

Supporting Information

Synthesis of a 3-Deoxy-D-manno-octulosonic acid (KDO) Building Block from D-Glucose via Fermentation

Gulden Camci-Unal, Rahman M. Mizanur, Yonghai Chai and Nicola L. B. Pohl*

G.C.-U. carried out and optimized the KDO production experiments and ran desalting columns. R.M.M. made the construct containing the three KDO-related genes and the ribose constitutive mutant cells. Y.C. protected and characterized the resulting KDO. N.L.B.P. conceived of and managed the project. G.C.-U. and N.L.B.P. wrote the manuscript including editorial comments from all four authors. All four authors contributed to this supporting information.

Table of Contents

General methods	S1
Construction of metabolically engineered <i>E. coli</i> strain	S2
TBA Assay	S3
Purification of KDO	S4
Preparation of the acetylated KDO methyl ester 3	S4
References	S5
NMR spectra of 3	S6
Table S1. Oligonucleotides used in this study.	S2
Figure S1. SDS-PAGE for expression of 3 KDO genes.	S3
Figure S2. KDO Calibration curve.	S3

General methods

Analytical thin layer chromatography (TLC) was performed using Sorbent Technologies 0.25 mm glass-backed silica gel HL TLC plates with UV254. Visualization was accomplished with UV light and staining with a 10% sulfuric acid in ethanol solution dip followed by heating. Flash chromatography was performed using 230–400 mesh silica gel (Type 60A) purchased from Sorbent Technologies. NMR spectroscopy (^1H , ^{13}C) was conducted using a Bruker DRX400 spectrometer at 400 MHz and 100 MHz respectively. Chemical shifts are reported in ppm relative to CDCl_3 (^1H = 7.27 ppm, ^{13}C = 77.23 ppm) or to D_2O (^1H = 4.63 ppm) as an internal reference and coupling constants are reported in Hz. Mass spectrometry was performed by the W. M. Keck Metabolomics Research Laboratory at Iowa State University using an Applied Biosystems QSTAR® XL Hybrid LC/MS/MS System. Melting points were recorded on a MEL-TEMP® capillary melting point apparatus without correction. The optical rotation measurement was taken on a JASCO Digital polarimeter DIP-370 at 598 nm at 20 °C, L= 0.5 dm.

Standard procedures to manipulate DNA, including plasmid DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, DNA ligation and transformation of *E. coli*, were performed by conventional methods (Sambrook et al. 1989). The PCR was carried out in an Eppendorf Mastercycler gradient thermocycler (Eppendorf Scientific Inc. Westbury, NY). Protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-

PAGE, Tris-HCl 10-20% gradients, Bio-Rad Laboratories, Hercules, CA). The gels were stained with Coomassie brilliant blue. Protein concentrations were determined with the Bio-Rad protein assay kit according to the method of Bradford (Bradford 1976) using bovine serum albumin as the standard.

Construction of metabolically engineered E. coli strain. A D-ribose constitutive mutant of E. coli BL21 (DE3) was created by repeated growing of cells in MOPS minimal medium (Teknova, Hollister, CA) supplemented with 0.2% D-ribose as the sole carbon source and was named as E. coli BL21 (DE3) DR001 (EDR001). A control was run in parallel using 0.2% D-glucose as the sole carbon source. The D-ribose mutant was then modified further to allow for gene expression from plasmids containing T7 promoter. In order to achieve a T7 promoter compatible mutant a T7 RNA polymerase gene under a lac-promoter was inserted into the chromosome of EDR001 using the IDE3 lysogenization kit (Novagen, San Diego, California) according to the manufacturer's instruction to generate the strain E. coli BL21 (DE3) DR002 (EDR002). The chemically competent EDR002 was then transformed with the plasmid pMNP 25 for IPTG induced expression of recombinant genes for the fermentative production of KDO. Transformants were selected at 37 °C and grown on LB medium supplemented with carbenicillin.

Table S1. Oligonucleotides used in this study.

Oligonucleotide	Description
yrbH-fwd	5'-aaacatatgtcgcacgtagagtacaaccggg -3'
yrbH-rev	5'-aaagaattcttacactacgcctgcacgcagtaaatcatgcatgtgtaacacacc- 3'
kdsA-fwd	5'- aaacatatgaacaaaaagtggtagcattggc g -3'
kdsA-rev	5'- aaagaattcttacttgctggtatccagttcttcgaaacc -3'
yrbI-fwd	5'- aaacatatgagcaaagcaggtgcgtcgttcgcgacc -3'
yrbI-rev	5'-aaagaattctcatatcgattgccctttggcttcacccagtttgc -3'
AvrII-fwd	5'-aattcctaggacagc-3'
AvrII-rev	5'-tcgagctgtcctagg-3'

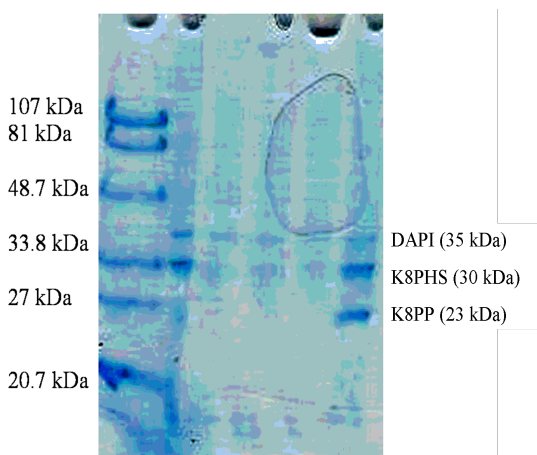


Figure S1. SDS-PAGE for expression of 3 KDO genes.

TBA Assay. The calibration curve was obtained for known concentrations of KDO using the previously reported thiobarbituric acid assay (Karkhanis 1978). Concentrations of D-ribose and D-glucose measured using the TBA assay are shown on the curve for comparison. The OD value from the assay is converted into an approximate amount of KDO present in the media using the calibration curve below.

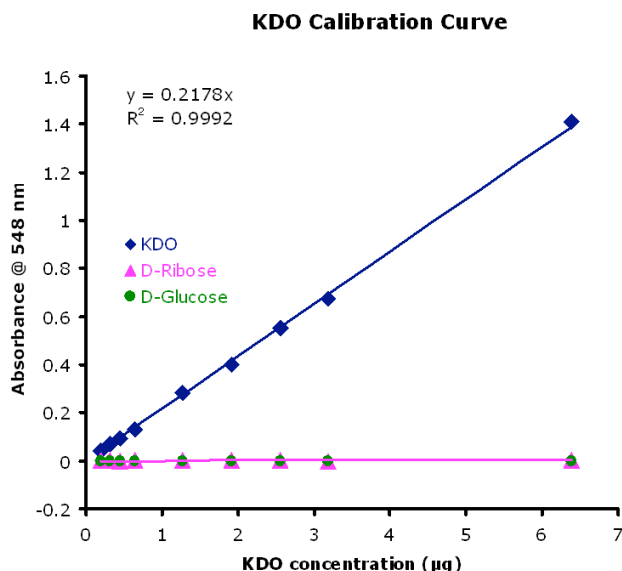


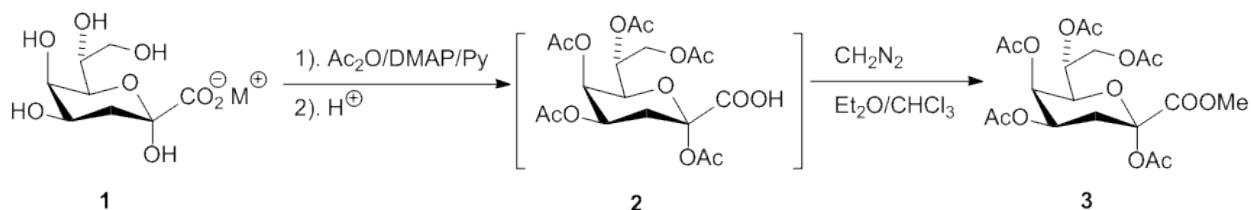
Figure S2. KDO Calibration curve.

Purification of KDO.

The lyophilized solid mixture containing KDO is partially purified using the formate form of strong anion exchange resin Bio-Rad AG[®]1-X8. KDO is eluted from the column by 1 M ammonium formate. The fractions containing KDO are combined and lyophilized from dI H₂O to afford a solid residue. This residue is then passed through a desalting column, Bio-Gel[®] P-2. The fractions containing KDO are lyophilized. ¹H NMR in D₂O matches that of an authentic sample from Sigma. QTOF electrospray ionization mass spectrometry of the final sample gave the expected [H-1]⁻ peak in the (-) ion mode.

HRMS (+ TOF) calcd for (M+⁻H) C₈H₁₃O₈⁻, 237.18; found 237.07

Preparation of the acetylated KDO methyl ester 3



Methyl 4,5,7,8-tetra-O-acetyl-3-deoxy- α -D-manno-2-octulopyranosonate (3) (Unger 1980, Charon 1979, Smith 1990)

To a suspension of the crude KDO (400 mg, after desalting column described above) in pyridine/acetic anhydride (v/v = 1:1, 5 mL) was added 4-dimethylamino pyridine (DMAP, 20 mg, 0.16 mmol). The reaction mixture was allowed to stir at room temperature under nitrogen atmosphere. After 8 h, pyridine/acetic anhydride was removed under reduced pressure to afford a brown oily residue (2.2 g). CHCl_3 (10 mL) and H_2O (5 mL) were added to the residue. The mixture was transferred into a separatory funnel and washed with 1N cold sulfuric acid aqueous solution (10 mL). The organic layer was separated and the aqueous layer was extracted with CHCl_3 (2 x 10 mL). The combined organic layers were dried over anhydrous MgSO_4 . The solid was removed by filtration and the solvent was removed under reduced pressure to provide a yellow syrup (50 mg). The yellow syrup was dried under high vacuum for 20 h and then directly used for the following methyl esterification. To a solution of the syrup in CHCl_3 (10 mL) was slowly added a solution of diazomethane (Anzalone 1985) in Et_2O at room temperature until yellow color persisted and no gas was produced. The solvent was removed under reduced pressure and the residue was purified by flash column chromatography ($\text{Et}_2\text{O}/\text{cyclohexane}$, 1:1, v/v) to provide a white solid (26 mg).

m.p. 156-158 °C

^1H NMR (400 MHz, CDCl_3): d 5.38 (s, 1H), 5.33-5.30 (m, 1H), 5.24-5.19 (m, 1H), 4.48 (dd, 1H, $J = 2, 12$ Hz), 4.17 (d, 1H, $J = 9.6$ Hz), 4.11 (dd, 1H, $J = 4, 12$ Hz), 3.80 (s, 3H), 2.31-2.17 (m, 2H), 2.14 (s, 3H), 2.11 (s, 3H), 2.04 (s, 3H), 1.99 (s, 6H).

^{13}C NMR (100 MHz, CDCl_3): d 170.6, 170.5, 170.2, 169.7, 168.1, 166.9, 97.6, 69.8, 67.4, 66.1, 64.1, 62.3, 53.4, 31.1, 20.9, 20.8

HRMS (+ TOF) calcd for ($\text{M}^+ + \text{Na}$) $\text{C}_{19}\text{H}_{26}\text{NaO}_{13}$, 485.1271; found 485.1033

Optical rotation: $[\alpha]_{\text{D}}^{20} = +81.9^\circ$ (c 0.035, chloroform)

Previously reported (Unger 1980): $[\alpha]_D^{20} = + 87.1^\circ$ (*c* 0.81, chloroform)

References

Anzalone, L.; Hirsch, J.A. *J. Org. Chem.* **1985**, *50*, 2607-2613.

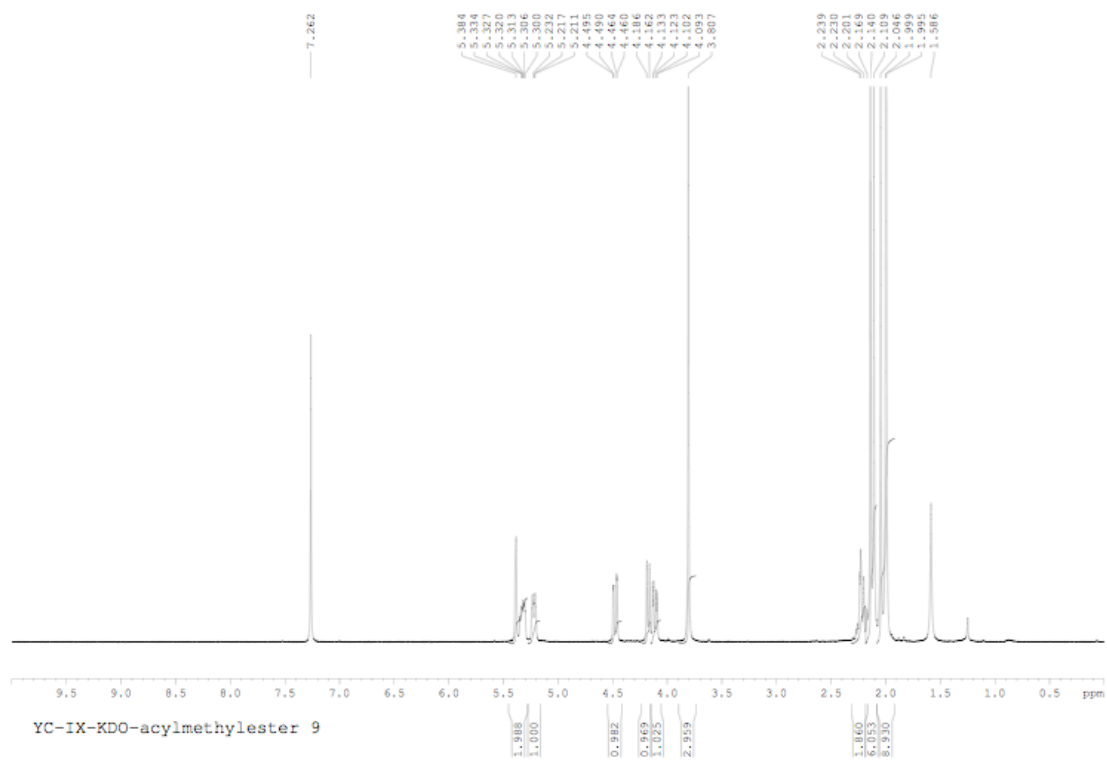
Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248-254.

Charon, D.; Szabo, L. *J. Chem. Soc. Perkin Trans. I* **1979**, 2369-2374.

Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular cloning: a laboratory manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: Plainview, NY, 1989.

Smith, D.B.; Wang, Z.; Schreiber, S.L. *Tetrahedron* **1990**, *46*, 4793-4808.

Unger, F.M.; Stix, D.; Schulz, G. *Carbohydr. Res.* **1980**, *80*, 191-195.

¹H NMR spectra of KDO methyl ester **3**

^{13}C NMR spectra of KDO methyl ester **3**