

Action of rat liver Gal β 1-4GlcNAc α (2-6)-sialyltransferase on Man β 1-4GlcNAc β -OME, GalNAc β 1-4GlcNAc β -OME, Glc β 1-4GlcNAc β -OME and GlcNAc β 1-4GlcNAc β -OME as synthetic substrates

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Incubation of synthetic Man β 1-4GlcNAc β -OME, GalNAc β 1-4GlcNAc β -OME, Glc β 1-4GlcNAc β -OME, and GlcNAc β 1-4GlcNAc β -OME with CMP-Neu5Ac and rat liver Gal β 1-4GlcNAc α (2-6)-sialyltransferase resulted in the formation of Neu5Ac α 2-6Man β 1-4GlcNAc β -OME, Neu5Ac α 2-6GalNAc β 1-4GlcNAc β -OME, Neu5Ac α 2-6Glc β 1-4GlcNAc β -OME and Neu5Ac α 2-6GlcNAc β 1-4GlcNAc β -OME, respectively. Under conditions which led to quantitative conversion of Gal β 1-4GlcNAc β -OEt into Neu5Ac α 2-6Gal β 1-4GlcNAc β -OEt, the aforementioned products were obtained in yields of 4%, 48%, 16% and 8%, respectively. HPLC on Partisil 10 SAX was used to isolate the various sialyltrisaccharides, and identification was carried out using 1- and 2-dimensional 500-MHz 1 H-NMR spectroscopy.

Keywords: α (2-6)-sialyltransferase; CMP-Neu5Ac:Gal β 1-4GlcNAc α (2-6)-*N*-acetylneuraminyltransferase; sialyloligosaccharides; 1 H-NMR spectroscopy

Abbreviations: 2D, 2-dimensional; CMP, cytidine 5'-monophosphate; CMP-Neu5Ac, cytidine 5'-monophospho- β -*N*-acetylneuraminic acid; COSY, correlation spectroscopy; DQF, double quantum filtered; HOHAHA, homonuclear Hartmann-Hahn; MLEV, composite pulse devised by M. Levitt; Neu5Ac, *N*-acetylneuraminic acid; Neu5Ac2en, 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid.

Introduction

In biological systems, substrate specific α (2-6)-sialyltransferases catalyse the transfer of Neu5Ac from CMP-Neu5Ac to the primary hydroxyl group of definite monosaccharide residues that form part of a carbohydrate chain, like galactose in Gal β 1-3/4GlcNAc β 1-R, *N*-acetylglucosamine in Gal β 1-3GlcNAc β 1-R and *N*-acetylgalactosamine in GalNAc α (1-*O*)Ser/Thr [1, 2]. The Neu5Ac α 2-6Glc element occurs less frequently [1], and recently the Neu5Ac α 2-6GalNAc β 1-4GlcNAc β 1-2Man α 1-R sequence has been found [3, 4], but so far no related specific α (2-6)-sialyltransferases have been characterized. The urine of patients with β -mannosidosis contains, apart from the major storage product Man β 1-4GlcNAc, the trisaccharide Neu5Ac α 2-6Man β 1-4GlcNAc with the unusual Neu5Ac α 2-6Man linkage [5]. It is supposed that this urinary compound is formed by the action of an α (2-6)-sialyltransferase on Man β 1-

4GlcNAc, since *in vitro* incubation of Man β 1-4GlcNAc with CMP-Neu5Ac in the presence of rat liver Gal β 1-4GlcNAc α (2-6)-sialyltransferase yielded Neu5Ac α 2-6Man β 1-4GlcNAc [6]. Studies on the properties of rat liver Gal β 1-4GlcNAc α (2-6)-sialyltransferase have shown that this enzyme has a large preference for the Gal β 1-4GlcNAc sequence as acceptor. The rates of incorporation of Neu5Ac using Gal β 1-3GlcNAc, Gal β 1-6GlcNAc and Gal β 1-4Glc are \leq 1% relative to Gal β 1-4GlcNAc [7], indicating that the Hex β 1-4GlcNAc element is of major importance for the activity.

In the framework of a programme focused on the potency of rat liver Gal β 1-4GlcNAc α (2-6)-sialyltransferase to transfer Neu5Ac to substrates which contain other β (1-4)-linked terminal monosaccharides than galactose, we report here the results of the enzymatic transfer reaction using the synthetic disaccharides R' β 1-4GlcNAc β -OME (R' = Man, GalNAc, Glc or GlcNAc) as acceptor. A preliminary report has been published [8].

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Materials and methods

Rat liver CMP-Neu5Ac: β -D-Gal-(1-4) β -D-GlcNAc α (2-6)-N-acetylneuraminyltransferase (EC 2.4.99.1) was obtained from Boehringer Mannheim, Germany, and CMP-Neu5Ac (sodium salt) from Sigma St. Louis, MD, USA. Gal β 1-4GlcNAc β -OEt was purchased from BioCarb Chemicals, Lund, Sweden.

Column chromatography was performed on Kieselgel 60 (Merck, <230 mesh) and fractions were monitored by TLC on Kieselgel 60 F₂₅₄ (Merck). Detection was effected by charring with sulfuric acid after examination under UV light. Optical rotations were measured at 20 °C using a Perkin-Elmer 241 polarimeter. In the work-up procedures, washings were carried out three times with appropriate quantities of water or aqueous 5% sodium hydrogencarbonate, unless indicated otherwise. Evaporations were conducted under reduced pressure at 40 °C (bath). All solvents were distilled from appropriate drying agents.

Methyl 2-acetamido-4-O-(2,3,4,6-tetra-O-acetyl- β -D-mannopyranosyl)-3,6-di-O-benzyl-2-deoxy- β -D-glucopyranoside (3)

A solution of ethyl 4-O-(3-O-allyl-4,6-O-isopropylidene- β -D-mannopyranosyl)-3,6-di-O-benzyl-2-deoxy-2-phthalimido-1-thio- β -D-glucopyranoside (316 mg, 0.39 mmol) [9] in 10 ml acetic acid:water, 3:2 by vol, was kept for 45 min at 70 °C, then concentrated, and co-concentrated with toluene (5 \times 10 ml). The residue was taken up in 10 ml pyridine:acetic anhydride, 1:1 by vol, and stirred for 16 h at room temperature. The solution was concentrated, and co-concentrated with toluene (3 \times 10 ml). A solution of the residue in toluene (10 ml), containing methanol (1 ml) and powdered 4 Å molecular sieves (2 g) was then stirred for 1 h under nitrogen. Methyl triflate (220 μ l, 2 mmol) [10] was added and the mixture was stirred for 16 h at room temperature, when TLC (dichloromethane:acetone, 9:1 by vol) showed an incomplete conversion. After the addition of further methyl triflate (250 μ l, 2.3 mmol), and heating for 1 h at 60 °C, the methyl glycoside formation was shown to be complete. Triethylamine (1 ml) was added, the mixture was diluted with dichloromethane (250 ml), filtered through Celite, washed with water, dried (MgSO₄), and concentrated. Column chromatography (dichloromethane:acetone, 9:1 by vol) of the residue gave 1, isolated as a white solid (277 mg, 85%), *R*_F 0.57.

To a mixture of 1 (270 mg, 0.32 mmol) and 1,4-diazabicyclo[2.2.2]octane (15 mg, 0.13 mmol) in 16 ml ethanol:toluene:water, 7:3:1 by vol, was added tris(triphenylphosphine)rhodium(I) chloride (42 mg, 45 μ mol) [11], and the mixture was boiled under reflux for 1.5 h, then cooled, and concentrated. The residue was dissolved in 12 ml acetone:water, 9:1 by vol, containing mercuric chloride (440 mg, 1.62 mmol) and mercuric oxide (5 mg, 0.02 μ mol), and the solution was stirred for 1 h, when TLC (dichloro-

methane:acetone, 9:1 by vol), showed the deallylation to be complete. The solvent was evaporated and the residue diluted with dichloromethane (250 ml). The solution was washed with aqueous 30% potassium bromide (2 \times 25 ml), and water, dried (MgSO₄), and concentrated. Column chromatography (dichloromethane:acetone, 9:1 by vol) of the residue gave 2, isolated as a syrup (153 mg, 60%), *R*_F 0.27.

A solution of 2 (153 mg, 0.19 mmol) and hydrazine acetate (524 mg, 5.7 mmol) [12] in ethanol (20 ml) was boiled under reflux for 16 h, then concentrated, and the residue was taken up in 10 ml pyridine:acetic anhydride, 1:1 by vol, and kept at room temperature for 16 h. Then TLC [light petroleum (BP 40–60 °C):ethyl acetate, 7:3 by vol] showed the acetylation to be complete, and the mixture was concentrated, and co-concentrated with toluene (3 \times 10 ml), diluted with ethyl acetate (250 ml), washed with water, dried (MgSO₄), and concentrated. Column chromatography [light petroleum (BP 40–60 °C):ethyl acetate, 1:4 by vol] of the residue gave 3, isolated as a syrup (139 mg, 98%). *R*_F 0.09 [light petroleum (BP 40–60 °C):ethyl acetate, 3:7 by vol]. NMR data (C²HCl₃): ¹³C, δ 170.5, 170.2, 170.0, 169.7 and 169.4 (5 COCH₃), 138.8, 137.7 and 128.4–127.5 (C₃H₆CH₂O), 100.7 (C-1'), 97.7 (C-1), 78.1, 77.1, 74.0, 72.0, 70.9, 68.9 and 65.7 (C-3,4,5,2',3',4',5'), 73.3, 68.4 (2 C) and 62.1 (C-6,6' and 2 PhCH₂O), 56.4 (C-2 and CH₃O), 23.3 (NHCOCH₃), 20.5 (COCH₃).

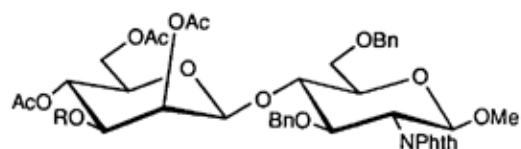
Methyl 2-acetamido-2-deoxy-4-O- β -D-mannopyranosyl- β -D-glucopyranoside (A1)

A solution of 3 (139 mg, 0.186 mmol) in 10 ml ethanol:ethyl acetate, 1:1 by vol, containing acetic acid (50 μ l) and 10% Pd-C (60 mg), was hydrogenolysed for 1 h at atmospheric pressure, then filtered, concentrated, and co-concentrated with toluene (2 \times 10 ml). The residue was dissolved in methanol (6 ml), and sodium methoxide was added until pH 8. The solution was stirred for 16 h, when TLC (n-butanol:water:acetic acid, 2:1:1 by vol) showed the de-O-acetylation to be complete, neutralized with Dowex 50W (H⁺) resin, filtered, and concentrated to give A1, isolated as a white solid (71 mg, 94%), [α]_D²⁵ -54° (c 0.1, water), literature [α]_D²⁵ -45.5° (c 0.725, methanol:water, 1:1 by vol) [13], *R*_F 0.41. For ¹H-NMR data, see Table 1.

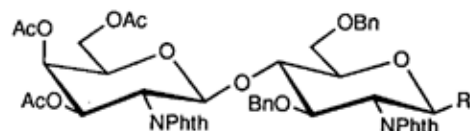
Analysis: Calculated for C₁₅H₂₇NO₁₁: C, 45.34; H, 6.85. Found: C, 45.42; H, 6.88.

Ethyl 4-O-(3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido- β -D-galactopyranosyl)-3,6-di-O-benzyl-2-deoxy-2-phthalimido-1-thio- β -D-glucopyranoside (4)

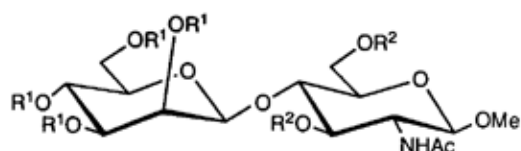
A solution of ethyl 3,6-di-O-benzyl-2-deoxy-2-phthalimido-1-thio- β -D-glucopyranoside (1.9 g, 3.6 mmol) [9] and 3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido- α -D-galactopyranosyl bromide (2.6 g, 5.3 mmol) [14] in 40 ml dichloromethane:toluene, 1:1 by vol, containing powdered 4 Å molecular sieves (7.5 g), was stirred for 30 min under nitrogen. A solution of silver triflate (1.7 g, 6.6 mmol) in



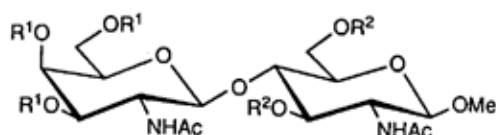
1 R = All
2 R = H



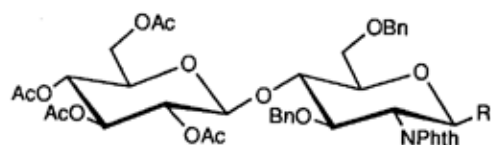
4 R = SEt
5 R = OMe



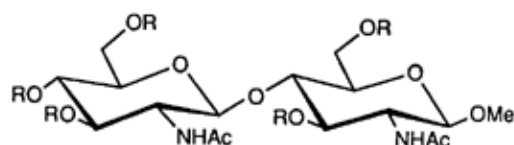
3 R¹ = Ac, R² = Bn
A1 R¹, R² = H



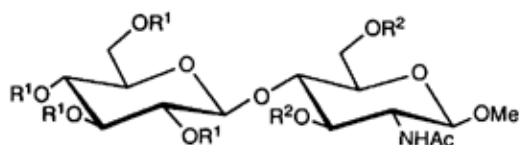
6 R¹ = Ac, R² = Bn
A2 R¹, R² = H



7 R = SEt
8 R = OMe



10 R = Ac
A4 R = H



9 R¹ = Ac, R² = Bn
A3 R¹, R² = H

toluene (40 ml) was added dropwise in the dark during 1 h at -40°C , and stirring was continued for 1 h at -40°C . Then TLC [light petroleum (BP $40-60^{\circ}\text{C}$):ethyl acetate, 3:2 by vol] showed the complete disappearance of the thioglycoside (R_F 0.55) and the formation of 4 (R_F 0.22). Pyridine (3 ml) was added, and the mixture was diluted with dichloromethane (500 ml), filtered through Celite, washed with aqueous 10% sodium thiosulfate (3×15 ml), and water, dried (MgSO_4), and concentrated. Column chromatography [light petroleum (BP $40-60^{\circ}\text{C}$):ethyl acetate, 3:2 by vol] of the residue gave 4, isolated as a syrup (2.8 g, 80%), $[\alpha]_D^{+7}$ (c 1, chloroform), R_F 0.22. NMR data (C^2HCl_3): ^1H , δ 7.93–7.65 and 7.36–6.91 (2 m, 18 H, 2 Ph and 2 Phth), 5.789 (dd, 1 H, H-3'), 5.503 (d, 1 H, H-1'), 5.375 (broad d, 1 H, H-4'), 5.100 (d, 1 H, H-1), 2.559 (m, 2 H, $\text{CH}_3\text{CH}_2\text{S}$), 2.070, 2.006 and 1.823 (3 s, each 3 H, 3 Ac), 1.110 (t, 3 H, $\text{CH}_3\text{CH}_2\text{S}$), $J_{1,2}$ 9.9, $J_{1',2'}$ 8.4, $J_{2',3'}$ 11.5, $J_{3',4'}$ 3.5 Hz; ^{13}C , δ 170.1 (2 C) and 169.6 (3 COCH_3), 168.2, 167.9 and 167.4 (2 C) (4 CO Phth), 138.4, 138.2, 134.3, 133.6, 131.5, 131.3, 128.2–126.9, 123.7 and 123.4 ($\text{C}_6\text{H}_5\text{CH}_2\text{O}$ and Phth),

97.1 (C-1'), 80.8, 78.6, 77.7, 75.8, 70.4, 67.7 and 66.4 (C-1,3,4,5,3',4',5'), 74.1, 72.6, 68.1 and 60.7 (C-6,6' and 2 PhCH_2O), 54.6 and 51.9 (C-2,2'), 23.6 ($\text{CH}_3\text{CH}_2\text{S}$), 20.5 (2 C) and 20.3 (3 COCH_3), 14.7 ($\text{CH}_3\text{CH}_2\text{S}$).

Analysis: Calculated for $\text{C}_{50}\text{H}_{50}\text{N}_2\text{O}_{15}\text{S}$: C, 63.15; H, 5.30. Found: C, 62.61; H, 5.29.

Methyl 4-O-(3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido- β -D-galactopyranosyl)-3,6-di-O-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside (5)

A solution of 4 (510 mg, 0.54 mmol) in toluene (13 ml) and methanol (330 μl), containing powdered 4 Å molecular sieves (1.3 g) was stirred for 30 min under nitrogen. Methyl triflate (300 μl , 2.7 mmol) [10] was added, and the mixture was stirred for 2 h at 50°C , when TLC [light petroleum (BP $40-60^{\circ}\text{C}$):ethyl acetate, 1:1 by vol] showed the disappearance of the starting compound and the formation of a new product. After the addition of triethylamine (1 ml), the mixture was diluted with dichloromethane (200 ml), filtered through Celite, washed with water, dried (MgSO_4),

and concentrated. Column chromatography (dichloromethane:acetone, 95:5 by vol) of the residue gave **5**, isolated as a syrup (430 mg, 87%), R_F 0.38. NMR data (C^2HCl_3): 1H , δ 7.92–7.62 and 7.48–6.84 (2 m, 18 H, 2 Ph and 2 Phth), 5.788 (dd, 1 H, H-3'), 5.496 (d, 1 H, H-1'), 5.373 (broad d, 1 H, H-4'), 4.899 (d, 1 H, H-1), 4.883 and 4.496 (2 d, each 1 H, $PhCH_2O$), 3.295 (s, 3 H, CH_3O), 2.077, 2.010 and 1.827 (3 s, each 3 H, 3 Ac), $J_{1,2}$ 8.4, $J_{1',2'}$ 8.4, $J_{2',3'}$ 11.5, $J_{3',4'}$ 3.4 Hz.

Methyl 2-acetamido-4-O-(2-acetamido-2-deoxy- β -D-galactopyranosyl)-2-deoxy- β -D-glucopyranoside (A2)

A solution of **5** (425 mg, 0.46 mmol) and hydrazine acetate (2.6 g, 28.2 mmol) [12] in ethanol (75 ml) was boiled under reflux for 16 h, then concentrated, and the residue was taken up in 20 ml pyridine:acetic anhydride, 1:1 by vol, and kept at room temperature for 16 h. Then TLC (dichloromethane:acetone, 7:3 by vol) showed the acetylation to be complete, and the mixture was concentrated, and co-concentrated with toluene (3 \times 10 ml), diluted with ethyl acetate (250 ml), washed with water, dried ($MgSO_4$), and concentrated. Column chromatography (dichloromethane:acetone, 7:3 by vol) of the residue gave **6**, isolated as a syrup (295 mg, 85%), R_F 0.26.

A solution of **6** (290 mg, 0.39 mmol) in 10 ml ethanol:ethyl acetate, 1:1 by vol, containing acetic acid (50 μ l) and 10% Pd-C (90 mg) was hydrogenolysed for 1 h at atmospheric pressure, then filtered, concentrated, and co-concentrated with toluene (2 \times 10 ml). Part of the residue (50 mg, 77 μ mol) was dissolved in methanol (5 ml), and sodium methoxide was added until pH 8. The solution was stirred for 16 h, when TLC (n-butanol:water:acetic acid, 2:1:1 by vol) showed the de-O-acetylation to be complete, neutralized with Dowex 50W (H^+) resin, filtered, and concentrated to give **A2**, isolated as a white solid (31 mg, 92%), $[\alpha]_D -3^\circ$ (c 0.3, water), R_F 0.36. NMR data (2H_2O): ^{13}C , δ 176.0 and 175.9 (2 $COCH_3$), 103.2 and 103.0 (C-1,1'), 80.5, 76.6, 75.8, 73.9, 72.0 and 68.9 (C-3,4,5,3',4',5'), 62.2 and 61.4 (C-6,6'), 58.4, 56.0 and 53.8 (C-2,2' and CH_3O), 23.5 ($COCH_3$). For 1H -NMR data, see Table 1.

Analysis: Calculated for $C_{17}H_{30}N_2O_{11} \cdot \frac{1}{2}H_2O$: C, 45.6; H, 7.0. Found: C, 45.2; H, 7.2.

Methyl 4-O-(2,3,4,6-tetra-O-acetyl- β -D-glucopyranosyl)-3,6-di-O-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside (8)

A solution of ethyl 3,6-di-O-benzyl-2-deoxy-2-phthalimido-1-thio- β -D-glucopyranoside (533 mg, 1.0 mmol) [9] and 2,3,4,6-tetra-O-acetyl- α -D-glucopyranosyl bromide (1.3 g, 3.2 mmol) in 20 ml dichloromethane:toluene, 1:1 by vol, containing powdered 4 Å molecular sieves (4 g), was stirred for 30 min under nitrogen. A solution of silver triflate (1.1 g, 4.3 mmol) in toluene (20 ml) was added dropwise in the dark during 2 h at $-40^\circ C$, and stirring was continued for 2 h at $-40^\circ C$. Then TLC [light petroleum (BP 40–60 $^\circ C$):

ethyl acetate, 3:2 by vol] showed the complete disappearance of the thioglycoside (R_F 0.55) and the formation of **7** (R_F 0.29). Pyridine (2.5 ml) was added, and the mixture was diluted with dichloromethane (100 ml), filtered through Celite, washed with aqueous 10% sodium thiosulfate (3 \times 15 ml), and water, dried ($MgSO_4$), and concentrated. Column chromatography [light petroleum (BP 40–60 $^\circ C$): ethyl acetate, 3:2 by vol] of the residue gave **7**, isolated as a syrup (839 mg, 97%), R_F 0.29.

A solution of **7** (830 mg, 0.96 mmol) in toluene (20 ml) and methanol (1 ml), containing powdered 4 Å molecular sieves (2.5 g) was stirred for 30 min under nitrogen. Methyl triflate (550 μ l, 5.0 mmol) [10] was added, and the mixture was stirred for 1 h at 50 $^\circ C$, when TLC [light petroleum (BP 40–60 $^\circ C$):ethyl acetate, 3:2 by vol] showed the disappearance of the starting compound and the formation of a new product. After the addition of triethylamine (1.5 ml), the mixture was diluted with dichloromethane (200 ml), filtered through Celite, washed with water, dried ($MgSO_4$), and concentrated. Column chromatography [light petroleum (BP 40–60 $^\circ C$):ethyl acetate, 3:2 by vol] of the residue gave **8**, isolated as a syrup (699 mg, 87%), R_F 0.24. NMR data (C^2HCl_3): 1H , δ 7.68–6.79 (m, 14 H, 2 Ph and Phth), 4.835 and 4.522 (2 d, each 1 H, $PhCH_2O$), 4.787 and 4.385 (2 d, each 1 H, $PhCH_2O$), 4.640 (d, 1 H, H-1), 3.371 (s, 3 H, CH_3O), 2.022, 2.012, 1.996 and 1.952 (4 s, each 3 H, 4 Ac), $J_{1,2}$ 7.7 Hz.

Methyl 2-acetamido-2-deoxy-4-O- β -D-glucopyranosyl- β -D-glucopyranoside (A3)

A solution of **8** (699 mg, 0.84 mmol) and hydrazine acetate (2.3 g, 25.0 mmol) [12] in ethanol (100 ml) was boiled under reflux for 16 h, then concentrated. The residue was taken up in 20 ml pyridine:acetic anhydride, 1:1 by vol, and kept at room temperature for 16 h. TLC [light petroleum (BP 40–60 $^\circ C$):ethyl acetate, 3:7 by vol] showed the acetylation to be complete, and the mixture was concentrated, and co-concentrated with toluene (3 \times 10 ml), diluted with ethyl acetate (250 ml), washed with water, dried ($MgSO_4$), and concentrated. Column chromatography [light petroleum (BP 40–60 $^\circ C$):ethyl acetate, 3:7 by vol] of the residue gave **9**, isolated as a white amorphous solid (610 mg, 97%), R_F 0.15.

A solution of **9** (610 mg, 0.82 mmol) in 20 ml ethanol:ethyl acetate, 1:1 by vol, containing acetic acid (50 μ l) and 10% Pd-C (200 mg), was hydrogenolysed for 1 h at atmospheric pressure, then filtered, concentrated, and co-concentrated with toluene (2 \times 10 ml). Part of the residue (60 mg, 0.11 mmol) was dissolved in methanol (6 ml), and sodium methoxide was added until pH 8. The solution was stirred for 16 h, when TLC (n-butanol:water:acetic acid, 2:1:1 by vol) showed the de-O-acetylation to be complete, neutralized with Dowex 50W (H^+) resin, filtered, and concentrated to give **A3**, isolated as a white solid (41 mg, 97%), $[\alpha]_D -18^\circ$ (c 0.1, water), R_F 0.43. For 1H -NMR data, see Table 1.

Analysis: Calculated for $C_{15}H_{27}NO_{11} \cdot \frac{1}{2}H_2O$: C, 44.33; H, 6.94. Found: C, 43.98; H, 6.96.

Methyl 2-acetamido-4-O-(2-acetamido-2-deoxy- β -D-glucopyranosyl)-2-deoxy- β -D-glucopyranoside (A4)

A solution of *N,N'*-diacetylchitobiose hexaacetate (Sigma) (50 mg, 74 μ mol) in acetic acid saturated with hydrogen bromide (33 wt%, 1.2 ml), was stirred for 16 h [15], then diluted with ice cold dichloromethane (50 ml), and washed with ice cold aqueous 5% sodium hydrogencarbonate, and water, dried ($CaCl_2$), and concentrated. A solution of the residue in dichloromethane (2 ml) was added to a stirred mixture of mercuric cyanide (100 mg, 0.4 mmol), methanol (0.5 ml), and 3 Å molecular sieves (100 mg) in dichloromethane (1 ml). After stirring for 16 h, when TLC (R_F 0.21, dichloromethane:acetone:methanol, 6:3:1 by vol) showed the methyl glycoside formation to be complete, the mixture was diluted with dichloromethane (50 ml), filtered through Celite, washed with aqueous 10% potassium iodide (3 \times 10 ml), and water, dried ($MgSO_4$), and concentrated. Column chromatography (dichloromethane:acetone:methanol, 6:3:1 by vol) of the residue gave **10**, isolated as a white solid (30 mg, 62%). The product was dissolved in methanol (4 ml), and sodium methoxide was added until pH 8. After stirring for 16 h, when TLC (n-butanol:water:acetic acid, 2:1:1 by vol) showed the de-*O*-acetylation to be complete, the solution was neutralized with Dowex 50W (H^+) resin, filtered, and concentrated to give **A4**, isolated as a white solid (15 mg, 74%), $[\alpha]_D^{25} -21^\circ$ (*c* 0.07, water), literature $[\alpha]_D^{26} -31^\circ$ (*c* 0.28, water) [16], literature $[\alpha]_D^{25} -27^\circ$ (*c* 0.5, water) [17], R_F 0.40. For 1H -NMR data, see Table 1.

Analysis: Calculated for $C_{17}H_{30}N_2O_{11}$: C, 46.57; H, 6.90. Found: C, 46.86; H, 7.09.

Incubations and isolation of the incubation products

The synthetic disaccharides (1.0 mg) were each dissolved in 1.0 ml 0.2 M Tris-maleate buffer, pH 6.7: glycerol, 4:1 by vol, containing 1.0 mg bovine serum albumin, and 2.5 mg CMP-Neu5Ac was added. The mixtures were incubated with 50 mU (25 μ l) α (2-6)-sialyltransferase for 24 h at 37 °C. To follow the course of the reaction, aliquots (10 μ l) of each of the solutions were taken for HPLC analysis at 0, 1, 2, 4, 8 and 24 h. The incubations were stopped by cooling to -20 °C.

HPLC analysis was performed isocratically on a Partisil 10 SAX column (250 mm \times 4.6 mm, Whatman) using 30 mM KH_2PO_4 , pH 4.7:acetonitrile, 25:75 by vol, as elution system, at a flow rate of 2 ml min^{-1} . The Kratos Spectroflow 400 solvent delivery system was equipped with a Kratos Spectroflow 757 absorbance detector connected to a Kratos SP 4290 integrator. Absorbance was monitored at 205 nm. After preparative runs, fractions were desalted on a column (40 cm \times 1.0 cm) of Bio-Gel P-2 (200–400 mesh). Quantifications were carried out on analytical runs, taking into account the number of C=O groups in the remaining acceptor and

the formed product, which are responsible for the UV absorbance [18].

TLC was performed on Merck Plastikfolien Kieselgel 60 (0.2 mm, 10 \times 10 cm) eluted with n-butanol:acetic acid: water, 2:1:1 by vol. The plates were stained with orcinol: H_2SO_4 to detect hexose-containing compounds, or with orcinol: $FeCl_3$:HCl to visualize sialic acid containing compounds [19].

1H -NMR spectroscopy

1H -NMR spectra (300, 360 or 500 MHz) of oligosaccharide derivatives were recorded at 25 °C on Bruker AC 300, HX 360 or AM 500 spectrometers, respectively. ^{13}C -NMR spectra (APT, 50 MHz) were recorded at 25 °C on a Bruker WP 200 spectrometer. Chemical shifts are given in ppm relative to internal Me_4Si (C^2HCl_3) for 1H , and to internal Me_4Si (C^2HCl_3 ; indirectly to C^2HCl_3 , δ 76.9) or external Me_4Si (2H_2O ; indirectly to internal acetone, δ 31.55) for ^{13}C .

Prior to 1H -NMR spectroscopy, underivatized oligosaccharides were repeatedly treated with 2H_2O , finally using 99.96 atom % 2H_2O (MSD isotopes) at p^2H 7 and room temperature. Resolution-enhanced 1H -NMR spectra of the underivatized oligosaccharides were recorded on a Bruker AM-500 spectrometer at a probe temperature of 27 °C or 37 °C, applying a selective pulse for HO^2H suppression [20]. Chemical shifts are expressed relative to internal acetone (δ 2.225) [21].

Two-dimensional (2D) double-quantum filtered 1H - 1H correlation spectroscopy (2D-DQF 1H - 1H COSY) [22] was performed using a spectral width of 3000 Hz in each dimension. The HO^2H signal was suppressed by presaturation during 1 s. In total, 330–512 spectra of 2048 data points were recorded, with 32–96 scans per t_1 value.

Two-dimensional (2D) homonuclear Hartmann-Hahn spectroscopy (2D-HOHAHA) [23] was carried out, using a MLEV-17 mixing sequence of 100 ms. The 90° pulse was adjusted to 25 μ s and the spectral width was 2500 or 3200 Hz in each dimension. The HO^2H signal was presaturated for 1 s. In total, 512 spectra of 2048 data points were recorded, with 32 or 64 scans per t_1 value.

Results and discussion

Compounds **A1–A4** were incubated with CMP-Neu5Ac and α (2-6)-sialyltransferase, applying conditions under which $Gal\beta 1-4GlcNAc\beta-OEt$ is quantitatively converted into $Neu5Ac\alpha 2-6Gal\beta 1-4GlcNAc\beta-OEt$, as checked by TLC of the incubation mixture after 24 h, showing the presence of a hexose and sialic acid positive band and the absence of acceptor. For each of the incubations of **A1–A4**, HPLC analyses of aliquots of the incubation mixture, taken during the course of the reaction, were carried out. As a typical example, the HPLC analysis of the incubation of $Glc\beta 1-4GlcNAc\beta-OMe$ is illustrated in Fig. 1. In each incubation, the CMP-Neu5Ac concentration gradually decreased until

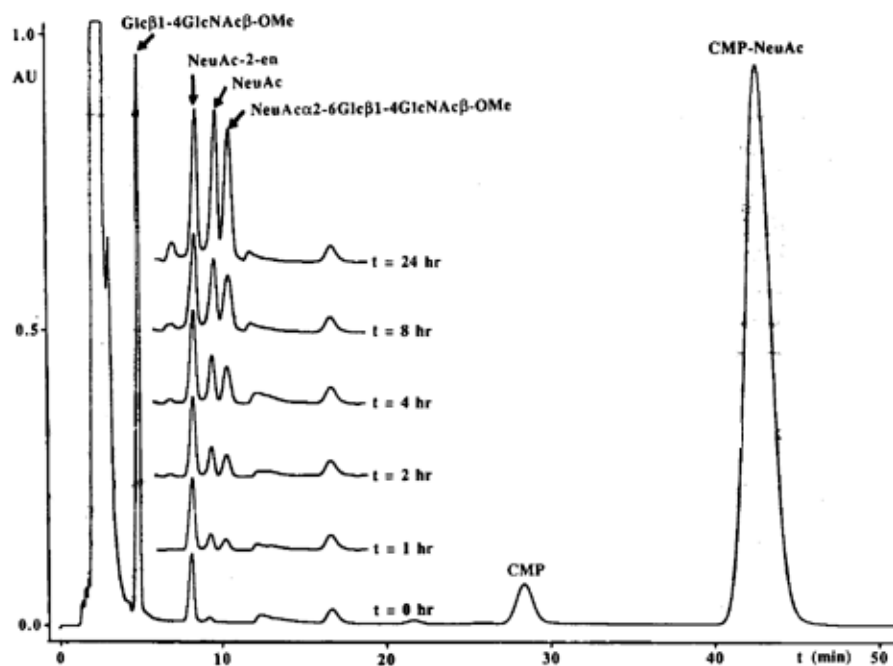


Figure 1. HPLC profile on Partisil 10 SAX of 10- μ l aliquots of the incubation mixture of Glc β 1-4GlcNAc β -OMe, CMP-Neu5Ac and rat liver Gal β 1-4GlcNAc α (2-6)-sialyltransferase, taken during the course of the reaction. The column was eluted with 25 mM KH $_2$ PO $_4$, pH 4.7:acetonitrile, 25:75 by vol, at 2 ml min $^{-1}$ with UV detection at 205 nm. The insert illustrates the time dependent formation of Neu5Ac α 2-6Glc β 1-4GlcNAc β -OMe.

almost zero after 24 h. This is mainly caused by the nonenzymatic hydrolysis of CMP-Neu5Ac, giving rise to the formation of Neu5Ac, Neu5Ac2en and CMP [24]. The peaks appearing after approximately 5, 8 and 9 min represent the acceptor, Neu5Ac2en, and Neu5Ac, respectively. In each case, the compound eluting at 10 min was collected from several HPLC runs and desalted on Bio-Gel P-2. The products obtained in this way from the incubation of A1–A4 were denoted P1–P4 respectively. TLC of P1–P4 showed in each case one band which was hexose and sialic acid positive. P1–P4 were analysed by 500-MHz 1 H-NMR spectroscopy (Fig. 2), and relevant NMR data are given in Table 1.

Compound P1, formed in 4% yield, was identified as Neu5Ac α 2-6Man β 1-4GlcNAc β -OMe. The 1 H-NMR spectrum of P1 shows structural-reporter-group signals characteristic for the presence of an α -linked Neu5Ac residue (H-3a, δ 1.721; H-3e, δ 2.687; NAc, δ 2.030). These values are comparable with those of the Neu5Ac residue in Neu5Ac α 2-6Man β 1-4GlcNAc, isolated from β -mannosidosis urine (H-3a, δ 1.724; H-3e, δ 2.698; NAc, δ 2.033) [5]. Going from A1 to P1, similar shifts for Man H-1 ($\Delta\delta$ -0.015), Man H-5 ($\Delta\delta$ +0.086) and GlcNAc NAc ($\Delta\delta$ +0.022) were observed as found upon α (2-6)-sialylation of Man β 1-4GlcNAc [6].

Compound P2, formed in 48% yield, was identified as Neu5Ac α 2-6GalNAc β 1-4GlcNAc β -OMe. In the 1 H-NMR spectrum of P2, the H-3a and H-3e signals of Neu5Ac at δ 1.698 and δ 2.658, respectively, are indicative for Neu5Ac

in α (2-6)-linkage to *N*-acetylgalactosamine. For the Neu5Ac α 2-6GalNAc β 1-4GlcNAc β 1-2Man α 1-3 element, Neu5Ac H-3a and H-3e signals were reported at δ 1.719 and δ 2.660, respectively [3]. Both sets of values are closely related to those generally found for Neu5Ac in α (2-6)-linkage to Gal β 1-4GlcNAc β 1-R (H-3a, δ 1.70–1.72; H-3e, δ 2.67) [21]. The chemical shifts of the GalNAc H-1 and NAc signals of P2 match those reported in [3]. In a similar way as observed for the step A1 \rightarrow P1, clear downfield shift effects were observed for GlcNAc H-1 ($\Delta\delta$ +0.026) and NAc ($\Delta\delta$ +0.030), going from A2 to P2.

Product P3, formed in 16% yield, was identified as Neu5Ac α 2-6Glc β 1-4GlcNAc β -OMe and was formed in 16% yield. Compared with the Neu5Ac structural-reporter-group signals in synthetic Neu5Ac α 2-6Glc β -OMe (H-3a, δ 1.752; H-3e, δ 2.713; NAc, δ 2.018, corrected for acetone at δ 2.225) [25], the H-3a (δ 1.709) and H-3e (δ 2.686) signals in P3 differ significantly. To prove the formation of P3, 2D-HOHAHA (Fig. 3) and 2D-COSY experiments were carried out on both A3 and P3, affording the complete assignment of the H-atoms of glucose in both compounds, whereby the H-6 $_{proR}$ and H-6 $_{proS}$ protons were assigned according to [25]. Compared with A3, Glc H-6 $_{proS}$ in P3 shows an upfield shift of $\Delta\delta$ -0.174, whereas downfield shifts are observed for H-5 ($\Delta\delta$ +0.10) and H-6 $_{proR}$ ($\Delta\delta$ +0.170). These shift effects are similar to those observed when Neu5Ac is α (2-6) linked to Glc β -OMe [25]. Furthermore, the downfield shift effects on the GlcNAc H-1 and NAc

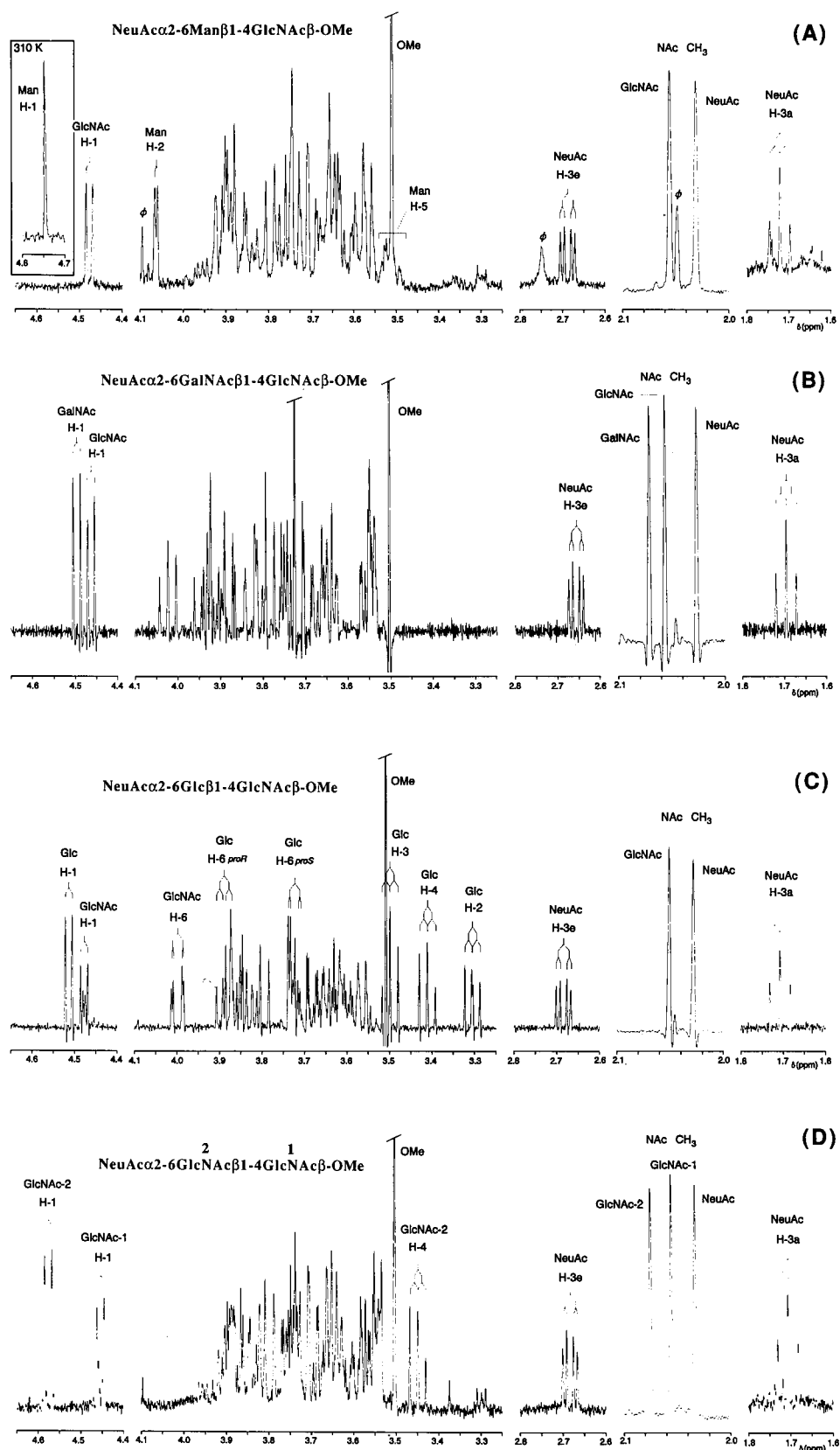
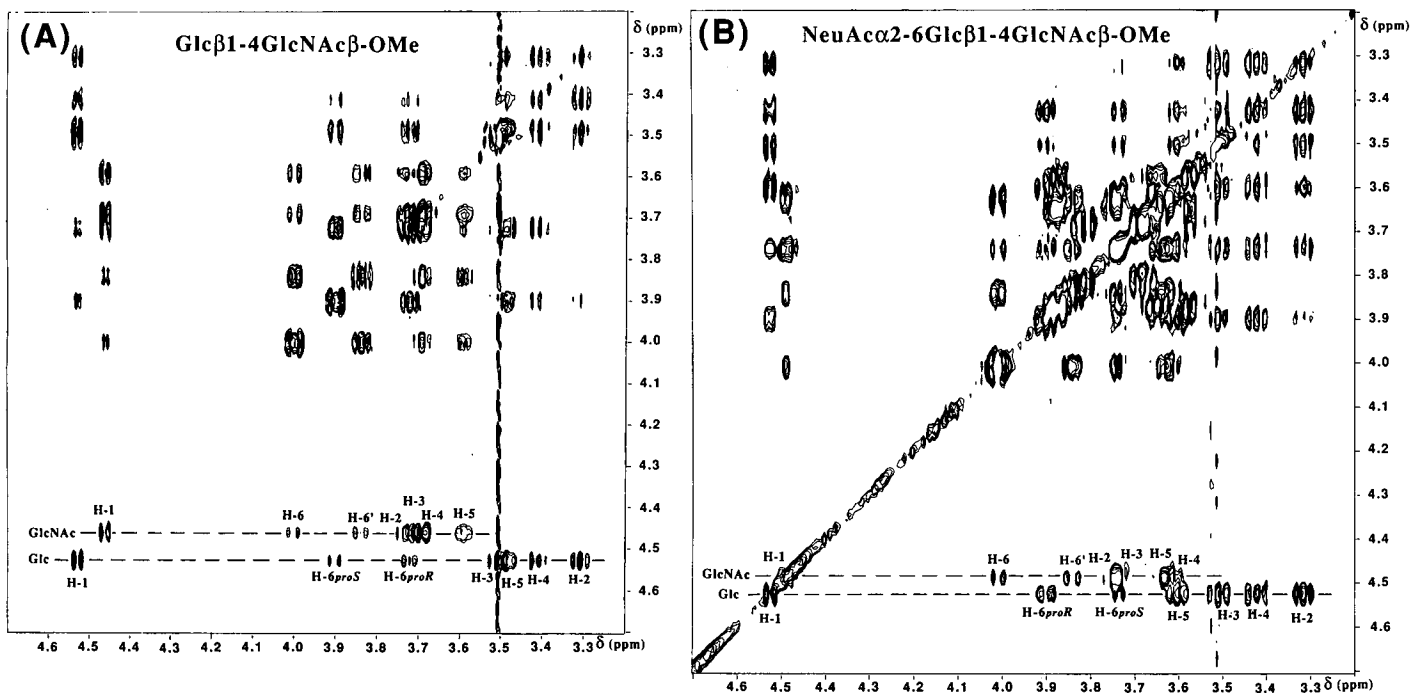


Figure 2. 500-MHz $^1\text{H-NMR}$ spectra of (A) P1, (B) P2, (C) P3 and (D) P4, recorded at 27 °C. Only relevant parts of the spectra are presented. The relative scale of the NAc-methyl protons differs from that of the rest of the spectrum. ϕ indicates a noncarbohydrate contaminant.

Table 1. $^1\text{H-NMR}$ Chemical shifts of structural-reporter-group protons of the constituent monosaccharides of R' β 1-4GlcNAc β -OMe (A1–A4) and Neu5Ac α 2-6R' β 1-4GlcNAc β -OMe (P1–P4). Chemical shifts are given at 27 °C and were measured in $^2\text{H}_2\text{O}$ relative to internal acetone (δ 2.225) [21].

Residue	Reporter group	Chemical shift (ppm) in R' β 1-4GlcNAc β -OMe (A1–A4) or Neu5Ac α 2-6R' β 1-4GlcNAc β -OMe (P1–P4)							
		R' = Man		R' = GalNAc		R' = Glc		R' = GlcNAc	
		A1	P1	A2	P2	A3	P3	A4	P4
GlcNAc	H-1	4.460	4.476	4.437	4.463	4.458	4.479	4.433	4.452
	NAc	2.033	2.055	2.028	2.058	2.030	2.052	2.027	2.054
	OMe	3.505	3.510	3.497	3.505	3.501	3.509	3.496	3.502
R'	H-1	4.768 ^a	4.753 ^a	4.517	4.497	4.526	4.515	4.581	4.575
	H-2	4.063	4.063	n.d. ^b	n.d.	3.307	3.307	n.d.	n.d.
	H-5	3.421	3.507	n.d.	n.d.	3.482	3.58 ^c	n.d.	n.d.
	H-6 $_{\text{proR}}$	n.d.	n.d.	n.d.	n.d.	3.720	3.890	n.d.	n.d.
	H-6 $_{\text{proS}}$	n.d.	n.d.	n.d.	n.d.	3.900	3.726	n.d.	n.d.
	NAc	–	–	2.069	2.073	–	–	2.069	2.073
Neu5Ac	H-3a	–	1.721	–	1.698	–	1.709	–	1.705
	H-3e	–	2.687	–	2.658	–	2.686	–	2.682
	NAc	–	2.030	–	2.028	–	2.030	–	2.031

^a Measured at 37 °C.^b n.d., not determined.^c Value obtained from a 2D-HOHAHA experiment.**Figure 3.** 2D-HOHAHA spectra of (A) Glc β 1-4GlcNAc β -OMe (A3) and (B) Neu5Ac α 2-6Glc β 1-4GlcNAc β -OMe (P3).

signals upon sialylation of A3 ($\Delta\delta +0.021$ and $\Delta\delta +0.022$, respectively) are comparable with those observed for the GlcNAc H-1 and NAc signals in the steps A1 \rightarrow P1 and A2 \rightarrow P2.

Product P4, formed in 8% yield, was identified as Neu5Ac α 2-6GlcNAc β 1-4GlcNAc β -OME. The set of Neu5Ac H-3a, H-3e and NAc signals at δ 1.705, δ 2.682 and δ 2.031, respectively, is similar to that in P3, indicating that Neu5Ac occurs in α (2-6)-linkage. It has to be noted that these values differ significantly from the set reported for Neu5Ac in α (2-6) linkage to the internal *N*-acetylglucosamine residue in the Gal β 1-3GlcNAc β 1-4Man element (H-3a, δ 1.76–1.77; H-3e, δ 2.72–2.73; NAc, δ 2.03) [26, 27]. The downfield shift effects on the H-1 ($\Delta\delta +0.019$) and NAc ($\Delta\delta +0.027$) signals of GlcNAc β -OME in the step A4 \rightarrow P4 are comparable with those observed for A1 \rightarrow P1, A2 \rightarrow P2 and A3 \rightarrow P3.

In summary, the highest yield of Neu5Ac transfer was obtained when using GalNAc β 1-4GlcNAc β -OME as an acceptor. The Neu5Ac α 2-6GalNAc β 1-4GlcNAc β 1-2Man α 1-R element has been found recently in *N*-glycans of human lutropin [3] and Bowes melanoma tissue plasminogen activator [4]. The possibility of transferring Neu5Ac to *N*-acetylgalactosamine in a relatively high yield *in vitro* raises the question of whether the same enzyme is responsible for the sialylation of both Gal β 1-4GlcNAc β -R and GalNAc β 1-4GlcNAc β -R elements *in vivo*. Neu5Ac α 2-6Glc β 1-4GlcNAc β -OME was also formed in relatively high yield. In addition to the method presented in this paper, the Neu5Ac α 2-6Glc element can be prepared by employing the reverse hydrolysis or transglycosylation activity of immobilized sialidase [28].

The finding that Neu5Ac can be transferred to the terminal monosaccharides galactose, mannose, *N*-acetylgalactosamine, glucose and *N*-acetylglucosamine (R') in R' β 1-4GlcNAc β -OME indicates that certain modifications at C-2 and/or C-4 of the terminal monosaccharide do not exclude the action of Gal β 1-4GlcNAc α (2-6)-sialyltransferase, but the activity is severely influenced in terms of yield. To obtain detailed information concerning the enzyme-acceptor interaction on the molecular level, currently studies are focused on the synthesis of a wider range of possible substrates containing structural variants of the galactose residue, as well as on the kinetics of the transfer reaction.

The potency of rat liver Gal β 1-4GlcNAc α (2-6)-sialyltransferase to transfer Neu5Ac in α (2-6)-linkage to other β (1-4)-linked monosaccharides than galactose as demonstrated here, can be exploited for the synthesis on a preparative scale of various sialylated compounds.

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References

1. Corfield AP, Schauer R (1982) In *Sialic Acids* (Schauer R, ed) pp 5–50. Vienna: Springer-Verlag.
2. Broquet P, Baubichon-Cortay H, George P, Louisot P (1991) *Int J Biochem* **23**:385–89.
3. Weisshaar G, Hiyama J, Renwick AGC, Nimtz H (1991) *Eur J Biochem* **195**:257–68.
4. Chan AL, Morris HR, Panico M, Etienne AT, Rogers ME, Gaffney P, Creighton-Kempsford L, Dell A (1991) *Glycobiology* **1**:173–85.
5. Van Pelt J, Dorland L, Duran M, Hokke CH, Kamerling JP, Vliegthart JFG (1990) *J Biol Chem* **265**:19685–89.
6. Van Pelt J, Dorland L, Duran M, Hokke CH, Kamerling JP, Vliegthart JFG (1989) *FEBS Lett* **256**:179–84.
7. Weinstein J, De Souza-e-Silva U, Paulson JC (1982) *J Biol Chem* **257**:13845–53.
8. Hokke CH, Van der Ven JGM, Kamerling JP, Vliegthart JFG (1991) *Glycoconjugate J* **8**:259.
9. Kerékgyártó J, Van der Ven JGM, Kamerling JP, Lipták A, Vliegthart JFG (1993) *Carbohydr Res* **238**:135–45.
10. Lönn H (1987) *J Carbohydr Chem* **6**:301–6.
11. Corey EJ, Suggs JW (1973) *J Org Chem* **38**:3224.
12. Lee H-H, Schwartz DA, Harris JF, Carver JP, Krepinsky JJ (1986) *Can J Chem* **64**:1912–18.
13. Bush CA, Dua VK, Ralapati S, Warren CD, Spik G, Strecker G, Montreuil J (1982) *J Biol Chem* **257**:8199–204.
14. Paulsen H, Bünsch A (1982) *Carbohydr Res* **100**:143–67.
15. Tarasiejska Z, Jeanloz RW (1958) *J Am Chem Soc* **80**:6325–27.
16. Zehavi U, Jeanloz RW (1968) *Carbohydr Res* **6**:129–37.
17. Grivet J-P, Delmotte F, Monsigny M (1978) *FEBS Lett* **88**:176–80.
18. Damm JBL, Voshol J, Hård K, Kamerling JP, Vliegthart JFG (1989) *Eur J Biochem* **180**:101–10.
19. Schauer R (1978) *Methods Enzymol* **50**:64–89.
20. Hård K, Van Zadelhoff G, Moonen P, Kamerling JP, Vliegthart JFG (1992) *Eur J Biochem* **209**:895–915.
21. Vliegthart JFG, Dorland L, Van Halbeek H (1983) *Adv Carbohydr Chem Biochem* **41**:209–374.
22. Rance M, Sørensen OW, Bodenhausen G, Wagner G, Ernst RR, Wüthrich K (1983) *Biochem Biophys Res Commun* **117**:479–85.
23. Bax A, Davis DG (1985) *J Magn Reson* **65**:355–60.
24. Beau J-M, Schauer R, Haverkamp J, Kamerling JP, Dorland L, Vliegthart JFG (1984) *Eur J Biochem* **140**:203–8.
25. Ohru H, Nishida Y, Itoh H, Meguro H (1991) *J Org Chem* **56**:1725–31.
26. Cumming DA, Hellerqvist CG, Harris-Brandts M, Michnick SW, Carver JP, Bendiak B (1988) *Biochemistry* **28**:6500–12.
27. Green ED, Adelt G, Baenziger JU, Wilson S, Van Halbeek H (1988) *J Biol Chem* **263**:18253–68.
28. Thiem J, Sauerbrei B (1991) *Angew Chem Int Ed Engl* **30**:1503–5.