A synthetic biology approach for the transformation of L-α-amino acids to the corresponding enantiopure (*R*)- or (*S*)-α-hydroxy acids

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SUPPORTING INFORMATION

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1 General information

1.1 General procedures

Chemical reagents were purchased from different commercial sources and used without further purification Melting points were taken on samples in open capillary tube and are uncorrected. Formate dehydrogenase (FDH) 2.1 U/mg was purchased from evocatal. ^{1}H and ^{13}C-NMR spectra were obtained using Bruker spectrometer (^{1}H, 300.13 MHz; ^{13}C 75.5 MHz). The chemical shifts are given in ppm and the coupling constants in Hertz (Hz). Mass spectra experiments (MS) were carried out by ESI- using Agilent 1260 HPLC coupled with by a 6120 quadrupole. Conversions for aromatic substrates were determined with a Shimadzu chromatograph UV detector at different wavelengths using a Luna C18 (25 cm × 4.6 mm I.D.). Conversions for aliphatic substrates were determined by NMR. Chiral high performance liquid chromatography (HPLC) analyses were carried out using a Shimadzu chromatograph UV detector at different wavelengths using a Chiralcel OJ (25 cm × 4.0 mm I.D.) for the determination of enantiomeric excesses. Chiral gas chromatography analysis (GC) were carried out using an Agilent GC7890A system and a CP-Chiralsil-dex CB column (25 m × 0.32 mm).

1.2 List of abbreviations

ara: arabinose promoter and araC repressor.

bla: β-lactamase.

ColE1: origin of replication ColE1.

FWD: Forward primer

dNTP: Deoxynucleotide solution mix.

DTT: Dithiothreitol.

FDH: Formate dehydrogenase.

FDH∗: Gene carrying the designated ultrastrong RBS.

D- or L-HIC: D- or L- selective isocaproate reductase.

IPTG: Isopropyl β-D-1-thiogalactopyranoside.

KPi buffer: potassium phosphate buffer.

L-AAD: L-amino acid deaminase.

lacI: lac repressor.

LB: lysogeny broth.

NAD: Nicotinamide dinucleotide oxidized form.

NADH: Nicotinamide dinucleotide reduced form.

OE-PCR: Overlap extension PCR.

paraC: araC promoter.

pB: arabinose-inducible promoter (pBAD).

pbla: β-lactamase promoter.

PBS: Phosphate buffered saline.

PCR: Polymerase chain reaction.

PEG 8000: Polyethylene glycol 8000.

RBS: Ribosome binding site from pET21a(+).

RBS∗: designed ultrastrong RBS.

REV: Reverse primer.

t7: IPTG-inducible T7/lacO promoter.

tB: terminator rrnBT2.

t*lac*I: lac repressor terminator.

tt7: T7 terminator; term., terminator.

2. DNA and protein sequences of the cascade enzymes

2.1 Formate dehydrogenase (FDH)

Source organism: *Candida boidinii*

Coding DNA sequence (the sequence was codon optimized for *E. coli*):

```
ATGAAAATTGTGCTGGTGCTGTATGATGCGGGCAAACATGCGGCGGATGAAGAAAAACTGTA
TGGCTGCACCGAAAATAAACTGGGCATTGCGAACTGGCTGAAAGATCAGGGCCATGAACTGA
TTACCACCTCTGATAAAGAAGGCGGCAACAGCGTTCTGGATCAGCATATTCCGGATGCGGAT
ATTATTATTACCACCCCGTTTCATCCGGCGTATATCACCAAAGAACGCATCGATAAAGCGAA
AAAACTGAAACTGGTGGTGGTGGCGGGCGTGGGCAGCGATCATATTGATCTGGATTATATCA
ACCAGACCGGTAAAAAAATTAGCGTGCTGGAAGTGACCGGCAGCAACGTGGTGAGCGTGGCG
GAACATGTGGTGATGACCATGCTGGTGCTGGTGCGTAACTTTGTGCCGGCGCATGAACAAAT
TATTAACCACGATTGGGAAGTGGCGGCGATTGCGAAAGATGCGTATGATATCGAAGGCAAAA
CCATTGCGACCATTGGCGCGGGTCGTATTGGCTATCGTGTGCTGGAACGTCTGGTGCCGTTT
AATCCGAAAGAACTGCTGTATTATGATTATCAGGCGCTGCCGAAAGATGCGGAAGAAAAAGT
GGGTGCGCGTCGTGTGGAAAACATTGAAGAACTGGTGGCGCAGGCGGATATTGTGACCGTGA
ACGCGCCGCTGCATGCGGGCACCAAAGGCCTGATCAACAAAGAGCTGCTGTCTAAGTTTAAA
AAAGGCGCGTGGCTGGTGAATACCGCGCGTGGCGCGATTTGCGTGGCCGAAGATGTTGCGGC
GGCGCTGGAAAGCGGTCAGCTGCGTGGCTATGGCGGTGATGTGTGGTTTCCGCAGCCGGCGC
CGAAAGATCATCCGTGGCGTGATATGCGTAACAAATATGGCGCGGGTAACGCCATGACCCCG
CATTATAGCGGCACCACCCTGGATGCGCAGACCCGTTATGCGCAGGGCACCAAAAACATTCT
GGAAAGCTTTTTCACCGGCAAATTTGATTATCGTCCGCAGGACATTATTCTGCTGAACGGCG
AATATGTGACCAAAGCGTATGGCAAACACGATAAAAAATAATAA
```

Protein sequence (GenBank accession CAA09466; UniProtKB ID O13437):

```
MKIVLVLYDAGKHAADEEKLYGCTENKLGIANWLKDQGHELITTSDKEGGNSVLDQHIPDAD
IIITTPFHPAYITKERIDKAKKLKLVVVAGVGSDHIDLDYINQTGKKISVLEVTGSNVVSVA
EHVVMTMLVLVRNFVPAHEQIINHDWEVAAIAKDAYDIEGKTIATIGAGRIGYRVLERLVPF
NPKELLYYDYQALPKDAEEKVGARRVENIEELVAQADIVTVNAPLHAGTKGLINKELLSKFK
KGAWLVNTARGAICVAEDVAAALESGQLRGYGGDVWFPQPAPKDHPWRDMRNKYGAGNAMTP
HYSGTTLDAQTRYAQGTKNILESFFTGKFDYRPQDIILLNGEYVTKAYGKHDKK
```

2.2 L-amino acid deaminase (L-AAD)

Source organism: *Proteus myxofaciens*

Coding DNA sequence:

```
ATGAACATTTCAAGGAGAAAGCTACTTTTAGGTGTTGGTGCTGCTGGCGTACTTGCTGGTGG
TGCGGCCACTTTAGTTCCAATGGTTCGCCGTGATGGTAAATTTGTTGAATCTAAATCAAGAG
CTTTATTTGTTGAAAGTACTGAGGGTGCCCTGCCATCAGAATCTGATGTGGTCATTATTGGA
GGTGGTATTCAAGGTATCATGACAGCGATTAATTTAGCTGAACGTGGTATGAGTGTCACCAT
TTTAGAAAAAGGCGAGGTTGCTGGAGAGCAATCAGGCCGCGCATACAGCCAAATCATTAGCT
```

ACCAAACGTCACCCGAAATTTTCCCATTGCATCATTACGGAAAAATTTTATGGCGTGGTATG
AACGAAAAAATTGGTGCTGATACCAGCTATCGCACACAAGGTCGAGTTGAAGCGCTTGCTGA
TGAAAAAGCATTAGATAGAGCGCAAGAATGGATCAAAACAGCCAAAGAAACAGCAGGATTTG
ATGTACCTTTAAATACTCGTATTATTAAGGGTGAAGAGTTATCAAATAGATTAGTAGGTGCA
CAAACACCTTGGACTGTTGCTGCTTTTGAAGAAGATTCTGGTTCTGTCGATCCTGAAACGGG
TACACCAACATTAGCGCGTTATGCTAAACAAATTGGTGTTAAAATCTATACTCATTGCGCAG
TAAGAGGTATTGAAACAGCAGGTGGTAAAATTTCTGATGTTGTCACTGAAAAAGGTGCAATA
AGAACATCTAACGTTGTTCTTGCTGGGGGTATTTGGTCACGTTTATTCATGGGGAATATGGG
GGTTGATCTTCCAACCTTGAATGTTTACTTATCACAACAACGTGTATCCGGTGTTCCAGGCG
CACCACGTGGTAATGTGCATTTACCAAATGGTATCCACTTTCGAGAACAAGCTGACGGCACT
TATGCTGTAGCCCCACGTATCTTCACAAGCTCCATTGTTAAAGATAGTTTCCTATTAGGGCC
TAAATTTATGCACTTATTAGGTGGTGGTGAGCTACCATTAGAATTCTCTATTGGTGAAGACT
TGTTTAATTCATTCAAAATGCCTACATCATGGAAATTAGACGAAAAATCACCTTTTGAGCAA
TATCGCATCGCGACTGCAACACAAAATACTGAGCATTTAGATGCTGTATTCCAAAGAATGAA
AACAGAATTCCCAGTATTTGAAAAATCACAAATTGTTGAACGTTGGGGTGCAGTTGTAAGTC
CAACATTTGATGAATTACCGATTATTTCAGAAGTAAAAGAGTACCCAGGTCTTGTTATCAAT
ACAGCGACAGTGTGGGGAATGACAGAAGGTCCTGCTGCCGGTGAAGTTACCGCAGATATTGT
GACGGGTAAAAAACCCGTCATTGATCCAACGCCGTTTAGTTTGGATCGTTTTAAGAAGTAA

Protein sequence:

MNISRRKLLLGVGAAGVLAGGAATLVPMVRRDGKFVESKSRALFVESTEGALPSESDVVIIG
GGIQGIMTAINLAERGMSVTILEKGEVAGEQSGRAYSQIISYQTSPEIFPLHHYGKILWRGM
NEKIGADTSYRTQGRVEALADEKALDRAQEWIKTAKETAGFDVPLNTRIIKGEELSNRLVGA
QTPWTVAAFEEDSGSVDPETGTPTLARYAKQIGVKIYTHCAVRGIETAGGKISDVVTEKGAI
RTSNVVLAGGIWSRLFMGNMGVDLPTLNVYLSQQRVSGVPGAPRGNVHLPNGIHFREQADGT
YAVAPRIFTSSIVKDSFLLGPKFMHLLGGGELPLEFSIGEDLFNSFKMPTSWKLDEKSPFEQ
YRIATATQNTEHLDAVFQRMKTEFPVFEKSQIVERWGAVVSPTFDELPIISEVKEYPGLVIN
TATVWGMTEGPAAGEVTADIVTGKKPVIDPTPFSLDRFKK

2.3 L-2-hydroxyisocaproate dehydrogenase (L-Hic)

Source organism: *Lactobacillus confusus DSM 201966*

Coding DNA sequence:

ATGGCACGTAAGATTGGAATTATCGGCCTTGGAAACGTTGGGGCTGC**C**GTAGCGCACGGATT
GATTGCACAAGGTGTAGCCGACGACTACGTCTTTATTGATGCAAACGAAGCAAAGGTGAAGG
CTGATCAAATTGATTTCCAAGACGCAATGGCGAACTTGGAAGCGCACGGTAACATTGTGATT
AACGATTGGGCAGCCTTGGCTGATGCTGATGTTGTGATTTCAACACTGGGGAACATCAAGTT
GCAACAAGACAACCCAACCGGTGACCGTTTTGCTGAGTTGAAGTTTACCAGCAGCATGGTGC
AATCAGTCGGCACAAACTTGAAGGAATCTGGTTTCCACGGCGTATTGGTCGTGATTTCAAAC
CCGGT**T**GACGTGATTACGGCCTTGTTCCAACACGTGACTGGTTTCCCAGCTCACAAGGTTAT
CGGAACCGGTACTTTGCTTGACACGGCGCGTATGCAACGTGCAGTTGGTGAGGCGTTTGATT
TGGA**C**CCACGTTCTGTTTCAGGTTACAACTTGGGTGAGCACGGTAACTCACAATTCGTAGCT
TGGTCAACGGTGCGCGTGATGGGTCAACCAATCGTGACGTTGGCTGATGCCGGCGATATTGA
CTTGGCGGCCATCGAAGAGGAAGCACGTAAGGGTGGCTTCACGGTCTTGAATGGTAAGGGCT
ACACGAGTTATGGTGTTGCAACGTCAGCAATCCGCATTGCCAAGGCTGTTATGGCTGACGCG
CATGCTGAATTGGTTGTCTCAAATCGTCGCGATGACATGGGAATGTACTTGTCATACCCAGC

GATTATTGGTCGCGATGGTGTCTTGGCAGAAACGACGCTTGATTTGACGACGGATGAGCAAG
AAAAGCT**C**TTGCAATCACGTGACTACATCCAACAACGTTTCGACGAAATTGTGGATACACTC
TAA

To remove restriction sites for PstI, SalI, BamHI and HindIII we introduced the mutations indicated in bold; all mutations were silent.

Protein sequence (UniProtKB ID P14295):

MARKIGIIGLGNVGAAVAHGLIAQGVADDYVFIDANEAKVKADQIDFQDAMANLEAHGNIVI
NDWAALADADVVISTLGNIKLQQDNPTGDRFAELKFTSSMVQSVGTNLKESGFHGVLVVISN
PVDVITALFQHVTGFPAHKVIGTGTLLDTARMQRAVGEAFDLDPRSVSGYNLGEHGNSQFVA
WSTVRVMGQPIVTLADAGDIDLAAIEEEARKGGFTVLNGKGYTSYGVATSAIRIAKAVMADA
HAELVVSNRRDDMGMYLSYPAIIGRDGVLAETTLDLTTDEQEKLLQSRDYIQQRFDEIVDTL

2.4 D-2-hydroxyisocaproate dehydrogenase (D-Hic)

Source organism: *Lactobacillus paracasei DSM 20008*

Coding DNA sequence:

ATGAAGATTATTGCTTACGGTGCTCGCGTTGACGAGATTCAATATTTCAAGCAATGGGCCAA
GGATACAGGCAACACACTTGAATACCATACAGAATTTCTCGATGAAAACACCGTTGAATGGG
CTAAAGGGTTTGATGGCATCAATTCATTGCAGACAACGCCATA**C**GCAGCCGGCGTTTTTGAA
AAAATGCACGCGTATGGTATCAAGTTCTTGACGATTCGGAATGTGGGTACGGATAACATTGA
TATGACTGCCATGAAGCAATACGGCATTCGTTTGAGCAATGTACCGGCTTATTCGCCAGCAG
CGATTGCTGAATTTGCTTTGACCGATACTTTGTACTTGCTACGTAATATGGGTAAAGTACAG
GCGCAACTACAGGCGGGCGATTATGAAAAAGCGGGCACCTTCATCGGTAAGGAACTCGGTCA
GCAAACCGTTGGCGTGATGGGCACCGGTCATATTGGACAGGTTGCTATCAAACTGTTCAAAG
GCTTTGGCGCCAAAGTGATTGCTTACGATCCTTATCCAATGAAGGGCGATCACCCAGATTTT
GACTATGTCAGCCTTGAAGACCTCTTTAAGCAAAGTGATGTCATTGATCTTCATGTTCCTGG
GATTGAACAAAATACCCACATTATCAATGAAGCGGCATTTAATTTGATGAAACCGGGTGCGA
TTGTGATCAACACGGCTCGGCCAAATCTGATTGACACGCAAGCCATGCTCAGCAATCTTAAG
TCTGGCAAGTTGGCCGGTGTCGGGATTGACACCTATGAATACGAAACCGAGGACTTGTTGAA
TCTCGCCAAGCACGGCAGCTTCAAGGA**C**CCGTTGTGGGACGAGCTGTTGGGGATGCCAAATG
TTGTCCTCAGCCCGCACATTGCCTACTACACCGAGACGGCTGTGCATAATATGGTTTACTTC
TCACTACAACATCTCGTTGATTTCTTGACCAAA**GG**C**G**AAACCAGCACGGAAGTTACTGGTCC
AGCAAA**G**TAG

To remove restriction sites for NdeI and BamHI we introduced the mutations indicated in bold; all mutations were silent. There is a reported conflict between the DNA coding sequence (GenBank accession M26929) and the amino acid sequence (UniProtKB ID P17584) of D-Hic of *L. paracasei*. To encode the amino acid sequence of UniProtKB ID P17584 we introduced two mutations (blue) and inserted two bases (red) at the 3'-terminus of the DNA sequence.

Protein sequence (UniProtKB ID P17584)

MKIIAYGARVDEIQYFKQWAKDTGNTLEYHTEFLDENTVEWAKGFDGINSLQTTPYAAGVFE
KMHAYGIKFLTIRNVGTDNIDMTAMKQYGIRLSNVPAYSPAAIAEFALTDTLYLLRNMGKVQ
AQLQAGDYEKAGTFIGKELGQQTVGVMGTGHIGQVAIKLFKGFGAKVIAYDPYPMKGDHPDF
DYVSLEDLFKQSDVIDLHVPGIEQNTHIINEAAFNLMKPGAIVINTARPNLIDTQAMLSNLK
SGKLAGVGIDTYEYETEDLLNLAKHGSFKDPLWDELLGMPNVVLSPHIAYYTETAVHNMVYF
SLQHLVDFLTKGETSTEVTGPAK

3. Cloning

3.1 Preparation of the pCAS constructs

3.1.a PCR amplification of pCAS vector parts and the cascade genes.

The parts comprising the pCAS vectors were PCR amplified from in-house templates using designed primers (Table S1) synthetized by Integrated DNA technologies (IDT, Coralville, IA). 1 ng of plasmid template was used for the amplification with 0.5 µM primers, 0.2 mM of each dNTP, 1X *Ex Taq* buffer and 1 U of *Ex Taq* polymerase (all TaKaRa Bio Inc, Otsu, Japan) in a final reaction volume of 50 µL. For PCR, the thermocycler (Applied Biosystems 2720 Thermal Cycler, Foster City, CA) was programmed as follows: 1 minute at 95 °C for denaturation of the template, followed by 5 cycles of 30 seconds at 95 °C, 30 seconds at 55 °C and 1 minute at 72 °C. This was followed by 25 cycles of 30 seconds at 95 °C, 30 seconds at 65 °C and 1 minute at 72 °C for elongation of the PCR product. The amplification was finished with a 7 minutes elongation at 72 °C and a holding step at 4 °C.

The cascade genes were PCR amplified from constructs subcloned into the pET21a(+) vector (Novagene) using designed primers (Table S2) and low error rate *Phusion* high-fidelity DNA polymerase (Thermo Scientific, Waltham, MA) or *Pwo* DNA polymerase (Roche, Basel, Switzerland). 1 ng of plasmid template was used for the amplification with 0.5 µM primers, 0.2 mM of each dNTP, 1X *Phusion* HF buffer or *Pwo* buffer and 1 U of corresponding polymerase in a final volume of 50 µL. The PCR program was 2 minutes at 94 °C for denaturation of the template, followed by 10 cycles of 15 seconds at 94 °C, 30 seconds at 55 °C and 45 seconds at 72 °C. This was followed by 20 cycles of 15 seconds at 94 °C, 30 seconds at 62 °C and 45 seconds at 72 °C for elongation of the product. The amplification was finished with a 5 minutes elongation at 72 °C and a holding step at 4 °C.

3.1.b Overlap-extension PCR (OE-PCR)

The first step of the OE-PCR[1] is the annealing of overlapping 5’ regions of the DNA fragments to be joined. This step was done in 50 µL final volume with 5 ng of the largest

DNA fragment and equimolar quantities of the others, 0.2 mM of each dNTPs, 1X *Phusion* HF buffer and 1 U of *Phusion* high-fidelity DNA polymerase (Thermo Scientific). The cycling program was as follows: 2 minutes at 94 °C for denaturation of the DNA fragments, then 5 cycles of 15 seconds at 94 °C, 30 seconds at 55 °C and 45 seconds at 72 °C in order to amplify annealed products. Then 7 minutes at 72 °C were applied for elongation and the program was held at 4 °C.

In the second step, the primers were added to the reaction in order to specifically amplify the desired combination of DNA fragments. 0.5 µM of designed primers (Table S2) were added to the reaction together with 1X *Phusion* HF buffer, 1.25 µL of dNTP mixture at a concentration of 2.5 mM each, and 1 U of *Phusion* high-fidelity DNA polymerase. The reaction mixture was filled up with sterile water to a final volume of 70 µL. Then the following program was run: 2 minutes at 94 °C for denaturation of the template followed by 10 cycles of 15 seconds at 94 °C, 30 seconds at 55 °C and 45 seconds at 72 °C. This was followed by 20 cycles of 15 seconds at 94 °C, 30 seconds at 62 °C and 45 seconds at 72 °C with an increment of 5 seconds per cycle for the elongation step at 72 °C. The program was finished by a 7 minutes step at 72 °C and a holding step at 4 °C.

3.2 Gibson assembly

We followed the method described by Gibson *et al.*[2]. Briefly, all DNA fragments contained 45 bp flanking homologies and were purified from agarose gels after PCR using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) prior to assembly. 100 ng of the smallest DNA fragment and equimolar quantities of all others were mixed with 15 µL of the Gibson reaction mix. The mix was composed of 1X ISO reaction buffer (5% (w/v) PEG-8000, 100 mM Tris/Cl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 0.2 mM of each dNTP, 1 mM NAD in sterile water), 10 U/µL *T5* exonuclease (Biozym Scientific GmbH, Hessisch Oldendorf, Germany), 2 U/µL *Phusion* high-fidelity DNA polymerase (Thermo Scientific), 40 U/µL *Taq* DNA ligase (New England BioLabs Inc., MA). The reaction mixture was then incubated for 1 h at 50 °C without shaking. The assembly product was desalted for 15 minutes on MCE MF-Millipore membrane filters with a pore size of 0.45 µm (Millipore, Darmstadt, Germany) and directly used for transformation of electrocompetent[3] *E. coli* Top10F' (F'[*lac*I^q Tn*10*(tet^R)] *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80lacZΔM15 Δ*lac*X74 *deo*R *nup*G *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L(Str^R) *end*A1 λ-). The colonies containing the correctly assembled DNA construct were selected by their ampicillin resistance on lysogeny broth (LB) agar plate

supplemented with ampicillin (100 mg/L). Plasmid DNA was isolated using the GeneJET Plasmid miniprep kit (Fermentas, Burlington, Canada). The assembled constructs were further analysed by restriction digest and selected clones were sequence verified by Microsynth (Microsynth, Balgach, Switzerland).

Table S1: Primers used for amplification of pCAS backbone vector parts.

primer	sequence (5' → 3')	part	orientation
pBP524	tttgtttgcaagcagcagattacgcgcagaaaaaaaggatctcaactcgagcctaatacgactcactataggggaattgtgagc	t7	FWD
pBP525	ggatccggtatatctccttgaattcggaattgttatccgctcacaattccggaattgttatccgctcacaattccc	t7	REV
pBP526	ggaattgtgagcggataacaattccgaattcaaggagatataccggatccctagcataaccccttggggc	RBS + tt7.	FWD
pBP527	tcgttccactgagcgtcagaccccgtagaaaagatcaaaggatcttcagatctcaaaaaaccccctcaagacccg	RBS + tt7.	REV
pBP528	tagcataaccccttggggcctctaaacgggtcttgaggggttttttgagatctgaagatcctttgatcttttctacggggtc	bla cassette	REV
pBP529	cgctatcatgccataccgcgaaaggttttgcaccattcgatggtgtcaagcttcgcggaaccccctatttgtttatttttc	bla cassette	FWD
pBP530	tacatatttgaatgtatttagaaaaataaacaaataggggttccgcgaagcttgacaccatcgaatggtgcaaaac	*lacI* cassette	FWD
pBP531	ccagatggagttctgaggtcattactggatctatcaacaggagtcgcggccgcttacgtgcagtcgatgataagctgtc	*lacI* cassette	REV
pBP532	cattaggcacaattctcatgtttgacagcttatcatcgactgcacgtaagcggccgcgactcctgttgatagatccagtaatgacc	lambda t0 term.	FWD
pBP533	gcgtcgatttttgtgatgctcgtcaggggggcggagcctatggaaactgcagattctcaccaataaaaaacgcccg	lambda t0 term.	REV
pBP534	gttcagaacgctcggttgccgccgggcgtttttttattggtgagaatctgcagtttccataggctccgcccc	ColE1	REV
pBP535	cggaattgttatccgctcacaattcccctatagtgagtcgtattaggctcgagttgagatccttttttttctgcgcgtaatc	ColE1	FWD
pBP536	atctgttgtttgtcggtgaacgctctcctgagtaggacaaatccgccgtcgactaatacgactcactataggggaattgtgagc	t7	FWD
pBP537	ctgcagggtatatctccttgaattcggaattgttatccgctcacaattccggaattgttatccgctcacaattccc	t7	REV
pBP538	ggaattgtgagcggataacaattccgaattcaaggagatataccctgcagctagcataaccccttggggc	RBS + tt7.	FWD
pBP539	cgctatcatgccataccgcgaaaggttttgcaccattcgatggtgtccccgggcccaaaaaaccccctcaagacccg	RBS + tt7.	REV
pBP540	agcataaccccttggggcctctaaacgggtcttgaggggttttttgggcccggggacaccatcgaatggtgcaaaac	*lacI* cassette	FWD
pBP541	tttttgtttgcaagcagcagattacgcgcagaaaaaaaggatctcaactcgagttacgtgcagtcgatgataagctgtc	*lacI* cassette	REV
pBP542	tacatatttgaatgtatttagaaaaataaacaaataggggttccgcgaagctttttccataggctccgcccc	ColE1	REV
pBP543	ttaggcacaattctcatgtttgacagcttatcatcgactgcacgtaactcgagttgagatccttttttttctgcgcgtaatc	ColE1	FWD
pBP544	tccggaattgttatccgctcacaattcccctatagtgagtcgtattagtcgacggcggatttgtcctactcaggagag	rrnB1 term.	FWD
pBP545	gtgccggttgtgaagaaaaagtgaatgatgtagccgtcaagttgtcataaaggcatcaaataaaacgaaaggctc	rrnB1 term.	REV
pBP546	ataaaacgaaaggcccagtctttcgactgagcctttcgttttatttgatgcctttatgacaacttgacggctacatcattcac	ara cassette	REV
pBP547	tcggggaaatgtgcgcggaagaaggccatcctgacggatggccttttttctagaaaaaaaacgggtatggagaaacagtagagag	ara cassette	FWD
pBP548	acgctttttatcgcaactctctactgtttctccatacccgtttttttttctagaaaaaggccatccgtcaggatg	rrnB2 term.	REV

pBP549	tgttcagaacgctcggttgccgccgggcgtttttattggtgagaatagatcttaaatcgatgcaggtggcacttttc	rrnB2 term.	FWD
pBP550	cgttccactgagcgtcagaccccgtagaaaagatcaaaggatcttcgcggccgcgactcctgttgatagatccagtaatgacc	lambda t0 term.	FWD
pBP551	cttcttccgcgcacatttccccgaaaagtgccacctgcatcgatttaagatctattctcaccaataaaaaacgcccg	lambda t0 term.	REV
pBP552	tccagatggagttctgaggtcattactggatctatcaacaggagtcgcggccgcgaagatcctttgatcttttctacggggtc	bla cassette	REV
pBP553	agcgtcgatttttgtgatgctcgtcaggggggcggagcctatggaaaaagcttcgcggaacccctatttgtttatttttc	bla cassette	FWD

Abbreviations are as follows: FWD, forward primer; REV, reverse primer. t7, IPTG-inducible T7/lacO promoter; tt7, T7 terminator; term., terminator; RBS, RBS from pET21a(+); ColE1, origin of replication ColE1; ara, arabinose promoter and araC repressor; bla, β-lactamase; *lacI*, lac repressor.

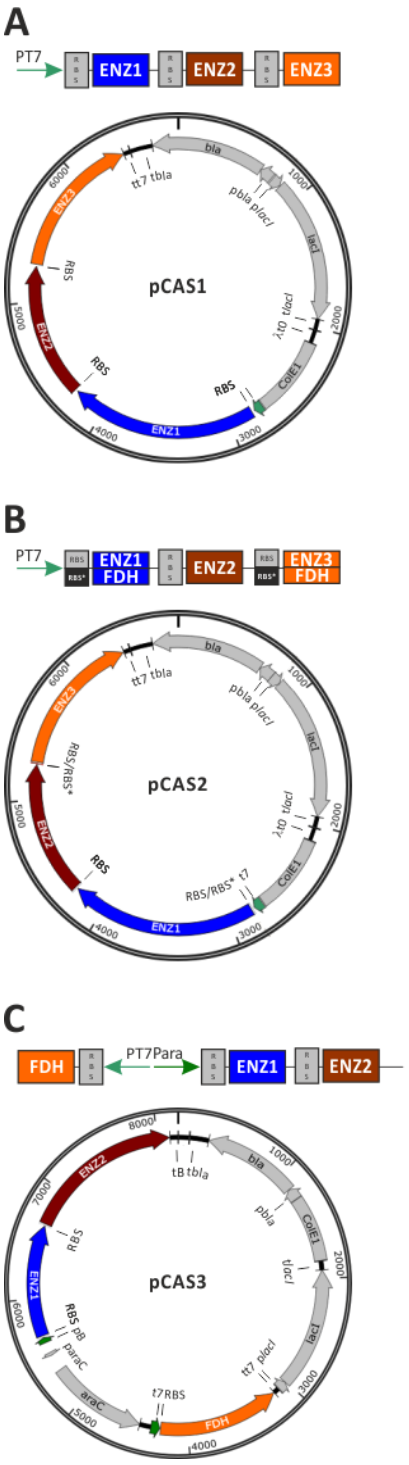
Table S2: Primers used for the amplification of cascade genes.

primer	plasmid	sequence (5' → 3')	part	orientation
pBP594	pCAS1.1	aggggaattgtgagcggataacaattccggaattgtgagcggataacaattccggatccgaattcaaggagatataccatgaacatttcaaggagaaagctacttttaggtg	L-AAD	FWD
pBP595	pCAS1.1	ctcgtcaacgcgagcaccgtaagcaataatcttcatggtatatctccttgaattcttacttcttaaaacgatccaaactaaacggcg	L-AAD	REV
pBP596	pCAS1.1	ccaacgccgtttagtttggatcgttttaagaagtaagaattcaaggagatataccatgaagattattgcttacggtgctcg	D-HicDH	FWD
pBP597	pCAS1.1	gtttgcccgcatcatacagcaccagcacaattttcatggtatatctccttgaattcctactttgctggaccagtaacttcc	D-HicDH	REV
pBP598	pCAS1.1	gaaaccagcacggaagttactggtccagcaaagtaggaattcaaggagatataccatgaaaattgtgctggtgctgtatgatg	FDH	FWD
pBP599	pCAS1.1	aaacccctcaagacccgtttagaggccccaaggggttatgctagcatatgttattatttttttatcgtgtttgccatacgctttg	FDH	REV
pBP600	pCAS1.2	gaaaccagcacggaagttactggtccagcaaagtaggaattcaaggagatataccatgaacatttcaaggagaaagctacttttaggtg	L-AAD	FWD
pBP601	pCAS1.2	aaacccctcaagacccgtttagaggccccaaggggttatgctagcatatgttacttcttaaaacgatccaaactaaacggcg	L-AAD	REV
pBP602	pCAS1.2	gaccaaagcgtatggcaaacacgataaaaaataataagaattcaaggagatataccatgaagattattgcttacggtgctcg	D-HicDH	FWD
pBP603	pCAS1.2	caacacctaaaagtagctttctccttgaaatgttcatggtatatctccttgaattcctactttgctggaccagtaacttccg	D-HicDH	REV
pBP604	pCAS1.2	aggggaattgtgagcggataacaattccggaattgtgagcggataacaattccggatccgaattcaaggagatataccatgaaaattgtgctggtgctgtatgatg	FDH	FWD
pBP605	pCAS1.2	ctcgtcaacgcgagcaccgtaagcaataatcttcatggtatatctccttgaattcttattatttttttatcgtgtttgccatacgctttg	FDH	REV
pBP606	pCAS1.3	aggggaattgtgagcggataacaattccggaattgtgagcggataacaattccggatccgaattcaaggagatataccatgaacatttcaaggagaaagctacttttaggtg	L-AAD	FWD
pBP607	pCAS1.3	gtttccaaggccgataattccaatcttacgtgccatggtatatctccttgaattcttacttcttaaaacgatccaaactaaacggcg	L-AAD	REV
pBP608	pCAS1.3	ccaacgccgtttagtttggatcgttttaagaagtaagaattcaaggagatataccatggcacgtaagattggaattatcgg	D-HicDH	FWD
pBP609	pCAS1.3	gtttgcccgcatcatacagcaccagcacaattttcatggtatatctccttgaattcttagagtgtatccacaatttcgtcgaaacg	D-HicDH	REV

pBP610	pCAS1.3	caacaacgtttcgacgaaattgtggatacactctaagaattcaaggagatataccatgaaaattgtgctggtgctgtatg atg	FDH	FWD
pBP611	pCAS1.3	aaacccctcaagacccgtttagaggccccaaggggttatgctagcatatgttattatttttatcgtgtttgccatacgc tttgg	FDH	REV
pBP612	pCAS1.4	caacaacgtttcgacgaaattgtggatacactctaagaattcaaggagatataccatgaacatttcaaggagaaagctac ttttagg	L-AAD	FWD
pBP613	pCAS1.4	aaacccctcaagacccgtttagaggccccaaggggttatgctagcatatgttacttcttaaaacgatccaaactaaacgg cg	L-AAD	REV
pBP614	pCAS1.4	gaccaaagcgtatggcaaacacgataaaaaataataagaattcaaggagatataccatggcacgtaagattggaattatc gg	D-HicDH	FWD
pBP615	pCAS1.4	caacacctaaaagtagctttctccttgaaatgttcatggtatatctccttgaattcttagagtgtatccacaatttcgtc gaaacg	D-HicDH	REV
pBP616	pCAS1.4	aggggaattgtgagcggataacaattccggaattgtgagcggataacaattccggatccgaattcaaggagatataccat gaaaattgtgctggtgctgtatgatg	FDH	FWD
pBP617	pCAS1.4	gtttccaaggccgataattccaatcttacgtgccatggtatatctccttgaattcttattatttttatcgtgtttgcca tacgctttg	FDH	REV
pBP618	pCAS3.2	gaaaccagcacggaagttactggtccagcaaagtaggagctcaaggagatataccatgaacatttcaaggagaaagctac ttttagg	L-AAD	FWD
pBP619	pCAS3.2	ggggaaatgtgcgcggaagaaggccatcctgacggatggccttttctagattacttcttaaaacgatccaaactaaacg gc	L-AAD	REV
pBP620	pCAS3.2	caactctctactgtttctccatacccgttttttttcatatgaaggagatataccatgaagattattgcttacggtgctcg	D-HicDH	FWD
pBP621	pCAS3.2	caacacctaaaagtagctttctccttgaaatgttcatggtatatctccttgagctcctactttgctggaccagtaacttc c	D-HicDH	REV
pBP622	pCAS3.2	caattccggaattgtgagcggataacaattccgaattcaaggagatataccatgaaaattgtgctggtgctgtatg	FDH	FWD
pBP623	pCAS3.2	caaaaaacccctcaagacccgtttagaggccccaaggggttatgctagctgcagttattatttttatcgtgtttgccat acgctttg	FDH	REV
pBP624	pCAS3.1	caactctctactgtttctccatacccgttttttttcatatgaaggagatataccatgaacatttcaaggagaaagctactt ttagg	L-AAD	FWD
pBP625	pCAS3.1	ctcgtcaacgcgagcaccgtaagcaataatcttcatggtatatctccttgagctcttacttcttaaaacgatccaaacta aacggc	L-AAD	REV
pBP626	pCAS3.1	ccaacgccgtttagtttggatcgttttaagaagtaagagctcaaggagatataccatgaagattattgcttacggtgctc g	D-HicDH	FWD
pBP627	pCAS3.1	ggggaaatgtgcgcggaagaaggccatcctgacggatggccttttctagactactttgctggaccagtaacttcc	D-HicDH	REV
pBP628	pCAS3.4	cgtttcgacgaaattgtggatacactctaactcgaggagctcaaggagatataccatgaacatttcaaggagaaagctac ttttaggtg	L-AAD	FWD
pBP629	pCAS3.4	caactctctactgtttctccatacccgttttttttcatatgaaggagatataccatggcacgtaagattggaattatcg	L-HicDH	FWD
pBP630	pCAS3.4	ccaacacctaaaagtagctttctccttgaaatgttcatggtatatctccttgagctcctcgagttagagtgtatccacaa	L-HicDH	REV

		tttcgtc		
pBP631	pCAS3.3	gtttccaaggccgataattccaatcttacgtgccatggtatatctccttgagctcttacttcttaaaacgatccaaactaaacggc	L-AAD	REV
pBP632	pCAS3.3	ccaacgccgtttagtttggatcgttttaagaagtaagagctcaaggagatataccatggcacgtaagattggaattatcgg	L-HicDH	FWD
pBP633	pCAS3.3	ggggaaatgtgcgcggaagaaggccatcctgacggatggccttttttctagactcgagttagagtgtatccacaatttcg	L-HicDH	REV
pBP795	pCAS2.1	ggaagttactggtccagcaaagtagtgtctctactataactacggttcaaactatgaaaattgtgctggtgctgtatgatg	FDH	FWD
pBP796	pCAS2.1	catacagcaccagcacaattttcatagtttgaaccgtagttatagtagagacactactttgctggaccagtaacttccgtgc	D-HicDH	REV
pBP799	pCAS2.2	ggaattgtgagcggataacaattccacaaatacgaataatactacggttaaatccatgaaaattgtgctggtgctgtatg	FDH	FWD
pBP797	pCAS2.2	cgacgaaattgtggatacactctaagcttcactcggagcgactctacggttactatatatgaaaattgtgctggtgctgtatgatg	FDH	FWD
pBP798	pCAS2.3	catacagcaccagcacaattttcatatatagtaaccgtagagtcgctccgagtgaagcttagagtgtatccacaatttcgtcgaaac	L-HicDH	REV
pBP799	pCAS2.4	ggaattgtgagcggataacaattccacaaatacgaataatactacggttaaatccatgaaaattgtgctggtgctgtatg	FDH	FWD

Abbreviations are as follows: FWD, forward primer; REV, reverse primer.



Construct	Enzyme ENZ1	Enzyme ENZ2	Enzyme ENZ3
pCAS1.1	L-AAD	D-Hic	FDH
pCAS1.2	FDH	D-Hic	L-AAD
pCAS1.3	L-AAD	L-Hic	FDH
pCAS1.4	FDH	L-Hic	L-AAD

Construct	Enzyme ENZ1	Enzyme ENZ2	Enzyme ENZ3
pCAS2.1	L-AAD	D-Hic	*FDH
pCAS2.2	*FDH	D-Hic	L-AAD
pCAS2.3	L-AAD	L-Hic	*FDH
pCAS2.4	*FDH	L-Hic	L-AAD

Construct	Enzyme ENZ1	Enzyme ENZ2
pCAS3.1	L-AAD	D-Hic
pCAS3.2	D-Hic	L-AAD
pCAS3.3	L-AAD	L-Hic
pCAS3.4	L-Hic	L-AAD

Figure S1. The different pCAS1, pCAS2 and pCAS3 cascade expression constructs. (**A**) On the pCAS1 constructs, all cascade genes are under the control of the T7/*lacO* promoter (t7). Each gene carries the same RBS sequence from the pET21a(+) plasmid. (**B**) The pCAS2 constructs are similar to the pCAS1 constructs except that the RBS sequence from pET21a(+) preceding the formate dehydrogenase (FDH) gene was exchanged for a designed ultrastrong RBS (RBS*) sequence. (**C**) On the pCAS3 constructs, the L-amino deaminase (L-AAD) and

one of each of the enantioselective isocaproate reductases (L/D-Hic) are under the control of the arabinose-inducible pBAD promoter while FDH is expressed from promoter T7/*lacO* promoter. All cascade genes carry the same RBS sequence from pET21a(+). The tables in A, B and C summarize the variations of the gene order in the polycistronic construct. ENZ1, 2, 3 indicates the enzyme at positions 1, 2 and 3, respectively; bla, β-lactamase gene; pbla, β-lactamase promoter; tbla, β-lactamase terminator; t7, T7/*lacO* promoter; tt7, T7 terminator; *lacI*, lac repressor; p*lacI*, lac repressor promoter; t*lacI*, lac repressor terminator; pB, arabinose-inducible promoter (pBAD); paraC, *araC* promoter; tB, terminator rrnBT2; RBS, RBS from pET21a(+); RBS*, designed ultrastrong RBS: *FDH, FDH gene carrying the designed ultrastrong RBS.

4. Biocatalyst preparation and analysis of protein expression

4.1 Protein Expression.

The cascade expression strains *E. coli*/pCAS were prepared by electroporating3 the different constructs into *E. coli* BL21(DE3) (F– *omp*T *gal dcm lon hsd*SB(rB- mB-) λ(DE3 [*lac*I *lac*UV5-T7 gene 1 *ind*1 *sam*7 *nin*5]). 100 mL LB-amp medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 100 mg/L ampicillin) in a baffled 1 L shake flask were inoculated with an overnight pre-culture to an initial OD_{600} of 0.1. The cultures were shaken at 120 rpm and 37 °C until they reached an OD_{600} of 0.6-0.8. Cascade expression from the pCAS1 and pCAS2 constructs was induced with 0.1 mM IPTG. We used 2% (w/v) arabinose in addition to 0.1 mM IPTG with the pCAS3 constructs. Protein expression was performed for either 24 h at 28 °C or 4 h at 37 °C and 120 rpm shaking. All the cells of the culture were harvested by low speed centrifugation (4 °C, 20 min, 4000 rpm), and cells were used to make different preparations. **Crude lysate:** for the preparation of crude lysate the cells were suspended in 10 mM potassium phosphate buffer pH 7 at 10% (w/v). The cells were disrupted by sonication (2 x 0.1 sec on 0.4 sec off, output 40%) with a brake of 1 min in-between the cycles, constantly on ice. After disruption the not clarified crude lysate was directly used for activity measurements. **Lyophilised whole cells:** a) for the PBS preparation cells were washed once with PBS (1.9 mM KH_2PO_4; 15 mM Na_2HPO_4; 150 mM NaCl; pH 7.0), resuspended in a volume of PBS corresponding to ¼ of the culture volume, shock frozen in liquid nitrogen and lyophilised. b) Alternatively, for the potassium phosphate preparation the cells were prepared in the same way using a volume of 10 mM potassium phosphate buffer (5 mM KH_2PO_4, 5 mM K_2HPO_4; pH 7) corresponding to $^1/_8$ of the culture volume instead of PBS. The lyophilised cells were stored at 4 °C and used as such for further experiments.

4.1.a Analysis of cascade gene expression in whole cell samples

After performing protein expression as described above all the cells of the culture were harvested by low speed centrifugation (see above, 3.1) and the pellet was resuspended in 25 mL of lysis buffer (50 mM NaH_2PO_4 H_2O, 300 mM NaCl, 10 mM imidazole, pH 8.0). The cells were disrupted by passing the cell suspension three times through the high pressure emulsifier EmulsiFlex C3 (Avestin, Canada). The insoluble and soluble protein fractions were separated by centrifugation at 4 °C, 40 min, 40,000 g. 6 M urea at a volume equivalent to that

of the soluble fraction was added for resuspension of the pellet. Bradford assay[4] was performed with both fractions for determination of the total protein content.

3 µg of total protein each of the soluble and insoluble protein fractions were separated on 10% SDS-polyacrylamide gels according to Laemmli[5]. Prestained PAGE-Ruler mix (Fermentas, Burlington, Canada) was applied to estimate the molecular weight of the proteins of interest. The SDS-polyacrylamide gels were stained with Coomassie solution [0.25% (w/v) Coomassie Brilliant Blue G250, 10% (v/v) acetic acid, 45% (v/v) ethanol] and destained in the same solution without the dye.

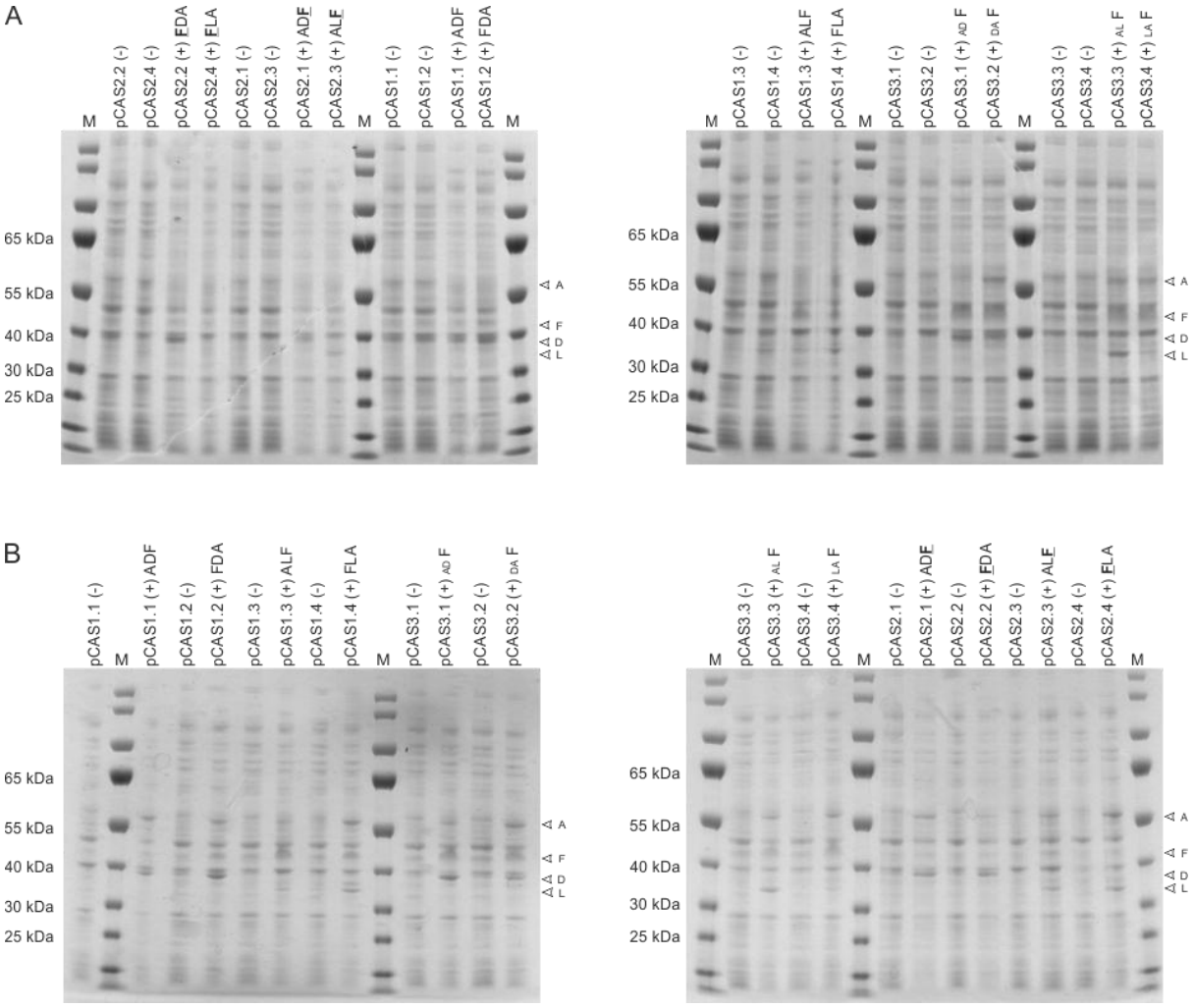


Figure S2. Expression of the different pCAS constructs. A) 24 h at 28 °C; B) 4 h at 37 °C. A, L-AAD; F, FDH; D, D-Hic; L, L-Hic. An underlined label in bold indicates expression with the ultrastrong RBS; a label in smaller font indicates expression from the arabinose-inducible pBAD promoter. Calculated molecular weights: L-AAD, 52.7 kDa; FDH, 40.6 kDa; D-HicDH, 37.1 kDa; L-HicDH, 36.7 kDa. (-), uninduced, (+), induced; M, molecular size marker.

5. Activity measurements

For the activity measurements the catalysts were co-expressed at 28 °C for 24 h in all cases.

5.1. Determination of HIC and FDH activity (spectrophotometrical)

The activities of FDH and HICs were determined by measuring the initial velocity change at 340 nm for the lyophilized preparations of constructs pCAS1, pCAS2 and pCAS3. Moreover for pCAS3.1 and 3.3 activities of crude lysate prepared as described in 3.1 were determined as well. FDH activities were measured as follows: 970 µL of formate solution (150 mM ammonium formate in potassium phosphate buffer, 100 mM, pH 7), and 20 µL of NAD^+ (100 mM in distilled water) were mixed in a 1 mL cuvette (Table S3). The reaction was started by the addition of 10 µL of enzyme solution (different enzyme preparations were used), and the production of NADH was monitored at 340 nm over a period of 1 min. One unit of activity was defined as the amount of NADH (µmol) produced per unit of time (min) and per the mass of the lyophilized cells (mg). To compare lyophilised cells and crude lysate activity was given per optical density at 600 nm (OD_{600}) (Figure S5).

For the determination of the HIC activity 970 µL of substrate solution (5 mM phenylpyruvic acid in potassium phosphate buffer, 100 mM pH 7) were mixed with 20 µL of NADH solution (12.5 mM in distilled water) in a 1 mL cuvette. After addition of the enzyme solution (10 µL, different enzyme preparations were used) the consumption of NADH was monitored over 1 min. Activity measurements were obtained in triplicate. One unit of activity was defined as the amount of NADH (µmol) consumed per unit of time (min) and per the mass of the lyophilized cells (mg). To compare lyophilised cells and crude lysate activity was given per optical density at 600 nm (OD_{600}) (Figure S5).

5.2. Determination of L-AAD activity (HPLC)

The activity of the L-AAD was determined for the oxidation of L-phenyl alanine by measuring the initial rate for the formation of phenyl pyruvic acid by HPLC (Table S4). The reaction was monitored over a period of 60 min. One unit of activity was defined as the amount of phenyl pyruvic acid (µmol) produced per unit of time (min) and per the mass of the lyophilized cells (mg).

Activities for both lyophilised preparations of pCAS3.1 and 3.3 as well as for the corresponding crude lysates were also determined (Figure S5). One unit of activity was defined as the amount of phenyl pyruvic acid (µmol) produced per unit of time (min) and per optical density at 600 nm (OD600).

Experiments were performed as follows: L-phe (100 mM), catalyst preparation (1 mg or 20 µL), 21 °C in KP_i (100 mM, pH 7, 1 mL), 170 rpm and 1 bar O_2 in a glass vial. Activity measurements were obtained in duplicate.

5.3 Results of the photometrical activity measurements

In order to compare the different whole cell catalysts individual activities for each enzyme were determined. According to the results summarized in table S3, the FDH always displays the lowest activity of the three cascade enzymes. To address this issue different constructs were prepared in particular focussing on an optimised FDH expression (see Figure S1). For the pCAS1 series the same promoter and RBS was used for all desired genes: here the FDH activity was only measurable when expressed in the following order: L-AAD, HIC and FDH (pCAS1.3, entry 3). A stronger RBS introduced in the second pCAS2 series did not result in any improved FDH activity (entry 5-8). However, the exclusive use of a strong promoter for the FDH expression finally led to FDH expression with a detectable activity (pCAS3 series). Comparing the activities of all three enzymes it is obvious that the rate of the reduction step is always determined by the recycling enzyme FDH. Nevertheless, we were pleased to see that the highest activities for the FDH obtained for construct pCAS3 led to cell catalysts where the rate of the oxidation and the reduction steps did not lead to accumulation of the α-keto acid intermediate.

Table S3. Initial rates for the FDH, L-AAD (mU/mg) and HICs in (U/mg) of the lyophilized cells for the PBS preparations of *E. coli*/pCAS 1, pCAS 2 and pCAS 3.

		reduction step		oxidation step
Entry	**Construct**	**Hic (U/mg)[a]**	**FDH (mU/mg)[a]**	**L-AAD (mU/mg)[b]**
1	pCAS1.1 (ADF)	0.50	<1	116
2	pCAS1.2 (FDA)	1.02	<1	87
3	pCAS1.3 (ALF)	0.54	11	62
4	pCAS1.4 (FLA)	0.18	<1	80
5	pCAS2.1 (AD**F**)	0.60	<1	82
6	pCAS2.2 (**F**DA)	0.78	<1	85
7	pCAS2.3 (AL**F**)	0.06	<1	96
8	pCAS2.4 (**F**LA)	<0.01	<1	114
9	pCAS3.1 (AD F)	1.71	12	59
10	pCAS3.2 (DA F)	1.01	9	71
11	pCAS3.3 (AL F)	0.69	19	98
12	pCAS3.4 (LA F)	0.08	5	103

[a] activities of HIC and FDH were determined using the spectophotometrical method given in 4.1.

[b] activities of the L-AAD were determined using HPLC according to 4.2; different conditions compared to determination of HIC and FDH activity were used.

5.4. Initial rate of the overall reaction, the oxidation and reduction steps for pCAS1.1, pCAS3.1 and pCAS3.3

Overall reaction

The activity for the overall transformation was determined by measuring the initial rate for the formation of phenyl lactic acid by HPLC. The reaction was monitored over a period of 60 min. One unit of activity was defined as the amount of phenyl lactic acid (µmol) produced per unit of time (min) and per the mass of the lyophilized cells (mg) under the following reaction conditions: L-phe (100 mM), HCO_2NH_4 (300 mM), NAD^+ (1 mM), lyophilised *E. coli*/pCAS cells (5 mg), 21 °C in KP_i (100 mM, pH 7, 1 mL), 170 rpm and 1 bar O_2 in a glass vial. Activity measurements were performed in duplicate (Table S4).

Oxidation step

The activity for the oxidation of L-phenyl alanine was determined by measuring the initial rate for the formation of phenyl pyruvic acid by HPLC. The reaction was monitored over a period of 60 min. One unit of activity was defined as the amount of phenyl pyruvic acid (µmol) produced per unit of time (min) and per the mass of the lyophilized cells (mg) under the following reaction conditions: L-phe (100 mM), lyophilised *E. coli*/pCAS cells (1 mg), 21 °C in KP_i (100 mM, pH 7, 1 mL), 170 rpm and 1 bar O_2 in a glass vial. Activity measurements were performed in duplicate (Table S4).

Reduction step

The activity for the reduction step was determined by measuring the initial rate for the formation of phenyl lactic acid by HPLC. The reaction was monitored over a period of 60 min. One unit of activity was defined as the amount of phenyl lactic acid (µmol) produced per unit of time (min) and per the mass of the lyophilized cells (mg) under the following reaction conditions: Phenyl pyruvic acid (5 mM), HCO_2NH_4 (300 mM), NAD^+ (1 mM), lyophilised *E. coli*/pCAS cells (0.5 mg), 21 °C in KP_i (100 mM, pH 7, 1 mL), 170 rpm in a glass vial. Activity measurements were performed in duplicate (Table S4).

With the aim of rationalizing the accumulation the intermediate ketoacid observed for pCAS1.1 and the balance between the oxidation and the reduction steps obtained for pCAS 3.1 and 3.3 we measured the intial rates for the overall cascade and the individual oxidation and reduction steps under comparable reaction conditions (Table S4). The activity values perfectly correlate with the experimental results obtained for the biotransformations. Thus, the accumulation of the intermediate keto acid **2a** was observed if the oxidation step was faster than the reduction, e.g, with pCAS1.1 (ADF, entry 1). In this case the overall rate is determined by the reduction step. On the other hand, the formation of keto acid **2a** was negligible when the reduction step was faster than the oxidation as for instance found for pCAS 3.1 or pCAS 3.3 (AD F or AL F, entries 2-3 respectively). Here, the overall reaction rate was determined by the oxidation step.

Table S4. Initial rates (mU/mg) of the lyophilized cells for the overall cascade, and the individual oxidation and reduction steps using lyophilized PBS preparations of *E.coli*/pCAS1.1, pCAS3.1 and pCAS3.3.

Entry	Construct	Overall rate (mU/mg)[a]	Oxidation(mU/mg)[a]	Reduction(mU/mg)[a]
1	pCAS 1.1	8	116	15
2	pCAS 3.1	55	59	142
3	pCAS 3.3	89	98	218

[a] See section 4.4 for the experimental details.

6. Analytical biotransformations

6.1. Biotransformations using *E.coli* pCAS1.1-1.4 with external supplementation of FDH

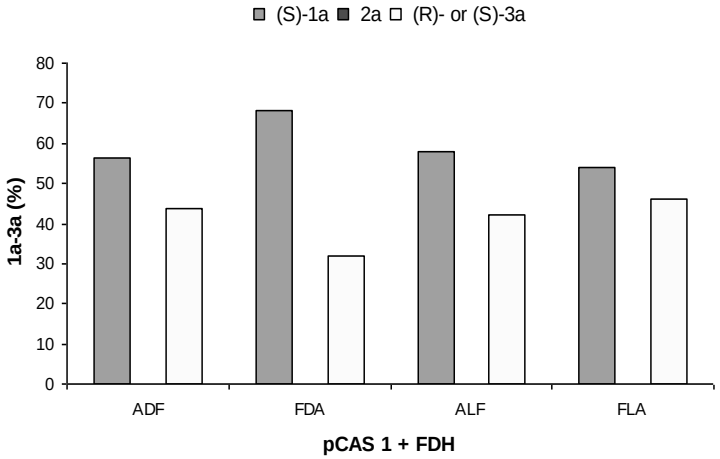


Figure S3. Percentage of **1a**-**3a** employing lyophilized *E. coli*/pCAS1.1-1.4 with external supplementation of FDH (3 mg, 6.2 U). Protein expression was induced at 28 °C for 24 h. Reaction conditions (*S*)-**1a** 100 mM, O_2 1 bar, *E. coli*/CAS1 (20 mg), FDH (2 mg, 6.2 U), NAD^+ (1 mM), HCO_2NH_4 (300 mM), KP_i (100 mM, pH 7), 1 h, 21 °C, 170 rpm.

6.2. Biotransformations using *E.coli* pCAS2.1-2.4

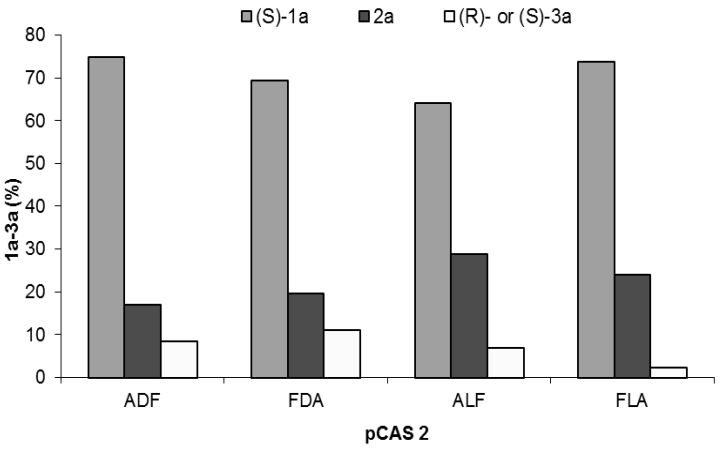


Figure S4. Percentage of **1a**-**3a** employing lyophilized *E. coli*/pCAS2.1-2.4. Protein expression was induced at 28 °C for 24 h. Reaction conditions (*S*)-**1a** 100 mM, O_2 1 bar, construct (10 mg), NAD^+ (1 mM), HCO_2NH_4 (300 mM), KP_i (100 mM, pH 7), 1 h, 21 °C, 170 rpm.

7. Effect of the biocatalyst preparation: PBS vs KP_i

The preparation of the lyophilized *E. coli* cells was found to be a key factor for the successful coupling of the oxidation and the reduction steps. For that reason, lyophilized *E. coli* cells of the construct 3.1 were prepared using a PBS as well as a KPi buffer (see section 3.1 for experimental details) and the individual activities were measured. Activity values were normalized per unit of OD_{600} to enable a proper comparison between the different preparations. The individual activities were in all cases higher when using the PBS- compared to the KP_i preparations (Figure S5). Moreover, the activities reached with the PBS preparation were similar to the ones obtained for fully disrupted cell (Figure S5 C).

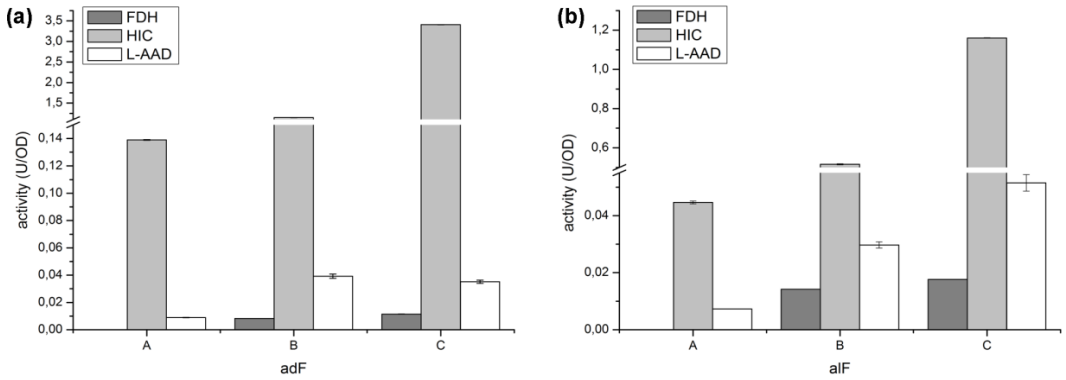


Figure S5. Enzyme activities (FDH, Hic and L-AAD) for constructs pCAS3.1 (a) and 3.3 (b) using different buffers for suspended cells prior lyophilisation: A: KP_i B: PBS C: Disrupted cells. Activities (U/OD_{600}) were determined spectrophotometrically for Hic and FDH (see section 4.1) and for the L-AAD the initial rates were determined by HPLC (see section 4.2).

As a consequence the kinetic profile of the cascade was followed using these two preparations of construct 3.1: PBS buffer (Figure S6) and KP_i buffer (Figure S7).

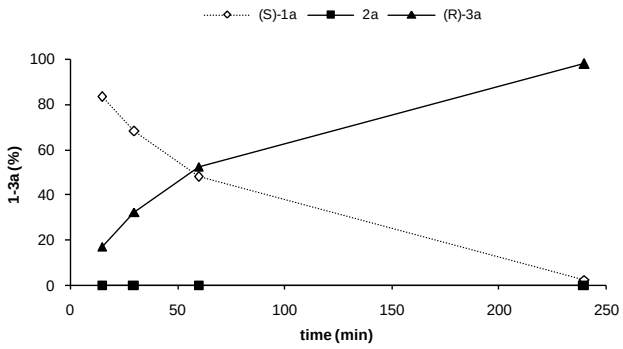


Figure S6. Percentage of **1a-3a** versus time using construct 3.1 as lyophilized preparation of PBS suspended cells. Reaction conditions (*S*)-**1a** 100 mM, O_2 1 bar, 3.1 (20 mg), NAD^+ (1 mM), HCO_2NH_4 (300 mM), KP_i (100 mM, pH 7) 1 h, 21 °C, 170 rpm.

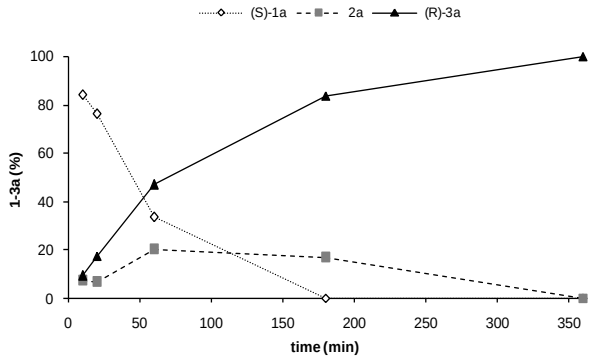


Figure S7. Percentage of **1a-3a** versus time using construct 3.1 as lyophilized preparation of cells suspended in KP_i buffer. Reaction conditions (*S*)-**1a** 100 mM, O_2 1 bar, 3.1 (6 mg), NAD^+ (1 mM), HCO_2NH_4 (300 mM), KP_i (100 mM, pH 7) 1 h, 21 °C, 170 rpm.

While for the PBS preparation the oxidation and the reduction steps were balanced since no intermediate ketoacid accumulated at any stage and obtaining >99% conversion after only 4 h (Figure S6). The K-phosphate preparation did not perform as well, here the coupling between the oxidation and the reduction steps is unbalanced resulting in the undesired accumulation of the intermediate ketoacid (Figure S7). In spite of this, the system also reached >99% conversion after 6 h without detecting the formation of side products because of the accumulation of the intermediate.

8. Biocatalytic preparative transformation

8.1. Time course of the biotransformation of L-1a employing lyophilized *E. coli* pCAS3.3 cells

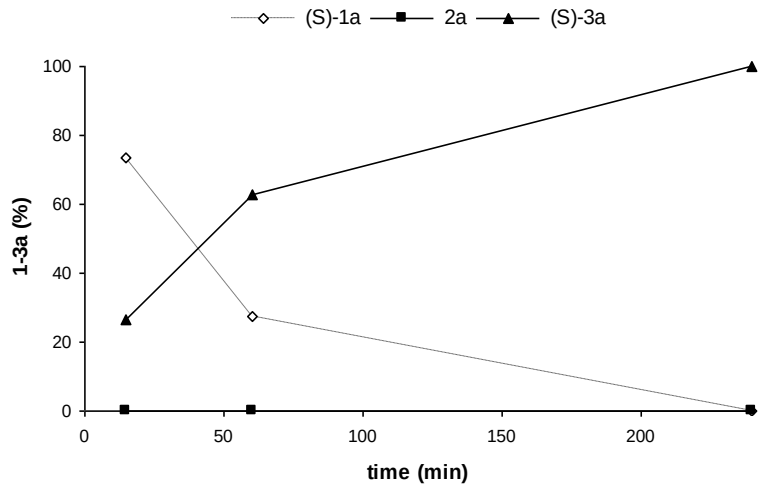


Figure S8. Time course of the transformation of L-**1a** employing lyophilized *E. coli*/pCAS3.3 cells. Reaction conditions (*S*)-**1a** 100 mM, O_2 1 bar, *E. coli*/pCAS3.1 (20 mg), NAD^+ (1 mM), HCO_2NH_4 (300 mM), 21 °C, 170 rpm.

8.2. Representative procedure for the synthesis of (*R*)- or (*S*)-2-hydroxy-3-phenylpropanoic acid [(*R*)- or (*S*)-3a]

Lyophilised *E. coli*/pCAS1.3, 3.1 or 3.3 cells (120 mg freeze dried *E. coli* cells containing overexpressed L-AAD, L- or D-Hic and FDH) were suspended in a K-phosphate buffer (3 mL, 100 mM, pH 7.0) containing the substrate (L-**1a**, 96.6 mg, 0.60 mmol, 200 mM), NAD^+ (1 mM) and NH_4HCOO (600 mM). The suspension was shaken at 170 rpm, 21 °C and 1 bar of oxygen pressure for 6 h. After this time, the reaction was acidified to pH 1 with aqueous HCl 4 M (1 mL), and extracted with EtOAc (3 × 25 mL). The organic phases were combined, dried over Na_2SO_4, and the resulting solid washed with *n*-heptane to remove minor impurities coming from the enzyme (3 × 5 mL) affording (*R*)- or (*S*)-**3a** as white solids (71-86 % yield, >99% *ee*).

8.3. Representative procedure for the synthesis of (*S*)-2-hydroxy-3-(4-hydroxyphenyl)propanoic acid [(*S*)-3c]

E. coli/pCAS3.3 cells (160 mg freeze dried *E. coli* cells containing overexpressed L-AAD, L-Hic and FDH) were added to a K-phosphate buffer (4 mL, 100 mM, pH 7.0) containing the substrate (L-**1c**, 144.9 mg, 0.80 mmol, 200 mM), NAD^+ (1 mM) and NH_4HCOO (600 mM). The suspension was shaken at 170 rpm, 21 °C and 1 bar of oxygen pressure for 6 h. After this time, the reaction was acidified to pH 1 with aqueous HCl 4 M (1 mL), and extracted with EtOAc (3 × 25 mL). The organic phases were combined, dried over Na_2SO_4, and the resulting solid washed with *n*-heptane to remove minor impurities coming from the enzyme (3 × 5 mL) affording (*S*)-**3c** as a slightly yellowish solid (121.4 mg, 86 % yield, 99% *ee*).

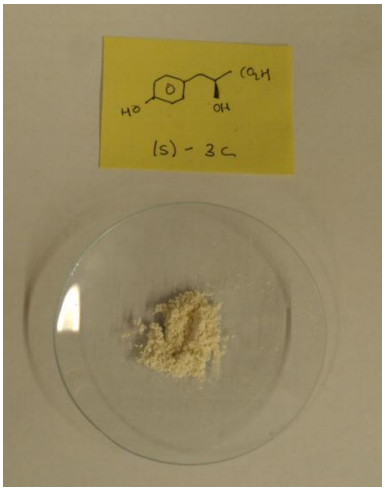


Figure S12. Enantiomerically pure (*S*)-**3c** isolated from the biotransformation of (*S*)-**1c** using pCAS3.3 as whole cell catalyst.

9. Product characterization

(*R*)- and (*S*)-2-hydroxy-3-phenylpropanoic acid (3a). Isolated yield: 86% for (*S*)-**3a** and 85% for (*R*)-**3a**. White solids. Spectroscopical and analytical data in agreement with those previously published.[6]

Spectroscopical data for (*R*)-2-hydroxy-4-methylpentanoic acid (3b). Isolated yield: 78% for (*R*)-**3b**. White solid. Spectroscopical and analytical data in agreement with those previously published.[6]

Spectroscopical data (*R*)- and (*S*)-2-hydroxy-3-(4-hydroxyphenyl)propanoic acid (3c).
Isolated yield: 86% for (*S*)-**3c** and 75% for (*R*)-**3c**. White solid. Spectroscopical and analytical data in agreement with those previously published.[6]

10. Conversion measurement

10.1 Aromatic substrates

Conversion values for the biotransformations of L-**1a** and L-**1c** were determined by reverse phase HPLC.

Sample preparation: A MeCN/H_2O solution (1 mL, 1:1) containing 0.1% TFA was added to an aliquot of the reaction (1 mL). The protein was removed by centrifugation and the solution was filtered trough a VIVAspin membrane polyethersulfon filters. The resulting solution was analysed by HPLC under the conditions stated below.

Column: Luna C18 5 μm; *Flow*: 1 mL/min; *Temperature*: 30 °C; *Gradient*: from 100% H_2O (0.1% TFA) to 100% MeCN (0.1% TFA) in 22 min. Wavelength: 254 nm for **1a** and 280 nm for **1c**.
Further analytical details were previously reported.[6]

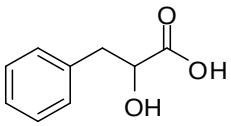
10.2 Aliphatic substrates

Conversion values for the biotransformations of L-**1b** were determined by NMR.

Sample preparation: Reactions (1 mL scale) were centrifuged at 15000 rpm for 5 min to spin down the cells. The so-obtained clear solution was lyophilized, re-dissolved in D_2O (0.8 mL) and the resulting solution was analyzed by ^{1}H-NMR (300 MHz, 64 scans).

Further details were previously reported.[6]

11. Chiral analytic on HPLC [2-hydroxy-3-phenylpropanoic acid, 3a]



Column: Chiralcel OJ

Eluent: *n*-hexane/2-propanol/TFA 96:4:0.1

Flow: 0.8 mL/min

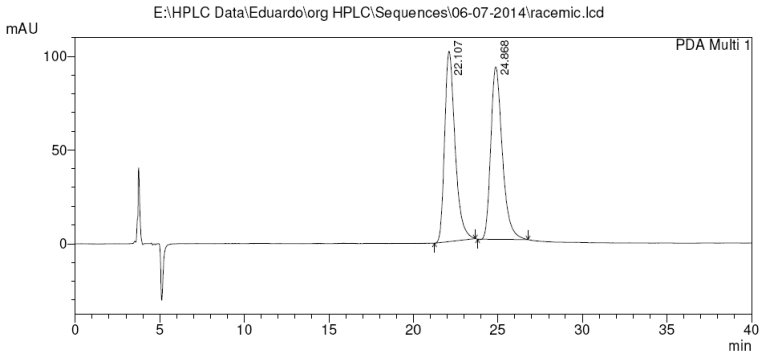
Temperature: 30 ºC

Rs: 1.2

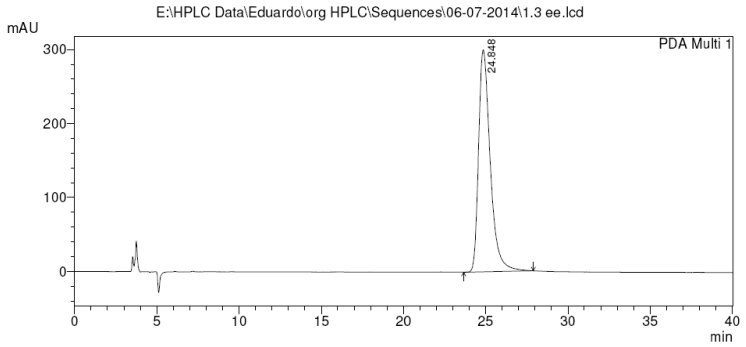
Retention times: t_R (*R*) = 32.2 min, t_R (*S*) = 37.3 min

Wavelength: 210 nm

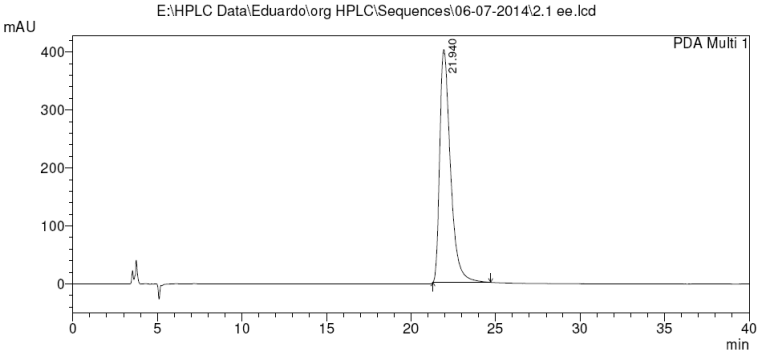
HPLC separation for both enantiomers of phenyllactic acid [(±)-3a]



(*S*)-3a in >99% ee (L-Hic catalyzed reduction)



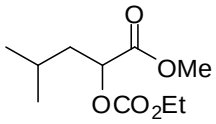
(*R*)-3a in >99% ee (D-Hic catalyzed reduction)



Derivatization protocol for *ee* measurement (3b): The hydroxy acid (4 mg) was dissolved in MeOH (700 μL, containing 5% DMAP) and ethyl chloroformate (150 μL) was added. The reaction was shaken at 50 °C and 700 rpm for 1 h. After that time, MeOH was removed under reduced pressure and HCl 2% was added (700 μL). The aqueous phase was extracted with EtOAc (3×700 μL), the organic phases combined and dried over Na_2SO_4. An aliquot (5 μL) was injected in the GC under the conditions stated below.

Derivatization protocol for *ee* measurement (3c): The hydroxy acid (4 mg) was dissolved in EtOAc (300 μL) and the solution was transferred to a suspension of Ac_2O (75 μL) and DMAP (10 mg). The reaction was shaken at 30 °C and 700 rpm for 1 h. After that time, the reaction was quenched with HCl 2% (700 μL) and extracted with EtOAc (3×700 μL). Organic phases were combined and dried over Na_2SO_4 and the solvent removed under reduced pressure. The so-obtained oil was redissolved in heptane:2-PrOH (80:20, 1 mL) and injected in the chiral HPLC (5 μL).

Chiral analytic on GC [2-hydroxy-4-methylpentanoic acid, 3b, derivatized with ethyl chloroformate]



Column: CP-Chiralsil-Dex CB

Injector temprature: 250 °C

Flow: 1.0 mL/min

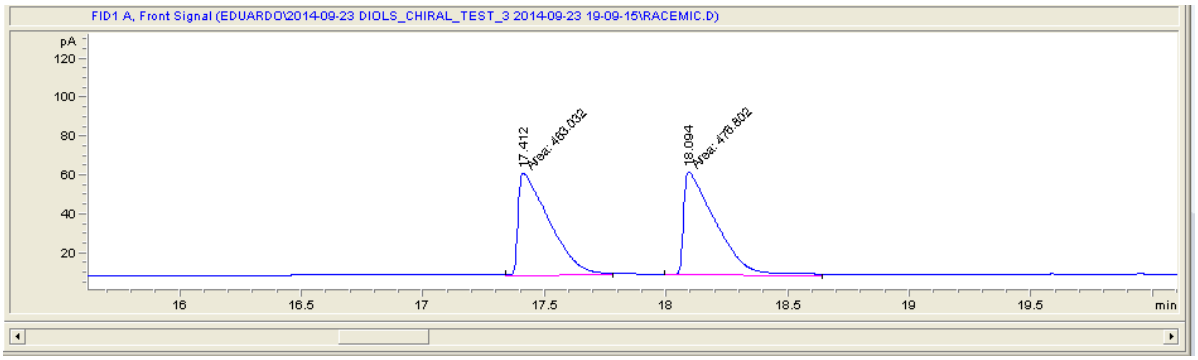
Detector Temperature: 250 ºC

Rs: 1.5

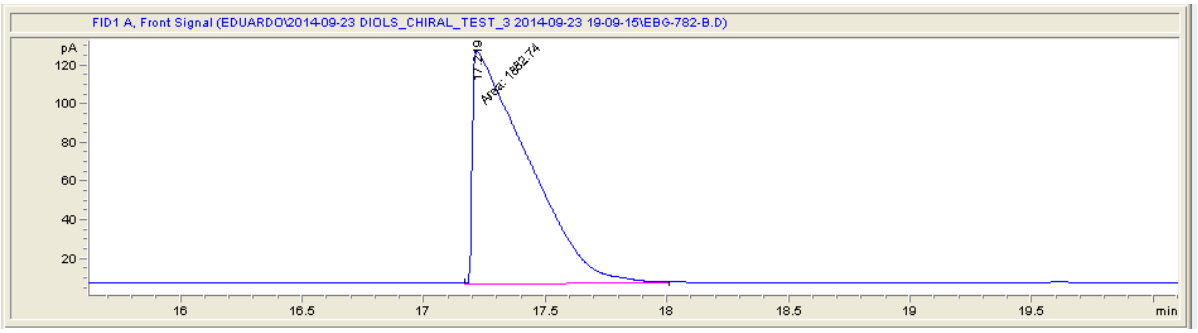
Retention times: t_R (*R*) = 17.4 min, t_R (*S*) = 18.1 min

Temperature programme: 60 °C (5 min) then 3 °C/min until 180 °C

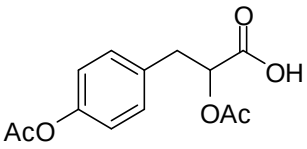
GC separation for both enantiomers of (±)-3b



(*R*)-3c >99% ee (D-Hic catalyzed reduction)



Chiral analytic on HPLC [2-hydroxy-3-(4-hydroxy)phenylpropanoic acid, 3c] derivatized as diacetate



Column: Chiralcel OJ

Eluent: *n*-hexane/2-propanol/TFA 80:20:0.1

Flow: 0.8 mL/min

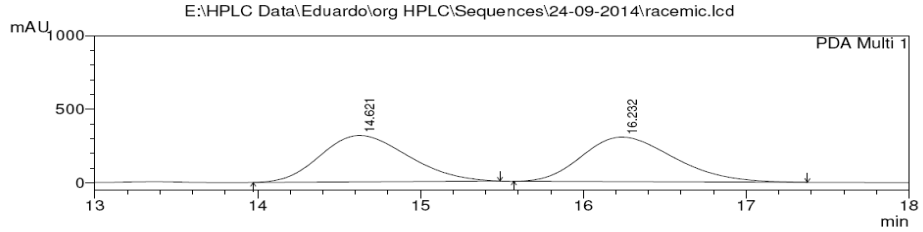
Temperature: 30 ºC

Rs: 1.1

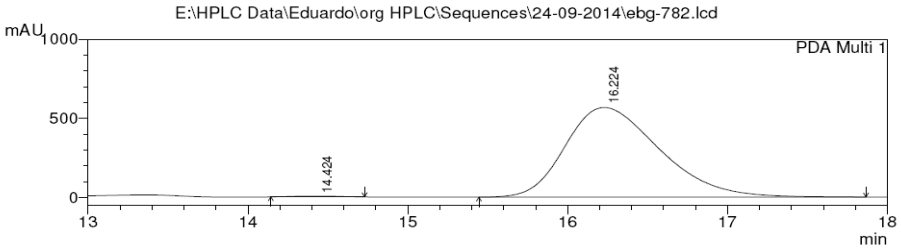
Retention times: t_R (*R*) = 14.6 min, t_R (*S*) = 16.2 min

Wavelength: 210 nm

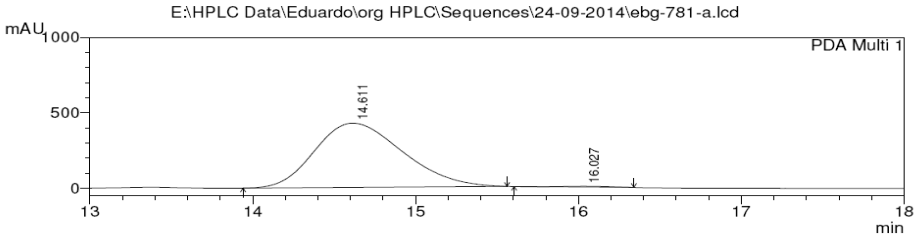
HPLC separation for both enantiomers of 2-acetoxy-3-(4-acetoxyphenyl)propanoic acid [(±)-3c]



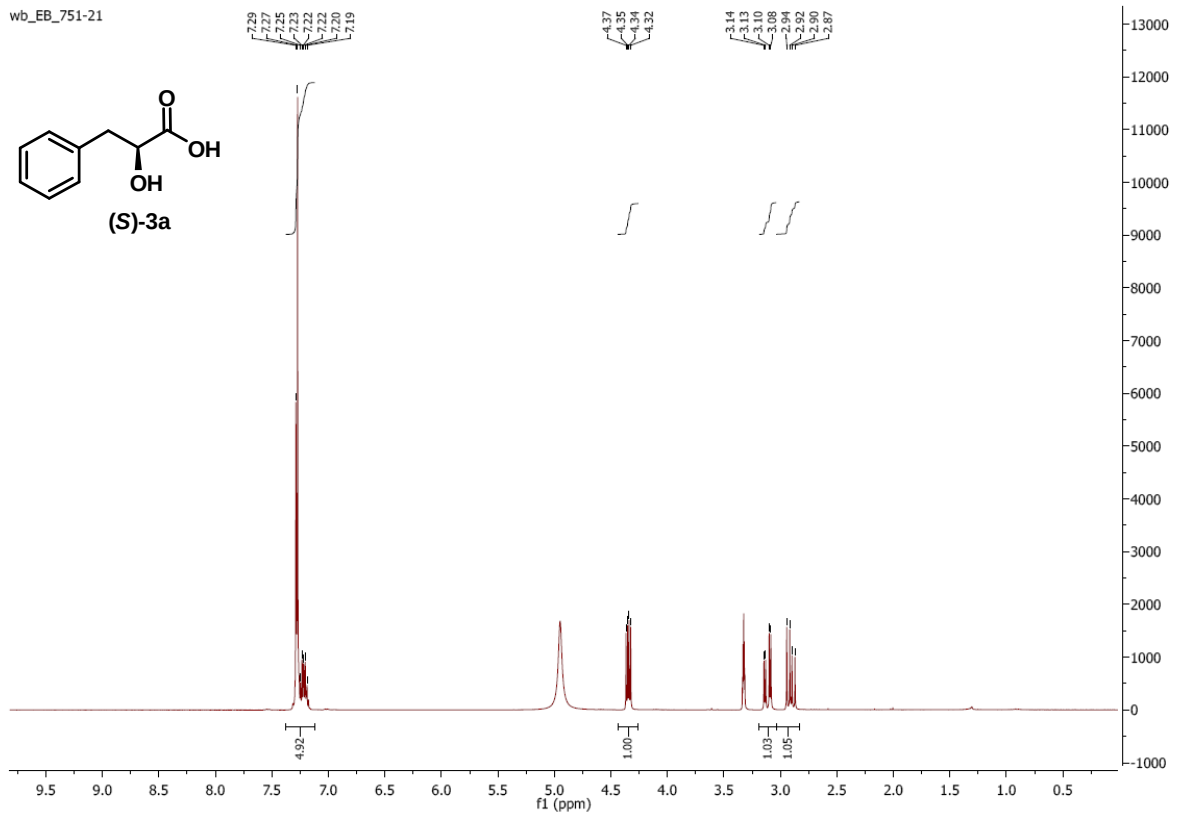
(*S*)-3c in 99% ee (L-Hic catalyzed reduction)

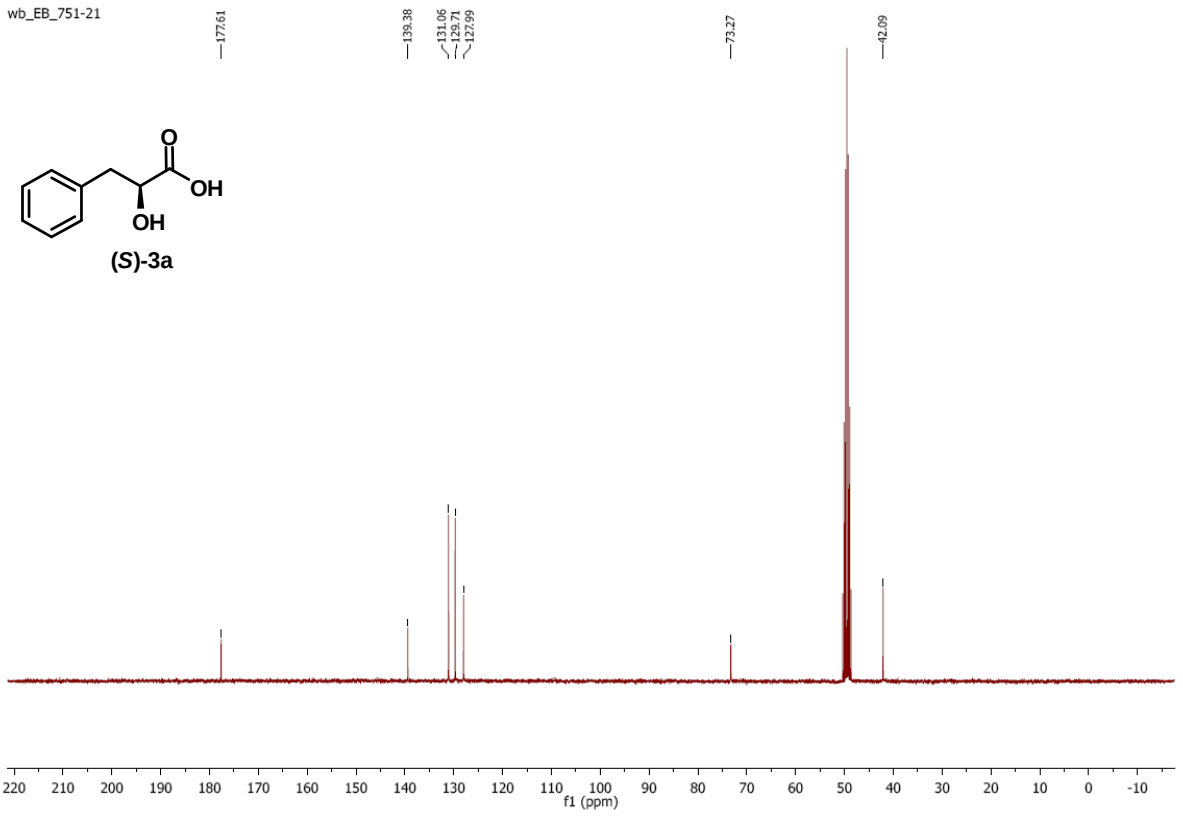


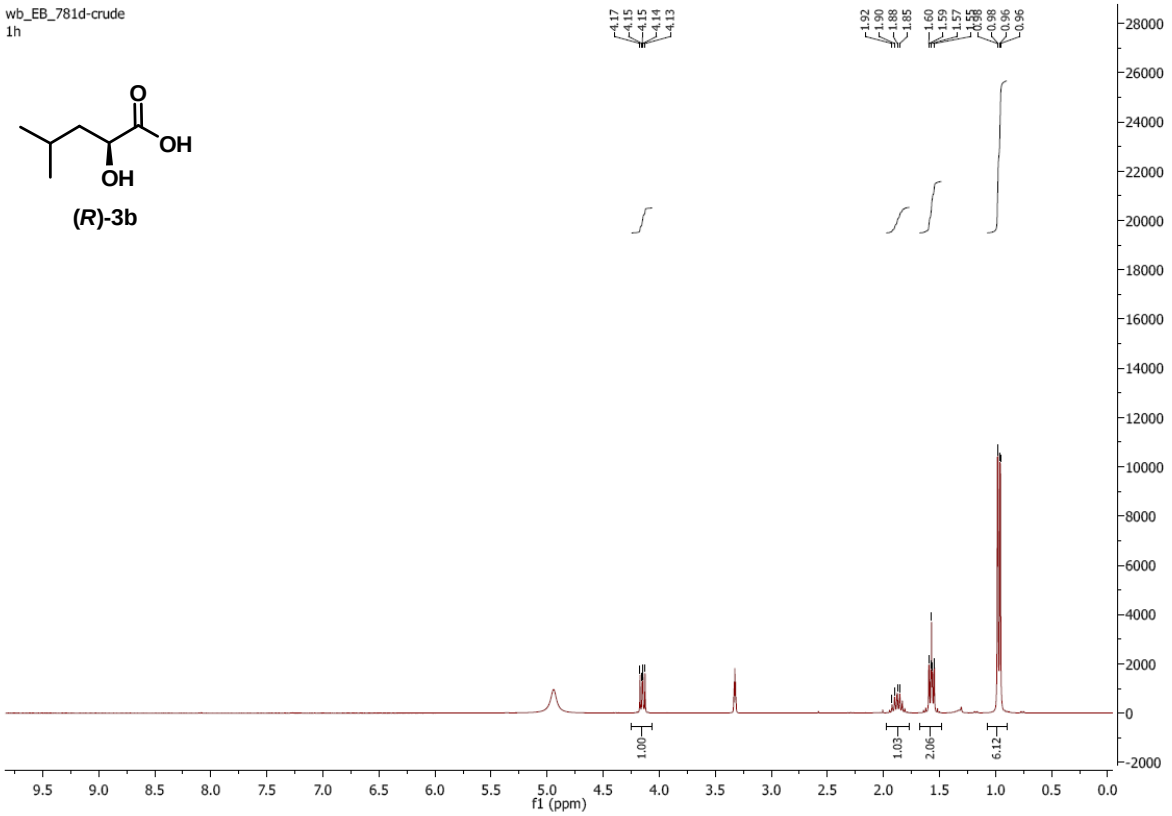
(*R*)-3c in 98% ee (D-Hic catalyzed reduction)

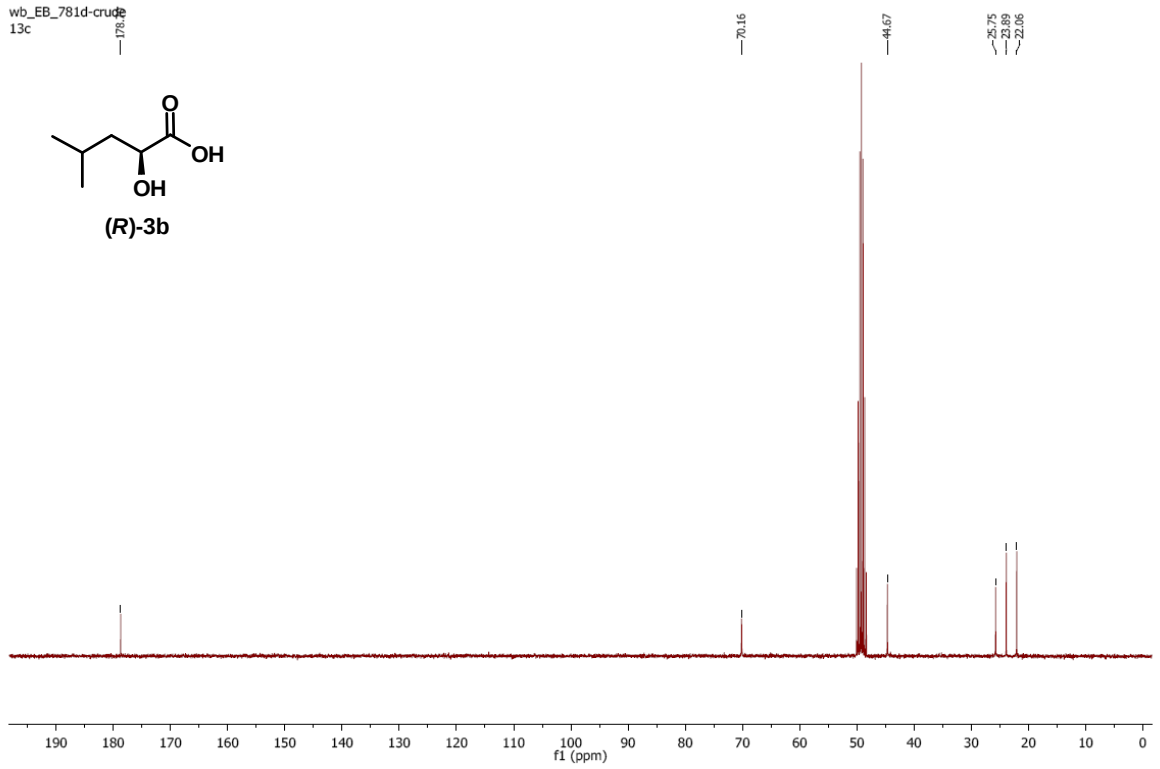


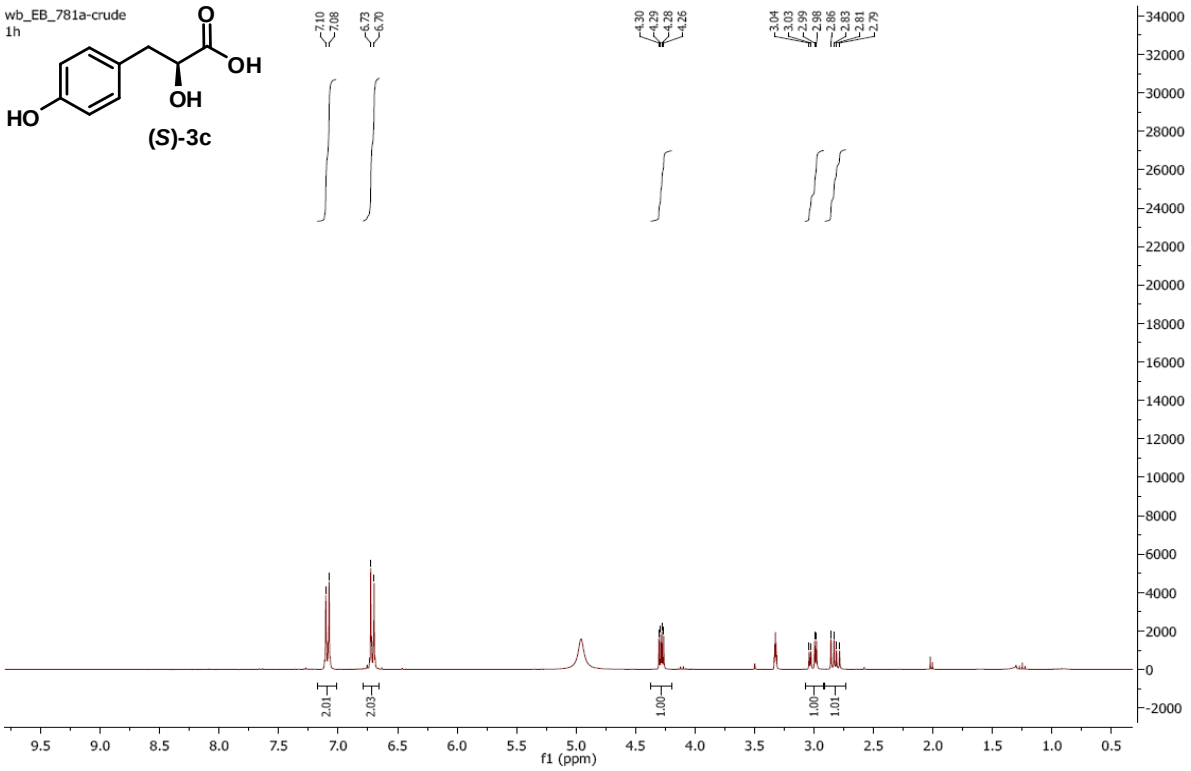
12. NMR spectra

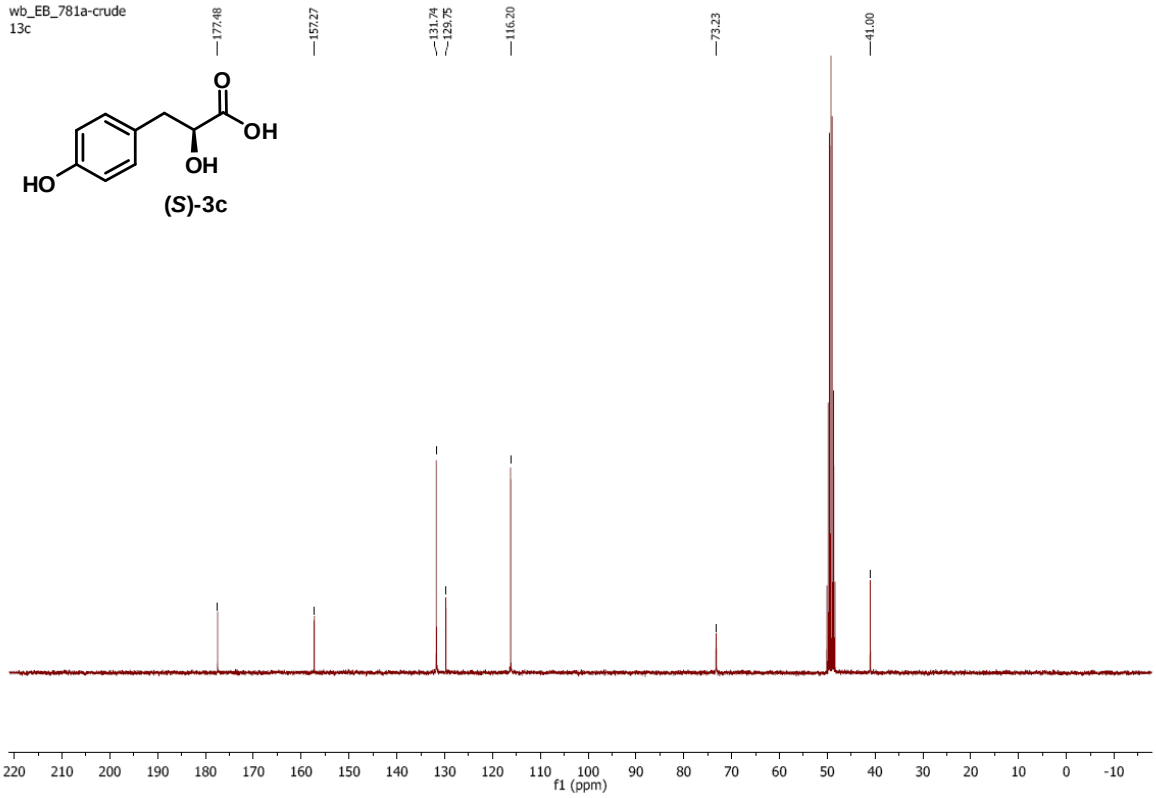





wb_EB_781d-crude
1h
O
OH
OH
(R)-3b
4.17
4.15
4.15
4.14
4.13
1.92
1.90
1.88
1.85
1.60
1.59
1.57
1.55
0.98
0.98
0.96
0.96
1.00
1.03
2.06
6.12
f1 (ppm)


wb_EB_781d-crude
13c
178.8
70.16
44.67
25.75
23.89
22.06
O
OH
OH
(R)-3b
f1 (ppm)


wb_EB_781a-crude
1h
(S)-3c
7.10
7.08
6.73
6.70
4.30
4.29
4.28
4.26
3.04
3.03
2.99
2.98
2.86
2.83
2.81
2.79
2.01
2.03
1.00
1.00
1.01
f1 (ppm)


wb_EB_781a-crude
13c
(S)-3c
177.48
157.27
131.74
129.75
116.20
73.23
41.00
f1 (ppm)

References

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[2] D. G. Gibson, L. Young, R. Y. Chuang, J. C. Venter, C. A. Hutchison, H. O. Smith, *Nat. Methods*, 2009, **6**, 343-345.
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