University of Groningen

Engineered Proline-based Carboligases With Improved Catalytic Properties

Guo, Chao

DOI:
[10.33612/diss.160297693](https://doi.org/10.33612/diss.160297693)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2021

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):
Guo, C. (2021). *Engineered Proline-based Carboligases With Improved Catalytic Properties*. [Thesis fully internal (DIV), University of Groningen]. University of Groningen. <https://doi.org/10.33612/diss.160297693>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

**Engineered Proline-based Carboligases With Improved
Catalytic Properties**

Chao Guo

2021

The research described in this thesis was carried out in the department of Pharmaceutical Biology (Groningen Research Institute of Pharmacy, University of Groningen, The Netherlands) and was financially supported by the European Union's Horizon 2020 research and innovation programme under grant agreement No 635595.

The research was carried out according to the requirements of the Graduate School of Science, Faculty of Mathematics and Natural Sciences, University of Groningen, The Netherlands.

Printing of this thesis was financially supported by the University Library and the Graduate School of Science, Faculty of Mathematics and Natural Sciences, University of Groningen, The Netherlands.

Cover design: Chao Guo

Layout and design: David de Groot, www.persoonlijkproefschrift.nl

Printed by: Ridderprint, www.ridderprint.nl

Copyright ©2021 Chao Guo. All rights are reserved. No parts of this thesis may be reproduced or transmitted in any form or by any means without the prior permission in writing of the author.

university of
 groningen

Engineered proline-based carboligases with improved catalytic properties

PhD thesis

to obtain the degree of PhD at the
University of Groningen
on the authority of the
Rector Magnificus Prof. C. Wijmenga
and in accordance with
the decision by the College of Deans.

This thesis will be defended in public on

Friday 12 March 2021 at 9.00 hours

by

Chao Guo

born on 19 November 1987
in Liaoning, China

Supervisors

Prof. G.J. Poelarends

Prof. W.J. Quax

Assessment Committee

Prof. M.W. Fraaije

Prof. G.J.W. Euverink

Prof. L. van Niftrik

Paranimfen

Guangcai Xu

Andreas Kunzendorf

CONTENTS

Aim and Outline of this Thesis	8
Chapter 1	13
Introduction to Protein Engineering of 4-Oxalocrotonate Tautomerase	
Chapter 2	27
Biocatalytic Asymmetric Michael Additions of Nitromethane to α,β -Unsaturated Aldehydes via Enzyme-bound Iminium Ion Intermediates	
Chapter 3	91
Enantioselective Aldol Addition of Acetaldehyde to Aromatic Aldehydes Catalyzed by Proline-based Carboligases	
Chapter 4	165
Tuning Enzyme Activity for Nonaqueous Solvents: Engineering of an Enantioselective ‘Michaelase’ for Catalysis in High Concentrations of Ethanol	
Chapter 5	187
Using Mutability Landscapes to Guide Enzyme Thermostabilization	
Chapter 6	207
Summary and Future Perspectives	
Nederlandse samenvatting	212
Acknowledgements	218

AIM AND OUTLINE OF THIS THESIS

Many enzymes have been reported to catalyze additional and completely distinct types of reactions relative to the natural activity they evolved for, a phenomenon that is known as catalytic promiscuity. Although promiscuous enzyme activities are generally several orders of magnitude lower than native enzyme activities, enzyme promiscuity has proven to be a successful starting point for the engineering of novel biocatalysts for chemical synthesis. Using enzyme promiscuity to develop new biocatalysts is especially attractive for abiological reactions and can contribute to expanding our knowledge of the parameters involved in natural and laboratory enzyme evolution.

A fascinating example of a catalytically promiscuous enzyme is 4-oxalocrotonate tautomerase (4-OT) from *Pseudomonas putida* mt-2, which utilizes an amino-terminal proline as key catalytic residue to promiscuously catalyze C-C bond-forming reactions, such as Michael additions and aldol condensations. The aim of the work described in this thesis was to further explore 4-OT for different synthetically useful C-C bond-forming reactions, as well as to improve its biocatalytic properties by mutability-landscape-guided protein engineering.

In **Chapter 1**, we review previous protein engineering studies on 4-OT, in which significant improvements in the promiscuous C-C bond-forming activities of this enzyme were achieved.

In **Chapter 2**, we report that the F50A mutant of 4-OT is able to efficiently promote enantioselective Michael additions of nitromethane to various α,β -unsaturated aldehydes to give γ -nitroaldehydes, important precursors to biologically active γ -aminobutyric acids. High conversions, high enantiocontrol, and good isolated product yields were achieved. The reactions proceed via reactive iminium ion intermediates, which opens up exciting possibilities for diverse synthetically useful enzymatic bond-forming reactions. In addition, a

cascade of three 4-OT (F50A)-catalyzed reactions followed by an enzymatic oxidation step enables one-pot assembly of γ -nitrobutyric acids from three simpler building blocks.

In **Chapter 3**, we report the biocatalytic aldol condensation of acetaldehyde with various aromatic aldehydes to give a number of aromatic α,β -unsaturated aldehydes using a previously engineered variant of 4-OT [4-OT(M45T/F50A)] as carboligase. Moreover, an efficient one-pot two-step chemoenzymatic route toward chiral aromatic 1,3-diols has been developed. This one-pot chemoenzymatic strategy successfully combined a highly enantioselective aldol addition step catalyzed by a proline-based carboligase [either 4-OT(M45T/F50A) or the 4-OT homologue TAUT015] with a chemical reduction step to convert enzymatically prepared aromatic β -hydroxyaldehydes into the corresponding 1,3-diols with high optical purity (e.r. up to >99:1) and in good isolated yield (up to 92%). These developed (chemo)enzymatic methodologies offer alternative synthetic choices to prepare a variety of important drug precursors.

In **Chapter 4** and **Chapter 5**, we applied a mutability-landscaped-guided protein engineering approach to enhance the stability of 4-OT. In **Chapter 4**, we generated a mutability landscape of 4-OT to identify “hotspot” positions at which mutations are beneficial for catalysis in high concentrations of ethanol. Randomization of one of the identified hotspot residues (Ala-33) in a highly enantioselective but ethanol-sensitive 4-OT variant (L8F/M45Y/F50A) resulted in an improved enzyme variant (L8F/A33I/M45Y/F50A) that showed high ethanol stability, allowing efficient and enantioselective catalysis of Michael addition reactions in 40% ethanol, permitting high substrate loading.

In **Chapter 5**, we generated a mutability landscape of 4-OT to identify “hotspot” positions at which mutations are beneficial for catalysis at elevated temperatures. This led to the identification of three single mutations (R11Y, R11I and A33D) within 4-OT that gave rise to significant increases in apparent melting temperature T_m (up to 20 °C) and in half-life at 80 °C (up to 112-fold).

Introduction of these beneficial mutations in an enantioselective but thermolabile 4-OT variant (M45Y/F50A) afforded improved triple-mutant enzyme variants showing an up to 39 °C increase in T_m value, with no reduction in catalytic activity or enantioselectivity. The studies reported in **Chapter 4** and **Chapter 5** illustrate the power of mutability-landscape-guided protein engineering for tuning enzyme activity in non-aqueous solvents and thermostabilizing enzymes.

Finally, in **Chapter 6** the work described in this thesis is summarized and suggestions for future research are presented.

1

Introduction to Protein Engineering of 4-Oxalocrotonate Tautomerase

Figure 1. Crystal structure of 4-OT WT (PDB: 4OTA).^[1] A) Hexameric structure of 4-OT WT. B) Structure of one dimer of 4-OT WT. C) Structure of one subunit of 4-OT WT. D) Close-up view of the active site showing the key catalytic residues.

4-Oxalocrotonate tautomerase (4-OT) from *Pseudomonas putida* mt-2 is composed of six identical subunits of only 62 amino acid residues each (Figure 1A). It belongs to the tautomerase superfamily, a group of homologous proteins that share a unique catalytic amino-terminal proline (Pro1) and a characteristic β - α - β structural fold (Figure 1B and 1C).^[1] 4-OT naturally catalyzes the tautomerization of 2-hydroxymuconate (**1**) to 2-oxohex-3-enedioate (**2**) in *Pseudomonas putida* mt-2, a catalytic step in the metabolism of aromatic hydrocarbons (Scheme 1). Three key catalytic residues are involved in the tautomerization of **1**. The Pro1 residue functions as a general base ($\text{p}K_a \approx 6.4$) that transfers the 2-hydroxyl proton of **1** to the C5-position to give **2**. The other two catalytic residues are Arg11 and Arg39. Arg11 is proposed to interact with the C-6 carboxylate group in a bidentate fashion, and Arg39 is proposed to interact

with the 2-hydroxyl group of **1** and a C-1 carboxylate oxygen. The interaction between Arg11 and the C-6 carboxylate group may draw electron density toward C-5 to facilitate protonation.

Scheme 1. Reaction naturally catalyzed by 4-oxalocrotonate tautomerase as part of a catabolic pathway for aromatic hydrocarbons in *Pseudomonas putida* mt-2.

Inspired by the tremendous success of aminocatalysis, 4-OT was found to promiscuously catalyze carbon-carbon (C-C) bond-forming reactions, including Michael and aldol additions *via* enamine intermediates (Scheme 2).^[9] The Pro1 residue of 4-OT first attacks the carbonyl carbon of an aliphatic aldehyde, such as acetaldehyde, producing a reactive enamine intermediate (Scheme 2).^[2] In 4-OT catalyzed aldol additions, the enamine intermediate acts as a nucleophile to attack the carbonyl carbon of an acceptor aldehyde, such as benzaldehyde, yielding β -hydroxy aldehydes, while the product might further undergo an enzyme-catalyzed dehydration step to give α,β -unsaturated aldehydes (Scheme 2).^[2] In 4-OT catalyzed Michael additions, the enamine intermediate was found to attack the β -carbon of nitroalkenes yielding γ -nitrobutyric aldehydes.

PROTEIN ENGINEERING OF 4-OXALOCROTONATE TAUTOMERASE FOR ALDOL REACTIONS

Scheme 2. Proposed mechanism of 4-OT-catalyzed Michael and aldol addition reactions.

Poelarends and coworkers discovered that 4-OT wild-type (WT) is able to catalyze the aldol condensation of acetaldehyde with benzaldehyde to yield cinnamaldehyde. The proposed catalytic mechanism involves formation of an enamine intermediate between acetaldehyde and the Pro1 residue of 4-OT, as shown by mass spectrometry and X-ray crystallography experiments.^[2-4] The low-level aldolase activity can be improved 16-fold by introducing a single point mutation (Leu8Arg, L8R) in the active site of 4-OT (Table 1, entry 1 and 2). Using a mechanism-inspired engineering approach, Zandvoort et al. generated a 4-OT mutant (Phe50Ala, F50A) with a strongly enhanced aldol condensation activity (600-fold in terms of $k_{\text{cat}}/K_{\text{m}}$, Table 1, entry 3).^[2] In the same study, it was demonstrated that 4-OT F50A catalyzes the dehydration step, converting **5** into **6**.

A systematic protein engineering strategy, a so-called mutability-landscape-guided protein engineering approach, was applied by Rahimi et al. to improve the aldolase activity of 4-OT [6,7]. Three ‘hotspot’ positions, His-6, Met-45 and Phe-50, at which single mutations greatly improve the aldolase activity of 4-OT for the cross-condensation of different aldehydes were identified.[6] The further combinatorial mutagenesis of ‘hotspot’ positions led to the generation of 4-OT mutants Met45Thr/Phe50Ala (M45T/F50A) and His6Phe/Met45Thr/Phe50Ala (H6F/M45T/F50A) with ~3300-fold and ~5276-fold improved aldolase activity in terms of $k_{\text{cat}}/K_{\text{m}}$ compared to that of 4-OT WT, respectively (Table 1, entry 8-10). The same strategy was applied to engineering 4-OT into a more efficient aldolase for self-condensation reactions of linear aliphatic aldehydes (Table 1, entry 11-13).[7] The generated 4-OT mutant Met45Tyr/Phe50Val (M45Y/F50V) was found to have improved self-coupling activity.

Another study on 4-OT WT and the 4-OT F50A mutant by Rahimi et al. showed that these enzymes are capable of accepting various carbonyl compounds as substrates for both inter- and intramolecular aldol reactions (Table 1, entry 4-7 and 14-17).[5] The ¹H-NMR spectroscopy monitored self-condensation of propionaldehyde showed that the yields of the corresponding product **6** were ~5% and 27% after 4 d for the reactions catalyzed by 4-OT WT and F50A, respectively (Table 1, entry 4 and 5). Importantly, no ¹H NMR signals corresponding to the presumed aldol product **5** were observed. In the same study, analysis of the ¹H NMR spectra of the 4-OT F50A-catalyzed reaction between propionaldehyde and benzaldehyde revealed accumulation of aldol cross-coupling product **5** to ~36% after 1d, which decreased to ~20% with an increase in yield of **6** to 15% after 14 d (Table 1, entry 7). These observations suggest that 4-OT F50A catalyzed a relatively fast aldol cross-coupling of propionaldehyde to benzaldehyde to give aldol product **5** followed by a slower dehydration of **5** to yield **6** (Table 1, entry 7). A similar observation was made for the 4-OT- and F50A-catalyzed intramolecular aldol reactions (Table 1, entry 14-17).

Table 1. Substrates, biocatalysts, product structures, relative $k_{\text{cat}}/K_{\text{m}}$ and product yields for aldol reactions catalyzed by wild-type 4-OT and 4-OT variants.

Entry	Substrate(s)	R ₁	R ₂	4-OTs	Products	Relative $k_{\text{cat}}/K_{\text{m}}$	t (d)	Yield (%) ^b	Ref ^c
1	3 and 4	Ph	H	WT	6	1	14	10	[3]
2	3 and 4	Ph	H	L8R	6	16	- ^a	-	[3]
3	3 and 4	Ph	H	F50A	6	600	1	16	[2]
4	3 and 4	Et	Me	WT	6	-	4	5	[5]
5	3 and 4	Et	Me	F50A	6	-	4	27	[5]
6	3 and 4	Ph	Me	WT	5 and 6	-	14	-	[5]
7	3 and 4	Ph	Me	F50A	5 and 6	-	14	20/15	[5]
8	3 and 4	Ph	H	F50V	6	636	-	-	[6]
9	3 and 4	Ph	H	M45T/F50A	6	3300	-	-	[6]
10	3 and 4	Ph	H	H6F/M45T/F50A	6	5276	-	-	[6]
11	3 and 4	Et	Me	M45Y/F50V	6	-	-	-	[7]
12	3 and 4	Me	H	M45Y/F50V	6	-	-	-	[7]
13	3 and 4	n-Pr	Et	M45Y/F50V	6	-	-	-	[7]
14	7 (n = 1)	-	-	WT	8 and 9	-	1	40/10	[5]
15	7 (n = 1)	-	-	F50A	8 and 9	-	1	67/23	[5]
16	7 (n = 2)	-	-	WT	8 and 9	-	0.9	42/8	[5]
17	7 (n = 2)	-	-	F50A	8 and 9	-	0.9	81/11	[5]

a. not applicable; b. yields according to literature records; c. References.

PROTEIN ENGINEERING OF 4-OXALOCROTONATE TAUTOMERASE FOR MICHAEL ADDITION REACTIONS

The 4-OT-catalyzed Michael addition of acetaldehyde to nitroalkenes *via* enamine intermediates produces γ -nitrobutyraldehydes as products, which are precursors for pharmaceutically active γ -aminobutyric acids (GABAs, Scheme 2).^[8] Zandvoort et al. first discovered that 4-OT is capable of promoting the Michael addition of acetaldehyde to nitroalkenes **10a** and **10b** (Table 2, entry 1 and 2).^[9] Compared with organocatalysis, these 4-OT-catalyzed Michael additions achieved higher enantioselectivity (up to 89% *ee*), while using relatively low catalyst loading (0.7 mol% compared to **10a** and **10b**) and water as reaction medium. Encouraged by these initial findings, a further study by Poelarends and co-workers demonstrated that 4-OT also accepts linear aldehydes, ranging from propanal to octanal, as donor substrates for addition to **10a**.^[10] As shown in Table 2 (entry 3 and 4), using **4b** or **4c** as donor substrate decreases the enantioselectivity and reaction rate, compared with using **4a** as donor. Indeed, increasing bulkiness of the aldehyde substrates did not influence the diastereoselectivity but diminished enantioselectivity and slowed down the reaction rate.^[10] In a following study, it was demonstrated that 4-OT is able to catalyze the asymmetric Michael addition of acetaldehyde (**1**) to a series of nitroalkenes, achieving good conversions and good product *ee* and yield (Table 2, entry 5-7).^[8] Given the broad substrate scope and high stereoselectivity of 4-OT, it would be of interest to improve the catalytic efficiency of 4-OT in Michael addition reactions by protein engineering.

A mutability-landscape-guided protein engineering approach was applied by van der Meer et al. in order to improve the ‘Michaelase’ activity and enantioselectivity of 4-OT.^[11] Analysis of the mutability landscape revealed four ‘hotspot’ positions, His-6, Ala-33, Met-45 and Phe-50, at which point mutations

significantly improved the specific activity of 4-OT for Michael additions. The best single mutants have 3-5-fold improved Michael addition activity of **4c** to **10a** compared to 4-OT WT. Notably, single mutations at position Ala-33 also have significantly improved activity of **4a** to **10a** (Table 2).

Table 2. Substrates, biocatalysts, products, reaction time, conversion and product enantiomeric excess and yield of the Michael addition of aldehydes to nitroalkenes catalyzed by wild-type 4-OT or 4-OT variants.

Entry	Substrates	4-OTs (mol% <i>a</i>)	Product (Abs. Conf.)	Conversion (%)	t (h)	Yield (%) <i>b</i>	ee (%)	Ref ^c
1	4a and 10a	WT (0.7)	11a (<i>S</i>)	46	3	41	89	[9]
2	4a and 10b	WT (0.7)	11b (<i>S</i>)	65	3	59	51	[9]
3	4b and 10a	WT (1.4)	11f (2 <i>R</i> ,3 <i>S</i>) <i>d</i>	- <i>e</i>	4	64	50	[10]
4	4c and 10a	WT (1.4)	11g (2 <i>R</i> ,3 <i>S</i>)	-	6	57	38	[10]
5	4a and 10c	WT (1.8)	11c (<i>S</i>)	>99	2	49	74	[8]
6	4a and 10d	WT (2.8)	11d (<i>S</i>)	>99	2.5	51	69	[8]
7	4a and 10e	WT (5.3)	11e (<i>R</i>)	-	0.4	74	98	[8]
8	4a and 10a	A33D (1.4)	11a (<i>S</i>)	>99	0.7	94	98	[11]
9	4a and 10d	A33D (2.8)	11d (<i>S</i>)	>99	1.2	81	96	[11]
10	4a and 10e	A33D (5.3)	11e (<i>R</i>)	>99	0.8	60	99	[11]

Continued:

Entry	Substrates	4-OTs (mol% ^a)	Product (Abs. Conf.)	Conversion (%)	t (h)	Yield (%) ^b	ee (%)	Refs
11	4a and 10a	M45Y/F50A (0.7)	11a (<i>R</i>)	>99	21	65	92	[11]
12	4a and 10d	M45Y/F50A (2.8)	11d (<i>R</i>)	>99	1.5	60	24	[11]
13	4a and 10e	M45Y/F50A (5.3)	11e (<i>S</i>)	>99	1.2	60	80	[11]
14	4a and 10a	L8Y/M45Y/F50A (2.5)	11a (<i>R</i>)	>99	1.3	87	96	[12]
15	4a and 10d	L8Y/M45Y/F50A (2.15)	11d (<i>R</i>)	>99	1	97	99	[12]
16	4a and 10e	L8Y/M45Y/F50A(1.4)	11e (<i>S</i>)	>99	0.8	63	99	[12]

a. Compared to nitroalkene; b. Isolated yield; c. References; d. The *d.r.* for **11f** and **11g** are 93:7 and 89:11, respectively; e. Not determined.

In the same study, mutability landscape navigation also led to the identification of ‘hotspot’ positions at which mutations have improved or inverted enantioselectivity for the Michael addition reaction. Several single mutations at residue positions Ala-33, Arg-39, Ala-57 and Arg-61 were found to improve enantioselectivity, while single mutations at positions His-6, Arg-11, Met-45, Phe-50 and Gly-54 have the most pronounced effect on inversion of enantioselectivity. Notably, mutant Ala33Asp (A33D) has both improved activity and enantioselectivity compared to 4-OT WT (Table 2, entry 8-9).^[11] Combinatorial mutagenesis of ‘hotspot’ positions led to the identification of mutant Met45Tyr/Phe50Ala (M45Y/F50A), which was able to catalyze the Michael addition of **4a** to versatile nitroalkenes to produce the pharmaceutically relevant enantiomers of the corresponding γ -nitrobutyraldehyde products with high *ee* (Table 2, entry 11-13).

In a following study by Biewenga et al., a structure-guided protein engineering approach was used to generate a 4-OT mutant, Leu8Tyr/Met45Tyr/Phe50Ala (L8Y/M45Y/F50A), that exhibited excellent enantioselectivity (yielding *S*-**11e** with 99% *ee*) and an ~6-fold activity improvement compared to M45Y/F50A (Table 2, entry 16).^[12] In the same study, a one-pot two-step biocatalytic cascade was also developed for the synthesis of the pharmaceutically relevant enantiomers

of γ -nitrobutyric acids, starting from simple precursors (i.e., acetaldehyde and nitroalkenes). Furthermore, a three-step chemoenzymatic cascade route for the synthesis of GABA analogues in one pot was achieved with high enantiopurity and high overall yields (Scheme 3).^[12] 4-OT L8Y/M45Y/F50A, an aldehyde dehydrogenase (ALDH) and a cofactor recycling system (NOX) were used to construct these chemoenzymatic routes.

Scheme 3. One-pot three-step chemoenzymatic cascade for synthesis of GABA analogues.

The highly reactive nature of acetaldehyde requires intricate handling, which can impede its usage in practical synthesis. Biewenga et al. therefore investigated several enzymatic routes to synthesize acetaldehyde in situ in one-pot cascade reactions with 4-OT.^[13] Two routes afforded practical acetaldehyde concentrations, using an environmental pollutant, *trans*-3-chloroacrylic acid, or an inexpensive bio-renewable, ethanol, as starting substrate. Importantly, these routes can be combined with 4-OT catalyzed Michael-type additions and aldol reactions in one pot. This methodology provides a stepping stone towards the development of larger enzymatic cascades for the practical synthesis of diverse chemical synthons. Particularly ethanol is an attractive precursor of acetaldehyde because it can simultaneously act as a co-solvent to solubilize the Michael- and aldol-acceptor substrates.

In 4-OT-catalyzed nitromethane additions to α,β -unsaturated aldehydes, the carbonyl carbon of the α,β -unsaturated aldehyde is first attacked by Prol to give a reactive iminium ion intermediate (Scheme 2), which then reacts with

nitromethane to give a γ -nitroaldehyde as final product (Chapter 2). 4-OT's ability to promiscuously catalyze asymmetric carbonyl transformations via iminium ion intermediates likely opens up new possibilities for various synthetically useful enzymatic bond-forming reactions.

During the period of finalizing this thesis, Poelarends and co-workers have established a colorimetric “turn-on” probe as a prescreening tool to facilitate engineering of 4-OT in iminium catalysis.^[14] The mechanism behind this system involves the formation of a brightly colored merocyanine-dye-type structure between the probe, 2-hydroxy-cinnamaldehyde, and the catalytic Prol of 4-OT upon complexation. Use of this system in a solid-phase prescreening assay resulted in the reduction of the screening effort up to 20-fold, and after two rounds of directed evolution, two 4-OT triple mutants were identified with up to 39-fold improvement in activity for the addition of nitromethane to cinnamaldehyde. The corresponding product was obtained in excellent isolated yield (up to 95%) and with high enantiopurity (> 99% *ee*).

The ability of 4-OT to promiscuously catalyze asymmetric carbonyl transformations via iminium ion intermediates was also exploited to promote C-O bond-forming reactions.^[15] In this case, different hydroperoxides (*t*-BuOOH and H₂O₂) were used as nucleophiles (instead of nitromethane) in addition to cinnamaldehyde. The resulting enamine intermediate subsequently undergoes ring closure to construct the final epoxide moiety. A mutability-landscape-guided protein engineering approach, followed by iterative combinatorial mutagenesis, was used by Xu et al. to construct a 4-OT mutant, Q4Y/M45I/F50A (YIA), that showed 60-fold enhancement in activity for the epoxidation of cinnamaldehyde by *t*-BuOOH compared to that of 4-OT WT.^[15] The authors further showed that 4-OT YIA accepts different hydroperoxides (*t*-BuOOH and H₂O₂) to accomplish enantiocomplementary epoxidations of various α,β -unsaturated aldehydes (citral and substituted cinnamaldehydes), providing access to both enantiomers of the

corresponding α,β -epoxy-aldehydes with high conversions (up to 98%), high enantioselectivity (up to 98% *ee*), and good product yields (50-80%).

In conclusion, 4-OT utilizes its unusual N-terminal proline to facilitate versatile non-native bond-forming reactions such as Michael additions, aldol reactions and recently discovered epoxidation reactions. Application of advanced high-throughput screening procedures in the directed evolution of 4-OT has proven very successful in ongoing research, allowing the development of more effective biocatalysts for chemical synthesis, bringing the technology closer to industrial application.

REFERENCES

- [1] A. B. Taylor, R. M. Czerwinski, W. H. Johnson Jr., C. P. Whitman, and M. L. Hackert, *Biochemistry* **1998**, *37*, 14692–14700.
- [2] E. Zandvoort, E. M. Geertsema, W. J. Quax, G. J. Poelarends, *ChemBioChem* **2012**, *13*, 1274–1277.
- [3] E. Zandvoort, B. J. Baas, W. J. Quax, G. J. Poelarends, *ChemBioChem* **2011**, *12*, 602–609.
- [4] H. Poddar, M. Rahimi, E. M. Geertsema, A. M. W. H. Thunnissen, G. J. Poelarends, *ChemBioChem* **2015**, *16*, 738–741.
- [5] 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**, *15*, 2809–2816.
- [6] M. Rahimi, J. Y. van der Meer, E. M. Geertsema, H. Poddar, B. J. Baas, G. J. Poelarends, *ChemBioChem* **2016**, 1225–1228.
- [7] M. Rahimi, J. Y. van der Meer, E. M. Geertsema, G. J. Poelarends, *ChemBioChem* **2017**, *18*, 1435–1441.
- [8] E. M. Geertsema, Y. Miao, P. G. Tepper, P. Dehaan, E. Zandvoort, G. J. Poelarends, *Chem. - A Eur. J.* **2013**, *19*, 14407–14410.
- [9] E. Zandvoort, E. M. Geertsema, B. J. Baas, W. J. Quax, G. J. Poelarends, *Angew. Chemie - Int. Ed.* **2012**, *51*, 1240–1243.
- [10] Y. Miao, E. M. Geertsema, P. G. Tepper, E. Zandvoort, G. J. Poelarends, *ChemBioChem* **2013**, *14*, 191–194.
- [11] 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.
- [12] L. Biewenga, T. Saravanan, A. Kunzendorf, Y. Van Der Meer, T. Pijning, P. Tepper, R. Van Merkerk, S. J. Charnock, A. W. H. Thunnissen, G. J. Poelarends, *ACS Catalysis* **2019**, *9*, 1503–1513.
- [13] L. Biewenga, A. Kunzendorf, G. J. Poelarends, *Chembiochem* **2020**, *21*, 1505–1509.
- [14] L. Biewenga, M. Crotti, M. Saifuddin, G. J. Poelarends, *ACS Omega* **2020**, *5*, 2397–2405.
- [15] G. Xu, M. Crotti, T. Saravanan, K. M. Kataja, G. J. Poelarends, *Angew. Chemie - Int. Ed.* **2020**, *59*, 10374–10378

2

Biocatalytic Asymmetric Michael Additions of Nitromethane to α,β -Unsaturated Aldehydes via Enzyme-bound Iminium Ion Intermediates

Chao Guo

Mohammad Saifuddin

Thangavelu Saravanan

Masih Sharifi

Gerrit J. Poelarends

Published in *ACS Catalysis* **2019**, 9 (5), 4369-4373.

ABSTRACT

The enzyme 4-oxalocrotonate tautomerase (4-OT) exploits an N-terminal proline as main catalytic residue to facilitate several promiscuous C-C bond-forming reactions via enzyme-bound enamine intermediates. Here we show that the active site of this enzyme can give rise to further synthetically useful catalytic promiscuity. Specifically, the F50A mutant of 4-OT was found to efficiently promote asymmetric Michael additions of nitromethane to various α,β -unsaturated aldehydes to give γ -nitroaldehydes, important precursors to biologically active γ -aminobutyric acids. High conversions, high enantiocontrol and good isolated product yields were achieved. The reactions likely proceed via iminium ion intermediates formed between the catalytic Pro-1 residue and the α,β -unsaturated aldehydes. In addition, a cascade of three 4-OT(F50A)-catalyzed reactions followed by an enzymatic oxidation step enables assembly of γ -nitrocarboxylic acids from three simple building blocks in one pot. Our results bridge organo- and biocatalysis, and emphasize the potential of enzyme promiscuity for the preparation of important chiral synthons.

KEYWORDS

Biocatalysis; Michael addition; asymmetric synthesis; enzyme catalysis; protein engineering

γ -Nitroaldehydes are important chiral building blocks for the preparation of biologically active γ -aminobutyric acids. The asymmetric synthesis of γ -nitroaldehydes from simple starting materials has become feasible due to outstanding developments within the organocatalysis field, particularly fueled by aminocatalysis.^[1] This is nicely illustrated by the work of Hayashi and co-workers, who reported that diphenylprolinol silyl ether can promote the asymmetric synthesis of γ -nitroaldehydes through alternative Michael-type reactions: enamine-mediated addition of aldehydes to nitroalkenes, and nitroalkane addition to α,β -unsaturated aldehydes activated as iminium ions.^[1d-f]

Scheme 1. Proposed mechanisms for the 4-OT catalyzed Michael additions of acetaldehyde to nitroalkenes (A) and nitromethane to α,β -unsaturated aldehydes (B) to yield γ -nitroaldehydes.

Inspired by these developments in the organocatalysis field, work from our laboratory focused on the development of a biocatalytic procedure for asymmetric synthesis of γ -nitroaldehydes. We reported that 4-oxalocrotonate tautomerase (4-OT), which utilizes a unique N-terminal proline as key catalytic residue, can promiscuously catalyze the Michael addition of acetaldehyde (as well as various other aldehydes) to nitroalkenes yielding enantioenriched γ -nitroaldehydes (Scheme 1A).^[2] The catalytic mechanism involves the formation of an enamine species between acetaldehyde and the Pro-1 residue ($pK_a \sim 6.4$).^[3,4] Hilvert and coworkers have reported a highly engineered computationally

designed artificial aldolase, RA95.5-8, which can catalyze the asymmetric synthesis of γ -nitroketones (but not γ -nitroaldehydes) via acetone addition to nitrostyrenes, and nitroalkane addition to conjugated ketones.^[5] However, a biocatalytic methodology for nitroalkane addition to α,β -unsaturated aldehydes to yield enantioenriched γ -nitroaldehydes is an as yet unmet challenge.

We previously reported that 4-OT catalyzes the aldol condensation of acetaldehyde with benzaldehyde to yield cinnamaldehyde.^[3,6] Considering that the active site of 4-OT can accommodate cinnamaldehyde, this aromatic α,β -unsaturated aldehyde was tested as potential Michael acceptor substrate. Cinnamaldehyde was expected to react with Pro-1, the catalytic amine, to form a covalently bound iminium ion intermediate, which could be attacked by nitromethane (Scheme 1B). This Michael reaction was performed in the presence of wild-type 4-OT in HEPES buffer (pH 7.3), containing 5% (v/v) EtOH, 200 mM of nitromethane (**1**, Scheme 2) and 3 mM of cinnamaldehyde (**2a**), and reaction progress was monitored by following the depletion of **2a** by UV-VIS spectrophotometry. Under these conditions, 50% of substrate **2a** was consumed in 24 h and the corresponding product **3a** was obtained in 35% isolated yield (as confirmed by ¹H NMR spectroscopy). Analysis of product **3a** by chiral HPLC revealed high enantiocontrol at the site of addition with formation of the (*R*)-configured product (e.r. of 86:14). Interestingly, we earlier reported that wild-type 4-OT catalyzes the Michael addition of acetaldehyde to *trans*- β -nitrostyrene to yield (*S*)-**3a** with an e.r. of 95:5.^[2a] Hence, 4-OT catalyzes the synthesis of γ -nitroaldehyde **3a** via two enantiocomplementary Michael reactions: enamine-mediated addition of acetaldehyde to *trans*- β -nitrostyrene, and nitromethane addition to cinnamaldehyde likely activated as iminium ion.

Scheme 2. Wild-type 4-OT catalyzed Michael addition of nitromethane (**1**) to cinnamaldehyde (**2a**) to yield γ -nitroaldehyde (*R*)-**3a**.

Encouraged by these initial findings, a systematic mutagenesis approach was applied to enhance this promiscuous Michael addition activity of 4-OT. For this, an earlier constructed collection of 4-OT genes coding for almost all possible single-mutant variants of 4-OT was used.^[7] Improved variants (>2-fold increase in activity) were identified by monitoring the depletion of **2a** in a spectrophotometric kinetic assay in multi-well plates. Given that several mutations at positions Met-45 and Ala-46 (M45G, M45H, M45S, A46H and A46S) result in a slight improvement in activity (~3-fold), three mutations at position Phe-50 (F50I, F50V and F50A) significantly enhanced the Michael addition activity. Assays with the purified mutant enzymes showed a 6-fold, 8-fold and 15-fold increase in activity for F50I, F50V and F50A, respectively (Figure S1). Further characterization of the Michael reaction between **1** and **2a** catalyzed by the best 4-OT variant (F50A) showed that besides increased activity, this mutant enzyme also has enhanced stereoselectivity, allowing the production of optically pure (*R*)-**3a** (e.r. 99:1) in high isolated yield of 92% (Table 1, entry 1; Figure S2-S4). These results underscore the potential of the highly promiscuous 4-OT enzyme for evolutionary optimization. At semi-preparative scale, the 4-OT(F50A) catalyzed Michael addition of **1** (50 mM, 152 mg in 50 mL) to **2a** (25 mM, 157 mg in 50 mL) gave product (*R*)-**3a** (96% conversion in 11 h) in good isolated yield (204.7 mg, 85% yield) and with high e.r. of 98:2 (Figure S34, S35).

Notably, the mutation F50A was previously found to improve the aldol condensation activity of 4-OT.^[6a] This mutation makes the active site pocket of 4-OT more accessible to the outside aqueous environment, without changing the pK_a of Pro-1 too much, and likely enhances the aldol condensation activity of 4-OT by promoting the final hydrolysis step in which product is released from Pro-1, which has been suggested to be rate-limiting.^[6a] Similarly, the F50A mutation may increase the Michael addition activity of 4-OT by making the active site more amenable for hydrolytic cleavage of the covalent enzyme-product intermediate.

Having established that 4-OT(F50A) can efficiently promote the asymmetric Michael addition of **1** to **2a**, a set of α,β -unsaturated aldehydes was prepared (see Support Information for details) and tested as Michael acceptor substrates. The results demonstrate that the 4-OT(F50A) enzyme has a broad substrate scope, accepting both aromatic and aliphatic Michael acceptor substrates, and catalyzes the addition of **1** to the α,β -unsaturated aldehydes **2b-k** to yield the corresponding γ -nitroaldehydes **3b-k** with excellent enantiopurity (e.r. up to >99:1) and in good isolated yield (61-96%) (Table 1, Figure S5-S33). Interestingly, the enzymatic Michael reactions with *meta*- and *para*-substituted cinnamaldehydes (**2c,d** and **2f-j**) provided the corresponding products as the (*R*)-configured enantiomers, while those with the *ortho*-substituted cinnamaldehydes (**2b** and **2e**) yielded the (*S*)-configured product enantiomers (Table 1, entries 2 and 5). This suggests that positioning substituents on the *ortho* position of the substrate promoted steric effects, which caused either substrate relocation in the enzyme active site or a stereofacial shielding effect. Notably, the consequence of *ortho*-substituents on the stereochemical outcome of organo- and biocatalytic reactions with aromatic aldol and Michael acceptor substrates has been observed before.^[2d,8]

We next investigated whether the mechanism of the 4-OT(F50A) catalyzed Michael reaction proceeds *via* iminium ion formation between Pro-1 and the α,β -unsaturated aldehyde substrate. A 4-OT(F50A) sample modified by **2a** in the

presence of NaBH_4 and an unmodified 4-OT(F50A) sample were digested with Glu-C (an endoproteinase from *Staphylococcus aureus* V8), and the generated peptides were characterized by LC-MS (Figure S43-S45). Comparing the peaks of the modified 4-OT(F50A) sample to those of the nonmodified 4-OT(F50A) sample revealed a modification of the fragment PIAQIHILE by a species with a mass of 116 Da. This corresponds to labeling by one cinnamaldehyde molecule. Characterization of the remaining peaks revealed no labeling of other fragments (Figure S44, S45). Within the N-terminal fragment Pro-1 to Glu-9, the most probable positions for alkylation are Pro-1 and His-6. To identify the labeled residue, the modified and unmodified peptides were analyzed by LC-MS/MS (Figure S46).

Table 1. 4-OT(F50A)-catalyzed nitromethane addition to α,β -unsaturated aldehydes 2a-2k using optimized reaction conditions.^a

nitromethane 1	α,β -unsaturated aldehyde 2a-2k				γ -nitroaldehydes 3a-3k	
	2a R = Ph				3a (precursor phenibut)	
	2b R = <i>o</i> -Cl-Ph				3b	
	2c R = <i>m</i> -Cl-Ph				3c	
	2d R = <i>p</i> -Cl-Ph				3d (precursor baclofen)	
	2e R = <i>o</i> -MeO-Ph				3e	
	2f R = <i>p</i> -MeO-Ph				3f	
	2g R = <i>p</i> -NO ₂ -Ph				3g	
	2h R = <i>p</i> -OH-Ph				3h	
	2i R = <i>p</i> -F-Ph				3i	
	2j R = 3-OH-4-MeO-Ph				3j (precursor rolipram)	
	2k R = isobutyl				3k (precursor pregabalin)	

Entry	α,β -unsaturated aldehyde	Product	t [h]	Conv. ^b (Yield) ^c	e.r. ^d	Abs.config. ^e
1	2a		7	99 (92)	99:1	R
2	2b		2	99 (90) ^f	>99:1	S
3	2c		6	98 (93)	98:2	R
4	2d		24	98 (93)	98:2	R

Continued:

Entry	α,β -unsaturated aldehyde	Product	t [h]	Conv. ^b (Yield) ^c	e.r. ^d	Abs.config. ^e
5	2e	3d	10	99 (96)	86:14	S
6	2f	3f	18	90 (80) ^g	98:2	R
7	2g	3g	18	85 (75) ^g	98:2	R
8	2h	3h	20	98 (71)	97:3	R
9	2i	3i	8	97 (89) ^g	>99:1	R
10	2j	3j	20	84 (73) ^g	91:9	R
11	2k	3k	18	95 (61) ^g	93:7	S

^aAll the reactions were performed in buffer [20 mM HEPES/5% (v/v) ethanol] at pH 6.5 with 4-OT F50A (72 μ M, except for **2g** and **2i** for which 36 μ M enzyme was used), **1** (25 mM) and **2a-k** (3 mM, except for **2g** which was used at 2 mM); ^bDetermined by ¹H NMR analysis; ^cIsolated yield (%). ^dDetermined by chiral HPLC or GC. ^eThe absolute configuration was determined by comparison of chiral HPLC or GC data with those previously reported (see Supporting Information for details). ^fApparent kinetic parameters determined with this substrate at a fixed nitromethane concentration of 25 mM: $k_{\text{cat}} = 0.05 (\pm 0.002)$ s⁻¹; $K_{\text{m}} = 367 (\pm 37)$ μ M. ^gFurther purified using flash column chromatography.

The spectrum of the ion corresponding to the unlabeled PIAQIHILE peptide showed the characteristic b5 ion resulting from the peptide fragment PIAQI. MS/MS analysis of the modified PIAQIHILE peptide revealed a mass increase of 116 Da for this b5 ion. Therefore, it can be concluded that Pro-1 is the sole site of modification by **2a**. This result supports the hypothesis that Pro-1

functions as an amine catalyst in the enzymatic addition reaction, increasing the electrophilicity of the Michael acceptor through Schiff base formation (Scheme 1B). Replacement of Pro-1 with an alanine in wild-type 4-OT led to a 32-fold decrease in activity for the addition of **1** to **2a** (Figure S1), providing further support for this mechanism. Work is in progress to determine the structure of 4-OT(F50A) covalently modified by **2a**.

We have previously reported that 4-OT(F50A) can catalyze the aldol condensation of acetaldehyde with benzaldehyde to give cinnamaldehyde.^[3,6] Here we show that the three different activities observed for the 4-OT(F50A) enzyme can be used to prepare γ -nitroaldehydes in a biocatalytic cascade involving sequential aldol addition of acetaldehyde to a suitable aromatic aldehyde, dehydration, and Michael addition of nitromethane. Inclusion of a suitable aldehyde dehydrogenase and cofactor-recycling NADH oxidase^[9] in the reaction mixture enabled efficient one-pot synthesis of γ -nitrocarboxylic acids (Scheme 3). Using acetaldehyde (**4**), benzaldehyde (**5a**) and nitromethane (**1**) as starting substrates, product (*R*)-**7a** was obtained in 53% isolated yield (65% overall conversion) and with an excellent e.r. of 99:1 (Figure S36-S38). Replacing substrate **5a** with **5b**, yielded product (*S*)-**7b** in 80% isolated yield (>99% overall conversion) and with an excellent e.r. of 99:1 (Figure S39-S42). This simple and effective cascade further demonstrates the tremendous potential of combining different enzymes to construct simple synthetic routes for preparation of important chemical products.

Scheme 3. Four-step biocatalytic cascade synthesis of γ -nitrobutyric acids **7a** and **7b** in one pot. The cascade reactions were performed with **1** (50 mM), **4** (150 mM) and either **5a** or **5b** (3 mM).

In summary, our results indicate that the active site of 4-OT can give rise to synthetically useful promiscuous activities. Like proline-based organocatalysts, 4-OT utilizes a prolyl amine to attack diverse aldehydes forming reactive enamine and iminium ion intermediates. Hence, this natural enzyme with its unique catalytic amino-terminal proline could possibly accelerate many of the bond-forming reactions promoted by organocatalysts. We have therefore initiated studies aimed at exploring alternative nucleophiles for addition to α,β -unsaturated aldehydes, which would allow for the enzymatic synthesis of additional products.

In contrast to difficult to prepare proline- and peptide-based organocatalysts, the enzyme 4-OT can be produced in large amounts by simple bacterial fermentation. Moreover, the enzymatic reaction proceeds in eco-friendly aqueous buffer rather than in organic solvent. In previous work, we have demonstrated that 4-OT can be engineered into a more efficient biocatalyst for the aldol condensation of acetaldehyde with benzaldehyde to yield cinnamaldehyde, with

a >5000-fold enhancement in catalytic efficiency ($k_{\text{cat}}/K_{\text{m}}$) and a >10⁷-fold change in reaction specificity.^[6b] It is therefore conceivable that the promiscuous activity of 4-OT(F50A) for the Michael addition of nitromethane to α,β -unsaturated aldehydes can be optimized by directed evolution to generate novel biocatalysts for practical synthesis of chiral precursors for important pharmaceuticals.

AUTHOR INFORMATION

Corresponding Author

*Corresponding author. Tel.: +31503633354; E-mail: g.j.poelarends@rug.nl; Web: <http://www.rug.nl/staff/g.j.poelarends/>

Author Contributions

Chao Guo and Mohammad Saifuddin contributed equally to this work.

ASSOCIATED CONTENT

Supporting Information. Additional experimental procedures and compound characterization.

ACKNOWLEDGMENT

This research was financially supported by the European Union's Horizon 2020 Research and Innovation Programme (grant 635595), the European Research Council (grant 713483) and the Netherlands Organization of Scientific Research (grant 724.016.002). We thank M.H. de Vries for support in acquiring mass spectrometry data, and Lieuwe Biewenga for insightful discussions.

REFERENCES

- [1] a) Wiesner, M.; Revell, J. D.; Wennemers, H. Tripeptides as Efficient Asymmetric Catalysts for 1,4-Addition Reactions of Aldehydes to Nitroolefins--A Rational Approach. *Angew. Chem. Int. Ed.* **2008**, *47*, 1871-1874; b) Wiesner, M.; Neuburger, M.; Wennemers, H. Tripeptides of the Type H-D-Pro-Pro-Xaa-NH₂ as Catalysts for Asymmetric 1,4-Addition Reactions: Structural Requirements for High Catalytic Efficiency. *Chem. Eur. J.* **2009**, *15*, 10103-10109; c) García-García, P.; Ladépêche, A.; Halder, R.; List, B. Catalytic Asymmetric Michael Reactions of Acetaldehyde. *Angew. Chem. Int. Ed.* **2008**, *47*, 4719-4721; d) Gotoh, H.; Ishikawa, H.; Hayashi, Y. Diphenylprolinol Silyl Ether as Catalyst of an Asymmetric, Catalytic, and Direct Michael Reaction of Nitroalkanes with α,β -Unsaturated Aldehydes. *Org. Lett.* **2007**, *9*, 5307-5309; e) Hayashi, Y.; Gotoh, H.; Hayashi, T.; Shoji, M. Diphenylprolinol Silyl Ethers as Efficient Organocatalysts for the Asymmetric Michael Reaction of Aldehydes and Nitroalkenes. *Angew. Chem. Int. Ed.* **2005**, *44*, 4212-4215; f) Hayashi, Y.; Itoh, T.; Ohkubo, M.; Ishikawa, H. Asymmetric Michael Reaction of Acetaldehyde Catalyzed by Diphenylprolinol Silyl Ether. *Angew. Chem. Int. Ed.* **2008**, *47*, 4722-4724; g) Jentzsch, K. I.; Min, T.; Etcheson, J. I.; Fettingner, J. C.; Franz, A. K. Silyl Fluoride Electrophiles for the Enantioselective Synthesis of Silylated Pyrrolidine Catalysts. *J. Org. Chem.* **2011**, *76*, 7065-7075; h) Qiao, Y.; He, J.; Ni, B.; Headley, A. D. Asymmetric Michael Reaction of Acetaldehyde with Nitroolefins Catalyzed by Highly Water-Compatible Organocatalysts in Aqueous Media. *Adv. Synth. Catal.* **2012**, *354*, 2849-2853; i) Alza, E.; Pericàs, M. A. A Highly Selective, Polymer-Supported Organocatalyst for Michael Additions with Enzyme-Like Behavior. *Adv. Synth. Catal.* **2009**, *351*, 3051-3056; j) Riente, P.; Mendoza, C.; Pericàs, M. A. Functionalization of Fe₃O₄ Magnetic Nanoparticles for Organocatalytic Michael Reactions. *J. Mater. Chem.* **2011**, *21*, 7350-7355; k) Donslund, B. S.; Johansen, T. K.; Poulsen, P. H.; Halskov, K. S.; Jørgensen, K. A. The Diarylprolinol Silyl Ethers: Ten Years After. *Angew. Chem. Int. Ed.* **2015**, *54*, 13860-13874; l) Hojabri, L.; Hartikka, A.; Moghaddam, F. M.; Arvidsson, P. I. A. New Imidazole-Containing Imidazolidinone Catalyst for Organocatalyzed Asymmetric Conjugate Addition of Nitroalkanes to Aldehydes. *Adv. Synth. Catal.* **2007**, *349*, 740-748; m) Palomo, C.; Landa, A.; Mielgo, A.; Oiarbide, M.; Puente, Á.; Vera, S. Water-Compatible Iminium Activation: Organocatalytic Michael Reactions of Carbon-Centered Nucleophiles with Enals. *Angew. Chem. Int. Ed.* **2007**, *46*, 8431-8435; n) Tsogoeva, S. B. Recent Advances in Asymmetric Organocatalytic 1,4-Conjugate Additions. *Eur. J. Org. Chem.* **2007**, 1701-1716; o) Almaşi, D.; Alonso, D. A.; Nájera, C. Organocatalytic Asymmetric Conjugate Additions. *Tetrahedron: Asymmetry* **2007**, *18*, 299-365; p) Mase, N. Enamine Catalysis of Michael Reactions. In *Science of Synthesis: Asymmetric Organocatalysis*; List, B.; Maruoka, K. Eds.; Thieme: Stuttgart, Germany, **2012**, 135-216; q) Nödling, A. R.; Świderek, K.; Castillo, R.; Hall, J. W.; Angelastro, A.; Morrill, L. C.; Jin, Y.; Tsai, Y. H.; Moliner, V.; Luk, L. Y. P. Reactivity and Selectivity of Iminium Organocatalysis Improved by a Protein Host. *Angew. Chem. Int. Ed.* **2018**, *57*, 12478-12482; r)

Betancort, J. M.; Barbas III, C. F. Catalytic Direct Asymmetric Michael Reactions: Taming Naked Aldehyde Donors. *Org. Lett.* **2001**, *3*, 3737-3740.

[2] a) Zandvoort, E.; Geertsema, E. M.; Baas, B. J.; Quax, W. J.; Poelarends, G. J. Bridging Between Organocatalysis and Biocatalysis: Asymmetric Addition of Acetaldehyde to β -Nitrostyrenes Catalyzed by a Promiscuous Proline-Based Tautomerase. *Angew. Chem. Int. Ed.* **2012**, *51*, 1240-1243; b) Miao, Y.; Geertsema, E. M.; Tepper, P. G.; Zandvoort, E.; Poelarends, G. J. Promiscuous Catalysis of Asymmetric Michael-Type Additions of Linear Aldehydes to β -Nitrostyrene by the Proline-Based Enzyme 4-Oxalocrotonate Tautomerase. *ChemBioChem* **2013**, *14*, 191-194; c) Geertsema, E. M.; Miao, Y.; Tepper, P. G.; de Haan, P.; Zandvoort, E.; Poelarends, G. J. Biocatalytic Michael-Type Additions of Acetaldehyde to Nitroolefins with the Proline-Based Enzyme 4-Oxalocrotonate Tautomerase Yielding Enantioenriched γ -Nitroaldehydes. *Chem. Eur. J.* **2013**, *19*, 14407-14410; d) Miao, Y.; Tepper, P. G.; Geertsema, E. M.; Poelarends, G. J. Stereochemical Control of Enzymatic Carbon–Carbon Bond-Forming Michael-Type Additions by “Substrate Engineering”. *Eur. J. Org. Chem.* **2016**, *32*, 5350-5354.

[3] Zandvoort, E.; Baas, B. J.; Quax, W. J.; Poelarends, G. J. Systematic Screening for Catalytic Promiscuity in 4-Oxalocrotonate Tautomerase: Enamine Formation and Aldolase Activity. *ChemBioChem* **2011**, *12*, 602-609.

[4] Poddar, H.; Rahimi, M.; Geertsema, E. M.; Thunnissen, A. M. W. H.; Poelarends, G. J. Evidence for the Formation of an Enamine Species During Aldol and Michael-type Addition Reactions Promiscuously Catalyzed by 4-Oxalocrotonate Tautomerase. *ChemBioChem* **2015**, *16*, 738-741.

[5] Garrabou, X.; Verez, R.; Hilvert, D. Enantiocomplementary Synthesis of γ -Nitroketones Using Designed and Evolved Carboligases. *J. Am. Chem. Soc.* **2017**, *139*, 103-106.

[6] a) Zandvoort, E.; Geertsema, E. M.; Quax, W. J.; Poelarends, G. J. Enhancement of the Promiscuous Aldolase and Dehydration Activities of 4-Oxalocrotonate Tautomerase by Protein Engineering. *ChemBioChem* **2012**, *13*, 1274-1277; b) Rahimi, M.; van der Meer, J. Y.; Geertsema, E. M.; Poddar, H.; Baas, B. J.; Poelarends, G. J. Mutations Closer to the Active Site Improve the Promiscuous Aldolase Activity of 4-Oxalocrotonate Tautomerase More Effectively than Distant Mutations. *ChemBioChem* **2016**, *17*, 1225-1228.

[7] van der Meer, J. Y.; Poddar, H.; Baas, J. B.; Miao, Y.; Rahimi, M.; Kunzendorf, M. A.; van Merkerk, R.; Tepper, P. G.; Geertsema, E. M.; Thunnissen, A. M. W. H.; Quax, W. J.; Poelarends, G. J. Using Mutability Landscapes of a Promiscuous Tautomerase to Guide the Engineering of Enantioselective Michaelases. *Nat. Commun.* **2016**, *7*, 10911.

[8] Martínez, A.; van Gemmeren, M.; List, B. Unexpected Beneficial Effect of ortho-Substituents on the (S)-Proline-Catalyzed Asymmetric Aldol Reaction of Acetone with Aromatic Aldehydes. *Synlett.* **2014**, *25*, 961-964.

- [9] Biewenga, L.; Saravanan, T.; Kunzendorf, A.; van der Meer, J. Y.; Pijning, T.; Tepper, P. G.; van Merkerk, R.; Charnock, S. J.; Thunnissen, A. M. W. H.; Poelarends, G. J. Enantioselective Synthesis of Pharmaceutically Active γ -Aminobutyric Acids Using a Tailor-Made Artificial Michaelase in One-Pot Cascade Reactions. *ACS Catal.* **2019**, *9*, 1503-1513.

SUPPORTING INFORMATION

1. Materials and Methods

1.1 Materials

Chemicals were purchased from Sigma Aldrich, Across, Merck or Fluka (unless noted otherwise) and were used without further purification. The α,β -unsaturated aldehydes **2b-k** were prepared using previously reported methods.^[1] Synthesis of racemic reference compounds, required for chiral analysis of enzymatic products, was done according to published protocols.^[2]

1.2 General methods

NMR spectra were recorded on a Bruker 500 MHz spectrometer. Enzymatic assays were performed on a V-650 or V-660 spectrophotometer from Jasco (IJsselstein, The Netherlands). Reverse phase HPLC was carried out using an in-house analytical HPLC equipped with a Shimazu LC-10 AT pump and a Shimazu SPD-M10A diode array detector. Gas chromatography was carried out with a HP 5890 series II gas chromatograph using a chiral GTA column (30 m $\times$ 0.25 mm $\times$ 0.12 μ ; Supelco).

1.3 Screening 4-OT single mutants for enhanced Michael addition activity

A systematic mutagenesis strategy was applied to identify residue positions at which mutations give a marked improvement in the activity of 4-OT for addition of **1** to **2a**. For this, a previously constructed collection of 1040 single 4-OT mutants was used.^[3] The proteins were produced in *E. coli* and cell free extracts (CFEs) were prepared according to a reported procedure.^[3] Briefly, 1.25 mL of LB medium supplemented with 100 μ g/mL ampicillin and 100 μ M IPTG was inoculated from a glycerol stock of the corresponding mutant and the culture was grown overnight at 37 $^{\circ}$ C in a 96-deep well plate. The cells from

the overnight cultures were harvested by centrifugation and lysed with 250 μ L of Bugbuster (Novagen), supplemented with 25 U/mL benzonase, for 40 min. After centrifugation, the obtained CFE was used for the activity assay. The reaction mixtures (100 μ L) consisted of CFE (40% v/v), **1** (25 mM), **2a** (0.25 mM) and ethanol (5% v/v) in 20 mM HEPES buffer (pH 6.5). The reaction was initiated by adding 5 μ L from a stock solution of **2a** (5 mM in ethanol). Prior to starting the measurements, the 96-well plates were shaken for 1 min at 500 rpm to ensure proper mixing. The depletion in the absorbance of **2a** was monitored at 292 nm for 3 h with 3 min time intervals.

1.4 Protein expression and purification

The expression and purification of wild-type 4-OT and 4-OT mutants were based on protocols described elsewhere.^[4] A sample of each purified protein was analyzed by ESI-MS to confirm that the protein had been processed correctly and the initiating methione had been removed. The purified protein was flash frozen in liquid nitrogen and stored at -80 °C until further use.

1.5 Chiral analysis

The enantiomeric ratio of the enzymatically synthesized compounds **3a**, **3d**, **3i** and **3k** was analyzed directly using HPLC or GC with a chiral stationary phase. The enantiomeric ratio of the compounds **3h** and **3j** was analyzed by chiral HPLC after derivatizing the enzymatic products into a cyclic acetal, prepared according to a literature procedure, and using chemically synthesized racemic cyclic acetal reference compounds for comparison.^[3] For compounds **3b**, **3c**, **3e**, **3f** and **3g**, the enantiomeric ratio was analyzed by chiral HPLC after reducing the aldehyde functionality of the enzymatic product into the corresponding alcohol using NaBH₄ according to a reported procedure and using chemically synthesized racemic γ -nitroalcohols as reference compounds.^[2a] Analytical conditions for each compound are given below.

1.6. Analytical scale reactions

Activity comparison of wild-type 4-OT (4-OT WT) and the variants 4-OT F50A, 4-OT F50I, 4-OT F50V, and 4-OT P1A for addition of **1** to **2a** was carried out by UV spectroscopic analysis on analytical scale (0.3 mL reaction volume). Purified enzyme (300 μ g) was incubated in a 1 mm cuvette with **1** (25 mM) and **2a** (1 mM) in 20 mM HEPES buffer (pH 6.5; 0.3 mL final volume). The reactions were monitored by following the decrease in absorbance at 290 nm, which corresponds to the depletion of substrate **2a**.

Figure S1. UV traces for monitoring the depletion of **2a** in the presence of purified 4-OT WT, 4-OT F50A, 4-OT F50I, 4-OT F50V and 4-OT P1A. The mutant enzymes F50I, F50V and F50A showed a 6-fold, 8-fold, and 15-fold increase in activity, respectively, based on the initial substrate depletion rates (from 0-13 min). Analysis of the corresponding products showed that the mutant enzymes F50I, F50V and F50A allow the production of (*R*)-**3a** with e.r. values of 99:1, 97:3 and 99:1, respectively.

1.7. Kinetic assay

Kinetic assays were performed at 20 °C in 20 mM HEPES buffer (pH 6.5) by following the decrease in absorbance at 290 nm, which corresponds to the depletion of substrate **2b**. An appropriate amount of purified 4-OT F50A enzyme (20 μ g) was incubated in a 1 mm cuvette with **1** (25 mM) and **2b** (varying concentrations ranging from 0.1 to 2 mM) in 20 mM HEPES buffer (pH 6.5; 0.3 mL final volume) at 20 °C. The initial rates (μ M/s) were plotted versus the concentrations (μ M) of substrate **2b**. SigmaPlot was used to fit the data to Michaelis-Menten kinetics to determine the kinetic parameters. The measurements were done in triplo.

2. Chemical synthesis of α,β -unsaturated aldehydes (**2b-k**)

Compound **2a** is commercially available. Synthesis of compounds **2b-k** was carried out *via* the Wittig reaction and according to reported procedures summarized in Scheme S1.

Scheme S1. Synthesis of α,β -unsaturated aldehydes **2b-g**, **2i**, and **2k** (Scheme S1A) and **2h**, **2j** (Scheme S1B) *via* the Wittig reaction. Abbreviation: WR, Wittig reagent.

(a) Synthesis of compounds **2b-g**, **2i** and **2k** (Scheme S1A)

Compounds **2b-g**^[1a-h], **2i**^[1i] and **2k**^[1j-k] were prepared according to literature procedures and their ¹H NMR spectra match with earlier reported NMR data.^[1a-k]

(b) Synthesis of compound **2e** (Scheme S1A)

To a stirred solution of (*E*)-3-(2-hydroxyphenyl)acrylaldehyde (1.0 eq) in 5 mL of dry DMF was added K₂CO₃ (1.5 eq) at 0 °C. Subsequently, MeI (1.5 eq) was added dropwise at the same temperature and the reaction mixture was further incubated for 1 h. After completion of the reaction (monitored by TLC with KMnO₄ staining), the reaction was quenched with saturated aqueous ammonium chloride, and the reaction mixture was extracted 3 times with ethyl acetate. The organic layers were combined, dried over Na₂SO₄ and concentrated under

vacuo. The crude product was purified by silica gel column chromatography using petroleum ether/ethyl acetate (90:10) as an eluent to give the corresponding cinnamaldehyde derivative **2e**. Yellow solid; yield = 95% (52 mg, starting from 50 mg of **2e***). The ^1H NMR spectrum matches with previously reported NMR data.^[1f]

(c). Synthesis of compounds **2h** and **2j** (Scheme S1B)

Compounds **2h** and **2j** were prepared according to the route shown in Scheme S1B, which involves the preparation of compounds **8h**, **8j**, **9h** and **9j**.

(i) Synthesis of compounds **8h** and **8j** (Scheme S1B)

The reported literature procedure was slightly modified.^[11-m] 4-OH-benzaldehyde (1.0 eq) was dissolved in 5 mL anhydrous DCM and treated with carbethoxymethylene triphenylphosphorane (1.2 eq) at room temperature. After the complete addition of all the starting material, the reaction mixture was stirred at room temperature for 12 h. After the complete consumption of the starting material (monitored by TLC with KMnO₄ staining), the reaction was quenched with saturated aqueous ammonium chloride, and the reaction mixture extracted three times with DCM. The organic layers were combined, dried over Na₂SO₄ and concentrated under *vacuo*. The crude product was purified by silica gel column chromatography using petroleum ether/ethyl acetate (90:10) as an eluent to give the corresponding α,β -unsaturated ester **8h**. Colorless solid; yield = 79% (248 mg, starting from 200 mg of **5h**). Similarly, compound **8j** was prepared by using the same procedure. Colorless solid; yield = 76% (222 mg, starting from 200 mg of **5j**). The ^1H NMR data of compounds **8h**^[11] and **8j**^[1m] match with earlier reported NMR data.

(ii) Synthesis of compound 9h and 9j (Scheme S1B)

The reported literature procedure was slightly modified.^[1n-o] To a stirred solution of ethyl (*E*)-3-(4-hydroxyphenyl)acrylate (1.0 eq) in 5 mL of dry DCM was added diisobutylaluminum hydride (DIBAL-H) (1.5 eq, 1.0 M in cyclohexane) dropwise at 0 °C. After the complete addition of all the starting material, the reaction mixture was stirred at 0 °C for 3 h followed by 1 h at room temperature. After the completion of the starting material (monitored by TLC with KMnO₄ staining), the reaction was quenched with saturated aqueous ammonium chloride, and the reaction mixture was extracted three times with DCM. The organic layers were combined, dried over Na₂SO₄ and concentrated under *vacuo*. The crude product was purified by silica gel column chromatography using petroleum ether/ethyl acetate (90:10) as an eluent to give the corresponding alcohol **9h**. Yellow solid; yield = 73% (104 mg, starting from 200 mg of **8h**). Similarly, compound **9j** was prepared by using the same procedure. Colorless oil; yield = 61% (100 mg, starting from 200 mg of **8j**). The ¹H NMR data of compounds **9h**^[1n] and **9j**^[1o] match with the reported NMR data.

(iii) Synthesis of compounds 2h and 2j

To a stirred solution of (*E*)-4-(3-hydroxyprop-1-en-1-yl)phenol (1.0 eq) in 5 mL of dry 1,4-dioxane was added DDQ (1.2 eq) dropwise at 0 °C. After the complete addition of all the starting material, the reaction mixture was stirred at room temperature for 30 min. After the completion of the starting material (monitored by TLC with KMnO₄ and DNP staining), the reaction was quenched with saturated aqueous NaHCO₃, and the reaction mixture was extracted 3 times with EtOAc. The organic layers were combined, dried over Na₂SO₄ and concentrated under *vacuo*. The crude product was purified by silica gel column chromatography using petroleum ether/ethyl acetate (90:10) as an eluent to give the corresponding cinnamaldehyde derivative **2h**. Yellow solid; yield = 66% (65 mg, starting from 100 mg of **9h**). Similarly, compound **2j** was prepared by using

above procedure. Light yellow solid; yield = 61% (60 mg, starting from 100 mg of **9j**). The ^1H NMR data of compounds **2h**^[10] and **2j**^[1p] match with those previously reported.

(d). ^1H NMR data of compounds 2b-2k, 2e*, 8h, 8j, 9h and 9j

(E)-3-(2-chlorophenyl)acrylaldehyde (2b). ^1H NMR (500 MHz, CDCl_3): δ 9.75 (d, $J = 7.7$ Hz, 1H), 7.93 (d, $J = 16.0$ Hz, 1H), 7.65 (dd, $J = 7.7, 1.7$ Hz, 1H), 7.45 (dd, $J = 8.0, 1.1$ Hz, 1H), 7.39 – 7.28 (m, 2H), 6.70 (dd, $J = 16.0, 7.7$ Hz, 1H).

(E)-3-(3-chlorophenyl)acrylaldehyde (2c). ^1H NMR (500 MHz, CDCl_3): δ 9.72 (d, $J = 7.6$ Hz, 1H), 7.55 (t, $J = 1.7$ Hz, 1H), 7.47 – 7.35 (m, 4H), 6.71 (dd, $J = 16.0, 7.6$ Hz, 1H).

(E)-3-(4-chlorophenyl)acrylaldehyde (2d). ^1H NMR (500 MHz, CDCl_3): δ 9.71 (d, $J = 7.6$ Hz, 1H), 7.53 – 7.48 (m, 2H), 7.46 – 7.39 (m, 3H), 6.69 (dd, $J = 16.0, 7.6$ Hz, 1H).

(E)-3-(2-hydroxyphenyl)acrylaldehyde (2e*). ^1H NMR (500 MHz, CDCl_3): δ 9.68 (d, $J = 8.0$ Hz, 1H), 7.79 (d, $J = 16.0$ Hz, 1H), 7.50 (dd, $J = 7.8, 1.4$ Hz, 1H), 7.31 (td, $J = 8.1, 1.6$ Hz, 1H), 7.07 – 6.93 (m, 2H), 6.89 (dd, 1H), 6.59 (s, 1H).

(E)-3-(2-methoxyphenyl)acrylaldehyde (2e). ^1H NMR (500 MHz, CDCl_3): δ 9.67 (d, $J = 7.9$ Hz, 1H), 7.82 (d, $J = 16.1$ Hz, 1H), 7.52 (dd, $J = 7.7, 1.6$ Hz, 1H), 7.40 (ddd, $J = 8.9, 7.5, 1.7$ Hz, 1H), 6.98 (t, $J = 7.5$ Hz, 1H), 6.93 (d, $J = 8.3$ Hz, 1H), 6.77 (dd, $J = 16.1, 7.9$ Hz, 1H), 3.89 (s, 3H).

(E)-3-(4-methoxyphenyl)acrylaldehyde (2f). ^1H NMR (500 MHz, CDCl_3): δ 9.65 (d, $J = 7.8$ Hz, 1H), 7.54 – 7.50 (m, 2H), 7.43 (d, $J = 15.8$ Hz, 1H), 6.97 – 6.92 (m, 2H), 6.61 (dd, $J = 15.8, 7.8$ Hz, 1H), 3.86 (s, 3H).

(E)-3-(4-nitrophenyl)acrylaldehyde (2g). ^1H NMR (500 MHz, CDCl_3): δ 9.78 (d, $J = 7.4$ Hz, 1H), 8.32 – 8.27 (m, 2H), 7.73 (d, $J = 8.8$ Hz, 2H), 7.53 (d, $J = 16.1$ Hz, 1H), 6.81 (dd, $J = 16.1, 7.4$ Hz, 1H).

(*E*)-3-(4-hydroxyphenyl)acrylaldehyde (2h). ^1H NMR (500 MHz, $\text{DMSO-}d_6$): δ 10.20 (s, 1H), 9.58 (d, $J = 7.9$ Hz, 1H), 7.67 – 7.57 (m, 3H), 6.84 (d, $J = 8.6$ Hz, 2H), 6.66 (dd, $J = 15.8, 7.9$ Hz, 1H).

(*E*)-3-(4-fluorophenyl)acrylaldehyde (2i). ^1H NMR (500 MHz, CDCl_3): δ 9.69 (d, $J = 6.6$ Hz, 1H), 7.57 (s, 2H), 7.45 (d, $J = 16.0$ Hz, 1H), 7.13 (t, $J = 7.2$ Hz, 2H), 6.65 (dd, $J = 15.8, 7.1$ Hz, 1H).

(*E*)-3-(3-hydroxy-4-methoxyphenyl)acrylaldehyde (2j). ^1H NMR (500 MHz, $\text{DMSO-}d_6$): δ 9.58 (d, $J = 7.8$ Hz, 1H), 9.30 (s, 1H), 7.59 (d, $J = 15.8$ Hz, 1H), 7.23 – 7.10 (m, 2H), 7.00 (d, $J = 8.3$ Hz, 1H), 6.60 (dd, $J = 15.8, 7.8$ Hz, 1H), 3.83 (s, 3H).

(*E*)-5-methylhex-2-enal (2k). ^1H NMR (500 MHz, CDCl_3): δ 9.54 (d, $J = 8.0$ Hz, 1H), 7.09 (dd, $J = 15.3, 10.0$ Hz, 1H), 6.08 (dd, $J = 15.4, 8.0$ Hz, 1H), 2.11 (t, $J = 6.7$ Hz, 2H), 1.74 (dh, $J = 13.3, 6.6$ Hz, 1H), 0.93 (d, $J = 6.7$ Hz, 6H).

Ethyl (*E*)-3-(4-hydroxyphenyl)acrylate (8h). ^1H NMR (500 MHz, CDCl_3): δ 7.64 (d, $J = 16.0$ Hz, 1H), 7.40 (d, $J = 8.6$ Hz, 2H), 7.02 (s, 1H), 6.88 (d, $J = 8.6$ Hz, 2H), 6.29 (d, $J = 15.9$ Hz, 1H), 4.27 (q, $J = 7.1$ Hz, 2H), 1.34 (t, $J = 7.1$ Hz, 3H).

Ethyl (*E*)-3-(3-hydroxy-4-methoxyphenyl)acrylate (8j). ^1H NMR (500 MHz, CDCl_3): δ 7.58 (d, $J = 15.9$ Hz, 1H), 7.13 (d, $J = 2.1$ Hz, 1H), 7.01 (dd, $J = 8.3, 2.1$ Hz, 1H), 6.82 (d, $J = 8.3$ Hz, 1H), 6.28 (d, $J = 15.9$ Hz, 1H), 5.81 (s, 1H), 4.24 (q, $J = 7.1$ Hz, 2H), 3.90 (s, 3H), 1.32 (t, $J = 7.1$ Hz, 3H).

(*E*)-4-(3-hydroxyprop-1-en-1-yl)phenol (9h). ^1H NMR (500 MHz, $\text{DMSO-}d_6$): δ 9.48 (s, 1H), 7.22 (d, $J = 8.5$ Hz, 2H), 6.70 (d, $J = 8.5$ Hz, 2H), 6.41 (d, $J = 16.0$ Hz, 1H), 6.12 (dt, $J = 15.9, 5.4$ Hz, 1H), 4.79 (t, $J = 5.5$ Hz, 1H), 4.05 (t, $J = 4.8$ Hz, 2H).

(*E*)-5-(3-hydroxyprop-1-en-1-yl)-2-methoxyphenol (9j). ^1H NMR (500 MHz, $\text{DMSO-}d_6$): δ 8.95 (s, 1H), 6.93 – 6.72 (m, 3H), 6.38 (d, $J = 15.9$ Hz, 1H), 6.19 – 6.04 (m, 1H), 4.78 (t, $J = 5.4$ Hz, 1H), 4.06 (t, $J = 4.7$ Hz, 2H), 3.75 (s, 3H).

3. Biocatalytic synthesis of γ -nitroaldehydes (**3a-k**) using 4-OT F50A

Reaction mixtures (20 mL) contained nitromethane (**1**, 25 mM), an α,β -unsaturated aldehyde (**2a-k**, 3 mM, except for **2g** which was used at 2 mM) and 4-OT F50A enzyme (72 μ M, except for **2g** and **2i** for which 36 μ M enzyme was used) in HEPES buffer (20 mM, pH 6.5). Stock solutions of **2a-k** were prepared in absolute ethanol. The reaction mixture was incubated at room temperature and the progress of the reaction was monitored by UV-VIS spectrophotometry (**2a-j**) or GC (**2k**). To monitor the progress of the enzyme catalyzed reactions of **2a-j** with **1**, aliquots (80 μ L) were taken from the reaction mixture, diluted with 160 μ L of 20 mM HEPES buffer (pH 6.5), and analyzed by UV-VIS spectrophotometry. To monitor the progress of the enzyme-catalyzed reaction of **2k** with **1**, aliquots (100 μ L) were taken from the reaction mixture, extracted with toluene (100 μ L), and analyzed by GC. After the completion of the reactions, each reaction mixture was extracted with ethyl acetate (3×40 mL). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4 , and concentrated under *vacuo*. The products **3a-e** and **3h** were obtained in high purity and did not require further column purification. The crude products of **3f**, **3g**, and **3i-k** were further purified by silica gel column chromatography (petroleum ether/ethyl acetate from 95:5 to 50:50).

(*R*)-4-nitro-3-phenylbutanal (3a). Yellow oil; yield = 92% (10.7 mg). ^1H NMR (500 MHz, CDCl_3): δ 9.71 (s, 1H), 7.37 – 7.33 (m, 2H), 7.31 – 7.27 (m, 1H), 7.25 – 7.22 (m, 2H), 4.68 (dd, $J = 12.5, 7.2$ Hz, 1H), 4.62 (dd, $J = 12.5, 7.5$ Hz, 1H), 4.08 (p, $J = 7.3$ Hz, 1H), 3.01 – 2.91 (m, 2H). The enantiomeric ratio (e.r.) of the enzymatic product **3a** was determined by chiral GC (Astec® CHIRALDEX G-TA column; $30\text{ m} \times 0.25\text{ mm} \times 0.12\text{ }\mu\text{m}$) using the following conditions: $10\text{ }^\circ\text{C}/\text{min}$ from $70\text{ }^\circ\text{C}$ to $170\text{ }^\circ\text{C}$, followed by 20 min at $170\text{ }^\circ\text{C}$; flame ionization detection: t_{R} (major) = 23.9 min , (minor) = 24.3 min . The absolute configuration of enzymatically prepared **3a** was assigned by comparing to previously reported data.^[3]

Figure S2. UV spectra monitoring the Michael addition of **1** to **2a** catalyzed by 4-OT F50A in buffer [HEPES 20 mM/5% (v/v) ethanol]] at pH 6.5.

Figure S3. ¹H NMR spectrum of enzymatic product **3a**.

Figure S4. Chiral GC analysis of enzymatic product **3a**.

(S)-3-(2-chlorophenyl)-4-nitrobutanal (3b). Colorless oil; yield = 90% (12.2 mg). ^1H NMR (500 MHz, CDCl_3): δ 9.74 (t, J = 1.1 Hz, 1H), 7.43 – 7.41 (m, 1H), 7.28 – 7.21 (m, 3H), 4.78 (dd, J = 12.8, 6.8 Hz, 1H), 4.73 (dd, J = 12.8, 6.8 Hz, 1H), 4.55 (p, J = 6.9 Hz, 1H), 3.12 – 3.00 (m, 2H). The enantiomeric ratio of the enzymatic product **3b** was determined (after converting the aldehyde functionality into the corresponding alcohol) using reverse phase HPLC on a Chiralpak® ID column (150 mm $\times$ 4.6 mm, Daicel) (MeCN/water = 5:95, 25°C) at a flow rate of 1 mL/min. UV detection at 220 nm: t_{R} (minor) = 125.9 min, (major) = 134.9 min. The assignment of the absolute configuration of enzymatically prepared **3b** was based on earlier reported chiral HPLC-data.^[2a]

Figure S5. UV spectra monitoring the Michael addition of **1** to **2b** catalyzed by 4-OT F50A in buffer [HEPES 20 mM/5% (v/v) ethanol]] at pH 6.5.

Figure S6. ^1H NMR spectrum of enzymatic product **3b**.

Figure S7. Chiral HPLC analysis of derivatized enzymatic product **3b**.

(*R*)-3-(3-chlorophenyl)-4-nitrobutanal (3c). Colorless oil; yield = 93% (12.7 mg). ^1H NMR (500 MHz, CDCl_3): δ 9.71 (s, 1H), 7.29 – 7.27 (m, 2H), 7.23 – 7.22 (m, 1H), 7.14 – 7.12 (m, 1H), 4.68 (dd, $J = 12.7, 6.9$ Hz, 1H), 4.60 (dd, $J = 12.7, 7.8$ Hz, 1H), 4.06 (p, $J = 7.1$ Hz, 1H), 2.96 (dt, $J = 7.0, 1.1$ Hz, 2H). The enantiomeric ratio of the enzymatic product **3c** was determined by reverse phase HPLC (after converting the aldehyde functionality into the corresponding alcohol) using a Chiralpak® ID column (150 mm $\times$ 4.6 mm, Daicel) (MeCN/water = 15:85, 25°C) at a flow rate of 1 mL/min. UV detection at 220 nm: t_{R} (minor) = 8.5 min, (major) = 9.5 min. The assignment of the absolute configuration of enzymatically prepared **3c** was based on earlier reported chiral HPLC data.^[2a]

Figure S8. UV spectra monitoring the Michael addition of **1** to **2c** catalyzed by 4-OT F50A in buffer [HEPES 20 mM/5% (v/v) ethanol]] at pH 6.5.

Figure S9. ¹H NMR spectrum of enzymatic product **3c**.

Figure S10. HPLC analysis of derivatized enzymatic product **3c**

(*R*)-3-(4-chlorophenyl)-4-nitrobutanal (3d). Light yellow oil; yield = 93% (12.7 mg). ^1H NMR (500 MHz, CDCl_3): δ 9.71 (s, 1H), 7.34 – 7.31 (m, 2H), 7.19 – 7.16 (m, 2H), 4.67 (dd, J = 12.6, 6.9 Hz, 1H), 4.59 (dd, J = 12.6, 7.9 Hz, 1H), 4.07 (p, J = 7.1 Hz, 1H), 2.95 (dd, J = 7.0, 0.9 Hz, 2H). The enantiomeric ratio of the enzymatic product **3d** was determined by reverse phase HPLC using a Chiralpak[®] AD-RH column (150 mm $\times$ 4.6 mm, Daicel) (MeCN/water = 32:68, 25°C) at a flow rate of 0.5 mL/min. UV detection at 220 nm: t_{R} (major) = 27.6 min, (minor) = 29.6 min. The assignment of the absolute configuration of enzymatically prepared **3d** was based on earlier reported chiral HPLC data.^[3]

Figure S11. UV spectra monitoring the Michael addition of **1** to **2d** catalyzed by 4-OT F50A in buffer [HEPES 20 mM/5% (v/v) ethanol] at pH 6.5.

Figure S12. ¹H NMR spectrum of enzymatic product **3d**.

Figure S13. HPLC analysis of the enzymatic product **3d**.

(S)-3-(2-methoxyphenyl)-4-nitrobutanal (3e). Light yellow oil; yield = 96% (12.8 mg). ^1H NMR (500 MHz, CDCl_3): δ 9.70 (s, 1H), 7.29 – 7.25 (m, 1H), 7.15 (dd, J = 7.6, 1.7 Hz, 1H), 6.94 – 6.88 (m, 2H), 4.76 (dd, J = 12.5, 7.0 Hz, 1H), 4.71 (dd, J = 12.5, 7.2 Hz, 1H), 4.29 (p, J = 7.1 Hz, 1H), 3.86 (s, 3H), 3.05 – 2.95 (m, 2H). The enantiomeric ratio of enzymatic product **3e** was determined by reverse phase HPLC (after converting the aldehyde functionality into the corresponding alcohol) using a Chiralpak® AD-RH column (150 mm $\times$ 4.6 mm, Daicel) (MeCN/water = 7.5:92.5, 25°C) at a flow rate of 1 mL/min. UV detection at 220 nm: t_{R} (minor) = 14.8 min, (major) = 16.2 min. The assignment of the absolute configuration of enzymatically prepared **3e** was based on earlier reported chiral HPLC data.^[2a]

Figure S14. UV spectra monitoring the Michael addition of **1** to **2e** catalyzed by 4-OT F50A in buffer [HEPES 20 mM/5% (v/v) ethanol] at pH 6.5.

Figure S15. ^1H NMR spectrum of enzymatic product **3e**.

Figure S16. HPLC analysis spectra of derivatized enzymatic product **3e**.

(*R*)-3-(4-methoxyphenyl)-4-nitrobutanal (3f). Light yellow oil; yield = 80% (10.7 mg). ^1H NMR (500 MHz, CDCl_3): δ 9.69 (t, J = 1.2 Hz, 1H), 7.15 – 7.14 (m, 2H), 6.87 – 6.86 (m, 2H), 4.64 (dd, J = 12.4, 7.2 Hz, 1H), 4.57 (dd, J = 12.4, 7.7 Hz, 1H), 4.03 (p, J = 7.3 Hz, 1H), 3.78 (s, 3H), 2.96 – 2.86 (m, 2H). The enantiomeric ratio of the enzymatic product **3f** was determined by reverse phase HPLC (after converting the aldehyde functionality into the corresponding alcohol) using a Chiralpak® AD-RH column (150 mm $\times$ 4.6 mm, Daicel) (MeCN/water = 15:85, 25°C) at a flow rate of 1 mL/min. UV detection at 220 nm: t_{R} (major) = 27.3 min, (minor) = 36.3 min. The assignment of the absolute configuration of enzymatically prepared **3f** was based on earlier reported chiral HPLC data.^[2a]

Figure S17. UV spectra monitoring the Michael addition of **1** to **2f** catalyzed by 4-OT F50A in buffer [HEPES 20 mM/5% (v/v) ethanol]] at pH 6.5.

Figure S18. ^1H NMR spectrum of enzymatic product **3f**.

Figure S19. HPLC analysis spectra of derivatized enzymatic product **3f**.

(*R*)-4-nitro-3-(4-nitrophenyl)butanal (3g). Light yellow oil; yield = 75% (7.1 mg). ^1H NMR (500 MHz, CDCl_3): δ 9.74 (s, 1H), 8.22 (d, J = 8.7 Hz, 2H), 7.45 (d, J = 8.7 Hz, 2H), 4.75 (dd, J = 12.9, 6.4 Hz, 1H), 4.67 (dd, J = 12.9, 8.2 Hz, 1H), 4.24 – 4.19 (m, 1H), 3.04 – 3.02 (m, 2H). The enantiomeric ratio of enzymatic product **3g** was determined by reverse phase HPLC (after converting the aldehyde functionality into the corresponding alcohol) using a Chiralpak[®] ID column (150 mm $\times$ 4.6 mm, Daicel) (MeCN/water = 15:85, 25°C) at a flow rate of 1 mL/min. UV detection at 220 nm: t_{R} (major) = 17.4 min, (minor) = 20.1 min. The assignment of the absolute configuration of enzymatically prepared **3g** was based on earlier reported chiral HPLC data.^[2a]

Figure S20. UV spectra monitoring the Michael addition of **1** to **2g** catalyzed by 4-OT F50A in buffer [HEPES 20 mM/5% (v/v) ethanol]] at pH 6.5.

Figure S21. ¹H NMR spectrum of enzymatic product **3g**. Figure S22. HPLC analysis of derivatized enzymatic product **3g**.

(*R*)-3-(4-hydroxyphenyl)-4-nitrobutanal (3h). Light yellow oil; yield = 71% (8.9 mg). ^1H NMR (500 MHz, CDCl_3): δ 9.70 (s, 1H), 7.12 – 7.09 (m, 2H), 6.82 – 6.78 (m, 2H), 4.84 (s, 1H), 4.64 (dd, J = 12.4, 7.1 Hz, 1H), 4.57 (dd, J = 12.4, 7.7 Hz, 1H), 4.02 (p, J = 7.3 Hz, 1H), 2.97 – 2.87 (m, 2H). The enantiomeric ratio of the enzymatic product **3h** was determined by reverse phase HPLC (after converting the aldehyde functionality into the corresponding hemi acetal) using a Chiralpak[®] AD-RH column (150 mm $\times$ 4.6 mm, Daicel) (MeCN/water = 30:70, 25°C) at a flow rate of 0.5 mL/min. UV detection at 210 nm: t_{R} (major) = 16.8 min, (minor) = 30.6 min. The assignment of the absolute configuration of enzymatically prepared **3h** was based on earlier reported chiral HPLC data.^[3]

Figure S23. UV spectra monitoring the Michael addition of **1** to **2h** catalyzed by 4-OT F50A in buffer [HEPES 20 mM/5% (v/v) ethanol]] at pH 6.5.

Figure S24. ^1H NMR spectrum of enzymatic product **3h**.

Figure S25. HPLC analysis of derivatized enzymatic product **3h**.

(*R*)-3-(4-fluorophenyl)-4-nitrobutanal (3i). Colorless oil; yield = 89% (11.3 mg). ^1H NMR (500 MHz, CDCl_3): δ 9.70 (s, 1H), 7.23 – 7.20 (m, 2H), 7.05 – 7.02 (m, 2H), 4.67 (dd, J = 12.6, 6.9 Hz, 1H), 4.59 (dd, J = 12.5, 7.9 Hz, 1H), 4.07 (p, J = 7.2 Hz, 1H), 2.94 (dd, J = 7.1, 1.0 Hz, 2H). The enantiomeric ratio of the enzymatic product **3i** was determined by reverse phase HPLC using a Chiralpak[®] AD-RH column (150 mm $\times$ 4.6 mm, Daicel) (MeCN/water = 15:85, 25°C) at a flow rate of 0.5 mL/min. UV detection at 210 nm: t_{R} (major) = 48.6 min, (minor) = 51.7 min. The assignment of the absolute configuration of enzymatically prepared **3i** was based on earlier reported chiral HPLC data.^[3]

Figure S26. UV spectra monitoring the Michael addition of **1** to **2i** catalyzed by 4-OT F50A in buffer [HEPES 20 mM/5% (v/v) ethanol]] at pH 6.5.

Figure S27. ¹H NMR spectrum of enzymatic product **3i** **Figure S28.** HPLC analysis of enzymatic product **3i**.

(*R*)-3-(3-hydroxy-4-methoxyphenyl)-4-nitrobutanal (3j). Colorless oil; yield = 73% (10.5 mg). ^1H NMR (500 MHz, CDCl_3): δ 9.69 (t, J = 1.2 Hz, 1H), 6.81 – 6.78 (m, 2H), 6.72 (dd, J = 8.2, 2.3 Hz, 1H), 5.63 (s, 1H), 4.62 (dd, J = 12.4, 7.2 Hz, 1H), 4.56 (dd, J = 12.4, 7.6 Hz, 1H), 3.99 (p, J = 7.3 Hz, 1H), 3.87 (s, 3H), 2.94 – 2.84 (m, 2H). The enantiomeric ratio of the enzymatic product **3j** was determined by reverse phase HPLC (after converting the aldehyde functionality into the corresponding cyclic acetal) using a Chiralpak[®] AD-RH column (150 mm $\times$ 4.6 mm, Daicel) (MeCN/water = 30:70, 25°C) at a flow rate of 0.5 mL/min. UV detection at 210 nm: t_{R} (major) = 21.2 min, (minor) = 30.2 min. The assignment of the absolute configuration of enzymatically prepared **3j** was based on earlier reported chiral HPLC data.^[3]

Figure S29. UV spectra monitoring the Michael addition of **1** to **2j** catalyzed by 4-OT F50A in buffer [HEPES 20 mM/5% (v/v) ethanol]] at pH 6.5.

Figure S30. ¹H NMR spectrum of enzymatic product **3j**.

Figure S31. HPLC analysis of derivatized enzymatic product **3j**.

(S)-5-methyl-3-(nitromethyl)hexanal (3k). Colorless oil; yield = 61% (6.3 mg). ^1H NMR (500 MHz, CDCl_3): δ 9.78 (s, 1H), 4.50 – 4.39 (m, 2H), 2.83 – 2.71 (m, 1H), 2.67 (ddd, $J = 18.4, 6.9, 0.7$ Hz, 1H), 2.57 (ddd, $J = 18.5, 5.6, 0.9$ Hz, 1H), 1.69 – 1.57 (m, 1H), 1.28 (dt, $J = 7.1, 3.5$ Hz, 2H), 0.94 (td, $J = 15.1, 6.8$ Hz, 6H). The enantiomeric ratio of enzymatic product **3k** was determined by chiral GC (Astec® CHIRALDEX G-TA column; $30\text{ m} \times 0.25\text{ mm} \times 0.12\text{ }\mu\text{m}$) under the following conditions: $10\text{ }^\circ\text{C}/\text{min}$ from $70\text{ }^\circ\text{C}$ to $170\text{ }^\circ\text{C}$, followed by 20 min at $170\text{ }^\circ\text{C}$. Flame ionization detection: t_{R} (major) = 22.3 min , (minor) = 22.6 min . The assignment of the absolute configuration of enzymatically prepared **3k** was based on earlier reported chiral GC data.^[3]

Figure S32. ^1H NMR spectrum of enzymatic product **3k**.

Figure S33. Chiral GC analysis of enzymatic product **3k**.

4. Preparative-scale synthesis of (*R*)-**3a**

The total volume of the reaction mixture was 50 mL (HEPES buffer, 20 mM, pH 6.5). A stock solution of **2a** (500 mM) in absolute ethanol was prepared. An aliquot of this stock solution (2.5 mL, 25 mM final concentration) was added to HEPES buffer (pH 6.5) containing nitromethane (**1**, 25 mM) and 4-OT F50A (350 μ M). The reaction mixture was incubated at room temperature and the progress of the reaction was monitored by UV-VIS spectrophotometry. For this, aliquots (10 μ L) were taken from the reaction mixture, diluted with 240 μ L of 20 mM HEPES buffer (pH 6.5), and then analyzed by UV-VIS spectrophotometry. After ~50% of the substrate **2a** was consumed, an additional 25 mM of nitromethane **1** was added into the reaction mixture. After the completion of the reaction, the reaction mixture was extracted with ethyl acetate (3 $\times$ 40 mL). The combined organic layers were washed with brine, dried over anhydrous Na₂SO₄, and

concentrated under *vacuo* to give crude product **3a** with 96% conversion (^1H NMR analysis). The crude product was further purified using flash column chromatography (silica gel, petroleum ether/ethyl acetate from 95:5 to 70:30) to give the pure compound (204.7 mg, 1.1 mmol, 85% yield). The enantiomeric ratio of purified product (*R*)-**3a** was determined using chiral GC as described above.

Figure S34. ^1H NMR spectrum of enzymatic product **3a**.

Figure S35. Chiral GC analysis of enzymatic product **3a**.

5. One-pot, four-step enzymatic cascade synthesis of γ -nitrobutyric acids (**7a-b**)

The reaction mixture (20 mL) contained **5a** (6.36 mg, 3 mM) or **5b** (8.43 mg, 3 mM), 4-OT F50A (219 μ M), and ethanol (5% v/v) in HEPES buffer (20 mM, pH 7.3). The reaction was initiated by the addition of **4** (1 mL from a freshly prepared 3 M stock solution, 150 mM final concentration), and the reaction mixture was incubated at room temperature. The progress of the reaction was monitored by GC for **5a** and UV-VIS spectrophotometry for **5b**. After the reaction was completed, the pH of the reaction mixture was adjusted to pH 6.5, and nitromethane (27 μ L, final concentration of 25 mM) was added and the

progress of the reaction was monitored by GC (consumption of **2a**) or UV-VIS spectrophotometry (consumption of **2b**). After the reaction was finished, the pH of the reaction mixture was adjusted to pH 8.5 followed by the addition of PRO-ALDH(003) (0.5 mg/mL), PRO-NOX(009) (1 mg/mL) and NAD⁺ (0.5 mM). After 30 min incubation, the pH was adjusted to pH 5.0, and the reaction mixture was extracted with ethyl acetate (3 × 40 mL). The combined organic layers were washed with brine, dried over anhydrous Na₂SO₄, and concentrated under *vacuo*. The crude product was purified by silica gel column chromatography (petroleum ether/ethyl acetate from 95:5 to 50:50) to obtain (*R*)-**7a** (6.7 mg, 53% yield) or (*S*)-**7b** (11.7 mg, 80% yield). The acid functionality of (*R*)-**7a** and (*S*)-**7b** was derivatized to the corresponding methyl ester and ethyl ester, respectively, based on the reported protocol for enantiomeric ratio analysis.^[5] For derivatization, the compound **7a** or **7b** (0.1 mmol) was dissolved in dichloromethane (1 mL) and methanol or ethanol (0.2 mL), and cooled down to 0 °C. To this cooled solution, EDC.HCl (0.15 mmol) was added, followed by DMAP (0.01 mmol). After overnight incubation, the reaction mixture was quenched with saturated aq. NH₄Cl and extracted with diethyl ether, and the combined organic layers were dried over anhydrous Na₂SO₄. The dried organic layer was concentrated under *vacuo* and the resulting product dissolved in acetonitrile for chiral HPLC analysis.

(*R*)-4-nitro-3-phenylbutanoic acid (7a). Yield = 53% (6.7 mg). ¹H NMR (500 MHz, CDCl₃): δ 7.38 – 7.26 (m, 3H), 7.26 – 7.20 (m, 2H), 4.72 (dd, *J* = 12.6, 7.1 Hz, 1H), 4.63 (dd, *J* = 12.6, 7.8 Hz, 1H), 3.97 (p, *J* = 7.4 Hz, 1H), 2.83 (dd, *J* = 7.4, 2.3 Hz, 2H). The NMR data match previously reported NMR data for this compound.^[5] The enantiopurity of derivatized **7a** was analyzed by reverse phase HPLC using a chiral column (Chiralpak-ID, 150 mm x 4.6 mm, Daicel®) (MeCN/water 30:70, 25 °C, 1 mL/min flow rate). Detection at 210 nm, retention time (*R*)-**7a**: 32.5 min, (*S*)-**7a**: 34.8 min. The assignment of the absolute configuration was based on earlier reported chiral HPLC data.^[5]

Figure S36. GC spectra monitoring the consumption of **5a** and **2a**, and the formation of **3a** in the one-pot, four-steps cascade synthesis of γ -nitrobutyric acid **7a**. A) 0 h. B) After 7 h, followed by addition of **1**. C) After 12 h.

Figure S37. ^1H NMR spectrum of **7a** obtained by enzymatic cascade synthesis.

Figure S38. Chiral HPLC analysis of derivatized enzymatic product **7a**.

(S)-3-(2-chlorophenyl)-4-nitrobutanoic acid (7b). Yield = 80% (11.7 mg). ^1H NMR (500 MHz, CDCl_3): δ 7.45 – 7.39 (m, 1H), 7.29 – 7.21 (m, 3H), 4.84 – 4.72 (m, 2H), 4.45 (p, J = 7.0 Hz, 1H), 3.01 – 2.87 (m, 2H); ^{13}C NMR (126 MHz, CDCl_3): δ 175.17, 135.21, 133.81, 130.52, 129.31, 128.14, 127.50, 77.32, 36.50, 35.51; HRMS (ESI+): calcd. for $\text{C}_{10}\text{H}_9\text{ClNO}_4$, 244.0371; found, 244.0367. The enantiopurity of derivatized **7b** was analyzed by reverse phase HPLC using a chiral column (Chiralpak-ID, 150 mm x 4.6 mm, Daicel®) (MeCN/water 25:75, 25 °C, 1 mL/min flow rate). Detection at 210 nm, retention time: (major) 18.9 min, (minor) 20.3 min. The assignment of the absolute configuration of enzymatically prepared **7b** was based on the unambiguously established absolute configuration of its precursor **3b**.

Figure S39. UV spectra monitoring the consumption of **5b** (A) and **2b** (B) in the one-pot four-steps cascade synthesis of γ -nitrobutyric acid **7b**.

Figure S40. ¹H NMR spectrum of **7b** obtained by enzymatic cascade synthesis.

Figure S41. ¹³C NMR spectrum of **7b** obtained by enzymatic cascade synthesis.

Figure S42. Chiral HPLC analysis of derivatized enzymatic product **7b**.

6. Mass spectral analysis of modified and unmodified 4-OT F50A

An amount of 4-OT F50A (0.5 mg) was incubated with cinnamaldehyde (**2a**, 10 mM) in 20 mM NaH_2PO_4 buffer (pH 7.3) for 1 h at 22 °C (total volume of 1 mL). In a separate control experiment, the same amount of 4-OT F50A was incubated without **2a** under otherwise identical conditions. Subsequently, NaBH_4 was added to both samples to give a final concentration of 25 mM, and the samples were incubated at 22 °C for 1 h. The buffer of the two incubation mixtures was exchanged against 20 mM NaH_2PO_4 buffer (pH 7.3) using separate pre-packed PD-10 Sephadex G-25 gelfiltration columns (GE Healthcare, Piscataway, NJ,

USA). The two purified 4-OT F50A proteins were analyzed by ESI-MS using the mass spectrometer system described below (Figure S43).

For the peptide mapping studies, 20 μL of each protein solution (0.5 mg/mL) was diluted with 20 μL ammonium bicarbonate (100 mM, pH 8). Then, 10 μL of a 10 ng/ μL solution of endoproteinase GluC (Promega) was added and the mixture was incubated for 3 h at 37 °C. Subsequently, the samples were acidified and injected into the LC-MS system, consisting of an Ultimate 3000 UHPLC equipped with a quaternary pump, an autosampler, and a column oven coupled by a HESI-II electrospray source to a Q-Exactive Orbitrap™-based mass spectrometer (all Thermo Scientific, San Jose, CA, USA). For LC separation, a BEH-C18 (1.7 μm particles, 50 $\times$ 2.1 mm) column (Waters, Milford, MA, USA) was used, with (A) water and (B) acetonitrile (both containing 0.1% v/v formic acid) as eluents. The following gradient was used: 5% B until 0.5 min, then linear to 90% B in 17.5 min. This composition was held for 2 min, after which a switch back to 5% B was performed in 0.1 min. After 2.9 min of equilibration, the next injection was performed. The LC flow rate was 350 $\mu\text{L}/\text{min}$, the LC column was kept at 60 °C and the injection volume was 10 μL . The HESI-II electrospray source was operated with the parameters recommended by the MS software for the LC flow rate used (spray voltage of 3.5 kV, positive mode); other parameters were sheath gas 40 AU, auxiliary gas 8 AU, cone gas 2 AU; capillary temperature 325 °C; heater temperature 350 °C. The samples were measured in positive mode from m/z 300-2000 at a resolution of 70000 @ m/z 200 and a Top5 data dependant (DDA) scan was performed at a resolution of 17500 at m/z 200. The system was controlled using the software packages Xcalibur 4.1, SII for Xcalibur 1.3 and Q-Exactive Tune 2.9 (all Thermo Scientific). For data processing Peaks studio X (Bioinformatics Solutions Inc., Waterloo, Ontario, Canada) was used. The identified peptide fragments are given in Figures S44 and S45. Selected ions of both samples were subjected to MS/MS analysis (Figure S46).

Figure S43. ESI-MS spectra of labeled (A) and unlabeled (B) 4-OT F50A. (A) 4-OT F50A incubated with 10 mM **2a** and afterwards treated with sodium borohydride (expected mass 6851 Da). (B) Unmodified 4-OT F50A treated with sodium borohydride (expected mass 6735 Da).

Peptide List

Peptide	-10lgP	Mass	Length	ppm	m/z	RT	Fraction	Scan	Source File	Intensity Sample 1	#Feature	#Feature Sample 1	Accession	PTM	AScor
AISRLDAPLTSVRVIITE	62.66	2040.1578	19	4.2	681.0627	6.36	1	2145	18mdv27u-label-GluC2-DDA_20181122170307.raw	1.11E9	9	9	us14-OT-1 8mdv2761 Cguo-4OT		
VSEAIRSLDAPLTSVRVIITE	58.33	2355.3008	22	0.9	1178.6587	6.68	1	2306	18mdv27u-label-GluC2-DDA_20181122170307.raw	9.88E8	8	8	us14-OT-1 8mdv2761 Cguo-4OT		
M(+15.89) AKGHAGIGGELASKVRR	51.63	1853.0054	18	1.4	464.2593	3.20	1	820	18mdv27u-label-GluC2-DDA_20181122170307.raw	3.55E7	14	14	us14-OT-1 8mdv2761 Cguo-4OT	Oxidation (M)	M1:Oxidation (M):10.00.00
MAKGHAGIGGELASKVRR	50.67	1837.0104	18	1.8	460.2607	3.30	1	850	18mdv27u-label-GluC2-DDA_20181122170307.raw	6.36E8	20	20	us14-OT-1 8mdv2761 Cguo-4OT		
MAKGHAGIGGE	40.24	1026.4917	11	1.0	514.2537	0.90	1	280	18mdv27u-label-GluC2-DDA_20181122170307.raw	6.4E8	31	31	us14-OT-1 8mdv2761 Cguo-4OT		
M(+15.99)AKGHAGIGGE	34.21	1042.4866	11	1.3	522.2512	0.66	1	184	18mdv27u-label-GluC2-DDA_20181122170307.raw	4.23E7	14	14	us14-OT-1 8mdv2761 Cguo-4OT	Oxidation (M)	M1:Oxidation (M):10.00.00
PIAQIHILE	29.59	1032.5968	9	3.5	517.3075	5.69	1	1874	18mdv27u-label-GluC2-DDA_20181122170307.raw	2.97E9	13	13	us14-OT-1 8mdv2761 Cguo-4OT		
TLIREVSE	28.92	945.5131	8	3.6	473.7655	3.95	1	1148	18mdv27u-label-GluC2-DDA_20181122170307.raw	1.15E8	10	10	us14-OT-1 8mdv2761 Cguo-4OT		
GRSDEQKE	24.71	947.4308	8	0.1	316.8176	0.33	1	53	18mdv27u-label-GluC2-DDA_20181122170307.raw	2.72E7	24	24	us14-OT-1 8mdv2761 Cguo-4OT		
PIAQIHILEGRSDE	24.36	1576.8209	14	1.1	526.6148	4.98	1	1561	18mdv27u-label-GluC2-DDA_20181122170307.raw	2.48E6	2	2	us14-OT-1 8mdv2761 Cguo-4OT		
P(+116.06)IAQIHILE	19.60	1148.6567	9	3.6	575.3377	7.32	1	2578	18mdv27u-label-GluC2-DDA_20181122170307.raw	6.93E8	2	2	us14-OT-1 8mdv2761 Cguo-4OT	C9H8-nterminus	P1:C9H8-nterminus:1000.00
LASKVRR	18.67	828.5294	7	0.6	415.2722	0.53	1	133	18mdv27u-label-GluC2-DDA_20181122170307.raw	2.41E8	5	5	us14-OT-1 8mdv2761 Cguo-4OT		
total 12 peptides															

Figure S44. Peptide mapping data of labeled 4-OT F50A digested with protease GluC.

Biocatalytic Asymmetric Michael Additions of Nitromethane to α,β -Unsaturated Aldehydes
via Enzyme-bound Iminium Ion Intermediates

Peptide List

Peptide	-10lgP	Mass	Length	ppm	m/z	RT	Fraction	Scan	Source File	Intensity Sample 2	#Feature	#Feature Sample 2	Accession	PTM	AScore
VSEAIRSLDAPLTSVRVIITE	63.12	2355.3008	22	0.5	1178.6582	6.66	3	2352	18mdv27u-uni-GluC- DDA_20181122163511.raw	5.46E9	10	10	us 4-OT-1 8mdv276 Cguo-40T		
AISRSLDAPLTSVRVIITE	62.16	2040.1578	19	0.2	1021.0864	6.32	3	2184	18mdv27u-uni-GluC- DDA_20181122163511.raw	1.47E10	19	19	us 4-OT-1 8mdv276 Cguo-40T		
MAKGHAGIGGELASKVRR	46.84	1837.0104	18	1.6	460.2606	3.30	3	925	18mdv27u-uni-GluC- DDA_20181122163511.raw	2.06E8	9	9	us 4-OT-1 8mdv276 Cguo-40T		
M(+15.99) AKGHAGIGGELASKVRR	43.36	1853.0054	18	0.4	464.2588	3.21	3	891	18mdv27u-uni-GluC- DDA_20181122163511.raw	1.31E7	6	6	us 4-OT-1 8mdv276 Cguo-40T	Oxidation (M)	M1:Oxi dation (M):10 00.00
MAKGHAGIGGE	39.29	1026.4917	11	1.0	514.2537	0.91	3	316	18mdv27u-uni-GluC- DDA_20181122163511.raw	2.31E9	31	31	us 4-OT-1 8mdv276 Cguo-40T		
M(+15.99)AKGHAGIGGE	33.25	1042.4866	11	3.8	522.2526	0.66	3	205	18mdv27u-uni-GluC- DDA_20181122163511.raw	2.3E8	14	14	us 4-OT-1 8mdv276 Cguo-40T	Oxidation (M)	M1:Oxi dation (M):10 00.00
PIAQIHILE	28.69	1032.5968	9	2.6	517.3070	5.66	3	1872	18mdv27u-uni-GluC- DDA_20181122163511.raw	1.74E10	35	35	us 4-OT-1 8mdv276 Cguo-40T		
TLIREVSE	28.52	945.5131	8	1.1	473.7643	3.95	3	1230	18mdv27u-uni-GluC- DDA_20181122163511.raw	1.72E8	8	8	us 4-OT-1 8mdv276 Cguo-40T		
GRSDEQKE	27.12	947.4308	8	1.1	316.8179	0.47	3	142	18mdv27u-uni-GluC- DDA_20181122163511.raw	1.36E8	25	25	us 4-OT-1 8mdv276 Cguo-40T		
PIAQIHILEGRSDE	23.66	1576.8209	14	0.5	526.6145	4.99	3	1640	18mdv27u-uni-GluC- DDA_20181122163511.raw	1.19E6	2	2	us 4-OT-1 8mdv276 Cguo-40T		
QKETLIRE	21.47	1015.5662	8	1.0	339.5297	2.86	3	749	18mdv27u-uni-GluC- DDA_20181122163511.raw	1.14E6	2	2	us 4-OT-1 8mdv276 Cguo-40T		
LASKVRR	16.69	828.5294	7	0.9	415.2723	0.53	3	157	18mdv27u-uni-GluC- DDA_20181122163511.raw	1.88E8	3	3	us 4-OT-1 8mdv276 Cguo-40T		
total 12 peptides															

Figure S45. Peptide mapping of unlabeled 4-OT F50A digested with protease GluC.

Figure S46. MS/MS analysis of modified and unmodified peptide ions. A) MS/MS spectrum of the ion corresponding to the modified peptide PIAQIHILE obtained from 4-OT F50A treated with **2a** and sodium borohydride. B) MS/MS spectrum of the ion corresponding to the unmodified peptide PIAQHILE obtained from 4-OT F50A treated with only sodium borohydride. The expected m/z of the b_5 ion (PIAQI) of the modified peptide is 639 Da, whereas that of the unmodified peptide is 523 Da.

7. References

- [1] a) Ramachandran, P. V.; Burghardt, T. E.; Reddy, M. V. R. Asymmetric Allylboration of α,β -Enals as a Surrogate for the Enantioselective Synthesis of Allylic Amines and α -Amino Acids. *J. Org. Chem.* **2005**, *70*, 2329-2331; b) Dong, H.; Shen, M.; Redford, J. E.; Stokes, B. J.; Pumphrey, A. L.; Driver, T. G. Transition Metal-Catalyzed Synthesis of Pyrroles from Dienyl Azides. *Org. Lett.* **2007**, *9*, 5191-5194; c) Joshi, P. R.; Nanubolu, J. B.; Menon, R. S. Oxygenative and Dehydrogenative [3 + 3] Benzannulation Reactions of α,β -Unsaturated Aldehydes and γ -Phosphonyl Crotonates Mediated by Air: Regioselective Synthesis of 4-Hydroxybiaryl-2-carboxylates. *Org. Lett.* **2016**, *18*, 752-755; d) Rodrigo, E.; García Ruano, J. L.; Cid, M. B. Organocatalytic Michael Addition/Intramolecular Julia-Kocienski Olefination for the Preparation of Nitrocyclohexenes. *J. Org. Chem.* **2013**, *78*, 10737-10746; e) Dong, S.; Frings, M.; Zhang, D.; Guo, Q.; Daniliuc, C. G.; Cheng, H.; Bolm, C. Organocatalytic Asymmetric Synthesis of *trans*- γ -Lactams. *Chem. Eur. J.* **2017**, *23*, 13888-13892; f) Lyu, L.; Jin, M. Y.; He, Q.; Xie, H.; Bian, Z.; Wang, J. Bi(OTf)₃-Catalyzed Addition of Isocyanides to 2*H*-Chromene Acetals: An Efficient Pathway for Accessing 2-Carboxamide-2*H*-Chromenes. *Org. Biomol. Chem.* **2016**, *14*, 8088; g) Zhang, M.; Zhai, Y.; Ru, S.; Zang, D.; Han, S.; Yu, H.; Wei, Y. Highly Practical and Efficient Preparation of Aldehydes and Ketones from Aerobic Oxidation of Alcohols with an Inorganic-Ligand Supported Iodine Catalyst. *Chem. Commun.* **2018**, *54*, 10164-10167; h) Meazza, M.; Light, M. E.; Mazzanti, A.; Rios, R. Synergistic Catalysis: *cis*-Cyclopropanation of Benzoxazoles. *Chem. Sci.* **2016**, *7*, 984-988; i) Kim, E.; Park, S.; Chang, S. Silylative Reductive Amination of α,β -Unsaturated Aldehydes: A Convenient Synthetic Route to β -Silylated Secondary Amines. *Chem. Eur. J.* **2018**, *24*, 5765-5769; j) Palais, L.; Babel, L.; Quintard, A.; Belot, S.; Alexakis, A. Copper-Catalyzed Enantioselective 1,4-Addition to α,β -Unsaturated Aldehydes. *Org. Lett.* **2010**, *12*, 1988-1991; k) Bergonzini, G.; Vera, S.; Melchiorre, P. Cooperative Organocatalysis for the Asymmetric γ -Alkylation of α -Branched Enals. *Angew. Chem. Int. Ed.* **2010**, *49*, 9685-9688; l) Farndon, J. J.; Ma, X.; Bower, J. F. Transition Metal Free C–N Bond Forming Dearomatizations and Aryl C–H Aminations by in Situ Release of a Hydroxylamine-Based Aminating Agent. *J. Am. Chem. Soc.* **2017**, *139*, 14005-14008; m) Fumeaux, R.; Menozzi-Smarrito, C.; Stalmach, A.; Munari, C.; Kraehenbuehl, K.; Steiling, H.; Crozier, A.; Williamson, G.; Barron, D. First Synthesis, Characterization, and Evidence for the Presence of Hydroxycinnamic Acid Sulfate and Glucuronide Conjugates in Human Biological Fluids as a Result of Coffee Consumption. *Org. Biomol. Chem.* **2010**, *8*, 5199-5211; n) Konrádová, D.; Kozubíková, H.; Doležal, K.; Pospíšil, J. Microwave-Assisted Synthesis of Phenylpropanoids and Coumarins: Total Synthesis of Osthol. *Eur. J. Org. Chem.* **2017**, 5204-5213; o) Tucker, H. β -Adrenergic blocking agents. 20. (3-Hydroxyprop-1-enyl)-Substituted 1-(Aryloxy)-3-(alkylamino)propan-2-ols. *J. Med. Chem.* **1980**, *23*, 1122-1126; p) Xing, C. (Kuality HerbceuticsT Llc) WO2016179587A1, **2016**.

- [2] a) Miao, Y.; Tepper, P. G.; Geertsema, E. M.; Poelarends, G. J. Stereochemical Control of Enzymatic Carbon-Carbon Bond-Forming Michael-Type Additions by “Substrate Engineering”. *Eur. J. Org. Chem.* **2016**, 5350-5354; b) West, T. H.; Daniels, D. S. B.; Slawin, A. M. Z.; Smith, A. D. Catalytic Enantioselective [2,3]-Rearrangements of Allylic Ammonium Ylides: A Mechanistic and Computational Study. *J. Am. Chem. Soc.* **2017**, *139*, 4366-4375; c) Whitlock, G. A.; Brennan, P. E.; Roberts, L. R.; Stobie, A. Potent and Selective α_{1A} Adrenoceptor Partial Agonists-Novel Imidazole Frameworks. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 3118-3121.
- [3] van der Meer, J. Y.; Poddar, H.; Baas, B. J.; Miao, Y.; Rahimi, M.; Kunzendorf, A.; van Merkerk, R.; Tepper, P. G.; Geertsema, E. M.; Thunnissen, A. M. W. H.; Quax, W. J.; Poelarends, G. J. Using Mutability Landscapes of a Promiscuous Tautomerase to Guide the Engineering of Enantioselective Michaelases. *Nat. Commun.* **2016**, *7*, 10911.
- [4] Zandvoort, E.; Geertsema, E. M.; Baas, B. J.; Quax, W. J.; Poelarends, G. J. Bridging Between Organocatalysis and Biocatalysis: Asymmetric Addition of Acetaldehyde to β -Nitrostyrenes Catalyzed by a Promiscuous Proline-Based Tautomerase. *Angew. Chem. Int. Ed.* **2012**, *51*, 1240-1243.
- [5] Biewenga, L.; Saravanan, T.; Kunzendorf, A.; van der Meer, Y.; Pijning, T.; Tepper, P.; van Merkerk, R.; Charnock, S. J.; Thunnissen, A. M. W. H.; Poelarends, G. J. Enantioselective Synthesis of Pharmaceutically Active γ -Aminobutyric Acids Using a Tailor-Made Artificial Michaelase in One-Pot Cascade Reactions. *ACS Catal.* **2019**, *9*, 1503-1513.

3

Enantioselective Aldol Addition of Acetaldehyde to Aromatic Aldehydes Catalyzed by Proline-based Carboligases

Mohammad Saifuddin

Chao Guo

Lieuwe Biewenga

Thangavelu Saravanan

Simon J. Charnock

Gerrit J. Poelarends

Published in *ACS Catalysis* **2020**, 10 (4), 2522-2527.

ABSTRACT

Aromatic β -hydroxyaldehydes, 1,3-diols, and α,β -unsaturated aldehydes are valuable precursors to biologically active natural products and drug molecules. Herein we report the biocatalytic aldol condensation of acetaldehyde with various aromatic aldehydes to give a number of aromatic α,β -unsaturated aldehydes using a previously engineered variant of 4-oxalocrotonate tautomerase [4-OT(M45T/F50A)] as carboligase. Moreover, an efficient one-pot two-step chemoenzymatic route towards chiral aromatic 1,3-diols has been developed. This one-pot chemoenzymatic strategy successfully combined a highly enantioselective aldol addition step catalyzed by a proline-based carboligase [4-OT(M45T/F50A) or TAUT015] with a chemical reduction step to convert enzymatically prepared aromatic β -hydroxyaldehydes into the corresponding 1,3-diols with high optical purity (e.r. up to >99:1) and in good isolated yield (51-92%). These developed (chemo)enzymatic methodologies offer alternative synthetic choices to prepare a variety of important drug precursors.

KEYWORDS

Biocatalysis; aldol reaction; aldolases; β -hydroxyaldehydes; carboligases

The aldol reaction is one of the most important methodologies for the straightforward construction of carbon-carbon bonds in synthetic organic chemistry.¹ This reaction allows complex chiral compounds to be rapidly assembled from simpler building blocks. Enzyme catalysis by using native and engineered aldolases offers a powerful strategy to perform this transformation.² With the discovery and engineering of novel aldolase activities, an increasing number of applications of aldolases in stereoselective synthesis have been reported.^{2e,2i} Despite the rapidly expanding biocatalytic toolbox, aldolases that can use various aromatic aldehydes as acceptors for acetaldehyde addition are rare.^{2f-h} Such aldolases would provide an attractive synthetic tool for the preparation of chiral precursors to various biologically active compounds and drug molecules.

Our group has previously reported that the enzyme 4-oxalocrotonate tautomerase (4-OT) from *Pseudomonas putida* mt-2 promiscuously catalyzes the cross-condensation of acetaldehyde (**1**) with benzaldehyde (**2a**) to give cinnamaldehyde (**4a**) (Scheme 1).³ It has been shown that 4-OT catalyzes both the initial aldol addition to yield 3-hydroxy-3-phenylpropanal (**3a**) as well as the subsequent rapid dehydration of **3a** to give **4a**, preventing the accumulation of chiral aldol adduct **3a** in the reaction mixture.^{3a} Rahimi and co-workers applied mutability landscape-guided protein engineering to further improve this promiscuous aldolase activity of 4-OT. This resulted in the identification of a double mutant, 4-OT M45T/F50A, with a remarkable 3300-fold improved catalytic efficiency (in terms of k_{cat}/K_m) over wild-type 4-OT.^{3b}

Scheme 1. 4-OT catalyzed aldol condensation of acetaldehyde (**1**) with benzaldehyde (**2a**) to yield cinnamaldehyde (**4a**).

Although cinnamaldehydes are valuable synthons in their own right, we were especially interested in the enzymatic preparation of chiral aromatic β -hydroxyaldehydes, and the corresponding 1,3-diols, which are important precursors to various natural products, bioactive compounds and drugs (Figure 1).^{4,5} We envisioned that these chiral aldol adducts could be constructed by the enzymatic addition of acetaldehyde to selected aromatic aldehydes, yielding β -hydroxyaldehydes that are not (or slowly) dehydrated, allowing their accumulation in the reaction mixture. Chemical reduction of the enzymatically prepared aromatic β -hydroxyaldehydes would provide access to the corresponding aromatic 1,3-diols, which are key intermediates in the synthesis of drugs such as Fluoxetine (Figure 1).⁵

Figure 1. Chiral aromatic β -hydroxyaldehydes and cinnamaldehydes are valuable precursors to biologically active compounds and drugs.

Herein we report the 4-OT(M45T/F50A)-catalyzed synthesis of various aromatic α,β -unsaturated aldehydes, starting from acetaldehyde and a number of aromatic aldehydes, in good isolated yields. We also report the one-pot, two-step chemoenzymatic asymmetric synthesis of various aromatic 1,3-diols with high optical purity (e.r. up to >99:1) and in good isolated yield (51-92%). This chemoenzymatic strategy highlights a highly stereoselective aldol addition of acetaldehyde to diverse aromatic aldehydes, catalyzed by either 4-OT(M45T/F50A) or the newly identified carbolligase TAUT015, yielding various

enantioenriched aromatic β -hydroxyaldehydes, which are chemically reduced into the corresponding 1,3-diols in the same pot. The applied carboligases accept a broad range of aromatic aldehydes for acetaldehyde additions, offering alternative synthetic choices to prepare important drug precursors.

We previously reported that an engineered variant of 4-OT (mutant M45T/F50A) can efficiently catalyze the aldol condensation of acetaldehyde (**1**) with benzaldehyde (**2a**) to yield cinnamaldehyde (**4a**).^{3b} This prompted us to start our investigations by testing a large set of twenty-five aromatic aldehydes (**2b-y** and **2ac**) as potential aldol acceptor substrates in the 4-OT(M45T/F50A)-catalyzed aldol reaction with donor substrate **1** (Scheme 2). The results demonstrate that the 4-OT(M45T/F50A) enzyme has a remarkably broad substrate scope, accepting nineteen aromatic aldehydes with electron withdrawing substituents (**2b-t**) as aldol acceptor substrates (Scheme 2, Figures S1-S19). On the contrary, aromatic aldehydes with electron-donating substituents (**2u-y** and **2ac**) were not, or very poorly, accepted by 4-OT(M45T/F50A) (Scheme 2, Figures S20-S24, S28). While this may be attributed to unfavorable equilibrium constants for these aromatic aldehydes in the aldol addition with acetaldehyde, the electron donating properties of the substituents on the aromatic ring likely also lead to weak activation of the carbonyl carbon for nucleophilic attack.^{2j,2l}

Scheme 2. Enzymatic aldol reaction of acetaldehyde (**1**) with various aromatic aldehydes (**2**) using promiscuous proline-based tautomerases as carboligases.

Out of the nineteen well-accepted aromatic aldehydes, fourteen substrates undergo an aldol condensation reaction with formation of the corresponding α,β -unsaturated aldehydes, whereas five substrates (**2k-n** and **2s**, see below) undergo conversion without significant formation of the corresponding α,β -unsaturated aldehydes (Figure S1-S19). To demonstrate the synthetic usefulness of 4-OT(M45T/F50A) for the preparation of α,β -unsaturated aldehydes, nine aromatic aldehydes (**2b-d**, **2f-j** and **2t**) that could be efficiently converted by 4-OT(M45T/F50A) were selected and used in semi-preparative scale synthesis (Scheme 3). The corresponding α,β -unsaturated aldehydes (**4b-d**, **4f-j** and **4t**) could be isolated in moderate to good yields (Scheme 3, Figures S30-S47). Notably, *ortho*-substituted benzaldehydes (e.g. **2b**, **2f**, **2h** and **2i**) were better accepted by 4-OT(M45T/F50A) than *para*-substituted benzaldehydes (**2p**, **2r**, **2o** and **2q**).⁶ These results underscore the potential of 4-OT(M45T/F50A) for the synthesis of versatile aromatic α,β -unsaturated aldehydes, which are important precursors to various abundantly prescribed drugs (Figure 1).^{4f}

Scheme 3. Synthesis of aromatic α,β -unsaturated aldehydes from simpler building blocks using the carboligase 4-OT(M45T/F50A). ^aReaction conditions: reaction mixtures consisted of 100 mM **1**, 3 mM **2** and 0.1 mg/mL 4-OT (M45T/F50A) in 20 mM sodium phosphate buffer, 5% DMSO v/v, pH 7.3. The reaction volume was 60 mL. ^bReaction time (48 h, 72 h for **2t**). ^cPurified by silica gel column chromatography.

Interestingly, for the 4-OT(M45T/F50A)-catalyzed aldol addition of **1** to **2k-n** and **2s**, depletion of the starting substrates was observed without significant accumulation of the corresponding α,β -unsaturated aldehydes (Figures S10-S13, S18). These results led us to hypothesize that in these instances the initially formed aldol adducts, β -hydroxyaldehydes **3k-n** and **3s**, were not (or slowly) dehydrated and accumulated in the reaction mixture. To confirm this hypothesis, an analytical scale experiment was performed using 4-OT(M45T/F50A) in NaPi buffer (0.3 mL, pH 7.3, 5% DMSO), containing **1** (50 mM) and **2k** (2 mM). Reaction progress was monitored by following the depletion of **2k** by UV-VIS spectrophotometry, and NaBH₄ was added to the reaction mixture (after 1 h) to convert the aldol adduct into the more stable 1,3-diol **5k**. Analysis of chemoenzymatic product **5k** by HPLC on a chiral stationary phase, using authentic standards with known absolute configuration, revealed high enantiocontrol at the site of addition with formation of the *R* enantiomer (e.r. = 94:6). These results demonstrate that the enzyme 4-OT(M45T/F50A) can indeed catalyze enantioselective aldol additions.

Having established that 4-OT(M45T/F50A) has potential for the asymmetric synthesis of enantioenriched aromatic β -hydroxyaldehydes, we first optimized the reaction conditions by varying the pH and the concentration of acetaldehyde, buffer and enzyme (Figure S88). Using the optimized conditions, the one-pot two-step chemoenzymatic cascade synthesis of aromatic 1,3-diols **5k-n** and **5s**, via enzymatically prepared β -hydroxyaldehydes **3k-n** and **3s**, was achieved with high enantioselectivity (e.r. up to 99:1) and in good isolated yield (63-92%) (Table 1, Figures S48-S62).

Table 1. Asymmetric chemoenzymatic synthesis of aromatic 1,3-diols using 4-OT(M45T/F50A) as carboligase.^a

Reaction scheme: $R-CHO$ (**2k-n, 2s**) + CH_3CHO (**1**) $\xrightarrow{4-OT(M45T/F50A)}$ $[R-CH(OH)-CH_2-CHO]$ (**3k-n, 3s**) $\xrightarrow{NaBH_4}$ $R-CH(OH)-CH_2-OH$ (**5k-n, 5s**)

Entry	Aldol acceptor	Product	Reaction time [h] ^b	Isolated yield (%) ^c	e.r. ^d	Abs. Conf.
1	2k	5k	6	92	91:9	<i>R</i> ^e
2	2l	5l	7	63	90:10	<i>R</i> ^e
3	2m	5m	2.5	88	90:10	<i>R</i> ^e
4	2n	5n	5	70	92:8	<i>R</i> ^f
5	2s	5s	1	65	99:1	<i>R</i> ^f

^a Assay conditions: acetaldehyde (**1**, 100 mM), aromatic aldehyde (**2k-n, 2s**, 2 mM) and purified 4-OT(M45T/F50A) (0.125–0.475 mg/mL) in 20 mM sodium phosphate buffer (MOPS for **2k**), with 5% DMSO (v/v) as cosolvent, at pH 8.0 (pH 7.3 for **2s** to increase its electrophilicity) and room temperature. The reaction volume was 40 mL. To reduce the aldehyde functionality of the enzymatic products **3k-n** and **3s**, NaBH₄ (30 mM) was added to the reaction mixture followed by incubation for 3 h at room temperature.

^b Reaction time of the enzymatic step. ^c Isolated yield of the aromatic 1,3-diol product.

^d Determined by HPLC analysis on a chiral stationary phase using chemically synthesized racemic standards. ^e The absolute configuration was determined by chiral HPLC using chemically prepared authentic standards with known (*R*) or (*S*) configuration. ^f The absolute configuration was assigned based on analogy.

To expand the number of enzymatically accessible β -hydroxyaldehyde products (e.g. **3b-d**, **3f** and **3g**) in principle two different approaches can be used: (i) the use of protein engineering to create new variants of 4-OT(M45T/F50A) that lack dehydration activity and possess unchanged or enhanced aldol addition activity, or (ii) the use of homologous enzymes that possess the desired aldol addition activity but lack dehydration activity. To selectively eliminate the dehydration activity of 4-OT(M45T/F50A), a structure-based approach is favored, which awaits the determination of the enzyme crystal structure complexed with one of the β -hydroxyaldehyde products (work in progress). In this study, we therefore have chosen to screen a panel of fifty commercially available 4-OT homologues for enzymes with the desired aldol addition activity to yield enantioenriched β -hydroxyaldehydes. For initial activity testing, we used donor substrate **1** and aldol acceptor substrate **2b**. Interestingly, out of the fifty tested 4-OT homologues, three enzymes (TAUT015, TAUT021 and TAUT028) were found to show significant activity towards the aldol addition of **1** to **2b** to give the desired product **3b** without promoting significant dehydration leading to formation of **4b** (Figure S29). Notably, the same reaction catalyzed by 4-OT(M45T/F50A) resulted in the formation of α,β -unsaturated aldehyde **4b** as the sole product, providing additional support that the β -hydroxyaldehyde dehydration step is enzyme catalyzed.

We next investigated the substrate scope of the best performing enzyme, TAUT015, which shows 28% overall sequence identity to 4-OT (Figure S89), in additions using **1** as donor substrate and a number of selected benzaldehyde derivatives as potential aldol acceptor substrates. The results show that, like 4-OT(M45T/F50A), TAUT015 does not accept benzaldehydes with electron donating substituents (**2u**, **2v**, **2z**, **2aa** and **2ab**) on the aromatic ring (Figures S25-S27). However, several benzaldehydes with electron withdrawing substituents (**2b-d**, **2f** and **2g**) were well accepted by TAUT015. Encouraged by these findings, semi-preparative scale reactions were carried out under optimized reaction

conditions. The reaction progress (**1** with **2b-d**, **2f** and **2g**) was monitored by UV-VIS spectrophotometry and the enzymatically prepared β -hydroxyaldehydes **3b-d**, **3f** and **3g** were reduced to the corresponding aromatic 1,3-diols (**5b-d**, **5f** and **5g**) using NaBH_4 . These one-pot, two-step chemoenzymatically prepared compounds **5b-d**, **5f** and **5g** were obtained in good isolated yields (up to 72%) and with high e.r. values (e.r. up to >99:1) (Table 2, Figures S63-S87). Note that enzymatic access to these useful chiral synthons requires the use of TAUT015 as carboligase and is not possible with 4-OT(M45T/F50A). Hence, the enzyme TAUT015 nicely supplements the toolbox of biocatalysts for the production of important chiral aromatic β -hydroxyaldehydes and corresponding 1,3-diols.

In conclusion, our results indicate that members of the 4-OT family of enzymes, which possess an uncommon catalytic amino-terminal proline, can function as novel carboligases, promoting the cross-aldol reaction of acetaldehyde with diverse aromatic aldehydes to give access to a range of important α,β -unsaturated aldehydes as well as enantioenriched β -hydroxyaldehydes. It is feasible that their promiscuous aldol addition activities, which are beyond the synthetic scope of currently known aldolases, can be further optimized by directed evolution or computational redesign. It is important to emphasize that the controlled cross-aldol addition of acetaldehyde is a particularly challenging synthetic aim because acetaldehyde is a relatively reactive and difficult to tame chemical.^{5,7} Remarkably, the promiscuous 4-OT enzymes accept acetaldehyde as a nucleophile in aldol additions, which is unparalleled among the aldolases, with the notable exception of 2-deoxy-D-ribose-5-phosphate aldolase (DERA) and d-fructose-6-phosphate aldolase (FSA), which are also able to use acetaldehyde as a nucleophilic substrate.^{2g,2k,2o} As such, these proline-based 4-OT enzymes nicely complement the toolbox of biocatalysts for (asymmetric) C-C bond-formation, and open up new opportunities to develop practical enzymatic processes for the more sustainable and step-economic synthesis of valuable drug precursors starting from simple building blocks. Further screening of related proline-

Table 2. Asymmetric chemoenzymatic synthesis of aromatic 1,3-diols using TAUT015 as carboligase.^a

Entry	Aldol acceptor	Product	Reaction time [h] ^b	Isolated yield (%) ^c	e.r. ^d	Abs. Conf. ^e
1			4.5	72	>99:1	<i>R</i>
2			8.3	51	94:6	<i>R</i>
3			3.3	62	99:1	<i>R</i>
4			6.5	69	99:1	<i>R</i>
5			11	56	89:11	<i>R</i>

^aAssay conditions: acetaldehyde (**1**, 100 mM), benzaldehyde derivative (**2b-d**, **2f-g**, 2 mM) and enzyme (crude cell extract, 0.33 mg/mL) in 20 mM sodium phosphate buffer, containing 5% EtOH (v/v) as cosolvent, at pH 6.5 and room temperature. The specific enzyme activity was determined to be 0.044 U/mg CFE. Unit definition: 1 U of enzyme converts 1 μ mol of **2b** in 1 minute; assay conditions: 20 mM NaPi pH 6.5, 5% EtOH, 100 mM **1**, 2 mM **2b**. The reaction volume was 40 mL. To reduce the aldehyde functionality of **3b-d** and **3f-g**, NaBH₄ (30 mM) was added to the reaction mixture followed by incubation for 3 h at room temperature. ^bReaction time of the enzymatic step. ^cIsolated yield of the aromatic 1,3-diol product. ^dDetermined by HPLC analysis on a chiral stationary phase using chemically synthesized racemic standards. ^eThe absolute configuration was determined by chiral HPLC using chemically prepared authentic standards with known (*R*) or (*S*) configuration.

based enzymes might prove to be a valuable approach to discover new aldolase activities that could be exploited to develop synthetically useful biocatalysts for carbon-carbon bond formation.

REFERENCES

- [1] (a) Gröger, H.; Vogl, E. M.; Shibasaki, M. New Catalytic Concepts for the Asymmetric Aldol Reaction. *Chem. Eur. J.* **1998**, *4*, 1137-1141; (b) Palomo, C.; Oiarbide, M.; García, J. M. Current Progress in the Asymmetric Aldol Addition Reaction. *Chem. Soc. Rev.* **2004**, *33*, 65-75; (c) Yamashita, Y.; Yasukawa, T.; Yoo, W. J.; Kitanosono, T.; Kobayashi, S. Catalytic Enantioselective Aldol Reactions. *Chem. Soc. Rev.* **2018**, *47*, 4388-4480.
- [2] (a) Wong, C. H.; Junceda, E. G.; Chen, L.; Blanco, O.; Gijssen, H. J. M.; Steensma, D. H. Recombinant 2-Deoxyribose-5-phosphate Aldolase in Organic Synthesis: Use of Sequential Two-Substrate and Three-Substrate Aldol Reactions. *J. Am. Chem. Soc.* **1995**, *117*, 3333-3339; (b) Fessner, W. D. Enzyme Mediated C-C Bond Formation. *Curr. Opin. Chem. Biol.* **1998**, *2*, 85-97; (c) Machajewski, T. D.; Wong, C. H. The Catalytic Asymmetric Aldol Reaction. *Angew. Chem. Int. Ed.* **2000**, *39*, 1352-1374; (d) DeSantis, G.; Liu, J.; Clark, D. P.; Heine, A.; Wilson, I. A.; Wong, C. H. Structure-Based Mutagenesis Approaches Toward Expanding the Substrate Specificity of D-2-Deoxyribose-5-phosphate Aldolase. *Bioorg. Med. Chem.* **2003**, *11*, 43-52; (e) Clapés, P.; Garrabou, X. Current Trends in Asymmetric Synthesis with Aldolases. *Adv. Synth. Catal.* **2011**, *353*, 2263-2283; (f) Fesko, K.; Khadjawi, M. G. Biocatalytic Methods for C-C Bond Formation. *ChemCatChem.* **2013**, *5*, 1248-1272; (g) Clapés, P.; Fessner, W. D.; Sprenger, G. A.; Samland, A. K. Recent Progress in Stereoselective Synthesis with Aldolases. *Curr. Opin. Chem. Biol.* **2010**, *14*, 154-167; (h) Chen, Q.; Chen, X.; Cui, Y.; Ren, J.; Lu, W.; Feng, J.; Wu, Q.; Zhu, D. A New D-Threonine Aldolase as a Promising Biocatalyst for Highly Stereoselective Preparation of Chiral Aromatic β -Hydroxy- α -Amino acids. *Catal. Sci. Technol.*, **2017**, *7*, 5964-5973; (i) Schmidt, N. G.; Eger, E.; Kroutil, W. Building Bridges: Biocatalytic C-C-Bond Formation toward Multifunctional Products. *ACS Catal.* **2016**, *6*, 4286-4311; (j) Obexer, R.; Godina, A.; Garrabou, X.; Mittl, P. R. E.; Baker, D.; Griffiths, A. D.; Hilvert, D. Emergence of a Catalytic Tetrad during Evolution of a Highly Active Artificial Aldolase. *Nat. Chem.* **2017**, *9*, 50-56; (k) Roldán, R.; Hernandez, K.; Joglar, J.; Bujons, J.; Parella, T.; Sánchez-Moreno, I.; Hélaine, V.; Lemaire, M.; Guérard-Hélaine, C.; Fessner, W. D.; Clapés, P. Biocatalytic Aldol Addition of Simple Aliphatic Nucleophiles to Hydroxyaldehydes. *ACS Catal.* **2018**, *8*, 8804-8809; (l) Xie, Z. B.; Wang, N.; Jiang, G. F.; Yu, X. Q. Biocatalytic Asymmetric Aldol Reaction in Buffer Solution. *Tetrahedron Lett.* **2013**, *54*, 945-948; (m) Roldán, R.; Hernández, K.; Joglar, J.; Bujons, J.; Parella, T.; Fessner, W. D.; Clapés, P. Aldolase-Catalyzed Asymmetric Synthesis of N-Heterocycles by Addition of Simple Aliphatic Nucleophiles to Aminoaldehydes. *Adv. Synth. Catal.* **2019**, *361*, 2673-2687; (n) Junker, S.; Roldan, R.; Joosten, H. J.; Clapés, P.; Fessner, W. D. Complete Switch of Reaction Specificity of an Aldolase by Directed Evolution In Vitro: Synthesis of Generic Aliphatic Aldol Products. *Angew. Chem. Int. Ed.* **2018**, *57*, 10153-10157;

(o) Roldán, R.; Sanchez-Moreno, I.; Scheidt, T.; Hélaïne, V.; Lemaire, M.; Parella, T.; Clapés, P.; Fessner, W. D.; Guérard-Hélaïne, C. Breaking the Dogma of Aldolase Specificity: Simple Aliphatic Ketones and Aldehydes are Nucleophiles for Fructose-6-phosphate Aldolase. *Chem. Eur. J.* **2017**, *23*, 5005-5009.

[3] (a) Zandvoort, E.; Geertsema, E. M.; Quax, W. J.; Poelarends, G. J. Enhancement of the Promiscuous Aldolase and Dehydration Activities of 4-Oxalocrotonate Tautomerase by Protein Engineering. *ChemBioChem* **2012**, *13*, 1274-1277; (b) Rahimi, M.; van der Meer, J. Y.; Geertsema, E. M.; Poddar, H.; Baas, B. J.; Poelarends, G. J. Mutations Closer to the Active Site Improve the Promiscuous Aldolase Activity of 4-Oxalocrotonate Tautomerase More Effectively than Distant Mutations. *ChemBioChem* **2016**, *17*, 1225-1228; (c) Rahimi, M.; van der Meer, J. Y.; Geertsema, E. M.; Poelarends, G. J. Engineering a Promiscuous Tautomerase into a More Efficient Aldolase for Self-Condensations of Linear Aliphatic Aldehydes. *ChemBioChem* **2017**, *18*, 1435-1441; (d) Rahimi, M.; Geertsema, E. M.; Miao, Y.; van der Meer, J. Y.; van den Bosch, T.; de Haan, P.; Zandvoort, E.; Poelarends, G. J. Inter- and Intramolecular Aldol Reactions Promiscuously Catalyzed by a Proline-based Tautomerase. *Org. Biomol. Chem.* **2017**, *15*, 2809-2816.

[4] (a) Rej, R. K.; Das, T.; Hazra, S.; Nanda, S. Chemoenzymatic Asymmetric Synthesis of Fluoxetine, Atomoxetine, Nisoxetine, and Duloxetine. *Tetrahedron: Asymmetry* **2013**, *24*, 913-918; (b) Wenthur, C. J.; Bennett, M. R.; Lindsley, C. W. Classics in Chemical Neuroscience: Fluoxetine (Prozac). *ACS Chem. Neurosci.* **2014**, *5*, 14-23; (c) de Fátima, Â.; Lapis, A. A. M.; Pilli, R. A. A Concise Total Synthesis of (R)-Fluoxetine, a Potent and Selective Serotonin Reuptake Inhibitor. *J. Braz. Chem. Soc.* **2005**, *16*, 495-499; (d) Roesner, S.; Aggarwal, V. K. Enantioselective synthesis of (R)-tolterodine using lithiation/borylation-protodeboronation methodology. *Can. J. Chem.* **2012**, *90*, 1-10; (e) Sabitha, G. Padmaja, P.; Yadav, J. S. A Concise Total Synthesis of Diospongingins A and B. *Helvetica Chimica Acta* **2008**, *91*, 2235-2239; (f) Guo, C.; Saifuddin, M.; Saravanan, T.; Sharifi, M.; Poelarends, G. J. Biocatalytic Asymmetric Michael Additions of Nitromethane to α,β -Unsaturated Aldehydes via Enzyme-bound Iminium Ion Intermediates. *ACS Catal.* **2019**, *9*, 4369-4373; (g) Fan, X.; Rodriguez-Esrich, C.; Wang, S.; Sayalero, S.; Pericas, M. A. Highly Enantioselective Cross-Aldol Reactions of Acetaldehyde Mediated by a Dual Catalytic System Operating under Site Isolation. *Chem. Eur. J.* **2014**, *20*, 13089-13093.

[5] Hayashi, Y.; Itoh, T.; Aratake, S.; Ishikawa, H. A Diarylprolinol in an Asymmetric, Catalytic, and Direct Crossed- Aldol Reaction of Acetaldehyde. *Angew. Chem. Int. Ed.* **2008**, *47*, 2082-2084.

[6] Martínez, A.; Van Gemmeren, M.; List, B. Unexpected Beneficial Effect of ortho-Substituents on the (S)-Proline-Catalyzed Asymmetric Aldol Reaction of Acetone with Aromatic Aldehydes. *Synlett* **2014**, *25*, 961-964.

[7] Yang, J. W.; Chandler, C.; Stadler, M.; Kampen, D.; List, B. Proline-Catalysed Mannich Reactions of Acetaldehyde. *Nature* **2008**, *452*, 453-455.

SUPPORTING INFORMATION

1. Materials and Methods

1.1 Materials

Chemicals were purchased from Sigma Aldrich, Across, Merck or Fluka (unless noted otherwise) and were used without further purification. The α,β -unsaturated aldehydes **2o-r** were prepared using a previously reported method.^[1a] Synthesis of racemic and enantiopure reference compounds, required for chiral analysis of enzymatic products, was done according to published protocols.^[2]

1.2 General methods

NMR spectra were recorded on a Bruker 500 MHz spectrometer. Enzymatic assays were performed on a V-650 or V-660 spectrophotometer from Jasco (IJsselstein, The Netherlands). Reverse phase HPLC was carried out using an in-house analytical HPLC equipped with a Shimadzu LC-10 AT pump and a Shimadzu SPD-M10A diode array detector.

1.3 Protein expression and purification

The expression and purification of 4-OT(M45T/F50A) mutant was based on a protocol described elsewhere.^[3] A sample of purified protein was analyzed by ESI-MS to confirm that the protein had been processed correctly and the initiating methionine had been removed. The purified protein was flash frozen in liquid nitrogen and stored at -80 °C until further use.

1.4 Chiral analysis

The enantiomeric ratio and absolute configuration of the chemoenzymatically synthesized compounds **5b-d**, **5f-g** and **5k-m** was analyzed by chiral HPLC using chemically synthesized racemic 1,3-diols and enantiopure 1,3-diols as

reference compounds.^[2a-b] For compounds **5n** and **5s**, the enantiomeric ratio was analyzed by chiral HPLC using chemically synthesized racemic 1,3-diols^[2a] and the absolute configuration was based on analogy.

1.5 Analytical scale reactions

Enzymatic and non-enzymatic reactions between substrates **1** and **2** were monitored by following the depletion in the absorbance corresponding to the consumption of **2**. Reaction conditions are given below each figure.

Figure S1. UV spectra monitoring the aldol reaction of **1** (50 mM) with **2b** (2 mM) catalyzed by 4-OT(M45T/F50A) (1 mg/mL) in buffer [20 mM NaPi/5% (v/v) EtOH, 0.3 mL] at pH 7.3. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

Figure S2. UV spectra monitoring the aldol reaction of **1** (50 mM) with **2c** (2 mM) catalyzed by 4-OT(M45T/F50A) (1 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO, 0.3 mL] at pH 7.3. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

Figure S3. UV spectra monitoring the aldol reaction of **1** (50 mM) with **2d** (2 mM) catalyzed by 4-OT(M45T/F50A) (1 mg/mL) in buffer [20 mM NaPi/5% (v/v) EtOH, 0.3 mL] at pH 7.3. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

Figure S4. UV spectra monitoring the aldol reaction of **1** (50 mM) with **2e** (2 mM) catalyzed by 4-OT(M45T/F50A) (1 mg/mL) in buffer [20 mM NaPi/5% (v/v) EtOH, 0.3 mL] at pH 7.3. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

Figure S5. UV spectra monitoring the aldol reaction of **1** (50 mM) with **2f** (2 mM) catalyzed by 4-OT(M45T/F50A) (1 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO, 0.3 mL] at pH 7.3. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

Figure S6. UV spectra monitoring the aldol reaction of **1** (50 mM) with **2g** (2 mM) catalyzed by 4-OT(M45T/F50A) (1 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO, 0.3 mL] at pH 7.3. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

Figure S7. UV spectra monitoring the aldol reaction of **1** (50 mM) with **2h** (2 mM) catalyzed by 4-OT(M45T/F50A) (1 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO, 0.3 mL] at pH 7.3. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

Figure S8. UV spectra monitoring the aldol reaction of **1** (50 mM) with **2i** (2 mM) catalyzed by 4-OT(M45T/F50A) (1 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO, 0.3 mL] at pH 7.3. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

Figure S9. UV spectra monitoring the aldol reaction of **1** (50 mM) with **2j** (2 mM) catalyzed by 4-OT(M45T/F50A) (1 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO, 0.3 mL] at pH 7.3. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

3

Figure S10. UV spectra monitoring the aldol reaction of **1** (50 mM) with **2k** (2 mM) catalyzed by 4-OT(M45T/F50A) (1 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO, 0.3 mL] at pH 7.3. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

Figure S11. UV spectra monitoring the aldol reaction of **1** (50 mM) with **2l** (2 mM) catalyzed by 4-OT(M45T/F50A) (1 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO, 0.3 mL] at pH 7.3. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

Figure S12. UV spectra monitoring the aldol reaction of **1** (50 mM) with **2m** (2 mM) catalyzed by 4-OT(M45T/F50A) (1 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO, 0.3 mL] at pH 7.3. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

Figure S13. UV spectra monitoring the aldol reaction of **1** (50 mM) with **2n** (2 mM) catalyzed by 4-OT(M45T/F50A) (1 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO, 0.3 mL] at pH 7.3. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

Figure S14. UV spectra monitoring the aldol reaction of **1** (50 mM) with **2o** (2 mM) catalyzed by 4-OT(M45T/F50A) (1 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO, 0.3 mL] at pH 7.3. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

Figure S15. UV spectra monitoring the aldol reaction of **1** (50 mM) with **2p** (2 mM) catalyzed by 4-OT(M45T/F50A) (1 mg/mL) in buffer [20 mM NaPi/5% (v/v) EtOH, 0.3 mL] at pH 7.3. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

3

Figure S16. UV spectra monitoring the aldol reaction of **1** (50 mM) with **2q** (2 mM) catalyzed by 4-OT(M45T/F50A) (1 mg/mL) in buffer [20 mM NaPi/5% (v/v) EtOH, 0.3 mL] at pH 7.3. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

Figure S17. UV spectra monitoring the aldol reaction of **1** (50 mM) with **2r** (2 mM) catalyzed by 4-OT(M45T/F50A) (1 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO, 0.3 mL] at pH 7.3. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

Figure S18. UV spectra monitoring the aldol reaction of **1** (50 mM) with **2s** (2 mM) catalyzed by 4-OT(M45T/F50A) (1 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO, 0.3 mL] at pH 7.3. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

Figure S19. UV spectra monitoring the aldol reaction of **1** (50 mM) with **2t** (2 mM) catalyzed by 4-OT(M45T/F50A) (1 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO, 0.3 mL] at pH 7.3. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

Figure S20. UV spectra monitoring the aldol reaction of **1** (50 mM) with **2u** (2 mM) catalyzed by 4-OT(M45T/F50A) (1 mg/mL) in buffer [20 mM NaPi/5% (v/v) EtOH, 0.3 mL] at pH 7.3. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

Figure S21. UV spectra monitoring the aldol reaction of **1** (50 mM) with **2v** (2 mM) catalyzed by 4-OT(M45T/F50A) (1 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO, 0.3 mL] at pH 7.3. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

3

Figure S22. UV spectra monitoring the aldol reaction of **1** (50 mM) with **2w** (2 mM) catalyzed by 4-OT(M45T/F50A) (1 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO, 0.3 mL] at pH 7.3. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

Figure S23. UV spectra monitoring the aldol reaction of **1** (50 mM) with **2x** (2 mM) catalyzed by 4-OT(M45T/F50A) (1 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO, 0.3 mL] at pH 7.3. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

Figure S24. UV spectra monitoring the aldol reaction of **1** (50 mM) with **2y** (2 mM) catalyzed by 4-OT(M45T/F50A) (1 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO, 0.3 mL] at pH 7.3. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

Figure S25. UV spectra monitoring the aldol reaction of **1** (50 mM) with **2z** (2 mM) catalyzed by TAUT015 (1 mg/mL) in buffer [20 mM NaPi/5% (v/v) EtOH, 0.3 mL] at pH 6.5. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

Figure S26. UV spectra monitoring the aldol reaction of **1** (50 mM) with **2aa** (2 mM) catalyzed by TAUT015 (1 mg/mL) in buffer [20 mM NaPi/5% (v/v) EtOH, 0.3 mL] at pH 6.5. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

Figure S27. UV spectra monitoring the aldol reaction of **1** (50 mM) with **2ab** (2 mM) catalyzed by TAUT015 (1 mg/mL) in buffer [20 mM NaPi/5% (v/v) EtOH, 0.3 mL] at pH 6.5. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

3

Figure S28. UV spectra monitoring the aldol reaction of **1** (50 mM) with **2ac** (2 mM) catalyzed by 4-OT(M45T/F50A) (1 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO, 0.3 mL] at pH 7.3. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

2. Screening of 4-OT homologues for aldol addition activity

Fifty 4-OT homologues (selected by Prozomix from in-house metagenomes) were provided as crude cell-free extracts (CFEs) by Prozomix Ltd (Station Court, Haltwhistle, Northumberland, UK), and used without any further purification. The aldol addition activity of the 4-OT homologues was determined using the donor substrate **1** and aldol acceptor substrate **2b**. Briefly, the reaction mixtures (300 μ L) consisted of CFE (1 mg/mL), **1** (50 mM), **2b** (2 mM) and ethanol (5% v/v) in 20 mM NaPi buffer (pH 6.5). The reaction was initiated by the addition

of 15 μL from a stock solution of **2b** (20 mM in ethanol). The progress of the reaction was monitored by following the depletion in absorbance at 249 nm, which corresponds to the consumption of **2b** (Figure S29).

Figure S29. Screening of 4-OT homologues for the aldol addition of **1** (50 mM) to **2b** (2 mM). **A.** General screening results. The conversion (%) was calculated based on the amount of **2b** consumed. **B, C** and **D.** UV spectra monitoring the aldol addition of **1** (50 mM) to **2b** (2 mM) catalyzed by TAUT015 (**B**), TAUT021 (**C**) and TAUT028 (**D**).

3. Biocatalytic synthesis of α,β -unsaturated aldehydes (**4b-d**, **4f-j** and **4t**) using 4-OT(M45T/F50A)

Reaction mixtures (60 mL) contained acetaldehyde (**1**, 100 mM), aromatic aldehydes (**2b-d**, **2f-j** and **2t**, 3 mM) and 4-OT(M45T/F50A) enzyme (0.1 mg/mL) in NaPi buffer (20 mM, pH 7.3). Stock solutions of **2b-d**, **2f-j** and **2t** were prepared in DMSO. The reaction mixture was incubated at room temperature and reaction progress was monitored by UV-vis spectrophotometry. After the completion of the reaction, the reaction mixture was extracted with ethyl acetate (3×40 mL). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4 , and concentrated under *vacuo*. The crude products **4b-d**, **4f-j** and **4t** were further purified by silica gel column chromatography (petroleum ether/ethyl acetate from 95:5 to 50:50).

(*E*)-3-(2-chlorophenyl)acrylaldehyde (4b**).**^[1a] White solid; yield = 81% (24 mg). ^1H NMR (500 MHz, CDCl_3) δ 9.76 (d, $J = 7.7$ Hz, 1H), 7.94 (d, $J = 16.0$ Hz, 1H), 7.66 (dd, $J = 7.7, 1.7$ Hz, 1H), 7.46 (dd, $J = 7.9, 1.3$ Hz, 1H), 7.39 – 7.29 (m, 2H), 6.70 (dd, $J = 16.0, 7.7$ Hz, 1H). The ^1H NMR data is in agreement with published data.^[1a]

Figure S30. UV spectra monitoring the aldol condensation of **1** (100 mM) with **2b** (3 mM) catalyzed by 4-OT(M45T/F50A) (0.1 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO] at pH 7.3.

Figure S31. ^1H NMR spectrum of enzymatic compound **4b**.

(E)-3-(3-chlorophenyl)acrylaldehyde (4c).^[1a] White solid; yield = 70% (21 mg). ^1H NMR (500 MHz, CDCl_3) δ 9.72 (d, J = 7.6 Hz, 1H), 7.55 (t, J = 1.7 Hz, 1H), 7.48 – 7.42 (m, 2H), 7.42 – 7.36 (m, 2H), 6.71 (dd, J = 16.0, 7.6 Hz, 1H). The ^1H NMR data is in agreement with published data.^[1a]

Figure S32. UV spectra monitoring the aldol condensation of **1** (100 mM) with **2c** (3 mM) catalyzed by 4-OT(M45T/F50A) (0.1 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO] at pH 7.3.

Figure S33. ^1H NMR spectrum of enzymatic compound **4c**.

(E)-3-(2,4-dichlorophenyl)acrylaldehyde (4d).^[1b] White solid; yield = 71% (25 mg). ^1H NMR (500 MHz, CDCl_3) δ 9.76 (d, J = 7.6 Hz, 1H), 7.86 (d, J = 16.0 Hz, 1H), 7.60 (d, J = 8.5 Hz, 1H), 7.49 (d, J = 2.1 Hz, 1H), 7.32 (dd, J = 8.5, 1.8 Hz, 1H), 6.69 (dd, J = 16.0, 7.6 Hz, 1H). The ^1H NMR data is in agreement with published data.^[1b]

Figure S34. UV spectra monitoring the aldol condensation of **1** (100 mM) with **2d** (3 mM) catalyzed by 4-OT(M45T/F50A) (0.1 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO] at pH 7.3.

Figure S35. ^1H NMR spectrum of enzymatic compound **4d**.

(E)-3-(2-nitrophenyl)acrylaldehyde (4f).^[1c] White solid; yield = 73% (23 mg). ^1H NMR (500 MHz, CDCl_3) δ 9.78 (d, $J = 7.4$ Hz, 1H), 8.42 (t, $J = 1.9$ Hz, 1H), 8.30 (ddd, $J = 8.2, 2.2, 0.9$ Hz, 1H), 7.90 (d, $J = 7.7$ Hz, 1H), 7.65 (t, $J = 8.0$ Hz, 1H), 7.54 (d, $J = 16.1$ Hz, 1H), 6.82 (dd, $J = 16.1, 7.4$ Hz, 1H). The ^1H NMR data is in agreement with published data.^[1c]

Figure S36. UV spectra monitoring the aldol condensation of **1** (100 mM) with **2f** (3 mM) catalyzed by 4-OT(M45T/F50A) (0.1 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO] at pH 7.3.

Figure S37. ^1H NMR spectrum of enzymatic compound **4f**.

(E)-3-(3-nitrophenyl)acrylaldehyde (4g).^[1d] White solid; yield = 61% (16 mg). ^1H NMR (500 MHz, CDCl_3) δ 9.79 (d, J = 7.7 Hz, 1H), 8.12 (d, J = 8.2 Hz, 1H), 8.05 (d, J = 15.9 Hz, 1H), 7.74 – 7.66 (m, 2H), 7.65 – 7.58 (m, 1H), 6.64 (dd, J = 15.8, 7.7 Hz, 1H). The ^1H NMR data is in agreement with published data.^[1d]

Figure S38. UV spectra monitoring the aldol condensation of **1** (100 mM) with **2g** (3 mM) catalyzed by 4-OT(M45T/F50A) (0.1 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO] at pH 7.3.

Figure S39. ¹H NMR spectrum of enzymatic compound **4g**.

(E)-3-(2-fluorophenyl)acrylaldehyde (4h).^[1b] White solid; yield = 79% (21 mg). ¹H NMR (500 MHz, CDCl₃) δ 9.72 (d, *J* = 7.7 Hz, 1H), 7.66 (d, *J* = 16.2 Hz, 1H), 7.59 (td, *J* = 7.6, 1.7 Hz, 1H), 7.43 (dddd, *J* = 8.3, 7.2, 5.3, 1.7 Hz, 1H), 7.25 – 7.19 (m, 1H), 7.15 (ddd, *J* = 10.5, 8.3, 1.0 Hz, 1H), 6.79 (dd, *J* = 16.2, 7.7 Hz, 1H). The ¹H NMR data is in agreement with published data.^[1b]

Figure S40. UV spectra monitoring the aldol condensation of **1** (100 mM) with **2h** (3 mM) catalyzed by 4-OT(M45T/F50A) (0.1 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO] at pH 7.3.

Figure S41. ^1H NMR spectrum of enzymatic compound **4h**.

(E)-3-(2-bromophenyl)acrylaldehyde (4i).^[1c] White solid; yield = 85% (32 mg). ^1H NMR (500 MHz, CDCl_3) δ 9.78 (d, J = 7.7 Hz, 1H), 7.91 (d, J = 15.9 Hz, 1H), 7.66 (dt, J = 7.7, 1.2 Hz, 2H), 7.41 – 7.35 (m, 1H), 7.29 (td, J = 7.8, 1.6 Hz, 1H), 6.68 (dd, J = 15.9, 7.7 Hz, 1H). The ^1H NMR data is in agreement with published data.^[1c]

Figure S42. UV spectra monitoring the aldol condensation of **1** (100 mM) with **2i** (3 mM) catalyzed by 4-OT(M45T/F50A) (0.1 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO] at pH 7.3.

Figure S43. ¹H NMR spectrum of enzymatic compound **4i**.

(E)-3-(2,3,5,6-tetrafluorophenyl)acrylaldehyde (4j). White solid; yield = 85% (31 mg). ¹H NMR (500 MHz, CDCl₃) δ 9.75 (d, *J* = 7.5 Hz, 1H), 7.52 (d, *J* = 16.5 Hz, 1H), 7.23 – 7.11 (m, 1H), 7.02 (dd, *J* = 16.5, 7.4 Hz, 1H). ¹³C NMR (126 MHz, CDCl₃) δ 193.61, 147.19, 146.00, 145.21, 143.97, 136.27, 135.72, 135.66, 135.59, 114.86, 108.21, 108.03, 107.85. HRMS (ESI⁺): calcd. for C₉H₅F₄O [M+H]⁺: 205.0277, found: 205.0268.

Figure S44. UV spectra monitoring the aldol condensation of **1** (100 mM) with **2j** (3 mM) catalyzed by 4-OT(M45T/F50A) (0.1 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO] at pH 7.3.

Figure S45. ¹H NMR (top) and ¹³C NMR (bottom) spectra of enzymatic compound **4j**.

(*E*)-3-(furan-2-yl)acrylaldehyde (4t).^[1c] White solid; yield = 23% (5 mg). ¹H NMR (500 MHz, CDCl₃) δ 9.61 (d, *J* = 7.9 Hz, 1H), 7.56 (d, *J* = 1.5 Hz, 1H), 7.22 (d, *J* = 15.7 Hz, 1H), 6.77 (d, *J* = 3.4 Hz, 1H), 6.58 (dd, *J* = 15.7, 7.9 Hz, 1H), 6.53 (dd, *J* = 3.4, 1.8 Hz, 1H). The ¹H NMR data is in agreement with published data.^[1c]

Figure S46. UV spectra monitoring the aldol condensation of **1** (100 mM) with **2t** (3 mM) catalyzed by 4-OT(M45T/F50A) (0.1 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO] at pH 7.3.

Figure S47. ¹H NMR spectrum of enzymatic compound **4t**.

4. Chemoenzymatic synthesis of 1,3-diols (**5k-n** and **5s**) using 4-OT(M45T/F50A)

The reaction was performed in NaPi buffer (MOPS buffer for **2k**) [20 mM, pH 8.0 (pH 7.3 for **2s**), 40 mL]. Stock solutions of **2k-n** and **2s** (40 mM) were prepared in DMSO. From the stock solution of aldol acceptor substrate, 2 mL (2 mM final concentration) was added to buffer containing acetaldehyde (**1**, 100 mM) and an appropriate amount of purified enzyme 4-OT(M45T/F50A) (0.125–0.475 mg/mL). The reaction mixture was incubated at room temperature and the progress of the reaction was monitored by UV-vis spectrophotometry, following the consumption of **2k-n** and **2s**. When there was no further depletion of the substrates **2k-n** and **2s**, NaBH₄ was added into the reaction mixture (final concentration 30 mM). After 3 h of incubation at rt, the reaction mixture was extracted with ethyl acetate (3 × 40 mL). The combined organic layers were washed with brine, dried over anhydrous Na₂SO₄, and concentrated under *vacuo*. The crude products **5k-n** and **5s** were further purified by silica gel column chromatography (using petroleum ether/ethyl acetate from 95:5 to 50:50). The aromatic aldehydes **2k-n** and **2s** were used at a concentration of 2 mM to allow for easy monitoring of their consumption by UV-vis spectrophotometry. Notably, slightly higher reaction rates can be achieved when using these aldol acceptor substrates at a concentration of 10 mM.

(R)-4-(1,3-dihydroxypropyl)benzonitrile (5k).^[4] Clear oil; yield = 92% (13 mg). ¹H NMR (500 MHz, CDCl₃) δ 7.64 (d, *J* = 8.2 Hz, 2H), 7.49 (d, *J* = 8.1 Hz, 2H), 5.09 – 4.97 (m, 1H), 3.89 (q, *J* = 4.7 Hz, 2H), 3.51 (d, *J* = 3.0 Hz, 1H), 2.31 (s, 1H), 1.94 (q, *J* = 5.6 Hz, 2H). ¹³C NMR (126 MHz, CDCl₃) δ 149.86, 132.45 (2xC), 126.47 (2xC), 118.97, 111.25, 73.67, 61.45, 40.35. The NMR data are in agreement with published data.^[4] The enantiomeric ratio of chemoenzymatic product **5k** was determined using reverse phase HPLC on a Chiralpak® ID column (150 mm × 4.6 mm, Daicel) (MeCN/water = 7:93, 20°C) at a flow rate of 1 mL/min. UV detection at 210 nm: t_R:(major) = 11.6 min, (minor) = 16.8 min. The absolute

configuration of **5k** was assigned by chiral HPLC using chemically prepared authentic standards with known configuration.

Figure S48. UV spectra monitoring the aldol addition of **1** (100 mM) to **2k** (2 mM) catalyzed by 4-OT(M45T/F50A) (0.475 mg/mL) in buffer [20 mM MOPS/5% (v/v) DMSO] at pH 8.0. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

Figure S49. ¹H NMR (top) and ¹³C NMR (bottom) spectra of chemoenzymatic product **5k**.

Figure S50. Chiral HPLC analysis of chemoenzymatic product **5k**.

(R)-1-(4-(trifluoromethyl)phenyl)propane-1,3-diol (5l).^[2b] White solid; yield = 63% (11 mg). ¹H NMR (500 MHz, CDCl₃) δ 7.61 (d, *J* = 8.2 Hz, 2H), 7.49 (d, *J* = 8.1 Hz, 2H), 5.10 – 5.00 (m, 1H), 3.90 (q, *J* = 4.9 Hz, 2H), 3.22 (d, *J* = 2.9 Hz, 1H), 2.22 (t, *J* = 4.1 Hz, 1H), 2.06 – 1.90 (m, 2H). ¹³C NMR (126 MHz, CDCl₃) δ 148.39, 129.95, 129.69, 126.05, 125.61, 125.58, 125.55, 125.52, 73.84, 61.52, 40.47. The NMR data are in agreement with published data.^[2b] The enantiomeric ratio of chemoenzymatic product **5l** was determined using normal phase HPLC on a Chiralpak® IC column (150 mm × 4.6 mm, Daicel) (Heptane/IPA = 95:5, 30°C) at a flow rate of 0.5 mL/min. UV detection at 210 nm: *t*_R:(minor) = 13.09 min, (major) = 14.73 min. The absolute configuration of chemoenzymatically prepared **5l** was assigned by chiral HPLC using chemically prepared authentic standards with known configuration.

Figure S51. UV spectra monitoring the aldol addition of **1** (100 mM) to **2l** (2 mM) catalyzed by 4-OT(M45T/F50A) (0.125 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO] at pH 8.0. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

Figure S52. ¹H NMR (top) and ¹³C NMR (bottom) spectra of chemoenzymatic product **5l**.

Figure S53. Chiral HPLC analysis of chemoenzymatic product **5l**.

(R)-1-(perfluorophenyl)propane-1,3-diol (5m).^[2b] White solid; yield = 88% (17 mg). ¹H NMR (500 MHz, CDCl₃) δ 5.33 (d, *J* = 8.7 Hz, 1H), 3.98 – 3.81 (m, 2H), 3.37 (s, 1H), 2.38 – 2.30 (m, 1H), 2.27 (s, 1H), 2.01 – 1.89 (m, 1H). ¹³C NMR (126 MHz, CDCl₃) δ 145.93, 143.97, 141.77, 139.75, 138.72, 136.71, 116.84, 65.43, 61.03, 38.08. The NMR data are in agreement with published data.^[2b] The enantiomeric ratio of chemoenzymatic product **5m** was determined using reverse phase HPLC on a Chiralpak® ID column (150 mm × 4.6 mm, Daicel) (MeCN/water = 7:93, 20°C) at a flow rate of 1 mL/min. UV detection at 210 nm: *t_R*:(major) = 14.7 min, (minor) = 23.8 min. The absolute configuration of chemoenzymatically prepared **5m** was assigned by chiral HPLC using chemically prepared authentic standards with known configuration.

Figure S54. UV spectra monitoring the aldol addition of **1** (100 mM) to **2m** (2 mM) catalyzed by 4-OT(M45T/F50A) (0.25 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO] at pH 8.0. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

Figure S55. ¹H NMR (top) and ¹³C NMR (bottom) spectra of chemoenzymatic product **5m**.

Figure S56. Chiral HPLC analysis of chemoenzymatic product **5m**.

(R)-5-(1,3-dihydroxypropyl)-2-fluorobenzonitrile (5n). White solid; yield = 70% (11 mg). ^1H NMR (500 MHz, CDCl_3) δ 7.67 (dd, J = 6.0, 2.2 Hz, 1H), 7.62 (ddd, J = 7.5, 5.1, 2.3 Hz, 1H), 7.20 (t, J = 8.7 Hz, 1H), 5.06 – 4.98 (m, 1H), 3.98 – 3.88 (m, 2H), 3.47 (d, J = 2.6 Hz, 1H), 2.04 (s, 1H), 2.02 – 1.88 (m, 2H). ^{13}C NMR (126 MHz, CDCl_3) δ 163.47, 161.41, 141.72, 132.54, 132.47, 130.79, 116.65, 116.49, 114.14, 72.97, 61.55, 40.44. HRMS (ESI $^+$): calcd. for $\text{C}_{10}\text{H}_{11}\text{FNO}_2$ $[\text{M}+\text{H}]^+$: 196.0774, found: 196.0766. The enantiomeric ratio of chemoenzymatic product **5n** was determined using reverse phase HPLC on a Chiralpak[®] ID column (150 mm $\times$ 4.6 mm, Daicel) (MeCN/water = 7:93, 20 $^\circ\text{C}$) at a flow rate of 1 mL/min. UV detection at 210 nm: t_{R} :(major) = 13.7 min, (minor) = 27.1 min. The absolute configuration of chemoenzymatically prepared **5n** was tentatively assigned based on analogy.

Figure S57. UV spectra monitoring the aldol addition of **1** (100 mM) to **2n** (2 mM) catalyzed by 4-OT(M45T/F50A) (0.25 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO] at pH 8.0. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

Figure S58. ^1H NMR (top) and ^{13}C NMR (bottom) spectra of chemoenzymatic product **5n**.

Figure S59. Chiral HPLC analysis of chemoenzymatic product **5n**.

(*R*)-1-(pyridin-2-yl)propane-1,3-diol (5s**)**.^[5] Clear oil; yield = 65% (8 mg). ¹H NMR (500 MHz, CDCl₃) δ 8.55 (d, *J* = 4.7 Hz, 1H), 7.72 (td, *J* = 7.6, 1.4 Hz, 1H), 7.32 (d, *J* = 7.9 Hz, 1H), 7.23 (dd, *J* = 7.4, 5.1 Hz, 1H), 5.00 (dd, *J* = 8.4, 3.2 Hz, 1H), 4.59 (s, 1H), 3.99 – 3.81 (m, 2H), 2.87 (s, 1H), 2.17 – 2.08 (m, 1H), 1.98 – 1.87 (m, 1H). ¹³C NMR (126 MHz, CDCl₃) δ 161.52, 148.30, 137.11, 122.60, 120.24, 72.78, 61.26, 39.99. The NMR data are in agreement with published data.^[5] The enantiomeric ratio of chemoenzymatic product **5s** was determined using reverse phase HPLC on a Chiralpak® ID column (150 mm × 4.6 mm, Daicel) (MeCN/water = 7:93, 20 °C) at a flow rate of 0.5 mL/min. UV detection at 210 nm: *t_R*: (major) = 7.3 min, (minor) = 9.2 min. The absolute configuration of chemoenzymatically prepared **5s** was tentatively assigned based on analogy.

Figure S61. ¹H NMR (top) and ¹³C NMR (bottom) spectra of chemoenzymatic product **5s**.

Figure S62. Chiral HPLC analysis of chemoenzymatic product **5s**.

5. Chemoenzymatic synthesis of 1,3-diols (**5b-d** and **5f-g**) using TAUT015

The reaction was performed in NaPi buffer (20 mM, pH 6.5, 40 mL). Stock solutions of **2b-d** and **2f-g** (40 mM) were prepared in absolute ethanol. From the stock solution, 2 mL (2 mM final concentration of the aldol acceptor substrate) was added to the NaPi buffer containing acetaldehyde (100 mM) and an appropriate amount of TAUT015 (crude extract, 0.33 mg/mL). The reaction mixture was incubated at room temperature and the progress of the reaction was monitored by UV-vis spectrophotometry, following the consumption of **2b-d** and **2f-g**. When there was no further depletion of the substrates **2b-d** and **2f-g**, NaBH₄ was added into the reaction mixture (final concentration 30 mM). After 3 h of incubation at rt, the reaction mixture was extracted with ethyl acetate (3 × 40 mL). The combined organic layers were washed with brine, dried over anhydrous Na₂SO₄, and concentrated under *vacuo*. The crude products **5b-d** and **5f-g** were further purified by silica gel column chromatography (using petroleum ether/ethyl acetate from 95:5 to 50:50).

(*R*)-1-(2-chlorophenyl)propane-1,3-diol (5b).^[2b] yellowish oil; yield = 72% (11 mg). ¹H NMR (500 MHz, CDCl₃) δ 7.62 (dd, J = 7.7, 1.4 Hz, 1H), 7.33 – 7.28 (m, 2H), 7.20 (td, J = 7.6, 1.7 Hz, 1H), 5.34 (dd, J = 8.8, 2.6 Hz, 1H), 3.93 – 3.83 (m, 2H), 3.45 (s, 1H), 2.68 (s, 1H), 2.09 – 1.99 (m, 1H), 1.94 – 1.84 (m, 1H). The ¹H NMR data is in agreement with published data.^[2b] The enantiomeric ratio (e.r.) of chemoenzymatic product **5b** was determined by reverse phase HPLC using a Chiralpak® ID column (150 mm $\times$ 4.6 mm, Daicel) (MeCN/water = 5:95, 25°C) at a flow rate of 1 mL/min. UV detection at 220 nm: t_R :(major) = 11.1 min, (minor) = 12.5 min.

Figure S63. UV spectra monitoring the aldol addition of **1** (100 mM) to **2b** (2 mM) catalyzed by TAU015 (0.33 mg/mL) in NaPi buffer [20 mM/5% (v/v) EtOH] at pH 6.5. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

Figure S64. ^1H NMR spectrum of chemoenzymatic product **5b**.

Figure S65. HPLC analysis of racemic **5b**.

Figure S66. HPLC analysis of authentic standard (*R*)-5b

Figure S67. HPLC analysis of chemoenzymatic product 5b.

(*R*)-1-(3-chlorophenyl)propane-1,3-diol (5c).^[6] yellowish oil; yield = 51% (8 mg). ¹H NMR (500 MHz, CDCl₃) δ 7.29 (t, J = 2.1 Hz, 1H), 7.24 – 7.18 (m, 2H), 7.14 – 7.11 (m, 1H), 4.78 (dd, J = 8.3, 4.4 Hz, 1H), 4.11 (s, 2H), 3.75 – 3.62 (m, 2H), 1.87 – 1.76 (m, 2H). The ¹H NMR data is in agreement with published data.^[6] The enantiomeric ratio of chemoenzymatic product 5c was determined by reverse phase HPLC using a Chiralpak® ID column (150 mm $\times$ 4.6 mm, Daicel) (MeCN/water = 10:90, 25°C) at a flow rate of 1 mL/min. UV detection at 220 nm: t_R :(major) = 9.7 min, (minor) = 14.1 min.

Figure S68. UV spectra monitoring the aldol addition of **1** (100 mM) to **2c** (2 mM) catalyzed by TAUT015 (0.33 mg/mL) in NaPi buffer [20 mM/5% (v/v) EtOH] at pH 6.5. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

Figure S69. ^1H NMR spectrum of chemoenzymatic product **5c**.

Figure S70. HPLC analysis of racemic **5c**. Figure S71. HPLC analysis of authentic standard (*S*)-**5c**.

Figure S72. HPLC analysis of chemoenzymatic product **5c**.

3

(R)-1-(2,4-dichlorophenyl)propane-1,3-diol (5d).^[7] yellowish oil; yield = 62% (11 mg). ¹H NMR (500 MHz, CDCl₃) δ 7.49 (d, *J* = 8.4 Hz, 1H), 7.30 (d, *J* = 2.0 Hz, 1H), 7.24 (dd, *J* = 8.4, 1.8 Hz, 1H), 5.22 (dd, *J* = 8.7 Hz, 1H), 4.21 (s, 1H), 3.88 – 3.77 (m, 2H), 3.29 (s, 1H), 2.01 – 1.89 (m, 1H), 1.86 – 1.72 (m, 1H). The ¹H NMR data is in agreement with published data.^[7] The enantiomeric ratio of chemoenzymatic product **5d** was determined by reverse phase HPLC using a Chiralpak® ID column (150 mm × 4.6 mm, Daicel) (MeCN/water = 10:90, 25°C) at a flow rate of 1 mL/min. UV detection at 220 nm: t_R:(major) = 17.9 min, (minor) = 19.1 min.

Figure S73. UV spectra monitoring the aldol addition of **1** (100 mM) to **2d** (2 mM) catalyzed by TAUT015 (0.33 mg/mL) in NaPi buffer [20 mM/5% (v/v) EtOH] at pH 6.5. **A**. Enzymatic reaction. **B**. Non-enzymatic reaction.

Figure S74. ¹H NMR spectrum of chemoenzymatic product **5d**.

Figure S75. HPLC analysis of racemic **5d**.

Figure S76. HPLC analysis of authentic standard (*S*)-5d.

Figure S77. HPLC analysis of chemoenzymatic product 5d.

(*R*)-1-(2-nitrophenyl)propane-1,3-diol (5f).^[2b] yellowish oil; yield = 69% (11 mg). ¹H NMR (500 MHz, CDCl₃) δ 7.92 (ddd, *J* = 11.9, 8.1, 1.2 Hz, 2H), 7.67 (td, *J* = 7.6, 1.1 Hz, 1H), 7.43 (td, *J* = 8.0, 7.5, 1.4 Hz, 1H), 5.51 (dd, *J* = 9.0, 2.5 Hz, 1H), 4.04 – 3.92 (m, 2H), 3.59 (s, 1H), 2.31 (s, 1H), 2.15 – 2.07 (m, 1H), 2.03 – 1.94 (m, 1H). The ¹H NMR data is in agreement with published data.

^[2b] The enantiomeric ratio of chemoenzymatic product 5f was determined by reverse phase HPLC using a Chiralpak® ID column (150 mm × 4.6 mm, Daicel)

(MeCN/water = 10:90, 25°C) at a flow rate of 1 mL/min. UV detection at 220 nm: t_R :(major) = 5.8 min, (minor) = 6.8 min.

Figure S78. UV spectra monitoring the aldol addition of **1** (100 mM) to **2f** (2 mM) catalyzed by TAUT015 (0.33 mg/mL) in NaPi buffer [20 mM/5% (v/v) EtOH] at pH 6.5. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

Figure S79. ^1H NMR spectrum of chemoenzymatic product **5f**.

Figure S80. HPLC analysis of racemic 5f.

Figure S81. HPLC analysis of authentic standard (R)-5f.

Figure S82. HPLC analysis of chemoenzymatic product **5f**.

(R)-1-(3-nitrophenyl)propane-1,3-diol (5g).^[2b] yellowish oil; yield = 56% (9 mg). ^1H NMR (500 MHz, CDCl_3) δ 8.24 (t, J = 2.0 Hz, 1H), 8.12 (dd, J = 8.3, 2.2 Hz, 1H), 7.72-7.70 (m, 1H), 7.52 (t, J = 8.0, 1.7 Hz, 1H), 5.16 – 5.02 (m, 1H), 3.98 – 3.85 (m, 2H), 3.71 (s, 1H), 2.44 (s, 1H), 1.98 (ddd, J = 10.2, 6.2, 4.0 Hz, 2H). The ^1H NMR data is in agreement with published data.^[2b] The enantiomeric ratio of enzymatic product **5g** was determined by reverse phase HPLC using a Chiralpak® ID column (150 mm $\times$ 4.6 mm, Daicel) (MeCN/water = 10:90, 25°C) at a flow rate of 1 mL/min. UV detection at 220 nm: t_R :(major) = 5.8 min, (minor) = 8.8 min.

Figure S83. UV spectra monitoring the aldol addition of **1** (100 mM) to **2g** (2 mM) catalyzed by TAUT015 (0.33 mg/mL) in NaPi buffer [20 mM/5% (v/v) EtOH] at pH 6.5. **A.** Enzymatic reaction. **B.** Non-enzymatic reaction.

Figure S84. ¹H NMR spectrum of chemoenzymatic product **5g**.

Figure S85. HPLC analysis of racemic **5g**. Figure S86. HPLC analysis of authentic standard (*S*)-**5g**. Figure S87. HPLC analysis of chemoenzymatic product **5g**.

Figure S88. Effect of pH, acetaldehyde (**1**) concentration, and buffer on the aldol addition of **1** to **2k**. **A.** Conversion of **2k** in the aldol addition of **1** (50 mM or 100 mM) to **2k** (2 mM) catalyzed by 4-OT(M45T/F50A) (1 mg/mL) in buffer [20 mM NaPi/5% (v/v) DMSO, 0.3 mL] at pH 7.3, 7.5 or 8.0. **B.** Formation of corresponding cinnamaldehyde in the aldol addition of **1** (100 mM) with **2k** (2 mM) catalyzed by 4-OT(M45T/F50A) (1 mg/mL) in buffer [20 mM NaPi or MOPS/5% (v/v) DMSO, 0.3 mL] at pH 8.0.

Figure S89. Sequence alignment of 4-OT from *P. putida* mt-2 and TAUT015. Indicated with a star (*) are positions 45 and 50 that were mutated to give 4-OT M45T/F50A. The sequence alignment was generated using the online Constraint-based Multiple Alignment Tool (COBALT, NCBI <https://www.ncbi.nlm.nih.gov/tools/cobalt/>) using default parameters.

6. Chemical synthesis of racemic 1,3-diols

6.1. Synthesis of compound **5k**

Compound **5k** was prepared according to the route shown above, which involves the preparation of a mixture of compounds **8a** and **8b**.

6.1.1. Synthesis of compound **8**

Compound **8**^[8a] was prepared according to a literature procedure and its ¹H NMR spectrum matches with earlier reported NMR data.^[8b]

6.1.2. Synthesis of compound **5k**

The reported literature procedure was slightly modified.^[9a] To a stirred solution of a mixture of **8a** and **8b** (1.0 equiv.) in dry MeOH (10 mL) at 0 °C was added NaBH₄ (3.0 equiv.) portion wise, and the reaction was run at room temp for 4 h. After completion of the reaction, as monitored by TLC (KMnO₄ test), the reaction mixture was extracted three times with EtOAc. The organic layers were combined, dried over Na₂SO₄ and evaporated under *vacuo*. The crude product was purified by silica gel column chromatography using petroleum ether/ethyl acetate (90:10) as an eluent to give the corresponding racemic 1,3-diol **5k**. Colorless liquid; yield = 86% (140 mg, starting from 200 mg of **8**). The ¹H NMR data of compound **5k**^[9b] match with earlier reported NMR data. ¹H NMR (500 MHz, CDCl₃) δ 7.66 – 7.63 (m, 2H), 7.50 (d, *J* = 8.2 Hz, 2H), 5.05 (t, *J* = 6.1 Hz, 1H), 3.90 (t, *J* = 5.5 Hz, 2H), 3.35 (s, 1H), 2.15 – 2.07 (m, 1H), 1.99 – 1.90 (m, 2H).

6.2. Synthesis of compounds 5m-n and 5s

Compounds **5m-n** and **5s** were prepared according to the route shown above, which involves the preparation of compounds **9m-n** and **9s**.

6.2.1. Synthesis of compounds 9m-n and 9s

Compounds **9m-n** and **9s** were prepared according to a literature procedure.^[2a]

Ethyl 3-hydroxy-3-(perfluorophenyl)propanoate (9m). ¹H NMR (500 MHz, CDCl₃) δ 5.59 – 5.46 (m, 1H), 4.26 – 4.17 (m, 2H), 3.27 (d, *J* = 5.3 Hz, 1H), 3.13 (dd, *J* = 16.7, 9.4 Hz, 1H), 2.75 (dd, *J* = 16.7, 4.1 Hz, 1H), 1.28 (t, *J* = 7.1 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 171.37, 146.12, 144.13, 142.08, 140.05, 138.74, 136.72, 115.25, 62.15, 61.42, 40.41, 14.12. HRMS (ESI⁺): calcd. for C₁₁H₈F₅O₂ [MH-H₂O]⁺: 267.0444, found: 267.0436.

Ethyl 3-(3-cyano-4-fluorophenyl)-3-hydroxypropanoate (9n). ¹H NMR (500 MHz, CDCl₃) δ 7.67 (dd, *J* = 6.0, 2.2 Hz, 1H), 7.65 – 7.59 (m, 1H), 7.21 (t, *J* = 8.6 Hz, 1H), 5.13 (dt, *J* = 8.0, 3.9 Hz, 1H), 4.20 (q, *J* = 7.1 Hz, 2H), 3.63 (d, *J* = 3.5 Hz, 1H), 2.75 – 2.59 (m, 2H), 1.28 (t, *J* = 7.1 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 172.06, 163.62, 161.56, 139.82, 132.59, 132.52, 130.90, 116.75, 116.60, 113.94, 68.82, 61.40, 43.09, 14.24. HRMS (ESI⁺): calcd. for C₁₂H₁₁FNO₂ [MH-H₂O]⁺: 220.0774, found: 220.0765.

Ethyl 3-hydroxy-3-(pyridin-2-yl)propanoate (9s).^[10] ¹H NMR (500 MHz, CDCl₃) δ 8.55 (d, *J* = 4.8 Hz, 1H), 7.70 (td, *J* = 7.7, 1.7 Hz, 1H), 7.42 (d, *J* = 7.9 Hz, 1H), 7.21 (dd, *J* = 7.4, 4.9 Hz, 1H), 5.18 (dt, *J* = 9.3, 5.0 Hz, 1H), 4.28 (d, *J* = 5.6 Hz, 1H), 4.18 (q, *J* = 7.1 Hz, 2H), 2.90 (dd, *J* = 16.0, 4.1 Hz, 1H), 2.76 (dd,

$J = 16.0, 8.4$ Hz, 1H), 1.25 (t, $J = 7.1$ Hz, 3H). The ^1H NMR data is in agreement with published data.^[10]

6.2.2. Synthesis of compounds **5m-n** and **5s**

The compounds **5m-n** and **5s** were obtained by reducing **9m-n** and **9s** (1.0 equiv.) using NaBH_4 (3.0 equiv.) following a procedure similar to that used for **5k**. The ^1H NMR data of compounds **5m**^[2b] and **5s**^[5] match with earlier reported NMR data.

1-(perfluorophenyl)propane-1,3-diol (5m). ^1H NMR (500 MHz, CDCl_3) δ 5.53 – 5.24 (m, 1H), 4.01 – 3.82 (m, 2H), 3.07 (s, 1H), 2.42 – 2.25 (m, 1H), 2.09 – 1.82 (m, 2H). The ^1H NMR data is in agreement with published data.^[2b]

5-(1,3-dihydroxypropyl)-2-fluorobenzonitrile (5n). ^1H NMR (500 MHz, CDCl_3) δ 7.61 (dd, $J = 6.0, 2.2$ Hz, 1H), 7.57 (ddd, $J = 7.5, 5.1, 2.2$ Hz, 1H), 7.16 (t, $J = 8.7$ Hz, 1H), 4.97 – 4.88 (m, 1H), 3.86 – 3.72 (m, $J = 5.7$ Hz, 2H), 3.65 (s, 2H), 1.86 (q, $J = 5.3$ Hz, 2H). ^{13}C NMR (126 MHz, CDCl_3) δ 163.45, 161.39, 141.77, 141.74, 132.57, 132.50, 130.78, 116.63, 116.47, 114.15, 72.81, 61.37, 40.43. HRMS (ESI⁺): calcd. for $\text{C}_{10}\text{H}_{11}\text{FNO}_2$ [$\text{M}+\text{H}$]: 196.0729, found: 196.0769.

1-(pyridin-2-yl)propane-1,3-diol (5s). ^1H NMR (500 MHz, CDCl_3) δ 8.43 (d, $J = 4.9$ Hz, 1H), 7.67 (td, $J = 7.7, 1.6$ Hz, 1H), 7.39 (d, $J = 7.9$ Hz, 1H), 7.16 (dd, $J = 7.2, 5.1$ Hz, 1H), 4.98 (dd, $J = 8.9, 3.7$ Hz, 1H), 3.90 – 3.76 (m, 2H), 2.09 – 1.98 (m, 1H), 1.96 – 1.81 (m, 1H). The ^1H NMR data is in agreement with published data.^[5]

6.3. Synthesis of compounds 5b-d and 5f-g

The compounds **5b-d** and **5f-g** were obtained by reducing **9t-x** (1.0 equiv.) using NaBH₄ (3.0 equiv.) following a procedure similar to that used for **5k**. The ¹H NMR data of compounds **5b**^[2b], **5c**^[11a], **5d**^[11b] and **5f-g**^[2b] match with earlier reported NMR data.

1-(2-chlorophenyl)propane-1,3-diol (5b). ¹H NMR (500 MHz, CDCl₃) δ 7.57 (dd, *J* = 7.7, 1.6 Hz, 1H), 7.27 (qd, *J* = 7.7, 1.2 Hz, 2H), 7.17 (td, *J* = 7.6, 1.7 Hz, 1H), 5.29 (dd, *J* = 8.8, 2.9 Hz, 1H), 3.86 – 3.77 (m, 2H), 3.63 (s, 2H), 2.04 – 1.94 (m, 1H), 1.88 – 1.77 (m, 1H). The ¹H NMR data is in agreement with published data.^[2b]

1-(3-chlorophenyl)propane-1,3-diol (5c). ¹H NMR (500 MHz, CDCl₃) δ 7.28 (s, 1H), 7.25 – 7.16 (m, 2H), 7.13 (dt, *J* = 6.7, 1.7 Hz, 1H), 4.78 (dd, *J* = 8.2, 4.4 Hz, 1H), 4.11 (s, 2H), 3.78 – 3.62 (m, 2H), 1.89 – 1.74 (m, 2H). The ¹H NMR data is in agreement with published data.^[11a]

1-(2,4-dichlorophenyl)propane-1,3-diol (5d). ¹H NMR (500 MHz, CDCl₃) δ 7.45 (d, *J* = 8.4 Hz, 1H), 7.28 (d, *J* = 2.1 Hz, 1H), 7.21 (dd, *J* = 8.4, 2.0 Hz, 1H), 5.18 (dd, *J* = 8.9, 2.4 Hz, 1H), 4.55 (s, 1H), 3.80 – 3.76 (m, 3H), 1.96 – 1.88 (m, 1H), 1.79 – 1.68 (m, 1H). The ¹H NMR data is in agreement with published data.^[11b]

1-(2-nitrophenyl)propane-1,3-diol (5f). ¹H NMR (500 MHz, CDCl₃) δ 7.86 – 7.80 (m, 2H), 7.62 – 7.55 (m, 1H), 7.38 – 7.31 (m, 1H), 5.42 (d, *J* = 7.7 Hz, 1H),

3.85 (m, 2H), 3.25 (s, 1H), 2.09 (s, 1H), 2.06 – 1.97 (m, 1H), 1.90 – 1.79 (m, 1H). The ^1H NMR data is in agreement with published data.^[2b]

1-(3-nitrophenyl)propane-1,3-diol (5g). ^1H NMR (500 MHz, CDCl_3) δ 8.12 (s, 1H), 7.98 (dd, $J = 8.1, 1.8$ Hz, 1H), 7.61 (d, $J = 7.7$ Hz, 1H), 7.42 (t, $J = 7.9$ Hz, 1H), 4.98 (t, $J = 6.1$ Hz, 1H), 4.54 (s, 1H), 3.83 – 3.71 (m, $J = 5.3$ Hz, 2H), 3.61 (s, 1H), 1.88 – 1.85 (m, 2H). The ^1H NMR data is in agreement with published data.^[2b]

6.4. Synthesis of compound 5l

Compound **5l** was prepared according to a literature procedure and its ^1H NMR data match with earlier reported data.^[12]

1-(4-(trifluoromethyl)phenyl)but-3-en-1-ol (10). ^1H NMR (500 MHz, CDCl_3) δ 7.61 (d, $J = 8.2$ Hz, 2H), 7.48 (d, $J = 8.1$ Hz, 2H), 5.87 – 5.71 (m, 1H), 5.23 – 5.14 (m, 2H), 4.81 (dd, $J = 8.0, 4.7$ Hz, 1H), 2.59 – 2.51 (m, 1H), 2.51 – 2.41 (m, 1H), 2.13 (s, 1H). The ^1H NMR data is in agreement with published data.^[12a]

4-(4-(trifluoromethyl)phenyl)butane-1,2,4-triol (11). ^1H NMR (500 MHz, CDCl_3) δ 7.58 (d, $J = 8.1$ Hz, 2H), 7.45 (d, $J = 8.1$ Hz, 2H), 5.00 (dd, $J = 10.0, 2.8$ Hz, 1H), 4.01 (ddd, $J = 9.6, 6.5, 3.2$ Hz, 1H), 3.62 (dd, $J = 11.2, 3.2$ Hz, 1H), 3.47 (dd, $J = 11.2, 6.8$ Hz, 1H), 1.85 (dt, $J = 14.6, 9.8$ Hz, 1H), 1.72 (dt, $J = 14.5, 2.8$ Hz, 1H). The ^1H NMR data is in agreement with published data.^[12b]

1-(4-(trifluoromethyl)phenyl)propane-1,3-diol (5l). ^1H NMR (500 MHz, CDCl_3) δ 7.61 (d, $J = 8.2$ Hz, 2H), 7.50 (d, $J = 8.1$ Hz, 2H), 5.05 (dd, $J = 8.1, 4.1$

Hz, 1H), 3.90 (t, $J = 5.6$ Hz, 2H), 2.03 – 1.91 (m, 2H). The ^1H NMR data is in agreement with published data.^[12d]

7. Chemical synthesis of enantiopure 1,3-diols using Jorgenson and Hayashi catalyst

7.1. Chemical synthesis of enantiopure 1,3-diols 5k-m, 5b and 5f-g

Compounds **5k-m**^[2b], **5b**^[2b] and **5f-g**^[2b] were prepared according to literature procedures and their ^1H NMR spectra match with earlier reported NMR data.^[2b]

(R)-4-(1,3-dihydroxypropyl)benzonitrile (5k). ^1H NMR (500 MHz, CDCl₃) δ 7.65 (d, $J = 8.2$ Hz, 2H), 7.50 (d, $J = 8.4$ Hz, 2H), 5.11 – 4.99 (m, 1H), 3.93 – 3.90 (m, 2H), 3.64 (dd, $J = 11.0, 3.1$ Hz, 1H), 3.40 (dd, $J = 11.0, 7.8$ Hz, 1H), 1.98 – 1.94 (m, 2H). The ^1H NMR data is in agreement with published data.^[2b]

(R)-1-(4-(trifluoromethyl)phenyl)propane-1,3-diol (5l). ^1H NMR (500 MHz, CDCl₃) δ 7.62 (d, $J = 8.1$ Hz, 2H), 7.50 (d, $J = 8.4$ Hz, 2H), 5.06 (dd, $J = 8.2, 4.1$ Hz, 1H), 3.94 – 3.87 (m, 2H), 2.00 – 1.96 (m, 2H). The ^1H NMR data is in agreement with published data.^[2b]

(R)-1-(perfluorophenyl)propane-1,3-diol (5m). ^1H NMR (500 MHz, CDCl₃) δ 5.40 – 5.32 (m, 1H), 4.00 – 3.87 (m, 2H), 3.02 (d, $J = 4.4$ Hz, 1H), 2.35 (ddt,

$J = 17.4, 9.2, 4.1$ Hz, 1H), 1.97 (ddt, $J = 14.4, 6.7, 3.9$ Hz, 1H), 1.81 (s, 1H). The ^1H NMR data is in agreement with published data. ^[2b]

(*R*)-1-(2-chlorophenyl)propane-1,3-diol (5b). ^1H NMR (500 MHz, CDCl_3) δ 7.62 (dd, $J = 7.7, 1.4$ Hz, 1H), 7.34 – 7.26 (m, 2H), 7.20 (td, $J = 7.6, 1.7$ Hz, 1H), 5.34 (dd, $J = 8.8, 2.7$ Hz, 1H), 3.93 – 3.85 (m, 2H), 3.45 (s, 1H), 2.68 (s, 1H), 2.06 – 2.01 (m, 1H), 1.95 – 1.84 (m, 1H). The ^1H NMR data is in agreement with published data. ^[2b]

(*R*)-1-(2-nitrophenyl)propane-1,3-diol (5f). ^1H NMR (500 MHz, CDCl_3) δ 7.93 (ddd, $J = 11.6, 8.0, 1.4$ Hz, 2H), 7.67 (td, $J = 7.7, 1.4$ Hz, 1H), 7.43 (ddd, $J = 8.5, 7.4, 1.5$ Hz, 1H), 5.52 (dd, $J = 9.0, 2.6$ Hz, 1H), 4.09 – 3.91 (m, 2H), 3.54 (s, 1H), 2.23 (s, 1H), 2.17 – 2.08 (m, 1H), 2.04 – 1.93 (m, 1H). The ^1H NMR data is in agreement with published data. ^[2b]

(*R*)-1-(3-nitrophenyl)propane-1,3-diol (5g). ^1H NMR (500 MHz, CDCl_3) δ 8.13 (s, 1H), 7.98 (ddd, $J = 8.2, 2.3, 1.0$ Hz, 1H), 7.60 (d, $J = 7.7$ Hz, 1H), 7.41 (t, $J = 7.9$ Hz, 1H), 4.94 (t, $J = 15.4, 9.7$ Hz, 1H), 4.91 (s, 1H), 4.03 (s, 1H), 3.80 – 3.67 (m, 2H), 1.89 – 1.83 (m, 2H). The ^1H NMR data is in agreement with published data. ^[2b]

7.2. Chemical synthesis of enantiopure 1,3-diols **5c-d** using Jorgenson and Hayashi catalyst

The compounds **5c**^[2b] and **5d**^[2b] were obtained by following a procedure similar to that used for **5k** using enantiocomplementary catalyst **B**, and their ¹H NMR spectra match with earlier reported NMR data.^[6,13]

(S)-1-(3-chlorophenyl)propane-1,3-diol (5c). ¹H NMR (500 MHz, CDCl₃) δ 7.35 (s, 1H), 7.30 – 7.17 (m, 3H), 4.88 (dd, *J* = 8.2, 4.2 Hz, 1H), 3.80 (m, 3H), 3.41 (s, 1H), 1.98 – 1.84 (m, 2H). The ¹H NMR data is in agreement with published data.^[6,13]

(S)-1-(2,4-dichlorophenyl)propane-1,3-diol (5d). ¹H NMR (500 MHz, CDCl₃) δ 7.57 (d, *J* = 8.4 Hz, 1H), 7.37 – 7.27 (m, 2H), 5.29 (d, *J* = 8.2 Hz, 1H), 3.96 – 3.85 (m, 2H), 3.78 (s, 1H), 2.76 (s, 1H), 2.07 – 1.97 (m, 1H), 1.91 – 1.80 (m, 1H). The ¹H NMR data is in agreement with published data.^[13]

8. References

- [1] a) Guo, C.; Saifuddin, M.; Saravanan, T.; Sharifi, M.; Poelarends, G. J. Biocatalytic Asymmetric Michael Additions of Nitromethane to α,β -Unsaturated Aldehydes via Enzyme-bound Iminium Ion Intermediates. *ACS Catal.* **2019**, *9*, 4369-4373; b) Wang, M. M.; Ning, X. S.; Qu, J. P.; Kang, Y. B. Dehydrogenative Synthesis of Linear α,β -Unsaturated Aldehydes with Oxygen at Room Temperature Enabled by tBuONO. *ACS Catal.* **2017**, *7*, 4000-4003; c) Hayashi, Y.; Sakamoto, D.; Okamura, Daichi. One-Pot Synthesis of (S)-Baclofen via Aldol Condensation of Acetaldehyde with Diphenylprolinol Silyl Ether Mediated Asymmetric Michael Reaction as a Key Step. *Org. Lett.* **2016**, *18*, 4-7; d) Yamazaki, S.; Sugiura, H.; Ohashi, S.; Ishizuka, K.; Saimu, R.; Mikata, Y.; Ogawa, A. Intramolecular [2 + 2] and [4 + 2] Cycloaddition Reactions of Cinnamylamides of Ethenetricarboxylate in Sequential Processes. *J. Org. Chem.* **2016**, *81*, 10863-10886; e) EL-Atawy, M. A.; Ferretti, F.; Ragaini, F. A Synthetic Methodology for Pyrroles from Nitrodienes. *Eur. J. Org. Chem.* **2018**, 4818-4825.
- [2] a) Koszelewski, D.; Zysk, M.; Brodzka, A.; Źądło, A.; Paprocki, D.; Ostaszewski, R. Evaluation of a New Protocol for Enzymatic Dynamic Kinetic Resolution of 3-Hydroxy-3-(aryl)propanoic Acids. *Org. Biomol. Chem.* **2015**, *13*, 11014-11020; b) Hayashi, Y.; Itoh, T.; Aratake, S.; Ishikawa, H. A Diarylprolinol in an Asymmetric, Catalytic, and Direct Crossed- Aldol Reaction of Acetaldehyde. *Angew. Chem. Int. Ed.* **2008**, *47*, 2082 -2084.
- [3] Zandvoort, E.; Geertsema, E. M.; Baas, B. J.; Quax, W. J.; Poelarends, G. J. Bridging Between Organocatalysis and Biocatalysis: Asymmetric Addition of Acetaldehyde to β -Nitrostyrenes Catalyzed by a Promiscuous Proline-Based Tautomerase. *Angew. Chem. Int. Ed.* **2012**, *51*, 1240-1243.
- [4] Qiao, Y.; Chen, Q.; Lin, S.; Ni, B.; Headley, A. D. Organocatalytic Direct Asymmetric Crossed-Aldol Reactions of Acetaldehyde in Aqueous Media. *J. Org. Chem.* **2013**, *78*, 2693 - 2697.
- [5] Kumar, M.; Shah, B. A.; Tanejaa, S. C. Tandem Catalysis by Lipase in a Vinyl Acetate Mediated Cross-Aldol Reaction. *Adv. Synth. Catal.* **2011**, *353*, 1207 - 1212.
- [6] Erion, M. D.; Reddy, K. R.; Boyer, S. H.; Matelich, M. C.; Gomez-Galeno, J.; Lemus, R. H.; Ugarkar, B. G.; Colby, T. J.; Schanzer, J.; van Poelje, P. D. Design, Synthesis, and Characterization of a Series of Cytochrome P450 3A-Activated Prodrugs (HepDirect Prodrugs) Useful for Targeting Phosph(on)ate-Based Drugs to the Liver. *J. Am. Chem. Soc.* **2004**, *126*, 5154-5163.
- [7] Fan, X.; Rodriguez-Esrich, C.; Wang, S.; Sayalero, S.; Pericas, M. A. Highly Enantioselective Cross-Aldol Reactions of Acetaldehyde Mediated by a Dual Catalytic System Operating under Site Isolation. *Chem. Eur. J.* **2014**, *20*, 13089 - 13093.

- [8] a) CARPINO, Albert, P. (PFIZER INC.) WO 2013/068875 A1, **2013**; b) Korsager, S.; Nielsen, D. U.; Taaning, R. H.; Skrydstrup, T. Access to β -Keto Esters by Palladium-Catalyzed Carbonylative Coupling of Aryl Halides with Monoester Potassium Malonates. *Angew. Chem. Int. Ed.* **2013**, *52*, 9763–9766.
- [9] a) Kim, H.; Lee, C. Nickel-Catalyzed Reductive Cyclization of Organohalides. *Org. Lett.* **2011**, *13*, 2050–2053; b) Banik, S. M.; Mennie, K. M.; Jacobsen, E. N. Catalytic 1,3-Difunctionalization via Oxidative C–C Bond Activation. *J. Am. Chem. Soc.* **2017**, *139*, 9152–9155.
- [10] Baudoux, J.; Lefebvre, P.; Legay, R.; Lasne, M. C.; Rouden, J. Environmentally Benign Metal-free Decarboxylative Aldol and Mannich Reactions. *Green Chem.*, **2010**, *12*, 252–259.
- [11] a) Borowiecki, P.; Wawro, A. M.; Winska, P.; Wielechowska, M.; Bretner, M. Synthesis of Novel Chiral TBBt Derivatives with Hydroxyl Moiety. Studies on Inhibition of Human Protein Kinase CK2a and Cytotoxicity Properties. *Eur. J. Med. Chem.* **2014**, *84*, 364–374; b) Reddy, K. R.; Matelich, M. C.; Ugarkar, B. G.; Gómez-Galeno, J. E.; DaRe, J.; Ollis, K.; Sun, Z.; Craig, W.; Colby, T. J.; Fujitaki, J. M.; Boyer, S. H.; van Poelje, P. D.; Erion, M. D. Pradefovir: A Prodrug That Targets Adefovir to the Liver for the Treatment of Hepatitis B. *J. Med. Chem.* **2008**, *51*, 666–676.
- [12] a) Dam, J. H.; Fristrup, P.; Madsen, R. Combined Experimental and Theoretical Mechanistic Investigation of the Barbier Allylation in Aqueous Media. *J. Org. Chem.* **2008**, *73*, 3228–3235; b) Theodorou, A.; Triandafillidi, I.; Kokotos, C. G. Green Organocatalytic Dihydroxylation of Alkenes. *Eur. J. Org. Chem.* **2017**, 1502–1509; c) Bosset, C.; Angibaud, P.; Stanfield, I.; Meerpoel, L.; Berthelot, D.; Guérinot, A.; Cossy, J. Iron-Catalyzed Synthesis of C2 Aryl- and N-Heteroaryl-Substituted Tetrahydropyrans. *J. Org. Chem.* **2015**, *80*, 12509–12525; d) Twilton, J.; Christensen, M.; DiRocco, D. A.; Ruck, R. T.; Davies, I. W.; MacMillan, D. W. C. Selective Hydrogen Atom Abstraction through Induced Bond Polarization: Direct α -Arylation of Alcohols through Photoredox, HAT, and Nickel Catalysis. *Angew. Chem. Int. Ed.* **2018**, *57*, 5369–5373.
- [13] Kumar, M.; Kumar, A.; Rizvi, M.; Mane, M.; Vanka, K.; Taneja, S. C.; Shah, B. A. Synthesis of α,β -Unsaturated δ -Lactones by Vinyl Acetate Mediated Asymmetric Cross-Aldol Reaction of Acetaldehyde: Mechanistic Insights. *Eur. J. Org. Chem.* **2014**, 5247–5255.

4

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

Chao Guo

Lieuwe Biewenga

Max Lubberink

Ronald van Merkerk

Gerrit J. Poelarends

Published in *ChembioChem* **2020**, *21*, 1499-1504.

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 γ -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).^[1,2] 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.^[3–7] For instance, the 4-OT catalyzed Michael-type addition of acetaldehyde (**3**) to nitroalkenes **4a** and **4b** yields γ -nitroaldehydes **5a** and **5b**, important precursors for the γ -aminobutyric acid analogues phenibut (*R*-**6a**) and pregabalin (*S*-**6b**), respectively (Scheme 1B).^[5] Hence, several enzyme engineering studies have been performed to improve the activity and enantioselectivity of 4-OT for this reaction.^[8,9]

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.^[10] 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**.^[11] 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^[8] 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),^[9] 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.^[8] 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.^[a]

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

^[a] Assay conditions: 3 mM **4b**, 100 mM **3**, 73 μ M 4-OT, 20 mM NaH₂PO₄ (pH 7.3), 0.3 mL reaction volume ^[b] Determined by GC using a chiral stationary phase; the absolute configuration was determined by literature comparison^[8,9] ^[c] Reaction progress was monitored by following the depletion in absorbance at 249 nm. ^[d] No data due to protein precipitation. ^[e] 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 NaH₂PO₄ 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.^[10] 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.^[12–14] Currently employed rational engineering strategies include stabilization of flexible regions, introduction of new cysteine bridges and modification of access tunnels.^[14–17]

An important strategy to guide enzyme-engineering efforts is to make use of mutability landscapes.^[18–21] 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.^[8] 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.^[9] Conversely, the single mutant 4-OT A33D markedly improved the enantioselectivity towards the opposite enantiomer *R*-**5b**.^[8]

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.^[22,23] 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.

ACKNOWLEDGEMENT

We acknowledge financial support from the Netherlands Organization of Scientific Research (VICI grant 724.016.002) and the European Union's Horizon 2020 Research and Innovation Programme under grant agreement No 635595 (CarbaZymes).

EXPERIMENTAL SECTION

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

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 NaH₂PO₄ buffer (pH 7.3), 100 μ L final volume. The reactions were performed in 96-well microtiter plates (MTP) (UV-star μ clear, Greiner Bio-one), covered with UV-transparent plate seals (VIEWsealTM, 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^[24] and 4-OT quadruple mutants^[9] 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 µg, 73 µM) was incubated in a 1 mm cuvette with **3** (50 mM) and **4a** (2 mM) in 20 mM NaH₂PO₄ (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'-CTGGTCAGCGGMNNATCCAGGGAGC-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 µg, 73 µM) was incubated in a 1 mm cuvette with **3** (100 mM) and **4b** (3 mM) in 20 mM NaH₂PO₄ buffer (pH 7.3; 0.3 mL final volume). After the reactions were completed, product **5b** was extracted with ethyl acetate (400 µL) 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.^[9]

Determination of T_{50}^{60} . 4-OT L8F/M45Y/F50A and 4-OT L8F/A33I/M45Y/F50A (50 μ L of 2 mg/mL in 20 mM NaH_2PO_4 , pH 7.3) were incubated in 0.2-mL PCR tubes at temperatures ranging from 30 $^{\circ}\text{C}$ to 90 $^{\circ}\text{C}$ for 60 min in a thermal cycler. After incubation, the enzymes were cooled on ice for 10 min followed by equilibration at 25 $^{\circ}\text{C}$ 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 μ L of enzyme supernatant, 50 mM **3**, 0.5 mM **4a**, 5% v/v ethanol in 20 mM NaH_2PO_4 buffer (pH 7.3), 100 μ L final volume. The ‘Michaelase’ activities were normalized to that obtained after 60 min incubation at 30 $^{\circ}\text{C}$.

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_2PO_4 , pH 7.3) were incubated in 20 mM NaH_2PO_4 buffer (pH 7.3) containing 10%, 30% or 50% v/v ethanol in a water bath of 25 $^{\circ}\text{C}$. Aliquots of enzyme (80 μ L) were taken at different time intervals and centrifuged to remove any aggregated protein. 50 μ 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_2PO_4 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 μ L **3**, 12 mL buffer (20 mM NaH_2PO_4 , 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 μ M, based on monomer concentration), and 40% (v/v) ethanol in 20 mM NaH_2PO_4 buffer (pH 6.5). The reaction progress was monitored using UV-spectrophotometric analysis. At timely intervals, a

sample of 30 μL was collected from the reaction mixture and diluted to 300 μL with 20 mM NaH_2PO_4 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_2SO_4 . 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^[5]. 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.

REFERENCES

- [1] S. Harayama, M. Rekik, K. L. Ngai, L. N. Ornston, *J. Bacteriol.* **1989**, *171*, 6251 – 6258.
- [2] C. P. Whitman, B. A. Aird, W. R. Gillespie, N. J. Stolowich, *J. Am. Chem. Soc.* **1991**, *113*, 3154–3162.
- [3] M. Rahimi, J. Y. van der Meer, E. M. Geertsema, H. Poddar, B. J. Baas, G. J. Poelarends, *ChemBioChem* **2016**, *17*, 1225–1228.
- [4] M. Rahimi, J. Y. van der Meer, E. M. Geertsema, G. J. Poelarends, *ChemBioChem* **2017**, *18*, 1435–1441.
- [5] E. M. Geertsema, Y. Miao, P. G. Tepper, P. de Haan, E. Zandvoort, G. J. Poelarends, *Chem. Eur. J.* **2013**, *19*, 14407–14410.
- [6] E. Zandvoort, E. M. Geertsema, B. J. Baas, W. J. Quax, G. J. Poelarends, *Angew. Chem. Int. Ed.* **2012**, *51*, 1240–1243.
- [7] C. Guo, M. Saifuddin, T. Saravanan, M. Sharifi, G. J. Poelarends, *ACS Catal.* **2019**, *9*, 4369–4373.
- [8] 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.
- [9] L. Biewenga, T. Saravanan, A. Kunzendorf, J. Y. van der Meer, T. Pijning, P. G. Tepper, R. van Merkerk, S. J. Charnock, A.M. W. H. Thunnissen, G. J. Poelarends, *ACS Catal.* **2019**, *9*, 1503–1513.
- [10] V. Stepankova, S. Bidmanova, T. Koudelakova, Z. Prokop, R. Chaloupkova, J. Damborsky, *ACS Catal.* **2013**, *3*, 2823–2836.
- [11] D. Prat, O. Pardigon, H. W. Flemming, S. Letestu, V. Ducandas, P. Isnard, E. Guntrum, T. Senac, S. Ruisseau, P. Cruciani, P. Hosek, *Org. Process Res. Dev.* **2013**, *17*, 1517–1525.
- [12] R. J. Kazlauskas, U. T. Bornscheuer, *Nat. Chem. Biol.* **2009**, *5*, 526–529.
- [13] F. H. Arnold, *FASEB J.* **1993**, *7*, 744–749.
- [14] V. G. H. Eijssink, A. Bjørk, S. Gåseidnes, R. Sirevåg, B. Synstad, B. van den Burg, G. Vriend, *J. Biotechnol.* **2004**, *113*, 105–120.
- [15] M. T. Reetz, J. D. Carballeira, A. Vogel, *Angew. Chem. Int. Ed.* **2006**, *45*, 7745–7751.

- [16] T. Koudelakova, R. Chaloupkova, J. Brezovsky, Z. Prokop, E. Sebestova, M. Hesseler, M. Khabiri, M. Plevaka, D. Kulik, I. Kuta Smatanova, P. Rezacova, R. Ettrich, U. T. Bornscheuer, J. Damborsky, *Angew. Chem. Int. Ed.* **2013**, *52*, 1959–1963.
- [17] M. Karpusas, W. A. Baase, M. Matsumura, B. W. Matthews, *Proc. Natl. Acad. Sci.* **1989**, *86*, 8237 – 8241.
- [18] A. Fulton, V. J. Frauenkron-Machedjou, P. Skoczinski, S. Wilhelm, L. Zhu, U. Schwaneberg, K. E. Jaeger, *ChemBioChem* **2015**, *16*, 930–936.
- [19] P. A. Romero, T. M. Tran, A. R. Abate, *Proc. Natl. Acad. Sci.* **2015**, *112*, 7159 – 7164.
- [20] N. Palackal, Y. Brennan, W. N. Callen, P. Dupree, G. Frey, F. Goubet, G. P. Hazlewood, S. Healey, Y. E. Kang, K. A. Kretz, E. Lee, X. Tan, G. L. Tomlinson, J. Verruto, V. W. K. Wong, E. J. Mathur, J. M. Short, D. E. Robertson, B. A. Steer, *Protein Sci.* **2004**, *13*, 494–503.
- [21] J. Y. van der Meer, L. Biewenga, G. J. Poelarends, *ChemBioChem* **2016**, *17*, 1792–1799.
- [22] B. Rasekh, K. Khajeh, B. Ranjbar, N. Mollania, B. Almasinia, H. Tirandaz, *Eng. Life Sci.* **2014**, *14*, 442–448.
- [23] T. Börner, S. Rämisch, S. Bartsch, A. Vogel, P. Adlercreutz, C. Grey, *ChemBioChem* **2017**, *18*, 1482–1486.
- [24] E. Zandvoort, B. J. Baas, W. J. Quax, G. J. Poelarends, *ChemBioChem* **2011**, *12*, 602–609.

Supporting Information

Figure S1. Activity of purified wild-type 4-OT and wild-type 4-OT in cell free extract for the Michael-type addition of **3** to **4a** in the presence of different ethanol concentrations.

Figure S2. Crystal structure of wild-type 4-OT (PDB: 4X1C). In light grey, a single monomer of 4-OT is indicated. Residues Pro-1, Ser-30 and Ala-33 are indicated as stick representation.

Figure S3. ^1H NMR spectrum of enzymatically obtained **5a** (4-nitro-3-phenylbutanal). ^1H NMR (500 MHz, Chloroform-*d*) δ 9.71 (s, 1H), 7.37 – 7.33 (m, 2H), 7.31 – 7.27 (m, 1H), 7.25 – 7.21 (m, 2H), 4.68 (dd, J = 12.5, 7.2 Hz, 1H), 4.62 (dd, J = 12.5, 7.6 Hz, 1H), 4.08 (p, J = 7.3 Hz, 1H), 3.01 – 2.96 (m, 1H), 2.96 – 2.90 (m, 1H).

Figure S4. HPLC chromatograms of derivatized racemic (A) and enzymatically (B) obtained **5a**.

5

Using Mutability Landscapes to Guide Enzyme Thermostabilization

Chao Guo

Yan Ni

Lieuwe Biewenga

Tjaard Pijning

Andy-Mark W.H. Thunnissen,

Gerrit J. Poelarends

Published in *ChembioChem* **2020**, 21, 1-7.

ABSTRACT

The thermostabilization of enzymes while retaining their activity and enantioselectivity for applied biocatalysis is an important topic in protein engineering. Rational and computational design strategies as well as directed evolution have been used successfully to thermostabilize enzymes. Here, we describe an alternative mutability-landscape approach that identified three single mutations (R11Y, R11I and A33D) within the enzyme 4-oxalocrotonate tautomerase (4-OT), which has potential as a biocatalyst for pharmaceutical synthesis, that gave rise to significant increases in apparent melting temperature T_m (up to 20 °C) and in half-life at 80 °C (up to 111-fold). Introduction of these beneficial mutations in an enantioselective but thermolabile 4-OT variant (M45Y/F50A) afforded improved triple-mutant enzyme variants showing an up to 39 °C increase in T_m value, with no reduction in catalytic activity or enantioselectivity. This study illustrates the power of mutability-landscape-guided protein engineering for thermostabilizing enzymes.

KEYWORDS

Protein engineering; thermostability; mutability landscape; biocatalysis

INTRODUCTION

Enzyme thermostability is an attractive feature for industrial biocatalysis because it allows for the use of higher process temperatures, which may be helpful to achieve higher reaction rates, improved substrate solubility, better mixing because of decreased viscosity of the solvent, and inhibition of microbial growth.

^[1] Thermostable enzymes are often also more stable towards high substrate, product and cosolvent concentrations, as well as other destabilizing reaction conditions.^[1c, 2] Moreover, in a laboratory setting, thermostable enzymes are the preferred starting points for generating highly active biocatalysts by directed evolution because the evolution of activity often comes at the cost of stability. ^[3] Given that most enzymes isolated from mesophilic microorganisms show relatively low stability, the thermostabilization of enzymes has become an increasingly important topic in protein engineering.^[1d, 3d, 4]

Various protein engineering strategies for improving enzyme stability have been reported, including error-prone PCR-based directed evolution, focused mutagenesis based on crystallographic B-factors, rational design based on the stabilization of flexible (loop) regions or improved interactions between subunits, ancestral sequence reconstruction, and advanced computational design strategies. ^[2b, 3a-3d, 5] Another interesting concept in enzyme engineering is the generation of large-scale mutational data in order to chart protein mutability landscapes, which is a powerful tool that can be used to identify functional “hotspots” at any place in the amino acid sequence of an enzyme.^[6] These “hotspot” positions can be used as targets for combinatorial mutagenesis to yield enzymes with improved properties. We have previously used this mutability landscape approach to engineer 4-oxalocrotonate tautomerase (4-OT), an enzyme that utilizes an N-terminal proline to promiscuously catalyze synthetically useful asymmetric carbonyl transformations via enamine and iminium ion intermediates,^[7] towards

improved Michael-type addition, aldol condensation and peroxygenase activity, as well as enhanced stereoselectivity and cosolvent stability.^[6a, 7c, 8]

In this study, we explored the use of mutability-landscape-guided protein engineering for improving the thermostability of 4-OT. To this end, 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 activity at elevated temperatures. Three mutations, A33D, R11Y and R11I, resulted in enzyme variants with remarkable increases in apparent melting temperature T_m and in half-life at 80 °C. Introduction of one of these beneficial mutations in a previously engineered enantioselective but thermolabile 4-OT variant (M45Y/F50A) afforded an improved enzyme variant (R11I/M45Y/F50A) showing a 39 °C increase in T_m value, with no reduction in activity or enantioselectivity.

RESULTS

In order to identify “hotspot” positions of 4-OT at which mutations increase the thermostability of the enzyme, a defined collection of 1040 single-mutant variants of 4-OT was screened using cell-free extracts (CFEs) prepared from cultures each expressing a different 4-OT mutant.^[6a] The single-mutant variants were incubated for 1 h at 25 °C or 80 °C, and the remaining ‘Michaelase’ activity (using acetaldehyde and *trans*- β -nitrostyrene as the substrates) after incubation at 80 °C, compared to that after incubation at 25 °C, was graphically represented in a mutability landscape for thermostability (Figure 1). Analysis of the mutability landscape revealed two “hotspot” positions, Arg-11 and Ala-33, at which single mutations resulted in enzyme variants that showed more than 50% remaining activity at the elevated temperature. Interestingly, mutant A33D, which has previously been shown to possess high enantioselectivity and

increased ‘Michaelase’ activity,^[6a] as well as improved stability towards high ethanol concentrations,^[8c] appears to also have enhanced thermostability.

In an attempt to further increase the thermostability of 4-OT, a combinatorial saturation mutagenesis library at “hotspot” positions Arg-11 and Ala-33 was constructed using wild-type 4-OT as the template. However, we could not produce enough soluble 4-OT protein in cultures grown in 96-well plates for thermostability assessments. Therefore, as an alternative strategy, the mutation A33D was combined with mutation R11I, R11Y, R11Q, R11L or R11F. These single mutations at position 11 were selected because they resulted in enzyme variants that showed more than 50% remaining activity at 80 °C (Figure 1). Appropriate amounts of soluble protein could be produced and purified from larger cell cultures grown in 5 L flasks with rigorous shaking. However, the double mutants showed lower activity and a decreased apparent melting temperature T_m compared to the best single mutants (Table S1), which indicates that combining these mutations at positions 11 and 33 does not result in a further increase in the thermostability of 4-OT.

Figure 1. Thermostability mutability landscape of 4-OT. The horizontal axis of the data matrix represents the residue position of 4-OT, starting with Ile-2 and ending with Arg-62. The vertical axis represents all 20 canonical amino acids. For clarity, 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 other squares indicates the residual ‘Michaelase’ activity of 4-OT mutants after 1 h incubation at 80 °C compared to that after 1 h incubation at 25 °C. Grey boxes indicate that the ‘Michaelase’ activity was too low to determine the remaining activity. A “hotspot” position is defined as an amino acid residue position at which several single mutations result in enzyme variants that show high (>50%) remaining activity at the elevated temperature.

The three single-mutant 4-OT variants A33D, R11I and R11Y were purified and investigated in more detail with regard to their stability at elevated temperatures. Upon incubation at 80 °C, the wild-type enzyme was inactivated within 60 min, whereas the mutant enzyme A33D retained ~40% activity after 360 min incubation (Figure 2A). Remarkably, the mutant enzymes R11I and R11Y retained ~90% activity after 360 min incubation at 80 °C (Figure 2A), and only after incubation at 100 °C for 240 min we could observe significant inactivation of these 4-OT variants (Figure 2B). The half-life ($t_{1/2}$) values of A33D, R11Y and R11I at 80 °C were 285, 1260 and 1980 min, which were about 16-, 71- and 111-fold higher than that of the wild-type enzyme respectively (Table 1).

Figure 2. Thermostability assessment of wild-type 4-OT and the three single-mutant 4-OT variants. A) Activity decay at 80 °C. B) Activity decay at 100 °C. C) Determination of T_{50}^{120} values. D) The first derivatives of each melting curve where each minimum indicates the apparent melting temperature T_m for wild-type 4-OT and the three 4-OT mutants.

Table 1. Properties of wild-type 4-OT and 4-OT mutants.^a

4-OT	$t_{1/2}$ (min) ^b	T_{50}^{120} (°C) ^c	T_m (°C)
WT	17.8 ± 1.5	68.7 ± 1.3	73.8 ± 0.1
A33D	285 ± 36	79.8 ± 1.4	77.9 ± 0.1
R11I	1980 ± 52	95.5 ± 0.5	94.0 ± 0.1
R11Y	1260 ± 73	88.4 ± 2.2	92.0 ± 0.2

^aValues represent the mean of three independent sets of experiments.

^b $t_{1/2}$ represents the half-life at 80 °C.

^cTemperature at which the enzyme lost 50% activity after incubation for 120 min.

Next, the enzyme thermostability was evaluated by determining the T_{50}^{120} (Figure 2C), the temperature at which the enzyme lost 50% of its activity after 120 min incubation. To this end, the remaining activities of wild-type 4-OT and the 4-OT mutants were assessed after incubating each enzyme at temperatures from 30 to 100 °C. Whereas no significant differences in remaining activity were observed with treatment below 50 °C, incubation at temperatures above 60 °C showed large variances in remaining activity for the different enzymes (Figure 2C). For example, the remaining activity of wild-type 4-OT was only 40% after treatment for 120 min at 70 °C, while the single-mutant 4-OT variant R11I retained ~100% of its activity after the same treatment. The curves shown in Figure 2C gave T_{50}^{120} values of 68.7 °C for wild-type, 79.8 °C for mutant A33D, 88 °C for mutant R11Y, and 95.5 °C for mutant R11I. Finally, the apparent melting temperature T_m of each enzyme was measured by differential scanning fluorimetry (Figure 2D). The resulting melting curves gave T_m values of 73.8 °C for wild-type, 77.9 °C for A33D, 92 °C for R11Y and 94 °C for R11I (Table 1).

Using acetaldehyde and *trans*- β -nitrostyrene as the substrates, we determined apparent kinetic parameters for wild-type 4-OT and the single-mutant 4-OT variants (Table 2). Whereas mutant A33D showed a 2.2-fold increase in k_{cat} , mutants R11I and R11Y showed a 1.6-fold and 3.5-fold, respectively, decrease in k_{cat} compared to that of the wild-type enzyme (Table 2). Notably, the mutations only have a minor effect on the stereoselectivity of 4-OT, with mutant A33D showing nearly perfect enantioselectivity (Table 2, Figure S1).

Table 2. Apparent kinetic parameters for the Michael-type addition of acetaldehyde to *trans*- β -nitrostyrene catalyzed by wild-type 4-OT and single-mutant 4-OT variants.^a

4-OT	K_m (mM)	k_{cat} (s ⁻¹) $\times 10^3$	k_{cat}/K_m s ⁻¹ M ⁻¹	Conf. ^b	<i>e.r.</i>
Wild type	0.38 $\pm$ 0.067	23.2 $\pm$ 1.4	61.0 $\pm$ 4.5	<i>S</i>	97:3
A33D	0.36 $\pm$ 0.028	50.6 $\pm$ 1.3	140.6 $\pm$ 15.7	<i>S</i>	>99:1
R11I	0.46 $\pm$ 0.039	14.0 $\pm$ 0.4	30.5 $\pm$ 3.7	<i>S</i>	95:5
R11Y	0.09 $\pm$ 0.005	6.5 $\pm$ 0.1	73.0 $\pm$ 5.3	<i>S</i>	98:2

a. Determination of apparent kinetic parameters was performed using various *trans*- β -nitrostyrene concentrations and 100 mM acetaldehyde at 25 °C and pH 7.3. The values shown indicate mean $\pm$ standard deviation from three parallel experiments.

b. Determination of absolute configuration was based on chiral GC and previously reported chiral GC data with authentic standards (Figure S1).^[6a, 8c]

Next, we investigated whether the identified single mutations that are beneficial for thermostability can confer high thermostability to 4-OT variant M45Y/F50A. This double-mutant variant was chosen because it is highly enantioselective, produces the pharmaceutically relevant *R* enantiomer of the γ -nitroaldehyde product, and is thermolabile (Table S1).^[6a] To this end, three new triple mutants were generated by the introduction of mutation R11I, R11Y or A33D into 4-OT M45Y/F50A as the starting template. Differential scanning fluorimetry experiments showed that all three triple mutants are significantly more thermostable than parental mutant 4-OT M45Y/F50A, with T_m values of 84 °C for A33D/M45Y/F50A, 92 °C for R11Y/M45Y/F50A, and 96 °C for R11I/M45Y/F50A (Table S1). Activity assays showed that A33D/M45Y/F50A (2.7-fold), R11Y/M45Y/F50A (1.7-fold), and R11I/M45Y/F50A (2.4-fold) have even slightly increased Michael-type addition activity compared to M45Y/F50A, while retaining the high enantioselectivity towards the desired *R*-enantiomer of the product (Table S1, Figure S2).

DISCUSSION

In this study, a mutability landscape approach was applied to discover two “hotspot” positions within the enzyme 4-OT, Arg-11 and Ala-33, at which single mutations resulted in enzyme variants that showed a significant increase in apparent melting temperature T_m (up to 20 °C) and in half-life at 80 °C (up to 111-fold). Although there was no positive effect of combining the mutations at positions 11 and 33 on the thermostability of 4-OT, we used the information from the mutability landscape to further engineer a previously constructed enantioselective 4-OT variant (M45Y/F50A) that exhibits relatively poor thermostability.^[6a] The introduction of the identified beneficial mutations (R11Y, R11I and A33D) in 4-OT variant M45Y/F50A afforded improved triple-mutant enzyme variants, with the best enzyme (R11I/M45Y/F50A) showing an 39 °C increase in T_m value with no reduction in catalytic activity or enantioselectivity. These results further demonstrate the power of mutability-landscape-guided protein engineering for improving enzyme properties.^[6b]

Interestingly, residue Ala-33 was previously identified as a “hotspot” position at which mutations are beneficial for catalysis in high concentrations of the cosolvent ethanol.^[8c] Particularly, the 4-OT variant A33D not only showed improved ethanol tolerance,^[8c] but also enhanced Michael-type addition activity and enantioselectivity.^[6a] This single point mutation thus has a beneficial effect on the activity, stereoselectivity, solvent stability and thermostability of 4-OT. Such functional plasticity is also observed for mutations at position Arg-11, with 4-OT variants R11I and R11Y now demonstrated to be extremely thermostable. While Arg-11 is essential for the native tautomerase activity of 4-OT, most mutations at this position have no effect on the Michael-type addition activity of the enzyme.^[6a] Because of their positive effects on activity and stability, these single mutations at positions Arg-11 and Ala-33 were used in combination with the enantioselective 4-OT variant M45Y/F50A to create active, enantioselective and thermostable

biocatalysts. Other identified beneficial mutations for thermostability, like I20S, L35P and S37D (Figure 1), have not been tested in combination with variant M45Y/F50A because previously constructed mutability landscapes showed that these single mutations have a negative effect on the Michael-type addition activity of 4-OT.^[6a] Hence, the construction of mutability landscapes for multiple properties of one enzyme provides the exciting opportunity to select mutations that are beneficial either for one or for several of these properties.^[6b]

Inspection of the crystal structure of wild-type 4-OT does not provide an obvious explanation for the improved thermostability caused by mutations at positions Arg-11 and Ala-33, illustrating the effectiveness of mutability-landscape navigation to identify functional “hotspot” positions. By determining crystal structures of 4-OT variants A33D and R11I, we aim to explain the multifunctional effects of these interesting point mutations. The large amount of mutational data generated within this study may be used as a unique training set to generate improved mutation prediction models for enhancing enzyme thermostability. More accurate computational predictions necessitates less experimental testing of enzyme mutants, accelerating enzyme thermostabilization.

EXPERIMENTAL SECTION

Production of cell-free extracts: The thermostability of all members of the 4-OT mutant collection was determined using cell-free extracts (CFEs) of *E. coli* BL21(DE3) cultures each expressing a different 4-OT mutant. These CFEs were prepared according to a reported procedure.^[6a, 7c] Concisely, 1.25 mL of LB medium supplemented with 100 µg/mL ampicillin and 100 µM isopropyl-β-D-1-thiogalactopyranoside (IPTG) was inoculated from a glycerol stock of the corresponding mutant and the culture was grown overnight at 37 °C in a 96-deep-well plate (Greiner Bio-one, 96 well Masterblock®). Each mutant was inoculated in duplicate. The deep-well plates were sealed with sterile, gas-permeable seals (Greiner Bio-one, BREATHseal™) and incubated overnight at 37 °C with shaking at 200 rpm. The cells from the overnight cultures were harvested by centrifugation (3,500 rpm for 30 min at 4 °C) and lysed with 300 µL of Bugbuster (Novagen), supplemented with 25 U/mL benzonase, for 40 min. After centrifugation (4,000 rpm for 60 min at 4 °C), the obtained CFE was used for the activity assay.

Construction of the mutability landscape of 4-OT for thermostability: The CFEs prepared from cells producing 4-OT single-mutant variants were transferred into 96-well PCR plates (semi-skirted, ThermoFisher Scientific, Waltham, Massachusetts, USA), after which the plates were sealed with thermoresistance mats (Thermo Scientific VersiCap™ Mats), resulting in plates with 50 µL CFE in each well. The plates were then incubated at 25 °C (control plate) or 80 °C for 1 h in a PCR thermocycler. The plates were then placed on ice for 20 min, followed by centrifugation at 4,000 rpm for 30 min, after which 20 µL supernatant of each CFE was transferred into a 96-well microtiter plate (MTP; UV-star µclear, Greiner Bio-one) and used for measuring residual enzyme activity.

The reaction mixture (100 μ L final volume) used to monitor the 4-OT-catalyzed Michael-type addition of acetaldehyde to *trans*- β -nitrostyrene consisted of the following: CFE (20% v/v), acetaldehyde (50 mM), *trans*- β -nitrostyrene (0.5 mM) and ethanol (5% v/v) in 20 mM NaH_2PO_4 buffer (pH 7.3). First, the appropriate amounts of CFE and buffer were added to each well of the MTP, using a Packard Multiprobe® II HT EX 8 tip robotic liquid handling system. The assay was then initiated by adding 5 μ L of a stock solution of *trans*- β -nitrostyrene (10 mM in ethanol) and 5 μ L of a stock solution of acetaldehyde (1 M in 20 mM NaH_2PO_4 buffer, pH 7.3) to each well of the MTP. The MTPs were sealed with UV-transparent plate seals (VIEWseal™, Greiner Bio-one) to minimize evaporation of reaction components. To ensure proper mixing of the reagents, the plate was shaken (30 s at 500 rpm) immediately after all reaction components were added. Reaction progress was monitored in a SPECTROstar Omega plate reader (BMG LABTECH, Isogen Life Science, de Meern, NL) by following the depletion in absorbance at 320 nm, corresponding to the concentration of *trans*- β -nitrostyrene, for 80 min with a 60 s data interval. The slope of the linear part of the curve was determined for each reaction. The remaining enzyme activity was determined by dividing the slope of the reaction at 80 °C by the slope of the reaction at 25 °C.

4-OT purification: The 4-OT enzyme, either wild-type or mutant, was produced in *E. coli* BL21(DE3) by using the pET20b(+) expression system. LB medium (5 mL) containing ampicillin (100 μ g/mL) was inoculated with cells from a glycerol stock of *E. coli* BL21(DE3) containing the appropriate expression vector by using a sterile loop. After overnight growth at 37 °C, this culture was used to inoculate fresh LB/ampicillin medium (1 L) in a 3 L Erlenmeyer flask. Cultures were grown overnight at 37 °C with vigorous shaking. Cells were harvested by centrifugation and stored at -20 °C. The purification of 4-OT variants (constructed using QuikChange technology) was based on previously reported procedures.^[6a,7a] All purified proteins were >90% pure as assessed by

SDS-PAGE (Figure S3). 4-OT variants were analyzed by electron spray ionization mass spectrometry (ESI-MS) to confirm that the proteins had been processed correctly and the initiating methionine had been removed.^[6a] The purified protein was flash-frozen in liquid nitrogen and stored at -80°C until further use.

4-Determination of T_{50}^{120} values: 4-OT variants (50 μ L of 2 mg/mL in 20 mM NaH_2PO_4 , pH 7.3) were incubated in 0.2 mL PCR tubes at temperatures ranging from 30 to 100 °C for 120 min in a thermal cycler. After this 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 enzymatic activity for the addition of acetaldehyde to *trans*- β -nitrostyrene was tested in a plate reader. The following conditions were used: 25 μ L enzyme supernatant, acetaldehyde (50 mM), *trans*- β -nitrostyrene (0.5 mM) and ethanol (5% v/v) in 20 mM NaH_2PO_4 buffer, pH 7.3 (final volume of 100 μ L). The enzymatic activities obtained after 120 min incubation at the different temperatures were normalized to that obtained after 120 min incubation at 30 °C. The T_{50}^{120} values were obtained by curve fitting.

Determination of $t_{1/2}$ values: The 4-OT variants (1.5 mL of 2 mg/mL in 20 mM NaH_2PO_4 , pH 7.3) were incubated at 80 °C or 100 °C in a water bath. Aliquots (100 μ L) were taken at different time points (from 0 to 360 min) and cooled immediately on ice for 10 min followed by equilibration at room temperature for 10 min. Samples were centrifuged to remove any precipitated protein. The residual enzymatic activity for the addition of acetaldehyde to *trans*- β -nitrostyrene was tested using either a V-650 or V-660 spectrophotometer from Jasco (IJsselstein, The Netherlands). The reaction mixture (0.3 mL final volume in a 1 mm cuvette) contained the following: 80 μ L of enzyme supernatant, acetaldehyde (50 mM), and *trans*- β -nitrostyrene (1.5 mM, from a 30 mM stock solution in 100% ethanol) in 20 mM NaH_2PO_4 buffer (pH 7.3). The $t_{1/2}$ values were determined by curve fitting.

Differential Scanning Fluorimetry (DSF): The apparent melting temperature T_m of the 4-OT variants was determined by using differential scanning fluorimetry (DSF). The fluorescence change was monitored using a MyiQ real-time PCR machine (Bio-Rad, Hercules, CA, USA) with an excitation wavelength at 490 nm and an emission wavelength at 575 nm. In iQ 96-well real-time PCR plates, purified enzyme (2 mg/mL in 20 mM NaH_2PO_4 , pH 6.5) was incubated with 50-fold diluted Sypro orange dye in a total volume of 50 μL over the temperature ramp from 30 $^\circ\text{C}$ to 100 $^\circ\text{C}$ (0.1 $^\circ\text{C}$ per cycle, 30 seconds/cycle). The final fluorescence signal was the average of three independent measurements. The apparent T_m values were obtained by plotting the first derivative of the measured fluorescence traces versus temperature.

Enzymatic synthesis: The enzyme-catalyzed Michael-type addition reactions were performed at 25 $^\circ\text{C}$. The reaction mixture (2 mL final volume in a sealed container) consisted of *trans*- β -nitrostyrene (1.5 mM) and acetaldehyde (50 mM) in 20 mM NaH_2PO_4 buffer (pH 7.3). The reactions were initiated by the addition of an appropriate amount of purified enzyme (150 μg). Reaction progress was monitored by UV spectroscopy. After the reaction was completed, the reaction mixture was extracted with 400 μL ethyl acetate and the product enantiomer ratio was determined by chiral GC as reported before.^[7c]

ACKNOWLEDGEMENT

We acknowledge financial support from the Netherlands Organization of Scientific Research (VICI grant 724.016.002 and ECHO grant 713.015.003) and the European Union's Horizon 2020 Research and Innovation Programme under grant agreement No 635595 (CarbaZymes).

REFERENCES

- [1] a) V. L. Schramm, *Annu. Rev. Biochem.* **1998**, *67*, 693-720; b) D. A. Kraut, K. S. Carroll, D. Herschlag, *Annu. Rev. Biochem.* **2003**, *72*, 517-571; c) A. Kumar, K. Dhar, S. S. Kanwar, P. K. Arora, *Biol. Proced. Online* **2016**, *18*, 2; d) F. Rigoldi, S. Donini, A. Redaelli, E. Parisini, A. Gautieri, *APL Bioeng.* **2018**, *2*, 011501.
- [2] a) S. Luetz, L. Giver, J. Lalonde, *Biotechnol. Bioeng.* **2008**, *101*, 647-653; b) T. Koudelakova, R. Chaloupkova, J. Brezovsky, Z. Prokop, E. Sebestova, M. Hesseler, M. Khabiri, M. Plevaka, D. Kulik, I. K. Smatanova, P. Rezacova, R. Ettrich, U. T. Bornscheuer, J. Damborsky, *Angew. Chem. Int. Ed. Engl.* **2013**, *52*, 1959-1963.
- [3] a) V. G. Eijssink, A. Bjork, S. Gaseidnes, R. Sirevag, B. Synstad, B. van den Burg, G. Vriend, *J. Biotechnol.* **2004**, *113*, 105-120; b) M. Furst, C. Martin, N. Loncar, M. W. Fraaije, *Methods Enzymol.* **2018**, *608*, 151-187; c) A. G. L. Giver, P. Freskgard, F. H. Arnold, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 12809-12813; d) H. P. Modarres, M. R. Mofrad, A. Sanati-Nezhad, *RSC Advances* **2016**, *6*, 115252-115270.
- [4] a) M. Naushad, Z. A. Alothman, A. B. Khan, M. Ali, *Int. J. Biol. Macromol.* **2012**, *51*, 555-560; b) V. G. Eijssink, S. Gaseidnes, T. V. Borchert, B. van den Burg, *Biomol. Eng.* **2005**, *22*, 21-30; c) S. Kumar, C. Tsai, R. Nussinov, *Protein Eng.* **2000**, *13*, 179-191.
- [5] a) A. Korkegian, M. E. Black, D. Baker, B. L. Stoddard, *Science* **2005**, *308*, 857-860; b) Z. Wu, W. Deng, Y. Tong, Q. Liao, D. Xin, H. Yu, J. Feng, L. Tang, *Appl. Microbiol. Biotechnol.* **2017**, *101*, 3201-3211; c) A. O. Magnusson, A. Szekrenyi, H. J. Joosten, J. Finnigan, S. Charnock, W. D. Fessner, *FEBS J.* **2019**, *286*, 184-204; d) H. Yu, Y. Yan, C. Zhang, P. A. Dalby, *Sci. Rep.* **2017**, *7*, 41212; e) I. V. Korendovych, *Methods Mol. Biol.* **2018**, *1685*, 15-23; f) A. Bosshart, S. Panke, M. Bechtold, *Angew. Chem. Int. Ed. Engl.* **2013**, *52*, 9673-9676; g) Z. Sun, Q. Liu, G. Qu, Y. Feng, M. T. Reetz, *Chem. Rev.* **2019**, *119*, 1626-1665; h) Y. Xie, J. An, G. Yang, G. Wu, Y. Zhang, L. Cui, Y. Feng, *J. Biol. Chem.* **2014**, *289*, 7994-8006; i) H. J. Wijma, M. J. L. J. Fürst, D. B. Janssen, *Methods Mol. Biol.* **2018**, *1685*, 69-85; j) L. Ge, D. Li, T. Wu, L. Zhao, G. Ding, Z. Wang, W. Xiao, *J. Biotechnol.* **2018**, *275*, 17-23; k) F. S. Aalbers, M. J. L. J. Fürst, S. Rovida, M. Trajkovic, J. Rubén Gómez Castellanos, S. Bartsch, A. Vogel, A. Mattevi, M. W. Fraaije, *eLife* **2020**, *9*, e54639; l) M. T. Reetz, J. D. Carballeira, *Nat. Protoc.* **2007**, *2*, 891-903; m) Y. Gumulya, J.-M. Baek, S.-J. Wun, R. E. S. Thomson, K. L. Harris, D. J. B. Hunter, J. B. Y. H. Behrendorff, J. Kulig, S. Zheng, X. Wu, B. Wu, J. E. Stok, J. J. De Voss, G. Schenk, U. Jurva, S. Andersson, E. M. Isin, M. Bodén, L. Guddat, E. M. J. Gillam, *Nat. Catal.* **2018**, *1*, 878-888; n) H. Yu, Y. Yan, C. Zhang, P. A. Dalby, *Sci. Rep.* **2017**, *7*, 41212.

- [6] a) 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; b) J. Y. van der Meer, L. Biewenga, G. J. Poelarends, *Chembiochem* **2016**, *17*, 1792-1799; c) A. Fulton, V. J. Frauenkron-Machedjou, P. Skoczinski, S. Wilhelm, L. Zhu, U. Schwaneberg, K. E. Jaeger, *Chembiochem* **2015**, *16*, 930-936.
- [7] a) E. Zandvoort, E. M. Geertsema, B. J. Baas, W. J. Quax, G. J. Poelarends, *Angew. Chem. Int. Ed. Engl.* **2012**, *51*, 1240-1243; b) H. Poddar, M. Rahimi, E. M. Geertsema, A. W. H. Thunnissen, G. J. Poelarends, *Chembiochem* **2015**, *16*, 738-741; c) C. Guo, M. Saifuddin, T. Saravanan, M. Sharifi, G. J. Poelarends, *ACS Catal.* **2019**, *9*, 4369-4373; d) Y. Miao, M. Rahimi, E. M. Geertsema, G. J. Poelarends, *Curr. Opin. Chem. Biol.* **2015**, *25*, 115-123.
- [8] a) M. Rahimi, J. Y. van der Meer, E. M. Geertsema, H. Poddar, B. J. Baas, G. J. Poelarends, *Chembiochem* **2016**, *17*, 1225-1228; b) M. Rahimi, J. Y. van der Meer, E. M. Geertsema, G. J. Poelarends, *Chembiochem* **2017**, *18*, 1435-1441; c) C. Guo, L. Biewenga, M. Lubberink, R. van Merkerk, G. J. Poelarends, *Chembiochem* **2020**, *21*, 1499-1504; d) G. Xu, M. Crotti, T. Saravanan, K. M. Kataja, G. J. Poelarends, *Angew. Chem. Int. Ed. Engl.* **2020**, *59*, 10374-10378.

SUPPORTING INFORMATION

Figure S1. Chiral GC chromatograms of the γ -nitroaldehyde products obtained by enzymatic acetaldehyde addition to *trans*- β -nitrostyrene. Racemic standard (A), product obtained using 4-OT wild type (B), product obtained using 4-OT variant A33D (C), product obtained using 4-OT variant R11I (D) and product obtained using 4-OT variant R11Y (E).

Figure S2. Chiral GC chromatograms of the γ -nitroaldehyde products obtained by enzymatic acetaldehyde addition to *trans*- β -nitrostyrene. Racemic standard (A), product obtained using 4-OT variant R11I/M45Y/F50A (B), product obtained using 4-OT variant R11Y/M45Y/F50A (C), and product obtained using 4-OT variant A33D/M45Y/F50A (D).

Figure S3. SDS-PAGE gels of purified enzymes. 4-OT wild-type (A), 4-OT variant A33D (B), 4-OT variant R11I (C), and 4-OT variant R11Y (D).

5

Table S1. Relative activity, enantioselectivity and stability of 4-OT variants.

Catalyst	Relative activity	<i>e.r.</i>	Abs. conf.	T_m (°C)
WT	1	97:3	<i>S</i>	74
A33D	2.3	>99:1	<i>S</i>	78
R11I	0.7	95:5	<i>S</i>	94
R11Y	0.4	98:2	<i>S</i>	92
R11I/A33D	0.2	99:1	<i>S</i>	60
R11L/A33D	0.2	97:2	<i>S</i>	64
R11Y/A33D	0.2	99:1	<i>S</i>	65
R11F/A33D	0.2	>99:1	<i>S</i>	76
R11Q/A33D	0.5	>99:1	<i>S</i>	51
M45Y/F50A	1.3	6:94	<i>R</i>	57
A33D/M45Y/F50A	3.5	8:92	<i>R</i>	84
R11I/M45Y/F50A	3.1	5:95	<i>R</i>	96
R11Y/M45Y/F50A	2.2	6:94	<i>R</i>	92

6

Summary and Future Perspectives

Enzymes have naturally evolved to catalyze a particular reaction on a particular substrate, usually with a high catalytic efficiency. In addition to their main activity, many enzymes were found to promiscuously catalyze other chemical reactions. These promiscuous activities are generally several orders of magnitude lower than the native activity. Nevertheless, enzyme promiscuity has proven to be a successful starting point for the engineering of novel biocatalysts for chemical synthesis. Exploiting enzyme promiscuity to develop new biocatalysts is especially attractive for abiological reactions and may provide new opportunities to develop practical enzymatic processes for the more sustainable and step-economic synthesis of important molecules.

4-Oxalocrotonate tautomerase (4-OT) from *Pseudomonas putida* mt-2 was found to promiscuously catalyze the Michael-type addition of acetaldehyde to nitroolefins and the aldol condensation of acetaldehyde with benzaldehyde, yielding γ -nitroaldehydes and cinnamaldehyde, respectively. Although the promiscuous C-C bond-forming activity and stereoselectivity of 4-OT wild-type (WT) is too low for practical applications, several rounds of protein engineering have been performed to improve the biocatalytic properties of 4-OT. In **Chapter 1**, we have reviewed previous efforts on engineering of 4-OT.

4-OT was previously reported to facilitate C-C bond-forming reactions via enzyme-bound enamine intermediates. In **Chapter 2** we describe the discovery of 4-OT catalyzed nitromethane addition to α,β -unsaturated aldehydes via enzyme-bound iminium ion intermediates. In this study, a mutability-landscape-guided protein engineering approach was applied to evolve this Michaelase activity of 4-OT. The obtained 4-OT mutant F50A has a 16-fold enhanced activity compared to that of 4-OT. To expand the synthetic utility of 4-OT F50A, various α,β -unsaturated aldehydes were tested as potential acceptor substrates. Interestingly, all tested α,β -unsaturated aldehydes were accepted by 4-OT as substrates for reactions with nitromethane to form highly enantioenriched γ -nitroaldehydes (up to >99% *ee*) with good to excellent yields (up to 96%). Interestingly, the

enzymatic nitromethane additions to *meta*- and *para*-substituted cinnamaldehydes provided the corresponding products as the (*R*)-configured enantiomers, while those with the *ortho*-substituted cinnamaldehydes yielded the (*S*)-configured product enantiomers. This suggests that positioning substituents on the *ortho* position of the aromatic substrate induced steric effects, which caused either substrate repositioning in the enzyme active site or a stereofacial shielding effect. Furthermore, a cascade of three 4-OT(F50A)-catalyzed reactions followed by an enzymatic oxidation step enabled one-pot assembly of γ -nitrocarboxylic acids from three simpler building blocks. Our results bridge organo- and biocatalysis and emphasize the potential of enzyme promiscuity for the synthesis of important chiral synthons.

4-OT was previously reported to promiscuously catalyze the aldol condensation of acetaldehyde with benzaldehyde yielding cinnamaldehyde. In **Chapter 3**, we reported the biocatalytic aldol condensation of acetaldehyde with various aromatic aldehydes to give a number of aromatic α,β -unsaturated aldehydes using a previously engineered variant of 4-oxalocrotonate tautomerase [4-OT(M45T/F50A)] as carbolligase. Moreover, an efficient one-pot two-step chemoenzymatic route toward chiral aromatic 1,3-diols has been developed. This one-pot chemoenzymatic strategy successfully combined a highly enantioselective aldol addition step catalyzed by a proline-based carbolligase [4-OT(M45T/F50A) or TAUT015] with a chemical reduction step to convert enzymatically prepared aromatic β -hydroxyaldehydes into the corresponding 1,3-diols with high optical purity (e.r. up to >99:1) and in good isolated yield (51–92%). These developed (chemo)enzymatic methodologies offer alternative synthetic choices to prepare a variety of important drug precursors.

Protein engineering was also applied to improve the thermo- and solvent-stability of 4-OT. In **Chapter 4**, we used a collection of nearly all single mutants of 4-oxalocrotonate tautomerase 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, Ser30 and Ala33, at which mutations are beneficial for catalysis in high ethanol concentrations. The “hotspot” position Ala33 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 γ -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.

In **Chapter 5**, we describe a similar mutability-landscape approach that identified three single mutations (R11Y, R11I and A33D) within the enzyme 4-oxalocrotonate tautomerase (4-OT) that gave rise to significant increases in apparent melting temperature T_m and in half-life at 80 °C. Introduction of these beneficial mutations in an enantioselective but thermolabile 4-OT variant (M45Y/F50A) afforded improved triple-mutant enzyme variants showing an up to 39 °C increase in T_m value, with no reduction in catalytic activity or enantioselectivity. This study illustrates the power of mutability-landscape-guided protein engineering for thermostabilizing enzymes.

General remarks and future perspectives

The chemical versatility of the 4-OT protein scaffold was explored for the identification of new promiscuous C-C bond-forming activities, including the Michael addition of nitromethane to α,β -unsaturated aldehydes (**Chapter 2**) and the aldol addition of acetaldehyde to aromatic aldehydes (**Chapter 3**). Protein engineering of 4-OT for enhancing its solvent- and thermo-stability has been described in **Chapter 4** and **Chapter 5**, respectively. Hence, in the studies described in the first part of this thesis we expanded the reaction and substrate

scope of 4-OT, and therefore its synthetic usefulness. In the studies reported in the second part of this thesis we demonstrated that synthetically useful 4-OT variants can be engineered to improve catalysis in high concentrations of ethanol, permitting high substrate loading, and at high temperatures, which may be helpful to achieve improved stability towards high substrate, product and cosolvent concentrations, as well as other destabilizing reaction conditions. Although the road to the application of this enzyme in practical organic synthesis may be long, the results presented here are promising, as they can serve as a foundation for the further redesign of 4-OT. Several engineering projects are ongoing in our laboratory, a few of which in collaboration with industrial partners, with the aim to optimize 4-OT for C-C bond-forming aldol and Michael-type addition reactions for pharmaceutical synthesis.

The most important innovative aspect of the work described in this thesis is the unprecedented discovery of 4-OT's ability to promiscuously catalyze asymmetric carbonyl transformations via iminium ion intermediates, which opens up new possibilities for various synthetically useful enzymatic bond-forming (e.g. C-C, C-O, C-S) reactions. It would therefore be interesting to test the ability of 4-OT to accept various chemically distinct nucleophiles in additions to different α,β -unsaturated aldehydes. Indeed, this systematic substrate screening strategy has proven very successful in ongoing research, allowing the development of a set of biocatalysts, based on the 4-OT scaffold, that can be used to promote various synthetically useful bond-forming reactions, including epoxidations, asymmetric thiol additions, and cyclopropanation reactions.

NEDERLANDSE SAMENVATTING

Enzymen zijn van nature geëvolueerd om een bepaalde reactie met een specifiek substraat te katalyseren, meestal met een hoge katalytische efficiëntie. Naast hun hoofdactiviteit blijken veel enzymen andere chemische reacties te katalyseren. Deze promiscue activiteiten zijn over het algemeen verscheidene orden van grootte lager dan de natuurlijke activiteit. Desalniettemin is de promiscuïteit van enzymen een succesvol startpunt gebleken voor de engineering van nieuwe biokatalysatoren voor chemische synthese. Het benutten van enzympromiscuïteit om nieuwe biokatalysatoren te ontwikkelen is vooral aantrekkelijk voor niet-natuurlijke reacties en kan nieuwe kansen bieden om praktische enzymatische processen te ontwikkelen voor de duurzamere en stap-economische synthese van belangrijke moleculen.

Het promiscue enzym 4-oxalocrotonaat tautomerase (4-OT) van de bacterie *Pseudomonas putida* mt-2 bleek de additie van acetaldehyde aan nitroalkenen alsmede de aldolcondensatie van acetaldehyde met benzaldehyde te katalyseren, resulterend in respectievelijk γ -nitroaldehyden en kaneelaldehyde. Hoewel de promiscue C-C-bindingsvormende activiteit en stereoselectiviteit van 4-OT te laag is voor praktische toepassingen, zijn er verschillende rondes van ‘protein engineering’ uitgevoerd om de biokatalytische eigenschappen van 4-OT te verbeteren. In **Hoofdstuk 1** hebben we eerdere inspanningen op het gebied van ‘protein engineering’ van 4-OT beschreven.

Het werd eerder gerapporteerd dat 4-OT in staat is om C-C-bindingsvormende reacties te katalyseren via enzym gebonden enamine-tussenproducten. In **Hoofdstuk 2** beschrijven we de ontdekking dat 4-OT de additie van nitromethaan aan α,β -onverzadigde aldehyden kan katalyseren via enzym gebonden iminium-tussenproducten. In deze studie werd vervolgens een systematische mutagenese methode toegepast om deze interessante katalytische activiteit van 4-OT te verbeteren. De verkregen 4-OT-mutant F50A heeft een 16-voudig verhoogde

activiteit in vergelijking met die van het wildtype 4-OT. Om de synthetische bruikbaarheid van 4-OT F50A uit te breiden, werden verschillende α,β -onverzadigde aldehyden getest als potentiële acceptor-substraten. Interessant genoeg werden alle geteste α,β -onverzadigde aldehyden door 4-OT geaccepteerd als substraten voor reacties met nitromethaan om sterk enantioverrijkte γ -nitroaldehyden te vormen (tot >99% ee) met goede tot uitstekende opbrengsten (tot 96%). Interessant is dat de enzymatische addities van nitromethaan aan meta- en para-gesubstitueerde kaneelaldehyden de overeenkomstige producten opleverden als de (*R*)-geconfigureerde enantiomeren, terwijl die met de ortho-gesubstitueerde kaneelaldehyden de (*S*)-geconfigureerde product-enantiomeren opleverden. Dit suggereert dat het positioneren van substituenten op de ortho-positie van het aromatische substraat sterische effecten induceerde, die ofwel herpositionering van het substraat in het actieve centrum van het enzym of een stereospecifiek afschermingseffect veroorzaakten. Bovendien maakte een cascade van drie 4-OT(F50A)-gekatalyseerde reacties gevolgd door een enzymatische oxidatiestap het mogelijk om in één pot γ -nitrocarbonsuren uit drie eenvoudigere bouwstenen te assembleren. Onze resultaten overbruggen organo- en biokatalyse en benadrukken het potentieel van enzympromiscuïteit voor de synthese van belangrijke chirale bouwstenen.

Van 4-OT werd eerder gemeld dat het de aldolcondensatie van acetaldehyde met benzaldehyde kan katalyseren, wat kaneelaldehyde oplevert. In **Hoofdstuk 3** beschrijven we de biokatalytische aldolcondensatie van acetaldehyde met verschillende aromatische aldehyden om een aantal aromatische α,β -onverzadigde aldehyden te produceren met behulp van een eerder ontwikkelde variant van 4-OT (mutant M45T/F50A) als artificieel carboligase. Bovendien is een efficiënte twee-staps chemo-enzymatische route naar aromatische 1,3-diolen ontwikkeld. Deze chemo-enzymatische strategie combineerde met succes in één pot een zeer enantioselectieve aldoladditiestap, gekatalyseerd door een proline-afhankelijk artificieel carboligase [4-OT (M45T/F50A) of TAUT015], met een chemische

reductiestap om enzymatisch bereide aromatische β -hydroxyaldehyden om te zetten in de corresponderende 1,3-diolen met hoge optische zuiverheid (enantiomeer ratio tot >99:1) en in goede geïsoleerde opbrengst (51-92%). Deze ontwikkelde (chemo)enzymatische synthese methoden bieden alternatieve keuzes om een verscheidenheid aan belangrijke moleculen die kunnen fungeren als farmaceutische bouwstenen te bereiden.

‘Protein engineering’ werd ook toegepast om de thermo- en oplosmiddelstabiliteit van 4-OT te verbeteren. In de studie beschreven in **Hoofdstuk 4** hebben we een verzameling van bijna alle afzonderlijke mutanten van 4-OT gebruikt om het effect van elke mutatie op het vermogen van dit enzym om zijn activiteit te behouden in verhoogde ethanolconcentraties te onderzoeken. Deze stelselmatige mutagenese methode maakte de identificatie mogelijk van twee belangrijke aminozuurposities, Ser30 en Ala33, waar mutaties gunstig zijn voor katalyse in hoge ethanolconcentraties. De ‘hotspot’-positie Ala33 werd vervolgens gerandomiseerd in een zeer enantioselectieve maar ethanolgevoelige 4-OT-variant (L8F/M45Y/F50A) om een verbeterde enzymvariant te genereren (L8F/A33I/M45Y/F50A) die een grote ethanolstabiliteit vertoonde. Deze verbeterde enzymvariant kan gebruikt worden om de enantioselectieve additie van acetaldehyde aan nitrostyreen te katalyseren in 40% ethanol, waardoor het gebruik van een hoge substraatconcentratie mogelijk is, om het gewenste γ -nitroaldehyde te produceren met een uitstekende opbrengst (89%) en enantiomeerzuiverheid (ee = 98%). Het gepresenteerde werk demonstreert de kracht van de gebruikte systematische mutagenese methode voor het efficiënt optimaliseren van enzymen voor biokatalyse in organische oplosmiddelen.

In **Hoofdstuk 5** beschrijven we een vergelijkbare systematische mutagenese methode waarmee drie enkele mutaties (R11Y, R11I en A33D) werden geïdentificeerd in 4-OT die aanleiding gaven tot significante verhogingen van de smelttemperatuur T_m en de levensduur bij 80 °C. Introductie van deze gunstige mutaties in een enantioselectieve maar thermolabiele 4-OT-variant (M45Y/F50A)

leverde verbeterde enzymvarianten op die een toename van de T_m -waarde tot zelfs 39 °C vertoonden, zonder vermindering van de katalytische activiteit of enantioselectiviteit. Deze studie benadrukt de kracht van de gebruikte systematische mutagenese methode voor het efficiënt thermostabiliseren van enzymen.

Algemene opmerkingen en toekomstperspectieven

Door gebruik te maken van de chemische veelzijdigheid van het 4-OT enzym hebben we nieuwe promiscue C-C-bindingsvormende activiteiten geïdentificeerd, waaronder de Michael-additie van nitromethaan aan α,β -onverzadigde aldehyden (**Hoofdstuk 2**) en de aldol-additie van acetaldehyde aan aromatische aldehyden (**Hoofdstuk 3**). Het engineeren van 4-OT voor het verbeteren van de oplosmiddel- en thermo-stabiliteit is beschreven in respectievelijk **Hoofdstuk 4** en **Hoofdstuk 5**. In de studies beschreven in het eerste deel van dit proefschrift hebben we dus de reactie- en substraatvang van 4-OT uitgebreid, en daarmee de synthetische bruikbaarheid van dit enzym verbeterd. In de studies gerapporteerd in het tweede deel van dit proefschrift hebben we aangetoond dat synthetisch bruikbare 4-OT-varianten kunnen worden ontworpen om de katalyse te verbeteren in hoge concentraties ethanol, waardoor een hogere substraat concentratie gebruikt kan worden, en bij hoge temperaturen, wat nuttig kan zijn om een verbeterde stabiliteit te bereiken in aanwezigheid van hoge substraat-, product- en oplosmiddel-concentraties, evenals andere destabiliserende reactieomstandigheden. Hoewel de weg naar de toepassing van het 4-OT enzym in praktische organische synthese nog lang kan zijn, zijn de hier gepresenteerde resultaten veelbelovend, aangezien ze als basis kunnen dienen voor het verder optimaliseren van de katalytische eigenschappen van 4-OT. In ons laboratorium lopen verschillende engineeringprojecten, waarvan enkele in samenwerking met industriële partners, met als doel om 4-OT te optimaliseren

voor C-C-bindingsvormende aldol- en Michael-additie reacties voor synthese van belangrijke farmaceutische stoffen.

Het voornaamste innovatieve aspect van het werk beschreven in dit proefschrift is de onvoorziene ontdekking van de capaciteit van 4-OT om asymmetrische carbonyltransformaties te katalyseren via ‘iminium ion’ tussenproducten, wat nieuwe mogelijkheden opent voor verschillende synthetisch nuttige enzymatische bindingsvormende (bijv. C-C, C-O, C-S) reacties. Het zou daarom interessant zijn om te onderzoeken of 4-OT chemisch verschillende nucleofielen kan accepteren als substraat in addities aan diverse α,β -onverzadigde aldehyden. Deze systematische strategie voor het screenen van substraten is inderdaad zeer succesvol gebleken in lopend onderzoek, waardoor de ontwikkeling mogelijk is van een reeks nieuwe biokatalysatoren, gebaseerd op 4-OT, die kunnen worden gebruikt om verschillende synthetisch bruikbare bindingsvormende reacties te bevorderen, zoals epoxidaties, asymmetrische thiol-addities en cyclopropanaties.

ACKNOWLEDGEMENT

By the opportunity of finalizing this thesis, I would like to acknowledge the contributions of the following people, without whom this project could never have been completed, much less been a success.

First of all, I would like to thank **Prof. Dr. Gerrit J. Poelarends**. Time flies away amazingly fast, I still remember that we had our first email connection back to the year of 2012. I sincerely appreciate you giving me this great opportunity to work in your group. I admire your passion, accuracy and motivation on science, your capability on learning, and your knowledge. Besides biocatalysis, the way of your working, thinking and talking is also teaching me how to be a real researcher. In addition to science and working, I think you are a very good joke maker. I really appreciate your constant support and continuous help throughout my PhD.

I would like to thank **Prof. Dr. Wim Quax** for providing me the opportunity to work in the Department of Chemical and Pharmaceutical Biology. I do not believe that Dutch is that tall until I met you in the winter of 2013, Beijing. Thanks for your kind suggestions and remarks during every discussion.

I would like to thank my family. I can never finish my thesis without your support. 爹娘,是你们一直以来的支持和爱才让我走到了现在。从我上大学至今,这十几年的时间里我在你们身边的日子一年比一年少,特别是父亲,你在欧洲几年我们都未曾见面。时光飞逝,转眼我也奔赴中年,我们与其感叹过去纯粹的时光,不如携手珍惜剩下的每一天。刘艺璇同学,真心感谢你这几年对我的照顾,不管我回家时心情多么的不好,看见你温柔可爱的模样,都会开开心心。

Next, I would like to thank my reading committee, **Prof. M.W. Fraaije**, **Prof. G.J.W. Euverink** and **Prof. L. van Niftrik**, for kindly accepting to read my thesis and also for your corrections.

I would like to thank my colleagues on the 9th floor. **Lieuwe**, I appreciate your suggestions and kind advices on my projects and it is happy to work with

you. **Jan-ytzen**, thanks for helping me a lot to start my PhD journey. I wish you the best in your future career and your life. **Zainal** and **Tije**, thanks for bringing Indonesian style to our lab and I hope you have bright future in your home country. **Andreas**, I did not believe Germany can drink a lot of beers until I had the first beer with you. **Michele**, I hope you enjoy my ‘former’ seat and you can get some lucky from it. **Alex**, **Andreas** and **Michele**, thank you so much for bringing so much fun to my Ph.D journey, I can never forget about it. **Haigen**, **Jielin** and **Guangcai**, thanks for talking to me in Chinese so that I did not forget my mother tongue. Thanks for your sharing and discussions when I was confused. **Marie** and **Ele**, I really admire your organization skills, your writing always look clean and clear and probably I can never learn that. Overall, thanks for all of you on creating a nice working atmosphere on the 9th floor, and I hope you enjoy the rest of your stay in Groningen for those who stay longer.

Saranvanan and **Saif**, Thanks for teaching me a lot of Chemistry and I really appreciate it. Without you guys I can never understand those ‘comprehensive’ chemistry. I admire your patience and your knowledge on chemistry. I sincerely wish you all the best with your future scientific career.

I would like to give my special thanks to the current and previous members who I have met during my study in our department: **Fabiola**, **Magda**, **Yizhou**, **Yafeng**, **Joko**, **Baojie**, **Xinyu**, **Fangyuan**, **Hao**, **Abel**, **Laura**, **Shanshan**, **Olivia**, **Jan**, **Zhangping**, **Martijn** and **Bin**, I enjoy every meal, drink, seminars and activities we had together. Thanks for bringing me the nice work atmosphere in our department.

I would like to thanks our nice technicians, **Pieter**, **Ronald**, **Rita** and **Mr. Cool**. **Pieter**, it is nice to have you on my side when I started learn chemical purification and synthesis, although you can make worse and the worst jokes. I appreciate all your input on HPLC, GC and everything in the chemistry lab. **Ronald**, thanks for your help with spectrometers, robot, making landscape and the music you played in the lab. And of course your jokes, I can never forget

about that. **Rita**, I can always get rapid feedbacks from you whenever I sent you an email. You are such a kind person and you barely say no. Thank you so much for helping me during my Ph.D study. **Mr. Cool**, people told me that you would like to hear **Dr. Cool** instead of **Mr. Cool**. Thanks for answering me a lot of questions and I really appreciate you help me with the gel filtration experiments. By the way, I was shocked by your dancing and drinking skills, unbelievable. Thanks again for all of you, **Pieter, Ronald, Rita** and **Dr. Cool**, you are always kindly help us whenever we have questions, I cannot image that doing a PhD without your help.

I would like to give my sincerely thanks to my friends who I met in Groningen, **Kai, Wenjia, Jingyao, Qian, Yu, Lin, Keni, Zhuangzhuang, Hugh, Coco, Tony, Michael, Tingchao, Yi Wu, Vincent** and **Tim**, I enjoy every crazy or cozy moments that I had with you, and because of you, I really have a nice life in Groningen and I will never forget it. **Yapei**, it's a pity that when you started your PhD and I was almost leaving but at least we had some happy moments, I hope you enjoy your PhD studies and do not give up on what you want.

.