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Carbohydrate Research 343 (2008) 2075–2082
Carbohydrate RESEARCH

Chemoenzymatic synthesis of CMP-*N*-acetyl-7-fluoro-7-deoxy-neuraminic acid

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Received 30 October 2007; received in revised form 31 January 2008; accepted 4 February 2008
Available online 10 February 2008

Presented at Eurocarb 14th Lübeck, Germany, September 2007

Abstract—7-Fluoro sialic acid was prepared and activated as cytidine monophosphate (CMP) ester. The synthesis started with D-glucose, which was efficiently converted into *N*-acetyl-4-fluoro-4-deoxy-D-mannosamine. Aldolase catalyzed transformation yielded the corresponding fluorinated sialic acid which was activated as CMP ester using three different synthetases in the presence as well as in the absence of pyrophosphatase which possesses inhibitory properties. Finally, conditions were optimized to perform a one-pot reaction starting from fluorinated mannosamine, which yielded the 7-fluoro-7-deoxy-CMP-sialic acid by incubation with three enzymes.

Keywords: CMP-7-F-sialic acid; Chemoenzymatic synthesis; Sugar nucleotide

1. Introduction

Sialic acids are a widely distributed class of negatively charged 9-carbon sugars with more than 40 natural occurring derivatives in vertebrates and bacteria.[1] The most important member of this family is *N*-acetyl-neuraminic acid. The synthesis of sialic acid and analogues has been extensively studied.[2,3] The enzymatic synthesis of *N*-acetyl-neuraminic acid (sialic acid, **1**) from *N*-acetyl-D-mannosamine or *N*-acetyl-D-glucosamine followed by activation as CMP-ester is well established.[4–6] Multienzyme systems have been optimized for this sequence,[7–9] which includes the regeneration of CMP to CTP.[10] To access new derivatives, every position of sialic acid was addressed during the past years[1–3] and several variations at position 7 of sialic acid were synthesized.[3,11,12] Several approaches follow chemoenzymatic routes using derivates of *N*-acetyl-D-mannosamine and pyruvate or pyruvate derivatives.[2,3] Also, several total synthesis approaches of native and artificial sialic acids were published.[2,3] Many sialic acid recognizing proteins exhibit strong interactions to the glycerol side chain of sialic acid, which makes this position important for modifications.[3]

2. Results and discussion

Here, we report an improved synthesis of CMP-*N*-acetyl-7-fluoro-7-deoxy-neuraminic acid (**1**)[12] starting from D-glucose. The synthesis of **1** (Scheme 1) starts with commercially available methyl glycoside **2**. After activation as triflate, azide introduction was readily accomplished using sodium azide in DMF following an approach published by Moravcova and coworkers[13] and Pipik and coworkers.[14] In our hands, the isolated

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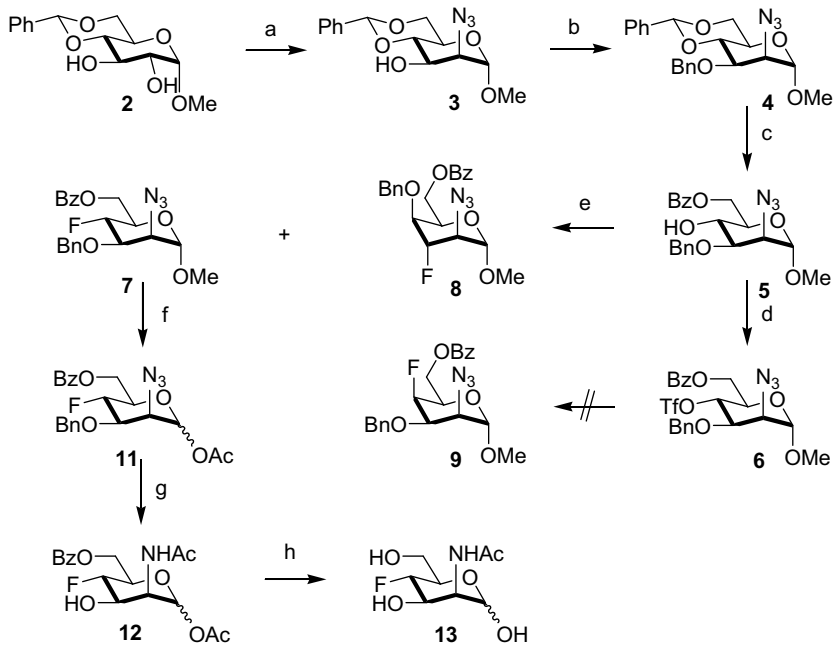
doi:10.1016/j.carres.2008.02.003

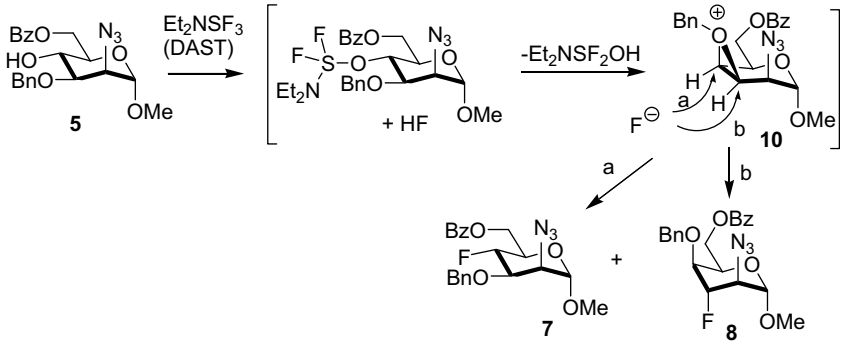


Scheme 1. Reagents and conditions: (a) Tf_2O, pyridine, CH_2Cl_2, −20 °C, 2 h; then NaN_3, 15-crown-5, DMF, 50 °C, 24 h, 93%; (b) BnBr, TBAI, NaH, DMF, 0 °C→20 °C, 14 h, 93%; (c) *p*-TSA, MeOH, 20 °C, 3 h; then BzCl, 2,6-lutidine, CH_2Cl_2, 0 °C, 2 h, 99% (d) Tf_2O, pyridine, CH_2Cl_2, −20 °C→−8 °C, 3 h; (e) DAST, CH_2Cl_2, −78 °C→20 °C, 4 d, 63% for **7**; 13% for **8**, 11% for **5**; (f) Ac_2O/H_2SO_4 (3v%), 0 °C, 1 h, α-anomer 63%, β-anomer 26%; (g) $Pd(OAc)_2$, EtOAc, Ac_2O, 20 °C, 5.6 bar, 4 × 24 h, 79%; (h) NaOMe, MeOH, 20 °C, 3 h.

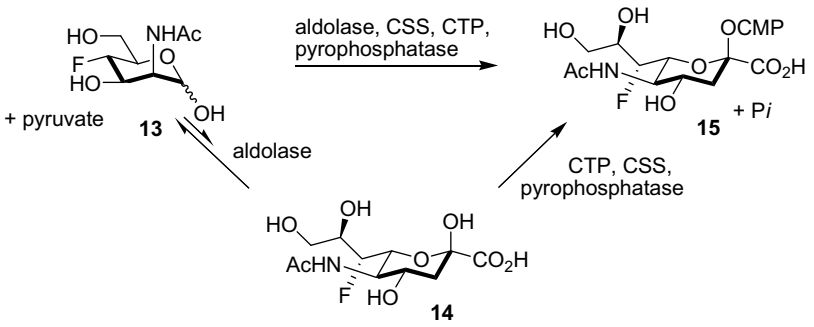
yield of *manno* azide **3** could be improved from 53% to 93% by the addition of crown ether (15-c-5), which also allowed us to reduce the amount of sodium azide and lower the reaction temperature.

To selectively address position 4 of the hexose, benzyl ether formation at 3-OH was carried out first. Hydrolysis of the benzylidene acetal **4** under acidic conditions (*p*TsOH in methanol) yielded the 4,6-diol, which could be selectively converted into benzoate **5** in 99% overall yield. Any attempts to fluorinate triflate **6** failed; however, the reaction of alcohol **5** with DAST in dichloromethane gave fluorinated hexose **7** in 63% yield. Surprisingly, product **9** which results from S_N2 inversion of configuration was not detected. In fact, we could only isolate the 3-fluoro sugar **8**. We tentatively assume that a DAST-promoted removal of the hydroxyl group in **5** furnishes the intermediate O-benzylated oxirane **10**, which was ring-opened by fluoride to the corresponding *manno*- and *ido*-configured hexoses **7** and **8**, respectively (Scheme 2). DAST-induced oxirane formation was also encountered during the synthesis of fluorinated furanosides by Mikhailopulo and Sivets.[15] Anomeric deprotection (H_2SO_4 in acetic acid anhydride) gave acetate **11** as anomeric mixture (89%; $\alpha/\beta = 2.4{:}1$), which was debenzylated at elevated pressure to yield compound **12**. Final transesterification was performed with MeOH/NaOMe and the resulting *N*-acetyl-4-deoxy-4-fluoro-D-mannosamine (**13**) was formed and could be employed for enzymatic syntheses without further purification.

Studies on the enzymatic synthesis of 7-deoxy-7-fluoro sialic acid (**14**) were initiated using commercially available sialic acid aldolase (Fluka). Reaction conditions[16] found for complete transformation of *N*-acetyl-mannosamine into *N*-acetyl-neuraminic acid were



Scheme 2. Proposed mechanism for the benzyloxy migration and formation of **7** and **8**.



Scheme 3. Aldolase and CMP-sialic acid synthetase reaction to CMP-7-fluoro sialic acid.

applied to *N*-acetyl-4-fluoro-mannosamine **13** and resulted in only 56% conversion to **14**. Therefore, we planned to shift the aldolase equilibrium by coupling the aldolase step with the CMP activation using CMP-sialic acid synthetase (CSS) (Scheme 3). We tested three different CSS, which were cloned from *Neisseria meningitidis* serogroup B (NmB),[17] mouse (amino acid 39–267 of the murine CSS with N-terminal NusA-StrepII-thrombin-tag in expression vector pET43)[18] and rainbow trout (amino acid 28–255 of rainbow trout CMP-Kdn-synthetase with N-terminal NusA-StrepII-thrombin-tag in expression vector pET43.Strep).[19] *N*-Acetyl-mannosamine served again as a model substrate to find optimized reaction conditions. Addition of pyrophosphatase was crucial for rapid transformation although higher conversion to CMP-*N*-acetyl-neuraminic acid was also achieved after a prolonged incubation time in the absence of this enzyme (Fig. 1). In contrast to these results, no formation of CMP glycoside **15** could be observed in the absence of pyrophosphatase, and only *N*-acetyl-4-fluoro-mannosamine **13** and 7-F-sialic acid **14** were present in the reaction mixture as judged by ESIMS. In comparison to the other CSS, the bacterial enzyme from

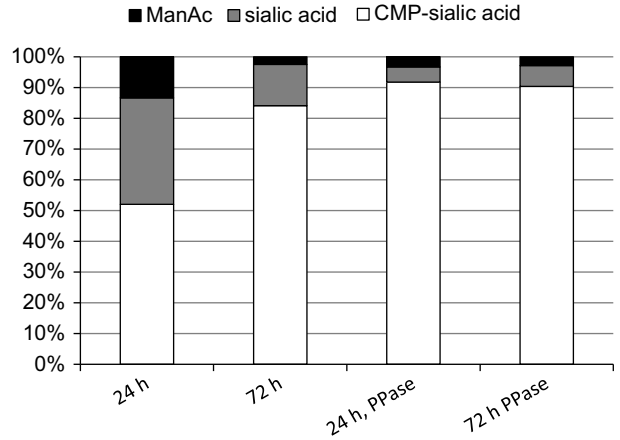


Figure 1. Optimization of reaction conditions for the one-pot two/three-enzyme system sialic acid aldolase/CMP-sialic acid synthetase/pyrophosphatase (PPase) (percentage of the different products with and without pyrophosphatase at pH 8.8 according to LC-ESIMS).

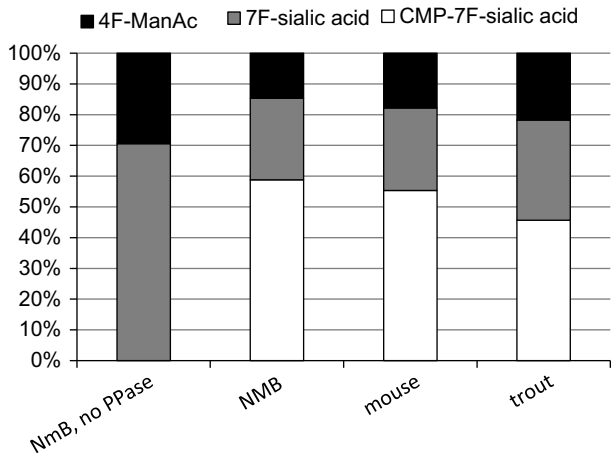


Figure 2. Optimization of reaction conditions for the synthesis of CMP-7-deoxy-7-fluoro sialic acid (percentage of the different products using different CMP-sialic acid synthetases at pH 8.8 according to LC-ESIMS).

NmB gave best results (Fig. 2). Thus, coupling aldolase, CSS and pyrophosphatase in a one-pot reaction did not improve the low efficiency of the aldolase reaction and hexosamine **13** was still detectable along with not activated 7-F-sialic acid **14**. Using purified **14** and CSS (NmB, pyrophosphatase) resulted in complete conversion to **15** while no free 7-F-sialic acid could be detected by ES-MS.

In summary, we present a flexible and high yielding synthesis of *N*-acetyl-4-fluoro-mannosamine **13** starting from D-glucose. Aldolase-catalyzed transformation to the corresponding 7-deoxy-7-fluoroneuraminic acid **14** was performed and three CMP-sialic acid synthetases were compared for CMP-activation. The present study is currently expanded to other neuraminic acid analogues in our laboratories.

3. Experimental

3.1. General methods

Optical rotations were measured with a Perkin Elmer 341 polarimeter. NMR spectra were recorded on Bruker

ARX-400 (^{1}H, 400 MHz; ^{13}C, 100 MHz) and Bruker AM-500 (^{1}H, 500 MHz; ^{13}C, 125 MHz) spectrometers. All spectra were measured using standard Bruker pulse sequences. ESI mass spectra were recorded on a LCT mass spectrometer (Micromass) with Lock-Spray dual ion source. The LCT-spectrometer was coupled with a Waters Alliance 2695 HPLC unit. All solvents used were of reagent grade and were further dried. Reactions were monitored by thin-layer chromatography (TLC) on Silica Gel 60 F^{254} (E. Merck, Darmstadt) and spots were detected either by UV-absorption or by charring with $KMnO_4$/NaOH in water. Preparative column chromatography was performed on Silica Gel 60 (E. Merck, Darmstadt). HPLC-purifications were carried out using a Merck/Hitachi LaChrom unit (L-7150 pump, L-7455 DAAD, Sedere Sedex-75 ELSD). Reagents were purified and dried by standard techniques.

3.2. Methyl 2-azido-4,6-*O*-benzylidene-2-deoxy-α-D-mannopyranoside (3)[13,14]

To a soln of methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (**2**) (5.27 g, 18.7 mmol) in CH_2Cl_2 (60 mL) was added pyridine (60 mL, 37.3 mmol) under argon. At −20 °C, trifluoromethanesulfonic acid anhydride (3.72 mL, 22.4 mmol) was added dropwise to the mixture and the temperature was kept at −20 °C for 2 h. The reaction mixture was quenched with brine (50 mL), washed with brine (3 × 50 mL) and dried over $MgSO_4$. After the removal of CH_2Cl_2, traces of water were removed by azeotropic distillation with toluene and the trifluoromethanesulfonic ester was dissolved in DMF (50 mL) under argon atmosphere without further purification. Sodium azide (3.64 g, 56.0 mmol) and 15-crown-5 ether (11.2 mL, 56.0 mmol) were added and the suspension was stirred at 50 °C for 25 h. After terminating the reaction by the addition of water (100 mL), the mixture was extracted with Et_2O (4 × 50 mL). The combined organic extracts were washed with water (3 × 50 mL) and brine (50 mL), dried over $MgSO_4$ and concentrated under diminished pressure. The crude product was purified by column chromatography (10:1–3:1 hexane–EtOAc) to give **3** (5.3 g, 17.3 mmol; 93%) as an amorphous white solid. $[\alpha]_D^{20}$ +59.1 (*c* 1, $CHCl_3$); R_f 0.10 (hexane–EtOAc = 3:1); ^{1}H NMR (400 MHz, $CDCl_3$, δ $CHCl_3$ 7.26) δ: 7.52–7.47 (m, 2H, Ph), 7.42–7.35 (m, 3H, Ph), 5.56 (s, 1H, C*H*Ph), 4.67 (d, J = 1.1 Hz, 1H, H-1), 4.27–4.22 (m, 1H, H-6a), 4.22 (ddd, J = 9.6, 4.1, 3.9 Hz, 1H, H-3), 3.91 (dd, J = 3.9, 1.1 Hz, 1H, H-2), 3.88 (dd, J = 9.6, 9.6 Hz, 1H, H-4), 3.82–3.72 (m, 2H, H-6b, H-5), 3.38 (s, 3H, OC*H*$_3$), 2.81 (d, J = 4.1 Hz, 1H, O*H*); ^{13}C NMR (100 MHz, $CDCl_3$, $\delta$$CDCl_3$ 77.16) δ: 137.2 (C-1 Ph), 129.4 (C-4 Ph), 128.5 (C-2 Ph), 126.4 (C-3 Ph), 102.4 (Ph*C*H), 100.2 (C-1), 79.1 (C-4), 68.9 (C-3), 68.8 (C-6), 63.7 (C-2), 63.4 (C-5), 55.3 (O*C*H$_3$).

3.3. Methyl 2-azido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-α-D-mannopyranoside (4)

A soln of **3** (100 mg, 0.325 mmol) in DMF (1 mL) was stirred under argon at 0 °C. Sodium hydride (19.6 mg, 0.488 mmol) was added portionwise followed by tetrabutylammonium bromide (12.0 mg, 0.033 mmol) and benzyl bromide (77 μL, 0.651 mmol). Stirring was continued at 0 °C for 2 h and at 20–22 °C for 16 h. The mixture was terminated by the addition of methanol (0.2 mL), followed by water (5 mL) and extracted with Et_2O (4 × 5 mL). Combined organic extracts were washed with water (3 × 5 mL) and brine (5 mL), dried over $MgSO_4$ and concentrated under diminished pressure. The crude product was purified by column chromatography (10:1–5:1 hexane–EtOAc) to give **4** (128 mg, 0.322 mmol; 99%) as an amorphous white solid. $[\alpha]_D^{20}$ +57.2 (*c* 1, $CHCl_3$); R_f 0.40 (hexane–EtOAc = 5:1); ^{1}H NMR (400 MHz, $CDCl_3$, δ $CHCl_3$ 7.26) δ: 7.53–7.29 (m, 10H, Ph), 5.65 (s, 1H, C*H*Ph), 4.91 (d, J = 12.1 Hz, 1H, C*H*$_2$Ph), 4.76 (d, J = 12.1 Hz, 1H, C*H*$_2$Ph), 4.68 (d, J = 1.7 Hz, 1H, H-1), 4.30 (dd, J = 10.0, 4.1 Hz, 1H, H-6a), 4.16–4.12 (m, 2H, H-4, H-3), 4.02–4.00 (m, 1H, H-2), 3.90–3.77 (m, 2H, H-6b, H-5), 3.37 (s, 3H, OC*H*$_3$); ^{13}C NMR (100 MHz, $CDCl_3$, δ $CDCl_3$ 77.16) δ: 138.1 (CHPh C-1), 137.5 (CH_2Ph C-1), 129.0–126.1 (Ph), 101.7 (*C*HPh), 100.2 (C-1), 79.2 (C-4), 75.8 (C-3), 73.3 (*C*H$_2$Ph), 68.8 (C-6), 63.8 (C-5), 62.7 (C-2), 55.1 (O*C*H$_3$).

3.4. Methyl 2-azido-6-*O*-benzoyl-3-*O*-benzyl-2-deoxy-α-D-mannopyranoside (5)

To a soln of **4** (12.39 g, 31.19 mmol) in MeOH (150 mL) was added *p*-toluenesulfonic acid (1.78 g, 9.36 mmol). After stirring at 20–22 °C for 3 h, the mixture was neutralized with $NaHCO_3$ and concentrated under diminished pressure. Traces of water and methanol were removed by azeotropic distillation with toluene and the resulting diol was dissolved in anhydr CH_2Cl_2 (150 mL) under argon atmosphere. After the addition of 2,6-lutidine (5.43 mL, 46.79 mmol) followed by benzoyl chloride (4.35 mL, 37.42 mmol) stirring was continued at 0 °C for 2 h. The mixture was washed with water (3 × 50 mL) and brine (50 mL). The organic extracts were dried over $MgSO_4$ and concentrated under diminished pressure. The crude product was purified by column chromatography (10:1–5:1 hexane–EtOAc) to give **5** (12.71 g, 30.75 mmol; 99%) as an amorphous white solid. $[\alpha]_D^{20}$ +20.5 (*c* 1, $CHCl_3$); R_f 0.34 (hexane–EtOAc = 2:1); ^{1}H NMR (400 MHz, C_6D_6, δ C_6D_5H 7.16) δ: 8.32–8.28 (m, 2H, Bz H-3), 7.42–7.39 (m, 2H, Bn H-3), 7.31–7.13 (m, 6H, Bn and Bz), 4.78 (dd, J = 11.9, 5.3 Hz, 1H, H-6a), 4.71 (dd, J = 11.9, 2.3 Hz, 1H, H-6b), 4.55 (d, J = 11.7 Hz, 1H, CH*H*′Ph), 4.47 (d, J = 11.7 Hz, 1H, C*H*H′Ph), 4.46 (d, J = 1.4 Hz,

1H, H-1), 4.09 (dd, $J = 9.7$, 9.4 Hz, 1H, H-4), 3.92 (dd, $J = 9.4$, 3.7 Hz, 1H, H-3), 3.88 (ddd, $J = 9.7$, 5.3, 2.3 Hz, 1H, H-5), 3.63 (dd, $J = 3.7$, 1.4 Hz, 1H, H-2), 3.03 (s, 3H, OCH_3); ^{13}C NMR (100 MHz, C_6D_6, δ C_6D_6 128.06) δ: 166.7 (*C*OPh), 138.3 (C-1 Bn), 133.0 (C-4 Bz), 130.7 (C-1 Bz), 130.1 (C-3 Bz), 128.8 (C-2 Bz), 128.6–127.8 (C-2, C3, C4 Bn), 99.5 (C-1), 79.7 (C-3), 72.6 (*C*H_2Ph), 71.1 (C-5), 67.4 (C-4), 64.1 (C-6), 60.9 (C-2), 54.5 (O*C*H_3); HRESIMS: calcd for $C_{21}H_{23}N_3O_6Na$: 436.1490; found: 436.1485 $[M+Na]^+$.

3.5. Methyl 2-azido-6-*O*-benzoyl-3-*O*-benzyl-2-deoxy-4-*O*-trifluoromethanesulfonyl-α-D-mannopyranoside (6)

To a soln of **5** (100 mg, 0.242 mmol) in anhydr CH_2Cl_2 (1.5 mL) under argon atmosphere was added pyridine (48.5 μL, 0.605 mmol). The mixture was stirred at −20 °C for 10 min and trifluoromethanesulfonic acid anhydride (56 μL, 0.339 mmol) was slowly added. Within 3 h the temperature was raised to −8 °C. The mixture was diluted with CH_2Cl_2 (10 mL), washed with brine (4 × 5 mL), dried over $MgSO_4$ and concentrated under diminished pressure. The crude product was purified by column chromatography (5:1 hexane–EtOAc) to give **6** (128 mg, 0.235 mmol; 97%) as a colourless oil. $[\alpha]_D^{20}$ +64.7 (*c* 1, $CHCl_3$); R_f 0.44 (hexane–EtOAc = 3:1); ^{1}H NMR (400 MHz, $CDCl_3$, δ $CHCl_3$ 7.26) δ: 8.10–8.08 (m, 2H, Bz H-3), 7.60–7.59 (m, 1H, Bz H-4), 7.50–7.36 (m, 7H, Ph and Bz), 5.29 (dd, $J = 9.9$, 9.4 Hz, 1H, H-4), 4.82 (d, $J = 11.3$ Hz, 1H, C*H*H′Ph), 4.74 (dd, $J = 12.5$, 2.0 Hz, 1H, H-6a), 4.71 (d, $J = 1.4$ Hz, 1H, H-1), 4.65 (d, $J = 11.3$ Hz, 1H, CH*H*′Ph), 4.40 (dd, $J = 12.5$, 4.3 Hz, 1H, H-6b), 4.17 (dd, $J = 9.4$, 3.6 Hz, 1H, H-3), 4.13 (ddd, $J = 9.9$, 4.3, 2.0 Hz, 1H, H-5), 3.84 (dd, $J = 3.6$, 1.4 Hz, 1H, H-2), 3.37 (s, 3H, OCH_3); ^{13}C NMR (100 MHz, $CDCl_3$, δ $CDCl_3$ 77.16) δ: 166.1 (*C*OPh), 136.5 (C-1 Bn), 133.4 (C-4 Bz), 129.9 (C-3 Bz), 129.6 (C-1 Bz), 128.8 (C-2 Bz), 128.7–128.6 (C-2, C3, C4, Bn), 118.5 (q, $J = 319.1$ Hz, *C*F_3), 99.2 (C-1), 80.2 (C-4), 76.2 (C-3), 73.4 (*C*H_2Ph), 68.0 (C-5), 62.2 (C-6), 61.6 (C-2), 55.6 (O*C*H_3).

3.6. Methyl 2-azido-6-*O*-benzoyl-3-*O*-benzyl-2,4-dideoxy-4-fluoro-α-D-mannopyranoside (7) and methyl 2-azido-6-*O*-benzoyl-4-*O*-benzyl-2,3-dideoxy-3-fluoro-α-D-idopyranoside (8)

In a Teflon™ flask, DAST (0.28 mL, 2.322 mmol) was dissolved in anhydr CH_2Cl_2 (1.5 mL). A soln of **5** (120 mg, 0.290 mmol) in anhydr CH_2Cl_2 (2 mL) was slowly added at −78 °C. The temperature was raised to 20 °C and stirring was continued for 4 d. The mixture was cooled to −20 °C, terminated by the addition of methanol (1 mL) and concentrated under diminished pressure. The residue was dissolved in CH_2Cl_2 (10 mL), washed with water (3 × 5 mL), dried over $MgSO_4$ and concentrated under diminished pressure. The crude product was purified by column chromatography (15:1–10:1 hexane–EtOAc) to yield **7** (76 mg, 0.183 mmol; 63%, amorphous white solid), **8** (16 mg, 0.038 mmol; 13%, amorphous white solid) and **5** (13 mg, 0.032 mmol; 11%, colourless oil).

Compound **7**: $[\alpha]_D^{20}$ +51.7 (*c* 1.1, $CHCl_3$); R_f 0.54 (hexane–EtOAc = 2:1); ^{1}H NMR (400 MHz, $CDCl_3$, δ $CHCl_3$ 7.26) δ: 8.07–8.05 (m, 2H, Bz H-3), 7.59–7.52 (m, 1H, Bz H-4), 7.45–7.41 (m, 2H, Bz H-2), 7.33–7.29 (m, 5H, Bn), 4.86 (ddd, $J = 51.0$, 9.6, 9.1 Hz, 1H, H-4), 4.83 (d, $J = 11.8$ Hz, 1H, C*H*H′Ph), 4.73 (d, $J = 11.8$ Hz, 1H, CH*H*′Ph), 4.72 (dd, $J = 1.7$, 1.7 Hz, 1H, H-1), 4.66 (ddd, $J = 12.0$, 2.2, 2.1 Hz, 1H, H-6a), 4.49 (dd, $J = 12.0$, 5.2 and 1.1 Hz, 1H, H-6b), 4.10 (ddd, $J = 13.4$, 9.1, 4.0 Hz, 1H, H-3), 4.00 (dddd, $J = 9.6$, 5.3, 5.2, 2.1 Hz, 1H, H-5), 3.96 (m, 1H, H-2), 3.38 (s, 3H, OCH_3); ^{13}C NMR (100 MHz, $CDCl_3$, δ $CDCl_3$ 77.16) δ: 166.4 (*C*OPh), 137.6 (C-1 Bn), 133.3 (C-4 Bz), 129.9 (C-3 Bz), 129.8 (C-1 Bz), 128.7 (C-2 Bz), 128.6–127.8 (C-2, C-3, C-4 Bn), 99.3 (d, $J_{F-C} = 1.2$ Hz, C-1), 88.3 (d, $J_{F-C} = 181.2$ Hz, C-4), 76.7 (d, $J_{F-C} = 17.4$ Hz, C-3), 73.3 (t, $J_{F-C} = 1.7$ Hz, *C*H_2Ph), 68.3 (d, $J_{F-C} = 24.3$ Hz, C-5), 63.1 (C-6), 62.2 (d, $J_{F-C} = 8.8$ Hz, C-2), 55.4 (O*C*H_3); HRESIMS: calcd for $C_{23}H_{25}N_4O_5FNa$: 479.1707; found: 479.1715 $[M+Na+CH_3CN]^+$.

Compound **8**: $[\alpha]_D^{20}$ +2.6 (*c* 0.4, $CHCl_3$); R_f 0.69 (hexane–EtOAc = 2:1); ^{1}H NMR (400 MHz, $CDCl_3$, δ $CHCl_3$ 7.26) δ: 8.07–8.05 (m, 2H, Bz H-3), 7.59–7.52 (m, 1H, Bz H-4), 7.45–7.41 (m, 2H, Bz H-2), 7.33–7.29 (m, 5H, Bn), 4.83 (d, $J = 11.9$ Hz, 1H, C*H*H′Ph), 4.74 (ddd, $J = 49.7$, 8.3, 5.5 Hz, 1H, H-3), 4.70 (d, $J = 5.3$ Hz, 1H, H-1), 4.63 (dd, $J = 12.0$, 8.0 Hz, 1H, H-6a), 4.62 (d, $J = 11.9$ Hz, 1H, CH*H*′Ph), 4.47 (dd, $J = 12.0$, 4.0 Hz, 1H, H-6b), 4.38 (ddd, $J = 8.0$, 4.7, 4.0 Hz, 1H, H-5), 3.86 (ddd, $J = 16.5$, 5.5, 4.7 Hz, 1H, H-4), 3.59 (ddd, $J = 13.2$, 8.3, 5.3 Hz, 1H, H-2), 3.42 (s, 3H, OCH_3); ^{13}C NMR (100 MHz, $CDCl_3$, δ $CDCl_3$ 77.16) δ: 166.3 (*C*OPh), 137.1 (C-1 Bn), 133.4 (C-4 Bz), 129.8 (C-1 Bz), 129.7 (C-3 Bz), 128.7 (C-2 Bz), 128.6–128.3 (C-2, C-3, C-4 Bn), 99.7 (d, $J_{F-C} = 7.1$ Hz, C-1), 92.1 (d, $J_{F-C} = 184.2$ Hz, C-3), 75.2 (d, $J_{F-C} = 20.9$ Hz, C-4), 73.1 (d, $J_{F-C} = 0.7$ Hz, *C*H_2Ph), 68.9 (d, $J_{F-C} = 6.3$ Hz, C-5), 62.6 (C-6), 62.2 (d, $J_{F-C} = 20.1$ Hz, C-2), 56.2 (O*C*H_3); HRESIMS: calcd for $C_{23}H_{25}N_4O_5FNa$: 479.1707; found: 479.1715 $[M+Na+CH_3CN]^+$.

3.7. 1-*O*-Acetyl-2-azido-6-*O*-benzoyl-3-*O*-benzyl-2,4-dideoxy-4-fluoro-α,β-D-mannopyranose (11)

Mannopyranoside **7** (750 mg, 1.805 mmol) was dissolved in acetic anhydride (9.9 mL) and concd H_2SO_4 (0.1 mL) at 0 °C and stirring was continued for 1 h. After neutralization with phosphate buffer (100 mM,

pH 7.0), the mixture was extracted with EtOAc (4 × 50 mL). The combined organic extracts were washed with brine (2 × 50 mL), dried over $MgSO_4$, concentrated under diminished pressure and co-distilled with toluene. The crude product was purified by column chromatography (20:1–10:1 hexane–EtOAc) to yield **11** (α-anomer: 504 mg, 1.137 mmol; 63%, and β-anomer: 210 mg, 0.474 mmol; 26%; both as amorphous white solids).

α-Anomer: $[\alpha]_D^{20}$ +44.8 (*c* 1.1, $CHCl_3$); R_f 0.37 (hexane–EtOAc = 2:1); ^{1}H NMR (400 MHz, $CDCl_3$, δ $CHCl_3$ 7.26) δ: 8.07–8.05 (m, 2H, Bz H-3), 7.59–7.55 (m, 1H, Bz H-4), 7.47–7.41 (m, 2H, Bz H-2), 7.43–7.41 (m, 5H, Bn), 6.07 (dd, $J = 2.2$, 2.2 Hz, 1H, H-1), 4.97 (ddd, $J = 51.1$, 9.5, 9.5 Hz, 1H, H-4), 4.89 (d, $J = 11.8$ Hz, 1H, C*H*H′Ph), 4.75 (d, $J = 11.8$ Hz, 1H, CH*H*′Ph), 4.63 (ddd, $J = 12.2$, 1.9, 1.9 Hz, 1H, H-6a), 4.49 (ddd, $J = 12.2$, 4.5, 1.1 Hz, 1H, H-6b), 4.14–4.07 (m, 2H, H-5, H-3), 3.94–3.92 (m, 1H, H-2), 2.11 (s, 3H, COC*H*$_3$); ^{13}C NMR (100 MHz, $CDCl_3$, δ $CDCl_3$ 77.16) δ: 168.3 (*C*OCH$_3$), 166.3 (*C*OPh), 137.3 (C-1 Bn), 133.3 (C-4 Bz), 129.9–127.9 (C-2, C-3, C-4 Bn, C1, C-2, C-3 Bz), 91.8 (d, $J_{F-C} = 1.2$ Hz, C-1), 87.8 (d, $J_{F-C} = 181.3$ Hz, C-4), 76.1 (d, $J_{F-C} = 18.0$ Hz, C-3), 73.5 (d, $J_{F-C} = 1.9$ Hz, *C*H$_2$Ph), 70.7 (d, $J_{F-C} = 24.9$ Hz, C-5), 62.6 (C-6), 61.3 (d, $J_{F-C} = 8.6$ Hz, C-2), 21.0 (CO*C*H$_3$); HRESIMS: calcd for $C_{22}H_{22}N_3O_6FNa$: 466.1390; found: 466.1390 $[M+Na]^+$.

β-Anomer: $[\alpha]_D^{20}$ −33.0 (*c* 1, $CHCl_3$); R_f 0.60 (hexane–EtOAc = 2:1); ^{1}H NMR (400 MHz, $CDCl_3$, δ $CHCl_3$ 7.26) δ: 8.07–8.04 (m, 2H, Bz H-3), 7.59–7.55 (m, 1H, Bz H-4), 7.47–7.43 (m, 2H, Bz H-2), 7.40–7.31 (m, 5H, Bn), 5.75 (d, $J = 1.7$ Hz, 1H, H-1), 4.86 (d, $J = 11.9$ Hz, 1H, C*H*H′Ph), 4.85 (ddd, $J = 50.6$, 9.1, 9.1 Hz, 1H, H-4), 4.74 (d, $J = 11.9$ Hz, 1H, CH*H*′Ph), 4.65 (ddd, $J = 12.2$, 1.9, 1.9 Hz, 1H, H-6a), 4.48 (ddd, $J = 12.2$, 5.3, 1.4 Hz, 1H, H-6b), 4.02–3.99 (m, 1H, H-2), 3.87–3.80 (m, 2H, H-5, H-3), 2.17 (s, 3H, COC*H*$_3$); ^{13}C NMR (100 MHz, $CDCl_3$, δ $CDCl_3$ 77.16) δ: 168.7 (*C*OCH$_3$), 166.3 (*C*OPh), 137.1 (C-1 Bn), 133.4 (C-4 Bz), 130.0 (C-3 Bz), 129.8 (C-1 Bz), 128.8 (C-2 Bz), 128.6–128.0 (C2, C3, C4 Bn), 91.6 (d, $J_{F-C} = 1.3$ Hz, C-1), 87.6 (d, $J_{F-C} = 182.3$ Hz, C-4), 77.5 (d, $J_{F-C} = 18.0$ Hz, C-3), 73.2 (t, $J_{F-C} = 1.9$ Hz, *C*H$_2$Ph), 73.0 (d, $J_{F-C} = 25.4$ Hz, C-5), 62.7 (C-6), 62.0 (d, $J_{F-C} = 8.8$ Hz, C-2), 20.9 (CO*C*H$_3$); HRESIMS: calcd for $C_{22}H_{22}N_3O_6FNa$: 466.1390; found: 466.1398 $[M+Na]^+$.

3.8. 2-Acetamido-1-*O*-acetyl-6-*O*-benzoyl-3-*O*-benzyl-2,4-dideoxy-4-fluoro-α-D-mannopyranose (12)

$Pd(OAc)_2$ (186 mg, 0.829 mmol) was added to a soln of **11** (α-anomer: 465 mg, 1.049 mmol) and acetic anhydride (985 μL, 10.49 mmol) in EtOAc (5 mL). The suspension was stirred at 20–22 °C for 22 h under an atmosphere of H_2 (5–5.6 bar). After filtration through a short pad of Celite™ and washing with EtOAc (50 mL), the crude product was dried under diminished pressure. It turned out to be necessary to repeat the whole reaction procedure four times. The product was purified by column chromatography (silica gel; EtOAc) to yield **12** (305 mg, 0.826 mmol; 79%) as an amorphous white solid. $[\alpha]_D^{20}$ +34.83 (*c* 1, $CHCl_3$); R_f 0.38 (EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$, δ $CHCl_3$ 7.26) δ: 8.01–7.99 (m, 2H, Bz H-3), 7.59–7.58 (m, 1H, Bz H-4), 7.45–7.41 (m, 2H, Bz H-2), 6.27 (d, $J = 7.5$ Hz, 1H, N*H*), 6.07 (dd, $J = 2.2$, 2.1 Hz, 1H, H-1), 4.66 (ddd, $J = 50.5$, 9.4, 9.4 Hz, 1H, H-4), 4.64 (dd, $J = 12.2$, 2.6, 1.5 Hz, 1H, H-6a), 4.54 (dd, $J = 12.2$, 5.4, 1.0 Hz, 1H, H-6b), 4.49 (dddd, $J = 7.5$, 4.9, 2.7, 2.1 Hz, 1H, H-2), 4.38 (ddd, $J = 14.5$, 9.4, 4.9 Hz, 1H, H-3), 3.92 (dddd, $J = 9.4$, 5.8, 5.4, 2.6 Hz, 1H, H-5), 2.13 (s, 3H, COC*H*$_3$), 2.01 (s, 3H, NHCOC*H*$_3$); ^{13}C NMR (100 MHz, $CDCl_3$, δ $CDCl_3$ 77.16) δ: 172.4 (NH*C*OCH$_3$), 168.5 (O*C*OCH$_3$), 166.3 (*C*OPh), 133.5 (Bz C-4), 129.8 (Bz C-3), 129.7 (Bz C-1), 128.6 (Bz C-2), 91.7 (d, $J_{F-C} = 1.0$ Hz, C-1), 88.3 (d, $J_{F-C} = 181.2$ Hz, C-4), 70.1 (d, $J_{F-C} = 24.7$ Hz, C-5), 68.4 (d, $J_{F-C} = 18.6$ Hz, C-3), 62.8 (C-6), 52.9 (d, $J_{F-C} = 8.4$ Hz, C-2), 23.1 (NHCO*C*H$_3$), 20.9 (OCO*C*H$_3$); HRESIMS: calcd for $C_{17}H_{20}NO_7FNa$: 392.1122; found: 392.1138 $[M+Na]^+$.

3.9. 2-Acetamido-2,4-dideoxy-4-fluoro-α-D-mannopyranose (13)

Pyranosyl acetate **12** (106 mg, 0.287 mmol) was dissolved in anhydr MeOH (2 mL) and freshly prepared sodium methoxide (0.018 mL, 1.6 M, 0.029 mmol) was added. After 3 h at 20–22 °C aqueous HCl (0.029 mL, 1 M, 0.029 mmol) was added and the product was dried under diminished pressure. Compound **13** (R_f 0.38, 5:1 EtOAc–MeOH) was directly used for the next reaction without further purification.

3.10. 5-Acetamido-3,5,7-trideoxy-7-fluoro-D-*glycero*-D-*galacto*-2-nonulopyranonic acid (14)

A soln of **13** (5.0 mg, 0.022 mmol), pyruvate (24.7 mg, 0.224 mmol), dithiothreitole (3.5 mg; 0.022 mmol) and aldolase (1 mg; EC 4.1.3.3; Fluka; 21.1 U/mg) in sodium phosphate buffer (0.5 mL; 0.1 M; pH 7.5) was incubated at 32 °C for 24 h. After the addition of another batch of pyruvate (24.7 mg, 0.224 mmol), reaction was continued at the same temperature for 24 h. The crude mixture was freeze-dried, dissolved in formic acid (0.4 mL, 0.1%) and purified by HPLC (column: Luna [Phenomenex] 5μ NH_2, 100 Å, 100 × 21.20 mm; mobile phase: formic acid [0.1%; 8 mL/min]; R_t 117 min) to yield **14** (3.9 mg, 0.012 mmol; 56%) as an amorphous white solid.

^{1}H NMR (500 MHz, D_2O) δ: 4.36 (dd, $J = 45.7$, 8.5 Hz, 1H, H-7), 3.96–3.83 (m, 3H, H-4, H-6, H-8), 3.77 (dd, $J = 10.3$, 10.3 Hz, 1H, H-5), 3.67 (ddd,

$J = 12.2$, 2.5, 2.5 Hz, 1H, H-9b), 3.52 (ddd, $J = 12.2$, 5.5, 2.1 Hz, 1H, H-9a), 2.26 (dd, $J = 12.3$, 4.7 Hz, 1H, H-3_{eq}), 1.85 (s, 3H, NHCOCH_3), 1.74 (dd, J = 12.3, 12.3 Hz, 1H, H-3_{ax}); ^{13}C NMR (100 MHz, D_2O): 178.2 (*C*OOH), 174.5 (NH*C*OCH$_3$), 96.1 (C-2), 88.8 (d, $J = 178.9$ Hz, C-7), (d, $J = 17.3$ Hz, C-6), 67.5 (d, $J = 26.6$ Hz, C-8), 67.1 (C-4), 62.1 (C-9), 51.7 (C-5), 38.9 (C-3), 21.8 (NHCO*C*H$_3$); HRESIMS: calcd for $C_{11}H_{17}NO_8F$: 310.0938; found: 310.0945 $[M-H]^-$.

3.11. Cytidine-5′-monophospho-5-acetamido-3,5,7-trideoxy-7-fluoro-D-*glycero*-D-*galacto*-2-nonulopyranonic acid (15) from 13

A soln of **13** (5.0 mg, 0.022 mmol), pyruvate (24.7 mg, 0.224 mmol), dithiothreitole (3.5 mg, 0.022 mmol) and cytidine-5′-triphosphate (13.9 mg, 0.025 mmol) in Tris–HCl buffer (1 mL; 0.1 M; pH 8.8) was supplied with $MgCl_2$ (40 μL, 0.5 M), aldolase (20 U; EC 4.1.3.3; Fluka; 21.1 U/mg), pyrophosphatase (5 U; EC 3.6.1.1; Fluka; 80 U/mg) and CMP-sialic acid synthetase (CSS; cloned from *Neisseria meningitidis* serogroup B, NmB[17] 5 μL, 1.21 mg/mL). The soln was incubated at 30 °C for 24 h and another portion of pyruvate (24.7 mg, 0.224 mmol), cytidine-5′-triphosphate (13.9 mg, 0.025 mmol), aldolase (1 mg; EC 4.1.3.3; Fluka; 21.1 U/mg), pyrophosphatase (5 U; EC 3.6.1.1; Fluka; 80 U/mg) and NmB CSS (5 μL, 1.21 mg/mL) was added. The reaction was continued at 30 °C for 24 h. The crude mixture was analyzed by LC–ESIMS.

HRESIMS: calcd for $C_{20}H_{29}FN_4O_{15}P$: 615.1351; found: 615.1348 $[M-H]^-$.

For complete ESIMS assignments, see Supplementary data.

3.12. Cytidine-5′-monophospho-5-acetamido-3,5,7-trideoxy-7-fluoro-D-*glycero*-D-*galacto*-2-nonulopyranonic acid (15) from 14

A soln of **14** (1.8 mg, 5.8 μmol), dithiothreitole (0.9 mg, 5.8 μmol) and cytidine-5′-triphosphate (4.2 mg, 7.5 μmol) in Tris–HCl buffer (0.5 mL; 0.1 M; pH 8.8) was supplied with $MgCl_2$ (20 μL, 0.5 M), pyrophosphatase (5 U; EC 3.6.1.1; Fluka; 80 U/mg) and NmB CSS[17] (5 μL, 1.21 mg/mL). The soln was incubated at 35 °C for 24 h. The crude mixture was analyzed by LC–ESIMS.

HRESIMS: calcd for $C_{20}H_{29}FN_4O_{15}P$: 615.1351; found: 615.1348 $[M-H]^-$.

For complete ESIMS see Supplementary data.

Acknowledgements

We thank Dipl.-Biochem. Melanie Oschlies for her expertise and for supplying samples of the CMP-sialic acid synthetases from mouse and rainbow trout. This work was supported by the Deutsche Forschungsgemeinschaft (Grants Ki 397/8-1 and Ki 397/9-1; GE 801/6-1) and the Fonds der Chemischen Industrie.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2008.02.003.

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