Supporting Information

Biocatalytic Aldol Addition of Simple Aliphatic Nucleophiles to Hydroxyaldehydes

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Materials

Synthetic oligonucleotides were purchased from Eurofins MWG Operon. Phosphonoglucone isomerase from baker’s yeast (S. cerevisiae), glucose-6-phosphate dehydrogenase from baker's yeast (S. cerevisiae), D,L-glyceraldehyde 3-phosphate (G3P), nicotinamide adenine dinucleotide phosphate (NADP⁺), antibiotics, acrylamide-bisacrylamide, buffer components, benzylxyethanlan, ethanol, propanone, butanone, 3-pentanone, cyclobutanone, cyclopentanone, cyclohexanone, 3,3-diethoxy-1-propanol, and 4,4-diethoxy-2-butanone, were purchased from Sigma-Aldrich. R-3-hydroxybutanal was a generous gift from Prof. Fessner (Technische Universität Darmstadt Organische Chemie & Biochemie, Germany). Culture media components for bacteria were from Pronadisa. Milli-Q grade water was used for analytical and preparative HPLC, buffer preparations and other assay solutions were obtained from an Arium™ Pro Ultrapure Water Purification System (Sartorius Stedim Biotech). All the other solvents used were of analytical grade. Bacterial strains, oligonucleotides and plasmids used in this study are listed in Table S1. The plasmid pQEfsa containing the gene for expression of FSA was constructed in our lab using routine procedures of molecular biology.

General procedures.

Mutagenesis.

All DNA manipulations and bacterial transformation were carried out according to the standard protocols or manufacturers’ instructions. D-fructose-6-phosphate aldolase from E. Coli (FSA) gene mutation was introduced with the QuickChange site-directed mutagenesis kit (QuickChange®, Stratagene), using the plasmid pQEfsa as template and performed according to the manufacturer’s protocols. E. coli Nova Blue competent cells (EMD Millipore) were used for transformation and plasmid preparation. The plasmid DNA was isolated with the High Pure Plasmid Isolation Kit (Roche). DNA sequencing analysis of the expressed proteins confirmed the expected mutations in the gene sequence.

Site directed mutagenesis.

The double variants FSA D6H/A165G, FSA D6H/L107A, FSA D6H/L163A, FSA D6E/A165G, FSA D6E/L107A, FSA D6E/L163A, FSA D6L/A165G, FSA D6L/L107A, FSA D6L/L163A were obtained using pQEfsa_D6H, pQEfsa_D6E and pQEfsa_D6L respectively as templates with the mutagenesis primers FSA A165G, FSA L107A and FSA L163A, forward and reverse (Table S1).
Table S1: Strain, plasmids and oligonucleotides used in this study. Mutagenized codons are highlighted in italic and bold, silent mutations are shown in italic.

| Plasmids | Relevant genetic characteristics | Ref./Origin |
|----------|---------------------------------|-------------|
| pQE40   | $P_{T7}$, Amp', ColE1 ori | Quiagen |
| pQEfsa  | fsagene (660bp) cloned in pQE40 (BseRI/HindIII), the C-terminal His-tag was deleted | |
| pQEfsaD6A | fsaD6A gene (660 bp) cloned in pQE40 | This study |
| pQEfsaD6E | fsaD6E gene (660 bp) cloned in pQE40 | This study |
| pQEfsaD6H | fsaD6H gene (660 bp) cloned in pQE40 | This study |
| pQEfsaD6L | fsaD6L gene (660 bp) cloned in pQE40 | This study |
| pQEfsaD6N | fsaD6N gene (660 bp) cloned in pQE40 | This study |
| pQEfsaD6Q | fsaD6Q gene (660 bp) cloned in pQE40 | This study |
| pQEfsaD6S | fsaD6S gene (660 bp) cloned in pQE40 | This study |
| pQEfsaD6T | fsaD6T gene (660 bp) cloned in pQE40 | This study |
| pQEfsaL107A | fsaL107A gene (660 bp) cloned in pQE40 | This study |
| pQEfsaL163A | fsaL163A gene (660 bp) cloned in pQE40 | This study |
| pQEfsaA165G | fsaA165G gene (660 bp) cloned in pQE40 | This study |
| pQEfsaK85M | fsaK85M gene (660 bp) cloned in pQE40 | This study |
| pQEfsaD6E/L107A | fsaD6E/L107A gene (660 bp) cloned in pQE | This study |
| pQEfsaD6E/L163A | fsaD6E/L163A gene (660 bp) cloned in pQE | This study |
| pQEfsaD6E/A165G | fsaD6E/A165G gene (660 bp) cloned in pQE | This study |
| pQEfsaD6H/L107A | fsaD6H/L107A gene (660 bp) cloned in pQE | This study |
| pQEfsaD6H/L163A | fsaD6H/L163A gene (660 bp) cloned in pQE | This study |
| pQEfsaD6H/A165G | fsaD6H/A165G gene (660 bp) cloned in pQE | This study |
| pQEfsaD6L/L107A | fsaD6L/L107A gene (660 bp) cloned in pQE | This study |
| pQEfsaD6L/L163A | fsaD6L/L163A gene (660 bp) cloned in pQE | This study |
| pQEfsaD6L/A165G | fsaD6L/A165G gene (660 bp) cloned in pQE | This study |

| Strains | Relevant genotype | Ref./Origin |
|---------|------------------|-------------|
| E. coli M15[pREP4] | nai"1, str"1, rif"1, thi" - lac +, ara" +, gal" - | Qiagen |
| E. coli Nova Blue | endA1, hsdR17(b' + , mB + ), supE44, thi-1, recA1, gyrA96, relA lac F' [proA B', lacI ZAM15::Tn10] (TetR) | EMD Millipore |

| Oligonucleotides | Oligonucleotide sequences (5' → 3') | Ref./Origin |
|------------------|------------------------------------|-------------|
| FSAD6A 5' - 3'   | ATGGAACTGTATCTGCGACACTCACAGGC    | This study  |
| FSAD6A 3' - 5'   | GCCGACAACGCTTAAAGTGCCGAGTATCCACT | This study  |
| FSAD6E 5' - 3'   | GGAAGCTGTATCCTGR4AGCAGCTCAGCTTGGG | This study  |
| FSAD6E 3' - 5'   | CGCAAACACGTCTGACATCCTCCAGTACG    | This study  |
| FSAD6H 5' - 3'   | GGAAGCTGTATCCTGR4AGCAGCTCAGCTTGGG | This study  |
| FSAD6H 3' - 5'   | CGCAAACACGTCTGACATCCTCCAGTACG    | This study  |
| FSAD6L 5' - 3'   | GGAAGCTGTATCCTGR4AGCAGCTCAGCTTGGG | This study  |
| FSAD6L 3' - 5'   | CGCAAACACGTCTGACATCCTCCAGTACG    | This study  |
| FSAD6N 5' - 3'   | ATGGAACTGTATCTGCGACACTCACAGGC    | This study  |
| FSAD6N 3' - 5'   | GGAAGCTGTATCCTGR4AGCAGCTCAGCTTGGG | This study  |
| FSAD6Q 5' - 3'   | GGAAGCTGTATCCTGR4AGCAGCTCAGCTTGGG | This study  |
| FSAD6Q 3' - 5'   | CGCAAACACGTCTGACATCCTCCAGTACG    | This study  |
| FSAD6S 5' - 3'   | GGAAGCTGTATCCTGR4AGCAGCTCAGCTTGGG | This study  |
| FSAD6S 3' - 5'   | CGCAAACACGTCTGACATCCTCCAGTACG    | This study  |
| FSAD6T 5' - 3'   | GGAAGCTGTATCCTGR4AGCAGCTCAGCTTGGG | This study  |
| FSAD6T 3' - 5'   | CGCAAACACGTCTGACATCCTCCAGTACG    | This study  |
| FSAL107A 5' - 3' | CGACCGGCAGAACCGGCGGATATGCGG    | This study  |
| FSAL107A 3' - 5' | ACCGGTCTGCTGGGTCGGGAATCCCTTCCC | This study  |
| FSAL163A 5' - 3' | GAAAGTGCGGGCAGCGAGTTTCAAAC    | This study  |
| FSAL163A 3' - 5' | GAAAGTGCGGGCAGCGAGTTTCAAAC    | This study  |
| FSAA165G 5' - 3' | GAAAGTGCGGGCAGCGAGTTTCAAAC    | This study  |
| FSAA165G 3' - 5' | GGAAGCTGTATCCTGR4AGCAGCTCAGCTTGGG | This study  |
**Protein expression and purification FSA catalysts.** The *E. coli* strain M-15 [pREP-4] (QIAGEN) was transformed with plasmid and grown in LB medium with ampicillin (100 µg mL\(^{-1}\)) plus kanamycin (25 µg L\(^{-1}\)) at 37°C on a rotary shaker at 250 rpm up to an optical density of 0.6 at 600 nm. For protein expression, temperature was lowered to 30 ºC and 1 mM final concentration of IPTG was added. After additional incubation of 12 h cells were harvested, suspended in starting GlyGly buffer (50mM, pH 8.5) containing dithiothreithol (DTT) (1 mM) and lysed using a TS 0.75 kW 40K Cell Disrupter (Constant Systems). Cellular debris was removed by centrifugation at 8228 × g for 30 min at 4 ºC. The clear supernatant was treated with heat-shock (70 ºC, 30 min), and centrifuged at 8228 × g for 30 min at 4 ºC. The supernatant was dialyzed against GlyGly buffer (5 mM, pH 8.5) containing DTT (0.1 mM) and finally, lyophilized. Protein concentrations were calculated with the Bradford method.
Figure S1. SDS-PAGE analysis of FSA D6X variants. Protein loads (≈ 2 µg) were applied to each lane of a 12% (v/v) SDS-PAGE gel. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250. A protein ladder from Thermo Pierce Unstained Protein Molecular weight marker was used as standard; a: negative control without IPTG; b: protein expression with IPTG, c: protein after the heat-shock (70 ºC, 30 min).

Figure S2. SDS-PAGE analysis of FSA double variants after the heat-shock (70 ºC, 30 min). Protein loads (≈ 2 µg) were applied to each lane of a 12% (v/v) SDS-PAGE gel. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250. A protein ladder from Thermo Pierce Unstained Protein Molecular weight marker was used as standard.
2-Deoxy-d-ribose-5-phosphate aldolase from *E. coli* (DERA\textsubscript{Ecoli}) catalyst: expression and purification. *E. coli* strain BL21-AI\textsuperscript{TM} from ThermoFisher Scientific was transformed with the plasmid pET-21a(+)\textsubscript{DERAEcoli}, (gift from Prof. Fessner Technische Universität Darmstadt, Organische Chemie & Biochemie, Germany), and grown in LB medium with ampicillin (100 µg mL\textsuperscript{-1}) at 37 ºC on a rotary shaker at 200 rpm. A final optical density at 600 nm (OD600) of 2–3 was usually achieved. An aliquot of the pre-culture (20 mL) was transferred into a shake-flask (2 L) containing LB (1 L) with ampicillin (100 µg mL\textsuperscript{-1}) and antifoam SE-15 (0.02% final concentration) and incubated at 37 ºC with shaking at 200 rpm. During the middle exponential phase growth (DO\textsubscript{600} ≈ 0.5), the temperature was decreased to 30 ºC to minimize potential inclusion bodies formation, then isopropyl-β-D-1-thiogalactopyranoside (IPTG; 1 mM final concentration) and L-arabinose (0.2% final concentration) was added. Cells from the induced-culture broths (5 L) were centrifuged at 7500 g for 30 min at 4 ºC. The pellet was re-suspended with starting sodium phosphate buffer (200 mL, 50 mM, pH 8.0), containing NaCl (300 mM) and imidazole (10 mM). Cells were lysed using a cell disrupter (Constant Systems). Cellular debris was removed by centrifugation at 30 000 g for 30 min. The clear supernatant was collected and purified by immobilized metal ion affinity chromatography (IMAC) using a FPLC system (Amersham biosciences). The crude supernatant was applied to a cooled HR 16/40 column (GE Healthcare) packed with HiTrap chelating support (50 mL bed volume; Amersham Biosciences) and washed with the start buffer (250 mL). The protein was eluted with sodium phosphate buffer (50 mM, pH 8.0) containing NaCl (300 mM) and imidazole (500 mM) at a flow rate of 3 mL min\textsuperscript{-1}. Fractions containing the recombinant protein were combined and dialyzed against sodium phosphate buffer (10 mM, pH 7.0) at 4 ºC. The dialyzed solution was frozen at –80 ºC and. The white solid obtained (yield: 80 mg L\textsuperscript{-1} of culture) was stored at –20 ºC. Specific activity DERA\textsubscript{Ecoli}, 0.32 U mg\textsuperscript{-1} lyophilized material, one international unit (1 U) of DERA\textsubscript{Ecoli} is that amount which catalyzes the cleavage of 1 µmol of 2-deoxy-d-ribose-5-phosphate (Dr5P) per minute at [Dr5P] = 0.6 mM, 50 mM triethanolamine buffer pH 7.5 at 30 ºC.
Figure S3. SDS-PAGE analysis of 2-deoxy-β-ribose-5-phosphate aldolase from *E. coli* (DERA<sub>Ecoli</sub>). Protein loads (≈ 2 μg) were applied to each lane of a 12% (v/v) SDS-PAGE gel. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250. A protein ladder from Thermo Pierce Unstained Protein Molecular weight marker was used as standard; a: negative control without IPTG; b: Total after lysis, before centrifugation, c: Supernatant after lysis and centrifugation, d: Pellet after lysis and centrifugation, e: Load before elution, f: Wash before elution, g: Eluted protein (purified).

**Activity assay of FSA catalysts** (Table S2): The assays were performed in a 96 well-plates. Total volume: 300 μL. The formation of D-fructose 6-phosphate from dihydroxypropanone (DHA) (300 mM) and D,L-glyceraldehyde 3-phosphate (6 mM) was monitored with a coupled assay using phosphoglucone isomerase and glucose-6-phosphate dehydrogenase in GlyGly buffer (50 mM, pH 8.5) containing DTT (1 mM) at 30 °C. The reduction of NADP (0.5 mM) was monitored at 340 nm for 5 minutes in a SpectraMax M5 Series Multi-Mode Microplate Reader (Molecular Devices, LLC, Sunnyvale CA, USA); the formation of 1 μmol of NADPH was set equivalent to 1 μmol of D-fructose 6-phosphate formed (one U will produce 1 μmol of D-fructose 6-phosphate (D-F6P) per minute).
### Table S2. Specific aldolase activity of the FSA wild-type and variants used in this work

| FSA variant    | Specific activity U mg\(^{-1}\) protein (Bradford) |
|----------------|-----------------------------------------------|
| FSA wt         | 45.4±0.1                                       |
| FSA D6A        | 0.06±0.01                                      |
| FSA D6E        | 0.73±0.01                                      |
| FSA D6H        | 0.45±0.02                                      |
| FSA D6L        | 0.03±0.01                                      |
| FSA D6N        | 46.1±0.1                                       |
| FSA D6Q        | 0.15±0.01                                      |
| FSA D6S        | 0.18±0.01                                      |
| FSA D6T        | 0.06±0.01                                      |
| D6E/L107A      | nd                                             |
| D6E/L163A      | nd                                             |
| D6E/A165G      | nd                                             |
| D6H/L107A      | nd                                             |
| D6H/L163A      | nd                                             |
| D6H/A165G      | nd                                             |
| D6L/L107A      | nd                                             |
| D6L/L163A      | nd                                             |
| D6L/A165G      | nd                                             |

nd: no activity detected

**TLC analyses.** Samples (10 µL) were mixed with MeOH (50 µL) and eluted with CHCl\(_3\):MeOH (5:1).

**HPLC analyses.** HPLC analyses were performed on an X-Bridge\(^{TM}\) C18, 5 µm, 4.6 ×250 mm column from Waters (Milford, USA). Samples (30 µL) were injected and eluted with the following conditions: solvent system (A) aqueous trifluoroacetic acid (TFA) (0.1% (v/v) and (B): TFA (0.095% (v/v)) in CH\(_3\)CN/H\(_2\)O (4:1), gradient elution from 10-100 %B in 30 min, flow rate 1 mL min\(^{-1}\), detection at 215 nm, column temperature 30 °C. The amount of aldol adduct product was quantified from the peak areas using and external standard methodology.

**NMR analysis.** High field \(^1\)H and \(^13\)C nuclear magnetic resonance (NMR) analyses were carried out using an AVANCE 500 BRUKER spectrometer equipped with a high-sensitive CryoProbe for D\(_2\)O and CD\(_3\)OD solutions. Full characterization of the described compounds was performed using typical gradient-enhanced 2D experiments: COSY, NOESY, HSQC and HMBC, recorded under routine conditions. When possible, NOE data were obtained from selective 1D NOESY versions using a single pulsed-field-gradient echo as a selective excitation method and a mixing time of 500 ms. Routine, \(^1\)H (400-500 MHz) and \(^13\)C (101 MHz) NMR spectra of compounds were recorded with Varian Mercury-400 and Varian Anova-500 spectrometers.
**Determination of the specific optical rotations** ([α]_D^20). Specific optical rotations were measured with a Perkin Elmer Model 341 (Überlingen, Germany) polarimeter (Na lamp, 589 nm). The products (5–20 mg) were dissolved in methanol (1 mL) and the samples were measured at 25 °C with a 0.1 dm cell.

**Chiral HPLC analyses.** Chiral HPLC separations were performed with a CHIRALPACK® ID, 5 µm, 4.6×250 mm column from Daicel (Illkirch, France). Samples (10 µL) were injected and eluted with hexane:isopropanol 90:10 (v/v) for 45 min, flow rate 1 mL min⁻¹, detection at 209 nm.

**Chiral GC analyses.** Chiral GC (Shimadzu GC-17A) separations were performed with a chiral GC Rt-βDEXsm column (2,3-di-O-methyl-6-O-tert-butyldimethylsilylbeta cyclodextrin added into 14% cyanopropylphenyl/86% dimethyl polysiloxane) from RESTEK, using nitrogen as carrier gas, program: 80°C, 4 K min⁻¹, 230°C Inj/Det 230°C.

**Preparation of 3-hydroxypropanal (2a).** 3,3-Diethoxy-1-propanol (6.75 mmol, 1 g) was dissolved in 2 M aqueous solution of H_2SO_4 (5 mL). The reaction was stirred at 250 rpm and 25 °C. The formation of the aldehyde was monitored by TLC (CHCl₃:MeOH 5:1 v/v). After 1.5 h the reaction was stopped by addition of CaCO₃ powder to neutralize the acid. The solution was filtered to remove precipitated CaSO₄ and washed with 50 mM triethanolamine buffer pH 7 (10 mL). The aldehyde solution (500 mM, 12 mL) was used in aliquots without further purification dissolved in water. The final concentration was determined by HPLC with propionaldehyde as standard.

**Preparation of (S)-3-hydroxybutanal (S-2b).** (S)-3-Hydroxybutanal was synthesized from 4,4-dimethoxybutan-2-one following the procedure described by Besse et al.⁶

**Preparation of selected racemic aldol adducts**

**Pyrrolidine catalyzed aldol addition of propanone to 3-hydroxypropanal.**

**rac-2-Methoxy-2-methyltetrahydro-2H-pyran-4-ol (rac-3b):** To a mixture of 3-hydroxypropanal (0.5 mmol, 1 mL of 500 mM aqueous stock solution) and propanone (4 mL), pyrrolidine (0.01 mmol) was added. Total reaction volume was 5 mL. Reaction mixture was stirred at 25 °C overnight. Propanone was evaporated and the reaction was lyophilized. This procedure was repeated five times to have enough material. The product was then purified by silica gel column chromatography and eluted with a step gradient of CHCl₃:MeOH from 1:0 to 93:7, yielding 80.2 mg (42%) of rac-3b. ¹H NMR (400 MHz, CD₃OD) δ 3.92 (tt, J = 11.2, 4.7 Hz, 1H), 3.64 (ddd, J = 11.5, 5.3, 1.7 Hz,
S11

1H), 3.55 (ddd, J = 12.9, 11.4, 2.3 Hz, 1H), 3.14 (s, 3H), 2.02 (ddd, J = 12.5, 4.7, 2.1 Hz, 1H), 1.80 (ddd, J = 12.5, 4.6, 2.2 Hz, 1H), 1.38 (tdd, J = 12.5, 11.3, 5.2 Hz, 1H), 1.32-1.27 (m, 1H), 1.27 (s, 3H).

13C NMR (101 MHz, CD3OD) δ 99.4, 63.5, 59.2, 46.5, 44.5, 34.2, 22.5. No signals from the open form of the compound were observed.

Synthesis of (2R,4S)-2-methoxy-2-methyltetrahydro-2H-pyran-4-ol (i.e. methyl glycoside derivative of 3b): 40 mg of 3b was dissolved in dried MeOH (21 mL). Dried Dowex H+ (20 mg) and molecular sieves 3 Å were added. The reaction mixture was stirred at 25 ºC for 24-48 hours. Then the solvent was evaporated and the product was purified by silica gel column chromatography and eluted with a step gradient of hexane:AcOEt:Et3N from 99:0:1 to 39:60:1, yielding 30 mg (75 %) of product.

Lithium diisopropylamide LDA methodology.7

rac-5-(Benzyloxy)-4-hydroxypentan-2-one (rac-6b): Lithium diisopropylamide (LDA) (700 µL of a 2 M stock solution in tetrahydrofuran (THF)) was added to anhydrous THF (8 mL) and the solution was cooled down to – 78 ºC. Then propanone (100 µL) was added and stirred for 20 minutes. After that, benzyloxyethanal (2c) (150 µL, 1 mmol) dissolved in anhydrous THF (2 mL) was added. The reaction was stirred for 2 hours at – 78 ºC. After that the reaction was left to warm up at room temperature and NaHCO3 (20 mL of 10% aqueous solution) was added. The product was extracted with AcOEt (3 x 15 mL), the organic phases were pooled, dried with anhydrous Na2SO4 and evaporated to dryness. The product was purified by silica gel column chromatography, eluted with a step gradient of hexane:AcOEt from 1:0 to 3:7, yielding 125.7 mg (60%) of rac-6b. The NMR spectra was identical from the one obtained by enzymatic catalysis (see below).

Enzymatic reactions. Screening

General procedure for aldol addition reactions. Reactions were conducted in 1 mL 96 well plates. Total reaction volume was 300 µL. Aldol additions of propanone (1b).

Protocol I. To a solution of 3-hydroxypropanal (2a) or (S)- or (R)-3-hydroxybutanal (S-2b, R-2b) (48 µL of a 500 mM aqueous stock solution, 80 mM final concentration), propanone (45 µL, 15%) was added. Protocol II. Benzylxoyethanal (2c) (80 mM final concentration) was dissolved in propanone (45 µL, 15% v/v). Then, for both protocols 50 mM triethanolamine buffer pH 8.0 (15 µL, of a 1 M stock solution) was added. The reaction was started by adding the lyophilized FSA preparation (3 mg mL⁻¹, 1 mg
dissolved in 192, and 240 µL for the protocol I and II, respectively). **Aldol addition of ketones 1c, 1d, 1e, 1f, and 1g, Protocol I.** To a solution of 3-hydroxypropanal (2a) or (S)- or (R)-3-hydroxybutanal (S-2b, or R-2b) (48 µL of a 500 mM aqueous stock solution, 80 mM final concentration), the ketone (15 µL) was added. **Protocol II.** Benzyloxyethanal (2c) (80 mM final concentration) was dissolved in the ketone (1c, 1d, 1e, 1f, or 1g) (15 µL). Then, for both protocols, 50 mM triethanolamine buffer pH 8.0 (15 µL of a 1 M stock solution) was added. The reaction was started by adding the lyophilized FSA preparation (3 mg mL\(^{-1}\), 1 mg dissolved in 222, and 270 µL of plain water for protocols I and II, respectively). **Aldol addition of ethanal (1a), Protocol I.** For aldehydes 2a, S-2b, R-2b, and 2c: To a solution of 3-hydroxypropanal (2a) or (S)- or (R)-3-hydroxybutanal (S-2b, or R-2b) (48 µL of a 500 mM aqueous stock solution, 80 mM final concentration), ethanal (1a) (30 µL of a 1 M stock solution, 100 mM final concentration) was added. **Protocol II.** To benzyloxyethanal (2c) (24 µmol), ethanal (30 µL of a 1 M stock solution) was added. Then, for both protocols, 50 mM triethanolamine buffer pH 8.0 (15 µL of a 1 M stock solution) was added. The reaction was started by adding the lyophilized FSA preparation (3 mg mL\(^{-1}\), 1 mg dissolved in 207, and 255 µL of plain water, for protocols I, and II, respectively). In all cases, reaction mixtures were shaken (1500 rpm) at 25 ºC. Reaction monitoring: **Protocol I.** Analysis by TLC: samples (10 µL) were diluted in MeOH (50 µL) and eluted with CHCl\(_3\): MeOH (5:1). **Protocol II:** Samples (50 µL) were diluted in MeOH (450 µL) and after centrifugation were analyzed by HPLC with a gradient elution system (10 to 100 % of B in 30 min).
**Table S3.** Screening of the aldol additions of nucleophiles 1a-g to electrophilic components 2a-c catalyzed by FSA variants.^[a]^  

| FSA     | 2a      | 1a  | 1b  | 1c  | 1d  | 1e  | 1f  | 1g  | 1a  | 1b  | 1c  | 1d  | 1e  | 1f  | 1g  | 2c      | 1a  | 1b  | 1c  | 1d  | 1e  | 1f  | 1g  |
|---------|---------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--------|-----|-----|-----|-----|-----|-----|-----|
| wild-type | —       | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —      | —   | —   | —   | —   | —   | —   | —   |
| D6A     | —       | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —      | —   | —   | —   | —   | —   | —   | —   |
| D6L     | —       | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —      | —   | —   | —   | —   | —   | —   | —   |
| D6N     | —       | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —      | —   | —   | —   | —   | —   | —   | —   |
| D6Q     | —       | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —      | —   | —   | —   | —   | —   | —   | —   |
| D6S     | —       | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —      | —   | —   | —   | —   | —   | —   | —   |
| D6T     | —       | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —      | —   | —   | —   | —   | —   | —   | —   |
| A165G   | —       | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —      | —   | —   | —   | —   | —   | —   | —   |
| L107A   | —       | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —      | —   | —   | —   | —   | —   | —   | —   |
| L163A   | —       | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —      | —   | —   | —   | —   | —   | —   | —   |
| D6H/A165G | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —      | —   | —   | —   | —   | —   | —   | —   |
| D6H/L107A | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —      | —   | —   | —   | —   | —   | —   | —   |
| D6H/L163A | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —      | —   | —   | —   | —   | —   | —   | —   |
| D6L/A165G | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —      | —   | —   | —   | —   | —   | —   | —   |
| D6L/L107A | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —      | —   | —   | —   | —   | —   | —   | —   |
| D6L/L163A | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —      | —   | —   | —   | —   | —   | —   | —   |
| D6E/A165G | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —      | —   | —   | —   | —   | —   | —   | —   |
| D6E/L107A | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —      | —   | —   | —   | —   | —   | —   | —   |
| D6E/L163A | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —      | —   | —   | —   | —   | —   | —   | —   |

[a] Reactions were analyzed after 24 h. Crosses indicate a visual estimation of the intensity of a new spot on TLC analysis (green squares). Quantitative determination of product formed was measured by HPLC when using electrophile 2c. Conditions: [1a] = 5% in the reaction with 2a; [1a] = 100 mM in the reaction with S- and R-2b; [1b] = 15% v/v and [1c-f] = 5% v/v; in all cases [2] = 80 mM. Reactions were conducted in aqueous 50 mM triethanolamine buffer pH 8 at 25 °C. nr.: No reaction detected. – No new spot observed

**Scale up of aldol addition of propanone to aldehydes**

**General procedure:** Reactions were conducted in a 50 mL screw capped conical-bottom polypropylene tubes. *Method 1.* To an enzyme (3 mg mL\(^{-1}\)) solution in plain water (12.8 mL), 1 M triethanolamine buffer, pH 8 (1 mL) was added. To this solution, the aldehyde (80 mM, 3.2 mL of 500 mM aqueous stock solution) and propanone (1b) (3 mL) were added. *Method 2.* To an enzyme solution (3 mg mL\(^{-1}\)) in plain water (16 mL), 1 M triethanolamine buffer, pH 8 (1 mL) was added. To this solution, benzyloxyethanal (2c) (80 mM) dissolved in 1b (3 mL) was added. For both Methods: Total reaction volume was 20 mL. Reaction mixtures were shaken (1000 rpm) at 25 °C for 24 h. After that, the reactions were quenched with MeOH (30 mL) to precipitate the
enzyme. Method 1. The mixture was filtered through Celite, the MeOH was evaporated and the aqueous residue was lyophilized. Method 2. The mixture was filtered through activated charcoal. MeOH was evaporated and the residue was lyophilized.

**Aldol addition of 1b to S-2b (Method 1): (2R,4R,6S)-2,6-dimethyltetrahydro-2H-pyran-2,4-diol (4b):** This compound was obtained following the procedure described above (Method 1). S-3-Hydroxybutanal (S-2b) (1.6 mmol) and FSA D6H were used. The product was purified by silica gel column chromatography and eluted with a step gradient of CHCl₃:MeOH from 1:0 to 95:5, yielding 60.7 mg (25%) of 4b. [α]D²⁰ = −58.8 (c = 1.12 in MeOH); ¹H NMR (400 MHz, CD₃OD) δ 3.96 (tt, J = 11.2, 4.7 Hz, 1H), 3.67 (ddd, J = 11.5, 6.4, 2.1 Hz, 1H), 2.03 (ddd, J = 12.5, 4.8, 1.9 Hz, 1H), 1.89 (ddt, J = 12.3, 4.4, 2.1 Hz, 1H), 1.29 (s, 3H), 1.25 (dd, J = 12.5, 11.2 Hz, 1H), 1.17 (d, J = 6.3 Hz, 3H), 1.05 (dd, J = 12.3, 11.4 Hz, 1H). ¹³C NMR (101 MHz, CD₃OD) δ 66.5, 65.2, 45.3, 43.0, 24.1, 21.7. ESI-TOF: calculated for [M+Na]⁺ C₇H₁₄O₃Na: 169.0840, found 169.0837.

**Aldol addition of 1b to R-2b (Method 1): (2S,4R,6R)-2,6-dimethyltetrahydro-2H-pyran-2,4-diol (5b):** This compound was obtained following the procedure described above (Method 1). (R)-3-Hydroxybutanal (R-2b) (1.6 mmol) and FSA D6N were used. The product was purified by silica gel column chromatography and eluted with a step gradient of CHCl₃:MeOH from 1:0 to 95:5, yielding 21.2 mg (10%) of 5b. [α]D²⁰ = +11.1 (c = 0.36 in MeOH); The spectral properties of this sample agreed with those reported in the literature.⁸ ¹H NMR (500 MHz, CD₃OD) δ 4.03 (m, 1H), 4.04 (m, 1H), 1.93 (ddd, J = 14.4, 2.9, 2.1 Hz, 1H), 1.73 (ddt, J = 13.7, 3.2, 2.2 Hz, 1H), 1.70 – 1.65 (m, 1H), 1.48 – 1.39 (m, 1H), 1.30 (s, 3H), 1.20 (d, J = 6.3 Hz, 3H). ¹³C NMR (101 MHz, CD₃OD) δ 100.0, 64.7, 60.8, 39.8, 38.8, 22.9, 20.1. ESI-TOF: calculated for [M+Na]⁺ C₆H₁₄O₃Na: 169.0840, found 169.0842.

**Aldol addition of 1b to 2a (Method 1): 2-methyltetrahydro-2H-pyran-2,4-diol (3b):** This compound was obtained following the procedure described above (Method 1) as a mixture of two anomers α(2S,4R) (α-3b) and β(2R,4R) (β-3b) in equilibrium with the acyclic form (R)-4,6-dihydroxyhexan-2-one. However, the composition changes with the time and after a month the major anomer was β(2R,4R) (β-3b). 3-Hydroxypropanal (2a) (1.6 mmol) and FSA D6H were used. The product was purified by silica gel column chromatography and eluted with a step gradient of CHCl₃:MeOH from 1:0 to 93:7, yielding 50.0 mg (32%) of 3b. [α]D²⁰ = −24.2 (c = 1.07 in MeOH); (2S,4R)-(α-
3b): $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 3.94 (m, 2H), 3.66 (ddd, $J = 11.6, 5.3, 1.8$ Hz, 1H), 3.58 (ddd, $J = 12.8, 11.4, 2.3$ Hz, 1H), 2.05 (ddd, $J = 12.6, 4.8, 2.1$ Hz, 1H), 1.82 (ddt, $J = 10.4, 4.6, 2.2$ Hz, 1H), 1.41 (m, 1H), 1.37 (s, 3H), 1.28 (m, 1H). $^{13}$C NMR (126 MHz, CD$_3$OD) $\delta$ 100.8, 65.0, 60.6, 45.9, 35.6, 23.9. ($2R$,$4R$) ($\beta$-3b): $^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 4.25–4.12 (m, 1H), 4.00 – 3.85 (m, 2H), 2.61 (dd, $J = 6.4, 2.8$ Hz, 2H), 2.18 (s, 3H), 1.74–1.62 (m, 2H). $^{13}$C NMR (101 MHz, CD$_3$OD) $\delta$ 210.5, 66.4, 59.8, 51.9, 40.6, 30.6. ESI-TOF: calculated for $[M+Na]^+$ C$_6$H$_{12}$O$_3$Na: 155.0684, found 155.0689.

Aldol addition of 1b to 2c (Method 2): (S)-5-(benzylxy)-4-hydroxypentan-2-one (6b): This compound was obtained following the procedure described above (Method 2). Benzylxyethanal (2c) (240 µL, 1.6 mmol) and FSA D6 Q were used. After 24 h an additional amount of FSA D6Q (3 mg ml$^{-1}$) was added and the reaction was shaken (1000 rpm) at 25 ºC for 24 h more. The product was purified by silica gel column chromatography and eluted with a step gradient of hexane:AcOEt from 1:0 to 1:1, yielding 256 mg (77%) of 6b. [$\alpha$]$_D^{20}$ = – 5.29 (c = 1.26 in MeOH); HPLC: 10 to 100% of B in 30 min, $t_R$ = 16 min; $^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 7.45 – 7.10 (m, 5H), 4.53 (d, $J = 1.9$ Hz, 2H), 4.27 – 4.17 (m, 1H), 3.50 – 3.37 (m, 2H), 2.72 – 2.54 (m, 2H), 2.16 (s, 3H). $^{13}$C NMR (101 MHz, CD$_3$OD) $\delta$ 210.1, 138.4, 127.9, 127.4, 127.2, 75.0, 74.3, 67.8, 49.6, 30.7.

Removal of the Bn group of 6b: synthesis of (S)-4,5-dihydroxypentan-2-one. The aldol adduct 6b (3 mM) was dissolved in H$_2$O:MeOH 1:1 (70 mL), then Pd/C (60 mg) was added. The mixture was shaken under H$_2$ (2.5 atm) overnight at room temperature. After that, the reaction was filtered and the solvent was evaporated. The title compound was characterized without any further purification. [$\alpha$]$_D^{20}$ = – 30.3 (c = 0.5 in CHCl$_3$) (lit.: for the R-enantiomer [$\alpha$]$_D^{20}$ = + 34.0 (c = 0.44, CHCl$_3$)); The spectral properties of this sample agreed with those reported in the literature. $^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 4.05 (dtd, $J = 8.3, 5.5, 4.5$ Hz, 1H), 3.45 (dd, $J = 5.5, 1.2$ Hz, 2H), 2.63 (dd, $J = 16.2, 4.5$ Hz, 1H), 2.55 (dd, $J = 16.2, 8.2$ Hz, 1H), 2.17 (s, 3H). $^{13}$C NMR (101 MHz, CD$_3$OD) $\delta$ 209.1, 68.1, 65.4, 46.7, 29.3. ESI-TOF: calculated for [M+2Na]$^+$ C$_8$H$_{10}$O$_3$Na$_2$: 165.0503, found 165.0505.
Scale up of aldol addition of ketones to aldehydes 1c, 1d, 1e, and 1f

General procedure:
The reaction was conducted in a 50 mL screw capped conical-bottom polypropylene tubes. Method 1. To an enzyme (3 mg mL$^{-1}$) solution in plain water (14.8 mL), 1 M triethanolamine buffer, pH 8 (1 mL) was added. To this solution, the aldehyde (80 mM, 3.2 mL of 500 mM aqueous stock solution) and ketone (1 mL) were added. Method 2. To an enzyme (3 mg mL$^{-1}$) solution in plain water (18 mL), 1 M triethanolamine buffer, pH 8 (1 mL) was added. To this solution, aldehyde (80 mM) and ketone (1 mL) were added. Methods 1 and 2: Total reaction volume was 20 mL. Reaction mixtures were shaken (1000 rpm) at 25 $^\circ$C for 24 h. After that, the reaction was stopped with MeOH (30 mL) to precipitate the enzyme. Method 1: the mixture was filtered through activated charcoal. Method 2 the mixture was filtered through Celite. Methods 1 and 2: MeOH was evaporated and the reaction was lyophilized.

**Aldol addition of 1e to $S$-2b (Method 1):** (1R,3S,5R,6R)-3-methyl-2-oxabicyclo[4.2.0]octane-1,5-diol (4e): This compound was obtained following the procedure described above (Method 1) in equilibrium with the acyclic adduct. ($S$)-3-Hydroxybutanal ($S$-2b) (1.6 mmol), cyclobutanone (1e) and FSA D6H were used. The product was purified by silica gel column chromatography and eluted with a step gradient of CHCl$_3$:MeOH from 1:0 to 95:5, yielding 55.3 mg (22%) of 4e. [α]$_D^{20}$ = +13.69 ($c$ = 2.0 in MeOH); $^1$H NMR (400 MHz, CD$_3$OD) δ 3.93 (dt, $J$ = 12.0, 6.3 Hz, 1H), 3.72 (dqd, $J$ = 11.1, 6.3, 1.4 Hz, 1H), 2.65 (ddd, $J$ = 9.9, 8.9, 6.5 Hz, 1H), 1.96 – 1.89 (m, 2H), 1.73 (ddddd, $J$ = 12.7, 5.9, 1.5, 0.7 Hz, 1H), 1.64 – 1.53 (m, 2H), 1.36 (dd, $J$ = 12.6, 11.4 Hz, 1H), 1.20 (d, $J$ = 6.3 Hz, 3H). $^{13}$C NMR (101 MHz, CD$_3$OD) δ 67.6, 65.7, 46.8, 38.3, 34.8, 21.8, 13.3. Open form of 4e: ($R$)-2-((1R,3S)-1,3-dihydroxybutyl)cyclobutan-1-one: $^1$H NMR (400 MHz, CD$_3$OD) δ 3.94 (m, 2H), 3.39 (ddddd, $J$ = 9.7, 7.1, 5.3, 2.7 Hz, 1H), 3.02 – 2.80 (m, 2H), 2.18 – 2.04 (m, 1H), 2.02 – 1.95 (m, 1H), 1.71 (m, 1H), 1.55 – 1.48 (m, 1H) 1.18 (d, $J$ = 1.7 Hz, 3H). $^{13}$C NMR (101 MHz, CD$_3$OD) δ 68.4, 67.5, 65.1, 45.6, 45.1, 24.3, 14.3. ESI-TOF: calculated for [M+Na]$^+$ C$_8$H$_{14}$O$_3$Na: 181.0840, found 181.0843.

**Aldol addition of 1f to $S$-2b (Method 1):** (2S,4R,4aR,7aR)-2-methylhexahydrocyclopenta[b]pyran-4,7a(2H)-diol (4f): This compound was obtained following the procedure described above (Method 1). (S)-3-Hydroxybutanal ($S$-2b) (1.6 mmol), cyclopentanone (1f) and FSA D6H were used. The product was purified by silica gel column chromatography and eluted with a step gradient of
CHCl₃:MeOH from 1:0 to 95:5, yielding 22.6 mg (8%) of 4f. [α]D²⁰ = −2.03 (c = 1.49 in MeOH); ¹H NMR (400 MHz, CD₃OD) δ 4.05 (dt, J = 11.8, 5.4 Hz, 1H), 3.71 (dqd, J = 12.7, 6.3, 2.1 Hz, 1H), 2.20 (h, J = 5.6 Hz, 1H), 1.94 – 1.86 (m, 1H), 1.78 – 1.57 (m, 6H), 1.43 – 1.30 (m, 1H), 1.18 (d, J = 6.3 Hz, 3H). ¹³C NMR (126 MHz, CD₃OD) δ 109.5 (d, J = 9.0 Hz), 65.9, 65.6, 47.1, 35.6, 34.6, 21.6, 20.3, 19.4. ESI-TOF: calculated for [M+Na]+ C₉H₁₆O₃Na: 195.0997, found 195.0991.

**Aldol addition of 1e to 2c (Method 2):** (R)-2-((S)-2-(benzylzyoxy)-1-hydroxyethyl)cyclobutan-1-one (6e) (75%) and (R)-2-((R)-2-(benzylzyoxy)-1-hydroxyethyl)cyclobutan-1-one (7e) (25%). These compounds were obtained following the procedure described above (Method 2). Benzylxyethanal (2c) (1.6 mmol), cyclobutanone (1e) and FSA D6Q were used. The mixture was purified by silica gel column chromatography and eluted with a step gradient of hexane:AcOEt from 1:0 to 3:7, yielding 46.0 mg (13%) of 6e:7e in 75:25 ratio. HPLC: 10 to 100 % of B in 30 min, tR = 18 min. Major 6e: ¹H NMR (400 MHz, CD₃OD) δ 7.38 – 7.26 (m, 5H), 4.53 (s, 2H), 3.92 (dt, J = 6.5, 5.0 Hz, 1H), 3.59 (dd, J = 9.8, 6.6 Hz, 1H), 3.55 (m, 1H), 3.53 (m, 1H), 2.93 (ddd, J = 10.1, 7.7, 2.7 Hz, 1H), 2.88 (ddd, J = 9.8, 6.2, 2.5 Hz, 1H), 2.15 – 1.96 (m, 2H). ¹³C NMR (101 MHz, CD₃OD) δ 212.0, 139.6, 129.3, 128.9, 128.7, 74.3, 73.5, 70.3, 64.1, 45.8, 13.9. Minor 7e: ¹H NMR (400 MHz, CD₃OD) δ 7.38-7.26 (m, 5H), 4.53 (s, 2H), 4.05 (dt, J = 6.3, 5.0 Hz, 1H), 3.47 (dd, J = 9.0, 5.2, 1H), 3.40 (dd, J = 9.8, 6.3, 1H), 3.05 (m, 1H), 2.81 (m, 1H), 2.15-1.96 (m, 2H). ¹³C NMR (101 MHz, CD₃OD) δ 212.3, 139.6, 129.3, 128.9, 128.7, 74.3, 73.6, 69.1, 64.3, 46.0, 13.1. ESI-TOF: calculated for [M+Na]+ C₁₃H₁₆O₃Na: 243.0997, found 243.0990.

**Scale up of aldol addition of ethanal to aldehydes**

**General procedure:**
The reaction was conducted in a 50 mL screw capped conical-bottom polypropylene tubes. To an enzyme (3 mg mL⁻¹) solution in plain water (15.7 mL), 1 M triethanolamine buffer, pH 8 (1 mL) was added. To this solution, aldehyde (80 mM, 3.2 mL of 500 mM aqueous stock solution) and ethanal (5%, 1 mL, using electrophile 2a and 100 mM, 112 µL using electrophiles 2b) were added. Total reaction volume was 20 mL. Reaction mixtures were shaken (1000 rpm) at 25 °C for 24 h. After that, the reaction was stopped with MeOH (30 mL) to precipitate the enzyme, and the mixture was filtered through activated charcoal. MeOH was evaporated and the reaction was lyophilized.
Aldol addition of 1a to S-2b (Method 1): (2S,4R,6S)-6-methyltetrahydro-2H-pyran-2,4-diol (α-anomer) (α4a) (2R,4R,6S)-6-methyltetrahydro-2H-pyran-2,4-diol (β-anomer) (β4a). These compounds were obtained as a mixture (∼ 1:1) following the procedure described above. S-3-Hydroxybutanal (S-2b) (1.6 mmol) and FSA D6H were used. The product was purified by silica gel column chromatography and eluted with a step gradient of CHCl₃:MeOH from 1:0 to 95:5, yielding 57.1 mg (27%) of α4a:β4a 1:1. [α]D₂₀ = -15.68 (c = 0.19 in MeOH). (α-anomer): ¹H NMR (400 MHz, CD₃OD) δ 4.65 (dd, J = 9.7, 2.1 Hz, 1H), 3.80 – 3.70 (m, 1H), 3.52 (ddd, J = 11.2, 6.3, 2.0 Hz, 1H), 2.09 (ddt, J = 12.0, 4.3, 2.0 Hz, 1H), 1.86 (ddt, J = 12.4, 4.2, 1.9 Hz, 1H), 1.22 (d, J = 6.2 Hz, 3H), 1.12 – 1.00 (m, 2H). ¹³C NMR (101 MHz, CD₃OD) δ 92.0, 67.9, 66.0, 41.7, 41.6, 20.2. (β-anomer): ¹H NMR (400 MHz, CD₃OD) δ 5.29 (d, J = 3.6 Hz, 1H), 4.17 – 3.98 (m, 2H), 2.02 – 1.90 (m, 3H), 1.40 (ddd, J = 12.4, 11.4, 3.6 Hz, 1H), 1.16 (d, J = 6.3 Hz, 3H). ¹³C NMR (101 MHz, CD₃OD) δ 93.9, 63.5, 62.6, 42.5, 39.2, 20.4. ESI-TOF: calculated for [M+H]+ C₆H₁₃O₃: 133.0864, found 133.0860.

Aldol addition of 1a to R-2b (Method 1): (2S,4R,6R)-6-methyltetrahydro-2H-pyran-2,4-diol (α-anomer) (α5a) (2R,4R,6R)-6-methyltetrahydro-2H-pyran-2,4-diol (β-anomer) (β5a). These compounds were obtained as a mixture of α:β anomers ≈ 4:1 following the procedure described above. R-3-Hydroxybutanal (R-2b) (1.6 mmol) and FSA D6H were used. The product was purified by silica gel column chromatography and eluted with a step gradient of CHCl₃:MeOH from 1:0 to 95:5, yielding 28.7 mg (14%) of α5a:β5a 4:1. [α]D₂₀ = -8.5 (c = 1.7 in MeOH); The ¹H and ¹³C NMR of this sample agreed with those reported in the literature.¹⁰ (α-anomer): ¹H NMR (400 MHz, CD₃OD) δ 5.02 (dd, J = 9.9, 2.2 Hz, 1H), 4.17 (t, J = 3.0 Hz, 1H), 3.99 (dqd, J = 12.6, 6.3, 2.2 Hz, 1H), 1.83 (ddt, J = 13.5, 3.1, 2.1 Hz, 1H), 1.63 – 1.55 (m, 1H), 1.43 (ddd, J = 13.3, 9.9, 3.0 Hz, 1H), 1.38 – 1.30 (m, 1H), 1.15 (d, J = 6.3 Hz, 3H). ¹³C NMR (101 MHz, CD₃OD) δ 91.7, 66.4, 64.8, 39.0, 39.0, 20.3. (β-anomer): ¹H NMR (400 MHz, CD₃OD) δ 5.14 (t, J = 3.0 Hz, 1H), 4.36 (ddd, J = 10.8, 6.5, 2.8 Hz, 1H), 4.11 – 4.08 (m, 1H), 1.77 (td, J = 3.4, 2.9, 1.4 Hz, 1H), 1.76 – 1.69 (m, 1H), 1.53 – 1.48 (m, 1H), 1.15 (d, J = 6.3 Hz, 3H). ¹³C NMR (101 MHz, CD₃OD) δ 92.1, 64.2, 59.5, 39.3, 35.5, 20.1. ESI-TOF: calculated for [M+H]+ C₆H₁₃O₃: 133.0864, found 133.0865.

Aldol addition of 1a to 2a (Method 1): (2S,4R)-tetrahydro-2H-pyran-2,4-diol (α-anomer) (α3a) and (2R,4R)-tetrahydro-2H-pyran-2,4-diol (β-anomer) (β3a): These compounds were obtained as a mixture (∼ 1:1) following the procedure described
above. 3-Hydroxypropanal (2a) (1.6 mmol) and FSA D6L/A165G were used. The product was purified by silica gel column chromatography and eluted with a step gradient of CHCl₃:MeOH from 1:0 to 95:5, yielding 41.5 mg (22%) of 3a (α:β 1:1). [α]₀^20= −2.93 (c = 0.62 in MeOH). (α-anomer): ^1H NMR (400 MHz, CD₃OD) δ 5.15 (dd, J = 4.4, 3.1 Hz, 1H), 4.06 (dd, J = 8.4, 4.4 Hz, 1H), 3.87 (dd, J = 8.8, 3.2 Hz, 1H), 3.72 (dd, J = 5.6, 4.3 Hz, 1H), 1.85 – 1.76 (m, 2H), 1.58 (ddd, J = 12.9, 8.5, 3.1 Hz, 1H), 1.49 (ddd, J = 12.9, 8.6, 4.1 Hz, 1H). ^13C NMR (101 MHz, CD₃OD) δ 91.9, 63.1, 58.6, 39.6, 33.9. (β-anomer): ^1H NMR (400 MHz, CD₃OD) δ 4.61 (dd, J = 9.0, 2.2 Hz, 1H), 3.98 – 3.93 (m, 1H), 3.72 (m, 1H), 3.40 (td, J = 11.9, 2.5 Hz, 1H), 2.07 (ddt, J = 12.1, 4.2, 2.0 Hz, 1H), 1.77 (m, 1H), 1.39 (m, 1H), 1.29 (m, 1H). ^13C NMR (101 MHz, CD₃OD) δ 94.2, 66.0, 61.1, 41.8, 34.1. ESI-TOF: calculated for [M+2Na]^+ C₅H₁₀O₃Na₂: 165.0503, found 165.0507.

Aldol addition of 1a to 2a catalyzed by DERA_Ecoli (Method 1): Synthesis of (2S,4R)-tetrahydro-2H-pyran-2,4-diol (α-anomer) (α3a) and (2R,4R)-tetrahydro-2H-pyran-2,4-diol (β-anomer) (β3a) using 2-deoxyribose-5-phosphate aldolase from E. coli (DERA_Ecoli) as catalyst. The anomeric mixture was obtained following the procedure described above. 3-Hydroxypropanal (2a) (1.6 mmol), ethanal (100 mM, 112 µL) and DERA_Ecoli (3 mg pure enzyme mL⁻¹) were used. The product was purified by silica gel column chromatography and eluted with a step gradient of CHCl₃:MeOH from 1:0 to 95:5, yielding 167.5 mg (82%) of 3a. [α]₀^20= −28.6 (c = 1 in MeOH) (Lit.: ^11 [α]₀^20= −19.0 (c = 0.5, CH₃OH). The ^1H and ^13C NMR of this sample agreed with those reported in the literature. ^1H NMR (400 MHz, CD₃OD) δ 5.15 (dd, J = 4.4, 3.1 Hz, 1H), 4.07 (tt, J = 8.3, 8.3, 4.0, 4.0 Hz, 1H), 3.90 – 3.84 (m, 1H), 3.72 – 3.66 (m, 1H), 1.80 (m, 2H), 1.63 – 1.54 (m, 1H), 1.49 (ddd, J = 13.0, 8.7, 4.1 Hz, 1H). ^13C NMR (101 MHz, CD₃OD) ^13C NMR (101 MHz, CD₃OD) δ 95.8, 67.0, 62.6, 43.6, 37.9. (β-Anomer) ^1H NMR (400 MHz, CD₃OD) δ 4.61 (dd, J = 9.0, 2.2 Hz, 1H), 3.98 – 3.90 (m, 1H), 3.76 – 3.70 (m, 1H), 3.40 (td, J = 11.9, 11.9, 2.4 Hz, 1H), 2.07 (ddt, J = 12.2, 4.3, 2.0, 2.0 Hz, 1H), 1.81 – 1.75 (m, 1H), 1.45 – 1.35 (m, 1H), 1.29 (ddd, J = 12.2, 10.7, 9.0 Hz, 1H). ^13C NMR (101 MHz, CD₃OD) δ 98.2, 70.0, 65.0, 45.7, 38.0.
Figure S4. NMR spectra (D$_2$O) of $\alpha$4a and $\beta$4a mixture: a) $^1$H; b) $^{13}$C; c) 2D $^1$H-$^1$H COSY; d) HSQC; e) HMBC and f) selective 1D-NOESY.
Figure S5. NMR spectra (D$_2$O) of 4b: a) $^1$H; b) $^{13}$C; c) 2D $^1$H-$^1$H COSY and d) HSQC
Figure S6. NMR spectra (D$_2$O) of 4e and its acyclic specie mixture: a) $^1$H; b) $^{13}$C; c) 2D $^1$H-$^1$H COSY and d) HSQC.
Figure S7. NMR spectra (D$_2$O) of 4f: a) $^1$H; b) $^{13}$C; c) 2D $^1$H-$^1$H COSY; d) HSQC; e) HMBC and f) 1D-NOESY.
Figure S8. NMR spectra (D$_2$O) of $\alpha$5a and $\beta$5a mixture: a) $^1$H; b) $^{13}$C; c) 2D $^1$H-$^1$H COSY and d) HSQC.
Figure S9. NMR spectra (D$_2$O) of pure $\alpha$5a and $\beta$5a mixture: a) $^1$H and b) $^{13}$C.
Figure S10. NMR spectra (D$_2$O) of 5b: a) $^1$H; b) 2D $^1$H-$^1$H COSY, c) HSQC; d) TOCSY; e) HMBC and f) selective 1D-NOESY.
Figure S11. NMR spectra (D$_2$O) of $\alpha$3a and $\beta$3a mixture: a) $^1$H; b) $^{13}$C; c) 2D $^1$H-$^1$H COSY and d) HSQC.
Figure S12. NMR spectra of (D₂O) of α3a and β3a mixture obtained via DERA from E. coli catalysis: a) ¹H and b) ¹³C.
Figure S13. NMR spectra (D$_2$O) of $\alpha$3b and $\beta$3b mixture in equilibrium with the corresponding acyclic product: a) $^1$H and $^{13}$C b) 2D $^1$H-$^1$H COSY and HSQC, c) $^1$H, $^{13}$C, 2D $^1$H-$^1$H COSY and HSQC recorded after two months to allow the anomers and acyclic product to equilibrate.
Figure S14. NMR spectra (D$_2$O) of the racemic mixture of rac-3b catalyzed obtained via pyrrolidine catalysis: a) $^1$H and b) $^{13}$C.
Figure S15. NMR spectra (D$_2$O) of methyl glycoside derivative of the racemic mixture of rac-3b: a) $^1$H; b) HSQC c) 2D NOESY.
Figure S16. GC chiral analysis of A) a racemic sample of methyl glycoside derivative of \textit{rac}-3b and B) a sample obtained by FSA D6H. Column: (2,3-di-\textit{O}-methyl-6-\textit{O}-\textit{tert}-butyl dimethylsilyl beta cyclodextrin, Program: 80°C, 4 K min\(^{-1}\), 230°C Inj/Det 230°C.
Figure S17. NMR spectra (CD$_3$OH) of 6b: a) $^1$H; b) $^{13}$C; c) 2D $^1$H-$^1$H COSY and d) HSQC.
Figure S18. Chiral HPLC analysis of A) a sample of rac-6b and B) a sample of S-6b obtained by FSA D6Q. Column: CHIRALPAK® ID (4.6 x 250 mm), isocratic elution 90:10 hexane:isopropanol, 1 mL min⁻¹, detection 209 nm. Sample: 10 μL, 2 mg mL⁻¹.
Figure S19. NMR spectra (D$_2$O) of unprotected derivative of S-6b: a) $^1$H; b) $^{13}$C; c) 2D $^1$H-$^1$H COSY and d) HSQC
Figure S20. NMR spectra (D$_2$O) of 6f and 7f mixture: a) $^1$H; b) $^{13}$C; c) 2D $^1$H-$^1$H COSY and d) HSQC.
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