Enantioselective Biocascade Catalysis With a Single Multifunctional Enzyme

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Abstract: Asymmetric catalytic cascade processes offer direct access to complex chiral molecules from simple substrates and in a single step. In biocatalysis, cascades are generally designed by combining multiple enzymes, each catalyzing individual steps of a sequence. Herein, we report a different strategy for biocascades based on a single multifunctional enzyme that can promote multiple stereoselective steps of a domino process by mastering distinct catalytic mechanisms of substrate activation in a sequential way. Specifically, we have used an engineered 4-oxalocrotonate tautomerase (4-OT) enzyme with the ability to form both enamines and iminium ions and combine their mechanisms of catalysis in a complex sequence. This approach allowed us to activate aldehydes and enals toward the synthesis of enantiopure cyclohexene carbaldehydes. The multifunctional 4-OT enzymes could promote both a two-component reaction and a triple cascade characterized by different mechanisms and activation sequences.

Introduction

Asymmetric cascade reactions are powerful synthetic tools for rapidly generating structural and stereochemical complexity.[1] In a single step and through the formation of multiple bonds, simple substrates are converted into complex products containing multiple stereocentres. Because of the synthetic benefits of cascade catalysis, chemists have recently focused on the design of increasingly sophisticated processes.[2] In biocatalysis, the combination of sequential enzymatic transformations to achieve cascade processes is a rapidly developing field.[3] Non-natural biocascades are generally designed by combining multiple enzymes, each catalyzing an individual step of a reaction sequence by means of a specific mechanism of substrate activation (Figure 1a, path *i*). Such multi-

enzyme systems have a remarkable synthetic potential,[4] but they are not simple to implement. Ensuring compatibilities in terms of reaction media and rates while achieving high selectivity often requires substantial protein engineering of each enzyme component.
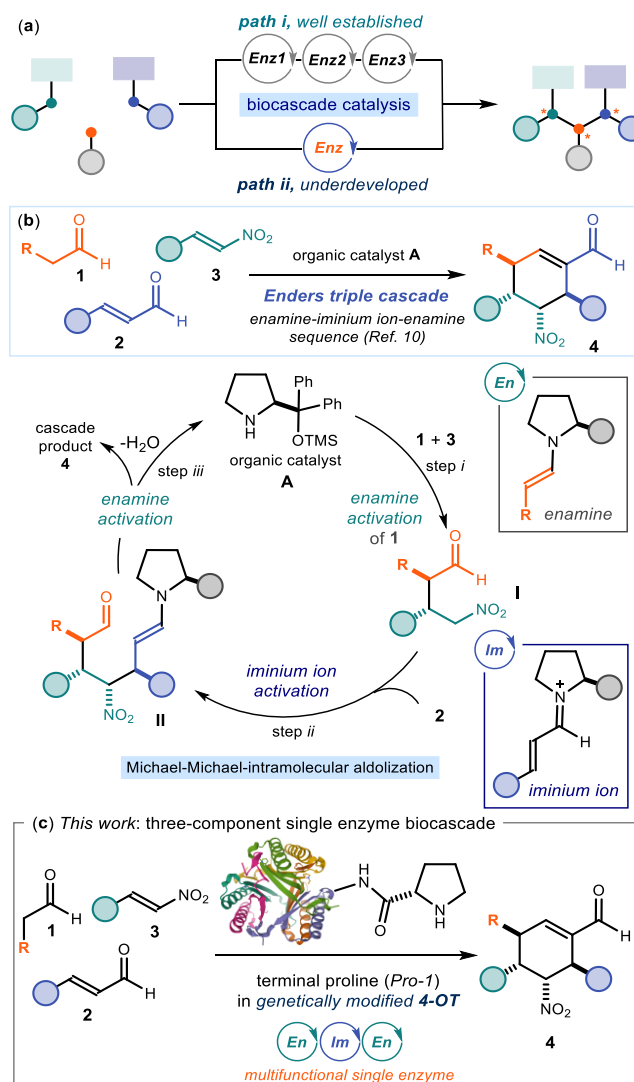

Figure 1. a) Established approach to design biocascade processes based on the combination of multiple enzymes (*path i*); the use of a single multifunctional enzyme that can master different catalytic mechanisms of substrate activation is far less developed (*path ii*). b) The triple organocascade developed by Dieter Enders, based on the ability of the amine catalyst **A** to promote an enamine-iminium ion-enamine activation sequence. c) Our approach exploits a genetically modified carboligase to promote complex biocascades, including the Enders triple cascade.

On these premises, developing biocascade processes based on a *single enzyme* that could couple simple substrates via multiple bond-forming events and control a variety of stereocentres would be highly desirable (Figure 1a, path *ii*). This one-pot, one-catalyst system would offer a more straightforward approach to biocascades, but it requires the identification of a *multifunctional* enzyme able to sequentially

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master orthogonal mechanisms of catalysis. So far, only few examples of single enzyme cascades have been reported,[5] the most remarkable based on a metagenomic imine reductase that could sequentially couple a conjugate reduction–reductive amination sequence to prepare enantiopure chiral amines.[6]

In this manuscript, we have used a class of multifunctional biocatalysts that can activate both aldehydes **1** and their α,β-unsaturated counterparts **2** using orthogonal catalytic mechanisms of substrate activation. The ability of this single enzyme to master a well-defined activation sequence enabled the development of two mechanistically distinct asymmetric biocascades. Our work was inspired by the successful applications of organocatalysis in cascade processes,[7] which led to highly efficient techniques for the one-step synthesis of stereochemically dense molecules, including natural products.[8] The success of organocascade catalysis relied on the ability of chiral secondary amine catalysts of type **A** to activate aldehydes **1** and enals **2** via enamine and iminium ion activation, respectively (Figure 1b).[9] One outstanding example was reported by the late Dieter Enders,[10] who demonstrated the ability of the amine catalyst **A** to realize an *enamine–iminium ion–enamine activation sequence*. The resulting three-component process (*Enders triple cascade*) proceeded by way of an enamine-catalyzed Michael addition of aldehyde **1** to nitroalkene **3** (step *i*) and subsequent iminium-ion-mediated Michael addition of the resulting nucleophilic intermediate **I** to enal **2** (step *ii*). A final enamine-catalyzed intramolecular aldol reaction within adduct **II**, followed by dehydration, afforded the complex cascade product **4** (step *iii*). The cyclohexene architecture within **4** was constructed with the creation of three bonds and four stereogenic centers in a single sequence.

Here, we demonstrate that biocatalysis can match and even surpass in efficiency the potential of organocascade catalysis (Figure 1c). Specifically, we used a genetically modified carboligase that can form both *enamine* and *iminium ion* intermediates.[11-13] The effective combination of these two mechanisms of catalysis allowed us to develop the enzymatic version of the Enders triple cascade along with a mechanistically different biocascade process.

Results and Discussion

Background and Design Plan. To successfully translate the catalytic machinery of organocascade catalysis into an enzymatic setting, we needed to identify a biocatalyst with the ability to readily form enamine and iminium ion intermediates from aldehyde substrates and repurpose them in polar pathways. We were inspired by recent works of Poelarends and co-workers, who demonstrated that natural 4-oxalocrotonate tautomerases (4-OT)[11] from *Pseudomonas putida* mt-2 (Pp-4OT) and their engineered variants could be used to successfully develop a variety of non-natural carbon-carbon bond-forming processes using the catalytic mechanisms of classical organocatalysis with high stereocontrol.[12,13] Importantly, the *N*-terminal Pro-1 residue, which is the main catalytic residue within the active site, secured the formation of both enamines and iminium ions. For example, 4-OT efficiently promoted, upon enamine formation from linear aliphatic aldehydes **1**, the asymmetric Michael addition to nitroalkenes **3** (Figure 2a).[12] The activation of enals **2** generated instead an electrophilic iminium ion, which could undergo stereoselective nucleophilic addition by nitromethane **5** (Figure 2b).[13] The catalytic activity and the stereoselectivity of 4-OT enzymes in these processes could be significantly improved by evolution campaigns,[12b,13] which demonstrated their mutational robustness. All these features, including the ability to bring two substrates in close proximity within their active sites, made 4-OT enzymes suitable starting points for our studies.
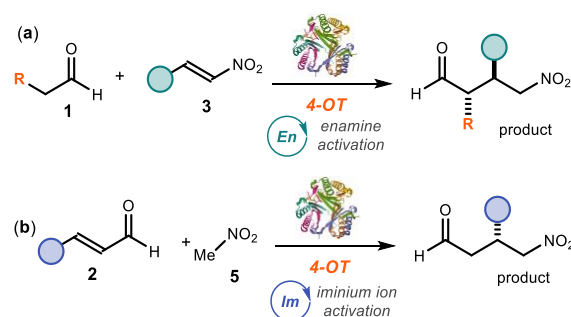

Figure 2. 4-Oxalocrotonate tautomerase (4-OT) in organocatalytic asymmetric processes. a) Enamine-mediated catalysis with aldehydes **1**. b) Iminium ion activation of enals **2**; note that both products correspond to intermediate **I** of the cascade in Figure 1b.

Model Reaction and Initial Studies. To test the feasibility of 4-OT enzymes to combine enamine and iminium ion activation in a single sequence and catalyze complex biocascade processes, we initially focused on a two-component reaction originally developed using organocatalyst **A** (Figure 3).[14] The cascade combines nitromethane **5** and enals **2** to afford *tri*-substituted cyclohexene carbaldehydes **4** bearing three stereogenic centers. The chemistry proceeds by means of the iminium-ion-mediated addition of **5** to enal **2** to form intermediate **I** (step *i* in Figure 3). The carbon α- to the nitro group of adduct **I** is characterized by a nucleophilic character, which can be leveraged to trigger a second Michael addition step to **2** via a second iminium ion activation (step *ii*). A final enamine-based intramolecular aldol condensation afforded product **4** (step *iii*). This two-component cascade was selected as the model reaction for different reasons: first, 4-OTs are known to promote the first Michael addition step leading to intermediate **I** upon iminium ion activation of enal **2**.[13] In addition, **I** is the same intermediate of the Enders triple cascade (see Figure 1b, where **I** is generated via enamine-mediated coupling of aldehyde **1** to nitroalkene **3**). In fact, the final part of the two-component cascade (the second iminium ion step followed by the aldol condensation, steps *ii* and *iii* respectively) follows the exact same mechanism of the more complex triple cascade, therefore offering a simplified testbed for our studies.

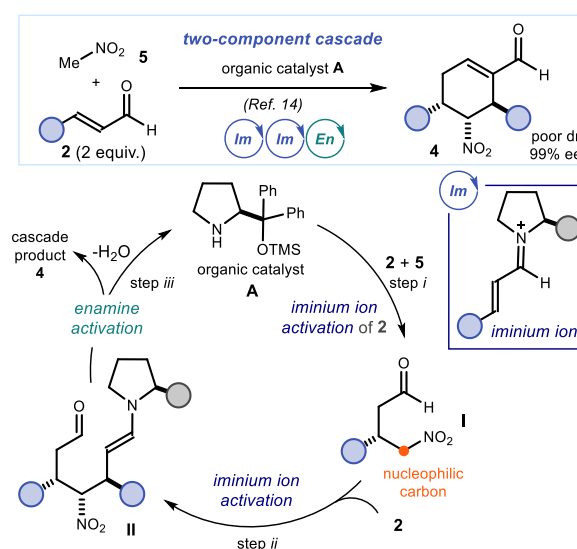*Figure 3*. The organocatalytic two-component cascade of enals **2** and nitromethane **5** via an iminium ion-iminium ion-enamine activation sequence that served as the model process, TMS: trimethylsilyl.
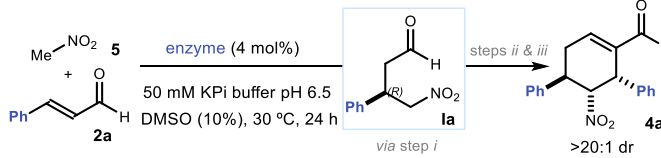
Identifying a single multifunctional enzyme capable of triggering the two-component process in Figure 3 would therefore offer a suitable ground to attack the real target of our studies, that is

developing the biocatalytic version of the Enders triple cascade. We therefore investigated the combination of nitromethane **5** and cinnamaldehyde **2a** as the model reaction (Table 1). We initially tested the activity of the wild type Pp-4OT (200 μM, 4 mol%, entry 1), which was incubated with cinnamaldehyde **2a** (5 mM) and **5** (50 mM) for 24 h in 50 mM potassium phosphate (KPi) buffer pH 6.5 containing 10% of DMSO at 30 ℃. While this enzyme could promote effectively the first iminium ion-based Michael addition step leading to adduct **Ia** with an *R* absolute configuration (44% analytical yield and 99% ee), we could detect only traces of the cascade product **4a**.

To increase the efficiency of the biocascade, we used PSI-BLAST,[15] an iterative method for protein sequence similarity search, to identify variants sharing >75% of sequence similarity with Pp-4OT. All the considered enzymes retained the *N*-terminal proline (Pro-1) because of this residue's critical catalytic role. A collection of 4-OT enzymes, including Ml-4OT from *Marinobacter lipolyticus* SM19 (85.7% sequence similarity with Pp-4OT), Tb-4OT from *Thiolapillus brandeum* (79.4% sequence similarity) and Ps-4OT from *Pseudomonas sagittaria* (93.7% sequence similarity), was prepared following reported procedures[16] (see section B in the Supporting Information (SI) for details). Activity and stereoselectivity tests revealed that these variants could only promote the first Michael addition step of the cascade leading to intermediate **Ia**, offering a reduced stereoselectivity than Pp-4OT (entries 2-4). We then focused on an engineered variant of deoxyribose-phosphate aldolase (DERA-MA), which was recently shown to outperform Pp-4OT in the iminium ion-mediated addition of **5** to **2a**.[17] We confirmed the ability of DERA-MA to produce intermediate **Ia** with excellent stereocontrol (99% ee, entry 5). However, the target cascade product **4a** was not formed at all.

To enable the cascade process, we sought to facilitate the second iminium ion-based Michael addition of intermediate **Ia** to **2a** by a relative increase of cinnamaldehyde stoichiometry, thus favoring iminium ion formation. The four 4-OTs (4 mol%, 200 μM) and DERA-MA enzymes were incubated with cinnamaldehyde **2a** (5 mM) and **5** (5 mM). Gratifyingly, Pp-4OT effectively promoted the biocascade leading to product **4a** in 49% yield and with high stereocontrol (>20:1 dr and 96% ee, entry 6). Also under these conditions, DERA-MA offered only traces of the cascade product **4a**, demonstrating the inability to further react intermediate **Ia** (entry 7). The other 4-OT-based enzymes also failed to promote the biocascade (entries 8-10). From a synthetic standpoint, it is important to note that the original organocatalytic method (Figure 3)[14] afforded product **4a** with a poor 1.8:1 dr (preference for the thermodynamic epimer). In contrast, the biocascade catalyzed by Pp-4OT offered the opposite diastereomer of **4a** with full diastereocontrol (epimer at the carbon bearing the nitro group, see section G in the SI for details). In addition, control experiments conducted without any enzyme did not result in any product formation. Collectively, these results highlight the ability of the enzyme to *govern each single step of the biocascade by combining iminium ion and enamine activation in a highly regulated fashion*. Specifically, Pp-4OT, acting as a multifunctional enzyme, can drive the first Michael addition step to then accommodate in its active site both the resulting intermediate **Ia** and a new molecule of cinnamaldehyde **2a** to master a second iminium-ion-mediated step. We also performed deuterium labelling experiments, detailed in section H1 of the SI, which proved that also the last enamine-mediated aldol cyclization is governed by the enzyme.

Table 1. Identification of a multifunctional catalyst that can drive a two-component biocascade and optimization studies.[a]
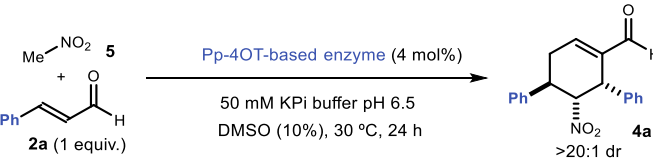

entry	enzyme	equiv. **2a**	equiv. **5**	**Ia** %yield / %ee[b]	**4a** yield [%][c]
1	Pp-4-OT	1	10	44 / 99	<5
2	Ml-4-OT	1	10	41 / 12	0
3	Tb-4-OT	1	10	27 / 22	0
4	Ps-4-OT	1	10	38 / 37	0
5	DERA-MA	1	10	50 / 99	0
6	Pp-4-OT	1	1	13 / 99	49 (96% ee)
7	DERA-MA	1	1	38 / 99	7[d]
8	Ml-4-OT	1	1	12 / 12	6[d]
9	Tb-4-OT	1	1	18 / 22	6[d]
10	Ps-4-OT	1	1	22 / 37	7[d]

[a] Reactions on a 2.5 μmol scale (5 mM) at 30 °C. Analytical yields of **Ia** and **4a** determined by GC-FID analysis (calibration using mesitylene as internal standard) and by HPLC analysis (calibration using 1,3,5-methoxybenzene as internal standard). [b] Ee value of **Ia** determined by UPC^2 analysis using a chiral IG-3 column. [c] Dr and ee for the cascade product **4a** measured by HPLC analysis. [d] Dr and ee not determined.

Based on these results, we selected Pp-4OT for further optimization studies.

Construction of Fused 4-OTs with Internal His-Tag. With the aim to simplify the preparation and purification of Pp-4OT, we sought to include a polyhistidine-tag (His-Tag) in the aminoacid sequence. Previous studies indicated that His-Tag incorporation to the C-terminus of 4-OT enzymes was detrimental for their catalytic activity.[18] We therefore aimed to include the His-Tag in the internal sequence. 4-OT, which belongs to the tautomerase superfamily, is composed of six short monomers of 62 amino acid residues each. The enzyme consists of a trimeric arrangement of interacting pairs of monomers. To expand the space available for genetic manipulations and internal His-Tag insertion, we first created a tandem-fused 4-OT by fusing the *C*-terminus of the first monomer with the *N*-terminus of the second monomer. The idea of a fused enzyme was informed by recent studies by Poelarends[19] and the occurrence of the naturally fused 4-OT from *Burkholderia lata*,[20] which shares 59% sequence similarity with Pp-4OT. The existence of this structural arrangement suggests that fusion events were precedented in the evolutionary history of the tautomerase superfamily. We therefore inserted the linker sequence *GAGGSL* at the *C*-terminus of Pp-4OT. This flexible glycine-rich linker consisting of six amino acid residues is similar to the *GAPPSL* linker found in the naturally fused enzyme. We substituted the proline residues with glycines since they offer increased flexibility when constructing artificial fusions.[21] We also removed the last three residues (*VRR*) in the sequence of Pp-4OT to further increase the flexibility of the linker while matching the length of the naturally fused 4-OT from *Burkholderia lata* (127 aa). The resulting Pp-4OT-F1 variant was then tested in the model cascade process (Table 2). When incubated with 5 mM of **1** and 5 mM of **2a** in 50 mM KPi pH 6.5 for 24h at 30℃, this fused enzyme afforded diastereomerically and enantiomerically pure product **4a** in slightly increased yield (55% yield) than the Pp-4OT progenitor (compare entries 1 and 2).

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Table 2. Further optimization of Pp-4OT-based enzymes.[a]

entry	enzyme	deviation	4a % yield	4a % ee
1	Pp-4-OT	none	49	96
2	Pp-4-OT-F$_1$	none	55	99
3	Pp-4-OT-F$_2$	none	8	n.d.
4	Pp-4-OT-F$_3$	none	57	99
5	Pp-4-OT-F$_3$	3 equiv. of **2a**	80	99
6	Pp-4-OT-F$_3$	3 equiv. of **2a**, EtOH instead of DMSO	75	99
7	Pp-4-OT-F$_3$	3 equiv. of **2a**, DMF instead of DMSO	80	99

[a] Reactions performed on a 2.5 µmol scale (5 mM) at 30 °C using 4-OT enzymes (4 mol %, 200 µM) and 1 equiv. of **5** and **2a**. Analytical yields and stereoselectivity (dr and ee) of **4a** determined by HPLC analysis on a chiral column (calibration using 1,3,5-methoxybenzene as internal standard); n.d., not determined.

Since the fused variant Pp-4OT-F1 was fully functional, we used homology models to visualize the enzyme's structure and better evaluate where to include the His-Tag (see section H3, Figure S14 in the SI for details). We targeted loop regions of the enzyme exposed to the aqueous environment. In particular, we constructed Pp-4OT-F2, which carries a His-Tag close to the newly formed linker (at positions 57-62); Pp-4OT-F3, containing an His-Tag at positions 12-17; and Pp-4OT-F4, which carries a *C*-terminal His-Tag at positions 130-135. All fused His-tagged Pp-4OTs were purified in one step using Nickel-NTA columns with yields of 45-50 mg gr^{-1} of cell pellet (see Figure S1 in the SI for purity of all fused variants and Table S1 for the aminoacid sequences). The fused enzymes were then tested for their ability to promote the two-component cascade. Pp-4OT-F2 led to product **4a** in a yield as low as 8% yield (Table 2, entry 3). Notably, Pp-4OT-F3 offered an increased 57% yield with perfect stereoselectivity (dr >20:1, >99% ee, entry 4). The Pp-4OT-F4 enzyme carrying a *C*-terminal His-Tag immediately precipitated after purification and we could not use it in any biotransformation. Since Pp-4OT-F3 offered the best results, we ran a final cycle of optimization to find that an increased amount of **2a** (3 equiv.) led to the formation of the cascade product **4a** in 80% yield and with total stereocontrol (entry 5). We also established that other co-solvents than DMSO, including ethanol (EtOH) or dimethylformamide (DMF), could be used without affecting the efficiency of the biocascade (entries 6-7).

Figure 4 offers a visual comparison of the three-dimensional arrangement of the natural Pp-4OT and the fused Pp-4OT-F3 containing a His-Tag, which was selected as the best performing multifunctional enzyme. The crystal structure of the wild type Pp-4OT (Figure 4a, PDB: 4x19) shows the homohexameric structure of the enzyme and the three pairs of monomers. Each monomer (blue or light green in Figure 4a) is organized as a β1-α-β2 unit. To identify the main structural differences, we created a homology model of Pp-4OT-F3 (Figure 4b), which suggested that the His-Tag is located at the loop connecting the β1-α1 of each of the β1-α-β2 monomeric units (Figure 4b, yellow). The His-Tag is therefore far from the catalytically active Pro-1 residue (in magenta). Another structural feature of Pp-4OT-F3 is a new loop created by the linker peptide (in red), which is located after the first β1-α-β2 monomer (in blue) and connects it with the second monomer (in light green).
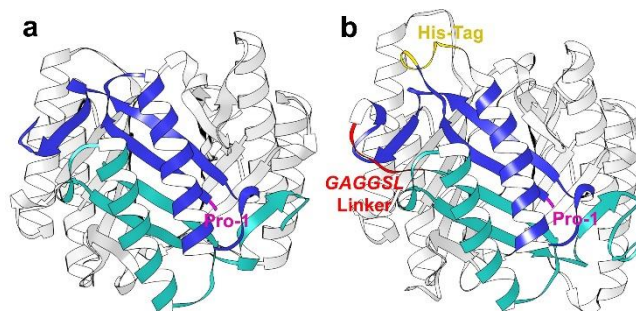

Figure 4. a) Crystal structure of the wild type Pp-4OT (PDB: 4X19) in which two β$_1$-α-β$_2$ monomers (blue and light green) form a dimer. A trimeric arrangement of dimers results in the homohexameric structure. b) Homology model of Pp-4OT-F3 in which the His-Tag (yellow, positions 12-17) is incorporated in the first loop between β$_1$-α of the β$_1$-α-β$_2$ monomer. The linker *GAGGSL* (red, positions 67-72) connects the two monomers. The catalytic Pro-1 residue is shown in magenta. Homology model created with YASARA structure; UCSF Chimera software was used for visualization.

Using the optimized conditions specified in entry 5 of Table 2, we examined the substrate tolerance of the fused Pp-4OT-F3 multifunctional enzyme (Figure 5). Various *ortho*, *meta*, and *para*-substituted cinnamaldehydes **2**, adorned with electron-rich and electron-poor substituents, were accepted in the active site, leading to the corresponding cascade products (**4a**-**4h**) with high yields (44-89%), good to excellent diastereoselectivity (dr ranging from 3:1 and >20:1) and complete enantioselectivity in all cases. The best accepted substrates were the *para*-fluoro- and *meta*-methoxy-cinnamaldehydes, which afforded products **4b** and **4c** in 89% and 88% yield, respectively, with complete stereoselection. When using a *meta*-substituted cinnamaldehyde, we could improve the yield of product **4g** up to 84% using DMF as co-solvent. *Ortho*-substituted cinnamaldehydes afforded products **4d** and **4f** in 63% and 44% yield, respectively. Finally, the Pp-4OT-F3 also accepted an heteroaromatic α,β-unsaturated aldehyde, but affording product **4i** in low yield (20%) and diastereoselectivity (3:1). Aliphatic enals (e.g. octenal) were unreactive under the optimal conditions.

The synthesis of the cascade products **4** could be performed on a semi-preparative scale using a 30 mL reaction volume (150 µmol of substrate **2**) and cell-free-extracts (50 mg of enzyme, 2.5 mg$_{enzyme}$ mL^{-1}). This procedure did not require any enzyme purification thus simplifying the process. We could isolate the cascade products **4a** and **4b** as single stereoisomers in 61% yield (28 mg) and 88% yield (46 mg), respectively, after purification by column chromatography. Crystallization of the isolated product **4f** allowed us to unambiguously infer the relative and absolute configuration of the major diastereomer by X-ray crystallographic analysis.[22] NMR conformational studies established the identity of the minor diastereomer of **4f** as the epimer with different configuration at the carbon bearing the nitro group (see section G in the SI for details).

All the cascade products **4** prepared so far have a 'symmetric' structure since they feature the same substituents at the C4 and C6 of the cyclohexene ring, arising from the enal substrate **2**. Differentiating the substituents within **4** would require the ability to selectively activate two different enals **2** in each of the two sequential iminium ion steps of the cascade. This target was not achievable using organocatalysis (Figure 3),[14] since there was no possibility for the chiral amine catalyst **A** to differentiate and specifically regulate the different steps of the cascade sequence. We explored if biocatalysis could offer a way to address this limitation, allowing for the preparation of non-symmetric products **4** with different substituents at C4 and C6. To achieve this target, we planned to separate in time

the initial two biocatalytic iminium ion steps by using two different enzymes working in sequence (Figure 5, route B).
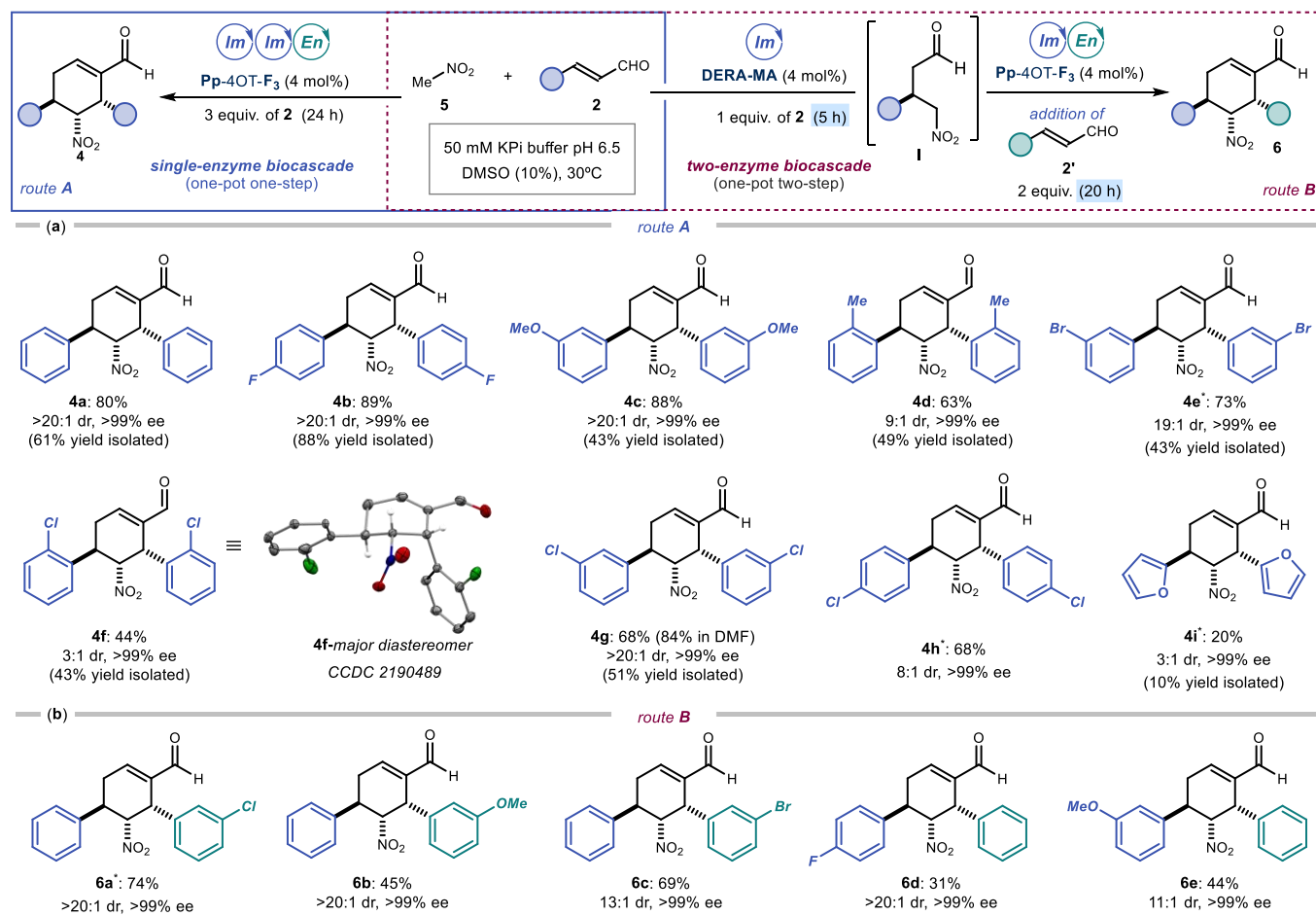

Figure 5. Biocatalytic two-component cascade reactions. a) Survey of the enals **2** that can participate in the two-component one-step process: reactions performed using Pp-4OT-F3 (4 mol%) on a 2.50 μmol scale of nitromethane **5** (5 mM) in phosphate buffer at pH 6.5 in 10% of DMSO. The reactions were run for 24 hours at 30 °C in a thermoshaker equipped with temperature control. Yields are given below each entry (average of two runs) as analytical yields (measured by HPLC analysis using 1,3,5-trimethoxybenzene as the internal standard). Yields in parenthesis refer to isolated products **4** obtained from 30 mL reactions with cell-free-extract containing Pp-4OT-F3 (2.5 mg mL⁻¹) on a 150 μmol scale; the dr and ee were measured via chiral HPLC analysis. b) Sequential one-pot two-enzyme cascade to prepare the non-symmetrical products **6**; reactions performed on a 2.50 μmol scale of **5** (5 mM) in two steps; *first step* performed in the presence of DERA-MA (4 mol%), enal **2** (5 mM) and **5** (5 mM) in phosphate buffer at pH 6.5 in 10% of DMSO for 5 h; *second step* performed by adding in situ 2 equivalents of a different enal **2'** (10 mM) together with Pp-4OT-F3 (4 mol%) and run for additional 20h. *Reactions run in DMF as co-solvent.

For this to be possible, a first enzyme should promote the addition of nitromethane **5** to an enal **2** and form intermediate **I**, but then stop at this first step of the biocascade. The judicious addition of the multifunctional Pp-4OT-F3 could then complete the cascade by accepting **I** as the substrate while promoting the second round of iminium ion catalysis with a different, freshly added enal **2'**. The final intramolecular aldol condensation under enamine activation would lead to the non-symmetric tri-substituted cyclohexene carbaldehyde products. This idea was based on our early observation that DERA-MA was a suitable catalyst only for the iminium-ion-mediated addition of **5** to cinnamaldehyde **2a** to afford intermediate **Ia**, without being able to form the cascade product **4a** (see entry 5 in Table 1). This sequential enzymatic cascade was successfully implemented by combining the action of DERA-MA and Pp-4OT-F3, both of them used at 4 mol% (Figure 5b). Once DERA-MA selectively promoted the formation of intermediate **I** by activating enal **2**, the sequential addition of Pp-4OT-F3 and a different enal **2'** led to the target non-symmetric cascade products **6**, bearing different substituents at C4 and C6, with moderate to good yields and almost complete stereocontrol. These results highlighted how *biocatalysis could enable cascade processes not achievable using other strategies*, including organocatalysis.

Developing the Biocatalytic Version of the Enders Triple Cascade. We then sought to evaluate the real potential of our multifunctional enzymes to promote mechanistically more complex biocascades. The Enders triple cascade discussed in Figure 1b offered a suitable testbed. This process is considered the most sophisticated and spectacular demonstration of the potential of organocatalysis to engineer complex cascades for the generation of stereochemical and molecular complexity in one single step from readily available substrates.[10] We reasoned that the Pp-4OT-F3 enzyme, which served as suitable catalyst of the bifunctional biocascade discussed in Figure 5, could also promote the three-component process of acetaldehyde **1a**, nitrostyrene **3a**, and cinnamaldehyde **2a** proceeding by way of an enamine-iminium ion-enamine sequence (Figure 6a). Our motivation arose from previous studies showing that 4-OT-based enzymes could promote the enamine-catalyzed Michael addition of acetaldehyde to **3a**,[12] which is the first step of the triple cascade. In addition, we recognized the ensuing product **Ia** as the same intermediate generated in the first step of the two-component cascade in Figure 5. We therefore expected intermediate **Ia** to follow the same iminium ion-enamine sequence as above, thus leading to product **4a** from different starting substrates. We confirmed that Pp-4OT-F3 could promote the enamine-driven addition of **1a** to **3a** leading to **Ia** (Figure 6b).

Surprisingly, however, we did not observe the formation of the cascade product **4a** when repeating the reaction in the presence of cinnamaldehyde **2a** (Figure 6c), which indicated that Pp-4OT-F3 was unable to promote the triple biocascade.
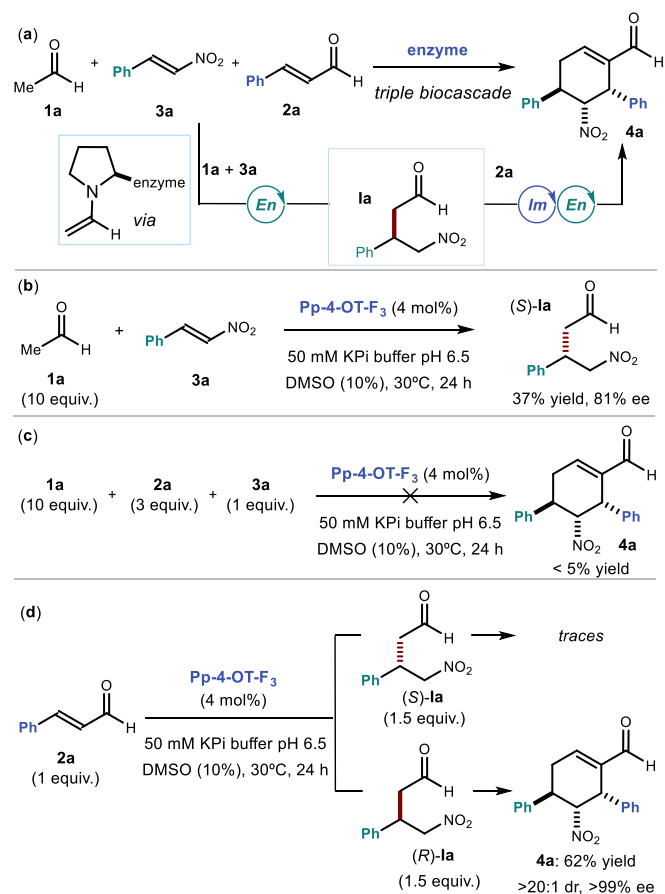

Figure 6. a) The biocatalytic version of the Enders triple cascade. b) Pp-4OT-F3 in the first enamine step of the triple cascade. c) Pp-4OT-F3 failed to promote the whole triple cascade. d) Strong matched-mismatched effect in the second iminium ion step of the cascade: Pp-4OT-F3 can only accept intermediate **Ia** with *R* absolute configuration.

Puzzled by this lack of reactivity, we looked for a possible explanation. We noticed that the Pp-4OT-catalyzed addition of **1a** to **3a** led to intermediate **Ia** with an (*S*) absolute configuration (Figure 6b), while the iminium-ion-based reaction between cinnamaldehyde **2a** and nitromethane **5** forged the opposite (*R*) enantiomer of the same intermediate **Ia** (see results in Table 1). We therefore wondered if the exceptional selectivity generally inherent to enzymatic catalysis could account for the inability of Pp-4OT to drive the triple cascade because of an extreme case of matched-mismatched effect.[23] In this scenario, the (*S*) enantiomer of intermediate **Ia** would not be accommodated by the enzyme in the position necessary for addition to the iminium ion, while only (*R*)-**Ia** would be accommodated productively in the active site. To probe this possibility, we prepared authentic samples of enantiopure (*S*) and (*R*) products **Ia**, which were then subjected to the second part of the cascade in the presence of cinnamaldehyde **2a** (Figure 6d). These experiments confirmed that Pp-4OT-F3 could accept readily (*R*)-**Ia**, leading to the cascade product **4a** in 62 % yield with full stereoselectivity. In contrast, the same biocatalytic reaction with (*S*)-**Ia** afforded only traces of **4a** (<5 %). Control reactions in the absence of the enzyme using (*R*)-**Ia** resulted in traces of product **3a** (<5 %), indicating that the enzyme is needed to catalyze the cascade.

To rationalize the outstanding selectivity of Pp-4OT-F3 for (*R*)-**Ia**, we docked both enantiomers of this pro-nucleophilic intermediate into the active site of the enzyme (Figure 7). We considered the enzyme in its reactive iminium ion form containing cinnamaldehyde **2a** bound to the Pro-1. Results showed that two binding poses are possible for both (*R*)-**Ia** and (*S*)-**Ia** (see section H3 in SI for more details). For a productive binding pose, an optimal distance between the reactive carbon of **Ia** and the β-carbon of the iminium ion should be around 3.6 Å (the sum of the Van der Waals radii).[24] For (*S*)-**Ia**, all the identified binding poses showed a distance above 6 Å, indicating a non-productive interaction of this enantiomer (Figures 7b and S15). In contrast, the binding poses of (*R*)-**Ia** established a distance (3.4 Å) suitable for a productive reaction (Figures 7a and S15).
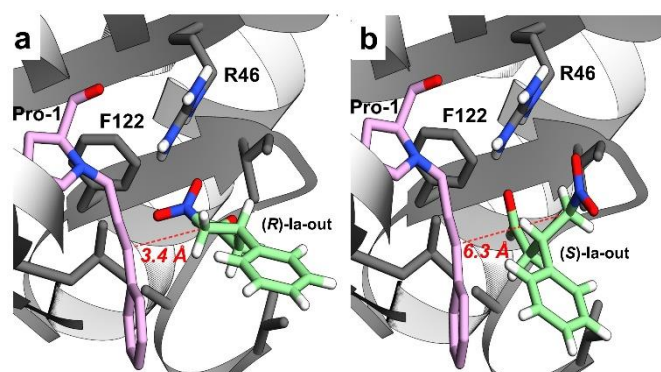

Figure 7. a) Docking of (*R*)-**Ia** in the active site of Pp-4OT-F3: binding energy: 4.00 Kcal mol⁻¹. b) Docking of (*S*)-**Ia** in the active site of Pp-4OT-F3: binding energy: 4.31 Kcal mol⁻¹. The iminium ion formed between Pro-1 and **2a** is shown in purple while intermediate **Ia** is in green. The dashed red line shows the distance between the two reactive carbons for C-C bond formation. R46 is shown because it stabilizes **Ia** in the active site of the enzymes. For simplicity the ribbon of Pro-1 is not shown. All docking simulations performed with YASARA structure. UCSF Chimera software was used for visualization.

Understanding the reasons behind the lack of reactivity of the triple cascade catalyzed by Pp-4OT-F3 allowed us to identify a solution. We thought that we could enable the process by identifying an enzyme that could promote the first enamine-based addition of acetaldehyde **1a** to nitrostyrene **3a** leading to product **Ia** with *R* selectivity. If the enzyme could then retain this intermediate (*R*)-**Ia** in its active site, it could trigger the second step of the triple cascade upon iminium ion activation of cinnamaldehyde **2a**. This idea was translated into experimental reality by using the enzyme Pp-4OT-F3^M117Y/F122A. We created this variant of Pp-4OT-F3 by introducing the mutations M117Y and F122A. The design of this enzyme was motivated by previous studies showing how similar mutations could induce the wild type Pp-4OT to catalyze the enamine-based addition of **1a** to **3a** with *R* selectivity.[12b] Gratifyingly, also Pp-4OT-F3^M117Y/F122A could promote this reaction leading to (*R*)-**Ia** in 85% ee (Figure 8a). More importantly, the new enzyme could effectively promote the Enders triple cascade leading to product **4a** in 28% yield and full stereocontrol (Figure 8b). Using butanal as the aldehyde component, the cascade product **7a** was obtained in higher yield (50%). The efficiency of the biocascade could be further increased using a higher amount of enzyme (6 mol %, **7a** formed in 64% yield). We then established that the enzyme could also tolerate substituted cinnamaldehydes **2** and nitroalkenes **3**, catalyzing the triple biocascade to afford the corresponding products **7b** and **7c** in yield and stereoselectivity comparable with the original organocatalytic system.[10]
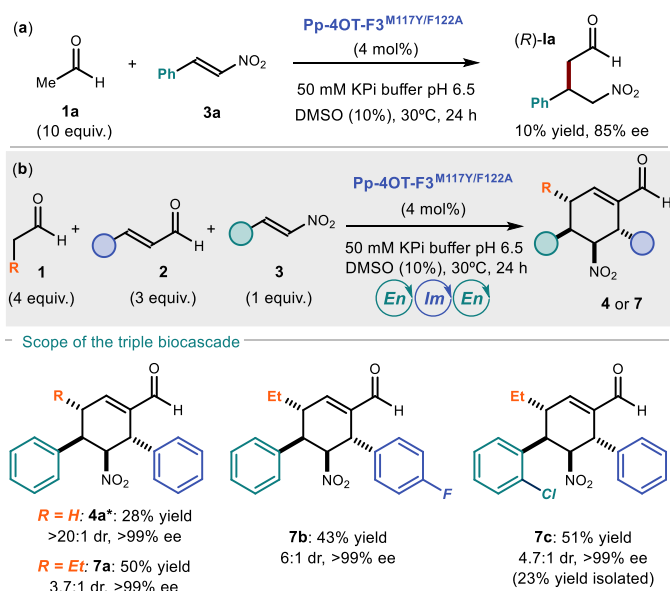
Figure 8. Developing the triple biocascade using a single multifunctional enzyme. a) Pp-4OT-F3[M117Y/F122A] in the first enamine step of the triple cascade. b) Scope of the triple biocascade catalyzed by the new variant Pp-4OT-F3[M117Y/F122A]: reactions performed on a 1.25 μmol scale of nitrostyrene **3a** (2.5 mM reaction concentration) for 24 hours at 30˚C in a thermoshaker equipped with temperature control. Yields are given as an average of two runs and determined by HPLC analysis using 1,3,5-trimethoxybenzene as the internal standard. The dr and ee were measured via chiral HPLC or UPC^2 analysis. The yield in parenthesis refers to the isolated product for the reaction on a 150 μmol scale. *Reaction run with 6 eq. of **1a**; product **4a** has an opposite relative configuration than products **7** at the carbon bearing the nitro group (**4a** same configuration as in Figure 5, see SI for details).

Conclusions

In summary, we have described the use of multifunctional enzymes that can promote two distinct biocascade processes characterized by different reaction sequences. The reported carboligases can form both enamine and iminium ion intermediates and combine them in well-defined sequences to activate aldehydes and enals toward the formation of enantiopure cyclohexene carbaldehydes bearing multiple stereogenic centers. We used the multifunctional enzymes to promote a two-component reaction of enals and nitromethane proceeding via an iminium ion–iminium ion–enamine activation sequence. This cascade offered an entry into thermodynamically unfavored trisubstituted products that could not be synthesized using established organocatalytic methods. In addition, we designed a tandem sequential process combining the action of two enzymes to prepare previously unattainable non-symmetric cyclohexene carbaldehydes. By engineering the active site of the enzyme, we could also develop a three-component cascade leading to tetra-substituted cyclic products. In this case, the enzyme mastered a different reaction sequence proceeding via enamine–iminium ion–enamine activation.

Overall, this study offers a rare example of a multifunctional biocatalyst that can promote multiple steps of a cascade in a stereoselective and highly regulated fashion. Our results highlight that biocatalytic strategies can match and even surpass in efficiency the potential of organocatalytic cascade catalysis. In addition, we have reported and characterized a fully functional genetically fused 4-OT enzyme bearing an internal His-Tag, which greatly simplified purification while setting the grounds for possible enzyme immobilization.[25]

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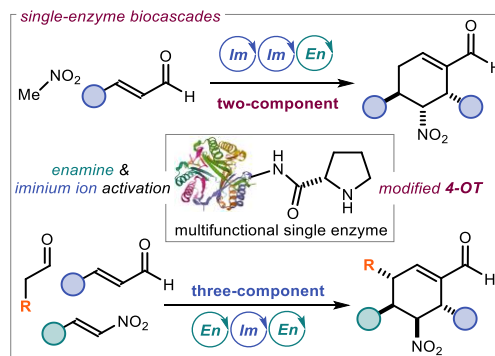
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Biocatalysis

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Enantioselective Biocascade Catalysis With a Single Multifunctional Enzyme

We report a single multifunctional enzyme that can promote biocatalytic cascades based on multiple stereoselective steps. Specifically, a 4-oxalocrotonate tautomerase (4-OT) enzyme can form enamine and iminium ion intermediates from aldehydes and enals to promote both a two-component reaction and a triple cascade characterized by different mechanisms and activation sequences.

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