Revealing Substrate Promiscuity of 1-Deoxy-D-Xylulose 5-Phosphate Synthase

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Supporting Information

General.
Unless otherwise noted, all reagents were purchased from Sigma-Aldrich. HPLC analyses of derivatized enzymatic reaction mixtures were performed on a Beckman Gold Nouveau System Gold with a Grace Alltima 3 μm C18 analytical Rocket® column (53 mm × 7 mm). Spectrophotometric analysis for concentration determination of DXP synthase and D-GAP was carried out on a Beckman DU 800 UV/Vis spectrophotometer. Mass spectrometric analyses were performed on an API 3000 triple quadrapole ESI mass spectrometer. All enzymatic and derivatization reactions utilizing aliphatic aldehydes as alternative substrates were carried out in low-retention microcentrifuge tubes to prevent adsorption of hydrophobic substrates to the reaction tubes.

Purification of recombinant 1-deoxy-D-xylulose 5-phosphate synthase (DXP synthase).
E. coli BL21 (DE3) cells harboring *dxs-pET37b* were grown to OD_{600} ~ 0.6 and induced with isopropyl β-D-thiogalactoside (IPTG, 100 μM) at 37°C. Shaking was continued for 5 hours. Bacterial cells were harvested by centrifugation at 4°C and stored at -20°C overnight. Thawed cell pellets were resuspended in protein purification buffer (3 mL of buffer per gram of cell pellet): 50 mM Tris, pH = 8.0, 5 mM $MgCl_2$, 1 mM thiamin diphosphate (ThDP), 10% glycerol (v/v), 1 mM β-mercaptoethanol, 100 μM PMSF, and 100 μM protease inhibitor cocktail (for Histidine-tagged proteins). Cells were then disrupted by sonication. The cell debris was removed by centrifugation at 4°C. The supernatant was incubated with Ni-NTA in 20 mM imidazole at 4°C for 1.5 hours. DXP synthase was then eluted from the resin over a stepwise gradient of 20 mM – 500 mM imidazole. Elutions were analyzed by SDS-polyacrylamide gel electrophoresis (12%) and stained with Coomassie Brilliant Blue G. Fractions containing a major band at 68.7 kDa (Figure S1) were combined and dialyzed at 4°C overnight against 1 L of 50 mM Tris, pH 8.0, 10% glycerol (v/v), 10 mM $MgCl_2$, and 1 mM ThDP. A second dialysis was carried out in 1 L of 50 mM Tris, pH 8.0, 10% glycerol, 10 mM $MgCl_2$, 1 mM ThDP, and 1 mM β-mercaptoethanol (4 h). Protein concentration was determined by Biorad Protein Assay with bovine serum albumin (BSA) as a standard. Protein was then flash frozen in liquid N_2 and stored at -80°C. Yielded 26.8 mg DXP synthase/L of culture.

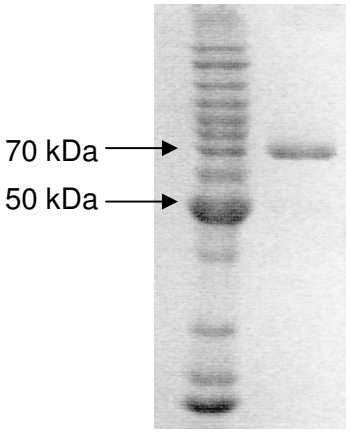


Figure S1. Purified DXP synthase. Left lane: MW ladder. Right Lane: C-His DXP synthase, 68.7 kDa.

Preparation of D,L-GAP from D,L-glyceraldehyde 3-phosphate diethyl acetal barium salt (Spectrum). D,L-GAP was prepared according to the general protocol outlined by Sigma (G5376 Datasheet). More concentrated solutions of D,L-GAP (60 mM) were achieved by reducing the volume of the DOWEX suspension.

Determination of D-GAP concentration. The concentration of D-GAP prior to addition to DXP synthase reaction mixtures was determined spectrophotometrically according to modified literature protocols.[1,2] Briefly, reaction mixtures containing 30 mM pyrophosphate buffer, pH 8.6, 200 μM NAD^+, 200 μM sodium arsenate, 5 mM L-cysteine, and 10 U of GAPDH were preincubated at 30°C for 10 minutes. D,L-GAP was added to a final concentration not exceeding 200 μM (100 μM D-GAP), and the reaction was monitored spectrophotometrically at λ_{max} = 340 nm until the reaction was complete. The concentration of D-GAP was calculated from the concentration of NADH formed (extinction coefficient = 6220 $M^{-1} \cdot cm^{-1}$).

General procedure for derivatization of substrates and products with 2,4-DNP. Carbonyl-containing substrates and products were derivatized as the hydrazones using at least 5-fold excess 2,4-dinitrophenylhydrazine (2,4-DNP) at room temperature for 5 minutes. To carry out these reactions, quenched enzymatic reactions were diluted 1-2-fold in 2,4-DNP (100 mM in 75% H_2SO_4). A comparison of derivatized aldehydes and ketones to propanal hydrazone (analytical standard purchased from Sigma) was used to confirm that derivatization reactions were complete within 2-3 minutes (Figure S2). To prevent degradation of phosphorylated species under acidic conditions, the solution pH was adjusted to pH 5-7 by addition of 1 M HEPES buffer, pH 8.0. Precipitated 2,4-DNP was removed by centrifugation, and samples were analyzed by HPLC (λ_{max} 365 nm).

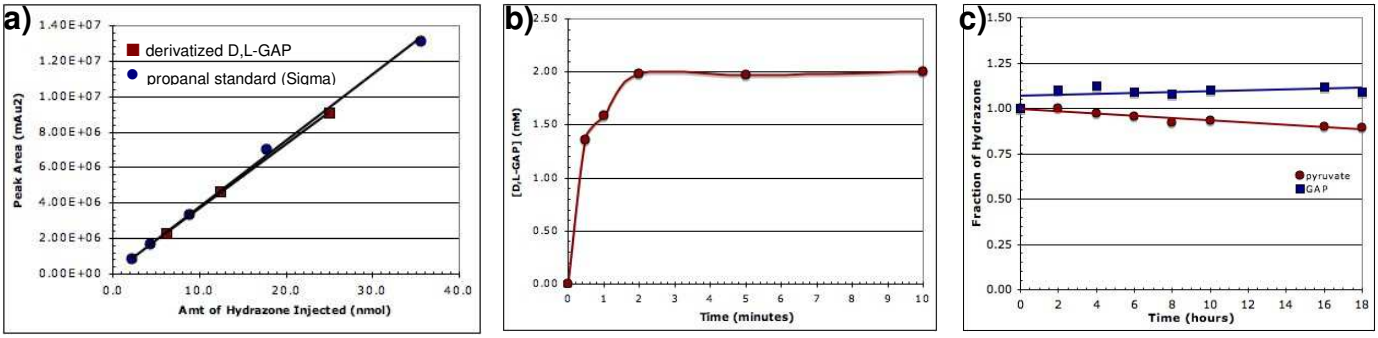


Figure S2. **a**) Comparison of two hydrazone standard curves, propanal hydrazone and GAP hydrazone. The concentration of D-GAP was first determined by GAPDH assay (see above). GAP was then derivatized according to the protocol outlined above and compared to the standard curve of propanal hydrazone. **b**) Derivatization of carbonyl containing compounds with excess 2,4-DNP is complete within 2 minutes at room temperature. **c**) Stability of D,L-GAP and pyruvate hydrazones after adjustment of pH to 5-7. Less than 11% depletion in pyruvate hydrazone was observed over 18 hours.

Confirmation of DXP synthase-catalyzed DXP Formation. Enzyme-catalyzed DXP formation was accomplished under the following conditions at 37°C: 100 mM HEPES, pH 8.00, 1 mg/mL BSA, 5 mM $MgCl_2$, 5 mM NaCl, 1 mM ThDP, 2.5 mM TCEP, 10 mM pyruvate, 1 mM D,L-GAP, 200 nM DXP synthase. After 30 minutes, Antarctic Phosphatase (10 U, AP) was added to the reaction mixture, and the appearance of GA and deoxy-xylulose (DX) hydrazones was observed following derivatization of quenched aliquots with 2,4-DNP. Authentic GA and DXP were derivatized with 2,4-DNP and each solution was co-injected with the AP-treated and derivatized solution, thereby confirming DXP product formation (Figure S3).

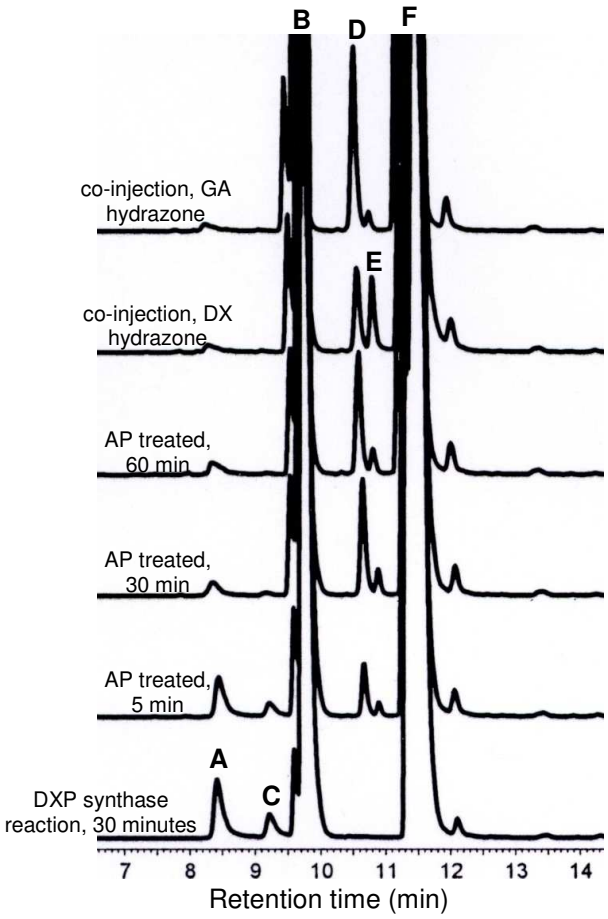


Figure S3. **A** = D,L-GAP hydrazone; **B** = pyruvate hydrazone; **C** = DXP hydrazone; **D** = GA hydrazone; **E** = DX hydrazone; **F** = 2,4-DNP.

Determination of kinetic parameters for D-glyceraldehyde 3-phosphate (D-GAP) and pyruvate. DXP synthase reaction mixtures containing 100 mM phosphate buffer, pH 8.0, 1 mg/mL BSA, 5 mM $MgCl_2$, 5 mM NaCl, 2.5 mM tris(2-caroxyethyl)phosphine (TCEP), 1 mM ThDP, D,L -GAP and pyruvate were preincubated at 37°C for 5 minutes in a final volume of 200 μL. DXP synthase (200 nM) was added to initiate reactions. Aliquots (60 μL) were quenched into cold MeOH (60 μL), and samples were incubated at 4°C for 20 minutes. Protein was removed by centrifugation and mixtures were derivatized as described above. The determination of K_m for D-GAP was carried out at a constant concentration of pyruvate (10 mM) and over a range of D-GAP concentrations (25.0 μM – 2.0 mM). Four samples were taken between 1.0 and 4.0 minutes for each concentration, and initial reaction rates were calculated from these linear data. The determination of K_m for pyruvate was carried out at a constant concentration of D-GAP (2.0 mM) and over a range of pyruvate concentrations (200 μM – 10 mM). For each concentration of pyruvate, four aliquots were removed and quenched between 0.5 and 3 min. Each experiment was carried out in triplicate. Derivatized samples were analyzed by HPLC. Reaction components were separated using the following conditions: Flow rate = 3 mL/min; Solvent A: 100 mM NH_4OAc, 0.05% TFA, pH 4.60; Solvent B: CH_3CN/0.05% TFA. Method: 0%-42% B over 9 minutes, then 42%-100% B over 0.1 min. The GAP and DXP hydrazone peak areas were measured, and the product concentration was determined as a percent of total peak area. Data analysis was carried out using GraFit version 5 from Erithacus Software.

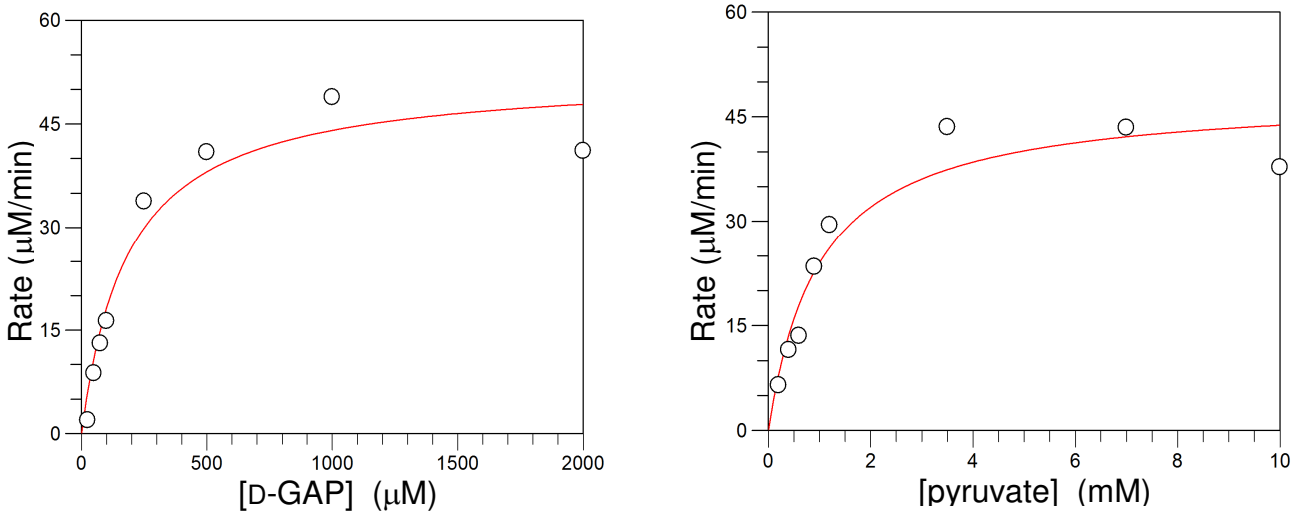


Figure S4. Representative Michaelis-Menten kinetic analysis of D-GAP (left) and pyruvate (right).

Determination of k_{cat}/K_m for D-glyceraldehyde (GA). The concentrations of aldehyde stock solutions were determined by comparison to analytical 2,4-dinitrophenylhydrazone standards as described above. Reaction mixtures containing 100 mM phosphate buffer, pH 8.0, 5 mM $MgCl_2$, 2.5 mM TCEP, 1 mM ThDP, saturating pyruvate, and concentrations of GA ranging from 2.0 – 3.0 mM were preincubated at 37°C for 5 minutes in a final reaction volume of 60 μL. DXP synthase (5 μM) was added to initiate the reaction. Three samples were quenched in an equal volume of cold MeOH between 5 and 10 minutes for each concentration. Quenched samples were incubated at 4°C for 20 minutes, and then clarified by centrifugation prior to derivatization and HPLC analysis as described above. DX and GA hydrazone peak areas were measured, and the product concentration was calculated as a percent of total peak area. Rates of DX formation were plotted as a function of GA concentration to determine k_{cat}/K_m.

Determination of k_{cat}/K_m for aliphatic aldehydes. Enzymatic reactions were carried out as described above for GA, using 10 μM DXP synthase. In these cases, the pH of derivatization reaction mixtures was adjusted by addition of 1M HEPES in 35% MeOH solutions, to enhance solubility of aldehyde hydrazones. The timecourse analysis was carried out between 10 and 30 minutes for each concentration of acetaldehyde, and between 30 and 60 minutes for all other aldehydes. Aliquots were quenched into an equal volume of cold MeOH. Prior to derivatization, quenches were diluted 5-fold into ddH_2O to enhance solubility during derivatization. HPLC analysis was performed using the following method: Flow rate = 3 mL/min; Solvent A: 100 mM NH_4OAc, 0.05% TFA, pH 4.60; Solvent B: MeOH + 0.05% TFA. 0%-90% B over 8 minutes, then 90%-97.5% B over 6 min. The concentrations of product formed were determined as a percent of total peak area in each case as described above. Calculation of k_{cat}/K_m was carried out as described above and performed in triplicate. In each case, the formation of acetoin was also observed, presumably via decarboxylation of acetolactate, indicating competitive binding of pyruvate with the second aldehyde substrate.

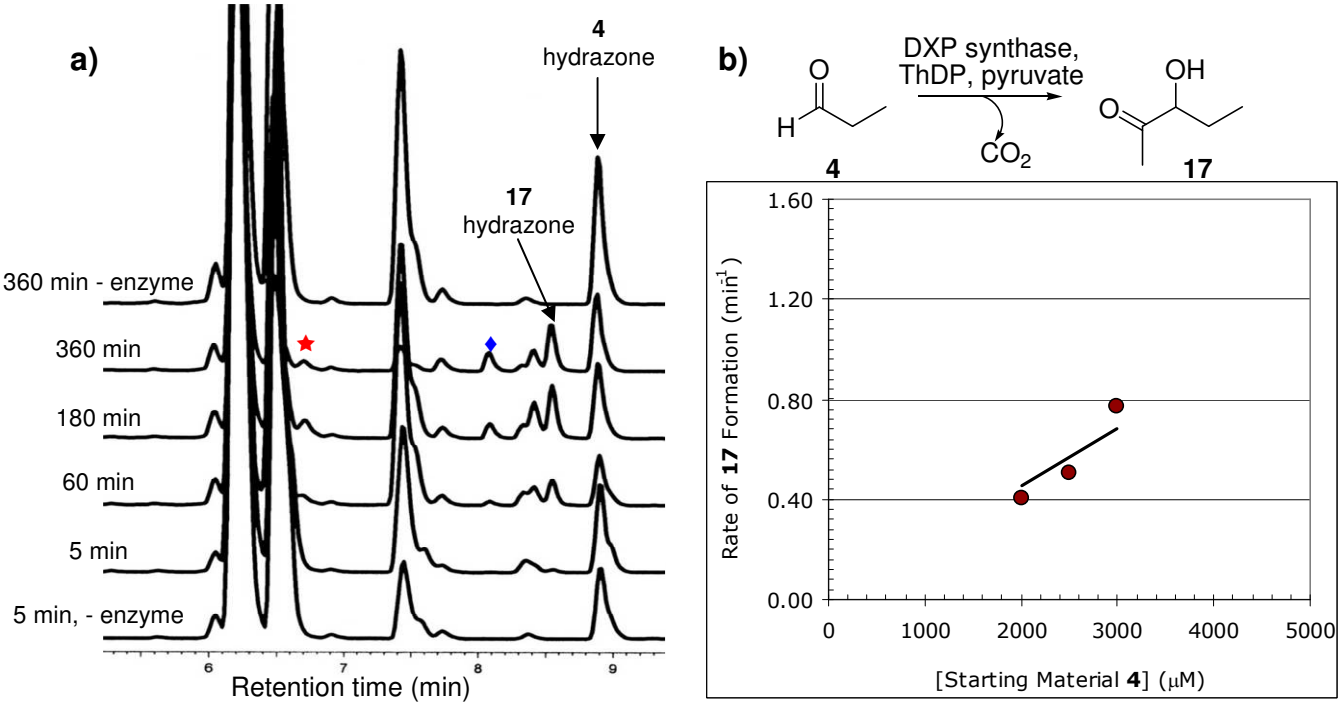


Figure S5. Analysis of propanal **4** as a substrate for DXP synthase. **a**) HPLC timecourse analysis. (♦) acetoin hydrazone; (★) acetolactate hydrazone **b**) Representative analysis of estimating k_{cat}/K_m for **4** at low substrate concentrations.

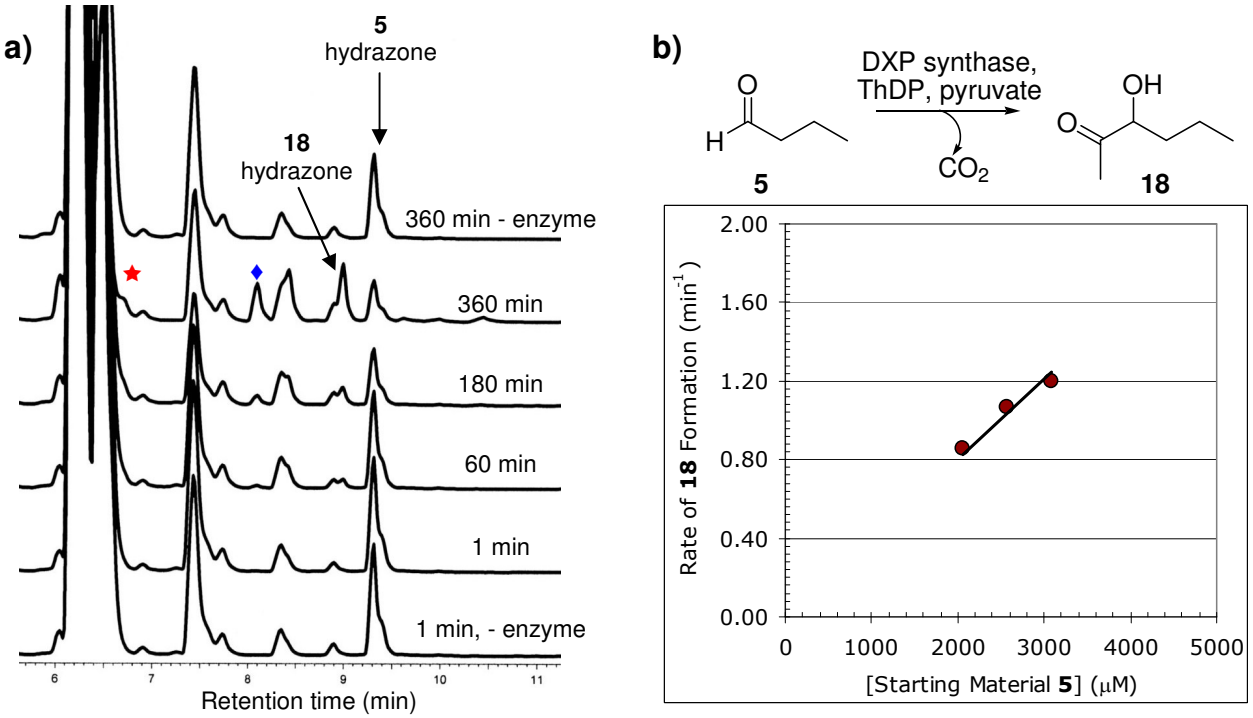


Figure S6. Analysis of butanal **5** as a substrate for DXP synthase. **a**) HPLC timecourse analysis. (♦) acetoin hydrazone; (★) acetolactate hydrazone. **b**) Representative analysis of estimating k_{cat}/K_m for **5** at low substrate concentrations.

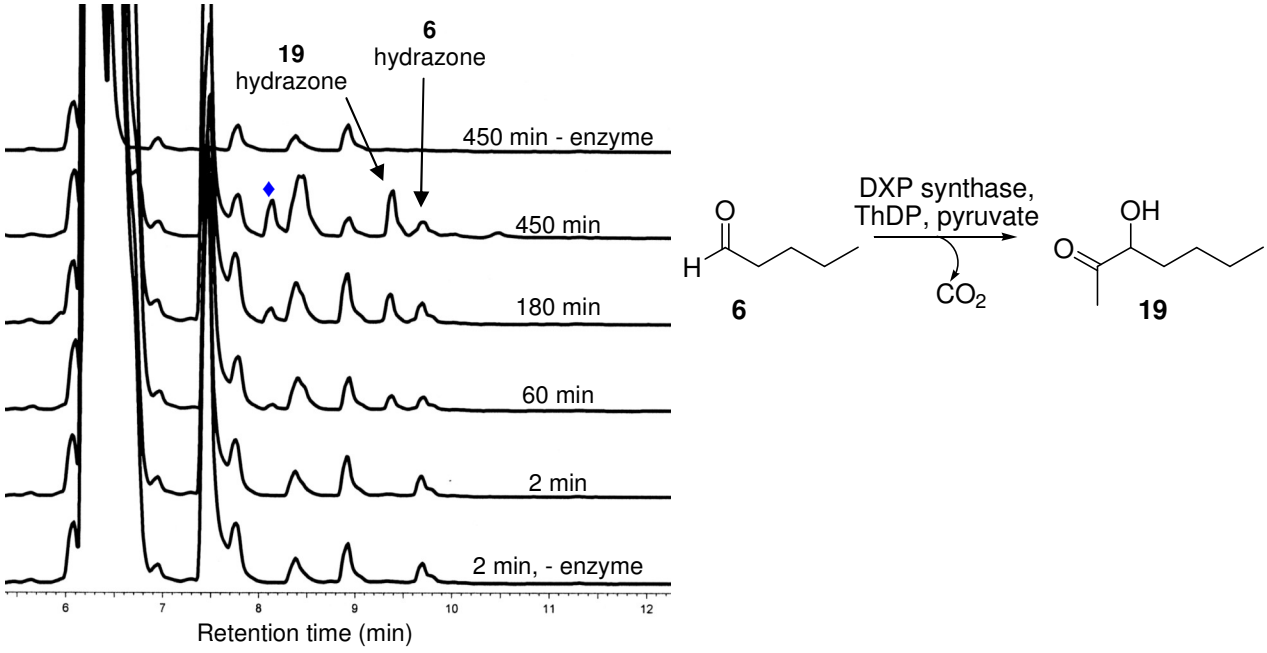


Figure S7. Analysis of pentanal **6** as a substrate for DXP synthase. (♦) acetoin hydrazone.

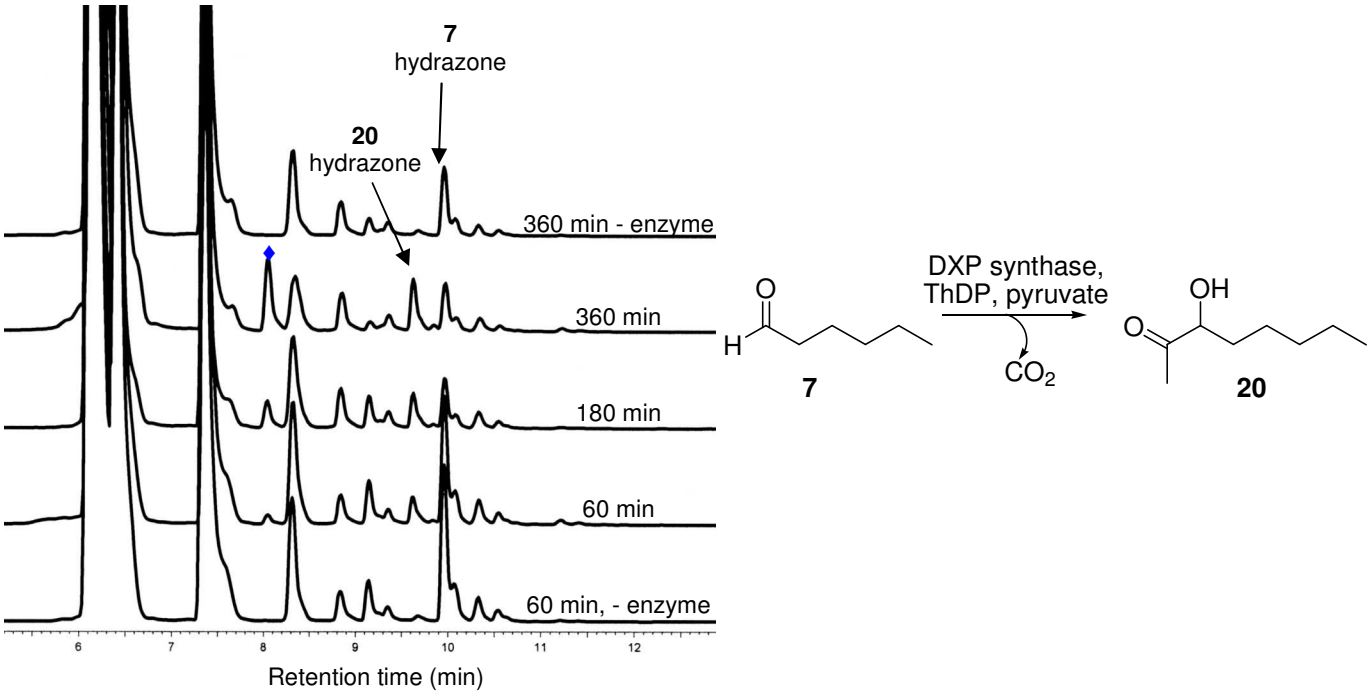


Figure S8. Analysis of hexanal **7** as a substrate for DXP synthase. (♦) acetoin hydrazone.

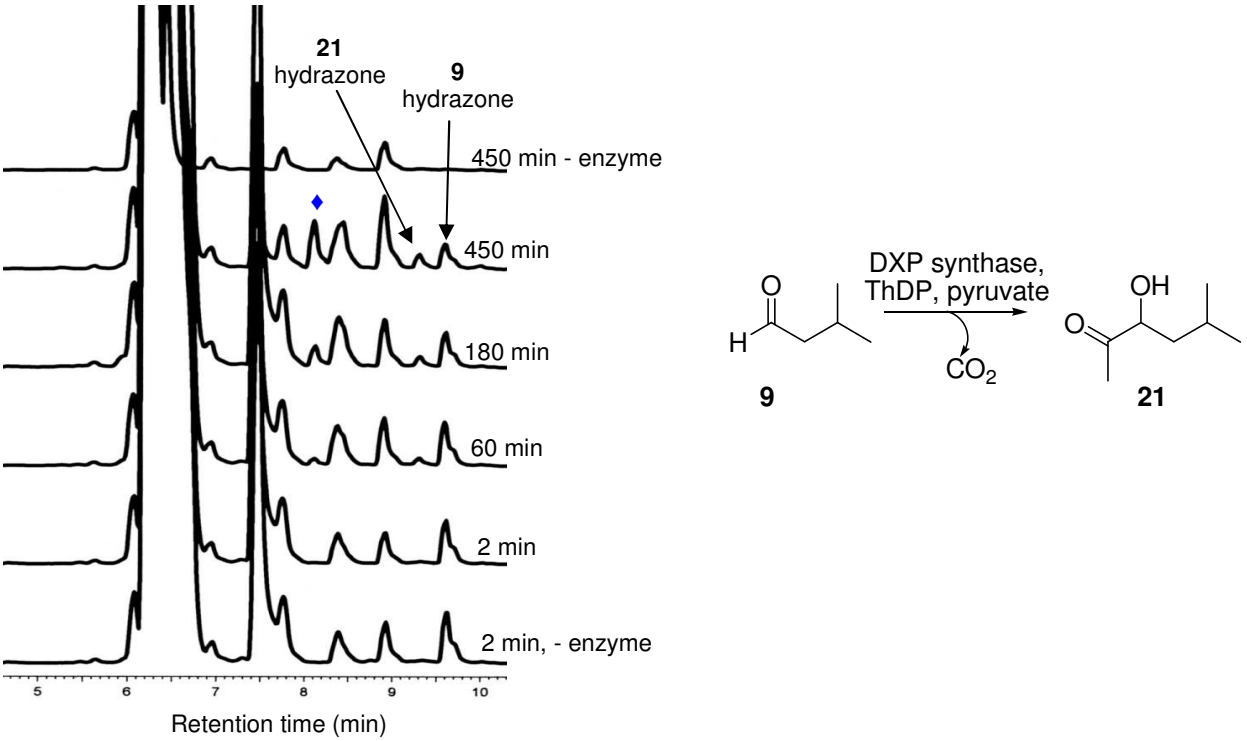


Figure S9. Analysis of a β-branched aldehyde **9** as a substrate for DXP synthase. (♦) acetoin hydrazone.

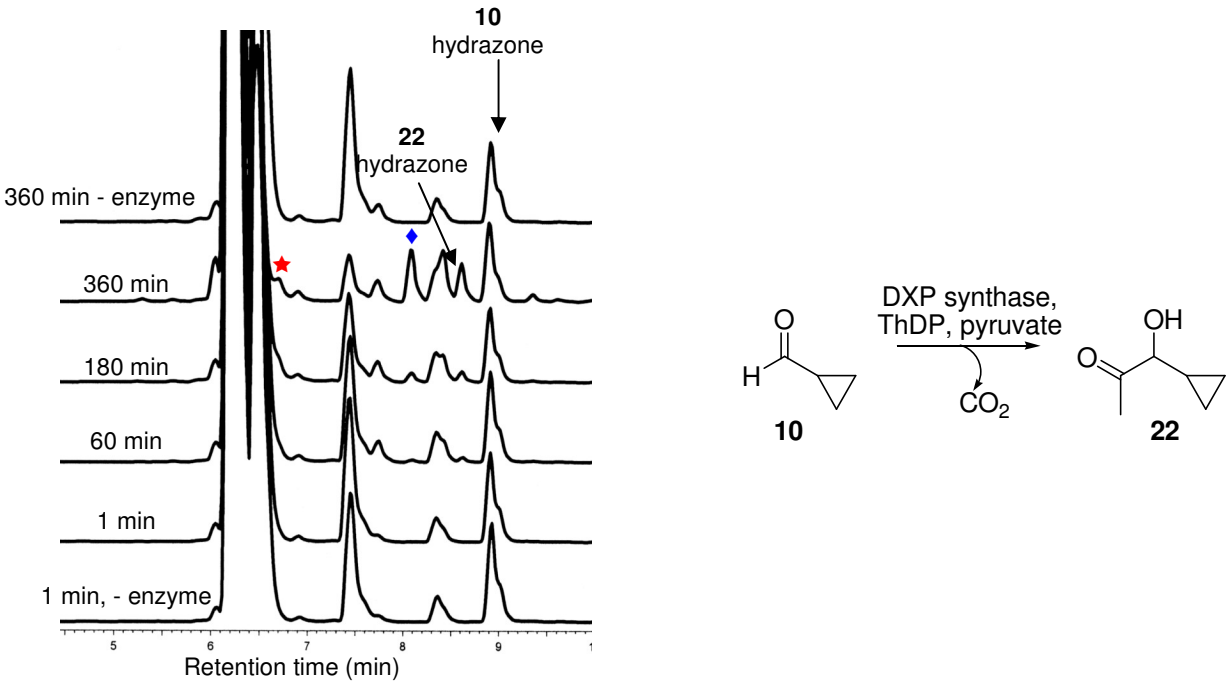


Figure S10. Analysis of cyclopropanecarboxaldehyde **10** as a substrate for DXP synthase. (♦) acetoin hydrazone; (★) acetolactate hydrazone.

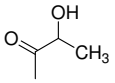
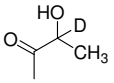
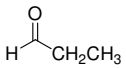
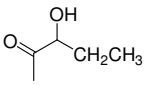
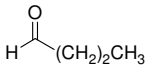
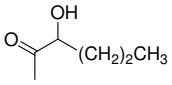
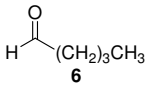
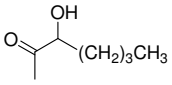
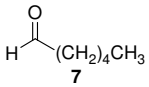
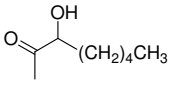
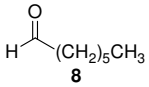
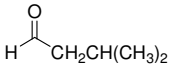
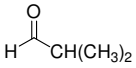
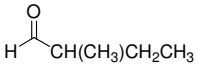
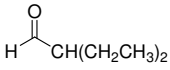
Substrate	Product	m/z [M+H]$^+$
 **3**		268.98
 **23**		273.04
 **4**		283.18
 **5**		297.36
 **6**		311.45
 **7**		325.20
 **8**	insoluble	*n.d.*
 **9**	minor product observed	*n.d.*
 **10**	minor product observed	*n.d.*
 **11**	no product observed	*n.d.*
 **12**	no product observed	*n.d.*
 **13**	no product observed	*n.d.*

Table S1. Summary of substrate specificity of DXP synthase toward alternative aliphatic aldehydes. In addition to D-GAP and GA (Table 1), kinetic parameters were determined for compounds **3**-**5**. Solubility prevented accurate determination of specificity constants for long chain aldehydes (**6** and **7**), although products were identified by mass spectrometry. Heptanal (**8**) was insoluble under our assay conditions. Minor products were observed for compounds **9** and **10**; however, product concentrations were significantly lower, preventing characterization by mass spectrometry. No product formation was observed when there was branching in the alpha position (compounds **11**-**13**). *n.d.* = not determined.

Characterization of enzyme-generated products.

DXP. DXP product formation in the presence of pyruvate and D-GAP was confirmed by comparison of the corresponding dephosphorylated product hydrazones to authentic DX hydrazone (Figure S3). In this case, Antarctic phosphatase (10 U) was used to dephosphorylated GAP and DXP to give GA and DX. GA and DX hydrazones co-eluted with authentic samples of each.

Acetoin. Acetoin produced in the presence of acetaldehyde and pyruvate was confirmed by comparison to an authentic hydrazone standard. Additionally, the acetoin hydrazone peak was purified by HPLC, desalted and analyzed by mass spectrometry (Table S1: $C_{10}H_{12}N_4O_5$ calculated = 268.08; observed $[M+H]^+$ = 268.98). Similarly, d4-acetoin hydrazone was characterized by mass spec analysis (Table S1: $C_{10}H_8D_4N_4O_5$ calculated = 272.11; observed $[M+H]^+$ = 273.04). In the reaction carried out with d4-acetaldehyde, both d4- and H4-acetoin products were observed, resulting from both d4-acetaldehyde and H3-pyruvate acting as acceptor substrates.

α-Hydroxy ketones. Enzyme-generated products of propanal, butanal, pentanal and hexanal were characterized by mass spectrometry as described above (Table S1).

compound **17** hydrazone: $C_{11}H_{14}N_4O_5$ calculated = 282.10; observed $[M+H]^+$ = 283.18
compound **18** hydrazone: $C_{12}H_{16}N_4O_5$ calculated = 296.11; observed $[M+H]^+$ = 297.36
compound **19** hydrazone: $C_{13}H_{18}N_4O_5$ calculated = 310.13; observed $[M+H]^+$ = 311.45
compound **20** hydrazone: $C_{14}H_{20}N_4O_5$ calculated = 324.14; observed $[M+H]^+$ = 325.20

[1] Krebs, E. *Methods Enzymol*. **1955**, *1*, 407-411.
[2] Amelunxen, R.; Carr, D. *Methods Enzymol.* **1975**, 41, 264-267.