Multienzyme System for Synthesis of the Sialylated Thomsen–Friedenreich Antigen Determinant

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Dedicated to Professor K. Heyns on the occasion of his 90th birthday

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A combined sequential use of β-galactosidase from bovine testes together with α2–3-sialyltransferase from porcine liver including cofactor regeneration transforms GalNAcα1–OThr by a multistep one-pot reaction into the sialylated Thomsen–Friedenreich antigen determinant Neu5Acα2–3Galβ1–3GalNAcα1-OThr.

Sialic acid presents an important structural motif of many different glycoconjugates involved in biological processes such as cell-cell recognition, interaction and differentiation. The toxic release of *N*-acetylneuraminic acid from sialoglycoproteins of the erythrocyte membrane causes an exposure of the Thomsen−Friedenreich antigen (Galβ1−3-GalNAcα1−OThr) leading to a destruction of the red cell membrane followed by cell lysis.[1] For development of an efficient therapeutic concept against this medical defect the synthesis of α2−3-sialylated T-antigen derivatives is required. So far it was only possible to solve the complex problem associated with chemical sialylation in certain examples convincingly.[2] Due to the lack of a neighboring group at C-3 of *N*-acetylneuraminic acid it is difficult to influence the stereochemistry at the anomeric center except by solvents.[2] Further, the marked reverse anomeric effect of the carboxyl group leads to the stereoelectronically preferred β-configuration. Thus, this area is ideally suited for the application of stereo- and regioselective biocatalysts.

The activated donor (CMP-Neu5Ac) has been synthesized enzymatically from CTP and Neu5Ac by catalysis of CMP-Neu5Ac synthase.[3][4] An improved method for the preparative scale operates by the enzymatic formation of the costly CTP from cheap CMP by using adenylate kinase and pyruvate kinase. Adenylate kinase transfers one phosphate group from ATP to CMP leading to CDP, which is phosphorylated by pyruvate kinase to give CTP.[5]

In transferase-catalyzed reactions, the activated neuraminic acid serves as precursor for the introduction of the terminal Neu5Ac residue into various oligosaccharides. This approach is particularly attractive because the chemical synthesis with Neu5Ac glycosyl donors often leads to the formation of undesired anomeric mixtures. The sialyltransferase step was recently incorporated into the above-mentioned recycling pathway, leading to the formation of activated neuraminic acid, which is transferred in a joined reaction to give the sialylated compound.[6]

The use of mixed catalytic systems with several enzymes can provide multiple benefits in terms of costs, effectiveness of the particular production process and possible structures.

A novel one-pot multienzyme system with cofactor regeneration for the synthesis of a sialylated heterooligosaccharide (Neu5Acα2−3Galβ1−3GalNAc) was demonstrated previously by Kren et al.[7] The combined sequential use of a glycosidase together with a glycosyltransferase including cofactor regeneration transforms complicated multistep reactions into a one-pot reaction, thus avoiding laborious purification of intermediates.

The synthesis of the sialylated T-antigen derivative **4** was performed on a semipreparative scale following the one-pot multienzyme system approach including cofactor regeneration (Scheme 1). The starting material was the monosaccharide amino acid conjugate **2** as acceptor substrate which was obtained either by classical glycosylation or under enzymatic condensation conditions.[8] The first step of the reaction cycle shown in Scheme 1 was galactosylation of the α-amino acid *N*-acetylgalactosaminide **2**[8] with β-galactosidase from bovine testes and *p*-nitrophenyl β-galactopyranoside as donor, leading to a β1−3-linked disaccharide amino acid conjugate **3**. The reverse hydrolysis of this intermediate **3** was blocked by its immediate α2−3-sialyltransferase-mediated conversion into the trisaccharide **4**, which is no longer a substrate for the glycosidase.

Although the pH optimum of α2−3-sialyltransferase is 6.5,[9] the sialylation reaction was performed at pH = 7.5 to prevent a decomposition of the activated neuraminic acid at lower pH. This pH was still tolerable for CMP-Neu5Ac synthase (pH optimum 9.0[10]), all the enzymes regenerating and β-galactosidase from bovine testes (pH optimum 4.3[11]).[7]

A modified HPLC-supported assay[12] enabled the determination of the sialylated product and was used to monitor

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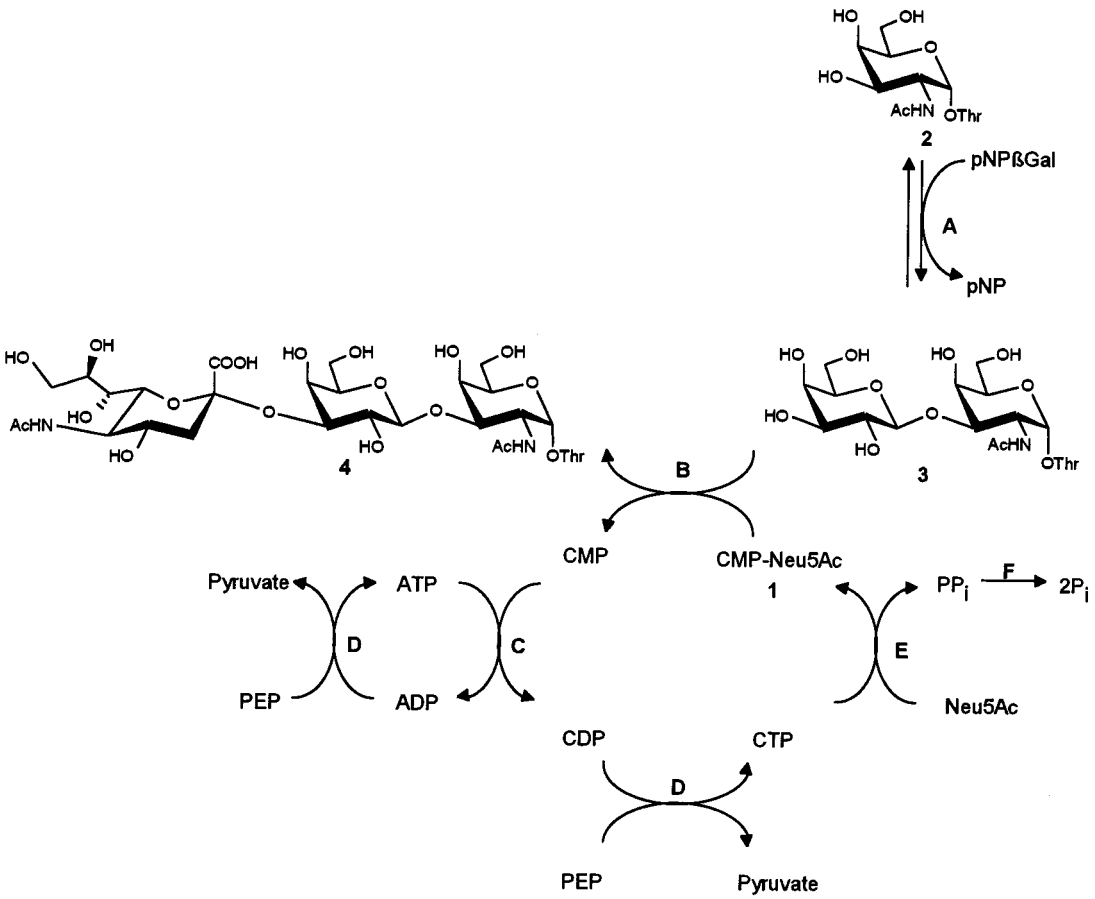
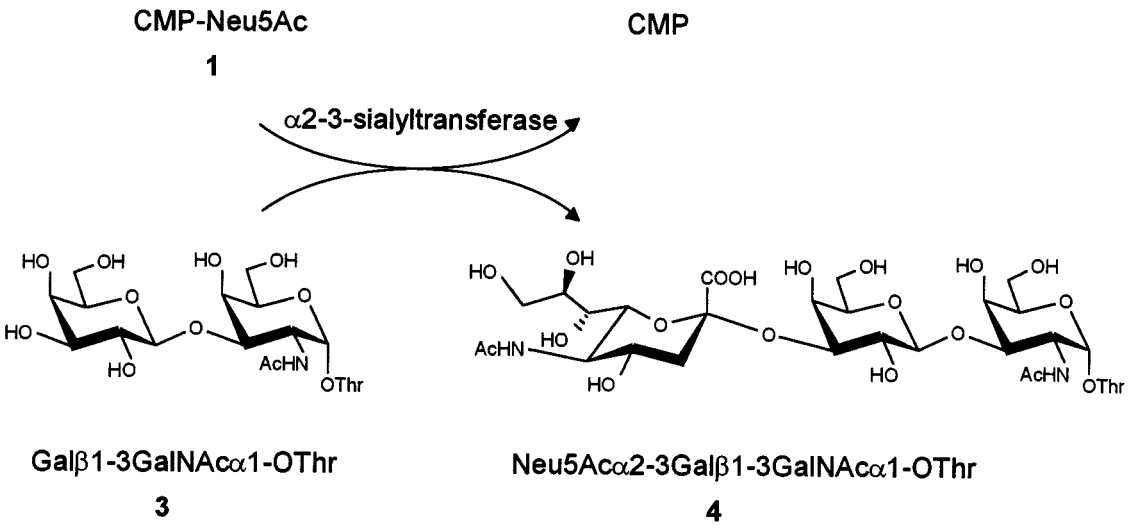


Scheme 1. Multienzyme system for the synthesis of the sialylated T-antigen determinant with integrated cofactor regeneration: **A**: β-galactosidase (bovine testes); **B**: α2−3-sialyltransferase (porcine liver); **C**: adenylate kinase; **D**: pyruvate kinase; **E**: CMP-Neu5Ac synthase (calf brain); **F**: inorganic pyrophosphorylase

the reaction. The starting material **2** was galactosylated and sialylated quantitatively after 48 h to give the sialylated T-antigen derivative **4** which could be eluted after 2.9 min using 96% phosphate buffer (pH = 5.2) and 4% acetonitrile on a reversed phase. The preparative-scale formation of the sialylated compound was done in a stepwise reaction cycle with isolation of the intermediates. These were detected with the above-described HPLC system as well as characterized by NMR spectroscopy and mass spectometry. The synthesis of the T-antigen determinant **3** described recently was based on the transgalactosylation of **2** by using β-galactosidase from bovine testes and *p*-nitrophenyl β-galactopyranoside as donor.[8] Subsequently, CMP-Neu5Ac (**1**) could be prepared enzymatically[13] and effectively purified with an HPLC system (LiChrosorb-NH_2). Then α2−3-sialyltransferase isolated from porcine liver[9] was used to introduce sialic acid to give the sialylated T-antigen determinant **4** (Scheme 2). The HPLC-assay system on a reversed phase was applied to analyze the reaction and served to confirm the identity of the sialylated product **4** synthesized either by the one-pot multienzyme system described above or by the multistep reaction cycle. The complete conversion of the starting material **3** could be detected after 72 h but the isolation of the sialylated compoud was affected by problems due to the dipolar amino acid aglycon. Finally, the optimal purification could be elaborated by using silica gel and an ethyl acetate/methanol/water mixture as eluent to give the product in 24% yield.

The structural analysis was performed through ^{1}H- und ^{13}C-NMR techniques. The glycosylated *N*-acetylneuraminic acid showed the characteristic low-field shift of the 3-Heq signal (δ = 2.72) and the high-field shift of the 3-Hax signal (δ = 1.74) relative to those of free Neu5Ac. The coupling constant $J_{1',2'}$ is 7.8 Hz, which is typical for a β-glycosidic linkage of galactose, whereas the α-glycosidic linkage of the neuraminic acid is due to the stereospecificity of the sialyltransferase and confirmed by the empirically known low-field shift of the 3-Heq signal. Comparison with previously published data of α2−3-sialylated T-antigen derivatives are in good accordance[7][9]. The 2−3-linkage of neuraminic acid to the T-antigen, as well as the 1−3-linkage of galactose onto GalNAc were confirmed by HMBC (heteronuclear multibond correlation) spectroscopy.

The one-pot reaction of the sialylated T-antigen derivative demonstrated another strategy for oligosaccharide synthesis based on a multienzyme system with regeneration of CMP-Neu5Ac in comparison to the traditional multistep reaction sequence.



Scheme 2. Sialylation of the T-antigen determinant

Experimental Section

General: Adenylate kinase (EC 2.7.4.3), pyruvate kinase (EC 2.7.1.40) and inorganic pyrophosphatase (EC 3.6.1.1) were from Sigma, Germany. β-Galactosidase from bovine testes (EC 3.2.1.23) was from Boehringer Mannheim, Germany. α2−3-Sialyltransferase (EC 2.4.99) was isolated from porcine liver[9] and CMP-Neu5Ac synthase (EC 2.7.7.43) from calf brain[3] as described previously.

Cytidine-5′-(5-acetamido-3,5-dideoxy-β-D-glycero-D-galacto-2-nonulopyranosonate Monophosphate)[13] (1): **N**-Acetylneuraminic acid (20 mg, 65 μmol) and CTP (31 mg, 65 μmol) were dissolved in 1.2 mL of Tris buffer [90% 1 M Tris buffer (pH = 9) and 10% 1 M $MgCl_2$ containing 3 mM 2-mercaptoethanol] and incubated with CMP-Neu5Ac synthase immobilized with BrCN-activated Sepharose (0.5 U) and inorganic pyrophosphatase (1 U) at 37°C for 36 h. The reaction was controlled by analytical HPLC on a LiChrosorb-NH_2 column (150 × 4.6 mm) with a linear gradient containing 40% acetonitrile und 10% 15 mM sodium phosphate buffer (pH = 5.2). The immobilized enzyme was centrifuged after 36 h. After lyophilization, the reaction mixture was separated by semi-preparative HPLC on a LiChrosorb-NH_2 column (250 × 16 mm) with 50% acetonitrile and 50% 15 mM sodium phosphate buffer (pH = 5.2) as eluent to obtain 67 mg (103 μmol, 67%) of a white sodium salt. − $[\alpha]_D$ = −36 (*c* = 1.0 in H_2O).[10] − ^{1}H NMR (400 MHz, D_2O):[10] δ = 7.95 (d, $J_{5'',6''}$ = 7.6 Hz, 1 H, 6′′-H), 6.11 (d, $J_{5'',6''}$ = 7.6 Hz, 1 H, 5′′-H), 5.98 (d, $J_{1',2'}$ = 4.1 Hz, 1 H, 1′-H), 4.35−4.22 (m, 5 H, 2′-H, 3′-H, 4′-H, 5a′-H, 5b′-H), 4.13 (d, $J_{5,6}$ = 10.2 Hz, 1 H, 6-H), 4.05 (ddd, $J_{3ax,4}$ = 12.9 Hz, $J_{3eq,4}$ = 5.1 Hz, $J_{4,5}$ = 10.4 Hz, 1 H, 4-H), 3.98−3.86 (m, 3 H, 5-H, 8-H, 9a-H), 3.62 (dd, $J_{8,9b}$ = 6.6 Hz, $J_{9a,9b}$ = 11.7 Hz, 1 H, 9b-H), 3.44 (dd, $J_{7,8}$ = 9.2 Hz, 1 H, 7-H), 2.48 (dd, $J_{3ax,3eq}$ = 12.9 Hz, $J_{3eq,4}$ = 5.1 Hz, 1 H, 3-Heq), 2.04 (s, 3 H, Ac), 1.64 (ddd, $J_{3ax,3eq}$ = 12.9 Hz, $J_{3ax,4}$ = 12.9 Hz, 1 H, 3-Hax).

HPLC-Supported Assay of the Sialylation Reaction: The eluent used was a linear gradient of 96% sodium phosphate (pH = 5.2) and 4% acetonitrile. The separation was carried out on a 5 μm, 124 × 4 mm RP-18 column and detection of compounds was monitored by UV. Samples from the enzymatic reaction were taken, desalted on a Biogel P2 column with water and passed through a filter prior to injection of an 20 μl aliquot.

***O*-{5-Acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosonate}-*O*-[2-acetamido-2-deoxy-3-*O*-(β-D-galactopyranosyl)-α-D-galactopyranosyl]-L-threonine (4):** Compound **3** (3 mg, 7.8 μmol) and CMP-Neu5Ac (7 mg, 0.011 mmol) were dissolved in 0.3 mL of 200 mM MES buffer (pH = 6.8) containing 500 mM NaCl, 200 mM $MnCl_2$, 10 mM DTE, 5 mM ATP and 1 mM Neu5Ac, diluted with 2.7 mL of water and the pH was adjusted to 7. The solution was incubated with α2−3-sialyltransferase from porcine liver (3−10 mU) at 37°C. The reaction was controlled applying the described HPLC assay. After 72 h, the solution was centrifuged and lyophylized. The separation was performed employing flash chromatography on silica gel (ethyl acetate/methanol/water, 7:4:3) to give 1.5 mg (1.9 μmol, 24%) of the sialylated compound. − $[\alpha]_D$ = +12 (*c* = 1.0 in H_2O). − ^{1}H NMR (500 MHz, D_2O): δ = 4.92 (d, $J_{1,2}$ = 3.7 Hz, 1 H, 1-H), 4.48 (d, $J_{1',2'}$ = 7.8 Hz, 1 H, 1′-H), 4.42 (dd, $J_{\beta CH,CH3}$ = 6.6 Hz, 1 H, ThrβCH), 4.25 (dd, $J_{1,2}$ = 3.7 Hz, $J_{2,3}$ = 11.3 Hz, 1 H, 2-H), 4.17 (d, $J_{3,4}$ = 2.5 Hz, 1 H, 4-H), 4.05−403 (m, 1 H, 5-H), 4.01 (dd, $J_{2,3}$ = 11.3 Hz, $J_{3,4}$ = 2.5 Hz, 1 H, 3-H), 3.88 (d, $J_{3',4'}$ = 3.1 Hz, 1 H, 4′-H), 3.62−3.60 (m, 1 H, 3′-H), 3.49 (dd, $J_{1',2'}$ = 7.8 Hz, $J_{2',3'}$ = 11.6 Hz, 1 H, 2′-H), 2.72 (dd, $J_{3''ax,3''eq}$ = 12.6 Hz, $J_{3''eq,4''}$ = 4.7 Hz, 1 H, 3-Heq), 1.99 (s, 3 H, CH_3CO), 1.98 (s, 3 H, CH_3CO), 1.74 (dd, $J_{3''ax,3''eq}$ = 12.6 Hz, $J_{3''ax,4''}$ = 12.6 Hz, 1 H, 3′′-Hax), 1.38 (d, 3 H, $ThrCH_3$). − ^{13}C NMR (500 MHz, D_2O): δ = 172.5 (C-1′′), 104.7 (C-1′), 100.0 (C-1, C-2′′), 77.3 (C-3), 74.8 (ThrβCH), 73.5 (C-3′), 69.4 (C-2′), 69.0 (C-4), 59.5 (ThrαCH), 48.8 (C-2), 40.8 (C-3′′), 21.3 ($COCH_3$), 19.3 ($ThrCH_3$). − ESI-MS; *m/z*: 774 $[M - H]^+$, 796 $[M + Na]^+$.

Multienzyme System for the Synthesis of Compound 4: Compound **2** (1 mg, 3.1 μmol), Neu5Ac (1 mg, 3.1 μmol), *p*-nitrophenyl β-D-galactopyranoside (1.4 mg, 4.6 μmol) and potassium phosphoenolpyruvate (1 mg, 3.5 μmol) were dissolved in 45 μl of 25 mM sodium cacodylate buffer (pH = 7.5) containing 10 mM $MgCl_2$, 5 mM $MnCl_2$, 20 mM KCl, 10 mM CMP, 0.05 mM CTP and 0.5 mM ATP and the pH was adjusted to 7.5. The solution was incubated with α2−3-sialyltransferase from porcine liver (1 mU), β-galactosidase from bovine testes (10 mU), CMP-Neu5Ac synthase immobilized with BrCN-activated Sepharose (5 mU), adenylate kinase (3 U), pyruvate kinase (5 U) and inorganic pyrophosphatase (22 mU). After 48 h the reaction mixture was analyzed by using the described HPLC-supported assay. The starting material was completely con-

verted to give the sialylated compound which was eluted after 2.9 min and which was identical to the product obtained with the multistep reaction method.

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