Supporting Information

Combination of UDP-Glc(NAc) 4'-Epimerase and Galactose Oxidase in a One-Pot Synthesis of Biotinylated Nucleotide Sugars


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Supplemental experimental section

Enzyme assay for the recombinant 4'-epimerases from S. cerevisiae and C. jejuni

A discontinuous assay was used to determine the activity of the conversion of UDP-Glc(NAc) to UDP-Gal(NAc). Diluted enzyme pools were mixed with 50 mM Tris-HCl, pH 8.7 and 1 mM UDP-hexoses to a total volume of 100 µl. The activity assay was incubated at 37 °C and samples were taken at various time points. The incubation was stopped by heating samples to 95 °C for 5 min. After centrifugation, samples were analyzed by capillary electrophoresis (CE) using a P/ACE MDQ apparatus from Beckman Coulter (Krefeld, Germany), equipped with a variable UV detector set to 254 nm. The conditions for the separation of NDP-sugars, NDP, NMP, and nucleosides by capillary zone electrophoresis were as follows: untreated fused-silica capillary (I.D. 75 µm, 60.2 cm total capillary length, 50 cm to the detector); separations were run at 25 kV (23 µA) at 25 °C using borate buffer (50 mM sodium tetraborate/64 mM borate), pH 9.0, samples were injected by pressure (5.0 s at 0.5 psi in the forward direction) and separated within 15 min total migration time. The concentration of the UDP-hexoses was determined by standard calibration curves using the Karat software (Beckmann-Coulter, USA).
Cloning of the gal10 gene from Saccharomyces cerevisiae

Chromosomal DNA from S. cerevisiae was isolated by the DNA isolation kit from Molzym, Germany. The purified DNA was chosen as template for amplifying gal10 (GenBank accession number X81324) by PCR with the two primers 5´ATGACAGCTCAGTTACAAA
GTGAAAGTACTTCT´3 and 5´TCAGGAAAATCTGTAGACAATCTTGGGCCGTAA`3. Site-specific PCR mutagenesis was used to create the restriction sites XhoI and BamHI (MBI Fermentas, Germany) for the insertion of the native gal10 gene into the expression vector. The isolated gal10 was chosen as template using the two primers 5’CCGCTCGAGCGGATGACAGCTCAGTTACAAA3’ and 5’CGGGATCCCGTCAGGAAATCTGTAGAC3’ for the amplification of gal10. The resulting fragment was digested by Xhol/BamHI and ligated with Xhol/BamHI digested pET14b vector to give expression vector pET14b-His6_GalX.

Production of UDP-Glc 4’-epimerase from S. cerevisiae

Transformants were grown in 100 ml Erlenmeyer flasks containing 20 ml LB medium with 100 µg/ml ampicillin. The cultures were incubated overnight at 37°C and 130 rpm. For protein production cells were grown in 5 L Erlenmeyer flasks with 1 L TB-medium with 100 µg/ml ampicillin and 0.1 mM IPTG at 25°C and 90 rpm. After 20 hours the cells were harvested by centrifugation and stored at –20°C.

Purification of the recombinant UDP-Glc 4’-epimerase from S. cerevisiae

A 40% (w/w) cell suspension in 50 mM HEPES-NaOH buffer pH 8.0 was disrupted by sonification and the supernatant collected after centrifugation used for the purification. A Ni²⁺-NTA column (Qiagen, Hilden, Germany) was used for purification directly from the crude extract. E. coli crude extract (30 ml) was loaded onto a column (1.6 cm x 10 cm, linear velocity 75 cm/h) which was previously equilibrated with 100 ml 50 mM HEPES-NaOH, pH 8.0 (buffer A), containing 0.3 M NaCl and 10 mM imidazole. After a washing step with 100 ml of buffer A additionally containing 20 mM imidazole the elution was done with buffer A containing 250 mM imidazole. The eluted fractions were assayed for UDP-Glucose-4’-epimerase activity as described above. The epimerase from C. jejuni was purified as described elsewhere.[18]

Influence of mercaptoethanol on the activity of recombinant UDP-Glc(NAc) 4’-epimerases from S. cerevisiae and C. jejuni

The enzyme pool of the C. jejuni enzyme contained 5 mM β-mercaptoethanol and was dialyzed against a buffer containing 20 mM MOPS pH 6.5, 200 mM NaCl, 1 mM EDTA. The buffer for
UDP-Glc 4’-epimerase from yeast was 50 mM HEPES-NaOH, pH 8.0 (buffer A), containing 0.3 M NaCl and 250 mM imidazole. Both enzymes were incubated at 30 °C without and with the addition of 5 mM β-mercaptoethanol. Samples were taken at various time-points and enzyme activities were analyzed by CE as described above.

**Influence of oxidative conditions on the activity of recombinant UDP-Glc(NAc) 4’-epimerases from C. jejuni**

The purified epimerase was treated as described above for the preparation of an enzyme preparation free from β-mercaptoethanol. The reactor for bubble-free aeration\(^{12}\) was filled with buffer (45 ml, 20 mM MOPS pH 6.5, 200 mM NaCl, 1 mM EDTA). Equilibration of the buffer solution with oxygen was achieved by pumping the solution through silicon tubes in a oxygen filled flask (0.1 mbar) at 30 °C for 10 min. Enzyme solution (900 U) was then added for a final volume of 50 ml, and samples were taken at various time-points for analysis. For control measurements, the same experiment was conducted without oxygen supply. Enzyme activities were analyzed via CE as described above.

**HPLC and CE analysis of UDP-Gal(NAc) derivatives**

Nucleosides, nucleoside mono- and diphosphates, nucleotide sugars and biotinylated nucleotide sugars were analyzed by reversed-phase HPLC (Hypersil ODS column (5 µm), Thermo Electron, USA) with a methanol concentration of 1% in bidist. H\(_2\)O at pH 7.0. HPLC was used to monitor the formation of biotinylated nucleotide sugars (Figure 1). The isolated products were also analyzed by CE as described above.

**CE analysis of UDP-6-biotinyl-Gal (7)/UDP-6-biotinyl-Glc (9)**

The peaks at 8.408 min and 8.600 min depict 9 and 7, respectively, in a ratio of 1.487:1.
Testing the epimerization of UDP-6-biotinyl-Gal (7) by the recombinant UDP-Glc(NAc) 4’-epimerase from *C. jejuni*

The epimerization of the pure compound UDP-6-biotinyl-Gal (7) and formation of 9 by Gne from *C. jejuni* was tested by incubation of 1 mM 7 with a diluted enzyme pool in sodium phosphate buffer (50 mM, pH 6.0, 100 µl total volume) at 37 °C. Samples were taken at various time points up to 50 h. After centrifugation, samples were analyzed by CE as described above, but at 15 kV and 15 °C whereby peaks are shifted to higher migration times.

CE analysis of the synthesized mixture of UDP-6-biotinyl-Gal (7)/UDP-6-biotinyl-Glc (9)
The peaks at 12.033 min and 12.158 min depict compound 9 and 7, respectively.

CE analysis of the pure compound UDP-6-biotinyl-Gal (7) [12]

CE analysis of UDP-6-biotinyl-Gal (7) after 50h incubation with Gne from C. jejuni.
The formation of 9 from 7 was not observed. We conclude that synthesis of the mixture of 7 and 9 (see above) is due the epimerization of UDP-6-aldo-Gal 5.
ESI-MS analysis of UDP-Gal(NAc) derivatives

All spectra were recorded on an LCT spectrometer (Mircomass) equipped with a lockspray dual-electrospray ion source using the following conditions: capillary voltage = 2700V, rf-filter = 150 V, source temperature = 120°C, solvent = water, solvent flow = 0.2 mL/min, desolvation gas = 460 L/h, reference ions = phosphoric acid. After recording the data, all spectra were processed according the manufacturers guidelines using the nearest phosphoric acid peak as reference. NS spectra are depicted below.
NMR-Analysis of UDP-6-biotinyl-GalNAc (8)

The signals were identified using standard 1D (\(^1\)H, \(^13\)C, DEPT90 and DEPT135) and 2D (H-H-COSY, HMBC and HMQC) experiments. The \(^13\)C-signals were indexed using the DEPT135 spectra as “+” (up, CH, CH\(_3\)), “−” (down, CH\(_2\)) and “o” (missing, quaternary C) The signals for the aliphatic biotin protons could not be clearly distinguished.


\(^{13}\)C-NMR (100 MHz, D\(_2\)O) \(\delta\): 176.94 (o, C\(_B\)-7), 175.55 (o, C\(_B\)-1), 175.29 (o, C\(_A\)), 166.53 (o, C\(_U\)-4), 165.67 (o, C\(_B\)-16), 152.12(o, C\(_U\)-2), 142.04 (+, C\(_U\)-6), 102.99 (+, C\(_U\)-5), 94.96 (+, d, \(J_{C-P}= 6.3\) Hz, C\(_A\)-1), 88.89 (+, C\(_R\)-1), 83.55 (+, d, \(J_{C-P}= 9.0\) Hz, C\(_R\)-4), 74.15 (+, C\(_R\)-2), 70.03 (+, C\(_R\)-3), 69.61 (+, C\(_A\)), 69.35 (+, C\(_A\)-5), 68.18 (+, C\(_A\)-3), 65.36 (-, d, \(J_{C-P}= 5.2\) Hz, C\(_R\)-5), 62.44 (+, C\(_B\)-13), 60.61 (+, C\(_B\)-14), 55.75 (+, C\(_B\)-12), 51.48 (-, C\(_A\)-6), 50.06 (+, d, \(J_{C-P}= 8.2\) Hz, C\(_A\)-2), 40.09 (-, C\(_B\)-15), 39.50 (-, C\(_B\)-6), 35.88 (-, C\(_B\)-8), 33.99 (-, C\(_B\)-2), 28.36, 28.24, 28.04, 25.95, 25.58, 25.13 (6\(^*\) -, C\(_B\)-3, C\(_B\)-4, C\(_B\)-5, C\(_B\)-9, C\(_B\)-10, C\(_B\)-12), 22.52 (+, C\(_A\)) ppm.
$^1$H-NMR (400 MHz) of UDP-6-biotinyl-GalNAc (8)

$^{13}$C-NMR (100 MHz) of UDP-6-biotinyl-GalNAc (8)
HMOC of UDP-6-biotinyl-GalNAc (8)
ESI-Tof of UDP-6-biotinyl-GalNAc (8)
NMR-Analysis of UDP-6-biotinyl-Gal (7)

The signals were identified using standard 1D (\(^1\)H, \(^{13}\)C, DEPT90 and DEPT135) and 2D (H-H-COSY, HMBC and HMQC) experiments. The \(^{13}\)C-signals were indexed using the DEPT135 spectra as “+” (up, CH, CH\(_3\), “-“ (down, CH\(_2\)) and “o” (missing, quaternary C) The signals for the aliphatic biotin protons could not be clearly distinguished.


\(^{13}\)C-NMR (100 MHz, D\(_2\)O) \(^\delta\): 176.98 (o, C\(_{B-7}\)), 175.57 (o, C\(_{B-1}\)), 166.62 (o, C\(_{U-4}\)), 165.71 (o, C\(_{B-16}\)), 152.21 (o, C\(_{U-2}\)), 142.02 (+, C\(_{U-6}\)), 103.04 (+, C\(_{U-5}\)), 96.05 (+, d, \(J_{C-P} = 6.3\) Hz, C\(_{A-1}\)), 88.82 (+, C\(_{R-1}\)), 83.59 (+, d, \(J_{C-P} = 9.0\) Hz, C\(_{R-4}\)), 74.17 (+, C\(_{R-2}\)), 70.36 (+, C\(_{A-4}\)), 70.03 (+, C\(_{R-3}\)), 69.76 (+, C\(_{A-3}\)), 69.26 (+, C\(_{A-5}\)), 68.69 (+, d, \(J_{C-P} = 9.0\) Hz, C\(_{A-2}\)), 65.36 (-, d, \(J_{C-P} = 5.2\) Hz, C\(_{R-5}\)), 62.45 (+, C\(_{B-13}\)), 60.63 (+, C\(_{B-14}\)), 55.76 (+, C\(_{B-12}\)), 54.13 (-, C\(_{A-6}\)), 40.10 (-, C\(_{B-15}\)), 39.52 (-, C\(_{B-6}\)), 35.90 (-, C\(_{B-8}\)), 34.00 (-, C\(_{B-2}\)), 28.36, 28.24, 28.04, 25.95, 25.59, 25.14 (6* -, C\(_{B-3}\), C\(_{B-4}\), C\(_{B-5}\), C\(_{B-9}\), C\(_{B-10}\), C\(_{B-12}\)) ppm.
$^1$H-NMR (400 MHz) of UDP-6-biotinyl-Gal (7)

$^{13}$C-NMR (100 MHz) of UDP-6-biotinyl-Gal (7)
HMQC of UDP-6-biotinyl-Gal (7)
NMR-Analysis of UDP-6-biotinyl-Glc (9)

Due to the fact that UDP-6-biotinyl-Glc (9) was formed as a mixture together with UDP-6-biotinyl-Gal (7) complete NMR-analysis was not possible. The identity of 9 was proven after subtracting of the $^1$H and $^{13}$C NMR spectra of 7 from the mixture of 7 and 9. The graphical data are shown below. All hexose signals of 9 are above and of 7 are below the baseline. UDP and biotin signals are not visible in the subtraction.

The following relevant data could be obtained proving the gluco-configured hexose.

$^1$H-NMR (400 MHz, D$_2$O) $\delta$: 5.14 (dd, $J= 6.9$ and $3.4$ Hz, 1H, H-1), 3.87 (ddd, $J= 9.8$, 8.4 and 2.0 Hz, 1H, H-5), 3.64 (t, $J= 9.8$ Hz, 1H, 3-H), 3.44 (dt, $J= 9.8$ and 3.4 Hz, 1H, 2-H), 3.21 (t, $J= 9.8$ Hz, 1H, 4-H) ppm.

$^{13}$C-NMR (100 MHz, D$_2$O) $\delta$: 95.5 (+, d, $J_{C,P}$= 6.3 Hz, C$_{A-1}$), 72.96 (+, C$_{A-3}$), 71.77 (+, d, $J_{C,P}$= 9.0 Hz, C$_{A-2}$), 71.49 (+, C$_{A-4}$), 70.49 (+, C$_{A-5}$), 51.67 (-, C$_{A-6}$) ppm.

In addition to the NMR results ESI-MS analysis of the mixture of 7 and 9 revealed just one species with the molecular composition C$_{31}$H$_{51}$N$_7$O$_{19}$P$_2$S (found: 918.2355; calculated for M-H$^+$:918.2357).
$^1$H-NMR (400 MHz) of UDP-6-biotinyl-Gal/ UDP-6-biotinyl-Glc
Overlay of $^{13}$C-NMR spectra of 7/9 and 7

![Dual Display Plot]

- Main Trace: c:\nmr-fid\fid\gdclugbc\010001.1R
- Second Trace: C:\NMR-FID\FID\GDCLUGLC\010001.1R

- x-Increment: 0.18 ppm
- y-Increment: 0.00
- y-Factor: 0.15
Overlay of $^1$H-NMR spectra of 7/9 and 7
H-H-COSY of UDP-6-biotinyl-Gal/UDP-6-biotinyl-Glc
ESI-Tof of UDP-6-biotinyl-Gal/UDP-6-biotinyl-Glc