Supporting Information

Combining Aldolases and Transaminases for the Synthesis of 2-Amino-4-Hydroxybutanoic acid

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Materials

Formaldehyde, L-homoserine, D-homoserine, L-Ala, sodium pyruvate, benzaldehyde and benzyl amine were purchased from Sigma-Aldrich. Reagents for molecular biology were from Life Thermo Scientific. High-density IDA-Agarose 6BCL nickel charged was from GE Healthcare Life Science. 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.

Methods

Cloning and expression of 2-keto-3-deoxy-L-rhamnonate aldolase (YfaU EC 4.1.2.53) from E. coli K-12. The gene rhmA from *E. coli* K-12 (NCBI database accession number NC_000913.3) was amplified by PCR from genomic DNA and cloned into pQE40MBP (MBP maltose binding protein) using KpnI and HindIII with de primers: Forward: 5′-AGGTACCATGAACGCATTATTAAGCAATCCC-3′. Reverse and complementary: 5′-CAATAAGCTTTCAATAACTACCTTTTATGCGTGGCC–3′ (Figure S1). The plasmid pQE40 MBP-YfaU was transformed into an *E. coli* strain M-15 [pREP-4] from QIAGEN and grown in LB medium with ampicillin (100 μg mL^{-1}) plus kanamycin (25 μg mL^{-1}) at 37 ºC on a rotary shaker at 200 rpm. A final optical density at 600 nm (OD600) of 2–3 was usually achieved. An aliquot of the pre-culture (12 mL) was transferred into a shake-flask (2 L) containing LB (600 mL) with ampicillin (100 μg mL^{-1}) plus kanamycin (25 μg mL^{-1}) and incubated at 37 ºC with shaking at 200 rpm. During the middle exponential phase growth (DO_{600} ≈ 0.5), the temperature was decreased to 20 ºC to minimize potential inclusion bodies formation and isopropyl-β-D-1-thiogalactopyranoside (IPTG; 1 mM final concentration) was added. Cells from the induced-culture broths (3 L) were centrifuged at 12 000 g for 30 min at 4 ºC. The pellet was re-suspended with starting sodium phosphate buffer (200 mL, 50 mM, pH 8.0), containing NaCl (300 mM) and imidazole (10 mM). Cells were lysed using a cell disrupter (Constant Systems). Cellular debris was removed by centrifugation at 30 000 g for 30 min. The clear supernatant was collected and purified by immobilized metal ion affinity chromatography (IMAC) using a FPLC system (Amersham biosciences). The crude supernatant was applied to a cooled HR 16/40 column (GE Healthcare) packed with HiTrap chelating support (50 mL bed volume; Amersham Biosciences) and washed with the start buffer (250 mL). The protein was eluted with sodium phosphate

buffer (50 mM, pH 8.0) containing NaCl (300 mM) and imidazole (500 mM) at a flow rate of 3 mL min^{-1}. Fractions containing the recombinant protein were combined and dialyzed against sodium phosphate buffer (10 mM, pH 7.0) at 4 ºC. The dialyzed solution was frozen at – 80 ºC and lyophilized. The white solid obtained (yield: 130 mg L^{-1} of culture) was stored at – 20 ºC. See Figure S2 for the Coomassie Blue-stained SDS-PAGE of MBP-YfaU.

MRGSHHHHHHGSGIMKIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVE
HPDKLEEKFPQVAATGDGPDIIFWAHDRFGGYAQSGLLAEITPDKAFQDKLYPF
TWDAVRYNGKLIAYPIAVEALSLIYNKDLLPNPPKTWEEIPALDKELKAKGKSA
LMFNLQEPYFTWPLIAADGGYAFKYENGKYDIKDVGVDNAGAKAGLTFLVDLI
KNKHMNADTDYSIAEAAFNKGETAMTINGPWAWSNIDTSKVNYGVTVLPTFK
GQPSKPFVGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVNKDKPLGAVAL
KSYEEELAKDPRIAATMENAQKGEIMPNIPQMSAFWYAVRTAVINAASGRQTV
DEALKDAQTSSGLEVLFQGPACGTMNALLSNPFKERLRKGEVQIGLWLSSTTA
YMAEIAATSGYDWLLIDGEHAPNTIQDLYHQLQAVAPYASQPVIRPVEGSKPLI
KQVLDIGAQTLLIPMVDTAEQARQVVSATRYPPYGERGVGASVARAARWGRIE
NYMAQVNDSLCLLVQVESKTALDNLDEILDVEGIDGVFIGPADLSASLGYPDN
AGHPEVQRIIETSIRRIRAAGKAAGFLAVAPDMAQQCLAWGANFVAVGVDTML
YSDALDQRLAMFKSGKNGPRIKGSY

Figure S1. Sequence of fused maltose binding protein (MBP) and 2-keto-3-deoxy-L-rhamnonate aldolase (YfaU) (MBP-YfaU). MBP (blue), YfaU (green), 6x His tag (red) and recognition and cleavage site for human rhinovirus 3C and PreScission proteases (orange).

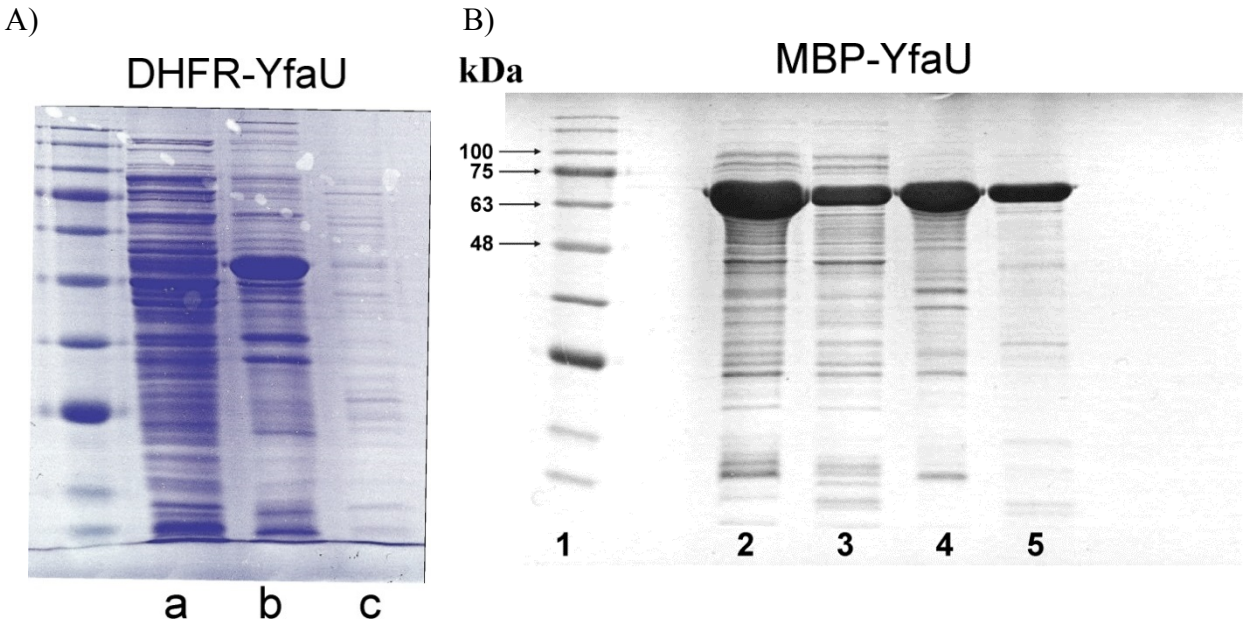


Figure S2. Coomassie Blue-stained SDS-PAGE of DHFR-YfaU (A) and MBP-YfaU (B). The gel was loaded with samples of DHFR-YfaU (A) from supernatant after centrifugation of cell debris (lane a), Pellet (lane b) and purified (lane c) and MBP-YfaU from crude extract (lane 2), supernatant of lysis (lane 3), pellet after lysis and centrifugation (lane 4), and eluate of affinity chromatography (IMAC) (lane 5). The molecular masses of the proteins in the standard (lane 1) are as indicated. The predicted molecular mass of MBP-YfaU is 72 kDa.

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 CH_3CN/H_2O 4:1, flow rate 1 mL min^{-1}, detection at 215 nm and column temperature at 30 °C. The amount of products and substrates were quantified from the peak areas using an external standard methodology.

Reaction monitoring for pyruvate (**1**), formaldehyde (**2**) and aldol adduct (**3**) were carried out as follows: samples were withdrawn from the reaction mixture (10 μL) and diluted with plain water, to obtain a concentration range between 3 and 50 mM. Then, the dilution (10 μL) 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 ºC for 5 min, samples were diluted in methanol (500 μL) and

after centrifugation analyzed by HPLC. Elution conditions: gradient elution from 10 to 100% B over 30 min (Figure S3).

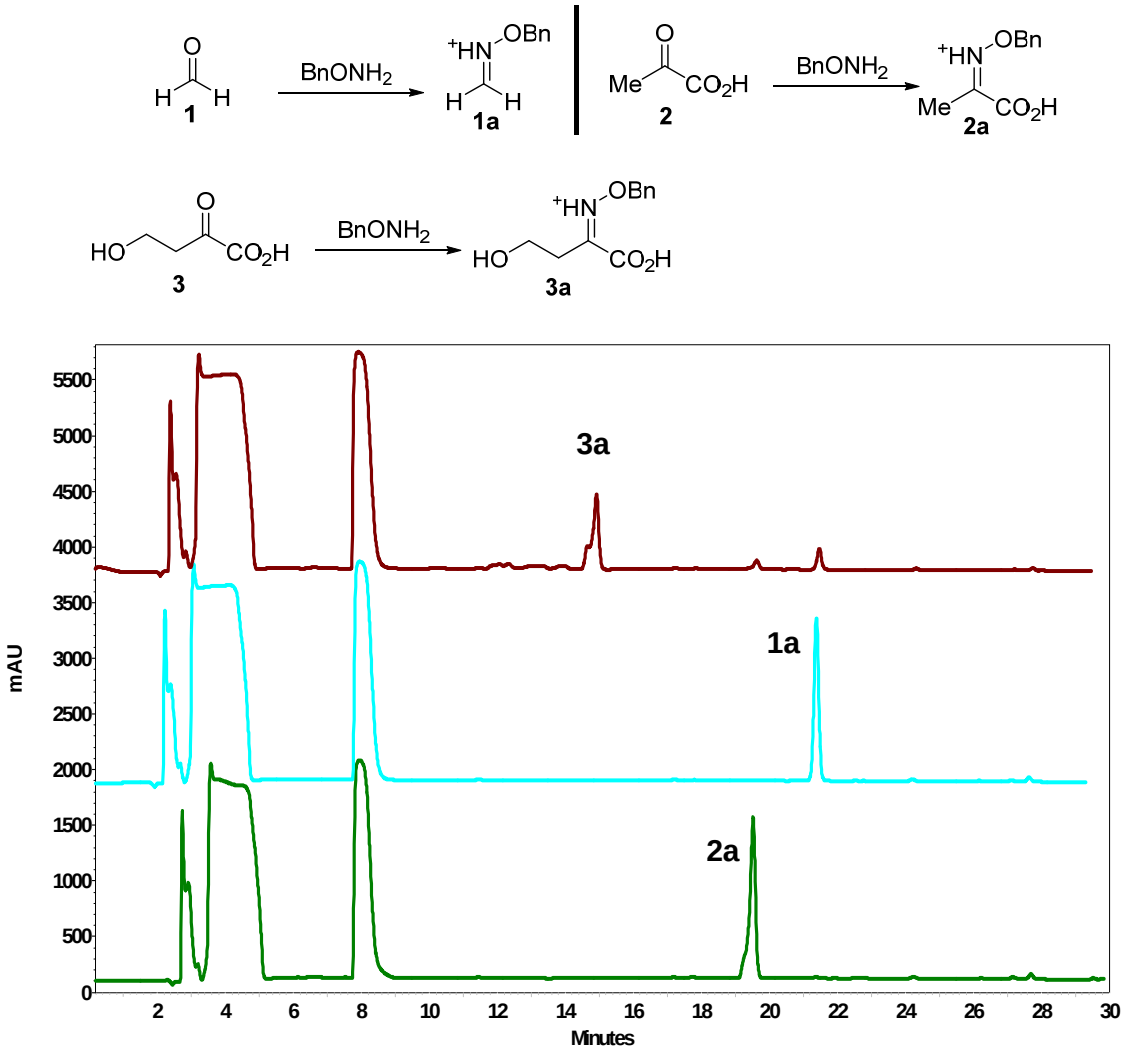


Figure S3. Example of HPLC chromatogram of *O*-benzylhydroxylamine derivatives of pyruvate (**1a**), formaldehyde (**2a**) and aldol adduct (**3a**).

Reaction monitoring for L-Ala (**4**) and L-homoserine (**5**) were carried out as follows: samples were withdrawn from the reaction mixture (10 µL) and diluted with plain water, to obtain a concentration range between 3 and 50 mM. Then the dilution (10 µL) was mixed with a solution of CbzOSu (50 µL of a 150 mM stock solution in acetonitrile). After incubation at 60 ºC for 60 min, samples were diluted in methanol (440 µL) and after centrifugation analyzed by HPLC. Elution conditions: gradient elution from 10 to 100 % B over 30 min (Figure S4).

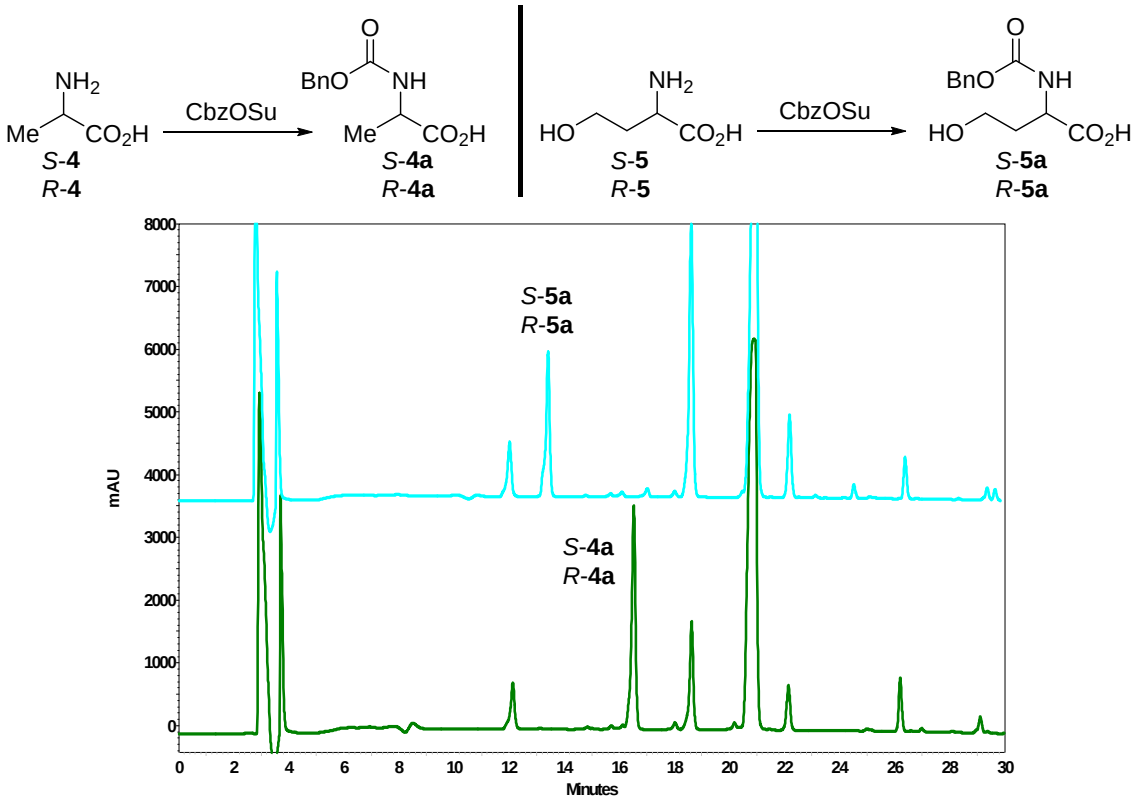


Figure S4. Examples of HPLC analysis of Cbz-L- or -D-Ala (*S*-**4a** or *R*-**4a**, respectively) and Cbz-L- or -D-homoserine (*S*-**5a** or *R*-**5a**, respectively) derivatives.

NMR analysis. Routine, ^{1}H (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, NOESY, HSQC, HMBC and TOCSY, recorded under routine conditions.

Chiral HPLC analysis. Enantiomeric excesses (*ee*) were determined with chiral HPLC analysis on a CHIRALPAK® ID, 46 x 250 mm column, 5 µm, flow rate 0.8 mL min^{-1} at 20 ºC and UV detection (254 nm). The solvent for isocratic elution was: hexane/EtOH/CH_2Cl_2, 7/2/1 (v/v/v). *Specific rotation* values were measured with a Perkin Elmer Model 341 (Überlingen, Germany).

Activity determination of MBP-YfaU-M^{2+}. The activity was determined using the aldol addition of pyruvate to formaldehyde by a discontinuous assay. One unit of activity was defined as the amount of MBP-YfaU, which catalyzes the formation of 1 µmol of 4-hydroxy-2-oxobutanoic acid (**3**) per min at 25 ºC in 50 mM sodium phosphate buffer pH 7.0, containing 1 mM of different cations (Ni^{2+}, Co^{2+} and Mg^{2+}). The assay procedure

was as follows: To a equimolar solution mixture of sodium pyruvate and formaldehyde (150 µL of a 133 mM solution in 50 mM sodium phosphate buffer pH 7.0 containing MCl_2 (1.3 mM)), a solution of the enzyme (50 µL, of stock solution between 5.4–0.08 mg of lyophilized powder mL^{-1}, in 50 mM sodium phosphate buffer pH 7.0) was added. The total reaction volume was 0.2 mL. Reaction monitoring was as follows: at 10 min, samples (10 µL) were analyzed by HPLC as described above. The results are presented in Table S1 and Figure S5.

Table S1. Activity of MBP-YfaU-M^{2+} with different metal cofactors.

Metal cofactor	Activity[a] (U mg^{-1})
Co^{2+}	10.0
Ni^{2+}	7.8
Mg^{2+}	3.9
Without M^{2+} added	2.5

[a]One unit of activity was defined as the amount of MBP-YfaU, which catalyzes the formation of 1 µmol of 4-hydroxy-2-oxobutanoic acid (**3**) per min at 25 ºC in 50 mM sodium phosphate buffer pH 7.0, with or without the addition of Ni^{2+}, Co^{2+} or Mg^{2+} (1 mM). The activity of the MBP-YfaU without M^{2+} added was lower than that with Mg^{2+} added due to that enzyme not being saturated by metal cofactor.

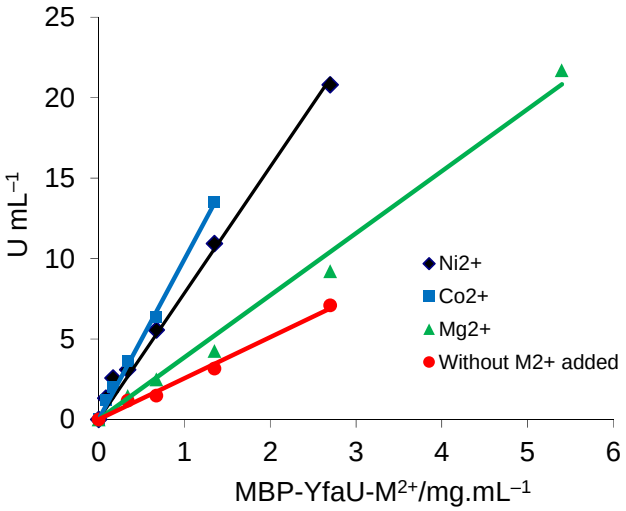


Figure S5. Determination of MBP-YfaU-M^{2+} activity. 4-Hydroxy-2-oxobutanoic acid (**3**) formed in 10 min (µmol min^{-1} mL^{-1}) against the MBP-YfaU-M^{2+} concentration in mg lyophilized powder mL^{-1}.

Activity determination of (S)-selective transaminases. TA20, 26, 39 and 51. The activity (Table S2) was determined using the reductive amination of pyruvate to L-alanine using benzylamine as amine donor by a fixed-time (discontinuous) assay. One unit of activity was defined as the amount of TA, which catalyzes the formation of 1 μmol of benzaldehyde per min at 25 ºC in sodium phosphate buffer (50 mM; pH 7.0, containing 1 mM of PLP). The assay procedure was as follows: To an equimolar solution of sodium pyruvate and benzylamine (172 μL of a 174 mM stock solution in 50 mM sodium phosphate buffer pH 7.0 containing PLP (1 mM)), a solution of the enzyme (128 μL of a stock solutions between 0.15–20.0 mg of lyophilized powder mL^{-1} in 50 mM sodium phosphate buffer pH 7.0 containing PLP (1 mM)) was added. Total final volume was 0.3 mL. Reaction monitoring was as follows: at 10 min, samples (100 μL) were withdrawn, diluted with methanol (400 μL), and directly analyzed and quantified by HPLC using an external standard method, gradient elution from 10 to 100 % B.

Table S2. Activity of (*S*)-Selective Prozomix TAs.

Prozomix TA	Activity[a] (U mg^{-1})
20	0.061
26	0.17
39	0.035
51	0.14

[a]One unit of activity was defined as the amount of TA, which catalyzes the formation of 1 μmol of benzaldehyde per min at 25 ºC in sodium phosphate buffer (50 mM; pH 7.0, containing 1 mM of PLP).

Activity determination of (R)-Selective transaminases. TA7, 17, and 43. The transamination reaction of benzylamine to pyruvate did not work with these transaminases. Therefore, the procedure used in this case was the following. The activity (Table S3) was determined using the reductive amination of 4-hydroxy-2-oxobutanoic acid (**3**) to D-homoserine using D-Ala as amine donor by a fixed-time (discontinuous) assay. One unit of activity was defined as the amount of TA, which catalyzes the formation of 1 μmol of pyruvate per min at 25 ºC in sodium phosphate buffer (50 mM; pH 7.0, containing 1 mM of PLP). The assay procedure was as

follows: To a solution of **3** and D-Ala (134 mM and 671 mM respectively in sodium phosphate buffer pH 7.0 containing PLP (1 mM)), a solution of the enzyme (51 μL of a stock solutions between 0.15–20.0 mg of lyophilized powder mL^{-1} in 50 mM sodium phosphate buffer pH 7.0 containing PLP (1 mM)) was added. Total final volume was 0.2 mL. Reaction monitoring was as follows: at 10 min, samples (10 μL) were withdrawn and analyzed by HPLC as describe above for pyruvate.

Table S3. Activity of (*R*)-Selective Prozomix TA.

Prozomix TA	Activity[a] (U mg^{-1})
7	2.4
17	5.8
43	2.3

[a]One unit of activity was defined as the amount of TA, which catalyzes the formation of 1 μmol of pyruvate per min at 25 ºC in sodium phosphate buffer (50 mM; pH 7.0, containing 1 mM of PLP).

Inhibitory effect of the amount of formaldehyde added (one portion) on the sodium 4-hydroxy-2-oxobutanoate (3) yield. Formaldehyde (0.05 to 1.5 mmol, 4 μL to 122 μL of a 12.3 M commercial aqueous solution, 0.1 to 2.4 M in the reaction mixture) was dissolved in 50 mM sodium phosphate buffer pH 7.0 (0.25 mL) containing sodium pyruvate (0.1 to 3.0 M). An equimolar amount of pyruvate and formaldehyde were used in all experiments. The reactions were started by addition of MBP-YfaU(Mg^{2+}) (0.25 mL, 2 mg lyophilized powder mL^{-1} in the reaction mixture). The reactions were stirred (1000 rpm vortex mixer) at 25 ºC and analyzed by HPLC after 24 h. Data of Figure 1 in main text were adjusted to inhibitory dose-response model with variable Hill slope using GraphPad Prism 5 software (Motulsky, H. (2007). In GraphPad Prism 5: Statistics Guide. *GraphPad Software Inc. Press, San Diego CA*, 94.). From the dose-response curve the IC_{50} value (i.e. the amount of formaldehyde added that decreased the maximum **3** yield after 24 h to 50%) was 1.4 M, initial concentration in reaction.

Influence of the metal cofactor in the aldol addition of pyruvate to formaldehyde. Aldol adducts 3 and 6 distribution as a function of the metal cofactor (Figure S6). Formaldehyde (1 mmol, 81 μL of the 12.3 M commercial aqueous solution, 1 M in the reaction mixture) was dissolved in 50 mM sodium phosphate buffer pH 7.0 (0.5 mL) containing sodium pyruvate (1 M). The reactions were started by addition of different divalent cations (Ni^{2+}, Mg^{2+}, Zn^{2+}, Co^{2+} and Mn^{2+} at 1 mM final concentration in the reaction mixture) and MBP-YfaU (0.5 mL, 2 mg lyophilized powder mL^{-1} in the reaction mixture). The reactions were stirred (1000 rpm vortex mixer) at 25 ºC and analyzed by NMR after 24 h. All reactions were conducted in deuterated water.

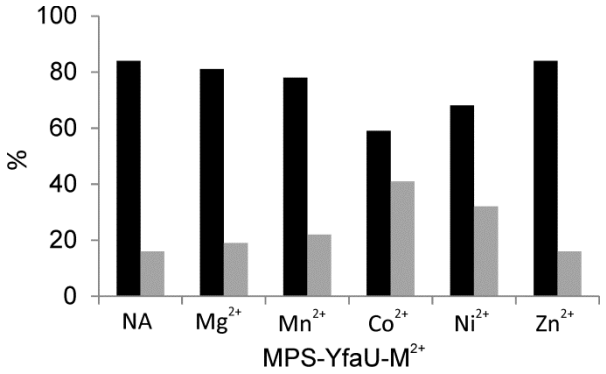


Figure S6. MBP-YfaU-M^{2+}-catalyzed aldol addition of pyruvate to formaldehyde. Influence of the metal cofactor on the product selectivity. Black bars represent compound **3**, grey bars compound **6**; NA: no metal cofactor added to the original MBP-YfaU(Mg^{2+}) enzyme sample, the enzyme partially keeps its natural Mg^{2+} cofactor from the bacterial growth medium, during purification with IMAC and dialysis against 10 mM sodium phosphate buffer pH 7.0 at 4 ºC.

Effect of metal on the aldol addition of 4-hydroxy-2-oxobutanoate (3) to formaldehyde *(Figure S7).* Formaldehyde (0.35 mmol, 28 μL from the commercial aqueous solution of 12.3 M) was added to a solution of **3** (500 μL of a 700 mM solution in 50 mM sodium phosphate buffer pH 7.0). The reactions were started by addition of the metal (0.5 mmol, 5 μL of a 0.1 M stock solution). Final volume 533 μL. The reactions were stirred (1000 rpm vortex mixer) at 25 ºC and analyzed by HPLC after 24 h.

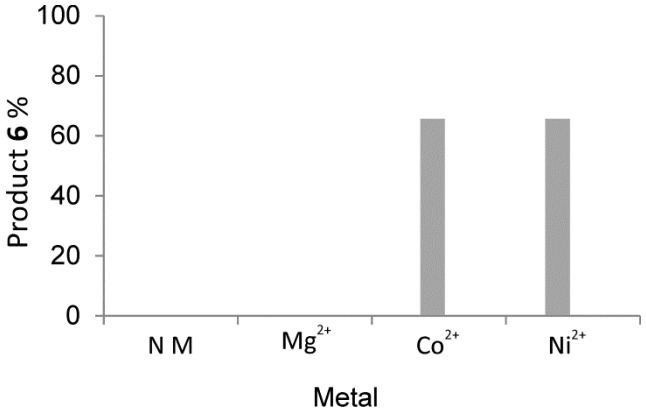
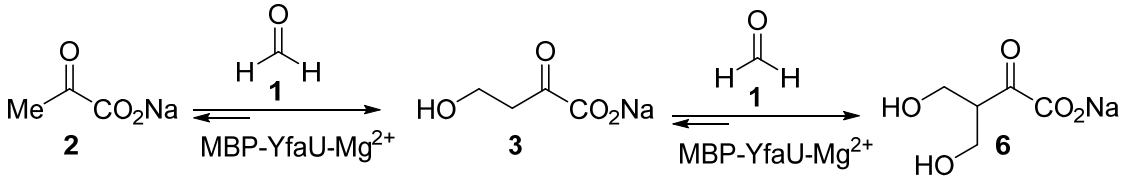


Figure S7. Metal-catalyzed aldol addition of aldol adduct **3** to formaldehyde (**1**). Influence of the metal on the **6** yield. N M: No metal added

Synthesis of sodium 4-hydroxy-2-oxobutanoate (3).

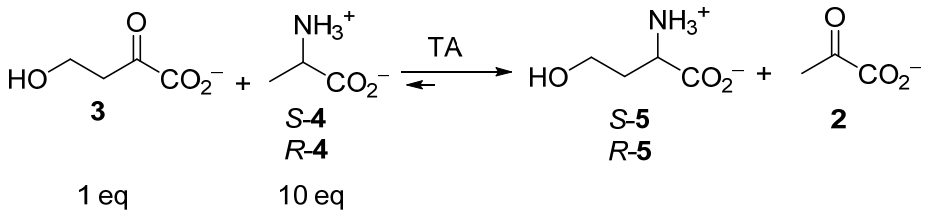


Reaction (7.7 mL total volume) was conducted in a Falcon Tube (15 mL). MBP-YfaU(Mg^{2+}) (15.4 mg of protein (i.e., 31 mg of lyophilized powder) 2 mg mL^{-1}, 77 U, 10 U mL^{-1}) was dissolved in sodium pyruvate (**2**) solution (7.1 mL of a 1.0 M stock solution at pH 6.5-7.0, adjusted with NaOH, 50 mM, 1 M in the reaction). The reaction was initiated by slow addition of formaldehyde (577 μL of a 12.3 M commercial aqueous solution, 115.4 μL each 2 h during 8 h) and shacked in a vortex mixer (1000 rpm) at 25 ºC. After 16 h the reaction was centrifuged (5000 g at 4 ºC for 30 min) and the MBP-YfaU in the supernatant was eliminated using an Amicon ultrafiltration unit (Millipore, USA, MWCO 10 kDa, 5000 g at 4 ºC for 60 min). Solution was frozen at –80 ºC and lyophilized to afford the title compound as a white solid (980 mg, 98% as a **3**:**6** mixture in a 9:1 ratio). Sodium 4-hydroxy-2-oxobutanoate (**3**) ^{1}H NMR (400 MHz, D_2O) δ 3.71 (t, *J* = 5.9 Hz, 2H), 2.88 (t, *J* = 5.9 Hz, 2H). ^{13}C NMR (101 MHz, D_2O) δ 200.6 (CO), 166.4 (CO_2^-), 56.0 (CH_2OH), 41.1 (CH_2). Sodium 2,2,4-trihydroxybutanoate (hydrate form of **3**). ^{1}H NMR (400 MHz, D_2O) δ 3.54 (t, *J* = 6.7 Hz, 2H), 1.95 (t, *J* = 6.7 Hz, 2H). ^{13}C NMR (101 MHz, D_2O) δ 174.4 (CO_2^-), 118.9 ($C(OH)_2$), 56.8 (CH_2OH), 40.1 (CH_2). Sodium 4-hydroxy-3-(hydroxymethyl)-2-

oxobutanoate (**6**) (10%). ^{1}H NMR (400 MHz, D_2O) δ 3.72 (m, 4H), 3.33 (m, 1H). ^{13}C NMR (101 MHz, D_2O) δ 58.6 (CH_2OH), 52.4 (CH).

Reaction conducted using MBP-YfaU-Ni^{2+} (15.4 mg of protein, 2 mg mL^{-1}, 77 U, 10 U mL^{-1}) pyruvate (1.7 M, final concentration in reaction) and formaldehyde (1.7 M, final concentration in reaction added in one portion) gave sodium 4-hydroxy-2-oxobutanoate (**3**) and sodium 4-hydroxy-3-(hydroxymethyl)-2-oxobutanoate (**6**) in a ~7:3 ratio.

Reductive amination of 4-hydroxy-2-oxobutanoate (3). Screening of Transaminases

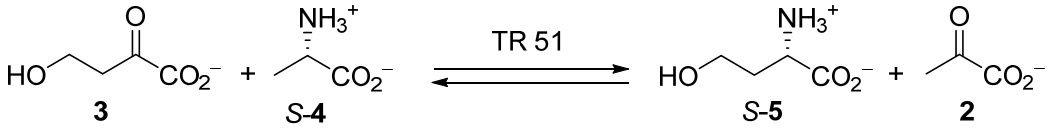


L-Ala donor (*S*-**4**). The procedure for sodium 4-hydroxy-2-oxobutanoate (**3**) production necessary for the screening of the transaminases, was identical as described above (i.e. using MBP-YfaU(Mg^{2+})) except that it was used directly in solution (0.7-0.8 M).

Reactions (300 μL total volume) were conducted in an Eppendorf tube (1 mL) and stirred in a vortex mixer (1000 rpm) at 25 ºC. Prozomix TA (2-3 mg of lyophilized solid) was dissolved in 50 mM sodium phosphate buffer pH 7.0 (104 μL) and L-Ala (150 μL of a 1.8 M solution in 50 mM sodium phosphate buffer pH 7.0, 0.9 M final concentration in reaction) was added. The reactions were started by the addition of sodium 4-hydroxy-2-ketobutanoate (**3**) (43 μL of a 0.7 M stock solution in 50 mM sodium phosphate buffer, pH 7.0, 0.1 M final concentration in reaction) and PLP (3 μL of a 0.1 M stock solution in water, 1 mM final concentration in reaction). After 24 h, samples (10 μL) were mixed with a solution of CbzOSu (150 μL of a 0.15 M solution in acetonitrile) and analyzed as described above. The percentage of product formation was determined from the peak areas of the HPLC chromatogram using an external standard method.

D-Alanine donor (*R*-**4**). Identical conditions than above but using D-Ala as amine donor

Determination of equilibrium constant (K_{eq}) for the reductive amination of 4-hydroxy-2-oxobutanoic acid (3) with L-Ala (4) catalyzed by transaminase TR 51 (Figure S8 and S9).



The equilibrium constant of the reaction of **3** and **4** to L-homoserine (**5**) and pyruvate (**2**) was determined according to the method described by Tufvesson et al. 2012.[1] Reactions (200 μL total volume) were carried out in 50 mM sodium phosphate buffer pH 7.0, PLP (1 mM) and Prozomix TA 51 (5 mg of solid, 25 mg mL^{-1}, 0.7 U, 3.5 U mL^{-1}). A preliminary estimation of the equilibrium constant was performed using the concentration data from (Figure S12A):

$$K_{eq}^{\ estimated} \approx \frac{[L-Homoserine]_{24h}[Pyruvate]_{24h}}{[L-Ala]_{24h}[2-oxoacid]_{24h}} \approx \frac{100mM * 100mM}{300mM * 100mM} \approx 0.3$$

Equation S1. Estimation of the transamination equilibrium constant $K_{eq}^{\ estimated}$ using the data from Figure S12A.

From the $K_{eq}^{\ estimated}$ we established the interval of reaction quotient (Q_{0h}) for the precise equilibrium constant determination and observing the change in the reaction quotient Q after 24 h.

$$\frac{K_{eq}^{\ estimated}}{10} \leq Q_{0h} \leq 10K_{eq}^{\ estimated}$$

Equation S2. Determination of the interval for Q_{0h}.

Initial substrate and product concentrations for seven reactions with different reaction quotients were selected (Table S4).

Table S4. Substrate and product concentration at the initial start point of the reaction and after 24 h.

	C/mM					C/mM			
Q_{0h}	**3**	**2**	**5**	**4**	Q_{24h}	**3**	**2**	**5**	**4**
3.00	10.0	128.0	5.9	25.0	0.73	15.1	120.6	2.7	30.8
1.50	20.0	64.0	23.4	50.0	0.74	26.4	59.7	14.1	52.6

0.75	30.0	32.0	70.3	100.0	0.76	31.1	34.2	53.6	91.3
0.38	40.0	16.0	140.6	150.0	0.79	35.8	29.6	121.1	140.4
0.19	50.0	8.0	234.4	200.0	0.82	38.2	27.8	205.8	185.1
0.094	60.0	4.0	351.6	250.0	0.82	45.6	28.3	331.8	259.2
0.047	70.0	2.0	492.2	300.0	0.85	50.5	28.6	445.8	295.8

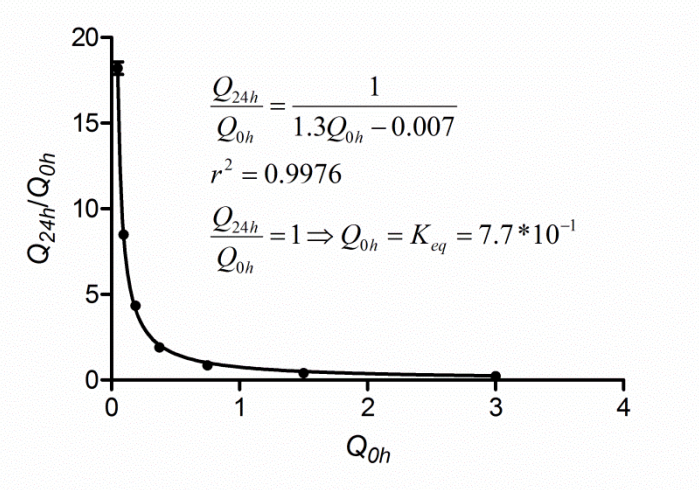


Figure S8. Experimental determination of K_{eq} for the transamination of **3** with L-Ala to yield L-homoserine and pyruvate. The reactants were mixed in different concentrations (Q_{0h}) and allowed to react for 24 h. Q_{24h}/Q_{0h} were plotted against Q_{0h} and the points were fitted to a hyperbole equation using nonlinear regression. The point at which Q_{24h}/Q_{0h} equals 1 was the K_{eq} of the reaction. Data were adjusted using GraphPad Prism 5 software (Motulsky, H. (2007). In GraphPad Prism 5: Statistics Guide. *GraphPad Software Inc. Press, San Diego CA*, 94.)

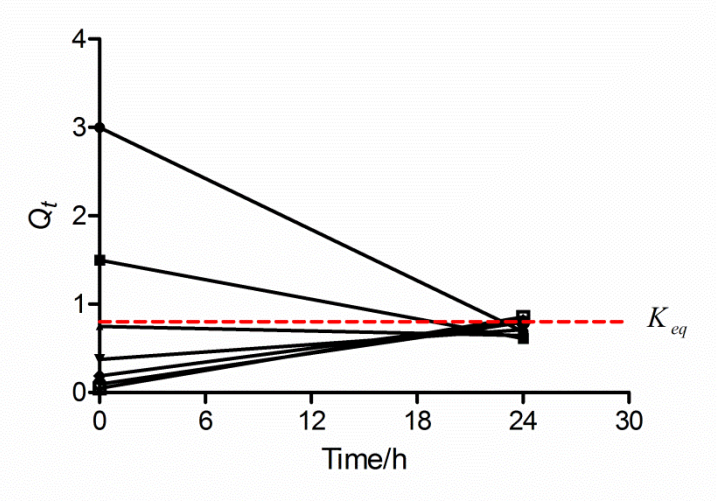
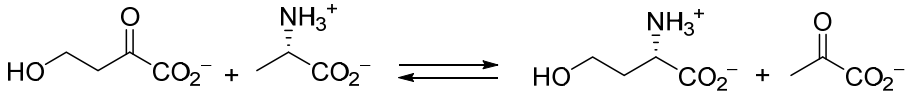
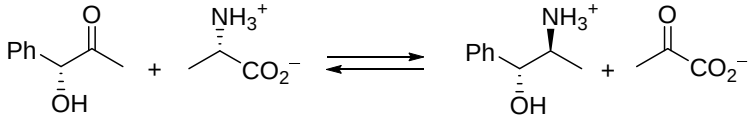
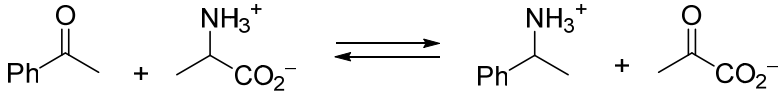
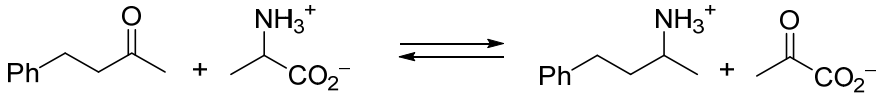
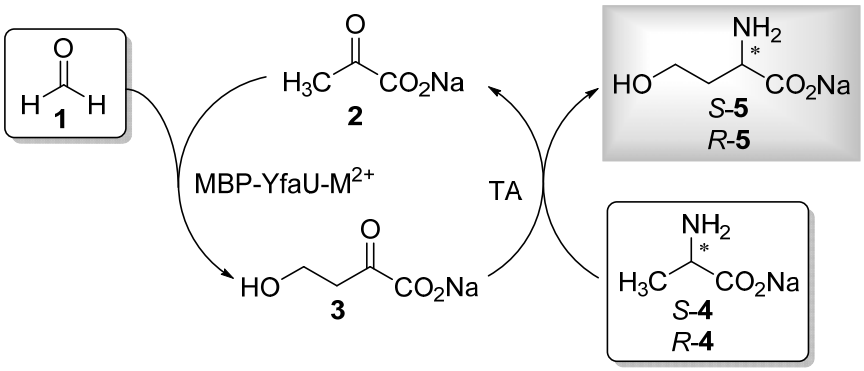


Figure S9. Experimental validation of K_{eq} for the transamination of **3** with L-Ala to yield L-homoserine and pyruvate. The solid lines represent different initial reactant quotients (Q) and the dotted line represents the experimentally determined K_{eq} value. After 24 h the reaction quotient converges in all reactions to K_{eq}.

Table S5. Comparison of equilibrium constant (K_{eq}) for the reductive amination of 2-oxoacid or ketones with L-Ala as amino donor.

Enzymatic reductive amination reaction	K_{eq}	Ref.
	$7.7\ 10^{-1}$	This work
	$2.1\ 10^{-3}$	Ref[2]
	$4.0\ 10^{-5}$	Ref[1]
	$6.1\ 10^{-4}$	Ref[1]

One-pot biocatalytic cyclic cascade synthesis of L-homoserine.



Scheme 1. Cyclic cascade one-pot biocatalytic synthesis of L- and D-homoserine. TA: transaminase.

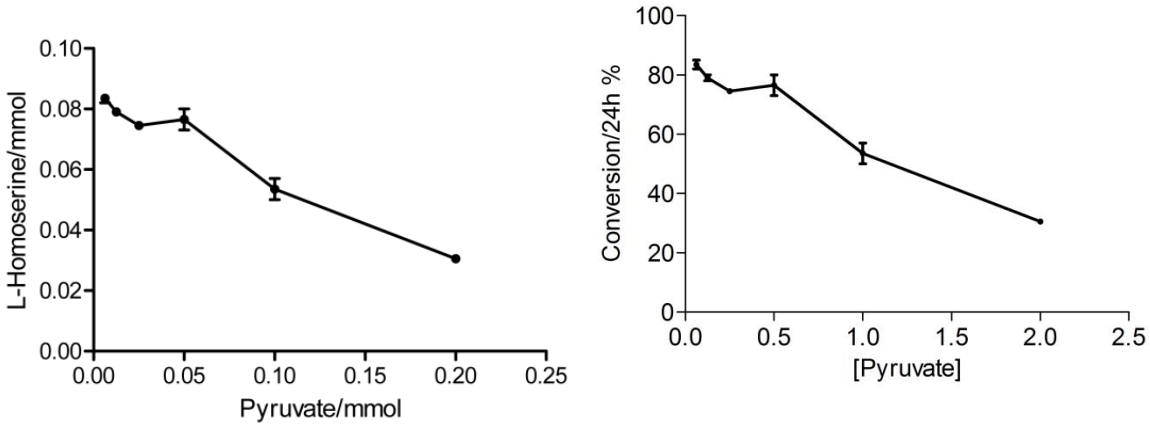


Figure S10. Effect of starting **2** concentration (mM) or amount (mmol) on L-homoserine (*S*-5) production. Conditions: reaction volume: 1.0 mL; *S*-**4** (0.1 mmol), **1** (0.1 mmol, added portion wise 0.0125 mmol each hour, 8 additions), PLP (1 mM), MBP-YfaU (2 mg, 10 U), TA 51 (2.8 U), in 50 mM sodium phosphate buffer pH 7.

Inhibitory effect of the amount of formaldehyde added each hour (portion wise) on the L-homoserine yield in the one-pot cyclic cascade systems after 24 h of reaction (Figure S11)

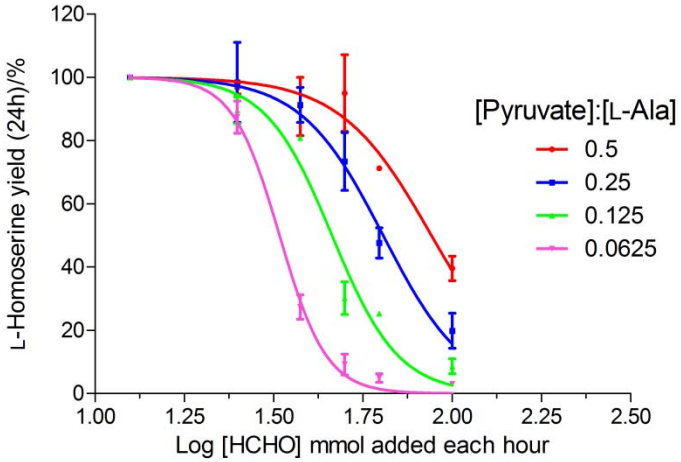


Figure S11. Effect of the amount of formaldehyde added each hour on the one-pot cyclic cascade synthesis of L-homoserine yield after 24 h of reaction with Prozomix TA 51. Normalized L-homoserine yield after 24 h vs the Log of the amount of formaldehyde added each hour for different $[pyruvate]_0$:$[L\text{-}Ala]_0$ ratio. From these curves a IC_{50} values were calculated. Data were adjusted to inhibitory dose-response model with variable Hill slope using GraphPad Prism 5 software (Motulsky, H. (2007). In GraphPad Prism 5: Statistics Guide. *GraphPad Software Inc. Press, San Diego CA*, 94.). The IC_{50} value is the amount of formaldehyde added each hour that decreased the maximum L-homoserine yield to 50% after 24 h for each $[pyruvate]_0$:$[L\text{-}Ala]_0$ ratio: IC_{50} (mmol each hour): 0.088 ($[pyruvate]_0$:$[L\text{-}Ala]_0$ = 0.5), 0.064 ($[pyruvate]_0$:$[L\text{-}Ala]_0$ = 0.25), 0.046 ($[pyruvate]_0$:$[L\text{-}Ala]_0$ = 0.125), 0.033 ($[pyruvate]_0$:$[L\text{-}Ala]_0$ = 0.0625).
For detailed conditions see below.

A) Synthesis of L-homoserine by portion wise addition of formaldehyde

The reaction was conducted in an Eppendorf tube (1.5 mL). The reaction volume was 1 mL. MBP-YfaU (4 mg, 10 U mL^{-1}) and transaminase Prozomix TA 51 (2.8 U mL^{-1}) were dissolved in 50 mM sodium phosphate buffer pH 7.0 (491 µL). Then, L-Ala (267 µL of a 1.5 M stock solution in 50 mM sodium phosphate buffer pH 7.0, 0.4 M final concentration in the reaction), sodium pyruvate (200 µL of a 1.0 M stock solution in 50 mM sodium phosphate buffer pH 7.0, 0.2 M final concentration

in reaction) and PLP (10 µL of a 100 mM stock solution in 50 mM sodium phosphate buffer pH 7.0, 1 mM final concentration in reaction) were added. The reaction was placed in a vortex mixer (1000 rpm) at 25 ºC. The reaction was initiated by slow addition of formaldehyde (4 µL of 12.3 M solution every 1 h, 4 additions of 0.05 mmol during 4 h (i.e., total 0.2 mmol) (Figure S12A), 8 additions, 32 µL total volume added (Figure S12B). Reaction monitoring was carried out by HPLC and the product was quantified using an external standard method (Figure S13). After 24 h the yield of L-homoserine was 50% with respect to the cumulative amount of formaldehyde added.

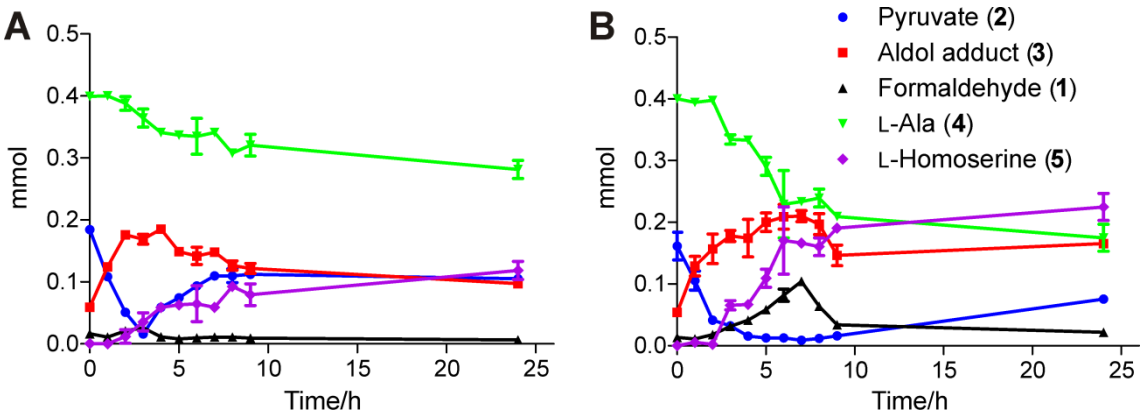


Figure S12. Progress of the biocatalytic cyclic cascade synthesis of L-homoserine with formaldehyde added portion wise. Reaction volume: 1 mL; pyruvate (0.2 mmol), L-Ala (0.4 mmol), MBP-YfaU-M^{2+} (10 U mL^{-1}) and TA 51 (2.8 U mL^{-1}) PLP (1 mM), in 50 mM sodium phosphate buffer pH 7.**A**) Formaldehyde: 4 additions of 0.05 mmol during 4 h (i.e., total 0.2 mmol); **B**) Formaldehyde: 8 additions of 0.05 mmol during 8 h (i.e., total 0.4 mmol). Samples were taken each hour immediately after the addition of formaldehyde.

B) Synthesis of L-homoserine by continuous addition of formaldehyde

The reaction was carried out in a Falcon tube (50 mL). Initial amount of pyruvate (2.1 mmol) and L-Ala (4.2 mmol) were dissolved in 50 mM sodium phosphate buffer pH 7.0 containing PLP (1 mM) (Table S6). Then, MBP-YfaU(Mg^{2+}) (105 U, 10 U mL^{-1} in the final volume), Prozomix TA 39 (74 U, 7 U mL^{-1}) were added. After that, formaldehyde was constantly added with a syringe pump at 0.57 mmol h^{-1} (0.18 mL h^{-1}) and varying the total addition time (i.e., volume added) in each experiment. The starting reaction volume was different for each experiment to adjust the volume of

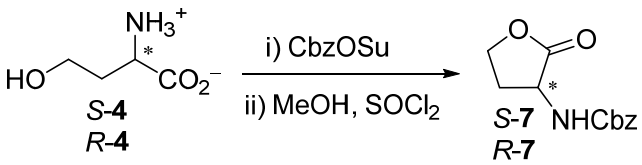
formaldehyde solution that has to be added and the final total volume. The total volume in all experiments was 10.5 mL.

Table S6. Conditions of the continuous addition of formaldehyde for the study of the effect of the formaldehyde on the yield of L-homoserine.

Entry	Total HCOH added (mmol)	Starting reaction volume (mL)[a]	Total addition time (h)	Total HCHO solution added (mL)	[HCHO] solution added (M)
1	2.1	9.8	4	0.7	3.0
2	4.2	9.2	7	1.3	3.2
3	6.3	8.5	11	2.0	3.2
4	7.3	8.0	14	2.5	2.9
5	8.4	7.8	15	2.7	3.1

[a]50 mM sodium phosphate buffer pH 7.0.

Isolation, purification and determination of enantiomeric excess of synthetized L- and D-homoserine. Formation of Cbz-L- and -D-homoserine lactone.



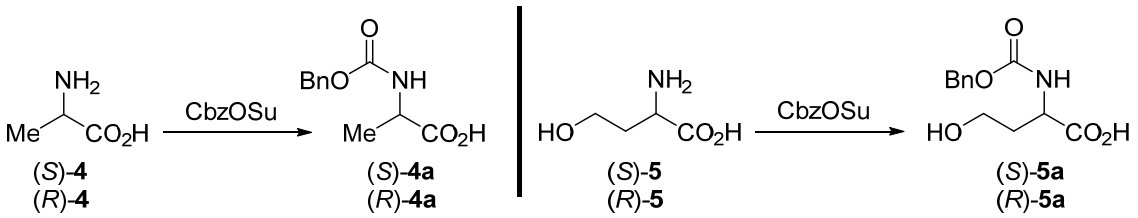
S-**4** *R*-**4** *S*-**7** *R*-**7**

* (*S*)- or (*R*)- depending on steroselectivity of the transaminase used

Scheme S2. Formation of Cbz-L- and -D-homoserine lactone from L- and D-homoserine respectively.

Example from the L-homoserine cyclic synthesis using the conditions of entry 5 (Table S6), the general procedure for purification, formation of Cbz-L-homoserine lactone and determination of enantiomeric excess was as follows. After 24 h, the reaction was filtered through active charcoal (in a filter funnel Pyrex 3, 5 cm Ø, filter bed 1 cm) and the pellet washed with $NaHCO_3$ 5% (3 x 50 mL). CbzOSu (1.04 g dissolved in CH_3CN (100 mL) was added to the filtrated (160 mL) and the reaction was stirred at 25 ºC. After 12 h, organic solvent was reduced under vacuum and pH of aqueous phase was adjusted to 2.0 with HCl (3 M). The aqueous solution was extracted with AcOEt (3 x 50

mL). The combined organic phases were dried over anhydrous $MgSO_4$ and concentrated under vacuum. The residue was dissolved in methanol (150 mL) cooled down at – 80 ºC, and thionyl chloride (1.2 mL) was added dropwise. After stirring for 12 h at 25 ºC, the solvent and volatile acids were removed under vacuum. The residue solid was absorbed onto silica gel (40 g) and loaded onto a silica column chromatography. The product was eluted with a step gradient of hexane:EtOAc: 100:0, 200 mL, 75:25, 200 mL, 50:50, 200 mL and 25:75, 800 mL. Pure fractions were pooled and the solvent reduced under vacuum affording the benzyl (2-oxotetrahydrofuran-3-yl)carbamate (S)-**7** as white solid: 551 mg, 56% yield (based on initial amount of L-Ala); *ee* > 99 %, t_R = 13.3 min. Chiral HPLC analysis (Figure S14): CHIRALPAK® ID 46 x 250 mm column, 5 μm, isocratic elution hexane/CH_2Cl_2/EtOH 70/10/20 (v/v/v), flow rate 0.8 mL min^{-1} at 20 ºC, UV detection 209 and 254 nm, t_R (*R*) = 11.6 min and t_R (*S*) = 13.8 min. $[\alpha]^{20}_D$ = – 40.1 (*c* = 0.6 in DMSO). Data of lactones from authentic samples of L- and D-homoserine: $[\alpha]^{20}_D$ (*R*) = + 40.8 and $[\alpha]^{20}_D$ (*S*) = – 40.4, respectively. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.77 (d, *J* = 8.5 Hz, 1H), 7.39 – 7.25 (m, 5H), 5.02 (s, 2H), 4.41 (dt, *J* = 11.3, 8.7, 8.7 Hz, 1H), 4.29 (td, *J* = 8.9, 8.8, 1.5 Hz, 1H), 4.16 (ddd, *J* = 10.9, 8.7, 6.2 Hz, 1H), 2.37 (dddd, *J* = 12.0, 9.0, 6.2, 1.5 Hz, 1H), 2.14 (qd, *J* = 11.4, 11.4, 11.3, 8.9 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 175.8 (CO), 156.2 (NHCO), 137.2 (Carom), 128.8 (Carom), 128.4 (Carom), 128.3 (Carom), 66.1 (-CH_2-), 65.5 (-CH_2-), 50.0 (-CH-), 28.5 (-CH_2-).



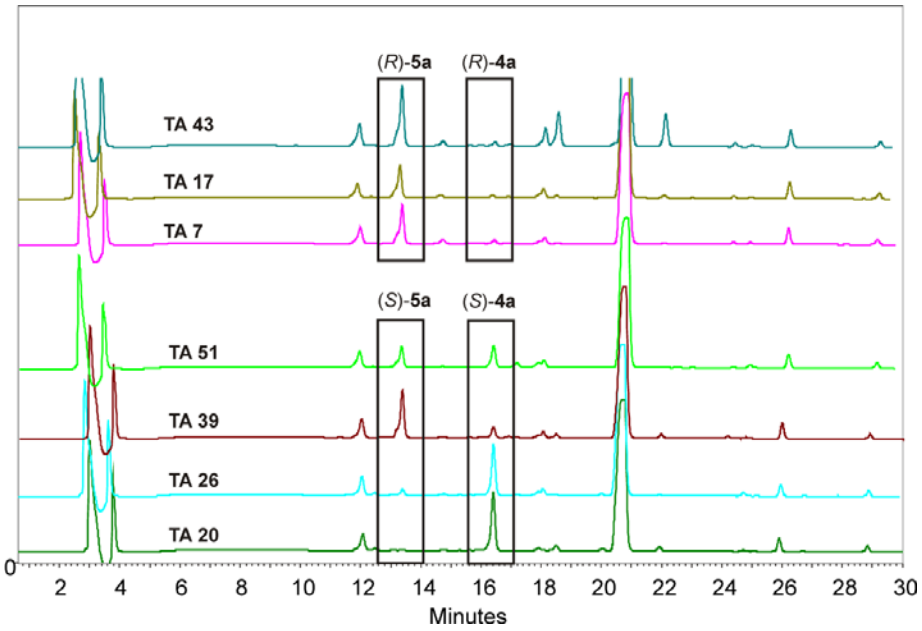


Figure S13. HPLC chromatogram of L-and D-homoserine (**5**) synthesis after 24 h of reaction with different Prozomix TA transaminases. In the chromatograms the peak of homoserine, (*R*)-**5a** and (*S*)-**5a**, and the alanine left, (*R*)-**4a** and (*S*)-**4a**, after 24 h is indicated. Reaction conditions.. Initial pyruvate (2.1 mmol) and L- or D-Ala (4.2 mmol), ($[Pyruvate]_0$:[L- or D-$Ala]_0$ ratio = 0.5), were dissolved in 50 mM sodium phosphate buffer pH 7.0 (7.8 mL) containing PLP (10.5 µmol, 1mM in the reaction), MBP-YfaU (100 U) and TA 20 (11.6 U) or TA 26 (33.6 U) or TA 39 (7.4 U) or TA 51 (37.8 U) or TA 7 (480 U) or TA 17 (1160 U) or TA 43 (460 U) depending on the experiment. Formaldehyde dissolved in 50 mM sodium phosphate buffer pH 7.0 was constantly added with a syringe pump at 0.57 mmol h^{-1} (0.18 mL h^{-1}) total addition time 15 h. The final volume was 10.5 mL.

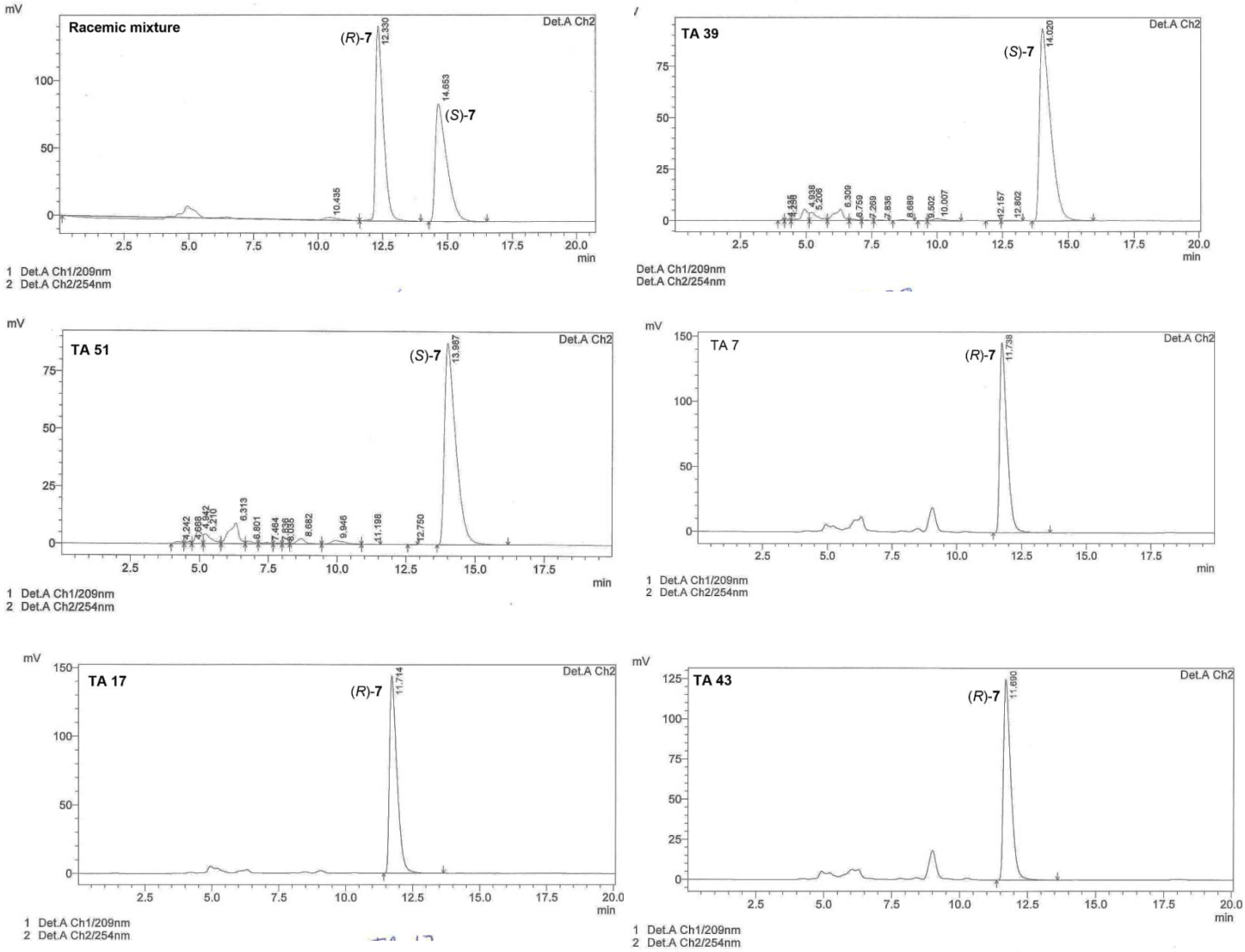
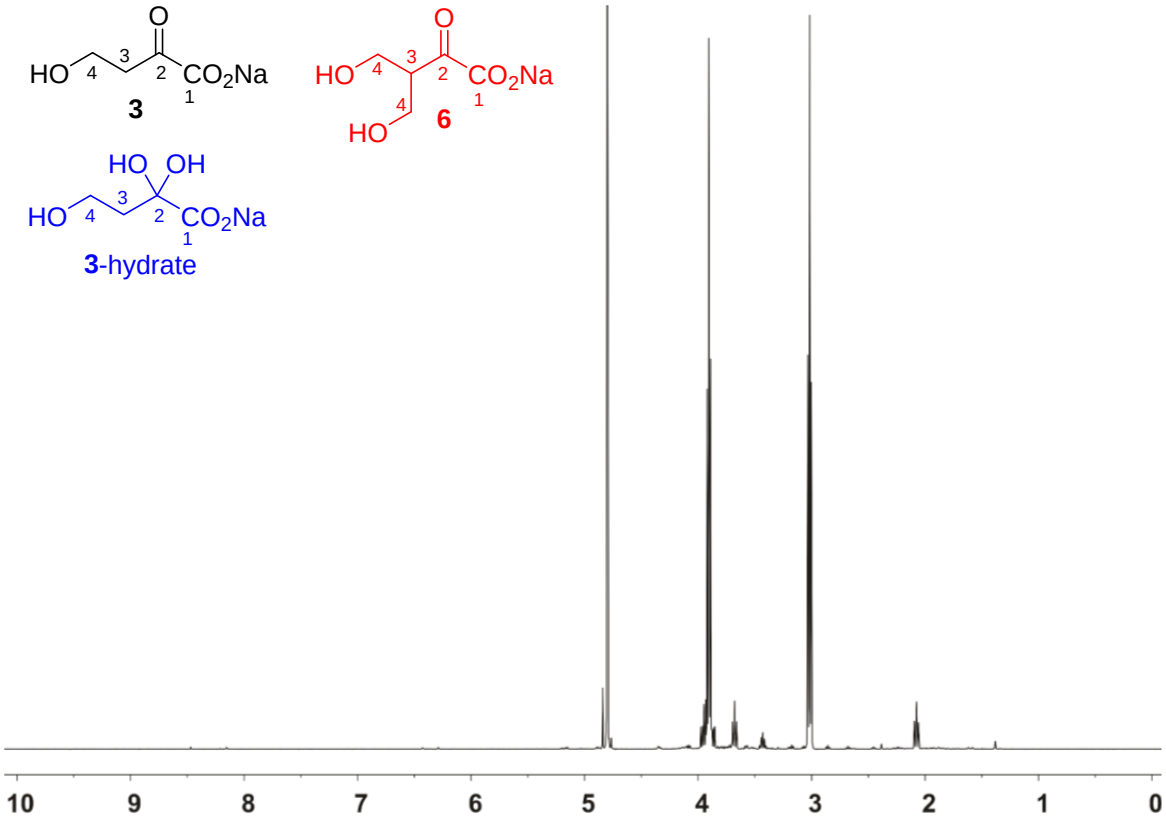


Figure S14. HPLC chromatograms of the *N*-Cbz-homoserine lactone derivatives (*S*)-**7** and (*R*)-**7** from the L- and D-homoserine obtained with the (*S*)- and (*R*)-selective transaminases. Analysis were performed in a CHIRALPAK® ID 46 x 250 mm column, 5 μm, with isocratic elution system consisting of hexane/CH_2Cl_2/EtOH 70/10/20 (v/v/v), flow rate 0.8 mL min^{-1} at 20 ºC, UV detection 209 and 254 nm, t_R (*R*) = 11.6 min and t_R (*S*) = 13.8 min.

a)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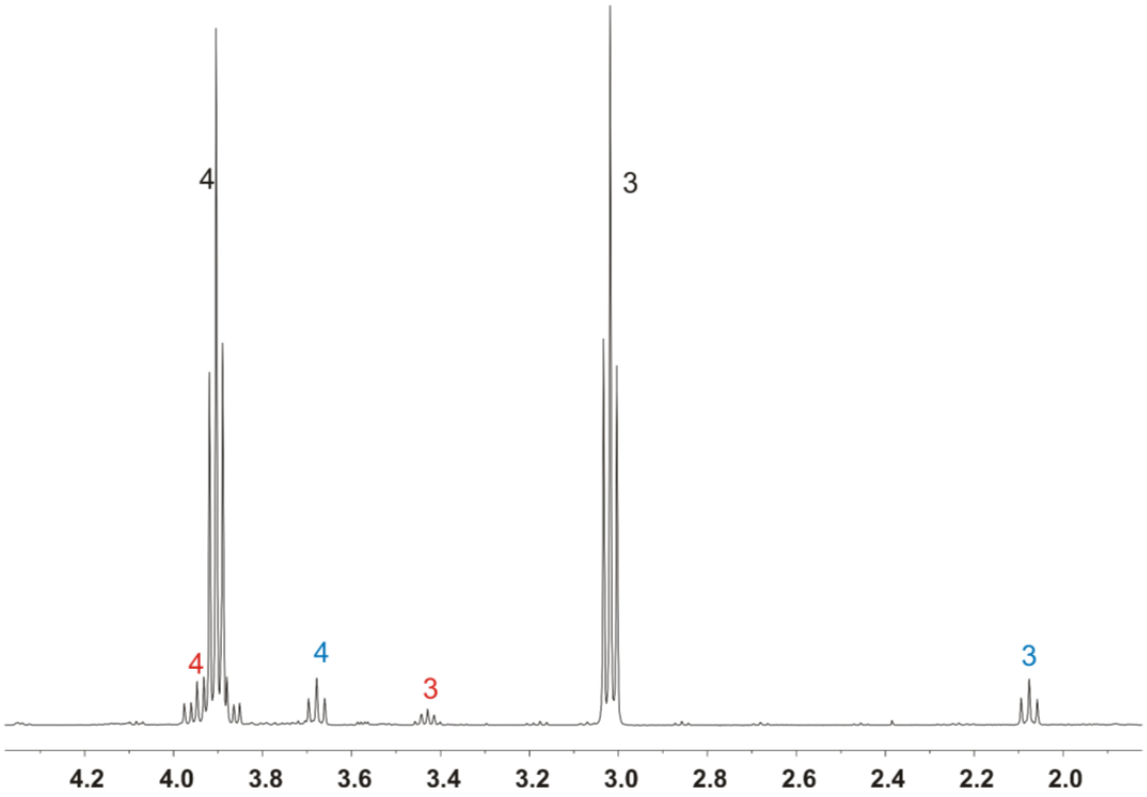
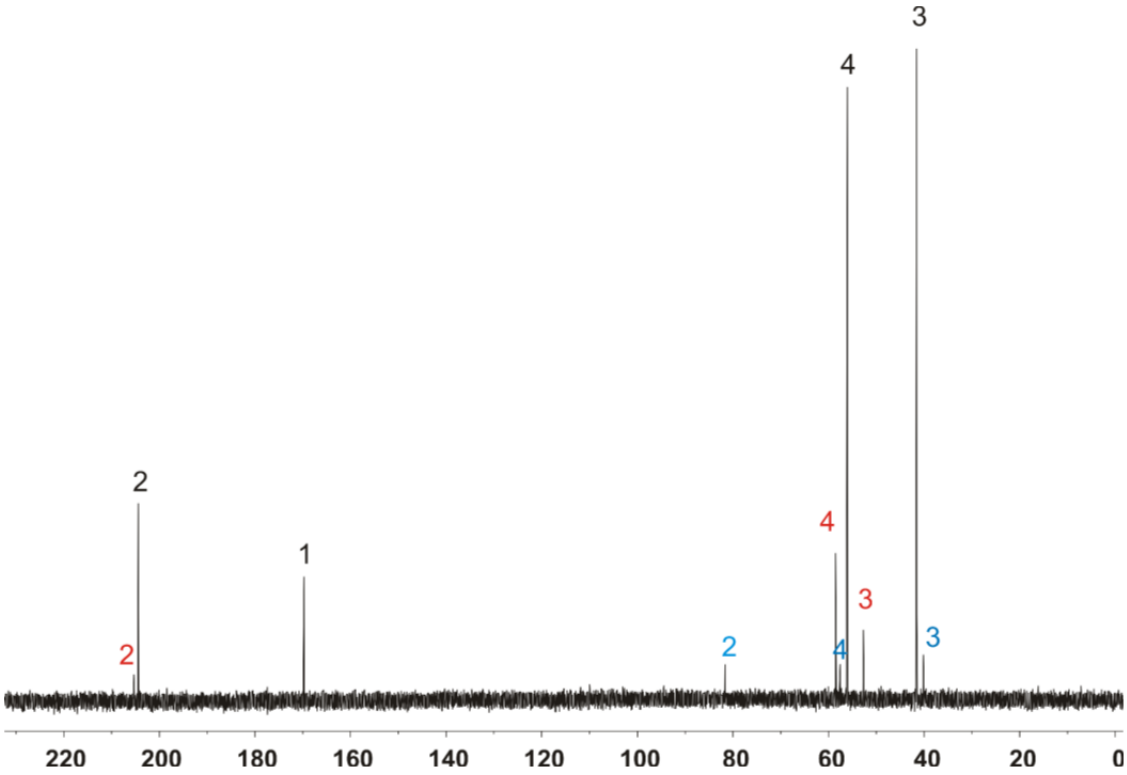
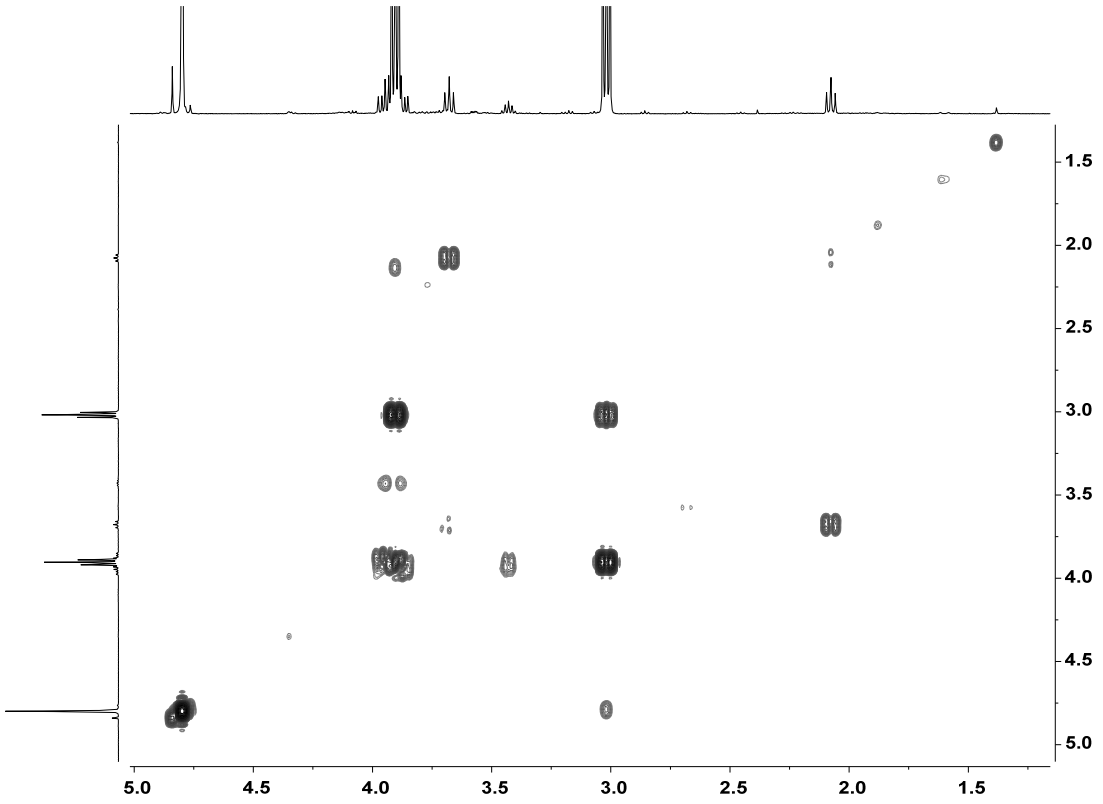
3
2
O
CO2Na
1
3
HO
4
3
2
O
CO2Na
1
4
HO
6
HO
OH
HO
4
3
2
CO2Na
1
3-hydrate
10
9
8
7
6
5
4
3
2
1
0


4
3
4
4
3
3
4.2
4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0

b)


3
4
2
1
4
3
3
2
2
4
220
200
180
160
140
120
100
80
60
40
20
0

c)


1.5
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2.5
3.0
3.5
4.0
4.5
5.0
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5

d)

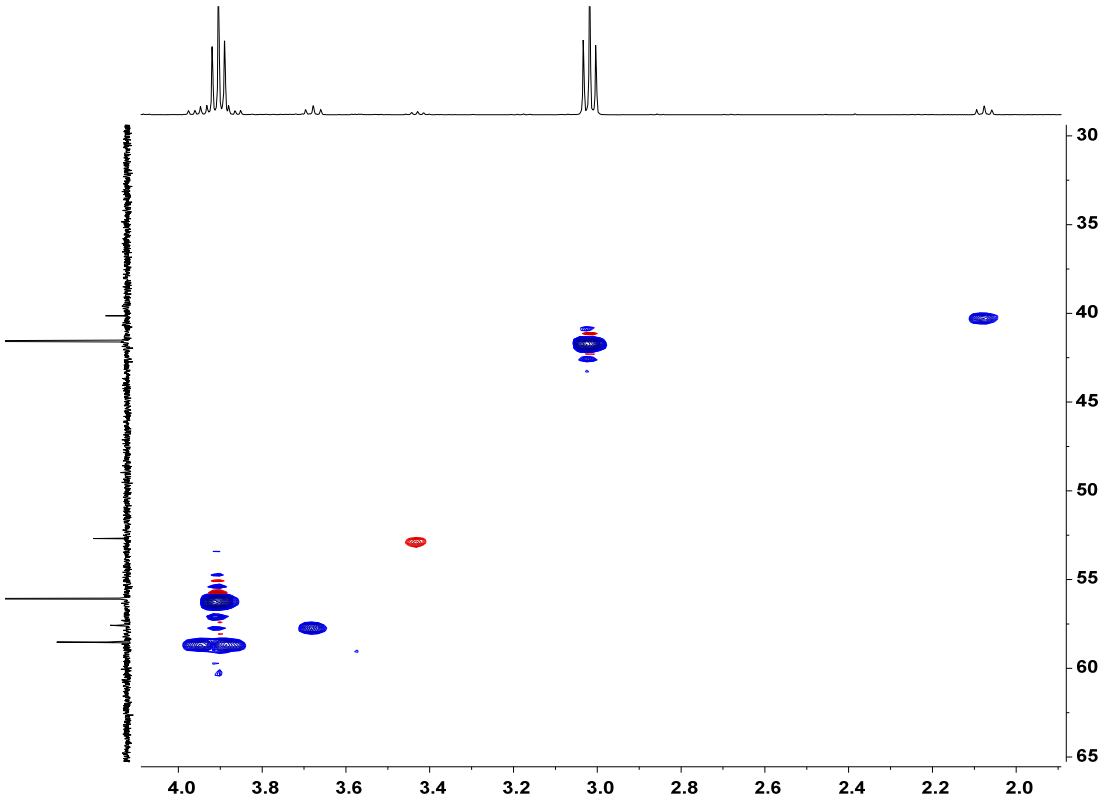
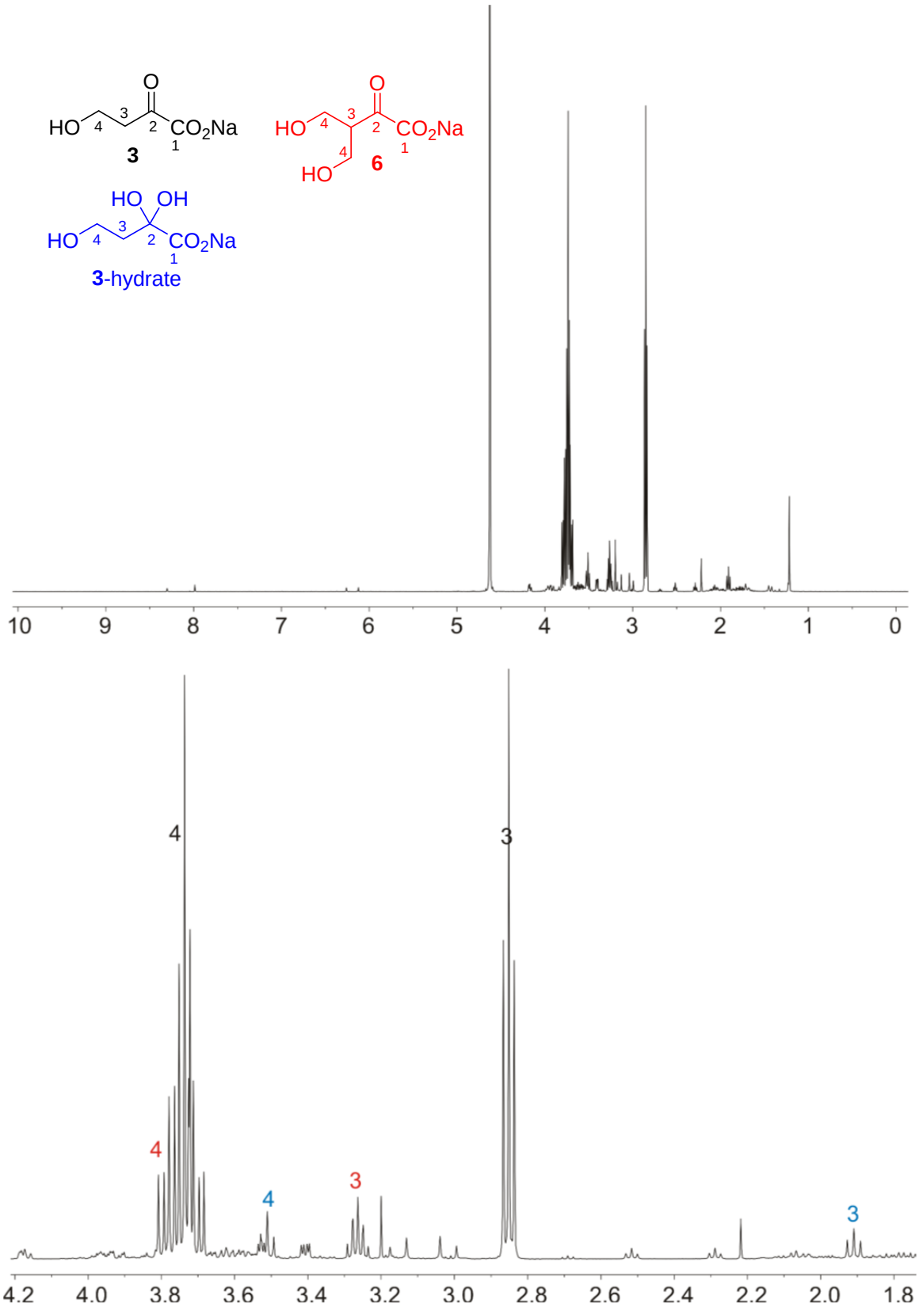


Figure S15. NMR spectra (D_2O) of compounds **3**, **3**-hydrate and **6** obtained using MBP-YfaU(Mg^{2+}) as catalyst; a) ^{1}H NMR, b) ^{13}C, c) 2D COSY, d) 2D HSQC.

a)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O
CO2Na
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3
HO
4
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2
O
CO2Na
1
4
HO
6
HO
OH
HO
4
3
2
CO2Na
1
3-hydrate
10
9
8
7
6
5
4
3
2
1
0
4
3
4
4
3
3
4.2
4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8

b)

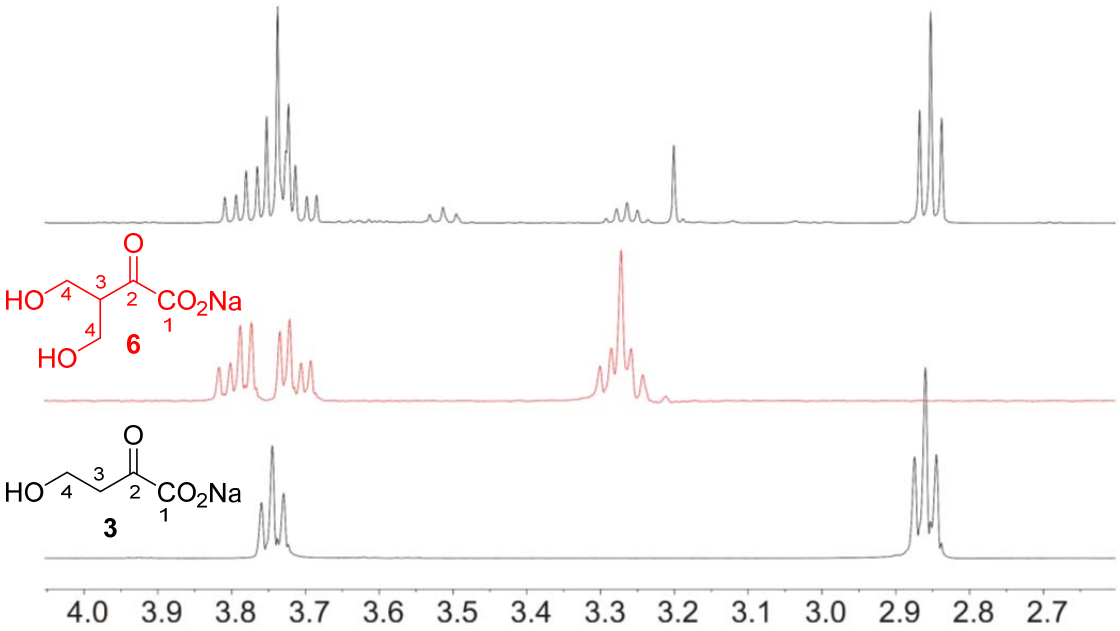
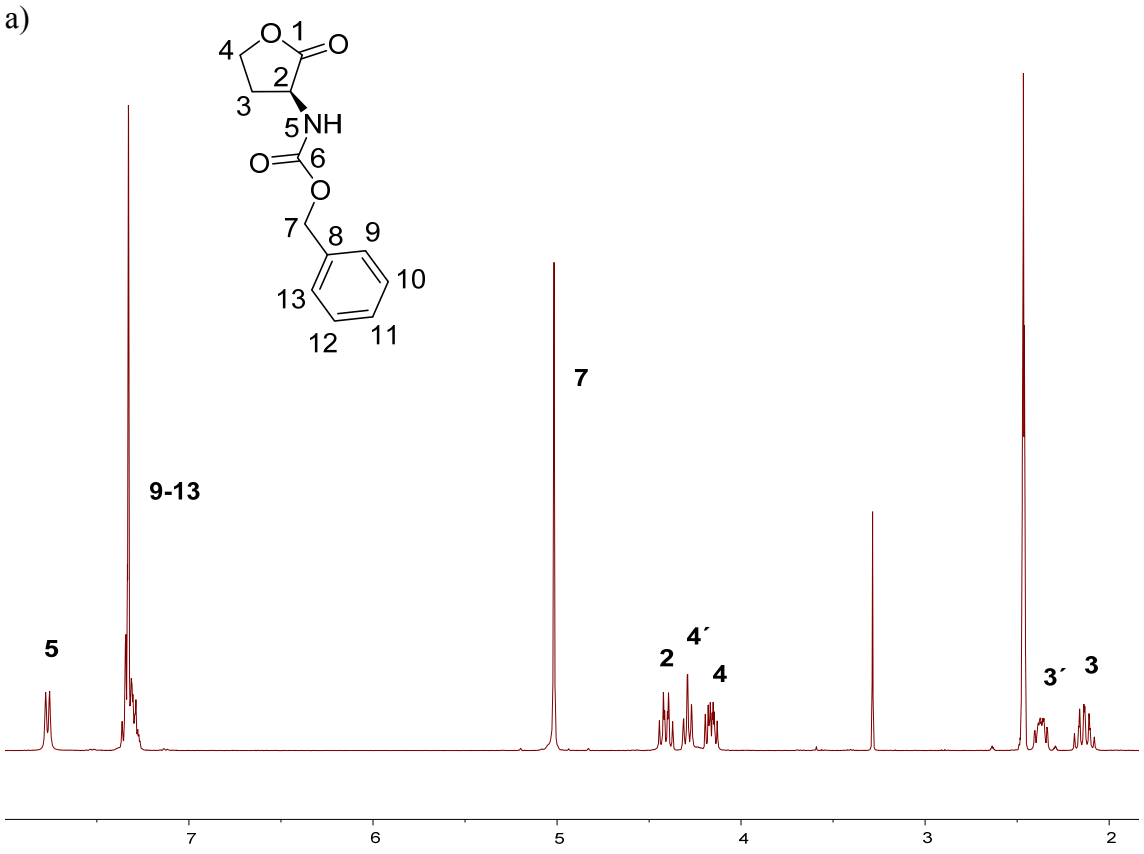


Figure S16. NMR spectra (D_2O) of compounds **3**, **3**-hydrate and **6** obtained using MBP-YfaU-Ni^{2+} as catalyst; a) ^1H NMR, b) 1D TOCSY.

a)


O
1
O
4
2
3
5NH
O
6
O
7
8
9
10
13
12
11
5
9-13
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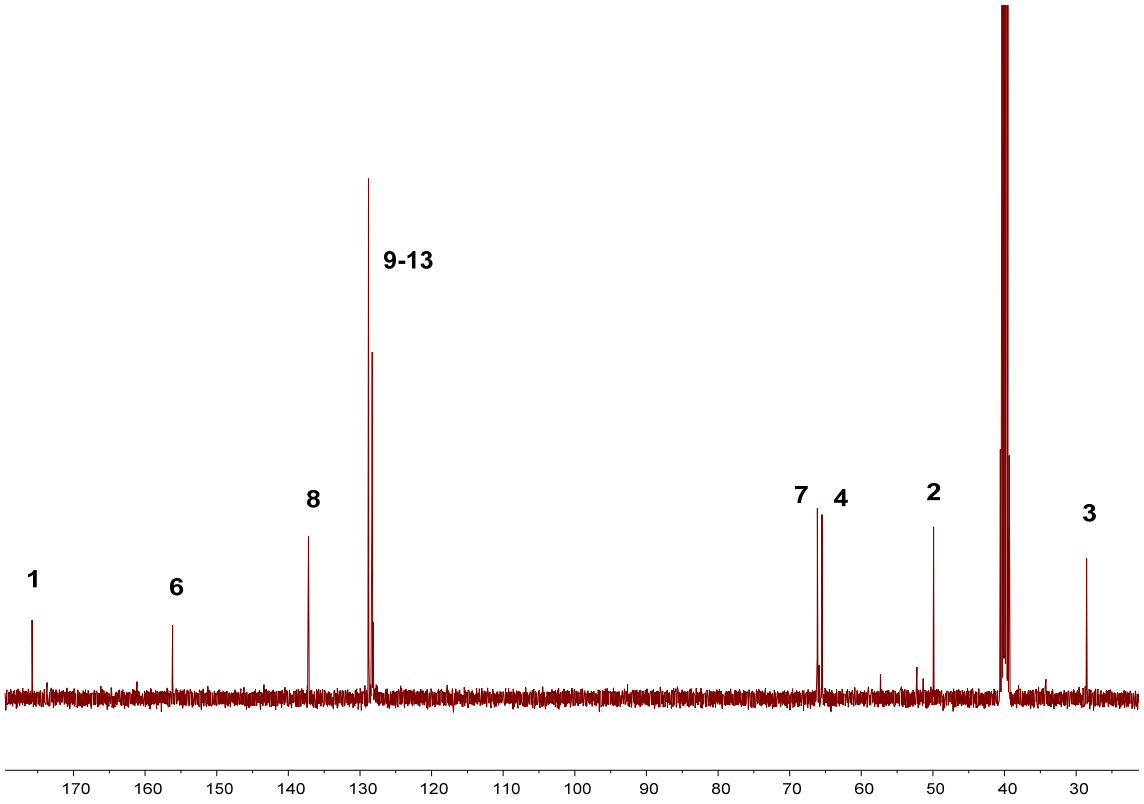
3´
3
7
6
5
4
3
2

b)


9-13
8
1
6
7
4
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140
130
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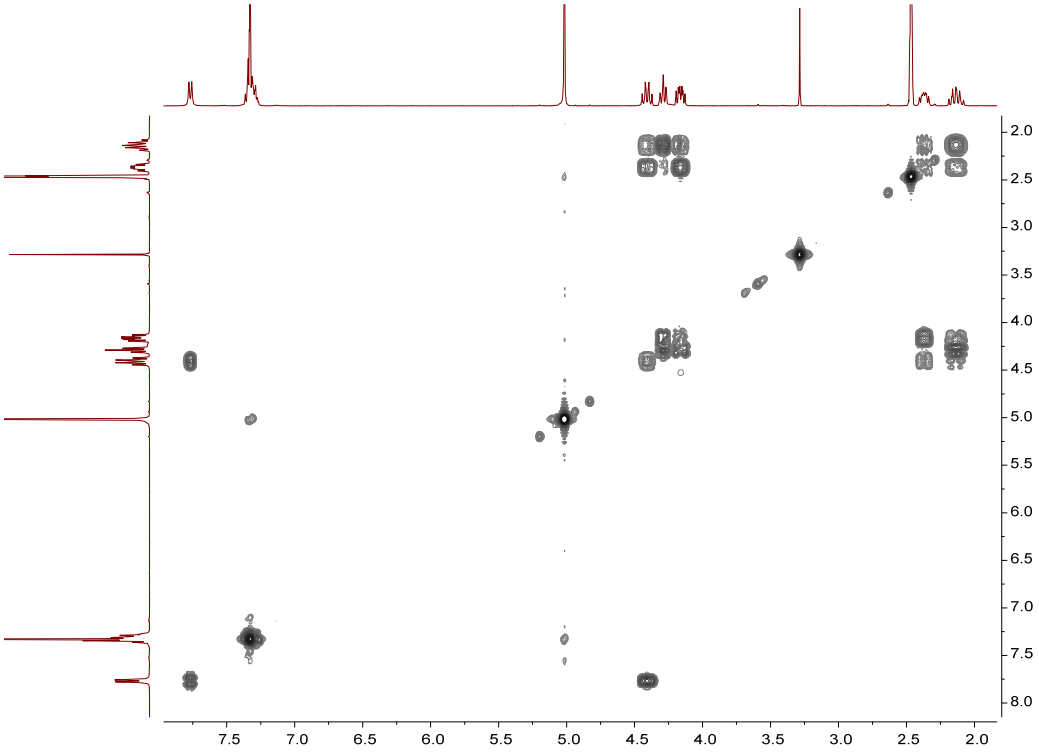
110
100
90
80
70
60
50
40
30

c)



d)

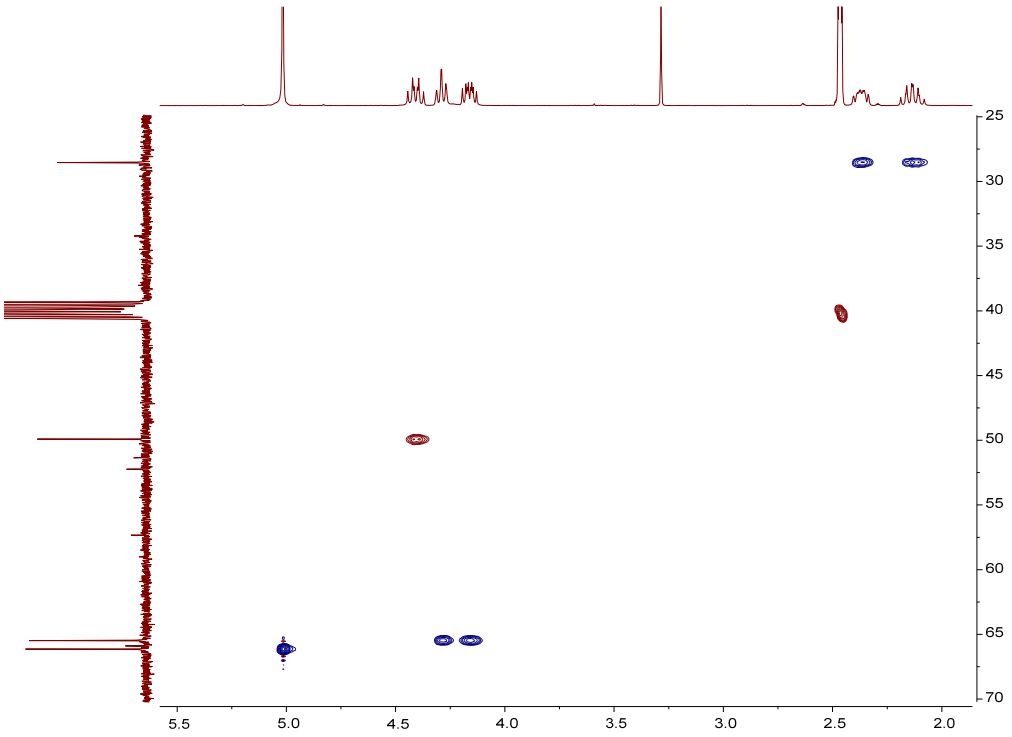


Figure S17. NMR spectra (DMSO-d_6) of Cbz-L-homoserine lactone **7**; a) ^{1}H NMR, b) ^{13}C, c) 2D COSY, d) 2D HSQC.

References

(1) Tufvesson, P.; Jensen, J. S.; Kroutil, W.; Woodley, J. M. *Biotechnol. Bioeng.* **2012**, *109*, 2159-2162.

(2) Sehl, T.; Hailes, H. C.; Ward, J. M.; Wardenga, R.; von Lieres, E.; Offermann, H.; Westphal, R.; Pohl, M.; Rother, D. *Angew. Chem. Int. Ed.* **2013**, *52*, 6772-6775.