A New Assay For Sialyltransferases Using Fluorescein-Labelled Acceptors

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A novel HPLC assay system for sialyltransferase activity based on the use of fluorescein-labelled acceptor oligosaccharides is described. The fluorescein-labelled disaccharides βGal-(1→4)-βGlc-OR (**4**), βGal-(1→4)-βGlcNAc-OR (**17**), and βGal-(1→3)-βGlcNAc-OR (**22**) where OR consists of a six carbon spacer with fluorescein attached, were synthesised. Synthetic standard products were produced chemo-enzymatically on a preparative scale to yield fluorescein-labelled trisaccharides. The use of reverse phase HPLC with an ion-pairing agent allowed the separation of starting materials from products and separation of the two isomeric trisaccharides αNeu5Ac-(2→3/6)-βGal-(1→4)-βGlcNAc-OR (**24** and **25**), so that the assay could be used to measure the different silalyltransferase activities in a mixture. The assay was successfully applied to the detection of sialyltransferase activity of commercially available enzymes and a crude preparation of bovine colostrum. The predominant sialyltransferase activity in bovine colostrum adds sialic acid α(2-6) to **17**.

Sialyltransferases belong to the family of glycosyltransferases which are responsible for the biosynthesis of the carbohydrate components in glycoproteins and glycolipids[1]. Glycosyltransferases transfer nucleotide-activated monosaccharides to sugar residues already bound to a protein or lipid moiety, or linked to an artificial spacer which resembles these moieties[2]. There are a number of different sialyltransferases from different sources that differ in their acceptor specificities, but all use cytidine monophosphate (CMP)-activated *N*-acetylneuraminic acid (Neu5Ac) as the donor substrate[3]. These enzymes are becoming increasingly important as analytical tools[4] as well as catalysts in the syntheses of complex oligosaccharides[5], and considerable effort has been directed at developing simple, reliable and inexpensive assays for them. Common assays for sialyltransferases rely upon monitoring the fate of the donor CMP-Neu5Ac by using radioactive[6] or fluorescently labelled Neu5Ac[7], or detecting the equimolar amount of liberated CMP[8]. A new assay based on isolation of the sialylated product, its acid hydrolysis and measurement of free Neu5Ac released with a colourimetric assay was reported

Our intention in carrying out this work was a reinvestigation of the sialyltransferases present in bovine colostrum, a cheap and readily available source of glycosyltransferases. To our knowledge the only sialyltransferase isolated from colostrum so far is an α2,6-sialyltransferase (α2,6-ST)[6,10]. However, early studies by Bartholomew et al.[11] on partially purified bovine colostrum showed evidence of formation both α(2 → 6)- and α(2 → 3)-sialylated lactose. No α(2 → 3) activity was detected for the purified α2,6-ST isolated from bovine colostrum[3,6,10]. We reasoned that a putative bovine α2,3-sialyltransferase could have been lost during purification of the other enzyme. We therefore required an assay system that was able to detect small amounts of sialyltransferase activity in crude protein preparations and that could differentiate between sialyltransferases. We chose to follow the course of the reaction using labelled acceptors which would allow the detection of sialyltransferase activity towards particular acceptors and could differentiate between enzymes with different acceptor specificities. This strategy has already been shown to be successful for galactosyl-[12] and fucosyltransferases[13].

Furthermore, every other glycosyltransferase that uses the same acceptor could be detected by simply changing the added nucleotide-activated donor. A set of glycosyltransferases could be detected one after the other by enzymatically converting the original labelled acceptor into other labelled product that would act as an acceptor for another glycosyltransferase. Thus detection of a sequence of coupled glycosyltransferases, as would be involved in the biosynthetic construction of complex oligosaccharides, would become possible by using just one labelled acceptor and adding the corresponding activated donors in quantitative amounts.

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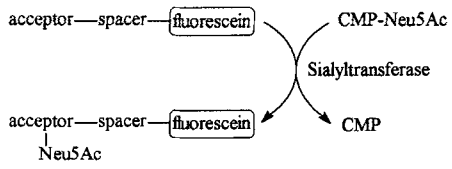
Results and Discussion

In order to establish our new assay method we chose to synthesize the known acceptor structures for the well studied α2,3- and α2,6-sialyltransferases (α2,3-ST, E.C. 2.4.99.6 and α2,6-ST, E.C. 2.4.99.1) isolated from rat liver[14]. These would then be evaluated as acceptor substrates for the enzymes in bovine colostrum which may not have the same acceptor specificities as the membrane-bound rat liver transferases.

Therefore the first targets for our chemical syntheses were fluorescein-labelled *N*-acetyllactosamine (LacNAc), an acceptor used by all three sialyltransferases, as well as lactose, an acceptor for the α2,6-ST from bovine colostrum[6] which is still utilized by the rat liver α2,6-ST and α2,3-ST[15] as shown in Scheme 1. We also aimed to synthesise a labelled version of the disaccharide βGal-(1 → 3)-βGlcNAc-OR because it is only utilized by the α2,3-ST from rat liver and not recognised by either of the two α2,6-ST enzymes, but may well be an acceptor for the putative α2,3-ST from colostrum.

Scheme 1



acceptor	sialyltransferases from rat liver	
	α2,6-ST	α2,3-ST
βGal-(1-4)- βGlc-OR	~	~
βGal-(1-4)-βGlcNAc-OR	+	+
βGal-(1-3)- βGlcNAc-OR	–	+

(+) good substrate, (~) poor substrate, (-) not a substrate

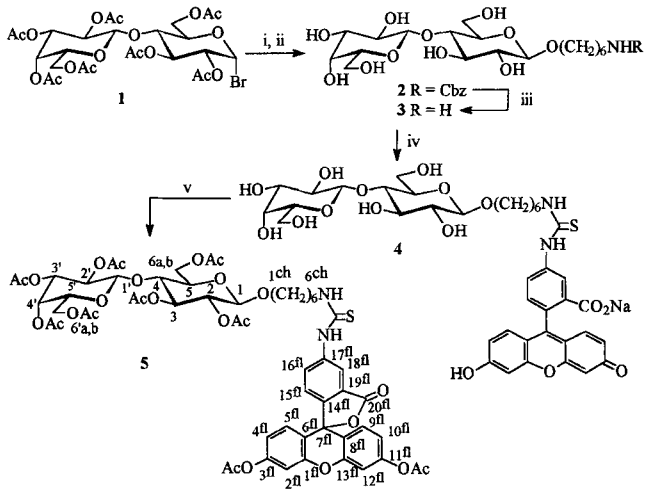
We chose to use fluorescein labelling of our acceptors. Successful application of Brossmer's assay system for sialyltransferases using fluorescein-labelled CMP-Neu5Ac[7] as a donor had demonstrated that the thiourea-linked fluorescein group was compatible with standard incubation conditions in terms of chemical stability, and provided satisfactory detection sensitivity. Labelling with fluorescein is usually performed by allowing a free amino group on the compound to be labelled to react with commercially available fluorescein isothiocyanate (isomer I) with formation of a new thiourea group[16].

The synthesis of the comparable rhodamine-labelled LacNAc, which was described in the recently published fucosyltransferase assay[13], involves first treating [8-(methoxycarbonyl)octyl]-βLacNAc with 1,2-diaminoethane and then coupling the remaining free amino group with isothiocyanate-activated rhodamine. To avoid a second coupling step we decided to use a hexanolamine spacer instead. Recent publications have demonstrated the suitability of oligosaccharides glycosidically bound to hexanolamine spacers regarding recognition and binding to glycosyltransferases[17] as well as selectin binding sites[18] in that such binding is not altered by the spacer. Furthermore, the three disaccharides **3**, **16**, and **21** are all described in literature[19,20].

Chemical Synthesis

The free amino group on the spacer arm is usually protected as a benzylcarbamate[20] or masked as an azido group[21] and is revealed only for the final coupling step. Because 6-[(benzyloxycarbonyl)amino]-1-hexanol is easily accessible[22] we choose to follow the first approach. Synthesis of (6-aminohexyl)-β-lactoside **3**, which was earlier described by using 6-(trichloroacetamido)-1-hexanol[19], was carried out by the reaction of 6-[(benzyloxycarbonyl)amino]-1-hexanol with hepta *O*-acetyl-α-lactosyl bromide[25], as depicted in Scheme 2, with silver triflate as catalyst in the presence of anhydrous disodium hydrogenphosphate to avoid orthoester formation[24]. The crude material obtained by flash chromatography was still contaminated with 6-[(benzyloxycarbonyl)amino]-1-hexanol but was subjected to deacetylation to yield pure **2** in a yield of 64% (from α-bromide **1**) by direct crystallisation from methanol. Quantitative hydrogenenolytic deprotection of **2** furnished **3** which in turn was treated without any further purification with fluorescein isothiocyanate in water/acetone and an equimolar amount of sodium carbonate (pH ~ 9). Unreacted or hydrolysed fluorescein isothiocyanate was removed by size exclusion chromatography on Sephadex LH20[25] using methanol/chloroform as eluant to give **4** in 89% yield. Later HPLC studies showed this compound to contain minor impurities (~5%) due to impure starting fluorescein (Aldrich specification: 90%) and/or light-induced decomposition during workup procedures. Chromatography on LiChroprep Rp 18 (Merck) provided **4** in >99% purity. For further characterisation and NMR signal assignment, a sample of **4** was peracetylated to give **5**.

Scheme 2. i) AgOTf, Na_2HPO_4, 2 equiv. of $HO(CH_2)_6NHCbz$, CH_2Cl_2 dry, 2 h, dark; ii) NaOMe, MeOH, overall i and ii 64%; iii) H_2, $Pd(OH)_2$ on charcoal, MeOH/H_2O (2:1, v/v), 98%; iv) 1.5 equiv. fluorescin isothiocyanate, Na_2CO_3, H_2O/acetone (2:1, v/v), 18 h, dark, 72%; v) Ac_2O, Py, 18 h, 74%

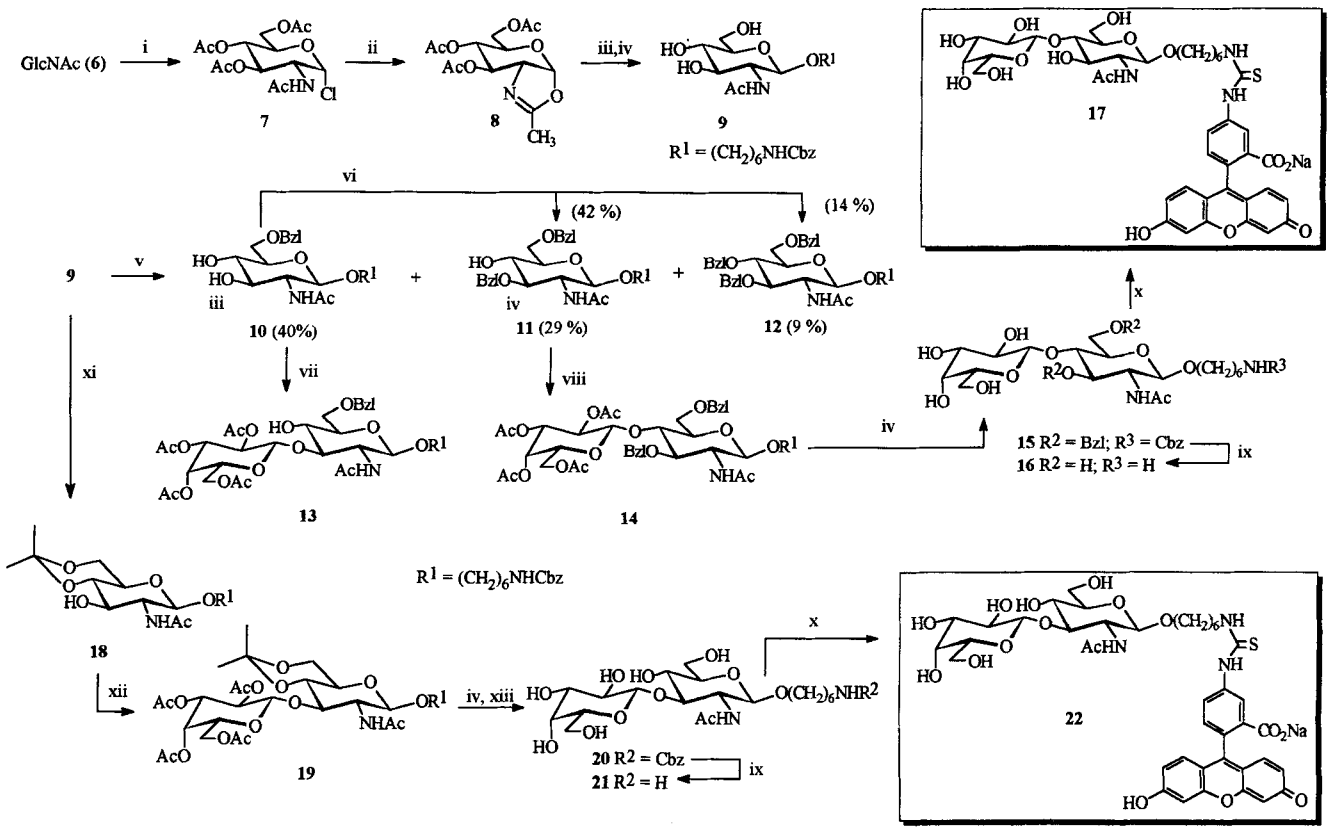


The *N*-acetylglucosamine (GlcNAc) β-glycoside **9** was prepared following the oxazoline route[26] starting with α-chloride **7**[27] which can be almost quantitatively converted to oxazoline **8**[28]. Reaction of **8** with 6-[(benzyloxycarbonyl)amino]-1-hexanol using camphor sulfonic acid as promotor[29] followed by deacetylation and crystallisation afforded pure **9** in 25% overall yield from **6** without involving any chromatographic purification[30]. Lee and co-workers reported partial benzylation of **9** to give the 3,6-di-, 3,4-di-, and 3,4,6-tribenzyl ethers in 43, 9, and 16% yield[20]. Under slightly modified conditions we obtained 6-mono, 3,6-di, and 3,4,6-tribenzyl ethers **10**, **11**, and **12** in 40, 29, and 9% yield, respectively. Our major product monobenzyl ether **10** could be subjected either to a second selective benzylation to yield another 43% of the desired 3,6-dibenzylated **11** or to galactosylation with peracetylated α-galactosyl trichloroacetimidate[31] in dichloromethane under boron trifluoride catalysis as shown in Scheme 3. The latter reaction led almost exclusively to the formation of the β(1 → 3)-linked disaccharide **13** in 46% yield. Traces of another disaccharide, most likely β(1 → 4)-linked, could be seen on TLC but not isolated in pure form. The predominance of the β(1 → 3)-linkage formation and low overall yield can be compared to those achieved with the corresponding 2-azido sugar as acceptor, where a 3:1 mixture of β(1 → 3)- and β(1 → 4)-linked products was obtained in a total of 86% yield, as described by Schmidt and co-workers[32]. Presumably the *N*-acetyl group lowers the reactivity of the acceptor leading to increased selectivity but lower yields. A higher yielding synthesis of β(1 → 3)-linked compounds is described below and was used for the preparation of the labelled acceptor **22**, but it should be noted that **13** has the ability to be modified at the 4-position of the GlcNAc residue, e.g. fucosylated, without any further manipulations.

The 3,6-dibenzylated GlcNAc derivative **11** was allowed to react with peracetylated α-galactosyl trichloroacetimidate to give 64% of the known LacNAc derivative **14**[20]. *O*- and *N*-deprotection led to **16**, which could be coupled with fluorescein isothiocyanate to yield **17** in 94% yield.

An alternative route towards the β(1 → 3)-linked disaccharide **22** used the approach published by Lee and co-workers[20] starting with isopropylidenation of **9** to give 4,6 blocked glycoside **18**. Boron trifluoride-catalysed galactosylation with peracetylated α-galactosyl trichloroacetimidate

Scheme 3. i) AcCl, 18 h; ii) NBu_4Cl, Na_2CO_3, molecular sieves 4 Å, CH_3CN dry, 2 h, 60 °C; iii) 1.5 equiv. of $HO(CH_2)_6NHCbz$, CSA, $C_2H_6Cl_2$ dry, 4 h, 60 °C; iv) NaOMe/MeOH, 18 h; overall yield i) to iv): 25%; (iv: with **14**: 84% of **15**); v) BaO, $Ba(OH)_2$, 4 equiv. of BnBr, DMF, 24 h, 0 °C; vi) BaO, $Ba(OH)_2$, 2 equiv. of BnBr, DMF, 24 h, 0 °C; vii) 1.2 equiv. of 2,3,4,6-tetra-*O*-acetyl-α-D-galactosyl trichloroacetimidate, BF_3, CH_2Cl_2 dry, 6 h, room temp., 46%; viii) 2 equiv. of 2,3,4,6-tetra-*O*-acetyl-α-D-galactosyl trichloroacetimidate, BF_3, CH_2Cl_2 dry, 50 h, 64%; ix) H_2, $Pd(OH)_2$-C, MeOH/H_2O (5:1, v/v), 4 h, 93% (of **15**) and 98% (of **21**); x) 1.5 equiv. fluorescein isothiocyanate Na_2CO_3, H_2O/acetone (2:1, v/v), 18 h, dark, 94% (for **17**) and 88% (for **22**); xi) 2,2-dimethoxypropane, DMF dry, molecular sieves 4 Å, *p*-TosOH, 12 h, room temp., 92%; xii) 1.5 equiv. of 2,3,4,6-tetra-*O*-acetyl-α-D-galactosyl trichloroacetimidate, BF_3, CH_2Cl_2 dry, 2 h, 76%; xiii) Amberlite IR 120 H^+ MeOH, 18 h, overall iv) and xiii) gives 92% of **20**



led to the formation of **19** in 76% yield. By altering the deprotection procedure to deacetylation first, followed by acid-catalysed acetal cleavage involving Amberlite IR 120 H^+ exchange resin, we obtained **20** in 92% overall yield. This was then subjected to hydrogenolytic deprotection of the terminal amino group and then coupling with fluorescein isothiocyanate to yield the fluorescein-labelled βGal-(1 → 3)-βGlcNAc-OR derivative **22** in 88% yield.

Enzymatic Studies

All three fluorescein-labelled disaccharides **4**, **17**, and **22** were tested for substrate recognition by the enzymes α2,6-ST (E.C. 2.4.99.1) and α2,3-ST (E.C. 2.4.99.6) isolated from rat liver.

Kinetic investigation gave the kinetic parameters for the labelled disaccharides which are compared to earlier published values[15] for the corresponding disaccharides, either as simple β-methyl glycosides or β-linked to an octylcarbonyl spacer arm (Table 1). All our labelled substrates showed as good or even slightly better acceptor capacities for one or both of the sialyltransferases as evidenced by lower K_m and relative V_{max} values. The presence of the fluorescein label does not reduce enzyme activity under normal incubation conditions (substrate concentrations around 0.2 mM), although substrate inhibition of the enzymes was detected at substrate concentrations higher than 0.2–3 mmol/l, which led to high error margins for theK_m of substrate **4**. The kinetic parameters for the lactose derivative **4** showed surprisingly good acceptor capacity for α2,6-ST but very poor acceptance by α2,3-ST. The relative turn-over rates of **4** with α2,3-ST were about 30% compared to a 100% for the octylcarbonyl-β-lactose compound listed in Table 1. Although this phenomenon might permit the use of **4** in a determination of which kind of sialyltransferase activity is present in an enzyme source, the comparatively weak interaction of **4** with α2,6-ST would result in an assay with poor sensitivity.

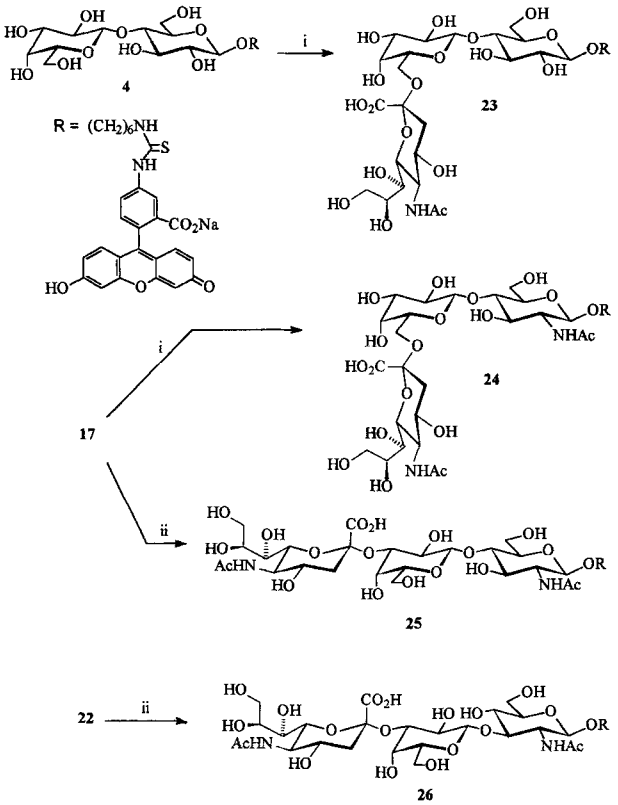
Table 1. Kinetic parameters for substrate recognition by the enzymes α2,6-ST and α2,3-ST. R^1 = $(CH_2)_6NHC(S)NH$-fluorescein; R^2 = $(CH_2)_8CO_2Me$

Acceptor	2,6-Sialyltransferase		2,3-Sialyltransferase	
	K_m (μmol/l)	V_{max} (rel. [a])	K_m (μmol/l)	V_{max} (rel. [a])
βGal-(1-4)-βGlc-OR^1 (**4**)	5700 ± 1368	0.59		
βGal-(1-4)-βGlc-OR^2 [16]			4300 ± 750	1.9
βGal-(1-4)-βGlcNAc-OR^1 (**17**)	157 ± 11	0.46	403 ± 58	0.58
βGal-(1-4)-βGlcNAc-OMe [16]	1660 ± 60	1.3	5370 ± 650	0.3
βGal-(1-4)-βGlcNAc-OR^2 [16]	400 ± 50	1.0	900 ± 270	0.3
βGal-(1-3)-βGlcNAc-OR^1 (**22**)	not a substrate		93 ± 11	0.47
βGal-(1-3)-βGlcNAc-OMe [16]	not a substrate		540 ± 40	2.1
βGal-(1-3)-βGlcNAc-OR^2 [16]	not a substrate		110 ± 10	1.0

[a] Relative rates are set for 2,6-ST at 1.0 for substrate βGal-(1-4)-βGlcNAc-OR^2 and for 2,3-ST at 1.0 for substrate βGal-(1-3)-βGlcNAc-OR^2.

To validate our HPLC-based assay system we required standard samples of starting materials and expected products. We thus undertook the preparative-scale chemo-enzymatic synthesis of the four products **23**–**26** that result from sialylation of **4**, **17**, and **22** by the known rat liver enzymes. The chemo-enzymatic synthesis of structures such as αNeu5Ac-(2 → 3/6)-βGal-(1 → 3/4)-βGlc(NAc)-OR utilizing sialyltransferases has been shown to be a very successful approach starting from free or spacer-linked disaccharides[33], or even from monosaccharides, involving additional galactosyltransferase[34] or galactosidase[35] enzymes. In our case, incubation of **4** and **17** with α2,6-ST and **17** and **22** with α2,3-ST led to the formation of the four sialylated compounds **23**–**26** as outlined in Scheme 4.

Scheme 4. i) CMP-Neu5Ac, 2,6-sialyltransferase (rat liver), alkaline phosphatase, cacodylate buffer system pH 7.0, 22 °C, 75% of **23** (after 5 d) and 67% of **24** (after 9 d); ii) CMP-Neu5Ac, 2,3-sialyltransferase (rat liver), alkaline phosphatase, cacodylate buffer system pH 7.0, 22 °C, 51% of **25** (after 10 d) and 56% of **26** (after 5 d)



The reactions were carried out at 22 °C in cacodylate buffer at pH 7.0 containing bovine serum albumin and Triton CF 54 detergent to optimise enzyme stability and alkaline phosphatase to remove liberated CMP which would otherwise inhibit the enzymatic reaction. Product isolation started, as earlier described for fucosyltransferase-catalysed reactions[36], by loading the crude incubation mixtures onto C-18 Sep-pak cartridges and eluting proteins and buffer salts with water. More hydrophobic components such as fluorescein-labelled compounds as well as the detergent were initially retained and could be eluted later with aceto-

nitrile/water. The detergent was removed by chromatography on Iatro beads, and products **23**–**26** were finally purified on LiChroprep Rp 18 with 10% acetonitrile in water. All compounds were identified by comparison of proton-NMR spectroscopic data with published data for the corresponding unlabelled trisaccharides[33]. For the α(2 → 3)-sialylated compounds **25** and **26** the α(2 → 3) linkage was assigned by the downfield shifts for the protons in 3′ and 4′ positions on the galactosyl ring at, and next to, the linkage position of the sialic acid[33].

HPLC on a reversed-phase column showed each of the resultant products **23**–**26** to be one single fluorescein-labelled component that was eluted earlier than the corresponding starting disaccharides **4**, **17**, and **22**. Addition of the ion-paring agent tetrabutylammonium hydrogensulfate[37] shifted all peaks for the sialylated compounds **23**–**26** to longer retention times than those for the corresponding non-sialylated **4**, **17**, and **22**.

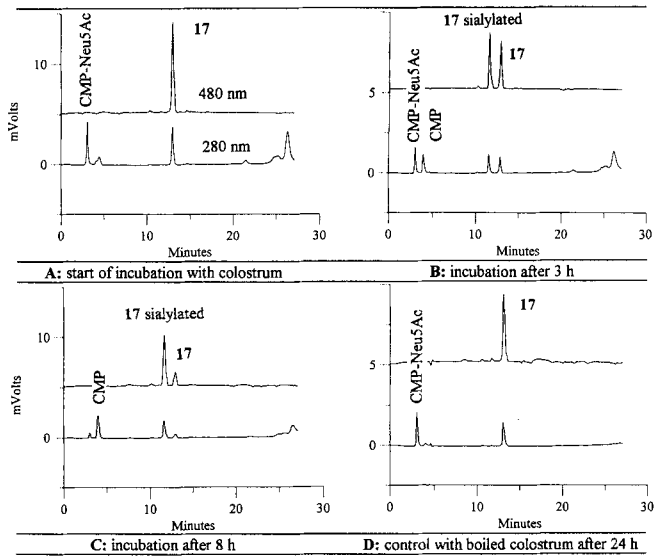
HPLC-Supported Assay

The known assay systems for sialyltransferase are all based on determination of the concentration of one or two incubation components either by coupling the transferase reaction to a selective enzymatic or chemical transformation of one component that allows quantification[8,9] or by separation of starting donor and acceptors from products and counting the production of labelled product[6,7]. Brossmer and co-workers[7] used HPLC separation on a size exclusion matrix for quantifying the transfer of their labelled CMP-Neu5Ac donor to the asialo-α_1-acid glycoprotein acceptor. The much lower molecular masses of our acceptors and products permitted us to conduct the HPLC separation on reversed-phase materials.

For our initial purpose, detection of sialyltransferase activity in crude colostrum preparations, detection by a UV-Vis diode array detector system was found to be sensitive enough to detect concentrations of 1–10 μmol/l of fluorescein-labelled materials, due to the strong and very specific absorption of the fluorescein residue at 480 nm in neutral or slightly alkaline medium. The advantage of using a diode array, rather than a fluorescence detector system, which might lead to some increase in sensitivity, is that the consumption of donor CMP-Neu5Ac could be observed simultaneously by monitoring at its absorption at 280 nm. Thus for the first time the changes in concentrations for all components of the enzymatic reaction could be detected at the same time.

To show the applicability of our new assay system, we first carried out tests with commercial α2,6-ST from rat liver. Incubation of acceptors **4**, **17**, and **22** with commercial α2,6-ST led to complete conversion of **17** to a product with a retention time identical to that of **24**, only slight reaction of **4** to **23** and, as expected, no reaction for **22** even after 24 h. Similar results were achieved with a crude preparation of colostrum, which was centrifuged and dialysed against the incubation buffer. The LacNAc derivative **17** incubated with dialysed colostrum was sialylated to about 50% after 3 h and to almost 80% after 8 h incubation, with complete consumption of donor CMP-Neu5Ac, as shown in traces A–C in Figure 1. In contrast, a control containing boiled and centrifuged colostrum remained unchanged regarding the acceptor **17** and donor CMP-Neu5Ac over the same period, as shown in trace D in Figure 1, indicating that all the observed changes were caused by enzymatic reactions.

Figure 1. Incubation of **17** with a crude colostrum preparation followed over a period of 8 h. Trace D shows an identical incubation after 24 h with colostrum that was boiled and centrifuged prior usage. A linear gradient of phosphate buffer (pH 7.0) and acetonitrile was used for the elution



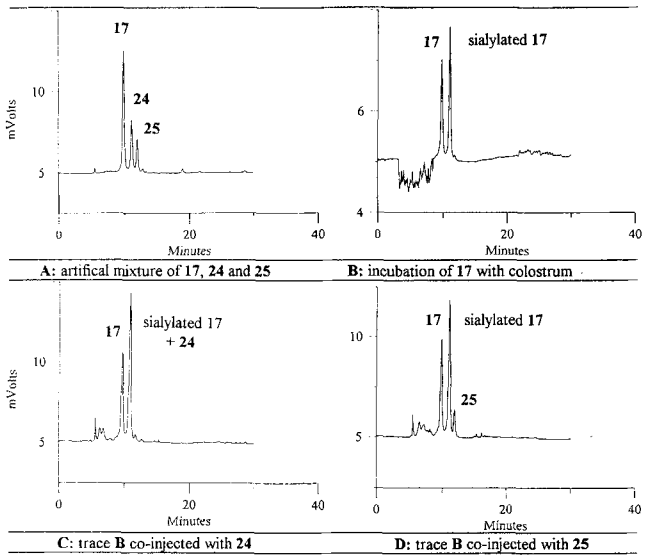
Again the lactose acceptor **4** showed only slow turnover whereas the βGal-(1 → 3)-βGlcNAc acceptor **22** did not show any detectable reaction with the colostrum preparation under the chosen conditions.

To determine whether the transferase activity in bovine colostrum caused an α(2 → 3) and/or an α(2 → 6) sialylation of LacNAc, we had to change the HPLC elution conditions. The linear gradient of phosphate buffer (pH 7.0) and acetonitrile initially used gave us a satisfactory separation of sialylated **24** or **25** and non-sialylated **17**, but only poor separation between regioisomeric sialylated **24** and **25**. Addition of tetrabutylammonium hydrogensulfate as an ion-pairing agent[37] and increasing the pH of the phosphate buffer to 8.0 shifted all sialylated compounds **23**–**26** to longer retention times than their corresponding non-sialylated starting materials **4**, **17**, and **22**. Good separation of **17**, **24**, and **25** could be achieved by applying these new conditions (trace A in Figure 2).

Reevaluation of the incubation of **17** with bovine colostrum employing the ion-pairing conditions in the HPLC analysis of the products (trace B in Figure 2) showed only one significant peak for sialylated **17**. Co-injections of the incubation mixture with **24** (trace C in Figure 2) and **25** (trace D in Figure 2) identified this compound as the (2 → 6)-sialylated LacNAc derivative **24**.

Further work is required to reach a final conclusion as to the presence at low levels of α2,3-ST activity towards LacNAc and/or the βGal-(1 → 3)-βGlcNAc acceptors in this colostrum preparation.

Figure 2. Incubation of **17** with a crude colostrum preparation analysed by using a linear gradient of phosphate buffer (pH 8.0) containing ion-pairing agent tetrabutylammonium hydrogensulfate and acetonitrile



A corresponding investigation regarding the nature of the lactose specific activity, which for crude colostrum was earlier reported[11] to give a 9:1 mixture of (2 → 6)- and (2 → 3)-sialyllactose, is also under way. However, given the modest absolute response for sialyltransferase activity in crude colostrum using lactose and the βGal-(1 → 3)-βGlcNAc-based acceptors, some concentration of the colostrum activities will be required.

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Experimental

NMR: Bruker AC300E, 300 MHz for ^1H and 75.5 MHz for ^{13}C, for **23**–**26** Varian Unity 500 operating at 500 MHz for ^1H. For spectra recorded in $CDCl_3$ or [D_4]methanol, TMS was used as an internal standard. For spectra recorded in D_2O, acetonitrile was used as internal standard at δ = 2.00 in ^1H and 2.0 for CH_3 in ^{13}C spectra. All signal assignments were made by ^1H-^1H and ^1H-^{13}C COSY methods. – Optical rotations: Perkin Elmer 241 automatic polarimeter, given in deg $cm^2\ g^{-1}$. – Melting points: Reichert Jung Thermovar hot-stage apparatus, values uncorrected. – High resolution mass spectra: Recorded by John Allen (Hort Research Ltd, Palmerston North, New Zealand) with a VG70-250S double focusing magnetic sector mass spectrometer (VG Analytical, Manchester UK). FAB mass spectra for fluorescein-labelled compounds **4**, **17**, **22**, and **23**–**26** were obtained in a reducing matrix containing dithiothreitol and dithioerythriol (5:1, w/w), and the analysed peak was [M + 2H]. – High-pressure liquid chromatography: Gilson HPLC System (John Morris Scientific Ltd, NZ) comprising 305/306 series pumps with associated manometric module and dynamic mixer for high-pressure mixing, 231 XL sampling auto-injector and 160 Diode Array Detector, under the control of Gilson UniPoint software. Data was collected at 280 and 480 nm and analysed by UniPoint.

Table 2. ^1H-NMR chemical shifts (δ) for all monosaccharides in [D_6]acetone, D_2O (1:1, v/v) (**9**), [D_4]methanol (**10**), $CDCl_3$ (**11**, **12**, **18**). – Protecting groups: **10**: (5H, m, 5 CH Ar, 6-Bzl), 4.52 (2H, s, CH_2-Ar, 6-Bzl); **11**: 7.25 (10H, m, 2 × 5 CH Ar, 3,6-Bzl), 4.69, 4.62 (1H, 1H, d, d, CH_2 3-Bzl), 4.50 (2H, AB, CH_2 6-Bzl); **12**: 7.25 (15H, m, 3 × 5 CH Ar, 3,4,6-Bzl), 4.60–4.34 (6H, m, 3 CH_2-Ar, 3,4,6-Bzl); **18**: 1.41, 1.35 (3H, 3H, s, s, 2 CH_3, isopropylidene)

	9	**10**	**11**	**12**	**18**
1-H	4.62 (d)	4.28 (d)	4.74 (d)	4.69 (d)	4.48 (d)
2-H	3.80 (dd)	3.53 (dd)	3.22 [dd(br)]	3.37 (m)	3.40 (m)
3-H	3.55 (m)	3.30 (m)	4.01 (dd~t)	3.98 (dd~t)	3.70 (m)
4-H	3.55 (m)	3.30 (m)	3.55 (dd~t)	3.53 (m)	3.50 (dd~t)
5-H	3.68 (m)	3.30 (m)	3.42 (ddd)	3.53 (m)	3.13 (m)
6a-H	4.01 (m)	3.75 (m)	3.66 (AB)	3.64 (m)	3.83 (dd)
6b-H	3.86 (dd)	3.57 (dd)	3.66 (AB)	3.64 (m)	3.70 (m)
$1a^{ch}$-H	4.01 (m)	3.75 (m)	3.73 (dq)	3.77 (dq)	3.70 (m)
$1b^{ch}$-H	3.68 (m)	3.30 (m)	3.35 (dq)	3.37 (m)	3.40 (m)
$2a,b^{ch}$-H	1.59 (m)	1.44 (m)	1.41 (m)	1.42 (m)	1.44 (m)
$3a,b^{ch}$-H	1.42 (m)	1.24 (m)	1.23 (m)	1.24 (m)	1.24 (m)
$4a,b^{ch}$-H	1.42 (m)	1.24 (m)	1.23 (m)	1.24 (m)	1.24 (m)
$5a,b^{ch}$-H	1.59 (m)	1.44 (m)	1.41 (m)	1.42 (m)	1.44 (m)
$6a,b^{ch}$-H	3.23 (t)	3.00 (t)	3.08 (t)	3.07 (t)	3.13 (m)
5CH Cbz	7.51 (m)	7.22 (m)	7.25 (m)	7.25 (m)	7.27 (m)
NH Cbz	exchanged	exchanged	4.76 (br)	4.83 (br)	4.83 (br)
CH_2 Cbz	5.19 (s)	4.95 (s)	5.00 (s)	5.00 (s)	5.02 (s)
N*H*-Ac	exchanged	exchanged	5.76 (br)	5.84 (br)	6.26 (br)
CH_3 (Ac)	2.14 (s)	1.86 (s)	1.80 (s)	1.76 (s)	1.94 (s)

Table 3. Coupling constants in Hz

	9	**10**	**11**	**12**	**18**
$J_{1,2}$	8.4	8.3	8.0	8.7	8.3
$J_{2,3}$	10.3	10.0	10.0	9.5	
$J_{3,4}$			9.0	9.5	9.5
$J_{4,5}$			8.5		9.5
$J_{5,6a}$			6.0		5.5
$J_{5,6b}$	5.0	6.0	4.8		
$J_{6a,6b}$	11.7	10.6			10.8

Table 4. ^{13}C-NMR chemical shifts (δ) for all disaccharides in [D_6]-acetone, D_2O (1:1, v/v) (**9**), [D_4]methanol (**10**), $CDCl_3$ (**11**, **12**, **18**). – *, #, indicate values that might be interchanged with each other. Always interchangeable are the values for C-2^{ch}, C-5^{ch} and C-3^{ch}, C-4^{ch}. – Protecting groups: **10**: 139.6 (Cq, Ar, 6-Bzl), 129.4–128.6 (5 CH Ar, 6 Bzl), 74.4 (CH_2-Ar, 6-Bzl); **11**: 138.3, 136.6 (2 Cq Ar, 3,6-Bzl), 128.3–127.6 (10 CH Ar, 3,6-Bzl), 74.1 (CH_2-Ar 3-Bzl), 73.6 (CH_2-Ar 6-Bzl); **12**: 138.4, 138.3, 137.4 (3 Cq Ar, 3,4,6-Bzl), 128.4–127.6 (15 CH Ar, 3,4,6-Bzl), 74.4, 74.4 (2 CH_2-Ar, 3,4-Bzl), 73.3 (CH_2-Ar, 6-Bzl); **18**: 99.7 (Cq, isopropylidene), 28.9, 18.9 (2 CH_3, isopropylidene)

	9	**10**	**11**	**12**	**18**
C-1	103.9	102.6	99.7	99.7	100.5
C-2	58.5	57.3	56.9	56.8	58.6
C-3	78.9*	76.9*	80.4	80.5	72.1
C-4	72.9*	72.2*	73.2	78.5*	74.3
C-5	76.8	76.0*	73.6	74.6*	67.0
C-6	63.7	70.9	70.6	68.9	61.9
C-1^{ch}	72.8	70.4	69.2	69.0	69.2
C-2^{ch}	31.8	30.8	29.6	29.6	30.8
C-3^{ch}	28.5	27.9	26.0	26.0	25.8
C-4^{ch}	27.7	26.6	25.3	25.3	25.0
C-5^{ch}	31.5	30.4	29.0	29.0	29.7
C-6^{ch}	44.0	41.6	40.7	40.7	40.0
CO (NAc)	176.0	173.6	170.4	170.2	172.0
CH_3 (Ac)	25.0	22.9	23.4	23.3	23.3
C=O (Cbz)	161.2	159.4	156.8	156.4	156.4
Cq (Cbz)	130.8	139.6	136.6	136.5	136.5
5 CH Ar (Cbz)	131.5-130.4	129.4-128.6	128.3-127.6	128.4-127.6	128.4-127.8
CH_2 (Cbz)	69.5	67.2	66.5	66.4	66.5

Table 5. ^{1}H-NMR chemical shifts (δ) for all disaccharides in [D$_6$]acetone, D$_2$O (1:1, v/v) (**2**, **20**), D$_2$O (**3**, **16**, **21**), [D$_4$]methanol (**4**, **15**, **17**, **22**), or CDCl$_3$ (**5**, **13**, **14**, **19**). – Protecting groups: **2**: 7.49 (5H, m, 5 CH Ar, Cbz), 5.18 (2H, s, CH$_2$-Ar, Cbz); **5**: 2.27, 2.24 (3H, 3H, s, s, 2 C*H*$_3$, OAc on C-3^{fl} and C-11^{fl}), 2.09, 2.07, 2.04, 1.98, 1.96, 1.93, 1.88 (each 3H, each s, 7 CH$_3$, OAc sugar); **13**: 7.25 (10H, m, 5 CH Ar, Cbz, 5 CH Ar, Bzl), 5.01 (2H, s, CH$_2$-Ar, Cbz), 4.74 (1H, br, NH, Cbz), 4.53 (2H, AB, CH$_2$-Ar, Bzl), 2.09, 2.07, 2.01, 1.96 (each 3H, each s, 4 CH$_3$, AcO-Gal); **14**: 7.25 (15H, m, 5 CH Ar, Cbz, 10 CH Ar, Bzl), 5.00 (2H, s, CH$_2$-Ar, Cbz), 4.74 (1H, br, NH, Cbz), 4.68, 4.62 (1H, 1H, d, d, CH$_2$-Ar, 3-Bzl), 4.53, 4.38 (1H, 1H, d, d, CH$_2$-Ar, 6-Bzl), 2.03, 1.94, 1.92, 1.90 (each 3H, each s, 4 CH$_3$, AcO-Gal); **15**: 7.30–7.15 (15H, m, 5H Ar, Cbz, 10H Ar, Bzl), 4.95 (CH$_2$-Ar, Cbz), 4.59, 4.35 (1H, 1H, d, d, CH$_2$-Ar, Bzl), 4.54 (2H, m, CH$_2$-Ar, Bzl); **19**: 7.28 (5H, m, 5 CH Ar, Cbz), 5.02 (2H, s, CH$_2$-Ar, Cbz), 2.09, 2.06, 1.99, 1.96 (each 3H, each s, 4 CH$_3$, AcO-Gal), 1.45, 1.34 (3H, 3H, s, s, 2 CH$_3$ isopropylidene); **20**: 7.51 (5H, m, 5 CH Ar, Cbz), 5.20 (2H, s, CH$_2$-Ar, Cbz). – Fluoresceinyl: **4**, **17**, **22**: 7.73 (1H, br, 18^{fl}-H), 7.55 [1H, d (br), 16^{fl}-H], 7.12 (3H, m, 15^{fl}-H, 2^{fl}-H, 12^{fl}-H), 6.54 (4H, m, 4^{fl}-H, 5^{fl}-H, 9^{fl}-H, 10^{fl}-H); **5**: 7.78 (1H, d, 18^{fl}-H), 7.46 (1H, dd, 16^{fl}-H), 7.18 (1H, d, 15^{fl}-H), 7.03 (2H, m, 2^{fl}-H, 12^{fl}-H), 6.81 (4H, m, 4^{fl}-H, 5^{fl}-H, 9^{fl}-H, 10^{fl}-H)

	2	**3**	**4**	**5**	**13**	**14**	**15**	**16**	**17**	**19**	**20**	**21**	**22**
1-H	4.59 (d)	4.37 (d)	4.25 (d)	4.37 (d)	4.81 (d)	4.58 (d)	4.35 (d)	4.45 (d)	4.31 (d)	5.01 (d)	4.72 (d)	4.48 (d)	4.38 (d)
2-H		3.49 (dd)		5.04 (dd)	2.92 (dd~t)	3.60 (m)	3.65 (m)			2.91 (dd~t)	3.95 (m)		3.64 (m)
3-H				5.12 (dd)	4.35 (dd~t)	3.88 (m)	3.90 (m)			4.40 (dd~t)	3.68 (m)*		3.75 (m)*
4-H	4.09-3.64	3.96-3.54	3.85-3.15	3.76 (m)	3.40 (m)	3.60 (m)	3.30 (m)	3.99-3.45	3.78-3.20	3.70 (m$_c$)	3.68 (m)*	3.87-3.43	3.64 (m)*
5-H	(13H, m)	(12H, m)	(16H, m)	3.57 (m)	3.40 (m)	3.60 (m)	3.65 (m)	(14H, m)	(16H, m)	3.27 (ddd~dt)	3.68 (m)*	(13H, m)	3.33 (m)*
6a-H				4.34 (m)	3.78 (m)	3.60 (m)	3.90 (m)			3.84 (dd)	3.95 (m)		3.64 (m)#
6b-H				4.03 (m)	3.59 (dd)	3.60 (m)	3.65 (m)			3.70 (m$_c$)	3.95 (m)		3.64 (m)#
1'-H	4.51 (d)	4.44 (d)	4.22 (d)	4.42 (d)	4.49 (d)	4.43 (d)	4.29 (d)	4.41 (d)	4.27 (d)	4.80 (d)	4.57 (d)	4.36 (d)	3.64 (m)*
2'-H	3.44 (dd~t)	3.23 (dd~t)		4.81 (dd)	5.17 (dd)	5.05 (d)	3.30 (m)			5.07 (dd)	368 (m)	3.87-3.43	3.64 (m)*
3'-H				4.88 (dd)	4.93 (dd)	4.82 (dd)	3.20 (m)			4.90 (dd)	3.95 (m)*	(13H, m)	3.64 (m)*
4'-H	4.09-3.64	3.96-3.54	3.85-3.15	5.27 (d)	5.29 (d)	5.23 (d)	3.20 (m)	3.99-3.45	3.78-3.20	5.28 (d)	3.68 (m)*	3.27 (d)	3.33 (m)*
5'-H	(13H, m)	(12H, m)	(16H, m)	3.76 (m)	3.92 (dd~t)	3.60 (m)	3.65 (m)	(14H, m)	(16H, m)	3.70 (m$_c$)	3.95 (m)*	3.87-3.43	3.33 (m)*
6a'-H				4.03 (m)	4.04 (AB)	3.99 (dd)	3.65 (m)			4.12 (dd)	3.95 (m)	(13H, m)	3.64 (m)#
6b'-H				4.03 (m)	4.04 (AB)	3.88 (m)	3.30 (m)			4.03 (dd)	3.95 (m)		3.33 (m)#
1a^{ch}-H	4.09-3.64	3.96-3.54	3.85-3.15	3.76 (m)	3.78 (m)	3.75 (dq)	3.65 (m)	3.99-3.45	3.78-3.20	3.70 (m$_c$)	3.95 (m)	3.87-3.43	3.75 (m)
1b^{ch}-H	(13H, m)	(12H, m)	(16H, m)	3.42 (dt)	3.40 (m)	3.34 (dt)	3.30 (m)	(14H, m)	(16H, m)	3.38 (dt)	3.68 (m)	(13H, m)	3.33 (m)
2a,b^{ch}-H	1.60 (m)	1.56 (m)	1.60 (m)	1.52 (m)	1.45 (m)	1.40 (m)	1.42 (m)	1.51 (m)	1.49 (m)	1.40 (m)	1.61 (m)	1.52 (m)	1.49 (m)
3a,b^{ch}-H	1.32 (m)	1.33 (m)	1.35 (m)	1.34 (m)	1.28 (m)	1.22 (m)	1.24 (m)	1.31 (m)	1.32 (m)	1.25 (m)	1.44 (m)	1.29 (m)	1.32 (m)
4a,b^{ch}-H	1.32 (m)	1.33 (m)	1.35 (m)	1.34 (m)	1.28 (m)	1.22 (m)	1.24 (m)	1.31 (m)	1.32 (m)	1.25 (m)	1.44 (m)	1.29 (m)	1.32 (m)
5a,b^{ch}-H	1.48 (m)	1.56 (m)	1.60 (m)	1.68 (m)	1.45 (m)	1.40 (m)	1.42 (m)	1.51 (m)	1.57 (m)	1.40 (m)	1.61 (m)	1.52 (m)	1.57 (m)
6a,b^{ch}-H	3.23 (t)	2.87 (t)	3.85-3.15	3.57 (m)	3.09 (m)	3.09 (m)	2.99 (t)	2.88 (t)	3.78-3.20	3.10 (t)	3.25 (t)	2.86 (t)	3.33 (m)
NH-Ac	---	---	---	---	6.19 (br)	5.95 (br)	exchanged	exchanged	exchanged	5.96 (br)	exchanged	exchanged	exchanged
CH$_3$ (Ac)	---	---	---	---	1.90 (s)	1.84 (s)	1.79 (s)	1.85 (s)	1.88 (s)	1.89 (s)	2.15 (s)	1.95 (s)	1.87 (s)

Table 6. Coupling constants in Hz. – Fluoresceinyl: **4**, **17**, **22**: $J_{15fl,16fl}$ = 7.1, $J_{16fl,18fl}$ < 1; **5**: $J_{15fl,16fl}$ = 8.0, $J_{16fl,18fl}$ = 1.8 Hz

	2	**3**	**4**	**5**	**13**	**14**	**15**	**16**	**17**	**19**	**20**	**21**	**22**
*J*1,2	7.4	7.7	7.4	7.8	8.2	7.8	8.0	8.1	8.3	7.0	8.2	7.7	8.0
*J*2,3		10.0		9.3	9.5					10.0			
*J*3,4				9.3	9.0					10.0			
*J*4,5										10.0			
*J*5,6a										5.5			
*J*5,6b					5.5					9.7			
*J*6a,6b					10.9					10.8			
*J*1',2'	7.8	8.6	7.8	8.0	8.0	8.0	7.7	7.8	7.6	8.0	7.4	7.6	7.6
*J*2',3'	8.0	9.0		9.5	10.4	10.5				10.0			
*J*3',4'				3.0	3.4	3.4				3.4		2.0	
*J*5',6a'					5.5	8.0				6.0			
*J*5',6b'					5.5					7.1			
*J*6a',6b'						11.3				11.2			

The columns used were a Nucleosil 5 μm, 250 mm × 4.6 mm C-18 or 3 μm, 150 mm × 4.6 mm C-18 (Chromospec Distributors Ltd, NZ) with a 20 mm × 2 mm C-18 guard column (Alltech Associates, Inc, NZ). – Column chromatography: silica gel S 32–63 μm (230–400 mesh ASTM, Riedel-de Haën), using the flash technique. For purification of **23**–**26**, Iatro Beads 6RS-8060 from Iatron Inc. Tokyo were used. As reversed-phase material LiChroprep RP-18 25–40 μm from Merck and for size exclusion chromatography with organic solvents, lipophilic Sephadex LH-20 from Sigma was used. – Enzyme isolation: Rat livers from Pel-Freez Biologicals (Rogers, Arkansas, U.S.A.), Cibacron Blue FG3A from Serva and Sepharose 6B from Pharmacia were used. For enzyme assays and kinetics, C-18 Sep-pak cartridges from Waters (Millipore), Ecolite scintillation fluid from ICN Biochemicals Ltd, CMP-[^{3}H]Neu5Ac (20 Ci/mmol) from American Radiolabeled Chemicals and CMP-Neu5Ac (sodium salt), CDP, Triton CF 54, bovine serum albumin (BSA) and alkaline phosphatase (bovine intestinal mucosa) from Sigma were used. Commercially available α2,6-sialyltransferase from rat liver and CMP-Neu5Ac (sodium salt) for the HPLC runs were purchased from Boehringer Mannheim Ltd. – For the labelling reaction: fluorescein isothiocyanate isomer I (90%) from Aldrich was used. – All other chemicals were purchased from Aldrich, Sigma, Merck or Acros and were used without further purification.

Extraction and Purification of Sialyltransferases from Rat Liver: α2,3- and α2,6-ST were both extracted from rat liver with Triton CF 54 and partially purified on a Cibacron Blue Sepharose column according to a published procedure[15,38]. The enzymes were separated on a column containing βGal-(1→3)-βGlcNAc-O(CH$_2$)$_8$-CO$_2$H acceptor covalently linked to Sepharose 6B. The enzymes were separated on a column containing βGal-(1→3)-βGlcNAc-O(CH$_2$)$_8$CO→ acceptor covalently linked via an amide bond to Sepharose 6B. The enzyme preparation was applied to the column

Table 7. ^{13}C-NMR chemical shifts (δ) for all disaccharides in [D$_6$]acetone, D$_2$O (1:1, v/v) (**2**, **20**), D$_2$O (**3**, **16**, **21**), [D$_4$]methanol (**4**, **15**, **17**, **22**), or CDCl$_3$ (**5**, **13**, **14**, **19**). – *, #, indicate values that might be interchangeable with each other. Always interchangeable are the values for C-2^{ch}, C-5^{ch} and C-3^{ch}, C-4^{ch}. – Protecting groups: **2**: 161.2 (C=O, Cbz), 139.8 (Cq Ar, Cbz), 131.5–130.4 (5 CH Ar, Cbz), 69.0 (CH$_2$-Ar); **5**: 170.1–168.2 (9 C=O, OAc), 30.8, 27.8 (2 CH$_3$, OAc on C-3^{fl} and C-11^{fl}), 21.0–20.4 (7 CH$_3$, OAc sugar); **13**: 170.6–169.3 (4 C=O, OAc), 156.6 (C=O, Cbz), 138.5, 136.3 (Cq, Cbz, Cq, Bzl), 128.6–127.6 (10 CH Ar, Cbz and Bzl), 73.5 (CH$_2$-Ar, Bzl), 66.7 (CH$_2$-Ar, Cbz), 20.9–20.5 (4 CH$_3$, AcO-Gal); **14**: 172.0–170.5 (4 C=O, OAc), 157.2 (C=O, Cbz), 138.0, 137.4, 136.6 (Cq, Cbz, 2 Cq, Bzl), 128.6–127.6 (15 CH Ar, Cbz and Bzl), 73.6, 73.5 (2 CH$_2$-Ar, Bzl), 66.7 (CH$_2$-Ar, Cbz), 20.5–19.8 (4 CH$_3$, AcO-Gal); **15**: 159.8 (C=O, Cbz), 139.7, 139.7, 139.6 (Cq, Cbz, 2 Cq, Bzl), 129.8–128.6 (15 CH Ar, Cbz and Bzl), 76.4, 74.3 (2 CH$_2$-Ar, Bzl), 67.3 (CH$_2$-Ar, Cbz); **19**: 169.8 (4 C=O, OAc), 156.0 (C=O, Cbz), 136.1 (Cq, Cbz), 128.4–127.6 (5 CH Ar, Cbz), 99.0 (Cq, isopropylidene), 66.3 (CH$_2$-Ar, Cbz), 28.9, 18.1 (2 CH$_3$, isopropylidene), 20.6–20.3 (4 CH$_3$, AcO-Gal); **20**: 160.4 (C=O, Cbz), 139.6 (Cq Ar, Cbz), 131.8–130.4 (5 CH Ar, Cbz), 69.5 (CH$_2$-Ar). – Fluorescinyl: **4**, **17**, **22**: 183.5 (C=S), 182.8 (CO$_2$Na), 159.8* (C-1^{fl}, C-3^{fl}, C-6^{fl}, C-7^{fl}, C-8^{fl}, C-11^{fl}, C-13^{fl}), 144.0 (C-14^{fl}), 141.4 (C-17^{fl}), 133.5 (C2^{fl}, C12^{fl}), 132.7 (C-18^{fl}), 132.1 (C-19^{fl}), 126.3 (C-15^{fl}), 124.9 (C-5^{fl}, C-9^{fl}, C-16^{fl}), 114.4* (C-1^{fl}, C-3^{fl}, C-6^{fl}, C-7^{fl}, C-8^{fl}, C-11^{fl}, C-13^{fl}), 105.5 (C-4^{fl}, C-10^{fl}); **5**: 183.2 (C=S), 173.8 (CO$_2$R), 152.6, 152.3, 152.2, 152.1, 151.5, 151.4 (C-1^{fl}, C-3^{fl}, C-6^{fl}, C-8^{fl}, C-11^{fl}, C-13^{fl}), 143.9 (C-14^{fl}), 136.9 (C-16^{fl}), 128.9 (C-5^{fl}, C-9^{fl}), 128.1 (C-19^{fl}), 127.5 (C-17^{fl}), 126.3 (C-18^{fl}), 125.0 (C-15^{fl}), 117.9, 117.7 (C-4^{fl}, C-10^{fl}), 110.5, 110.4 (C-12^{fl}, C-2^{fl}), 81.8 (C-7^{fl})

	2	**3**	**4**	**5**	**13**	**14**	**15**	**16**	**17**	**19**	**20**	**21**	**22**
C-1	105.7	105.8	105.0	101.0	98.9	100.0	102.1	105.8	106.0	99.1	103.5	103.6	102.7
C-2	75.3*	75.7	76.4*	69.1	57.9	57.6	56.4	58.0	57.7	58.2	57.4	57.3	56.1
C-3	77.3*	77.6*	77.0*	72.8	83.2	76.7	77.5	77.6*	77.4*	76.8	85.6*	85.1*	85.1*
C-4	81.3*	81.4*	80.6*	76.2	69.7	74.4*	82.4	81.4*	81.8*	73.8	78.3*	78.0*	77.4*
C-5	77.2*	77.4*	76.5*	72.5	75.3	73.6*	76.3*	75.4*	75.7*	66.2	75.4*	75.2*	74.4*
C-6	63.5#	63.9#	62.6#	62.0	69.8	70.5	69.4	63.9#	63.5#	61.9	63.8	63.7#	62.5#
C-1'	104.8	104.9	104.2	100.5	101.5	99.9	104.2	104.0	103.7	99.8	106.4	106.2	105.2
C-2'	75.4	73.8	72.7*	71.7	69.0	69.5	73.0	73.8*	73.5*	69.5	73.5*	73.3*	72.2*
C-3'	73.5*	75.4*	74.5*	70.6	71.1	70.7	77.4#	75.3*	75.2*	71.0	71.6*	71.4*	70.4*
C-4'	77.9*	78.2*	77.1*	66.6	67.1	66.9	74.9#	78.2*	78.0*	66.8	78.1*	77.9*	76.8*
C-5'	71.2*	71.4*	70.4*	70.9	71.2	70.4*	70.4*	71.4*	71.4*	70.4	71.3*	71.2*	70.2*
C-6'	62.8#	63.0#	61.9#	60.7	61.6	60.8	63.0	63.0#	62.9#	61.1	63.8	63.4#	62.3#
C-1^{ch}	73.3	73.3	71.3	69.8	69.5	69.3	70.5	73.3	71.6	69.6	72.6	73.0	70.3
C-2^{ch}	31.5	31.4	30.6	29.1	29.7	29.8	30.9	31.2	31.9	29.5	32.3	31.0	30.3
C-3^{ch}	28.3	28.2	27.7	26.6	26.0	26.4	27.5	28.1	28.6	25.9	28.6	27.9	27.5
C-4^{ch}	27.5	27.5	26.8	25.4	25.5	25.8	26.7	27.5	27.7	25.2	27.7	27.3	26.6
C-5^{ch}	31.4	30.5	30.0	28.0	29.1	29.6	30.5	30.3	30.9	29.0	31.4	30.0	29.8
C-6^{ch}	44.0	42.5	46.2	46.6	41.0	38.1	41.7	42.1	46.9	40.5	43.2	42.2	48.6
CO (NAc)	---	---	---	---	170.9	172.0	173.1	176.8	174.6	169.8	176.5	177.1	173.2
CH$_3$ (Ac)	---	---	---	---	23.8	23.1	23.1	25.0	24.1	23.4	25.1	24.9	23.2

Table 8. ^{1}H-NMR chemical shifts (δ) and coupling constants in Hz for all sialylated compounds (**23**, **24**, **25**, **26**) in D$_2$O. – Fluoresceinyl for all compounds **23**–**26**: 7.75 (1 H, s, 18^{fl}-H), 7.68 (1 H, d, 16fl-H), 7.37 (3 H, m, 15^{fl}-H, 4^{fl}-H*, 9^{fl}-H*), 6.87–6.82 (4 H, m, 2^{fl}-H*, 9^{fl}-H*, 10^{fl}-H*, 12^{fl}-H*). – * Proton might be interchanged due to isomerisation

	23	**24**	**25**	**26**
1-H	4.44 (d), $J_{1,2}$ 7.6	4.53 (d), $J_{1,2}$ 8.0	4.51 (d), $J_{1,2}$ 8.0	4.49 (d), $J_{1,2}$ 7.5
1'-H	4.41 (d), $J_{1',2'}$ 7.8	4.4i (d), $J_{1',2'}$ 7.5	4.44 (d), $J_{1',2'}$ 7.5	4.48 (d), $J_{1',2'}$ 7.9
3'-H	3.95-3.52	4.02-3.48	4.08 (dd), $J_{2',3'}$ 9.6, $J_{3',4'}$ 2.9	4.05 (dd), $J_{2',3'}$ 9.7, $J_{3',4'}$ 3.0
4'-H	3.95-3.52	4.02-3.48	3.93 (d)	3.89 (d)
sugar H	3.95-3.52	4.02-3.48	3.86-3.46	3.76-3.45
multiplet	(23 H, m)	(23 H, m)	(21 H, m)	(21 H, m)
3''ax-H	2.72 (dd)	2.64 (dd)	2.73 (dd)	2.75 (dd)
$J_{3''ax,3''eq}$	12.2	12.2	12.2	12.4
$J_{3''ax,4''}$	4.4	4.4	4.4	4.7
3''eq-H	1.75 (dd~t)	1.71 (dd~t)	1.77(dd~t)	1.78 (dd~t)
$J_{3''eq,4''}$	12.4	12.2	12.2	12.4
NHAc	2.03 (s)	2.05 (s)	2.02 (s)	2.00 (s)
NHAc	–	1.99 (s)	2.02 (s)	1.96 (s)
1a,b^{ch}-H	3.95-3.52	4.02-3.48	3.86-3.46	3.76-3.45
2a,b^{ch}-H	1.43 (m)	1.54 (m)	1.56 (m)	1.57 (m)
3a,b^{ch}-H	1.33 (m)	1.35 (m)	1.34 (m)	1.37 (m)
4a,b^{ch}-H	1.33 (m)	1.35 (m)	1.34 (m)	1.37 (m)
5a,b^{ch}-H	1.68 (m)	1.65 (m)	1.62 (m)	1.65 (m)
6a,b^{ch}-H	3.95-3.52	4.02-3.48	3.86-3.46	3.76-3.45

and eluted with a buffer containing CDP, under which conditions α2,6-ST was not retained. The retarded α2,3-ST was then eluted by washing with a buffer containing lactose but no CDP.

The α2,3-ST activity was determined by a radiochemical assay using ^{3}H-radiolabelled donor in which the radiolabelled product is separated from radiolabelled donor on a C-18 Sep-pak cartridge as earlier described[8,36]. The standard assay for α2,3-ST contained 1.8 mM βGal-(1→3)-βGlcNAc-O(CH$_2$)$_8$CO$_2$CH$_3$ as acceptor, 550 μM CMP-Neu5Ac including CMP-[^{3}H]Neu5Ac (65000 dpm) as donor substrates and approximately 9 μU of 2,3-ST in a total volume of 20 μl of incubation buffer A containing 40 mM sodium cacodylate (pH 7.0), 10 mM MnCl$_2$, 0.1% (w/v) Triton CF 54, and 0.2 mg/ml of BSA. Assay tubes were incubated for 15 min at 37 °C in a water bath, and reactions were quenched by the addition of 0.6 ml of water and applied immediately to C-18 Sep-pak cartridges. The cartridges were washed with 50 ml of water, and then the radiolabelled product was eluted with 4 ml of methanol. The methanol eluants were added to 10 ml of Ecolite scintillation cocktail and counted in a Beckman scintillation counter.

The flow-through and wash fractions of the βGal-(1→3)-βGlcNAc-O(CH$_2$)$_8$CO$_2$H Sepharose column containing α2,6-ST activity were pooled and dialysed against buffer B containing 10 mM sodium cacodylate (pH 6.5), 0.1% (w/v) Triton CF 54, 25% glycerol, and 150 mM NaCl. The dialysed fractions were loaded onto a Cibacron Blue F3GA Sepharose column (2.5 cm × 14 cm), which was equilibrated in the same buffer, at a flow rate of ~2 ml/min. The column was washed with buffer B containing 250 mM NaCl until no protein, monitored by absorbance at 280 nm, could be detected in the eluted fractions. Elution of the enzyme was achieved with two column volumes (140 ml) of buffer B containing an additional 5 mM CDP, and 15 ml fractions were collected. All fractions containing α2,6-ST activity were pooled, concentrated 10 fold in an Amicon cell on a PM-10 membrane and dialysed against buffer B containing 300 mM NaCl, to remove the CDP. The enzyme was stored in the same buffer containing 50% glycerol at −25 °C.

The yield from the loaded 600 mU of α2,6-ST which was obtained after the separation of the α2,3-ST (starting from 300 g of

rat liver, 1200 mU of α2,6-ST) was 450 mU. The standard assay for α2,6-ST contained 0.81 mM βGal-(1→4)-βGlcNAc-O(CH_2)$_8$-CO_2CH_3 as acceptor, 550 μM CMP-Neu5Ac including CMP-[^{3}H]Neu5Ac (90000 dpm) as donor substrates and approximately 11 μU of α2,6-ST in a total volume of 20 ml of incubation buffer A. Assay tubes were incubated for 45 min at 37°C in a water bath, and processed as described for the α2,3-ST standard assay.

Kinetic Studies: Kinetic parameters for α2,3-ST an α2,6-ST were determined for the acceptors **4**, **17**, and **22** with the radiochemical assay as described above. Acceptor K_m assays contained a saturating amount of CMP-Neu5Ac and were carried out at 6–7 different acceptor concentrations. Assays were performed in duplicate and contained typically 550 μM CMP-Neu5Ac, including CMP-[^{3}H]Neu5Ac (75000 dpm), 13 μU of α2,3-ST or 17 μU of α2,6-ST in a total volume of 20 μl of incubation buffer A. Assay tubes were incubated for 1 to 2 h at 37°C in a water bath and processed as described for the standard assays above. The final concentrations of fluorescein-labelled acceptors were 2.06–13.19 mM for **4** and α2,6-ST, 0.028–1.8 mM for **17** and α2,6-ST, 0.028–1.8 mM for **17** and α2,3-ST and 0.056–0.36 mM for **22** and α2,3-ST. Kinetic data listed in Table 1 were obtained by fitting the initial rate data to the Michaelis-Menten equation using nonlinear regression analysis (Sigma Plot 4.10). It should be noted that substrate inhibition was observed for **4** and 2,6-ST above 3 mM, **17** and α2,6-ST above 1.5 mM and **22** and α2,3-ST above 0.2 mM. For this reason it was ensured that the final concentrations of the acceptors in the preparative scale syntheses were lower than the concentration at which substrate inhibition was seen in the K_m determinations.

HPLC Separation and Analytical Incubation Conditions: All salts and the organic solvents were HPLC grade, water was Milli Q quality, solutions were filtered through 0.45-μm membranes before use. The initial eluant used was a linear gradient of phosphate buffer at pH 7.0 in acetonitrile. Buffer I was made up by mixing 50 mM of potassium dihydrogen orthophosphate with 50 mM dipotassium hydrogen orthophosphate until pH 7.0 and diluting to 25 mM with water. Buffer II consisted of 1 volume of 50 mM phosphate buffer mixed with 2 volumes of acetonitrile. The separation was carried out on a 5-μm, 250 mm × 4.6 mm C-18 column using a linear gradient from 20% buffer II to 75% buffer II in 22 min starting 2 min after injection.

Altering the pH of the aqueous buffers to 8.0 and the addition of tetrabutylammonium hydrogensulfate as an ion pairing agent[37] was required to separate the isomerically sialylated LacNAc derivatives. A 50-mM solution of potassium dihydrogen orthophosphate was added to 50 mM dipotassium hydrogen orthophosphate until pH 8.0, and tetrabutylammonium hydrogensulfate was added to give a 10-mM solution. Buffer Ia and IIa were made by compiling 1 volume of the resulting solution mixed with 1 volume of Milli Q water or with 2 volumes of acetonitrile, respectively. The separation was carried out on a 3-μm, 150 mm × 4.6 mm C-18 column using a linear gradient from 30% of buffer IIa to 75% of buffer IIa in 22 min starting 2 min after injection. Standard samples were made up to the appropriate concentration in Milli Q water. Samples from the enzymatic reactions (80 μl) were taken, treated as described below and passed through a 0.45 μm filter, prior to injection of an 40 μl aliquot.

Incubation of **17** *with Commercial α2,6-ST from Rat Liver:* A mixture containing 0.22 mM fluoresceinyl-LacNAc **17**, 0.32 mM CMP-Neu5Ac, 38 mM sodium cacodylate (pH 6.5), 8 mM $MnCl_2$, 0.08% (w/v) BSA, 0.4% (v/v) Triton CF 54, and 8 mU[38,39] of α2,6-ST in a total volume of 400 μl was incubated at 37°C, and 80-μl samples taken at regular intervals. The samples were snap-frozen immediately in liquid nitrogen and held at −35°C until being thawed for HPLC analysis.

Preparation of Crude Colostrum Extract: Colostrum from the first two milkings after calving was frozen as soon as possible after collection (typically less than 4 h) and held at −35°C until required, showing no loss of activity over a period of one year. The colostrum was allowed to thaw at 4°C for about 16 h, and 18 ml was centrifuged at 13000 rpm for 10 min. The central aqueous layer (~7 ml) was dialysed (10000 molecular mass cut off membrane) against 250 ml of a buffer containing 20 mM sodium cacodylate (pH 6.5), 4 mM $MnCl_2$ for 17 h, then the buffer was replaced and dialysis continued for a further 8 h, all at 4°C. The retentate was centrifuged again and the central aqueous layer (~9 ml) stored at 4°C until used for the incubation.

Incubation of **17** *with a Crude Bovine Colostrum Preparation:* A sample of the crude colostrum preparation (320 μl) was added to an incubation mixture to give a final volume of 400 μl containing 0.22 mM fluoreceinyl-LacNAc **17**, 0.32 mM CMP-Neu5Ac, 20 mM sodium cacodylate (pH 6.5), 5 mM $MnCl_2$, 0.01% (w/v) BSA, and 0.05% (v/v) Triton CF 54. The mixture was incubated at 37°C, and 80-μl samples were taken at regular intervals. The samples were snap-frozen immediately in liquid nitrogen and held at −35°C until being thawed for HPLC analysis.

Chemical Syntheses

6-[(Benzyloxycarbonyl)amino]hexyl β-D-Lactoside (**2**): 2,2′,3,3′,4′,6,6′-Hepta-*O*-acetyl-α-D-lactosyl bromide (**1**)[23] (4.2 g, 6 mmol) was dissolved in dry dichloromethane (50 ml). Anhydrous disodium hydrogenphosphate (3 g, 21 mmol), molecular sieves 4 Å, and a solution of 6-[(benzyloxycarbonyl)amino]-1-hexanol[22] (3 g, 11.9 mmol) in dry dichloromethane (20 ml) were added. The mixture was kept under argon and cooled to 0°C. Previously dried silver triflate (1.65 g, 6.3 mmol) was added, and the mixture was allowed to warm up to room temp. and stirred for an additional 3 h. Solids were filtered off over celite, washed several times with dichloromethane, and the combined solutions were twice washed with water, aqueous sodium thiosulfate, and again water, dried with magnesium sulfate and evaporated in vacuo. The obtained material was purified by flash chromatography with toluene/ethyl acetate (3:2, v/v) as eluant to yield the crude heptaacetate of **2** still containing traces of starting 6-[(benzyloxycarbonyl)amino]-1-hexanol. The crude material was dissolved in dry methanol (100 ml) and treated with a freshly prepared 1 M solution of sodium methanolate in dry methanol (5 ml) for about 16 h. The solution was neutralised by addition of Amberlite IR 120 H^+. The ion exchange resin was filtered off, and the solvent was removed in vacuo. Crystallisation from methanol yielded **2** (2.2 g, 64%), m.p. 149°C, $[\alpha]_D^{20}$ = +25.7 (c = 0.6 in H_2O). – HR-FAB-MS: calcd. for MH^+ ($C_{26}H_{42}NO_{13}$) 576.2656; found 576.2662.

6-Aminohexyl β-D-Lactoside (**3**): Compound **2** (100 mg, 0.17 mmol) was dissolved in methanol, water (20 ml, 1:1,. v/v) and 20% palladium(II) hydroxide on charcoal (20 mg) were added. The mixture was kept in a hydrogen atmosphere with vigorously stirring for 4 h. The catalyst was filtered off and the solvents evaporated in vacuo and finally removed by freeze drying to obtain pure **3** (74 mg, 98%), m.p. 145°C $[\alpha]_D^{20}$ = −25.6 (c = 1.0 in H_2O). – HR-FAB-MS: calcd. for MH^+ ($C_{18}H_{36}NO_{11}$) 442.2288; found 442.2268.

6-(3-Fluoresceinylthioureido)hexyl β-D-Lactoside (**4**): Compound **3** (20 mg, 47 μmol) was dissolved in water (5 ml) containing sodium carbonate (7 mg, 66 μmol). A solution of fluorescein isothiocyanate isomer I (24 mg, 62 μmol) in acetone/water (10 ml, 1:1, v/v) was

added dropwise at 0 °C. The mixture was allowed to warm to room temp. and was stirred for another 18 h with the exclusion of light. Solvents were evaporated in vacuo and finally removed by freeze drying. Desalting and removal of access fluorescein was achieved by size exclusion chromatography on Sephadex LH 20 with methanol/chloroform (1:1, v/v) as eluant to obtain **4** (36 mg, 89%) as a bright orange foam. The obtained product was about ~95% pure by HPLC (phosphate buffer, pH 7.0, acetonitrile) and could be finally purified by chromatography on LiChroprep Rp 18 (Merck) using successive elution with water/acetonitrile (15:1, 10:1, 5:1, v/v). $[\alpha]_D^{20} = -19.2$ ($c = 0.65$ in H_2O). – HR-FAB-MS: calcd. for reduced $[M + 3H]^+$ ($C_{39}H_{48}N_2NaO_{16}S$) 855.2622; found 855.2618.

6-(3-Fluoresceinylthioureido)hexyl 2,2',3,3',4',6,6'-Hepta-O-acetyl-β-D-lactoside (**5**): Compound **4** (20 mg, 0.024 mmol) was dissolved in pyridine (2 ml). Acetic anhydride (1 ml) was added, and the mixture was stirred for 18 h. Dichloromethane (5 ml) was added, and the organic layer was washed with aqueous sodium hydrogencarbonate and twice with water, dried with magnesium sulfate and evaporated in vacuo. Final purification by column chromatography with dichloromethane/ethanol (30:1, v/v) afforded **5** (22 mg, 74%) as an amorphous white solid, $[\alpha]_D^{20} = -2.2$ ($c = 0.25$ in CH_2Cl_2). – HR-FAB-MS: calcd. for MH^+ ($C_{57}H_{65}N_2O_{25}S$) 1209.3597; found 1209.3588.

Selective Benzylation of **9**: A solution of **9** (0.45 g, 1 mmol) in dry DMF (20 ml) was cooled to 0 °C, and anhydrous barium oxide (1 g, 6.5 mmol) and barium hydroxide octahydrate (0.5 g, 1.6 mmol) were added. Benzyl bromide (480 µl, 4 mmol) was added dropwise in two portions after 30 min and 6 h and stirring continued for a total of 24 h at 0 °C. The suspension was poured into dichloromethane (100 ml), and the solids were removed by filtration over celite followed by washing with dichloromethane. The solvents were evaporated to a volume of about 20 ml. Ethyl acetate (100 ml) was added, and the organic layer was washed three times with water and brine, dried with magnesium sulfate and evaporated in vacuo. Column chromatography with a solvent gradient of dichloromethane/ethanol (50:1, 30:1, 8:1, v/v) afforded first *6-[(benzyloxycarbonyl)amino]hexyl 2-acetamido-3,4,6-tri-O-benzyl-2-deoxy-β-D-glucopyranoside* (**12**) (84 mg, 9%) as a white gum (ref.[120]: m.p. 151 °C), $[\alpha]_D^{20} = -2.8$ ($c = 0.95$ in CH_2Cl_2). – HR-FAB-MS: calcd. for MH^+ ($C_{43}H_{53}N_2O_8$) 725.3801; found 725.3790, followed by *6-[(benzyloxycarbonyl)amino]hexyl 2-acetamido-3,6-di-O-benzyl-2-deoxy-β-D-glucopyranoside* (**11**) (185 mg, 29%) as white crystals, m.p. 117 °C, $[\alpha]_D^{20} = -15.7$ ($c = 1.0$ in MeOH) (ref.[120]: no data given). – HR-FAB-MS: calcd. for MH^+ ($C_{36}H_{47}N_2O_8$) 635.3332; found 635.3317. Finally, *6-[(Benzyloxycarbonyl)amino]hexyl 2-acetamido-6-O-benzyl-2-deoxy-β-D-glucopyranoside* (**10**) (218 mg, 40%) was obtained as white crystals m.p. 123 °C, $[\alpha]_D^{20} = -12.4$ ($c = 0.57$ in MeOH). – HR-FAB-MS: calcd. for MH^+ ($C_{29}H_{41}N_2O_8$) 545.2863; found 545.2869.

Selective Benzylation of **10**: Compound **10** (200 mg, 0.36 mmol) was treated as described above for **9**, but only 2 equiv. benzyl bromide (90 µl, 0.72 mmol) was used. The workup procedure and chromatography as described above afforded **12** (36 mg, 14%) next to **11** (96 mg, 42%) and unreacted **10** (32 mg, 16%).

6-[(Benzyloxycarbonyl)amino]hexyl O-(2,3,4,6-Tetra-O-acetyl-β-D-galactopyranosyl)-(1→3)-2-acetamido-6-O-benzyl-2-deoxy-β-D-glucopyranoside (**13**): A solution of **10** (50 mg, 90 µmol) and 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl trichloroacetimidate[131] (54 mg, 110 µmol) in dry dichloromethane (20 ml) was treated with boron trifluoride–ether (10 µl) at 0 °C and stirred for 1 h at 0 °C and an additional 4 h at room temp. Triethylamine (50 µl) was added, and the solvents were removed in vacuo. Purification was achieved by column chromatography using dichloromethane/ethanol (40:1, v/v) as eluant to yield **13** (36 mg, 46%) as a white foam, $[\alpha]_D^{20} = -5.8$ ($c = 0.8$ in CH_2Cl_2), HR-FAB-MS: calcd. for MH^+ ($C_{43}H_{58}N_2O_{17}$) 875.3801; found 875.3791, next to 8 mg of a mixture of **13** and its presumed (1→4) isomer followed by recovered **10** (17 mg, 34%).

6-[(Benzyloxycarbonyl)amino]hexyl O-(2,3,4,6-Tetra-O-acetyl-β-D-galactopyranosyl)-(1→4)-2-acetamido-3,6-di-O-benzyl-2-deoxy-β-D-glucopyranoside (**14**): A solution of **11** (190 mg, 0.3 mmol) and 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl trichloroacetimidate (295 mg, 0.6 mmol) in dry dichloromethane (20 ml) was treated with boron trifluoride–ether (25 µl) and stirred for 50 h. Triethylamine (100 µl) was added, and the solvents were removed in vacuo. Purification was achieved by column chromatography using toluene/ethyl acetate (3:2, v/v) followed by dichloromethane/methanol (50:1, 30:1, v/v) as eluant to yield **16** (185 mg, 64%) as a white foam, $[\alpha]_D^{20} = -14.8$ ($c = 0.75$ in CH_2Cl_2). – HR-FAB-MS: calcd. for MH^+ ($C_{50}H_{65}N_2O_{17}$) 965.4283; found 965.4299.

6-[(Benzyloxycarbonyl)amino]hexyl O-(β-D-Galactopyranosyl)-(1→4)-2-acetamido-3,6-di-O-benzyl-2-deoxy-β-D-glucopyranoside (**15**): Compound **14** (115 mg, 0.12 mmol) was dissolved in dry methanol (20 ml) and the solution treated with a 1 M solution of sodium methanolate in methanol (1 ml) for 18 h. The solution was neutralised by the addition of Amberlite IR 120 H^+. The ion exchange resin was filtered off and the solvent evaporated in vacuo to yield **15** (80 mg, 84%) as a colourless foam, $[\alpha]_D^{20} = -3.2$ ($c = 0.75$ in MeOH). – HR-FAB-MS: calcd. for MH^+ ($C_{42}H_{57}N_2O_{13}$) 797.3860; found 797.3860.

6-Aminohexyl O-(β-D-Galactopyranosyl)-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (**16**): Compound **15** (46 mg, 57.8 µmol) was treated as described for **3** to yield **16** (26 mg, 93%) as a colourless foam, $[\alpha]_D^{20} = -21.5$ ($c = 0.4$ in H_2O). – HR-FAB-MS: calcd. for MH^+ ($C_{20}H_{39}N_2O_{11}$) 483.2553; found 483.2548.

6-(3-Fluoresceinylthioureido)hexyl O-(β-D-Galactopyranosyl)-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (**17**): Glucopyranoside **16** (20 mg, 41.5 µmol) was treated as described for **4** with fluorescein isothiocyanate isomer I (24 mg, 62 µmol). Chromatography on Sephadex LH 20 with chloroform/methanol/water (65:30:5, v/v/v) as eluant yielded **17** (34.7 mg, 94%) as a bright orange foam. The obtained product was about 95% pure as judged by HPLC analysis (phosphate buffer, pH 7.0, acetonitrile) and was finally purified by chromatography on LiChroprep Rp 18 using succesive elution with water/acetonitrile (15:1, 10:1, 5:1, v/v), $[\alpha]_D^{20} = -21.9$ ($c = 0.85$ in MeOH). – HR-FAB-MS: calcd. for reduced $[M + 3H]^+$ ($C_{41}H_{49}N_3NaO_{16}S$) 894.2731; found 894.2721.

6-[(Benzyloxycarbonyl)amino]hexyl O-(2,3,4,6-Tetra-O-acetyl-β-D-galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-4,6-di-O-isopropylidine-β-D-glucopyranoside (**19**): A solution of **18**[20] (200 mg, 0.4 mmol) and 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl trichloroacetimidate (296 mg, 0.6 mmol) in dry dichloromethane (20 ml) was cooled to −20 °C and treated with boron trifluoride–ether (10 µl). The solution was allowed to warm up to room temp. and stirred for 4 h. Triethylamine (100 µl) was added, and the solvents were removed in vacuo. Purification was achieved by column chromatography using toluene/ethyl acetate (3:2, v/v) followed by dichloromethane/ethanol (30:1, v/v) as eluant to yield **23** (250 mg, 76%) as a white foam, $[\alpha]_D^{20} = -7.8$ ($c = 1.3$ in CH_2Cl_2). – HR-FAB-MS: calcd. for MH^+ ($C_{39}H_{56}N_2O_{17}$) 825.3657; found 825.3678.

6-[(Benzyloxycarbonyl)amino]hexyl O-(β-D-Galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-β-D-glucopyranoside (**20**): Derivative

19 (180 mg, 0.21 mmol) was dissolved in dry methanol (20 ml) and deacetylated as described for **14**. After neutralisation with Amberlite IR 120 H^+, stirring was extended for about 16 h to cleave off the isopropylidene group. The ion exchange resin was filtered off, and the solvent was evaporated in vacuo to yield **20** (119 mg, 92%) as an amorphous solid, that could be crystallised from ethanol, m.p. 206°C dec., $[\alpha]_D^{20} = -18.8$ ($c = 0.85$ in H_2O, acetone; 1:1; v/v). – HR-FAB-MS: calcd. for MH^+ ($C_{28}H_{45}N_2O_{13}$) 617.2921; found 617.2938.

6-Aminohexyl O-(β-D-Galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-β-D-glucopyranoside (**21**): Compound **20** (20 mg, 32 μmol) was treated as described for **3** to yield **21** (15 mg, 98%) as a colourless foam, $[\alpha]_D^{20} = -29.6$ ($c = 0.7$ in H_2O). – HR-FAB-MS: calcd. for MH^+ ($C_{20}H_{39}N_2O_{11}$) 483.2553; found 483.2565.

6-(3-Fluoresceinylthioureido)hexyl O-(β-D-Galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-β-D-glucopyranoside (**22**): Amine **21** (14 mg, 29 μmol) was treated as described for **17** with fluorescein isothiocyanate isomer I (20 mg, 50 μmol) to yield **22** (23 mg, 88%) as a bright orange foam, $[\alpha]_D^{20} = +8.1$ ($c = 0.85$ in MeOH). – HR-FAB-MS: calcd. for reduced $[M + 3H]^+$ ($C_{41}H_{49}N_3NaO_{16}S$) 894.2731; found 894.2736.

Reactions with α2,6-Sialyltransferase from Rat Liver: A typical reaction mixture contained fluorescein-labelled acceptor, 1 equivalent of CMP-Neu5Ac, 10–14 U of alkaline phosphatase, and 28 mU of rat liver α2,6-ST. The incubation buffer (4 ml) contained 40 mM (50 mM for **24**) sodium cacodylate (pH 7.0), 10 mg/ml of BSA, 1% w/v Triton CF 54, 100 mM NaCl and 30 mM $MnCl_2$. This mixture was incubated in a plastic test tube at room temp., rotating gently. Tubes were covered with aluminium foil to avoid photodegradation of the fluorescein-labelled compounds. Further half molar equivalents of CMP-Neu5Ac were added every 2 d over the course of the incubation, for a total of 5 d for **23** and 9 d for **24**. The reaction mixture was divided into three lots and loaded onto three C-18 Sep-pak cartridges. Each cartridge was washed with an equal amount of water, and the flow-through and washings were loaded onto a second and in the same way onto a third cartridge until no product could be detected in the washings. Each cartridge was washed with water (~50 ml) before the hydrophobic components were eluted with water/acetonitrile (~10 ml, 1:1, v/v). The combined solutions were evaporated and freeze-dried. Further purirification was achieved by chromatography on Iatro beads with chloroform/methanol/water (6:3:1, v/v/v) and on LiChroprep Rp 18 with water/acetonitrile (10:1, v/v).

6-(3-Fluoresceinylthioureido)hexyl O-(5-Acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonic acid)-(2→6)-β-D-lactoside (**23**): Compound **4** (4.3 mg, 5.1 mmol) was treated with CMP-Neu5Ac (total amount added: 6.7 mg, 10.7 mmol) as described above to furnish pure **23** (4.3 mg, 75%) as a bright orange foam. – HR-FAB-MS: calcd. for reduced $[M + 3H]^+$ ($C_{48}H_{64}N_3Na_2O_{24}S$) 1144.3363; found 1144.3396.

6-(3-Fluoresceinylthioureido)hexyl O-(5-Acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonic acid)-(2→6)-O-(β-D-galactopyranosyl)-(1→4)-O-2-acetamido-2-deoxy-β-D-glucopyranoside (**24**): Compound **17** (3.1 mg, 3.5 mmol) was treated with CMP-Neu5Ac (total amount added: 6.6 mg, 10.5 mmol) as described above to furnish pure **24** (2.7 mg, 66%) as a bright orange foam. – HR-FAB-MS: calcd. for reduced $[M + 3H]^+$ $C_{50}H_{67}N_4Na_2O_{24}S$ 1185.3661; found 1185.3745.

Reactions with α2,3-Sialyltransferase from Rat Liver: A typical reaction mixture contained fluorescein-labelled acceptor, 1 equivalent of CMP-Neu5Ac, 7–10 U of alkaline phosphatase, and 7.4 mU (4.9 mU for **26**) of rat liver α2,3-ST. The incubation buffer (4.5 ml for **25** and 17.5 ml for **26**) contained 40 mM (64 mM for **26**) sodium cacodylate (pH 7.0), 10 mg/ml of BSA, 1% w/v Triton CF 54, 100 mM NaCl, and 30 mM $MnCl_2$. The reaction mixture was incubated as described for the α2,6-ST reactions for a total of 10 d for **25** and 5 d for **26**. Further half molar equivalents of CMP-Neu5Ac were added every 2 d and one further addition of α2,3-ST (1.2 mU after 8 d for **25** and 4.9 mU after 2 d for **26** was made. The reactions were processed as described for the α2,6-ST reactions.

6-(3-Fluoresceinylthioureido)hexyl O-(5-Acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonic acid)-(2→3)-O-(β-D-galactopyranosyl)-(1→4)-O-2-acetamido-2-deoxy-β-D-glucopyranoside (**25**): Compound **17** (2.4 mg, 2.7 mmol) was treated with CMP-Neu5Ac (total amount added: 4.2 mg, 6.5 mmol) as described above for the α2,3-ST reactions to furnish pure **25** (1.6 mg, 51%) as a bright orange foam. – HR-FAB-MS: calcd. for reduced $[M + 3H]^+$ ($C_{50}H_{67}N_4Na_2O_{24}S$) 1185.3661; found 1185.3648.

6-(3-Fluoresceinylthioureido)hexyl O-(5-Acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonic acid)-(2→3)-O-(β-D-galactopyranosyl)-(1→3)-O-2-acetamido-2-deoxy-β-D-glucopyranoside (**26**): Compound **22** (3.2 mg, 3.6 mmol) was treated with CMP-Neu5Ac (total amount added. 4.8 mg, 7.5 mmol) as described above for the α2,3-ST reactions to furnish pure **26** (2.3 mg, 56%) as a bright orange foam. – HR-FAB-MS: calcd. for reduced $[M + 3H]^+$ ($C_{50}H_{67}N_4Na_2O_{24}S$) 1185.3661; found 1185.3624.

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