Supporting Information

Chemoenzymatic Hydroxymethylation of Carboxylic Acids by Tandem Stereodivergent Biocatalytic Aldol reaction and Chemical Decarboxylation

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Content

Materials	2
General methods.....	2
Molecular biology techniques and protein expression and purification	5
Mutagenesis techniques	7
Activity determination of KPHMT-M ²⁺	14
Synthesis of starting substrates.	15
NMR spectra of ethyl 2-oxoesters and 2-oxoacids	20
Screening experiments and determination initial reaction rate.....	48
Reaction monitoring for the synthesis of products S-4a-h	50
Reaction monitoring for the synthesis of products R-4a-j	72
Enzymatic synthesis of 2-(4'-bromophenyl)-2-oxoethyl ester of 4-hydroxy-2-oxocarboxylic acid derivatives.....	92
NMR spectra of 2-(4'-bromophenyl)-2-oxoethyl ester derivatives (4a-k)	104
Synthesis of benzyl and 4-nitrobenzyl ester derivatives	126
NMR spectra of benzyl and 4-NO ₂ benzyl ester derivatives	130
Synthesis of derivatives for absolute stereochemistry determination	136
NMR spectra of carboxylic acid methyl esters and carboxylic acid derivatives.....	140
Synthesis of racemic mixtures of products 4b-k	150
Synthesis of racemic mixtures of products rac-5a and rac-6a	153

Determination of absolute configuration	153
X-ray structure determinations.....	154
Chiral HPLC analysis.....	161
Computational Methods.	177
References.....	179

Materials

Synthetic oligonucleotides were purchased from Eurofins Genomics. Antibiotics, isopropyl- β -D-1-thiogalactoside (IPTG) and 3-morpholinopropane-1-sulfonic acid (MOPS) were from Carl Roth. Culture media components for *E. coli* were from Pronadisa (Madrid, Spain). Molecular biology reagents were from Thermo Scientific. High-density IDA-Agarose 6BCL nickel charged was from GE Healthcare Life Science. Bacterial strains, oligonucleotides and plasmids used in this study are listed in Table S1. Methanal, dimethylformamide (DMF), 2,4'-dibromoacetophenone, benzyl bromide, 4-nitrobenzyl bromide, sodium 2-oxobutanoate, sodium 2-oxopentanoate, sodium 4-methyl-2-oxopentanoate, sodium (*S*)-4-hydroxy-3-methyl-2-oxobutanoate and sodium (*R*)-4-hydroxy-3-methyl-2-oxobutanoate were from Sigma-Aldrich. Other reagents were from Sigma-Aldrich. Substrates **1d-k** used in this study were synthesized in our lab. Water for analytical HPLC and for the preparation of buffers and other assay solutions was obtained from an Arium pro ultrapure water purification system (SartoriusStedim Biotech). All other solvents used were of analytical grade.

General methods

Thin layer chromatography was performed using precoated silica gel plates with or without fluorescent indicator UV₂₅₄ (Macherey-Nagel GmbH & Co. KG, Kieselgel 60). Column chromatography was performed in a glass column (AFORA, 5880/2, 47x4.5) packed with silica gel (100 g, 35-70 μ m, 200-500 mesh, Merck). Stains were detected on TLC plates using UV₂₅₄ fluorescence or developed with ceric ammonium molybdate (CAM) stain ($\text{Ce}(\text{SO}_4)_2$ (10 g L⁻¹) and $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ (50 g L⁻¹) in H₂SO₄ 2 M).

Specific rotation: Specific rotation values were measured with a Perkin Elmer Model 341 (Überlingen, Germany).

Melting points. Melting points were determined using a BUCHI fully automatic melting point B-545, heating rate: 0.5 °C/min between 30 °C to 150 °C.

NMR analysis. Routine, ^1H (400 MHz) and ^{13}C (101 MHz) NMR spectra of compounds were recorded with a Varian Mercury-400 spectrometer. Full characterization of the described compounds was performed using typical gradient-enhanced 2D experiments: COSY and HSQC recorded under routine conditions.

HPLC analysis. HPLC analyses were performed on a RP-HPLC XBridge C18, 5 μm , 4.6×250 mm column (Waters). The solvent system used was: solvent (A): 0.1% (v/v) trifluoroacetic acid (TFA) in H_2O and solvent (B): 0.095% (v/v) TFA in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ 4:1, flow rate 1 mL min^{-1} , detection at 215 nm and column temperature at $30\text{ }^\circ\text{C}$. The amount of products and substrates were quantified from the peak areas using an external standard methodology.

HPLC monitoring of 2-oxoacids (**1**), methanal (**2**) and aldol adducts (**3**) during the aldol addition reactions was carried out as follows: Samples from the reaction mixture (10 μL) were withdrawn and diluted with plain water to adjust the concentration between the limits of linearity (i.e., from 3 to 100 mM). Then, an aliquot sample (10 μL) of the diluted solution was mixed with a solution of *O*-benzylhydroxylamine hydrochloride (50 μL of a 0.13 mM stock solution in pyridine:methanol:water 33:15:2). After incubation at $25\text{ }^\circ\text{C}$ for 5 min, samples were diluted with methanol (500 μL), centrifuged (20 000 g, 5 min) and analyzed by HPLC. Elution conditions for $\text{R}^1 = \text{a-j}$, gradient from 10 to 100% B over 30 min and for $\text{R}^1 = \text{k}$, gradient from 10 to 100% B over 45 min.

Scheme S1. Pre-column derivatization reaction of carbonyl compounds with *O*-benzylhydroxylamine.

The HPLC reaction monitoring for the synthesis of 2-(4-bromophenyl)-2-oxoethyl esters (**4**) was carried out as follows: samples (10 μL) were withdrawn from the reaction mixture, diluted with methanol (500 μL) and after centrifugation (20 000 g, 5 min)

analyzed by HPLC. Elution conditions for $R^1 = \mathbf{a-j}$, gradient from 10 to 100% B over 30 min. and for $R^1 = \mathbf{k}$, gradient from 10 to 100% B over 45 min.

Chiral HPLC analysis. Enantiomeric excesses (*ee*) were determined using chiral HPLC analysis on 46 x 250 mm columns, 5 μm particle size and 254 nm or 209 nm UV detection. Column type, specific elution conditions and flow rates are described for each compound.

Molecular biology techniques and protein expression and purification

Table S1. Strain, plasmids and oligonucleotides used in this study. Mutagenized codons are highlighted in italic and bold.

Plasmids	Relevant genetic characteristics
pQE60 ^a	
pQE60 <i>panB</i> ^b	<i>panB</i> gene (795 bp) from <i>E. coli</i> K-12 (NCBI data base accession number NC_000913.3) cloned in pQE60 (NcoI and BglII)
pQE60 <i>panB</i> L42A	Plasmid pQE60 <i>panB</i> as template (QuickChange site-directed mutagenesis)
pQE60 <i>panB</i> I212A	<i>panB</i> gene as template (megaprimer site-directed mutagenesis)
pQE60 <i>panB</i> L42A/I212A	<i>panB</i> L42A gene as template (megaprimer site-directed mutagenesis)
pQE60 <i>panB</i> I202A	<i>panB</i> gene as template (megaprimer site-directed mutagenesis)
pQE40 <i>malE</i> ^c - <i>rmhA</i> ^d	¹
pQE40 <i>malE</i> - <i>rmhA</i> W23V	²
pQE40 <i>malE</i> - <i>rmhA</i> W23V/L216A	²
Strains	Relevant genotype
<i>E. coli</i> Nova Blue (used for plasmid preparation)	<i>endA1</i> , <i>hsdR17</i> (rB ⁺ , mB ⁺), <i>supE44</i> , <i>thi1</i> , <i>recA1</i> , <i>gyrA96</i> , <i>relA1</i> , <i>lac F'</i> [proA ⁺ B ⁺ , <i>lacI</i> ^q ZΔM15::Tn10] (<i>Tet</i> ^R).
<i>E. coli</i> M15 [pREP4] (used for protein expression)	<i>nal</i> ^s , <i>str</i> ^s , <i>rif</i> ^s , <i>thi</i> ⁻ , <i>lac</i> ⁻ , <i>ara</i> ⁺ , <i>gal</i> ⁺ , <i>mtl</i> ⁻ , F ⁻ , <i>recA</i> ⁺ , <i>uvr</i> ⁺ , <i>lon</i> ⁺ .
Oligonucleotides	Sequences
L42A	CGT(L)→GCG(A) Forward: 5'ACGTCATGG CG GTGGGCGATTCGCTGGG 3' Reverse: 5'ATCGCCCAC CG CCATGACGTTAAGCCCTTC 3'
I212A	ATC(I)→GCG(A) Forward: 5'CTGACGGGCAG CG CTCGTGATGCAC 3'
I202A	ATT(I)→GCG(A) Forward: 5'ATCCCGGTTATTGG CG CGGGCGCAGGCA 3'
F1	Forward: 5'ATGAAACCGACCACC 3'
F2	Forward: 5'ATCGATTAACCATGGACATGAAACCGACCACC 3'
R	Forward: 5'GCAGGTACAAGATCTATGGAAACTGTGTTC 3'

^aQIAexpressionist, A. (2002). A handbook for high-level expression and purification of 6xhis-tagged proteins. *Qiagen. P1-125*. ^b*panB* gene codes for 3-methyl-2-oxobutanoate hydroxymethyltransferase (KPHMT, EC 2.1.2.11). ^c*malE* gene codes for maltose binding protein (MBP). This version of the gene does not encode a signal sequence, so fusion protein remains in the cytosol. ^d*rmhA* gene codes for 2-keto-3-deoxy-L-rhamnonate aldolase (YfaU, EC 4.1.2.53)

The plasmid containing the gene for expression of MBP-YfaU and its variants was constructed in our lab as described in a previous work.¹

Cloning of 3-methyl-2-oxobutanoate hydroxymethyltransferase gene (*panB*) from *E. coli* K-12 in pQE60 plasmid. The gene *panB* from *E. coli* K-12 (NCBI data base accession number NC 000913.3) was amplified by PCR from genomic DNA and inserted into pQE60 using FastDigest NcoI and BglII (Thermo Scientific) as restriction enzymes with the primers: forward: 5'-ATCGATTAACCATGGACATGAAACCGACCACC-3'; reverse and complementary: 5'-GCAGGTACAAGATCTATGGAAACTGTGTTC-3'. **A)** Bases sequence of the *panB* gene insert in pQE60 and **B)** and amino acid sequence of protein construct. Start methionine incorporated by open reading frame in pQE60 (black), 3-methyl-2-oxobutanoate hydroxymethyltransferase (KPHMT, blue) and 6x His tag (red).

A

ATGGACATGAAACCGACCACCATCTCCTTACTGCAGAAGTACAAACAGGAAAAAA
ACGTTTCGCGACCATCACCGCTTATGACTATAGCTTCGCCAAACTCTTTGCTGATG
AAGGGCTTAACGTCATGCTGGTGGGCGATTTCGCTGGGCATGACGGTTCAGGGGCAC
GACTCCACCCTGCCAGTTACCGTTGCCGATATCGCCTACCACACTGCCGCCGTACG
TCGCGGCGCACCAAACCTGCCTGCTGCTGGCTGACCTGCCGTTTATGGCGTATGCCA
CGCCGGAACAAGCCTTCGAAAACGCCGCAACGGTTATGCGTGCCGGTGCTAACATG
GTCAAAATTGAAGGCGGTGAGTGGCTGGTAGAAACCGTACAAATGCTGACCGAACG
TGCCGTTCTGTATGTGGTCACTTAGGTTTAACACCACAGTCAGTGAATATTTTCG
GTGGCTACAAAGTTCAGGGGCGCGGCGATGAAGCGGGCGATCAACTGCTCAGCGAT
GCATTAGCCTTAGAAGCTGCTGGGGCACAGCTGCTGGTGCTGGAATGCGTGCCGGT
TGAAGTGGCAAAACGTATTACCGAAGCACTGGCGATCCCGGTTATTGGCATTGGCG
CAGGCAACGTCACCTGACGGGCAGATCCTCGTGATGCACGACGCCTTTGGTATTACC
GGCGGTCACATTCCTAAATTCGCTAAAAATTTCTCGCCGAAACGGGCGACATCCG
CGCGGCTGTGCGGCAGTATATGGCTGAAGTGGAGTCCGGCGTTTATCCGGGCGAAG
AACACAGTTTCCATAGATCTCATCACCATCACCATCAC

B

MDMKPTTISLLQKYKQEKRFATITAYDYSFAKLFADEGLNVMLVGDSLGMTVQGH
DSTLPVTVADIAYHTAAVRRGAPNCLLLADLPFMAYATPEQAFENAATVMRAGANM
VKIEGGEWLTVQMLTERAVPVCGLGLTPQSVNIFGGYKVQGRGDEAGDQLLSD
ALALEAAGAQLLVLECVVELAKRITEALAIPIVIGIGAGNVTGQILVMHDAFGIT
GGHIPKFAKNFLAETGDIRAAVRQYMAEVESGVYPGEEHSFHRSHHHHHH

Mutagenesis techniques

a) QuickChange site-directed mutagenesis

The *panB* gene mutation L42A was introduced with the QuickChange site-directed mutagenesis strategies using the plasmid pQE60 *panB* as template and performed according to the manufacturer's protocols (QuickChange®, Stratagene; QuikChange® Site-Directed Mutagenesis Kit. Instruction manual Catalog #200518 and #200519 Revision A. *Stratagene. p 1-20*), with some modifications in PCR procedure (Table S2). Chemically competent *E. coli* Nova Blue strain cells (Merckmillipore) were transformed with DNA preparation (without any purification step). The plasmid DNA was isolated with the GeneJET Plasmid Miniprep Kit (Thermo Scientific) and the expected mutations were confirmed by DNA sequencing.

Table S2. QuickChange site-directed mutagenesis PCR protocol.

Components	V/ μ L	Final concentration (50 μ L total volume)	PCR cycles		
Water, nuclease free	35.5		Step	Temperature/ $^{\circ}$ C	Time/min
5X Green Buffer ^a	10		Initial denaturation	98	3
dNTP mix (10 mM each)	1	0.2 mM each	35 PCR cycles	98	0.5
Forward primer (125 ng μ L ⁻¹)	1	2.5 ng μ L ⁻¹		55	1
Reverse primer (125 ng μ L ⁻¹)	1	2.5 ng μ L ⁻¹		72	12
Template DNA (10 ng μ L ⁻¹)	1	0.2 ng μ L ⁻¹	Final extension	72	30
DNA Polymerase (2 U μ L ⁻¹) ^b	0.5	0.02 U μ L ⁻¹		4	hold

^a5X Phusion Green HF Buffer (Thermo Scientific). ^bPhusion Green High-Fidelity DNA Polymerase (Thermo Scientific).

b) Megaprimer site-directed mutagenesis

The *panB* gene mutations I212A and I202A were introduced with the Megaprimer site-directed mutagenesis strategies (Figure S1) with PCR procedure described in (Table S3).

Figure S1. Schematic illustration of the megaprimer method for site-directed mutagenesis. The first-round PCR (PCR 1) was performed using external forward (F1) and reverse primer (R) with BglIII restriction site to amplify the *panB* gene. The second-round PCR (PCR 2) was performed using the mutagenic primer (M) and external primer (R) to amplify the mutagenic “megaprimer” fragment from the DNA template. The third-round PCR (PCR 3) was performed using external primer (F2) with NcoI restriction site and "megaprimer" (purified product of second-round PCR).

Table S3. Megaprimer site-directed mutagenesis PCR protocol. a) PCR 1, b) PCR 2 and c) PCR 3.

a) PCR 1

Components	V/ μ L	Final concentration (50 μ L total volume)	PCR cycles		
			Step	Temperature/ $^{\circ}$ C	Time/sec
Water, nuclease free	35.5		Initial denaturation	98	30
5X Green Buffer ^a	10				
dNTP mix (10 mM each)	1	0.2 mM each	35 PCR cycles	98	10
F1, forward primer (25 μ M)	1	0.5 μ M		55	10
R, reverse primer (25 μ M)	1	0.5 μ M		72	16
Template DNA (1 ng μ L ⁻¹)	1	0.02 ng μ L ⁻¹	Final extension	72	5
DNA Polymerase (2 U μ L ⁻¹) ^b	0.5	0.02 U μ L ⁻¹		4	hold

b) PCR 2

Components	V/ μ L	Final concentration (50 μ L total volume)	PCR cycles		
			Step	Temperature/ $^{\circ}$ C	Time/sec
Water, nuclease free	35.5		Initial denaturation	98	30
5X Green Buffer ^a	10				
dNTP mix (10 mM each)	1	0.2 mM each	35 PCR cycles	98	10
M, mutagenic primer (25 μ M)	1	0.5 μ M		55	10
R, reverse primer (25 μ M)	1	0.5 μ M		72	16
Template DNA (1 ng μ L ⁻¹)	1	0.02 ng μ L ⁻¹	Final extension	72	5
DNA Polymerase (2 U μ L ⁻¹) ^b	0.5	0.02 U μ L ⁻¹		4	hold

c) PCR 3

Components	V/ μ L	Final concentration (50 μ L total volume)	PCR cycles		
Water, nuclease free	Up to 50		Step	Temperature/ $^{\circ}$ C	Time/sec
5X Green Buffer ^a	10		Initial denaturation	98	30
dNTP mix (10 mM each)	1	0.2 mM each	35 PCR cycles	98	10
F2, forward primer (25 μ M)	1	0.5 μ M		55	10
Megaprimer (product of PCR2)	- ^b	Up to 0.5 μ M		72	16
Template DNA (18 pg μ L ⁻¹)	1	0.36 pg μ L ⁻¹	Final extension	72	5
DNA Polymerase (2 U μ L ⁻¹) ^c	0.5	0.02 U μ L ⁻¹		4	hold

^a5X Phusion Green HF Buffer (Thermo Scientific). ^bThe concentration of Megaprimer depends on the efficiency of the PCR2. Megaprimer size: 173 bp (I212A) and 207 bp (I202A). ^cPhusion Green High-Fidelity DNA Polymerase (Thermo Scientific).

Product of PCR3 and pQE60 were digested with FastDigest NcoI and BglII (Thermo Scientific) and fragments were purified from agarose gel according to the manufacturer's protocol (Thermo Scientific, GeneJET Gel Extraction and DNA Cleanup Micro Kit, instruction manual Catalog #K0831 and #K0832). Ligation of DNA fragments was performed using T4 DNA Ligase (Thermo Scientific) according to the manufacturer's protocol. Chemically competent *E. coli* Nova Blue (Merckmillipore) strain cells were transformed with ligation reaction. The plasmid DNA was isolated with the GeneJET Plasmid Miniprep Kit (Thermo Scientific) and the expected mutations in the gene sequence were confirmed by DNA sequencing.

Protein production and purification. Competent *E. coli* M-15[pREP-4] strain cells from QIAGEN, were transformed with plasmid pQE60 *panB* and grown in LB medium with ampicillin (100 μ g mL⁻¹) and kanamycin (25 μ g mL⁻¹) at 37 $^{\circ}$ C, on a rotary shaker at 200 rpm. A final optical density at 600 nm (OD₆₀₀) of 3–4 was usually achieved. An aliquot of the pre-culture (20 mL) was transferred into a baffled shaker flask (2 L) containing LB medium (1000 mL) plus ampicillin (100 μ g mL⁻¹), kanamycin (25 μ g mL⁻¹) and antifoam SE-15 (0.02% v/v), and incubated at 37 $^{\circ}$ C with shaking at 200

rpm. During the middle exponential phase growth ($DO_{600} \approx 0.5-0.8$), the temperature was decreased to 30 °C to minimize inclusion bodies formation and then, isopropyl- β -D-1-thiogalactopyranoside (IPTG; 1 mM final concentration) was added. After 12-16 h, cells from the induced-culture broths (5 L, 20-25 g of cells) were centrifuged (2500 g for 45 min at 4 °C). The pellet was re-suspended in starting buffer (400 mL) consisting of 50 mM sodium phosphate buffer pH 8.0, containing NaCl (300 mM), imidazole (10 mM) and glycerol (10%). Cells were lysed using a cell disrupter (Constant Systems) and cellular debris were removed by centrifugation at 35 000 g for 45 min at 4 °C. The clear supernatant was applied to a cooled HR 16/40 column (GE Healthcare) packed with Ni SepharoseTM High Performance (50 mL bed volume, GE Healthcare) and washed with starting buffer (400 mL) at 3 mL min⁻¹. The protein was eluted with gradient from 10 mM to 500 mM of imidazole at 5 mL min⁻¹. Fractions containing the recombinant protein were pooled and dialyzed against 20 mM MOPS buffer pH 7.0 containing NaCl (50 mM) and glycerol (50%) at 4 °C (3x1 L 24 h each). The dialyzed solution obtained (60-100 mL) was stored at – 20 °C (Figure S2). Protein concentration was determined using Pierce® 660 nm Protein Assay Reagent (Thermo Scientific) in 96 wells plate according to the manufacturer's protocols. The glycerol in dialysis buffer minimizes protein precipitation after purification.

Wild-type MBP-YfaU and variants were expressed and purified as described in previous publications.¹⁻³

Figure S2. Analysis of KPHMT purification steps by Coomassie Blue-stained SDS-PAGE. Wild-type (**A**), L42A variant (**B**), I212A variant (**C**), L42A/I212A variant (**D**) and I202A variant (**E**). The gel was loaded with samples from supernatant of lysis (lane 1), pellet after lysis (lane 2), flow-through fraction (lane 3), wash fraction (lane 4) and elution and dialyzed fraction (lane 5). The molecular masses of the proteins in the Standard Molecular Weight Marker (ST) are as indicated. The predicted molecular mass of KPHMT is 29.5 kDa.

Table S4. Yield of KPHMT variants after purification

KPHMT variant	Yield ^a
Wild-type	172
L42A	102
I212A	148
L42A/I212A	156
I202A	170
^a mg protein L ⁻¹ medium culture	

Electrospray mass spectrometry of proteins. Glycerinated proteins were diluted in water/formic acid (0.1% v/v) (1 mg protein mL⁻¹, final concentration in the solution) and glycerol and salts were removed with PD MiniTrap G-10 of (GE Healthcare). The samples were eluted in water/formic acid (0.1% v/v, 0.5 mL) and injected (5 µL) to an Acquity UPLC BEH C4, 1.7 µm, 2.1 x 50 mm column connected to a mass spectrometer ESI-TOF instrument (LCT PremierWaters, Milford, MA, USA), equipped with a 4 GHz time-to-digital converter (TDC) with a dual ESI source (LockSpray). The second sprayer provided the lock mass calibration with leucine enkephalin (m/z 556.2771). The ESI-TOF was operated in the W-optics mode, thus providing a mass resolution of at least 10 000 full width at half maximum (FWHM). The acquisition time per spectrum was set to 0.2 s, and the mass range was from 500 to 1800 Da. Data were acquired using a cone voltage of 50 V, capillary voltage of 3000 V, desolvation temperature of 35 °C, and source temperature of 100 °C. The desolvation gas flow was set at 400 L h⁻¹ and the cone gas flow was set at 30 L h⁻¹. The solvent system used for the elution was: solvent (A): 0.1% formic acid in CH₃CN/H₂O (70/30, v/v) and solvent (B): 0.1% (v/v) formic acid in H₂O, gradient elution from 28% A to 100% A in 25 min, 100% A for 2 min and 28% A for 18 min, flow rate 0.2 mL min⁻¹. MassLynx 4.1 software (Waters. Milford, MA, USA) was used for data acquisition and processing. Magtran software⁴, kindly provided by Dr. Zhongqi Zhang (Amgen, Inc.; Thousand Oaks, CA), was used for molecular weight deconvolution from ESI-MS spectra of proteins.

Table S5: Molecular weight determination by ESI-MS of KPHMT variants.

Enzyme variant	Molecular weight calculated/Da ^a	Molecular weight measured/Da	Difference/Da
Wild-type	29549.83	29549,68	0.15
L42A	29507.75	29509,36	1.61
I212A	29507.75	29505,78	1.97
L42A/I212A	29465.67	29463,42	2.25
I202A	29507.75	29508,22	0.47

^a Molecular weight was calculated using ProtParam (<http://expasy.org/tools/protparam.html>).

Activity determination of KPHMT-M²⁺

The activity was determined using the aldol addition of sodium 2-oxobutanoate (**1a**) to methanal (**2**) by a discontinuous assay. One unit of activity was defined as the amount of KPHMT, which catalyzes the formation of 1 μmol of **R-3a** per min at 25 °C in 20 mM MOPS buffer pH 7.0, containing 1 mM of different cations (Ni^{2+} , Co^{2+} , Mg^{2+} , Zn^{2+} , Mn^{2+} and Cu^{2+}). The assay procedure was as follows: To an equimolar solution mixture of **1a** and **2** (150 μL of a 133 mM solution in 20 mM MOPS pH 7.0 containing MCl_2 (1.3 mM)), a solution of the enzyme (50 μL , of stock solution between 10.7–0.17 mg protein mL^{-1} in 20 mM MOPS buffer pH 7.0) was added. The total reaction volume was 0.2 mL. Reaction monitoring was as follows: at 30 min, samples (10 μL) were analyzed by HPLC as described above. The results are presented in Figure S3 and Table S6.

Figure S3: Activity of KPHMT-M²⁺ with different metal cofactors. One unit of activity was defined as the amount of KPHMT that catalyzes the formation of 1 μmol of sodium (*R*)-4-hydroxy-3-methyl-2-oxobutanoate per min at 25 °C in 20 mM MOPS buffer pH 7.0. Activities were determined in duplicate and the error bars are the standard error of the mean.

Table S6: Relative activity of KPHMT-M²⁺ with different metal cofactors.

Metal cofactor (1 mM)	Relative activity ^a
Mg^{2+}	1
Co^{2+}	1.8 ± 0.1
Ni^{2+}	1.5 ± 0.1
Zn^{2+}	0.9 ± 0.2
Mn^{2+}	0.8 ± 0.1
Cu^{2+}	- ^b
EDTA	- ^b
Without M^{2+} added	0.10 ± 0.01

^aRelative to Mg^{2+} (natural metal cofactor).

^bActivity not detected. Activities were determined in duplicate ± standard error of the mean (SE).

Synthesis of starting substrates.

Method A

2-oxohexanoic acid (1d)

Typical procedure: A dried three-necked round bottomed flask equipped with a reflux condenser was charged with magnesium (2.13 g, 87.58 mmol, 1.2 eq) under N_2 atmosphere. A small crystal of iodine was added and heated until the iodine was sublimated. After cooling down at room temperature, a solution of 1-bromobutane (10.0 g, 72.98 mmol, 1.0 eq) in THF (100 mL) was added drop-wise over the course of 30 minutes, so that the mixture was continuously boiling. The reaction was refluxed for 2 h, cooled down to room temperature and added drop-wise to a solution of diethyl oxalate (9.06 g, 62.01 mmol, 0.85 eq) in THF (50 mL) at $-78\text{ }^\circ\text{C}$. After 4 h, the reaction was quenched by the addition in a saturated ammonium chloride solution (400 mL) and extracted with Et_2O (3 x 200 mL). The organic phases were combined, washed with brine (100 mL) dried over anhydrous magnesium sulfate, filtered and the solvent removed under vacuum. The product was loaded onto a silica column chromatography and was eluted with a step gradient of hexane: Et_2O : 100:0, 200 mL, 98:2, 400 mL, 95:5, 500 mL and 90:10, 500 mL. Pure fractions were pooled and the solvent removed under vacuum affording the 2-oxoester intermediate as a yellow oil (8.75 g, 89%). The spectral properties of this product agreed with those reported in the literature.⁵ ^1H NMR (400 MHz, CDCl_3) δ 4.29 (q, $J = 2 \times 7.2, 7.1$ Hz, 2H), 2.80 (t, $J = 2 \times 7.3$ Hz, 2H), 1.59 (p, $J = 2 \times 7.6, 2 \times 7.5$ Hz, 2H), 1.41 – 1.26 (m, 5H), 0.90 (t, $J = 2 \times 7.4$ Hz, 3H). ^{13}C NMR (101 MHz, CDCl_3) δ 194.8, 161.3, 62.3, 38.9, 25.0, 22.1, 14.0, 13.7.

The 2-oxoester intermediate (8.75 g, 55.31 mmol) was dissolved in 2-methyltetrahydrofuran (2-MeTHF, 55 mL). Then, 1.0 M sodium phosphate buffer pH 7.0 (32 mL) and Novozym 435 (0.64 g, 20 mg mL⁻¹ final concentration in aqueous phase) were added. The reaction, in a closed vessel (250 mL), was conducted at 25 °C and orbital shaker. After 24 h, the starting material was not detected on TLC. The reaction was then filtered and NaHCO₃ 5% (300 mL) was added. The mixture was washed with Et₂O (3 x 100 mL). Then, the aqueous solution was acidified at pH = 1.0 (HCl 37%) and the product was extracted with Et₂O (3 x 100 mL). The organic phases were combined, dried over anhydrous magnesium sulfate, filtered and the solvent removed under vacuum affording the titled compound (**1d**) as a colorless oil that crystallized as a yellow solid after overnight storage at – 20 °C (6.04 g, 84%, isolated yield over two steps: 75%). This material was used without any further purification. The spectral properties of this product agreed with those reported in the literature.⁶ ¹H NMR (400 MHz, CDCl₃) δ 2.93 (t, *J* = 2x7.3 Hz, 2H), 1.63 (p, *J* = 2x7.5, 2x7.4 Hz, 2H), 1.36 (dq, *J* = 14.6, 2x7.4, 7.2 Hz, 2H), 0.92 (t, *J* = 2x7.4 Hz, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 196.0, 159.3, 36.9, 25.1, 22.0, 13.7.

2-Oxoheptanoic acid (**1e**)

The titled compound (**1e**) was obtained as a colorless oil that crystallized as a yellow solid after overnight storage at – 20 °C (8.45 g, 80%, isolated yield over two steps: 74%) was prepared following the procedure described for **1d**. The spectral properties of this product agreed with those reported in the literature.⁷ ¹H NMR (400 MHz, CDCl₃) δ 2.90 (t, *J* = 2x7.3 Hz, 2H), 1.64 (p, *J* = 2x7.2, 2x7.1 Hz, 2H), 1.37 – 1.24 (m, 4H), 0.88 (t, *J* = 2x6.8 Hz, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 195.9, 159.9, 37.4, 31.0, 22.7, 22.2, 13.8.

2-Oxohex-5-enoic acid (**1f**)

The titled compound (**1f**) was obtained as a colorless oil that crystallized as a yellow solid after overnight storage at $-20\text{ }^{\circ}\text{C}$ (5.37 g, 73%, isolated yield over two steps: 67%) was prepared following the procedure described for **1d**. The spectral properties of this product agreed with those reported in the literature.⁸ ^1H NMR (400 MHz, CDCl_3) δ 5.79 (ddt, $J = 16.8, 10.2, 2 \times 6.5$ Hz, 1H), 5.13 – 4.96 (m, 2H), 3.04 (t, $J = 2 \times 7.2$ Hz, 2H), 2.4 (qt, $J = 3 \times 7.2, 2 \times 1.4$ Hz, 2H). ^{13}C NMR (101 MHz, CDCl_3) δ 195.1, 159.4, 135.5, 116.3, 36.5, 26.9.

2-Oxohept-6-enoic acid (**1g**)

The titled compound (**1g**) was obtained as a colorless oil that crystallized as yellow solid after overnight storage at $-20\text{ }^{\circ}\text{C}$ (6.06 g, 89%, isolated yield over two steps: 75%) was prepared following the procedure described for **1d**. ^1H NMR (400 MHz, CDCl_3) δ 5.73 (ddt, $J = 16.9, 10.2, 2 \times 6.7$ Hz, 1H), 5.07 – 4.95 (m, 2H), 2.94 (t, $J = 2 \times 7.2$ Hz, 2H), 2.10 (q, $J = 3 \times 7.1$ Hz, 2H), 1.76 (p, $J = 2 \times 7.3, 2 \times 7.2$ Hz, 2H). ^{13}C NMR (101 MHz, CDCl_3) δ 195.8, 159.3, 137.1, 116.0, 36.4, 32.7, 22.2.

2-Oxo-oct-7-enoic acid (**1h**)

The titled compound (**1h**) was obtained as a colorless oil that crystallized as a yellow solid after overnight storage at $-20\text{ }^{\circ}\text{C}$ (5.58 g, 87%, isolated yield over two steps: 69%) was prepared following the procedure described for **1d**. ^1H NMR (400 MHz, CDCl_3) δ 5.76 (ddt, $J = 16.9, 10.2, 2 \times 6.7$ Hz, 1H), 5.05 – 4.89 (m, 2H), 2.93 (t, $J = 2 \times 7.2$ Hz, 2H), 2.06 (q, $J = 2 \times 7.1, 6.9$ Hz, 2H), 1.66 (p, $J = 4 \times 7.3$ Hz, 2H), 1.43 (p, $J = 2 \times 7.5, 2 \times 7.3$ Hz, 2H). ^{13}C NMR (101 MHz, CDCl_3) δ 195.6, 159.8, 138.0, 115.0, 37.2, 33.2, 28.0, 22.4.

3-Cyclohexyl-2-oxopropanoic acid (**1k**)

The titled compound (**1k**) was obtained as a white solid (8.80 g, 74%, isolated yield over two steps: 67%) was prepared following the procedure described for **1d**. The spectral properties of this product agreed with those reported in the literature.⁹ ¹H NMR (400 MHz, CDCl₃) δ 2.80 (d, *J* = 6.8 Hz, 2H), 1.90 (dt, *J* = 14.8, 2x7.0, 3.4, 3.4 Hz, 1H), 1.74 – 1.59 (m, 5H), 1.38 – 1.21 (m, 2H), 1.20 – 1.07 (m, 1H), 1.06 – 0.92 (m, 2H). ¹³C NMR (101 MHz, CDCl₃) δ 195.7, 159.3, 44.3, 33.7, 33.0, 25.9, 25.9.

Method B

3-Cyclopropyl-2-oxopropanoic acid (**1i**)

Typical procedure: A three-necked round bottomed flask was charged with toluene (100 mL) and NaH (1.54 g, 38.58 mmol, 1.2 eq, 60% dispersion in mineral oil) was added under N₂ atmosphere. A solution of (bromomethyl)cyclopropane (4.34 g, 32.15 mmol, 1.0 eq) and ethyl 1,3-dithiane-2-carboxylate (6.18 g, 32.15 mmol, 1.0 eq) in DMF (100 mL) was added drop-wise over the course of 30 min at 4 °C. The mixture was stirred 1h and at room temperature overnight. The reaction was quenched by the addition of NaH₂PO₄ 5% solution (100 mL) and the organic phase was separated, washed with water (3 x 100 mL), NaHCO₃ 5% (3 x 100 mL) and brine (3 x 100 mL). Finally, the organic phase was dried over anhydrous magnesium sulfate and filtered. The filtrate was concentrated under vacuum to give colorless viscous oil, which was not

characterized. In the next reaction step, the crude product was dissolved in acetone (50 ml) and was added dropwise to a solution of *N*-bromosuccinimide (45.77 g, 257.16 mmol, 8 eq) in acetone:water 94:6 v/v (100 mL) mixture and stirred at $-10\text{ }^{\circ}\text{C}$. The solution quickly turned red (bromine) but soon faded to yellow-orange. The reaction was stirred for 10 min and quenched with a saturated sodium sulfite solution (200 mL). The mixture was extracted with Et_2O (3 x 100 mL), then the organic phases were combined, washed with NaHCO_3 5% (3 x 100 mL), brine (3 x 100 mL), dried over anhydrous magnesium sulfate, filtered and the solvent removed under vacuum to give a colorless viscous oil that was not characterized. In the last reaction step, the crude material was dissolved in 2-methyltetrahydrofuran (2-MeTHF, 32 mL). Then, 1.0 M sodium phosphate buffer pH 7.0 (32 mL) and, Novozym 435 (0.64 g, 20 mg mL^{-1} final concentration in aqueous phase) were added. The reaction, in a closed vessel (250 mL), was conducted at $25\text{ }^{\circ}\text{C}$ and orbital shaker. After 24 h, the starting material was not detected on TLC and the reaction was filtered and NaHCO_3 5% (300 mL) was added. The mixture was washed with Et_2O (3x100 mL). Then, the aqueous solution was acidified at pH = 1.0 (HCl 37%) and the product was extracted with Et_2O (3x100 mL). The organic phases were combined, dried over anhydrous magnesium sulfate, filtered and the solvent removed under vacuum affording the titled compound (**1i**) as a yellow oil that crystallized as a yellow solid after overnight storage at $-20\text{ }^{\circ}\text{C}$ (1.59 g, isolated yield over three steps: 39%). This material was used without any further purification. ^1H NMR (400 MHz, CDCl_3) δ 2.82 (d, $J = 6.9\text{ Hz}$, 2H), 1.13 – 0.97 (m, 1H), 0.60 (dt, $J = 8.0, 2 \times 6.0\text{ Hz}$, 2H), 0.18 (dt, $J = 6.2, 2 \times 4.8\text{ Hz}$, 2H). ^{13}C NMR (101 MHz, CDCl_3) δ 195.3, 159.6, 42.1, 5.3, 4.4.

3-Cyclobutyl-2-oxopropanoic acid (**1j**)

The titled compound (**1j**) was obtained as a colorless oil that crystallized as a yellow solid after overnight storage at $-20\text{ }^{\circ}\text{C}$ (2.50 g, isolated yield over three steps: 70%) was prepared following the procedure described for **1i**. ^1H NMR (400 MHz, CDCl_3) δ 3.04 (d, $J = 7.2\text{ Hz}$, 2H), 2.73 (hept, $J = 6 \times 7.7\text{ Hz}$, 1H), 2.21 – 2.09 (m, 2H), 1.88 (td, $J = 2 \times 14.7, 11.7, 9.2, 6.4\text{ Hz}$, 2H), 1.69 (pd, $J = 9.2, 3 \times 9.0, 2.4\text{ Hz}$, 2H). ^{13}C NMR (101 MHz, CDCl_3) δ 195.1, 159.2, 43.8, 30.8, 28.3, 18.8.

NMR spectra of ethyl 2-oxoesters and 2-oxoacids

a)

b)

Figure S4. NMR spectra (CDCl_3) of **S1d**: a) ^1H and b) ^{13}C .

a)

b)

Figure S5. NMR spectra (CDCl_3) of **1d**: a) ^1H and b) ^{13}C .

a)

b)

Figure S6. NMR spectra (CDCl_3) of **S1e**: a) ^1H and b) ^{13}C .

a)

b)

Figure S7. NMR spectra (CDCl_3) of **1e**: a) ^1H and b) ^{13}C .

a)

b)

Figure S8. NMR spectra (CDCl_3) of **S1f**: a) ^1H and b) ^{13}C .

a)

b)

Figure S9. NMR spectra (CDCl_3) of **1f**: a) ^1H and b) ^{13}C .

a)

b)

Figure S10. NMR spectra (CDCl_3) of **S1g**: a) ^1H and b) ^{13}C .

a)

b)

Figure S11. NMR spectra (CDCl_3) of **1g**: a) ^1H and b) ^{13}C .

a)

b)

Figure S12. NMR spectra (CDCl_3) of **S1h**: a) ^1H and b) ^{13}C .

a)

b)

Figure S13. NMR spectra (CDCl_3) of **1h**: a) ^1H and b) ^{13}C .

a)

3

b)

Figure S14. NMR spectra (CDCl_3) of **1i**: a) ^1H and b) ^{13}C .

a)

b)

Figure S15. NMR spectra (CDCl_3) of **1j**: a) ^1H and b) ^{13}C .

a)

b)

Figure S16. NMR spectra (CDCl_3) of **S1k**: a) ^1H and b) ^{13}C .

a)

Figure S17. NMR spectra (CDCl_3) of **1k**: a) ^1H and b) ^{13}C .

Substrates (1a-k) stock solution preparation

Stock solutions of 2-oxoacids were prepared in H₂O, pH adjusted to 7.0 with NaOH (1M) and stored at 4 °C. Under these conditions precipitation of sodium salt compounds **1d-k** was observed. Before using, stock solutions (**1d-k**) were carefully treated with the heat gun under stirring until a clear solution was obtained.

R ¹	Stock concentration/M
1a	2.0
1b	1.0
1c	1.5
1d	1.0
1e	1.5
1f	2.0
1g	2.0
1h	1.0
1i	1.5
1j	1.0
1k	0.8

Screening experiments and determination initial reaction rate

Reaction time course of aldol addition of 2-oxoacids (**1a-k**) to methanal (**2**) catalyzed by wild-type MBP-YfaU and variants thereof was determined as follows: To a solution of YfaU (4 mg protein mL⁻¹ in the reaction, 0.06 mol%), plain water and the oxoacid (**1a-k**, 0.05 mmol, 1 eq, 0.1 M in the reaction) were added. NiCl₂ (1.3 μL of a 0.1 M stock solution, 0.25 mM in the reaction) was added. The reaction was started by adding methanal (**2**, 3.8 μL of a 13.3 M commercial solution, 0.05 mmol, 1 eq, 0.1 M in the reaction). The total reaction volume was 0.5 mL. The reaction was monitored by HPLC as described above every 10 min during the first hour, then every hour during 6 h and finally at 24 h.

Reaction time course of aldol addition of 2-oxoacids (**1a-k**) to methanal (**2**) catalyzed with KPHMT wild-type and variants thereof was determined as follows: To a solution of KPHMT (glycerinated protein solution, 4 mg protein mL⁻¹ in the reaction, 0.14 mol%), the 2-oxoacid (**1a-k**, 0.05 mmol, 1 eq, 0.1 M in the reaction) was added. Plain

water and CoCl_2 (3 μL of a 0.1 M stock solution, 0.6 mM in the reaction) were added. The reaction was started by adding methanal (**2**, 3.8 μL of a 13.3 M commercial solution, 0.05 mmol, 1 eq, 0.1 M in the reaction). The total reaction volume was 0.5 mL. The reaction was monitored by HPLC as described above every 10 min during the first hour, then every hour during 6 h and finally at 24 h.

The initial reaction rates were calculated at the beginning of the reaction (i.e. samples taken every 10 min during the first hour keeping reaction conversions <10%) to ensure a linear dependence of the product formation versus time. The product formed for the screening was calculated at 24 h. Concentrations of the aldol products were calculated with an external standard method from peak areas.

Reaction monitoring for the synthesis of products S-4a-h

Figure S18. Aldol addition of sodium 2-oxobutanoate (**1a**) to methanal (**2**) catalyzed by MBP-YfaU wild-type (Ni^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 4 h (> 95% conversion).

MBP-YfaU	v_o^a mM min^{-1}	Conv 24 h %
wild-type	2.5 ± 0.3	> 95
Control	$1.7 \cdot 10^{-2} \pm 1.0 \cdot 10^{-3}$	16

^a[**1a**] = [**2**] = 100 mM, [enzyme] = 4 mg mL⁻¹.

Figure S19. Reaction time course of aldol addition of sodium 2-oxobutanoate (**1a**) to methanal (**2**) catalyzed by MBP-YfaU wild-type (Ni^{2+}) and control experiment performed under the same conditions without the enzyme. Values are the meaning of two determinations. The $\pm$ are the values of the standard error of the mean of two independent experiments under the same reaction conditions.

Figure S20. Effect of methanal concentration on the enantiomeric excess of *S*-4a.

Figure S21. HPLC monitoring of oxidative decarboxylation of *S*-3a (a) and synthesis of 2-(4'-bromophenyl)-2-oxoethyl ester *S*-4a (b).

Figure S22. Aldol addition of sodium 2-oxopentanoate (**1b**) to methanal (**2**) catalyzed by MBP-YfaU W23V (Ni^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 4 h (> 95% conversion).

Figure S23. Reaction time course of aldol addition of sodium 2-oxopentanoate (**1b**) to methanal (**2**) catalyzed by MBP-YfaU (Ni^{2+}) wild-type, W23V and W23V/L216A variants. Control experiment was performed under the same conditions without the enzyme. Values are the meaning of two determinations. The $\pm$ are the values of the standard error of the mean of two independent experiments under the same reaction conditions.

Figure S24. Effect of methanal concentration on the enantiomeric excess of *S*-4b.

Figure S25. HPLC monitoring of oxidative decarboxylation of *S*-3b (a) and synthesis of 2-(4'-bromophenyl)-2-oxoethyl ester *S*-4b (b).

Figure S26. Aldol addition of sodium 4-methyl-2-oxopentanoate (**1c**) to methanal (**2**) catalyzed by MBP-YfaU W23V (Ni^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (> 95% conversion).

MBP-YfaU	v_o^a mM min^{-1}	Conv 24 h %
wild-type	— ^b	— ^b
W23V	0.9 ± 0.1	93
W23V/L216A	0.2 ± 0.1	90
Control	— ^b	— ^b

^a[**1c**] = [**2**] = 100 mM, [enzyme] = 4 mg mL^{-1} .

^bProduct not detected.

Figure S27. Reaction time course of aldol addition of sodium 4-methyl-2-oxopentanoate (**1c**) to methanal (**2**) catalyzed by MBP-YfaU (Ni^{2+}) wild-type, W23V and W23V/L216A variants. Control experiment was performed under the same conditions without the enzyme. Values are the meaning of two determinations. The $\pm$ are the values of the standard error of the mean of two independent experiments under the same reaction conditions.

Figure S28. Effect of methanal concentration on the yield of *S*-**3c** in the aldol addition of **2** to **1c** catalyzed by MBP-YfaU W23V (Ni²⁺).

Figure S29. HPLC monitoring of oxidative decarboxylation of *S*-**3c** (a) and synthesis of 4'-bromophenacyl ester *S*-**4c** (b).

Figure S30. Aldol addition of sodium 2-oxohexanoate (**1d**) to methanal (**2**) catalyzed by MBP-YfaU W23V (Ni^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (> 95% conversion).

MBP-YfaU	v_o^a mM min^{-1}	Conv 24 h %
wild-type	$1.3 \cdot 10^{-2} \pm 2.0 \cdot 10^{-3}$	26
W23V	0.7 ± 0.1	>95
W23V/L216A	0.5 ± 0.1	88
Control	— ^b	— ^b

^a[**1d**] = [**2**] = 100 mM, [enzyme] = 4 mg mL⁻¹.

^bProduct not detected.

Figure S31. Reaction time course of aldol addition of sodium 2-oxohexanoate (**1d**) to methanal (**2**) catalyzed by MBP-YfaU (Ni^{2+}) wild-type, W23V and W23V/L216A variants. Control experiment was performed under the same conditions without the enzyme. Values are the meaning of two determinations. The $\pm$ are the values of the standard error of the mean of two independent experiments under the same reaction conditions.

Figure S32. Effect of methanal concentration on the yield of *S*-**3d** in the aldol addition of **2** to **1d** catalyzed by MBP-YfaU W23V (Ni²⁺).

Figure S33. HPLC monitoring of oxidative decarboxylation of *S*-**3d** (a) and synthesis of 4'-bromophenacyl ester *S*-**4d** (b).

Figure S34. Aldol addition of sodium 2-oxoheptanoate (**1e**) to methanal (**2**) catalyzed by MBP-YfaU W23V (Ni^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (> 95% conversion).

MBP-YfaU	v_o^a mM min^{-1}	Conv 24 h %
wild-type	$4.2 \cdot 10^{-2} \pm 4.0 \cdot 10^{-3}$	26
W23V	1.1 ± 0.1	>95
W23V/L216A	0.2 ± 0.1	78
Control	$1.8 \cdot 10^{-3} \pm 1.0 \cdot 10^{-4}$	<5

^a[**1e**] = [**2**] = 100 mM, [enzyme] = 4 mg mL⁻¹.

Figure S35. Reaction time course of aldol addition of sodium 2-oxoheptanoate (**1e**) to methanal (**2**) catalyzed by MBP-YfaU (Ni^{2+}) wild-type, W23V and W23V/L216A variants. Control experiment was performed under the same conditions without the enzyme. Values are the meaning of two determinations. The $\pm$ are the values of the standard error of the mean of two independent experiments under the same reaction conditions.

Figure S36. Effect of methanal concentration on the yield of *S-3e* in the aldol addition of **2** to **1e** catalyzed by MBP-YfaU W23V (Ni²⁺).

Figure S37. HPLC monitoring of oxidative decarboxylation of *S-3e* (a) and synthesis of 4'-bromophenacyl ester *S-4e* (b).

Figure S38. Aldol addition of sodium 2-oxohex-5-enoate (**1f**) to methanal (**2**) catalyzed by MBP-YfaU W23V (Ni^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (> 95% conversion).

MBP-YfaU	v_o^a mM min^{-1}	Conv 24 h %
wild-type	$6.7 \cdot 10^{-2} \pm 3.0 \cdot 10^{-2}$	42
W23V	1.1 ± 0.1	>95
W23V/L216A	0.7 ± 0.1	>95
Control	$3.4 \cdot 10^{-3} \pm 3.0 \cdot 10^{-4}$	5

^a[**1f**] = [**2**] = 100 mM, [enzyme] = 4 mg mL⁻¹.

Figure S39. Reaction time course of aldol addition of sodium 2-oxohex-5-enoate (**1f**) to methanal (**2**) catalyzed by MBP-YfaU (Ni^{2+}) wild-type, W23V and W23V/L216A variants. Control experiment was performed under the same conditions without the enzyme. Values are the meaning of two determinations. The $\pm$ are the values of the standard error of the mean of two independent experiments under the same reaction conditions.

Figure S40. Effect of methanal concentration on the yield of *S*-**3f** in the aldol addition of **2** to **1f** catalyzed by MBP-YfaU W23V (Ni²⁺).

Figure S41. HPLC monitoring of oxidative decarboxylation of *S*-**3f** (a) and synthesis of 4'-bromophenacyl ester *S*-**4f** (b).

Figure S42. Aldol addition of sodium 2-oxohept-6-enoate (**1g**) to methanal (**2**) catalyzed by MBP-YfaU W23V (Ni^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (> 95% conversion).

MBP-YfaU	v_o^a mM min^{-1}	Conv 24 h %
wild-type	$5.8 \cdot 10^{-2} \pm 1.0 \cdot 10^{-2}$	37
W23V	2.3 ± 0.2	>95
W23V/L216A	1.5 ± 0.2	>95
Control	$4.9 \cdot 10^{-2} \pm 1.0 \cdot 10^{-2}$	9

^a[**1g**] = [**2**] = 100 mM, [enzyme] = 4 mg mL⁻¹.

Figure S43. Reaction time course of aldol addition of sodium 2-oxohept-6-enoate (**1g**) to methanal (**2**) catalyzed by MBP-YfaU (Ni^{2+}) wild-type, W23V and W23V/L216A variants. Control experiment was performed under the same conditions without the enzyme. Values are the meaning of two determinations. The $\pm$ are the values of the standard error of the mean of two independent experiments under the same reaction conditions.

Figure S44. Effect of methanal concentration on the yield of *S*-**3g** in the aldol addition of **2** to **1g** catalyzed by MBP-YfaU W23V (Ni²⁺).

Figure S45. HPLC monitoring of oxidative decarboxylation of *S*-**3g** (a) and synthesis of 4'-bromophenacyl ester *S*-**4g** (b).

Figure S46. Aldol addition of sodium 2-oxooct-7-enoate (**1h**) to methanal (**2**) catalyzed by MBP-YfaU W23V (Ni^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (> 95% conversion).

MBP-YfaU	v_o^a mM min^{-1}	Conv 24 h %
wild-type	$4.9 \cdot 10^{-2} \pm 2.0 \cdot 10^{-3}$	22
W23V	1.1 ± 0.1	90
W23V/L216A	0.4 ± 0.1	84
Control	$5.6 \cdot 10^{-3} \pm 2.0 \cdot 10^{-4}$	<5

^a[**1h**] = [**2**] = 100 mM, [enzyme] = 4 mg mL⁻¹.

Figure S47. Reaction time course of aldol addition of sodium 2-oxooct-7-enoate (**1h**) to methanal (**2**) catalyzed by MBP-YfaU (Ni^{2+}) wild-type, W23V and W23V/L216A variants. Control experiment was performed under the same conditions without the enzyme. Values are the meaning of two determinations. The $\pm$ are the values of the standard error of the mean of two independent experiments under the same reaction conditions.

Figure S48. Effect of methanal concentration on the yield of *S-3h* in the aldol addition of **2** to **1h** catalyzed by MBP-YfaU W23V (Ni²⁺).

Figure S49. HPLC monitoring of oxidative decarboxylation of *S-3h* (a) and synthesis of 4'-bromophenacyl ester *S-4h* (b).

Figure S50. Aldol addition of sodium 3-cyclopropyl-2-oxopropanoate (**1i**) to methanal (**2**) catalyzed by MBP-YfaU W23V (Ni^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (> 95% conversion).

MBP-YfaU	v_o^a mM min^{-1}	Conv 24 h %
wild-type	0.18 ± 0.02	86
W23V	12.2 ± 0.5	>95
W23V/L216A	9.1 ± 0.3	>95
Control	$1.5 \cdot 10^{-2} \pm 1.0 \cdot 10^{-3}$	19

^a[**1i**] = [**2**] = 100 mM, [enzyme] = 4 mg mL⁻¹.

Figure S51. Reaction time course of aldol addition of sodium 3-cyclopropyl-2-oxopropanoate (**1i**) to methanal (**2**) catalyzed by MBP-YfaU (Ni^{2+}) wild-type, W23V and W23V/L216A variants. Control experiment was performed under the same conditions without the enzyme. Values are the meaning of two determinations. The $\pm$ are the values of the standard error of the mean of two independent experiments under the same reaction conditions.

Figure S52. Effect of methanal concentration on the yield of *S*-**3i** in the aldol addition of **2** to **1i** catalyzed by MBP-YfaU W23V (Ni²⁺).

Figure S53. HPLC monitoring of oxidative decarboxylation of *S*-**3i** (a) and synthesis of 4'-bromophenacyl ester *S*-**4i** (b).

Figure S54. Aldol addition of sodium 3-cyclobutyl-2-oxopropanoate (**1j**) to methanal (**2**) catalyzed by MBP-YfaU W23V (Ni^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (> 95% conversion).

MBP-YfaU	v_o^a mM min ⁻¹	Conv 24 h %
wild-type	$2.0 \cdot 10^{-2} \pm 2.0 \cdot 10^{-3}$	21
W23V	2.1 ± 0.1	>95
W23V/L216A	1.5 ± 0.1	92
Control	$-^b$	$-^b$

^a[**1j**] = [**2**] = 100 mM, [enzyme] = 4 mg mL⁻¹.

^bProduct not detected.

Figure S55. Reaction time course of aldol addition of sodium 3-cyclobutyl-2-oxopropanoate (**1j**) to methanal (**2**) catalyzed by MBP-YfaU (Ni^{2+}) wild-type, W23V and W23V/L216A variants. Control experiment was performed under the same conditions without the enzyme. Values are the meaning of two determinations. The $\pm$ are the values of the standard error of the mean of two independent experiments under the same reaction conditions.

Figure S56. Effect of methanal concentration on the yield of *S*-**3j** in the aldol addition of **2** to **1j** catalyzed by MBP-YfaU W23V (Ni²⁺).

Figure S57. HPLC monitoring of oxidative decarboxylation of *S*-**3j** (a) and synthesis of 4'-bromophenacyl ester *S*-**4j** (b).

Figure S58. Aldol addition of sodium 3-cyclohexyl-2-oxopropanoate (**1k**) to methanal (**2**) catalyzed by MBP-YfaU W23V (Ni^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (> 95% conversion).

● MBP-YfaU wild-type ■ MBP-YfaU W23V
 ▲ MBP-YfaU W23V/L216A ▼ Control experiment

MBP-YfaU	v_o^a mM min^{-1}	Conv 24 h %
wild-type	$1.1 \cdot 10^{-2} \pm 2.0 \cdot 10^{-3}$	10
W23V	1.6 ± 0.2	85
W23V/L216A	0.6 ± 0.1	74
Control	$6.8 \cdot 10^{-3} \pm 6.0 \cdot 10^{-4}$	7

^a[**1k**] = [**2**] = 100 mM, [enzyme] = 4 mg mL⁻¹.

Figure S59. Reaction time course of aldol addition of sodium 3-cyclohexyl-2-oxopropanoate (**1k**) to methanal (**2**) catalyzed by MBP-YfaU (Ni^{2+}) wild-type, W23V and W23V/L216A variants. Control experiment was performed under the same conditions without the enzyme. Values are the meaning of two determinations. The $\pm$ are the values of the standard error of the mean of two independent experiments under the same reaction conditions.

Figure S60. Effect of methanal concentration on the yield of *S-3k* in the aldol addition of **2** to **1k** catalyzed by MBP-YfaU W23V (Ni²⁺).

Figure S61. HPLC monitoring of oxidative decarboxylation of *S-3k* (a) and synthesis of 4'-bromophenacyl ester *S-4k* (b).

Reaction monitoring for the synthesis of products *R*-4a-j

Figure S62. Aldol addition of sodium 2-oxobutanoate (**1a**) to methanal (**2**) catalyzed by KPHMT wild-type (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 3 h (90% conversion).

KPHMT	v_o^a mM min^{-1}	Conv 24 h %
wild-type	3.3 ± 0.7	>95
Control	$1.3 \cdot 10^{-2} \pm 1.0 \cdot 10^{-3}$	19

^a[**1a**] = [**2**] = 100 mM, [enzyme] = 4 mg mL⁻¹.

Figure S63. Reaction time course of aldol addition of sodium 2-oxobutanoate (**1a**) to methanal (**2**) catalyzed by KPHMT wild-type (Co^{2+}) and control experiment performed under the same conditions without the enzyme. Values are the meaning of two determinations. The $\pm$ are the values of the standard error of the mean of two independent experiments under the same reaction conditions.

Figure S64. Effect of methanal concentration on the enantiomeric excess of *R*-4a.

Figure S65. HPLC monitoring of oxidative decarboxylation of *R*-3a (a) and synthesis of 4'-bromophenacyl ester *R*-4a (b).

Figure S66. Aldol addition of sodium 2-oxopentanoate (**1b**) to methanal (**2**) catalyzed by KPHMT wild-type (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 6 h (95% conversion).

KPHMT	v_o^a mM min^{-1}	Conv 24 h %
wild-type	1.9 ± 0.2	95
Control	$1.1 \cdot 10^{-2} \pm 1.0 \cdot 10^{-3}$	12

^a[**1b**] = [**2**] = 100 mM, [enzyme] = 4 mg mL⁻¹.

Figure S67. Reaction time course of aldol addition of sodium 2-oxobutanoate (**1b**) to methanal (**2**) catalyzed by KPHMT wild-type (Co^{2+}) and control experiment performed under the same conditions without the enzyme. Values are the meaning of two determinations. The $\pm$ are the values of the standard error of the mean of two independent experiments under the same reaction conditions.

Figure S68. Effect of methanal concentration on the enantiomeric excess of *R*-**4b**.

Figure S69. HPLC monitoring of oxidative decarboxylation of *R*-**3b** (a) and synthesis of 4'-bromophenacyl ester *R*-**4b** (b).

Figure S70. Aldol addition of sodium 4-methyl-2-oxopentanoate (**1c**) to methanal (**2**) catalyzed by KPHMT I202A (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (70% conversion).

KPHMT	v_o^a mM min^{-1}	Conv 24 h %
wild-type	$7.4 \cdot 10^{-3} \pm 1.0 \cdot 10^{-3}$	10
L42A	$-^b$	$-^b$
I212A	$8.0 \cdot 10^{-3} \pm 1.0 \cdot 10^{-3}$	12
L42A/I212A	$5.0 \cdot 10^{-2} \pm 1.0 \cdot 10^{-2}$	19
I202A	0.2 ± 0.1	57
Control	$-^b$	$-^b$

^a[**1c**] = [**2**] = 100 mM, [enzyme] = 4 mg mL⁻¹.

^bProduct not detected.

Figure S71. Reaction time course of aldol addition of sodium 4-methyl-2-oxopentanoate (**1c**) to methanal (**2**) catalyzed by KPHMT (Co^{2+}) wild-type, L42A, I212A, L42A/I212A and L202A variants. Control experiment was performed under the same conditions without the enzyme. Values are the meaning of two determinations. The $\pm$ are the values of the standard error of the mean of two independent experiments under the same reaction conditions.

Figure S72. Effect of methanal concentration on the yield of *R*-**3c** in the aldol addition of **2** to **1c** catalyzed by KPHMT I202A (Co²⁺).

Figure S73. HPLC monitoring of oxidative decarboxylation of *R*-**3c** (a) and synthesis of 4'-bromophenacyl ester *R*-**4c** (b).

Figure S74. Aldol addition of sodium 2-oxohexanoate (**1d**) to methanal (**2**) catalyzed by KPHMT I212A (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (85% conversion).

KPHMT	v_o^a mM min^{-1}	Conv 24 h %
wild-type	$2.0 \cdot 10^{-2} \pm 1.0 \cdot 10^{-2}$	20
L42A	— ^b	— ^b
I212A	0.4 ± 0.1	88
L42A/I212A	0.3 ± 0.1	87
Control	— ^b	— ^b

^a[**1d**] = [**2**] = 100 mM, [enzyme] = 4 mg mL⁻¹.

^bProduct not detected.

Figure S75. Reaction time course of aldol addition of sodium 2-oxohexanoate (**1d**) to methanal (**2**) catalyzed by KPHMT (Co^{2+}) wild-type, L42A, I212A and L42A/I212A variants. Control experiment was performed under the same conditions without the enzyme. Values are the meaning of two determinations. The $\pm$ are the values of the standard error of the mean of two independent experiments under the same reaction conditions.

Figure S76. Effect of methanal concentration on the yield of *R*-**3d** in the aldol addition of **2** to **1d** catalyzed by KPHMT I212A (Co²⁺).

Figure S77. HPLC monitoring of oxidative decarboxylation of *R*-**3d** (a) and synthesis of 4'-bromophenacyl ester *R*-**4d** (b).

Figure S78. Aldol addition of sodium 2-oxoheptanoate (**1e**) to methanal (**2**) catalyzed by KPHMT L42A/I212A (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (88% conversion).

KPHMT	v_o^a mM min^{-1}	Conv 24 h %
wild-type	$3.0 \cdot 10^{-3} \pm 1.0 \cdot 10^{-3}$	7
L42A	— ^b	— ^b
I212A	0.6 ± 0.1	83
L42A/I212A	0.3 ± 0.1	87
Control	— ^b	— ^b

^a[**1e**] = [**2**] = 100 mM, [enzyme] = 4 mg mL⁻¹.

^bProduct not detected.

Figure S79. Reaction time course of aldol addition of sodium 2-oxoheptanoate (**1e**) to methanal (**2**) catalyzed by KPHMT (Co^{2+}) wild-type, L42A, I212A, L42A/I212A and I202A variants. Control experiment was performed under the same conditions without the enzyme. Values are the meaning of two determinations. The $\pm$ are the values of the standard error of the mean of two independent experiments under the same reaction conditions.

Figure S80. Effect of methanal concentration on the yield of *R*-**3e** in the aldol addition of **2** to **1e** catalyzed by KPHMT L42A/I212A (Co²⁺).

Figure S81. HPLC monitoring of oxidative decarboxylation of *R*-**3e** (a) and synthesis of 4'-bromophenacyl ester *R*-**4e** (b).

Figure S82. Aldol addition of sodium 2-oxohex-5-enoate (**1f**) to methanal (**2**) catalyzed by KPHMT I212A (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (87% conversion).

KPHMT	v_o^a mM min^{-1}	Conv 24 h %
wild-type	0.2 ± 0.1	80
L42A	$4.0 \cdot 10^{-2} \pm 1.0 \cdot 10^{-2}$	26
I212A	1.1 ± 0.2	92
L42A/I212A	0.6 ± 0.1	>95
Control	— ^b	— ^b

^a[**1f**] = [**2**] = 100 mM, [enzyme] = 4 mg mL⁻¹.

^bProduct not detected.

Figure S83. Reaction time course of aldol addition of sodium 2-oxohex-5-enoate (**1f**) to methanal (**2**) catalyzed by KPHMT (Co^{2+}) wild-type, L42A, I212A and L42A/I212A variants. Control experiment was performed under the same conditions without the enzyme. Values are the meaning of two determinations. The $\pm$ are the values of the standard error of the mean of two independent experiments under the same reaction conditions.

Figure S84. Effect of methanal concentration on the yield of *R*-**3f** in the aldol addition of **2** to **1f** catalyzed by KPHMT I212A (Co²⁺).

Figure S85. HPLC monitoring of oxidative decarboxylation of *R*-**3f** (a) and synthesis of 4'-bromophenacyl ester *R*-**4f** (b).

Figure S86. Aldol addition of sodium 2-oxohept-6-enoate (**1g**) to methanal (**2**) catalyzed by KPHMT I212A (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (93% conversion).

KPHMT	v_o^a mM min^{-1}	Conv 24 h %
wild-type	$7.0 \cdot 10^{-2} \pm 2.0 \cdot 10^{-2}$	28
L42A	— ^b	— ^b
I212A	2.2 ± 0.3	93
L42A/I212A	1.0 ± 0.1	86
Control	— ^b	— ^b

^a[**1g**] = [**2**] = 100 mM, [enzyme] = 4 mg mL⁻¹.

^bProduct not detected.

Figure S87. Reaction time course of aldol addition of sodium 2-oxohept-6-enoate (**1g**) to methanal (**2**) catalyzed by KPHMT (Co^{2+}) wild-type, L42A, I212A and L42A/I212A variants. Control experiment was performed under the same conditions without the enzyme. Values are the meaning of two determinations. The $\pm$ are the values of the standard error of the mean of two independent experiments under the same reaction conditions.

Figure S88. Effect of methanal concentration on the yield of *R*-**3g** in the aldol addition of **2** to **1g** catalyzed by KPHMT I212A (Co²⁺).

Figure S89. HPLC monitoring of oxidative decarboxylation reaction of *R*-**3g** (a) and synthesis of 4'-bromophenacyl ester *R*-**4g** (b).

Figure S90. Aldol addition of sodium 2-oxooct-7-enoate (**1h**) to methanal (**2**) catalyzed by KPHMT L42A/I212A (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (92% conversion).

● KPHMT wild-type ■ KPHMT L42A
 ▲ KPHMT I212A ▼ KPHMT L42A/I212A
 ◆ Control experiment

KPHMT	v_o^a mM min^{-1}	Conv 24 h %
wild-type	$9.0 \cdot 10^{-3} \pm 1.0 \cdot 10^{-3}$	6
L42A	$1.0 \cdot 10^{-2} \pm 5.0 \cdot 10^{-3}$	5
I212A	1.5 ± 0.2	88
L42A/I212A	1.0 ± 0.2	90
Control	— ^b	— ^b

^a[**1h**] = [**2**] = 100 mM, [enzyme] = 4 mg mL⁻¹.

^bProduct not detected.

Figure S91. Reaction time course of aldol addition of sodium 2-oxooct-7-enoate (**1h**) to methanal (**2**) catalyzed by KPHMT (Co^{2+}) wild-type, L42A, I212A and L42A/I212A variants. Control experiment was performed under the same conditions without the enzyme. Values are the meaning of two determinations. The $\pm$ are the values of the standard error of the mean of two independent experiments under the same reaction conditions.

Figure S92. Effect of methanal concentration on the yield of *R-3h* in the aldol addition of **2** to **1h** catalyzed by KPHMT L42A/I212A (Co^{2+}).

Figure S93. HPLC monitoring of oxidative decarboxylation of *R-3h* (a) and synthesis of 2-(4'-bromophenyl)-2-oxoethyl ester *R-4h* (b).

Figure S94. Aldol addition of sodium 3-cyclopropyl-2-oxopropanoate (**1i**) to methanal (**2**) catalyzed by KPHMT wild-type (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (>95% conversion).

KPHMT	v_o^a mM min^{-1}	Conv 24 h %
wild-type	1.8 ± 0.2	>95
L42A	$5.0 \cdot 10^{-2} \pm 1.0 \cdot 10^{-2}$	29
I212A	1.9 ± 0.3	94
L42A/I212A	0.5 ± 0.1	88
I202A	3.0 ± 0.3	94
Control	$7.0 \cdot 10^{-3} \pm 3.0 \cdot 10^{-4}$	9

^a[**1i**] = [**2**] = 100 mM, [enzyme] = 4 mg mL⁻¹.

Figure S95. Reaction time course of aldol addition of sodium 3-cyclopropyl-2-oxopropanoate (**1i**) to methanal (**2**) catalyzed by KPHMT (Co^{2+}) wild-type, L42A, I212A, L42A/I212A and I202A variants. Control experiment was performed under the same conditions without the enzyme. Values are the meaning of two determinations. The $\pm$ are the values of the standard error of the mean of two independent experiments under the same reaction conditions.

Figure S96. Effect of methanal concentration on the yield of *R*-**3i** in the aldol addition of **2** to **1i** catalyzed by KPHMT wild-type (Co^{2+}).

Figure S97. HPLC monitoring of oxidative decarboxylation of *R*-**3i** (a) and synthesis of 2-(4'-bromophenyl)-2-oxoethyl ester *R*-**4i** (b).

Figure S98. Aldol addition of sodium 3-cyclobutyl-2-oxopropanoate (**1j**) to methanal (**2**) catalyzed by KPHMT I202A (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (90% conversion).

KPHMT	v_o^a mM min^{-1}	Conv 24 h %
wild-type	$8.0 \cdot 10^{-2} \pm 1.0 \cdot 10^{-2}$	24
L42A	$1.0 \cdot 10^{-2} \pm 5.0 \cdot 10^{-3}$	14
I212A	$9.0 \cdot 10^{-2} \pm 2.0 \cdot 10^{-2}$	22
L42A/I212A	$6.0 \cdot 10^{-2} \pm 1.0 \cdot 10^{-2}$	20
I202A	0.2 ± 0.1	79
Control	$-^b$	$-^b$

^a[**1j**] = [**2**] = 100 mM, [enzyme] = 4 mg mL⁻¹.

^bProduct not detected.

Figure S99. Reaction time course of aldol addition of sodium 3-cyclobutyl-2-oxopropanoate (**1j**) to methanal (**2**) catalyzed by KPHMT (Co^{2+}) wild-type, L42A, I212A, L42A/I212A and I202A variants. Control experiment was performed under the same conditions without the enzyme. Values are the meaning of two determinations. The $\pm$ are the values of the standard error of the mean of two independent experiments under the same reaction conditions.

Figure S100. Effect of methanal concentration on the yield of *R*-**3j** in the aldol addition of **2** to **1j** catalyzed by KPHMT I202A (Co²⁺).

Figure S101. HPLC monitoring of oxidative decarboxylation of *R*-**3j** (a) and synthesis of 4'-bromophenacyl ester *R*-**4j** (b).

Enzymatic synthesis of 2-(4'-bromophenyl)-2-oxoethyl ester of 4-hydroxy-2-oxocarboxylic acid derivatives

2-(4'-bromophenyl)-2-oxoethyl (S)-3-hydroxy-2-methylpropanoate (S-4a)

Typical procedure: Reaction (2.0 mmol scale, 2.0 mL total volume) was carried out at room temperature with magnetic stirring (250 rpm). The reaction was conducted in a round-bottom flask of 100 mL capacity to avoid spilling of the reaction media due to the huge amount of foam generated when adding the catalase to eliminate the excess of H_2O_2 (see below). MBP-YfaU wild-type (16.0 mg of lyophilized powder, 4.0 mg protein mL^{-1} in the reaction, 0.006 mol%) was dissolved in a solution of sodium 2-oxobutanoate (**1a**, 1.0 mL of a 2.0 M stock solution pH 7.0, 2.0 mmol, 1 eq, 1 M in the reaction). No buffer was employed, the 2-oxoacid at pH 7.0 acted as a buffer. Plain water (0.85 mL) and NiCl_2 (5 μL of a 0.1 M stock solution, 0.25 mM in the reaction, 4 eq respect to the biocatalysts) were added. The reaction was started by adding methanal (**2**, 0.15 mL of a 13.3 M commercial solution, 2 mmol, 1 eq, 1 M in the reaction). The reaction was monitored by HPLC and after 4 h hydrogen peroxide (1.14 mL of a 8.8 M commercial solution, 10 mmol, 5 eq) was added. When the 2-oxoacid (**S-3a**) was not detected by HPLC, catalase from bovine liver (50 mg) dissolved in 1 mL of 10 mM sodium phosphate buffer, pH 7.0 was added. The reaction mixture was diluted with methanol (20 mL), filtered through Celite[®] and the pellet was washed with methanol (3x50 mL). Glycerol (2-3 mL) was added to solubilize the sodium salt residue once the solvent was removed under vacuum. Then, the mixture was diluted with DMF (10 mL) and 2,4'-dibromoacetophenone (556 mg, 2.0 mmol, 1 eq) was added. EtOAc (200 mL) was added to the reaction mixture and washed with H_2O (3x50 mL). The combined organic phases were dried over anhyd MgSO_4 , absorbed onto silica gel (100 mL) and loaded onto a silica gel column. Product was eluted with a step gradient of hexane:EtOAc: 100:0, 200 mL, 90:10, 200 mL, 80:20, 200 mL, 70:30, 200 mL, 60:40,

200mL and 50:50, 600 mL. Pure fractions were pooled and the solvent removed under vacuum affording *S*-**4a** as a white solid (482 mg, 80%). $[\alpha]_D^{20} = +19.0$ ($c = 1$ in MeOH). Chiral HPLC analysis: CHIRALCELL[®] OD, isocratic elution hexane/isopropanol 80/20 (v/v), flow rate 0.7 mL min⁻¹, t_r (*S*) = 16.0, t_r (*R*) = 17.55, 98% *ee*.

2-(4'-bromophenyl)-2-oxoethyl (*R*)-3-hydroxy-2-methylpropanoate (*R*-4a)

Typical procedure: Reaction (0.8 mmol scale, 2.0 mL total volume) was carried out at room temperature with magnetic stirring (250 rpm). The reaction was conducted in a round-bottom flask of 100 mL capacity to avoid spilling of the reaction media due to the huge amount of foam generated when adding the catalase to eliminate the excess of H₂O₂ (see below). To a solution of KPHMT wild-type (0.75 mL of a 10 mg mL⁻¹ stock glycerinated protein solution, 4 mg mL⁻¹ in the reaction, 0.03 mol%), sodium 2-oxobutanoate (**1a**, 0.8 mL of a 1 M stock solution pH 7.0, 0.8 mmol, 1 eq, 0.4 M in the reaction) was added. Plain water (0.37 mL) and CoCl₂ (12 μL of a 0.1 M stock solution, 0.6 mM in the reaction, 4 eq respect to the biocatalysts) were added. A homogeneous white colloidal solution appeared. The reaction was started by adding methanal (**2**, 60 μL of a 13.3 M commercial solution, 0.8 mmol, 1 eq, 0.4 M in the reaction). The reaction was monitored by HPLC and after 6 h hydrogen peroxide (0.5 mL of a 8.8 M commercial solution, 4 mmol, 5 eq) was added. When the 2-oxo acid (*R*-**3a**) was not detected by HPLC, catalase from bovine liver (50 mg) dissolved in 1 mL of 10 mM sodium phosphate buffer, pH 7.0 was added. The reaction mixture was diluted with methanol (20 mL), filtered through Celite[®] and the pellet was washed with methanol (3x50 mL). The solvent was removed under vacuum. Synthesis of 4'-phenacyl ester was performed as described for *S*-**4a**. The product was obtained as a white solid (214 mg, 88%). $[\alpha]_D^{20} = -18.5$ ($c = 1$ in MeOH). Chiral HPLC analysis: CHIRALCELL[®] OD, isocratic elution hexane/isopropanol 80/20 (v/v), flow rate 0.7 mL min⁻¹, t_r (*S*) = 16.0, t_r (*R*) = 17.55, 95% *ee*.

NMR of *S*-**4a** and *R*-**4a**: ^1H NMR (400 MHz, CDCl_3) δ 7.77 (d, J = 8.8 Hz, 2H), 7.64 (d, J = 8.7 Hz, 2H), 5.46 (d, J = 16.5 Hz, 1H), 5.30 (d, J = 16.5 Hz, 1H), 3.86 (dd, J = 11.4, 4.3 Hz, 1H), 3.77 (dd, J = 11.4, 7.4 Hz, 1H), 2.86 (pd, J = 7.2, 3x7.1, 4.3 Hz, 1H), 1.23 (d, J = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, CDCl_3) δ 192.1, 174.7, 132.4, 132.3, 129.6, 129.3, 65.7, 65.3, 42.3, 13.0.

ESI-TOF m/z for *S*-**4a** and *R*-**4a**: Calcd for $[\text{M}+\text{Na}^+]$ $\text{C}_{12}\text{H}_{13}\text{O}_4\text{NaBr}$: 322.9895, found $[\text{M}+\text{Na}^+]$: 322.9889. Melting point: 67.3-67.9 $^\circ\text{C}$.

2-(4'-bromophenyl)-2-oxoethyl (*S*)-2-(hydroxymethyl)butanoate (*S*-**4b**)

The title compound was prepared using MBP-YfaU W23V variant and the procedure described for *S*-**4a** (2.0 mmol scale, 2.0 mL total volume). The product was obtained as a white solid (512 mg, 81%). $[\alpha]_{\text{D}}^{20}$ = + 4.3 (c = 1 in MeOH). Chiral HPLC analysis: CHIRALPAK[®] IC, isocratic elution hexane/isopropanol 80/20 (v/v), flow rate 0.7 mL min^{-1} , t_{r} (*S*) = 19.85, t_{r} (*R*) = 22.47, 99% *ee*.

2-(4'-bromophenyl)-2-oxoethyl (*R*)-2-(hydroxymethyl)butanoate (*R*-**4b**)

The title compound was prepared using KPHMT wild-type and the procedure described for *R*-**4a** (0.8 mmol scale, 4.0 mL total volume). The product was obtained as a white solid (157 mg, 63%). $[\alpha]_{\text{D}}^{20}$ = − 5.2 (c = 1 in MeOH). Chiral HPLC analysis isocratic elution: CHIRALPAK[®] IC, hexane/isopropanol 80/20 (v/v), flow rate 0.7 mL min^{-1} , t_{r} (*S*) = 19.85, t_{r} (*R*) = 22.47, 95% *ee*.

NMR of *S*-**4b** and *R*-**4b**: ^1H NMR (400 MHz, CDCl_3) δ 7.77 (d, J = 8.7 Hz, 2H), 7.63 (d, J = 8.7 Hz, 2H), 5.43 (d, J = 16.5 Hz, 1H), 5.34 (d, J = 16.5 Hz, 1H), 3.89 – 3.78 (m, 2H), 2.69 (dtd, J = 8.0, 6.7, 6.5, 5.1 Hz, 1H), 1.84 – 1.68 (m, 1H), 1.66 – 1.51 (m, 1H),

0.99 (t, $J = 2 \times 7.5$ Hz, 3H). ^{13}C NMR (101 MHz, CDCl_3) δ 192.1, 174.2, 132.4, 132.3, 129.6, 129.3, 65.7, 63.7, 50.0, 21.2, 11.8.

ESI-TOF m/z for *S* and *R*-**4b**: Calcd for $[2\text{M}+\text{Na}^+]$ $\text{C}_{26}\text{H}_{30}\text{O}_8\text{NaBr}_2$: 651.0205, found $[2\text{M}+\text{Na}^+]$: 651.0229. Melting point: 51.3-51.7 °C.

2-(4'-bromophenyl)-2-oxoethyl (*S*)-2-(hydroxymethyl)-3-methylbutanoate (*S*-4c)

The title compound was prepared using MBP-YfaU W23V variant and the procedure described for *S*-**4a** (2.0 mmol scale, 2.0 mL total volume). The product was obtained as a white solid (488 mg, 75%). $[\alpha]_D^{20} = -6.1$ ($c = 4$ in CHCl_3). Chiral HPLC analysis: CHIRALPAK[®] ID, isocratic elution hexane/isopropanol 75/25 (v/v), flow rate 0.7 mL min^{-1} , t_r (*R*) = 19.51, t_r (*S*) = 22.33, 99% *ee*.

2-(4'-bromophenyl)-2-oxoethyl (*R*)-2-(hydroxymethyl)-3-methylbutanoate (*R*-4c)

The title compound was prepared using KPHMT I202A variant and the procedure described for *R*-**4a** (2.0 mmol scale, 20 mL total volume). The product was obtained as a white solid (321 mg, 49%). $[\alpha]_D^{20} = +5.1$ ($c = 4$ in CHCl_3). Chiral HPLC analysis: CHIRALPAK[®] ID, isocratic elution hexane/isopropanol 75/25 (v/v), flow rate 0.7 mL min^{-1} , t_r (*R*) = 19.51, t_r (*S*) = 22.33, 92% *ee*.

NMR of *S*-**4c** and *R*-**4c**: ^1H NMR (400 MHz, CDCl_3) δ 7.77 (d, $J = 8.6$ Hz, 2H), 7.63 (d, $J = 8.5$ Hz, 2H), 5.46 (d, $J = 16.5$ Hz, 1H), 5.32 (d, $J = 16.5$ Hz, 1H), 4.06 – 3.69 (m, 2H), 2.54 (td, $J = 2 \times 8.4$, 4.2 Hz, 1H), 2.22 – 1.74 (m, 1H), 1.01 (d, $J = 2.2$ Hz, 3H), 0.99 (d, $J = 2.2$ Hz, 3H). ^{13}C NMR (101 MHz, CDCl_3) δ 192.1, 173.9, 132.5, 132.3, 129.6, 129.3, 65.6, 62.4, 55.6, 27.4, 21.0, 20.1.

ESI-TOF m/z for *S*-**4c** and *R*-**4c**: Calcd for $[M+Na^+]$ $C_{14}H_{17}O_4NaBr$: 351.0208, found $[M+Na^+]$: 351.0159. Melting point: 82.0-82.9 °C.

2-(4'-bromophenyl)-2-oxoethyl (S)-2-(hydroxymethyl)pentanoate (*S*-4d**)**

The title compound was prepared using MBP-YfaU W23V variant and the procedure described for *S*-**4a** (3.0 mmol scale, 3.0 mL total volume). The product was obtained as a white solid (669 mg, 68%). $[\alpha]_D^{20} = +2.8$ ($c = 2$ in MeOH). Chiral HPLC analysis: CHIRALPAK[®] IC, isocratic elution hexane/isopropanol 75/25 (v/v), flow rate 0.7 mL min^{-1} , t_r (*S*) = 16.13, t_r (*R*) = 18.81, 99% *ee*.

2-(4'-bromophenyl)-2-oxoethyl (R)-2-(hydroxymethyl)pentanoate (*R*-4d**)**

The title compound was prepared using KPHMT I212A variant and the procedure described for *R*-**4a** (1.5 mmol scale, 15 mL total volume). The product was obtained as a white solid (291 mg, 60%). $[\alpha]_D^{20} = -2.9$ ($c = 2$ in MeOH). Chiral HPLC analysis: CHIRALPAK[®] IC, isocratic elution hexane/isopropanol 75/25 (v/v), flow rate 0.7 mL min^{-1} , t_r (*S*) = 16.13, t_r (*R*) = 18.81, 95% *ee*.

NMR of *S*-**4d** and *R*-**4d**: 1H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, $J = 8.5$ Hz, 2H), 7.63 (d, $J = 8.4$ Hz, 2H), 5.42 (d, $J = 16.5$ Hz, 1H), 5.34 (d, $J = 16.5$ Hz, 1H), 3.86 – 3.80 (m, 2H), 2.77 (dq, $J = 8.3, 6.4, 2 \times 6.1$ Hz, 1H), 1.71 (dq, $J = 13.2, 7.6, 2 \times 7.3$ Hz, 1H), 1.49 (ddt, $J = 12.9, 9.6, 2 \times 6.3$ Hz, 1H), 1.44 – 1.34 (m, 2H), 0.93 (t, $J = 2 \times 7.2$ Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 192.2, 174.4, 132.4, 132.3, 129.6, 129.3, 65.7, 64.0, 48.2, 30.0, 20.5, 13.9.

ESI-TOF m/z for *S*-**4d** and *R*-**4d**: Calcd for $[M+Na^+]$ $C_{14}H_{17}O_4NaBr$: 351.0208, found $[M+Na^+]$: 351.0159. Melting point: 38.1-38.2 °C.

2-(4'-bromophenyl)-2-oxoethyl (S)-2-(hydroxymethyl)hexanoate (S-4e)

The title compound was prepared using MBP-YfaU W23V variant and the procedure described for *S*-**4a** (3.0 mmol scale, 5.0 mL total volume). The product was obtained as a white solid (650 mg, 65%). $[\alpha]_D^{20} = -1.9$ ($c = 4$ in iPrOH). Chiral HPLC analysis: CHIRALPAK[®] IC, isocratic elution hexane/isopropanol 75/25 (v/v), flow rate 0.7 mL min⁻¹, t_r (*S*) = 15.93, t_r (*R*) = 19.09, 99% *ee*.

2-(4'-bromophenyl)-2-oxoethyl (R)-2-(hydroxymethyl)hexanoate (R-4e)

The title compound was prepared using the enzyme variant KPHMT L42A/I212A and the procedure described for *R*-**4a** (1.5 mmol scale, 15 mL total volume). The product was obtained as a white solid (291 mg, 57%). $[\alpha]_D^{20} = +2.2$ ($c = 4$ in iPrOH). Chiral HPLC analysis: CHIRALPAK[®] IC, isocratic elution hexane/isopropanol 75/25 (v/v), flow rate 0.7 mL min⁻¹, t_r (*S*) = 15.93, t_r (*R*) = 19.09, 93% *ee*.

NMR of *S*-**4e** and *R*-**4e**: ¹H NMR (400 MHz, CDCl₃) δ 7.77 (d, $J = 8.7$ Hz, 2H), 7.63 (d, $J = 8.7$ Hz, 2H), 5.43 (d, $J = 16.5$ Hz, 1H), 5.34 (d, $J = 16.5$ Hz, 1H), 3.90 – 3.76 (m, 2H), 2.75 (dtd, $J = 7.9, 2 \times 6.6, 5.3$ Hz, 1H), 1.72 (dtd, $J = 15.4, 8.2, 7.9, 4.2$ Hz, 1H), 1.59 – 1.43 (m, 1H), 1.4 – 1.3 (m, 4H), 0.89 (t, $J = 2 \times 6.9$ Hz, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 192.2, 174.4, 132.4, 132.3, 129.6, 129.3, 65.7, 64.0, 48.5, 29.4, 27.6, 22.6, 13.9.

ESI-TOF m/z for *S*-**4e** and *R*-**4e**: Calcd for $[M+Na]^+$ C₁₅H₁₉O₄NaBr: 365.0379, found $[M+Na]^+$: 365.0364. Melting point: 72.1-73.5 °C.

2-(4'-bromophenyl)-2-oxoethyl (S)-2-(hydroxymethyl)pent-4-enoate (S-4f)

The title compound was prepared using MBP-YfaU W23V variant and the procedure described for *S*-4a (3.0 mmol scale, 3.0 mL total volume). The product was obtained as a white solid (719 mg, 73%). $[\alpha]_D^{20} = -2.8$ ($c = 2$ in iPrOH). Chiral HPLC analysis: CHIRALPAK[®] IC, isocratic elution hexane/isopropanol 80/20 (v/v), flow rate 0.7 mL min⁻¹, t_r (*S*) = 22.28, t_r (*R*) = 24.55, 98% *ee*.

2-(4'-bromophenyl)-2-oxoethyl (*R*)-2-(hydroxymethyl)pent-4-enoate (*R*-4f)

The title compound was prepared using KPHMT I212A variant and the procedure described for *R*-4a (2.0 mmol scale, 10 mL total volume). The product was obtained as a white solid (445 mg, 68%). $[\alpha]_D^{20} = +2.8$ ($c = 2$ in iPrOH). Chiral HPLC analysis: CHIRALPAK[®] IC, isocratic elution hexane/isopropanol 80/20 (v/v), flow rate 0.7 mL min⁻¹, t_r (*S*) = 22.28, t_r (*R*) = 24.55, 96% *ee*.

NMR of *S*-4f and *R*-4f: ¹H NMR (400 MHz, CDCl₃) δ 7.77 (d, $J = 8.3$ Hz, 2H), 7.63 (d, $J = 8.3$ Hz, 2H), 5.80 (ddt, $J = 17.2, 10.1, 2 \times 6.9$ Hz, 1H), 5.46 (d, $J = 16.5$ Hz, 1H), 5.31 (d, $J = 16.5$ Hz, 1H), 5.12 (dq, $J = 17.0, 3 \times 1.7$ Hz, 1H), 5.06 (dq, $J = 10.4, 1.4, 2 \times 1.3$ Hz, 1H), 3.90 (dd, $J = 11.4, 4.2$ Hz, 1H), 3.83 (dd, $J = 11.5, 7.3$ Hz, 1H), 2.84 (qd, $J = 3 \times 7.2, 4.3$ Hz, 1H), 2.51 (dt, $J = 14.3, 2 \times 6.9$ Hz, 1H), 2.32 (dt, $J = 14.5, 2 \times 7.0$ Hz, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 192.1, 173.6, 134.8, 132.4, 129.7, 129.3, 117.3, 65.8, 63.4, 47.8, 32.1.

ESI-TOF m/z for *S*-4f and *R*-4f: Calcd for $[M+Na]^+$ C₁₄H₁₅O₄NaBr: 349.0051, found $[M+Na]^+$: 349.0070. Melting point: 50.4-50.6 °C.

2-(4'-bromophenyl)-2-oxoethyl (*S*)-2-(hydroxymethyl)hex-5-enoate (*S*-4g)

The title compound was prepared using MBP-YfaU W23V variant and the procedure described for *S*-**4a** (2.4 mmol scale, 3.0 mL total volume). The product was obtained as a white solid (563 mg, 69%). $[\alpha]_D^{20} = -8.4$ ($c = 4$ in iPrOH). Chiral HPLC analysis: CHIRALPAK[®] IC, isocratic elution hexane/isopropanol 80/20 (v/v), flow rate 0.7 mL min⁻¹, t_r (*S*) = 18.68, t_r (*R*) = 22.56, 95% *ee*.

2-(4'-bromophenyl)-2-oxoethyl (*R*)-2-(hydroxymethyl)hex-5-enoate (*R*-4g)

The title compound was prepared using KPHMT I212A variant and the procedure described for *R*-**4a** (2.0 mmol scale, 10.0 mL total volume). The product was obtained as a white solid (467 mg, 68%). $[\alpha]_D^{20} = +8.2$ ($c = 4$ in iPrOH). Chiral HPLC analysis: CHIRALPAK[®] IC, isocratic elution hexane/isopropanol 80/20 (v/v), flow rate 0.7 mL min⁻¹, t_r (*S*) = 18.68, t_r (*R*) = 22.56, 97% *ee*.

NMR of *S*-**4g** and *R*-**4g**: ¹H NMR (400 MHz, CDCl₃) δ 7.77 (d, $J = 8.4$ Hz, 2H), 7.64 (d, $J = 8.4$ Hz, 2H), 5.78 (ddt, $J = 16.9, 10.2, 2 \times 6.7$ Hz, 1H), 5.44 (d, $J = 16.5$ Hz, 1H), 5.33 (d, $J = 16.5$ Hz, 1H), 5.04 (dq, $J = 17.2, 3 \times 1.6$ Hz, 1H), 4.99 (dtd, $J = 10.1, 2 \times 1.7, 0.9$ Hz, 1H), 3.98 – 3.70 (m, 2H), 2.78 (tdd, $J = 2 \times 7.8, 6.1, 4.3$ Hz, 1H), 2.15 (dt, $J = 9.0, 2 \times 6.6$ Hz, 2H), 1.86 (dq, $J = 13.5, 3 \times 7.7$ Hz, 1H), 1.62 (dtd, $J = 13.9, 2 \times 7.7, 6.0$ Hz, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 192.1, 174.1, 137.5, 132.4, 129.7, 129.3, 115.5, 65.7, 63.9, 47.6, 31.3, 27.0.

ESI-TOF m/z for *S*-**4g** and *R*-**4g**: Calcd for $[M+Na^+]$ C₁₅H₁₇O₄NaBr: 363.0235, found $[M+Na^+]$: 363.0208. Melting point: 44.6-45.2 °C.

2-(4'-bromophenyl)-2-oxoethyl (*S*)-2-(hydroxymethyl)hept-6-enoate (*S*-4h)

The title compound was prepared using MBP-YfaU W23V variant and the procedure described for *S*-**4a** (3.0 mmol scale, 5.0 mL total volume). The product was obtained as a white solid (723 mg, 68%). $[\alpha]_D^{20} = -3.5$ ($c = 4$ in *i*PrOH). Chiral HPLC analysis: CHIRALPAK[®] IC, isocratic elution hexane/isopropanol 80/20 (v/v), flow rate 0.7 mL min⁻¹, t_r (*S*) = 17.91, t_r (*R*) = 21.79, 78% *ee*.

2-(4'-bromophenyl)-2-oxoethyl (*R*)-2-(hydroxymethyl)hept-6-enoate (*R*-4h)

The title compound was prepared using KPHMT L42A/I212A variant and the procedure described for *R*-**4a** (2.0 mmol scale, 10 mL total volume). The product was obtained as a white solid (454 mg, 64%). $[\alpha]_D^{20} = +4.1$ ($c = 4$ in *i*PrOH). Chiral HPLC analysis: CHIRALPAK[®] IC, isocratic elution hexane/isopropanol 80/20 (v/v), flow rate 0.7 mL min⁻¹, t_r (*S*) = 17.91, t_r (*R*) = 21.79, 93% *ee*.

NMR of *S*-**4h** and *R*-**4h**: ¹H NMR (400 MHz, CDCl₃) δ 7.77 (d, $J = 8.6$ Hz, 2H), 7.64 (d, $J = 8.6$ Hz, 2H), 5.78 (ddt, $J = 16.9, 10.2, 2 \times 6.7$ Hz, 1H), 5.43 (d, $J = 16.5$ Hz, 1H), 5.34 (d, $J = 16.5$ Hz, 1H), 5.00 (dq, $J = 17.1, 3 \times 1.6$ Hz, 1H), 4.95 (ddt, $J = 10.7, 2.3, 2 \times 1.1$ Hz, 1H), 3.90 – 3.77 (m, 2H), 2.76 (ddt, $J = 10.8, 7.3, 2 \times 3.8$ Hz, 1H), 2.07 (q, $J = 2 \times 6.9, 6.8$ Hz, 2H), 1.73 (ddd, $J = 15.0, 9.4, 5.8$ Hz, 1H), 1.55 (dt, $J = 8.9, 2 \times 6.4$ Hz, 1H), 1.53 – 1.40 (m, 2H). ¹³C NMR (101 MHz, CDCl₃) δ 192.1, 174.2, 138.2, 132.4, 132.3, 129.7, 129.3, 114.9, 65.7, 64.0, 48.3, 33.5, 27.4, 26.5.

ESI-TOF *m/z* for *S*-**4h** and *R*-**4h**: Calcd for $[M+Na]^+$ C₁₆H₁₉O₄NaBr: 377.0362, found $[M+Na]^+$: 377.0364. Melting point: 60.3-60.6 °C.

2-(4'-bromophenyl)-2-oxoethyl (*S*)-2-cyclopropyl-3-hydroxypropanoate (*S*-4i)

The title compound was prepared using MBP-YfaU W23V variant and the procedure described for *S*-4a (3.0 mmol scale, 3.0 mL total volume). The product was obtained as a white solid (717 mg, 73%). $[\alpha]_D^{20} = -25.4$ ($c = 4$ in iPrOH). Chiral HPLC analysis: CHIRALPAK[®] IC, isocratic elution hexane/isopropanol 80/20 (v/v), flow rate 1.0 mL min⁻¹, t_r (*S*) = 18.85, t_r (*R*) = 20.50, 98% *ee*.

2-(4'-bromophenyl)-2-oxoethyl (*R*)-2-cyclopropyl-3-hydroxypropanoate (*R*-4i)

The title compound was prepared using the enzyme KPHMT wild-type and the procedure described for *R*-4a (2.0 mmol scale, 5.0 mL total volume). The product was obtained as a white solid (444 mg, 68%). $[\alpha]_D^{20} = +23.2$ ($c = 4$ in iPrOH). Chiral HPLC analysis: CHIRALPAK[®] IC, isocratic elution hexane/isopropanol 80/20 (v/v), flow rate 1.0 mL min⁻¹, t_r (*S*) = 18.85, t_r (*R*) = 20.50, 87% *ee*.

NMR of *S*-4i and *R*-4i: ¹H NMR (400 MHz, CDCl₃) δ 7.78 (d, $J = 8.5$ Hz, 2H), 7.64 (d, $J = 8.5$ Hz, 2H), 5.43 (d, $J = 16.3$ Hz, 1H), 5.38 (d, $J = 16.3$ Hz, 1H), 4.06 – 3.84 (m, 2H), 2.00 (ddd, $J = 10.1, 7.6, 4.8$ Hz, 1H), 1.04 (dddd, $J = 13.0, 9.9, 8.1, 4.9$ Hz, 1H), 0.67 – 0.50 (m, 2H), 0.42 – 0.30 (m, 1H), 0.26 – 0.17 (m, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 192.1, 173.8, 132.4, 132.3, 129.6, 129.3, 65.7, 64.3, 53.5, 9.6, 3.9, 3.6.

ESI-TOF *m/z* for *S*-4i and *R*-4i: Calcd for $[M+Na^+]$ C₁₄H₁₅O₄NaBr: 349.0048, found $[M+Na^+]$: 349.0051. Melting point: 76.9-78.9 °C.

2-(4'-bromophenyl)-2-oxoethyl (*S*)-2-cyclobutyl-3-hydroxypropanoate (*S*-4j)

The title compound was prepared using MBP-YfaU W23V variant and the procedure described for *S*-**4a** (3.0 mmol scale, 3.8 mL total volume). The product was obtained as a white solid (814 mg, 80%). $[\alpha]_D^{20} = -12.1$ ($c = 3$ in iPrOH). Chiral HPLC analysis: CHIRALPAK[®] IC, isocratic elution hexane/isopropanol 95/5 (v/v), flow rate 1.0 mL min⁻¹, t_r (*S*) = 67.68, t_r (*R*) = 73.46, 98% *ee*.

2-(4'-bromophenyl)-2-oxoethyl (*R*)-2-cyclobutyl-3-hydroxypropanoate (*R*-4j**)**

The title compound was prepared using KPHMT I202A variant and the procedure described for *R*-**4a** (2.0 mmol scale, 5.0 mL total volume). The product was obtained as a white solid (328 mg, 48%). $[\alpha]_D^{20} = +7.2$ ($c = 3$ in iPrOH). Chiral HPLC analysis: CHIRALPAK[®] IC, isocratic elution hexane/isopropanol 95/5 (v/v), flow rate 1.0 mL min⁻¹, t_r (*S*) = 67.68, t_r (*R*) = 73.46, 75% *ee*.

NMR of *S*-**4j** and *R*-**4j**: ¹H NMR (400 MHz, CDCl₃) δ 7.76 (d, $J = 8.5$ Hz, 2H), 7.63 (d, $J = 8.4$ Hz, 2H), 5.40 (d, $J = 16.5$ Hz, 1H), 5.33 (d, $J = 16.5$ Hz, 1H), 3.79 (dd, $J = 11.4$, 4.0 Hz, 1H), 3.74 (dd, $J = 11.3$, 7.5 Hz, 1H), 2.76 (ddd, $J = 11.4$, 7.5, 4.1 Hz, 1H), 2.70 – 2.56 (m, 1H), 2.18 – 2.03 (m, 2H), 1.99 – 1.71 (m, 4H). ¹³C NMR (101 MHz, CDCl₃) δ 192.1, 173.3, 132.4, 132.3, 129.6, 129.3, 65.6, 62.0, 54.7, 33.9, 27.3, 26.9, 18.5.

ESI-TOF m/z for *S*-**4j** and *R*-**4j**: Calcd for $[M+Na^+]$ C₁₅H₁₇O₄NaBr: 363.0193, found $[M+Na^+]$: 363.0208. Melting point: 98.0-99.0 °C.

2-(4'-bromophenyl)-2-oxoethyl (*S*)-2-cyclohexyl-3-hydroxypropanoate (*S*-4k**)**

The title compound was prepared using MBP-YfaU W23V variant and the procedure described for *S*-**4a** (2 mmol scale, 6.7 mL total volume). The product was obtained as a white solid (542 mg, 73%). $[\alpha]_D^{20} = -1.8$ ($c = 2.4$ in iPrOH). Chiral HPLC analysis: CHIRALPAK[®] IC, isocratic elution hexane/isopropanol 95/5 (v/v), flow rate 1.0 mL min⁻¹, t_r (*S*) = 77.94, t_r (*R*) = 83.96, 96% *ee*. NMR: ¹H NMR (400 MHz, CDCl₃) δ 7.77

(d, $J = 8.7$ Hz, 2H), 7.63 (d, $J = 8.7$ Hz, 2H), 5.46 (d, $J = 16.5$ Hz, 1H), 5.30 (d, $J = 16.5$ Hz, 1H), 3.93 (dd, $J = 11.3, 8.7$ Hz, 1H), 3.86 (dd, $J = 11.3, 4.2$ Hz, 1H), 2.59 (td, $J = 2 \times 8.4, 4.2$ Hz, 1H), 1.91 – 1.57 (m, 6H), 1.38 – 0.95 (m, 5H). ^{13}C NMR (101 MHz, CDCl_3) δ 192.2, 173.9, 132.5, 132.3, 129.6, 129.3, 65.5, 62.1, 54.8, 36.8, 31.4, 30.4, 26.2. ESI-TOF m/z : Calcd for $[\text{M}+\text{Na}^+]$ $\text{C}_{17}\text{H}_{21}\text{O}_4\text{NaBr}$: 391.0540, found $[\text{M}+\text{Na}^+]$: 391.0521. Melting point: 118.9-119.1 $^\circ\text{C}$.

NMR spectra of 2-(4'-bromophenyl)-2-oxoethyl ester derivatives (4a-k)

The NMR spectra of the aldol adduct derivatives **4a-k** obtained with MBP-YfaU and KPHMT wild-type and variants were undistinguishable. For the sake of simplicity only the *S*-enantiomers are shown.

a)

b)

Figure S102. NMR spectra (CDCl_3) of *S*-4a: a) ^1H and b) ^{13}C .

a)

b)

Figure S103. NMR spectra (CDCl_3) of *S*-4b: a) ^1H and b) ^{13}C .

a)

b)

Figure S104. NMR spectra (CDCl_3) of *S*-4c: a) ^1H and b) ^{13}C .

a)

b)

Figure S105. NMR spectra (CDCl_3) of *S*-4d: a) ^1H and b) ^{13}C .

a)

b)

Figure S106. NMR spectra (CDCl_3) of *S*-4e: a) ^1H and b) ^{13}C .

a)

b)

Figure S107. NMR spectra (CDCl_3) of *S*-4f: a) ^1H and b) ^{13}C .

a)

b)

Figure S108. NMR spectra (CDCl_3) of *S*-4g: a) ^1H and b) ^{13}C .

a)

b)

Figure S109. NMR spectra (CDCl_3) of *S*-4h: a) ^1H and b) ^{13}C .

a)

b)

Figure S110. NMR spectra (CDCl_3) of *S*-4i: a) ^1H and b) ^{13}C .

a)

b)

Figure S111. NMR spectra (CDCl_3) of *S*-4j: a) ^1H and b) ^{13}C .

a)

b)

Figure S112. NMR spectra (CDCl_3) of *S*-4k: a) ^1H and b) ^{13}C .

Synthesis of benzyl and 4-nitrobenzyl ester derivatives

Enzymatic synthesis of benzyl and 4-nitrobenzyl ester of (*S*)-3-hydroxy-2-methylpropanoate (*S*-5a and *S*-6a)

The aldol additions catalyzed by MBP-YfaU wild-type and the subsequent oxidative decarboxylation of *S*-3a were performed as described for *S*-4a. Then, anhydrous DMF (50 mL) and CsCl (168 mg, 0.1 mmol, 0.2 eq) were added under N₂ atmosphere. The reactions were started by adding BnBr (654 mg, 5 mmol, 1 eq) or 4-NO₂C₆H₄CH₂Br (1.1 g, 5 mmol, 1 eq) and heated at 60 °C during 2 h. EtOAc (200 mL) was added to the reaction mixtures and washed with H₂O (3x50 mL). The organic phases were dried over anhydrous MgSO₄, absorbed onto silica gel (100 mL) and loaded onto a silica gel column. Products were eluted with a step gradient of hexane:AcOEt: 100:0, 200 mL, 90:10, 200 mL, 80:20, 200 mL, 70:30, 400 mL and 60:40, 800mL. Pure fractions were pooled, and the solvent removed under vacuum affording the title compounds.

Benzyl (*S*)-3-hydroxy-2-methylpropanoate (*S*-5a)

The title compound was obtained as a yellow oil (852 mg, 60% over three steps). $[\alpha]_{\text{D}}^{20} = +18.6$ ($c = 0.85$ in MeOH) [lit: $[\alpha]_{\text{D}}^{20} = -24.2$, $c = 0.85$ in MeOH, 90% *ee*, *R*].¹⁰ Chiral HPLC analysis: CHIRALPACK® IB, isocratic elution hexane/isopropanol 98/2 (v/v), flow rate 1 mL min⁻¹, t_{r} (*S*) = 18.64, t_{r} (*R*) = 19.89, > 99% *ee*. ¹H NMR (400 MHz, CDCl₃) δ 7.42 – 7.27 (m, 5H), 5.14 (s, 2H), 3.88 – 3.59 (m, 2H), 2.71 (pd, $J = 4 \times 7.2$, 4.8 Hz, 1H), 1.19 (d, $J = 7.2$ Hz, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 175.4, 135.8, 128.6, 128.3, 128.0, 66.4, 64.5, 41.8, 13.4. ESI-TOF *m/z*: Calcd for [M+H⁺] C₁₁H₁₅O₃: 195.1027, found [M+H⁺]:195.1021.

4-Nitrobenzyl (*S*)-3-hydroxy-2-methylpropanoate (*S*-6a)

The title compound was obtained as a colorless oil which crystalized as white solid after overnight storage at $-20\text{ }^{\circ}\text{C}$ (1.15 g, 66% over three steps). $[\alpha]_{\text{D}}^{20} = +13.6$ ($c = 1$ in CHCl_3). Chiral HPLC analysis: CHIRALPACK[®] IB, isocratic elution hexane/isopropanol 95/5 (v/v), flow rate 1 mL min^{-1} , $t_{\text{r}}(S) = 31.55$, $t_{\text{r}}(R) = 32.97$, 98% *ee*. ^1H NMR (400 MHz, CDCl_3) δ 7.78 (d, $J = 8.5\text{ Hz}$, 2H), 7.64 (d, $J = 8.5\text{ Hz}$, 2H), 5.43 (d, $J = 16.3\text{ Hz}$, 1H), 5.38 (d, $J = 16.3\text{ Hz}$, 1H), 4.06 – 3.84 (m, 2H), 2.00 (ddd, $J = 10.1, 7.6, 4.8\text{ Hz}$, 1H), 1.04 (m, 1H), 0.67 – 0.50 (m, 2H), 0.42 – 0.30 (m, 1H), 0.26 – 0.17 (m, 1H). ^{13}C NMR (101 MHz, CDCl_3) δ 192.1, 173.8, 132.4, 132.3, 129.6, 129.3, 65.7, 64.3, 53.5, 9.6, 3.9, 3.6. ESI-TOF m/z : Calcd for $[2\text{M}+\text{Na}^+]$ $\text{C}_{22}\text{H}_{26}\text{N}_2\text{O}_{10}\text{Na}$: 501.1508, found $[2\text{M}+\text{Na}^+]$: 501.1485. Melting point: $56.1\text{--}56.9\text{ }^{\circ}\text{C}$.

Synthesis of benzyl and 4-nitrobenzyl esters of (*R*)-3-hydroxy-2-methylpropanoate (*R*-5a and *R*-6a) and (*R*)-2-(hydroxymethyl)hex-5-enoate (*R*-5g)

Aldol products were synthesized with KPHMT wild-type (*R*-5a and *R*-6a) and KPHMT I212A (*R*-5g) following the procedure described above for *R*-4a. The esterifications were performed by adding anhydrous DMF (50 mL) and CsCl (210 mg, 1.25 mmol, 0.5 eq) under N_2 atmosphere. The reactions were started by adding BnBr (854 mg, 5 mmol, 2 eq) or $p\text{-NO}_2\text{C}_6\text{H}_4\text{CH}_2\text{Br}$ (1.1 g, 5 mmol, 2 eq) in the mixture and were conducted at $25\text{ }^{\circ}\text{C}$ overnight. EtOAc (200 mL) was added to the reaction mixtures and washed with H_2O (3x50 mL). The organic phases were dried over anhydrous MgSO_4 , absorbed onto silica gel (100 mL) and loaded onto a silica gel column. Products were eluted with a step gradient of hexane:AcOEt: 100:0, 200 mL, 90:10, 200 mL, 80:20, 200 mL, 70:30, 400 mL and 60:40, 800mL. Pure fractions were pooled, and the solvent removed under vacuum affording the title compounds.

Benzyl (*R*)-3-hydroxy-2-methylpropanoate (*R*-5a)

R-5a was obtained as a yellow oil (188 mg, 40% over three steps). $[\alpha]_D^{20} = -16.9$ ($c = 0.85$ in MeOH) [lit: $[\alpha]_D^{20} = -24.2$, $c = 0.85$ in MeOH, 90% *ee*, *R*].¹⁰ Chiral HPLC analysis: CHIRALPACK[®] IB, isocratic elution hexane/isopropanol 98/2 (v/v), flow rate 1 mL min⁻¹, t_r (*S*) = 18.64, t_r (*R*) = 19.89, > 99% *ee* (82% *ee* 2h at 60 °C). The spectral properties of this product are coincided with those of *S*-5a.

4-Nitrobenzyl (*R*)-3-hydroxy-2-methylpropanoate (*R*-6a)

R-6a was obtained as a colorless oil which crystalized as white solid after overnight storage at -20 °C (355 mg, 59% over three steps). $[\alpha]_D^{20} = -13.4$ ($c = 1$ in MeOH). Chiral HPLC analysis: CHIRALPACK[®] IB, isocratic elution hexane/isopropanol 95/5 (v/v), flow rate 1 mL min⁻¹, t_r (*S*) = 31.55, t_r (*R*) = 32.97, > 98% *ee*. The spectral properties of this product are coincided with those of *S*-6a.

Benzyl (*R*)-2-(hydroxymethyl)hex-5-enoate (*R*-5g)

R-5g was obtained as colorless oil (559 mg, 60% over three steps). $[\alpha]_D^{20} = +17.5$ ($c = 3$ in iPrOH). Chiral HPLC analysis: CHIRALPACK[®] ID, isocratic elution hexane/isopropanol 90/10 (v/v), flow rate 1 mL min⁻¹, t_r (*S*) = 8.15, t_r (*R*) = 8.94, 97% *ee*. ¹H NMR (400 MHz, CDCl₃) δ 7.42 – 7.29 (m, 5H), 5.74 (ddt, $J = 16.9, 10.2, 2 \times 6.7$ Hz, 1H), 5.17 (d, $J = 12.3$ Hz, 1H), 5.13 (d, $J = 12.3$ Hz, 1H), 5.05 – 4.89 (m, 2H), 3.81 – 3.72 (m, 2H), 2.64 (qd, $J = 2 \times 6.7, 6.6, 4.9$ Hz, 1H), 2.07 (m, 2H), 1.78 (dq, $J = 15.3, 7.6, 2 \times 7.5$ Hz, 1H), 1.63 (dq, $J = 14.1, 2 \times 7.6, 7.5$ Hz, 1H). ¹³C NMR (101 MHz, CDCl₃)

δ 175.0, 137.4, 128.6, 128.3, 128.1, 115.5, 66.4, 62.9, 46.7, 31.2, 27.5. ESI-TOF m/z:
Calcd for $[2M+Na^+]$ $C_{28}H_{36}O_6Na$: 491.2435, found $[2M+Na^+]$: 491.2410.

NMR spectra of benzyl and 4-NO₂ benzyl ester derivatives

The NMR spectra of enantiomeric aldol adducts derivatives obtained with MBP-YfaU and KPHMT variants were undistinguishable. For the sake of simplicity only the *S*-enantiomers are shown.

a)

b)

Figure S113. NMR spectra (CDCl_3) of *S*-5a: a) ^1H and b) ^{13}C .

a)

b)

Figure S114. NMR spectra (CDCl₃) of *S*-6a: a) ¹H and b) ¹³C.

a)

b)

Figure S115. NMR spectra (CDCl_3) of *R*-5g: a) ^1H and b) ^{13}C .

Synthesis of derivatives for absolute stereochemistry determination

Enzymatic synthesis of methyl (*S*)-2-(hydroxymethyl)butanoate (*S*-7b)

Typical procedure: The aldol addition catalyzed by MBP-YfaU W23V and the subsequent oxidative decarboxylation of **3b** was performed as described for *S*-4a (without adding glycerol for salt solubilization). The sodium salt of carboxylic acid was resuspended in MeOH (200 mL), the mixture was cooled down to $-80\text{ }^{\circ}\text{C}$ and SOCl_2 (1.46 mL, 20 mmol, 5eq) was added dropwise. After stirring for 12 h at rt, sodium bicarbonate (200 mL, 5%) was added and the MeOH was removed under vacuum. The aqueous solution was extracted with AcOEt (3x100 mL), the combined organic phases were dried over anhydrous MgSO_4 , filtered and the solvent was removed under vacuum. The product (yellow oil, 203 mg, 38%) was used without any further purification. $[\alpha]_{\text{D}}^{20} = -3.8$ ($c = 1$ in CHCl_3) [lit: $[\alpha]_{\text{D}}^{20} = -5.9$, $c = 1$ in CHCl_3 , >95% *ee*, *S*].¹¹ The spectral properties of this product agreed with those reported in the literature.¹² ^1H NMR (400 MHz, CDCl_3) δ 3.81 – 3.71 (m, 2H), 3.70 (s, 3H), 2.50 (qd, $J = 3 \times 7.2$, 4.5 Hz, 1H), 1.75 – 1.49 (m, 2H), 0.92 (t, $J = 2 \times 7.5$ Hz, 3H). ^{13}C NMR (101 MHz, CDCl_3) δ 175.74, 62.76, 51.68, 48.86, 21.62, 11.65.

Enzymatic synthesis of (*S*)-2-(hydroxymethyl) carboxylic acid (*S*-8c-e)

(*S*)-2-(Hydroxymethyl)-3-methylbutanoic acid (*S*-8c)

Typical procedure: The aldol addition catalyzed by MBP-YfaU W23V and the subsequent oxidative decarboxylation of **3c** was performed as described above (without adding glycerol for salt solubilization). After removing the MeOH under vacuum, H_2O

(200 mL) was added to the mixture. The solution was acidified at pH 1.0 (HCl 1 M) and saturated with NaCl. The product was extracted with Et₂O (3x100 mL) and the combined organic phases were dried over anhydrous MgSO₄, filtered and the solvent removed under vacuum. The product (yellow oil, 324 mg, 70%) was used without any further purification. $[\alpha]^{20}_{\text{D}} = +4.1$ ($c = 4$, in CHCl₃) [lit: $[\alpha]^{20}_{\text{D}} = -5.4$, $c = 4$ in CHCl₃, 98% *ee*, *R*].¹³ The spectral properties of this product agreed with those reported in the literature.¹³ ¹H NMR (400 MHz, CDCl₃) δ 3.86 (dd, $J = 11.1, 8.5$ Hz, 1H), 3.78 (dd, $J = 11.2, 4.0$ Hz, 1H), 2.42 (td, $J = 8.1, 7.8, 4.0$ Hz, 1H), 2.12 – 1.96 (m, 1H), 0.99 (d, $J = 4.5$ Hz, 3H), 0.97 (d, $J = 4.4$ Hz, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 180.0, 61.3, 54.0, 27.6, 20.5, 20.1.

(*S*)-2-(Hydroxymethyl)pentanoic acid (*S*-8d)

The title compound (yellow oil, 354 mg, 76%) was prepared following the procedure described for *S*-8c. $[\alpha]^{20}_{\text{D}} = +2.8$ ($c = 10$ in CHCl₃) [lit:¹³9, $[\alpha]^{20}_{\text{D}} = -3.0$, $c = 10$ in CHCl₃, 92% *ee*, *R*]. The spectral properties of this product agreed with those reported in the literature.¹³ ¹H NMR (400 MHz, CDCl₃) δ 3.82 – 3.73 (m, 2H), 2.62 (p, $J = 2 \times 7.0, 2 \times 6.9$ Hz, 1H), 1.65 (dq, $J = 13.7, 8.0, 2 \times 7.8$ Hz, 1H), 1.51 (dq, $J = 13.4, 2 \times 6.9, 6.5$ Hz, 1H), 1.38 (h, $J = 3 \times 7.5, 2 \times 7.3$ Hz, 2H), 0.92 (t, $J = 2 \times 7.3$ Hz, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 180.6, 62.9, 47.1, 30.3, 20.4, 13.9.

(*S*)-2-(Hydroxymethyl)hexanoic acid (*S*-8e)

The title compound (yellow oil, 164 mg, 75%) was prepared following the procedure described for *S*-8c. $[\alpha]^{20}_{\text{D}} = -5.9$ ($c = 1$ in MeOH) [lit:¹⁴ $[\alpha]^{20}_{\text{D}} = +6.5$, $c = 1$ in MeOH, 99% *ee*, *R*]. The spectral properties of this product agreed with those reported in the literature.¹⁴ ¹H NMR (400 MHz, CDCl₃) δ 3.80 – 3.74 (m, 2H), 2.60 (m, 1H), 1.67 (dq, $J = 15.0, 7.4, 2 \times 7.3$ Hz, 1H), 1.54 (dq, $J = 13.1, 2 \times 7.1, 6.4$ Hz, 1H), 1.38 – 1.25 (m,

4H), 0.89 (t, $J = 2 \times 7.0$ Hz, 3H). ^{13}C NMR (101 MHz, CDCl_3) δ 180.5, 62.9, 47.3, 29.3, 27.9, 22.5, 13.8.

(S)-2-(Hydroxymethyl)pent-4-enoic acid (*S*-8f)

The title compound (yellow oil, 261 mg, 67%) was prepared following the procedure described for *S*-8c. ^1H NMR (400 MHz, CDCl_3) δ 5.77 (ddt, $J = 17.5, 10.2, 2 \times 7.4$ Hz, 1H), 5.17 – 4.88 (m, 1H), 3.79 (d, $J = 5.8$ Hz, 2H), 2.75 – 2.64 (m, 1H), 2.52 – 2.40 (m, 1H), 2.39 – 2.27 (m, 1H). ^{13}C NMR (101 MHz, CDCl_3) δ 179.5, 134.4, 117.7, 62.3, 46.7, 32.4.

Catalytic hydrogenation of compounds *S*-8f and *R*-5g

Product *S*-8f (120 mg, 1 mmol) was resuspended with Et_2O (100 mL), the solution was saturated with N_2 and Pd/C (191 mg, 10 mol%, 10% Pd, 50% humidity) was added keeping the reaction under N_2 . Then, H_2 (1 atm) was added under stirring and the reaction was left overnight at room temperature. The crude material was filtered through Celite[®] and the pellet was washed with Et_2O (3x50 mL). Then, the solvent was removed under vacuum obtaining the product *S*-8d (yellow oil, 108 mg, 91%) that was used without any further purification. $[\alpha]_{\text{D}}^{20} = +1.8$ ($c = 10$ in CHCl_3) [lit.¹³ $[\alpha]_{\text{D}}^{20} = -3.0$, $c = 10$ in CHCl_3 , 92% *ee*, *R*]. The spectral properties of this product agreed with *S*-8d and with those reported in the literature.¹³

The title compound (yellow oil, 145 mg, 94%) was prepared following the procedure described for *S*-8f. $[\alpha]_{\text{D}}^{20} = +6.0$ ($c = 1$ in MeOH) [lit: $[\alpha]_{\text{D}}^{20} = +6.5$, $c = 1$ in MeOH,

99% *ee*, *R*].¹⁴ The spectral properties of this product agreed with *S-8e* and with those reported in the literature.¹⁴

NMR spectra of carboxylic acid methyl esters and carboxylic acid derivatives

a)

b)

Figure S116. NMR spectra (CDCl_3) of *S*-7b: a) ^1H and b) ^{13}C .

a)

b)

Figure S117. NMR spectra (CDCl_3) of *S*-8c: a) ^1H and b) ^{13}C .

a)

b)

Figure S118. NMR spectra (CDCl_3) of *S*-8d: a) ^1H and b) ^{13}C .

a)

b)

Figure S119. NMR spectra (CDCl_3) of *S*-8e: a) ^1H and b) ^{13}C .

a)

b)

Figure S120. NMR spectra (CDCl_3) of *S*-8f: a) ^1H and b) ^{13}C .

Synthesis of racemic mixtures of products 4b-k

Typical procedure: Reactions (3 mL total volume) were conducted at room temperature in a round-bottom flask (100 mL) with magnetic stirring (250 rpm). Substrates **1b-k** (3 mmol, 1 eq, 1 M in the reaction) were dissolved in sodium phosphate buffer (0.15 mL of a 1 M stock solution at pH 7.0, 50 mM in the reaction) and water was added. The reactions were started by adding NiCl₂ (30 μL of a 0.1 M stock solution in water, 1 mM in the reaction) and methanal (**2**, 0.225 mL of a 13.3 M commercial solution, 3 mmol, 1 eq, 1 M in the reaction). The reactions were monitored by HPLC until no evolution of substrates and products were detected. Decarboxylation of aldol adducts, and the subsequent esterification were performed as described for the enzymatic reaction. The NMR spectra and properties of racemic aldol adducts (**rac-4b-k**) were undistinguishable from those obtained with MBP-YfaU and KPHMT variants.

Figure S121. Reaction time course of the aldol addition of a) **1b**, b) **1c** c) **1d**, d) **1e**, e) **1f**, f) **1g**, g) **1h**, h) **1i**, i) **1j** and j) **1k** to methanal (**2**) catalyzed by Ni²⁺ (1 mM) in sodium phosphate buffer (50 mM, pH 7.0) and control experiment. Control experiment performed under identical conditions of the enzymatic reactions but without enzyme.

Synthesis of racemic mixtures of products *rac*-5a and *rac*-6a

Typical procedure: Aldol addition reaction was performed as described for the racemic phenacyl ester products. Decarboxylation of aldol adducts, and the subsequent esterification were performed as described for the enzymatic reaction. The spectral properties of the racemic products (*rac*-5,6) agreed with the enzymatically prepared samples.

Determination of absolute configuration

The absolute stereochemistry of compounds *R* and *S*-4a was unequivocally determined using chiral HPLC. The phenacyl ester of each pure enantiomer was synthesized from commercial (*R*) and (*S*)- β -hydroxyisobutyric acid sodium salt. The phenacyl esters were processed and purified as described for the enzymatic reactions. The NMR spectra were undistinguishable of those from enzymatic synthesis.

The absolute stereochemistry of *R* and *S*-4b-g was assigned by transforming the aldol adduct *S*-3b into β -hydroxy carboxylic methyl ester *S*-7b and *S*-3c-f and *R*-5g into β -hydroxy carboxylic acids *S*-8c-f and *R*-8e, respectively. The sign of specific rotation was compared with the corresponding literature values.

Table S7: Sign of specific rotation of compounds *S*-3b-e and *R*-3g compared with the corresponding literature values.

Aldol adduct	This work ^a [α] ²⁰ _D	Literature values [α] ²⁰ _D
<i>S</i> -3b transformed into <i>S</i> -7b	− 3.8 (<i>S</i> -7b)	− 5.9 (<i>S</i>) (>95% <i>ee</i>) ¹¹
<i>S</i> -3c transformed into <i>S</i> -8c	+ 4.1 (<i>S</i> -8c)	− 5.4 (<i>R</i>) (98% <i>ee</i>) ¹³
<i>S</i> -3d transformed into <i>S</i> -8d	+ 2.8 (<i>S</i> -8d)	− 3.0 (<i>R</i>) (92% <i>ee</i>) ¹³
<i>S</i> -3f transformed into <i>S</i> -8d	+ 1.8 (<i>S</i> -8d)	
<i>S</i> -3e transformed into <i>S</i> -8e	− 5.9 (<i>S</i> -8e)	+ 6.5 (<i>R</i>) (99% <i>ee</i>) ¹⁴
<i>R</i> -3g transformed into <i>R</i> -8e	+ 6.0 (<i>R</i> -8e)	

^aDifferences between literature values may be due to the presence of solvent in the pure product.

X-ray structure determinations

For compounds **4i-k**, the absolute stereochemistry was determined by X-ray diffraction. Crystallization: compound *S-4i* (100 mg) was dissolved in (iPr)₂O (3 mL) and *S-4j,k* (100 mg) were dissolved in Et₂O (2 mL and 3 mL respectively). Crystals were obtained by evaporation in glass vials (6 mL, 4.5 cm, Ø 1.3 cm) after 24 h at 25 °C. Based on the high stereopreference observed for the enzymes MBP-YfaU and KPHMT, the absolute configuration of products *S-4h* and *R-4h* respectively was inferred.

Crystallographic methods

Data for all structures were collected on a STOE IPDS II two-circle diffractometer with a Genix Microfocus tube with mirror optics using MoK α radiation ($\lambda = 0.71073$ Å). The data were scaled using the frame scaling procedure in the *X-AREA* program system (Stoe & Cie, 2002). The structures were solved by direct methods using the program *SHELXS* (Sheldrick, 2008) and refined against F^2 with full-matrix least-squares techniques using the program *SHELXL* (Sheldrick, 2008).

S-4i shows pseudo-symmetry. The *ADDSYM* routine in *PLATON*¹⁵ detects that 94% of the structure fulfill the symmetry of the space group *P2₁/c*. However, refinement in this space group results in disorder and requires that the crystal would contain a racemic mixture. The Flack-x-parameter of 0.008(12), on the other hand, clearly indicates that the crystal contains only one enantiomer. The H atoms bonded to O were isotropically refined with the O-H distances restrained to 0.84(1) Å.

In *S-4j*, the H atoms bonded to O were isotropically refined with the O-H distances restrained to 0.84(2) Å. The absolute configuration could be unequivocally determined: Flack-x-parameter = 0.01(5).

In *S-4k*, the H atoms bonded to O were isotropically refined with the O-H distances restrained to 0.84(4) Å. The absolute configuration could be unequivocally determined: Flack-x-parameter = -0.009(7).

CCDC reference numbers: CCDC-1901294 (*S-4i*), CCDC-1901295 (*S-4j*), CCDC-1901296 (*S-4k*).

Stoe & Cie, *X-AREA*. Diffractometer control program system. Stoe & Cie, Darmstadt, Germany, 2002.¹⁵⁻¹⁶

Table S8. Crystal data and structure refinement for *S-4i*.

Empirical formula	$\text{C}_{14} \text{H}_{15} \text{Br O}_4$	
Formula weight	327.17	
Temperature	173(2) K	
Wavelength	0.71073 Å	
Crystal system	Monoclinic	
Space group	$P 2_1$	
Unit cell dimensions	$a = 12.2875(7)$ Å	$\alpha = 90^\circ$.
	$b = 8.8923(4)$ Å	$\beta = 98.116(4)^\circ$.
	$c = 12.7600(6)$ Å	$\gamma = 90^\circ$.
Volume	1380.25(12) Å ³	
Z	4	
Density (calculated)	1.574 Mg/m ³	
Absorption coefficient	2.985 mm ⁻¹	
F(000)	664	
Crystal size	0.210 x 0.180 x 0.160 mm ³	
Theta range for data collection	3.350 to 27.593°.	
Index ranges	$-16 \leq h \leq 15$, $-11 \leq k \leq 11$, $-16 \leq l \leq 16$	
Reflections collected	21734	
Independent reflections	6367 [R(int) = 0.0413]	
Completeness to theta = 25.000°	99.7 %	
Absorption correction	Semi-empirical from equivalents	
Max. and min. transmission	1.000 and 0.644	
Refinement method	Full-matrix least-squares on F ²	
Data / restraints / parameters	6367 / 3 / 351	
Goodness-of-fit on F ²	1.046	
Final R indices [I > 2sigma(I)]	R1 = 0.0429, wR2 = 0.1010	
R indices (all data)	R1 = 0.0539, wR2 = 0.1060	
Absolute structure parameter	0.008(12)	
Largest diff. peak and hole	0.844 and -0.772 e.Å ⁻³

Figure S122. Perspective view of molecule 1 in the asymmetric unit of *S-4i*. Displacement ellipsoids are drawn at the 50 % probability level. The dashed line indicates a hydrogen bond.

Figure S123. Perspective view of molecule 2 in the asymmetric unit of *S-4i*. Displacement ellipsoids are drawn at the 50 % probability level. The dashed line indicates a hydrogen bond.

Table S9. Crystal data and structure refinement for *S-4j*.

Empirical formula	$\text{C}_{15} \text{H}_{17} \text{Br O}_4$	
Formula weight	341.20	
Temperature	173(2) K	
Wavelength	0.71073 Å	
Crystal system	Triclinic	
Space group	$P 1$	
Unit cell dimensions	$a = 6.9684(9) \text{ Å}$	$\alpha = 93.786(10)^\circ$.
	$b = 8.3965(12) \text{ Å}$	$\beta = 98.075(9)^\circ$.
	$c = 14.2033(15) \text{ Å}$	$\gamma = 112.946(10)^\circ$.
Volume	$751.01(17) \text{ Å}^3$	
Z	2	
Density (calculated)	1.509 Mg/m^3	
Absorption coefficient	2.746 mm^{-1}	
F(000)	348	
Crystal size	$0.230 \times 0.170 \times 0.140 \text{ mm}^3$	
Theta range for data collection	3.294 to 25.699° .	
Index ranges	$-8 \leq h \leq 8$, $-9 \leq k \leq 10$, $-16 \leq l \leq 17$	
Reflections collected	5635	
Independent reflections	4464 [$R(\text{int}) = 0.0455$]	
Completeness to $\theta = 25.000^\circ$	99.1 %	
Absorption correction	Semi-empirical from equivalents	
Max. and min. transmission	1.000 and 0.429	
Refinement method	Full-matrix least-squares on F^2	
Data / restraints / parameters	4464 / 5 / 367	
Goodness-of-fit on F^2	0.999	
Final R indices [$I > 2\sigma(I)$]	$R1 = 0.0549$, $wR2 = 0.1336$	
R indices (all data)	$R1 = 0.0679$, $wR2 = 0.1402$	
Absolute structure parameter	0.002(17)	
Largest diff. peak and hole	0.468 and -0.453 e.Å^{-3}

Figure S124. Perspective view of molecule 1 in the asymmetric unit of *S-4j*. Displacement ellipsoids are drawn at the 50 % probability level. The dashed line indicates a hydrogen bond.

Figure S125. Perspective view of molecule 2 in the asymmetric unit of *S-4j*. Displacement ellipsoids are drawn at the 50 % probability level. The dashed line indicates a hydrogen bond.

Table S10. Crystal data and structure refinement for **S-4k**.

Empirical formula	C ₁₇ H ₂₁ Br O ₄	
Formula weight	369.25	
Temperature	173(2) K	
Wavelength	0.71073 Å	
Crystal system	Orthorhombic	
Space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁	
Unit cell dimensions	a = 9.2251(3) Å	α = 90°.
	b = 11.8147(4) Å	β = 90°.
	c = 30.5485(11) Å	γ = 90°.
Volume	3329.5(2) Å ³	
Z	8	
Density (calculated)	1.473 Mg/m ³	
Absorption coefficient	2.484 mm ⁻¹	
F(000)	1520	
Crystal size	0.220 x 0.220 x 0.180 mm ³	
Theta range for data collection	1.848 to 26.197°.	
Index ranges	-11 ≤ h ≤ 10, -14 ≤ k ≤ 14, -37 ≤ l ≤ 37	
Reflections collected	32456	
Independent reflections	6665 [R(int) = 0.0448]	
Completeness to theta = 25.000°	99.9 %	
Absorption correction	Semi-empirical from equivalents	
Max. and min. transmission	1.000 and 0.739	
Refinement method	Full-matrix least-squares on F ²	
Data / restraints / parameters	6665 / 2 / 405	
Goodness-of-fit on F ²	1.054	
Final R indices [I > 2σ(I)]	R1 = 0.0429, wR2 = 0.0984	
R indices (all data)	R1 = 0.0510, wR2 = 0.1019	
Absolute structure parameter	-0.009(7)	
Largest diff. peak and hole	0.349 and -0.493 e.Å ⁻³

Figure S126. Perspective view of molecule 1 in the asymmetric unit of *S*-4k. Displacement ellipsoids are drawn at the 50 % probability level.

Figure S127. Perspective view of molecule 2 in the asymmetric unit of *S*-4k. Displacement ellipsoids are drawn at the 50 % probability level.

Chiral HPLC analysis

Figure S128: Chiral HPLC analysis chromatogram of *S*-4a synthesized with MBP-YFAU(Ni²⁺) wild-type (a) and *R*-4a synthesized with KPHMT wild-type (b). Conditions: CHIRALCELL® OD 46 x 250 mm column, 5 μ m, flow rate 0.7 mL min⁻¹ at 20 °C and UV detection (254 nm). Isocratic elution hexane:iPrOH 80:20.

a)

b)

Figure S129: Chiral HPLC analysis chromatogram of *S*-**4b** synthesized with MBP-YFAU(Ni²⁺) wild-type (a) and *R*-**4b** synthesized with KPHMT wild-type (b). Conditions: CHIRALPACK® IC 46 x 250 mm column, 5 μ m, flow rate 0.7 mL min⁻¹ at 20 °C and UV detection (254 nm). Isocratic elution hexane:iPrOH 80:20.

Figure S130: Chiral HPLC analysis chromatogram of *S*-**4c** synthesized with MBP-YfaU W23V (a) and *R*-**4c** synthesized with KPHMT I202A (b). Conditions: CHIRALPACK® ID 46 x 250 mm column, 5 μ m, flow rate 0.7 mL min⁻¹ at 20 °C and UV detection (254 nm). Isocratic elution hexane:iPrOH 75:25.

a)

b)

Figure S131: Chiral HPLC analysis chromatogram of *S*-4d synthesized with MBP-YfaU W23V.(a) and *R*-4d synthesized with KPHMT I212A (b). Conditions: CHIRALPACK® IC 46 x 250 mm column, 5 μ m, flow rate 0.7 mL min⁻¹ at 20 °C and UV detection (254 nm). Isocratic elution hexane:iPrOH 75:25.

a)

b)

Figure S132: Chiral HPLC analysis chromatogram of *S*-4e synthesized with MBP-YfaU W23V (a) and *R*-4e synthesized with KPHMT L42A/I212A (b). Conditions: CHIRALPACK® IC 46 x 250 mm column, 5 μ m, flow rate 0.7 mL min⁻¹ at 20 °C and UV detection (254 nm). Isocratic elution hexane:iPrOH 75:25.

a)

b)

c)

Figure S133: Chiral HPLC analysis chromatogram of *S*-4f synthesized with MBP-YfaU W23V (a), *S*-4f synthesized with MBP-YfaU W23V (b) and *R*-4f synthesized with KPHMT I212A (c). Conditions: CHIRALPACK® IC 46 x 250 mm column, 5 μ m, flow rate 0.7 mL min⁻¹ at 20 °C and UV detection (254 nm). Isocratic elution hexane:iPrOH 80:20.

Figure S134: Chiral HPLC analysis chromatogram of *S*-**4g** synthesized with MBP-YfaU W23V (a) and *R*-**4g** synthesized with KPHMT I212A (b). Conditions: CHIRALPACK® IC 46 x 250 mm column, 5 μ m, flow rate 0.7 mL min⁻¹ at 20 °C and UV detection (254 nm). Isocratic elution hexane:iPrOH 80:20.

Figure S135: Chiral HPLC analysis chromatogram of *S*-4h synthesized with MBP-YfaU W23V (a) and *R*-4h synthesized with KPHMT L42A/I212A (b). Conditions: CHIRALPACK® IC 46 x 250 mm column, 5 μ m, flow rate 0.7 mL min⁻¹ at 20 °C and UV detection (254 nm). Isocratic elution hexane:iPrOH 80:20.

a)

b)

Figure S136: Chiral HPLC analysis chromatogram of *S*-4i synthesized with MBP-YfaU W23V (a), *S*-4i synthesized with MBP-YfaU wild-type (b) and *R*-4i synthesized with KPHMT wild-type (c). Conditions: CHIRALPACK® IC 46 x 250 mm column, 5 μ m, flow rate 1 mL min⁻¹ at 20 °C and UV detection (254 nm). Isocratic elution hexane:iPrOH 80:20.

Figure S137: Chiral HPLC analysis chromatogram of *S*-**4j** synthesized with MBP-YfaU W23V (a) and *R*-**4j** synthesized with KPHMT I202A (b). Conditions: CHIRALPACK® IC 46 x 250 mm column, 5 μm , flow rate 1 mL min⁻¹ at 20 °C and UV detection (254 nm). Isocratic elution hexane:iPrOH 95:5.

Figure S138: Chiral HPLC analysis chromatogram of *S*-4k synthesized with MBP-YfaU W23V. Conditions: CHIRALPACK® IC 46 x 250 mm column, 5 μ m, flow rate 1 mL min⁻¹ at 20 °C and UV detection (254 nm). Isocratic elution hexane:iPrOH 95:5.

a)

b)

Figure S139: Chiral HPLC analysis chromatogram of *S*-5a synthesized with MBP-YfaU wild-type (a) and *R*-5a synthesized with KPHMT wild-type (b). Conditions: CHIRALPACK® IB 46 x 250 mm column, 5 μ m, flow rate 1 mL min⁻¹ at 20 °C and UV detection (209 nm). Isocratic elution hexane:iPrOH 98:2.

Figure S140: Chiral HPLC analysis chromatogram of *R*-5g synthesized with KPHMT I212A. Conditions: CHIRALPACK® ID 46 x 250 mm column, 5 μ m, flow rate 1 mL min⁻¹ at 20 °C and UV detection (209 nm). Isocratic elution hexane:iPrOH 90:10.

a)

b)

Figure S141: Chiral HPLC analysis chromatogram of a) **S-6a** synthesized with MBP-YfaU wild-type (a) and **R-6a** synthesized with KPHMT wild-type (b). Conditions: CHIRALPACK® IB 46 x 250 mm column, 5 μ m, flow rate 1 mL min⁻¹ at 20 °C and UV detection (209 nm). Isocratic elution hexane:iPrOH 95:5.

Computational Methods.

Protein complexes were modeled with the package Schrödinger Suite 2018-3,¹⁷ through its graphical interface Maestro.¹⁸ The Protein Preparation Wizard¹⁹⁻²⁰ included in Maestro was used to prepare the protein structures by removing solvent molecules and ions (except the essential cation and the two coordinated waters), adding hydrogens, setting protonation states²¹ and running a restrained minimization using the OPLS3 force-field.²² The program MacroModel²³ with the same force field and GB/SA water solvation conditions²⁴ was used for further molecular mechanics calculations. The program QSite²⁵⁻²⁷ was used for the QM/MM calculations.

Molecular models of the pre-reactive nucleophilic enolate intermediates and methanal bound into the active site of YfaU and KPHMT proteins were generated starting from the crystal structure of YfaU in complex with pyruvate (PDB 2VWT)²⁸ and of KPHMT in complex with 3-methyl-2-oxobutanoic acid (PDB 1M3U),²⁹ following a similar protocol to that previously described.¹⁻² The asymmetric unit of the YfaU structure comprises three monomers with three active sites that are located at the interface between units. Therefore, the full trimer was considered in our calculations although we focused on a single active site, i.e. that between chains A and B. On the other hand, the KPHMT structure is decameric but each monomer has its own active site which is not participated by residues of any other monomer, thus in this case only chain A was considered for our calculations. Mutations on both protein structures were introduced within Maestro, followed by restrained minimization. The structure of the pyruvate or 3-methyl-2-oxobutanoic acid molecules bound into the active sites of YfaU or KPHMT, respectively, were modified using Maestro to build the corresponding nucleophile molecules. Methanal was manually placed in the active site of each enzyme and adequately oriented to satisfy the conditions of (i) having its C-atom at approximately 2.4 Å of the reactive enolate C-atom of the nucleophile, and (ii) forming hydrogen bond interactions with residue R74 and a metal-coordinated water in YfaU or with the protonated E181 residue and another metal-coordinated water in KPHMT (residue E181 has been postulated as the catalytic base that abstracts the C3 proton of the nucleophile, to form the enolate, and could also participate on the protonation of the alcoxide derived from the electrophilic carbonyl, once the C-C bond is formed). The structures of the complexes were then minimized with QSite at the DFT B3LYP/6-31G** level of

theory, establishing a distance constraint between the two reactive C-atoms to keep them at a distance of 2.4 Å. The QM/MM boundary was defined by placement of hydrogen caps between the C α and C β atoms of residues E153 and D179 of Yfau and between those of residues D45 and D84 of KPHMT. The Mg²⁺ cation and the two coordinated waters, as well as the nucleophile enolate and the methanal molecule, were also included as part of the QM region. All residues with atoms within 6 Å of the QM region were simultaneously optimized using the OPLS 2005 force-field,³⁰⁻³¹ while residues which were further away were kept frozen. Furthermore, to find the best binding conformation for each nucleophile a conformational search was performed. For the nucleophiles with the less flexible R¹ substituents (i.e. **1c,1i-k**), the conformational scan option of QSite was used to rotate the C3-C4 bond in 40 degrees increments, followed by QM/MM optimization of the rest of the structure with the already described settings. The resulting structures were then re-optimized allowing full flexibility for the nucleophile, as well as for the rest of the QM region, as described above. For the nucleophiles with the more flexible R¹ substituents (ie. **1d-h**), the mixed MCMM/LMCS method³² implemented in MacroModel was used to find the best poses for the R¹ substituents, while the rest of the system was kept frozen. Then, the best conformers detected by this search were QM/MM optimized as above.

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