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Engineering a Promiscuous Tautomerase into a More Efficient Aldolase for Self-Condensations of Linear Aliphatic Aldehydes

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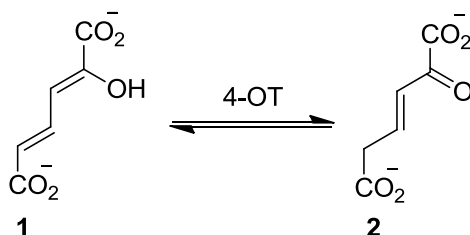
Keywords: 4-oxalocrotonate tautomerase, catalytic promiscuity, aldolase activity,
condensation reaction, protein engineering

ABSTRACT

The enzyme 4-oxalocrotonate tautomerase (4-OT) from *Pseudomonas putida* mt-2 takes part in a catabolic pathway for aromatic hydrocarbons, where it catalyzes the conversion of 2-hydroxyhexa-2,4-dienedioate into 2-oxohexa-3-enedioate. This tautomerase can also promiscuously catalyze carbon-carbon bond-forming reactions, including various types of aldol reactions, utilizing its amino-terminal proline as a key catalytic residue. Here, we have used a systematic mutagenesis strategy to identify two ‘hotspot’ positions in 4-OT (Met-45 and Phe-50) at which single mutations give a marked improvement in aldolase activity for the self-condensation of propanal. Activity screening of a focused library in which these two ‘hotspot’ positions were varied led to the discovery of a 4-OT variant (M45Y/F50V) with strongly enhanced aldolase activity in the self-condensation of linear aliphatic aldehydes, such as acetaldehyde, propanal and butanal, yielding α,β -unsaturated aldehydes. In the presence of both propanal and benzaldehyde, this double mutant, unlike the previously constructed single mutant F50A, mainly catalyzes the self-condensation of propanal rather than the cross-condensation of propanal and benzaldehyde, indicating that it indeed has an altered substrate specificity. This M45Y/F50V variant of 4-OT could serve as a starting template to create new biocatalysts that lack dehydration activity and possess further enhanced aldolase activity, enabling the efficient enzymatic self-coupling of aliphatic aldehydes.

INTRODUCTION

The enzyme 4-oxalocrotonate tautomerase (4-OT) is composed of six identical subunits of only 62 amino acid residues each. It is a member of the tautomerase superfamily, a group of homologous proteins having a β - α - β structural fold and a catalytic amino-terminal proline (Pro-1) in common.^[1-3] 4-OT takes part in a catabolic pathway for aromatic hydrocarbons in *Pseudomonas putida* mt-2, where it catalyzes the conversion of 2-hydroxyhexa-2,4-dienedioate (**1**, Scheme 1) into 2-oxohexa-3-enedioate (**2**).^[4,5] In this tautomerization reaction, Pro-1 acts as a general base (pK_a of Pro-1 ~ 6.4) abstracting the 2-hydroxyl proton of **1** and transferring it to the C5-position to give **2**.^[6]

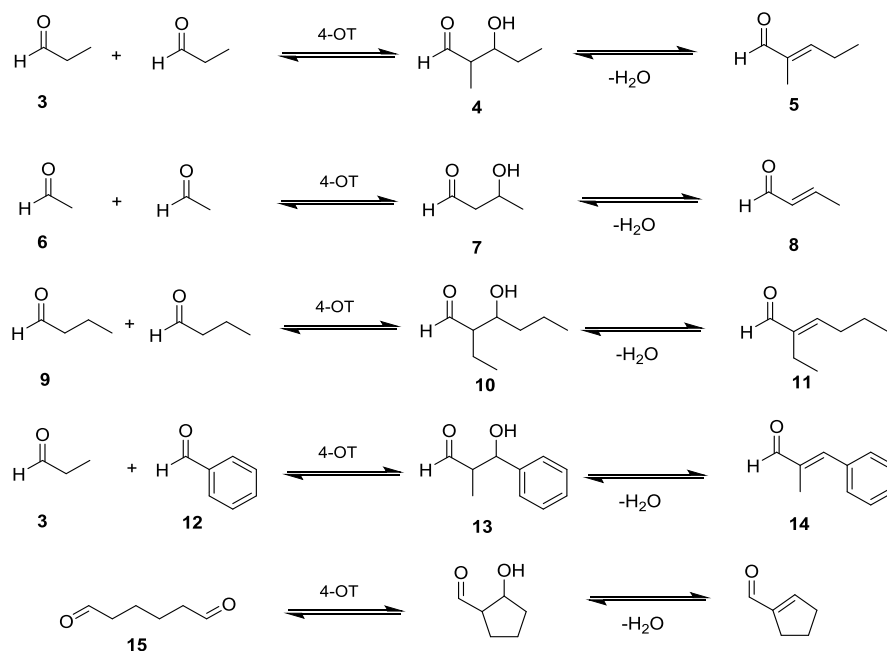


Scheme 1. Proton-transfer reaction naturally catalyzed by 4-OT.

In addition to its natural tautomerase activity, 4-OT has been shown to promiscuously catalyze several carbon-carbon bond-forming reactions.^[7a, 8-16] These include various types of aldol reactions such as the self-condensation of propanal (**3** in Scheme 2), the cross-condensation of acetaldehyde (**6**) with benzaldehyde (**12**), the cross-coupling of propanal (**3**) and benzaldehyde (**12**), and the intramolecular cyclization of hexanedial (**15**) or heptanedial.^[10,12,16] For the self-condensation of **3**, as well as the cross-condensation of **6** and **12**, it has been demonstrated that 4-

OT catalyzes both the initial aldol-coupling step to yield the β -hydroxyaldehyde intermediate and the subsequent dehydration step to yield the final α,β -unsaturated aldehyde product.^[12,16] In the proposed mechanism for the aldolase activity of 4-OT, the active site Pro-1 residue functions as a nucleophile, rather than a base, and reacts with the carbonyl functionality of the aldehyde to form a covalent enamine intermediate.^[10,17] This intermediate reacts with another aldehyde in an inter- or intramolecular aldol addition, after which the final product (the aldol compound or the corresponding dehydrated compound) is released from the active site upon hydrolysis.

Enzyme promiscuity has great promise as a source of synthetically useful catalytic transformations,^[2,7] and could be exploited as starting point to create new biocatalysts for challenging aldol reactions.^[18,19] In previous work, we have constructed several active site mutants of 4-OT, including the variant F50A, which possess improved aldolase activity for the cross-condensation of **6** with **12**.^[12,20] Here, we report the engineering of 4-OT into a more efficient aldolase for self-condensation reactions of linear aliphatic aldehydes such as acetaldehyde (**6**), propanal (**3**) and butanal (**9**) (Scheme 2). For this, a large collection of single mutants of 4-OT was first screened for single mutations that give a strong improvement in the desired aldolase activity, after which identified ‘hotspot’ positions were subjected to combinatorial mutagenesis.



Scheme 2. Examples of aldol reactions promiscuously catalyzed by 4-OT.

RESULTS AND DISCUSSION

We applied a systematic mutagenesis strategy to identify residue positions at which mutations give a marked improvement in the aldolase activity of 4-OT. For this, a collection of 1040 single mutants of 4-OT, covering at least 15 of the 19 possible variants at each residue position (from Ile-2 to Arg-62), was used.^[21] Single mutants of the Pro-1 residue were not included in the collection, because Pro-1 is a key catalytic residue and mutations at this position lead to incorrect demethionylation of the protein.^[22] The complete collection of 4-OT mutants was screened for activity for the self-condensation of **3**, using conditions that allowed for the detection of variants with strongly improved aldolase activity only. The results are graphically represented in a heatmap (Figure 1).

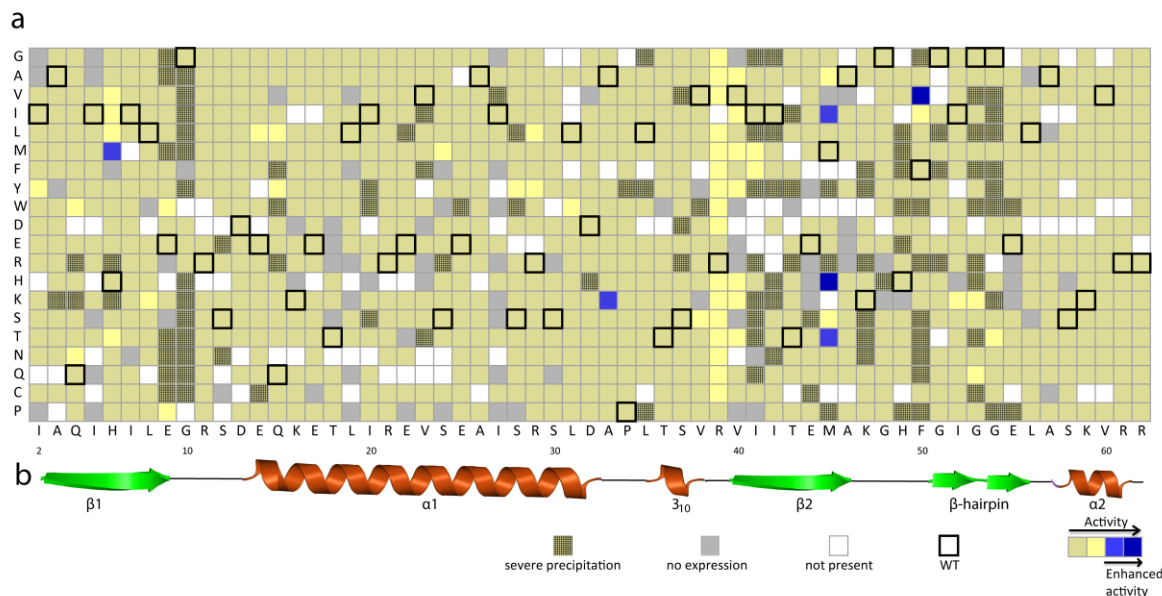


Figure 1. Heatmap depicting the aldolase activity of single mutants of 4-OT for the self-condensation of propanal. a, The horizontal axis of the data matrix depicts the wild-type sequence of 4-OT and the vertical axis depicts the 20 possible amino acid residues. The wild-type amino acid residue at each position is indicated by bold squares. White boxes represent single mutants which were not present in the collection, grey boxes represent mutants which were not produced above the detection limit (0.5 mg/ml in the CFE), and boxes with a crosshatch pattern indicate mutants which precipitated under the screening conditions, preventing activity measurements. The ability of each single mutant of 4-OT to catalyze the self-condensation of **3** was monitored by UV-spectroscopy, following the formation of **5** ($\lambda_{\max} = 234$ nm). b, The secondary-structure elements of 4-OT.

Two residue positions at which single mutations lead to large improvements in the aldolase activity of 4-OT were identified. The first ‘hotspot’ position is Met-45; replacement of this residue with Thr, His or Ile resulted in a pronounced improvement in aldolase activity. The second ‘hotspot’ position is Phe-50; substitution of this residue by Val strongly improved the aldolase activity of 4-OT. In addition to these beneficial mutations, it was found that the replacement of His-6 with Met, and the mutation of Ala-33 to Lys, also significantly improved the aldolase activity of 4-OT. Mutant F50V showed the highest aldolase activity among all variants. Progress curves of the aldol self-condensation of **3** (50 mM) catalyzed by purified

enzymes confirmed the enhanced activity of mutant F50V when compared to wild-type 4-OT or the previously constructed mutant F50A^[12] (Figure 2).

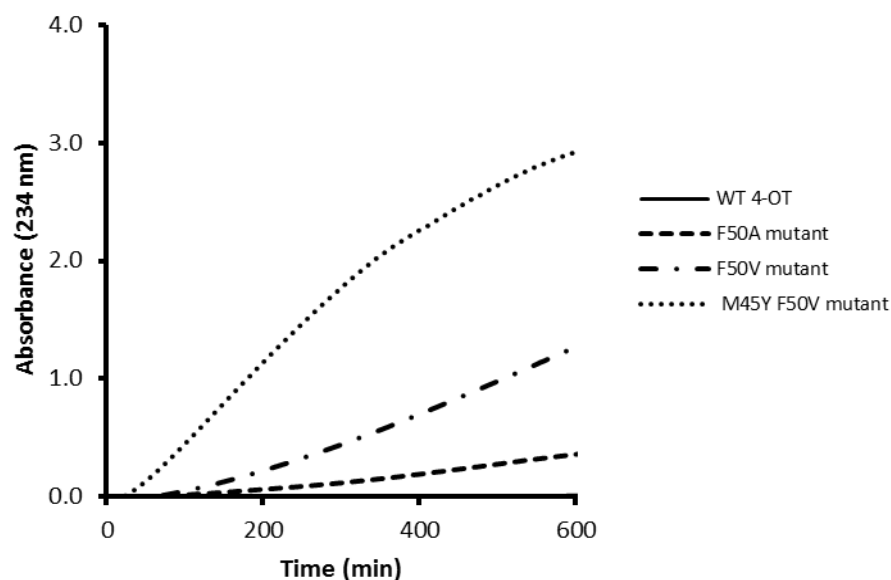


Figure 2. UV traces monitoring the formation of **5** during the self-condensation of **3** catalyzed by wild-type 4-OT (low activity), mutant F50A, mutant F50V, or mutant M45Y/F50V.

To further improve the aldolase activity of 4-OT, we constructed a double-site library in which the two ‘hotspot positions’ Met-45 and Phe-50 were simultaneously randomized by using NNK-codon degeneracy,^[23,24] covering all 20 possible amino acids, for both positions. In addition to this double-site library, we constructed a triple-site library in which positions His-6, Met-45, and Phe-50 were simultaneously randomized. The NNK-codon degeneracy was used for randomization of positions His-6 and Met-45, whereas Phe-50 was randomized using a NYK-codon degeneracy.^[23,24] The NYK-codon degeneracy was chosen for position Phe-50 because it reduces the library size by covering only the codons of nine different aliphatic and polar amino acid residues, including those residues which result in an enhanced aldolase activity, as well as

the wild-type residue. The two libraries were used to transform *Escherichia coli* BL21(DE3) cells. The double-site library was screened by evaluating ~800 transformants, whereas ~3500 transformants of the triple-site library were screened for the aldol self-condensation of **3**.

Screening of the double-site library resulted in the identification of a double mutant, M45Y/F50V, with strongly enhanced aldolase activity (~30-fold improvement compared to wild-type 4-OT), whereas screening of the triple-site library did not yield a mutant with higher activity than that of mutant M45Y/F50V. The progress curves of the aldol self-condensation of **3** (50 mM) catalyzed by purified enzymes confirmed the enhanced activity of mutant M45Y/F50V when compared to the single mutants F50V and F50A (Figures 2 and S1). Mutant ¹H NMR spectroscopic analysis confirmed the formation of **5** as product of the aldol self-condensation of **3** catalyzed by mutant M45Y/F50V (Figure S2). Hence, 4-OT can be engineered into a more efficient aldolase for the self-condensation of **3** to yield **5** by exploring small libraries in which only 'hotspot' positions are varied.

Having established that mutant M45Y/F50V has improved aldolase activity for the self-condensation of **3**, it was tested whether this mutant also has enhanced aldolase activity towards the self-condensation of aldehydes **6** and **9**. Accordingly, in separate experiments, the purified enzymes wild-type 4-OT, P1A, F50A and M45Y/F50V (150 μM each) were incubated with either **6** or **9** (50 mM each) and the reactions were monitored by UV spectroscopy. The results indicate that the self-condensation of **6** or **9** to give **8** or **11** is enzyme-catalyzed, with variant M45Y/F50V having strongly enhanced activity when compared to wild-type 4-OT or mutant F50A (Figures 3 and 4). ¹H NMR spectroscopic analysis confirmed the formation of both **7** and **8** as products of the enzyme-catalyzed self-condensation of **6** (Figure S3 and S4), and the formation of **11** as a product of the enzyme-catalyzed self-condensation of **9** (Figure S5 and S6).

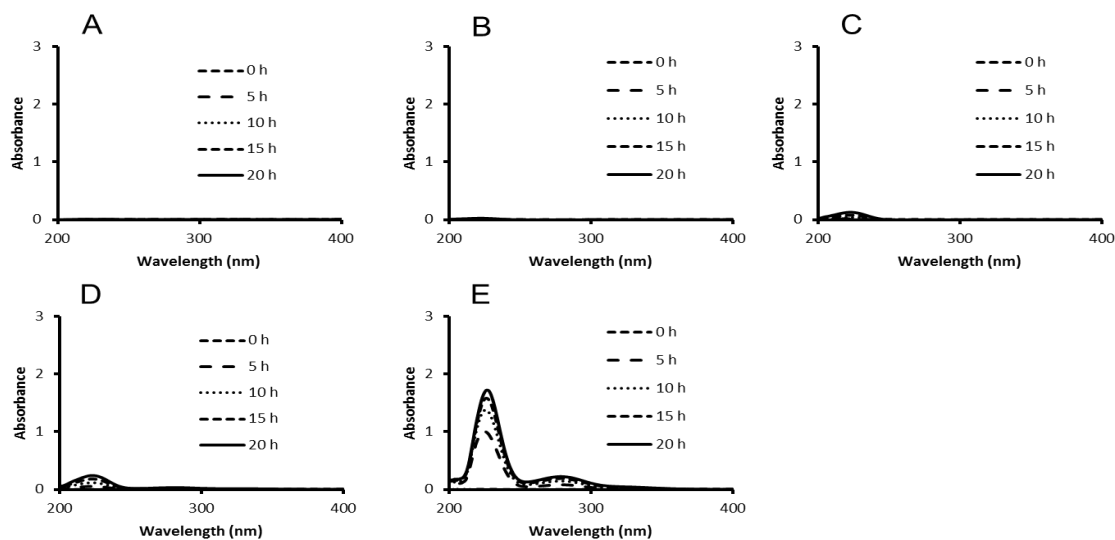


Figure 3. UV spectra recorded after incubation of **6** with A) no enzyme (control sample); B) 4-OT P1A; C) wild-type 4-OT; D) 4-OT F50A; and E) 4-OT M45Y/F50V. Formation of **8** ($\lambda_{\text{max}} = 227$ nm) was monitored in the course of the reactions.

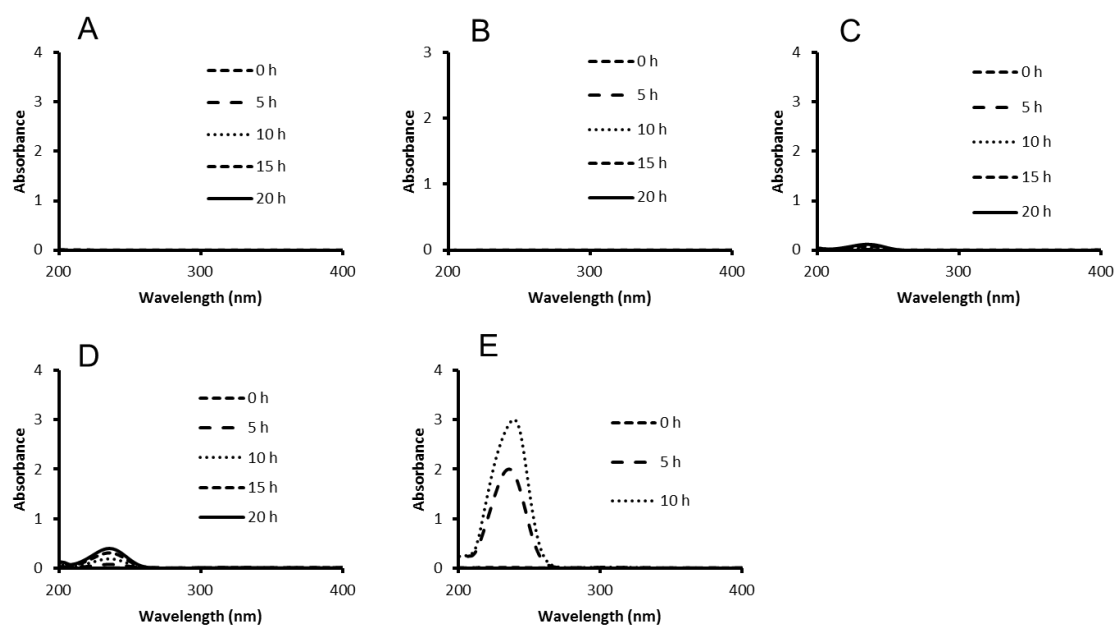


Figure 4. UV spectra recorded after incubation of **9** with A) no enzyme (control sample); B) 4-OT P1A; C) wild-type 4-OT; D) 4-OT F50A; and E) 4-OT M45Y/F50V. Formation of **11** ($\lambda_{\text{max}} = 240$ nm) was monitored in the course of the reactions.

Next, we compared the ability of mutants F50A and M45Y/F50V to catalyze an aldol cross-condensation reaction using **3** and **12** as the substrates. Accordingly, in separate experiments, the mutant proteins (150 μ M) were incubated with **3** (50 mM) and **12** (2 mM) and the reactions were followed by UV spectroscopy. The UV spectra of the reaction mixture incubated with mutant F50A showed a decrease in absorbance at 250 nm (Figure 5A), indicating the depletion of **12** as the result of a cross-coupling reaction. Interestingly, the UV spectra of the reaction mixture with mutant M45Y/F50V showed a negligible decrease in absorbance at 250 nm (Figure 5B). Instead, an increase in absorbance at 234 nm was observed, corresponding to the formation of **5** as the result of the self-condensation of **3**. ^1H NMR spectroscopic analysis of the reaction mixtures confirmed that **13** and **14** are the main products in the reaction mixture incubated with mutant F50A (indicative of cross-coupling), whereas **5** was the main product in the reaction mixture incubated with mutant M45Y/F50V (Figure S7). Hence, compared to the previously constructed mutant F50A,^[12] mutant M45Y/F50V has an altered substrate specificity and prefers the self-condensation of **3** over the cross-coupling of **3** and **12**. This result is fully consistent with the fact that mutant M45Y/F50V was engineered for enhanced activity towards the self-condensation of **3**.

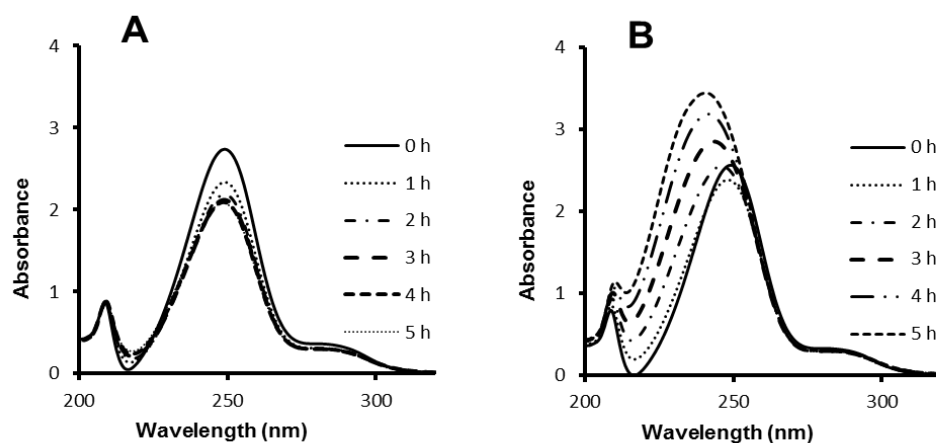


Figure 5. UV spectra recorded after incubation of **3** and **12** with A) 4-OT F50A; and B) 4-OT M45Y/F50V.

In conclusion, we demonstrated that the promiscuous enzyme 4-OT can be engineered into a more efficient aldolase (variant M45Y/F50V) for self-condensations of aliphatic aldehydes by exploring small libraries in which only two ‘hotspot’ positions (Met-45 and Phe-50) are varied. Notably, in a recent study, the same residue positions were identified as ‘hotspots’ for improving 4-OT’s aldolase activity for the cross-condensation of aldehydes **6** and **12**, yielding a different 4-OT variant (M45T/F50A) after combinatorial mutagenesis and activity screening.^[20] Hence, 4-OT can be tailored to catalyze a specific aldol reaction. Work is in progress to determine the crystal structure of 4-OT variant M45Y/F50V in complex with product **5**, **8** or **11**. Such enzyme-product complex structures could guide the design of new 4-OT variants that completely lack dehydration activity and possess further enhanced aldolase activity, enabling the efficient enzymatic self-coupling of small aliphatic aldehydes. With only a few natural aldolases available that can catalyze the self-coupling of aldehydes, including 2-deoxyribose-5-phosphate aldolase (DERA)^[18b] and D-fructose-6-phosphate aldolase (FSA),^[19b] the engineering of a promiscuous enzyme may prove to be a useful approach to create new biocatalysts for these challenging aldolizations.

MATERIALS AND METHODS

Materials

The sources for the buffers, solvents, components of Luria-Bertani (LB) media as well as the materials, enzymes and reagents exploited in molecular biology procedures are reported elsewhere.^[25]

General methods

Standard molecular biology techniques were performed based on methods described elsewhere.^[26] Protein was analyzed by polyacrylamide gel electrophoresis (PAGE) using pre-casted 10% polyacrylamide gels (NuPAGE[®] Novex[®] 10% Bis-Tris). Coomassie brilliant blue was used to stain the gels. Protein concentrations were determined using the Waddell method.^[27] Enzymatic assays were performed either on a V-650 or V-660 spectrophotometer from Jasco (IJsselstein, The Netherlands) or on a SPECTROstar Omega plate reader (BMG LABTECH, Isogen Life Science, de Meern, NL). ¹H NMR spectra were recorded on a Varian Inova 500 (500 MHz) spectrometer using a pulse sequence for selective presaturation of the water signal. Chemical shifts for protons are reported in parts per million scale and are referenced to H₂O ($\delta = 4.80$).

Expression and purification of wild-type 4-OT and 4-OT mutants

Wild-type 4-OT and 4-OT mutants were produced in *E. coli* BL21 (DE3) as native proteins without an affinity tag, using either a pET20b(+) or pJexpress 414 expression vector. The construction of the expression vectors for wild-type 4-OT and 4-OT mutants P1A and F50A, as well as the purification procedure for 4-OT, were reported previously.^[10,12]

Cell-free extract (CFE) preparation of *E. coli* cells expressing the single 4-OT mutants

A defined collection of 1040 single mutant 4-OT genes, each cloned individually into a pJexpress 414 vector, was purchased from DNA2.0 (Menlo Park, CA). An aliquot of each pJexpress 414 vector with a unique mutant 4-OT gene was transformed individually into *E. coli* BL21 (DE3), as described elsewhere.^[21] Each *E. coli* BL21(DE3) transformant harboring a pJexpress 414 vector with a unique mutant 4-OT gene was stored at -80°C until further use. The expression of the

mutant 4-OT genes in *E. coli* BL21(DE3), the preparation of CFEs, and the assessment of the concentrations of the 4-OT mutants are described elsewhere.^[21]

Determination of the aldolase activity of the single 4-OT mutants

The produced CFEs were used to determine the aldolase activity of the single 4-OT mutants using UV-spectroscopy. The UV-spectroscopic measurements were performed in 96-wells micro titer plates (MTP) (UV-star µclear, Greiner Bio-one). The final reaction mixtures consisted of CFE (30% v/v) and propanal (**3**, 40 mM) in 10 mM NaH₂PO₄ buffer (pH 7.3). The volume of these reaction mixtures was 100 µl and the MTPs were sealed with UV-transparent plate seals (VIEWseal™, Greiner Bio-one) to prevent evaporation. The MTPs were incubated for 16 h at 25°C during which the reaction progress was monitored by UV spectroscopy (220-500 nm). Formation of product **5** ($\lambda_{\text{max}} = 234$ nm) was quantified based on the increase in absorbance at 234 nm. To eliminate false positives in which the increase in absorbance was a result of protein precipitation (in these cases there is an increase in absorbance over the whole range of the UV-spectrum), reaction mixtures in which there was a high increase in absorbance at 350 nm ($\Delta A_{350} > 0.5$) were assigned as “precipitation” (Figure 1). At this wavelength product **5** has no UV-absorbance. The aldolase activity of wild-type 4-OT was used as a control and single mutants with a marked improvement in aldolase activity could be identified. Using Microsoft excel 2010, the data were represented as colors in the data matrix to create a visually interpretable heat map (Figure 1).

Construction of double and triple mutant libraries

The construction of the double and triple mutant libraries has been described elsewhere.^[20]

UV spectroscopic assay for the self-condensation of propanal

The self-condensation of propanal (**3**) was monitored by following the increase in absorbance at 234 nm, which corresponds to the formation of 2-methyl-2-pentenal (**5**). The enzyme (150 μ M) was incubated in a 1 mm cuvette with **3** (50 mM) in 20 mM NaH₂PO₄ buffer (pH 7.3; 0.3 mL final volume), and the reaction was followed for 20 h at room temperature. UV spectra were recorded from 200 to 400 nm (Figure S1 and Figure 2).

Redissolving WT 4-OT and 4-OT mutants in NaD₂PO₄ buffer

A VIVASPIN concentrator (from Sartorius Stedim Goettingen, Germany) with a cut-off filter of 5000 Da was washed four times with H₂O by centrifugation (4000 rpm, 20 min). Subsequently, the concentrator was charged with a solution of 4-OT (either WT or mutant; 300 μ L with a concentration of $\sim$ 10 mg/mL in 20 mM NaH₂PO₄ buffer, pH 7.3) and centrifuged (4000 rpm, 30 min). The enzyme was retained on the filter and redissolved in NaD₂PO₄ (200 μ L, 20 mM; pD 7.6) and centrifuged (4000 rpm, 30 min). Once more, the remaining enzyme on the filter was redissolved in NaD₂PO₄ (300 μ L, 20 mM; pD 7.6) after which the final enzyme concentration was determined. The exchange of NaH₂PO₄ with NaD₂PO₄ buffer was carried out only for the enzyme preparations used in the ¹H NMR spectroscopic assay for the self-condensation of **3** or cross-coupling of **3** and **12**.

¹H NMR spectroscopic assay for the self-condensation of propanal

4-OT wild-type or mutant (290 μ M) was incubated with **3** (30 mM) at room temperature in NaD₂PO₄ buffer (20 mM; pD 7.6, final volume of 650 μ L in an NMR tube), containing 18-crown-6 ether as internal standard (2.15 mM). A control sample was prepared containing all

components except for the enzyme. ^1H NMR spectra were recorded ~ 1 h after the start of the incubation, and subsequently after 1, 4, 8 and 14 days.

Compound 3: ^1H NMR (500 MHz, 20 mM NaD_2PO_4 ; pD 7.6): δ 9.69 (t, $J = 1.3$ Hz, 1H), 2.57 (dq, $J = 7.3, 1.3$ Hz, 2H), 1.06 (t, $J = 7.3$ Hz, 3H).

Hydrated form of 3: ^1H NMR (500 MHz, 20 mM NaD_2PO_4 ; pD 7.6): δ 4.96 (t, $J = 5.5$ Hz, 1H), 1.59 (dq, $J = 7.5, 5.5$ Hz, 2H), 0.92 (t, $J = 7.5$ Hz, 3H).

Compound 5: ^1H NMR (500 MHz, 20 mM NaD_2PO_4 ; pD 7.6): δ 9.29 (s, 1H), 6.81 (t, $J = 7.6$ Hz, 1H), 2.41 (dq, $J = 7.6, 7.6$ Hz, 2H), 1.71 (s, 3H), 1.09 (t, $J = 7.6$ Hz, 3H).

Internal standard 18-crown-6 (1,4,7,10,13,16-hexaoxacyclooctadecane): ^1H NMR (500 MHz, 20 mM NaD_2PO_4 ; pD 7.6): $\delta = 3.68$ (s, 24H).

UV spectroscopic assay for the self-condensation of 6

The self-condensation of **6** was monitored by following the increase in absorbance at 227 nm, which corresponds to the formation of **8**. The enzyme (150 μM) was incubated in a 1 mm cuvette with **6** (50 mM) in 20 mM NaH_2PO_4 buffer (pH 7.3; 0.3 mL final volume), and the reaction was followed for 20 h at room temperature. UV spectra were recorded from 200–400 nm (Figure 3).

^1H NMR spectroscopic assay for the self-condensation of 6

In separate experiments, 4-OT wild-type or mutant (290 μM , in 20 mM NaH_2PO_4) was incubated with **6** (50 mM) at room temperature in NaD_2PO_4 buffer (20 mM; pD 7.6, final volume of 650 μL in an NMR tube), containing 18-crown-6 ether as internal standard (2.15 mM). A control sample

was prepared containing all components except for the enzyme. ^1H NMR spectra were recorded ~2 h after the start of the incubation and then after 1, 4 and 7 days.

Compound 6: ^1H NMR (500 MHz, 20 mM NaD_2PO_4 ; pD 7.6): δ 9.66 (q, J = 3.0 Hz, 1H), 2.23 (d, J = 3.0 Hz, 3H).

Hydrated form of 6: ^1H NMR (500 MHz, 20 mM NaD_2PO_4 ; pD 7.6): δ 5.23 (q, J = 5.3 Hz, 1H), 1.32 (d, J = 5.3 Hz, 3H).

Compound 7: ^1H NMR (500 MHz, 20 mM NaD_2PO_4 ; pD 7.6): δ 9.69 (t, J = 5.9 Hz, 1H), 5.16 (m, 1H), 2.03 (m, 2H), 1.18 (d, J = 5.9 Hz, 3H).

Compound 8: ^1H NMR (500 MHz, 20 mM NaD_2PO_4 ; pD 7.6): δ 9.37 (d, J = 8.3 Hz, 1H), 7.19 (m, 1H), 6.21 (m, 1H), 2.05 (d, J = 7.0 Hz, 3H).

UV spectroscopic assay for the self-condensation of **9**

The self-condensation of **9** was monitored by following the increase in absorbance at 240 nm, which corresponds to the formation of **11**. The enzyme (150 μM) was incubated in a 1 mm cuvette with **9** (50 mM) in 20 mM NaH_2PO_4 buffer (pH 7.3; 0.3 mL final volume), and the reaction was followed for 20 h at room temperature. UV spectra were recorded from 200-400 nm (Figure 4).

^1H NMR spectroscopic assay for the self-condensation of **9**

In separate experiments, 4-OT wild-type or mutant (290 μM , in 20 mM NaH_2PO_4) was incubated with **9** (50 mM) at room temperature in NaD_2PO_4 buffer (20 mM; pD 7.6, final volume of 650 μL in an NMR tube), containing 18-crown-6 ether as internal standard (2.15 mM). A control sample

was prepared containing all components except for the enzyme. ^1H NMR spectra were recorded ~2 h after the start of the incubation and then after 1, 4 and 7 days.

Compound 9: ^1H NMR (500 MHz, 20 mM NaD_2PO_4 ; pD 7.6): δ 9.65-9.67 (m, 1H), 2.50 (m, 2H), 1.59-1.67 (m, 2H), 0.88-0.94 (m, 3H).

Hydrated form of 9: ^1H NMR (500 MHz, 20 mM NaD_2PO_4 ; pD 7.6): δ 5.03 (t, J = 5.5 Hz, 1H), 1.31-1.43 (m, 2H), 1.53-1.59 (m, 2H), 0.88-0.94 (m, 3H).

Compound 11: ^1H NMR (500 MHz, 20 mM NaD_2PO_4 ; pD 7.6): ^1H NMR (500 MHz, 20 mM NaD_2PO_4 ; pD 7.6): δ 9.26 (s, 1 H), 6.75 (t, J = 7.5 Hz, 1H), 2.42 (q, J = 7.3 Hz, 2H), 2.25 (m, 2H), 1.55 (m, 2H), 0.90-0.97 (m, 3H), 0.90-0.97 (m, 3H)

UV spectroscopic assay for the cross-coupling of 3 and 12

The cross-coupling of **3** and **12** was monitored by following the decrease in absorbance at 250 nm ($\lambda_{\text{max},12}$ = 250 nm), which corresponds to the depletion of **12**. Simultaneously, the increase in absorbance at 234 nm, which corresponds to the formation of **5**, was monitored. The enzyme (150 μM) was incubated in a 1 mm cuvette with **3** (50 mM) and **12** (2 mM) in 20 mM NaH_2PO_4 buffer (pH 7.3; 0.3 mL final volume) and the reaction was followed for 20 h at room temperature. UV spectra were recorded from 200 to 400 nm.

^1H NMR spectroscopic assay for the cross-coupling of 3 and 12

In separate experiments, 4-OT wild-type or mutant (290 μM) was incubated with **3** (30 mM) and **6** (15 mM) in NaD_2PO_4 buffer (20 mM; pD 7.6, final volume of 650 μL in an NMR tube), containing 18-crown-6 ether as internal standard (2.15 mM). A control sample was prepared with

all the components except for the enzyme. ^1H NMR spectra were recorded ~ 2 h after the start of the incubation, and then after 1, 4, 8 and 14 days.

Compound 12: ^1H NMR (500 MHz, 20 mM NaD_2PO_4 ; pD 7.6): δ 9.94 (s, 1H), 7.97 (d, $J = 7.9$ Hz, 2H), 7.76 (d, $J = 7.5$ Hz, 1H), 7.63 (dd, $J = 7.9, 7.5$ Hz, 2H).

Compound 13: ^1H NMR (500 MHz, 20 mM NaD_2PO_4 ; pD 7.6): δ 9.74 (s, 1H), 7.48 – 7.36 (m, 5H), 5.23 (s, 1H), 1.03 (s, 3H).

Hydrated form of 13: ^1H NMR (500 MHz, 20 mM NaD_2PO_4 ; pD 7.6): δ 7.48 – 7.36 (m, 5H), 5.21 (s, 1H), 4.94 (s, 1H), 0.94 (s, 3H).

Compound 14: ^1H NMR (500 MHz, 20 mM NaD_2PO_4 ; pD 7.6): δ 9.49 (s, 1H), 7.67 (d, $J = 7.5$ Hz, 2H), 7.55 – 7.50 (m, 4H), 2.03 (s, 3H).

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References:

- [1] G. J. Poelarends, V. Puthan Veetil, C. P. Whitman, *Cell. Mol. Life Sci.* **2008**, 65, 3606–3618.
- [2] B.-J. Baas, E. Zandvoort, E. M. Geertsema, G. J. Poelarends, *ChemBioChem.* **2013**, 14, 917–926.
- [3] C. P. Whitman, *Arch. Biochem. Biophys.* **2002**, 402, 1–13.

- [4] S. Harayama, M. Rekik, K. L. Ngai, L. N. Ornston, *J. Bacteriol.* **1989**, *171*, 6251–6258.
- [5] C. P. Whitman, B. A. Aird, W. R. Gillespie, N. J. Stolowich, *J. Am. Chem. Soc.* **1991**, *113*, 3154–3162.
- [6] J. T. Stivers, C. Abeygunawardana, A. S. Mildvan, G. Hajipour, C. P. Whitman, *Biochemistry* **1996**, *35*, 814–823.
- [7] a) Y. Miao, M. Rahimi, E. M. Geertsema, G. J. Poelarends, *Curr. Opin. Chem. Biol.* **2015**, *25*, 115–23; b) C. K. Prier, F. H. Arnold, *J. Am. Chem. Soc.* **2015**, *137*, 13992–14006; c) H. Renata, Z. J. Wang, F. H. Arnold, *Angew. Chem. Int. Ed.* **2015**, *54*, 3351–3367; d) X. Garrabou, T. Beck, D. Hilvert, *Angew. Chem. Int. Ed.* **2015**, *54*, 5609–5612; e) A. Pabis, S. C. L. Kamerlin, **2016**, *37*, 14–21.
- [8] E. Zandvoort, E. M. Geertsema, B.-J. Baas, W. J. Quax, G. J. Poelarends, *ChemBioChem.* **2012**, *13*, 1869–1873.
- [9] Y. Miao, E. M. Geertsema, P. G. Tepper, E. Zandvoort, G. J. Poelarends, *ChemBioChem.* **2013**, *14*, 191–194.
- [10] E. Zandvoort, B.-J. Baas, W. J. Quax, G. J. Poelarends, *ChemBioChem.* **2011**, *12*, 602–609.
- [11] E. Zandvoort, E. M. Geertsema, B.-J. Baas, W. J. Quax, G. J. Poelarends, *Angew. Chem. Int. Ed. Engl.* **2012**, *51*, 1240–1243.
- [12] E. Zandvoort, E. M. Geertsema, W. J. Quax, G. J. Poelarends, *ChemBioChem.* **2012**, *13*, 1274–1277.
- [13] E. M. Geertsema, Y. Miao, P. G. Tepper, P. de Haan, E. Zandvoort, G. J. Poelarends, *Chem. Eur. J.* **2013**, *19*, 14407–14410.
- [14] E. M. Geertsema, Y. Miao, G. J. Poelarends, in *Practical Methods in Biocatalysis and Biotransformations*, Vol. 3 (Eds: J. Whittall, P. Sutton, W. Kroutil), Wiley, UK., **2016**.
- [15] E. M. Geertsema, G. J. Poelarends, in *Science of Synthesis: Biocatalysis in Organic Synthesis*, Vol. 2 (Eds: K. Faber, W. D. Fessner, N. Turner), Thieme Chemistry, Stuttgart, Germany, p.313–333, **2014**.
- [16] M. Rahimi, E. M. Geertsema, Y. Miao, J.-Y. van der Meer, T. van den Bosch, P. de Haan, E. Zandvoort, G. J. Poelarends, *Org. Biomol. Chem.* **2017**, DOI: 10.1039/C7OB00302A.
- [17] H. Poddar, M. Rahimi, E. M. Geertsema, A.-M. W. H. Thunnissen, G. J. Poelarends, *ChemBioChem.*, **2015**, *16*, 738–741.

- [18] a) T. D. Machajewski, C.-H. Wong, *Angew. Chem. Int. Ed. Engl.* **2000**, 39, 1352–1375; b) C.F. Barbas III, Y.-F. Wang, C.-H. Wong, *J. Am. Chem. Soc.* **1990**, 112, 2013–2014.
- [19] a) P. Clapés, W.-D. Fessner, G. A Sprenger, A. K. Samland, *Curr. Opin. Chem. Biol.* **2010**, 14, 154–167; b) X. Garrabou, J. A. Castillo, C. Guérard-Hélaine, T. Parella, J. Joglar, M. Lemaire, P. Clapés, *Angew. Chem. Int. Ed.* **2009**, 48, 5521–5525.
- [20] M. Rahimi, J.-Y. van der Meer, E. M. Geertsema, H. Poddar, B.-J. Baas, G. J. Poelarends, *ChemBioChem*. **2016**, 17, 1225–1228.
- [21] J.-Y. van der Meer, H. Poddar, B.-J. Baas, Y. Miao, M. Rahimi, A. Kunzendorf, R. van Merkerk, P. G. Tepper, E. M. Geertsema, A.-M. W. H. Thunnissen, W. J. Quax, G. J. Poelarends, *Nat. Commun.* **2016**, 7:10911 doi: 10.1038/ncomms10911.
- [22] B.-J. Baas, E. Zandvoort, A. A. Wasiele, G. J. Poelarends, *FEBS Open Bio* **2014**, 4, 651–658.
- [23] M. T. Reetz, M. Bocola, J. D. Carballeira, D. Zha, A. Vogel, *Angew. Chem. Int. Ed. Engl.* **2005**, 44, 4192–4196.
- [24] M. T. Reetz, D. Kahakeaw, R. Lohmer, *ChemBioChem*. **2008**, 9, 1797–1804.
- [25] H. Raj, B. Weiner, V. Puthan Veetil, C. R. Reis, W. J. Quax, D. B. Janssen, B. L. Feringa, G. J. Poelarends, *ChemBioChem*. **2009**, 10, 2236–2245.
- [26] J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, **1989**.
- [27] W. J. Waddell, *J. Lab. Clin. Med.* **1956**, 48, 311–314.

Table of Contents

Engineering a promiscuous enzyme: we have used a systematic mutagenesis strategy, followed by combinatorial mutagenesis of identified ‘hotspot’ positions and activity screening, to greatly enhance the aldolase activity of 4-oxalocrotonate tautomerase (4-OT) for the self-condensation of linear aliphatic aldehydes yielding α,β -unsaturated aldehydes.

Aldol self-condensations catalyzed by an engineered 4-OT variant

