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Chemoenzymatic galactosialylation with integrated cofactor regeneration

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As a precursor for the chemical synthesis of sialylated oligosaccharides, the trisaccharide glycoside Neu5Ac α(2–8)Galβ(1–4)GlcNAcβ(1–*O*)-pent-4-ene was synthesized starting from GlcNAcβ(1–*O*)-pent-4-ene, UDP-glucose and *N*-acetylneuraminic acid in a one pot reaction employing galactosyltransferase and α(2–6)sialyltransferase in a complete cofactor regeneration system.

Keywords: *N*-Acetylneuraminic acid, galactosyltransferase, α(2–6)sialyltransferase, cofactor regeneration.

Abbreviations: Neu5Ac, *N*-acetylneuraminic acid; CMP-Neu5Ac, cytidine 5′-monophosphosialate; CMP, cytidine 5′-monophosphate; CDP, cytidine 5′-diphosphate; CTP, cytidine 5′-triphosphate; Gal, galactose; GlcNAc, *N*-acetylglucosamine; UDP, uridine 5′-diphosphate; UDP-Glc, uridine-5′-diphosphoglucose; UDP-Gal, uridine-5′-diphosphogalactose; PEP, phosphoenolpyruvate.

Introduction

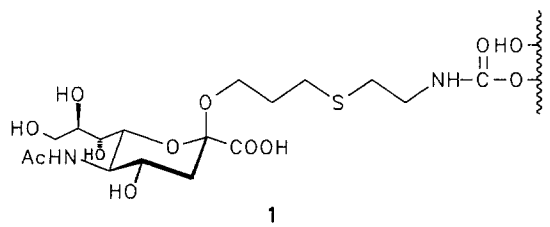
α-Sialooligosaccharides are located on the periphery of the glycocalix, and thus they are involved in biological processes such as cell–cell recognition, interaction and differentiation [1]. The use of activated derivatives of *N*-acetylneuraminic acid (Neu5Ac, **3**) as glycosyl donors in classical glycosylation chemistry often gives poor stereoselectivity, and anomeric mixtures of sialosides are obtained [2]. More complex derivatives of Neu5Ac were used in the chemical synthesis of sialooligosaccharides in order to promote the formation of natural α-sialosides and to increase the yields in glycosylation of secondary alcohols [3]. Generally, by enzyme catalyzed syntheses high stereo- and regioselectivities can be achieved. In particular, by use of galactosyl- and sialyltransferases, the syntheses of a number of complex oligosaccharides were reported [4]. Techniques for detection, isolation and characterization of reaction products exist, but the amount of product formed is limited by the availability of the glycosyltransferases. Therefore it is essential to realize a high transfer rate of the activated glycosyl donor in order to obtain larger quantities of complex carbohydrates.

Recently Wong *et al.* published the enzymatic preparation of sialylated lactosamine derivatives [5, 6]. Previously we had reported our preliminary corresponding efforts [7, 8]. Here we wish to disclose our approach to an efficient enzymatic synthesis of the nonphysiological trisaccharide Neu5Acα(2–6)Galβ(1–4)GlcNAcβ(1–*O*)pent-4-ene (**17**) in a 'one pot' reaction as mentioned previously.

Results and discussion

Isolation of the sialate cytidyltransferase

A solution of crude sialate cytidyltransferase was prepared by homogenization and ultracentrifugation of calf brain. The enzyme activity in all approaches could be determined by the method of Kean *et al.* [9]. As a standard method for semipurification, ammonium sulfate precipitation [10] was applied followed by an affinity chromatography [11] which gave the pure enzyme. The stationary phase consisted of CNBr-activated Sepharose 4B coupled with β-[3-(2-aminoethylthio)propyl]-*N*-acetylneuraminic acid (**1**).



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This affinity ligand β-[3-(2-aminoethylthio)propyl]-*N*-acetylneuraminic acid was characterized by ^{1}H NMR-spectroscopy. In contrast to the possible α-anomer it shows a remarkable downfield shift regarding the resonance signal assigned to its 3e-H proton, and thus proves the β-configuration at the anomeric center [12].

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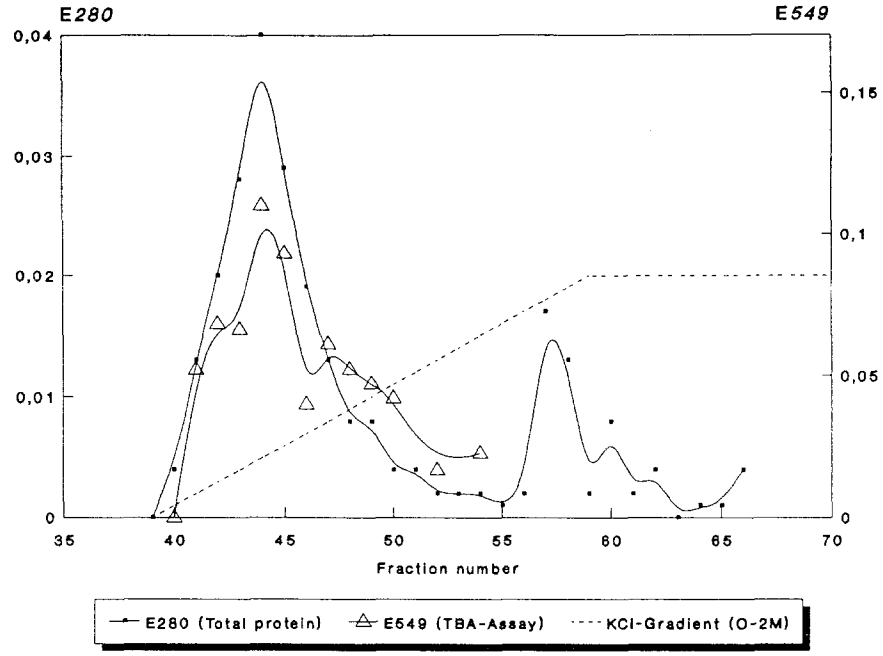
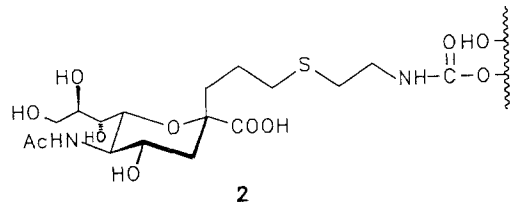


Figure 1. Fractionation of sialate cytidyltransferase on a β-[3-(2-aminoethylthio)propyl-*N*-acetylneuraminic acid column. The column was eluted with a KCl gradient (0.0–1.0 M) in aqueous ammonia (pH 9.0, 10 ml h^{-1}, 0.5 ml per fraction). Active fractions were detected by a thiobarbituric acid assay.

Figure 1 illustrates the results of a typical analytical run on the *N*-acetylneuraminic acid affinity adsorbent with elution of the protein by a KCl gradient, which gave an enzyme sample of about 1.5 mU total activity (25% recovery). Compared with the crude extract a sixfold purification was achieved in this step, and the final activity obtained on this analytical scale was 5 mU mg^{-1}. In a semipreparative approach the previously used ligand gave 670 mU mg^{-1} from trout liver [13]. It could be assumed that purification with this material may operate by an ion exchange mechanism. However, indirect evidence was acquired with the corresponding C-glycosidic material **2** [14] which did not give any purification at all, even though the ion exchange should operate in a similar way.



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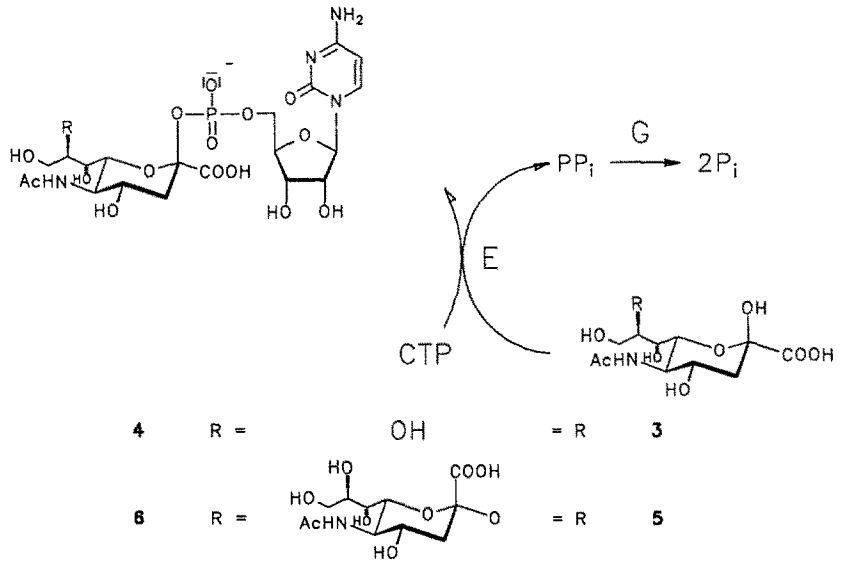
Preparative enzymatic synthesis of cytidine-5′-monophosphosialate (**4**)

The first preparative synthesis of cytidine-5′-monophosphosialate (CMP-Neu5Ac, **4**) was reported by Kean *et al.* [9] as early as 1966. Since then a number of improvements have been described over the years [10, 15] and presently the activated neuraminic acid has been reported to be accessible on the gram scale [32]. The synthetic reaction uses Neu5Ac (**3**) and cytidine 5′-triphosphate (CTP) to give CMP-Neu5Ac (**4**) catalysed by the enzyme sialate cytidyltransferase.

The pH conditions had to be correlated to the two maxima of activity of the enzyme regarding the ions involved. In the presence of manganese(II) ions (6 mM) the maximum of activity was observed at pH 8.0. However, in this range the degradation of nucleotide sugars occur, and thus magnesium(II) ions (25 mM) at pH 9.5 were preferred [10]. In order to prevent the inhibition of sialate cytidyltransferase by pyrophosphate, the enzyme inorganic pyrophosphatase was added. Thus, pyrophosphate was converted irreversibly to phosphate and the equilibrium shifted towards the desired products. For determination of the content of CMP-Neu5Ac (**4**) the sialate cytidyltransferase assay as described [9] could be used. In the presence of a manganese(II) buffer the yield of **4** was 27%, and with a magnesium(II) buffer 76% was obtained.

Immobilization of this enzyme was achieved on silica gel [16] and on a synthetic copolymer of vinyl acetate and *N*,*N*′-divinylethylene urea (VA-epoxy) [17]. The first gave an activity yield of about 50% and the product **4** could be obtained in 70% yield. During the immobilization on VA-epoxy denaturation was observed, and thus the activity dropped to a mere 5%. Thus a preparative synthesis of **4** with this material was not useful.

As the next step, the coupling of the synthesis of **4** with regeneration of CTP by adenylate and pyruvate kinase was achieved. These inexpensive enzymes were added in excess



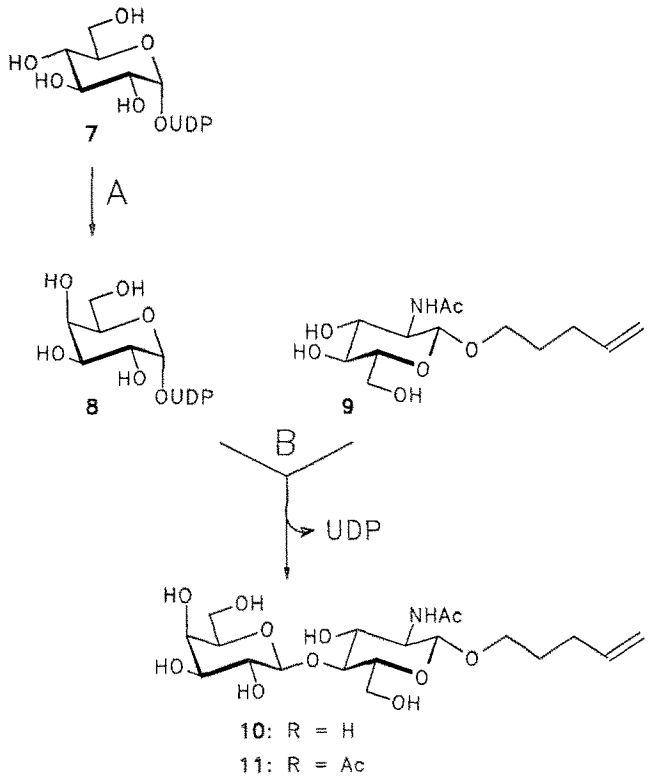
E: sialate cytidyltransferase
G: inorganic pyrophosphatase

in order to overcome the phosphatase activity in the system and to guarantee a sufficient CTP concentration. Until now this approach has given the activated Neu5Ac **4** in 15.2% yield [18], and the mediocre yield may be due to residual phosphatases.

It was of interest to use the above described enzyme system for the synthesis of an important class of oligosaccharides which contain the Neu5Acα(2–8)Neu5Ac sequence, such as group B meningococcal polysaccharides [19], brain tissues [20], and tumour-associated gangliosides [21]. The idea was to use Neu5Acα(2–8)Neu5Ac (**5**) as substrate and transfer it to the activated compound **6** which, in turn, should be tested for use in the sialyltransferase step to construct these structures. Starting with colominic acid, a polymer of Neu5Acα(2–8)Neu5Ac residues, a modified acid hydrolysis according to the method of Jennings *et al.* [22] gave compound **5** in 15.2% (by weight) isolated yield following ion exchange chromatography. However, on the employment of **5** in the activation step no turnover was observed. On the other hand, no inhibitory effects could be demonstrated and thus it is assumed that sialate cytidyltransferase cannot recognize structure **5**.

Synthesis of the acceptor Galβ(1–4)GlcNAcβ(1–O)-pent-4-ene (**10**)

The galactosylation to form Galβ(1–4)GlcNAcβ(1–*O*)-pent-4-ene (**10**) was accomplished by a standard procedure [23–28]. The activated form of galactose required for the transferase reaction, uridine 5′-diphosphate galactose (UDP-Gal, **8**), is very expensive and was therefore generated *in situ* from UDP-glucose (**7**) in a reaction catalyzed by UDP-galactose 4-epimerase. The galactose transfer from UDP-Gal (**8**) to the respective acceptor thus occurred with release of UDP. Galactosylation of chemically synthesized

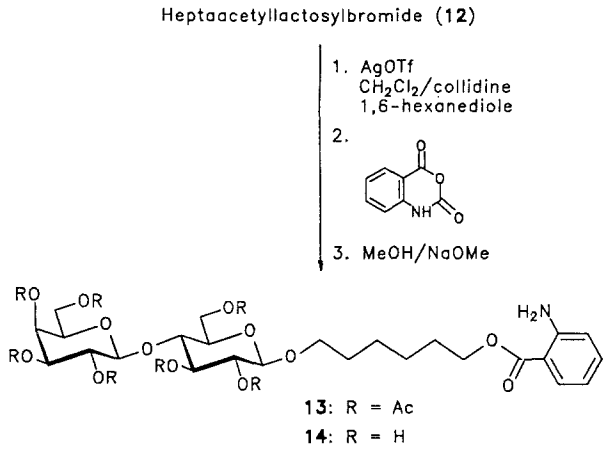


A: UDP-glucose-4′-epimerase
B: bovine galactosyltransferase

GlcNAcβ(1–*O*)pent-4-ene (**9**) according to the above procedure afforded **10** in 31% yield.

Isolation of the α(2–6)sialyltransferase

As described previously, cytidine-5′-diphospho-hexanolamine Sepharose was used as an affinity adsorbent to purify

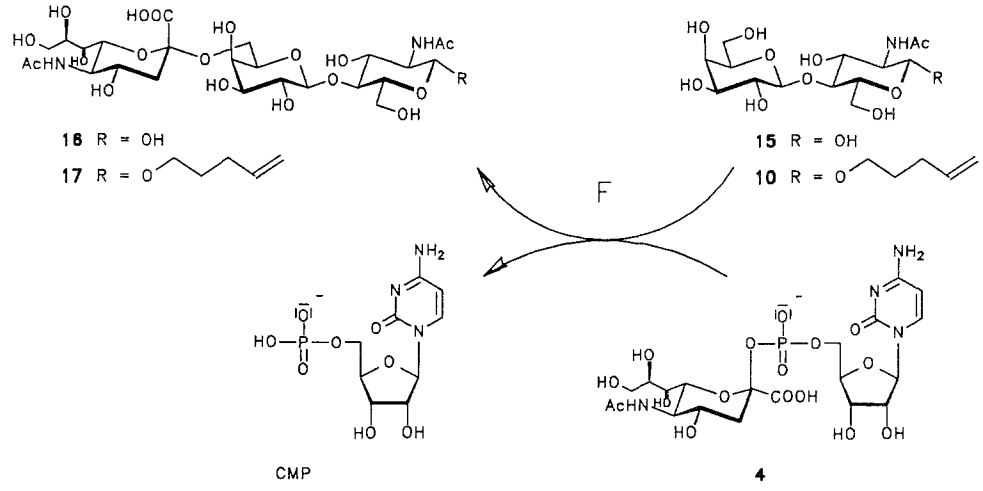


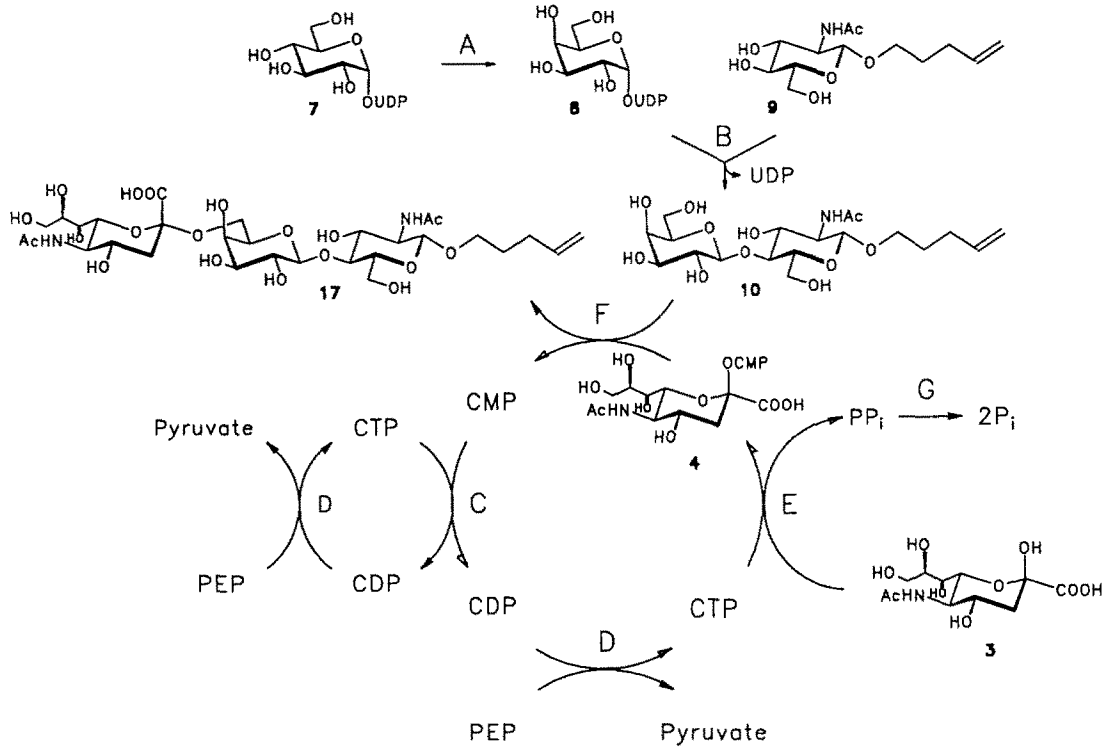
an α(2–6)sialyltransferase from bovine colostrum [29]. Upon binding of the enzyme to the adsorbent, elution was achieved nonspecifically with 0.5–1.0 M sodium chloride. The activity was assayed by measurement of the sialylated product in the enzyme reaction mixture that contains CMP-sialic acid and an acceptor. Usually assays are performed with radioactively labelled CMP-sialic acid, and sialylated products are separated by precipitation, gel filtration or ion exchange chromatography and then measured by scintillation. An alternative was shown by Sato *et al.* [30] using a fluorogenic acceptor for sialyltransferase, and its use in a simple and sensitive assay by HPLC. We now report an easily synthesized new fluorogenic compound and its use in a sialyltransferase assay. Starting from heptaacetyllactosylbromide (**12**), the fluorogenic spacer was added under Koenigs–Knorr conditions to yield after deacetylation [6″-*O*-(2‴-aminobenzoyl)hexyl]-4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranoside (**14**) in 76%. We expected from the above results that **14** could be used as acceptor for the assay of sialyltransferase in bovine colostrum. The amount of sialylated **14** formed with respect to reaction time and enzyme concentration was examined and the transfer rate remained constant for 4 h. Thus, the assay could be reliably employed for monitoring sialyltransferase reactions.

Galactosialylation reaction including an integrated cofactor regeneration

It has been shown previously that *N*-acetyllactosamine (**15**) could be converted to sialyllactosamine (**16**) by the use of an α(2–6)sialyltransferase from bovine colostrum. This single step is handicapped by the inhibitor cytidine 5′-monophosphate (CMP) formed in the transferase step, so the trisaccharide **16** could be obtained in only 52% yield [15]. In order to avoid this problem, the activation was coupled with the sialyltransfer. In this enzymatic system, CMP was converted to cytidine 5′-diphosphate (CDP) catalysed by adenylate kinase in the presence of CTP. CDP was further converted to CTP with phosphoenol pyruvate (PEP) catalyzed by pyruvate kinase. CTP was then reacted with Neu5Ac (**3**) catalyzed by sialate cytidyltransferase to give the product **4**. The byproduct inorganic pyrophosphate was decomposed by pyrophosphatase. Sialylation of Galβ(1–4)GlcNAcβ(1–*O*)pent-4-ene (**10**) was accomplished by CMP-Neu5Ac (**4**) and α(2–6)sialyltransferase. The released CMP was again converted to CDP, to CTP, and to CMP-Neu5Ac. By this sialylation with intrinsic regeneration of the cofactor which only needs catalytic amounts of CMP the trisaccharide **17** was isolated in 76% yield.

Finally we could show that all previously described steps can be combined in a 'single pot' reaction which gave compound **17** in an overall yield of 24%. The reason for the reduced yield in comparison with the single sialylation cycle could be the formation of uridine 5′-diphosphate (UDP) during the galactosylation, which inhibits this transferase. In order to prevent this, a separate regeneration system for UDP into UDP-Glc will have to be incorporated.





A: UDP-glucose-4'-epimerase
B: bovine galactosyltransferase
C: adenylate kinase
D: pyruvate kinase
E: sialate cytidyltransferase
F: α(2->6)sialyltransferase
G: inorganic pyrophosphatase

Materials and methods

Materials

Adenylate kinase (EC 2.7.4.3), pyruvate kinase (EC 2.7.1.40), UDP-glucose-4'-epimerase (EC 5.1.3.2), and inorganic pyrophosphatase (EC 3.6.1.1) were from Sigma, Germany, bovine galactosyltransferase (EC 2.4.1.22) was from Boehringer Mannheim, Germany. Solutions of enzymes were concentrated by ultrafiltration using Amicon concentrators with PM-10 Diaflo membranes at 50–70 lb in^{-2}. ^{1}H-NMR spectra were recorded with a Bruker AMX-400 (400 MHz) instrument. All reactions were monitored by TLC on silica gel FG_{254} (Merck) with detection by UV light or charring with sulfuric acid. The α(2–6)sialyltransferase was isolated from bovine colostrum as described previously [29]. The glycosides were separated by gel chromatography on Sephadex G-10 (Pharmacia).

Purification of sialate cytidyltransferase

The sialate cytidyltransferase (EC 2.7.7.43) was first semi-purified from fresh calf brain by ammonium sulfate precipitation [9]. For the subsequent affinity chromatography, a column of β-[3-(2-aminoethylthio)propyl]-*N*-acetylneuraminic acid coupled to CNBr-activated Sepharose 4B (**1**) (3 ml wet gel) was pre-equilibrated with 1 mM aqueous ammonia, pH 9.0, containing 1 mM 2-mercaptoethanol and NaN_3 (0.02%). The column was loaded with a solution of the semi-purified enzyme (1 ml, 10 ml h^{-1}) and washed with the aqueous ammonia mentioned above. When the UV absorbance at 280 nm was about zero the enzyme was eluted with a KCl gradient (0.0–1.0 M) in the aqueous ammonia (10 ml, 10 ml h^{-1}, 0.5 ml per fraction). Active fractions were detected by a thiobarbituric acid assay [9] and combined.

Enzymatic synthesis of cytidine 5'-monophosphosialate (**4**)

N-Acetylneuraminic acid (**3**, 40 mg, 130 μmol) and cytidine 5'-triphosphate (100 mg, 0.22 mmol) were dissolved in incubation buffer. Then sialate cytidyltransferase (4 U) and inorganic pyrophosphatase (10 U) were added, the reaction incubated at 37 °C and regularly monitored by TLC

on cellulose plates. Further amounts of CTP (100 mg, 0.22 mmol, each) were added in three individual portions every 60 min. After 4 h the enzymes were removed by ultrafiltration and the product purified by gel filtration (Sephadex G-10, 150 cm × 2.5 cm column) and eluting with aqueous ammonia, pH 8.5.

Application of incubation buffer A, 62 mM tris(hydroxymethyl)aminomethane and 6 mM manganese(II) chloride, at pH 8.0, yield: 10 mg (27% based on Neu5Ac). Application of incubation buffer B, 62 mM tris(hydroxymethyl)aminomethane and 25 mM magnesium(II) chloride, at pH 9.5, yield: 31 mg (76%, based on Neu5Ac), literature [9]: 79%.

^{1}H-NMR (^2H_2O): δ = 8.00 (d, H-6″), 6.15 (d, H-5″), 5.98 (d, H-1′), 4.20–4.30 (m, H-2′ to H-5′), 1.67 (dd, H-3a), 2.48 (dd, H-3e), 4.07 (ddd, H-4), 3.90 (mc, 3H, H-5, H-8, H-9a), 4.10 (dd, H-6), 3.45 (dd, H-7), 3.62 (dd, H-9b), 2.04 (s, NAc); $J_{5'',6''}$ = 7.6, $J_{1',2'}$ = 4.3, $J_{3a,3e}$ = 13.2, $J_{3a,P}$ = 6.1, $J_{3a,4}$ = 4.7, $J_{3e,4}$ = 11.3, $J_{3e,P}$ < 0.5, $J_{4,5}$ = 10.4, $J_{5,6}$ = 10.3, $J_{6,7}$ = 0.8, $J_{7,8}$ = 9.5, $J_{8,9b}$ = 6.3, $J_{9a,9b}$ = 11.7 Hz.

In situ enzymatic synthesis of cytidine 5′-triphosphate (CTP) and cytidine 5′-monophosphosialate (**4**)

A solution of Neu5Ac (**3**, 20 mg, 65 µmol), CMP (200 mg, 0.48 mmol), and potassium phosphoenol pyruvate (300 mg, 1.3 mmol) in degassed incubation buffer (20 ml) [100 mM tris(hydroxymethyl)aminomethane, 25 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.1% sodium azide at pH 8.0] was treated with sialate cytidyltransferase (1 U), inorganic pyrophosphatase (10 U), pyruvate kinase (1000 U), and adenylate kinase (2500 U). After 3 d shaking at room temperature the reaction was quenched by ultrafiltration and worked up as described above. The yield was 3 mg (15.2% based on Neu5Ac).

Acid hydrolysis of colominic acid

A solution of colominic acid (240 mg, 14 µmol) and water (75 ml) was adjusted with 2 N HCl to pH 3.8. The mixture was stirred at 79 °C for 1.5 h, cooled with ice and neutralized with 2 N NaOH. After lyophilization, products were separated by ion exchange chromatography using a linear gradient of 1N NaCl:0.01 N tris-buffer, pH 7.6, and subsequent desalting on Sephadex G-10. The yields were Neu5Ac (**3**), 8.0 mg (260.0 µmol), 3.3% (per weight): $(Neu5Ac)_2$ (**5**); 36.4 mg (58.8 µmol), 15.2% (per weight).

^{1}H-NMR (400 MHz, ^2H_2O): **3**: δ = 2.29 (dd, 1H, H-3_{eq}), 1.87 (dd, 1H, H-3_{ax}), 4.10 (ddd, 1H, H-4), 3.94 (dd, 1H, H-5), 4.05 (dd, 1H, H-6), 3.55 (dd, 1H, H-7), 3.77 (ddd, 1H, H-8), 3.63 (dd, 1H, H-9a), 3.85 (dd, 1H, H-9b), 2.07 (s, 3H NAc); $J_{3ax,3eq}$ = 13.1, $J_{3ax,4}$ = 11.8, $J_{3eq,4}$ = 4.7, $J_{4,5}$ = 10.4, $J_{5,6}$ = 10.4, $J_{6,7}$ = 0.8, $J_{7,8}$ = 9.2, $J_{8,9a}$ = 6.3, $J_{8,9b}$ = 2.5, $J_{9a,9b}$ = 11.7 Hz.

^{1}H-NMR (400 MHz, ^2H_2O) **5**: δ = 2.18 (dd, 1H, H-3_{eq}), 1.65 (dd ~ t, 1H, H-3_{ax}), 2.73 (dd, 1H, H-$3'_{eq}$), 1.54 (dd ~ t, 1H, H-$3'_{ax}$), 4.07-3.31 (m, 14H, H-4′, H-5′, H-6′, H-7′, H-8′, H-9a′, H-9b′, H-4, H-5, H-6, H-7, H-8, H-9a, H-9b), 1.89 (s, 3H, NAc), 1.92 (s, 3H, NAc); $J_{3'ax,3'eq}$ = 12.5, $J_{3'ax,4'}$ = 11.9, $J_{3'eq,4'}$ = 3.5, $J_{3ax,3eq}$ = 13.0, $J_{3ax,4}$ = 12.0, $J_{3eq,4}$ = 4.5 Hz.

Pentenyl 2-acetamido-2-deoxy-4-O-(β-D-galactopyranosyl)-β-D-glucopyranoside (**10**)

The incubation buffer was 100 mM tris-(hydroxymethyl)-aminomethane: 5 mM $MgCl_2$: 0.02% NaN_3, pH 7.5.

UDP-glucose (**7**) (260 mg, 0.40 mmol) and **9** (232 mg, 0.80 mmol) were dissolved in incubation buffer (20 ml) and the solution was deoxygenized with helium. Bovine serum albumin (20 mg), α-lactalbumin (10 mg), UDP-galactose-4-epimerase (10 U) and galactosyltransferase (5 U) were added and the mixture was incubated at 37 °C. The reaction was followed by a UDP-assay [31] and stopped after 2 d with a monitored yield of 40%. After lyophilization, separation followed by gel chromatography on Sephadex G-10.

Isolated yield: 58 mg (31%), m.p. 183 °C, $[\alpha]_D^{20}$ + 34.3° (*c* 1.4, H_2O). ^{1}H-NMR (400 MHz, ^2H_2O): δ = 4.53 (d, 1H, H-1), 4.59 (d, 1H, H-1′), 3.60 (dd, 1H, H-2′), 3.67 (dd ~ t, 1H, H-3′), 3.96 (dt, $H_{pentenyl}$-1a), 3.57 (dt, 1H, $H_{pentenyl}$-1b), 1.59–1.73 (m, 2H, $H_{pentenyl}$-2), 2.06–2.12 (m, 2H, $H_{pentenyl}$-3), 5.95 (m, 1H, $H_{pentenyl}$-4), 5.05–5.26 (m, 2H, $H_{pentenyl}$-5a, $H_{pentenyl}$-5b), 2.09 (s, 3H, NAc); $J_{1',2'}$ = 7.5, $J_{2',3'}$ = 9.5, $J_{1,2}$ = 8.0, $J_{pentenyl\text{-}1a,\ pentenyl\text{-}1b}$ = 10.5, $J_{pentenyl\text{-}1a,2}$ = 6.0, $J_{pentenyl\text{-}1b,2}$ = 6.5 Hz.

For detailed characterization, **10** (10 mg) was acetylated by the standard procedure with pyridine:acetic anhydride (3:1) to give pentenyl 2-acetamido-3,6-di-*O*-acetyl-2-deoxy-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-β-D-glucopyranoside (**11**).

^{1}H-NMR (400 MHz, C^2HCl_3) **11**: δ = 4.38 (d, 1H, H-1), 3.96 (ddd, 1H, H-2), 5.01 (dd ~ t, 1H, H-3), 3.72 (dd ~ t, 1H, H-4), 3.56 (ddd, 1H, H-5), 3.98–4.11 (m, 3H, H-6a, H-6b, H-6′b), 4.44 (d, 1H, H-1′), 5.05 (dd, 1H, H-2′), 4.91 (dd, 1H, H-3′), 5.29 (dd, 1H, H-4′), 3.79–3.84 (m, 1H, H-5′), 4.43 (dd, 1H, H-6′a), 3.76 (dt, 1H, $H_{pentenyl}$-1a), 3.38 (dt, 1H, $H_{pentenyl}$-1b), 1.62–1.73 (m, 2H, $H_{pentenyl}$-2), 2.08–2.14 (m, 2H, $H_{pentenyl}$-3), 5.71 (m, 1H, $H_{pentenyl}$-4), 4.85–4.96 (m, 2H, $H_{pentenyl}$-5a, $H_{pentenyl}$-5b), 5.58 (d, 1H, NH), 2.03 (s, 3H, NAc); $J_{1',2'}$ = 8.0, $J_{2',3'}$ = 10.5, $J_{3',4'}$ = 3.5, $J_{4',5'}$ = 8.5, $J_{5',6'a}$ = 3.0, $J_{6'a,6'b}$ = 12.0, $J_{1,2}$ = 7.5, $J_{4,5}$ = 8.5, $J_{5,6a}$ = 2.5, $J_{5,6b}$ = 5.0, $J_{6a,6b}$ = 12.0, $J_{pentenyl\text{-}1a,\ pentenyl\text{-}1b}$ = 9.5, $J_{pentenyl\text{-}1a,2}$ = 6.5, $J_{pentenyl\text{-}1b,2}$ = 7.0 Hz.

[6″-O-(2‴-Aminobenzoyl)hexyl]-2,3,6-tri-O-acetyl-4-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-β-D-glucopyranoside (**13**)

Heptaacetyllactosylbromide (**12**, 1.80 g, 2.5 mmol), hexane-1,6-diol (308 mg, 2.6 mmol) and 4 Å molecular sieves were dissolved in anhydrous dichloromethane (60 ml) under nitrogen. After cooling to −40 °C, silver triflate (693 mg, 2.7 mmol) and collidine (442 µl, 3.1 mmol) were added and stirred for 1 h. After 4 h the mixture was warmed to −10 °C, molecular sieves and catalyst were separated and the

product isolated by chromatography on silica gel with toluene:acetone (7:3). Dihydroxy-2,4-dioxo-3,1-benzoxazine (815 mg, 5.0 mmol) in EtOH:H_2O (1:1, 20 ml) were added at pH 10.0. After neutralization the product was separated as described above.

Yield: 1.39 g (76%), $[\alpha]_D^{20}$ + 32° (*c* 1.0, $CHCl_3$). ^{1}H-NMR (400 MHz, C^2HCl_3): δ = 4.78 (d, 1H, H-1), 4.81 (dd, 1H, H-2), 5.22 (dd ~ t, 1H, H-3), 3.61–3.89 (m, 4H, H-4, H-5, H-5′, H_{hexyl}-1″a), 3.92–4.19 (m, 4H, H-6a, H-6b, H-6′a, H-6′b), 4.61 (d, 1H, H-1′), 5.08–5.14 (m, 1H, H-2′), 5.02 (dd, 1H, H-3′), 5.39 (dd, 1H, H-4′), 3.53 (t, 1H, H_{hexyl}-1″b), 3.47 (t, 2H, H_{hexyl}-6″), 1.43–1.75 (m, 8H, H_{hexyl}-2″-7″), 1.91–7.82 (d, 1H, H_{aryl}-3‴), 7.17 (d, 1H, H_{aryl}-6‴), 6.09–6.16 (m, 2H, H_{aryl}-4‴/5‴), 1.88–2.09 (m, 21H, OAc); $J_{1',2'}$ = 8.5, $J_{2',3'}$ = 10.0, $J_{3',4'}$ = 3.5, $J_{1,2}$ = 8.0, $J_{2,3}$ = 9.5 Hz.

[6″-O-(2‴-Aminobenzoyl)hexyl]-4-O-(β-D-galactopyranosyl)-β-D-glucopyranoside (**14**)

13 (1.39 g, 1.9 mmol) was dissolved in anhydrous methanol (50 ml) with a catalytic amount of NaOMe. After 24 h at room temperature, neutralization was effected with dry ice. Separation was performed by HPLC (see sialyltransferase assay).

Yield: 0.98 g (93%), $[\alpha]_D^{20}$ + 97.6° (*c* 1.5, MeOH), ε_{280} 14.3 $cm^2\ mmol^{-1}$, ε_{330} 5.2 $cm^2\ mmol^{-1}$. ^{1}H-NMR (400 MHz, ^2H_2O): δ = 4.69 (d, 1H, H-1), 4.63 (d, 1H, H-1′), 3.48 (t, 2H, H_{hexyl}-1″a/1″b), 1.49–1.76 (m, 8H, H_{hexyl}-2″-7″), 7.82 (d, 1H, H_{aryl}-3‴), 7.17 (d, 1H, H_{aryl}-6‴), 6.09–6.16 (m, 2H, H_{aryl}-4‴/5‴); $J_{1',2'}$ = 8.5, $J_{1,2}$ = 8.0 Hz.

β-D-Galactoside α(2–6)-sialyltransferase assay

The incubation buffer was 10 mM sodium cacodylate; 2 mM $MnCl_2$ pH 6.5, the solution of **14** was 5.0 mmol (1.9 mg ml^{-1}) in incubation buffer and of CMP-Neu5Ac (**4**) was 7.0 mmol (5.3 mg ml^{-1}) in aqueous ammonia, pH 7.5. The assay mixture was incubation buffer, 900 µl: solution of **14**, 70 µl: CMP-Neu5Ac (**4**), 30 µl: enzyme, x µl.

The mixture was incubated at 37 °C for 30 min and the reaction terminated by cooling in liquid nitrogen, and subsequent ultrafiltration. Separation followed on a Lichrosorb-NH_2 column (20 cm × 0.5 cm, 5 µm, Knauer) with a 0.1 M NH_4OAc buffer, pH = 4.5, as eluant and a flow rate of 1.5 ml min^{-1}. Elution time for **14**, 10.2 min; for sialylated **14**, 11.9 min. The HPLC peaks were integrated by use of Merck–Hitachi software.

Pentenyl 2-acetamido-2-deoxy-4-O-[6-O-(5-acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonate)-β-D-galactopyranosyl]-β-D-glycopyranoside (**17**)

Without integrated galactosylation. The incubation buffer was 10 mM sodium cacodylate: 2 mM $MnCl_2$: 0.02% NaN_3, pH 7.4. Neu5Ac (**3**, 22 mg, 70 µmol), **10** (37 mg, 66 µmol), CMP-Neu5Ac (**4**) (10 mg, 24 µmol) and PEP (300 mg, 1.3 mmol) were dissolved in degassed incubation buffer (10 ml). Subsequently, bovine serum albumin (10 mg) and the following enzymes were added: β-D-galactoside-α(2–6)sialyltransferase (0.6 U), sialate cytidyltransferase (1.0 U), inorganic pyrophosphatase (10 U), pyruvate kinase (1000 U), adenylate kinase (2500 U). The reaction mixture was incubated at 37 °C and monitored by TLC (Lichrosorb-NH_2, ethanol:1 M ammonium acetate). After 48 h, lyophilization and product separation on Sephadex G-10 with a 0.01 M ammonium hydrogencarbonate eluent gave, after evaporation, 37 mg (76%) **17**.

With integrated galactosylation. The incubation buffer was 10 mM sodium cacodylate: 2 mM $MnCl_2$: 0.02% NaN_3, pH 7.4. Neu5Ac (**3**, 12 mg, 38 µmol), pentenyl derivative **10** (11 mg, 36 µmol), CMP-Neu5Ac (**4**, 10 mg, 24 µmol), PEP (300 mg, 1.3 mmol) and UDP-Glc (**7**, 52 mg, 80 µmol) were dissolved in degassed incubation buffer (10 ml). Bovine serum albumin (10 mg), α-lactalbumin (10 mg) and the following enzymes were added: UDP-galactose-4-epimerase (5.0 U), galactosyltransferase (2.0 U), β-D-galactoside-α(2–6)sialyltransferase (0.6 U), sialate cytidyltransferase (1.0 U), inorganic pyrophosphatase (10 U), pyruvate kinase (1000 U), adenylate kinase (2500 U). Incubation and separation were performed as described above. The yield was 6 mg (24%) of a colourless amorphous solid, $[\alpha]_D^{20}$ − 47.3° (*c* 1.7, H_2O). ^{1}H-NMR (400 MHz), ^2H_2O): δ = 4.48 (d, 1H, H-1′), 4.58 (d, 1H, H-1″), 2.70 (dd, 1H, H-$3'''_{eq}$), 1.76 (dd ~ t, 1H, H-$3'''_{ax}$), 1.71–1.64 (m, 2H, $H_{pentenyl}$-2), 2.01–2.13 (m, 8H, $H_{pentenyl}$-3, 2N*Ac*), 5.92 (m, 1H, $H_{pentenyl}$-4), 5.01–5.12 (m, 2H, $H_{pentenyl}$-5a/5b): $J_{3'''ax,\ 3'''eq}$ = 12.8, $J_{1'',2''}$ = 8.5, $J_{1',2'}$ = 8.0 Hz.

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References

1. Schauer R (1982) *Adv Carbohydr Chem Biochem* **40**:131–43.
2. Iijima H, Ogawa T (1989) *Carbohydr Res* **186**:95–106.
3. Okamoto K, Goto T (1990) *Tetrahedron* **46**:3987–97.
4. Toone EJ, Simon ES, Bednarski MD, Whitesides GM (1989) *Tetrahedron* **45**:5365–422.
5. Ichikawa Y, Shen GJ, Wong CH (1991) *J Am Chem Soc* **113**:4698–700
6. Ichikawa Y, Liu JLC, Shen GJ, Wong CH (1991) *J Am Chem Soc* **113**:6300–2.
7. Thiem J (1989) *197th ACS Spring Meeting*, Dallas, Abstr Carb 46.
8. Thiem J (1990) *199th ACS Spring Meeting*, Boston, Abstr Carb 60.
9. Kean L, Roseman S (1966) *Methods Enzymol* **8**:208–15.
10. Higa HH, Paulson JC (1985) *J Biol Chem (1985)* **260**:8838–49.

11. Thiem J, Stangier P, Matschulat P, Paulsen H (1992) *Carbohydr Res* **225**:147–50.
12. Dabrowski U, Friebolin H, Brossmer R, Supp M (1979) *Tetrahedron Lett* **4**:4637–40.
13. Schmelter T, Ivanov S, Stangier P, Thiem J, Schauer R (1993) *Biol Chem Hoppe-Seyles*, in press.
14. Paulsen H. Matschulat P (1991) *Liebigs Ann Chem* 487–95.
15. Thiem J, Treder W (1986) *Angew Chem Int Ed Engl* **25**:1096–97.
16. Weetall HH (1976) *Methods Enzymol* **44**:134–48.
17. Burg K, Mauz O, Noetzel S, Sauber K (1988) *Angew Makromol Chem* **157**:105–12.
18. Thiem J, Stangier P (1990) *Liebigs Ann Chem* 1101–5.
19. Jennings HJ, Katzenellenbogen E, Lugowski C, Michon F, Roy R, Casper DL (1984) *Pure Appl Chem* **56**:893–901.
20. Finne J (1985) *Trends Biochem Sci* **10**:129–37.
21. Cahn LD, Irie RF, Singh R, Cassidenti A, Paulson JC (1982) *Proc Natl Acad Sci USA* **79**:7629–38.
22. Jennings HJ, Roy R, Michon F (1985) *J Immunol* **134**:2561–73.
23. Wong CH, Haynie SL, Whitesides GM (1982) *J Org Chem* **47**:5416–23.
24. Thiem J, Wiemann T (1990) *Angew Chem Int Ed Engl* **29**:80–82.
25. Palcic MM, Srivastava OP, Hindsgaul O (1987) *Carbohydr Res* **159**:315–24.
26. Augé C, Fernandez-Fernandez R, Gautheron C (1990) *Carbohydr Res* **200**:257–68.
27. Thiem J, Wiemann T (1991) *Angew Chem Int Ed Engl* **30**:1163–65.
28. Thiem J, Wiemann T (1992) *Synthesis* 141–45.
29. Paulson JC, Beranek WE, Hill RL (1977) *J Biol Chem* **252**:2356–62.
30. Sato T, Omichi K, Ikenaka T (1988) *J Biochem* (*Tokyo*) **104**:18–21.
31. Graßl M (1970) In *Methoden der enzymatischen Analyse*, Vol 2, p 2071 (Bergmeyer HU ed.). Verlag Chemie, Weinheim.