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Supporting Information

Oxalyl-CoA Decarboxylase Enables Nucleophilic One-Carbon Extension of Aldehydes to Chiral α-Hydroxy Acids

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Supplementary Information – Table of Contents

Chemicals

Unless stated otherwise, standard laboratory reagents were obtained from Sigma-Aldrich® (Steinheim, Germany) or Carl Roth GmbH & Co. KG (Karlsruhe, Germany) with the highest purity available. Propionaldehyde, Vanillin, (*R*)- and (*S*)-mandelic acid were obtained from Tokyo Chemical Industry (Zwijndrecht, Belgium). 4-chloromandelic acid was obtained from Alfa Aesar (ThermoFisher (Kandel) GmbH; Kandel, Germany).

Experimental Procedures

Formyl-CoA synthesis

Formyl-CoA was synthesized as described previously.[1] After Extraction with diethylether formyl-CoA was purified by preparative HPLC-MS with an acetonitrile gradient in 25 mM ammonium formate pH 4.2. The fractions containing the product were lyophilized and stored at -20 °C. Formyl-CoA was dissolved in aq. HCl (pH 4). The concentration was determined by enzymatic depletion with PduP,[2] following NADH consumption at 340 nm.

Oxalyl-CoA synthesis

Oxalyl-CoA was synthesized enzymatically with OXS. A 5 mL reaction containing 50 mg CoA (0.064 mmol, 1 eq.), 52 mg ATP (0.086 mmol, 1.3 eq.), 10 mg disodium oxalate (0.075 mmol, 1.2 eq.) in buffer (100 mM MES-KOH pH 6.8, 15 mM $MgCl_2$) was started by adding OXS to a final concentration of 0.4 mg/mL and incubated at 30 °C for 1 hour. The reaction was quenched with 250 µL formic acid and the enzyme removed by centrifugation (4,000 × g, 4 °C, 10 min). Oxalyl-CoA was purified by preparative HPLC-MS with an acetonitrile gradient in 25 mM ammonium formate pH 4.2. The fractions containing the product were lyophilized and stored at -20 °C. Oxalyl-CoA was dissolved in 10 mM acetate buffer pH 4.5. The concentration was determined by enzymatic depletion with PanE2, following NADPH consumption at 340 nm.

Mandelyl-CoA synthesis

Mandelyl-CoA and (*S*)-mandelyl-CoA were synthesized chemically with the carbonyldiimidazole (CDI) CoA-acylation method described previously.[3] 21 mg CDI (0.127 mmol, 4 eq.) was dissolved in 1 mL tetrahydrofuran, mandelic acid or (*S*)-mandelic acid was added (0.127 mmol, 4 eq.) and the mixture stirred at 22 °C for 15 min. 25 mg CoA (0.032 mmol, 1 eq.) was dissolved in 1 mL 0.5 M $NaHCO_3$ and added to the reaction mixture, followed by stirring at 22 °C for 30 min. THF was removed by applying vacuum (100 mbar) for 5 min. The mixture was then purified by preparative HPLC with a methanol gradient in 25 mM ammonium formate pH 8.0. The fractions containing the product were lyophilized and stored at -20 °C. Mandelyl-CoA was dissolved in 10 mM acetate buffer pH 4.5. The concentration was determined spectrophotometrically, using the extinction coefficient for saturated CoA esters (ε_{260nm} =

16.4 cm^{-1} mM^{-1}). The concentration was confirmed by enzymatic depletion with YciA, detecting the liberated CoA with Ellman's reagent (ε_{412nm} = 14.15 cm^{-1} mM^{-1}).[4]

Cloning and Mutagenesis

Oligonucleotides were obtained from Eurofins Genomics (Ebersbach, Germany). *oxc* (MexAM1_META1p0990), *oxs* (MexAM1_META1p2130) and *panE2* (MexAM1_META1p3141) were PCR-amplified from *Methylorubrum extorquens* chromosomal DNA using the corresponding primers (Table S1). The purified PCR products were digested with NdeI and BamHI and ligated into pET-16b. Correct cloning was confirmed by sequencing (Eurofins Genomics). Human *hacl1* (Gene ID: 26061) was obtained by gene synthesis (Table S2), performed by BaseClear (Leiden, The Netherlands). The gene was codon optimized for *E. coli* and sub-cloned into pET-16b. *adk*, *paaI*, *tesB* and *yciA* were obtained from the ASKA collection.[5] Point mutations were introduced into *oxc* by PCR using mismatch primers (Table S1). A 50 µL reaction contained 60 ng of pET-16b_OXC_{Me}, 0.25 µM forward and reverse primer, 200 µM dNTP, 5 µL 10x Reaction Buffer, 1 µL Phusion polymerase (2 U/µL). Template plasmid was removed by DpnI digest (10 U) at 37 °C immediately after PCR amplification. Mutations were confirmed by sequencing.

Protein Production and Purification

All proteins except $HACL_{Hs}$ were heterologously produced in *E. coli* BL21 (DE3). 500 mL TB containing 100 µg/mL ampicillin (OXC_{Me}, OXS, PduP, and PanE2) or 34 µg/mL chloramphenicol (AdK, PaaI, TesB and YciA) was inoculated with freshly-transformed cells and incubated at 37 °C. After reaching an OD_{600} of 0.8 expression was induced by adding IPTG to a final concentration of 0.25 mM and the incubation temperature was lowered to 25 °C. $HACL_{Hs}$ was produced in *E. coli* ArcticExpress (DE3). 1 L LB containing 100 µg/mL ampicillin and 15 µg/mL gentamycin was inoculated with freshly-transformed cells and incubated at 37 °C. After reaching an OD_{600} of 0.8 the culture was cooled on ice for 15 min. Then expression was induced by adding IPTG to a final concentration of 0.1 mM and the incubation temperature was lowered to 15 °C. Cells were harvested after 16 h (24 h for $HACL_{Hs}$) by centrifugation (4500× g, 10 min) and resuspended in buffer A (500 mM KCl, 50 mM HEPES-KOH pH 7.6). If not used immediately, cell pellets were flash-frozen in liquid nitrogen and stored at −20 °C. The cell lysate obtained by sonication was clarified by centrifugation 75,000× g at 4 °C for 45 min. The supernatant was filtered through a 0.4 µm syringe tip filter (Sarstedt, Nümbrecht, Germany). Ni-affinity purification was performed with an Äkta FPLC system from GE Healthcare (GE Healthcare, Freiburg, Germany). The filtered soluble lysate was loaded onto a 1 mL Ni-Sepharose Fast Flow column (HisTrap FF, GE Healthcare, Little Chalfont, UK) that had been equilibrated with 10 mL buffer A. After washing with 20 mL 85% buffer A, 15% buffer B (500 mM KCl, 50 mM HEPES-KOH pH 7.6, 500 mM imidazole), the protein was eluted with 100% buffer B. Fractions containing purified protein were pooled and the buffer was exchanged to storage buffer (150 mM KCl, 50 mM HEPES-KOH pH 7.6) with a desalting column (HiTrap, GE

Healthcare). Proteins were concentrated by ultrafiltration (Amicon Ultra). Concentration was determined on a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA) using the extinction coefficient at 280 nm, as calculated by protparam (https://web.expasy.org/protparam/). Enzyme purity was confirmed by SDS-PAGE. The purified proteins were stored in 50 vol% glycerol at −20 °C. OXC_{Me} wild-type and mutants were flash-frozen in liquid nitrogen and stored at −80 °C.

LC-MS analyses

Samples were prepared for LC-MS analysis by quenching an aliquot of a reaction with formic acid (final concentration 4%) and centrifuging for 10 min at 17,000 rcf, to remove precipitated proteins. LC-MS data were analyzed and quantified using MassHunter Qualitative Navigator and Quantitative Analysis software (Agilent, Waldbronn, Germany).

LC-MS detection of CoA esters

Samples were diluted 1:10 in H_2O. UPLC-high resolution MS of CoA-esters was performed as described previously.[6] CoA-esters were analyzed using an Agilent 6550 iFunnel Q-TOF LC-MS system equipped with an electrospray ionization source set to positive ionization mode. Compounds were separated on a RP-18 column (50 mm x 2.1 mm, particle size 1.7 µm, Kinetex EVO C18, Phenomenex) using a mobile phase system comprised of 50 mM ammonium formate pH 8.1 (A) and methanol (B). Chromatographic separation was carried out using the following gradient condition at a flow rate of 250 µL/min: 0 min 2.5% B; 2.5 min 2.5% B; 8 min 23% B; 10 min 80 %B; 11 min 80%; 12 min 2.5% B; 12.5 min 0% B. The column oven was set to 40 °C and autosampler was maintained at 10 °C. Standard injection volume was 1 µL. Capillary voltage was set at 3.5 kV and nitrogen gas was used as nebulizing (20 psig), drying (13 L/min, 225 °C) and sheath gas (12 L/min, 400°C). The TOF was calibrated using an ESI-L Low Concentration Tuning Mix (Agilent) before measurement (residuals less than 2 ppm for five reference ions) and was recalibrated during a run using 922.0908 *m/z* as reference mass. The scan range for MS and MS/MS data is 100-1000 *m/z* and 50-1000 *m/z* respectively. Collision energy used for MS/MS fragmentation was 35 eV.

LC-MS detection of mandelic acid derivatives

Samples were diluted 1:10 in H_2O. UPLC-high resolution MS analyses were performed on an Agilent 6550 iFunnel QTOF LC/MS system equipped with an electrospray ionization source to negative ionization mode. The analytes were isocratically chromatographed on a chiral column (100 mm x 2.1 mm, particle size 2.7 µm, Poroshell 120 Chiral-T, Agilent) kept at ambient tempature using a mobile phase system comprised of 30:70 20 mM ammonium formate pH 4 / methanol at a flow rate of 250 µL/min for 10 min. Samples were held at 10°C and injection volume was 1 µL. Capillary voltage was set at 3.5 kV and nitrogen gas was used as nebulizing (20 psig), drying (13 L/min, 225 °C) and sheath gas

(12 L/min, 400°C). MS data were acquired with a scan range of 100-1100 *m/z*. For quantification the calculated *m/z* value of $[M-H]^-$ was used to obtain the extracted ion count from the total ion count.

Enzyme assays with LC-MS detection

Unless noted otherwise, all LC-MS assays were carried out at 30 °C in reaction buffer consisting of 50 mM TES-KOH pH 6.8, 10 mM $MgCl_2$. 0.5 mM ADP, 0.15 mM ThDP.

OXC_{Me} and $HACL_{Hs}$ aldehyde screen

In reaction buffer, 1 mM formyl-CoA and 10 mM aldehyde (formaldehyde, acetaldehyde, glycolaldehyde, propionaldehyde, glyceraldehyde, glyoxylate, succinic semialdehyde, benzaldehyde, and phenylacetaldehyde) was mixed with 5 µM OXC_{Me} and $HACL_{Hs}$, respectively. The reaction was stopped after 1 h and products analyzed with the CoA ester method described above.

YciA substrate screen

Thioesterase activity of YciA was determined by adding 2 µM YciA to the reaction buffer containing 25 mM benzaldehyde and 0.5 mM formyl-CoA, oxalyl-CoA and mandelyl-CoA, respectively. Samples were taken after 0, 1, 5 and 30 min and analyzed with the CoA ester method described above.

OXS- OXC_{Me}-YciA cascade prototyping

In a 1.5 mL microfuge tube, reaction buffer containing 0.5 mM CoA, 10 mM ATP, 25 mM benzaldehyde, 2 µM YciA, 5 µM OXC_{Me} and 5 µM OXS were mixed and the reaction was initiated by the addition of 10 mM disodium oxalate. For the negative controls each enzyme was omitted in a separate reaction. Samples were taken after 0, 3, 15, 40 min, 1, 2, 3 and 22 h and analyzed with the mandelic acid derivatives method described above. For quantification commercial, racemic mandelic acid was diluted in reaction buffer to appropriate concentrations to obtain a calibration curve (Figure S8).

OXS- OXC_{Me}-YciA cascade aldehyde scope

The aldehyde substrate screen of the OXS-$OXC_{Me\text{-}Y497A}$-YciA cascade was carried out as described above, except that the aromatic aldehydes were prepared as 33 mM stocks in 20 vol% DMSO and diluted to final concentration of 25 mM into the assay. Samples were analyzed with the mandelic acid derivatives method described above. Mandelic acid (**3a**), 4-chloromandelic acid (**3c**), 2-chloromandelic acid (**3d**) and 3-phenyllactic acid (**3b**) were quantified by comparison to commercial standards (Figure S8).

OXS- OXC_{Me}-YciA cascade on semi-preparative scale

In a glass vial (25 mL reaction volume) 50 mM TES-KOH pH 6.8, 10 mM $MgCl_2$, 0.5 mM ADP (0.0125 mmol, 5.3 mg), 0.15 mM ThDP (0.00375 mmol, 1.7 mg), 0.5 mM CoA (0.0125 mmol, 9.8 mg), 25 mM disodium oxalate (0.625 mmol, 84 mg), 25 mM benzaldehyde (0.625 mmol, 66 mg), 2 µM YciA, 10 µM $OXC_{Me\text{-}Y497A}$, 5 µM OXS, 1.3 µM adenylate kinase and 25 units creatine kinase (Roth) were mixed and the reaction was initiated by the addition of 10 mM creatine phosphate. The same amount of creatine phosphate was added after 1, 2, 4 and 6 h to reach a final concentration of 50 mM (1.25 mmol, 409 mg). The vials were incubated without shaking at 30 °C for 24 h. Then the pH was lowered to 3 by adding HCl. The quenched reaction was saturated with NaCl and extracted with 25 mL diethylether. The organic phase was dried over $MgSO_4$ and filtered. After evaporation of the ether under vacuum, 50 mg (0.331 mmol, 53%) of a white solid remained, which was confirmed to be mandelic acid by UV (Figure S9), HPLC (Figure S10), and NMR (Figure S11). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.4 (m, 5H); 5.05 (s, 1H). ^{13}C NMR (300 MHz, DMSO-d_6) δ 174.5, 140.7, 128.6, 128.1, 127.1, 72.9.

Spectrophotometric enzyme assays

Assays were performed on a Cary-60 UV/Vis spectrophotometer (Agilent) at 30°C using quartz cuvettes (10 mm path length; Hellma, Müllheim, Germany). For the determination of steady-state kinetic parameters, each substrate concentration was measured in triplicates and the obtained curves were fit using GraphPad Prism 7. Hyperbolic curves were fit to the Michaelis-Menten equation to obtain apparent k_{cat} and K_M values.

Michaelis-Menten kinetics of OXS

Oxalyl-CoA production was followed by coupling OXS to purified PanE2, an NADPH-dependent oxalyl-CoA reductase from *M. extorquens*.[7] An assay containing 50 mM potassium phosphate pH 6.5, 0.3 mM NADPH, 10 mM $MgCl_2$, 1 mM ATP, 2.5 mM CoA, 1.2 µM PanE2, 176 nM OXS was preincubated for 2 min and the reaction started by adding disodium oxalate to a final concentration of 5, 10, 25, 100, 250, 1,000 µM, respectively. Reaction procedure was monitored by following the oxidation of NADPH at 340 nm.

Michaelis-Menten kinetics OXC_{Me}

Formyl-CoA production was followed by coupling OXC to purified PduP, a promiscuous CoA-dependent aldehyde dehydrogenase that reduces formyl-CoA to formaldehyde under NADH consumption.[2] An assay containing 50 mM MES-KOH pH 6.5, 0.3 mM NADH, 10 mM $MgCl_2$, 0.5 mM ADP, 0.15 mM ThDP, 5 µM PduP, and OXC_{Me} (concentration depending on the mutant) was preincubated for 2 min and the reaction started by adding oxalyl-CoA (concentrations depending on the mutant). Reaction procedure

was monitored by following the oxidation of NADH at 340 nm. Michaelis-Menten graphs are shown in Figure S3.

Supplementary Tables

Table S1. Primers used for cloning of OXC and OXS and site-directed mutagenesis of OXC.

Primer name	Nucleotide sequence (5‘ to 3‘) (restriction sites or mismatches are underlined)
oxc_fw_NdeI	GTTCACATATGACCGTCCAGGCCCAG
oxc_rv_BamHI	CGCTGGATCCTCACTTCTTCTTCAAGGTGCTC
oxs_fw_NdeI	GTTCACATATGACGATGCTTCTGCC
oxs_rv_BamHI	CAATGGATCCTCAGACCAGCCCGAG
panE2_fw_NdeI	GCGCACATATGAGCATCGCGATCGTCG
panE2_rv_BamHI	CAGAGGATCCTCATGCTCCCTGGATCGC
oxc_Y497A_fw	CAACAACGGCATCGCTCGCGGCACCGAC
oxc_Y497A_rv	GTCGGTGCCGCGAGCGATGCCGTTGTTG
oxc_S568A_fw	CCGGCAGCGAGGCCGGCAATATCGG
oxc_S568A_rv	CCGATATTGCCGGCCTCGCTGCCGG

Table S2. Synthetic gene sequences

Gene name	DNA sequence
hacl1 (Gene ID 26061)	ATGCCGGACAGTAACTTCGCAGAGCGCAGCGAGGAGCAGGTGTCTGGTGCTAAAGTCATCGCTC AGGCCCTGAAAACGCAAGATGTGGAGTACATATTTGGCATCGTAGGCATCCCAGTGACCGAAATC GCCATTGCTGCCCAGCAGCTAGGCATCAAGTACATCGGGATGAGGAATGAGCAAGCGGCTTGTT ATGCTGCCTCCGCGATTGGATATCTGACAAGCAGGCCAGGAGTCTGCCTTGTTGTTTCTGGCCCA GGTCTCATCCATGCCTTGGGCGGTATGGCAAATGCAAACATGAACTGCTGGCCCTTGCTTGTGAT TGGTGGTTCCTCTGAAAGAAACCAAGAAACAATGGGAGCTTTCCAGGAGTTTCCTCAGGTTGAAG CTTGTAGATTATATACCAAGTTCTCTGCCCGCYCAAGCAGCATAGAAGCTATTCCTTTTGTTATTGA AAAGGCAGTGAGAAGCAGTATCTATGGTCGTCCAGGTGCTTGCTATGTTGACATACCAGCAGATT TTGTGAACCTTCAGGTGAATGTGAATTCTATAAAGTACATGGAACGCTGCATGTCACCTCCTATTA GCATGGCAGAAACCTCTGCTGTGTGCACGGCGGCTTCTGTTATTAGGAATGCCAAACAACCCCTT CTTATCATCGGGAAAGGTGCTGCTTACGCTCATGCAGAAGAGAGTATCAAGAAATTGGTGGAGCA ATATAAACTGCCATTTTTGCCCACCCCTATGGGAAAGGGTGTTGTCCCTGACAACCATCCATACTG TGTAGGTGCAGCCAGATCCAGGGCTTTGCAATTTGCTGATGTAATTGTGTTATTTGGTGCCAGACT AAATTGGATTTTACATTTTGGACTGCCTCCAAGATATCAGCCAGATGTGAAGTTTATCCAGGTTGAT ATCTGTGCAGAAGAATTGGGGAATAATGTAAAGCCCGCTGTTACTTTGCTAGGAAACATACATGCT GTCACTAAGCAGCTTTTAGAGGAACTTGATAAAACACCATGGCAGTATCCTCCAGAGAGCAAGTG GTGGAAAACTCTGAGAGAAAAAATGAAGAGCAATGAAGCTGCATCCAAGGAACTAGCTTCTAAAA AATCCCTGCCTATGAATTATTACACAGTATTCTACCATGTTCAAGAACAACTACCTAGAGACTGTTT CGTGGTAAGTGAAGGAGCAAATACTATGGACATTGGACGGACTGTGCTTCAGAACTACCTTCCTC GTCACAGGCTTGATGCTGGTACTTTCGGAACAATGGGAGTTGGTTTGGGATTTGCTATTGCAGCT GCCGTGGTGGCTAAAGATAGAAGCCCTGGGCATTGGATCATCTGTGTGGAAGGAGACAGTGCAT TTGGGTTTTCTGGCATGGAGGTAGAAACCATCTGCAGGTACAACTTGCCAATCATACTGTTGGTAG TGAATAACAATGGAATTTACCAAGGTTTTGATACAGATACTTGGAAAGAAATGTTAAAATTTCAAGA TGCTACTGCAGTGGTCCCTCCAATGTGTTTGCTGCCAAATTCACATTATGAGCAAGTCATGACTGC ATTTGGAGGCAAAGGGTATTTTGTACAAACACCAGAAGAACTCCAAAAATCCCTGGAGCAGAGCC TAGCAGACACAACTAAACCTTCTCTTATCAACATCATGATTGAGCCACAAGCCACACGGAAGGCC CAGGATTTTCATTGGCTGACCCGCTCTAATATGTAA
hacl1 codon optimized for *E. coli*	ATGCCGGATTCAAACTTTGCTGAACGTTCAGAAGAACAGGTTTCTGGCGCAAAAGTGATTGCGCA AGCGCTAAAAACCCAGGATGTCGAATATATCTTCGGTATAGTGGGTATTCCGGTGACGGAAATCG CCATCGCGGCTCAGCAACTCGGTATTAAATACATCGGTATGCGTAATGAGCAGGCGGCTTGTTAC GCAGCAAGCGCGATTGGCTATCTGACCTCAAGACCGGGGGTATGCCTTGTTGTCAGCGGCCCGG GCCTGATTCATGCCCTGGGTGGTATGGCGAATGCTAACATGAACTGCTGGCCGCTGTTAGTTATA GGCGGCAGCAGTGAACGCAATCAGGAGACCATGGGCGCATTCCAAGAGTTTCCTCAGGTGGAAG CCTGCCGCCTGTATACCAAATTTTCTGCTAGACCTTCGTCAATAGAAGCCATCCCCTTCGTGATTG AAAAAGCGGTCCGTAGTTCTATCTATGGGCGCCCTGGGGCTTGCTATGTTGACATTCCCGCCGAC TTCGTAAACCTCCAAGTGAATGTCAATTCCATTAAATACATGGAACGCTGTATGTCGCCGCCGATC AGTATGGCCGAGACCAGCGCCGTGTGCACAGCGGCGAGTGTTATCCGCAACGCCAAACAGCCAT TACTAATAATTGGGAAAGGAGCCGCCTATGCTCACGCTGAAGAGTCCATCAAAAAACTGGTGGAA CAATACAAGTTGCCCTTTTTACCTACTCCCATGGGTAAAGGTGTTGTACCTGATAATCATCCATACT GCGTCGGTGCAGCCCGTTCCCGCGCACTACAGTTCGCTGACGTGATTGTGCTGTTTGGTGCGCG TTTAAACTGGATACTCCACTTCGGTTTACCGCCACGCTACCAGCCGGATGTGAAATTTATACAGGT TGACATCTGTGCCGAAGAACTGGGCAATAATGTGAAACCAGCAGTCACTTTGCTGGGGAACATCC ATGCAGTCACCAAACAACTGCTTGAAGAGCTGGACAAGACGCCGTGGCAATATCCGCCAGAGTCA AAATGGTGGAAGACGCTACGTGAGAAAATGAAGAGCAACGAAGCGGCTTCGAAAGAGTTGGCCT CCAAAAAGTCATTGCCTATGAATTACTATACCGTCTTTTATCATGTCCAAGAGCAGCTGCCGCGGG ATTGCTTTGTCGTGTCGGAGGGCGCCAACACCATGGATATAGGTCGCACTGTCCTTCAGAACTAT CTGCCCCGGCATCGCCTGGACGCTGGTACCTTCGGAACTATGGGCGTAGGGCTGGGCTTTGCTA TTGCGGCGGCAGTGGTAGCAAAGGATCGCAGCCCGGGGCAGTGGATTATTTGTGTAGAAGGGGA CTCCGCATTTGGATTCTCTGGTATGGAGGTAGAGACAATATGCCGTTATAATTTGCCAATTATCCT TCTAGTGGTGAATAACAATGGTATTTATCAGGGCTTTGATACAGATACGTGGAAGGAAATGCTGAA ATTTCAAGATGCCACAGCCGTGGTACCCCCCATGTGTTTGCTGCCGAACTCTCATTACGAGCAAG TGATGACAGCATTCGGCGGCAAGGGTTACTTTGTGCAGACACCGGAAGAACTTCAAAAGAGCCTT CGCCAGAGCTTGGCGGACACCACGAAACCGAGTCTGATTAATATAATGATTGAACCACAGGCCAC AAGGAAGGCACAAGACTTTCACTGGCTCACGCGTTCAAATATGTAA

Supplementary Figures

Figure S1. MS/MS spectra of the 2-hydroxyacyl-CoA thioester products. Calculated *m/z* values of the fragmentation products are indicated on top. Spectra on the left show the parent ion. Spectra on the right show the fragmentation products of the parent ion (blue asterisk). The numbers in the spectrum indicate measured *m/z* values. Mandelyl-CoA was produced from deuterated benzaldehyde; the deuterium is retained on the α-carbon in the product.

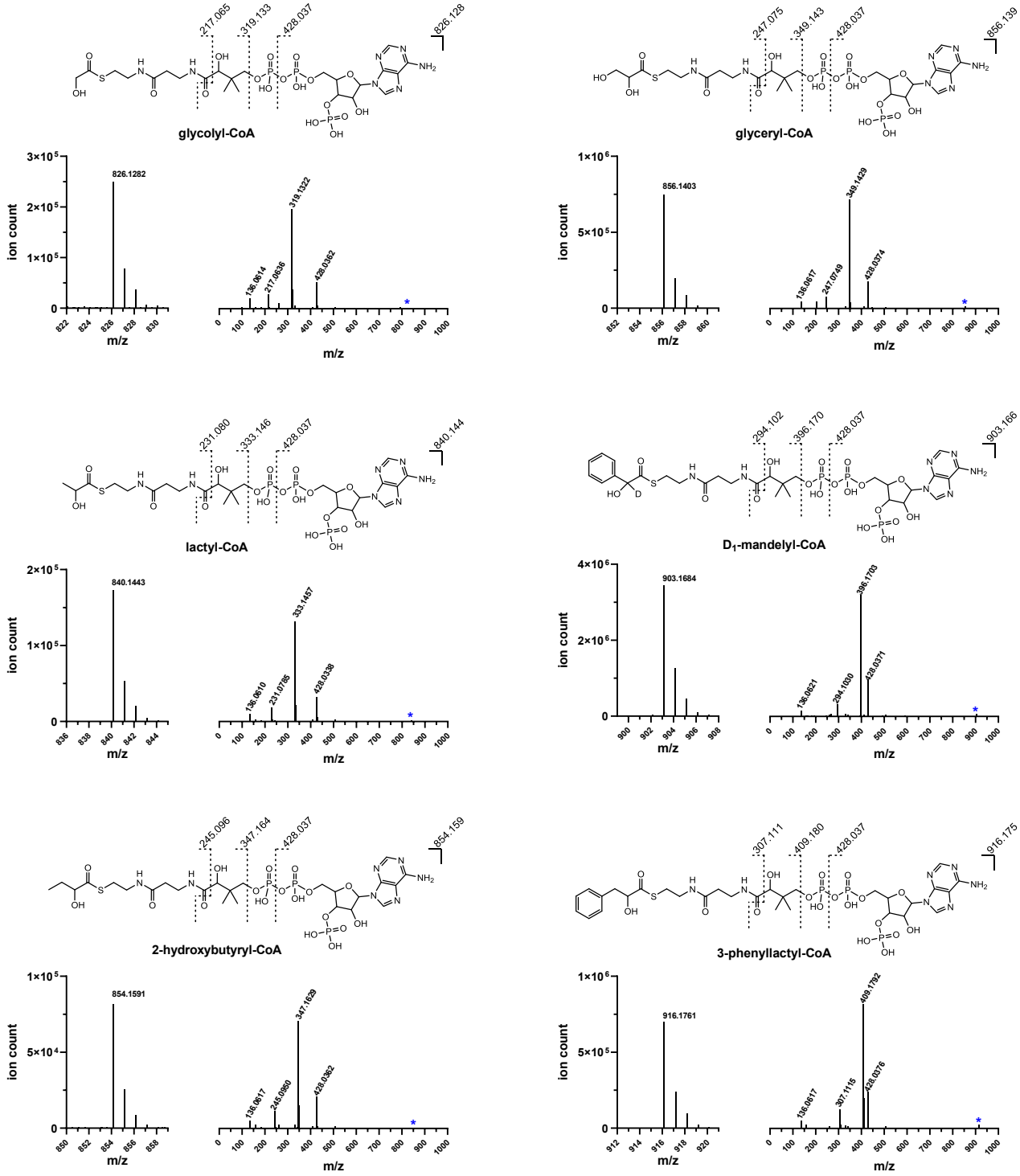


Figure S2. LC-MS analysis of oxalyl-CoA (1 mM) decarboxylation catalyzed by OXC_{Me}, $HACL_{Hs}$ and no enzyme control. The slope of HACL corresponds to a turnover number of approximately <1 min^{-1}.

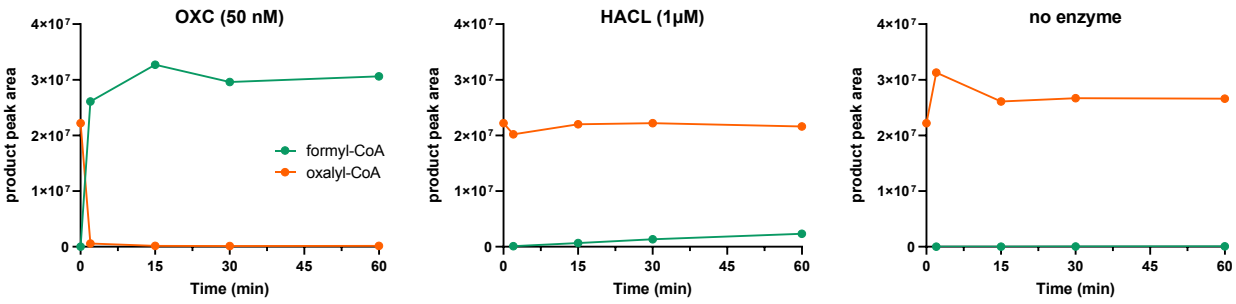


Figure S3. Michaelis-Menten graphs of OXC_{Me} and mutants thereof. Error bars show standard deviation of three replicates.

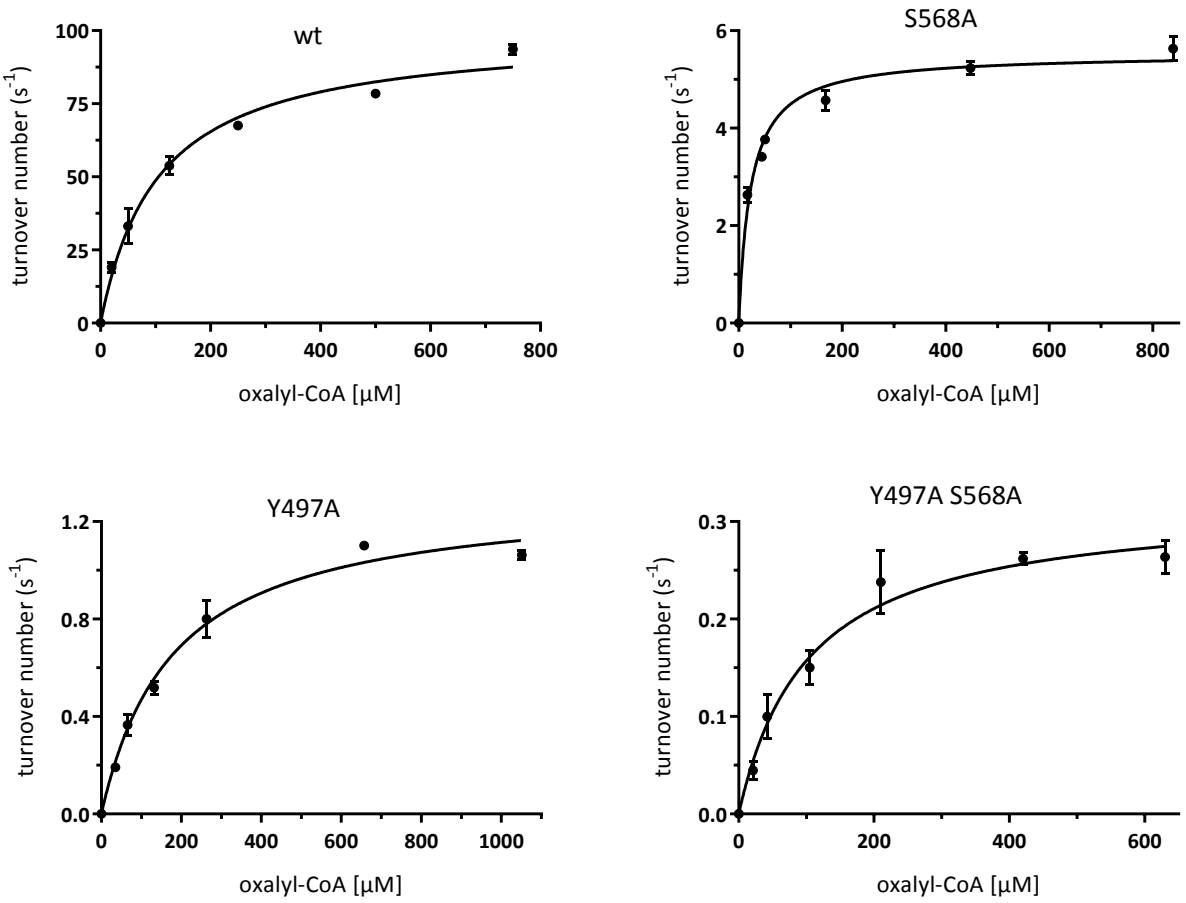
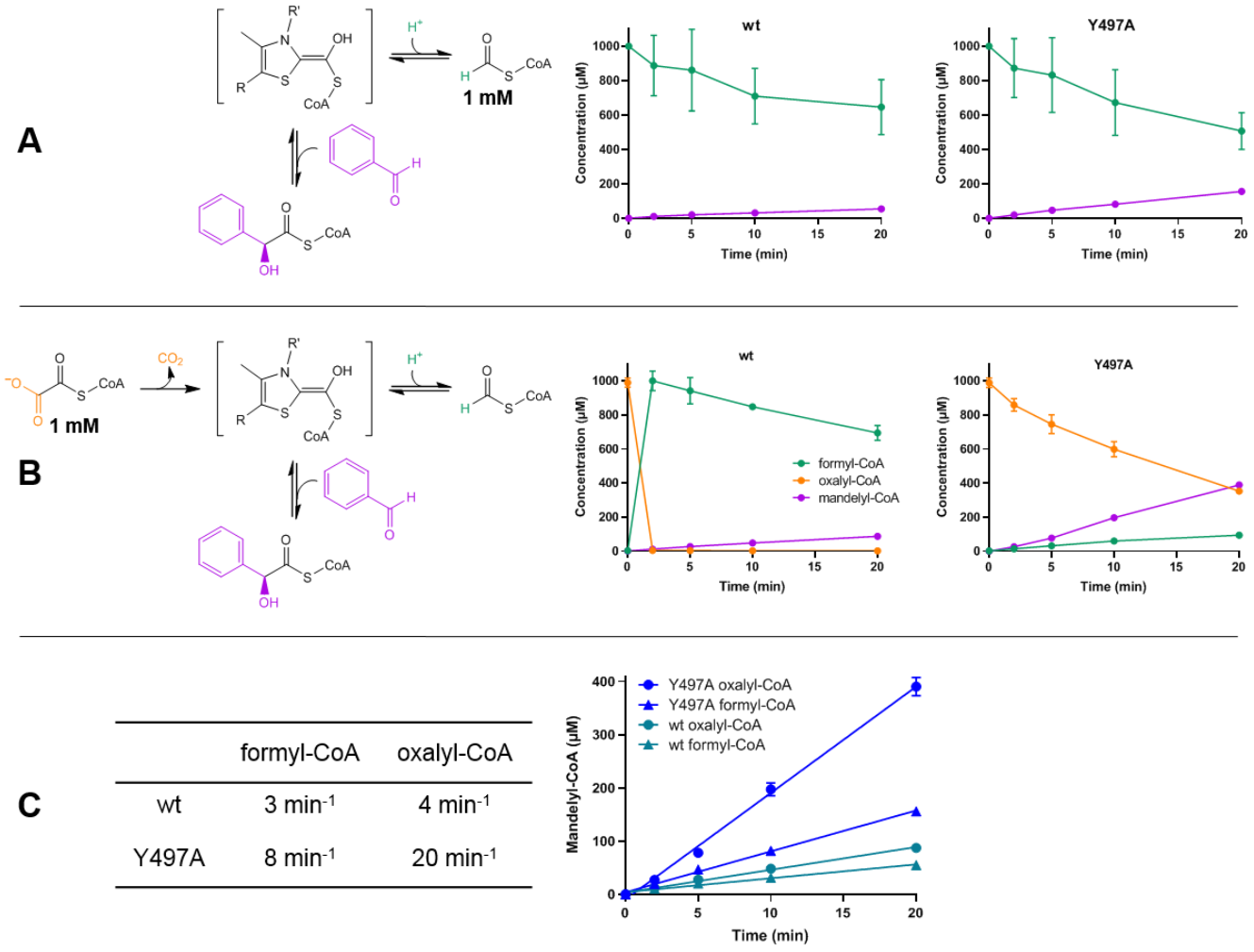


Figure S4. Competing reaction pathways of OXC. Reactions contained 1 µM OXC_{Me}, 25 mM benzaldehyde and were started by adding 1 mM formyl-CoA (A) or 1 mM oxalyl-CoA (B). C) Apparent turnover numbers were determined by linear regression of the mandelyl-CoA formation rate over 20 min. Error bars show standard deviation of three replicates. Concentration of CoA-esters was determined by comparison to a standard curve obtained from chemically synthesized formyl-CoA, oxalyl-CoA and mandelyl-CoA, respectively.



C

	formyl-CoA	oxalyl-CoA
wt	3 min^{-1}	4 min^{-1}
Y497A	8 min^{-1}	20 min^{-1}

Figure S5. Screen for mandelyl-CoA thioesterase activity. CoA formation was detected with the Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid)), which reacts with free thiols under release of 2-nitro-5-thiobenzoate (ε_{412nm} = 14.15 mM^{-1} cm^{-1}). Assays were carried out at 30 °C in 50 mM MES-KOH pH 6.8 and contained 1 mM Ellman's reagent and 0.5 µM YciA or 0.5 µM TesB or 0.5 µM PaaI. The reaction was started by adding 0.1 mM mandelyl-CoA (time point indicated with the arrow).

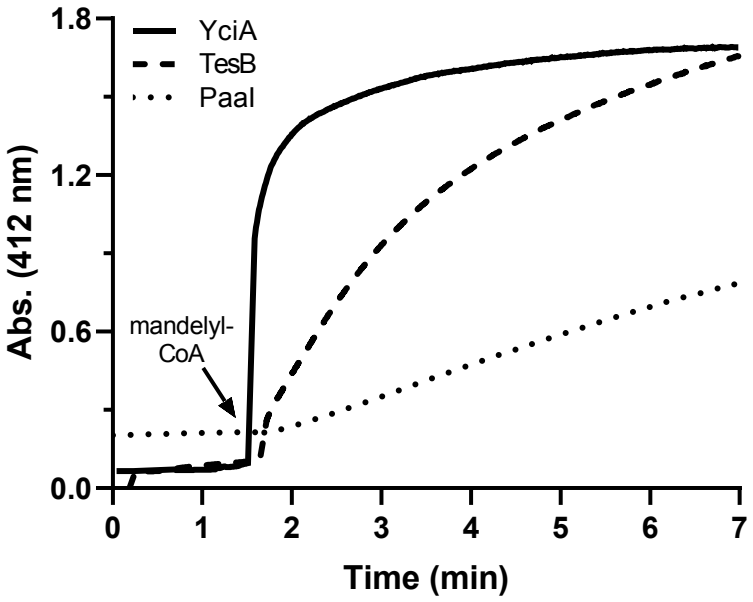


Figure S6. Thioesterase activity of YciA towards oxalyl-CoA, formyl-CoA and racemic mandelyl-CoA. Assays were carried out at 30 °C in reaction buffer consisting of 50 mM TES-KOH pH 6.8, 10 mM $MgCl_2$, 0.5 mM ADP, 0.15 mM ThDP and 25 mM benzaldehyde, 2 µM YciA (**A**) or no YciA (**B**). The reaction was started by adding either 0.5 mM formyl-CoA or 0.5 mM oxalyl-CoA or 0.5 mM mandelyl-CoA. Samples were taken after 0, 1, 5 and 30 minutes and analyzed with the LC-MS detection of CoA esters. CoA esters were quantified by comparison to synthetic standards of formyl-CoA, oxalyl-CoA and mandelyl-CoA, respectively. Error bars show standard deviation of two replicates.

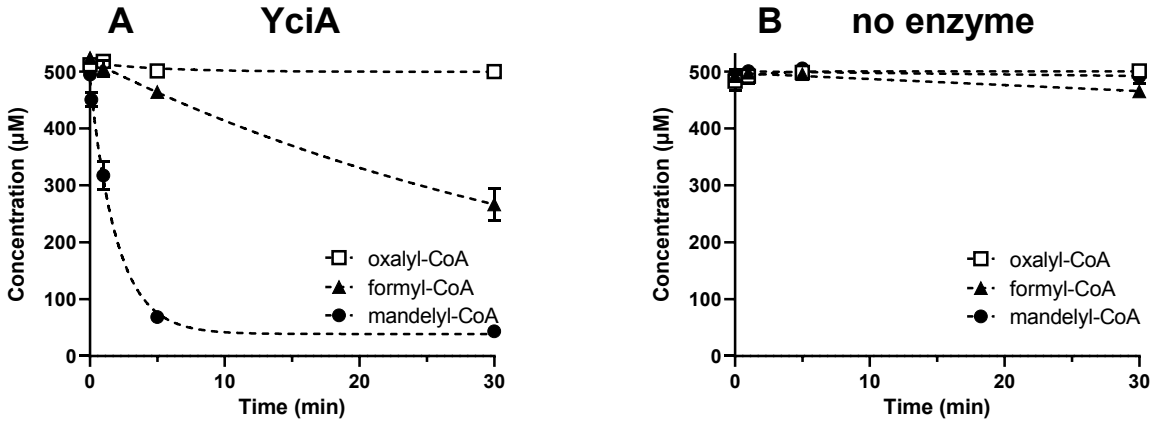


Figure S7. Chiral LC-MS product analysis of the aldehyde scope (Figure 2). On the left side are chromatograms of the last time point (24 h), showing extracted ion counts. The chemical structure of the product and its $[M\text{-}H]^-$ *m/z* used to obtain the extracted ion count are shown in each chromatogram. Dashed lines show commercial standards at a concentration of 10 mM. Plots on the right side show the time courses of the reactions. The lines show a one-phase association fit to the data. MA, mandelic acid. 4-CMA, 4-chloromandelic acid. (*S*)-2-CMA, (*S*)-2-chloromandelic acid. PLA, 3-phenyllactic acid.

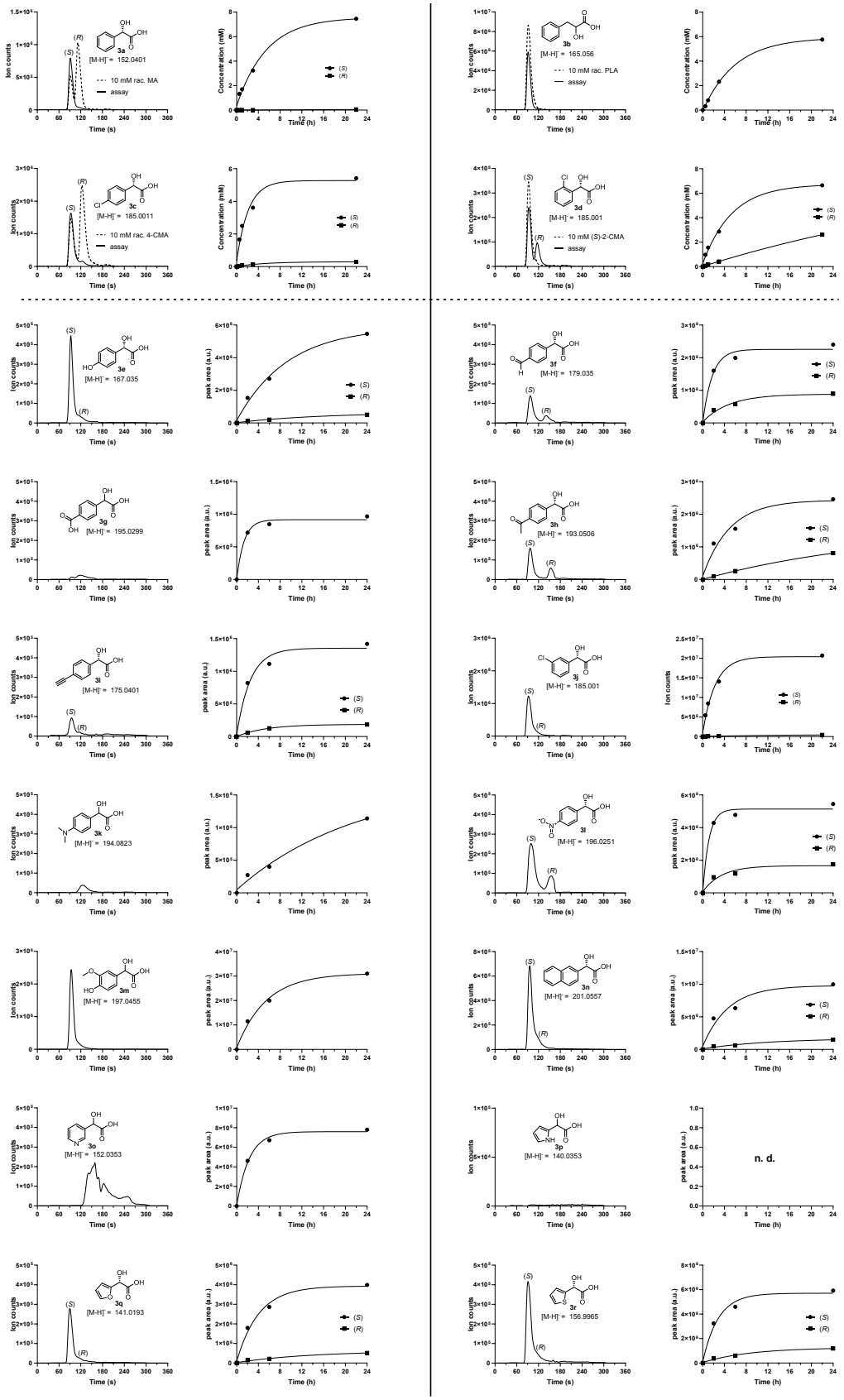
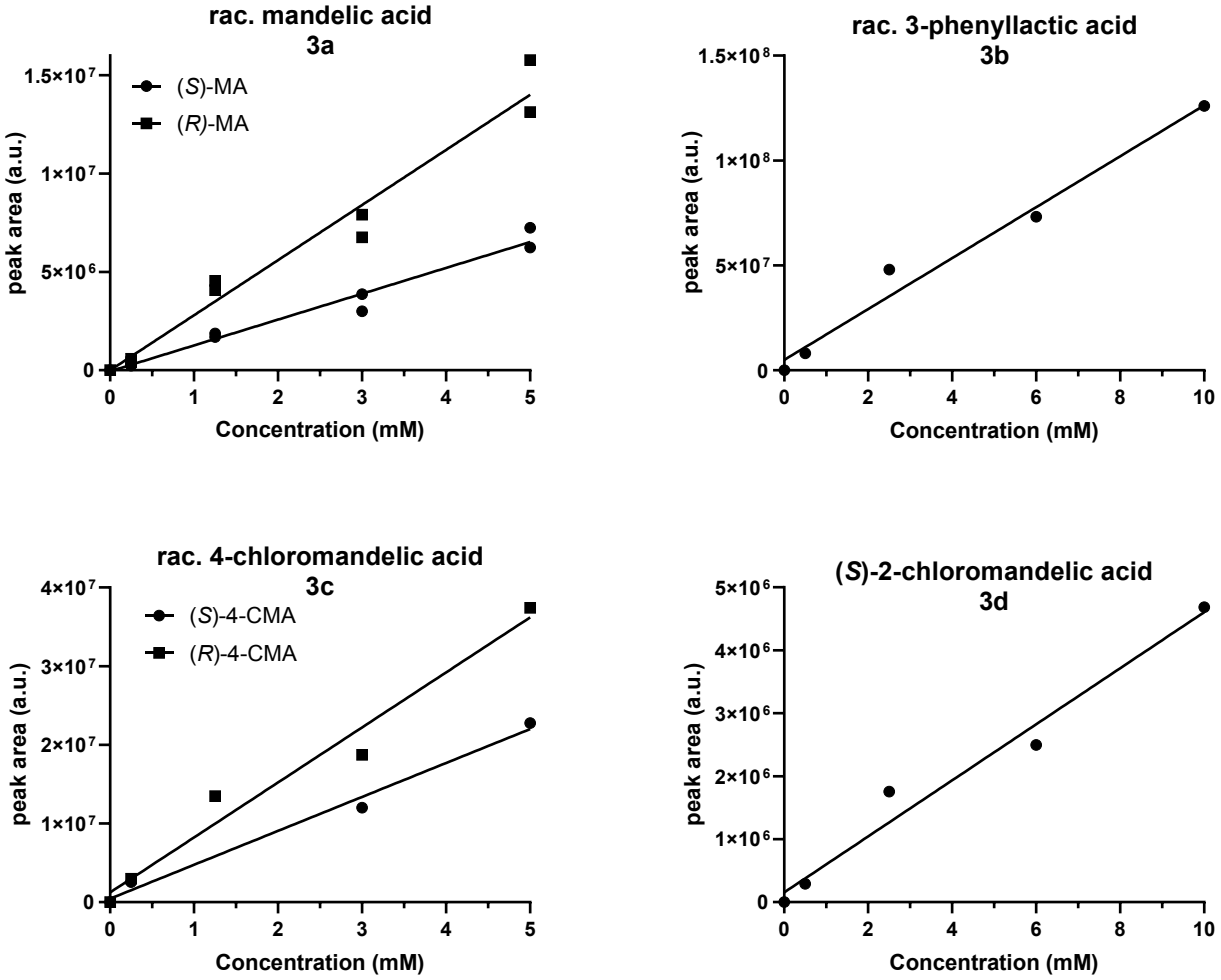


Figure S8. Calibration curves of commercially obtained racemic mandelic acid, racemic 3-phenyllactic acid, racemic 4-chloromandelic acid and (*S*)-2-chloromandelic acid. The lines show a linear fit to the data, slope (A) and Y-intercept (B) are indicated in the table below. Concentration of α-hydroxy acids in the reaction samples was calculated with the equation *concentration* = (*peak area* – B) / A.



Compound	A	B	R^2
(*S*)-**3a**	1313446	-51928	0.9760
(*R*)-**3a**	2801078	-3823	0.9707
3b	12133853	4946676	0.9797
(*S*)-**3c**	4312421	441083	0.9882
(*R*)-**3c**	6991962	1236119	0.9692
(*S*)-**3d**	445091	153684	0.9731

Figure S9. UV spectrum of the mandelic acid semi-preparative synthesis.

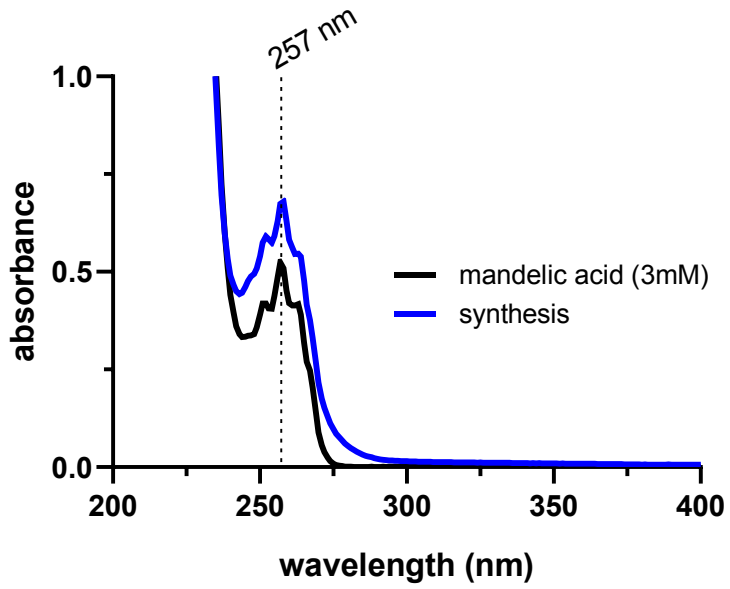


Figure S10. HPLC UV (210 nm) chromatogram of the mandelic acid semi-preparative synthesis.

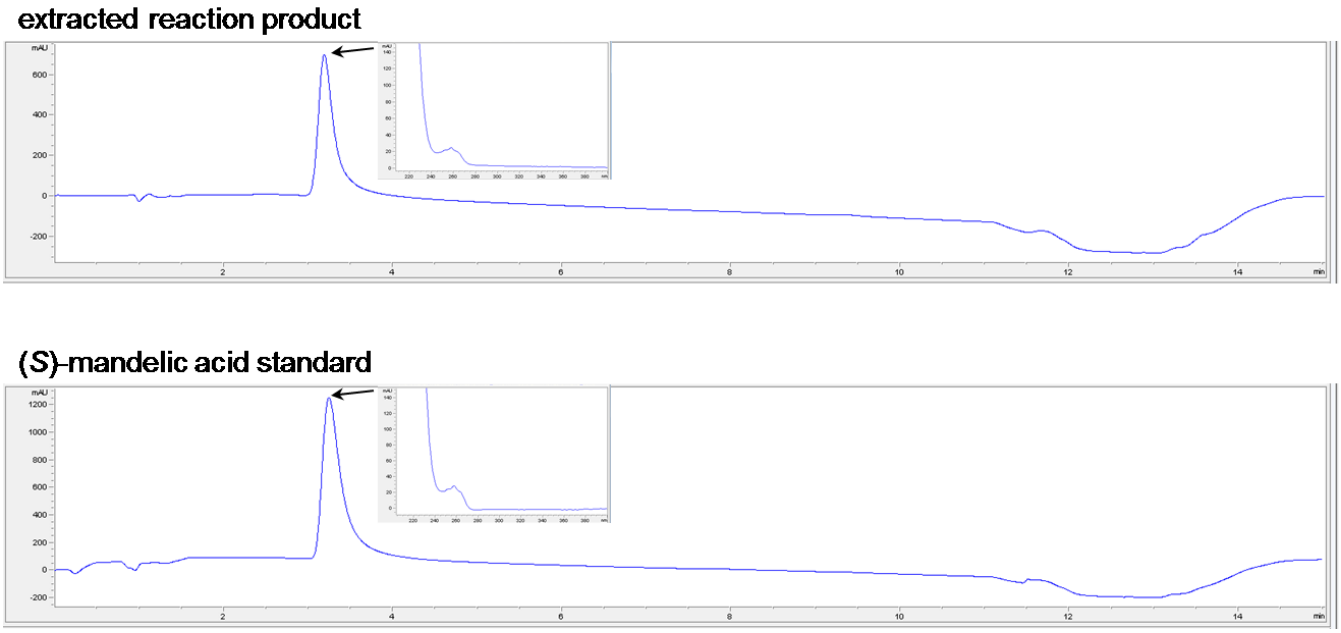
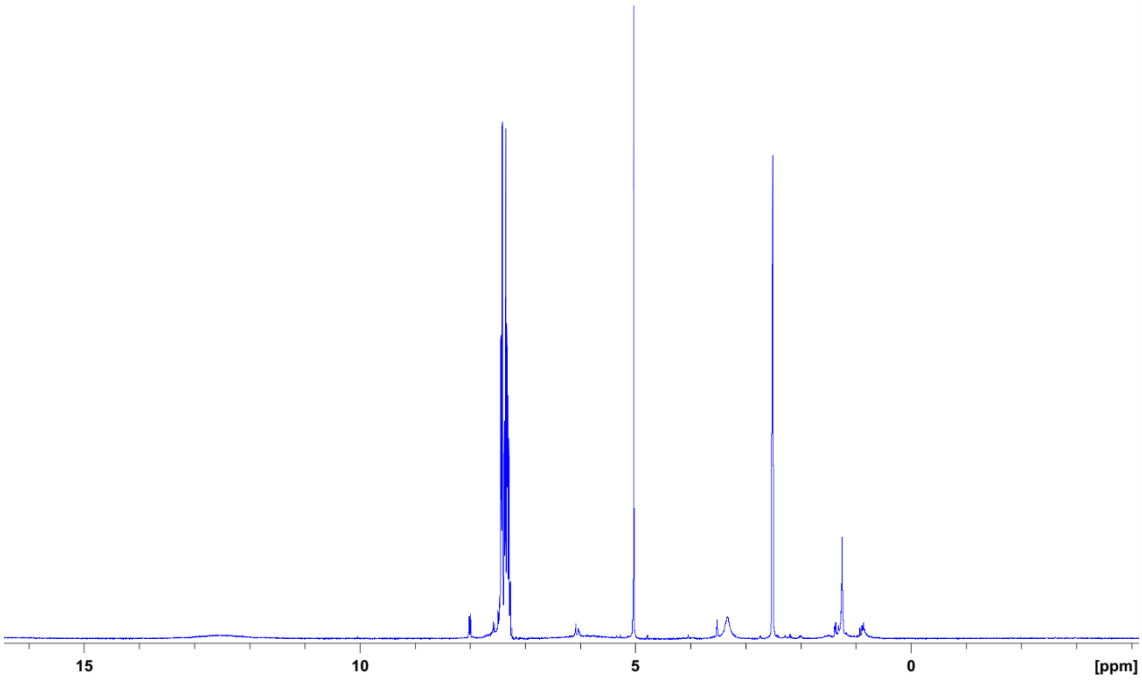
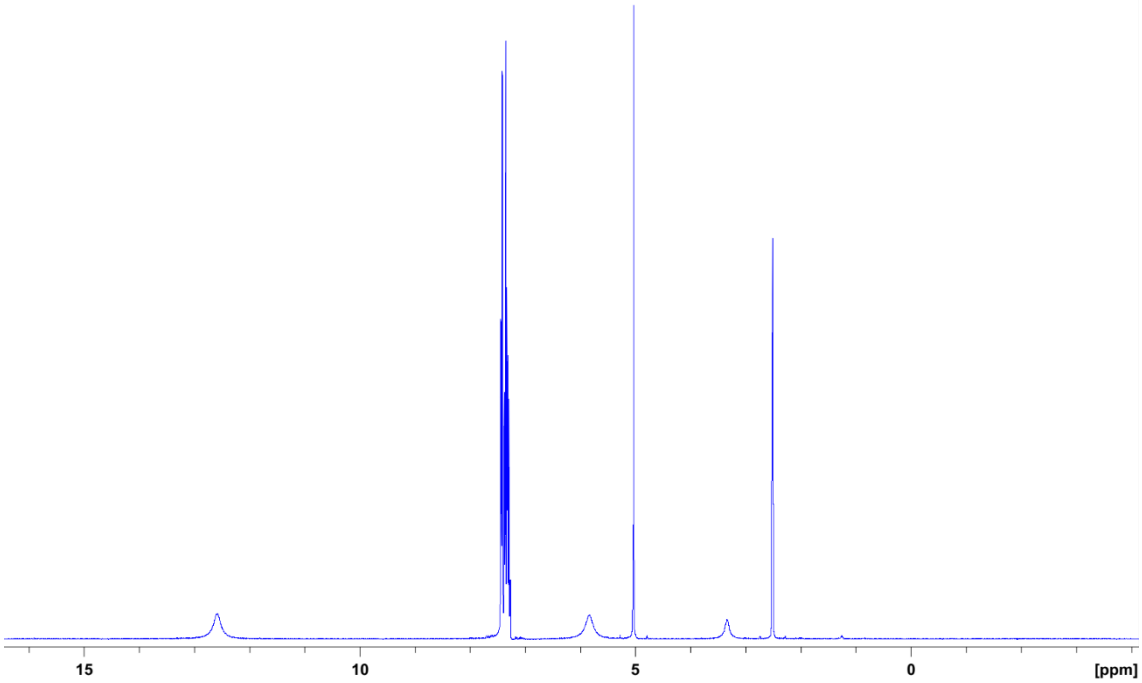


Figure S11. NMR spectra recorded in DMSO-d_6, 300 MHz. A) ^{1}H NMR of the extracted reaction product. B) ^{1}H NMR of commercial (*S*)-mandelic acid. C) ^{13}C NMR of the isolated reaction product. D) ^{13}C NMR of commercial (*S*)-mandelic acid.

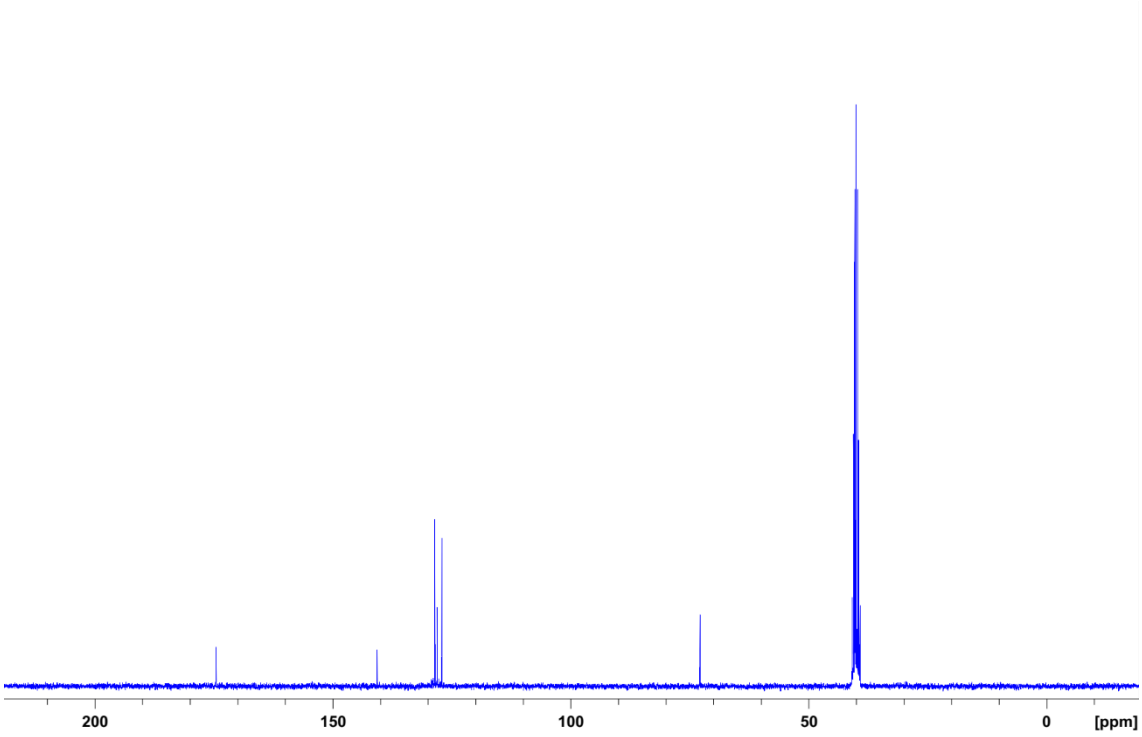
A



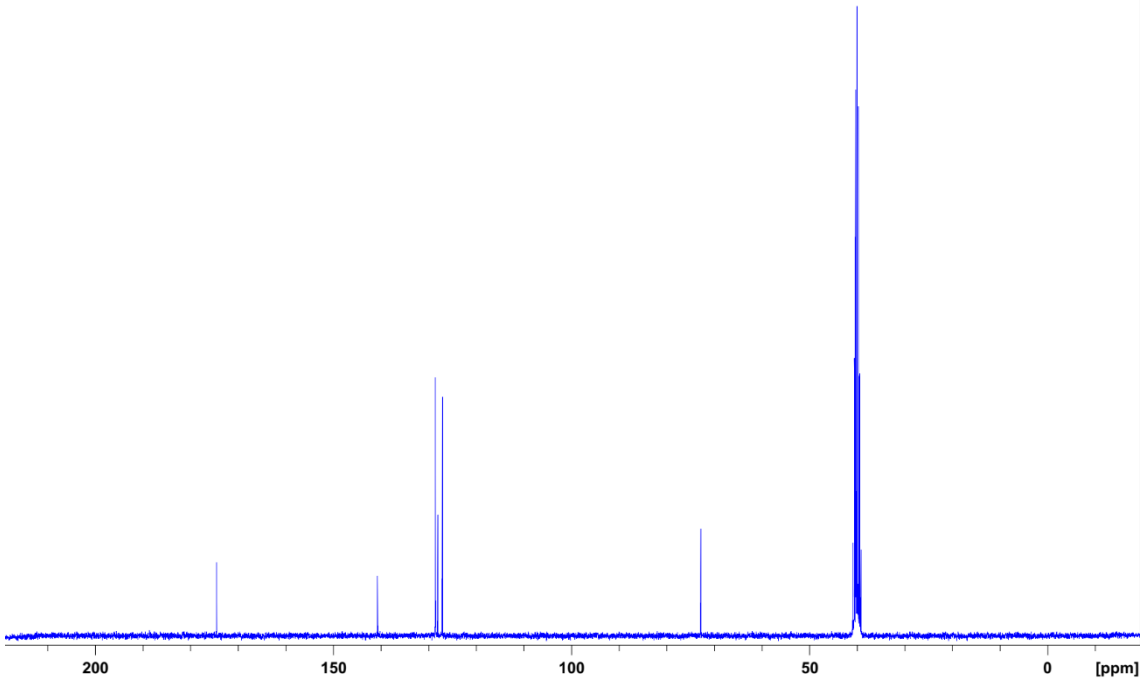
B



C



D



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