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New sialyltransferase inhibitors based on CMP-quinic acid: development of a new sialyltransferase assay

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Quinic acid (4) was transformed into phosphitamides 6, 14, and 15, which could be readily linked to 5′-O-unprotected cytidine derivative 7; ensuing oxidation of the obtained phosphite triesters with *tert*-butylhydroperoxide furnished the corresponding phosphate triesters 8, 16, and 17, respectively. Hydrogenolytic debenzylation of the phosphate moiety, base catalysed removal of acetyl protective groups, and basic hydrolysis of the methylester of the quinic acid moiety furnished CMP-Neu5Ac analogues 1–3. In order to measure their inhibition of sialyltransferases, a nonradioactive sialyltransferase assay [employed for *α*(2-6)-sialyltransferase from rat liver (EC 2.4.99.1)] based on reversed-phase HPLC separation of UV-labelled acceptor 20 (p-nitrophenyl glycoside of *N*-acetyllactosamine) from the UV-labelled product 21 (p-nitrophenyl glycoside of sialyl *α*(2-6′)-*N*-acetyllactosamine) and p-nitrophenylalanine as internal standard was developed. The assay reproduced the reported K_M values for CMP-Neu5Ac and *N*-acetyllactosamine and the K_i values for CDP. 1 and 2 turned out to be potent sialyltransferase inhibitors.

***Keywords*: CMP-Neu5Ac analogues, synthesis, sialyltransferase inhibitors, sialyltransferase assay, *α*(2-6)-sialyltransferase**

Introduction

Sialic acids play an important role in quite a few biological processes, such as cell adhesion and inflammation [1]. Additionally, several reports indicate that there is a correlation between cell surface sialic acid or sialyltransferase activity and the growth [2] or metastatic potential of tumour cells [3, 4]. Thus, it is desirable to develop specific inhibitors for sialyltransferases, in order to elucidate the influence of sialyl residues in biological systems. Because the various sialyltransferases, independent of their source and their acceptor specificity, all employ cytidine monophosphate *N*-acetylneuraminic acid (CMP-Neu5Ac, **A** in Scheme 1) as the donor substrate [5], donor analogous inhibitors of high affinity seem to be particularly important. Up to now only a few donor and acceptor analogous sialyltransferase inhibitors have been reported [6, 7]. We present the synthesis of new CMP-Neu5Ac analogues (Scheme 1, **1–3**) which are based on quinic acid (Scheme 2, **4**) and derivatives [8]. The inhibition constants (K_i values) of these compounds were measured based on a new assay system utilizing a UV-labelled acceptor leading to a UV-labelled product which can be readily separated by RP-HPLC and then quantified by UV-spectroscopy.

Results and discussion

Synthesis of sialyltransferase inhibitors based on quinic acid

An efficient inhibitor requires high binding affinity to the active site (generally provided by substrate and/or transition state analogues with competitive binding) and high stability under physiological conditions. Following previous reports, binding of donor analogues to sialyltransferases requires the CMP moiety or at least a nucleoside monophosphate residue [9], yet structural variations in the phosphate group and the 4-, 5-, or 9-position of the Neu5Ac residue [10–12] are tolerated. Additionally, in order to block the transferase potential of the enzymes, stability of the glycosidic bond is required. All these demands can be almost ideally fulfilled by quinic acid and derivatives having the CMP moiety attached to the tertiary hydroxy group. Therefore, we initiated a programme for the synthesis of this type of compound [8] – typical examples are **1–3** (Scheme 1) – and to investigate their sialyltransferase inhibition properties.

Use of quinic acid (Scheme 2, **4**) for the synthesis of CMP-Neu5Ac analogues requires selective protection of the

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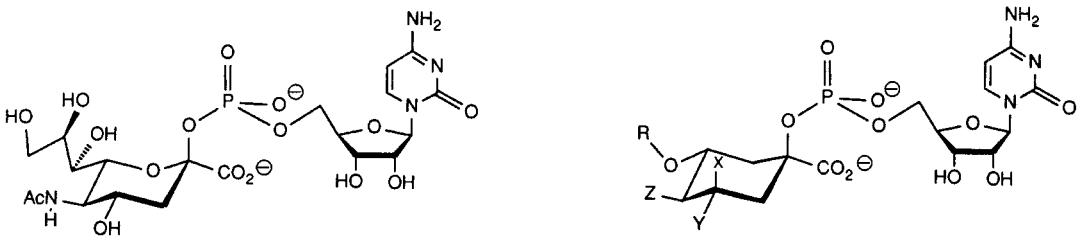


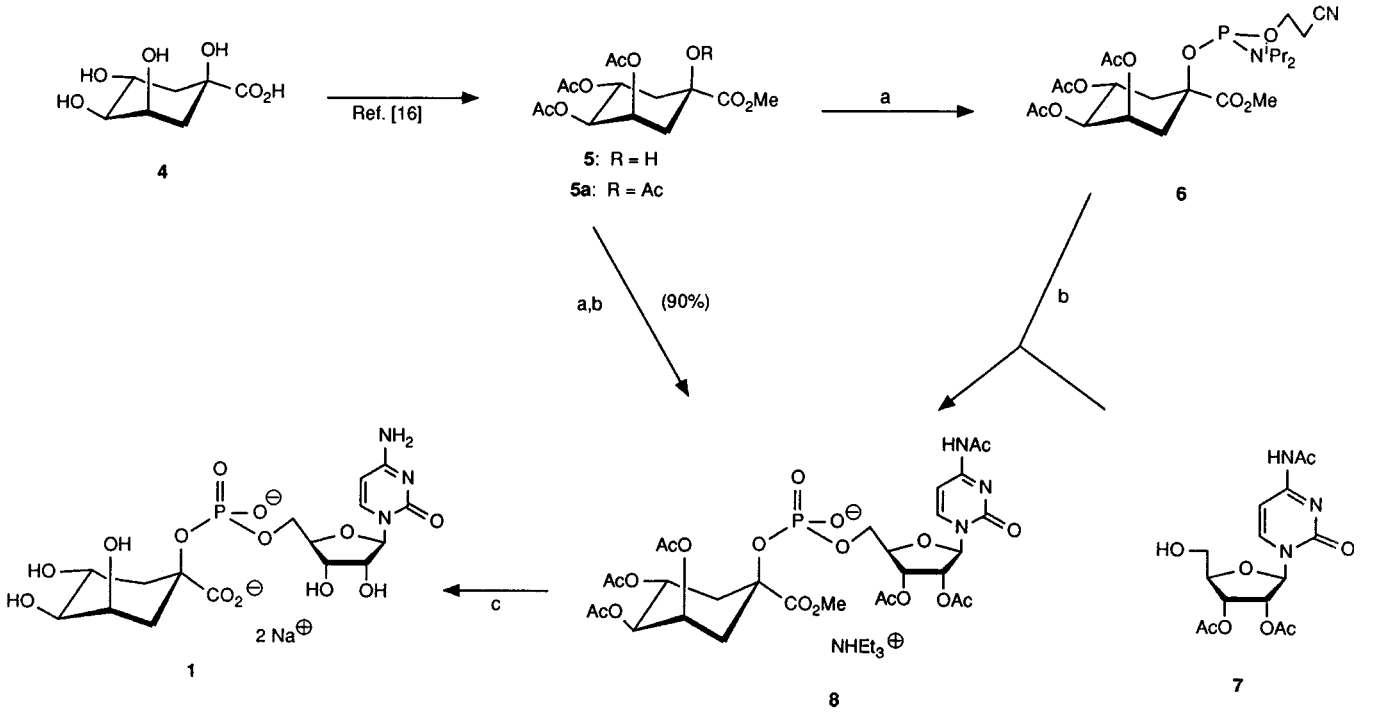
A

1: X = Z = OH, Y = R = H

2: X = R = H, Y = NHAc, Z = OH

3: X = R = H, Y = $\overset{\oplus}{N}H_3$, Z = OH

Scheme 1.



Scheme 2. (a) $(^iPr_2N)_2POCH_2CH_2CN$, diisopropylammonium tetrazolide, CH_3CN (75%). (b) i: 1-H-tetrazole, CH_3CN; ii: t-BuOOH; iii: NEt_3/SiO_2 (90%). (c) i: NaOMe, MeOH; Amberlite IRC 176; ii: NaOH, MeOH/H_2O; Amberlite IRC 176, (65%).

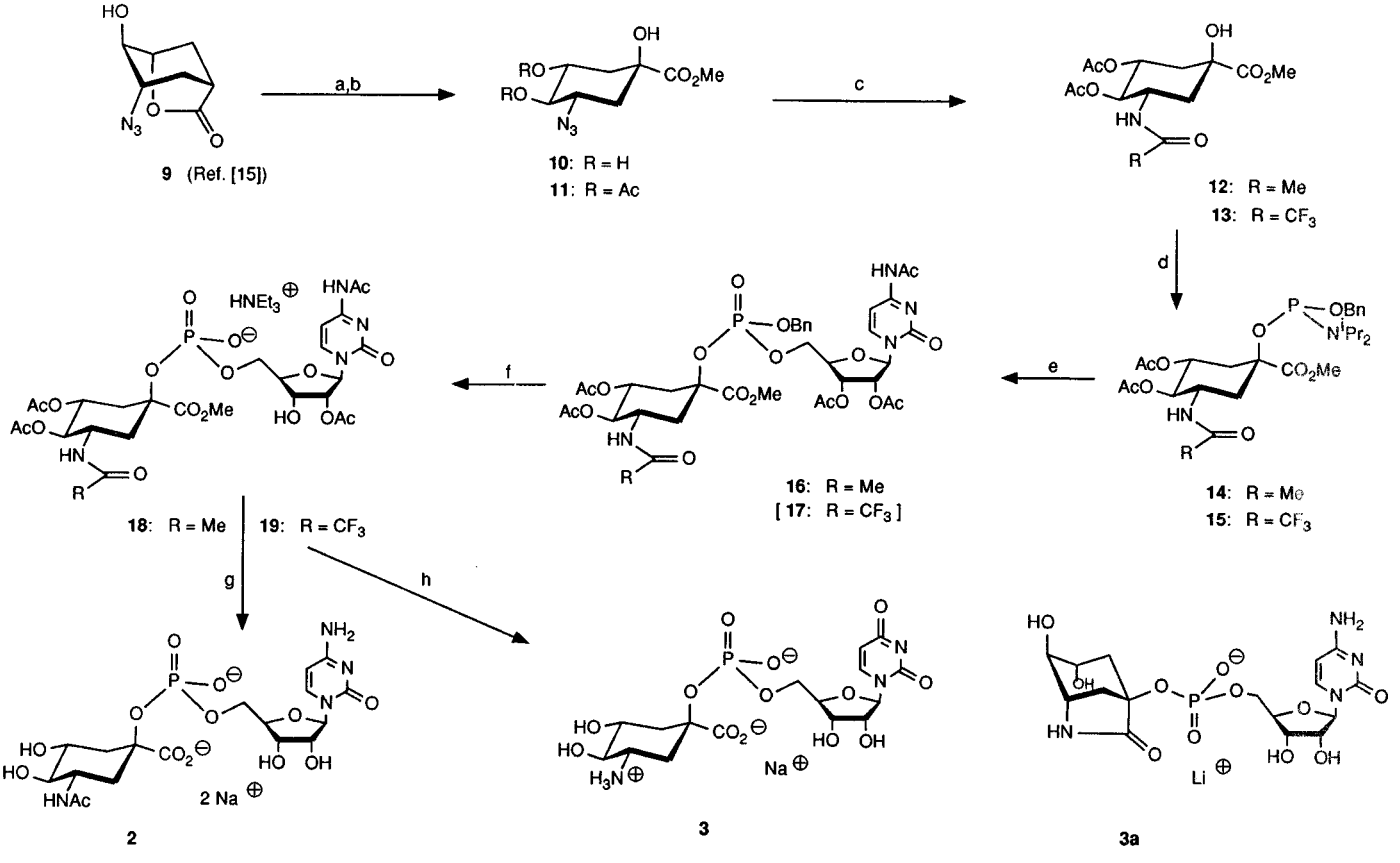
hydroxy groups in 3-, 4-, and 5-position. Due to their different relative positioning and stereochemical orientation, chemoselective reactions have been already developed for this endeavour [13–15]. For instance, the readily available methylester [16] can be selectively transformed into 3,4,5-tri-*O*-acetyl derivative **5** in good yield [16]; fully *O*-acetylated derivative **5a**, obtained as minor byproduct, could be separated. For the attachment of the CMP residue, the classical phosphite amide methodology [17] was chosen. To this aim, **5** was treated with 2-cyanoethoxy-bis(diisopropylamino)phosphane [18] in the presence of tetrazole/diisopropylamine furnishing phosphite diester derivative **6** in good yield (75%); upon reaction with 5′-O-unprotected cytidine **7** [19] in the presence of tetrazole, oxidation of the intermediate phosphite triester with *tert*-butylhydroperoxide, and ensuing treatment with triethylamine then afforded the desired phosphate diester **8**, which could be fully structurally assigned by NMR-spectroscopy. An overall 90% yield of **8** could be obtained when the reaction sequence starting from **5** was carried out as one-pot procedure. Treatment of the acetyl protected methylester derivative **8** first with NaOMe in methanol and then with NaOH in methanol/water furnished target molecule **1** as disodium salt, which could be structurally assigned by NMR and mass spectrometry.

For the introduction of amino groups into the quinic acid residue, differentiation between the hydroxy groups in 3-, 4-, and 5-position was required. To this aim, **4** was transformed

into 3-azido derivative **9** following known procedures [15] (Scheme 3). Opening of the lactone moiety with NaOMe in methanol gave methylester **10** which was readily transformed into the 4,5-di-*O*-acetyl derivative **11**. Transformation of the azido group into the amino group was performed by hydrogenation in the presence of palladium on carbon as catalyst; ensuing treatment with acetic anhydride or with trifluoroacetic anhydride furnished *N*-acetyl derivatives **12** and **13**, respectively. Both compounds were tranformed into phosphitamide diesters **14** and **15** with benzyloxy-chlorodiisopropylaminophosphane [20] in the presence of Hünig's base. Condensation of **14** with **7** in the presence of tetrazole and ensuing oxidation with *tert*-butylhydroperoxide gave fully protected CMP-Neu5Ac analogue **16**; hydrogenolytic debenzylation afforded *N*-acetyl protected phosphorous diester **18** as triethylammonium salt. Formation of **18** from **14** and **7** could also be carried out as a one pot procedure. Similarly, from **15** and **7**, via phosphorous triester **17** as the intermediate, *N*-trifluoroacetyl protected phosphorous diester **19** was obtained. Treatment of **18** with NaOMe in methanol, in order to remove the *O*-acetyl groups and the *N*-acetyl group at the cytosine moiety, and then with NaOH in methanol/water led to the CMP-Neu5Ac analogue **2** in high yield. When **19** was treated with NaOMe in methanol the *O*-acetyl groups were removed. The ensuing reaction with LiOH in methanol/water led also to loss of the *N*-trifluoroacetyl group, thus releasing a free amine functionality, which – before hydrolysis of the methylester moiety was completed – resulted in competing lactam formation; therefore, besides target molecule **3** (sodium salt) also lactam **3a** was obtained (lithium salt). The structures of these compounds were also ascertained by NMR spectroscopy.

Determination of K_i values of **1–3** with the help of a new sialyltransferase assay system

The known sialyltransferase assay systems use either radioactive [21] or fluorescence [22] labelled CMP-Neu5Ac; another approach is coupling of the sialyltransfer to an NADH oxidation in a multienzyme system [23]. A recent assay is based on isolation of the sialylated product, its acid hydrolysis, and measurement of released Neu5Ac by colorimetry [24]. Also, UV-labelled acceptor structures were recently employed as basis for a sialyltransferase assay [25]; but, this assay is not convenient for the determination of kinetic parameters.



Scheme 3. (a) NaOMe, MeOH 0 °C → RT, Amberlite IR 120 H^+ (88%). (b) Ac_2O, pyridine (61%). (c) **12**: Pd/C, H_2, MeOH; Ac_2O (81%); **13**: Pd/C, H_2, $NaHCO_3$, TFAA; NEt_3 (72%). (d) **14**: Cl(^{i}Pr$_2$N)P(OBn), ^{i}Pr$_2$EtN, CH_3CN (84%); **15**: Cl(^{i}Pr$_2$N)P(OBn), ^{i}Pr$_2$EtN, CH_3CN (92%). (e) **7**, 1-H-tetrazole, CH_3CN; t-BuOOH, **16**: (71%). (f) **18**: H_2, Pd/C, MeOH; NEt_3 (93%); **19**: H_2, Pd/C, MeOH; NEt_3 (62%). (g) i: NaOMe, MeOH; IRC 176; ii: NaOH, MeOH/H_2O, IRC 176 (85%). (h) i: NaOMe/MeOH, IRC 176, ii: LiOH, MeOH/H_2O, IRC 176 **3**: (70%); **3a**: (29%).

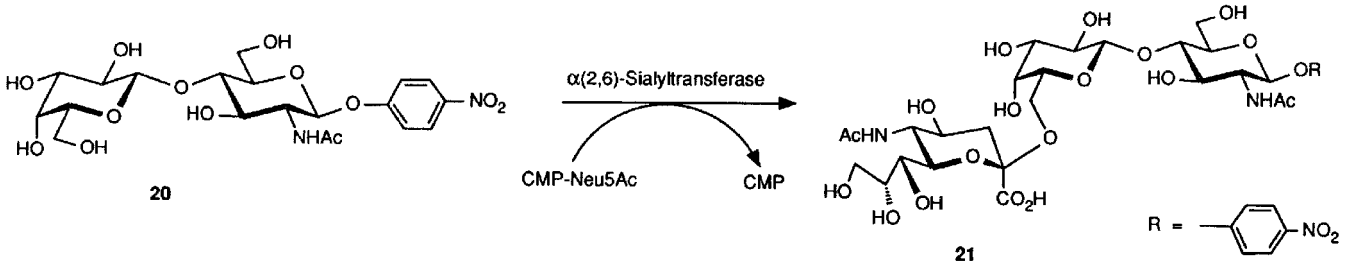
Therefore, we looked for a sialyltransferase assay system which permits the testing of potential inhibitors in a direct yet inexpensive manner. It should be nonradioactive, therefore either the acceptor or the donor should be UV- or fluorescence labelled. Due to the ease of synthesis and handling a UV-labelled acceptor seemed to be advantageous; RP-HPLC was envisaged for the separation from the UV-labelled product and for convenient quantification of the acceptor and product concentrations an internal standard had to be selected.

It was reported that *N*-acetyllactosamine serves as acceptor for α(2-6)-sialyltransferase from rat liver (Ec 2.4.99.1) [26]. Because of availability, this enzyme and this acceptor were chosen for the studies. Thus, a UV-labelled *N*-acetyllactosamine derivative was required. From the known UV-labels for saccharides (such as umbelliferyl [27], fluoresceinyl [25], or p-nitrophenyl [28]), p-nitrophenyl was chosen because it can be readily introduced, it has an intensive absorption at 300 nm, it seems to be tolerated by glycosyltransferases [28, 29], and a great number of p-nitrophenyl glycosides, for use as acceptors with other glycosyltransferases, are commercially available.

p-Nitrophenyl *N*-acetyllactosamine (**20**, Scheme 4) was obtained from *N*-acetyllactosamine in three steps following a known procedure [30]. In order to demonstrate that **20** is a substrate for α(2-6)-sialyltransferase from rat liver, p-nitrophenyl 6′-O-sialyl-*N*-acetyllactosamine (**21**) was enzymatically synthesized from **20** and CMP-Neu5Ac [31], isolated by RP-HPLC (yield: 0.9 mg, 50%), and structurally assigned by FAB-MS. For the quantification of the enzymatic transformations, p-nitrophenylalanine turned out to be suitable as internal standard because it exhibited a UV-absorption at 300 nm, it was stable under the assay conditions, it was easily separable from the acceptor **20** and the product **21** by RP-HPLC, and it did not interfer with the enzymatic reaction.

First, this assay system was used to determine the K_M values of **20**; Figure 1 shows a typical RP-HPLC chromatogram of an assay solution, containing **20**, **21**, and p-nitrophenylalanine. Compared with *N*-acetyllactosamine, and the corresponding methyl and methoxycarbonyloctyl glycosides [32], **20** exhibited practically the same acceptor properties (see Table 1).

The data in Table 1 are based on six different concentrations (4 mM to 0.125 mM) of acceptor **20** and a constant



Scheme 4. Enzymatic sialylation of acceptor **20**.

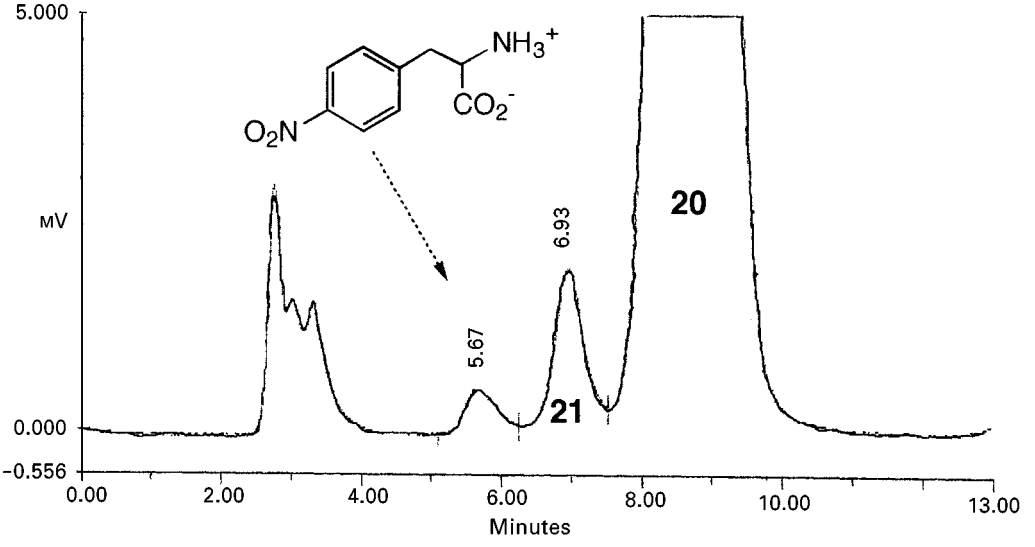


Figure 1. HPLC chromatogram of an assay mixture using acetonitrile/ 0.0375 M triethylammonium phosphate (pH 7.0–7.2) 15:85 as eluent, showing the separation of internal standard, product **21** and acceptor **20**. The peaks between 2 and 4 min refer to CMP and CMP-Neu5Ac.

donor concentration (CMP-Neu5Ac 0.56 mM). Aliquots ($\sim$20 µl) were taken from the reaction mixture every 15 min, the compounds separated by RP-HPLC, and the reaction rates calculated from the ratio of the integrals of product **21** and standard (p-nitrophenylalanine); an autosampler was employed for RP-HPLC separation.

The donor concentration was varied (CMP-Neu5Ac: 400 µM to 6.25 µM) and the acceptor concentration was kept constant (**20**: 1 mM) for the collection of inhibition data. Thus, for CMP-Neu5Ac a K_M value of 0.046 ± 0.007 was determined, which corresponded well with a reported value (0.045 mM) [33]. The inhibition data for CDP, a known competitive inhibitor of sialyltransferase [9, 34], were then reproduced with this assay system (Table 2); it gave reliable inhibition data, when at least three different inhibitors (best: $4 \times K_i$ to $0.4 \times K_i$) and five different donor concentrations were employed; for new inhibitors (compounds **1–3**, for data, see Table 2) was also necessary to verify that no peak overlap in the RP-HPLC separation occured. Table 2 shows that compounds **1** and **2** are interesting inhibitors for α(2-6)-sialyltransferase from rat liver. The K_i values found are in the range of CDP which is thus far the most potent sialyltransferase inhibitor. However, introduction of a positive charge as in compound **3**, strongly reduces the affinity for the sialyltransferase.

In conclusion, quinic acid can be readily transformed into CMP-Neu5Ac analogues, which, as expected, turned out to be efficient sialyltransferase inhibitors. The sialyltransferase assay used is based on a UV-labelled acceptor yielding a UV-labelled product which is separated by RP-HPLC and quantified with the help of an internal standard. The assay system is nonradioactive, it uses readily accessible starting materials and it can be employed for the measurement of donor, acceptor, and inhibitor affinities. Extension of this simple yet efficient assay principle to other glycosyltransferases is under investigation.

Table 1. Kinetic parameters for different acceptors of α-(2,6)-sialyltransferase from rat liver.

Acceptor	*K_M [mmol]*	*Reference*
βGal-(1-4)-βGlcNAc-OR[1] **20**[a]	1.5 ± 0.25	
βGal-(1-4)-βGlcNAc-OH	0.80 ± 0.05	[32]
βGal-(1-4)-βGlcNAc-OR[2b]	0.40 ± 0.05	[32]
βGal-(1-4)-βGlcNAc-OMe	1.66 ± 0.06	[32]

[a] R^1 = p-nitrophenyl.
[b] $R^2 = (CH_2)_8CO_2Me$.

Materials and methods

(a) Chemical syntheses

General methods

Solvents were purified and dried in the usual way. Melting points (uncorrected): metal block. ^{1}H-, ^{13}C- and ^{31}P-NMR spectra (22 °C, TMS or the resonance of the deuterated solvent as internal standard): Bruker AC 250, DRX 600 and Jeol JNM-GX 400. ^{1}H and ^{13}C resonances were assigned by homonuclear or heteronuclear resonance. MALDI-MS: Kratos Kompact Maldi 1, as matrices 2,5-dihydroxybenzoic acid (DHB) or 6-aza-2-thiothymine (ATT) were used. FAB-MS: Finnigan MAT 312/AMD 5000; 790 eV, 70 °C. Optical rotations: Perkin-Elmer polarimeter 241/MS; 1 dm cell; 22 °C. Thin layer Chromatography: Merck plastic plates, silica gel 60 F_{254} or Merck glass plates, reversed phase C_{18}; detection by treatment with a solution of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (20 g) and $Ce(SO_4)_2$ (0.4 g) in 10% sulfuric acid (400 ml). Flash chromatography: J.T. Baker silica gel 60 (0.040–0.063 mm) at a pressure of 0.3 bar. Preparative HPLC separations were performed on an Autochrom System with a Shimadzu LC 8A preparative pump and a Rainin Dynamax UV 1 detector. The column used was a Lichrosorb RP-18, 7 µm, 250 mm × 16 mm (Knauer GmbH, Germany). As mobile phase mixtures of acetonitrile and 0.05 M triethylammonium bicarbonate (pH 7.2–7.5) were used at a flow of 9 ml min^{-1}.

Disodium cytidine-5′-(quinic acid)-1-monophosphate (**1**)

To a solution of the peracetylated triethylammonium salt **8** (50 mg, 0.057 mmol) in dry methanol (5 ml) a 0.5 M solution of sodium methoxide in dry methanol (0.6 ml) was

Table 2. Inhibition constants of CDP and inhibition results with **1–3**.

Inhibitor	*α-(2-6)-sialyltransferase*	*K_i [µM]*	*Inhibitition mode*	*Reference*
CDP	Bovine colostrum	10	Competitive	[34]
CDP	Human serum	19	Competitive	[9]
CDP[a]	Rat liver	10 ± 2	Competitive	
1[b]	Rat liver	44 ± 7	Competitive	
2[b]	Rat liver	84 ± 14	Competitive	
3[b]	Rat liver	1400 ± 300	Competitive	

[a] Concentrations of CDP used for determination of K_i: 40 µM, 20 µM and 5 µM.
[b] Concentrations used for determination of K_i: **1**, 0.5 mM, 0.1 mM, and 0.05 mM; **2**, 1 mM, 0.5 mM, and 0.25 mM; **3**, 2 mM and 0.75 mM.

added. After stirring for 2 h at room temperature the solution was neutralized with Amberlite IRC 176, filtered and evaporated. The residue was dissolved in methanol:water 1:1 (4 ml), sodium hydroxide (40 mg) was added and after 2 h of stirring the reaction was complete. The solution was neutralized with Amberlite IRC 176, filtered, the pH adjusted to 8 with 1 M NaOH, and evaporated. After lyophilization, the residue was purified by precipitation from water with ethanol, yielding **1** as a colourless precipitate (20 mg, 65%). $R_F = 0.28$ (ethyl acetate:MeOH:NH_3:CH_3CO_2H:H_2O 5:5:1:1:1). $[\alpha]_D$ 5.4 (c 0.5, H_2O). ^{1}H-NMR (600 MHz, D_2O): δ 1.91–1.99 (m, 2 H, 2a″-, 6a″-H), 2.18–2.24 (m, 1 H, 2b″-H), 2.30–2.36 (m, 1 H, 6b″-H), 3.40–3.49 (dd, $^3J_{4'',5''} = 7$ Hz, $^3J_{4'',3''} = 3.1$ Hz, 1 H, 4″-H), 3.90–3.99 (m, 2 H, 3″-, 5″-H), 4.07–4.15 (m, 3 H, 4′-, 5a′-, 5b′-H), 4.15–4.23 (m, 2 H, 2′-, 3′-H), 5.86 (d, $^3J_{1',2'} = 3.4$ Hz, 1 H, 1′-H), 6.01 (d, $^3J_{5,6} = 7.5$, 1 H, 5-H), 7.88 (d, $^3J_{6,5} = 7.5$, 1 H, 6-H). ^{13}C-NMR (150.9 MHz, D_2O): $\delta = 37.30$ (2″-C, $^3J_{C,P} = 6$ Hz), 38.13 (6″-C, $^3J_{C,P} = 6$ Hz), 64.17 (5′-C, $^2J_{C,P} = 5.5$ Hz), 67.29 (5″-C), 68.17 (3″-C), 69.10 (3′-C), 74.21 (2′-, 4″-C), 82.77 (4′-C, $^3J_{C,P} = 8$ Hz), 82.92 (1″-C, $^2J_{C,P} = 7$ Hz), 89.19 (1′-C), 96.46 (5-H), 141.46 (6-H), 157.74 (4-C), 166.18 (2-C), 178.69 (Carbonyl-C). ^{31}P-NMR (161.7 MHz, d6-DMSO): δ 0.22. FAB-MS (negative mode, matrix: glycerol): 496 $[M-2Na + H]^-$, 518 $[M-Na]^-$.

*Disodium cytidine-5′-[(1*S,*3*S,*4*S,*5*R*)-3-acetamido-1,4,5-trihydroxy-cyclohexane-1-carboxylate]-1-monophosphate* (**2**)

To a solution of **18** (50 mg, 0.057 mmol) in dry methanol (5 ml) a 0.5 M solution of sodium methoxide in dry methanol (0.2 ml) was added. After stirring for 1 h the solution was neutralized by addition of Amberlite IRC 176, filtered, and evaporated. The residue was dissolved in methanol:water 1:1 (5 ml) and sodium hydroxide (40 mg) was added. After stirring for 18 h the solution was neutralized by addition of Amberlite IRC 176, filtered and concentrated in vacuum. Precipitation of the residue from water with ethanol yielded after lyophilization **2** as a colourless foam (28 mg, 85%). $R_F = 0.53$ (ethyl acetate:MeOH:1 m NH_4OAc 1:1:1). $[\alpha]_D$ + 17.2 (c 0.5 in H_2O). ^{1}H-NMR (600 MHz, D_2O): δ 1.64 (ddd, $^2J_{2ax'',2eq''} \approx {}^3J_{2ax'',3''} = 13.1$ Hz, $^4J_{6ax'',P} = 3.6$ Hz, 1 H, 2ax″-H), 1.72 (dd, $^2J_{6ax'',6eq''} \approx {}^3J_{6ax'',5''} = 12.9$ Hz, 1 H, 6ax″-H), 1.86 (s, 3 H, *N*-acetyl), 2.10–2.17 (ddd, $^2J_{2eq'',2ax''} = 13.8$ Hz, $^4J_{2eq'',6eq''} \approx {}^3J_{2eq'',3''} = 3.6$ Hz, 1 H, 2eq-H), 2.39–2.44 (ddd, $^2J_{6eq'',6ax''} = 13.3$ Hz, $^4J_{6eq'',2eq''} \approx {}^3J_{6eq'',5''} = 3.6$ Hz, 1 H, 6eq-H), 3.21 (dd, $^3J_{4'',3''} \approx {}^3J_{4'',5''} = 9.7$ Hz, 1 H, 4″-H), 3.82 (ddd, $^3J_{5'',6ax''} = 12.9$ Hz, $^3J_{5'',3''} = 9.3$ Hz, $^3J_{5'',6eq''} = 4.6$ Hz, 1 H, 5″-H), 3.94 (ddd, $^3J_{3'',2ax''} = 12.5$ Hz, $^3J_{3'',4''} = 10$ Hz, $^3J_{3'',2eq''} = 4.3$ Hz, 1 H, 3″-H), 4.08–4.18 (m, 3 H, 4′-, 5a′-, 5b′-H), 4.20 (dd, $^3J_{2',1'} = 4.1$ Hz, $^3J_{2',3'} = 4.6$ Hz, 1 H, 2′-H), 4.25 (m, 1 H, 3′-H), 5.87 (d, $^3J_{1',2'} = 4.1$ Hz, 1 H, 1′-H), 6.00 (d, $^3J_{5,6} = 7.6$ Hz, 1H, 5-H), 7.9 (d, $^3J_{6,5} = 7.6$ Hz, 1H, 6-H). ^{13}C-NMR (150.9 MHz, D_2O): δ 22.09 (acetyl-CH_3), 38.99 (2″-C, $^3J_{C,P} = 7$ Hz), 39.77 (6″-C, $^3J_{C,P} = 4$ Hz), 49.26 (3″-C), 64.03 (5′-C, $^2J_{C,P} = 4$ Hz), 69.13 (3′-C), 69.95 (5″-C), 74.28 (2′-C), 77.34 (4″-C), 81.56 (1″-C, $^2J_{C,P} = 6$ Hz), 82.84 (4′-C, $^3J_{C,P} = 8$ Hz), 89.19 (1′-C), 96.40 (5-C), 141.60 (6-C), 157.73 (4-C), 166.13 (2-C), 173.80, 178.23 (Carbonyl-C). ^{31}P-NMR (161.7 MHz, D_2O): δ -2.99. MALDI-MS (negative mode, matrix ATT): 538 $[M-2 Na + H]^-$.

*Sodium cytidine-5′-[(1*S,*3*S,*4*S,*5*R*)-3-amino-1,4,5-trihydroxy cyclohexane-1-carboxylate]-1-monophosphate* (**3**)
*Lithium cytidine-5′-[(1*S,*3*S,*4*S,*5*R*)-3-amino-1,4,5-trihydroxy cyclohexane-1-carboxylicacid-1,3-lactame]-1-monophosphate* (**3a**)

To a solution of triethylammonium salt **19** (100 mg, 0.10 mmol) in dry methanol (10 ml) 0.5 m sodium methoxide solution in dry methanol (0.2 ml) was added. After stirring for 1 h at room temperature the solution was neutralized with Amberlite IRC 176 H^+ resin, filtered and evaporated to dryness. The residue was dissolved in water:methanol 1:1 (4 ml) and lithium hydroxide (1.1 mmol, 46 mg) was added. After stirring for 15 h at room temperature the solution was neutralized with Amberlite IRC 176 H^+ resin, filtered, the pH adjusted to 8 and then lyophilized. The mixture of **3** and the lactam **3a** was separated by preparative HPLC (0.05 m triethylammonium bicarbonate: acetonitrile 99:1). Ion exchange with Amberlite IR 120 Na^+ or Li^+ and lyophilization yielded **3** (36 mg, 70%) as a mono sodium salt and **3a** (14 mg, 29%) as mono lithium salt.

3: $R_F = 0.19$ (ethyl acetate:MeOH:1 m NH_4OAc 1:1:1). $[\alpha]_D$ 7.2 (c 0.5 in H_2O). ^{1}H-NMR (600 MHz, D_2O): δ 1.66 (ddd, $^2J_{6ax'',6eq''} \approx {}^3J_{6ax'',5''} = 12.7$ Hz, $^4J_{6ax'',P} = 2.9$ Hz, 1 H, 6ax-H), 1.83 (dd, $^2J_{2ax'',2eq''} \approx {}^3J_{2ax'',3''} = 12.4$ Hz, 1 H, 2ax-H), 2.32–2.4 (m, 1H, 6eq″-H), 2.41–2.46 (m, 1 H, 2eq″-H), 3.25–3.34 (m, 2 H, 3″-, 4″-H), 3.8 (ddd, $^3J_{5'',6ax''} = 11.7$ Hz, $^3J_{5'',4''} = 8.5$ Hz, $^3J_{5'',6eq''} = 4.7$ Hz, 1 H, 5″-H), 4.06–4.10 (m, 1 H, 5a′-H), 4.12–4.16 (m, 2 H, 4′-, 5b′-H), 4.20–4.24 (m, 2 H, 2′-, 3′-H), 5.87 (d, $^3J_{1',2'} = 4.18$ Hz, 1 H, 1′-H), 6.00 (d, $^3J_{5,6} = 7.6$ Hz, 1 H, 6-H), 7.87 (d, $^3J_{6,5} = 7.6$ Hz, 1 H, 6-H). ^{13}C-NMR (150.9 MHz, D_2O): $\delta = 36.95$ (2″-C, $3J_{C,P} = 6$ Hz), 40.53 (6″-C, $3J_{C,P} = 6$ Hz), 50.68 (3″-C), 64.34 (5′-C, $2J_{C,P} = 5.5$ Hz), 69.19 (3′-C), 69.49 (5″-C), 74.14 (2′-C), 76.20 (4″-C), 81.10 (1″-C, $2J_{C,P} = 5$ Hz), 82.82 (4′-C, $3J_{C,P} = 8$ Hz), 89.21 (1′-C), 96.47 (5-C), 141.55 (6-C), 157.75 (4-C), 166.17 (2-C), 177.51 (Carbonyl-C). ^{31}P-NMR (161.7 MHz, D_2O): δ-3.32. FAB-MS (negative mode, matrix: glycerol): 495 [M-2Na + H], 517 $[M-Na]^-$.

3a: $R_F = 0.61$ (ethyl acetate:MeOH:1 m NH_4OAc 1:1:1). ^{1}H-NMR (600 MHz, d^6-DMSO): δ 1.85 (d, $J = 13.4$ Hz, 1 H, 6a″-H), 2.30–2.34 (m, 3 H, 2ax″-, 2eq″-, 6b″-H), 3.4–3.5 (m, 2 H, 3″-, 4″-H), 3.70–4.00 (m, 6 H, 2′-, 3′-, 4′-, 5a′-, 5b′-, 5″-H), 4.66 (bs, 1 H, 5″-OH), 5.18 (bs, 1 H, 4″-OH), 5.34 (bs, 2 H, 2′-, 3′-OH), 5.74 (d, $^3J_{5,6} = 7.4$ Hz, 1 H, 5-H), 5.81 (d, $^3J_{1',2'} = 4.4$ Hz, 1 H, 1′-H), 7.03/7.27 (2 bs, 2 H, NH2), 7.61 (s, 1 H, NH-Lactame), 7.81 (d, $^3J_{6,5} = 7.4$ Hz, 1 H, 6-H). ^{13}C-NMR (150.9 MHz, d^6-DMSO): $\delta = 36.5$ (2″-C), 39.5 (6″-C), 52.34 (3″-C), 64.09 (5′-C), 69.24 (4″-C), 70.01 (3′-C), 70.73 (5″-C), 74.05 (2′-C), 76.38 (1″-C), 82.79 (4′-C), 88.56 (1′-C), 94.26 (5-C), 141.39 (6-C), 155.57 (4-C), 165.59 (2-C),

177.31 (CO). MALDI-MS (positive mode, matrix: ATT): 487 $[M+H]^+$, 493 $[M+Li]^+$.

*3,4,5-Tri-*O*-acetyl-quinic acid methylester-1-*O*-[*N,N*-diisopropyl-(2-cyanoethoxy)] phosphitamide* (**6**)

To a solution of **5** [16] (250 mg, 0.75 mmol) in dry dichloromethane:acetonitrile 1:1 (10 ml) (2-cyanoethoxy)-bis(diisopropylamino)-phosphane [18] (454 mg, 1.5 mmol) and diisopropylammonium tetrazolide (77 mg, 0.45 mmol) was added under an argon atmosphere. After 3 d of stirring at room temperature the reaction was complete and it was quenched with saturated sodium bicarbonate solution (15 ml). The layers were separated and the aqueous layer was extracted with dichloromethane (15 ml), dried with $MgSO_4$, and evaporated. This afforded the crude phosphitamide **6** (450 mg) as a colourless oil. Flash chromatography (toluene:ethyl acetate 3:1) yielded **6** (300 mg, 75%) as a colourless oil. $R_F = 0.63$ (toluene:ethyl acetate 1:1). ^{1}H-NMR (250 MHz, $CDCl_3$): δ 1.1–1.3 (m, 12 H, $CH(CH_3)_2$), 1.95–2.1 (3 s, 9 H, 3 × acetyl), 2.1–2.45 (m, 4 H, 2-, 2′-, 6-, 6′-H), 2.72 (t, $^3J = 6.8$, 2 H, CH_2CH_2CN), 3.63 [m, 2 H, $CH(CH_3)_2$], 3.7 (s, 3 H, Me-Ester), 4.0 (q, $^3J = {}^3J_{P,H} = 6.8$ Hz, 2 H, CH_2CH_2CN), 5.0–5.12 (m, 1 H, 4-H), 5.2–5.4 (m, 2 H, 3-, 5-H). ^{31}P-NMR (161.7 MHz, $CDCl_3$): δ 145.03, 145.49.

*Triethylammonium (*N^4,O*2′*,O*3′-triacetyl-cytidine)-5′-(3,4,5-tri-*O*-*acetyl-quinic acid methylester)-1-monophosphate* (**8**)

To a solution of the crude phosphitamide **6** (400 mg, 0.75 mmol) and cytidine **7** [19] (277 mg, 0.75 mmol) in dry acetonitrile (7 ml) tetrazole (80 mg, 1.13 mmol) was added. After 15 h of stirring at room temperature the suspension was cooled to −10 °C and a 3 M solution of tert-butylhydroperoxide in dry toluene (0.5 ml, 1.5 mmol) was added. After stirring for 1 h at −10 °C and for 2 h at room temperature, three drops of triethylamine were added and the solution was evaporated. The residue was loaded on a flash silica gel column (toluene:acetone 1:1 + 2% NEt_3) and left there overnight in order to remove the cyanoethoxy group. Elution of the column (ethyl acetate:methanol 4:1 + 2% NEt_3) afforded the triethylammonium salt **8** (585 mg, 90%). $R_F = 0.15$ (silica gel:ethyl acetate:methanol:NEt_3 4:1 + 2%). $R_F = 0.31$ (RP-18: ethanol:water 3:1). $[\alpha]_D + 9.6$ (c 0.5 in methanol). ^{1}H-NMR (250 MHz, d4-MeOH): δ 1.31 (t, $^3J = 7.3$ Hz, 9 H, $HN(CH_2CH_3)_3$), 2.00–2.15 (s, 15 H, 5 × *O*-acetyl), 2.18 (s, 3 H, *N*-acetyl), 2.33–2.44 (m, 3 H, 2a″-, 2b″-, 6ax″-H), 2.59 (dd, $^2J_{6eq'',6ax''} = 13.2$ Hz, $^3J_{6eq'',5''} = 4$ Hz, 1 H, 6eq″-H), 3.19 [q, $^3J = 7.3$ Hz, 6 H, $HN(CH_2CH_3)_3$], 3.79 (s, 3 H, Me-Ester), 4.13 (ddd, $^2J_{5a',5b'} = 12.0$ Hz, $^3J_{5a',4'} = 2.45$ Hz, $^3J_{5a',P} = 5.4$ Hz, 1 H, 5a′-H), 4.26 (ddd, $^2J_{5a',5b'} = 12.0$ Hz, $^3J_{5b',4'} = 2.45$ Hz, $^3J_{5b',P} = 5.4$ Hz, 1 H, 5b′-H), 4.42 (m, 1 H, 4′-H), 5.1–5.2 (m, 2 H, 3′-, 4″-H), 5.36–5.53 (m, 3 H, 2′-, 3″-, 5″, -H), 6.21 (d, $^3J_{1',2'} = 4.4$ Hz, 1 H, 1′-H), 7.56 (d, $^3J_{5,6} = 7.5$ Hz, 1 H, 5-H), 8.47 (d, $^3J_{6,5} = 7.5$ Hz, 1 H, 6-H). ^{31}P-NMR (161.7 MHz, d4-MeOH): δ-3.88. MALDI-MS (negative mode, matrix: ATT): 763 $[M\text{-}NHEt_3]^-$, 883 $[M+H_2O]^-$. $C_{35}H_{53}N_4O_{19}P \times 0.5\,H_2O$ (864.8): calcd C 48.11, H 6.22, N 6.41; found C 48.20, H 6.34, N 6.41.

*Methyl (1*S*,3*S*,4*S*,5*R*)-3-azido-1,4,5-trihydroxycyclohexane-1-carboxylate* (**10**)

To a solution of azide **9** [15] (1 g, 5.02 mmol) in dry methanol (10 ml) was added a 0.5 M solution of sodium methoxide in dry methanol (10 ml) at 0 °C. After stirring for 1.5 h at room temperature the solution was neutralized with Amberlite IR 120 H^+ resin, filtered, and concentrated under vacuum. Flash chromatography (toluene:acetone 2:1) afforded the methylester **10** (1.01 g, 88%) as a colourless oil. $R_F = 0.17$ (toluene:acetone 2:1). $[\alpha]_D + 28.0$ (c 1 in $CHCl_3$). ^{1}H-NMR (250 MHz, $CDCl_3$): δ 1.80 (dd, $^2J_{2ax,2eq} = 12.8$ Hz, $^3J_{2ax,3} = 6.4$ Hz, 1 H, 2ax-H), 1.85 (dd, $^2J_{6ax,6eq} = 12.2$ Hz, $^3J_{6ax,5} = 7.0$ Hz, 1 H, 6ax-H), 1.99–2.06 (m, 2 H, 2eq-, 6eq-H), 3.37 (dd, $^3J_{4,3} = {}^3J_{4,5} = 9.4$ Hz, 1 H, 4-H), 3.65–3.89 (m, 5 H, 1-, 4-, 5-OH, 3-, 5-H), 3.79 (s, 3 H, Me-ester). $C_8H_{13}N_3O_5 \times 0.5\,H_2O$ (240.22): calcd C 40.00, H 5.87, N 17.48; found C 40.13, H 5.48, N 17.02.

*Methyl (1*S*,3*S*,4*S*,5*R*)-4,5-di-*O*-acetyl-3-azido-1,4,5-trihydroxy-cyclohexane-1-carboxylate* (**11**)

Compound **10** (840 mg, 3.63 mmol) was dissolved in dry pyridine (10 ml) and acetic anhydride (1.1 ml) was added. After stirring for 20 h at room temperature the solution was coevaporated with toluene (3 × 20 ml). The residue was purified by flash chromatography (toluene:acetone 10:1) to afford the di-*O*-acetyl compound **11** (700 mg, 61%) and 29% of the tri-*O*-acetyl compound. $R_F = 0.19$ (toluene:acetone 10:1). $[\alpha]_D$–2.5 (c 1 in $CHCl_3$). ^{1}H-NMR (250 MHz, $CDCl_3$): δ 1.81–1.98 (m, 2 H, 2ax-, 6ax-H), 1.99/2.09 (s, 6 H, 2 × *O*-acetyl), 2.01–2.17 (m, 2 H, 2eq-, 6eq-H), 3.31 (s, 1 H, 1-OH), 3.80 (s, 3 H, Me-ester), 3.89 (ddd, $^3J_{3,4} = 9.9$ Hz, $^3J_{3,2ax} = 12.2$ Hz, $^3J_{3,2eq} = 4.8$ Hz, 1 H, 3-H), 5.02 (dd, $^3J_{4,5} = {}^3J_{4,3} = 9.9$ Hz, 1 H, 4-H), 5.21 (ddd, $^3J_{5.4} = 9.9$ Hz, $^3J_{5,6ax} = 11.45$ Hz, $^3J_{5,6eq} = 5$ Hz, 1 H, 5-H). $C_{12}H_{17}N_3O_7$ (315.28): calcd C 45.72, H 5.43, N 13.33; found C 45.50, H 5.52, N 13.4.

*Methyl (1*S*,3*S*,4*S*,5*R*)-4,5-di-*O*-acetyl-3-acetamido-1,4,5-trihydroxycyclohexane-1-carboxylate* (**12**)

To a solution of azide **11** (1 g, 3.17 mmol) in dry methanol (80 ml) 10% Pd/C (300 mg) was added. After 20 min of hydrogenation acetic anhydride (1 ml) was added. The suspension is filtered through a pad of Celite after stirring for an additional hour. The filtrate was concentrated under vacuum and recrystallized from toluene:acetone 1:1 and diethyl ether to yield **12** as a colourless solid (0.85 g, 81%) M.p. 166–167 °C. $R_F = 0.23$ (toluene:acetone 1:1). $[\alpha]_D$ –9.7 (c 1 in $CHCl_3$). ^{1}H-NMR (250 MHz, $CDCl_3$): δ 1.72–1.85 (m, 2 H, 2ax-, 6ax-H), 1.88/1.97 (s, 6 H, 2 × *O*-acetyl), 2.03 (s, 3 H, *N*-acetyl), 2.14–2.27 (m, 2 H, 2eq-, 6eq-H), 3.74 (s, 3 H, Me-ester), 3.80 (s, 1 H, 1-OH), 4.42 (dddd, $^3J_{3,2ax} =$

11.5 Hz, $^3J_{3,4}$ = 10.5 Hz, $^3J_{3,NH}$ = 8.4 Hz, $^3J_{3,2eq}$ = 4 Hz, 1 H, 3-H), 4.90 (dd, $^3J_{4,3}$ = 10.5 Hz, $^3J_{4,5}$ = 9.7 Hz, 1 H, 4-H), 5.29 (ddd, $^3J_{5.6ax}$ = 11.5 Hz, $^3J_{5,4}$ = 9.7 Hz, $^3J_{5,6eq}$ = 5 Hz, 1 H, 5-H), 5.87 (d, $^3J_{NH,3}$ = 8.4 Hz, 1 H, NH). $C_{14}H_{21}NO_8$ (331.32): calcd C 0.75, H 6.38, N 4.23; found C 50.84, H 6.39, N 4.42.

*Methyl (1*S,*3*S,*4*S,*5*R*)-4,5-di*-O-*acetyl-3-trifluoroacetamido-1,4,5-trihydroxycyclohexane carboxylate* (**13**)

To a solution of azide **11** (418 mg, 1.32 mmol) in dry ethyl acetate (20 ml) 10% Pd/C (100 mg), $NaHCO_3$ (400 mg) and trifluoroacetic anhydride (559 µl, 3.96 mmol) were added. After 4 h of hydrogenation triethylamine was added until the solution was alkaline (pH ~8) and then stirred overnight. The suspension was filtered through a pad of Celite and the filtrate was concentrated in vacuum. The residue was purified by flash chromatography (toluene:acetone 8:1–5:1) to yield the trifluoroacteamide **13** (367 mg, 72%) as a colourless solid M.p. 209–210 °C. R_F = 0.26 (toluene: acetone 4:1). $[\alpha]_D$ –23.8 (c 1 in $CHCl_3$). ^{1}H-NMR (250 MHz, $CDCl_3$): δ 1.84–1.97 (m, 2 H, 2ax-, 6ax-H), 2.00/2.06 (s, 6 H, 2 × *O*-acetyl), 2.15–2.28 (m, 2 H, 2eq-, 6eq-H), 3.31 (s, 1 H, 1-OH), 3.79 (s, 3 H, Me-ester), 4.44–4.51 (m, 1 H, 3-H), 5.00 (dd, $^3J_{4,3}$ = 10.5 Hz, $^3J_{4,5}$ = 9.7 Hz, 1 H, 4-H), 5.32 (ddd, $^3J_{5.6ax}$ = 11.5 Hz, $^3J_{5,4}$ = 9.7 Hz, $^3J_{5,6eq}$ = 5 Hz, 1 H, 5-H), 6.78 (bd, $^3J_{NH,3}$ = 8 Hz, 1 H, NH). MALDI-MS (positive mode, matrix: DHB): 409 $[M + Na]^+$, 425 $[M + K]^+$. $C_{14}H_{18}F_3NO_8$ (385.29): calcd C 43.64, H 4.70, N 3.64; found C 43.63, H 4.70, N 3.79.

*Methyl (1*S,*3*S,*4*S,*5*R*)-4,5-di*-O-*acetyl-3-acetamido-1,4,5-trihydroxycyclohexane-1-carboxylate-1*-O-*[*N,N-*diisopropyl-(benzyloxy)]-phosphitamide* (**14**)

To a solution of the alcohol **12** (200 mg, 0.6 mmol) in dry acetonitrile (8 ml) were added diisopropylethylamine (226 µl, 1.32 mmol) and benzyloxy-chloro-diisopropylamino-phosphane [20] (328 mg, 1.2 mmol) under an inert atmosphere. After stirring for 20 h, the reaction mixture was evaporated. Flash chromatography of the residue (toluene: acetone 5:1) yielded the phosphitamide **14** (285 mg, 84%) as a colourless foam (mixture of diastereomeres). R_F = 0.64 (toluene:acetone 1:1). ^{1}H-NMR (250 MHz, $CDCl_3$): δ 1.1–1.3 (m, 12 H, 4 CHC*H*$_3$), 1.6-1.8 (m, 2 H, 2ax-, 6ax-H), 1.86/1.89/1.95/1.99 (4 s, 6 H, 2 *O*-acetyl), 2.03 (s, 3 H, *N*-acetyl), 2.4–2.7 (m, 2 H, 2eq-, 6eq-H), 3.52/3.54 (2 s, 3 H, Me-ester), 3.56–3.79 (m, 2 H, 2 C*H*CH$_3$), 4.45–4.56 (m, 2 H, Benzyl-C*H*$_2$), 4.64–4.73 (2 m, 1 H, 3-H), 4.88/4.92 (2 dd, $^3J_{4,3}$ = 10.5 Hz, $^3J_{4,5}$ = 9.7 Hz, 1 H, 4-H), 5.29–5.46 (m, 1 H, 5 H), 5.67/5.83 (2 bs, $^3J_{NH,3}$ = 8.8 Hz, 1 H, NH), 7.27–7.34 (m, 5 H, phenyl-H).

*Methyl (1*S,*3*S,*4*S,*5*R*)-4,5-di*-O-*acetyl-3-trifluoroacetamido-1,4,5-trihydroxy cyclohexane-1-carboxylate-1*-O-*[*N,N-*diisopropyl-(benzyloxy)]-phosphitamide* (**15**)

As described for compound **14** the alcohol **13** (200 mg, 0.52 mmol) was converted to the posphitamide **15** in the presence of diisopropylethylamine (195 µl, 1.14 mmol) and benzyloxy-chloro-diisopropylamino-phosphane [20] (285 mg, 1.04 mmol) in dry acetonitrile (8 ml). The reaction mixture was stirred for 5 h and flash chromatography (toluene: acetone 11:1) yielded the phosphitamide **15** (300 mg, 92%) as a colourless oil (mixture of diastereomeres). R_F = 0.72 (toluene:acetone 2:1). ^{1}H-NMR (250 MHz, $CDCl_3$): δ 1.07–1.33 (m, 12 H, 4 CHC*H*$_3$), 1.68–1.92 (m, 2 H, 2ax-, 6ax-H), 1.95/1.98/2.02/2.04 (4 s, 6 H, 2 *O*-acetyl), 2.4–2.7 (m, 2 H, 2eq-, 6eq-H), 3.53/3.56 (2 s, 3 H, Me-ester), 3.58–3.77 (m, 2 H, 2 C*H*CH$_3$), 4.40–4.57 (m, 2 H, Benzyl-C*H*$_2$), 4.60–4.73 (m, 1 H, 3-H), 4.90/4.96 (2 dd, $^3J_{4,3}$ = $^3J_{4,5}$ = 10.5 Hz, 1 H, 4-H), 5.25–5.50 (m, 1 H, 5-H), 6.42/6.67 (2 bs, 1 H, $^3J_{NH,3}$ = 8 Hz), 7.20–7.37 (m, 5 H, phenyl-H).

*(*N^4, O2′, O3′*-Triacetylcytidine)-5′-[(1*S,*3*S,*4*S,*5*R*)-4,5-di*-O-*acetyl-3-acetamido-1,4,5-trihydroxycyclohexane-1-carboxylicacidmethylester]-1-benzyloxy-monophosphate* (**16**)

To a solution of phosphitamide **14** (285 mg, 0.5 mmol) and cytidine **7** [19] (185 mg, 0.5 mmol) in dry acetonitrile (10 ml) was added 1-H-tetrazole (53 mg, 0.75 mmol) under an inert atmosphere. After stirring for 2 h t-BuOOH (3 M in toluene, 0.4 ml) was added and the suspension was stirred again for 1 h. After addition of three drops of triethylamine the solvents were evaporated. Flash chromatography (toluene:acetone 1:2–1:3) of the residue yielded the protected phosphate **16** (301 mg, 71%) as a colourless foam (mixture of diastereomeres). R_F = 0.16 (toluene:acetone 1:2). ^{1}H-NMR (250 MHz, $CDCl_3$): δ 1.85–2.19 (m, 20 H, 4 *O*-acetyl, 2 *N*-acetyl, 2ax″-, 6ax″-H), 2.5–2.7 (m, 2 H, 2eq″-, 6eq″-H), 3.69/3.71 (2 s, 3 H, Me-ester), 4.2–4.35 (m, 3 H, 5a′-, 5b′-, 4-H), 4.35–4.5 (m, 1 H, 3″-H), 4.98/5.02 (2 dd, $^3J_{4'',5''}$ = $^3J_{4'',3''}$ = 10.5 Hz, 1 H, 4″-H), 5.1–5.4 (m, 5 H, 2′-, 3′-, 5″-H, benzyl-C*H*$_2$), 6.09/6.11 (2 d, $^3J_{1',2'}$ = 4.6 Hz, 1 H, 1′-H), 6.45–6.55 (m, 1 H, NH), 7.2–7.4 (m, 6 H, 5-, phenyl-H), 8.01/8.04 (2 d, $^3J_{6,5}$ = 4.5 Hz, 1 H, 6-H). ^{31}P-NMR (242.9 MHz, $CDCl_3$): δ 0.11. MALDI-MS (positive mode, matrix: DHB): 853 $[M + H]^+$, 876 $[M + Na]^+$, 892 $[M + K]^+$. $C_{36}H_{45}N_4O_{18}P \times 1\,H_2O$ (852.74): calcd C 49.65, H 5.43, N 6.43; found C 49.75, H 5.30, N 6.86.

*Triethylammonium (*N^4, O2′, O3′*-triacetylcytidine)-5′-[(1*S, *3*S,*4*S,*5*R*)-4,5-di*-O-*acetyl-3-acetamido-1,4,5-trihydroxycyclohexane-1-carboxylic acidmethylester]-1-monophosphate* (**18**)

To a solution of **16** (100 mg, 0.12 mmol) in methanol:ethyl acetate (8 ml) 10% Pd/C (15 mg) was added. After 10 min of hydrogenation, five drops of triethylamine were added and the suspension was filtered through a pad of Celite. The filtrate was concentrated under vacuum and the residue was purified by flash chromatography (ethyl acetate:MeOH: NEt_3 3:1 + 1%) to yield after lyophilization the triethylammonium salt **18** (96 mg, 93%) as a colourless foam. R_F = 0.38 (silica gel: ethyl acetate:MeOH:NEt_3 1:1 + 1%). R_F = 0.55 (RP-18: EtOH:H_2O 1: 3). $[\alpha]_D$ + 20.2 (c 1 in MeOH). ^{1}H-NMR (250 MHz, d4-MeOD): δ 1.30 (t, ^3J = 7.3 Hz, 9 H,

$HN(CH_2CH_3)_3$), 1.85/1.95/1.96/2.05 (4 s, 12 H, 4 *O*-acetyl), 2.09/2.17 (2 s, 6 H, 2 *N*-acetyl), 1.8–2.1 (m, 2 H, 2ax″-, 6ax″-H), 2.4–2.7 (2 m, 2 H, 2eq″-, 6eq″-H), 3.18 (q, $^3J = 7.3$ Hz, 6 H, $HN(CH_2CH_3)_3$ 3.75 (s, 3 H, Me-ester), 4.2–4.5 (m, 4 H, 5a′-, 5b′-, 4′-H, 3″-H), 4.98 (dd, $^3J_{4,3} = {}^3J_{4,5} = 10.1$ Hz, 1 H, 4″-H), 5.32 (ddd, $^3J_{5'',6ax''} = 11.2$ Hz, $^3J_{5'',4''} = 10.2$ Hz, $^3J_{5'',6eq''} = 5$ Hz, 1 H, 5″-H) 5.48/5.51 (2 d, $^3J_{2',3'} = 4.5$ Hz, 2 H, 3′-, 2′ -H), 6.18 (d, $^3J_{1',2'} = 4.0$ Hz, 1 H, 1′-H), 7.52 (d, $^3J_{5,6} = 7.5$ Hz, 1 H, 5-H), 8.48 (d, $^3J_{6,5} = 7.5$ Hz, 1 H, 6-H). ^{31}P-NMR (242.9 MHz, d4-MeOD): δ 0.16. MALDI-MS (negative mode, matrix: ATT): 762 $[M\text{-}NHEt_3]^-$. $C_{35}H_{54}N_5O_{18}P$ (863.81) × 1.5 H_2O: calcd C 47.19, H 6.44, N 7.86; found C 47.29, H 6.25, N 8.01.

Triethylammonium (N^4, *O2′, O3′-triacetylcytidine)-5′-[(1S, 3S,4S,5R)-4,5-di-*O*-acetyl-3-trifluoroacetamido-1,4,5-trihydroxy-cyclohexane-1-carboxylicacidmethylester]-1-monophosphate* (**19**)

As described for compound **16**, the phosphitamide **15** (296 mg, 0.47 mmol) was coupled with cytidine **7** (194 mg, 0.52 mmol) using tetrazole (49 mg, 0.7 mmol) in dry acetonitrile (10 ml). Stirring for 5 h and oxidation with t-BuOOH (3 M in toluene, 0.4 ml) yielded after flash chromatography (toluene:acetone 1:1) the protected phosphate **17** contaminated with a small amount of cytidine **7**. A suspension of the protected phosphate **17** in methanol:ethyl acetate 1:1 (15 ml) and 10% Pd/C (30 mg) was hydrogenated for 15 min. The suspension was neutralized with NEt_3, filtered through Celite, and evaporated. Flash chromatography (ethyl acetate:methanol:NEt_3 4.5:1 + 1%) of the residue and lyophilization yielded the triethylammonium salt **19** (270 mg, 62%) as a colourless foam. $R_F = 0.22$ (ethyl acetate: MeOH:NEt_3 3:1 + 1%). $[\alpha]_D + 10.6$ (c 1 in MeOH). ^{1}H-NMR (250 MHz, d4-MeOD): δ 1.31 [t, $^3J = 7.3$ Hz, 9 H, $HN(CH_2CH_3)_3$], 1.93/1.96/2.06/2.07/2.16 (5 s, 15 H, 5 acetyl), 1.81–2.25 (m, 2 H, 2ax″-, 6ax″-H), 2.47–2.69 (2 m, 2 H, 2eq″-, 6eq″-H), 3.20 [q, $^3J = 7.3$ Hz, 6 H, $HN(CH_2CH_3)_3$], 3.76 (s, 3 H, Me-ester), 4.17–4.37 (m, 2 H, 5a′-, 5b′-H), 4.40–4.55 (m, 2 H, 4′-, 3″-H), 5.06 (dd, $^3J_{4'',3''} \approx {}^3J_{4'',5''} \approx 10$ Hz, 1 H, 4″-H), 5.36 (ddd, $^3J_{5'',6ax''} = 11.6$ Hz, $^3J_{5'',4''} = 9.7$ Hz, $^3J_{5'',6eq''} = 5$ Hz, 1 H, 5″-H), 5.46–5.53 (m, 2 H, 2′-, 3′-H), 6.16 (d, $^3J_{1',2'} = 3.9$ Hz, 1 H, 1′-H), 7.52 (d, $^3J_{5,6} = 7.5$ Hz, 1 H, 5-H), 8.49 (d, $^3J_{6,5} = 7.5$ Hz, 1 H, 6-H). ^{31}P-NMR (161.7 MHz, d4-MeOD): δ-3.12. MALDI-MS (negative mode, matrix: ATT): 816 $[M\text{-}NHEt_3]^-$. $C_{35}H_{51}F_3N_5O_{18}P$ (917.77) × 1 H_2O: calcd C 44.92, H 5.71, N 7.48; found: C 44.82, H 5.82, N 7.60.

(b) Sialyltransferase assay

Materials

α(2,6)-Sialyltransferase from rat liver (EC 2.4.99.1), alkaline phosphatase (EC 3.1.3.1), p-DL-nitrophenylalanine and bovine serum albumin (Fraction V) were purchased from Sigma. Cacodylic acid, sodium salt trihydrate, Triton X-100 and CDP trisodium salt were from Fluka. CMP-Neu5Ac was synthesized according to Schmidt *et al.* [31]; p-nitrophenyl *N*-acetyl-lactosamine **20** was synthesized according to a method of Matta *et al.* [30] and purified by RP18-flash chromatography.

Analytical HPLC analysis

HPLC analysis was performed on a Merck-Hitachi system with an L 7200 autosampler and an L 4000 UV-detector. Data were collected at 300 nm and analysed by Merck-Hitachi HPLC Manager (Ver. 2). The column used was a Eurospher 100-C18 5 μm, 250 mm × 4.0 mm with a 11 mm × 4.0 mm guard column (Knauer GmbH, Germany). A premixed 15:85-mixture of acetonitrile and 0.0375 M triethylammonium phosphate (pH 7.0–7.2) was used as mobile phase at 0.8 ml min^{-1}. Acetonitrile was HPLC grade, water was Milli Q quality, solutions were filtered through 0.45 μm membranes and degassed with helium before use.

Calculation of kinetic parameters

The inital rates were calculated from the least square analysis of the ratio of the areas of internal standard to product **21** plotted against time.

Kinetic parameters were calculated using the computer program Leonora of A. Cornish-Bowden, by fitting the initial rates to the Michaelis-Menten equation using relative and robust weighting of the experimental data [35].

Preparative sialylation, preparation of Neu5Acα(2-6)-Galβ(1-4)-GlcNAcβOC$_6$H$_4$pNO$_2$ (**21**)

p-Nitrophenyl *N*-acetyl-lactosamine **20** (1 mg, 2 μmol) was disolved in 1 ml of 25 mM sodium cacodylate (pH 6.5) containing 1% Triton X 100, 10 mg ml^{-1} BSA, 100 mM NaCl, 30 mM $MnCl_2$. CMP-Neu5Ac (1.32 mg, 2 μmol), 5 mU α-(2,6)-sialyltransferase from rat liver and 10 U alkaline phosphatase were added. The mixture was incubated at room temperature. After 2 and 4 days 0.5 eq. CMP-Neu5Ac were added. The reaction was monitored by analytical HPLC. After 7 days the mixture was filtered through a short C-18 column which was eluted with 20 ml of methanol: water 1:4. The solvents were evaporated and the residue was purified by preparative HPLC as described above. After lyophilization, the colourless triethylammonium salt (0.9 mg, 50%) of **21** was isolated.

FAM-MS (negative mode, matrix: glycerol): 794 $[M\text{-}NHEt_3]$

Kinetic sialyltransferase assay

All assays were designed to limit the CMP-Neu5Ac consumption to 10–15%, in order to get reliable initial rates.

Determination of acceptor K_M

The K_M of acceptor **20** was determined using six different concentrations from 4 mM to 0.125 mM at a saturating concentration of CMP-Neu5Ac. The assay mixtures contained 25 mM sodium cacodylate (pH 6.5), 0.5% Triton X 100,

1 mg ml^{-1} BSA, 560 μM CMP-Neu5Ac, 1.25 μg ml^{-1} p-nitrophenyl-DL-alanine and 333 μU α-(2,6)-sialyltransferase from rat liver in a total volume of 80 μl. The assay tubes were incubated at 37 °C and every 15 min an aliquot of 20 μl was taken and heated in boiling water for 3 min. Every sample was analysed by HPLC. Kinetic data listed in Table 1 were obtained by fitting the initial rate data to the Michaelis-Menten equation.

Determination of donor K_M

The K_M of donor CMP-Neu5Ac was determined using five different concentrations of CMP-Neu5Ac from 0.2 mM to 0.025 mM at a constant concentration of the acceptor **20**. The assay mixtures contained 25 mM sodium cacodylate (pH 6.5), 0.5% Triton X 100, 1 mg ml^{-1} BSA, 1 mM acceptor **20**, 1.25 μg ml^{-1} p-nitrophenyl-DL-alanine and 166–333 μU α-(2,6)-sialyltransferase from rat liver in a total volume of 80 μL. The assay tubes were incubated at 37 °C and every 20 min an aliquot of 20 μl was taken and heated in boiling water for 3 min. Every sample was analysed by HPLC. Kinetic data were obtained by fitting the initial rate data to the Michaelis-Menten equation.

Inhibition studies

The kinetic parameters of CDP, **1**, **2** and **3** were determined using five different concentrations of CMP-Neu5Ac from 0.4 mM to 0.0375 mM at a fixed concentration of the acceptor **20** and the concentration of inhibitor mentioned in Tables 2 and 3. The assay mixtures contained 25 mM sodium cacodylate (pH 6.5), 0.5% Triton X 100, 1 mg ml^{-1} BSA, 1 mM acceptor **20**, different concentrations of the inhibitors CDP, **1**, **2** and **3** (see Table 2), 1.25 μg/ml^{-1} p-nitrophenyl-DL-alanine and 166–333 μU α-(2,6)-sialyltransferase from rat liver in a total volume of 80 μl. The assay tubes were incubated at 37 °C and every 20 min an aliquot of 20 μl was taken and heated in boiling water for 3 min. Every sample was analysed by HPLC. Kinetic data listed in Table 2 were obtained by fitting the initial rate data to the Michaelis-Menten equation.

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