Enzyme Catalysis

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Bridging between Organocatalysis and Biocatalysis: Asymmetric Addition of Acetaldehyde to β-Nitrostyrenes Catalyzed by a Promiscuous Proline-Based Tautomerase**

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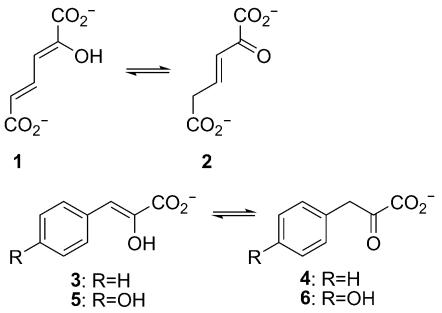
In recent years, organocatalysis has become one of the main areas in asymmetric catalysis of carbon–carbon bond-forming reactions.[1] The fast evolution of the organocatalysis field has been particularly fueled by aminocatalysis, in which secondary and primary amines react with carbonyl compounds to give enamine and iminium ion intermediates. The field was completely transformed during the last two decades by the seminal contributions of List,[2] MacMillan,[3] Yamaguchi,[4] and co-workers. The natural chiral amino acid proline and derivatives thereof were found to be powerful organocatalysts. These secondary amines are applied in substoichiometric quantities and afford high product yields and enantioselectivities in fundamental carbon–carbon bond-forming reactions such as aldolizations,[1,2,3b] Michael additions,[1,4,5] Mannich reactions,[1,6] and Knoevenagel condensations.[1,7]

Inspired by the versatile success of proline and its derivatives as organocatalysts, we examined whether the enzyme 4-oxalocrotonate tautomerase (4-OT),[8] which carries a catalytic amino-terminal proline (Pro = P), might be suitable to promiscuously catalyze carbon–carbon bond-forming reactions. Herein, we describe the discovery and characterization of two 4-OT-catalyzed asymmetric carbon–carbon bond-forming Michael-type addition reactions. Considering our reported 4-OT-catalyzed aldolizations,[9] this work is a pivotal step forward towards our aim to bridge organocatalysis and biocatalysis by developing a new class of biocatalysts that use the powerful proline-based enamine mechanism of organocatalysts[1] but that take advantage of the water solubility and relatively high catalytic rates available with enzymes. A few elegant studies on promiscuous enzyme-catalyzed carbon–carbon bond-forming Michael additions have been reported, but most of these reactions proceed in organic solvents and with moderate stereocontrol.[10]

4-OT is a stable enzyme composed of six identical subunits of only 62 amino acid residues each.[11] It belongs to the tautomerase superfamily, a group of homologous proteins that share a conserved catalytic amino-terminal proline and a characteristic β-α-β structural fold.[8,12] 4-OT takes part in a degradation pathway for aromatic hydrocarbons in *Pseudomonas putida* mt-2, where it catalyzes the tautomerization of 2-hydroxy-2,4-hexadienedioate (**1**) into 2-oxo-3-hexenedioate (**2**, Scheme 1).[13] The key catalytic residues of 4-OT are Pro-1,



Scheme 1. Natural tautomerization reactions catalyzed by 4-OT.

Arg-11, and Arg-39 (Arg = R = arginine). Residue Pro-1 functions as a general base ($pK_a \approx 6.4$) that transfers the 2-hydroxy proton of **1** to the C5-position to give **2**.[14] Residues Arg-11 and Arg-39 are important for binding of **1** and interact with the C6 and C1 carboxylate groups of **1**, respectively.[15] 4-OT also accepts phenylenolpyruvate (**3**) and *p*-hydroxyphenylenolpyruvate (**5**) as substrates for tautomerization, giving phenylpyruvate (**4**) and *p*-hydroxyphenylpyruvate (**6**) as products, respectively (Scheme 1).[16]

To investigate whether 4-OT exhibits promiscuous carbon–carbon bond-forming activities, the Michael-type addition of acetaldehyde (**7**) to *trans*-nitrostyrene (**8**) and *p*-hydroxy-*trans*-nitrostyrene (**10**) were selected as model reactions (Scheme 2) for a number of reasons. First, previous labeling experiments have indicated that residue Pro-1 of 4-OT rapidly attacks the carbonyl carbon atom of **7**.[9] The resulting enamine has nucleophilic character and may act as a donor. Second, the possible acceptors **8** and **10** show structural resemblance to compounds **3** and **5** (Scheme 1), two known substrates of 4-OT.[14,16] Third, the presumed product of the reaction between **7** and **8** is 4-nitro-3-phenyl-

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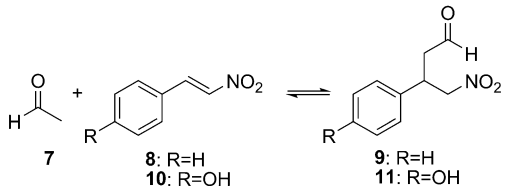

Scheme 2. Non-natural Michael-type addition reactions catalyzed by 4-OT.

butanal (**9**), an important precursor for the antidepressant phenibut (4-amino-3-phenylbutanoic acid).[5c,d]

The Michael-type addition reaction of **7** and **8** (λ_{max} = 320 nm) was monitored by UV spectroscopy. Incubation of 4-OT (73 μM) with **7** (50 mM) and **8** (1.3 mM) resulted in a decrease in the absorbance at 320 nm (A_{320}), thus indicating the disappearance of **8** (Figure 1a and Figure S1A in the Supporting Information). To ensure that the depletion of **8** is enzyme-catalyzed, **7** and **8** were also incubated without 4-OT. This experiment did not result in a similar decrease in the A_{320} value (Figure 1b and Figure S1A in the Supporting Information) as observed for the incubation with 4-OT. The 4-OT sample used in the assay was highly purified. To eliminate the concern that a contaminating enzyme from the expression strain could be responsible for the observed activity, a 4-OT sample free of cellular enzymes was prepared by total chemical synthesis.[17] Indeed, incubation of **7** and **8** with chemically synthesized 4-OT resulted in the same decrease in the A_{320} value as observed for the incubation with recombinant 4-OT (Figure 1c and Figure S1A in the Supporting Information). To exclude the possibility of nucleophilic addition of water or ethanol, present as main solvent and cosolvent, respectively, to **8**, 4-OT was incubated with **8** in the absence of **7**. The small decrease in the A_{320} value over time (Figure 1d) was similar to that observed for the incubation of **7** and **8** without enzyme (Figure 1b), thus demonstrating that 4-OT does not catalyze the addition of water or ethanol to **8** but solely catalyzes the addition of **7** to **8**.

To identify the product of the 4-OT-catalyzed Michael-type reaction between **7** and **8**, a preparative scale reaction was performed with **7** (50 mM), **8** (2 mM, 18 mg), and 4-OT (0.7 mol%, 14.7 μM) in NaH_2PO_4 buffer (20 mM, pH 7.3, 60 mL). After three hours reaction time and a standard work up, ^{1}H NMR spectroscopy indicated complete depletion of **8** and 46% conversion into **9**. Subsequent purification of **9** (Figure S2 in the Supporting Information) and analysis by HPLC on a chiral stationary phase (Figure S3 in the Supporting Information) revealed that 4-OT is highly stereoselective, producing the *S* enantiomer of **9** with 89% *ee*.

After we established that 4-OT catalyzes the addition of **7** to **8** to give **9**, kinetic parameters were determined. The rate of the 4-OT-catalyzed reaction was dependent on the concentrations of both **7** and **8**. Apparent kinetic parameters were estimated at a fixed concentration (50 mM) of **7** and various concentrations of **8**. 4-OT catalyzes the addition reaction with a k_{cat} value of $1.7 \times 10^{-2}\,s^{-1}$ and a K_m value of 250 μM, thus resulting in a k_{cat}/K_m value of $68\,M^{-1}s^{-1}$.

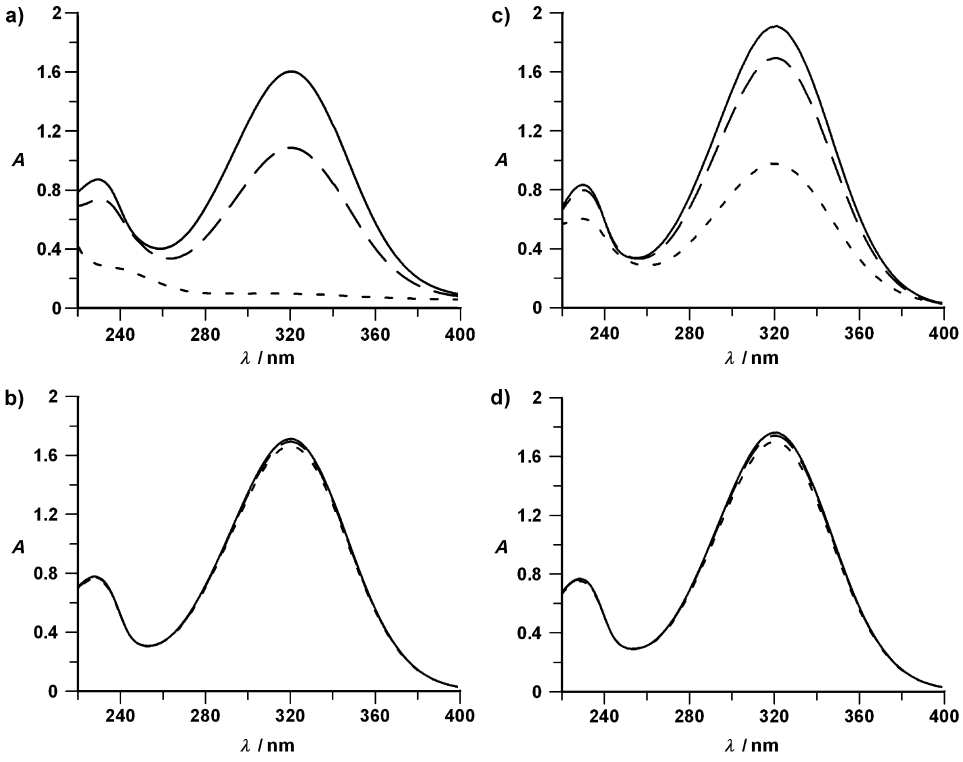

Figure 1. UV spectra showing the depletion of nitrostyrene (**8**) in the presence of a) acetaldehyde (**7**) and recombinant 4-OT, b) acetaldehyde (**7**), c) acetaldehyde (**7**) and synthetic 4-OT, and d) recombinant 4-OT. —— 0 min, – – – 4 min, ----- 20 min.

We next investigated the importance of residue Pro-1 to the Michael-type addition activity of 4-OT by mutagenesis and labeling experiments. First, residue Pro-1 of 4-OT was mutated to an alanine (Ala = A). This 4-OT P1A mutant (29.4 μM) was incubated with **7** (50 mM) and **8** (2 mM) in NaH_2PO_4 buffer (20 mM, pH 7.3), and the depletion of **8** was monitored by UV spectroscopy (Figure S1B in the Supporting Information). A similar experiment was performed with 4-OT inhibited by 3-bromopyruvate, an irreversible inhibitor that alkylates residue Pro-1 (Figure S1A in the Supporting Information).[18] Both mutant and alkylated 4-OT almost completely lost their activity, thereby indicating that residue Pro-1 is crucial for the enzymatic Michael-type addition of **7** to **8**. To evaluate the importance of the two active-site arginine residues (Arg-11 and Arg-39) for the promiscu-

ous Michael-type addition activity of 4-OT, the corresponding alanine mutants were prepared. In separate reactions, 4-OT R11A and 4-OT R39A (29.4 μM each) were incubated with **7** (50 mM) and **8** (2 mM) in NaH_2PO_4 buffer (20 mM, pH 7.3), and depletion of **8** was monitored by UV spectroscopy (Figure S1B in the Supporting Information). Mutant 4-OT R39A almost completely lost activity, while mutant 4-OT R11A showed a greatly reduced activity. This finding suggests that both arginine residues are crucial for the promiscuous activity of 4-OT. The importance of residues Pro-1, Arg-11, and Arg-39 strongly suggests that the addition of **7** to **8** takes place in the same active site as the natural tautomerization reactions (Scheme 1) catalyzed by 4-OT.[14–16]

In a possible mechanism that may explain the Michael-type addition activity of 4-OT, residue Pro-1 acts as nucleophile and attacks the carbonyl carbon atom of **7** (Scheme S1 in the Supporting Information).[1,9] The formed iminium ion is deprotonated, thereby resulting in an enamine intermediate. This nucleophilic species reacts with **8** in a Michael-type addition. Residue Arg-11 likely ensures correct substrate binding by coordinating the nitro functionality of **8** in analogy to the interaction of residue Arg-11 with the C6 carboxylate group of the natural substrate **1**. Residue Arg-39 might act as a general acid catalyst, delivering a proton at the Cα carbon atom of **8**. The final product **9** is released from the Pro-1 residue of 4-OT by hydrolysis.

UV spectroscopic assays demonstrated that compound **10** (Scheme 2) is also accepted as a substrate by 4-OT (Figures S4 and S5 in the Supporting Information). The 4-OT-catalyzed addition of **7** to **10** gives predominantly the *S* enantiomer of 4-nitro-3-(4-hydroxyphenyl)butanal (**11**; Figures S6 and S7 in the Supporting Information). A preparative-scale reaction gave **11** in 65% yield and with 51% *ee*. The apparent kinetic parameters for the 4-OT-catalyzed addition of **7** to **10** ($K_m = 1.6$ mM for **10**; $k_{cat} = 5.9 \times 10^{-2}\ s^{-1}$; $k_{cat}/K_m = 37\ M^{-1}s^{-1}$) are similar to those determined for the addition of **7** to **8**. As expected, the active-site residues Pro-1, Arg-11, and Arg-39 are also important for the 4-OT-catalyzed addition of **7** to **10** (Figure S5 in the Supporting Information).

In summary, we have shown that the proline-based tautomerase 4-OT is an efficient catalyst for the asymmetric Michael-type additions of acetaldehyde (**7**) to nitrostyrenes **8** and **10**. Product **9** was obtained in 46% yield and good enantiomeric excess (89% *ee*), whereas *p*-hydroxy derivative **11** was furnished in 65% yield and with 51% *ee*. The characteristic Pro-1 residue of 4-OT is crucial for activity, and catalysis likely takes place via an enamine intermediate.[19] Our results compare well to the corresponding organocatalytic strategies reported (Table 1).[5c] Underivatized (*S*)-proline yielded **9** from **7** and **8** in low yield (10%) and *ee* (30%), whereas the more advanced proline-based catalyst diphenylprolinol silyl ether gave **9** in 51% yield and with 92% *ee*. Our enzymatic system complements the classic organocatalytic approach in two ways. First, when compared to the amount of catalyst generally used in organocatalysis (10–20 mol%), the catalyst loading of 0.7 mol% 4-OT in our setup is low. Indeed, for promiscuous non-natural reactions, 4-OT exhibits pronounced catalytic efficiencies of $k_{cat}/K_m = 68\ M^{-1}s^{-1}$ (addition of **7** to **8**) and $37\ M^{-1}s^{-1}$ (addition of **7** to **10**). Second, the enzymatic reactions were not performed in organic solvent, but in an environmentally benign aqueous buffer. Together with our reported 4-OT-catalyzed aldol condensations,[9] these results serve as an excellent starting point for directed evolution experiments to further broaden the substrate and reaction scope of 4-OT and to improve reaction rates. If successful, these efforts may help to increase the number and diversity of biocatalysts that can be used for (large industrial scale) asymmetric carbon–carbon bond-forming reactions.

Table 1: Comparison of organo- and biocatalytic synthesis of **9** and **11**.

Substrates	Product	Catalyst	Catalyst loading [mol%]	Solvent	Reaction time [h]	Yield [%]	*ee* [%]
7 and **8**[a]	**9**	(*S*)-proline	20	MeCN	15	10	30
7 and **8**[a]	**9**	diphenylprolinol silyl ether	20	MeCN	15	51	92
7 and **8**[b]	**9**	4-OT	0.7	H_2O	3	46	89
7 and **10**[b]	**11**	4-OT	0.7	H_2O	3	65	51

[a] Data taken from reference [5c]. [b] Data described herein.

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[19] In theory, the reaction could proceed via the enolate anion of **7** (a mechanism in which residue Pro-1 would function as a general base catalyst removing a proton from **7**) instead of via the derived enamine (a mechanism in which residue Pro-1 acts as nucleophile). Because residue Pro-1 rapidly reacts with **7** to form an imine or enamine, the alternative mechanism involving an enolate anion seems unlikely.

