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# The use of immobilised glycosyltransferases in the synthesis of sialyloligosaccharides*

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ABSTRACT

The CMP-sialic acids, cytidine 5'-(5-acetamido-3,5-dideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid monophosphate) (**1**) and cytidine 5'-(5-acetamido-9-*O*-acetyl-3,5-dideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid monophosphate) (**2**) were prepared from CMP, phosphoenolpyruvate, *N*-acetylneuraminic acid or its 9-acetate, and a catalytic amount of ATP in the presence of immobilised pyruvate kinase, nucleoside monophosphate kinase, inorganic pyrophosphatase, and CMP-sialic acid synthetase. CMP-NeuAc (**1**) was used as a donor of *N*-acetylneuraminic acid in the reaction catalysed by immobilised porcine liver β-D-Gal*p*-(1→4)-α-D-Glc*p*NAc-(2→6)-sialyltransferase. α-D-Neu*p*5Ac-(2→6)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man-OMe (**5**) was obtained on a 0.1-mmol scale by enzymic sialylation of β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man-OMe (**4**), prepared by enzymic galactosylation of β-D-Glc*p*NAc-(1→2)-α-D-Man-OMe (**3**). Likewise, using **2**, α-D-Neu*p*-5,9$Ac_2$-(2→6)-β-D-Gal*p*-(1→4)-D-Glc*p*NAc (**7**) was obtained from *N*-acetyl-lactosamine (**6**).

## INTRODUCTION

Being located on the periphery of glycoconjugates, α-sialyloligosaccharides are involved in many biological processes[1]. There have been major developments in the chemical synthesis of neutral oligosaccharides in the past decade, but much less progress has been made with sialyloligosaccharides. Sialylation still remains a challenge for chemists. The use of common derivatives of *N*-acetylneuraminic acid as glycosyl donors usually results in poor stereoselectivity[2] and equimolar αβ-mixtures of sialosides are often obtained. More complex derivatives of *N*-acetylneuraminic acid[3] have been used in chemical synthesis of sialyloligosaccharides in order to promote the formation of the natural α-sialoside and to increase the yields in condensations with secondary alcohols.

Our work on enzymic galactosylation[4] prompted an investigation of this approach for sialylation. Enzymic synthesis of sialyloligosaccharides involves sialyltransferases that catalyse the transfer of *N*-acetylneuraminic acid or, more generally, sialic acid from the nucleotide–sugar donor to the oligosaccharide acceptor with the release of CMP.

*Contribution from the research group of Professor Serge David.

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Several sialyltransferases with different specificities have been characterised and purified. Each enzyme is specific and recognizes not only the sugar acceptor but also the penultimate sugar and the connecting linkage[5]. Sabesan *et al.*[6] reported the preparation of ten sialyloligosaccharides on a 10–20 μmol scale catalysed by three different sialyltransferases purified from rat liver or porcine submaxillary gland. The use of sialyltransferases isolated from human placenta and regenerating rat liver led to the synthesis of a disialotetrasaccharide of the M and N blood-group determinant on a μmol scale[7]. The feasibility of the synthesis of sialylated *N*-acetyl-lactosamine on a semi-preparative scale has been reported by Thiem *et al.*[8].

We now report the enzymic sialylation of β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man-OMe (**4**) and β-D-Gal*p*-(1→4)-D-Glc*p*NAc (**6**), using for the first time an immobilised β-D-Gal*p*-(1→4)-α-D-Glc*p*NAc-(2→6)-sialyltransferase (EC 2.4.99.1) obtained in partially purified form from porcine liver. With CMP-Neu5Ac (**1**), **4** was converted into α-D-Neu*p*5Ac-(2→6)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man-OMe (**5**), the methyl glycoside of the compound previously synthesised by Paulsen *et al.*[9]. This sequence occurs commonly at the non-reducing end of oligosaccharide chains of *N*-glycoproteins of the lactosamine type. With CMP-Neu5,9$Ac_2$ (**2**), **6** was converted into α-D-Neu*p*5,9$Ac_2$-(2→6)-β-D-Gal*p*-(1→4)-D-Glc*p*NAc (**7**). Resialylation experiments on sialidase-treated human erythrocytes have shown that **7** could be one of the receptors of the influenza C virus[10].

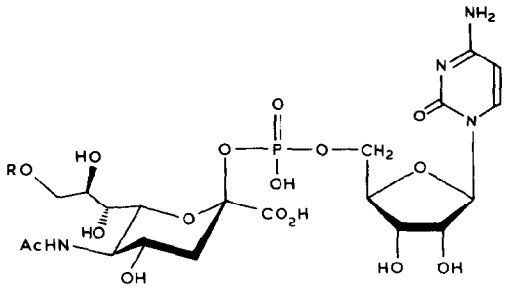
## RESULTS AND DISCUSSION

*Preparation of CMP-sialic acids.* — Nucleotide donors are key intermediates in the reactions catalysed by glycosyltransferases, and CMP-sialic acids can be obtained by the CMP-sialic acid synthetase-catalysed condensation of sialic acid with CTP.

Several enzymic syntheses of CMP-NeuAc (**1**), using either soluble[11–14] or immobilised[15] CMP-sialic acid synthetase, have been reported in which a large excess of CTP was used. Since sialic acids are now available readily[16], CTP is the limiting factor in the preparation of CMP-sialic acids. Therefore, a method was developed that would allow the straightforward synthesis of CMP-sialic acids from CMP. Preliminary reports[17,18] of the method have been published and the syntheses of CMP-Neu5Ac (**1**) and CMP-Neu5,9$Ac_2$ (**2**) are now described in detail. Simon *et al.*[19] have reported a similar enzymic preparation of **1**.

The system involved four enzymes immobilised on agarose previously activated with cyanogen bromide. CMP was phosphorylated to give CDP, using nucleoside monophosphokinase (EC 2.7.4.4) and a catalytic amount of ATP, which was continuously regenerated, and CDP was converted into CTP by the action of pyruvate kinase (EC 2.7.1.40) and phosphoenolpyruvate. This immobilised two-enzyme system was stable and its re-use allowed the preparation of CTP on a g scale.

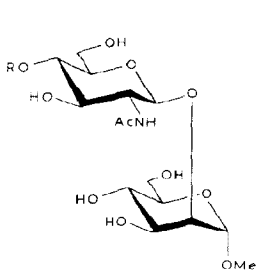
CMP-sialic acid synthetase (EC 2.7.7.43) was prepared from calf brain by a slight modification of the published procedure[14]. The precipitate obtained with ammonium sulfate was taken up in the buffer used for the immobilisation step, in which the yield was



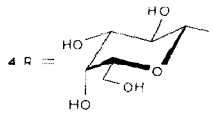
1 R = H
2 R = Ac

> 100%. Some inhibition in the soluble enzymic preparation may explain this result which was also mentioned by Corfield *et al.*[15] A nucleotidase that hydrolysed CMP into cytidine was contaminating the preparation of CMP-sialic acid synthetase, so that it could not be used together with pyruvate kinase and nucleoside monophosphokinase. Therefore, the filtrate that contained CTP was incubated with a stoichiometric amount of sialic acid and CMP-sialic acid synthetase. Inorganic pyrophosphatase (EC 3.6.1.1), which hydrolyses pyrophosphate, an inhibitory product of the reaction, was added. In this way, CMP-Neu5Ac (**1**, 52%) and CMP-Neu5,9$Ac_2$ (**2**, 40%) were obtained, respectively, from *N*-acetylneuraminic acid and its 9-acetate, respectively, after purification on DEAE-Sephadex A-25. The synthesis of **2** was achieved at pH 7.5 under the conditions described by Higa *et al.*[14], which did not cause *O*-deacetylation. The $^1$H-n.m.r. spectra of **1** and **2** were identical with those reported[14].

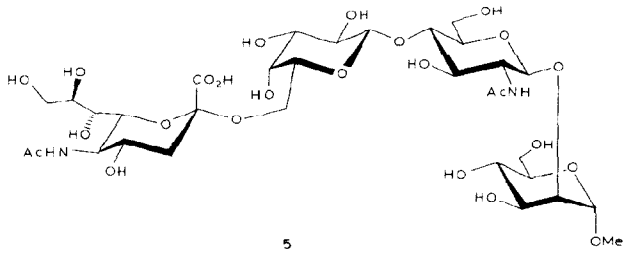
*Preparation of immobilised β-D-Galp-(1→4)-α-D-GlcpNAc-(2→6)-sialyltransferase from porcine liver.* — This enzyme has been purified to homogeneity from rat liver[20] and its presence in porcine liver has been reported[21]. The latter material, which is readily available, was found to be a good source (40 $U.kg^{-1}$) of the enzyme that was extracted with Triton X-100 and concentrated by adsorption on a column of CDP–hexanolamine–agarose (10 $\mu mol.mL^{-1}$ of gel) according to the procedure described for the rat liver enzyme[20]. The dialysed eluate was then applied to a column of CDP–hexanolamine–agarose (2 $\mu mol.mL^{-1}$ of gel) which was eluted with a gradient of CDP. The α-(2→6)-sialyltransferase was finally concentrated on SP-C50 Sephadex and obtained in 7–10% overall yield with a specific activity of 0.3 $U.mg^{-1}$ and a purification factor of 1000. At this stage, the enzyme was concentrated sufficiently (8 mL, 0.5 U) and, prior to immobilisation, it was diluted with bovine serum albumin and dialysed against the buffer used in the immobilisation step. The coupling with agarose was performed in the presence of CDP in yields of 25–70%, depending on the conditions. The immobilised enzyme was stable for at least five months at −20° in 10mM sodium cacodylate buffer (pH 6.5) containing 0.5M NaCl, 1% of Triton X-100, and 50% of glycerol.



3 R = H



4 R =

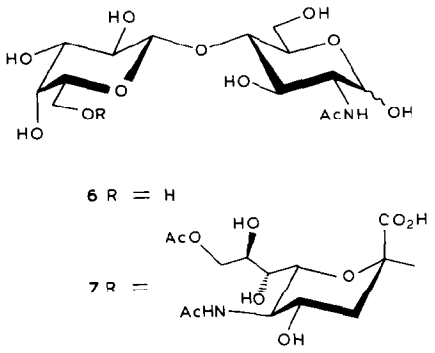


5

*Enzymic synthesis of sialyloligosaccharides.* — The disaccharide glycoside **3** (ref. 22) was galactosylated with the immobilised multi-enzyme system[23] which regenerates UDP-α-D-galactose *in situ*. After 4 days, the yield was 45% (h.p.l.c.) but was not increased further even after the addition of a further catalytic amount of UTP. The enzymes were collected and stored in the appropriate buffer, and the filtrate was deionised in order to eliminate the accumulated phosphates that might have caused some inhibition, and recycled. The concentrations of the substrates and co-factors were then diluted twice with respect to the first galactosylation. The mixture was stirred for 4 days until reaction was complete, and the trisaccharide glycoside **4** (48%) was obtained after column chromatography on silica gel. Compound **4** is the methyl glycoside of the compound already synthesised[24]. The chemical shifts of the H-1 resonances corresponded to those reported for the Gal*p* and Glc*p*NAc residues[24] and for the Man*p* residue[22].

Compound **4** (10mM) was incubated with a stoichiometric amount of CMP-Neu5Ac (**1**, added in two portions) in the presence of the immobilised sialyltransferase for 24 h in 50mM sodium cacodylate buffer (pH 7) containing 50mM NaCl. $MnCl_2$ was added to 20mM in order to minimize the inhibition by CMP, a product of the reaction. The tetrasaccharide glycoside **5** (46%) was obtained pure after gel filtration on Bio-Gel P-2. Whereas only 10 mg of the free tetrasaccharide corresponding to **5** were obtained in the chemical synthesis[9], 75 mg of **5** were prepared by the enzymic synthesis. The $^1$H-n.m.r. spectrum of 5 contained signals for three anomeric protons and for H-3*a*,3*e* of the Neu5*p*Ac residue. Their chemical shifts accorded with the values reported[25] for the natural compounds.

*N*-Acetyl-lactosamine (**6**) was treated with a stoichiometric amount of CMP-Neu5,9$Ac_2$ (**2**) in the presence of the immobilised sialyltransferase, as described for the synthesis of **5**, to give the trisaccharide **7** (57%) after gel filtration on Bio-Gel P-2. The $^1$H-n.m.r. spectrum of **7** contained signals for the deshielded H-9,9′ of the Neu*p*Ac residue in addition to a signal at 2.09 p.p.m. (s) attributed to OAc. H.p.l.c. revealed ~5% of contaminating *O*-deacetylated compound. 9-Acetates of sialic acids are labile




compounds[16]. Pure **7**, which could be prepared by flash chromatography (8:2 1-propanol–water), has not yet been synthesised chemically. 9-*O*-Acetylsialyl-lactose, an analogue of **7**, has been obtained by selective acetylation of sialyl-lactose[26].

The $^{13}$C-n.m.r. data of **5** and **7** are listed in Table I. The proposed assignments accord with the data reported[5,27]. As a result of acetylation at HO-9, the C-9 resonance of the α-D-Neu5*p*Ac residue is shifted downfield by 3.07 p.p.m. (α-effect) and the C-8 resonance is shifted upfield by 2.39 p.p.m. (β-effect).

Enzymic sialylation was equally efficient with **1** and **2**. Moreover, 0.4 U of the immobilised sialyltransferase was required for the preparation of 0.1 mmol each of **5** and **7**, and was still active, whereas 2.5 U of the soluble enzyme were necessary[8] to synthesise 0.05 mmol. Clearly, enzymic synthesis based on the immobilised sialyltransferase can compete with chemical syntheses, and sialyltransferases should become more easily available by cloning techniques[28].

## EXPERIMENTAL

*General.* — $^{13}$C-N.m.r. (62.9 MHz, internal 1,4-dioxane, 67.4 p.p.m. from the signal for $Me_4Si$) and $^1$H-n.m.r. spectra (250 MHz, external 0.2% $Me_4Si$ in $CDCl_3$) were recorded with a Bruker AM-250 spectrometer for solutions in $D_2O$. Optical rotations were measured with a Roussel–Jouan electronic, digital micropolarimeter. H.p.l.c. was carried out with a Waters model 590 pump, a Rheodyne 20-μL injection loop, a Waters 410 differential refractometer, a Spectra Physics SP 4290 integrator, and a reversed phase $C_{18}$ radial-pak cartridge (10 μm, 100 × 8 mm, Waters) or a column of Spherisorb $NH_2$ (5 μm, 250 × 4.6 mm, SFC France).

Pyruvate kinase (type I), UDP-D-glucose pyrophosphorylase, inorganic pyrophosphatase, and UDP-D-galactose 4-epimerase were purchased from Sigma, and nucleoside monophosphokinase from Boehringer. The 4-β-D-galactosyltransferase was prepared from cow colostrum and immobilised, as for the other enzymes of the galactosylation cycle, on Ultrogel A4 previously activated with cyanogen bromide[23]. Ultrogel A4 (4% agarose) was purchased from I.B.F. CDP–hexanolamine–agarose was prepared as described[29]. *N*-Acetylneuraminic acid was obtained by enzymic synthesis[16].

TABLE I

Assignments of $^{13}C$ chemical shifts for sialyloligosaccharides

| *Sugar unit of compound* | *C-1* | *C-2* | *C-3* | *C-4* | *C-5* | *C-6* | *C-7* | *C-8* | *C-9* | *NCOCH$_3$* | *OCOCH$_3$* | *NCOCH$_3$* | *OCOCH$_3$* | *OMe* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **5** | | | | | | | | | | | | | | |
| α-D-Man*p* | 98.65 | 76.92 | 70.36 | 68.05 | 73.41[a] | 62.35 | | | | | | | | 55.39 |
| β-D-Glc*p*NAc | 99.99 | 55.59 | 72.87[a] | 81.43 | 75.20 | 60.97 | | | | 175.54 | | 23.19 | | |
| β-D-Gal*p* | 104.31 | 71.50 | 73.18 | 69.15 | 74.47 | 64.11 | | | | | | | | |
| α-D-Neu*p*5Ac | 174.31 | 100.92 | 40.84 | 68.98 | 52.66 | 73.31 | 68.98 | 72.49 | 63.42 | 175.54 | | 22.84 | | |
| **7** | | | | | | | | | | | | | | |
| β-D-Glc*p*NAc | 95.49 | 56.75 | 73.19 | 81.50 | 75.34 | 61.14 | | | | 175.63 | | 23.11 | | |
| α-D-Glc*p*NAc | 91.38 | 54.23 | 70.10[b] | 81.73 | 70.79 | 60.98 | | | | 175.63 | | 23.11 | | |
| β-D-Gal*p* | 104.29 | 71.51 | 73.19 | 69.20 | 74.52 | 64.31 | | | | | | | | |
| α-D-Neu*p*5Ac | 174.23 | 101.02 | 40.91 | 69.04 | 52.64 | 73.19 | 69.04 | 69.93[b] | 66.49 | 175.63 | 175.17 | 22.84 | 21.07 | |

[a,b] Assignments may be interchanged.

*N*-Acetyl-lactosamine (**6**) was a generous gift from Dr A. Veyrières. T.l.c. was performed on silica gel and PEI-cellulose F (Merck), with detection by u.v. light or charring with sulphuric acid. Silica gel (Merck, 70–230 mesh) was used for column chromatography. All the buffers were prepared with twice-distilled water.

*Enzymic synthesis of CTP.* — A solution of nucleoside monophosphokinase (20 mg, 1.8 U) in 1:1 0.1M citrate buffer (pH 6.5)–glycerol (0.4 mL) was diluted with 3:1 0.1M $NaHCO_3$ buffer (pH 8.2)–glycerol (6 mL) containing 0.5M NaCl, mM ATP, and 0.5mM CTP, and stirred overnight at 4° under nitrogen with Ultrogel A4 (2 mL) freshly activated by BrCN (60 $mg.mL^{-1}$ of gel). The gel was then collected, washed with M NaCl, twice-distilled water, and 0.1M Tris buffer (pH 7.5), and stored in this buffer. The enzymic activity was determined using a spectrophotometric assay[30] that had been adapted for immobilised enzyme (0.6 U was bound to the agarose).

Immobilised nucleoside monophosphokinase (0.6 U) and pyruvate kinase (10 U) were stirred gently at 37° under nitrogen with CMP (0.5 mmol), ATP (0.05 mmol), and phosphoenolpyruvate (1.5 mmol) in 0.1M Tris buffer (pH 7.5) containing 35mM KCl, 2mM $MgCl_2$, 3mM 2-mercaptoethanol, mM thymol, and 0.1mM EDTA. The reaction was monitored by t.l.c. on PEI-cellulose with successive elutions with LiCl: 0.3M (1 min), M (12 min), and 1.6M (47 min). After 2 days, the gel was collected and washed with 0.1M Tris buffer (pH 7.5), and the combined filtrate and washings were used directly for the synthesis of CMP-sialic acids. The gel could be reused.

*Preparation of immobilised CMP-sialic acid synthetase.* — Fresh calf brain (600 g, 40 U) was homogenised with 0.01M sodium pyrophosphate (1 L) in a Waring Blendor for two periods of 1 min, each with an interval of 1 h. The homogenate was centrifuged at 15000*g* for 20 min, and the pellet was suspended in 0.4M KCl (400 mL), with the use of a Potter–Elvehjem homogenizer, and centrifuged at 30000*g* for 20 min. The supernatant solution contained the enzyme (10 U). The pellet was extracted for a second time with the same amount of 0.4M KCl, and a second batch of enzyme was obtained (20 U). Each supernatant solution was treated[14], with ammonium sulfate, and each precipitate was taken up in the buffer used for the immobilisation step [0.1M $NaHCO_3$ (pH 8.8) containing 0.5M NaCl], to give two enzyme preparations with specific activities of 0.012 and 0.038 $U.mg^{-1}$ of protein, respectively. The enzymic activity was determined using the standard procedure[11] that had been adapted for immobilised enzyme. One unit of enzyme was defined as the quantity that converted 1 μmol of *N*-acetylneuraminic acid into CMP-NeuAc per min. For immobilisation, 25% of the soluble CMP-sialic acid synthetase (100 mL, 5 U) was stirred overnight at 4° under nitrogen with Ultrogel A4 (50 mL) freshly activated by BrCN (100 $mg.mL^{-1}$ of gel). The gel was washed with M NaCl, twice-distilled water, and 0.1M Tris buffer (pH 9) containing 3mM 2-mercaptoethanol, and then stored in suspension in this buffer (9 U of enzymic activity were bound to the agarose).

*Partial purification of the β-D-Galp-(1→4)-α-D-GlcpNAc-(2→6)-sialyltransferase from porcine liver.* — Glassware was siliconised[20]. Fractions from the column were collected in plastic tubes. All operations were performed at 4°. Proteins were estimated by the modified Lowry procedure[31]. The activity of the sialyltransferase was determined

by a radiochemical assay. Assay mixtures (25 $\mu$L) contained *N*-acetyl-lactosamine (0.5 $\mu$mol), bovine serum albumin (25 $\mu$g), enzyme extract, and CMP-[$^{14}$C]Neu5Ac (40 000 c.p.m., 13 nmol) in 30mM sodium cacodylate buffer (pH 7.5) containing 2.5% of Triton X-100. After incubation at 37° (5–30 min), the radioactive product was isolated on a Pasteur-pipette column of Dowex 1-X8 ($PO_4^{2-}$) resin (200–400 mesh). One unit of enzyme was defined as the amount of enzyme which catalysed the transfer of 1 $\mu$mol of *N*-acetylneuraminic acid per min under the above incubation conditions. When assaying column fractions containing CDP, 20mM $MnCl_2$ was added to the standard assay in order to prevent inhibition of activity by CDP.

Fresh porcine liver (500 g) was homogenised with 25mM Na cacodylate buffer containing 20mM $MnCl_2$ (1 L) in a Waring Blendor for 4 periods of 10 s each, with intervals of 10 min. The homogenate was centrifuged at 5000 r.p.m. for 60 min, the pellet was resuspended in the same buffer (600 mL), and the treatment was repeated. After centrifugation, the pellet was homogenised as above with 25mM Na cacodylate buffer (pH 6.0) containing 10mM $MnCl_2$ and 75mM NaCl, then extracted twice with Triton X-100 as described for the rat liver sialyltransferase[20]. Triton extracts I and II were treated[20] with a solution of 0.5M EDTA (pH 7.7) before adsorption on CDP–hexanolamine–agarose. Triton extract I (720 mL) was adsorbed on a column (4.5 × 6 cm) of CDP–hexanolamine–agarose (10 $\mu$mol.mL$^{-1}$ of gel) previously equilibrated in 10mM Na cacodylate buffer (pH 6.5) containing 0.1M NaCl, 1% of Triton X-100, and 25% of glycerol. Unless stated otherwise, all the subsequent buffers contained the last two components. The column was washed with this buffer (1 L) and then equilibrated with 30mM Na cacodylate buffer (pH 5.8) containing 0.1M NaCl (100 mL). The sialyltransferase was eluted from the column with this buffer containing 1.5M NaCl. Triton extract II was treated in the same way. Fractions containing enzyme activity (150 mL) were combined, dialysed against 10mM Na cacodylate buffer (pH 6.5) (2 × 4 L), and then applied to a column (2.5 × 14 cm) of CDP–hexanolamine–agarose (2 $\mu$mol.mL$^{-1}$ of gel) equilibrated in 10mM Na cacodylate buffer (pH 6.5) containing 0.1M NaCl. The column was washed with this buffer (200 mL) and equilibrated with 30mM Na cacodylate buffer (pH 5.8) containing 0.1M NaCl. The sialyltransferase was eluted with a linear CDP gradient, with 75 ml of the above buffer as starting buffer and 75 mL of 30mM Na cacodylate buffer (pH 5.8) containing 0.075M NaCl and 2mM CDP as the limit buffer. The combined fractions (50 mL) that contained sialyltransferase were diluted 4-fold in 10mM Na cacodylate buffer (pH 5.5) containing 25mM NaCl and 1% of Triton X-100, and loaded on a column (0.4 × 4 cm) of SP-C50 Sephadex equilibrated in the same buffer, which was washed with this buffer (5 mL). The enzyme was eluted with 30mM Na cacodylate buffer (pH 6.0) containing M NaCl. Fractions containing the partially purified sialyltransferase were combined (8 mL) and used for the immobilisation.

*Immobilisation of the sialyltransferase.* — To the foregoing solution of the purified sialyltransferase was added bovine serum albumin (0.5 mg.mL$^{-1}$), and the solution was dialysed during 3 h against 0.1M Na phosphate buffer (pH 7.8) containing 25% of glycerol (2 × 100 mL). Prior to dialysis, the membrane was saturated with bovine serum albumin. The dialysate was stirred overnight at 4° under nitrogen with Ultrogel A4 (0.25

vol.), freshly activated by BrCN (60 mg.mL$^{-1}$ of gel), in the presence of mM CDP. The gel was washed with twice-distilled water and 10mM Na cacodylate buffer (pH 6.5) containing 0.5M NaCl, 1% of Triton X-100, and 25% of glycerol, and then stored at −20° after the addition of ice-cold glycerol (0.5 vol.). For use, the immobilised preparation was collected on sintered glass and washed with the buffer to be used for incubation with the enzyme. The immobilised sialyltransferase was assayed as for the soluble enzyme, except that the assay mixture was multiplied by a factor of 8 and CMP-[$^{14}$C]Neu5Ac of a lower specific activity (40 000 c.p.m./42 nmol) was used. The assay mixture was stirred continously during the incubation.

*Cytidine 5'-(5-acetamido-3,5-dideoxy-β-*D*-glycero-*D*-galacto-2-nonulopyranosylonic acid monophosphate)* (**1**, *CMP-Neu5Ac*). — Immobilised CMP-sialic acid synthetase (3.7 U) and inorganic pyrophosphatase (6 U) were added to the crude preparation of CTP (0.5 mmol), together with *N*-acetylneuraminic acid (0.5 mmol). The substrate was adjusted to 2mM by dilution with 0.1M Tris buffer (pH 9). The pH was adjusted to 9 and the $MgCl_2$ concentration to 35mM. 2-Mercaptoethanol and thymol were kept 3mM and 1mM, respectively, and the mixture was stirred gently at 37° under nitrogen. The reaction was monitored by t.l.c. on PEI-cellulose, as described for CTP synthesis, and on silica gel (7:3 1-propanol–water). After 10 h, the yield of **1** was 60%, as estimated by the thiobarbituric acid assay[11], and the reaction was stopped. The gel was collected and washed with 0.1M Tris buffer (pH 9), and the combined filtrate and washings were purified by chromatography on a refrigerated column (3 × 45 cm) of DEAE-Sephadex A-25 ($HCO_3^-$ form). Elution with a gradient of 0–0.75M triethylammonium hydrogencarbonate (pH 7.8) gave **1** as its bis(triethylammonium) salt (234 mg, 52%), $[\alpha]_D^{20}$ −18° (*c* 1.9, water), $R_F$ 0.53 (7:3 1-propanol–water). $^1$H-n.m.r. data ($D_2O$): $\delta$ 1.65 (m, 1 H, H-3*a*), 2.05 (s, 3 H, NAc), 2.50 (dd, 1 H, $J_{3e,3a}$ 12.5, $J_{3e,4}$ 5 Hz, H-3*e*), 5.97 (d, 1 H, $J_{1,2}$ 4.5 Hz, H-1 ribose), 6.10 (d, 1 H, $J_{5,6}$ 7.5 Hz, H-5 cytosine), 7.97 (d, 1 H, H-6 cytosine).

*Cytidine 5'-(5-acetamido-9-*O*-acetyl-3,5-dideoxy-β-*D*-glycero-*D*-galacto-2-nonulopyranosylonic acid monophosphate)* (**2**, *CMP-Neu5,9Ac$_2$*). — Immobilised CMP-sialic acid synthetase (9 U) and inorganic pyrophosphatase (5 U) were added to the crude preparation of CTP (0.5 mmol) together with 9-*O*-acetyl-*N*-acetylneuraminic acid (0.5 mmol). The substrate was ajusted to 2mM by dilution with 0.1M Tris buffer (pH 7.5), and $MnCl_2$ was added to 6.25mM. 2-Mercaptoethanol and thymol were kept 3mM and mM, respectively, and the mixture was stirred gently at 37° under nitrogen. The reaction was monitored by t.l.c. on PEI-cellulose or on silica gel, as for the synthesis of CMP-Neu5Ac. The thiobarbituric acid assay[11] indicated a 52% yield of **2** after 8 h when the reaction was stopped. The gel was collected, and washed with 0.1M Tris buffer (pH 7.5), and the combined filtrate and washings were purified by chromatography on DEAE-Sephadex A-25, as described above, to give **2** as its bis(triethylammonium) salt (190 mg, 40%), $R_t$ 0.60 (7.3 1-propanol–water), $[\alpha]_D^{20}$ −4° (*c* 1.5, water). $^1$H-N.m.r. data ($D_2O$): $\delta$ 1.64 (m, 1 H, $J_{3a,3e} = J_{3a,4} = 13$, $J_{3a,P}$ 5.7 Hz, H-3*a*), 2.04 (s, 3 H, NAc), 2.08 (s, 3 H, OAc), 2.46 (dd, 1 H, $J_{3e,4}$ 4.7 Hz, H-3*e*), 3.49 (d, 1 H, $J_{8,7}$ 9 Hz, H-7), 3.94 (t, 1 H, $J_{5,6} = J_{5,4} = 10$ Hz, H-5) 4.04 (m, 1 H, H-8), 6.0 (d, 1 H, $J_{1,2}$ 4.5 Hz, H-1 ribose), 6.12 (d, 1 H, $J_{5,6}$ 7.5 Hz, H-5 cytosine), 7.94 (d, 1 H, H-6 cytosine).

Methyl O-*β*-D-*galactopyranosyl*-(*1→4*)-O-(*2-acetamido-2*-deoxy-*β*-D-*glucopyranosyl*)-(*1→2*)-*α*-D-*mannopyranoside* (**4**). — To a solution of **3** (229 mg, 0.66 mmol), *α*-D-glucopyranosyl phosphate (241 mg, 0.66 mmol), phosphoenolpyruvate (137 mg, 0.66 mmol), and UDP-*α*-D-glucose (10 mg, 0.015 mmol) in water (70 mL) were added $MnCl_2$ (40 mg, 0.2 mmol), $MgCl_2$ (81 mg, 0.4 mmol), KCl (447 mg, 6 mmol), dithiothreitol (154 mg, 1 mmol), and $NaN_3$ (10 mg). The pH of the solution was adjusted to 8.0 with M NaOH, and the following immobilised enzymes bound to agarose (30 mL) were added: *β*-D-galactosyltransferase (2.8 U), pyruvate kinase (42 U), UDP-D-glucose pyrophosphorylase (5 U), inorganic pyrophosphatase (14 U), and UDP-D-galactose-4-epimerase (3 U). The reaction was allowed to proceed at 30° under nitrogen with gentle shaking and the pH was controlled at 8 using a pH-stat. The reaction was monitored by h.p.l.c. (using a reversed phase $C_{18}$ radial-pak cartridge and elution with water). There was 45% conversion of **3** into **4** after 4 d. UTP (4.5 mg, 0.007 mmol) was added but no further reaction was observed. The gel was collected, washed with twice-distilled water, and stored in 0.1M Tris buffer (pH 8.0) containing mM dithiothreitol. The filtrate and washings were deionised using, successively, Dowex 1-X8 ($HCOO^-$) and 50-X8 ($H^+$) resins, and concentrated to dryness. A solution of the residue (284 mg) in twice-distilled water was submitted to a new galactosylation cycle, using *α*-D-glucopyranosyl phosphate (133 mg, 0.36 mmol), phosphoenolpyruvate (75 mg, 0.36 mmol), $NAD^+$ (40 mg, 0.055 mmol), $MnCl_2$ (22 mg, 0.11 mmol), $MgCl_2$ (45 mg, 0.22 mmol), KCl (246 mg, 3.3 mmol), dithiothreitol (85 mg, 0.55 mmol), and $NaN_3$ (5.5 mg). This solution was adjusted to pH 8.0 and re-treated with the same immobilised enzymes (in a total volume of 100 mL). After 4 days, the gel was collected and treated as described above. The residue obtained after deionisation was purified on a column of silica gel (2:2:1 2-propanol–ethyl acetate–water) to give **3** (28 mg, 11%), a mixture (40 mg) of **3** and **4**, and **4** (160 mg, 48%), $[\alpha]_D^{20}$ −13° (*c* 0.7, water), $R_F$ 0.35 (3:3:2 2-propanol–ethyl acetate–water). $^1$H-N.m.r. data ($D_2O$): $\delta$ 2.03 (s, 3 H, NAc), 3.39 (s, 3 H, OMe), 4.04 (m, 1 H, H-2a), 4.45 (d, 1 H, $J_{1,2}$ 8 Hz, H-1c), 4.57 (d, 1 H, $J_{1,2}$ 8 Hz, H-1b), 4.76 (d, 1 H, $J_{1,2}$ 1.5 Hz, H-1a).

*Methyl* 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-*glucopyranosyl*)-(*1→2*)-*α*-D-*mannopyranoside* (**5**). — A solution of **4** (109 mg, 0.2 mmol), CMP-Neu5Ac bis(triethylammonium) salt **1** (115 mg, 0.138 mmol), and $MnCl_2$ (80 mg, 0.4 mmol) in 50mM Na cacodylate buffer (pH 7.0, 20 mL) containing 50mM NaCl was incubated at 37°, under nitrogen in a siliconised vessel, with immobilised sialyltransferase (0.4 U, 4 mL of gel). The reaction was monitored by h.p.l.c. [using a column of Spherisorb $NH_2$ and elution with 7:3, acetonitrile–10mM potassium phosphate (pH 6.0)]. After 5 h, more **1** (0.05 mmol) was added and the incubation was continued for 19 h. The gel was collected, washed with 50mM Na cacodylate buffer (pH 7.0) containing 50mM NaCl, and stored in 10mM Na cacodylate buffer (pH 6.5) containing 0.5mM NaCl, 1% of Triton X-100, and 50% of glycerol. The filtrate and washings were freeze-dried, and a solution of the residue in water (8 mL) was applied to a column (2.5 × 40 cm) of Bio-Gel P-2 (200–400 mesh) equilibrated and eluted with water. Appropiate fractions

were combined and freeze-dried to give **5** (75 mg, 46% based on **1**), $[\alpha]_D^{20}$ −20° (*c* 1, water). $^1$H-N.m.r. data ($D_2O$): $\delta$ 1.70 (t, 1 H, $J_{3a,4} = J_{3a,3e}$ = 12 Hz, H-3*a*d), 2.00 (s, 3 H, NAc), 2.04 (s, 3 H, NAc), 2.64 (dd, 1 H, $J_{3e,4}$ 4.5 Hz, H-3*e*d), 4.04 (m, 1 H, H-2a), 4.42 (d, 1 H, $J_{1,2}$ 8 Hz, H-1c), 4.58 (d, 1 H, $J_{1,2}$ 8 Hz, H-1b), 4.76 (d, 1 H, $J_{1,2}$ 1.5 Hz, H-1a).

*Methyl* O-*(5-acetamido-9-*O-*acetyl-3,5-dideoxy-α-*D-glycero-D-galacto-*2-nonulopyranosylonic acid)-(2→6)-*O-*β-*D-*galactopyranosyl-(1→4)-2-acetamido-2-deoxy-*D-*glucopyranose* (**7**). — A solution of **6** (61 mg, 0.16 mmol), CMP-Neu5,9$Ac_2$ bis(triethylammonium) salt **2** (90 mg, 0.095 mmol), and $MnCl_2$ (56 mg, 0.28 mmol) in 50mM Na cacodylate buffer (pH 7.0, 14 mL) containing 50mM NaCl was incubated at 37°, under nitrogen in a siliconised vessel, with immobilised sialyltransferase (0.4 U, 4 mL of gel). The reaction was monitored by h.p.l.c. as described above. After 4 h, more **2** (0.065 mmol) was added and the incubation was continued for 20 h. H.p.l.c. then showed the formation of 65% of **7** and the presence of ~15% of *O*-deacetylated product. The gel was collected, washed with 50mM Na cacodylate buffer (pH 7.0) containing 50mM NaCl, and stored in 10mM NaCl containing 1% of Triton X-100 and 50% of glycerol. The filtrate and washings were freeze-dried, and a solution of the residue in water (4.5 mL) was applied to a column (2.5 × 40 cm) of Bio-Gel P-2 (200–400 mesh), equilibrated and eluted with water, to afford **7** (66 mg, 57%), $[\alpha]_D^{20}$ +1.2° (*c* 0.8, water). $^1$H-N.m.r. data ($D_2O$): $\delta$ 1.67 (t, 1 H, $J_{3a,4} = J_{3a,3e}$ = 12 Hz, H-3*a*c), 1.98 (s, 3 H, NAc), 2.02 (s, 3 H, NAc), 2.09 (s, 3 H, OAc), 2.63 (dd, 1 H, $J_{3e,4}$ 4.5 Hz, H-3*e*c), 4.16 (dd, 1 H, $J_{9,9'}$ 12, $J_{9',8}$ 6 Hz, H-9′c),. 4.37 (dd, 1 H, $J_{9,8}$ 2 Hz, H-9c), 4.41 (d, 1 H, $J_{1,2}$ 8 Hz, H-1b), 4.71 (d, 0.6 H $J_{1,2}$ 8 Hz, H-1β*a*), 5.15 (d, 0.4 H, $J_{1,2}$ 2.5 Hz, H-1α*a*). H.p.l.c. showed **7** to be contaminated with ~5% of the *O*-deacetylated compound. An aliquot of **7** (20 mg) was purified by flash chromatography on silica gel (8:2 1-propanol–water) to give amorphous **7** (15 mg) (containing traces of soluble silicic acid).

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