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Enzymatic synthesis of 3-deoxy-D-manno-octulosonic acid (KDO) and its application for LPS assembly

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ABSTRACT

The studies of 3-deoxy-D-manno-octulosonic acid (KDO) have been hindered due to its limited availability. Herein, an efficient enzymatic system for the facile synthesis of KDO from easy-to-get starting materials is described. In this one-pot three-enzyme (OPME) system, D-ribulose 5-phosphate, which was prepared from D-xylose, was employed as starting materials. The reaction process involves the isomerization of D-ribulose 5-phosphate to D-arabinose 5-phosphate catalyzed by D-arabinose 5-phosphate isomerase (KdsD), the aldol condensation of D-arabinose 5-phosphate and phosphoenolpyruvate (PEP) catalyzed by KDO 8-phosphate synthetase (KdsA), and the hydrolysis of KDO-8-phosphate catalyzed by KDO 8-phosphate phosphatase (KdsC). By using this OPME system, 72% isolated yield was obtained. The obtained KDO was further transferred to lipid A by KDO transferase from *Escherichia coli* (WaaA).

Lipopolysaccharides (LPS), also known as endotoxins, are large molecules that anchored in the outer membrane of Gram-negative bacteria by lipid A, to which a nonrepeating core oligosaccharide and a distal polysaccharide termed as O-antigen are attached (Fig. 1).[1] Nonrepeating core oligosaccharide part contains 3-deoxy-D-manno-octulosonic acid (KDO) and heptose and is highly conserved in different bacteria.[2] KDO is the only sugar that found in all known core structures, although in some cases a derivative, D-glycero-D-talo-2-octulosonic acid (KO), is also present.[2] KDO was also found in capsular polysaccharides of many bacteria. For example, the repeating unit of *Neisseria meningitides* serogroup E capsule consists of alternating D-galactosamine and KDO residues.[3] *Escherichia coli* K12 capsule contains rhamnose and KDO residues.[4] Besides, KDO was found in plant and green algae.[5–8] Concerning the importance of KDO in kinds of biological processes, enzymes that involved in KDO biosynthetic pathway are exciting targets for the development of new classes of antibiotics.[9,10] Core polysaccharides of LPS are also the potential vaccines against bacterial infection. Many KDO-containing polysaccharides have been synthesized and evaluated in recent years.[11–14] The fact that immunizations with many of these polysaccharides lead to antibody responses provides an impetus to explore further KDO-containing polysaccharides as a vaccine candidate.[14] Nevertheless, such studies have been hampered by the lack of efficient and convenient preparation methods for KDO preparation.

Chemical methods for KDO synthesis have been developed over the past decades,[15–21] but the tedious protection/de-protection steps can be complicated and suffer from low yield. Alternatively, enzymatic syntheses employing KDO aldolase,[22] sialic acid aldolase,[23,24] KDO phosphate synthetase[25] proceed regio- and stereoselectively without protection. KDO aldolase and sialic acid aldolase could condense arabinose and pyruvate into KDO directly, but both enzymes suffer from low specific activity,[22,23] making these processes impractical for the scalable synthesis of KDO. In contrast with both aldolases, KDO 8-phosphate synthetase showed significantly higher specific activity,[25–27] and more than 120 mg of protein could be obtained from one liter of LB culture medium by using pET protein expression system (data in this work). KDO 8-phosphate synthetase catalyzes the aldol condensation of D-arabinose 5-phosphate and phosphoenolpyruvate (PEP), resulting in KDO 8-phosphate, which can be hydrolyzed to afford KDO by phosphatase.[25] The only block of this process for large scale synthesis is the low accessibility of D-arabinose 5-phosphate. Commercially available D-arabinose 5-phosphate is extremely expensive ($643/25 mg, Sigma–Aldrich) for preparative scale synthesis. Moreover, D-arabinose 5-phosphate has been difficult to prepare in quantity because there is a lack of kinase that could efficiently phosphorylate D-arabinose at C-5 position directly. Bednarski et al. used hexokinase and ATP-regeneration system to produce D-arabinose 5-phosphate for KDO synthesis.[25] Nevertheless, the low specific activity of hexokinase towards D-arabinose requires a large amount of hexokinase. To avoid using expensive starting materials, Pohl and co-workers have developed a biological 'living

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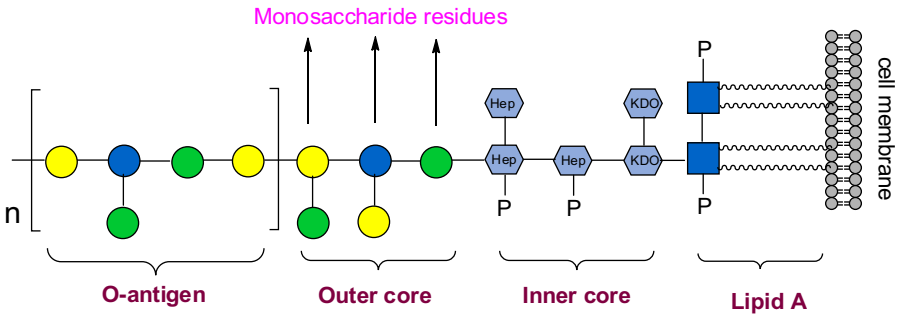
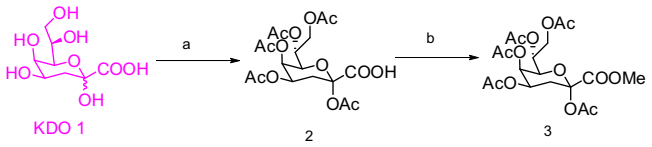


Figure 1. The structure of *E. coli* LPS.

factory', by which KDO was produced from glucose through cell fermentation.[28] Although hundreds milligram of KDO could be produced in one liter of medium, the purification of the final product from fermentation broth can be complicated. Therefore, an efficient and convenient method to readily provide KDO in considerable amounts is highly attractive in enabling the studies of KDO.

Herein, an efficient enzymatic strategy for the facile synthesis of KDO from easy-to-get starting materials is described (Scheme 1). In the first stage, D-ribulose 5-phosphate was prepared from D-xylose in multi-gram scale. In the second stage, D-ribulose 5-phosphate was incubated with D-arabinose 5-phosphate isomerase (KdsD), KDO 8-phosphate synthetase (KdsA), and KDO 8-phosphate phosphatase (KdsC) in one-pot fashion to produce KDO. The obtained KDO was further transferred into lipid A by KDO transferase from *E. coli* (WaaA) (Scheme 3).

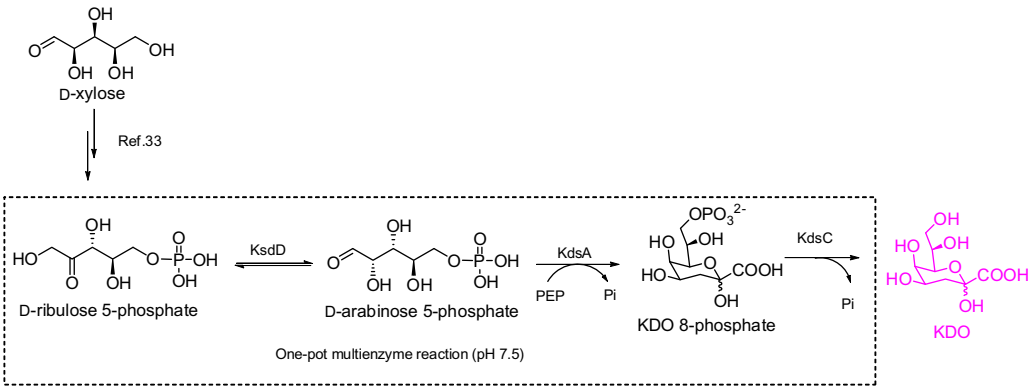
D-Arabinose 5-phosphate is a rare sugar phosphate, and there is a lack of kinase that could efficiently phosphorylate D-arabinose directly. Therefore, D-arabinose 5-phosphate has been difficult to prepare in quantity, and the commercially available product is extremely expensive. Meanwhile, many synthetic methods have been explored for the synthesis of D-ribulose 5-phosphate, which is a key intermediate in pentose phosphate pathway (PPP) and widely exists in bacteria, plants, and animals.[29] The reported methods for the synthesis of D-ribulose 5-phosphate relies on the isomerization of D-ribose 5-phosphate,[30] the phosphorylation of D-ribulose,[31] and the oxidization of D-gluconate 6-phosphate.[32] Although scalable product could be produced by using these methods, these processes still suffer from expensive starting materials, low yields, or a complicated purification step. As a consequence, commercially available D-ribulose 5-phosphate is also extremely expensive ($1245/25 mg, Sigma–Aldrich). Recently, we have developed an efficient and convenient platform for the facile synthesis of phosphorylated ketopentoses,[33] in which the synthesis of



Scheme 2. Synthesis of the pentaacetate methyl ester of KDO. Reagents and conditions: (a) Ac_2O, DMAP, pyridine, rt; (b) $TMSCHN_2$, DCM/MeOH.

D-ribulose 5-phosphate was also included. In this strategy, D-ribulose was prepared from D-xylose by a one-pot two-enzyme system in first reaction stage,[34] and then D-ribulose was phosphorylated by using L-ribulose kinase at C-5 position. The product was purified by using silver nitrate precipitation.[33] Having got a considerable amount of D-ribulose 5-phosphate in hand in this work (multi-gram), we try to use a sequential one-pot three-enzyme (OP3E) system containing KdsD, KdsA, and KdsC to synthesize KDO (Scheme 1).

The requirement of several enzyme-catalyzed reactions being carried in one-pot is that the enzymes must explicitly recognize their individual substrate. Otherwise, the cross-reactions will result in unpredictable by-products and increase the purification difficulties. KDO 8-phosphate synthetase could specifically recognize D-arabinose 5-phosphate but not D-ribulose 5-phosphate,[27] making our design (Scheme 1) reasonable. KdsC is highly active to hydrolyze the phosphate group of KDO 8-phosphate, while only trace activity towards D-arabinose 5-phosphate and PEP was observed (thousands of times lower than KDO 8-phosphate),[35] indicates its potential applications in OPME reaction. However, its substrate specificity towards D-ribulose 5-phosphate is unknown. To test the substrate specificity of KdsC towards D-ribulose 5-phosphate, D-ribulose 5-phosphate was incubated with KdsC in excess amount for three hours, while D-ribulose



Scheme 1. One-pot multienzyme system for the production of KDO.