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Systematic Screening for Catalytic Promiscuity in 4-Oxalocrotonate Tautomerase: Enamine Formation and Aldolase Activity

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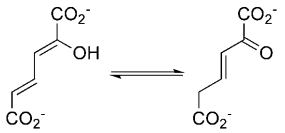
The enzyme 4-oxalocrotonate tautomerase (4-OT) is part of a catabolic pathway for aromatic hydrocarbons in *Pseudomonas putida* mt-2, where it catalyzes the conversion of 2-hydroxy-2,4-hexadienedioate (**1**) to 2-oxo-3-hexenedioate (**2**). 4-OT is a member of the tautomerase superfamily, a group of homologous proteins that are characterized by a β-α-β structural fold and a catalytic amino-terminal proline. In the mechanism of 4-OT, Pro1 is a general base that abstracts the 2-hydroxyl proton of **1** for delivery to the C-5 position to yield **2**. Here, 4-OT was explored for nucleophilic catalysis based on the mechanistic reasoning that its Pro1 residue has the correct protonation state ($pK_a \sim 6.4$) to be able to act as a nucleophile at pH 7.3. By using inhibition studies and mass spectrometry experiments it was first demonstrated that 4-OT can use Pro1 as a nucleophile to form an imine/enamine with various aldehyde and ketone compounds. The chemical potential of the smallest enamine (generated from acetaldehyde) was then explored for further reactions by using a small set of selected electrophiles. This systematic screening approach led to the discovery of a new promiscuous activity in wild-type 4-OT: the enzyme catalyzes the aldol condensation of acetaldehyde with benzaldehyde to form cinnamaldehyde. This low-level aldolase activity can be improved 16-fold with a single point mutation (L8R) in 4-OT's active site. The proposed mechanism of the reaction mimicks that used by natural class-I aldolases and designed catalytic aldolase antibodies. An important difference, however, is that these natural and designed aldolases use the primary amine of a lysine residue to form enamines with carbonyl substrates, whereas 4-OT uses the secondary amine of an active-site proline as the nucleophile catalyst. Further systematic screening of 4-OT and related proline-based biocatalysts might prove to be a useful approach to discover new promiscuous carbonyl transformation activities that could be exploited to develop new biocatalysts for carbon-carbon bond formation.

Introduction

The notion that many enzymes are catalytically promiscuous and can catalyze one or more alternative reactions in addition to the one they evolved for has become a source of inspiration for the design of useful biocatalysts.[1] However, in order to exploit the promiscuous activities of existing enzymes to develop new biocatalysts, an important consideration is how the usually low-level promiscuous activities of enzymes might be systematically characterized. So far most promiscuous activities have been discovered either by chance or by looking for a specific reaction based on an enzyme's close relatives.[2,3] Herein, we have used a systematic screening strategy to discover new promiscuous activities in 4-oxalocrotonate tautomerase (4-OT). It is based on the mechanistic reasoning that the catalytic amino-terminal proline of this enzyme provides a unique chemical functionality in the active site that might be suitable for enamine catalysis.

4-OT is part of a catabolic pathway for aromatic hydrocarbons in *Pseudomonas putida* mt-2, in which it catalyzes the conversion of 2-hydroxy-2,4-hexadienedioate (**1**) to 2-oxo-3-hexenedioate (**2**; Scheme 1).[4] The enzyme is a member of the tautomerase superfamily, a group of homologous proteins that are characterized by a conserved catalytic amino-terminal proline embedded within a β-α-β structural fold.[5] In the mechanism of 4-OT, Pro1 is a general base that abstracts the 2-hydroxyl proton of **1** for delivery to the C-5 position, yielding **2**.[6]



1 2

Scheme 1. The tautomerization reaction catalyzed by 4-OT.

Pro1 can function as a general base because the prolyl nitrogen has a pK_a of ~6.4 and exists largely as the uncharged species at pH 7.3. Two other key catalytic residues are Arg11 and Arg39.[7] Arg39 is proposed to interact with the 2-hydroxyl group of **1** and a C-1 carboxylate oxygen, whereas Arg11 is proposed to interact with the C-6 carboxylate group in a bidentate fashion. The latter interaction might draw electron density toward C-5 for protonation by Pro1.

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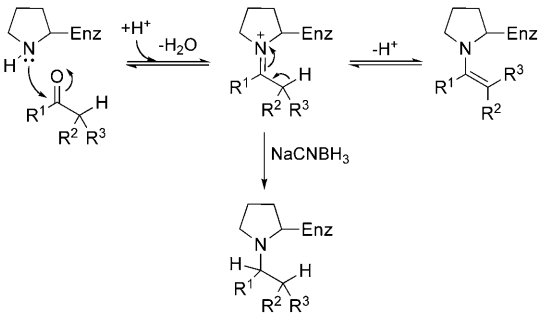
Dawson and co-workers demonstrated that by mutating Pro1 to an alanine, 4-OT can be changed from an acid/base tautomerase into an enzyme that decarboxylates oxaloacetate through a nucleophilic imine mechanism.[8] This change in catalytic activity and mechanism is consistent with the expectation that the active-site primary amine of Ala1 would be less basic and more conformationally flexible than the secondary amine of Pro1. This enables the nucleophilic addition of Ala1 to oxaloacetate to form an iminium ion intermediate (i.e., a protonated Schiff base), which facilitates decarboxylation. The decarboxylated product, pyruvate, is released from Ala1 by hydrolysis.[8]

Different from this earlier work on mutant 4-OT, we set out to test whether the active-site secondary amine of Pro1 in wild-type 4-OT might be suitable for nucleophilic catalysis. We reasoned that although Pro1 functions as a base catalyst in 4-OT's natural activity, it has the correct protonation state (pK_a ~ 6.4) to be able to act as a nucleophile at pH 7.3.[9] Furthermore, being the only natural amino acid with a secondary amine group, Pro1 might facilitate the reversible generation of enamines from carbonyl compounds (Scheme 2). Indeed, secondary



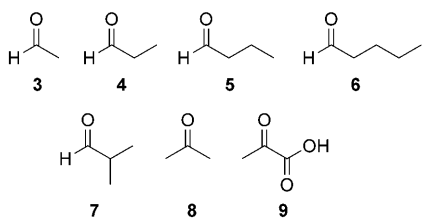
Scheme 2. Proposed mechanism of enamine formation in the active site of 4-OT. Reduction of the imine intermediate by $NaCNBH_3$ leads to irreversible covalent modification and inactivation of the enzyme.

amines react with carbonyl compounds to favor the formation of enamine intermediates, whereas primary amines favor the imine tautomers.[10] The enzymatically generated enamine then could undergo a wide range of further reactions by using different electrophiles, which might allow for the discovery of several new activities within the active site of wild-type 4-OT. This mechanistic reasoning is strengthened by a large body of literature that highlights the success of proline and related cyclic secondary amines as organocatalysts in asymmetric enamine catalysis.[11]

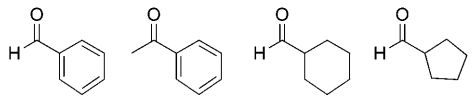
Results and Discussion

To identify reactive carbonyl compounds that might give rise to useful enamines, 4-OT was incubated with various aldehydes and ketones (in separate reactions) and the mixtures were treated with $NaCNBH_3$. Reduction of the imine intermediate will covalently link the carbonyl compound to the enzyme and result in its inactivation (Scheme 2).[8,12] When 4-OT was treated with $NaCNBH_3$ in the presence of the selected aldehydes **3**–**7** (Scheme 3), enzymatic activity was almost completely lost (Table 1). Gel filtration chromatography did not restore activity, which is indicative of irreversible covalent modification. Treatment of the enzyme with the aldehyde or $NaCNBH_3$ alone did not result in the loss of enzymatic activity. These observations suggest that an imine can form between 4-OT and aldehydes **3**–**7**. Although these compounds are reactive aldehydes with the potential for forming imines or enamines with nearby amines, it is significant that inactivation and covalent modification of 4-OT also occurs with the less-reactive ketones **8** and **9** (Scheme 3, Table 1). The higher reactivity of **9** likely reflects its structural resemblance to the pyruvoyl moiety of 4-OT's natural substrate **2**.

Carbonyl compounds tested as enamine donor



Electrophiles tested in enamine catalysis



Scheme 3. Carbonyl compounds tested as enamine donors (**3**–**9**) or as acceptors (**10**–**13**).

Table 1. Inactivation and covalent modification of 4-OT by selected carbonyl compounds in the presence of $NaCNBH_3$.

Enamine donor	Inactivation [%]	$Mass_{calcd}$ [Da][a]	$Mass_{obs}$ [Da]	Covalent labeling [%][b]
3	95	6839	6839	>99
4	>99	6853	6853	>99
5	>99	6867	6867	>99
6	97	6881	6811,[c] 6881	60
7	79	6867	6867	95
8	23	6854	6811,[c] 6854	29
9	>99	6883	6883	>99

[a] Mass corresponding to reduced imine complex of donor substrate and 4-OT. [b] Estimated from relative peak heights in ESI-MS spectra. [c] Mass of unmodified 4-OT.

The inactivated protein samples were analyzed by ESI-MS to determine whether the mass is consistent with the mechanism shown in Scheme 2 and to ascertain whether single or multiple modifications had occurred. The deconvoluted spectrum of the 4-OT sample inactivated by either **3**–**5**, **7**, or **9** displayed one major peak corresponding to the mass expected for the enzyme modified by a single molecule of the respective compound and reduced by $NaCNBH_3$ (Table 1). Analysis of the 4-OT sample inactivated by either **6** or **8** showed two peaks, one

corresponding to the mass of unmodified 4-OT and the other to the mass expected for the enzyme modified by a single molecule of **6** or **8**, respectively, and reduced by $NaCNBH_3$ (Table 1). These results indicate that the reaction between 4-OT and the carbonyl compounds is specific (and not the consequence of multiple nonspecific encounters between 4-OT and carbonyl compound) and consistent with the proposed mechanism of enamine formation in the enzyme active site (Scheme 2).

We next investigated whether imine/enamine formation is an active site process that involves Pro1. A 4-OT sample inactivated by **3** and a control sample (unmodified 4-OT) were digested with endoproteinase Glu-C, and the resulting peptide mixtures were analyzed by nano-LC–MS. A comparison of the peaks of the 4-OT sample inactivated by **3** in the presence of $NaCNBH_3$ to those of the unmodified 4-OT sample revealed a single modification by a species having a mass of 28 Da on the fragment Pro1 to Glu9 (Table 2). The mass of this species corresponds to labeling by one acetaldehyde molecule. Analysis of the remaining peaks showed no modification of other fragments.

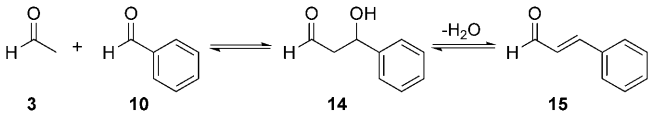
Table 2. Identification of Pro1 as the sole site of labeling by **3** by using Glu-C digestion and nano-LC-MS and MS/MS analyses.

Peptide fragment	$Mass_{calcd}$ [Da][a]	$Mass_{obs}$ [Da][b]
PIAQIHILE	1033.6[c]	1032.5[c]
PIAQIH	660.4	660.4
PIAQ	410.2	410.2
PI	211.1	211.1
C_2H_4-PIAQIHILE	1061.6[c]	1060.6[c]
C_2H_4-PIAQIH	688.4	688.4
C_2H_4-PIAQ	438.2	438.2
C_2H_4-PI	239.1	239.1

[a] These values are calculated using the average molecular mass. [b] The reported masses correspond to the b-ions. [c] These values correspond to the total mass of the parent ion.

Within the amino-terminal fragment Pro1 to Glu9, the most likely targets for alkylation are Pro1 and His6. To identify the alkylated amino acid residue, the unmodified and modified peptides were subjected to nano-LC–MS/MS analysis. The spectrum of the ion corresponding to the unlabeled peptide (PIAQIHILE) displayed characteristic b-ions resulting from the peptide fragments PIAQIH, PIAQ, and PI. MS/MS analysis of the ion corresponding to the modified peptide (C_2H_4-PIAQIHILE) revealed an increase in mass of 28 Da for these b-ions (Table 2). Because one of these fragment ions is generated by the dipeptide Pro-Ile, we conclude that the active site Pro1 residue is the sole site of modification by **3**. While the precise site of labeling of the other reactive carbonyl compounds (**4**–**9**) has not been determined by mass spectrometry, it can be inferred from these results that compounds **4**–**9** likely form imines/enamines with Pro1.

Encouraged by these findings, the chemical potential of the smallest enamine (generated from **3**) was explored for further reactions by using four selected electrophiles (**10**–**13**, Scheme 3). These electrophiles were chosen as model substrates for screening because they show structural resemblance to the phenyl portion of phenylpyruvate, a known substrate of 4-OT.[6b] Accordingly, 4-OT (0.4 mg; 90 μM) was incubated with **3** (50 mM) and each of the electrophiles (50 mM), and the four reactions were followed by ^{1}H NMR spectroscopy. After incubation of **3** and **10** with 4-OT for 14 days at 22 °C (in 20 mM phosphate buffer, pH 7.3), the intensity of the signals corresponding to these aldehyde substrates diminished and new signals corresponding to cinnamaldehyde (**15**, Scheme 4) appeared (Figure S1 A in the Supporting Information). Integration of the signals indicates that ~10% of **3** and **10** had been converted to **15**. Whereas **15** has characteristic ^{1}H NMR spectroscopic signals (at 6.70 and 9.44 ppm), its identity in the incubation mixture described above was further confirmed by GC/MS analysis (Figure S2). In addition to the two substrate molecules, a major product was observed and identified as **15** based both on retention time comparison and the detection of fragment ions with masses identical to those found with an authentic standard.



Scheme 4. The aldol condensation reaction catalyzed by 4-OT.

The effect of enzyme concentration on the amount of **15** produced was also investigated. Accordingly, **3** and **10** (each at 20 mM) were incubated with either 90 or 180 μM of 4-OT, and the two reactions were followed by ^{1}H NMR spectroscopy. By using 90 μM of 4-OT, ~4 and ~10% of **3** and **10** were converted to **15** after 7 and 14 days, respectively. By using 180 μM of 4-OT, ~9 and ~16% of **3** and **10** were converted to **15** after 7 and 14 days, respectively. These results show that doubling the enzyme concentration doubles the amount of product formed, strongly suggesting that the reaction is enzyme catalyzed. Notably, no significant enzyme inhibition occurs at these aldehyde concentrations.

Control experiments further demonstrate that the aldol condensation reaction between **3** and **10** is an enzyme-catalyzed process. GC/MS and ^{1}H NMR spectroscopic analyses of **3** and **10** (each at 50 mM) incubated in 20 mM phosphate buffer (pH 7.3) for 14 days at 22 °C showed no formation of **15**; this rules out a nonenzymatic aldol condensation (Figure S1 B). Hence, the results show that the 4-OT-catalyzed aldol condensation of **3** with **10** generates **15**. A likely scenario for the formation of **15** from these compounds involves the initial enzymatic cross coupling of **3** and **10** to generate the aldol product **14** (Scheme 4). Subsequent chemical or enzymatic dehydration of **14** yields **15**. The incubations of **3** and each of the electrophiles **11**–**13** with 4-OT showed no detectable conversion, demonstrating the high selectivity of 4-OT for substrate **10**. As expected, incubation of only **10** (without **3**) with 4-OT in 20 mM phosphate buffer (pH 7.3) for 14 days at 22 °C also

showed no conversion, which demonstrates that both substrates (**3** and **10**) are required for product formation.

The preparation of 4-OT used in these experiments was highly purified, but it remained possible that a contaminating enzyme from the *E. coli* BL21(DE3) expression host could be responsible for the observed aldolase activity. To eliminate this concern, three control experiments were performed. First, incubation of 4-OT with 3-bromopyruvate, an active-site-directed irreversible inhibitor that alkylates Pro1,[13] led to single-site modification of 4-OT (as shown by ESI-MS) and the concomitant loss of the aldolase activity (Figure S3C). Second, a mock purification was performed from BL21(DE3) cells harboring an empty pET20b(+) vector. Incubation of an aliquot of this purification with **3** and **10** for 14 days at 22 °C did not result in the formation of **15**. Third, a 4-OT sample free of contaminating cellular enzymes was prepared by total chemical synthesis.[14] Incubation of **3** and **10** with synthetically prepared 4-OT for 14 days at 22 °C yielded product **15** in an amount comparable to that formed in the reaction catalyzed by purified recombinant 4-OT (Figure S3D).

We next investigated the importance of Pro1, Arg11, and Arg39 to 4-OT's aldolase activity by analyzing the kinetic properties of the corresponding alanine mutants. ^{1}H NMR spectroscopic analysis revealed that after a 14 day incubation period at 22 °C, the R39A-4-OT-catalyzed reaction results in ~8% of **15**, whereas the P1A-4-OT and R11A-4-OT-catalyzed reactions gave only a trace amount of **15** (<1%; Figure S3). These results provide further evidence indicating that 4-OT is responsible for the observed aldolase activity and that Pro1 and Arg11 are critical for the activity, but Arg39 is not essential for the activity.

It has previously been reported that 4-OT exhibits a promiscuous dehalogenase activity that can be significantly increased (50-fold in terms of k_{cat}/K_m) by the replacement of the active-site residue Leu-8 with an arginine.[15] This observation prompted us to test whether this mutation might also affect the promiscuous aldolase activity of 4-OT. Accordingly, the L8R-4-OT mutant was constructed, purified, and tested for its ability to catalyze the aldol condensation of **3** with **10**. To perform accurate rate measurements, an UV spectroscopy assay was developed that follows the decrease in substrate absorbance at 250 nm and the increase in product absorbance at 290 nm. Incubation of **3** and **10** with L8R-4-OT resulted in a decrease in absorbance corresponding to **10** (λ_{max} = 250 nm), accompanied by the appearance of a new absorbance peak at 290 nm, which corresponds to **15** (Figure 1, lower panel). When the rate of this reaction is compared to that catalyzed by wild-type 4-OT (Figure 1, upper panel), it is clear that the L8R-4-OT mutant has an improved aldolase activity. Analysis of the reaction by ^{1}H NMR spectroscopy verified that the product of the L8R-4-OT catalyzed aldol condensation of **3** with **10** is **15**, as established for wild-type 4-OT (Figure S3F). Again, no product formation was detected for the control reaction without enzyme.

Having established that the L8R-4-OT mutant has a significantly improved aldolase activity, kinetic parameters were determined. The rates of the 4-OT and L8R-4-OT-catalyzed reactions were dependent on the concentrations of both **3** and **10** (Figure S4). Apparent k_{cat}/K_m values were estimated at a fixed concentration of **3** (50 mM) and varying concentrations of **10**. A comparison of these values shows that the L8R-4-OT mutant ($k_{cat}/K_m = 1.4 \times 10^{-2}\,M^{-1}s^{-1}$) is 16-fold more efficient in catalyzing the aldol condensation of **3** with **10** than wild-type 4-OT ($k_{cat}/K_m = 8.5 \times 10^{-4}\,M^{-1}s^{-1}$). The improved aldolase activity of L8R-4-OT can also be clearly visualized by using a phloroglucinol-based colorimetric assay,[16] which monitors the formation of **15** (Figure S5).

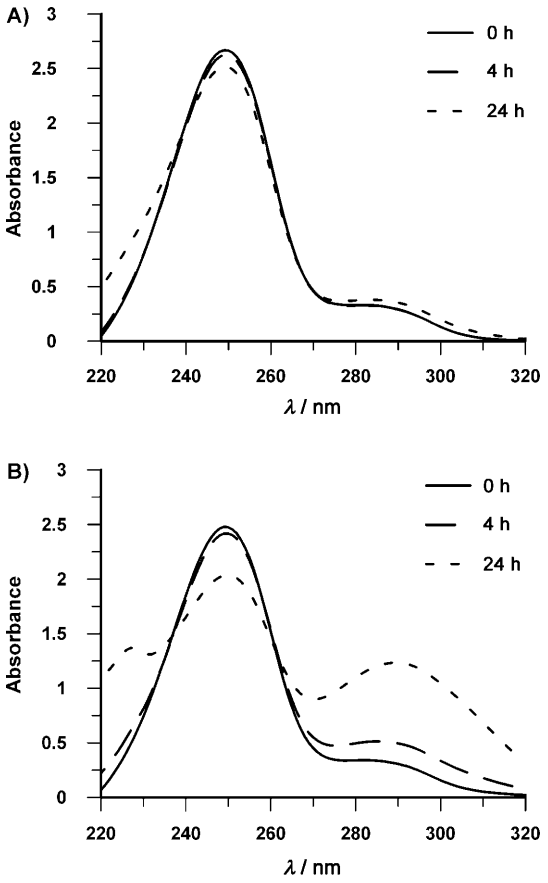


Figure 1. UV spectra monitoring the aldol condensation of **3** with **10** catalyzed by either A) 4-OT or B) L8R-4-OT.

In a possible mechanism that might explain the observed aldolase activity, Pro1 functions as a nucleophile and attacks the carbonyl carbon of **3** to form an iminium ion (Scheme S1). Deprotonation of this ion leads to the generation of the reactive enamine intermediate, which is equivalent to a nucleophilic carbanion. Nucleophilic addition of the enamine to the carbonyl carbon of **10** gives a modified iminium ion. Dehydration and hydrolysis of this iminium ion intermediate yields the final product **15**. The negative charge that develops on the carbonyl oxygen of **10** (and/or **3**) could be stabilized by the active site arginine (i.e., Arg11), which is known to be critical for the activity. This arginine could also serve as the source for a proton that would assist in formation of the hydroxyl group of the iminium ion intermediate. The additional arginine residue in the L8R-4-OT mutant might assist Arg11 in charge stabiliza-

tion, explaining the increased aldolase activity of this mutant enzyme.[17]

Lineweaver–Burk plots at varying concentrations of **3** and different fixed values of **10** give a series of intersecting lines (Figure S4). This pattern is characteristic of a sequential bi-substrate mechanism. Because only substrate **3** can serve as enamine donor (**10** does not have enolizable hydrogens and can only act as enamine acceptor in the aldol reaction), the mechanism is expected to be sequential ordered rather than sequential random. This is supported by the relative rate of imine formation between 4-OT and **3** or **10**. After incubation of 4-OT with either **3** or **10** (each at 1 mM) for 1 h at 22 °C, and subsequent reduction with $NaCNBH_3$, 4-OT is found to be labeled only partially by **10**, whereas covalent modification by **3** is almost complete (Figure S6). Taken together, the kinetic and labeling results are consistent with a sequentially ordered mechanism for the 4-OT catalyzed condensation of **3** with **10**.

Notably, the UV spectra monitoring the aldol reactions (Figure 1) also show a time-dependent increase in absorbance at ~227 nm. Control experiments in which the nonenzymatic self-condensation of **3** was followed by UV and ^{1}H NMR spectroscopy demonstrate that this absorbance corresponds to the formation of but-2-enal (**16**, Scheme S2). Although 4-OT might weakly catalyze this reaction, the activity appears to be more significant for the L8R-4-OT mutant (Figure 1). Indeed, additional signals corresponding to **16** were also observed in the ^{1}H NMR spectra monitoring the incubations of **3** and **10** with either 4-OT (Figure S1) or the L8R-4-OT mutant (Figure S3F). Additional experiments with synthetically prepared 4-OT and L8R-4-OT are underway to verify whether these enzymes (rather than a contaminating protein) also catalyze the self-condensation of **3**.

In conclusion, we have used a systematic screening strategy to discover new promiscuous activities in wild-type 4-OT. The key strength of this strategy is the observation that the same N-terminal proline that is used to catalyze proton transfer (in 4-OT's natural activity) can be used to generate a range of structurally distinct enamines that might undergo reactions with various electrophiles. As a proof of principle experiment, we have shown that the aldol condensation of **3** with **10** is catalyzed by 4-OT, and that this activity can be improved (16-fold) by a single point mutation (L8R) in the enzyme's active site. The proposed mechanism of this reaction mimicks that used by natural class-I aldolases,[18] catalytic aldolase antibodies,[19] and designed aldolase peptides.[20] However, an important mechanistic difference is that these catalysts use the primary amine of a lysine residue to form enamines with carbonyl substrates, whereas 4-OT uses the secondary amine of an active-site proline as the nucleophile catalyst.

Systematic screening of a protein scaffold in which an active-site proline residue is present as a nucleophile and that has the reactivity to form enamines might prove to be a useful approach to discover new promiscuous carbonyl transformation activities that could serve as starting activities to develop new biocatalysts for carbon–carbon bond formation. This approach does not copy something in Nature (i.e., proline-based enamine catalysis is not known to occur in nature), but is based on the synthetic requirements of the desired bond-forming reactions.[21] Work is in progress to explore new enamine donors for 4-OT and to exploit the chemical potential of the various enamines that can be formed in 4-OT's active site to find new promiscuous aldol, alkylation, and Michael addition reactions in this fascinating enzyme. In addition, we have initiated studies aimed at screening known and putative tautomerase superfamily members (that share a nucleophilic active site proline) with various aldehyde and ketone probes for the presence of intrinsic, promiscuous carbonyl-transformation activities. Directed evolution experiments could then be used to enhance the desired activities to a practical level.

Experimental Section

Materials: All chemicals were obtained from Sigma–Aldrich unless stated otherwise. The sources for the biochemicals, buffers, solvents, components of Luria–Bertani (LB) medium as well as the materials, enzymes, and reagents used in the molecular biology procedures are reported elsewhere.[22] Sequencing grade endoproteinase Glu-C (protease V-8) was purchased from Roche Diagnostics. High-purity synthetic 4-OT (lyophilized, with Met-45 replaced by Nle to prevent oxidation upon sample handling) was obtained from GenScript USA Inc. (Piscataway, NY) and folded into the active homohexamer as described before.[23] 2-Hydroxy-2,4-hexadienedioate (commonly known as 2-hydroxymuconate) was kindly provided by Prof. Dr. Christian P. Whitman (University of Texas at Austin).

General methods: Techniques for restriction enzyme digestions, ligation, transformation, and other standard molecular biology manipulations were based on methods described elsewhere.[24] The PCR was carried out in a DNA thermal cycler (model GS-1) obtained from Biolegio (Nijmegen, The Netherlands). DNA sequencing was performed by Macrogen (Seoul, Korea). Protein was analyzed by polyacrylamide gel electrophoresis (PAGE) by using sodium dodecyl sulfate (SDS) gels containing polyacrylamide (10%). The gels were stained with Coomassie brilliant blue. Protein concentrations were determined by the Waddell method.[25] Kinetic data were obtained on a V-650 spectrophotometer from Jasco (IJsselstein, The Netherlands). ^{1}H NMR spectra were recorded on a Varian Inova 500 (500 MHz) spectrometer by using a pulse sequence for selective presaturation of the water signal. Chemical shifts for protons are reported in parts per million scale (δ scale) downfield from tetramethylsilane and are referenced to protium (H_2O: $\delta = 4.67$). GC-MS spectra were recorded on an in-house Shimadzu GC-17A/GCMS-QP5000 system. The masses of 4-OT and 4-OT mutants were determined by ESI-MS by using a Sciex API 3000 triple quadrupole mass spectrometer (AB Sciex, Concord, Canada). The masses of peptide fragments in samples of Glu-C-digested 4-OT were determined by nano-LC-MS/MS by using a QSTAR XL mass spectrometer (AB/MDS-Sciex, Toronto, Canada) coupled to an Agilent 1100 nanoflow system (Waldbronn, Germany). These mass spectrometers are housed in the Mass Spectrometry Facility Core in the Department of Pharmacy at the University of Groningen.

Construction of the expression vector for the production of 4-OT: The gene encoding wild-type 4-OT was amplified from plasmid pET3b(4-OT)[15] by using the following primers: 5′-A TAG CAG GTA CAT ATG CCT ATT GCC CAG ATC CAC AT-3′ (primer F, NdeI site underlined) and 5′- G TGA TGT TAT GGA TCC TCA GCG TCT GAC CTT GCT GGC CAG TTC G-3′ (primer R, BamHI site underlined). The PCR

product was gel-purified, digested with NdeI and BamHI, and cloned in frame with the ATG start codon of the pET20b(+) vector. The newly constructed expression vector was named pET20b(4-OT).

Construction of the 4-OT mutants P1A, R11A, R39A, and L8R: The four 4-OT mutants were constructed by PCR by using the coding sequence for 4-OT in plasmid pET20b(4-OT) as the template. To introduce the P1A, L8R, and R11A mutations, the following oligonucleotides were used as forward primers: 5′-A TAG CAG GTA CAT ATG **GCC** ATT GCC CAG ATC CAC ATC CTT G-3′, 5′-A TAG CAG GTC CAT ATG CCT ATT GCC CAG ATC CAC ATC **CGC** GAA GGC CGC AGC-3′, and 5′-A TAG CAG GTA CAT ATG CCT ATT GCC CAG ATC CAC ATC CTT GAA GGC **GCC** AGC GAC GAG CAG-3′, respectively, in which the NdeI site is underlined and the mutated codon is in bold. These primers correspond to the 5′-end of the wild-type coding sequence and were used in combination with primer R. For the construction of the R39A mutant the following oligonucleotide was used as the reverse primer: 5′-A TGT TAT GGA TCC TCA GCG TCT GAC CTT GCT GGC CAG TTC GCC GCC GAT GCC GAA GTG GCC CTT GGC CAT CTC CGT GAT AAT CAC **GGC** CAC GCT GG-3′, in which the BamHI site is underlined and the mutated codon is in bold. This primer corresponds to the 3′-end of the wild-type coding sequence and was used in combination with primer F. The resulting PCR products were gel-purified, digested, and cloned in-frame with the ATG start codon of the pET20b(+) vector. All genes were fully sequenced to assure that only the desired mutations were introduced.

Expression and purification of 4-OT wild-type and mutants: The 4-OT enzyme, either wild-type or mutant, was produced in *E. coli* BL21(DE3) by using the pET20b(+) expression system. LB medium (5 mL) containing ampicillin (Ap, 100 $\mu g\,mL^{-1}$) was inoculated with cells from a glycerol stock of *E. coli* BL21(DE3) containing the appropriate expression vector by using a sterile loop. After overnight growth at 37 °C, this culture was used to inoculate fresh LB/Ap medium (1 L) in a 3 L Erlenmeyer flask. Cultures were grown overnight at 37 °C with vigorous shaking to an OD_{600} of ~4.5. Cells were harvested by centrifugation (20 min, 4500 rpm), washed with 10 mM NaH_2PO_4 buffer (pH 8.0, buffer A) and stored at −20 °C.

The 4-OT protein, either wild type or mutant, was purified by using disposable hand-packed columns. Typically, in this protocol, cells from 0.5 L of culture were suspended in ~10 mL of buffer A, sonicated, and centrifuged (45 min, 15000 rpm). Subsequently, the supernatant was loaded onto a DEAE-sepharose column (10×1.0 cm filled with ~8 mL of resin) that had been previously equilibrated with buffer A. The column was first washed with buffer A (3× 10 mL), and then the protein was eluted by gravity flow by using buffer A containing 0.5 M Na_2SO_4 (8 mL). Fractions (~1.5 mL) were collected, and 4-OT was identified by SDS-PAGE. The appropriate fractions were pooled and made 1.6 M in $(NH_4)_2SO_4$. After stirring for 2 h at 4 °C, the precipitate was removed by centrifugation (20 min at 20000 *g*), and the supernatant was filtered and loaded onto a phenyl-sepharose column (10×1.0 cm filled with ~8 mL of resin) that had been previously equilibrated with buffer A containing 1.6 M $(NH_4)_2SO_4$. The column was first washed with the loading buffer (3×10 mL) and then the protein was eluted by gravity flow by using buffer A (8 mL). Fractions (~1.5 mL) were collected and analyzed as described above, and those that contained purified 4-OT protein were combined and the buffer was exchanged against 20 mM NaH_2PO_4 buffer (pH 7.3) by using a pre-packed PD-10 Sephadex G-25 gelfiltration column. The purified protein was stored at −80 °C until further use.

Wild-type 4-OT and the P1A and R11A mutant proteins had little interaction with the phenyl-sepharose column and eluted as homogeneous proteins (>95% purity as assessed by SDS-PAGE) in the first washing step. The R39A mutant interacted more strongly with the phenyl-sepharose column and eluted in the second and third washing step, as well as in one of the first elution fractions. The R39A protein that eluted in the washing steps (~85% purity) was used in the assays. The L8R mutant eluted from the DEAE-sepharose column in the second washing step as homogenous protein (>95% purity), and no further purification on the phenyl-sepharose column was performed.

The masses of the purified wild-type and mutant proteins were determined with ESI-MS to confirm that the proteins had been processed correctly and the initiating methionine had been removed. The observed monomer mass for wild-type 4-OT was 6811 Da (calcd 6810 Da). The observed monomer mass for the P1A mutant was 6786 Da (calcd 6785 Da), that of the R11A mutant was 6727 Da (calcd 6726 Da), and that of the R39A mutant was 6727 Da (calcd 6726 Da).

In addition, a mock purification was performed from BL21(DE3) cells harboring an empty pET20b(+) vector according to the procedure described above for wild-type 4-OT. An aliquot of this sample was used as a control in the colorimetric and ^1H NMR assays for aldolase activity (see below).

Spectrophotometric assay for tautomerase activity: The ketonization of 2-hydroxymuconate (**1**) by 4-OT was monitored by following the depletion of the absorbance of **1** at 288 nm ($\varepsilon = 20\ mM^{-1}cm^{-1}$) in 20 mM NaH_2PO_4 buffer (pH 7.3). A small aliquot (1 μL) of a 1.1 μM (monomer concentration) stock solution of 4-OT was added to a cuvette containing 1 mL of buffer. To initiate the assay, 2 μL of a stock solution of **1** (50 mM in ethanol) was added.

Labeling of 4-OT with 3-bromopyruvate: 4-OT (2 mg) was incubated with 3-bromopyruvate (3-BP; final concentration 20 mM) in 20 mM NaH_2PO_4 buffer (pH 7.3) for 1 h at 22 °C (total volume of 1 mL). Subsequently, $NaBH_4$ was added to a final concentration of 25 mM and the sample was incubated at 22 °C for 1 h. In a separate control experiment, the same quantity of 4-OT was incubated without inhibitor under otherwise identical conditions. The two incubation mixtures were loaded onto separate PD-10 Sephadex G-25 gel filtration columns, which had previously been equilibrated with 20 mM NaH_2PO_4 buffer (pH 7.3). The proteins were eluted by gravity flow by using the same buffer. Fractions (~0.5 mL) were analyzed for the presence of protein by UV absorbance at 214 nm. The appropriate fractions containing the purified proteins were combined and assayed for tautomerase activity as described above. The 4-OT sample treated with 3-BP had no residual tautomerase activity. Incubation of 4-OT without inhibitor under the same conditions had no effect on activity. The covalent modification of 4-OT resulting from the incubation with 3-BP was confirmed by ESI-MS. The observed monomer mass for the covalently modified 4-OT was 6901 Da (calcd 6900 Da). This 4-OT protein inactivated by 3-BP was used as a control in the ^1H NMR spectroscopic assay for aldolase activity (see below).

Sodium cyanoborohydride treatment of 4-OT in the presence of carbonyl compounds (3–10, 15): 4-OT (1 mg) was incubated with 1 mM of compounds **3**–**7**, **10**, or **15** or 10 mM of compounds **8** or **9** in a final volume of 1 mL of 20 mM NaH_2PO_4 buffer (pH 7.3) for 1–3 h at 22 °C (in separate reactions). Subsequently, an aliquot of a 100 mM stock solution of $NaCNBH_3$ in H_2O was added to give a final concentration of 25 mM. After incubation for 1 h at 22 °C, the buffer was exchanged against 5 mM NH_4HCO_2 buffer (pH 8.0) using

a pre-packed PD-10 Sephadex G-25 gelfiltration column. The purified 4-OT proteins were assayed for residual tautomerase activity using the spectrophotometric assay described above. To assess the extent and specificity of the covalent labeling, the purified proteins were also analyzed by ESI-MS.

Control reactions containing enzyme, buffer, and carbonyl compound, or enzyme, buffer, and $NaCNBH_3$ were carried out under identical conditions. These mixtures did not lead to inactivation of 4-OT.

Mass spectral analysis of modified 4-OT and peptide mapping: 4-OT (0.5 mg) was incubated with 1 mM of acetaldehyde (**3**) in 20 mM NaH_2PO_4 buffer (pH 7.3) for 1 h at 22 °C (total volume of 1 mL). A second 4-OT sample was not treated with **3** and was used as the control sample. An aliquot from a 100 mM stock solution of $NaCNBH_3$ in H_2O was added to both samples to give a final concentration of 25 mM. After incubation for 1 h at 22 °C, the buffer was exchanged against 10 mM NaH_2PO_4 buffer (pH 8.0) by using a pre-packed PD-10 sephadex G-25 gel-filtration column. The two purified 4-OT proteins were assayed for tautomerase activity, analyzed by ESI-MS, and used in the following peptide mapping experiments.

For the peptide-mapping studies, unmodified 4-OT and 4-OT modified by **3** (50 μg) were vacuum-dried. The individual protein pellets from the two samples were dissolved in 10 M guanidine–HCl (10 μL) and incubated for 2 h at 37 °C. Subsequently, the samples were diluted with NH_4HCO_3 buffer (90 μL, 100 mM; pH 8.0) and incubated for 2 d at 37 °C with protease GluC (2.5 μL from a 10 mg mL^{-1} stock solution in H_2O). These two digested samples were analyzed by nano-LC–MS to identify the labeled peptide fragment. Selected ions of both samples were subjected to MS/MS analysis.

^{1}H NMR spectroscopic screening for carbonyl transformations by 4-OT: A reaction mixture of **3** (50 mM) and either benzaldehyde (**10**), acetophenone (**11**), cyclohexanecarboxyaldehyde (**12**) or cyclopentanecarboxyaldehyde (**13**; each at ~50 mM) in NaH_2PO_4 buffer (0.55 mL, 20 mM; pH 7.3) was placed in an NMR tube, along with D_2O (0.05 mL) and 4-OT (0.4 mg; 0.05 mL from a 8 mg mL^{-1} solution). Similar mixtures without 4-OT (the control samples) were also prepared to analyze the nonenzymatic (uncatalyzed) reaction. ^{1}H NMR spectra were recorded directly after mixing, and then after 1, 7, and 14 d.

Compound 3 and its hydrate: ^{1}H NMR (500 MHz, 20 mM phosphate/D_2O buffer, pH 7.3): $\delta = 1.19$ (d, $J = 4.5$ Hz, 3H), 2.09 (d, $J = 3.0$ Hz, 3H), 5.11 (q, $J = 5.0$ Hz, 1H), 9.52 ppm (q, $J = 3.0$ Hz, 1H).

Compound 10: ^{1}H NMR (500 MHz, 20 mM phosphate/D_2O buffer, pH 7.3): $\delta = 7.46$ (t, $J = 7.5$ Hz, 2H), 7.60 (t, $J = 7.5$ Hz, 1H), 7.79 (d, $J = 8.0$ Hz, 2H), 9.77 ppm (s, 1H).

Compound 11: ^{1}H NMR (500 MHz, 20 mM phosphate/D_2O buffer, pH 7.3): $\delta = 2.52$ (s, 3H), 7.42 (t, $J = 7.5$ Hz, 2H), 7.55 (t, $J = 7.5$ Hz, 1H), 7.85 ppm (d, $J = 8.5$ Hz, 2H).

Compound 12: ^{1}H NMR (500 MHz, 20 mM phosphate/D_2O buffer, pH 7.3): $\delta = 1.06$–1.40 (m, 6H), 1.50–1.70 (m, 4H), 1.77 (m, 1H), 9.41 ppm (s, 1H).

Compound 13: ^{1}H NMR (500 MHz, 20 mM phosphate/D_2O buffer, pH 7.3): $\delta = 1.20$–1.54 (m, 6H), 1.58–1.76 (m, 2H), 2.27 (m, 1H), 9.45 ppm (d, $J = 2.0$ Hz, 1H).

In the incubation containing **3**, **10**, and 4-OT, extra signals were detected after 1 d, indicating the formation of cinnamaldehyde (**15**). The ^{1}H NMR signals for *trans*-**15** are as follows: ^{1}H NMR (500 MHz, 20 mM phosphate/D_2O buffer, pH 7.3): $\delta = 6.70$ (dd, $J = 8$ Hz, 1H), 7.29 (m, 1H), 7.38 (m, 2H), 7.59 (d, $J = 6.5$ Hz, 2H), 7.66 (d, $J = 16$ Hz, 1H), 9.44 ppm (d, $J = 8$ Hz, 1H). Furthermore, in all samples (with or without enzyme) the following extra signals were detected after 1 week: ^{1}H NMR (500 MHz, 20 mM phosphate/D_2O buffer, pH 7.3): $\delta = 1.91$ (d, $J = 7.0$ Hz, 3H), 6.07 (dd, $J = 8.0$ Hz, 1H), 7.03 (m, 1H), 9.22 ppm (d, $J = 8.5$ Hz, 1H). These signals correspond to the formation of 2-butenal (**16**). This self-condensation product of **3** is formed in slightly higher amounts in the incubations containing 4-OT as compared to the control reactions without enzyme. [Reference spectra of **3** (and its hydrate), **10**, **15**, and **16** are given in Figure S7].

Detection of 15 by UV spectroscopy and GC/MS analysis: Although **15** has characteristic ^{1}H NMR spectroscopic signals, its identity in the incubation mixture described above was further confirmed by UV spectroscopy and GC/MS analysis. Accordingly, after 14 d of incubation, a small aliquot was removed from the mixture containing **3**, **10**, and 4-OT and diluted 200-fold in 20 mM NaH_2PO_4 buffer (pH 7.3). In addition, a small aliquot was removed from the control mixture (**3** and **10** incubated without enzyme) and also diluted 200-fold in the same buffer. Subsequently, UV/Vis spectra were recorded from the diluted samples. Apart from the characteristic absorbance peak of **10** ($\lambda_{max} = 250$ nm, $\varepsilon_{250} = 15$ mM^{-1} cm^{-1}), the sample containing 4-OT showed extra peaks at $\lambda_{max} = 290$ nm (**15**, $\varepsilon_{290} = 26.7$ mM^{-1} cm^{-1}) and around $\lambda_{max} = 227$ nm (**16**, $\varepsilon_{227} = 19$ mM^{-1} cm^{-1}). The absorbance peak corresponding to **15** was lacking in the control sample without enzyme.

For detection of **15** by GC/MS analysis, the remaining part (ca. 0.6 mL) of the reaction mixture containing **3**, **10**, and 4-OT was removed from the NMR tube and extracted with 1.8 mL of ethylacetate. The ethylacetate layer was dried over $MgSO_4$ and subsequently analyzed by GC/MS. The control sample (**3** and **10** incubated without enzyme) was prepared and analyzed in the same way, but did not show the presence of **15**.

^{1}H NMR spectroscopy assay for aldolase activity: ^{1}H NMR spectra monitoring the aldol condensation of **3** with **10** catalyzed by either wild-type 4-OT, L8R-4-OT, P1A-4-OT, R11A-4-OT, R39A-4-OT, 4-OT inactivated by 3-BP, or synthetic 4-OT, were recorded as follows. In an NMR tube, the enzyme (90 μM) was incubated with **3** and **10** (50 mM each, unless stated otherwise) in 0.6 mL of 20 mM NaH_2PO_4 buffer (pH 7.3) at 22 °C. In addition, to each tube 0.05 mL of D_2O was added. In two additional control experiments, **3** and **10** were incubated without enzyme or with an aliquot from a mock purification under otherwise identical conditions. The first ^{1}H NMR spectrum was recorded immediately after mixing, and then after 7 and 14 days. The formation of **15** is indicative of the presence of aldolase activity. The ^{1}H NMR signals for **3** (and its hydrate), **10** and **15** are described above.

Colorimetric assay for aldolase activity: Purified wild-type 4-OT, 4-OT mutants, and synthetic 4-OT were assayed for aldolase activity by monitoring production of cinnamaldehyde (**15**) upon incubation with acetaldehyde (**3**) and benzaldehyde (**10**). Accordingly, wild-type 4-OT, P1A-4-OT, R11A-4-OT, R39A-4-OT, L8R-4-OT, or synthetic 4-OT (200 μg) were incubated (in separate vials) with **3** and **10** (30 mM each) in NaH_2PO_4 buffer (1.2 mL, 20 mM; pH 7.3) at 22 °C. In two separate control experiments, **3** and **10** were incubated without enzyme or with an aliquot of a mock purification. After incubation of the reaction mixtures at 22 °C for 3 d, a sample (50 μL) was removed and mixed with 0.2% (*w/v*) phloroglucinol (150 μL) in HCl/EtOH (25:75%, *v/v*). Compound **15** forms a short-

lived yellow-colored complex with phloroglucinol, indicative of the presence of aldolase activity.

UV spectrophotometric assay for aldolase activity: The kinetic assays were performed at 22 °C by following the increase in absorbance at 290 nm, which corresponds to the formation of **15**. An aliquot of 4-OT was added to NaH_2PO_4 buffer (0.3 mL, 20 mM; pH 7.3) in a 1 mm cuvette, yielding a final enzyme concentration of 587 μM (146 μM for the L8R-4-OT mutant). The enzyme activity was assayed by the addition of **3** (at a fixed concentration of 50 mM) and **10** (in concentrations varying from 0.5 to 10 mM). The initial rates [mM s^{-1}] were plotted versus the concentration of **10** [mM] (Figure S4). The slope of this plot is a straight line that equals $(k_{cat} \times [E])/K_m$. Dividing the slope by the enzyme concentration results in a value for the apparent k_{cat}/K_m (for benzaldehyde).

Additional kinetic assays using the L8R-4-OT mutant were performed to demonstrate that the initial rate is dependent on both substrate concentrations, and to verify that the kinetic mechanism is sequential. An aliquot of L8R-4-OT was added to NaH_2PO_4 buffer (0.3 mL, 20 mM; pH 7.3) in a 1 mm cuvette, yielding a final enzyme concentration of 292 μM. The enzyme activity was assayed by the addition of **3** (in concentrations varying from 10 to 50 mM) and **10** (in concentrations varying from 1 to 8 mM). The resulting data were plotted as the reciprocal value of the initial rate [s mM^{-1}] versus the reciprocal value of the concentration of **3** [mM^{-1}] (Figure S4).

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[1] a) U. T. Bornscheuer, R. J. Kazlauskas, *Angew. Chem.* **2004**, *116*, 6156 – 6165; *Angew. Chem. Int. Ed.* **2004**, *43*, 6032 – 6040; b) R. J. Kazlauskas, *Curr. Opin. Chem. Biol.* **2005**, *9*, 195 – 201.

[2] A. Yarnell, *CENEAR* **2003**, *81*, 33 – 35.

[3] a) D. R. J. Palmer, J. B. Garrett, V. Sharma, R. Meganathan, P. C. Babbitt, J. A. Gerlt, *Biochemistry* **1999**, *38*, 4252 – 4258; b) L. C. James, D. S. Tawfik, *Protein Sci.* **2001**, *10*, 2600 – 2607; c) P. J. O'Brien, D. Herschlag, *Biochemistry* **2001**, *40*, 5691 – 5699; d) S. C. Wang, W. H. Johnson, Jr., C. P. Whitman, *J. Am. Chem. Soc.* **2003**, *125*, 14282 – 14283; e) D. M. Schmidt, E. C. Mundorff, M. Dojka, E. Bermudez, J. E. Ness, S. Govindarajan, P. C. Babbitt, J. Minshull, J. A. Gerlt, *Biochemistry* **2003**, *42*, 8387 – 8393; f) G. J. Poelarends, H. Serrano, W. H. Johnson, Jr., D. W. Hoffman, C. P. Whitman, *J. Am. Chem. Soc.* **2004**, *126*, 15658 – 15659; g) W. S. Yew, J. Akana, E. L. Wise, I. Rayment, J. A. Gerlt, *Biochemistry* **2005**, *44*, 1807 – 1815; h) C. Roodveldt, D. S. Tawfik, *Biochemistry* **2005**, *44*, 12728 – 12736; i) O. Khersonsky, C. Roodveldt, D. S. Tawfik, *Curr. Opin. Chem. Biol.* **2006**, *10*, 498 – 508; j) L. F. Olguin, S. E. Askew, A. C. O'Donoghue, F. Hollfelder, *J. Am. Chem. Soc.* **2008**, *130*, 16547 – 16555; k) B. van Loo, S. Jonas, A. C. Babtie, A. Benjdia, O. Berteau, M. Hyvönen, F. Hollfelder, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 2740 – 2745.

[4] a) S. Harayama, M. Rekik, K. L. Ngai, L. N. Ornston, *J. Bacteriol.* **1989**, *171*, 6251 – 6258; b) C. P. Whitman, B. A. Aird, W. R. Gillespie, N. J. Stolowich, *J. Am. Chem. Soc.* **1991**, *113*, 3154 – 3162; L. H. Chen, G. L. Kenyon, F. Curtin, S. Harayama, M. E. Bembenek, G. Hajipour, C. P. Whitman, *J. Biol. Chem.* **1992**, *267*, 17716 – 17721.

[5] a) C. P. Whitman, *Arch. Biochem. Biophys.* **2002**, *402*, 1 – 13; b) G. J. Poelarends, V. P. Veetil, C. P. Whitman, *Cell. Mol. Life Sci.* **2008**, *65*, 3606 – 3618.

[6] a) S. C. Wang, W. H. Johnson, Jr., R. M. Czerwinski, S. L. Stamps, C. P. Whitman, *Biochemistry* **2007**, *46*, 11919 – 11929; b) E. A. Burks, C. D. Fleming, A. D. Mesecar, C. P. Whitman, S. D. Pegan, *Biochemistry* **2010**, *49*, 5016 – 5027.

[7] T. K. Harris, R. M. Czerwinski, W. H. Johnson, Jr., P. M. Legler, C. Abeygunawardana, M. A. Massiah, J. T. Stivers, C. P. Whitman, A. S. Mildvan, *Biochemistry* **1999**, *38*, 12343 – 12357.

[8] A. Brik, L. J. D'Souza, E. Keinan, F. Grynszpan, P. E. Dawson, *ChemBioChem* **2002**, *3*, 845 – 851.

[9] J. T. Stivers, C. Abeygunawardana, A. S. Mildvan, G. Hajipour, C. P. Whitman, *Biochemistry* **1996**, *35*, 814 – 823.

[10] P. Y. Bruice, *J. Am. Chem. Soc.* **1989**, *111*, 962 – 970.

[11] S. Mukherjee, J. W. Yang, S. Hoffmann, B. List, *Chem. Rev.* **2007**, *107*, 5471 – 5569, and references therein.

[12] I. Fridovich, F. H. Westheimer, *J. Am. Chem. Soc.* **1962**, *84*, 3208 – 3209.

[13] J. T. Stivers, C. Abeygunawardana, A. S. Mildvan, G. Hajipour, C. P. Whitman, L. H. Chen, *Biochemistry* **1996**, *35*, 803 – 813.

[14] 4-OT was synthesized by GenScript USA Inc. (Piscataway, NY).

[15] G. J. Poelarends, J. J. Almrud, H. Serrano, J. E. Darty, W. H. Johnson, Jr., M. L. Hackert, C. P. Whitman, *Biochemistry* **2006**, *45*, 7700 – 7708.

[16] a) F. Pomar, F. Merino, A. Ros Barceló, *Protoplasma* **2002**, *220*, 17 – 28; b) W. Zha, S. B. Rubin-Pitel, H. Zhao, *Mol. BioSyst.* **2008**, *4*, 246 – 248.

[17] An alternative mechanism in which acetaldehyde reacts via the derived enolate anion rather than via an enamine intermediate cannot be excluded. In such a mechanism, the critical Pro1 residue would function as a general base catalyst that removes a proton from acetaldehyde to give the reactive enolate. However, this alternate mechanism seems unlikely in view of the ability of Pro1 to rapidly react with acetaldehyde to form an iminium ion.

[18] T. D. Machajewski, C.-H. Wong, *Angew. Chem.* **2000**, *112*, 1406 – 1430; *Angew. Chem. Int. Ed.* **2000**, *39*, 1352 – 1374, and references therein.

[19] J. Wagner, R. A. Lerner, C. F. Barbas III, *Science* **1995**, *270*, 1797 – 1800.

[20] F. Tanaka, R. Fuller, C. F. Barbas III, *Biochemistry* **2005**, *44*, 7583 – 7592.

[21] B. List, *Acc. Chem. Res.* **2004**, *37*, 548 – 557.

[22] H. Raj, B. Weiner, V. Puthan Veetil, C. R. Reis, W. J. Quax, D. B. Janssen, B. L. Feringa, G. J. Poelarends, *ChemBioChem* **2009**, *10*, 2236 – 2245.

[23] M. C. Fitzgerald, I. Chernushevich, K. G. Standing, S. B. H. Kent, C. P. Whitman, *J. Am. Chem. Soc.* **1995**, *117*, 11075 – 11080.

[24] J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, **1989**.

[25] W. J. Waddell, *J. Lab. Clin. Med.* **1956**, *48*, 311 – 314.

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