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Probing the substrate specificity of four different sialyltransferases using synthetic β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→O)$(CH_2)_7CH_3$ analogues
General activating effect of replacing *N*-acetylglucosamine by *N*-propionylglucosamine

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Abstract

The acceptor specificities of ST3Gal III, ST3Gal IV, ST6Gal I and ST6Gal II were investigated using a panel of β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→O)$(CH_2)_7CH_3$ analogues. Modifications introduced at either C2, C3, C4, C5, or C6 of terminal D-Gal, as well as *N*-propionylation instead of *N*-acetylation of subterminal D-GlcN were tested for their influence on the α-2,3- and α-2,6-sialyltransferase acceptor activities. Both ST3Gal enzymes displayed the same narrow acceptor specificity, and only accept reduction of the Gal C2 hydroxyl function. The ST6Gal enzymes, however, do not have the same acceptor specificity. ST6Gal II seems less tolerant towards modifications at Gal C3 and C4 than ST6Gal I, and prefers β-D-Gal*p*NAc-(1→4)-β-D-Glc*p*NAc (LacdiNAc) as an acceptor substrate, as shown by replacing the Gal C2 hydroxyl group with an *N*-acetyl function. Finally, a particularly striking feature of all tested sialyltransferases is the activating effect of replacing the *N*-acetyl function of subterminal GlcNAc by an *N*-propionyl function.

Keywords: Sialyltransferases; Substrate specificity; Synthetic analogue

1. Introduction

In mammals, sialylation of glycoconjugate glycans, using CMP-Neu5Ac as donor, is mediated by a group of enzymes called sialyltransferases (STs). To date, 20 STs have been reported, cloned and characterized from various vertebrate species (for a review, see [1] and references cited therein). They all have been described as type II membrane-bound glycoproteins with highly conserved motifs L, S, VS (long, short, very short), and motif III in their catalytic domain [2–5]. The various STs are named according to the monosaccharide and position where *N*-acetylneuraminic acid (Neu5Ac) is attached. For example, ST3Gal I–VI sialylate β-D-galactopyranosyl (Gal) residues via an α2–3 linkage, and ST6Gal I–II via an α2–6 linkage; ST6GalNAc I–VI sialylate Ser/Thr-linked *N*-acetyl-β-D-galactosaminyl (GalNAc) residues via an α2–6 linkage;

Abbreviations: Alt*p*, L-altropyranosyl; CMP-Neu5Ac, cytidine monophosphate *N*-acetylneuraminic acid; Gal*p*, D-galactopyranosyl; Glc*p*NAc, *N*-acetyl-D-glucosaminopyranosyl; Gul*p*, D-gulopyranosyl; Man*p*, D-mannopyranosyl; Neu5Ac, *N*-acetylneuraminic acid (sialic acid); ST3Gal, CMP-Neu5Ac: Galβ1-R α-2,3-sialyltransferase; ST6Gal, CMP-Neu5Ac: Galβ1-R α-2,6-sialyltransferase

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ST8Sia I–V sialylate another sialic acid residue via an α2–8 linkage [6].

A Neu5Ac residue linked to Gal in α2–3 or α2–6 linkage is one of the most common outer epitopes of N-linked glycans, O-linked glycans or glycolipids. Regardless of the kind of glycoconjugates, this sialylation is observed in the widespread type II [Gal(β1−4)GlcNAc] chains as well as in the more tissue-specific type I [Gal(β1−3)GlcNAc] chains [7,8]. A deeper understanding of the specific epitope topology required by ST3Gal and ST6Gal was reached by studying type I synthetic disaccharides [9,10], type II isolated or synthetic N-glycan related disaccharides [11,12], and different sets of synthetic disaccharides with structural modifications on specific positions of terminal Gal or of subterminal GlcNAc in β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc glycosides [13,14]. Based on the influence of aglycon hydrophobicity when transferring Neu5Ac to Gal [13,15] and on the possibility of recognizing a more extended part in the native glycan chains, type II trisaccharides related to N-glycan were previously designed with diverse modifications introduced at Gal C3, C4, C5, and C6 in order to achieve a more systematic approach when exploring the relevance of each position in the sialylation process [16,17]. Studies with human and rat liver ST6Gal I revealed that these enzymes require Gal HO6 and the *N*-acetyl function of GlcNAc [13], but can tolerate few modifications introduced at Gal C2, C3, or C4 [13,14,18]. On the other hand, rat liver ST3Gal III displays a much narrower specificity, since only mimics modified at O4 can be sialylated [13,18]. Recently, an additional set of analogues of β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→O) $(CH_2)_7CH_3$ with modifications at Gal C2 or C6, as well as at GlcNAc C2 were synthesized [19].

Here, we report on the acceptor specificities of recombinant forms of rat ST3Gal III (ST3Gal III), human ST3Gal IV (ST3Gal IV), human ST6Gal I (ST6Gal I), and the recently reported human ST6Gal II (ST6Gal II) [20–22], using a panel of β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→O) $(CH_2)_7CH_3$ analogues modified at Gal C2, C3, C4, C5, and C6, as well as at GlcNAc C2, as substrate [16,17,19].

2. Materials and methods

2.1. General

The synthesis of compounds **1**–**19** (Fig. 1) has been described earlier [16,17,19]. CMP-[^{14}C]-Neu5Ac was purchased from Amersham (Uppsala, Sweden) (11.15 TBq/mol) and Sep-Pak C_{18} cartridges from Waters (Milford, USA).

To express soluble, active and His_6-tagged forms of rat ST3Gal III, human ST3Gal IV, and human ST6Gal I, the N-terminal sequence including the cytoplasmic and transmembrane domains were eliminated and replaced by the peptide signal sequence of the viral gene ecdysone-S-glycosyltransferase (EGT) [23] and six His codons. Rat ST3Gal III (clone ST3N-1, [24]), human ST3Gal IV (GenBank accession number L23767, [25]), and human ST6Gal I (GenBank accession number X17247), were thus expressed without their first 34, 54, and 51 amino acids, respectively. Construction of recombinant baculoviruses in *Spodoptera frugiperda Sf-9* was achieved as described previously [21,26], and large-scale productions of soluble ST3Gal III, ST3Gal IV, and ST6Gal I were set in 400 mL roller bottles in serum free medium. Despite N-terminus His6-tagged enzymes, the conditioned culture media were directly used as the enzyme sources for sialyltransferase assays and kinetic parameter determinations without previous purification. The risk of activity loss during the Immobilized Metal-Ion Affinity Partitioning (IMAP) purification steps and the putative inhibitory effect of imidazole, as observed with FucT III [27], substantiated the choice of culture media over purified enzymes as enzymatic source in our assays. Large-scale production of soluble Flag-tagged ST6Gal II (GenBank accession number AJ512141), deleted of the first 33 amino acids, was performed by transient transfection of COS-7 cells as reported earlier [21], and the conditioned culture media were used as enzyme sources. Negative controls were performed using ST6Gal II-free media from COS-7 cells transfected with empty vector.

2.2. Acceptor specificity assays

Substrate specificities, using compounds **1**–**19** as acceptors, were determined for ST3Gal III, ST3Gal IV, ST6Gal I and ST6Gal II. Briefly, standard incubations were carried out with 1 mM of acceptor substrate, 0.1 M sodium cacodylate pH 6.2, 10 mM $MnCl_2$, 0.2% Triton-CF54, and 55 μM CMP-[^{14}C]-Neu5Ac (0.75 TBq/mol; 100 000 dpm), and 23 μL of the enzyme source in a final volume of 40 μL. Samples were kept for 2 h at 37 °C, then reactions were stopped by addition of 1 mL ice-cold water and products were isolated by hydrophobic chromatography on SepPak C_{18} cartridges as previously described [28]. Each measurement was performed at least in duplicate; controls without acceptor were used as blanks. Under such conditions, the enzymatic activity towards compound **1** corresponding to 100% have been calculated being 0.163 nmol/mL/min for ST3Gal III, 0.0007 nmol/mL/min for ST3Gal IV, 0.053 nmol/mL/min for ST6Gal I, and 0.019 nmol/mL/min for ST6Gal II, respectively.

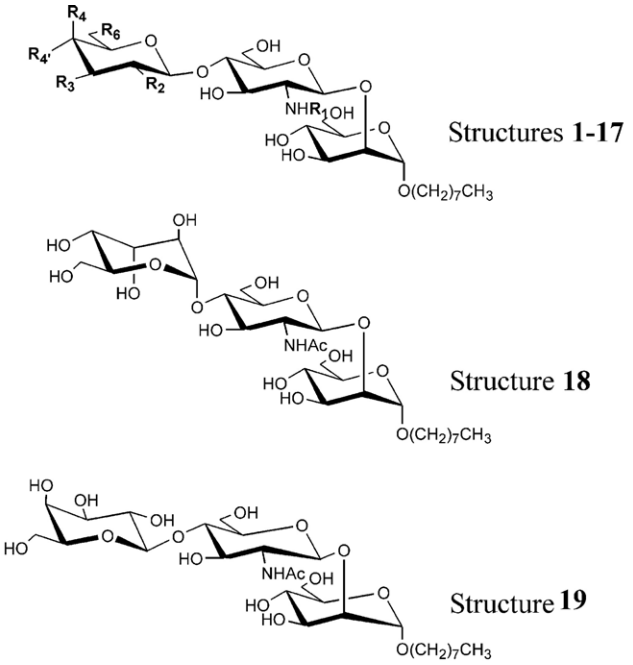
2.3. Determination of kinetic parameters

Kinetic parameters were determined for ST3Gal III and ST3Gal IV with compounds **1**–**4**, and for ST6Gal I and ST6Gal II with compounds **1**–**8**, following incubation conditions as described above, except that 10 μL instead of 23 μL of crude enzyme extract were used for ST3Gal III and ST6Gal I. A set of 10 points varying from 0 to 2 mM of each compound was used with ST3Gal III, and a set of 12 points varying from 0 to 5 mM with ST6Gal I. For ST6Gal II and ST3Gal IV, sets of 7 points varying from 0 to 2 mM were used. Assays were performed in triplicate for ST3Gal III and in duplicate for ST6Gal I, ST6Gal II, and ST3Gal IV. Due to low activity of the enzymatic extracts, incubation times for ST3Gal IV were set to 6 h and not 2 h, after checking the linearity of the enzymatic response. In the case of ST6Gal II, traces of endogenous ST activity from COS-7 cells were overcome by subtracting the relative transfer determined in blank media containing 0, 0.5, 1 and 2 mM of acceptors from the relative transfer determined in the ST6Gal II assays. Since the enzymes were used as crude enzyme fractions, the kinetic parameter K_m and V_{max} are apparent constants and are named app K_m and app V_{max}. These values were calculated by fitting the data to the Michaelis–Menten equation [13,18] using the software Graphpad Prism 4.

3. Results

3.1. Synthetic substrates for sialyltransferases

The structures of β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→O) $(CH_2)_7CH_3$ **1** and its analogues **2**–**19**, used in the ST studies, are summarized in Fig. 1. Hydroxyl functions of the terminal Gal residue were modified at C2 (**3**–**8**; OH→H, OH→NHAc, OH→NHPr), at C3 (**9**, **10**; OH→H, OH→F), at C4 (**11**–**13**; OH→H, OH→F, D-Gal→D-Glc), at C5 (**18**; D-Gal→L-Alt), or at C6 (**14**–**17**; OH→H, OH→NH_2), and D-Gal was replaced by L-Gal (**19**). Part of the series with modifications on the Gal unit contained an *N*-propionyl instead of an *N*-acetyl group in the subterminal GlcNAc residue (**2**, **4**, **6**, **8**, **15**, and **17**).



Code	Structure	R_1	R_2	R_3	R_4	$R_{4'}$	R_6
1	β-D-Gal-β-D-GlcNAc-R	Ac	OH	OH	OH	H	OH
2	β-D-Gal-β-D-GlcNPr-R	Pr	OH	OH	OH	H	OH
3	2-deoxy-β-D-Gal-β-D-GlcNAc-R	Ac	H	OH	OH	H	OH
4	2-deoxy-β-D-Gal-β-D-GlcNPr-R	Pr	H	OH	OH	H	OH
5	2-acetamido-2-deoxy-β-D-Gal-β-D-GlcNAc-R	Ac	NHAc	OH	OH	H	OH
6	2-acetamido-2-deoxy-β-D-Gal-β-D-GlcNPr-R	Pr	NHAc	OH	OH	H	OH
7	2-deoxy-2-propionamido-β-D-Gal-β-D-GlcNAc-R	Ac	NHPr	OH	OH	H	OH
8	2-deoxy-2-propionamido-β-D-Gal-β-D-GlcNPr-R	Pr	NHPr	OH	OH	H	OH
9	3-deoxy-β-D-Gal-β-D-GlcNAc-R	Ac	OH	H	OH	H	OH
10	3-deoxy-3-fluoro-β-D-Gal-β-D-GlcNAc-R	Ac	OH	F	OH	H	OH
11	4-deoxy-β-D-Gal-β-D-GlcNAc-R	Ac	OH	OH	H	H	OH
12	4-deoxy-4-fluoro-β-D-Gal-β-D-GlcNAc-R	Ac	OH	OH	F	H	OH
13	β-D-Glc-β-D-GlcNAc-R (4 epimer)	Ac	OH	OH	H	OH	OH
14	6-deoxy-β-D-Gal-β-D-GlcNAc-R	Ac	OH	OH	OH	H	H
15	6-deoxy-β-D-Gal-β-D-GlcNPr-R	Pr	OH	OH	OH	H	H
16	6-amino-6-deoxy-β-D-Gal-β-D-GlcNAc-R	Ac	OH	OH	OH	H	NH_2
17	6-amino-6-deoxy-β-D-Gal-β-D-GlcNPr-R	Pr	OH	OH	OH	H	NH_2
18	α-L-Alt-β-D-GlcNAc-R (5 epimer)	-	-	-	-	-	-
19	β-L-Gal-β-D-GlcNAc-R (enantiomer)	-	-	-	-	-	-

Fig. 1. Structures of tested synthetic trisaccharide octyl glycosides. The syntheses of these compounds have been described earlier [16,17,19]. R = (1 → 2)-α-D-Man*p*-(1 → O) $(CH_2)_7CH_3$.

3.2. Specificity of substrate for ST3Gal III, ST3Gal IV, ST6Gal I, and ST6Gal II

The acceptor specificities of the recombinant enzymes towards the various trisaccharide octyl glycosides, using CMP-[^{14}C]-Neu5Ac as donor, are presented in Table 1. The relative rate of transfer with non-modified compound **1** as acceptor was set to 100% for each enzyme to overcome the variations of transfer rate observed between the different sialyltransferases. As the results for ST3Gal III and ST6Gal

Table 1
Acceptor specificity of ST3Gal III, ST3Gal IV, ST6Gal I, and ST6Gal II with various acceptors derived from the structure β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→O)$(CH_2)_7CH_3$

Acceptor	ST3Gal III (%)	ST3Gal IV (%)	ST6Gal I (%)	ST6Gal II (%)
1 β-D-Gal-β-D-GlcNAc-R	100	100	100	100
2 β-D-Gal-β-D-GlcNPr-R	188	203	480	221
3 2-deoxy-β-D-Gal-β-D-GlcNAc-R	18	96	101	158
4 2-deoxy-β-D-Gal-β-D-GlcNPr-R	31	125	409	242
5 2-acetamido-2-deoxy-β-D-Gal-β-D-GlcNAc-R	<2	12.5	25	183
6 2-acetamido-2-deoxy-β-D-Gal-β-D-GlcNPr-R	<2	7.5	123	198
7 2-deoxy-2-propionamido-β-D-Gal-β-D-GlcNAc-R	<2	9.5	30	238
8 2-deoxy-2-propionamido-β-D-Gal-β-D-GlcNPr-R	2.8	7	144	358
9 3-deoxy-β-D-Gal-β-D-GlcNAc-R	*0*[a]	5	*13*[a]	7
10 3-deoxy-3-fluoro-β-D-Gal-β-D-GlcNAc-R	*0*[a]	7	*8*[a]	7
11 4-deoxy-β-D-Gal-β-D-GlcNAc-R	*2*[a]	5	*29*[a]	7
12 4-deoxy-4-fluoro-β-D-Gal-β-D-GlcNAc-R	*7*[a]	9	*17*[a]	<2
13 β-D-Glc-β-D-GlcNAc-R	*0*[a]	7	*5*[a]	<2
14 6-deoxy-β-D-Gal-β-D-GlcNAc-R	6	14	6	10
15 6-deoxy-β-D-Gal-β-D-GlcNPr-R	2.5	9	<2	<2
16 6-amino-6-deoxy-β-D-Gal-β-D-GlcNAc-R	2.5	18.5	<2	<2
17 6-amino-6-deoxy-β-D-Gal-β-D-GlcNPr-R	2.5	14	3	7
18 α-L-Alt-β-D-GlcNAc-R	*0*[a]	9	<1[a]	<2
19 β-L-Gal-β-D-GlcNAc-R	*0*[a]	10	*0*[a]	<2

Values are expressed as a percentage of the incorporation of Neu5Ac into the parent compound **1**, and are referred as relative rate of transfer. The enzymatic activity towards compound **1** corresponding to 100% for ST3Gal III, ST3 Gal IV, ST6Gal I and ST6Gal II were calculated to be 0.163, 0.0007, 0.053, and 0.019 nmol/mL/min, respectively. For ST3Gal III, ST6Gal I, and ST6Gal II, relative transfers <10% correspond to background activity. For ST3Gal IV, the relevance threshold is set at 20% due to the low enzymatic activity. R = (1→2)-α-D-Man*p*-(1→O)$(CH_2)_7CH_3$.

[a] Values reported in italic were determined in a previous study by van Dorst et al. [18].

I with compounds **9**–**13** and **18**–**19** have already been reported [18], these enzymes were only tested with acceptors **1**–**8** and **14**–**17**; ST3Gal IV and ST6Gal II were tested with compounds **1**–**19**. For more clarity, previous data reported by van Dorst et al. [18] have been included in Table 1.

3.2.1. ST3Gal III (sialylation of Gal HO3)

To be effective, investigations with **11**–**13**, **18**–**19**, and some other analogues (D-Gal→D-Gul; HO3→$NH_2$3) had demonstrated that ST3Gal III requires an intact OH group at Gal C3 (equatorially oriented), whereas Gal HO4 tolerates only a few modifications, e.g. methylation and to a lower extent fluoration [18]. Calculation of the specific activities of ST3Gal III for **2**–**8** and **14**–**17** show that sialylation is only observed for **2**–**4**; low noise activity (<6%) is detected for **5**–**8** and **14**–**17** (Table 1). It turned out that replacement of the GlcNAc *N*-acetyl group in **1** by an *N*-propionyl group (**2**) leads to an increase of the transfer rate by a factor of 1.8 (**1**→**2**, 100%→188%). Interestingly, although the presence of a deoxy function at Gal C2 reduces the enzyme activity by a factor of 5.5 (**1**→**3**, 100%→18%), indicating that this modification is tolerated to some extent, the replacement of the GlcNAc *N*-acetyl group in **3** by an *N*-propionyl group (**4**) gives rise to a similar increase of the transfer rate by a factor of 1.7 (**3**→**4**, 18%→31%; compared with **1**). An *N*-acetyl (**1**→**5/6**) or an *N*-propionyl (**1**→**7/8**) function at Gal C2 is not accepted by the enzyme. The importance of the Gal HO6 group is illustrated by the inactivity of **14**–**17**, being modified at Gal C6.

3.2.2. ST3Gal IV (sialylation of Gal HO3)

As the enzymatic activity of ST3Gal IV was very low, leading to a high background, transfer rates <20% were considered as negligible. As evidenced in Table 1, compounds modified at Gal C4 (**11**–**13**), C6 (**14**–**17**), C5 (**18**), or containing L-Gal (**19**) do not show relevant activity, suggesting that OH functions (with the right stereochemistry) at C3, C4, C5, and C6 are required for sialylation. Also an *N*-acetyl (**5**, **6**) or an *N*-propionyl (**7**, **8**) group at Gal C2 completely suppresses sialic acid transfer. However, reduction of Gal HO2 is tolerated without much influence on the enzyme activity (**1**→**3**, 100→96%). Interestingly, as found already for ST3Gal III, the replacement of subterminal GlcNAc by GlcNPr increases the transfer rate by a factor of 2.0 (**2**, 203%; **4**, 125%; compared with **1**).

3.2.3. ST6Gal I (sialylation of Gal HO6)

As reported earlier [18], hST6Gal I tolerates specific modifications of Gal HO3 (**9**, **10**; MeO3 or $NH_2$3 not accepted) and HO4 (**11**, **12**; MeO4 not accepted), but requires the right stereochemistry at Gal C-3 (D-Gul not accepted), C4 (**13** not accepted), C5 (**18** not accepted), and the right absolute configuration for Gal (**19** not accepted) (Table 1). In the present study, it has been found that Gal C6 amino analogues of **1** (**16**, **17**) are not sialylated; only background is recorded (values <7%). On the other hand, reduction of Gal HO2 in **1** is completely tolerated (**3**, 101%), whereas replacement of Gal HO2 by an NHAc (**5**, 25%) or an NHPr (**7**, 30%) group gives rise to a reduced rate of transfer. Finally, replacing the *N*-acetyl by an *N*-propionyl group in the GlcNAc unit of **1**, **3**, **5**, and **7**

turned out to have striking activating effects (**2**, 480%; **4**, 409%; **6**, 123%; **8**, 144%).

3.2.4. ST6Gal II (sialylation of Gal HO6)

Acceptor specificity studies with the recently cloned ST6Gal II [20–22] reveal that the OH functions at Gal C3 (**9**, **10**) and C4 (**11**, **12**), as well as the stereochemistry at Gal C4 (**13**) play an important role in the recognition by the enzyme; the introduced modifications lead to a complete loss of activity (background transfer rate measured until 10%). The same holds for the amination of Gal C6 (**16**, **17**), the epimerization of Gal HO5 (**18**) and the replacement of D-Gal by L-Gal (**19**). Interestingly, ST6Gal II shows a higher specificity when Gal HO2 is reduced (**3**, 158%; compared with **1**) or replaced by an NHAc (**5**, 183%) or an NHPr (**7**, 238%) group. Finally, replacement of subterminal GlcNAc by GlcNPr leads to an increase of the transfer rate by a factor of at least ~1.5 (**1**→**2**, 100%→221%; **3**→**4**, 158%→242%; **7**→**8**, 238→358%, with the exception of **5**→**6**, where a slightly smaller increase of ~1.1, 183%→198%, is observed).

3.3. Kinetic parameters

Kinetic parameters were calculated for ST3Gal with compounds **1**–**4** and for ST6Gal with compounds **1**–**8**. The results are summarized in Table 2 for ST3Gal and in Table 3 for ST6Gal. Calculations were performed using nonlinear fitting approach software [29].

3.3.1. ST3Gal III

The data obtained for ST3Gal III with the GlcNPr-containing compounds **2** and **4** reveal that the global increase of relative efficiency (app V_{max}/app K_m) results from a variation in the maximum velocity (app V_{max}) and the catalytic process itself, more than from a serious change of affinity of the enzyme for the substrate. The app V_{max} values increase 1.7 times going from **1** to **2** (0.68 and 1.22 nmol/mL/min, respectively) and 1.6 times going from **3** to **4** (0.021 and 0.034 nmol/mL/min, respectively), while the app K_m values remain in the same range for the same considered compounds (3.98 and 4.50 mM for **1** and **2**, respectively; 0.99 and 1.59 mM for **3** and **4**, respectively). Going from **1** to **3** (reduction of Gal HO2), the relative efficiency app V_{max}/app K_m of ST3Gal III drops 8 times. Here, the catalytic properties of the enzyme are mainly affected, as app V_{max} drops 32 times. Due to the low measured transfer rate of **3** compared to **1** (Table 1), the increase of affinity (app K_m) of 4 times going from **1** to **3**, is likely to be overestimated at the molecular level. However, this will not influence the conclusions, that modifications of the substrates rather change the catalytic properties of ST3Gal III (app V_{max}) than the affinity for the substrate. Even if modifications of the affinity (app K_m) cannot be excluded, it does not seem to be the main factor influencing the activity of the enzyme and therefore the efficiency of the reaction.

3.3.2. ST3Gal IV

When incubated with benzyl *N*-acetyl-β-lactosaminide, ST3Gal IV shows a substantial increase in the app V_{max}/app K_m value of **1** from 0.0035 to 0.0412. The presence of an (1→2)-α-D-Man*p*-(1→O) $(CH_2)_7CH_3$ unit at the reducing end of *N*-acetyl-β-lactosaminide instead of a benzyl group leads to a clear decrease in app V_{max} by a factor of 5 (0.014→0.0028 nmol/mL/min), whereas the app K_m values appear less affected. This finding may suggest that the enzyme has a preference for glycans differing from N-glycan antennae or that a distinct influence of the benzyl spacer is detected. Unfortunately, the large standard deviations resulting from the low activity of the enzyme prevent clear analysis of kinetic parameters when the *N*-acetyl group in the GlcNAc residue is replaced by an *N*-propionyl group (**1**→**2**) or when Gal HO2 is reduced (**1**→**3**). Considering the values of the kinetic parameters calculated for benzyl *N*-acetyl-β-lactosaminide, the use of unfavorable substrates rather than a defective level of expression may cause the reduced enzyme activities observed for **1**–**4**.

3.3.3. ST6Gal I

As shown above for ST6Gal I, replacement of the NHAc group of subterminal GlcNAc by an NHPr group has an activating effect on the reaction. Going from **1** to **2**, the relative enzymatic efficiency app V_{max}/app K_m is increased 7 times (0.016→0.112). Similar increases hold for **3** to **4** (0.019→0.094), **5** to **6** (0.004→0.026), and **7** to **8** (0.006→0.027). The app V_{max} values increase 1.5–3 times (0.063 and 0.21 nmol/mL/min for **1** and **2**, respectively; 0.099 and 0.15 nmol/mL/min for **3** and **4**, respectively; 0.034

Table 2
Kinetic parameters of ST3Gal III and ST3Gal IV for various synthetic acceptors derived from the structure β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→O)$(CH_2)_7CH_3$ and for benzyl *N*-acetyllactosaminide, β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→O)C_6H_5

Acceptor	ST3Gal III			ST3Gal IV		
	app K_m mM	app V_{max} nmol/mL/min	app V_{max}/app K_m	app K_m mM	app V_{max} nmol/mL/min	app V_{max}/app K_m
1 β-D-Gal-β-D-GlcNAc-R	3.98±1.6	0.68±0.2	0.171±0.013	0.81±0.44	0.0028±0.0006	0.0035±0.0024
2 β-D-Gal-β-D-GlcNPr-R	4.5±2.5	1.22±0.53	0.271±0.074	0.57±0.22	0.0035±0.0005	0.0061±0.0025
3 2-deoxy-β-D-Gal-β-D-GlcNAc-R	0.99±0.45	0.021±0.004	0.021±0.010	0.15±0.07	0.0025±0.0002	0.0167±0.0121
4 2-deoxy-β-D-Gal-β-D-GlcNPr-R	1.59±0.53	0.034±0.006	0.022±0.004	0.27±0.09	0.002±0.0002	0.0074±0.0025
β-D-Gal-β-D-GlcNAc-OBn	nd	nd	nd	0.34±0.16	0.014±0.002	0.0412±0.0254

nd, not determined in the present study.
R=(1→2)-α-D-Man*p*-(1→O)$(CH_2)_7CH_3$.

Table 3
Kinetic parameters of ST6Gal I and ST6Gal II for various synthetic acceptors derived from the structure β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→O)$(CH_2)_7CH_3$. R=(1→2)-α-D-Man*p*-(1→O)$(CH_2)_7CH_3$

Acceptor	ST6Gal I			ST6Gal II		
	app K_m mM	app V_{max} nmol/mL/min	app V_{max}/app K_m	app K_m mM	app V_{max} nmol/mL/min	app V_{max}/app K_m
1 β-D-Gal-β-D-GlcNAc-R	3.84±0.78	0.063±0.008	0.016±0.002	1.84±0.38	0.032±0.004	0.017±0.002
2 β-D-Gal-β-D-GlcNPr-R	1.88±0.3	0.21±0.02	0.112±0.008	1.4±0.3	0.06±0.007	0.043±0.005
3 2-deoxy-β-D-Gal-β-D-GlcNAc-R	5.13±1.4	0.099±0.018	0.019±0.003	0.48±0.03	0.032±0.001	0.067±0.004
4 2-deoxy-β-D-Gal-β-D-GlcNPr-R	1.59±0.29	0.15±0.01	0.094±0.014	0.67±0.07	0.064±0.003	0.096±0.009
5 2-acetamido-2-deoxy-β-D-Gal-β-D-GlcNAc-R	8.65±1.7	0.034±0.005	0.004±0.0003	1.12±0.69	0.043±0.013	0.038±0.032
6 2-acetamido-2-deoxy-β-D-Gal-β-D-GlcNPr-R	2.99±0.6	0.071±0.008	0.024±0.002	0.76±0.23	0.052±0.006	0.068±0.019
7 2-deoxy-2-propionamido-β-D-Gal-β-D-GlcNAc-R	6.99±1.6	0.042±0.006	0.006±0.0004	0.7±0.16	0.06±0.005	0.086±0.016
8 2-deoxy-2-propionamido-β-D-Gal-β-D-GlcNPr-R	3.63±0.84	0.096±0.016	0.026±0.003	0.5±0.09	0.08±0.005	0.16±0.023

and 0.071 nmol/mL/min for **5** and **6**, respectively; 0.042 and 0.096 nmol/mL/min for **7** and **8**, respectively), while the app K_m values decrease 2–3 times (3.84 and 1.88 mM for **1** and **2**; 5.13 and 1.59 mM for **3** and **4**; 8.65 and 2.99 mM for **5** and **6**; 6.99 and 3.63 mM for **7** and **8**). Thus, the increase of the activity observed by *N*-propionylation of the subterminal residue results from a combined increase of affinity (app K_m) and catalytic properties (app V_{max}). When Gal is replaced by GalNAc or GalNPr, the app V_{max}/app K_m values decrease 3–4 times (0.016, 0.004 and 0.006 for **1**, **5**, and **7**, respectively). Here, the app V_{max} values also decrease (0.063, 0.034 and 0.042 nmol/mL/min for **1**, **5** and **7**, respectively) and the app K_m values increase (3.84, 8.65 and 6.99 mM for **1**, **5** and **7**, respectively).

3.3.4. ST6Gal II

Also for ST6Gal II, an activating effect on the Neu5Ac transfer is observed when replacing the *N*-acetyl group of GlcNAc by an *N*-propionyl group, but the influence is lower than for ST6Gal I. Going from **1** to **2**, a 2.5-increase is observed for the app V_{max}/app K_m value (0.017→0.043). Similar findings hold for **3** to **4** (0.067→0.096), **5** to **6** (0.038→0.068), and **7** to **8** (0.086→0.16). In the presence of the *N*-propionyl group, the app V_{max} values are multiplied by ~1.5–2 (**1** → **2**, 0.032→0.06; **3** → **4**, 0.032→0.064; **5** → **6**, 0.043→0.052; and **7** → **8**, 0.06→0.08), but the app K_m values do not undergo significant changes per set. Replacing the Gal residue by a GalNAc residue (**1**→**5**) results in an increase of the relative enzymatic efficiency app V_{max}/app K_m (0.017→0.038); the app V_{max} value increases, whereas the app K_m values hardly vary. Consequently, the increased activity of ST6Gal II towards *N*-propionylation (GlcNAc→GlcNPr) and *N*-acetylation (Gal→GalNAc) originates mostly from an enhanced catalytic process more than any changes in the affinity of the enzymes for its substrate.

4. Discussion

In this study, the substrate specificities of four sialyltransferases belonging either to the ST3Gal family or to the ST6Gal family were investigated. Different structural variants of β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→O)$(CH_2)_7CH_3$, having modifications at C2, C3, C4, C5, and/or C6 of the terminal Gal unit and modifications at C2 of the subterminal GlcNAc residue [16,17,19], were used as potential substrate for ST3Gal III, ST3Gal IV, ST6Gal I or ST6Gal II. Because of the extensive literature published on the rat liver ST3Gal III acceptor specificity [9,13,18,24] and the non-availability of the human clone during our studies, a rat ortholog of the enzyme was preferred. The earlier choice of mimicking trisaccharide β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→O)R elements of N-glycan antennae [16–18] instead of only disaccharide β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→O)R elements [13] was based on the observation that the hydrophobicity of aglycons is an important kinetic factor when transferring Neu5Ac to Gal. Furthermore, the possibility that sialyltransferases recognize more extended parts of the native acceptors cannot be excluded so far.

The most striking results of the present investigation are the activating effects of replacing the *N*-acetyl group by an *N*-propionyl group on the subterminal GlcNAc unit in type II N-glycan like mimics, found for all four ST enzymes tested, despite their subfamily or despite their substrate specificity. This finding was reported earlier when comparing the disaccharides β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→O) $(CH_2)_8COOCH_3$ and β-D-Gal*p*-(1→4)-β-D-Glc*p*NPr-(1→O) $(CH_2)_8COOCH_3$ [13], using rat liver ST3Gal and ST6Gal. It was suggested that the methyl group of the *N*-acetyl function is likely to be involved in some hydrophobic interaction, being enhanced in the *N*-propionyl analogue. Our study demonstrates that this seems to be a general feature, giving even possibilities to repair a reduced acceptor specificity, decreased by replacements in the Gal substitution pattern.

The acceptor specificities of both ST3Gal III and ST3Gal IV were shown to be very similar for all studied N-glycan like type II trisaccharide octyl glycosides. Considering the substrate specificities and the tissue specific expression of ST3Gal enzymes [30], it can be reminded that ST3Gal III accepts preferentially type I (β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc) over type II structures, while ST3Gal IV is active on both type II and type III (β-D-Gal*p*-(1→3)-β-D-Gal*p*NAc) glycans [25]. For α2–3 sialylation, Gal C3 needs an OH group, whereas for Gal HO2 and Gal HO4 most of the tested modifications were not tolerated, and the tested modifications for Gal HO5 and HO6

were not tolerated at all [18, this study]. The Gal HO4 function was shown to be a hydrogen-bond acceptor [18]. The requirement of an intact D-*galacto*-3,4,6-triol system for optimal transfer is in accordance with earlier work by Wlasichuk et al. [13] on disaccharide mimics using rat liver ST3Gal. However, our studies show that reduction of Gal HO2 is accepted by ST3Gal IV, but to a much lower degree by ST3Gal III. The kinetic data collected for both ST3Gal III and ST3Gal IV suggest that the variations influence much more the catalytic process than the binding of the substrates, as the app K_m values are relatively unaffected compared with the app V_{max} values. The use of a linear-fitting "Eadie–Hofstee" plot based-approach in the work of Wlasichuk et al. [13] can explain why both app K_m and app V_{max} vary in the determination of the kinetic parameters of rat liver ST3Gal III. In the compelling question about the ST3Gal IV function, the negative effect of an (α1–2)-linked Man at the reducing site of *N*-acetyllactosamine strongly suggests that this enzyme might not be involved in the N-glycan processing, but rather in the O-glycan or glycolipid biosynthesis. Nevertheless, more tests on O-glycan and glycolipid analogues are required to ascertain the physiological role of ST3Gal IV.

The acceptor specificities of ST6Gal I and ST6Gal II showed interesting differences for the studied trisaccharide octyl glycosides. For ST6Gal I, an OH group at Gal C6 is required; the positions C2, C3, and C4 of Gal tolerate some modifications [18, this study], whereby deoxygenation at C2 has no influence at all (this study). Based on the studied analogues, it could be shown that both Gal HO3 and HO4 contribute importantly to the hydrogen bonding in the active site, being hydrogen-bond donating functions [18]. No real contradictions with earlier work by Wlasichuk et al. [13] on disaccharide mimics using rat liver ST6Gal, showing the importance of Gal HO6 together with GlcNAc NHAc2, were detected. Previously, the influence of the substitution of GlcNAc C6 has been reported [13]. Using rat liver ST6Gal and a series of mimics of Gal(β1–4)GlcNAc(β1-OMe, replacement of Gal by GalNAc (C2), Glc (C4), GlcNAc (C2 and C4), and Man (C2 and C4) reduced the activities to 48%, 16%, 8%, and 4%, respectively [12]. The recently cloned ST6Gal II shows much narrower acceptor specificity, as besides the required OH group at Gal C6, only the modifications of the substitution at Gal C2 are accepted. Kinetic parameters of ST6Gal I revealed that both the binding of the substrate and the catalytic process are influenced, as both app V_{max} and app K_m are affected [18, this study], whereas for ST6Gal II, the app K_m values are relatively unaffected compared with the app V_{max} values. As mentioned above for rat liver ST3Gal III, the variations with the kinetic parameters reported by Wlasichuk et al. [13] for rat liver ST6Gal I, being mainly in the substrate affinity app K_m changes, might result from the difference in the calculation method used.

Both α-Neu5Ac-(2→6)-β-D-Gal*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→R and α-Neu5Ac-(2→3)-β-D-Gal*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→R motifs are naturally occurring elements of animal glycoconjugate glycans. Typical examples of the (α2–6)-sialylation are found in human lutropin [31], Bowes melanoma tissue plasminogen activator [32], human urokinase [33], bovine mammary gland derived glycoproteins [34–36], and recombinant human protein C expressed in human kidney 293 cells [37]. Typical examples of the (α2–3)-sialylation are shown for a thrombin-like serine protease, ancrod, from the viper *Agkistrodon rhodostoma* [38] and a thrombin-like enzyme, batroxobin, from the snake *Bothrops atrox moojeris* [39]. The present studies showed decreased acceptor specificity for ST6Gal I in case of replacing Gal by GalNAc, but strongly increased acceptor specificity for STGal II. In accordance with this observation and the activating effect of the *N*-propionylation, it might be interesting to test the effect of a free amino function at GlcNAc C2. Under such conditions, ST6Gal II should present decreased acceptor specificity. The increased activity of ST6Gal II for LacdiNAc structures raises the question about the native function of this ST6, being probably a ST6GalNAc enzyme. This is in agreement with the substrate specificity of the recently cloned *Drosophila melanogaster* α-2,6-sialyltransferase, which also prefers the LacdiNAc disaccharide motif over *N*-acetyllactosamine [40]. The *D. melanogaster* gene appears to be orthologous to the ancestor present before the split of ST6Gal I and II subfamilies, and ST6Gal II could have maintained the ancestral function [41]. So far, the expression of ST6Gal II mRNA has been reported to occur in pituitary gland [21], as well as in fetal brain, small intestine, and colon [22]. In this context, the synthesis of analogues especially active for one of the two ST6Gal enzymes could help in the discrimination of either ST6Gal I or ST6Gal II activity.

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