Supporting Information

Unlocking Asymmetric Michael Additions in an Archetypical Class I Aldolase by Directed Evolution

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General information

All commercially available chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) or TCI Europe N.V. (Zwijndrecht, Belgium) unless otherwise stated. Organic solvents were purchased from Biosolve (Valkenswaard, The Netherlands) and Sigma-Aldrich Chemical Co. Oligonucleotides were purchased from Eurofins Scientific (Ebersberg, Germany) and Biolegio B.V. (Nijmegen, The Netherlands). Spectrophotometric measurements were performed on a V-650 or V-660 spectrophotometer from Jasco (IJsselstein, The Netherlands), and measurements in a 96-well format were performed on a SPECTROstar Omega plate reader (BMG LABTECH, Isogen Life Science, de Meern, The Netherlands). HPLC was performed with a Shimadzu LC-10AT HPLC with a Shimadzu SPD-M20A diode array detector. GC-MS analysis was performed on a Shimadzu GC-MS-QP2010 SE. NMR spectra were recorded on a Bruker DRX-500 (500 MHz) spectrometer at the Drug Design laboratory of the University of Groningen. Chemical shifts (δ) are reported in parts per million (ppm) and are referenced to CHCl₃ (δ = 7.26 ppm).
Synthesis of α,β-unsaturated aldehyde 1f

The synthesis of α,β-unsaturated aldehyde 1f was performed according to a (slightly modified) previously reported procedure. The 1H NMR spectroscopic data of 1f are in agreement with previously reported data.

Synthesis of racemic reference compounds 3a-k

Racemic 3a-j, which served as reference compounds in chiral-phase HPLC analysis, were synthesized according to a (slightly modified) reported literature procedure by using pyrrolidine instead of a chiral organocatalyst. Racemic 3k was synthesized using the same procedure with an equimolar amount of (S)- and (R)-(+)-α,α-diphenyl-2-pyrrolidinemethanol trimethylsilyl ether as a catalyst. The 1H NMR spectroscopic data of racemic 3a-k are in agreement with previously reported data. Typically, the aldehyde functionality of compounds 3a-j was reduced (red.) in situ with NaBH₄ to the corresponding alcohol before chiral-phase HPLC analysis.
Screening of wild-type DERA for Michael-type additions

The activity of wild-type DERA from *Escherichia coli* for the Michael-type addition of cinnamaldehyde 1a and nitromethane 2 was screened in analytical scale reactions. 17.4 μM wild-type DERA was incubated in 20 mM sodium phosphate buffer, pH 6.5 supplemented with 3 % v/v DMSO, 2 mM cinnamaldehyde, and 20 mM nitromethane at room temperature in a final reaction volume of 1 ml. To avoid non-enzymatic background reactions at higher pH values, the enzymatic assay was performed at pH 6.5. After 30 h, the reaction mixture was extracted with 300 µl ethyl acetate (EtOAc) and analyzed by GC-MS (Figure S1a). Under otherwise identical conditions, reactions were performed with DERA K167L and without the addition of enzyme. The enantiomeric ratio of 3a obtained from the reaction catalyzed by wild-type DERA was determined by GC analysis using a ChiralDex G-TA column (30 m × 0.25 mm, df 0.12 μm, Astec) (70 °C to 170 °C at 10 °C/min; 170 °C, 25 min, 2.18 ml/min). Flame ionization detection: \( t_R: R-3a = 25.4 \text{ min}, S-3a = 25.9 \text{ min} \), led to the following enantiomeric ratios (R:S): racemic \( 3a = 50:50 \) and enzymatic \( 3a = 96:4 \) (Figure S1b). The absolute configuration was assigned based on previously published data.⁷
Figure S1. a) GC-MS chromatogram of reference compound 3a and reactions performed without enzyme, with knockout mutant DERA K167L and wild-type DERA (DERA-wt). b) Chiral GC chromatogram of racemic and enzymatically (wild-type DERA) obtained 3a.
Error-prone PCR (epPCR)

The gene coding for *E. coli* DER*Α* with a C-terminal His-tag was PCR amplified by error-prone PCR (epPCR, cloning primers: AKUpET20bDERA_fwd and AKUpET20bDERA_rev, Table S1) with GoTaq DNA polymerase (Promega Corporation, Madison, WI, USA) using a previously reported procedure. The resulting linear epPCR fragments and the vector pET20b(+) or pET26b(+) were digested with NdeI and XhoI restriction endonuclease (Thermo Fisher Scientific, Waltham, MA, USA), the vector backbone was dephosphorylated, the DNA was purified using a PCR purification kit (Macherey-Nagel, Düren, Germany) and ligated using T4 DNA ligase (Thermo Fisher Scientific, Waltham, MA, USA). The ligation product was purified using a PCR purification kit and used to transform electrocompetent *E. coli* DH5α. Transformants were selected by outgrowth in LB medium, supplemented with 100 µg/ml ampicillin (first round) or 30 µg/ml kanamycin (round 2-11), the plasmid DNA was isolated, and subsequently transformed into *E. coli* BL21(DE3). Before the screening, typically, the library quality was accessed by sequencing the plasmid DNA from several single colonies.

Staggered extension PCR (StEP)

Variants showing increased activity were further diversified by recombination using staggered extension PCR (StEP), followed by epPCR. Briefly, the genes were amplified in a standard PCR with Q5 High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA), StEP was performed with 65 cycles and 5 s annealing/elongation at 55 °C with GoTaq DNA polymerase. The obtained DNA product was used as a template for epPCR, and the gel-purified (Macherey-Nagel, Düren, Germany) DNA library genes were digested and ligated into cut pET26b(+), as described above.
Cassette mutagenesis

During the 9th round of directed evolution, cassette mutagenesis with spiked oligonucleotides was employed to target loops 1, 2, 6, and 7 of the inner core of DERA. The first half of the gene was amplified in a standard PCR using cloning primer AKUPET20bDERA_fwd and a backward spiked oligonucleotide primer (e.g. AKU-DERA15-25r), while the second part of the gene was amplified using AKU-pET20bDERA_rev and the forward spiked oligonucleotide primer (e.g. AKU-DERA15-25f). The obtained DNA was purified from a 0.9 % w/v agarose gel using a commercial gel-purification kit, and the two gene fragments were mixed in equimolar amounts to serve as a template for an overlap extension PCR using primers AKU-pET20bDERA_fwd and AKU-pET20bDERA_rev. The assembled DNA was digested with NdeI and XhoI, gel purified, and ligated into cut pET26b(+), as described above.

Site-saturation and site-directed mutagenesis

During the random mutagenesis rounds, specific active site residues were targeted with either site-saturation or site-directed mutagenesis. Site-saturation mutagenesis or single DNA codon substitutions were introduced into the DERA gene using the QuickChange technology. For the PCR reactions, pET26b(+) containing the DERA gene served as a template. The whole length plasmid was amplified using Q5 High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA) in a 50 μl reaction volume. The following PCR program was used: 98 °C, 30 s (initial denaturation), followed by 18 cycles of 98 °C for 10 s, 60 °C (modified according to the primers Tm) for 30 s, 72 °C for 3 min, and a final elongation step at 72 °C for 5 min. Subsequently, the PCR product was purified, and 1 μl of DpnI (Thermo Fisher Scientific, Waltham, MA, USA) was added to the reaction mixture and incubated at 37 °C for 1 h. The obtained DNA was used to transform E. coli DH5α cells, which were spread on LB agar plates supplemented with 30 μg/ml kanamycin. After outgrowth overnight, the colonies were resuspended in LB medium and the plasmid DNA isolated and used to transform E. coli BL21(DE3).
### Table S1. Primers used for library construction.

| Type | Round | Name                  | Sequence                                      |
|------|-------|-----------------------|----------------------------------------------|
| CP   | -     | AKU-pET20bDERA_fwd   | TTGTTTA ACTTTAAGGAAGGAGATATACATATG          |
| CP   | -     | AKU-pET20bDERA_rev   | GGTGGTGGTGCTGAG                            |
| SSM  | 4     | AKU-DERA52S-C47NNKf   | CGGCAATACCGCCGTATC CnnKATCTATCTCCGATCTATCC  |
| SSM  | 4     | AKU-DERA52S-C47NNKr   | GGA TAGACGCGAGATATMNNGAAGGCTGGATGAAATGCCC   |
| SSM  | 6     | AKU-DERA-T170NNKf     | CGAACCTCATCAAAACCTCTC TNNKGGTAAGGCTGGATGACG |
| SSM  | 6     | AKU-DERA-T170NNKr     | GCGTTCACAGCCACTTTAC CnnKAGGTTTGGATGAAATGCCC |
| SSM  | 8     | AKU-DERA-18NNKf       | GTGTTTTGACGCAATCGGGAT CnnKGCTAGCTGATATGACG |
| SSM  | 8     | AKU-DERA-18NNKr       | CACTGAAATGGAAGGCTGGNKAACCCTGAATGCGACGACAC  |
| SSM  | 8     | AKU-DERA-22NNKf       | TTCTCGTGGTGGTCGCTMNN ATCCGGGGAAGGATGACG    |
| SSM  | 8     | AKU-DERA-22NNKr       | GGACCTGCACCCATGAATNNGKAGACACACCGAGAACAG    |
| SSM  | 8     | AKU-DERA-47NNKf       | GGA TAGACGCGAGATATMNNGAAGGCTGGATGAAATGCCC  |
| SSM  | 8     | AKU-DERA-47NNKr       | CGGCAATACCGCCGTATC CnnKATCTATCTCCGATCTATCC  |
| SSM  | 8     | AKU-DERA-52NNKf       | TTTTTGACGCAATCGGGATMNN GAAGGCTGGATGAAATGCCC |
| SSM  | 8     | AKU-DERA-52NNKr       | GCTACGATATCTATCTCTCGC CnnKATCCCGGTTGCTGCAAAACA  |
| SSM  | 8     | AKU-DERA-197NNKf      | CCGTTAGTTTGAACCAAMNN TTTTTCTACGCCCATATCCGAGTC |
| SSM  | 8     | AKU-DERA-197NNKr      | GATCCGTTGATATGCGGCTAGAATANNKGGTGGGTTCACAATACGG |
| SSM  | 8     | AKU-DERA-202NNKf      | CCGACAGCCGCCTG MNN 5 TTGAACCAAGAGGTATTCCGAGGACCC |
| SSM  | 8     | AKU-DERA-202NNKr      | GGCGTAGAAATACCGGTTTGTTCAAANNKACCGGCGGCGCTGAC |
| SSM  | 8     | AKU-DERA-203NNKf      | AGTAGCAGCAGCGGC CnnNTAGTTTAGAAACCAAGGATATTCCGAGTC |
| SSM  | 8     | AKU-DERA-203NNKr      | GCGTAGAAATACCGGTTTGTTCAAATNNKGGCGGGCGGCTGACT |
| SSM  | 8     | AKU-DERA-213NNKf      | CTGCGATGCGGGAGATATTMMNN GCGATCTTTCCGCGATGACG |
| SSM  | 8     | AKU-DERA-213NNKr      | GCGTACTCGGAGATGCGGNKAAATATCTCGCACTCGACG     |
| SSM  | 8     | AKU-DERA-230NNKf      | CCAACGGGTAGTGGC CnnMN ATCTGCCAGTCAGCAGCAG    |
| SSM  | 8     | AKU-DERA-230NNKr      | CCGTGCTGCTGGCAGTTGGCAATANNKGGCGGGCGGCTGACT |
| SSM  | 8     | AKU-DERA-239NNKf      | CAACAGCCCTCCGAGCMNN GGAAGGACCAAAACCCGAGTA    |
| SSM  | 8     | AKU-DERA-239NNKr      | TACCGCTTTTGTGGCTTCC CnnKCTGCGTGGCAGCGCGTGTG |
| SSM  | 8     | AKU-DERA-230NNKf      | CCAACGGGTAGTGGC CnnMN ATCTGCCAGTCAGCAGCAG    |
| SSM  | 8     | AKU-DERA-239NNKr      | TACCGCTTTTGTGGCTTCC CnnKCTGCGTGGCAGCGCGTGTG |
| SSM  | 8     | AKU-DERA-19NNKf       | CGGTGCTGCTGGCAGTTGGCAATANNKGGCGGGCGGCTGACT |
| SSM  | 8     | AKU-DERA-19NNKr       | GAAATTTAGGAACCTGGATCC CnnKCTGAGATGGCGAGACCCG |
| SSM  | 8     | AKU-DERA-166NNKf      | CACTTTACCAGGTAGGTTTMMNN GAAATGGCGCCACCGCTGTTGAT |
| SSM  | 8     | AKU-DERA-166NNKr      | ATCAAAGCAGGGTGCGGACTTCC CnnKAAACCTCAGGGTAAAACTG |
| SSM  | 8     | AKU-DERA-171NNKf      | CGTCGGCCACAGCCACTTTMMNN GATTAGAGGTTTGGATGAAATC |
| SSM  | 8     | AKU-DERA-171NNKr      | GACTTCATCAAACCTCTACC CnnKAAAGTGCGCTGGAACCGACG |
SSM 8 AKU-DERA-172NNKf GCGTCGCGTTCACAGCCACMNNACCGGTAGAGGTTTTGATG
SSM 8 AKU-DERA-172NNKr CATCAAAACCTCTACCGGTNNKGTGGCTGTAACGCCGACGC
SSM 8 AKU-DERA-185NNKf ATACCCGATCATCTCCATMNNGATGCCGCGCTTTCCGG
SSM 8 AKU-DERA-185NNKr CCGGAAAGCGCGCGCATCNNKATGGAAGTATCGTGAT
SSM 8 AKU-DERA-200NNKf CGCACCGCCCGTTAGTTTMNNCAACCGGATTTTCTACGC
SSM 8 AKU-DERA-200NNKr GCGTAGAAACATCGTTGTTNNKAAACTAACGGGCACGC
SSM 8 AKU-DERA-236NNKf GACGGCCGGAGCMNNAAAGCGGTAGTGAGCGCTATCTG
SSM 8 AKU-DERA-236NNKr CAGATACCGTCATACCCCGTTMNNKGTCCGCGGCTGC
SSM 8 AKU-DERA-238NNKf CAGATACCGTCGCTTCCATMNNKGTGGCTGTGAACGCGACGC
SSM 8 AKU-DERA-238NNKr GCGTAGAAAAATCCGTTGGTNNKAAACTAACGGGCACGC
CM 9 AKU-DERA15-25f ATGGACCTGTCCACCCTGAATGGCGACTACACC  
CM 9 AKU-DERA15-25r GGGTGGACAGGTCCAT  
CM 9 AKU-DERA44-51f GCCGCTATCAGTATCTATCGCTCT  
CM 9 AKU-DERA44-51r GATAGATACCTGATACGCCG  
CM 9 AKU-DERA164-176f ATGGACCTGTCCACCCTGAATGGCGACTACACC  
CM 9 AKU-DERA164-176r GGGTGGACAGGTCCAT  
CM 9 AKU-DERA198-208f GTTGGTTTCAAAGTGACGGGCGTGCGT  
CM 9 AKU-DERA198-208r CCGTACCTTGAAACCCAC  
SSM 11 AKU-DERA49NNKr ATAGACCGGGMNNGATACCTGATACGCCGCTGTA  
SSM 11 AKU-DERA49NNKf CTATCAGTATC TNTCCCGATATTG  
SSM 11 AKU-DERA50NNKr AGAGCGMNNATAGATACCTGATAGCGCGGCTATCGC  
SSM 11 AKU-DERA50NNKf CGCCGCTATCAGTATCTATNNKCGCTCTATCCTCGAT  
SSM 11 AKU-DERA102NNKr AGCCGATGAAGTTMNNKGTGGTGTTCCCGTGAT  
SSM 11 AKU-DERA102NNKf GACGTCAACTCNNKCGCAGTACATTCG  
SSM 11 AKU-DERA104NNKr AGTTGACGTGNNKTCCGCTACCGCGCGCTGATG  
SSM 11 AKU-DERA104NNKf TGCTGAAAGTNKAACTACCGGCGAACTGAAA  
SSM 11 AKU-DERA105NNKr CCGGTTTCGATMNNCATTTCAGCAGTACATTCG  
SSM 11 AKU-DERA105NNKf TGCTGAAAGTNKAACTACCGGCGAACTGAAA  
SSM 11 AKU-DERA137NNKr TCGATGATCACMNNCAGCTACATTCGCTGATG  
SSM 11 AKU-DERA137NNKf ATGTACGTGNNKTCCGCTACCGCGCGCTGATG  
SSM 11 AKU-DERA139NNKr CCGGTTTCGATMNNCATTTCAGCAGTACATTCG  
SSM 11 AKU-DERA139NNKf TGCTGAAAGTNKAACTACCGGCGAACTGAAA  
SSM 11 AKU-DERA141NNKr GTTCGCGGTMNNGATGATCCTTTCAGCAGTACATTCG  
SSM 11 AKU-DERA141NNKf AAAGTGATACCTNNKACCGCGAACTGAAACGGACGA  
SSM 11 AKU-DERA142NNKr GTTCGCCMNNTTCGATGATCCTTTCAGCAGTACATTCG
| SSM   | AKU-DERA142NNKf | CTGCTGAAAGTGATCATCGAAANNKGCCGAACCTGAAAGAC |
|-------|----------------|------------------------------------------|
| SSM   | AKU-DERA168NNKr | GTTAGAMNNTTTGATGAAATCCGCACCCCGCTTTGAT    |
| SSM   | AKU-DERA168NNKf | GTGCGGACCTCATCAAANNKCTACCGGTGTTTTAGGG    |
| SSM   | AKU-DERA169NNKr | ACTAAACCGGTMAANNGGTTTGTGTAAGTCCGCCACC    |
| SSM   | AKU-DERA169NNKf | CTCATCAAAAAANNKACCCTGTTTAGTGCTGTA       |
| SSM   | AKU-DERA21NNKr | TGATAGTGCCMNNGAGGTGGACAGGTCC             |
| SSM   | AKU-DERA21NNKf | GTCCACCGTGNNGCGACTACACCGAGC              |
| SSM   | AKU-DERA237NNKr | CCGGAMNNACCAAGCGGTAGTGACCGCGC           |
| SSM   | AKU-DERA237NNKf | CACTACCGCTTTGTNNKTCGCCCTGCT             |
| SSM   | AKU-DERA176NNKr | CCGCGTCGCNNACAGCCACTAAACCG              |
| SSM   | AKU-DERA176NNKf | AGTGGGCTGTTNNKGGCGACCCGGAAAGCG          |
| SSM   | AKU-DERA207NNKr | TTCCGCAGTMMNNCGCGCGCCCGTCA              |
| SSM   | AKU-DERA207NNKf | GGGCCGCGCNNKACTGCGGAAGATGC              |
| SSM   | AKU-DERA201NNKr | GCCCCGTCACMNNGAAACCAAAGGATTITTT         |
| SSM   | AKU-DERA201NNKf | GGGTGTTTNNKGTAGCGGGCGCCGGC             |
| SSM   | AKU-DERA200NNKr | CCGTGCATTTTMNNACCAACGGGATTITTTACTCGC   |
| SSM   | AKU-DERA200NNKf | AAATCCGTGTTTNNKAAAGTGACCGGGCGCCGCG     |
| SDM   | AKU-DERAS18Tr  | CATTACGGGTTGCTAGTTCTACATCAATT          |
| SDM   | AKU-DERAS18Tf  | TGAATGGCAGTAGCTACCGCTGTAAATG            |
| SDM   | AKU-DERAG22Dr  | CGGTGTAATTACCTACTACGGGTGACAGG          |
| SDM   | AKU-DERAG22Tf  | CACCCCTGAATGATGACTACACCGACG           |
| SDM   | AKU-DERAY24Dr  | TTCTGTCGATTGCTGCGTCTACGGGTTGAC          |
| SDM   | AKU-DERAY24Tf  | TGAATGGCAGTAGCTACCGCTGTAAATG            |
| SDM   | AKU-DERAS47Cr  | GCGAGGATAGATCAGATAGCCGGGCGGTATTGCC     |
| SDM   | AKU-DERAS47Tf  | ACCGCGCGATCTCTCGATCTCATCTACG           |
| SDM   | AKU-DERAS52Fr  | GCAATCGGGAATAACGCGAGGATAGATCGT         |
| SDM   | AKU-DERAS52Tf  | TCTATCCCTGCTTTATCCCGGATTGCTCGA         |
| SDM   | AKU-DERAL172Kr | TTCACAGCCACTTTACCGGTAGAGGTITTGA        |
| SDM   | AKU-DERAL172Tf | CCTCTACCGGTAAAGTGGCGTGTGACCGGAC        |
| SDM   | AKU-DERAS197Tr | GAAACCAACGGGATTITTTTCTACGCCCATATCAC    |
| SDM   | AKU-DERAS197Tf | TGGGCGTAGAAAAACCGGTGTTTCAAAATG         |
| SDM   | AKU-DERAV202Pr | GCCCCGTGTTTGAACCAACCGGATTITTTT         |
| SDM   | AKU-DERAV202Pf | ATCCGTTGTTTCAACCGACGGCGGCC             |
| SDM   | AKU-DERAT203Ar | GCGCGCCGCCACTTTGAAACCA                  |
| SDM   | AKU-DERAT203Af | TTCAAAGTGCGGGGCCGGCGCGCG               |
| SDM           | Primers          | Sequences                      |
|---------------|------------------|--------------------------------|
|               | AKU-DERAA206Vr   | AGTACGCAGCGCAGCGCCT            |
|               | AKU-DERAA206Vf   | GCCGCCGTCGACTGCGG              |
|               | AKU-DERAG239Sr   | TGGCCAGCGGCTGGAAGCAGCAAAAG     |
|               | AKU-DERAG239Sf   | GGGCTTCCAGGCTGGAAGGCC          |
|               | AKU-DERAK167Lr   | ACCGTTAGAGCTAGGTGACCTGCAGCAAGC|
|               | AKU-DERAK167Lf   | GCGGACTTCATCCTGACCTACGGGTATTAGT|

Note: cassette mutagenesis (CM), cloning primers (CP), site-saturation mutagenesis (SSM), site-directed mutagenesis (SDM); ° A = (ACTG 92.5:2.5:2.5:2.5), C = (CTAG 92.5:2.5:2.5:2.5), T = (TCAG 92.5:2.5:2.5:2.5) and G = (GTCA 92.5:2.5:2.5:2.5).
**Screening**

Before measuring the activity of DERA in cell-free extract (CFE), an agar plate pre-screening was performed according to previously reported procedures.11 Electropotent E. coli BL21(DE3) cells were transformed with library DNA and plated on LB agar plates supplemented with either 100 µg/ml ampicillin (first round) or 30 µg/ml kanamycin (round 2-11) and 0.2 % w/v lactose. After incubation at 37 °C for approximately 16 h, the agar plates were stored at room temperature for 3-5 h. A heated solution (~ 60 °C) of 0.6 % w/v agarose, 0.5 % v/v DMSO and 0.1 mg/ml 2-hydroxycinnamaldehyde in 10 mM sodium phosphate, pH 7.3 was poured on the agar plates. The plates were incubated for 10 min at room temperature to allow the agarose to solidify. Typically, colonies with red staining appeared after 1 min, and approximately 800-1400 stained colonies per library were picked with sterile toothpicks, which were used to inoculate 1 ml LB medium supplemented with 100 µg/ml ampicillin (first round) or 30 µg/ml kanamycin (round 2-11) and 0.2 % w/v lactose in 96-deep well plates (96-well Masterblock, Greiner Bio-one, Kremsmünster, Austria). Two wells were inoculated with a clean, sterile toothpick (negative control), and six wells were inoculated with the most active variants from the previous mutagenesis rounds in triplicate as a positive control and reference. The 96-deep well plates were sealed with sterile gas-permeable seals (Breathe-Easy, Diversified Biotech, Boston, MA, USA) and incubated with shaking at 200 rpm for 6 h at 37 °C, followed by 30 °C overnight. 30 µl of the bacterial culture were mixed with a sterile solution of 30 µl 50 % w/v glycerol and stored for later use at -80 °C. The remainder of the bacterial culture was harvested and resuspended in 50 µl BugBuster (EMD Biosciences, Madison, WI, USA) containing 0.5 µl/ml benzonase. After 20 min of incubation at room temperature under vigorous shaking, the CFE was obtained by clearing the lysates by centrifugation. Before centrifugation, the lysed cells were diluted with 50 µl to 1 ml 20 mM HEPES, 100 mM NaCl, pH 6.5. For the screening, the final reaction mixture for monitoring the addition of nitromethane 2 to cinnamaldehyde 1a consisted of 40 % v/v CFE, 0.25 mM 1a, 20 mM 2, 3 % v/v DMSO in 20 mM HEPES, 100 mM NaCl, pH 6.5, in 100 µl final volume. The reaction was performed in a 96-well plate (UV-star F-bottom microplate, Greiner Bio-one, Kremsmünster, Austria), and the reaction progress was followed in a plate reader by measuring the decrease in absorbance at 290 nm. The variants with the highest increase in activity compared to the reference were picked from the glycerol stock for plasmid isolation, DNA sequencing, and further mutagenesis.
Throughout the directed evolution process, the following adjustments were made to compensate for the increased enzyme activity in the CFE:

**Round 1-11:** The CFE was diluted stepwise from 50 µl up to 1 ml with 20 mM HEPES, 100 mM NaCl, pH 6.5.

**Round 2:** The expression system was changed from pET20b to pET26b for better expression control.

**Round 8:** The volume of CFE in the reaction was lowered to 20 % v/v.

**Round 9:** The 96-well Masterblocks contained 600 µl LB medium supplemented with 0.2 % w/v lactose, 0.05 % w/v glucose, and 30 µg/ml kanamycin; the 96-well Masterblocks were inoculated with 20 µl from an overnight culture, and the cells were grown for 2 h at 37 °C, followed by 25 °C overnight.

**Round 10:** 0.3 mM 1a and 100 mM 2 were used in the activity screening.
Directed evolution

Figure S2 gives a schematic overview of the directed evolution program to optimize wild-type *E. coli* DERA for the Michael addition of 2 to 1a. Amino acid substitutions of distinctive purified DERA variants are listed in Table S2.

**Round 1:** Wild-type DERA was used as a template to create a library by epPCR. Approximately 50,000-75,000 colonies were pre-screened with 2-hydroxycinnamaldehyde on agar plates. Approximately 50 positive hits from this pre-screening were subsequently screened by UV-vis for the addition of 2 to 1a.

**Round 2:** The DNA of 8 variants from round 1, which showed improved activities compared to wild-type DERA, were recombined by a StEP, and the DNA product served as the template in an epPCR. Approximately 600 colonies from the resulting library were screened by UV-vis for the addition of 2 to 1a.

**Round 3:** The DNA of 17 variants from round 2, which showed improved activities, were recombined by a StEP, and the DNA product served as the template in an epPCR. Approximately 1000 colonies from the resulting library were screened by UV-vis for the addition of 2 to 1a. Analysis of selected variants in an UV-vis based activity assay with purified proteins yielded DERA-34H1 and DERA-38D10, which showed similar enhanced activities compared to wild-type DERA.

**Round 4:** The DNA of DERA-34H1 and DERA-38D10 was recombined by a StEP, and the DNA product served as the template in an epPCR. Approximately 1000 colonies from the resulting library were screened by UV-vis for the addition of 2 to 1a. In a focused library, position 47 was targeted in DERA-34H1 and DERA-38D10 with SSM using NNK degenerate codons. For each focused library, approximately 90 colonies were screened.

**Round 5:** The DNA of 11 variants from round 4, which showed improved activities compared to variants from the previous round, were recombined by a StEP, and the DNA product served as the template in an epPCR. Approximately 1400 colonies from the resulting library were screened by UV-vis for the addition of 2 to 1a. Analysis of selected variants in an UV-vis based activity assay with purified proteins yielded DERA-43C10, DERA-58B1, and DERA-59D3, which showed similar enhanced activities compared to wild-type DERA.
Round 6: The DNA of DERA-43C10, DERA-58B1, and DERA-59D3 were recombined in one library by a StEP, and the DNA product served as a template to construct a second library by epPCR. Approximately 1400 colonies from the resulting libraries were screened by UV-vis for the addition of 2 to 1a. In a focused library, position 170 was targeted in DERA-43C10 and DERA-58B1 with SSM using NNK degenerate codons. About 90 colonies were screened for each focused library.

Round 7: The DNA of 15 variants from round 6, which showed improved activities compared to variants from the previous round, were recombined by a StEP, and the DNA product served as the template in an epPCR. Approximately 1800 colonies from the resulting library were screened by UV-vis for the addition of 2 to 1a. Analysis of selected variants in an UV-vis based activity assay with purified proteins yielded DERA-713C12.

Round 8: The DNA of variant DERA-713C12 served as a template for site-saturation mutagenesis with NNK degenerate codons targeting the following positions: 18, 19, 22, 47, 52, 166, 171, 172, 185, 197, 200, 202, 203, 213, 230, 236, 238 and 239. The focused libraries were pooled, and a total of 900 colonies were screened by UV-vis for the addition of 2 to 1a. Analysis of selected variants in an UV-vis based activity assay with purified proteins yielded DERA-87B11.

Round 9: The DNA of 6 variants from round 8, which showed improved activities compared to variants from the previous round, were recombined by a StEP, and approximately 700 colonies of the resulting library were screened by UV-vis for the addition of 2 to 1a. Variant DERA-87B11 served as a template for cassette mutagenesis with spiked oligonucleotides, which targeted loops 1 (position 15 - 25), 2 (position 44 - 51), 6 (position 164 - 176), and 7 (position 198 - 208), of the inner core of DERA. A total of about 2800 colonies were screened from these libraries by UV-vis for the addition of 2 to 1a and yielded variants DERA-964C8, DERA-966F5, DERA-9610C8, and DERA-971B3.

Round 10: The DNA of variants DERA-964C8, DERA-966F5, DERA-9610C8, and DERA-971B3 were recombined by a StEP, and the DNA product served as the template in an epPCR. Approximately 2000 colonies from the resulting library were screened by UV-vis for the addition of 2 to 1a. Analysis of selected variants in an UV-vis based activity assay with purified proteins yielded DERA-971B3.
proteins yielded DERA-1111H11, which showed enhanced activity compared to the variant from the previous round.

**Round 11:** The DNA of variant DERA-1111H11 served as a template for site-saturation mutagenesis with NNK degenerate codons targeting the following positions: 21, 49, 50, 102, 104, 105, 137, 139, 141, 142, 168, 169, 176, 200, 201, 207 and 237. For each focused library, about 90 colonies were screened by UV-vis for the addition of 2 to 1a. Analysis of selected variants in an UV-vis based activity assay with purified proteins yielded the best variant DERA-MA.
**Figure S2.** Schematic overview of the directed evolution campaign to optimize DERA for the Michael addition of 2 to 1a. epPCR: error-prone PCR, StEP: staggered-extension PCR, SSM: site-saturation mutagenesis.
**Table S2.** Amino acid substitutions compared to wild-type DERA of selected DERA variants. Amino acid substitutions that newly emerged and are retained in the final variant are highlighted in **bold**. *Variants used in Figure 1b and c in the main text.*

| Round | Name         | Amino acid substitutions compared to wild-type DERA                                      |
|-------|--------------|-------------------------------------------------------------------------------------------|
| 1     | DERA-11C2*   | D22G, F52S, T74S, I159V, **T197S**                                                       |
| 3     | DERA-34H1*   | D22G, **C47S**, F52S, T74S, A230T, **S239G**                                               |
| 3     | DERA-38D10   | D22G, **D24Y**, F52S, T197S, P202L, **A203T**                                              |
| 4     | DERA-43C10   | D22G, C47S, F52S, T197S, P202L, A203T, A230T, S239G                                     |
| 5     | DERA-58B1*   | **T18S**, D22G, C47S, F52S, T197S, P202L, A203T, A230T, S239G                           |
| 5     | DERA-59D3    | T2S, D22G, D24Y, C47S, F52S, T197S, P202L, A203T                                       |
| 7     | DERA-713C12* | T18S, D22G, D24Y, C47S, F52S, T197S, P202L, A203T, S239G                               |
| 8     | DERA-87B11*  | T18S, D22G, D24Y, C47S, F52S, T197S, **P202V**, A203T, S239G                           |
| 9     | DERA-964C8   | T18S, D22G, D24Y, C47S, F52S, K172R, T197S, P202V, A203T, S239G                         |
| 9     | DERA-966F5   | T18S, D22G, D24Y, C47S, F52S, K172I, T197S, P202V, A203T, S239G                         |
| 9     | DERA-9610C8  | T18S, D22G, D24Y, C47S, F52S, **K172L**, T197S, P202V, A203T, S239G                   |
| 9     | DERA-971B3   | T18S, D22G, D24Y, C47S, F52S, T197S, P202V, A203T, **V206A**, S239G                   |
| 10    | DERA-1111H11* | T18S, D22G, D24Y, C47S, F52S, **K172L**, T197S, P202V, A203T, **V206A**, S239G       |
| 11    | DERA-MA*     | T18S, D22G, D24Y, C47S, F52S, **T142S**, K172L, T197S, P202V, A203T, **V206A**, S239G |
Protein expression and purification

Individual DERA variants with a C-terminal His-tag were expressed and purified from *E. coli* BL21(DE3) using the pET20b(+) or pET26b(+) expression systems. Typically, 100 ml terrific broth medium, supplemented with the appropriate antibiotic (100 µg/ml ampicillin or 30 µg/ml kanamycin), was inoculated with 1 ml from an overnight culture. The cells were incubated at 37 °C, 200 rpm until ~ 0.5 OD (2-3 h) was reached, after which the expression was induced by adding 100 µM isopropyl-β-D-thiogalactopyranoside (IPTG), and the culture was further incubated overnight at 25 °C. After harvesting the cells, the pellet was resuspended in 15 ml 50 mM potassium phosphate, 20 mM imidazole, pH 7, and the cells lysed by ultrasonication. The lysate was cleared by centrifugation, Ni-sepharose (1 ml) was added to the lysate and incubated at 4 °C under slow rotation for 30 min. The Ni-sepharose and lysate mixture was loaded on a column and washed with 10 ml 50 mM potassium phosphate, 30 mM imidazole, pH 7, followed by elution with 3 ml 50 mM potassium phosphate, 300 mM imidazole, pH 7. The buffer was exchanged to 20 mM potassium phosphate, pH 7, by using a PD-10 sephadex G-25 gel-filtration column (GE Healthcare, Chicago, IL, USA). The protein concentration was determined by measuring the absorbance at 280 nm with the theoretical molecular weight and extinction coefficient calculated with ProtParm (ExPASy, https://web.expasy.org/protparam/). The protein solution was snap-frozen in liquid nitrogen and stored at -20 °C until further use.
Spectrophotometric measurement of enzyme activity

Michael addition activity

All spectrophotometric measurements were performed by following the decrease in absorbance of cinnamaldehyde 1a at 290 nm (ε = 23598 M⁻¹ cm⁻¹) after the addition of 100 mM nitromethane 2 in 20 mM HEPES, 100 mM NaCl, pH 6.5 and 3 % v/v DMSO at 29 °C (1 ml reaction volume). After the addition of 2, 300 µl of the reaction mixture was transferred into a 1 mm cuvette for the spectrophotometric analysis. The initial rates of the enzymatic reactions were corrected for the buffer-catalyzed background reaction.

For the activity comparison of DERA variants (main text Figure 1b), the initial rate of the reaction catalyzed by wild-type DERA or evolved DERA variants was determined using 5 µM purified enzyme and 1 mM cinnamaldehyde 1a. Measurements for wild-type DERA and DERA-MA were performed with two independent protein batches. Measurements for the other DERA variants were performed in duplicates from the same protein batch.

The apparent steady-state parameters k_{cat,app} and K_{M,app} were determined from reactions with 1 µM DERA-MA and 25 µM – 1 mM cinnamaldehyde 1a. The initial rates obtained from duplicate measurements of two independent protein batches were corrected for the buffer-catalyzed background reaction and fitted by non-linear regression to the Michaelis-Menten equation \( \frac{v_0}{[E]_0} = \frac{k_{cat,app}[cinnamaldehyde]}{K_{M,app}[cinnamaldehyde]} \) using SigmaPlot 14 (Figure S3).

To determine the buffer catalyzed background reaction rate, the depletion of cinnamaldehyde 1a (25 µM - 1mM) after the addition of 100 mM 2 was followed at 290 nm in 20 mM HEPES, 100 mM NaCl, pH 6.5, and 3 % v/v DMSO at 29 °C. The initial rates of three independent measurements were fitted to the pseudo-first-order rate equation \( v_0 = k_{buffer}[cinnamaldehyde] \) by non-linear regression using SigmaPlot 14 (Figure S4).
Figure S3. Michaelis-Menten plot for the addition of **2** to **1a** catalyzed by DERA-MA. The concentration of **2** was fixed at 100 mM and the concentration of **1a** varied between 25 µM – 1 mM. The measurement was performed in duplicates with purified enzyme from two independent protein batches. Error bars correspond to the calculated standard deviation.
Figure S4. Buffer catalyzed background reaction for the addition of 2 to 1a was determined in 20 mM HEPES, 100 mM NaCl, pH 6.5, and 3 % v/v DMSO at 29 °C. Error bars correspond to the standard deviation of three independent measurements.

Figure S5. Relative initial rate $v_0$ of the reaction catalyzed by DERA-MA variants normalized to the initial rate of the reaction catalyzed by DERA-MA. Initial rates were measured in duplicates with 5 μM DERA, 1 mM 1a and 100 mM 2 in 20 mM HEPES, 100 mM NaCl, pH 6.5 and 3 % v/v DMSO at 29 °C. Error bars correspond to the standard deviation of two measurements.
Retro-aldol activity

The retro-aldol activity of wild-type DERA and DERA-MA with 2-deoxy-D-ribose-5-phosphate as the substrate to produce acetaldehyde and D-glyceraldehyde-3-phosphate was measured in a coupled enzyme assay using α-Glycerophosphate Dehydrogenase-Triosephosphate Isomerase from rabbit muscle (GPD/TPI, G1881, Sigma-Aldrich, St. Louis, MO, USA).12,13 The final reaction mixture (0.8 ml) contained the following: 0.4 mM 2-deoxy-D-ribose-5-phosphate (Sigma-Aldrich, St. Louis, MO, USA), 0.3 mM NADH (abcr, Karlsruhe, Germany), 1 U/ml GPD/TPI, and 0.05 mg/ml DERA variant in 50 mM HEPES pH 6.8. The reaction progress was monitored at 20 °C by measuring the depletion in absorbance of NADH at 340 nm.

![Figure S6](image_url)

**Figure S6.** Reaction progress curves of the DERA wild-type and DERA-MA catalyzed retro-aldol reaction with the natural substrate 2-deoxy-D-ribose-5-phosphate. The reaction is monitored by following the consumption of NADH at 340 nm in a coupled enzyme assay with GPD/TPI.
Condensation of 1a with DERA-MA

The condensation of cinnamaldehyde 1a with DERA-MA was monitored by measuring the absorbance spectrum of 5 µM 1a in the presence of 25 µM DERA-MA or 25 µM DERA-MA K167L in 10 mM HEPES, 100 mM NaCl, pH 6.5 and 3 % v/v DMSO in a 1 cm quartz cuvette.

Figure S7. Absorbance spectrum of 5 µM cinnamaldehyde 1a in the presence of 25 µM DERA-MA or 25 µM DERA-MA K167L in 10 mM HEPES, 100 mM NaCl, pH 6.5, and 3 % v/v DMSO.
Covalent labelling of DERA-MA with 1a

To covalently label DERA with cinnamaldehyde 1a, 1 mg DERA-MA was incubated with 0.6 mM 1a in 20 mM HEPES, 100 mM NaCl, pH 6.5 and 3 % v/v DMSO at room temperature, 1 ml final volume. After 5 min, 200 µl 150 mM NaBH3CN in H2O were added, and the reaction was incubated for 2 h. The buffer was exchanged against 20 mM HEPES, 100 mM NaCl, pH 6.5 by using a centrifugal concentrator (Vivaspin 2, 5,000 MWCO, Sartorius, Göttingen, Germany). An aliquot of the protein solution was analyzed by ESI-MS. The remaining fraction was digested with endoproteinase Glu-C and a sample of the digestion product was analyzed by LC-MS/MS similar to previously published methods. As a control, the experiment was performed under otherwise identical experimental conditions but without adding cinnamaldehyde.

Figure S8. a) Mass spectrometry (ESI-MS) after chemical reduction of DERA-MA (calcd. 28643.71 Da, found 28643.67 Da); b) ESI-MS after chemical reduction of DERA-MA in the presence of 1a (calcd. 28760.78 Da, found 28761.03 Da). The lowered protein mass (major peak) observed in both spectra is caused by removal of the N-terminal methionine (-132 Da).
Table S3. Peptide mapping data of DERA-MA after digestion with Glu-C. For clarity, only peptides with posttranslational modification (PTM) are listed.

| Peptide | -10lgP | Mass       | Length | ppm | m/z         | z   | RT     | Scan  | Area Sample 2 | Start | End | PTM     |
|---------|--------|------------|--------|-----|-------------|-----|--------|-------|----------------|-------|-----|---------|
| E.VDVVFPYRALM(+15.99)AGNE.Q | 134.58 | 1695.829   | 15     | -0.1| 848.9099    | 2   | 42.61  | 13951 | 9.72E+08       | 101   | 115 | Oxidation (M) |
| M.TDLKASSRLAUKLM(+15.99)D.L | 122.52 | 1676.913   | 15     | -5.3| 839.4471    | 2   | 31.92  | 8911  | 3.65E+08       | 2     | 16  | Oxidation (M) |
| M(+15.99)TDLKASSRLAUKMD.L | 105.32 | 1807.954   | 16     | -12.1| 904.96      | 2   | 34.48  | 10146 | 1.33E+08       | 1     | 16  | Oxidation (M) |
| E.VDVVFPPYRALM(+15.99)AGNEQVGF.D.L | 97.28  | 2242.073   | 20     | -0.6| 1122.027    | 2   | 45.9   | 15548 | 3.43E+07       | 101   | 120 | Oxidation (M) |
| E.VDVVFPYRALM(+15.99)AGNEQV.G | 91.18  | 1922.956   | 17     | -0.2| 962.4709    | 2   | 43.38  | 14484 | 9.34E+06       | 101   | 117 | Oxidation (M) |
| E.VDVVFPPYRALM(+15.99).A | 88.16  | 1324.685   | 11     | 3.2 | 663.3422    | 2   | 41.96  | 13692 | 3.33E+07       | 101   | 111 | Oxidation (M) |
| MTDLKASSRLAUKLM(+15.99)D.L | 80.36  | 1807.954   | 16     | -0.8| 603.6492    | 3   | 32.8   | 9306  | 1.68E+08       | 1     | 16  | Oxidation (M) |
| M(+15.99)TDLKASSLR.A | 73.67  | 1136.586   | 10     | 0.1 | 569.292     | 2   | 22.75  | 4663  | 8.00E+06       | 1     | 10  | Oxidation (M) |
| M(+15.99)TDLKASSRLA | 68.14  | 1448.802   | 13     | -1.6| 483.9335    | 3   | 25.64  | 6026  | 3.35E+06       | 1     | 13  | Oxidation (M) |
| M(+15.99)TDLKASSLR | 62.77  | 982.4849   | 9      | 0.4 | 491.2427    | 2   | 28.38  | 7261  | 1.72E+07       | 1     | 9   | Oxidation (M) |
| E.VRDMM(+15.99)GVEK.V | 58.19  | 1148.586   | 10     | -0.4| 575.2916    | 2   | 19.45  | 3407  | 2.87E+07       | 188   | 197 | Oxidation (M) |
| E.VRDMM(+15.99)GVE.K | 53.79  | 933.4589   | 8      | -0.2| 467.7298    | 2   | 22.29  | 4436  | 4.71E+08       | 188   | 195 | Oxidation (M) |
| E.SARIMM(+15.99)E.V | 53.34  | 852.3834   | 7      | -6.9| 427.1898    | 2   | 22.91  | 4791  | 1.46E+09       | 181   | 187 | Oxidation (M) |
| E.SARIM(+15.99)ME.V | 48.58  | 852.3834   | 7      | -6  | 427.1902    | 2   | 26.36  | 6351  | 1.06E+09       | 181   | 187 | Oxidation (M) |
| E.SARIM(+15.99)ME.V | 44.39  | 868.3783   | 7      | 0.3 | 435.1902    | 2   | 19.45  | 3981  | 1.41E+08       | 181   | 187 | Oxidation (M) |
| E.VDVVFPPYRALM(+15.99)AGNEQVGF.DLVKACKEACAAAN.V | 44.12  | 3514.705   | 33     | 0.1 | 879.6707    | 4   | 42.21  | 13765 | 1.67E+07       | 101   | 133 | Oxidation (M) |
| M.TDLKASSRLAUKLM(+15.99)D.LST.L | 42.77  | 1978.077   | 18     | 10.2| 660.3633    | 3   | 44.14  | 14720 | 1.29E+06       | 2     | 19  | Oxidation (M) |
Table S4. Peptide mapping data of DERA-MA labelled with 1a after digestion with Glu-C. For clarity, only peptides with posttranslational modification (PTM) are listed.

| Peptide | -10lgP | Mass | Length | ppm | m/z | z | RT | Scan | Area Sample 1 | Start | End | PTM |
|---------|--------|------|--------|-----|-----|---|-----|-----|----------|------|------|-----|
| E.VDVVFPRALM(+15.99)AGNE.Q | 127.96 | 1695.829 | 15 | 2.4 | 848.9189 | 2 | 41.9 | 13210 | 5.95E+08 | 101 | 115 | Oxidation (M) |
| M.TDLKASSRALKLM(+15.99)D.L | 102.49 | 1676.913 | 15 | -1.7 | 839.4576 | 2 | 31.4 | 8264 | 9.87E+07 | 2 | 16 | Oxidation (M) |
| E.VDVVFPRALM(+15.99)A | 98.97 | 1324.685 | 11 | 2.5 | 963.3477 | 2 | 41.47 | 13576 | 3.86E+07 | 101 | 111 | Oxidation (M) |
| D.FIK(+116.06)TSTGLVAVNATPE.S | 92.57 | 1762.95 | 16 | 2.4 | 882.4796 | 2 | 41.87 | 13045 | 1.18E+08 | 165 | 180 | cinnamaldehyde |
| E.ISIKAGADFIK(+116.06)GTST.G | 92.26 | 1566.866 | 14 | -1.7 | 784.4343 | 2 | 39.93 | 12442 | 7.52E+08 | 157 | 170 | cinnamaldehyde |
| E.VDVVFPRALM(+15.99)AGNEQ.V | 91.39 | 1952.956 | 17 | 0.9 | 962.4807 | 2 | 42.88 | 13873 | 1.14E+07 | 101 | 117 | Oxidation (M) |
| E.VDVVFPRALM(+15.99)A.G | 89.63 | 1395.722 | 12 | -0.4 | 698.8641 | 2 | 42.92 | 13629 | 1.45E+07 | 101 | 112 | Oxidation (M) |
| M(+15.99)TDLKASSRALKLM.D.L | 89.31 | 1807.954 | 16 | 1.6 | 603.656 | 3 | 33.18 | 9638 | 5.67E+07 | 1 | 16 | Oxidation (M) |
| MTDLKASSRALKLM(+15.99)D.L | 89.27 | 1807.954 | 16 | 0.3 | 904.9792 | 2 | 32.78 | 8884 | 7.44E+07 | 1 | 16 | Oxidation (M) |
| G.ADFFK(+116.06)TSTGLVAVNATPE.S | 88.92 | 1949.015 | 18 | 5.1 | 975.514 | 2 | 44.55 | 14301 | 1.70E+06 | 163 | 180 | cinnamaldehyde |
| E.VDVVFPRALM(+15.99)AGNEQVGFD.L | 84.11 | 2242.073 | 20 | -0.4 | 1122.037 | 2 | 45.53 | 14874 | 4.49E+06 | 101 | 120 | Oxidation (M) |
| E.ISIKAGADFNG(+116.06)GTST.T | 81.7 | 1465.818 | 13 | 0.4 | 733.9124 | 2 | 39.5 | 12044 | 2.93E+07 | 157 | 169 | cinnamaldehyde |
| M(+15.99)TDLKASSRALK.X | 80.13 | 1320.707 | 12 | -4.6 | 441.2385 | 3 | 29.59 | 7408 | 1.90E+06 | 1 | 12 | Oxidation (M) |
| E.ISIKAGADFNG(+116.06)GTST.S | 74.24 | 1378.786 | 12 | 3.4 | 690.3987 | 2 | 39.3 | 11926 | 1.59E+07 | 157 | 168 | cinnamaldehyde |
| E.ISIKAGADFNG(+116.06)TTSTG.L | 72.74 | 1736.971 | 16 | 4.7 | 579.9971 | 3 | 44.81 | 14380 | 1.23E+06 | 157 | 172 | cinnamaldehyde |
| E.DAQQ(+116.06)YLAIDE.L | 70.64 | 1351.666 | 11 | 3 | 676.8357 | 2 | 44.27 | 14136 | 2.38E+06 | 211 | 221 | cinnamaldehyde |
| M(+15.99)TDLKASS.R.A | 66.47 | 1136.586 | 10 | -5.5 | 569.2939 | 2 | 22.65 | 4397 | 3.46E+05 | 1 | 10 | Oxidation (M) |
| M(+15.99)TDLKASS.R | 66.45 | 980.4849 | 9 | -8.3 | 491.2429 | 2 | 26.8 | 6978 | 2.75E+07 | 1 | 9 | Oxidation (M) |
| E.VDVVFPRALM(+15.99)AGNE.E | 61.71 | 1566.786 | 14 | 4 | 784.3992 | 2 | 41.83 | 13086 | 5.71E+05 | 101 | 114 | Oxidation (M) |
| M(+15.99)TDLKASSRALKLM(+15.99)D.L | 60.11 | 1823.949 | 16 | -0.9 | 608.9861 | 3 | 31.33 | 4244 | 4.40E+06 | 1 | 16 | Oxidation (M) |
| E.VIRDM(+15.99)GVE.K | 55.44 | 933.4589 | 8 | -9.5 | 467.7296 | 2 | 22.42 | 4722 | 1.31E+08 | 188 | 195 | Oxidation (M) |
| E.VDVVFPRALM(+15.99)AGNEQVGFD.LVKACKEACAAN.V | 54.16 | 3514.705 | 33 | 0.3 | 879.6767 | 4 | 42.1 | 13193 | 6.84E+06 | 101 | 133 | Oxidation (M) |
| M(+15.99)TDLKASSRALK.L | 50.11 | 1448.802 | 13 | -8.5 | 483.9444 | 2 | 25.5 | 5621 | 3.25E+05 | 1 | 13 | Oxidation (M) |
| K.AGADFIK(+116.06)TSTGLVAVNATPE.S | 50.03 | 2077.073 | 20 | 0.3 | 1039.538 | 2 | 45.17 | 14554 | 3.69E+05 | 161 | 180 | cinnamaldehyde |
| D.VDVVFPRALM(+15.99)AGNE.Q | 49.75 | 1481.734 | 13 | 0.8 | 741.8705 | 2 | 37.67 | 11204 | 5.20E+05 | 103 | 115 | Oxidation (M) |
| E.SARIM(+15.99)AE.+15.99)E.V | 47.97 | 868.3783 | 7 | -5.7 | 435.1915 | 2 | 19.39 | 3338 | 2.72E+07 | 181 | 187 | Oxidation (M) |
| E.VIRDM(+15.99)GVE.KS.V | 45.52 | 1148.586 | 10 | -8.5 | 383.8638 | 3 | 19.42 | 3329 | 2.14E+06 | 188 | 197 | Oxidation (M) |
| L.GHSDGK(+116.06)SASSYLE.H | 45.35 | 1432.642 | 13 | -3.6 | 712.3214 | 2 | 29.85 | 7564 | 1.92E+07 | 249 | 261 | cinnamaldehyde |
X-ray crystallography

Purified variant DERA-MA was thawed and diluted to 16 mg/ml in 10 mM potassium phosphate buffer, pH 7. Screening for initial crystallization conditions was carried out in 96-well sitting-drop plates at 293 K with the help of a Mosquito robot (TTP Labtech) and using various commercial screens. Crystallization hits were optimized manually using hanging-drop vapor diffusion experiments. Final crystals were grown using 13% PEG 3350, 10% isopropanol and 0.1 M HEPES pH 7.5 as reservoir solution, in hanging-drops prepared by mixing 1 µl protein solution with 1 µl reservoir solution. Crystals grew in a few days to an average size of 0.2 x 0.2 x 0.1 µm³. Prior to X-ray data collection the crystals were flash-cooled in liquid nitrogen using 16% PEG3350, 10% isopropanol, 0.1 mM HEPES pH 7.5, supplemented with 25% glycerol, as cryoprotectant solution. To trap the DERA-MA-cinnamylidene Schiff base intermediate, crystals were soaked for 5 minutes in a crystallization solution saturated with cinnamaldehyde, followed by flash-cooling in liquid nitrogen. For both crystals, diffraction data were recorded at the MASSIF-1 beamline at the ESRF, Grenoble. Data to 1.23 Å resolution were recorded for a single apo DERA-MA crystal, and subsequently re-processed with the program XDS and with the AIMLESS routines from the CCP4 software suite. The apo DERA-MA crystal belonged to space group P2₁ and contained two polypeptide chains in the asymmetric unit with a solvent content of 39.4 %. Initial phases and electron maps for the apo DERA-MA model were obtained by molecular replacement with the program Phaser, using PDB entry 1P1X as a search model. The resulting model was improved by several rounds of model building and refinement, using the programs Coot and REFMAC5, respectively, and included placement and validation of water molecules and refinement of anisotropic B-factors. The best crystal of the DERA-MA-cinnamylidene complex diffracted to 1.90 Å resolution, but required X-ray data processing in space group P1 with twelve polypeptide chains in the asymmetric unit (solvent content of 46.9%). Positions and orientations of the polypeptide chains in the asymmetric unit were obtained by molecular replacement using an apo DERA-MA molecule as search model. In all twelve polypeptide chains additional electron density was observed extending from the side chain of Lys167, indicative of the formation of a cinnamaldehyde-derived enzyme–Schiff-base intermediate. Refinement of the DERA-MA/Lys-167-cinnamylidene model, including non-crystallographic symmetry restraints and anisotropic atomic B-factors, was carried out using Phenix_refine, using a topology and parameter file for the lysine-linked cinnamylidene that was obtained from the CCP4 monomer.
library database. Validation of the final structures of apo DERA-MA and DERA-MA Lys167-cinnamylidene was performed with the wwPDB Validation Server at https://validate.wwpdb.org. PyMOL (Schrödinger) was used for structure analysis and figure preparation. Coordinates and structure factors were deposited at the PDB with entry codes 7P75 (apo DERA-MA) and 7P76 (DERA-MA/Lys167-cinnamylidene). A summary of the data collection and refinement statistics are available in Table S5.
Table S5. Crystallographic statistics of apo DERA-MA and DERA-MA/Lys-167-cinnamylidene.

|                                | apo DERA-MA | DERA-MA/Lys-167-cinnamylidene |
|--------------------------------|-------------|--------------------------------|
| **Data collection**            |             |                                |
| ESRF beamline                  | MASSIF-1    | MASSIF-1                       |
| Wavelength (Å)                 | 0.9655      | 0.9655                         |
| Resolution (Å)                 | 47 – 1.23 (1.25-1.23) | 80 – 1.90 (1.93-1.90) |
| Space group                    | P2₁         | P1                             |
| Unit cell dimensions,  a, b, c, (Å) | 47.6, 67.5, 73.7 | 80.3, 92.8, 106.9 |
|                               | 90.0, 101.0, 90.0 | 89.0, 88.8, 89.1 |
| Number of observations         | 293475 (13078) | 3743113 (18962)               |
| Number of unique reflections   | 128757 (5862) | 229190 (11296)                |
| CC(1/2)                        | 0.990 (0.539) | 0.980 (0.719)                 |
| <I/σ>                          | 6.9 (1.2)    | 2.5 (1.3)                      |
| Rmerge                         | 0.068 (0.723) | 0.112 (0.587)                 |
| Completeness (%)               | 96.9 (89.6)  | 94.3 (93.8)                    |
| Multiplicity                   | 2.3 (2.2)    | 1.6 (1.7)                      |
| **Refinement**                 |             |                                |
| Resolution (Å)                 | 47 – 1.35    | 80 – 1.90                      |
| Rwork/Rfree                    | 0.148/0.171  | 0.235/0.283                    |
| No. of protein chains in AU²   | 2           | 12                             |
| No. of non-H atoms in AU, protein/solvent/other | 3798/513 | 22223/1275/114               |
| Average B-factors, protein/solvent/other (Å²) | 17.7/32.3 | 19.3/28.2/23.5               |
| RMSD bond lengths (Å)/bond angles (*) | 0.0070/1.4 | 0.0078/0.9                    |
| Ramachandran preferred/outliers (%) | 97.8/0.0 | 97.7/0.0                      |
| PDB entry                      | 7P75        | 7P76                           |

<sup>a</sup>High resolution shell in parentheses; <sup>b</sup>AU is asymmetric unit.
Figure S9. Comparison of the active sites in wild-type DERA (from *E. coli*) and DERA-MA. a) Wild-type DERA (cyan) after forming a Schiff base complex with 2-deoxy-D-ribose-5-phosphate (carbons in yellow, PDB entry 1JCJ). b,c,d) DERA-MA (orange) after forming a Schiff base complex with cinnamaldehyde (carbons in yellow, PDB entry 7P76), shown in different views. Dashed black lines indicate selected hydrogen bonds. The molecular surfaces in grey define the boundaries of the active site pockets. The cinnamylidene moiety occupies a new subpocket in the active site of DERA-MA, created by the displacement of Leu-20 and the C47S mutation. The cinnamylidene-binding pocket is lined at one side by the side chains of Tyr-49, Val-73 and Phe-76, which stabilize the phenyl ring and acyl carbons via aromatic and hydrophobic contacts. At the other side the active site is largely accessible to solvent and reactive nucleophiles like nitromethane. Also, there is sufficient space in the cinnamylidene-binding pocket to accommodate substituents on the aromatic ring of cinnamaldehyde, consistent with the observed activities of DERA-MA on selected cinnamaldehyde derivatives.
Figure S10. Interactions of the Lys-167-cinnamylidene Schiff base intermediate at the DERA-MA active site. Hydrogen bonds and hydrophobic contacts are shown as blue and black dashed lines, respectively. Residues highlighted in orange contact the cinnamylidene moiety at its front face, residues highlighted in green at its back face. The Schiff-base intermediate is stabilized via electron resonance resulting in a partial positive charge on the C3 carbon of the cinnamylidene moiety. Actually, destabilization of the positive charge on the amino group of Lys-167 by the nearby Lys-201 may increase the significance of the right resonance structure. The positive charge at the C3 carbon, combined with steric hindrance of Tyr-49, Val-73 and Phe-76 at its si-face, will support an attack by nitromethane at the re-face of the cinnamylidene C3 carbon, explaining the R-enantioselective formation of γ-nitroaldehydes.
Substrate scope of DERA-MA

To a 15 ml Greiner tube were added: 1 mM 1a-k, 20 mM 2, 5 µM DERA-MA, and 3 % v/v DMSO, in a final volume of 4 ml 20 mM HEPES, 100 mM NaCl, pH 6.5. After the reaction was initiated by the addition of 2, 300 µl reaction mixture were transferred into a 1 mm quartz cuvette and the substrate depletion was monitored by following the decrease in absorbance at 290 nm (1a-f,h,i), 310 nm (1j), or 325 nm (1g) at 29 °C. The reaction process with substrate 1k was monitored by GC-MS. The remainder of the reaction mixture was placed in an incubator at 29 °C. After UV-vis analysis indicated completion of the reaction, or after 24 hours, 700 µl reaction mixture were extracted with 500 µl EtOAc and analyzed by GC-FID. The product in the remaining reaction mixture was reduced \textit{in situ} with NaBH₄, extracted with 2 x 1 ml EtOAc, concentrated \textit{in vacuo}, and analyzed by chiral-phase HPLC.
4-nitro-3-phenylbutanal 3a

The enantiomeric ratio was determined by normal phase HPLC with reduced 3a using a CHIRALPAK® IB column (150 mm × 4.6 mm, Daicel) (isopropanol/heptane 10:90, 0.5 ml/min, 30 °C). UV detection at 220 nm: \( t_R \) -R-3a = 18.2 min, red.-S-3a = 20.6 min, led to the following enantiomeric ratio (R:S): racemic red.-3a = 50:50 and enzymatic red.-3a = 99:1. The absolute configuration of 3a was assigned based on earlier reported chiral-phase HPLC data.\(^{22}\)

**Figure S11.** HPLC chromatogram of racemic and enzymatically obtained reduced 3a.
3-(2-chlorophenyl)-4-nitrobutanal 3b

The enantiomeric ratio was determined by normal phase HPLC with reduced 3b by using a CHIRALPAK® IC column (150 mm × 4.6 mm, Daicel) (isopropanol/heptane 5:95, 1 ml/min, 30 °C). UV detection at 220 nm: t_R: red.-R-3b = 19.4 min, red.-S-3b = 21.6 min, led to the following enantiomeric ratio (R:S): racemic red.-3b = 50:50 and enzymatic red.-3b = 99:1. The absolute configuration of 3b was assigned based on earlier reported chiral-phase HPLC data.\textsuperscript{23}

![HPLC chromatogram of racemic and enzymatically obtained reduced 3b.]

**Figure S12.** HPLC chromatogram of racemic and enzymatically obtained reduced 3b.
3-(3-chlorophenyl)-4-nitrobutanal 3c

The enantiomeric ratio was determined by normal phase HPLC with reduced 3c by using a CHIRALCEL® OD-H column (150 mm × 4.6 mm, Daicel) (isopropanol/heptane 8:92, 1 ml/min, 30 °C). UV detection at 220 nm: t<sub>R</sub>: red.-R-3c = 14.4 min, red.-S-3c = 15.7 min, led to the following enantiomeric ratio (R:S): racemic red.-3c = 50:50 and enzymatic red.-3c = 99:1. The absolute configuration of 3c was assigned based on earlier reported chiral-phase HPLC data.\textsuperscript{23}

![HPLC chromatogram of racemic and enzymatically obtained reduced 3c.](image)

**Figure S13.** HPLC chromatogram of racemic and enzymatically obtained reduced 3c.
3-(4-chlorophenyl)-4-nitrobutanal 3d

The enantiomeric ratio was determined by normal phase HPLC with reduced 3d by using a CHIRALPAK® IC column (150 mm × 4.6 mm, Daicel) (isopropanol/heptane 5:95, 1 ml/min, 30 °C). UV detection at 220 nm: tᵣ: red.-R-3d = 19.5 min, red.-S-3d = 21.8 min, led to the following enantiomeric ratio (R:S): racemic red.-3d = 49:51 and enzymatic red.-3d = 99:1. The absolute configuration of 3d was assigned based on earlier reported chiral-phase HPLC data.²³

![HPLC chromatogram of racemic and enzymatically obtained reduced 3d.](image)

**Figure S14.** HPLC chromatogram of racemic and enzymatically obtained reduced 3d.
3-(2-methoxyphenyl)-4-nitrobutanal 3e
The enantiomeric ratio was determined by normal phase HPLC with reduced 3e by using a CHIRALCEL® OD-H column (150 mm × 4.6 mm, Daicel) (isopropanol/heptane 8:92, 1 ml/min, 30 °C). UV detection at 210 nm: tR: red-\(R\)-3e = 14.0 min, red-\(S\)-3e = 16.4 min, led to the following enantiomeric ratio (R:S): racemic red-3e = 51:49 and enzymatic red-3e = 86:14. The absolute configuration of 3e was assigned based on earlier reported chiral-phase HPLC data.24

![HPLC chromatogram of racemic and enzymatically obtained reduced 3e.](image)

**Figure S15.** HPLC chromatogram of racemic and enzymatically obtained reduced 3e.
3-(3-methoxyphenyl)-4-nitrobutanal 3f

The enantiomeric ratio was determined by normal phase HPLC with reduced 3f by using a CHIRALPAK® IC column (150 mm × 4.6 mm, Daicel) (isopropanol/hexane 10:90, 1 ml/min, 25 °C). UV detection at 220 nm: t<sub>R</sub>: red.-R-3f = 22.1 min, red.-S-3f = 19.7 min, led to the following enantiomeric ratio (R:S): racemic red.-3f = 50:50 and enzymatic red.-3f = 97:3. The absolute configuration of 3f was assigned based on earlier reported chiral-phase HPLC data.\textsuperscript{25}

**Figure S16.** HPLC chromatogram of racemic and enzymatically obtained reduced 3f.
3-(4-methoxyphenyl)-4-nitrobutanal 3g

The enantiomeric ratio was determined by normal phase HPLC with reduced 3g by using a CHIRALPAK® IC column (150 mm × 4.6 mm, Daicel) (isopropanol/heptane 10:90, 1 ml/min, 30 °C). UV detection at 210 nm: t_R: red.-R-3g = 15.9 min, red.-S-3g = 18.1 min, led to the following enantiomeric ratio (R:S): racemic red.-3g = 50:50 and enzymatic red.-3g = 91:9. The absolute configuration of 3g was assigned based on earlier reported chiral-phase HPLC data.\textsuperscript{26}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{hplc_chromatogram.png}
\caption{HPLC chromatogram of racemic and enzymatically obtained reduced 3g.}
\end{figure}
3-(4-fluorophenyl)-4-nitrobutanal 3h

The enantiomeric ratio was determined by normal HPLC with reduced 3h by using a CHIRALPAK® IC column (150 mm × 4.6 mm, Daicel) (isopropanol/heptane 10:90, 1 ml/min, 30 °C). UV detection at 210 nm: $t_R$: red.-R-3h = 9.5 min, red.-S-3h = 10.9 min, led to the following enantiomeric ratio (R:S): racemic red.-3h = 50:50 and enzymatic red.-3h = 99:1. The absolute configuration of 3h was assigned based on earlier reported chiral-phase HPLC data.²⁷

Figure S18. HPLC chromatogram of racemic and enzymatically obtained reduced 3h.
3-(4-bromophenyl)-4-nitrobutanal 3i

The enantiomeric ratio was determined by normal phase HPLC with reduced 3i by using a CHIRALPAK® IC column (150 mm × 4.6 mm, Daicel) (isopropanol/heptane 8:92, 1 ml/min, 30 °C). UV detection at 210 nm: tR: red.-R-3i = 12.4 min, red.-S-3i = 13.8 min, led to the following enantiomeric ratio (R:S): racemic red.-3i = 51:49 and enzymatic red.-3i = 99:1. The absolute configuration of 3i was assigned based on earlier reported chiral-phase HPLC data.28

Figure S19. HPLC chromatogram of racemic and enzymatically obtained reduced 3i.
4-nitro-3-(4-nitrophenyl)butanal 3j

The enantiomeric ratio was determined by reverse phase HPLC with reduced 3j by using a CHIRALPAK® ID column (150 mm × 4.6 mm, Daicel) (acetonitrile/water 20:80, 1 ml/min, 25 °C). UV detection at 210 nm: t_R: red.-R-3j = 16.8 min, red.-S-3j = 19.8 min, led to the following enantiomeric ratio (R:S): racemic red.-3j = 50:50 and enzymatic red.-3j = 99:1. The absolute configuration of 3j was assigned based on earlier reported chiral-phase HPLC data.\(^5\)

![HPLC chromatogram of racemic and enzymatically obtained reduced 3j.](image)

**Figure S20.** HPLC chromatogram of racemic and enzymatically obtained reduced 3j.
4-methyl-3-(nitromethyl)pentanal 3k

The enantiomeric ratio was determined by GC analysis using a chiral Chiraldex G-TA column (30 m × 0.25 mm, df 0.12 μm, Astec) (130 °C isothermal, 40 min, 1.97 ml/min). Flame ionisation detection: $t_{R}: R$-3k = 22.4 min, S-3k = 26.5 min, led to the following enantiomeric ratio ($R:S$): racemic 3k = 48:52, organocatalytic 3k = 99:1, and enzymatic 3k = 82:18. The absolute configuration was assigned by comparison to 3k obtained with ($R$)-(+)-α,α-diphenyl-2-pyrrolidinemethanol trimethylsilyl ether as a catalyst, which yields the major enantiomer of γ-nitroaldehydes in the $R$-configuration.²
Figure S21. Gas chromatogram of racemic, organocatalytic, and enzymatically obtained 3k.
Semi-preparative scale reactions

4-nitro-3-phenylbutanal 3a

To a 50 ml Greiner tube were added: 5 mM cinnamaldehyde, 20 mM nitromethane, 3 % v/v DMSO and 4 µM DERA-MA in 10 mM HEPES, 100 mM NaCl, pH 6.5 in a total volume of 37.8 ml. The Greiner tube was placed in a water bath at 29 °C, and the reaction progress was monitored by GC-MS analysis at timely intervals. After 16 h, the reaction mixture was extracted 3-times with 15 ml EtOAc, the combined organic layers were washed with 20 ml brine, dried over anhydrous Na₂SO₄, and concentrated in vacuo. The product was purified by silica column chromatography in ethyl acetate/petroleum ether (1:10) to yield 3a (24 mg, 66 % isolated yield). The ¹H NMR spectroscopic data of 3a are in agreement with previously reported data.² The enantiomeric ratio was determined by normal phase HPLC with reduced 3a using a CHIRALPAK® IB column (150 mm × 4.6 mm, Daicel) (isopropanol/heptane 10:90, 0.5 ml/min, 30 °C). UV detection at 220 nm: tₕ: red.-R-3a = 17.7 min, red.-S-3a = 20.0 min, led to the following enantiomeric ratio (R:S): racemic red.-3a = 50:50 and enzymatic red.-3a = 99:1. The absolute configuration of 3a was assigned based on earlier reported chiral-phase HPLC data.²²
Figure S22. $^1$H NMR spectrum of enzymatically produced 3a (500 MHz, CDCl$_3$).
Figure S23. HPLC chromatogram of racemic and enzymatically obtained reduced 3a.
3-(4-fluorophenyl)-4-nitrobutanal 3h

To a 50 ml Greiner tube were added: 5 mM 3-(4-fluorophenyl)acrylaldehyde 1h, 20 mM nitromethane, 3 % v/v DMSO and 4 µM DERA-MA in 10 mM HEPES, 100 mM NaCl, pH 6.5 in a total volume of 33.3 ml. The Greiner tube was placed in a water bath at 29 °C, and the reaction progress was monitored by GC-MS analysis at timely intervals. After 12 h, the reaction mixture was extracted 3-times with 15 ml EtOAc, the combined organic layers were washed with 20 ml brine, dried over anhydrous Na₂SO₄, and concentrated in vacuo. The product was purified by silica column chromatography in ethyl acetate/petroleum ether (1:5) to yield 3h (22.7 mg, 65 % isolated yield). The ¹H NMR spectroscopic data of 3h are in agreement with previously reported data. The enantiomeric ratio was determined by reverse phase HPLC using a CHIRALPAK® AD-RH column (150 mm × 4.6 mm, Daicel) (acetonitrile/water 30:70, 0.5 ml/min, 25 °C). UV detection at 220 nm: t_R: R-3h = 38.5 min, S-3h = 40.9 min, led to the following enantiomeric ratio (R:S): racemic 3h = 50:50 and enzymatic 3h = 99:1. The absolute configuration of 3h was assigned based on earlier reported chiral-phase HPLC data.

**Figure S24.** ¹H NMR spectrum of enzymatically produced 3h (500 MHz, CDCl₃).
Figure S25. HPLC chromatogram of racemic and enzymatically obtained 3h.
3-(4-chlorophenyl)-4-nitrobutanal 3d

To a 50 ml Greiner tube were added: 5 mM 3-(4-chlorophenyl)acrylaldehyde 1d, 20 mM nitromethane, 3 % v/v DMSO and 8 µM DERA-MA in 10 mM HEPES, 100 mM NaCl, pH 6.5 in a total volume of 30.0 ml. The Greiner tube was placed in a water bath at 29 °C, and the reaction progress was monitored by GC-MS analysis at timely intervals. After 16.5 h, the reaction mixture was extracted 3-times with 15 ml EtOAc, the combined organic layers were washed with 20 ml brine, dried over anhydrous Na$_2$SO$_4$, and concentrated in vacuo. The product was purified by silica column chromatography in ethyl acetate/pentane (1:5) to yield 3d (19.0 mg, 56 % isolated yield). The $^1$H NMR spectroscopic data of 3d are in agreement with previously reported data. The enantiomeric ratio was determined by normal phase HPLC with reduced 3d by using a CHIRALPAK® IC column (150 mm × 4.6 mm, Daicel) (isopropanol/heptane 5:95, 1 ml/min, 30 °C). UV detection at 220 nm: t$_R$: red.-R-3d = 19.6 min, red.-S-3d = 21.7 min, led to the following enantiomeric ratio (R:S): racemic red.-3d = 50:50 and enzymatic red.-3d = 99:1. The absolute configuration of 3d was assigned based on earlier reported chiral-phase HPLC data.

Figure S26. $^1$H NMR spectrum of enzymatically produced 3d (500 MHz, CDCl$_3$).
Figure S27. HPLC chromatogram of racemic and enzymatically obtained reduced 3d.
DNA and protein sequences

**Table S6.** DNA and amino acid sequences of *E. coli* wild-type DERA and variant DERA-MA. Amino acids that are mutated in the variant DERA-MA compared to wild-type DERA are highlighted in **blue**.

| DERA variant | Type | Sequence |
|--------------|------|----------|
| wild-type    | DNA  | ATGACTGATCCTGAAAAGCAGACCTGCACCTGAAATTGATGGAACCTGACCAACCATCCTG ATAGCAGCAGCACCGAGACGAGAAAGTAATTGCTCTGTGTCATCA CGGCAATCCGCCGCTATCTGTTATCTTCTCCTCGTTATACCGGATATTGCTCGCAAAACACTGA AAGAAGCGAGGCCACCCGGAATCTCGATTTGCAATCCCGGCGCAGAGAATGATAGTGAACAGCAGCCAACGCGC GTGGACGTGGTGTTCCAAGCGACGCCTACCCGCTTTGCTGTCATCGGCAAAACACTGA AAGAAGCGAGGCCACCCGGAATCTCGATTTGCAATCCCGGCGCAGAGAATGATAGTGAACAGCAGCCAACGCGC GTGGACGTGGTGTTCCAAGCGACGCCTACCCGCTTTGCTGTCATCGGCAAAACACTGA AAGAAGCGAGGCCACCCGGAATCTCGATTTGCAATCCCGGCGCAGAGAATGATAGTGAACAGCAGCCAACGCGC |
| DERA-MA      | DNA  | ATGACTGATCCTGAAAAGCAGACCTGCACCTGAAATTGATGGAACCTGACCAACCATCCTG ATAGCAGCAGCACCGAGACGAGAAAGTAATTGCTCTGTGTCATCA CGGCAATCCGCCGCTATCTGTTATCTTCTCCTCGTTATACCGGATATTGCTCGCAAAACACTGA AAGAAGCGAGGCCACCCGGAATCTCGATTTGCAATCCCGGCGCAGAGAATGATAGTGAACAGCAGCCAACGCGC GTGGACGTGGTGTTCCAAGCGACGCCTACCCGCTTTGCTGTCATCGGCAAAACACTGA AAGAAGCGAGGCCACCCGGAATCTCGATTTGCAATCCCGGCGCAGAGAATGATAGTGAACAGCAGCCAACGCGC GTGGACGTGGTGTTCCAAGCGACGCCTACCCGCTTTGCTGTCATCGGCAAAACACTGA AAGAAGCGAGGCCACCCGGAATCTCGATTTGCAATCCCGGCGCAGAGAATGATAGTGAACAGCAGCCAACGCGC |
| Protein      |      | MTDLKASSRLKMLDLYTLDNDDTDKVIACLGJQAKTPVGNTAAICYPREFPIARKTLKEQGT PEIARIATVNFPHNGDDELALTAACAAAYGADQEVDFYPYRALMAGNEQVGFDDLVAKCAEA CAAAANVLLKVEETGEKDEAIKACEISIKAGAFLFSTKGKAVNATPESARAMMENVIRMDG VKEKTGFKPAAGVRTADYQKLYAIADFELGADWADARHRFGASSILLSLKLALGHDGDKS ASSYLEHHHHHH |
| Protein      |      | MTDLKASSRLKMLDLYTLDNDDTDKVIACLGJQAKTPVGNTAAICYPREFPIARKTLKEQGT PEIARIATVNFPHNGDDELALTAACAAAYGADQEVDFYPYRALMAGNEQVGFDDLVAKCAEA CAAAANVLLKVEETGEKDEAIKACEISIKAGAFLFSTKGKAVNATPESARAMMENVIRMDG VKEKTGFKPAAGVRTADYQKLYAIADFELGADWADARHRFGASSILLSLKLALGHDGDKS ASSYLEHHHHHH |


Saturation of DERA-MA with nitromethane 2

Figure S28. Spectrophotometric measurement of the activity of DERA-MA (0.5 µM) assayed with 20 mM 2, 50 mM 2, or 100 mM 2 and 1 mM 1a in 20 mM HEPES, 100 mM NaCl, pH 6.5 with 3 % DMSO. In a control experiment the uncatalyzed reaction (without enzyme) with 100 mM 2 and 1 mM 1a was measured under otherwise identical conditions.
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