Pyruvate aldolases in chiral carbon–carbon bond formation

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A procedure for the preparation of optically pure α-keto-γ-hydroxy carboxylic acids through stereospecific aldol addition catalyzed by pyruvate aldolases from the Entner–Doudoroff and the DeLey–Doudoroff glycolytic pathways is described. This highly versatile fragment serves as a precursor for a variety of commonly encountered functionalities, including β-hydroxy aldehydes and carboxylic acids, α-amino-γ-hydroxy carboxylic acids and α,γ-dihydroxy carboxylic acids. The protocol described here uses recombinant His_6-tagged KDPG aldolase for the synthesis of (*S*)-4-hydroxy-2-keto-4-(2′-pyridyl)butyrate. A protocol for evaluating enantiomeric excess through formation of the γ-lactone of the dithioacetal followed by chiral-phase gas–liquid chromatography is also described. Enzyme expression and enzymatic synthesis can be accomplished in approximately 1 week. The enzymatic aldol addition proceeds in nearly quantitative yields with enantiomeric excesses greater than 99.7%.

INTRODUCTION

In addition to managing the flow of small molecules that create and support life, enzymes have found great utility in chemical synthesis[1–3]. With the exception of only a few categories, every mechanistic class of reactions known to the organic chemist has an enzymatic equivalent. Enzymes operate with enormous efficiency, providing rate enhancements up to 10^{12} over the uncatalyzed reaction, and proceed in aqueous solution, at or near room temperature and neutral pH. Of greatest advantage, enzymes are chiral catalysts, and in many instances carry out reactions stereospecifically. Roughly 60% of all pharmaceutical agents at market today are chiral, and the well-known problems associated with the use of racemic drugs has led to a growing regulatory pressure for the sale and use of enantiomerically pure formulations[4–8]. The chiral nature of enzymes facilitates both enantiomerically pure synthesis and the resolution of racemates: today, every major pharmaceutical producer has in place or makes use of a biocatalysis unit.

All synthesis ultimately rests on carbon–carbon bond formation. Although a number of carbon–carbon bond formation strategies are known in nature, the enzymatic aldol reaction has found the greatest use in organic synthesis[9,10]. Originally reported by Whitesides[11–14] and Effenberger[15–17], and later elaborated by Wong[18–21], Fessner[22–24] and others[25–27], the dihydroxyacetone phosphate glycolytic aldolases provide access to virtually any 1,3,4-trihydroxy-2-butanone skeleton. Although the enzymes show a strict requirement for dihydroxyacetone phosphate as the nucleophilic component, the enzymes accept virtually any aldehyde as the electrophilic component, providing a powerful set of catalysts. Thus, for example, the use of azide-containing electrophilic substrates facilitated the synthesis of an enormous diversity of azasugar glycosidase inhibitors[28]. Still, difficulties associated with the preparation of dihydroxyacetone phosphate, mixed kinetic and thermodynamic diastereoselectivity and the production of a highly but not differentially functionalized carbon skeleton have limited the broader use of the DHAP aldolases in organic synthesis.

In contrast, aldolases of the Entner–Doudoroff and DeLey–Doudoroff glycolytic pathways catalyze aldol addition of pyruvate to an electrophilic aldehyde to produce a 2-keto-4-hydroxybutyrate skeleton (**Fig. 1**). This moiety is both highly and differentially functionalized, lending itself to ready synthetic elaboration to, for example, α-amino-γ-hydroxybutyrates, α,γ-diydroxybutyrates and α-hydroxy aldehydes and acids.

2-Keto-3-deoxy-6-phosphogluconate aldolase (KDPG) is a type I pyruvate aldolase of the Entner–Doudoroff glycolytic pathway, which metabolizes glucose through sequential conversion to KDPG followed by retro-aldol cleavage to the key metabolites pyruvate and glyceraldehyde-3-phosphate (G3P)[29]. The equilibrium characterizing KDPG–pyruvate/G3P interconversion lies far toward synthesis[30], and during aldol addition, KDPG aldolase catalyzes the addition of pyruvate to electrophilic aldehydes with *si*-facial selectivity (**Fig. 1**). In contrast to other pyruvate aldolases, KDPG aldolase displays complete kinetic stereoselectivity, further enhancing its utility as a synthetic catalyst[31]. The related enzyme 2-keto-3-deoxy-6-phosphogalactonate (KDPGal) aldolase catalyzes the analogous reaction, but with *re*-facial selectivity, providing access to both stereochemical sequences[32]. The enzyme shows reasonably broad substrate specificity with regard to the electrophilic moiety, accepting straight-chain, branched and cyclic aldehydes, provided they possess polar functionality at C2, C3 or C4. Like the DHAP aldolases, both KDPG and KDPGal aldolase show more restricted substrate specificity with regard to the nucleophilic component, accepting in addition to pyruvate, 2-oxobutyrate and fluoropyruvate.

In the course of developing an enzymatic synthesis of the unnatural amino acid (2*S*,4*S*) 2-amino-4-hydroxy-4-(2-pyridyl) butyrate, we developed an enzymatic strategy to prepare the key intermediate (*S*)-4-hydroxy-2-keto-4-(2′-pyridyl)butyrate[33,34]. The synthesis involves aldol addition between 2-pyridinecarboxaldehyde

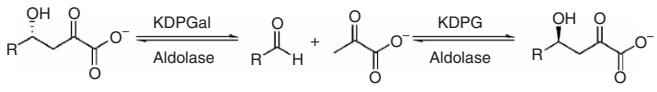

Figure 1 | Pyruvate aldolases in synthesis.

and pyruvate catalyzed by KDPG aldolase (**Fig. 2**), and is illustrative of the use of KDPG and KDPGal aldolases for stereocontrolled carbon–carbon bond formation. Formation of the dithioacetal accompanies formation of the corresponding γ-lactone: this species is sufficiently volatile to permit determination of enantiomeric excess by chiral-phase gas–liquid chromatography.

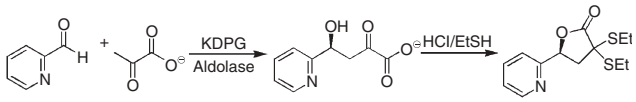

Figure 2 | Synthesis of (*S*)-4-hydroxy-2-keto-4-(2′-pyridyl)butyrate and lactone formation.

Experimental design

This protocol describes the expression and application of KDPG aldolase for stereocontrolled carbon–carbon bond formation. Although we describe the protocol for the specific case of 2-pyridinecarboxaldehyde as the electrophilic substrate, the protocol is applicable to other aldehydic substrates. Similarly, substitution of KDPGal aldolase furnishes the analogous series of products epimeric at C4. The protocol described here is directly applicable to substrates other than 2-pyridinecarboxaldehyde, although reaction times and enzyme concentrations must be scaled to reflect relative reactivities. The protocols are also applicable for related enzymes—for example, KDPGal aldolase—although substrate specificities must first be evaluated to determine the feasibility of each transformation.

MATERIALS

REAGENTS

- Kanamycin sulfate (Fluka, cat. no. 60615)
- Terrific broth (EMD Chemicals Inc., cat. no. 1.01629.5007)
- HEPES (Sigma, cat. no. H3375)
- Luria broth (EMD Chemicals Inc., cat. no. 1.10285.5007)
- Agar (Difco, cat. no. 214530)
- Trizma hydrochloride (Sigma, cat. no. T5941)
- Imidazole (Sigma-Aldrich, cat. no. I2399)
- EDTA (Aldrich, cat. no. 431788)
- Nickel(II) sulfate hexahydrate (Sigma-Aldrich, cat. no. 467901)
- His●Bind Resin (Novagen, cat. no. 69670-5)
- pET28b plasmid (Novagen, cat. no. 69865-3)
- KDPG-pet (available from the authors on request[36])
- KDPG aldolase (available from the authors on request[36])
- BL21 CodonPlus (DE3)-RIL cells (Stratagene, cat. no. 230245)
- SOC media
- IPTG (Calbiochem, cat. no. 420322)
- 12% (w/v) Tris-HCl gel (Bio-Rad, cat. no. 161-1102)
- Amberlyst 131 (Sigma, cat. no. A2461)
- Sodium chloride (Sigma-Aldrich, cat. no. 204439)
- Sodium hydroxide (Sigma-Aldrich, cat. no. 480878)
- Sodium pyruvate (Sigma-Aldrich, cat. no. P8574)
- 2-pyridinecarboxaldehyde (Aldrich, cat. no. P62003)
- Hydrochloric acid (Sigma-Aldrich, cat. no. 435570)
- Oxalacetic acid (Aldrich, cat. no. 171255)
- Magnesium sulfate (Aldrich, cat. no. 203726)
- Methylene chloride (Aldrich, cat. no. 676853)
- Ethanethiol (Aldrich, cat. no. E3708)
- Lithium chloride (Sigma-Aldrich, cat. no. 310498)
- Ethanol, 200 proof (Aldrich, cat. no. 459836)
- Distilled water

EQUIPMENT

- 50 ml tube top filter (Corning, cat. no. 430320)
- 50 ml centrifuge tube (Corning, cat. no. 430829)
- 250 ml filter system (Corning, cat. no. 430281)
- 250 ml Erlenmeyer flask (Corning, cat. no. 4980-250)
- 4 liter Erlenmeyer flask (Corning, cat. no. 4980-4L)
- pH meter (Thermo Orion, cat. no. 0420A0)
- Econo-Column column (Bio-Rad, cat. no. 737-2531)
- Petri dish (BD Falcon, cat. no. 351009)
- HP8453 UV–visible system (Hewlett Packard)
- 150 ml lyophilizer jar (Labcono, cat. no. 7540300)
- 600 ml lyophilizer jar (Labcono, cat. no. 7540800)
- Lyophilizer (Labcono, cat. no. 7754500)
- Incubator shaker (New Brunswick Scientific Inc.)
- Incubator oven (Lab-line Instrument Inc.)
- C18 reverse-phase column (Isco)
- C18 reverse-phase thin-layer chromatography (TLC) plate (EMD Chemicals Inc., cat. no. 15683-3)
- Varian CP-3800 Gas Chromotograph
- Chromopack Chirasil-L-Val column (25 m × 0.25 mm)
- CombiFlash column runner (Retrieve)
- Mini-Protean 3 electrophoresis module (Bio-Rad, cat. no. 165-3302)
- French pressure cell press (SLM Aminco Instrument Inc.)
- Rotary evaporator (Buchi)
- Sorvall RC 5C Plus centrifuge (DuPont Instruments)

PROCEDURE

Protein expression of KDPG aldolase

1| A plasmid containing the gene KDPG-*pet*[36], which encodes His_6-tagged KDPG aldolase, is chemically transformed into a BL21 CodonPlus (DE3)-RIL cell according to published procedures[35].

2| Plate 50 µl of the transformation mixture on an LB/Kan (50 µg ml^{-1}) plate and incubate overnight at 37 °C.

■ **PAUSE POINT** Plated cells are left in the incubator overnight at 37 °C.

3| Introduce a single isolated colony from the LB/Kan plate to 50 ml of TB/Kan (50 µg ml^{-1}) and incubate the culture at 37 °C for 16 h.

■ **PAUSE POINT** To be left in the shaker overnight at 37 °C.

4| Transfer 10 ml of inoculated culture to 1 liter of TB/Kan (50 µg ml^{-1}) and shake at 37 °C until an OD_{600} of 0.8 is reached.

5| Induce protein expression by adding IPTG (230 mg $liter^{-1}$, to 1 mM) to the cell culture to induce protein expression. IPTG binds to the *Lac* repressor, inducing protein production. The induced culture is shaken at 37 °C for an additional 4 h.

6| Collect the cell culture by centrifugation at 4,000*g* for 10 min at 4 °C, and freeze the pellet at −20 °C overnight.
■ **PAUSE POINT** The cell pellet can be stored at −20 °C for up to 3 months with minimal loss in enzyme activity.

7| Thaw the pellet on ice and resuspended cells in 30 ml of 20 mM HEPES, pH 7.5.

8| Lyse the resuspended cells in a French press at 12,000 psi. Three consecutive passes through the French press ensures complete cell lysis. Alternately, sonication can be used to lyse the cell pellet. Optimal enzyme yields are achieved when lysis is conducted at 4 °C.

9| Remove cellular debris by centrifugation at 14,500*g* for 20 min at 4 °C.

10| Condition the Ni^{2+} affinity resin by washing with distilled water and charging with nickel(II) sulfate. Then, load the cellular supernatant onto the Ni^{2+} affinity column and elute His_6-tagged enzyme according to published procedures[35]. The protein is eluted with 50 ml of elution buffer and collected in a 50 ml centrifuge tube.

11| Determine the purity of the expressed protein by analysis on 12% SDS-polyacrylamide gel electrophoresis.

12| Remove imidazole by dialysis of the eluted protein in a 10,000 MWCO cellulose membrane against three changes of 20 mM HEPES, pH 7.5 (2 liters each) at 4 °C for a total of 24 h.
■ **PAUSE POINT** The protein can be stored at 4 °C for up to 6 months without a significant loss in activity.

13| The protein concentration can be estimated by measuring A_{280} using the extinction coefficient of 16,516 M^{-1} cm^{-1}. Typical yields for pure KDPG aldolase are about 120–150 mg $liter^{-1}$. The specific activity of the protein is typically 300–600 U mg^{-1} (one unit of enzyme catalyzes the conversion of 1 mol of KDPG[37] to pyruvate and glyceraldehyde-3-phosphate per minute at pH 7.0). If desired, the enzyme can be assayed in the retroaldol direction using a coupled lactate dehydrogenase assay[29]. In this assay, the disappearance of pyruvate is monitored by a decrease in absorbance at 340 nm as NADH is converted into NAD^+. Typical kinetic parameters for conversion of KDPG by KDPG aldolase are K_m of 0.1 mM, k_{cat} of 80 s^{-1} and k_{cat}/K_m of 800 mM^{-1} s^{-1}.

Synthesis of (*S*)-4-hydroxy-2-keto-4-(2′-pyridyl)butyrate

14| Dissolve 4 ml (36 mmol) of freshly distilled 2-pyridinecarboxaldehyde in 150 ml of 20 mM HEPES, pH 7.5.
! **CAUTION** 2-pyridinecarboxaldehyde can cause irritation to skin, eye and/or throat.

15| Filter the solution through a 0.22 μm filter using a Corning filter system and set this solution aside.

16| Dissolve sodium pyruvate (3.8 g, 34 mmol) in 30 ml of 20 mM HEPES, pH 7.5, in a 50 ml centrifuge tube.
! **CAUTION** Sodium pyruvate can cause skin irritation.

17| Filter this solution through a 0.22 μm filter into a Corning 50 ml tube top filter.

18| Add 5 ml of the sodium pyruvate solution to the 2-pyridinecarboxaldehyde solution and set the rest of the sodium pyruvate solution aside.

19| Add 2–5 mg of KDPG aldolase to the Corning 250 ml reaction vessel and incubate the reaction mixture for 30 min at room temperature.

20| Add an additional 5 ml of sodium pyruvate solution to the reaction vessel and incubate for an additional 30 min.

21| Repeat Step 20 four more times until the pyruvate solution has been added.

22| Incubate the reaction for a further 24 h. During this incubation, the reaction mixture will develop a faint yellowish color.
■ **PAUSE POINT** The reaction proceeds at room temperature overnight.

23| Transfer the reaction to a 600 ml lyophilizer bottle and freeze the material to liquid nitrogen temperature. Once frozen, lyophilize the reaction mixture.
■ **PAUSE POINT** Lyophilization typically proceeds overnight.

24| Transfer the crude material to a 1 liter Erlenmeyer flask and add 250 ml of absolute ethanol to the crude reaction mixture. Stir the mixture at room temperature for 30 min.

25| Filter the insoluble precipitate through a Buchner funnel and into a 500 ml Erlenmeyer flask.
▲ **CRITICAL STEP** The insoluble materials are salts; the desired product remains soluble in the ethanol solution.

26| Concentrate the ethanol solution under reduced pressure using a rotary evaporator.
▲ **CRITICAL STEP** Heating the solution during concentration results in product decomposition, evidenced by the appearance of a dark brown color.

27| Redissolve the crude solid in distilled water (60 ml) containing LiCl (1.5 g, 0.035 mmol).

28| Transfer the solution to a 150 ml lyophilizer jar, freeze the sample to liquid nitrogen temperature and lyophilize overnight.
■ **PAUSE POINT** The reaction remains on the lyophilizer overnight or until all the water has been removed.

29| Dissolve the crude material in 50 ml of absolute ethanol with stirring to break up any aggregates. The material is then placed at −20 °C for 4 h to facilitate precipitation.

30| The product as the insoluble lithium salt is then filtered and washed with cold absolute ethanol to recover the desired product.

31| The crude material can be further purified by reverse-phase chromatography. Load crude product on a C18 column (roughly 1 g crude mixture to 130 g support) and elute with distilled water at the rate of 70 ml min^{-1}. Collect 10 ml fractions; product is eluted near fractions 15–30. (The C18 column is conditioned with distilled water for 15 min before the sample is loaded onto the column.)

32| Product-containing fractions are identified by spotting on C18 reverse-phase TLC plate and developed in 100% distilled water. The TLC plate is observed under a UV lamp at 254 or 365 nm and all UV-active fractions are collected.

33| Pool product-containing fractions and lyophilize to yield product as a white powder.

Synthesis of 2,2-dithioethyl-4-(2′-pyridyl)-4-butyro-γ-lactone
34| Dissolve 400 mg of (*S*)-4-hydroxy-2-keto-4-(2′-pyridyl)butyrate in 6 ml of ethanethiol and 2 ml of concentrated HCl. Stir the mixture for 24 h at room temperature.
! **CAUTION** Ethanethiol has a strong stench and is a skin irritant. Concentrated hydrochloric acid will cause skin burns.
■ **PAUSE POINT** Reaction can be left stirring overnight.

35| Remove excess ethanethiol by distillation.

36| Extract the resulting solid three times with 10 ml of methylene chloride. Combine the organic extracts, wash with water and dry the organic phase over $MgSO_4$. The organic extracts are then concentrated, leaving a yellow oil.

37| The product is analyzed by chiral gas chromatography using a chiral Chromopack Chirasil-L-Val column (25 × 0.25 m) at a temperature of 160 °C and a pressure of 15 psi to determine the enantioselectivity. A racemic product was synthesized as a calibration standard (**Box 1**).
? **TROUBLESHOOTING**

BOX 1 | SYNTHESIS OF RACEMIC 4-HYDROXY-2-KETO-4-(2′-PYRIDYL)BUTYRATE (CHEMICAL)

Oxalacetic acid (4.5 g, 35 mmol) was dissolved in 3 M NaOH (20 ml) at 0 °C and brought to pH 9.0. 2-pyridinecarboxaldehyde (3.75 g, 35 mmol) was added and the resulting solution was adjusted to pH 9.0. The reaction was stirred for 1 h and the pH maintained at 9.0 by periodic addition of 3 M NaOH. When the pH stabilized at 9.0, the solution was allowed to stir at room temperature for an additional 1 h. The pH of the reaction was lowered to 3.5 by the addition of strongly acidic (Amberlyst 131) ion exchange (H^+) resin. Spontaneous decarboxylation accompanied acidification. The solution was adjusted to pH 7.0 and lyophilized. The crude material was stirred in 750 ml of ethanol for 30 min and then filtered. The material was washed with ethanol and precipitated with LiCl as described above. 4-Hydroxy-2-keto-4-(2±-pyridyl)butyrate was converted to racemic 2,2-dithioethyl-4-(2′-pyridyl)-4-butyro-γ-lactone as described above[38].

● **TIMING**
Protein expression
Step 1: 2 h
Step 2: 16 h
Step 3: 16 h
Steps 4–6: 8 h
Step 7–9: 1 h

Steps 10 and 11: 5 h
Step 12: 24 h
Step 13: 20 min

(*S*)-4-hydroxy-2-keto-4-(2′-pyridyl)butyrate synthesis
Steps 14–21: 8 h
Step 22: 24 h
Step 23: 24 h
Steps 24–27: 4 h
Step 28: 24 h
Steps 29 and 30: 5 h
Steps 31 and 32: 3 h
Step 33: 24 h

2,2-dithioethyl-4-(2′-pyridyl)-4-butyro-γ-lactone synthesis
Step 34: 24 h
Steps 35 and 36: 2 h
Step 37: 2 h

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Problem	Possible reason	Solution
Low KDPG aldolase yield	Bad cell line of BL21 CodonPlus (DE3)-RIL was used	Use a fresh cell stock BL21 CondonPlus (DE3)-RIL
Insufficiently pure KDPG aldolase	Overloaded affinity columns	Use at least 10 ml of resin for 80 mg of crude protein
	Insufficiently conditioned resins	Condition resins completely against 10 volumes of Ni^{2+} buffer
	Insufficient wash before protein elution	Elute column with 10 bed volumes of wash buffer before beginning elution
Low (*S*)-4-hydroxy-2-keto-4-(2′-pyridyl) butyrate yield	Impure 2-pyridinecarboxaldehyde	Use freshly distilled 2-pyridinecarboxaldehyde
	Inactive KDPG aldolase	Re-express the KDPG aldolase
Color formation in product	Excessive heat applied during product concentration	Do not heat beyond 30 °C to avoid formation of elimination product
Cannot find product	Isolated the precipitated salts instead of the ethanol fraction	Product is in the ethanol fraction; isolate this fraction instead of the salt fraction

ANTICIPATED RESULTS

Typical yields

Typical protein expression yields of KDPG aldolase should be between 180 and 200 mg per liter of growth. Typical isolated yield of (*S*)-4-hydroxy-2-keto-4-(2′-pyridyl)butyrate is 80–90% after reverse-phase C18 purification.

Analytical data

(*S*)-4-hydroxy-2-keto-4-(2′-pyridyl)butyrate
^{1}H NMR (400 MHz, D_2O) δ 2.93 (dd, 1H, $J = 5.5, 15.6$ Hz), 3.14 (dd, 1H, $J = 8.0, 15.0$ Hz), 5.21 (dd, 1H, $J = 6.0, 8.0$ Hz), 7.41 (t, 1H, $J = 6.5$ Hz), 7.56 (d, 1H, $J = 8.0$ Hz), 7.93 (t, 1H, $J = 7.7$), 8.40 (d, 1H, $J = 5.5$ Hz). ^{13}NMR (400 MHz, D_2O) δ 41.8, 63.5, 120.0, 123.1, 138.0, 140.9, 155.1, 167.3, 168.0 ppm. IR (KBr) ν 1,725, 1,715, 1,640 cm^{-1}.

2,2-dithioethyl-4-(2′-pyridyl)-4-butyro-γ-lactone
^{1}H NMR (300 MHz, $CDCl_3$) δ 1.21 (q, 6H, J =7.7 Hz), 2.56–2.94 (m, 6H), 5.64 (dd, 1H, $J = 6.3, 9.3$ Hz), 7.21 (dd, 1H, $J = 4.1, 7.0$ Hz), 7.50 (d, 1H, $J = 8.0$ Hz), 7.76 (dt, 1H, $J = 1.9, 7.8$ Hz), 8.59 (d, 1H, J 4.9 Hz). ^{13}C NMR (300 Hz, $CDCl_3$) δ 13.8, 13.9, 24.3, 24.8, 46.0, 58.3, 78.1, 120.6, 123.5, 137.5, 148.8, 155.9, 168.3 ppm.

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COMPETING INTERESTS STATEMENT The authors declare no competing financial interests.

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