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Arabinose 5-phosphate analogues as mechanistic probes for *Neisseria meningitidis* 3-deoxy-D-*manno*-octulosonate 8-phosphate synthase

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ABSTRACT

3-Deoxy-D-*manno*-octulosonate 8-phosphate (KDO8P) synthase catalyses the condensation reaction between phosphoenolpyruvate and D-arabinose 5-phosphate (D-A5P) in a key step in lipopolysaccharide biosynthesis in Gram-negative bacteria. The KDO8P synthase from *Neisseria meningitidis* was cloned into *Escherichia coli*, overexpressed and purified. A variety of D-A5P stereoisomers were tested as substrates, of these only D-A5P and L-X5P were substrates. The Asn59Ala mutant of *N. meningitidis* KDO8P synthase was constructed and this mutant retained less than 1% of the wild-type activity. These results are consistent with a catalytic mechanism for this enzyme in which the C2 and C3 hydroxyl groups of D-A5P and Asn59 are critical.

1. Introduction

3-Deoxy-D-*manno*-octulosonate 8-phosphate synthase (KDO8P) synthase (EC 2.5.1.55) catalyses the condensation reaction between phosphoenolpyruvate (PEP) and D-arabinose 5-phosphate (D-A5P) to generate KDO8P and inorganic phosphate (see Fig. 1). This reaction is the first committed step in the biosynthesis of 3-deoxy-D-*manno*-octulosonate (KDO), a site-specific component of the inner core of the lipopolysaccharide (LPS), and is essential for LPS formation.[1] As the LPS is found in most Gram-negative bacteria and is necessary for their viability, the enzymes responsible, including KDO8P synthase, have been identified as attractive targets for the development of novel antimicrobial compounds.

The reaction mechanism of KDO8P synthase has already received considerable attention. It has been shown that the condensation follows an ordered sequential kinetic mechanism in which PEP binds to the enzyme before D-A5P and the product KDO8P is released last.[2] Labeling studies have shown that the reaction is highly stereospecific with the *si* face of PEP coupling with the *re* face of D-A5P, and that the reaction involves the unusual cleavage of the C–O bond of PEP rather than O–P bond cleavage.[3,4] A linear biphosphate reaction intermediate arising from attack of water at C2 of PEP following attack by C3 of PEP on the electrophilic carbonyl carbon of acyclic D-A5P has been identified using time-resolved electrospray ionisation mass spectrometry.[5] Nevertheless, the precise catalytic events that lead to this intermediate are still unresolved. These initial studies were performed primarily with the KDO8P synthase from *Escherichia coli*, an enzyme, that is, unaffected by the presence of metal ions or metal ion chelators. However, more recently it has been shown that the KDO8P synthases from *Aquifex aeolicus*[6], *Helicobacter pylori*[7], *Chlamydia psittaci*[7] and *Aquifex pyrophilus*[8] require the presence of a metal ion for activity, raising the possibility that two distinct families of KDO8P synthases with different catalytic mechanisms exist.

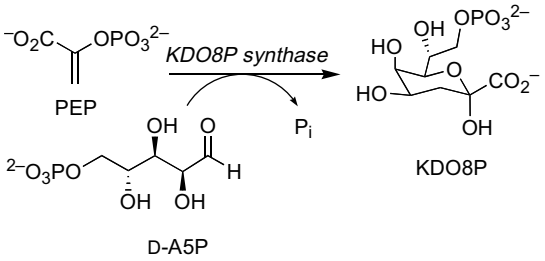

Figure 1. Reaction catalysed by KDO8P synthase.

Structures of both metal-dependent (*A. aeolicus*) and metal-independent (*E. coli*) KDO8P synthases have been determined.[9,10] Both enzymes crystallise as homotetramers with (β/α_8-barrel subunits containing four independent active sites. Almost all key active site residues appear to be conserved in both proteins with the only notable difference being at the metal-binding site. Three of the four residues responsible for metal coordination in

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A. aeolicus KDO8P synthase are found in the metal-independent *E. coli* KDO8P synthase. The fourth ligand, a Cys, is substituted by an Asn in the metal-independent *E. coli* enzyme.[7] Sequence analysis indicates that all KDO8P synthases whose activity has been shown to be metal-dependent have this Cys, whereas the Asn is found in the equivalent position in characterised metal-independent enzymes, indicating that this amino-acid may be the prime determinant of metal dependency. Recent elegant studies have shown that single reciprocal mutations between the Cys and Asn interconvert metal-dependent and metal-independent enzyme activity, although with heavily compromised activity relative to the parent enzyme.[11] As the ability to bind metal is readily dispensable it is likely that both metal-independent and metal-dependent enzymes share a common reaction mechanism, and that the metal (when present) plays a structural rather than catalytic role.

KDO8P synthase is closely related to the enzyme that catalyses the first step of the shikimate pathway, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAH7P) synthase; DAH7P synthase catalyses the formation of DAH7P from PEP and the four-carbon sugar D-erythrose 4-phosphate (D-E4P) in an analogous reaction. DAH7P and KDO8P synthases share many mechanistic and structural features and on the basis of this similarity and detailed phylogenetic analyses it has been proposed that these enzymes share a common ancestry.[7]

Whereas DAH7P synthase appears to be relatively insensitive to changes in its aldehydic substrate KDO8P synthases are reported to exhibit greater substrate stringency.[12–14] Previous studies have shown that the C2 epimer of D-A5P, ribose 5-phosphate (D-R5P),[15,16] is not a substrate for either metal-dependent or metal-independent KDO8P synthases and 2- and 3-deoxy analogues of D-A5P are either very poor substrates or not utilised at all as D-A5P alternatives in the KDO8P synthase catalysed reaction.[2,12,16,17] In contrast, acyclic 4-deoxy-D-A5P is reported to be a substrate for the *E. coli* KDO8P synthase.[2]

Neisseria meningitidis, a Gram-negative bacterium that colonises the nasopharynx of about 10% of healthy humans, is responsible for two serious human diseases, pyogenic meningitis and meningococcal septicemia.[18,19] Sequence alignment of the *N. meningitidis* KDO8P synthase with other characterised KDO8P synthases reveals an Asn residue is present rather than the metal-binding Cys suggesting this enzyme is metal-independent. In this study we describe the characterisation of the KDO8P synthase from *N. meningitidis* and address the specific roles that the C2, C3 and C4 hydroxyl groups of substrate D-A5P play in the KDO8P synthase reaction. To this end we have prepared the D-A5P diastereomers D-lyxose 5-phosphate and D- and L-xylose 5-phosphate and examined the ability of these compounds and D-R5P to act as substrates for *N. meningitidis* KDO8P synthase. Our findings illuminate a catalytic mechanism for KDO8P synthase in which the correct configurations of the C2 and C3 hydroxyl groups, but not the C4 hydroxyl group, are essential for enzymic reaction.

2. Results

2.1. Cloning, purification and properties of *N. meningitidis* KDO8P synthase

The KDO8P synthase from *N. meningitidis* was successfully cloned into the pT7-7 plasmid and subsequent SDS–PAGE analysis determined that the protein was overexpressed in *E. coli* BL21(DE3) cells and found predominantly in the soluble fraction. Only two purification steps were required to yield pure KDO8P synthase. Substantial separation from contaminants was achieved by anion exchange chromatography in the first purification step (Table 1). Hydrophobic interaction chromatography yielded a further increase in KDO8P synthase purity with only a small loss of protein and a single band of ~ 30,000 Da was visible by SDS–PAGE. The *Nme*N59A mutant was also overexpressed and again found to be predominantly soluble. Once purified the activity of the mutant was determined and was found to have only 0.5% of the wild-type activity for substrate D-A5P.

Table 1
Purification of *N. meningitidis* wild-type KDO8P synthase

Step	Total protein (mg)	Total enzyme (U)	Calculated specific activity ($U\ mg^{-1}$)[a]	Yield (%)	Approximate purity
Crude cell lysate	240	140	0.6	100	1.00
Source™ 15Q	66	115	1.7	28	3.0
Source™ 15Phe and Concentration	48	96	2.0	20	3.5

[a] Specific activity was determined at 25 °C and pH 7.5.

Consistent with the sequence analysis suggesting that this enzyme has an asparagine residue (Asn23) rather than the metal-binding cysteine, the enzyme does not appear to require a metal for catalysis. Assays enriched with Mn^{2+} did not alter activity, and enzyme activity was unchanged by treatment with 1 mM EDTA (data not shown).

2.2. Preparation of D-A5P substrate analogues

A series of A5P analogues was prepared by a combination of chemical and enzymatic syntheses in order to investigate the influence of hydroxyl group configuration on the KDO8P synthase catalysed reaction. D-Lyxose 5-phosphate (D-L5P) was prepared enzymatically from D-lyxose using hexokinase in an analogous reaction to that used for the preparation of D-A5P from D-arabinose (Fig. 2). ^{31}P NMR spectroscopy was used to follow the progress of these reactions.

D-Xylose 5-phosphate (D-X5P) and L-xylose 5-phosphate (L-X5P) were synthesised from D- and L-xylose, respectively, using the procedure outlined in Scheme 1.

2.3. Substrate specificity and kinetics of *N. meningitidis* KDO8P synthase

In order to investigate the substrate specificity and catalytic mechanism of this enzyme a range of phosphorylated aldopentoses were tested as substrates and kinetics were determined for those that showed activity (Table 2). The compounds D-R5P, D-L5P and D-X5P, with altered configuration at C2, C3 or both C2 and C3 relative to D-A5P were not substrates for *N. meningitidis* KDO8P synthase. However, L-X5P, in which only the C4 position is inverted relative to natural substrate D-A5P, was utilised as a substrate by this enzyme, albeit to a lesser extent than D-A5P as evidenced by the 10-fold decrease in the k_{cat}/K_m value. This change in enzyme efficiency resulted from an approximately fivefold increase in the K_m for the phosphorylated monosaccharide. The product of the reaction between PEP and L-X5P was purified and characterised. Consistent with the predicted structure, where the altered stereocentre is outside the pyranose or furanose ring, the ^1H NMR spectrum of this compound, 3-deoxy-L-*gulo*-octulosonate 8-phosphate, was very similar to that of the natural product KDO8P (Fig. 3).

3. Discussion

3.1. Mechanistic implications of substrate specificity

These results show that in order to act as a substrate for KDO8P synthase the correct configuration of the C2 and C3 positions is

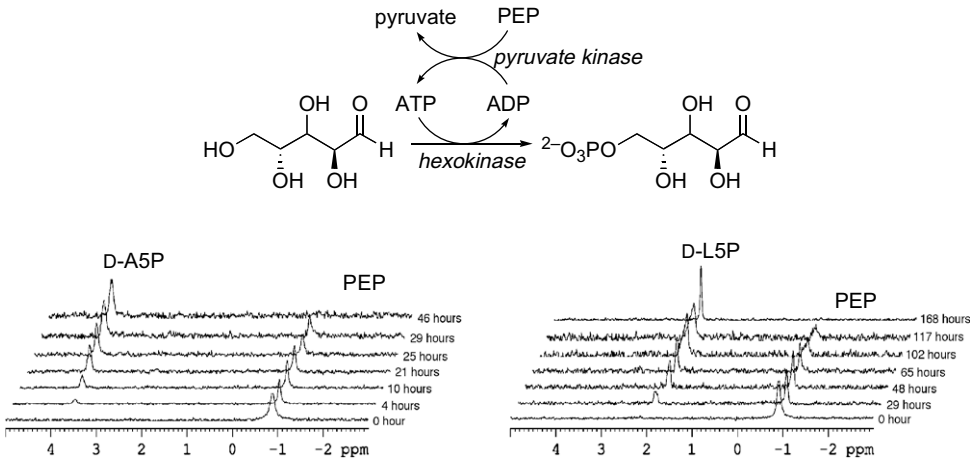
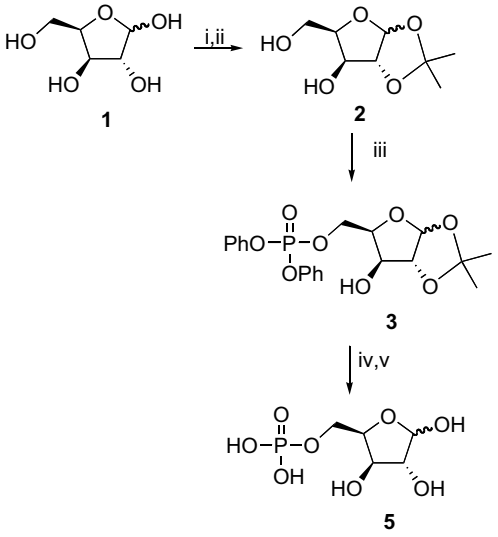


Figure 2. Hexokinase-catalysed preparation of D-A5P and of D-L5P followed by ^{31}P NMR. The reactions were performed at pH 7.6 and at room temperature (~20 °C).

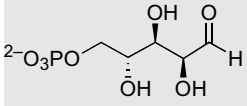
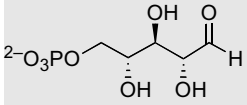
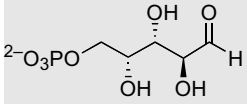
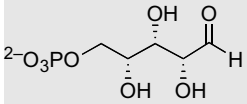
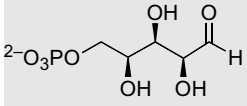


Scheme 1. Synthesis of D-xylose 5-phosphate. Reagents and conditions: (i) Me_2CO, H_2SO_4, $CuSO_4$; (ii) 0.2% HCl; (iii) $ClPO(OPh)_2$, imidazole, CH_2Cl_2, 31%; (iv) Pt_2O, H_2, 100%; (v) H_2O, △, 72%.

crucial, whereas the enzyme is prepared to accept a substrate, L-X5P with the opposite C4 configuration to that of the natural substrate, D-A5P. These results are consistent with the earlier observations showing that 2-deoxy-D-R5P (2-deoxy-D-A5P) is at best an extremely poor substrate for the *E. coli* enzyme, and that 3-deoxy-D-A5P is not accepted as a substrate.[1,16] The C2 and C3 hydroxyl groups of D-A5P are clearly required and it is essential that they be in the correct configuration. Similarly, it has been previously shown that 4-deoxy-D-A5P is a substrate for *E. coli* KDO8P synthase[2], and this observation in combination with the results with L-X5P presented here suggest that KDO8P synthase is ambivalent to changes at C4.

The available structures of *A. aeolicus* and *E. coli* KDO8P synthases and the recently proposed mechanism for this enzyme can be used to rationalise these substrate specificity findings. Despite the variation in metal dependency, the *A. aeolicus* and *E. coli* enzymes have been shown to share similar active sites, and these enzymes have, respectively 43% and 68% identity with the KDO8P synthase from *N. meningitidis*. The structure of the *A. aeolicus* KDO8P synthase bound to both substrates has also been solved allowing an examination of the interactions that the hydroxyl groups of the natural substrate D-A5P have with enzyme residues (Fig. 4). The key step in the reaction mechanism catalysed by this enzyme is attack by C3 of PEP on C1 of

Table 2
Substrate specificity and kinetic parameters *of N. meningitidis* KDO8P synthase

Substrate structures	k_{cat} (s^{-1})	K_m (PEP) (μM)	K_m (mono.) (μM)	k_{cat}/K_m (mono.) (s^{-1} μM^{-1})
D-A5P	2.7 ± 0.6	<1[b]	12 ± 1[f]	
				0.23 ± 0.07
D-R5P[e]				
			NS[a]	
D-L5P[c]				
			NS	
D-X5P[d]				
			NS	
L-X5P	1.1 ± 0.1	2.5 ± 0.1[b]	57 ± 2[f]	
			0.019 ± 0.003	

Assays were performed at pH 7.5 and 30 °C.
[a] NS - compound was determined not to be a substrate.
[b] To determine kinetic parameters for PEP, the assay mixtures contained 1–8 μM PEP with 80 μM D-A5P or 1–2 μM PEP with 300 μM L-X5P.
[c,d,e] Assay mixtures contained 160 μM PEP and either D-L5P (1.5–4 mM), D-X5P (0.7–2 mM), or D-R5P (1–3 mM; Sigma) with 0.016–0.032 U KDO8P synthase.
[f] To determine kinetic parameters for phosphorylated monosaccharides, the reaction mixtures contained 160 μM PEP with 2–300 μM D-A5P or 7–370 μM L-X5P.

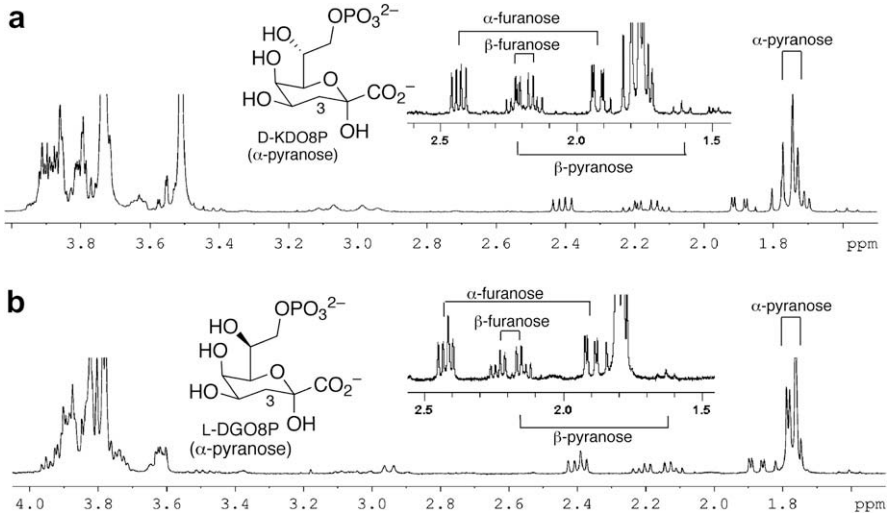


Figure 3. ^{1}H NMR spectra (500 MHz) of (a) KDO8P and (b) 3-deoxy-L-*gulo*-octulosonate 8-phosphate (L-DGO8P), products of the reactions between (a) PEP and D-A5P, or (b) PEP and L-X5P, respectively, catalysed by *N. meningitidis* KDO8P synthase. Inserts show an expansion of the signals corresponding to the H3 protons in pyranose and furanose forms.

D-A5P, and subsequent attack at C2 of PEP by water to form an intermediate, as detected using time-resolved ESI.[5] This initial addition step requires careful placement and activation of the carbonyl moiety of D-A5P in order to ensure the nucleophilic C3 of PEP approaches C1 of D-A5P from the correct angle. Structures indicate that the C2 hydroxyl of D-A5P coordinates (via a water molecule) to the metal in the metal-dependent *A. aeolicus* enzyme and an overlay of structures suggests that the C2 will interact with the absolutely conserved asparagine found instead of cysteine in the metal-independent KDO8P synthases. This interaction between C2 hydroxyl and enzyme is important for controlling the dihedral angle that helps determine positioning of the carbonyl moiety. Altering the stereochemistry at this position would upset this, and consistent with observations made previously, it would be predicted that altering the configuration would have a more significant effect on the ability of D-A5P to act as a substrate than removing this hydroxyl group completely. In line with this, the structure of *A. aeolicus* KDO8P synthase in complex with PEP and D-R5P shows the C2 hydroxyl is closer to the metal and that there is significant displacement of C1 of D-R5P. The structure of the *A. aeolicus* enzyme with D-A5P bound indicates that the C3 hydroxyl group interacts with an asparagine residue (Asn48 in *A. aeolicus*, Asn62 in *E. coli* and Asn59 in *N. meningitidis*) that also H-bonds to the carbonyl. This residue is absolutely conserved in all KDO8P synthases sequenced to date and this interaction would be expected to be critical to (and have a direct bearing on) the correct positioning of the carbonyl moiety of D-A5P. Hence inversion (this study) or loss of the C3 hydroxyl group dramatically influences catalysis.[1] In line with this we have found that the *Nme*N59A mutation of *N. meningitidis* KDO8P synthase has less than 1% activity of the wild-type with natural substrate D-A5P. In contrast, the C4 hydroxyl group of D-A5P makes no obvious interactions that might influence the placement of the carbonyl moiety, and therefore inversion at this centre is not as catastrophic.

4. Conclusion

In summary, our results with modified substrates and with the N59A mutant *N. meningitidis* enzyme are consistent with a mechanism for KDO8P synthase in which the protein interactions with the C2 and C3 but not the C4 hydroxyl groups are essential for the correct positioning of D-A5P's carbonyl group for attack on PEP's olefin.

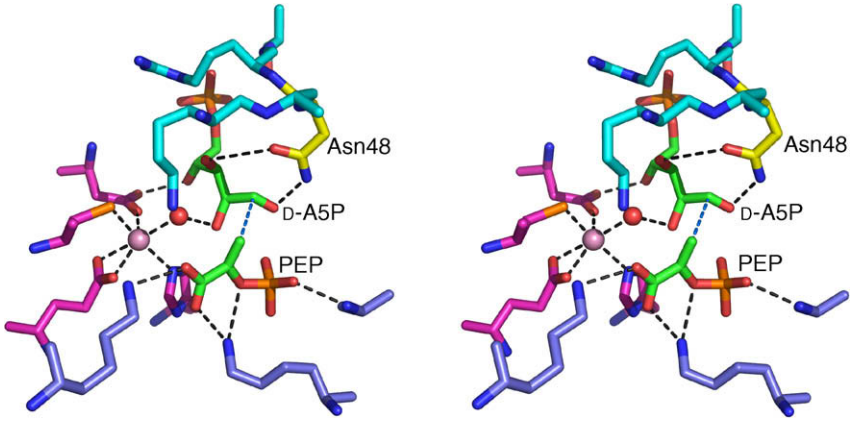


Figure 4. Stereo diagram showing the active site of *A. aeolicus* KDO8PS (PDB 1FWW[9]). Metal and metal ligands are in magenta and PEP ligands are shown in slate. Substrates, PEP and D-A5P are shown in green. L-A5P ligands are shown in cyan. Asn48 of *A. aeolicus* KDO8PS, which corresponds to Asn59 of *N. meningitidis* KDO8PS, is shown in yellow (note that Asn48 is rotated 180° compared to the published structure for sensible carbonyl (D-A5P)C=O. . .HN(Asn59) hydrogen bond).

5. Experimental

5.1. Cloning and expression of *N. meningitidis* KDO8P synthase

An open reading frame, annotated as encoding KDO8P synthase (*kdsA) from N. meningitidis* serotype B strain MC58 (ATCC BAA-355), was retrieved with flanking 5′ and 3′ regions from Genbank (http://www.ncbi.nlm.nih.gov/entrez/; Genbank Accession No. AE002098, gi: 7226523; Protein Accession No. AAF41659). Standard PCR methodologies were employed to amplify the gene sequence from *N. meningitidis* genomic DNA (ATCC) using the primers NmeK*Nde*F (5′-GGTGGTCATATGGATATTAAAATCAACGACA TCAC) and NmeK*Bam*R (5′-CGAGGATCCTTTACTC**A**ATTGTTAAAATC GGTTGTGA) to introduce NdeI and BamHI restriction sites (underlined) into the 862 bp PCR product. The synonymous substitution of **A** for G in NmeK*Bam*R was introduced to decrease the strong secondary structure predicted for this oligonucleotide. The amplified PCR product was recovered directly using a High Pure PCR product purification kit (Roche), digested with NdeI and BamHI, ethanol precipitated and ligated into pT7-7,[20] which had been previously digested with the same endonucleases. The resulting construct was used to transform *E. coli* TG1 (Stratagene) cells. The sequence of the construct was confirmed using both sense and antisense strands as templates before the pT7-7-Nme*kdsA* plasmid was transformed into *E. coli* BL21(DE3) cells (Novagen).

Site-directed mutagenesis was performed using the QuikChange® II site-directed mutagenesis kit (Stratagene) to create the Asn59Ala (*Nme*N59A) mutant of *N. meningitidis* KDO8P synthase. Mutagenic primers NmeKN59AF 5′-CTCTTTCGACAAGGCA**GC A**CGTTCCTCCATCCATTC and NmeKN59AR 5′-GAATGGATGGAGGAA CG**TGC**TGCCTTGTCGAAAGAG (mutation shown in bold) were used to convert the asparagine to alanine. The sequence of the mutant was verified, after which the plasmid was transformed into *E. coli* BL21(DE3) cells, then the mutant was overexpressed and purified as described below.

5.2. Protein purification

The recombinant *kdsA* and the *Nme*N59A mutant genes were both expressed using the same procedure. Overnight starter cultures of *E. coli* BL21(DE3) cells harbouring either pT7-7-Nme*kdsA* or pT7-7-*Nme*N59A grown in 10 mL Luria Bertani (LB) broth supplemented with ampicillin (0.1 mg mL^{-1}) were used the following day to inoculate two 1 L of LB broth containing 0.1 mg mL^{-1} ampicillin. The cultures were grown at 37 °C with vigorous shaking to an OD_{600} of 0.4–0.6, whereupon isopropyl thio-β-D-galactoside (IPTG) was added (final concentration 1 mM). The cultures were grown for a further 4 h at 37 °C then harvested by centrifugation (4400*g*, 20 min, 4 °C). Purification of the KDO8P synthase *Nme*N59A mutant was identical to that of the Nme*kdsA* gene product. Cell pellets were resuspended in a minimal volume (~8 mL) of cold lysis buffer (10 mM BTP, pH 7.5, 1 mM DTT, 200 mM KCl and 200 μM PEP) and lysed by sonication on ice (15 × 15 s with 1 min intervals between bursts). Cellular debris were removed by centrifugation (26,700*g*, 20 min, 4 °C) and the supernatant diluted fivefold with Buffer A (10 mM BTP, pH 7.5, 1 mM DTT). The diluted protein was then loaded onto a Source™ 15Q column (Amersham; 10 × 90 mm; 4 °C) pre-equilibrated with Buffer A at a flow rate of 2 mL min^{-1}. After washing the column with Buffer A (2 column volumes, CV), a linear gradient from 0 to 0.11 M NaCl (5 CV) was applied, then KDO8P synthase was eluted by a shallow linear gradient from 0.11 to 0.18 M NaCl (6.5 CV) during which 3 mL fractions were collected. Protein was analysed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) using Laemmli's buffer system under denaturing and reducing conditions.[21] Fractions that were determined to contain mainly the KDO8P synthase were pooled and solid $(NH_4)_2SO_4$ was added (final concentration 1 M). The protein was loaded onto a Source™ 15Phe column (Amersham, 10 × 105 mm; ambient temperature) pre-equilibrated with Buffer B (10 mM BTP, pH 7.5, 1 mM DTT, 1 M $(NH_4)_2SO_4$) at a flow rate of 1 mL min^{-1}. Nonspecific protein was removed first by washing the column with Buffer B (3.5 CV) after which a sharp linear gradient was applied from 1 to 0.68 M $(NH_4)_2SO_4$ for 0.6 CV. KDO8P synthase was eluted by a linear gradient from 0.68 to 0.3 M $(NH_4)_2SO_4$ (~10 CV) during which 3 mL fractions were collected. Protein was again analysed by SDS–PAGE and fractions that were determined to be pure KDO8P synthase (or N59A_KDO8P synthase) were pooled and both concentrated and desalted using a prewashed 20 mL Vivaspin concentrator (10,000 MWCO; Vivascience). Aliquots (100 μL) of the purified enzymes were flash-frozen and stored at −80 °C. Protein concentrations were determined by the Bradford method using bovine serum albumin as standard.[22]

5.3. Enzyme assays

Enzyme activity was determined by monitoring the consumption of PEP at 232 nm ($\varepsilon = 2.8 \times 10^3\ M^{-1}\ cm^{-1}$) using a continuous spectrophotometric assay at 30 °C.[23] Assays to determine substrate specificity contained 160 μM PEP, 0.016 U KDO8P synthase and either D-A5P (100 μM), or D-L5P (1.5–4 mM), or L-X5P (300 μM), or D-X5P (0.7–2 mM), or D-R5P (1–3 mM; Sigma) in 50 mM BTP buffer (pH 7.5) in a total volume of 1 mL. Assays were initiated with the addition of enzyme. Also assessed was the effect of EDTA on the activity of wild-type KDO8P synthase over time. An aliquot of purified KDO8P synthase was removed prior to mixing the remainder 1:1 with 2 mM EDTA. Assays (1 mL) containing 100 μM PEP, 50 mM BTP buffer (pH 7.5), and either 0.005 U untreated KDO8P synthase or EDTA-treated protein were initiated with 100 μM D-A5P. Activity of the proteins was checked after 5, 10, 15, 30, and 60 min as well as 2, 4, 8 and 24 h. For determination of the effect of metal ion, assays were carried out in the presence of $MnSO_4.H_2O$ (Sigma). Each 1 mL assay consisted of 100 μM PEP, 100 μM D-A5P, 0.006 U KDO8P synthase in 50 mM BTP (pH 7.5) with or without 90 μM $MnSO_4$ and initiated by the addition of D-A5P. Assays to determine the activity of the mutant (N59A) contained 200 μM PEP and 0.017 U *Nme*N59A in 50 mM BTP (pH 7.5) and were initiated with 100 μM D-A5P. All assays were preincubated at 30 °C for 5–6 min prior to initiation and were performed in duplicate.

5.4. Michaelis–Menten kinetics

To determine the kinetics for PEP use, reaction mixtures contained either, PEP (1–8) μM and 80 μM D-A5P, or PEP (1–20 μM) and 300 μM L-X5P in 50 mM BTP (pH 7.5). To determine the parameters for the use of the aldose phosphate co-substrate assays consisted of PEP (160 μM) and either D-A5P (2-300 μM) or L-X5P (7.4–370 μM) in 50 mM BTP (pH 7.5). Reaction mixes were preincubated for 5–6 min at 30 °C prior to initiation and brought to the final 1 mL volume with the addition of enzyme (0.016 U *N. meningitidis* KDO8P synthase). All kinetic experiments were carried out in duplicate and the kinetic parameters were determined by fitting the data to the Michaelis–Menten equation using Enzfitter (Biosoft).

5.5. Preparation of D-xylose 5-phosphate

(−)-1,2-*O*-isopropylidene-α-D-xylofuranose **2** was prepared from D-xylose **1** in two steps according to previously reported procedures.[24,25]

5.6. Synthesis of (−)-1,2-*O*-isopropylidene–D-diphenylphospho xylofuranose 3

(−)-1,2-*O*-Isopropylidene-α-D-xylofuranose **3** (0.95 g, 5 mmol) was dissolved in 150 mL of dry CH_2Cl_2. Imidazole (1.36 g, 0.02 mol) and diphenylchlorophosphate (1.4 mL, 7 mmol) were added at 0 °C after which the reaction mixture was stirred under N_2 for 6 h. The reaction was stopped by adding 10 mL H_2O and 10% (v/v) HCl (10 mL). The aqueous layer was extracted three times with CH_2Cl_2 (50 mL) and the combined organic extracts were washed with saturated $NaHCO_3$ (20 mL), H_2O (2 × 20 mL), and then saturated NaCl (20 mL). The organic layer was dried with $MgSO_4$ and concentrated. Purification by flash chromatography (3:2 (v/v) ethyl acetate/hexane) gave **3** (0.65 g, 31%) as a colourless syrup.

R_F (hexane/EtOAc, 1:1) = 0.5.

m/*z* (+ve FAB) $(M+H^+)$ $C_{20}H_{24}O_8P$, calcd 423.12088 found 423.1208.

^1H NMR (400 MHz, $CDCl_3$) δ 7.3 (m, 10H, Ar*H*), 5.85 (d, *J* = 3.5 Hz, 1H), 4.5 (m, 2H), 4.28 (m, 2H), 4.07 (d, *J* = 1.9 Hz, 1H), 1.45 (s, 3H), 1.27 (s, 3H) ppm; ^{13}C NMR (125 MHz, $CDCl_3$) δ 150.2 (d, *J* = 15 Hz), 130.0, 125.8 (d, *J* = 13.5 Hz), 120.1 (d, *J* = 14.3 Hz), 115.4, 105.0, 85.1, 78.8, 73.8, 65.2, 26.9, 26.2 ppm.

5.7. Synthesis of (−)-1,2-*O*-isopropylidene–D-5-phospho xylofuranose 4

(−)-1,2-*O*-Isopropylidene-α-D-5-diphenylphospho xylofuranose **3** (0.65 g, 1.5 mmol) was dissolved in methanol and Pt_2O (0.34 g, 1.5 mmol) was added. The reaction mixture was then stirred overnight under a complete H_2 atmosphere at room temperature. Removal of solvent in vacuo gave **4** (0.42 g, 100%).

^1H NMR (400 MHz, $CDCl_3$) δ 5.80 (d, 1 H, *J* = 3.7 Hz), 4.45 (d, 1 H, *J* = 3.7 Hz), 4.19 (m, 1 H), 4.07 (d, 1 H, *J* = 2.8 Hz), 3.95 (m, 1 H), 3.83 (m, 1 H), 1.27 (s, 3 H), 1.11 (s, 3 H) ppm. ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 113.0, 104.8, 84.8, 79.9, 73.9, 64.0, 25.8, 25.3 ppm.

5.8. Synthesis of D-xylose 5-phosphate 5

(−)-1,2-*O*-Isopropylidene-α-D-5-phospho xylofuranose **4** (0.42 g, 1.56 mmol) was dissolved in 15 mL of H_2O and stirred for two days at 50 °C. The progress of the reaction was followed by ^1H NMR. The D-X5P mixture was loaded onto a Source™ 15Q column (Amersham; 10 × 95 mm; 4 °C) pre-equilibrated with H_2O at a flow rate of 2 mL min^{-1}. After washing the column with 1.3 CV of H_2O, a linear gradient from 0 to 0.5 M NH_4CO_3 (20 CV) was applied which eluted the D-X5P. Fractions containing D-X5P (determined by the loss of PEP in an assay catalysed by *E. coli* DAH7P synthase) were pooled and freeze-dried, giving **5** (0.26 g, 72%).

m/*z* (−ve ESI) $(M-H)^-$ $C_5H_{10}O_8P$; calcd 229.0113 found 229.0113.

5.9. Preparation of L-xylose 5-phosphate

L-X5P (0.2 g, 13% overall yield) was prepared starting with L-xylose (1 g, 6.67 mmol) following the same procedures for the synthesis of D-X5P. NMR spectra from the intermediate compounds appeared to be same as those recorded for the synthesis of D-X5P.

m/*z* (−ve ESI) $(M-H)^-$ $C_5H_{10}O_8P$; calcd 229.0113 found 229.0110.

5.10. Preparation of D-arabinose 5-phosphate and D-lyxose 5-phosphate[26]

D-Arabinose 5-phosphate and D-lyxose 5-phosphate were prepared using similar procedures. PEP (0.08 g, 0.34 mmol) and ATP (5.5 mg, 10 μmol) were added to D-arabinose (0.05 g, 0.3 mmol) dissolved in 5 mL H_2O. $MgSO_4$ (14 mg, 0.17 mmol) and KCl (8.4 mg, 0.13 mmol) were then added. The pH was adjusted to 7.6 with 10% NaOH after which 2-mercaptoethanol (10 μL, 0.15 mmol) and pyruvate kinase (218 U) were added. The reaction was initiated by the addition of hexokinase (270 U) and left to stir at room temperature. The progress of the reaction was followed by ^{31}P NMR. The reaction for D-A5P was completed after 46 h. For the D-lyxose 5-phosphate reaction (0.05 g, 0.3 mmol), more $MgSO_4$ (14 mg, 0.17 mmol) and hexokinase (109 U) were required and were added after 25 h to the reaction mixture, which was then allowed to stir for a further 28 h before more $MgSO_4$ (100 mg, 0.83 mmol), pyruvate kinase (436 U), and hexokinase (540 U) were introduced. After an additional five days, the reaction for D-L5P was completed. Protein in D-A5P and D-L5P mixtures was removed using centrifugal filtration devices (10,000 MWCO; Vivaspin), and D-A5P and D-L5P were purified by anion exchange chromatography as described for D-xylose 5-phosphate. Fractions containing D-A5P or D-L5P were identified by PEP consumption when incubated with *N. meningitidis* KDO8P synthase or by using Bial's reagent.[27] These fractions were pooled and freeze-dried, giving D-A5P (73 mg, 95%) and D-L5P (58 mg, 76%).

m/*z* (−ve ESI, D-A5P) $(M-H)^-$ $C_5H_{10}O_8P$ calcd 229.0113, found 229.0119.

m/*z* (−ve ESI, D-L5P) $(M-H)^-$ $C_5H_{10}O_8P$; calcd 229.0113, found 229.0115.

5.11. KDO8P and 3-deoxy-L-gulo-octulosonate 8-phosphate (DGO8P) preparation and isolation

Large scale enzymatic reactions were performed using *N. meningitidis* KDO8P synthase to generate both KDO8P from PEP and D-A5P, and DGO8P from PEP and L-X5P. For KDO8P, D-A5P (11 mg, 47.3 μmol) and PEP (11 mg, 53.4 μmol) were dissolved in H_2O (8 mL) and the pH was adjusted to pH 7 using 1 M NaOH. The reaction was initiated by the addition of KDO8P synthase (0.6 mg) and the loss of PEP at 232 nm was monitored. After 2 to 3 h, the loss of PEP had ceased, whereupon the enzyme was removed as described above. The same procedure was used to generate DGO8P with L-X5P (2 mg, 9 μmol) and PEP (2 mg, 9.7 μmol) dissolved in 6 mL H_2O. The products of both reactions were purified using anion exchange chromatography (using the established method described above but with a linear gradient of 0–1 M NH_4HCO_3 (23 CV) applied to elute the phosphorylated monosaccharides). The thiobarbituric acid assay was used to identify fractions that contained the 3-deoxyaldulosonic acid product. Fractions that tested positive were pooled and freeze-dried (giving 15 mg of KDO8P and 1.5 mg of DGO8P). The products were stored at −80 °C prior to NMR and mass spectral analysis.

m/*z* (−ve ESI, KDO8P) M^- $C_8H_{14}O_{11}P$; calcd 317.0274, found 317.0277; found $(M-H+Na)^-$ 329.0246; found $(M^- - H_2O)$ 299.0197.

m/*z* (−ve ESI, DGO8P) M^- $C_8H_{14}O_{11}P$; calcd 317.0274, found 317.0273; found $(M+Na-H)^-$ 329.0242; found $(M^- - H_2O)$ 299.0193.

5.12. Thiobarbituric acid assay[13,28]

Aliquots (100 μL) of the reaction mixture were mixed with 50 μL H_2O and 100 μL $NaIO_4$ (25 mM solution in 0.125 N H_2SO_4) and heated for 1 h at 60 °C. Excess oxidising agent was removed by the addition of 200 μL sodium metaarsenite (2% w/v in 0.5 N HCl). Once the yellow colouration disappeared, thiobarbituric acid (1 mL of 0.36% w/v solution adjusted to pH 9 with NaOH) was added and the reaction mixture was heated at 100 °C for 10 min.

After the sample was cooled to 25 °C the absorbance was measured at 549 nm ($\varepsilon = 1.03 \times 10^5\ M^{-1}\ cm^{-1}$).

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