A Simple Synthesis of Sugar Nucleoside Diphosphates by Chemical Coupling in Water**

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anie_201205433_sm_miscellaneous_information.pdf
1. General Information ........................................................................................................................................3
2. Activation of UMP in D2O with ImIm monitored by 31P-NMR ..................................................5
3. Activation of UMP in H2O with ImIm monitored by 1H-NMR ......................................................7
4. Enzymatic reaction with UDP-Gal using galactosyltransferases ...........................................8
   a) Preparation of synthetic UDP-Gal crude mixture
   b) Enzymatic reaction with galactosyltransferases
   c) Inhibition assay of UMP and UMP-dimer
   d) Milligram scale synthesis of LacNAc-TMR using synthetic UDP-Gal and β-1,4-GalT
5. Enzymatic reaction with ADP-Glc using barley starch synthase I, followed by
   fluorescent labeling ......................................................................................................................................11
   a) Preparation of synthetic ADP-Glc crude mixture
   b) Enzymatic reaction with ADP-Glc using barley starch synthase I
   c) Fluorescent labeling and UPLC-MS analysis
6. NMR and MS spectra ..................................................................................................................................14
   a) 1H and 31P{1H}-NMR and MS spectra of UDP-Glc ammonium salt
   b) 1H and 31P{1H}-NMR and MS spectra of UDP-Gal ammonium salt
   c) 1H and 31P{1H}-NMR and MS spectra of ADP-Glc ammonium salt
   d) 1H and 31P{1H}-NMR and MS spectra of GDP-Man ammonium salt
   e) 31P-NMR spectrum of UDP-Gal crude mixture
   f) 31P-NMR spectrum of ADP-Glc crude mixture
   g) 1H-NMR spectrum of LacNAc-TMR
   h) 1H-NMR and MS spectra of ImIm prepared by reaction of DMC with 2.0 eq imidazole
   i) 1H and 31P-NMR spectra of reaction mixture of UMP with 2.0 eq DMC after 24 h at 37
      °C
7. References ...............................................................................................................................................24/
1. General information

ESI MS spectra were recorded on a Bruker Esquire 3000-Plus Ion Trap spectrometer with samples injected as solutions in acetonitrile/water (1/1). MALDI-TOF MS spectra were recorded on a Bruker Microflex spectrometer using 2,5-dihydroxybenzoic acid [10 mg dissolved in 1.0 mL of 50/50 (v/v) acetonitrile/water with 0.1% TFA] as a matrix. \(^1\)H and \(^31\)P-NMR spectra were recorded on a Bruker Advance DRX-400 spectrometer using the residue proton resonance of the solvent as the internal standard and using 85% phosphoric acid as an external standard. Proton decoupled \(^31\)P-NMR spectra were recorded using ethlenediaminetetraacetic acid as a chelating reagent. UV/Vis spectra were recorded using a Thermo Scientific NanoDrop 2000 spectrophotometer.

CE analysis was performed on a PrinCE 560 capillary electrophoresis system with a uncoated fused-silica capillary (75 μm i.d. with a length of 60 cm). The running buffer consisted of borate (50 mM, pH 9.3) and sodium dodecyl sulfate (150 mM). TMR-labeled compounds were analyzed at 25 kV and quantitated using an Argos 250B fluorescence detector (Flux Instruments: Basel, Switzerland), equipped with an excitation filter of 546.1/10 nm and an emission filter of 570 nm. The data were processed by PrinCE 7.0 software (Prince Technologies: Emmen, Netherlands).

UPLC-MS analysis was performed on a Waters Acquity UPLC-MS system with fluorescence detection (\(\lambda_{ex} = 320\) nm; \(\lambda_{em} = 420\) nm) using a Waters Acquity UPLC GST Amide Column (1.7 μm, 2.1×150 mm). Buffer A (10 mM ammonium formate, pH 4.5) and B (acetonitrile) were used. Mobile phase flow rate was set at 0.2 mL/min, with elution starting at 22% buffer A and 78% buffer B for 5 min, followed by a linear gradient to 55% buffer B in 38 min. The eluted peaks were identified using an online mass-spectrometer, Waters Micromass Quattro Premier Tandem quadrupole mass spectrometer. The instrument was operated using an electrospray source in positive mode. The ionization source conditions were as follows: capillary voltage of 3.5 kV, source block temperature of 120 °C and solvent evaporation temperature of 250 °C. The cone
voltage was 50 V. The data acquisition and processing were performed using MassLynx V4.1 software (Micromass: Manchester, UK).

2-Chloro-1,3-dimethylimidazolinium chloride (DMC), imidazole, α-d-galactose 1-phosphate (Gal-1-P) dipotassium salt pentahydrate, guanosine 5’-monophosphate (GMP) disodium salt hydrate (22% loss on drying) and ADP-Glc disodium salt were purchased from Sigma-Aldrich (St. Louis, MO). α-d-Glucose 1-phosphate (Glc-1-P) dipotassium salt hydrate (12% water content), α-d-mannose 1-phosphate (Man-1-P) dippotassium salt (0.02% loss on drying), uridine 5’-monophosphate (UMP) disodium salt (22% water content) and adenosine 5’-monophosphate (AMP) disodium salt (21% loss on drying) were purchased from Carbosynth (Compton, UK). UDP-Gal disodium salt was purchased from Merck Millpore (Darmstadt, Germany). Deuterium oxide (D, 99.9%) was purchased from Cambridge Isotope Laboratories (Andover, MA). Calf intestinal alkaline phosphatase (AP, 20 U/μL) was purchased from Life Technologies (Carlsbad, CA). Bovine β-1,4-galactosyltransferase (β-1,4-GalT)\textsuperscript{1} or human blood group B galactosyltransferase (GTB)\textsuperscript{2} were expressed as described previously. Barley starch synthase 1 was cloned and expressed in E. coli using standard cloning techniques. Its preparation will be reported elsewhere.
2. Activation of UMP in D$_2$O with ImIm monitored by $^{31}$P-NMR

DMC (0.50 mmol), imidazole (1.0 mmol) and UMP disodium salt (0.25 mmol) were placed in an eppendorf tube with a stir bar. D$_2$O (250 $\mu$L) was added. At various time points (15 min, 30 min, 1 h, 2 h, 4 h, 8 h and 16 h) with stirring at 37 °C, 30 $\mu$L of the reaction mixture was taken and mixed with 570 $\mu$L of D$_2$O. The progress of UMP activation was monitored by $^{31}$P-NMR as shown in Figure I. ESI-MS analysis of the reaction mixture was carried out after activation for 1 h (Figure II).

**Figure I.** Monitoring of UMP activation in D$_2$O by $^{31}$P-NMR. 4: UMP, 5: UMP-Im, 7: UMP-dimer
Figure II. ESI-MS spectrum of the reaction mixture of UMP (4) with DMC (1) and imidazole (2) after 1 h.
3. Activation of UMP in H₂O with ImIm monitored by ¹H-NMR

DMC (0.50 mmol), imidazole (1.0 mmol) and UMP disodium salt (0.25 mmol) were placed in an eppendorf tube with a stir bar. H₂O (250 µL) was added. At various time points (15 min, 30 min, 1 h, 1.5 h, 2 h, 4 h and 6 h) with stirring at 37 °C, 30 µL of the reaction mixture was taken and mixed with 570 µL of D₂O. The progress of UMP activation was monitored by ¹H-NMR as shown in Figure III. After the reaction, UMP-dimer (7) was isolated by ion exchange chromatography on DEAE Sephadel.

**Figure III.** Monitoring of UMP activation in H₂O by ¹H-NMR. 3: ImIm, 4: UMP, 5: UMP-Im, 7: UMP-dimer
4. Enzymatic reaction with UDP-Gal using galactosyltransferases

a) Preparation of synthetic UDP-Gal crude mixture

DMC (9.0 mg, 53 μmol), imidazole (7.3 mg, 107 μmol) and UMP disodium salt (13.3 mg, 28 μmol) were placed in an eppendorf tube with a stir bar. D2O (13 μL) was added. The mixture was stirred at 37 °C for 1 h, followed by addition of Gal-1-P dipotassium salt (3.0 mg, 7.0 μmol). After further stirring at 37 °C for 18 h, the reaction mixture was diluted with D2O (585 μL) and subjected to ^1H and ^31P NMR experiments to identify and quantify components. After the NMR measurement, the mixture was diluted with 700 μL of water and directly used for subsequent enzymatic reactions. The final resultant solution (1.3 mL) contained UDP-Gal (3.2 mM), GalP (1.6 mM), galactose 1,2-cyclic phosphate (0.6 mM), UMP (5.5 mM), UMP dimer (6.5 mM), 1,3-dimethyl-2-imidazolidinone (41 mM), imidazole-HCl (82 mM).

b) Enzymatic reaction with galactosyltransferases

The incubation mixture (pH 7.0) consisted of a total volume (12 μL); acceptor (150 μM, GlcNAc-TMR or FucGal-TMR), MOPS (50 mM), MnCl2 (20 mM), bovine serum albumin (1.0 mg/mL), galactosyltransferase (β-1,4-GalT: 0.033 μg or GTB: 0.035 μg), and the above mixture containing synthetic or commercial UDP-Gal (150 μM). At various time points (10, 20, 40, 60, 80, 100 min and 12 h) during incubation at 37 °C, 1.0 μL of the reaction mixture was taken and mixed with 300 μL of cold water. Product formations were monitored by MALDI-TOF-MS and CE.
Figure IV. Analysis of enzymatic reaction using crude synthetic UDP-Gal. The galactosyltransferases used were A) β-1,4-galactosyltransferase (bovine) and B) GTB (human). After 12 h incubation, complete product formation was confirmed by a) MALDI-TOF MS and b) capillary electrophoresis. c) represents the comparison of initial reaction rates with crude synthetic UDP-Gal (red line) and commercial UDP-Gal (blue).

c) Inhibition assay of UMP and UMP-dimer

Inhibitory activities of UMP and UMP-dimer for β-1,4-GalT and GTB were measured using a Sep-Pak assay as previously reported. Table I summarizes the inhibition constants (Ki) and concentrations of UMP and UMP-dimer used for the assay.
Table I. Inhibitory activity of UMP and UMP-dimer against β-1,4-GalT and GTB

<table>
<thead>
<tr>
<th></th>
<th>$K_m$ [μM]</th>
<th>$K_i$ [μM]</th>
<th>$K_i$ [μM]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UDP-Gal</td>
<td>UMP</td>
<td>UMP-dimer</td>
</tr>
<tr>
<td>β-1,4-GalT B. taurus</td>
<td>22</td>
<td>149.3</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(80, 160, 240, 320, 400)</td>
<td>(25, 50, 75, 100, 125)</td>
</tr>
<tr>
<td>GTB H. Sapiens</td>
<td>27</td>
<td>9.9</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(15, 30, 45, 60, 75)</td>
<td>(25, 50, 75, 100, 125)</td>
</tr>
</tbody>
</table>

* The concentrations (μM) of UMP and UMP-dimer used in Dixon plots for Ki determinations are in parentheses.

d) Milligram scale synthesis of LacNAc-TMR using synthetic UDP-Gal and β-1,4-GalT

The incubation mixture (pH 7.0) consisted of a total volume (1.0 mL); GlcNAc-TMR (1.7 mg, 2.0 μmol), the above synthetic UDP-Gal (2.6 μmol), MOPS (50 mM), MnCl$_2$ (20 mM), bovine serum albumin (1.0 mg/mL), β-1,4-GalT (19.5 μg). After incubation at 37°C for 6 h, β-1,4-GalT (9.8 μg in 15 μL) and the mixture containing synthetic UDP-Gal (1.5 μmol in 390 μL) were added. After a further additional incubation for 12 h, the reaction mixture was loaded on a C18 Sep-pak cartridges with water (10 mL) and the cartridges were flushed with water (100 mL). LacNAc-TMR was eluted with MeOH (30 mL) giving 1.8 mg (1.8 μmol, 90%). $^1$H-NMR (400 MHz, CD$_3$OD): δ8.52, 8.05, 7.36, 7.26, 7.02 and 7.94 (9H, TMR), 4.39 (d, 1H, $J_{1,2} = 8.4$ Hz, H-1$^{GlcNAc}$), 4.38 (d, 1H, $J=7.5$ Hz, H-1$^{Gal}$), 3.91-3.29 (m, 26H), 2.86-2.83 (m, 1H), 2.52-2.46 (m, 2H), 2.25-2.20 (m, 2H), 2.03-1.95 (m, 1H), 1.96 (s, 3H, -C(=O)CH$_3^{GlcNAc}$), 1.61-1.57 (m, 2H), 1.57-1.48 (m, 2H), 1.40-1.31 (br, 8H); m/z (MALDI-TOF): found [M+H]$^+$ 994.77, C$_{50}$H$_{68}$N$_5$O$_{16}$ calcd for [M+H]$^+$ 994.47.
5. Enzymatic reaction with ADP-Glc using barley starch synthase I, followed by fluorescent labeling

a) Preparation of synthetic ADP-Glc crude mixture

DMC (24.0 mg, 142 μmol), imidazole (19.2 mg, 282 μmol) and AMP disodium salt (35.7 mg, 72 μmol) were placed in an eppendorf tube with a stir bar. D2O (35 μL) was added. The mixture was stirred at 37 °C for 1 h, followed by addition of Glc-1-P dipotassium salt (6.7 mg, 18 μmol). After further stirring at 37 °C for 18 h, the reaction mixture was diluted with D2O (565 μL) and subjected to 1H and 31P-NMR experiments to identify and quantify components. After the NMR measurement, the mixture was diluted with 2.9 mL of water and directly used for subsequent enzymatic reaction. The resultant solution contained ADP-Glc (2.1 mM), Glc-1-P (2.1 mM), glucose 1,2-cyclic phosphate (1.0 mM), AMP (6.1 mM), AMP-dimer (7.0 mM), 1,3-dimethyl-2-imidazolidinone (41 mM), imidazole-HCl (81 mM).

b) Enzymatic reaction with ADP-Glc using barley starch synthase I

The incubation mixture (pH 8.0) consisted of a total volume (25 μL); maltopentaose (2.0 mM), bicine (22 mM), DTT (2.0 mM), barley starch synthase I (9.2 μg), and the above crude synthetic ADP-Glc (2.0 mM) or commercial ADP-Glc (2.0 mM). After incubation at 37 °C for 12 h, the reaction mixtures were subjected to a reducing end specific fluorescent labeling method to quantify the products.
c) Fluorescent labeling and UPLC-MS analysis²

A portion of each reaction mixture (10 µL, total 5 nmol of sugar) was added to a GlycanTip.² After the capture and labeling steps, labeled sugars were cleaved from the tips using a solution of aq. ammonia and hydroxylamine. The filtrates were concentrated on a Speed-Vac and re-dissolved in 100µL of 50% aq. MeOH and 5 µL of each solution were used for analysis by UPLC-MS.
Figure V. UPLC chromatograms of fluorescent labeled maltopentaose before (A) and after enzymatic reaction with barley starch synthase I using synthetic ADP-Glc (B) or commercial ADP-Glc (C).
6. NMR and MS spectra

a-1) $^1$H-NMR spectrum of UDP-Glc ammonium salt

![H-NMR spectrum of UDP-Glc ammonium salt](image1)

a-2) $^{31}$P{$^1$H}-NMR spectrum of UDP-Glc ammonium salt

![P-$^1$H-NMR spectrum of UDP-Glc ammonium salt](image2)

$J_{P,P} = 20.7$ Hz
a-3) ESI MS spectrum of UDP-Glc ammonium salt

b-1) $^1$H-NMR spectrum of UDP-Gal ammonium salt
b-2) $^{31}\text{P}^{1\text{H}}$-NMR spectrum of UDP-Gal ammonium salt

\[ J_{P,P} = 20.7 \text{ Hz} \]

b-3) ESI-MS spectrum of UDP-Gal ammonium salt
c-1) $^1$H-NMR spectrum of ADP-Glc ammonium salt

![ADP-Glc spectrum](image1)

c-2) $^{31}$P{$^1$H}-NMR spectrum of ADP-Glc ammonium salt

![ADP-Glc spectrum](image2)

$J_{PP} = 20.7$ Hz
c-3) ESI-MS spectrum of ADP-Glc ammonium salt

d-1) $^1$H-NMR spectrum of GDP-Man ammonium salt
d-2) $^{31}\text{P}^{\{1\text{H}\}}$-NMR spectrum of GDP-Man ammonium salt

$d-3$) ESI-MS spectrum of GDP-Man ammonium salt
e) $^{31}$P-NMR spectrum of UDP-Gal crude mixture

f) $^{31}$P-NMR spectrum of ADP-Glc crude mixture
g) $^1$H-NMR spectrum of LacNAc TMR

![LacNAc-TMR 1H-NMR spectrum]

h-1) $^1$H-NMR spectrum of ImIm prepared by reaction of DMC with 2.0 eq imidazole

![ImIm (3): Reaction of DMC (1) with 2.0 eq imidazole (2)]
h-2) ESI-MS spectrum of ImIm prepared by reaction of DMC with 2.0 eq imidazole

i-1) $^1$H-NMR spectrum of reaction mixture of UMP with 2.0 eq DMC after 24 h at 37 °C
i-2) $^1$H-NMR spectrum of reaction mixture of UMP with 2.0 eq DMC after 24 h at 37 °C
(Expansion)

![H-NMR spectrum of reaction mixture of UMP with 2.0 eq DMC after 24 h at 37 °C](image)

i-3) $^{31}$P-NMR spectrum of reaction mixture of UMP with 2.0 eq DMC after 24 h at 37 °C

![P-NMR spectrum of reaction mixture of UMP with 2.0 eq DMC after 24 h at 37 °C](image)
7. References


2. A modification of a reported solid-phase oligosaccharide labeling protocol was used. Briefly, 200μL pipet tips containing a fixed 2 mg plug of monolithic silica were purchased from GL Sciences (Tokyo, Japan) and aminated under standard conditions using triethoxy aminopropyl silane. The functionalized tips were then chemically modified to provide a hydroxylamine-based silica with the chemical structure shown in 4c, that we refer to as a “GlycanTip”. Capture of reducing oligosaccharides, blocking of unreacted hydroxylamines, reduction, reductive amination and basic cleavage used procedures similar to those described in A. Lohse, R. Martins, M. R. Jørgensen, O. Hindsgaul. *Angew. Chem. Int. Ed.* **2006**, 45, 4167-4172.