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Combined Chemical and Enzymatic Synthesis of the Sialylated Non Reducing Terminal Sequence of GM_{1b} Glycolylated Ganglioside, a Potential Human Tumor Marker

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Abstract—*N*-Glycolylglucosamine **8** was synthesized in 4 steps from anisal glucosamine, via the new crystalline monochloracetyl derivatives **3**, **4** and **7**. *N*-Glycolylneuraminic acid **10** was prepared in 59 % yield starting from pyruvate and a mixture of **8** and its manno epimer **9** in a 2:3 ratio, with immobilized sialic acid aldolase. Neu5Gc **10** was converted into CMP–NeuGc **11** in the presence of immobilized calf brain CMP–sialate synthetase. Finally **11** was used as a donor in the transfer to the acceptor β-D-Gal-(1–3)-β-D-GalNAc-OBn **12** catalyzed by a preparation of porcine liver (2–3)-α-sialyltransferase, roughly purified by a chromatography on Cibacron Blue–agarose. α-Neu5Gc-(2–3)-β-D-Gal-(1–3)-β-D-GalNAc-OBn **13** isolated in 56 % yield was deprotected to give the non-reducing terminal sequence of GM_{1b} glycolylated ganglioside, which might be expressed in human tumors.

Introduction

N-Acetylneuraminic acid (Neu5Ac) is the biosynthetic precursor of all other members of the sialic acids family,[1] which includes *N*-glycolylneuraminic acid (Neu5Gc) resulting from hydroxylation of the *N*-acetyl group. It is noteworthy that *in vivo* conversion of Neu5Ac into Neu5Gc occurs exclusively at the level of activated sialic acid, the CMP–*N*-acetylneuraminic acid, by action of *N*-acetylneuraminate monooxygenase.[2,3] The relative contents of Neu5Ac and Neu5Gc depend on the organism and tissue. For example high proportions of Neu5Gc are found in mouse serum glycoproteins and in erythrocyte membranes from pig, horse and cow.[4] Neu5Gc also represents a differentiation antigen during rat intestine development [5] and a tumor-associated compound appearing in O-linked oligosaccharides of rat mammary adenocarcinoma cells.[6] Moreover Neu5Gc can be considered as an oncofetal antigen in humans and chickens: indeed it is expressed in fetal human tissue[7] and it has been detected in certain human tumors, whereas it is not found in normal adult human tissue. Neu5Gc turned out to be the essential portion of the determinant of the tumor-associated Hanganutziu–Deicher antigen, characterized from human colon cancer tissues[8] and detected in human retinoblastoma cell lines.[9] However the content of Neu5Gc in such cancerous tissues is extremely low, as has recently been reported;[10] this might explain why the expression of Neu5Gc containing gangliosides in human tumors is still controversial.[11]

As part of our research program related to the synthesis of tumor-associated carbohydrate antigens, we herein describe the first synthesis of α-Neu5Gc-(2–3)-β-D-Gal-(1–3)-D-GalNAc **1**, which has been isolated from porcine submaxillary mucin as an oligosaccharide alditol.[12] The availability of this oligosaccharide may be hepful in inhibition tests, using either murine monoclonal antibodies reacting specifically with gangliosides having α-Neu5Gc-(2–3)Gal as terminal sequences,[13] or lectins binding specifically to Neu5Gc, like the lectin recently purified from the hemolymph of the marine crab *Scylla serrata*.[14]

We first report the chemo-enzymatic synthesis of *N*-glycolylneuraminic acid **10** starting from *N*-glycolylglucosamine **8** synthesized according to a new procedure and involving sialic acid aldolase [EC 4.1.33], then the activation of Neu5Gc as CMP–*N*-glycolylneuraminic acid **11** (CMP–Neu5Gc) with the help of CMP–sialate synthetase [EC 2.7.7.43]. In the third enzymatic step, this nucleotide–sugar was used for the transfer of Neu5Gc to the acceptor β-D-Gal-(1–3)-β-D-GalNAc-OBn **12** catalyzed by porcine liver (2–3)-α-sialyltransferase [EC 2.4.99. 4].[15]

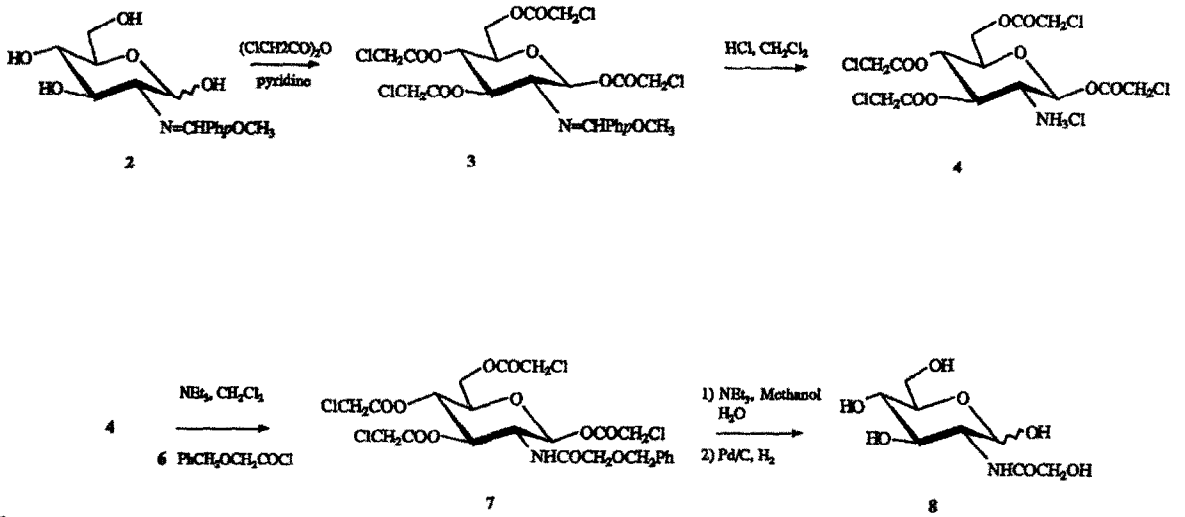
Results and Discussion

Two of us[16] have already reported the possibility of enzymatically synthesizing *N*-glycolylneuraminic acid **10** from an epimeric mixture of *N*-glycolylmannosamine **9** and glucosamine **8** but the preparation of **8** had to be improved. A straightforward synthesis of **8**, easily feasible on a large scale is described here. Monochloroacetyl group was selected as the protecting group on the glucosamine derivative; indeed it can be removed in milder conditions than an acetyl group, avoiding trouble in the deprotection step of peracetylated *N*-glycolylglucosamine, thus affording **8** in a better overall yield. The synthesis was achieved by treatment of anisal glucosamine **2** with monochloroacetic anhydride in pyridine–dichloromethane at 0 °C. The crystalline β-monochloroacetyl **3** was isolated in 68 % yield. Acid hydrolysis of the Schiff base liberated the free amine which crystallized in the reaction mixture as

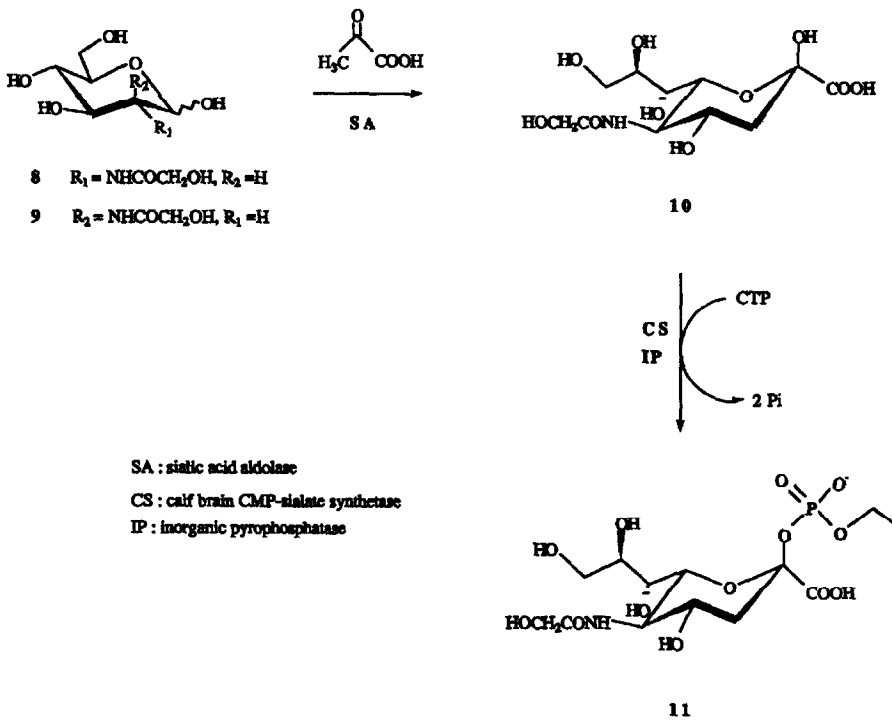
its hydrochloride **4** in 80 % yield. 2-Benzyloxyacetic acid **5** was readily prepared in 66 % yield from a stoichiometric amount of monochloroacetic acid and benzyl alcohol in the presence of two equivalents of sodium hydride in tetrahydrofuran. Acid **5** was converted into its potassium salt and treated with a solution of oxalyl chloride in dichloromethane, affording acid chloride **6** which was used directly in the condensation with glucosamine hydrochloride **4**, in the presence of triethylamine. The reaction proceeded very quickly at room temperature and the condensation product **7** was isolated by crystallization in 67 % yield. Dechloroacetylation could be then achieved at 0 °C, affording after hydrogenolysis pure crystalline *N*-glycolylglucosamine **8** in 82 % yield (Scheme I). Compound **8** was epimerized in alkaline medium, as previously reported.[16] Crystallization of the gluco compound **8** led to a residual syrup containing 60 % of manno compound **9**; it is noteworthy that epimerization of *N*-acetylglucosamine led, after crystallization of the *N*-acetylglucosamine (much easier than the one of the glycolyl analog) to a much higher proportion of manno compound in the residual syrup.[17] The mixture of *N*-glycolylglucosamine **8** and *N*-glycolylmannosamine **9** in a 2:3 ratio and pyruvate were incubated with sialic aldolase immobilized on Eupergit 250L (Scheme II). Immobilization was achieved by stirring the sialic acid aldolase dissolved in 1 M phosphate buffer pH 7.4 with Eupergit 250L at room temperature for 3 days in the presence of pyruvate. In these conditions, 87 % of proteins were bound to the gel and the optimum immobilization yield regarding enzyme activity was 40 %. The same immobilized enzymatic preparation was re-used in three successive runs affording altogether 1 g of *N*-glycolylneuraminic acid **10**. The activated neuraminic acid **11** was prepared by condensation of compound **10** and CTP used in stoichiometric amount in the presence of calf brain CMP–sialic acid synthethase[17] immobilized on agarose previously activated with cyanogen bromide, according to the published procedure,[18,19] and soluble membrane-enclosed inorganic pyrophosphatase[20] (Scheme II).The advantage of the calf brain enzyme compared with the microbial enzymes[21,22] is its broader specificity. CMP–NeuGc **11** was isolated in 60 % yield and the synthesis could be repeated twice with the same enzymatic preparations. The ^{1}H NMR spectrum of **11** was identical with the literature.[17]

The nucleotide–sugar **11** was then used as a donor in the transfer reaction catalyzed by a preparation of porcine liver (2–3)-α-sialyltransferase. We previously reported the partial purification of this enzyme and its use for transferring Neu5Ac from CMP–NeuAc to the disaccharide β-D-Gal-(1–3)-D-GlcNAc isomer of the natural acceptor β-D-Gal-(1–3)-D-GalNAc.[23] This enzyme has now been cloned and expressed in COS-1 cells.[24] The enzymatic preparation used here, was purified by a chromatography on Cibacron Blue F3GA–agarose, used as an alternative to the first affinity chromatography on CDP–hexanolamine–agarose.[24] The major advantages of this procedure, first introduced by Brossmer's group for another sialyltransferase[25] are the low cost of the ligand compared with CDP–hexanolamine and the simplicity of the coupling reaction between ligand and agarose. In this way (2–3)-α-sialyltransferase was purified 5 to 8 fold in 55 % yield. At this stage, contamination by (2–6)-α-sialyltransferase[26] was found to be very low, this probably being due to the much higher instability of the (2–6)-α-sialyltransferase compared with the (2–3) one. This slight contamination however was not troublesome because the acceptor **12** was not a substrate of (2–6)-α-sialyltransferase. This preparation could be lyophilized in the presence of CTP to stabilize the enzyme; the lyophilized powder was taken up in water and used after dialysis for synthetic purposes.

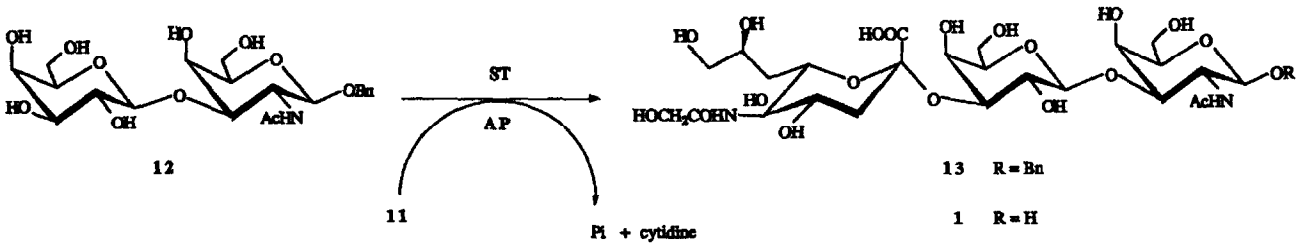
The disaccharide acceptor **12** and a stoichiometric amount of CMP–NeuGc **11** were incubated with the enzymatic preparation and calf intestinal alkaline phosphatase according to Unverzagt *et al.*[17] (Scheme III). From TLC it clearly appeared that after 24 h the reaction had proceeded to a large extent. A small extra amount of **11** was then added and incubation was pursued for 24 h. The sialyldisaccharide was purified by anion-exchange-



Scheme I.



Scheme II.



Scheme III.

chromatography; elution with volatile triethylammonium hydrogencarbonate afforded a large fraction of pure α-Neu5Gc-(2–3)-β-D-Gal-(1–3)-β-D-GalNAc-OBn **13** and two other fractions contaminated respectively by *N*-glycolylneuraminic acid **10** and 2-deoxy-2,3-didehydro-*N*-glycolylneuraminic acid, which required a further purification by flash chromatography on silica gel. Compound **13** was finally obtained in 56 % overall yield and was fully characterized by ^{13}C and ^{1}H NMR spectroscopy, showing typical H-3a and H-3e chemical shifts for a sialyl residue linked in α-(2–3) to galactose. Finally, compound **13** was hydrogenolyzed with 10 % palladium on carbon in aqueous ethanol to give the target compound **1** in quantitative yield. This is the non reducing terminal sequence of GM_{1b} glycolylated ganglioside, which has been found in mouse[13] and might be expressed in human tumors.

Experimental

General

Melting points are uncorrected. Optical rotations were measured with a Jasco digital micropolarimeter. ^{1}H NMR spectra were recorded at 250 MHz with a Bruker AM-250 spectrometer, the chemical shifts are given relative to the signal of tetramethylsilane as external standard (0.2 % solution in $CDCl_3$) for solution in D_2O. ^{13}C NMR spectra were recorded at 50 MHz with a Bruker AM-200 spectrometer; 1,4-dioxane was used as the external standard (δ 66.64 ppm). Reactions were followed by TLC on silica gel plates with fluorescence indicator (Merck). Detection was done by UV absorption and by spraying the plates with 5 % ethanolic sulfuric acid. Silica gel Merck (6–35 μm) was used for flash chromatography. Elemental

analyses were performed by the Laboratoire Central de Micro-analyse du CNRS. Sialic acid aldolase was obtained from Toyobo, inorganic pyrophosphatase and calf intestinal alkaline phosphatase from Sigma. Eupergit 250L was purchased from Röhm Pharma. Cibacron Blue F3GA was obtained from Serva. Ultrogel A4 (4 % agarose) used for coupling with Cibacron Blue F3GA was purchased from IBF.

1, 3, 4, 6-Tetra-O-chloracetyl-2-deoxy-2-N-p-methoxy-phenylmethylidene-β-D-glucopyranose (3)

To the Schiff base **2**,[28] (8.57 g, 32 mmol) in suspension in pyridine (43 mL) and dry CH_2Cl_2 (25 mL) cooled to 0 °C, was added $(ClCH_2CO)_2O$ (30.38 g, 177 mmol) in 30 min. The mixture was kept for 30 min at 0 °C, then diluted with CH_2Cl_2 (100 mL), extracted with cold 2 N HCl, washed with water and evaporated to dryness. Chromatography (dichloromethane) of the residue gave the perchloracetyl **2** which crystallized from ethanol (68 %, 13.8 g), mp 111 °C; $[\alpha]_D^{28}$ +127 (*c* 1.3, dichloromethane) ; ^1H NMR ($CDCl_3$): δ 3.57 (dd, 1H, $J_{1,2}$ = 8.5, $J_{2,3}$ = 9.5 Hz, H-2), 3.85 (s, 3H, OCH_3), 3.87 (s, 2H, $COCH_2Cl$), 4.04 (s, 4H, 2 $COCH_2Cl$), 4.09 (m, 1H, H-5), 4.14 (m, 3 H, $COCH_2Cl$), 4.32 (dd, 1H, $J_{5,6}$ = 2, $J_{6,6'}$ = 12.5 Hz, H-6), 4.47 (dd, 1H, $J_{5,6'}$ = 4.5 Hz, H-6'), 5.22 (t, 1H, $J_{3,4}$ = $J_{4,5}$ = 9.5 Hz, H-4), 5.53 (t, 1H, H-3), 5.98 (d, 1H, H-1), 6.92 (d, 2H, Ph), 7.65 (d, 2H, Ph), 8.18 (s, 1H, PhCH). Anal. calcd for $C_{22}H_{23}NO_{10}Cl_4$: C, 43.80; H, 3.84; N, 2.32; O, 26.52. Found: C, 43.73; H, 3.83; N, 2.12; O, 26.76.

2-Amino-1, 3, 4, 6-tetra-O-chloracetyl-2-deoxy-β-D-glucopyranose hydrochloride (**4**)

8 N HCl (1.76 mL) was added to a solution of derivative **3** (8.47 g, 13.37 mmol) in CH_2Cl_2 (50 mL), heated at 40 °C. The mixture was vigorously stirred for 15 min. Crystallization of hydrochloride **4** occurred and the mixture was allowed to cool to room temperature. Filtration of the solution gave pure **4** (80 %, 6.97 g), mp 189 °C, $[\alpha]_D^{28}$ +35 (*c* 1.8, DMSO); ^1H NMR (DMSO) δ: 3.68 (t, 1H, $J_{1,2}$ = $J_{2,3}$ = 8.5 Hz, H-2), 4.2–4.56 (m, 9H, H-6, H-6', H-5, 3 $COCH_2Cl$), 4.68 (s, 2H, $COCH_2Cl$), 5.14 (t, 1H, $J_{3,4}$ = 8.5 Hz, H-4), 5.54 (t, 1H, H-3), 6.09 (d, 1H, H-1), 8.94 (s, 3H, NH_3^+). Anal. calcd for $C_{15}H_{18}NO_9Cl_5$: C, 32.22; H, 3.48; N, 2.68; O, 27.60; Cl, 34.02. Found: C, 32.33; H, 3.44; N, 2.68; O, 27.74; Cl, 34.03.

2-Benzyloxyacetyl chloride (**6**)

Monochloracetic acid (24.66 g, 261 mmol) and benzyl alcohol (27 mL, 261 mmol) were stirred in tetrahydrofuran (350 mL). NaH (522 mmol) was added portionwise, the mixture stirred at room temperature for 2 h and then heated at reflux overnight. Water was added, tetrahydrofuran was evaporated and the aqueous phase was first extracted with CH_2Cl_2, then acidified with 4 N HCl. Extraction with CH_2Cl_2, washing with water to neutrality and evaporation gave 2-benzyloxyacetic acid **5** (28.5 g, 66 %); ^1H NMR ($CDCl_3$): δ 4.15 (s, 2H, CH_2CO), 4.63 (s, 2H, PhCH_2), 7.35 (m, 5H, Ph).

KOH (9.59 g, 171 mmol), as a powder, was added to the solution of the acid **5** (28.5 g, 171 mmol) in CH_2Cl_2; the mixture was stirred overnight at room temperature, evaporated to dryness and dried. The potassium salt was suspended in dry CH_2Cl_2 and a solution of oxalyl chloride (15 mL, 170 mmol) in dry CH_2Cl_2 was slowly added to this suspension cooled to 0 °C. The mixture was stirred for 1 day at 0 °C. Evaporation of the solvent afforded crude acid chloride **6** (31 g) which was used as such in the condensation with **4**; ^1H NMR ($CDCl_3$): δ 4.41 (s, 2H, CH_2CO), 4.65 (s, 2H, PhCH_2), 7.32 (m, 5H, Ph).

2-[2-(Benzyloxy)-acetamido]-1, 3, 4, 6-tetra-O-chloracetyl-2-deoxy-β-D-glucopyranose (7)

The acid chloride **6** (prepared from 12.25 mmol of **5**) was added to a solution of **4** (4.33 g, 8.17 mmol) and triethylamine (3.4 mL) in CH_2Cl_2. After 30 min at room temperature, the reaction mixture was diluted with CH_2Cl_2 and the organic phase was washed with cold 1M aqueous NaOH, then with water. Evaporation of the solvent afforded compound **7** which crystallized from ethanol (3.53 g, 67 %), mp 162 °C, $[\alpha]_D^{28}$ + 22 (*c* 1.55, dichloromethane); ^1H NMR ($CDCl_3$) : δ 3.93 (s, 2H, CH_2 Ph), 4.02 (s, 4H, 2 $COCH_2Cl$), 4.10 (s, 2H, $COCH_2Cl$), 4.13 (s, 2H, $COCH_2Cl$), 4.22–4.66 (m, 3H, H-6, H-6', H-5), 4.53 (s, 2H, $COCH_2Cl$), 5.21 (t, 1H, $J_{3,4}$ = $J_{4,5}$ = 9.5 Hz, H-4), 5.47 (t, 1H, $J_{2,3}$ = 9.5 Hz, H-3), 5.94 (d, 1H, $J_{1,2}$ = 8.5 Hz, H-1), 6.82 (d, 1H, $J_{NH,2}$ = 9Hz, NH), 7.32 (m, 5H, Ph). Anal. calcd for $C_{23}H_{25}NO_{11}Cl_4$: C, 43.62; H, 3.98; N, 2.21; O, 27.79. Found: C, 43.41; H, 3.92; N, 2.36; O, 28.05.

2-Deoxy-2-[2-(hydroxy)-acetamido]-D-glucopyranose (8)

A mixture of triethylamine–water–methanol (1:1:8; 250 mL) was added to a solution of **7** (13.7 g, 22 mmol) in acetone, cooled to 0 °C. The mixture was kept overnight in the cold. Evaporation to dryness gave 2-[2-(benzyloxy)-acetamido]-2-deoxy-D-glucopyranose, homogenous by TLC (quantitative yield); ^1H NMR (D_2O): δ 3.4–3.97 (m, 6H, H-2, H-3, H-4, H-5, H-6, H-6'), 4.06 (d, 1H, J_{gem} = 12Hz, CH_aCO), 4.16 (d, 1H, CH_bCO), 4.62 (s, 2H, CH_2Ph), 4.74 (d, 0.53H, $J_{1,2}$ = 8.5 Hz, H-1β), 5.16 (d, 0.47H, $J_{1,2}$ = 3.5 Hz, H-1α), 7.41 (m, 5H, Ph).

The above compound (7.2 g) was dissolved in ethanol (100 mL) and hydrogenated at atmospheric pressure over 10 % Pd/C (2.65 g) for 1 day. The catalyst was filtered off and evaporation afforded pure **8** which crystallized on concentration (4.25 g, 82 %), $[\alpha]_D^{28}$ +56 → +30.5 (*c* 1.9, water) mp 187 °C (litt : 184–190 °C);[29] ^1H NMR (D_2O): δ 4.10 (s, 2H, CH_2), 4.76 (d, 0.5H, $J_{1,2}$ = 8 Hz, H-1β), 5.18 (d, 0.5H, $J_{1,2}$ = 3.5 Hz, H-1α).

Epimerization of 2-deoxy-2-[2-(hydroxy)-acetamido]-D-glucopyranose (8) to 2-deoxy-2-[2-(hydroxy)-acetamido]-D-mannopyranose (**9**)

5 M Sodium hydroxide (1.5 mL) was added to an aqueous solution of **8** (3.39 g, 15 mL). The mixture was left at room

temperature for 3 days, then deionized with Amberlite IR 120 resin, and filtrated. The filtrate was concentrated; a first crop of **8** crystallized on concentration (1.15 g). The mother-liquor was again concentrated, a second crop and then a third crop of **8** were obtained (0.86 g). The ratio of **8** to **9** in the residual syrup was estimated from ^{1}H NMR (D_2O) : δ 4.08, 4.09, 4.12 (3s, 2H, CH_2), 4.31 (dd, 0.3H, $J_{2,3}$ = 4.5 Hz, H-2, β-**9**), 4.44 (dd, 0.3H, $J_{2,3}$ = 4.5 Hz, H-2, α-**9**), 4.74 (d, 0.2H, $J_{1,2}$ = 8 Hz, H-1, β-**8**), 5.02 (d, 0.3H, $J_{1,2}$ = 2Hz, H-1, β-**9**), 5.10 (d, 0.3H, $J_{1,2}$ = 1.5 Hz, H-1, α-**9**), 5.10 (d, 0.2H, $J_{1,2}$ = 3.5 Hz, H-1, α-**8**).

*3,5-Dideoxy-5-[2-(hydroxy)-acetamido]-D-*glycero-*D-*galacto-*nonulopyranosylonic acid (10)*

The manno:gluco mixture of **9** and **8** (706 mg) in a 3:2 ratio was incubated with immobilized sialic acid aldolase (8 Units:U), sodium pyruvate (1.6 g), dithiothreitol (0.02 mmol) and NaN_3 (0.02 %) in 0.05 M potassium phosphate pH 7 (20 mL) at 37 °C under nitrogen and with gentle stirring. The reaction was monitored by estimation of neuraminic acid based on the colorimetric reaction with orcinol reagent. After 4 days the enzyme was removed by filtration and compound **10** was purified from the filtrate by anion exchange chromatography on Dowex 1-X8 (formate, 100–200 mesh) using a gradient of formic acid as eluent (340 mg, 59 %), $[\alpha]_D^{28}$ –25 (*c* 0.85, water); ^{1}H NMR (D_2O) δ: 1.83 (t, 1H, $J_{3a,3e} = J_{3a,4}$ = 13 Hz, H-3a), 2.27 (dd, 1H, $J_{3e,4}$ = 4.5 Hz, H-3e), 3.51 (d, 1H, $J_{7,8}$ = 9Hz, H-7), 3.56 (dd, 1H, $J_{9,9'}$ = 11.5, $J_{9,8}$ = 6 Hz, H-9), 3.72 (m, 1H, H-8), 3.80 (dd, 1H, $J_{9',8}$ = 2.5 Hz, H-9'), 3.96 (t, 1H, $J_{4,5} = J_{5,6}$ = 10 Hz, H-5), 4.20–4.04 (m, 4H, CH_2, H-4, H-6); ^{13}C NMR (D_2O) : δ 38.91 (C-3), 51.76 (C-5), 60.98 ($COCH_2OH$), 63.13 (C-9), 66.51 (C-4), 68.14 (C-7), 70.18 (C-6, C-8), 95.40 (C-2), 173.53 (C-1), 175.65 (C=O).

*Cytidine 5'-[3,5-dideoxy-5-(2-hydroxy)-acetamido-β-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid monophosphate] (11)*

Immobilized CMP–sialic acid synthetase (20 U) was incubated with CTP (0.46 mmol) and compound **10** (150 mg, 0.46 mmol) in the presence of thymol (0.1 mmol), 2-mercaptoethanol (0.3 mmol), $MgCl_2$ (3.5 mmol), in 0.1 M Tris buffer pH 9 (final volume: 100 mL). Inorganic pyrophosphatase (20 U) enclosed in a dialysis bag was added and the mixture was gently stirred at 37 °C under nitrogen. The reaction was monitored by HPLC, using a Spherisorb NH_2 column and elution with 7:3, CH_3CN–10 mM phosphate buffer pH 6. After 10 h the reaction was stopped. Enzymes were removed and the filtrate was purified by anion-exchange chromatography on a refrigerated column of DEAE-Sephadex A-25 (HCO_3^-). Elution with a gradient of 0–0.75 M triethylammonium hydrogencarbonate pH 7.8 gave **11** as its bis (triethylammonium) salt (230 mg, 60 %), $[\alpha]_D^{28}$ –9 (*c* 0.9, water); ^{1}H NMR (D_2O): δ 1.24 (t, 18H, 2 $N(CH_2CH_3)_3$), 1.61 (ddd, 1H, $J_{3e,3a} = J_{3a,4}$ = 13, $J_{3a,P}$ = 6 Hz, H-3a), 2.46 (dd, 1H, $J_{3e,4}$ = 4.5 Hz, H-3e), 3.16 (q, 12H, 2 $N(CH_2CH_3)_3$), 3.39 (d, 1H, $J_{7,8}$ = 9 Hz, H-7), 3.58 (dd, 1H, $J_{9,9'}$ = 12, $J_{9,8}$ = 6.5 Hz, H-9), 3.82 (m, 2H, H-8, H-9'), 3.97 (t, 1H, $J_{4,5} = J_{5,6}$ = 10 Hz, H-5), 4.07 (m, 4H, CH_2, H-4, H-6), 4.15–4.37 (m, 5 H, H-2, H-3, H-4, H-5, H-5' ribose), 5.94 (d, 1H, $J_{1,2}$ = 4.5 Hz, H-1 ribose), 6.10 (d, 1H, $J_{5,6}$ = 7.5 Hz, H-5 cytosine), 7.98 (d, 1H, H-6 cytosine).

*Benzyl-O-[3,5-dideoxy-5-(2-hydroxy)-acetamido-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid]-(2→3)-O-β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-β-D-galactopyranoside (13)*

Disaccharide glycoside **12**,[30] (100 mg, 0.21 mmol) and nucleotide–sugar **11** (166 mg, 0.20 mmol) were incubated at 37 °C with α-(2–3) sialyl-transferase (0.07 U) in 50 mM Na cacodylate buffer pH 7.5 (7 mL) containing 50 mM NaCl, 0.05 mM CTP, 0.5 % Triton X-100 and 20 mM $MnCl_2$, in the presence of calf intestine phosphatase (5U). The reaction was monitored by TLC on silica gel (7:3, 1-propanol–water) : **12** R_f 0.73, **13** R_f 0.65. After 1 day more donor **11** (0.05 mmol) was added and the incubation was continued for 1 day. Proteins were precipitated by addition of ethanol and removed by centrifugation. The supernatant was chromatographed on a column (1.5 × 11 cm) of Dowex 1-X8 (HCO_3^-, 200–400 mesh). After washing with water, elution with a stepwise gradient of triethylammonium hydrogencarbonate pH 8 afforded, in the first fractions of 0.2 M buffer, pure sialylated disaccharide **13** (53 mg) as the triethylammonium salt, then a mixture of **13** and **10** (60 mg) and a mixture of **13** and 2,3-dehydroNeuGc (40 mg). Rechromatography of both last fractions on a silica gel column first eluted with 19:1, then 4:1, 1-propanol–water, afforded more pure **13** (50 mg) (56 % overall yield). $[\alpha]_D^{28}$ –8.5 (*c* 0.8, water); ^{1}H NMR (D_2O) : δ 1.21 (t, 9 H, $N(CH_2CH_3)_3$), 1.74 (t, 1H, $J_{3"a,3"e}$ = $J_{3"a,4}$ = 12.5 Hz, H-3"a), 1.86 (s, 3H, NAc), 2.71 (dd, 1H, $J_{3"e,4}$ = 4.5 Hz, H-3"e), 3.10 (q, 6 H, $N(CH_2CH_3)_3$), 4.41 (d, 1H, $J_{1',2'}$ = 8 Hz, H-1'), 4.48 (d, 1H, $J_{1,2}$ = 8 Hz, H-1), 4.64 (d, 1H, J_{gem} = 12 Hz, CH_2 Ph), 4.85 (d, 1H, CH_2 Ph), 7.38 (m, 5H, Ph); ^{13}C NMR (D_2O): δ 8.35 ($N(CH_2CH_3)_3$), 22.32 ($NHCOCH_3$), 39.86 (C-3"), 46.70 ($N(CH_2CH_3)_3$), 51.22 (C-5"), 51.44 (C-2), 61.05 (C-6, C-6', $COCH_2$), 62.52 (C-9"), 67.42 (C-4'), 67.93, 68.05 (C-4", C-7"), 68.20 (C-4), 69.07 (C-2'), 71.42, 71.94, 72.58 (CH_2Ph, C-8", C-6"), 74.88 (C-5', C-5), 75.62 (C-3'), 80.05 (C-3), 99.76 (C-2"), 100.19 (C-1), 104.60 (C-1'), 128.50, 128.68, 128.78 (Ph), 136.87 (Ph), 174.03 (C-1"), 174.69 (C=O) and 175.80 (C=O).

*O-[3,5-Dideoxy-5-(2-hydroxy)-acetamido-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)-O-β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-D-galactopyranose (1)*

Disaccharide glycoside **13** (45 mg) and 10 % Pd–C (45 mg) in 90 % ethanol (5 mL) were stirred under hydrogen (45 Psi) for 24 h. The solution was then filtered through celite and the filtrate concentrated to give **1** (40 mg, quantitative yield); $[\alpha]_D^{28}$ + 4 (*c* 0.5, water); ^{1}H NMR (D_2O) δ 1.76 (t, 1H, $J_{3"a,3"e} = J_{3"a,4}$ = 12.5 Hz, H-3"a),

1.97 (s, 3H, NAc), 2.72 (dd, 1H, $J_{3''e,4}$ = 4.5 Hz, H-3"e), 4.02 (s, 2H, CH_2), 4.46 (d, 0.53H, $J_{1',2'}$ = 8 Hz, H-1'β), 4.52 (d, 0.47 H, $J_{1',2'}$ = 8 Hz, H-1'α), 4.64 (d, 0.53H, $J_{1,2}$ = 8 Hz, H-1β), 5.18 (d, 0.47H, $J_{1,2}$ = 8 Hz, H-1α).

Preparation of crude β-D-Gal-(1–3)-D-GalNAc:CMP-NeuAc–(2–3)-α-sialyltransferase from porcine liver by chromatography on Cibacron Blue F3GA–agarose.

The membrane-bound enzyme was first extracted with 1.4 % Triton X-100,[23] then the Triton extract (50 mL, 0.35 U, specific activity : 0.43 mU mg^{-1}) was adsorbed on a column (3 × 7 cm) of Cibacron Blue F3GA–agarose (4 μmol mL^{-1}) prepared according to the published procedure.[31] The column was previously equilibrated with 10 mM sodium cacodylate buffer pH 6 containing 0.1 M NaCl, 1 % Triton X-100 and 25 % glycerol. After washing with this buffer, the column was again loaded with the same amount of Triton extract, washed, and loaded a third time. The enzyme was eluted from the gel with a stepwise gradient of NaCl (0.5 M, 1 M, 1.5 M) in the above cacodylate buffer but without glycerol (0.55 U, specific activity: 2.25 mU mg^{-1}). The 1 M NaCl eluate (70 mL) was dialyzed against 50 mM sodium cacodylate buffer pH 7.5 containing 50 mM NaCl, 0.5 % Triton X-100 and 50 μM CTP and lyophilized; the lyophilizate was taken up in water (14 mL) and again dialyzed before use against the above buffer.

Sialic acid aldolase immobilisation on Eupergit 250L

Eupergit 250L (400 mg) was added to a solution of sialic acid aldolase (8 mg, 64 U) in 1 M potassium phosphate buffer pH 7.4 (3.2 mL) containing 40 mM sodium pyruvate and 0.02 % NaN_3; the suspension was stirred for 3 days at room temperature under N_2. The gel was washed with 0.1 M potassium phosphate buffer pH 7 (10 mL) and stored at 4 °C in this buffer in the presence of 40 mM pyruvate and 1 mM dithiothreitol.

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