Flexible enzymatic and chemo-enzymatic approaches to a broad range of uridine diphospho-sugars

James C. Errey, Balaram Mukhopadhyay, K. P. Ravindranathan Kartha and Robert A. Field

Centre for Carbohydrate Chemistry, School of Chemical Sciences and Pharmacy, University of East Anglia, Norwich, UK NR4 7TJ

Fax: +44-1603-592003; Email: r.a.field@uea.ac.uk

Bacterial strains and plasmids.
E. coli K-12, sub-strain MG1655 (ATCC# 47076) was purchased from ATCC. Plasmid vector pET15b and E. coli competent Novablue and Origami cells were from Novagen Inc.

Cloning, over-expression and purification of individual enzymes.
DNA manipulations were performed essentially as described by Wang and co-workers. The galK gene and galPUT gene were cloned from E. coli K-12 and inserted into Nde1 and BamH1 restriction sites of the pET15b vector. The resulting plasmids pET15b-galK and pET15b-galPUT, respectively, were transformed into E. coli cloning strain Novablue and expression strain BL21 (DE3) Origami. Selected clones were characterized by restriction mapping. The expression and purification of individual enzymes from cell lysates were as described previously.

Enzymatic activity assay for galactokinase.
This radiochemical assay measures the phosphorylation of [6-3H]-galactose; the phosphorylated product is separated from the assay mixture with the aid of an anion exchange resin. The reaction mixture (50µl) contains 10µl each of: galactose (1mM, containing 100Bq [3H]-galactose - Amersham); assay buffer (50mM triethanolamine pH 7.0); ATP (1mM); co-factors (1mM DTT, 5mM MgCl2); protein sample. Molarities quoted are the final concentrations in the assay. The reaction mixture was incubated for 20 minutes at 37°C, diluted with Milli Q water (1ml) and applied to a column containing 0.5ml of QAE Q-25-120 Sephadex anion exchange resin. The columns were washed with Milli Q water (2ml) and the flow through was collected. The columns were then eluted with 1M NH4OAc (2ml) and the flow through was collected. Scintillation fluid (8ml) (Fisher High safe 8) was added to the samples collected, and the mixture was counted for 2 minutes using a liquid scintillation counter.

Enzymatic activity assay for galactose-1-phosphate uridyltransferase.
Galactose-1-phosphate uridyltransferase was diluted in assay buffer (50mM HEPES, 10mM
MgCl\(_2\) and 10mM KCl adjusted to pH 7.4) to give a stock solution of 3.33mg/ml. A stock solution of glucose-1-phosphate (2mM) and uridine triphosphate (2mM) was prepared by dissolving the substrate in assay buffer. The substrate was subsequently diluted in assay buffer to give a substrate concentration of 50\(\mu\)M upon the enzyme being added to the reaction mixture. The substrate was aliquoted (100\(\mu\)l) into the microtubes and enzyme (5\(\mu\)g) added to the substrate. The reaction mixture was then vortexed and incubated (37°C) for 30mins and subsequently quenched by boiling the microtube for 30 seconds. The reaction mixture (20\(\mu\)l) was then applied to a Phenomenex Luna 5\(\mu\) C18 HPLC column (4.6 x 150 mm) and eluted isocratically at flow rate of 1ml/min with phosphate buffer (50mM potassium phosphate, 2.5mM tetrabutylammonium hydrogensulfate, adjusted to pH 6.9).\(^3\) Sugar nucleotides were detected at 265nm.

Sugar-1-phosphates

General methods for product characterization
\(^{1}\)H NMR spectra were recorded either on a Varian Gemini spectrometer at 300MHz or a Varian Unity Plus spectrometer at 400MHz and were referenced to the internal CH\(_3\)OH (\(\delta\)H 3.35ppm) or H\(_2\)O (\(\delta\)H 4.75ppm). Coupling constants are given in Hz. Only partial (diagnostic) NMR data are given; other spectral features were in accord with the proposed structures. \(^{13}\)C NMR spectra were recorded either on a Varian Unity Plus spectrometer at 100MHz and were referenced to the internal CH\(_3\)OH (\(\delta\)C 49.0ppm). Electrospray mass spectra were recorded on a Micromass Quattro II Spectrometer.

UDP 2-deoxy-D-galactopyranose
UDP 2-deoxy-D-galactopyranose was prepared according to the typical 1-pot enzymatic procedure, to yield the target compound as a white solid (19.4mg, 98%). \(^{1}\)H NMR (D\(_2\)O, 400 MHz): \(\delta\) 7.93 (d, 1H \(J_{5,6}=8.0\) Hz, H-6), 5.97 (d, 1H, \(J_{1',2'}=3.6\) Hz, H-1'), 5.94 (d, 1H, \(J_{5,6}=8.0\) Hz, H-5), 5.72 (m, 1H, \(J_{1'',P}6.8\) Hz, H-1''), 3.87 (d, 1H, \(J_{3'',4''}3.2\) Hz, H-3''), 2.03-1.89 (m, 2H, H-2a'', H-2b''). ES-MS: Calcd for [C\(_{15}\)H\(_{24}\)N\(_2\)O\(_{16}\)P\(_2\)] 550.3, obs m/s 549.50 (M-1). Analytical data in accordance with literature data.\(^4\)

UDP 2-deoxy-2-fluoro-D-galactopyranose
UDP 2-deoxy-2-fluoro-D-galactopyranose was prepared according to the typical 1-pot enzymatic procedure, to yield the target compound as a white solid (19.8mg, 97%). \(^{1}\)H NMR (D\(_2\)O, 400 MHz): \(\delta\) 7.96 (d, 1H, \(J_{5,6}=8.0\) Hz, H-6), 5.98 (d, 1H, \(J_{1',2'}=4.0\) Hz, H-1'), 5.93 (d, 1H, \(J_{5,6}=8.0\) Hz, H-5), 5.63 (dd, 1H, \(J_{1'',P}=7.6\) Hz, \(J_{1'',2''}=4.0\) Hz, H-1''), 4.64 (ddt, 1H, \(J_{1'',1''}=7.6\) Hz, \(J_{1'',2''}=9.6\) Hz, \(J_{2'',3''}=4.0\) Hz, H-2''), 4.36 (d, 2H, \(J_{2',3'}=4.0\) Hz, H-2' and H-3'), 4.27 (m, 1H, H-4'), 4.22-4.16 (m, 4H, H-5a', H-5b', H-3'' and H-5''), 4.12 (t, 1H, \(J_{3'',4''}=3.8\) Hz, H-4''), 3.77-3.68 (m, 2H, H-6a'' and H-6b''). ES-MS: Calcd for [C\(_{15}\)H\(_{23}\)FN\(_2\)O\(_{16}\)P\(_2\)] 568.29, obs m/s 567.06 (M-1). Analytical data in accordance with literature data.\(^5\)

UDP 2-amino-2-deoxy-D-galactopyranose
UDP 2-amino-2-deoxy-D-galactopyranose was prepared according to the typical 1-pot enzymatic procedure, to yield the target compound as an off white solid (19.4mg, 95%). \(^{1}\)H NMR (D\(_2\)O, 400 MHz): \(\delta\) 7.92 (d, 1H, \(J_{5,6}=8.0\) Hz, H-6), 5.96 (d, 1H, \(J_{1':2'}=4.4\) Hz, H1'), 5.94
UDP N-acetyl-D-galactosamine

UDP N-acetyl-D-galactosamine was prepared according to the typical 1-pot enzymatic procedure, with the exception that 400 U of galK and 300 U of galPUT were used and incubation was for 24 hours, to yield the target compound as a white solid (4.2 mg, 19%).

UDP 3-deoxy-3-fluoro-D-galactopyranose

UDP 3-deoxy-3-fluoro-D-galactopyranose was prepared according to the typical 1-pot enzymatic procedure, with the exception that 400 U of galK and 300 U of galPUT were used and incubation was for 12 hours, to yield the target compound as a white solid (11.3 mg, 57%).

UDP 6-deoxy-D-galactopyranose

UDP 6-deoxy-D-galactopyranose was prepared according to the typical 1-pot enzymatic procedure, with the exception that 400 U of galK and 300 U of galPUT were used and incubation was for 24 hours, to yield the target compound as a white solid (8.7 mg, 47%).

UDP L-arabinopyranose

UDP L-arabinopyranose was prepared according to the typical 1-pot enzymatic procedure, with the exception that 400 U of galK and 300 U of galPUT were used and incubation was for 12 hours, to yield the target compound as a white solid (8.7 mg, 47%).

UDP 6-deoxy-6-fluoro-D-galactopyranose

UDP 6-deoxy-6-fluoro-D-galactopyranose was prepared according to the typical 1-pot enzymatic procedure, with the exception that 400 U of galK and 300 U of galPUT were used and incubation was for 24 hours, to yield the target compound as a white solid (12.5 mg, 64%).

UDP 6-deoxy-6-fluoro-D-galactopyranose was prepared according to the typical 1-pot enzymatic procedure, with the exception that 400 U of galK and 300 U of galPUT were used and incubation was for 12 hours, to yield the target compound as a white solid (4.2 mg, 19%).

UDP L-arabinopyranose

UDP L-arabinopyranose was prepared according to the typical 1-pot enzymatic procedure, with the exception that 400 U of galK and 300 U of galPUT were used and incubation was for 12 hours, to yield the target compound as a white solid (8.7 mg, 47%).
UDP 2-azido-2-deoxy-D-galactopyranose
UDP 2-azido-2-deoxy-D-galactopyranose was prepared using the typical 2-pot chemo-enzymatic procedure with the exception that 600 U of galPUT was used, to yield the target compound as an off-white solid (13.3 mg, 64%).  

ES-MS: Calcd for [C14H22N2O16P2] 536.04, obs m/s 535.20 (M-1).  Analytical data in accordance with literature data.9,10

UDP D-mannopyranose
UDP D-mannose was prepared using the typical 2-pot chemo-enzymatic procedure to yield the target compound as an off-white solid (16.6 mg, 93%).  

ES-MS: Calcd for [C15H23N5O16P2] 591.07, obs m/s 590.50 (M-1).  Analytical data in accordance with literature data.11

UDP N-acetyl-D-glucosamine
UDP N-acetyl-D-glucosamine was prepared using the typical 2-pot chemo-enzymatic procedure to yield the target compound as an off-white solid (2.8 mg, 30%).  

ES-MS: Calcd for [C17H27N3O17P2] 607.08, obs m/s 606.50.  Analytical data in accordance with literature data.14

UDP D-xylose
UDP D-xylose was prepared using the typical 2-pot chemo-enzymatic procedure to yield the target compound as a white solid (16.2 mg, 89%).  

ES-MS: Calcd for [C14H22N2O16P2] 536.04, obs m/s 535.50.  Analytical data in accordance with literature data.15

UDP D-galactofuranose
UDP D-galactofuranose was prepared using the typical 2-pot chemo-enzymatic procedure, to yield the target compound as a white solid (16.1 mg, 79%).  

ES-MS: Calcd for [C15H24N2O17P2] 566.06, obs m/s 565.50.  Analytical data in accordance with literature data.16
UDP L-fucopyranose was prepared using the typical 2-pot chemo-enzymatic procedure to yield the target compound as an off-white solid (2.4mg, 12%). $^1$H NMR (D$_2$O, 400 MHz) $\delta$ 7.92 (d, 1H, $J_{5,6} = 8.4$ Hz, H-6) 5.99 (d, 1H, $J_{1',2'} = 5.2$ Hz, H-1') 5.95 (d, 1H, $J_{5,6}, H-5$), 5.42 (dd, 1H, $J_{1'',2''} = 3.0$ Hz, $J_{1'',P} = 6.8$ Hz, H-1''), 4.35-4.23 (m, 6H, H-2', H-3', H-4', H-5', H-5' and H-5''), 3.95 (dd, 1H, $J_{2'',3''} = 8.4$ Hz, $J_{3'',4''} = 3.2$ Hz, H-3''), 3.88 (br d, 1H, H-4''), 3.78 (dt, 1H, $J_{1'',2'', J_2''} = 3.0$ Hz, H-2''), 1.19 (d, 3H, $J_{5'',6''} = 6.8$ Hz, H-6''). ES-MS: Calcd for [C$_{15}$H$_{24}$N$_2$O$_{16}$P$_2$] 550.06, obs m/s 549.70. Analytical data in accordance with literature data.

References