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# Tuning Enzyme Activity for Nonaqueous Solvents: Engineering of an Enantioselective ‘Michaelase’ for Catalysis in High Concentrations of Ethanol

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## ABSTRACT

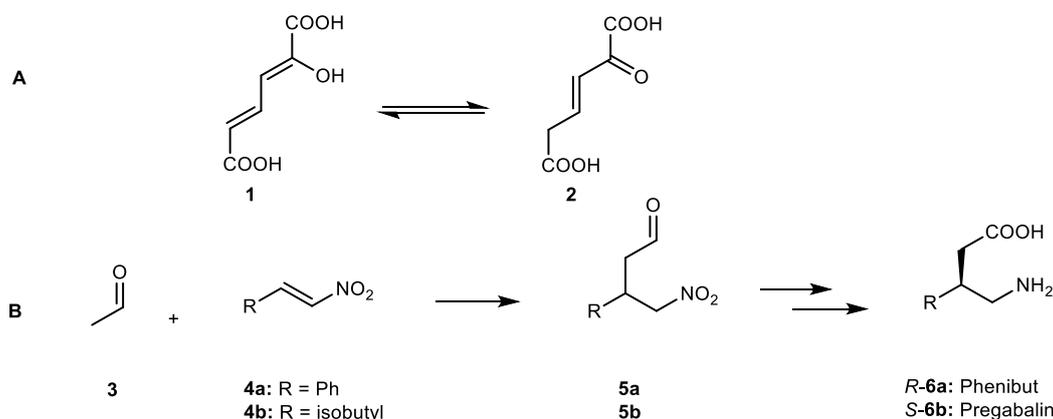
Enzymes have evolved to function under aqueous conditions and may not exhibit features essential for biocatalytic application, such as the ability to function in high concentrations of an organic solvent. Consequently, protein engineering is often required to tune an enzyme for catalysis in non-aqueous solvents. In this study, we have used a collection of nearly all single mutants of 4-oxalocrotonate tautomerase, which promiscuously catalyzes synthetically useful Michael-type additions of acetaldehyde to various nitroolefins, to investigate the effect of each mutation on the ability of this enzyme to retain its ‘Michaelase’ activity in elevated concentrations of ethanol. Examination of this mutability landscape allowed the identification of two hotspot positions, Ser-30 and Ala-33, at which mutations are beneficial for catalysis in high ethanol concentrations. The ‘hotspot’ position Ala-33 was then randomized in a highly enantioselective, but ethanol-sensitive 4-OT variant (L8F/M45Y/F50A) to generate an improved enzyme variant (L8F/A33I/M45Y/F50A) that showed great ethanol stability and efficiently catalyzes the enantioselective addition of acetaldehyde to nitrostyrene in 40% ethanol (permitting high substrate loading) to give the desired  $\gamma$ -nitroaldehyde product in excellent isolated yield (89%) and enantiopurity (ee = 98%). The presented work demonstrates the power of mutability-landscape-guided enzyme engineering for efficient biocatalysis in non-aqueous solvents.

## Keywords

Enzyme engineering; biocatalysis; solvents; mutability landscape; Michael addition

## Introduction

The enzyme 4-oxalocrotonate tautomerase (4-OT) from *Pseudomonas putida* mt-2 catalyzes the tautomerization of 2-hydroxy-2,4-hexadienedioate (**1**) to 2-oxo-3-hexenedioate (**2**) as part of a metabolic pathway for the degradation of aromatic hydrocarbons (Scheme 1A).<sup>[1,2]</sup> In addition, 4-OT can promiscuously catalyze several C-C bond-forming reactions, including Michael-type additions and aldol condensations, yielding precursors for important classes of pharmaceuticals.<sup>[3–7]</sup> For instance, the 4-OT catalyzed Michael-type addition of acetaldehyde (**3**) to nitroalkenes **4a** and **4b** yields  $\gamma$ -nitroaldehydes **5a** and **5b**, important precursors for the  $\gamma$ -aminobutyric acid analogues phenibut (*R*-**6a**) and pregabalin (*S*-**6b**), respectively (Scheme 1B).<sup>[5]</sup> Hence, several enzyme engineering studies have been performed to improve the activity and enantioselectivity of 4-OT for this reaction.<sup>[8,9]</sup>



**Scheme 1.** A) Tautomerization reaction naturally catalyzed by 4-OT. B) Michael-type addition of acetaldehyde (**3**) to nitroalkenes **4a** and **4b**, promiscuously catalyzed by 4-OT. Products **5a** and **5b** are precursors for phenibut (*R*-**6a**) and pregabalin (*S*-**6b**), respectively.

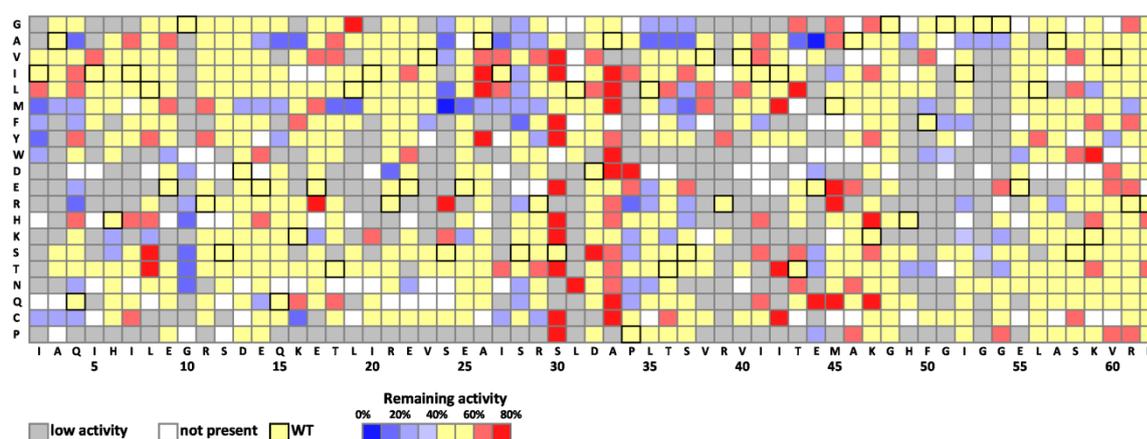
Solubilization of substrates **4a** and **4b** requires the use of cosolvents. Since enzymes have evolved to function under aqueous conditions, high concentrations of cosolvents can significantly affect their catalytic performance and eventually result in enzyme

precipitation.<sup>[10]</sup> In this study, we used a collection of nearly all single-mutant variants of 4-OT to investigate the effect of each mutation on the ability of the enzyme to retain its ‘Michaelase’ activity in elevated concentrations of ethanol. Ethanol was selected as cosolvent because it is readily accessible from biorenewable sources and can also function as a precursor for **3**.<sup>[11]</sup> Randomization of the identified ‘hotspot’ position Ala-33 in the context of a previously engineered highly enantioselective, but ethanol-sensitive, 4-OT variant (L8F/M45Y/F50A) afforded an improved enzyme variant (4-OT L8F/A33I/M45Y/F50A) with high ethanol stability, allowing efficient and enantioselective Michael-type addition reactions in 40% (v/v) ethanol. As such, our work provides an interesting example of how targeted mutagenesis of a single amino acid can radically modify the cosolvent stability of an enzyme, allowing efficient catalysis in high concentrations of ethanol.

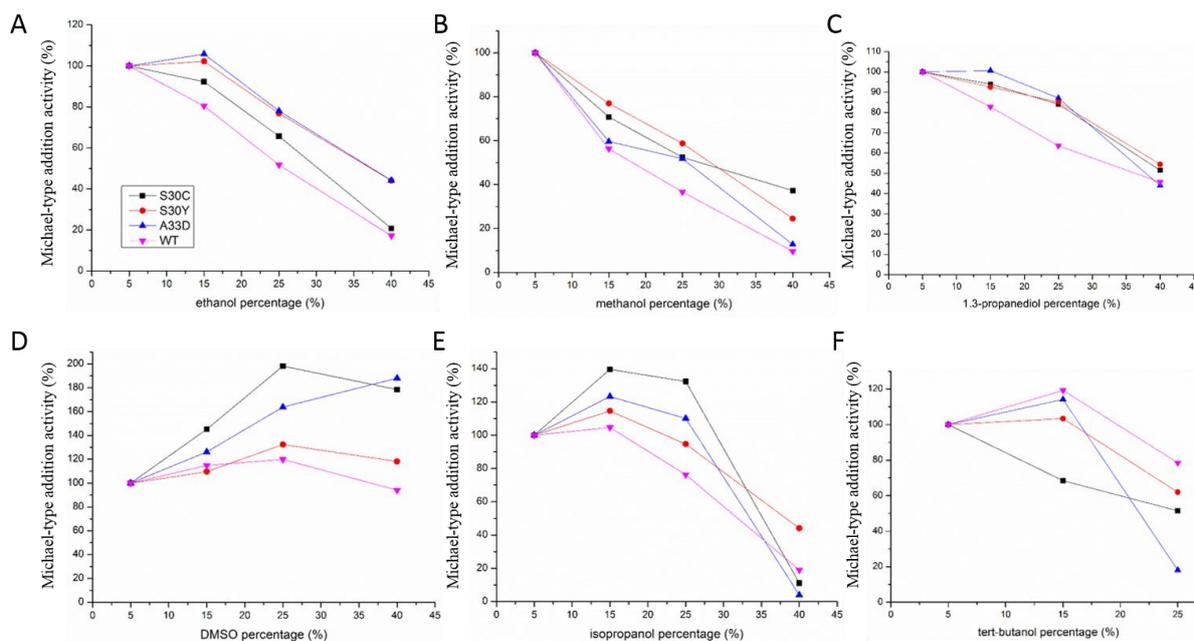
## Results

In order to identify ‘hotspot’ positions of 4-OT at which mutations are beneficial for catalysis in high concentrations of ethanol, a defined collection of 1040 single-mutant variants of 4-OT<sup>[8]</sup> was screened using cell-free extracts (CFEs) prepared from cultures each expressing a different 4-OT mutant. The Michael-type addition of **3** to **4a** was used as a model reaction in screening owing to the marked absorbance of **4a** at 320 nm. Control experiments demonstrated no significant difference between the effect of ethanol on the reaction catalyzed by purified 4-OT or 4-OT present in CFE (Figure S1). The ‘Michaelase’ activity of each single-mutant variant of 4-OT was measured using either 5% or 25% ethanol as cosolvent and the remaining activity at 25% ethanol, compared to that at 5% ethanol, was graphically represented in a mutability landscape for solvent tolerance (Figure 1). Increasing the concentration of ethanol from 5% to 25% reduced the ‘Michaelase’ activity of wild-type 4-OT by approximately 50% (Figures 1 and 2A). Interestingly, analysis of the ethanol-tolerance

mutability landscape revealed two ‘hotspot’ positions, Ser-30 and Ala-33, at which single mutations resulted in enzyme variants that showed more than 70% residual ‘Michaelase’ activity at 25% ethanol (Figure 1). Notably, the crystal structure of wild-type 4-OT does not provide an immediate explanation for the improved ethanol tolerance caused by mutations at these two positions (Figure S2), illustrating the importance of mutability-landscape navigation to identify functional ‘hotspot’ positions. Three single mutants, 4-OT S30C, S30Y and A33D, which showed high ethanol tolerance, were purified and the effect of ethanol and other cosolvents on the ‘Michaelase’ activity was tested (Figure 2). Interestingly, 4-OT S30C, S30Y and A33D also showed tolerance towards other cosolvents such as DMSO and isopropanol, suggesting that these mutations convey general cosolvent resistance. Notably, while the 4-OT variants perform well up to 40% DMSO, visible protein precipitation with concomitant loss of activity was observed at DMSO concentrations  $\geq 50\%$  (v/v).



**Figure 1. Ethanol-tolerance mutability landscape of 4-OT.** The horizontal axis of the data matrix represents the residue positions of 4-OT. The vertical axis represents all 20 canonical amino acids. The wild-type amino acid at each position is indicated with a bold square. White squares indicate that this mutant is not present in the collection. The color of the square indicates the residual ‘Michaelase’ activity of a specific single-mutant variant of 4-OT for the addition of **3** to **4a** in 25% ethanol, compared to that in 5% ethanol. Grey boxes indicate that the ‘Michaelase’ activity was too low to determine the remaining activity.



**Figure 2.** Michael-type addition of **3** to **4a** catalyzed by purified wild-type 4-OT or 4-OT mutants in the presence of different cosolvent concentrations. A) ethanol, B) methanol, C) 1,3-propanediol, D) DMSO, E) isopropanol, F) *tert*-butanol. The activity of each mutant is normalized to the activity in the presence of 5% cosolvent.

We next investigated if we could use the information from the solvent-tolerance mutability landscape to engineer a previously constructed highly enantioselective 4-OT variant, L8F/M45Y/F50A (4-OT FYA),<sup>[9]</sup> to function in high concentrations of ethanol. As single mutants at ‘hotspot’ position Ala-33 generally exhibited higher ‘Michaelase’ activity than those at ‘hotspot’ position Ser-30, we focused our mutagenesis strategy on position Ala-33.<sup>[8]</sup> In the context of 4-OT FYA, residue Ala-33 was mutated to all possible amino acids and the nineteen enzyme variants were expressed and purified to homogeneity. Initially, we tested all variants for visible precipitation upon incubation (1 h) of the enzyme with increasing concentrations of ethanol (up to 50%). The parental enzyme 4-OT FYA rapidly precipitated when incubated with ethanol concentrations equal to or greater than 10% (Figure 3D). Interestingly, enzyme variants with isoleucine (A33I/FYA), leucine (A33L/FYA) or valine (A33V/FYA) at position 33 could tolerate up to 50% ethanol without any visible protein

precipitation after 1 hour of incubation. Notably, substitution of Ala-33 to aspartate, glutamate or cysteine in the context of 4-OT FYA also strongly improved the stability of the enzyme in high concentrations of ethanol, tolerating up to 40% ethanol without visible protein precipitation.

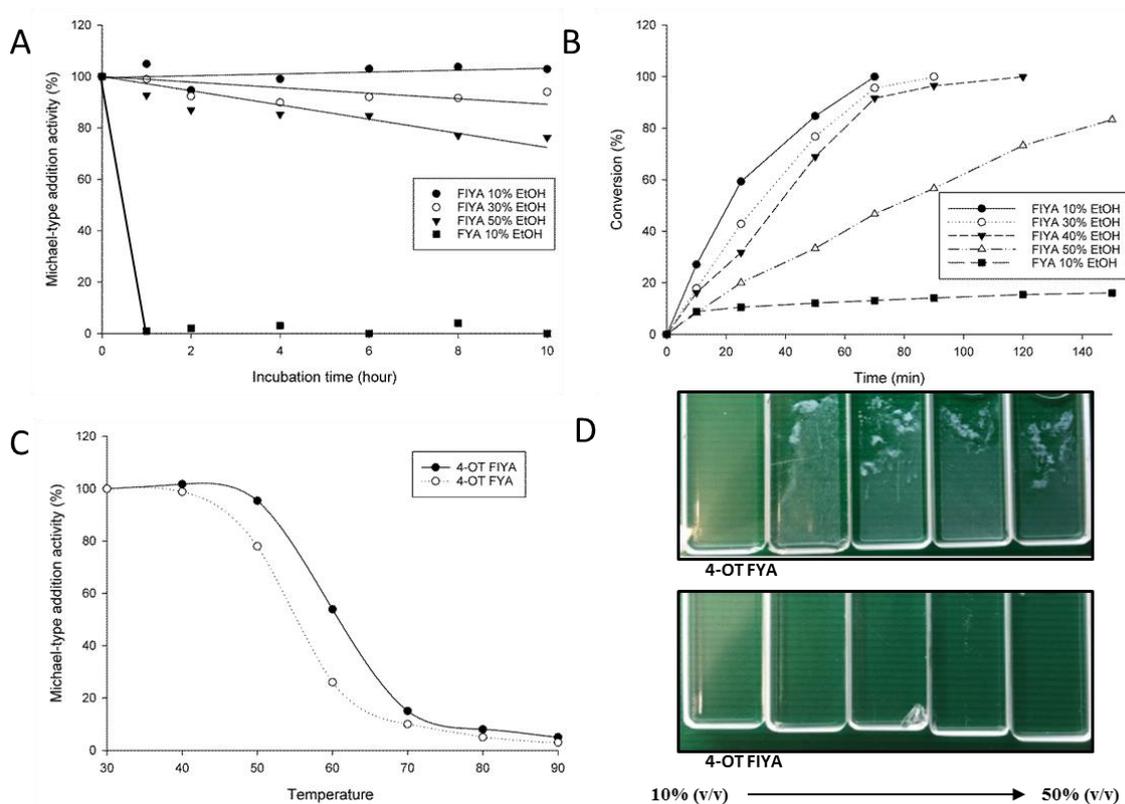
Next, we tested the activity and enantioselectivity of these six quadruple mutants in the presence of 5%, 30% or 50% ethanol. The Michael-type addition of **3** to **4b** was used as model reaction because the optical purity of product **5b** can easily be analyzed by gas chromatography. All six quadruple mutants proved to be highly enantioselective giving nearly enantiopure product **5b**, and, importantly, increasing ethanol concentrations do not negatively affect enzyme enantioselectivity (Table 1). In the presence of 30% ethanol, the reactions with the six quadruple mutants were completed within 35-70 min, whereas the reaction with the parental enzyme (4-OT FYA) showed no conversion due to rapid protein precipitated (Table 1).

**Table 1. Biocatalytic addition of **3** to **4b** catalyzed by 4-OT mutants in different ethanol concentrations.<sup>[a]</sup>**

Enzymes	5% v/v ethanol		30% v/v ethanol		50% v/v ethanol	
	ee <sup>[b]</sup>	Reaction time (min) <sup>[c]</sup>	ee <sup>[b]</sup>	Reaction time (min) <sup>[c]</sup>	ee <sup>[b]</sup>	Reaction time (min) <sup>[c]</sup>
FYA	98 ( <i>S</i> )	40	_ <sup>[d]</sup>	_ <sup>[d]</sup>	_ <sup>[d]</sup>	_ <sup>[d]</sup>
A33D/FYA	98 ( <i>S</i> )	50	98 ( <i>S</i> )	70	92 ( <i>S</i> )	> 360 <sup>[e]</sup>
A33E/FYA	96 ( <i>S</i> )	30	96 ( <i>S</i> )	60	98 ( <i>S</i> )	> 360 <sup>[e]</sup>
A33C/FYA	98 ( <i>S</i> )	55	98 ( <i>S</i> )	70	_ <sup>[d]</sup>	_ <sup>[d]</sup>
A33I/FYA	98 ( <i>S</i> )	30	98 ( <i>S</i> )	35	98 ( <i>S</i> )	90
A33L/FYA	98 ( <i>S</i> )	35	98 ( <i>S</i> )	50	96 ( <i>S</i> )	180
A33V/FYA	98 ( <i>S</i> )	35	98 ( <i>S</i> )	45	98 ( <i>S</i> )	120

<sup>[a]</sup> Assay conditions: 3 mM **4b**, 100 mM **3**, 73  $\mu$ M 4-OT, 20 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 7.3), 0.3 mL reaction volume <sup>[b]</sup> Determined by GC using a chiral stationary phase; the absolute configuration was determined by literature comparison<sup>[8,9]</sup> <sup>[c]</sup> Reaction progress was monitored by following the depletion in absorbance at 249 nm. <sup>[d]</sup> No data due to protein precipitation. <sup>[e]</sup> Reaction was not finished after 360 min.

The best mutant, 4-OT A33I/FYA, catalyzed the Michael-type addition reaction practically as efficient in 40% ethanol as in 10% ethanol (Figure 3B). Moreover, pre-incubation of 4-OT A33I/FYA in 50% ethanol for 10 h resulted in only 25% loss of activity (Figure 3A). On the contrary, the parental enzyme (4-OT FYA) rapidly lost its activity upon incubation in 10% ethanol (Figure 3A, B). Interestingly, an increase in the  $T_{50}^{60}$  of approximately 6 °C was observed for 4-OT A33I/FYA compared to 4-OT FYA, indicating that 4-OT A33I/FYA is also somewhat more thermostable than 4-OT FYA (Figure 3C). Finally, to further demonstrate the synthetic usefulness of 4-OT A33I/FYA, a semi-preparative scale reaction was performed, using 40% ethanol as cosolvent, which allowed for the solubilization of 15 mM **4a**. Using a 6.7-fold excess of **3** over **4a**, the reaction was finished within 200 min. Product *R*-**5a** was obtained in excellent isolated yield (89%) and enantiopurity (ee = 98%) (Figures S3 and S4). Taken together, these results demonstrate that 4-OT can be engineered to efficiently catalyze enantioselective Michael-type reactions in ethanol concentrations up to 40%.



**Figure 3.** Characterization of 4-OT A33I/FYA (FIYA). A) Enzyme activity for the Michael-type addition of **3** to **4a** after pre-incubation (1, 2, 4, 6, 8 or 10 h) in the presence of 10, 30 or 50% ethanol. The data is normalized to the activity of the enzyme without pre-incubation. B) Progress curves of 4-OT A33I/FYA- and 4-OT FYA-catalyzed Michael-type additions in the presence of different ethanol concentrations. C) Temperature-induced inactivation profiles of 4-OT A33I/FYA and 4-OT FYA. The enzyme activity after incubation for 60 min at 30 °C was set as 100%. D) Photograph of cuvettes in which 4-OT FYA (190 μg/mL, top five cuvettes) and 4-OT A33I/FYA (190 μg/mL, bottom five cuvettes) were incubated for 1 h in 20 mM NaH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.3) containing 10%, 20%, 30%, 40% or 50% (v/v) ethanol (cuvette 1 to 5, respectively, from left to right). 4-OT FYA shows rapid precipitation in buffer containing >10% ethanol.

## Discussion

Enzymes are highly attractive catalysts for organic synthesis because of their unparalleled enantio-, regio- and chemoselectivity. Given that enzymes have evolved to operate in the mild aqueous environment of the cell, they are usually not fit for preparative biocatalysis in the presence of high concentrations of organic cosolvents required for substrate solubilization.<sup>[10]</sup>

A solution to this problem is the engineering of enzymes to improve their cosolvent tolerance. Rational enzyme engineering towards increased cosolvent tolerance is still very challenging due to our relatively poor understanding of the interactions between enzymes and solvent molecules.<sup>[12–14]</sup> Currently employed rational engineering strategies include stabilization of flexible regions, introduction of new cysteine bridges and modification of access tunnels.<sup>[14–17]</sup>

An important strategy to guide enzyme-engineering efforts is to make use of mutability landscapes.<sup>[18–21]</sup> By screening a large collection of nearly all single mutants of an enzyme, important information is obtained on single mutations or residue positions that influence a desired characteristic of the enzyme. Here we have applied mutability-landscape guided enzyme engineering to improve the ethanol tolerance of 4-OT. Screening of a collection of nearly all single-mutant variants of 4-OT revealed that mutations at particularly positions Ser-30 and Ala-33 resulted in improved ethanol tolerance. Interestingly, a previously reported 4-OT variant with 3.5-fold increased ‘Michaelase’ activity, 4-OT A33D, also showed improved ethanol tolerance.<sup>[8]</sup> We used the information from the ethanol-tolerance mutability landscape to further engineer a previously constructed highly enantioselective 4-OT variant (FYA) that exhibits poor ethanol stability. All 19 possible variants at position Ala-33 in the context of 4-OT FYA were constructed, expressed and purified. Remarkably, from this small, focused set of quadruple mutants, six mutants showed strongly improved ethanol tolerance. It is interesting to note that all six variants, including 4-OT A33D/FYA, are highly enantioselective towards the synthesis of *S*-**5b**, similar to the parental mutant 4-OT FYA.<sup>[9]</sup>

Conversely, the single mutant 4-OT A33D markedly improved the enantioselectivity towards the opposite enantiomer *R-5b*.<sup>[8]</sup>

Incubation of the best mutant, 4-OT A33I/FYA, for 10 h in the presence of 50% ethanol resulted in only 25% loss of activity, whereas the parental enzyme 4-OT FYA lost all its activity upon incubation for 1 h in 10% ethanol. 4-OT A33I/FYA also showed an increase in thermostability compared to 4-OT FYA, an effect that has also been observed for other enzymes that have been engineered towards increased solvent tolerance.<sup>[22,23]</sup> We further show that 4-OT A33I/FYA can be used to efficiently catalyze the Michael-type addition of **3** to **4a** in the presence of 40% ethanol, which permitted the use of a higher substrate loading (up to 15 mM **4a**). Product *R-5a* could be obtained in good isolated yield (89%) and with excellent enantiopurity (ee = 98%).

In conclusion, our results demonstrate the power of mutability landscapes to guide engineering efforts to improve the cosolvent tolerance of enzymes. By specifically targeting the identified ‘hotspot’ position Ala-33, we could engineer an ethanol-sensitive mutant, 4-OT FYA, into a highly ethanol-resistant mutant 4-OT A33I/FYA. Further tuning of 4-OT A33I/FYA might lead to new synthetic opportunities in almost neat organic solvents.

## Experimental section

**Production of cell-free extract.** Cell-free extracts (CFE) of 4-OT single mutants were prepared according to a reported procedure.<sup>[8]</sup>

**Construction of the ethanol-tolerance mutability landscape.** The CFEs prepared from cells producing 4-OT single-mutant variants were used in two reactions, containing either 5% or 25% v/v ethanol. The following reaction conditions were used: CFE (20% v/v), **3** (50 mM), **4a** (0.5 mM) in 20 mM NaH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.3), 100 μL final volume. The reactions were performed in 96-well microtiter plates (MTP) (UV-star μclear, Greiner Bio-one), covered with UV-transparent plate seals (VIEWseal™, Greiner Bio-one). To ensure proper mixing of the reagents, the plate was shaken (60 s at 500 rpm) immediately after all reaction components were added. The reaction progress was monitored in a plate reader by measuring the depletion in absorbance at 320 nm, corresponding to the concentration of **4a**, for 60 min with a 60 s data interval. The slope of the linear part of the curve was determined for both the reactions. The remaining enzymatic activity was determined by dividing the slope of the reaction in 25% v/v ethanol by the slope of the reaction in 5% v/v ethanol.

**4-OT purification.** The purification of 4-OT single mutants<sup>[24]</sup> and 4-OT quadruple mutants<sup>[9]</sup> are based on previously reported procedures. All purified proteins were >90% pure as assessed by SDS-PAGE. All purified mutants were analyzed by electron spray ionization (ESI) mass spectrometry to confirm the correct mass of the enzyme. The purified protein was flash-frozen in liquid nitrogen and stored at -80 °C until further use.

**UV-spectroscopic assay for the enzymatic activity of 4-OT single mutants in different organic solvents.** The enzymatic activities of the 4-OT mutants and wild-type 4-OT was monitored by following the decrease in absorbance at 320 nm, which corresponds to the

depletion of **4a**. Purified enzyme (150  $\mu$ g, 73  $\mu$ M) was incubated in a 1 mm cuvette with **3** (50 mM) and **4a** (2 mM) in 20 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 7.3; 0.3 mL final volume).

**Construction of nineteen Ala-33 mutants of 4-OT L8F/M45Y/F50A.** Ala-33 was randomized by Quikchange technology using the gene encoding 4-OT L8F/M45Y/F50A cloned in the pET20b vector as the template. The following two primers were used: 5'-GCTCCCTGGATNNKCCGCTGACCAG-3' and 5'-CTGGTCAGCGMNNATCCAGGGAGC-3'. After transformation of the DNA into *E. coli* cells, random colonies were picked from an agar plate, and the mutant 4-OT genes were sequenced by MacroGen Europe (Meibergdreef 31, 1105AZ, Amsterdam, the Netherlands) until all of the 19 quadruple mutants were obtained.

**Activity assays of the six best quadruple mutants.** The enzymatic activities of the 4-OT quadruple mutants and 4-OT L8F/M45Y/F50A were monitored by following the decrease in absorbance at 249 nm, which corresponds to the depletion of **4b**. Purified enzyme (150  $\mu$ g, 73  $\mu$ M) was incubated in a 1 mm cuvette with **3** (100 mM) and **4b** (3 mM) in 20 mM NaH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.3; 0.3 mL final volume). After the reactions were completed, product **5b** was extracted with ethyl acetate (400  $\mu$ L) and analyzed by gas chromatography using an Astec CHIRALDEX G-TA column, isocratic 125 °C. Retention time *S-5b*: 25.6 min, retention time *R-5b*: 26.9. The assignment of the absolute configuration was based on earlier reported data.<sup>[9]</sup>

**Determination of  $T_{50}^{60}$ .** 4-OT L8F/M45Y/F50A and 4-OT L8F/A33I/M45Y/F50A (50  $\mu$ L of 2 mg/mL in 20 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.3) were incubated in 0.2-mL PCR tubes at temperatures ranging from 30 °C to 90 °C for 60 min in a thermal cycler. After incubation, the enzymes were cooled on ice for 10 min followed by equilibration at 25 °C for 10 min. Samples were centrifuged to remove any precipitated protein. The residual 'Michaelase' activity for the addition of **3** to **4a** was tested in a plate reader. Following conditions were used: 25  $\mu$ L of enzyme supernatant, 50 mM **3**, 0.5 mM **4a**, 5% v/v ethanol in 20 mM NaH<sub>2</sub>PO<sub>4</sub> buffer (pH

7.3), 100  $\mu$ l final volume. The 'Michaelase' activities were normalized to that obtained after 60 min incubation at 30 °C.

**Stability of 4-OT L8F/M45Y/F50A and 4-OT L8F/A33I/M45Y/F50A upon incubation with increasing ethanol concentrations.** 4-OT L8F/M45Y/F50A and 4-OT L8F/A33I/M45Y/F50A (1 mL of 1.5 mg/mL in 20 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.3) were incubated in 20 mM NaH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.3) containing 10%, 30% or 50% v/v ethanol in a water bath of 25 °C. Aliquots of enzyme (80  $\mu$ L) were taken at different time intervals and centrifuged to remove any aggregated protein. 50  $\mu$ L of the supernatant was used to test the residual enzymatic activity. The reaction mixture consisted of the following: **3** (50 mM), **4a** (2 mM, from a 40 mM stock solution in 100% (v/v) ethanol) in 20 mM NaH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.3), 0.3 mL final volume. Depletion of **4a** was monitored by following the decrease in absorbance at 320 nm in time. The activities were normalized to the activity measured without incubation of the enzyme.

**Semi-preparative scale synthesis.** To a 50 mL round bottom flask was added: 6 mL ethanol, 112  $\mu$ L **3**, 12 mL buffer (20 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 6.5) containing 4-OT L8F/A33I/M45Y/F50A. The reaction was initiated by the addition of 2 mL ethanol containing 150 mM **4a**. The final concentrations were: **3** (100 mM), **4a** (15 mM), 4-OT L8F/A33I/M45Y/F50A (75  $\mu$ M, based on monomer concentration), and 40% (v/v) ethanol in 20 mM NaH<sub>2</sub>PO<sub>4</sub> buffer (pH 6.5). The reaction progress was monitored using UV-spectrophotometric analysis. At timely intervals, a sample of 30  $\mu$ L was collected from the reaction mixture and diluted to 300  $\mu$ L with 20 mM NaH<sub>2</sub>PO<sub>4</sub> buffer and a full spectrum from 200 nm to 500 nm was recorded. After 200 min, the reaction was finished. The reaction mixture was extracted 3x with ethyl acetate. The combined organic layers were washed with brine and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. The

organic layer was concentrated in vacuo, yielding **5a** without any further purification (51.5 mg, 89% yield). The aldehyde functionality of **5a** was derivatized to a cyclic acetal according to a reported procedure<sup>[5]</sup>. The enantiopurity of derivatized **5a** was determined by reverse phase HPLC using a chiralpak AD-RH column (150 mm x 4.6 mm, Daicel) MeCN/water 70:30. Retention time *R*-**5a**: 7.8 min, *S*-**5a**: 10.8 min.

### Acknowledgement

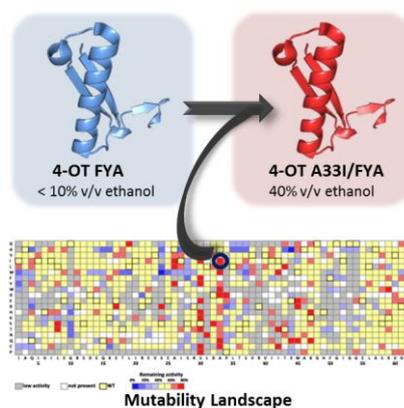
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## TOC graphic and text



**Loving alcohol!** In this study, we generated a mutability landscape of 4-oxalocrotonate tautomerase (4-OT) to identify ‘hotspot’ positions at which mutations are beneficial for catalysis in high concentrations of ethanol. Randomization of one of these ‘hotspot’ residues in a highly enantioselective, but ethanol-sensitive, enzyme variant (4-OT FYA) resulted in an improved enzyme variant (4-OT A33I/FYA) with high ethanol stability, allowing efficient and enantioselective catalysis of Michael reactions in 40% ethanol.