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A Thermostable Aldolase for the Synthesis of 3-Deoxy-2-ulosonic Acids

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This paper is dedicated to Professor J. Grant Buchanan on the occasion of his 80th birthday.

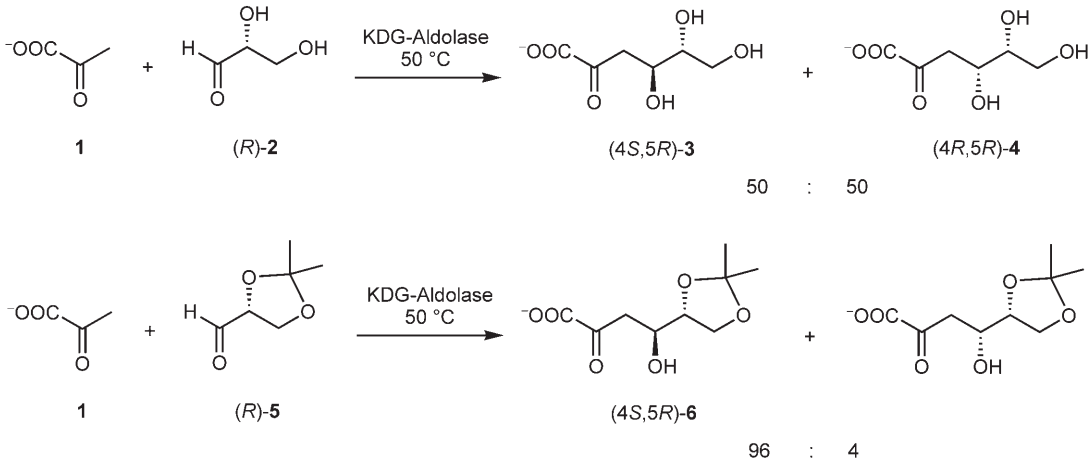
Abstract: A stereochemically promiscuous 2-keto-3-deoxygluconate aldolase has been used as an efficient biocatalyst to catalyse the aldol reaction of pyruvate with C_3- and C_4-aldoses to afford *syn*- and *anti*-3-deoxy-2-ulosonic acids in poor to good *de*. A continuous flow bioreactor containing immobilised aldolase has been developed that enables gram quantities of C_6- and C_7-3-deoxyhept-2-ulosonic acids to be produced in an efficient manner.

Keywords: aldol reaction; aldolase; 3-deoxy-2-ulosonic acids; diastereoselectivity; pyruvate

3-Deoxy-2-ulosonic acids are an important class of natural product that exhibit a number of key biological functions.[1] For example, (4*S*,5*R*)-D-2-keto-3-deoxygluconate (D-KDG) and (4*R*,5*R*)-D-2-keto-3-deoxygalactonate (D-KDGal) are intermediates of microbial hexose catabolism,[2] whilst D-KDG is a metabolic product of human erythrocytes whose levels are often elevated in diabetic patients.[3] The 7-phospho derivative of (4*R*,5*R*,6*R*)-3-deoxy-D-*arabino*-hept-2-ulosonate (DAH) is an intermediate in the shikimic acid pathway, which is employed for aromatic amino acid biosynthesis in bacteria, fungi and plants.[4] 3-Deoxy-D-*manno*-2-octulosonate (KDO) forms a critical link between lipid A and hydrophilic polysaccharide subunits in the outer membrane lipopolysaccharide of Gram-negative bacteria.[5] 5-Acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulosonate (*N*-acetylneuraminate; sialic acid) occurs at the terminus of oligosaccharide chains in membrane-bound glycoproteins and glycolipids, which perform an essential function in cell-cell recognition, cell-adhesion and tumour metastasis.[6] Given this biological importance, analogues of 3-deoxy-2-ulosonic acid have also been developed as bioactive compounds, including anti-microbials,[7] herbicides,[8] anti-virals[9] and anti-tumour compounds.[10]

Consequently, considerable attention has been devoted to the development of efficient synthetic methodology for the preparation of 3-deoxy-2-ulosonic acids and their analogues.[11] One of the most efficient approaches involves the use of pyruvate aldolases as versatile biocatalysts to catalyse stereoselective aldol reactions of pyruvate with aldose acceptors.[12] These type I aldolases use the ε-amino group of active-site lysine residues to generate Schiff base intermediates that facilitate the delivery of enamine equivalents of pyruvate to different aldehyde substrates. This has enabled the direct asymmetric synthesis of a wide range of 3-deoxy-2-ulosonic acids without recourse to protecting group chemistry.[13]

2-Keto-3-deoxygluconate aldolase (KDG-aldolase) from the hyperthermophile *Sulfolobus solfataricus*, is a type I pyruvate aldolase that can be efficiently expressed as a recombinant protein in *Escherichia coli*.[14] We have shown that this thermostable aldolase exhibits no diastereocontrol for the aldol condensation of its natural substrates, pyruvate **1** and D-glyceraldehyde **2**, which affords approximately equal amounts of D-KDG **3** and D-KDGal **4** in good yield.[15,16] A substrate engineering technique has been developed to engineer stereocontrol into the aldol reaction of C_3-aldoses using D-glyceraldehyde acetonide **5** as an alternative substrate, which enables the stereoselective synthesis of D-KDG acetonide **6** in >92% *de* (Scheme 1).[7] This KDG-aldolase displays a relatively broad specificity profile towards non-phosphorylated aldehydes and we now report herein on an investigation into its activity towards a range of C_3-, C_4- and C_5-aldoses,[18] and its use as an immobilised biocatalyst within a recyclable bioreactor for the



Scheme 1. KDG-aldolase-catalysed aldol reaction of pyruvate with D-glyceraldehyde **2** and D-glyceraldehyde acetonide **5**.

efficient preparation of C_6- and C_7-3-deoxy-2-ulosonic acids.

The KDG-aldolase was assayed as a biocatalyst for the aldol condensation of pyruvate with a range of C_3-, C_4- and C_5-aldose substrates using a modified thiobarbituric acid assay to measure the formation of 3-deoxy-2-ulosonic acids over time.[19] The resultant activities revealed that both the (*R*)- and (*S*)-enantiomers of glyceraldehyde were excellent substrates for the aldolase, with activities around 10 μmol·min^{-1}·mg^{-1} in each case. Reverse phase HPLC analysis of the aldol products arising from use of D-glyceraldehyde and L-glyceraldehyde revealed that both reactions proceeded with essentially no diastereocontrol, affording approximately 50:50 mixtures of their respective KDG and KDGal diastereoisomers (Table 1).

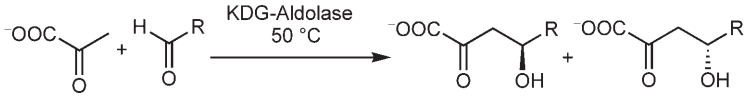
The KDG-aldolase also demonstrated synthetically useful levels of reactivity towards all four stereoisomeric C_4-aldoses assayed, displaying activities of between 0.80 and 4.95 μmol·min^{-1}·mg^{-1} for aldol reaction of the enantiomers of threose and erythrose with pyruvate. Reverse phase HPLC analysis of the crude reaction products arising from these aldol reactions revealed that mixtures of their respective *syn*- and *anti*-aldol diastereoisomers had been formed in varying ratios. For example, the aldol reaction of L-erythrose with pyruvate was poorly selective, affording a mixture of diastereoisomeric 3-deoxy-hept-2-ulosonic acids in a ratio of 60:40. However, the aldol condensation reaction of L-threose with pyruvate was highly stereoselective, affording a major aldol diastereoisomer in 90:10 *dr*.

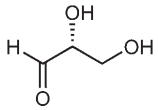
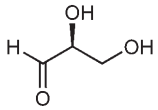
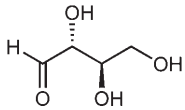
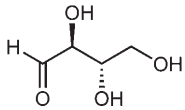
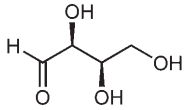
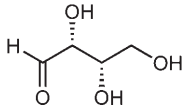
Therefore, it is clear that the relative and absolute configurations of the C-2 and C-3 hydroxy groups of these C_4-aldose substrates play an important role in controlling the facial selectivity of their KDG-aldolase-catalysed reactions with pyruvate (Table 1). The KDG-aldolase displayed 100-fold lower levels of reactivity towards all eight C_5-aldoses screened,[20] displaying activities of between 0.03 and 0.06 μmol·min^{-1}·mg^{-1} in each case. Unfortunately, the low level of reactivity observed for these C5-aldoses prevented the diastereoselectivity of their aldol reactions from being determined.

In order to carry out preparative reactions it was decided to immobilise the aldolase on to a solid support so that it could be used as a recyclable biocatalyst for continuous operation. Therefore, recombinant KDG-aldolase was efficiently expressed containing a histidine tag at its *N*-terminus, which enabled it to be immobilised *via* metal chelation to Ni^{2+} ions on a His-bind resin. Ten grams of KDG-aldolase His-bind resin were packed into a column to create a bioreactor containing approximately 60 mg of immobilised enzyme. Pumping an aqueous solution of C_3- or C_4-aldehyde and pyruvate through the KDG-aldolase column, using a thermal jacket to maintain its temperature at 50 °C, enabled gram quantities of the corresponding 3-deoxy-2-ulosonic acids to be obtained. For example, this bioreactor was employed to continuously transform 100 mM (*rac*)-glyceraldehyde **2** (9 g·L^{-1})[21] and 200 mM pyruvate **1** (22 g·L^{-1}, two-fold molar excess) at a flow rate of 1 mL·min^{-1}. This afforded > 1.0 g·h^{-1} of a 50:50 mixture of (*rac*)-KDG **3** and (*rac*)-KDGal **4**, with no loss in aldolase activity occurring over a period of 60 h continuous use.

Diastereoisomeric mixtures of *syn*- and *anti*-3-deoxy-2-ulosonic acids produced in these KDG-aldolase-catalysed reactions could be separated on a preparative scale by Dowex 1X8-formate anion exchange chromatography. For example, partial separation of 2.0 g of a 50:50 mixture of D-KDG and D-KDGal[22] was achieved to afford diastereoisomerically pure D-KDG (250 mg, $[\alpha]_D^{25}$: −34.1 {*c* 1.3, H_2O), Lit. $[\alpha]_D^{25}$: −33.1 (*c* 1.3, H_2O)[22a]} and D-KDGal {174 mg, $[\alpha]_D^{25}$: +7.3 (*c* 2, H_2O), Lit. $[\alpha]_D^{25}$: +7.9 (*c* 1.65, H_2O)[23]}.[24] Therefore, whilst KDG-aldolase-catalysed aldol reac-

Table 1. Activity and diastereoselectivity of KDG-aldolase-catalysed aldol reaction of pyruvate with C_3- and C_4-aldoses.

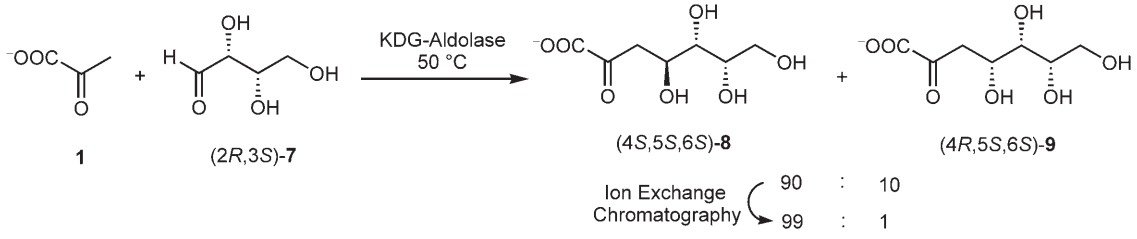


Aldehydes (RCHO)		Rate [μmol·min^{-1}·mg^{-1}]	Ratio of Aldol Diastereoisomers[a,b]
D-Glyceraldehyde		11.4	45:55[c]
L-Glyceraldehyde		9.71	50:50
D-Erythrose		1.36	35:65[d]
L-Erythrose		0.80	60:40[d]
D-Threose		1.55	40:60[d]
L-Threose		4.95	90:10[c]

[a] Ratio of diastereoisomers determined by HPLC analysis using a Bio-Rad Aminex HPX-87H organic analysis column linked to an RID-10 A refractive index detector.
[b] Ratio reflects order of elution of major diastereoisomer during HPLC analysis.
[c] Major diastereoisomer has 4(*S*)-configuration.
[d] Absolute configuration of major diastereoisomer not determined.

tions of D-threose and both enantiomers of erythrose proceed with a lack of stereocontrol, the ability to separate the resultant mixtures of *syn*-/*anti*-aldol diastereoisomers means that KDG-aldolase can be used to access seven out of the eight possible stereoisomers of the 3-deoxyhept-2-ulosonic acids.[25]

Since we had shown that L-threose was an excellent substrate for the KDG-aldolase that afforded a highly diastereoselective aldol reaction, we employed it as a substrate to carry out a preparative aldol reaction using the KDG-aldolase column bioreactor. This resulted in a first synthesis of (4*S*,5*S*,6*S*)-3-deoxy-L-*lyxo*-hept-2-ulosonate **8** in 70% yield and a diastereoisomeric ratio of >99:1 after purification by ion-exchange chromatography (Scheme 2). The stereochemistry of the newly formed stereocentre of this 3-deoxy-2-ulosonic acid **8** was assigned as (4*S*) by comparison of its ^{1}H NMR spectra with the spectra previously reported for *ent*-**8**,[25a] and *via* direct comparison with the ^{1}H NMR spectra of D-KDG **3** and D-KDGal **4** which clearly revealed its similarity to D-KDG which also contains a (4*S*)-stereocentre.



Scheme 2. Preparative aldol reaction of KDG-aldolase using L-threose as substrate.

In conclusion, we have demonstrated that a stereochemically promiscuous KDG-aldolase from the hyperthermophile *Sulfolobus solfataricus* may be used as an efficient biocatalyst for the aldol reaction of pyruvate with C_3- and C_4-aldoses to afford *syn*- and *anti*-3-deoxy-2-ulosonic acids with poor to good levels of diastereocontrol.

Experimental Section

Expression and Immobilization of Recombinant KDG-Aldolase

S. solfataricus KDGA was expressed with a polyhistidine tag (MGHHHHHHHHHHSSGHIDDDDKH) on the N-terminus of the protein, using the pET-19b expression vector with the gene cloned into the *Nde*I and *Bam*HI restriction sites. *E. coli* BL21(DE3) was transformed using the recommended protocol and 1-L cultures were incubated overnight at 37 °C without induction in LB media containing 50 $\mu g \cdot mL^{-1}$ carbenicillin. Cells were harvested by centrifugation and an extract was made using BugBuster/Benzonase. His-bind resin was used to purify and immobilise KDGA in a single step, following the recommended manufacturer's protocol (Novagen), but omitting the elution of bound protein with imidazole. To assess the success of the immobilisation process, a small amount of resin was treated with imidazole and the eluted enzyme sample analysed by the thiobarbituric acid assay and on a 10% (w/v) sodium dodecyl sulphate-polyacrylamide electrophoresis gel.

Representative Experimental Protocol

L-Threose (0.25 g, 50 mmol) and 0.47 g of pyruvate (100 mmol) were dissolved in distilled water (42 mL). The resulting solution was continuously pumped through the column bioreactor at 0.5 $mL \cdot min^{-1}$ with the temperature maintained at 50 °C by a thermal jacket connected to a water bath. Substrate conversion was monitored by HPLC analysis using a Bio-Rad Aminex HPX-87H organic analysis column linked to an RID-10 A refractive index detector, which showed distinct peaks for pyruvate **1** ($R_t = 13.03$ min), L-threose (2*R*,3*S*)-**7** ($R_t = 13.64$ min), (4*S*,5*S*,6*S*)-3-deoxy-L-*lyxo*-hept-2-ulosonate **8** ($R_t = 10.73$ min) and (4*R*,5*S*,6*S*)-3-deoxy-L-*xylo*-hept-2-ulosonate **9** ($R_t = 11.20$ min). HPLC peak areas revealed that 95% of L-threose had been consumed, forming (4*S*,5*S*,6*S*)-**8** in a *dr* of 90:10 after 6 h. The column was then washed with 50 mL of distilled water which was then pooled with the reaction product. The combined fractions were then lyophilised and purified by Dowex 1X8-formate anion exchange chromatography using a 0–-0.6 M formic acid elution gradient. Selected fractions were combined and dried to afford (4*S*,5*S*,6*S*)-3-deoxy-L-*lyxo*-hept-2-ulosonate **8** in >99:1 dr; yield: 307 mg (70%); $[\alpha]_D^{25}$: −33.0 (*c* 2.0, H_2O), Lit. $[\alpha]_D^{25}$: +37.0 (*c* 2.25, H_2O) for (4*R*,5*R*,6*R*)-3-deoxy-D-*lyxo*-hept-2-ulosonate *ent*-**8**; HR-MS: $m/z = 207.0507$, calcd. for $C_7H_{11}O_7$: 207.0510.

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