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2-Keto-3-deoxy-6-phosphogluconate Aldolases as Catalysts for Stereocontrolled Carbon−Carbon Bond Formation

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Abstract: The pyruvate aldolases use pyruvate as the nucleophilic component in stereoselective aldol condensations, producing a 4-hydroxy-2-ketobutyrate framework. We have examined the 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolases from *Pseudomonas putida*, *Escherichia coli*, and *Zymomonas mobilis* for utility as synthetic reagents. Unlike other pyruvate aldolases examined to date, the KDPG aldolases accept short-chain, non-carbohydrate electrophilic aldehydes as substrates, providing a general methodology for the construction of the 4-hydroxy-2-ketobutyrate skeleton. The three aldolases differ markedly with respect to enzyme stability, pH optima, stability in organic cosolvent mixtures, substrate specificity, and diastereoselectivity during aldol condensation. All three enzymes show broad substrate specificity with regard to the electrophilic component. The primary requirements for substrate activity appear to be minimal steric hindrance and the presence of electron-withdrawing substituents at C2. The aldolases from *Pseudomonas* and *Escherichia* are also specific for the D-stereochemical configuration at C2, while the enzyme from *Zymomonas* displays no stereochemical discrimination with regard to the electrophilic substrate. Nucleophiles other than pyruvate are accepted as nucleophilic substrates by all three enzymes, provided the electrophile is sufficiently reactive. In preparative scale reactions with three unnatural electrophiles, the three enzymes show varying degrees of stereochemical fidelity. In most cases, a single diastereomer of the aldol adduct was produced, although in one case, a diastereomeric excess of 50% was observed. In all cases, the diastereoselectivity is exclusively kinetic in origin, despite the reversibility of some reactions. The enzymes are remarkably tolerant of added cosolvent: all three showed >60% of native activity in 30% DMSO and DMF. By appropriate choice of enzyme, the KDPG aldolases offer exceptional utility for stereocontrolled carbon−carbon bond formation under a wide range of experimental conditions.

Introduction

After over 100 years of routine use in organic synthesis, enzymes are by now firmly established as powerful tools in the armamentarium of the organic chemist. Although much of the early use of enzymes in organic synthesis involved oxidoreductases and the hydrolytic enzymes, enzyme-catalyzed bond formation has more recently become an area of intense interest.[1−10] Among enzymes that form carbon−carbon or carbon−heteroatom bonds, the aldolases, those enzymes that catalyze stereoselective carbon−carbon bond formation, are of particular interest. Three broad groups of aldolases can be identified according to nucleophile type (Scheme 1). The best

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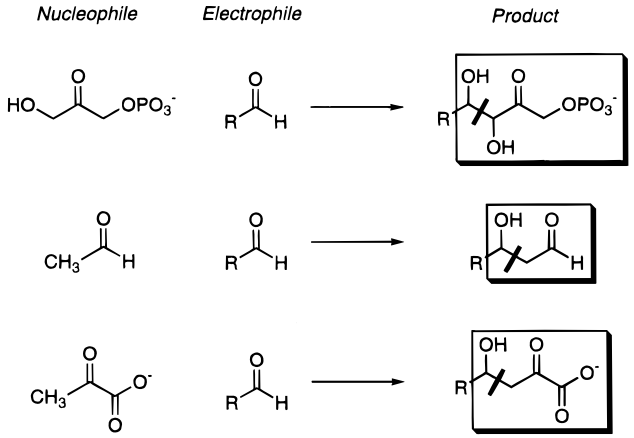
Scheme 1. General Representation of Aldol Condensations Catalyzed by the Dihydroxyacetone Phosphate (DHAP, top), Acetaldehyde (center), and Pyruvate (bottom) Aldolases[a]

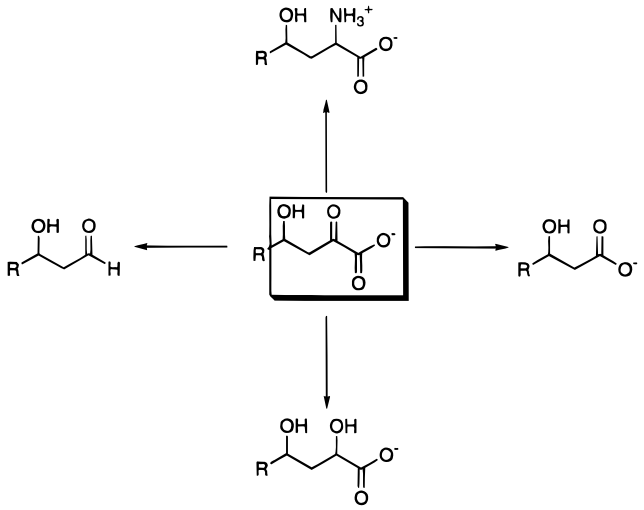


[a] The hash mark in each product denotes the bond formed during condensation.

studied group, those using dihydroxyacetone phosphate (DHAP) as the nucleophile, includes enzymes capable of forming all four possible diastereomers of a 1,3,4-trihydroxy-2-butanone skeleton.[3] This group of enzymes has been used extensively in the preparation of amino sugars of the nojirimycin type[11−16] and to lesser extent in the preparation of precursors to cyclitols.[17] The second group, those using acetaldehyde as the nucleophilic component, contains a single enzyme, 2-deoxyribose 5-phosphate aldolase (DERA).[18−21]

Despite the power of the DHAP aldolases, their utility in synthesis is limited, primarily due to the nature of the products generated. While the 1,3,4-trihydroxy-2-butanone skeleton is *densely* functionalized, it is not *differentially* functionalized. The classical synthetic manipulations required to, for example, differentiate the two secondary alcohols, detract markedly from the power of enzymatic synthesis, namely the ability to carry out complex synthetic transformations in aqueous solution without the need for protecting group chemistry. As a result of these limitations, the development of new carbon−carbon bond forming catalysts remains an important goal of applied enzymology.

Scheme 2. Accessible Transformations from Pyruvate Aldolase Adducts



The third and by far the largest group of aldolases utilizes pyruvate or phosphoenolpyruvate as the nucleophilic component, and catalyzes formation of a 4-hydroxy-2-ketobutyrate framework. In principle, this class of aldolases obviates most of the limitations of the DHAP aldolases. The conserved four-carbon fragment prepared in all pyruvate aldolase-catalyzed condensations is both densely and differentially functionalized. Thus, in each adduct, four different oxidation states of carbon are contained in four contiguous carbons. The framework prepared by the pyruvate aldolases thus provides opportunities for the preparation of α-amino-γ-hydroxycarboxylic acids, β-hydroxycarboxylic acids, α,γ-dihydroxycarboxylic acids, and 2-deoxyaldose sugars (Scheme 2). In most cases these transformations can be effected enzymatically or through classical methodologies in aqueous solution without the need for protecting groups.

The best utilized member of the pyruvate aldolases, *N*-acetylneuraminic aldolase (EC 4.1.3.3), has been used extensively to prepare modified sialic acids.[22−36] *In vivo*, NeuAc aldolase catalyzes the reversible condensation of pyruvate and *N*-acetylmannosamine, to produce *N*-acetylneuraminic acid. While *N*-acetylneuraminic aldolase accepts a range of unnatural electrophilic substrates, it does not accept lower-carbon homologues: aldose substrates of less than four carbons are not accepted.[25] Furthermore, the enzyme requires polar functionality at C3 of the electophile.[25] Under kinetic control, *N*-acetylneuraminic aldolase catalyzes *si*-face attack of pyruvate,

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producing products with the *S*-configuration at the new stereogenic center. Most NeuAc aldolase-catalyzed reactions operate under thermodynamic control, and the stereochemical outcome of the reaction depends on the configuration of the reactants.[31,33,35] L-Sugars frequently give diastereomeric mixtures resulting from both *re*- and *si*-facial attack, although some L-sugars give complete *re*-facial diastereoselectivity.[31,33,35]

2-Keto-3-deoxyoctulosonate aldolase (KDO aldolase, EC 4.1.2.23) has been used to prepare both natural and unnatural aldonate sugars.[37] *In vivo*, KDO aldolase catalyzes reversible condensation of pyruvate with D-arabinose to form 3-deoxy-D-*manno*-octulosonate. As is the case for NeuAc aldolase, product formation is generally under thermodynamic control and product mixtures are frequently encountered. Under kinetic control, the enzyme catalyzes *re*-face addition of pyruvate to prochiral electrophiles.[37] The enzyme accepts a variety of polyhydroxylated aldehydes, although lower carbon homologues are accepted only slowly.[37]

Recently, we reported the use of 2-keto-3-deoxy-6-phosphogluconate aldolase (KDPG aldolase, EC 4.1.2.14) from *Pseudomonas putida* (PP) as a reagent for stereocontrolled carbon−carbon bond formation.[38] KDPG aldolase catalyzes *si*-face addition of pyruvate to an electrophilic aldehyde to generate 4-hydroxy-2-ketobutyrates with the *S*-configuration at the newly formed stereogenic center.[38] The enzyme appeared superior to either NeuAc or KDO aldolases as a general catalyst for carbon−carbon bond formation, accepting small non-carbohydrate electrophiles as substrates. Here, we present a more complete report of the suitability of this enzyme for organic synthesis, as well as the utility of the corresponding enzymes from *Escherichia coli* (EC) and *Zymomonas mobilis* (ZM).

Results and Discussion

Enzyme Isolation. KDPG aldolase from *P. putida* was isolated from a commercially-available strain (ATCC 12633). This organism provides 200 U/L of fermentation broth (one unit of enzyme catalyzes cleavage of 1 μmol of 2-keto-3-deoxy-6-phosphogluconate to pyruvate and D-glyceraldehyde 3-phosphate per minute at pH 7.5, 25 °C).

Two sources of *Z. mobilis* KDPG aldolase were investigated. The enzyme was initially isolated from a commercially available strain of *Zymomonas* (ATCC 31821), which furnishes 650 U of aldolase/L of cell culture. Because of problems with enzyme instability during purification, we examined the same aldolase cloned and expressed in *E. coli*. High-copy plasmid pTC 162 was incorporated in *E. coli* DF 214, an *eda*$^-$ mutant strain that does not express a KDPG aldolase. The resulting *E. coli* strain produced the *Zymomonas* aldolase at a level of 2100 U/L of cell culture. The enzyme behaved identically to that from wild-type strain *Zymomonas* during purification, eliminating the possibility of a contaminating activity as the origin of the enzyme instability. Because of the higher total activity available from the recombinant strain, enzyme from that organism was utilized throughout.

KDPG aldolase is expressed by wild-type *E. coli* at a level of 80 U/L of cell culture (ATCC 11303), approximately 40% that of *P. putida* grown on glucose.[39] This low level of expression is clearly not useful for preparative-scale chemistry. We therefore incorporated the high copy plasmid pTC 190, which contains the *eda* gene, into the *eda*$^-$ *E. coli* strain DF214.

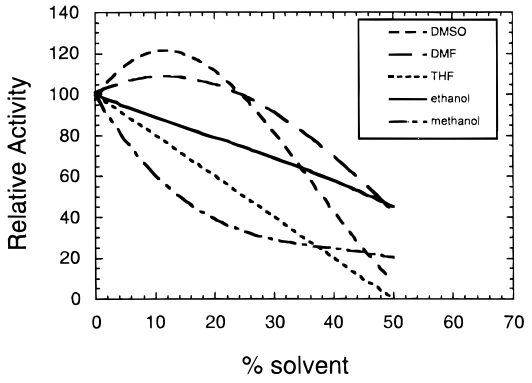

Figure 1. Effect of organic solvent on *P. putida* KDPG aldolase activity.

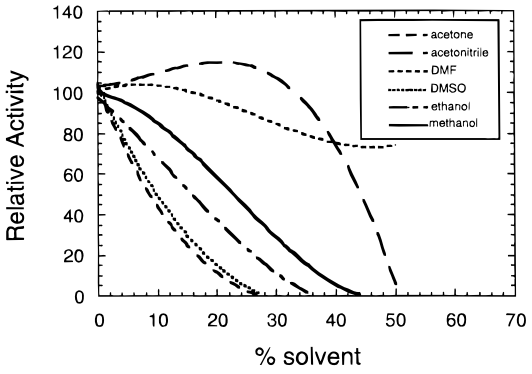

Figure 2. Effect of organic solvent on *Z. mobilis* KDPG aldolase activity.

The resulting strain produces the *Escherichia* aldolase at a level of 1350 U/L of cell culture and was used throughout.

Aldolases from all three sources were purified by differential dye-ligand chromatography. The details of this isolation have been reported elsewhere by us and others.[40,41] Briefly, all three aldolases can be effectively purified using a combination of Procion Yellow MX-GR as the "positive" and Procion Navy H-ER as the "negative" ligands on a Sepharose CL-4B support. In all cases, elution with a mixture of pyruvate and (±)-α-glycerophosphate gave the highest specific activities, although the enzymes can also be eluted nonspecifically with 1 M sodium chloride.

Enzyme Stability. Despite similarities in amino acid sequences, the three enzymes show several remarkable differences. The enzyme from *P. putida* is stable during isolation, during lyophilization, and for periods of at least years when stored at −78 °C as a lyophilized powder. The enzyme from *E. coli* is stable during isolation and can be concentrated to at least 0.7 mg/mL (278 U/mL) as a solution in buffer but loses all activity during freezing and lyophilization. *Z. mobilis* aldolase is stable as a crude suspension following ammonium sulfate fractionation of the crude cell extract. Following differential dye-ligand chromatography the enzyme rapidly loses activity either on standing in buffer or during freezing and lyophilization. Approximately 50% of the activity is lost during 12 h on standing in solution, and essentially all activity is lost during freezing.

Solvent Stability and pH Optima. KDPG aldolases from all three sources are remarkably stable in water-miscible organic solvents (Figures 1−3). The activity of KDPG aldolase is increased by up to 20% by the addition of DMSO and DMF as cosolvents. KDPG aldolases from *E. coli* and *Z. mobilis* retain 80% of their original activity at 40% DMSO. The ability of

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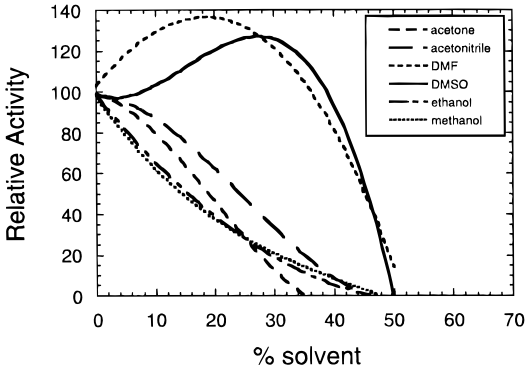


Figure 3. Effect of organic solvent on *E. coli* KDPG aldolase activity.

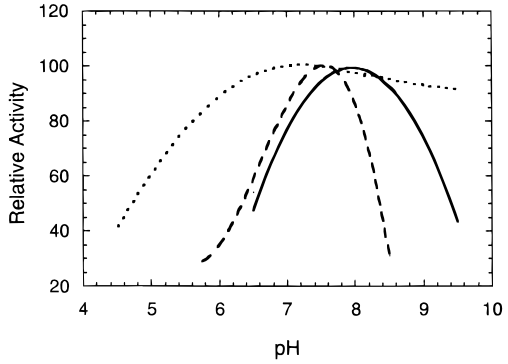


Figure 4. pH−activity profiles for PP (solid line), ZM (dashes), and EC (dots) KDPG aldolases.

these aldolases to operate under these conditions allows the use of electrophilic aldehydes that are not readily water soluble.

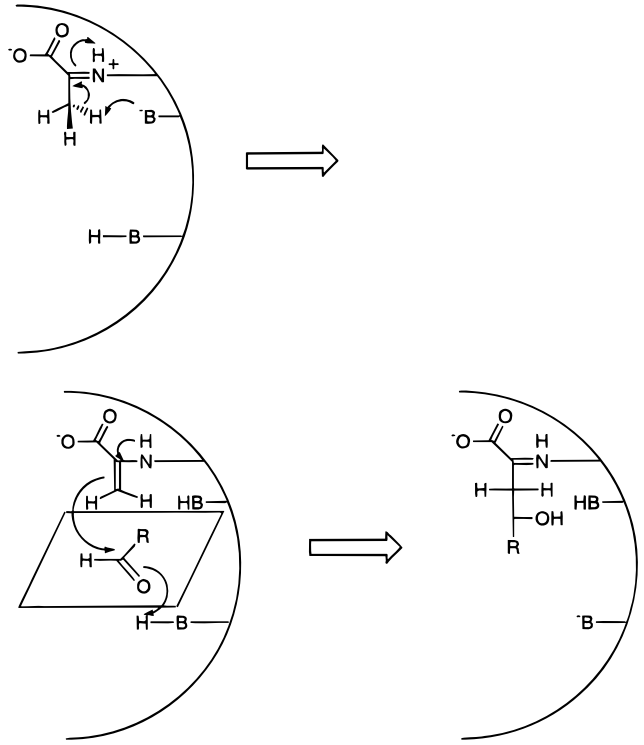
The three enzymes show markedly different pH optima (Figure 4). The enzymes from both *Zymomonas* and *Pseudomonas*[42] show classical bell-shaped pH−activity curves with maxima near pH 7.5−8. Both curves are indicative of a requirement for two enzyme ionizable residues, one ionized and one protonated, that presumably act as general base and general acid catalysts during condensation. The KDPG aldolases are type I aldolases and proceed *via* a Schiff-base intermediate (Scheme 3). In contrast, the enzyme from *Escherichia* shows a single transition pH−activity curve and apparently utilizes solvent as the general acid during condensation.

The behavior of the three enzymes provides an exceptionally broad pH range over which KDPG aldolase can be utilized. Activities of >50% maximum activity are available with all three enzymes over the range pH 6.5−8.5, while at least one of the three shows >50% maximum activity over the pH range 5.0 to >9.0.

Substrate Specificity. To better establish the utility of pyruvate aldolases as tools for stereocontrolled carbon−carbon bond formation, we explored the substrate specificity of the three aldolases with a variety of unnatural electrophiles.

The results contained in Table 1 clearly show that the substrate specificity of all three enzymes is usefully broad for organic synthesis. The three aldolases show many similarities but several significant differences with regard to substrate specificity. In all three cases, removal of the phosphate group from the natural electrophile D-glyceraldehyde 3-phosphate has a profound effect on the rate of conversion: D-glyceraldehyde is converted to 2-keto-3-deoxygluconate at roughly 1% the rate at which D-glyceraldehyde 3-phosphate is converted to 2-keto-3-deoxy-6-phosphogluconate. While negative charge at C-3 of the electrophile appears to be optimal, the charge may be

Scheme 3. Mechanism of Pyruvate Aldolase-Catalyzed Aldol Condensation



profitably located at either C-2 or C-4. Thus, two of the best unnatural electrophiles are D-erythrose 4-phosphate and glyoxylate, which are both accepted by all three aldolases at >25% of the rate of D-glyceraldehyde 3-phosphate.

Arsenate buffer has in some instances been shown to mimic a required phosphate group in enzymatic transformation, presumably through the formation of kinetically-labile arsenate esters.[43,44] Measurements of the rate of condensation of glyceraldehyde in phosphate and arsenate buffer showed no enhancement of rate in the arsenate buffer. The relative rates of arsenate ester formation and decomposition relative to that of substrate binding are crucial and apparently fall outside the required range here.

Simple aliphatic aldehydes are not accepted as substrates by any of the aldolases, in contrast to both the dihydroxyacetone phosphate aldolases and deoxyribose aldolase.[18,19,45,46] Benzaldehyde and substituted benzaldehydes are also non-substrates for all three aldolases. Alternatively, a variety of other cyclic aldehydes are substrates for all three aldolases. 2- and 3-Furaldehyde and 3-thiophenecarboxaldehyde are accepted by one or more aldolases, although 2-thiophenecarboxaldehyde is not accepted by any. Hindered substrates are not accepted: 2,3-*O*-isopropylidene-D-glyceraldehyde is not accepted by any of the three enzymes.

The pyridine carboxaldehydes are very good substrates for all three aldolases, although 3-pyridinecarboxaldehyde is not accepted by the *Pseudomonas* enzyme. At pH 7.5, where all assays were run, the pyridinecarboxaldehydes are not significantly protonated, and the observed difference in rates of reaction, *i.e.*, 2 > 4 > 3, is likely more indicative of differential

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Table 1. Substrate Specificity of Pyruvate Aldolases from *P. putida*, *E. coli*, and *Z. mobilis*

nucleophile	electrophile	PP	EC	ZM
pyruvate	D-glyceraldehyde	100	100	100
	L-glyceraldehyde	−	−	+
	DL-glyceraldehyde	++	+++	+++
	D-lactaldehyde	++	++	+
	L-lactaldehyde	−	−	+
	D-erythrose	+	+	++
	D-threose	−	+	+
	L-erythrose	−	−	+
	L-threose	++	+	+
	D-erythrose 4-phosphate	++++	++++	++++
	D-ribose	−	+	+
	D-ribose 5-phosphate	+	+	++
	chloroacetaldehyde	++++	+	++++
	2,3-*O*-isopropylidine D-glyceraldehyde	−	−	−
	glycolaldehyde	+	+	++
	glyoxylate	++++	++++	++++
	2-pyridine carboxaldehyde	++++	++++	++++
	3-pyridine carboxaldehyde	−	++	+++
	4-pyridine carboxaldehyde	+	++++	++++
	2-thiophene carboxaldehyde	−	−	−
	3-thiophene carboxaldehyde	−	−	+
	2-furaldehyde	−	+	++
	3-furaldehyde	−	−	+
	2-chlorobenzaldehyde	−	−	−
	3-chlorobenzaldehyde	−	−	−
	4-chlorobenzaldehyde	−	−	−
	benzaldehyde	−	−	−
	valeraldehyde	−	−	−
	butyraldehyde	−	−	−
	acrolein	−	−	−
2-ketobutyrate	D-glyeraldehyde 3-phosphate	+++	++++	+
3-hydroxypyruvate	D-glyeraldehyde 3-phosphate	+	+	+
3-fluoropyruvate	D-glyeraldehyde 3-phosphate	+	+	++

− (non-substrate)

+	0−25%	+++	50−100%
++	25−50%	++++	>100%

hydrogen bonding to the enzyme than to the electron-withdrawing nature of the ring nitrogen.

Despite many similarities in substrate specificity, the three aldolases also show several significant differences. In general, the aldolases from *P. putida* and *E. coli* have very similar and relatively restrictive substrate specificities, while the aldolase from *Z. mobilis* shows less selectivity, accepting a broader range of substrates. For example, the *Zymomonas* aldolase alone accepts both 3-furaldehyde and 3-thiophene carboxaldehyde. The most significant departure in substrate specificities by the *Zymomonas* aldolase from the other two is an apparently relaxed stereochemical requirement for the configuration at C2 of the electrophile. Both the *Pseudomonas* and *Escherichia* enzymes are highly specific for the D-configuration at C2 of the electrophilic substrate. Both enzymes also appear to be slightly inhibited by the wrong enantiomer, and D-glyceraldehyde is utilized by both enzymes 2−3-fold faster than the racemate. In contrast to either the *Pseudomonas* or *Escherichia* aldolases, the *Zymomonas* aldolase accepts both enantiomers of glyceraldehyde and lactaldehyde and all four tetrose stereoisomers.

The differing stereochemical requirements for the three enzymes provide both power and flexibility in the construction of aldol adducts. If the *R*-configuration is required at C5 of the adduct, aldol condensation can be catalyzed by either the *Pseudomonas* or *Escherichia* enzymes using racemic aldehydes. Aldol condensation will then proceed with a concomitant kinetic resolution, effectively setting two stereocenters during condensation. Alternatively, if the *S*-configuration is required at C5 of the adduct, the *Zymomonas* aldolase is the enzyme of choice, although in this instance enantiomerically pure electrophiles are required to avoid diastereomeric mixtures.

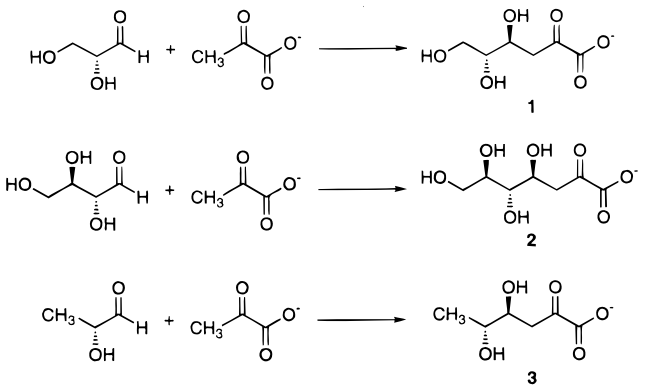
All three aldolases accept substrates other than pyruvate as the nucleophile, providing the electrophilic component is sufficiently reactive. 2-Ketobutyrate, fluoropyruvate, and hydroxypyruvate are accepted by all three enzymes, with D-glyceraldehyde 3-phosphate as the electrophile. This behavior is in contrast to the other synthetically useful pyruvate aldolases, which accept only pyruvate as the nucleophile.[25,37] Acceptance of substituted pyruvates greatly increases the utility of the pyruvate aldolases, permitting incorporation of functionality at C3 of the aldol adduct. At this time we have not determined the stereochemical course of the addition with substituted pyruvates.

In summary, a negatively charged residue at position 2, 3, or 4 of the electrophile, or at least substituents capable of acting as hydrogen bond acceptors, and minimal steric hindrance at C2 are the major factors influencing the substrate specificities of KDPG aldolases from *P. putida*, *E. coli*, and *Z. mobilis*. In addition, both the *Pseudomonas* and *Escherichia* aldolases require the D-configuration at C2 of the aldehyde substrate.

Preparative Scale Reactions. While assays are useful in delineating the range of substrates accepted by an enzyme, preparative-scale reactions are clearly required to unambiguously establish product identity and stereochemical fidelity during condensation. For example, Fessner and co-workers have reported less than complete diastereofacial selectivity by dihydroxyacetone phosphate aldolases with some unnatural electrophiles.[47−49] To establish the level of asymmetric induction

(47) Fessner, W.-D.; Sinerius, G.; Schneider, A.; Dreyer, M.; Schulz, G. E.; Badía, J.; Aguilar, J. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 555.
(48) Fessner, W.-D.; Eyrisch, O. *Angew. Chem., Int. Ed. Engl.* **1992**, *21*, 56.

Scheme 4. Preparative-Scale KDPG Aldolase-Catalyzed Condensations



available with the three KDPG aldolases, we carried out preparative-scale reactions using D-glyceraldehyde, D-erythrose, and D-lactaldehyde as electrophilic substrates (Scheme 4), producing 3-deoxy-D-*erythro*-2-hexulosonate (2-keto-3-deoxygluconate, KDG, **1**), 3-deoxy-D-*ribo*-2-heptulosonate (**2**), and 3,6-dideoxy-D-*erythro*-2-hexulosonate (**3**), respectively. Reaction scales, quantity of enzyme used, reaction time, isolated product yields, and diastereoselectivities are shown in Table 2.

The stereochemical configuration of the adduct of pyruvate and D-glyceraldehyde was determined by comparison with authentic 2-keto-3-deoxygluconate prepared from glucose 6-phosphate (Scheme 5). A crude preparation of *P. putida* 6-phosphogluconate dehydratase was used to eliminate the elements of water across the C2/C3 bond of 6-phosphogluconate, to yield 2-keto-3-deoxy-6-phosphogluconate. Adventitious aldolase present in the crude enzyme preparation was destroyed prior to use with pyruvate and sodium cyanoborohydride. Enzymatic dephosphorylation provided the required authentic 2-keto-3-deoxygluconate.[38,50]

3-Deoxy-D-*erythro*-2-hexulosonate (**1**) exists in solution as a mixture of α- and *β*-anomers of both pyranose and furanose forms, in accord with previously reported spectra.[51] As expected, the α-anomer of the pyranose form is the dominant species, while roughly equimolar amounts of the two furanoses are observed. In contrast to 3-deoxy-D-*erythro*-2-hexulosonate 6-phosphate, no open-chain form of the sugar is observed. This behavior is undoubtedly reflective of the additional cyclic form available to the unphosphorylated material. At pH 7.0, no lactones were observed.

The diastereoselectivity of all enzymatic aldol condensations were determined by ^{13}C NMR examination of the upfield C3 methylene signal, according to the method of Augé.[33] Examination of 3-deoxy-D-*erythro*-2-hexulosonate produced by *Pseudomonas* and *Escherichia* aldolases showed only the expected four signals, providing diastereomeric excesses of >97%. Alternatively, the ^{13}C spectrum of the adduct produced by the *Zymomonas* aldolase shows eight resonances in the region 30−45 ppm, clearly indicating the presence of the D-*threo* diastereomer. The diastereomeric excess of the expected D-*erythro* product was 50%.

The absolute configuration of 3-deoxy-D-*ribo*-2-heptulosonate (**2**) was determined by nonoxidative decarboxylation of the enzymatic adduct to 2-deoxy-D-allose (**4**, Scheme 6). The decarboxylation was carried out by non-stereoselective reductive amination, followed by oxidative decarboxylation with ninhydrin. The product of the decarboxylation was spectroscopically identical to authentic commercial material.

^{13}C NMR spectra of 3-deoxy-D-*ribo*-2-heptulosonate shows three resonances in the region 30−45 ppm, at 36.65, 43.42, 44.19 ppm, indicating the presence of a mixture of α-pyranose and both α- and *β*-furanose anomers, in a roughly 1:1:1 ratio. Two additional forms were observed in 2D ^{1}H NMR spectra, presumably the *β*-pyranose and open-chain forms. The latter two are each populated to roughly 1.5%. Again, no lactone products were observed. The adducts produced by all three aldolases showed no evidence of diastereomeric impurities, providing diastereomeric excesses in all three cases of >97%.

We initially attempted to determine the absolute configuration of the adduct of D-lactaldehyde and pyruvate, 3,6-dideoxy-D-*erythro*-hexulosonate (**3**), by comparison of rotations with previously reported values. In 1968, Portsmouth prepared all four diastereomers of 3,6-dideoxyhexulosonate and assigned the stereochemical orientation of the products on the basis of 1-D NMR assignments.[52] Portsmouth assigned the specific rotation of the D-*erythro* product as −11.5° and of the D-*threo* product as +18.2°. We obtained specific rotations for all three enzymatic adducts of +17°, suggesting formation of the unexpected *R*-stereochemistry at the newly formed stereogenic center. To unambiguously assign the stereochemical course of the enzyme-catalyzed condensation, we carried out NOE studies on the diacetylated methyl ester derivative **5** (Figure 5). Using enantiomerically pure D-lactaldehyde as the electrophilic substrate, the enzymatic aldol condensation could conceivably produce two of the four possible diastereomers. The observed NOE from the C6 methyl protons to the C4 proton unambiguously establishes the stereochemistry as the expected D-*erythro* configuration.

The 30−45 ppm region of the ^{13}C NMR spectrum of 3,6-dideoxy-D-*erythro*-2-hexulosonate shows a single resonance at 43.26 ppm and a peak of ∼15% relative intensity at 42.09 ppm (Figure 6). The upfield ^{13}C resonance represents open-chain form. The remainder of the spectrum shows 10 major resonances, indicative of two equilibrating species, and a smaller set of lines, again corresponding to the open-chain form. The product thus exists as a roughly 1:1 mixture of α- and *β*-furanoses, with 15% open-chain product. NMR examination of the ^{13}C NMR spectrum of all three enzyme-catalyzed adducts showed the presence of a single diastereomer, indicating diastereomeric excess levels of >97%.

In retroaldol condensations, KDPG aldolase is specific for the open-chain form of 2-keto-3-deoxy-6-phosphogluconate, present to approximately 9% in aqueous solution near neutral pH. This behavior is in contrast to NeuAc and KDO aldolases, both of which bind cyclic substrates. Specificity for the open-chain form of the aldol adduct in retroaldol reaction has profound implications for both the reversibility or the reaction and the mechanism of diastereoselectivity of KDPG aldolase-catalyzed condensations. To the extent that the product of an aldol condensation does not populate the open-chain form to an appreciable extent (relative to the substrate K_m), formation of these products is irreversible. Thus, for example, 3-deoxy-D-*erythro*-hexulosonate cannot be cleaved to pyruvate and glyceraldehyde even in the presence of lactate dehydrogenase, which forces the equilibrium toward retroaldol condensation (Scheme 7). In such cases the observed diastereoselectivity in aldol condensation is purely kinetic.

Alternatively, products which populate the open-chain form at concentrations nearing or greater than K_m for retroaldol cleavage could in principle be formed under either kinetic or

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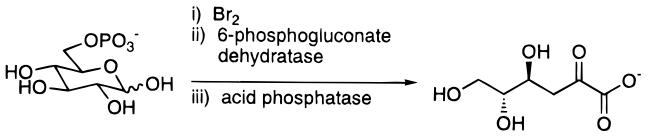
(50) O'Connell, E. L.; Meloche, H. P. *Methods Enzymol.* **1982**, *89*, 98.
(51) Plantier-Royon, R.; Cardona, F.; Anker, D.; Condemine, G.; Nasser. W.; Robert-Baudouy, J. *J. Carbohydr. Chem.* **1991**, *10*, 787.
(52) Portsmouth, D. *Carbohydr. Res.* **1968**, *8*, 193.

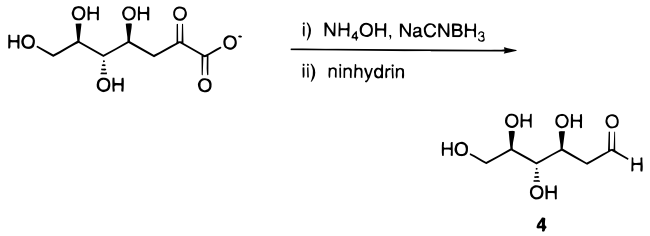
Table 2. Preparative-Scale Enzymatic Aldol Condensations

enzyme	electrophile	scale (mmol of electrophile)	units of enzyme	coversion (%)	de (%)	% yield
Pseudomonas	D-glyceraldehyde	8.9	300	25	>97	24
	D-erythrose	2.0	250	18	>97	12
	D- lactaldehyde	7.5	300	30	>97	30
Escherichia	D-glyceraldehyde	3.9	560	24	>97	14
	D-erythrose	2.0	560	15	>97	13
	D-lactaldehyde	4.0	400	22	>97	20
Zymomonas	D-glyceraldehyde	3.9	905	46	50	37
	D-erythrose	2.0	900	23	>97	18
	D-lactaldehyde	4.0	900	13	>97	10

Scheme 5. Preparation of 2-Keto-3-deoxygluconate (3-Deoxy-D-*erythro*-2-hexulosonate) from Glucose 6-Phosphate



Scheme 6. Nonoxidative Decarboxylation of 3-Deoxy-D-*ribo*-2-heptulosonate to 2-Deoxy-D-*ribo*-hexose (2-Deoxyallose)



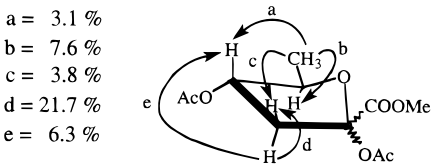


Figure 5. NOE contacts for the methyl ester diacetate derivative of 3,6-dideoxy-D-*erythro*-2-hexulosonate.

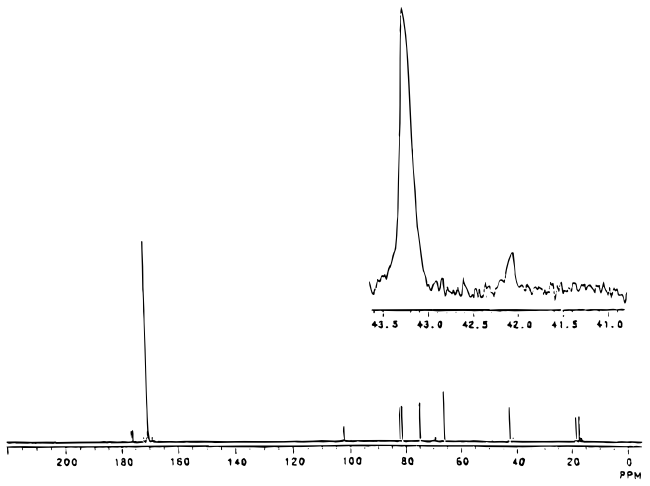
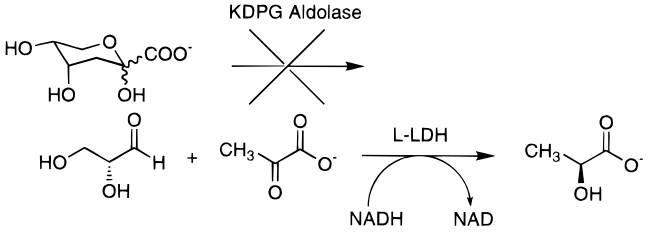


Figure 6. ^{13}C NMR spectrum for 3,6-dideoxy-D-*erythro*-2-hexulosonate. The upfield region (insert) indicates the presence of straight-chain ketose. The resonance at 171.3 ppm is formate.

thermodynamic control. This situation clearly holds for 3,6-dideoxy-D-*erythro*-2-hexulosonate. Enzymatic condensation of pyruvate with enantiomerically-pure D-lactaldehyde could produce two diastereomers that MM3 calculations show are essentially equienergetic. The observed production of a single

Scheme 7. 2-Keto-3-deoxygluconate Cannot Be Cleaved by KDPG Aldolase, Even in the Presence of L-Lactate Dehydrogenase (L-LDH), Which Forces the Equilibrium to the Right as Drawn



diastereomeric product implies that the enzymatic aldol condensation remains entirely under kinetic control, despite the reversibility of the reaction.

This behavior stands in contrast to NeuAc and KDO aldolases, which operate under kinetic or thermodynamic control, depending on the extent of conversion and the stereochemical configuration of the substrates.[25,31,33,37] While the generality of the kinetic diastereoselectivity observed here remains to be more fully delineated, the behavior provides a high degree of predictability in KDPG aldolase-catalyzed condensation.

Together, the results of preparative-scale condensations show that KDPG aldolases from all three sources are effective catalysts for stereoselective carbon−carbon bond formation. The least selective enzyme in terms of substrate specificity, that from *Zymomonas*, shows less than complete specificity with one unnatural substrate, D-glyceraldehyde. The reasons for the reduced stereoselectivity with an electrophilic substrate so closely related to the natural electrophile are unclear but highlight the importance of careful evaluation of diastereoselectivities in all enzyme-catalyzed aldol condensations.

In conclusion, KDPG aldolase is a useful catalyst for stereoselective carbon−carbon bond formation in the preparation of a wide range of 2-keto-4-hydroxybutyrate skeletons. We are currently exploring methodologies for enhancing rates of reaction of unnatural electrophile substrates and pursuing syntheses of several natural products utilizing enzymatic carbon−carbon bond formation and will report our results in due course.

Experimental Section

General Procedures. UV kinetic assays were performed on a Hewlett-Packard HP-8452A UV/vis spectrophotometer. ^1H NMR spectra were recorded on either a General Electric QE-300 or Varian Unity 500 instrument, operating at 300.150 or 499.928 MHz, respectively. ^{13}C NMR spectra were recorded on a General Electric QE-300 instrument operating at 75.48 MHz. ^1H 1-D NOE and 2-D $^1H-^1H$ COSY spectra were recorded on a Varian Unity 500 operating at 499.928 MHz. ^1H NMR spectra are referenced to HOD at 4.63 ppm. ^{13}C NMR are referenced to *p*-dioxane at 66.64 ppm. Plasmids pTC 162[53] and pTC 190[54] were generous gifts from T. Conway, School of

(53) Conway, T.; Fliege, R.; Jones-Kilpatrick, D.; Liu, J.; Barnell, W. O.; Egan, S. E. *Mol. Microbiol.* **1991**, *5*, 2901.

Biological Sciences, University of Nebraska. *E.* coli strain DF 214[55] was a gift from B. Bachmann, *E. coli* Genetic Stock Center, Yale University.

Growth of Bacterial Strains. *E. coli* strains containing plasmids pTC 162 and pTC 190 were grown according to previously published procedures.[40] *P. putida* was grown according to previously published procedures.[38]

Enzyme Purification. EC and PP KDPG aldolases were purified by differential dye-ligand chromatography by previously reported procedures.[40] KDPG aldolase from ZM was purified by protamine sulfate precipitation, ammonium sulfate fractionation, and ultrafiltration (to remove ammonium sulfate) and was then utilized for assays and preparative-scale syntheses without dye column purification.

Enzymatic Activity. KDPG aldolase activity was determined by utilizing the standard coupled assay with L-lactic dehydrogenase (EC 1.1.1.27, Type II from rabbit muscle).[56] KH_2PO_4 (20 mM, pH 7.5, 3.0 mL), NADH (50 μL, 15 mg/mL), KDPG aldolase (5−50 μL), and L-lactic dehydrogenase (EC 1.1.1.27, Type II from rabbit muscle, 10 μL) were added to a disposable cuvette. KDPG (50 μL, 100 mg/mL) was added to the sample to initiate the reaction. KDPG aldolase activity was determined from the initial slope of the absorbance curve during the first 10 s of the reaction.

Enzymatic Assay for Pyruvate. Pyruvate was determined by utilizing a modification of the standard assay with rabbit muscle L-lactic dehydrogenase.[57] KH_2PO_4 (20 mM, pH 7.5, 3.0 mL), NADH (50 μL, 15 mg/mL), and the reaction sample (175 μL) were added to a disposable cuvette. An initial UV absorbance value at 340 nM was recorded. L-Lactic dehydrogenase (EC 1.1.1.27, Type II from rabbit muscle, 5 μL) was added to the sample. After stabilization, the final absorbance value was recorded.

Unnatural Electrophile Assays. Unnatural electrophile assays were performed on a 3 mL scale with electrophile and nucleophile concentrations both at 50 mM. Typically, 100−150 units of aldolase was used for each assay. DMSO (20% (v/v)) was added as a cosolvent when 2-furaldehyde, 3-furaldehyde, 2-thiophenecarboxaldehyde, 3-thiophenecarboxaldehyde, 2-chlorobenzaldehyde, 3-chlorobenzaldehyde, and 4-chlorobenzaldehyde were used as the electrophile. Aliquots (100 μL) were taken at timely intervals. The aldolase in the aliquots was destroyed by treatment with 7% perchloric acid (30 μL). The samples were neutralized and diluted with 1 M NaOH (20 μL) and 20 mM KH_2PO_4, pH 7.5 (1000 μL), respectively.

Unnatural Nucleophile Assays. Unnatural nucleophile assays were performed on a 3 mL scale with nucleophile and electrophile concentrations between 10 and 15 mM. Aliquots (100 μL) were taken at timely intervals. The aldolase in the aliquots was destroyed by treatment with 7% perchloric acid (30 μL). The samples were neutralized and diluted with 1 M NaOH (20 μL) and 20 mM KH_2PO_4, pH 7.5 (1000 μL), respectively.

Enzymatic Assays in Arsenate Buffer. To a 2 mL solution of arsenate (100 mM, pH 7.5) containing sodium pyruvate (50 mM, 100 μmol) and D-glyceraldehyde (50 mM, 100 μmol) was added KDPG aldolase (20 U). Aliquots (100 μL) were taken at timely intervals. The aldolase in the aliquots was destroyed by treatment with 7% perchloric acid (30 μL). The samples were neutralized and diluted with 1 M NaOH (20 μL) and 20 mM KH_2PO_4, pH 7.5 (1000 μL), respectively.

Synthesis of 2-Keto-3-deoxygluconate (KDG) (EC Aldolase). D-Glyceraldehyde (500 mg, 3.89 mmol), 20 mM KH_2PO_4, pH 7.5 (74.5 mL), sodium azide (77.8 mg), and sodium pyruvate (428.1 mg, 3.89 mmol) were added to an Erlenmeyer flask. Aldol condensation was initiated by the addition of KDPG aldolase (556 U). The solution was stirred for 4 days. The reaction mixture was lyophilized. The lyophilized powder was dissolved in deionized water and then applied to an anion exchange column (AG-1X8, formate form). The product was eluted with a linear gradient of 0−1 M formic acid. Fractions containing the product were pooled and concentrated *in vacuo*. The residue was stripped from water *in vacuo* several times to ensure removal of residual formic acid. The oil was dissolved in deionized water and neutralized with NaOH. The water was removed *in vacuo* to yield 286.2 mg (36.8%) of the desired product as the sodium salt: $[\alpha]_D^{20}$ −21.2 (*c* 5.67, H_2O); spectral data (^{1}H NMR and ^{13}C NMR) are identical to those of literature spectra.[38,51]

Synthesis of 2-Keto-3-deoxygluconate (KDG) (ZM Aldolase). 2-Keto-3-deoxygluconate was prepared using *Zymomonas* aldolase in a fashion identical to that prepared using *Escherichia* aldolase, with the following modifications: D-glyceraldehyde (500 mg, 3.89 mmol), 20 mM KH_2PO_4, pH 7.5 (76 mL), sodium azide (77.8 mg), and sodium pyruvate (428.9 mg, 3.90 mmol) were added to an Erlenmeyer flask. The reaction was started by the addition of KDPG aldolase (0.5 mL, 905 U). The solution was stirred for 4 days. Chromatographic workup yielded 109.5 mg (14.1%) of the desired product as the sodium salt: $[\alpha]_D^{20}$ −8.6 (*c* 2.01, H_2O). ^{1}H spectra were uninterpretable.

Synthesis of 2-Keto-3-deoxygluconate (PP Aldolase) (1). 2-Keto-3-deoxygluconate was prepared using *Pseudomonas* aldolase in a fashion identical to that prepared using *Escherichia* aldolase, with the following modifications: D-glyceraldehyde (0.8 g, 8.9 mmol), 30 mM KH_2PO_4, pH 7.5 (90 mL), and sodium pyruvate (1.0 g, 8.9 mmol) were added to an Erlenmeyer flask. Condensation was initiated by addition of KDPG aldolase (300 U) in a dialysis bag. The reaction was stirred for 2 days, at which point the reaction had gone to 25% completion. The product was applied to an anion exchange column (AG-1X8, formate form) and eluted with a linear gradient (0−1 M, 1 L). The product-containing fractions were pooled, solvent was removed *in vacuo*, and the resulting clear oil was converted to the sodium salt by neutralization with NaOH. Removal of the solvent yielded a pale yellow oil (500 mg, 32%): $[\alpha]_D^{20}$ −13.2 (*c* 25.7, H_2O); spectral data (^{1}H NMR and ^{13}C NMR) are identical to those of literature spectra.[38,51]

Synthesis of 3,6-Dideoxy-D-*erythro*-2-hexulosonate (EC Aldolase). D-Lactaldehyde (15 mL, 4.04 mmol), prepared from L-threonine,[58] 20 mM KH_2PO_4, pH 7.5 (64 mL), sodium azide (82.6 mg), and sodium pyruvate (451.2 mg, 4.10 mmol) were added to an Erlenmeyer flask. Condensation was initiated by addition of KDPG aldolase (400 U). The solution was stirred for 4 days, and the reaction mixture was lyophilized. The resulting powder was dissolved in deionized water and applied to an anion exchange column (AG-1X8, formate form). The product was eluted with a linear gradient of 0−1 M formic acid. The fractions containing product were pooled and concentrated *in vacuo*. The residue was stripped from water *in vacuo* several times to ensure removal of residual formic acid. The resulting oil was converted to the sodium salt (NaOH) to yield 72.8 mg (9.8%) of desired product: $[\alpha]_D^{20}$ +13.0 (*c* 0.76, H_2O); ^{1}H NMR (500 MHz) for furanose I δ 1.136 (3H, d, J = 6.2 Hz), 2.101 (1H, dd, J = 6.6, 13.6 Hz), 2.205 (1H, dd, J = 6.6, 13.6 Hz), 3.861−3.908 (1H, m), 3.940−3.992 (1H, m); ^{1}H NMR for furanose II δ 1.098 (3H, d, J = 6.2 Hz), 1.791 (1H, dd, J = 5.1, 13.9 Hz), 2.467 (1H, dd, J = 7.7, 13.9 Hz), 3.861−3.908 (1H, m), 3.940−3.992 (1H, m); ^{1}H NMR for straight chain δ 1.002 (3H, d, J = 6.2 Hz), 2.672 (1H, dd, J = 9.2, 17.0 Hz), 2.885 (1H, d br, J = 17.6 Hz), 3.574−3.622 (1H, m), 3.861−3.908 (1H, m) ppm; ^{13}C NMR δ 16.57, 18.00, 19.16, 42.07, 43.26, 69.26, 70.00, 75.54, 75.80, 82.00, 82.91, 102.93, 103.11, 176.71, 177.38 ppm.

Synthesis of 3,6-Dideoxy-D-*erythro*-2-hexulosonate (ZM Aldolase). 3,6-Dideoxy-D-*erythro*-2-hexulosonate was prepared with *Zymomonas* aldolase in a fashion identical to that prepared with *Escherichia* aldolase, with the following modifications: D-lactaldehyde (15 mL, 4.0 mmol), 20 mM KH_2PO_4, pH 7.5 (62 mL), sodium azide (82.8 mg), and sodium pyruvate (451.2 mg, 4.1 mmol) were added to an Erlenmeyer flask. Condensation was initiated by the addition of KDPG aldolase (914 U). The solution was stirred for 4 days. Upon workup, 145 mg (19.5%) of the desired product was isolated as the sodium salt: $[\alpha]_D^{20}$ +17.4 (*c* 2.77, H_2O); spectral characteristics are identical to those for material prepared with *Escherichia* aldolase.

Synthesis of 3,6-Dideoxy-D-*erythro*-2-hexulosonate (PP Aldolase). 3,6-Dideoxy-D-*erythro*-2-hexulosonate was prepared with *Pseudomonas*

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aldolase in a fashion identical to that prepared with *Escherichia* aldolase, to yield 219 mg (30%): $[\alpha]_D^{20}$ +14.3 (*c* 0.30, H_2O). Spectral characteristics are identical to those for material prepared with *Escherichia* aldolase.

Synthesis of 3-Deoxy-D-*ribo*-2-heptulosonate (ZM Aldolase). D-Erythrose (Sigma, 500 mg, 2.1 mmol), 20 mM KH_2PO_4, pH 7.5 (35 mL), sodium azide (45 mg) and sodium pyruvate (231 mg, 2.1 mmol) were added to an Erlenmeyer flask. Condensation was initiated by the addition of KDPG aldolase (905 U). The solution was stirred for 4 days, and the reaction mixture was lyophilized. The lyophilized powder was dissolved in deionized water and then applied to an anion exchange column (AG-1X8, formate form). The product was eluted with a linear gradient of 0−1 M formic acid. Fractions containing product were pooled and concentrated *in vacuo*. The residue was stripped from water *in vacuo* several times to ensure removal of residual formic acid. The resulting oil was converted to the sodium salt (NaOH) to yield 61.6 mg (12.8%) of the desired product as the sodium salt: $[\alpha]_D^{20}$ +36.8 (*c* 0.79, H_2O); ^{1}H NMR (500 MHz) for furanose I δ 2.125 (1H, dd, J = 5.12, 13.92 Hz), 2.205 (1H, dd, J = 6.59, 13.91 Hz), 3.432−3.489 (1H, m), 3.522−3.624 (1H, m), 3.766 (1H, dd, J = 4.03, 6.59 Hz), 4.369 (1H, ddd, J = 5.50, 10.44, 4.76 Hz) ppm; ^{1}H NMR for furanose II δ 1.877 (1H, ddd, J = 15.02, 14.84, 2.57 Hz), 2.358 (1H, dd, J = 7.33, 14.29 Hz), 3.432−3.489 (1H, m), 3.644−3.675 (1H, m), 3.973 (1H, dd, J = 4.40, 4.22 Hz), 4.333 (1H, ddd, J = 6.23, 6.59, 3.30 Hz) ppm; ^{1}H NMR for α-pyranose δ 1.877 (1H, ddd, J = 15.0, 14.8, 2.6 Hz), 1.983 (1H, dd, J = 14.7, 3.3 Hz), 3.536 (1H, dd, J = 2.9, 10.3 Hz), 3.644−3.675 (1H, m), 3.860 (1H, dd, J = 3.3, 3.3 Hz), 4.023 (1H, ddd, J = 3.1, 3.1, 3.2 Hz) ppm; ^{1}H NMR for straight chain δ 1.792−1.815 (1H, m), 2.583 (1H, m) ppm; ^{13}C NMR (75 MHz) δ 36.66, 43.42, 44.20, 61.08, 62.33, 66.08, 67.65, 69.28, 71.05, 71.71, 72.11, 72.32, 86.44, 87.09, 96.09, 103.79, 103.93, 176.72, 176.76, 176.79 ppm.

Synthesis of 3-Deoxy-D-*ribo*-2-heptulosonate (EC Aldolase). 3-Deoxy-D-*ribo*-2-heptulosonate was prepared from pyruvate and D-erythrose using *Escherichia* aldolase in a fashion identical to that prepared with *Zymomonas* aldolase, with the following modifications: D-erythrose (500 mg, 2.08 mmol), 20 mM KH_2PO_4, pH 7.5 (33.5 mL), sodium azide (47.0 mg), and sodium pyruvate (231.2 mg, 2.10 mmol) were added to an Erlenmeyer flask. Condensation was initiated by addition of KDPG aldolase (556 U). The solution was stirred for 4 days. After standard workup, 87.6 mg (18.2%) of the desired product was isolated as the sodium salt: $[\alpha]_D^{20}$ +32.0 (*c* 0.33, H_2O); spectral characteristics are identical to those for material prepared with *Zymomonas* aldolase.

Synthesis of 3-Deoxy-D-*ribo*-2-heptulosonate (PP Aldolase). 3-Deoxy-D-*ribo*-2-heptulosonate was prepared from pyruvate and D-erythrose using *Escherichia* aldolase in a fashion identical to that prepared with *Zymomonas* aldolase, with the following modifications: D-erythrose (500 mg, 2.1 mmol), 20 mM KH_2PO_4, pH 7.5 (29 mL), sodium azide (52.0 mg), and sodium pyruvate (233.5 mg, 2.1 mmol) were added to an Erlenmeyer flask. The reaction was started by the addition of KDPG aldolase (10 mL, 549 U). The solution was stirred for 4 days. After standard workup, 58.6 mg (12.3%) of the desired product was isolated as the sodium salt: $[\alpha]_D^{20}$ +30.5 (*c* 1.03, H_2O); spectral characteristics are identical to those for material prepared with *Zymomonas* aldolase.

Synthesis of 2-Keto-3-deoxygluconate from D-Glucose 6-Phosphate. 2-Keto-3-deoxy-6-phosphogluconate (KDPG) was prepared from glucose 6-phosphate according to the method of O'Connell and Meloche.[50] KDPG (240 mg, 0.77 mmol) was dissolved in deionized water and dephosphorylated by the addition of acid phosphatase (EC 3.1.3.2, sweet potato, 5 U) at room temperature. The solution was stirred overnight and was then applied to an anion exchange column (AG-1X8, formate form). KDG was eluted from the column with a linear gradient of formic acid (0−1 M). The fractions containing KDG were pooled, and the solvent was removed *in vacuo*. The resulting clear oil was converted to the sodium salt by neutralization with NaOH. Removal of the solvent yielded a pale yellow oil (109 mg, 80% yield). Spectral data (^{1}H and ^{13}C NMR) are identical to those previously reported.

Synthesis of 2-Deoxy-D-allose. 3-Deoxy-D-ribo-2-heptulosonate (**2**) was converted to 2-deoxy-D-allose by reductive amination according to Borch *et al*.[59] **2**, as the sodium salt (24.15 mg, 0.105 mmol), was dissolved in water (0.5 mL). Methanol (1.5 mL), NH_4Br (49.1 mg, 0.5 mmol) and $NaCNBH_3$ (17.3 mg, 0.25 mmol) were added, followed by additional methanol (5 mL). The reaction mixture, was stirred for 48 h at room temperature. Concentrated HCl (0.5 mL) was added to the mixture and it was stirred for 1 h. The solvent was removed *in vacuo*. The residue was dissolved in deionized water (7 mL) and passed down a Dowex HCR-S (H^+) cation exchange column. The desired amino acid product was eluted with 1 M NH_4OH. The eluant was concentrated *in vacuo* and lyophilized. The resulting solid was oxidatively decarboxylated according to the procedure of Durrwachter *et al*.[58] The product obtained is spectroscopically identical to commercial available 2-deoxy-D-allose (2-deoxy-D-*ribo*-hexopyranose, Sigma).

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