The Putative Diels-Alderase Macrophomate Synthase is an Efficient Aldolase

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Supporting Information

Materials and General Methods. All reactions were conducted in oven– (135 °C) or flame-dried glassware under an inert atmosphere of dry N_2 unless otherwise stated. NMR were recorded on Bruker AV600 (^{1}H 600 MHz, ^{13}C 150.9 MHz), Bruker DRX 500 (^{1}H 500 MHz, ^{13}C 125 MHz), Bruker DRX (^{1}H 400 MHz, ^{13}C 100 MHz), ARX 300 (^{1}H 300 MHz, ^{13}C 75 MHz), or Varian Gemini 300 (^{1}H 300 MHz, ^{13}C 75 MHz) NMR spectrometers. All ^{13}C-NMR spectra are ^{1}H-broadband decoupled and were measured at room temperature if not stated otherwise, and the ^{13}C resonance of the chloroform was used as an internal standard ($^{13}CDCl_3$: 77.16). Chemical shifts are reported in ppm from tetramethylsilane with the solvent resonance resulting from incomplete deuteration as the internal standard ($CDCl_3$: 7.26). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet or combinations thereof), coupling constants, and integration. Multiplicity was assigned according to a method described by Hoye.[1] Enantiomer ratios were determined by ^{1}H NMR of derivatized adducts (see the individual substrate entries for details) or chiral HPLC analysis. HPLC: Daicel AD column (4.6 mm x 250 mm). Mass spectra were recorded on a Finnigan TSQ7000 Triple-Quad mass spectrometer (ESI) or on a HiRes-ESI IonSpec Ultima FTMS-spectrometer (Ionspec, Lake Forest, CA, USA). ESI spectra of small organic molecules were usually measured in MeOH. Calculated masses were based on average isotope composition or on single isotope masses for high resolution spectra.

Chemicals were purchased from Sigma, Acros, ABCR, Merck, Aldrich or Fluka and were used without further purification, unless otherwise noted. Buffer salts were purchased from Sigma, J.T. Baker, or Fluka and used without further purification. Oxaloacetic acid was purchased from Fluka or Sigma and recrystallized from ethyl acetate before use. NMR solvents and internal standards, 3-trimethylsilyl-proprionate-d_4 sodium salt (TSP) and tetramethyl silane, were purchased from Armar (Doettingen, Switzerland) and Cambridge Isotope Laboratories (MA, USA), respectively. Silica gel chromatography was driven with compressed air and performed with silical gel *60* from *Fluka* (grain size 40-63 μm) at room temperature.

Solvents were dried as follows: THF was freshly distilled over Na/benzophenone before use. Et_2O was filtered through a basic alumina plug directly before use. CH_2Cl_2 was distilled over CaH_2. DMF was stirred over KOH pellets overnight and then distilled under reduced pressure over CaH_2 at 40 °C.

Plasmids. The pETMPS plasmid encoding the gene for MPS[2] was provided by Prof. Hideaki Oikawa, Hokkaido University, Japan. A commercially available pET22b(+) vector (Novagen) was used for construction of the pETMPS22-2 plasmid. Experimental details for construction and introduction of the plasmid into BL21 cells have already been described.[3]

Protein production. Competent *E. coli* cells (BL21) were transformed with pETMPS22-2 or the corresponding mutant plasmid. The proteins were overproduced upon induction with 2.5 mM IPTG in 250 ml cultures grown at 25 ºC in LB medium supplemented with ampicillin (200 mg/l). Cell pellets were suspended in 7.5 ml loading buffer (50 mM sodium phosphate 300 mM NaCl, pH 7.0), 2 mg of the protease-inhibitor PEFA-bloc were added and the cells were lysed by incubation with 10 mg lysozyme at 4 °C followed by ultrasonication. Cleared cell lysates were incubated with 4 ml Ni^{2+}-NTA-agarose resin (Qiagen), which was pre-equilibrated with loading buffer containing 10 mM imidazole, pH 7.0. After 10 min incubation, this suspension was loaded onto Ni-chromatography columns that were then washed with 30 ml each of 10, 20, and 40 mM imidazole in loading buffer. The desired protein was eluted as a pure fraction with 12 ml of buffer containing 200 mM imidazole. Protein purity was assessed by 20% SDS-PAGE to be >98%, protein identity was confirmed by LC-MS and plasmid sequencing after a plasmid miniprep of a 5 mL portion of the cell culture. Typically, a 250 ml culture yielded between 20 and 40 mg of purified protein, depending on the mutant. A typical yield for the wild type enzyme was ca. 50 mg of pure protein per 250 ml of cell culture. The proteins were dialyzed into 20 mM potassium phosphate buffer, pH 7.0, at 4 ºC, sterile filtered and then stored in the cold prior to use.

LC-MS was performed on a Spectra System HPLC connected to a diode array detector (UV6000LP, Thermo Separation Products) and an ion-trap mass spectrometer (LCQdeca, Finnigan) with the same columns as for analytical RP-HPLC (see below).

Two buffer systems were routinely used for reactions with macrophomate synthase. Buffer system A was used for the kinetic analysis of decarboxylation of oxaloacetate, and buffer system B was used for all analytical and preparative scale aldol reactions except when stated otherwise.

MPS Reaction buffer A (50 mM PIPES, 5 mM $MgCl_2$, pH 7.0): 16.2 g of PIPES monosodium salt and 1.015 g of $MgCl_2$ • 12 H2O were dissolved in 1 L UPW. The pH was adjusted to 7.0 with 1 M NaOH. The buffer was sterile filtered and stored at 4 °C.

MPS Reaction buffer B (50 mM phosphate buffer, 5 mM $MgCl_2$, pH 7.0): 0.413 g NaH_2PO_4 • 2 H_2O, 17.07 g Na2HPO4 • 12 H2O and 1.015 g of $MgCl_2$ • 12 H_2O were dissolved in 1 L UPW. If necessary, the pH was adjusted to 7.0 with 1 M NaOH or 1 M HCl. The buffer was sterile filtered and stored at 4 °C.

UV data were collected on a Perkin-Elmer Lambda series UV/VIS spectrophotometer (Lambda 16, Lambda 20, or Lambda 40). Measurements were usually

carried out at 30 °C, unless stated otherwise. Scan speed was 480 nm*s^{-1} and the bandwidth was set to 2 nm.

Decarboxylation kinetics. The MPS-catalyzed decarboxylation of oxaloacetate was assayed by monitoring the absorption decrease at 305 nm ($\Delta\varepsilon$305 = 173 $M^{-1}cm^{-1}$). Reactions were carried out in 50 mM PIPES buffer, pH 7.0 with 5 mM $MgCl_2$ at 30°C. Enzyme concentration was 150 nM. The enzyme solution was pre-incubated for 3 min at 30° C and then the reaction was initiated by addition of oxaloacetate. Oxaloacetate concentration varied between 25 μM and 4 mM. Initial velocities were determined by linear regression and data was fitted to the Michaelis-Menten equation $v_0 = k_{cat}$ [E][S]/(K_m + [S]), where v_0 is the initial velocity, [S] is substrate concentration and [E] is total enzyme concentration.

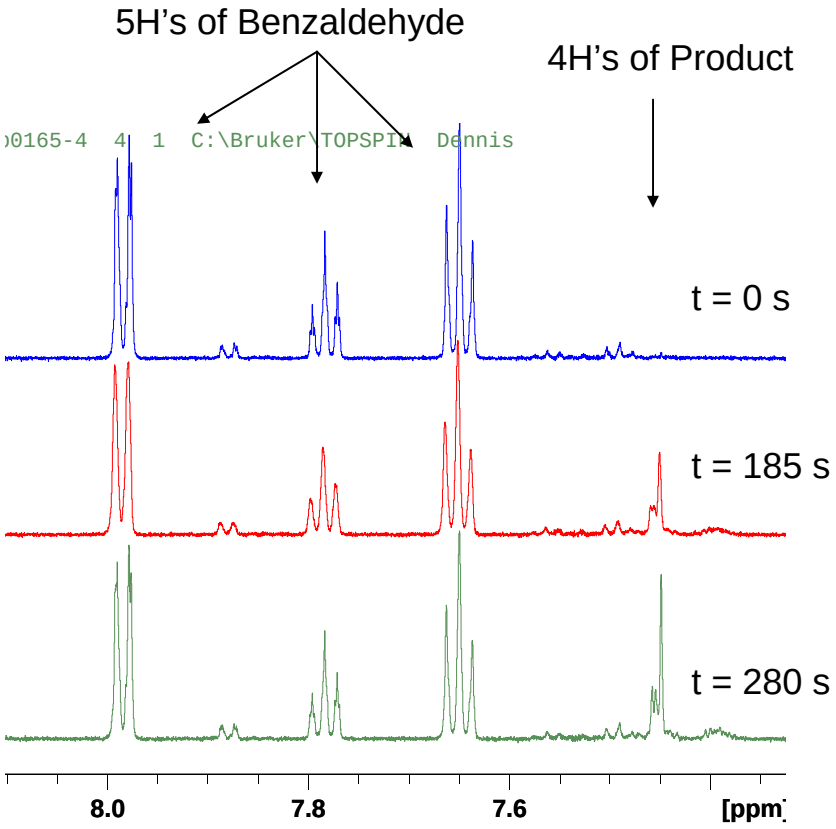
RP-HPLC was performed on a Waters HPLC system with 220 & 254 nm UV detection. For analytical runs, a C8 column (Macherey-Nagel Nucleosil 250 mm × 4.6 mm, 300 Å, 5 μm), a C18 column (Macherey-Nagel Nucleosil 250 mm × 4.6 mm, 100 Å, 5 μm), or a short C18 column (Waters Polarity 100 mm × 4.6 mm, 100 Å, 3 μm) at a flow rate of 1 ml/min were used. Peptides and small organic molecules were eluted with linear gradients of solvents A and B (A = acetonitrile containing 0.05% TFA, B = H_2O containing 0.1% TFA). Preparative RP-HPLC separations were performed using a C8 column (Macherey-Nagel Nucleosil 250 mm × 21 mm, 300 Å, 7 μ) or a C18 column (Vydac 250 mm × 22 mm, 300 Å, 10 μ) at a flow rate of 10 or 8 ml/min. Linear gradients of solvents A and B (A = CH_3CN, B = H_2O containing 0.1% TFA) were used. Gradients for analytical and preparative RP-HPLC are given as %A/%B to %A/%B followed by the time. If not stated otherwise, quoted retention times refer to the gradients 10/90 to 35/65 in 25 min at a flow rate of 1 ml / min for analytical HPLC and 10/90 to 45/55 in 55 min at a flow rate of 10 ml / min for preparative HPLC. In cases where it was desirable to maximize the yield after preparative RP-HPLC the products were eluted with pure water to prevent undesired side reactions such as elimination to **6a** or cyclization to the lactone.

Test for MPS-catalyzed elimination of 5a to give 6a. A sample of 2-oxo-4-hydroxy-4-phenyl-butanoic acid (**5a**) was dissolved in 50 mM phosphate buffer, 5 mM $MgCl_2$, pH 7.0 and was treated with MPS (300 nM). The signal increase at 340 nm (conjugated enone **6a** absorbs strongly in this region while **5a** is silent) was monitored over 1 h and compared to a control reaction that contained none of the enzyme. There was a slow and identical increase in absorbance in both the enzymatic and control reactions indicating no catalysis by MPS.

Procedure for the aldol kinetics outlined in Figure 1. The reaction of benzaldehyde and oxaloacetate in the presence of MPS was monitored by ^{1}H-NMR spectroscopy on a Bruker AV600 instrument set to 300 K. Kinetic experiments were carried out in 50 mM phosphate buffer, 5 mM $MgCl_2$, pH 7.0 in 9:1 H_2O:D_2O. For each kinetic run 4 μl of a 20 mM solution of sodium-3-trimethylsilyltetradeuteriopropionate (TSP) (in reaction buffer) and the appropriate amounts of the benzaldehyde and oxaloacetate solutions (prepared as stock solutions in the reaction buffer) were added to a clean high-precision Wilmad 520-PP NMR tube; the total volume of each sample was 800 μl. The sample was introduced into the spectrometer and allowed to equilibrate for five minutes before shimming. Water suppression was then optimized on the equilibrated sample using presaturation and a spectrum was recorded to verify the initial conditions. The sample was removed from the

spectrometer and the enzyme solution added (6.4 µl of a 12.5 µM solution for a final concentration of 100 nM) followed by six careful inversions (t_0 set to first inversion). The sample was introduced again and data collection was immediately initiated. Consumption of benzaldehyde and the appearance of product were quantified by measuring the change in the integral of the aromatic protons of both the benzaldehyde starting material and the aldol product **5a** relative to the integral of the internal standard (TSP, *s*, 9H, 0 ppm, 0.1 mM) (see Figure S1 for details). Initial velocities were determined by linear regression using the program Kaleidagraph and the data were fitted to the Michaelis-Menten equation: $v_0 = k_{cat}$ [E][S]/(K_m + [S]).

Figure S1. Representative set of NMR spectra used to determine initial velocities for aldol kinetics



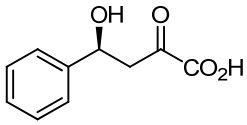
Retro-aldol activity of MPS with 5a. The retro-aldol reaction was analyzed with an NADH/lactate dehydrogenase (LDH) coupled assay. Eight kinetic runs were collected in a concentration range for **5a** of 25 µM to 900 µM and an enzyme concentration of 300 nM. The assay was run in MPS reaction buffer A (50 mM PIPES buffer, see General notes section for further details) containing 0.2 mM NADH and 1.5 µg of LDH. Ketoacid **5a** was added to the reaction buffer containing the assay components and the sample was monitored for 3 minutes to insure no background was observed. The enzyme was then added to bring the total volume to 800 µL and the absorption increase at 340 nm was monitored for five minutes. Initial velocities were determined by linear regression using the program Kaleidagraph and the data were fitted to the Michaelis-Menten equation: v_0

= k_{cat} [E][S]/(K_m + [S]). The background reaction was determined by NMR analysis under the same conditions (except that the buffer contains 10% D_2O) by measuring the appearance of products (benzaldehyde and the elimination product) and the disappearance of starting material as a function of time. Concentrations were determined by comparison with the known concentration of the internal standard TSP (sodium-3-trimethylsilyltetradeuteriopropionate, [TSP] = 2 mM).

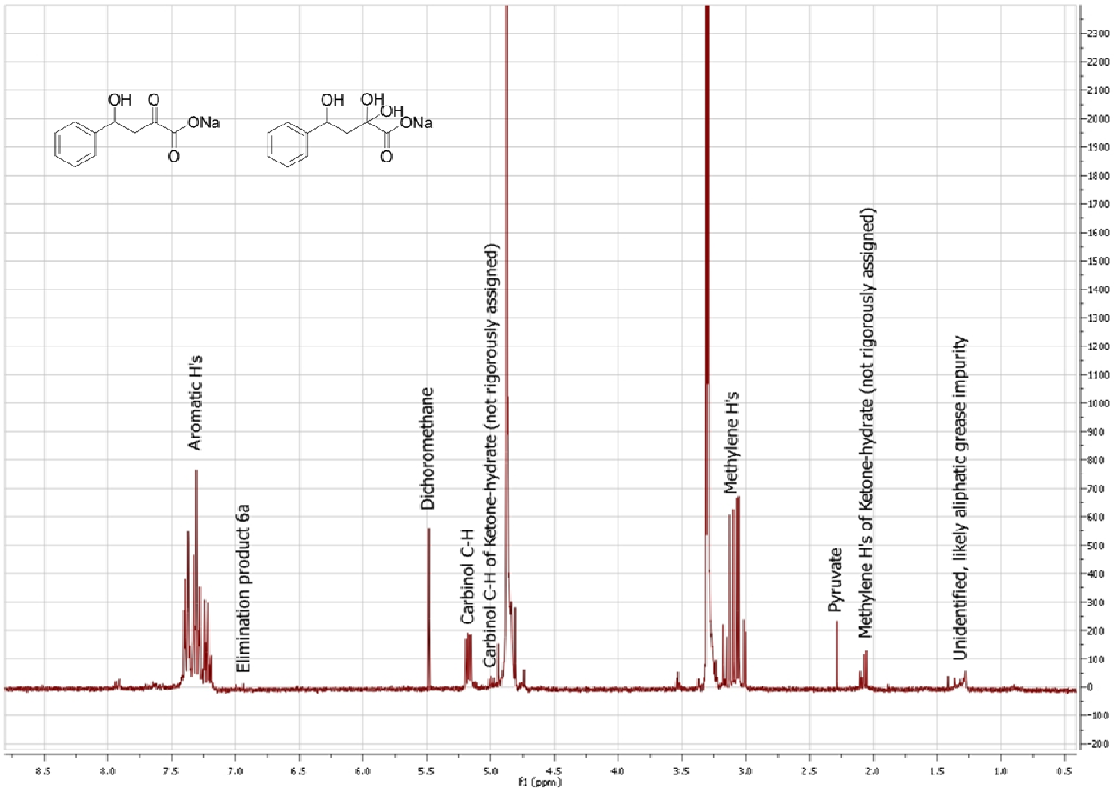
Compounds prepared by MPS catalyzed aldol reactions. NOTE: Although drawn in the acid form, those compounds not purified by acidic RP-HPLC were typically isolated as the sodium salt.

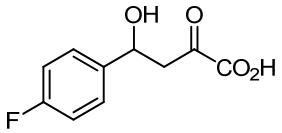
Analytical scale aldol reactions. The reaction of oxaloacetate with various aldehydes in the presence and absence of MPS was carried out in 50 mM phosphate buffer with 5 mM $MgCl_2$ at pH 7.0 at room temperature. The two reactions were run in parallel, one with enzyme and a control containing an equivalent volume of buffer. A 1 M solution of aldehyde in acetonitrile (10 µl) was added to 710 µl of MPS reaction buffer. Then, 80 µl of a 10 µM MPS solution in MPS reaction buffer was added to one sample (final MPS concentration 800 nM) and 80 µl of buffer to the control, and the samples were incubated for 10 min at room temperature. The reaction was initiated by addition of 100 µl of a 60 mM solution of oxaloacetate in MPS reaction buffer and both reaction vessels were gently shaken. After 1 h another 100 µl of 60 mM oxaloacetate were added to give the final reaction volume of 1 ml. After 2 h, both samples were frozen in liquid nitrogen and lyophilized. The remaining solid was dissolved in 200 µl of 49.5% acetonitrile, 49.5% water and 1% TFA. This solution was then analyzed by analytical RP-HPLC, using a linear gradient starting from 90% water and 10% acetonitrile, going to 65% water in 15 min and then to 10% water in another 10 min and also by LC-MS starting from 90% water going to 40% water in 50 min. For analysis of these reactions, the analytical RP-HPLC eluants (H_2O and CH_3CN) contained 0.5% TFA in order to improve separation of the 2-oxo-carboxylic acid derivatives which are the products of the aldol reaction. This leads to some loss of water to give the elimination products **6** and this was considered when determining conversion.

Preparative scale aldol reactions procedure. 0.5 ml of a 1 M aldehyde solution in acetonitrile was added to 17.5 ml of 50 mM phosphate buffer, 5 mM $MgCl_2$, pH 7.0. 1 ml of a 100 µM enzyme solution (in the same buffer) was added to give a final MPS concentration of 5 µM and the mixture was incubated for 10 min at room temperature. The reaction was then initiated by addition of 1 ml of a 60 mM solution of oxaloacetate (in the reaction buffer). Additional aliquots of oxaloacetate were added every 60 min. In total, 7 ml of oxaloacetate solution were added over 7 h. At the end of the reaction, the sample was frozen and lyophilized. The solid was then dissolved in methanol and filtered to remove inorganic salts. The ethanol was evaporated and the solid was dissolved in CD_3OD for NMR analysis and quantitation by comparing with an internal standard. The products were also weighed to verify the NMR yield (typically 10% lower yields by NMR indicating possible NMR-silent impurities in the dry-mass). If analytically pure products were required preparative RP-HPLC (100% water, C-18 column, 8 mL/min, retention time: 12.5 min) could be used. The use of an acidic mobile phase led to both elimination and cyclization side-products.

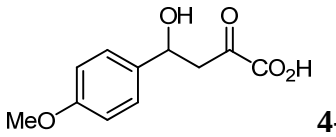


(S)-4-Hydroxy-2-oxo-4-phenylbutanoic acid (5a). Benzaldehyde and oxaloacetate were reacted as described above to deliver **5a** in 72 % yield. A labelled ^{1}H-NMR spectrum of the crude reaction mixture in CD_3OD is shown below. ^{1}H-NMR (300 MHz, CD_3OD): 7.2-7.4 (m, 5H), 5.2 (dd, J = 8.4, 4.5 Hz, 1H), 3.2 (dd, J = 36, 8.4 Hz, 1H), 3.1 (dd, J = 36, 4.5 Hz, 1H). HRMS-EI(+): Calc. for $C_{10}H_8O_4$ [M+H]: 193.0501. Found: 193.0505. The elimination product **6a** was also characterized. ^{1}H-NMR (300 MHz, CD_3OD): 7.69 (d, J = 16.5 Hz, 1H), 7.2-7.4 (m, 4H), 7.65 (m, 1H), 6.98 (d, J = 16.5 Hz, 1H). ^{13}C-NMR (75 MHz, CD_3OD): 119.9, 129.7, 129.9, 129.9, 130.0, 130.0, 132.5, 144.2, 171.6, 196.2. MS HRMS-EI(+): Calc. for $C_{10}H_8O_3$ [M^+]: 176.0473. Found: 176.0473. Analytical RP-HPLC retention times (conditions as described in General Notes section): (**5a**): 13.8 min. (**cyclization**): 15.5 min. (**6a**): 18.5 min.

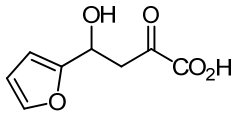




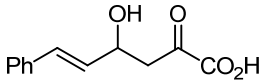
4-(4-fluorophenyl)-4-Hydroxy-2-oxobutanoic acid (5b). 4-fluorobenzaldehyde and oxaloacetate were reacted as described above to deliver a 9:1 mixture of **5b** and **6b** in 52 % yield. The identity of **5b** was confirmed by MS and ^{1}H NMR. ^{1}H-NMR (300 MHz, CD_3OD): 7.33 (dd, J = 8.7, 5.5 Hz, 2H) 7.09 (dd, J = 8.7, 8.7 Hz, 2H), 5.17 (dd, J = 8.3, 4.6 Hz, 2H), 3.13 (dd, J = 16.2, 8.4 Hz, 1H), 3.03 (dd, J = 16.2, 4.7 Hz, 1H). HRMS-EI(+): Calc. for $C_{10}H_8O_4F$ [M-H]: 211.0407. Found: 211.0412. Analytical RP-HPLC retention times (conditions as described in General Notes section): (**6b**): 19.9 min. (4-fluoro-benzaldehyde, **4b**): 16.7 min.



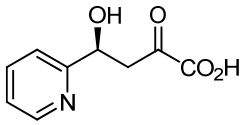
4-Hydroxy-4-(4-methoxyphenyl)-2-oxobutanoic acid (5c) and elimination product (6c). 4-Methoxybenzaldehyde and oxaloacetic acid were combined as described above to deliver a 1:3 mixture of **5c** and **6c** in 30% yield along with 30% recovered starting material. The small amount of **5c** obtained precluded its characterization.



4-(furan-2-yl)-4-Hydroxy-2-oxobutanoic acid (5d). 2-furancarboxaldehyde and oxaloacetic acid were combined as described above to deliver a 3:1 mixture of **5d** and **6d** in 49% yield. **5d**: ^{1}H-NMR (300 MHz, CD_3OD): 7.42 (dd, J = 1.8, 0.8 Hz, 1H), 6.33 (dd, J = 3.2, 1.8 Hz, 1H), 6.28 (dd, J = 3.2, 0.8 Hz, 1H), 5.18 (dd, J = 7.4, 5.7 Hz, 1H), 3.23 (d, J = 7.5 Hz, 1H), 3.22 (d, J = 5.7 Hz, 1H). ^{13}C-NMR (75 MHz, CD_3OD): 46.7, 64.2, 107.0, 111.2, 143.2, 157.2, 172.0, 205.0.



(E)-4-Hydroxy-2-oxo-6-phenylhex-5-enoic acid (5e). Cinnamaldehyde and oxaloacetate were combined as described earlier to deliver the product **5e** in 35% yield (50% conv). ^{1}H-NMR (300 MHz, CD_3OD): 7.59-7.05 (m, 5H), 6.63 (d, J = 15.9 Hz, 1H), 6.29 (dd, J = 15.9, 6.1 Hz, 1H), 4.77 (dddd, J = 6.6, 6.1, 6.1, 1.3 Hz, 1H), 3.00 (d, J = 6.6 Hz, 1H), 3.00 (d, J = 6.1 Hz, 1H).



(S)-4-Hydroxy-2-oxo-4-(pyridin-2-yl)butanoic acid (5f). 2-Pyridinecarboxaldehyde and oxaloacetate were combined as described earlier to deliver the product **5f** in 95% yield. This compound was identical in all respects to the reported characterization data.[4] The enantioselectivity (36%, see Figure S2) was determined by first treating **5f** with a 1:1 mixture of ethanethiol and fuming HCl (to give 0.2 M **5f**) and the resulting lactone was separated by chiral HPLC using a Daicel Chiralpak® AD column (97:3 Hexane : Isopropanol, 1 mL/min, 220 nm detection) Retention times: Major: 18.9 min, Minor: 22.2 min. The absolute stereochemistry was determined by comparison of the optical rotation to the known value: $[\alpha]_D^{lit}$ = -39, $[\alpha]_D$ = -15.8 (sample of 36% ee, c = 0.17).

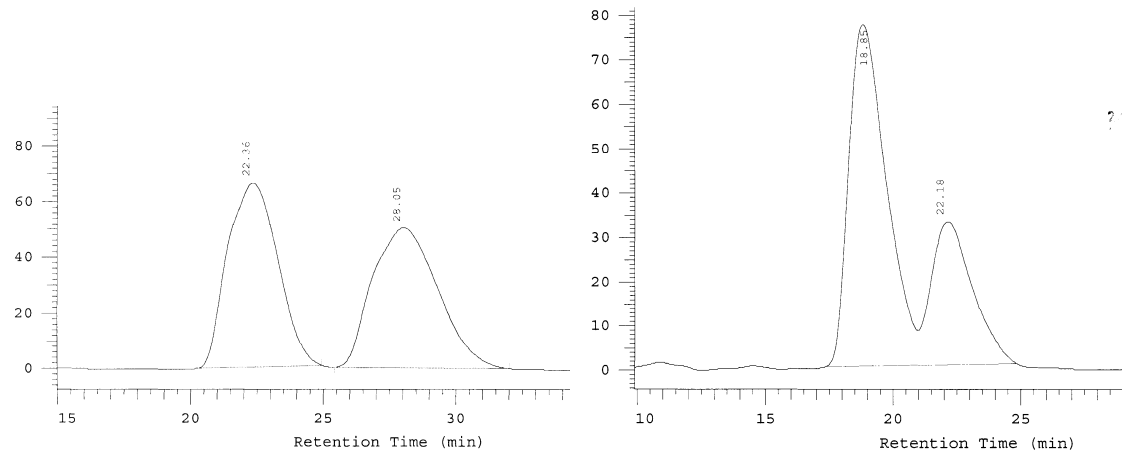
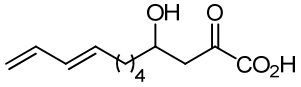
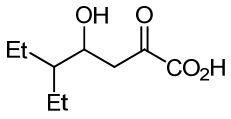


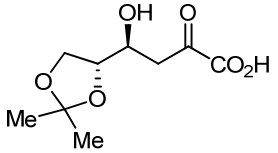
Figure S2. *Left*: Authentic racemic lactone. *Right*: Product obtained from enzymatic reaction, 36% ee.



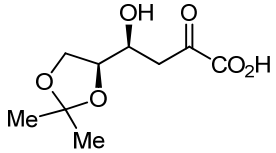
(E)-4-Hydroxy-2-oxododeca-9,11-dienoic acid (5g). 4g and oxaloacetate were combined as described earlier to deliver the product **5g** in 48% yield. Analytical RP-HPLC retention times (conditions as described in General Notes section): (**5g**): 23.1 min (**4g**): 24.3 min. ^{1}H-NMR (300 MHz, CD_3OD): 6.29 (ddd, J = 17.0, 10.2, 10.2 Hz, 1H), 6.15-5.98 (m, 1H), 5.78-5.62 (m, 1H), 5.05 (d, J = 17.0 Hz, 1H), 4.96 (d, J = 10.2 Hz, 1H Note: partially obscured by methanol solvent resonance), 4.05 (m, 1H), 2.86 (dd, J = 15.1, 4.0 Hz, 1H), 2.79 (dd, J = 15.1, 6.5 Hz, 1H), 2.15-2.03 (m, 2H), 1.55–1.33 (m, 6H). ^{13}C-NMR (75 MHz, CD_3OD): 26.3, 30.4, 33.6, 38.3, 48.7, 68.6, 115.0, 132.5, 136.1, 138.7, 170.6, 205.3. HRMS-ESI(-): calc. 225.1132; found 225.1131.



5-Ethyl-4-hydroxy-2-oxoheptanoic acid (5h). 4h and oxaloacetate were combined as described earlier to deliver the product **5h** in 45% yield. ^{1}H-NMR (300 MHz, CD_3OD): 4.15 (ddd, J = 8.5, 4.3, 4.3 Hz, 1H), 2.83 (dd, J = 15.2, 3.5 Hz, 1H), 2.76 (dd, J = 15.2, 7.5 Hz, 1H), 1.50-1.20 (m, 4H), 0.97-0.86 (m, 6H).



(S)-4-((R)-2,2-Dimethyl-1,3-dioxolan-4-yl)-4-hydroxy-2-oxobutanoic acid (8a)



(S)-4-((S)-2,2-dimethyl-1,3-dioxolan-4-yl)-4-hydroxy-2-oxobutanoic acid (8b). The general procedure was followed with glyceraldehyde

derivatives **7a** or **7b** to deliver **8a** or **8b** respectively. Their spectral properties were identical to the literature report.[5]

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(1) T. R. Hoye, H. Zhao, *J. Org. Chem.* **2002**, *67*, 4014–4016.

(2) Watanabe, K.; Oikawa, H.; Yagi, K.; Ohashi, S.; Mie, T.; Ichihara, A.; Honma, M. *J. Biochem.* **2000**, *127*, 467-473.

(3) Serafimov, J.; Lehmann, H. C.; Oikawa, H.; Hilvert, D. *Chem. Commun.* **2007**, 1701-1703.

(4) Henderson, D. P; Shelton, M. C.; Cotterill, I. C.; Toone, E. J. *J. Org. Chem.* **1997**, *62*, 7910-7911.

(5) Lamble, H. J.; Danson, M. J.; Hough, D. W.; Bull, S. D. *Chem. Commun.* **2005**, 124-126.