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The synthetic utility of KDPGal aldolase

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Abstract

We describe the isolation and purification of KDPGal aldolase from two sources. To facilitate this isolation, a facile five-step chemical synthesis of 2-keto-3-deoxy-6-phosphogalactonate (KDPGal) (**1**) from commercially available D-galactono-1,4-lactone (**3**) is described. The key steps are the β-elimination of the protected aldonolactone **4** to furnish the desired 2,3-unsaturated ketone **5** and selective phosphorylation of the primary hydroxyl moiety of the diol **7**. The purity of the KDPGal sample was $>95\%$ as determined by enzymatic assay using crude KDPGal aldolase from *Pseudomonas saccharophila*. An initial substrate specificity and pH–activity profile of KDPGal aldolase are described.

Keywords: 2-Keto-3-deoxy-6-phosphogalactonate (KDPGal); P. saccharophila; Aldol condensation; Pyruvate aldolases; *P. cepacia*; *E. coli*

1. Introduction

Enzymatic aldol condensation is a powerful method for stereocontrolled carbon–carbon bond formation in organic synthesis [1–6]. The aldolases are broadly divided into four groups according to nucleophile type; specifically dihydroxyacetone phosphate, acetaldehyde, glycine, and pyruvate or phosphoenolpyruvate aldolases. Of these groups, the dihydroxyacetone phosphate aldolases have been most extensively examined for synthetic utility [7–10]. More recently, the utility of the pyruvate and phosphoenolpyruvate aldolases has been explored [11–20]. In particular, *N*-acetylneuraminic acid aldolase (EC 4.1.3.3) [11–19] and 2-keto-3-deoxyoctulosonate aldolase (EC 4.1.2.23) [20] have been investigated. In both cases, the substrate specificity is sufficiently broad to allow the preparation of analogues of the natural products. The diastereoselectivity of both enzymes is under thermodynamic control and consequently, both the stereochemical sense and purity of the products depend critically on the configuration of the electrophilic aldehyde [15,17,19,20].

We are currently exploring the utility of the pyruvate aldolases as catalysts in organic synthesis. The pyruvate aldolases 2-keto-3-deoxy-6-phosphogluconate aldolase (KDPG aldolase, EC 4.1.2.14) and 2-keto-3-deoxy-6-phosphogalactonate aldolase (KDPGal aldolase, EC 4.1.2.21) are key enzymes in bacterial oxidative degradation of glucose and galactose, respectively. In the synthetic sense, the aldolases catalyze the addition of pyruvate with D-glyceraldehyde-3-phosphate to furnish either

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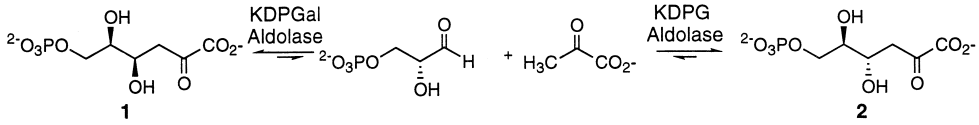
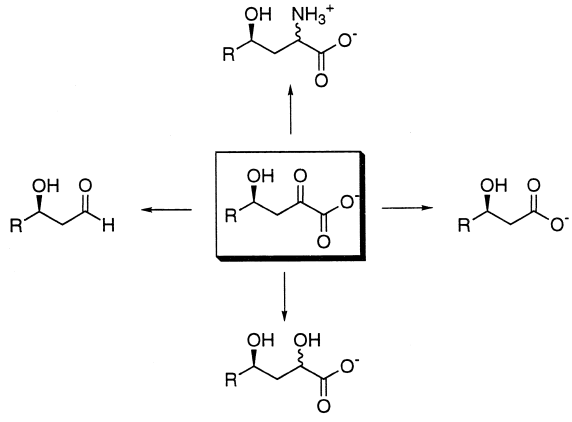


Scheme 1. KDPG and KDPGal aldolase catalyzed reactions.

KDPGal **1** or KDPG **2** (Scheme 1) [21]. The products of pyruvate aldolase-catalyzed addition are 4-substituted 4-hydroxy-2-ketobutyrates, a framework both densely and differentially functionalized. This substitution pattern allows facile transformation of enzymatic aldol products to a variety of complex natural products (Scheme 2).

To date, work in our laboratory has focused primarily on the synthetic utility of KDPG aldolase from several microbial sources [22–26]. KDPG aldolase generates 4-hydroxy-2-ketobutyrates with the *S* configuration at the newly formed stereogenic center via *si*-face addition of pyruvate to an electrophilic aldehyde [22,23]. Furthermore, KDPG aldolase from some, but not all, sources accepts only aldehydes possessing the *R* stereochemical configuration at C2. Concomitant kinetic resolution and stereospecific recognition of diastereotopic faces produce a vicinal diol with the D-*erythro* configuration at C4/C5 [23].

To broaden the scope and power of pyruvate aldolase catalyzed addition, we are now exploring the synthetic utility of KDPGal aldolase. KDPGal aldolase generates aldol adducts with the complementary *R* stereochemical configuration at the new stereogenic center [21]. Assuming the aldolase effects a kinetic resolution of racemic aldehyde electrophiles similar to KDPG aldolase, KDPGal aldolase will provide access to vicinal diols with the D-*threo* configuration.



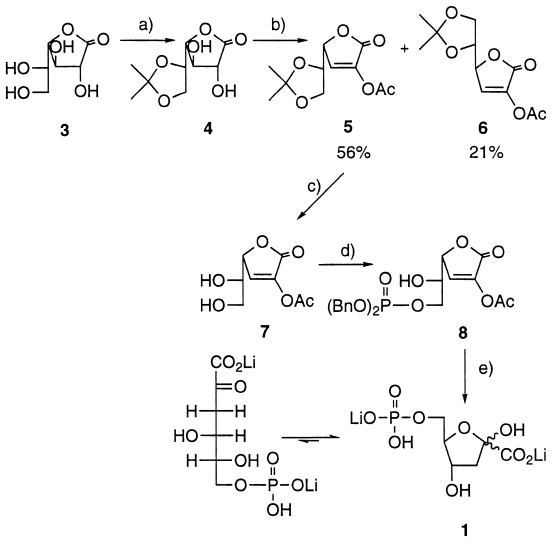
Scheme 2. Synthetic manipulations of KDPG aldolase products.

2. Results and discussion

2.1. Synthesis of KDPGal

KDPGal aldolase has been identified in a variety of sources, including *Pseudomonas saccharophila* [27], *Azotobacter vinelandii* [28], *P. fluorescens* [29], *Rhizobium meliloti* [30], *Helicobacter pylori* [31], *Caulobacter crescentus* [32], *P. cepacia* [33] and *Escherichia coli* [34]. In all instances, KDPGal aldolase is an inducible enzyme, expressed only when cells are grown on galactose or galactonate as the sole carbon source. Alternatively, KDPG aldolase is a constitutive enzyme; consequently KDPGal aldolase must be purified from the closely related KDPG aldolase [27]. Although such a purification has been reported by Meloche et al., assay of individual enzymes requires access to pure KDPGal [27]. Two syntheses of KDPGal have been reported. A synthesis by Meloche et al. utilizes KDPGal aldolase for aldol addition of pyruvate to D-glyceraldehyde-3-phosphate [35]. Additionally, a single chemical synthesis of KDPGal has been reported [36]. The synthesis proceeds in an overall yield of under 0.5% and provides a product only 50% pure by enzymatic assay. Here, we report a facile five-step chemical synthesis of KDPGal (Scheme 3).

The synthesis proceeds from commercially available D-galactono-1,4-lactone (**3**). The transformation requires two essential conversions:

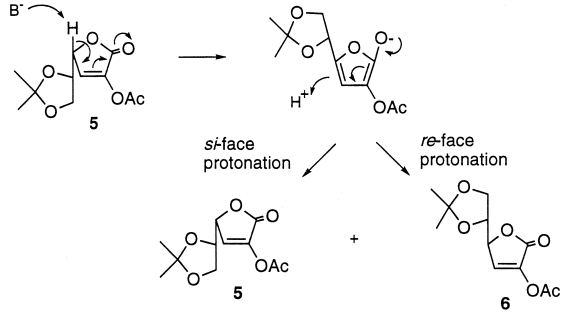


Reagents:
a) acetone, H_2SO_4, 99%; b) pyridine, acetic anhydride,CH_2Cl_2, 50 °C;
c) 80% glacial acetic acid, 95%; d) DDP, 1*H* -tetrazole, THF, then *m*CPBA,
40%; e) H_2, 10% Pd/C, THF then aq. 50 mM LiOH, 82%.

Scheme 3. Chemical synthesis of KDPGal.

elimination of water across the C2–C3 bond to set the 2-keto-3-deoxy moiety, and selective phosphorylation of the primary hydroxyl moiety. Although acetylated aldonolactones readily undergo β-elimination to give 2,3-unsaturated lactones, the elimination of acetylated 1,4-lactones is problematic since the initially formed 2,3-unsaturated lactones readily undergo further eliminations [37].

In an effort to minimize this side reaction, the lactone **3** was treated with sulfuric acid in acetone to furnish the 5,6-*O*-isopropylidine **4** as the sole product in excellent yield. This product



Scheme 4. By-product of β-elimination reaction.

was treated with pyridine and acetic anhydride at 50°C for 48 h, producing the desired β-elimination product **5** in a single step. The major by-product is the *gluco*-epimer **6**, presumably arising from the corresponding furan (Scheme 4).

Selective phosphorylation of the primary alcohol following removal of the isopropylidene was achieved utilizing dibenzyl-*N*,*N*-diethylphosphoramidite and 1*H*-tetrazole in THF at 0°C [38]. Oxidation to the phosphate **8** was accomplished with 85% 3-chloroperoxybenzoic acid in methylene chloride at -60°C, and removal of the benzyl ether phosphate protecting groups by hydrogenolysis yielded the C2 protected KDPGal δ-lactone. Simultaneous hydrolysis of the C2 acetate and δ-lactone were effected by 50-mM aq. LiOH, to furnish KDPGal **1** in 82% yield. Care must be taken in this step to avoid basic pH (>7.5) which leads to retroaldol cleavage of KDPGal. Together, this route provides KDPGal in five steps with an overall yield of 17%. The purity of KDPGal by enzymatic assay with *P. saccharophila* crude extracts was $>95\%$ [27]. As expected, KDPG aldolase from *E. coli* did not cleave KDPGal.

2.2. Isolation of KDPGal aldolase

2.2.1. P. saccharophila

Meloche and O'Connell [27] reported an isolation of KDPGal aldolase from *P. saccharophila* that produces roughly 35 U of KDPGal aldolase and 20 U of KDPG aldolase per gram of cell paste. Because KDPG aldolase is a significant contaminant in *P. saccharophila* grown on galactose, the reported isolation involves five chromatographic steps. The overall reported yield of KDPGal aldolase during purification is 14%, and the enzyme has a final specific activity of 130 U mg^{-1} [27].

Although Meloche et al. were able to achieve cell yields of 34 g l^{-1} using a 400-l fermentor, on an 8-l scale we were unable to attain cell yields of greater than 8 g l^{-1}. At this level of production, an 8-l growth provides roughly 300

U of pure enzyme after a laborious separation. Furthermore, we were unable to achieve complete separation of KDPGal and KDPG aldolases: the highest ratio of activities observed was roughly 50:1. The consequences of incomplete separation are unclear. If the enzymatic aldol addition is reversible, the presence of two catalysts with opposite stereoselectivities would yield a mixture of products determined solely by the difference in product free energies. Because these differences are typically small, the types of product mixtures observed with *N*-acetylneuraminic acid aldolase are expected [15,17,19]. Alternatively, if enzymatic aldol addition is irreversible, the mixture of stereoisomers produced by a mixture of KDPG and KDPGal aldolases would depend solely on the relative activities of the two proteins. In previous work, we have noted that KDPG aldolase-catalyzed additions are irreversible [24]. Presumably the failure to observe retro-aldol addition is the result of product cyclization to lactone and/or hemiketal derivatives: unlike both *N*-acetylneuraminic and 2-keto-3-deoxyoctulosonate aldolases, KDPG aldolase is specific for the open-chain form of the substrate [24].

Ultimately, due to the low yield of enzyme, the arduous separation of KDPGal aldolase from KDPG aldolase and our inability to completely remove traces of KDPG aldolase, we abandoned *P. saccharophila* as a source of KDPGal aldolase.

2.2.2. P. cepacia

In order to circumvent the laborious separation of KDPG and KDPGal aldolases, we investigated KDPGal aldolase from an eda$^-$ mutant of *P. cepacia*, reported originally by Lessie et al. [33,39]. This organism grows to a cell density of 2.4 g l^{-1} on a glycerol medium augmented with 1% galactose. Crude cell extracts contained 13 U of KDPGal aldolase per gram of cell paste at a specific activity of 0.39 U mg^{-1}. KDPG aldolase was undetectable in the same extracts. KDPGal aldolase was purified to a specific activity of 4.2 U mg^{-1} with an overall yield of 86% in a single chromatographic step on Sepharose CL-4B derivatized with a Procion Navy H-ER triazine dye [23]. The protein can be stored as a lyophilized powder at −78°C for periods of at least months with no loss of activity. The enzyme shows a broad bell-shaped

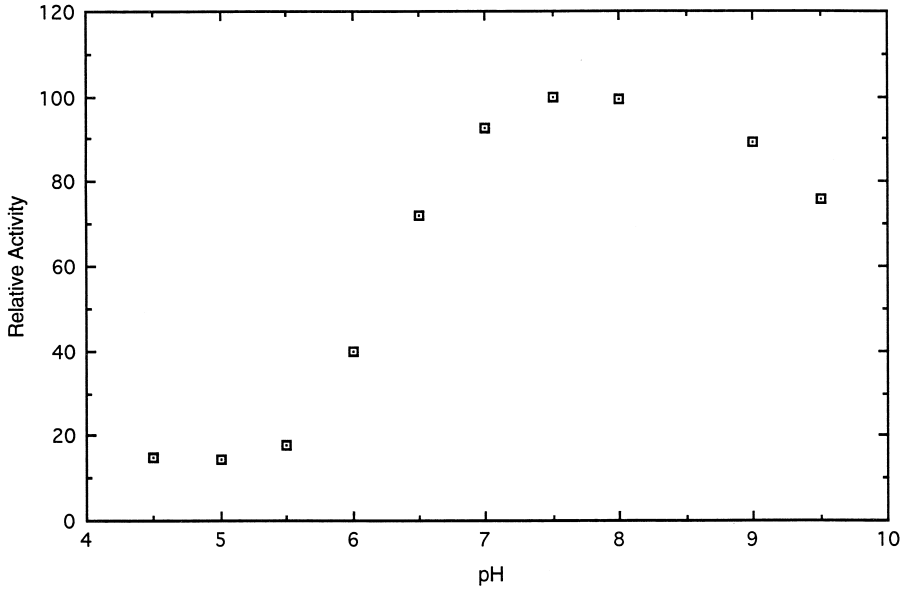


Fig. 1. pH–activity profile for KDPGal aldolase from *P. cepacia* eda$^-$.

Table 1
Substrate specificity of KDPGal aldolase from *P. cepacia* eda$^-$ strain 249-27

Electrophile	Nucleophile	V_{rel}
D-Glyceraldehyde	pyruvate	100%
L-Glyceraldehyde		0%
Glyoxylate		101%
D-Erythrose		56%
Chloroacetaldehyde		41%
L-Threose		75%
D-Ribose		76%
D-Ribose-5-phosphate		180%
2-Pyridine carboxaldehyde		78%
Benzaldehyde		0%
Valeraldehyde		3%
D-Glyceraldehyde-3-phosphate	3-fluoropyruvate	0%
D-Glyceraldehyde-3-phosphate	2-oxobutyrate	0%

pH-activity profile, retaining at least 50% of maximum activity over a pH range of 6.5–9.5 (Fig. 1) [40].

The enzyme shows a broad substrate specificity similar to that exhibited by KDPG aldolase (Table 1) [22,24]. As with KDPG aldolase, removal of the phosphate at C3 of D-glyceraldehyde-3-phosphate leads to a loss of activity of roughly 100-fold [24]. However, a range of substrates containing polar functionality at C2 or C3 are accepted at synthetically useful rates. While substrate specificity studies are useful for delineating the range of compounds theoretically accessible, preparative scale reactions are necessary to unambiguously determine product identity and the level of stereoselectivity available with KDPGal aldolase. We have conducted preparative-scale reactions using D-glyceraldehyde and 2-pyridinecarboxaldehyde as electrophilic substrates: in both instances, enzymatic aldol addition yields the expected products arising solely from *re*-face addition of pyruvate to the aldehyde. Details of these syntheses are reported elsewhere [40].

2.2.3. E. coli

KDPGal aldolase has also been reported from *E. coli* [34]. In the course of our investigations of KDPG aldolase, we observed production of KDPGal aldolase by the *E. coli* eda$^-$ mutant DF214 containing the *E. coli* eda high copy plasmid pTC 190 [41]. When grown on glucose or gluconate, this strain produces KDPG aldolase at a level of 13,500 U l^{-1}. Alternatively, growth of this same organism on Luria broth augmented with 1% galactose produces KDPG aldolase at 2025 U l^{-1} in addition to KDPGal aldolase at a rate of 375 U l^{-1}. The specific activity of KDPGal aldolase in the ammonium sulfate pellet following cell disruption was 4.6 U mg^{-1}. Clearly this source of KDPGal aldolase is superior to either *P. saccharophila* or mutant *P. cepacia* in terms of total enzyme yield [27,40]. Unfortunately, the large amount of KDPG aldolase expressed by the pTC 190 plasmid makes the crude preparation unsuitable for synthetic use. Attempted growth of DF214 absent the pTC 190 plasmid produced low cell masses, impeding investigations of the eda$^-$ strain.

The origin of the tremendous overproduction of KDPGal aldolase is unclear. KDPG and KDPGal aldolases are far removed on the *E. coli* genome, eliminating the possibility of adventitious incorporation of the KDPGal gene into the pTC 190 plasmid [42]. Apparently overexpression of the eda gene product or resulting change in metabolism upregulates production of KDPGal aldolase in some fashion.

We attempted purification of *E. coli* KDPGal aldolase by differential dye-ligand chromatography [23,43]. Because *E. coli* KDPG aldolase is not retained by the Procion Navy H-ER dye, a mixture of KDPG and KDPGal aldolases were passed over this ligand on a Sepharose support. As expected, KDPG aldolase was not retained by the column [23]. No KDPGal aldolase was observed in the wash eluent. Unfortunately, treatment of the column with a mixture of pyruvate and ($\pm$)-α-glycerophosphate failed to elute KDPGal aldolase. At this point, it is unclear whether the aldolase is unstable during purification or if the protein is not eluted by the substrate mimics. Accordingly, the protocol was abandoned.

In conclusion, we have developed a chemical synthesis of KDPGal that facilitates isolation of

KDPGal aldolase from mixtures of this enzyme and KDPG aldolase. We have identified a ready source of KDPGal aldolase and demonstrated its suitability for organic synthesis. We continue to explore other sources of KDPGal aldolase and the synthetic utility of the pyruvate aldolases and will report our findings in due course.

3. Experimental

3.1. General procedures

THF was distilled from sodium/benzophenone prior to use. Petroleum ether refers to the fraction boiling in the range 35–60°C. Chromatography refers to the method of Still et al. [44] using Silica Gel 60 F_{254}, 230–400 mesh. Dibenzyl-*N*-*N*-diethylphosphoramidite (DDP) was synthesized according to the method of Wong et al. [38]. Procion dyes were a gift of ICI Zeneca Colors, Charlotte, NC. All reagents and chemicals were purchased from Aldrich or Sigma Chemical and used without purification. *P. cepacia* eda$^-$ strain 249-27 was donated by P.V. Phibbs, Jr. (East Carolina University) [33,39]. Plasmid pTC 190 was a generous gift from T. Conway (University of Nebraska) [42]. *E. coli* DF214 was a gift from B. Bachmann, *E. coli* Genetic Stock Center, Yale University [45].

^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian Unity 400 instrument. Chemical shifts (δ in ppm) are given relative to HOD at 4.63 ppm or $CDCl_3$ at 7.26 ppm. ^{13}C NMR are referenced to *p*-dioxane at 66.664 ppm. Kinetic assays were performed on a Hewlett-Packard HP-8452A UV/VIS spectrophotometer. Protein concentrations were determined by the method of Layne.

*3.2. 5,6-O-isopropylidene-D-galactono-1,4-lactone (**4**)*

Concentrated sulfuric acid (8 drops) was added to D-galactono-1,4-lactone **3** (5.0 g, 28.07 mmol) in dry acetone (200 ml) under an atmosphere of argon. The reaction mixture was stirred at room temperature for 1 h. The pH of the solution was adjusted to 7.0 by the addition of $NaHCO_3$ and the solid filtered off. The filtrate was concentrated in vacuo to yield **4** (6.06 g, 99%) as a colorless syrup. ^{1}H NMR ($CDCl_3$) δ 5.10–4.68 (brs, 2H), 4.58 (d, $J = 8.5$ Hz, 1H), 4.40–4.27 (m, 2H), 4.25–4.10 (m, 2H), 4.01 (dd, $J = 8.5$, 6.5 Hz, 1H), 1.41 (s, 3H), 1.37 (s, 3H) ppm; ^{13}C NMR ($CDCl_3$) δ 174.26, 109.67, 79.33, 73.77, 73.54, 73.46, 64.31, 25.39, 24.64 ppm; IR λ 3359, 1783 cm^{-1}.

*3.3. 2-O-acetyl-3-deoxy-5,6-O-isopropylidene-D-threo-hex-2-enono-1,4-lactone (**5**) and 2-O-acetyl-3-deoxy-5,6-O-isopropylidene-D-erythro-hex-2-enono-1,4-lactone (**6**)*

Pyridine (24 ml) and acetic anhydride (14 ml) were added to a solution of the diol (**3**) (6.0 g, 27.5 mmol) in dry methylene chloride (30 ml) under an atmosphere of argon. The reaction mixture was stirred for 48 h at 50°C. The reaction was taken up in dichloromethane (50 ml) and washed with water (3×75 ml), sat. aq. sodium hydrogencarbonate (3×75 ml), sat. aq. copper sulfate (3×75 ml) and brine (2×50 ml). The solvent was dried ($MgSO_4$), filtered and concentrated in vacuo to yield a viscous, black oil. Chromatography [1:1 ethyl acetate–petroleum ether] of the crude product afforded the desired product **5** (3.70 g, 56%) as a white solid. m.p. 94–95°C (EtOH); ^{1}H NMR ($CDCl_3$) δ 7.16 (d, $J = 1.8$ Hz, 1H), 5.02 (dd, $J = 3.8$, 1.8 Hz, 1H), 4.37–4.28 (m, 1H), 4.05 (dd, $J = 8.8$, 7.2 Hz, 1H), 3.83 (dd, $J = 8.8$, 5.5 Hz, 1H), 2.24 (s, 3H), 1.35 (s, 3H), 1.28 (s, 3H) ppm; ^{13}C NMR ($CDCl_3$) δ 166.25, 165.70, 138.00, 129.46, 109.81, 77.43, 73.75, 63.94, 25.21, 24.28, 20.14 ppm; IR λ 1767, 1652 cm^{-1}. Anal. Calcd. for $C_{11}H_{14}O_6$: C 54.53, H 5.83. Found: C 54.28, H 5.61; and the epimer **6** (1.4 g, 21%) as a white solid. m.p. 78–80°C (EtOH); ^{1}H NMR ($CDCl_3$) δ 7.38 (d, $J = 1.8$ Hz, 1H), 4.86 (dd, $J = 8.0$, 1.8 Hz, 1H), 4.19–4.06 (m, 2H), 4.02–3.95 (m, 1H), 2.32 (s, 3H),

1.46 (s, 3H), 1.35 (s, 3H) ppm; ^{13}C NMR ($CDCl_3$) δ 166.19, 165.66, 137.67, 131.53, 109.82, 78.29, 75.61, 65.90, 26.02, 24.16, 20.15 ppm; IR λ 1782, 1647 cm^{-1}; FAB HRMS obsd. 243.0869, calcd. for $C_{11}H_{15}O_6$ 243.0869.

3.4. 2-O-acetyl-3-deoxy-D-threo-hex-2-enono-1,4-lactone (7)

A mixture of glacial acetic acid (8 ml), water (2 ml) and the lactone (5) (1.10 g, 4.55 mmol) was stirred at room temperature for 16 h. The solvent was concentrated in vacuo to give a pale yellow oil. Chromatography (ethyl acetate) of the crude product afforded the diol **7** (873 mg, 95%) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 7.31 (d, $J = 1.8$ Hz, 1H), 5.18 (dd, $J = 4.0$, 1.8 Hz, 1H), 4.38 (brs, 2H), 3.96–3.87 (m, 1H), 3.80–3.67 (m, 2H), 2.30 (s, 3H) ppm; ^{13}C NMR ($CDCl_3$) δ 166.90, 166.75, 137.15, 131.84, 79.48, 70.95, 62.35, 20.06 ppm; IR λ 3409, 1763, 1649 cm^{-1}; FAB HRMS obsd. 203.0556, calcd. for $C_8H_{11}O_6$ 203.0556.

3.5. 2-O-acetyl-3-deoxy-6-dibenzyl-oxyphosphate-D-threo-hex-2-enono-1,4-lactone (8)

Dibenzyl-*N*-*N*-diethylphosphoramidite (DDP) (1.14 g, 3.6 mmol) was added to a solution of the diol (7) (560 mg, 2.77 mmol) and 1*H*-tetrazole (393 mg, 5.54 mmol) in dry THF (20 ml) at 0°C under an atmosphere of argon and the mixture was stirred for 30 min. The reaction mixture was cooled to -60°C and a solution of 85% 3-chloroperoxybenzoic acid (1.12 g, 5.54 mmol) in methylene chloride (10 ml) added. After stirring 15 min at room temperature, 10% aq. $NaHSO_3$ (20 ml) was added and the mixture stirred for a further 10 min. Diethyl ether (100 ml) was added and the aqueous phase discarded. The ethereal phase was washed with 10% aq. $NaHSO_3$ (2×30 ml), 5% aq. $NaHCO_3$ (2×30 ml), dried ($MgSO_4$), filtered and concentrated in vacuo to give a colorless oil. Chromatography [1:1 methylene chloride–ethyl acetate] of the crude product yielded **8** (510 mg, 40%) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 7.30–7.26 (m, 10H), 7.12 (d, $J = 2.0$ Hz, 1H), 5.03–4.95 (m, 4H), 4.92 (dd, $J = 2.0$, 3.5 Hz, 1H), 3.99 (dd, $J = 5.2$, 9.3 Hz, 2H), 3.94–3.88 (m, 1H), 3.42–3.24 (brs, 1H), 2.21 (s, 3H) ppm; ^{13}C NMR ($CDCl_3$) δ 166.31, 166.02, 137.42, 134.72, 134.64, 130.25, 130.15, 127.99, 127.59, 127.45, 78.20, 69.19, 68.85, 67.09, 20.12 ppm; ^{31}P NMR (CH_2Cl_2) δ -0.69 ppm; IR λ 3352, 1775, 1650 cm^{-1}; FAB HRMS obsd. 463.1158, calcd. for $C_{22}H_{24}O_9P$ 463.1158.

3.6. 2-Keto-3-deoxy-6-phosphogalactonate (1)

The lactone **8** (270 mg, 0.584 mmol) was dissolved in THF (20 ml) and 10% Pd/C (25 mg). The solution was hydrogenated at atmospheric pressure for 15 min then filtered through celite. The solvent was concentrated in vacuo and water (15 ml) added. The pH was adjusted to 7.5 using aq. 50-mM LiOH and the reaction mixture stirred at room temperature until no further decrease in pH was observed (the pH was maintained at 7.5 by the addition of aq. 50-mM LiOH). The pH was adjusted to 7.0 with aq. 50-mM HCl and the solution lyophilized to give KDPGal **1** (130 mg, 82%) as a pale yellow solid. ^{1}H NMR (D_2O) δ 4.26–4.14 (m, 1H), 4.06–3.88 (m, 1H), 3.82–3.40 (m, 2H), 2.24–1.78 (m, 2H) ppm; ^{13}C NMR (D_2O) δ 177.01, 176.76, 103.51, 103.10, 83.58, 83.48, 82.45, 82.35, 71.63, 70.42, 62.27 ppm; ^{31}P NMR (D_2O) δ 4.96, 4.82 ppm; IR λ 3390, 1722, 1627 cm^{-1}.

3.7. P. saccharophila KDPG and KDPGal aldolase isolation

P. saccharophila ATCC 4.1.2.21 was grown on galactose as described by Meloche and O'Connell [27]. The cells were harvested by centrifugation then suspended in KH_2PO_4 buffer (20 mM, pH 7.5, 1.5 volumes) and cooled to 0°C. The cells were disrupted by sonication for a total of 5 min. Protamine sulfate (2%, pH 5.5,

0.2 volumes) was added to the crude extracts and the mixture stirred for 15 min at room temperature. The mixture was then centrifuged for 30 min ($22{,}000 \times g$) and the solids discarded. The *P. saccharophila* extracts were used for assays without further purification.

3.8. KDPGal purity by enzymatic assay

KH_2PO_4 buffer (20 mM, pH 7.5, 3.0 ml), NADH (50 μl, 15 mg/ml), L-lactic dehydrogenase (EC 1.1.1.27, Type II from rabbit muscle, 10 μl), and *P. saccharophila* cellular extracts, containing both KDPGal aldolase and KDPG aldolase (25 μl) were added to a disposable cuvette. An initial UV absorbance value at 340 nm was recorded. KDPGal (10 μl, 100 mg/ml) was added to the sample to initiate the reaction. After stabilization, the final absorbance value was recorded [46].

3.9. Determination of KDPGal aldolase activity

KDPGal aldolase activity was determined by a modification of a coupled assay with L-lactic dehydrogenase (L-LDH, EC 1.1.1.27, type II, rabbit muscle) as previously described by Meloche and Wood [46]. 20-mM KH_2PO_4 (pH 7.5, 3.0 ml), NADH (50 μl, 15 mg/ml), L-LDH (10 μl, 100 U) and KDPGal aldolase (50 μl) were added to a 4.5 ml disposable cuvette. The reaction was initiated by the addition of KDPGal (50 μl, 100 mg/ml, Li^+ salt). pH-Activity studies were conducted in a similar fashion with 20-mM KH_2PO_4 buffer at various pH values.

3.10. Growth and purification of P. cepacia KDPGal aldolase

P. cepacia eda$^-$ strain 249-27 was grown according to published procedures on glycerol/1% galactose followed by growth on the same medium in 4-l Erlenmeyer flasks at 37°C [33,39]. The cells were pelleted by centrifugation ($19{,}690 \times g$, 10 min) and suspended in 20-mM KH_2PO_4, pH 6.5 with 1-mM PMSF. The cells were disrupted by sonication with a Heat Systems Ultrasonics sonicator/cell disrupter (macro tip, power level 10) at 0°C for a total of 5 min. The resulting extract was treated with 2% protamine sulfate, pH 5.5 (0.2 volumes) for 10 to 15 min at room temperature and then centrifuged ($37{,}050 \times g$, 30 min). The resulting supernatant was made to 25% saturation with solid $(NH_4)_2SO_4$ and the solid removed by centrifugation ($37{,}050 \times g$, 20 min). The supernatant was made to 50% saturation with solid $(NH_4)_2SO_4$ and the solid removed by centrifugation ($37{,}050 \times g$, 20 min). The supernatant was then made to 65% saturation with solid $(NH_4)_2SO_4$ and the pellet following centrifugation ($37{,}050 \times g$, 20 min) was suspended in 20-mM KH_2PO_4, pH 6.5 and dialyzed overnight against 20 volumes of the same buffer. The extract (≤ 1 g of protein) was placed on the Procion Navy H-ER column [23,43]. The column was washed with 20-mM KH_2PO_4, pH 6.5 (100 ml) then with 30-mM α-DL-glycerophosphate, pH 6.5 (50 ml). The aldolase was selectively eluted with 20-mM sodium pyruvate in 30-mM α-DL-glycerophosphate, pH 6.5 (50 ml) in 84% yield.

3.11. Growth and purification of E. coli KDPGal aldolase

E. coli was grown according to published procedures with an additional 1% galactose followed by growth on the same medium in 4-l Erlenmeyer flasks at 37°C [41,42,45]. Isolation of KDPG and KDPGal aldolases proceeded as with the *P. cepacia* strain. KDPG aldolase was not retained on the Procion Navy H-ER dye column while KDPGal aldolase was retained. The substrate mimic mixture did not elute any KDPGal aldolase.

3.12. Unnatural nucleophile and electrophile assays

Assays were conducted on a 3-ml scale with nucleophile and electrophile concentrations at

50 mM. Twenty units of *P. cepacia* KDPGal aldolase were used for each assay. The 100-μl aliquots were taken at timely intervals and the aldolase in the aliquots destroyed with 7% perchloric acid (30 μl). The samples were neutralized with 1-M NaOH (20 μl) and diluted with 20-mM KH_2PO_4, pH 6.5 (1000 μl). The reactions were followed by monitoring the disappearance of the nucleophile with L-LDH [46].

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