Synergistic Catalysis of Tandem Michael Addition/Enantioselective Protonation Reactions by an Artificial Enzyme

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Abstract

Enantioselective protonation is conceptually one of the most attractive methods to generate an α-chiral center. However, enantioselective protonation presents major challenges, especially in water as a solvent. Herein, we report an artificial enzyme catalyzed tandem Michael addition and enantioselective protonation reaction of α-substituted acroleins with 2-acyl imidazole derivatives in water. The artificial enzyme uses a synergistic combination of two abiological catalytic sites: a genetically encoded non-canonical p-aminophenylalanine residue and a Lewis acid Cu(II) complex. The exquisite stereochemical control achieved in the protonation of the transient enamine intermediate generated by conjugate addition of the Michael donor is illustrated by the >20:1 dr and up to >99% ee obtained for the products. These results illustrate the potential of exploiting synergistic catalysis in artificial enzymes for challenging reactions.
Tertiary carbon stereocenters are ubiquitous in biologically active natural products and pharmaceuticals. Enantioselective protonation is conceptually one of the most efficient methods for generating a tertiary carbon stereocenter.\textsuperscript{1-4} However, enantioselective transfer of a proton presents tremendous challenges, especially in aqueous solvents: protons are difficult to control due to their small size, protons in water are highly mobile, proton transfer is generally very fast and the products can be prone to racemization.\textsuperscript{5-7} Nature has evolved several efficient enzymes such as decarboxylases and esterases that catalyse enantioselective protonation reactions.\textsuperscript{8-11} Approaches for non-enzymatic enantioselective protonation mainly rely on the stereoselective protonation of prochiral enolates. These are either pre-prepared or formed in-situ in transition metal catalyzed or organocatalytic conjugate addition reactions, which are mostly performed in organic solvents.\textsuperscript{12-17} Complementary to enolate chemistry, enantioselective protonation of enamine intermediates is an attractive alternative.\textsuperscript{18,19,20,21} Here, we report tandem Michael addition/enantioselective protonation reactions in water catalyzed by an artificial enzyme with excellent enantioselectivity in proton transfer to an enamine intermediate. Key to the activity and stereoselectivity of the artificial enzyme is the synergistic combination of two abiological catalytic groups that activate both the Michael donor and acceptor.

Artificial enzymes, which are proteins equipped with abiological catalytic groups, have emerged as a powerful approach towards the creation of enzymes for new-to-nature reactions.\textsuperscript{22-24} The well-defined secondary coordination sphere around the catalytic sites and substrates provided by the protein offers fascinating opportunities to optimize both the catalytic activity and selectivity of the artificial enzymes. Some examples of tandem Friedel-Crafts alkylations / enantioselective
protonation reactions catalyzed by artificial metalloenzymes have been reported, albeit with moderate ee’s.\textsuperscript{25–27}  
Recently, we have introduced a novel design of an artificial enzyme that contained two abiological catalytic sites that could engage in synergistic catalysis of Michael addition reactions.\textsuperscript{28} Synergistic catalysis is a powerful concept that involves simultaneous activation of two substrates in a bimolecular reaction, for example, an electrophile and nucleophile, by separate catalytic moieties.\textsuperscript{29–31} The synergistic artificial enzyme was based on the Lactococcal multidrug resistance Regulator (LmrR).\textsuperscript{32–34} It contained a genetically encoded non-canonical p-aminophenylalanine (pAF) residue which can function as nucleophilic catalyst\textsuperscript{35} and a Lewis acidic Cu(II)-complex of 1,10-phenanthroline (Cu(II)-phen), bound between the indole rings of two tryptophan residues in the hydrophobic cavity of the dimer interface of LmrR.\textsuperscript{36,37} The simultaneous action of these two catalytic sites, which are in close proximity and in a well-defined orientation with respect to each other, resulted in a high catalytic activity and excellent enantioselectivity in the catalyzed Michael addition reaction. Having established the power of the synergistic catalysis concept in artificial enzymes, we sought to apply this concept to the catalysis of the challenging tandem Michael addition / enantioselective protonation reaction in water.
This work

Figure 1. Top: enantioselective protonation of enolate or enamine intermediates; Bottom: tandem Michael addition and enantioselective protonation catalyzed by an LmrR-based artificial enzyme via synergistic combination of two catalytic sites: an unnatural pAF residue that activates the α-substituted enal via iminium ion formation and a Lewis acid Cu(II) complex that catalyzes the enolization of the Michael donor. After Michael addition reaction, the resulting enamine intermediate undergoes enantioselective protonation.

The artificial enzyme was assembled as described before.28 In this study, we used the variant LmrR_V15pAF, which contains a non-canonical pAF residue at position 15 inside the hydrophobic pore. pAF was introduced indirectly: first p-azidophenylalanine was introduced via amber stop codon suppression using the dedicated orthogonal translation system (pEVOL-pAzF), followed by Staudinger reduction of the azide to the amine with TCEP during purification.35,38 We have found before that this indirect method gives rise to higher yields of incorporation than direct incorporation of pAF.35 The Lewis acidic site was then introduced through supramolecular self-assembly by combining LmrR_V15pAF with Cu(II)-phen in a buffered solution.36 In this
design, an α-substituted acrolein is activated through iminium ion formation with the aniline residue, while the nucleophilic enolate is formed by activation of the Michael donor by the Lewis acidic Cu(II)-phen complex. This results in a Michael addition to the activated acrolein derivative, followed by an enantioselective proton-transfer reaction to the transient enamine intermediate to give the chiral aldehyde product (Figure 1).

Initial reactivity tests were performed with 2-acyl imidazole (1a) as Michael donor and methacrolein (2a) as Michael acceptor. LmrR_V15pAF/Cu(II)-phen was prepared by self-assembly from 6 mol % Cu(II)-phen (60μM) with 5 mol % LmrR_V15pAF (50μM), in MES buffer at pH 6.5 for 1 h. No product was formed when omitting one of the components from the artificial enzyme, that is, using only LmrR_V15pAF or Cu(II)-phen or the combination of wild type LmrR, without pAF residue, and Cu(II)-phen (Table 1, entry 1−3). When using the artificial enzyme, the Michael addition/enantioselective protonation product 3a was obtained with 26% yield, 3:1 dr and 89% and 32% ee for the major and minor diastereomer, respectively (Table 1, entry 5). At shorter reaction times higher diastereoselectivity and enantioselectivity was obtained, suggesting a slow racemization of the chiral product occurs over time (Table 1, entry 6). This is well-known for ketones and aldehydes containing a stereocenter at the α position.
Table 1. Results of tandem Michael addition/enantioselective protonation catalyzed by LmrR_V15pAF/Cu(II)-phen

| Entry | Catalysts | Yield (%)<sup>a</sup> | dr<sup>b</sup> | ee (%)<sup>b,c</sup> |
|-------|-----------|------------------------|---------------|----------------------|
| 1     | LmrR_V15pAF / | <1 | ND | ND |
| 2     | / Cu(II)-phen | <1 | ND | ND |
| 3     | LmrR Cu(II)-phen | <1 | ND | ND |
| 5     | LmrR_V15pAF Cu(II)-phen | 26±3 | 3 : 1 | 89±0/32±1 |
| 6<sup>d</sup> | LmrR_V15pAF Cu(II)-phen | 14±2 | 6 : 1 | 93±0/24±2 |

Typical conditions: 1.2 equiv Cu(II)-phen (6 mol%; 60 μM) loading with respect to LmrR_V15pAF or LmrR (5 mol%; 50 μM), 1 mM 1a, 10 mM 2a, in 20 mM MES buffer (pH 6.5), 150 mM NaCl, at 4 °C for 48 h, unless noted otherwise. Yield and ee values are the average of at least three independent experiments. <sup>a</sup>Yields are determined by HPLC analysis. <sup>b</sup>ee and dr values were determined by chiral HPLC. <sup>c</sup>ee values of major/minor diastereomer. <sup>d</sup>Reactions carried out for 16 hours. Errors represent standard deviation based on at least three independent experiments. ND = not determined.

Encouraged by these results, we performed this reaction with para-methoxy substituted ketone 1b as Michael donor, which in our previous study was found to be more reactive (Table 2). After optimization of the reaction conditions, using a combination of 2.5 mol % LmrR_V15pAF with 3 mol % Cu(II)-phen, the reaction with ketone 1b resulted in a higher dr value and excellent enantioselectivities for both diastereomers of the product with 99% and 72% ee, respectively (Table 2, entry 2). The catalyst loading could be lowered to 1 mol% without affecting the stereoselectivity of the reaction (Table 2, entries 1, 3). When we removed the Cu(II)-phen binding site by mutation of the central tryptophan residues to alanine, that is, mutant LmrR_V15pAF_W96A, a strong decrease of yield and stereoselectivity was observed. These results support the importance of the precise positioning of the Cu(II)-bound enolate with respect to the activated enal (Table 2, entry 4).
Mutations of the methionine at position 8 were known to potentially improve the catalytic activity of the artificial enzyme.\textsuperscript{28,39} Hence, some M8 mutants of LmrR\textsubscript{V15pAF} were evaluated in catalysis (Table 2, entry 5–7). The variant LmrR\textsubscript{V15pAF M8L} exhibited both improved stereoselectivity and reactivity compared to LmrR\textsubscript{V15pAF} with 65% yield, >20 : 1 dr and >99% ee for the major diastereomer. Higher reaction temperatures and longer reaction times were found to have a negative effect on the diastereoselectivity and reactivity (Table 2, entry 8, 9). Using slightly higher concentration of artificial enzyme improved the reaction, resulting in >99% ee, >20 : 1 dr and 90% yield (Table 2, entry 10).

Table 2. Optimization of reaction conditions and mutagenesis study of artificial enzymes

| Entry | Catalysts | Yield (%)\textsuperscript{a} | dr\textsuperscript{b} | ee (%)\textsuperscript{b,c} |
|-------|-----------|-----------------|----------------|------------------|
| 1\textsuperscript{c} | LmrR\textsubscript{V15pAF} Cu(II)-phen | 44 ± 2 | 10 : 1 | 99 ± 0/73 ± 1 |
| 2 | LmrR\textsubscript{V15pAF} Cu(II)-phen | 28 ± 3 | 10 : 1 | 99 ± 0/72 ± 1 |
| 3\textsuperscript{e} | LmrR\textsubscript{V15pAF W96A} Cu(II)-phen | 16 ± 1 | 9 : 1 | 99 ± 0/68 ± 2 |
| 4 | LmrR\textsubscript{V15pAF M8W} Cu(II)-phen | 5 ± 1 | 3 : 1 | 18 ± 1/–66 ± 2 |
| 5 | LmrR\textsubscript{V15pAF M8I} Cu(II)-phen | 40 ± 1 | 15 : 1 | 99 ± 0 |
| 6 | LmrR\textsubscript{V15pAF M8L} Cu(II)-phen | 58 ± 3 | 18 : 1 | >99 |
| 7 | LmrR\textsubscript{V15pAF M8L} Cu(II)-phen | 65 ± 2 | 20 : 1 | >99 |
| 8\textsuperscript{f} | LmrR\textsubscript{V15pAF M8L} Cu(II)-phen | 33 ± 2 | 5 : 1 | 99 ± 0/75 ± 1 |
| 9\textsuperscript{g} | LmrR\textsubscript{V15pAF M8L} Cu(II)-phen | 88 ± 1 | 8 : 1 | 99 ± 0/74 ± 2 |

Typical conditions: 1.2 equiv Cu(II)-phen (3 mol%; 30 μM) loading with respect to LmrR\textsubscript{V15pAF} or variants (2.5 mol%; 25 μM), 1 mM \textbf{1b}, 10 mM \textbf{2a}, in 20 mM MES buffer (pH 5.5), 150 mM NaCl, at 4 °C for 16 h, unless noted otherwise. Yield and ee values are the average of at least three independent experiments. \textsuperscript{a} Yields are determined by HPLC. \textsuperscript{b} ee and dr values are determined by chiral HPLC. \textsuperscript{c} Ee values for major/minor diastereomer; in cases where the dr was high, the ee of the major diastereomer was not determined. \textsuperscript{e} Reaction with 5 mol% protein and 6 mol% Cu(II)-phen loading. \textsuperscript{f} Reaction with 1 mol% protein and 1.2 mol% Cu(II)-phen loading. \textsuperscript{i} Reaction performed at 18 °C. \textsuperscript{g} Reaction performed for 48 h. Errors represent standard deviation based on at least three independent experiments.
The substrate scope of the tandem Michael addition / enantioselective protonation was investigated by variation of the α-substituted enal and of the 2-acyl imidazole or 2-acyl pyridine with LmrR_V15pAF_M8L with Cu(II)-phen, using the optimized conditions (scheme 1). Using the para-chlorophenyl (1c) and 3-thiophene substituted 2-acyl imidazole 1d gave the products 3c and 3d, respectively, with excellent ee’s, albeit with a slightly lower dr compared to 3a and 3b. Replacing the 2-acyl-imidazole moiety with 2-acyl pyridine (4) gave the product 5 with moderate results. In addition to methacrolein, α-benzyl and α-ethyl acrolein were also well-tolerated in this reaction, giving products 3f, 3g and 3h, with excellent ee, dr and yield.
Scheme 1. Substrate scope of tandem Michael addition/enantioselective protonation catalyzed by LmrR_V15pAF_M8L-based artificial enzymes

Typical conditions: 1.2 equiv Cu(II)-phen (6 mol%; 60 μM) loading with respect to LmrR_V15pAF_M8L (5 mol%; 50 μM), 1 mM 1, 10 mM 2, in 20 mM MES buffer (pH 5.5), 150 mM NaCl, at 4 °C for 16 h, unless noted otherwise. Yield and ee values are the average of at least three independent experiments. Error values represent the standard deviations. aReactions for 7 hours. bReaction with 1 mM 1, 2 mM 2, in 20 mM MES buffer (pH 5.5), 150 mM NaCl, at 4 °C for 24 h. ee and dr values were determined after conversion by Wittig reaction. The ee of the minor diastereomers are not shown in this figure but in supporting information Scheme S1.
Generally, it was found that both diastereoselectivity and enantioselectivity decreased somewhat with longer reaction time, indicative of racemization of the product (Table S3). This was not unexpected in view of the known lability of α stereocenters. Control experiments involving incubating enantioenriched 3g with protein LmrR_V15pAF_M8L, LmrR and pH 5.5 MES buffer separately suggested that both the artificial enzyme and the medium contribute to the racemization process.

In conclusion, here we have shown that the tandem Michael addition / enantioselective protonation reaction in water is catalyzed efficiently by an LmrR-based artificial enzyme that exploits synergistic catalysis by two abiological catalytic groups. The combination of using a genetically encoded non-canonical amino acid to activate the electrophile and a supramolecularly bound metal complex to activate the nucleophile and deliver it with high precision to one prochiral face of the activated electrophile makes it possible to perform challenging reactions, such as the reaction presented here, with excellent diastereo- and enantio-selectivities. These results illustrate the power of synergistic artificial enzymes and we envision this concept to be broadly applicable for the biocatalysis of new-to-nature reactions.

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Competing interests

The authors declare no competing interests.
References

1. Fehr, C. Enantioselective Protonation of Enolates and Enols. *Angew. Chem. Int. Ed.* 35, 2566–2587 (1996).

2. Mohr, J. T., Hong, A. Y. & Stoltz, B. M. Enantioselective protonation. *Nat. Chem.* 1, 359–369 (2009).

3. Oudeyer, S., Brière, J. F. & Levacher, V. Progress in catalytic asymmetric protonation. *European J. Org. Chem.* 2014, 6103–6119 (2014).

4. Phelan, J. P. & Ellman, J. A. Conjugate addition-enantioselective protonation reactions. *Beilstein J. Org. Chem.* 12, 1203–1228 (2016).

5. Sibi, M. P., Coulomb, J. & Stanley, L. M. Enantioselective enolate protonations: Friedel-crafts reactions with α-substituted acrylates. *Angew. Chem. Int. Ed.* 47, 9913–9915 (2008).

6. Kieffer, M. E., Repka, L. M. & Reisman, S. E. Enantioselective synthesis of tryptophan derivatives by a tandem friedel-crafts conjugate addition/asymmetric protonation reaction. *J. Am. Chem. Soc.* 134, 5131–5137 (2012).

7. Li, Y. P. *et al.* Chiral Spiro Phosphoric Acid-Catalyzed Friedel-Crafts Conjugate Addition/Enantioselective Protonation Reactions. *ACS Catal.* 9, 6522–6529 (2019).

8. Matsumoto, K., Tsutsumi, S., Ihori, T. & Ohta, H. Enzyme-Mediated Enantioface-Differentiating Hydrolysis of α-Substituted Cycloalkanone Enol Esters. *J. Am. Chem. Soc.* 112, 9614–9619 (1990).

9. Hirata, T., Shimoda, K. & Kawano, T. Asymmetric hydrolysis of enol esters with two esterases from Marchantia polymorpha. *Tetrahedron Asymmetry* 11, 1063–1066 (1999).

10. MIYAMOTO, K. & OHTA, H. Purification and properties of a novel arylmalonate decarboxylase from Alcaligenes bronchisepticus KU 1201. *Eur. J. Biochem.* 210, 475–481 (1992).

11. Matoishi, K., Ueda, M., Miyamoto, K. & Ohta, H. Mechanism of asymmetric decarboxylation of α-aryl-α-methylmalonate catalyzed by arylmalonate decarboxylase originated from Alcaligenes bronchisepticus. *J. Mol. Catal. B Enzym.* 27, 161–168 (2004).

12. Sibi, M. P. & Patil, K. Enantioselective H-atom transfer reactions: A new methodology for the synthesis of β2-amino acids. *Angew. Chem. Int. Ed.* 43, 1235–1238 (2004).

13. Poisson, T., Yamashita, Y. & Kobayashi, S. Catalytic asymmetric protonation of chiral calcium enolates via 1,4-addition of malonates. *J. Am. Chem. Soc.* 132, 7890–7892 (2010).

14. Mohr, J. T., Nishimata, T., Behenna, D. C. & Stoltz, B. M. Catalytic enantioselective decarboxylative protonation. *J. Am. Chem. Soc.* 128, 11348–11349 (2006).

15. Leow, D., Lin, S., Chittimalla, S. K., Fu, X. & Tan, C. H. Enantioselective protonation catalyzed by a chiral bicyclic guanidine derivative. *Angew. Chem. Int. Ed.* 47, 5641–5645
16. Vora, H. U. & Rovis, T. N-Heterocyclic Carbene Catalyzed Asymmetric Hydration: Direct Synthesis of α-Protop and α-Deuterio α-Chloro and α-Fluoro Carboxylic Acids. *J. Am. Chem. Soc.* **132**, 2860–2861 (2010).

17. Kitanosono, T., Sakai, M., Zhu, L. & Kobayashi, S. Enantioselective Aza-Michael/protonation reactions in water. *Chem. Lett.* **48**, 783–786 (2019).

18. Fu, N., Zhang, L. & Luo, S. Catalytic asymmetric enamine protonation reaction. *Org. Biomol. Chem.* **16**, 510–520 (2018).

19. Fu, N., Zhang, L., Luo, S. & Cheng, J. P. Chiral primary-amine-catalyzed conjugate addition to α-substituted vinyl ketones/aldehydes: Divergent stereocontrol modes on enamine protonation. *Chem. - A Eur. J.* **19**, 15669–15681 (2013).

20. Fu, N., Zhang, L., Li, J., Luo, S. & Cheng, J. P. Chiral primary amine catalyzed enantioselective protonation via an enamine intermediate. *Angew. Chem. Int. Ed.* **50**, 11451–11455 (2011).

21. Zhang, L., Fu, N. & Luo, S. Pushing the limits of aminocatalysis: Enantioselective transformations of α-branched β-ketocarbonyls and vinyl ketones by chiral primary amines. *Acc. Chem. Res.* **48**, 986–997 (2015).

22. Hilvert, D. Design of Protein Catalysts. *Annu. Rev. Biochem.* **82**, 447–470 (2013).

23. Schwizer, F. *et al.* Artificial Metalloenzymes: Reaction Scope and Optimization Strategies. *Chem. Rev.* **118**, 142–231 (2018).

24. Nanda, V. & Koder, R. L. Designing artificial enzymes by intuition and computation. *Nat. Chem.* **2**, 15–24 (2010).

25. García-Fernández, A., Megens, R. P., Villarino, L. & Roelfes, G. DNA-Accelerated Copper Catalysis of Friedel-Crafts Conjugate Addition/Enantioselective Protonation Reactions in Water. *J. Am. Chem. Soc.* **138**, 16308–16314 (2016).

26. Mansot, J. *et al.* A rational quest for selectivity through precise ligand-positioning in tandem DNA-catalysed Friedel-Crafts alkylation/asymmetric protonation. *Chem. Sci.* **10**, 2875–2881 (2019).

27. Villarino, L. *et al.* Cofactor Binding Dynamics Influence the Catalytic Activity and Selectivity of an Artificial Metalloenzyme. *ACS Catal.* **10**, 11783–11790 (2020).

28. Zhou, Z. & Roelfes, G. Synergistic catalysis in an artificial enzyme by simultaneous action of two abiological catalytic sites. *Nat. Catal.* **3**, 289–294 (2020).

29. Allen, A. E. & MacMillan, D. W. C. Synergistic catalysis: A powerful synthetic strategy for new reaction development. *Chem. Sci.* **3**, 633–658 (2012).

30. Patil, N. T., Shinde, V. S. & Gajula, B. A one-pot catalysis: The strategic classification
with some recent examples. *Org. Biomol. Chem.* **10**, 211–224 (2012).

31. Afewerki, S. & Córdova, A. Combinations of Aminocatalysts and Metal Catalysts: A Powerful Cooperative Approach in Selective Organic Synthesis. *Chem. Rev.* **116**, 13512–13570 (2016).

32. Agustiandari, H., Lubelski, J., Van Den Berg Van Saparoea, H. B., Kuipers, O. P. & Driessen, A. J. M. LmrR is a transcriptional repressor of expression of the multidrug ABC transporter LmrCD in Lactococcus lactis. *J. Bacteriol.* **190**, 759–763 (2008).

33. Madoori, P. K., Agustiandari, H., Driessen, A. J. M. & Thunnissen, A. M. W. H. Structure of the transcriptional regulator LmrR and its mechanism of multidrug recognition. *EMBO J.* **28**, 156–166 (2009).

34. Roelfes, G. LmrR: A Privileged Scaffold for Artificial Metalloenzymes. *Acc. Chem. Res.* **52**, 545–556 (2019).

35. Drienovská, I., Mayer, C., Dulson, C. & Roelfes, G. A designer enzyme for hydrazone and oxime formation featuring an unnatural catalytic aniline residue. *Nat. Chem.* **10**, 946–952 (2018).

36. Bos, J., Browne, W. R., Driessen, A. J. M. & Roelfes, G. Supramolecular Assembly of Artificial Metalloenzymes Based on the Dimeric Protein LmrR as Promiscuous Scaffold. *J. Am. Chem. Soc.* **137**, 9796–9799 (2015).

37. Villarino, L. *et al.* Dynamics of Metal Complex Binding in Relation to Catalytic Activity and Selectivity of an Artificial Metalloenzyme. *ACS Catal.* **10**, 11783-11790 (2020).

38. Mayer, C., Dulson, C., Reddem, E., Thunnissen, A. M. W. H. & Roelfes, G. Directed Evolution of a Designer Enzyme Featuring an Unnatural Catalytic Amino Acid. *Angew. Chem. Int. Ed.* **58**, 2083–2087 (2019).

39. Villarino, L. *et al.* An Artificial Heme Enzyme for Cyclopropanation Reactions. *Angew. Chem. Int. Ed.* **57**, 7785–7789 (2018).
Supplementary Information

Synergestic Catalysis of Tandem Michael Addition/Enantioselective Protonation Reactions by an Artificial Enzyme

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1. General Procedures

General:

Unless otherwise noted, chemicals were purchased from Sigma Aldrich or TCI EUROPE and used without further purification. Flash chromatography was performed using silica gel 60 Å (Merck, 230-400 mesh). Thin-layer chromatography was performed on silica plates. Compounds were visualized by UV light (254 nm), using KMnO₄. ¹H-NMR and ¹³C-NMR spectra were recorded on a Varian 400 MHz in CDCl₃. ¹H-NMR data are reported as follows: chemical shifts, multiplicity (br = broad, d = doublet, dd = doublet of doublet, ddd = doublet of doublet of doublets, dq = doublet of quartets, m = multiplet, q = quartet, s = singlet, t = triplet), coupling constants (Hz), and integration. Mass spectra (HRMS) were recorded on an Orbitrap XL (Thermo Fisher Scientific; ESI pos. mode). Enantiomeric excess determinations and yields were measured by normal phase (np) HPLC analysis (Chiralpak-ADH) using UV-detection (Shimadzu SCL-10Avp).

Protein expression and purification: LmrR_pAF variants were produced and purified as previously described.³⁴ The identity of proteins and the successful reduction of pAzF were determined by mass spectrometry and the purity of the protein confirmed by SDS PAGE. Protein concentration was determined by correcting the calculated extinction coefficients for LmrR variants for the absorbance of pAF (ε₂⁸₀ = 1333 M⁻¹ cm⁻¹).

Catalytical reaction setup and product characterization

The catalytic solution was prepared by combining 12 μL Cu(1,10-phenanthroline)(NO₃)₂ (60 μM, 6 % catalyst loading) prepared in 50% : 50% DMF/MES pH 5.5 buffer with LmrR_V15pAF_ or LmrR_V15pAF variants (50 μM, 5 % catalyst loading) in a final volume of 276 μL MES buffer (20 mM MES, 250 mM
NaCl, pH 5.5) and incubating at 4 °C for one hour. To this mixture, 12 μL of a fresh stock solution of substrate 1 in DMF/MES buffer (50:50, 25 mM, final concentration in reaction mixture 1 mM) and 12 μL of a fresh stock solution of substrate 2 in DMF/MES buffer (50:50, 250 mM, final concentration in reaction mixture 10 mM) were added. The reaction was mixed for 7 to 24 hours by continuous inversion at 4 °C. Then the product was extracted with 900 μL of ethyl acetate and 100 μL 1 mM 2-phenylquinoline solution in ethyl acetate as internal standard. The organic layers were dried on Na₂SO₄, and the solvent was evaporated under reduced pressure. The product was re-dissolved in 120 μl of a heptane:propan-2-ol mixture (9:1) and the yield and enantiomeric excess (for product 3a, 3b, 3c, 3e and 5) were determined with np-HPLC, using a calibration curve (see Section 5).

The enantiomeric excess of products 3d, 3f, 3g were determined as follows: after evaporation under reduced pressure, the product was re-dissolved in 400 μl ethyl acetate and then added to 100 μl Wittig reagent (Ethoxycarbonylmethyl)triphenylphosphonium bromide (100 mM in ethyl acetate) solution. The Wittig reaction was performed by continuously shaking at 40 °C for 2 hours. The solvent was evaporated under reduced pressure. The product was re-dissolved in 120 μl of a heptane:propan-2-ol mixture (9:1) and the diastereomeric- and enantiomeric excess were determined with np-HPLC.
2. Supporting Tables and Scheme

**Table S1. Screening conditions of MA/EP reactions with methacrolein (2a) and 1a**

![Chemical structure](image)

| Entry | Catalysts          | Yield (%)<sup>a</sup> | dr  | ee (%)<sup>b</sup> |
|-------|--------------------|------------------------|-----|---------------------|
| 1     | LmrR_V15pAF        | <1                     | ND  | ND                  |
| 2     | /                  | <1                     | ND  | ND                  |
| 3     | LmrR               | <1                     | ND  | ND                  |
| 4     | /                  | <1                     | ND  | ND                  |
| 5     | LmrR_V15pAF        | 26 ± 3                 | 3:1 | 89 ± 0/32 ± 1       |
| 6<sup>c</sup> | LmrR_V15pAF       | 14 ± 2                 | 6:1 | 93 ± 0/24 ± 2       |
| 7<sup>c</sup> | LmrR_V15pAF_M8L   | 48 ± 2                 | 8:1 | 98 ± 0/62 ± 2       |
| 8<sup>d</sup> | Macmillan catalyst | 68                     | 1:1 | 11/22               |

Typical conditions: 1.2 equiv Cu(II)-phen (6 mol%; 60 μM) loading with respect to LmrR_V15pAF or LmrR (5 mol%; 50 μM), 1 mM 1a, 10 mM 2a, in 20 mM MES buffer (pH 6.5), 150 mM NaCl, at 4 °C for 48 h. Yield and ee values are the average of at least three independent experiments. Errors represent standard deviations.<sup>a</sup>Yield are determined by HPLC according to the calibration curve. <sup>b</sup>ee and dr values are determined by chiral HPLC. <sup>c</sup>Reaction for 16 hours. <sup>d</sup>Reaction set up with catalyst (2S,5S)-(−)-2-tert-Butyl-3-methyl-5-benzyl-4-imidazolidinone (20 mol%) loading at 40 °C in CH<sub>3</sub>OH for 48 h.
Table S2. Screening conditions of MA/EP reactions with methacrolein (2a) and 1b

![Chemical structure](image)

| Entry | LmrR variants + Cu(II)-phen | Yield (%) | dr | ee (%) |
|-------|-----------------------------|-----------|----|--------|
| 1     | LmrR_V15pAF                 | 45 ± 1    | 9 : 1 | 99 ± 0/76 ± 1 |
| 2<sup>c</sup> | LmrR_V15pAF             | 63 ± 2    | 1.2 : 1 | 83 ± 0/65 ± 1 |
| 3<sup>d</sup> | LmrR_V15pAF             | 23 ± 1    | 12 : 1 | 99 ± 0/70 ± 2 |
| 4<sup>d,e</sup> | LmrR_V15pAF             | 38 ± 1    | 10 : 1 | 99 ± 0/72 ± 2 |
| 5<sup>d,f</sup> | LmrR_V15pAF             | 30 ± 1    | 12 : 1 | 99 ± 0/70 ± 1 |
| 6<sup>d,g</sup> | LmrR_V15pAF             | 18 ± 1    | 12 : 1 | 99 ± 0/76 ± 1 |
| 6<sup>d,h</sup> | LmrR_V15pAF             | 16 ± 1    | 9 : 1  | 99 ± 0/68 ± 2 |
| 7<sup>d,e</sup> | LmrR_V15pAF_W96A         | 5 ± 1     | 3 : 1  | 18 ± 1/–66 ± 2 |
| 8<sup>d,e</sup> | LmrR_V15pAF_M8W          | 40 ± 1    | 15 : 1 | 99 ± 0 |
| 9<sup>d,e</sup> | LmrR_V15pAF_M8I          | 58 ± 3    | 18 : 1 | >99 |
| 10<sup>d,e</sup> | LmrR_V15pAF_M8L         | 65 ± 2    | 20 : 1 | >99 |
| 11<sup>d,e,i</sup> | LmrR_V15pAF_M8L       | 33 ± 2    | 5 : 1  | 99 ± 0/75 ± 1 |
| 12<sup>d,e,j</sup> | LmrR_V15pAF_M8L       | 88 ± 1    | 8 : 1  | 99 ± 0/74 ± 2 |
| 13<sup>e</sup> | LmrR_V15pAF_M8L         | 90 ± 2    | >20 : 1 | >99 |

Typical conditions: 1.2 equiv Cu(II)-phen (6 mol %; 40 μM) loading with respect to LmrR_V15pAF variants (5 mol %; 50 μM), 1 mM 1a, 10 mM 2b, in 20 mM MOPS buffer (pH 6.5), 150 mM NaCl, at 4 °C for 16 h. Errors represent standard deviation based on at least three independent experiments.

<sup>a</sup>Yield are determined by HPLC according to the calibration curve.

<sup>b</sup>ee and dr values are determined by chiral HPLC.

<sup>c</sup>Reaction for 72 hours.

<sup>d</sup>Reaction with 2.5 mol % protein and 3 mol % Cu(II)-phen loading.

<sup>e</sup>Reaction in 20 mM MES buffer (pH 5.5), 150 mM NaCl.

<sup>f</sup>Reaction in 20 mM MES buffer (pH 6.0), 150 mM NaCl.

<sup>g</sup>Reaction in 20 mM MOPS buffer (pH 7.2), 150 mM NaCl.

<sup>h</sup>Reaction with 1 mol % protein and 1.2 mol % Cu(II)-phen loading.

<sup>i</sup>Reaction at room temperature.

<sup>j</sup>Reaction for 48 hours.
Table S3. Racemization experiments under several conditions

\[
\begin{align*}
\text{Conditions} & \quad \text{ee (\%)} & \quad \text{dr} \\
5 \text{ mol} \% \text{LmrR}_{-}\text{V15pAF}_{-}\text{M8L in pH 5.5 buffer} & \quad 93 & \quad 4:1 \\
5 \text{ mol} \% \text{LmrR in pH 5.5 buffer} & \quad 89 & \quad 3:1 \\
\text{pH 5.5 buffer} & \quad 97 & \quad 5:1 \\
\end{align*}
\]

Control experiments were conducted by incubating enantioenriched 3g which was directly collected from enzyme catalysis reaction after extraction and evaporation under several conditions in 300 μL scale with protein LmrR_{-}V15pAF_{-}M8L, LmrR and pH 5.5 MES buffer separately for 24 hours at 4 °C. The results suggested that the artificial enzyme as well as the medium both contribute to the racemization process.
Scheme S1. Substrate scope of tandem MA/EP catalyzed by LmrR_V15pAF_M8L

Typical conditions: 1.2 equiv Cu(II)-phen (6 mol%; 60 μM) loading with respect to LmrR_V15pAF_M8L (5 mol%; 50 μM), 1 mM 1, 10 mM 2, in 20 mM MES buffer (pH 5.5), 150 mM NaCl, at 4 °C for 16 h, unless noted otherwise. Yield and ee values are determined by chiral HPLC. Errors represent standard deviation based on at least three independent experiments. *Reactions for 7 hours. **Reaction with 1 mM 1, 2 mM 2, in 20 mM MES buffer (pH 5.5), 150 mM NaCl, at 4 °C for 24 h. †ee and dr values were determined after conversion by Wittig reaction.
3. Chemical synthesis

General procedure for the preparation of Michael addition/enantioselective protonation reaction products as reference material: To the mixture of the 2-acyl-1-methylimidazole (0.2 M) and 2 equivalents of the aldehyde (0.4 M) in 1 mL MeOH, was added 20 mol % pyridine. The reaction was stirred at 45 °C for 72 hours. Then the product was purified by column chromatography (silica gel, gradient of Heptane:EtOAc = 8:1 to 3:1). Most of the racemic reference products (3a, 3b, 3c, 3d, 3e, 3f, 5) were obtained as an inseparable mixture of two diastereomers. There are two sets of proton or carbon signals in the NMR spectrum for some protons and carbons. Only product 3g was isolated as a major diastereomer.

Derivatization of product 3d, 3f, 3g by Wittig reaction: To the purified product dissolved in 1 ml ethyl acetate was added 2 equivalent Wittig reagent (Ethoxycarbonylmethyl)triphenylphosphonium bromide. The wittig reaction was performed with continuously shaking at 40 °C for 2 hours. Then the product was purified by column chromatography (silica gel, gradient of Heptane:EtOAc = 12:1 to 5:1).
2-methyl-5-(1-methyl-1H-imidazol-2-yl)-5-oxo-4-phenylpentanal (3a). Purified by column chromatography (SiO$_2$, Heptane:EtOAc = 8:1 to 3:1), to afford the product as a colorless oil. Yield: 16 mg, (61%). $^1$H NMR (400 MHz, CDCl$_3$) δ 9.55 (s, 1H), 7.44–7.40 (m, 2H), 7.30–7.26 (m, 2H), 7.22–7.19 (m, 1H), 7.12 (s, 1H), 6.97 (s, 1H), 5.32–5.24 (m, 1H), 3.92 (s, 3H), 2.45–2.38 (m, 1H), 2.22–2.20 (m, 1H), 2.07–2.00 (m, 1H), 1.13–1.10 (m, 3H); Visible peaks of minor diastereoisomer: 9.60 (s, 1H), 2.63–2.57 (m, 1H), 2.30–2.28 (m, 1H), 1.87–1.82 (m, 1H); $^{13}$C NMR (101 MHz, CDCl$_3$) δ 206.9, 206.8, 194.4, 194.3, 145.3, 145.2, 141.4, 140.8, 132.1, 132.0, 131.4, 131.3, 131.3, 131.3, 130.2, 130.1, 129.9, 129.8, 52.6, 52.5, 47.1, 46.9, 38.8, 38.8, 36.2, 35.6, 16.5, 15.9. HRMS (ESI$^+$) calculated for C$_{16}$H$_{20}$N$_2$O$_2$ (M+H$^+$) = 271.1441, found = 271.1439.

The ee of the enzymatic reaction was determined by HPLC analysis (Chiralpak-ADH, n-heptane:iPrOH 99:1, 0.5 ml/min). Retention times: major diastereomer, 75.7 min and 82.5 min; minor diastereomer, 74.1 min and 90.5 min.
4-(4-methoxyphenyl)-2-methyl-5-(1-methyl-1H-imidazol-2-yl)-5-oxopentalanal  (3b).

Purified by column chromatography (SiO$_2$, Heptane:EtOAc = 8:1 to 3:1), to afford the product as a colorless oil. Yield: 21 mg, (68%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 9.56 (s, 1H), 7.37–7.33 (m, 2H), 7.13 (s, 1H), 6.98 (s, 1H), 6.84–6.81 (m, 2H), 5.25–5.20 (m, 1H), 3.93 (s, 3H), 3.75 (s, 3H), 2.45–2.37 (m, 1H), 2.25–2.20 (m, 1H), 2.03–1.96 (m, 1H), 1.14–1.10 (m, 3H); Visible peaks of minor diastereoisomer: 9.60 (s, 1H), 2.60–2.55 (m, 1H), 2.31–2.29 (m, 1H), 1.86–1.82 (m, 1H); $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 206.9, 206.8, 194.6, 194.5, 161.5, 161.4, 145.1, 133.3, 132.6, 132.5, 132.3, 132.0, 131.9, 130.1, 130.0, 116.8, 116.7, 57.8, 51.7, 51.6, 47.0, 46.9, 38.8, 38.8, 36.2, 35.6, 16.5, 15.9. HRMS (ESI$^+$) calculated for C$_{17}$H$_{22}$N$_2$O$_3$ (M+H$^+$) = 301.1546, found = 301.1548. The ee of the enzymatic reaction was determined by HPLC analysis (Chiralpak-ADH, n-heptane:iPrOH 99:1, 0.5 ml/min). Retention times: major diastereomer, 152.2 min and 167.6 min; minor diastereomer, 159.5 min and 199.6 min.
4-(4-chlorophenyl)-2-methyl-5-(1-methyl-1H-imidazol-2-yl)-5-oxopentanal (3c).

Purified by column chromatography (SiO₂, Heptane:EtOAc = 8:1 to 5:1), to afford the major isomer as a colorless oil. Yield: 24 mg, (72%). ¹H NMR (400 MHz, CDCl₃) δ 9.56 (s, 1H), 7.37–7.34 (m, 2H), 7.26–7.23 (m, 2H), 7.12 (s, 1H), 6.99 (s, 1H), 5.29–5.22 (m, 1H), 3.92 (s, 3H), 2.39–2.34 (m, 1H), 2.25–2.18 (m, 1H), 2.07–2.00 (m, 1H), 1.13–1.10 (m, 3H); Visible peaks of minor diastereoisomer: 9.59 (s, 1H), 2.62–2.54 (m, 1H), 2.30–2.25 (m, 1H), 1.83–1.76 (m, 1H); ¹³C NMR (101 MHz, CDCl₃) δ 206.6, 206.5, 193.9, 193.8, 144.9, 139.9, 139.3, 135.8, 135.7, 132.7, 132.6, 132.2, 132.1, 131.5, 131.5, 130.3, 130.3, 52.0, 51.9, 47.0, 46.9, 38.8, 38.8, 36.0, 35.5, 16.5, 15.9. HRMS (ESI⁺) calculated for C₁₆H₁₉ClN₂O₂ (M⁺H⁺) = 305.1051, found = 305.1050. The ee of the enzymatic reaction was determined by HPLC analysis (Chiralpak-ADH, n-heptane:iPrOH 99:1, 0.5 ml/min). Retention times: major diastereomer, 84.9 min and 97.5 min; minor diastereomer, 90.7 min and 117.2 min.
2-methyl-5-(1-methyl-1H-imidazol-2-yl)-5-oxo-4-(thiophen-3-yl)pentanal (3d).

Purified by column chromatography (SiO₂, Heptane:EtOAc = 8:1 to 3:1), to afford the product as a colorless oil. Yield: 19 mg, (66%). ¹H NMR (400 MHz, CDCl₃) δ 9.55 (s, 1H), 7.22–7.21 (m, 2H), 7.15–7.12 (m, 2H), 7.00 (s, 1H), 5.47–5.39 (m, 1H), 3.94 (s, 3H), 2.43–2.36 (m, 1H), 2.27–2.22 (m, 1H), 2.04–1.97 (m, 1H), 1.13–1.10 (m, 3H);

Visible peaks of minor diastereoisomer: 9.58 (s, 1H), 2.61–2.54 (m, 1H), 2.31–2.27 (m, 1H), 1.89–1.82 (m, 1H); ¹³C NMR (101 MHz, CDCl₃) δ 206.7, 206.6, 193.9, 193.8, 145.1, 145.0, 141.6, 141.0, 132.1, 132.0, 130.3, 130.2, 130.1, 128.5, 128.4, 125.7, 125.5, 48.0, 48.0, 47.0, 47.0, 38.8, 38.8, 36.0, 35.7, 16.4, 16.0. HRMS (ESI⁺) calculated for C₁₄H₁₈N₂O₂S (M+H⁺) = 277.1005, found = 277.1005. The ee of enzymatic reaction was determined by the conversion of aldehyde to corresponding ethyl acrylate via Wittig reaction with Ph₃PCHCO₂Et by HPLC analysis (Chiralpak-ADH, n-heptane:iPrOH 99:1, 0.5 ml/min). Retention times: major diastereomer, 111.8 min and 119.1 min; minor diastereomer, 132.7 min and 139.6 min. HRMS of the derivatization product by Wittig reaction (ESI⁺) calculated for C₁₈H₂₄N₂O₃S (M+H⁺) = 347.1424, found = 347.1420.
2-ethyl-4-(4-methoxyphenyl)-5-(1-methyl-1H-imidazol-2-yl)-5-oxopentanal (3e).

Purified by column chromatography (SiO$_2$, Heptane:EtOAc = 8:1 to 2:1), to afford the product as a colorless oil. Yield: 24 mg, (75%), 1:1 dr. For some protons and carbons there are two sets of proton or carbon signals in the NMR spectrum. $^1$H NMR (400 MHz, CDCl$_3$) δ 9.56 (s, 1H), 9.48 (s, 1H), 7.33–7.30 (m, 4H), 7.10 (s, 2H), 6.96 (s, 2H), 6.96–6.79 (m, 4H), 5.17–5.13 (m, 2H), 3.91 (s, 6H), 3.73 (s, 6H), 2.44–2.38 (m, 1H), 2.23–2.09 (m, 4H), 1.99–1.92 (m, 1H), 1.69–1.51 (m, 4H), 0.89–0.85 (m, 6H); $^{13}$C NMR (101 MHz, CDCl$_3$) δ 207.2, 207.1, 194.7, 194.6, 161.4, 161.3, 145.2, 133.2, 133.0, 132.4, 132.0, 130.1, 130.0, 116.7, 57.8, 53.8, 53.7, 51.9, 51.9, 38.8, 33.9, 33.7, 24.9, 24.3, 13.8, 13.7. HRMS (ESI$^+$) calculated for C$_{18}$H$_{24}$N$_2$O$_3$ (M+H$^+$) = 315.1703, found = 315.1705. The ee of the enzymatic reaction was determined by HPLC analysis (Chiralpak-ADH, n-heptane:iPrOH 99:1, 0.5 ml/min). Retention times: major diastereomer, 149.8 min and 160.2 min; minor diastereomer, 142.1 min and 184.5 min.
2-benzyl-4-(4-methoxyphenyl)-5-(1-methyl-1H-imidazol-2-yl)-5-oxopentanal (3f).

Purified by column chromatography (SiO₂, Heptane:EtOAc = 8:1 to 3:1), to afford the major isomer as a colorless oil. Yield: 30 mg, (81%), 1:1.2 dr. ¹H NMR (400 MHz, CDCl₃) δ 9.65 (s, 1H), 9.51 (s, 1H), 7.32–7.30 (m, 2H), 7.24–7.10 (m, 12H), 7.06–7.04 (m, 2H), 6.95 (s, 2H), 6.82–6.76 (m, 4H), 5.25–5.19 (m, 2H), 3.90 (s, 6H), 3.74 (s, 6H), 2.98–2.89 (m, 4H), 2.76–2.74 (m, 1H), 2.58–2.57 (m, 1H), 2.42–2.36 (m, 1H), 2.30–2.24 (m, 1H), 2.21–2.17 (m, 1H), 2.01–1.99 (m, 1H); ¹³C NMR (101 MHz, CDCl₃) δ 206.4, 206.4, 194.6, 194.4, 161.5, 161.4, 145.1, 141.0, 140.8, 132.8, 132.7, 132.5, 132.4, 132.0, 132.0, 131.9, 131.7, 131.6, 131.4, 131.1, 130.9, 130.7, 130.1, 130.0, 129.0, 129.0, 116.8, 116.7, 57.8, 57.8, 54.0, 53.7, 51.8, 38.8, 38.7, 38.1, 37.8, 34.2, 33.7; HRMS (ESI⁺) calculated for C₂₃H₂₆N₂O₃ (M+H⁺) = 377.1859, found = 377.1858.

The ee of enzymatic reaction was determined by the conversion of aldehyde to corresponding ethyl acrylate via Wittig reaction with Ph₃PCHCO₂Et by HPLC analysis (Chiralpak-ADH, n-heptane:iPrOH 98:2, 1.0 ml/min). Retention times: major diastereomer, 63.1 min and 72.9 min; minor diastereomer, 38.9 min and 55.7 min. HRMS of the derivatization product by Wittig reaction (ESI⁺) calculated for C₂₇H₃₂N₂O₄ (M+H⁺) = 447.2278, found = 447.2270.
2-benzyl-4-(4-chlorophenyl)-5-(1-methyl-1H-imidazol-2-yl)-5-oxopentanal \(^{(3g)}\). Purified by column chromatography (SiO\(_2\), Heptane:EtOAc = 8:1 to 3:1), to afford the major diastereoisomer as a colorless oil. Yield: 15 mg, (39%). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 9.56 (s, 1H), 7.33–7.31 (m, 2H), 7.26–7.17 (m, 5H), 7.12–7.10 (m, 3H), 6.98 (s, 1H), 5.27 (d, \(J = 12\) Hz, 1H), 3.91 (s, 3H), 2.97–2.84 (m, 2H), 2.59–2.55 (m, 1H), 2.24–2.20 (m, 2H); \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \(\delta\) 206.1, 193.7, 140.8, 139.6, 135.8, 132.7, 132.2, 131.6, 131.5, 131.2, 130.3, 129.1, 53.9, 51.9, 38.8, 37.8, 34.1. HRMS (ESI\(^+\)) calculated for C\(_{22}\)H\(_{23}\)ClN\(_2\)O\(_2\) (M+H\(^+\)) = 381.1364, found = 381.1368. The ee of the enzymatic reaction was determined by the conversion of aldehyde to corresponding ethyl acrylate via Wittig reaction with Ph\(_3\)PCHCO\(_2\)Et by HPLC analysis (Chiralpak-ADH, n-heptane:iPrOH 97:3, 0.5 ml/min). Retention times: major diastereomer, 60.1 min and 67.1 min; minor diastereomer, 70.8 min and 86.3 min. HRMS of the derivatization product by Wittig reaction (ESI\(^+\)) calculated for C\(_{26}\)H\(_{29}\)ClN\(_2\)O\(_3\) (M+H\(^+\)) = 451.1783, found = 451.1778.
**2-methyl-5-oxo-4-phenyl-5-(pyridin-2-yl)pentanal (5).** Purified by column chromatography (SiO₂, Heptane:EtOAc = 10:1 to 5:1), to afford the product as a light yellow oil. Yield: 22 mg, (82%). ¹H NMR (400 MHz, CDCl₃) δ 9.55 (s, 1H), 8.64–8.62 (m, 1H), 7.98–7.96 (m, 1H), 7.76–7.72 (m, 1H), 7.39–7.35 (m, 3H), 7.27–7.22 (m, 2H), 7.18–7.15 (m, 1H), 5.53–5.49 (m, 1H), 2.44–2.37 (m, 1H), 2.28–2.22 (m, 1H), 2.13–2.08 (m, 1H), 1.14 (m, 3H); Visible peaks of minor diastereoisomer: 9.62 (s, 1H), 2.62–2.56 (m, 1H), 2.32–2.28 (m, 1H), 1.95–1.90 (m, 1H), 1.10 (m, 3H); ¹³C NMR (101 MHz, CDCl₃) δ 207.0, 207.0, 203.2, 155.3, 151.5, 151.5, 141.1, 140.7, 139.5, 139.4, 131.6, 131.6, 131.3, 131.3, 129.8, 129.7, 129.7, 129.7, 125.4, 125.3, 50.7, 50.6, 47.0, 47.0, 36.3, 35.9, 16.6, 16.1. HRMS (ESI⁺) calculated for C₁₇H₁₉NO₂ (M+H⁺) = 268.1332, found = 268.1331. The ee of the enzymatic reaction was determined by HPLC analysis (Chiralpak-ADH, n-heptane:iPrOH 99:1, 0.5 ml/min). Retention times: major diastereomer, 41.8 min and 45.8 min; minor diastereomer, 49.8 min and 52.6 min.
4. Calibration Curves

The calibration curves were made by simulating the same reaction scale and solvents composition as the enzymatic reaction but with 0.2 to 1.0 mM corresponding product. The work up are the same as the enzyme catalyzed reactions.
3c \quad y = 0.0327x \quad R^2 = 0.9959

3d \quad y = 0.0409x \quad R^2 = 0.9707

3e \quad y = 0.0317x \quad R^2 = 0.9981
5. Sequence information

DNA Sequence of LmrR_V15pAF

ATGGGTGCCGAATCCCGAAAGAAATGCTGCTCAAAACCAATTTAGATCC
TGCTGAATGTCTCTGAAACAAGGGCGATAAACATATGTGATGGCATTATCAAACAG
GTGAAAGAAAGCCGAGCGAAGCTGAAATGGGAATCTGAAGATGACACCCCGGCTAT
ACGATTTTTTGATCGTCTGGAACAGGACGGCATTATCAGCTCTTACTGGGTTGA
TGAAAGTCAAGGCCTGCCTGCAAAATATATTACCGTCTGACCAGAAATCCCGGAT
GAAAACATGCGCCGCTGCCGTTCAATCTCTGGAGTTCGTGTGGAGCACAATATCTTG
AAAATCTGGAAGCAAACAAAAAATCTGAAGCGATCAAATCTAGAGGTGGCA
GCGGTGGCTGGAGGCCACCCGCAGTTCCGA AAAATAA

Amino acid sequence of LmrR_V15pAF

GAEIPKEMLRARQTNXILLNLKQGDNYVYGIIKQVKESN
GEMELNEATLYTIFDRLEQDGISSYWGDSEQGRKRKYR
LTEIGHENMLAFESWSRVDKIENLEANKKSARSGG
SGGSWSHPQFEK Stop

DNA Sequence of LmrR_V15pAF_M8L

ATGGGTGCCGAATCCCGAAAGAAACATGCTGCTCAAAACCAATTTAGATCC
TGCTGAATGTCTCTGAAACAAGGGCGATAAACATATGTGATGGCATTATCAAACAG
GTGAAAGAAAGCCGAGCGAAGCTGAAATGGGAATCTGAAGATGACACCCCGGCTAT
ACGATTTTTTGATCGTCTGGAACAGGACGGCATTATCAGCTCTTACTGGGTTGA
TGAAAGTCAAGGCCTGCCTGCAAAATATATTACCGTCTGACCAGAAATCCCGGAT
GAAAACATGCGCCGCTGCCGTTCAATCTCTGGAGTTCGTGTGGAGCACAATATCTTG
AAAATCTGGAAGCAAACAAAAAATCTGAAGCGATCAAATCTAGAGGTGGCA
GCGGTGGCTGGAGGCCACCCGCAGTTCCGA AAAATAA

Amino acid sequence of LmrR_V15pAF_M8L

GAEIPKELLRAQTNXILLNLKQGDNYVYGIIKQVKESN
GEMELNEATLYTIFDRLEQDGISSYWGDSEQGRKRKYR
LTEIGHENMLAFESWSRVDKIENLEANKKSARSGG
SGGSWSHPQFEK Stop
6. HPLC chromatograms

|                  | Rac-3a       |                      | Chiral-3a      |                      |       | ee (%) |
|------------------|--------------|---------------------|----------------|---------------------|-------|--------|
|                  | Ret. Time    | Area                | Ret. Time      | Area                | dr    |        |
| Diastereomer 1   | 75.7         | 1907284             | 75.8           | 247139              | 8:1   | 98     |
|                  | 80.9         | 1806707             | 82.5           | 22137249            |       |        |
| Diastereomer 2   | 73.5         | 2681546             | 74.1           | 524380              | 55    |        |
|                  | 90.5         | 2632089             | 90.3           | 1598465             |       |        |
Chiralpak-AD-H, n-heptane:iPrOH 99:1, 0.5 mL/min, 278 nm

|                | Ret. Time | Area     | Ret. Time | Area     | dr  | ee (%) |
|----------------|-----------|----------|-----------|----------|-----|--------|
| **Diastereomer 1** | 151.8     | 21225541 | 152.2     | 19830    | >20:1 | >99    |
|                | 167.4     | 22614321 | 167.6     | 30143232 |      |        |
| **Diastereomer 2** | 158.5     | 24676859 | 159.5     | 120481   | 82  |        |
|                | 198.8     | 24428814 | 199.6     | 1185660  |      |        |
### Chiralpak-AD-H, n-heptane/iPrOH 99:1, 0.5 mL/min, 282 nm

| Diastereomer 1 | 86.9 | 4578923 | 84.9 | 351544 | 8:1 | 98 |
|----------------|------|----------|------|--------|-----|-----|
| Diastereomer 2 | 99.8 | 4672052  | 97.5 | 34152416 |     |     |
|                | 93.1 | 4851048  | 90.7 | 1160863 |     | 50  |
|                | 119.4| 4984563  | 117.2| 3236190 |     |     |
Chiralpak-AD-H, n-heptane:PrOH 99:1, 0.5 mL/min, 280 nm

N=N\text{CH}_3

\text{S}

\text{Wittig product of 3d}

\begin{tabular}{|c|c|c|c|c|c|}
\hline
 & Ret. Time & Area & Ret. Time & Area & dr & ee (%) \\
\hline
\text{Rac-product} & & & & & & \\
\hline
Diastereomer 1 & 111.9 & 4815067 & 111.8 & 19016562 & 9:1 & 96 \\
 & 119.2 & 4616010 & 119.1 & 362588 & & \\
\hline
Diastereomer 2 & 132.1 & 9656250 & 132.7 & 1039924 & & \\
 & 139.6 & 9523909 & 139.6 & 1033347 & & \\
\hline
\end{tabular}

Wittig product of 3d
Chiralpak-AD-H, n-heptane/IPrOH 99:1, 0.5 mL/min, 278 nm

|         | Rac-3e | Chiral-3e |
|---------|--------|-----------|
|         | Ret. Time | Area | Ret. Time | Area | dr | ee (%) |
| Diastereomer 1 | 148.9 | 17943570 | 149.8 | 89735 | 9:1 | >99   |
|          | 159.0 | 17879259 | 160.2 | 47154876 |
| Diastereomer 2 | 141.1 | 18291092 | 142.1 | 1054450 | | 60 |
**Chiralpak-AD-H, n-heptane/iPrOH 98:2, 1.0 mL/min, 280 nm**

|                  | Ret. Time | Area     | Ret. Time | Area     | dr  | ee (%) |
|------------------|-----------|----------|-----------|----------|-----|--------|
| **Rac-product**  |           |          |           |          |     |        |
|                  |           |          |           |          |     |        |
| Diastereomer 1   | 38.9      | 10480115 |           |          |     | /      |
|                  | 55.7      | 10306548 |           |          |     |        |
| **Diastereomer 2** | 62.8  | 15271617 | 63.1      | 18482    | >20:1 | >99    |
|                  | 72.6      | 14938244 | 72.9      | 10182110 |     |        |
### Chiralpak-AD-H, n-heptane/iPrOH 97:3, 0.5 mL/min, 282 nm

| Diastereomer | Ret. Time (min) | Area (counts) | Ret. Time (min) | Area (counts) | dr | ee (%) |
|--------------|----------------|--------------|----------------|--------------|----|--------|
| **Diastereomer 1** | 61.4           | 26666434     | 60.1           | 39552        | >20:1 | >99    |
|               | 67.8           | 26638468     | 67.1           | 20291448     |     |        |
| **Diastereomer 2** | 71.8           | 17430537     | 70.8           | 401344       |     | 28     |
|               | 87.7           | 17477524     | 86.3           | 622195       |     |        |
### Chiralpak-AD-H, n-heptane/iPrOH 99:1, 0.5 mL/min, 263 nm

| Diastereomer | Ret. Time | Area   | Ret. Time | Area   | dr  | ee (%) |
|--------------|-----------|--------|-----------|--------|-----|--------|
| Diastereomer 1 | 42.0      | 14799929 | 41.8      | 7423964 | 5:1 | 70     |
|               | 46.0      | 15317279 | 45.8      | 1468504 |     |        |
| Diastereomer 2 | 49.9      | 14021460 | 49.8      | 426974  |     | 49     |
|               | 52.6      | 14564812 | 52.6      | 1199920 |     |        |
7. NMR spectra

![NMR Spectra Image]

$\text{3a}$
8. Supplementary references

1. Chin, J. W. et al. Addition of p-Azido- l-phenylalanine to the Genetic Code of Escherichia coli. *J. Am. Chem. Soc.* **124**, 9026–9027 (2002).

2. Bos, J., Browne, W. R., Driessen, A. J. M. & Roelfes, G. Supramolecular Assembly of Artificial Metalloenzymes Based on the Dimeric Protein LmrR as Promiscuous Scaffold. *J. Am. Chem. Soc.* **137**, 9796–9799 (2015).

3. Drienovská, I., Mayer, C., Dulson, C. & Roelfes, G. A designer enzyme for hydrazone and oxime formation featuring an unnatural catalytic aniline residue. *Nat. Chem.* **10**, 946–952 (2018).

4. Zhou, Z. & Roelfes, G. Synergistic catalysis in an artificial enzyme by simultaneous action of two abiological catalytic sites. *Nat. Catal.* **3**, 289–294 (2020).