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PAPER

Synthesis of a 3-deoxy-D-manno-octulosonic acid (KDO) building block from D-glucose *via* fermentation†‡

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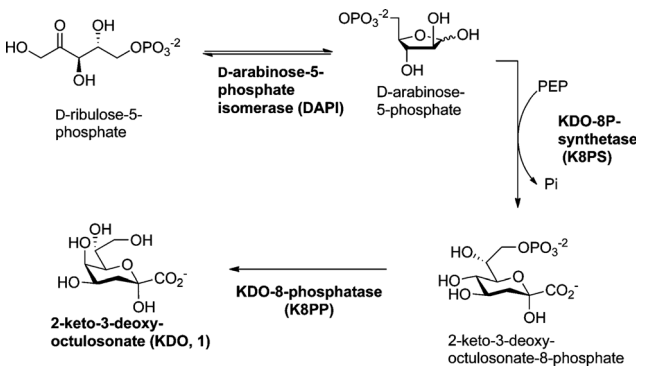
Herein we report the first synthesis of a 3-deoxy-D-manno-octulosonic acid (KDO) building block starting from glucose through pathway engineering of *Escherichia coli* and subsequent chemical modifications to provide an alternative method to produce KDO, found in plant and bacterial oligosaccharides.

KDO (3-deoxy-D-manno-octulosonic acid) is an unusual 8-carbon acidic sugar that is essential for the growth of many pathogenic bacteria affecting plants and animals.[1–3] For example, in Gram-negative bacteria such as *Brucella* – which causes severe infections in humans and livestock – KDO is a crucial part of the lipid A-based lipopolysaccharides.[4] KDO has also been discovered in plants and green algae, although its biological roles in these organisms are still unclear.[5–13] Despite its biological ubiquity, synthetic efforts to incorporate KDO into larger structures to explore structure/function relationships have been limited.[14] KDO is commercially available, but is over 2 million times more expensive than glucose per mole. Given this expense, multiple chemical and enzymatic syntheses of KDO have been developed over several decades.[15–39] The only route that has proven amenable to producing this 8-carbon sugar in sufficient quantities for further synthetic transformations relies on the Cornforth procedure[40] for aldol condensation of D-arabinose and oxaloacetic acid followed by decarboxylation.[41] Although the 66% reported yield of this procedure is moderate, the starting materials are also expensive, thereby limiting full exploration of the chemistry and biology of KDO and development of possible therapeutics based on KDO. Herein we report the first synthesis of KDO starting from glucose through pathway engineering of *Escherichia coli* to provide a relatively low cost alternative method to produce KDO for incorporation as a chemical building block in future syntheses of larger KDO-containing structures.

E. coli is a common host for chemical production through fermentation and conveniently already makes KDO for its own membranes.[42] The enzymes for KDO biosynthesis are known (Schemes 1, 2) as they have already attracted interest as potential targets for antibacterial compounds against Gram-negative bacteria.[43,44] When KDO synthesis stops in a cell, lipopolysaccharide (LPS) synthesis stops and this event eventually leads to cell growth arrest.[45] However, ideally the labor of isolating and stabilizing these enzymes *in vitro* could be avoided with a whole cell system. Because *E. coli* normally makes KDO for direct incorporation into its LPS and not as a free sugar, it was uncertain at the outset if overproduction of this carbohydrate would be toxic to the cultured cells. There is no known KDO exporter protein and therefore the sugar could potentially build up inside the cell and serve to inhibit the biosynthetic pathways related to KDO. Also, ideally KDO would be secreted into the media for ease of isolation. Fortunately, related work in the production of sialic acid in *E. coli*[46] showed the production of that carbohydrate in the media despite lack of a known export protein. Finally, overexpression of the genes should only produce KDO itself and not related analogs from non-standard cellular substrates for a cost-effective purification.

To test if overexpression of KDO biosynthetic genes would lead to the production of secreted KDO, a plasmid was designed to encode three *E. coli* genes that are necessary for KDO biosynthesis from ribose: D-arabinose 5-phosphate isomerase (DAPI, *yrb*H),[47] KDO-8-phosphate synthetase (KPS, *kds*A),[48]



Scheme 1 Pathway for the biosynthesis of KDO.

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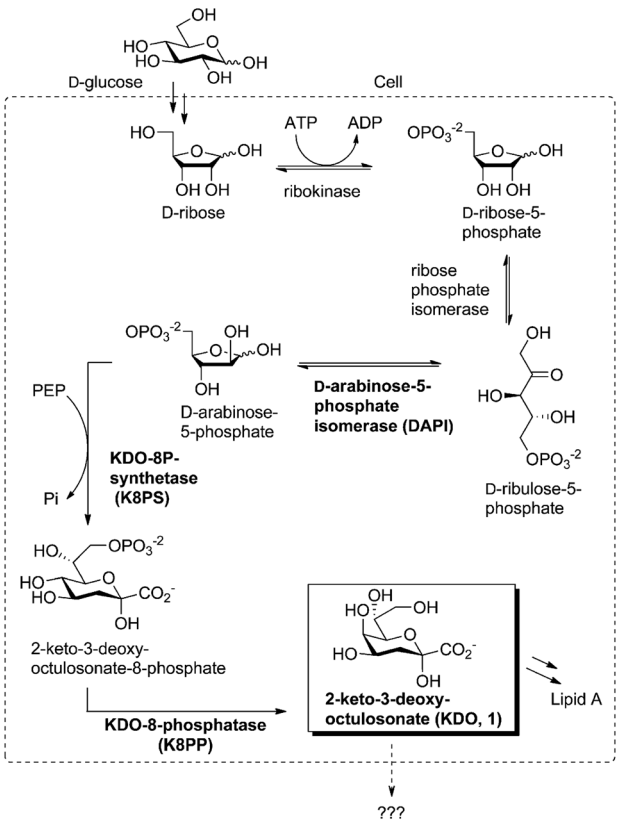
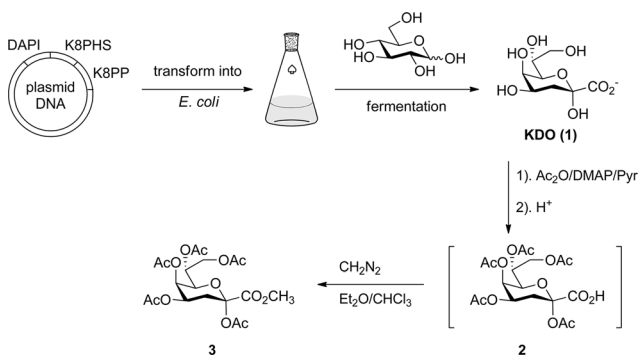


Scheme 2 Production of KDO in the bacterial cell.



Scheme 3 Production and protection of KDO to form an organic soluble building block.

and KDO-8-phosphatase (KPP, *yrbI*)[49] (Scheme 3). The plasmid containing the three KDO biosynthesis genes for IPTG-induced expression of recombinant genes for the fermentative production of KDO is outlined in Scheme 3. The first enzyme interconverts D-ribulose-5-phosphate and D-arabinose-5-phosphate. The resulting D-arabinose-5-phosphate is converted into 2-keto-3-deoxy-octulonate-8-phosphate by the addition of KDO-8-phosphate synthetase and phosphoenolpyruvate (PEP) yielding the product and inorganic phosphate (P_i). Finally, an enzyme is included to cleave the phosphate to render the substrate less charged and therefore more likely to be transported to the extracellular media (Scheme 1). The new plasmid was transformed into an *E. coli* strain for IPTG-induction of expression of the three genes.

Table 1 Production of KDO, mg L^{-1}

	t = 1 h	*t* = 48 h	*t* = 96 h
E. coli with KDO genes, IPTG induced	ND[a]	122	334
E. coli with KDO genes, no IPTG induction	ND	51	101
E. coli without KDO genes (pUC 19), IPTG induced	ND	ND	ND
E. coli with DAPI gene, IPTG induced	ND	ND	ND
E. coli with K8PHS gene, IPTG induced	ND	ND	ND
E. coli with (KPHS + K8PP) genes, IPTG induced	ND	ND	ND

[a] ND not detected.

Fortunately, SDS-PAGE analysis confirmed expression of all three expected enzymes based on their expected molecular weights (see ESI†).

Given that the proteins were expressed, the production of KDO itself was probed next. Gratifyingly, initial experiments using D-ribose as the carbon source in shake-flask experiments using the newly engineered bacteria clearly showed the presence of KDO in the fermentation media. Apparently, the sugar could be removed from the inside of the bacterial cell. The approximate amount of KDO in the supernatant solution was determined by the known thiobarbituric acid assay.[50] Given this success, we then tried to replace D-ribose with the 100-fold cheaper carbon source D-glucose. Apparently, conversion of the glucose into the required KDO precursors is not yield-limiting as glucose worked as efficiently as D-ribose in the production of KDO. The fermentation experiments were repeated for *E. coli* containing the carbenicillin resistance gene but without the KDO-related genes (pUC 19) as a control. The results indicated no evidence of KDO production in the fermentation media without the presence of the plasmid containing the three KDO biosynthetic genes (Table 1). In addition, we investigated the effect of IPTG induction on the production of KDO given the possibility of leaky protein expression from the T7 promoter. Although the bacteria still produces KDO to an extent without IPTG induction, yields are about 3-fold less than that of IPTG-induced cells. With IPTG induction, the procedure could yield 334 mg of KDO per liter of culture – yields that are lower but in the same range as those found for the recent production of sialic acid in *E. coli*.[46] Moreover, we have done fermentation experiments using *E. coli* with plasmids that contain DAPI, K8PHS and (K8PHS + K8PP) genes separately.

KDO production is found to be non-detectable in all of the cases, an observation that indicates that all these three KDO-related genes are needed in the *E. coli* strain to overproduce KDO. This fermentation route is perhaps not as amenable to the synthesis of KDO-like sugars as an *in vitro* route; however, the cost of these fermentation experiments is lower than the reported oxaloacetic acid procedure[37] as our minimal fermentation medium includes just basic salts for bacterial growth and our carbon source – glucose – is also very inexpensive. These findings are in line with other results in that the most cost-effective method to synthesize such molecules is fermentation as abundant, inexpensive, and renewable starting materials can be used.[51,52] Furthermore, the oxaloacetic acid method uses the

toxic heavy metal $NiCl_2$ which is known to be harmful to both humans and the environment.[53,54] The fermentation method for KDO production does not involve any toxic heavy metals in any stage of the production.

To confirm the identity and yield of the product and also to demonstrate that the fermentation product could be readily transformed into the known protected form of KDO **3** needed for further synthetic manipulations in organic solvents, the product was freeze-dried and subjected to anion exchange chromatography. The resulting product co-migrated on a thin layer silica gel chromatography plate with an authentic KDO sample and showed a proton NMR spectrum consistent with the known product. High-resolution quadrupole time-of-flight (QTOF) electrospray ionization mass spectrometry of the final KDO sample gave the expected $[H-1]^-$ peak in the (−) ion mode. Subjection of the crude fermentation mixture to acetylation followed by methyl esterification conditions (Scheme 2) resulted in the isolation of the known peracetylated KDO methyl ester **3** despite the presence of salts in the original starting material to confirm the yield of KDO in the fermentation. Proton and carbon NMR as well as HRMS and optical rotation of this product indeed confirmed the identity of the fermentation product. Fortunately, no evidence was found for the production of any KDO-like analogs that would have significantly complicated the purification efforts.

In summary, production of KDO in fermentation media using whole cells could be attained with *E. coli* cells engineered to overproduce the three enzymes involved in the biosynthetic production of this 8-carbon sugar. Production of KDO did not appear toxic to the cells and the 334 mg KDO yields per liter of culture using glucose as a carbon source should reduce the cost of obtaining KDO and therefore help spur the development of the chemistry and biology of this ubiquitous sugar. In addition, work can now also commence on modification of KDO for the design of inhibitors of the KDO biosynthetic pathway as a new antibiotic strategy against multidrug resistant Gram-negative bacteria.[55] Future work will include incorporation of KDO in combinatorial oligosaccharide synthesis. In addition, the lectin binding ability of this unique 8-C sugar can be systematically investigated.

Experimental section

Materials and equipment

Enzymes and reagents used for the molecular biology procedures, DNA ladders and deoxynucleotide triphosphates (dNTPs) were purchased from Promega (Madison, WI) or New England Biolabs (Beverly, MA). Oligonucleotides for DNA amplification were synthesized by Integrated DNA Technologies (Coralville, IA). Protein molecular weight standards were obtained from BioRad (Hercules, CA). The QIAQuick gel extraction kit was obtained from Qiagen (Valencia, CA), and the Zero blunt PCR cloning kit was purchased from Invitrogen (Carlsbad, CA). Periodic acid (H_5IO_6), thiobarbituric acid (TBA), sodium meta arsenite ($NaAsO_2$) and the ammonium salt of 2-keto-3-deoxyoctonate (KDO) were purchased from Sigma (St. Louis, MO). Sulfuric acid (H_2SO_4), hydrochloric acid (HCl), potassium phosphate dibasic (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), ammonium sulfate ($(NH_4)_2SO_4$), magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$), dimethyl sulfoxide (C_2H_6OS), sodium hydroxide (NaOH) and D-glucose were obtained from Fisher (Pittsburgh, PA). Isopropyl-beta-D-thiogalactopyranoside (IPTG) and carbenecillin were purchased from Lab Scientific (Livingston, NJ). Bio-Gel® P-2 Gel (45–90 μm) and AG®1-X8 resin (100–200 mesh) formate form were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). Ammonium formate (NH_4COOH) was obtained from Fluka (St. Louis, MO). All other chemicals were received from Aldrich (Milwaukee, WI). All chemicals were used without further purification.

A Buchi rotary evaporator (West Chester, PA) was used to remove water from the fermentation medium. Thermo IEC Centra CL3R and Thermo IEC Micromax RF (Needham Heights, MA) instruments were used for centrifugation purposes. A VWR Orbital Incubator Shaker Model 1575R (West Chester, PA) was used to grow cells. A Fisher (Pittsburgh, PA) brand heat block was used to boil samples for the TBA assay. A Spectronic 20 Genesys UV Spectrophotometer (Waltham, MA) was used to take optical density measurements at 548 nm and 600 nm. Deionized water (18.0 MHz) was obtained from a Barnstead/Thermolyne Water Purification system (Dubuque, IA).

Bacterial strains and growth conditions

Escherichia coli K12 was used to isolate chromosomal DNA. *E. coli* BL21 (DE3) was used to create a D-ribose constitutive mutant (EDR001) which was then used for expression of recombinant genes after introducing T7 RNA polymerase to create the strain EDR002. Oneshot Top10 competent cells (Invitrogen, Carlsbad, CA), *Escherichia coli* XL-10Blue (Stratagene, La Jolla, CA) and PCR Blunt vectors (Invitrogen) were used for direct cloning of PCR products. *Escherichia coli* cells were grown on Luria Bertani (Sigma) medium at 37 °C in an incubator shaker at 225 rpm. When required, the antibiotics carbenicillin or kanamycin was added at 50 μg mL^{-1} to make the selective media.

PCR amplification and construction of expression vector

The primers were designed to construct the D-arabinose 5-phosphate isomerase (*yrb*H), KDO 8-phosphate synthase (*kds*A) and KDO 8-phosphate phosphatase (*yrb*I) expression plasmids. The lists of primers and oligonucleotides used in this study are given in Table S1.† The chromosomal DNA of *E. coli* K12 was amplified in three separate reactions using the forward and reverse primers designed for *yrb*H, *kds*A and *yrb*I genes. The amplification reaction mixtures contained standard *Pfu* DNA polymerase buffer, 375 μM of dNTPs, 3 ng of each primer, 4 ng of total genomic DNA and 2.5 units of *Pfu* DNA polymerase. The cycling parameters were as follows 94 °C for 2 min 40 s followed by 30 cycles of 94 °C for 30 s, 58 °C for 45 s and 72 °C for 2 min 15 s, with a final elongation step of 72 °C for 15 min. The amplified DNA from each reaction was checked by agarose gel electrophoresis and purified using gel extraction kit before cloned into zero-blunt vector and was transformed into OneShot Top10 and *E. coli* XL10 competent cells to check the correct

insert. The resulting constructs were digested with appropriate restriction enzymes for cloning into an *Avr*II introduced modified pET21a vector. The *Avr*II restriction site was introduced into the pET21a vector right after the *EcoR*I site using the synthetic oligonucleotides (*Avr*IIF and *Avr*IIR) listed in Table S1.† The two complementary oligonucleotides were mixed in T4 ligase buffer in equal molar concentration and heated at 95 °C for 2 min before incubating at room temperature for 40 min for annealing. The annealed dinucleotide was then ligated into an *EcoR*I-*Xho*I digested pET21a vector which was named pMNP26. Each gene was individually cloned into pMNP26 to generate plasmids pMNP21 (pMNP26-*yrb*H), pMNP22 (pMNP26-*kds*A) and pMNP23 (pMNP26-*yrb*I) and were subsequently subcloned under the T7-promoter of the vector pMNP26 to generate the plasmids pMNP24 (pMNP26-*kds*A*yrb*I) and pMNP25 (pMNP26-*kds*A*yrb*I*yrb*H) using standard molecular cloning techniques.

Cell growth and fermentation

E. coli cells harboring the recombinant KDO biosynthetic genes were grown overnight at 37 °C, 240 rpm in LB broth. Cells were then harvested by centrifuging the overnight culture at 10 000 rpm for 10 min. The minimum fermentation medium contained 49.5 mL autoclaved mixture of potassium hydrogen phosphate (0.331 g), potassium dihydrogen phosphate (0.15 g), magnesium sulfate (0.0085 g) and ammonium sulfate (0.200 g), 50 μL of 50 mg mL^{-1} carbenecillin, 0.5 mL 15% (w/v) sterile D-ribose or D-glucose solution and cells from a 15 mL overnight culture. A total volume of 50 mL fermentation was incubated at 37 °C, 240 rpm. 20 μL of 1 M IPTG was added once OD_{600} reached 0.6–0.8. The fermentation experiment was stopped after 96 h. 0.5 mL samples were taken and 0.5 mL of 15% D-ribose or D-glucose was added to the flask each day. Cells were then centrifuged at 3600 rpm for 40 min and discarded; the supernatant contained the KDO. The KDO in the final solution was determined by the thiobarbituric acid method.[56] TLC of the fermentation product showed consistent results as that of the commercial KDO sample. TLC of the final medium was run in *n*-butanol–acetic acid–water (v/v/v 2 : 1 : 1) and stained with 10% sulfuric acid in ethanol. Finally, the product was freeze-dried to obtain the solid KDO product with 334 mg yield. ^{1}H NMR of the solid residue in D_2O is consistent with an authentic sample from Sigma.

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