from CMP-Neu5Ac to HO6′ in the accumulated disaccharide Man(β1-4)-GlcNAc. Additional studies by us [7, 8] and by others [9] have shown that the purified rat liver Gal(β1-4)GlcNAc α-2,6-sialyltransferase, though specific for the β-1,4-linkage in the *N*-acetyllactosamine epitope in N-glycans, tolerated modifications in the accepting terminal monosaccharide *in vitro*, producing varying yields of sialyloligosaccharides. These results indicated that some of the hydroxyl groups of the terminal monosaccharide are of minor importance for effective sialylation, at least by the applied α-2,6-sialyltransferase, and prompted us to a program aimed at the exploration of the specific topology required by sialyltransferases.

In a first approach, the acceptor specificity of enzymes involved in the sialylation of N-linked-type oligosaccharides was studied, using a purified rat liver Gal(β1-4)GlcNAc α-2,6-sialyltransferase, a recombinant full-length form of the same enzyme from human liver, and a soluble form of recombinant rat liver Gal(β1-3/4)GlcNAc α-2,3-sialyltransferase. To obtain detailed information about the allowed structural modifications, the synthetic trisaccharide Gal(β1-4)GlcNAc(β1-2)Man(α1-O)-$(CH_2)_7CH_3$ and analogues containing structural variants of D-galactose [10, 11] were employed as substrates (for structures, see Fig. 1). These trisaccharides represent a complete branch of a *N*-acetyllactosamine glycan, mimicking the requirements of the active sites of the enzymes as well as possible. Hydroxyl groups at either C3 or C4 of the galactose residue were substituted by hydrogen or fluorine, by amino- or *O*-methyl groups, or were inverted, to determine their involvement in binding and catalytic activity. In addition, trisaccharides containing α-L-altrose (an isomer of β-D-galactose with an inverted hydroxymethyl group at C5) or β-L-galactose instead of β-D-galactose at the non-reducing terminus were used as probes.

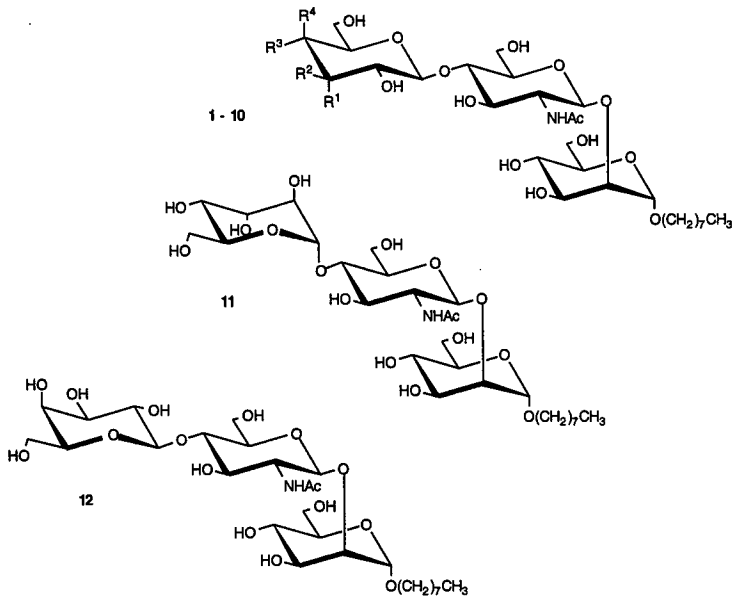
EXPERIMENTAL PROCEDURES

Materials. The synthesis of the oligosaccharides **1–12** (Fig. 1) has been recently described [10, 11]. These compounds all contain an octyl aglycon to facilitate the separation of radio-

Correspondence to J. P. Kamerling, Bijvoet Center, Department of Bio-Organic Chemistry, Utrecht University, P.O. Box 80.075, NL-3508 TB Utrecht, The Netherlands
Fax: +31 30 2540980.

Abbreviations. Alt, altrose; CHARMm, Molecular Simulations Inc. version of chemistry Harvard macromolecular mechanics; CHEAT, carbohydrate hydroxyls represented by extended atoms; 1D, 1-dimensional; Gul, gulose; Hex, hexose; ST, sialyltransferase.

Enzymes. CMP-Neu5Ac:Gal(β1-4)GlcNAc-R α-2,6-sialyltransferase (EC 2.4.99.1); CMP-Neu5Ac:Gal(β1-3/4)GlcNAc-R α-2,3-sialyltransferase (EC 2.4.99.6); orthophosphoric monoester phosphohydrolase, alkaline phosphatase (EC 3.1.3.1).



Code	Structure	R^1	R^2	R^3	R^4
1	β-D-Gal-R	H	OH	H	OH
2	3-deoxy-β-D-Gal-R	H	H	H	OH
3	3-deoxy-3-fluoro-β-D-Gal-R	H	F	H	OH
4	β-D-Gul-R (3-epimer)	OH	H	H	OH
5	3-*O*-methyl-β-D-Gal-R	H	OMe	H	OH
6	3-amino-3-deoxy-β-D-Gal-R	H	NH_2	H	OH
7	4-deoxy-β-D-Gal-R	H	OH	H	H
8	4-deoxy-4-fluoro-β-D-Gal-R	H	OH	H	F
9	β-D-Glc-R (4-epimer)	H	OH	OH	H
10	4-*O*-methyl-β-D-Gal-R	H	OH	H	OMe
11	α-L-Alt-R (5-epimer)	–	–	–	–
12	β-L-Gal-R (enantiomer)	–	–	–	–

Fig. 1. Structures of tested trisaccharides of the general type Hex*p*-(1→4)-*β*-D-Glc*p*NAc-(1→2)-*α*-D-Man*p*-(1→O)(CH_2)$_7CH_3$. The synthesis of these compounds is described in [10, 11]. R, (1→4)-*β*-D-Glc*p*NAc-(1→2)-*α*-D-Man*p*-(1→O)(CH_2)$_7CH_3$.

labelled product from unreacted CMP-Neu5Ac by reverse-phase chromatography in the sialyltransferase assays (*vide infra*). Rat liver Gal(*β*1-4)GlcNAc *α*-2,6-sialyltransferase (Boehringer Mannheim) had an activity of 0.25 U/ml; one unit (U) of enzyme activity is defined as the amount of enzyme catalyzing the transfer of 1 µmol Neu5Ac/min using the incubation conditions as indicated below and **1** as an acceptor at a concentration of 2 mM. A crude preparation of recombinant full-length human liver Gal(*β*1-4)GlcNAc *α*-2,6-sialyltransferase, with an activity of 3.5 mU/ml when assayed with 2 mM **1**, was isolated from a *Saccharomyces cerevisiae* cell culture as previously described [12]. This enzyme was tested without purification, since no interfering endogenous sialyltransferase activity is present in yeast cells. Recombinant N-terminal-truncated rat liver Gal(*β*1-3/4)-GlcNAc *α*-2,3-sialyltransferase [13] was a purified soluble enzyme, kindly donated by Ciba AG. The activity of this enzyme was 0.25 U/ml, using **1** as an acceptor at a concentration of 2 mM. CMP-[^{14}C]Neu5Ac (Amersham, specific activity 293 Ci/mol) was diluted to an appropriate activity with non-radioactive CMP-Neu5Ac (Oxford Glycosystems, UK). CMP-Neu5Ac used for large-scale incubations was a gift of Dr U. Kragl (Forschungszentrum Jülich GmbH, Germany). Calf intestinal alkaline phosphatase was supplied by Sigma. Sep-Pak C_{18} cartridges were purchased from Waters Associates.

Sialyltransferase assays. The standard incubation mixture contained, in a total volume of 50 µl, 2 mM of a specific trisaccharide derivative (**1**–**12**), 7.5 nmol CMP-[^{14}C]Neu5Ac (7000 cpm/nmol), 50 mM sodium cacodylate pH 6.5, 0.5% (mass/vol.) Triton X-100, 50 µg BSA, and a specific enzyme preparation (0.05–0.07 mU). Samples were incubated for 30 min at 37°C, and the reaction was stopped by addition of 1.0 ml ice-cold water. The mixtures were loaded onto conditioned Sep-Pak C_{18} cartridges [14]. Following removal of unreacted CMP-Neu5Ac and eventually related degradation products [15] by washing with water (20 ml), radiolabelled product and non-labelled acceptor were eluted with methanol (10 ml). Solutions were mixed with 10 ml Insta-Gel Plus (Packard) and analyzed for radioactivity by liquid scintillation counting. Controls were routinely incubated in the absence of either enzyme or exogenous acceptor, and all assays were performed at least in duplicate.

Determination of kinetic parameters. Relative rates for each acceptor, using rat liver *α*-2,6-ST (sialyltransferase), recombinant human liver *α*-2,6-ST, or recombinant rat liver *α*-2,3-

ST, were calculated as a percentage of the incorporation of Neu5Ac into the D-Gal-containing trisaccharide **1**. Under the conditions used, the formation of product with **1** as the acceptor was shown to be linear in time. K_m values were determined within a range of 8−10 acceptor concentrations around a previously determined crude value. Rate data were fit to the Michaelis-Menten equation using the Enzfitter® software package.

Inhibition studies with rat liver α-2,6-ST and recombinant rat liver α-2,3-ST. Compound **1** was used as an acceptor at a concentration of 1.0 mM in the standard sialyltransferase assay in the absence or presence of a potential inhibitor at a concentration of 1.0 or 3.0 mM. The inhibition was measured as the ability to reduce the incorporation of Neu5Ac by either rat liver α-2,6-ST or recombinant rat liver α-2,3-ST into **1**.

Large-scale incubations with rat liver α-2,6-ST and recombinant rat liver α-2,3-ST. The incubation mixture contained in a volume of 1 ml: 2−5 mg of a trisaccharide acceptor, CMP-Neu5Ac (3.5−10 mg), 50 mM sodium cacodylate pH 6.5, 0.5% (mass/vol.) Triton X-100, 1 mg BSA, calf intestinal alkaline phosphatase (18 U), and a specific sialyltransferase preparation (5 mU). Calf intestinal alkaline phosphatase was included to decompose the released CMP [16], a strong inhibitor of sialyltransferase. In the case of trisaccharide **1**, the mixture was kept for 2 and 3 days at 37°C with rat liver α-2,6-ST and recombinant rat liver α-2,3-ST, respectively. The other acceptors were incubated for 7 days at 37°C, irrespective of the enzyme used; calf intestinal alkaline phosphatase and CMP-Neu5Ac were added in several batches at regular intervals of 2 days, the latter to account for the relatively rapid hydrolysis of the sugar-nucleotide. The reaction was stopped by freezing and thawing and the sample was loaded onto a conditioned Sep-Pak C_{18} cartridge. After washing with 30 ml water, the column was eluted with 10 ml methanol. The organic solvent was evaporated, and the residue was fractionated on a Resource Q anion-exchange column (30×6.4 mm, Pharmacia FPLC system). After elution with 2 ml water, a linear concentration gradient of 0−50 mM NaCl in 8 ml water, at a flow rate of 4 ml/min, was applied. The elution pattern was monitored at 214 nm. Collected fractions were lyophilized and desalted on a Sep-Pak C_{18} cartridge as described above. Following evaporation of the methanol eluate, the residue was dissolved in water (2 ml) and lyophilized.

Large-scale incubation with recombinant human liver α-2,6-ST. The incubation mixture contained in a volume of 1 ml: 2.3 mg compound **7**, CMP-Neu5Ac (10 mg), 50 mM sodium cacodylate pH 6.5, 0.5% (mass/vol.) Triton X-100, calf intestinal alkaline phosphatase (18 U), and a crude preparation of recombinant human liver α-2,6-ST (5 mU). The mixture was treated for 7 days at 37°C as described above and, after freezing and thawing, filtered through a 0.22-µm pore filter (Ultrafree-MC, Millipore). The filtrate was further processed in a similar way as described above.

500-MHz ^{1}H-NMR spectroscopy. Prior to ^{1}H-NMR spectroscopy, carbohydrate samples were treated twice with ^2H_2O (99.9% ^{2}H, Isotec Inc., USA) with intermediate lyophilization. One-dimensional (1D) ^{1}H-NMR spectra were recorded on a Bruker AMX-500 spectrometer (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University) at a probe temperature of 27°C. Chemical shifts (δ) are expressed in ppm relative to internal acetone (δ 2.225) [17].

Computer simulations. Molecular dynamics simulations were performed using CHEAT (carbohydrate hydroxyls represented by extended atoms), a CHARMm (Molecular Simulations Inc. version of chemistry Harvard macromolecular mechanics) based force field for carbohydrates [18], 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., USA). The atoms of the *O*-methyl and *N*-acetyl substituents were implemented on the basis of the existing general valence force field (CHARMm). *Φ/Ψ*-dependent iso-energy contour plots of disaccharide methyl glycosides were generated by rotating ϕ and ψ independently from −180° to 180° at 10° intervals. At each increment, the dihedrals were maintained and the disaccharide was minimized to a maximum derivative of the energy function to the atomic positions of 0.04 kJ · mol^{-1} · nm^{-1}. A contour plot as a function of ϕ and ψ was constructed showing the energy levels at 4.2 kJ · mol^{-1} intervals up to 50 kJ · mol^{-1} above the absolute minimum energy value. For each disaccharide derivative, the conformation with the lowest energy was chosen from its contour plot and further minimized without constraints. The calculations were performed on a local Silicon Graphics Workstation. The interglycosidic torsion angles ϕ and ψ are defined as O5−C1−O1−C*x* and C1−O1−C*x*−C(*x*−1), respectively, where C*x* and C(*x*−1) are aglyconic atoms (IUPAC convention [19]).

Table 1. Acceptor specificity of sialyltransferases with various trisaccharide derivatives of the type Hex*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→O)(CH$_2$)$_7$CH$_3$. The substrate concentration was 2 mM for all compounds tested. Relative rates for each acceptor are expressed as a percentage of the incorporation of Neu5Ac into the parent compound **1**. 100% activity corresponds to 1.0 nmol · min^{-1} · ml^{-1} for rat liver α-2,6-sialyltransferase (RL α-2,6-ST), 2.2 nmol · min^{-1} · ml^{-1} for recombinant human liver α-2,6-sialyltransferase (HL α-2,6-rST) and 1.2 nmol · min^{-1} · ml^{-1} for recombinant rat liver α-2,3-sialyltransferase (RL α-2,3-rST). R, (1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→O)-(CH$_2$)$_7$CH$_3$.

	Acceptor	Relative rate of sialylation of		
		RL α-2,6-ST	HL α-2,6-rST	RL α-2,3-rST
		%		
1	β-D-Gal-R	100	100	100
2	3-Deoxy-β-D-Gal-R	7	13	0
3	3-Deoxy-3-fluoro-β-D-Gal-R	2	8	0
4	β-D-Gul-R	1	<1	0
5	3-*O*-Methyl-β-D-Gal-R	1	<1	0
6	3-Amino-3-deoxy-β-D-Gal-R	2	<1	0
7	4-Deoxy-β-D-Gal-R	40	29	2
8	4-Deoxy-4-fluoro-β-D-Gal-R	23	17	7
9	β-D-Glc-R	6	5	0
10	4-*O*-Methyl-β-D-Gal-R	2	2	51
11	α-L-Alt-R	<1	<1	0
12	β-L-Gal-R	0	0	0

RESULTS

Acceptor specificities of sialyltransferases. The acceptor specificities of rat liver α-2,6-ST, recombinant human liver α-2,6-ST, and recombinant rat liver α-2,3-ST towards the panel of trisaccharide octyl glycosides **1**−**12** (Fig. 1) are presented in Table 1. Kinetic constants were determined for effective acceptors, and are listed in Table 2.

Gal(β1-4)GlcNAc α-2,6-sialyltransferases. The K_m values for the native trisaccharide **1** obtained with rat liver α-2,6-ST and recombinant human liver α-2,6-ST were 0.81 mM and

Table 2. Kinetic parameters of sialyltransferases with various acceptors of the type Hex*p*-(1→4)-*β*-D-Glc*p*NAc-(1→2)-*α*-D-Man*p*-(1→O)(CH$_2$)$_7$CH$_3$. V_{max}/K_m, kinetic efficiency. Dashes are indicated where measurement of kinetic constants was not practical due to low acceptor activity. RL *α*-2,6-ST, rat liver *α*-2,6-sialyltransferase; HL *α*-2,6-rST, recombinant human liver *α*-2,6-sialyltransferase; RL *α*-2,3-rST, recombinant rat liver *α*-2,3-sialyltransferase; R, (1→4)-*β*-D-Glc*p*NAc-(1→2)-*α*-D-Man*p*-(1→O)(CH$_2$)$_7$CH$_3$.

Acceptor	RL *α*-2,6-ST			HL *α*-2,6-rST			RL *α*-2,3-rST		
	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m
	mM	nmol · min^{-1} · ml^{-1}		mM	nmol · min^{-1} · ml^{-1}		mM	nmol · min^{-1} · ml^{-1}	
1 *β*-D-Gal-R	0.81	1.24	1.53	1.12	2.70	2.41	0.49	1.19	2.42
2 3-Deoxy-*β*-D-Gal-R	1.43	0.34	0.24	6.25	0.62	0.10	–	–	–
3 3-Deoxy-3-fluoro-*β*-D-Gal-R	–	–	–	3.61	0.21	0.06	–	–	–
7 4-Deoxy-*β*-D-Gal-R	3.78	2.06	0.54	4.67	0.69	0.15	–	–	–
8 4-Deoxy-4-fluoro-*β*-D-Gal-R	2.89	0.53	0.18	0.67	0.16	0.24	–	–	–
9 *β*-D-Glc-R	7.24	0.61	0.08	17.9	0.39	0.02	–	–	–
10 4-*O*-Methyl-*β*-D-Gal-R	–	–	–	–	–	–	0.17	1.21	7.12

1.12 mM, respectively (Table 2), whereas a value of 0.55 mM has been reported for the 8-methoxycarbonyloctyl glycoside analogue of **1** [9]. As is evident from Table 1, all substrate analogues, except **12**, were accepted to some extent by both enzymes, although a substantial decrease in activity with respect to **1** was observed for each effective acceptor. Substitution of the hydroxyl group at C3 by fluorine (**3**) was tolerated much better by recombinant human liver *α*-2,6-ST than by rat liver *α*-2,6-ST. Nevertheless, after a 4-h incubation with the latter enzyme, using **3** at a concentration of 10 mM, a significant incorporation of Neu5Ac was also observed (data not shown). Under similar conditions, the analogues with a D-gulose (**4**) or a 3-*O*-methyl-galactose residue (**5**) remained poor acceptors. Modifications at C4 of galactose were well accepted, especially the sterically conservative substitutions of HO4 by hydrogen (**7**) or fluorine (**8**). Both *α*-2,6-sialyltransferases were able to sialylate the trisaccharide derivative with *α*-L-altrose at the non-reducing terminus (**11**), though at a very low rate. When **11** was incubated for 6 h at a concentration of 10 mM, a significant incorporation of sialic acid was observed. In contrast, the *β*-L-galactose-containing compound **12** remained completely ineffective as an acceptor under these conditions (data not shown).

Gal(β1-3/4)GlcNAc α-2,3-sialyltransferase. Recombinant rat liver *α*-2,3-ST displayed a narrower specificity pattern than the *α*-2,6-sialyltransferases (Table 1). Trisaccharide derivative **4** has a potential reaction site, but was not sialylated. At C4 of galactose, some modification was allowed, and particularly the introduction of an axial bulky methyl group at this position (**10**) produced an effective substrate, with a threefold increase of the kinetic efficiency V_{max}/K_m due to a lower K_m value (0.17 mM) as compared to that of the parent trisaccharide **1** (Table 2). Neither the analogue with an inverted hydroxymethyl group at C5 of galactose (**11**) nor the derivative containing the enantiomer of D-galactose (**12**) was a substrate for recombinant rat liver *α*-2,3-ST.

Inhibition of *α*-2,3- and *α*-2,6-sialyltransferases. Mixed substrate experiments were performed with **1** and trisaccharide analogues of **1** having a relative sialylation rate of ≤ 2%. The maximum concentration of the potential inhibitor was restricted to 3.0 mM, since high concentrations of the native substrate **1** (> 12 mM) gave rise to substrate inhibition (data not shown). All trisaccharide analogues that were evaluated showed inhibitory activity towards both recombinant rat liver *α*-2,3-ST and rat liver *α*-2,6-ST (Table 3), indicating that they were bound by the enzymes. The best inhibitor for rat liver *α*-2,6-ST was the amino derivative **6**, showing 47% inhibition at an equimolar ratio of **1** and **6**. The C3-modified trisaccharides were good inhibitors for recombinant rat liver *α*-2,3-ST. The amino derivative **6** was particularly effective, and abolished the incorporation of Neu5Ac into **1** at a threefold excess of inhibitor. Interestingly, the substrate analogue with a *β*-L-galactose residue (**12**) was a good inhibitor for recombinant rat liver *α*-2,3-ST as well, producing an inhibition of 81% when used in a 1:1 ratio with **1**.

Table 3. Inhibition of sialyltransferases. The relative inhibitor capacity of acceptor analogues towards rat liver *α*-2,6-sialyltransferase (RL *α*-2,6-ST) and recombinant rat liver *α*-2,3-sialyltransferase (RL *α*-2,3-rST) is expressed as the inhibition of enzyme activity measured with *β*-D-Gal*p*-(1→4)-*β*-D-Glc*p*NAc-(1→2)-*α*-D-Man*p*-(1→O)(CH$_2$)$_7$CH$_3$ (**1**) at 1.0 mM. The concentration of potential inhibitor was either 1.0 mM or 3.0 mM. Dashes are indicated for compounds that were effective acceptors for rat liver *α*-2,6-sialyltransferase. R, (1→4)-*β*-D-Glc*p*NAc-(1→2)-*α*-D-Man*p*-(1→O)(CH$_2$)$_7$CH$_3$; n.d., not determined.

Inhibitor	Inhibition of activity of			
	RL *α*-2,6 ST at		RL *α*-2,3-rST at	
	1.0 mM	3.0 mM	1.0 mM	3.0 mM
	%			
2 3-Deoxy-*β*-D-Gal-R	–	–	42	90
3 3-Deoxy-3-fluoro-*β*-D-Gal-R	–	–	27	59
4 *β*-D-Gul-R	29	54	58	88
5 3-*O*-Methyl-*β*-D-Gal-R	34	64	58	83
6 3-Amino-3-deoxy-*β*-D-Gal-R	47	n.d.	95	99
7 4-Deoxy-*β*-D-Gal-R	–	–	17	45
9 *β*-D-Glc-R	–	–	60	92
11 *α*-L-Alt-R	19	20	35	73
12 *β*-L-Gal-R	31	40	81	n.d.

Structural analysis of sialyloligosaccharides produced by sialyltransferases. The substrates **1**, **2**, **7**, **8**, **9**, and **10** were used in large-scale experiments in order to analyse the sialylated products formed and to evaluate the applicability of rat liver *α*-2,6-ST, recombinant human liver *α*-2,6-ST, and recombinant rat liver *α*-2,3-ST as synthetic tools. A summary of these glycosylation reactions is presented in Table 4. Incubation of the parent trisaccharide **1** with a two- to threefold excess of CMP-Neu5Ac in the presence of either rat liver *α*-2,6-ST or recombinant rat liver *α*-2,3-ST resulted, by TLC analysis, in a complete

Table 4. Enzymatic synthesis of sialyloligosaccharides. Reactions were performed as described in Experimental Procedures; incubation time is indicated in days (d) at 37°C. RL α-2,6-ST, rat liver α-2,6-sialyltransferase; HL α-2,6-rST, recombinant human liver α-2,6-sialyltransferase; RL α-2,3-rST, recombinant rat liver α-2,3-sialyltransferase; R, (1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→O)$(CH_2)_7CH_3$.

Acceptor			Donor	Enzyme	Time	Product			
code	structure	amount				code	structure	amount	yield
		mg/μmol	μmol		d			mg	%
1	β-D-Gal-R	2.1/3.2	7.7	RL α-2,6-ST	2	**13**	α-Neu5Ac-(2→6)-β-D-Gal-R	2.4	79
2	3-Deoxy-β-D-Gal-R	2.2/3.4	15.0	RL α-2,6-ST	7	**14**	α-Neu5Ac-(2→6)-3-deoxy-β-D-Gal-R	2.1	63
7	4-Deoxy-β-D-Gal-R	2.4/3.7	15.2	RL α-2,6-ST	7	**15**	α-Neu5Ac-(2→6)-4-deoxy-β-D-Gal-R	2.2	63
8	4-Deoxy-4-fluoro-β-D-Gal-R	2.1/3.2	15.4	RL α-2,6-ST	7	**16**	α-Neu5Ac-(2→6)-4-deoxy-4-fluoro-β-D-Gal-R	2.5	79
9	β-D-Glc-R	2.3/3.5	15.1	RL α-2,6-ST	7	**17**	α-Neu5Ac-(2→6)-β-D-Glc-R	1.8	53
10	4-*O*-Methyl-β-D-Gal-R	5.1/7.6	15.2	RL α-2,6-ST	7	**18**	α-Neu5Ac-(2→6)-4-*O*-methyl-β-D-Gal-R	0.9	13
7	4-Deoxy-β-D-Gal-R	2.3/3.6	15.1	HL α-2,6-rST	7	**15**	α-Neu5Ac-(2→6)-4-deoxy-β-D-Gal-R	2.3	67
1	β-D-Gal-R	2.3/3.5	9.5	RL α-2,3-rST	3	**19**	α-Neu5Ac-(2→3)-β-D-Gal-R	2.8	81
8	4-Deoxy-4-fluoro-β-D-Gal-R	2.5/3.8	15.2	RL α-2,3-rST	7	**20**	α-Neu5Ac-(2→3)-4-deoxy-4-fluoro-β-D-Gal-R	1.1	31
10	4-*O*-Methyl-β-D-Gal-R	2.0/3.0	15.3	RL α-2,3-rST	7	**21**	α-Neu5Ac-(2→3)-4-*O*-methyl-β-D-Gal-R	1.7	57

disappearance of **1** and the formation of **13** or **19** after 2 and 3 days, respectively. The structures of **13** (isolated in 79% yield) and **19** (81%) were established by 500-MHz ^{1}H-NMR spectroscopy, and relevant NMR data are listed in Table 5, along with the chemical shift values of acceptor trisaccharide **1** [10]. The (α2-6) or (α2-3) linkage of the newly introduced Neu5Ac residue was evident from the chemical shifts of the H3 atoms (**13**, $H3_{eq}$, δ 2.670; $H3_{ax}$, δ 1.718; **19**, $H3_{eq}$, δ 2.757; $H3_{ax}$, δ 1.798) [17, 20].

The incubation of the substrate analogues of **1** with rat liver α-2,6-ST, recombinant human liver α-2,6-ST, or recombinant rat liver α-2,3-ST was stopped after 7 days, regardless of incomplete sialylation, to limit the use of expensive CMP-Neu5Ac and to allow a direct comparison of the reaction yields. Under these conditions, the rat liver α-2,6-ST-catalyzed sialylation of the poor substrates **2**, **7**, **8**, and **9** afforded the tetrasaccharides **14–17** in good yields (53–79%). Likewise, the 4-*O*-methyl-galactose-containing derivative **10** was converted into tetrasaccharide **18** by rat liver α-2,6-ST in an acceptable yield of 13%, in spite of a relative sialylation-rate of 2% only (Table 1). The concentration of **10** used in this incubation was higher than for the other substrate analogues, because the relative rate of sialylation of **10** was found to increase at higher concentrations (data not shown). The ^{1}H-NMR spectra of **14–18** (Table 5, acceptor **10** included for comparison) all contained the structural-reporter-group signals indicative of the presence of an α-2,6-linked Neu5Ac residue ($H3_{eq}$, δ 2.663–2.690; $H3_{ax}$, δ 1.718–1.729; NAc, δ 2.029–2.032) [17, 20]. To establish the utility of the recombinant α-2,6-sialyltransferase, compound **7** was incubated with CMP-Neu5Ac using a crude yeast cell extract containing recombinant human liver α-2,6-ST activity. After workup, a single tetrasaccharide product **15** was obtained in a yield of 67%, similar to that obtained with the rat liver enzyme.

The number of sialyloligosaccharides produced with recombinant rat liver α-2,3-sialyltransferase was limited, due to the strict substrate specificity of this enzyme. Starting from acceptors **8** and **10**, tetrasaccharides **20** and **21** were obtained in yields of 31% and 57%, respectively. The introduction of a Neu5Ac residue in α-2,3-linkage was evident from the chemical shifts of the Neu5Ac H3 atoms ($H3_{eq}$, δ 2.767/83; $H3_{ax}$, δ 1.772/95). Moreover, **20** and **21** showed, like the parent tetrasaccharide **19**, a signal characteristic for Gal H3 at low field (δ 4.11–4.25) [17, 20].

DISCUSSION

Modified oligosaccharides have been used in a number of studies to explore the substrate specificities of sialyltransferases [8, 9, 21–25]. In view of the complexity of chemical oligosaccharide synthesis, these studies usually include the minimal acceptor structure and derivatives thereof. However, Gal(β1-4)-GlcNAc α-2,6-sialyltransferase is known to interact with parts of the acceptor structure in *N*-acetyllactosamine-type glycans remote from the site of glycosylation, extending to parts of the core region [26, 27]. In the present study, a complete branch of a *N*-acetyllactosamine-type N-glycan (**1**) and a systematic series of analogues were employed as substrates.

Specificity of Gal(β1-4)GlcNAc α-2,6-sialyltransferase. Previously it has been shown, using a number of modified *N*-acetyllactosamine-type disaccharides, that Gal HO6 together with the amide group of the penultimate GlcNAc residue are the only groups indispensible for an effective transfer of Neu5Ac, provided that they can be properly positioned in the enzyme active site [9]. Nevertheless, the enzyme is known to exhibit a high *in vivo* specificity and does not accept Gal(β1-3)GlcNAc nor Gal(β1-6)GlcNAc as a substrate [28]. Therefore, the active site of α-2,6-sialyltransferase must be closely contouring the substrate for an efficient transfer of Neu5Ac. Consistent with this, the replacement of HO3 or HO4 of galactose in **1** by sterically conservative substituents like hydrogen or fluorine was accepted much better than methylation or epimerization of these hydroxyl functions. The significant decrease in activity with all effective acceptors modified at either C3 or C4 suggests important contributions from HO3 and HO4 to hydrogen bonding. Whether these hydroxyl groups are involved as hydrogen-bond donors or acceptors may be derived from experiments with the fluoro analogues **3** and **8**, because fluorine cannot act as a hydrogen-bond donor but can serve as an acceptor [29]. Relative sialylation rates of both fluorinated analogues were lower than those of the deoxygenated compounds (Table 1), suggesting that HO3 and HO4 are hydrogen-bond donating. Interestingly, comparison of the K_m values for the deoxy and fluoro derivatives indicates a higher affinity of the enzyme for the latter, especially in the case of the human liver enzyme. This might be the result of some structural changes in the active site of the enzyme accompanying the binding of the fluorinated analogues. Such a feature has been previously observed in the binding of 6-deoxy-6-fluoro-D-galactose by the L-arabinoside binding protein [30].

Table 5. Relevant 500-MHz ^{1}H-NMR chemical shift values (δ) of structural-reporter-group protons for tetrasaccharides 13–21, together with those of asialo precursors 1 and 10. Chemical shifts are given relative to internal acetone (δ 2.225) and at p^{2}H 7 [17]. The data for acceptors **1** and **10** are taken from [10]. For tetrasaccharide **14** the value for $H3_{ax}$ (Gal) is indicated; $H3_{eq}$ resonates at 2.218 ppm. For tetrasaccharide **15** the value for $H4_{eq}$ (Gal) is indicated; $H4_{ax}$ resonates at 1.417 ppm. For tetrasaccharide **17** values are reported for a β-Glc residue instead of a β-Gal residue.

Residue	Reporter group	Acceptor		α-2,6-sialylation						α-2,3-sialylation		
		1	**10**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
		ppm										
α-D-Man*p*	H1	4.861	4.860	4.881	4.886	4.882	4.883	4.876	4.879	4.861	4.860	4.860
	H2	4.043	4.039	4.050	4.051	4.050	4.050	4.049	4.048	4.045	4.043	4.042
β-D-Glc*p*NAc	H1	4.583	4.578	4.608	4.614	4.604	4.604	4.597	4.602	4.568	4.576	4.573
	NAc	2.050	2.049	2.069	2.073	2.066	2.065	2.065	2.066	2.047	2.047	2.046
β-D-Gal*p*	H1	4.468	4.436	4.447	4.447	4.431	4.554	4.516	4.419	4.554	4.634	4.518
	H2	–	–	–	–	3.210	–	3.305	–	–	–	–
	H3	–	–	–	1.754	–	–	–	–	4.114	4.254	4.208
	H4	3.927	–	3.927	–	2.005	4.834	3.423	–	3.958	4.830	–
	CH_3O	–	3.516	–	–	–	–	–	3.492	–	–	3.510
α-Neu5Ac	$H3_{eq}$	–	–	2.670	2.663	2.665	2.680	2.690	2.679	2.757	2.783	2.767
	$H3_{ax}$	–	–	1.718	1.728	1.718	1.721	1.720	1.729	1.798	1.772	1.795
	NAc	–	–	2.029	2.029	2.029	2.031	2.032	2.031	2.031	2.030	2.033
Octyl	CH_3	0.860	0.860	0.861	0.861	0.861	0.861	0.861	0.860	0.860	0.859	0.859

In spite of the importance of Gal HO3 and HO4 in the catalytic process, the enzyme was able to accomplish the transfer of Neu5Ac to oligosaccharides with terminal residues lacking a β-D-*galacto* configuration. The sialylation of trisaccharide **11**, containing α-L-altrose instead of β-D-galactose, suggests that this compound exhibits to some extent a proper orientation of Alt HO6 and the amide group of GlcNAc in the active site. Since **12** was completely ineffective as a substrate, the L-Gal(β1-4)-GlcNAc structure in compound **12** apparently lacks the required topography. In order to confirm this assumption, energy maps of the disaccharide derivatives D-Gal(β1-4)GlcNAc(β1-O)Me, L-Alt(α1-4)GlcNAc(β1-O)Me, and L-Gal(β1-4)GlcNAc(β1-O)-Me, comprising the variable parts of **1**, **11**, and **12**, respectively, were constructed. The ϕ/ψ-dependent iso-energy contour plots of these fragments are depicted in Fig. 2 (top), where absolute minima are indicated for each disaccharide element. The minimum energy conformation for each disaccharide was estimated from its respective contour plot, and a new energy minimization was performed with no constraints set on the torsion angles ϕ and ψ, starting from this estimated conformation. The resulting structures are indicated in Fig. 2 (bottom). It appeared that the hydroxymethyl groups of D-Gal and L-Alt are located in a similar orientation with respect to the NHAc moiety of the GlcNAc residue (cf. Fig. 2A and B), namely at one side of the disaccharide (see also [31] for Fig. 2A). In contrast, these groups are positioned at opposite sides in the L-Gal(β1-4)GlcNAc element (Fig. 2C).

Specificity of Gal(β1-3/4)GlcNAc α-2,3-sialyltransferase. The α-2,3-sialyltransferase acting on N-glycans is capable of using both Gal(β1-3)GlcNAc (type I) and Gal(β1-4)GlcNAc (type II) structures as a substrate, suggesting a relatively loose specificity as compared to the α-2,6-sialyltransferase. Using deoxygenated substrate analogues [9], the enzyme has been previously shown to require an intact D-*galacto*-3,4,6-triol system on the accepting galactose unit, together with some unidentified additional structural features [9], for an effective transfer of Neu5Ac. The present data are supportive of the requirement for a D-*galacto*-3,4-diol system for catalytic action, but for binding this topography is not essential. The substrates with an inverted hydroxyl function at either C3 (**4**) or C4 (**9**) were not sialylated, but nevertheless were effective as inhibitors (*vide infra*). HO4 appears to be a hydrogen-bond acceptor, since the 4-*O*-methyl derivative **10** was a good substrate for the enzyme. In addition, the 4-fluoro derivative (**8**) was a better substrate than the deoxygenated analogue (**7**).

Inhibition of α-2,3- and α-2,6-sialyltransferase. Aberrant sialylation of the cell surface has been associated with the growth and metastatic potential of tumor cells [5, 32]. The development of sialyltransferase inhibitors may offer interesting prospects in relation to cancer chemotherapeutic agents. Some donor-based inhibitors, such as *O*- and *S*-sialylnucleosides have been described, as well as their antitumor and antimetastatic activity [33]. In the present study, some interesting features concerning the acceptor structure were established. All ineffective acceptors were inhibitory towards the rat liver α-2,6-sialyltransferase, indicating that the introduction of modifications in the galactose moiety did not prevent binding. Likewise, the α-2,3-sialyltransferase was inhibited by all non-substrates. A decrease in binding affinity that results from the absence of a single hydroxyl group of galactose may be counteracted by a contribution of the hydroxyl groups of the other monosaccharide residues, since the trisaccharides offer a relatively large template for recognition. This is supported by the observation that the 3-deoxy-Gal-containing trisaccharide **2** proved to be a good inhibitor of α-2,3-sialyltransferase, whereas deoxygenation at HO3 of galactose of the related disaccharide Gal(β1-4)GlcNAc(β1-O)$(CH_2)_8$-$COOCH_3$ has been found to result in a complete loss of recognition and binding by the enzyme [34]. The mannosyl residue in the trisaccharides studied here may therefore constitute at least some of the previously mentioned additional structural features [9] required by the α-2,3-sialyltransferase.

As tight binding is required for effective inhibitors, a relevant size of an acceptor analogue thus seems to be important. Enhancement of affinity may be realized in particular through replacement of hydroxyl functions by amino groups, as illustrated by the strong inhibition of α-2,3-sialyltransferase by the 3-amino-Gal-containing trisaccharide **6** as compared to the other inhibitory compounds. It should be noted that methyl 3′-

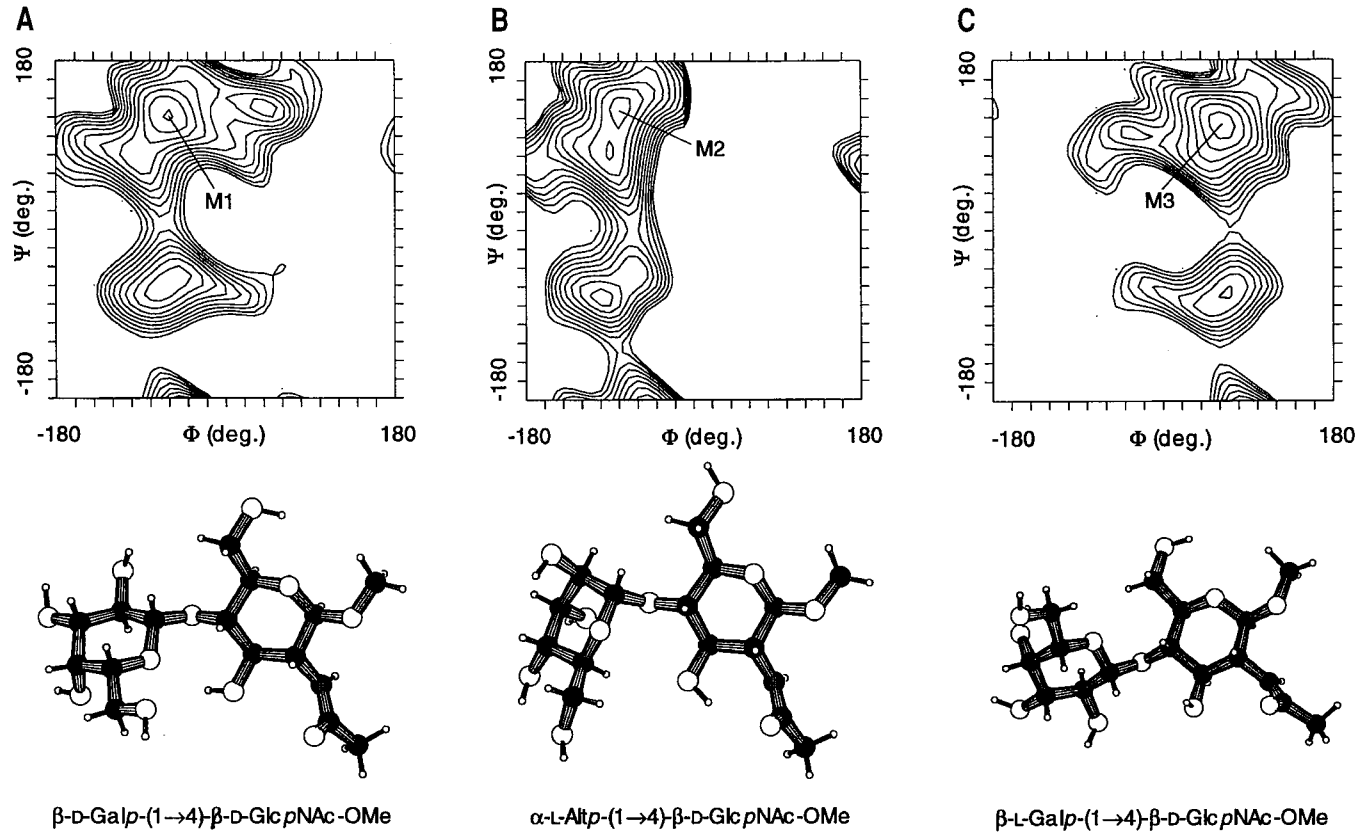


Fig. 2. Iso-energy contour plots (top) and conformations (bottom) of *β*-D-Gal*p*-(1→4)-*β*-D-Glc*p*NAc-OMe (A), *α*-L-Alt*p*-(1→4)-*β*-D-Glc*p*NAc-OMe (B), and *β*-L-Gal*p*-(1→4)-*β*-D-Glc*p*NAc-OMe (C). Contours are shown as a function of the ϕ and ψ interglycosidic torsion angles, and are plotted at regular intervals of 4.2 kJ·mol^{-1} up to 50 kJ · mol^{-1} above the absolute minimum energy values M1, M2, and M3, respectively. The disaccharides are depicted in the minimum energy conformation corresponding to these minima, with interglycosidic torsion angles of ϕ/ψ = −61.7°/122.1° (A), ϕ/ψ = −78.2°/131.3° (B), and ϕ/ψ = 62.7°/112.8° (C).

amino-3′-deoxy-*N*-acetyllactosaminide has been shown to inhibit UDP-Gal:Gal(*β*1-4)GlcNAc-R *α*-1,3-galactosyltransferase, with a K_i value significantly lower than the K_m for the corresponding acceptor Gal(*β*1-4)GlcNAc(*β*1-O)Me [35]. Amino groups are protonated under the assay conditions (pH 6.5) [36, 37] and may result in strong interactions with a negatively charged amino acid residue in the active site. A specific involvement of a basic residue in binding has been suggested for a number of glycosyltransferases, including *α*-2,3-sialyltransferase [34]. Such a residue would accept the proton from the hydroxyl group undergoing glycosylation during the glycosyl transfer reaction.

Synthetic applications of α-2,6- and α-2,3-sialyltransferase. The synthesis on a milligram scale of a number of modified sialyloligosaccharides indicates that sialyltransferases can be exploited as biocatalysts in the synthesis of interesting non-natural compounds, which is particularly valuable in view of the difficulties in chemical sialylation [1, 38, 39]. Importantly, none of the alterations of the galactose residue influenced the regiospecificity of either *α*-2,6- or *α*-2,3-sialyltransferases, as was evident from the product characterization by ^{1}H-NMR spectroscopy. Comparison of the specificity of the recombinant human liver *α*-2,6-sialyltransferase with the rat liver enzyme revealed only minor differences, allowing the use of the recombinant enzyme as a synthetic tool. Interestingly, a large-scale expression of this enzyme in *Saccharomyces cerevisiae*, yielding 47 U, has been achieved [40]. Using this enzyme and other glycosyltransferases, future studies will be directed towards the chemo-enzymatic synthesis of modified carbohydrate ligands, and their suitability as probes for studying molecular recognition phenomena.

The authors thank Dr P. H. Kruiskamp for recording NMR spectra. Thanks are also due to Dr U. Kragl (Forschungszentrum Jülich GmbH, Germany) for a gift of CMP-Neu5Ac.

REFERENCES

1. Schauer, R. & Kamerling, J. P. (1996), in *Glycoproteins* (Montreuil, J., Vliegenthart, J. F. G. & Schachter, H., eds) New Comprehensive Biochemistry, vol. 29b, Elsevier, Amsterdam, in the press.
2. Lis, H. & Sharon, N. (1993) *Eur. J. Biochem. 218*, 1–27.
3. Varki, A. (1994) *Proc. Natl Acad. Sci. USA 91*, 7390–7397.
4. Rutishauser, U., Acheson, A., Hall, A. K., Mann, D. M. & Sunshine, J. (1988) *Science 240*, 53–57.
5. Hakomori, S. (1989) *Adv. Cancer Res. 52*, 257–331.
6. Van Pelt, J., Dorland, L., Duran, M., Hokke, C. H., Kamerling, J. P. & Vliegenthart, J. F. G. (1990) *J. Biol. Chem. 265*, 19685–19689.
7. Van Pelt, J., Dorland, L., Duran, M., Hokke, C. H., Kamerling, J. P. & Vliegenthart, J. F. G. (1989) *FEBS Lett. 256*, 179–184.
8. Hokke, C. H., van der Ven, J. G. M., Kamerling, J. P. & Vliegenthart, J. F. G. (1993) *Glycoconjugate J. 10*, 82–90.
9. Wlasichuk, K. B., Kashem, M. A., Nikrad, P. V., Bird, P., Jiang, C. & Venot, A. P. (1993) *J. Biol. Chem. 268*, 13971–13977.
10. Van Dorst, J. A. L. M., van Heusden, C. J., Voskamp, A. F., Kamerling, J. P. & Vliegenthart, J. F. G. (1996) *Carbohydr. Res. 291*, 63–83.

11. Van Dorst, J. A. L. M., van Heusden, C. J., Tikkanen, J. M., Kamerling, J. P. & Vliegenthart, J. F. G. (1996), *Carbohydr. Res.*, in press.
12. Krezdorn, C. H., Kleene, R. B., Watzele, M., Ivanov, S. X., Hokke, C. H., Kamerling, J. P. & Berger, E. G. (1994) *Eur. J. Biochem. 220*, 809–817.
13. Gosselin, S., Alhussaini, M., Streiff, M. B., Takabayashi, K. & Palcic, M. M. (1994) *Anal. Biochem. 220*, 92–97.
14. Palcic, M. M., Heerze, L. D., Pierce, M. & Hindsgaul, O. (1988) *Glycoconjugate J. 5*, 49–63.
15. Beau, J. M., Schauer, R., Haverkamp, J., Kamerling, J. P., Dorland, L. & Vliegenthart, J. F. G. (1984) *Eur. J. Biochem. 140*, 203–208.
16. Unverzagt, C., Kunz, H. & Paulson, J. C. (1990) *J. Am. Chem. Soc. 112*, 9308–9309.
17. Vliegenthart, J. F. G., Dorland, L. & van Halbeek, H. (1983) *Adv. Carbohydr. Chem. Biochem. 41*, 209–374.
18. Grootenhuis, P. D. J. & Haasnoot, C. A. G. (1993) *Mol. Sim. 10*, 75–95.
19. IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN). Symbols for specifying the conformation of polysaccharide chains (1983) *Eur. J. Biochem. 131*, 81–88.
20. Kamerling, J. P. & Vliegenthart, J. F. G. (1992) *Biol. Magn. Reson. 10*, 1–194.
21. Kajihara, Y., Hashimoto, H., Kodama, H., Wakabayashi, T. & Sato, K. (1993) *J. Carbohydr. Chem. 12*, 991–995.
22. Kajihara, Y., Kodama, H., Wakabayashi, T., Sato, K. & Hashimoto, H. (1993) *Carbohydr. Res. 247*, 179–193.
23. Ito, Y., Gaudino, J. J. & Paulson, J. C. (1993) *Pure Appl. Chem. 65*, 753–762.
24. Kuhns, W., Rutz, V., Paulsen, H., Matta, K. L., Baker, M. A., Barner, M., Granovsky, M. & Brockhausen, I. (1993) *Glycoconjugate J. 10*, 381–394.
25. Chandrasekaran, E. V., Jain, R. K., Larsen, R. D., Wlasichuk, K. B. & Matta, K. L. (1995) *Biochemistry 34*, 2925–2936.
26. Joziasse, D. H., Schiphorst, W. E. C. M., van den Eijnden, D. H., van Kuik, J. A., van Halbeek, H. & Vliegenthart, J. F. G. (1985) *J. Biol. Chem. 260*, 714–719.
27. Nemansky, M., Schiphorst, W. E. C. M. & van den Eijnden, D. H. (1995) *FEBS Lett. 363*, 280–284.
28. Weinstein, J., De Souza-e-Silva, U. & Paulson, J. C. (1977) *J. Biol. Chem. 252*, 2363–2371.
29. Street, I. P., Armstrong, C. R. & Withers, S. G. (1986) *Biochemistry 25*, 6021–6027.
30. Vermersch, P. S., Tesmer, J. J. G. & Quiocho, F. A. (1992) *J. Mol. Biol. 226*, 923–929.
31. Imberty, A., Delage, M. M., Bourne, Y., Cambillau, C. & Perez, S. (1991) *Glycoconjugate J. 8*, 456–483.
32. Backman, I., Erbing, B., Jansson, P. E. & Kenne, L. (1988) *J. Chem. Soc. Perkin Trans. I*, 889–898.
33. Khan, S. H. & Matta, K. L. (1992) in *Glycoconjugates; composition, structure, and function* (Allen, H. J. & Kisailus, E. C., eds) pp. 361–378, Marcel Dekker, New York.
34. Hindsgaul, O., Kaur, K. J., Srivastava, G., Blaszczyk-Thurin, M., Crawley, S. C., Heerze, L. D. & Palcic, M. M. (1991) *J. Biol. Chem. 266*, 17858–17862.
35. Helland, A. C., Hindsgaul, O., Palcic, M. M., Stults, C. L. M. & Macher, B. A. (1995) *Carbohydr. Res. 276*, 91–98.
36. Field, R. A., Neville, D. C. A., Smith, R. W. & Ferguson, M. A. J. (1994) *Bioorg. Med. Chem. Lett. 4*, 391–394.
37. Gross, H. J. & Brossmer, R. (1988) *Glycoconjugate J. 5*, 411–417.
38. DeNinno, M. P. (1991) *Synthesis*, 583–593.
39. Okamoto, K. & Goto, T. (1990) *Tetrahedron 46*, 5835–5857.
40. Borsig, L., Ivanov, S. X., Herrmann, G. F., Kragl, U., Wandrey, C. & Berger, E. G. (1995) *Biochem. Biophys. Res. Commun. 210*, 14–20.